


Cellular immunological changes in patients with LADA are a mixture of those seen in patients with type 1 and type 2 diabetes

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Accepted for publication 1 March 2019
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Introduction

Latent autoimmune diabetes mellitus in the adult (LADA) [1] accounts for 9% of all adult onset diabetes [1]. Phenotypically, LADA patients share characteristics of both type 1 diabetes (T1D) (antibodies against the insulin-producing β cells) and type 2 diabetes (T2D) patients (aged >35 years and non-insulin-dependent at onset) [2,3]. LADA also shares genetic features of both T1D and T2D [4]. Despite the presence of autoantibodies, predominantly against glutamic acid decarboxylase (GAD), the disease progress is much slower than in T1D. At present, the focus on the immunological characterization of LADA has concerned the presence of autoantibodies, with only

Summary

There is currently scarce knowledge of the immunological profile of patients with latent autoimmune diabetes mellitus in the adult (LADA) when compared with healthy controls (HC) and patients with classical type 1 diabetes (T1D) and type 2 diabetes (T2D). The objective of this study was to investigate the cellular immunological profile of LADA patients and compare to HC and patients with T1D and T2D. All patients and age-matched HC were recruited from Uppsala County. Peripheral blood mononuclear cells were isolated from freshly collected blood to determine the proportions of immune cells by flow cytometry. Plasma concentrations of the cytokine interleukin (IL)-35 were measured by enzyme-linked immunosorbent assay (ELISA). The proportion of CD11c⁺CD123⁻ antigen-presenting cells (APCs) was lower, while the proportions of CD11c⁺CD123⁺ APCs and IL-35⁺ tolerogenic APCs were higher in LADA patients than in T1D patients. The proportion of CD3⁻CD56^{high}-CD16⁺ natural killer (NK) cells was higher in LADA patients than in both HC and T2D patients. The frequency of IL-35⁺ regulatory T cells and plasma IL-35 concentrations in LADA patients were similar to those in T1D and T2D patients, but lower than in HC. The proportion of regulatory B cells in LADA patients was higher than in healthy controls, T1D and T2D patients, and the frequency of IL-35⁺ regulatory B cells was higher than in T1D patients. LADA presents a mixed cellular immunological pattern with features overlapping with both T1D and T2D.

Keywords: Cellular immunology, IL-35, LADA, type 1 diabetes, type 2 diabetes

a few studies of other components of the immune system, such as low-grade proinflammatory markers or cellular immunology [4–6]. So far there are no published reports describing the cellular immunological profile that characterizes LADA patients in comparison to healthy individuals, T1D as well as T2D patients, simultaneously.

Innate immune cells, such as dendritic cells (DCs), are the main players during early development of autoimmune diabetes [7–10]. Allen *et al.* have reported that plasmacytoid dendritic cells (pDCs) are expanded during the early development of T1D and enhance the islet autoantigen presentation to T cells through an immune complex capture [11]. In line with this, it has been shown that interferon (IFN)- α -producing pDCs augment the T helper

type 1 (Th1) response in T1D and the number of IFN- α -producing pDCs are increased in T1D patients compared to healthy controls [12]. Therefore, it is of interest to investigate CD11c⁺CD123⁻ human leucocyte antigen (HLA)-DR⁺Lin⁻ (CD11c⁺CD123⁻) antigen-presenting cells (APCs), CD11c⁺CD123⁺HLA-DR⁺Lin⁻ (CD11c⁺CD123⁺) APCs and CD11c⁻CD123⁺HLA-DR⁺Lin⁻ (CD11c⁻CD123⁺) pDCs in LADA patients. Herein, these subsets of DCs were analysed using flow cytometry, as described previously [13]. Neutrophils have been found to be reduced in recent-onset T1D [14,15], and CD15^{low} neutrophils (low-density granular neutrophils) have also been shown to have a pathogenic role in systemic autoimmunity [16]. In accordance with this, Neuwrith *et al.* have reported a role of CD15^{low} cells in T1D development [17]. Alterations in the frequency of CD16 monocytes has been associated with diabetes complications [18].

Natural killer (NK) cells participate in innate immune responses and in animal models of T1D, it has been reported that depletion of NK cells prevents the development of T1D [19,20]. An altered frequency of total number of NK cells has been found in LADA, but not in T2D, patients [21,22]. Furthermore, it has been reported in children with T1D that NK cells have defects in signalling and function that might contribute to the pathogenesis of T1D [23]. NK cell subsets could be defined based on the relative expression of CD16 and CD56, i.e. CD56^{dim} (CD56^{low}CD16⁺ and CD56^{low}CD16⁻) and CD56^{bright} (CD56^{high}CD16⁺ and CD56^{high}CD16⁻), as reviewed by Poli *et al.* [24]. CD56^{dim} NK cells are considered to be mature NK cells and CD56^{bright} cells are immature cells. CD56^{dim} NK cells have cytotoxic properties while CD56^{bright} NK cells are proinflammatory, as they produce IFN- γ [25]. In the present study we determined the proportion of both CD56^{dim} and CD56^{bright} NK cell subpopulations using flow cytometry.

Adaptive immune cells such as T (CD4⁺ and CD8⁺) and B (CD19⁺) cells also play a major role in autoimmunity and metabolic disorders [26–30]. An altered phenotype of CD4⁺, CD8⁺ and CD19⁺ cells has been reported in LADA patients [5,31–33]. Therefore, in this study we determined the proportions of these cells in LADA patients when compared to T1D and T2D patients. Immune cells that produce anti-inflammatory cytokines, e.g. interleukin (IL)-10, transforming growth factor (TGF)- β and IL-35, are known as regulatory immune cells, i.e. regulatory T (T_{reg}) cells, regulatory B (B_{reg}) cells and tolerogenic APCs [34–36]. T_{reg} cells have received a great deal of attention, as these cells play a major role in maintaining the homeostasis of the immune system and preventing the development of autoimmune diseases [37,38]. A reduced number of T_{reg} cells has been reported in different autoimmune diseases, including T1D [39]. T_{reg} cells can be identified by using the cell surface

markers CD4, CD25 and CD127 and the intracellular forkhead box (Fox) transcription factor FoxP3 [40]. B_{reg} cells can be identified by using the cell surface markers CD19, CD24, CD38 and CD40 [41]. T_{reg} cells, B_{reg} cells and APCs produce IL-35 [34–36,42–44]. This cytokine is a novel anti-inflammatory cytokine, which belongs to the IL-12 cytokine family, and has two subunits, Ebi3 and IL-12p35 [42,43]. It has previously been reported that IL-35 suppresses autoimmunity and plays a putative role in tumour biology [35,36,42,45–47]. We have found that the systemic administration of IL-35 prevented the development of autoimmune diabetes in the multiple low-dose streptozotocin (MLDSTZ) mouse model, and effectively reversed established disease in both the MLDSTZ and non-obese diabetic mouse models of T1D by maintaining the normal suppressive phenotype of T_{reg} cells [46]. We also found that IL-35 levels were higher in T1D patients with residual C-peptide compared to T1D patients with no measurable residual C-peptide [48]. Thus, our previous findings indicate that IL-35 may prevent the loss of β cell mass. The possible alteration in the frequencies of IL-35-producing cells in LADA patients has not yet been investigated.

In this study we aimed to determine the proportions of different innate, adaptive and regulatory immune cells in order to elucidate the immunological profile that characterizes LADA in comparison to healthy controls, T1D and T2D patients.

Materials and methods

Informed consent

The Uppsala County regional ethics board approved this study, and the reported investigations were carried out in accordance with the principles of the Declaration of Helsinki, as revised in 2000. All participants were provided with oral and written information about the study, and signed a written consent.

Inclusion

The All New Diabetes in Uppsala (ANDiU) study (www.andiu.se) is an ongoing study with the aim of describing the incidence of diabetes subgroups in Uppsala County, Sweden (population approximately 300 000). Patients newly diagnosed with diabetes (ADA criteria) are included. Upon registration patients are classified into diabetes subtypes according to criteria shared with the All New Diabetics in Scania (ANDIS) study (<http://andis.ludc.med.lu.se>; Principle Investigator: Leif Groop) in southern Sweden. Criteria for LADA diagnosis are age ≥ 35 years, GAD antibody (GADA) positivity (>20 IE/ml) and fasting (f) C-peptide levels ≥ 0.3 nmol/l. T1D criteria are GADA

positivity and fC-peptide <0.3 nmol/l, whereas for T2D age ≥ 35 years, GADA negativity and fC-peptide >0.72 nmol/l are used as diagnostic criteria. For all three diagnoses there should be no history of pancreatitis, pancreatic cancer or other causes of secondary diabetes. There are no unified criteria for LADA diagnosis and classification, but the criteria used in ANDiU and ANDIS are in line with previous literature [3]. The exception is fC-peptide, which is used in ANDiU and ANDIS as an indicator of remaining insulin production to separate LADA from T1D [49,50]. It replaces the insulin therapy criterion (i.e. no insulin therapy within 6–12 months of diagnosis) that is often used, which is open to subjectivity as it reflects the assessment made by the treating physician. From ANDiU we recruited patients classified as LADA and T2D. T1D patients and healthy controls were recruited at Uppsala University Hospital and Eriksberg Primary Health Care Clinic, Uppsala, respectively. T1D and T2D patients, as well as healthy controls, were age-, sex- and body mass index (BMI)-matched to the LADA patients. Exclusion criteria were ongoing hormonal treatment (ATC code H01-03), use of non-steroid anti-inflammatory drugs (NSAID, ATC code M01), ongoing chemotherapy treatment (ATC code L01-04) and other autoimmune disease (e.g. hypo/hyperthyroidism, rheumatic disease, vitiligo, psoriasis, coeliac disease, Addison's disease and inflammatory bowel disease). Table 1 provides characteristics of the included patients and Supporting information, Fig. S1 shows a flow-chart of the inclusion process of the study.

Routine laboratory parameters

Blood samples were collected after overnight fasting. All routine laboratory parameters were analysed at the Central Clinical Chemistry Laboratory at Uppsala University Hospital, which is certified by the External Quality Assessment Service (EQUALIS) (equalis.se/en). On inclusion, blood haemoglobin [51], blood leucocyte count (Lkc), plasma C-reactive protein (CRP), serum transglutaminase [immunoglobulin (Ig)A] antibodies, serum thyroxine (T4), serum triiodothyronine (T3), serum thyroid-stimulating hormone (TSH), serum TSH-receptor [18] antibody, serum thyroid peroxidase antibody, plasma IgA antibody, serum cortisol, 21-hydroxylase [18] antibody, serum GADA, fasting serum C-peptide, fasting plasma glucose and blood haemoglobinA1c (HbA1c) were analysed. Glomerular filtration rate (GFR) was estimated based on creatinine-levels calculated with the MDRD formula [52].

Flow cytometry

Freshly isolated peripheral blood mononuclear cells (PBMCs) were prepared using Histopaque-1077 (Sigma, St Louis, MO, USA), as described earlier [48]. PBMCs were stained with antibodies for surface markers (Supporting information, Table S1). The cells were thereafter fixed and permeabilized with fixation permeabilization buffer (eBioscience, San Diego, CA, USA) for intracellular markers (Supporting information, Table S1). The samples were run on LSR II Fortesa (Becton Dickinson, Franklin Lakes, NJ, USA) using DivaDacker software [53] and 1 million events

Table 1. Characteristics of the research subjects; patients were matched for age, sex and BMI

	Controls (n = 13)	T1D (n = 16)	LADA (n = 14)	T2D (n = 16)
Age (years)	61.6 \pm 2.9	66.0 \pm 1.4	65.7 \pm 2.4	64.0 \pm 2.0
Sex (M%/F%)	62%/38%	73%/27%	57%/43%	70%/30%
BMI (kg/m ²)	28.9 \pm 4.0	26.5 \pm 0.7	27.9 \pm 0.5	29.1 \pm 1.0
Age at onset	n.a.	19.7 \pm 1.8	63.6 \pm 1.5 ^b	60.0 \pm 2.0 ^b
Disease duration	n.a.	46.1 \pm 2.2	4.9 \pm 0.3 ^b	5.3 \pm 0.3 ^{a,b}
HbA1c (mmol/mol)	35.8 \pm 0.7	58.9 \pm 3.2 ^a	45.8 \pm 2.3 ^b	52.27 \pm 4.0 ^a
fC-peptide (nmol/l)	1.10 \pm 0.11	0.22 \pm 0.13 ^a	0.95 \pm 0.14 ^b	1.30 \pm 0.12 ^{b,c}
fp-glucose (mmol/l)	5.9 \pm 0.1	11.7 \pm 1.0 ^a	8.0 \pm 0.5 ^b	9.1 \pm 0.6 ^{a,b}
GAD antibody IgG (IE/ml)	0 \pm 0	508 \pm 205	337 \pm 190	1 \pm 0
IA-2 antibody IgG (IE/ml)	1 \pm 0	25 \pm 7	387 \pm 295	1 \pm 0
Hb (g/l)	145 \pm 2	142 \pm 3	145 \pm 3	141 \pm 4
Lkc (10 ⁹ /l)	6.0 \pm 0.4	5.8 \pm 0.4	6.6 \pm 0.8	5.4 \pm 0.2
CRP (mg/l)	1.2 \pm 0.4	1.6 \pm 0.3	1.3 \pm 0.2	2.4 \pm 0.8

All values are given as mean \pm standard error of the mean (s.e.m.). Multiple comparison of means by analysis of variance (ANOVA), Tukey's *post-hoc* test.

^aP-value versus controls < 0.05; ^bP-value versus T1D < 0.05; ^cP-value versus LADA < 0.05.

GADA = glutamate decarboxylase 65 antibody; eGFR = creatinine estimated glomerular filtration rate; TSH = thyroid-stimulating hormone; LADA = latent autoimmune diabetes mellitus in the adult (LADA); Ig = immunoglobulin; BMI = body mass index; n.a. = not available; HbA1c = haemoglobinA1c; CRP = C-reactive peptide; fC-peptide = fasting C-peptide; fp-glucose = fasting plasma glucose; Lkc = leucocyte count.

were counted for analysis. Fluorescence minus one (FMO), isotype and single-stained controls were used for gating strategies. The flow cytometry standard (FCS) files were analysed on FlowLogic software (Inivai Technologies, Mentone, Australia). Representative gating strategies are shown in Supporting information, Fig. S2–S5.

IL-35 measurements in plasma

Plasma IL-35 concentrations were determined by using an enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Clone Corporation, Wuhan, China).

Statistics

Differences in means between healthy controls, T1D and T2D patients compared to LADA patients were calculated by one-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test for immunological profiles. Comparisons between all groups were performed by ANOVA with Tukey's *post-hoc* test for research subjects' characteristics (Table 1). A *P*-value < 0.05 was considered as a significant difference between groups. Statistical analyses were carried out in GraphPad Prism version 6.0™.

Results

Innate immune cell responses

We observed a lower proportion of CD11c⁺CD123⁻ APCs in LADA patients than in healthy controls and T1D patients (Fig. 1a). Furthermore, the proportion of CD11c⁺CD123⁻ APCs among HLA-DR⁺Lin⁻ cells was lower in LADA patients than in T1D patients (Fig. 1b), whereas the proportions of pDCs and pDCs among HLA-DR⁺Lin⁻ were not altered in LADA patients (Fig. 1c,d). We did not observe any difference in the proportion of CD11c⁺CD123⁺ APCs (Fig. 1e), but the proportion of CD11c⁺CD123⁺ cells among HLA-DR⁺Lin⁻ was higher in patients with LADA than in patients with T1D (Fig. 1f).

We did not find any alteration in the proportions of CD15^{low} neutrophils and CD16⁺ monocytes in LADA patients (Supporting information, Fig. S6a,b). We also examined different subsets of NK cells and did not find any alteration in LADA patients compared to the other groups regarding the proportions of CD3⁻CD56^{low}CD16⁺, CD3⁻CD56^{low}CD16⁻ and CD3⁻CD56^{high}CD16⁻ NK cells (Fig. 2a–c). However, the proportion of CD3⁻CD56^{high}CD16⁺

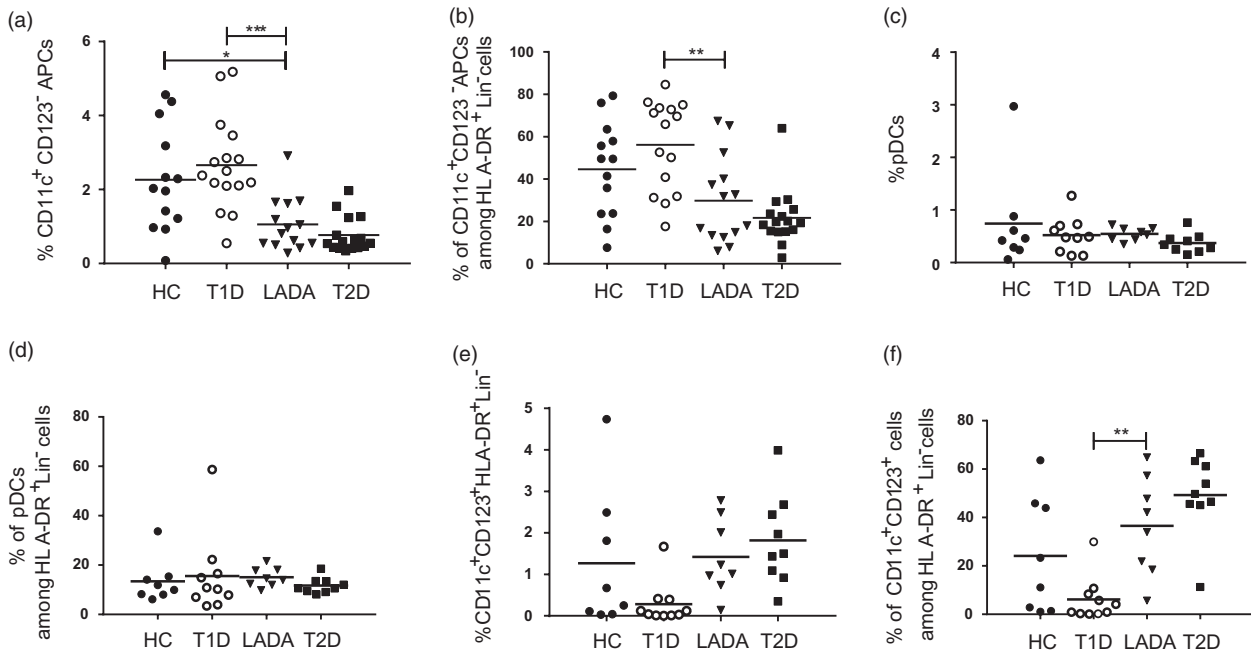


Fig. 1. The proportions of CD11c⁺CD123⁻ antigen-presenting cells (APCs) are decreased while CD11c⁺CD123⁺ APCs are increased in latent autoimmune diabetes mellitus in the adult (LADA) compared to type 1 diabetes patients. Proportions of (a) CD11c⁺CD123⁻ human leucocyte antigen (HLA)-DR⁺Lin⁻ APCs in total live cells, (b) CD11c⁺CD123⁻ APCs among HLA-DR⁺Lin⁻ cells, which were gated on HLA-DR⁺Lin⁻ cells, (c) CD11c⁺CD123⁺HLA-DR⁺Lin⁻ plasmacytoid dendritic cells (pDCs) in total live cells, (d) CD11c⁺CD123⁺ pDCs among HLA-DR⁺Lin⁻ cells, which were gated on HLA-DR⁺Lin⁻ cells, (e) CD11c⁺CD123⁺HLA-DR⁺Lin⁻ APCs in total live cells and (f) CD11c⁺CD123⁺ APCs among HLA-DR⁺Lin⁻ cells, which were gated on HLA-DR⁺Lin⁻ cells. The proportions of these cells were determined by using a flow cytometer. HC = healthy controls. One-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test comparison to patients with LADA was used, *n* = 13–16/group. **P* < 0.05; ***P* < 0.01.

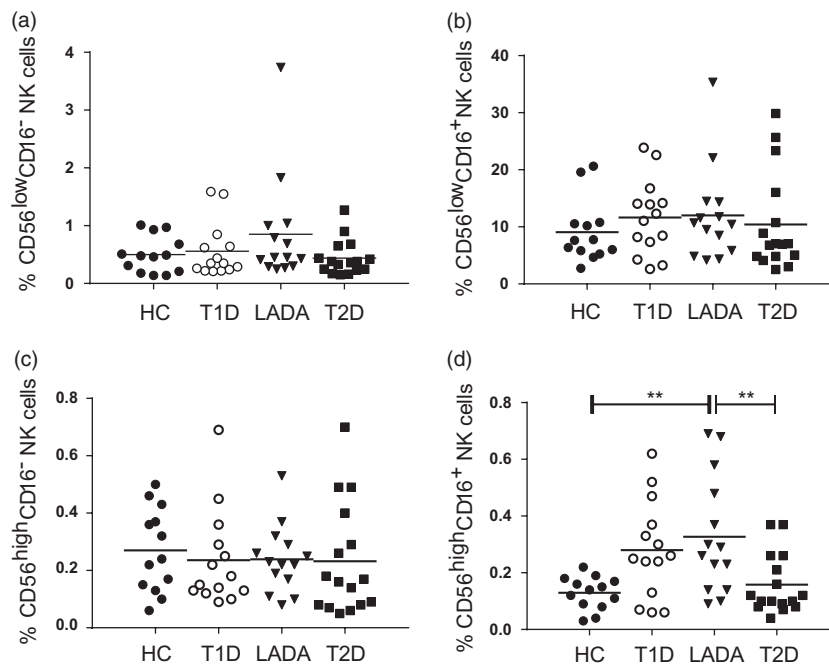


Fig. 2. The proportion of CD3⁻CD56^{high}CD16⁺ natural killer (NK) cells was increased in latent autoimmune diabetes mellitus in the adult (LADA) patients. The proportions of (a) CD3⁻CD56^{low}CD16⁻ NK cells in total live cells, (b) CD3⁻CD56^{low}CD16⁺ NK cells in total live cells, (c) CD3⁻CD56^{high}CD16⁻ NK cells in total live cells and (d) CD3⁻CD56^{high}CD16⁺ NK cells in total live cells were determined using a flow cytometer. HC = healthy controls. One-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test comparison to patients with LADA was used, $n = 13-16$ /group; ** $P < 0.01$.

NK cells was higher in LADA patients compared to both healthy controls and T2D patients (Fig. 2d). We did not observe any alteration in the proportions of CD3⁺CD56⁺ NK T cells between the groups (Supporting information, Fig. S6c).

Adaptive immune cell responses

The proportions of CD3⁺, CD4⁺CD25⁻ and CD8⁺ T cells were not altered in LADA patients when compared to the other groups (Supporting information, Fig. S7a-c). Similarly, the proportion of CD19⁺ cells was not altered (Supporting information, Fig. S7d).

Regulatory immune cell responses

The proportions of CD4⁺CD25⁺CD127⁻FoxP3⁺ cells (T_{reg} cells) and CD4⁺CD25⁺CD127⁻FoxP3⁺Helios⁺thymic-derived T cells (tT_{reg}) were similar in all four groups (Supporting information, Fig. S8a,b). Similarly, the proportions of T_{reg} cells as well as the tT_{reg} cells among CD4⁺ T cells were similar in all four groups (Supporting information, Fig. S8c,d). However, the proportions of IL-35⁺ cells among T_{reg} and tT_{reg} cells in patients with LADA were similar in patients with T1D and T2D, but lower when compared to healthy controls (Fig. 3a,b).

We also analysed the proportion of CD19⁺CD24⁺CD40⁺CD38⁺ cells (B_{reg} cells), and observed a higher proportion of these cells in LADA patients when compared to healthy controls and patients with T1D and T2D (Fig. 3c). The proportion of IL-35⁺ cells among B_{reg} cells was higher in LADA patients when compared to T1D patients (Fig. 3d). Subsequently, we analysed the proportion of IL-35⁺ tolerogenic APCs. Interestingly, the proportion of IL-35⁺ cells among APCs was higher in patients with LADA compared to patients with T1D (Fig. 3e). Patients with LADA had IL-35 concentrations in blood plasma that were similar to those in patients with T1D and T2D, but lower compared to those in healthy controls (Fig. 3f).

Discussion

We determined the proportions of different immune cells to elucidate the immunological patterns that distinguish patients with LADA compared not only to healthy individuals, but also to patients with T1D and T2D. With regard to the innate and adaptive immune cells, we found that LADA shares common features with both T1D and T2D. The proportion of CD11c⁺CD123⁻ APCs in LADA patients was between that observed in T1D and T2D patients. Similarly, the proportion of

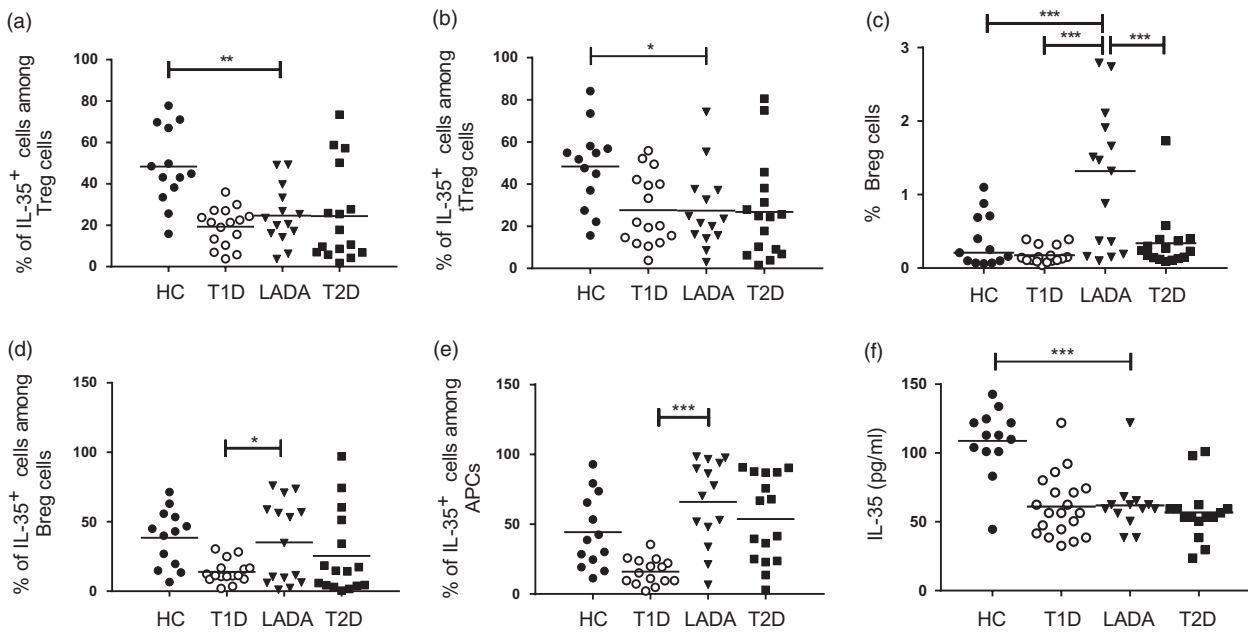


Fig. 3. The proportion of interleukin (IL)-35⁺ regulatory T cells (T_{reg}) is decreased, but proportions of regulatory B cells (B_{reg}) and IL-35-producing B_{reg} cells and tolerogenic antigen-presenting cells (APCs) are increased in latent autoimmune diabetes mellitus in the adult (LADA) patients. Proportions of (a) IL-35⁺ cells among $CD4^+CD25^+CD127^-$ forkhead box protein 3 (FoxP3)⁺ T_{reg} cells, (b) IL-35⁺ cells among $CD4^+CD25^+CD127^-$ FoxP3⁺Helios⁺ thymic-derived T_{regs} (tT_{regs}), (c) $CD19^+CD24^+CD38^+CD40^+$ B_{reg} cells in total live cells, (d) IL-35⁺ cells among $CD19^+CD24^+CD40^+CD38^+$ B_{reg} cells and (e) IL-35⁺ cells among $CD11c^+CD123^-$ human leucocyte antigen (HLA)-DR⁺Lin⁻ APCs. The proportions of these cells were determined using a flow cytometer. (f) IL-35 plasma concentrations were measured by enzyme-linked immunosorbent assay (ELISA). HC = healthy controls. One-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test comparison to patients with LADA was used, $n = 13-16$ /group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

$CD11c^+CD123^+$ cells among the HLA-DR⁺Lin⁻ was highest in T2D patients and lowest in T1D patients, whereas the proportion in LADA patients was between that of T1D and T2D patients.

Neutrophils have previously been claimed to play a role in the early development of both T1D [14,15,54] and T2D [55]. In the present study, we did not find any alteration in the proportion of $CD15^{low}$ neutrophils in LADA patients when compared to the other study groups. It cannot be excluded that early transient alterations during disease development as described for T1D [14,15] had occurred, as our study subjects with LADA had a mean disease duration of almost 5 years.

It has been reported previously that the proportion of $CD3^-CD56^+CD16^+$ NK cells is decreased in LADA patients [22]. Instead, we observed an increased proportion of $CD3^-CD56^{high}CD16^+$ NK cells in LADA patients compared to both healthy controls and T2D patients. The reason for the discrepancy between the studies is unknown, but it could be because we used freshly prepared PBMCs instead of cryopreserved cell preparations. $CD56^{high}$ or $CD56^{bright}$ NK cells produce IFN- γ [25]. The proportions of these NK cells were similar in LADA and T1D patients. This result illustrates that, like T1D, LADA is also a Th1 response disorder.

The frequency of FoxP3⁺ T_{reg} cells was similar in all groups. However, the proportion of IL-35⁺ cells among T_{reg} cells in patients with LADA was similar to those in patients with T1D and T2D, but lower when compared to healthy controls. This may suggest a common functional defect of T_{reg} cells in LADA, T1D and T2D patients. This idea was further supported because the circulating concentrations of IL-35 in patients with LADA were similar to patients with T1D and T2D, but lower than in than healthy controls. We have previously shown that decreased IL-35 production by T_{reg} cells is associated with a functional defect [46]. Also, previous findings of a decreased frequency of FoxP3⁺ cells among $CD4^+CD25^{intermediate}$ cells in LADA patients when compared to healthy controls [5], and a decreased *Foxp3* gene expression in $CD4^+$ T cells in LADA patients, are considered with defective T_{reg} cell functionality [56]. In patients with T1D, it has been reported that T_{reg} cells lose their FoxP3 expression due to DNA methylation in the *Foxp3* gene, which may cause a defective function in T_{reg} cells and result in a decreased number of T_{reg} cells [57]. Interestingly, Li *et al.* also reported an abnormal DNA methylation in peripheral $CD4^+$ T cells of LADA patients [33], which indicates a dysfunction of T_{reg} cells [58].

B_{reg} cells play an important role in maintaining the autoimmune and/or inflammatory response [35,36]. We found that the frequency of B_{reg} cells was higher in LADA patients compared to all other groups. These results are in agreement with a previous report, where Deng *et al.* found the lowest frequency of IL-10-producing B cells in T1D patients when compared to LADA and T2D patients [32]. Our observed lower proportion of IL-35⁺ cells among B_{reg} cells in T1D than LADA patients suggests that the response of IL-35⁺ B_{reg} cells in LADA patients is similar to that of T2D patients, which might protect against β cell destruction by immunological insults in LADA patients. Although the proportions of IL-35⁺ B_{reg} cells and IL-35⁺ tolerogenic APCs were higher in LADA than in T1D patients, the circulating concentrations of IL-35 were similar in LADA and T1D patients. This could be due to other IL-35-producing immune cells, such as iTr35 [59] and macrophages [60]. These particular populations have not been investigated in the present study.

APCs (CD123⁻ APCs) and pDCs (CD123⁺ APCs) prime T cells to react against the insulin-producing β cells. In the present study, we found that the numbers of APCs were lower in LADA than in T1D patients. This indicates that the initial immune response is lower in LADA patients, which might cause a delay in the development of an autoimmune response. Tolerogenic APCs protect β cells in animal models of T1D [44], and Dixon *et al.* reported that tolerogenic APCs produce IL-35 in human peripheral blood [34]. We analysed the frequency of IL-35⁺ cells among APCs in our study and found that IL-35⁺ cells were more frequent in LADA than in T1D patients, which may contribute to a milder disease development. In a previous study, we found that systemic treatment with IL-35 in two different animal models of T1D prevented destruction of β cells [46]. However, as this is an association study it is impossible to determine whether or not the immunological differences are causal.

We observed changes in the proportions of different immune cells in LADA patients compared to healthy controls and other diabetes patients. Our conclusion is that the changes in APC and B_{reg} cell numbers in LADA patients are more similar to those also observed in T2D patients, whereas the changes in NK cell numbers are more similar to those found in T1D patients. There are no differences in IL-35 concentrations and IL-35⁺ T_{reg} cell numbers in between LADA, T1D and T2D patients, but in all three conditions these are lower than the healthy individuals. Thus, our analysis of several subsets of immune cells reveals that LADA patients present a mixture of cellular immunological changes with features overlapping with both T1D and T2D.

Acknowledgements

The technical assistance from research nurses Violeta Armijo Del Valle, Rebecka Hilmius and Karin Kjellström is gratefully acknowledged. The present study was supported financially by the Swedish Research Council (2017-01343, 921-2014-7054), EXODIAB, the Swedish Diabetes Foundation, Diabetes Wellness Sverige, the Swedish Child Diabetes Fund, SEB Diabetesfonden, O.E. och Edla Johanssons vetenskapliga stiftelse and the Novo Nordisk Foundation.

Disclosures

The authors declare that there are no conflicts of interest associated with this paper.

Author contributions

M. M. and K. S. contributed to the design of the study, conducted experiments, acquired and analysed data, and participated in writing the manuscript. Z. L., D. E., J. S. and S. S. contributed to the design of the study, analysed data and participated in the writing of the manuscript. P. O. C. designed the study, analysed data and wrote the manuscript. All authors read and approved the final version. K. S. is the guarantor of this work and, as such, had access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

- 1 Hawa MI, Kolb H, Schloot N *et al.* Adult-onset autoimmune diabetes in Europe is prevalent with a broad clinical phenotype: action LADA 7. *Diabetes Care* 2013; **36**:908–13.
- 2 Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. *Diabetes* 1993; **42**:359–62.
- 3 Tuomi T, Santoro N, Caprio S, Cai M, Weng J, Groop L. The many faces of diabetes: a disease with increasing heterogeneity. *Lancet* 2014; **383**:1084–94.
- 4 Laugesen E, Ostergaard JA, Leslie RD, Danish Diabetes Academy Workshop and Workshop speakers. Latent autoimmune diabetes of the adult: current knowledge and uncertainty. *Diabet Med* 2015; **32**:843–52.
- 5 Radenkovic M, Silver C, Arvastsson J *et al.* Altered regulatory T cell phenotype in latent autoimmune diabetes of the adults (LADA). *Clin Exp Immunol* 2016; **186**:46–56.
- 6 Castelblanco E, Hernandez M, Castelblanco A *et al.* Low-grade inflammatory marker profile may help to differentiate patients with LADA, classic adult-onset type 1 diabetes, and type 2 diabetes. *Diabetes Care* 2018; **41**:862–8.

- 7 Price JD, Beauchamp NM, Rahir G *et al.* CD8⁺ dendritic cell-mediated tolerance of autoreactive CD4⁺ T cells is deficient in NOD mice and can be corrected by blocking CD40L. *J Leukoc Biol* 2014; **95**:325–36.
- 8 Calderon B, Carrero JA, Miller MJ, Unanue ER. Cellular and molecular events in the localization of diabetogenic T cells to islets of Langerhans. *Proc Natl Acad Sci USA* 2011; **108**:1561–6.
- 9 Price JD, Hotta-Iwamura C, Zhao Y, Beauchamp NM, Tarbell KV. DCIR2+ cDC2 DCs and Zbtb32 restore CD4⁺ T-cell tolerance and inhibit diabetes. *Diabetes* 2015; **64**:3521–31.
- 10 Billiard F, Lobry C, Darrasse-Jeze G *et al.* Dll4-Notch signaling in Flt3-independent dendritic cell development and autoimmunity in mice. *J Exp Med* 2012; **209**:1011–28.
- 11 Allen JS, Pang K, Skowera A *et al.* Plasmacytoid dendritic cells are proportionally expanded at diagnosis of type 1 diabetes and enhance islet autoantigen presentation to T-cells through immune complex capture. *Diabetes* 2009; **58**:138–45.
- 12 Xia CQ, Peng R, Chernatynskaya AV *et al.* Increased IFN- α -producing plasmacytoid dendritic cells (pDCs) in human Th1-mediated type 1 diabetes: pDCs augment Th1 responses through IFN- α production. *J Immunol* 2014; **193**:1024–34.
- 13 Rovati B, Mariucci S, Manzoni M, Bencardino K, Danova M. Flow cytometric detection of circulating dendritic cells in healthy subjects. *Eur J Histochem* 2008; **52**:45–52.
- 14 Harsunen MH, Puff R, D'Orlando O *et al.* Reduced blood leukocyte and neutrophil numbers in the pathogenesis of type 1 diabetes. *Horm Metab Res* 2013; **45**:467–70.
- 15 Valle A, Giamporcaro GM, Scavini M *et al.* Reduction of circulating neutrophils precedes and accompanies type 1 diabetes. *Diabetes* 2013; **62**:2072–7.
- 16 Carmona-Rivera C, Kaplan MJ. Low-density granulocytes: a distinct class of neutrophils in systemic autoimmunity. *Semin Immunopathol* 2013; **35**:455–63.
- 17 Neuwirth A, Dobes J, Ujezdaska J *et al.* Eosinophils from patients with type 1 diabetes mellitus express high level of myeloid α -defensins and myeloperoxidase. *Cell Immunol* 2012; **273**:158–63.
- 18 Min D, Brooks B, Wong J *et al.* Alterations in monocyte CD16 in association with diabetes complications. *Mediat Inflamm* 2012; **2012**:649083.
- 19 Rodacki M, Milech A, de Oliveira JE. NK cells and type 1 diabetes. *Clin Dev Immunol* 2006; **13**:101–7.
- 20 Maruyama T, Watanabe K, Yanagawa T *et al.* The suppressive effect of anti-asialo GM1 antibody on low-dose streptozotocin-induced diabetes in CD-1 mice. *Diabetes Res* 1991; **16**:171–5.
- 21 Piatkiewicz P, Milek T, Bernat-Karpinska M, Ohams M, Czech A, Ciostek P. The dysfunction of NK cells in patients with type 2 diabetes and colon cancer. *Arch Immunol Ther Exp (Warsz)* 2013; **61**:245–53.
- 22 Akesson C, Uvebrant K, Oderup C *et al.* Altered natural killer (NK) cell frequency and phenotype in latent autoimmune diabetes in adults (LADA) prior to insulin deficiency. *Clin Exp Immunol* 2010; **161**:48–56.
- 23 Qin H, Lee IF, Panagiotopoulos C *et al.* Natural killer cells from children with type 1 diabetes have defects in NKG2D-dependent function and signaling. *Diabetes* 2011; **60**:857–66.
- 24 Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology* 2009; **126**:458–65.
- 25 Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001; **22**:633–40.
- 26 Xia C, Rao X, Zhong J. Role of T lymphocytes in type 2 diabetes and diabetes-associated inflammation. *J Diabetes Res* 2017; **2017**:6494795.
- 27 Menart-Houtermans B, Rutter R, Nowotny B *et al.* Leukocyte profiles differ between type 1 and type 2 diabetes and are associated with metabolic phenotypes: results from the German Diabetes Study (GDS). *Diabetes Care* 2014; **37**:2326–33.
- 28 Mallone R, Martinuzzi E, Blancou P *et al.* CD8⁺ T-cell responses identify beta-cell autoimmunity in human type 1 diabetes. *Diabetes* 2007; **56**:613–21.
- 29 Zmora N, Bashiardes S, Levy M, Elinav E. The role of the immune system in metabolic health and disease. *Cell Metab* 2017; **25**:506–21.
- 30 Hampe CS. B cell in autoimmune diseases. *Scientifica (Cairo)* 2012; **2012**:215308.
- 31 Sachdeva N, Paul M, Badal D *et al.* Preproinsulin specific CD8⁺ T cells in subjects with latent autoimmune diabetes show lower frequency and different pathophysiological characteristics than those with type 1 diabetes. *Clin Immunol* 2015; **157**:78–90.
- 32 Deng C, Xiang Y, Tan T *et al.* Altered peripheral B-lymphocyte subsets in type 1 diabetes and latent autoimmune diabetes in adults. *Diabetes Care* 2016; **39**:434–40.
- 33 Li Y, Zhao M, Hou C *et al.* Abnormal DNA methylation in CD4⁺ T cells from people with latent autoimmune diabetes in adults. *Diabetes Res Clin Pract* 2011; **94**:242–8.
- 34 Dixon KO, van der Kooij SW, Vignali DA, van Kooten C. Human tolerogenic dendritic cells produce IL-35 in the absence of other IL-12 family members. *Eur J Immunol* 2015; **45**:1736–47.
- 35 Shen P, Roch T, Lampropoulou V *et al.* IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature* 2014; **507**:366–70.
- 36 Wang RX, Yu CR, Dambuzza IM *et al.* Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med* 2014; **20**:633–41.
- 37 Bluestone JA, Tang Q. How do CD4⁺CD25⁺ regulatory T cells control autoimmunity? *Curr Opin Immunol* 2005; **17**:638–42.
- 38 Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* 2008; **8**:523–32.

- 39 Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. *Lancet* 2014; **383**:69–82.
- 40 Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* 2010; **10**:490–500.
- 41 Stolp J, Turka LA, Wood KJ. B cells with immune-regulating function in transplantation. *Nat Rev Nephrol* 2014; **10**:389–97.
- 42 Niedbala W, Wei XQ, Cai B *et al.* IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol* 2007; **37**:3021–9.
- 43 Collison LW, Workman CJ, Kuo TT *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 2007; **450**:566–9.
- 44 Creusot RJ, Giannoukakis N, Trucco M, Clare-Salzler MJ, Fathman CG. It's time to bring dendritic cell therapy to type 1 diabetes. *Diabetes* 2014; **63**:20–30.
- 45 Li X, Singh K, Luo Z *et al.* Pro-tumoral immune cell alterations in wild type and Shb-deficient mice in response to 4T1 breast carcinomas. *Oncotarget* 2018; **9**:18720–33.
- 46 Singh K, Kadesjo E, Lindroos J *et al.* Interleukin-35 administration counteracts established murine type 1 diabetes – possible involvement of regulatory T cells. *Sci Rep* 2015; **5**:12633.
- 47 Turnis ME, Sawant DV, Szymczak-Workman AL *et al.* Interleukin-35 limits anti-tumor immunity. *Immunity* 2016; **44**:316–29.
- 48 Espes D, Singh K, Sandler S, Carlsson PO. Increased interleukin-35 levels in patients with type 1 diabetes with remaining C-peptide. *Diabetes Care* 2017; **40**:1090–5.
- 49 Rasouli B, Andersson T, Carlsson PO *et al.* Smoking and the risk of LADA: results from a Swedish population-based case-control study. *Diabetes Care* 2016; **39**:794–800.
- 50 Hjort R, Alfredsson L, Carlsson PO *et al.* Low birthweight is associated with an increased risk of LADA and type 2 diabetes: results from a Swedish case-control study. *Diabetologia* 2015; **58**:2525–32.
- 51 Zhou X, Bailey-Bucktrout SL, Jeker LT *et al.* Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells *in vivo*. *Nat Immunol* 2009; **10**:1000–7.
- 52 Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999; **130**:461–70.
- 53 Kleffel S, Vergani A, Tezza S *et al.* Interleukin-10+ regulatory B cells arise within antigen-experienced CD40+ B cells to maintain tolerance to islet autoantigens. *Diabetes* 2015; **64**:158–71.
- 54 Diana J, Simoni Y, Furio L *et al.* Crosstalk between neutrophils, B-1a cells and plasmacytoid dendritic cells initiates autoimmune diabetes. *Nat Med* 2013; **19**:65–73.
- 55 Lou M, Luo P, Tang R *et al.* Relationship between neutrophil-lymphocyte ratio and insulin resistance in newly diagnosed type 2 diabetes mellitus patients. *BMC Endocr Disord* 2015; **15**:9.
- 56 Yang ZF, Zhou ZG, Tang WL *et al.* Decrease of FOXP3 mRNA in CD4+ T cells in latent autoimmune diabetes in adult. *Zhonghua Yi Xue Za Zhi* 2006; **86**:2533–6.
- 57 Cabrera SM, Rigby MR, Mirmira RG. Targeting regulatory T cells in the treatment of type 1 diabetes mellitus. *Curr Mol Med* 2012; **12**:1261–72.
- 58 Long SA, Buckner JH. CD4+FOXP3+ T regulatory cells in human autoimmunity: more than a numbers game. *J Immunol* 2011; **187**:2061–6.
- 59 Collison LW, Chaturvedi V, Henderson AL *et al.* IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol* 2010; **11**:1093–101.
- 60 Lee CC, Lin JC, Hwang WL *et al.* Macrophage-secreted interleukin-35 regulates cancer cell plasticity to facilitate metastatic colonization. *Nat Commun* 2018; **9**:3763.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Flow-chart of the inclusion process of study. Of the 17 registered LADA patients of the All New Diabetes in Uppsala (ANDiU) study, 13 were included. Additional patients with type 1 diabetes (T1D, $n = 7$), and type 2 (T2D, $n = 13$) diabetes) were recruited from ANDiU. Another 11 participants with T1D were included from the department of Endocrinology, Uppsala University hospital, Uppsala (UAS). Healthy controls (HC, $n = 13$) were recruited upon arrival (with no infection or systemic inflammation) at the Eriksberg Primary Healthcare Centre (EVC). Study subjects were matched for sex, age and body mass index (BMI).

Fig. S2. The representative gating strategies for tolerogenic APCs. The live cells were gated based on forward scatter (FSC) and side scatter (SSC). Cells were further gated for HLA-DR and Lin mix expression. HLA-DR⁺Lin⁻ cells were thereafter gated for the expression of CD11c, CD123 or CD11c and CD123. CD11c⁺CD123⁻HLA-DR⁺Lin⁻ cells were gated for IL-12p35 and Ebi3 expression and gate was drawn by using FMO together with isotype controls to analyze IL-35⁺ cells.

Fig. S3. The representative gating strategies for NK cells. The live cells were gated based on forward scatter (FSC) and side scatter (SSC). Cells were then gated for CD3 and CD56 expression. CD56^{high} and CD56^{low} cells were thereafter gated for CD16 expression.

Fig. S4. The representative gating strategies for Breg cells. The live cells were gated based on forward scatter (FSC) and side scatter (SSC). They were then gated for CD19 expression.

CD19⁺ cells were gated for the expression of CD24 and CD40. CD19⁺CD24⁺CD40⁺ cells were gated for CD38 expression, CD19⁺CD24⁺CD40⁺CD38⁺ cells were thereafter gated for IL-12p35 and Ebi3 expression. FMO together with isotype controls were used for negative control to determine IL-12p35⁺Ebi3⁺ (IL-35⁺) cells.

Fig. S5. Representative gating strategies for Treg cells. The live cells were gated based on side scatter (SSC) and forward scatter (FSC). The cells were then gated for the expression of CD4 and CD25. CD4⁺CD25⁺ cells were further gated for the expression of CD127. CD4⁺CD25⁺CD127⁻ cells were gated for Foxp3 expression. CD4⁺CD25⁺CD127⁻Foxp3⁺ cells were gated for the expression of IL-12p35 and Ebi3. IL-12p35 and Ebi3 gates were made based on fluorescence minus one control together with isotype controls (this control was used as a negative control).

Fig. S6. The proportions of neutrophils, monocytes and NKT cells. The proportions of (a) CD15⁺ neutrophils in total live cells, (b) CD16⁺ monocytes in total live cells and (c) CD3⁺CD56⁺ NKT cells in total live cells, were determined by using a flow cytometer. HC denotes healthy controls. One-way ANOVA with Dunnett's post-hoc test

comparison to patients with LADA was used, $n = 13-16$ /group.

Fig. S7. The proportions of T-cells and B-lymphocytes. The proportions of (a) CD3⁺CD56⁻ T-cells in total live cells, (b) CD4⁺CD25⁻ T-cells in total live cells, (c) CD8⁺ T-cells in total live cells, (d) CD19⁺ B-lymphocytes in total live cells, were determined by using a flow cytometer. HC denotes healthy controls. One-way ANOVA with Dunnett's post-hoc test comparison to patients with LADA was used, $n = 13-16$ /group.

Fig. S8. The proportions of Treg and tTreg cells among CD4⁺ T cells. The proportion of (a) CD4⁺CD25⁺CD127⁻Foxp3⁺ Treg cells in total live cells, (b) CD4⁺CD25⁺CD127⁻Foxp3⁺Helios⁺ tTreg cells in total live cells, (c) The proportions of CD4⁺CD25⁺CD127⁻Foxp3⁺ Treg cells among CD4⁺ T-cells and (d) CD4⁺CD25⁺CD127⁻Foxp3⁺Helios⁺ tTregs among CD4⁺ T-cells. The proportions of these cells were determined by using a flow cytometer. HC denotes healthy controls. One-way ANOVA with Dunnett's post-hoc test was used for comparisons, $n = 13-16$ /group.

Table S1. Antibodies for surface and intracellular antigens used in flow cytometry staining.