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GM-CSF producing autoreactive CD4$^+$ T cells in type 1 diabetes

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ABSTRACT

The phenotype of autoreactive T cells in type 1 diabetes is described as Th1, Th17 and/or Th21, but is largely uncharacterized. We combined multi-parameter cytokine profiling and proliferation, and identified GM-CSF producing cells as a component of the response to beta cell autoantigens proinsulin and GAD65. Overall cytokine profiles of CD4$^+$ T cell were not altered in type 1 diabetes. In contrast, patients with recent onset type 1 diabetes had increased frequencies of proinsulin-responsive CD4$^+$ CD45RA$^-$ T cells producing GM-CSF ($p = 0.002$), IFNγ ($p = 0.004$), IL-17A ($p = 0.008$), IL-21 ($p = 0.011$), and IL-22 ($p = 0.007$), and GAD65-responsive CD4$^+$ CD45RA$^-$ T cells producing IL-21 ($p = 0.039$). CD4$^+$ T cells with a GM-CSF$^+$IFNγ$^-$IL-17A$^-$IL-21$^-$IL-22$^-$ phenotype were increased in patients for responses to both proinsulin ($p = 0.006$) and GAD65 ($p = 0.037$). GM-CSF producing T cells are a novel phenotype in the repertoire of T helper cells in type 1 diabetes and consolidate a Th1/Th17 pro-inflammatory pathogenesis in the disease.

1. Introduction

Type 1 diabetes is a T cell-mediated autoimmune disease, resulting in the destruction of insulin-producing beta cells in the pancreas. Beta cell autoantigen targeting CD4$^+$ and CD8$^+$ T cells have been identified in the peripheral blood of patients at and before the onset of symptomatic disease [1–3], and T cells that are specific for beta cell autoantigens have been isolated from the pancreatic islets of patients [4–6]. The phenotype of the beta cell autoantigen targeting CD4$^+$ T cells in patients has been indicated as T helper (Th)1 [1,7], and Th17 [8–10], along with circulating IL-21 producing follicular helper T cell [11,12], with the limitation that only a restricted range of cytokines was determined in these studies. Response to antigen can also be measured using proliferation [13–15] and it has been shown that patients have an increased responsiveness to beta cell autoantigens in their antigen-experienced CD45RO$^+$ CD4$^+$ T cell compartment [16]. Here, we have combined proliferation and multi-parameter intracellular cytokine measurements in order to define the phenotype of beta cell autoantigen-targeting CD4$^+$ T cells that are found in type 1 diabetes.

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2. Methods

2.1. Subjects

Cytokine production by CD4$^+$ T cells was measured after short polyclonal stimulation and in antigen (proinsulin, GAD65, insulin, tetanus toxoid) responsive memory CD4$^+$ T cells after 5 days of stimulation. Each experimental setting compared patients with recent onset type 1 diabetes to age matched control individuals (Supplementary table S1). All methods were performed in accordance with relevant guidelines and regulations and all samples were obtained with informed consent as part of the DiMelli study [17] and the TeenDiab study [18]. Protocols were approved by the Ethikkommission der Bayerischen Landesärztekammer (No. 08043) and by the Ethikkommission der Fakultät für Medizin der Technischen Universität München (No. 2149/08).

2.2. Quantification of cytokine producing CD4$^+$ T cells

Frozen PBMCs were thawed with pre-warmed DMEM (4,5 g/l Glucose, w/o L-Glutamine; Lonza) supplemented with Benzonase (25 Units/ml; Novagen 99% purity) and viability was assessed by trypan blue staining. For the measurement of total CD4$^+$ T cell cytokine production in setting 1, 2×10^6 cells were polyclonally stimulated with PMA and ionomycin in the presence of Brefeldin A (leukocyte activation

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cocktail with GolgiPlug; final dilution 1:500; BD Bioscience) for 6 h. Samples were then incubated for additional 5 min in the presence of 40 μl DNase I (Final concentration 60.000 U/ml; Sigma-Aldrich) at 37 °C, 5% CO_2 and 95% humidity, washed in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS; Gibco) and stained for 30 min with UV Viability Dye eFluor455UV (eBioscience) on ice. For simultaneous staining of surface and intracellular markers, samples were permeabilized and blocked in 50 μl of PBS containing 0.1% (*w*/*v*) Saponin, 1% BSA, 0.05% NaN_3 and 5% (w/v) Nonfat dried milk powder (PBS-S milk buffer) for 2 h on ice. The following monoclonal antibodies were used: anti-CD3 Horizon V500 (clone UCHT1; BD Bioscience), anti-CD4 Qdot605 (clone S3.5; Life Technologies), anti-CD8 Brilliant Violet 785 (clone RPA-T8; BioLegend), anti-IFNγ APC-eFluor780 (clone 4S.B3; eBioscience), anti-IL-17A PE-Cy7 (clone eBio64DEC17; eBioscience), anti-IL-22 PE (clone 22URTI; eBioscience), anti-IL-2 Brilliant Violet 650 (clone MQ1-17H12; BioLegend), anti-IL-4 Alexa Fluor 488 (clone MP4-25D2; BioLegend), anti-TNFα Brilliant Violet 711 (clone Mab11; BioLegend), anti- GM-CSF PerCP-Cy5.5 (clone BVD2-21C11; BD Bioscience), anti-IL-21 Alexa Fluor 647 (clone 3A3-N2.1; BD Bioscience), and anti-CD45-RA APC-H7 (clone HI100; BD Bioscience). For intracellular cytokine staining, the following respective isotype control antibodies were used: APC-eFluor780 (Mouse; IgG1; κ; clone P3.6.2.8.1; eBioscience), PE-Cy7 (Mouse; IgG1; κ; clone MOPC-21; BioLegend), PE (Mouse; IgG1; κ; clone MOPC-21; BioLegend), Brilliant Violet 650 (Rat; IgG2a; κ; clone RTK2758; BioLegend), Alexa Fluor 488 (Rat; IgG1; κ; clone RTK2071; BioLegend), Brilliant Violet 711 (Mouse; IgG1; κ; clone MOPC-21; BioLegend), PerCP-Cy5.5 (Rat; IgG2a; κ; clone RTK2758; BioLegend) and Alexa Fluor 647 (Mouse; IgG1; κ; clone MOPC-21; BioLegend). Cells were stained for 30 min on ice and antibody staining was terminated by washing the cells in 2 ml PBS-Saponin buffer. All samples were directly acquired on a BD LSR-II and cytokine expression analyzed with FlowJo software (Version 10; TreeStar Inc.).

2.3. $CD4^+$ T cell proliferation and cytokine production

For the analysis of cytokine production in antigen-responsive $CD4^+$ T cells, thawed PBMCs were washed twice with PBS and labeled with Cell Proliferation Dye eFluor670 (5 μM in PBS for 10 min at 37 °C; eBioscience). Staining was terminated by adding RPMI 1640 containing 15% human serum AB at 4 °C. 2×10^5 eFluor670-labeled PBMCs were added to each well of a round-bottom 96-well microtiter plate and incubated in RPMI 1640 (Invitrogen) supplemented with 5% human serum AB (Invitrogen), 2 mM L-glutamine (Lonza) and 100 U/ml Penicillin/Streptomycin (Lonza) at 37 °C, 5% CO_2 and 95% humidity in the presence of proinsulin (10 μg/ml, Lilly), GAD65 (10 μg/ml; Diamyd Diagnostics), insulin (50 μg/ml; Lilly) or, as control antigen, tetanus toxoid (1 μl/ml; Novartis) for 5 days. Cells were subsequently stimulated with pre-diluted leukocyte activation cocktail with GolgiPlug (final dilution 1:500; BD Bioscience) for 5 h, harvested and stained using the following monoclonal antibodies: anti-CD4 Brilliant Violet 510 (clone SK3; BD Horizon) and anti-CD45RA PE-Cy5 (clone HI100; eBioscience). Cells were stained for 20 min at 4 °C in PBS containing 0.5% BSA, washed in PBS and stained for 15 min at room temperature with Zombie NIR (BioLegend) for cell viability. Cells were fixed and permeabilized for 20 min using Cytofix/Cytoperm buffer solution (BD Bioscience) and subsequently stained intracellularly in Perm/Wash buffer (BD Bioscience) with monoclonal anti-IL-10 Alexa Fluor 488 (clone JES3-9D7; BioLegend), anti-GM-CSF PerCP-Cy5.5 (clone BVD2-21C11; BioLegend), anti-IL-17A Brilliant Violet 605 (clone BL168; BioLegend), anti-IL-21 PE (clone 3A3-N2; eBioscience), anti-IL-22 eFluor450 (clone 22URTI; eBioscience) and anti-IFNγ PE-Cy7 (clone B27; BD Bioscience) for 20 min at room temperature. After washing, all samples were acquired within 24 h on a Becton Dickinson LSRFortessa flow cytometer and analyzed with FlowJo software (Version 10; TreeStar Inc.). For each stimulus at least two replicates were analyzed and responsiveness of $CD4^+CD45RA^-$ T cells was identified as proliferation dye decline in the presence of respective antigens. Proliferation to antigen is expressed as the proportion of dye-diluted $CD45RA^-CD4^+$ T cells after subtraction of values from medium alone control wells. Cytokine production in proliferating cells is expressed as the proportion of total $CD45RA^-CD4^+$ T cells that were both positive for the respective cytokine and had dye-diluted after subtraction of values from medium alone control wells.

2.4. Gene expression analysis

Gene expression of the single cell sorted $CD4^+CD45RA^-$ T cells was performed as previously described [19]. Raw data were analyzed using KNIME 2.9.4 [20] and R version 3.2.2 (Vienna). Ct values were pre-processed using a linear model to correct for potential confounding effects, which can mask biological variability, as previously described [21].

2.5. Statistical analysis

Comparisons between groups were made using the Mann-Whitney *U* test for polyclonally stimulated cytokine responses and Students *t*-test for cytokine profiles of antigen-responsive T cells. For all tests, a two-tailed *p*-value of <0.05 divided by the number of comparisons was considered to be significant. This corresponded to $p < 0.0083$ for individual cytokines of antigen responsive cells ($n = 6$ cytokines), and $p < 0.007$ for cytokine profiles of proinsulin-responsive ($n = 7$ cytokine profiles) or $p < 0.012$ for cytokine profiles of GAD65-responsive ($n = 4$ cytokine profiles). In order to identify cytokine profile patterns of proinsulin-responsive $CD4^+CD45RA^-$ T cells, we performed t-distributed Stochastic Neighbor Embedding (t-SNE) analysis [22] using Rtsne [23], where the input for each samples was its frequency for each of the 7 most abundant cytokine profiles observed. To model the bi-modal expression of single cells, the Hurdle model, a semi-continuous modeling framework, was applied to the pre-processed data [24]. This allowed us to assess the differential expression profiles with respect to the frequency of expression and the positive expression mean via a Likelihood-ratio test [25]. All analyses were performed using GraphPad Prism 4 (GraphPad), SPSS (IBM) or R Version 3.2.2 (Vienna).

3. Results

3.1. Cytokine profiles of $CD4^+$ T cells of patients at type 1 diabetes onset

Cytokine production by total $CD4^+$ T cells was quantified after short-term polyclonal activation with PMA and ionomycin (Fig. 1, Supplementary Fig. S1). IL-2^+ (median, 35.7%, IQR, 32.6% to 40.5%) and TNFα$^+$ cells (median, 19.5%, IQR, 15.5% to 21.8%) were the most abundant cytokine producing $CD4^+$ T cells. GM-CSF^+ $CD4^+$ T cells (median, 5.2%, IQR, 3.7% to 6.9%) were also observed and were as abundant as IFNγ$^+$ $CD4^+$ T cells (median, 5.0%, IQR, 3.2% to 6.5%). GM-CSF production by $CD4^+$ T cells was usually concomitant with IL-2 and/or TNFα production, but was also seen with other effector cytokines such as IFNγ (Fig. 1B). Cells producing IL-4 (median, 1.4%, IQR, 1.0% to 2.0%), IL-17A (median, 0.5%, IQR, 0.3% to 0.8%), IL-21 (median, 0.4%, IQR, 0.3% to 0.6%), or IL-22 (median, 0.6%, IQR, 0.4% to 0.8%) were relatively infrequent (Fig. 1A). No difference in $CD4^+$ T cell cytokine profiles (Fig. 1A and B) or cytokine production expressed as MFI of positive cells (Fig. 1C) was observed between patients at onset of T1D and age-matched control children. Similarly, no difference was observed in cytokine profiles when frequencies were expressed as a proportion of the total $CD45RO^+$ $CD4^+$ T cell population (Supplementary Fig. S2).

3.2. Cytokine profiles of antigen-responsive memory $CD4^+$ T cells

Cytokine profiles of antigen-responsive $CD4^+$ T cells were subsequently examined after 5-day stimulation with beta cell autoantigens (Fig. 2; Supplementary Fig. S3). Consistent with previous reports [13,15,16], patients had increased memory T cell responses to proinsulin

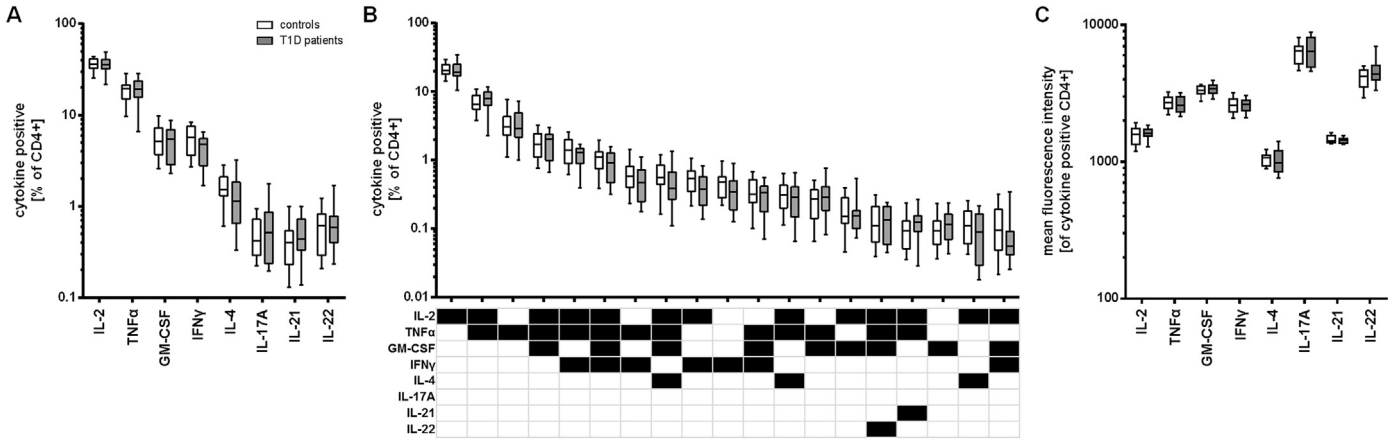

Fig. 1. Cytokine profiles of $CD4^+$ T cells. A) Frequency of cytokine producing $CD4^+$ T cells determined by intracellular cytokine staining after short-term stimulation with PMA and ionomycin in islet autoantibody-negative controls (open, $n = 23$) and patients at onset of type 1 diabetes (shaded; $n = 18$). B) Boolean analysis was used to identify the most dominant cytokine profiles characterized by exclusive or combinatory production of respective cytokines. C) Mean fluorescence intensity was used to reflect expression levels in cytokine positive $CD4^+$ T cells. Indicated in each boxplot are the median and the 10th–90th percentile of data. No differences were observed between groups (Mann-Whitney *U* test).

($p = 0.0002$), GAD65 ($p < 0.0001$) and insulin ($p = 0.0326$) as compared to controls when measured by $CD45RA^-CD4^+$ T cell proliferation (Fig.2; Supplementary Fig. S3). No differences between groups were observed in the proliferative response to tetanus toxoid (Supplementary Fig. S4).

Cytokine production in proinsulin-, GAD65-, and tetanus toxoid-responsive $CD4^+CD45RA^-$ T cells of all subjects was dominated by GM-CSF, IFNγ and IL-21 (Fig. 3; Supplementary Fig. S3), with less responsive cells positive for IL-17A or IL-22 ($p < 0.001$ for each antigen in each dataset). IL-10 producing antigen responsive $CD4^+CD45RA^-$ T cells were rare.

Patients had increased frequencies of proinsulin-responsive $CD4^+$ $CD45RA^-$ T cells producing GM-CSF ($p = 0.002$), IFNγ ($p = 0.004$), IL-17A ($p = 0.008$), and IL-22 ($p = 0.007$) as compared to control children. While increased frequencies of proinsulin-responsive $CD4^+$ $CD45RA^-$ T cells producing IL-21 ($p = 0.011$) was observed in patients as compared to controls, this was no longer significant when multiple testing ($n = 6$ cytokines) was considered. Patients also had increased frequencies of IL-21^+ ($p = 0.039$) GAD65-responsive $CD4^+CD45RA^-$ T cells (Fig. 3), which was no longer significant after considering multiple testing. No differences in cytokine positive tetanus-responsive $CD4^+CD45RA^-$ T cells were observed between patients at onset of type 1 diabetes and control children (Supplementary Fig. S4). Cytokine expression as described by mean fluorescence intensity (MFI) of cytokine positive cells did not differ between any of the groups (Supplementary Fig. S5). A relationship between proinsulin- and GAD65-responsive $CD4^+CD45RA^-$ T cells was observed for GM-CSF producing cells and IFNγ producing cells (Supplementary Fig. S6).

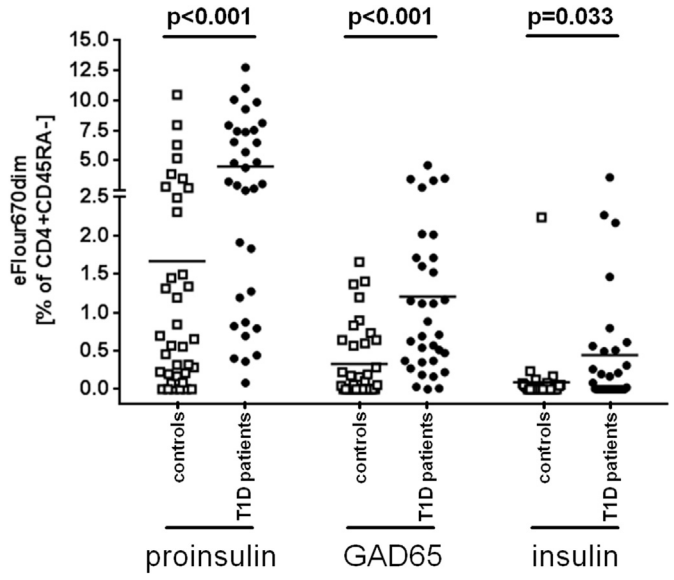

Fig. 2. Proliferation of memory $CD4^+$ T cells in presence of diabetes-related antigens. Antigen-responsive $CD4^+CD45RA^-$ T cells in islet autoantibody-negative children (open squares, $n = 36$), and patients with T1D (circles, $n = 33$) were identified via flow cytometry as a decrease in proliferation dye eFluor670 intensity. Frequencies of responsive $CD4^+CD45RA^-$ T cells in the presence of diabetes-related antigens proinsulin, GAD65 and insulin are expressed as the proportion of dye-diluted $CD45RA^-CD4^+$ T cells within total $CD45RA^-CD4^+$ T cells after subtraction of values from medium alone control wells (y axis). Indicated in each scatter plot is the mean and *p*-values. *P*-values were obtained using the Student's unpaired *t*-test.

Since we observed increased frequencies of GM-CSF, IFNγ, IL-17A, IL-21, and IL-22 secreting $CD4^+CD45RA^-$ T cell to proinsulin in patients at onset of T1D, we examined their combinations to determine which multi-parameter cytokine profiles were increased in patient proinsulin- and GAD65-responsive $CD4^+CD45RA^-$ T cells. Mean responses to proinsulin in patients were above 0.05% for 7 combinations among proinsulin-responsive T cells and for 4 combinations for GAD65-responsive $CD4^+CD45RA^-$ T cells (Fig. 4). Response profiles containing GM-CSF^+, IFNγ^+, and IL-21^+ $CD4^+CD45RA^-$ T cells were the most abundant for both antigens. For responses to proinsulin, 4 of the 7 profiles were increased in the patients as compared to controls. Of these, GM-CSF^+ only ($p = 0.006$) and IFNγ^+ only ($p = 0.0035$), responses remained significant after considering multiple testing ($n = 7$). The GM-CSF^+ only response was also the most abundant and increased ($p = 0.037$) in patients for GAD65-responsive $CD4^+CD45RA^-$ T cells. A multidimensional data analysis was performed on the 7 most abundant cytokine profiles of the proinsulin-responsive $CD4^+$ $CD45RA^-$ T cells for the 69 samples analyzed (Fig. 5). The tSNE analysis identified four potential clusters. Clusters 1 (green; $n = 18$ samples) and 2 (red; $n = 21$ samples) contained samples with little or no cytokine positive responsive cells. The majority (27 of 39; 69.2%) of samples in these clusters were from control individuals. Clusters 3 and 4 were dominated by samples from patients with type 1 diabetes (21 of 30; 70%; Fig. 5B). A feature of samples in clusters 3 and 4 was the presence of multiple cytokine profiles in their proinsulin responses. Prominent profiles that were only seen in the patient samples in cluster 3 included IFNγ^+ only phenotype, while cluster 4 included both GM-CSF^+ only and GM-CSF^+ IFNγ^+ combinatory phenotypes (Fig. 5C).

In order to further characterize antigen-responsive $CD4^+$ T cells that express GM-CSF, proinsulin-responsive $CD4^+CD45RA^-$ T cells ($n =$

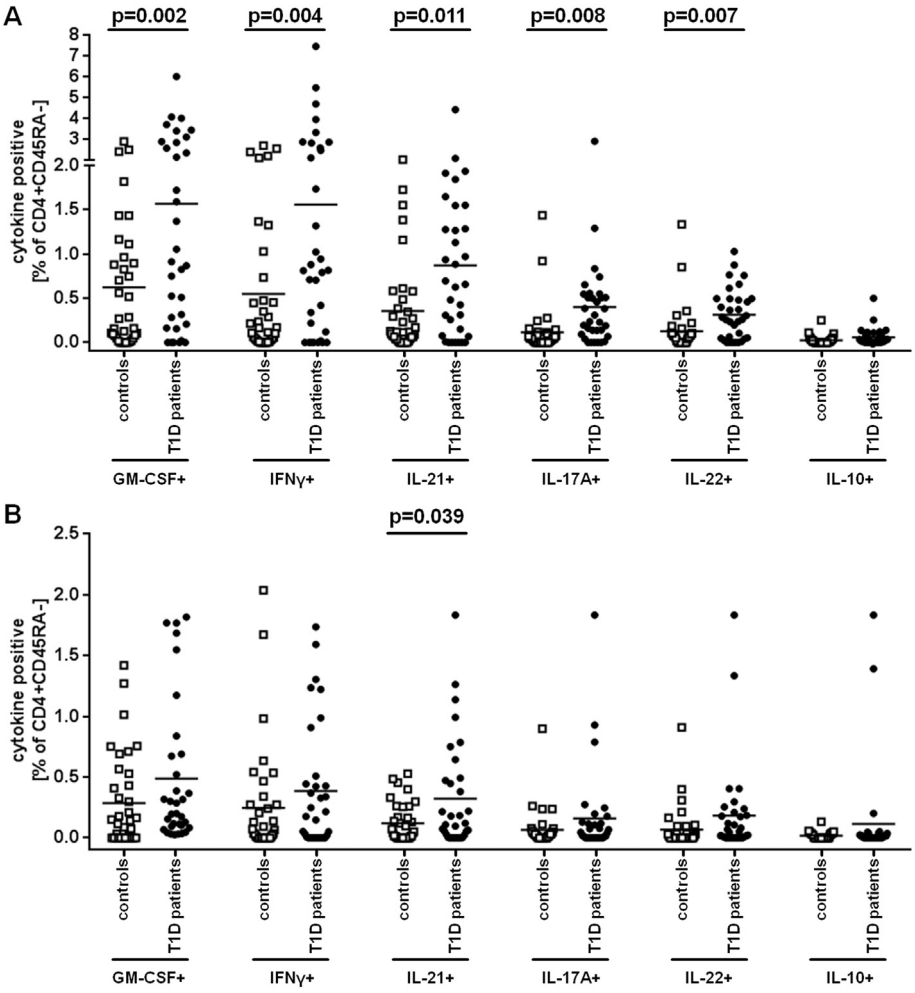

Fig. 3. Production of cytokines by antigen-responsive memory $CD4^+$ T cells. Flow cytometry identification of cytokine production in $CD4^+CD45RA^-$ T cells responsive to A) proinsulin, and B) GAD65 in islet autoantibody-negative control children (open squares, n = 36), and patients with T1D (circles, n = 33). Cytokine production in proliferating cells is expressed as the proportion of total $CD45RA^-CD4^+$ T cells that were both positive for the respective cytokine and had dye-diluted after subtraction of values from medium alone control wells (y axis). Indicated in each scatter plot is the mean and p-values. P-values were obtained using Student's unpaired *t*-test.

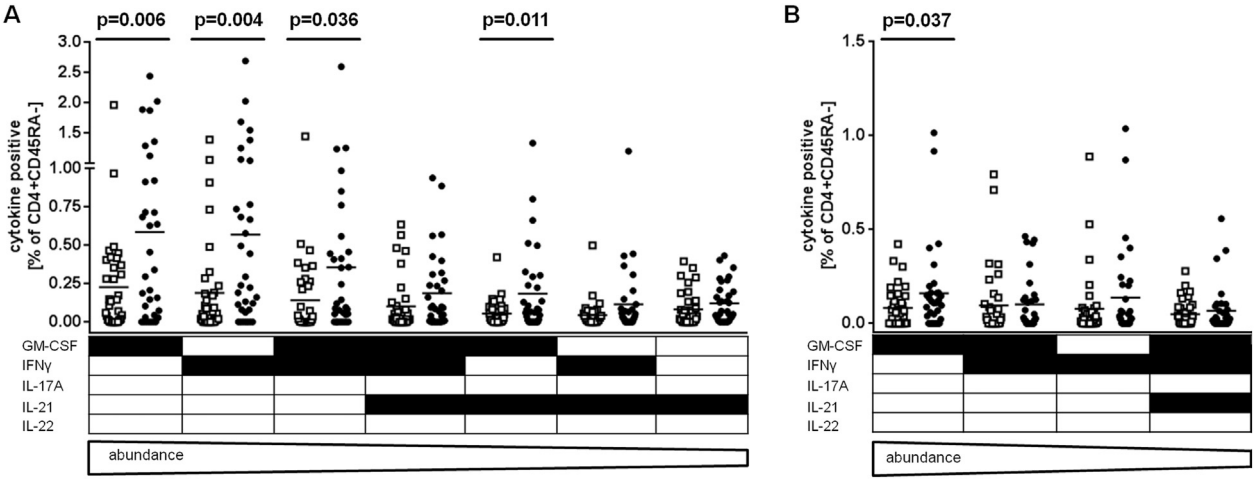

Fig. 4. Multi-parameter cytokine signature of beta cell antigen-responsive memory $CD4^+$ T cells. Boolean analysis was used to identify the most dominant cytokine profiles of A) proinsulin- or B) GAD65-responsive $CD4^+CD45RA^-$ T cells characterized by exclusive or combinatory production of respective cytokines. The frequency of antigen-responsive $CD4^+CD45RA^-$ T cells with a particular cytokine combination after subtraction of frequencies in the medium wells is shown on the y axis for islet autoantibody-negative children (open squares, n = 36) and patients with T1D (circles, n = 33). Abundance order was determined from the median rank of the cytokine profile of responsive cells for each of the samples tested. The combinations from most abundant (left) to least abundant (right) are shown for combinations where the mean response was >0.05% in the patients (7 combinations for proinsulin-responsive cells and 4 combinations for GAD65-responsive cells). Indicated in each scatter plot is the mean and p-values. P-values were obtained using Student's unpaired *t*-test.

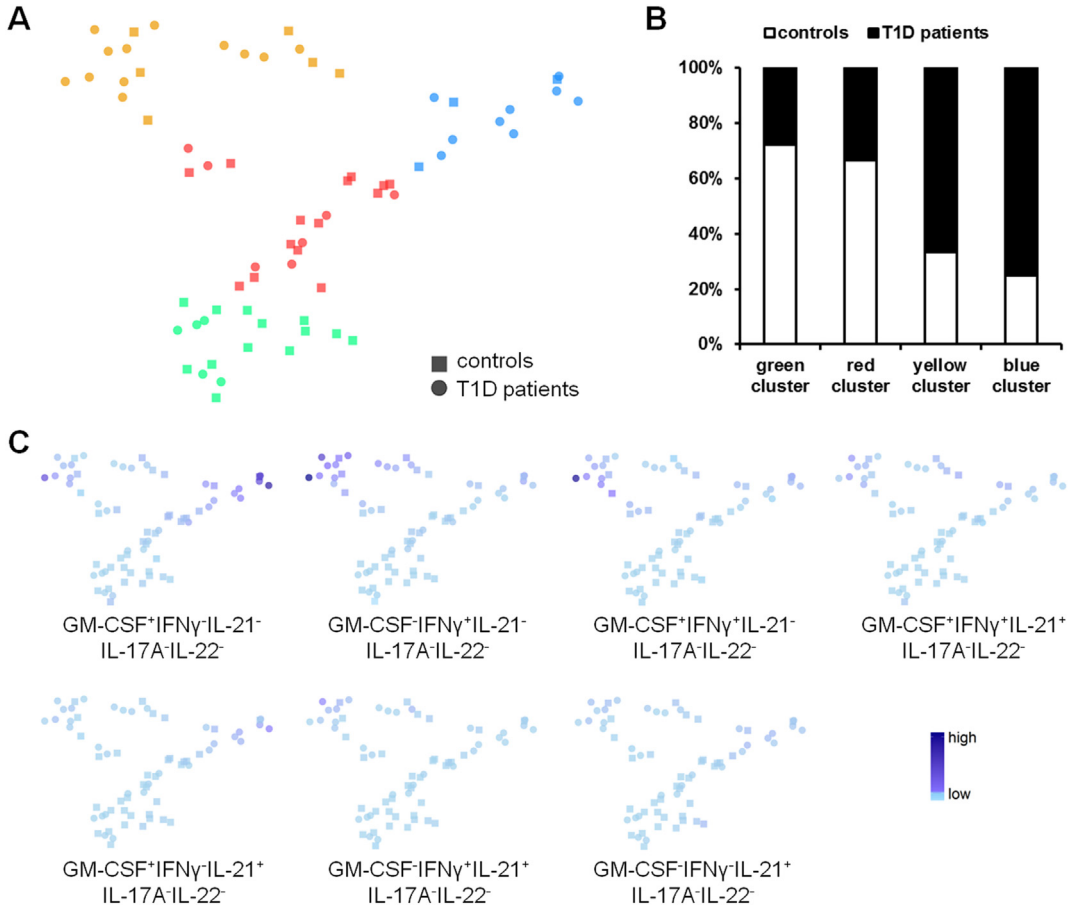

Fig. 5. tSNE analysis of proinsulin-responsive memory $CD4^+$ T cell cytokine profiles. For each sample the frequency of the 7 most abundant cytokine profiles observed in proinsulin-responsive $CD4^+CD45RA^-$ T cells were analyzed using tSNE analysis. A) tSNE visualized four major clusters, defined as clusters 1 (green, 18 samples), 2 (red, 21 samples), 3 (yellow, 18 samples), and 4 (blue, 12 samples) in samples from controls (squares) and patients with type 1 diabetes (circles). B) Distribution of patients with T1D (green cluster, $n = 5$; red cluster, $n = 7$; yellow cluster, $n = 12$; blue cluster, $n = 9$) and islet autoantibody-negative control children (green cluster, $n = 13$; red cluster, $n = 14$; yellow cluster, $n = 6$; blue cluster, n = 3) among different clusters. C) Expression intensities (light blue, negative; dark blue, highest frequency) of each of the 7 most abundant cytokine profiles observed in proinsulin-responsive $CD4^+CD45RA^-$ T cells.

104) were isolated from three patients for single cell gene expression analysis. *GMCSF* gene expression was observed in 19 (18%) cells, of which 10 expressed *IFNG* ($n = 9$) or *IL21* ($n = 6$; Fig. 6A). As compared to cells that were *GMCSF* negative, the cells expressing the *GMCSF* gene had lower expression of *FOXP3*, *CD27*, *CD25*, *CCR7*, and *CD137*, and increased expression of *CD154* (all $p < 0.05$, Fig. 6B).

4. Discussion

Immune mediated diseases are often classified by cytokine bias in innate or adaptive immune responses. We report cytokine profiles of islet autoantigen-responsive $CD4^+$ T cells in type 1 diabetes and show that the cells have profiles dominated by GM-CSF, IFNγ, and/or IL-21, but also IL-17A and IL-22. Patients had increased memory $CD4^+$ T cell responses to proinsulin and to GAD65 as compared to controls, and the increase for proinsulin-responsive and GAD65-responsive $CD4^+CD45RA^-$ T cells was consistently seen within the GM-CSF^+ producing cells, and in cells with a GM-CSF^+ $IFN\gamma^-$ $IL17A^-$ $IL\text{-}21^-$ $IL\text{-}22^-$ phenotype. The findings are consistent with a pro-inflammatory Th1/Th17 adaptive T cell response to beta cell autoantigens in type 1 diabetes and introduce GM-CSF-producing $CD4^+$ T cells as a new entity in the pathogenesis of type 1 diabetes.

Unlike previous reports, the study used the combination of proliferation and multi-parameter cytokine production to measure responses to beta cell autoantigens. This provided the opportunity to assess cytokine combinations of responsive $CD4^+$ T cells. We also focused on the $CD4^+CD45RA^-$ T cell population as this contains antigen-experienced cells. A limitation of the assay is that the proliferating responding cells will include bystander responders. Moreover, we did not use $CD45RO^+$ enriched cells for the assays, and although our experience indicates that CD45RA is not lost from naïve T cells that respond to antigen over the 5 day culture, it remains possible that some of the responding $CD45RA^-$ cells were naïve $CD4^+$ T cells prior to in vitro stimulation with antigen. It is also possible that the responses to proinsulin and insulin observed in the patients were in part promoted by insulin injection. However, it is notable that, an increased GM-CSF^+ $CD4^+CD45RA^-$ T cell response was observed in patients for both GAD65 and proinsulin. Finally, a large proportion of cells that proliferated in the presence of antigen were negative for the cytokines measured. In view of the findings for polyclonally stimulated total $CD4^+$ T cells, it is likely that many of these cells are IL-2 and/or TNFα producing $CD4^+$ T cells, and the inclusion of these cytokines should be considered for future panels.

IFNγ, IL-21, IL-17A and IL-22 have been reported as type 1 diabetes-relevant effector molecules [1,10,11,26]. Our findings suggest that autoantigen-responsive cells in type 1 diabetes produce these cytokines. A novel finding is that GM-CSF producing cells are also prominent in the response. GM-CSF producing $CD4^+$ T cells are an interesting recently described subset [27]. They have both a Th1 and a Th17 association, and include cells from both of these subsets [28]. They are also increased in autoimmune and inflammatory diseases including multiple sclerosis [29], and synovial $CD4^+$ T cells are an abundant source of GM-CSF that promotes the differentiation of inflammatory dendritic cells in

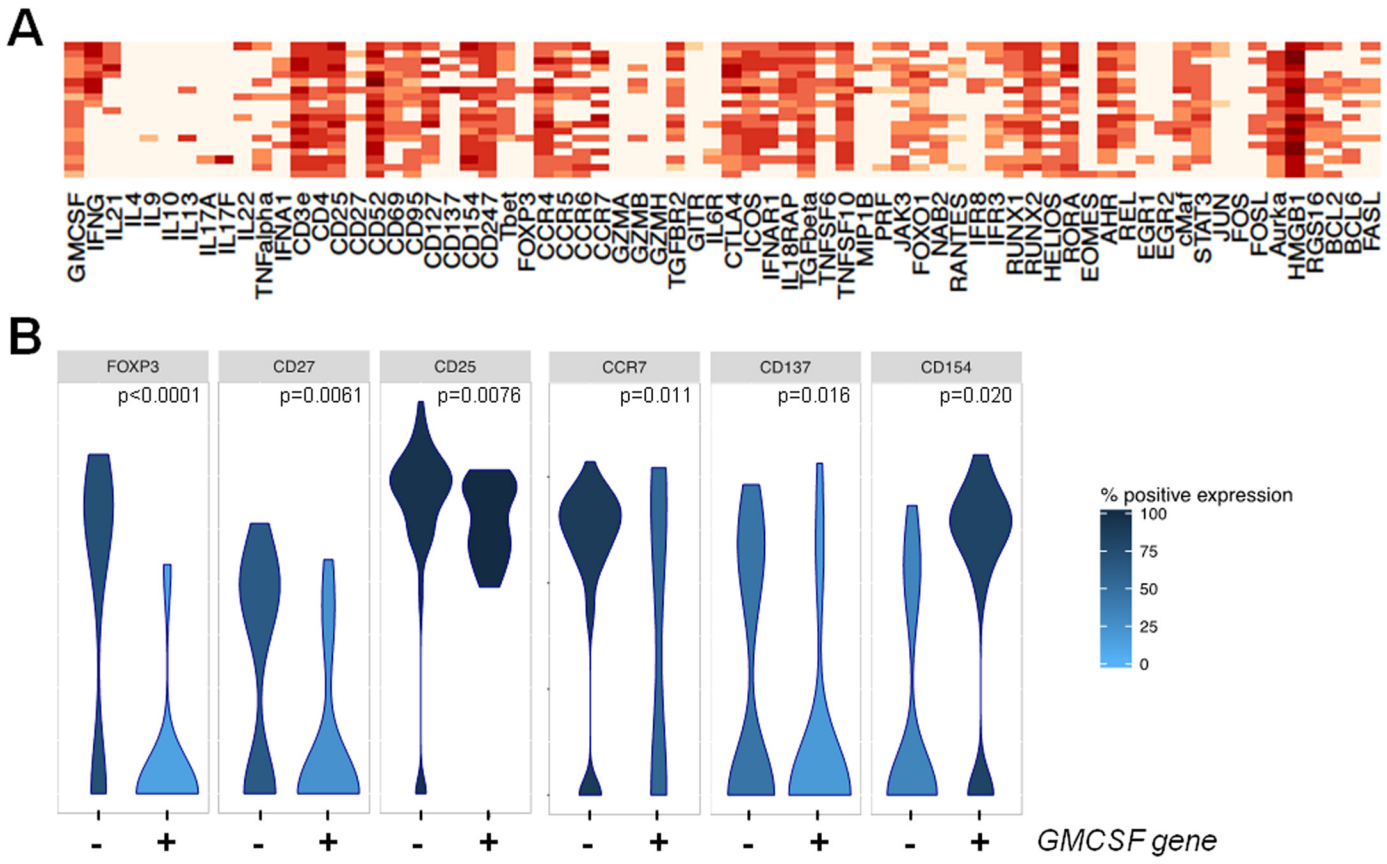

Fig. 6. Gene transcription profiles of proinsulin-responsive CD4$^+$CD45RA$^-$ T cells. Proinsulin-responsive CD4$^+$CD45RA$^-$ T cells from three patients with type 1 diabetes were single cell-sorted and processed for multi-parameter gene expression. Of 104 cells processed, 19 cells had *GMCSF* gene expression. A) The gene expression profiles of these 19 cells are shown as a heatmap. B) Violin plots representing the distribution of Ct expression values for each gene showing significance at $p < 0.0005$ (Likelihood-ratio test) in cells with (+) or without (−; $n = 85$) *GMCSF* gene expression. The proportion of cells expressing the gene ranged from light blue (0%) to dark blue (100%).

patients with rheumatoid arthritis [30]. T cell derived GM-CSF also has a non-redundant role in EAE [31,32]. Hence, our finding of increased GM-CSF$^+$ IL-17A$^+$ CD4$^+$ T cells that respond to beta cell autoantigen is of particular interest. In mice, GM-CSF production by Th17 cells is promoted by IL-23 [28] and potentially also IL-1β [33]. However, in man, IL-23 appears to be inhibitory for GM-SCF production in T cells, while IL-12 may enhance production [34]. STAT5 is critical in the differentiation of naïve CD4$^+$ T cells to GM-CSF producing CD4$^+$ T cells [34]. STAT5 is used in IL-2 and IL-7 signaling, and both pathways are reported to be relevant to autoimmune diabetes in mouse [35,36] and man [37,38]. It has been repeatedly demonstrated, that GM-CSF plays an important and critical role in various autoimmune diseases by triggering differentiation of pathogenic Th17 cells [39]. Our novel findings of an activated and non-regulatory phenotype of proinsulin-responsive, GM-CSF-producing CD4$^+$ T cells in T1D patients support this role. GM-CSF producing CD4$^+$ T cells represented around 25% of the memory CD4$^+$ T cell component in children and adolescents (Supplementary Fig. S2). Of these GM-CSF$^+$ CD4$^+$ T cells, the IFNγ$^+$ T cells are the most abundant effector cytokine phenotype (median, 32.2%, range, 18.7% to 47.6%). We did not observe a general increase in GM-CSF producing cells in patients with type 1 diabetes. Moreover, we did not see an increase in any of the cytokine producing cell subsets in the patients, including IL-21 producing CD4$^+$ T cells, which were previously reported to be increased in adult patients with T1D [11,12]. Our observation of an increase in IL-21$^+$ autoantigen-responsive CD4$^+$ T cells in patients, however, supports a role for IL-21 in the disease.

In conclusion, we have developed a multi-parameter cytokine and proliferation response assay for the detection of antigen-responsive CD4$^+$ T cells and have found that T cells responsive to beta cell autoantigens include a GM-CSF producing component and that patients with type 1 diabetes have increased frequencies of GM-CSF$^+$ IFNγ$^-$ IL-17A$^-$ IL-21$^-$ IL-22$^-$ proinsulin- and GAD65-responsive CD4$^+$ CD45RA$^-$ T cells. These findings raise the possibility of therapeutically targeting T cell GM-CSF production in type 1 diabetes.

Author contributions

J.K., A.-G.Z., and E.B. contributed to the conduct of the study and the acquisition, analysis, interpretation of data, and drafted, reviewed and approved the manuscript. A.G., D.K., J.R., M.H., M.H., A.E. and C.O. contributed to the acquisition, analysis and interpretation of data, and reviewed and approved the manuscript. E.B. is the guarantor of this work.

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Conflict of interest declaration

The authors declare no conflict of interest.

Competing financial interests

None of the authors has declared potential financial conflict of interest related to this manuscript.

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Appendix A. Supplementary data

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References

[1] S. Arif, T.I. Tree, T.P. Astill, J.M. Tremble, A.J. Bishop, C.M. Dayan, B.O. Roep, M. Peakman, Autoreactive T Cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health, J. Clin. Invest. 113 (2004) 451–463, https://doi.org/10.1172/JCI19585.
[2] M. Hedman, M. Faresjo, S. Axelsson, J. Ludvigsson, R. Casas, Impaired CD4 and CD8 T cell phenotype and reduced chemokine secretion in recent-onset type 1 diabetic children, Clin. Exp. Immunol. 153 (2008) 360–368, https://doi.org/10.1111/j.1365-2249.2008.03720.x.
[3] B.O. Roep, M. Peakman, Antigen targets of type 1 diabetes autoimmunity, Cold Spring Harb. Perspect. Med. 2 (2012)https://doi.org/10.1101/cshperspect.a007781.
[4] K.T. Coppieters, F. Dotta, N. Amirian, P.D. Campbell, T.W. Kay, M.A. Atkinson, B.O. Roep, M.G. von Herrath, Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients, J. Exp. Med. 209 (2012) 51–60, https://doi.org/10.1084/jem.20111187.
[5] V. Pathiraja, J.P. Kuehlich, P.D. Campbell, B. Krishnamurthy, T. Loudovaris, P.T. Coates, T.C. Brodnicki, P.J. O'Connell, K. Kedzierska, C. Rodda, P. Bergman, E. Hill, A.W. Purcell, N.L. Dudek, H.E. Thomas, T.W. Kay, S.I. Mannering, Proinsulin-specific, HLA-DQ8, and HLA-DQ8-transdimer-restricted CD4+ T cells infiltrate islets in type 1 diabetes, Diabetes 64 (2015) 172–180, https://doi.org/10.2337/db14-0858.
[6] J.A. Babon, M.E. DeNicola, D.M. Blodgett, I. Crevecoeur, T.S. Buttrick, R. Maehr, R. Bottino, A. Naji, J. Kaddis, W. Elyaman, E.A. James, R. Haliyur, M. Brissova, L. Overbergh, C. Mathieu, T. Delong, K. Haskins, A. Pugliese, M. Campbell-Thompson, C. Mathews, M.A. Atkinson, A.C. Powers, D.M. Harlan, S.C. Kent, Analysis of self-antigen specificity of islet-infiltrating T cells from human donors with type 1 diabetes, Nat. Med. 22 (2016) 1482–1487, https://doi.org/10.1038/nm.4203.
[7] L.G. Petrich de Marquesini, J. Fu, K.J. Connor, A.J. Bishop, N.E. McLintock, C. Pope, F.S. Wong, C.M. Dayan, IFN-gamma and IL-10 islet-antigen-specific T cell responses in autoantibody-negative first-degree relatives of patients with type 1 diabetes, Diabetologia 53 (2010) 1451–1460, https://doi.org/10.1007/s00125-010-1739-3.
[8] K. Alnek, K. Kisand, K. Heilman, A. Peet, K. Varik, R. Uibo, Increased blood levels of growth factors, Proinflammatory cytokines, and Th17 cytokines in patients with newly diagnosed type 1 diabetes, PLoS One 10 (2015)https://doi.org/10.1371/journal.pone.0142976.
[9] A.K. Marwaha, S.Q. Crome, C. Panagiotopoulos, K.B. Berg, H. Qin, Q. Ouyang, L. Xu, J.J. Priatel, M.K. Levings, R. Tan, Cutting edge: increased IL-17-secreting T cells in children with new-onset type 1 diabetes, J. Immunol. 185 (2010) 3814–3818, https://doi.org/10.4049/jimmunol.1001860.
[10] J. Honkanen, J.K. Nieminen, R. Gao, K. Luopajarvi, H.M. Salo, J. Ilonen, M. Knip, T. Otonkoski, O. Vaarala, IL-17 immunity in human type 1 diabetes, J. Immunol. 185 (2010) 1959–1967, https://doi.org/10.4049/jimmunol.1000788.
[11] R. Kenefeck, C.J. Wang, T. Kapadi, L. Wardzinski, K. Attridge, L.E. Clough, F. Heuts, A. Kogimtzis, S. Patel, M. Rosenthal, M. Ono, D.M. Sansom, P. Narendran, L.S. Walker, Follicular helper T cell signature in type 1 diabetes, J. Clin. Invest. 125 (2015) 292–303, https://doi.org/10.1172/JCI76238.
[12] R.C. Ferreira, H.Z. Simons, W.S. Thompson, A.J. Cutler, X.C. Dopico, D.J. Smyth, M. Mashar, H. Schuilenburg, N.M. Walker, D.B. Dunger, C. Wallace, J.A. Todd, L.S. Wicker, M.L. Pekalski, IL-21 production by CD4+ effector T cells and frequency of circulating follicular helper T cells are increased in type 1 diabetes patients, Diabetologia 58 (2015) 781–790, https://doi.org/10.1007/s00125-015-3509-8.
[13] S.I. Mannering, J.S. Morris, N.L. Stone, K.P. Jensen, V.A.N.E. PM, L.C. Harrison, CD4+ T cell proliferation in response to GAD and proinsulin in healthy, pre-diabetic, and diabetic donors, Ann. N. Y. Acad. Sci. 1037 (2004) 16–21, https://doi.org/10.1196/annals.1337.003.
[14] S.I. Mannering, F.S. Wong, I. Durinovic-Bello, B. Brooks-Worrell, T.I. Tree, C.M. Cilio, N.C. Schloot, R. Mallone, T.C.W.C. ImmunologyOf diabetes society, Current approaches to measuring human islet-antigen specific T cell function in type 1 diabetes, Clin. Exp. Immunol. 162 (2010) 197–209, https://doi.org/10.1111/j.1365-2249.2010.04237.x.
[15] T.I. Tree, G. Duinkerken, S. Willemen, R.R. de Vries, B.O. Roep, HLA-DQ-regulated T-cell responses to islet cell autoantigens insulin and GAD65, Diabetes 53 (2004) 1692–1699, https://doi.org/10.2337/diabetes.53.7.1692.
[16] P. Monti, M. Scirpoli, A. Rigamonti, A. Mayr, A. Jaeger, R. Bonfanti, G. Chiumello, A.G. Ziegler, E. Bonifacio, Evidence for in vivo primed and expanded autoreactive T cells as a specific feature of patients with type 1 diabetes, J. Immunol. 179 (2007) 5785–5792, https://doi.org/10.4049/jimmunol.179.9.5785.
[17] L. Thuemer, K. Adler, E. Bonifacio, F. Hofmann, M. Keller, C. Milz, A. Munte, A.G. Ziegler, German new onset diabetes in the young incident cohort study: DiMelli study design and first-year results, Rev. Diabet. Stud. 7 (2010) 202–208, https://doi.org/10.1900/RDS.2010.7.202.
[18] A.G. Ziegler, F. Meier-Stiegen, C. Winkler, E. Bonifacio, T.S. Group, Prospective evaluation of risk factors for the development of islet autoimmunity and type 1 diabetes during puberty—TEENDIAB: study design, Pediatr. Diabetes 13 (2012) 419–424, https://doi.org/10.1111/j.1399-5448.2011.00763.x.
[19] A.K. Heninger, A. Eugster, D. Kuehn, F. Buettner, M. Kuhn, A. Lindner, S. Dietz, S. Jergens, C. Wilhelm, A. Beyerlein, A.G. Ziegler, E. Bonifacio, A divergent population of autoantigen-responsive CD4+ T cells in infants prior to beta cell autoimmunity, Sci. Transl. Med. 9 (2017)https://doi.org/10.1126/scitranslmed.aaf8848.
[20] M.R. Berthold, N. Cebron, F. Dill, T.R. Gabriel, T. Kötter, T. Meinl, P. Ohl, C. Sieb, K. Thiel, B. Wiswedel, KNIME: The Konstanz information miner, in: C. Preisach, H. Burkhardt, L. Schmidt-Thieme, R. Decker (Eds.), Data Analysis, Machine Learning and Applications, Springer, Berlin 2007, pp. 319–326.
[21] F. Buettner, K.N. Natarajan, F.P. Casale, V. Proserpio, A. Scialdone, F.J. Theis, S.A. Teichmann, J.C. Marioni, O. Stegle, Computational analysis of cell-to-cell heterogeneity in single-cell RNA-sequencing data reveals hidden subpopulations of cells, Nat. Biotechnol. 33 (2015) 155–160, https://doi.org/10.1038/nbt.3102.
[22] G. Finak, A. McDavid, M. Yajima, J. Deng, V. Gersuk, A.K. Shalek, C.K. Slichter, H.W. Miller, M.J. McElrath, M. Prlic, P.S. Linsley, R. Gottardo, MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data, Genome Biol. 16 (2015)https://doi.org/10.1186/s13059-015-0844-5.
[23] F. Pedregosa, G. Olivier, M. Blondel, P. Prettenhofer, R. Weiss, V. Dubourg, J. Vanderplas, A. Passos, D. Cournapeau, M. Brucher, M. Perrot, E. Duchesnay, Scikit-learn: machine learning in python, J. Mach. Learn. Res. 12 (2011) 2825–2830.
[24] A. McDavid, G. Finak, M. Yajima, MAST: Model-based Analysis of Single Cell Transcriptomics. R Package Version 0.931, 2015.
[25] A. McDavid, L. Dennis, P. Danaher, G. Finak, M. Krouse, A. Wang, P. Webster, J. Beechem, R. Gottardo, Modeling bi-modality improves characterization of cell cycle on gene expression in single cells, PLoS Comput. Biol. 10 (2014)https://doi.org/10.1371/journal.pcbi.1003696.
[26] L.S. Walker, M. von Herrath, CD4 T cell differentiation in type 1 diabetes, Clin. Exp. Immunol. 183 (2016) 16–29, https://doi.org/10.1111/cei.12672.
[27] W. Sheng, T cell-derived GM-CSF, regulation of expression and function, Immun. Res. 11 (2015)https://doi.org/10.4172/1745-7580.1000098.
[28] L. Codarri, G. Gyulveszi, V. Tosevski, L. Hesske, A. Fontana, L. Magnenat, T. Suter, B. Becher, RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation, Nat. Immunol. 12 (2011) 560–567, https://doi.org/10.1038/ni.2027.
[29] J. Rasouli, B. Ciric, J. Imitola, P. Gonnella, D. Hwang, K. Mahajan, E.R. Mari, F. Safavi, T.P. Leist, G.X. Zhang, A. Rostami, Expression of GM-CSF in T cells is increased in multiple sclerosis and suppressed by IFN-beta therapy, J. Immunol. 194 (2015) 5085–5093, https://doi.org/10.4049/jimmunol.1403243.
[30] G. Reynolds, J.R. Gibbon, A.G. Pratt, M.J. Wood, D. Coady, G. Raftery, A.R. Lorenzi, A. Gray, A. Filer, C.D. Buckley, M.A. Haniffa, J.D. Isaacs, C.M. Hilkens, Synovial CD4+ T-cell-derived GM-CSF supports the differentiation of an inflammatory dendritic cell population in rheumatoid arthritis, Ann. Rheum. Dis. 75 (2016) 899–907, https://doi.org/10.1136/annrheumdis-2014-206578.
[31] E.D. Ponomarev, L.P. Shriver, K. Maresz, J. Pedras-Vasconcelos, D. Verthelyi, B.N. Dittel, GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis, J. Immunol. 178 (2006) 39–48, https://doi.org/10.4049/jimmunol.178.1.39.
[32] H.M. Grifka-Walk, D.A. Giles, B.M. Segal, IL-12-polarized Th1 cells produce GM-CSF and induce EAE independent of IL-23, Eur. J. Immunol. 45 (2015) 2780–2786, https://doi.org/10.1002/eji.201545800.
[33] M. El-Behi, B. Ciric, H. Dai, Y. Yan, M. Cullimore, F. Safavi, G.X. Zhang, B.N. Dittel, A. Rostami, The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF, Nat. Immunol. 12 (2011) 568–575, https://doi.org/10.1038/ni.2031.
[34] R. Noster, R. Riedel, M.F. Mashreghi, H. Radbruch, L. Harms, C. Haftmann, H.D. Chang, A. Radbruch, C.E. Zielinski, IL-17 and GM-CSF expression are antagonistically regulated by human T helper cells, Sci. Transl. Med. 6 (2014)https://doi.org/10.1126/scitranslmed.3008706.
[35] Y. Grinberg-Bleyer, A. Baeyens, S. You, R. Elhage, G. Fourcade, S. Gregoire, N. Cagnard, W. Carpentier, Q. Tang, J. Bluestone, L. Chatenoud, D. Klatzmann, B.L. Salomon, E. Piaggio, IL-2 reverses established type 1 diabetes in NOD mice by a

local effect on pancreatic regulatory T cells, J. Exp. Med. 207 (2010) 1871–1878, https://doi.org/10.1084/jem.20100209.
[36] C. Penaranda, W. Kuswanto, J. Hofmann, R. Kenefeck, P. Narendran, L.S. Walker, J.A. Bluestone, A.K. Abbas, H. Dooms, IL-7 receptor blockade reverses autoimmune diabetes by promoting inhibition of effector/memory T cells, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 12668–12673, https://doi.org/10.1073/pnas.1203692109.
[37] M.A. Hulme, C.H. Wasserfall, M.A. Atkinson, T.M. Brusko, Central role for interleukin-2 in type 1 diabetes, Diabetes 61 (2012) 14–22, https://doi.org/10.2337/db11-1213.
[38] M. Evangelou, D.J. Smyth, M.D. Fortune, O.S. Burren, N.M. Walker, H. Guo, S. Onengut-Gumuscu, W.M. Chen, P. Concannon, S.S. Rich, J.A. Todd, C. Wallace, A method for gene-based pathway analysis using genomewide association study summary statistics reveals nine new type 1 diabetes associations, Genet. Epidemiol. 38 (2014) 661–670, https://doi.org/10.1002/gepi.21853.
[39] A. Shiomi, T. Usui, T. Mimori, GM-CSF as a therapeutic target in autoimmune diseases, Inflamm. Regen. 36 (2016)https://doi.org/10.1186/s41232-016-0014-5.