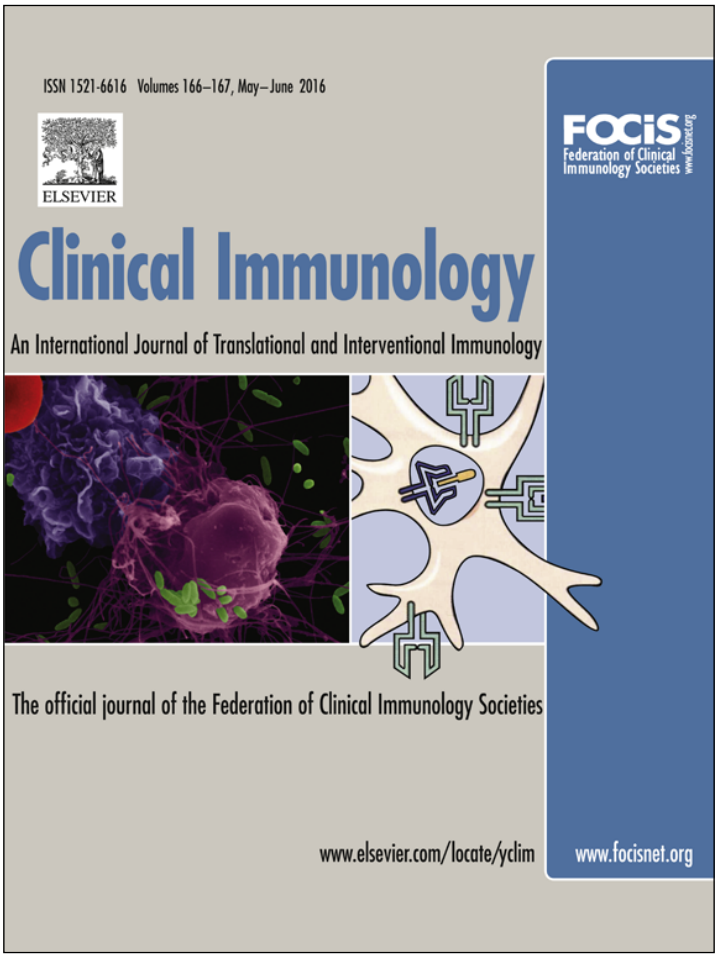

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A pilot study showing associations between frequency of CD4$^+$ memory cell subsets at diagnosis and duration of partial remission in type 1 diabetes

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ABSTRACT

In some patients with type 1 diabetes the dose of insulin required to achieve euglycemia is substantially reduced soon after diagnosis. This partial remission is associated with β-cell function and good glucose control. The purpose of this study was to assess whether frequencies of CD4$^+$ T cell subsets in children newly diagnosed with type 1 diabetes are associated with length of partial remission. We found that the frequency of CD4$^+$ memory cells, activated Treg cells and CD25$^+$ cells that express a high density of the IL-7 receptor, CD127 (CD127^{hi}) are strongly associated with length of partial remission. Prediction of length of remission via Cox regression is significantly enhanced when CD25$^+$ CD127^{hi} cell frequency is combined with either Insulin Dependent Adjusted A1c (IDAA1c), or glycosylated hemoglobin (HbA1c), or C-peptide levels at diagnosis. CD25$^+$ CD127^{hi} cells do not express Foxp3, LAG-3 and CD49b, indicating that they are neither Treg nor Tr1 cells.

1. Introduction

Type 1 diabetes is a progressive heterogeneous autoimmune disease caused by the destruction of insulin secreting β-cells by T cells. Most patients have a period of improved glucose control soon after diagnosis. The partial remission period is the period during which patients still respond to low levels of insulin (<0.5 U/Kg body weight) to achieve euglycemia [1–4]. This partial remission period, also termed the honeymoon period, is variable, lasting from several weeks to over a year [5–6].

Clinical studies suggest that immunotherapy is most effective if started early post-diagnosis [7–10] at a time when patients generally have greater residual β-cell mass. This might be explained if therapeutic intervention prevents or slows down the ongoing destruction of the remaining β-cells, or enhances ongoing mechanisms to improve β-cell mass and inhibit autoimmunity. β-cell mass retention results in good glucose control that can in turn further reduce β-cell damage by limiting glucose toxicity. It is reasonable then to suggest that patients with a prolonged period of improved glucose control and partial remission might be particularly responsive to therapy. However, to date there is no way to predict which newly diagnosed patients will go on to have a long or short period of partial remission. This pilot study was designed to test whether the immune cell profile at diagnosis correlates with length of partial remission in newly diagnosed patients with type 1 diabetes.

The immune cell subsets measured include CD4$^+$ naïve, memory and regulatory (Treg) cell subsets, and CD25$^+$ CD127^{hi} cells. To measure partial remission both insulin dose and HbA1c are combined in a standard formula to provide a single value, the Insulin Dose Adjusted A1c (IDAA1c). The IDAA1c value reflects the quality of glucose control. An IDAA1c value equal to or <9 is given to indicate partial remission [11].

Our data show that of the cell subsets tested, the relative frequency of CD4$^+$ memory cells, activated Treg cells and CD4$^+$ CD25$^+$ CD127^{hi} cells are most significantly associated with length of partial remission. Notably, both activated Treg cells and CD25$^+$ CD127^{hi} cells are CD4$^+$ memory cells. The predictive value of CD25$^+$ CD127^{hi} cells, but not other cell subsets, is strongly enhanced when combined with either the HbA1c, IDAA1c or C-peptide levels at diagnosis. CD25$^+$ CD127^{hi} cells do not express Foxp3, the transcription factor for Treg cells [14–17], neither do they express LAG-3 and CD49b, markers that identify Tr1 regulatory cells [23], indicating that they are neither Treg nor Tr1 cells. However, CD25$^+$ CD127^{hi} cells express a high density of

Abbreviations: T1D, type 1 diabetes; BGL, blood glucose level; HbA1c, glycosylated hemoglobin; IDAA1c, insulin dose adjusted A1c; Treg, Foxp3$^+$ regulatory T cell; PBMC, peripheral blood mononuclear cells; IRB, Institutional Review Board; mAb, monoclonal antibody; AUC, area under the curve; HA, hyaluronan; MFI, mean fluorescence intensity; CM, central memory; TM, transitional memory; EM, effector memory; EMRA, CD45RA$^+$ effector memory.

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CD44 and the CD44 variant v6, signaling through which promotes expression of Foxp3, IL-2, TGF-β and IL-10 [12–13] all critical requirements for the development and function of Treg [14–21] and Tr1 [22]. These data strongly suggest that the relative frequency of $CD4^+$ memory cell subsets that are associated with immune regulation can predict length of remission in patients with T1D, and warrant further investigation in a validation study with a large cohort of patients.

2. Materials and methods

2.1. Patient population

Archived peripheral blood mononuclear cells (PBMC) from 9 female and 10 male newly diagnosed type 1 diabetic patients were obtained from TrialNet Ancillary Studies. Patients were between 9 and 16 years of age and enrolled in the placebo groups of TrialNet clinical trials. Two PBMC samples from each patient were analyzed, one taken at baseline (within 3 months of diagnosis) and one 3 months later. The study was blinded. Clinical parameters were evaluated at baseline and again at 3, 6, 9, 12, 18, and 24 months post-baseline.

2.2. Healthy subject population

Whole blood from healthy donors was obtained from the Normal Blood Donor Program at The Scripps Research Institute (TSRI). Human Subjects protocols and consent forms were reviewed and approved by both TSRI IRB and San Diego Biomedical Research Institute (SDBRI) IRB. Whole blood was collected in heparin and processed within 2 h. PBMC were isolated using standard methods and either used immediately or frozen in liquid nitrogen, as indicated for each experiment.

2.3. Measurement of partial remission and β-cell function using IDAA1c and C-peptide AUC

A standard formula, HbA1c (%) + (4 × insulin dose (U/kg per 24 h), is used to take into account both insulin requirement and HbA1c levels in a single value, the Insulin Dose Adjusted A1c (IDAA1c). An IDAA1c equal to or <9 indicates the partial remission period [11]. Partial remission measured using IDAA1c is associated with a C-peptide AUC_{120} level of at least 108, equivalent to a mean C-peptide level of 0.9 ng/ml/min or 0.3 pmol/ml [11]. In this study the end of partial remission is between the last visit when IDAA1c is equal to or <9 and the first visit when IDAA1c is >9. A more accurate estimate of time of end of partial remission was determined by interpolating between these two observed values. Length of remission is the time between diagnosis and end of remission. Stimulated C-peptide AUC was calculated over 120 min using the trapezoidal rule, with observed C-peptide values at 0, 15, 30, 60, 90, and 120 min.

2.4. Type 1 diabetes PBMC analysis by flow cytometry

Vials of PBMC from patients with T1D were thawed and stained with each of the following panels of mAbs. Healthy subject PBMC were thawed and used as positive controls for mAb staining in each experiment. To distinguish dominantly naïve ($CD45RA^+$, $CD45RO^-$) and memory ($CD45RA^-$, $CD45RO^+$) cells within the total CD4 compartment we used either APC-conjugated anti-CD4 (BioLegend, clone OKT4) with PerCP-Cy5-5-conjugated anti-CD3 (BioLegend, clone OKT3), PE-conjugated anti-CD45RO (BioLegend, clone UCHL1), and APC-H7-conjugated anti-CD45RA (BD Biosciences, clone HI100). (Note: anti-CCR7 and anti-CD62L were not used on frozen PBMC because in our hands the relative frequency of cells expressing either CCR7 and CD62L is consistently less on frozen/thawed cells than it is on fresh cells.) To identify $CD4^+$ $CD25^+$ $CD127^{hi}$ and Tregs ($CD25^+$ $CD127^{low}$) we used the BD Biosciences Treg cocktail (FITC-conjugated anti-CD4 (clone SH3), Alexa Fluor 647-conjugated anti-CD127 (clone HIL-7R-M21), and PE-Cy7-conjugated anti-CD25 (clone 2 A3)), with PerCP-Cy5-5-conjugated anti-CD3. Activated and resting Treg were identified using FITC-conjugated anti-CD4 (BioLegend, clone OKT4), APC-H7-conjugated anti-CD45RA (BD Biosciences, clone HI100) and PerCP-Cy5.5-conjugated anti-Foxp3 (BD Biosciences, clone 236a/E7). To identify Th1-type effector cells and Treg subsets thawed PBMC were washed twice in RPMI (Invitrogen) with 10% human AB serum and rested at 37 °C overnight. Cells were resuspended in RMPI with 10% human AB serum, HEPES (Gibco BRL), glutamine, penicillin, streptomycin (Irvine Scientific), and 2-mercaptoethanol (Sigma-Aldrich) and cultured in 24 well plates at a concentration of $1–3 \times 10^6$ cell per ml with 50 ng/ml PMA (Sigma) and 1 μM Ionomycin (Sigma). 1 μl of Brefeldin A (BD Bioscience) per ml medium was added at the beginning of the culture. After 4 h cultured cells were washed twice. Th1-type effector cells were identified by their expression of T-bet and either IFN-γ, or IL-2, or TNF-α, using APC-conjugated anti-CD4 (BioLegend, clone OKT4), BV421-conjugated anti-T-bet (BioLegend, clone 4B10), and one of PE-conjugated anti-TNF-α (eBioscience, clone MAb11), anti-IL-2 (eBioscience, clone MQ1-17H12), or anti-IFN-γ (eBioscience, clone 4S·B3). Treg subsets expressing either IL-4, or IL-2, or IFN-γ, or TNF-α, were identified using APC-conjugated anti-CD4, PerCP-Cy5.5-conjugated anti-Foxp3 (BD Biosciences, clone 236a/E7), and either PE-conjugated anti-IL-4 (eBioscience, clone 8D4–8), or anti-TNFα, anti-IL-2, or anti-IFN-γ as described above.

Data were acquired on an LSRFortessa. Isotype controls were used in every experiment and for every antigen-specific antibody. Of all cell subsets analyzed, only $CD4^+$ $CD45RO^+$ memory cells, activated Tregs and $CD4^+$ $CD25^+$ $CD127^{hi}$, are associated with length of partial remission. Therefore, in the manuscript we focus our attention on these cell subsets.

2.5. Further phenotype of $CD25^+$ $CD127^{hi}$ cells by flow cytometry

Using freshly isolated PBMC from healthy subjects the relative frequency of naïve ($CD45RA^+$, $CD45RO^-$, $CCR7^+$, $CD62L^+$, $CD28^+$), central memory (CM; $CD45RA^-$, $CD45RO^+$, $CCR7^+$, $CD28^+$, $CD62L^{+/-}$), transitional memory (TM; $CD45RA^-$, $CD45RO^+$, $CCR7^-$, $CD28^+$), and effector memory (EM; $CD45RA^-$, $CD45RO^+$, $CCR7^-$, $CD28^-$, $CD62L^{+/-}$) in $CD4^+$ $CD25^+$ $CD127^{hi}$ was determined by cell surface phenotype using BV510-conjugated CCR7 (BioLegend, clone GO43H7), BV421-conjugated anti-CD62L (BioLegend, clone DREG-56), PE-conjugated anti-CD28 (BioLegend, clone CD28.2), and APC-H7-conjugated anti-CD45RA. To determine the expression of CD44, CD44 variants, Foxp3, LAG-3 and CD49b on $CD4^+$ $CD25^+$ $CD127^{hi}$ cells either BV510-conjugated anti-CD44 (BioLegend, clone IM7), or biotin-conjugated anti-CD44v4 (eBioscience, clone VFF-11), or biotin-conjugated anti-CD44v6 (eBioscience, clone VFF-18), or PerCP-Cy5.5-conjugated Foxp3, or PE-conjugated anti-LAG-3 (BD Bioscience, clone T47–530), or BV421-conjugated anti-CD49b (BD Bioscience, clone 12F1) were used. Cells labeled with biotin-conjugated mAbs were incubated with BV510-conjugated streptavidin. Sample acquisition was as described for frozen PBMC samples

Registry identifiers for the antibodies used for target antigens in Flow Cytometry experiments are as follows: CD45RO-PE–AB_571946; CD4-APC–AB_571946; CD4-FITC–AB571951; CD3-PerCP-Cy5.5–AB_2561628; CD45RA-APC-H7–AB_1727497; CD62L-BV421–AB_2562914; CCR7-BV510–AB_2563866; CD44-BV510–AB-2,561,391; CD28-PE–AB_314309; LAG-3-PE–AB_2571727; CD49b-BV421–AB_2571728; Treg cocktail–AB_1645496; T-bet-BV421–AB_10896427; TNF-α-PE–AB_466207; IL-2-PE–AB_466151; IFN-γ-PE–AB_1272026; IL-4-PE–AB_1548817; CD44v6-biotin–AB_10596818; CD44v4-biotin–AB_10596812, Foxp3-PerCP-Cy5.5–AB_10714077.

2.6. Statistics

Descriptive statistics are reported on the clinical variables. Associations of clinical variables with length of remission were assessed

initially through Pearson correlations, as length of partial remission was available for each subject. Univariate and multivariate Cox proportional hazards regression analyses were then performed with length of remission as the outcome variable. Covariates included the various cell subset frequencies at baseline (time 0), along with baseline IDAA1c, HbA1c, C-peptide levels and insulin dose requirement (U/kg/24 h). These results are reported as hazard ratios, with 95% confidence intervals. We performed all subsets regressions, but limited consideration to models with only two covariates, because of the relative paucity of data. All analyses were performed with Graphpad Prism and SPSS V21.0 (IBM Corporation, 2012).

3. Results

3.1. Changes in IDAA1c and C-peptide AUC with time

IDAA1c (see Table 1A in Ref [23]) and C-peptide AUC (see Table 1B in Ref [23]) were measured for each patient within 3 months of diagnosis (baseline), and then at 3, 6, 9, 12, 18 and 24 months post-baseline. In Fig. 1 we show the increase in IDAA1c reflecting a decline in glucose control (Fig. 1A), and a decline in stimulated C-peptide release, measured as C-peptide AUC (Fig. 1B) over the first 24 months post-diagnosis for each patient.

3.2. Immune cell subsets measured in patients newly diagnosed with T1D

Of the cell subsets tested only $CD4^+$ $CD25^+$ $CD127^{hi}$, $CD45RO^+$ memory cells and activated Treg frequencies are significantly associated with length of partial remission. Therefore, we will limit the data shown to these three cell subsets. $CD4^+$ $CD45RO^+$ memory and $CD45RA^+$ naive cells (Fig. 2A), activated and resting Treg (Fig. 2B) and $CD4^+$ $CD25^+$ $CD127^{hi}$ and Treg (Fig. 2C), were identified and their relative frequencies in patient PBMC at baseline and at 3 months post-baseline determined. Patients were stratified for analysis (see Table 2 in Ref [23]) into those with good glycemic control (having a length of partial remission longer than 1 month, $n = 12$, Fig. 2D) and those with poor glycemic control (having a length of partial remission shorter than 1 month, $n = 7$, Fig. 2E).

3.3. The relative frequency of $CD4^+$ $CD45RO^+$ memory cells, activated Treg cells, and $CD25^+$ $CD127^{hi}$ cells strongly correlates with length of partial remission

The correlation between frequencies of immune cell subsets measured at baseline with length of partial remission was determined in patients with good glycemic control. The length of partial remission correlates significantly with relative frequency of $CD45RO^+$ memory cells (Fig. 3A), activated Treg (Fig. 3B), and $CD25^+$ $CD127^{hi}$ cells (Fig. 3C and D). Although the outlier shown in Fig. 3C (red circle, subject X) improved the correlation between the frequency of $CD25^+$ $CD127^{hi}$ cells and length of remission, a correlation of 0.61 was achieved without it (Fig. 3D). In contrast, the relative frequency of Tregs (data not shown), $CD45RA^+$ naive cells (data not shown), resting Treg (data not shown), baseline IDAA1c (Fig. 3E) and C-peptide AUC (Fig. 3F) do not significantly correlate with length of remission. The relative frequency of activated Treg, but no other cell subset, also correlates with length of time HbA1c levels are maintained at or below 7% (see Figure 1 in Ref [23]). The relative frequency of $CD45RO^+$ memory cells, and $CD25^+$ $CD127^{hi}$ cells also correlated with length of remission when measured at 3 months post-baseline (see Figure 2 in Ref [23]). No correlation was noted between the relative frequency of any cell subsets tested and either body mass index (BMI), age at diagnosis, or insulin dose requirement at any time tested (data not shown). These data show that relative frequency of certain immune cell subsets at diagnosis does correlate with rate of disease progression after diagnosis.

3.4. The correlation between $CD25^+$ $CD127^{hi}$ cell frequency, but not $CD45RO^+$ and activated Treg cells, and length of partial remission increases when used as a covariate with either baseline HbA1c, or baseline IDAA1c or baseline C-peptide AUC

As a way to address whether poor glucose control and β-cell function at diagnosis might affect the correlations between cell subsets and length of partial remission, we included all patients ($n = 19$) in a Cox regression analysis and used baseline HbA1c, IDAA1c, C-peptide AUC, and insulin requirement as covariates with $CD45RO^+$, activated Treg, and $CD25^+$ $CD127^{hi}$ cell subset frequency to determine whether any combination of values more strongly correlated along with length of partial remission than any single marker. In this regard, the range of remission period was 0 to 20.5 months, with a median of 5.4 months, and inter-quartile range 0 to 8.3 months. Given the relative paucity of data, we limited consideration to regression models incorporating all subsets of one or two covariates. The three most significant models (predictors) were nearly equivalent in terms of goodness of fit, and all three involved $CD25^+$ $CD127^{hi}$ cell frequency: (i) model 1, with covariates baseline $CD25^+$ $CD127^{hi}$ and baseline HbA1c levels, $\chi^2_2 = 17.52$, $p = 0.0002$; (ii) model 2, with covariates baseline $CD25^+$ $CD127^{hi}$ and baseline IDAA1c levels, $\chi^2_2 = 14.91$, $p = 0.0006$; and (iii) model 3, with covariates baseline $CD25^+$ $CD127^{hi}$ and baseline C-peptide levels, $\chi^2_2 = 14.64$, $p = 0.0007$.

Regression coefficients from the three models are given in Table 1. The regression coefficient B (or, exp(B)) is the change in the log hazard (or, hazard) for a one unit change in the corresponding covariate when

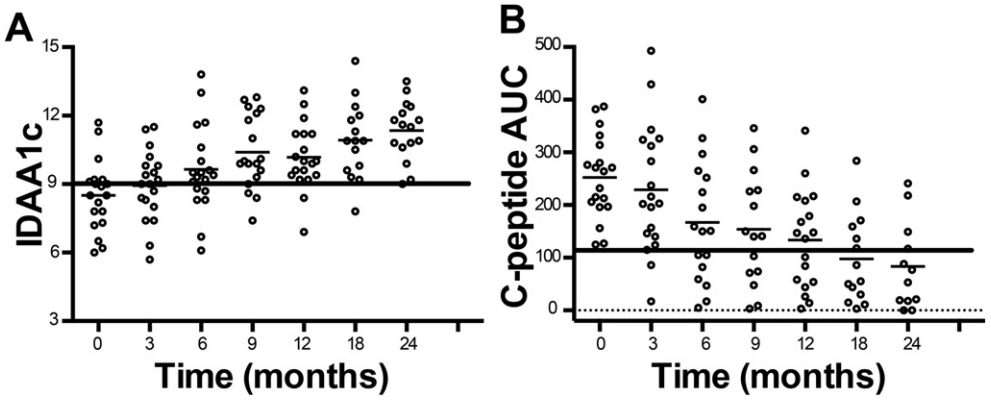

Fig 1. Changes in IDAA1c and C-peptide AUC with time IDAA1c (A) and C-peptide AUC (B) were calculated for each patient ($n = 19$) within 3 months of diagnosis (baseline, time 0), and then at 3, 6, 9, 12, 18 and 24 months post-baseline. Each symbol represents a patient. Patients with an IDAA1c above 9 indicated by a horizontal line are not in partial remission at that time point (A). At time points when there are fewer than 19 patients represented, data for either HbA1c (A), or C-peptide (B) was not available.

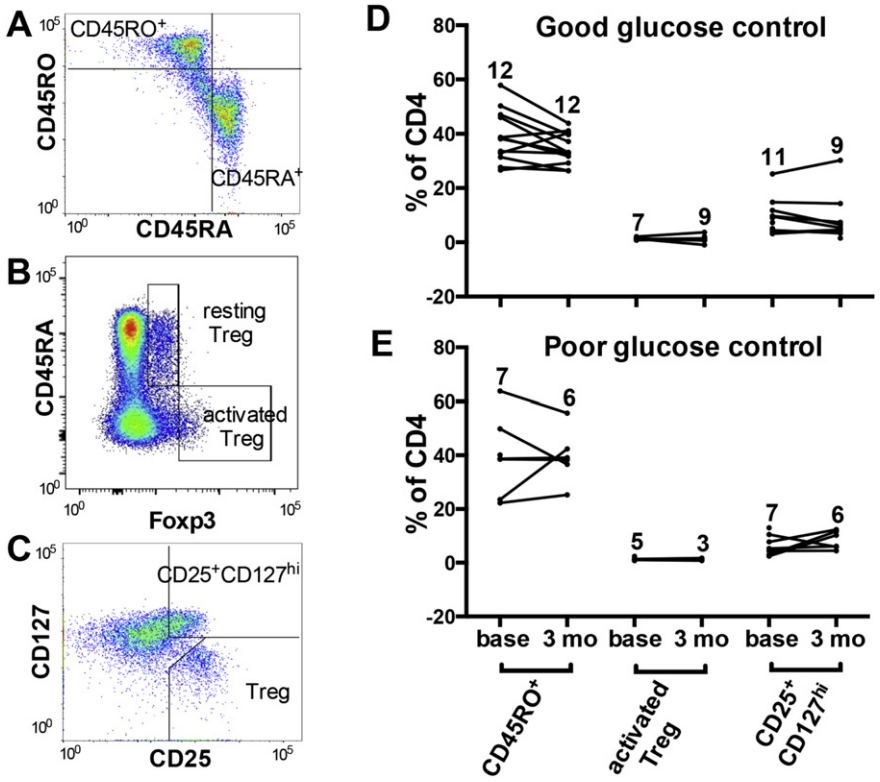

Fig 2. Immune cell subsets measured in patients newly diagnosed with T1D PBMC isolated from 19 patients at baseline were labeled for either CD3, CD4, CD25, and CD127, or CD3, CD4, CD45RA, CD45RO and Foxp3. Dot plot A is gated on $CD3^+$ $CD4^+$ cells and shows co-expression of CD45RO and CD45RA. $CD45RO^+$ $CD45RA^-$ memory cells are in the upper left quadrant. The plot in panel B is also gated on $CD3^+$ $CD4^+$ T cells and shows the activated Treg region. The co-expression of CD25 and CD127 on gated $CD3^+$ $CD4^+$ cells is shown as a dot plot (C). The upper right gate shows the $CD25^+$ $CD127^{hi}$ cells while the lower right gate shows the Treg population. Panels D and E show the relative frequency of all three cell subsets at baseline (base) and at 3 months post-baseline (3 months) in patients with either good (D) or poor (E) glycemic control during the first three moths post diagnosis. Each symbol at each time point represents an individual patient. The lines link the same patient at the two time points tested. The numbers of patients tested for each cell subset at each time point are shown on the figure.

the other covariate is held constant. Here, hazard refers to the event, ending of the partial remission period. The regression coefficient for $CD25^+$ $CD127^{hi}$ cells is virtually identical in the three models: there is roughly a 20% decline in the hazard for a unit change (increase) in relative frequency of $CD25^+$ $CD127^{hi}$ cells. High $CD25^+$ $CD127^{hi}$ cell frequency and C-peptide levels are protective, but high HbA1c or IDAA1c values are associated with increased hazard (shorter partial remission period).

These data show that of the parameters tested the combination $CD25^+$ $CD127^{hi}$ cell frequency with either baseline HbA1c, or IDAA1c, or C-peptide AUC provide the strongest predictor of length of remission. No other cell subsets, either alone or in combination with other parameters, provide a stronger predictor.

3.5. $CD25^+$ $CD127^{hi}$ cells are central and transitional memory cells

Using PBMC isolated from healthy adults we further investigated the phenotype of $CD25^+$ $CD127^{hi}$ cells (Fig. 4A). PBMC were labeled for combinations of markers that distinguished naïve, central memory, transitional memory and effector memory cells. The majority (>98%) of $CD25^+$ $CD127^{hi}$ cells are memory cells with about a 3:2 ratio of CM:TM (Fig. 4B).

3.6. $CD25^+$ $CD127^{hi}$ cells are neither $Foxp3^+$ Treg nor Tr1 cells

Both $Foxp3^+$ Treg and Tr1 regulatory cells have been implicated in protection from T1D. Although Treg generally express a low density CD127 suggesting that $CD25^+$ $CD127^{hi}$ cells are not Treg we further evaluated their Treg content. Co-expression of LAG-3^+ and $CD49b^+$, markers that identify Tr1 cells [24], was also determined. PBMC from healthy adult donors were labeled for CD3, CD4, CD25, CD127, and either Foxp3, or LAG-3 and CD49b and the expression of Foxp3 (Fig. 5A and B) and LAG-3 and CD49b (Fig. 5C–H) in total $CD4^+$, $CD25^+$ $CD127^{hi}$, and Treg (identified as $CD25^+$ $CD127^{low}$) was determined. The percent $CD25^+$ $CD127^{hi}$ cells that are $Foxp3^+$ is <4% (Fig. 5B). The frequency of cells that express either LAG-3 (Fig. 5 C and F) or CD49b (Fig. 5D and G) is variable within $CD25^+$ $CD127^{hi}$ cell population but not different from that in total CD4 and Treg. The frequency of Tr1 cells within $CD25^+$ $CD127^{hi}$ cells is significantly higher than that in total $CD4^+$ and Treg cells, although it is very low at or below 1% (Fig. 5E and H). These data show that $CD25^+$ $CD127^{hi}$ cells are neither $Foxp3^+$ Treg nor Tr1 cells.

3.7. $CD25^+$ $CD127^{hi}$ cells express CD44v6 and a high density of CD44

Signaling via CD44 and the CD44v6 variant can promote expression of Foxp3, IL-2, TGF-β and IL-10 [12–13], factors that are critical for the development and function of Treg [14–21] and Tr1 [22]. $CD25^+$ non-Treg cells in PBMC from healthy subjects were investigated for their expression of CD44 and CD44v6 as well as CD44v4. CD44v4 is expressed on resting naïve cells and therefore we predicted that $CD25^+$ non-Treg cells would not express CD44v4 [13]. Our data show that CD44v6 is expressed on 64.9 ± 10.9% of $CD25^+$ non-Treg while CD44v4 is expressed on <3% (Fig. 6A and B). Moreover, $CD25^+$ $CD127^{hi}$ cells express a significantly higher density of CD44 than do Treg cells (Fig. 6C and D). These data might suggest a mechanistic link between the relative frequency of $CD25^+$ non-Treg cells and protection from diabetes progression.

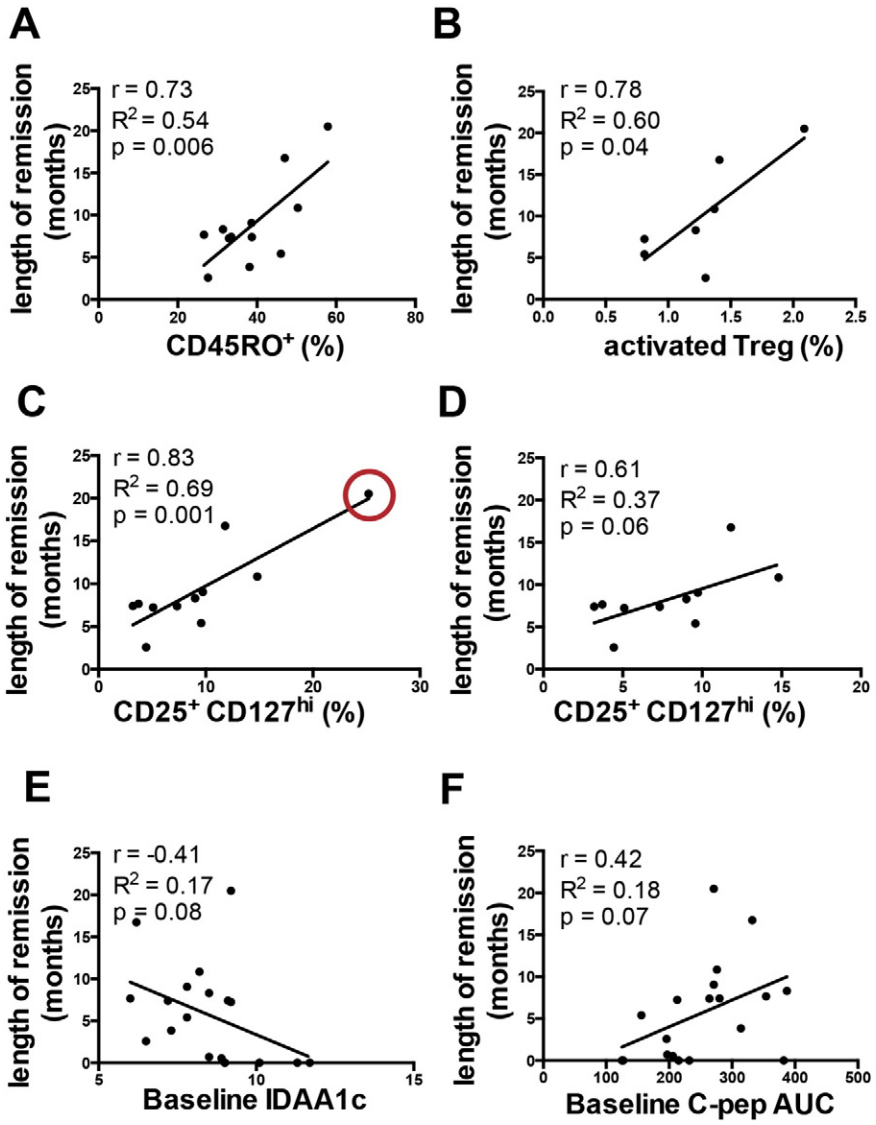

Fig 3. The relative frequency of $CD4^+$ $CD45RO^+$ memory cells, activated Treg cells, and $CD25^+$ $CD127^{hi}$ cells correlates with length of remission. The relationship between the relative frequency of $CD45RO^+$ cells (A), activated Treg cells (B), $CD25^+$ $CD127^{hi}$ cells (C and D), baseline IDAA1c (E) and baseline C-peptide AUC (F) with length of partial remission. Each symbol represents a patient. Pearson's Correlation Coefficient (r), R squared (R^2) and statistical significance (p) are shown on each panel.

3.8. The relative frequency of $CD4^+$ $CD45RO^+$ and activated Treg cells correlates significantly with frequency of $CD25^+$ $CD127^{hi}$

The correlation between the relative frequency of $CD4^+$ $CD45RO^+$ cells, activated Treg and $CD25^+$ $CD127^{hi}$ cells was determined for the samples described in Fig. 2D. The relative frequency of $CD45RO^+$ cells and activated Treg correlates significantly with the relative frequency of $CD25^+$ $CD127^{hi}$ cells (Fig. 7).

4. Discussion

In this study we show a significant correlation between the relative frequency of $CD4^+$ $CD45RO^+$ memory cells, activated Treg and $CD4^+$ $CD25^+$ $CD127^{hi}$ cells in patients newly diagnosed with type 1 diabetes and length of remission. Of these the strongest predictor of length of remission is the relative frequency of $CD25^+$ $CD127^{hi}$ cells when used as a covariate with HbA1c. The improved predictive strength of $CD25^+$ $CD127^{hi}$ for length of remission when using baseline HbA1c, as well as IDAA1c and C-peptide, levels as covariates in a Cox regression analysis suggests a relationship between the relative frequency of this cell subset with β-cell function and glucose control early after diagnosis. Some patients with poor glucose control have a high frequency of $CD25^+$ $CD127^{hi}$ cells. It is possible that high blood glucose levels either inhibit any protective effect of the $CD25^+$ $CD127^{hi}$ population, or prevent their depletion.

A growing literature is dedicated to studies reporting associations between circulating autoreactive $CD4^+$ and $CD8^+$ T cells and type 1 diabetes pathogenesis [25–28]. The report that autoreactive $CD8^+$ T cells identified in islets from patients with type 1 diabetes have specificities previously detected in the circulation strongly suggests that pathogenic T cells can be identified in the circulation [25]. In our study the relative frequency of $CD4^+$ $CD45RO^+$ memory cells, activated Treg, and $CD4^+$ $CD25^+$ $CD127^{hi}$ cell population in the circulation does not correlate with higher C-peptide levels, but instead with better glucose control. It is not yet clear whether these immune cell subsets play a role in, or are affected by, disease progression.

A critical role for Tregs in controlling inflammation is most clearly shown by the multi-organ inflammatory disorder, Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome that ensues if Foxp3 is genetically deficient [29–30]. Tregs can develop in the periphery (induced or iTregs) [31–32] as well as in the thymus (natural or

Table 1
The correlation between CD25$^+$ CD127^{hi} cell frequency and length of partial remission increases when used as a covariate with either baseline HbA1c, or baseline IDAA1c or baseline C-peptide AUC.

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Regression variables, model 1								
CD4$^+$ CD25$^+$ CD127^{hi}	−0.215	0.081	7.025	1	0.008	0.806	0.687	0.945
HbA1c	1.204	0.471	6.528	1	0.011	3.333	1.324	8.395
Regression variables, model 2								
CD4$^+$ CD25$^+$ CD127^{hi}	−0.208	0.075	7.653	1	0.006	0.812	0.684	0.923
IDAA1c	0.474	0.206	5.291	1	0.021	1.606	1.086	2.456
Regression variables, model 3								
CD4$^+$ CD25$^+$ CD127^{hi}	−0.192	0.078	6.069	1	0.014	0.826	0.709	0.962
CPEP	−0.011	0.005	4.650	1	0.031	0.989	0.980	0.999

Notes: B denotes the regression coefficient for the corresponding covariate in a Cox model of time to end of honeymoon. SE denotes the standard error of the regression coefficient. Wald, df (degrees of freedom), and Sig (p-value) denote chi-square tests of the null hypothesis that the parameter estimate for that covariate is 0. It is convenient to consider Exp(B) and its associated confidence interval: Exp(B) connotes the change in the hazard for a one unit change in the covariate.

nTregs) [29] and the expression of Foxp3 by Tregs is an absolute requirement for their suppressor function [14–17]. There are clear differences in the Treg compartment in patients with type 1 diabetes compared to healthy subjects [33–34], and modulating regulatory cell number can alleviate type 1 diabetes in the NOD mouse model for type 1 diabetes [35–36]. Although increasing Treg numbers is not always associated with protection after immunomodulation [37], in recent clinical trials, several patients treated with ex vivo expanded polyclonal Treg had higher C-peptide levels than untreated controls supporting a role for Treg in protection from disease progression [38–39]. Tr1 cells also have anti-inflammatory potential for therapeutic intervention in type 1 diabetes [40]. If modulation of Treg function and Tr1 numbers and function in patients with type 1 diabetes results in improved clinical outcome the potential for CD44 expressing CD4$^+$ T cells to promote Treg function and Tr1 cell development will warrant further investigation.

Several lines of evidence suggest a mechanistic link between CD25$^+$ CD127^{hi}, immune regulation, and the control of autoimmunity and inflammation. Thus, i) the relative frequency of CD25$^+$ CD127^{hi} correlates significantly with the relative frequency of activated Treg, ii) two thirds of CD25$^+$ CD127^{hi} are central memory cells, a cell subset that can differentiate to functional Tregs [41], and iii) CD25$^+$ non-Tregs express a high density of CD44 and the CD44 variant CD44v6 signaling through which can induce the expression of Foxp3, as well as IL-2, TGF-β, and IL-10, factors that increase Treg function [12–13]. CD44 signaling can also induce the development of a second IL-10-expressing regulatory T cell, Tr1 [42–43]. In healthy subjects, CD25$^+$ CD127^{hi} cells are not themselves Treg cells or Tr1 cells. Although we cannot exclude the possibility that CD25$^+$ CD127^{hi} cells from patients with T1D are different in this respect, these data suggest that CD25$^+$ CD127^{hi} cells might promote glucose control and inflammation by enhancing activated Treg numbers and promoting the development of Tr1 cells via CD44 and CD44v6 signaling.

Signaling via cell surface CD25, CD127 and CD44 expressed by CD25$^+$ CD127^{hi} provides alternative pathways for regulating the relative frequency of this cell population. In inflammatory environments, including in patients with type 1 diabetes, soluble CD127 and CD25 (sCD127 and sCD25) released from activated T cells act as IL-7 and IL-2 antagonists, blocking IL-7 and IL-2 mediated signaling and the expansion of CD127$^+$ and CD25$^+$ T cells [44–46]. This leaves CD127$^+$ and CD25$^+$ cells vulnerable to depletion [44–46]. However, hyperglycemia can result in glycation of sCD127 making it ineffective as an IL-7 antagonist in patients with poor glucose control [44]. On the other hand, CD44 co-stimulation induces CD25 expression promoting CD25 signaling in the presence of lower circulating levels of IL-2 [13]. In addition, CD44, and its variant CD44v6, both receptors for hyaluronan (HA), inhibit apoptosis on cross-linking CD44 [12,47–48]. During inflammation HA is broken down into a low m.wt form that is ineffective in cross-linking CD44 [49]. It is possible that the relative frequency of CD25$^+$ CD127^{hi} reflects the level of inflammation and circulating inflammatory mediators in patients with type 1 diabetes.

Previously published studies have shown immunological changes during the remission period in patients with type 1 diabetes, but whether these changes are causally related to remission remains unclear [50]. Thus, an early study showed an increase in T cell proliferative responses to insulin secretory granules in a small number of children with type 1 diabetes during the remission period [51]. Later studies reported that Foxp3 expression at diagnosis predicted poor future glycemic control

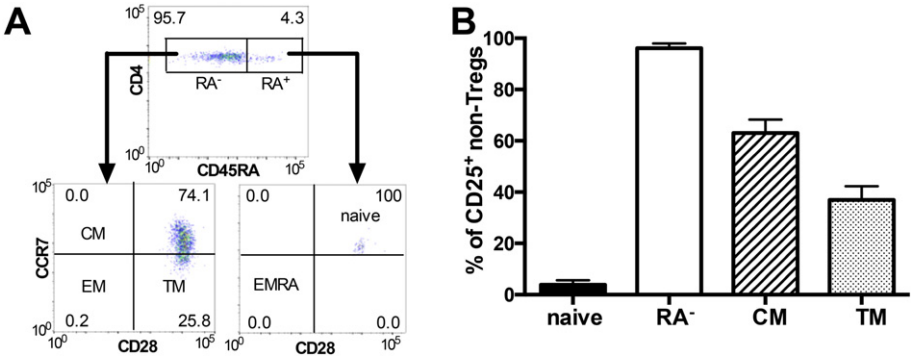

Fig 4. In healthy individuals CD25$^+$ CD127^{hi} cells are central and transitional memory cells. PBMC freshly isolated from healthy adults were co-stained for CD3, CD4, CD25, CD127, CD45RA, CCR7 and CD28 ($n = 8$). Panel A top dot plot identifies CD4$^+$ CD45RA$^-$ (RA$^-$) and CD4$^+$ CD45RA$^+$ (RA$^+$) cells gated CD4$^+$ CD3$^+$ cells. The lower left plot is gated on RA$^-$ cells and identifies central memory (CM), transitional memory (TM) and effector memory (EM) cells. The lower right plot is gated on RA$^+$ cells and identifies naïve and effector memory cells that express RA (EMRA). Panel B shows the mean ± SD relative frequency of naïve, memory (RA$^-$) CM, TM and EM cells in CD25$^+$ CD127^{hi}. No EMRA cells were seen in healthy donors.

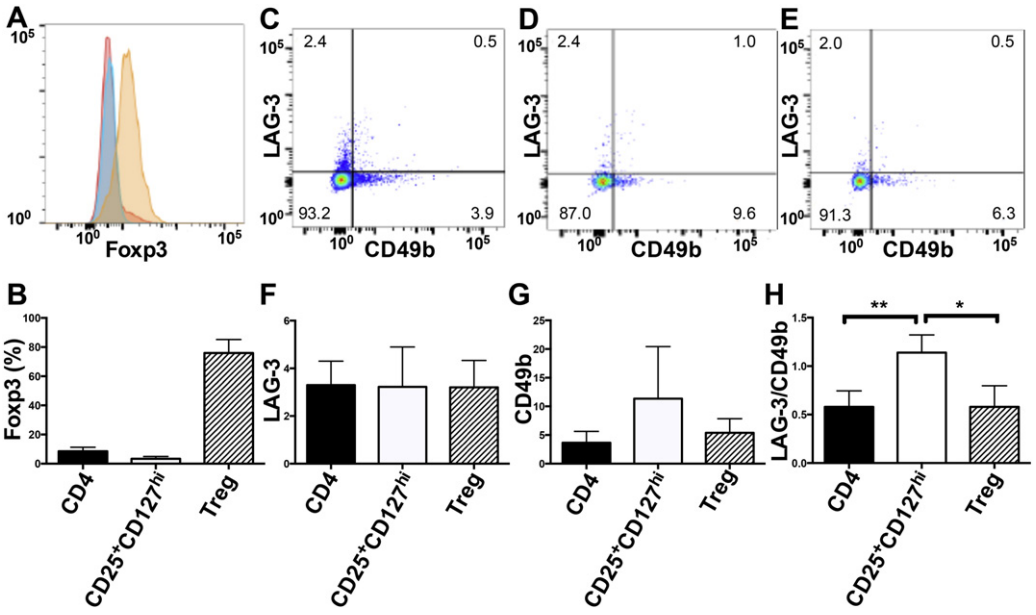

Fig 5. CD25$^+$ CD127^{hi} cells are neither Foxp3$^+$ Treg nor Tr1 cells. PBMC from healthy donors ($n = 5$) were thawed and labeled for CD3, CD4, CD25, CD127, and either Foxp3, or LAG-3 and CD49b. (A) A representative example of Foxp3 expression on gated CD4$^+$ (pink, CD3$^+$ CD4$^+$), CD25$^+$ CD127^{hi} (blue, CD3$^+$ CD4$^+$ CD25$^+$ CD127^{hi}) and Treg (orange, CD3$^+$ CD4$^+$ CD25$^+$ CD127^{low}) cells, and the percent Foxp3$^+$ cells within each subset from all donors is shown in B. The dot plots show a representative example of the co-expression of LAG-3 and CD49b gated on either CD4$^+$ (C), or CD25$^+$ CD127^{hi} (D), or Treg (E) cells and the percent single LAG-3$^+$ (F), CD49b$^+$ (G) and LAG-3/CD49b double positive (H) cells within each cell subset from all donors. The data are shown as mean ± SD and are pooled from 2 separate experiments. Significant differences shown in panel H were calculated using Mann Whitney, ** $p = 0.008$, * $p = 0.02$.

[52] and that Treg suppressor function transiently decreased during the remission period while effector cytokine secretion increased [53]. The published data overall point to heightened T cell activity during remission, a condition necessary for the co-development of Tregs and peripheral tolerance as well as effector cell function [50]. Our data are consistent with a non-antigen-specific non-Treg CD4$^+$ T cell change in the immune cell profile in patients with type 1 diabetes. This might suggest that strategies to re-establish immune homeostasis are important in the treatment of type 1 diabetes.

Amongst the many susceptibility loci for autoimmune disease there are several that link CD25, the high affinity IL-2 receptor (IL-2RA), with disease risk. Susceptible IL-2RA haplotypes show reduced signaling to IL-2 in Tregs in type 1 diabetic and multiple sclerosis patients [33] as well as healthy individuals [54]. Moreover, individuals with protective

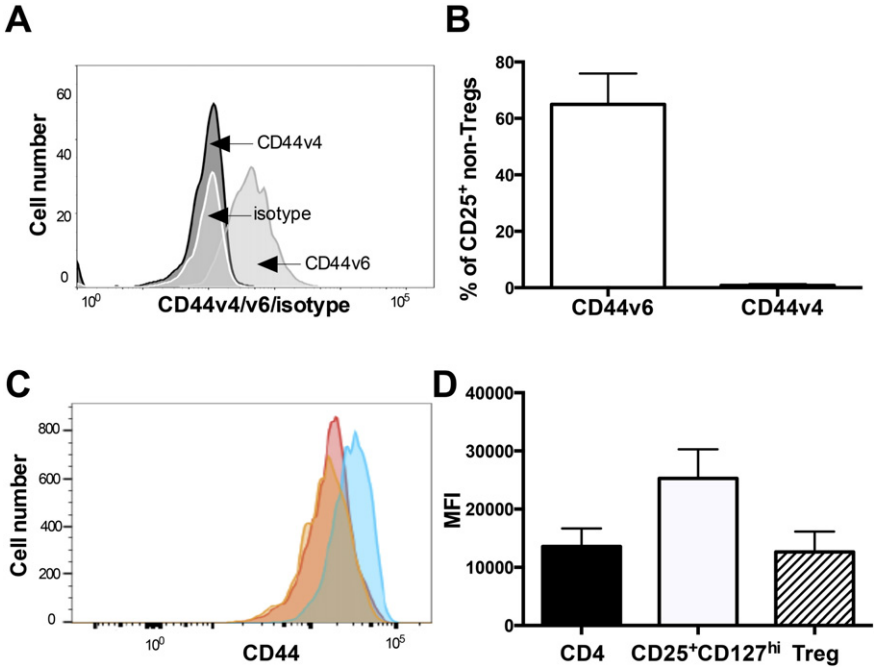

Fig 6. CD25$^+$ CD127^{hi} cells express CD44v6 and a high density of CD44. PBMC freshly isolated from healthy adults were co-stained for CD3, CD4, CD25, CD127, and either CD44v4 and CD44v6 ($n = 6$) or CD44 ($n = 5$). Panel A shows a representative example of CD44v6 and CD44v4 expression, as well as isotype control staining, on CD25$^+$ CD127^{hi} cells. The isotype for the CD44v6- and CD44v4-specific antibodies used is the same. The mean ± SD CD44v6 and CD44v4 expression on CD25$^+$ CD127^{hi} cells is shown in B. Panels C and D show expression of CD44 on total CD4$^+$ (pink), CD25$^+$ CD127^{hi} (blue) and Treg (orange) cells (C), and mean fluorescence intensity (MFI) of CD44 on each cell subset (D).

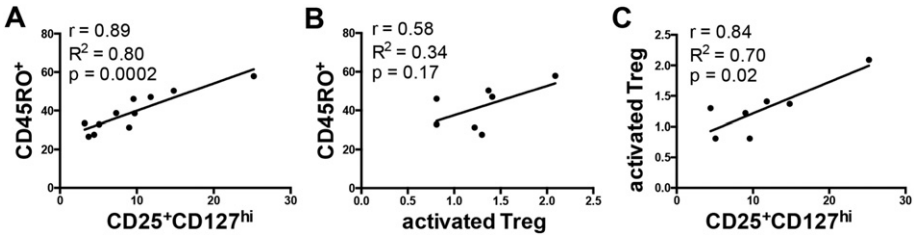

Fig 7. The relative frequency of $CD4^+$ $CD45RO^+$ and activated Treg cells correlates significantly with frequency of $CD25^+$ $CD127^{hi}$ cells. The relationship between the relative frequency of $CD45RO^+$, activated Treg, and $CD25^+$ $CD127^{hi}$ cells in patients with recent onset T1D who have good glycemic control (ages 8–16 years). Each symbol represents a patient. Pearson's Correlation Coefficient (r), R squared (R^2), and statistical significance (p) are shown on each panel.

IL-2RA haplotypes express significantly higher expression of CD25 on non-Treg, but not Treg $CD4^+$ T cells, compared to non-protective haplotypes [55]. This might suggest a protective role for CD25 on non-Treg $CD4^+$ T cells in type 1 diabetes. Whether the low relative frequency of $CD25^+$ $CD127^{hi}$ cells in patients with type 1 diabetes who have a short remission period is linked to the IL-2RA haplotype associated with reduced expression of CD25 on non-Treg cells is yet to be determined. Although the $CD25^+$ $CD127^{hi}$ cell population is not generally recognized as a defined cell population it has previously been visualized on human $CD4^+$ $CD3^+$ T cells co-labeled for CD25 and CD127 (http://m.bdbiosciences.com/documents/T_Cell_BD_Human_Regulatory_Cocktail.pdf).

5. Conclusions

This pilot study has identified the relative frequency of $CD4^+$ $CD45RO^+$ cells, activated Treg, and $CD25^+$ $CD127^{hi}$ cells as potential predictors of length of remission in patients with T1D. The predictive potential for $CD25^+$ $CD127^{hi}$ cells increases dramatically when used as a covariate with either baseline HbA1c, or baseline IDAA1c, or baseline *C*-peptide suggesting a relationship between glucose control and β-cell function. $CD25^+$ $CD127^{hi}$ cells express CD44v6 and a high density of CD44, signaling through which promotes the expression of Foxp3, IL-2, TGF-β, and IL-10 suggesting a potential mechanistic link between the presence of $CD25^+$ $CD127^{hi}$ cells, Treg, and protection from disease progression. Whether this cell subset plays an active role in maintaining partial remission, either by enhancing regulatory T cell function, or by some other mechanism, remains to be determined. These data warrant further investigation in a validation study with a large cohort of patients.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2016.04.012.

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