Transient partial remission, a period of low insulin requirement experienced by most patients soon after diagnosis has been associated with mechanisms of immune regulation. A better understanding of such natural mechanisms of immune regulation might identify new targets for immunotherapies that reverse T1D. In this study, using Cox model multivariate analysis we validate our previous findings that patients (n = 84) with the highest frequency of CD4+ CD25+CD127^{hi} (127-hi) cells at diagnosis experience the longest partial remission and we show that the 127-hi cell population is a mix of Th1- and Th2-type cells with a significant bias towards anti-inflammatory Th2-type cells. In addition, we extend these findings to show that patients with the highest frequency of 127-hi cells at diagnosis are significantly more likely to maintain beta-cell function. Moreover, in patients treated with Alefacept in the TIDAL clinical trial, the probability of responding favorably to the anti-inflammatory drug was significantly higher in those with a higher frequency of 127-hi cells at diagnosis than those with a lower 127-hi cell frequency. These data are consistent with the hypothesis that 127-hi cells maintain an anti-inflammatory environment that is permissive for partial remission, beta-cell survival and response to anti-inflammatory immunotherapy.
CD4⁺CD25⁺CD127hi cell frequency predicts disease progression in type 1 diabetes

Aditi Narsale¹, Breanna Lam¹, Rosa Moya¹, TingTing Lu², Alessandra Mandelli³, Irene Gotuzzo³, Benedetta Pessina³, Gian Maria Giamporcaro³, Rhonda Geoffrey⁴, Kerry Buchanan⁵,⁶, Mark Harris⁵,⁶, Anne-Sophie Bergot⁵, Ranjeny Thomas⁵, Martin J. Hessner⁴, Manuela Battaglia³,⁷, Elisavet Serti², Joanna D. Davies¹

¹San Diego Biomedical Research Institute, 10865 Road to the Cure, San Diego, California, USA

²Immune Tolerance Network, Bethesda, MD, USA

³San Raffaele Diabetes Research Institute, IRCCS San Raffaele Hospital, Milano, Italy

⁴Department of Pediatrics, The Medical College of Wisconsin, Milwaukee, Wisconsin, USA

⁵Diamantina Institute, University of Queensland, Australia

⁶Department of Pediatric Endocrinology, Queensland Children’s Hospital, Australia

⁷Current affiliation: Telethon Foundation, Milano, Italy

Corresponding author: Joanna D. Davies, ¹San Diego Biomedical Research Institute, 10865 Road to the Cure, Suite 100, San Diego, CA 92121, U.S.A. Tel: (858) 200-7048; FAX: (858) 200-7096; Email: jdavies@sdbri.org

The authors have declared that no conflict of interest exists.
Abstract

Transient partial remission, a period of low insulin requirement experienced by most patients soon after diagnosis has been associated with mechanisms of immune regulation. A better understanding of such natural mechanisms of immune regulation might identify new targets for immunotherapies that reverse T1D. In this study, using Cox model multivariate analysis we validate our previous findings that patients (n = 84) with the highest frequency of CD4⁺ CD25⁺CD127hi (127-hi) cells at diagnosis experience the longest partial remission and we show that the 127-hi cell population is a mix of Th1- and Th2-type cells with a significant bias towards anti-inflammatory Th2-type cells. In addition, we extend these findings to show that patients with the highest frequency of 127-hi cells at diagnosis are significantly more likely to maintain β-cell function. Moreover, in patients treated with Alefacept in the T1DAL clinical trial, the probability of responding favorably to the anti-inflammatory drug was significantly higher in those with a higher frequency of 127-hi cells at diagnosis than those with a lower 127-hi cell frequency. These data are consistent with the hypothesis that 127-hi cells maintain an anti-inflammatory environment that is permissive for partial remission, β-cell survival and response to anti-inflammatory immunotherapy.
**Introduction**

Type 1 diabetes (T1D) is a progressive heterogeneous autoimmune disease resulting in the destruction of insulin secreting β-cells by T cells (1). Despite enormous effort there is still no therapy to stop and reverse the disease. However, there are sufficient examples of immunotherapies that transiently preserve β-cell function in sub-populations of patients to encourage the notion that disease progression can be modulated by targeting the immune system (2-6). In an effort to identify novel immune mechanisms that might be targeted therapeutically to treat and reverse T1D our group has focused attention on a well-recognized natural phenomenon called partial remission, a period of improved glucose control experienced by many T1D patients soon after diagnosis (7-10). Partial remission can last from several weeks to over a year (11,12). The clinical significance for the partial remission period is that patients who experience partial remission have a significantly reduced risk of complications (13) including, chronic microvascular complications (14), a lower risk of severe hypoglycemia (15,16), diabetic retinopathy (17) and an increase in growth in prepubescent children (18). Therefore, identifying mechanisms that promote and extend partial remission might also identify new targets for novel therapies to reduce short and long-term complications.

The mechanism for partial remission is not known. One idea is that it is actively controlled by a form of immune regulation. Consistent with this idea, we have identified a novel CD4+ T cell population that expresses CD25 and a high density of CD127 that is present at a higher frequency in children with a long remission compared to children with short or no remission (19,20). In
healthy people, CD25^+CD127^{hi} (127-hi) cells are predominantly memory cells with a mix of anti-inflammatory, IL-4, IL-10, IL-13 and IL-5 secreting Th2 cells and pro-inflammatory, IFN-γ and IL-2 secreting Th1 type cells, with a bias towards Th2 (21). Th1 and Th2 cell function is defined by the cytokines that they secrete (22,23). Thus, Th2 cytokines promote the differentiation to, and the expansion of Th2 cells, and inhibit the differentiation to and expansion of Th1 cells, and vice versa (24-28). The Th2 bias in 127-hi cells might then suggest that in patients with T1D, 127-hi cells play an active role in prolonging remission by promoting an anti-inflammatory microenvironment.

The correlation between 127-hi cell frequency and remission length combined with the Th2 bias in 127-hi cell function provided the justification to test the validity of these findings in a larger new study. In collaboration with the Immune Tolerance Network (ITN) USA, the Medical College of Wisconsin (MCW) USA, San Raffaele Diabetes Research Institute (SRDRI) in Italy and the University of Queensland in Australia (UQ) we tested the validity of our original finding that the relative frequency of 127-hi cells correlates with length of partial remission.

CD4^+ and CD8^+ T cells fall into three main functional groups. Pro-inflammatory cells can be either IFN-γ secreting Th1 (CD4^+ T cells) and Tc1 (CD8^+ T cells) cells or IL-17 and IL-22 secreting Th17/Tc17 cells, anti-inflammatory Th2/Tc2 cells secrete IL-4, IL-5 and IL-13 and regulatory cells secrete IL-10 and TGF-β (29). Lineage-specific cytokines and transcription factors can positively influence their own expansion and negatively influence the expansion of other lineages. Specifically, in mice, it is well established that Th1 cells are positively induced by IL-12 and IFN-γ but negatively influenced by IL-4, while Th2 cells are positively regulated by IL-4 and negatively regulated by the Th1 cytokine IL-12 (22-27). In addition, the Th2–type transcription factor GATA-3 (30) plays a critical role in promoting the Th2 response and in inhibiting Th1 differentiation (31-
Likewise, both IL-4 and IFN-γ inhibit the development of Th17 cells (35,36) and during an immune response this results in a negative association between Th17 and both Th1 and Th2 responses. This type of immune regulation, or deviation, caused by the polarization towards and away from lineage commitment, can enhance either the pro-inflammatory, or anti-inflammatory immune responses. Th1/Tc1 cells are known to be pathogenic effector cells in T1D (37-39).

Pre-committed Th2 and Th1-type cells influence polarization of T cells that are in close proximity, for example during recirculation through inflamed tissue and lymph nodes (LN). Within inflamed tissue pre-committed cells are thought to commit to a specific lineage on encounter with specific antigen (40). To recirculate through LN where primary responses to antigen generally take place (41) T cells must express homing receptors, CCR7 and CD62L, also the cell surface markers that distinguish naïve and central memory (CM) T cells from effector memory (EM) T cells (42,43). As cells differentiate from naïve and CM to EM expression of both CCR7 and CD62L is reduced giving the cells access to peripheral tissue (42,43). By combining cytokine profiles with the expression of cell surface markers the potential function of T cell subsets that are associated with clinical outcomes can be predicted. In our study, the frequency of the 127-hi cell population that is preferentially made up of IL-4 secreting pre-Th2 memory cells is associated with length of partial remission and β-cell function. We suggest that 127-hi cells are actively involved in delaying disease progression by inhibiting the expansion and differentiation of potentially pathogenic autoimmune Th1/Tc1 cells.

Retrospective analyses of clinical trial data show that T1D patients are more likely to respond well to immunotherapy if it is given soon after diagnosis (3-6). However, even within those patients treated within 3 months of diagnosis not all patients respond equally. This heterogeneity in response to immunotherapy is not fully understood (44). We suggest the hypothesis that of the
patients treated soon after diagnosis, those in partial remission will respond better to treatment because the mechanism of partial remission period provides a permissive environment for immunotherapy. Furthermore, we might expect that patients with the highest frequency of 127-hi cells and therefore predicted to have the longest partial remission and the highest level of protection would respond best to immunotherapy. As part of this study we tested whether 127-hi cell frequency at diagnosis correlates with response to the anti-inflammatory drug Alefacept, in T1D patients recruited to the T1DAL study (2,45,46).

**Results**

The source, number, gender and age of the participants in all Figures and Tables are summarized in Table 1. For this study, the first time point for blood sample collection is within 3 months of diagnosis. Throughout this manuscript this time point is referred to as “baseline”.

**127-hi cells are equivalently quantified at different research sites**

Investigators at all 4 research sites, ITN, SRDRI, MCW, and UQ were asked to identify flow cytometry data from their own group generated using PBMC from people within three months of diagnosis with T1D stained with a panel of antibodies that were specific for CD3 CD4 CD25 CD127 (n = 12 for ITN, n = 39 for SRDRI, n = 22 for MCW and n = 11 for UQ). Our group sent each collaborative group a schematic (Figure 1) that describes how to identify 127-hi, CD25-neg and Treg cells. Each group was asked to record the relative frequency of 127-hi cells within the total CD4⁺ T cell population using their own raw flow cytometry data. The same data were blinded and sent to our group at SDBRI so that we could quantify the same cell subset using the same flow
cytometry data. All investigators also quantified the relative frequency of established T cell populations, CD4+ T cells and Treg cells. The data were then exchanged, unblinded, and the frequency of 127-hi cells, CD4+ T and Treg cells in each sample compared between investigators at different sites for consistency using linear regression (Figure 2). The mean fluorescence for CD25 and CD127 on 127-hi cells analyzed at the 4 different sites is shown in Supplemental Figure 1.

The relative frequency of 127-hi cells at baseline correlates with length of partial remission

Data were pooled from ITN, SRDRI, MCW and UQ to determine correlations between baseline measures of 127-hi cell frequency, age, BMI, IDAA1c and gender with LoR. When using cox model single covariate analysis adjusted for study variability 127-hi cell frequency at diagnosis correlated with LoR (p = 0.0227; n = 84), while age, BMI, baseline IDAA1c and gender do not (p = 0.1929; p = 0.3323; p = 0.7029; p = 0.9790 respectively). Multivariate analysis also identified baseline 127-hi cell frequency as the only significant correlate with length of partial remission (LoR) (Table 2). However, the best fit model for correlation with LoR included 127-hi cell frequency with BMI (Table 3). Using a multivariate analysis add back strategy to test the effect of either BMI, or age, or gender, or baseline IDAA1c on the influence of 127-hi cell frequency on LoR, only BMI improved the correlation with LoR (Table 4).

To test whether there is a relationship between 127-hi cell frequency and either age, or BMI, or gender, in addition to IDAA1c, a linear regression of covariates was performed (Table 5). Baseline 127-hi cell frequency and age are significantly predictive of each other, as are baseline 127-hi cell frequency and BMI (Table 5). Moreover, at higher levels of BMI, the influence of age on 127-hi
increases and at older age at diagnosis, the influence of BMI on 127-hi cell frequency increases (Table 6). Baseline IDAA1c and gender do not significantly predict 127-hi cell frequency (Table 5).

The effect of 127-hi cell frequency at baseline, age at diagnosis, and baseline IDAA1c on survival probability was also determined where the limit of survival is the end of remission. End of remission occurs significantly earlier in patients with a baseline 127-hi cell frequency below the mean compared to those with a 127-hi cell frequency above the mean (Figure 3A; p = 0.02). To examine the effect of age, patients were stratified by being either older than 17 years, between 9 and 17 years and younger than 9 years at diagnosis. Patients diagnosed with T1D when they were older than 17 had a significantly longer end of remission compared to children younger than 9 years (Figure 3B; p = 0.01). There was no significant difference in end of remission between the group aged between 9 and 17 years at diagnosis and either of the other groups. Using the log-rank test for trend, there is also a significant association between earlier end of remission in younger patients (Figure 3B; p = 0.003). The end of remission was not different in patients stratified by having an IDAA1c level at baseline of either less than 7.5, or between 7.5 and 9, or greater than 9 (Figure 3C). Figure 3D shows the relative frequency of 127-hi cells for all samples used in the analyses shown in Figure 3A, B and C from ITN, SRDRI, MCW and UQ.

The probability of preserving β-cell function is greater in patients with a higher relative frequency of 127-hi cells at diagnosis

We next tested the relationship between 127-hi cell frequency at baseline and future β-cell function by measuring the effect of 127-hi cell frequency on probability of preserving β-cell function where
the limit of survival is fasting C-peptide level at 12 months post-diagnosis (Figure 4A and B). The fasting C-peptide level at 12 months post-diagnosis in patients with good glucose control is significantly lower in patients with a baseline 127-hi cell frequency below the mean compared to those with a 127-hi cell frequency above the mean (Figure 4 A; n = 28; p = 0.04). However, when the analysis included all patients tested (n = 42), including those with poor glucose control (n = 14) the relationship was no longer significant.

Fasting C-peptide is less commonly used to assess β-cell function than stimulated C-peptide. In our study fasting C-peptide was the measurement of choice because stimulated C-peptide was measured in only 12 of the 84 participants whereas fasting C-peptide was measured in 42 patients. To determine the relationship between levels of fasting and stimulated C-peptide, we compared these two measurements in the same blood samples collected at baseline. Only patients with good glucose control were included. The data show a strong correlation between levels of fasting C-peptide and stimulated C-peptide AUC in this population (Figure 4C; r = 0.71, p<0.0001).

**The relative frequency of 127-hi cells decreases over time post-diagnosis**

The relative frequency of 127-hi cells was determined at 6, 12, and 24 months and compared to baseline values to determine whether there is a decline in this cell population over time. Changes in IDAA1c and C-peptide levels were also determined. The relative frequency of 127-hi cells is reduced by 12 and 24-months post-baseline (Figure 5A and B). As expected, IDAA1c levels increase (Figure 5C) and C-peptide levels decline with time after diagnosis (Figure 5D).

**127-hi cells from people with T1D are predominantly memory cells with a Th2 bias**
In healthy individuals 127-hi cells are a mixture of naïve, central memory (CM), effector memory (EM), pre-committed Th1 and Th2 cells (21). To determine whether 127-hi cells from patients with T1D have the same mixed phenotype the relative frequency of naïve, CM and EM cells within the 127-hi cell compartment in PBMC from T1D patients was determined. The same cell subsets were also quantified in the CD25-neg and total CD4+ T cell compartments. More than 85% of 127-hi cells are memory cells and the majority of these are EM (Figure 6A). In contrast, within the CD25-neg (Figure 6B) and total CD4+ (Figure 6C) cell populations more than 50% of the cells have a naïve cell phenotype and of the memory phenotype cells, CM are in the majority (Figure 6C). We further characterized the 127-hi memory cells from T1D patients by quantifying the relative frequency of pre-Th2 (CXCR5-CXCR3-CCR4+) and pre-Th1 (CXCR5-CXCR3+CCR4-) cells and comparing to CD25-neg and total CD4+ memory T cells. As seen in healthy people, 127-hi memory cells have a significantly higher pre-Th2 frequency than either the CD25-neg or the total CD4+ memory cell populations whereas the frequency of pre-Th1 cells is not different between the 127-hi and CD25-neg memory cell compartments (Figure 6D).

**127-hi pre-Th2 memory cells secrete significantly more Th2-type cytokines than CD25-neg memory cells** The capacity of 127-hi and CD25-neg pre-Th2 memory cells to secrete Th2-type cytokines was tested by sorting pre-Th2 127-hi memory and pre-Th2 CD25-neg memory cells, and measuring Th2 and Th1-type cytokines after stimulation for 48 hours in vitro. 127-hi pre-Th2 cells secreted significantly more IL-4, IL-13, IL-5 and IL-10 (Figure 7A-D), and significantly less IFN-γ than CD25-neg pre-Th2 cells (Figure 7E). Levels of IL-2, IL-6 and TNF-α are not different between groups (Figure 7F-H). As part of the same experiment pre-Th1 memory cells were also sorted. However, insufficient numbers of cells were available for analysis (Supplemental Table 1).
The 127-hi cell population contains significantly more GATA-3+ cells and fewer T-bet+ cells than CD25-neg cells. PBMC from T1D patients, collected at baseline, were stimulated with PMA and ionomycin for 4 hours. After stimulation cells were labeled for cell surface CD3 CD4 CD45RO CD25 and CD127, and intracellular expression of GATA-3, T-bet and RORγt, and transcription factor expression in 127-hi memory cells was compared with than in CD25-neg cells. The frequency of 127-hi memory cells that express GATA-3 is significantly higher than the frequency of 127-hi cells that express either T-bet or RORγt (Figure 8A). In addition, the frequency of GATA-3+ cells is significantly higher in 127-hi cells than in CD25-neg cells, whereas, the frequency of T-bet positive cells is higher in CD25-neg cells (Figure 8A). The frequency of RORγt+ cells was not different in the two cell subsets. Figure 8B shows representative expression of GATA-3, T-bet and RORγt (Figure 8B). No correlation was seen between the relative frequency of 127-hi cells that express either GATA-3, or T-bet, or RORγt and the relative frequency of 127-hi cells (Supplemental Figure 2).

**Treatment with Alefacept depletes circulating 127-hi EM cells**

Alefacept was originally used as an anti-inflammatory drug for patients with psoriasis (47-50). Alefacept works by inhibiting CD2-mediated co-stimulation and by depleting CD2 expressing cells (51,52). Because CD2 is expressed at a high level on EM cells, which are known to cause pathology in T1D, Alefacept was tested, with some success, as an immunotherapy to reverse T1D (2,45,46). Further characterization of 127-hi cells shows that 127-hi EM cells express a higher density of CD2 than do 127-hi naïve and CM cells (Figure 9A), identifying 127-hi EM cells as a
potential target for Alefacept. In the T1DAL clinical trial, treated T1D patients were given two 12-week courses of Alefacept, the first beginning at baseline and the second beginning at 24 weeks post-baseline. Our data show that Alefacept significantly depleted 127-hi cells after both the first (tested at 3 months post-baseline), and second round of treatment (tested at 9 months post-baseline; Figure 9B). 127-hi EM cells are the main target for Alefacept but they appear to recover quickly after the second treatment is completed (Figure 9C). In contrast, the frequency of CM cells remained unaffected (Figure 9D). The relative frequency of naïve cells increased as EM frequency decreased (Figure 9E).

**The probability of remaining in remission (survival probability) is greater in Alefacept-treated patients who have a higher baseline relative frequency of 127-hi cells**

We compared the probability of remaining in remission in Alefacept-treated patients with a baseline frequency of 127-hi cells above the mean compared to below the mean and found that those above the mean were significantly more likely to remain in remission at 2 years post-baseline compared to those below the mean (Figure 10A). The same was true for 127-hi CM cells (Figure 10B), but not for 127-hi EM cells (Figure 10C), or 127-hi naïve cells (Figure 10D).

**Discussion**

This study validates our previously published finding that the probability of remaining in remission for patients newly diagnosed with T1D is significantly greater for patients who have the highest relative frequency of 127-hi cells at diagnosis. In addition, we extend these findings to show that patients with the highest frequency of 127-hi cells have the greatest probability of preserving β-
cell function. Furthermore, as β-cell function declines over time post-diagnosis, 127-hi cell frequency is reduced, again associating with disease progression. We suggest that, if 127-hi cells are mechanistically involved in promoting and maintaining partial remission a reduction in their frequency as β-cell function declines might be expected. As seen previously for 127-hi cells in healthy people (21), 127-hi cells in patients with T1D are a mix of naïve, CM and EM, pre-Th1 and pre-Th2 cells with a bias towards Th2. The Th2 bias suggests the possibility that 127-hi cells play an active role in prolonging partial remission by deviating potentially pathogenic Th1 type cells towards the anti-inflammatory Th2-type. In addition, the potential for 127-hi cell frequency to predict response to immunotherapy is suggested by the finding that patients with a higher baseline 127-hi cell frequency have a greater probability of remaining in remission at 2 years post-diagnosis after treatment with the anti-inflammatory drug Alefacept.

127-hi cells were first identified by us by their expression of CD25 with a high density of CD127 (19). They also express a high density of CD44 and CD44v6 (19). The finding that 127-hi cells can be equivalently identified and quantified by different research groups validates the 127-hi cell population as a measurable component of the CD4+ T cell compartment. The frequency of circulating 127-hi cells in patients with T1D might be controlled by their expression of CD25, CD127, CD44 and CD44v6. Thus, CD25 and CD127 mediated signaling after binding to circulating IL-2 and IL-7, respectively, promotes the survival and expansion of T cell populations (53). However, in inflammatory environments, including in patients with T1D, activated T cells shed CD25 and CD127 into the circulation where they bind their respective cytokines lowering the concentration of circulating IL-2 and IL-7 (54-56). During hyperglycemia, sCD127 is glycated making it inaccessible to IL-7 and ineffective as an inhibitor of IL-7 signaling (54). CD44 and CD44v6 are receptors for the extracellular matrix component hyaluronan (HA) signaling through
which induces CD25 expression (57) and inhibiting cell apoptosis (58-60). However, during inflammation HA breaks down into low molecular weight forms that fail to cross link CD44 and CD44v6 (61). Taken together these data suggest that the level of 127-hi cells in patients with T1D reflects the level of inflammation.

The Th2 bias of 127-hi cells was originally reported in healthy people (21) and shown again here in patients with T1D. 127-hi cells in healthy (21) and in patients with T1D, shown in this study, secrete significantly higher quantities of the Th2 cytokine IL-4 than other CD4 memory cells giving them the potential to be more potent inhibitors of pathogenic Th1/Tc1 type responses. This Th2 bias combined with the knowledge that a higher frequency of 127-hi cells correlates with longer partial remission and preserved β-cell function suggests that 127-hi cells might play an active role in delaying disease progression by inhibiting the expansion of Th1 cells via Th2 cell-mediated immune deviation (22-24). 127-hi cells might also regulate disease progression via their expression of CD44v6, signaling through which can induce the expression of Foxp3, IL-2, TGF-β and IL-10 (57,58), factors that can promote both Treg cells and Tr1 cells (62,63). This provides additional potential mechanisms by which 127-hi cells might protect from disease progression.

Immune deviation by Th2 cells, and immune regulation by Treg and Tr1 cells requires close proximity between the regulatory cell and the effector cells. Such regulatory function takes place in lymphoid organs, including lymph nodes (LN) where T cells priming takes place (41), and in the peripheral tissue (40). CM cells, including 127-hi CM cells, express the homing receptors CCR7 and CD62L allowing them to circulate between the blood and lymphatic system via LN (42,43). It is possible that while in the LN, 127-hi CM cells create a Th2-type microenvironment capable of deviating the differentiation of primary autoantigen-reactive T cell responses towards the less inflammatory Th2 type and away from the pro-inflammatory Th1-type.
Previously published studies have shown significant associations between age (11,64-68) and partial remission. Consistent with these findings we show that the probability of remaining in remission (survival probability) is greater in patients who are older at diagnosis. However, when we use multivariate analysis to test the relative strength of the relationships between age and gender, 127-hi cell frequency, BMI and baseline IDAA1c with LoR, when all of these parameters are combined 127-hi cell frequency is the only covariate that predicts length of partial remission. Moreover, 127-hi cell frequency is a significant predictor of age, and vice versa, which is consistent with the notion that 127-hi cell frequency and function might explain the association between partial remission and age.

Consistent with the concept that partial remission is actively controlled by a form of immune regulation, the duration of persistent insulin secretion is negatively related to levels of inflammation and positively associated with abundances of circulating activated regulatory T cells measured near clinical onset (69). Moreover, several investigators have investigated the possibility that levels of circulating cytokines, chemokines, other molecules and immune cell subsets can be used as biomarkers to predict partial remission. Elevated levels of IL-6, a Th2-type cytokine that is pro-inflammatory when in its bound to soluble IL-6 receptor and anti-inflammatory when not (70), is positively associated with remission (71). Elevated levels of IL-10, also a Th2-type cytokine, is also positively associated with remission (72) while serum levels of IFN-γ are negatively associated with remission (73). It is possible that levels of these cytokines reflect a change in the frequencies of circulating 127-hi Th2 and Th1 cells. The frequency of apoptotic Treg is negatively related to remission (74). Although these associations are significant, none are sufficiently accurate to be considered biomarkers. Similarly, although baseline 127-hi cell frequency is significantly associated with length of partial remission, and probability of remaining
in remission and preserving β-cell function, suggesting a mechanistic link, the associations are not sufficiently strong for 127-hi cell frequency to be used as a biomarker for clinical outcome. An additional advantage to better understanding the mechanism of partial remission is that markers that accurately reflect that mechanism might be used as biomarkers to predict the beginning, duration and end of the remission period.

Alefacept is an LFA-3 dimer fused to the Fc portion of IgG1 and was first used as an anti-inflammatory drug to treat psoriasis (47-50). Alefacept depletes its target cells by binding to CD2, which is expressed at different levels on EM, CM and naïve T cells in the order EM>CM>naïve. Those cells that express the highest density are depleted the most effectively (51,52). Because EM cells are a known pathogenic effector cell in T1D Alefacept was also identified as a treatment for T1D (2,45,46). Alefacept did not significantly delay T1D progression in the treated group as a whole compared to the placebo group, however, it did preserve β-cell function in several patients for at least 2 years (2). Retrospective analysis of flow cytometry data shows that Alefacept depletes both EM and CM cells but that EM cells were depleted more efficiently (2). In contrast, Treg were not depleted (2). Reanalysis of these data by our group revealed that 127-hi cells, preferentially 127-hi EM cells, were also significantly depleted but there was little evidence of an effect on 127-hi CM and 127-hi naïve cells. Moreover, further analysis showed that the probability of remaining in remission after Alefacept treatment was significantly greater in those patients with the highest frequency of both total 127-hi cells and 127-hi CM cells at baseline, but there was no relationship with 127-hi EM and 127-hi naïve cells. These data suggest that the correlation between 127-hi cells and protection from disease progression is linked to either the 127-hi cell population as a whole, or with CM cells.
Further research will expose the cellular and molecular pathways that drive the association between 127-hi cells and partial remission and verify whether 127-hi cells play a role in prolonging the duration of remission and by which mechanism. Elements within mechanistic pathways that explain the relationship between 127-hi cells and disease progression might be used as biomarkers to select the patients most likely to respond favorable to immunotherapy.

**Methods**

**Patient population:** Flow cytometry data and samples were obtained from patients in four different sources for this study, ITN, SRDRI, MCW and UQ. Table 1 shows the source of patient data and samples described in Figures 2 through 10 and Tables 2 through 4. Prior to this collaborative study, PBMC from the T1D patients recruited by ITN, SRDRI, MCW and UQ were labeled at the respective collaborator laboratories with monoclonal antibodies specific for CD3, CD4, CD25 and CD127 to identify CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory cells (Treg) using flow cytometry. Treg were identified by their expression of CD3, CD4 and CD25 and a low density of CD127 (28). The 127-hi cell population is identified with the same antibody panel used to identify Treg, except that 127-hi cells are identified by their expression of CD3, CD4, CD25 and a high density of CD127 (19). For the same patients, each collaborator site also measured and recorded insulin dose, HbA1c, and C-peptide levels at either 3 or 6 monthly intervals for at least 2 years.

**Healthy subject population:** Whole blood from healthy donors was obtained by SDBRI 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 SDBRI IRB.
Whole blood was collected in heparin and processed within 2 hours. PBMC were isolated using standard methods and frozen in liquid nitrogen.

**Measurement of partial remission and β-cell function using IDAA1c and C-peptide AUC:** A standard formula, HbA1c (%) + (4 x insulin dose (U/kg per 24 hours), 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 less than 9 indicates the partial remission period (\(^{75}\)). In this study the end of partial remission is between the last visit when IDAA1c is equal to or less than 9 and the first visit when IDAA1c is greater than 9. **Length of remission** is the time between diagnosis and end of remission. Stimulated C-peptide AUC was calculated over 120 minutes using the trapezoidal rule, with observed C-peptide values at 0, 15, 30, 60, 90, and 120 minutes. **Fasting C-peptide** was measured in pmol/mL.

**Good and poor glucose control:** For some experiments, patients were stratified for analysis into those with either good glycemic control, defined as having a length of partial remission longer than 1 month, or poor glycemic control, defined as having a length of partial remission shorter than 1 month.

**T cell subsets identified by Flow Cytometry:** Fluorochromes, vendors, catalog numbers and registry identifiers for all antibodies used in Flow Cytometry experiments, including isotype control antibodies, are as shown in Supplemental Table 1. Vials of PBMC from patients with T1D were thawed and stained with the antibody panels listed in Supplemental Table 2. Healthy subject PBMC were thawed and used as positive controls for mAb staining in each experiment.

To identify CD4\(^+\), CD4\(^+\) CD25\(^+\) CD127\(^{hi}\) (127-hi), CD4\(^+\) CD25\(^-\) (CD25-neg) T cells and Tregs (CD25\(^+\) CD127\(^{low}\)) we used either the BD Biosciences Treg cocktail (BD Biosciences Cat
or with individual antibodies for CD3, CD4, CD25 and CD127. The MFI for CD3, CD4, CD25 and CD127 on 127-hi, CD25-neg and Treg cells analyzed at each of the four collaborative sites is shown in Supplemental Figure 1. No differences were seen in the relative frequency of 127-hi, CD25-neg and Treg cells from thawed PBMC compared to freshly isolated PBMC (Supplemental Figure 3). The relative frequency of naïve (CD45RA⁺, CD45RO⁻, CCR7⁺), central memory (CM; CD45RA⁻, CD45RO⁺, CCR7⁻), and effector memory (EM; CD45RA⁻, CD45RO⁺, CCR7⁻) in total CD4⁺, 127-hi and CD25-neg was determined by cell surface phenotype using anti-CCR7, anti-CD45RA and anti-CD45RO. Treg were identified as CD3⁺ CD4⁺ CD25hi CD127low as previously described (28). To identify Th1 and Th2 cell subsets within CD4⁺, 127-hi and CD25-neg memory cell populations, PBMC were also labeled for CD45RO, CXCR5, CXCR3, and CCR4 to identify pre-committed Th1 (CD45RO⁺ CXCR5⁻ CXCR3⁺ CCR4⁻) and pre-committed Th2 (CD45RO⁺ CXCR5⁻ CXCR3⁻ CCR4⁺) (76,77) memory cells. To identify cells that express transcription factors for either the Th2 (GATA-3), Th1 (T-bet) or Th17 (RORγt) cells subsets, PBMC were washed twice in RPMI (Invitrogen) with 10% human AB serum and rested at 37°C overnight. Cells were resuspended in RPMI 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 x 10⁶ cell per ml with 50ng/ml PMA (Sigma) and 1µM Ionomycin (Sigma). 1 µl of Brefeldin A (BD Bioscience) per ml medium was added at the beginning of the culture. After 4 hours cultured cells were washed twice. Intracellular expression of either GATA-3, or T-bet, or RORγt was measured. Data were acquired on an LSRII (ITN), Canto II (SRDRI), LSR Fortessa and LSR II (MCW), Fortessa X20 (UQ) and LSR Fortessa or CytoFlex S (SDBRI) and analyzed using FlowJo version 10 (Ashland, OR). Isotype controls were used in every experiment and for every antigen-specific antibody.
Cell subset purification by sorting: Pre-Th1 and pre-Th2 CD25⁻ memory and CD25⁺CD127^{hi} memory cells were identified using the antibodies described in the section above. The 4 cell populations were sorted on a BD FACSARia high-speed cell sorter. Gates used to sort cell subsets are shown in Supplemental Figure 4.

Measurement of T cell cytokine secretion: Sorted T cell subsets were incubated at 5,000 cells per well in 96 well plates for 48 hours with 2 µl per well anti-CD3/CD28 coated beads (BD Biosciences), 10% human serum (Gemini), HEPES (Gibco BRL), glutamine, penicillin, streptomycin (Irvine Scientific), and 2-mercaptoethanol (Sigma-Aldrich) in RPMI (Invitrogen). Different plates were set up for different time points. After 48 hours of culture, 100 µl of culture supernatant was collected and concentrations of IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and TNF-α were determined by Flow Cytometry using and Biolegend LegendPlex Human Th2 panel.

Statistics: The relationship between the relative frequency of cell subsets determined at different research sites was determined using both Spearman correlation and linear regression. Associations between T cell subsets and probability of remaining in remission and probability of preserving β-cell function was assessed using the Log-rank (Mantel-Cox) test and the Log-rank test for trend. Univariate and multivariate Cox proportional hazards regression analyses were also used to assess the relationship between T cell subsets and clinical variables with LoR as the outcome variable. Covariates included the 127^{hi} cell subset frequency at baseline (time 0), age at diagnosis, gender, baseline IDAA1c, and body mass index (BMI). These results are reported as hazard ratios, with 95% confidence intervals. Multivariate analysis was performed for up to five variables and adjusted for study variability. The relationship between covariates was determined with linear regression. The relationship between levels of fasting C-peptide and stimulated C-peptide AUC was determined using Spearman correlation. Changes in 127^{hi} cell frequency, IDAA1c and C-
peptide levels at 6, 12 and 24 months compared to baseline were determined using the paired student t test. ANOVA followed by Sidak’s multiple comparison test was used to compare Th2:Th1 ratios between more than two cell populations. Differences in cytokine secretion between cells subsets was determined using Wilcoxon matched-pairs signed rank test differences in transcription factors expression was calculated using Welch’s ANOVA (p = 0.003) and Wilcoxon matched-pairs signed rank test. The effect of Alefacept on the frequency of circulating 127-hi cells over 24 months was assessed using repeated measure ANOVA. All analyses were performed with Graphpad Prism and SPSS V21.0 (IBM Corporation, 2012). A p value of less than 0.05 is considered statistically significant.

**Study approval**

All data reported in this study were either collected by each collaborative organization as part of studies conducted prior to the study described in this manuscript, or, generated at SDBRI by reanalysis of previously generated data, or generated at SDBRI from staining vials of frozen PBMC samples obtained from ITN and collected by ITN as part of their prior studies. The sample and data analysis completed specifically for this study, as described in this manuscript, was approved by the San Diego Biomedical Research Institute IRB under Exemption 4.

**Author contributions**

A.N. generated the data for Figure 6, reanalyzed data for all Figures and Tables, contributed to data analysis and to the writing of the manuscript. B.L contributed to data generated for Figures 7 and 8. R.M. contributed to data generated for Figure 1. T.T.L. analyzed data shown in Figures 3,
4, 5, 9 and 10, and Tables 2, 3 and 4. A.M. contributed to flow cytometry data analysis for Figure 2. I.G. and B.P. contributed to clinical data collection for patients from SRDRI. G.G. collected clinical samples and performed flow cytometry staining. R.G. contributed to data generated for Figure 3 and Tables 2, 3 and 4. A-S.B and J.B and M.H. contributed to data used in Figures 3 and 6D and Tables 2, 3 and 4. R.T. provided data from U.Q. M.H. provided data from MCW. M.B. contributed to identifying the clinical collaborators and provided data from SRDRI. E.S. coordinated the transfer of data and samples from ITN, contributed to the design of statistical analysis, and to the writing of the manuscript. ITN provided samples and data. J.D.D. designed the research study, coordinated the collaborative team, samples and data. J.D.D. also analyzed the data and wrote the manuscript. All authors reviewed and approved the final manuscript.

**Acknowledgements**

Funding for this study was provided by NHMRC grant 1071822 to RT and NCI R01 CA185349 grant to JDD. We would like to thank Dr. Jerry Nepom for helpful discussion during the preparation of this manuscript. Portions of the research reported in this publication were performed using samples from clinical trials of the Immune Tolerance Network, which is supported by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health under Award Number UM1AI109565. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
References


67. Bowden SA, Duck MM, Hoffman RP. Young children (< 5 yr) and adolescents (> 12 yr) with type 1 diabetes mellitus have low rate of partial remission: Diabetic ketoacidosis is an important risk factor. *Pediatr Diabetes*. 2008;9(3 PART 1):197-201.


Figure 1. A schematic to identify 127-hi cells. Frozen PBMC isolated from healthy adults were labeled for CD3 CD4 CD25 and CD127. Co-expression of CD25 and CD127 on gated CD3+ CD4+ cells is presented in logical (A) and logarithmic (B) scale. (C) Isotype control staining for both CD25 and CD127 expression is shown in red. For all three plots (A-C), 127-hi cells are in the box in the upper right-hand quadrant and co-express CD127 and CD25 (CD25+ CD127hi), Treg cells are in the box in the lower right quadrant and express a low density of CD127 and a high density of CD25 and CD25-neg (25-neg) cells are in the upper and lower left quadrants. The data are representative of more than 10 healthy donor PBMC samples.
Figure 2. 127-hi cells are equivalently quantified at different research sites  Flow cytometry data generated by investigators at ITN (A, B and C), SRDRI (D, E and F), MCW (G, H and I) and UQ (J, K and L) were used to determine whether the frequency of total CD4⁺ (A, D, G and J), 127-hi (B, E, H and K) and Treg (C, F, I and L) cells is equivalent when quantified by ITN and SRDRI (A-C), SRDRI and SDBRI (D-F), MCW and SDBRI (G-I) and UQ and SDBRI (J-L). The data were generated using PBMC from T1D patients. Samples were analyzed at baseline from the 12 patients at ITN (n = 12), 39 from SRDRI (n = 39), 22 from MCW (n = 22) and 11 from UQ (n = 11). PBMC were labeled for CD3 CD4 CD25 and CD127. Each symbol represents an individual time point for each patient. The relationship between the relative frequency of each cell subset determined at different sites was determined using both Spearman correlation (r) and linear regression ($R^2$). Values for r, $R^2$ and the p values are shown on each panel.
Figure 3. The probability of remaining in remission (survival probability) is greater in patients who have a higher baseline relative frequency of 127-hi cells and are older at diagnosis. The effect of the relative frequency of 127-hi cells (A), age (B), and IDAA1c level (C) at baseline on the probability of staying in remission was determined. Patients were stratified by (A) having a relative frequency of 127-hi cells either equal to or greater than the mean (n = 40, solid line), or lower than the mean (n = 44, dashed line), or (B) being either older than 17 years at diagnosis (n = 8, solid line), or between 9 and 17 years (n = 50, dashed line), or younger than 9 years (n = 26, solid line), or (C) by having a baseline IDAA1c either lower than 7.5 (n = 8, solid line) or between 7.5 and 9 (n = 11, dotted line), or higher than 9 (n = 62, dashed line). Statistical significance was determined using Log-rank (Mantel-Cox) test. For panel (A) p = 0.02 for 127-hi cell frequency greater than the mean compared to below the mean. Panel (B) p = 0.01 for panel for the age group greater than 17 years of age compared to the group younger than 9 years, and no significant differences between other groups. Panel (C) no significant differences between groups. (D) Relative frequency of baseline 127-hi for all samples used in this figure. The log-rank test for trend was also used for
panel (B) – \( p = 0.003 \), and panel (C) – \( p = 0.13 \) (not significant). Differences between groups are considered significant when \( p < 0.05 \).

**Figure 4.** The probability of preserving \( \beta \)-cell function is greater in patients with a higher relative frequency of 127-hi cells at diagnosis. (A) The relative frequency of 127-hi cells was determined in PBMC collected by ITN (\( n = 9 \)) and SRDRI (\( n = 19 \)) at baseline from T1D patients with good glucose control. Fasting C-peptide levels were measured in each patient at 12 months post-diagnosis. Patients were stratified into groups based on having either equal to or greater than the mean frequency of 127-hi cells (\( n = 13 \), solid line), or lower than the mean frequency of 127-hi cells (\( n = 15 \), dashed line) at baseline. Statistical significance was determined using the Log-rank (Mantel-Cox) test. (B) The relative frequency of 127-hi cells at baseline for patient data used in the analysis shown in panel (A). Each symbol represents an individual patient. (C) In a different cohort of patients with good glucose control fasting C-peptide levels and stimulated C-peptide AUC were measured at baseline and the correlation between the 2 C-peptide values determined using Spearman correlation (\( n = 36 \)).
Figure 5. The relative frequency of 127-hi cells decreases with time post-diagnosis. The frequency of 127-hi cells (A, B), IDAA1c levels (C) and stimulated C-peptide AUC (D) were determined at baseline (time 0 months), and at 6, 12 and 24 months post-baseline (n = 12). The fold change for each parameter was compared to baseline using the paired student t test. *** and **** indicates a p value of 0.0009-0.0001 and <0.0001 respectively. Panel (B) shows the relative frequency of each individual at each time point. A p value of 0.05 and below is considered significant.
Figure 6. 127-hi cells from people with T1D are predominantly memory cells with a Th2 bias. The frequency of naïve, CM and EM cells in either 127-hi cells or CD25-neg cells or total CD4⁺ T cells in PBMC collected at baseline from patients with T1D was determined (n = 48). The pie chart shows the mean of each subset within 127-hi (A), CD25-neg (B) and total CD4⁺ (C) cells, and the bar graphs show the mean ± SEM for each cell subset within each population. (D) In a separate experiment, PBMC from T1D patients (n = 21) collected at baseline were evaluated for the relative frequency of pre-committed Th2 cells and pre-committed Th1 cells (D) within 127-hi memory cells, CD25-neg memory cells and CD4⁺ memory cells. Statistical differences between groups for all panels was calculated using ANOVA (p<0.0001) followed by Sidak’s multiple comparison test. Data are shown as mean ± SEM. **** indicates a p value <0.0001.
Figure 7. 127-hi pre-Th2 memory cells secrete significantly more Th2-type cytokines than CD25-neg memory cells. PBMC collected at baseline from patients with T1D (n = 10) were labeled for CD3, CD4, CD45RO, CXCR5, CXCR3, CCR4, CD25, and CD127 and the 127-hi pre-Th2 (closed circles) and CD25-neg pre-Th2 (open circles) cells were sorted. Cells were stimulated with anti-CD3/anti-CD28 beads for 48 hours and supernatants harvested. IL-4 (A), IL-13 (B), IL-5 (C), IL-10 (D), IFN-γ (E), IL-2 (F), IL-6 (G), and TNF-α (H) levels were measured. Statistical significance was calculated using Wilcoxon matched-pairs signed rank test. Data are shown as mean ± SEM. ** indicates p = 0.009-0.001.
Figure 8. The 127-hi cell population contains significantly more GATA-3$^+$ cells and fewer T-bet$^+$ cells than CD25-neg cells. PBMC from T1D patients, collected at baseline, were stimulated for 4 hours with PMA and ionomycin. Stimulated cells were labeled for CD3 CD4 CD45RO CD25 and CD127 and either intracellular GATA-3 (closed circles, n = 9), or T-bet (open circles, n = 9), or RORγt (closed triangles, n = 6). A. Relative frequency of GATA-3, T-bet and RORγt in either 127-hi cells or CD25-neg cells. B. Co-expression of CD127 and CD25 on CD3$^+$CD4$^+$CD45RO$^+$ memory cells. Rectangles show the gating strategy to identify either CD25-neg or 127-hi cells. C-E are representative plots showing expression of GATA-3 (C), T-bet (D) and RORγt (E) in 127-hi and CD25-neg cells. Statistical significance in A was calculated using Welch’s ANOVA (p = 0.003) and Wilcoxon matched-pairs signed rank test. Data are shown as mean ± SEM. ** indicates p = 0.009-0.001 and * indicates p = 0.05-0.01.
Figure 9. Treatment with Alefacept depletes circulating 127-hi EM cells (A) Data generated from T1DAL study participants by ITN using PBMC collected at baseline (before treatment began) from both the placebo and treated groups were reanalyzed at SDBRI to determine the level of cell surface expression of CD2 on 127-hi EM, CM and naïve cells compared to EM, CM and naïve cells in the total CD4+ T cell population, respectively (n = 48). The relative frequency of total 127-hi cells (B), 127-hi EM cells (C), 127-hi CM cells (D) and 127-hi naïve cells (E) was determined at either 3, or 6-month intervals from baseline to 2 years post-baseline in the T1DAL study treated (n = 33) and placebo (n = 16) groups. Analysis of data shown in (B) was performed using repeated measure ANOVA for months 3, 6, 9, 12, 18 and 24. **, *** and **** indicates a p value of 0.009-0.001, 0.0009-0.0001 and <0.0001 respectively.
Figure 10. The probability of remaining in remission (survival probability) is greater in Alefacept-treated patients who have a higher baseline relative frequency of 127-hi cells. Patients were stratified based on having either lower than the mean (dashed line) or equal to or greater than the mean (solid line) relative frequency of total 127-hi cells (A, n = 11>mean, n = 20<mean), 127-hi CM cells (B, n = 13>mean, n = 18<mean), 127-hi EM cells (C, n = 16>mean, n = 15<mean), and 127-hi naïve cells (D, n = 9>mean, n = 22<mean) at baseline. Statistical significance was determined using the Log-rank (Mantel Cox) test.
Table 1. Source, number, gender and age of samples and data used

<table>
<thead>
<tr>
<th>Figure and Table number ¹</th>
<th>Sample source ²</th>
<th>Data source ³</th>
<th>Number ⁴</th>
<th>Male</th>
<th>Female</th>
<th>Age ⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>SDBRI</td>
<td>SDBRI</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>19-29</td>
</tr>
<tr>
<td>Figure 2</td>
<td>ITN⁶ SRDRI</td>
<td>ITN SRDRI</td>
<td>16</td>
<td>12</td>
<td>4</td>
<td>13-32</td>
</tr>
<tr>
<td></td>
<td>MCW UQ</td>
<td>MCW UQ</td>
<td>39</td>
<td>18</td>
<td>21</td>
<td>1-16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22</td>
<td>10</td>
<td>12</td>
<td>5-17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td>4</td>
<td>7</td>
<td>9-15</td>
</tr>
<tr>
<td>Figure 3 and Tables 2-6</td>
<td>ITN⁶ SRDRI</td>
<td>ITN SRDRI</td>
<td>12</td>
<td>9</td>
<td>3</td>
<td>13-32</td>
</tr>
<tr>
<td></td>
<td>MCW UQ</td>
<td>MCW UQ</td>
<td>39</td>
<td>18</td>
<td>21</td>
<td>1-16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22</td>
<td>10</td>
<td>12</td>
<td>5-17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td>4</td>
<td>7</td>
<td>9-15</td>
</tr>
<tr>
<td>Figure 4A</td>
<td>ITN⁷ SRDRI</td>
<td>ITN SRDRI</td>
<td>9</td>
<td>7</td>
<td>2</td>
<td>13-32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19</td>
<td>12</td>
<td>7</td>
<td>2-15</td>
</tr>
<tr>
<td>Figure 4B</td>
<td>ITN⁷</td>
<td>ITN</td>
<td>36</td>
<td>23</td>
<td>9</td>
<td>12-34</td>
</tr>
<tr>
<td>Figure 5</td>
<td>ITN⁷</td>
<td>ITN</td>
<td>12</td>
<td>9</td>
<td>3</td>
<td>13-32</td>
</tr>
<tr>
<td>Figure 6A, 6B and C</td>
<td>ITN⁷</td>
<td>ITN</td>
<td>48</td>
<td>27</td>
<td>21</td>
<td>12-34</td>
</tr>
<tr>
<td>Figure 6D</td>
<td>UQ</td>
<td>UQ</td>
<td>11</td>
<td>4</td>
<td>7</td>
<td>9-15</td>
</tr>
<tr>
<td></td>
<td>ITN⁸ SDBRI</td>
<td>SDBRI</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>12-16</td>
</tr>
<tr>
<td>Figure 7 and 8</td>
<td>ITN⁷ SDBRI</td>
<td>SDBRI</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>13-24</td>
</tr>
<tr>
<td>Figure 9A</td>
<td>ITN⁷</td>
<td>ITN</td>
<td>48</td>
<td>27</td>
<td>21</td>
<td>12-34</td>
</tr>
<tr>
<td>Figure 9B, C, D and E</td>
<td>ITN⁷</td>
<td>ITN</td>
<td>49</td>
<td>27</td>
<td>21</td>
<td>12-34</td>
</tr>
<tr>
<td>Figure 10</td>
<td>ITN⁸</td>
<td>ITN</td>
<td>31</td>
<td>16</td>
<td>15</td>
<td>12-34</td>
</tr>
</tbody>
</table>

1. There are a total of 8 figures and 6 tables (including this table). All available data were used to address the questions posed in each figure and table.

2. Frozen PBMC or whole blood were collected from one of 5 different collaborative groups, SDBRI, ITN, SRDRI, MCW and UQ.

3. Raw flow cytometry data were collected from one of 5 different collaborative groups, SDBRI, ITN, SRDRI, MCW and UQ.

4. The number of either healthy subjects or T1D patients in each test.

5. Age in years at diagnosis.

6. T1DAL study flow cytometry data from the placebo group. In Figures 2, 3 and 5 data from 12 placebo patients were used. These were the only patients in the T1DAL placebo group for which IDAA1c data were available. For Figure 4A data from 9 placebo patients were used because only 9 had good glucose control and C-peptide data (n = 9).

7. T1DAL study flow cytometry data from both treated and placebo groups.

8. T1DAL study PBMC samples (2 from the treated and 2 from the placebo groups) plus START study PBMC samples from 6 of the treated group.
9. Frozen PBMC samples from the T1DAL study, placebo group
10. T1DAL study flow cytometry data from the treated group

Table 2. Cox model multivariate analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Parameter Estimate</th>
<th>p value</th>
<th>Hazard Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25^CD127^{hi}</td>
<td>-0.06207</td>
<td>0.0227</td>
<td>0.940</td>
</tr>
<tr>
<td>BMI^6</td>
<td>-0.04905</td>
<td>0.3323</td>
<td>0.952</td>
</tr>
<tr>
<td>Age^7</td>
<td>-0.04260</td>
<td>0.1929</td>
<td>0.958</td>
</tr>
<tr>
<td>Baseline IDAA1c^8</td>
<td>0.02038</td>
<td>0.7029</td>
<td>1.021</td>
</tr>
<tr>
<td>Gender (female)</td>
<td>0.00774</td>
<td>0.9790</td>
<td>1.008</td>
</tr>
</tbody>
</table>

1. Measurement recorded for each patient (n = 84)
2. The most likely value of the parameter
3. Chi-square tests of the null hypothesis that the parameter estimate for that covariate is 0
4. The change in hazard for a one unit change in the covariate
5. Relative frequency of 127-hi cells at baseline
6. Body mass index at baseline
7. Age at diagnosis in years
8. IDAA1c at baseline
**Table 3. Best fit model**

<table>
<thead>
<tr>
<th>Parameter $^{1}$</th>
<th>Parameter Estimate $^{2}$</th>
<th>p value $^{3}$</th>
<th>Hazard Ratio $^{4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25+CD127$^{hi}$</td>
<td>-0.07185</td>
<td>0.0278</td>
<td>0.931</td>
</tr>
<tr>
<td>BMI$^{5}$</td>
<td>0.00455</td>
<td>0.9347</td>
<td>1.005</td>
</tr>
</tbody>
</table>

1. Measurement recorded for each patient  
2. The most likely value of the parameter  
3. Chi-square tests of the null hypothesis that the parameter estimate for that covariate is 0  
4. The change in hazard for a one unit change in the covariate  
5. Relative frequency of 127-hi cells at baseline  
6. Body mass index at baseline
### Table 4. Multivariate analysis add back

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Parameter Estimate</th>
<th>p value</th>
<th>Hazard Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25⁺CD127⁺ hi</td>
<td>-0.06207</td>
<td>0.0227</td>
<td>0.940</td>
</tr>
<tr>
<td>CD25⁺CD127⁺ hi BMI⁺</td>
<td>-0.07185</td>
<td>0.0278</td>
<td>0.931</td>
</tr>
<tr>
<td>CD25⁺CD127⁺ hi Age⁺</td>
<td>-0.06447 0.00468</td>
<td>0.0588</td>
<td>0.938</td>
</tr>
<tr>
<td>CD25⁺CD127⁺ hi Baseline IDAA1c⁺</td>
<td>-0.06382 0.03119</td>
<td>0.0211</td>
<td>0.938</td>
</tr>
<tr>
<td>CD25⁺CD127⁺ hi Gender</td>
<td>-0.06529 0.17499</td>
<td>0.0189</td>
<td>0.937</td>
</tr>
</tbody>
</table>

1. Measurement recorded for each patient
2. The most likely value of the parameter
3. Chi-square tests of the null hypothesis that the parameter estimate for that covariate is 0
4. The change in hazard for a one unit change in the covariate
5. Relative frequency of 127-hi cells at baseline
6. Body mass index at baseline
7. Age at diagnosis in years
8. IDAA1c at baseline
Table 5. Linear regression of covariates

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1</td>
<td>82</td>
<td>9.52</td>
<td>0.0028</td>
</tr>
<tr>
<td>BMI</td>
<td>1</td>
<td>68</td>
<td>6.94</td>
<td>0.0105</td>
</tr>
<tr>
<td>Baseline IDAA1c</td>
<td>1</td>
<td>78</td>
<td>0.20</td>
<td>0.6587</td>
</tr>
<tr>
<td>Gender (female)</td>
<td>1</td>
<td>82</td>
<td>0.38</td>
<td>0.5372</td>
</tr>
</tbody>
</table>

1. Parameters tested as effectors of 127-hi cell frequency
2. The number of degrees of freedom
3. The denominator degrees of freedom
4. The F value is the result of the test where the null hypothesis is that all of the regression coefficients are equal to zero
5. The null hypothesis is that the predictor has no effect on the outcome variable evaluated with regard to this p value
6. Age at diagnosis in years
7. Body mass index
8. IDAA1c at baseline
Table 6. The effect of interaction between age and BMI in their relationship with 127-hi cell frequency

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 6</td>
<td>1</td>
<td>66</td>
<td>10.91</td>
<td>0.0020</td>
</tr>
<tr>
<td>BMI 7</td>
<td>1</td>
<td>66</td>
<td>10.35</td>
<td>0.0015</td>
</tr>
<tr>
<td>BMI Age 8</td>
<td>1</td>
<td>66</td>
<td>8.75</td>
<td>0.0043</td>
</tr>
</tbody>
</table>

1. Parameters tested as effectors of 127-hi cell frequency
2. The number of degrees of freedom
3. The denominator degrees of freedom
4. The F value is the result of the test where the null hypothesis is that all of the regression coefficients are equal to zero
5. The null hypothesis is that the predictor has no effect on the outcome variable evaluated with regard to this p value
6. Age at diagnosis in years
7. Body mass index
8. Interaction between age and BMI