Title: Regulatory T Cell Responses in Participants with Type 1 Diabetes after a Single-Dose of Interleukin-2: A Non-Randomized, Open Label, Adaptive Dose-Finding Trial


Affiliations:
1 JDRF/Wellcome Trust Diabetes and Inflammation Laboratory, Department of Medical Genetics, National Institute for Health Research Cambridge Biomedical Research Centre, Cambridge Institute for Medical Research, University of Cambridge, Cambridge Biomedical Campus, Cambridge, UK.
2 National Institute for Health Research Cambridge Clinical Trials Unit, Cambridge University Hospitals NHS Foundation Trust, Cambridge Biomedical Campus, Cambridge, UK.
3 Department of Immunobiology, Faculty of Life Sciences & Medicine, King's College London, London, U.K. National Institute of Health Research Biomedical Research Centre at Guy's and St Thomas' National Health Service Foundation Trust and King's College London, London, UK.
Wellcome Trust/MRC Institute of Metabolic Science, Department of Medicine, National Institute for Health Research Cambridge Biomedical Research Centre, Cambridge Biomedical Campus, Cambridge, UK.

Division of Virology, Department of Pathology, University of Cambridge, Addenbrooke’s Hospital, Cambridge, UK.

Public Health England, Clinical Microbiology and Public Health Laboratory, Addenbrooke’s Hospital, Cambridge, UK.

MRC Biostatistics Unit Hub for Trials Methodology Research, Cambridge Institute of Public Health, Cambridge, UK.

† These authors contributed equally to the work

‡ Present address, Department of Mathematics, Imperial College London, South Kensington Campus, London, UK

∞ Present address, Experimental Medicine and Immunotherapeutics, Department of Medicine, National Institute for Health Research Cambridge Biomedical Research Centre, Cambridge Institute for Medical Research, University of Cambridge, Cambridge Biomedical Campus, Cambridge, UK.

* fw211@cam.ac.uk and jat34@cam.ac.uk
Abstract

Background

Interleukin-2 (IL-2) has an essential role in the expansion and function of CD4+ regulatory T cells (Tregs). Tregs reduce tissue damage by limiting the immune response following infection and regulate autoreactive CD4+ effector T cells (Teffs) to prevent autoimmune diseases, such as type 1 diabetes (T1D). Genetic susceptibility to T1D causes alterations in the IL-2 pathway, which supports Tregs as a cellular therapeutic target. Proleukin (recombinant human IL-2), which is administered at high doses to activate the immune system in cancer immunotherapy, is now being repositioned to treat autoinflammatory disorders at lower doses by targeting Tregs.

Methods and Findings

To define the Proleukin dose response for Tregs and to find doses that increase Tregs physiologically for treatment of T1D, we took a statistical and systematic approach by analyzing the pharmacokinetics and pharmacodynamics of single doses of subcutaneous Proleukin in the “Adaptive study of IL-2 dose on regulatory T cells in type 1 diabetes” (DILT1D) a single centre non-randomised, open label, adaptive dose-finding trial with 40 adult participants with recently-diagnosed T1D. The primary endpoint was the maximum percentage increase in Tregs (defined as CD3+CD4+CD25^{high}CD127^{low}) from their baseline frequency in each participant measured over the 7 days following treatment. There was an initial learning phase with five pairs of participants, each pair receiving one of five pre-assigned single doses from 0.04 x 10^6 to 1.5 x 10^6 IU/m^2, in order to model the dose response curve. Results from each participant were then incorporated into interim statistical modelling to target the two doses most likely to induce 10% and 20% increases in Treg frequencies. Primary analysis of the evaluable population (N = 39) found that the optimal doses of Proleukin to induce 10% and 20% increases in Tregs were 0.101 x 10^6 IU/m^2 (standard
error, SE, = 0.078 and 95% confidence interval, 95% CI, = -0.0520, 0.254) and 0.497 x 10^6 IU/m^2
(SE = 0.092 and 95% CI = 0.316, 0.678), respectively. On analysis of secondary outcomes, using
a highly sensitive IL-2 assay, we observed plasma concentrations of the drug at 90 minutes that
exceeded the hypothetical Treg-specific therapeutic window determined in vitro (0.015-0.24
IU/ml), even at the lowest doses (0.04 x 10^6 and 0.045 x 10^6 IU/m^2) administered. We observed a
rapid decrease in Treg frequency in the circulation at 90 minutes and at day 1, which was dose
dependent (mean decrease 11.6%, SE = 2.39% and range 10% - 48.2%, N = 37), rebounding at
day 2 and increasing to frequencies above baseline over 7 days. Teffs, natural killer cells and
eosinophils also responded, with their frequencies rapidly and dose-dependently decreased in the
blood, then returning to, or exceeding, pretreatment levels. Furthermore, we detected a dose-
dependent down modulation of one of the two signaling subunits of the IL-2 receptor, the β chain
(CD122), on Tregs and a reduction in their sensitivity to Proleukin, at 90 minutes (mean decrease
-58%, SE = 2.8%, 9.8% - 85.5%, N = 33), day 1 and 2 post treatment. Due to blood volume
requirements as well as ethical and practical considerations the study was limited to adults and to
analysis of peripheral blood only.

Conclusions
The DILT1D trial results, most notably an early altered trafficking and desensitisation of Tregs
induced by a single ultra-low dose of Proleukin that resolve within 2-3 days, inform the design of
the next trial to determine a repeat dosing regimen aimed at establishing a steady-state Treg
frequency increase of 20-50% with the eventual goal of preventing T1D.

Trial Registrations
International Standard Randomized Controlled Trial Number Register (ISRCTN27852285) and at
ClinicalTrials.gov (NCT01827735)
Author summary

Why was this study done?

- Insulin replacement, the only approved therapy for the treatment of type 1 diabetes (T1D), treats the symptoms but not the underlying cause of disease, namely immune-mediated destruction of insulin-producing β cells of the pancreas.

- We selected Proleukin, recombinant interleukin-2 (IL-2), as a candidate immunotherapy to prevent or delay autoimmunity because previous genetic and phenotypic analyses indicate a major role for the IL-2 pathway in the development of T1D.

- IL-2 is critical for maintaining the function of T regulatory cells (Tregs) that prevent autoimmunity. Therefore, the first step in our approach to T1D prevention was to determine the single doses of Proleukin that increase Treg numbers within the physiological range, aiming to mimic the protection against T1D afforded by the risk-reducing alleles of the IL-2 pathway.

What did the research do and find?

We successfully employed a state-of-the-art dose-finding, open label, adaptive clinical trial design to determine the doses of Proleukin in 40 patients with T1D to raise Treg frequencies by 10% and 20%,

- Increased Treg frequencies induced by Proleukin were preceded by a peak of Proleukin in the blood at 90 minutes that caused a transient dose-dependent decrease of Tregs and other cell subsets in the circulation.
Following treatment with a dose of Proleukin, Tregs had a decreased sensitivity to IL-2 that returned to baseline on day 3 after treatment.

What Do These Findings Mean?

- We showed that it is possible to conduct an adaptive dose-finding trial to estimate two drug doses based on an immune cell biomarker as a primary outcome.

- Partial desensitisation of Tregs might help explain why some patients in previous trials with daily dosing regimens are non-responders to Proleukin, suggesting that in our future trials we would not administer drug on a daily basis.
Introduction

Type 1 diabetes (T1D) is the second most common chronic diseases of children and yet insulin replacement is the only therapy currently approved to treat the disease. Insulin replacement corrects insulin deficiency and hyperglycemia but does not treat the underlying autoimmune T lymphocyte-mediated destruction of the insulin-producing β cells of the pancreatic islets [1]. The result is a lifelong requirement for intensive insulin treatment that increases the risk of hypoglycemia and stops the majority of patients from achieving adequate metabolic control to prevent the long term complications of retinopathy, neuropathy and/or nephropathy [2, 3]. Given these clinical outcomes there have been intensive efforts over the last four decades to develop immunotherapies that suppress β-cell autoimmunity in order to preserve endogenous insulin production using standard trial methodologies [4-8]. While these approaches have achieved some success, an alternative strategy to identify potential immunotherapies is to use pathophysiological insights from human genetic associations to identify modifiable pathways [9, 10].

Our interest in modulation of the IL-2 pathway as a potential T1D therapy was initiated by mapping of a major susceptibility locus in the non-obese diabetic (NOD) mouse model to the IL-2 gene [11]. The mechanism for T1D susceptibility in NOD mice was identified as a reduction of Treg function through reduced IL-2 production by the NOD susceptibility allele [12]. In humans allelic variation of the IL-2 receptor gene, IL2RA, encoding the α subunit, CD25 was identified as a susceptibility determinant for T1D [13]. Genome-wide association studies identified a number of other T1D susceptibility genes in the IL-2 pathway, encoding critical proteins mediating immune activation and regulation (IL-2, IL-21, BACH2, PTPN2, IL-10) [14, 15]. Analysis of the effect of the common susceptibility allele of the major causal variant in the IL2RA region showed that it decreased expression of CD25 on the surface of effector CD4+ T cells (Teffs) and regulatory
CD4+ T cells (Tregs) [16, 17]. Furthermore, the IL2-IL21 T1D susceptibility region has been found to be associated with decreased numbers of IL-10 producing islet-specific CD4+ Teffs [18]. Thus deficiencies in the IL-2 pathway and in genes expressed in CD4+ T cells, including Tregs, could contribute to disruption of the homeostatic equilibrium between IL-2-dependent Tregs and Teffs, which produce IL-2 to sustain the Tregs and enable them to regulate Teff activity during infections and the maintenance of self-tolerance in health [19-23].

High-dose Proleukin (recombinant human IL-2) is currently licensed for the treatment of adults with metastatic renal cell cancer and metastatic melanoma administered intravenously at 600,000 IU/kg every 8 hours for 5 days followed by 9 days rest and a further two treatment cycles, if clinically tolerated [24, 25]. Proleukin at these doses induces the activation and proliferation of Teffs and natural killer cells (NK) to destroy tumor cells. However, it was found that high-dose Proleukin also expands the Treg pool such that patients with melanoma with enhanced expansion of Tregs had a worse outcome compared to those with fewer Tregs [26]. These observations, combined with the results of several preclinical murine studies in which Proleukin selectively increased Tregs and prevented or reversed autoimmune diabetes, have focused efforts on the development of much lower doses of Proleukin to treat T1D and other immune disorders [27, 28].

Most recently, proof of concept of the clinical benefit of low-dose Proleukin has been reported for chronic Graft versus Host Disease (cGVHD) [29, 30], Hepatitis C virus induced vasculitis [31], systemic lupus erythematosus (SLE) [32] and alopecia areata [33]. In T1D a small safety study was conducted in which relatively high doses of Proleukin (4.5 × 10^6 IU, 3 times a week for 1 month) were combined with rapamycin resulting in a transient loss of endogenous insulin production in participants that was reversed on withdrawal of the therapy [34]. This has led to discontinuation of this combination [35]. In contrast, using lower doses of Proleukin alone
(range $0.33 \times 10^6 - 3.0 \times 10^6$ IU) administered daily for 5 days caused a transient dose-dependent increase in Tregs without impairment of pancreatic β-cell function over this time period [36]. The treatment regimen developed from this dose-finding study has now been carried forward into the DIABIL-2 clinical trial to access the efficacy and safety of ultra-low doses of recombinant human IL-2 in children and adults with T1D [37].

These studies and trials share an initial induction phase of daily or every other day IL-2 administration, or long-term daily dosing, modelled on cancer therapy and a standard approach in drug delivery. In contrast, our goal is to establish a regular dosing regimen that could be given to children and adults shortly after diagnosis of T1D over a long period of time that maintains residual β-cell function by protecting β cells from autoimmune Teffs through improving Treg function within physiological ranges. Our underlying rationale is that we aim to mimic the protection from disease afforded by the risk-reducing alleles of the IL-2 pathway genes by establishing in experimental medicine studies a dosing regimen that induces and maintains a stable, steady-state increase in Tregs within the normal physiological range, in the hope that long-term treatment does not compromise immune defence against pathogens, responses to vaccines or cancer immunosurveillance. This approach might avoid the potential complications and risks associated with prolonged administration of immunosuppressive treatments that preclude their use in the T1D prevention setting [38, 39]. More intensive treatment regimens may be required for autoinflammatory disorders in which the tissue damage caused by the ongoing immune response causes pathology that may be life threatening without immunosuppression, such as in SLE and cGVHD. In contrast, the immune system of patients diagnosed with T1D is relatively immunocompetent with only small differences found on analysis of lymphocyte phenotypes between cases and controls [18, 40, 41].
Our first step in optimizing the delivery of Proleukin in T1D is to understand the kinetics of the biological effects of a single dose in adults with T1D. Here, in the “Adaptive study of IL-2 dose on regulatory T cells in type 1 diabetes” (DILT1D), we aimed to investigate the effects of single-dose Proleukin by initiating our observations of multiple biomarkers before treatment (baseline) and at 90 minutes post-drug administration with a 9 week follow up. The study used an adaptive design to initially estimate the Treg dose response to Proleukin and then to identify the two doses of Proleukin that increase Tregs minimally (10% relative to pretreatment) or maximally (20%) while remaining within the normal Treg frequency range.

Materials and Methods

Adaptive study design

The DILT1D trial was a 9 week, single-centre, non-randomized, open label, adaptive dose-finding trial that was conducted at National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre, Addenbrooke’s Hospital, Cambridge, United Kingdom. The study included 12 visits: after a screening visit, the Proleukin dose was administered on the treatment day 0 and the participants were followed up, including blood sampling, 90 minutes after administration and subsequently on days 1, 2, 3, 4, 7, 9, 14, 21 with a final follow up visit on day 60. According to protocol amendments visits between the 7th and 21st days were given 48 hours flexibility, and a safety visit on day 5 was removed. During protocol development statistical simulations suggested that a sample size of 40 would give an informative estimate of the dose and target responses. The trial consisted of two phases: a learning phase (with ten individuals) and adaptive phase (with 30 individuals). In the learning phase, the participants received $0.04 \times 10^6$, $0.16 \times 10^6$, $0.6 \times 10^6$, $1.0 \times 10^6$, $1.5 \times 10^6$ IU/m² of Proleukin in ascending order with each dose administered to two
participants before escalation. Once the tenth participant had completed 7 days of follow up the clinical Treg and safety data were extracted from the trial database and a preplanned interim analysis was carried out by the clinical trial statisticians by fitting a candidate set of parametric models to estimate the dose response curve. A statistical analysis report was generated and delivered to the Dose Determining Committee (DDC; physician (SN, ME or FWL), statistician (ME, SB or APM) and scientist (JAT or LSW) within 7 working days in order to select the next doses to achieve targets of Treg increases from baseline of 10% (minimal Treg increase) and 20% (maximal). The DDC also decided on the allocated doses for the first participants of the adaptive phase. As soon as each participant of the adaptive phase had completed the 7th day visit, the accumulated data of the primary endpoint were analysed by the statisticians of the study. The DDC was responsible for reviewing the interim analyses, accumulated safety data as well as allocating doses for the subsequent individuals of the adaptive phase. For each model, a set of doses to assign to the next patient cohort was identified that maximized the predicted reduction in the area of the 95% confidence region around the two estimated target doses (i.e. minimizing the determinant of the covariance matrix) at the following interim analysis. The DDC resolved the infrequent cases where the models recommended different sets of doses to assign. The DILT1D protocol and novel governance structures that were developed to make regular dose decisions by committee were published prior to completion of the study and final analysis of the endpoints [42].

**Primary endpoint**

The primary endpoint is based on the percentage of CD4 Treg (defined as CD3+CD4+CD25^{high}CD127^{low}) cells within the CD3+CD4+ T cell gate following treatment with Proleukin as measured by fluorescence-activated cell sorting (FACS). The maximum value
observed in each participant's profile of the follow-up period will be identified and the percentage change from the baseline value defines the primary endpoint.

Secondary endpoints

The following secondary endpoints were measured following treatment with Proleukin:

1. Change in full blood count (FBC).
2. Change in Treg number, phenotype and proliferation measured by FACS.
3. Change in cytokines and soluble receptors.
4. Change in Treg cell epigenetic profile (methylation status).
5. Change in Teff number, proliferation and phenotype measured by FACS.
6. Change in lymphocyte subset cell number, proliferation and phenotype subsets and NK and Natural Killer T (NKT) cells measured by FACS.
7. Change in metabolic control as measured by self-monitoring of blood glucose, laboratory measurement of blood glucose and glycated haemoglobin (HbA1c) and C-peptide.

Exploratory endpoints

The following exploratory endpoints were measured following treatment with Proleukin:

1. Change in intracellular T and NK cell signalling was measured \textit{in vitro} by FACS.
2. \textit{In vitro} dose response to IL-2 was measured to assess changes in intracellular T-cell signalling.
3. Change in Treg function was measured by a T cell suppression assay.
Safety assessments

The safety and tolerability of the treatment was assessed in trial participants by clinical history, insulin use, physical examination, temperature, blood pressure, heart rate, 12-lead electrocardiograms, glucose, HbA1c, clinical laboratory tests and adverse event recording.

Study participants, consent procedure and safety assessments

Potential participants were eligible for the study if they had a T1D duration of less than two years, one autoantibody positive (anti-islet cell, anti-GAD, anti-IA2, anti-ZnT8), aged 18 to 50 years and living in the European Union. The date of diagnosis of T1D was established by referring physicians, diabetes specialist nurses, review of register records and self-reporting by potential participants. Potential participants were excluded from the study if they had a history or evidence on screening of severe organ dysfunction, unstable diabetes, pregnancy, malignancy (within 5 years), hepatitis B or C, human immune deficiency virus, organ transplantation and/or donation of more than 500 ml of blood in the 2 months prior to treatment.

Potential participants interested in enrolling in DILT1D were provided with a patient information sheet and an informed consent form to review. Individuals were given a minimum of 24 hours to consider the information provided and then were contacted to determine if they remained interested in participating in the study or if they had any further queries. Interested potential participants were then invited to attend for an appointment where the Chief Investigator (CI) or delegate discussed the study with the participant, who then provided written informed consent before undergoing screening and any other trial related procedures.
Ethical approval, sponsorship and trial registrations

The trial was sponsored by the University of Cambridge and Cambridge University Hospitals NHS Foundation Trust. Ethical approval for the study was granted by the Health Research Authority, National Research Ethics Service, England (approval number: 13/EE/0020) on 18th of February 2013. The study was registered at the International Standard Randomized Controlled Trial Number Register (ISRCTN27852285) on the 26th of March 2013 and at ClinicalTrials.gov (NCT01827735) on the 4th of April 2013.

Clinical Immunophenotyping

All clinical flow cytometry (FACS) was performed following good clinical practice at the Department of Clinical Immunology, Addenbrooke’s Hospital, Cambridge, UK within 4 hours of phlebotomy. Operators were blinded to the Proleukin dose allocated. 2.6 ml of peripheral whole blood was collected into EDTA tubes and 50 µl stained with specific fluorochrome-conjugated antibodies at room temperature for 15 min to identify Tregs as CD3+CD4+CD25highCD127low T cells. The clones used were anti-CD3 (clone SK7, phycoerythrin [PE]-Cy7 labeled; BD Biosciences), anti-CD4 (clone RPA-T4, FITC-labeled; BD Biosciences), anti-CD127 (clone HIL-7R-M21, PE-labeled; BD Biosciences), anti-CD25 (clone M-A251 and 2A3, allophycocyanin [APC]-labeled, BD Biosciences), anti-CD45RA (clone HI100, APC-Cy7-labeled, Biolegend) and anti-CD62L (clone DREG-56, PerCP/Cy5.5 labeled, Biolegend). Red cells were then lysed (BD FACS Lysing Solution), the cells washed and resuspended in BD Cell Fix and then immediately analyzed on a BD Canto II flow cytometer utilizing FACSDiva software (BD Biosciences). In parallel a whole blood 6-color BD TBNK Multitest™ assay using BD Trucount Tubes according to the manufacturers’ instructions (BD Biosciences) was run to determine the relative and absolute concentration of lymphocyte subpopulations, including T, B and NK cells.
For each participant, 30 ml peripheral whole blood was collected into Lithium Heparin tubes and processed according to the workflow (S1 Fig). To determine the frequencies and phenotypes of NK and T cell subsets in whole blood within 4 hours of phlebotomy, multicolour FACS was performed using the antibodies shown in S1 and S2 tables. A full standard operating procedure is provided in Supplementary (S1 Materials). For surface staining, 150 µl of whole blood was incubated with fluorochrome-conjugated antibodies at room temperature for 45 min. Red cells were then lysed (BD FACS Lysing Solution), washed and then analyzed on a BD Fortessa flow cytometer. For intracellular staining, cells were treated with Fixation & Permeabilization reagent (eBioscience) after labelling with surface antibodies, washed twice with Permeabilization Buffer and then incubated with an intracellular antibody panel (shown in grey in S1 Table) at 4°C for 45 min. Samples were analyzed on a BD Fortessa flow cytometer using FACSDiva software (BD Biosciences) and FlowJo (Treestar, Inc). Where possible, subset frequencies such as % Treg were expressed as an average obtained from the analysis of tubes 1-6 (S1 Table). To measure the effects in vitro of Proleukin on CD25 and CD122 expression on lymphocyte subsets, heparinized, venous blood (25 mls) from healthy volunteers was divided into ten 2 ml aliquots. Aliquots of whole blood were cultured with either 50 units/ml Proleukin or medium alone (equivalent volume PBS + 2% BSA) at time points 0, 15, 30, 90 and 180 minutes. Samples were incubated at 37 °C while being rotated at 35 degrees above horizontal. All samples were processed at 180 minutes for surface staining as per the described standard operating procedure, except, following addition of the antibody panel samples were quenched on ice for 2 minutes and incubated at 4 °C to prevent ongoing stimulation. The number of participants contributing to each secondary outcome analysis
can vary due to missing data. The missing data with description of the quality control process are summarized in (S2 Materials).

**Cell sorting**

For *in vitro* functional assays, within 4 hours of phlebotomy 3 ml whole blood samples were stained with fluorochrome-conjugated antibodies (S3 Table) for 1.5 hours at room temperature followed by the addition of 30 ml RBC Lysis Buffer (eBioscience) for 10 minutes. Cells were washed and then resuspended in 500 µl XVIVO + 1% AB serum for cell sorting with a BD FACS Aria II flow cytometer and FACSDiva software (BD Biosciences). CD4+ Tregs and Teffs were sorted by the expression pattern of CD25 and CD127: Tregs defined as CD25\textsuperscript{high}, CD127\textsuperscript{low} and Teff (non-Tregs) as CD25\textsuperscript{low-medium}, CD127\textsuperscript{low-high}. Naïve and memory Teffs and Tregs were defined using CD45RA. Central and effector memory Teffs were defined by the presence or absence of CD62L, respectively.

**Flow cytometric analysis for pSTAT5**

pSTAT5 staining was carried out as previously described [17]. Whole blood was either directly lysed and fixed *ex vivo* (Lyse/Fix buffer, BD Biosciences) or stimulated for 30 minutes at 37°C with the indicated concentrations of Proleukin diluted in X-VIVO 15 + 1% human AB serum (Lonza) then lysed and fixed. In some instances, 300 µl of blood was incubated for 2 hours at 37°C alone, with anti-human IL-2 antibody (20 µg/ml) (clone: MQ1-17H12, purified NA/LE, BD Biosciences) or isotype control antibody (20 µg/ml) (clone RTK2758, purified NA/LE, BD Biosciences) prior to addition of lyse/fix buffer. After washing, cells were permeabilized with methanol (>99.9%, Sigma-Aldrich), washed and stained with fluorescence-conjugated antibodies detailed in S4 Table. Cells were acquired using a BD Fortessa flow cytometer (BD Biosciences) using FACSDiva software. To generate normalized results per lymphocyte cell subset for *in vitro*
Proleukin dose titration studies, the pSTAT5 MFI of cells not incubated with Proleukin was subtracted from the pSTAT5 MFI at each Proleukin dose and then divided by the pSTAT5 MFI observed in the maximal response of that cell subset.

pSTAT5 staining of Proleukin stimulated cryopreserved peripheral blood mononuclear cells (PBMCs) was carried out as previously described [43] in a batch manner where each batch included 14 samples from participants and two biological controls that were kept constant for all experiments to enable normalization of data influenced by day-to-day staining variation. Assays were conducted using selected participants across the dose groups at relevant time points in regard to Treg responses. PBMCs were stained with anti-CD4-APC-eFluor780 (clone SK3, eBioscience), anti-CD25-PE (clone 2A3, M-A251, BD Biosciences), anti-CD45RA-PE-Cy7 (clone HI100, BioLegend), anti-FOXP3-Alexa Fluor 488 (clone 236A/E7, BD Biosciences) and pSTAT5 (pY694)-Alexa Fluor 647 (clone 47, BD Biosciences) for 1 hour at 4-8 °C. Data acquisition was performed on a BD FACSCanto II (BD Biosciences) and analyzed using FlowJo (Treestar).

**In vitro suppression assays**

Suppression assays were performed in selected participants with Treg responses in V-bottom 96-well plates using cryopreserved PBMCs by coculturing 500 sorted CD4^+CD25^{int-}

^k^CD127^+CD45RA^- mTeffs in the presence or absence of CD4^+CD25^{hi}CD127^lo Tregs at various ratios (Treg:Teff, 0:1 to 1:8) with 1 x 10^3 CD19^+CD4^- B cells. Samples were stimulated with PHA (4 µg/ml; Alere) and incubated at 37°C, 5% CO_2 for 6 days. Proliferation was assessed by the addition of 0.5 µCi/well [^3^H] thymidine (PerkinElmer, Waltham, MA) for the final 20 h of coculture. All conditions were run in 12 replicates and proliferation readings (CPM) averaged. Any samples with averaged proliferation less than 1,000 CPM from the mTeff wells alone were
excluded. The percentage suppression in each culture was calculated using the following formula:

\[ \text{% suppression} = 100 - \left( \frac{\text{CPM in the presence of Tregs}}{\text{CPM in the absence of Tregs}} \right) \times 100 \]

**Methylation Status**

The methylation status of the FOXP3 Treg-specific demethylated region (TSDR) was analysed using a next-generation sequencing method that assesses the TSDR at single-base resolution [44].

**Cytokine measurements**

Plasma C-reactive protein (CRP) was measured using a custom V-PLEX Human CRP kit according to manufacturer’s instructions (Meso Scale Diagnostics, MSD, Rockville, Maryland, USA) and read on the MSD Sector Imager 6000. Plasma IL-2 was measured using the MSD S-PLEX Human IL-2 assay [45] (limit of quantitation 2 fg/ml) at MSD. In order to convert the plasma fg/ml IL-2 levels provided by MSD to plasma Proleukin IU/ml levels, readings from the MSD S-PLEX Human IL-2 assay at the 90 minutes post-Proleukin administration point for all DILT1D participants were compared to results from an in-house Human IL-2 DELFIA-based immunoassay [46] that has a limit of quantitation of 10 pg/ml using Proleukin (specific activity 16.4 IU/ng) as the standard curve. A linear relationship was observed between the two assays; a conversion of 3.4-fold was required to convert MSD S-PLEX Human IL-2 assay readings to those obtained using a Proleukin standard curve. This conversion factor was applied to all other samples tested at MSD, all of which had been below the limit of detection or quantification in the in-house assay. Plasma soluble CD25 (sCD25) was measured in duplicate using an in house sandwich immunoassay as described in [47].
Statistical analysis

Pre-defined populations of the study

Three populations were defined in this study: Analysis, Evaluable and Safety. The Analysis population includes all participants who received a single dose of Proleukin with a follow-up period of 7 days and the primary endpoint was observed. The Evaluable population includes all participants who received a single dose of Proleukin, the primary endpoint was observed and were not withdrawn from the study on clinical grounds. The Safety population includes all participants who received a single dose of Proleukin and were followed up for any period of time during the trial.

Primary Endpoint Analysis – Interim analyses

The primary endpoint analysis was carried out at all conducted interim analyses of the trial as well as at the end of the trial using all accumulated data of the Evaluable population. The aim of the primary analysis was to find the best model that describes the dose-response curve, and to identify the doses that achieve the minimum Treg increase (10%) and the maximum Treg increase (20%): targets selected by the DDC of the trial. The relationship of the primary endpoint with dose was explored by fitting a number of candidate models. The candidate models include the linear, the quadratic, the cubic, the logistic and the Emax (with 3 and 4 parameters). The Aikake Information Criterion (AIC) and the deviance of each model were computed as measures of adequacies of fit. The proposed target doses of each model with their standard errors and with a 95% confidence interval were also computed. The primary endpoint analysis at the end of the trial was conducted on both the Evaluable and Analysis populations (S1 Analysis).

Secondary Endpoints Analyses
For each time point and for each response/phenotype we defined two measures: the absolute change and the percentage change, with the absolute change defined as the difference between the time point measurements with the baseline (pretreatment) measurement. The percentage change is defined as the ratio of the aforementioned absolute change with baseline. Based both on the absolute and on the percentage change we defined a number of derived-variables, including the 90 minute measurement (where available), the day 1 measurement and the maximum observed value from day 1 (or from 90 minutes where available) to day 7 times 100. One of the key questions of interest answered is whether the absolute (or percentage) change at a particular time point of interest is zero. We tested this hypothesis using a t-test statistic. Equivalently we have used paired t-tests to compare two responses at a time point of interest as reported in the results. The effect of dose on the derived variables computed using the percentage change was explored by fitting both a linear model and a cubic model. The model with the smallest AIC was chosen as the most representative model for fitting the data. A p-value of association of dose with the response was computed by comparing through an analysis of variance (ANOVA) the two aforementioned models with the model represented only by the intercept term. In the case whether the linear model is the best we report both the dose coefficient as well as the dose p-value, in the case of the cubic model we report only the dose p-value. The effect of dose on the derived variables computed using the absolute change was explored by comparing a series of models that included the effect of baseline of each tested response. The best model was chosen as the one with the smallest AIC. For the cases were the interaction and additive models were the best ones they were compared with the baseline model for finding the effect of dose. A dose p-value was calculated through ANOVA. Similarly, for the models where the dose model was the best, a dose p-value was computed by comparing the dose model with the null model through ANOVA. For Treg CD25 MFI, pSTAT5
responses we were interested in understanding how their measurements changed per day. Using the percentage change of all time points between the 90 minute measurement and day 60 (or day 3 for pSTAT5) we modelled the relationship with dose either through a linear model given by or through a cubic model both of them with no intercept term included. For each response we chose the model with the smallest AIC across the tested days. We subsequently used the best model for predicting the response and a 95% confidence around it for each time point for the two chosen doses derived from the primary endpoint analysis.

**Average response plots and summary statistics**

We have divided the range of doses administered into five dose groups. These five groups are:

- 0.04 - 0.045 x 10^6
- 0.16 - 0.38 x 10^6
- 0.38 - 0.61 x 10^6
- 0.62 - 0.99 x 10^6
- 1.0 - 1.5 x 10^6 IU/m²

with 12, 8, 9, 7 and 4 participants of the Analysis population, respectively. For each time point of either the absolute change or the percentage change we averaged the time measurements of the participants within each dose group. This averaging was performed from the day 1 measurements (or from 90 minutes if applicable) to the day 7 measurements. For the individuals where their visit on day 7 was truncated to an alternative day (± 48 hours, according to protocol amendment), their day 7 measurement was linearly interpolated using the closest measurements to day 7 either way which was subsequently used in the averaged response graphs. Throughout the manuscript the following statistics are reported for each phenotype: mean (standard error; min – max) and the number of individuals (N).
Results

Participant analysis populations, characteristics and study design

DILT1D was an adaptive dose-finding study to increase Tregs as a proportion of total CD4\(^+\) T cells within the physiological range with a sample size of 40. In total, 45 participants were enrolled in the study between March 22\(^{nd}\), 2013 and May 15th 2014, with the first participant recruited on the 8\(^{th}\) of April 2013. Five were ineligible for the study owing to the absence of a minimum of a single T1D-associated antibody. Forty individuals were treated with a single dose of Proleukin (aldesleukin, Novartis) with one participant withdrawn owing to the development of a norovirus infection. Thirty-nine participants were included in the Evaluable population whilst 40 are included in the Safety and Analysis populations (Fig 1A). The demographic characteristics and autoantibody status at screening of the Safety and Analysis populations are shown in Table 1. Participants were recruited from the European Union to the study site at University of Cambridge, UK with 38 from the UK, one from Ireland and one from France [48]. Most participants had recently-diagnosed T1D (N = 36; duration 3 - 24 months) with four newly diagnosed (< 3 months).

The DILT1D study team employed a response adaptive design with a learning phase to find the shape of the dose response curve and an adaptive phase to find the two Proleukin doses that increase the target Treg frequency by 10% and 20% in the best statistical model (Fig 1B and C).

Fig 1 DILT1D study profile and adaptive design. (A) Flow chart showing the allocation of participants to the three predefined study populations: Evaluable, Safety and Analysis. (B) The study was conducted in two phases, a learning phase (140 days) and an adaptive phase (240 days). Individual participants are represented by a horizontal line, the length corresponding to the time from treatment until their final visit. In the learning phase, the first ten participants received a 0.04, 0.16, 0.6, 1.0, or 1.5 \( \times 10^6 \) IU/m\(^2\) dose of Proleukin (color represents dose allocated) in ascending order with each dose being administered to two participants before escalation. In the adaptive phase the Dose Determining Committee (S3 Materials)
met on 19 occasions to review the interim safety data and allocate dose(s) based on analysis of the
accumulated Treg data (shaded area) from all treated participants at that time. (C) The schematic of the
study design illustrating that each participant who passed screening was administered a single dose of
Proleukin and followed for 60 days (11 visits). During the adaptive phase, Treg data for every participant
up to day 7 was included in an interim analysis and then further dose(s) were allocated to the next
participant(s).

Demography (n=40)

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean (SE**, Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity: White</td>
<td>40</td>
<td>--</td>
</tr>
<tr>
<td>Sex: Male/ Female</td>
<td>30/10</td>
<td>--</td>
</tr>
<tr>
<td>Age years</td>
<td>40</td>
<td>30.54 (1.32, 18.35 – 49.08)</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>40</td>
<td>23.88 (0.59, 16.7 – 32)</td>
</tr>
<tr>
<td>Body Surface Area*</td>
<td>40</td>
<td>1.91 (0.03, 1.55 – 2.23)</td>
</tr>
<tr>
<td>Duration of disease (in months)</td>
<td>40</td>
<td>10.74 (0.96, 0.86 – 23.7)</td>
</tr>
</tbody>
</table>

Autoantibody status (n=40)

<table>
<thead>
<tr>
<th>Autoantibody type</th>
<th>N</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Islet</td>
<td>14</td>
<td>--</td>
</tr>
<tr>
<td>Anti-GAD</td>
<td>39</td>
<td>--</td>
</tr>
<tr>
<td>Anti-IA2</td>
<td>19</td>
<td>--</td>
</tr>
<tr>
<td>Anti-ZnT8</td>
<td>26</td>
<td>--</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Autoantibody number</th>
<th>N</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>One positive antibody</td>
<td>11</td>
<td>--</td>
</tr>
<tr>
<td>Two positive antibodies</td>
<td>12</td>
<td>--</td>
</tr>
<tr>
<td>Three positive antibodies</td>
<td>7</td>
<td>--</td>
</tr>
<tr>
<td>Four positive antibodies</td>
<td>10</td>
<td>--</td>
</tr>
</tbody>
</table>

* DuBois formula **SE = standard error

Table 1 Participant characteristics and autoantibody status

We found no differences between baseline haematological parameters and the final visit (complete
blood count, S5 Table; FACS analysis, S6 Table). We noted that there was some clinical evidence
of an improvement in metabolic control with a decrease in the mean glucose and HbA1c
accompanied by reduction in the dose of basal insulin, which is often observed in T1D trials where patients receive a higher degree of specialist care and disease management. There was no evidence of a decrease in random non-fasting C-peptide or the development of thyroid dysfunction (S7 Table).

**Primary endpoint**

The primary endpoint was the maximum percentage increase from the baseline of Tregs in each participant measured over the 7 days following treatment (Fig 2A) in response to adaptive dosing (Fig 2B). Tregs showed a dose response to a single administration of Proleukin with the higher doses leading to larger increases (Fig 2C). This relationship was analysed in several candidate models, with the cubic model being found to be the best one to describe the dose-response curve as it had the smallest deviance and visually fitted the data the best (S2 Analysis). Analysis of the Evaluable population for the primary endpoint found that the optimal doses of Proleukin to induce 10% and 20% increases in Tregs were \(0.101 \times 10^6\) IU/m\(^2\) (standard error, SE, = 0.078 and 95% confidence interval, CI, = -0.052, 0.254) and \(0.497 \times 10^6\) IU/m\(^2\) (SE = 0.092 and 95% CI = 0.316, 0.678), respectively (Fig 2C). The lower confidence limit for the dose to increase Tregs by 10% is negative as a result of the estimated target dose being close to zero.

**Fig 2** T regulatory cell primary endpoint (A) Tregs were defined as the percentage of CD3\(^+\)CD4\(^+\)CD25\(^{high}\)CD127\(^{low}\) cells within the CD3\(^+\)CD4\(^+\) gate measured (B) Individual participant dose allocations and dose groups showing convergence of the study to doses that achieve the two defined Treg targets. (C) A cubic model described the Treg dose response to Proleukin best with dashed lines showing the 10% and 20% Tregs target doses. The shaded areas represent 95% confidence intervals (CI). (Baseline or pretreatment Treg (% CD4 T cells) 6.60% (0.25, 3.5-10.7), N=39) indicating mean (SE; min – max) and the number of individuals averaged (N).
Safety and tolerability

Single doses of Proleukin were well tolerated at all doses with no serious adverse events (AEs) reported (Table 2). Non-serious AEs were clinically graded on severity as mild (easily tolerated), moderate (discomfort) or severe (unable to carry out usual activities) and relatedness to Proleukin administration (causality). There were 45 unexpected non-serious AEs reported by 28 participants in the safety population. Sixteen had a single event, nine had two events and three had three or more events, all resolving without residual effects. Three events (two episodes of nasal congestion on day one and one immediate injection site reaction) were assessed as related to Proleukin and were classified as adverse reactions. A single participant had a severe AE that consisted of a transient self-limiting gastrointestinal event commencing 30 hours after administration of a dose of Proleukin (S2 Fig A). Prior to administration of Proleukin, clinical history, examination and laboratory investigations were normal apart from a low neutrophil count [2.3 x 10^9/L (normal range: 4 x 10^9/L -11 x 10^9/L)]. The clinical presentation and course were consistent with a norovirus infection. To confirm the clinical diagnosis and identify the pathogen, we carried out serology testing for antibodies to norovirus and detected the induction of GII.4 IgG antibody titers at day 9 (S2 Fig B), peaking at day 21 post-infection and declining thereafter, confirming that the participant had been infected with norovirus. Most participants had an expected AE at the injection site consisting a non-itchy, local (1-5 cm) non-painful erythematous rash on day 1 followed by a subcutaneous nodule on day 2 that resolved on average by day 10 (SE=2; range, 1-58 days; S3 Fig). Proleukin had no effect on renal, bone or liver biochemistries during the study (S8 Table).
### Unexpected Adverse Events and Reactions (n=45)

<table>
<thead>
<tr>
<th>Category</th>
<th>Details</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Common Cold (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asthma exacerbation (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hand abrasion (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Headache (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>1</td>
</tr>
<tr>
<td>Relatedness with IL-2</td>
<td>Unrelated</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Unlikely</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Possibly *</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Nasal congestion (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Almost certainly *</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Acute site reaction (1)</td>
<td></td>
</tr>
<tr>
<td>Expected adverse events – Injection site reaction (n=45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type</td>
<td></td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Erythema</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Nodule</td>
<td>29</td>
</tr>
</tbody>
</table>

*Adverse reaction. Values in parentheses are the numbers within each category detail.

**Table 2 Summary of non serious adverse events and reactions (Safety population)**

#### Secondary and exploratory endpoints

**Transient decrease in lymphocytes in blood**

Full blood counts (FBC) analysis stratified by dose showed a decrease in lymphocyte counts in participants treated with doses greater than $0.045 \times 10^6$ IU/m$^2$ on day 1 with a recovery by day 2 (Fig 3A S5 Table). To investigate if the pretreatment lymphocyte count affected the day 1 response a set of candidate models that included the effects of both baseline lymphocyte count and dose was analyzed. The change in lymphocyte counts was found to be associated with dose through a model that depended both on baseline and dose (N=39; dose $p = 0.006$; Fig 3B) with the relationship being negative to baseline meaning the higher the pretreatment lymphocytes the greater the reduction in count on day 1 (S3 analysis). The greatest decrease in lymphocytes was observed in
participants who received the highest doses \((1.0 \times 10^6 \text{ and } 1.5 \times 10^6 \text{ IU/m}^2; \ N = 4)\), with one participant developing a transient asymptomatic lymphopenia \((\text{count } < 1 \times 10^9/L)\) that resolved by day 2. The white blood cell and neutrophil count decreased on average by approximately 10% without a dose response on day 1 while the monocyte and basophil counts were unchanged by Proleukin treatment (S5 Table).

**Fig 3 Lymphocyte responses to a dose of Proleukin (A)** Average response curves of the absolute change of lymphocytes counts across the five dose groups (baseline lymphocytes \(1.78 \times 10^9/L\) \((0.08; 0.95-3.84),\ N=39\) (B) Three dimensional plot of dose, baseline and change in lymphocytes on day 1 with lines representing the vertical projections of points (coloured by dose) on the dose/baseline axis. The surface grid represents the regression model for change in lymphocyte on day 1 (colour scale) showing that the decrease in lymphocytes depends both on dose and pretreatment count.

*Early transient increase in eosinophils is related to both Proleukin dose and pretreatment count*

There was an asymptomatic decrease in eosinophils of approximately 15% at 90 minutes followed by an increase on day 1 that resolved by days 3-4 with six participants developing a transient eosinophilia \((\text{count } > 0.4 \times 10^9/L)\) (Fig 4A, S5 Table). Given the increase and development of eosinophilia we analyzed further the eosinophil counts to explore if the change was related to both dose and baseline counts. We found that the change of eosinophil count on day 1 was dependent on the additive effects of dose through a model that included both the baseline and dose effects \((N = 39, \text{ dose } p = 0.026; \text{ Fig 4B})\). We found that the relationship was positive with a higher pretreatment eosinophil count resulting in a greater increase in eosinophils on day 1.

**Fig 4 Eosinophil response depends on baseline counts and Proleukin dose. (A)** Eosinophil counts showed an initial transient decrease at 90 minutes in a hyperacute response to Proleukin followed by a dose-dependent increase on day 1 with a return to baseline by days 3-4 (average baseline of eosinophils \(0.15 \times 10^9 / L\)).
$10^9/L$ (0.03; 0.04-0.86); $N = 39$). (B) Three dimensional plot of dose, baseline and change in eosinophil counts on day 1 with lines representing the vertical projections of points (coloured by dose) on the dose/baseline axis. The change in eosinophil count is affected by both dose and baseline using a linear dose response model with the grid showing the regression model (colour scale) for increase in eosinophils on day 1 (colour scale), (absolute change in eosinophil count on day 1 = -0.0058 + 0.0693 (dose) + 0.1748 (baseline)).

**Rapid circulatory changes in Treg numbers**

Utilizing clinical grade FACS assays we observed a rapid decrease on day 1 after Proleukin administration in the frequency of Tregs (Fig 5A, S6 Table) and in Treg numbers (Fig 5B). This was followed by increases in Tregs over baseline reaching a maximum by day 3 (SE = 0.31 days; range 1-7 days; $N = 39$) and then a gradual return to, or towards, baseline by day 7 for most participants. At the highest doses there were transient decreases in the counts of CD8+ T cells (S4 Fig A), B lymphocytes (CD19+CD3-; S4 Fig B) and NK cells (CD3-CD56+CD16+CD19-; S4 Fig C) on day 1, recovering to baseline by day 4.

Mechanistic studies were performed in parallel with the clinical grade FACS measurements (Fig 5C), with strong agreement between the measurements of Treg frequency from the two independent sources ($N = 377$, t-test $p = 2.2 \times 10^{-16}$; S5 Fig). Henceforth, the data generated by these mechanistic assays is presented, except otherwise stated.

Measurement of Tregs in whole blood samples after 90 minutes revealed that the reduction was rapid and sustained, remaining below baseline on day 1 at all doses greater than the lowest dose range (Fig 5C). At 90 minutes the decline in Tregs was not dose dependent, but by day 1, the reduction in Tregs was dose dependent with higher doses of Proleukin leading to greater declines in Tregs ($N = 37$, dose $p = 1.32 \times 10^{-5}$; Fig 5D).
Using a recently developed IL-2 assay that is 5000-fold more sensitive than conventional assays we established that baseline levels of IL-2 in the DILT1D participants were 12.17 – 64.07 fg/ml (0.0007 - 0.0036 IU/ml) (Fig 5E), similar to those reported for healthy individuals [45]. At 90 minutes following subcutaneous dosing, which is near the time of peak blood concentrations of Proleukin using this route of administration [49], IL-2 plasma levels ranged from 0.35-27.46 IU/ml depending on the dose delivered and averaged 5.73 IU/ml (SE = 1.07; N = 37) (Fig 5E). There was a linear relationship between the dose of Proleukin administered and the plasma concentration of IL-2 at day 1 in vivo (Fig 5F). All Proleukin doses produced IL-2 blood levels at 90 minutes sufficient to increase pSTAT5 in a portion of Teff and NK CD56\textsuperscript{bright} (CD56\textsuperscript{bright}TCR\textsuperscript{α/β}) cells in addition to a majority of the Tregs (Fig 5G); this finding was confirmed by assessing pSTAT5 levels in various cell types ex vivo at 90 minutes following Proleukin dosing. Proleukin plasma levels remained above the 0.015 IU/ml concentration required to cause signaling in naïve (nTregs) and memory Tregs (mTregs) for up to four days, and above the 0.24 IU/ml concentration required for memory Teff (mTeff) and NK CD56\textsuperscript{bright} cell activation for up to one day, depending on dose. Thus, although Tregs have an approximately 10-fold higher affinity for Proleukin than do Teff and NK CD56\textsuperscript{bright} cells, a completely Treg-specific Proleukin plasma level was not achieved, even at the lowest doses administered.

**Fig 5 Hyperacute Treg response to Proleukin.** (A) Treg proportions as a % of CD4\textsuperscript{+} T cells (average Treg level 6.6% (0.24; 3.5 - 10.7); N = 39) and (B) Treg counts following injection of Proleukin as measured by the clinical grade FACS assay in conjunction with the BD TBNK Multitest\textsuperscript{TM} assay are shown (average baseline Treg count 0.06 x 10\textsuperscript{9}/L (0.01; 0.02-0.14); N=39). (C) Tregs as a % of CD4\textsuperscript{+} T cells were measured in the mechanistic FACS assay (average Treg level 6.99% (0.27; 3.93 - 10.74); N = 37). (D) The decline of Tregs in blood on day 1 fits a cubic model (shaded area presents a 95% confidence interval (CI), N = 37).
(E) Plasma IL-2 levels following Proleukin dosing. The lines mark the 0.015 and 0.24 IU/ml concentrations that are the threshold levels of Proleukin at which Tregs, and Teff and NK CD56^{bright} cells, respond, respectively (Fig 5G). (F) Relationship between the dose of Proleukin administered and the plasma concentration of IL-2 at day 1 in vivo. (G) Proleukin dose-response curves generated in whole blood from DILT1D participants for pSTAT5 responses within individual cell populations on day 60 post-treatment (mean with bars showing 95% CI; N = 39). [(A)-(C) averaged response plots across the five dose groups. (D) and (F) show the best fitted models with 95% CI.]

**Change in Treg phenotype**

In order to determine if there was a change in Treg phenotype in blood after treatment we analyzed expression of CD25 and CD122 on Tregs before and after treatment. At baseline mTregs have approximately on average a 55% higher expression of CD25 than nTregs (S6 Fig (parts A and B)) and 90% higher levels of CD122 (S6 Fig (parts C and D). Because of this heterogeneity between Treg subsets we assessed mTregs and nTregs separately as well as together as total Tregs. At higher Proleukin doses there were initial small decreases of CD25 expression on mTregs at 90 minutes (Fig 6A) followed by a large dose-dependent increase on day 1 in response to treatment (N = 37, dose coefficient = 35.24, dose \( p = 6.68 \times 10^{-8}; \) Fig 6B). The increase in CD25 was maximal on day 1.59 (0.13; 1-4 days; N = 37) and was dose dependent in total Tregs (N = 37, dose coefficient = 35.40, \( p = 3.02 \times 10^{-8}; \) S7 Fig). A greater increase was observed in the mTreg compared to the nTreg subset (maximum observed percentage change of the CD25 MFI: mTreg = 43.1% change (3.68; 1.52 - 106) and nTreg = 28.4% change (2.99; 1.78 - 89.19); N=37; t-test \( p = 8.15 \times 10^{-9}\)). This mTreg increase was sustained over at least four days for doses greater than 0.045 x 10^6 IU/m^2 (Fig 6A). In contrast to changes observed for Treg CD25 expression, CD122 levels on
the mTregs remaining in the blood were substantially lower at 90 minutes post-dosing and this was dose dependent at day 1 (N=33, dose $p = 1.17 \times 10^{-6}$; Fig 6 C and D). This lower CD122 expression was still prevalent at day 2 with CD122 levels on mTregs returning to baseline by day 3 at all doses except at the two highest, $1.0 \times 10^{6}$ and $1.5 \times 10^{6}$ IU/m² with similar changes in CD122 observed in nTregs (S6 Fig D). A rapid disappearance of surface CD122 was also observed \textit{in vitro} when Tregs were incubated with Proleukin (S8 Fig A).

We investigated if there had been functional changes in the Treg subpopulations remaining in blood after Proleukin treatment focusing on phenotypes that could be altered by lower CD122 cell-surface levels. Whole blood samples at baseline and 90 minutes post-dosing were compared for their ability to respond to Proleukin at a concentration that saturates the high affinity IL-2 receptor (1000 IU/ml). We found that the mTregs responded to the high concentration of Proleukin \textit{in vitro} with lower pSTAT5 levels as compared to mTregs at baseline ($N = 36, p = 1.28 \times 10^{-9}$; Fig 6E). In order to replicate and extend our findings we measured pSTAT5 in Tregs subsets from cryopreserved PBMCs isolated at baseline and day 1 that were incubated with 0.4 IU/ml Proleukin to provide a submaximal stimulation. In the mTreg subset we observed that dosing with Proleukin resulted in a transient dose-dependent decrease in the percentage of mTregs that became pSTAT5$^+$ on day 1 post-dosing ($N = 21$, dose $p = 9.60 \times 10^{-4}$; Fig 6F). In contrast, although nTregs did show a reduction in pSTAT5 at 90 minutes post-administration of Proleukin ($N = 37, p = 0.0002$; Fig 6G), it was less profound than that observed for mTregs and there was not a consistent reduction in signaling when PBMC were stimulated with 0.4 IU/ml of Proleukin (Fig 6H).

\textbf{Fig 6 Phenotypes of the residual circulating Tregs at day 1 (A and B)} CD25 expression is increased on memory Tregs (mTregs) (average baseline CD25 MFI on mTreg $= 7412$ (SE $= 181.08$; range $= 5119 - 9393$), $N = 37$). \textbf{(C and D)} Concurrently there was a dose-dependent reduction in CD122 on mTregs in blood
(baseline CD122 MFI on mTreg = 444.2 (13.98; 288 - 616), N=33). (E) There is a reduction in pSTAT5 levels in mTregs incubated with a saturating concentration of Proleukin (1000 IU/ml) in vitro when assessing blood obtained 90 minutes post-dosing of Proleukin. (F) At day 1 post-dosing, there is a dose-dependent reduction in the percentage of mTregs that are pSTAT5+ following incubation with 0.4 IU/ml Proleukin in vitro (% of pretreatment time point mTregs that are pSTAT5+ following Proleukin incubation: 56.25% (SE = 1.60; range = 43.23 - 71.03) N = 22). (G) There was a reduction in pSTAT5 levels in nTregs assessed 90 minutes post-dosing when the cells are incubated with a saturating dose of Proleukin (1000 IU/ml) in vitro. (H) At day 1 post-dosing, there was not a consistent change from baseline in the percentage of nTregs that are pSTAT5+ following incubation with 0.4 IU/ml Proleukin in vitro (baseline % of nTregs that are pSTAT5+ following incubation with 0.4 IU/ml Proleukin: 58.01% (1.65; 40.83 - 69.88) N = 21). [(A) and (C) averaged response plots across the five dose groups. (B), (D) and (E) show the best fitted models with 95% CI]

**Sustained Treg phenotypes and functional responses**

As measured by ex vivo pSTAT5 levels, mTregs were more responsive to in vivo Proleukin treatment than nTregs at 90 minutes (N = 36; t-test comparison p = 1.46 \times 10^{-9}; Fig 7A and S9 Fig A). The pSTAT5 response in mTreg at 90 minutes and at day 1 was related to dose suggesting the maximum response had been observed (N = 36, p = 0.001 and N = 36, p = 8.32 \times 10^{-9}, respectively) (Fig 7B). The day 1 ex vivo pSTAT5 signal could be neutralized in vitro by the addition of anti-IL-2 antibodies to the assay (S9 Fig B), which confirms that it is the Proleukin remaining in the blood that is responsible for the elevated levels of pSTAT5 ex vivo.

Similarly, a dose-dependent relationship was found for CTLA-4 MFI (N = 31; p = 4.92 \times 10^{-6}) (Fig 7C and D), Treg FOXP3 MFI (N = 33, p = 3.58 \times 10^{-5}, dose coefficient= 71.18; Fig 7E
and F), and Ki-67 expression (mTregs) (N = 33, p = 0.03, dose coefficient = 39.14; Fig 7G) on day 1, with maximum increases on average by day 1.61 (0.27; 0.1 - 7 days), 1.74 (0.29; 0.1 - 8 days), and 2.16 (0.27; 0.1 - 7 days), respectively. The findings on day 1 were consistent with an independent analysis of cryopreserved PBMCs from day 3 post-dosing, when the Treg count in the blood is greatest. The circulating mTregs and nTregs were found more responsive to Proleukin with higher CD25 (N = 15, p = 3.76 x 10^{-5} and N = 15, p = 2.83 x 10^{-6}, respectively; S9 Fig (parts C and D)) and FOXP3 expression than at baseline (N = 15, p = 0.01 and N = 15, p = 0.005, respectively; S9 Fig (parts E and F)). The overall effect of a dose of Proleukin on Tregs by day 3 was to have increased the number of fully suppressive, demethylated FOXP3+ Tregs, which we presume have trafficked through tissues and been activated by self-antigens (Fig 7 H, I and K).

We used the data generated in the study to develop a predictive model for each of these Treg responses and applied it to investigate the effects of the optimal doses of Proleukin to induce 10% and 20% increases in Tregs, 0.101 x 10^6 IU and 0.497 x 10^6 IU/m^2, respectively. At 0.101 x 10^6 IU/m^2 Treg pSTAT5 peaks at 90 minutes and then returns to baseline by day 2, whilst CD25 MFI and Treg frequencies are increased for 3 days. The 0.497 x 10^6 IU/m^2 dose had a more sustained predicted response showing elevated Treg pSTAT5 for up to three days and total Treg frequencies not quite at baseline at 7 days with an increase in CD25 expression for up to 7 days (Fig 7J).

**Fig 7 In vivo Treg phenotypes and functional responses to Proleukin (A and B) mTr**eys had their maximum pSTAT5 response to treatment at 90 minutes and a detectable response is sustained for up to four days at the higher doses and is dose dependent on day 1 with a cubic dose response (average baseline pSTAT5 MFI = 7.36 (0.33; 4.64 - 12.53); N = 36). **(C-F) Following activation mTregs had a dose-dependent increase in CTLA-4 and FOXP3 expression returning to baseline by days 3-4 post-dosing (mTreg CTLA-4 MFI = 1539**
(G) Concurrent with these changes on day 1 there was an increase in proliferation of mTregs in blood (baseline Ki-67+ mTreg = 15.27% (0.86; 7.10 - 30.20), N=33) (H) Intracellular staining of Tregs from whole blood for FOXP3 showed an increase in FOXP3+ Tregs on day 3 (FOXP3+ Tregs/CD4+ T cells = 6.44% (0.25; 4.03 - 10.3); N = 37). (I) Analysis of FOXP3 gene demethylation on total Tregs, CD62Llow (effector memory, EM) and CD62Lhigh (central memory, CM) CD4+ memory T cells sorted from whole blood at pretreatment, post-treatment (day 3) and the last visit (day 60) showing stability of this Treg phenotype. (K) Tregs expanded in vivo at day 3 post-Proleukin suppressed in vitro proliferation of autologous T effectors equivalently to Tregs at day in a suppression assay across the dose range (Treg:Teff ratio) tested. Error bars in (I) and (K) represent SEs. (J) Predictive cubic models based on the study data for CD25, pSTAT5 and Treg responses at the doses identified to increase Tregs by 10% and 20%. The error bars present the 95% confidence intervals around the predictions by these models.

**T effector cell responses**

There were rapid dose-dependent changes in the proportions of mTeffs following Proleukin treatment, with increases at 90 minutes at doses less than $0.61 \times 10^6$ IU/m$^2$ and decreases below this dose suggesting that there may be a dose threshold for this response ($N = 37, p = 6.40 \times 10^{-5}$, dose coefficient = -9.23; Fig 8A, B). At the two highest doses ($1.0 \times 10^6$ and $1.5 \times 10^6$ IU/m$^2$; $N = 4$), there was a sustained increase in mTeffs from days 3-7 (Fig 8A, S10 Fig (parts A and B). pSTAT5 levels in mTeffs increased at 90 minutes, which was dose dependent ($N = 36$, dose $p = 2.71 \times 10^{-7}$; Fig 8C). The increase in pSTAT5 remained detectable on day 1 at all doses greater than $0.045 \times 10^6$ IU/m$^2$ with the two highest doses giving a larger and more prolonged response, an observation verified by the linear dose relationship on day 1 ($N = 36$, $p = 6.24 \times 10^{-4}$, dose
There was a transient small increase in CD69 expression at all doses 90 minutes post-treatment for both mTreg and mTeff (S10 Fig (parts C and D)). There was a small transient reduction in IL-2 sensitivity at 90 minutes in mTeff cells compared to Tregs at all doses that returned to baseline by day 1 (N = 37, t-test $p = 8.18 \times 10^{-5}$; S10 Fig (parts E and F)). There was a rapid dose-dependent reduction in expression of CD25 on mTeffs in blood that remained below baseline until day 3 except in participants who received the two highest doses who had a sustained increase above baseline from day 3 onwards (N = 37, $p = 5.15 \times 10^{-6}$; Fig 8E, F). For CD122 on mTeffs in blood there was initially a dose-dependent decrease (N = 33, $p = 1.46 \times 10^{-3}$; Fig 8G, H) followed by an increase in participants on days 1-4 who received the larger doses. Similar patterns of CD25 and CD122 expression changes were observed in vitro when mTeffs were incubated with Proleukin (S8 Fig B). To determine if there was evidence of proliferation of mTeffs, we measured Ki-67 and found that there was a linear dose relationship (N = 33, $p = 1.93 \times 10^{-3}$, dose coefficient = 145.71; Fig 8I, J).

**Fig 8 Effects of Proleukin on T effector cell number, phenotypes and proliferation.** (A) mTeffs were responsive to Proleukin with their frequencies as a percentage of non-Treg CD4+ T cells altered (B) resulting in opposing effects with lower doses leading to higher mTeff and higher doses leading to reduced frequencies (average baseline mTeff (% mTeffs of non-Treg CD4+ T cells) 61.1% (1.85 SE; range 38.61 - 87.35) N = 37) (C and D) There was increased pSTAT5 in mTeff [mTeff pSTAT5 7 MFI (0.3; 4 -13); N = 36]. (E and F) Concurrently there was a dose-dependent decrease in CD25 (average baseline mTeff CD25 1055 MFI (31; 676 - 1436); N = 37), and (G and H) in CD122 (137 MFI (7; 59 - 248); N = 33). (I and J) There was a dose-dependent increase in proliferation of mTeffs as measured by an increase in Ki-67+ mTeff over the 7 days following treatment (baseline Ki-67+ Teffs = 2.92% (0.32; 0.75-10.4); N = 33).
**NK, NK CD56^{bright} and NKT cell responses**

Treatment at all Proleukin doses caused a decline of total NK cells in circulation at 90 minutes, with the largest decrease in the NK CD56^{bright} cells compared to the CD56^{dim} TCR^{a/ß} (NK CD56^{dim}) cells (N = 38; t-test comparison $p = 1.00 \times 10^{-10}$) (Fig 9A, S11 Fig A). The decline in NK CD56^{bright} cells at 90 minutes was dose dependent (N=38; dose $p = 0.0005$; Fig 9B, NK CD56^{bright} cells were the most responsive NK subset to treatment with a greater increase in NK CD56^{bright} cells compared to NK CD56^{dim} cells (N=32; t-test comparison $p = 6.83 \times 10^{-11}$) (Fig 9A, S11 Fig A) and a larger pSTAT5 response (Fig 9C, S11 Fig B).

At baseline NK CD56^{bright} cells had approximately 250% more expression of CD25 and 100% more CD122 than the NK CD56^{dim} cells (S11 Fig C). Following treatment, there was a rapid dose-dependent reduction in expression of CD25 on the NK CD56^{bright} cells remaining in the blood (Fig 9D, E; N = 37; dose $p = 2.09 \times 10^{-6}$) and this change was greater than the NK CD56^{dim} at 90 minutes (S11 Fig D; N = 37; t-test $p = 5.37 \times 10^{-9}$). In contrast to the Treg response, a substantial decrease in CD25 expression on NK CD56^{bright} cells was observed that remained below baseline for up to 4 days after treatment (Fig 9D). CD122 on NK CD56^{bright} cells transiently decreased by a much more modest amount at 90 minutes as compared to CD25 followed by on day 1, in contrast to CD122 on Tregs (Fig 6C), a dose-dependent linear increase (N = 38; $p = 0.02$; dose coefficient $= 15.10$) that returned to baseline by day 3 (Fig 9F, G). Similar patterns of CD25 and CD122 expression changes were observed in vitro when NK CD56^{bright} cells were incubated with Proleukin (S8 Fig C). To assess the functional effects of treatment on NK homeostasis we measured the proliferation marker Ki-67 and observed that the greatest increase was in the NK CD56^{bright} cells at all doses administered (N = 30; t-test comparison $p = 0.0012$) (Fig 9H, S11 Fig).
E). However, at the $1.0 \times 10^6 - 1.5 \times 10^6$ IU/m² dose range there was a reduction in the specificity since proliferation of both NK subsets was similar (~300% increase; Fig 9H; S11 Fig E).

There was a dose dependent change in the frequency of NKT (CD56⁺TCRα/β⁺) cells in circulation following Proleukin treatment, with a decrease at 90 minutes at doses less than $0.61 \times 10^6$ IU/m² and little change (~10%) at higher doses. Only at the two highest doses, $1.0 \times 10^6$ and $1.5 \times 10^6$ IU/m² was there evidence of a sustained increase (~100%) with a decline towards baseline by day 7 (S11 Fig (parts F and G).

**Fig 9 Effects of Proleukin on NK CD56<sup>bright</sup> cell number, phenotypes and proliferation.** (A and B) NK CD56<sup>bright</sup> cells showed a rapid dose-dependent decline with the majority of cells not in circulation at 90 minutes (NK CD56<sup>bright</sup> cells (% lymphocytes) 0.41 % (0.03; 0.09-0.96); N=38). (C) Concurrent with this decline is a dose-dependent increase in NK CD56<sup>bright</sup> cell pSTAT5 levels (baseline pSTAT5 MFI = 16.55 (0.7; 9.51 - 27.87); N = 37). (D and E) There was a sustained dose-dependent reduction in expression of CD25 (642 MFI (32; 255 - 1148) N = 38) on NK CD56<sup>bright</sup> cells and (F) a transient reduction in CD122 at 90 minutes (G) followed by a linear dose-dependent increase on day 1 (baseline CD122 MFI = 6605 (213; 3786 - 9554); N = 38). (H) The outcome of treatment was increased proliferation of NK CD56<sup>bright</sup> cells (baseline percentage of Ki-67⁺ NK CD56<sup>bright</sup> cells = 9.86% (0.9; 3.35 - 25.9); N = 30).

**Changes in inflammatory markers soluble CD25 and C-reactive protein**

There was a decrease in serum soluble CD25 (sCD25) at 90 minutes followed by an increase in levels of this immune activation marker at day 1 (S12 Fig A). A linear relationship was found between dose and the percentage change of sCD25 on day 1 (N = 39, dose coefficient = 13.94, $p = 0.005$; S12 Fig B). The highest doses (1.0 and $1.5 \times 10^6$ IU/m²) had the longest duration of
response taking 7 days to return to baseline. We measured CRP to determine if there was evidence of an acute phase response following treatment and observed that there was a dose-dependent increase in CRP on day one, where doses greater than $0.62 \times 10^6$ IU/ml are estimated to increase CRP greater than 100% (S12 Fig (parts C and D)).

**Increases in chemokine expression on Tregs**

CXCR3 and CCR6 are chemokine receptors that contribute to the trafficking of T cells between the circulation and tissues [50]. In regard to the proportion of mTregs expressing CXCR3 and CCR6, we found no differences in response to Proleukin between chemokine-defined subsets of Tregs (S13 Fig and S14 Fig). However, the expression of CXCR3 was increased on a per cell basis on mTregs in a dose-dependent manner, peaking at day 1, and maintained for greater than four days at the two highest doses ($N = 35$ dose $p = 4 \times 10^{-3}$; Fig 10A, B; S14 Fig (parts G and H)). CCR6 on mTregs also increased, peaking at day one ($N = 37$; $p = 1.48 \times 10^{-4}$) and was sustained above baseline for greater than 7 days at the higher doses (Fig 10C, D; S14 Fig (parts I and J)).

**Fig 10 Proleukin upregulates CXCR3 and CCR6 on T regulatory cells. (A and B)** Dose-dependent sustained increase in expression of CXCR3 on mTregs (CXCR3 average baseline MFI 2252 (45.08; 1783 - 3193) $N = 35$) (C and D) The increase in CCR6 expression by mTregs was maximal and dose dependent on Day 1 (CCR6 MFI 1523 (31; 1106 - 1973) $N = 37$).

**Discussion**

Our aim is to develop an immunomodulatory therapy for T1D that increases immune regulation within physiological levels to inhibit autoreactive T cells whilst preserving immune responses to pathogens and cancer immunosurveillance. Such a therapy could be tested initially in newly-diagnosed patients many of whom retain sufficient insulin production to prevent diabetic
complications [51, 52], and if successful, leading to the treatment of autoimmunity to prevent the
diagnosis of T1D. Critical to this strategy is to first characterize the Treg dose-response, the
duration, and the effects of a single ultra-low dose of Proleukin on the human immune system. In
DILT1D we have determined the Treg dose response to a single dose of subcutaneous Proleukin
and defined two doses required to increase Treg frequencies by predefined amounts, in a formal
clinical governance framework, with an adaptive statistical design.

The single dose of Proleukin was well tolerated other than a small self-limiting injection
site reaction, a common adverse event observed in almost every treated participant except in one
in whom there was no increase in plasma Proleukin at 90 minutes following administration of an
extremely low volume dose (0.02 ml). We did not observe any pseudo-influenza like syndrome
(malaise, myalgia, arthralgia, shivering or fever) that have been observed in participants
administered 5 day or daily dosing regimens [36, 53] though there were two episodes of rhinitis
that were possibly related to drug administration. A norovirus infection developed in a single
participant following treatment that, retrospectively, was found to have been subclinically infected
before treatment and fully recovered mounting a robust immune response to virus. The early time
point and intensive daily monitoring in DILT1D enabled us to establish that lymphocyte counts
have a transient dose-dependent decline on day 1 without the development of persistent
lymphopenia that is characteristic of the high-dose Proleukin oncology protocols [54]. Persistent
lymphopenia would not be a favourable feature of a repeat dosing regimen but in DILT1D we
observed that the decrease in lymphocytes was dose dependent and a transient asymptomatic
lymphopenia (count < 1 x 10^9/L) was observed in only a single participant treated with the highest
dose administered (1.5 x 10^6/m²). The two doses that DILT1D identified increased Tregs by 10%
and 20% are ultra-low and have a very low risk of lymphopenia. In contrast, eosinophils increased
on day 1 after treatment and we determined that if a participant has a high baseline eosinophil count then Proleukin can induce transient eosinophilia, suggesting selection of participants with lower eosinophil counts might be useful in stratification to treatment in future Proleukin trials to maintain the count within the normal healthy range.

DILT1D was a mechanistic trial designed to determine immune outcomes of treatment and not to achieve clinical efficacy [55]. Participants had no evidence of the development of thyroid dysfunction or a decline in C-peptide that has previously been observed with frequent administration of repeat IL-2 doses with rapamycin in participants with T1D [34]. Another feature of our study is the use of whole blood FACS analysis rather than cryopreserved PBMCs. Fresh whole blood facilitated rapid analysis, reduced the manipulation of the samples and allowed us to analyse the expression of surface receptors such as the chemokine receptors that might be lost during the freezing, storage and thawing of PBMCs. However, owing to the variation in T cell numbers in the blood between individuals, lower numbers of T cells were available for analysis in blood samples from some patients making it difficult to define the response in rare subsets. Owing to blood volume requirements for our analyses, as well as ethical and practical considerations the study was limited to adults and to analysis of peripheral blood only.

In the process of achieving the two primary endpoints (10% and 20% maximum increases in Treg frequencies from baseline) we also analysed the pharmacokinetics of subcutaneous Proleukin and the body’s reaction, revealing hyperacute decreases in the frequencies of Tregs and of other cells in the blood, returning to, or exceeding baseline frequencies, 24 hours or days later, depending on Proleukin dose and cell type. These decreases could be due to Proleukin enhancing retention of cells in the tissues and/or egress of cells from the circulation into the tissues.
In the first hours after administration there was limited Treg selectivity, with responses from Teffs and NK cells owing to high concentrations of the drug for the first few hours consistent with its half-like \textit{in vivo} of 4-5 hours following subcutaneous administration [49]. These initial IL-2 concentrations are greater than those reported in patients with sepsis [45]. When we had used a conventional IL-2 assay and not the supersensitive IL-2 assay [46] we were able to observe the peak IL-2 concentrations at 90 minutes, but no quantifiable readings above background after this time point were obtained. Utilization of the much more sensitive assay allowed us to identify sustained physiologically-active concentrations of Proleukin up to four days after dosing in the case of Tregs and between one and two days for mTeffs and NK CD56\textsuperscript{bright} cells. Nevertheless, there is still some level of Treg selectivity, and it remains to be determined the equilibrium the immune system will reach if steady-state increases of Tregs in the order of 20-50\% could achieved by regular Proleukin injections in our follow up study, DILfrequency (NCT02265809) [56]. The finding at 24 hours post-dosing that the higher doses of Proleukin (> 0.38 x 10\(^6\) IU/m\(^2\)) resulted in sufficient concentrations of IL-2 to stimulate Teffs, alter their circulation and proliferate should inform the design of dosing regimens.

Another finding that impacts consideration of dosing regimens came from analysis of the expression of the IL-2 receptor subunits on Tregs, NK and Teffs. We detected a rapid down modulation of the signal-transducing subunit, the \(\beta\) chain/CD122, on Tregs associated with a reduction in the sensitivity of these cells to IL-2. It is possible that in a daily dosing induction phase of IL-2 treatment a combination of Treg desensitisation and Teff activation could increase insulitis and exacerbate T cell/cytokine-mediated destruction of \(\beta\) cells in T1D patients. Taken together our results suggest a regimen with doses in the range of 0.1 x 10\(^6\) to 0.5 x 10\(^6\) IU/m\(^2\) and we predict that a favourable interval might be greater than every two days, but probably not as
much as 1 or 2 weeks, if a steady-state Treg increase is the aim. Desensitisation could partly explain the non-responsiveness of Tregs in some patients receiving daily doses of Proleukin of 1.0 x 10^6 IU or more [36]. We acknowledge that regular dosing to achieve steady-state increases of Tregs of 20-50%, if this is achievable in DILfrequency, may not prevent β-cell destruction, and a phase 2 efficacy trial will be required to answer this question.

The prolonged, dose-dependent duration of Treg response to a single dose of Proleukin was unexpected. At the higher doses of Proleukin, mTregs remain elevated for greater than 7 days in the absence of IL-2 plasma levels above baseline suggesting that trafficking through tissues and exposure to self-antigens helps to sustain the Treg increase. Exposure of Tregs to IL-2 in vitro is not sufficient to cause proliferation. Activation and cell division of Tregs requires antigen and T cell receptor signalling. In mouse lymph nodes removal of either T cell receptor signalling or the IL-2 supplied from Teffs leads to reduced Treg function and uncontrolled effector T cells [22].

Finally, in addition to the effects on Tregs by Proleukin, NK cells that express high levels of CD56 and CD122 and low levels of CD25 are highly sensitive to Proleukin in vivo (Fig 9 A-H). These NK CD56^{bright} cells could have immunoregulatory function so their induction by Proleukin may be a desirable phenotype in suppression of T-cell mediated autoimmunity [57] and perhaps also in the regulation of the Teff responses that we observed when plasma Proleukin concentrations were high.

Having observed Proleukin-induced an alteration in cell trafficking we recalled that a decline in T cells from the peripheral blood has also been reported in T1D efficacy C-peptide preservation trials of anti-CD3 monoclonal antibody, which after a single treatment course (14 days) showed indications of C-peptide preservation in T1D participants one year after treatment [4, 58]. This response has been characterized previously in a humanized mouse model and shown
to require migration of CCR6+ CD4+ T cells to the gut to acquire a regulatory phenotype, including increased CCR6 expression and IL-10 production by FOXP3+ Tregs [59]. It therefore seems possible that anti-CD3-induced Treg migration during treatment may be due in part to IL-2 production by anti-CD3-stimulated T cells *in vivo*. Endogenous IL-2 production in response to anti-CD3 treatment was reported in the original preclinical NOD mouse study [60] and the cytokine was increased in plasma samples from a portion of patients in clinical trials of Teplizumab (humanized non-Fc binding antibody to anti-CD3) in T1D, which was also accompanied by an increase of CD25+ CD4+ T cells [61].

The DILT1D trial has characterized the effects of single ultra-low doses of Proleukin on circulating immune cells and defined two doses that increased Tregs within the normal range. The next step in our systematic experimental medicine approach is to bring these two doses forward in a second adaptive study to determine the optimal dose and frequency of administration to reach a steady-state of increased Tregs whilst minimizing Teff activation [56]. We do not think this can be modelled, given the pleiotropic response to Proleukin observed and has to be determined using an efficient statistical design, with mechanistic analyses to characterize the effects of repeated doses of Proleukin on the immune system.
Supporting information

Supplementary documents

S1 Text. DILT1D trial protocol
S2 Text. CONSORT statement
S3 Text. Health Research Authority - Favourable ethical opinion with conditions
S4 Text. Health Research Authority - Acknowledgement of receipt of additional documentation and confirmation of ethical approval

Supplementary materials

S1 Materials. DILT1D: Standard operating procedure Flow Cytometry Staining and Cell Sorting
S2 Materials. Mechanistic flow quality control process and missing mechanistic data summary
S3 Materials. Dose Determining Committee statistical analysis report from the adaptive phase of DILT1D (Interim Report DILT1D Trial)

Supplementary analyses

S1 Analysis. Primary Endpoint Analysis Population
S2 Analysis. Primary endpoint - Fitted models and their parameters
S3 Analysis. Analysis of lymphocytes and eosinophil counts from Diabetes - genes, autoimmunity and prevention (D-GAP) and Cambridge BioResource (CBR) DIL cohorts

Supplementary data

S1 Data. Supporting data figure 1
S2 Data. Supporting data figure 2
S3 Data. Supporting data figure 3
S4 Data. Supporting data figure 4
S5 Data. Supporting data figure 5
S6 Data. Supporting data figure 6
S7 Data. Supporting data figure 7
S8 Data. Supporting data figure 8
S9 Data. Supporting data figure 9
S10 Data. Supporting data figure 10

Supplementary tables

S1 Table. Antibody combinations for surface (tubes 1-6) and intra-cellular staining (tube 7)
S2 Table. Detailed antibody/clone information
S3 Table. Antibody combinations for cell sorting
S4 Table. Antibody combinations and information for pSTAT5 assay
S5 Table. Full blood counts baseline, day 1 and final visit
S6 Table. Clinical FACS analysis of TNBK assay at baseline, day 1, and final visit
S7 Table. Metabolic measures & Thyroid function tests at baseline and final visit
S8 Table. Renal, bone and liver biochemistries at baseline and final visit

Supplementary figures

S1 Fig. DILT1D sample workflow for each participant’s trial visit
S2 Fig. Clinical course and anti-norovirus GII.4 antibody response of Norovirus infected participant.
S3 Fig. Injection site reactions
S4 Fig. Changes in CD8+ T, B and NK cell counts in response to Proleukin
S5 Fig. Correlation between clinical and mechanistic FACS analysis of Tregs.
S6 Fig. CD25 and CD122 expression on memory and naïve Tregs from baseline to day 7 post-treatment.
S7 Fig. Linear increase in CD25 expression on Tregs in response to increased Proleukin dose
S8 Fig. Effects in vitro of Proleukin on CD25 and CD122 expression on NK CD56bright, memory T regulatory, and T effector cells
S9 Fig. Increased pSTAT5, CD25 and FOXP3 levels in T regulatory cell subsets in blood following a dose of Proleukin
S10 Fig. The effects of a Proleukin dose on memory T effector cell frequency and CD69+ T regulatory and effector cells and pSTAT5 response in mTeff cells
S11 Fig. Natural killer cell responses to treatment and baseline expression of CD25 and CD122

S12 Fig. Soluble CD25 and C-reactive protein responses

S13 Fig. Changes in frequency of CXCR3^+CCR6^+ and CXCR3^-CCR6^- memory Tregs

S14 Fig. CXCR3^+ and CCR6^+ memory and naive T regulatory cell responses to treatment

Acknowledgments: The authors acknowledge the assistance of the National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre and the Cambridge Clinical Trial Unit (CCTU) Sabine Klager, Sridev Nagarajan and Paula Kareclas for trial coordination; the NIHR/Wellcome Trust Clinical Research Facility, Addenbrooke’s Centre for Clinical Investigation (ACCI) for clinical facilities; Philip Knott, Graham Wood and team for set up and conduct of the clinical FACS at the Department of Clinical Immunology, Addenbrooke’s Hospital, Cambridge, UK; Dr. Kevin O'Shaughnessy, Clinical Pharmacology, University of Cambridge, independent chair of the DILT1D trial steering committee; Sarah Nutland, Kelly Beer, Jamie Rice, Chris Coner and Simon Hacking at the Cambridge BioResource for assistance with study setup and publicity. We gratefully acknowledge the participation of all NIHR Cambridge BioResource volunteers. Meeta Maisuria-Armer, Simon Duley, Jennifer Denesha and Trupti Mistry for sample processing and analysis, Lynne Adshead, Amie Ashley, Anna Simpson and Niall Taylor for laboratory administration and procurement support, Vin Everett and Sundeep Nanuwa for logistical and web development, Catherine Guy, Criona O’Brien and the Experimental Medicine group at the JDRF/Wellcome Trust Diabetes and Inflammation Laboratory, Cambridge Institute for Medical Research; Jenny Ly at MSD Assay Services for assistance with S-PLEX assays; Larry Peterson for the analyses of the pharmacokinetics of Proleukin. The authors thank the study volunteers and the staff at participating D-GAP (Diabetes Genes, Autoimmunity and Prevention) study hospital sites. This research was supported by the Cambridge NIHR BRC Cell Phenotyping
Hub. In particular, we wish to thank Anna Petrunkina Harrison, Natalia Sarvinykh and Chris Bowman for their advice and support in flow cytometry and cell sorting. We thank Maeve Waldron-Lynch and Eleonora Seelig for the critical review of the manuscript.

For assistance with identification of potential participants we acknowledge the ADDRESS-2 study: Desmond Johnston, Helen Walkey; the D-GAP study: David Dunger; Addenbrooke’s Hospital, Cambridge University Hospitals NHS Foundation Trust: Steve O’Rahilly, Tony Coll, Latika Sibal, David Simmons, Amanda Adler, Sarah Finer, Fleur Talbot, Ben Challis, Smitha Nalla, Margaret Hales, Katy Davenport, Shannon Farnham; Barnet Hospital, Royal Free London NHS Foundation Trust: Kate Mahaffey, Jackie Seenanda; Colchester Hospital University NHS Foundation Trust: Charles Bodmer, Aine Turner; East and North Hertfordshire NHS Trust: Ken Darzy; Guys and St Thomas NHS Foundation Trust: Fun Liu; Harrogate and District NHS Foundation Trust: Laura Dinning; Hinchingbrooke Health Care NHS Trust: Singhan Krishnan, Sandra Bovan; Ipswich Hospital NHS Trust: Gerry Rayman, Jo Rosier, Jon Hassler-Hurst; Imperial College Healthcare NHS Trust: Anne Dornhorst; Leicester Diabetes Centre at the University Hospitals of Leicester NHS Foundation Trust: Helen Bray; Northampton General Hospital NHS Trust: Dr Charles Fox, Andrea Kempa; Peterborough and Stamford Hospitals NHS Foundation Trust: Muhammad Butt, Alison Pearson; Royal Devon and Exeter NHS Foundation Trust: Lisella Wilkinson; University Hospitals Birmingham NHS Foundation Trust: Parth Narendran; University Hospital Southampton NHS Foundation Trust: Mayank Patel; West Hertfordshire Hospitals NHS Trust: Colin Johnston, Elaine Walker, Karen Markwell, Kathryn Constantin; West Suffolk NHS Foundation Trust: John Clark, Sarah Hewitt; Worcester Acute Hospitals NHS Trust: Lisa Smith, Jane Wilson; and Queen Mary University of London and Barts
Health NHS Trust: David Leslie, Anthimalar Saravanan. The generous participation and contributions of the participants in DILT1D are very gratefully acknowledged.

References

10.1038/ng2068. PubMed PMID: 17554260; PubMed Central PMCID: PMCPMC2492393.


A. 45 participants screened
   - 5 ineligible

B. Safety population
   - 40 treated

Analysis population
   - Primary endpoint
   - 1 withdrawn adverse event

Evaluate population
   - 39 observed for primary and secondary endpoints

C. Interim analysis sequence
   - Days
   - Learning phase
   - Adaptive phase

Participants
   - Dose 0.04
   - 1.5
   - Interim analysis sequence

Screening
   - 0 1 2 3 4 7 9 14 21 60
   - IL-2
   - Adapt
   - Next participant
   - IL-2
Figure

A) % change in NK CD56iso^bright^ lymphocytes from baseline

B) % change in NK CD56iso^bright^ pSTAT5

C) % change in NK CD56iso^bright^ CD25 MFI day 1

D) % change in NK CD56iso^bright^ CD122 MFI

E) % change in NK CD56iso^bright^ CD25 MFI

F) % change in NK CD56iso^bright^ CD122 MFI day 1

G) % change in NK CD56iso^bright^ CD25 MFI

H) % change in NK Ki67^CD56^ from baseline

Click here to download Figure Fig9.tif
Click here to access/download
Supporting Information
S2_text.doc
Click here to access/download
Supporting Information
S1_Materials.pdf
Click here to access/download
Supporting Information
S2_Materials.pdf
Click here to access/download Supporting Information S3_Materials.pdf
Click here to access/download
Supporting Information
S1_Analysis.pdf
Click here to access/download
Supporting Information
S2_Analysis.pdf
Click here to access/download Supporting Information S3_Analysis.pdf
Click here to access/download Supporting Information
S2_Data.xlsx
Click here to access/download
Supporting Information
S3_Data.xlsx
Click here to access/download
**Supporting Information**
S4_Data.xlsx
Click here to access/download
Supporting Information
S5_Data.xlsx
Supporting Information

Click here to access/download
Supporting Information
S6_Data.xlsx
Click here to access/download Supporting Information S7_Data.xlsx
Click here to access/download
**Supporting Information**
S8_Data.xlsx
Click here to access/download
Supporting Information
S10_Data.xlsx
Click here to access/download
**Supporting Information**
*S3_Table.pdf*
Click here to access/download
Supporting Information
S4_Table.pdf
Click here to access/download

Supporting Information

S5_Table.pdf
Click here to access/download
Supporting Information
S6_Table.pdf
Click here to access/download
**Supporting Information**
S8_Table.pdf
Click here to access/download
Supporting Information
S1_Fig.pdf
Click here to access/download
Supporting Information
S2_Fig.pdf
Click here to access/download

Supporting Information
S3_Fig.pdf
Click here to access/download
Supporting Information
S4_Fig.pdf
Click here to access/download

**Supporting Information**

S6_Fig.pdf
Click here to access/download
Supporting Information
S7_Fig.pdf
Click here to access/download Supporting Information S8_Fig.pdf
Click here to access/download
Supporting Information
S9_Fig.pdf
Click here to access/download

**Supporting Information**

S11_Fig.pdf
Click here to access/download
Supporting Information
S12_Fig.pdf
Click here to access/download

**Supporting Information**

S13_Fig.pdf