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PH.D. THESIS

T1D PROGRESSION IS ASSOCIATED WITH LOSS OF CD3+CD56+ REGULATORY CELLS

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Indice

1. INTRODUCTION

The term "diabetes mellitus" refers to a complex metabolic disorder characterized by chronic hyperglycemia. In particular, type 1 diabetes mellitus (T1D) is a metabolic disorder characterized by chronic hyperglycemia secondary to partial or total insulin deficiency due to the autoimmune destruction of pancreatic cells secreting this hormone (1). Three-quarters of all T1D cases are diagnosed in individuals under the age of 18.

2. EPIDEMIOLOGY OF TYPE 1 DIABETES MELLITUS

T1D is the most common form of diabetes in children and adolescents and it has a prevalence of 5-10%. It is estimated an incidence of about 96,000 cases per year among children under the age of 15. The incidence of T1D, however, varies greatly between different ethnic groups, with the highest incidence in Finland, Northern Europe and Canada. There is an approximately 20-fold difference in the incidence of T1D among the Caucasian population living in Europe; the incidence rates are correlated with the frequency of HLA susceptibility haplotypes in the general population. Out of the 500,000 children living in Europe who are affected by T1D, it is estimated that 26% are European, 22% from North America and the Caribbean regions. In Asia, the incidence of T1D is very low, it is estimated that in Japan it is about 2 per 100,000 people / year, in China 3.1 per 100,000, Taiwan about 5 per 100,000 (1). In contrast to most autoimmune disorders, which disproportionately affect females, gender differences in the incidence of T1D are found in some, but not all, populations (1). In recent decades, an increase in the incidence of T1D has been observed, mainly in countries where the incidence rate was lower (2, 3, 4, 5, 6, 7, 8). In addition, there was an increase of the incidence among children under the age of 5. It is estimated that the incidence of T1D in 2010 was about 40% higher than in 1997. The EURODIAB study (9) which allowed the creation of a T1D registry involving 44 centers between Europe and Israel, estimated an increase of 3-4% per year of the incidence rate of T1D. As regards the various age groups, there is an increase of the incidence rate of the disease of 6.3% between 0 and 4 years, 3.1% between 5 and 9 years and 2.4% between 10 and 14 years.

3. PATHOGENESIS OF TYPE 1 DIABETES MELLITUS

T1D is a multifactorial autoimmune disease characterized by CD8+ T cell-mediated destruction of pancreatic beta cells, which produce insulin. This autoimmune destruction occurs in genetically predisposed individuals.

Like other autoimmune diseases, the pathogenesis of T1D results from a combination of genetic susceptibility and environmental factors. According to the literature, the HLA locus on chromosome 6p21 (called IDDM1) contributes at least 45% of genetic susceptibility to T1D. Caucasians affected by T1D have HLA-DR3 / DR4 haplotype or both in 90-95% of cases (10).

There are also particular HLA haplotypes that seem to protect against the onset of T1D (for example DR2). In addition, about 20 non-HLA loci help to determine the predisposition to develop T1D; in particular, the function of only two of these loci is currently known: IDDM2 on chromosome 11p5.5 and IDDM12 on chromosome 2q33.

Regarding environmental factors, despite the numerous efforts of the researchers, the triggers responsible for the disease have not been identified with certainty. For example, the American DAISY study and the German BABYDIAB study concluded that there is no correlation between the onset of T1D and various potential environmental triggers, i.e. early exposure to cow's milk proteins, breastfeeding, viral infections, and timing of vaccinations.

These studies are, however, in contrast with some Finnish reports that suggest a positive correlation between these environmental factors and the onset of the disease (11). For example, the Finnish MIDIA study assign considerable importance to viral infections (mumps, rubella, coxsackie B, CMV); the viruses appear to damage pancreatic islets by promoting the release of self-proteins which would constitute the antigens triggering cell-mediated destruction or could favor a molecular mimicry mechanism whereby anti-virus antibodies would react not only towards viral proteins but also against self-proteins (12).

A new pathogenetic hypothesis states that environmental factors could act as disease "modifiers" rather than triggers. The "traditional" pathogenetic theory states that environmental factors act as triggers for the onset of T1D in genetically predisposed individuals. A new pathogenetic hypothesis is instead affirming itself in the scientific community, this theory states that environmental factors could act to promote and/or mitigate the disease during the different stages, with effects that depend on both the timing and the quantity of exhibitions (figure 1). This new model may explain, in part, why the frequency of T1D has increased dramatically over the past three decades as health care and hygiene standards have improved.

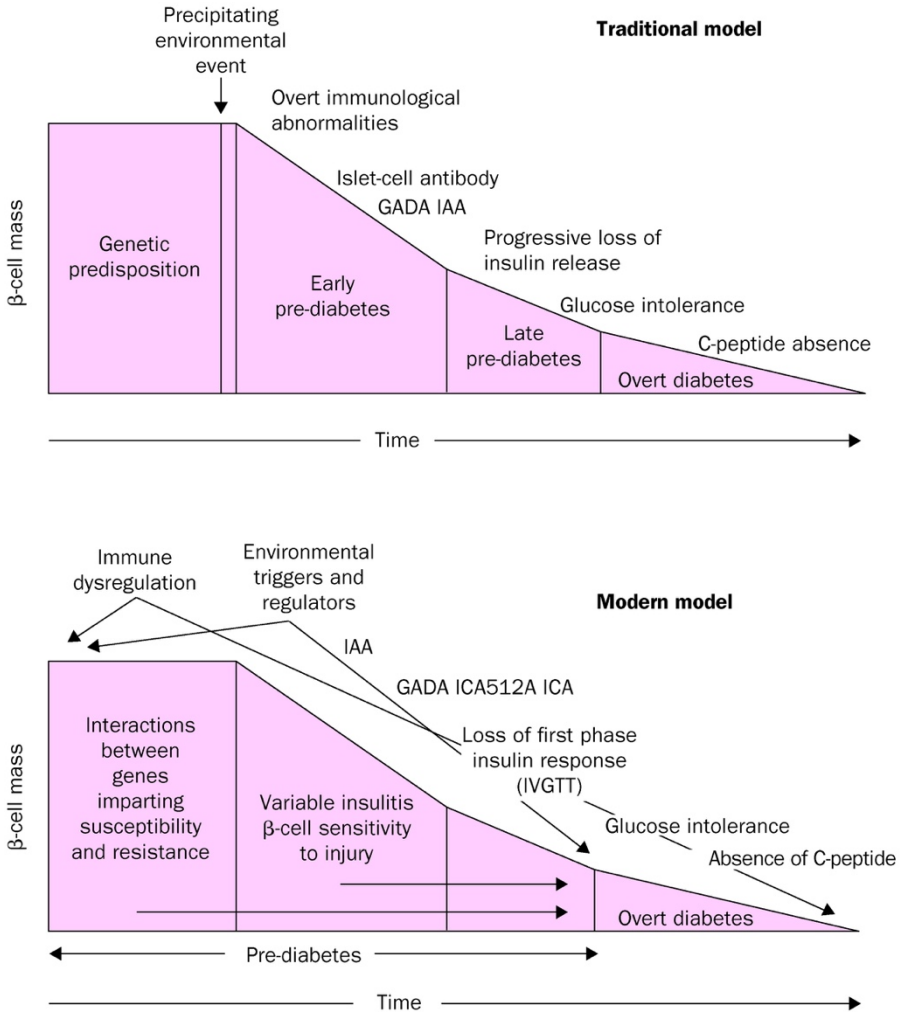


Figure 1

3.1 Cell-mediated immunity

The classical pathogenetic hypothesis considers cell-mediated adaptive immunity at the center of the development of T1D. After birth, a complex and delicate balance is established between self-reactive T cells against beta cells and regulatory T cells (T reg). If this state of immunotolerance is threatened by some factors (environmental factors), in genetically predisposed subjects, the T regs are no longer able to control the self-reactive T lymphocytes. This results in activation of B lymphocytes which begin to produce antibodies against beta cell antigens, and activation of cytotoxic CD8+ T lymphocytes that induce apoptosis (13).

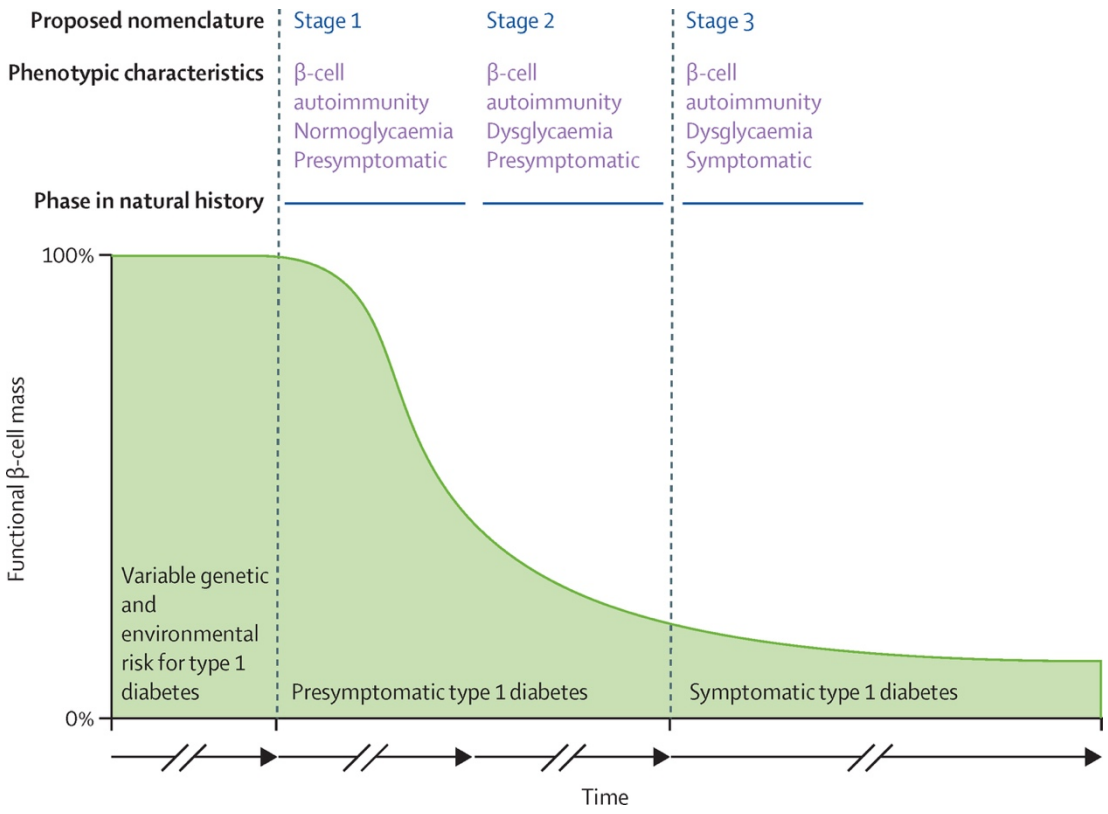


Figure 2

There are two main groups of regulatory T lymphocytes, classified on the basis of the expression of the forkhead box transcription factor (FOXP3). FOXP3+ T reg cells maintain immunological tolerance through the suppression of autoreactive T lymphocytes (14); CD4 + FOXP3- cells also have regulatory activity and many studies are showing that the enhancement of these cells could be of great importance for the prevention of T1D (14).

3.2 Humoral immunity

Generally clinical onset of overt diabetes is preceded by a prodromal period characterized by the production of specific antibodies and minimal metabolic alterations. Many studies have shown that the presence of one or more antibodies against beta cells leads to a significant increase in the risk of developing T1D in the following years (15).

T1D offers a clear example of "epitope spreading", that is, in the course of the disease the autoimmune response tends to expand due to the progressive exposure of beta cells autoantigens; for this reason, if initially the autoimmune attack is directed towards only some constituents of the attacked tissue, this then tends to turn towards an ever greater number of constituents. The autoantibodies most currently used for diagnostic purposes are:

- Anti-glutamic acid decarboxylase antibodies (GAD)
- Islet antigen 2 antibodies (IA-2)
- Insulin autoantibodies (IAA)
- Zinc Transporter 8 antibodies (ZnT8)

Many other autoantigens have been described (ICA69, IGRP, ChgA, GFAP, etc.) which, however, are currently of little clinical importance. The four autoantibodies listed above are currently the best and most reliable markers for the prediction of overt T1D and are also the first detectable sign of pre-diabetes as an autoimmune process that leads to the destruction of beta cell mass and then to hyperglycemia. Rarely, T1D can develop even in the absence of detectable autoantibodies against beta cells (T1D type b).

4. NEW PATHOGENETIC HYPOTHESIS: INFLAMMATORY THEORY AND META-IMMUNOLOGY

The new hypotheses involve not only the mechanisms of adaptive immunity (humoral and cell-mediated) in the pathogenesis of T1D, but also the mechanisms of innate immunity. As widely discussed in Odegaard's paper (16), in overweight / obese subjects, the accumulation in the various tissues (including the pancreas) of activated innate immunity cells (mainly macrophages and NK cells) favors the release of inflammatory-mediators, such as IL-1beta and TNF-alpha which cause insulitis and beta-cell damage.

Odegaard also states that in the last decade there was an increase of the incidence of T1D and obesity (16). Many papers underline an important link between these two diseases, in particular, children with T1D seem to have a greater weight in the first year of life than healthy subjects and the increase in body mass index (BMI) correlates strongly with an earlier onset of diabetes. Insulin resistance linked to overweight appears to be a predictor of the development of T1D, regardless of the HLA haplotype.

Meta-immunology has been developed in the last decade and allows us to find a link between metabolism and immunity; T1D studies examining meta-immunological parameters lead to discrepant results and non-definitive conclusions. In addition, these studies have been performed on insufficiently numerous patient samples, they analyzed few meta-immunological parameters, and no studies examined parameters that could predict residual beta-cell function in pediatric diabetic subjects (17).

Understanding the pathogenesis of T1D has greatly improved in recent years, but the risk markers for disease progression and beta cell depletion are not yet known. These markers could be useful for monitoring the disease, improving its prognosis and evaluating therapeutic efficacy. In a study conducted by the Pediatric Diabetology Group of the University of Naples "Federico II" on diabetic pediatric subjects at the onset of the disease and followed for 24 months, some meta-immunological markers were identified (17).

In particular:

- CD8+ T lymphocytes appear to increase in the blood of subjects who can be defined as pre-diabetic (subjects with HLA compatible with T1D and positivity of specific autoantibodies but with normoglycemia);
- memory T lymphocytes increase at the onset of the disease and then decrease in the following 24 months, in agreement with the evidence that GAD65-specific T cells show a memory phenotype in diabetic subjects and probably decrease over time because of the progressive depletion of clones of specific T cells for pancreatic islets;
- plasmacytoid dendritic cells (pDCs) are numerous in subjects with diabetes at onset; this aspect is interesting because in presence of specific autoantibodies, pDCs process and present islet antigens to TCD4 lymphocytes to amplify and maintain the T cell response;
- T cell population, characterized by the co-expression of CD3 and CD56 molecules, are predictors of residual C-peptide secretion 12 months after diagnosis of T1D;
- Leptin/Leptin receptor axis is altered at the onset of diabetes. Leptin seems to favor diabetes development in NOD mice while in humans it seems to have an insulin-like action (18;19); its soluble receptor (sLepR) can therefore modulate the effects of Leptin on target organs by inhibiting its binding to membrane receptors. In this way, the bioavailability of the circulating Leptin increases and its clearance increased too. Low levels of Leptin and high levels of its soluble receptor were found at the onset of T1D. Other groups also found this balance of leptin/sLepR axis in diabetic children with metabolic decompensation. sLepR levels could be an early marker of T1D, because sLepR levels decrease with the progression of the disease but still remain high in sick children compared to healthy controls. The circulating levels of sLepR seem to be inversely proportional to the beta-cell reserve, measured in terms of c-peptide at the onset of the disease. The complete understanding of the Lep/sLepR axis could open new therapeutic perspectives for patients with T1D, by administering leptin in combination with insulin therapy.

5. AIM OF THE STUDY

An unresolved issue in T1D is the lack of biomarkers of immunological self-tolerance for disease monitoring.

Aim of the present study is:

- to demonstrate that peripheral frequency of a regulatory T cell population, characterized by the co-expression of CD3 and CD56 molecules (TR3-56 cells), is reduced in subjects with new-onset of T1D;
- to evaluate if the low frequency of circulating TR3-56 cells is associated with reduced β-cell function and with the presence of diabetic ketoacidosis;
- to demonstrate that TR3-56 cells can suppress CD8+ T cell functions *in vitro* by reducing levels of intracellular reactive oxygen species.

6. MATHERIALS

6.1 Healthy and T1D subjects

The study was conduct in various cohorts of subjects:

- Italian cohort: recruited at the Dipartimento di Scienze Mediche Traslazionali, Sezione di Pediatria, Università di Napoli "Federico II", including subjects at T1D onset and individuals one year after T1D diagnosis. This cohort includes also "at-risk" subjects, that are siblings of T1D children, with at least two autoantibodies positive, and "healthy" children;

- Swedish cohort: recruited at Crown Princess Victoria Children´s Hospital, University Hospital, Linköping, Sweden, including T1D subjects; PBMCs were isolated and frozen at the Division of Pediatrics, Department of Biomedical and Clinical Sciences, Linköping University, Sweden;

- third independent cohort: subjects who developed other autoimmune diseases (AID); in particular celiac disease (CD) or autoimmune thyroiditis (AIT), before or after T1D diagnosis. These subjects were recruited at European Laboratory for the Investigation of Food-Induced Disease (ELFID), Università di Napoli "Federico II".

Diagnosis of T1D was defined according to the Global International Diabetes Federation/International Society for Pediatric and Adolescent Diabetes (ISPAD) Guidelines for Diabetes in Childhood and Adolescence (1) and included symptoms of diabetes in addition to casual plasma glucose concentration ≥11.1 mmol/L (200 mg/dl), or fasting plasma glucose ≥7.0 mmol/l (≥126 mg/dl), or 2 hours post load glucose ≥11.1 mmol/l (≥200 mg/dl) during an oral glucose tolerance test, and glycated haemoglobin (HbA_{1c}) ≥6.5. CD was diagnosed in accordance with the 1990 European Society for Pediatric Gastroenterology Hepatology and Nutrition guidelines (20).

Diagnosis of AIT was based on the presence of high levels of antithyroid antibodies (anti-thyroperoxidase and/or anti-thyroglobulin), normal or low thyroid function (T4, TSH), together with a heterogeneity and hypoechogenity of thyroid parenchyma at ultrasound examination (21).

Blood samples from individuals with recent-onset of T1D was achieved 10 days after glucose levels stabilization by treatment with exogenous insulin (glucose values between 3.5-10 mmol/l or 80-180 mg/dl) and all of them were positive for at least two anti-islet autoantibody.

Healthy subjects were matched for sex, age and BMI with T1D subjects and selected by the following criteria: fasting blood glucose of <5.5 mmol/L (<100 mg/dl), negative personal and familial history of autoimmune disorders, and negativity for islet autoantibodies at the 99th percentile. T1D and healthy subjects with recent vaccinations or infections were excluded from the study.

Institutional Review Board of the Ethics Committee of University of Naples "Federico II" approved the study (Prot. N. 200/16 and N.161/18). Approval by the Research Ethics Committee by Linköping University was obtained (Dnr 02-482). All adult human subjects, or parents of participating children, provided written informed consent. We have complied with all relevant ethical regulations.

6.2. Laboratory testing

Blood samples from T1D subjects, at-risk siblings and from healthy individuals were withdrawn at 8.00 a.m. into heparinized BD Vacutainers and processed within the following 4 hours. Serum or plasma were obtained after centrifugation and kept at −80°C until use. Fasting c-peptide levels were measured in duplicate serum samples, at the same time for all samples, using a commercial ELISA kit (Merck Millipore Corporation). Results for each assay were validated, and a high- and low-level control sample were included. Glucose levels were measured using enzymatic hexokinase method and HbA1c by high-performance liquid chromatography (HLC-723 G7 TOSOH, Bioscience). Islet autoantibodies (GAD, IA-2A, IAA, ZnT8), transglutaminase IgA and antithyroid antibodies were measured by commercial ELISA (Pantec). Whole

blood cells were analyzed with a clinical-grade haemocytometer to determine absolute lymphocyte numbers in each sample. Remaining part of blood samples was processed and after Ficoll-Hypaque (GE-Healthcare) gradient centrifugation, PBMCs were obtained.

T1D Swedish cohort blood samples were processed at Division of Pediatrics, Department of Clinical and Experimental Medicine, Medical Faculty Linköping University, Sweden. PBMCs were obtained and cryopreserved in liquid nitrogen. An aliquot of them were shipped to our laboratory at IEOS-CNR and kept in liquid nitrogen until use. Nitrogen cryopreserved PBMCs from Swedish cohort were thawed as follows: cryovials containing frozen cells were removed from liquid nitrogen storage and placed into a 37°C water bath; the vials were gently swirling in the 37°C water bath until there was a small amount of ice left in the vial. Pre-warmed complete growth medium (RPMI 10% FBS) drop wise into the cryovial containing the thawed cells. After centrifugation cells were re-suspend in complete growth medium and utilized for flow cytometry staining. Viability was always assessed after defrosting and was on average > 85%.

6.2.1 Flow cytometry and cell isolation

PBMCs from human healthy donors, T1D subjects and at-risk siblings of T1D were stained with the following antibodies for the evaluation of TR3-56 cells: FITC anti-human CD3 (BD Pharmingen, clone UCHT1), PE-Cy7 anti-human CD56 (BD Pharmingen, clone B159).

In order to evaluate TR3-56 death cell, PBMCs were stained with the following antibodies: FITC human Annexin V (BD Pharmingen), PE-Cy7 anti-human CD56 (BD Pharmingen, clone B159), APC anti-human CD3 (BD Pharmingen, clone UCHT1), propidium iodide (BD Pharmingen); Annexin V buffer (BD Pharmingen) was used for the staining according the manufactories' instructions.

Multiparametric flow cytometry was used for the evaluation of surface markers on TR3-56 cells from PBMCs: FITC or APC anti-human CD3 (BD Pharmingen, clone UCHT1), PE or APC-H7 anti-human CD4 (BD Pharmingen, clone RPA-T4), BV421 anti-human

CD8 (BD Pharmingen, clone RPA-T8), PE anti-human CD16 (BD Pharmingen, clone 3G8), BV510 anti-human CD27 (BD Pharmingen, clone M-T271), PE anti-human CD28 (BD Pharmingen, clone CD28.2), APC anti-human CD45 (BD Pharmingen, clone HI30), FITC anti-human CD45RA (Miltenyi Biotec, clone REA562), APC anti-human CD45RO (BD Pharmingen, clone UCHL1), PeCy7 or APC anti-human CD56 (BD Pharmingen, clone B159; BD Biosciences, clone NCAM16.2), APC anti-human CD94 (BD Pharmingen, clone HP-3D9), BB700 anti-human CCR7 (BD Horizon, clone 3D12), BV510 anti-human CXCR3 (BD Optibuild, clone 1C6/CXCR3), BB700 anti-human CXCR4 (BD Horizon, clone 12G5), BV510 anti-human DNAM-1 (BD Optibuild, clone DX11), BB700 anti-human NKG2A (BD Optibuild, clone 131411), BV510 anti-human NKG2C (BD Optibuild, clone 134591), APC anti-human NKG2D (BD Pharmingen, clone 1D11), PE labelled CD1d tetramers loaded with α-galactosyl ceramide (ProImmune), PE labelled CD1d negative control tetramers (ProImmune), FITC anti-human Vα24 (Beckman Coulter, clone C15), BV421 anti-human Granzyme B (BD Horizon, clone GB11), PECy7 anti–human CD25 (BD Pharmingen, clone M-A251), BV421 anti–human PD-1 (BD Horizon, clone EH12-1), PE anti-human FoxP3- all (BD Pharmingen, clone 259D/C7), APC anti–human CD152/CTLA-4 (BD Pharmingen, clone BN13), APC anti-human CD39 (BD Pharmingen, clone TU66), BV421 anti-human GITR (BD Horizon, clone V27-580). FITC and PE labelled mAbs against TCR Vβ epitopes; anti-human Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2; Vβ5.3, Vβ7.1, Vβ7.2, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ1.3, Vβ22 and Vβ23 all from Beckman Coulter. Granzyme B expression was performed by using the fixation/permeabilization solution kit BD Cytofix-Cytoperm (BD Biosciences), according the manufacturer's instructions. Staining for intracellular factors was performed by using fixation and permeabilization FoxP3 buffer kit (BD Pharmingen), according the manufacturer's instructions. Samples were acquired by using a two lasers equipped FACSCanto II (BD Bioscience); at least $3x10^4$ events in the lymphocyte gate. For the evaluation of positive events, fluorescence minus one (FMO) controls were used for setting the gate; non-viable cells were detected by 7-AAD viability staining (BD Pharmingen). Cytofluorimetric analyses were performed by using FlowJo Software (FlowJo, LLC).

Human TR3-56, $CD3^{-}$ $CD56^{+}$ (NK), $CD3^{+}CD56^{-}$, $CD4^{+}$ and $CD8^{+}$ T cells were isolated from PBMCs of human healthy donors and T1D subjects by high-performance cell sorting (BD FACS-Jazz, BD Bioscience) in the IEOS-CNR, after staining with the following antibodies: FITC anti-human CD3 (BD PharMingen, clone UCHT1), APC anti-human CD56 (BD Biosciences, clone NCAM16.2), APC anti-human CD4 (BD Pharmingen, clone RPA-T4), APC anti-human CD8 (BD Pharmingen, clone RPA-T8) or by magnetic cell separation with microbeads $CD3^{+}CD56^{+}$ isolation Kit (Miltenyi Biotec), Dynabeads™ CD8 Positive Isolation Kit (Invitrogen, Thermo Fisher Scientific) and Dynabeads™ Regulatory $CD4^{+}CD25^{+}$ T Cell Kit for $CD4^{+}$ cell isolation (Invitrogen, Thermo Fisher Scientific). Purity of isolated cells was 95%-99%.

6.2.2 Proliferation assays

Flow-sorted $CD4^{+}$ and $CD8^{+}$ T cells were labelled with 5,6-carboxyfluorescein-diacetate-succinimidyl ester (CFSE) (Thermo Fischer Scientific) before the culture. To assess cell proliferation, $3x10^{4}$ $CD4^{+}$ or $CD8^{+}$ cells were cultured for 72 hours in the presence of TR3-56 cells (or $CD3^{+}CD56^{-}$ control cells) stimulated with anti-CD3 plus anti-CD28 microbeads (0.2 beads/cell) (Gibco, Thermo Fisher Scientific) at different cell *ratio* (1:1, 1:2, 1:4, 1:8). All tests were performed in the presence of RPMI 1640 medium supplemented with 5% heat inactivated AB human serum (Euroclone). CFSE analyses were performed using BD FACSCanto II (BD Biosciences) and FlowJo software V.10 (FlowJo, LLC).

6.2.3. Cytotoxicity assays

To obtain CTLs directed against allogeneic targets, flow sorted $CD8^{+}$cells (purity > 95%) from adult healthy donor (effectors) were cultured with 30 Gy-irradiated allogeneic PBMCs (stimulators) for 10 days with regular hrIL-2 supplementation (20 IU/ml); allogeneic targets were obtained by anti-CD3 treatment and hrIL-2 expansion of stimulator PBMCs; specific cytotoxicity of effector cells was measured by using the 5,6-carboxyfluoresceindiacetate (CFDA) cytotoxicity assay (Molecular Probes, Eugene). Target cells were labelled with CFDA mixed with effector cells at different E:T *ratio* and incubated at 37°C for 3 hours in 96-well round-bottom plates (Falcon,

Becton Dickinson). The specific lysis of target cells was calculated as follows: % specific lysis = (CT-TE/CT) x100, where CT indicates mean number of fluorescent target cells in control tubes and TE indicates mean number of fluorescent cells in target plus effector tubes. TR3-56 cells (or $CD8^+$ control cells) and effector CTLs were co-cultured (at 1:1 ratio) in order to evaluate the ability of TR3-56 cells to suppress lytic capacity of effector CTLs against the CFDA-labeled allogeneic target.

6.2.4. Degranulation assay, CD10/LAMP-1 expression and INF-γ production

To obtain activated CTLs, flow-sorted $CD8^+$ T cells were cultured for 36 hours in RPMI-1640 (Gibco, Life Technologies) supplemented with 5% AB human serum (Euroclone) in the presence of hrIL-2 (Roche) at 200 IU/ml. After 36 hours, CTLs were labelled with BV421-conjugated anti-human CD8 and then cultured alone or in the presence of freshly flow-sorted TR3-56, NK and $CD8^+$ T lymphocytes at different ratio, with or without TCR-stimulation (1 bead/cell) in 96-well round-bottom plates (Falcon, Becton Dickinson). PE-conjugated anti-human CD107a/LAMP-1 (BD Pharmingen, clone H4A3) was added to the cell culture for the whole culture period (4 hours).

To avoid extracellular cytokine export, the cultures were performed in the presence of 5 μg/ml of Brefeldin-A (Sigma-Aldrich); in particular for CD107a/LAMP-1 experiments Brefeldin-A was added in the last 3 hours of culture. For IFN-γ production, CTLs were cultured as described above, while 5 μg/ml of Brefeldin-A was added to the cell culture for the whole culture period (4 hours). Then to evaluated IFN-γ expression, samples were fixed and permeabilized (Cytofix-Cytoperm, BD Bioscience) and stained for PE-conjugated anti-human IFN-γ (BD Pharmingen, clone B27), following the manufacturer's instructions.

For transwell experiments, the co-culture of TR3-56 cells with CTLs was performed in the above condition using transwell inserts (Corning Life Sciences) in 24-well round-bottom plates (Falcon, Becton Dickinson). For the degranulation assay in the presence of CD56 blocking soluble human recombinant cell adhesion molecule NCAM-1/CD56 (R&D Systems Inc.) was used (10 ng/ml). The control 345.134 IgG2a mAb, recognizing

a glycoprotein widely expressed on human leucocytes was a kind gift of Dr. S. Ferrone, and was used as described above.

In all the experiments, non-viable cells were detected by 7-AAD viability staining and both CD107a/LAMP-1 expression and IFN-γ production were evaluated in labelled-CTLs, using fluorescence values of unstimulated CTLs (medium) as negative values to identify positive gate. All the experiments were performed in autologous condition except when indicated. Experiments in adult healthy subjects were performed co-culturing $1x10^5$ CTLs and $1x10^5$ TR3-56 or control cells; experiments in T1D and control children were performed co-culturing at least $3x10^4$ CTLs and $3x10^4$ TR3-56 or control cells due to the reduced volume of blood withdrawn from children and also due to the reduce frequency of this population in T1D.

6.2.5. ROS production

For intracellular ROS production CTLs were stained with 2',7'-dichlorodihydrofluorescein diacetate (DCF) (Sigma-Aldrich) and cultured alone or with TR3-56 or control cells in the presence of anti-CD3 plus anti-CD28. ROS production was detected after 5, 20 e 40 min of culture. Induction of intracellular ROS was obtained by treating CTLs with menadione (0.05 μM). Mitochondrial ROS was measured by MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Fisher Scientific), according to manufactore's instructions. DCF and mitoSoX levels were evaluated by flow cytometry using BD FACSCanto II (BD Biosciences) and FlowJo software (FlowJo, LLC).

6.2.6. Seahorse analyses

Metabolic profile was evaluated in TR3-56, NK, $CD3^+CD56^-$ and $CD8^+$cells from adult healthy subjects, in the presence of anti-CD3 plus anti-CD28 microbeads (1 bead/cell) (Gibco, Thermo Fisher Scientific) for 1 hour. Real time measurements of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were performed by an XFe-96 Analyzer (Agilent Technologies). Specifically, cells were plated in XFe-96 plates (Agilent Technologies) at the concentration of 2×10^5 cells/well and cultured with RPMI-1640 medium supplemented with 5% AB human serum. ECAR was measured in

XF DMEM medium (Agilent Technologies) in basal condition and in response to 10 mM glucose, 5 μM oligomycin and 100 mM of 2DG (all from Sigma-Aldrich). OCR was measured in XF DMEM medium (supplemented with 10 mM glucose, 2 mM L-glutamin, and 1 mM sodium pyruvate), under basal conditions and in response to 5 μM oligomycin, 1.5 μM of carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) and 1 μM of antimycin A and rotenone (all from Sigma-Aldrich). Experiments with the Seahorse were done with the following assay conditions: 3 minutes mixture; 3 minutes wait; and 3 minutes measurement.

6.2.7. Transcriptome analysis

For microarray analysis flow sorted cell populations (TR3-56, NK, CD3+CD56-, CD8+) isolated from healthy adults, were quantified through microarray-based human Affymetrix Clariom S Assays (Eurofins Genomics), which provides extensive coverage of all known well-annotated genes (21448 gene probes for 19525 annotated unique genes). The raw intensity values were background corrected, log2 transformed and quantile normalized using the Robust Multi-array average (RMA) algorithm. Data were imported and analyzed using MultiExperimentViewer (MeV). Sample similarity was described by multivariate Principal Component Analysis (PCA) and Pearson's correlation. For supervised sample clustering, significant genes were selected by one-way ANOVA, followed by Pearson's correlation. In order to identify a TR3-56 cell specific gene expression pattern, we selected genes for having a consistent log2 fold change (either > +1 or < -1) compared to all other evaluated populations (NK, CD3+CD56- and CD8+ cells) and a significant Student’s t-test ($p<0.05$) for all three comparisons: TR3-56 vs NK, TR3-56 vs CD3+CD56- and TR3-56 vs CD8+ T cells.

For NGS analysis, RNA sequencing was performed by IGA technology (IGATech) services (Udine, Italy). Total RNA was extracted from TR3-56 cells isolated from either healthy subjects (n=3) or recent-onset T1D subjects (n=3) using the RNeasy Micro Kit from QIAGEN, according to manufacturer’s instructions. RNA samples were then quantified and quality tested by Agilent 2100 Bioanalyzer RNA assay (Agilent technologies, Santa Clara, CA) or Caliper (PerkinElmer, Waltham, MA). Libraries were prepared by the ‘Ovation SoLo RNA-seq Library Preparation kit’ (NuGEN, San Carlos,

CA), following the manufacturer's instructions and checked with both Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and Agilent Bioanalyzer DNA assay or Caliper (PerkinElmer, Waltham, MA). Sequencing was performed on single-end 75 bp mode on NextSeq 500 (Illumina, San Diego, CA) and number of reads ranged from 29.1x106 to 32.5x106. Raw data were processed by Bcl2Fastq 2.0.2 version of the Illumina pipeline for both format conversion and de-multiplexing and lower quality bases and adapters were removed by ERNE Version 1.4.644 and Cutadapt 1.1645 software. Reads were then deduplicated based on unique molecular identifier (UMI) composed of 8 random bases for unambiguous identification of unique library molecules by IGATech proprietary script; and aligned on reference GRCh38 genome/transcriptome with STAR3 2.646. Full-length transcripts representing multiple spliced variants for each gene locus were assembled and quantified by Stringtie 1.3.4d47. RNA-Seq data was preprocessed by counting the overlap of reads with genes through htseq-count 0.9.148 and DESeq2 1.14.149 was used to perform comparisons between expression levels of genes and transcripts. Normalization was performed using the median-of-ratios method50 and statistical significance determined using a Wald test49.

6.2.8. Cytokine assessment

A total of 40.000 flow-sorted TR3-56 cells from healthy and T1D subjects were cultured with RPMI-1640 medium supplemented with 5% serum autologous in the presence of anti-CD3 plus anti-CD28 microbeads (0.1 bead/cell) (Gibco, Thermo Fisher Scientific). After 48 hours supernatant were collected and stored at -20° C until use. Cytokine production was analyzed using the bead-based multianalyte immunoassay (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's recommendations, and then was measured by Multiplex technology (Luminex 200, Luminex). xPONENT 3.1 software (Luminex) was used for data acquisition.

6.3 Statistical analysis

Modelling and statistical analysis of data were carried by *JMP Statistical Discovery* software 6.0.3 (SAS, North Carolina, USA), and *GraphPad Prism 7 software* (GraphPad, California, USA). Comparisons were performed by Mann-

Whitney U-test, Student's *t*-test, one-way ANOVA and two-way ANOVA-corrected for multiple comparison using Bonferroni test and Wilcoxon matched pairs test. Correlation analyses were performed by Pearson's correlation. A linear model was used for the adjustment of the comparison for sex, age and BMI variables. To identify outliers, ROUT (Q=0.1%) method has been applied.

For all analyses, we used two-tailed tests, with $p<0.05$ values denoting statistical significance. A univariate logistic regression modeling was fitted to predict DKA at T1D diagnosis: T1D subjects were dichotomized on the basis of the presence (Yes) or absence (No) of DKA at disease diagnosis. Prognostic validity of the fitted models was evaluated by receiver operating characteristic (ROC) curve analysis and measured using the area under the ROC curve (AUC).

7. RESULTS AND DISCUSSION

General characteristics of the subjects enrolled in the study are reported in the following tables.

Table 1. Baseline characteristics of healthy controls and T1D subjects at diagnosis from pre-puberty Italian cohort. Data are expressed as mean ± SD.

Baseline characteristics	Healthy subjects	T1D recent at onset subjects	*p* value
Number of subjects	113	128	-
Age (years)	9.25 ± 3.59	8.21 ± 3.77	p=0.0417
Gender (M/F)	59/54	69/59	-
Body Mass Index (Kg/m^2)	18.65 ± 4.42	16.96 ± 3.25	p=0.0086
C-peptide (ng/mL)	-	0.51 ± 0.37	-
Ketoacidosis at diagnosis (Yes/No/NA)	-	37/72/19	-
Glycated Haemoglobin (%)	-	11.07 ± 2.59	-
Insulin dose (IU/Kg/d)	-	0.64 ± 0.34	-
Other autoimmune disorder (Yes/No)	0/113	0/128	-

Table 2. Baseline characteristics of post-puberty young adult healthy controls and post-puberty young adults from T1D Italian cohort. Data are expressed as mean ± SD.

Baseline characteristics	Healthy subjects	T1D recent at onset subjects	*p* value
Number of subjects	14	19	-
Age (years)	16.19 ± 0.95	15.91 ± 0.86	*NS* (p=0.4548)
Gender (M/F)	10/4	13/6	-
Body Mass Index (Kg/m^2)	21.42 ± 5.30	22.47 ± 3.73	*NS* (p=0.2869)
C-peptide (ng/mL)	-	0.74 ± 0.46	-
Ketoacidosis at diagnosis (Yes/No)	-	6/13	-
Glycated Haemoglobin (%)	-	11.04 ± 3.30	-
Insulin dose (IU/Kg/d)	-	0.52 ± 0.34	-
Other autoimmune disorder (Yes/No)	0/14	0/19	-

Table 3. Baseline characteristics of subjects at T1D onset from Swedish cohort. Data are expressed as mean ± SD.

T1D Validation Swedish Cohort	
Baseline Characteristics	**T1D recent at onset Subjects**
Number of subjects	36
Age (years)	9.75 ± 4.20
Gender (M/F)	22/14
Body Mass Index (Kg/m^2)	18.46 ± 3.15
C-peptide (ng/mL)	0.31 ± 0.21
Ketoacidosis at diagnosis (Yes/No)	2/34
Glycated Haemoglobin (%)	9.78 ± 2.70
Insulin dose (IU/Kg/d)	0.61 ± 0.26
Other autoimmune disorder (Yes/No)	1/35

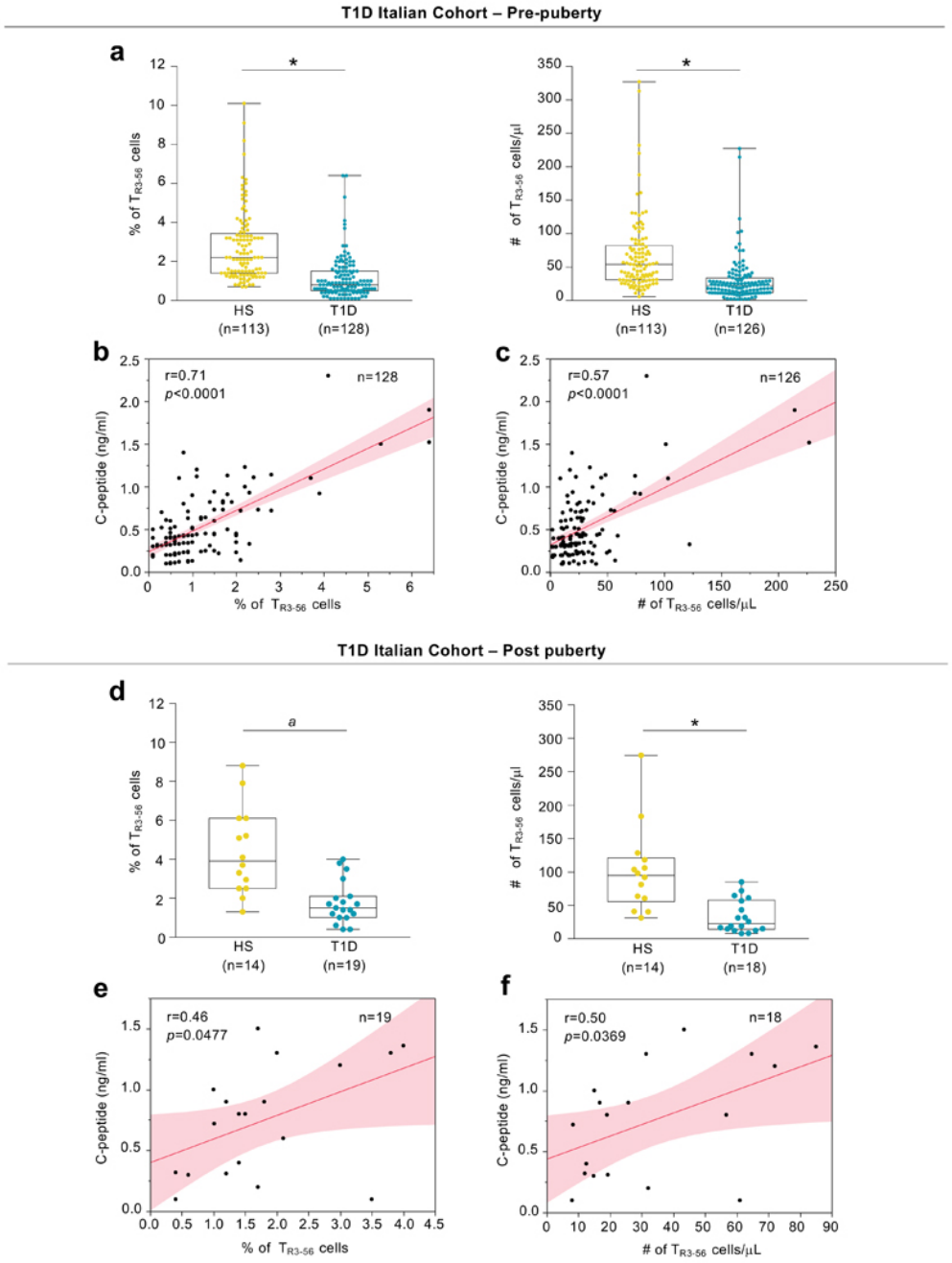
Table 4. Baseline characteristics of T1D subjects at diagnosis recruited in the T1D cohort- autoimmune conditions before or after T1D. Data are expressed as mean ± SD.

T1D Cohort - autoimmune conditions before or after T1D diagnosis			
Baseline characteristics	**T1D subjects with autoimmune conditions before T1D diagnosis**	**T1D subjects with autoimmune conditions after T1D diagnosis**	***p* value**
Number of subjects	21	23	-
Age (years)	11.53 ± 4.45	7.57 ± 4.44	p=0.047
Gender (M/F)	10/11	8/15	-
Body Mass Index (Kg/m^2)	18.97 ± 3.46	17.36 ± 2.55	*NS* (p=0.0915)
C-peptide (ng/mL)	0.60 ± 0.56	0.64 ± 0.51	*NS* (p=0.3971)
Ketoacidosis at diagnosis (Yes/No)	10/11	7/16	-
Glycated Haemoglobin (%)	11.87 ± 3.12	10.73 ± 1.95	*NS* (p=0.1455)
Insulin dose (IU/Kg/d)	0.58 ± 0.27	0.59 ± 0.25	*NS* (p=0.7877)
Other autoimmune disorder (CD/AIT)	7/14	14/7	-

To gain further insight into the physio-pathological relevance and the potential regulatory function of TR3-56 cells, we first enumerated circulating TR3-56 cells in Italian cohort of pre-puberty T1D children at disease onset (n=128), in comparison with healthy children (n=113).

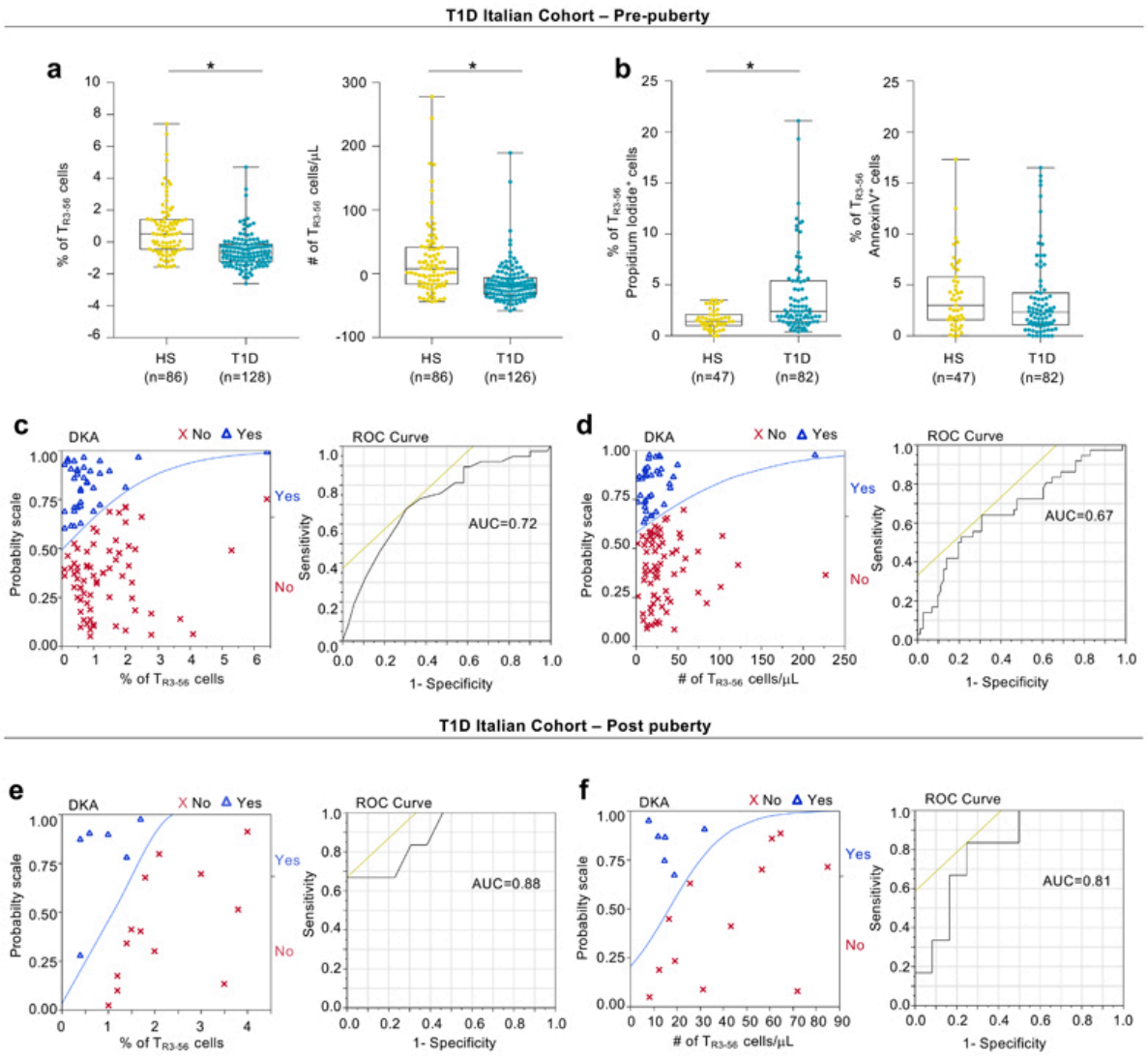
We found that T1D children had reduced percentage and absolute number of TR3-56 cells compared with healthy controls (Figure 3a).

Figure 3



The observed differences were maintained also after adjusting the comparison for sex, age and body mass index (BMI) (Figure 4a). The lower frequency of circulating TR3-56 cells in T1D subjects associated, at least in part, with their increased rate of necrotic death (1.5% ± 0.14, 3.9% ± 0.44 for healthy and T1D subjects, respectively), while no difference was observed in apoptosis (Figure 4b).

Figure 4

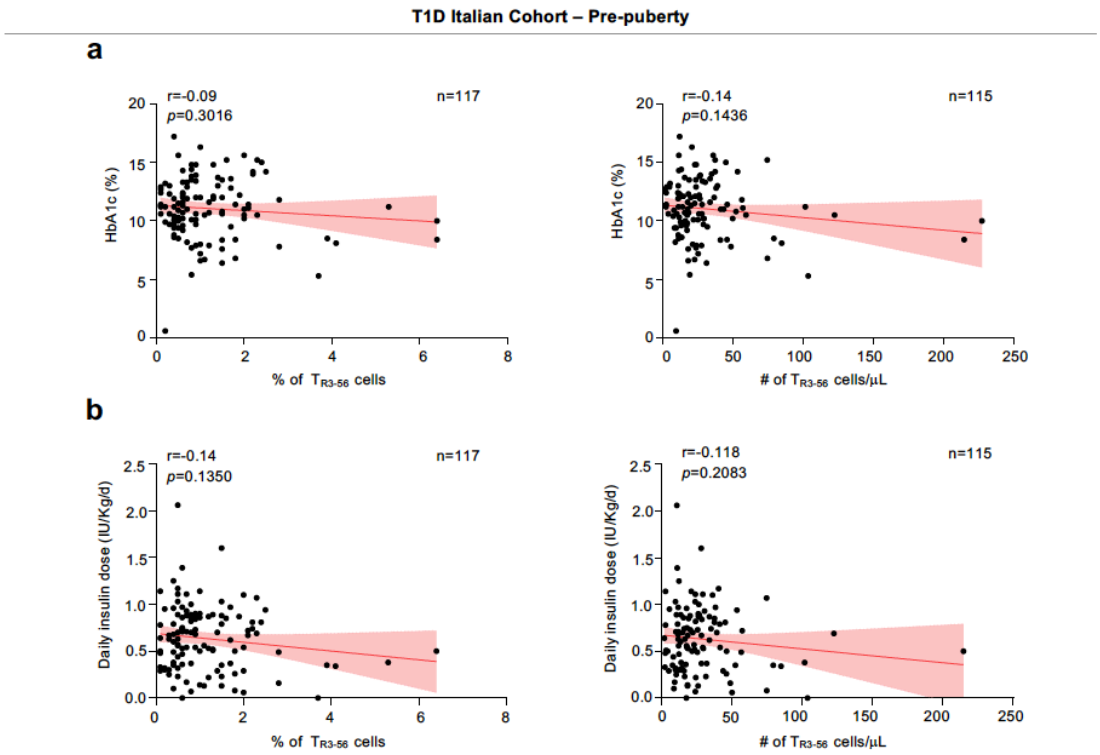


Next, we asked whether TR3-56 cells associated with residual pancreatic β-cell function (measured as circulating fasting c-peptide) in T1D at disease onset. To this end we performed a bivariate analysis that revealed a positive correlation between peripheral

frequency and absolute number of TR3-56 cells and fasting c-peptide levels (r=0.71, p<0.0001; r=0.57, p<0.0001, respectively) (Figure 3b,c).

As diabetic ketoacidosis (DKA), haemoglobin A1c (HbA1c) and daily insulin dose strongly influence T1D complication overtime [22,23], we applied a logistic regression modeling on these parameters and revealed that low percentages of TR3-56 cells were able to predict DKA at disease onset (Figure 4c). Prognostic validity of the fitted model was evaluated by ROC curve analysis and measured using the area under the curve (AUC) (Figure 4c). Low absolute counts of TR3-56 cells also associated with the presence of DKA (Figure 4d). Finally, the frequency and absolute numbers of TR3-56 cells did not associate either with HbA1c values or with daily insulin dose (Figure 5).

Figure 5



We corroborated our findings also in post-puberty young T1D adults at diagnosis (Italian cohort, n=19) (Table 2). Specifically, we observed that TR3-56 cell frequency and absolute number were reduced compared to age-matched healthy subjects (n=14) (Figure 3d), positively correlated with plasma levels of fasting c-peptide ($r=0.46$, $p=0.047$; $r=0.50$, $p=0.0369$, respectively) (Figure 3e,f) and negatively associated with presence of DKA (Figure 4e,f).

Then, to further validate TR3-56 cells as traceable biomarker of T1D progression, we analyzed an independent cohort of children with recent-onset T1D (n=36) recruited at Linköping University Hospital, Sweden (Table 3). In this Swedish cohort, bivariate analysis further confirmed that the frequency of circulating TR3-56 cells positively correlated with fasting c-peptide ($r=0.63$, $p<0.0001$) (Figure 6a).

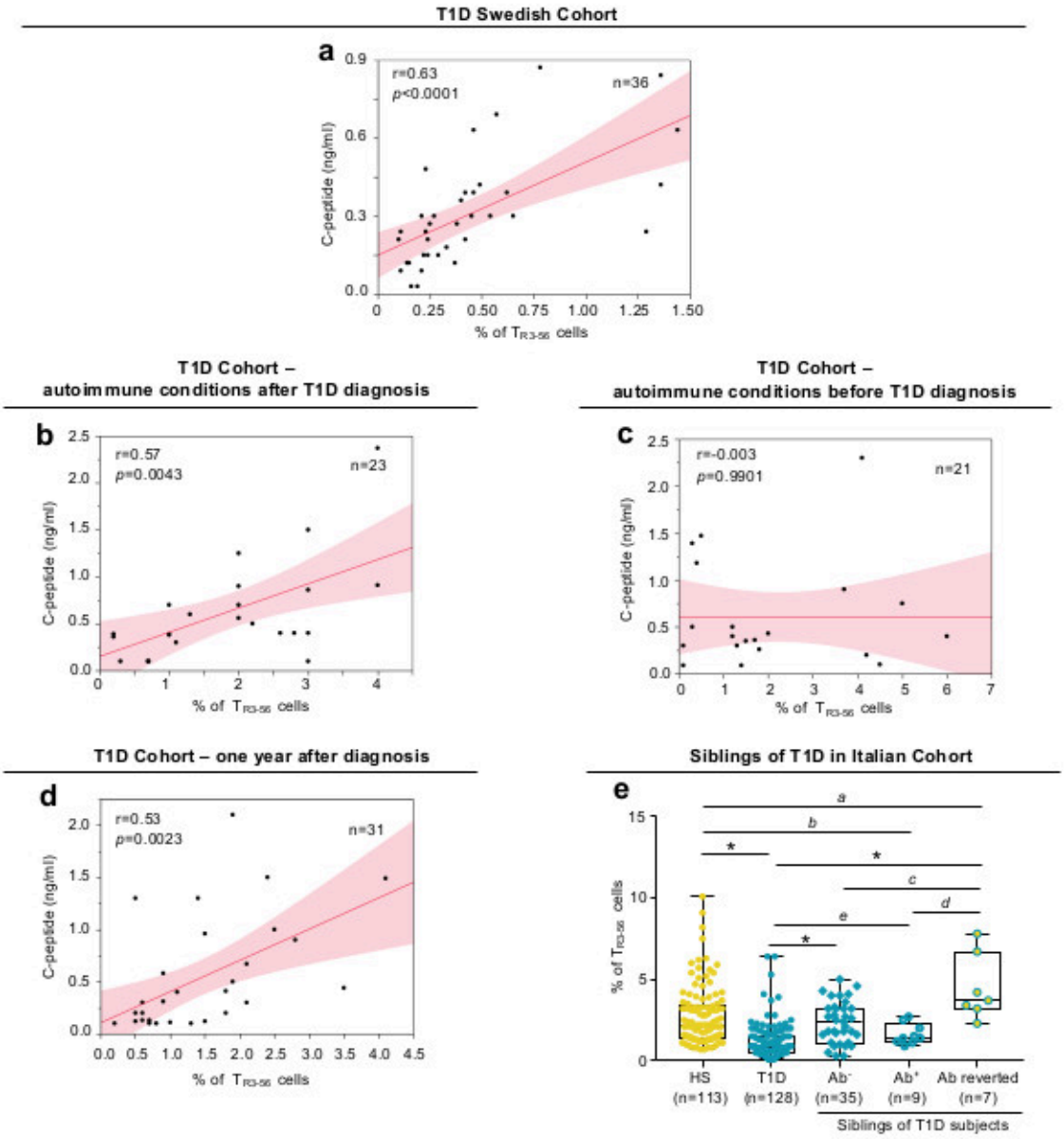
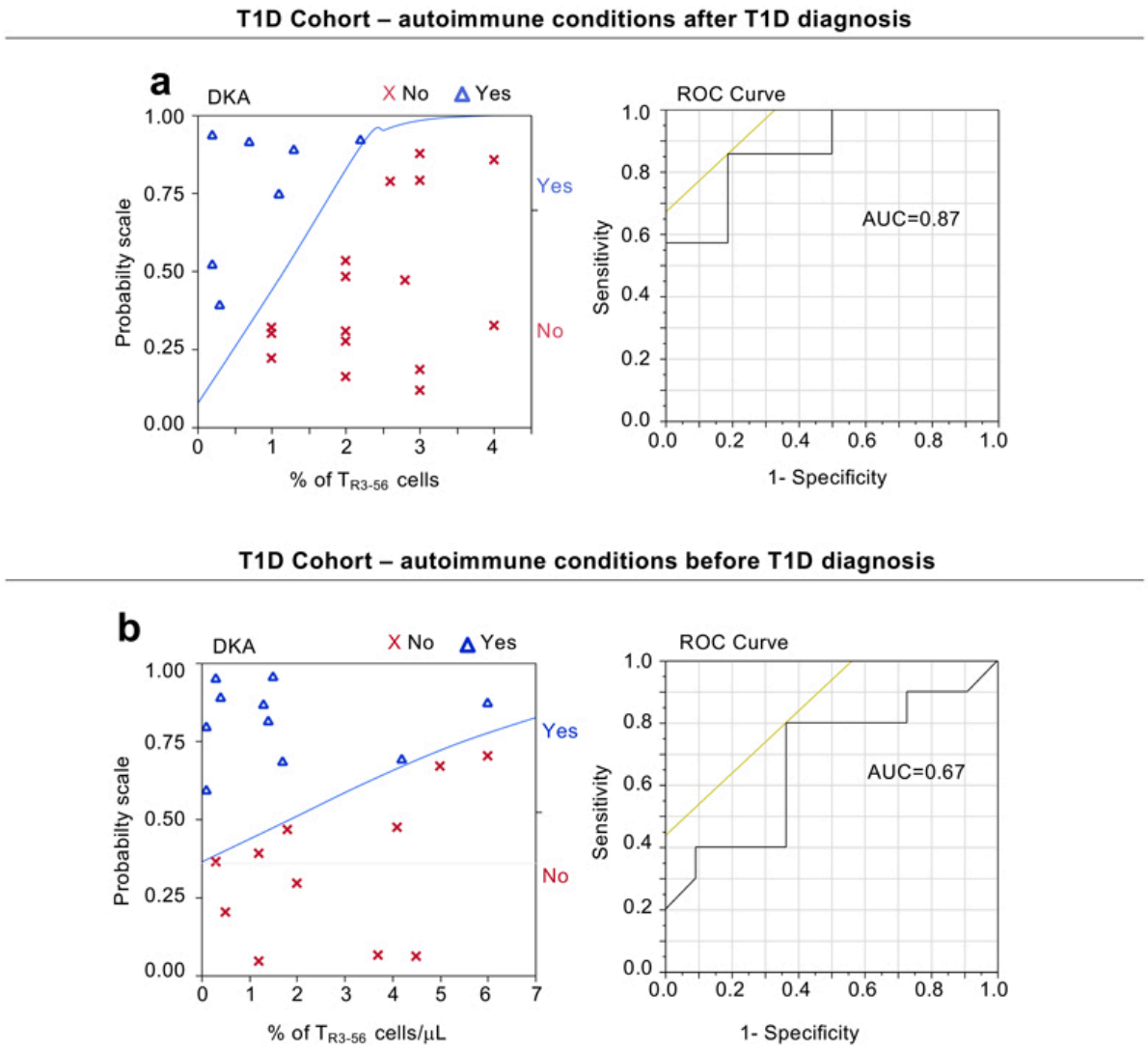

T1D Swedish Cohort
a
r=0.63
p<0.0001
n=36
C-peptide (ng/ml)
% of TR3-56 cells
T1D Cohort –
autoimmune conditions after T1D diagnosis
b
r=0.57
p=0.0043
n=23
C-peptide (ng/ml)
% of TR3-56 cells
T1D Cohort –
autoimmune conditions before T1D diagnosis
c
r=-0.003
p=0.9901
n=21
C-peptide (ng/ml)
% of TR3-56 cells
T1D Cohort – one year after diagnosis
d
r=0.53
p=0.0023
n=31
C-peptide (ng/ml)
% of TR3-56 cells
Siblings of T1D in Italian Cohort
e
% of TR3-56 cells
HS (n=113)
T1D (n=128)
Ab- (n=35)
Ab+ (n=9)
Ab reverted (n=7)
Siblings of T1D subjects

Figure 6

Next, we measured specificity of our findings in a third independent cohort of T1D subjects (n=44) recruited at ELFID (Table 4), in which T1D at diagnosis was associated or not with another autoimmune disease (AIT or CD). Strikingly, in 23 out of 44 children at T1D diagnosis (going to develop also CD or AIT in the following three years), bivariate analysis confirmed the positive correlation between TR3-56 cells and fasting c-peptide levels (r=0.57, p=0.0043) (Figure 6b). Logistic regression modeling established that peripheral percentages of TR3-56 cells indicated the presence of DKA (Figure 7a). On the contrary, in 21 out of the 44 children that at T1D diagnosis were already affected by either CD or AIT, TR3-56 cells did not show statistical correlation with fasting c-peptide levels (r=-0.003, p=0.9901) (Fig. 2c), and weakly associated with the presence of DKA (Figure 7b).

Figure 7



To exclude that the association between TR3-56 cells and c-peptide relied on metabolic alterations, i.e. hyperglycaemia and DKA, both typical of T1D onset, we assessed their frequency also in T1D subjects (n=31) one year after T1D diagnosis when metabolic alterations have been stabilized. In these subjects, we found that TR3-56 cell frequency positively correlated with plasma levels of fasting c-peptide (r=0.53, p=0.0023) and reflected residual β-cell mass (Figure 6d).

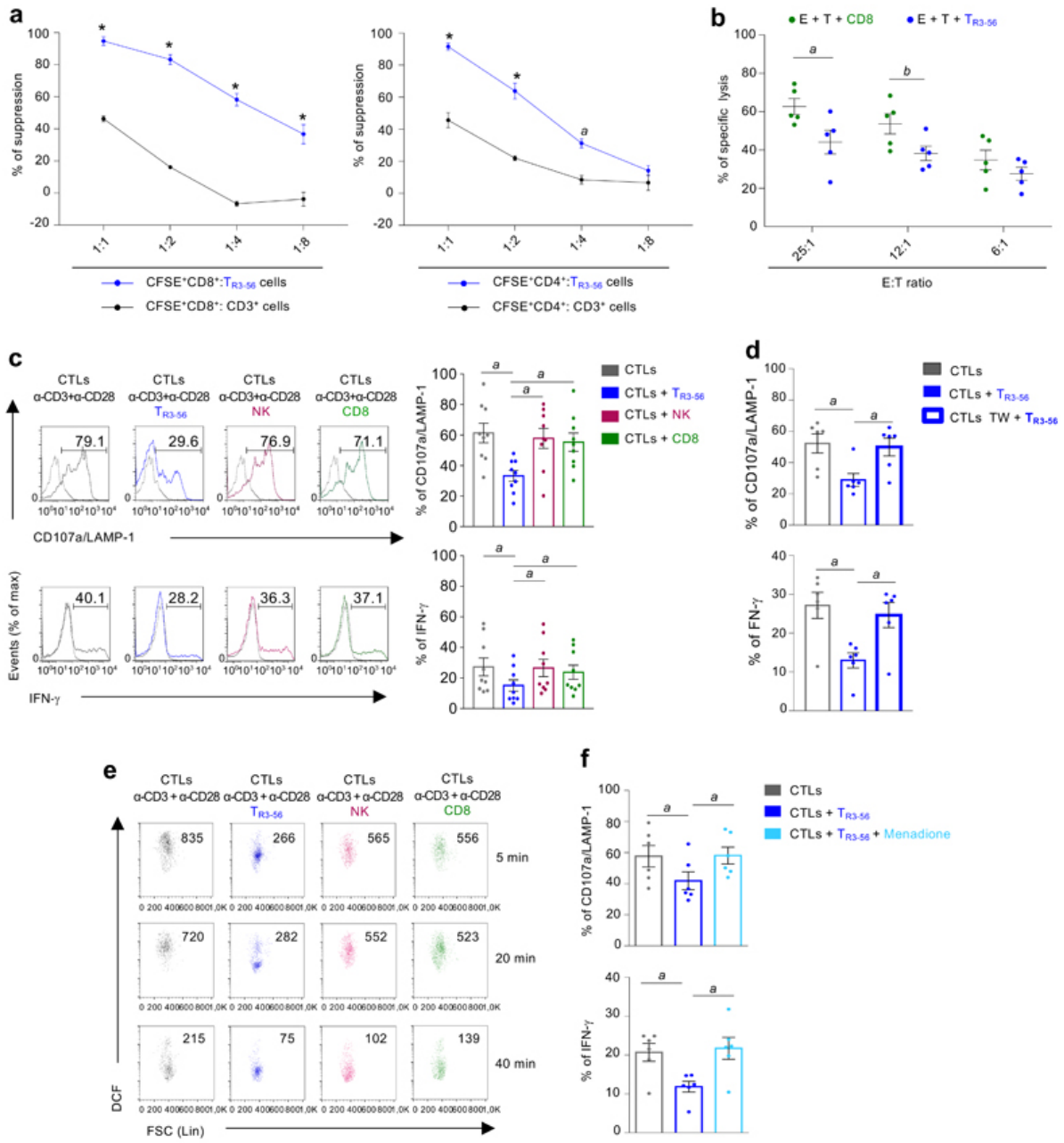
To investigate whether frequency of TR3-56 cells also associated with pre-symptomatic stages of T1D, we measured the frequency of TR3-56 cells in 51 at-risk subjects,

siblings of T1D individuals from our Italian cohort followed over time every six months from 2015. This included 35 autoantibody negative (Ab-), 9 autoantibody positive (Ab^+) and 7 autoantibody positive that reverted into autoantibody negative (Ab reverted) subjects. Interestingly, we observed that frequency of TR3-56 cells was significantly higher in "Ab reverted" subjects compared with healthy, Ab^- and Ab^+ children (Figure 6e). We also noticed a significant reduction of TR3-56 cells in Ab^+ subjects with respect to healthy individuals (Figure 6e). In all, peripheral frequency of TR3-56 cells could act as specific non-invasive biomarker able to reflect disease progression.

Since high frequency of TR3-56 cells associated with a preserved residual β-cell reservoir, we hypothesized a possible, unexplored, immune regulatory role for this cellular subset. To test this hypothesis, we characterized TR3-56 cells in adult healthy donors and subsequently we assessed their function, surface phenotype and molecular profile in T1D children. Specifically, we measured the capacity of TR3-56 cells to affect in vitro proliferation of T cell receptor (TCR)-stimulated human $CD8^+$ and $CD4^+$ T cells from adult healthy donors. Strikingly, we observed that TR3-56 cells inhibited proliferation of both $CD8^+$ and $CD4^+$ T cells (Figure 8a), with the main suppressive effect on the proliferation of the $CD8^+$ subset (Figure 8a). These findings prompted us to focus on the ability of TR3-56 cells to suppress cytotoxic functions of $CD8^+$ T lymphocytes. We evaluated the ability of TR3-56 cells to control cytotoxicity of human $CD8^+$ T cells against allogeneic target too. Specifically, TR3-56 cells, compared with control cells, suppressed lytic capacity of $CD8^+$ effector cells at different effector:target ratio (Figure 8b). Next, we further explore the regulatory activity of TR3-56 cells on cytolytic T lymphocytes (CTLs), generated from $CD8^+$ T cells stimulated with human recombinant (hr) IL-2 in vitro [24, 25]. CTLs were co-cultured with TR3-56 cells or control cells and stimulated for 4 hours via TCR to evaluate cytotoxic activity (measured by CD107a/LAMP-1 expression [26, 27]) and IFN-γ production by CTLs. TR3-56 cells significantly suppressed CTL effector functions, while addition of either Natural Killer

(NK) or $CD8^+$ T cells (as internal control), was unable to affect CD107a/LAMP-1 expression and IFN-γ production by CTLs (Figure 8c).

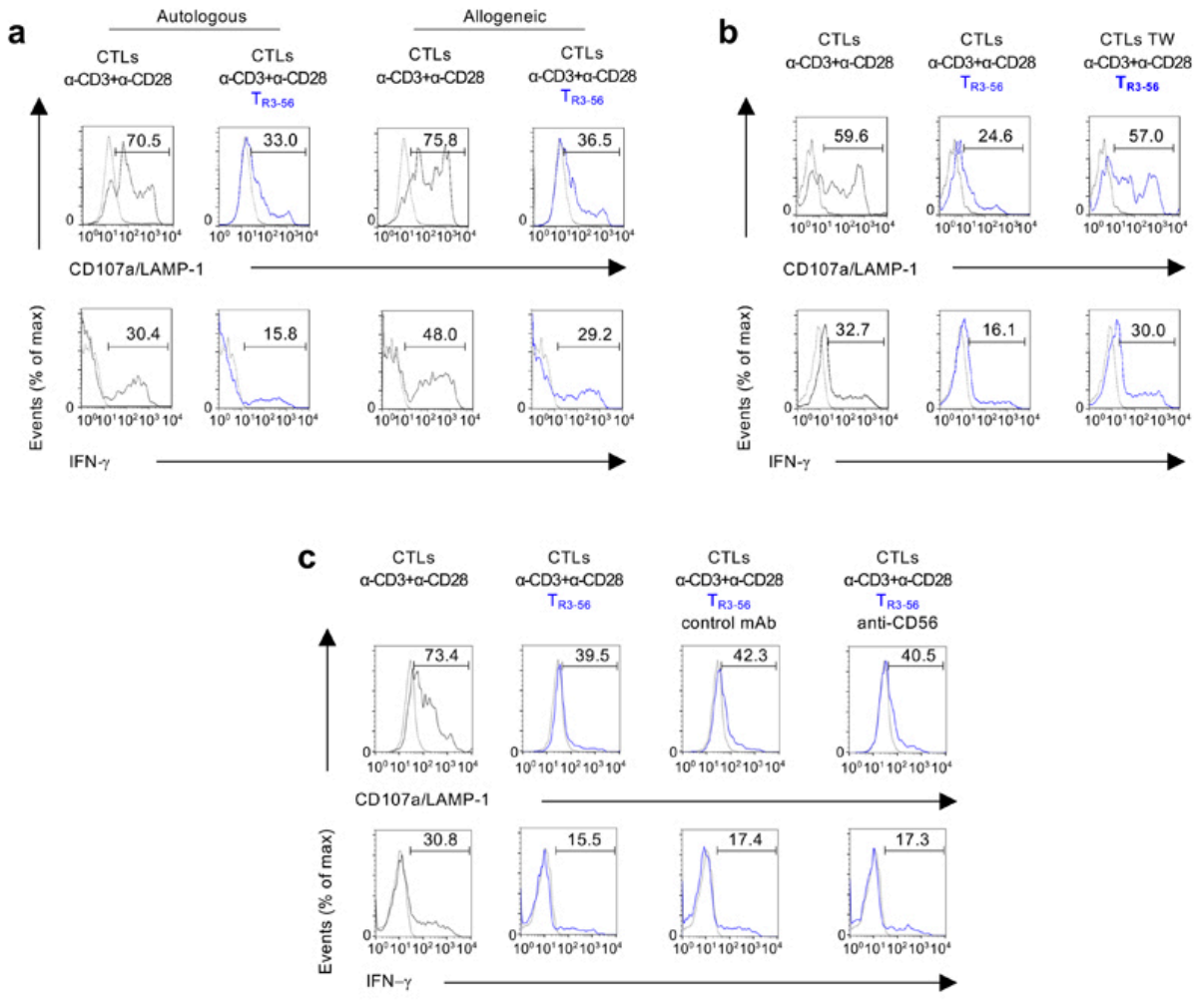
Figure 8



We found that TR3-56 cell suppressive functions were maintained also in co-culture with allogeneic CTLs (Figure 9).

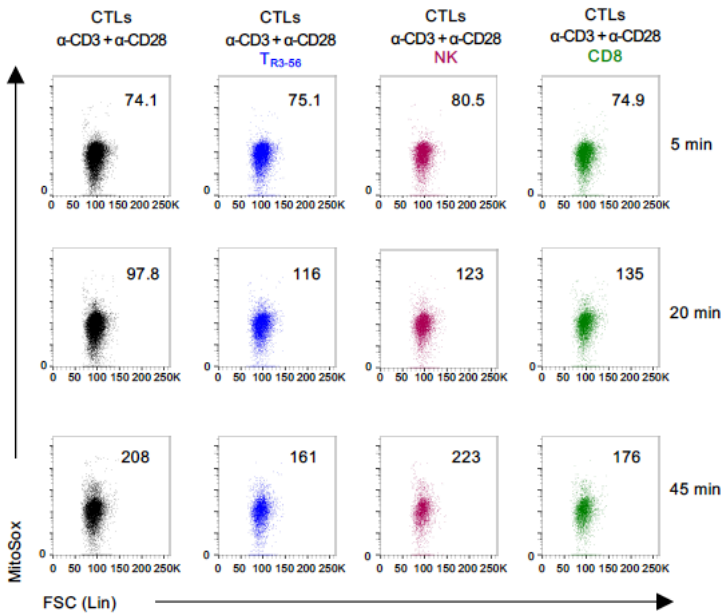
To identify the molecular mechanisms of TR3-56 cell suppression, we assessed whether this function relied on either cell-to-cell contact, secretion of soluble factors or both. Trans-well experiments revealed that TR3-56 cells were unable to exert regulatory activity when separated from CTLs (Figure 8d and Figure 9b). Therefore, their contact-mediated suppressive activity was independent on the expression of CD56 molecules (Figure 9c).

Figure 9



ROS-mediated signaling has been frequently associated with degranulation processes and IFN-γ production by CTLs [28,29]. We studied dynamic changes of cytosolic and mitochondrial ROS levels upon TCR-stimulation of CTLs cultured with TR3-56 cells. Cytosolic CTLs ROS levels, evaluated by 2',7'-dichlorodihydrofluorescein diacetate (DCF) staining, were significantly reduced by TR3-56 cells (Figure 8e); control cells (NK or $CD8^+$ T cells) did not influence cellular ROS levels in CTLs (Figure 8e). Conversely, TR3-56 cells did not affect mitochondrial-derived ROS in CTLs, as testified by mitoSOX staining (Figure 10).

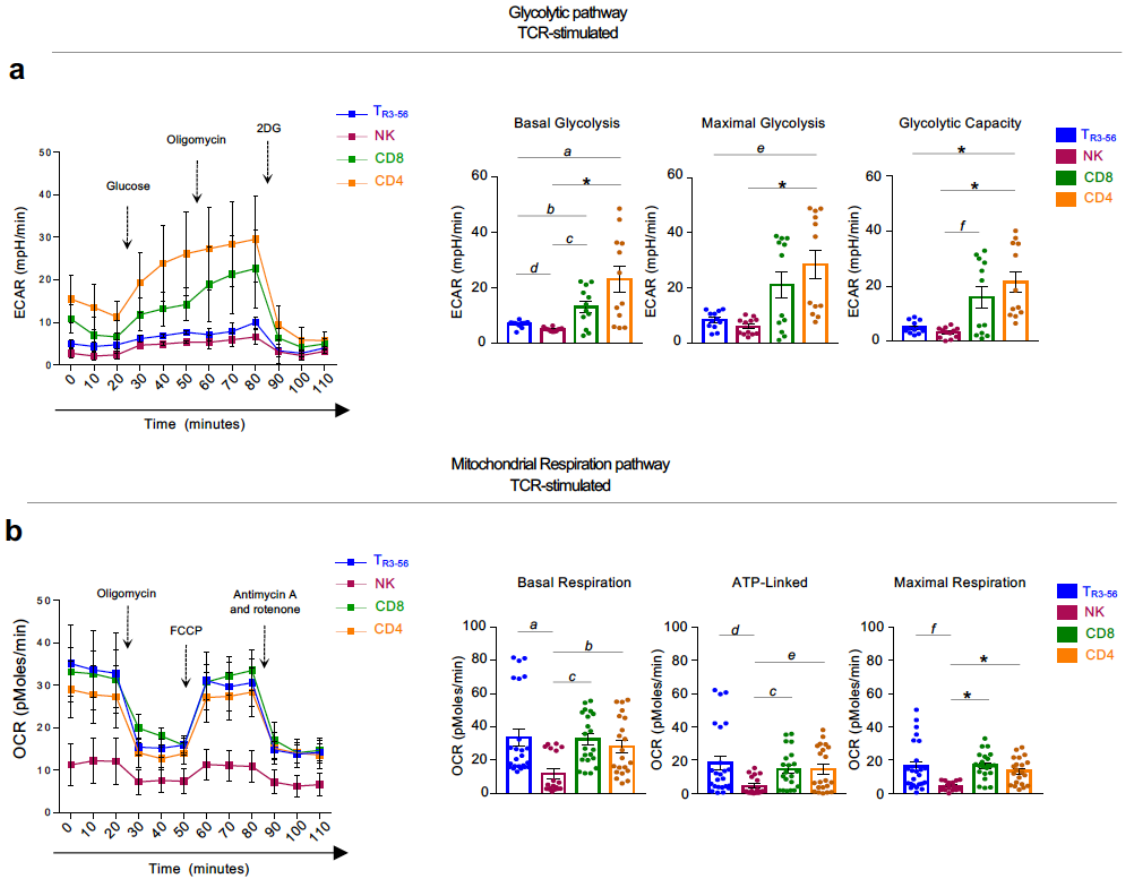
Figure 10



To confirm the role of cellular ROS in mediating TR3-56 cell regulatory activity, we took advantage of the ability of menadione, an analogue of 1,4- naphthoquinone, to generate intracellular ROS via redox cycling [30,31]. TR3-56 cells are unable to suppress CD107a/LAMP-1 expression and IFN-γ of menadione pre-treated CTLs (Figure 8f). To note, treatment with the ROS-inhibitor, N-acetyl-L-cysteine (NAC), completely blocked CTL activation [32], suggesting that TR3-56 cells control $CD8^+$ responses by modulating cytosolic ROS.

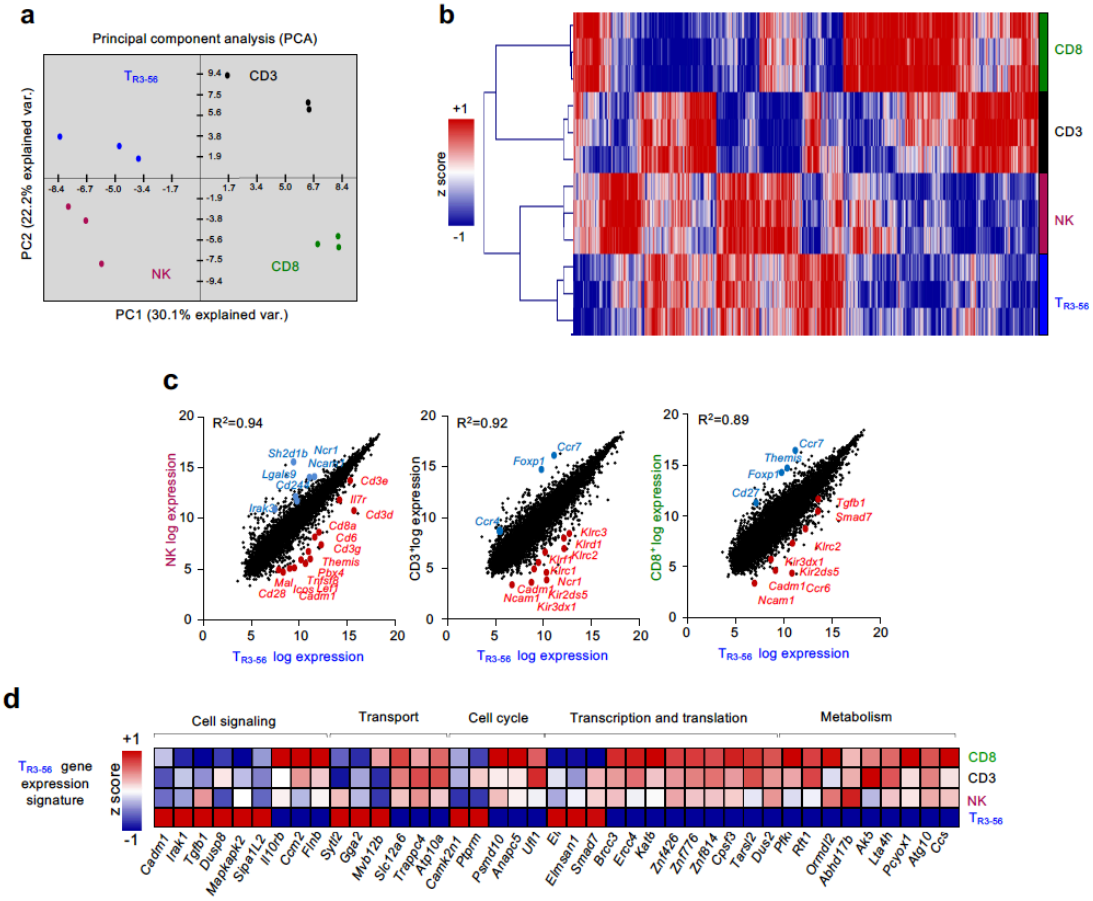
Finally, TR3-56 cells were also characterized for metabolic features (glycolysis and oxidative phosphorylation) and their transcriptional signature. Seahorse analysis revealed that upon TCR stimulation, TR3-56 cells have a distinct metabolic phenotype compared to NK, $CD8^+$ and $CD4^+$ cells, as preferentially utilizing OXPHOS as the main cellular bionergetic source (Figure 11).

Figure 11



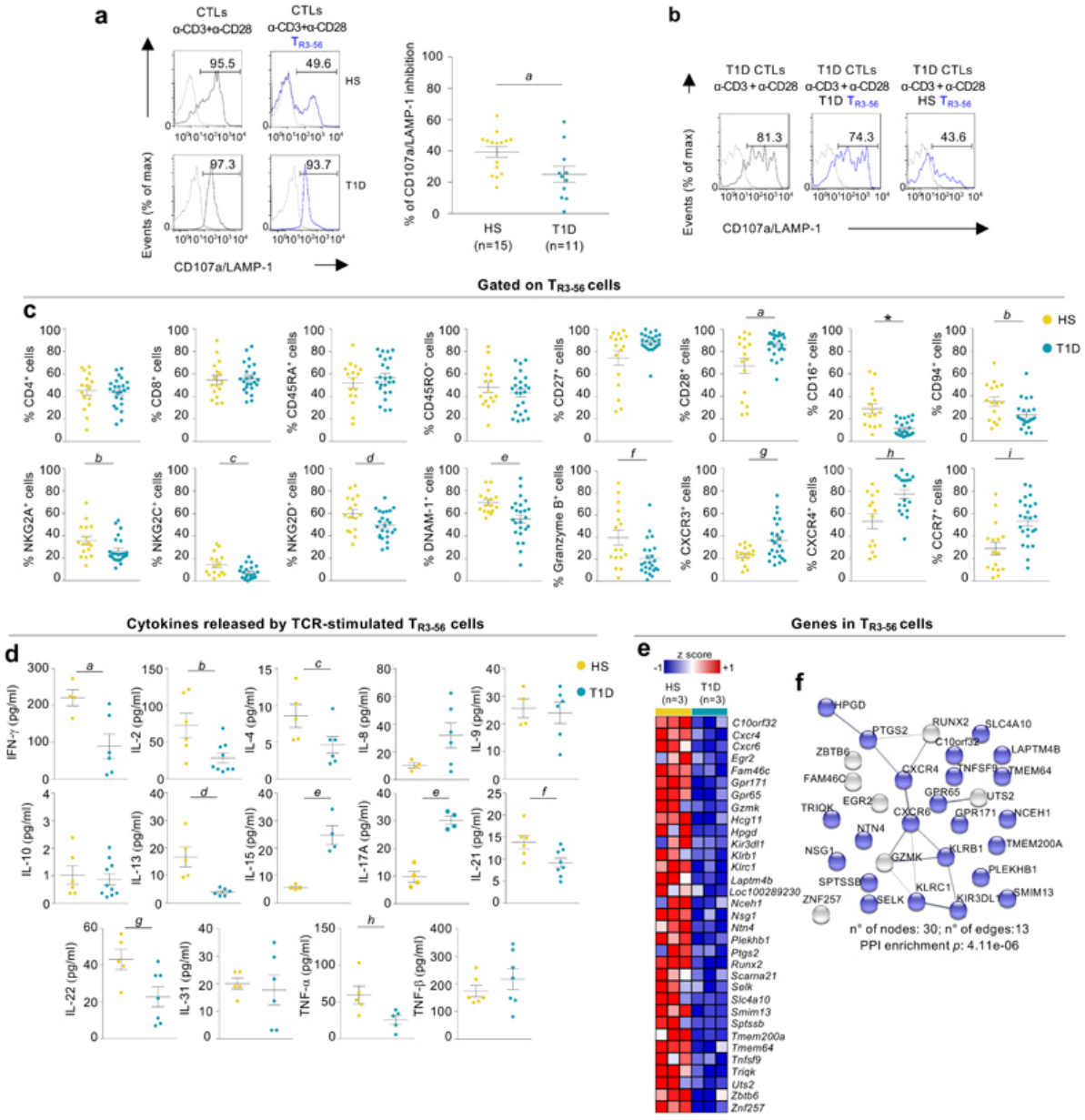
Microarray analysis of RNA from Tr3-56 cells revealed their distinct transcriptomic signature, compared to NK, $CD3^{+}CD56^{-}$ and $CD8^{+}$ subsets (Figure 12).

Figure 12



TR3-56 cells isolated from newly diagnosed T1D subjects had a decreased ability to modulate TCR-dependent CD107a/LAMP-1 expression of autologous CTLs (Figure 13a). This impaired suppressive function was not due to the presence of suppression-resistant $CD8^{+}$ T cells in T1D subjects, since CTLs from T1D resulted to be sensitive to regulatory activity of TR3-56 cells from healthy individuals (Figure 13b).

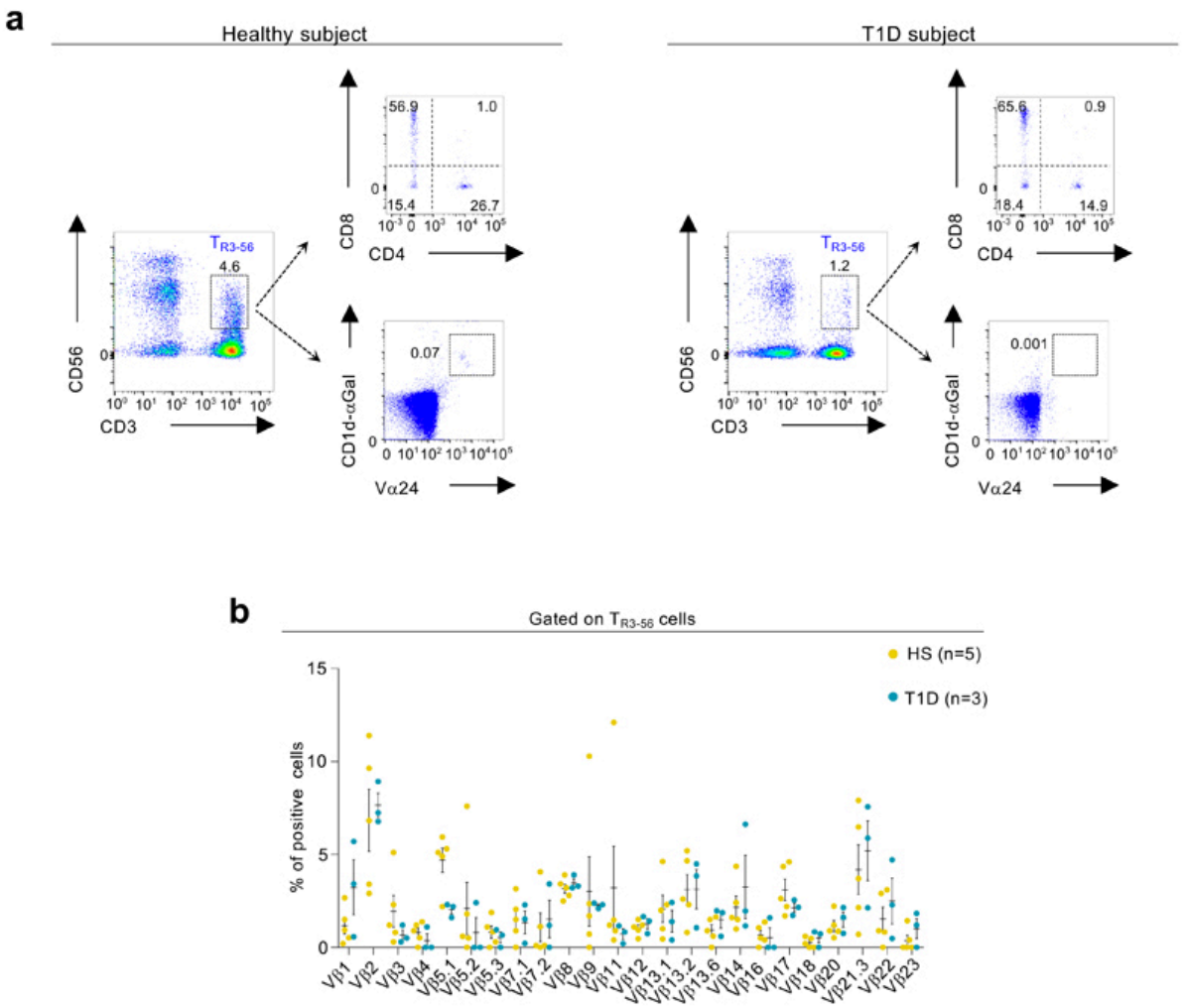
Figure 13



These results indicate that suppressive capability of TR3-56 cells is impaired in T1D children at diagnosis.

Surface phenotypic analysis revealed that TR3-56 cells from recent-onset T1D children were comparable to healthy controls for CD4, CD8, CD45RA, CD45RO and CD27 expression while CD28 surface levels were significantly higher in TR3-56 cells from T1D subjects (Figure 13c and Figure 14a).

Figure 14



Also, TR3-56 cells from T1D children had reduced surface expression of activating/inhibitor receptors (CD94, NKG2A, NKG2C, NKG2D, DNAM-1 and CD16) and cytotoxicity-related molecule (Granzyme-B), compared to healthy children (Figure 13c). On the other hand, TR3-56 cells from recent-onset T1D children expressed increased surface levels of chemokine receptors homing cells in the pancreas, such as CXCR3, CXCR4 and CCR7 (Figure 13c). Low or moderate levels of main T_{Reg} cell-associated markers, such as CD25, the transcription factor forkhead box P3 (FoxP3), CTLA-4, CD39, GITR and PD-1 were expressed on TR3-56 cells from both control and T1D subjects. Finally, FACS analysis revealed that TR3-56 cells from both healthy controls and T1D subjects are distinct from the invariant (i)NKT subset [33,34], as they

are not CD1d-restricted**,** do not express Vα24/Vβ11 TCR chains and display a heterogeneous β TCR repertoire (Figure 14a, b).

Multiplex cytokine analysis showed that TR3-56 cells from new onset T1D individuals released, upon 48 hours of TCR stimulation, reduced amount of IFN-γ, IL-2, IL-4, IL-13, IL-21, IL-22, TNF-α compared with healthy children (Figure 13d); on the other side, TR3-56 cells from T1D children secreted increased amounts of IL-15 and IL-17A (Figure 13d), while no significant differences were observed for other cytokines such as IL-8, IL-9, IL-10, IL-31, TNF-β (Figure 13d).

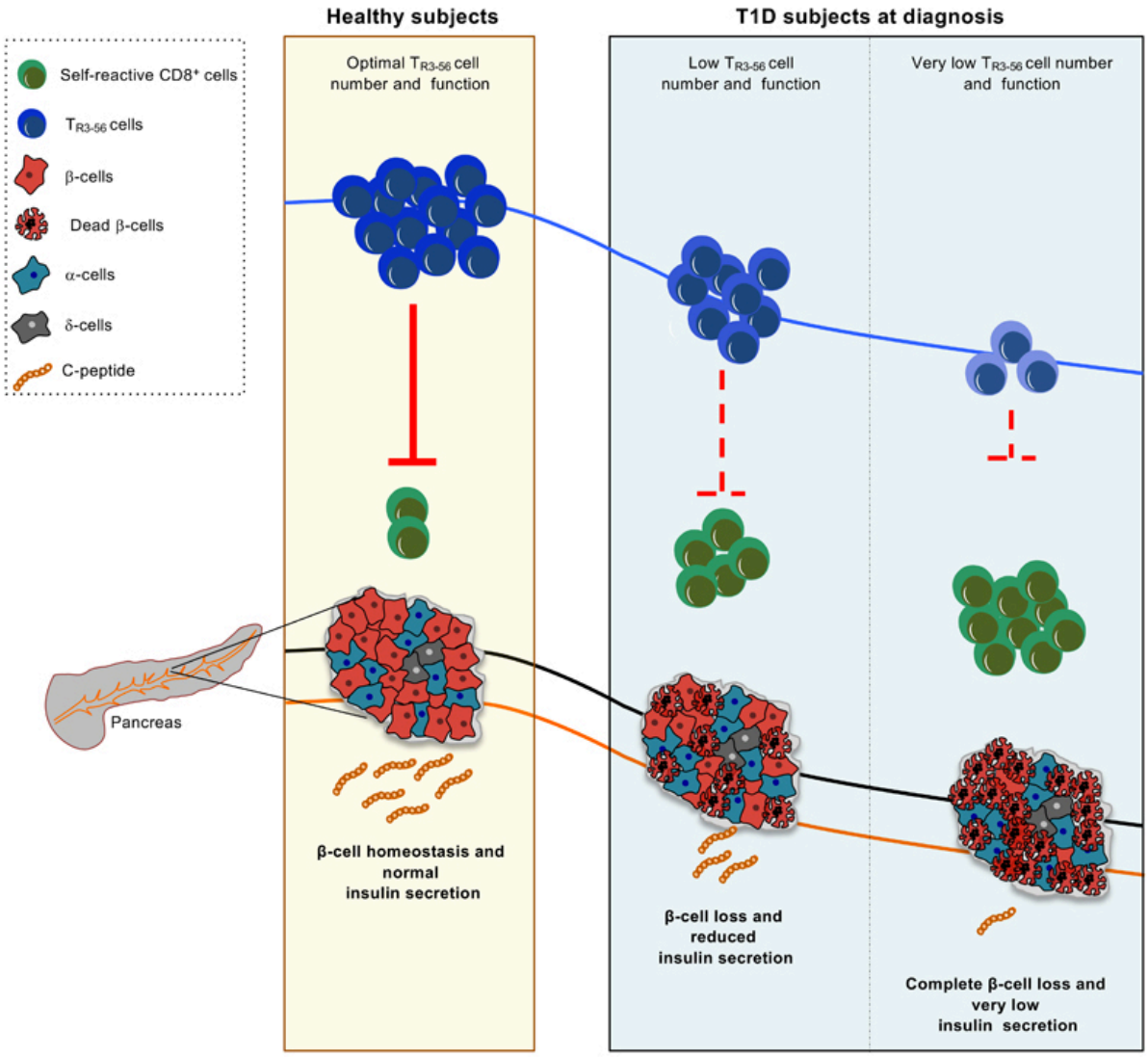
Furthermore, an un-biased high-throughput analysis (RNA-seq) of the transcriptome expressed by TR3-56 cells from T1D children in comparison with age- and sex-matched healthy controls revealed the dysregulation of several genes. In particular, we concentrated our attention on genes (n=33, see Figure 13e) whose mean level was found decreased of more than two folds in T1D cells compared to the healthy counterpart: the majority of these genes (n=23) encoded for proteins functionally linked to the membrane, suggesting a rearrangement of the cell surface in T1D (Figure 13f). Specifically, TR3-56 cells from newly diagnosed T1D expressed lower levels of the G protein-coupled receptor 65 (GPR65) gene, that has been genetically associated with autoimmune disorders [35], KLRB1 (alias CD161) and KLRC1 (alias NKG2A), two killer cell lectin like receptors, described to function as inhibitory determinants in human NK cells [36,37]. Further, we also spotted in T1D TR3-56 cells decreased expression of genes encoding for proteins related to regulatory functions, such as Lysosomal Protein Transmembrane 4 Beta (LAPTM4B) [38] and hydroxyprostaglandin dehydrogenase (HPGD) [39].

8. CONCLUSIONS

In summary, this study reveals that TR3-56 cells may represent a disease biomarker with a previous undisclosed role in human T1D. In three independent cohorts - from Italy and Sweden - of new onset T1D subjects, we found that lower frequency of this cellular subset associates with reduced insulin-secreting capacity and with undesirable disease outcome, such as DKA. Furthermore, we found that TR3-56 cells possess a certain degree of specificity for T1D as their enumeration failed to predict disease progression when T1D was preceded by another autoimmune disease as confounding factor. Moreover, TR3-56 cells associated to c-peptide levels also later from T1D diagnosis (one year later), when metabolic alterations have been normalized. In all, our results also revealed functional, phenotypic and molecular impairments in Tr3-56 cells isolated at T1D onset suggesting a "general" dysregulation of this cellular subset in T1D, also confirmed by reduced expression of either inhibitory/activating receptors and of genes encoding for proteins involved in canonical T_{Reg} cell-mediated suppressive functions (i.e. LPTMB4 and HPGD). In an integrate view, defects of TR3-56 cells associate with attack of pancreatic β-cells by islet-specific auto-reactive $CD8^+$ T cell clones, impacting on residual insulin production and influencing T1D progression. Further, TR3-56 cell counts may represent a valuable criterion to monitor disease progression also improving stratification of individuals for T1D trials and identify at-risk pre-diabetic subjects during the asymptomatic phase of the disease. It is clear that more research is needed to further strengthen our findings, and studies are in progress also in other autoimmune disorders to expand the role of TR3-56 cells in immunological self- tolerance and their potential translational relevance in a wider perspective. In conclusion, we propose a model in which in healthy conditions, TR3-56 cells might participate to immune regulation to preserve tissue integrity of insulin-producing β-cells. An alteration in number and/or function of this cellular subset could lead to β-cell damage and loss of endogenous insulin production (measured as fasting c-peptide), thus allowing the seed of autoimmunity to take root (Figure 15).

This study allowed us to publish a scientific article on “Nature metabolism” on February 2020.

Figure 15



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