Title
IL-13 pathway alterations impair iNKT cell-mediated regulation of T effector cells in Type 1 Diabetes

Running title: IL-13 secretion mediates regulation by iNKT

Authors
Lorena Usero¹, Ana Sánchez¹, Eduarda Pizarro², Cristina Xufré¹, Mercè Martí¹, Dolores Jaraquemada¹, Carme Roura-Mir¹

Affiliation
¹Immunology Unit, Institut de Biotecnologia i Biomedicina and Dept of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.
²Unitat d’Endocrinologia, Hospital de Mataró, Carretera de Cirera s/n, Mataró, 08304 Barcelona, Spain

Corresponding author
Carme Roura-Mir
Immunology Unit
Institut de Biotecnologia i Biomedicina
Universitat Autònoma de Barcelona
08193 Bellaterra, Barcelona
Spain
E-mail address: carme.roura@uab.cat
Tel.: +34 935812801; fax: +34 935812011

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ABSTRACT

Many studies have shown that human NKT cells can promote immunity to pathogens but their regulatory function is still being investigated. Invariant NKT cells (iNKT) have been shown to be very effective in preventing Type 1 diabetes in the NOD mouse model. Activation of plasmacytoid dendritic cells, modulation of B cell responses and immune deviation, were proposed to be responsible for the suppressive effect of iNKT cells. In the present work, we have studied the regulatory capacity of human iNKT cells from control and T1D patients at disease clinical onset. We demonstrated that control iNKT cells suppressed the proliferation of T effector cells through a cell contact-independent mechanism. Interestingly, suppression was dependent on the secretion of IL-13 by iNKT cells as an antibody blocking this cytokine resulted on the abrogation of Teff cell suppression. However, T1D-derived iNKT cells showed impaired regulation that could be attributed to the decrease in IL-13 secretion. Thus, alteration of the IL-13 pathway at disease onset may lead to the progression of the autoimmune response in T1D. Advances in the study of iNKT cells and the selection of agonists potentiating IL-13 secretion should permit the development of new therapeutic strategies to prevent the development of T1D.
INTRODUCTION

Type 1 diabetes (T1D) results from an autoimmune attack leading to the destruction of the insulin-secreting pancreatic β-cells. Infiltration of islets by T, B cells and macrophages, insulitis, is a pathognomonic sign for T1D at disease onset (1,2), as well as autoantibodies toward islet proteins (3). β-cell destruction is suggested to be mediated by CD4+ and CD8+ T cells (4) but the mechanisms, likely multifactorial, are not completely understood yet. Despite a strong emphasis placed on the role of effector T cells in the disease development and progression, deficiencies in multiple immune pathways have also been associated with T1D, such as decreased regulation by T regulatory cells (Tregs) or defects in the iNKT cell population (5).

Type I or invariant iNKT (iNKT) cells are a population of T cells expressing markers for NK and T cell lineages that display characteristics different from conventional T cells: (i) iNKT cells specifically recognize lipid-based antigens presented by the MHC-I-like CD1d molecule; (ii) virtually all human iNKT cells express a TCR with the Vα24Jα18 chain mostly paired with Vβ11; and (iii) iNKT cells express an activated/memory phenotype so they can rapidly exert effector functions without further TCR activation (6). iNKT cells from human peripheral blood mononuclear cells (PBMCs) in healthy donors typically range from 0.04 to 1.3% of the lymphocytes (7). In addition, human iNKT cells have been subdivided by their expression of CD4 or CD8, defining CD4+, CD8+ and CD4−CD8− DN iNKT cell subsets, constituting 27%, 24% and 49% of peripheral iNKT cells, respectively (8). These populations show different cytokine profiles (9).

While many studies showed that iNKT cells promote immunity to pathogens, their capacity to secrete IL-4 suggested a potential regulatory function of some autoimmune responses. In the NOD mouse model of T1D, iNKT cells provide a protective effect by releasing IL-4, inducing Th2-type responses to islet autoantigens (10,11). Adoptive transfer experiments revealed that iNKT cells potently inhibited the differentiation of islet-specific transferred T effector cells in the
pancreatic lymph node (PLN) (12). This was explained by the ability of iNKT cells to recruit tolerogenic dendritic cells favoring the presence of Tregs (13). These were necessary for iNKT cells to transfer protection from T1D (14). In contrast, data from human T1D are scarce. Clinical studies have shown low frequencies and functional defects of iNKT cells that might contribute to the disease development, although there is no consensus about the nature of the iNKT cells defects (7). Some studies pointed to similar defects of iNKT cells in T1D patients and at risk groups (15,16).

Following activation, iNKT cells promptly produce large amounts of various cytokines, thereby providing signals to other immune cells, including DCs, NK cells and conventional T and B cells (17,18). It has been proposed that the strength of the TCR downstream signaling, the integration of cytokine receptors signaling and the cellular qualities of the lipid presenting APC determine the expression of particular transcription factors favoring specific iNKT effector phenotypes (19,20,21).

The composition of the T-cell cytokine profile is an important factor in the outcome of the autoimmune response. Pro-inflammatory cytokines are abundantly secreted by in situ effector T lymphocytes and macrophages in T1D, whereas the secretion of anti-inflammatory cytokines is not predominant (22). Interleukin-13 is a cytokine primarily produced by Th2 lymphocytes, with powerful anti-inflammatory properties that retard or prevent T1D in NOD mice (23). IL-13 binds to two cell surface receptors, type 1 (IL13Rα1/IL4Rα) and type 2 (IL13Rα2). The IL13Rα1 monomer binds IL-13 before dimerizing with the IL-4Rα chain to transduce intracellular signals through STAT6 phosphorylation (24). IL13Rα2 binds IL-13 with higher affinity than IL13Rα1 but its signaling properties are less known. Recent reports showed that IL13Rα2 binds to several intracellular signaling molecules, suggesting specific signaling functions (25,26,27).

In the present manuscript, we hypothesized that iNKT cells may play a regulatory role in human T1D and investigated the cellular requirements and molecular mechanisms underlying iNKT cell
mediated regulation in healthy donors (HD) and T1D patients. We have demonstrated that (i) iNKT cells have regulatory effects on Teffs, (ii) the regulatory mechanism is mediated by their ability to secrete IL-13, (iii) suppression by iNKT cells is impaired in T1D patients compared to controls and, (iv) the reduced regulatory capacity of iNKT cells from T1D patients correlates with a defective IL-13 production. In summary, the results show that a diminished IL-13 secretion by T1D-derived iNKT cells may explain the impaired regulation of the autoimmune response described in human T1D.

RESEARCH DESIGN AND METHODS

Samples

Teff and iNKT cells were purified from PBMCs from healthy donor volunteers (HD) (n=14) used as control samples and T1D patients (n=18) (Table 1). Heparinized blood samples were taken after signing an informed consent approved by the ethical committee of the relevant institutions. Blood was drawn from patients at clinical disease onset, before receiving any treatment.

Selection and in vitro expansion of iNKT cells

iNKT cells were obtained from PBMCs by Ficoll Paque density gradient centrifugation. iNKT cells were selected based on the expression of the TCR α-chain Vα24Jα18 (anti-iNKT Microbeads Human, Milteny Biotec) using the Automacs system (Macs, Milteny Biotec). A fraction of the iNKT purified cells was cultured in IMDM medium (Iscove's Modified Dulbecco's Medium), supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100µg/ml) (all from Sigma-Aldrich) and human serum (8%). iNKT cells were expanded in the presence of 30Gy-irradiated-autologous PBMCs (3x10^6 cells/well), rhIL-2 (20U/ml, NIH) and 3x10^5 cells/well of irradiated C1Rd cells (lymphoblastoid B cell line, C1R cells, stably expressing CD1d) previously pulsed with 100ng/ml of αGalactosylCeramide for 1h
at 37°C (αGC, Enzo Life Sciences). After three days of culture, medium was replaced with fresh medium containing rhIL-2 (20U/ml). Cells were expanded for a minimum of 2 weeks before being used in the assays. Cells were phenotyped using specific monoclonal antibodies, purified by cell sorting and cryopreserved until use.

Teff cells (CD4+ CD25−) were isolated in a two-step negative selection process. First, based on CD4 expression and then on the absence of CD25, using the CD4+ T Cell Isolation Kit II human and the CD25 Microbeads Human kit (both from Milteny Biotec).

**Antibodies and flow cytometry analysis**

Cell phenotype was analyzed by flow cytometry (FACSCanto II, BD Biosciences) using specific MoAbs. For iNKT cells: CD3-PE-Cy7 (UCHT1, Biolegend), TCR α-chain Vα24Jα18-PE (6B11, Milteny Biotec), CD4α-APC (RPA-T4, Biolegend), CD8β-PE-Cy5 (2ST8.SH7, Beckman Coulter) and TCRVβ11-FITC (C21, Beckman Coulter). Purity of the Vα24Jα18CD3Vβ11+ cells before and after in vitro expansion was typically over 98%. Two combinations of MoAbs were used for Teff cell characterization: 1) CD3-Alexa488 (UCHT1, BD Pharmingen), CD4-PERCP (SK3, Becton&Dickinson) and CD25-PE (3G10, Milteny Biotec) and, 2) CD4-PERCP, CD25-PE and Foxp3-Alexa488 (PCH101, eBioscience), following manufacturer’s protocol. The purity of CD3+CD4+CD25−Foxp3− Teff cells was 80% average. IL-13 secretion was measured using the anti-IL13 antibody (JES10-5A2.2, Biolegend). Expression of IL-13 receptors was measured using specific antibodies to IL13Rα1 (SS12B, Biologend) and IL13Rα2 (SHM38, Biolegend).

**Verification of iNKT cell function**

iNKT cell function was assayed by measuring TNFα secretion in cultures with 30Gy- irradiated autologous PBMCs (1x10⁵cell/well), 45Gy-irradiated C1R− (mock transfectd C1R cells) or C1Rd cells (C1R cells expressing CD1d) (2x10⁴ cell/well) as APCs pulsed 1h at 37ºC with αGC. As positive controls, cells were stimulated with αCD3/CD28-coated beads (Dynabeads, Life Technologies). Culture supernatants were collected after 48 h. TNFα production was measured
by ELISA (Human TNFα Antibody Pair, Invitrogen, Life Technologies). Concentration was quantified in a Victor3 plate reader system (Perkin-Elmer).

**Cell suppression assays**

iNKT cells regulatory properties were analyzed by *in vitro* suppression assays. Different ratios of iNKT:Teff cells (0.25:1, 0.5:1, 1:1, 2:1), where 1=10⁴ cells/well, were seeded in triplicate in 96-well plates (Nunc, LabClinics) and stimulated with anti-CD3/CD28-coated beads (cell:bead ratio 2:1) (Dynabeads, Life Technologies) and autologous irradiated PBMCs (5×10⁴ cells/well). Cultures of each cell type (1×10⁴ cells/well) were used as controls. Plates were incubated 4 days at 37°C-5% CO₂. Teff proliferation was measured by H³-thymidine incorporation pulsing with 1 µCi/well of ³[H]-methyl-thymidine (Perkin-Elmer) 16 h before harvesting and analyzing on a beta counter (1450 Microbeta, Wallac Trimx, Liquid scintillation & Luminescence Counter). Percent suppression was calculated as follows: 100 − [(100 × cpm ratio iNKT:Teff cells)/ per cpm Teff cells]. All cultures were done in triplicate.

The requirement for cell-cell contact was assayed using a transwell system. Autologous 30Gy-irradiated PBMCs (4×10⁵ cells/well) were cultured in transwell 24-well plates (0.4 µm pore, LabClinics) using a 1:1 ratio of iNKT:Teff cells, where 1=10⁵ cells. Teffs were seeded on the lower chamber while iNKT cells were added to the lower or the upper chamber, together with feeder cells. As controls, each cell type was cultured alone. After stimulation as above, 24h and 48h supernatants were collected to analyze cytokine secretion.

**IL-13 assays**

To confirm the role of IL-13, an anti-IL13 neutralizing antibody (JES10-5A2, Biolegend) was added to suppression assays. iNKT and Teff cells alone or mixed at different ratios were incubated in the presence of increasing concentrations of the antibody or 30 µg/ml of control IgG₁ (Rat IgG₁, κ, Biolegend). Culture supernatants were collected after 48h. For some experiments, different concentrations of human recombinant IL-13 (rh-IL-13, Biolegend) were
added to the Teff cultures. In all cases, αCD3CD28-coated beads were used as stimulus. Teff proliferation was measured by H\(^3\)-thymidine incorporation after 4 day culture in all conditions.

**Cytokine secretion analysis**

Cytokine secretion was measured by Cytometric Bead Array (CBA, Becton Dickinson) on 24h culture supernatants (IL-2) or 48h culture supernatants (TNFα, IFNγ, IL-4, IL-10, IL-13 and IL-17A). Data were analyzed using FCAP Array™ Software Version 3.0 (Becton Dickinson). For intracellular staining, iNKT cells were incubated with 20 ng/ml PMA and 1 µg/ml Ionomycin for 6 hours at 37ºC and 1 µg/ml of brefeldin A was added the last 4 hours. Cells were collected, fixed with 4% paraformaldehyde and permeabilized with 0,1% Saponin. Cells were blocked with human serum + 0,3% saponin before an anti-IL13 antibody was added. IL-13 production was measured by flow cytometry.

**RNA extraction and cDNA Synthesis**

RNA was extracted from iNKT and Teff cell subpopulations from PBMCs of HD and T1D patients. RNA was extracted using the RNeasy Plus Mini kit (QIAGEN) and subsequently quantified using the Nanodrop 1000 (Thermo Scientific). Integrity of the RNA (RIN) was assessed with the LabChip 2100 BioAnalyzer (Agilent Technologies). Samples with RIN > 7 were considered for the experiments. cDNA was synthesized from total RNA using Superscript III (200U/µl, Invitrogen) following manufacturers’ protocol.

**Conventional PCR and Sequence analysis**

IL13, IL13Rα1 and IL13Rα2 expression was analyzed by RT-PCR with previously described primers (28,29,30). Primers specific for CD3γ were: Fw: 5'-CCCAATGACCAGCTCTACCA-3' and Rev: 5'-GGAACTGAATAGGAGGAGAACAC-3'. PCR reactions were performed at an annealing temperature of 59ºC for IL13Rα1 and IL-13, of 57ºC for IL13Rα2 and 58ºC for CD3γ. Primers to characterize iNKT cells: 1) Vα24 (Fw: 5'- CTGGATGCAGAACAAAGCAGA GC-
3’) and the Jα18 TCR segment (Rv: 5’- AGGCCAGACAGTCAACTGAGTTCC-3’); 2) Vβ11 (Fw: 5’-ACAGTCTCCAGAATAAGGACG-3’ and the Cβ (Rv: 5’-CTTCTGATGGCTCA AACA-3’). PCR reactions were performed at an annealing temperature of 65ºC for the TCRα chain and of 55ºC for the TCRβ chain (31). PCR products were electrophoresed on 2% agarose gel. The bands obtained were cut and purified using the kit “GFX PCR DNA and Gel Band Purification Kit” (GE Healthcare), and sequenced at the Genomics and Bioinformatic Service at UAB (ABI 3130XL genetic analyzer) using the BigDye 1.1 or BigDye 3.1 system, depending of the base pair numbers for each fragment.

To quantify the expression of IL13Rα1 and IL13Rα2, gels were analyzed by densitometry using GelAnalyzer 2010 software. Each band was quantified extrapolating from a standard concentration curve of known molecular weight fragments (Molecular Weight and Mass of 100bp DNA Ladder, Genegraft). Relative receptor expression by T cells was normalized with the CD3γ expression.

IL-13 receptor sequences were analyzed using the BLAST software (Basic Local Alignment Search Tool) and ClustalW2 alignment software. Sequence assignment for the V and J segments of the TCR α and β chains was performed with the IMGT-V-Quest software (ImMunoGeneTics).

Statistical analysis
Statistical analysis was done with T-student or One-way Anova tests using GraphPad Prism 4 software. A p value <0.05 was considered statistically significant.

RESULTS

Ex vivo and in vitro-expanded human iNKT cells suppress T effector cell proliferation

The capacity of human iNKT cells of regulating Teff proliferation was tested. PBMC-derived iNKT cells were cultured with CD4⁺CD25⁻ autologous Teff at different ratios. Regulation was assessed on a cell proliferation assay upon stimulation with anti-CD3/CD28-coated beads. Teff
proliferation was inhibited when iNKT cells were added to the culture (Fig. 1a, left). At high iNKT:Teff ratio, inhibition reached 40% and the difference was statistically significant (Fig. 1a, right). Proliferation of iNKT cells alone was similar to that of feeder cells.

We next analyzed the suppression function of *in vitro* expanded iNKT cells. Expression of TCRVα24Jα18/Vβ11 was measured by flow cytometry (suppl. fig. 1a) and αGC specificity and CD1d restriction by TNFα production (suppl. Fig. 1b). iNKT cells obtained and expanded from control PBMCs (n=9) maintained their capacity to suppress Teff proliferation. A statistically significant higher suppression activity was observed at 2:1 iNKT:Teff cell ratio when compared to proliferation by Teffs alone (Fig. 1b, left). Therefore, we demonstrated that human iNKT cells suppressed the proliferation of Teffs.

**IL-13 secreted by iNKT cells mediates suppression of autologous T effectors**

To define the mechanism behind iNKT cell-mediated suppression, production of IL-2 was measured in iNKT:Teff coculture supernatants. As shown in Figure 2, IL-2 secretion was dependent on the activation of Teffs and addition of iNKT cells to the cultures significantly reduced the secretion. Irradiation of Teffs abrogated IL-2 production. However, irradiated iNKT cells did not suppress IL-2 production by Teff. The correlation between a decreased Teff proliferation in the presence of iNKT cells and the reduction on IL-2 secretion in the cocultures indicated that one mechanism of iNKT cell suppression was the inhibition of IL-2 secretion by Teff cells.

To assess cell-cell contact requirements for suppression, proliferation was assayed on a transwell system. iNKT cells were alternatively loaded on the upper or the lower chamber of the transwells where Teffs were seeded. IL-2 secretion was inhibited by iNKT cells in a cell-cell contact independent manner, i.e., IL-2 reduction was equivalent whether iNKT cells were loaded on the upper or the lower chamber (Fig. 2). This indicated that suppression of Teff cell proliferation by iNKT cells was mediated by a soluble factor secreted by iNKT cells.
To find out if cytokines were mediating suppression, we measured cytokine secretion in iNKT:Teff cocultures and single cell type cultures. Individual stimulation of Teff and iNKT cells produced an array of cytokines including IFNγ and TNFα but not IL-17A or IL-10 (Fig. 3a and data not shown). Interestingly, iNKT cells secreted high levels of immunomodulatory cytokines IL-4 and IL-13 that increased in iNKT:Teff cocultures (Fig. 3b) in a cell-cell contact-independent manner. The most prominent increase was on IL-13 production. No IL-13 was produced by unstimulated ex vivo or in vitro-expanded iNKT cells (data not shown).

To assess the importance of IL-13 involvement on suppression, we measured proliferation in iNKT:Teff cell cocultures, adding increasing concentrations of an IL-13-blocking antibody (Fig. 4a). The addition of the antibody partially restored proliferation of Teffs while no recovery was observed if an irrelevant antibody was added instead. The same pattern was observed for IL-2 production by the same Teff cells (Figure 4b). These results strongly indicated that IL-13 was responsible for the suppression. Further experiments showed that the addition of anti IL-4 to the above cocultures did not modify the effect of anti-IL-13 antibody (Figure 4c).

**iNKT cells from T1D patients show deficient Teff suppression**

We next analyzed the suppressor capacity of T1D patients’ iNKT cells isolated at disease onset. In vitro expanded T1D patients’ iNKT cells showed impaired ability to suppress the proliferation of autologous Teffs as measured by a negative percentage of cell suppression (Fig. 5a and 5b). Of note, at a 2:1 iNKT:Teff ratio at which healthy donor-derived iNKT cells reached a mean Teff suppression of 66%, T1D-derived iNKT cells did not have any effect on the proliferation of autologous Teffs and the differences were statistically significant (Fig. 5c). No correlation was found between the suppression capacity of each patient’s iNKT cells and the patients’ age of onset.

We then investigated if the lack of suppression was due to an impairment of iNKT or Teff cells from patients. The data showed that control iNKT cells inhibited the proliferation of Teffs from
T1D patients at similar levels as they inhibited control Teffs. In addition, T1D-derived iNKT cells were not able to suppress the proliferation of allogeneic controls' Teffs. Therefore, suppression impairment was not due to a defect on patients' Teffs but to an intrinsic defect of T1D patients' iNKT cells (Fig. 6a,b).

Deficient suppressor function of T1D patients' iNKT cells is associated to decreased IL-13

Because regulation by iNKT cells depended upon IL-13 secretion, we measured the T1D-derived iNKT cell IL-13 secretion in culture supernatants from stimulated control and patients' iNKT cells. The results showed that iNKT cells from T1D patients secreted significantly lower levels of IL-13 (Fig. 7a, left). Analysis by flow cytometry by intracellular staining very clearly illustrated the differences observed (Fig. 7a, right). Furthermore, IL-13 secretion did not increase when patients' iNKT and Teff cells were cultured together as shown before for healthy donor cells (data not shown). All this correlated with the mRNA analysis for IL-13 in human pancreas showing that IL-13 expression was substantially lower in a T1D pancreas than in control samples (supplementary figure 2).

To assess if human IL-13 would restore the suppression function of T1D-derived iNKT cells, rh-IL-13 was added to the cocultures. This enhanced the suppression of Teff proliferation by T1D patients’ iNKT cells in a dose-dependent manner (Fig. 7b), further confirming the involvement of IL-13 as a major mechanism of iNKT-mediated suppression.

IL-13 can directly act on Teff cells through the IL-13 receptor

If IL-13 has a direct effect on Teff cells, these should express IL-13 receptor. Signaling through type 1 IL13Rα1 is essential for type 2 IL13Rα2 because it induces its expression (26). mRNA expression for IL13R chain genes was analyzed by RT-PCR on Teff cells, showing that both IL13Rα1 and IL13Rα2 were expressed but with a completely different expression pattern between patients and controls. Teff cells from T1D patients showed a higher expression of IL13Rα1 compared to control Teff cells. On the contrary, they expressed a significantly lower
level of IL13Rα2 than controls (Fig. 8a). The same differences were observed when the receptor expression was analyzed by flow cytometry (Figure 8b and 8c). These differences were not detected in iNKT cells from controls or T1D patients (data not shown).

Altogether, our data showed that defects related to the IL-13 pathway were responsible for the impaired Teff cell regulation by T1D-derived iNKT cells.

**DISCUSSION**

We have investigated the function of iNKT cells in the context of human T1D onset, seeking to identify the mechanisms underlying their ability to suppress autoreactive CD4+ T cells. iNKT cells activated by glycolipids produce a wide variety of cytokines and interact with other immune cells, so they can regulate the response in disease conditions. iNKT cell-mediated protection has been demonstrated in NOD mouse T1D, although the mechanisms are undefined. For the first time, in the present manuscript (i) we demonstrate the functional suppression activity of human iNKT cells and provide novel information on the direct regulatory effect of human αGC-activated iNKT cells on CD4+ effector cells; (ii) we identify the IL-13 pathway as an essential component of the mechanism underlying regulation by iNKT cells; and more importantly, (iii) we demonstrate the impairment of iNKT cell-mediated regulation in T1D patients at disease onset and propose that this defect is related to deficiencies in the IL-13 pathway.

*Ex vivo* isolated CD4+ iNKT cells from the peripheral blood of healthy donors suppressed the proliferation of autologous T CD4+ effector cells, irrespective of their antigenic specificity. Further, iNKT cells expanded in vitro with αGC maintained their dose-dependent suppressor capacity. This expansion was required because iNKT cell frequency in human peripheral blood is low (7). Interestingly, suppression by iNKT cells worked better on resting naïve and memory T cells than on *in vitro* expanded Teff cells (data not shown). This agreed with published data on
mouse iNKT cells proposing that they preferentially act at the level of activation and/or
differentiation of naive T cells (12).

Our data showed that Teff suppression by human iNKT cells did not require cell-cell contact, in
contrast to contact-dependent Treg-mediated regulation (32,33). We also demonstrated that IL-
13 secreted by activated iNKT cells was the main effector of suppression, reducing both Teff
proliferation and IL-2 secretion.

A most relevant finding was that the suppressor function was impaired in T1D patients’ iNKT
cells at disease onset. Little information is available on the potential role of human iNKT cells in
T1D pathogenesis. Studies have pointed out to a decreased frequency of CD4+ iNKT cells
having a role on the development of T1D but there is not a real consensus on the existence of
frequency alterations in these patients (34). For instance, an analysis using CD1d-tetramers as
readout concluded that there was no alteration of the number of iNKT cells or in the production
of IL-4 by PBMCs from T1D patients compared to controls (35). In contrast, an earlier study had
shown both diminished iNKT cells frequency and IL-4 production in T1D patients (36). Studies
in the NOD mouse have shown that functional defects and a reduced frequency of iNKT cells
contributed to T1D development (37,38). Besides, restoring iNKT cell numbers reduced T1D
incidence or slowed down its progression (39,40).

Our study demonstrate that human iNKT cells are an important source of IL-13 but that iNKT
cells from T1D patients have a remarkably reduced IL-13 production that could account for their
suppression disability. Altered IL-13 secretion has been reported in children at T1D onset as well
as in high-risk relatives of affected children (22,41). The origin of such deficiency has not been
found but our findings suggest that defective iNKT cells may be a main contributor to the
diminished serum levels of IL-13 at T1D onset.

iNKT cells are found in the pancreas from T1D patients (Usero et al, manuscript in preparation)
therefore they can be influenced by the cytokine milieu in the tissue. The enterovirus
Coxsackievirus-B4 was considered of etiological significance in T1D since it led to functional impairments and β-cell damage (42). There are also evidences that human islet cells can sustain an enteroviral infection in patients with T1D. IFNα, a cytokine secreted in response to such viral infections, is known to increase IL-4 synthesis while strongly downregulating IL-13 production (43). This, together with the finding that infection of human islets with Coxsackievirus-B4 causes downregulation of IL-13 (44), suggests that enterovirus alteration of the islets inflammatory milieu in T1D may favor β-cell loss by impairing suppression of Teff by iNKT cells. Further, Coxsackievirus-B4 infection may abrogate IL-13-mediated beta cell protection. IL-13 is a cytoprotective agent of pancreatic β-cells, because it maintains β-cell viability after treatment with a range of cytotoxic insults (45). Thus, we can speculate that low levels of IL-13 at onset may contribute to the final deregulation of the autoimmune attack leading to the destruction of the β-cells. In this context, it was shown that early administration of rIL-13 to NOD mice prevented insulitis and diabetes development while later prophylaxis conferred long-lasting protection against T1D and blocked progression of insulitis (23). IL-13 is thus likely to be of primary importance in diabetes pathogenesis and any reduction in IL-13 level may serve to exacerbate the disease effects on islet cell viability and function. Future studies are warranted to define IL-13 signaling pathway as potential therapeutic target of early intervention in patients with T1D.

The differences in IL-13 secretion by patients and controls' iNKT cells could depend on factors such as the APCs activating iNKT cells in vivo that may prevent the expansion of regulatory iNKT cell subsets in T1D. The quality of the TCR signal alone or the integration of signals from different receptors could be part of the explanation (21). In this context, we have observed that suppression by iNKT cells was abrogated when expanded in vitro with PHA instead of αGC. This correlated with lower secretion of IL-13 (Usero et al unpublished observations). Thus,
alterations in the TCR signaling events in iNKT cells from T1D patients may be involved on the absence of IL-13-mediated regulatory function.

Activation of IL13Rα1 by IL-13 phosphorylates STAT6 that induces IL13Rα2 transcription (46,47). Our data and others’ support a role for IL-13 signaling in Teff cell alterations in T1D (48,49). Indeed, T1D patients’ Teff cells expressed high levels of IL13Rα1 and low levels of IL13Rα2 compared to controls and this can modulate IL-13 signaling in vivo (25,26). Albeit being initially considered a decoy receptor, IL13Rα2-mediated signaling has been involved in the activation of the TGFβ1 promoter, underscoring its role as a signaling receptor (26). Further, IL13Rα2 receptor can act as a negative regulator of IL-13 effects. The IL13Rα2, expressed after exposure to IL-13, exits rapidly to the cell membrane from intracellular locations to capture and internalize extracellular IL-13 (25). Because IL13Rα2 is a high affinity receptor, this process reduces the IL-13 available to bind IL13Rα1, in a negative feedback loop. Thus, a control pattern with high IL13Rα2 expression would promote regulation, whereas T1D patients’ predominant IL13Rα1 signaling would prevent the regulatory action of IL13Rα2. Interestingly, we have preliminary data suggesting that Teff cells from control and patients increase the expression of IL13Rα2 when cultured in the presence of rhIL13.

Mechanisms proposed for iNKT-mediated regulation of Teff cells in the NOD model involved the action of intermediate cells but not a direct effect of iNKT-derived IL-13 on Teff cells (50,51). These differences could be explained by the differentiation state of NOD Teff and iNKT cells, compared to human. However, NOD mice show high basal production of IL-13, so that the mechanism proposed for human iNKT cell-mediated regulation could go undetected in this model (52).

Overall, this study describes an IL-13-mediated suppression mechanism in human iNKT cells. In the context of T1D onset, a decreased secretion of IL-13 by iNKT cells from T1D patients leads to an impaired regulation of T effector cells that favor the development of the autoimmune
disease. Although the use of αGalCer for treatment or prevention of human T1D has not been conclusively tested yet, the use of αGalCer analogues favoring IL-13 secretion may be a useful approach to boost iNKT cells suppressive function to reach T1D prevention.

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L.U. researched, analyzed data and wrote the manuscript; A.S. researched data; E.P. recruited and monitored patients; C.X. contributed to suppression assays experimental design; M.M. reviewed/edited the manuscript; D.J. contributed to discussion and reviewed/edited manuscript; C.R. analyzed data and wrote the manuscript. The content of this article is solely the responsibility of the authors. C.R. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. No potential conflicts of interest relevant to this article were reported. The authors are grateful to study volunteers for their participation and to staff at participating Hospital de Mataró for their assistance in the recruitment of participants. The authors thank Marta Catalfamo, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, for help with manuscript preparation.

Abbreviations: APC, antigen presenting cell; DC, dendritic cell, T1D, Type 1 diabetes; iNKT cells, Invariant Natural Killer T cell; Teff, Effector T cells; Treg, T regulatory cells; αGC, alpha-Galactosylceramide.
FIGURE LEGENDS

Figure 1. iNKT cells have the capacity to suppress the proliferation of autologous Teff cells.
a) Ex vivo iNKT cell-mediated suppression. Left, proliferation of human Teff cells from HD cultured in triplicate at different ratios with autologous freshly isolated iNKT cells, as described in research design and methods. Proliferation was assayed by [3H] thymidine incorporation. The graph shows data from one experiment. Right, mean suppression of Teff cells by different ratios of iNKT cells. Results represent the average of three experiments. Suppression increased significantly in a iNKT:Teff cell ratio dependent manner (p=0.0364). b) In vitro expanded iNKT cell-mediated suppression. Left, proliferation of human Teff cells with iNKT cells at different cell ratios, showing data from one experiment, representative of nine experiments performed. Decrease of proliferation was iNKT:Teff cell ratio dependent. Right, mean percent suppression of Teff cell proliferation by iNKT cells (n=9). F, feeder cells.

Figure 2. iNKT cells reduce the secretion of IL-2 by autologous Teff cells independently on cell-cell contact. IL-2 secretion measured in 24h supernatants from cocultures containing different combinations of Teff cells and in vitro expanded iNKTs. (1) indicates that the two cell types were seeded in different chambers of a transwell system. Otherwise they were co-cultured. (*) indicates irradiated cells. Data represent the mean±SD of triplicates of all experiments performed (n=5). F, feeder cells.

Figure 3. iNKT cells secrete immunomodulatory and effector cytokines. Proinflammatory (a) and immunomodulatory (b) cytokine secretion by Teff, in vitro expanded iNKT or iNKT:Teff cell cocultures. Cytokines were measured on 24h (IL-2) or 48h culture supernatants (TNFα, IFNγ, IL-4, IL-10, IL-13 and IL-17A) by CBA. Data represent the average production of
cytokines by 5 different donors. Each individual measurement corresponds to triplicate cultures. F, feeder cells. * p<0.05; ** p<0.01.

**Figure 4. Blocking IL-13 abrogates Teff cell proliferation suppression.** a) Cell suppression assays performed in the presence or absence of increasing concentrations of a blocking antibody to IL-13 (3, 30, 90 µg/ml) at an iNKT:Teff ratio of 1:1. As a control, 30 µg/ml of Isotype IgG1 was used. Left, a representative example of 5 donors tested. Right, mean percent suppression of all samples analyzed (n=5) at an iNKT:Teff ratio of 1:1. c) IL-2 production by Teff cells in the same conditions as in 4a. d) Cell suppression assay in the presence or absence of anti-IL4 (3, 30 µg/ml) added to the 30 µg/ml anti-IL13 antibody at a 1:1 iNKT:Teff ratio. * p<0.05.

**Figure 5. iNKT cells from T1D patients do not suppress the proliferation of autologous Teff cells.** a) Proliferation of Teff cells from T1D patients in the presence of autologous in vitro expanded iNKT cells at different ratios. Data shown are from one representative experiment of 10 performed in 5 patients’ samples. b) Mean percent suppression of 5 T1D patients’ Teff cell proliferation by iNKT cells. c) Summary comparison of the suppression of proliferation of Teff cells by iNKT cells from HD (light grey) (n=8) and T1D patients (dark grey) (n=5), at different iNKT:Teff ratio. Differences between HD and T1D iNKT-mediated suppression were statistically significant at both iNKT:Teff cell ratios (0.5:1, p=0.0056 and 1:1, p=0.0048 HD vs T1D).

**Figure 6. iNKT cells from T1D patients do not suppress the proliferation of allogeneic Teff cells derived from HD or T1D patients.** Percent suppression of Teff cells proliferation from HD (n=2) and T1D (n=2) patients with iNKT cells from HD (left) or T1D patients (right) at a 1:1
iNKT:Teff ratio. Suppression by iNKT cells was significantly different between iNKT<sub>HD</sub>:Teff<sub>HD</sub> vs iNKT<sub>HD</sub>:Teff<sub>T1D</sub>, p=0.0201 and iNKT<sub>T1D</sub>:Teff<sub>T1D</sub> vs iNKT<sub>T1D</sub>:Teff<sub>HD</sub>, p=0.0379.

**Figure 7.** iNKT cells from T1D patients show defective IL-13 secretion. a) Left, IL-13 secretion by iNKT cells from HD (n=15) and T1D patients (n=10), analyzed by CBA. Secretion was measured in 48h supernatants from *in vitro* expanded iNKT cells (10000 cells/well) stimulated with αCD3/CD28-coated beads in the presence of irradiated autologous PBMCs as feeder cells. Secretion by HD iNKT cells was significantly higher than by T1D-derived iNKT cells (p=0.0164). Right, an example of IL-13 production by iNKT cells from HD and T1D, measured by intracellular staining and flow cytometry analysis. b) Recombinant human IL-13 restored T1D-derived iNKT cells suppressor function. The graph shows the proliferation of T1D Teff cells after adding increasing concentrations of rh-IL-13 to the iNKT:Teff cocultures at different iNKT:Teff cells ratios (0.5:1 triangle and 1:1 circle, iNKT:Teff ratio). The decrease in proliferation was significant (p=0.0286) and dose-dependent.

**Figure 8.** Teff cells express the IL-13 receptor. a) Analysis of the expression of IL13Rα1 and IL13Rα2 chains by RT-PCR on Teff cells from HD (n=4) and T1D patients (n=6). PCR products were resolved in 2% agarose gel and quantified. Results are shown as relative expression mean values. Differences in IL13Rα2 expression between T1D patients and controls were statistically significant (p=0.0476). b) Flow cytometry analysis of the expression of IL13Rα1 and IL13Rα2 chains by Teff cells from HD (n=3) and T1D patients (n=3). Differences in IL13Rα2 expression between T1D patients and controls were statistically significant (p=0.0465). Light grey columns represent HD and dark grey columns, T1D patients. c) Representative dot plots corresponding to the flow cytometry analysis of the expression of IL-13Rα1 and IL-13Rα2 by Teff cells from HD and T1D patients.
Table 1. T1D patients’ data.

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</tbody>
</table>

Subject number (n), HbA1c in NGSP (%) and IFCC (mmol/mol) units, and number of patients with Antibodies (pos) to GAD or insulin. nd, not determined

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63x31mm (300 x 300 DPI)
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48x26mm (300 x 300 DPI)
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Supplementary figures

Supplementary figure 1

a) Phenotype of iNKT cells. Flow cytometry analysis of expanded human iNKT cells. Staining with antibodies to CD3, CD4, Vα24Jα18 and the Vβ11 TCR chains. b) iNKT cells expanded in vitro are αGC-specific in a dose dependent manner. TNFα secretion by iNKT cells in cocultures of irradiated autologous PBMCs (n=8), C1R- or C1Rd as APCs and different concentrations of αGC. As positive control, cells were stimulated with αCD3/CD28 coated beads or PHA-L.

Supplementary figure 1. Phenotype and specificity of in vitro expanded iNKT cells.

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Supplementary figure 2

Supplementary figure 2: IL13 expression in human pancreas at different stages of T1D development. Analysis of IL13 mRNA expression in human pancreas at pre-T1D, T1D-onset and long term T1D (n=1), performed by conventional PCR. Quantification was performed by gel band densitometry analysis.