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## Decreased circulating iNKT cell numbers in refractory coeliac disease

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Received 14 August 2007; accepted with revision 21 September 2007

Available online 31 October 2007

### KEYWORDS

iNKT cells;  
Refractory coeliac  
disease;  
Coeliac disease

### Abstract

**Introduction:** A small proportion of coeliac disease (CD) patients become refractory (RCD) to a gluten-free diet (GFD) showing uncontrolled immune-mediated enteropathy. Some of these patients exhibit a high risk to develop enteropathy-associated T-cell lymphoma (EATL).

**Aim:** The aim of the study was to find whether a lack of circulating homeostatic T cells, such as Treg,  $T\gamma\delta^+$  or iNKT cells would be associated with the development of RCD or EATL.

**Patients and methods:** We investigated in a total of 137 CD patients [28 untreated, 80 responsive to GFD and 29 RCD (14 type I and 15 type II)] and 73 age-matched healthy volunteers the frequency of Treg,  $T\gamma\delta^+$  and iNKT lymphocytes by flow cytometric analysis of peripheral blood.

**Results:** Circulating  $T\gamma\delta^+$  cell and Treg frequencies in RCD were comparable to those in healthy controls. However, RCD patients had significantly reduced numbers of circulating iNKT cells, as compared to age-matched patients responding to a GFD. This reduction was similar in RCD patients with and without aberrant intraepithelial lymphocytes and in patients with EATL. Circulating iNKT cell numbers were not reduced in untreated coeliac patients. GFD treated patients had a significantly increased proportion of  $CD4^+$  iNKT cells.

**Conclusion:** Follow-up studies are necessary to determine whether  $CD4^+$  iNKT cells control the immune response against gluten and their absence contributes to the progression to RCD and EATL.

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### Introduction

Coeliac disease (CD) is an immune-mediated enteropathy caused by the ingestion of wheat and other gluten-

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containing cereals (rye, barley and probably oats) in genetically predisposed individuals [1] leading to intestinal villous atrophy. This is characterized by crypt hyperplasia and increase of intraepithelial lymphocytes (IELs). The current treatment is a life-long strict gluten-free diet (GFD) resulting in a complete remission of the symptoms and returning to a normal small intestinal mucosa. However, a small proportion of CD patients becomes unresponsive to a GFD, a stage known as refractory CD (RCD) [2]. This is characterized by an uncontrolled, gluten-independent, intestinal immune reaction. As a result, the IEL of the diseased epithelium can continue to undergo NK-like reprogramming [3] with T cell receptor (TCR)-independent IFN- $\gamma$  production and cytotoxicity. Additionally, an aberrant IEL population, lacking surface expression of CD3 and CD8, appears in a subgroup of RCD patients (type II) which have a high risk to develop an enteropathy-associated T-cell lymphoma (EATL), whereas RCD type I patients show normal expression of T-cell antigens and have a better prognosis [2,4].

CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells [Tregs; CD3<sup>+</sup>, CD4<sup>+</sup>, CD25<sup>+</sup> and intracellular transcription factor Forkhead Box P3<sup>+</sup> (FoxP3<sup>+</sup>)], invariant NKT cells (iNKT; CD3<sup>+</sup>, TCR V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup>) and to a lesser extent TCR  $\gamma\delta$ <sup>+</sup> lymphocytes (T $\gamma\delta$ ; CD3<sup>+</sup>, TCR  $\gamma\delta$ <sup>+</sup>) are lymphocyte populations that help to maintain immune homeostasis [5–10]. Tregs [11] elicit their function by suppressing IL-2 production and T-cell proliferation [12,13]. Intraepithelial T $\gamma\delta$  cells appear to play a key role in oral tolerance by inducing Tregs [14,9]. After TCR-triggering, T $\gamma\delta$  cells rapidly but transiently express the lymph node-homing receptor CCR7. Once in the lymph nodes, they may act as professional antigen presenting cells inducing proliferation and differentiation of naïve T cells [15].

Human iNKT cells express classical NK cell markers as well as an invariant T cell receptor (TCR V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup>), which recognizes antigens presented by the MHC class I-like molecule CD1d [13,16–19]. iNKT cells can also be sub-divided in CD4<sup>+</sup> and CD4<sup>-</sup> (most of these CD4<sup>-</sup>CD8<sup>-</sup>) cells. CD4<sup>-</sup>CD8<sup>-</sup> iNKT cells predominantly produce TH1 cytokines (IFN $\gamma$  and TNF $\alpha$ ) whereas CD4<sup>+</sup> iNKT cells can produce both TH1 and TH2 (IL-4 and IL-13) cytokines [20,8]. Because of their unique

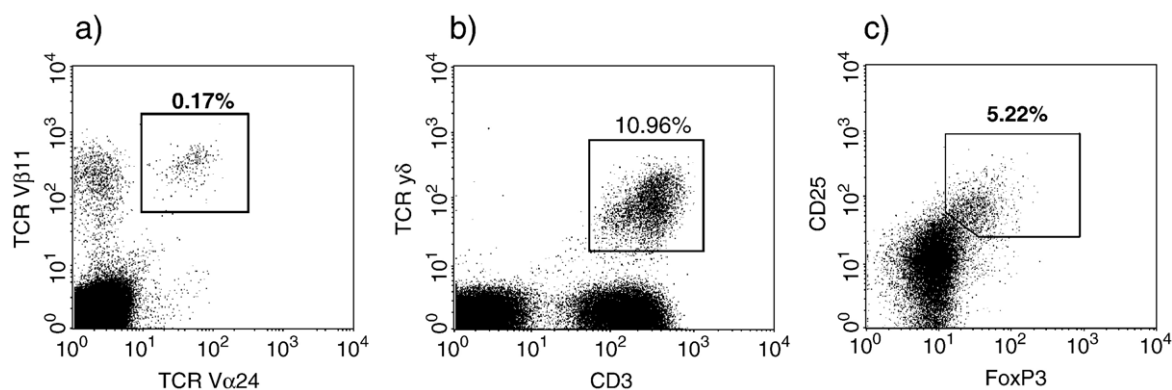
capacity to rapidly produce large quantities of both TH1 (IFN $\gamma$ ) and TH2 (IL-4) cytokines upon stimulation [21,22], iNKT probably play a key role either in the protection against tumors or in preventing autoimmune disease [18,23,24].

Although circulating iNKT, T $\gamma\delta$  and Treg cell numbers are relatively low (approximately 0.02–0.2% of the CD3<sup>+</sup> T-cells are iNKT cells [24], 2–10% of the CD3<sup>+</sup> cells are T $\gamma\delta$  cells [15] and 2–5% of CD4<sup>+</sup> T-cells are Tregs [11]), numeric deficiencies have been reported in autoimmune disease and in malignancy [18,23,25–27]. Reduced circulating numbers of both iNKT cells and T $\gamma\delta$ <sup>+</sup> cells have been reported in CD [28,29]. However, these studies have not been performed in relation to the development of uncontrolled autoimmunity as seen in RCD or to the development of malignancies as EATL. In addition, since iNKT can be either regulatory or pro-inflammatory, expression of the regulation-associated proteins FoxP3 and CTLA4 in iNKT cells might provide new information on the actual regulatory function.

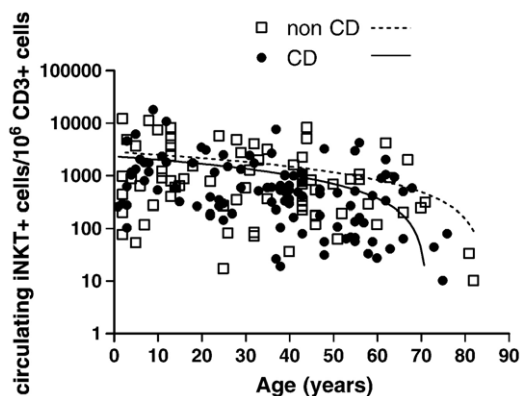
The hypothesis proposed in this study is that a lack of regulatory T cells, including CD4<sup>+</sup> iNKT cells, Treg and T $\gamma\delta$  cells, predisposes to the development of RCD. To this end, circulating levels of these cells were assayed in a group of RCD patients (both type I and type II), as well as in active (untreated) and GFD-treated CD patients and in age-matched healthy controls. Moreover, the intracellular levels of the regulatory proteins CTLA4 and FoxP3 were determined in iNKT cells.

## Materials and methods

We studied a total of 137 CD patients, 28 untreated (mean age 26.5 years; range 1–75 years, 36% males), 80 responsive to GFD (mean age 38.2 years; range 3–76 years 22% males) and 29 refractory CD (RCD) not responding to a GFD (mean age 57.50 years; range 45–68 years 38% males) and 73 age-matched healthy volunteers without known autoimmune diseases or malignancies (mean age 32.2 years; range 2–82 years; 36% males). RCD patients were divided in RCD type I (14 patients, mean age 57.5 years; range 47–68 years; 36% males) and RCD type II (15 patients, mean age: 60.6 years;



**Figure 1** Analysis of circulating regulatory cells from one representative donor. Live lymphocytes were gated based on forward and sideward scatter. (a) iNKT cell analysis. Example of staining of PBMCs for V $\alpha$ 24 and V $\beta$ 11 staining on gated CD3<sup>+</sup> lymphocytes. iNKTs were defined as CD3<sup>+</sup> V $\alpha$ 24<sup>+</sup> and V $\beta$ 11<sup>+</sup> (b) TCR T $\gamma\delta$ <sup>+</sup> analysis. Example of staining of PBMCs for T $\gamma\delta$  and CD3 expression on gated live lymphocytes. TCR T $\gamma\delta$ <sup>+</sup> cells were defined as CD3<sup>+</sup> T $\gamma\delta$ <sup>+</sup>. (c) Treg analysis. Example of staining of gated CD3<sup>+</sup>CD4<sup>+</sup> T-cells for surface CD25 and intracellular and FoxP3 expression. Treg cells were defined as surface CD3<sup>+</sup> CD4<sup>+</sup> CD25<sup>high</sup> and intracellular FoxP3<sup>+</sup>.



**Figure 2** Effect of age on circulating iNKT cell frequencies in healthy controls without known autoimmune diseases or malignancies (non-CD) and coeliac disease (CD) patients. Scatter diagrams with regression lines show an age-dependent reduction in circulating V $\alpha$ 24V $\beta$ 11 iNKT cells/10<sup>6</sup> T-cells in controls (Spearman's  $r = -0.238$ ,  $p = 0.0430$ ) and CD patients (Spearman's  $r = -0.4104$ ,  $p < 0.0001$ ).

range 45–68 years; 39% males) according to the absence or presence (>20% of IEL) of aberrant intraepithelial lymphocytes, respectively [30]. At the time of diagnosis, all CD patients, responding and non-responding, had positive endomysium antibodies, carried the HLA-DQ2 and/or DQ8 as well as typical mucosal changes in the duodenal biopsy. Responding GFD-CD patients were characterized by a clinical, serological and pathological recovery upon GFD while non-responding patients (RCD) only had serological recovery upon the GFD. At the time of performing the blood test for this study, active CD patients had positive serology and villous atrophy but all responding GFD-CD and non-responding RCD patients had negative serology for at least 1 year. The study was approved by the medical ethical committee of the VU University Medical Center, Amsterdam.

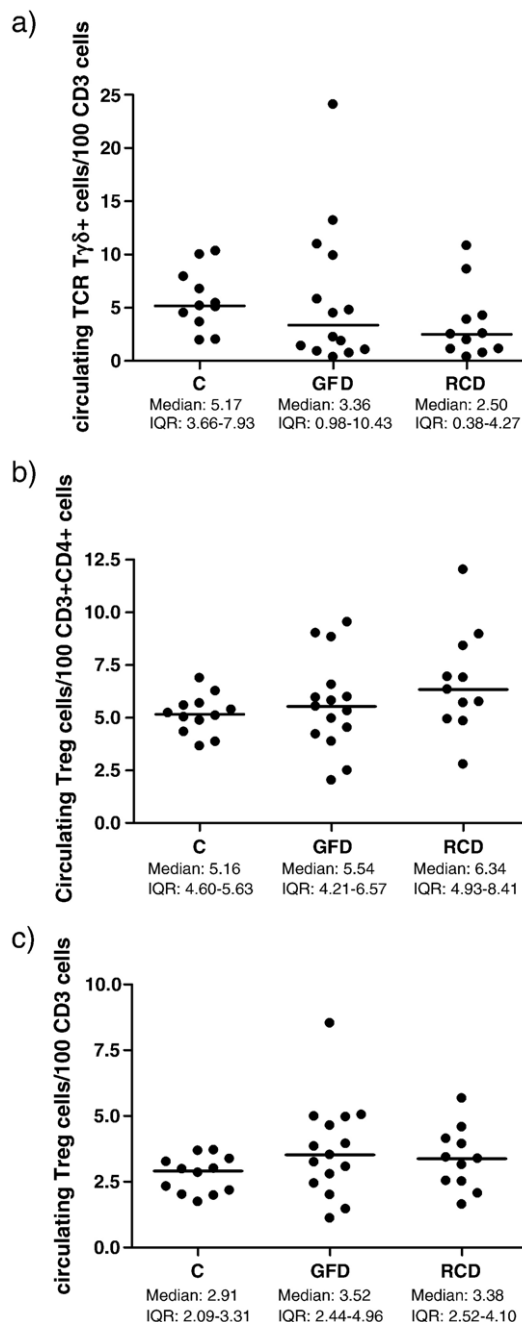
### Flow cytometric analysis of peripheral blood

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-hypaque density gradient centrifugation and the circulating cell numbers of the regulatory cell subsets were determined by flow cytometry on a FACS Calibur (BD Biosciences). iNKT cells were identified by coexpression of CD3 (APC-labelled, BD Biosciences), V $\alpha$ 24 (FITC-labelled, Immunotech) and V $\beta$ 11 (PE-labelled, Immunotech). Intracellular levels of CTLA4 (APC-labelled, BD Biosciences) and FoxP3 (APC-labelled, eBioscience) were also determined in iNKT cells combined with surface expression of CD4 (PerCP-labelled, BD Biosciences). Tregs were identified by coexpression of CD3 (PerCP-labelled, BD Biosciences), CD4 (FITC-labelled) and high expression of CD25 (APC-labelled, BD Biosciences) as well as intracellular FoxP3 (PE-labelled, eBioscience). Intracellular staining was done according to the manufacturer's instructions. T $\gamma\delta$  cells were identified by coexpression of CD3 (PerCP-labelled) and  $\gamma\delta$  TCR (PE-labelled). Appropriate isotype controls were used in all experiments.

A minimum of 100,000 viable lymphocytes were acquired per patient for Treg, T $\gamma\delta$  and iNKT cell determination.

### Statistical analysis

Correlation analyses and two-tailed non-parametric statistical analyses were performed using the Kruskal–Wallis one-



**Figure 3** Percentages of circulating T $\gamma\delta$ <sup>+</sup> and Tregs in peripheral blood of healthy controls without known autoimmune diseases or malignancies (C), GFD-responding (GFD) and GFD-non-responding (RCD) CD patients. (a) T $\gamma\delta$ <sup>+</sup> cells within CD3<sup>+</sup> T-cell population, (b) Tregs within CD3<sup>+</sup>CD4<sup>+</sup> T-cell population and (c) within the CD3<sup>+</sup> population. No statistically significant differences were observed (Kruskal–Wallis test,  $p = 0.1910$ ,  $p = 0.1804$  and  $p = 0.2185$ , respectively). Horizontal bars indicate median values. IQR: interquartile range.

way analysis of variance test and the Mann–Whitney  $U$  test.  $P < 0.05$  was considered significant.

## Results

### Phenotypical analysis of regulatory T-cells subsets

Examples of phenotypical analysis of circulating iNKTs, Tregs and  $T\gamma\delta$  cells by flow cytometry are shown in Fig. 1. Live lymphocytes were gated based on forward sideward scatter. Unless otherwise stated, iNKT, Treg and  $T\gamma\delta$  cell frequencies are given as a fraction of the  $CD3^+$  population.

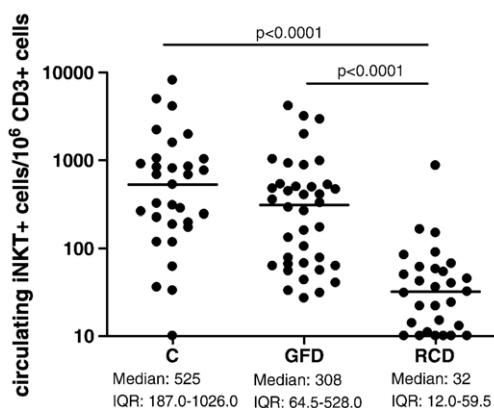
Since it is known that some regulatory T cell subset frequencies decrease with age [31] and may be influenced by gender, linear regression analysis was performed. No gender effect was observed for any subset, while an age related decline was observed for iNKT cell frequencies both in healthy controls (Spearman's  $r = -0.2376$ ,  $p = 0.0430$ ) and CD patients (Spearman's  $r = -0.4104$ ,  $p < 0.0001$ ; Fig. 2).

### Circulating $T\gamma\delta$ and Treg cell numbers are normal in CD and RCD

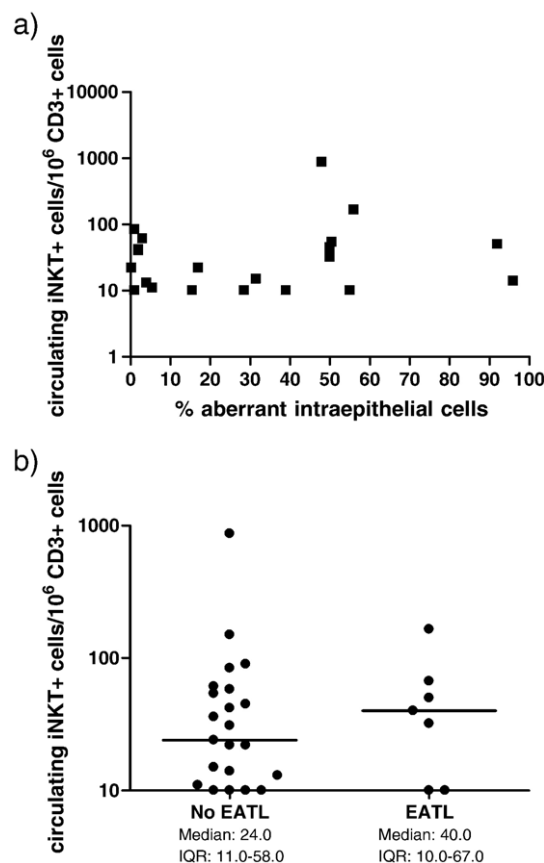
No significant differences in circulating  $T\gamma\delta$  cell frequencies between responding CD, RCD and healthy controls were observed (Fig. 3a). Circulating levels of  $CD4^+CD25^+FoxP3^+$  Tregs were determined within both the  $CD3^+CD4^+$  (Fig. 3b) subset and the total  $CD3^+$  (Fig. 3c) population. No differences in circulating Treg frequencies were observed in any case. Finally, within the RCD group, there was no correlation between the circulating levels of either Treg or  $T\gamma\delta$  cells and the percentage of aberrant IELs (data not shown).

### iNKT cell numbers are selectively decreased in RCD

iNKT cell frequencies in RCD patients, which were generally older than the control group (mean age 57.5 years, range 45–



**Figure 4** Circulating iNKT numbers per million of circulating  $CD3^+$  cells in peripheral blood of GFD-non-responding (RCD) and -responding (GFD) CD patients and healthy controls without known autoimmune diseases or malignancies (C) (over 40 years). Statistically significant differences are shown (two-tailed Mann–Whitney  $U$  test; Krustall–Wallis test  $p < 0.0001$ ). Horizontal bars indicate median values. IQR: interquartile range.

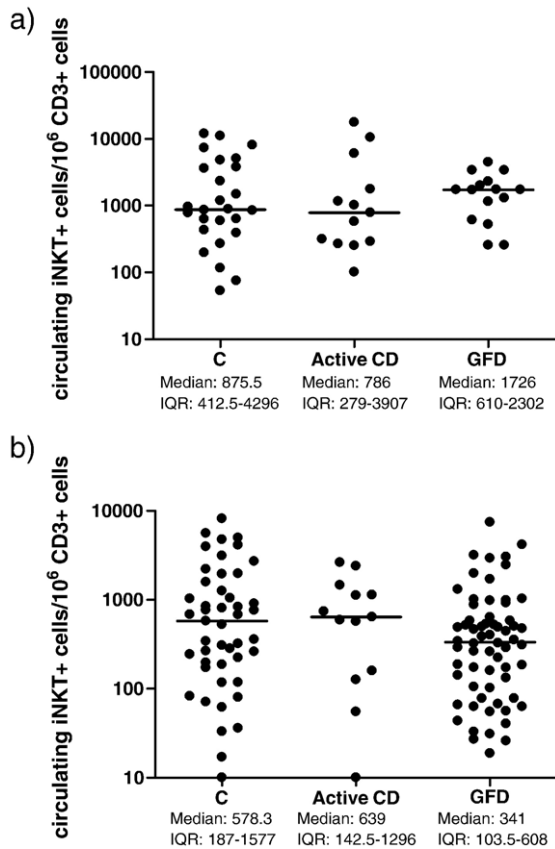


**Figure 5** No relation between iNKT cell frequency and aberrant intraepithelial cells or the development of enteropathy-associated T-cell lymphoma (EATL) in refractory coeliac disease (RCD). (a) iNKT cell numbers frequency related to the percentage of aberrant (surface  $CD3^-$ , intracellular  $CD3^+$ ) intraepithelial cells in RCD patients (Spearman's  $r = 0.2177$ ,  $p = 0.3303$ ) and (b) comparison of circulating iNKT cell frequency among RCD patients with or without EATL (two-tailed Mann–Whitney  $U$  test,  $p = 0.6588$ ). Horizontal bars indicate median values. IQR: interquartile range.

68 years), were compared to iNKT cell frequencies in a group of age-matched responding CD patients (38 individuals, mean age 55.3 years, range 41–76 years) and healthy controls (31 individuals, mean age 53.8 years, range 41–82 years) to correct for the age-related effect on circulating iNKT cell numbers. Fig. 4 shows that the iNKT cell population was reduced in RCD patients (median 32.0) compared to responding CD patients ( $p < 0.0001$ , median 308.0) and healthy controls ( $p < 0.0001$ , median 525.0)

### Reduction in iNKT cell numbers is not related to the presence of aberrant IEL or EATL in RCD

To investigate whether low iNKT cell numbers in RCD would be associated with the presence of potentially pre-malignant, aberrant IEL (surface  $CD3^-$ , intracellular  $CD3^+$ ), we compared iNKT cell numbers with the percentage of aberrant IEL in RCD patients (Fig. 5a). No significant correlation was found and consequently no difference was found in the circulating numbers of iNKT cells between RCD I and RCD II



**Figure 6** Frequency of circulating iNKT cells (number of iNKT cells per million of circulating CD3+ T-cells) in peripheral blood of healthy controls without known autoimmune diseases or malignancies (C), untreated patients with active CD (active CD) and GFD-responding patients (GFD): (a) children below 20 years and (b) adults over 20 years. No statistically significant differences were found (Kruskal–Wallis test,  $p=0.7008$  and  $p=0.1467$  respectively). Horizontal bars indicate median values. IQR: interquartile range.

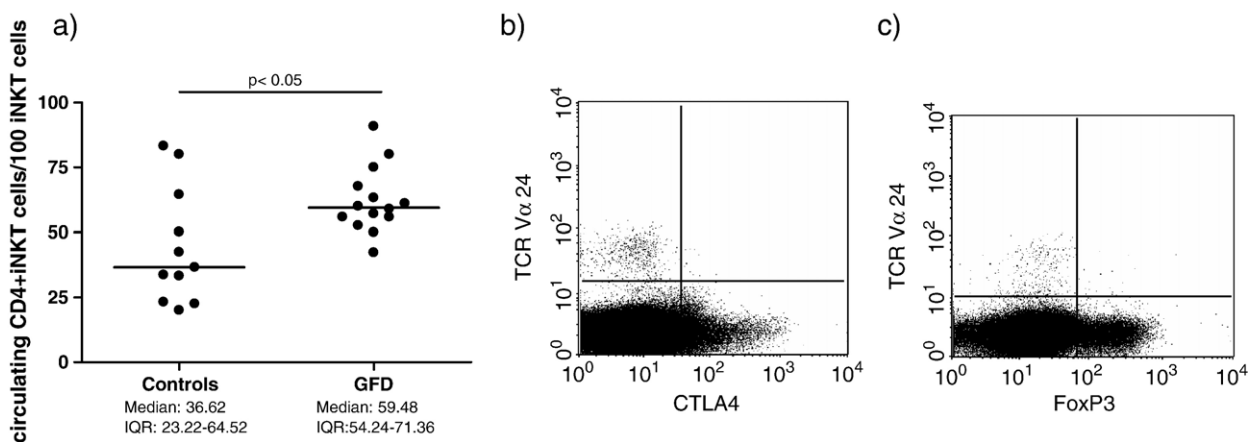
(RCD I median: 33.5, IQR 10.5–59.5; RCD II median: 32.0, IQR 13.0–67.0). Moreover, no further circulating iNKT cell depletion occurred in the 7 RCD patients that developed an EATL (Fig. 5b).

### Circulating iNKT cell numbers are normal in untreated or treated CD

We then checked in how far the numeric defect in iNKT cells was characteristic for the refractory state of coeliac patients or whether patients with active coeliac disease before treatment would already have reduced iNKT cell numbers, as was recently reported by Grose et al. [28]. Separate evaluations were performed for children (below 20 years) and adults (over 20 years), after confirming that there was no iNKT depletion within the first 20 years of life (healthy control group: Spearman's  $r=0.1526$ ,  $p=0.4567$ ; CD: Spearman's  $r=0.2539$ ,  $p=0.1924$ ). Both treated and untreated CD children (Fig. 6a) as well as adult patients (Fig. 6b) appeared to have normal circulating iNKT cell numbers as compared to healthy age-matched controls. Since large individual differences were observed, 4 individual patients with relatively low, intermediate and high iNKT levels were followed longitudinally before and 1 year after the start of the GFD. In all patients, the iNKT levels appeared to be remarkably stable (data not shown) during this year.

### The regulatory phenotype of iNKT in CD

Although numbers of iNKT cells were unaffected in active and treated CD, we next investigated to what extent the iNKT subset (CD4+) with regulatory functions was affected in the different coeliac patients. Unfortunately, iNKT cell numbers of patients with RCD were too low to allow for accurate assessment of their phenotype. Therefore we compared the phenotype (CD4+ or CD4-) of the iNKT cells and their expression of the regulation associated molecules FoxP3 and CTLA4 in uncomplicated CD (on a GFD) and in age-matched healthy controls. CD patients on a GFD showed a higher



**Figure 7** Analysis of iNKT cell phenotype. (a) Percentage of circulating CD4+iNKT cells in peripheral blood of GFD-responding CD patients (GFD) and healthy volunteers without known autoimmune diseases or malignancies (C) (two-tailed Mann–Whitney  $U$  test,  $p=0.0375$ ). Horizontal bars indicate median values. IQR: interquartile range,  $n$ : sample size. (b) Staining of intracellular CTLA4 and (c) FoxP3 in iNKT cells.

proportion of CD4<sup>+</sup> iNKT cells (Fig. 7a; median 59%) than healthy controls ( $p=0.0375$ , median 37%). Regarding the expression of the Treg-associated proteins, Fig. 7 illustrates that circulating iNKT cells in both CD patients and healthy controls, did not express CTLA4 or FoxP3, regardless of their CD4 expression (Figs. 7b and c).

## Discussion

iNKT cells are thought to be important in tumor immune surveillance as well as in the regulation of autoimmune disease [7,23]. This view is not only supported by numerical defects in circulating iNKT cells in autoimmune disease and malignancy [17,25], or because they protect against type 1 diabetes in NOD mice after interaction with Treg cells [32], but also by the fact that such reduced iNKT cell numbers were found to be associated with a poor prognosis in head and neck cancer [24]. It is, however, still a matter of debate whether a lack of adequate iNKT cells should be considered as a risk factor for, or rather as a consequence of the disease process. To address this question, CD patients represent a unique population showing the whole pathogenetic spectrum from a state of gluten hypersensitivity with reversible villous atrophy to a state of gluten-independent autoimmune disease in RCD and from RCD I with relatively good prognosis to RCD II with a high risk for malignant disease and eventually to the development of EATL [30].

Our results are compatible with the hypothesis that iNKT cells may prevent progression to RCD. However, to evaluate whether a numerical defect actually precedes a state of refractory disease, patients on a GFD with low iNKT cell numbers should be followed-up to assess their risk for developing RCD. Since only a very small proportion of patients on GFD develops refractory disease, this will be a very inefficient approach. On the other hand, it is also clear from our results that other factors play a role, since low iNKT cell numbers are, at least in some patients, compatible with good responsiveness on a GFD.

Regarding the risk of progression from a state of refractory CD to the development of pre-malignant and malignant disease, we tested whether the iNKT defects would be related to the presence of aberrant IEL, a characteristic of RCD II patients, which is associated with a relatively short survival time [30]. No such relation was found, however, probably because of the already very low, often hardly detectable iNKT cell numbers in RCD patients. Moreover, in patients who developed EATL, no further depletion (if at all possible because of the already low levels) of circulating iNKT had occurred. Still, our data suggest that the reduced numbers of iNKT cells in RCD somehow predispose for, or at least reflect a state of progressive, uncontrolled autoimmune destruction, since patients with active, untreated coeliac disease, both children and adults, had normal levels of iNKT cells as compared to age-matched healthy controls. This is in line with earlier results from our group [17], but in contrast with reports from Grose et al. [28], who described a peripheral numerical and functional deficiency of iNKT cells in both active and treated CD patients, accompanied by reduced iNKT cell numbers in the intestinal mucosa. It is not clear what causes this discrepancy, since both studies included relatively large numbers of patients, age-matched

controls and similar methodology. We evaluated the CD3<sup>+</sup>V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup> population in several clinical studies and found high correlations with  $\alpha$ GalCer/CD1d tetramer-positive iNKT cells [33]. Furthermore, Grose et al. [28] also found a significant reduction in V $\alpha$ 24<sup>+</sup> V $\beta$ 11<sup>+</sup> double-positive cells, indicating that the discrepancy cannot be explained by different methodological approaches. The only difference remains in the fact that Grose et al. [28] performed parametric statistical analyses although in the present study, iNKT cells did not have a normal distribution so non-parametric statistics were used in our study. We do not know whether concomitant Crohn's Disease or Ulcerative Colitis [27] or other autoimmune diseases, known to be associated with decreased iNKT numbers [17] and that had been excluded from our study have been included in that of Grose et al.

Finally, the phenotype of the iNKT cells was evaluated as support for a regulatory function of these cells. Unfortunately in RCD patients iNKT levels were too low to allow for accurate phenotyping. However, in CD patients responding to a GFD a higher frequency of CD4<sup>+</sup> iNKT was found than in age-matched healthy controls. Interestingly, gliadin-specific regulatory T cells were found in coeliac patients on a GFD as well [34], indicating that regulatory cell numbers can increase during a GFD, or that individuals with higher frequencies of regulatory cells are more likely to respond to a GFD. Remarkably we did not find molecular support for a regulatory function of the iNKT cells, since no expression of intracellular FOXP3 and CTLA4 was observed in either the CD4<sup>+</sup> or CD4<sup>-</sup> iNKT subset. This might suggest a different regulatory mechanism in iNKT cells than in Tregs, although it cannot be excluded that these regulation-associated proteins can be induced in iNKT cells upon activation.

Treatment of RCD involves immunomodulating drugs [35–37] and, in RCD II, chemotherapy and autologous hematopoietic stem cell transplantation [38]. However, since none of these therapies has been fully effective so far, additional treatments supporting the homeostasis of the immune system could be of vital importance. Recently, Gianfrani et al. [34] suggested a future therapeutic approach based on *in vitro* expanded gliadin-specific regulatory T cells with a specific gut-homing capacity, which could be reintroduced into RCD patients. Since we have clearly demonstrated a selective depletion of iNKT cells in RCD, new therapeutic strategies should also aim at reconstitution of adequate numbers of appropriately polarized iNKT cells [23].

With respect to other circulating regulatory T cells, like Treg and T $\gamma$  $\delta$  cells, we did not find abnormal levels, neither in responsive nor in refractory CD. Also, no differences were found between RCD I and II patients, although the RCD patient subgroups here were rather small. Tregs are, like iNKT cells, believed to prevent the development of autoimmune disease, while, on the other hand, tumors might benefit from them due to downregulation of anti-tumor immune responsiveness [39]. Despite normal levels in the circulation, however, defects locally in the intestinal mucosa with respect to numbers or regulatory capacity cannot be excluded. CD is known to be characterised by a permanent increase of TCR $\gamma$  $\delta$  IELs with a concomitant elevation of infiltrating TCR $\alpha\beta$ <sup>+</sup> cells during the active stage of the process [40–42]. However, the TCR $\alpha\beta$ <sup>+</sup> cells decrease in response to gluten withdrawal, whereas for TCR $\gamma$  $\delta$ <sup>+</sup> cells this may take years to occur [43].

Relatively high levels of mucosal T $\gamma\delta$  [44,45] and recently of gliadin specific regulatory T cells [34] have been described in CD in remission. In other intestinal diseases like colitis, CD4<sup>+</sup>CD25<sup>+</sup> Tregs were shown to prevent disease development [46]. Therefore, future investigation of not only mucosal iNKT cells but mucosal Tregs and T $\gamma\delta$  cells as well might shed more light on their role in maintaining intestinal homeostasis in CD patients.

In summary, our study demonstrates that from the circulating regulatory T cells tested, only the iNKT cell numbers have become selectively reduced in coeliac disease refractory to the gluten free diet. New therapeutic strategies aiming at the expansion of appropriately polarized iNKT cells [23] may be effective in preventing disease progression in refractory coeliac patients.

## Acknowledgments

This work has been partially funded by the Spanish Ministry of Education (FPU, AP2002-2696), the Spanish Ministry of Health (FIS, PI020895; 02/3068) and the Junta de Castilla y Leon (SAN1052-VA02/05 VA057/04).

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