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IL-15 controls T cell functions through its influence on CD30 and OX40 antigens in Celiac Disease

N. Periolo [a], L. Guillén [a], M.L. Arruvito [a], N.S. Alegre [a], S.I. Niveloni [b], J.H. Hwang [b], J.C. Bai [b], A.C. Cherñavsky [a,*]

[a] Instituto de Inmunología, Genética y Metabolismo, Hospital de Clínicas "José de San Martín", Universidad de Buenos Aires, Buenos Aires, Argentina
[b] Sección Intestino Delgado, Departamento de Medicina, Hospital de Gastroenterología "Dr. Carlos Bonorino Udaondo", Buenos Aires, Argentina

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ABSTRACT

Aim: To evaluate the ability of interleukin (IL)-15 to control T cell functions through its influence on CD30 and OX40 expressing cells in Celiac Disease (CD). In peripheral blood (PB), by examining the expression of OX40 in conventional effectors cells and T cells with a phenotypic specialization of regulatory cells [$CD4^+CD25^{high}$ forkhead box protein 3 $(Foxp3)^+$], and the co stimulation of IFN-γ and IL-4 production within CD30 and OX40 positive subsets of T cells. At the duodenal mucosa, by assessing the expression of CD30 and OX40 in intraepithelial (IE) and lamina propria (LP) lymphocytes (IEL, LPL).
Patients and methods: PB and duodenal mucosal biopsies were obtained from 38 patients with classic CD (Cel) and 38 healthy controls (HC). Analysis of cell surface and/or intracellular antigens was performed in anti-CD3-treated PB mononuclear cells (PBMC) before and after treatment with recombinant IL-15 (rIL-15), and in IE and LP cellular suspensions prepared from duodenal biopsies pre-treated with/without rIL-15.
Results: A subpopulation of $CD3^+OX40^+$ T blasts was induced in Cel and HC by a 3 days treatment of PBMC with anti-CD3 and decreased its size thereafter, regardless of the presence of rIL-15. However, the addition of rIL-15 to T blasts distinctively induced the survival of T cells with a regulatory phenotype that expresses OX40 antigen in Cel ($p < 0.05$). Celiac patients showed higher frequencies of IFN-γ-producing $CD3^+CD30^+$ blasts before and after treatment with rIL-15 ($p < 0.05$, vs. HC). IL-15 increased the frequencies of $CD3^+CD30^+$ LPL (HC: $p < 0.05$, Cel: $p < 0.05$) but not of $CD3^+OX40^+$ LPL, and CD30 or OX40 positive IEL.
Conclusions: The distinctive control of $OX40^+$ cells with a T regulatory phenotype mediated by the influence of IL-15 comes out as new function of this cytokine in the context of CD. The higher production of IFN-γ by a subpopulation of peripheral $CD3^+CD30^+$ cells contributes to the type I biased immune response.

1. Introduction

Celiac Disease (CD) is a T cell mediated small intestinal hypersensitivity to wheat and other gluten-containing cereals occurring in genetically predisposed individuals [1]. This Th1-biased chronic disorder is characterized by the production of large amounts of interferon gamma (IFN-γ) by CD4 positive T cells, driven by gluten peptides at the small intestinal mucosa [2,3]. Gluten peptides also induce the activation of intraepithelial lymphocytes (IEL) through epithelium-derived IL-15 [4]. The production of IL-15 by epithelial cells, dendritic cells and macrophages at the duodenal mucosa and also IL-15 serum levels are increased in patients with classical CD (Cel) [4].

IL-15 is likely to be produced in waves linked to gluten intake [5]. Although gliadin triggers an IL-15 mediated innate response in all individuals [6], the existence of a lower IL-15 immunological threshold may explain the origin of a secondary immune response developed in CD [7]. Interleukin 15 induces the proliferation of IEL [8] and lamina propria (LP) lymphocytes (LPL), the TCR-independent synthesis of interferon (IFN)-γ and the up regulation of CD25 expression in LPL [9]. The proinflammatory nature of IL-15 is due to direct effects such as the stimulation of innate IFN-γ production that potentially shifts the subsequent adaptive T cell

Abbreviations: CD, Celiac Disease; Cel, patients with classical CD; HC, healthy controls; Smads, small body size mothers against decapentaplegic proteins; Lck, lymphocyte-specific protein tyrosine kinases; Syk, spleen tyrosine kinases; NGFR/TNFR, nerve growth factor receptor/tumor necrosis factor receptor super family; Tregs, T regulatory cells; Foxp3, forkhead box protein 3; LP, Lamina propria; LPL, lamina propria lymphocytes; IEL, intraepithelial lymphocytes; IFN-γ, interferon gamma; IL, interleukin; EmA, anti-endomysial antibodies; rIL-15, recombinant human IL-15; tTG, tissue transglutaminase; PMA, phorbol myristate acetate; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; FC, flow cytometry; Na, not available.

* Corresponding author. Address: Hospital de Clínicas "José de San Martín", Avda Córdoba 2351, Piso 3, Sala 4, Ciudad Autónoma de Buenos Aires CP1120, Argentina. Tel.: +54 011 5950 8756; fax: +54 011 5950 8758.
E-mail address: accher@fibertel.com.ar (A.C. Cherñavsky).

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responses towards Th1 [10], and to indirect effects such as the impairment of TGF-β/small body size mothers against decapentaplegic proteins (Smads) signaling in T cells [11] and a co-adjuvating effect on mucosal dendritic cells provoking inflammatory immunity to dietary antigens [12]. Upon interaction of T cells with IL-15, downstream effects include the induction of bcl-2, the activation of the MAP kinase pathway, the phosphorylation of lymphocyte-specific protein tyrosine kinases (lck) and spleen tyrosine kinases (syk) [13], and the expression of several members of the tumor necrosis factor receptor super family (TNFRSF). Thus, surface expression of OX40 (CD134, TNFRSF4) remains unaltered, CD27 is down regulated, and CD30 (TNFRSF8) is up regulated by IL-15 in T lymphoblasts from healthy controls (HC) [14].

The interaction between members of the TNFRSF expressed on T cells with their cognate ligands is involved in the co stimulation of TCR signals. Although both molecules promote proliferation and cytokines production (i.e. IFN-γ and IL-4), these molecules might be functionally distinct given that CD30L triggers a weak co stimulation of conventional T cells compared with OX40L [15]. The interaction of these molecules with their ligands leads to the recruitment of TNFR-associated factor 2 (TRAF2). Besides interacting with upstream proteins like CD30 and OX40, TRAF2 interacts with downstream signaling proteins such as IL-15R alpha chain [16]. It is well established that the presence of definite culture conditions during T cell activation is needed to *influence the cytokine profile* expressed by these cells. For instance that STAT5-dependent cytokines such as IL-15 contribute to induce IL-4Rα expression, priming cells for Th2 differentiation and helping to maintain this state [17].

Thus the induction of Th1/Th2 differentiation comes from the integration of multiple intracellular signals and for these reasons; we aimed to study the regulation of cytokines production in CD30 and OX40 positive subsets of peripheral T cells by IL-15 in the context of CD.

The OX40 antigen is constitutively expressed in forkhead box protein 3 (Foxp3) positive natural and inducible T regulatory cells (Tregs) whereas both CD30 and OX40 are inducible antigens in resting conventional T cells [18]. T regulatory cells are essential to the harmless function of the immune system. The interaction of Tregs with dendritic cells in lymphoid and non lymphoid tissues may grant Tregs access to trans-presented IL-15. As a consequence, intracellular signaling through IL-15 receptor can help to support Tregs survival [19] and regulate surface OX40 expression in Tregs.

Our aim was to evaluate the ability of IL-15 to control T cell functions through its influence on CD30 and OX40 expressing cells in CD, both, in PB by examining the expression of OX40 in conventional effectors T cells and T lymphocytes with a regulatory phenotype and the co stimulation of IFN-γ and IL-4 production within CD30 and OX40 positive subsets of T cells, and at the duodenal mucosa by assessing the expression of CD30 and OX40 in IEL and LPL.

2. Patients and methods

Patients

We assessed a total of 38 Cel and 38 unrelated healthy volunteers who underwent upper endoscopies as HC. Duodenal biopsies were obtained at diagnosis of CD or during screening procedures for abdominal symptoms respectively. All Cel and HC were enrolled at the Small Intestinal Section of the "Dr. C.B. Udaondo" Gastroenterology Hospital in Buenos Aires after they were informed of the aim of the study and gave their written informed consent to be included. The study was approved by the Ethics and Research Committees of the Gastroenterology Hospital informed of internationally endorsed standards for the application of the Helsinki Declaration.

Diagnosis of CD was based on clinical, serological and histological conventional criteria such as the presence of clinical features including a characteristic celiac enteropathy [20], increased duodenal IEL density [21], positive anti-endomysial (EmA) and anti-tissue transglutaminase (tTG) antibodies [22], and human leucocytes antigens (HLA)-DQ2 genotyping [23]. Autoimmune enteropathies were excluded by screening of anti-enterocyte autoantibodies determined by indirect immunofluorescence using human small intestinal substrate [24].

2.1. Monoclonal antibodies and reagents

The fluorochrome-conjugated mouse monoclonal (mAbs) against human (hu) antigens IgG1 anti-CD3 Peridinin chlorophyll protein Complex (PerCP) (clone SK7), IgG1 anti-CD25 phycoerythrin (PE) (clone 2A3), IgG1 anti-CD103 fluorescein isothiocyanate (FITC) (clone Ber-ACT8), IgG1 anti-CD4 APC (clone RPA-T4), IgG1 anti-CD8 PerCP (clone SK-1), IgG1 anti-CD69 FITC (clone FN50) and isotype controls were obtained from Becton Dickinson (BD) (San Jose, CA, USA). Monoclonal Abs IgG2a anti-CD45RO PE (clone UCHL1), IgG1 anti-CD30 FITC (clone BerH8), IgG1 anti-OX40 FITC (clone ACT 35) were obtained from Dako Corp. (Santa Barbara, CA, USA). IgG1 Foxp3 PE (clone 259D/C7) was obtained from BD. IgG1 PE-Cyanine7 (clone BC96) was obtained from eBioscience (San Diego, CA, USA). For cell activation, IgG1 anti-CD3 (clone UCHT1) (Immunotech, Marseille, France) and a recombinant human IL-15 (rIL-15) obtained from R&D Systems (Minneapolis, MN, USA) were used. IgG1 anti-huIL-15 (clone 34,505) was also obtained from R&D Systems. Ficoll–Hypaque was purchased from Pharmacia Biotech (Uppsala, Sweden), RPMI 1640 medium was obtained from Life Technologies (Gaithersburg, MD, USA), propidium iodide was obtained from Santa Cruz Biotechnology (CA, USA) and gentamicin, glutamine, gelatin, ionomycin, Phorbol Myristate Acetate (PMA), dithiothreitol, collagenase A and Brefeldin A were purchased from Sigma Chemical Co (Saint Louis, Mo, USA). The fixation kit, permeabilization buffer for intracytoplasmic staining and mAbs for intracellular cytokines detection IgG2A anti-IFN-g PE (clone 4S.B3) and IgG1 anti-IL-4 PE (clone 8D4-8) were purchased from BD.

2.2. Cell isolation and activation

Heparinized blood samples (10 ml) were obtained immediately after duodenal biopsy and peripheral blood mononuclear cells (PBMC) were prepared by Ficoll–Hypaque density gradient centrifugation at 2000 rpm for 20 min. PBMC were washed twice in phosphate-buffered saline (PBS), counted and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum, 2 mmol/l L-glutamine, and 50 mg/ml gentamicin. For PBMC activation, 1×10^6 cells/ml of complete RPMI 1640 medium supplemented with 10% heat-inactivated huAB were dispensed into 24-well microtiter plates (Costar, Cambridge, MA, USA) previously inclubated with 1 mg/ml anti-CD3 mAb (clone UCHT1) for 2 h at 37 °C in a humid atmosphere containing 5% CO2 and harvested 3 days later. The T blasts cultures obtained after a 3 days incubation of PBMC with anti-CD3 were measured by flow cytometry (FC) for different surface and intracellular antigens as indicated, or received an additional 3 days incubation with RPMI 1640 alone or with 50 ng/ml recombinant IL-15 (rIL-15) before being measured.

2.3. Isolation of IEL and LPL from duodenal biopsy samples

Duodenal biopsy samples from patients with CD and HC were washed and cultured for 3 hours (h) with 50 ng/ml rIL-15, or left at baseline condition in complete RPMI 1640 alone as an internal

control for each patient. At the end of the incubation period, tissues were extensively washed with isotonic Hank's buffered salt solution (HBSS) and duodenal cellular suspensions were prepared from biopsy specimens as previously described [25]. Epithelial suspensions containing IEL were obtained by incubating the tissue in 5 ml of Ca^{2+} and Mg^{2+}-free HBSS pH 7.2 containing 1 mM EDTA and 1 mM dithiothreitol with moderate magnetic stirring for 1 h at 37 °C and 200 rpm. The supernatant was harvested, and the cells were pelleted at 2000 rpm for 5 min and resuspended in PBS pH 7.2 before immunostaining for CD30 and OX40. This procedure yielded a median of 0.5×10^6 cells (range 0.4–1.0) with a median viability of 70% (range 55–85). As a marker for human IELs, CD103 (also called human mucosal lymphocyte antigen 1, integrin alpha E beta 7) was studied. More than 95% of $CD3^+$IEL were $CD103^+$ as determined by immunofluorescence (not shown). Lamina propria (LP) cell suspensions containing LPL were obtained by cutting the remainder of the tissue into small pieces followed by incubation with 1 mg/ml collagenase A in RPMI 1640, 2 mmol/l L-glutamine, and 50 mg/ml gentamicin. The mixture was subjected to moderate magnetic stirring for 2 h at 37 °C and 200 rpm and yielded a median of 1.8×10^6 cells (range 0.4–3.1) with a median viability of 90% (range 75–95). After harvesting, the cells were washed in PBS and kept briefly on ice in RPMI 1640, 2 mmol/l L-glutamine, and 50 mg/ml gentamicin before immunostaining.

2.4. Flow cytometry

For staining of cell surface antigens, freshly isolated IEL-containing epithelial suspensions, LPL-containing LP suspensions and T blasts cultures were washed twice with 1 ml of PBS supplemented with 10% mouse serum for 10 min to block non-specific binding followed by staining with mAbs anti-human CD3, -CD4, -CD8, -CD25, -CD30, -OX40 and -CD103. For intracellular IL-4 and IFN-g cytokines staining, T blasts were incubated with 50 ng/ml PMA and 1 μg/ml calcium ionophore, with 10 μg/ml Brefeldin A for 4 h at room temperature. After cell surface staining, the cells were fixed at 4 °C for 20 min, washed and permeabilized. Anti-cytokine mAbs were added to the cells and incubated at 4 °C for 40 min in the presence of 50 μl of permeabilization buffer. To perform the FC-based analysis, lymphocytes were gated on forward- and side-scatter properties to exclude dead and/or granular cells. A region of CD3 positive cells was selected on side-scatter properties vs. CD3 expression, and IFN-γ or IL-4- producing $CD30^+$ cells were assessed within $CD3^+CD30^+$ or $CD3^+OX40^+$ cells, respectively.

For Foxp3 intracellular staining, cells were first stained with mAbs anti-human CD4 APC, -CD8 PerCP, -CD25 PE-Cy7 and -OX40 FITC simultaneously for 20 min at room temperature, followed by washing in PBS. Afterwards, cells were fixed and permeabilized stained with anti-human Foxp3 PE according to

Table 1
Demographical features, clinical, laboratory and histological parameters in patients with classic CD.

Patient No.	Gender/age (years)	Marsh histological classification type	IELs / 100 epithelial cells	Anti-tTG tests at diagnosis[a]	EmA tests at diagnosis[b]
1	F/55	IIIc	70.0	+	+
2	F/44	IIIc	52.0	–	+
3	F/38	IIIc	70.0	+	+
4	F/40	IIIc	34.0	+	+
5	M/25	IIIc	38.3	+	+
6	F/39	IIIc	58.6	+	+
7	F/31	IIIc	39.0	+	+
8	F/30	IIIc	45.0	+	+
9	F/29	IIIc	32.0	+	+
10	F/45	IIIc	30.0	Na	+
11	F/19	IIIc	24.0	+	-
12	F/23	IIIc	36.0	+	+
13	F/35	IIIb	30.3	–	–
14	F/28	IIIb	28.0	+	Na
15	F/24	IIIa	39.0	+	+
16	F/55	IIIa	38.0	+	+
17	F/60	IIIa	30.0	+	+
18	F/69	IIIc	45.0	+	–
19	F/33	IIIc	44.0	+	Na
20	F/56	IIIc	Na	+	–
21	F/47	IIIc	Na	–	+
22	F/31	IIIc	40.0	+	–
23	F/37	IIIa	36.0	+	+
24	F/45	IIIc	40.4	+	+
25	M/43	IIIc	27.0	+	+
26	F/76	IIIc	40.0	+	+
27	M/56	IIIc	40.0	+	+
28	M/59	IIIc	41.0	+	Na
29	F/42	IIIa	35.0	+	–
30	M/54	IIIb	28.0	–	+
31	F/61	IIIc	63.0	+	+
32	F/67	IIIc	54.0	+	+
33	F/59	IIIc	52.0	+	+
34	F/76	IIIc	40.0	+	+
35	M/65	IIIc	45.0	+	+
36	M/58	IIIa	43.0	+	+
37	F/29	IIIb	51.0	+	+
38	F/70	IIIc	49.0	+	+

CD = Celiac Disease, F = female, M = male. EmA = anti-endomisial antibodies, anti-tTG = anti-tissue transglutamise antibodies.
[a] IgA and IgG subtypes of anti-tTG were determined by ELISA using a commercial kit (22).
[b] Immunoglobulin A (IgA) and immunoglobulin G (IgG) subtypes of EmA were determined by indirect immunofluorescence on monkey esophagus substrate. Only the greater damage is reported as the description of the Marsh histological classification type. Na: not available.

the manufacturer's instructions, and analyzed immediately. To perform the FC-based analysis, a region of CD4 positive cells was selected on side-scatter properties vs. CD4 expression, and OX40 expression was assessed within $CD4^+CD25^+Foxp3^+$ subpopulation.

For the analysis of duodenal cell suspensions, 0.5 mg/ml propidium iodide was added just before acquisition to exclude dead cells. A total of 15.000 and 30.000 events from duodenal cell suspensions and PBMC, respectively, were acquired. Acquisition and analysis were performed on a FACSCalibur flow cytometer (BD) using the Windows Multiple Document Interface for Flow Cytometry software version 2.8 (Scripps Research Institute, La Jolla, Florida, USA).

2.5. Statistical analyses

The GraphPad Prism software (San Diego, CA, USA) was used for all analyses. The Mann–Whitney U test and Kruskal–Wallis variance analysis with Bonferroni's post test were used to compare data between two groups or between three or more groups as indicated. Results were expressed as median values, lower and upper quartiles (25th–75th percentile) as corresponding. The Wilcoxon signed rank test was used to compare paired data within experimental groups. *P*-values <0.05 were considered as significant.

3. Results

3.1. Demographical features, clinical, laboratory and histological parameters

Demography, clinical features, laboratory, and histological information of the patients with CD included in this study are shown in Table 1. Ninety percent of the patients were genotyped as HLA-DQ2 positive. At diagnosis of CD, all of them had typical histological characteristics and a clinical presentation with signs and symptoms of malabsorption such as diarrhoea, steatorrhoea, and weight loss or growth failure defined as a classical presentation [26]. Eighty percent of the patients were anti-tTG IgA or EmA IgA -positive.

3.2. The influence of IL-15 on the expression of OX40 in T lymphoblasts

We have previously demonstrated that rIL-15 augments the frequency of $CD3^+CD30^+$ peripheral lymphoblasts in Cel and HC. Furthermore, we have also demonstrated that the influence of rIL-15 on CD30 expression is greater in Cel [11]. To explore likely differences in the control of T cell functions by IL-15, we firstly evaluated the kinetics of OX40 surface expression in T

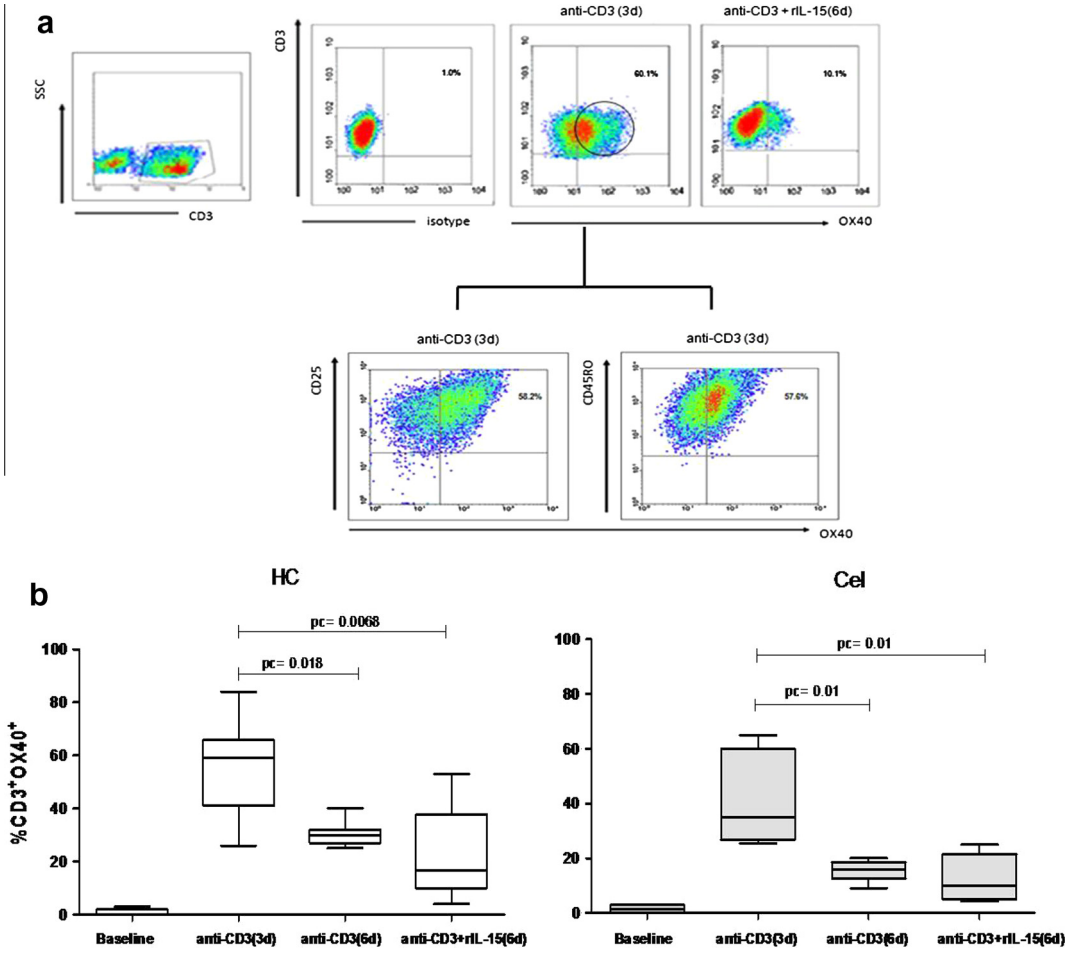

Fig. 1. The influence of IL-15 on OX40 expression in T lymphoblasts. Freshly isolated PBMC were activated with anti-CD3 + RPMI 1640 [anti-CD3 (3d) and anti-CD3 (6d)] or with anti-CD3 + rIL-15 [(anti-CD3 + rIL-15 (6d)], stained with anti-CD3 PerCP, -OX40 FITC, -CD25 PE, or with anti-CD3 PerCP, -OX40 FITC and -CD45RO PE, and assessed by FC as described in Methods (a) The analysis performed in one representative donor from the healthy control (HC) group is shown. Lymphocytes were gated on forward and side-scatter properties to exclude dead and/or granular cells. The region of CD3 positive cells is shown in the left panel. OX40 expression within CD3 positive cells assessed after anti-CD3 (3d) and anti-CD3 + rIL-15 (6d) respectively is shown in the upper right panel. The analysis of CD25 and CD45RO markers within $CD3^+OX40^+$ cells obtained after treatment with anti-CD3 for 3d is shown in the lower right panel. (b) The box and whiskers show non-parametric statistics: median, lower and upper quartiles, and confidence interval around the median values for the percentage of $CD3^+OX40^+$ cells among CD3 positive cells obtained from 10 HC and 5 patients with classic CD (Cel) (Table 1, patients 1–5) after treatment with anti-CD3 for 3 and 6 days, or with anti-CD3 + rIL-15 are shown. *P*-values were calculated using Kruskal–Wallis variance analysis with Bonferroni's post test for multiple comparisons. Pc: corrected P values.

lymphoblasts from Cel and HC. Since OX40 is not expressed in resting, conventional effectors T cells, PBMC were firstly activated with anti-CD3. A subpopulation of $CD3^+OX40^+$ cells was induced after 3 days of treatment. In agreement with previously reported data [27], OX40 expression was found largely within $CD4^+$ T blasts ($85.30 \pm 4.10\%$ of total OX40 positive $CD3^+$ blasts) (data not shown). This subpopulation of $CD4^+$ T blasts expressed CD25 and CD45RO surface markers as shown in Fig. 1a. Following an additional incubation of T blasts from Cel and HC for 3 days with RPMI 1640 alone, decreased numbers of $CD3^+OX40^+$ cells were observed. The treatment of T lymphoblasts with rIL-15 did not avoid the drop in the frequencies of $CD3^+OX40^+$ cells already observed following incubation with RPMI 1640 alone (Fig. 1b).

3.3. The influence of IL-15 on OX40 expression in peripheral $CD4^+$ cells with a T regulatory phenotype

OX40 antigen is constitutively expressed in $Foxp3^+$ natural and inducible Tregs. However, negligible percentages of $CD3^+OX40^+$ PBMC are found unless the cells are previously activated. For this reason, we evaluated OX40 expression in a subset of T cells with regulatory phenotype induced after anti-CD3 treatment of PBMC. Since the utilization of Foxp3 expression as a marker of Tregs needs an additional functional analysis [28], we evaluated OX40 expression in conventional activated T cells ($CD4^+CD25^+$ $Foxp3^-$) and T cells with a regulatory phenotype ($CD4^+CD25^{high}$ $Foxp3^+$). A representative FC-based analysis is shown (Fig. 2a). The frequencies of $CD4^+CD25^{high}$ $Foxp3^+$ cells among T blasts did not differ between Cel and HC. However, the addition of rIL-15 significantly increased the frequency of cells with a T regulatory phenotype that expresses $OX40^+$ in Cel but not in HC (Fig. 2b).

3.4. The influence of IL-15 on cytokine production in $CD3^+CD30^+$ and $CD3^+OX40^+$ peripheral subpopulations

To evaluate the co stimulatory function of IL-15 on cytokines production by peripheral $CD3^+CD30^+$ and $CD3^+OX40^+$ subpopulations, we analyzed the percentages of both IFN-γ- and IL-4- producing $CD3^+CD30^+$ or $CD3^+OX40^+$ T blasts in Cel and HC, before and after the addition of rIL-15 to T blasts cultures. Celiac patients showed a 7.5 folds greater median value of IFN-γ-producing $CD3^+CD30^+$ cells in T blasts cultures ($p = 0.01$, vs. HC) and also a 4.5 folds greater median value of IFN-γ-producing $CD3^+CD30^+$ cells after treatment of T blasts with rIL-15 ($p = 0.01$, vs. HC). The addition of rIL-15 to T blasts cultures up regulated the number of IFN-γ-producing $CD3^+CD30^+$ cells in HC ($p < 0.05$) but not in Cel (Fig. 3a). On the contrary, we observed similar frequencies of IFN-γ-producing $CD3^+OX40^+$ cells, $IL\text{-}4^+$ $CD3^+CD30^+$ and $IL\text{-}4^+$ $CD3^+OX40^+$ cells among total blasts from Cel and HC, regardless of the addition of rIL-15 (Fig. 3b–d).

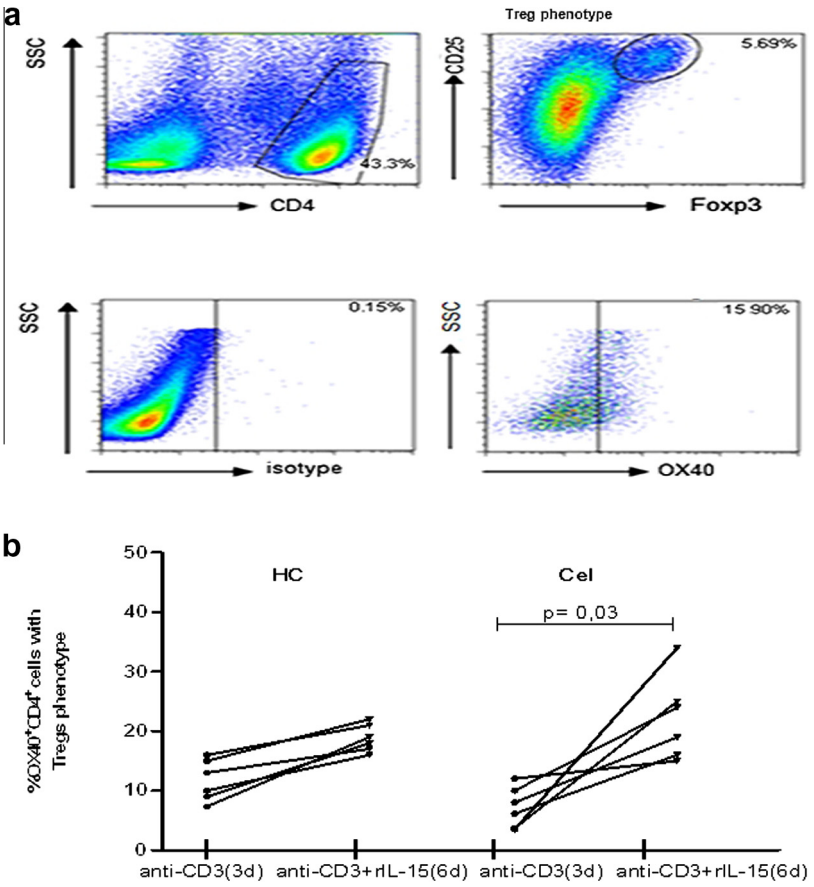

Fig. 2. The influence of IL-15 on OX40 expression in peripheral cells with a T regulatory phenotype. Freshly isolated PBMC were activated with anti-CD3 + RPMI 1640 for 6 days [anti-CD3(6d)] or with anti-CD3 for 3 days followed by an additional 3 days period of incubation with rIL-15[anti-CD3 + rIL-15(6d)], stained with anti- CD4 APC, -CD8 PerCP, -CD25 Cy7, -OX40 FITC and -Foxp3 PE and assessed by FC as described in Methods. (a) The analysis performed in one representative donor from the healthy control (HC) group assessed after treatment with anti-CD3 + rIL-15 is shown. Lymphocytes were gated on forward and side-scatter properties to exclude dead and/or granular cells. The region of CD4 positive cells, the analysis of CD25 vs. Foxp3 expression within the CD4 positive region and the analysis of OX40 expression within these cells in side-scatter properties vs. OX40 expression graph are shown. (b) Each line belongs to a healthy control (HC) or a patient with classic CD (Cel). Lines link the % $OX40^+$ $CD4^+$ cells with T regulatory phenotype obtained without (anti-CD3) and with the addition of rIL-15 (anti-CD3 + rIL-15) in 6 HC and 6 Cel (Table 1, patients 6–11). *P* values were calculated using the Wilcoxon signed rank test.

3.5. The influence of IL-15 on OX40 and CD30 expression at the duodenal mucosa

After ex vivo stimulation of duodenal biopsy samples from Cel and HC with gliadin, CD30 antigen was similarly expressed in LPL and IEL [29]. In the present study, we aimed to evaluate the likely influence of IL-15 on the control of LPL and IEL functions. To this end, duodenal biopsy samples were divided in two fractions. One fraction was incubated for 3 h in the presence of rIL-15 and the other remained at baseline condition until preparing the LP and epithelial cellular suspensions. We used biopsies coming from different patients for the assessment of CD30 and OX40 expression in IEL and LPL (Fig. 4). CD30 and OX40 antigens expression was studied by FC both at baseline and after the addition of rIL-15, and data were then analyzed as paired samples. The addition of rIL-15 to biopsy cultures increased the percentages of $CD3^+CD30^+$ LPL within the two study groups (Fig. 4b), but it failed to modify the expression of OX40 (Fig. 4c). Cel and HC showed similar percentages of $CD3^+CD30^+$ IEL and $CD3^+$ $OX40^+$ IEL (4.02 ± 1.08 vs. 4.90 ± 1.25, p = ns and 5.10 ± 2.09 vs. 5.80 ± 2.25, p = ns) within the epithelial cellular suspensions at baseline condition, and also after the addition of rIL-15(not shown).

4. Discussion

Our study reveals the presence of a subpopulation of activated $CD3^+CD30^+$ memory cells as source of IFN-γ that is increased in the context of CD. Since the addition of exogenous rIL-15 to T lymphoblasts did not induce further differentiation towards a Th1/cytotoxic profile in Cel, we cannot assume that the increased subpopulation of IFN-γ-producing $CD3^+CD30^+$ cells is under the in vivo influence of IL-15. The rIL-15- ex vivo mediated generation of peripheral T cells with a regulatory phenotype that expresses OX40 allows us to infer a new function of IL-15 in the context of CD.

The $CD3^+OX40^+$ subset of peripheral T blasts identified in the present study was characterized by triple staining for CD3/OX40/CD45RO and CD3/OX40/CD25 antigens. The similar frequencies observed by both staining strongly suggested that OX40 antigen is expressed in activated $CD45RO^+CD25^+$ T cells. The addition of rIL-15 to T-lymphoblasts from HC and Cel did not avoid the drop in the frequencies of $CD3^+OX40^+$ subpopulations observed following the incubation of lymphoblasts with RPMI 1640 alone. The signaling through OX40 provides a high level of activation and survival of T cells [30]. Since IL-15 also promotes cell activation and protects from cell death, the influence of IL-15 on OX40 positive blasts may be interpreted as a common T cell response against the survival of an excessive number of effectors and memory cells.

Interestingly, IL-15 induced the survival of peripheral $OX40^+$ T cells with a regulatory phenotype from Cel. This result may be linked to a higher expression of the IL-15 receptor alpha chain described in Tregs from Cel [31] and probably related to the lower IL-15 response threshold [31]. The failure of IL-15 to up regulate CD30 expression in LPL and also to induce production of IFN-γ by peripheral T blasts from Cel argues against the existence of a global lower IL-15 immunological threshold in CD. Nonetheless we cannot discard a saturation of stimuli in the last case.

Interleukin-15 helps effectors T lymphocytes to become resistant to suppression by Tregs in the context of CD though the mechanisms involved are not completely understood [31,32]. Besides, OX40 expression has been identified as a key negative regulator of $Foxp3^+$ Tregs [33]. From our results regarding the effect of IL-15 on peripheral T cells in Cel, we speculate that up regulation of OX40 might impair the putative suppressive activity of T cells with a regulatory phenotype. However, this hypothesis deserves further investigation.

As memory cells retain the Th1, Th2 or cytotoxic commitment that was imprinted at priming when stimulated under neutral conditions [34], in the present study T lymphoblasts were incubated with PMA and ionomycin to characterize the intracellular expression of IFN-γ and IL-4. Given that IFN-γ production is expressed as the percentage of IFN-γ- producing $CD3^+CD30^+$ cells of total $CD3^+CD30^+$ cells, it is unlikely that differences among treatments (i.e. presence or absence of rIL-15) or among experimental groups may be secondary to different absolute numbers of $CD3^+CD30^+$

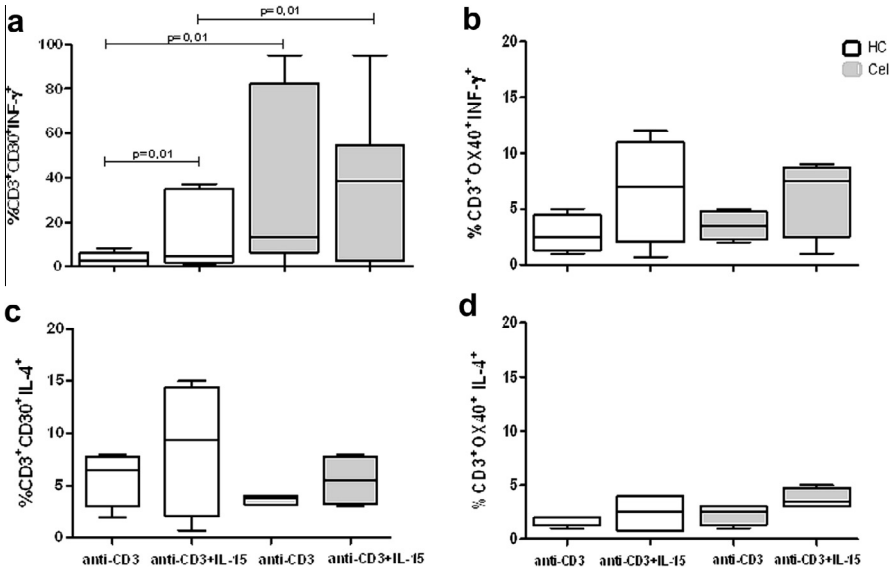

Fig. 3. The influence of IL-15 on cytokine production in $CD3^+CD30^+$ and $CD3^+OX40^+$ peripheral subpopulations. Freshly isolated PBMC from 12 HC and 11 Cel (patients 12–22, Table 1) were activated with anti-CD3 + RPMI 1640 (anti-CD3) or with anti-CD3 + rIL-15 (anti-CD3 + rIL-15), stained with anti-CD3 PerCP, -CD30 FITC and -IFN-γ PE (a), anti-CD3 PerCP, -OX40 FITC and -IFN-γ PE (b), anti-CD3 PerCP, -CD30 FITC and -IL-4 PE (c) or anti-CD3 PerCP, -OX40 FITC and -IL-4 PE (d), and assessed by FC as described in Methods. The box and whiskers show non-parametric statistics: median, lower and upper quartiles, and confidence interval around the median values for IFN-γ- or IL-4-producing $CD30^+$ cells in total $CD3^+CD30^+$ cells (a and c), or IFN-γ- and IL-4- producing $OX40^+$ cells in total $CD3^+OX40^+$ cells (b and d). *P* values were calculated using the two-tailed Mann–Whitney U test for comparisons performed between two groups.

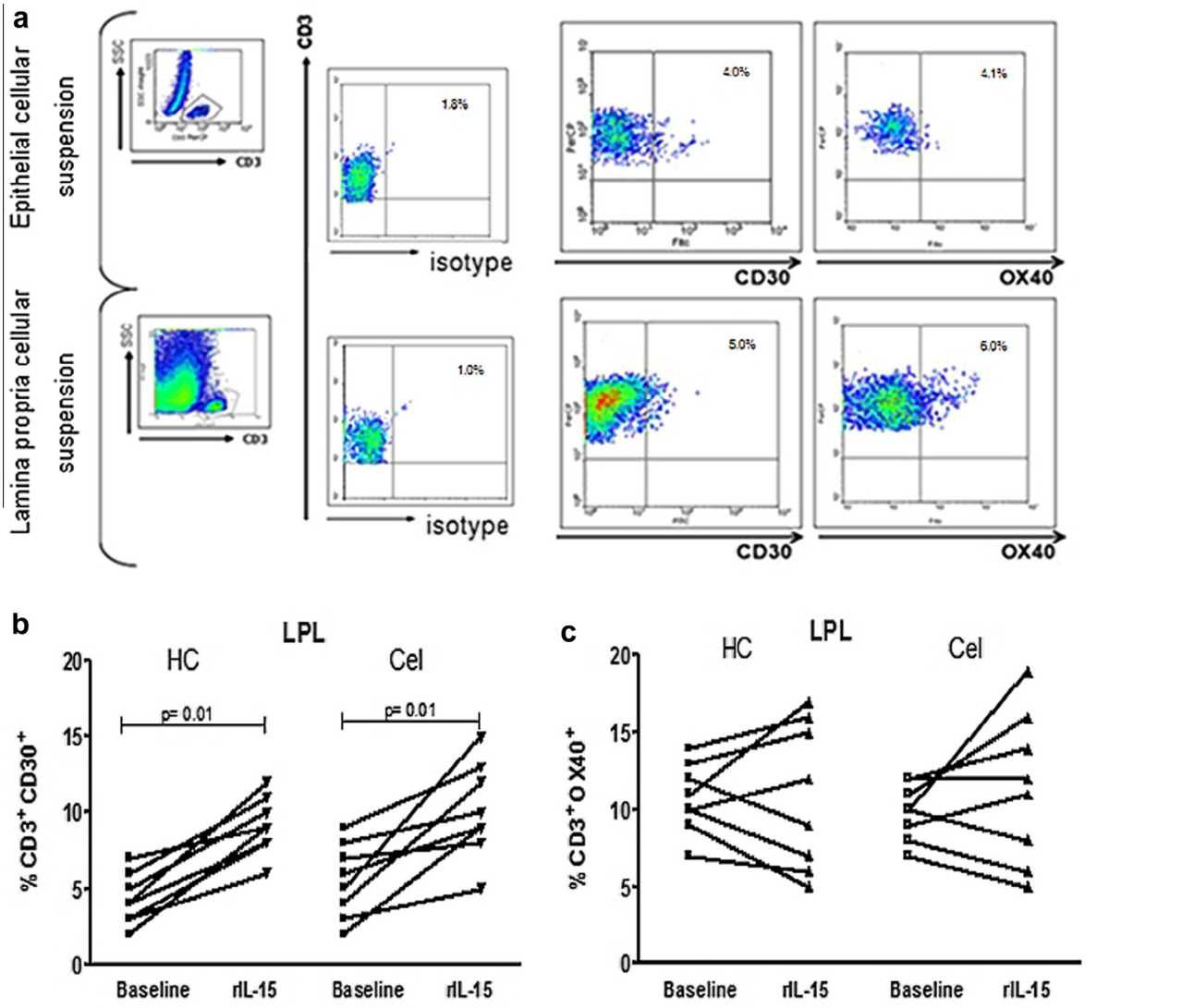

Fig. 4. The influence of IL-15 on OX40 and CD30 expression at the duodenal mucosa. Epithelial and lamina propria cellular suspensions were prepared after treatment of duodenal biopsies for 3 h with rIL-15 or RPMI 1640 (baseline), stained with anti-CD3 PerCP and -CD30 FITC or anti-CD3 PerCP and -OX40 FITC, and assessed by FC as described in Methods. (a) Representative regions of CD3 positive cells in epithelial and lamina propria cellular suspensions gated on side-scatter properties vs. CD3 expression (left panel). CD30 and OX40 expression analyzed within regions of CD3 positive epithelial and lamina propria cells in one representative donor from the Cel group, assessed at baseline condition, is shown (right panel). (b) Each line belongs to a healthy control (HC) or a patient with classic CD (Cel). Lines link the% $CD3^+CD30^+$ among $CD3^+$ LPL obtained at baseline and after treatment with rIL-15 in 8 HC and 8 Cel (patients 23–30, Table 1). (c) Each line belongs to an individual HC or Cel. Lines link the % $CD3^+OX40^+$ among $CD3^+$ LPL obtained at baseline and after treatment with rIL-15 in 8 HC and 8 Cel (patients 31–38, Table 1). *P* values were calculated using the Wilcoxon signed rank test.

cells. Instead, the increased percentage of circulating $CD3^+CD3^+$ cells retaining a Th1 or cytotoxic phenotype after treatment of PBMC with anti-CD3 probably reveal a higher number of cells that have been previously switched on to produce IFN-γ in Cel, in agreement with the spontaneous production of IFN-γ by PBMC previously reported [35]. IL-4 production by T lymphoblasts at baseline may be related to a constitutional feature of all the investigated individuals. It may also be possible that exogenously added rIL-15 is not sufficient to drive IL-4 production regardless that type 2 culturing conditions are set by the addition of IL-4 to the culture [36].

Isolated LPL and IEL were analyzed after a short period of ex vivo incubation of biopsy specimens with rIL-15. The interplay between local factors at the duodenal mucosa may be responsible for the similar expression of CD30 and OX40 observed at baseline conditions in all individuals. Our results exclude the possibility that these antigens can be considered as markers of a differential response to a previous exposure to gluten in vivo.

Whereas the addition of rIL-15 up regulated CD30 expression in LPL from all individuals, OX40 expression was not altered. Both the present results and our previous observations [11] indicate that IL-15 promotes the expression of CD30 in both peripheral T cells and LPL whereas it lacks any influence on OX40 expression in peripheral conventional effectors T cells and mucosal T cells. Cell lines of IEL emerging from the culture of duodenal biopsies from patients with refractory CD in the presence of IL-15 exhibit CD30 expression [37]. However, the lack of influences of rIL-15 on the expression of CD30 in IEL herein observed was not an unpredictable result. Aberrant and T cell receptor positive IEL display remarkable differences at the transcriptional level [38] and, thus, their putative response to cytokine mediated stimulation might not be comparable.

5. Conclusion

The survival of $OX40^+$ cells with a regulatory phenotype promoted by IL-15 comes out as new function of this cytokine in the context of CD. The production of IFN-γ by an increased subpopulation of peripheral $CD3^+CD30^+$ memory T blasts associated with CD contributes to the type I biased immune response.

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