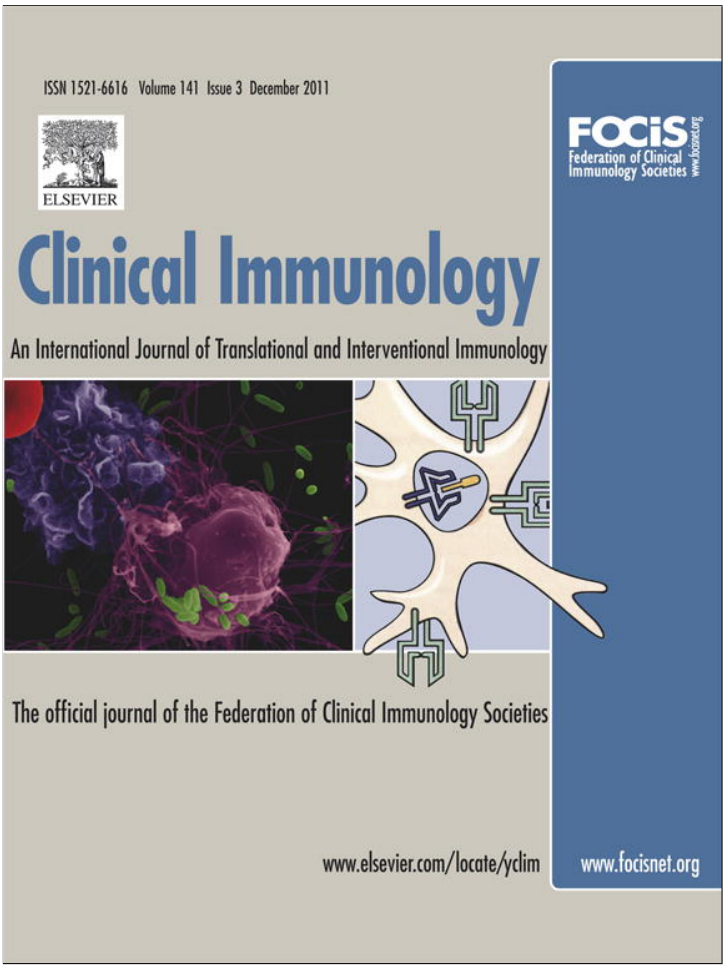

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CD4+ T Lymphocytes with follicular helper phenotype (T_{FH}) in patients with *SH2D1A* deficiency (XLP)

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Abstract Peripheral blood mononuclear cells with T_{FH} phenotype from two asymptomatic XLP patients were studied. Normal/high numbers of CXCR5+, CD4+ T cells coexpressing PD-1 were demonstrated. Peripheral blood mononuclear cells (PBMC) from these patients responded to sub-optimal PHA/IL-2 stimulation upregulating ICOS and CD40L and increasing intracellular expression of IL-10, IL-21 and IL-4 by CD4+ T_{FH} cells. However when compared to N, the time profile of activation and cytokine synthesis was different in XLP and N. While ICOS and CD40L expression in N decreased after 6–8 days, it continued to increase or was maintained in XLP cultures. Intracellular IL-10, IL-21 and IL-4 reached higher values in XLP than N after 8 days. Rather than the absence of T_{FH} cells or their intrinsic inability to respond to stimuli, differences in the time profile of their response could contribute to impair their role as helpers of B lymphocytes.

1. Introduction

X-linked lymphoproliferative disease (XLP) is a rare immunodeficiency associated to mutations in the *SH2D1A* gene (also known as *DHSP a*nd *SAP*) controlling the synthesis of the SLAM-associated adaptor protein SAP [1]. Affected individuals fail to respond efficiently to the initial exposure to Epstein Barr virus, and this leads to severe infectious mononucleosis, killing around 50% of the infected individuals [2]. Those who survive EBV infection, as well as XLP who were not infected

Abbreviations: AIM, acute infectious mononucleosis; CD40L, CD40 ligand; CXCR5, CXC-chemokine receptor 5; EBV, Epstein Barr virus; FCS, fetal calf serum; FH, Ficoll-Hypaque; FITC, Fluorescein isothiocyanate; GC, germinal center; ICOS, inducible; T-cell, costimulator; IL-2, Interleukin 2; IL-4, Interleukin 4; IL-10, Interleukin 10; IL-21, Interleukin 21; N, Normal controls; PBMC, Peripheral blood mononuclear cells; PE, phycoerythrin; PerCP, perdidin chlorophyll protein; PD-1, Programmed-death 1; PHA, Phytohemagglutinin; SLAM, Signalling lymphocytic activation molecule; SAP, SLAM-associated adaptor protein; T_{FH}, follicular helper T lymphocytes; Th2, T helper 2; VCA, EBV viral capside antigen; XLP, X-linked lymphoproliferative disease patients.

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with EBV, may develop hypogammaglobulinemia or dysgammaglobulinemia. The frequency of lymphoproliferative disease, mainly non-Hodgkin's B cell lymphoma, is higher in XLP patients than in non-*SH2D1A* deficient individuals. Reduced numbers of B lymphocytes with a memory phenotype and failure to integrate an adequate memory antibody response have been demonstrated in these individuals [3, 4]. It is thought that inability of follicular helper T lymphocytes (T_{FH}), to cooperate with B lymphocytes generating the formation of germinal center (GC) reaction in the follicular lymph nodes underlies the B-lymphocyte memory defect [5]. This CD4+ T cell subset provides cytokines that are important for the generation of a correct GC reaction leading to antibody synthesis, such as IL-21, IL-10 and IL-4 [6]. In *SH2D1A*-deficient mice, rather than intrinsic B cell defects or failure in the production of cytokines, the inability of T_{FH} to form stable B–T conjugates could underlie the GC reaction defect [7]. Most studies on T_{FH} function are centered on their role at the site of generation of the GC reaction [8]. This can be assessed directly in the mouse model [7, 8], but the study of the role of T_{FH} in human XLP is not simple. Tonsillar human T_{FH} cells are characterized by high expression of the CXC-chemokine receptor 5 (CXCR5) and the inducible T-cell costimulator (ICOS) [9]. This cell population differs from other tonsillar and peripheral blood T cell subsets in stimulatory activity, proliferative capacity, susceptibility to apoptosis and gene expression [10]. No such studies are available in XLP patients. However, it has proposed that some of the CXCR5+ human CD4 T lymphocytes present in peripheral blood, may represent the relevant T_{FH} cells that are involved in the GC reaction [11, 12]. The simultaneous expressions of PD-1 [11] on CXCR5+ CD4 T cells, as well as the ability to express ICOS [10] and to produce IL-10 and IL-21 [13, 14] are helpful as markers of these follicular T cells. The validity of CD57 [15, 16], as a surrogate marker of T_{FH} cells in peripheral blood, is not widely accepted, since its expression co-segregates with that of CXCR5 but not with T_{FH} functional activity [10] and high CD57 expression is associated to activation or senescence [17]. Previously, it was reported that both the expression of ICOS on PHA-stimulated XLP PBMC and the synthesis of interleukin-10 (IL-10) in XLP CD4+ T lymphocytes that were cultured under Th2-stimulation conditions, were impaired [4]. This was in agreement with previous results indicating that Th2 CD4+ T cells were reduced in XLP [3, 4]. However, neither the synthesis of IL-10 nor the expression of ICOS, were specifically searched in the subpopulation of CD4+ T cells with a T_{FH} phenotype (CXCR5+). In *SH2D1A*-deficient mice, the study of T_{FH} led to contradictory conclusions, as some groups noted normal T_{FH} development [7, 18] migrating into follicular areas of the lymph nodes and upregulating the expression of activation markers that were consistent with normal priming of the T_{FH} cell phenotype [19], and others observed impaired development [20, 21]. Recent work by Deenick et al. [22, 23], has shown that antigen-loaded presenting cells can overcome the deficiency of B cells that were unable to form stable B–T contacts with Sap−/− T lymphocytes, restoring T_{FH} formation. In human XLP, it is accepted that T cells fail to provide help to cognate B lymphocytes [4]. However, it has been shown that CXCR5+ T lymphocytes are present in XLP patients [22-Figure S3E].

We will now show that in XLP patients, within the CD4+ T cell population, the percentage of T_{FH} cells circulating in the blood was similar to that of normal controls (N). ICOS induced by a non specific T cell stimulus (PHA), was initially lower than that of controls (N) in XLP patients. However, after 5 days of culture it reached high levels that were maintained for 2 weeks. The percentages of CD4+ T cells expressing CD40L after PHA stimulation were similar in XLP and N at 2 days of culture. Again, after 5–8 days, while the expression of surface CD40L was reduced in N, it persisted or increased in both XLP patients. Likewise, the production of intracellular cytokines involved in the antibody response (IL-10, IL-21 and IL-4) followed a different kinetic pattern in XLP than in N, reaching more rapidly levels that could be even higher in XLP than those of N T cells. These results suggest that, in spite of their ability to produce these cytokines, differences in the time profile of the T_{FH} response to T cell stimuli might be associated to failure of XLP CD4+ T cells to collaborate with B cells in the assembly of a correct humoral memory response, failing to deliver the necessary cytokines in a coordinated way in order to raise a normal GC reaction.

2. Materials and methods

2.1. XLP patients #9 and #4

Two surviving hypogammaglobulinemic siblings of an established XLP family were studied. The inactivating mutation identified in this family resulted from the substitution of a G for a C nucleotide at position 383 within *SH2D1A* exon 1 [3]. Patient #4, now 45 years old, developed a tonsilar lymphoma at 4 years of age while patient #9 (32 years old) developed severe acute infectious mononucleosis (AIM) at 26 years of age and was successfully treated with humanized anti-CD20 monoclonal antibody (Rituximab) [24] in combination with acyclovir. Both patients are hypogammaglobulinemic and receive monthly intravenous IgG infusions. EBV infection had been confirmed before [3, 24]. The blood samples used in these investigations were obtained before IgG infusion. Informed consent was obtained. Blood samples from normal control individuals (N, n=20 asymptomatic adults, 25–40 years of age with positive EBV serology (IgG anti-VCA+1:16–1:32), indicative of past EBV infection) were drawn and processed at the same time than XLP samples.

2.2. Peripheral blood mononuclear cells (PBMC) isolation

PBMC were obtained by Ficoll-Hypaque (FH) centrifugation of heparinized blood and resuspended to 1×10^6/ml in RPMI tissue culture medium containing 10% fetal calf serum (GIBCO, Grand Island, USA), streptomycin and penicillin (RPMI-FCS). PBMC cultures were carried out in round bottom 5 ml polystyrene tubes (Falcon) containing 2×10^6 PBMC that were suspended in 2 ml RPMI-FCS [25].

2.3. Cell surface phenotype of PBMC from XLP patients and N controls

Peripheral blood samples from all subjects were collected on Heparin. A three color assay was used. Aliquotes of 100 μl

were incubated with monoclonal antibodies according to the manufacturer's recommendations. Samples were then lysed using FACSLysing solution (Becton Dickinson) during 10 min at room temperature. After centrifuging, cells were washed. Flow-cytometric analysis of surface markers was performed at 488 nm on a FAScan cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon ion laser and analyzed with CellQuest software. Lymphocytes were selected according to size (FSC) and side (SSC) scatter profiles (R1). In each assay, we assessed at least 20000 lymphocytes. FH isolated PBMC isolated peripheral blood mononuclear cells (PBMC) were also processed. Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, perdidin chlorophyll protein (PerCP)-labeled monoclonal antibodies were used according to the manufacturers' instructions. Anti-CD4, CXC-chemokine receptor 5 (CXCR5), inducible T-cell costimulator (ICOS), CD40 ligand (CD40L) and Programmed-death 1 (PD-1) were purchased from BD PharMingen (BD Biosciences, San Jose, CA). The phenotype of viable PBMC from XLP and N donors was analyzed after different periods of non stimulated or PHA-IL-2 stimulated culture (2 to 16 days of culture). Lymphocyte viability was calculated taking into account the proportion of live lymphocytes in the SSC/FSC dot plots (R1). Appropiate isotype controls were used to define the positive populations.

2.4. Intracellular IL-10, IL-21 and IL-4 in XLP and N CD4± T lymphocytes after PHA stimulated culture

PBMC (10^6/ml) were stimulated sub optimally with 2.5 μg/ml phytohemmagglutinin (PHA-P, Sigma, L1668) and 5 UI/ml interleukin-2 (IL-2, Peprotech, AF-200-02) and cultured at 37 °C in the CO_2 incubator for 2 to 16 days. In samples that had to be maintained for more than 5 days in culture, IL-2 and fresh culture medium (RPMI-FCS) was replenished every 5 days. Twelve hours before harvesting the cell pellets, these were suspended in RPMI-FCS (10^6/ml) and 10 μl of 2 μM monensin solution (BD Pharmingen) were added and the cell suspensions were incubated at 37 °C for 12 h. After washing, the cells were stained with CD4, CXCR5 monoclonal antibodies for 20 min at room temperature, and then fixed and permeabilized with the Fix and Perm cell permeabilization reagents (Caltag Laboratories, Burlingame, CA). Cells were finally stained with anti-IL-10, anti IL-21 or IL-4 antibodies (BD Pharmingen) and the appropriate isotype controls for 45 min at 4 °C, washed again and the percentage of IL-10+, IL-21+ or IL-4+ lymphocytes in the CD4 gated region (IL-10+, IL-21+ orIL-4+/CD4) was recorded.

Considering the percentage of total intracellular IL-10, IL-21 or IL-4 positive CD4+ T cells as 100% of the CD4+ intracellular cytokine producers, the relative contribution of the CXCR5+ cell subpopulation to overall cytokine production was calculated.

2.5. Statistical analysis

All statistics were performed using Prism software (version 4.0; GraphPad Software). The unpaired *t* test or the Mann–Whitney test were used for statistical comparisons between data sets from XLP patients and N. P values of less than 0.05 were considered significant.

3. Results

3.1. Expression of T_{FH} markers in peripheral blood CD4±XLP T lymphocytes of XLP and N controls

We had previously shown that the CD4+ T cells count was below the normal limits, while the CD8+ T cell numbers were increased in XLP #4 and #9 [26]. In order to determine if within the CD4 T lymphocyte compartment, cells with a T_{FH}-compatible phenotype were also reduced in XLP, we analyzed the expression of CXCR5 and PD-1 in peripheral blood T lymphocytes. Confirming our previous results, both the percentage of peripheral blood CD4+ T lymphocytes and their absolute numbers were lower in XLP than in N (Figs. 1A and B). However, the percentage of CXCR5+ in the CD4+ T cell population was not reduced in XLP. Moreover, in XLP #4, this value was higher than in N and in XLP #9 (Fig. 1C). Because the absolute numbers of CD4+ T lymphocytes were lower in XLP than in N, the CD4+, CXCR5+ lymphocyte counts were lower than N, in particular in XLP #9 (Fig. 1D). The percentage of PD-1 expressing CD4+ T cells was similar in the two XLP patients and higher than that of N (Fig. 1E) but the absolute number of PD-1+ CD4+ T cells was higher in #9 than in #4 and N (Fig. 1F). As it had been previously shown that peripheral blood CD4+, CXCR5+ T cells co-expressing PD-1 reflect the T_{FH} cells present at the lymph node follicles [11], we analyzed this subset in XLP #4 and #9. The results shown in Figs. 1G and H, demonstrate that there was no reduction in the percentage or absolute number of these cells in XLP patients. When comparing the two XLP patients, there were significantly higher CD4+, CXCR5+, PD-1+ lymphocytes in XLP #4 than in #9. Examples of the flow cytometry analysis of these markers are shown in Fig. 2.

3.2. ICOS and CD40L expression after activation in stimulated PBMC culture of CD4± T lymphocytes from XLP and N

We then analyzed the ability of CD4+ XLP T cells to respond to stimuli that increase the expression of molecules important in T–B cell cooperation, such as CD40L and ICOS. After several days of PBMC stimulated culture with suboptimal concentrations of PHA and IL-2, N PBMC responded with maximum expression of both CD40L and ICOS at 48 hs, and expression decreased thereafter. In contrast, CD40L expression after stimulation increased and was maintained in XLP patients after more than 7 days of culture (Fig. 3). As for ICOS, it was initially lower in XLP than in N at 48 h. However, its expression increased in CD4+ T cells of XLP patients up to 9 days of culture, while it decreased in N (Fig. 4). These results indicate that XLP CD4+ T lymphocytes are able to respond to non specific T cell stimuli by increasing the expression of CD40 L and ICOS, although with a different kinetic profile than N CD4+ T cells. Analysis of the co-expression of PD-1 and CXCR5 in CD4+ T lymphocytes after stimulated culture, revealed that this population was enriched in XLP after 12 days when compared to N (Fig. 5).

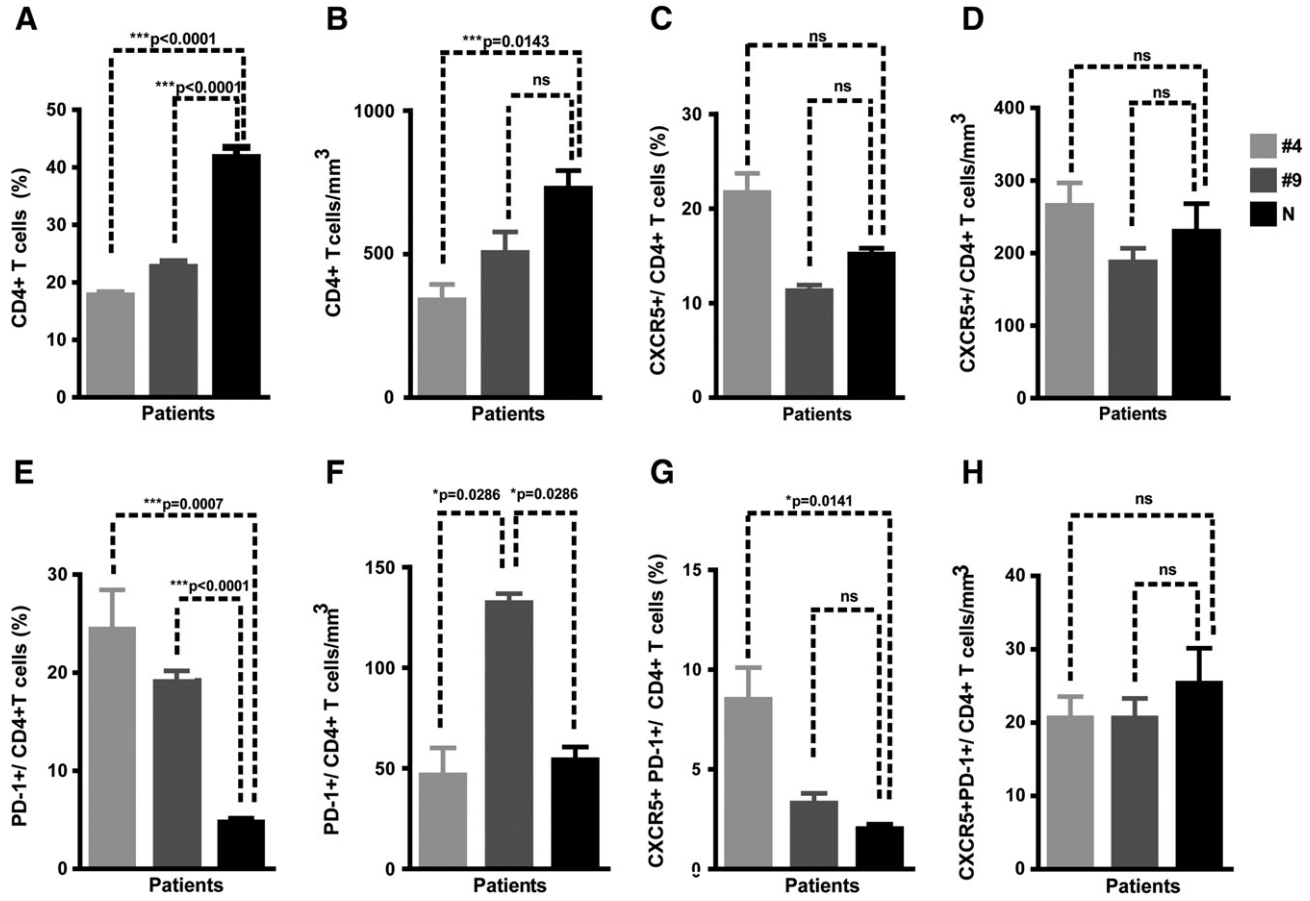

Figure 1 T_{FH} phenotype of CD4+ T cells in patients with XLP. Peripheral blood samples from XLP patients (#4 and #9) and N controls were analyzed by flow cytometry using anti CD4 (PerCP), CXCR5 (FITC) and PD-1 antibodies (PE), as described in Materials and methods. A: percentage values in the viable lymphocyte region. Mean ± Standard Error of 6 different experiments are given. Statistical differences, *t* test: #4 versus N, ***p<0.0001; #9 versus N, ***p<0.0001; C, E and G: percentage values (%) in the CD4 region. Mean ± Standard Error of 6 different experiments are given; C: lymphocytes co-expressing CXCR5 and CD4; Statistical differences, *t* test: #4 versus N, ns; #9 versus N, ns. E: lymphocytes co-expressing PD-1 and CD4; Statistical differences, *t* test: #4 versus N, ***p=0.0007; #9 versus N, ***p<0.0001; G: lymphocytes co-expressing CXCR5, PD-1 in the CD4 region; Statistical differences, *t* test: #4 versus N, *p=0.0141; #9 versus N, ns. B: lymphocyte count (cells/mm^3) in the CD4 region. Mean ± Standard Error of 6 different experiments are given; Statistical differences, *t* test: #4 versus N, ***p=0.0143; #9 versus N, ns. D: lymphocyte count (cells/mm^3) co-expressing CXCR5 and CD4; Statistical differences, *t* test: #4 versus N, ns; #9 versus N, ns. F: lymphocyte count (cells/mm^3) co-expressing PD-1 and CD4; Statistical differences, *t* test: #4 versus N, *p=0.0286; #9 versus N, *p=0.0286. H: lymphocyte count (cells/mm^3) co-expressing CXCR5, PD-1 in the CD4+ region; Statistical differences, *t* test: #4 versus N, ns; #9 versus N, ns.

3.3. Intracellular IL-10, IL-21 and IL-4 in XLP and N CD4± T lymphocytes after PHA stimulated culture

Regarding the ability of CD4+ T lymphocytes to respond by increasing the synthesis of IL-10, IL-21 and IL-4 that are central to the generation of an adequate humoral response, we analyzed the induction of these cytokines in CD4 T cells from N and XLP patients after PHA-IL-2 stimulated culture. The results of Fig. 6 demonstrate that XLP CD4+ T cells are able to respond to the T cell stimulus by increasing the intracellular expression of IL-10, IL-21 and IL-4, at levels that were even higher than those achieved by N CD4+ T cells. We then examined the contribution of cells with a T_{FH}-associated lymphocyte phenotype to IL-10, IL-21 and IL-4 production after stimulus. The results of Table 1 indicate that most IL-10+, IL-21+ or IL-4+ CD4+ T cells after PHA-IL-2 stimulation, were restricted to the CXCR5+, CD4+ subpopulation both in N and in XLP, suggesting that CD4 T cells involved in IL-10, IL-21 and IL-4 generation were mainly T_{FH} and that these cells were not intrinsically unable to respond to stimulation synthesizing these cytokines.

4. Discussion

It has been proposed that SAP-deficient T cells are unable to interact efficiently with B cells at the lymph node follicles leading to impaired formation and maintenance of the GC reaction. An adequate GC reaction is necessary to mount long lasting and mature antibody responses [12]. Since T_{FH} are main actors in this T–B interaction, immunological failure at this level could be the consequence of T_{FH} defects [20]. In ICOS-deficient patients the number of circulating cells with a T_{FH} phenotype were significantly reduced [16],

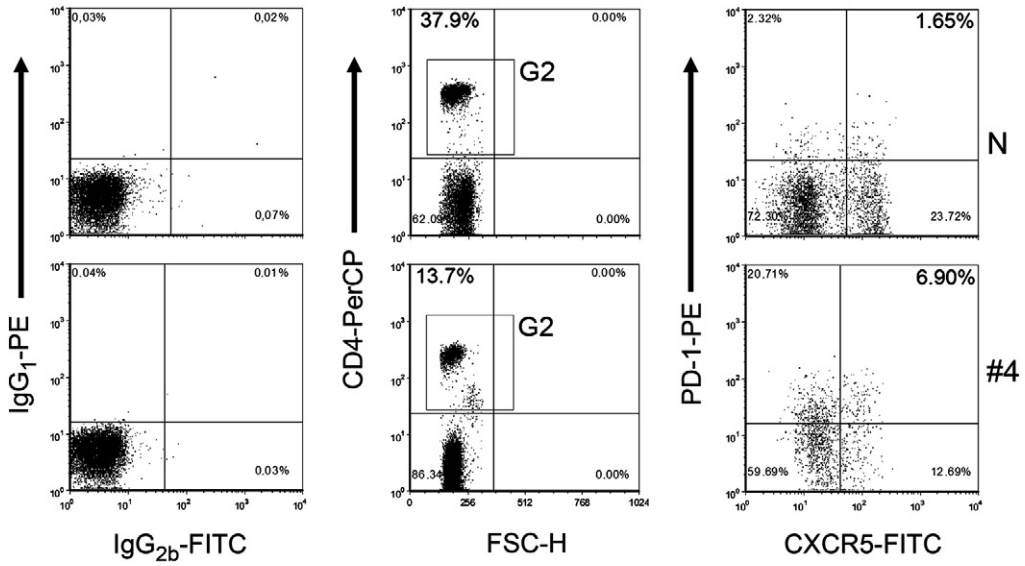

Figure 2 Flow cytometry analysis of T_{FH} markers in peripheral blood of N and XLP (#4). An example of a cytometry analysis of T_{FH} markers CXCR5 and PD-1, performed in peripheral blood as described in Materials and methods is shown for N and XLP #4 is shown. A viable lymphocyte region (Gate 1, not shown) was established on the basis of SSC and FSC. Isotype controls were done (left panel) and a CD4 lymphocyte gate (Gate 2) was then established for N and XLP #4 (center panel). The percentage of cells staining with anti PD-1, anti CXCR5 or both in the CD4 gate was determined (right panel).

indicating that ICOS played a central role in the differentiation of CXCR5+ memory CD4 T cells. Likewise, in *ICOS*−/− mice the decrease of CXCR5+ CD4 T cells reflected the lack of T_{FH} cells in lymphoid organs [16]. However, in contrast to *ICOS*−/− mice, in *SH2D1A*-deficient mice, T_{FH} were present and could upregulate CD40L after activation [27]. In human XLP, evidence for T_{FH} failure is mostly indirect, since direct evaluation of T_{FH} at the lymph node level is generally not possible. However, the presence of T_{FH}-like CD4+CXCR5+ cells has recently been reported in the blood of XLP patients [22].

In the present study, we evaluated if T_{FH} cells were present and functional in two asymptomatic XLP patients with hypogammaglobulinemia that had been followed for several years. Our results demonstrate that cells with phenotype associated to the T_{FH} subpopulation are present and functional in peripheral blood of XLP patients. Thus, the percentage of CXCR5+ T lymphocytes within the CD4 population was

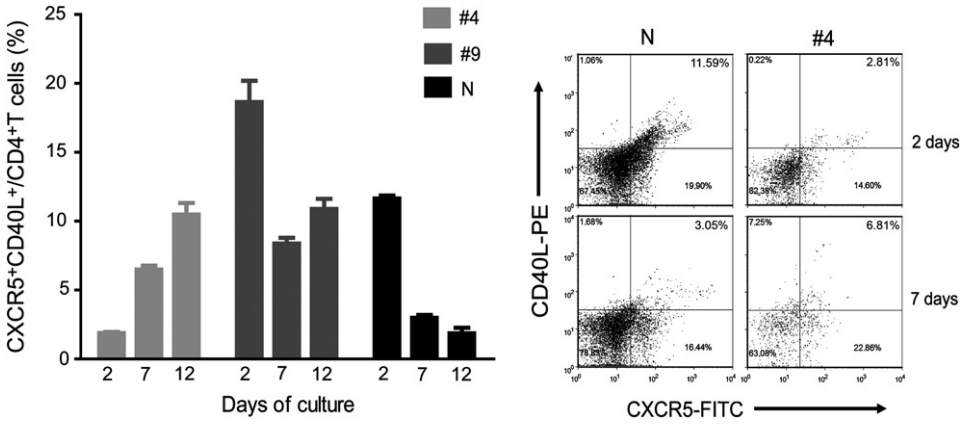

Figure 3 Co-expression of CXCR5 and CD40L in CD4+ T lymphocytes after sub optimal PHA + IL-2 stimulated PBMC culture. Analysis of CXCR5, CD40L co-expression was done as described in Materials and methods. After 2, 7 and 12 days the phenotype of CD4+ T cells was determined and the percentages of co-expression of CXCR5 and CD40L in the CD4+ T cell region are given (Mean ± Standard error of 5–6 different experiments). Statistical differences, *t* test: At day 2 of culture: #4 versus #9, *p = 0.05; #4 versus N, *p = 0.05; #9 versus N, *p = 0.05; at day 12 of culture: #4 versus N, *p = 0.05; #9 versus N, *p = 0.05. Examples of the flow cytometry analysis of these markers from #4 XLP and N controls are shown. A viable lymphocyte region was established on the basis of SSC and FSC and the CD4 lymphocyte gate was then established for N and XLP #4. The percentage of cells staining with anti CD40L, anti CXCR5 or both in the CD4 gate was determined after 2 days of culture (upper panel) and after 7 days of culture (lower panel).

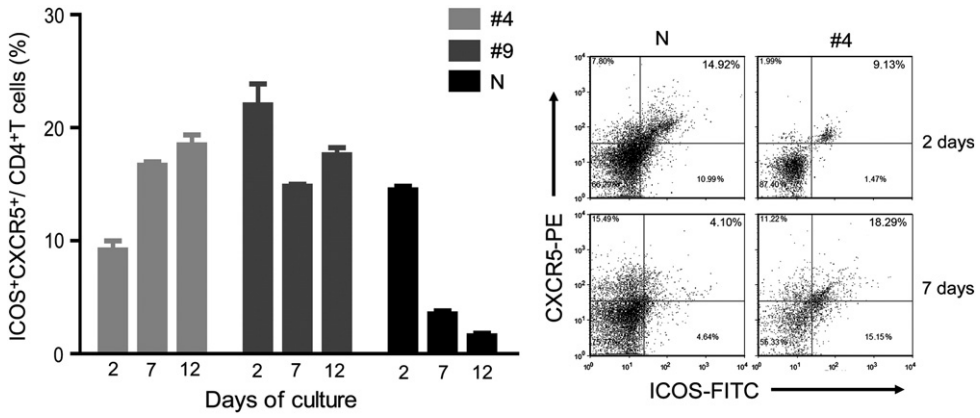

Figure 4 Co-expression of ICOS and CXCR5 in CD4+ T lymphocytes after sub optimal PHA + IL-2 stimulated PBMC culture. Analysis of CXCR5 and CD40L co-expression was done as described in Materials and methods. After 2, 7 and 12 days the phenotype of CD4+ T cells was determined and the percentages of co-expression of CXCR5 and ICOS in the CD4+ T cell region are given (Mean ± Standard error of 5–6 different experiments). Statistical differences, *t* test: At day 2 of culture: #4 versus #9, *p = 0.05; at day 7 of culture: #4 versus N, *p = 0.05; #9 versus N, *p = 0.05; at day 12 of culture: #4 versus N, *p = 0.05; #9 versus N, *p = 0.05. Examples of the flow cytometry analysis of these markers from #4 XLP and N controls are shown. A viable lymphocyte region was established on the basis of SSC and FSC and the CD4 lymphocyte gate was then established for N and XLP #4. The percentage of cells staining with anti ICOS, anti CXCR5 or both in the CD4 gate was determined after 2 days of culture (upper panel) and after 7 days of culture (lower panel).

comparable or higher in XLP than in N. Moreover, both the percentage and absolute number of CD4+ T cells co-expressing CXCR5 and PD-1, which is considered to reflect the T_{FH} subpopulation involved in the GC reaction at the lymph nodes [11], were equal or higher in XLP patients than in N. Interestingly, significant differences in the CXCR5+, PD-1+ and CD4+ values were observed between the two patients with the same *SH2D1A*-mutation and different clinical history. CXCR5+, PD-1+ and CD4+ values were higher in XLP#4 than in XLP#9. Differences independent of the *SH2D1A*-mutation, had been observed previously in the phenotype of memory effector CD8 T cells [26], suggesting that factors associated to the onset of EBV infection or the treatment received by each patient could alter in the long term their individual response.

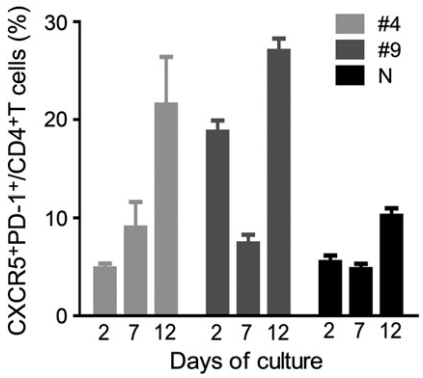

Figure 5 Co-expression of CXCR5 and PD-1 in CD4+ T lymphocytes from XLP and N after sub optimal PHA + IL-2 stimulated PBMC culture. Analysis of CXCR5, and PD-1 co-expression in CD4+ T lymphocytes was done as described in Materials and methods. After 2, 7 and 12 days the phenotype of CD4+ T cells was determined and the percentages of co-expression of CXCR5 and PD-1 in the CD4+ T cell region are given. (Mean ± Standard error, 5–6 experiments). Statistical differences, *t* test: At day 2 of culture: #4 versus #9, *p = 0.05; at day 12 of culture: #4 versus N, *p = 0.05; #9 versus N, *p = 0.05.

The issue of evaluating T_{FH} cells outside the secondary organs complicates human studies. This is important in the evaluation of T_{FH} cells since in humans it is not easy to access the tissue sites (secondary lymphoid organs) were their function is exerted. As for studies of T_{FH} cell function, they are mainly restricted to in vitro assays of their response to stimuli leading to upregulation of molecules involved in T–B cooperation and cytokines necessary for a mature B cell response. In this study, PBMC from XLP patients responded to T lymphocyte stimuli upregulating CD40L and ICOS. However, the time course of the reaction differed from that of N controls. In a previous report, deficient upregulation of ICOS was proposed as a one of the factors involved in the impaired antibody response of XLP patients [4]. As shown in Figs. 3 and 4, while ICOS and CD40L expression decreased in N CD4+ T cells after 48 h, they increased and were maintained after longer periods of culture in XLP patients. These results suggest that lack of downregulation of CD40L and/or ICOS in response to T cell stimuli, are factors to take into account when analyzing immunological failure in XLP. Failure to down regulate CD40L had also been observed in Sap−/− mice [27] and it is known to impair the generation of a memory antibody response in mice [28]. In Sap−/− mouse T cells, more CD40L was present on T cells after 1–2 days of stimulation than in wild type mice, coincidently with failure to mount a memory antibody response. Infused Th2 cytokines could reduce CD40L expression in Sap−/− T lymphocytes, but it did not restore their ability to provide B cell help in vivo [27]. CD40L is down regulated after its interaction with CD40, and

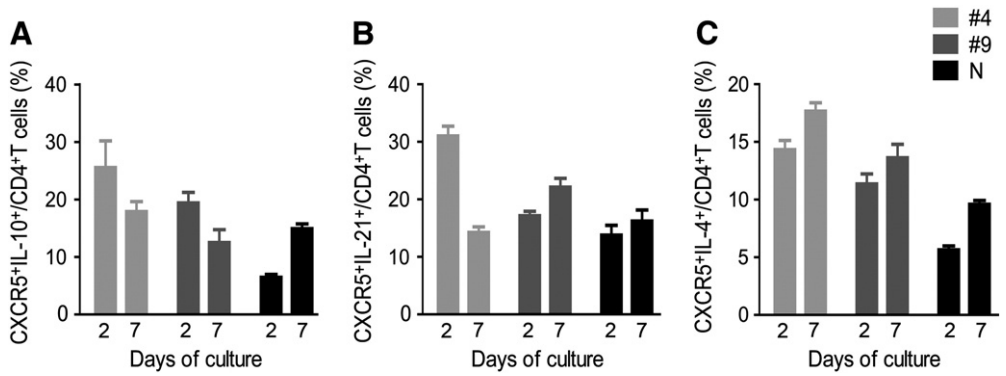

Figure 6 Co-expression of surface CXCR5 and intracellular IL-10, IL-21 and IL-4 after PHA/IL-2 in stimulated PBMC culture in CD4+ T lymphocytes from XLP patients (#4 and #9) and N controls. Analysis of surface CXCR5 and CD4 expression and of intracellular IL-10, IL-21 or IL-4 after 7 and 12 days of culture, was determined as described in Materials and methods. Percentage values referred to the CD4+ region are given (Mean ± Standard error, 5–7 experiments).

its persistence after culture in XLP T_{FH} cells could be related to the reduced number of B lymphocytes in these patients. Concerning induction of ICOS, it has been reported that XLP CD4+ T cells stimulated in vitro under Th2 polarizing conditions had reduced expression of ICOS [4]. This type of stimulation differs from the one used in our experiments which may account for the different results. Taken together, these results suggest that in addition to Th2 defects that may influence T–B interaction [14], other factors could play a major role in the ability of T_{FH} cells to participate efficiently in the assembly of the normal antibody response.

Since T_{FH} cells could be present in XLP, but might not be able to produce cytokines that are necessary for T–B cell cooperation, we assayed the generation of IL-10, IL-4 and IL-21 during PHA-IL-2 stimulated PBMC culture. IL-10 has been shown to participate in the generation of B cell responses in XLP [4], IL-21 is an important mediator of T_{FH} action [12] and the role of T_{FH} IL-4 at the GC site has been previously demonstrated [29 Reinhardt 2009]. In the present study we show that, as N CD4+ T_{FH} lymphocytes, XLP CD4+ T_{FH} cells are able to up regulate intracellular IL-10, IL-4 and IL-21 expression upon stimulation (Fig. 6). Again, the kinetics of the response differed in N and XLP. Maximum XLP responses were observed earlier and were higher than that of N. The fact that T cells from XLP patients respond to stimulation in vitro, does not imply that they will perform correctly in vivo. A relative reduction of cells expressing IL-21 had been reported in lymph nodes of *SH2D1A*−/− mice and while they could cooperate with B cells in vitro, they were unable to do so in vivo [18]. IL-4 production by both N and XLP T_{FH} cells after PHA + IL-2 stimulated PBMC culture (Fig. 6), contrasts with deficient IL-4 production by Th2 polarized CD4 of XLP patients [4] and *Sap*−/− mice [30]. However, in mice, it has been shown that T_{FH} cells constituted the major IL-4 secreting cells in the lymph nodes and were different to IL-4 secreting cells in peripheral tissues [29]. Nevertheless, the time profile of the response to different stimuli may be also an important issue to consider when evaluating the assembly of the immune response in XLP. Most recent studies performed on *SH2D1A*−/− deficient mice highlight the fact that, more than alterations of the cytokine synthesis in these animals, SAP is important for the maintenance and duration of the T–B cell conjugates at the follicles [12]. Our results in human XLP point to the same sense, since rather than absence of T_{FH} cells or their inability to respond to stimulation upregulating CD40L, ICOS, IL-10, IL-4 or IL-21, the absence of SAP, can impair T–B interactions that normally lead to the assembly of the humoral immune response through different mechanisms as suggested before [7, 20]. In addition, alterations in the regulation and time course expression of these proteins in XLP T_{FH}, could also contribute to failure of T–B cell cooperation.

Table 1 CD4+ intracellular IL-10, IL-21 and IL-4 positive cells: CXCR5+, CD4+ cells are the main source of these cytokines after PHA/IL-2 stimulation of PBMC.

Patient	Intracellular Cytokine (%) CD4+ phenotype		
	IL-10+, CXCR5+	IL-21+, CXCR5+	IL-4+, CXCR5+
XLP#4	88.9 ± 6	78.8 ± 9	96.9 ± 6
XLP#9	78.9 ± 12	86.3 ± 11	98.4 ± 5
N	89.2 ± 6	72.5 ± 7	92.3 ± 8

Intracellular IL-10, IL-21 or IL-4 positive cells were identified by flow cytometry as described in Materials and methods. The percentage of total intracellular IL-10, IL-21 or IL-4 positive CD4+ T cells was considered as 100% of CD4+ intracellular cytokine for XLP#4, XLP#9 or N. Then, the relative contribution of the CXCR5+ cell subpopulation was calculated after 7 days of PHA/IL-2 stimulated culture: % intracellular cytokine from cells co-expressing CXCR5 and CD4 in reference to total CD4+ lymphocytes stained for each cytokine. Statistical differences of #4, #9 and N for each cytokine were not significant. Total intracellular CD4+ IL-10+ cells (X ± ES, n = 3) were: XLP#4 = 25.04 ± 9%; XLP#9 = 10.4 ± 4% and N = 4.1 ± 2%. Total intracellular CD4+ IL-21+ cells (X ± ES, n = 3) were: XLP#4 = 15.2 ± 6.5%; XLP#9 = 6.3 ± 3% and N = 5.1 ± 1%. Total intracellular CD4+ IL-4+ cells (X ± ES, n = 3) were: XLP#4 = 14.6 ± 1.78%; XLP#9 = 12.3 ± 0.7% and N = 7.5 ± 3.4%.

Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

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