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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