

# Inherited CD70 deficiency in humans reveals a critical role for the CD70–CD27 pathway in immunity to Epstein–Barr virus infection

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**Epstein–Barr virus (EBV) infection in humans is a major trigger of malignant and nonmalignant B cell proliferations. CD27 is a co-stimulatory molecule of T cells, and inherited CD27 deficiency is characterized by high susceptibility to EBV infection, though the underlying pathological mechanisms have not yet been identified. In this study, we report a patient suffering from recurrent EBV-induced B cell proliferations including Hodgkin's lymphoma because of a deficiency in CD70, the ligand of CD27. We show that EBV-specific T lymphocytes did not expand properly when stimulated with CD70-deficient EBV-infected B cells, whereas expression of CD70 in B cells restored expansion, indicating that CD70 on B cells but not on T cells is required for efficient proliferation of T cells. CD70 was found to be up-regulated on B cells when activated and during EBV infection. The proliferation of T cells triggered by CD70-expressing B cells was dependent on CD27 and CD3 on T cells. Importantly, CD27-deficient T cells failed to proliferate when stimulated with CD70-expressing B cells. Thus, the CD70–CD27 pathway appears to be a crucial component of EBV-specific T cell immunity and more generally for the immune surveillance of B cells and may be a target for immunotherapy of B cell malignancies.**

## INTRODUCTION

EBV is a  $\gamma$ -herpes virus that infects most humans and has a marked tropism for B lymphocytes. Importantly, EBV is known to be one of the strongest triggers of intrinsically uncontrolled B cell proliferation and lymphomagenesis. Rare genetic diseases specifically predispose to defective control of EBV infection, leading to virus-associated hemophagocytic lymphohistiocytosis (HLH) syndrome and lymphoproliferative disorders such as Hodgkin's and non-Hodgkin's lymphomas (Veillette et al., 2013; Cohen, 2015). At present, mutations in *CTPS1*, *SH2D1A*, *MAGT1*, *ITK*, and *CD27* have been

associated with high penetrance of EBV infection with up to 70% of patients having developed diseases and lymphomas related to persistent EBV infection (van Montfrans et al., 2012; Li et al., 2014; Martin et al., 2014; Tangye, 2014; Alkhairy et al., 2015; Bienemann et al., 2015). Studies of these primary immunodeficiencies uncovered crucial pathways involved in T cell response toward EBV-infected B lymphocytes and more generally in T cell functions. In healthy individuals, efficiency of the immune response to EBV is indeed mainly dependent on the massive expansion of specific CD8<sup>+</sup> cytotoxic T cells that eliminate EBV-infected B cells (Callan et al., 2000; Long et al., 2011). In *CTPS1*, *SH2D1A*, and *MAGT1* deficiencies, CD8<sup>+</sup> T cell responses toward EBV-infected B lymphocytes are impaired as the result of defects in either cell-mediated cytotoxicity and/or expansion of

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Abbreviations used: 3D, three-dimensional; APC, allophycocyanin; BV, Brilliant Violet; CRISPR, clustered regularly interspaced short palindrome repeat; EBER, Epstein–Barr-encoded RNA; HLH, hemophagocytic lymphohistiocytosis; LCL, lymphoblastoid cell line; SAP, SLAM-associated protein; sgRNA, single-guide RNA; SLAM, signaling lymphocytic activation molecule; XLP, X-linked lymphoproliferative.

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specific cytotoxic CD8<sup>+</sup> T cells. X-linked lymphoproliferative syndrome 1 (XLP-1), characterized by EBV-induced HLH and occurrence of B lymphomas, is caused by mutations in *SH2D1A* coding the signaling lymphocytic activation molecule (SLAM)-associated protein (SAP). In XLP-1, the CD8<sup>+</sup> T cell-cytotoxicity response toward EBV-infected B cells is specifically compromised and abnormal because of impaired activity of SLAM receptors, which depend on SAP for their function (Snow et al., 2009; Hislop et al., 2010; Palendira et al., 2011). *MAGT1* codes for a transmembrane Mg<sup>2+</sup> transporter involved in TCR signaling and expression of NKG2D, an important cytolytic activating cell receptor expressed by CD8<sup>+</sup> T cells (Li et al., 2011; Chaigne-Delalande et al., 2013). Thus, the NKG2D and SLAM-SAP pathways represent important components of the immune response to EBV. ITK deficiency is caused by mutations in *ITK*, a well-characterized tyrosine kinase of the Bruton tyrosine kinase family involved in T cell activation through its ability to activate PLC- $\gamma$ 1 (Andreotti et al., 2010). In the absence of ITK, PLC- $\gamma$ 1 activation in response to TCR activation is compromised resulting in decreased Ca<sup>2+</sup> mobilization (Liu et al., 1998; Linka et al., 2012). Itk-deficient mice exhibit defective CD8<sup>+</sup> T cell expansion during antiviral responses (Atherly et al., 2006). However, the exact mechanisms underlying the defective immune response to EBV in ITK-deficient patients are not known. A deficiency in CD27 has been recently identified in 17 patients who all developed EBV-driven lymphoproliferative disorders, supportive of a key role of CD27 in immunity against EBV, although its exact mechanism of action has not been delineated so far (Goodwin et al., 1993; Hendriks et al., 2000; van Montfrans et al., 2012; Salzer et al., 2013; Alkhairy et al., 2015). The ligand of CD27, CD70, is expressed on some B lymphocyte and dendritic cell subpopulations and is transiently found on most immune cells when activated (van de Ven and Borst, 2015). CD70 is also present on a variety of cancer cells including neoplasias of B cell origin (Jacobs et al., 2015). CD27 and CD70 are homodimer type I and homotrimer type II membrane proteins, respectively (Goodwin et al., 1993). Both belong to the TNF superfamily. Studies of mouse models established that the CD27-CD70 pathway plays an important role in the generation and maintenance of T cell immunity, in particular during antiviral responses (Hendriks et al., 2000; van Gisbergen et al., 2011; Munitic et al., 2013; Welten et al., 2013).

Herein, we report the identification of the first patient with CD70 deficiency. Like CD27-deficient patients, this patient presented with recurrent EBV-driven lymphoproliferative disorder and had EBV-positive lymphoma. We further show that CD70, when expressed on B cells, plays a key role to trigger the proliferation of T cells, including EBV-specific T cells. Collectively, these results demonstrate that the CD70-CD27 axis is required for an efficient and proper immune response against EBV and more generally for the immune surveillance of B cells by promoting antigen-specific T cell expansion.

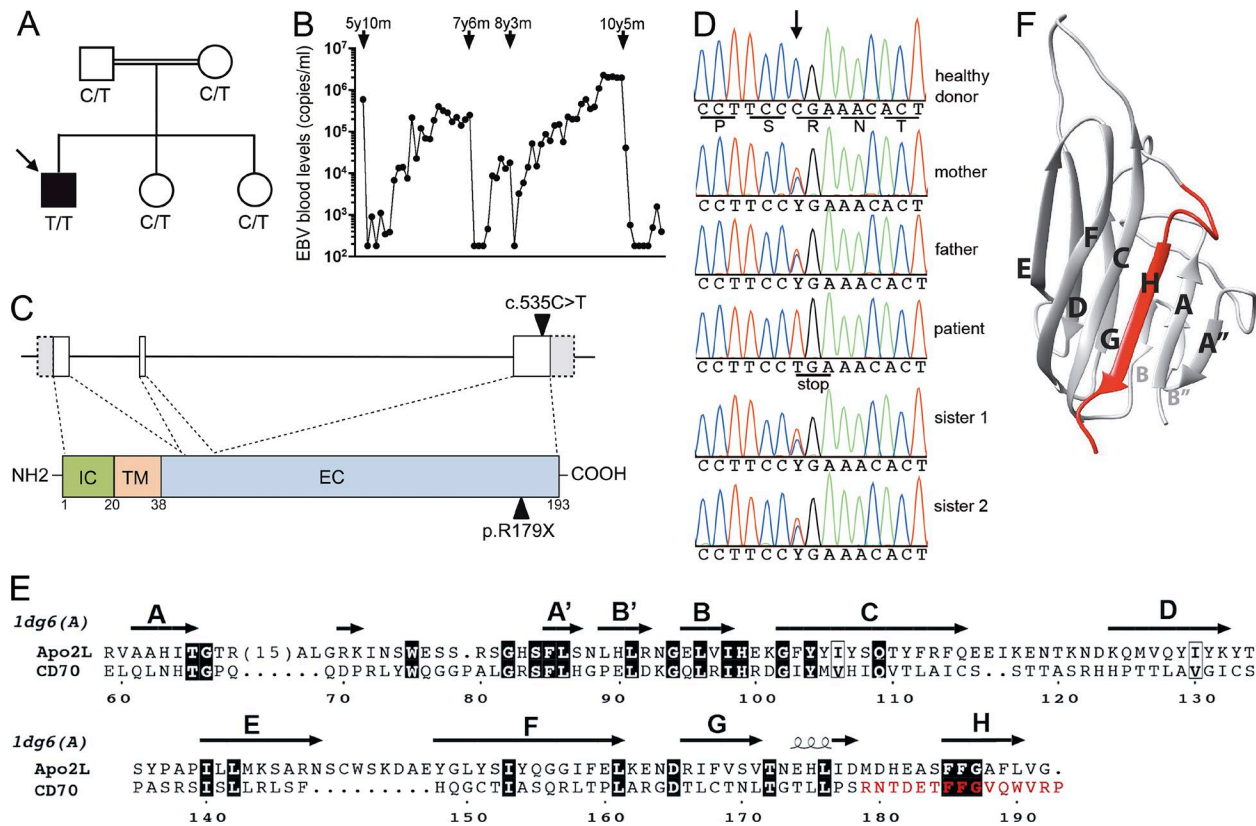
## RESULTS

### Clinical and immunological features

We investigated an 8-yr-old male of Egyptian origin with chronic EBV infection and lymphoma. His parents are first cousins (Fig. 1 A). At the age of 3 yr and 8 mo, he was diagnosed with EBV-positive nodular sclerosing Hodgkin's lymphoma. He received chemotherapy and radiotherapy and achieved a complete response with no relapse since then. Since the age of 4.5 yr, he had recurrent fever, lymphadenopathies, and hepatosplenomegaly, which were associated with a high load of EBV in blood and polyclonal expansion of B cells in lymph nodes and spleen (Fig. 1 B). His symptoms were relieved after the initiation of anti-CD20 therapy, leading to a decrease in EBV viral load, but reappeared requiring repeated anti-CD20 infusions. No other viral and bacterial infections were noted. Finally, he recently received allogeneic hematopoietic stem cell transplantation. Immunological investigations performed at the age of 5 yr before anti-CD20 therapy revealed significant but minor abnormalities including decreased counts of naive T, NK, invariant NKT, mucosal-associated invariant T, and memory B cells and reduced serum IgM and IgA levels (Table 1). T cell proliferation to CD3, PHA, and antigen stimulations was normal. Based on these observations, it was suspected that the high susceptibility to EBV infection associated with early onset Hodgkin's lymphoma was the consequence of an inborn error leading to immunodeficiency in the patient.

### Identification of homozygous nonsense mutation in CD70

To identify the genetic basis of the immunodeficiency in the patient, we first tested known autosomal genetic defects causing susceptibility to EBV infection. No mutation in *ITK*, *CD27*, *MAGT1*, and *CTPS1* was detected, excluding their causative role. We next performed whole-exome sequencing. Because of the consanguinity of the parents, we focused our analysis on homozygous genetic variations. Only one homozygous variation was predicted to be deleterious and localized in the *CD70* gene (Fig. 1 C). This variation consisting of a nonsense mutation (p.R179X; c.535C>T) in the third exon was only present as a heterozygous mutation in a public database of exomes and in our institute's database with a frequency of  $1.48 \times 10^{-5}$  (see the Exome sequencing and analysis section of Materials and methods). Sequencing of *CD70* by the Sanger method in the kindred confirmed that the patient was homozygous for the c.535C>T mutation, whereas his parents and two healthy sisters were heterozygous carriers (Fig. 1 D). At the protein level, the p.R179X mutation removes the last 15 C-terminal amino acids of CD70. Three-dimensional (3D) structure modeling of the human CD70 protein using as templates the TNF-like receptor Apo2L/TNF-related apoptosis-inducing ligand (TRAIL) 3D structures (Protein Data Bank accession nos. 1DG6 and 1D0G) showed that the last internal  $\beta$ -strand H (strand  $\beta$ H in red in Fig. 1 F) was lost in the truncated CD70<sup>R179X</sup> protein (Fig. 1, E and F). Of note, in Apo2L, this strand is involved in the contacts formed be-



**Figure 1. Identification of a mutation in *CD70* in a patient suffering from defective immunity to EBV.** (A) Pedigree of the family in which the c.535 C>T mutation in *CD70* was identified. The arrow indicates the proband who was analyzed by whole-exome sequencing. The genotype of each individual is indicated. (B) EBV load in the blood of the patient is shown as the number of EBV copies detected by PCR at different time points (black circles). Arrows correspond to the anti-CD20 treatments received by the patient with the age (y, year; m, month) of patient at the time of the treatment. (C) Schematic representation of intron–exon organization of the *CD70* gene with the coding exons in white, and their correspondence at protein level with the different domains of CD70 are shown, including the intracytoplasmic (IC), transmembrane (TM), and extracellular (EC) domains. The mutation is indicated by black triangles at the gene and protein levels. (D) DNA electropherograms of the family showing the region containing the C>T mutation in *CD70* and the corresponding amino acid translation. The position of the mutation is indicated by an arrow, and the stop codon caused by the C>T mutation is indicated. (E) Alignment of the human CD70 sequence with that of Apo2L, whose 3D structure is known. Observed secondary structures are shown above the sequences. The position of the missing amino acids in the truncated protein is shown in red. (F) Ribbon representation of the 3D structure model of human CD70. The model was built using the experimental 3D structure of Apo2L (Protein Data Bank accession no. 1DG6) based on the alignment shown in E. The position of the missing strand in the truncated protein is shown in red.

tween the different subunits of the homotrimer (Hymowitz et al., 1999). Importantly, CD70 is known to be the ligand for the CD27 molecule. Deficiency in CD27 causes a high susceptibility to EBV infection and the associated lymphoproliferative disorders (van Montfrans et al., 2012; Salzer et al., 2013; Alkhairi et al., 2015). Thus, we considered CD70 as a strong candidate gene underlying the immunodeficiency of the patient.

#### Loss of expression and lack of binding to CD27 of the CD70-mutant protein

The c.535C>T mutation had no impact on the amount of CD70 mRNA detected by quantitative PCR in PHA-stimulated T cells and in EBV-transformed B cells from the patient (further designated as lymphoblastoid cell line [LCL]; not depicted). At the protein level, the anti-CD70 antibody

failed to detect CD70 by flow cytometry at the cell surface of PHA-stimulated T cells of the patient. In contrast, expression of CD70 was detectable on a fraction of PHA-stimulated T cells from healthy donors at day 8 (Fig. 2 A). This proportion of CD70<sup>+</sup> T cells increased in culture, with a large proportion of T cells expressing CD70 at day 15. Similarly, CD70 was not detected on LCLs derived from the patient, in contrast to LCLs from healthy donors that expressed high levels of CD70 on their surface (Fig. 2 B, top). Defective expression of CD70 was confirmed by analyzing the capacity of CD70<sup>R179X</sup> to bind to CD27. A fusion protein containing the extracellular domain of CD27 (Fc-CD27) failed to bind on the surface of PHA-T cell blasts and LCLs from the patient, whereas its binding on control cells was detected (Fig. 2 B, bottom). Similar results were obtained when wild-type CD70

or CD70<sup>R179X</sup> proteins were transiently expressed in HEK-293 T cells (Fig. 2 C). Expression of CD70 and CD70<sup>R179X</sup> in HEK-293 T cells was further examined by Western blotting using N-terminal FLAG-tagged CD70 forms (Fig. 2 D). The mutant CD70<sup>R179X</sup> was weakly expressed compared with wild-type CD70. Collectively, these results indicate that the p.R179X mutation in CD70 compromises its expression and has a deleterious effect on its ability to recognize its cognate ligand CD27. Therefore, we conclude that the CD70 deficiency mimics CD27 deficiency and, thus, likely accounts for the high susceptibility to EBV infection of the patient.

**High levels of CD70 on activated B cells and EBV-infected B cells**

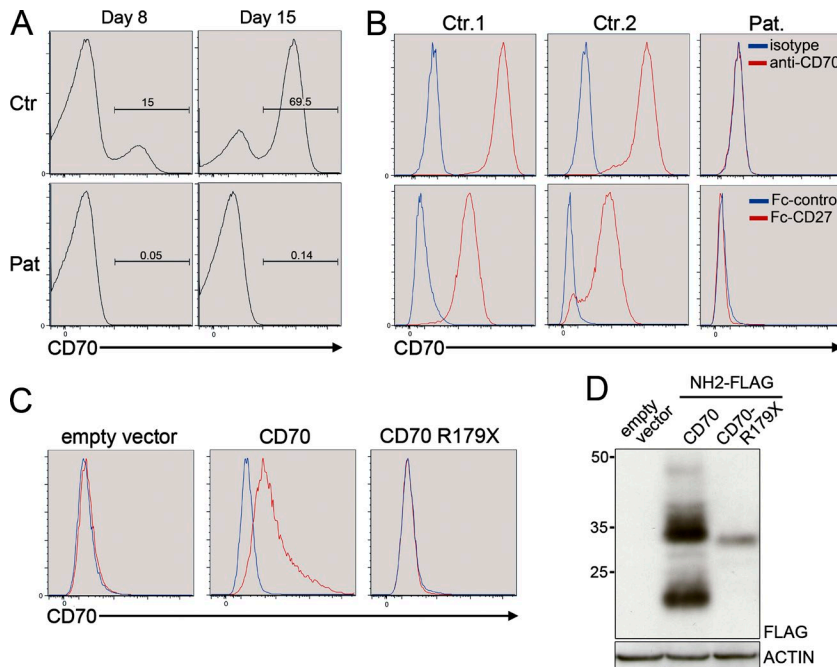
To better understand the role of CD70 in anti-EBV immunity, we first analyzed the expression of CD70 in PBMCs of several healthy donors. In humans, CD70 expression has

been shown to be restricted to some DCs and B cell subsets, whereas CD27 is expressed on most T cells and memory B cells (Nolte et al., 2009; van de Ven and Borst, 2015). Indeed, expression of CD70 was barely detectable on CD4 and CD8 T cells, monocytes, DCs, and NK cells, with the noticeable exception of a small fraction of B cells (Fig. 3, A and B). This contrasts with the high levels of CD27 on the surface of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes and a small fraction of B cells. CD70 expression on B cells was rapidly up-regulated upon activation by a combination of PMA and ionomycin (Fig. 3 C). At day 3 of stimulation, >80% of B cells expressed large amounts of CD70 in contrast to T cells. Similarly to activated B cells, all EBV-transformed B cell lines that we tested expressed high levels of CD70 (not depicted). These data suggest that the expression of CD70 is inducible in B cells in the course of EBV infection. To test this possibility, we analyzed the expression of CD70 in tonsils from individ-

Table 1. Immunological features of PBMCs from the CD70-deficient patient

	Age-matched normal values	5 yr/9 yr
Leukocytes (cells × mm <sup>-3</sup> )	4,400–15,500	7,000/7,200
Neutrophils (cells × mm <sup>-3</sup> )	1,800–8,000	4,200/4,600
Monocytes (cells × mm <sup>-3</sup> )	200–1,000	500/900
Lymphocytes (cells × mm <sup>-3</sup> )	1,900–3,700	2,100/2,300
<b>T cells</b>		
CD3 <sup>+</sup> (cells × mm <sup>-3</sup> )	1,200–2,600	1,617/1,863
CD4 <sup>+</sup> (cells × mm <sup>-3</sup> )	650–1,500	672/805
CD8 <sup>+</sup> (cells × mm <sup>-3</sup> )	370–1,100	714/644
CD4/CD8 ratio	0.9–2.6	1.7/1.3
TCRα/β (%)	26–85	68/65
TCRγ/δ (%)	0.2–14	8/8
CD31 <sup>+</sup> CD45RA <sup>+</sup> /CD4 <sup>+</sup> (recent naive thymic emigrant; %)	43–55	52/56
CD45RO <sup>+</sup> /CD4 <sup>+</sup> (memory; %)	13–30	19/22
CCR7 <sup>+</sup> CD45RA <sup>+</sup> /CD8 <sup>+</sup> (naïve; %)	52–68	<b>32/36</b>
CCR7 <sup>+</sup> CD45RA <sup>-</sup> /CD8 <sup>+</sup> (central memory; %)	2–4	2/2.5
CCR7 <sup>-</sup> CD27 <sup>-</sup> CD45RA <sup>-</sup> /CD8 <sup>+</sup> (effector memory; %)	11–20	10/11
CCR7 <sup>+</sup> CD27 <sup>-</sup> CD45RA <sup>-</sup> /CD8 <sup>+</sup> (exhausted effector memory; EMRA; %)	1–18	0.2/10
CD127 <sup>low</sup> CD25 <sup>+</sup> /CD4 <sup>+</sup> (regulatory; %)	2–8	3/5
Va7 <sup>+</sup> CD161 <sup>+</sup> /CD3 <sup>+</sup> (MAIT; %)	1–8	<b>0.3/0.4</b>
Va24 <sup>+</sup> Vb11 <sup>+</sup> CD161 <sup>+</sup> /CD3 <sup>+</sup> (iNKT; %)	>0.02	<b>0.006/0.004</b>
<b>T cell proliferation (cpm × 10<sup>-3</sup>)</b>		
PHA (6.25 mg ml <sup>-1</sup> )	>50	58.5/132
OKT3 (50 ng ml <sup>-1</sup> )	>30	90/79.5
<i>Tetanus toxoid</i>	>10	15.5/49
<b>NK cells</b>		
CD16 <sup>+</sup> CD56 <sup>+</sup> (cells × mm <sup>-3</sup> )	100–480	<b>63/92</b>
CD16 <sup>+</sup> CD56 <sup>+</sup> (%)	4–17	<b>3/4</b>
<b>B cells</b>		
CD19 <sup>+</sup> (cells × mm <sup>-3</sup> )	270–860	399/322
CD19 <sup>+</sup> (%)	13–27	22/17
CD21 <sup>+</sup> CD27 <sup>+</sup> /CD19 <sup>+</sup> (memory; %)	>10	<b>3/1</b>
IgD <sup>+</sup> IgM <sup>+</sup> /CD19 <sup>+</sup> CD21 <sup>+</sup> CD27 <sup>+</sup> (marginal zone; %)	31–51	<b>26/NA</b>
IgD <sup>-</sup> IgM <sup>-</sup> /CD19 <sup>+</sup> CD21 <sup>+</sup> CD27 <sup>+</sup> (switched; %)	21–49	38/NA
<b>Immunoglobulin levels (g × L<sup>-1</sup>; 5 yr old)</b>		
IgG (1/2/3)	4/0.4/0.17	5/0.45/0.81
IgM	0.5–1.1	0.51
IgA	0.7–1.3	<b>0.12</b>

Different immunological parameters of the patient were tested from blood and PBMCs: numbers of blood cell populations, different T cell subsets, B cell subsets, and natural killers from PBMCs (tested by flow cytometry), T cell proliferation in response to different stimuli (evaluated by incorporation of [<sup>3</sup>H]thymidine), and serum (immunoglobulin subclasses). Values in bold correspond to values below the age-matched normal values. MAIT, mucosal-associated invariant T cell.



**Figure 2. The R179X mutation in CD70 prevents binding to CD27.** (A) FACS histograms showing CD70 expression detected with anti-CD70 antibody on PHA-stimulated T cells from the patient (Pat.) or a healthy control (Ctr.) at days 8 and 15 of culture. Numbers inside the histograms correspond to the percentage of positive cells. One representative of three independent experiments is shown. (B) FACS histograms showing CD70 expression on LCLs from the patient or two healthy control individuals (Ctr.1 and Ctr.2) detected with anti-CD70 antibody (top) or revealed by the ability of CD70 to bind CD27-Fc fusion protein (bottom). One representative of three independent experiments is shown. (C) Histograms showing the binding of a CD27-Fc fusion protein on HEK 293 T cells transfected with an empty expression vector (empty vector) or a vector containing cDNAs coding of either NH2-FLAG-tagged CD70 (CD70) or NH2-FLAG-tagged R179X CD70 (CD70 R179X). One representative of two independent experiments is shown. (D) Same as C, except that CD70 expression was analyzed by Western blotting in cell lysates using anti-FLAG antibody. Molecular weights in kilodaltons are shown on the left. One representative of two independent experiments is shown.

uals with infectious mononucleosis. Although expression of CD70 staining was not detectable in tonsils of healthy donors, CD70 was found on large cells that were positive for Epstein-Barr-encoded RNA (EBER) and LMP-1 in three individuals with infectious mononucleosis (Fig. 3, D and E; and not depicted). These cells correspond to EBV-infected B cells as they were stained with anti-PAX5 antibody, a specific marker for B cells (Fig. 3 E). In contrast, CD27 expression was not associated with PAX5 and EBER staining and accumulated in cells located in the T cell areas (Fig. 3 E and not depicted). Collectively, these data indicate that expression of CD70 is up-regulated in activated and in EBV-infected B cells, whereas most T cells express high amounts of CD27 at resting state. Therefore, CD27-CD70 interactions can occur between CD27-expressing T cells and CD70-expressing B cells during the course of an EBV infection and may play an important role in the control of EBV-activated B cells by T cells.

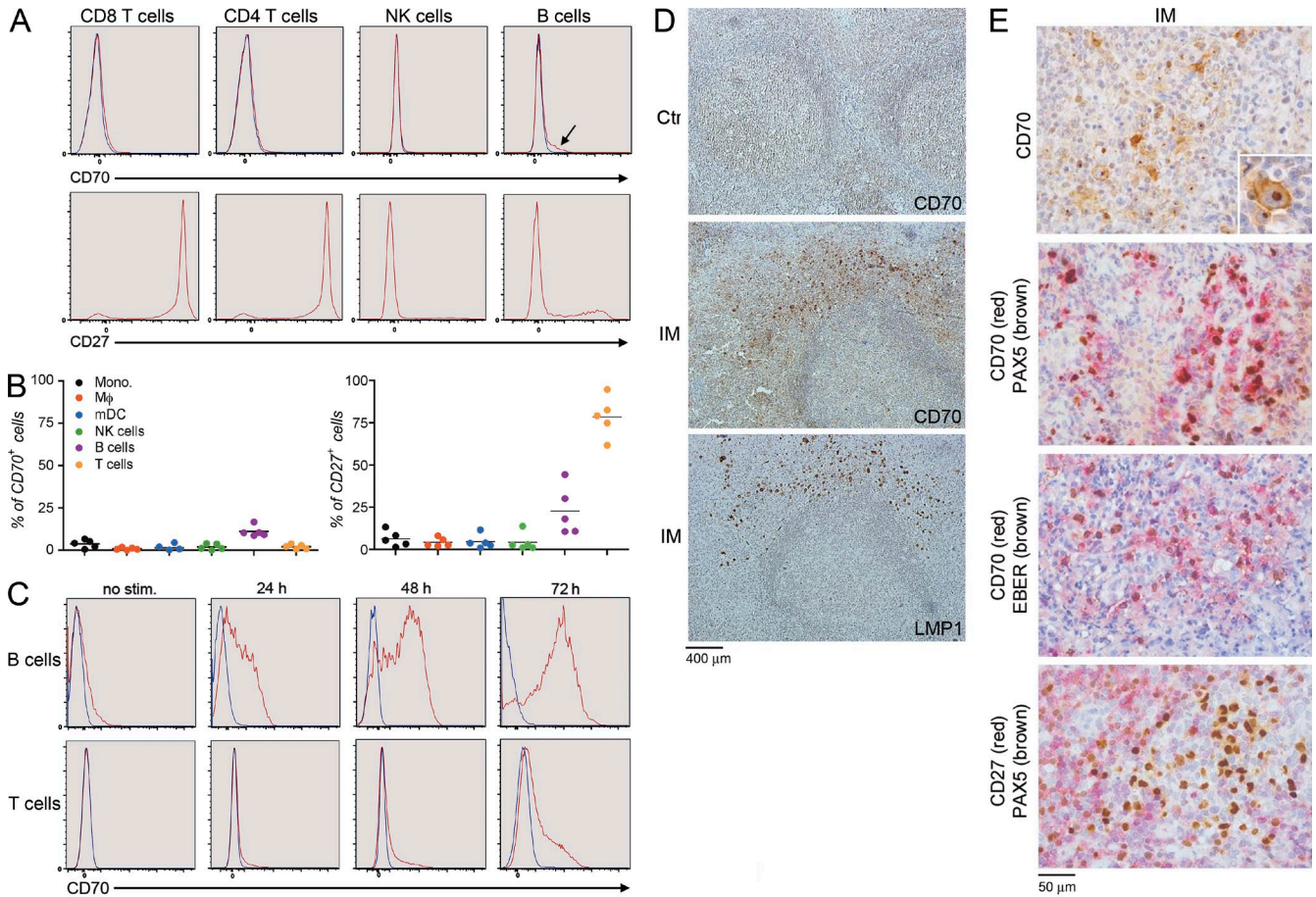
#### Decreased cytolytic response of patient T cells against autologous EBV-transformed B cells

In mice, CD70 and CD27 play an important role in antigen-specific T cell responses, in particular during antiviral responses (Arens et al., 2001; Munitic et al., 2013). Hence, T cell responses against EBV might be impaired in the absence of CD70. To test this hypothesis, we analyzed T cell responses against EBV from PBMCs of the patient or healthy controls that were co-cultured with irradiated autologous LCLs. Proportions of CD4<sup>+</sup> and CD8<sup>+</sup> as well as expression of activation markers were comparable when analyzed after 4–5 wk of co-culture. After 4 wk of co-culture, cell-cytotoxicity response and IFN- $\gamma$  production by T cells was assessed

against autologous EBV-transformed B cell lines (Fig. 4 A and not depicted). Compared with control T cell cultures, T cells of the patient exhibited a markedly decreased cytotoxic activity and IFN- $\gamma$  production, indicative of impaired T cell responses to EBV. To formally prove the link between CD70 deficiency on EBV-transformed B cells and defective T cell responses against EBV-transformed B cells, we performed reconstitution experiments by transducing LCLs of the patient with a lentiviral vector containing a cDNA coding wild-type CD70 (or an empty vector), which induced surface expression of CD70 to levels comparable with those seen on LCLs from control donors (Fig. 4 B). In these conditions, the cytolytic response of patient's T cells toward autologous CD70-expressing LCLs was restored to a magnitude similar to that of control T cells (Fig. 4 C). Collectively, these results demonstrate that the lack of CD70 on patient's B cells results in impaired T cell cytolytic responses toward EBV-infected B cells. However, the intrinsic cytotoxicity function of patient's T cells was preserved, whereas ectopic CD70 expression on target cells did not increase killing by T cells (not depicted).

#### Impaired proliferation of patient EBV-specific T cells

We next tested the possibility that impaired T cell cytotoxicity and cytokine production toward autologous CD70-deficient LCLs in the patient was the consequence of a defect in EBV-specific T cell expansion. HLA genotyping of the patient showed that he was a carrier of HLA-A\*01, for which no tetramer reagents were available to directly assess EBV-specific T cells. However, EBV-induced proliferating T cells were detectable by CellTrace violet dye dilution and expression of the activation marker CD25 among PBMCs

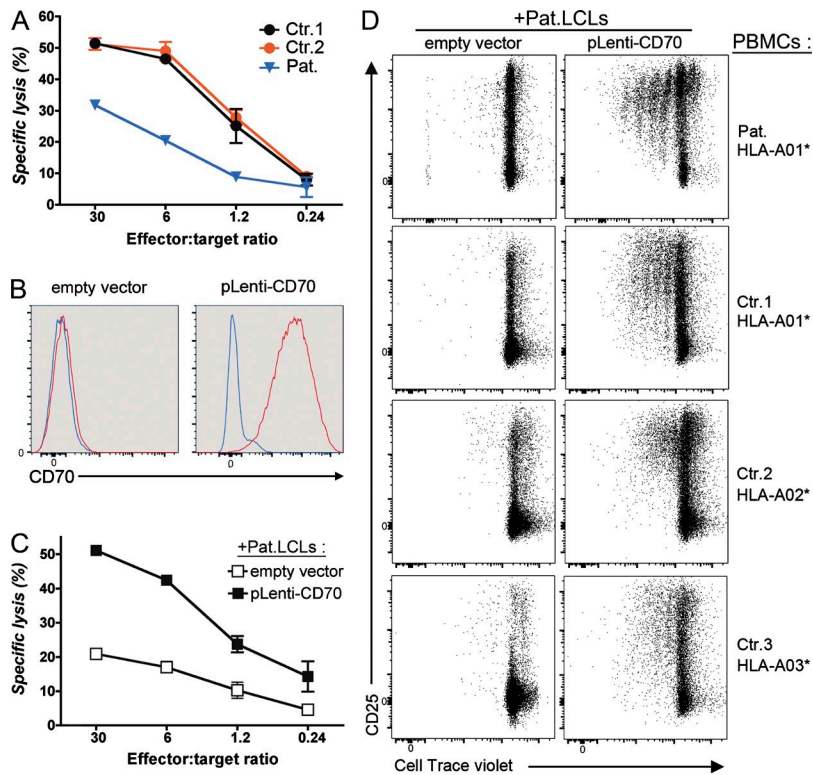


**Figure 3. CD70 expression is up-regulated in activated B lymphocytes and EBV-infected B cells.** (A) FACS histograms showing the expression of CD70 (top) or CD27 (bottom) on the different lymphoid subpopulations from PBMCs of one healthy donor. (Top) The control isotype is shown by the blue line, whereas the red line corresponds to anti-CD70 or -CD27 staining. The arrow indicates the population of B cells expressing CD70. One representative of three independent experiments is shown. (B) Percentage of CD70- or CD27-expressing cell populations among PBMCs of five healthy donors from FACS analysis stained with markers for macrophages (Mφ), monocytes (Mono), dendritic cells (mDC), T cells, B cells, and NK cells. Data from two independent experiments are shown. *n* = 5. (C) FACS histograms showing the expression of CD70 on B cells and T cells from PBMCs of a healthy individual that have been activated for different periods of time with PMA + ionomycin. The blue line corresponds to the isotype, whereas the red line corresponds to the anti-CD70 antibody. One representative of three independent experiments is shown. stim., stimulation. (D) Analysis of CD70 expression on tonsils of one healthy control (Ctr) and one individual with infectious mononucleosis (IM). Immunostaining for CD70 and LMP-1 expression is shown. (E) Immunostaining of tonsils from one individual with infectious mononucleosis for CD70, PAX5 (B cell marker), EBV probe, and CD27 expression. Large cells are CD70 positive (inset) with a membrane and an intracytoplasmic (dot) staining (top). CD70-positive cells are PAX5<sup>+</sup> and EBV<sup>+</sup> (EBER; middle), and CD27 is not expressed on the PAX5<sup>+</sup> cells (bottom). One representative of two experiments with two different individuals with IM tested.

of the patient, when co-cultured with autologous LCLs, in which CD70 expression was restored, but not with CD70-deficient parental LCLs (Fig. 4 D, top). Proliferating T cells from an HLA-A\*01-expressing healthy donor were also observed when co-cultured with HLA-A\*01 CD70-expressing LCLs of the patient but not with HLA-A\*01 CD70-deficient LCLs (top middle). In contrast, in nonautologous conditions, less proliferating T cells were detected among PBMCs from healthy donor carriers of HLA-A\*02 and HLA-A\*03 alleles (bottom middle and bottom).

**Defective T cell expansion of EBV-specific T cells in the absence of CD70 on B cells**

Next, to prove the role of CD70 in anti-EBV immunity, CD70-deficient LCLs were derived from PBMCs of a healthy HLA-A\*02 individual by gene inactivation. Most HLA-A\*02 individuals develop EBV-specific T cells against the GLCTLV AMV peptide (also termed GLC epitope), which is derived from the EBV-lytic cycle protein BMLF1 (Sauce et al., 2009). CD70-deficient LCLs were obtained by blunting CD70 expression using CRISPR (clustered regularly interspaced short



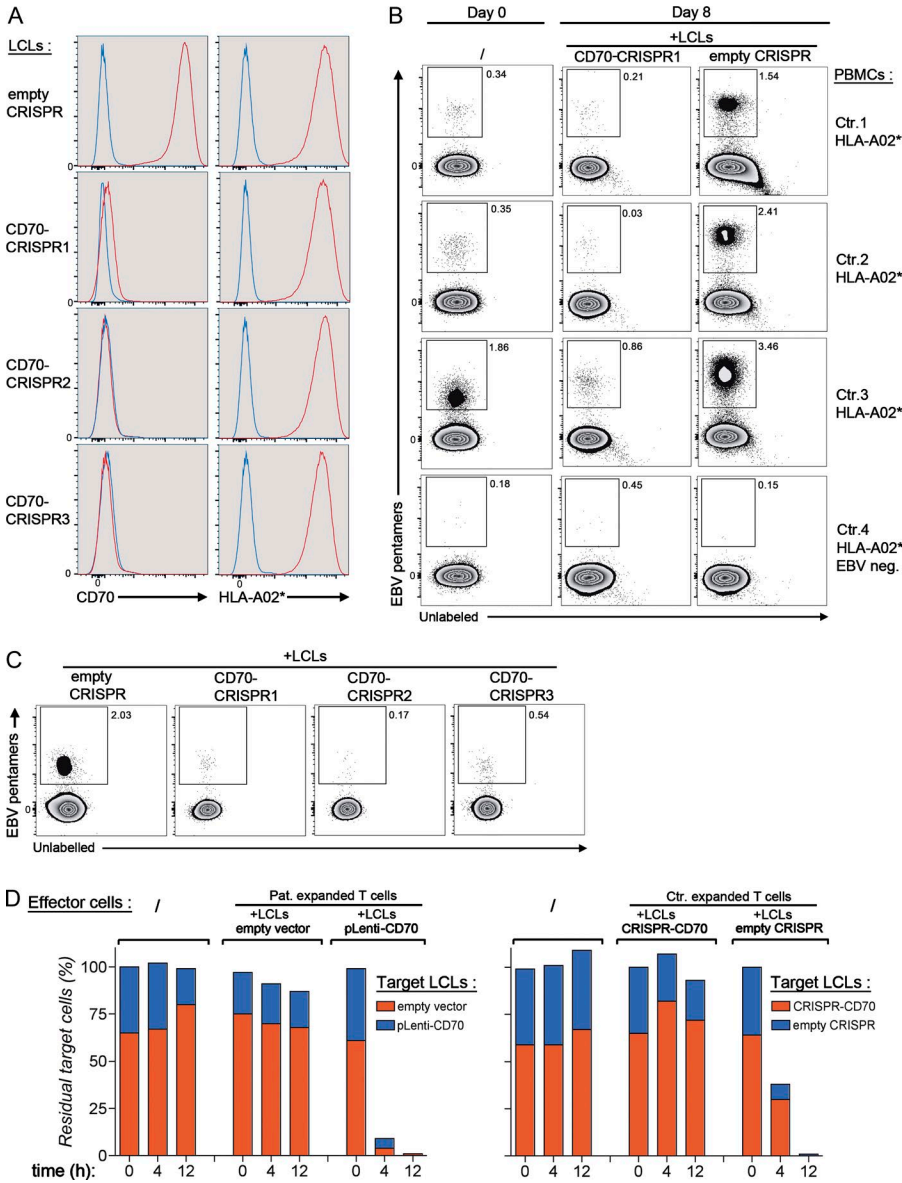
**Figure 4. Decreased cytolytic and proliferative responses to autologous CD70-deficient LCLs of the patient are restored by expression of CD70 in LCLs.** (A) Cytotoxic response of T cells from two control individuals (Ctr.1 and Ctr.2) and the CD70-deficient patient (Pat.) against autologous LCLs as measured by Cr<sup>51</sup> release at the indicated effector-to-target ratios. T cells have been co-cultured with the autologous LCLs for 4 wk before the test. One representative of two independent experiments and triplicates with SD are shown. (B) FACS histograms of CD70 expression on LCLs of the patient that have been infected with an empty lentiviral vector (empty vector) or a vector containing a cDNA coding wild-type CD70 (pLenti vector). One representative of five independent experiments is shown. (C) Same as A except that T cells from the patient were co-cultured with autologous CD70-deficient LCLs of the patient (Pat.LCLs) in which CD70 expression was restored (pLenti-CD70) or not (empty vector). Duplicates with SD are shown. (D) Proliferation of T cells from PBMCs of the patient and HLA-A matched (Ctr.1 HLA-A01\*) or nonmatched (Ctr.2 HLA-A02\* and Ctr.3 HLA-A03\*) healthy controls that were co-cultured for 8 d in the presence of irradiated CD70-deficient LCLs of the patient in which CD70 expression was restored or not. Representative dot plots of violet dye dilution and CD25 expression gated on CD3<sup>+</sup> cells are shown. Numbers inside the gates correspond to the percentage of dividing cells. One representative of five independent experiments is shown.

palindrome repeat)-associated nuclease Cas9 technology (Fig. 5 A). Three different CRISPR-Cas9 constructs containing RNA guides targeting exon 3 of *CD70* were transiently transfected in the LCLs. After having been selected and sorted to enrich for CD70-negative cells, three stable CD70-deficient (CD70KO) LCLs were obtained (hereinafter referred as CD70KO-CRISPR1, CD70KO-CRISPR2, and CD70KO-CRISPR3). These cell lines expressed comparable amounts of HLA-A\*02 molecules on their surface when compared with those of the wild-type parental LCLs or LCLs transfected with an empty CRISPR-Cas9 construct. Then, we examined the expansion of EBV-specific T cells of the HLA-A\*02 healthy donor in co-culture experiments with autologous irradiated wild-type LCLs (transfected with an empty CRISPR-Cas9 construct) or CD70KO-CRISPR1 LCLs. EBV-specific T cells were detectable at day 0 using a mix of pentamers for GLC, CLG, and FLY epitopes, and after co-culture with wild-type CD70-expressing LCLs (empty CRISPR LCLs), their proportion increased at day 8 of culture by fivefold (Fig. 5 B, left and right). Most of these cells expressed CD25 and were strongly proliferative (not depicted). In striking contrast, culture of T cells with autologous CD70KO-CRISPR1 LCLs did not result in any expansion of EBV-specific T cells (Fig. 5 B, middle). Similar results were obtained with PBMCs from two other healthy HLA-A\*02 donors (Ctr. 2 and Ctr. 3). No such expansion was observed with T cells from an EBV-naïve donor (Ctr. 4; Fig. 5 B). Absence of expansion was also observed when HLA-A\*02

PBMCs were co-cultured with other CD70-deficient LCLs, CD70KO-CRISPR2 and CD70KO-CRISPR3 (Fig. 5 C). Thus, our observations demonstrate that CD70 is a key factor required for the expansion of EBV-specific T cells. Furthermore, control and patient T cells expanded in the presence of autologous CD70-expressing LCLs were able to kill efficiently both CD70-expressing and CD70-deficient LCLs, indicating that CD70 deficiency does not per se preclude cytotoxic activity of T cells (Fig. 5 D). In contrast, T cells that were co-cultured with autologous CD70-deficient LCLs had no killing activity, demonstrating that specific effector T cells require CD70 for expansion.

#### CD70 on B cells provides a co-stimulatory signal required for TCR-mediated proliferation

To further extend our observation and to formally prove that CD70 deficiency results in blunted proliferation and activation in response to TCR stimulation by B-LCLs, we analyzed T cell proliferative responses after stimulation with irradiated LCLs expressing or not expressing CD70 in the presence of anti-CD3 antibody (Fig. 6). In these conditions, anti-CD3 antibody presumably binds to B-LCLs through the Fc $\gamma$  receptor CD32, similarly expressed on CD70-deficient and CD70-expressing LCLs (not depicted). Importantly, in these conditions activation of T cells was not restricted to HLA-A molecules expressed by irradiated LCLs. At day 8, only a low proportion of T cells from the patient or a healthy donor were detectable in the co-culture with CD70-deficient LCLs from



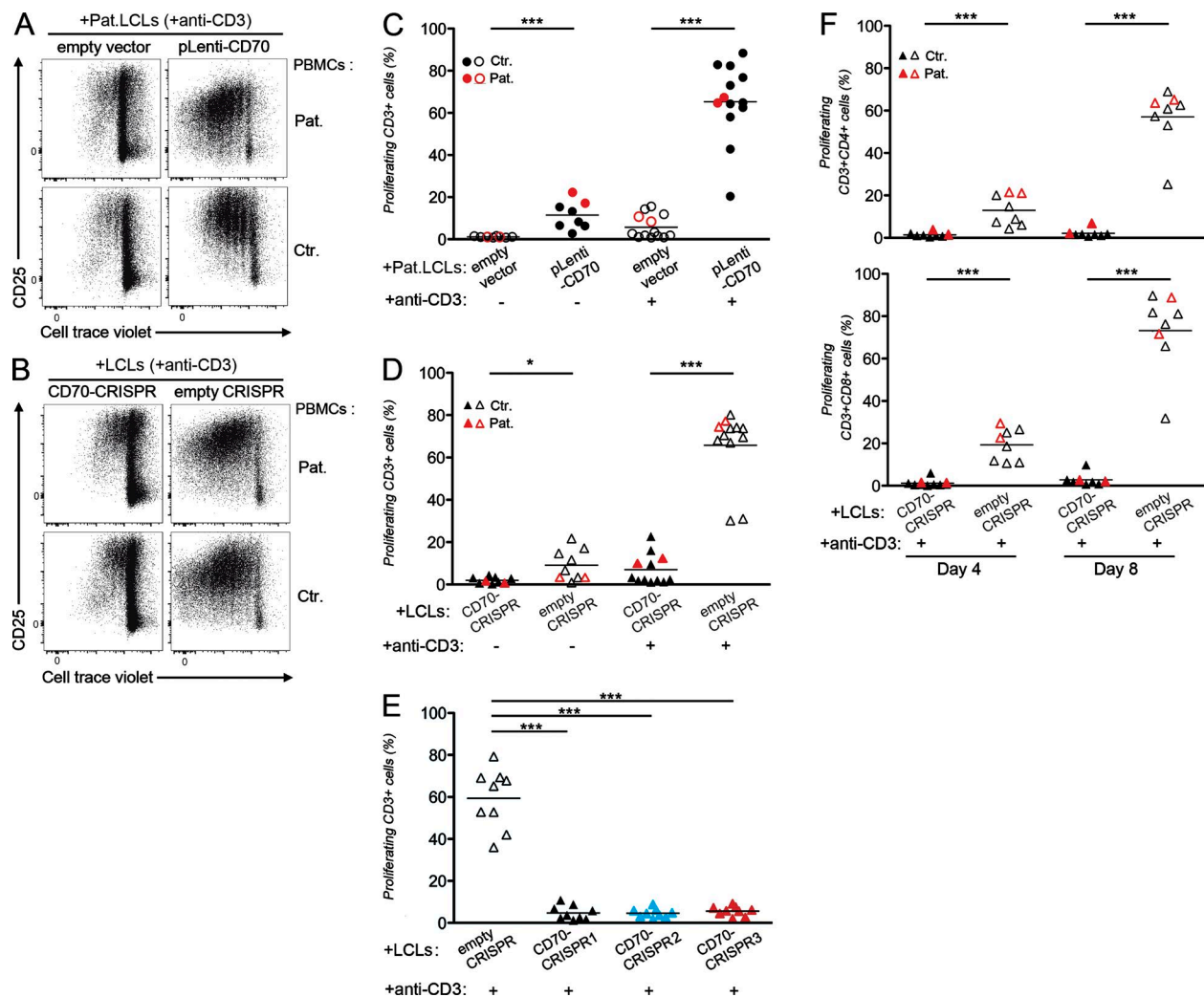
**Figure 5. CD70 on LCLs is required for patient and control T cell expansion of EBV-specific T cells.** (A) FACS histograms showing the expression of CD70 and HLA-A02\* on LCLs from an HLA-A02\* healthy donor, in which the CD70 gene was knocked out by CRISPR-Cas9-targeting exon 3 with three different RNA guides (CD70-CRISPR1, CD70-CRISPR2, and CD70-CRISPR3). One representative of four independent experiments is shown. (B) Dot plots from FACS analysis showing EBV-specific T cells gated on CD8<sup>+</sup>CD3<sup>+</sup> T cells stained with EBV-specific HLA-A02\* pentamers among PBMCs that have been co-cultured for 8 d or not in the presence of irradiated HLA-A02\* CD70-expressing LCLs or HLA-A02\* CD70-deficient CD70-CRISPR1 LCLs. PBMCs from the patient and four healthy donors (Ctr.1, Ctr.2, Ctr.3, and Ctr.4). Control 4 has not been infected by EBV and had a negative EBV serology (EBV neg.). Numbers in the plots indicate the percentage of EBV pentamer-positive cells in the gates. One representative of four independent experiments is shown. (C) Same as B, except that the presence of EBV-specific T cells of healthy donor control 1 was tested in the presence of two other irradiated HLA-A02\* CD70-deficient LCLs, CD70-CRISPR2 and CD70-CRISPR3, obtained by CRISPR-Cas9. Numbers in the plots indicate the percentage of EBV-pentamer-positive cells in the gates. One representative of two independent experiments is shown. (D) Analysis of cytolytic activity of T cells expanded from PBMCs of the CD70-deficient patient (Pat. expanded T cells) and one healthy control (Ctr. expanded T cells) for 8–15 d with irradiated autologous CD70-expressing (pLenti-CD70 or empty CRISPR) or CD70-deficient (empty vector or CD70-CRISPR) LCL cells. Cytolytic activity of T cells was then tested against a mixture of autologous CD70-expressing (blue) or CD70-deficient (red) LCL cells as target cells (Target LCLs) at an effector-to-target ratio of 1:1 of 1 for 0, 4, and 12 h. Residual target cells were evaluated by FACS analysis. Data are represented in percentages of cells normalized to the percentages at time 0. One representative of two independent experiments is shown.

the patient (Fig. 6 A) or with CD70-deficient LCLs obtained by CRISPR (CD70-CRISPR1 LCLs; Fig. 6 B). In contrast, when PBMCs were co-cultured with LCLs from the patient in which the expression of CD70 was restored or CD70-expressing LCLs from a healthy donor (empty CRISPR LCLs), most T cells from both the patient and the healthy donor were able to divide (Fig. 6, A and B).

These data were further extended with the analysis of several different healthy donors harboring different HLA

haplotypes, including HLA-A1, HLA-A2, and HLA-A3 (Fig. 6, C and D), and by using the two other CD70-deficient LCLs, CD70-CRISPR2 or CD70-CRISPR3 (Fig. 6 E). In these conditions, proliferation of T cells was dependent on CD3 stimulation, and in most individuals, a vast majority of T cells achieved at least one division when co-cultured with CD70-expressing LCLs but not with CD70-deficient LCLs. Dividing T cells included both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, although the proportion of CD8<sup>+</sup> cells was higher, indicating



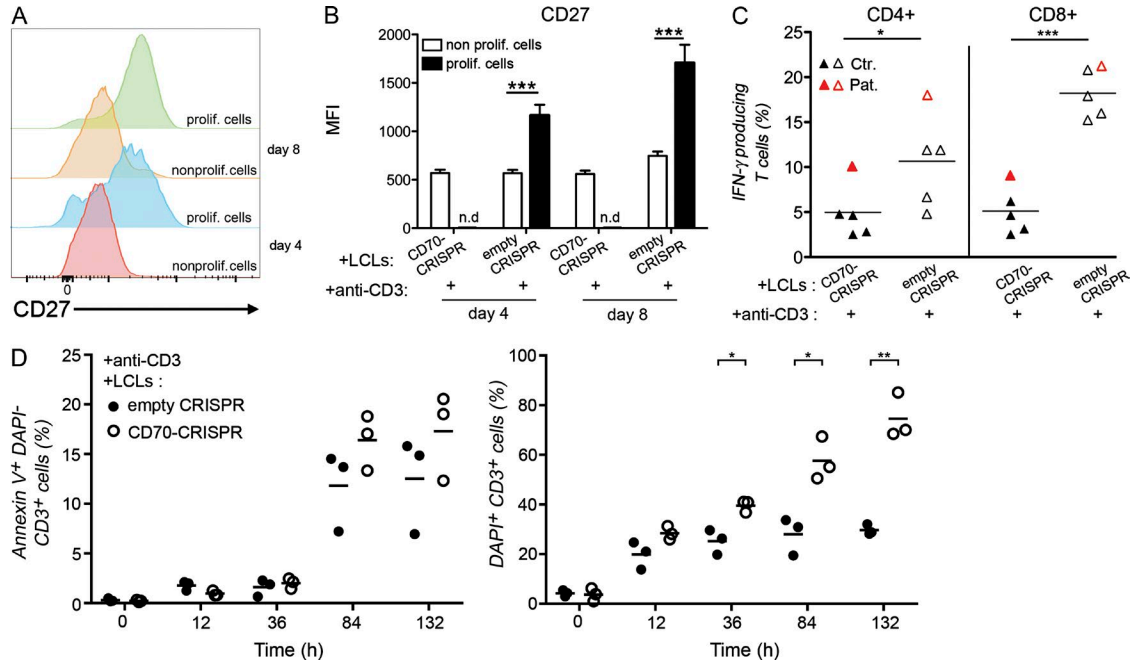


**Figure 6. CD3-mediated T cell proliferation by LCLs is dependent on their expression of CD70.** (A) Proliferation of T cells from PBMCs from the CD70-deficient patient (Pat.) or an HLA-A02\* healthy donor (Ctr.) cultured during 8 d with irradiated LCLs (+LCLs) from the patient expressing (pLenti-CD70) or not expressing (empty vector) CD70. Irradiated LCLs were preincubated with anti-CD3 antibody (+anti-CD3) before to be added to the PBMCs. Representative dot plots of violet dye dilution and CD25 expression gated on CD3<sup>+</sup> cells from one of four independent experiments are shown. (B) Same as A, except that PBMCs were cultured with irradiated LCLs from one healthy donor expressing (empty CRISPR) or not expressing (CD70-CRISPR) CD70 preincubated with anti-CD3 antibody. One representative of four independent experiments is shown. (C and D) Same as in A and B, except that several donors and the patient were tested, and proliferation was also examined in the absence of anti-CD3 ( $n = 8$  in C;  $n = 9$  in D) or in presence of anti-CD3 antibody ( $n = 13$  in C;  $n = 12$  in D) during the preincubation with LCLs. Percentage of proliferating CD3<sup>+</sup> T cells corresponds to cells having at least achieved one division based on the dilution of the violet dye. Percentages from FACS analysis histograms of violet dye staining are shown. PBMCs from seven (C) or six (D) different healthy donors are shown. Patient PBMC results are shown in red. Data are from five independent experiments. (E) Same as B, except that six different donors were tested, and PBMCs were co-cultured with two other irradiated CD70-deficient LCLs (CD70-CRISPR2 or CD70-CRISPR3) preincubated with anti-CD3 antibody. Percentage of proliferating CD3<sup>+</sup> T cells corresponds to cells having at least achieved one division based on the dilution of the violet dye. Data from two independent experiments are shown.  $n = 9$ . (F) Same as B, except that PBMCs of six different donors and the patient were tested for the proliferation of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells at day 4 and day 8 after being incubated with irradiated LCLs from one healthy donor expressing (empty CRISPR) or not expressing (CD70-CRISPR) CD70 preincubated with anti-CD3 antibody. One representative of three independent experiments is shown. (C–F) Unpaired Student's *t* tests were used. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0001$ .

that CD4<sup>+</sup> had a reduced capacity to proliferate compared with CD8<sup>+</sup> T cells. (Fig. 6 F).

CD70-dependent proliferating T cells were further characterized. A large proportion of these proliferating T cells expressed high levels of CD27, CD25, and CD45RO but not

CD70 and CD62L (Fig. 7, A and B; and not depicted). Proliferating T cells displayed an effector phenotype because they had the capacity to secrete IFN- $\gamma$  and TNF- $\alpha$  upon restimulation by anti-CD3 antibodies (Fig. 7 C and not depicted). In contrast, nonproliferating T cells in the co-cultures with

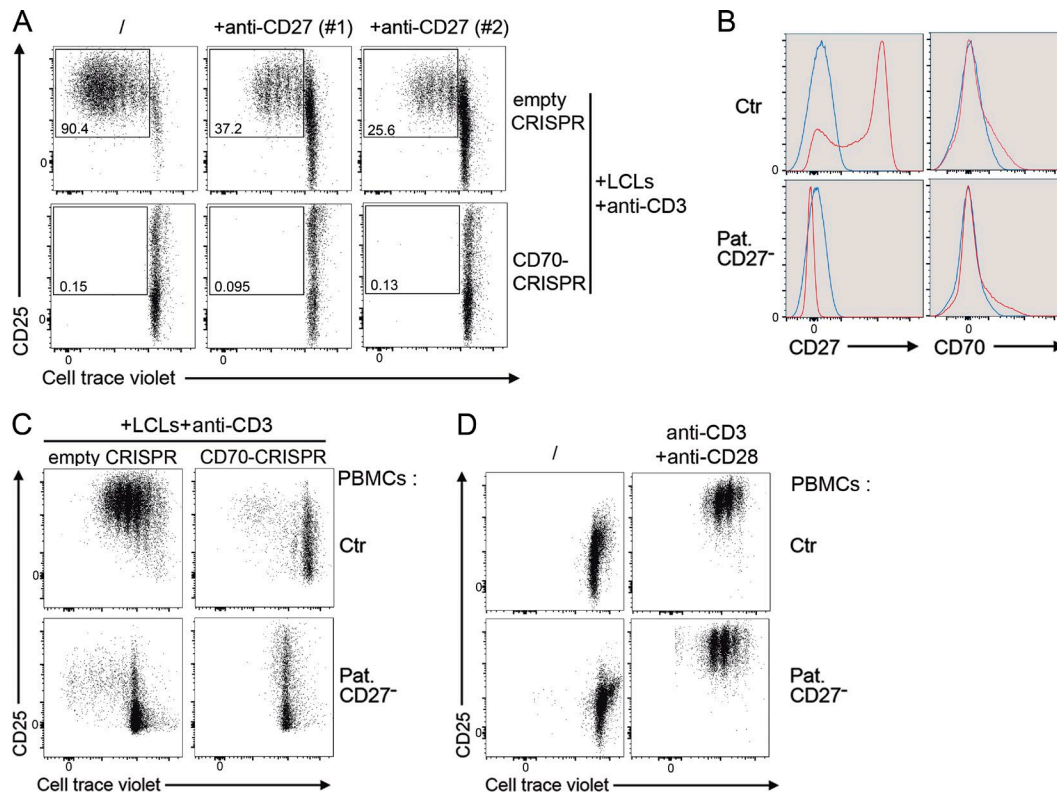


**Figure 7. Proliferating T cells that expand in response to CD70 expressed CD27 and are effector cells.** (A) Example of FACS histograms of CD27 expression at days 4 and 8 on T cells from PBMCs of one healthy donor that have been cultured in the presence of irradiated CD70-expressing LCLs (empty CRISPR) preincubated with anti-CD3 antibody. Proliferation is analyzed the same as in Fig. 6 B. Proliferating (prolif.) T cells gated on CD3<sup>+</sup> cells correspond to cells having at least achieved one division measured by CellTrace violet dye dilution. One representative experiment of five independent experiments is shown. (B) Mean fluorescence intensity (MFI) of CD27 expression calculated from FACS histograms corresponding to A. Means with SEM are presented. Data are from five independent experiments. (C) Intracellular IFN- $\gamma$  expression from FACS analysis in CD4<sup>+</sup> or CD8<sup>+</sup> T cells from PBMCs of four different control (Ctr.) individuals and the patient (Pat.; red;  $n = 5$ ) that have been cultured in the presence of irradiated LCLs (+LCLs) expressing (empty CRISPR) or not expressing (CD70-CRISPR) CD70 preincubated with anti-CD3 antibody (+anti-CD3). Before being tested for cytokine expression by FACS, T cells were restimulated with anti-CD3 antibody for 12 h. (D) Apoptosis and cell death of CD3<sup>+</sup> T cells from FACS analysis with staining with annexin V and DAPI of PBMCs from three different healthy controls that have been cultured in the presence of irradiated LCLs expressing or not expressing CD70 preincubated with anti-CD3 antibody for different periods of time. Left and right panels correspond to apoptotic cells (annexinV<sup>+</sup>DAPI<sup>-</sup>CD3<sup>+</sup> cells) and dead cells (DAPI<sup>+</sup>CD3<sup>+</sup> cells), respectively. Each point corresponds to a mean of duplicates. (B–D) Unpaired Student's *t* tests were used. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ ; \*\*\*,  $P < 0.0001$ .

CD70-deficient LCLs (CD70-CRISPR) did not express activation markers and did not up-regulate CD27 expression (Fig. 7 B). Importantly, no increase in apoptosis was noticed in the co-cultures with CD70-deficient LCLs at least until 36 h of culture, as shown by the proportion of annexin V-positive DAPI-negative cells, which did not significantly differ from that observed in co-cultures with CD70-expressing LCLs, suggesting that CD70 is not directly involved in the regulation of apoptosis (Fig. 7 D, left). However, when global cell death was examined by DAPI staining, we detected significant accumulation of dead cells from 36 h of culture in the co-cultures with CD70-deficient LCLs in comparison with co-cultures with CD70-expressing LCLs, likely corresponding to nonproliferative T cells that ultimately died independently of active apoptotic stimuli (Fig. 7 D, right).

We next verified that the ligand of CD70, the co-signaling molecule CD27 on T cells, was involved in CD70-dependent T cell proliferation. Addition of anti-CD27 antibodies substantially reduced CD70-dependent proliferation as expected (Fig. 8 A). CD27 and CD70 deficiencies appear as pheno-

copies. However, the exact mechanism underlying the defective immune response to EBV in CD27-deficient patients is currently not known. Collectively, our data obviously suggest that co-signals provided by CD70-expressing B cells are defective in CD27-deficient T cells. To formally prove it, we tested CD70-dependent T cell proliferation of PBMCs from a newly identified patient carrier of a homozygous mutation p.W110X in *CD27*. The W110X mutation abolished the surface expression of CD27 on PBMCs and T cell blasts of the patient (Fig. 8 B and not depicted). As expected, proliferation of CD27-deficient T cells in response to irradiated CD70-expressing B-LCLs in the presence of anti-CD3 antibody was markedly reduced, whereas CD27-deficient T cells normally proliferate in response to stimulation with anti-CD3 plus anti-CD28 antibodies (Fig. 8 D). These results demonstrate that CD70 when expressed on B cells provides an essential co-stimulatory signal via CD27 for TCR-mediated proliferation of T cells. Finally, strengthening the unique role of CD70 on B cells to activate T cell proliferation, we found that CD70-negative (CD70-CRISPR) and CD70-



**Figure 8. CD27 on T cells is required for CD70-mediated T cell proliferation by LCLs.** (A) Proliferation of T cells from PBMCs of one healthy donor were cultured for 8 d in the presence of irradiated LCLs (+LCLs) expressing (empty CRISPR) or not expressing (CD70-CRISPR) CD70 preincubated with anti-CD3 antibody in the presence or not (I) of 10  $\mu$ g/ml anti-CD27 antibody (clones LG.3A10 [#1] or O323 [#2]). Numbers in the gates indicate the percentage of dividing cells. Representative dot plots of violet dye dilution and CD25 expression gated on CD3<sup>+</sup> cells from one of three independent experiments are shown. (B) FACS histograms of CD27 expression on T cell blasts from healthy control (Ctr.) and a CD27-deficient patient (Pat. CD27<sup>-</sup>). One representative of three independent experiments is shown. (C) Proliferation of T cells from PBMCs of a control and a CD27-deficient patient cultured for 8 d in the presence of irradiated LCLs expressing or not expressing CD70 preincubated with anti-CD3 antibody (+anti-CD3). Representative dot plots of violet dye dilution and CD25 expression gated on CD3<sup>+</sup> cells from one of two independent experiments are shown. (D) Proliferation of T cells from PBMCs of a control and a CD27-deficient patient stimulated for 8 d by the coated beads with anti-CD3 and anti-CD28 antibodies. Dot plots of violet dye dilution and CD25 expression gated on CD3<sup>+</sup> cells from one of two independent experiments are shown.

expressing LCLs expressed comparable levels of known molecules expressed by B cells or antigen-presenting cells involved in co-stimulatory or regulation of T cell responses including B7 molecules CD80 and CD86 and TNF family members OX40L (TNFSF4), CD40 (TNFRSF5), B cell-activating factor receptor (TNFRSF13C), transmembrane activator and CAML interactor (TNFRSF13B), CD137L (TNFSF9), and FAS (TNFRSF6; not depicted).

## DISCUSSION

In this study, we report for the first time a genetic deficiency in *CD70* in a patient with defective immunity to EBV infection associated with uncontrolled lymphoproliferation of infected B cells. We decipher the pathway involving CD70 and its ligand CD27 and provide evidence that CD70 and CD27 form a functional cluster that plays a crucial role in the immune surveillance of B cells by T cells. When expressed on B cells, CD70 delivers signals to T cells through CD27,

those being necessary for T cell proliferation maintenance and hence execution of effector programs.

The patient had recurrent B cell lymphoproliferation associated with a high viral load of EBV and developed EBV-associated Hodgkin's lymphoma. His susceptibility to infections appears to be restricted to EBV, as until today, he has not suffered from other viral or bacterial infections. Based on a single patient who in addition is still young, it is obviously difficult to firmly conclude the clinical consequences associated with CD70 deficiency. However, genetic defects in CD27, which is the only known ligand for CD70, have been reported in 17 patients (van Montfrans et al., 2012; Salzer et al., 2013; Alkhairy et al., 2015). Notably, all of them developed EBV-related disorders as initial and main clinical manifestations, including lymphoproliferation disorders like Hodgkin's lymphoma and diffuse large B cell lymphoma. Therefore, the clinical symptoms of the CD70-deficient patient are similar if not identical to those associated with CD27 deficiency.

Hence, CD70 deficiency appears to be a phenocopy of CD27 deficiency. Phenotypes of CD27- and CD70-deficient mice are also comparable, and both are characterized by diminished antiviral responses (Hendriks et al., 2000; van Gisbergen et al., 2011; Welten et al., 2013). Interestingly enough, the clinical phenotype associated with ITK deficiency is also very close to the phenotype of CD27 and CD70 deficiencies (Bienemann et al., 2015).

Our results indicate that the CD70–CD27 axis represents a key factor of the immune response to EBV in humans. We showed that CD70 on EBV-infected B cells provides crucial signals to EBV-specific CTLs for their expansion. We observed that CD70 is strongly up-regulated in activated B cells and EBV-infected B cells. CD70 is also constitutively expressed by many EBV-dependent and non-EBV-dependent B cell malignancies (Jacobs et al., 2015). The peculiar predisposition to EBV infection associated with CD27 and CD70 deficiencies likely results from the unique tropism of EBV to B cells and its capacity to induce B cell proliferation and transformation. An efficient *in vivo* immune response to EBV requires rapid and massive expansion of effector cells to provide a matching amount of CTLs to eliminate highly proliferating EBV-infected B cells. Thus, the CD70–CD27 pathway represents a critical component of this response.

Numerous studies in mice have documented the role of the CD27–CD70 axis in the generation and maintenance of CD8<sup>+</sup> T cell responses, including establishment of long-term memory (Hendriks et al., 2000). A recent description of CD70-deficient mice confirmed the particular importance of CD70 in the initiation of primary CD8<sup>+</sup> T cell responses (Munitic et al., 2013). Accordingly, acquisition of T cell immunity toward EBV-infected B cells appears to be defective in the CD70-deficient patient, correlating with the absence of a proper expansion and proliferation of EBV-specific effector CD8<sup>+</sup> T cells *in vitro*. However, memory T cell immunity to EBV is likely preserved in the absence of CD70 because specific anti-EBV T cells could be expanded from PBMCs of the patient when co-cultured with autologous CD70-expressing LCLs. Collectively, these results pinpoint a specific role of CD70–CD27 interactions in triggering cell division of activated T cells without affecting their intrinsic capacity to execute their cytotoxic and IFN- $\gamma$ -producing functions and to differentiate into memory cells.

In accordance with a previous study, our observations demonstrate that CD27 behaves as a co-stimulatory molecule of TCR-dependent lymphocyte activation (Nolte et al., 2009). The co-signals delivered by CD27 are crucial for sustained T cell expansion as recently highlighted (Marchingo et al., 2014). However, the precise mechanism involved in CD27 signaling has not yet been clearly defined, although a role for TNF receptor-associated factor molecules has been reported (Akiba et al., 1998). Interestingly, stimulation of human T cells with anti-CD27 antibody or soluble CD70 has been reported to result in calcium mobilization in naive T cells and  $\gamma\delta$  T cells (Kobata et al., 1994; deBarros et al., 2011). Considering

that it is well established that ITK is involved in TCR signaling by its ability to activate PLC- $\gamma$ 1, Ca<sup>2+</sup> flux, and extracellular signal-regulated kinases (Liu et al., 1998; Andreotti et al., 2010), it is tempting to speculate that ITK might be required for CD27 signaling in T cells. This might explain the close similarity of the clinical phenotypes associated with CD70, CD27, and ITK deficiencies. Strengthening this hypothesis, CD27 contained in its intracellular domain several proline and tyrosine residues, which may form potential docking sites for the SH3 and SH2 domains of ITK (Bunnell et al., 1996) or other signaling molecules that have been involved in calcium mobilization in T cells.

Although our observations establish that CD70 on B cells is a key player in T cell immunity toward proliferating B cells, CD70 is also expressed on other hematopoietic cells including subpopulations of activated T cells and DCs. In the different models previously tested, CD27-dependent priming and maintenance of T cell responses were shown to involve interactions with CD70 expressed on DCs (Schildknecht et al., 2007; Ballesteros-Tato et al., 2010). Thus, it is also conceivable that the absence of CD70 on DCs contributes to the lack of control of EBV infection in the patient, in particular during the priming phase. Because CD27- and CD70-deficient patients did not develop major susceptibility to other infections, CD70 on DCs seems not to be absolutely required for immune responses to other pathogens. This is also supported by the fact that antigen-specific T cell proliferation in response to toxoid tetanus antigen was found to be normal in the patient (Table 1). Partial redundancy with other TNFR family members, such as 4-1BB/CD137 and OX40, that are expressed by T cells could provide similar signals to T cells as those delivered by CD27 when engaged by their ligand during interactions with DCs (Watts, 2005; Nolte et al., 2009). However, we cannot exclude that CD70 may be required for immune responses to infections other than EBV. Identification of other CD70-deficient patients will help to resolve this issue.

Interestingly enough, the mechanism underlying the defective immunity to EBV in the SAP deficiency caused by mutations in *SH2D1A* (or XLP-1 syndrome) is distinct and is associated with a different clinical phenotype than the one associated with CD27 and CD70 deficiencies. In SAP deficiency, impaired T–B cell interactions through SLAM receptors play a pivotal role in susceptibility to EBV infection, resulting in HLH syndrome associated with an expansion of CD8<sup>+</sup> T cells in the majority of patients (Tangye, 2014). Our results indicate that the CD27–CD70 axis also plays an important role during T–B cell interactions in the course of EBV infection. However, the impaired control of EBV infection in patients with CD27 and CD70 deficiencies did not lead readily to HLH. In the absence of SAP, effector-memory EBV-specific CD8<sup>+</sup> T cells have been shown to exhibit decreased capacity to kill EBV-infected B cells (Hislop et al., 2010; Palendira et al., 2011), whereas cell expansion is normal or even increased because of defective activation-

induced cell death (Snow et al., 2009), in sharp contrast to CD27 and CD70 deficiencies. Pathogenesis of XLP syndrome is thought to result from immunopathology caused by CD8<sup>+</sup> T cells unable to kill B cell targets. This is consistent with the pathophysiology of HLH, which is known to depend on the accumulation of activated T cells producing large amounts of IFN- $\gamma$  as a result of their inability to kill infected cells (Schmid et al., 2010). On the contrary, in CD27 and CD70 deficiencies, accumulation of activated T cells in the course of EBV infection is deficient, resulting from their impaired capacity to proliferate. These distinct molecular defects pinpoint to two critical steps in the induction of T cell response to EBV, i.e., cell expansion and cytotoxic effector function that depend on different molecules/pathways, CD70/CD27 and SLAM/SAP, respectively, for their execution.

Collectively, our observations suggest that CD70 on B cells acts as a semaphore to signal abnormal B cell proliferation to T cells even in the absence of EBV as a trigger of B cell proliferation. This fits well with the observation that somatic mutations in *CD70*, including large deletions, have been identified in B cell lymphomas such as diffuse large B cell lymphomas and Burkitt's lymphomas (Giefing et al., 2008; Scholtysik et al., 2012; Bertrand et al., 2013). Notably, a recent study identified *CD70* as one of the most frequently mutated genes in a series of diffuse large B cell lymphomas (de Miranda et al., 2014). Accumulation of mutations in *CD70* may represent a mechanism for malignant B cells to escape immune surveillance by T cells. Our data and previous studies in mice indicate that ectopic expression of CD70 on nonlymphoid tumor cells or CD70 expression in B cells (our study) can evoke T cell-mediated immunity against tumor cells irrespectively of their expression of CD70 (Lorenz et al., 1999; Kelly et al., 2002; Aulwurm et al., 2006). Thus, CD70-CD27 could represent an important target to induce antitumoral vaccination. Restoration or induction of CD70 expression in some lymphoma cells lacking CD70 might represent a therapeutic approach to induce a global and potent antitumoral immunity.

## MATERIALS AND METHODS

### Ethics

Informed consent was obtained from donors, patients, and families of patients. The study and protocols conform to the 1975 declaration of Helsinki as well as to local legislation and ethical guidelines from the Comité de Protection des Personnes de l'Île de France II, Hôpital Necker-Enfants Malades, Paris.

### Patients

Individuals with infectious mononucleosis were diagnosed on tonsil biopsies and confirmed with anti-VCA and anti-EBNA IgM-positive serology. The patient carrier of the homozygous c.329G>A, p.W110X, in *CD27* is issued from consanguineous parents of Tunisian origin and suffered from a recurrent EBV-driven lymphoproliferative disease. The *CD27* defect was diagnosed based on the lack of CD27 expression on B cells. The four exons of *CD27* were further sequenced.

### Clinical features of the CD70-deficient patient

At the age of 3 yr and 8 mo, the patient was diagnosed with nodular sclerosing Hodgkin's lymphoma stage 2. He was treated with chemotherapy and radiotherapy and achieved a complete response. Since the age of 4.5 yr, he presented a recurrent fever, lymphadenopathies, and hepatosplenomegaly, which were associated with a high level of EBV viral load in his blood. Relapse of Hodgkin's lymphoma was excluded by biopsy. Bone marrow biopsy specimens were analyzed and found to be normal without evidence of hemophagocytosis. His symptoms were relieved after the initiation of anti-CD20 (rituximab) therapy, and EBV viral load decreased but was never found to be negative. He had repeated anti-CD20 treatments, but EBV viral load remained high even though it was transiently diminished during the few months after each cure. Importantly, the patient had no other viral and bacterial infections. The patient was under treatment with intravenous immunoglobulins every 3 wk and prophylactic antibiotics and remained in good clinical condition despite persisting high EBV replication. The last lymphoproliferative episode occurred at the age of 10 yr and 4 mo. He presented again with recurrent fever, weight loss, and lymphoproliferative syndrome with EBV viral load increased to 6.4 log. A positron emission tomography scan revealed multiple hypermetabolic fixations (cavum and numerous lymph nodes; cervical groups IB, II, III, and IV, peritracheal, celiac, gastro-hepatic, mesenteric, retroperitoneal, retroaortic, retrocaval, ileac, and inguinal). A biopsy from an inguinal lymph node was performed showing no histological evidence of Hodgkin's lymphoma but showing EBV-associated B cell lymphoproliferation with clonal rearrangements of the IgH and IgK locus. The patient received again four courses of anti-CD20 treatment leading to clinical improvement, complete regression of the lymphoproliferative syndrome, and negatification of the EBV load. Very recently, he received allogeneic hematopoietic stem cell transplantation at the age of 10 yr and 10 mo and is well since.

### Exome sequencing and analysis

Exome capture was performed according to the manufacturer's protocol using an exome enrichment kit (TruSeq; Illumina) and sequencing of 100-bp paired-end reads on a HiSeq2500 sequencing system (Illumina). Approximately 10 Gb of sequence were obtained for each subject such that 90% of the coding bases of the exome defined by the consensus coding sequence project were covered by at least 10 reads. Adaptor sequences and quality-trimmed reads were removed using the Fastx toolkit, and then, a custom script was used to ensure that only read pairs with both mates present were subsequently used. Reads were aligned to hg19 with BWA31, and duplicate reads were marked using Picard (Broad Institute) and excluded from downstream analyses. Single nucleotide variants and short insertions and deletions (indels) were determined using SAMtools pileup and varFilter32. With the base alignment quality adjustment disabled, they were then quality filtered to require at least 20% of reads supporting

the variant call. Variants were annotated using both ANN OVAR33 and custom scripts to identify whether they affected protein coding sequences and whether they had previously been seen in the public databases of exomes and the 7,566 exomes previously sequenced at our center. The CD70 variation identified in the patient (19:6586078G/A), a homozygous nonsense mutation c.535C>T p.Arg179X, was present in the database of the Exome Aggregation Consortium (ExAC) and in our institute's database as a heterozygous mutation with a frequency of  $1.48 \times 10^{-5}$  (2/134 542 allele). It was not reported in other available public databases of exomes (dbSNP, the 1000 Genomes, and the NHLBI Exome Sequencing Project).

### DNA sequencing

Genomic DNA from peripheral blood cells of the patient, the parents, and other family members was isolated according to standard methods. Oligonucleotide primers in the introns flanking exon 3 of *CD70* and exon 3 of *CD27* were used to amplify genomic DNA: *CD70* forward, 5'-CCA CCACGTCCAACCTATTC-3'; *CD70* reverse, 5'-TCT CTTGTCCTGCCACCACT-3'; *CD27* forward, 5'-ATG GAAAGGGAAGCACGTC-3'; and *CD27* reverse, 5'-TTGGCCAACTCCTCTCCTAA-3'.

PCR products were amplified using high-fidelity Platinum Taq DNA Polymerase (Invitrogen) according to the manufacturer's recommendations, purified with a gel extraction kit (QIAquick; QIAGEN), sequenced using the ABI PRISM Big-Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) according to the manufacturer's recommendations, and analyzed with genetic analyzer (3500xL; Applied Biosystems). All collected sequences were analyzed using 4peaks software (version 1.7.2) or DNADynamo (BlueTractorSoftware).

### Immunohistochemistry

Staining and in situ hybridization were performed on an automated stainer (Bond Max; Leica Biosystems). The presence of EBV was demonstrated by in situ hybridization for the small RNA-encoding regions 1 and 2 (EBER). Antibodies used and dilution were: anti-CD70 (rabbit polyclonal; 1/200; Abgent), anti-PAX5 (clone DAK-PAX5; 1/30; Dako), anti-CD27 (clone 137B4; 1/50; Abcam), anti-CD8 (clone C8/144B; 1/200; Dako), and anti-LMP1 (clone CS.1-4; 1/200; Dako).

### Cell culture

Whole blood samples were collected from the patient and control donors. PBMCs were isolated by Ficoll-Paque density gradient (Lymphoprep; Proteogenix) from blood samples using standard procedures. Expansion of T cell blasts were obtained by incubating PBMCs for 72 h with  $2.5 \mu\text{g ml}^{-1}$  PHA (Sigma-Aldrich) in Panserin 401 (Pan Biotech) supplemented with 5% human male AB serum (BioWest),  $100 \text{ U ml}^{-1}$  penicillin, and  $100 \mu\text{g ml}^{-1}$  streptomycin. After 3 d, dead cells were removed by Ficoll-Paque density gradient, and blasts were maintained in culture with 100 or  $1,000 \text{ UI ml}^{-1}$  IL-2.

### EBV-transformed LCLs and EBV-specific T cell lines

EBV-transformed LCLs were generated from fresh or frozen PBMCs of the patient and control donors. The PBMCs were incubated with supernatant from B95-8 cells in the presence of  $1 \mu\text{g/ml}$  cyclosporine A (Sigma-Aldrich) as previously described (Savoldo et al., 2002). EBV-specific T cell lines were generated from the patient and control healthy donors. PBMCs were co-cultured with 40-Gy irradiated autologous LCLs at an effector-to-stimulator ratio of 40:1. After 9–14 d, viable cells were stimulated with 40-Gy irradiated autologous LCLs at an effector-to-stimulator ratio of 4:1 for 5–7 d and then analyzed for cytotoxicity.

### Gene expression analysis

Total mRNA was extracted from EBV-B cell lines and activated T cell lines of the patient and control donors with a Pure Link RNA Mini kit (Thermo Fisher Scientific) and treated with DNase I. Reverse transcription was performed with a first-strand synthesis system (SuperScript II; Thermo Fisher Scientific). Real-time quantitative PCRs for *CD70* and actin were performed in triplicate using a real-time PCR system (Viia 7; Thermo Fisher Scientific), SYBR Select Master Mix (Thermo Fisher Scientific), and the following primers: *CD70* forward primer, 5'-GGGTGG GACGTAGCTGAG-3' and *CD70* reverse primer, 5'-AAT GGTACAACCTTGGTGGAAG-3'.

### Cell cytotoxicity assays

The cytolytic activity of CTLs was evaluated with a standard 4-h  $\text{Cr}^{51}$  release assay. In brief,  $10^5$  cell target cells labeled with  $\text{Cr}^{51}$  were incubated with effector cells for 4 h at  $37^\circ\text{C}$  at the indicated effector-to-target ratios.  $30 \mu\text{l}$  of supernatant was then collected and counted with a MicroBetaTrilux and JET counter (PerkinElmer) with  $180 \mu\text{l}$  OptiPhase Supermix cocktail (Perkin Elmer). Specific lysis (percentage) was calculated according to the formula:  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ . Cytolytic activity of T cells expanded for 8 d from PBMCs was also evaluated in co-cultures against autologous CellTrace violet-labeled *CD70*-expressing LCLs and CFSE-labeled *CD70*-deficient LCLs at an effector-to-target ratio of 1:1 for 0, 4, and 12 h. Residual target cells were evaluated by FACS analysis. Data are represented in percentages of cells normalized to the percentages at time 0.

### Stimulation and proliferation assays

PHA-stimulated T cells were washed and cultured without IL-2 for 72 h to synchronize the cells. Then PHA-stimulated T cells or PBMCs were cultured during 4–8 d in complete medium alone or in the presence of anti-CD3/CD28-coated beads (Invitrogen). In the co-culture proliferation experiment, PHA-stimulated T cells or PBMCs plus or minus  $10 \mu\text{g ml}^{-1}$  anti-CD27 antibody (clone LG.3A10 from eBioscience or O323 from BioLegend) were co-cultured in the presence of 45 Gy irradiated autologous or allogeneous LCLs that had been pre-

viously incubated with 1  $\mu\text{g ml}^{-1}$  anti-CD3 antibody (OKT3) or not for 1 h. Cells were co-cultured at a ratio of one T cell or PBMC for 10 LCLs (ratio 1/10). Cell proliferation was monitored by labeling T cells or PBMCs with the CellTrace violet dye (violet proliferation dye 450; BD) before stimulation or co-culture with LCLs, according to the manufacturer's instructions. After 8 d of culture, cells were harvested, and CellTrace violet dye dilution was assessed by flow cytometry.

### Flow cytometry

Cell staining and flow cytometry-based phenotypic analyses of PBMCs, blast T cells, and cell lines were performed according to standard flow cytometry methods. The following mAbs with the identification numbers in parentheses were conjugated to FITC, PE, PE-cyanin 5 (PE-Cy5), PE-Cy5.5, PE-Cy7, peridinin-chlorophyll (PerCP), PerCP-Cy5.5, allophycocyanin (APC), APC-Cy7, APC-Vio7, Alexa Fluor 700, Brilliant Violet 421 (BV421), BV510, BV650, and BV785: anti-CD3 (UCHT1), anti-CD4 (OKT4), anti-CD8 (RPA-T8), anti-CD14 (M5E2), anti-CD19 (HIB19), anti-CD25 (BC96), anti-CD27 (LG.3A10, M-T271, and O323), anti-CD28 (CD28.2), anti-CD31 (WM59), anti-CD56 (HCD56), anti-CD70 (113-16), anti-CD107a/b (H4A3/HAB4), anti-CD161 (HP-3G10), anti-CD197 (G043H7), and anti-TCR  $\text{V}\alpha 7.2$  (3C10) from Sony Biotechnology Inc.; anti-CD16 (3G8), anti-CD45RA (HI100), anti-CD45RO (UCHL1), anti-CD95 (DX2), anti-TCR  $\alpha/\beta$  (VT31), anti-IgD (IA6-2), and anti-IgM (G20-127) from BD; and anti-CD21 (BL13), anti-TCR  $\gamma\delta$  (IMMU510), anti-TCR  $\text{V}\alpha 24$  (C15), and anti-TCR  $\text{V}\beta 11$  (X21) from Beckman Coulter. Binding of CD27 to CD70-expressing cells was assessed by incubation with human CD27- $\mu\text{lg}$ /biotin fusion protein (AnCell Corporation) according to the manufacturer's protocol and standard flow cytometry methods. Binding of CD27- $\mu\text{lg}$  to cells was detected with PE-conjugated streptavidin (BD). All data were collected on FACS Canto II or LSR Fortessa cytometers (BD) and analyzed using FlowJo software (version 10.0.8r1; Tree Star).

### Cytokine production and detection

For intracellular staining of cytokines, cells were restimulated overnight with irradiated LCLs beforehand preincubated with 1  $\mu\text{g ml}^{-1}$  OKT3 and in the presence brefeldin A (GolgiPlug; BD). Cells are then fixed and permeabilized using the cytofix/cytoperm plus kit (BD) according to the manufacturer's instructions. Next, cells were labeled with PE/Cy7-anti-TNF (mouse IgG1; MAb11), APC-anti-IFN- $\gamma$  (mouse IgG1; 4S.B3), and isotype-matched monoclonal antibodies purchased from BioLegend and analyzed by flow cytometry. Thereafter, cells were collected, washed, and stained with BV785-anti-CD3, BV650-PE-anti-CD8, and BV510-anti-CD4 mAbs and analyzed by flow cytometry.

### EBV-specific T cell detection

EBV-specific  $\text{CD8}^+$  T cells from PBMCs were co-cultured with 45-Gy irradiated LCLs for 8–10 d and were detected

using a mix of unlabeled EBV HLA-A2:01 Pro5 Pentamers (Proimmune) mixed with R-PE Pro5 Fluorotag in addition with BV785-anti-CD3, BV650-PE-anti-CD8, and BV510-anti-CD4 monoclonal antibodies. The EBV HLA-A2:01 Pro5 Pentamers mix contains three different pentamers presenting FLYALLALL (residues 356–364 from LMP2), CLGGLL TMV (residues 426–434 from LMP2), or GLCTLVAML (residues 259–267 from BMLF-1), peptides derived from LMP2 and BMLF1 proteins of EBV. After their isolation, EBV-specific T cells' activated phenotype was characterized using PE/Cy7-anti-CD25 and FITC-CD45RA antibodies. All staining was done according to the manufacturer's instructions.

### Cell transfection and CRISPR Cas9 genome editing

Full-length cDNA encoding wild-type CD70 and CD70 R179X were obtained by RT-PCR from blasts of control donors and the patient, respectively, using the following primers: forward primer, 5'-TCTCGGCAGCGCTCC-3' and reverse primer, 5'-ACCCTAATCAGCAGCAGTGG-3'. The cDNAs were verified by sequencing and inserted into a bicistronic lentiviral expression vector encoding GFP as a reporter (pLenti7.3/V5-TOPO; Invitrogen) or an N-terminal Flag-tagged expression vector (p3XFLAG-myc-CMV-26; Sigma-Aldrich). Patient's LCLs were transduced with the pLenti7.3/V5-TOPO containing wild-type CD70 or the empty vector as previously described (Martin et al., 2014). The lentiCRISPR plasmid was a gift from F. Zhang (Massachusetts Institute of Technology, Boston, MA; plasmid no. 49535; Addgene). All single-guide RNAs (sgRNAs) were designed using the CRISPR Design Tool (Massachusetts Institute of Technology). Three pairs of 24 bp forward (F) and reverse (R) of oligonucleotides targeting different sequences in exon 3 of *CD70* were synthesized (Eurogentec) with a 4-bp overhang to enable cloning into the BsmBI site in reverse oligonucleotides and a 4-bp overhang containing the PAM sequence in forward oligonucleotides. sgRNA sequences were: G1F, 5'-CACCGGCTACGTATC CATCGTGA-3', G1R, 5'-AAACTCACGATGGATACG TAGCC-3'; G2F, 5'-CACCGTACACATCCAGGTGAC GC-3', G2R, 5'-AAACGCGTCACCTGGATGTGTAC-3'; G3F, 5'-CACCGCAGGCTGATGCTACGGG-3'; and G3R, 5'-AAACCCCGTAGCATCAGCCTGC-3'. Pairs of synthesized oligos was annealed, phosphorylated, ligated to a linearized vector, and transformed into Stbl3 bacteria (Thermo Fisher Scientific). sgRNA insertion was confirmed by Sanger sequencing using a sequencing primer (5'-TTTCTTGGG TAGTTTGCAGTTTT-3'). The lentiCRISPR plasmids were transduced by infection in LCLs. LCL CD70-positive and CD70-negative populations were enriched by sorting.

### Immunoblotting

80  $\mu\text{g}$  of proteins were separated by SDS-PAGE and transferred on polyvinylidene fluoride membranes (EMD Millipore). Membranes were blocked with milk for 1 h before incubation with primary antibodies for 90 min. Anti-FLAG (clone M2) from Sigma-Aldrich was used for immunoblot-

ting and was revealed with anti-mouse HRP-conjugated secondary antibodies from GE Healthcare. Enhanced chemiluminescent Western blotting substrate (Thermo Fisher Scientific) was used for detection.

### Statistics

P-values were calculated with Student's *t* test using PRISM (GraphPad Software).

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