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Research Paper

HIV-Specific B Cell Frequency Correlates with Neutralization Breadth in Patients Naturally Controlling HIV-Infection

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ABSTRACT

HIV-specific broadly neutralizing antibodies (bnAbs) have been isolated from patients with high viremia but also from HIV controllers that repress HIV-1 replication. In these elite controllers (ECs), multiple parameters contribute to viral suppression, including genetic factors and immune responses. Defining the immune correlates associated with the generation of bnAbs may help in designing efficient immunotherapies. In this study, in ECs either positive or negative for the HLA-B*57 protective allele, in treated HIV-infected and HIV-negative individuals, we characterized memory B cell compartments and HIV-specific memory B cells responses using flow cytometry and ELISPOT. ECs preserved their memory B cell compartments and in contrast to treated patients, maintained detectable HIV-specific memory B cell responses. All ECs presented IgG1 + HIV-specific memory B cells but some individuals also preserved IgG2 + or IgG3 + responses. Importantly, we also analyzed the capacity of sera from ECs to neutralize a panel of HIV strains including transmitted/founder virus. 29% and 21% of HLA-B*57 + and HLA-B*57 − ECs, respectively, neutralized at least 40% of the viral strains tested. Remarkably, in HLA-B*57 + ECs the frequency of HIV-Env-specific memory B cells correlated positively with the neutralization breadth suggesting that preservation of HIV-specific memory B cells might contribute to the neutralizing responses in these patients.

Abbreviations: HIV, human immunodeficiency virus; Env, HIV envelope protein; cART, combined antiretroviral therapy; EC, elite controller; IgG, immunoglobulin G; (n)Ab, (neutralizing) antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; CTL, cytotoxic T cell; T/F, transmitted/founder virus; PBMC, peripheral blood mononuclear cells; ASC, antibody secreting cell; AM, activated memory B cells; RM, resting memory B cells; IM, intermediate memory B cells; MZ-like B cells, marginal zone-like B cells; TLM B cells, tissue like memory B cells.

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1. Introduction

HIV-1 (HIV) infection alters B cell differentiation resulting in spontaneous immunoglobulin secretion, hypergammaglobulinemia (Lane et al., 1983) and decrease in memory B cell frequencies (Moir et al., 2008; Hu et al., 2015; Buckner et al., 2013). HIV-specific antibodies (Abs), with the capacity to neutralize the autologous virus, appear several months after infection. However, these Abs poorly neutralize heterologous HIV strains (Tomaras et al., 2008; Moog et al., 1997; Wei et al., 2003; Deeks et al., 2006; Richman et al., 2003; Gray et al., 2007). Cross-reactive neutralizing Abs, are produced only 2 to 4 years after seroconversion (Gray et al., 2011; Mikell et al., 2011; Richman et al., 2003) and at low titers in most individuals (Hraber et al., 2014). Only 20% of patients harbor high titers of cross-reactive neutralizing Abs (Doria-Rose et al., 2010). Among them, 1% were identified as elite neutralizers based on the capacity of their plasma to neutralize, across clades, a large panel of HIV strains (Li et al., 2007; Simek et al., 2009). Broadly neutralizing monoclonal Abs (bnAbs) were cloned from HIV-specific memory B cells isolated from these patients (Scheid et al., 2009; Mouquet, 2014; Sok and Burton, 2016). Understanding how these bnAbs are generated in HIV-infected individuals could lead the path to the development of an antibody-based vaccine. In viremic patients, the breadth of neutralization has been associated with higher viral loads (Doria-Rose et al., 2010; Piantadosi et al., 2009; Deeks et al., 2006; Sajadi et al., 2011; Doria-Rose et al., 2009; Sather et al., 2009; Rodriguez et al., 2007), duration of viral exposure and viral diversity (Rusert et al., 2016).

HIV-infected individuals who naturally control HIV infection without combined antiretroviral therapy (cART) (Saez-Cirion and Pancino, 2013), in particular elite controllers (ECs, <1% of HIV-infected individuals) who maintain very low to undetectable viremia (Lambotte and Delfraissy, 2005; Grabar et al., 2009) represent a unique chance to study immune responses potentially involved in viral suppression (Walker and Yu, 2013). A fraction of ECs exhibit potent cytotoxic CD8+ T cell responses against HIV-infected cells (Sáez-Cirión et al., 2007; Betts et al., 2006; Hersperger et al., 2011), often associated with the expression of the HLA-B*57 allele (Migueles et al., 2000; Lambotte and Delfraissy, 2005; Betts et al., 2006). HIV-specific CD4+ T cells of ECs express high avidity T cell receptors (TCRs) suggesting that T cell helper responses contribute to HIV-control (Benati et al., 2016). In contrast, several studies have shown that ECs present lower cross-neutralizing Ab responses as compared to viremic individuals (Lambotte et al., 2009; Pereyra et al., 2008; Bailey et al., 2006; Sajadi et al., 2011). However, among ECs, there is a marked heterogeneity, some presenting broad cross-neutralizing capacities while others show minimal or no neutralization (Lambotte et al., 2009; Scheid et al., 2009; Pereyra et al., 2008; Bailey et al., 2006; Sajadi et al., 2011). Non-neutralizing Ab responses might also exert significant antiviral activities (Chung et al., 2015). In particular, titers of Abs executing antibody-dependent cell-mediated cytotoxicity (ADCC) have been shown to be higher in ECs (Lambotte et al., 2009) and predominant in HLA-B*57− ECs as compared to HLA-B57+ ECs (Lambotte et al., 2013). More recently, HIV-control has been linked to the capacity of the sera from ECs to perform multiple effector functions (Ackerman et al., 2016). Indeed, depending on their isotype, Abs exhibit different effector functions such as Fcγ receptors (FcγR) binding, initiation of ADCC and activation of the complement cascades. Although the immunoglobulin G1 (IgG1) subclass dominates HIV-specific responses, the proportion of IgG isotypes might vary depending on individuals, the HLA status and the clinical parameters (Binley et al., 2008, Banerjee et al., 2010, Ackerman et al., 2016, French et al., 2013). Ackerman et al. showed that the sera from ECs exhibiting strong polyfunctional antiviral activities are enriched in Abs of IgG1 and IgG3 subclasses (Ackerman et al., 2016). IgG2 Abs to the HIV Gag protein have been associated with long-term nonprogression (Martinez et al., 2005; Ngo-Giang-Huong et al., 2001) and seem more abundant in HLA-B*57− ECs compared to HLA-B*57+ ECs (French et al., 2013). Therefore, the quality of Ab responses, defined by the diversity of Ab subclasses should be considered when characterizing Ab responses elicited by HIV infection and vaccine candidates.

A successful vaccine should lead to the generation of long-lived plasma cells and memory B cells that are thought to be essential for sustained humoral immunity. However, during chronic infection, the persistence of viral antigens alters B cell differentiation into memory cells (Colineau et al., 2015). Memory B cells can be divided in 4 subpopulations: activated memory (AM, CD27+CD21−), resting memory (RM, CD27+CD21+), intermediate memory (IM, CD27−CD21+) and tissue-like memory B cells (TLM, CD27−CD21−) (Moir et al., 2008). AM and TLM B cells (the latter corresponding to anergic cells) are overrepresented in untreated HIV-infected patients (Moir et al., 2008; Pensieroso et al., 2013) and are associated with higher levels of viremia (Kardava et al., 2014). In contrast, RM cells that contribute to maintaining humoral responses, are decreased upon infection (Good et al., 2009; Moir and Fauci, 2013). Remarkably, HIV-specific B cells are enriched in TLM and AM B cell subsets but decreased in RM cells (Kardava et al., 2014). Compared to the levels in HIV-negative individuals, cART restores TLM and AM B cell proportions and only partially the RM compartment (Moir et al., 2008; Moir et al., 2010; Pensieroso et al., 2013). Although ECs do not clear the infection, studying their memory B cell responses could help understand the maintenance of long lasting humoral immunity in the presence of low to undetectable antigen loads. In ECs, RM and AM B cell proportions are higher compared to treated HIV-infected patients but no differences were observed concerning the percentage of TLM cells (Pensieroso et al., 2013). However, in ECs, the frequency of HIV-specific TLM B cells is reduced compared to treated HIV-infected patients (Buckner et al., 2016). Taken together, these results suggest that ECs preserve their memory B cell compartments but also exhibit features of viremic individuals (increased AM cells). Whether this preservation of the memory B cell subsets, in ECs, is associated with the maintenance and/or a higher frequency of HIV-specific memory B cells expressing various Ab subclasses remains an open question. In addition, potential correlations between HIV-specific memory B cell frequencies and the neutralization breadth in sera have not been investigated so far. Two studies previously analyzed potential correlations between Ab responses and Ab secreting cells (Bussmann et al., 2010; Doria-Rose et al., 2009). Bussman et al. asked whether the frequency of Env/gp120-specific B cells might correlate with the Ab titers to Env/gp120 protein (Bussmann et al., 2010) while Doria-Rose et al. studied the frequency of plasmablasts that spontaneously secret HIV-specific Abs and the breadth of neutralization (Doria-Rose et al., 2009). Both studies failed to observe any correlation between these HIV-specific B cell responses and the Ab profiles.

In the present study, we characterized memory B cell responses in a cohort of ECs either positive or negative for the HLA-B*57 protective allele. We analyzed whether the preservation of B cell compartments might be linked to the capacity of B cells to secrete HIV-specific Abs. We compared B cell responses in HLA-B*57+ and HLA-B*57− ECs with either that of aviremic patients undergoing successful cART or HIV-negative individuals. We observed a global preservation of memory B cell compartments in ECs with a proportion of TLM comparable to what was observed in cART and HIV-negative individuals. Interestingly, HIV-specific B cells were detected in 82% of ECs. In contrast, only 7% of cART patients presented HIV-specific responses whereas all groups exhibited similar levels of Influenza-specific B cell responses. HIV-specific responses consisted mainly of IgG1 secreting B cells although HIV-specific IgG2 and IgG3 secreting B cells were detected in a third of ECs. Next we analyzed whether these B cell responses might correlate with the capacity of patients' sera to neutralize HIV. For this purpose, we used mostly difficult-to-neutralize tier-2 transmitted/founder (T/F) viruses. 89% of sera from ECs neutralized at least one HIV strain tested and 8% blocked infection of at least 40% of difficult-to-neutralize tier-2 T/F viruses. Remarkably, among HLA-B*57+ ECs, the frequency of Env-specific memory B cells correlated positively with the capacity to neutralize T/F HIV strains, suggesting that these cells might contribute

to the neutralizing responses in this group of ECs. Overall, through the analysis of Env-specific memory B cell frequencies, the isotype diversity and the neutralization breadth, our results reveal major differences between HLA-B*57 + and HLA-B*57 − ECs.

2. Materials and Methods

2.1. Patients and Samples

EC (n = 37) were recruited from the CO21 CODEX cohort implemented by the ANRS (Agence nationale de recherches sur le SIDA et les hépatites virales). Whole blood and PBMC were cryopreserved at enrolment. Ten million PBMCs from ECs were available for this study. ECs were defined as HIV-infected individuals maintaining viral loads (VL) under 400 copies of HIV RNA/mL without cART for >5 years. ECs were divided in 2 groups: HLA-B*57 + (n = 18) or HLA-B*57 − (n = 19). HIV-infected efficiently treated patients (cART) (n = 13) were recruited at Kremlin Bicêtre Hospital. They were treated for at least 1 year (mean of 10 years) and had an undetectable viral load using standard assays. HIV-negative individuals (n = 12) were anonymous blood donors (Établissement Français du sang). A detailed description of the patients is provided in Table 1, including the median and interquartile range for age (at the time of the study), CD4 T cell count and RNA load for each group. HIV-RNA loads were measured on site with different real-time PCR-based assays; depending on the date of enrolment in the cohort and the assay routinely used on each site, the VL detection limit varied

Table 1
Clinical and epidemiological characteristics of the study groups.

		#	Sex	Age	Infection (Years)	HLA-B(1)	HLA-B(2)	VL RNA (copies/mL)	us VL RNA (copies/mL)	Proviral DNA (copies/ 10^6 PBMC)	CD4 count (cells/mm3)	Nadir CD4 (cells/mm3)	cART	cART (years)	Treatment	Color code
EC Median (IQR)				49 (33-63)	16 (6-29)					15 (2-351)	825 (279-1400)					
	*B*57+*			50 (40-63)	20 (8-29)					7 (4-351)	862 (279-1400)					
Elite Controllers (EC)	HLA-B*57+	1	F	41	11	18	57	43	43	4	702	-	NO	-	-	
		2	M	48	8	53	57	< 40	3	351	801	-	NO	-	-	
		3	F	56	18	44	57	< 10	< 1	4	1400	-	NO	-	-	
		4	F	63	10	52	57	< 10	< 1	15	630	-	NO	-	-	
		5	F	57	17	57	NA	20	NA	7	999	-	NO	-	-	
		6	F	55	26	18	57	< 20	< 1	4	770	-	NO	-	-	
		7	M	51	26	51	57	42	17	24	824	-	NO	-	-	
		8	F	44	25	57	70(71)	240	NA	33	826	-	NO	-	-	
		9	M	50	26	44	57	< 20	< 13	NA	1201	-	NO	-	-	
		11	M	63	27	7	57	60	135	213	1022	-	NO	-	-	
		12	F	49	29	8	57	< 40	< 1	4	512	-	NO	-	-	
		13	M	51	24	7	57	47	NA	4	1020	-	NO	-	-	
		14	F	43	10	53	57	166	NA	27	862	-	NO	-	-	
		15	M	44	9	27	57	23	NA	4	979	-	NO	-	-	
		16	F	40	20	57	NA	< 20	< 1	16	880	-	NO	-	-	
		17	M	62	27	7	57	< 40	NA	NA	1074	-	NO	-	-	
		18	F	48	19	14(65)	57	< 20	< 1	4	279	-	NO	-	-	
		19	F	52	24	5703	15(63)	*(< 40)*			1153	-	NO	-	-	
	*B*57-*			48 (33-59)	13 (6-27)					23 (2-159)	723 (432-1311)					
	HLA-B*57-	20	F	34	13	40(60)	15(62)	< 40	NA	3	1078	-	NO	-	-	
		21	F	53	6	35	52	< 40	< 1	4	694	-	NO	-	-	
		22	F	40	6	18	NA	< 10	2	83	1130	-	NO	-	-	
		23	F	37	12	8	76	168	NA	159	529	-	NO	-	-	
		24	F	36	6	72		153	NA	10	432	-	NO	-	-	
		25	F	48	24	27/81	71	61	NA	46	823	-	NO	-	-	
		26	M	40	23	27	45	< 40	1	13	585	-	NO	-	-	
		27	F	57	26	27	51	< 40	< 1	43	1060	-	NO	-	-	
		28	F	49	8	44	NA	< 20	< 1	126	853	-	NO	-	-	
		29	M	59	11	40(60)	15(62)	71	NA	26	519	-	NO	-	-	
		30	F	50	13	44	45	50	NA	19	723	-	NO	-	-	
		31	M	50	21	39	41	103	NA	109	504	-	NO	-	-	
		32	F	50	26	51	41	< 20	< 1	4	1311	-	NO	-	-	
		33	F	37	14	44	82	62	NA	3	500	-	NO	-	-	
		34	M	33	7	49	50	413	NA	42	600	-	NO	-	-	
		35	M	38	7	18	44	90	NA	127	1122	-	NO	-	-	
		36	M	52	10	35	40(60)	< 50	1	2	1234	-	NO	-	-	
		37	M	50	16	13	14	32	NA	3	1085	-	NO	-	-	
		38	M	48	27	27	51	< 20	22	23	561	-	NO	-	-	
cART Median (IQR)				50 (31-66)	16 (2-30)						570 (288-787)	219 (24-592)		12 (1-17)		
Aviremic treated HIV-infected patients (cART)		42	M	66	25	NA	NA	undetect.		NA	561	50	YES	15	Isentress+Norvir+Prezista	
		43	F	58	27	NA	NA	undetect.		NA	448	24	YES	17	Kivexa+Viramune	
		44	F	31	2	NA	NA	undetect.		NA	570	369	YES	2	Kivexa+Norvir+Reyataz	
		45	M	54	17	NA	NA	undetect.		NA	787	251	YES	16	Invitress+Kaletra	
		46	M	50	16	NA	NA	undetect.		NA	572	222	YES	16	Isentress+Truvada	
		47	M	58	9	NA	NA	undetect.		NA	580	342	YES	1	Isentress+Kivexa	
		48	M	42	30	NA	NA	undetect.		NA	415	173	YES	9	Isentress+Truvada	
		49	F	35	4	NA	NA	undetect.		NA	288	149	YES	4	Atripla	
		50	M	49	21	NA	NA	undetect.		NA	609	219	YES	12	Kivexa+Viramune	
		51	M	51	14	NA	NA	undetect.		NA	330	193	YES	13	Atripla	
		52	M	57	27	NA	NA	undetect.		1740	335	172	YES	17	Kivexa+Viramune	
		53	F	42	13	NA	NA	undetect.		NA	587	537	YES	5	Atripla	
		54	M	46	10	7	56	undetect.		290	765	592	YES	10	Truvada+Reyataz	

[a] NA, data not available; M, male; F, female; VL, viral load; us VL, ultra sensitive VL; (-) not applicable.

from 50 to 10 copies/mL. To better quantify low levels of viral replication, VL were also determined using an ultrasensitive, real-time PCR technique (GENERIC HIV, Biocentric, Bandol, France) with a threshold ranging from 1 to 13 copies/mL, depending on the available plasma volume. Total cells associated HIV-1 DNA levels were quantified using the real time PCR GENERIC HIV-DNA assay (Biocentric, Bandol, France) (Avettand-Fenoel et al., 2009).

2.2. Ethic Statement

All the subjects provided their written informed consent to participate in the study. The CO21 CODEX cohort and this sub-study were funded and sponsored by ANRS and approved by the Ile de France VII Ethics Committee. The study was conducted according to the principles expressed in the Declaration of Helsinki.

2.3. Flow Cytometry

Cell viability was evaluated using LIVE/DEAD® (ThermoFisher Scientific) and the following Abs were used: CD19-APCCy7 (SJ25C1), CD21-APC (B-ly4), CD27-PE (M-T271), IgD-PECF594 (IA6-2), IgG-BV605 (G18-145), CD38-V450 (HB7) (all from BD biosciences), IgM-AF700 (CH2, Exbio) and IgG2-AF488 (HP6002, Southern Biotech). Staining assays were performed using standard procedures in PBS containing 0.5% BSA and 2 mM EDTA (20 min at 4 °C). Samples were processed on a Fortessa cytometer using FACSDiva software (BD Biosciences) and further analyzed using FlowJo2 software (Tree Star).

2.4. Differentiation of B cells into Antibody Secreting Cells

PBMCs were thawed and cultured in Yssel medium supplemented with 1% human AB serum (Institut Jacque Boy), 0.5 μg/mL of TLR7/8 ligand (R848, InVivoGen) and 100 U/mL rhIL-2 (Miltenyi Biotec). Cells were cultured at 1×10^6 cells/mL. After 6 days, cells were harvested, the proportion of B cells evaluated using flow cytometry (using anti-CD19 antibody, not shown) and the frequency of HIV-specific B cells evaluated using B cell-ELISPOT assay (Pinna et al., 2009).

2.5. B Cell-ELISPOT Assay

ELISPOT plates (Millipore MSIPN4550) were pre-wet with 35% ethanol (1 min), washed with PBS and coated overnight at 4 °C with 15 μg/mL anti-IgG antibodies (Mabtech, MT91/145) or viral antigens diluted in PBS. HIV antigens included a trimeric cleavage-deficient recombinant glycoprotein from the YU-2 clade B (tier-2) HIV viral strain (gp140Yu2b, originally described in (Yang et al., 2000)) produced in HEK293T-derived cells by transient transfection as previously described (Lorin and Mouquet, 2015), gp41S30 (Licence N°WO2012101509 A2) and gp160THO (92THO23), oligomeric envelope glycoprotein produced from a hybrid HIV env gp120 from CRF01_AE (92TH023) and subtype B (LAI) gp41, which is deleted in the principal immunodominant domain (PID), expressed by vaccinia virus in BHK21 cells (Thongcharoen et al., 2007). HIV antigens were coated at 10 μg/mL. Influenza antigens (5 μg/mL, 2015 VAXIGRIP vaccine, Sanofi Pasteur Msd) and keyhole limpet hemocyanin (KLH, 10 μg/mL, Sigma-Aldrich) were used as positive and negative controls, respectively. Plates were washed with PBS and saturated with RPMI containing 10% FBS. Six days post activation, 1500 to 3000 or 1.5×10^5 to 3×10^5 B cells/well were plated for total IgG or antigen-specific detections, respectively, and incubated overnight at 37 °C in RPMI + 10% FBS. Plates were then washed with PBS + 0.05% Tween-20 prior incubation with biotinylated anti-IgG (1 μg/mL, MT78/145, Mabtech), anti-IgG1 (1 μg/mL, G17-1, BD), anti-IgG2 (0.2 μg/mL, HP6200, Mabtech) or anti-IgG3 (0.2 μg/mL, HP6050, Southern Biotech) Abs (2 h, RT). Elispots were revealed using alkaline-phosphatase coupled streptavidin (0.5 U/mL, Roche Diagnostics, 1 h RT) and 50 μL BCIP/NBT substrate (15 min, Sigma). The reaction was stopped using water. The number of spots was counted using AID reader (Autoimmun Diagnostika GmbH). For each experimental condition, the Elispot was performed mostly in triplicates and at least in duplicates. Frequency of antigen-specific B cells was calculated taking account the number of CD19 + B cells plated.

2.6. HIV-Specific IgG Ab Detection by Elisa

96-well plates were coated overnight with a sheep anti-human IgG (1 μg/mL in carbonate buffer, Binding Site) for the detection of total IgGs, or with gp140Yu2b or gp41S30 (2 μg/mL in carbonate buffer) for the detection of anti-gp140 or anti-gp41 IgGs, respectively. Plates were washed and saturated with PBS containing 5% BSA and incubated with diluted sera (2 h, at 37 °C). Plates were then washed and a secondary goat anti-human IgG-HRP (HorseRadish Peroxidase) added (1 h at 37 °C, 0.2 μg/mL in PBS, Southern Biotech). Finally, TMB (3,3′, 5,5′ TetraMethylBenzidine) substrate was added. After 30 min, the reaction was stopped (using 25 μL of 1 M H_2SO_4 per well) and the optical density (OD) read at 450 nm (reference 650 nm). The ratios of HIV-specific IgG were calculated as (OD of anti-gp140 or anti-gp41 IgGs × serum dilution) / (OD of total IgG x serum dilution).

2.7. Viruses and TZM-bl Neutralization Assays

For neutralization, pseudoviruses were produced by cotransfecting 293T cells with HIV-1 env expression plasmid SF162.LS, QH0692.42 and YU2 and the env-deficient HIV-1 backbone plasmid (pSG3ΔEnv). Infectious molecular clones: CH058, CH077, CH106, RHPA, THRO4156.18, REJO 4541.67 and TRJO4551.58 were produced by transfection on 293T cells. These different strains were obtained through the NIH AIDS reagent program.

Sera were tested for their ability to neutralize HIV-1 using TZM-bl neutralization as described previously (Li et al., 2005). As negative controls, HIV-negative sera (purchased at Etablissement Français du Sang) were used and the capacity of EC's sera to neutralize MuLV was assessed. One tier-1 reference strains (SF162.LS), eight tier-2 strains (YU2, QH0692.42, CH058, CH077, CH106, RHPA, THRO4156.18 and REJO4541.67) and one tier-3 strain (TRJO4551.58) were used. CH058, CH077, CH106, RHPA, THRO4156.18 and REJO4541.67 and TRJO4551.58 are T/F viruses. The 50% inhibitory reciprocal dilution (IRD50) was defined as the sample reciprocal dilution that caused a 50% reduction in relative luminescence units (RLU) (Li et al., 2005).

2.8. Statistics

Statistical significances (p-values) were determined using a Kruskal-Wallis test or a Mann-Whitney test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) and associations between continuous variables were evaluated using Spearman rank order correlation test using Prism software (GraphPad) and "Visualization of a Correlation Matrix" R package version 0.77 (https://CRAN.R-project.org/package=corrplot).

3. Results

We characterized memory B cell responses and HIV cross-neutralization potential in a cohort of ECs either positive or negative for the HLA-B*57 protective allele. We compared HLA-B*57 + and HLA-B*57 − ECs with aviremic patients undergoing successful cART or HIV-negative individuals (Table 1).

3.1. Memory B Cell Compartments are Preserved in EC

Using flow cytometry, we first analyzed the peripheral memory B cell compartments (as shown in Supplemental Fig. 1). The proportion of CD19 + B cells was slightly higher, although not significantly, in ECs compared to HIV-negative donors (Fig. 1a). ECs, cART and HIV-negative

donors presented no significant differences concerning the proportions of AM, IM and RM B cells (Fig. 1b) or other populations studied (total memory B cells, naïve B cells, IgG+ and IgG2+ memory B cells, plasmablasts and Marginal Zone-like (MZ-like) B cells (Supplemental Fig. 2). In contrast, a significant increase of TLM B cell proportion was detected in HLA-B*57− ECs compared to HIV-negative individuals (Fig. 1b, $p = 0.0247$). Overall, with the exception of the TLM B cells that were slightly expanded in HLA-B57− ECs, the B cell compartments seemed preserved in ECs.

3.2. EC Maintain HIV-Specific Memory B Cell Responses with Stronger Proliferative Capacity

We then evaluated the frequency of HIV-specific memory B cells using ELISPOT (Fig. 2 and Supplemental Fig. 3). The frequencies of B cells secreting total IgG, IgG1 or IgG3 were not significantly different between the 3 groups (Supplemental Fig. 3). In contrast, we noticed a reduced frequency of IgG2 secreting B cells in ECs compared to HIV-negative donors ($p = 0.0131$, Supplemental Fig. 3c). To detect antigen-specific B cell responses, we used different HIV envelopes (HIV Env): gp140Yu2b, gp41S30, gp160THO and Influenza antigens or KLH as positive and negative controls, respectively. The gp140Yu2b trimers can capture the B-cells and bind to non-neutralizing and cross-neutralizing Abs (Scheid et al., 2009; Mouquet et al., 2011) of all specificities and more importantly, of all types of bNAbs and clonal variants (Scheid et al., 2011; Mouquet et al., 2012), except PG16-like Abs. Gp41S30 is the target of Abs to gp41 such as bNAbs against the MPER (e.g. 2F5, 4E10 and 10E8) or non-NAbs against the PID (e.g. 7B2). Gp160THO was used in the RV144 vaccine trial that showed an estimated 31% vaccine efficacy (Rerks-Ngarm et al., 2009). It allows the capture of Abs directed to gp120 and to gp41 except Abs directed to the PID and PG16-like Abs. As an additional control, the frequency of HIV-specific B cells was evaluated in healthy HIV-negative donors (Fig. 2a). Remarkably, 82% of ECs (27/33 ECs tested) but only 7% of cART patients (1/13) exhibited a positive response against HIV Envs (Fig. 2a). This very low frequency of responders among cART patients was consistent with other studies describing that upon initiation of cART the frequency of HIV-specific B cells drops to low or undetectable levels (Morris et al., 1998; Bussmann et al., 2010; Buckner et al., 2016). This difference is strictly specific to HIV antigens since both groups reacted to Influenza antigens (63% and 75% reacting ECs and cART-patients, respectively) (Fig. 2b). Therefore, although both ECs and cART patients have undetectable viral loads, Env-specific memory B cells were detected mainly in ECs.

3.3. HIV-Specific Memory B Cell Responses are Mainly of the IgG1 Isotype

We next analyzed the specificity and isotype of HIV-specific secreted Abs in ECs (Fig. 3). Patients with detectable antibody responses to gp140 also reacted against gp41 and gp160 (Fig. 3A). In ECs, IgG+ B cells specific for gp140, gp41 and gp160 represented respectively 0.24, 0.20 and 0.13 mean % of IgG+ B cells (Fig. 3A). In ECs, the frequencies of IgG+ Influenza-specific and HIV Env-specific B cells were in the same order of magnitude (Fig. 3a). Note that in ECs, cART and HIV-negative donors, we did not observe a significant difference in the frequency of Influenza-specific B cells (Supplemental Fig. 4).

HIV Env- and Flu-specific B cell responses in ECs were mainly mediated by IgG1 Abs, which represented nearly 50% of responses (Fig. 3b). In addition, all patients reacting to HIV Env antigens exhibited an HIV-specific IgG1 response. In contrast, only 35% and 26% of ECs showed

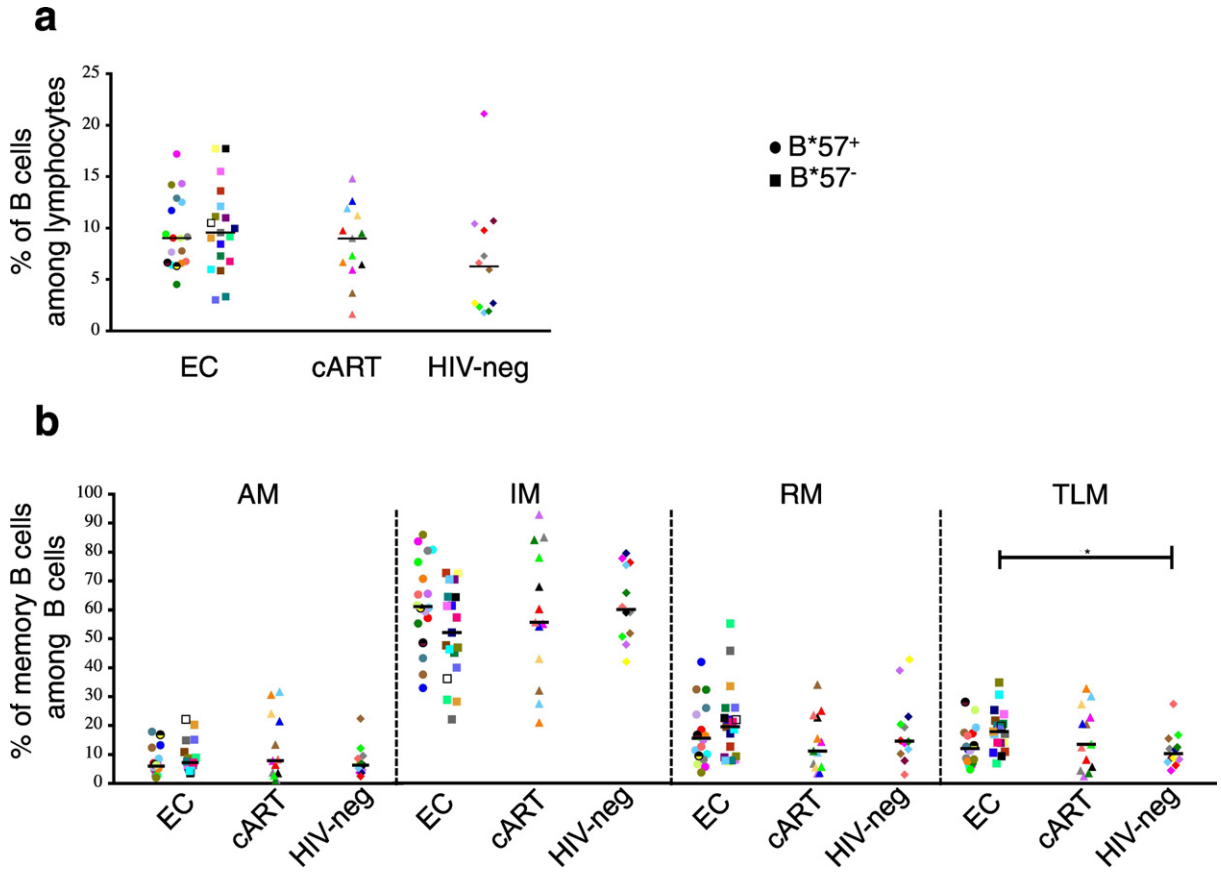

Fig. 1. Memory B cell compartments are preserved in ECs. In PBMC from ECs (n = 37), cART (n = 13) and HIV-negative donors (n = 12): (a) frequency of CD19+ B cells among lymphocytes and (b) frequencies of AM (CD27+CD21−), RM (CD27+CD21+), IM (CD27−CD21+) and TLM (CD27−CD21−) B cells among CD19+ B cells. Each individual is represented by a specific dot on each graph (shape and color). Circle: HLA-B*57+ EC; Square: HLA-B*57− ECs. The statistical significance was calculated using a Kruskal-Wallis test followed by a Dunn's test (*$p < 0.05$). Bars indicate median values.

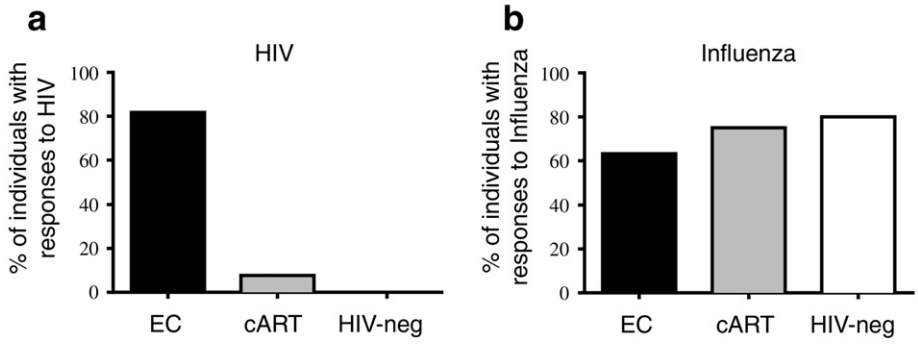

Fig. 2. ECs maintain HIV-specific memory B cell responses. Percentage of ECs (n = 33), cART (n = 13) and HIV-negative (n = 6) donors presenting memory B cell responses against (a) at least one HIV antigen ($gp140_{Yu2b}$, $gp41_{S30}$ or $gp160_{THO}$) and (b) Influenza vaccine antigens (2015 VAXIGRIP vaccine). B cell memory responses were evaluated by B cell ELISPOT.

gp140-specific IgG2 + or IgG3 + B cell responses, respectively (Fig. 3c–d). Note that IgG2 + and IgG3 + responses were not necessarily detected in the same patients (Fig. 3c–d).

Finally, whatever the targeted antigens, the proportion of ECs presenting IgG, IgG1, IgG2 and IgG3 Env-specific memory B cell responses and the magnitude of these responses were similar in HLA-B*57 − and HLA-B*57 + ECs (Fig. 3).

*3.4. In HLA-B*57 + ECs, the Frequency of Env-Specific Memory B Cells Correlates with the Neutralization Breadth*

We then asked whether HIV-specific B cell memory responses might be associated with the capacity of ECs to neutralize HIV-1 infection. To this end, we tested the ability of the sera from ECs to neutralize a reference (tier-1) strain, 8 difficult-to-neutralize tier-2 strains (including 7 transmitted founder (T/F) strains) and one highly difficult-to-neutralize tier-3 T/F strain (Fig. 4). Sera from most ECs (87%) neutralized the neutralization-sensitive $HIV\text{-}1_{SF162}$ strain. 25% of sera neutralized at least 4 HIV strains out of the 10 tested (Fig. 4). 48% blocked infection of at least one tier-2 virus. 36% blocked at least one tier-2 T/F virus (Fig. 4b) and 8% blocked at least 40% of tier-2 T/F strains (Fig. 4b). This demonstrates that some ECs effectively neutralize difficult-to-neutralize tier-2 T/F. Interestingly, when examining the neutralizing potential, the sera from HLA-B*57 + ECs had a twofold higher capacity to neutralize T/F strains than the sera from HLA-B*57 - ECs (Fig. 4a, average of 14% T/F neutralized versus 7% for HLA-B*57 + and HLA-B*57 − ECs, respectively), although this difference was not statistically significant ($p = 0.19$). In addition, 30% of the HLA-B*57 + versus 16% of the HLA-B*57 − ECs' sera neutralized >20% of the T/F viruses tested (Fig. 4b). This difference was almost exclusively due to the capacity of HLA-B*57 + ECs' sera to neutralize the T/F virus REJO.

To further analyze Ab responses in ECs, we examined potential correlations between the frequency of Env-specific B cells, evaluated in ELISPOT, and the percentage of neutralized strains for each individual (Fig. 5). In ECs, the capacity to neutralize all HIV strains or T/F viruses was neither associated with the frequency of anti-gp140 nor anti-gp41 secreting B cells (among IgG + B cells), for all IgG subclasses analyzed (Fig. 5a and not shown). However, by separating ECs into HLA-B*57 + and HLA-B*57 − donors, we showed that, among HLA-B*57 + ECs the frequency of gp140- and gp41-specific B cells correlated with

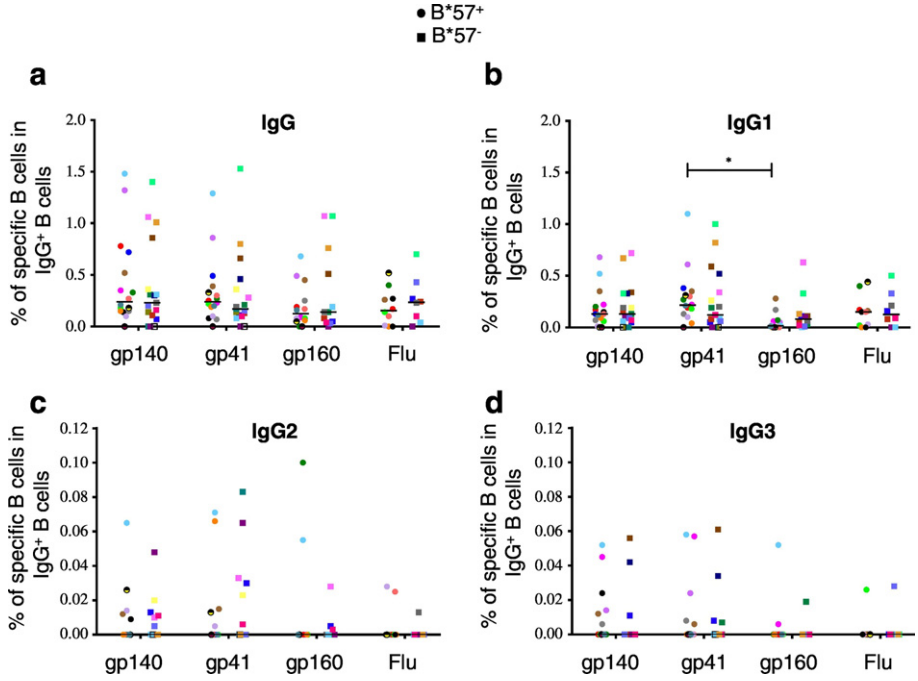

Fig. 3. In ECs, HIV-specific memory B cell responses are mainly of the IgG1 isotype. Percentage of antibody secreting B cells specific for HIV-Env antigens (gp140Yu2b, gp41S30 or gp160THO) and Influenza vaccine antigen (Flu, 2015 VAXIGRIP vaccine) presented according to the Ab isotype: (a) total IgG +, (b) IgG1 +, (c) IgG2 + and (d) IgG3 + antigen-specific ASC. Each individual is represented by a specific dot on each graph (shape and color). Statistical significance was calculated using a Kruskal-Wallis test followed by a Dunn's test (*$p < 0.05$). Bars indicate median values.

a

		Tier-1	Tier-2								Tier-3		Neutralized strains (all)			Neutralized Tier-2 strains			Neutralized T/F strains		
	ID	SF162	YU2	QHO	CH058	CH077	CH106	RHPA	REJO	THRO	TRJO	MuLV	/10	%	Average	/8	%	Average	/7	%	Average
HLA-B*57+	1	300											1	10		0	0		0	0	
	2	1000											1	10		0	0		0	0	
	3					21							1	10		1	12.5		1	14.3	
	4	500											1	10		0	0		0	0	
	5	1000		30	11	21		40					4	40		3	37.5		2	28.6	
	6	1000											1	10		0	0		0	0	
	7	1000	20			60		80	20				5	50		4	50		3	42.9	
	8	1000				195			80				3	30		2	25		2	28.6	
	9	1000		80				20					3	30	24%	2	25	18%	1	14.3	14%
	11	1000	60	200	51	51	20	150	40				8	80		7	87.5		5	71.4	
	12	80											1	10		0	0		0	0	
	13	1000	60	90					40				4	40		3	37.5		1	14.3	
	14	120											1	10		0	0		0	0	
	15												0	0		0	0		0	0	
	16	500											1	10		0	0		0	0	
	17	1000		150				20					4	40		3	37.5		2	28.6	
	18	1000											1	10		0	0		0	0	
HLA-B*57-	20												0	0		0	0		0	0	
	21												0	0		0	0		0	0	
	22												0	0		0	0		0	0	
	23	1000		30									2	20		1	12.5		0	0	
	24	400											1	10		0	0		0	0	
	25	1000	40	100	110	11		100					5	50		4	50		2	28.6	
	26	1000		30		50		70					4	40		3	37.5		2	28.6	
	27	1000		40									2	20		1	12.5		0	0	
	28	500	20	110	80	80		110					6	60		5	62.5		3	42.9	
	29	500											1	10	21%	0	0	15%	0	0	7%
	30	400		30	11	11		20					3	30		2	25		1	14.3	
	31	500		30									2	20		1	12.5		0	0	
	32	1000											1	10		0	0		0	0	
	33	1000		30									2	20		1	12.5		0	0	
	34	1000											1	10		0	0		0	0	
	35	500											1	10		0	0		0	0	
	36	20											1	10		0	0		0	0	
	37	1000	90	90									3	30		2	25		0	0	
	38	1000	80	150		30							4	40		3	37.5		1	14.3	
	median IRD50	1000	50	80	51	40	20	70	40												

Neg
IC50 < 20
20 ≤ IC50 < 100
100 ≤ IC50 < 300
300 ≤ IC50 < 900
IC50 ≥ 1000

b

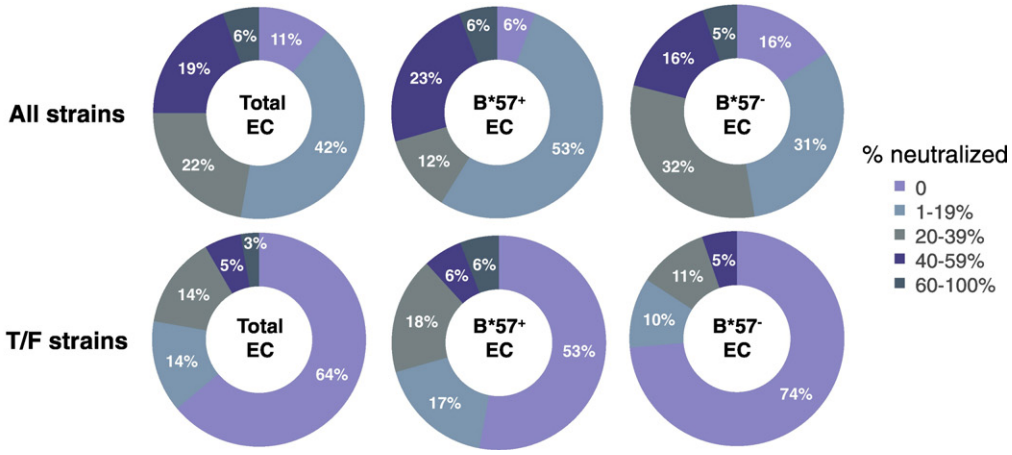

Fig. 4. Capacity of ECs to neutralize tier-2 T/F HIV strains in the TZM-bl assay. Sera from ECs (n = 36) were tested against one lab and 8 difficult-to-neutralize tier-2 HIV strains, including 7 T/F strains. The capacity to neutralize MuLV was used as a negative control. (A) Neutralization data are shown as the reciprocal serum dilution that neutralized 50% of the infection (IRD50) tested. A color code (right) indicates the potency of neutralization based on IRD50. The gray color indicates a decreased infection (at 1/20 dilution) that did not reach consistently 50% inhibition. An absence of color means that no neutralization was found at the lowest dilution tested (1/20). (B) Neutralization breadth expressed as percentage of neutralized strains for all strains (top panel) and T/F strains (lower panel) in all ECs (Total ECs, left rings), HLA-B*57 + (B*57 + ECs, middle rings) and HLA-B*57 − ECs (B*57 − ECs, right rings). The numbers indicate the % of patients for each fraction.

the capacity to neutralize all HIV strains ($p = 0.01$) including T/F viruses ($p = 0.02$) (Fig. 5a–b, blue square). Dissecting Env-specific isotypic responses in HLA-B*57 + ECs, we observed that gp41-specific IgG1 + B cell frequencies also correlated with the neutralization of all HIV strains ($p = 0.02$) and T/F strains ($p = 0.02$) (Fig. 5b, blue rectangle). In addition in these ECs, gp140-specific B cell frequencies correlated with the IRD50 for 6 out of 8 tier-2 T/F neutralized (Fig. 4a and not shown). Interestingly in HLA-B*57 + ECs, Env-specific IgG3 + memory B cell frequencies correlated positively with both total IgG and IgG1 Env-specific responses without being directly associated to the neutralization potential (Fig. 5b).

In contrast, in HLA-B*57 − ECs, the frequency of Env-specific B cells did not correlate positively with any of the neutralizing parameters analyzed. In fact in HLA-B*57 − ECs, an inverse correlation was observed between the frequency of gp140-specific IgG B cells, or of gp140- and gp41-specific IgG1 B cells and the number of T/F viruses neutralized (Fig. 5b, red rectangles, $p < 0.05$). A significant inverse correlation was also observed between the frequencies of gp140- and gp41-specific

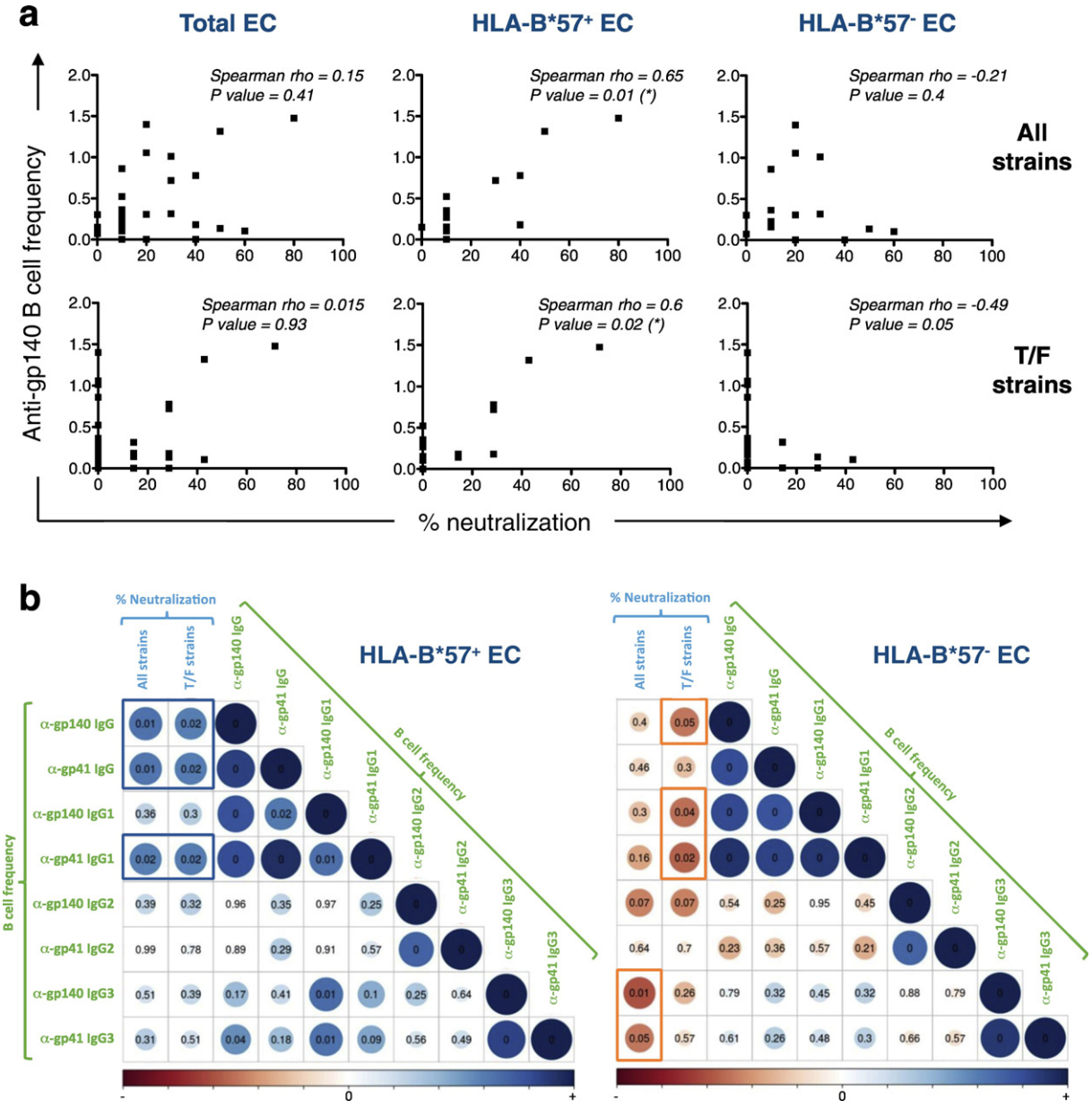

Fig. 5. In HLA-B*57 + ECs, the frequency of HIV-specific B cells correlates with the neutralization of tier-2 T/F virus. (a) Association of HIV gp140-specific B cell frequency (among IgG + B cells) and percent of neutralization for all HIV strains tested (top panel) or T/F HIV strains (bottom panel) for all ECs (Total EC), HLA-B*57 + and HLA-B*57 − ECs. (*) $p < 0.05$. (b) Spearman correlation matrices between the frequency of gp140- and gp41-specific B cell of various IgG isotypes and the percentage of neutralized strains (All strains) or T/F strains and the frequency of Env-specific B cell of various IgG subtypes. Left panel HLA-B*57 + and right panel HLA-B*57 − ECs. Strength and significance are represented as size and color intensity: blue for positive correlation and red for negative correlation. The numbers are p values.

IgG3 + B cells and the total number of viruses neutralized (Fig. 5b, red rectangle, $p < 0.05$). Note that these opposite correlations observed in HLA-B*57 + and HLA-B*57 − ECs, between Env-specific memory B cell frequencies and the neutralization potentials, did not mirror the quantity of Env-specific IgGs or IgG subclasses present in their sera (Supplemental Fig. 6 and not shown). Indeed, using Elisa, we detected similar levels of Env-specific IgGs in the sera of both groups (Supplemental Fig. 6a). In addition, no correlation was found between the frequency of Env-specific memory B cells and the quantity of Env-specific IgGs present in the sera (Supplemental Fig. 6b).

Altogether, these results show that HLA-B*57 + and HLA-B*57 − ECs have similar sustained frequency of HIV-specific B cells responses, similar HIV-specific Abs responses and non-statistically different neutralizing capacities. Despite these findings, the strong correlation observed in HLA-B*57 + ECs between the frequency of Env-specific memory B cells and the neutralizing parameters suggests that the quality of the Env-specific Abs induced differ with that of HLA-B*57 − ECs. In HLA-B*57 + ECs, the maintenance of HIV-specific memory B cells may sustain the production of functionally relevant neutralizing Ab responses.

4. Discussion

In this study, we show that, in contrast to treated patients, ECs naturally preserve their memory B cell compartments and maintain HIV-specific memory B cell responses despite low to undetectable viral loads. HIV-specific B cells mainly express the IgG1 + Ab isotype and some ECs also express anti-HIV IgG2 and IgG3 Abs. The sera from a fraction of ECs exhibit a broad cross-neutralization capacity against difficult-to-neutralize tier-2 T/F viruses. Remarkably, the frequency of Env-specific B cells, in HLA-B*57 + ECs, correlates with a broader

cross-neutralizing capacity. Our results suggest that, in HLA-B*57 + ECs, memory B cell responses might contribute to the maintenance of broad neutralization capacities and perhaps to the natural control of HIV infection.

Ex vivo analysis of B cell compartments in ECs, cART and HIV-negative donors showed a global preservation of the B cell subsets. This is in contrast to other studies that have observed a slight decrease of RM B cells in cART patients (Pensieroso et al., 2013; Buckner et al., 2016) compared to HIV-negative donors and an increase of AM B cells in ECs (Pensieroso et al., 2013). However, we noticed a clear increase of TLM B cell proportion in HLA-B*57 − compared to HLA-B*57 + ECs and to HIV-negative donors. This expansion of TLM B cells cannot be explained by a higher residual replication in HLA-B*57 − ECs compared to HLA-B*57 + individuals, as neither the levels of plasma RNA nor cell associated viral DNA were significantly different between the two groups. Unfortunately, owing to the limited amount of cells available for this study, we could not directly analyze the phenotype of HIV-specific B cells using for instance gp140-fluorescent probes and flow cytometry. We analyzed the frequency of HIV Env-specific memory B cell responses using B-cell ELISPOT, that requires fewer cells but whose results correlate with the frequency obtained using fluorescent antigens and flow cytometry (Buckner et al., 2016).

Remarkably, despite very low to undetectable viral loads, the majority of ECs presented Env-specific memory B cell responses. We obtained similar results using HIV Gag p24 as antigens (not shown). In ECs, the magnitude of gp140-specific IgG + memory B cell responses were 0.24%, a result slightly higher than reported by Bussman et al. (0.1% of memory B cell responses specific to gp120) using a cohort of 10 controllers with a median viral load above 400 RNA copy/ml (Bussmann et al., 2010). In contrast, we showed that memory B cells from cART patients, under successful virological control, rarely reacted to HIV antigens. This observation is consistent with other studies describing that upon initiation of cART the frequency of HIV-specific Ab secreting cells is strongly reduced to low or undetectable levels (Morris et al., 1998; Bussmann et al., 2010; Buckner et al., 2016; Fondere et al., 2004). Interestingly, using six ECs, Buckner et al. recently showed that the initiation of cART led to a decrease of HIV-specific memory B frequencies (Buckner et al., 2016). Therefore, although in our study, the frequency of HIV-specific memory B cells in ECs did not correlate with any virological parameters (Table 1), the maintenance of HIV-specific memory B cell responses might be due to a persistent low viral replication in the blood and/or tissues (Hatano et al., 2009; Pereyra et al., 2009).

Several studies have highlighted that ECs present heterogeneous cross-neutralizing Ab responses, with some ECs exhibiting broad cross-neutralizing capacities while others show minimal or no neutralization (Deeks et al., 2006; Scheid et al., 2009; Pereyra et al., 2008; Bailey et al., 2006; Sajadi et al., 2011; Doria-Rose et al., 2009; Braibant et al., 2008). In this work, we also observed some heterogeneity between ECs and we identified 8% of ECs with broader cross-neutralizing activities. This value is slightly lower than the 12% of ECs with broad cross-neutralization responses reported in previous studies (Sajadi et al., 2011; Scheid et al., 2009). As previously observed in ECs (Palmer et al., 2016; Ranasinghe et al., 2015), we did not find a correlation between residual viral loads and cross-neutralization potentials. In two studies on ECs (defined as controllers with viral loads below 2000 RNA copy/ml), the breadth of cross-neutralization was linked to an expansion of terminally differentiated CD57 + CD8 + T cells (Palmer et al., 2016) and an enhancement of HIV-specific CD4 + T cell responses (Ranasinghe et al., 2015). Recently, Martin-Gayo et al. observed in controllers a correlation between the enrichment of CXCR5 + CXCR3 + PD-1low CD4 + Tfh-like cells and the neutralization breadth (Martin-Gayo et al., 2017) while Dugast et al. identified a unique inflammatory profile that might be linked to the evolution of the neutralization breadth (Dugast et al., 2017).

Remarkably, we show here that in HLA-B*57 + ECs, the frequency of Env-specific B cells, correlated positively with the breadth of viruses neutralized. Previous studies analyzed potential correlations between antibody secreting cells and Ab responses (Bussmann et al., 2010; Doria-Rose et al., 2009). We confirmed the findings from Bussman et al. that Env-specific B cell frequencies do not correlate with the Ab titers to Env in the sera of ECs (Bussmann et al., 2010). Doria-Rose et al. did not observe an association between the frequency of plasmablasts spontaneously secreting HIV-specific Abs and the breadth of neutralization (Doria-Rose et al., 2009). In our study, we focused on long-lived memory B cells that provide not only an archive of contemporaneous HIV-specific Ab secreting cells but also of historic of Ab responses to the infection. In HLA-B*57 + ECs, we found a positive correlation between Env-specific memory B cell responses and the neutralizing breadth. However, it is important to note that this association was lost or correlated inversely when considering the global ECs population or HLA-B*57 − ECs, respectively.

Taking this study together with previously published work, we are proposing that in HLA-B*57 + ECs, an early and spontaneous control of viral replication (Goujard et al., 2009), probably mediated by CTL (Sáez-Cirión et al., 2007) and innate components of immunity (Barblu et al., 2012), might favor an early preservation of CD4 + Tfh cells and the establishment of efficient memory B cell responses sustaining the generation of potent antiviral responses including broad cross-neutralization. It is tempting to speculate that as recently observed in a unique HLA-B*57 + individual (Freund et al., 2017), broad neutralization in HLA-B*57 + ECs may also contribute to the control of HIV infection.

Abs can also mediate antiviral functions independently of their ability to neutralize viruses, for instance through the binding of FcR and the initiation of ADCC. Interestingly, when comparing 9 nonneutralizing and 5 broadly neutralizing monoclonal Abs, a recent publication reported that bnAbs, due to their enhanced capacity to recognize HIV-infected cells, mediate more potent ADCC than non-neutralizing Abs (Bruel et al., 2017). However, in sera from patients, neutralization-independent Ab activities have been previously associated with long-term control of HIV infection (Lambotte et al., 2009; Lambotte et al., 2013; Baum et al., 1996). In particular, using HIV-infected cells, Lambotte et al. observed that HLA-B*57 − ECs present significantly higher ADCC Ab titers than HLA-B*57 +. They suggested that ADCC plays a role in the immune control of HIV, especially in HLA-B*57 − ECs (Lambotte et al., 2013). In the present study, in HLA*B57 − ECs, we reveal an inverse correlation between Env-specific memory B cell frequencies and the neutralization breadth. Therefore in both group of ECs, whether HIV-specific memory B cell frequency might be associated with the potency of non-neutralizing antiviral Ab functions, such as ADCC of infected cells, needs to be further addressed. In a study, using the sera of 9 controllers and 11 progressors, Smalls-Mantey et al. analyzed potential correlations between ADCC of infected cells and various parameters including neutralization (Smalls-Mantey et al., 2012). They observed similar ADCC Ab titers in both groups and did not find a correlation between ADCC and neutralization activities (Smalls-Mantey et al., 2012). However, the controler cohort was limited in size and they did not compare the ADCC and neutralization profiles based on the HLA-B genotype (Smalls-Mantey et al., 2012).

Ackerman et al. proposed that the capacity to exert multiple antiviral functions might be linked to HIV-control (Ackerman et al., 2016). Indeed, depending on their isotype, Abs exert different antiviral functions. For instance, in contrast to IgG2 and IgG4, IgG1 and IgG3 bind strongly to FcR on phagocytic cells inducing efficient effector activity. Ackerman et al. showed that the sera from ECs exhibiting strong polyfunctional antiviral activities are enriched in Abs of IgG1 and IgG3 subtypes (Ackerman et al., 2016). In contrast, in viremic controllers, anti-HIV IgG2 production has been previously associated with HIV-control and slow progression (Martinez et al., 2005; Ngo-Giang-Huong et al., 2001). In this work, we show that in ECs, Env-specific memory B cell responses were mainly composed of IgG1 Abs. Only few ECs presented IgG2 + or IgG3 + responses. Interestingly in HLA-B*57 + ECs, Env-specific IgG3 + memory B cell frequencies correlated positively with both

total IgG and IgG1 Env-specific responses. In contrast, in HLA-B*57 − ECs, Env-specific IgG3 + memory B cell frequencies were negatively associated with the neutralization breadth of HIV. These observations suggest that depending on the HLA genotype (e.g. HLA-B*57 +/−) different Ab isotypes and/or functions might be involved in immune control of HIV infection.

To summarize, our work highlights the facts that ECs maintain HIV-specific memory B cell responses associated to effective antiviral humoral activities and that Env-specific memory B cell responses are positively associated with the neutralization breadth in HLA-B*57 + ECs. We propose that promoting HIV-specific B cell polyfunctional responses by therapeutic vaccination might be highly beneficial in cART treated patients.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2017.05.029.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: CR OL BA CM AM.
Funding acquisition: CR OL BA CM AM.
Investigation: AR JK BS SE CR AS GL VAF FB CR OL BA SGD CM AM.
Methodology: AR JK BS SE CR AS GL VAF FB LH HM CR OL BA SGD CM AM.
Project administration: FB CR OL BA SGD CM AM.
Resources: FB LH HM CR OL BA CM AM.
Software: AR JK NP CM AM.
Supervision: CM SGD AM.
Validation: CM AM.
Writing – original draft: AR AM.
Making of figures: AR JK CM AM.
Writing – review & editing: AR JK BS CR AS VAF FB LH HM CR OL BA SGF CM AM.

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