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J Clin Immunol. Author manuscript; available in PMC 2014 January 01.

Published in final edited form as:
J Clin Immunol. 2013 January ; 33(1): 14–21. doi:10.1007/s10875-012-9749-1.

EVER2 Deficiency is Associated with Mild T-cell Abnormalities

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Competing interest statement The authors declare that they have no competing financial interests.

Electronic supplementary material The online version of this article (doi:10.1007/s10875-012-9749-1) contains supplementary material, which is available to authorized users.

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Abstract

Epidermodysplasia verruciformis (EV) is a rare genodermatosis characterized by persistent flat warts or pityriasis versicolor-like lesions caused by betapapillomaviruses (EV-HPVs). Autosomal recessive EVER1 and EVER2 deficiencies account for EV in most patients. The mechanisms by which mutations in these partners of the Zinc transporter ZnT1 impair host defense against EV-HPVs are still poorly understood. Keratinocytes of EVER-deficient patients display an alteration of zinc homeostasis and an enhanced proliferative activity. Since EVER proteins are highly expressed in T lymphocytes, we aimed to assess the impact of EVER2 deficiency on T-cell development and function. We studied circulating lymphocyte populations in three adult EV patients sharing the same *EVER2* mutation (T150fsX3). We found a normal count of $CD4^+$ and $CD8^+$ T cells and a normal proliferative capacity in response to anti-CD3 stimulation. However, we observed a significant increase of memory $CD4^+$ and effector memory $CD8^+$ T cells, a bias of the TCR Vαβ and Vγδ repertoires and an increase of skin-homing $CD4^+$ T-cell subsets. Our findings suggest that EVER2-deficient patients display mild T-cell abnormalities. It remains unclear whether these abnormalities result from EVER deficiency, chronic EV-HPV infection, or both.

Keywords

Epidermodysplasia verruciformis; EVER; immune deficiencies; Tcells

Introduction

Epidermodysplasia verruciformis (EV) is a rare, lifelong dermatosis characterized by a selective susceptibility to keratinocyte-tropic human betapapillomaviruses (EV-HPVs) (OMIM 226400) [1]. EV patients develop disseminated and persistent flat warts or pityriasis versicolor-like lesions in childhood and are at high risk of developing HPV-5-associated non-melanoma skin cancers (NMSC) early in their adult life [2]. EV-causing HPVs are known to cause asymptomatic infections in the general population. The mechanism underlying persistent symptomatic infections by EV-HPVs in EV patients has long remained unclear [3]. In the late 70s and 80s, several reports described some deficiencies of cell-mediated immunity in patients with EV. Decreased T-cell counts, a defective T-cell proliferation in response to phytohemaglutinin (PHA) and cutaneous anergy to a variety of antigens had been observed in most patients [4–8]. An increased Natural Killer (NK) activity was also reported in some patients with EV [9, 10]. However, despite these immunological abnormalities, most EV patients display no other clinical signs, no other infections in particular [3]. Because these immunological observations were not consistent, and despite the unquestionable inheritance of an infectious phenotype, EV was not considered as primary immunodeficiency until the identification of inactivating mutations in *EVER1* (TMC6) and *EVER2* (TMC8) in 2002 as the two first genetic etiologies of EV [3, 11–13] These two genes are expressed in keratinocytes, in which the EVER proteins form a complex with the Zinc (Zn) transporter ZnT1 [14, 15]. In these cells, inactivating mutations in *EVER1* or *EVER2* affect intracellular Zn distribution and the activity of Zn-dependent transcription factors [14]. However, both *EVER1* and *EVER2* are strongly expressed in circulating lymphocytes, including $CD4^+$ and $CD8^+$ T cells, B cells and NK cells

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(www.biogps.org) [16]. It has been shown that Zn homeostasis is also important in all leukocytes [17], notably in T cells, where the activation-induced Zn influx contributes to T-cell receptor signaling by increasing ZAP70 phosphorylation [18]. Recently, Lazarczyk *et al* reported that T-cell activation by CD3 and CD28 stimulation led to a decreased mRNA expression of *EVER* genes and to an increase of free Zn^{2+} ions which might suggest that EVER deficiency impairs T-cell activation via an imbalance of Zn homeostasis [19]. We recently discovered RHOH and MST1 deficiencies characterized by a profound T-cell defect in patients with persistent EV-HPV infections and other clinical manifestations (unlike in EV patients) [20, 21]. We therefore investigated T-cells in three unrelated EVER2-deficient patients.

Material and Methods

Patients

The clinical history of the three unrelated patients with EV is summarized in Table I. Patient P2 is the only sporadic case. Several members of the families of P1 and P3 also have EV. P1 and P3 have been reported in previous papers [5, 7, 9, 10]. Briefly, these three patients have developed a typical form of EV since childhood and all of them developed in situ Bowen's carcinomas [22]. They were all infected with several HPVs, including HPV-5, common to all patients. They carry the same homozygous mutation in *EVER2* (Table I, our unpublished results (P. Cassonnet, M. Favre, S. Jablonska, S. Majewski, G. Orth and N. Ramoz)).

Flow Cytometry on Whole-Blood Samples

Immunological analysis of the T-, B-, and NK-cell compartments on whole blood samples was performed by flow cytometry with monoclonal antibodies against the surface markers CD3, CD4, CD8, CD19, CD16, CD56, CD45RA, CD45RO, CCR7 and CD31 (Becton Dickinson), as described elsewhere [20]. Blood samples were collected into EDTA and stained by incubation for 20 minutes with 1 % BSA in PBS, on ice, in the dark. Red blood cells were then lysed in FACS lysis solution (0.01 M CO_3HNa; 1 mM EDTA; 0.14 M $ClNH_4$), in three successive cycles of incubation for 5 minutes at room temperature in the dark. Samples were washed in 1× PBS (Gibco) and resuspended in 4 % paraformaldehyde in PBS for analysis on a BD FACS-canto machine (BD Bioscience).

Flow Cytometry on Cryopreserved PBMCs

Immunological analysis of tissue-homing subsets was performed with the following antibodies: the BD Horizon V450-conjugated anti-CD3 antibody (BD, Biosciences, San Jose, CA, UCHT1) was used for the gating of $CD3^+$ cells; the antibodies CD4-APC (Biolegend, RPA-T4), CD8-PE-Cy7 (Biolegend, SK1), CLA-FITC (MACS, HECA-452), CCR4-PE (BD, 1 G1), CCR6-PE (BD, 11A9), CCR10-PE (R&D Systems, 314305), αE (CD103)-PE (Biolegend, Ber-ACT8), α4-FITC (MACS, MZ18-24A9), β7-PE (Biolegend, FIB504) and the mouse IgG1-PE (BD), rat IgM-FITC (Biolegend), mouse IgG2b-FITC (Biolegend), rat IgG2a-PE (Biolegend) isotype controls were used to assess the different subsets. Dead cells were excluded with the Aqua Live/Dead marker (Invitrogen, L34957).

Thymidine Incorporation Assay

PBMCs were incubated for three days alone or with PHA (2.5 μg/ml), or for three days (P3) or four days (P1) with the monoclonal soluble anti-CD3 antibody OKT3 (10, 25, 50 ng/ml), or for six days with candidin (50 μg/ml). The anti-CD3 mAb OKT3 (IgG2a) has been described elsewhere [20]. Cultures were pulsed with tritiated thymidine for the last 18 hours of the incubation period. The incorporated radioactivity was determined with a Matrix 96

beta counter (Canberra Packard, Frankfurt/Main, Germany). Cell proliferation was assessed by determining the cpm for [^{3}H] thymidine incorporation, as previously described [20].

Determination of Vα, β, γ, δ Gene Usage and Immunoscope Analysis

Vα, β, γ and δ gene usage were determined and Immunoscope analysis was performed on cDNA samples, as previously described [20].

Statistical Analysis

Non-parametric two-tailed exact Wilcoxon test as implemented in the stats package of R software was used to compare the percentages of the various tissue-homing T-cell subsets between patients and healthy controls. Differences were considered significant if p-values were <0.05, for all comparisons.

Results

We carried out general immunological phenotyping on whole-blood samples from three unrelated EVER2-deficient patients sharing the same mutation (T150fsX3) to assess the development of various leukocyte subsets. The patients displayed normal counts of myeloid cells, including monocytes and polymorphonuclear cells (Table II). The frequencies of total B, T, NK cells and $CD4^+$ and $CD8^+$ T-cell subpopulations were within the normal ranges (Table II). The naive $CD45RA^+CD4^+$ T-cell compartment of two EVER2-deficient patients (P1 and P2) had a lower proportion compared to the healthy control range (Table III). P1 and P2 displayed a slightly decreased proportion of recent thymic T-cell emigrants ($CD31^+CD45RA^+CD4^+$). However, the three patients showed an enlarged memory $CD45RO^+CD4^+$ T-cell compartment (Table III). In the same way, P2 and P3 showed a decrease of naive $CD8^+$ T cells ($CCR7^+CD45RA^+CD8^+$) (Table III). In contrast, $CD8^+$ memory T cells were increased in all patients. Among the memory $CD8^+$ T-cell subsets, the central memory ($CCR7^+CD45RA^-$) subset was normal in the three patients, whereas the revertant memory ($CCR7^-CD45RA^+$) and the effector memory ($CCR7^-CD45RA^-$) subsets were increased in P2 and in all patients, respectively. Altogether, the only consistent abnormalities, found in all patients were an over-representation of the memory $CD4^+$ and effector memory $CD8^+$ T-cell subsets.

We next assessed the patients' T-cell proliferative capacity upon mitogenic or antigenic stimulation. The T cells of P1 and P2 showed a normal proliferation whereas the T cells of P3 showed a moderately impaired proliferation upon activation with PHA. The T cells from two patients tested (P1 and P3) proliferated normally upon anti-CD3 stimulation (Table IV). The three patients showed a normal or subnormal T-cell response to candidin (Table IV). An immunoscope analysis of the TCR Vαβ and Vγδ repertoires showed an abnormal profile for the Vα1-, Vα30-, Vβ4-, Vγ2-4, Vγ9-, Vδ2-families in the two patients tested (P1 and P2) (Fig. 1). This implied restricted TCR usage, with the clonal expansion of certain families, consistent with the expansion of the effector memory T-cell compartment in the patients.

Due to the specific cutaneous phenotype, we also studied the proportions of skin-homing T-cell subsets in the patients' peripheral blood. The most specific skin-homing marker of human T cells is the Cutaneous Lymphocyte Antigen (CLA) [23, 24]. The CLA^+CD3^+ T-cell subset was slightly increased in the patients' peripheral blood, due to a significant and specific increase of CLA^+ T cells in the $CD4^+$ T-cell compartment (Fig. 2a). We next assessed the expression of other skin-homing markers, the chemokine receptors CCR4, CCR6 and CCR10. CCR6 and CCR4 were expressed in the same proportions in EVER2-deficient patients and healthy controls (Supplementary Figures 1a, b). In contrast, the

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proportion of $CCR10^+$ T cells was significantly higher in the $CD3^+$ T-cell subpopulation due to a higher proportion in the $CD4^+$ T-cell subpopulation (Supplementary Figure 1c). Moreover, the CLA^+CCR4^+, CLA^+CCR10^+ T-cell subsets were also increased in the T-cell compartment, due to a significant increase in the $CD4^+$ T-cell subpopulation (Fig. 2b, c, d). The αE integrin chain is also associated with skin homing [25]. The proportions of αE^+ and αE^+CLA^+ T cells were not significantly different between EVER2-deficient patients and controls (Supplementary Figure 2a, b). Finally, we investigated gut-homing $\alpha 4^+\beta 7^+$ T cells, which were strongly decreased in RHOH-deficient patients and we observed no difference between patients and controls (Supplementary Figure 2c). Altogether, there was a significant and selective increase of the CLA^+, CLA^+CCR4^+ and CLA^+CCR10^+ skin-homing $CD4^+$ T-cell subsets in EVER2-deficient patients.

Discussion

The T-cell abnormalities detected in the three EVER2-deficient patients studied are mild. Indeed, naive $CD4^+$ and $CD8^+$ T cells are present in a normal proportion in one patient and in lower proportions in two patients, excluding a major defect during thymopoiesis. As previously reported in some EV patients [4–8], one patient (P3) displays a moderately decreased T-cell proliferation upon PHA stimulation but P1, who was described with a decreased or a normal T-cell proliferation [5], and P2 show a normal proliferation upon PHA stimulation in our own assay. This result is similar to the report on an EVER1-deficient patient who had a normal proliferation of T cells upon PHA stimulation [26]. The two patients tested (P1 and P3) have also a normal T-cell proliferation upon anti-CD3 stimulation. All patients show a normal proliferation upon candidin stimulation. These immunological features are clearly different from those observed in RHOH- or MST1-deficient patients with persistent EV-HPV infections (and other clinical manifestations, unlike EV patients) (Table V) [20, 21]. Decreased naive T cells and impaired T-cell proliferation upon stimulation with mitogens and anti-CD3 have been reported in both deficiencies [20, 21]. EVER2 deficiency therefore does not seem to significantly impair T-cell development and T-cell function, in contrast to RHOH and MST1 deficiencies.

By contrast, all EVER2-deficient patients display an excess of memory $CD4^+$ and effector memory $CD8^+$ T cells. Furthermore, the two patients tested also display a bias of the TCRαβ and TCRγδ repertoires, suggesting clonal expansions. An increase of memory T cells and a strong bias of the TCR repertoire had also been described in RHOH- and MST1-deficient patients (Table V) [20, 21, 27, 28]. The overrepresented memory T cells in both deficiencies had also an exhaustion phenotype, probably reflecting at least in part chronic viral infection [20, 21]. The increase of memory T cells in EVER2-deficient patients is also probably a consequence of a sustained T-cell stimulation as a result of chronic EV-HPV infection rather than a direct consequence of the EVER2 deficiency. This has already been described in patients with other chronic viral infections or with other primary immunodeficiencies without EV, such as DOCK8-deficient patients [29, 30].

Interestingly, we also report a significant increase of skin-homing $CD4^+$ T-cells (CLA^+, CLA^+ $CCR4^+$, CLA^+CCR10^+), but not of skin-homing $CD8^+$ T-cells, in the three EVER2-deficient patients. CCR10 is expressed only by a subset of skin-homing T cells, whereas CCR4 is expressed by all skin-homing T cells, but also by other circulating T cells, including gut-homing T cells [31]. We show here a specific increase of skin-homing CLA^+CCR4^+ cells within the $CCR4^+$ population. A decrease of memory CLA^+ T cells but also the presence of naive $CD4^+CLA^+$ T-cells have been described in RHOH-deficient patients, whereas CLA is only expressed by memory T cells in healthy individuals (Table V) [23], suggesting a dysregulation of CLA expression in the $CD4^+$ T-cell compartment in these patients [20]. In contrast, we do not find a decrease of $\beta 7^+$ and $\alpha 4^+\beta 7^+$ T-cell subsets,

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which was reported in RHOH deficient-patients, in EVER2-deficient patients (Table V) [20]. Consequently, the decrease of $\beta 7^+$ T cells is unlikely to be a consequence of chronic EV-HPV infections in RHOH-deficient patients, as the EVER2-deficient patients do not share these immunological features. Altogether, these observations suggest that the $CD4^+CLA^+$ T–cell subset may be involved in the pathogenesis of EV, or may be altered by chronic HPV infection in EV patients, or both.

To conclude, EVER2-deficient patients have a mild T-cell phenotype characterized by an increase of some memory T-cell subsets, suggesting that the maintenance of Zn homeostasis by EVER proteins is not essential for the normal development and activation of T cells. This result is consistent with the EV-restricted phenotype of these patients. However, we also observe an alteration in the skin-homing T cells subsets in EVER2- and RHOH-deficient patients. It will be important to characterize the tissue-homing T cells in MST1-deficient patients as well in order to identify the link between the abnormal tissue-homing marker expression pattern observed in EV patients and the pathogenesis of this disease. Thereafter, further investigations will be necessary to better assess the contributions of keratinocytes and T lymphocytes in the development and the persistence of EV lesions in EVER2-deficient patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the members of both branches of the Saint Giles Laboratory of Human Genetics of Infectious Diseases, but also Aminata Diabate, Corinne Jacques, Chantal Harre and Stephanie N'Daga for excellent technical assistance, and Svetlana Mazel for flow cytometry expertise. We warmly thank the patients for their participation. This work was supported by grants from INSERM, University Paris Descartes, the Rockefeller University, the St. Giles Foundation, and the Rockefeller University Center for Clinical and Translational Science grant number UL1RR024143.

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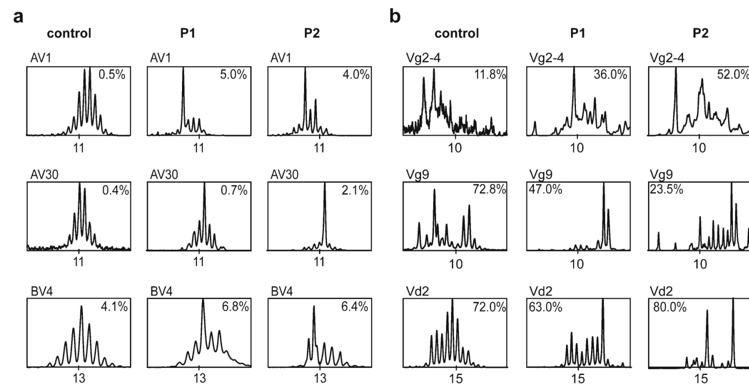

Fig. 1.

Patients P1 and P2 have abnormal Vαβ and Vγδ distributions. Immunoscope profiles of **a** TCRα and TCRβ and **b** TCRγ and TCRδ on cDNA samples obtained from the patients and controls following RNA extraction from PBMCs. Only profiles for Vα, Vβ, TCRγδ differing between patients and controls, with some clonal expansions, are shown. The x-axis indicates the CDR3 length (number of amino acids), and the y-axis shows the fluorescence intensity of the run-off products, in arbitrary units. The percentages indicate relative frequency of usage

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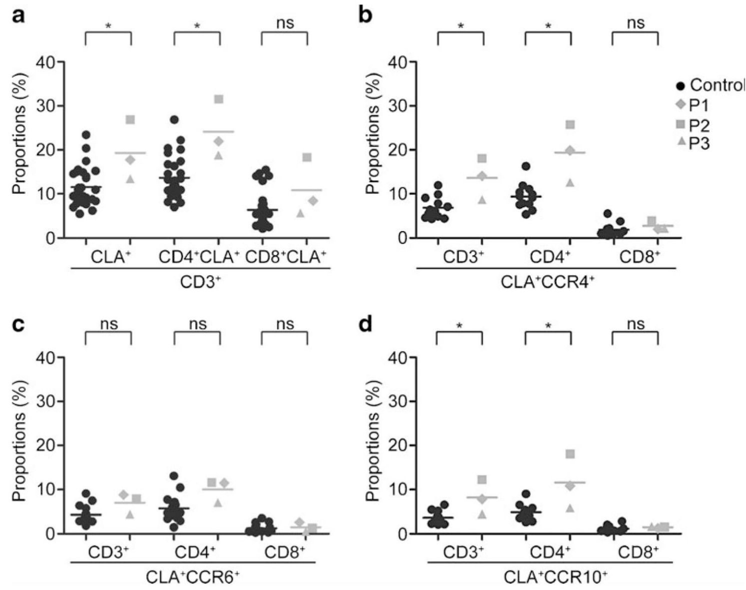

Fig. 2.

EVER2-deficient patients have an increase of skin-homing CLA$^+$CD4$^+$ T cells. Skin-homing T-cell subsets were assessed on live CD3$^+$-gated cryopreserved PBMCs from the patients (P1 values indicated by gray diamonds, P2 values indicated by gray squares, P3 values indicated by gray up triangles) and healthy controls (indicated by black circles) by flow cytometry **a** Skin-homing CLA$^+$ proportions were assessed on live CD3$^-$, CD4$^-$ and CD8$^-$ gated PBMCs for the patients and 28 healthy controls. **b** Skin-homing CLA$^+$CCR4$^+$ proportions were assessed on live CD3$^-$, CD4$^-$ and CD8$^-$ gated PBMCs for the patients and 12 healthy controls, respectively **c** Skin-homing CLA$^+$CCR6$^+$ proportions were assessed on live CD3$^-$, CD4$^-$ and CD8$^-$ gated PBMCs for the patients and 17 healthy controls and **d** CLA$^+$CCR10$^+$ proportions were assessed on live CD3$^-$, CD4$^-$ and CD8$^-$ gated PBMCs for the patients and 12 healthy controls. (*=p <0.05; ns=non significant)

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Table I

Patients' clinical and general phenotype

	P1	P2	P3
Age (year of birth)	54y (1957)	58y (1953)	56y (1955)
Gender	F	F	M
Age of onset	7y	4y	8y
Index[a] (EV "extent")	++	++/+++	++
HPV type	3a,5,8,12,19, 24,36	5,12,36	5,8,20,23,36
Cutaneous Malignancies	+	+	+
(age of onset)	(15y)	(35y)	(20y)
	Bowen	Bowen	Bowen
EVER2 mutation	T150fsX3	T150fsX3	T150fsX3
	Homozygous	Homozygous	Homozygous

a: ++: Multiple carcinomas in situ, ++/+++: multiple carcinomas, invasive at some sites

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Table II

Patients' immunological phenotype, from the analysis of whole-blood samples

	P1	P2	P3	Normal range
Polymorphonuclear neutrophils (10^9/l)*	2.7	2.2	ND	1.5-7
Polymorphonuclear eosinophils (10^9/l)*	0	0.1	ND	0-0.3
Polymorphonuclear basophils (10^9/l)*	0	0	ND	0-0.2
Monocytes (10^9/l)*	0.3	0.4	ND	0.2-1
Lymphocyte (10^9/l)*	1.7	2.7	1.6	1.2-3.3
T cells				
$CD3^+$ (%)	67	70	76	51-82
$CD4^+$ (%)	39	40	43	31-63
$CD8^+$ (%)	20	26	27	9-34
B cells				
$CD19^+$ (%)	17	12	16	4-21
NK cells				
$CD16^+CD56^+$ (%)	16	17	10	5-33

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Table III

Frequencies of the different CD4$^+$ and CD8$^+$ naive and memory subsets in whole-blood samples

	P1	P2	P3	Normal range[a]
CD4$^+$ Subset				
CD4$^+$CD45RA$^+$ (%)	35	25	41	41-55
CD4$^+$CD45RO$^+$ (%)	65	75	59	40-62
CD31$^+$CD45RA$^+$ /CD4$^+$ (%)	19	11	24	20-28
CD8$^+$ Subset				
CCR7$^+$CD45RA$^+$/CD8$^+$ (%)	33	4	20	25-51
CCR7$^+$CD45RA$^-$ /CD8$^+$ (%)	11	3	5	4-9
CCR7$^-$CD45RA$^-$ /CD8$^+$ (%)	39	58	53	25-35
CCR7$^-$CD45RA$^+$ /CD8$^+$ (%)	18	35	22	15-31

[a]Internal controls (N010, age >40 years)

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Table IV

T-cell proliferation in response to mitogen, antigen and anti-CD3 stimulations

Stimulus	P1	P2	P3	Normal range
3-day culture				
PHA 2.5ug/ml	126	55	21	>50
Number of days in culture	4		3	
OKT3 50 ng/ml	41	ND	43	>30
OKT3 25 ng/ml	29	ND	46	>30
OKT3 10 ng/ml	28	ND	49	>30
6-day culture				
Candidin	53	9	22.5	>10

3H-TdR incorporation in cpm/10^3

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Table V

EVER2 deficiency compared to other deficiencies conferring susceptibility to EV-HPV infections

Deficiencies	EVER2	RHOH	MST1
Clinical phenotype	EV,	Persistent EV-HPV infections,	Persistent EV-HPV infections,
	High risk of NMSC	α-HPV cutaneous warts,	α-HPV cutaneous warts,
		MCV, HSV1 infections,	bronchitis, pneumonia,
		Burkitt lymphoma,	CMC, autoimmunity, growth retardation
		broncho-pulmonary disease	
T-cell phenotype			
Naive/ Memory	High memory CD4/CD8 (effector)	Very low naive/High memory	Lack of naive/High memory
Response to OKT3	Normal	impaired	impaired
Response to antigens	Variable	variable	variable
Response to mitogen (PHA)	Normal	impaired	moderately impaired
Tissue-homing subsets	High $CLA^{+}CD4^{+}$,	low CLA^{+},	ND
	High $CCR10^{+}$ $CD4^{+}$	Increase CLA^{+} naive	
	Normal α E^{+},	Normal α E^{+},	
	Normal $\alpha4^{+}\beta7^{+}$	low $\alpha4\beta7^{+}$	
Natural Killer cells phenotype	Normal ADCC	ND	ND
	Increased cytotoxic activity against K562 (P1, P3)		

MCV molluscum contagosium virus; *HSV1* Herpes simplex virus 1; *CMC* cutaneomucocutaneous candidiasis; *ADCC* antibody-dependent cell-mediated cytotoxicity; *NMSC* non-melanoma skin cancer; *ND* not done