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EVER Proteins, Key Elements of the Natural Anti-Human Papillomavirus Barrier, Are Regulated upon T-Cell Activation

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Introduction

Papillomaviruses are widespread infectious agents, transmitted by sexual or cutaneous contacts. Human papillomaviruses (HPV) cause a variety of mucosal and skin lesions ranging from benign proliferations to invasive carcinomas. The clinical manifestations of infection are determined by host-related factors that define the natural anti-HPV barrier. Key elements of this barrier are the EVER1 and EVER2 proteins, as deficiency in either one of the EVER proteins leads to Epidermodysplasia Verruciformis (EV), a genodermatosis associated with HPV-induced skin carcinoma. Although EVERs have been shown to regulate zinc homeostasis in keratinocytes, their expression and function in other cell types that may participate to the anti-HPV barrier remain to be investigated. In this work, we demonstrate that EVER genes are expressed in different tissues, and most notably in lymphocytes. Interestingly, in contrast to the skin, where EVER2 transcripts are hardly detectable, EVER genes are both abundantly expressed in murine and human T cells. Activation of CD4⁺ and CD8⁺ T cells via the TCR triggers a rapid and profound decrease in EVER expression, accompanied by an accumulation of free Zn²⁺ ions. Thus, EVER proteins may be involved in the regulation of cellular zinc homeostasis in lymphocytes. Consistent with this hypothesis, we show that the concentration of Zn²⁺ ions is elevated in lymphoblastoid cells or primary T cells from EVER-deficient patients. Interestingly, we also show that Zn²⁺ excess blocks T-cell activation and proliferation. Therefore, EVER proteins appear as key components of the activation-dependent regulation of Zn²⁺ concentration in T cells. However, the impact of EVER-deficiency in T cells on EV pathogenesis remains to be elucidated.

Abstract

Human papillomaviruses (HPV) cause a variety of mucosal and skin lesions ranging from benign proliferations to invasive carcinomas. The clinical manifestations of infection are determined by host-related factors that define the natural anti-HPV barrier. Key elements of this barrier are the EVER1 and EVER2 proteins, as deficiency in either one of the EVER proteins leads to Epidermodysplasia Verruciformis (EV), a genodermatosis associated with HPV-induced skin carcinoma. Although EVERs have been shown to regulate zinc homeostasis in keratinocytes, their expression and function in other cell types that may participate to the anti-HPV barrier remain to be investigated. In this work, we demonstrate that EVER genes are expressed in different tissues, and most notably in lymphocytes. Interestingly, in contrast to the skin, where EVER2 transcripts are hardly detectable, EVER genes are both abundantly expressed in murine and human T cells. Activation of CD4⁺ and CD8⁺ T cells via the TCR triggers a rapid and profound decrease in EVER expression, accompanied by an accumulation of free Zn²⁺ ions. Thus, EVER proteins may be involved in the regulation of cellular zinc homeostasis in lymphocytes. Consistent with this hypothesis, we show that the concentration of Zn²⁺ ions is elevated in lymphoblastoid cells or primary T cells from EVER-deficient patients. Interestingly, we also show that Zn²⁺ excess blocks T-cell activation and proliferation. Therefore, EVER proteins appear as key components of the activation-dependent regulation of Zn²⁺ concentration in T cells. However, the impact of EVER-deficiency in T cells on EV pathogenesis remains to be elucidated.

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Indeed, several clinical observations indirectly suggest that the defect in the natural anti-HPV barrier in EV patients is not limited to epidermis but may potentially involve the adaptive immune system [5]. Notably, EV patients consistently exhibit immune abnormalities, mainly related to defective cell-mediated responses [6,16]. The responses to HPV antigens and common skin sensitizers (e.g., dinitrochlorobenzene) are compromised in EV patients [16–10]. Moreover, EV-like skin eruptions associated with HPV have been only in some instances reported in severely immunocompromised patients, pointing to the involvement of very specific mechanisms in the control of β-HPVs [19–23].

The causes of the immunologic defects in EV remain controversial [6,10]. They could be directly due to the impact of EVER-deficiency in immune cells or secondary to the massive lifelong HPV skin infection [24]. Therefore, we decided to assess the expression and function of EVERs in lymphocytes.

**Results and Discussion**

We first analyzed the expression of EVER genes in a panel of freshly collected murine tissues, and in murine and human lymphocyte subsets. Using both classical RT-PCR (data not shown) and quantitative RT-PCR (qRT-PCR), we observed that EVER1 and EVER2 were clearly expressed in spleen and thymus (Fig. 1A), as well as in purified murine (Fig. 1B) and human (Fig. S1A, B) lymphocyte populations. EVER1, and to a lesser extent EVER2, were also expressed in brain, heart, kidney, liver and skin (Fig. 1A). The identity of the amplified bands was confirmed by direct sequencing of the RT-PCR products or by cloning and sequencing of the qRT-PCR products (data not shown). It is noteworthy that the highest expression of EVER1 and EVER2 genes was not found in the skin but rather in lymphoid organs (Fig. 1A). The expression of EVER1 and EVER2 did not differ significantly between B and T cells (Fig. 1B), or between CD8+ and CD4+ T cells (Fig. 1B, Fig. S1B). Moreover, the amount of EVER1 and EVER2 transcripts, as assessed by qRT-PCR, was similar in CD8+ and CD4+ T cell subsets. The mean EVER2/EVER1 expression ratio was 0.96 (n = 4) and 0.77 (n = 6) in CD8+ and CD4+ T cells, respectively. Interestingly, a strikingly different pattern of EVER gene expression was observed in the skin where EVER1 was preferentially expressed whereas EVER2 was barely detectable (mean EVER2/EVER1 expression ratio 0.07; n = 3).

EVER1- or EVER2-deficiency leads to a clinically identical EV phenotype in humans [7–9]. This observation has two important implications. First, it suggests that EVER1 and EVER2 have non-redundant functions. Indeed, the absence of either one of the EVER proteins leads to the dysfunction of the whole EVER complex resulting in zinc imbalance [11]. Second, the cell type(s)/tissue(s) where both genes are expressed, and not those with a selective expression of either EVER1 or EVER2, are most probably essential in EV pathogenesis. In this context, it is tempting to speculate that the lymphoid tissue, which is characterized by a high expression of both EVER1 and EVER2 genes, rather than the skin, where the constitutive expression of EVER2 is extremely low (Fig. 1A), could be most relevant to EV pathogenesis.

We next verified by western blot that EVER proteins were expressed in spleen and freshly purified CD4+ or CD8+ T cells. A single band of approximately 100 kDa was detected using a commercially available antibody against murine EVER1 (Fig. 1C, D). To ensure that the antibody used was specific, and that it could discriminate between the two related EVER1 and EVER2 proteins, we employed two complementary strategies. First, the expression of EVER1 was confirmed with a second antibody recognizing a distinct epitope of EVER1 (Osense; OSR00223W). A single band of approximately 100 kDa was detected with this antibody in spleen and in CD8+ T cell lysates (data not shown). Second, we tested whether these two anti-EVER1 antibodies specifically recognized EVER1. To this end, 293 T cells were transfected with plasmids encoding either EVER1-FLAG or EVER2-FLAG fusion proteins. The fusion proteins were subsequently precipitated using the anti-FLAG antibody and western blot analyses were performed. We were able to detect both EVER1-FLAG and EVER2-FLAG fusion proteins with the anti-FLAG antibody. However, the two anti-EVER1 antibodies recognized a single band (corresponding to MW ≈ 85 kDa) in cells expressing EVER1-FLAG, but not in cells expressing EVER2-FLAG (Fig. 1E), demonstrating their specificity. We attempted to verify by the same approach the expression of the EVER2 protein in T cells but the anti-EVER2 antibody tested (Abcam, ab70002) did not work in our hands (data not shown).

Since EVER proteins form a complex with ZnT-1 [4,11], a well-characterized zinc transporter whose expression is enhanced by Zn2+ ions through the MTF-1 transcription factor [25,26], we evaluated whether transcription of EVER1 and EVER2 in T cells could also be regulated by zinc. Whereas expression of two zinc-inducible genes (MT-2 and ZnT-1) was clearly increased 2 or 6 hours after exposure to a non-toxic concentration of zinc (100 μM), expression of EVER genes remained stable following 6- and even 24-hour exposure to zinc (Fig. 2A).

We next tested the impact of in vitro T-cell activation and differentiation on EVER expression. EVER1 and EVER2 were constitutively expressed in purified naive CD8+ (Fig. 2B) and CD4+ (Fig. 2C) T cells. Interestingly, activation of these T cells using anti-CD3 and anti-CD28 antibodies led to a rapid and profound decrease in EVER1 and EVER2 mRNA levels. The drop in EVER1 and EVER2 expression was detected as early as 3 hours after activation (Fig. 2B, C). This decreased expression of EVER genes was maintained for at least 3 days (Fig. 2B, C). A similar EVER1 and EVER2 mRNA down-regulation was found in human T cells following TCR/CD28-dependent activation (Fig. S2A, B). The TCR and CD28 exerted a synergistic effect on the down-regulation of EVER genes, as triggering of either TCR or CD28 alone had little or no effect (Fig. 2D).

The exact mechanism of the down-regulation of EVER expression in response to T-cell activation remains unknown. Interestingly, the reduction in the amount of EVER transcripts is rapid, suggesting that EVER mRNA might be relatively unstable. Probably, due to its rapid turnover, the suppression of the transcription of the corresponding gene would result in such a rapid reduction in the mRNA quantity. Regardless of the exact molecular basis of this observation, the role of EVER1 and EVER2 in T cells and the functional significance of the modulation of their expression constitute an important unanswered question.

Cellular zinc homeostasis is tightly controlled by an elaborate system composed of different zinc sensors (like MTF-1), transporters (ZnT and ZIP families), and buffering proteins such as metallothionens [29]. It has been suggested that ZnT-1 and the EVER/ZnT-1 complex, like the other members of the ZnT family, could be involved in the transport of Zn2+ ions out of the
EVER Gene Expression in T-Cells

Figure A: Expression levels of EVER1 and EVER2 in different tissues. The expression is measured in percentage with error bars indicating variability.

Figure B: Expression levels of EVER1 and EVER2 in T CD8 cells, T CD4 cells, and B cells. The expression is measured in percentage with error bars indicating variability.

Figure C: Electrophoresis analysis of markers and splenocytes. The bands are shown at 100 kDa.

Figure D: Electrophoresis analysis of CD8+ T cells and CD4+ T cells. The bands are shown at 100 kDa.

Figure E: Western Blot analysis using anti-FLAG and anti-EVER1 antibodies. The results are summarized in a table.
cytoplasm, in order to maintain the low Zn\(^{2+}\) cytoplasmic level in spite of the large gradient of this metal across the plasma membrane [4,11,29]. Based on our data, we propose that the EVER complex is involved in the regulation of zinc balance in T cells, and that down-modulation of the EVER expression during T-cell activation could impact zinc homeostasis. In the course of CD8\(^{+}\) and CD4\(^{+}\) T-cell activation, we observed a decreased expression of EVER1 and EVER2 as well as of ZnT-1 (data not shown), with concomitant up-regulation of ZIP10 (Fig. 3A, B) and ZIP6 (Fig. S3A, B) expression. ZIP10 and ZIP6 are plasma membrane transporters responsible for Zn\(^{2+}\) influx into the cell, and in particular in T cells [30,31]. This coordinated reorganization of zinc transporter expression is compatible with a program of zinc accumulation in the cytosol of activated T cells. Indeed, we confirmed that the total amount of free Zn\(^{2+}\) ions per cell was significantly increased 24 h after TCR- and CD28-dependent

**Figure 1.** EVER1 and EVER2 are expressed in lymphocytes. A. Expression of EVER1 and EVER2 genes in different mouse organs was assessed by qRT-PCR. Expression in the spleen was set as 100%. For each organ at least 3 independent experiments were performed. B. Expression of EVER1 and EVER2 genes in murine T and B cells was determined by qRT-PCR. At least 3 independent experiments were performed for each cell type. Expression of EVER1 protein in murine splenocytes (C) and purified T cells (D) was determined by western blot. An EVER1-specific antibody (Abcam; ab67326) was used. A single band at \( \approx 100 \) kDa was detected (predicted EVER1 MW = 91 kDa). E. 293 T cells were transfected with plasmids encoding either EVER1-FLAG or EVER2-FLAG fusion protein. The fusion protein was immunoprecipitated with anti-FLAG antibody and subsequently a western blot was performed using the anti-FLAG antibody or two different anti-EVER1 antibodies (Ab n°1 - Abcam; ab67326; Ab n°2 - Osenses; OSR00223W).

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**Figure 2.** EVER1 and EVER2 expression is strongly down-regulated in T cells following TCR-mediated activation. A. Purified murine naive CD8\(^{+}\) T cells were incubated in 100 \( \mu \)M zinc for the indicated period of time. Thereafter the expression of metallothionein 2 (MT-2), zinc transporter 1 (ZnT-1) as well as EVER1 and EVER2 was assessed by qRT-PCR. Data are from 3 independent experiments. Purified murine naive CD8\(^{+}\) T cells (B) or CD4\(^{+}\) T cells (C) were activated with immobilized anti-CD3 and anti-CD28 Abs, in the presence of IL-2 (1 ng/ml) and IL-12 (10 ng/ml). Expression of EVER genes in the course of the activation was determined by qRT-PCR at the indicated time points. Data are representative of 3 independent experiments. D. Purified murine naive CD8\(^{+}\) T cells were incubated in medium or activated by anti-CD3 (CD3), anti-CD28 (CD28) or by a combination of anti-CD3 and anti-CD28 (CD3/CD28) Abs. After 24 h, the expression of EVER1 and EVER2 was determined by qRT-PCR. Freshly purified naive CD8\(^{+}\) T cells (ex vivo) were used as the reference 100% value. Data are from 3 independent experiments. E. Purified naive TCR-transgenic CD8\(^{+}\) T cells were activated by their cognate HA512–520 peptide in the presence of irradiated T cell-depleted syngeneic splenocytes as antigen-presenting cells. At the indicated time points, CD8\(^{+}\) T cells recovered by MACS purification, and expression of EVER1 and EVER2 in these cells was determined by qRT-PCR. Data are representative of 3 independent experiments.

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activation (Fig. 3C, D), and maintained at this level for at least 72 h (data not shown). The mechanism and functional significance of such an accumulation remain uncertain given the very limited knowledge on the physiological role of free Zn\(^{2+}\) ions in T cells. However, this zinc accumulation could be related to the massive changes in lymphocyte volume (about 10 times, based on the changes of the forward side scatter in flow cytometry) and metabolic activity during blast formation. Thus, at least two non-mutually exclusive hypotheses could be formulated. First, zinc accumulation could be a response to the increased needs of this metal for the newly synthesized zinc-binding proteins. Second, it might constitute a compensatory mechanism in order to maintain a constant free Zn\(^{2+}\) ion concentration during the massive enlargement of the stimulated T cells.

Recent findings, showing that Zn\(^{2+}\) ions play an important role in signal transduction and can even serve as a classical second messenger [32–34], highlight the need for tight control of the intracellular free zinc pool. Indeed, we observed that the aforementioned modulation in zinc transporter expression in activated CD4+ and CD8+ T cells is accompanied by massive transcription of MT-2 (Fig. 3E, F), encoding for the major zinc-buffering metallothionein [35,36]. Finally, despite the increase in the total zinc content per cell following TCR/CD28 triggering (Fig. 3C, D), the intracellular concentration of free zinc remained remarkably stable (Fig. 3G). In this context, it is tempting to speculate that the lack of either EVER proteins could disrupt the EVER/ZnT-1 complex and consequently impose zinc imbalance in T cells as previously demonstrated in keratinocytes [11]. To assess the impact of the EVER-deficiency on zinc homeostasis in lymphocytes, we first measured the concentrations of free Zn\(^{2+}\) in EBV-transformed B cells from an EV patient (EVER1 Del275A) and from a healthy relative using FluoZin-3, a zinc ion-specific fluorescent probe, and a flow cytometric assay. In five independent measurements, the zinc concentration was higher in EVER-deficient than in control cell lines (Fig. 4A). These results suggest that EVERs could be involved in the regulation of cellular zinc homeostasis.

To then address the contribution of EVER proteins for zinc balance in primary lymphocytes, untransformed T cell lines were generated from PBMC of two healthy individuals and two patients, one with full-blown EV with homozygous EVER2 T150fdX3 mutation and one heterozygous for the EVER2 T150fdX3 mutation. From a clinical point of view, this heterozygous patient clearly presented transiently EV-like eruptions and was HPV3 and HPV5 positive in the lesions (see Figure 3. Zinc homeostasis in T cells. Purified murine naive CD8+ (A) or CD4+ T cells (B) were activated with immobilized anti-CD3 and anti-CD28 Abs and expression of the ZIP10 zinc transporter and EVER1 genes was determined by qRT-PCR at the indicated time points. Data are from 3 independent experiments. Total amount of free zinc was determined in murine CD8+ (C) or CD4+ T cells (D) after 24 h incubation in standard medium (medium), in medium supplemented with IL-2 (IL-2), or with anti-CD3/anti-CD28 Abs in the presence of IL-2 (CD3/CD28). The content of free zinc was measured by flow cytometry with FluoZin-3, a zinc ion-specific indicator. The total amount of free zinc per cell was calculated as described in materials and methods and normalized for the unstimulated cells (medium) values (100%). Purified naive CD8+ (E) or CD4+ T cells (F) were stimulated by immobilized anti-CD3 and anti-CD28 Abs in the presence of IL-2 and IL-12. The expression of metallothionein 2 (MT-2) was assessed by qRT-PCR. Data are representative of 3 independent experiments. G. Murine naive CD8+- or CD4+- T cells were activated (CD3/CD28) or not (control) by immobilized anti-CD3 and anti-CD28 Abs for 24 h. The concentration of free zinc was then measured by flow cytometry with FluoZin-3. H. Purified murine naive CD8+- T cells were activated with immobilized anti-CD3 and anti-CD28 Abs. At the indicated time the zinc ionophore pyrithione (PY) was added to the medium at different concentrations. Radiolabeled thymidine was added 24 hrs after initiation of the cell culture, and proliferation was assessed on the basis of the thymidine incorporation 24 h later.

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Materials and Methods. Interestingly, high HPV5 viral load was recently described in non-symptomatic family members of an EV patient with heterozygous EVER2 13-nucleotide deletion [37]. These data suggest that heterozygous EVER2 mutation, and the resulting decrease in protein expression, favors HPV5 replication. This may explain the transient EV-like clinical presentation of this heterozygous patient. Using the FluoZin-3 based cytometric assay, two independent measurements of free zinc in CD4+T cells were performed, side-by-side for all the lines. The mean free Zn2+ concentration in EV-derived CD4+ T cells was higher than in CD4+ T cells from healthy donors (Fig. 4B). This higher concentration of free zinc in EVER-deficient CD4+ T cells was maintained following overnight incubation of the cells with a nontoxic zinc concentration (100 μM) (Fig. 4C). Although derived from a small number of patients, these data suggest that EVER proteins might be involved in the control of zinc homeostasis in lymphocytes. At least two different types of intracellular zinc signaling occur in T cells. One happens extremely rapidly after TCR triggering as a result of zinc transporter activity. The other depends on transcriptional changes in expression of zinc importers and exporters that increase the cellular zinc content in a delayed and durable manner. In this study, we have monitored the second type of zinc signal but it would be most interesting to assess whether there is any impact of EVER-deficiency on the early and focal T cell zinc fluxes upon TCR activation. Further studies on a larger cohort of EV patients and/or on EVER knock-out mice should help settle this issue.

Since the concentration of free Zn2+ is tightly controlled during lymphocyte activation (Fig. 3G) and EVER-deficiency might increase the concentration of unbound zinc ions (Fig. 4), one burning question relates to the biological consequence of the zinc imbalance in T cells. During T-cell activation zinc acts at different levels. Zn2+ participates in the interaction between Lck and CD4 and CD8 co-receptors, stabilizes Lck dimerization, and is likely involved in the activation of protein kinase C [38–40]. It has been shown that Zn2+ is released from lysosomes upon T-cell activation [32] and that it participates to IL-2R-mediated signaling through the regulation of ERK activity [32]. Moreover, zinc influx from extracellular sources is induced within minutes of TCR triggering in primary T cells, with compartmentalized high zinc concentration under the zone of the T cell/APC contact. This local increase in cytoplasmic zinc influences proximal TCR signaling resulting in enhanced proliferative responses to suboptimal stimuli [31]. On the other hand, Tanaka and colleagues have reported that excess of zinc suppresses mitogen-activated IL-2 production in lymphocytes [41]. Collectively, the current data indicate that Zn2+ ions play a clear role in signal transduction and T-cell activation, and any deviation in free zinc concentration is likely to impair these processes [42]. This, in turn, highlights the importance of the system that protects the cells from the high concentration of Zn2+ in the extra-cellular milieu. Interestingly, when we disrupted this zinc homeostasis by incubating T cells with non-toxic concentrations of a zinc ionophore (pyrithione), the CD3/CD28-dependent proliferation was inhibited, as demonstrated by thymidine incorporation (Fig. 3H) and CFSE dilution assay (data not shown).

Naïve T cells appear more sensitive to zinc than activated T cells (Fig. 3H), even though this inhibition is only temporary lasting cells enter into mitosis 24 hours after exposure to pyrithione (data not shown). The reason of the higher sensitivity to zinc of naive T cells remains to be elucidated. It is likely that activated T cells are more resistant to excess of zinc, since they have already reorganized the system controlling zinc homeostasis, and notably...
exhibit increased expression of zinc buffering proteins (metallothioneins). Taking into account the known properties of ZnT-1 as a zinc transporter, and our observations of increased concentration of Zn\(^{2+}\) in EVER-deficient lymphocytes (Fig. 4), it seems probable that the ZnT-1/EVER complex participates in the maintenance of low levels of free zinc in lymphocytes. It is tempting to speculate that EVER-deficiency, by shifting zinc homeostasis, could have an impact on T-cell activation, especially in naive T cells, in which EVER expression is the highest. Certainly, the clinical manifestations of EVER-deficiency suggest that such an impact does not interfere with crucial steps of T-cell activation, as EV patients respond normally to pathogens other than HPV. Indeed, we did not observe any striking differences in the rate of culture expansion during preparation of the primary T cell lines from EV patients and healthy controls (data not shown).

In conclusion, we have demonstrated that EVER genes are expressed at high levels in T and B cells. Their expression in T cells is sharply decreased upon TCR/CD28 stimulation, as a part of the global activation-dependent reorganization of the molecular machinery controlling cellular zinc homeostasis. Indeed, the elevated intracellular amount of free Zn\(^{2+}\) ions is associated with decreased expression of \(\alpha\text{ZnT-1}\) and increased expression of \(\alpha\beta_{10}\) and \(\gamma\beta_6\) in activated T cells. The preliminary data from EV patient-derived cell lines suggest that EVER might be involved in maintaining a constant low level of free zinc in lymphocytes. Altogether, these results support our previously formulated hypothesis [4] that the natural anti-HPV barrier, of which EVER proteins constitute an essential part, might comprise at least two arms: keratinocytes and lymphocytes. Although still speculative, we suggest that EVER-deficiency in T cells may contribute to susceptibility to HPV infection, possibly in combination with EVER-deficiency in keratinocytes. However, the exact mechanism of the impact of EVER-deficiency on T cell function as well as the reason for the extreme selectivity of this barrier towards HPV remains to be elucidated.

**Materials and Methods**

**Mice**

For all experiments 8 to 12 week-old female BALB/c mice were used. For antigen-driven T-cell activation, we used the CL4-TCR transgenic BALB/c mice, which express a TCR specific for the influenza virus HA121–230 peptide in the context of H2-K\(^{b}\) on most CD8\(^{+}\) T cells [27,28]. All breeding and experimental procedures were carried out in accordance with European Union guidelines and were approved by the regional ethics committee N\^MP/18/26/04/04.

**T Cell Purification and Culture**

For T cell cultures, spleens and lymph nodes were collected and naïve CD4\(^{+}\) or CD8\(^{+}\) T cells (CD4\(^{+}\) CD25\(^{-}\) CD62L\(^{+}\) and CD8\(^{+}\) CD25\(^{-}\) CD62L\(^{+}\)) were purified by MACS (Miltenyi Biotec) according to the manufacturer’s protocol (\(\geq 92\%\) or 94% purity, respectively). The purified naïve T cells were activated by immobilized anti-CD3 (BD Biosciences, clone 145-2C11), anti-CD28 (BD Biosciences, clone 37.51) antibodies individually, or combination CD3/CD28 beads were used (Dynabeads mouse CD3/CD28 T cell expander, Invitrogen). Antigen-specific stimulation was performed with CL4-TCR T cells co-cultured with irradiated, T cell-depleted, syngeneic splenocytes loaded with the cognate peptide. For the subsequent PCR analysis transgenic T cells were isolated by MACS (Miltenyi Biotec) using biotinylated Thy1.2 mAb (BD Pharmingen, clone 53-2.1) combined with streptavidin microbeads (Miltenyi Biotec).

For human T cell cultures, PBMC were obtained from buffy coat preparations from anonymous healthy donors, from the Purpan university hospital blood bank (Toulouse, France). Naïve CD8\(^{+}\) T cells were sorted by MACS from PBMC with naïve CD4\(^{+}\) T cell isolation kit (Miltenyi Biotec). Human CD8\(^{+}\) T cells (TCR+; CD4\(^{+}\); CD145RCHigh and TCR+; CD8\(^{+}\); CD145RCLow), were purified by FACS. The cells were thereafter stimulated by anti-CD3 and anti-CD28 beads (Dynabeads human T activator CD3/CD28, Invitrogen).

**EBV-transformed Human Lymphocytes**

The lines have previously been generated [9] using PBMC from an EV suffering patient (EVER2 Del275A, B. Bouadjar, P. Cassonnet, M. Favre, G. Orth, M. Ramoz, unpublished results) or from healthy relatives (homozygous EVER2 wt) by transformation with EBV. The cells were cultured in standard RPMI (Invitrogen) medium supplemented with 10% FBS (Invitrogen). The EVER2 genotype of each of the lines was confirmed by sequencing directly before the experiments.

**Primary Human T Cell Lines**

One patient presented with full-blown EV and was homozygous for the EVER2 T150fdX3 mutation (P. Cassonnet, M. Favre, S. Jablonska, S. Majewski, G. Orth, M. Ramoz, unpublished results). Her daughter was heterozygous for the EVER2 T150fdX3 mutation and had developed transient EV-like symptoms. Her first skin lesions were plane warts on the dorsum of the hands that appeared at the age of 7 yrs. The lesions were treated successfully by liquid nitrogen freezing. However at the age of 14 years new extensive skin lesions developed on the dorsum of the hands and feet, as well as on the trunk. These lesions were much more abundant, some of them larger than typical plane warts. Some of the lesions were confluent with uneven polycyclic outlines. The lesions persisted for about 2 years and disappeared after several courses of cryotherapy. In the skin lesions DNA of HPV3 and HPV5 was detected. Polyclonal T cell lines were generated from PBMC of the 2 patients or healthy controls, as previously described [43]. Briefly, mixed lymphocyte reaction cultures of PBMC (5 x 10\(^5\) cells/ml) and irradiated allogenic PBMC from 2 different donors (10\(^6\) cells/ml) were performed. The lines were cultured in IMDM (Cambrex Bio Science) supplemented with 1 µg/ml PHA, and 100 IU/ml recombinant human IL-2 (Chiron), 10% YSSEL medium (Dyaclone), 5% FBS (Cambrex Bio science), and penicillin/streptomycin (Bristol-Myers Squibb).

T cells were restimulated as described every 2 weeks. T cells were used in this study after one stimulation cycle. The study was approved by the Ethics Committee at the Medical University of Warsaw. Written informed consent was received from all participants.

**RT-PCR**

RNA from the cell cultures or organs was isolated by RNeasy mini kit (Qiagen), and reverse transcription (SuperScript III first-strand synthesis system for RT-PCR, Invitrogen) was performed, according to the manufacturer’s instructions. The obtained cDNA was used as a template in classical PCR (RT-PCR; Taq Polymerase, Q-BioGene) or in quantitative PCR (qRT-PCR; qPCR mastermix plus for SYBRgreen, Eurogentec). The primers used for the assessment of the EVER genes expression were designed using primer3 software (http://trodol.wi.mit.edu/primer3/) and their validity was confirmed by direct sequencing of the PCR product (RT-PCR) or by the sequencing of the qRT-PCR products cloned into the standard vector (Topo TA cloning kit for sequencing, Invitrogen). The primers used for qRT-PCR on
mouse samples were Ever1_F : 5’CTCAGCTGAGGAGCTGAG3’, Ever1_R : 5’CTCTAGAAGGTGAGGAGCAG3’; Ever2_F : 5’GGCTCTACCGGGAACCTGCG3’, Ever2_R : 5’AGAGGCCTCCTGCTGTA3’; Znt1_F : 5’GGCCACACCCACGAATTTTG3’, Znt1_R : 5’CTTCGGGCTTCCAGATTCTGA3’; MT-2_F : 5’AACTCTTTCAACCCGAGTCTCGT3’, MT-2_R : 5’AGATGACATTTGCAATTGTTGAT3’; Zip6_F : 5’GATGGTGATCATGGGGCAGAC3’, Zip6_R : 5’CTGTGTTTGCTAAAGGCTGG3’; Zip10_F : 5’GAGCCGGAAGAGGATGCAG3’, Zip10_R : 5’TCCACAGATGATGTCAGTG3’; HPRT_F : 5’TGACACTGGTAAACAAATGCAA3’, HPRT_R : 5’AACAAAGCTCTGGCTGTATACAA3’. The primers used for qRT-PCR on human cells were Ever1_F : 5’GGTTGCGCTGCTTCAACTAC3’, Ever1_R : 5’CCGGAAAGATGGACGAGCTG3’; Ever2_F : 5’CAAGAAGTACCCCTCTGGAAG3’, Ever2_B : 5’GAGGAGTGTATGGTCTGGA3’; GAPDH_F : 5’ACGGATTGGTGGTCATTGGGC3’, GAPDH_B : 5’TTGACGGTGGCATGAAATTGG3’.

Transfections
293 T cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS. They were transfected with plasmid encoding EVER1-FLAG or EVER2-FLAG fusion protein using FuGENE-6 (Roche), according to the manufacturer’s instructions. The cells were cultured for 24 h before analysis of EVER protein expression.

Western Blot Analysis
Primary T cells or transfected 293 cells were lysed using RIPA buffer (10 mM Tris HCl pH 7.5, 50 mM NaCl, 1% Triton X100, 30 mM Na2PO4, 5 mM MnCl2, 10% Glycerol, 0.1% SDS, 50 mM NaF, 1 mM Na3VO4, 1 mM DTT, and Roche mini complete protease inhibitor). The crude lysates (or precipitated proteins, depending on the experiment) were migrated on 10% polyacrylamide gels (Novex, Invitrogen) and transferred onto nitrocellulose membranes (Amersham Biosciences). The following primary antibodies were used: polyclonal Ab anti-EVER1 (Oenses ref: OSR00223W), polyclonal Ab anti-EVER1 (Abcam ref: ab67326), anti-β-actin (Sigma, clone AC-15) and anti-FLAG (Oenses ref: OSR00223W), polyclonal Ab anti-EVER1 (Abcam primary antibodies were used: polyclonal Ab anti-EVER1 nitrocellulose membranes (Amersham Biosciences). The following proteins, depending on the experiment) were precipitated by zinc chelator (TPEN; 100 μM - Sigma – ref: 87641). The “maximum” control cells with zinc ionophore (pyrithione; 50 μM - Sigma – ref: 63944) and 100 μM ZnSO4 (Sigma, ref: Z-0251). The zinc concentration was calculated on the basis of the median fluorescence intensity, according to a previously published formula [43]. The changes in the total cellular free zinc content during T-cell activation was assessed by taking into account the changes in the zinc concentration and the modifications of the mean cell volume (estimated according to the formula: VA = (FSC1)3/(FSC2)3 x 100 x, where VA is a mean volume change in the time and FSC1 and FSC2 are forward side scatter values measure at two different time points).

H3-thymidine Incorporation Assay
Purified murine naïve CD8+ T cells were seeded in 96-well plates and stimulated using anti-CD3 and anti-CD28 beads (Dynabeads mouse CD3/CD28 T cell expander, Invitrogen). At the start of the culture or after 3, 6 or 24 h of stimulation, the zinc ionophore (pyrithione, Sigma, ref: 63844) was added at the indicated concentrations. After 24 h, H3-thymidine was added followed by 18 h incubation. Cell associated radioactivity was measured with a Beta counter (PerkinElmer, 1450 LSC & Luminescence counter, MicroBeta Trilux).

Supporting Information
Figure S1 Expression of EVER1 and EVER2 in human T cells. Expression of EVER1 and EVER2 genes was assessed in freshly MACS-purified human CD4+ or CD8+ T cells by (A) conventional RT-PCR (2 independent experiments) or (B) qRT-PCR (3 independent experiments). (TIF)

Figure S2 EVER1 and EVER2 expression is strongly down-regulated in human T cells following TCR-mediated activation. Freshly purified human naïve CD8+ (A) or CD4+ T cells (B) were activated with immobilized anti-CD3 and anti-CD28 Abs. Expression of EVER genes was determined by qRT-PCR at the indicated time points in the course of activation. Data are from 3 independent experiments. (TIF)

Figure S3 ZIP6 zinc transporter expression is up-regulated in T cells following TCR-mediated activation. Purified murine naïve CD8+ (A) or CD4+ T cells (B) were activated with immobilized anti-CD3 and anti-CD28 Abs and expression of the ZIP6 zinc transporter, EVER1 and EVER2 genes was determined by qRT-PCR at the indicated time points. Data are from 3 independent experiments. (TIF)

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Author Contributions
Conceived and designed the experiments: ML MH LD SM MF RSL. Performed the experiments: ML CD MH LD BP IV. Analyzed the data: ML LD RSL. Contributed reagents/materials/analysis tools: SM. Wrote the paper: ML RSL.
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