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Epithelial stem cells as mucosal antigen-delivering cells: A novel AIDS vaccine approach

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Abstract

A key obstacle limiting development of an effective AIDS vaccine is the inability to deliver antigen for a sufficient period of time resulting in weak and transient protection. HIV transmission occurs predominantly across mucosal surfaces; therefore, an ideal vaccine strategy would be to target HIV at mucosal entry sites to prevent infection. Such a novel strategy relies on the activation of mucosal immune response via presentation of viral antigens by the mucosal epithelial cells. The use of a terminally differentiated epithelial cell promoter to drive expression of antigens leading to viral protein production in the upper layers of the epithelium is central to the success of this approach. Our results show that when administered intradermally to mice, a GFP-reporter gene under the transcriptional control of the involucrin promoter is expressed in the upper layers of the epidermis and, although transduced cells were very low in number, high and sustained anti-GFP antibody production is observed *in vivo*. A subsequent experiment investigates the effectiveness of GFP-tagged replication-competent SIVdeltaNef and GFP-tagged replication-deficient SIVdeltaVifdeltaNef constructs under the transcriptional control of the involucrin promoter. Optimal conditions for production of pseudotyped VSV-G viral particles destined to transduce basal epithelial stem cells at the mucosal sites of entry of SIV in our animal model were determined. Altogether, the data demonstrate the feasibility of an epithelium-based vaccine containing involucrin-driven viral antigen encoding sequences that integrate into epithelial stem cells and show long-term expression in the upper layer of the epithelium even after multiple cycle

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of epithelia renewal. Such epithelium-based vaccine should elicit a long-term immunity against HIV/SIV infection at the site of entry of the virus.

Keywords

Simian Immunodeficiency Virus; Vaccine; Mucosal; Antigen; Delivery; Epithelial Stem Cells

1. Introduction

Despite recent advances in developing immunogenic-induced HIV/AIDS vaccines [1], achieving protection against HIV infection remains elusive. The HIV virus presents barriers to effective immunity including antigenic variability; resistance to neutralizing antibodies; down regulation of MHC class I and CD4 on infected cells; and, preferential destruction of viral-specific CD4⁺ lymphocytes [2, 3].

Developing an effective vaccine restricting viral replication at the mucosal portal of entry remains an important goal since HIV transmission occurs predominantly across genital and rectal mucosal surfaces. The presence of HIV-specific T lymphocytes in the mucosa and at sites of early viral replication is an important factor for vaccine efficacy, as it would inhibit HIV spread into adjacent lymph nodes. Such approach requires i) a life-long stimulation of the immune system with viral antigens providing a strong barrier to viral replication; and, ii) a targeted immune response at sites of primary HIV replication (vaginal or rectal mucosa). Research using attenuated Simian Immunodeficiency Virus (SIV) in macaques often accomplishes effective and durable protection against pathogenic SIV challenge. One promising approach utilizes a replication-defective SIV limited to a single cycle of infection [4–7].

This manuscript details a novel vaccine approach based on the ability of therapeutic lentiviral vectors integrated in epidermal or mucosal epithelial stem cells to induce virus-specific cellular immune responses at mucosal sites of viral entry. The strategy employs the well-characterized Keratinocyte-specific involucrin locus (INV) promoter that ensures tissue specific expression in terminally differentiated epithelial cells [8]. By replacing viral enhancer elements with the minimal INV promoter, both tissue-specific expression and high viral titers can be achieved.

We have developed GFP-tagged replication-competent SIVmacdeltaNef and replication-deficient SIVmacdeltaVifdeltaNef constructs under the transcriptional control of the involucrin promoter (pINV).

The mechanisms controlling the involucrin transcription in the epidermis and mucosa are well conserved, as the human pINV is active in mouse epidermis and vaginal/ectocervix epithelium. This allows the use of a mouse model to develop the preliminary groundwork for our vaccine approach. We anticipated that pINV would drive expression of SIV-derived constructs in terminally differentiated epithelial cells. Long-term antigen expression in the epithelium upper layers will occur even after multiple epithelia renewal cycles, thus eliciting a long-term immunity against HIV/SIV infection at viral entry sites. We show that the

involucrin-driven lentiviral vector is exclusively expressed in the epithelium upper layer, and mediates long-lasting antibody production in mice. We also demonstrate that an involucrin-driven SIV is preferentially expressed in human calcium-differentiated keratinocytes.

2. Materials and methods

2.1. Construction designs

Recombinant DNA plasmids and vaccine vectors were constructed using restriction endonucleases and, ligations performed using Ligafast Rapid DNA Ligation protocol (New England Biolabs). OneShot TOP10 Chemically Competent *E. coli* (Invitrogen) were used for plasmid DNA amplification. Bacteria were routinely grown in Luria Broth (LB) supplemented with ampicillin (final concentration: 100 µg/ml). Plasmid DNA isolation was achieved using EndoFree Plasmid Mega Kits (Qiagen).

2.1.1. Construction of Involucrin promoter-driven vectors—The minimal involucrin promoter [10] was synthesized by overlapping PCR and replaced the pPGK promoter in vector pRRL.SIN.cPPT.pPGK-GFP.WPRE [40], using Cla-I/BamH-I endonuclease restriction and subsequent ligation, kindly provided by Dr Trono (EPFL, Switzerland).

2.1.2. SIV vaccine construction—The IRES-GFP fragment from pBlueScript IRES-GFP plasmid (Invitrogen) was amplified by PCR using specific primers containing XhoI restriction sites and cloned into the SIVmac239megalo3' plasmid [35] between positions 9500 and 9690 to generate plasmid pSIVmac239megalo3'/IRES-GFP. The nef gene in plasmids pSIVmac239megalo5' and pSIVmac239megalo3'/IRES-GFP was deleted and replaced by inserting the STR fragment from pSIVmac239/STR plasmid [41] between the EcoR-I/Not-I or Not-I/Nhe-I restriction sites, respectively, giving rise to pSIVmac239megalo/STR5' and pSIVmac239megalo/STR3'/IRES-GFP.

To avoid the TAR/Tat transcriptional control, we inactivated TAR sequence by homology to HIV using the following primers: 5'-

GCGGCCGCTGCGCAGAGGCAGAAAGAGCCATTGGAGGTTCTCTCCAGCACTAG C and 5'-AGGAGGAGCATTGGTGTTCCTGCTAGACTCTCACC. This fragment was subcloned and introduced at the Fsp-I and Nar-I sites of plasmids pSIVmac239megalo/STR5' and pSIVmac239megalo/STR3'/IRES-GFP creating plasmids pSIVmegaloSTR5'/TAR* and pSIVmegaloSTR3'/IRES-GFP/TAR*, respectively. A full-length construct was reconstituted after ligation of its 5'- and 3'-halves together. The resulting ubiquitously transcriptionally regulated construct was referred to as pCMV-IE/SIV/deltaNef/IRES-GFP.

The 570bp involucrin promoter was cloned in place of the 5'-CMV promoter of pSIVmac239megalo5' (NotI/FspI restriction sites) and pSIVmac239megalo3' (NotI/FspI restriction sites) plasmids. A full-length epithelia-specific transcriptionally regulated construct was reconstituted after ligation of its 5'- and 3'- halves (named: pInv/SIV/deltaNef/IRES-GFP).

To obtain replication-deficient viral constructs, the *vif* genes from the 5' moiety of pCMV-IE/SIV/deltaNef/IRES-GFP and pInv/SIV/deltaNef/IRES-GFP plasmids (pSP72 backbone) were deleted by replacing the PacI/SphI fragment with the PacI/SphI fragment of pSIVdeltaVif5', kindly provided by Dr. Desrosiers [42]. The resulting recombinant plasmids were named pCMV/SIV5'/deltaVif and pInv/SIV5'/deltaVif.

Full-length constructs were obtained by ligation of the 3' moiety of pCMV-IE/SIV/deltaNef/IRES-GFP or pInv/SIV/deltaNef/IRES-GFP plasmids (SphI/EcoRI). The full-length replication-deficient viral constructs were named pCMV-SIV (pCMV/SIV/deltaVif/deltaNef/IRES-GFP) and pINV-SIV (pInv/SIV/deltaVif/deltaNef/IRES-GFP).

2.2. CFA immunization and viral transduction of mice epidermis

Mice were immunized by footpad subcutaneous injection of emulsified Complete Freund's Adjuvant (CFA) with 200µg of His-tagged purified GFP in PBS (50/50; v/v). Viral transduction was performed as previously described [43]. Briefly, FVB mouse shaved backs were dermabraded using a felt wheel. Day 3 post-abrasion, animals were inoculated with 50µl (10⁸ pfu) of: VSV-pseudotyped pRRL.SIN.cPPT.pINV-GFP.WPRE; pRRL.SIN.cPPT.pPGK-GFP.WPRE (heat inactivated, 30min at 56°C); pRRL.SIN.cPPT.pINV-GFP.WPRE; or control pRRL.SIN.cPPT.pPGK-GFP.WPRE viruses.

2.3. Histological analysis

Day 7 post-inoculation, mice were sacrificed. The inoculation site was snap frozen in OCT compound. Eight µm-cryosections were fixed (10min, 4% paraformaldehyde), rinsed (PBS) and examined by fluorescent microscopy.

2.4. Anti-GFP antibody quantification in mice serum

The amount of anti-GFP antibody in mice serum was determined by an in-house ELISA using recombinant GFP protein [44] coated on maxisorp plates (Nalge Nunc, Rochester, USA) in a 1.5mM carbonate/bicarbonate pH=9.6 buffer. The serum was diluted 100- to -500-fold and incubated (1 hour, room temperature) in a 1.5mM carbonate/bicarbonate buffer. Anti-GFP immunoglobulins were quantitated after incubation (1 hour, room temperature) with goat anti-mouse Ig kappa light chain-HRP (Abcam, USA), and subsequent color development.

2.5. Human keratinocytes differentiation

Normal Human Epidermal Keratinocytes (NHEK, PromoCell, Germany) were cultured in keratinocyte growth medium 2 (PromoCell, Germany), according to manufacturer's instructions (Merck Millipore, Germany). For NHEK terminal differentiation, we used 1mM concentration of CaCl₂ (PromoCell). Clone 16B4 was used for cytokeratin-6 antibody detection (PromoCell).

2.6. Visualization and quantitation of GFP-expression

Light and fluorescent microscopy were performed using a Zeiss microscope. Flow cytometry experiments were performed on FACSCalibur (CellQuest software).

2.7. Viral stock production

HEK-293 cells were maintained as adherent cultures in DMEM supplemented with 10% FBS and 500ug/ml Geneticin. Plasmids were purified using EndoFree Plasmids Maxi Kit (Qiagen). HEK-293 cell cultures (75cm² flasks) were co-transfected with 15ug of each plasmid pCMV-IE/SIV/deltaNef/IRES-GFP and pLP/VSVG (Invitrogen) or pInv/SIV/deltaNef/IRES-GFP and pLP/VSVG, using Lipofectamine 2000 according to manufacture protocol (Invitrogen). Co-transfection using VSVG plasmid encoding for envelope G glycoprotein from VSV, produced pseudotyped retrovirus with a broader range of infectable cell types [11]. After overnight incubation, the media was changed and cells incubated for an additional 48 hours. The media was then removed, passed through a 0.45microm filter and concentrated using a MiniKrosFlo Research II Tangential Flow Filtration (TFF) System (Spectrum Laboratories). A polyethersulfone hollow fiber membrane module was used with a 500Kd molecular weight cutoff. The p27 titration, before and after concentration, was determined using SIV p27 Antigen Capture Assay (Advanced BioScience Laboratories).

3. Results

3.1. *In vivo* promoter activity assay

Involucrin is a well-characterized differentiation marker in keratinocytes [9]. When inoculated in transgenic mice, a minimal human involucrin promoter leads to the reporter gene expression in the epidermis upper layers [10]. Transient expression of the reporter gene driven from pINV is observed in the epidermis once introduced via topical transduction [10]. To test the pINV efficacy to drive a lentivirus construct *in vivo*, we performed transfections with two constructs: one driven by the ubiquitously expressed PGK promoter (pRRL.SIN.cPPT.pPGK-GFP.WPRE) and the other by pINV (pRRL.SIN.cPPT.pINV-GFP.WPRE). Mice epidermal cells were infected with VSV-pseudotyped HIV vector containing each construct. Results of topical delivery of the involucrin-GFP reporter plasmid are shown in Figure 1 (lower panel). As expected, GFP-expression of the involucrin-driven vector was seen in the *stratum corneum* of mice epithelia following epidermic inoculation.

The efficacy of the involucrin minimal promoter-GFP construct to elicit an immune response was evaluated by ELISA for the presence of anti-GFP antibodies in mice serum. A significant increase in anti-GFP antibodies was detected over time in mice serum inoculated with pINV driven vector compared to PGK promoter driven vector or Complete Freund Adjuvant alone (Figure 2). These results suggest that the involucrin minimal promoter used as a transcriptional regulatory element allow high and sustained expression of GFP in the epidermal upper layers.

3.2. Construction and *in vitro* assessment of SIV-derived vector

The strategy relies on the use of near full length SIV genome constructs under transcriptional control of the involucrin minimal promoter. The *Nef* gene has been deleted and replaced by GFP gene in order to follow antigen expression. For safety concern, deleting the *Vif* gene made the replication-deficient viral constructs. Figure 3 illustrates the mucosal SIV-derived vectors overall generation.

Viral stocks of the SIV-derived vector constructs were obtained by co-transfection of HEK 293T with various plasmids, as described above. Viruses were pseudo-typed by Vesicular Stomatitis Virus G glycoprotein (VSV-G) allowing production of viral particles [11]. The production of VSV-G pseudotyped viral particles was followed by monitoring p27 expression in culture supernatants using SIV p27 Antigen Capture Assay. The p27 titration of culture media before concentration was 6 ng/ml for both SIVpCMV and SIVpInv constructs, and at 35ng/ml and 90ng/ml, respectively, after concentration (Krosflo).

3.3. Transduction of human keratinocytes with the involucrin promoter driven SIV-derived vector

Infectious viral particles were used in transduction experiments in normal human epidermal keratinocytes (NHEK). In calcium-free conditions, only a few of the dividing NHEK (up to 10%) differentiated spontaneously. The addition of 1mM of calcium in culture media stopped cell division and induced massive stem cell terminal differentiation into keratinocytes [12]. To confirm that the involucrin minimal promoter was driving GFP expression in the vector, NHEK were transfected with the same involucrin promoter-driven vector as in mice (same as Figure 1, named pRRL.SIN.cPPT.pINV-GFP.WPRE). As expected, GFP expression was detected in these cells (Figure 4B). SIV-pINV-derived vector-transfected cells revealed GFP expression by fluorescent microscopy (Figure 4C) and flow cytometry (figure 4D). Interestingly, the percentage of cells expressing GFP was significantly increased with calcium addition in culture media, suggesting an increase in viral protein expression upon keratinocyte differentiation.

These results confirm the ability of SIV vector under the control of pINV to drive and increase gene expression in keratinocytes upon their differentiation.

4. Discussion

Of the vaccine approaches analyzed in the SIV/macques model, vaccination with live attenuated lentiviruses has consistently yielded the most effective protection against challenge with pathogenic SIV strains. The initial study by Daniel et al. [13] documented protection against intravenous challenge with 1000 monkey infectious doses (MID₅₀) of SIVmac251 three years after vaccination. Subsequent studies performed by other groups confirmed the ability of attenuated SIV strains to induce protective immunity [6, 7]. Experiments conducted at the New England Primate Research Center (NEPRC, MA) demonstrated that a more attenuated SIVmac239Delta3 strain deficient in *Nef*, *Vpr* and upstream sequence (US) of the LTR, protected against SIV challenge [14] and induced sterile protection against heterologous vaginal challenge [15]. However, safety is the overriding concern for live attenuated vaccines, and thus far all attempts have failed to demonstrate a safety level commensurate with humans use.

As an experimental vaccine approach designed to retain many of the live attenuated SIV features without risk of reversion to a pathogenic phenotype, we and others have developed genetic approaches to produce SIV strains limited to single cycles of infection [4, 6, 7, 16, 17]. Previous studies demonstrated that immunization of macaques with single-cycle SIV (scSIV) trans-complemented with VSV-G elicited potent virus-specific T lymphocyte

responses [17] comparable in magnitude to T-cell responses elicited by optimized prime-boost regimens including recombinant DNA and viral vectors [18–22]. However, despite the presentation of native trimeric SIV envelope glycoprotein on infected cells surface and virions, none of the scSIV-immunized macaques developed antibody responses neutralizing SIV_{mac}239 [17].

Skin epidermis is a stratified squamous epithelium mainly composed of keratinocytes that surrounds the entire body. Keratinocytes in the proliferative basal cell layer upregulate transcription of cornified envelope precursor proteins such as involucrin [23]. All stratified squamous epithelia including vaginal and oral epithelium present the same pattern of differentiation, differing chiefly by their number of epithelial layers, degree of keratinization and mucous production. While the stages of squamous differentiation are well characterized, the transcription factors regulating these differentiation-specific genes have only been recently described. Involucrin [24–28] is a well-characterized differentiation marker in keratinocytes with defined promoters. Although transcriptional control is incompletely understood, the tissue-specificity of keratinocyte genes is strict for involucrin [29].

Retroviral vectors are widely used to transduce exogenous genes into a variety of cells providing an efficient gene transfer tool for gene therapy applications. These vectors have been used with considerable success for transduction of epidermal stem cells *in vitro* and *in vivo*. Transcription from the long terminal repeat (LTR) is dependent on viral promoter/enhancer elements located in the U3 region of the 5'LTR allowing constitutive expression of the transferred gene in most cell types including keratinocytes [10, 30, 31]. Several strategies have been employed to confer tissue- or cell-specific expression to retroviral vectors. These include: insertion of a tissue-specific promoter in an internal position within the retroviral vectors, construction of self-inactivating vectors in which viral enhancer elements are deleted thereby allowing expression only from the internal promoter, and insertion of a complete minigene into the LTR upstream from the U3 region [32]. However, the above mentioned strategies usually decreased viral titer and often failed to induce strict tissue-specific expression [33]. In contrast, attempts to redirect LTR transcriptional activity by replacing the viral enhancer with heterologous control elements from cellular or viral genes, have been successful for HIV [34] and SIV [35]. This strategy should allow transgene expression in a specific tissue or cell without significant loss in the viral titer as observed for non-lentiviral retroviruses [36, 37]. However, from our experience, the size of tissue-specific enhancers remains a major limitation of this approach. Most candidate promoters exceed the 1500bp size limit in order to achieve correct tissue specificity [38] [39]. The involucrin promoter is well suited as it can be shortened without loss of tissue specificity by fusing the promoter distal region directly to the involucrin minimal promoter [8].

This study demonstrates the initial criteria essential for using mucosal lining epithelial cells as a tool for gene therapy-based vaccine strategy. GFP-tagged replication-competent SIV_{mac}deltaNef and GFP-tagged replication-deficient SIV_{mac}deltaVifdeltaNef constructs were generated under the transcriptional control of human pINV. We demonstrated that pINV, used in a GFP-reporter construct, could drive GFP expression in mice epidermis upper layers after epidermal inoculation. Moreover, our results demonstrate that GFP

expression under transcriptional control of pINV can elicit a high and sustained anti-GFP antibody production.

A vaccine approach providing a life-long viral antigen stimulation of the immune system at site of primary HIV replication should achieve solid long-term protection. We showed that epithelial stem cells could be used as permanent sources of viral antigen and their differentiated offspring as antigen producing-presenting cells. Placing a single cycle SIV genome under pINV control is a safer strategy compared to traditional attenuated lentivirus vaccines. Once administered into target epithelial stem cells from different tissues, the basal layer cells will divide and differentiate, thus triggering SIV antigen expression at mucosal sites. For safety considerations, the use of a replication-deficient construct is necessary.

We demonstrated that GFP used as a surrogate marker integrated into the involucrin promoter-driven SIV, is expressed in normal human epidermal keratinocyte (NHEK). As expected adding a differentiation factor into the culture enhances GFP expression.

The data demonstrates the feasibility of an epithelium-based vaccine containing involucrin-driven viral antigen encoding sequences. It is clear that pINV, when used as a transactivating element in a GFP reporter construct, allows GFP-expression in the epithelium upper layers *in vivo* after integration in epidermal cells following dermabrasion. Upon activation of the involucrin-promoter, GFP expression in the epithelium upper layers leads to maintain production of plasma anti-GFP antibodies. This is most likely due to the constant antigen delivery to lymph nodes by dermal professional APC. Even if efficient in mouse, this approach still remains to be tested in primates where the dermal architecture is quite different from the mouse in terms of thickness or antigen-presenting cells type.

Figure 5 illustrates our novel vaccine strategy as a proof-of-concept. After integration of the involucrin-driven viral constructs into basal layer stem cells, these cells divide and their differentiation trigger SIV antigen expression in the epithelia upper corneal layers. Upon epithelial cell differentiation, pINV drives SIV antigens expression. In the epidermis upper layer, SIV antigens are continuously freed in the mucosa lumen thus stimulating dendritic cells via cross priming and triggering long-lasting immunity.

Based on these observations, it is anticipated that the involucrin-promoter viral constructs will integrate after transduction into epithelial stem cells. The differentiated epithelial cells will show long-term expression of viral antigens even after multiple epithelia renewal cycles, thus eliciting a long-term immunity against HIV/SIV infection at viral entry mucosal sites.

5. Conclusion

Long-lasting formulations and novel delivery methods for mucosal vaccine offering ease of use and subsequent enhanced tissue adherence must be further developed. Our strategy proposed a novel approach in the development of an efficient vaccine against HIV infection by i) eliciting a life-long stimulation of the immune system with viral antigens providing a strong barrier to viral replication; and, ii) targeting immune responses at the site of primary HIV replication. A potential drawback of this strategy may be due to continuous antigen

exposure, which may lead to immune exhaustion as described for chronic viral infection [45]. However this possibility is inherent to antigen long-term delivery.

This proof-of-concept study should provide valuable information on the ability of such a vaccine strategy to induce T cell responses at mucosal sites, improve our knowledge of the role of mucosal immune responses against SIV and investigate other potential routes toward an eventual successful HIV vaccine. This strategy should offer the opportunity to generate a single-dose vaccine, which will be particularly appropriate for developing countries.

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Abbreviations used in this paper

AIDS	Acquired Immunodeficiency Syndrome
BD	Becton Dickinson Immunocytometry Systems
Bp	base pair
CMV	cytomegalovirus
CTL	Cytotoxic T Lymphocytes
DNA	Deoxyribonucleic acid
HIV	Human Immunodeficiency Virus
INV	involucrin
K	keratin
MMP-9	Matrix Metalloproteinase-9
MID	Monkey Infectious Dose
MVA	Modified Vaccinia Ankara
pCMV	CMV promoter
pINV	involucrin promoter
SIV	simian immunodeficiency virus
US	Upstream Sequence
VSV-G	Vesicular Stomatitis Virus G glycoprotein

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Highlights

- * Differentiation-dependant promoter driven lentivirus vector
- * Simian Immunodeficiency differentiation-dependant promoter
- * Terminally differentiated directed Simian Immunodeficiency vaccine

Figure 1.

Expression of pRRL.SIN.cPPT.pINV-GFP.WPRE plasmid in mice after epidermic inoculation. This construct (A) has been generated to test the efficacy of the involucrin minimal promoter (pInv) in driving the expression of GFP used as reporter gene in *in vivo* promoter activity assay. The upper panel represents the actual generation of the construct by replacement of the phosphoglycerate kinase promoter (pPGK) by the involucrin minimal promoter (pINV) in the pRRL.SIN.cPPT.pPGK-GFP.WPRE plasmid. Green Fluorescent Protein (GFP)-expression of pRRL.SIN.cPPT.pINV-GFP.WPRE plasmid in mice is shown in the lower panels following epidermic inoculation. (B) Figuration of the different layers of the sample taken at the site of inoculation. (C) Light microscopy of the sample showing the dermis and epidermis layers. (D) Observation of the expression of the GFP at the inoculation site of the pRRL.SIN.cPPT.pINV-GFP.WPRE plasmid. The representative pictures were taken one week post-inoculation and shows the reporter gene expression in the *stratum corneum* of the epithelia.

Figure 2.

Quantitation of anti-GFP antibodies in mice serum following inoculation of the pRRL.SIN.cPPT.pINV-GFP.WPRE or pRRL.SIN.cPPT.pPGK-GFP.WPRE virus. The level of anti-GFP antibodies was quantified in mice serum over a period of 5-months post epidermal inoculation. The total concentration of anti-GFP antibodies is show as microgram of antibody per ml of serum over 5 months post inoculation. Data are representative of 5 groups inoculated with: pRRL.SIN.cPPT.pPGK-GFP.WPRE (n=2), pRRL.SIN.cPPT.INV-GFP.WPRE (n=2), inactivated pRRL.SIN.cPPT.pPGK-GFP.WPRE (n=2), inactivated pRRL.SIN.cPPT.pINV-GFP.WPRE (n=2), or recombinant GFP in Complete Freund Adjuvant (n=2).

Figure 3.
Schematic representation of the pINV-SIV and pCMV-SIV plasmids.

Figure 4.

Normal Human Epidermal Keratinocytes (NHEK) were cultured in epidermal growth medium on fibronectin-coated plates. The keratinocyte nature of the cells was characterized by cytokeratin 6 expression (A). The NHEK were infected or not by the involucrin driven promoter vector (pRRL.SIN.cPPT.pINV-GFP.WPRE, Figure 1). A representative picture obtained two days after infection by fluorescence microscopy is reported in (B). The SIV under the control of the involucrin promoter (pINV-SIV, figure 3) was used to infect NHEK cells. GFP expression was recorded by fluorescence microscopy three days following

infection in the presence of calcium as terminal differentiating agent (C). Representative flow cytometry of infected NHEK is reported (D). Flow cytometry experiments were performed using in parallel two batches with or without calcium. The analysis was performed 72 hours post-transduction.

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Figure 5.

Schematic representation of the vaccine approach. After integration of the involucrin-driven viral constructs into basal layer stem cells (1), these cells will divide and differentiate triggering SIV antigens expression in the upper corneal layers of the epidermis. The level of SIV antigens expression is represented by the darkening green shades on the figure (left panel), from the basal layer stem cells (blue shade, black line) to the differentiated epidermal cells of the epithelia stratum corneum (dark green shade, red line). Arrows depict the orientation of the cells differentiation. The expression of SIV antigens will be transcriptionally regulated by the involucrin promoter, and will be depending on the differentiation of the epidermal cells of the epithelia stratum corneum. The right panel of the figure shows the overall schematic representation of the vaccine approach. VSV-G pseudotyped viral particles containing the involucrin-driven SIV construct is used as vector to target basal stem cells (2). As the basal stem cells divide to generate the epithelia, the epithelia cells are differentiating. The involucrin promoter is composed of binding sites for transcription factors (3) that drive the expression of the involucrin that is expressed in differentiated epithelial cells. Similarly, the involucrin promoter of the involucrin-driven SIV constructs will control the transcription of SIV genes (4) thus leading to the expression of SIV proteins/antigens (5) upon epithelial cell differentiation. Differentiated cells of the upper layers of the epithelia are expressing SIV proteins/antigens that are continuously

exposed by the differentiated epithelial cells (6) and released at the mucosa upon *stratum corneum* cell death and breakdown (7), thus eliciting long lasting immunity.

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