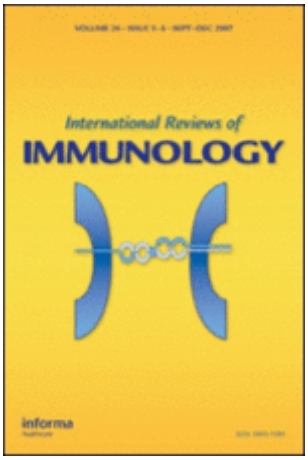

shRNA targeting caspase-3 inhibits apoptosis and cell detachment induced by Pemphigus Vulgaris autoantibodies

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shRNA targeting caspase-3 inhibits apoptosis and cell detachment induced by Pemphigus Vulgaris autoantibodies

Running Head: shRNA in Pemphigus

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Summary Sentence

Pemphigus autoantibodies cause acantholysis and apoptosis; the shRNA for caspase-3 prevents cell detachment and apoptosis induced by pemphigus IgG.

Abstract

Pemphigus is an organ-specific autoimmune disease that affects the skin and mucous membranes. It is induced by the deposition of pemphigus IgG autoantibodies, which mainly target Dsg1 and 3 and cause a loss of cell adhesion in a phenomenon known as acantholysis, and clinically is reflected as intraepidermal blistering. The present work assessed the effect of pemphigus vulgaris IgG (PV-IgG) on cell adhesion and caspase 3-dependent apoptosis in HaCaT cells. The expression of caspase-3 induced by PV-IgG was silenced in cells pre-treated with caspase 3-shRNA. PV-IgG induced cell detachment and apoptotic changes as demonstrated by the annexin-FITC assays. Treatment of cell cultures with normal IgG (control; N-IgG) did not have relevant effects on the aforementioned parameters. Then, the effect of PV-IgG on cells previously treated with shRNA was tested. The results demonstrated that shRNA reduced apoptotic features and the relative expression of caspase-3 measured by qRT-PCR, which showed a decrease of 96%. In conclusion shRNA prevented cell detachment and apoptosis of HaCaT cells induced by PV-IgG. The presented results further our understanding of the molecular pathophysiologic mechanisms involved in pemphigus diseases.

Keywords:

Pemphigus vulgaris; Caspase-3; Apoptosis; shRNA; Cell detachment

Plain Language Summary

Pemphigus is a group of autoimmune blistering diseases of the skin and mucous membranes such as the mouth; the lesions are caused by the action of autoantibodies that are directed against desmogleins, which are proteins that bind epithelial cells and produce cell detachment, this is called acantholysis and is characteristic of the disease, and then the cells die. The main mechanisms by which blisters develop begin with the binding of Pemphigus IgG (PV-IgG), which causes interference in the assembly between desmogleins, and this initiates intracellular signals through the p38MAPK pathway that culminate with cell shrinkage and apoptosis. In this work, programmed cell death was blocked with a silencer of the caspase 3 gene in cultured cells, which prevents the pathological effects induced by the pemphigus antibody. This investigation expands the knowledge about this enigmatic autoimmune disease.

INTRODUCTION

Pemphigus is an autoimmune disease characterized by the formation of intraepidermal blisters on the skin and/or mucous membranes that are triggered by the accumulation of IgG autoantibodies that target the desmosomal proteins of keratinocytes, resulting in the loss of intercellular junctions and acantholysis [1-3].

There are different clinical forms of pemphigus. 1) Pemphigus vulgaris (PV) is characterized by the presence of autoantibodies against Desmoglein 3 (Dsg3); if it involves the skin and mucous membranes, antibodies against Dsg3 and Dsg1 are present; the blister is located above the basal cell layer of the epidermis [4-6]. 2) Pemphigus foliaceous (PF) lacks oral involvement, autoantibodies are directed only against desmoglein 1 (Dsg1), and the blister is located in the upper part of the epidermis. There are two variants of PF, the endemic (fogo salvagem) and the nonendemic variant. 3) Pemphigus erythematosus or the Senear-Usher type is usually accompanied by anti-Dsg1 and antinuclear antibodies, and positive lupus band test [7]. 4) Paraneoplastic pemphigus (PNP) is a blistering disease with accompanying tumours and autoantibodies against desmoplakin I and II, periplakin, and plectin, among others, are present. [3-6]. 5) Pemphigus vegetans is a very rare variant of PV characterized by the presence of anti-Dsg1 and Dsg3, anti-desmocolin (Dsc) and periplakin autoantibodies. 6) Pemphigus can also be drug-induced. Finally, 7) Neonatal pemphigus is induced by the placental transfer of maternal pemphigus IgG [8].

Therapy for pemphigus encompasses medications to reduce blisters and decrease autoantibody production. Conventional therapy includes systemic corticosteroids and

immunosuppressive drugs; however, biological agents such as rituximab are effective for unresponsiveness pemphigus [9,10].

It has been shown that the main mechanism of pemphigus vulgaris pathology is direct steric interference of homophilic and heterophilic Dsg:Dsg and Dsc:Dsg binding. All other mechanisms studied, including p38MAPK signalling dependent and apoptosis, are probably an epiphenomenon of these steric direct effects of PV-IgG, and it has been proposed that acantholytic cells have apoptosis as final destiny. This assumption is based on TUNEL assay results, which are positive not only in the blister but also in the epidermis around the bleb [11]. Apoptosis in PV is mainly triggered by the extrinsic pathway and involves cell death receptors of the TNF family, including Fas (CD95) and its cognate ligands, also involves the EGFR receptor [12,13]. Therefore, sera from patients with active pemphigus show high levels of FasL compared to those in healthy individuals. Other experimental evidence that acantholysis and secondary apoptosis are linked is that anti-FasL antibody prevents the activation of caspase-8 and the excision of Dsg in human keratinocytes induced by PV-IgG. This finding suggests that FasL plays a secondary role in the mechanisms of blistering; concurrently, the activation of pro-apoptotic proteins such as Bax reinforces apoptosis by the intrinsic pathway, all of which are triggered by the PV-IgG effect [14,15].

Acantholysis is additionally promoted by apoptolysis, which involves signal transduction from membrane receptors such as EGFR and causes retraction of tonofilaments and cell shrinkage [16].

The present study is focused on inhibiting caspase-3, which we hypothesized would reduce acantholysis [15,17]. Our study was based on results obtained by other investigators in a knockout mouse model that demonstrates a lack of keratinocyte fragility and amelioration of blistering in keratinocyte-specific caspase-3-deficient mice ($casp3^{EKO}$) injected with a potent anti-Dsg3-specific antibody [16]. Additionally, our own data and the results of others support the possible role of caspase-3 in acantholysis, since the inhibition of caspase-3 by Ac-DEVD-cmk prevents the formation of intraepidermal blisters in neonatal BALB/c mice injected with PV-IgG [18,19]. The aforementioned results suggest that inhibition of caspase-3 activity is attractive mechanism for understand part of pathophysiology of pemphigus. For this reason, the present work further analyzed whether the silencing of caspase-3 by shRNA could modify cell adhesion, decrease apoptosis and affect keratinocyte proliferation *in vitro*.

MATERIALS AND METHODS

PV-IgG characterization

A positive serum for anti-epithelial antibodies obtained from a female patient 28 years old with pemphigus vulgaris was used for IgG purification, she had extensive blistering in mouth and trunk, with an skin biopsy by H&E showing intraepidermal blister and acantholytic cells, sampling was obtained during plasmapheresis. Anti-epithelial reactivity of pemphigus vulgaris serum was determined by indirect immunofluorescence (IFI) using cow's nose as an antigenic source. Goat FITC-labelled polyclonal anti-human IgG was used as the secondary antibody (Sigma, St. Luis, MO, USA). After incubation and washing with

PBS, the slides were evaluated under epifluorescence microscopy (Olympus BX40). Anti-desmoglein 3 antibody values were measured by ELISA (Euroimmun, Medizinische Labordiagnostika AG). In all assays, control human serum was included. All assays were performed in triplicate. In addition, the serum of a healthy control of the same age and gender was included, without evidence of autoimmune, cardiovascular or metabolic disease.

PV-IgG purification

The IgG was purified as follows: First, the starting material was plasma, then the gamma globulin fraction was precipitated by ammonium sulphate, and the precipitates were extensively dialyzed against distilled water using a cut-off membrane MWCO of 12 kDa. After complete salt removal, the gamma globulin precipitate was dialyzed against PBS, then it was diluted in binding buffer 0.02 M sodium phosphate pH 7.0, gamma globulin was purified by affinity chromatography using a *HiTrap*®Protein G prepacked column (Ge Healthcare), and the IgG bound to the G protein was eluted by 0.1 M glycine-HCl in pH 2.7 buffer then the effluent was neutralized with buffer of 1M Tris pH 9.0, and dialyzed against PBS as previously described with stirring for 48 hours at 4°C. The fractions obtained were characterized by 10% SDS-PAGE [20] and tested for anti-epithelial antibodies.

Cell culture

The HaCaT human keratinocyte cell line was obtained from the American Type Culture Collection (CRL12191-ATCC, Manassas, VA, USA), grown in cell monolayers on well plates (Sterile Nunclon^{TM}; Corning; Microscopy Chamber Ibidi) and cultured in high glucose DMEM (DMEM-HG, Gibco, Grand Island, NY, USA) supplemented with 10%

FBS, 1X antibiotic-antifungal, 1X sodium pyruvate and 1X L-glutamine (Gibco). The cells were incubated at 37°C in a 5% CO_2 atmosphere (Incubator NuAire, USA).

Cell Adhesion assays.

We used the attachment assay which detect the flattering of adherent cells by phase contrast microscopy [21], and those results were confirmed by colorimetric detection of bound cells to immobilized substrate using 0.1% (w/v) crystal violet, 200 mM MES (4-Morpholineethanesulfonic acid hydrate; 2-(N-Morpholino) ethanesulfonic acid hydrate) [22], also adherent and non-adherent cells were counted by cell cytometry (see ahead).

Caspase-3 shRNA expressing vector

We used the pGSH1-GFP vector (Gene Silencer®, 092205 MV) linearized by *BamHI* and *NotI* and was ready for ligation. Figure 1.

Oligo design for hairpin shRNA

Sequences of the caspase-3 gene (CASP3-NCBI) were selected using guide design software for oligo synthesis (Ambion, Austin Texas, USA). The oligonucleotides were automatically designed by the software and were then synthesized at a concentration of 0.05 μg (Life Technologies, Invitrogen). Sense 5'-GATCCGAAAGCACTGGAATGACATCGAAGCTTGGATGTCATTCCAGTGCTTTTTTTTTGGAAGC-3' and antisense 3'-GGCCGCTTCCAAAAAAAAAGCACTGGAATGACATCCAAGCTTCGATGTCATTCCAGTGCTTTCG-5'. Oligos were adjusted to reach a final concentration of 1 μg/μL and

then annealed and ligated by T4 DNA ligase into pGSH1-GFP. The ligase reaction mix was used for the transformation procedure [23].

Transformation of E. coli (F- recA1 endA1 hsdR17 supE44 thi-1 gyrA96relA1 ϕ80lacZΔM15 Δ (lacZYA-argF) U169)

Casp3-shRNA expression vectors were propagated into SmartCells™ chemically competent *E. coli*, and cells and vectors were mixed at 42°C for 45 seconds and then incubated in SOC medium at 37°C for 1 hour. Then, the transformation mix was spread on LB/agar plates containing 50 mg/mL kanamycin and incubated overnight at 37°C. Positive transformants were selected from colonies with GFP expression under blue light-emitting diode illumination.

Isolation of Casp3-siRNA expression vectors from E. coli transformants

Colonies were picked, and recombinant plasmids were extracted by alkaline lysis with SDS and digested (*Hind III*). Miniprepped DNA was run on a 1% ultrapure agarose gel. Positive shRNA-containing clones displayed a linear plasmid on the agarose gel, while negative clones appeared similar to the supercoiled plasmid after *Hind III* digestion. Positive clones were selected and grown overnight in LB media containing 50 mg/mL kanamycin to prepare sufficient quantities of plasmid DNA for transfection. Bacteria were incubated in an incubator/shaker overnight at 37ºC with constant shaking 250 rpm, then collected and centrifuged at 5,200 xg for 5 minutes, then cells were treated with deep blue lysis buffer, after that neutralized by the clearing buffer, samples were centrifuged in zymo-spin, the zyppy was washed, then the plasmid DNA was eluted by Tris-HCl, pH 8.5 and 0.1 mM EDTA (Zyppy™ Plasmid Miniprep Kit, Zymo Research).

Transfection of caspase-3 shRNA

Once the HaCaT cells were confluent at a density of 500, 1,000 and 2,000 cells per well, they were transfected with a cationic polymer as the transfection agent (*jet-PEI*, Polyplus Transfection) N/P = 5 and 4 µg of DNA (siRNA) in 100 µL of 150 mM NaCl. Triplicate cultures were subjected to the following conditions: Unsilenced. 1) Negative control (KSFM medium), 2) Purified IgG from Normal Human Serum (N-IgG; 1 mg/mL), 3) Purified IgG from Pemphigus vulgaris (PV-IgG; 1 mg/mL) 4) siRNA+ PV-IgG, 5) Positive apoptosis-inducing control camptothecin (CPT 4 µg/mL) (C9911-Sigma, Saint Louis, Missouri, 6) siRNA+CPT. The cells were incubated with caspase-3 inhibitors for one hour and then the inhibitor removed and the cells were washed with 1X PBS. Subsequently, 1 mL DMEM high glucose was added with the apoptosis inducer (CPT and PV-IgG) and the cells were incubated for 6 hours at 37°C in a 5% CO_2 atmosphere.

Cell viability and apoptosis monitoring

Cell cytometry

This assay was performed using a Guava Personal Cell Analysis Check System, Technologies PCA, as follows: A suspension of the adherent and non-adherent cells was prepared by mixing 50 µL of the cells with 450 µL of the Guava Via Count staining reagent (Cat. No. 4000-0040), and cell viability results were analyzed using CytoSoft 2.1.4.

Labelled annexin affinity assay

Apoptotic PS exposure was examined in HaCaT cells as follows: The culture medium was removed from the different wells with the treatment, and the cells were washed with PBS

1X pH 7.2 (Gibco®), fixed with 4% paraformaldehyde, and tagged with 100 µL of annexin-V-Fluos labelling solution (Cat. No. 11 858 777 001, Annexin-V-Fluos Staining Kit, Roche Diagnostics). After 10 minutes of incubation, apoptotic cells were analyzed by inverted fluorescence microscopy with an excitation range of 450-500 nm (Olympus IX71; DP71 camera; DP Manager 3.1.1.208 software). The green areas were quantified in pixels using Image-Pro® Plus vs 7.0.

Caspase-3 gene expression analysis by qPCR-RT

Total RNA was purified by a Pure Link™ RNA mini-Kit (Ambion, Life Technologies). 1 µg of total RNA was added to a total reaction mixture of 25 µL with SuperScript™ One-Step RT-PCR and Platinum® Taq (Invitrogen, Life Technologies). The reactions were carried out in a Pixo Helixis system (Illumina) using Fast SYBR® Master Mix (Applied Biosystems) detection chemistry with a final volume of 20 µL. The samples were amplified at 95°C for 2 minutes, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 60°C. The oligos used were Caspase-3: F-5'-TTGTGGAATTGATGCGTGAT-3' and R-3'-GGCTCAGAAGCACACAAACA-5'. As a housekeeping control, the α-tubulin (F-5'-CTTCGTCTCCGCCATCAG-3' and R-3'-TTGCCAATCTGGACACCA-5') gene was amplified separately, and the experiments were performed in triplicate. The expression levels of caspase-3 were normalized to that of α-tubulin, and each experiment was processed with its housekeeping control. Relative gene expression levels were determined using the comparative Ct method [24].

Statistical analysis

The experiments were performed in triplicate and analyzed using ANOVA biostatistical analysis, and silenced and unsilenced samples analysed by unpaired t test with GraphPad Prism version 9.2 (GraphPad, San Diego, CA, USA), $p<0.05$ was considered statistically significant.

RESULTS

PV-IgG induces loss of cellular adhesion and apoptosis of HaCaT cells

PV-IgG characterization

PV-IgG was isolated from patient plasma with clinical, serologic and histologic confirmation of the disease. This sample was taken during the clinical phase of disease activity with an anti-epithelial antibody titre of 1:2560 (negative value <1:160), anti-Dsg1 level of 254 U and anti-Dsg3 level of 281 U (negative value <20). Purified PV-IgG preserved its anti-epithelial activity.

Cell viability

HaCaT cells were grown under different experimental conditions as previously described, and the cells without treatment had reached 61% viability. Similar results were obtained with those incubated with N-IgG, which had no effect on cell viability; in contrast, PV-IgG induced a decline in viability of ~46% ($p<0.0001$), and this effect was reversed by pre-treatment with caspase-3 shRNA, which increased viability to ~60% ($p<0.0001$. The positive control comprising apoptosis induced by camptothecin used as a death control

decreased cell viability to ~51%. Unexpectedly, pre-treatment with shRNA had no effect on viability, indicating that the topoisomerase 1 poison possesses caspase-independent cell death effects (Figure 2. A. Viability).

Cell adhesion

The polarity of each cell and its interaction with neighbouring cells via the extracellular matrix is important for keratinocyte function, and the desmosomal molecular complex maintains this property. According to our present results, desmosomal-dependent junctions were disrupted by PV-IgG. The cell adherence under basal conditions without treatment was ~64 adherent cells/field, and this rate dropped to ~36 adherent cells/field with the PV-IgG autoantibody effect ($p<0.0004$). This detachment effect was similar in cells treated with CPT; in both cases, cell adherence was increased by pre-treatment with caspase-3 shRNA. (Figure 2. B. Adhesion).

Caspase-3 silencing reduces apoptosis induced by PV-IgG

Apoptosis

Under basal conditions, the rate of apoptotic HaCaT cells as well as the cells incubated with N-IgG was ~21%, and this rate was increased to ~33.7% by the PV-IgG effect ($p<0.0001$). As expected, the cells pre-treated with caspase-3 shRNA reduced apoptosis to ~20%, and the rate of apoptosis were comparable to the basal condition. On the other hand, the CPT positive control increased the apoptotic rate one-fold, and this effect was attenuated under the caspase-3 silencing effect (Figure 2. C. Apoptosis).

Phosphatidylserine (PS) exposure on the cell surface

The translocation of phosphatidylserine to the outer leaflet of the cell membrane is an early sign of cells undergoing apoptosis; we used a time course experiment to determine that 6 hours was the earliest time point at which apoptotic changes could be observed at the plasma membrane. To distinguish living cells from apoptotic by microscopy, cells were counterstained with propidium iodide (red signal), and apoptotic PS membranes were detected by annexin-FITC reagent (green signal). As expected, cells under basal conditions without treatment and those treated with N-IgG did not exhibit PS-annexin staining ~18 and 27.7, respectively. In sharp contrast, the cells treated with PV-IgG displayed a strong green signal indicative of apoptosis ~124 ($p<0.0001$) which was reversed by siRNA. As expected, the control cells in which apoptosis was induced by camptothecin also showed an intense annexin-FITC signal ~109. Interestingly, phosphatidyl serine exposure was prevented by the silencing of caspase-3, as depicted in Figure 2. D. PS exposure, Figure 2. E (Caspase 3 expression) and Figure 3. A, B and C.

shRNA reduced the expression of caspase-3

Caspase-3 siRNA reduced the expression of caspase-3; under basal conditions its expression level was ~1 ± 0.21-fold that of shRNA-treated cells. This expression level was similar in cells treated with N-IgG; however, with PV-IgG, the gene expression level of caspase-3 increased 3.3 ± 2.21-fold, and as expected, caspase-3 transcription silencing reduced its expression to 0.11 ± 0.03. Cells treated with CPT behaved similarly to those treated with PV-IgG, since the Topoisomerase 1 poison increased the expression of caspase-3 to 2.92 ± 1.08, and after silencing the caspase-3 gene, its transcription was no longer increased. Figure 2. E.

Inducer and inhibitor of Caspase 3

Camptothecin was used as inducer of apoptosis, therefore its effects under cell adhesion and viability was comparable to the effect obtained with PV-IgG.

Taken together, our data demonstrate that PV-IgG reduces cell adhesion and viability and increases cell detachment. These data suggest that apoptosis is linked to loss of cell adhesion since most of these effects were neutralized by the silencing of caspase-3 gene transcription.

DISCUSSION

In the present work, a shRNA approach against caspase-3 was used *in vitro* to demonstrate the possible role of caspase-3 in acantholysis, which is the pathogenic hallmark of pemphigus. This tool was chosen because it has the advantage of suppressing a target gene to demonstrate one of the pathogenic pathways of pemphigus, as shown in the results of other investigators who silenced the desmoplakin and desmoglein 3 genes and demonstrated the structural role of both proteins in cell attachment, proliferation and differentiation of human immortalized keratinocytes [25-28]. In addition, these results indirectly indicate the role of desmogleins as the main target of pemphigus autoantibodies and suggest that these are involved in activating caspase-3 cell death pathway.

Apoptosis of keratinocytes can be induced by UVB irradiation that increases the expression of TNF family cell death receptors and activates the extrinsic apoptosis pathway [29,30]. Additionally, UVB irradiation also activates the mitochondrial pathway, which releases

cytochrome c and subsequently activates caspases-8 and 9, which in turn increase effector caspases 3 and 7, leading to apoptosis [31-33]. Interestingly, the inhibition of initiator caspases does not prevent acantholysis in a mouse model of pemphigus; however, the inactivation of caspase-3 prevents acantholysis [18].

Since Anhalt et al [34], demonstrated the pathogenic blistering effect of PV-IgG, different pathophysiological models triggered by PV-IgG have been explored to determine the consequence of PV-IgG binding to Dsg1 and 3. This issue has been the subject of a large body of research, as different experimental and theoretical approach models have emerged to clarify the mechanism of acantholysis, however the notion accepted is that the main mechanism of pemphigus vulgaris pathology is direct steric interference by pemphigus autoantibodies of homophilic and heterophilic Dsg:Dsg and Dsc:Dsg binding [32-37].

We used HaCaT cells that mainly express desmoglein 1 and 3 to explore role of caspase-3 in the lost of cell adhesion. Our results demonstrated that PV-IgG induced cell detachment and apoptosis. The deleterious effect on keratinocyte survival induced by the pemphigus autoantibody was comparable to the effect induced by camptothecin, which was used as control to induce apoptosis. Our results coincide with those obtained in other studies that show that apoptosis in acantholytic cells is mediated by PV-IgG [38-40]. In contrast, other investigators found no evidence of apoptosis in acantholytic pemphigus cells [41] and suggested that apoptosis is not necessary to induce cell shedding; however, previously the apoptotic effect was attributed to the use of high concentrations of PV-IgG (750 µg/mL) [42]. In this study, a high concentration of autoantibody was purposely used (1000 µg/mL) since one of our goals was to observe the apoptotic effect mediated by PV-IgG in this issue. It is conceivable that autoantibody concentration may influence the apoptotic rate, since a

high concentration of PV-IgG in patients is always correlated with disease activity. Furthermore, the extent of blistering is directly related to the molar concentration of the autoantibody, as early was demonstrated [34]. The autoantibody induces blisters in a concentration-dependent manner, and our results are in agreement with those reported by others [38]. We must note that the effect of PV-IgG is specific and interfere the steric interaction between IgG and the extracellular matrix, since N-IgG at a concentration of 1000 μg/mL did not induce apoptosis or cell detachment. Another interesting finding that supports this notion is that the biological agent rituximab depletes autoreactive B-cells and pathogenic autoantibodies and consequently induces clinical remission, or the PV-anti–Idiotype effect, that decrease the blister formation [43,44].

Thus, with the hypothesis that acantholysis and apoptosis are close related, we propose that the inhibition of caspase-3 activity could be an attractive to demonstrate the adjuvant role of caspase-3 in pemphigus pathogenesis. Based on our results, silencing caspase-3 by shRNA significantly reduces the level of apoptosis induced by PV-IgG in HaCaT cells, which supports our hypothesis and confirms that caspase-3 plays a role in the cell detachment process. The present data are in agreement with our previous observations *in vivo* in which we demonstrated that the inhibition of caspase-3 by Ac-DEVD-cmk prevents blister formation induced by PV-IgG in neonatal BALB/c mice [19].

CONCLUSIONS

In conclusion, our studies show that shRNA is capable of reducing the gene expression of caspase-3, which decreases cell detachment *in vitro* and reduces apoptosis induced by PV-IgG.

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Declaration of interest

The authors report no conflict of interest.

Author Contributions

Conceptualization: Deyanira Pacheco-Tovar and Esperanza Avalos-Díaz. Data extraction: Maria-Guadalupe Pacheco-Tovar, Santiago Saavedra-Alonso, Juan-José Bollain-y-Goytia. Funding acquisition: Cristina Rodríguez-Padilla. Writing—original draft: Pablo Zapata Benavides. Writing—review and editing: Rafael Herrera-Esparza. All authors read and approved the manuscript.

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Figure legends

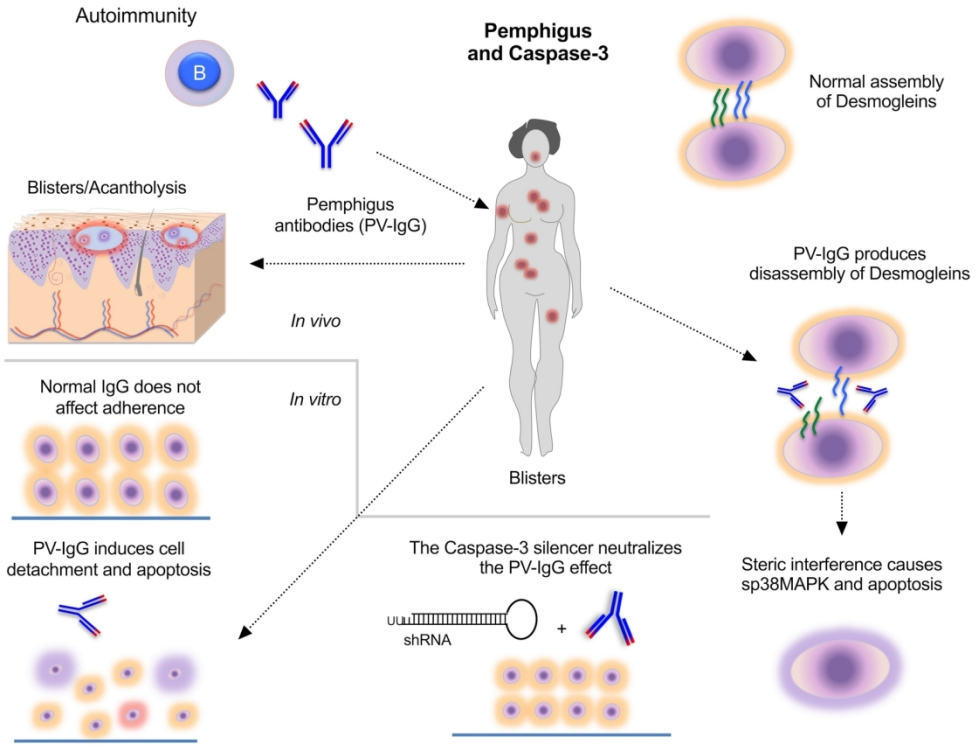
FIGURE 1. Schematic map of pGSH1-GFP vector (Gene Silencer®, 092205 MV). The shRNA expression cassettes were cloned downstream of the H1 promoter using the *BamH1/ Not1* restriction sites. Kanamicin resistance gene (Kan/Neo) and GFP markers are shown in black. This vector is based on a pUC vector backbone designed for high efficiency propagation in into SmartCellsTM chemically competent *E. coli*. In the bottom of the scheme is shown the short hairpin used to selectively inhibit caspase-3, and is formed after sense and antisense sequences (middle) are linked by a loop form after binding each other, producing a double-stranded RNA that can silence in this case caspase-3 gene.

Figure 2. Graphs showing the changes expressed by the HaCaT cells treated with N-IgG, PV-IgG, shRNA+PV-IgG, CPT and shRNA+CPT for cells under basal conditions (**A**) Viability, (**B**) Adhesion, (**C**) Apoptosis, (**D**) PS exposure measured in green pixels units and (**E**) expression of Caspase 3. The significant value is $p<0.05$.

Figure 3. Fluorescence of HaCaT cells counterstained with propidium iodide (red signal) control, the cells are under basal conditions. **A**. Control cells. **B**. Apoptotic PS membranes were detected by annexin-FITC reagent (green signal), and the PV-IgG induces PS exposure. **C.** shRNA-Cas-3 reduces PS exposure by inhibit caspase-3.

Abbreviations

CPT: camptothecin; DEVD-cmk: caspase 3 inhibitor; DMEM-HG: Dulbecco's Modified Eagle Medium high glucose; Dsc: desmocolin; Dsg1: desmoglein 1; Dsg3: Desmoglein 3; EGFR: epidermal growth factor receptor; FITC: fluorescein isothiocyanate; H&E: hematoxylin & eosin; IFI: indirect immunofluorescence; KSFM: Keratinocyte serum-free medium; MES: 4-Morpholineethanesulfonic acid hydrate; MWCO: molecular weight cut-off membrane; N-IgG: normal IgG; PF: pemphigus foliaceous; PNP: paraneoplastic pemphigus; PV-IgG: pemphigus vulgaris-IgG; PV: pemphigus vulgaris; qRT-PCR: quantitative reverse transcriptase polymerase chain reaction; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA: short hairpin RNA; siRNA: small interfering RNA.



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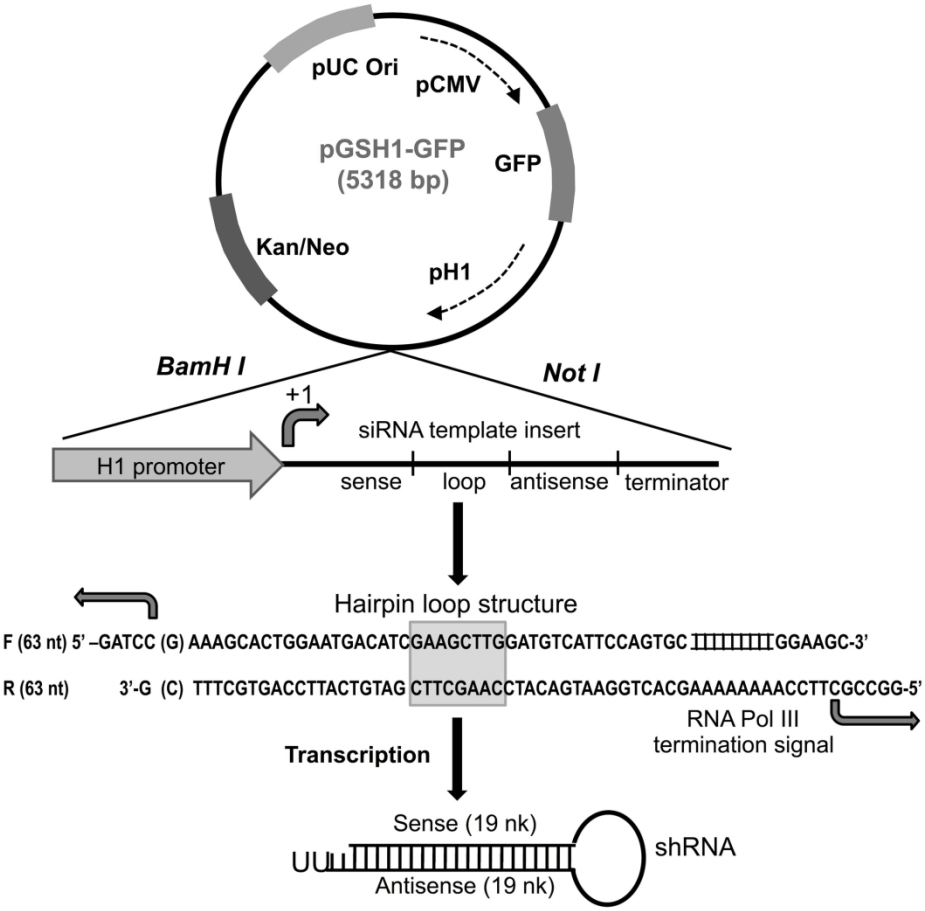

Figure 1

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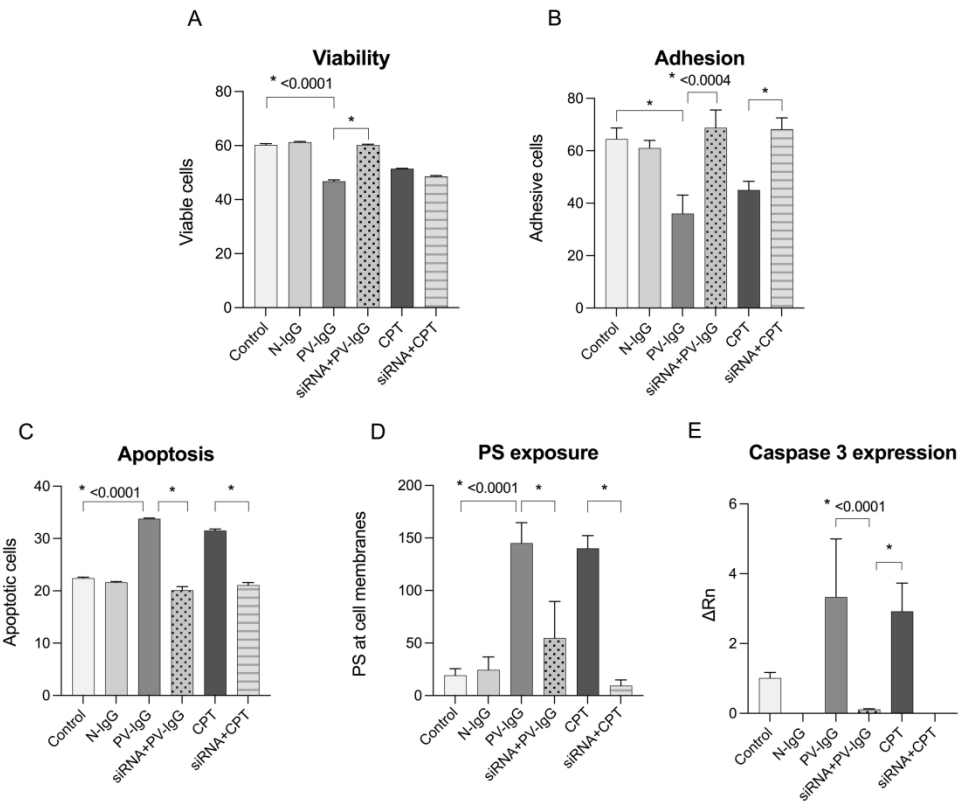

Figure 2

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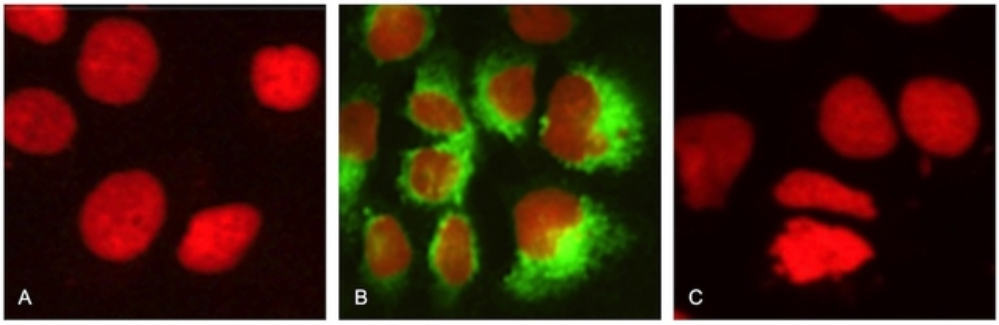

Figure 3

27x8mm (600 x 600 DPI)