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**Immunopotential of the antibody response against influenza HA with apoptotic bodies generated by rabies virus G-ERA protein-driven apoptosis**

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**Short title:** immunopotential of apoptotic bodies

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## ABSTRACT

Apoptosis is considered to be a way of eliminating unwanted cells without causing major inflammation. Nevertheless, several lines of evidence show that apoptotic cell-derived antigens can be strong immunogens. The rabies virus glycoprotein G-ERA is an apoptotic molecule. We tested the ability of G-ERA to potentiate a B cell response against an exogenous antigen (influenza hemagglutinin, HA). We found that co-expression of G-ERA and HA in apoptotic bodies increased both the primary and memory HA-specific immune responses. The immunopotential of G-ERA is apoptosis-mediated but not necrosis-mediated. Our data indicate that G-ERA-mediated apoptosis might be useful to improve the immunogenicity of live vaccines.

## INTRODUCTION

Apoptosis is a key phenomenon in the regulation of cell population size and lifespan, and plays an important role in normal tissue homeostasis and certain pathological conditions, including cancer [1]. In the past few years, a growing number of intracellular microbes (viruses, bacteria, protozoan parasites) have been shown to inhibit or to promote host cell apoptosis [2]. The mechanisms by which dying cells are recognized by scavenger cells, macrophages and dendritic cells (DC), and how these cells present the apoptotic cell antigens are the subject of intense investigation [3]. Furthermore, the role of apoptosis in the induction or prevention of an immune response is still unclear. Indeed, some groups have demonstrated that the uptake of apoptotic cells by DC results in T cell tolerance [4-6]. Conversely, other groups have shown that DC that have phagocytosed apoptotic bodies from virus-infected cells can present viral antigens to cytotoxic T cells and induce a cytotoxic response [7,8]. In other systems, antigens produced by apoptotic cells increase the immunogenicity of the antigen [9,10]. Apoptotic bodies containing the antigen of interest have been obtained by different means such as enforced expression of Fas, cytochrome C, BAX or caspases [11-14].

In the context of an infectious disease, the ultimate aim is to develop cheaper, more effective and more rapidly acting vaccines [15-17]. Some of these features could be achieved by stimulating the humoral immune response. The aim of this study was to evaluate whether the introduction of a pro-apoptotic molecule into a vaccine enhances the B cell response against a vaccine antigen. We have previously shown that the attenuated rabies virus strain ERA triggers mitochondrial/caspase-dependent programmed cell death in different types of human cell [18-20] and that G-ERA is the key factor responsible for triggering apoptosis [20,21]. Here, we tested whether G-ERA can improve the immunogenicity of a recombinant live vaccine. We tested whether G-ERA potentiates the immunogenicity of a vaccine preparation

composed of cells infected by recombinant vaccinia virus (VV) expressing the major immunogenic protein of influenza virus, hemagglutinin (HA).

To investigate whether G-ERA-mediated apoptosis of cells infected with a recombinant VV containing the influenza virus HA can enhance the immunogenicity of the HA vaccine, we constructed recombinant VV expressing either HA (recombinant virus 1) or G-ERA (recombinant virus 2) as well as a recombinant VV expressing no transgene (recombinant virus 3), mouse lymphoblastoid EL4 cells were infected with different combinations of these recombinant viruses, HA-specific antibody (Ab) responses were compared in mice immunized with cells co-infected with the two recombinant viruses or with one recombinant virus only and finally the respective roles of apoptosis and necrosis in the G-ERA-mediated immunopotentialiation were evaluated.

We provide evidence that G-ERA-driven apoptosis of antigen-presenting cells improves the immunogenicity of a vaccine antigen. This finding could be relevant for inserting pro-apoptotic polypeptides such as G-ERA into live vaccines or DNA/RNA vaccines.

## **MATERIALS AND METHODS**

**Cells and viruses:** The mouse lymphoma cells EL4 (ATCC # TIB-39) were established from a lymphoma in a C57BL6 mouse treated with 9,10-dimethyl-1,2-benzanthracene [22]. Cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin and 5% fetal bovine serum (FBS).

African green monkey kidney fibroblast CV-1 cells (ATCC # CCL-70) and human osteosarcoma 143B cells (ATCC #CRL-8303) were grown in DMEM medium supplemented with 100 mM Na-Pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 5% FBS. All cell lines were cultivated at 37°C in a 5% CO<sub>2</sub> atmosphere and split twice a week. Stocks of

recombinant VV (Copenhagen strain) were prepared on CV-1 cells as described by Kieny et al [23].

**Construction of recombinant VV:** Recombinant VV were constructed and isolated as previously described [23,24]. Transfer plasmids, derived from pTG 186poly [23] were used to insert foreign sequences at the vaccinia TK locus. In the ptg-186-poly vector, the foreign gene was under the control of the P7.5 promoter, which had been inserted into the VV *tk* gene. Therefore, recombinant viruses exhibited a [TK<sup>-</sup>] phenotype. Briefly, the rabies virus G-ERA gene was cloned as previously described [20], recovered from pRev-TRE.G.ERA by cutting with *Bam*HI/*Hpa*I and cloned into pTG 186poly in between the *Bam*HI and *Sma*I sites downstream from the vaccinia early-late promoter P7.5. The HA gene of the influenza A/PR/8/34 virus was amplified by RT-PCR from viral RNA of the mouse adapted A/PR/8/34 (H1/N1) influenza virus strain and the resulting DNA fragment was inserted at the *Bam*HI site of pTG 186poly. The sequences of the inserts were controlled. 143B cells were infected with the helper temperature-sensitive ts7 VV and then co-transfected with wild-type VV DNA (Copenhagen strain) and either parental (in order to obtain VV TG186) or recombinant pTG 186 poly transfer plasmids (in order to obtain VV G-ERA and VV HA respectively). After selection at nonpermissive temperature for the helper virus (40°C), recombinant viruses resistant to 5-bromodeoxyuridine treatment were selected by two rounds of plaque purification on 143Btk<sup>-</sup> and screened by PCR for presence of the transgene. Flow cytometry was performed to check cell-surface expression [20]. Therefore, three recombinant VV were used in this study: VV G-ERA (expressing rabies G-ERA glycoprotein), VV HA (expressing influenza HA) and VV TG186 (no insert and no expression of foreign protein).

**Antibodies (Ab), monoclonal antibodies (mAb) and reagents.** Cy<sup>TM</sup>2-conjugated Streptavidin or FITC-conjugated anti-mouse IgG were purchased from Jackson ImmunoResearch (distributed by Immunotech/Beckman Coulter, Orsay, France). The Alexa Fluor ® 594 F(ab')<sub>2</sub> fragment of goat anti-rabbit IgG (H+L) was from Molecular Probes (USA). Biotinylated anti-RV-G protein mAb 8-2 [25] and the rabbit A/PR/8/34 Ab were produced in the laboratory. Anti-vaccinia virus (strain Copenhagen) mouse Ab was a gift from Yves Rivière, Institut Pasteur. Cellfix was supplied by Becton-Dickinson Biosciences (Pont de Claix, France). Fluoromount-G was purchased from Cliniscience (France). Streptomycin, penicillin, Hepes, Na-Pyruvate and FBS were purchased from Invitrogen (Scotland, UK). Propidium iodide was obtained from R&D Systems Europe (Abingdon, UK). Z-VAD was purchased from Bachem (France).

**Immunostaining.** HA and G-ERA are expressed at the cell membrane, therefore they were detected by immunostaining as described by Prehaud *et al.* [20]. Cells were incubated for 1h at 37°C with anti-RV-G protein mAb, or anti-VV or anti-HA antibodies diluted in culture medium and then incubated with Cy<sup>TM</sup>2-conjugated Streptavidin, FITC-conjugated anti-mouse or Alexa Fluor ® 594 F(ab')<sub>2</sub> fragment of goat anti-rabbit IgG. For flow cytometry, cells were washed several times in staining buffer (1% FBS, 0.1% Na Azide in phosphate buffer saline, PBS) and fixed in Cellfix (1/10 in staining buffer). Viral proteins were detected in a cell population (10<sup>4</sup> cells) that was gated to exclude dead cells and cell debris. Expression of viral proteins in cell culture was measured by determining the frequency of events with FITC/Alexa 594 signals higher than a given threshold.

**Detection of cell death: Detection of apoptosis was measured by three different techniques:**

**a)** Morphological changes were assessed by measuring side and forward light scattering (SSC

and FSC, respectively) in flow cytometry and used to identify apoptotic EL4 T cells as previously described for Jurkat T cells [20]. b) ) The nuclear morphology of normal and apoptotic cells was assessed by staining with Hoechst 33342 (1 µg/ml) and examination under a Zeiss UV microscope. c) DNA fragmentation was assessed by TUNEL as previously described [26] with minor modifications [18]. Apoptotic cells were analyzed by flow cytometry. Apoptosis was blocked by 100 µM Z-VAD.

***Alternatively necrosis was measured as followed.*** Cells stained with propidium iodide (15 min incubation) were measured by flow cytometry. A positive control of necrosis was obtained by treating EL4 cells with acetone.

***Preparation of apoptotic bodies:*** EL4 cells were infected or co-infected with recombinant VVs at a multiplicity of infection (MOI) of one to three, which infects more than 90% of cells. In co-infection experiments, the multiplicity of infection was calculated so that the amount of HA antigen was the same as in single infections. At 24h post-infection, cells were harvested and apoptosis, necrosis, levels of infection and levels of the proteins were determined for each vaccine batch. If all these criteria were satisfactory, the cells were spun down at 2000g. The pellet was resuspended in PBS and then frozen at –80°C for several hours before being thawed in a 37°C water bath. The released VV virions were inactivated as described by Loewinger and Katz [27]. Briefly, the vaccine preparations were placed on cell culture Petri dishes and were irradiated with UV-C ( $\lambda$  254nm) for 3h. The absence of infectious VV was checked by titration on CV-1 cells. The presence of apoptotic bodies in the vaccine preparation was confirmed by microscopy.

***Mice:*** Experiments were performed with 8- to 16-week-old male C57BL/6 (B6) mice obtained from Janvier (St Berthevin, France). Mice were housed in a pathogen-free animal



facility. Mice (8 per group) were inoculated by the intraperitoneal (i.p.) route with apoptotic bodies originated from  $0.5 \times 10^6$  cells infected with the following inactivated virus preparations in 400  $\mu$ l of phosphate saline buffer (PBS): VV G-ERA/TG (co-infection with VV G-ERA and VV TG 186), VV HA/TG (co-infection with VV HA and VV TG 186), VV G-ERA/HA (co-infection with VV G-ERA and VV HA), VV G-ERA & VV HA (cells infected with VV G-ERA mixed with cells infected with VV HA immediately before injection). No chemical adjuvant was added in the vaccine doses. A booster was given 6 weeks later: i.p. injection of 200  $\mu$ l of a 1/10 dilution of a commercial influenza vaccine (Vaxigrip, Aventis Pasteur, France).

**ELISA.** Blood samples were collected from retro-orbital plexus 12 days after vaccination. Serum was separated by centrifugation. Microtiter plates were coated overnight with 250 ng/well of influenza virus (PR8/44) or rabies virus (ERA) in 0.05 M carbonate buffer pH 9.6 at 4°C. Plates were blocked with 10% FBS-PBS-Tween for 30 min and then washed with PBS-Tween. Dilutions (1/100 and 1/300) of serum and 10-fold serial dilutions of an internal reference were then added and incubated for 2h at 37°C. Plates were thoroughly washed with PBS-Tween and incubated with biotinylated anti-mouse IgG Ab. Bound Ab were then detected with streptavidin-horseradish peroxidase conjugate and ABTS. The concentrations of specific Ab were determined using the linear portion of the curve obtained with the internal standards. Titers are expressed in arbitrary units per ml (AU/ml). The internal reference for influenza ELISA was a pool of sera from mice immunized with a mouse-adapted PR8/44 influenza virus strain. The internal reference for rabies ELISA was purified anti-rabies virus IgG.

**Statistical analysis.** Differences between groups were analyzed using the Student's T test and the Mann-Whitney test. Differences were considered statistically significant for  $p < 0.05$  and  $p < 0.01$  respectively.

## RESULTS

### Vectorized G-ERA induces apoptosis of EL4 cells

To investigate whether the pro-apoptotic property of G-ERA was conserved in VV G-ERA, EL4 cells were infected with VV TG 186, VV HA or VV G-ERA at a multiplicity of infection of 3 and the cells were harvested 24 h later. In these conditions, the levels of infection were identical in all three cases: VV TG186 (95%), VV HA (87%) and VV G-ERA (95%). We used three different analysis methods to compare apoptosis. Firstly, we measured the induction of apoptosis by using flow cytometry analysis to compare side and forward light scattering. Cells undergoing apoptosis were found in the R2 population. We found that 48% of cells infected with VV G-ERA were in the R2 population compared to 8% of those infected with VV TG 186 and 25% of those infected with VV HA (Fig 1A and white bars Fig 1B). Secondly, we assessed apoptosis by using TUNEL to monitor nuclear fragmentation. We found that 43% of EL4 cells infected with VV G-ERA were TUNEL-positive (43%), compared to 20% of those infected with VV HA and 7% of those infected with VV TG186 (Fig 1B, hatched bars). Thirdly, we analyzed cell nucleus fragmentation (typical of a late phase of apoptosis) by Hoechst staining (Fig 2B). Thus, the pro-apoptotic property of G-ERA is conserved in VV G-ERA, as previously demonstrated in other experimental models [20]. Our results demonstrate the specificity of this mechanism as HA did not act as a major pro-apoptotic protein when expressed in the same conditions.

As some poxviruses trigger a major cytopathic effect [28], we analyzed the extent of necrosis in VV-infected EL4 cells. EL4 cells were infected either with VV TG 186, VV HA or VV G-ERA, or co-infected with VV G-ERA and VV HA (VV G-ERA/HA) at a multiplicity of infection of 3. We measured necrosis 24h later by propidium iodide staining and flow cytometry. The number of necrotic cells obtained after acetone treatment was used as a positive control and considered to represent 100% necrosis. The percentage of necrotic cells was estimated to be 3.7%, 3.5% and 4.3% that of the positive control following infection with VV TG 186, VV G-ERA and VV HA respectively (Fig 1C). The percentage of necrotic cells peaked at 10% relative to the positive control following VV G-ERA/HA infection. These data indicate that necrosis, unlike apoptosis, is a limited phenomenon in the VV-infected cultures.

**HA-expressing apoptotic bodies are generated by co-infection with VVG-ERA and VV HA.** To test whether G-ERA-mediated apoptosis could enhance the immune response against the HA antigen, we checked that cells co-infected with VV G-ERA and VV HA co-expressed rabies and influenza virus antigens and formed apoptotic bodies. We infected EL4 cells with VV G-ERA and/or VV HA at a multiplicity of infection of 3. Cells were harvested 24h post-infection. We monitored G-ERA and HA expression by flow cytometry. Large proportions of cells expressed G-ERA and HA after single infections with VV G-ERA (95%, left panel) and VV HA (87%, middle panel, Fig 2A) respectively. Following co-infection, 84% of cells (right panel Fig 2A) co-expressed G-ERA and HA antigens.

Microscopy revealed large numbers of apoptotic bodies expressing HA in co-infected cultures. A representative co-infected apoptotic cell is shown in Fig 2B. Nuclear fragmentation is clearly visible (left panel) as are G-ERA (middle panel) and HA (right panel). G-ERA and HA did not co-localize, even though they are both abundant (merge panel).

These data showed that co-infection triggers the production of apoptotic bodies expressing HA and suggest that G-ERA plays a major role in this production.

### **Apoptotic bodies expressing HA generated by G-ERA immunopotentiate the influenza Antibody response**

We immunized B6 mice with apoptotic bodies generated as described above. We assessed the Ab responses against HA and against rabies virus 12 days later.

Mice were initially immunized with vaccine preparations obtained either by infection of EL4 cells with VV G-ERA and VV HA (VV G-ERA/HA) or with VV HA and VV TG 186 (VVHA/TG). To test the importance of concomitant expression of HA and G-ERA, we prepared a fourth vaccine: EL4 cells infected with VV G-ERA were mixed with EL4 cells infected with VV HA (VV G-ERA & VV HA) immediately before immunization. The same amount of HA antigen was injected in all cases (such that 40% of cells expressed HA as measured by flow cytometry). HA-specific Ab titers were measured by ELISA (Fig 3A). The level of Ab was highest in mice immunized with the VV G-ERA/HA vaccine preparation. Although a similar amount of HA antigen was injected in all cases, the Ab response was significantly stronger ( $p < 0.01$ ) in VV G-ERA/HA-injected mice than in mice that received VV HA/TG alone or VV G-ERA & VV HA. Interestingly, the most effective vaccine preparation was also the most apoptotic. Indeed, according to TUNEL, 39%, 8% and 15% of cells infected with VV G-ERA/HA, VV HA and VV G-ERA & VV HA were apoptotic respectively. This suggests that immunopotentiation is linked to the pro-apoptotic properties of G-ERA. Furthermore, the HA titers did not increase when HA and G-ERA were not concomitantly expressed as the Ab response was lower with VV G-ERA & VV HA than for VV G-ERA/HA vaccine preparation. This indicates that G-ERA only immunopotentiate the

HA response if the pro-apoptotic property of G-ERA is exerted in cells that express the HA antigen.

We next studied the effect of HA expression on the Ab response to rabies virus to determine whether the immunopotential effect of G-ERA was specific or whether it also occurred with VV HA. Mice were injected with vaccine preparations containing equal amounts of G-ERA antigen (such that 85% of cells were infected as measured by flow cytometry) resulting from the infection of EL4 cells either with VV G-ERA and VV TG 186 (VV G-ERA/TG) or with VV G-ERA and VV HA (VV G-ERA/HA). Rabies virus Ab responses were not statistically different ( $p>0.05$ ) in the two groups of mice (Fig 3B). Thus, HA expression cannot enhance the Ab response against G-ERA. This indicates that, unlike the apoptotic bodies generated by G-ERA, the apoptotic bodies generated by HA expression (Fig 1B) cannot immunopotential an antibody response against an exogenous antigen.

Finally, we focused on the role of apoptotic bodies generated by G-ERA on the influenza memory immune response. Mice were injected with VV G-ERA, VV HA or VV G-ERA & VV HA. Six weeks later, they were immunized with a commercial influenza vaccine. The HA Ab response after the boost was higher ( $p<0.05$ ) for the mice that first had received VV G-ERA/HA than for those that had received VV HA alone (Fig 3C). Hence the G-ERA-mediated immunopotential is long-lived.

### **Blocking apoptosis reduces the immunopotential effect**

We then studied whether G-ERA-mediated immunopotential was strictly linked to the occurrence of apoptosis. EL4 cells were infected with VV G-ERA and VV HA at a multiplicity of infection of 3 and the cells were treated or not with Z-VAD, a pan-caspase inhibitor. In this system, Z-VAD had no effect on the recombinant VV infectious process as the same percentages of infection were obtained in treated and untreated cultures (data not

shown). Similarly, treatment of EL4 cells with Z-VAD did not modify G-ERA or HA expression which remained unchanged with or without treatment (data not shown). In presence of Z-VAD, apoptosis driven by VV G-ERA/HA was drastically reduced (2.9 % in presence of Z-VAD and 58.2 % without, Fig 4). Seven days after injection of mice with the two types of vaccine preparation, the level of HA Ab was significantly higher ( $p < 0.05$ ) when apoptosis was not blocked than when apoptosis was blocked (Fig 4). This means that blocking apoptosis reduces G-ERA-mediated immunopotentiality, demonstrating that this property of G-ERA requires a caspase-dependent apoptotic process.

In conclusion, G-ERA-mediated immunopotentiality is linked to the generation of apoptotic bodies co-presenting G-ERA and HA.

## **DISCUSSION**

Our data demonstrate that G-ERA, a pro-apoptotic molecule, can immunopotentiality antibody responses to live vaccines. This effect seems to last over the time as shown by the boost experiment. This immunopotentiality was specifically driven by G-ERA; expression of HA had no effect on the immune response directed against rabies virus. We showed that co-expression of G-ERA and HA was crucial. We provided evidence that apoptosis is a key factor of immunopotentiality because it was decreased by ZVAD. Uptake of apoptotic cells and bodies has been described as a powerful mechanism of immunopotentiality [3]. Thus, we hypothesize that fragmentation of a cell expressing a vaccine antigen by G-ERA generates apoptotic bodies that function as powerful vaccine antigen-presenting structures. Apoptosis-driven immunopotentiality is controlled by several factors, including the nature of the apoptotic stimulus, the nature of cells that engulf apoptotic cells, the microenvironment in which cells are dying and the number of dying cells [3]. Different molecules expressed by the dying cells, phagocytes, macrophages and dendritic cells are involved in uptake of dying cells

[29]. The best characterized modification of the plasma membrane of apoptotic cells is the loss of phospholipid asymmetry resulting in the exposure of phosphatidylserine (PS) from the inner face on the outer face of the cell membrane. Exposure of PS provides a sufficient signal to inhibit DC maturation and to modulate adaptive immune responses [30]. Exposure of PS may disable G-ERA immunopotentiality because G-ERA-mediated apoptosis exposes PS on the dying cell surface [20]. Conversely, antibodies to blebs or apoptotic bodies have been shown to affect the recognition of apoptotic cells by cells of the innate immune system. This may modulate the uptake of antigen complexes that are packed in the dying cells, thus modifying tolerance to foreign antigens [31]. This finding may help to explain the data gathered by Sasaki *et al.* [13,14] who showed that “bland” apoptosis can elicit enhanced immune responses. The rabies virus glycoprotein is the main viral antigen. It induces virus-neutralizing antibodies that are the major effectors of the immune response against rabies [32]. Rabies virus G-ERA has been vectorized in VV vectors and shown to be an excellent vaccine: it contributed to the control of rabies in European countries [33]. We can assume that following the injection of our vaccines, antibodies directed against G-ERA act as the immunopotentiality effectors that stimulate a strong B cell response against HA as long as this antigen is properly packed in apoptotic bodies. It would be interesting to test the efficacy of the humoral immune response against HA following injection of VV G-ERA/HA apoptotic bodies into mice already primed with the manufactured rabies vaccine.

G-ERA immunopotentiality was drastically decreased, but not abrogated, after ZVAD treatment. Caspase-independent pathways may account for the remaining immunopotentiality effect. Caspase activation is not required for all types of cell death [34]. Furthermore, caspase activation was recently shown to counteract some deleterious processes associated with cell death in some mammalian systems [35]. Overexpression of Bax or Bak induces cell death without the involvement of caspases [36-38], suggesting that factors other than caspases can

also mediate apoptosis. Indeed, we have reported that the rabies virus strain ERA induces not only a caspase-dependent apoptosis pathway in the human lymphoblastoid Jurkat T cell line but also a caspase-independent pathway involving the apoptosis inducing factor (AIF) [19]. Further studies are needed to determine whether AIF-mediated apoptosis plays a role in the immunopotential effect. Necrosis, a type of uncontrolled cell death releasing noxious components [39,40] that is not counteracted by ZVAD treatment, can also trigger a potent immune response [3]. In particular, necrotic cells can passively release the High mobility group box 1 protein (HMGB1) as well as heat shock proteins (HSPs), which are known to be pro-inflammatory molecules [41-43]. However, the denatured proteins generated by necrosis may be inadequate for vaccine protection. If necrosis is involved in immunopotential, the antibody titers induced after immunization with VV G-ERA & VV HA and in VV G-ERA/HA should have been similar. This was not the case, showing that necrosis does not play a role in G-ERA immunopotential.

Our results showed that G-ERA is an immunopotential molecule for B cell response and could be used to enhance vaccine efficacy. These data look promising and will urge us to further analyze the effect of G-ERA driven apoptosis on the cellular immunity response directed against HA and to test its effect on other vaccine antigens, in particular those will be necessary to be classified as poor vaccine immunogens. Also, further work is obviously required to determine precisely which mechanisms are triggered by G-ERA as the balance between apoptosis and immunopotential or tolerance are complex [44]. This work might contribute to the design of new type of improvement of low immunogenicity potential vaccines and to design a new generation of vaccines for infectious diseases.



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## Figure Legends

### **Figure 1: G-ERA recombinant VV virus triggers apoptosis but not necrosis.**

EL4 cells were infected with the recombinant VV viruses expressing either G-ERA (VV G-ERA), HA (VV HA) or no foreign gene (VV TG186). Apoptosis and necrosis were determined 24h post infection. Results are representative of three experiments. A) Induction of apoptosis was measured by flow cytometry analysis comparing side (SSC) and forward (FSC) light scattering in EL4 cells infected either with VV TG186 or VV G-ERA. This analysis allows to distinguish the live cells (R1) from the dying cells (R2). The numbers represent the percentage of R2 cells that were apoptotic, 8% in non-infected cells and 48% in G-ERA expressing cells. B) Apoptosis in VV TG186, VV HA or VV G-ERA infected EL4 cells as measured by determining the percentage of R2 cells in the cell population (white bars) or by determining the percentage of cells with fragmented nuclei stained by the TUNEL technique (hatched bars ). C) Presence of necrotic cells was determined in EL4 cells infected with VV TG186, VV G-ERA and VV HA or co-infected with VV G-ERA and VV HA by measuring the percentage of propidium iodide positive cells. Cells treated with acetone were used as a positive control.

### **Figure 2: VV G-ERA and VV HA co-infected EL4 cells co-express HA and G-ERA antigens and undergo apoptosis**

EL4 cells were infected for 24h with VV G-ERA and/or VV HA and then stained with anti-G-ERA mAb or anti-HA Ab. Cells were also stained with Hoescht 33342 to visualize nucleus fragmentation.

A) Percentage of cells expressing G-ERA or HA were measured by flow cytometry. White numbers represent the percentage of cells expressing G-ERA and HA. Black numbers represent the percentages of cells expressing HA or G-ERA. The levels of expression were determined to be 95% and 87% in VV G-ERA (left panel) and VV HA (middle panel) infected EL4 cells respectively. After coinfection, (right panel), 84% of the cells express the two antigens. B) EL4 cells co-infected with VV G-ERA and VV HA were stained and analyzed by UV microscopy after Hoescht 33342 (first left panel, note the fragmented blue nucleus), G-ERA (green in second left panel) and HA (red in first right panel) antibody staining. G-ERA and HA antigens can be detected in the same cell with a fragmented nucleus a marker for a late stage of apoptosis (merge). It can be noted that G-ERA and HA antigens do not co-localize in the infected cell (merge, second right panel).

### **Figure 3: Immunopotential of VV G-ERA**

B6 mice were immunized with vaccine doses prepared from EL4 cells infected with the recombinant VVs. The humoral immune response against HA or G-ERA was monitored in mouse sera by specific ELISAs.

A) Mice (n=8) were immunized with EL4 cells co-infected with VV G-ERA and VV HA (VV G-ERA/HA) or VV HA and VV TG186 (VVHA/TG), or with EL4 cells infected either by VV G-ERA and VV HA and mixed immediately before injection (VV G-ERA & VV HA). Apoptosis was quantified by TUNEL (numbers). Specific anti-HA response (expressed as AU/ml) in sera was monitored 12 days post vaccination. B) Mice (n=8) were immunized with VV G-ERA/TG or VV G-ERA/HA vaccine preparations. Specific rabies virus response (expressed as AU/ml) was quantified in sera 12 days post vaccination. C) Mice (n=8) were immunized with VV G-ERA/HA or VV G-ERA&VV HA. Six weeks later, mice were boosted with a commercial anti influenza vaccine. The memory immune response was determined by measuring the HA Ab titer 7 days later.

### **Figure 4: The immunopotential effect of vaccinia virus-expressed G-ERA is linked to its ability to trigger apoptosis**

Mice (n=8) were immunized with VV G-ERA/HA with or without the pan-caspase inhibitor Z-VAD. Apoptosis was quantified by TUNEL (numbers). The HA-specific Ab response was assessed 7 days post injection.

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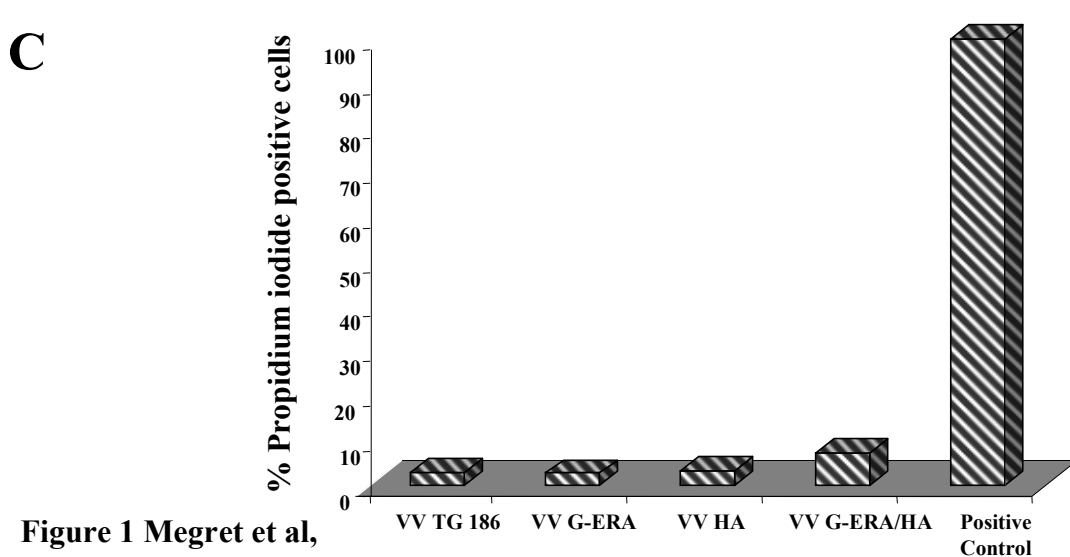
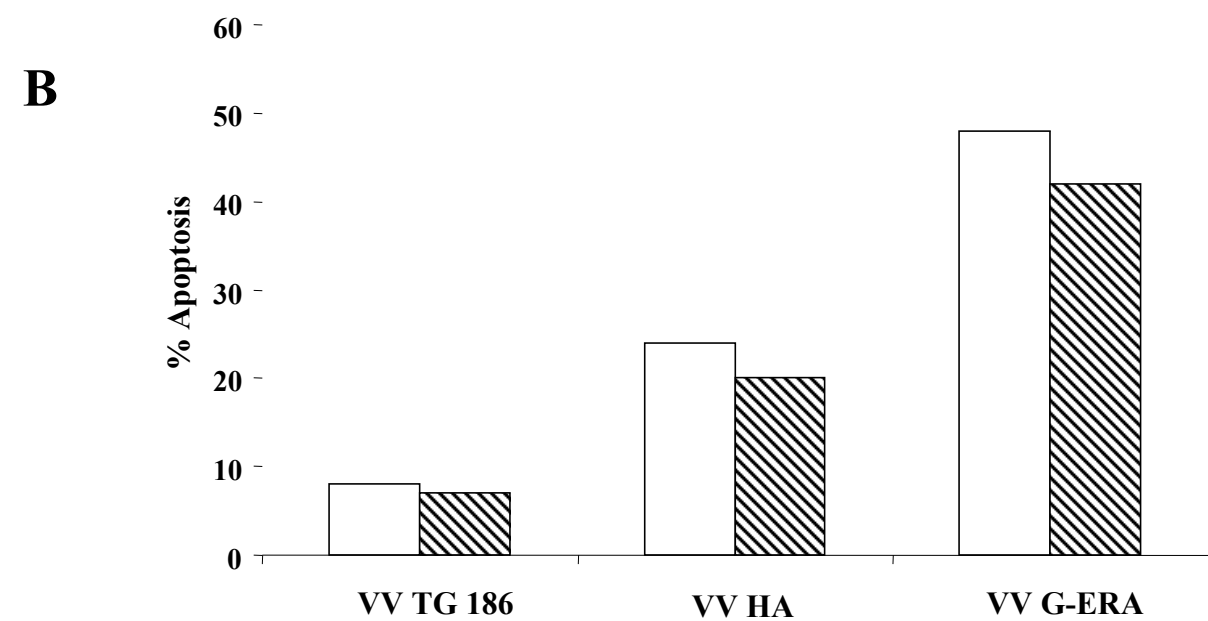
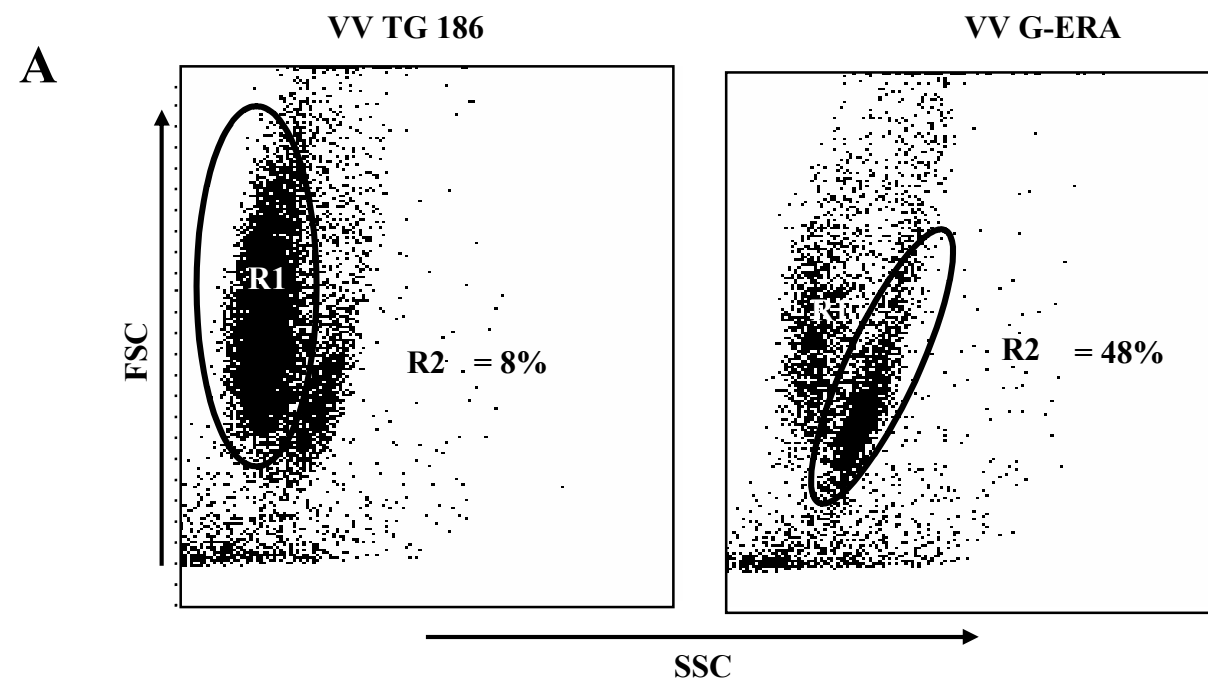


Figure 1 Megret et al,

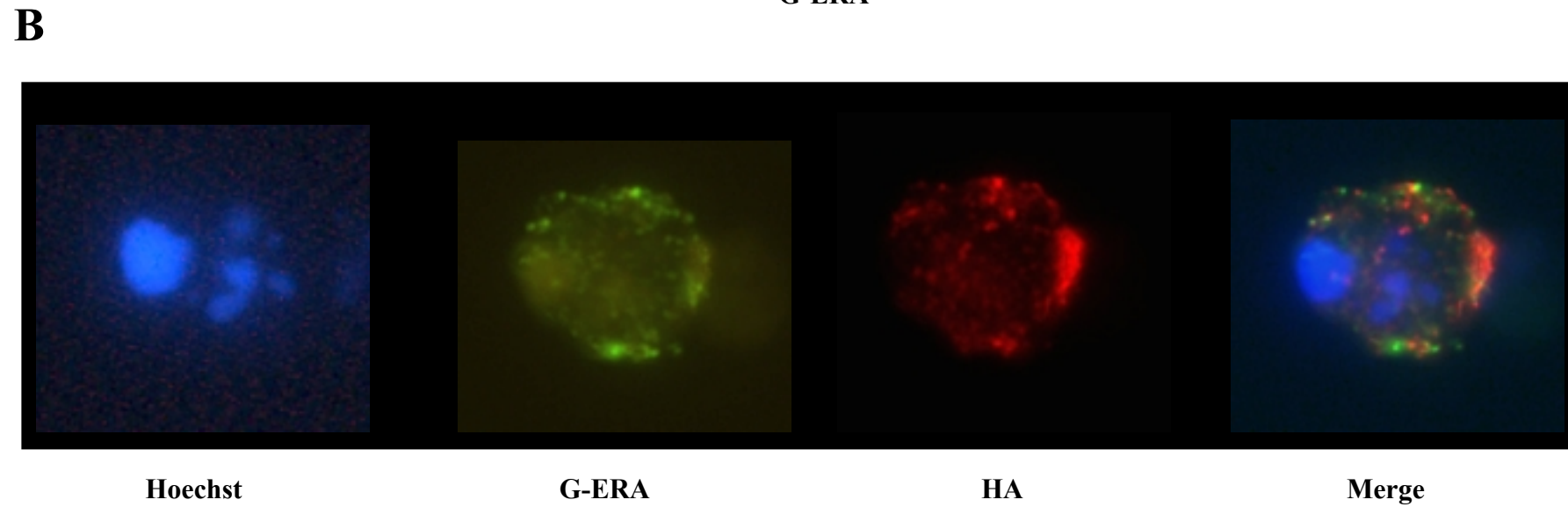
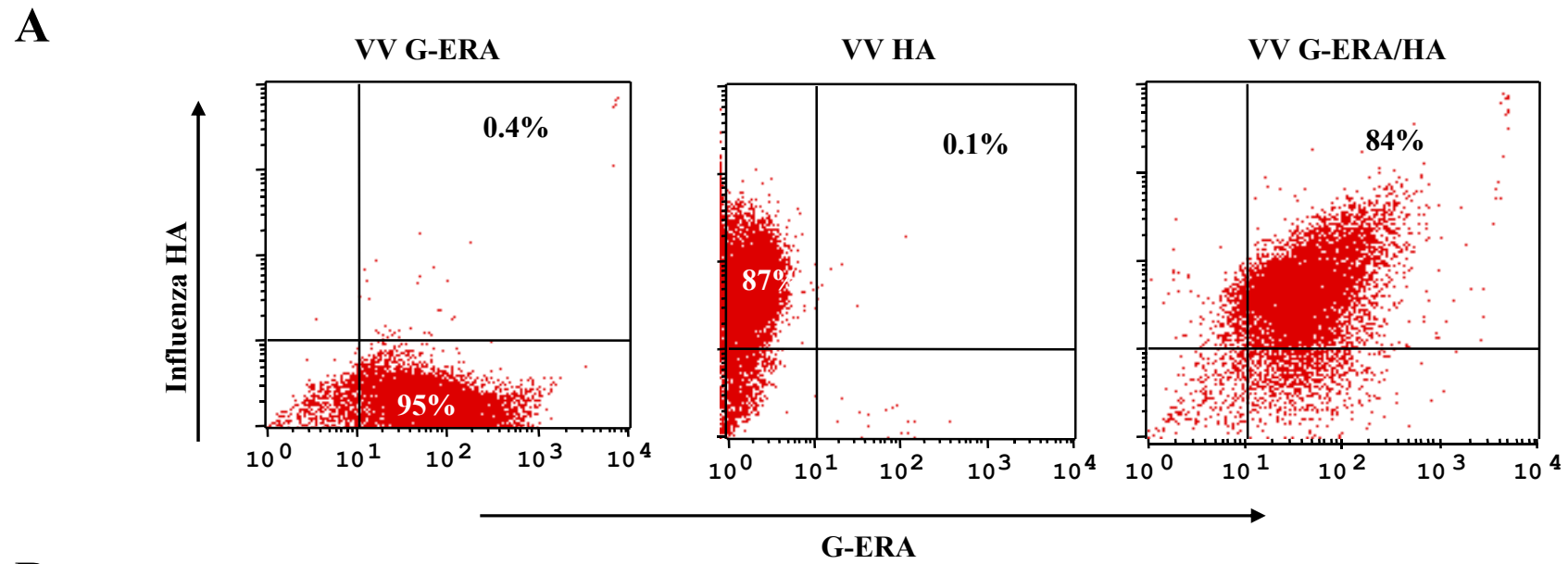


Figure 2, Megret et al,

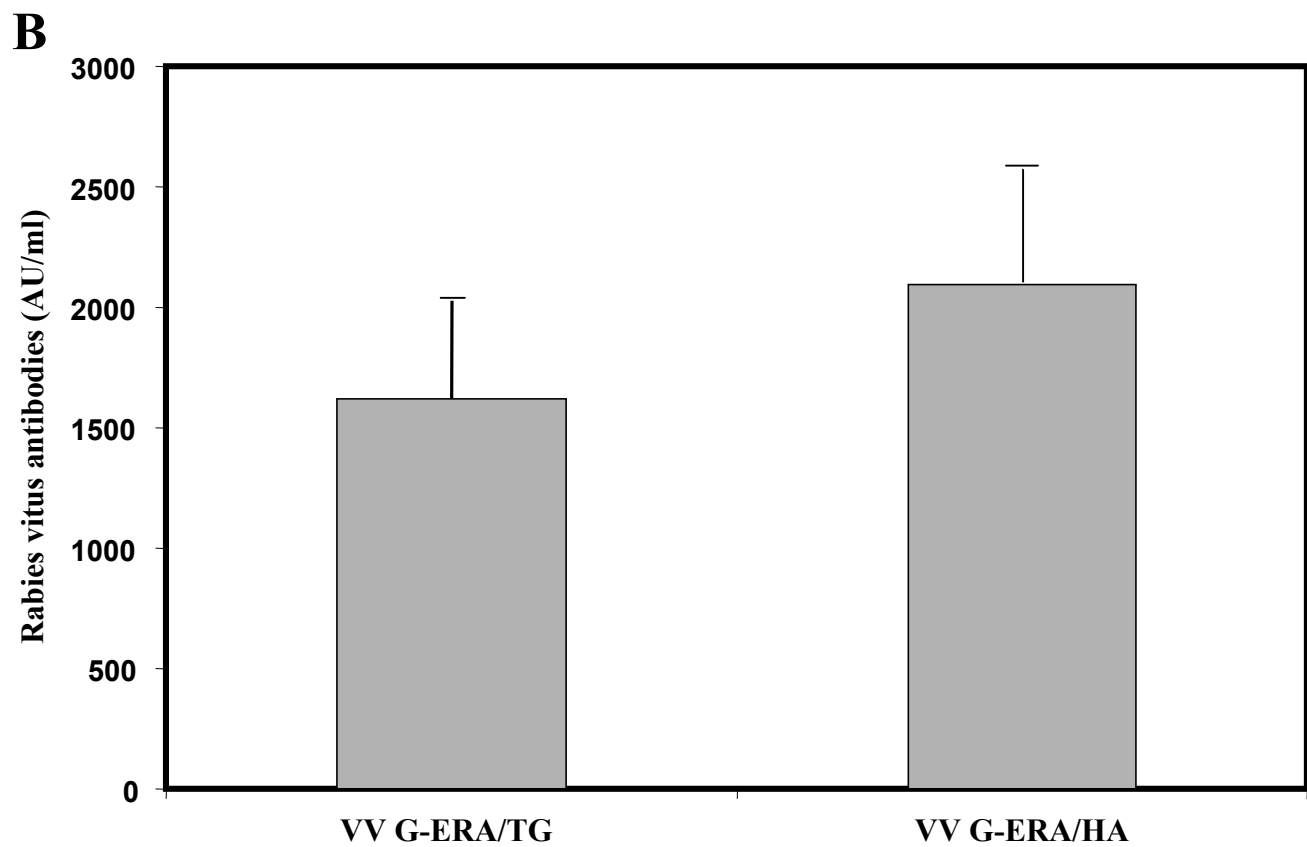
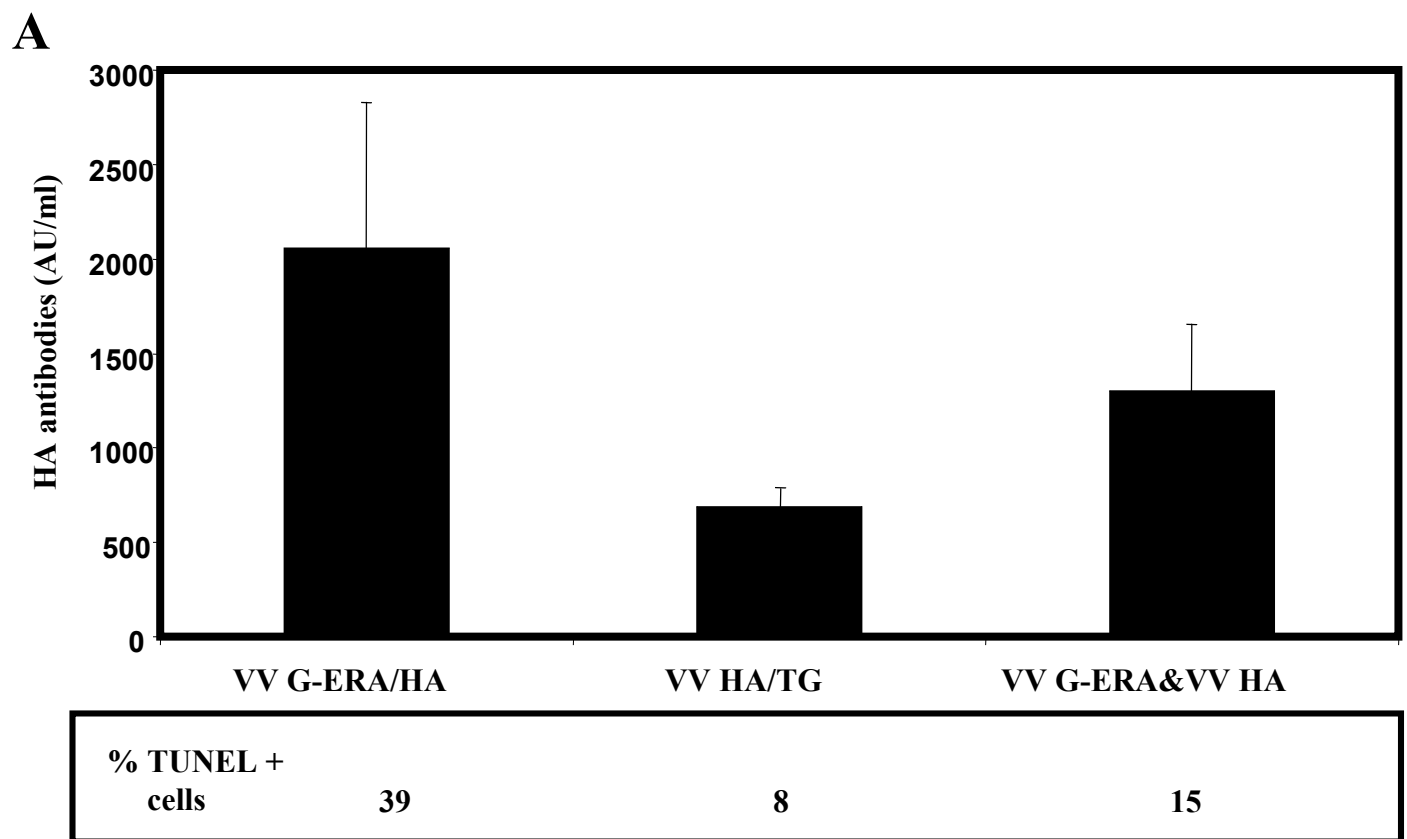


Figure 3 Megret et al,



**C**

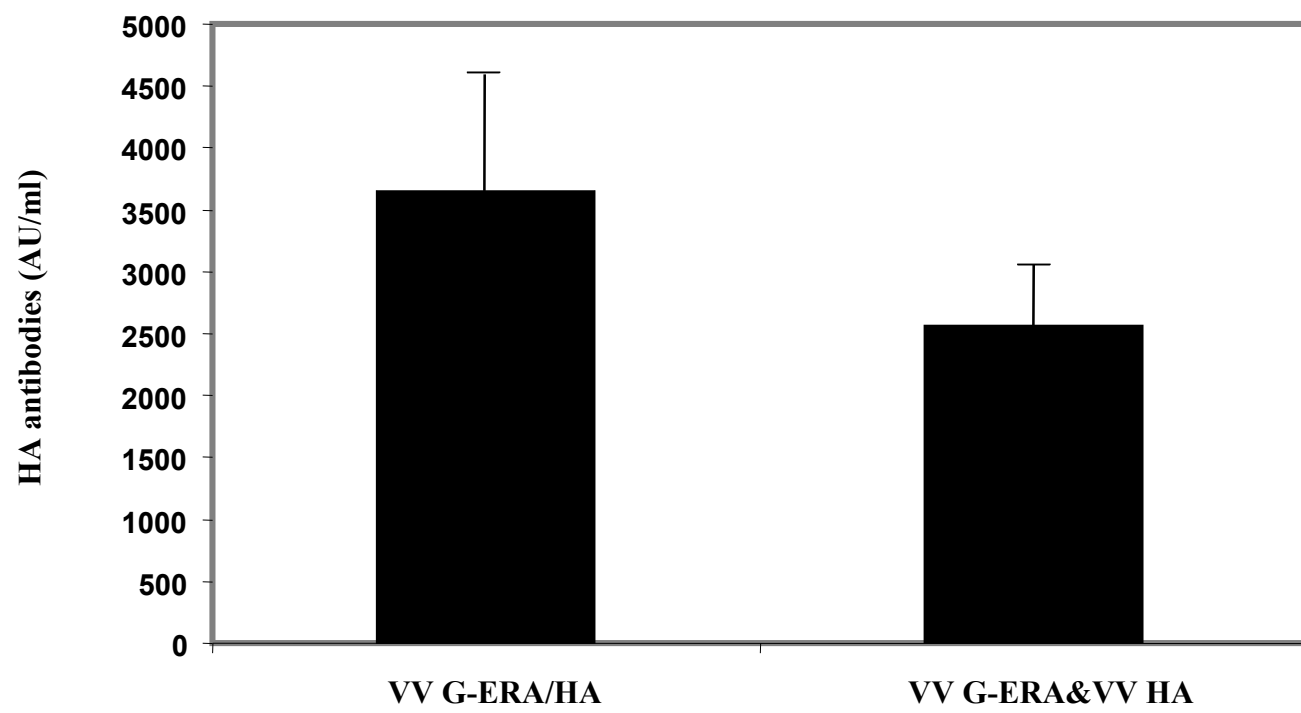
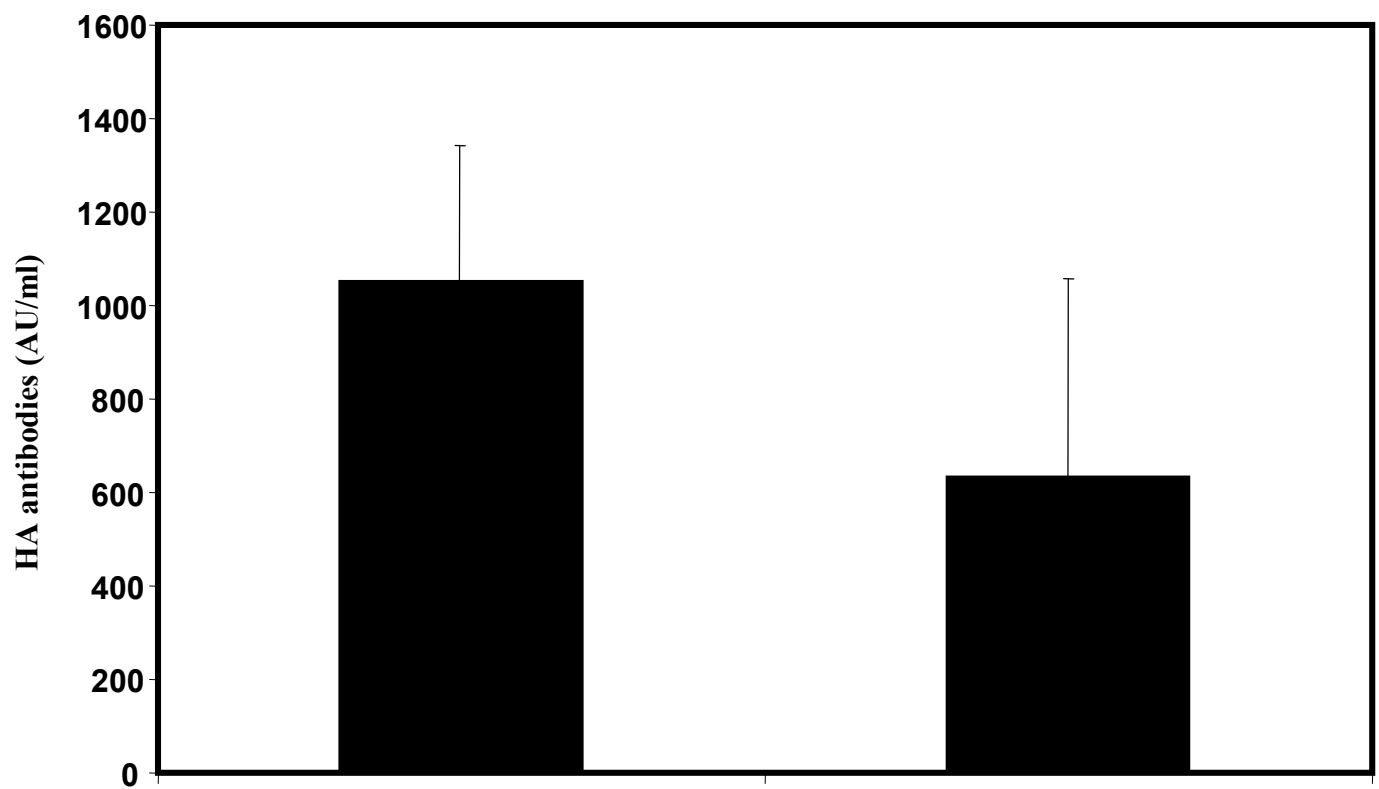


Figure 3 Megret et al,



	VV G-ERA/HA	VV G-ERA/HA
	no ZVAD	ZVAD
% TUNEL + cells	58.2%	2.9%

Figure 4 Megret et al,