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# Preclinical development of HIvax: human survivin Highly Immunogenic vaccines


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**Preclinical development of HIvax: human survivin Highly Immunogenic vaccines**

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## ABSTRACT

Our previous work involved the development of a recombinant fowlpox virus encoding survivin (FP-surv) vaccine that was evaluated for efficacy in mesothelioma mouse models. Results showed that FP-surv vaccination generated significant immune responses, which led to delayed tumor growth and improved animal survival. We have extended those previous findings in the current study, which involves the pre-clinical development of an optimized version of FP-surv designed for human immunization (HIvax). Survivin-derived peptides for the most common haplotypes in the human population were identified and their immunogenicity confirmed in co-culture experiments using dendritic cells and T cells isolated from healthy donors. Peptides confirmed to induce $CD8^+$ and $CD4^+$ T cells activation in humans were then included in 2 transgenes optimized for presentation of processed peptides on MHC-I (HIvax1) and MHC-II (HIvax2). Fowlpox vectors expressing the HIvax transgenes were then generated and their efficacy was evaluated with subsequent co-culture experiments to measure interferon-$\gamma$ and granzyme B secretion. In these experiments, both antigen specific $CD4^+$ and $CD8^+$ T cells were activated by HIvax vaccines with resultant cytotoxic activity against survivin-overexpressing mesothelioma cancer cells. These results provide a rationale for clinical testing of HIvax1 and HIvax2 vaccines in patients with survivin-expressing cancers.

**Keywords:** mesothelioma, cancer vaccines, fowlpox, T-Lymphocytes, immunodominant epitopes, EpiMatrix, tregitopes.

## INTRODUCTION

Despite recent progress in surgical, chemotherapeutic, and radiotherapy approaches, several cancers are still difficult to treat and cure, especially in patients with advanced stages of the disease. Therefore, new therapeutic strategies are needed and immunotherapeutic approaches targeting tumor-associated antigens (TAAs) are among the most prominent approaches recently developed. $CD8^+$ cytotoxic T lymphocytes (CTLs) are the most important effector cells for antitumor immune responses. CTLs recognize TAA-derived peptides that are presented on the tumor cell surface in association with major histocompatibility complex (MHC) class I molecules, leading to tumor cell killing.[1] $CD4^+$ T helpers (Th) cells provide assistance to CTLs in the form of soluble mediators like cytokines, which enhance the CTL expansion and cytolytic function.[2] Initial activation of naïve antigen-specific lymphocytes is mediated by specialized MHC-positive cells, particularly dendritic cells (DC). Clinical evidence for the effectiveness of antitumor immune responses has been obtained in several clinical settings.[3-5]

Survivin has been shown to fulfill major criteria for prime target antigens included in broad applicable anti-cancer vaccines: 1) tumor cells depend on its actions for survival or proliferation, 2) expression is strong in multiple tumor types but rarely detectable in normal tissues, 3) immunogenicity has been demonstrated in patients with different cancers, and 4) peptides restricted by different HLA molecules are known. Survivin is expressed in a majority of tumor cells at all stages, from premalignant to metastatic lesions, in solid tumors and hematopoietic malignancies, and the gene encoding survivin is the fourth most abundantly expressed gene in melanomas and cancers of the

colon, lung, brain, and breast. [6-8] Expression of survivin is typically limited to developing tissues but becomes reactivated in adult tissues during the neoplastic transformation, contributing to apoptosis evasion and molecular pathways driving unrestricted proliferation and angiogenesis in tumor cells.[9] Survivin expression is a marker of poor prognosis and/or resistance to therapy in multiple cancers.[10] In patients with a variety of cancers, survivin-specific cell mediated and humoral immune responses have been shown.[11-14] Preclinical and clinical data from survivin vaccination studies suggest that these vaccines can induce immune responses and do not raise substantial safety concerns. Multiple HLA class I-binding peptides and one HLA class II-binding peptide of survivin have been identified, some of which have demonstrated immunogenicity in clinical trials.[15, 16]

Malignant mesothelioma (MM) is an aggressive and deadly cancer. It originates from a chronic inflammatory process arising from asbestos or erionite exposure. The median patient survival is 9-12 months from diagnosis and intervention with trimodality therapy (chemotherapy, surgical resection and thoracic radiation) only extends survival by an average of 5 months. Survivin overexpression has been reported in MM in different studies.[17, 18] Our previous work involved the development of a novel recombinant fowlpox virus encoding survivin (FP-surv) vaccine that was evaluated for efficacy in different MM mouse models. Mice were injected subcutaneously or intraperitoneally with murine fiber-induced MM tumor cells and vaccinated with FP-surv. Results showed that FP-surv vaccination generated significant immune responses in both models, which led to delayed tumor growth and improved animal survival.[17]

We have extended those previous findings in the current study, which involves the pre-clinical development of an optimized version of FP-surv designed for human immunization (HIvax). With the purpose of identifying survivin-derived peptides that are highly immunogenic for the most common HLAs in the human population, the survivin protein was analyzed using an innovative epitope-prediction algorithm called EpiMatrix.[19-22] The immunogenicity of the predicted peptides was confirmed in co-culture experiments using autologous DC and T cells isolated from human healthy donors. The sequences encoding peptides, confirmed to induce CTLs activation in these co-cultures, were then inserted in a transgene optimized for presentation of processed peptides on MHC-I (HIvax1). Similarly, sequences for peptides found to activate Th cells were included in a transgene engineered to present antigens on MHC-II (HIvax2). One of the predicted MHC-I restricted peptides, (survivin 20-28), previously reported as an HLA binder,[5, 15] did not activate human CTLs in our co-culture experiments; hence it was excluded by our vaccine design. Recombinant fowlpox vectors expressing the HIvax transgenes were then generated and their efficacy was evaluated with subsequent co-culture experiments to measure interferon-$\gamma$ (IFN$\gamma$) and granzyme B secretion in human $CD8^+$ and $CD4^+$ T lymphocytes isolated from donors with different haplotypes.

## RESULTS

### In silico epitope mapping and peptide selection

The human survivin protein was computationally screened for class I and class II MHC epitopes using EpiMatrix. The protein sequence was parsed into 9-mer sequences, each overlapping the next by 8 amino acids, respectively, for a total of 134 9-mers. Each of these peptides was then scored for Class I HLA motif matches to the A*0101, A*0201, A*0301, A*2402, B*0702 and B*4403 alleles. More than 50 EpiMatrix hits (Z-score > 1.64; top 5% of scores) were discovered (data not shown). Among these, 4 sequences, positively scored for the considered alleles, were selected for experimental validation (Supplemental table 1). For Class II, epitope dense regions containing sequences predicted to bind multiple Class II HLA alleles (DRB1*0101, *0301, *0401, *0701, *1101, *1301 and *1501) were also identified. Three of these regions containing "hits" for several analyzed alleles were selected and the resulting peptide sequences were synthesized for experimental validation (Supplemental table 2).

### Human survivin-derived peptides induce activation of primary human $CD3^+$ T cells

Seven peptides (Hsurv1-7) representing sequences considered immunogenic in a major number of alleles were synthesized (Table 1). These EpiMatrix predicted peptides were used to prime human DC from naïve healthy donors. Autologous T cells were co-cultured with the DC for a 2 wks period, followed by reactivation with DC loaded with the same peptide in the presence of monensin, to allow evaluation of IFN$\gamma$ production. All peptides but one (Hsurv4) stimulated a significant number of specific IFN$\gamma^+$ $CD3^+$ T

lymphocytes in the donors (Figure 1). In contrast, overall responses to Hsurv4 peptide did not significantly differ from controls, which consisted of T cells stimulated with unpulsed DC (No pep) or irrelevant peptide pulsed DC (Irr pep). These experiments were reproduced at least in triplicate in ten healthy donors, each having a different haplotype, to assess the efficacy of the peptides in various HLA settings. Data representing $CD3^+$ T cell responses to survivin peptides for each individual donor are included in Supplemental Table 3.



**Selected survivin peptides induce IFNγ secretion in human $CD4^+$ and $CD8^+$ T cells**

We next evaluated separately the activation of $CD3^+$ T cell subpopulations: $CD3^+/CD4^+$ T cells and $CD3^+/CD8^+$ T cells. Lymphocytes purified from peripheral blood were cultured with autologous DC loaded with peptides designed for MHC-II (Hsurv1, 2, or 3) or MHC-I presentation (Hsurv4, 5, 6 or 7). IFNγ production was evaluated by intracellular flow cytometric analyses on $CD4^+$ or $CD8^+$ T cells (Fig.2). Percentages of $IFN\gamma^+$ T cells induced by the survivin peptides were compared with T cells stimulated with unpulsed DC (No pep), or with irrelevant peptide pulsed DC (Irr pep). Peptides designed for MHC-II presentation (Hsurv1, 2, or 3) activated peptide-specific $CD4^+$ T lymphocytes in 90% of donors (Table 2). Peptides designed for MHC-I (Hsurv5, 6 or 7) activated $CD8^+$ T lymphocytes responses in 100% of donors. Peptide Hsurv4, an antigen reported to induce immune responses in both mice and humans,[5, 23, 24] only stimulated T cells from a single donor only. Experiments were also performed to confirm that T cell responses were not induced by confounding artifacts related to the source of

peptide such as salts or buffer contaminants such as endotoxin. Production of IFNγ in T cells were simultaneously evaluated using the peptides described above and Hsurv peptides independently synthetized (i.s.) by a second commercial source. In these experiments we evaluated $CD4^+$ T cell responses after stimulation with Hsurv2 peptide and $CD8^+$ responses after stimulation with Hsurv4 or Hsurv5 compared to Hsurv2 (i.s.), Hsurv4 (i.s.) and Hsurv5 (i.s.). Similar $CD4^+$ T cell responses were stimulated by Hsurv2 and Hsurv2 (i.s.) and also similar $CD8^+$ T cell responses were induced by Hsurv5 and Hsurv5 (i.s.). Both Hsurv4 and Hsurv4 (i.s.) did not induce significant $CD8^+$ T cell responses (Supplemental Fig.1). These results suggested that the T cell IFNγ responses were elicited by the peptides themselves.

**Removal of survivin 20-28 peptide (Hsurv4) from stimulatory peptides leads to stronger and more consistent *in vitro* immunization**

The survivin peptide 20-28, designed Hsurv4 in our experiments, was the only peptide selected from the EpiMatrix analysis that did not induce significant $CD3^+$ T cell overall responses in our population of naïve human donors. This 9-mer was predicted to bind to multiple MHC-I alleles, but it only induced $CD8^+$ T cell activation from one human subject out of ten. To better evaluate the contribution of Hsurv4 in a hypothetical setting of multi-antigen vaccination, we compared pools of survivin peptides containing Hsurv4 (Hsurv1-7) to those without it (Hsurv 1,2,3,5,6,7). DC were stimulated with these peptide pools and used to prime autologous T cells. Table 3 shows the results of $CD4^+$ and $CD8^+$ T cells from four healthy donors with the two different peptide pools. In 75% of the

experiments, the pool without Hsurv4 induced $CD4^+$ and $CD8^+$ T cell responses. In comparison, the pool Hsurv1-7 that includes all the studied peptides induced both types of T cell responses in 50% of the experiments. In cells derived from one of the analyzed subjects the inclusion of Hsurv4 in the peptide pool led to lower numbers of IFNγ-secreting $CD4^+$ and $CD8^+$ T cells (Supplemental Fig.2). These results, together with those obtained using the single Hsurv4 peptide, suggest that the exclusion of this epitope may produce better immunization responses in humans.



**Development of HIvax1 and HIvax2 and analysis of their expression in human primary DC**

We constructed two fowlpox-based DNA vaccines, HIvax1 and HIvax2, each containing a distinct set of HLA class I and class II epitopes (Figure 3A). In HIvax1, sequences encoding Hsurv5, 6 and 7 were engineered into the vaccine matching the same order in which they are presented in the wild-type protein. Spacer sequences (Ala-Ala-Tyr) were included to promote the interaction between epitopes and transporters of antigen processing (TAP) that facilitate MHC-I loading in the endoplasmic reticulum (ER). [25] To further guide the HIvax1 encoded polypeptides to the ER and facilitate MHC-I loading, the immunoglobulin K (IgK) leader sequence was positioned upstream of epitope sequences, [26] resulting in trafficking of the peptide-MHC-I complex to the cell surface for interaction with the T cell receptor (TCR) on $CD8^+$ T cells (Figure 3D). In designing the HIvax2 antigen we included the tissue plasminogen activator (tPA) leader sequence upstream of epitope sequences to direct translation products to the secretory pathway.

The inclusion of Gly-Pro-Gly-Pro-Gly spacers between peptides, avoids the formation of immunodominant junctional epitopes.[27] In both HIvax1 and HIvax2, V5 tag sequences were included at the C-terminus to facilitate polypeptides detection.

RT-PCR was used to evaluate the expression of HIvax transgenes in mature human DC infected with recombinant fowlpox vectors codifying for HIvax1 or HIvax2. Fowlpox vectors without the transgenes (FP-ctrl) were used as control. DC infected with HIvax1 or HIvax2 produced the corresponding mRNA, whereas DC infected with FP-ctrl were negative (Fig.3B). The production of the HIvax1 and HIvax2 polypeptides was then evaluated through immunofluorescence staining of infected DC using anti-V5tag antibodies. Results showed the presence of the polypeptides produced by HIvax1 and HIvax2 in the cytoplasm of infected cells (Fig.3C).

### *In vitro* immunization of human DC with HIvax1 or HIvax2 induces IFNγ production in autologous T cells

The efficacy of HIvax vaccines in stimulating survivin specific T cells was evaluated using immune cells from two naïve healthy donors with different haplotypes (Fig. 4 and Supplemental Fig. 3). Monocyte-derived DC were infected with recombinant fowlpox vectors encoding HIvax1 or HIvax2, whereas FP-ctrl was used to infect DC in controls. Autologous T cells were cultured with the DC for 2 wks and then were reactivated with DC loaded with a pool of Hsurv5, 6, 7 peptides for HIvax1 or a pool of Hsurv1, 2, 3 peptides for HIvax2. In both cases control DC (No pep) were used as controls. Flow cytometric analysis revealed that $CD8^+$ T cells stimulated by HIvax1 responded to DC

loaded with Hsurv5, 6, 7 by producing IFNγ (Fig. 4A). In contrast, T cells initially incubated with FP-ctrl and reactivated by DC loaded with the same peptides did not produce IFNγ. Immune cells from this specific donor (Table 2, Donor no.5) were used to test HIvax2 and results showed that $CD4^+$ T cells stimulated by this vaccine reacted to DC loaded with Hsurv1, 2, 3 by producing IFNγ (Fig. 4B). DC loaded with the same peptides did not activate T cells stimulated by FP-ctrl. In immune cells from a donor with a different haplotype (Table 2, Donor no.8), HIvax1 induced $CD8^+$ T cells while HIvax2 did not induce a IFNγ response. This may reflect differences in immunogenicity of HIvax2 epitopes for different MHC-II haplotypes.

**T cells activated by HIvax1 and HIvax2 exhibit cytotoxic activity towards MM cells**

In previous studies we demonstrated that human malignant mesothelioma (MM) cells express high levels of survivin.[17] To further evaluate the efficacy of our HIvax approach, experiments were conducted in which survivin overexpressing ADA MM cells were co-cultured with T cells activated by HIvax vaccines. Both HIvax 1 and 2 vaccines were used together to simultaneously activate both $CD4^+$ and $CD8^+$ T cells and thereby increase the cytotoxic potential of CTLs. T cells stimulated with HIvax or with the control vaccine FP-ctrl were also assayed versus survivin-negative, primary autologous skin fibroblasts. To evaluate the cytotoxic activity of CTLs against MM cells or fibroblasts, secretion of granzyme B was measured by ELISPOT. Significantly higher granzyme B was detected for T cells activated by HIvax, co-cultured with ADA MM cells, but not with

primary fibroblasts (Fig.5). Altogether, these results suggest specific CTL function against MM generated by HIvax.

## DISCUSSION

In this study we describe the development of novel multi-epitope vaccines (HIvax1 and HIvax2) specifically designed to induce anti-tumor immune responses for eventual use in clinical trials. These data were built upon our previously published work demonstrating that fowlpox vectors expressing full-length survivin were capable of activating T cell responses against MM cells in mice. Full tumor regression was induced in a significant number of vaccinated mice, even if some mice retained MM tumors.[17] It has been recently demonstrated that human "self" proteins may contain regulatory T cell epitopes. These sequences (tregitopes) activate T regulatory cells and lead to the suppression of $CD4^+$ and $CD8^+$ T cell responses. Therefore, the efficacy of vaccines expressing entire human antigens may be limited by the presence of immune-suppressive tregitopes that counteract the effects of the other immune-stimulatory epitopes.[28-30] Our current study was designed to construct vaccines containing only stimulatory epitopes by testing the immunogenicity of candidate peptides with DC and T cells co-culture experiments. A sophisticated algorithm (EpiMatrix) identified three potential MHC-II-binding peptides (Hsurv1, 2, 3) and four potential MHC-I-binding peptides (Hsurv4, 5, 6, 7). Multiple experiments were conducted using immune cells isolated from ten healthy naïve donors with different haplotypes. All of these peptides, with the exception of Hsurv4, induced $CD3^+$ T cell responses as determined by IFN$\gamma$ production (Fig.1). Analysis of $CD3^+/CD8^+$ T cell subpopulations indicated that Hsurv4, a peptide predicted to induce CTL responses, was effective in cells from one donor out

of ten. The remaining peptides induced $CD4^+$ and $CD8^+$ T cells responses in 50-90% of subjects (Table 2). The survivin peptides used in this study have been identified for their potential of binding MHC molecules from the most common human HLA alleles (Supplemental tables 1 and 2). Analyses of the donors showed that most haplotypes belonged to these common HLA alleles. Some less common alleles were present in certain donors, but comparisons between EpiMatrix predictions and effective T cell responses only included donors with common HLA alleles. Although the sample size was too small to draw any definitive conclusion, a qualitative assessment of EpiMatrix predictions and observed effective T cell responses suggested that Hsurv4 peptide, corresponding to the epitope 20-28 of survivin, may represent an immune-regulatory T epitope. Our experiments using peptide pools with or without Hsurv4 suggested that often, although not always, the exclusion of this peptide from vaccines may lead to stronger IFN$\gamma$ responses. However, further evaluation of $CD4^+$ $CD25^+$ $FOXp3^+$ regulatory T cells in these experiments did not reveal significant tregitope properties of Hsurv4 (data not shown).

Despite an insufficient number of donors in this study is to define the immunostimulatory vs. immunosuppressive nature of Hsurv4 (survivin 20-28), the data obtained in our initial experiments warranted exclusion of Hsurv4 from the HIvax1. Since suppressive epitopes are likely present in full-length antigens, the efficacy of the candidate peptides should be evaluated by *in vitro* immunization studies prior to vaccine production. Ideally, personalized vaccine development tailored to include only those peptides found to be immunostimulatory for a particular patient would allow vaccines optimized for individuals.

In designing HIvax1, MHC-I peptides were separated by Ala-Ala-Tyr spacers and targeted to the ER by including an IgK signal sequence. This strategy drives the correct presentation of 9-mer peptides in the groove of MHC-I and has been successfully used in several vaccine studies.[25, 31] For HIvax2, epitopes were instead separated by Gly-Pro-Gly-Pro-Gly spacers and fused with a plasminogen activator signal sequence (tPA) to promote their presentation on MHC-II. Expression of the HIvax2 transgene should result in a polypeptide that is secreted, subsequently re-engulfed, and degraded through exogenous antigen processing pathways within lysosomes. Resultant peptides are loaded into the grooves of MHC-II and transported to the plasma membrane for presentation to $CD4^+$ T cells. In addition, the HIvax2 peptides may be also be loaded into MHC-I through cross-presentation pathways.[21, 22] In brief, prematurely truncated or abnormally folded proteins are exported to the cytosol, processed by the proteasome and resultant peptides loaded on MHC-I in the ER (Fig. 3). This pathway may also be involved in presentation of HIvax1 epitopes to MHC-II as previously demonstrated. [32-34]

The synthetic transgenes used in this study were cloned in recombinant FP vectors. Vaccines based on recombinant vectors like FP induce a strong inflammatory response, directed mainly towards the vector-encoded proteins. Pox viral vectors are among the most heavily exploited in vaccine development,[17] and FP has shown particularly promising results as a vaccine for infectious diseases and cancer.[35] FP infection leads to effective antigen presentation by human antigen presenting cells, and the generation of antigen-specific CTLs.[36] An advantage of FP for vaccination is the fact that this virus does not productively replicate in mammalian cells, but still leads to effective expression of epitopes as supported by the current study.

Since DC represent the most effective antigen presenting cells, this study used human monocyte-derived DC infected with HIvax vaccines to confirm the production of mRNA and proteins encoded by HIvax transgenes and presentation of antigens to cognate T cells. Infected DC from two different donors were used to prime autologous T cells. In these experiments, both antigen specific $CD4^+$ and $CD8^+$ T cells were activated in one of the two donors, while the other donor displayed only the activation of $CD8^+$ T cells by HIvax1. While it is not entirely clear why the differences were found between these two individuals, some evidence from the data involving the latter individual's $IFN\gamma^+$ $CD4^+$ T cells measured after incubation with DC loaded with MHC-II peptides. In particular, this last donor presented immune responses for 1 peptide out of 3 as shown in Table 2, (Donor no. 8). In comparison, the first donor, for which HIvax2 activated a significant number of $IFN\gamma^+$ $CD4^+$ T cells, had responses for all 3 Hsurv peptides (Donor no. 5). T cells, isolated from this donor, were stimulated using DC infected with both HIvax vaccines and evaluated for their cytotoxic ability against MM cells. These cancer cells over-express survivin and represent targets for HIvax-stimulated T cells that recognize survivin peptides within MHC-I and initiate a cytotoxic activity with secretion of granzyme B. Importantly, this vaccination does not promote CTL activity against healthy cells as supported by our data showing that T cells stimulated by HIvax were not activated by the co-culture with autologous fibroblasts. Taken together, these results suggest that HIvax vaccines induce specific CD4+ and CD8+ T cell responses in humans and promote specific killing of MM tumor cells *in vitro*. These activated lymphocytes should not induce autoimmune responses in humans since their reactivity was evaluated as not significant against autologous “self” cells.

These studies provide a rationale and framework for clinical testing of HIvax1 and HIvax2 vaccines in patients with survivin-expressing cancers. To understand whether patients with MM or other solid tumors will have the capacity to respond to vaccination in a manner similar to that seen *in vitro* with healthy donor cells, further clinically based investigations are required.

## MATERIALS AND METHODS

### Epitope selection and peptide synthesis

Survivin epitopes were scored for binding affinity, across a range of common HLA, using the EpiMatrix algorithm.[37] Top-scoring sequences were synthesized as 9 mer peptides for presentation on MHC-I, and as 15-18 mer for MHC-II (Table I). Synthetic peptides were manufactured by Peptide 2.0 (Chantilly, VA) or Elim Biopharm (Hayward, CA) using fluo-renylmethoxycarbonyl chemistry and solid-phase synthesis and purified by high-pressure liquid chromatography. The quality of the peptides was assessed by high-performance liquid chromatography analysis. Peptide purity was >90% as ascertained by analytical reversed phase HPLC. Individual peptides were dissolved in PBS and used at a final concentration of 10 µg/ml. The following irrelevant control peptides were used: eGFP peptide (aa. 200–208) for MHC- I and LCMV peptide (aa. 61-80) for MHC- II.

### Mesothelioma cell line and primary cells

The human MM cell line ADA was characterized and generously provided by Dr. H. Pass.[38] ADA cells were cultured in Ham's F12 culture medium containing 10% FBS and

penicillin-streptomycin. Whole blood was drawn from healthy volunteers who gave informed consent according to our protocol approved by the University Hawaii Committee on Human Subjects Institutional Review Board (CHS#19442). Peripheral blood mononuclear cells (PBMC) from these HLA-typed healthy donors were obtained by Ficoll density centrifugation.

***In vitro* immunization and human T cell cultures.**

To generate monocyte-derived dendritic cells (DC) from PBMCs, adherent cells were cultured for 7 days in X-VIVO 15 (Lonza, #04-744Q) containing 500 units/mL recombinant human interleukin 4 (IL-4) and 1,000 units/mL recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) (both from BioLegend, #574002 and #572902). On days 2 and 5 of incubation, media was replaced with fresh medium with IL-4 and GM-CSF. The mature DC expressed associated antigens, such as CD80, CD83, CD86, and HLA class I and class II, on their cell surfaces as confirmed by flow cytometry (data not shown). On day 5, DC were incubated with one of these conditions: 1) with 10 μg/mL individual peptides for 48 h; 2) with 10 μg/mL of a mixture of different peptides for 48 h; 3) with $10^7$ p.f.u./mL recombinant fowlpox vectors for 2 h. On day 6, 10 ng/mL tumor necrosis factor α (TNFα) (BioLegend, #570102) was added. On day 7, T cells were isolated from PBMCs from the same donors using plastic adherence to remove monocytes and magnetic anti-CD19 beads (Miltenyi, #130-050-301) to remove B cells. Purified T cells were incubated at a 100:1 ratio with the DC for 1 wk, then for another wk in the presence of X-VIVO 15 with 500 units/mL IL-2 (BioLegend, #589102). On day 14, T cells were plated with freshly isolated and matured DC and stimulated for 1 wk in the same conditions. On day 21, T cells were stimulated

in presence of monensin (eBioscience, #00-4505-51) with matured DC previously loaded with: 1) 10 μg/mL individual peptides or 2) 10 μg/mL of a mixture of different peptides. After 7 h, IFNγ production of these cells was analysed using a flow cytometer.

**Flow cytometric analysis for intracellular IFN-γ**

T cells were stained with the following monoclonal antibodies (mAb): PE/Cy5 conjugated anti-human CD3 (BioLegend, #300410) mAb, APC conjugated anti-human CD4 (BioLegend, #344613) and anti-human CD8 (BioLegend, #344721) mAb, washed and treated with a fixation/permeabilization kit (Life technologies, #GAS003). Following washes the cells were incubated with Pycoerythrin (PE) conjugated anti-human IFNγ (BioLegend, #506506) mAb. To discriminate between live and dead cells a LIVE/DEAD staining kit (Life technologies, #L-34975) was used. Data (300,000 events/sample) were collected using the FACScalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Treestar, Ashland, OR). In each experiment, the gate for background IFNγ-producing cells was set in no-peptide control samples at ≤ 0.02%. Events registering above this threshold value in peptide-stimulated samples were considered "positive" and indicated in data tables as "+" (> background); or "++" (two times > background); or "+++", (three times > background).

**HIvax vaccines engineering**

Epitope sequences were concatenated to form two multi-epitope genes: HIvax1, containing HLA Class I epitopes, and HIvax2, containing HLA Class II epitopes (Fig. 3A). All the peptides encoded by these two DNA vaccines were identified by immunoinformatics methods and their immunogenicity confirmed with the *in vitro* immunization experiments described above. To avoid creation of novel epitopes at epitope junctions, an algorithm which iteratively re-orders epitopes to reduce junctional immunogenicity (VaccineCAD), was used to optimize epitope order [21]. In addition, where re-ordering by VaccineCAD did not sufficiently reduce potential junctional immunogenicity, spacer sequences (Ala-Ala-Tyr for HIvax1, and Gly-Pro-Gly-Pro-Gly for HIvax2) were engineered between some epitopes to optimize antigen presentation. A Kozak sequence was positioned upstream of the coding sequence for efficient translation initiation. In HIvax1, to target the peptides to the MHC-I processing pathway, the immunoglobulin K (IgK) leader sequence (MGWSCIILFLVATATGVHS) was placed upstream of epitope sequences. In HIvax2, to target the immunogens to the MHC-II processing pathway, the tissue plasminogen activator (tPA) leader sequence (MQMSPALTCLVLGLALVFGEGSA) was placed upstream of epitope sequences to direct translation products to the secretory pathway. A V5 tag was incorporated downstream of both epitope sequences followed by two stop codons. Genes were synthesized by GeneScript (Piscataway, NJ) and cloned into a cassette coding for enhanced GFP (eGFP) in the fowlpox Transfer Plasmid Green (TPG) [39, 40]. Two recombinant fowlpox vectors, expressing the two transgenes, were then generated as described previously.[17]

## Immunofluorescence

Mature DC infected with FP-ctrl, HIvax1 or HIvax2 were cultured in culture slides (Falcon, #354104). Two days after infection, cells were fixed with methanol, blocked with 0.2% gelatin and incubated overnight at 4°C with 1:100 mouse anti-V5 antibodies (Santa Cruz Biotechnology, #sc-81594). Slides were then incubated for 2 h with 1:300 anti-mouse IgG conjugated with Alexa Fluor® 488 (BioLegend, #405319). Expression of V5 was evaluated with a Cameleon 3-Channel AOTF LSM confocal microscope (Nikon, Melville, NY) in cells counterstained with Propidium Iodide (PI).

## Reverse transcription PCR

Total RNA was extracted from DC infected with FP-ctrl, HIvax1 or HIvax2 using the RNeasy™ kit (Qiagen, #75162). RNA samples were treated with 0.05 unit/ml of DNase I (Qiagen, #79254) at 20 °C for 15 min. Total RNA (5 µg) was converted into first strand cDNA using random hexamers and High Capacity cDNA Synthesis kit (Life Technologies, #4368813). The expression of HIvax1 and HIvax2 transgenes was detected by PCR using the Taq polymerase kit (Qiagen, # 18038042) in a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer, Waltham, MA) followed by agarose gel electrophoresis. The sequences of the PCR primers were as follow: HIvax1 forward (5'-ATG GGG TGG TCC TGT ATT ATT CTG-3'), HIvax1 reverse (5'-GGC TTG CCC TTG TTG TTG GTT TCT-3'), HIvax2 forward (5'-CTG TGC GGA GCC GTG TTC GTC-3') and HIvax2 reverse (5'-CTC CAG GGC CAG GTC CAA TCT T-3'). As positive controls, $10^5$ p.f.u of HIvax FP vectors were added to the PCR reaction.

**HLA Typing**

cDNA was generated from RNA samples using Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies, # 18080051) with Oligo(dT)20 primer. HLA amplicon was created using cDNA amplified with KOD HotStart DNA polymerase (EMD, #71086) and fusion primers specially designed for 454 Sequencing analysis. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, #A63880) and quantified using Quant-IT PicoGreen dsAssay kit (Life Technologies, #P11496), followed by subsequent normalization and pooling as a preparation for emulsion-based clonal amplification PCR (emPCR). The emPCR allows for each strand of DNA produced in the HLA-specific PCR to be replicated clonally on a single bead using emPCR Reagents and Kit (Roche, Nutley, NJ). The emulsion mix was aliquoted to the wells of a 96-well PCR plate and placed in a Gene Amp PCR Sytem 9700 (Life Technologies) and a standard emPCR protocol was carried out over the course of 6 hours. Beads were then recovered, prepared and individually sequenced using 454 GS Junior sequencing system (Roche).

Sequencing reads were de-multiplexed by sample and trimmed from the adaptor, MID, and primer sequences using the Integroomer application (http://courge.ics.hawaii.edu/inte/groomer/). The resulting sequences were then subtyped through local alignments against the HLA database [41] using the USERCH program.[42]

## Granzyme B ELISPOT assay

To measure T cell cytotoxic ability, granzyme B secretion was analyzed using the Human Granzyme B ELISpot Kit (R&D system, #EL2906). $10^5$ T cells (effectors), stimulated with DC infected with $10^5$ p.f.u. of either FP-ctrl or a mixture of both HIvax vaccines, were added in wells followed by 5 X $10^3$ ADA cells or primary skin fibroblasts (targets) per well in 100 μL. Negative controls consisted of effector cells in the absence of target cells, target cells in the absence of effector cells and media only. After 4 h of incubation, detection of granzyme B spot was performed following the manufacturer directions. Dead T cells we removed with a dead cell removal kit (Miltenyi) before incubation with MM cells to enhance specificity. Survivin levels between fibroblast and MM cells were detected by western blot using an anti-survivin antibody (Santa Cruz Biotechnology, #sc-17779).

## Statistical analyses

For each blood donor, two experiments have been conducted with each condition analyzed in triplicate. All statistical tests were performed using GraphPad Prism (GraphPad, La Jolla, CA). Means of two groups were compared using two-tailed unpaired Student's t test. Statistical significance values are indicated in the figures.





## ACKNOWLEDGMENTS

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**Table 1. List of survivin peptides evaluated in DC-T cell co-cultures.**

| Name | Epitope | Sequence |
|---|---|---|
| Hsurv1 | Survivin class II (8-24) | acetyl-PAWQPFLKDHRISTFKN-amide |
| Hsurv2 | Survivin class II (83-97) | acetyl-GAAFLSVKKQFEELT-amide |
| Hsurv3 | Survivin class II (96-113) | acetyl-LTLGEFLKLDRERAKNKI-amide |
| Hsurv4 | Survivin class I (20-28) | STFKNWPFL-OH |
| Hsurv5 | Survivin class I (6-14) | LPPAWQPFL-OH |
| Hsurv6 | Survivin class I (47-55) | PTENEPDLA-OH |
| Hsurv7 | Survivin class I (112-120) | KIAKETNNK-OH |

## Table 2. T cell responses after co-culture with DC loaded with Hsurv peptides

| Donor no. | HLA A | HLA B | HLA DRB1 | $CD3^+/CD4^+$ response[A] | | | $CD3^+/CD8^+$ response[A] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Hsurv1 | Hsurv2 | Hsurv3 | Hsurv4 | Hsurv5 | Hsurv6 | Hsurv 7 |
| 1 | 02:01, 11:01 | 35:01, 13:02 | 01:01, 07:01 | + | ++ | + | - | ++ | - | - |
| 2 | 24:02, 03:01 | 07:21, 35:01 | 11:01, 15:01 | +++ | - | +++ | - | + | +++ | - |
| 3 | 02:01, 03:01 | 07:02, 35:01 | 11:03, 15:01 | - | +++ | - | - | + | - | + |
| 4 | 68:01, 26:01 | 35:01, 51:01 | 11:03, 16:01 | - | + | + | - | +++ | ++ | ++ |
| 5 | 01:01, 11:01 | 08:01, 42:01 | 03:01, - | ++ | ++ | + | - | + | + | - |
| 6 | 29:01, 29:02 | 35:01, 35:03 | 11:02, 15:03 | +++ | - | ++ | - | - | + | + |
| 7 | 02:01, 01:01 | 07:02, 08:01 | 03:01, 15:01 | - | ++ | - | - | - | +++ | +++ |
| 8 | 03:01, 30:01 | 13:02, 44:03 | 04:04, 15:03 | ++ | - | - | - | +++ | +++ | - |
| 9 | 11:01, 30:01 | 13:02, 35:01 | 04:05, 07:01 | + | - | - | - | ++ | + | ++ |
| 10 | 02:01, 33:03 | 44:03, 15:01 | 04:01, 07:01 | - | - | - | +++ | +++ | - | + |
| | | | | 60%* | 50%* | 50%* | 10%* | 80%* | 90%* | 70%* |

[A] Flow cytometry results comparing T cell reactivity with DC pulsed with stimulating peptide versus autologous not pulsed DC.

(-), ≤ background; (+), > background; (++), two times > background; (+++), three times > background.

* Percentage of experiments with positive results

**Table 3. T cell responses after co-culture with DC loaded with Hsurv peptides pools.**

| Donor no. | HLA A | HLA B | HLA DRB1 | $CD3^+/CD4^+$ response^ | | $CD3^+/CD8^+$ response^ | |
|---|---|---|---|---|---|---|---|
| | | | | Hsurv 1-7 | Hsurv 1,2,3,5,6,7 | Hsurv 1-7 | Hsurv 1,2,3,5,6,7 |
| 1 | 03:01, 30:01 | 13:02, 44:03 | 04:04, 15:03 | - | + | - | + |
| 2 | 11:01, 30:01 | 13:02, 35:01 | 04:05, 07:01 | + | +++ | + | ++ |
| 3 | 02:01, 33:03 | 44:03, 15:01 | 04:01, 07:01 | - | + | + | - |
| 4 | 02:01, 03:01 | 07:02, 35:01 | 11:03, 15:01 | + | - | - | + |
| | | | | 50%* | 75%* | 50%* | 75%* |

[A] Flow cytometry results comparing T cell reactivity with DC pulsed with stimulating peptide pools versus autologous not pulsed DC.

(-), ≤ background; (+), > background; (++), two times > background; (+++), three times > background.

* Percentage of experiments with positive results.

## FIGURES LEGENDS

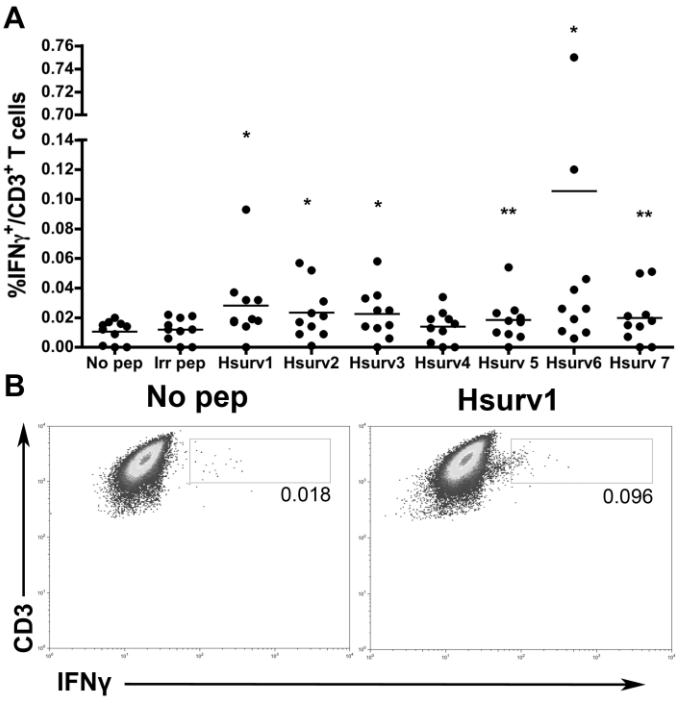


Fig 1.

**Fig. 1. Selected survivin peptides induce production of Interferon γ in T cells isolated from naïve healthy donors.** Blood monocyte-derived dendritic cells (DC), cultured from 10 healthy human donors were pulsed with survivin peptides (Hsurv 1-7) and used in co-culture experiments with autologous T cells. Intracellular IFN-γ in T cells was evaluated by flow cytometric analyses. (A) Percentages of IFN-γ expressing $CD3^+$ T cells induced by each survivin peptide. DC loaded with an irrelevant peptide or not pulsed with any peptide were used to stimulate T cells as controls. Data are presented as mean of IFN-$\gamma^+$ $CD3^+$ T cells ± S.E., with means from "Hsurv" conditions compared with control "No Pep" using a Student's t-test and significance at $*P < 0.05$, $**P < 0.1$. (B) Representative data from flow cytometric analysis of IFN-γ expressing $CD3^+$ T cells. T cells were pulsed with DC not loaded (No Pep) or stimulated with Hsurv6 peptide.

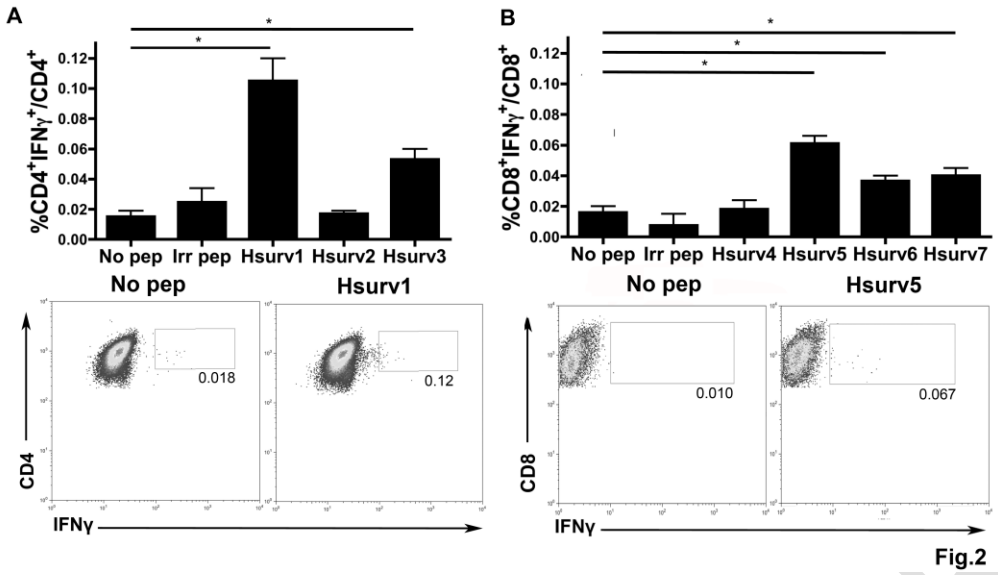


**Fig. 2. Autologous dendritic cells loaded with survivin peptides induce activation of CD4$^+$ and CD8$^+$ T cells.** Blood monocyte-derived dendritic cells (DC) were pulsed with survivin peptides and used in co-culture experiments with autologous T cells. Intracellular IFN-γ in CD4$^+$ and CD8$^+$ T cells was evaluated by flow cytometric analyses. (A) Top. Percentages of IFN-γ expressing CD4$^+$ T cells induced by survivin peptides designed for MHC-II (Hsurv 1-3). Donor haplotype: HLA DRB1 11:02, 15:03. DC loaded with an irrelevant peptide or not pulsed with any peptide were used to stimulate T cells as controls. Data are presented as mean of IFN-γ$^+$ CD4$^+$ T cells ± S.E., with means compared using a Student's t-test and significance at *$P$ < 0.05; Bottom. Representative data from flow cytometric analysis of IFN-γ expressing CD4$^+$ T cells. T cells were pulsed with DC not loaded (No Pep) or stimulated with Hsurv2 peptide. (B) Top. Percentages of IFN-γ expressing CD8$^+$ T cells induced by survivin peptides designed for MHC-I (Hsurv 4-7). Donor haplotype: HLA A 68:01, 26:01; HLA B 35:01, 51:01. Data are presented as mean of IFN-γ$^+$ CD8$^+$ T cells ± S.E., with means compared using a Student's t-test and significance at *$P$ < 0.05; Bottom. Representative data from flow cytometric analysis of

IFN-γ expressing $CD8^+$ T cells. T cells were pulsed with DC not loaded (No Pep) or stimulated with Hsurv5 peptide.

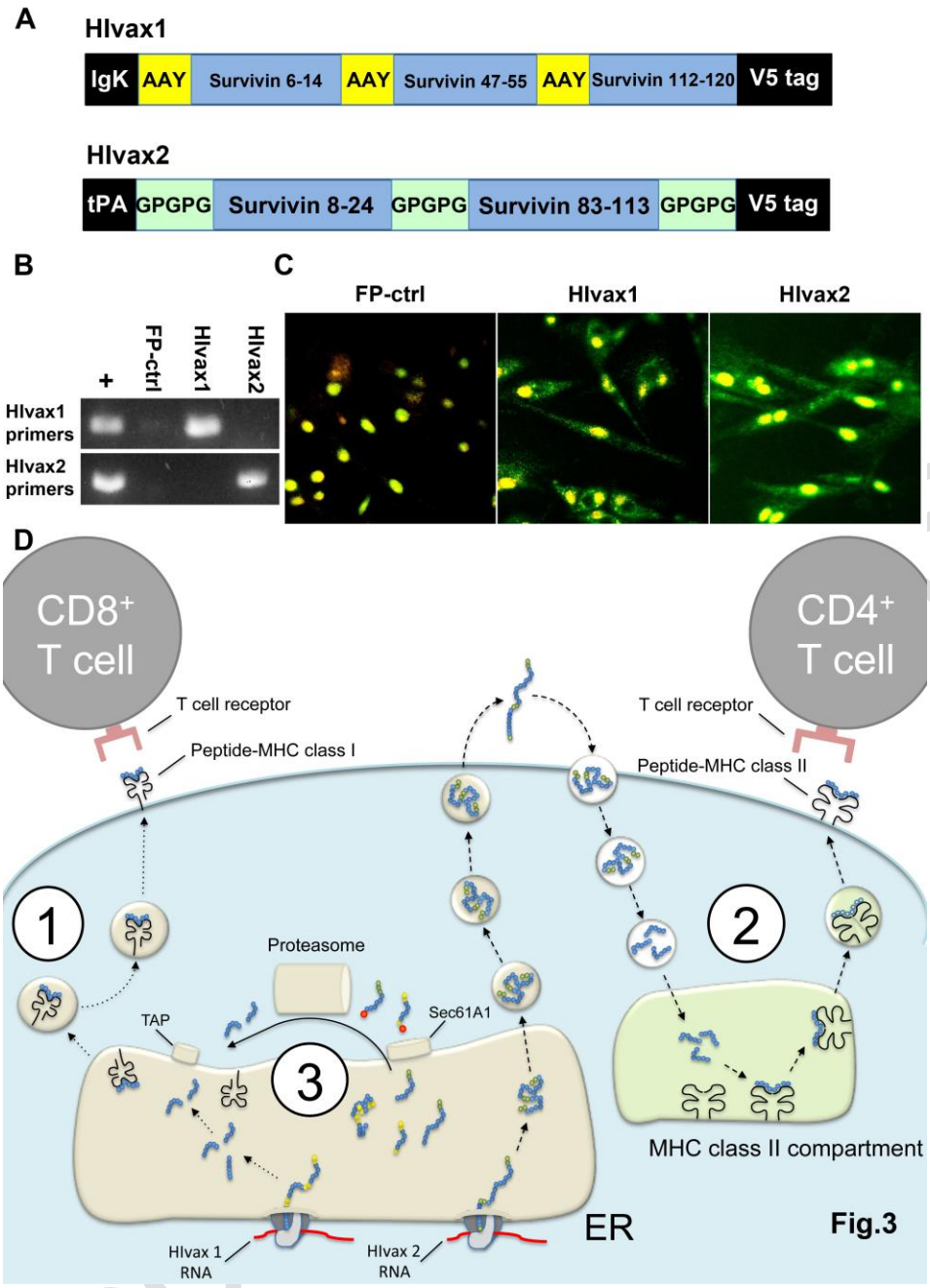


Fig.3

**Fig. 3. Survivin epitopes have been included in HIvax1 e HIvax2.** (A) HIvax 1 and 2 diagrams. Epitopes from Hsurv 5-7 were included in HIvax1, separated by AAY spacers and targeted to the ER by including an IgK signal sequence. This strategy facilitates the correct presentation of these peptides on MHC-I. For HIvax2, epitopes from Hsurv 1-3 were separated by GPGPG spacers and fused with a plasminogen activator signal

sequence (tPA) to facilitate presentation on MHC-II. (B) Detection of specific RNAs produced by DC infected with HIvax. RT-PCR was performed on RNA extracted from DC infected with HIvax1 or HIvax2, using primers specific for HIvax1 or HIvax2 transgenes. Positive control: DNA from HIvax vectors. Negative control: cDNA made from RNA from FP-ctrl infected DC. (C) Immunofluorescence staining to detect V5 in DC infected with FP-ctrl or HIvax. Fluorochrome-conjugated antibodies were used to detect V5 and the cell nuclei counterstained with propidium iodide. (D) Peptides produced by HIvax1 and HIvax2 are differentially processed and loaded into MHCs: (1) Antigens produced from HIvax1 RNA are processed by ER-resident peptidases and peptides mounted on MHC-I. These molecules are then transported to the plasma membrane for presentation to $CD8^+$ T cells; (2) Polypeptide encoded by the HIvax2 transgene is secreted, re-engulfed and degraded in lysosomes. Resultant peptides are mounted on MHC-II and transported to the plasma membrane for presentation to $CD4^+$ T cells; (3) Prematurely truncated or abnormally folded proteins produced by both HIvax1 and HIvax2 are exported to the cytosol, ubiquitinated and processed by the proteasome. Resultant peptides are transported back in the ER and loaded on MHC of class I. Peptides resulting from proteasome cleavage may be also mounted on MHC of class II with a mechanism that does not require TAP or ER transportation.

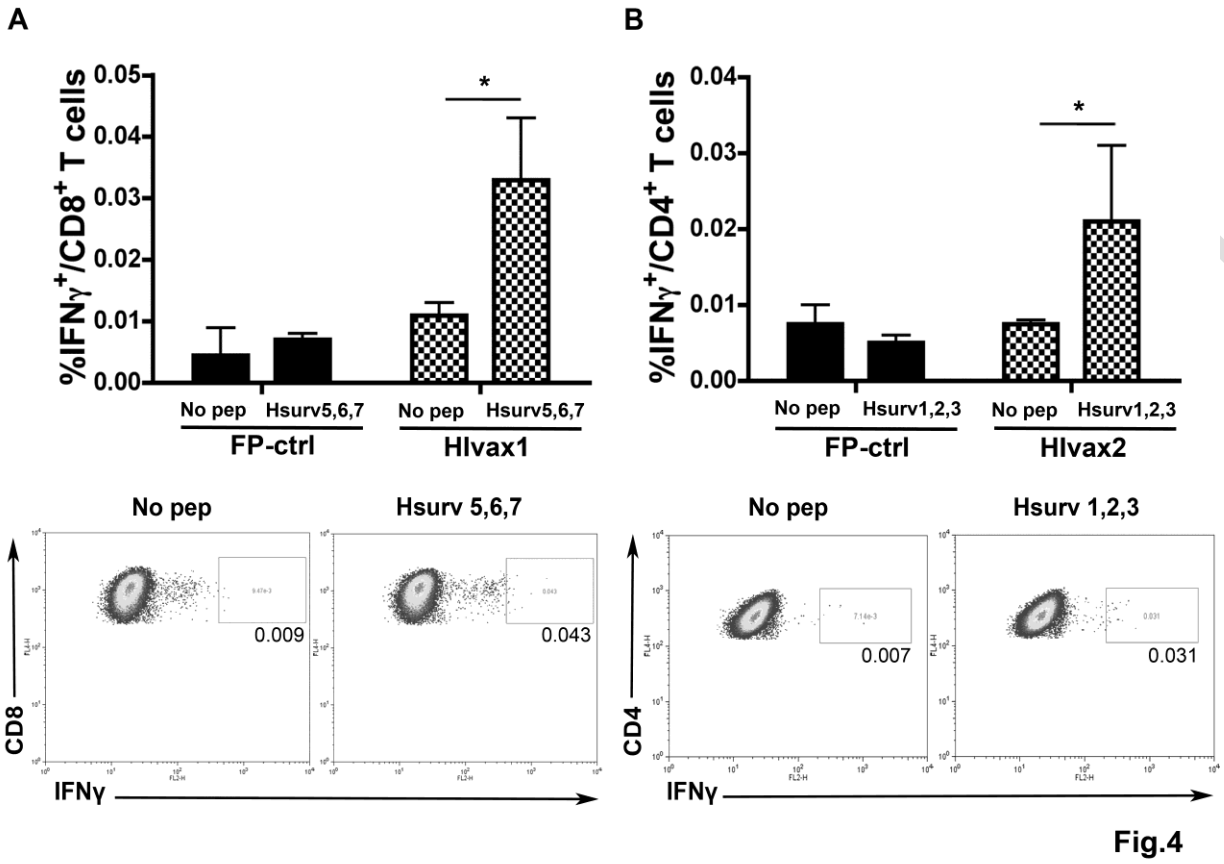


Fig.4

**Fig. 4. HIvax1 and HIvax2 induce activation of CD8$^+$ and CD4$^+$ T cells, respectively, in a donor with haplotype HLA-A 01:01, 11:01; HLA-B 08:01, 42:01; HLA-DRB1 03:01.** Human dendritic cells (DC) were infected with HIvax1 or HIvax2 and co-cultured for 2 wks with autologous T cells. T cells were then pulsed with new DC, not loaded or loaded with pool of survivin peptides and evaluated for intracellular IFN-γ by flow cytometric analyses. (A) Top. Percentages of IFN-γ expressing CD8$^+$ T cells induced by DC infected with Fp-ctrl or HIvax1. DC not loaded or loaded with the pool of peptides expressed by HIvax1, were used to activate T cells before the analyses. Data are presented as mean of IFN-γ$^+$ CD8$^+$ T cells ± S.E., with means compared using a Student's t-test and significance at *$P$ < 0.05; Bottom. Representative data from flow

cytometric analysis of IFN-γ expressing $CD8^+$ T cells generated with HIvax1. T cells were pulsed with DC not loaded or loaded with the pool Hsurv 5,6,7. (B) Top. Percentages of IFN-γ expressing $CD4^+$ T cells induced with Fp-ctrl or HIvax2. DC not loaded or loaded with the pool of peptides expressed by HIvax2, were used to pulse T cells before the cytometric analyses. Data are presented as mean of IFN-$\gamma^+$ $CD4^+$ T cells ± S.E., with means compared using a Student's t-test and significance at $*P < 0.05$; Bottom. Representative data from flow cytometric analysis of IFN-γ expressing $CD4^+$ T cells generated with HIvax2. T cells were pulsed with DC not loaded or loaded with the pool Hsurv 1,2,3.

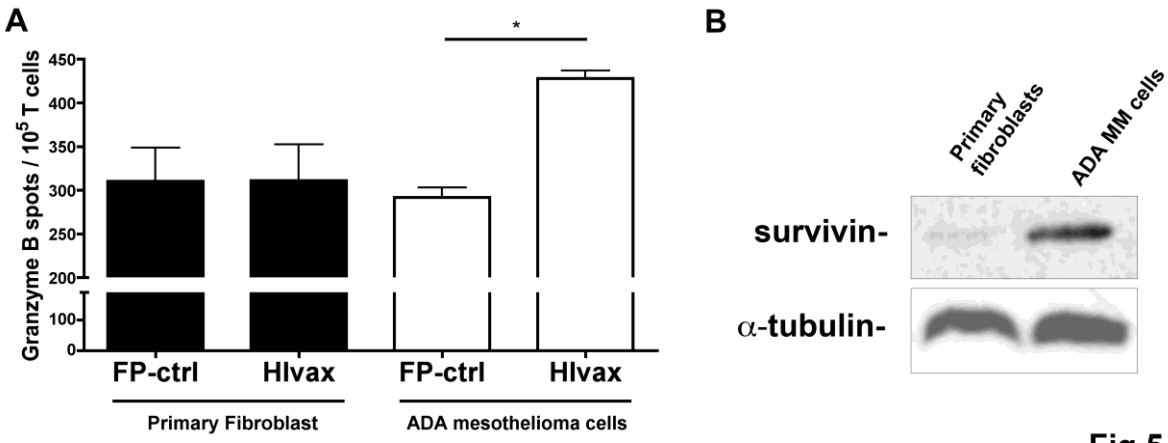


Fig.5

**Fig. 5. Cytolytic potential of HIvax-activated T cells against malignant mesothelioma cells overexpressing survivin.** (A) ELISPOT analysis of released granzyme B was performed after stimulation by DC infected with FP-ctrl or HIvax1/HIvax2 simultaneously (HIvax). T cells were co-cultured for 4 h with ADA human malignant mesothelioma (MM) cells or primary fibroblasts. Results shown are numbers of spots per $10^4$ T cells. Bars correspond to the mean ± S.E. of results obtained from 2 individual experiments, with means compared using a Student's t-test and significance at $*P < 0.05$. (B) Western blot analysis of lysates from primary fibroblasts and ADA MM cells to detect Survivin. Equal loading was compared using anti-αtubulin antibodies.