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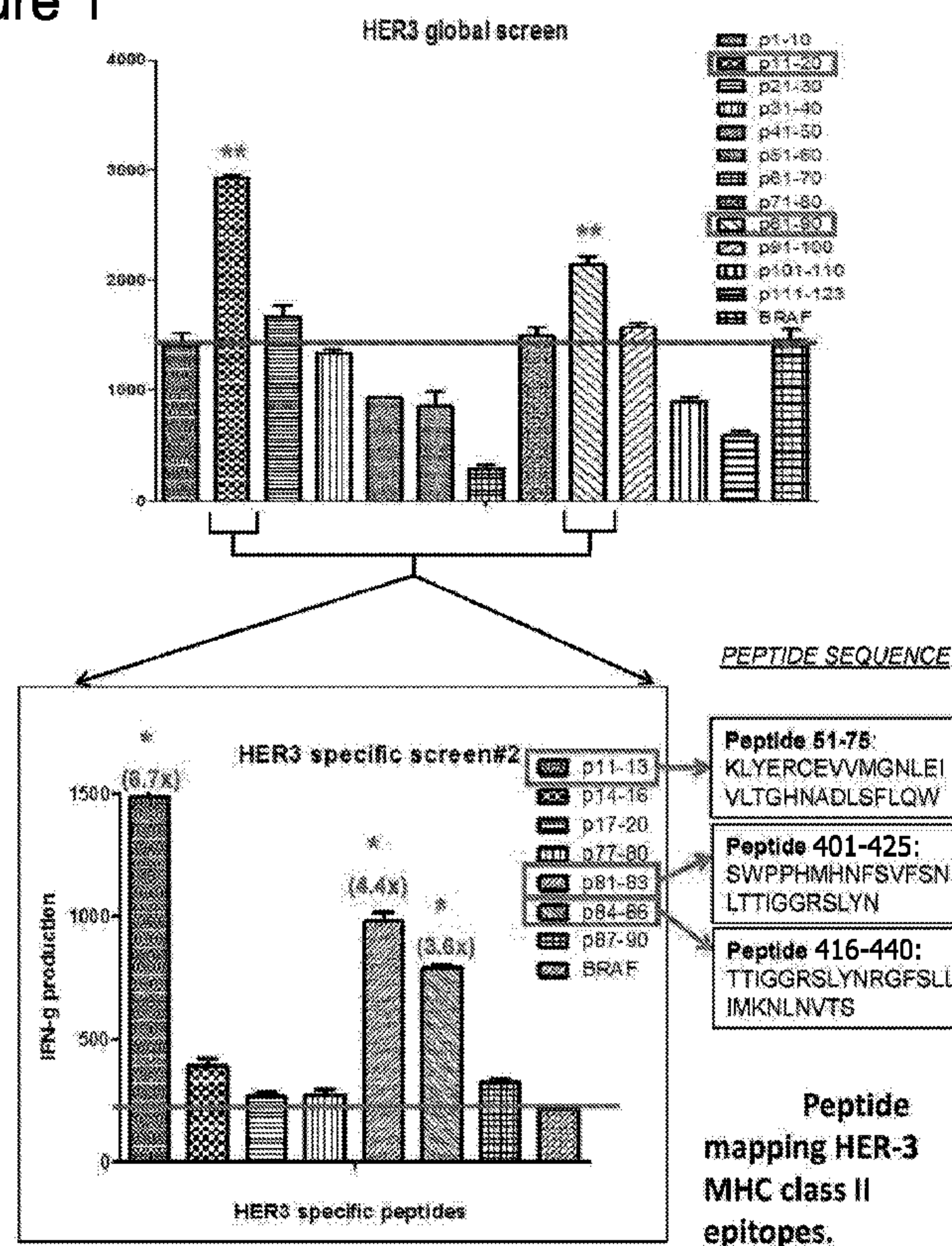
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(54) **Titre : IDENTIFICATION DE PEPTIDES IMMUNOGENES DE CLASSE II DU COMPLEXE MAJEUR D'HISTOCOMPATIBILITE POUR UNE IMMUNOTHERAPIE**
 (54) **Title: IDENTIFICATION OF IMMUNOGENIC MHC CLASS II PEPTIDES FOR IMMUNE-BASED THERAPY**

Figure 1



(57) **Abrégé/Abstract:**

The invention provides compositions, methods, and vaccines that may stimulate the immune system and that may be used for treating malignancies associated with overexpression of the HER-3 protein. Such compositions include epitopes of the HER-3 protein.

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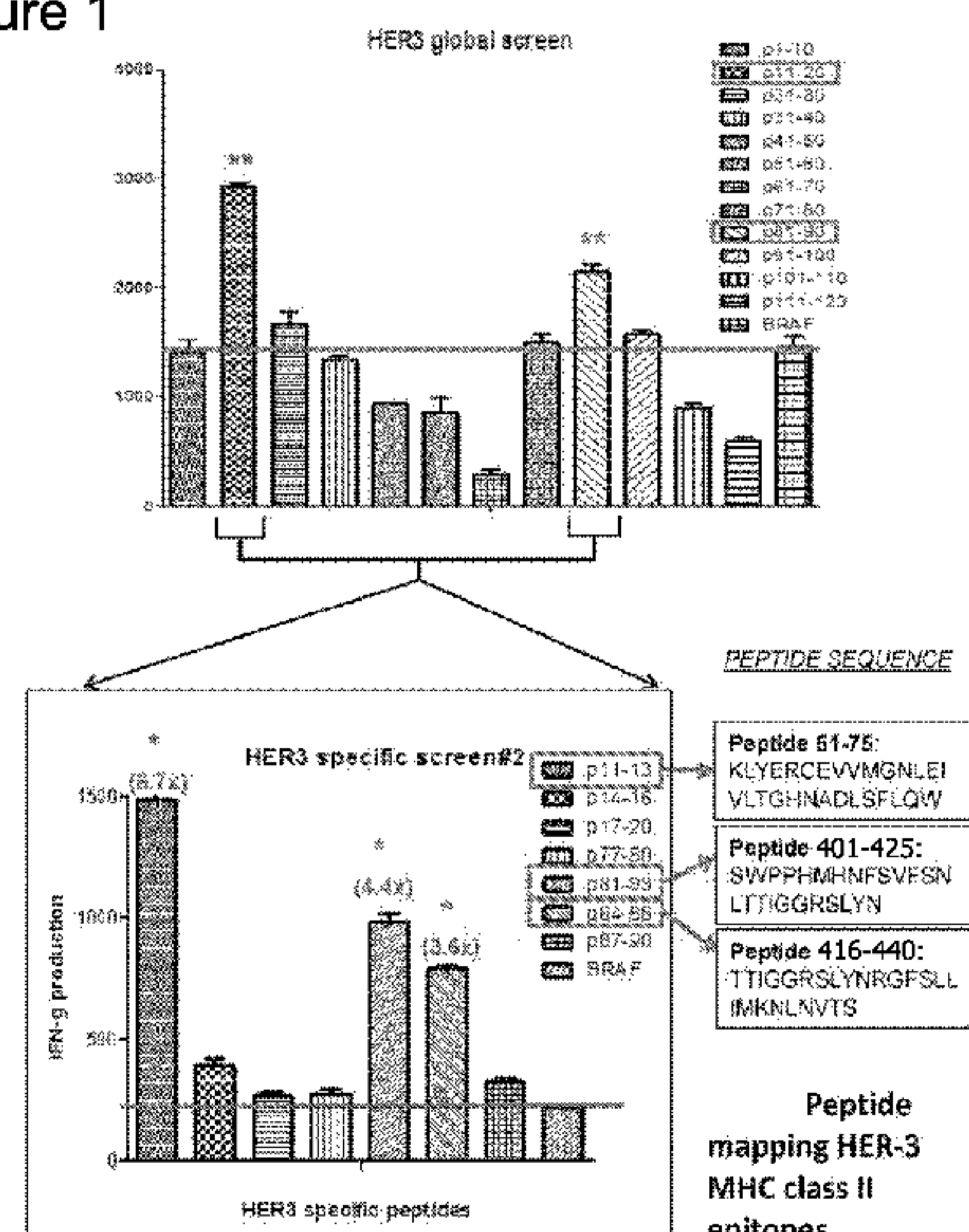
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(54) Title: IDENTIFICATION OF IMMUNOGENIC MHC CLASS II PEPTIDES FOR IMMUNE-BASED THERAPY

Figure 1



(57) Abstract: The invention provides compositions, methods, and vaccines that may stimulate the immune system and that may be used for treating malignancies associated with overexpression of the HER-3 protein. Such compositions include epitopes of the HER-3 protein.

IDENTIFICATION OF IMMUNOGENIC MHC CLASS II PEPTIDES FOR IMMUNE-BASED THERAPY

CROSS-REFERENCE TO RELATED APPLICATION

5 This application is a continuation-in-part application of Serial No. PCT/US15/41034 filed July 17, 2015 which in turn claims priority and benefit from U.S. Provisional Application Serial No. 62/076,789, filed November 7, 2014, and U.S. Provisional Application Serial No. 62/025,681, filed July 17, 2014, the contents of each of which are incorporated by reference herein in their entireties.

10

BACKGROUND

In 25-30% of breast cancers, amplification and overexpression of the growth factor receptor gene HER2 (human epidermal growth factor receptor-2, also known as neu/erbB2) is associated with enhanced tumor aggressiveness and a high risk of relapse and death (Slamon, D., et al., 1987, Science 235:177; Yarden, Y., 2001, Oncology 1:1). This oncogene encodes a 185 kilodalton (kDa) transmembrane receptor tyrosine kinase. As one of the four members of the human epidermal growth factor receptor (EGFR) family, HER2 distinguishes itself in several ways. First, HER2 is an orphan receptor. No high-affinity ligand has been identified. Second, HER2 is a preferred partner for other EGFR family members (HER1/EGFR, HER3, and HER4) for the formation of heterodimers, which show high ligand affinity and superior signaling activity. Third, full-length HER2 undergoes proteolytic cleavage, releasing a soluble extracellular domain (ECD). Shedding of the ECD has been shown to represent an alternative activation mechanism of full-length HER2 both in vitro and in vivo, as it leaves a membrane-anchored fragment with kinase activity. The central role of HER2 in EGFR family signaling correlates with its involvement in the oncogenesis of several types of cancers, such as breast, ovarian, colon, and gastric cancers, regardless of its expression level (Slamon, D., et al., 1989, Science 244:707; Hynes, N., et al., 1994, Biochem. Biophys. Acta, 1198:165). HER2 may also render tumor cells resistant to certain chemotherapeutics (Pegram, M., et al., 1997, Oncogene 15:537). Given its vital role in tumorigenesis, HER2 is an important target for cancer therapeutics.

The human EGF receptor (HER) family of receptor tyrosine kinases regulates a large variety of biological processes including cell proliferation, - migration, -invasion and -survival. The family consists of four members: EGFR (HER1), HER2 (neu or ErbB2), HER3 (ErbB3) and HER4 (ErbB4). To date, eleven
5 ligands have been reported including epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor .alpha. (TGF α), amphiregulin (AR), epiregulin, betacellulin and the heregulins. These ligands bind directly to their cognate receptors, which leads to the formation of receptor homo- or heterodimers that trigger the activation of multiple signaling pathways. Dysregulation
10 of members of the HER-family either by activating mutations, receptor over expression or aberrant ligand release leads to the development of a variety of human tumors. HER3 is over expressed in breast-, ovarian- and lung cancer and this genetic feature has been correlated with poor prognosis. Upon activation by heregulins, HER3 dimerizes with HER2 and EGFR to form potent oncogenic receptor heterodimers.
15 Within this complex, HER3 preferentially recruits PI3 kinase to its cytoplasmic docking sites thereby regulating cell proliferation and -survival. So far it was assumed that HER3 is kinase-inactive due to apparently aberrant sequence characteristics in its kinase domain and that it requires heterodimerization with a kinase-intact member of the HER-family in order to initiate signaling events. Consistent with this, it was
20 shown that HER2 requires HER3 to drive breast tumor cell proliferation. However, recent findings of showed that HER3 is able to phosphorylate Pyk2 which results in the activation of the MAPK pathway in human glioma cells. Furthermore, monoclonal antibodies specific for HER3 can inhibit the proliferation and migration of cancer cell lines. Interestingly, it was shown recently that cancer cells escape HER-family
25 inhibitor therapy by up-regulation of HER3 signaling and that HER3 inhibition abrogates HER2-driven tamoxifen resistance in breast cancer cells. Moreover, resistance to Gefitinib (Iressa) therapy, an EGFR small molecule inhibitor, was shown to be connected to HER3 signal activation.

HER3 is a receptor protein that plays an important role in regulating
30 normal cell growth. HER3 lacks an intrinsic kinase activity and relies on the presence of HER2 to transduce signals across the cell membrane. As initially transcribed, the pre-mRNA for HER3 contains 28 exons and 27 introns. The fully spliced HER3 mRNA from which the introns have been spliced out is composed of 28 exons.

Targeted therapy has emerged as the cornerstone of cancer therapeutics in the last decade. Members of the EGF receptor family - namely EGFR (or HER1) and ErbB2 (or HER2/*neu*) - have evolved as particularly attractive targets, since these receptor tyrosine kinases (RTK) are deregulated in a multitude of cancers. The oncogenic functions of another member of the EGF receptor family - ErbB3 or HER3- have only been recently scrutinized due its major role in mediating resistance to HER2 and PI3K pathway-directed therapies. Activating mutations in and/or overexpression of HER3 has been identified in a number of different tumor types, including breast, gastric, colon, bladder cancer, and melanoma, and portend a worse overall prognosis in these tumors.

Despite advances in the field, it is still uncertain whether effective immune responses can be generated in humans using cell- or protein-based vaccine strategies targeting HER-3. Accordingly, there is a need in the art to have additional immunotherapeutic approaches for treating or preventing breast cancer and other malignancies with which overexpression of the HER-3 protein is associated. The present invention fulfills this need.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of preferred embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figure 1 shows immunogenic peptides from HER-3 that exhibit the ability to activate CD4 T cells across many patients (SEQ ID NOS 1-3, respectively, in order of appearance).

Figure 2 shows a HER3 global screen with groups of 10 peptide fragments. Figure 2 also shows HER3 screen with single peptides (SEQ ID NOS 4-7, respectively, in order of appearance).

Figure 3 shows a HER3 global screen with groups of 10 peptide fragments. Figure 3 also shows HER3 screen with single peptides (SEQ ID NOS 4 and 7, respectively, in order of appearance).

Figure 4 shows a HER3 global screen with groups of 10 peptide fragments. Figure 4 also shows HER3 screen with single peptides (SEQ ID NOS 1-3, respectively, in order of appearance).

Figure 5 shows IFN- γ production from different HER3 peptides.

5 Figure 6 shows IFN- γ production from different HER3 peptides.

Figure 7 shows IFN- γ production from a "REVERSE" screen, starting with previously identified peptides, sensitizing to peptides and HER3 extracellular domain.

10 Figure 8 shows IFN- γ production from a "REVERSE" screen, starting with previously identified peptides, sensitizing to peptides and HER3 extracellular domain.

Figure 9 shows IFN- γ production from a "REVERSE" screen in a patient not previously sensitized with HER extracellular domain, starting with peptides, sensitizing to peptides and HER3 extracellular domain.

15 Figure 10 shows IFN- γ production from a "REVERSE" screen in a patient not previously sensitized with HER extracellular domain, starting with whole peptide library, sensitizing to peptides and HER3 extracellular domain.

20 Figure 11 shows IFN- γ production from a "REVERSE" screen in a patient not previously sensitized with HER extracellular domain, starting with whole peptide library, sensitizing to peptides and HER3 extracellular domain.

Figure 12 shows IFN- γ production from a "REVERSE" screen in a patient not previously sensitized with HER extracellular domain, starting with peptides, sensitizing to peptides and HER3 extracellular domain.

25 Figure 13 shows a sequential peptide screen in donor # UPCC 15107-24.

Figure 14 shows a sequential peptide screen in donor # UPCC 15107-38.

Figure 15 shows "REVERSE" sensitization in donor # UPCC 15107-38 and UPCC 15107-24.

30 Figure 16 shows that immunogenic HER3 epitope-pulsed DC1 sensitized CD4⁺ Th1 and overcame anti-HER3 immune tolerance in donor # UPCC 15107-30 and UPCC 15107-32 (both patients with known anti-HER3 non-reactivity to identified HER3 peptides and/or native HER3 ECD).

Figure 17 shows immunogenic CD4+ HER3 epitopes demonstrate MHC class II promiscuity.

Figure 18 shows that when activated HER-3 CD4+ cells are placed next to HER-3 expressing cells in a chamber, the HER-3 CD4+ cells cause apoptosis or death of HER-3 expressing cells breast cancer cells.

Figure 19 shows methods for identification of immunogenic Class II-promiscuous HER3 CD4+ peptides using the ECD of HER3 as a tumor antigen in order to generate anti-HER3 Th1 cellular immunity.

Figure 20 shows confirmation of immunogenicity of identified CD4+ HER3 ECD epitopes by "reverse" sensitization. A HER3 ECD screen was performed with single peptides shown.

Figure 21 shows additional results of confirmation of immunogenicity of identified CD4+ HER3 ECD epitopes by "reverse" sensitization.

Figure 22 shows photographs of immunohistochemistry scoring of HER staining.

Figures 23A and 23B are histograms showing rate of HER family overexpression in Barrett's esophagus with low-grade dysplasia (LGD) or high-grade dysplasia (HGD) (Figure 22A) , and high-grade dysplastic Barrett's lesions with (HGD with carcinoma) or without associated invasive cancer (HGD) (Figure 22B).

20

DETAILED DESCRIPTION

The present invention provides isolated peptides of the HER family of proteins as well as other receptor tyrosine kinases. In one embodiment, the invention provides isolated peptides of one or more of HER-1, HER-3, and c-MET protein. In one embodiment, a peptide of the invention represents an epitope of HER-1. In one embodiment, the peptide of the invention represents an epitope of HER-3. In one embodiment, the peptide of the invention represents an epitope of c-MET.

In some embodiments, the epitope of the corresponding HER family of proteins as well as other receptor tyrosine kinases is immunogenic. The present invention additionally provides compositions that include one or more peptides of the invention. In one embodiment, the invention provides a chimeric peptide, wherein the chimeric peptide comprises one or more peptides of the invention.

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In one embodiment, the invention includes a composition comprising a multivalent peptide. The multivalent peptide includes two or more of the peptides of the invention.

5 Methods of stimulating an immune response and methods of treating cancer in a subject are additionally provided. Vaccines are also provided for therapeutic and prophylactic use. The peptides of the invention, either alone or in the context of chimeric peptides, as described herein, are capable of invoking an immune response. In one embodiment, the immune response is a humoral response. In another embodiment, the immune response is a cell-mediated response. According to some 10 embodiments, the peptides of the invention confer a protective effect.

In another embodiment HER3 expression can be used as a marker of tumor progression in premalignant lesions of the gastroesophageal junction.

Definitions

15 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

20 Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry, and nucleic acid chemistry and hybridization are those well-known and commonly employed in the art.

Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally performed according to conventional 25 methods in the art and various general references (e.g., Sambrook and Russell, 2012, Molecular Cloning, A Laboratory Approach, Cold Spring Harbor Press, Cold Spring Harbor, NY, and Ausubel et al., 2012, Current Protocols in Molecular Biology, John Wiley & Sons, NY), which are provided throughout this document.

30 The nomenclature used herein and the laboratory procedures used in analytical chemistry and organic syntheses described below are those well-known and commonly employed in the art. Standard techniques or modifications thereof are used for chemical syntheses and chemical analyses.

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

5 "About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of $\pm 20\%$, or $\pm 10\%$, or $\pm 5\%$, or $\pm 1\%$, or $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

10 The term "abnormal" when used in the context of organisms, tissues, cells or components thereof, refers to those organisms, tissues, cells or components thereof that differ in at least one observable or detectable characteristic (*e.g.*, age, treatment, time of day, etc.) from those organisms, tissues, cells or components thereof that display the "normal" (expected) respective characteristic. Characteristics which are normal or expected for one cell or tissue type, might be abnormal for a different cell or tissue type.

15 "Adjuvant therapy" for breast cancer as used herein refers to any treatment given after primary therapy (*i.e.*, surgery) to increase the chance of long-term survival. "Neoadjuvant therapy" is treatment given before primary therapy.

20 The term "antigen" or "ag" as used herein is defined as a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a
25 protein that elicits an immune response therefore encodes an "antigen" as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene. It is readily apparent that the present invention includes, but is not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are
30 arranged in various combinations to elicit the desired immune response. Moreover, a skilled artisan will understand that an antigen need not be encoded by a "gene" at all. It is readily apparent that an antigen can be generated synthesized or can be derived from a biological sample. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a biological fluid.

“An antigen presenting cell” (APC) is a cell that are capable of activating T cells, and includes, but is not limited to, monocytes/macrophages, B cells and dendritic cells (DCs).

“Antigen-loaded APC” or an “antigen-pulsed APC” includes an APC, which has been exposed to an antigen and activated by the antigen. For example, an APC may become Ag-loaded *in vitro*, e.g., during culture in the presence of an antigen. The APC may also be loaded *in vivo* by exposure to an antigen. An “antigen-loaded APC” is traditionally prepared in one of two ways: (1) small peptide fragments, known as antigenic peptides, are “pulsed” directly onto the outside of the APCs; or (2) the APC is incubated with whole proteins or protein particles which are then ingested by the APC. These proteins are digested into small peptide fragments by the APC and are eventually transported to and presented on the APC surface. In addition, the antigen-loaded APC can also be generated by introducing a polynucleotide encoding an antigen into the cell.

The term “anti-tumor effect” as used herein, refers to a biological effect which can be manifested by a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in the number of metastases, an increase in life expectancy, or amelioration of various physiological symptoms associated with the cancerous condition. An “anti-tumor effect” can also be manifested by the ability of the peptides, polynucleotides, cells and antibodies of the invention in prevention of the occurrence of tumor in the first place.

The term “autoimmune disease” as used herein is defined as a disorder that results from an autoimmune response. An autoimmune disease is the result of an inappropriate and excessive response to a self-antigen. Examples of autoimmune diseases include but are not limited to, Addison’s disease, alopecia areata, ankylosing spondylitis, autoimmune hepatitis, autoimmune parotitis, Crohn’s disease, diabetes (Type I), dystrophic epidermolysis bullosa, epididymitis, glomerulonephritis, Graves’ disease, Guillain-Barr syndrome, Hashimoto’s disease, hemolytic anemia, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis, pemphigus vulgaris, psoriasis, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren’s syndrome, spondyloarthropathies, thyroiditis, vasculitis, vitiligo, myxedema, pernicious anemia, ulcerative colitis, among others.

As used herein, the term "autologous" is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

5 The term "B cell" as used herein is defined as a cell derived from the bone marrow and/or spleen. B cells can develop into plasma cells which produce antibodies.

The term "cancer" as used herein is defined as a hyperproliferation of cells whose unique trait--loss of normal control--results in unregulated growth, lack of differentiation, local tissue invasion, and/or metastasis. Examples include but are not
10 limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer, germ-cell tumors, and the like.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's
15 health continues to deteriorate.

A "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

20 A disease or disorder is "alleviated" if the severity or frequency of at least one sign or symptom of the disease or disorder experienced by a patient is reduced.

"Effective amount" or "therapeutically effective amount" are used interchangeably herein, and refer to an amount of a compound, formulation, material,
25 or composition, as described herein effective to achieve a particular biological result. Such results may include, but are not limited to, the inhibition of virus infection as determined by any means suitable in the art.

As used herein "endogenous" refers to any material from or produced inside an organism, cell, tissue or system.

30 As used herein, the term "exogenous" refers to any material introduced from or produced outside an organism, cell, tissue or system.

A "HER receptor" is a receptor protein tyrosine kinase which belongs to the HER receptor family and includes EGFR (ErbB1, HER1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) receptors. The HER receptor will generally

comprise an extracellular domain, which may bind an HER ligand and/or dimerize with another HER receptor molecule; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The HER receptor may be a “native sequence” HER receptor or an “amino acid sequence variant” thereof. Preferably the HER receptor is a native sequence human HER receptor.

The “HER pathway” refers to the signaling network mediated by the HER receptor family.

“HER activation” refers to activation, or phosphorylation, of any one or more HER receptors. Generally, HER activation results in signal transduction (e.g. that caused by an intracellular kinase domain of a HER receptor phosphorylating tyrosine residues in the HER receptor or a substrate polypeptide). HER activation may be mediated by HER ligand binding to a HER dimer comprising the HER receptor of interest. HER ligand binding to a HER dimer may activate a kinase domain of one or more of the HER receptors in the dimer and thereby results in phosphorylation of tyrosine residues in one or more of the HER receptors and/or phosphorylation of tyrosine residues in additional substrate polypeptides(s), such as Akt or MAPK intracellular kinases.

“HER3” and “ErbB3” refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. PNAS (USA) 86:9193-9197 (1989).

“HER3 extracellular domain” or “HER3ECD” refers to a domain of HER3 that is outside of a cell, either anchored to a cell membrane, or in circulation, including fragments thereof. In one embodiment, the extracellular domain of HER3 may comprise four domains: Domain I, Domain II, Domain III, and Domain IV. In one embodiment, the HER3ECD comprises amino acids 1-636 (numbering including signal peptide). In one embodiment, HER3 domain III comprises amino acids 328-532 (numbering including signal peptide).

“Homologous” as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by

adenine, then they are completely or 100% homologous at that position. The percent homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% identical, if 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 5'ATTGCC3' and 5'TATGGC3' share 50% homology.

In addition, when the terms "homology" or "identity" are used herein to refer to the nucleic acids and proteins, it should be construed to be applied to homology or identity at both the nucleic acid and the amino acid sequence levels.

The term "hyperproliferative disease" is defined as a disease that results from a hyperproliferation of cells. Exemplary hyperproliferative diseases include, but are not limited to, cancer or autoimmune diseases. Other hyperproliferative diseases may include vascular occlusion, restenosis, atherosclerosis, or inflammatory bowel disease, for example.

As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the compositions and methods of the invention. The instructional material of the kit of the invention may, for example, be affixed to a container which contains the nucleic acid, peptide, and/or composition of the invention or be shipped together with a container which contains the nucleic acid, peptide, and/or composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the compound be used cooperatively by the recipient.

"Immune response" as used herein means the activation of a host's immune system, e.g., that of a mammal, in response to the introduction of antigen. The immune response can be in the form of a cellular or humoral response, or both.

"Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

By the term "modulating," as used herein, is meant mediating a detectable increase or decrease in the level of a response in a subject compared with the level of a response in the subject in the absence of a treatment or compound, and/or compared with the level of a response in an otherwise identical but untreated
5 subject. The term encompasses perturbing and/or affecting a native signal or response thereby mediating a beneficial therapeutic response in a subject, preferably, a human.

A "peptide," "protein," or "polypeptide" as used herein can mean a linked sequence of amino acids and can be natural, synthetic, or a modification or combination of natural and synthetic.

10 As used herein, a "population" includes reference to an isolated culture comprising a homogenous, a substantially homogenous, or a heterogeneous culture of cells. Generally, a "population" may also be regarded as an "isolated" culture of cells.

As used herein, a "recombinant cell" is a host cell that comprises a recombinant polynucleotide.

15 "Sample" or "biological sample" as used herein means a biological material from a subject, including but is not limited to organ, tissue, exosome, blood, plasma, saliva, urine and other body fluid. A sample can be any source of material obtained from a subject.

"Signal 1" as used herein generally refers to the first biochemical
20 signal passed from an activated DC to a T cell. Signal 1 is provided by an antigen expressed at the surface of the DC and is sensed by the T cell through the T cell receptor.

"Signal 2" as used herein generally refers to the second signal provided by DCs to T cells. Signal 2 is provided by "costimulatory" molecules on the activated
25 DC, usually CD80 and/or CD86 (although there are other co-stimulatory molecules known), and is sensed by the T cell through the surface receptor CD28.

"Signal 3" as used herein generally refers to the signal generated from soluble proteins (usually cytokines) produced by the activated DC. These are sensed through receptors on the T lymphocyte. The 3rd signal instructs the T cell as to which
30 phenotypical or functional features they should acquire to best deal with the current threat.

By the term "specifically binds," as used herein, is meant a molecule, such as an antibody, which recognizes and binds to another molecule or feature, but does not substantially recognize or bind other molecules or features in a sample.

The terms "subject," "patient," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether *in vitro* or *in situ*, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

5 The term "T cell" as used herein is defined as a thymus-derived cell that participates in a variety of cell-mediated immune reactions.

The term "T-helper" as used herein with reference to cells indicates a sub-group of lymphocytes (a type of white blood cell or leukocyte) including different cell types identifiable by a skilled person. In particular, T-helper cell according to the present disclosure include effector Th cells (such as Th1, Th2 and Th17). These Th
10 cells secrete cytokines, proteins or peptides that stimulate or interact with other leukocytes.

"Th1 T cell" as used herein refers to a T cell that produces high levels of the cytokine IFN- γ and is thought to be highly effective against certain disease-
15 causing microbes that live inside host cells, and cancer as well.

"Th17 T cell" as used herein refers to a T cell that produces high levels of the cytokines IL-17 and IL-22 and is thought to be highly effective against disease-causing microbes that live on mucosal surfaces.

"Therapeutically effective amount" is an amount of a compound of the invention, that when administered to a patient, ameliorates a symptom of the disease.
20 The amount of a compound of the invention which constitutes a "therapeutically effective amount" will vary depending on the compound, the disease state and its severity, the age of the patient to be treated, and the like. The therapeutically effective amount can be determined routinely by one of ordinary skill in the art having regard
25 to his own knowledge and to this disclosure.

The terms "treat," "treating," and "treatment," refer to therapeutic or preventative measures described herein. The methods of "treatment" employ administration to a subject, in need of such treatment, a composition of the present invention, for example, a subject afflicted a disease or disorder, or a subject who
30 ultimately may acquire such a disease or disorder, in order to prevent, cure, delay, reduce the severity of, or ameliorate one or more symptoms of the disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment.

The term "vaccine" as used herein is defined as a material used to provoke an immune response after administration of the material to an animal, preferably a mammal, and more preferably a human. Upon introduction into a subject, the vaccine is able to provoke an immune response including, but not limited to, the production of antibodies, cytokines and/or other cellular responses.

"Variant" with respect to a peptide or polypeptide that differs in amino acid sequence by the insertion, deletion, or conservative substitution of amino acids, but retain at least one biological activity. Variant can also mean a protein with an amino acid sequence that is substantially identical to a referenced protein with an amino acid sequence that retains at least one biological activity. A conservative substitution of an amino acid, i.e., replacing an amino acid with a different amino acid of similar properties (e.g., hydrophilicity, degree and distribution of charged regions) is recognized in the art as typically involving a minor change. These minor changes can be identified, in part, by considering the hydrophobic index of amino acids, as understood in the art. Kyte et al., *J. Mol. Biol.* 157:105-132 (1982). The hydrophobic index of an amino acid is based on a consideration of its hydrophobicity and charge. It is known in the art that amino acids of similar hydrophobic indexes can be substituted and still retain protein function. In one aspect, amino acids having hydrophobic indexes of ± 2 are substituted. The hydrophilicity of amino acids can also be used to reveal substitutions that would result in proteins retaining biological function. A consideration of the hydrophilicity of amino acids in the context of a peptide permits calculation of the greatest local average hydrophilicity of that peptide, a useful measure that has been reported to correlate well with antigenicity and immunogenicity. U.S. Patent No. 4,554,101, incorporated fully herein by reference. Substitution of amino acids having similar hydrophilicity values can result in peptides retaining biological activity, for example immunogenicity, as is understood in the art. Substitutions can be performed with amino acids having hydrophilicity values within ± 2 of each other. Both the hydrophobicity index and the hydrophilicity value of amino acids are influenced by the particular side chain of that amino acid. Consistent with that observation, amino acid substitutions that are compatible with biological function are understood to depend on the relative similarity of the amino acids, and particularly the side chains of those amino acids, as revealed by the hydrophobicity, hydrophilicity, charge, size, and other properties.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

The invention provides an immunological composition comprising a peptide of a HER family of proteins as well as other receptor tyrosine kinases (RTKs). In one embodiment, the invention provides isolated peptides of one or more of HER-1, HER-3, and c-MET protein. In one embodiment, the peptides of the invention are useful in eliciting an immune response. A composition comprising a peptide of the invention is useful as a prophylactic therapeutic agent for initial protection as well as useful as a therapeutic agent for treatment of an ongoing condition.

The present invention also provides methods for treating or preventing cancer. Such methods involve the step of administering to a subject in need thereof a peptide or combinations of peptides of the invention. Administration of such peptide(s) results in the induction of anti-tumor immunity. Thus, the present invention provides methods for inducing anti-tumor immunity in a subject, such methods involving the step of administering to the subject the peptide or combination of peptides of the invention, as well as pharmaceutical compositions and cellular compositions derived thereof.

The invention encompasses a method for inducing a T cell response to in a mammal. The method comprises administering an antigen presenting cell (APC) that specifically induces proliferation of a T cell. In one embodiment, method comprises administering a dendritic cell vaccine pulsed with a peptide of the invention to thereby specifically induce proliferation of a T cell against the antigen corresponding to the peptide.

In one embodiment, APCs pulsed with the peptide of the invention can be used to culture expand T cells. Once sufficient numbers of antigen-specific T cells are obtained using the APC to expand the T cell, the antigen-specific T cells so obtained are administered to the mammal, thereby inducing an antigen specific T cell response in the mammal.

The invention includes a preparation of activated DCs. In one embodiment, the DC preparations are greater than 90% pure. In another embodiment, the DC preparations are fully activated. For example, the DCs are activated with a DC activation regimen comprising contacting the DC with a TLR agonist (e.g., LPS). In another embodiment, the DCs are activated with a calcium mobilizing treatment in conjunction with other DC activation regimens (e.g., activating agents) that enhance different 3rd signal cytokines.

The present invention includes mature, antigen loaded DCs activated by any DC activation regimen. The DCs of the present invention produce desirable levels of cytokines and chemokines. In one embodiment, the invention provides a method to pulse and activate cells, whereby the cells maintain the active state following cryopreservation. A benefit of the DC preparation of the invention is that the cells are efficiently cryopreserved from a single leukapheresis (patient collection) into an initial vaccine plus multiple "booster" doses (e.g., 10 or more) that can be thawed as needed at remote treatment locations without any specialized cell processing facilities or further required quality control testing.

The present invention also relates to the cryopreservation of these activated DCs in a manner that retains their potency and functionality in presenting antigen as well as their production of various cytokines and chemokines after thawing, such that the cryopreserved and subsequently thawed activated DCs are as clinically effective as freshly harvested and activated DCs.

As contemplated herein, the present invention provides a method for generating and cryopreserving DCs with superior functionality in producing stronger signals to T cells, and thus resulting in a more potent DC-based vaccine. By effectively cryopreserving such cells, samples can be stored and thawed for later use, thereby reducing the need for repeated pheresis and elutriation processes during vaccine production. Being able to freeze DCs and then thaw them out later is an advantage because it means that a single round of vaccine production can be divided into small parts, frozen away, and then administered one at a time to a patient over the

course of weeks, months, or years to give "booster" vaccinations that strengthen immunity.

The present embodiments also include use of HER3 expression as a marker of tumor progression in premalignant lesions of the gastroesophageal junction, also known as Barrett's esophagus. The marker has prognostic and therapeutic uses in invasive esophagogastric carcinoma.

Compositions

The present invention provides isolated peptides of the HER family of proteins as well as other receptor tyrosine kinases. In one embodiment, the invention provides isolated peptides of one or more of HER-1, HER-3, and c-MET protein. In one embodiment, the peptides of the invention represent epitopes of the corresponding HER or c-MET protein. In some embodiments, the epitopes of the corresponding HER or c-MET protein are immunogenic.

The present invention provides compositions that include one or more peptides of the invention. The present invention also provides compositions that include one or more chimeric peptides. In one embodiment, the chimeric peptides include one or more of the epitopes of the corresponding HER or c-MET protein.

Additionally, compositions having one or more multivalent peptides are provided. These multivalent peptides include two or more of the epitopes of the invention.

Methods of stimulating an immune response and methods of treating cancer in a subject using the compositions of the invention are included in the invention. Vaccines are also provided for therapeutic and prophylactic use. The epitopes of the invention, either alone or in the context of chimeric peptides, as described herein, is capable of invoking an immune response. In one embodiment, the immune response is a humoral response. In another embodiment, the immune response is a cell mediated response. According to some embodiments, the epitopes or peptides of the invention confer a protective effect.

In one embodiment, the HER-3 epitopes or otherwise peptides of the invention include:

p11-13 (Peptide 51-75): KLYERCEVVMGNLEIVLTIGHNADLSFLQW (SEQ ID NO: 1);

p81-83 (Peptide 401-425): SWPPHMHNFVFSNLTIGGRSLYN (SEQ ID NO: 2);

- p84-86 (Peptide 416-440): TTIGGRSLYNRGFSLLIMKNLNVTS (SEQ ID NO: 3);
p12 (Peptide 56-70): CEVVMGNLEIVLTGH (SEQ ID NO: 4);
p81 (Peptide 401-415): SWPPMHNFVFSNL (SEQ ID NO: 5);
p84 (Peptide 416-430): TTIGGRSLYNRGFSL (SEQ ID NO: 6);
5 p91 (Peptide 451-465): AGRYISANRQLCYH (SEQ ID NO: 7);

The HER-3 peptides or any peptide of the invention may be cyclized or linear. When cyclized, the epitopes may be cyclized in any suitable manner. For example, disulfide bonds may be formed between selected cysteine (Cys) pairs in
10 order to provide a desired conformation. It is believed that the formation of cyclized epitopes may provide conformations that improve the humoral response, thus improving the protective effect.

The HER-3 epitope identified by SEQ ID NO: 4 represents positions 56-70 of the HER-3 protein. The HER-3 epitope identified by SEQ ID NO: 5
15 represents positions 401-415 of the HER-3 protein. The HER-3 epitope identified by SEQ ID NO: 6 represents positions 416-430 of the HER-3 protein. The HER-3 epitope identified by SEQ ID NO: 7 represents positions 451-465 of the HER-3 protein.

As described herein, the HER-3 epitopes of the invention also
20 encompass peptides that are functional equivalents of the peptides identified by SEQ ID NOs. Such functional equivalents have an altered sequence in which one or more of the amino acids in the corresponding HER-3 epitope sequence is substituted or in which one or more amino acids are deleted from or added to the corresponding reference sequence. For example 1 to 3 amino acids may be added to the amino
25 terminus, carboxy terminus, or both. In some examples, the HER-3 epitopes are glycosylated.

In other examples, the HER-3 epitopes may be the retro-inverso isomers of the HER-3 epitopes. The retro-inverso modification comprises the reversal of all amide bonds within the peptide backbone. This reversal may be achieved by
30 reversing the direction of the sequence and inverting the chirality of each amino acid residue by using D-amino acids instead of the L-amino acids. This retro-inverso isomer form may retain planarity and conformation restriction of at least some of the peptide bonds.

Non-conservative amino acid substitutions and/or conservative substitutions may be made. Substitutions are conservative amino acid substitutions when the substituted amino acid has similar structural or chemical properties with the corresponding amino acid in the reference sequence. By way of example,

5 conservative amino acid substitutions involve substitution of one aliphatic or hydrophobic amino acids, e.g., alanine, valine, leucine and isoleucine, with another; substitution of one hydroxyl-containing amino acid, e.g., serine and threonine, with another; substitution of one acidic residue, e.g., glutamic acid or aspartic acid, with another; replacement of one amide-containing residue, e.g., asparagine and glutamine,

10 with another; replacement of one aromatic residue, e.g., phenylalanine and tyrosine, with another; replacement of one basic residue, e.g., lysine, arginine and histidine, with another; and replacement of one small amino acid, e.g., alanine, serine, threonine, methionine, and glycine, with another.

In some examples, the deletions and additions are located at the amino

15 terminus, the carboxy terminus, or both, of one of the sequences of the peptides of the invention. For example, the HER-3 epitope equivalent has an amino acid sequence which is at least 70% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the corresponding

20 HER-3 epitope sequences. Sequences which are at least 90% identical have no more than 1 alteration, i.e., any combination of deletions, additions or substitutions, per 10 amino acids of the reference sequence. Percent identity is determined by comparing the amino acid sequence of the variant with the reference sequence using known or to be developed programs in the art.

25 For functional equivalents that are longer than a corresponding HER-3 epitope sequence, the functional equivalent may have a sequence which is at least 90% identical to the HER-3 epitope sequence and the sequences which flank the HER-3 epitope sequences in the wild-type HER-3 protein.

Functional equivalents of the HER-3 epitopes may be identified by

30 modifying the sequence of the epitope and then assaying the resulting polypeptide for the ability to stimulate an immune response, e.g., production of antibodies. Such antibodies may be found in a variety of body fluids including sera and ascites. Briefly, a body fluid sample is isolated from a warm-blooded animal, such as a human, for whom it is desired to determine whether antibodies specific for HER-3 polypeptide

are present. The body fluid is incubated with HER-3 polypeptide under conditions and for a time sufficient to permit immunocomplexes to form between the polypeptide and antibodies specific for the protein and then assayed, preferably using an ELISA technique.

5 In accordance with other embodiments of the present invention, chimeric peptides and compositions comprising one or more chimeric peptides are provided. According to various embodiments, the chimeric peptides comprise a HER-3 epitope, another epitope, and a linker joining the HER-3 epitope to the other epitope. In one embodiment, the other epitope can include but is not limited to another
10 HER-3 epitope, a HER-1 epitope, a HER-2 epitope, and a c-Met epitope. It will be further understood that any suitable linker may be used. For example, depending upon the epitope used, the HER-3 epitope may be linked to either the amino or the carboxy terminus of the other epitope. The location and selection of the other epitope depends on the structural characteristics of the HER-3 epitope, whether alpha helical or beta-
15 turn or strand.

 In one embodiment, the linker may be a peptide of from about 2 to about 15 amino acids, about 2 to about 10 amino acids, or from about 2 to about 6 amino acids in length. The chimeric peptides may be linear or cyclized. Additionally, the HER-3 epitopes, the other epitopes, and/or the linker may be in retro-inverso
20 form. Thus the HER-3 epitope along could be in retro inverso form. Alternatively, the HER-3 epitope and the other epitope could be in retro inverso form. In another example, the HER-3 epitope, the other epitope, and the linker could be in retro inverso form.

 In another embodiment, the peptides of the invention can be in a
25 mixture together instead of being in a form of a chimeric peptide. In any event, the compositions of the invention comprising the peptides may be useful agents to pulse antigen presenting cells (e.g., dendritic cells) for the generation of cellular vaccines. In another embodiment, the compositions of the invention comprising the peptides may be useful immunogens for inducing production of antibodies. The compositions
30 of the invention may also be used to immunize a subject and retard or prevent tumor development. The compositions of the invention may be used in vaccines to provide a protective effect.

 In accordance with additional embodiments of the present invention, compositions comprising a mixture of two or more of the peptides or chimeric

peptides of the invention are provided. In some examples, the HER-3 epitope of each of the two or more chimeric peptides are different. In other examples, one of the HER-3 epitopes is selected from SEQ ID NOs: 1-7.

Peptides, including chimeric peptides, of the present invention can be prepared using well known techniques. For example, the peptides can be prepared synthetically, using either recombinant DNA technology or chemical synthesis. Peptides of the present invention may be synthesized individually or as longer polypeptides composed of two or more peptides. The peptides of the present invention are preferably isolated, i.e., substantially free of other naturally occurring host cell proteins and fragments thereof.

The peptide and chimeric peptides of the invention may be synthesized using commercially available peptide synthesizers. For example, the chemical methods described in Kaumaya et al., "De Novo" Engineering of Peptide Immunogenic and Antigenic Determinants as Potential Vaccines, in *Peptides, Design, Synthesis and Biological Activity* (1994), pp 133-164, which is specifically incorporated herein by reference, may be used. For example, HER-3 epitopes may be synthesized co-linearly with the other epitope to form a chimeric peptide. Peptide synthesis may be performed using Fmoc/t-But chemistry. The peptides and chimeric peptides may be cyclized in any suitable manner. For example, disulfide bonds may be achieved using differentially protected cysteine residues, iodine oxidation, the addition of water to boost removal of Ac group and the concomitant formation of a disulfide bond, and/or the silyl chloride-sulfoxide method.

The peptides and chimeric peptides may also be produced using cell-free translation systems and RNA molecules derived from DNA constructs that encode the epitope or peptide. Alternatively, the epitopes or chimeric peptides are made by transfecting host cells with expression vectors that comprise a DNA sequence that encodes the respective epitope or chimeric peptide and then inducing expression of the polypeptide in the host cells. For recombinant production, recombinant constructs comprising one or more of the sequences which encode the epitope, chimeric peptide, or a variant thereof are introduced into host cells by conventional methods such as calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape lading, ballistic introduction or infection.

The peptides of the present invention may contain modifications, such as glycosylation, side chain oxidation, or phosphorylation; so long as the modifications do not destroy the biological activity of the peptides. Other modifications include incorporation of D-amino acids or other amino acid mimetics that can be used, for example, to increase the serum half-life of the peptides.

The peptides of the invention can be prepared as a combination, which includes two or more of peptides of the invention, for use as a vaccine for a disease, e.g. cancers. The peptides may be in a cocktail or may be conjugated to each other using standard techniques. For example, the peptides can be expressed as a single polypeptide sequence. The peptides in the combination may be the same or different.

The present invention should also be construed to encompass "mutants," "derivatives," and "variants" of the peptides of the invention (or of the DNA encoding the same) which mutants, derivatives and variants are peptides which are altered in one or more amino acids (or, when referring to the nucleotide sequence encoding the same, are altered in one or more base pairs) such that the resulting peptide (or DNA) is not identical to the sequences recited herein, but has the same biological property as the peptides disclosed herein.

The invention also provides a polynucleotide encoding at least one peptide selected from a peptide having the sequence of any one or more of SEQ ID NOs 1-7. The nucleic acid sequences include both the DNA sequence that is transcribed into RNA and the RNA sequence that is translated into a peptide. According to other embodiments, the polynucleotides of the invention are inferred from the amino acid sequence of the peptides of the invention. As is known in the art several alternative polynucleotides are possible due to redundant codons, while retaining the biological activity of the translated peptides.

Further, the invention encompasses an isolated nucleic acid encoding a peptide having substantial homology to the peptides disclosed herein. Preferably, the nucleotide sequence of an isolated nucleic acid encoding a peptide of the invention is "substantially homologous", that is, is about 60% homologous, more preferably about 70% homologous, even more preferably about 80% homologous, more preferably about 90% homologous, even more preferably, about 95% homologous, and even more preferably about 99% homologous to a nucleotide sequence of an isolated nucleic acid encoding a peptide of the invention.

It is to be understood explicitly that the scope of the present invention encompasses homologs, analogs, variants, derivatives and salts, including shorter and longer peptides and polynucleotides, as well as peptide and polynucleotide analogs with one or more amino acid or nucleic acid substitution, as well as amino acid or
5 nucleic acid derivatives, non-natural amino or nucleic acids and synthetic amino or nucleic acids as are known in the art, with the stipulation that these modifications must preserve the biological activity of the original molecule. Specifically any active fragments of the active peptides as well as extensions, conjugates and mixtures are disclosed according to the principles of the present invention.

10 The invention should be construed to include any and all isolated nucleic acids which are homologous to the nucleic acids described and referenced herein, provided these homologous DNAs have the biological activity of the peptides disclosed herein.

The skilled artisan would understand that the nucleic acids of the
15 invention encompass an RNA or a DNA sequence encoding a peptide of the invention, and any modified forms thereof, including chemical modifications of the DNA or RNA which render the nucleotide sequence more stable when it is cell free or when it is associated with a cell. Chemical modifications of nucleotides may also be used to enhance the efficiency with which a nucleotide sequence is taken up by a cell
20 or the efficiency with which it is expressed in a cell. Any and all combinations of modifications of the nucleotide sequences are contemplated in the present invention.

Further, any number of procedures may be used for the generation of mutant, derivative or variant forms of a protein of the invention using recombinant DNA methodology well known in the art such as, for example, that described in
25 Sambrook and Russell, *supra*, and Ausubel et al., *supra*. Procedures for the introduction of amino acid changes in a peptide or polypeptide by altering the DNA sequence encoding the polypeptide are well known in the art and are also described in these, and other, treatises.

The nucleic acids encoding the peptides of the invention can be
30 incorporated into suitable vectors e.g. retroviral vectors. These vectors are well known in the art. The nucleic acids or the vectors containing them usefully can be transferred into a desired cell, which cell is preferably from a patient. Advantageously, the invention provides an off-the-shelf composition allowing rapid modification of a

patient's own cells (or those of another mammal) to rapidly and easily produce modified cells having excellent cancer cell killing properties.

Vectors

5 In other related aspects, the invention includes an isolated nucleic acid encoding one or more of peptides having a sequence selected from the group consisting of SEQ ID NOs: 1-7.

10 In one embodiment, the invention includes a nucleic acid sequence encoding one or more peptides of the invention operably linked to a nucleic acid comprising a promoter/regulatory sequence such that the nucleic acid is preferably capable of directing expression of the protein encoded by the nucleic acid. Thus, the invention encompasses expression vectors and methods for the introduction of exogenous DNA into cells with concomitant expression of the exogenous DNA in the cells such as those described, for example, in Sambrook et al. (2012, Molecular
15 Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York). The incorporation of a desired polynucleotide into a vector and the choice of vectors is well-known in the art as described in, for example, Sambrook et al., *supra*, and Ausubel et al., *supra*.

20 The polynucleotide can be cloned into a number of types of vectors. However, the present invention should not be construed to be limited to any particular vector. Instead, the present invention should be construed to encompass a wide plethora of vectors which are readily available and/or well-known in the art. For example, the polynucleotide of the invention can be cloned into a vector including,
25 but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

In specific embodiments, the expression vector is selected from the group consisting of a viral vector, a bacterial vector and a mammalian cell vector.
30 Numerous expression vector systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-vector based systems can be employed for use with the present invention to produce polynucleotides, or their cognate polypeptides. Many such systems are commercially and widely available.

Further, the expression vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook et al. (2012), and in Ausubel et al. (1997), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers. (See, e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193.

For expression of the desired nucleotide sequences of the invention, at least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements, i.e., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

A promoter may be one naturally associated with a gene or polynucleotide sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a polynucleotide sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding polynucleotide segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a polynucleotide

sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a polynucleotide sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other
5 prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including
10 PCR™, in connection with the compositions disclosed herein (U.S. Patent 4,683,202, U.S. Patent 5,928,906). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer
15 that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know how to use promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook et al. (2012). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate
20 conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

A promoter sequence exemplified in the experimental examples presented herein is the immediate early cytomegalovirus (CMV) promoter sequence.
25 This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR)
30 promoter, Moloney virus promoter, the avian leukemia virus promoter, Epstein-Barr virus immediate early promoter, Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the muscle creatine promoter. Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are

also contemplated as part of the invention. The use of an inducible promoter in the invention provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of
5 inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter. Further, the invention includes the use of a tissue specific promoter, which promoter is active only in a desired tissue.

In order to assess the expression of the nucleotide sequences encoding
10 the peptides of the invention, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other embodiments, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection
15 procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are known in the art and include, for example, antibiotic-resistance genes, such as neo and the like.

Reporter genes are used for identifying potentially transfected cells and
20 for evaluating the functionality of regulatory sequences. Reporter genes that encode for easily assayable proteins are well known in the art. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time
25 after the DNA has been introduced into the recipient cells.

Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (see, e.g., Ui-Tei et al., 2000 FEBS Lett. 479:79-82). Suitable expression systems are well known and may be prepared using well
30 known techniques or obtained commercially. Internal deletion constructs may be generated using unique internal restriction sites or by partial digestion of non-unique restriction sites. Constructs may then be transfected into cells that display high levels of siRNA polynucleotide and/or polypeptide expression. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter

gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter-driven transcription.

5 Vaccine

In one embodiment, the present invention is directed to a vaccine comprising a peptide of the invention. The vaccine of the invention can provide any combination of particular peptides for the particular prevention or treatment of the cancer of a subject that is in need of treatment.

10 The vaccine of the invention can induce antigen-specific T cell and/or high titer antibody responses, thereby inducing or eliciting an immune response that is directed to or reactive against the cancer or tumor expressing the antigen. In some embodiments, the induced or elicited immune response can be a cellular, humoral, or both cellular and humoral immune responses. In some embodiments, the induced or
15 elicited cellular immune response can include induction or secretion of interferon-gamma (IFN- γ) and/or tumor necrosis factor alpha (TNF- α).

In one embodiment, the present invention is directed to an anti-cancer vaccine. The vaccine can comprise one or more cancer antigens. The vaccine can prevent tumor growth. The vaccine can reduce tumor growth. The vaccine can
20 prevent metastasis of tumor cells. Depending upon the cancer antigen, the vaccine can be targeted to treat breast cancer, liver cancer, prostate cancer, melanomas, blood cancers, head and neck cancer, glioblastoma, recurrent respiratory papillomatosis, anal cancer, cervical cancer, brain cancer, and the like.

In a particular embodiment, the vaccine can mediate clearance or
25 prevent growth of tumor cells by inducing (1) humoral immunity via B cell responses to generate desirable antibodies; (2) increase cytotoxic T lymphocyte such as CD8⁺ (CTL) to attack and kill tumor cells; (3) increase T helper cell responses; (4) and increase inflammatory responses via IFN- γ and TNF- α or preferably all of the aforementioned. The vaccine can increase tumor free survival by 30%, 31%, 32%,
30 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, and 45%. The vaccine can reduce tumor mass by 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, and 60% after immunization.

The vaccine can increase a cellular immune response in a subject administered the vaccine by about 50-fold to about 6000-fold, about 50-fold to about 5500-fold, about 50-fold to about 5000-fold, about 50-fold to about 4500-fold, about 100-fold to about 6000-fold, about 150-fold to about 6000-fold, about 200-fold to about 6000-fold, about 250-fold to about 6000-fold, or about 300-fold to about 6000-fold as compared to a cellular immune response in a subject not administered the vaccine. In some embodiments the vaccine can increase the cellular immune response in the subject administered the vaccine by about 50-fold, 100-fold, 150-fold, 200-fold, 250-fold, 300-fold, 350-fold, 400-fold, 450-fold, 500-fold, 550-fold, 600-fold, 650-fold, 700-fold, 750-fold, 800-fold, 850-fold, 900-fold, 950-fold, 1000-fold, 1100-fold, 1200-fold, 1300-fold, 1400-fold, 1500-fold, 1600-fold, 1700-fold, 1800-fold, 1900-fold, 2000-fold, 2100-fold, 2200-fold, 2300-fold, 2400-fold, 2500-fold, 2600-fold, 2700-fold, 2800-fold, 2900-fold, 3000-fold, 3100-fold, 3200-fold, 3300-fold, 3400-fold, 3500-fold, 3600-fold, 3700-fold, 3800-fold, 3900-fold, 4000-fold, 4100-fold, 4200-fold, 4300-fold, 4400-fold, 4500-fold, 4600-fold, 4700-fold, 4800-fold, 4900-fold, 5000-fold, 5100-fold, 5200-fold, 5300-fold, 5400-fold, 5500-fold, 5600-fold, 5700-fold, 5800-fold, 5900-fold, or 6000-fold as compared to the cellular immune response in the subject not administered the vaccine.

The vaccine can increase interferon gamma (IFN- γ) levels in a subject administered the vaccine by about 50-fold to about 6000-fold, about 50-fold to about 5500-fold, about 50-fold to about 5000-fold, about 50-fold to about 4500-fold, about 100-fold to about 6000-fold, about 150-fold to about 6000-fold, about 200-fold to about 6000-fold, about 250-fold to about 6000-fold, or about 300-fold to about 6000-fold as compared to IFN- γ levels in a subject not administered the vaccine. In some embodiments the vaccine can increase IFN- γ levels in the subject administered the vaccine by about 50-fold, 100-fold, 150-fold, 200-fold, 250-fold, 300-fold, 350-fold, 400-fold, 450-fold, 500-fold, 550-fold, 600-fold, 650-fold, 700-fold, 750-fold, 800-fold, 850-fold, 900-fold, 950-fold, 1000-fold, 1100-fold, 1200-fold, 1300-fold, 1400-fold, 1500-fold, 1600-fold, 1700-fold, 1800-fold, 1900-fold, 2000-fold, 2100-fold, 2200-fold, 2300-fold, 2400-fold, 2500-fold, 2600-fold, 2700-fold, 2800-fold, 2900-fold, 3000-fold, 3100-fold, 3200-fold, 3300-fold, 3400-fold, 3500-fold, 3600-fold, 3700-fold, 3800-fold, 3900-fold, 4000-fold, 4100-fold, 4200-fold, 4300-fold, 4400-fold, 4500-fold, 4600-fold, 4700-fold, 4800-fold, 4900-fold, 5000-fold, 5100-fold, 5200-fold, 5300-fold, 5400-fold, 5500-fold, 5600-fold, 5700-fold, 5800-fold, 5900-

fold, or 6000-fold as compared to IFN- γ levels in the subject not administered the vaccine.

The vaccine of the present invention can have features required of effective vaccines such as being safe so that the vaccine itself does not cause illness or death; being protective against illness; inducing neutralizing antibody; inducing protective T cell responses; and providing ease of administration, few side effects, biological stability, and low cost per dose. The vaccine can accomplish some or all of these features by containing the cancer antigen as discussed below.

10 Generation of a loaded (pulsed) immune cell

The present invention includes a cell that has been exposed or otherwise "pulsed" with an antigen or otherwise a peptide of the invention. For example, an APC, such as a DC, may become Ag-loaded *in vitro*, e.g., by culture *ex vivo* in the presence of an antigen, or *in vivo* by exposure to an antigen.

15 A person skilled in the art would also readily understand that an APC can be "pulsed" in a manner that exposes the APC to an antigen for a time sufficient to promote presentation of that antigen on the surface of the APC. For example, an APC can be exposed to an antigen in the form of small peptide fragments, known as antigenic peptides, which are "pulsed" directly onto the outside of the APCs (Mehta-Damani *et al.*, 1994); or APCs can be incubated with whole proteins or protein particles which are then ingested by the APCs. These whole proteins are digested into small peptide fragments by the APC and eventually carried to and presented on the APC surface (Cohen *et al.*, 1994). Antigen in peptide form may be exposed to the cell by standard "pulsing" techniques described herein.

25 Without wishing to be bound by any particular theory, the antigen in the form of a foreign or an autoantigen is processed by the APC of the invention in order to retain the immunogenic form of the antigen. The immunogenic form of the antigen implies processing of the antigen through fragmentation to produce a form of the antigen that can be recognized by and stimulate immune cells, for example T cells.
30 Preferably, such a foreign or an autoantigen is a protein which is processed into a peptide by the APC. The relevant peptide which is produced by the APC may be extracted and purified for use as an immunogenic composition. Peptides processed by the APC may also be used to induce tolerance to the proteins processed by the APC.

The antigen-loaded APC, otherwise known as a "pulsed APC" of the invention, is produced by exposure of the APC to an antigen either *in vitro* or *in vivo*. In the case where the APC is pulsed *in vitro*, the APC can be plated on a culture dish and exposed to an antigen in a sufficient amount and for a sufficient period of time to
5 allow the antigen to bind to the APC. The amount and time necessary to achieve binding of the antigen to the APC may be determined by using methods known in the art or otherwise disclosed herein. Other methods known to those of skill in the art, for example immunoassays or binding assays, may be used to detect the presence of antigen on the APC following exposure to the antigen.

10 In a further embodiment of the invention, the APC may be transfected with a vector which allows for the expression of a specific protein by the APC. The protein which is expressed by the APC may then be processed and presented on the cell surface. The transfected APC may then be used as an immunogenic composition to produce an immune response to the protein encoded by the vector.

15 As discussed elsewhere herein, vectors may be prepared to include a specific polynucleotide which encodes and expresses a protein to which an immunogenic response is desired. Preferably, retroviral vectors are used to infect the cells. More preferably, adenoviral vectors are used to infect the cells.

In another embodiment, a vector may be targeted to an APC by
20 modifying the viral vector to encode a protein or portions thereof that is recognized by a receptor on the APC, whereby occupation of the APC receptor by the vector will initiate endocytosis of the vector, allowing for processing and presentation of the antigen encoded by the nucleic acid of the viral vector. The nucleic acid which is delivered by the virus may be native to the virus, which when expressed on the APC
25 encodes viral proteins which are then processed and presented on the MHC receptor of the APC.

As contemplated herein, various methods can be used for transfecting a polynucleotide into a host cell. The methods include, but are not limited to, calcium phosphate precipitation, lipofection, particle bombardment, microinjection,
30 electroporation, colloidal dispersion systems (i.e. macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes). These methods are understood in the art and are described in published literature so as to enable one skilled in the art to perform these methods.

In another embodiment, a polynucleotide encoding an antigen can be cloned into an expression vector and the vector can be introduced into an APC to otherwise generate a loaded APC. Various types of vectors and methods of introducing nucleic acids into a cell are discussed in the available published literature. For example, the expression vector can be transferred into a host cell by physical, chemical or biological means. See, for example, Sambrook et al. (2012, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York). It is readily understood that the introduction of the expression vector comprising a polynucleotide encoding an antigen yields a pulsed cell.

The present invention includes various methods for pulsing APCs including, but not limited to, loading APCs with whole antigen in the form of a protein, cDNA or mRNA. However, the invention should not be construed to be limited to the specific form of the antigen used for pulsing the APC. Rather, the invention encompasses other methods known in the art for generating an antigen loaded APC. Preferably, the APC is transfected with mRNA encoding a defined antigen. mRNA corresponding to a gene product whose sequence is known can be rapidly generated *in vitro* using appropriate primers and reverse transcriptase-polymerase chain reaction (RT-PCR) coupled with transcription reactions. Transfection of an APC with an mRNA provides an advantage over other antigen-loading techniques for generating a pulsed APC. For example, the ability to amplify RNA from a microscopic amount of tissue, i.e. tumor tissue, extends the use of the APC for vaccination to a large number of patients.

For an antigenic composition to be useful as a vaccine, the antigenic composition must induce an immune response to the antigen in a cell, tissue or mammal (e.g., a human). As used herein, an "immunological composition" may comprise an antigen (e.g., a peptide or polypeptide), a nucleic acid encoding an antigen (e.g., an antigen expression vector), or a cell expressing or presenting an antigen or cellular component. In particular embodiments the antigenic composition comprises or encodes all or part of any antigen described herein, or an immunologically functional equivalent thereof. In other embodiments, the antigenic composition is in a mixture that comprises an additional immunostimulatory agent or nucleic acids encoding such an agent. Immunostimulatory agents include but are not limited to an additional antigen, an immunomodulator, an antigen presenting cell or

an adjuvant. In other embodiments, one or more of the additional agent(s) is covalently bonded to the antigen or an immunostimulatory agent, in any combination. In certain embodiments, the antigenic composition is conjugated to or comprises an HLA anchor motif amino acids.

5 A vaccine, as contemplated herein, may vary in its composition of nucleic acid and/or cellular components. In a non-limiting example, a nucleic acid encoding an antigen might also be formulated with an adjuvant. Of course, it will be understood that various compositions described herein may further comprise additional components. For example, one or more vaccine components may be
10 comprised in a lipid or liposome. In another non-limiting example, a vaccine may comprise one or more adjuvants. A vaccine of the present invention, and its various components, may be prepared and/or administered by any method disclosed herein or as would be known to one of ordinary skill in the art, in light of the present disclosure.

It is understood that an antigenic composition of the present invention
15 may be made by a method that is well known in the art, including but not limited to chemical synthesis by solid phase synthesis and purification away from the other products of the chemical reactions by HPLC, or production by the expression of a nucleic acid sequence (e.g., a DNA sequence) encoding a peptide or polypeptide comprising an antigen of the present invention in an *in vitro* translation system or in a
20 living cell. In addition, an antigenic composition can comprise a cellular component isolated from a biological sample. The antigenic composition isolated and extensively dialyzed to remove one or more undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle. It is further understood that additional amino acids, mutations, chemical modification and such like, if any,
25 that are made in a vaccine component will preferably not substantially interfere with the antibody recognition of the epitopic sequence.

Antigen Presenting Cell Therapy

The invention encompasses a method of producing a population of
30 APCs (e.g., dendritic cells; DCs) that present the peptides of the invention on their surface that may be subsequently used in therapy. Such a method may be carried out *ex vivo* on a sample of cells that have been obtained from a patient. The APCs produced in this way therefore form a pharmaceutical agent that can be used in the treatment or prevention of cancer. The cells should be accepted by the immune system

of the individual because they derive from that individual. Delivery of cells that have been produced in this way to the individual from whom they were originally obtained, thus forms a therapeutic embodiment of the invention.

DCs are derived from pluripotent monocytes that serve as antigen-presenting cells (APCs). DCs are ubiquitous in peripheral tissues, where they are prepared to capture antigens. Upon antigen capture, DCs process the antigen into small peptides and move towards secondary lymphoid organs. It is within the lymphoid organs that DCs present antigen peptides to naive T cells, thereby initiating a cascade of signals that polarizes T cell differentiation. Upon exposure, DCs present antigen molecules bound to either MHC class I or class II binding peptides and activate CD8⁺ or CD4⁺ T cells, respectively (Steinman, 1991, *Annu. Rev. Immunol.* 9:271–296; Banchereau et al., 1998, *Nature* 392:245–252; Steinman, et al., 2007, *Nature* 449:419–426; Ginhoux et al., 2007, *J. Exp. Med.* 204:3133–3146; Banerjee et al., 2006, *Blood* 108:2655–2661; Sallusto et al., 1999, *J. Exp. Med.* 189:611–614; Reid et al., 2000, *Curr. Opin. Immunol.* 12:114–121; Bykovskaia et al., 1999, *J. Leukoc. Biol.* 66:659–666; Clark et al., 2000, *Microbes Infect.* 2:257–272).

DCs are responsible for the induction, coordination and regulation of the adaptive immune response and also serve to orchestrate communication between effectors of the innate arm and the adaptive arm of the immune system. These features have made DCs strong candidates for immunotherapy. DCs have a unique capacity to sample the environment through macropinocytosis and receptor-mediated endocytosis (Gerner et al., 2008, *J. Immunol.* 181:155–164; Stoitzner et al., 2008, *Cancer Immunol. Immunother.* 57:1665–1673; Lanzevecchia A., 1996, *Curr. Opin. Immunol.* 8:348–354; Delamarre et al., 2005, *Science*, 307(5715):1630–1634).

DCs also require maturation signals to enhance their antigen-presenting capacity. DCs upregulate the expression of surface molecules, such as CD80 and CD86 (also known as second signal molecules) by providing additional maturation signals, such as TNF- α , CD40L or calcium signaling agents (Czerniecki et al., 1997, *J. Immunol.* 159:3823–3837; Bedrosian et al. 2000, *J. Immunother.* 23:311–320; Mailliard et al., 2004, *Cancer Res.* 64,5934–5937; Brossart et al., 1998, *Blood* 92:4238–4247; Jin et al., 2004, *Hum. Immunol.* 65:93–103). It has been established that a mixture of cytokines, including TNF- α , IL-1 β , IL-6 and prostaglandin E2 (PGE2), have the ability to mature DC (Jonuleit, et al., 2000, *Arch. Derm. Res.*

292:325–332). DCs can also be matured with calcium ionophore prior to being pulsed with antigen.

In addition to pathogen-recognition receptors, such as PKR and MDA-5 (Kalali et al., 2008, *J. Immunol.* 181:2694–2704; Nallagatla et al., 2008, *RNA Biol.* 5(3):140–144), DCs also contain a series of receptors, known as Toll-like receptors (TLRs), that are also capable of sensing danger from pathogens. When these TLRs are triggered, a series of activational changes are induced in DCs, which lead to maturation and signaling of T cells (Boullart et al. 2008, *Cancer Immunol. Immunother.* 57(11):1589–1597; Kaisho et al., 2003, *Curr. Mol. Med.* 3(4):373–385; Pulendran et al., 2001, *Science* 293(5528):253–256; Napolitani et al., 2005, *Nat. Immunol.* 6(8):769–776). DCs can activate and extend the various arms of the cell-mediated response, such as natural killer γ - δ T and α - β T cells and, once activated, DCs retain their immunizing capacity (Steinman, 1991, *Annu. Rev. Immunol.* 9:271–296; Banchereau et al., 1998, *Nature* 392:245–252; Reid et al., 2000, *Curr. Opin. Immunol.* 12:114–121; Bykovskaia et al., 1999, *J. Leukoc. Biol.* 66:659–666; Clark et al., 2000, *Microbes Infect.* 2:257–272).

The present invention also provides methods of inducing antigen presenting cells (APCs) using one or more peptides of the invention. The APCs can be induced by inducing dendritic cells from the peripheral blood monocytes and then contacting (stimulating) them with one or more peptides of this invention *in vitro*, *ex vivo* or *in vivo*. When peptides of the present invention are administered to the mammal in need thereof, APCs that have the peptides of this invention immobilized to them are induced in the body of the mammal. Alternatively, after immobilizing the peptides of this invention to the APCs, the cells can be administered to the subject as a vaccine. For example, the *ex vivo* administration may include the steps of: collecting APCs from a mammal, and contacting the APCs with a peptide of the present invention.

The present invention also provides APCs presenting complexes formed between HLA antigens and one or more peptides of this invention. The APCs, obtained through contact with the peptides of this invention or the nucleotides encoding such peptides, are preferably derived from subjects who are the target of treatment and/or prevention, and can be administered as vaccines, alone or in combination with other drugs, including the peptides, exosomes, or T cells of the present invention.

The present invention provides compositions and methods for stimulating APC, preferably DCs, in the context of immunotherapy to stimulate the immune response in a mammal. DCs can be manipulated by stimulating them with a peptide or combination of peptides of the invention and causing the DCs to mature so that they stimulate anti-tumor immunity in a mammal in need thereof.

In one embodiment, the invention includes a method for inducing a T cell response in a mammal. The method comprising administering an APC, such as a DC, wherein the APC has been activated by contacting the APC with a peptide or combination of peptides of the invention thereby generating a peptide-loaded APC.

In one embodiment, the invention relates to novel APCs produced and methods for their use to, *inter alia*, expand a desired T cell, to activate T cells, to expand specific T cell, as well as numerous therapeutic uses relating to expansion and stimulation of T cells using the peptide-load APC and peptides of the invention. In some instances, the OCT4 stimulated DCs can be used to expand peptide-specific T cells.

The present invention relates to the discovery that a DC contacted with a peptide or combination of peptides of the invention can be used to induce expansion of peptide-specific T cells. A skilled artisan would recognize that the DCs contacted with the peptides of the invention are considered primed or otherwise peptide-loaded. The peptide-loaded DCs of the invention are useful for eliciting an immune response against a desired antigen, for example HER-3. Accordingly, the peptide-load DCs of the invention can be used to treat a disease associated with unregulated expression of HER-3.

25 Methods for Treating a Disease

The present invention also encompasses methods of treatment and/or prevention of a disease caused by pathogenic microorganisms, autoimmune disorder and/or a hyperproliferative disease.

Diseases that may be treated or prevented by use of the present invention include diseases caused by viruses, bacteria, yeast, parasites, protozoa, cancer cells and the like. The pharmaceutical composition of the present invention may be used as a generalized immune enhancer (DC activating composition or system) and as such has utility in treating diseases. Exemplary diseases that can be treated and/or prevented utilizing the pharmaceutical composition of the present

invention include, but are not limited to infections of viral etiology such as HIV, influenza, Herpes, viral hepatitis, Epstein Bar, polio, viral encephalitis, measles, chicken pox, Papilloma virus etc.; or infections of bacterial etiology such as pneumonia, tuberculosis, syphilis, etc.; or infections of parasitic etiology such as
 5 malaria, trypanosomiasis, leishmaniasis, trichomoniasis, amoebiasis, etc.

Preneoplastic or hyperplastic states that may be treated or prevented using the pharmaceutical composition of the present invention (transduced DCs, expression vector, expression construct, etc.) of the present invention include but are not limited to preneoplastic or hyperplastic states such as colon polyps, Crohn's
 10 disease, ulcerative colitis, breast lesions and the like.

Cancers that may be treated using the composition of the present invention of the present invention include, but are not limited to primary or metastatic melanoma, adenocarcinoma, squamous cell carcinoma, adenosquamous cell carcinoma, thymoma, lymphoma, sarcoma, lung cancer, liver cancer, non-Hodgkin's
 15 lymphoma, Hodgkin's lymphoma, leukemias, uterine cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, colon cancer, multiple myeloma, neuroblastoma, gastrointestinal cancer, brain cancer, bladder cancer, cervical cancer and the like.

Other hyperproliferative diseases that may be treated using DC activation system of the present invention include, but are not limited to rheumatoid
 20 arthritis, inflammatory bowel disease, osteoarthritis, leiomyomas, adenomas, lipomas, hemangiomas, fibromas, vascular occlusion, restenosis, atherosclerosis, pre-neoplastic lesions (such as adenomatous hyperplasia and prostatic intraepithelial neoplasia), carcinoma in situ, oral hairy leukoplakia, or psoriasis.

Autoimmune disorders that may be treated using the composition of the present invention include, but are not limited to, AIDS, Addison's disease, adult respiratory distress syndrome, allergies, anemia, asthma, atherosclerosis, bronchitis, cholecystitis, Crohn's disease, ulcerative colitis, atopic dermatitis, dermatomyositis, diabetes mellitus, emphysema, erythema nodosum, atrophic gastritis,
 25 glomerulonephritis, gout, Graves' disease, hypereosinophilia, irritable bowel syndrome, lupus erythematosus, multiple sclerosis, myasthenia gravis, myocardial or pericardial inflammation, osteoarthritis, osteoporosis, pancreatitis, polymyositis, rheumatoid arthritis, scleroderma, Sjogren's syndrome, and autoimmune thyroiditis;
 30

complications of cancer, hemodialysis, and extracorporeal circulation; viral, bacterial, fungal, parasitic, protozoal, and helminthic infections; and trauma.

In the method of treatment, the administration of the composition of the invention may be for either "prophylactic" or "therapeutic" purpose. When provided prophylactically, the composition of the present invention is provided in advance of any symptom, although in particular embodiments the vaccine is provided following the onset of one or more symptoms to prevent further symptoms from developing or to prevent present symptoms from becoming worse. The prophylactic administration of composition serves to prevent or ameliorate any subsequent infection or disease. When provided therapeutically, the pharmaceutical composition is provided at or after the onset of a symptom of infection or disease. Thus, the present invention may be provided either prior to the anticipated exposure to a disease-causing agent or disease state or after the initiation of the infection or disease.

An effective amount of the composition would be the amount that achieves this selected result of enhancing the immune response, and such an amount could be determined as a matter of routine by a person skilled in the art. For example, an effective amount of for treating an immune system deficiency against cancer or pathogen could be that amount necessary to cause activation of the immune system, resulting in the development of an antigen specific immune response upon exposure to antigen. The term is also synonymous with "sufficient amount."

The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular composition being administered, the size of the subject, and/or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular composition of the present invention without necessitating undue experimentation.

Vaccine Formulations

The present invention further includes vaccine formulations suitable for use in immunotherapy. In certain embodiments, vaccine formulations are used for the prevention and/or treatment of a disease, such as cancer and infectious diseases. In one embodiment, the administration to a patient of a vaccine in accordance with the present invention for the prevention and/or treatment of cancer can take place before or after a surgical procedure to remove the cancer, before or after a chemotherapeutic

procedure for the treatment of cancer, and before or after radiation therapy for the treatment of cancer and any combination thereof. In other embodiments, the vaccine formulations may be administered to a patient in conjunction or combination with another composition or pharmaceutical product. It should be appreciated that the present invention can also be used to prevent cancer in individuals without cancer, but who might be at risk of developing cancer.

The administration of a cancer vaccine prepared in accordance with the present invention, is broadly applicable to the prevention or treatment of cancer, determined in part by the selection of antigens forming part of the cancer vaccine. Cancers that can be suitably treated in accordance with the practices of the present invention include, without limitation, cancers of the lung, breast, ovary, cervix, colon, head and neck, pancreas, prostate, stomach, bladder, kidney, bone, liver, esophagus, brain, testicle, uterus and the various leukemias and lymphomas.

In one embodiment, vaccines in accordance with this invention can be derived from the tumor or cancer cells to be treated. For example, in the treatment of lung cancer, the lung cancer cells would be treated as described hereinabove to produce a lung cancer vaccine. Similarly, breast cancer vaccine, colon cancer vaccine, pancreas cancer vaccine, stomach cancer vaccine, bladder cancer vaccine, kidney cancer vaccine and the like, would be produced and employed as immunotherapeutic agents in accordance with the practices for the prevention and/or treatment of the tumor or cancer cell from which the vaccine was produced.

In another embodiment, vaccines in accordance with the present invention could, as stated, also be prepared to treat various infectious diseases which affect mammals, by collecting the relevant antigens shed into a culture medium by the pathogen. As there is heterogeneity in the type of immunogenic and protective antigens expressed by different varieties of organisms causing the same disease, polyvalent vaccines can be prepared by preparing the vaccine from a pool of organisms expressing the different antigens of importance.

In another embodiment of the present invention, the vaccine can be administered by intranodal injection into groin nodes. Alternatively, and depending on the vaccine target, the vaccine can be intradermally or subcutaneously administered to the extremities, arms and legs, of the patients being treated. Although this approach is generally satisfactory for melanoma and other cancers, including the prevention or

treatment of infectious diseases, other routes of administration, such as intramuscularly or into the blood stream may also be used.

Additionally, the vaccine can be given together with adjuvants and/or immuno-modulators to boost the activity of the vaccine and the patient's response.

5 Such adjuvants and/or immuno-modulators are understood by those skilled in the art, and are readily described in available published literature.

As contemplated herein, and depending on the type of vaccine being generated, the production of vaccine can, if desired, be scaled up by culturing cells in bioreactors or fermentors or other such vessels or devices suitable for the growing of
10 cells in bulk. In such apparatus, the culture medium would be collected regularly, frequently or continuously to recover therefrom any materials or antigens before such materials or antigens are degraded in the culture medium.

If desired, devices or compositions containing the vaccine or antigens produced and recovered, in accordance with the present invention, and suitable for
15 sustained or intermittent release could be, in effect, implanted in the body or topically applied thereto for a relatively slow or timed release of such materials into the body.

Other steps in vaccine preparation can be individualized to satisfy the requirements of particular vaccines. Such additional steps will be understood by those skilled in the art. For example, certain collected antigenic materials may be
20 concentrated and in some cases treated with detergent and ultracentrifuged to remove transplantation alloantigens.

HER3 Expression as a Biomarker for Diagnosis and Treatment of Disease

In another embodiment HER3 expression can serve as a biomarker for occult invasive disease in patients with Barrett's esophagus and high-grade dysplasia
25 (HGD). Additionally contemplated herein are therapeutics for targeting HER3 or CMET that may afford secondary prevention of gastroesophageal carcinoma in some patients.

These methods described herein are by no means all-inclusive, and
30 further methods to suit the specific application will be apparent to the ordinary skilled artisan. Moreover, the effective amount of the compositions can be further approximated through analogy to compounds known to exert the desired effect.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following
5 examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. The
10 following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Example 1: Creating peptide vaccines against other receptor tyrosine kinases that
15 cause breast cancer and other solid cancers

Experiments were designed to develop alternative therapies against patients designated as BRCA mutation carriers. That is, there is an unmet need for younger patients genetically at risk for breast cancer who are seeking alternatives to bilateral mastectomy.

20 Women with the breast cancer gene mutations BRCA1/BRCA2 have a 70% lifetime risk of developing breast cancer, and BRCA1 mutation carriers often develop triple-negative breast cancer. Experiments were designed to develop vaccines for this group and evaluate their safety in an immune-inducing trial, which is the first attempt ever at vaccination for primary prevention of breast cancer. BRCA2 mutation
25 carriers will also be included to see if estrogen receptor^{positive} breast cancer can be prevented using the multivalent vaccine of the invention.

Experiments were designed to study receptor tyrosine kinase expression in breast cancers and DCIS from BRCA mutation carriers. It was observed that tumors from the BRCA mutation carriers frequently over-expressed the c-MET
30 oncogene and HER-3 early on while the tumors from non-mutation or sporadic patients expressed HER-2 and HER-3. This is important because targets for tumor immunotherapy that can be used to develop vaccines for sporadic and BRCA mutation carriers it is now known based on the disclosure presented herein. This is the first distinguishing feature that can be targeted using immune response for prevention.

Accordingly, the invention includes compositions and methods for developing vaccines and uses thereof for prevention as an alternative to bilateral mastectomies.

Creating peptide vaccines

5 The HER family consists of four related signaling molecules—HER-1, HER-2, HER-3, and HER-4—that are involved in a variety of cancers. It is known that over-expression of HER-2 is found in 20% to 30% of breast cancers. The results presented herein demonstrate that other HER family members are involved in both early and invasive breast cancer, as well as other cancers. For example, HER-1 is
10 expressed on a small number of breast cancers, generally those that are triple negative. c-MET is a growth factor receptor involved in recurrence of many cancers that activates HER-3. HER-3 is over-expressed in colon, prostate, breast and melanoma. HER-3 is expressed in a large number of DCIS lesions and breast cancers. HER-3 can be detected in the residual DCIS at the time of surgery in some patients
15 who received a HER-2 vaccine. As a result of these findings, the potential to target these molecules in addition to HER-2 in breast cancer is believed to be beneficial.

 Immunogenic peptides from HER-3 have been identified (Figures 1 and 2) as follows:

p11-13 (Peptide 51-75): KLYERCEVVMGNLEIVLTGHNADLSFLQW (SEQ ID
20 NO: 1);
p81-83 (Peptide 401-425): SWPPHMHNFSVFSNLTTIGGRSLYN (SEQ ID NO: 2);
p84-86 (Peptide 416-440): TTIGGRSLYNRGFSLIMKNLNVTS (SEQ ID NO: 3);
p12 (Peptide 56-70): CEVVMGNLEIVLTGH (SEQ ID NO: 4);
p81 (Peptide 401-415): SWPPHMHNFSVFSNL (SEQ ID NO: 5);
25 p84 (Peptide 416-430): TTIGGRSLYNRGFSL (SEQ ID NO: 6); and
p91 (Peptide 451-465): AGRIYISANRQLCYH (SEQ ID NO: 7).

 The results presented herein demonstrate that these peptides can activate CD4 T cells across many patients. The peptides can be used to pulse dendritic cells and educate T cells to recognize HER-3. HER-3 is expressed in triple-negative
30 breast cancer and can impart resistance to anti-estrogen in ER^{positive} breast cancers. HER-3 is also expressed in other cancers, including melanoma, lung, colon, prostate cancer, and metastatic brain tumors. Without wishing to be bound by any particular theory, peptides from the intracellular part of the molecule may also be advantageous.

Based on the disclosure presented herein, immunogenic peptides for HER-1 and the c-MET receptor tyrosine kinase molecules can be screened and identified based on the procedure that identified immunogenic peptides for HER-3. The immunogenic peptides of the invention can be used to prepare a multivalent preventive vaccine for breast cancer as well as other cancers.

The results presented herein show the identification of the role of HER-2's sister proteins in breast cancer. These sister proteins can be effectively targeted and vaccines for other solid tumors can be developed. Peptides that can be used to target HER-1 and HER-3 have been developed. In DCIS specifically, specific anti-HER-1, HER-2, and HER-3 responses in patients before and after vaccination have been identified, which provides support for the development of a multivalent vaccine that can be used to prevent early cancer or treat women who have DCIS. The compositions of the invention is useful to treat other cancers including but not limited to colon cancer, melanoma, brain tumors, lung cancer, ovarian cancer, and other tumors.

Melanoma

Melanoma is an aggressive skin cancer that can be deadly if not caught early. Experiments were conducted in mice using a standard dendritic cell vaccine wherein the dendritic cell was engineered to exhibit a mutated protein (BRAF) that causes about 70% of melanomas. Vaccination with these dendritic cells protected the mice from challenge with melanoma cells, demonstrating that it is possible to develop vaccines for melanoma. Without wishing to be bound by any particular theory, combinations of BRAF and HER-3 targeting may be useful for treating melanomas as well as other cancers including but not limited to solid cancers, such as colon, pancreatic, and lung cancers, and other gastrointestinal tumors.

In addition, it has been shown that melanoma tumors use B cells to escape immune surveillance, and therefore it is believed that eliminating certain B cells can improve therapy. Experiments can be designed to assess whether altering the tumor microenvironment to a Th1-type response can help to prevent escape.

In some instances, the vaccine of the invention can be used to treat melanoma that has spread. In some instances, the invention provides therapies to eliminate remaining cells that often become resistant to drug therapy.

Example 2: Novel Strategy to Identify MHC Class II-Promiscuous CD4⁺ Peptides from Tumor Antigens for Utilization in Vaccination

Although cytotoxic CD8⁺ T lymphocytes (CTL) were historically considered primary effectors of antitumor immunity, solely boosting CTL responses with CD8⁺ vaccines in various tumor types has yielded unpredictable clinical results, possibly because CTLs function suboptimally without adequate CD4⁺ T-lymphocyte help. CD4⁺ T-helper type 1 (Th1) cells secrete INF- γ /TNF- α , inducing tumor senescence and apoptosis. As such, successful incorporation of CD4⁺ epitopes into cancer vaccine construction and generation of durable antigen-specific CD4⁺ immunity remains a challenge. Using the extracellular domain (ECD) of HER3 as a candidate "oncdriver" tumor antigen, experiments were performed to identify immunogenic HER3 CD4⁺ peptides that demonstrate Class II promiscuity and generate anti-HER3 CD4⁺ immunity for inclusion in a vaccine construct.

The materials and methods employed in these experiments are now described.

Materials and Methods

Experiments were designed to identify immunogenic Class II-promiscuous HER3 CD4⁺ peptides using the ECD of HER3 as a tumor antigen in order to generate anti-HER3 Th1 cellular immunity.

Protocol Overview

A library of 15-mer long peptides that overlap by 5 amino acids was created from the HER3 ECD. These peptides were pulsed onto monocyte-derived DCs from donors and were matured to type 1-polarized (DC1; IL-12-secreting) phenotype. The DC1s were harvested and co-cultured with purified CD4⁺ T cells from subjects who had known anti-HER-3 Th1 responses from our DCIS vaccine study. Large pools of 10 peptides were used and the identification process was progressively narrowed down to single reactive epitopes as measured by interferon gamma (IFN- γ) secretion of the CD4⁺ T cells. Upon screening 5-6 subjects, 4 peptides were identified that seemed to react across most donors i.e., HER3₅₆₋₇₀ (SEQ ID NO: 4), HER3₄₀₁₋₄₁₅ (SEQ ID NO: 5), HER3₄₁₆₋₄₃₀ (SEQ ID NO: 6), and HER3₄₅₁₋₄₆₅ (SEQ ID NO: 7). Subjects with no evidence of reactivity to CD4⁺ T cell recognition of HER-3 extracellular domain were identified and their DC1s were pulsed with the four HER-3 peptides and the pulsed DC1s were cultured with CD4 T cells for a week and then tested for

reactivity against HER-2 peptide and reaction to extracellular HER-3 protein. In all cases, at least 1 peptide led to recognition of both the peptide pulsed on monocytes and the whole HER-3 protein suggesting that primary sensitization had taken place ex vivo. It was also shown that healthy donors can react to these peptides and in triple negative breast cancer patients where there is a loss of anti-HER-3 Th1 responses. See, also, Gala, K., et al., Clin. Cancer Res 2014; 20: 1410-1416 and Datta, J., et al., "Progressive Loss of Anti-HER2 CD4+ T-helper Type 1 Response in Breast Tumorigenesis and the Potential for Immune Restoration". OncoImmunology (in press).

10 Protocol Highlights as further illustrated in Figure 19:

- A library comprising 123 overlapping 15 amino acid-long peptide fragments that overlapped by 5 amino acids was generated from the HER3 extracellular domain (ECD).
- Autologous monocyte-derived dendritic cells (DC) from donors were rapidly matured to a type 1-polarized (DC1 → IL-12 secreting) phenotype via GM-CSF, IFN- γ and LPS, and pulsed with relevant peptides (e.g., HER3 ECD or HER3 CD4⁺ peptides, where indicated). DC1 polarize Th1 responses via elaboration of IL-12.
- Harvested DC1s were allosensitized with purified CD4⁺ T-cells in 8-10 day co-cultures.
- Sensitized CD4⁺ T-cells (a large fraction of which are expected to become antigen-specific) were restimulated against immature DCs (iDC) that were pulsed with a specific CD4⁺ peptide of interest (e.g., HER3 library peptide clusters) or irrelevant class II peptide control.
- The supernatant from these co-cultures were then harvested. Th1 responses, measured by IFN- γ ELISA, were considered antigen-specific if IFN- γ production was at least twice that of irrelevant control.
- HLA-DR, DP, DQ typing was performed on donors by the Clinical Immunology laboratory at the Hospital of the University of Pennsylvania in order to assess MHC class II promiscuity of CD4⁺ Th1 responses.

A library comprising 123 overlapping 15 amino acid-long peptide fragments was generated from the HER3-ECD. Autologous monocyte-derived DCs

from donors were matured to DC1s, and pulsed with HER3-ECD. Harvested DC1s were co-cultured with purified CD4 T cells. After 10 days, sensitized CD4 T cells were restimulated against immature DCs (iDC) that were pulsed with HER3 library peptide clusters or irrelevant CD4 control peptide. Th1 responses, measured by IFN- γ ELISA, were considered antigen-specific if IFN- γ production was at least twice that of irrelevant control.

Experiments were performed in a 3-step process: 1) breast cancer patients with *known* anti-HER3 ECD reactivity following HER2-pulsed DC1 vaccine were obtained in order to identify immunogenic CD4+ peptides; 2) the immunogenicity of these peptides were confirmed in the same patients by a process of "reverse" sensitization; 3) patients with known anti-HER3 ECD non-reactivity following vaccination were obtained and used to identify CD4+ peptides to see if the cells were sensitized to the native HER3 ECD, thus overcoming/abrogating self-antigen (i.e., HER3) tolerance.

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The results of the experiments are now described.

Sequential screening of HER3 ECD peptide library to identify immunogenic epitopes recognized by HER3 ECD-sensitized CD4+ Th1 cells

Th1 sensitization was initially performed in 5 breast cancer patients with known anti-HER3 ECD reactivity in order to identify single immunogenic HER3 CD4+ epitopes. To achieve this, HER3 ECD-sensitized CD4+ Th1 were sequentially restimulated against 10-peptide clusters (1-10, 11-20, ... etc.), narrowed to 3-peptide clusters (1-3, 3-6, 7-10, ... etc.), and ultimately to single immunogenic HER3 peptides. Representative screens are shown in Figures 2, 13 and 14. Four immunogenic peptides - HER3(56-70) (SEQ ID NO: 4), HER3(401-415) (SEQ ID NO: 5), HER3(416-430) (SEQ ID NO: 6), and HER3(451-465) (SEQ ID NO: 7) - were reproducibly identified and promiscuous across HLA-DR, DP, and DQ subtypes. When Th1 cells from 4 *non-HER3 reactive* donors were sensitized using DC1s pulsed with the four identified HER3 peptides, and subsequently challenged to recognize HER3 ECD-pulsed iDCs, all donors demonstrated successful sensitization not only to individual immunogenic HER3 peptides, but also recognized native HER3-ECD.

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The results presented herein demonstrate that DC1 pulsed with an overlapping tumor antigen-derived peptide library can identify promiscuous class II peptides for CD4 T cell vaccine development. In this study, immunogenic HER3 CD4 peptides effectively overcome immune tolerance to self-tumor antigens. Utilization of these HER3 CD4 peptides in vaccine construction can be applied to patients harboring HER3-overexpressing cancers. Additionally, these results represent a novel strategy to rapidly and reproducibly identify class II-promiscuous immunogenic CD4 epitopes from any tumor antigen for cancer immunotherapy using a DC1-Th1 platform. Table 1 below shows initial identification of immunogenic CD4+ HER3 ECD peptides in patients with known anti-HER3 reactivity. Table 2 shows the amino acid sequences of the four immunogenic HER3 CD4+ epitopes identified by the sequential screening.

TABLE 1 - Four immunogenic peptides – HER3₅₆₋₇₀ (SEQ ID NO: 4), HER3₄₀₁₋₄₁₅ (SEQ ID NO: 5), HER3₄₁₆₋₄₃₀ (SEQ ID NO: 6), HER3₄₅₁₋₄₆₅ (SEQ ID NO: 7) – were reproducibly identified across 5 donors previously sensitized to HER3 ECD

Donor #	HER3 ₅₆₋₇₀	HER3 ₄₀₁₋₄₁₅	HER3 ₄₁₆₋₄₃₀	HER3 ₄₅₁₋₄₆₅
15107-38	✓			✓
15107-24	✓	✓	✓	
15107-26	✓	✓	✓	✓
26113-03	✓	✓	✓	✓
15107-31			✓	✓

TABLE 2 - Amino acid sequences of immunogenic HER3 CD4+ epitopes

HER3 ₅₆₋₇₀	CEVVMGNLEIVLTGH (SEQ ID NO: 4)
HER3 ₄₀₁₋₄₁₅	SWPPMHNFVFSNL (SEQ ID NO: 5)
HER3 ₄₁₆₋₄₃₀	TTIGGRSLYNRGFSL (SEQ ID NO: 6)
HER3 ₄₅₁₋₄₆₅	AGRIYISANRQLCYH (SEQ ID NO: 7)

Confirmation of immunogenicity of identified CD4+ HER3 ECD epitopes by "reverse" sensitization – i.e. ability of individual epitope-sensitized CD4+ Th1 to recognize native HER3 ECD

Figure 15 shows that in the donors with known HER3 ECD reactivity, CD4+ T-cells were sensitized with respective donor-specific immunogenic HER3 epitope-pulsed DC1s, and restimulated against iDCs pulsed with respective HER3 epitope and native HER3 ECD.

Figures 20 and 21 show additional results of "reverse" sensitization

CD4+ Th1 sensitized with immunogenic HER3 epitope-pulsed DC1 appears to abrogate anti-HER3 immune self-tolerance

As seen in Figure 16, when CD4+ Th1 cells from four HER3 ECD nonreactive donors were sensitized using DC1s pulsed with the four identified HER3 peptides, and subsequently challenged to recognize HER3 ECD-pulsed iDCs, all donors demonstrated successful sensitization not only to individual HER3 epitopes, but also recognized native HER3 ECD.

CD4+ HER3 epitopes demonstrate MHC class II promiscuity

Using the extracellular domain (ECD) of HER3 as a candidate "oncdriver" tumor antigen, experiments were performed to identify immunogenic HER3 CD4+ peptides that demonstrate Class II promiscuity and generate anti-HER3 CD4+ immunity that can be used in a vaccine construct as seen in Figure 17.

Peptides From Tumor Antigens

The results presented herein demonstrate that:

- DC1 pulsed with an overlapping tumor antigen-derived peptide library can identify promiscuous MHC class II peptides for CD4+ T-cell vaccine development.
- Immunogenic HER3 CD4+ peptides effectively overcome immune tolerance to self-tumor antigens.
- These results represent a novel strategy to rapidly and reproducibly identify class II-promiscuous immunogenic CD4+ epitopes from any tumor antigen for cancer immunotherapy using a DC1-CD4+ Th1 platform.

- Utilization of these HER3 CD4+ peptides in vaccine construction warrants investigation in patients harboring HER3-overexpressing cancers.

Example 3: HER3 Expression is a Marker of Tumor Progression in Premalignant Lesions of the Gastroesophageal Junction

Over-expression of receptor tyrosine kinases (RTKs) including members of the HER family, has prognostic and therapeutic significance in invasive esophagogastric carcinoma. RTK expression in premalignant gastroesophageal lesions has not been extensively explored previously.

- Barrett's esophagus, or the presence of metaplastic columnar epithelium in the distal esophagus, predisposes to the development of esophageal adenocarcinoma. (Cameron, A.J., et al., *Gastroenterology* 109(5):1541-6 (1995).) While the histologic transition from dysplasia to invasive malignancy is well characterized, carcinogenesis in metaplastic cells involves genetic alterations that are incompletely understood.
- Several recent reports have identified human epidermal growth factor receptor 2 (HER2) expression in a subset of Barrett's esophagus lesions with dysplasia. (Almhanna, K., et al., *Appl. Immunohistochem. Mol. Morphol.* July 16 2015) (Almhanna, et al.); Fassan, M., et al., *Histopathology* 61(5): 769-76 (2012) (Fassan, et al.); and Rossi, E., et al., *J. Cell. Mol. Med.* 13(9B):3826-33 (2009) (Rossi, et al.).
- Furthermore, the rate of HER2 expression correlates with degree of dysplasia, implicating related pathways in tumorigenesis.

- Over-expression of receptor tyrosine kinase (RTK) molecules, including members of the HER family (HER1, HER2, and HER3) and cMET, the mesenchymal-epithelial transition factor, have been demonstrated in many of the more common malignancies, including breast, lung, and gastrointestinal cancers (Yokata, J., et al., *Lancet* 1:765-767 (1986) as well as in esophagogastric carcinomas. The identification of HER2 overexpression in a subset of breast carcinomas, the association of HER2 overexpression with more aggressive biology and effective targeting of HER2 with a monoclonal antibody were pivotal events in the evolution of targeted therapies for the treatment of solid tumors. (Joensuu, H., et al., *N. Eng. J. Med.* 354(8):809-20 (2006).) This experience has provided a foundation for further efforts to target RTK molecules in the treatment of other malignancies.

HER2 overexpression has been demonstrated in a minority of gastric cancers and has been targeted with trastuzumab in the metastatic setting with a modest impact

on outcome. (Bang, Y.J., et al., *Lancet* 376(9742):687-97 (2010) (Bang, et al.))
HER2 overexpression is more frequently expressed in proximal gastric and
gastroesophageal junction compared to more distal gastric adenocarcinomas
(Rajagopal, I., et al., *J. Clin. Diagn. Res.* 9(3):EC06-10 (2015)) and has been targeted
5 with trastuzumab in the metastatic setting with a modest impact on outcome. (Bang,
et al. and Fichter, C.D., et al., *Int. J. Cancer* 135(7):1517-30 (2014) (Fichter, et al.))
HER1 and HER3 expression in gastric carcinomas have been associated with poor
prognosis in most studies. (Kandel, C., et al., *J. Clin. Pathol.* 67(4):307-12 (2014)
and Hayashi, M., et al., *Clin. Cancer Res.* 14(23):7843-9 (2008).) Overexpression of
10 cMET is associated with poor prognosis in esophageal adenocarcinoma and inhibition
of cMET-dependent signaling regulates the activity of HER1 and HER3. (Liu, X., et
al., *Clin. Cancer Res.* 17(22):7127-38 (2011).) These data provide rationale for
further efforts to characterize RTK expression in esophagogastric cancers and
precursor lesions. The studies described below are aimed to characterize RTK
15 expression in dysplastic lesions of the gastroesophageal junction in efforts to identify
potential targets for treatment and primary prevention.

METHODS

Following Institutional Review Board approval, the clinical records and
histologic specimens from 73 patients with Barrett's esophagus with dysplasia (low-
20 grade dysplasia (LGD), n=32, or high-grade dysplasia(HGD), n=59) were
retrospectively reviewed. Formalin-fixed paraffin-embedded tissue blocks from
stored endoscopic biopsy and mucosal resection specimens from 2003-2012 were
sectioned at 5µm on plus slides (Fisher Scientific, Waltham, MA) and subsequently
deparaffinized and rehydrated. All biopsy materials were immunostained for HER1
25 (clone H11; 1:50; DAKO), HER2 (HercepTest, DAKO, Carpinteria, CA) and HER3
(clone RTI.2; 1:30; Santa Cruz Biotechnology, Dallas, TX) (Leica Bond-III
instrument) and evaluated under the microscope (Leica Bond-III) by a single
pathologist. Membrane 3+ HER staining was considered positive, as was membrane
2+ HER2 staining in $\geq 10\%$ of tumor cells as seen in Figure 22. cMET
30 immunohistochemistry was performed in 42 cases when sufficient tissue was
available; moderate or strong membranous staining in $\geq 50\%$ of tumor cells was
considered positive. RTK overexpression was correlated with clinical data to evaluate
for associations with invasive carcinoma, either paired dysplasia-adenocarcinoma

biopsy specimens or the diagnosis of adenocarcinoma on subsequent biopsy specimens.

Statistical Analysis

Two tailed tests were used for all analyses. Descriptive statistics are presented as frequencies for categorical variables and median (interquartile range (IQR) for continuous variables. Pearson's χ^2 or Fisher's exact tests and Wilcoxon rank-sum test were used to analyze categorical and continuous variables, respectively. P-values ≤ 0.05 were considered statistically significant; all tests were two-sided. Analyses were carried out using SPSS v22.0 (IBM, Armonk, NY).

RESULTS

A total of 73 patients with Barrett's esophagus with low-grade dysplasia (n=32) or high-grade dysplasia (n=59) were identified and analyzed for HER1, HER2, HER3 and cMET expression by immunohistochemistry. Median age of the cohort was 65 years (IQR 60-73 years); 81.9% were male and 87.5% were Caucasian. The rate of alcohol use in the cohort was 14.3% and the rate of active cigarette use was 6.3%, yet 55.6% were former smokers. 26.4% had a family history of malignancy. There were no significant differences between the LGD and HGD cohorts in the measured clinical and demographic variables as seen below in Table 1.

		LGD Median (IQR) or no. of patients (%)	HGD Median (IQR) or no. of patients (%)	p-value
Age, years		64.0 (60.0-77.5)	66.0 (63.0-72.0)	0.621
Sex, male		19 (73.1)	41 (87.2)	0.130
Caucasian race		23 (95.8)	41 (93.2)	0.755
Cigarette use	Current	2 (9.1)	2 (4.8)	0.681
	Former	13 (59.1)	23 (54.8)	
Alcohol use		2 (9.1)	7 (16.7)	0.683
Positive family history		6 (28.6)	13 (32.5)	0.753

20

High-grade dysplasia (HGD) was associated with overexpression of HER1 (22.4% vs. 3.1%, $p=0.016$), HER2 (5.3% vs. 0.0%, $p=0.187$) and HER3 (45.6% vs. 9.4%, $p<0.001$) compared to low-grade dysplasia (LGD).

Foci of invasive esophageal adenocarcinoma were associated with dysplastic lesions in 6 cases, all of which arose in association with HGD (HGD: 10.2% vs. LGD: 0.0%, $p < 0.001$). An additional 9 patients were diagnosed with invasive esophageal adenocarcinoma on subsequent biopsy specimens (HGD: 17.0% vs. LGD: 0.0%, $p = 0.017$). There was a significant association of HER3, but not HER1 or HER2 (increase in HER1 (26.7% vs. 20.5%, $p = 0.616$) and HER2 (14.3% vs. 2.3%, $p = 0.077$), overexpression in HGD lesions compares with those without foci of invasive carcinoma (71.4% vs. 38.6%, $p = 0.032$) as seen in Figures 23A and 23B.

Overexpression of cMET was observed in 18 of 42 (42.9%) evaluated specimens and was increasingly observed in HGD compared to LGD specimens (58.3% vs. 36.7%, $p = 0.200$) and was most often co-expressed with HER3 (62.5% of HER3-positive specimens vs. 38.2% of HER3-negative specimens ($p = 0.212$)). Similar trends were not observed in HER1-positive ($p = 0.729$) or HER2-positive ($p = \text{NA}$) specimens. One of the 42 (5.6%) patients had invasive carcinoma identified; cMET was overexpressed in this patient ($p = 0.243$).

DISCUSSION:

This analysis of RTK expression in dysplastic lesions of the gastroesophageal junction confirms that (1) HER family proteins are upregulated in Barrett's esophagus with dysplasia; (2) the frequency of HER family and cMET overexpression is positively correlated with the degree of dysplasia; and (3) HER protein upregulation, particularly in dysplastic lesions, is associated with an increased incidence of associated invasive cancer.

HER3 may therefore serve as a biomarker for occult invasive disease in patients with Barrett's esophagus and HGD. Additionally, therapeutics targeting HER3 or CMET may afford secondary prevention of gastroesophageal carcinoma in subsets of patients.

Previous evaluations of HER expression in Barrett's esophagus have been limited to an assessment of HER2, which is overexpressed in a minority of cases. *See, Almhanna, et al., Fassan, et al., and Rossi, et al.* HER2 overexpression in this study was present in 3.3% of biopsy specimens, lower than the rate of HER1 or HER3 overexpression. This pattern is consistent with HER family protein expression in invasive gastroesophageal junction cancers, where HER3 is overexpressed more commonly than HER2. Fichter, et al. Increasing HER3 protein overexpression with progression from LGD to HGD and frequent overexpression of HER3 in particular,

represent novel, though not unanticipated findings. Homo- and hetero-dimerization of HER receptors drive signal activation; clustered overexpression of multiple members of the HER family have been observed in other tumor types. In conjunction, activated c-MET positively regulates the activity of HER1 and HER3¹¹. Indeed, the interplay
5 between these receptors have provided rationale for multivalent therapeutic approaches targeting multiple receptor tyrosine kinases. (Baselga, J., et al., *N. Eng. J. Med.* 366(2):109-19 (2012); Waddell, T., et al., *Lancet Oncol.* 14(6):481-489 (2013))

The present data also suggest an opportunity for targeted secondary prevention of gastroesophageal carcinoma that has not yet been explored. Previously targeted
10 HER2 expression in ductal carcinoma in situ (DCIS) of the breast has been targeted with promising results. See, U.S. Ser. No. 14/ 658,095 filed March 13, 2015; U.S. Ser. No. 14/985,303 filed December 30, 2015; Datta, J., et al., *Oncotmmunology* 4:8 e1022301 (2015) DOI:10.1080/2162402X.2015.1022301; Datta, J., et al., *Breast Cancer Res.* 17(1):71 (2015). Such an approach remains a more distant goal for
15 gastrointestinal malignancies. Notwithstanding, current treatment options for Barrett's esophagus, including endoscopic resection and ablative modalities and radical surgery all have significant limitations. Alternative strategies that spare morbidity and mitigate the risk of invasive carcinoma are needed.

This analysis of RTK expression in dysplastic lesions of the gastroesophageal
20 junction confirms that (1) HER family proteins are upregulated in Barrett's esophagus with dysplasia; (2) the frequency of HER family and cMET overexpression is positively correlated with the degree of dysplasia; and (3) HER protein upregulation, particularly in dysplastic lesions, is associated with an increased incidence of associated invasive cancer.

25 HER3 may therefore serve as a biomarker for occult invasive disease in patients with Barrett's esophagus and HGD. Additionally, therapeutics targeting HER3 or CMET may afford secondary prevention of gastroesophageal carcinoma in subsets of patients.

In summary, the present data indicate a relationship between frequent
30 overexpression of HER3 in high-grade dysplastic lesions of the gastroesophageal junction, especially those with occult invasive carcinoma and malignant transformation. These findings may justify a more aggressive management approach for HER3-expressing dysplastic lesions and provide rationale for the future

application of HER3-targeted therapeutics in an early disease setting as will be readily appreciated by those skilled in the art.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

- 5 While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. An isolated peptide selected from the group consisting of p11-13 (Peptide 51-75): KLYERCEVVMGNLEIVLTGHNADLSFLQW (SEQ ID NO. 1), p81-83 (Peptide 401-425): SWPPHMHNFVFSNLTIGGRSLYN (SEQ ID NO. 2), p84-86 (Peptide 416-440): TTIGGRSLYNRGFSLIMKNLNVTS (SEQ ID NO. 3), p12 (Peptide 56-70): CEVVMGNLEIVLTGH (SEQ ID NO. 4), p81 (Peptide 401-415): SWPPHMHNFVFSNL (SEQ ID NO. 5), p84 (Peptide 416-430): TTIGGRSLYNRGFSL (SEQ ID NO. 6), and p91 (Peptide 451-465): AGRYISANRQLCYH (SEQ ID NO. 7).
2. An immunomodulating agent comprising one or more peptides of claim 1.
3. A vaccine comprising one or more peptides of claim 1 and a pharmaceutically acceptable salt.
4. The vaccine of claim 3 further comprising an adjuvant.
5. A cell, wherein the cell has been contacted with one or more peptides of claim 1.
6. The cell of claim 5, wherein said cell is an antigen presenting cell.
7. The cell of claim 5, wherein said cell is a T cell.
8. A method of eliciting an immune response in a subject comprising administering to the subject the composition of claim 1.
9. A method of treating cancer in a subject comprising administering to the subject one or more peptides of claim 1.
10. The method of claim 9, wherein said subject is a human and has cancer.
11. The method of claim 10, wherein said cancer is selected from the group consisting of breast cancer, ovarian cancer, lung cancer, prostate cancer, colon

cancer, melanoma, pancreatic cancer, gastrointestinal cancer, brain cancer, and any combination thereof.

12. A method of activating a cell comprising contacting said cell with one or more peptides of claim 1.

13. The method of claim 12, wherein said cell is an antigen presenting cell.

14. The method of claim 12, wherein said cell is a T cell.

15. A method of generating a peptide loaded, activated dendritic cells (DC) for use in immunotherapy, comprising:

pulsing said DC with one or more peptide of claim 1;

activating said DC with at least one TLR agonist.

16. The method of claim 15 comprising contacting said DC with an agent that elevates the intracellular calcium concentration in said DC.

17. The method of claim 15, wherein said agent comprises a calcium ionophore.

18. The method of claim 15, further comprising cryopreserving said DC, wherein when said DC is thawed, and said DC produces an effective amount of at least one cytokine to generate a T cell response.

19. A cell generated from the method of claim 15.

20. A vaccine comprising a cell generated from the method of claim 15.

21. The vaccine of claim 20, wherein said vaccine is in a form of an injectable multi-dose vaccine.

22. A method of eliciting an immune response in a mammal, comprising administering a population of cells generated from the method of claim 20 to a mammal in need thereof.

23. A method of treating a disease or disorder in a mammal, comprising administering a population of cells generated from the method of claim 20 to a mammal in need thereof.

24. A biomarker for detecting tumor progression in premalignant lesions of the gastroesophageal junction in a subject having Barrett's esophagus which comprises detecting overexpression of HER3 in said subject.

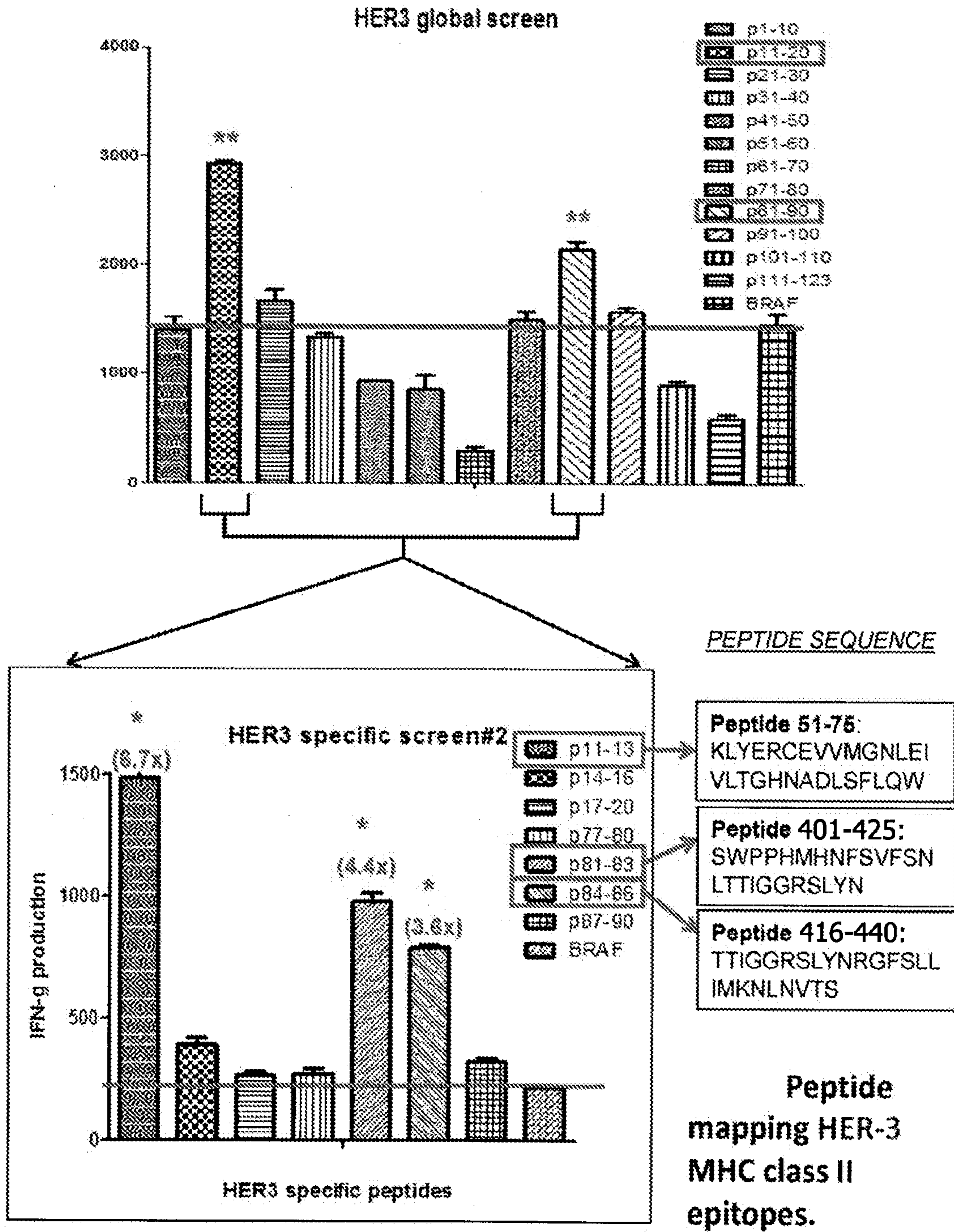


Figure 1

2/23

Patient UPCC 15107-24

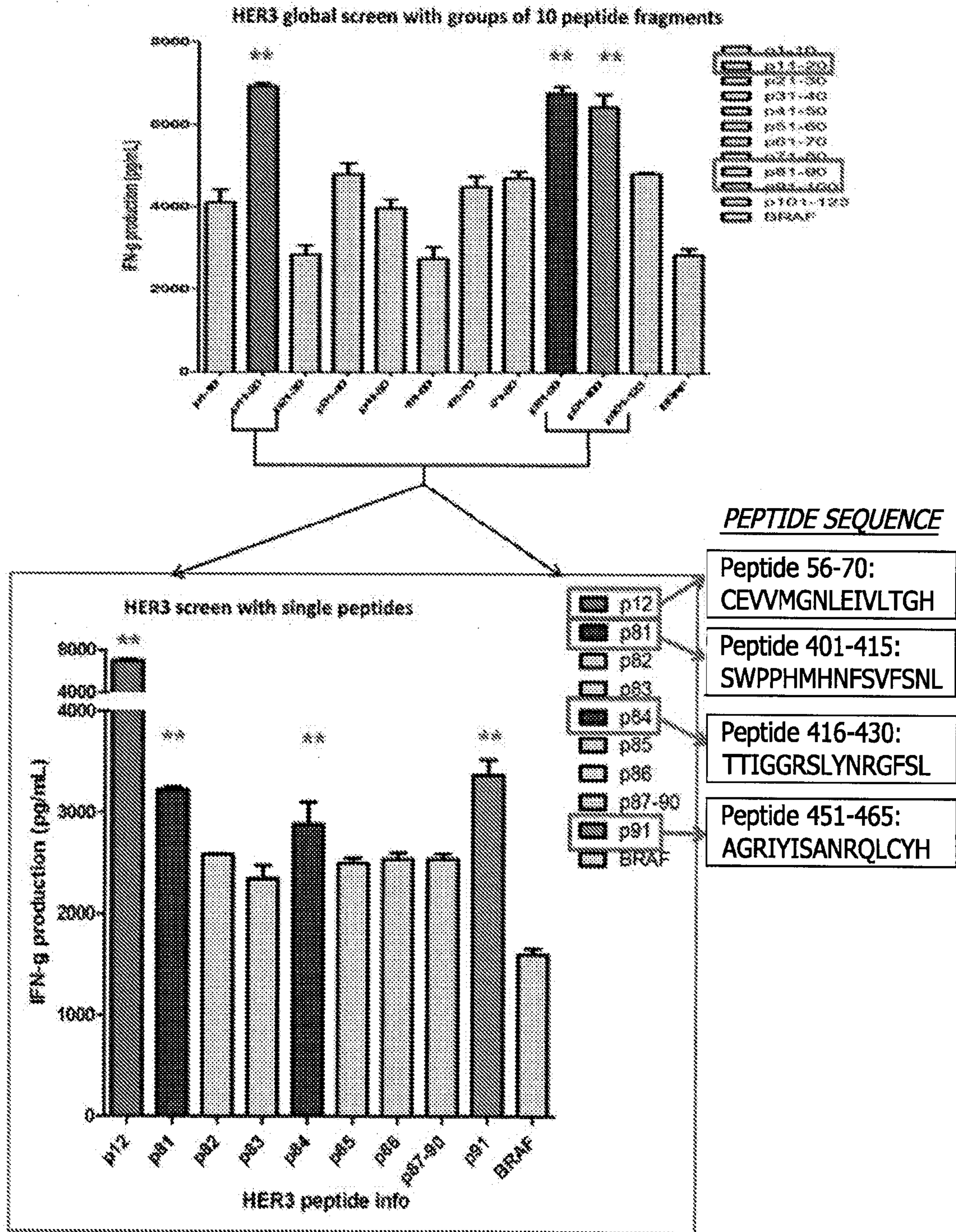


Figure 2

3/23

Protocol UPCC 15107-38: previously sensitized to HER3 ECD

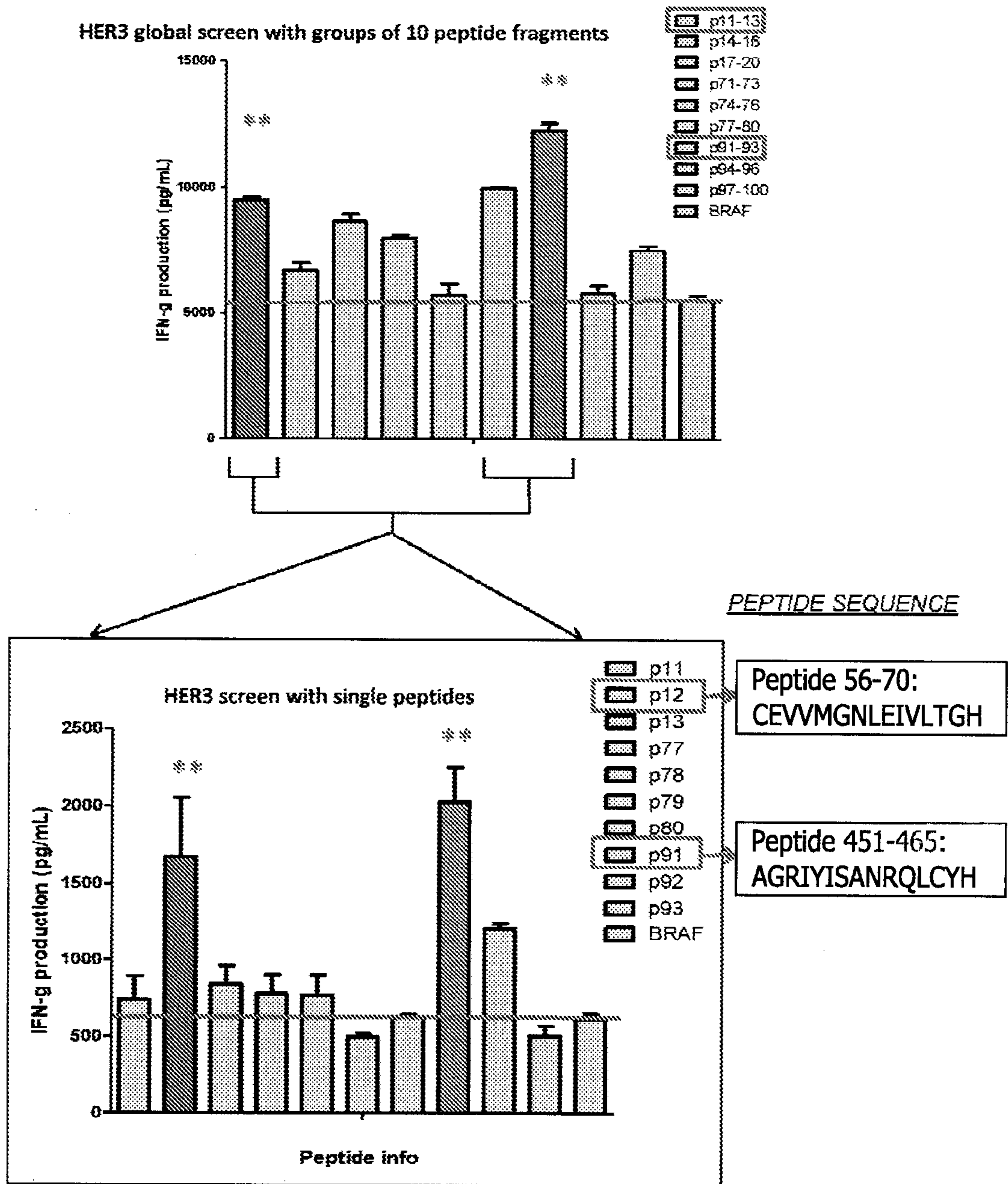


Figure 3

4/23

Protocol UPCC 15107-26: previously sensitized to HER3 ECD

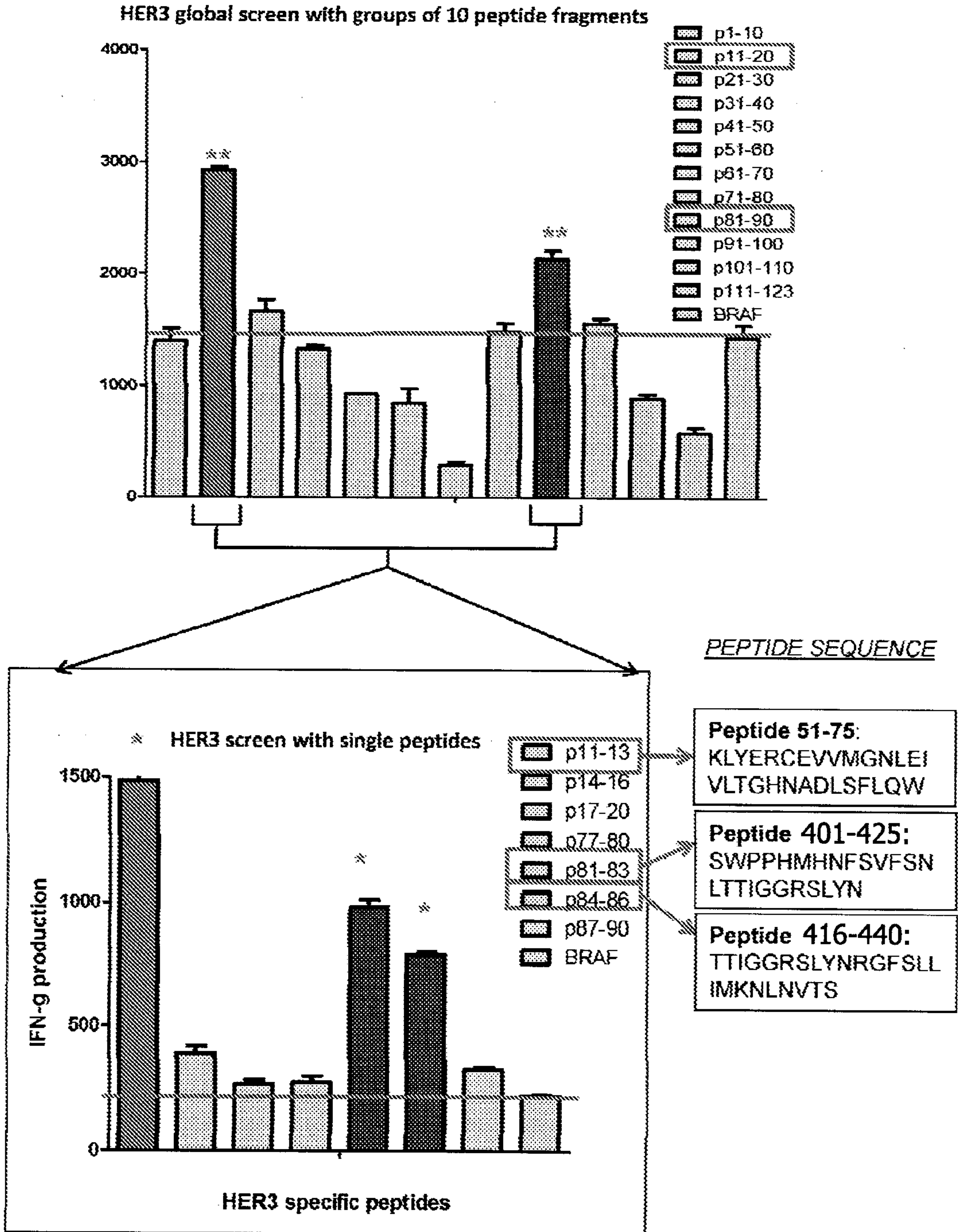


Figure 4

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Invasive breast cancer donor MN- previously sensitized to HER3

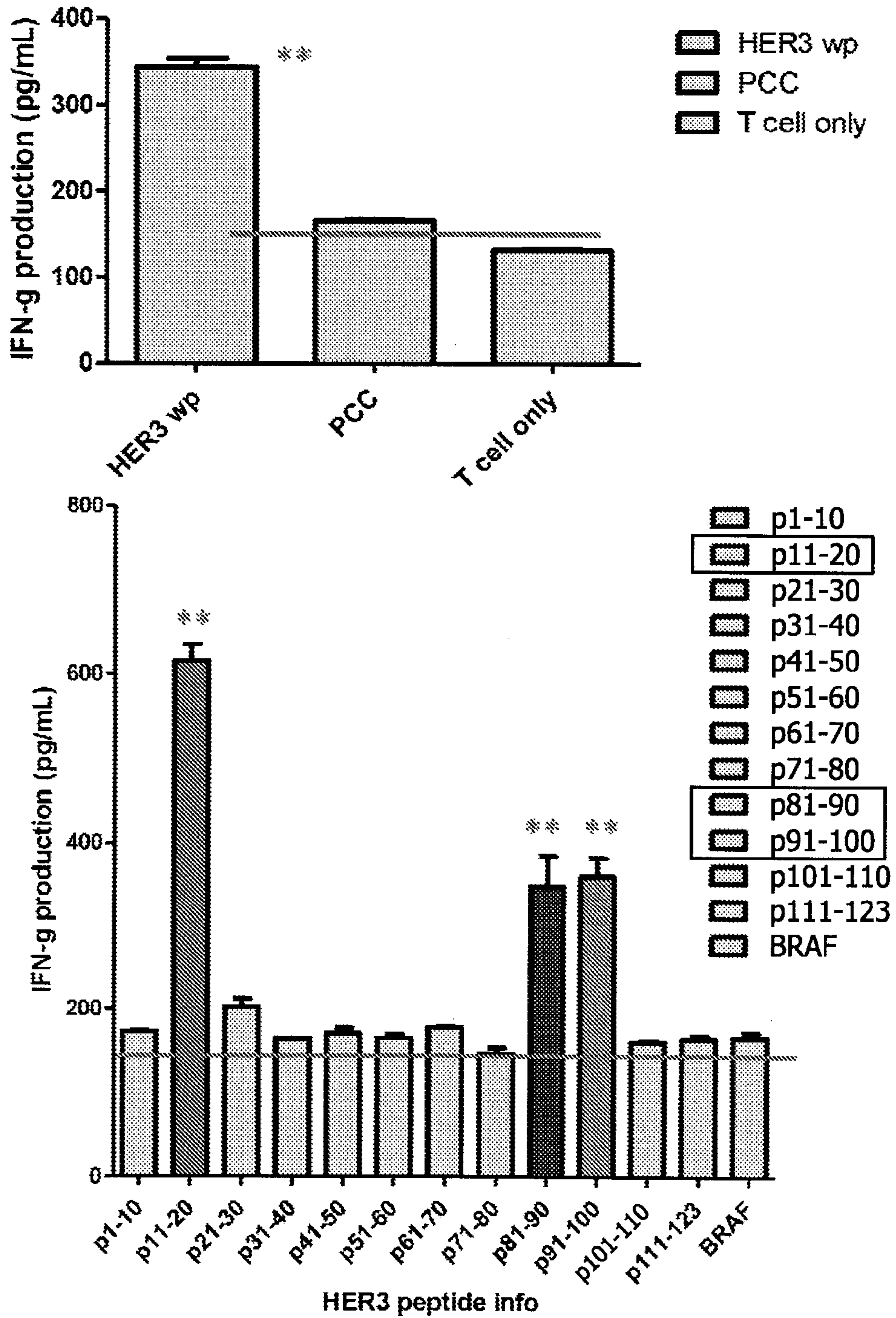


Figure 5

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Protocol UPCC 15107-31

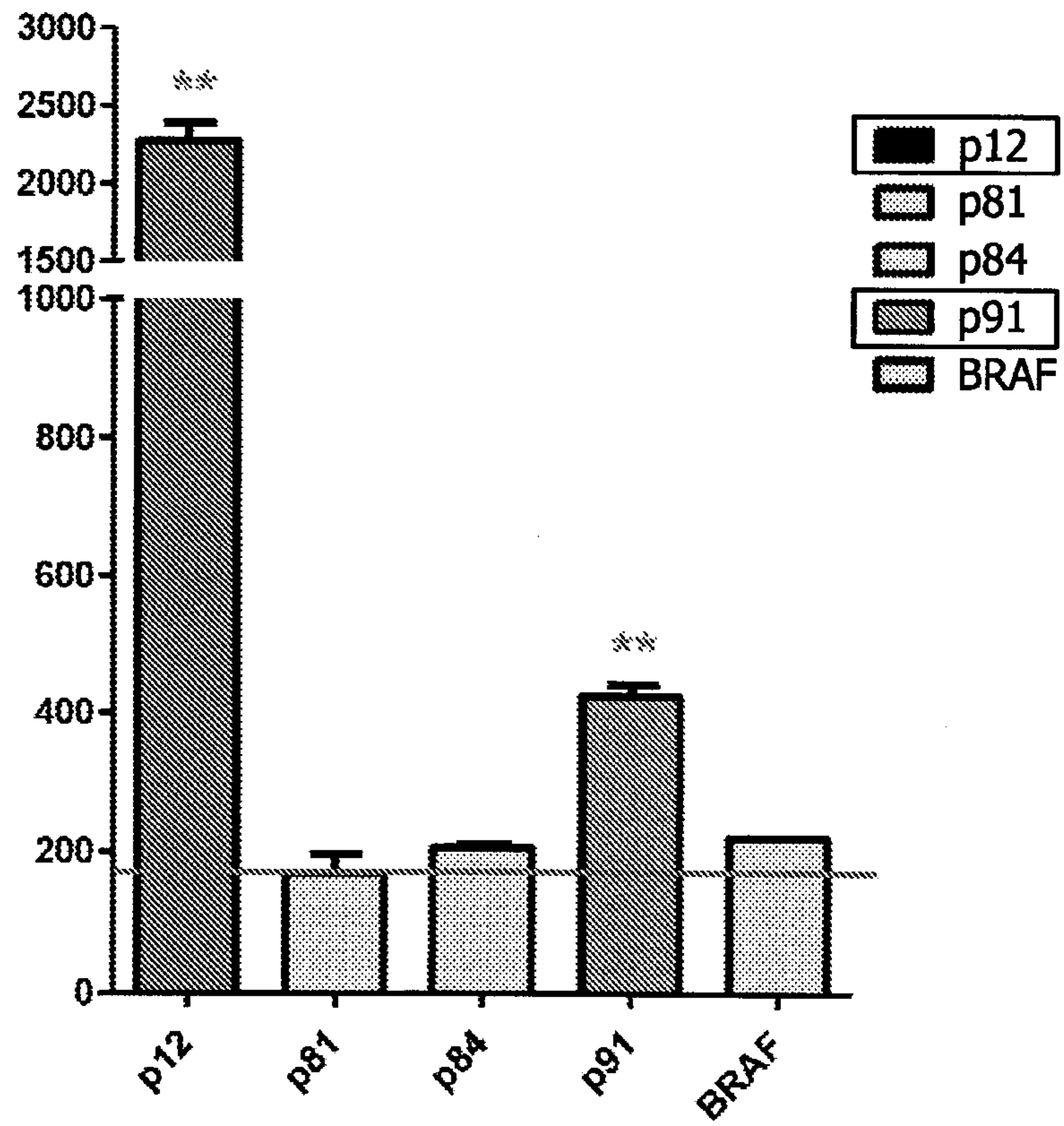
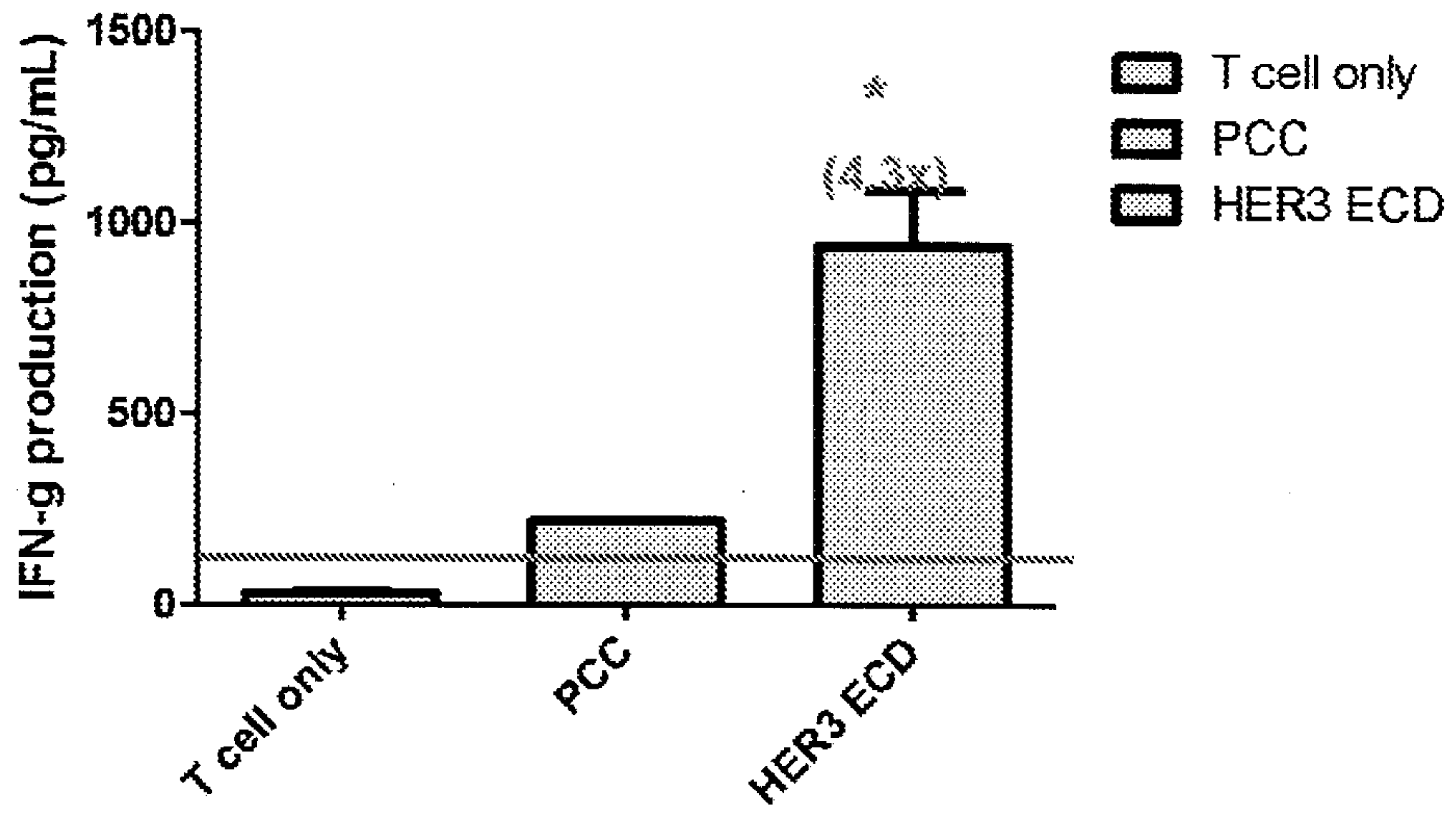


Figure 6

7/23

Protocol UPCC 15107-38

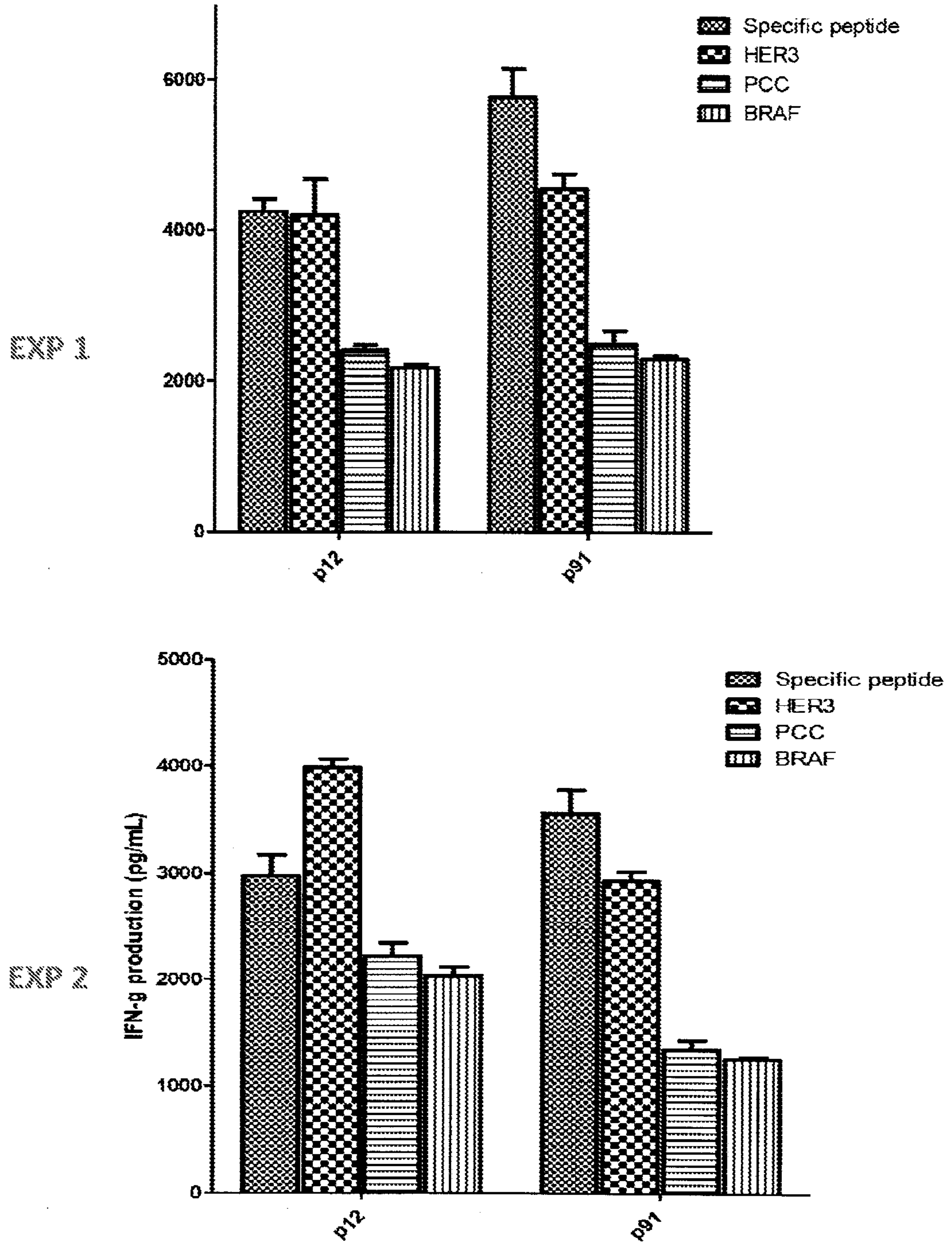


Figure 7

Protocol UPCC 15107-24

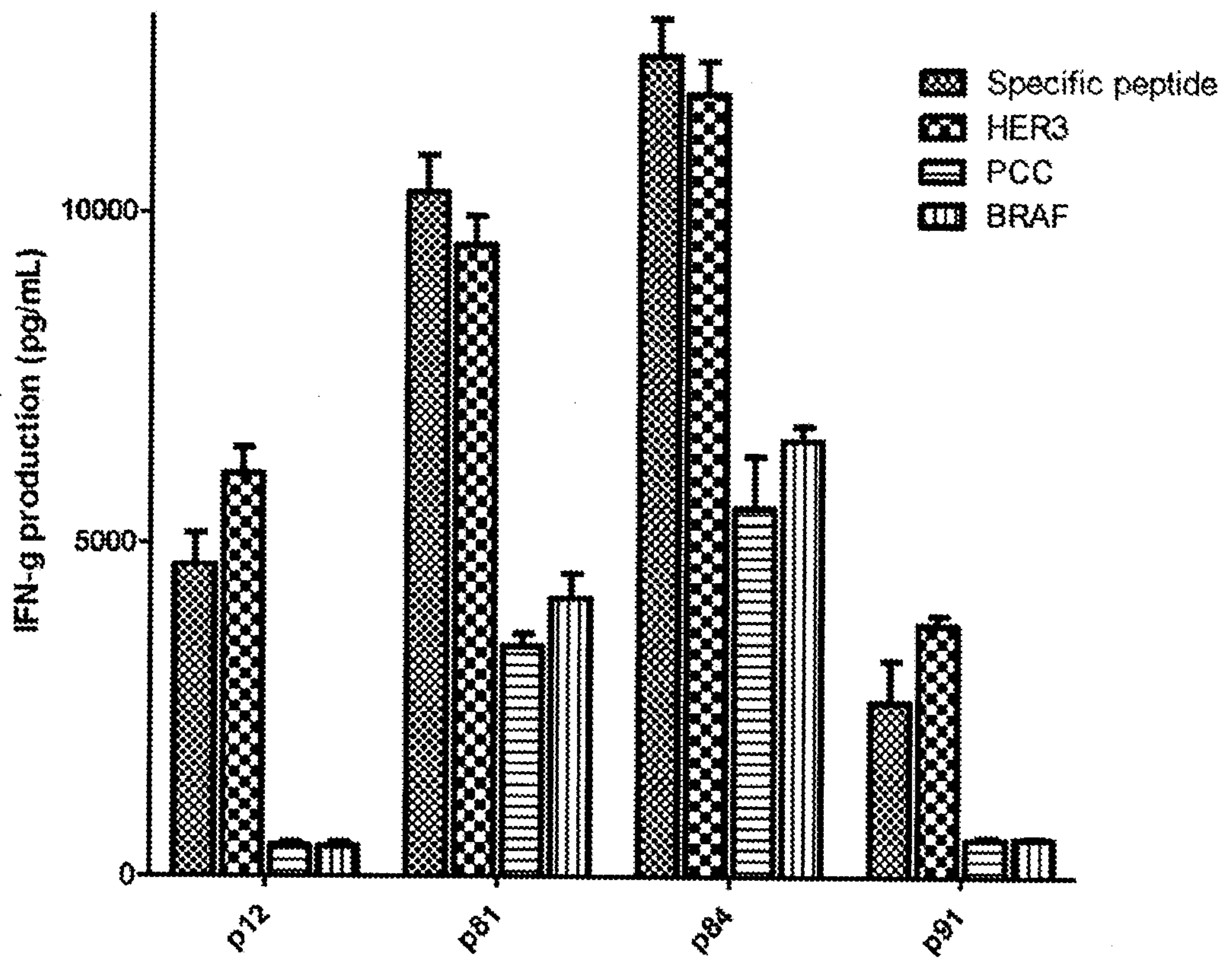


Figure 8

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"BREAKING TOLERANCE"

Protocol UPCC 15107-30

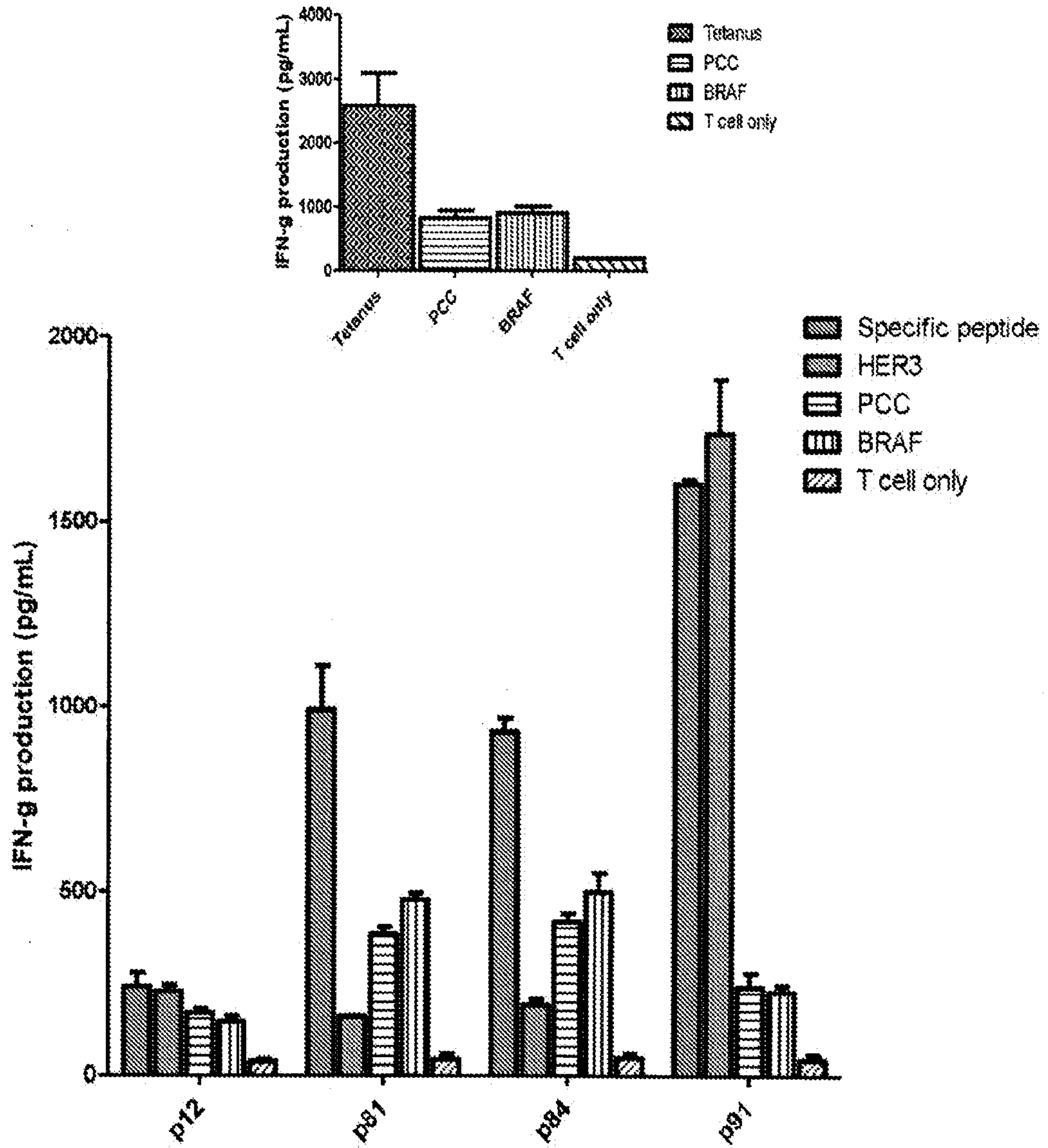


Figure 9

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"BREAKING TOLERANCE"

Protocol UPCC 15107-30

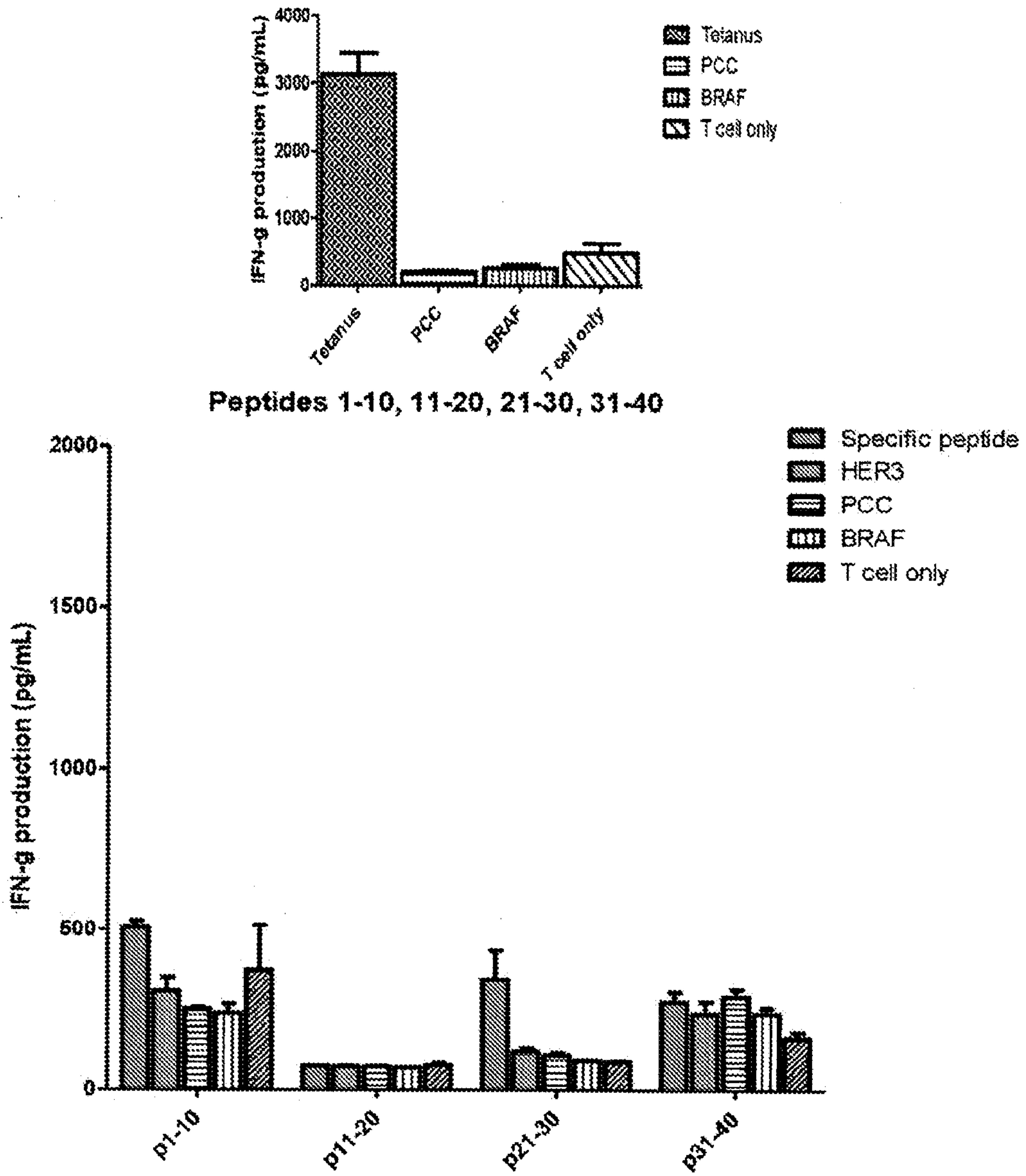


Figure 10

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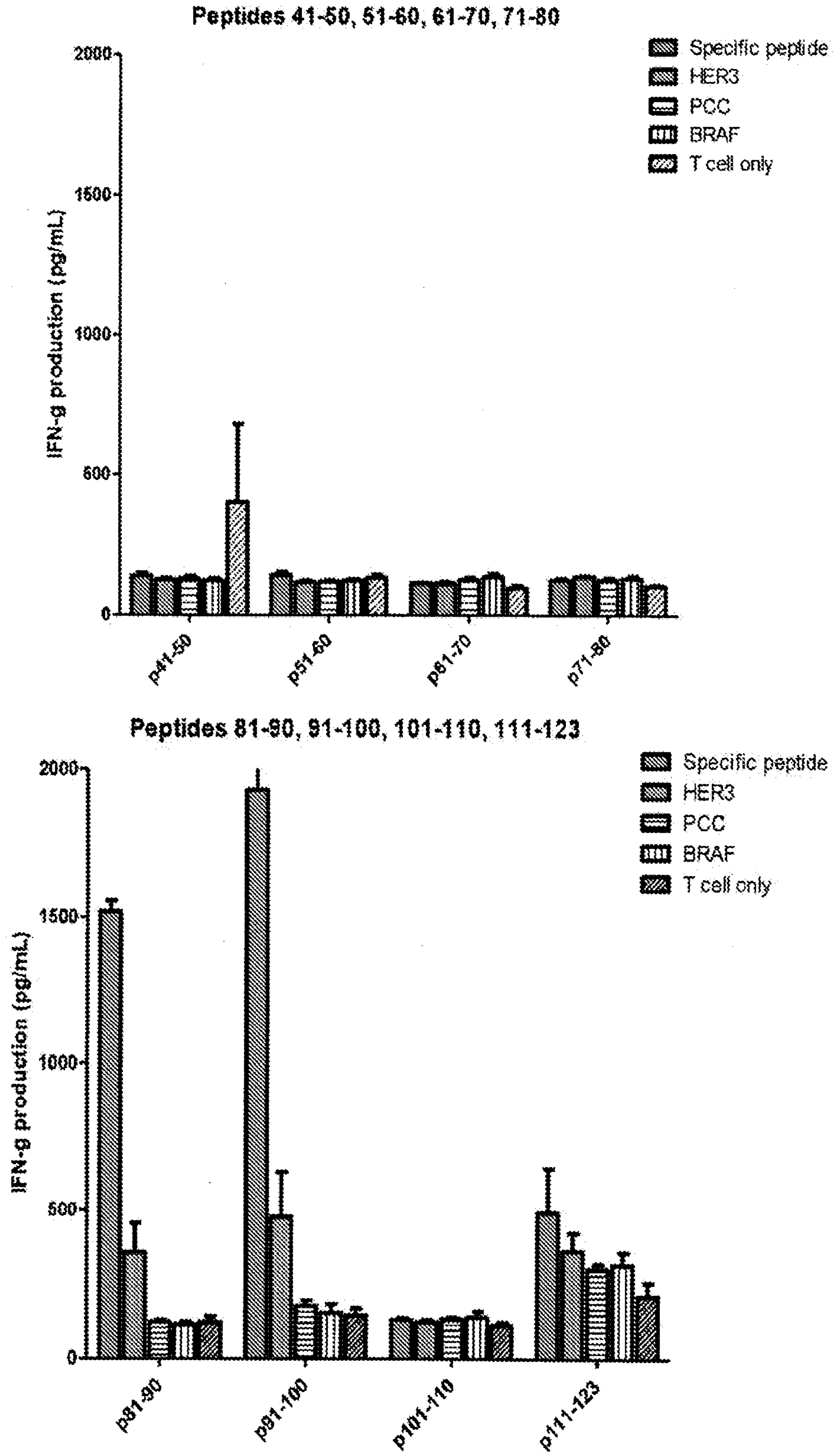


Figure 11

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"BREAKING TOLERANCE"

Protocol UPCC 15107-32

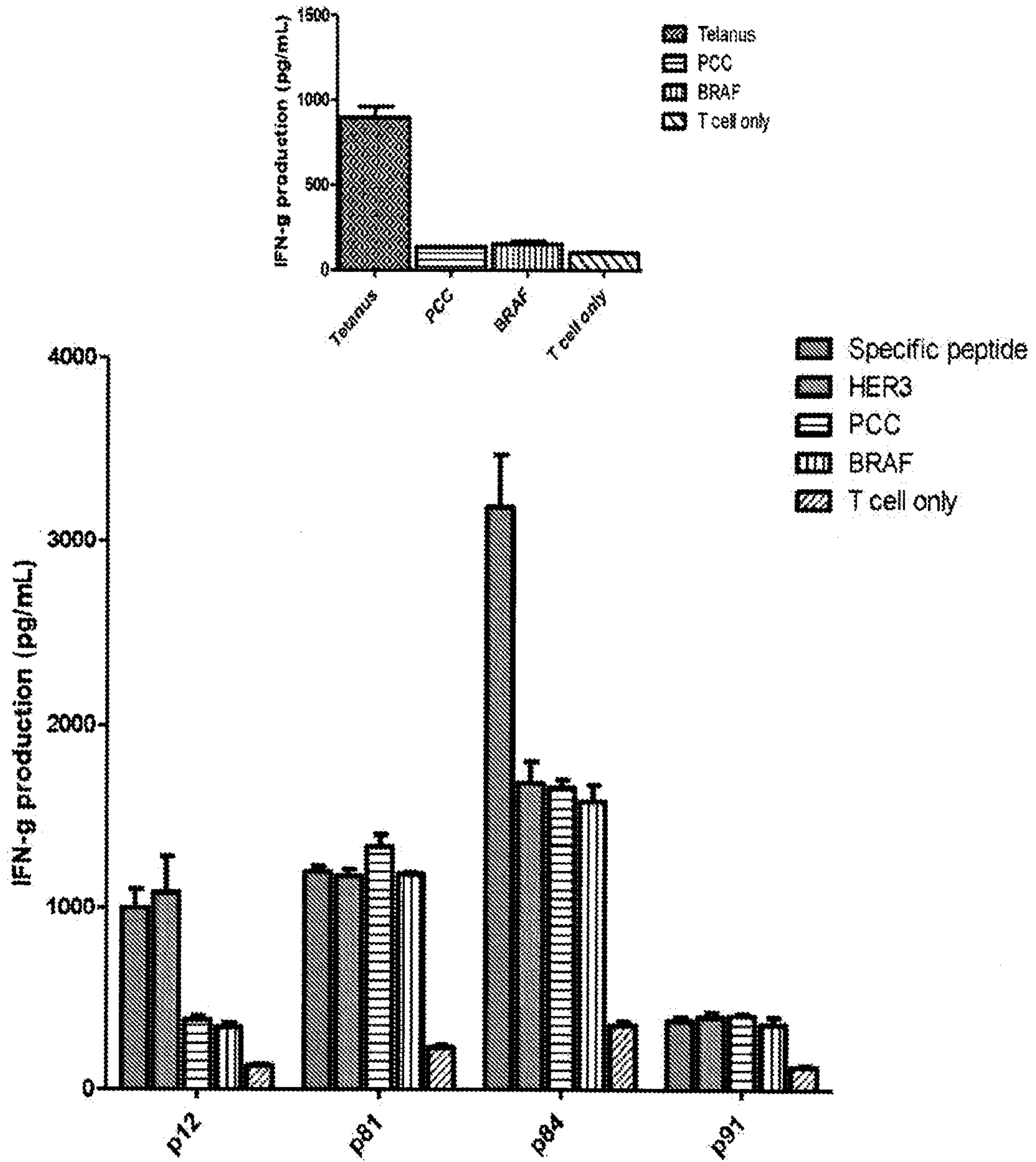


Figure 12

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UPCC 15107-24

HER3 library screen with groups of 10 peptide fragments

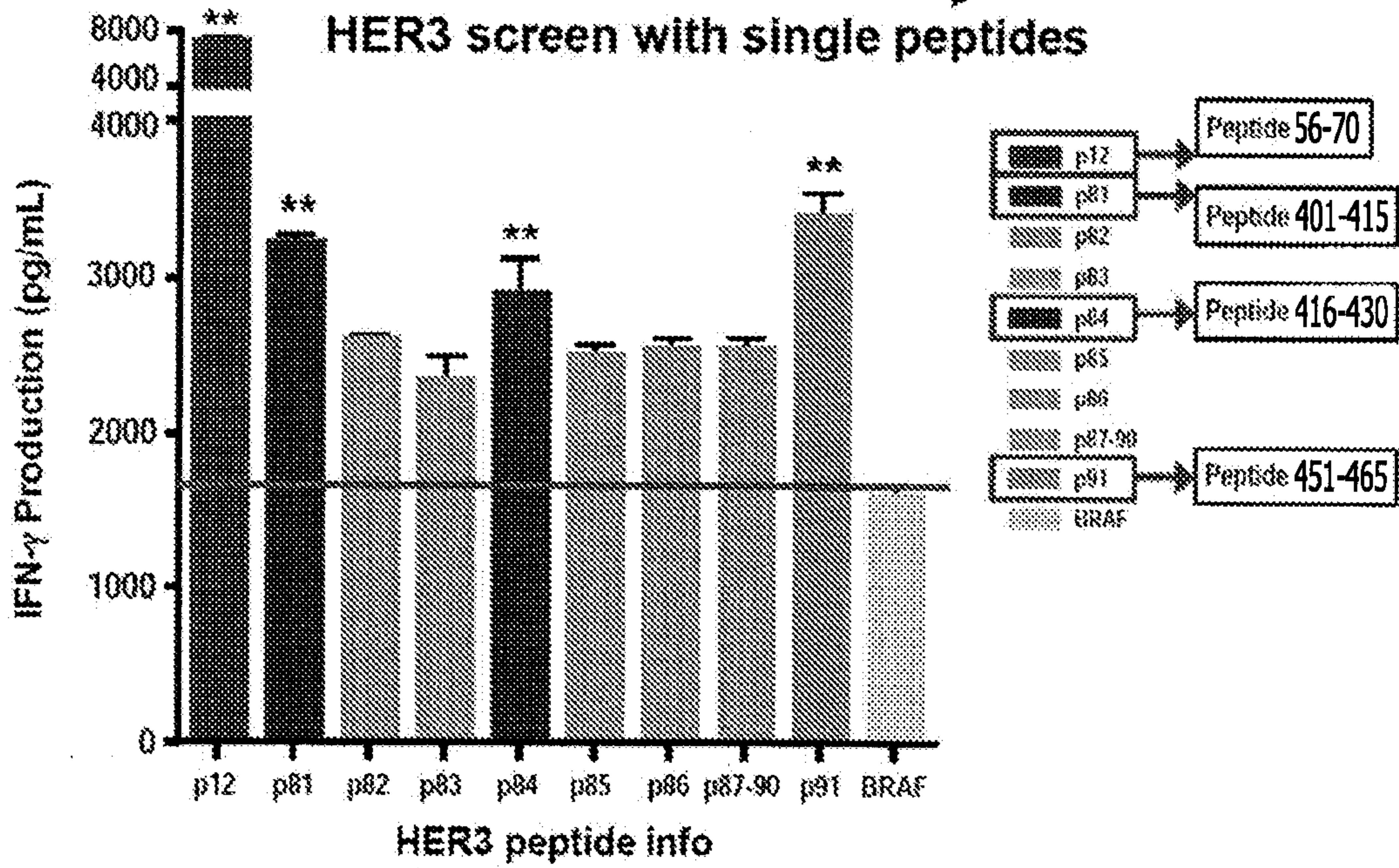
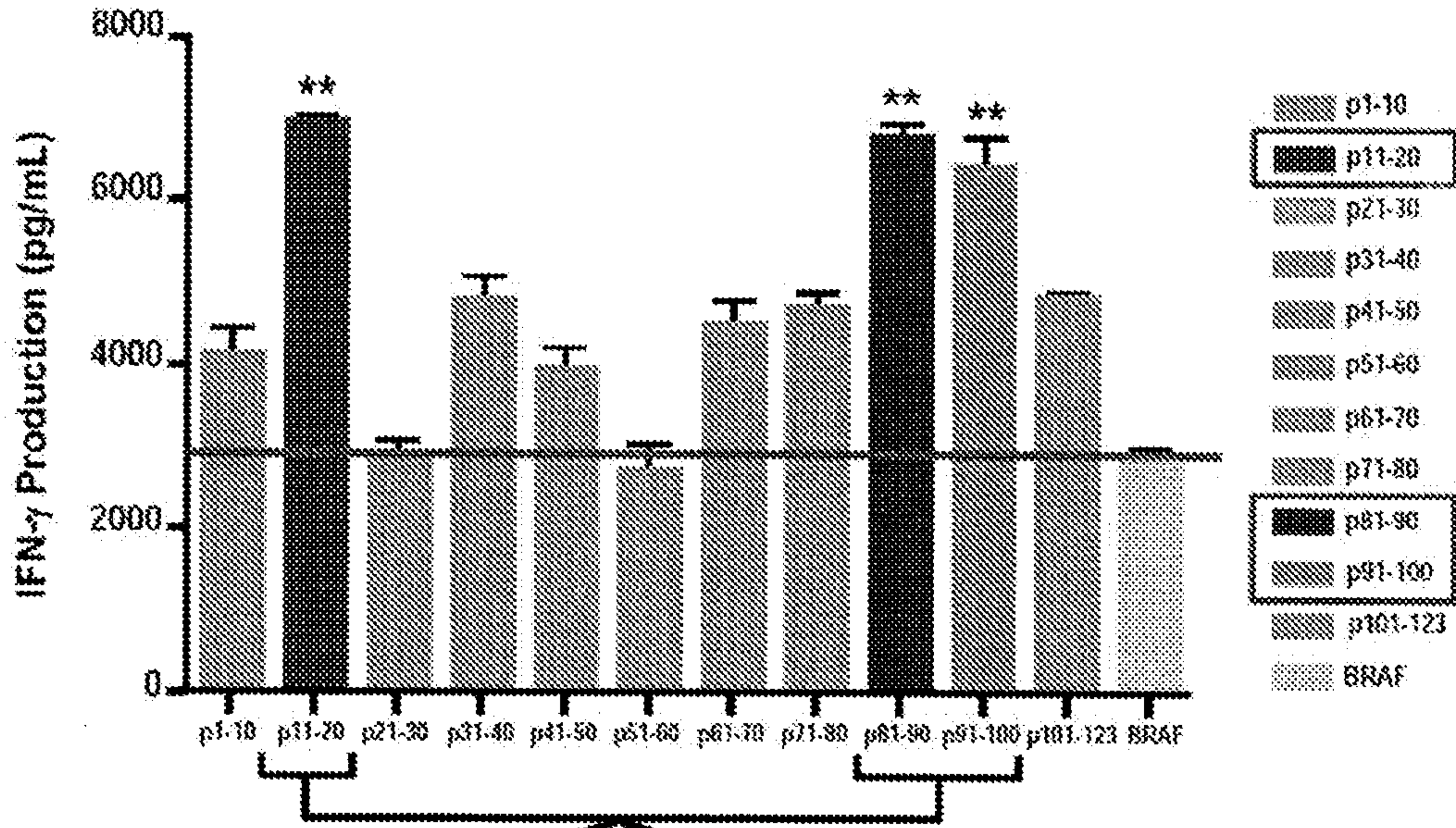


Figure 13

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UPCC 15107-38

HER3 library screen with groups of 10 peptide fragments

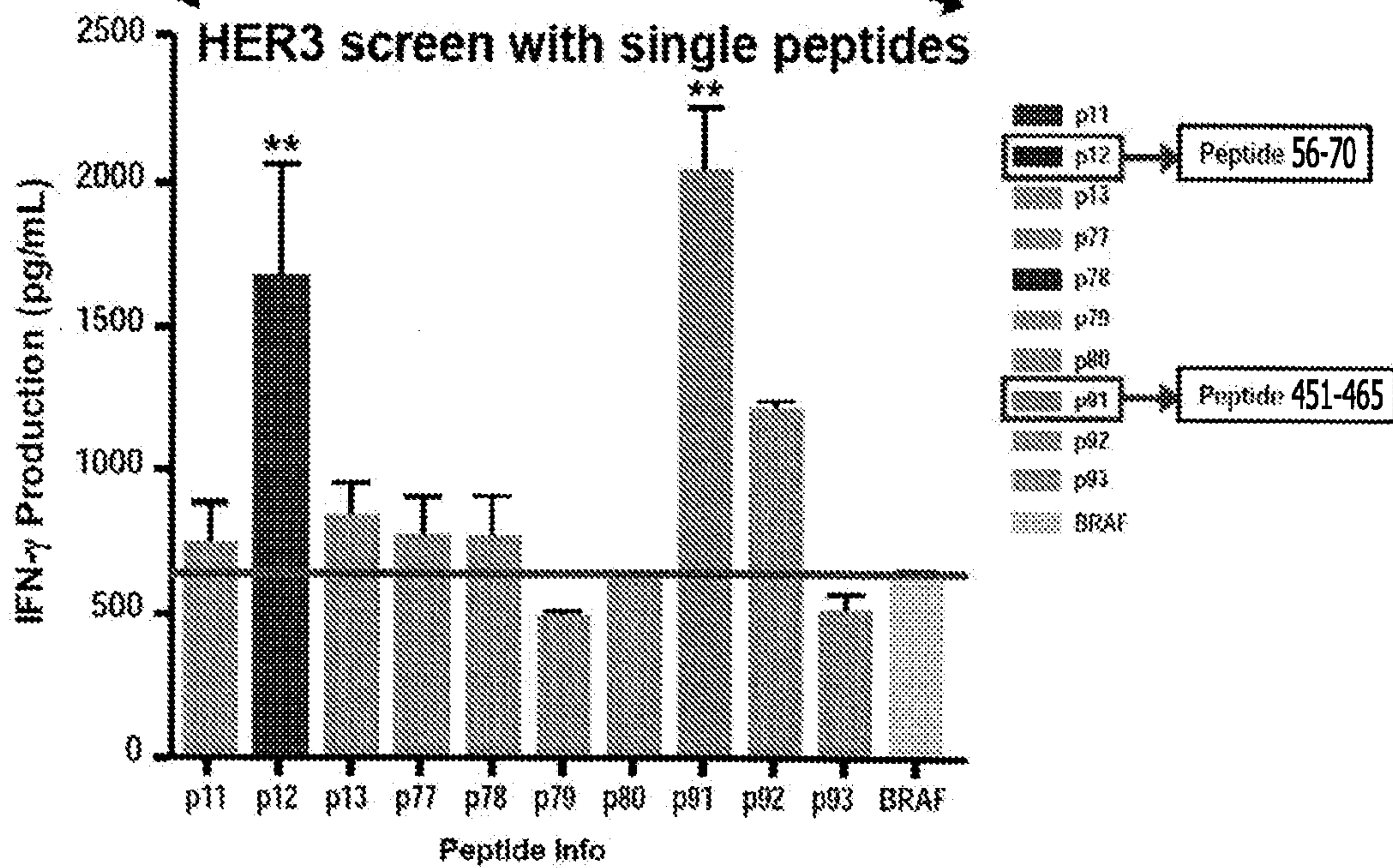
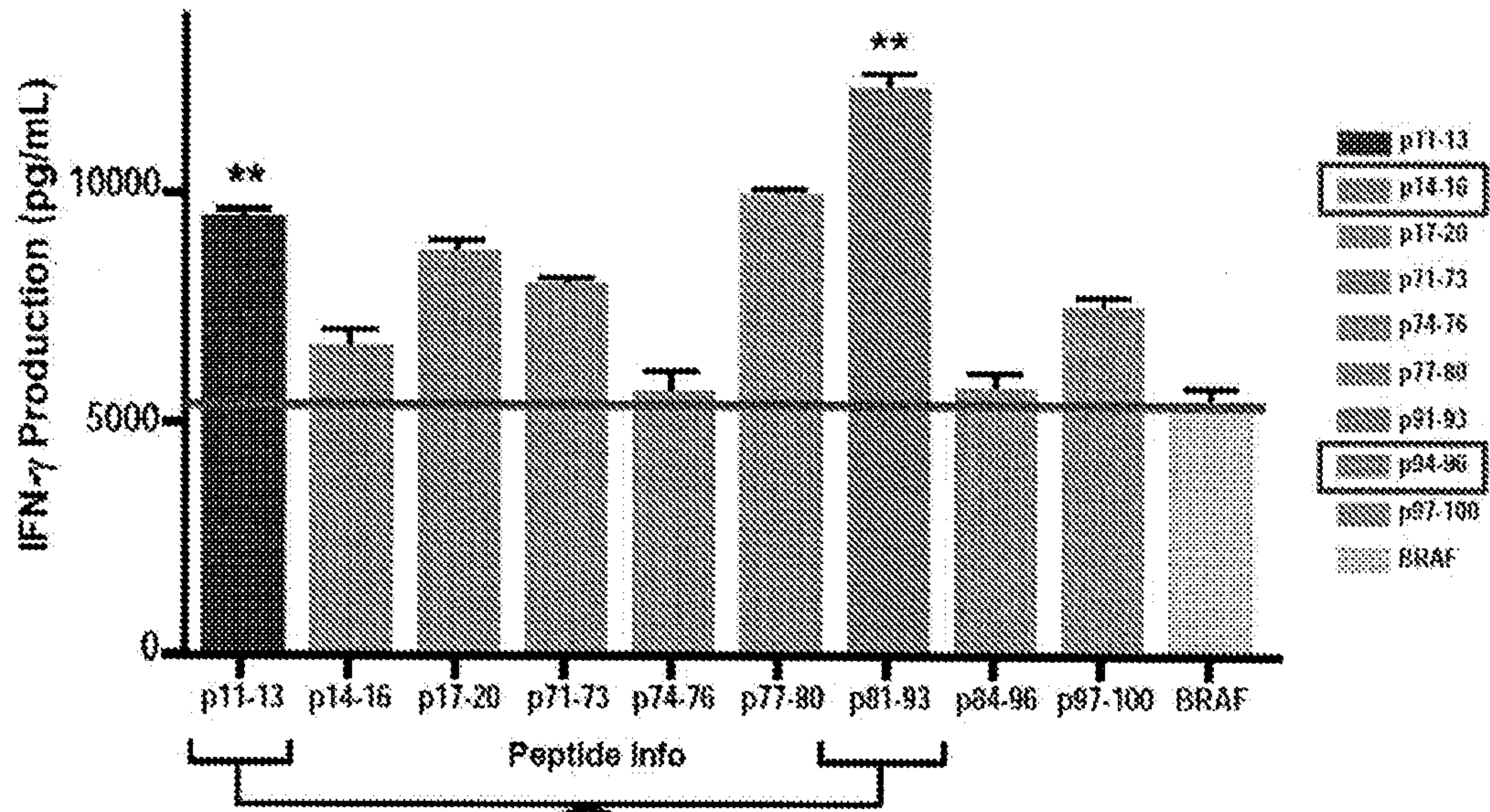


Figure 14

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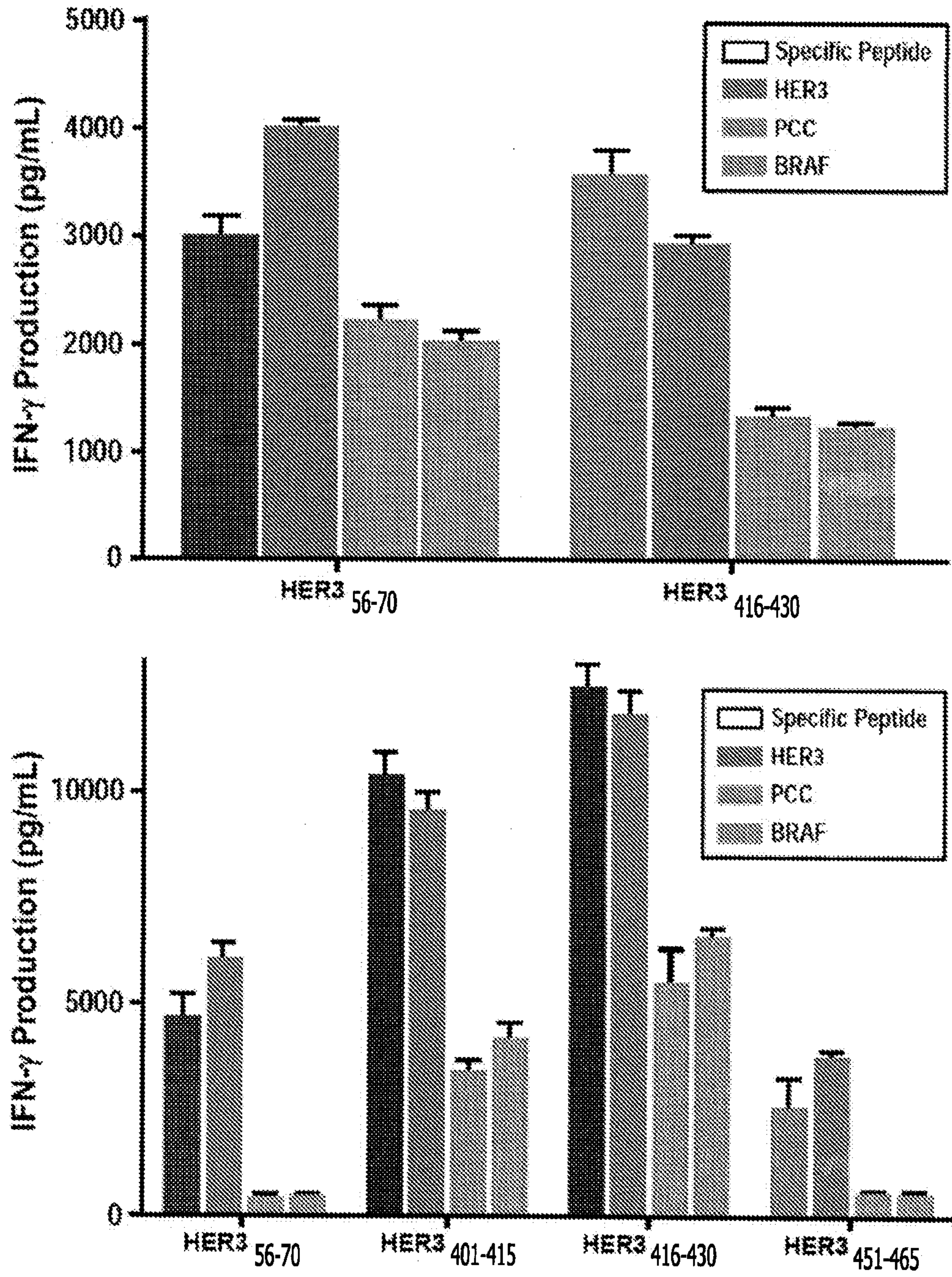


Figure 15

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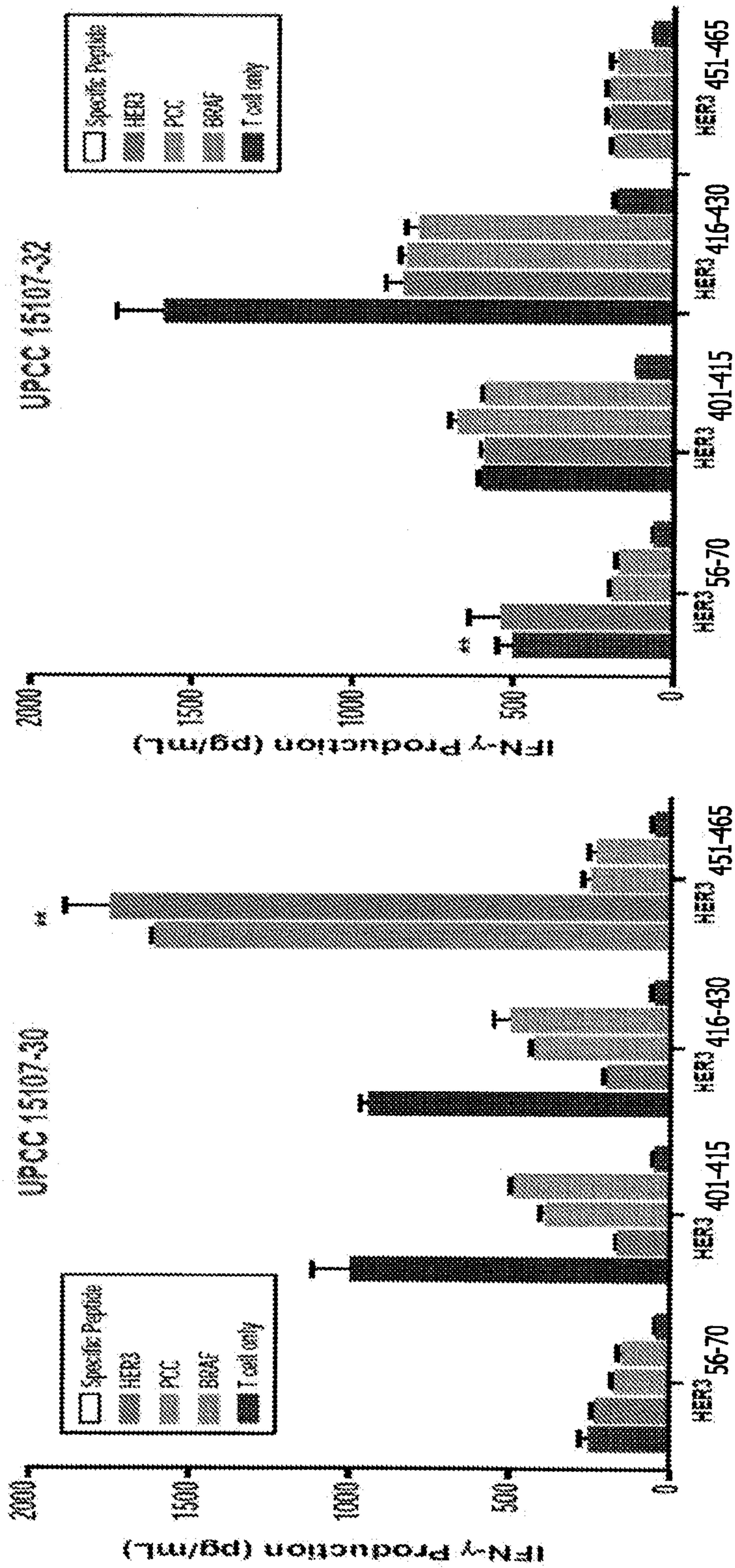


Figure 16

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HER3 epitope	DR							DQ					DP(B1)				
	1	4	7	10	13	16	17	2	5	6	7	8	03:01	03:05	04:01	13:01	14:01
HER3 ₅₆₋₇₀	Diagonal				Diagonal							Diagonal	Diagonal	Diagonal	Diagonal	Diagonal	Diagonal
HER3 ₄₀₁₋₄₁₅	Dark				Dark							Dark	Dark	Dark	Dark	Dark	Dark
HER3 ₄₁₆₋₄₃₀	Diagonal				Diagonal							Diagonal	Diagonal	Diagonal	Diagonal	Diagonal	Diagonal
HER3 ₄₅₁₋₄₆₅	Diagonal				Diagonal							Diagonal	Diagonal	Diagonal	Diagonal	Diagonal	Diagonal

Figure 17

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MDA-MB-468 06-04-14
MW31

50x10³ MDA-MB-468 cells co-cultured in transwell with

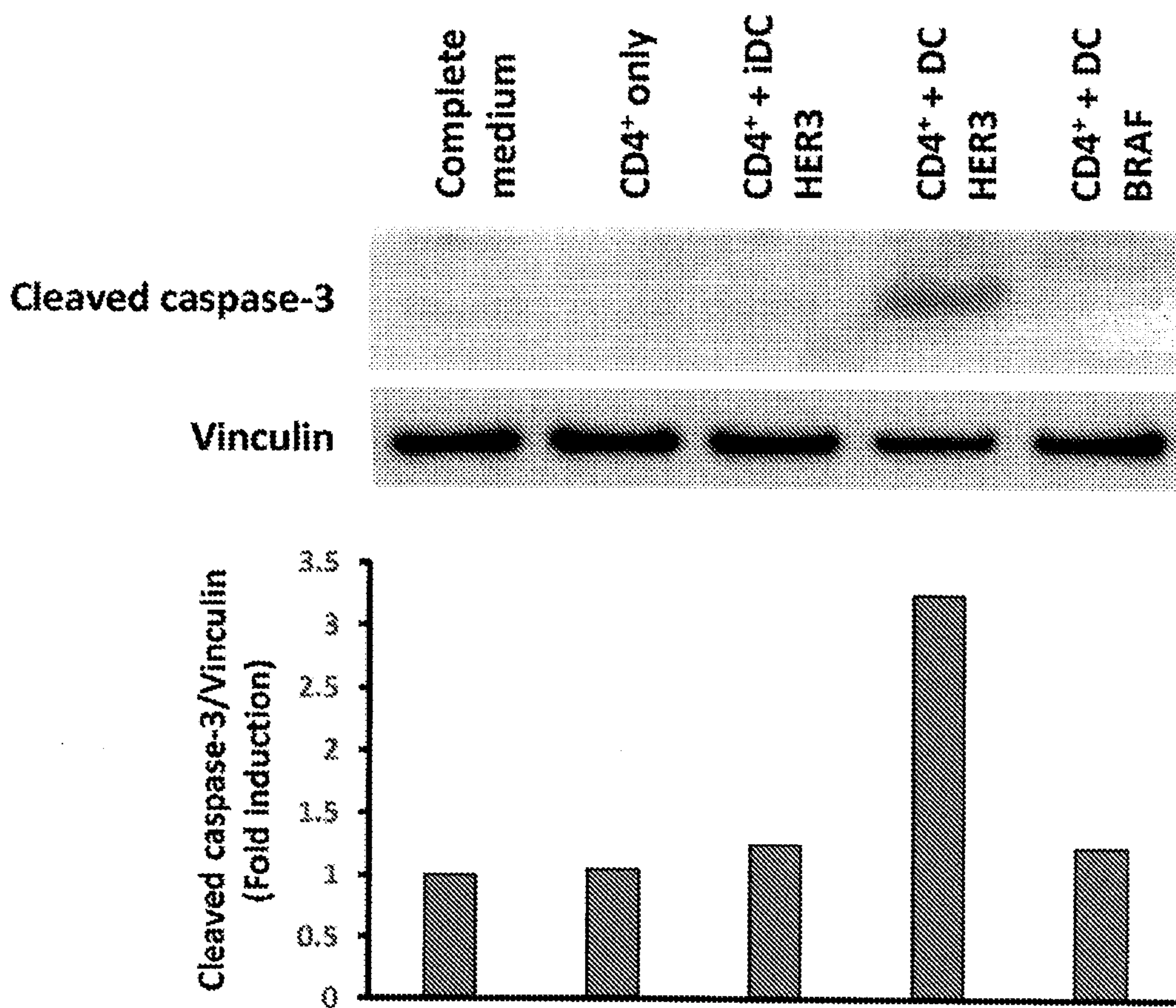


Figure 18

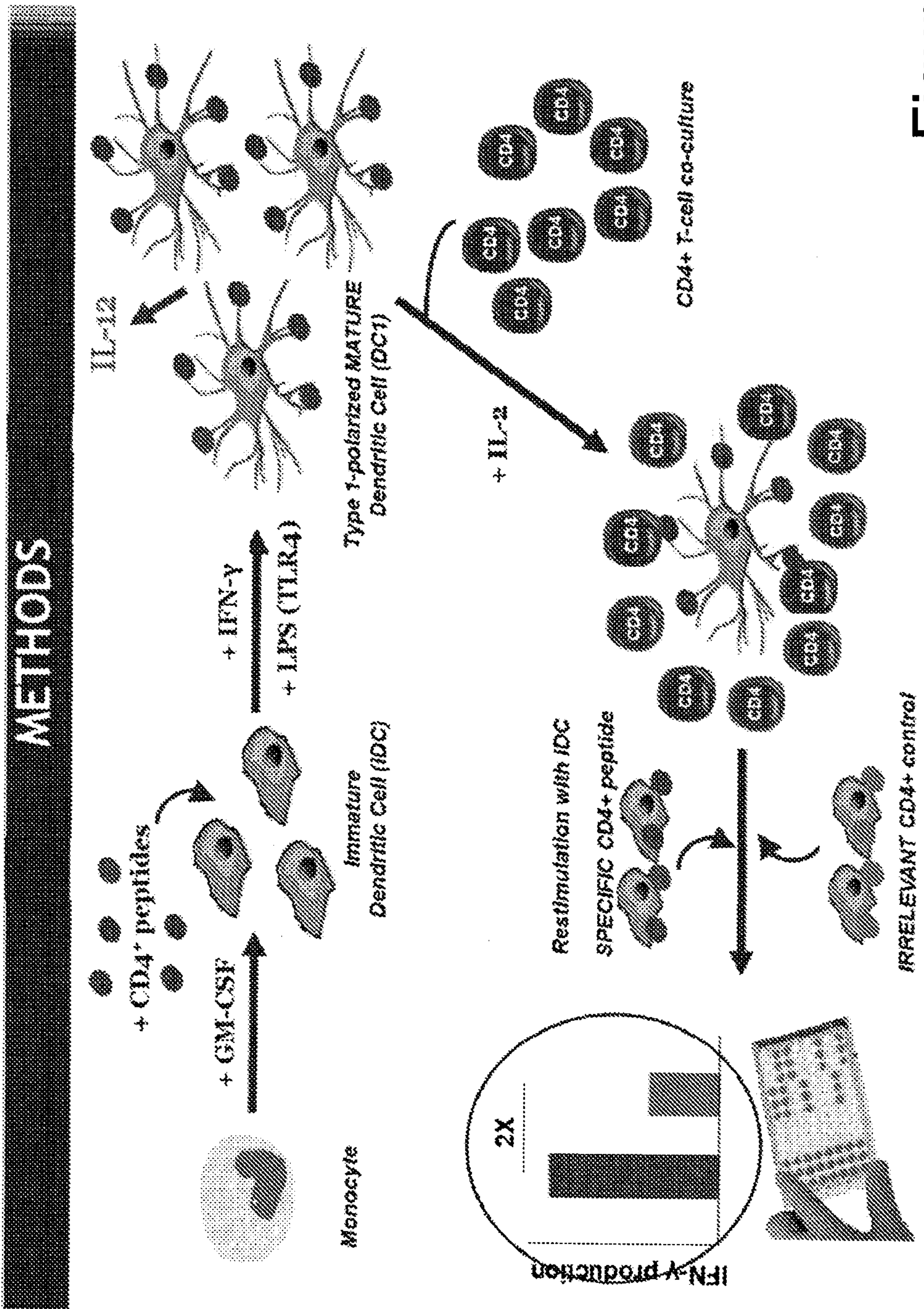


Figure 19

RESULTS "REVERSE" SENSITIZATION

B. Confirmation of immunogenicity of identified CD4+ HER3 ECD epitopes by "reverse" sensitization

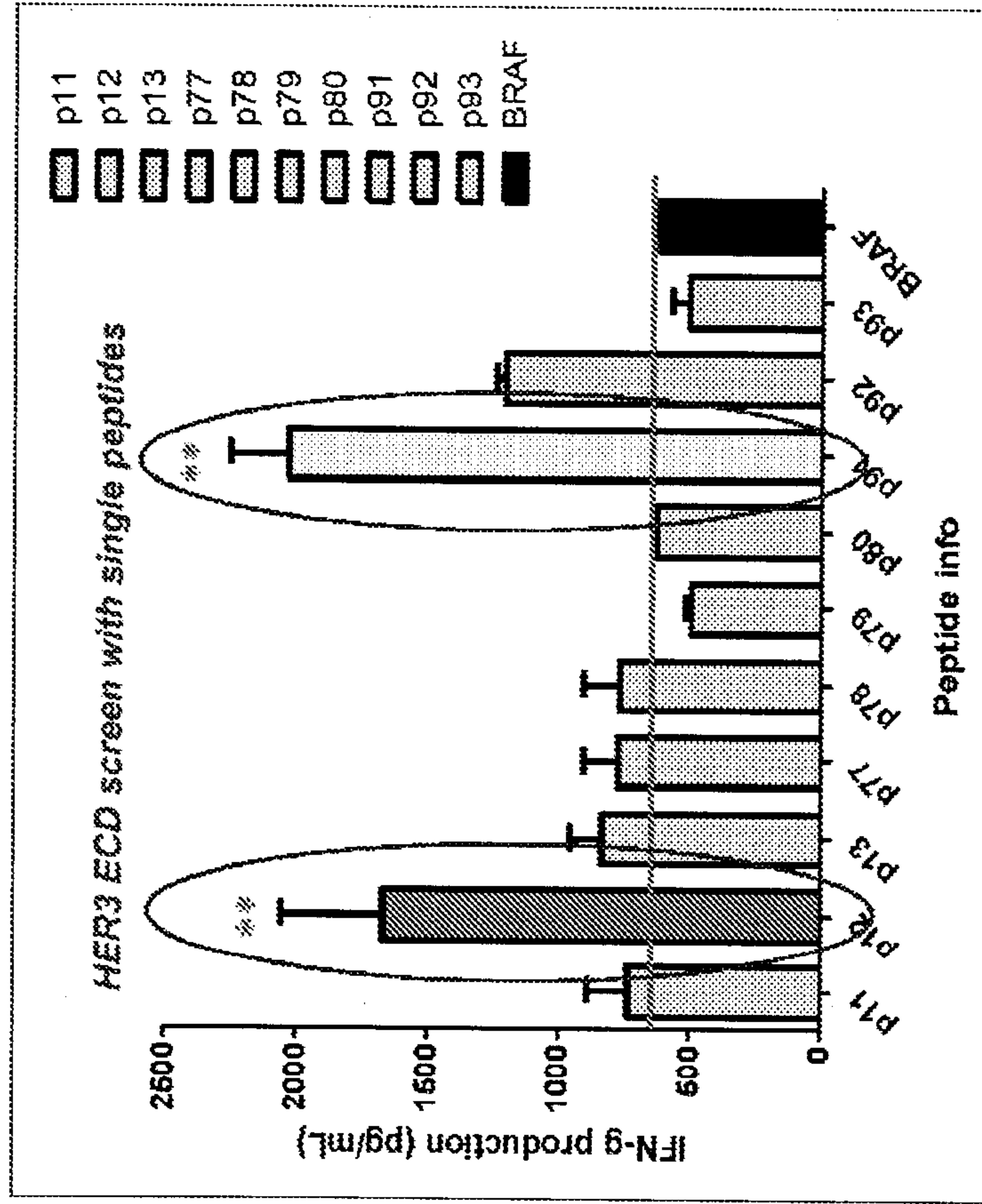


Figure 20

RESULTS: "REVERSE" SENSITIZATION

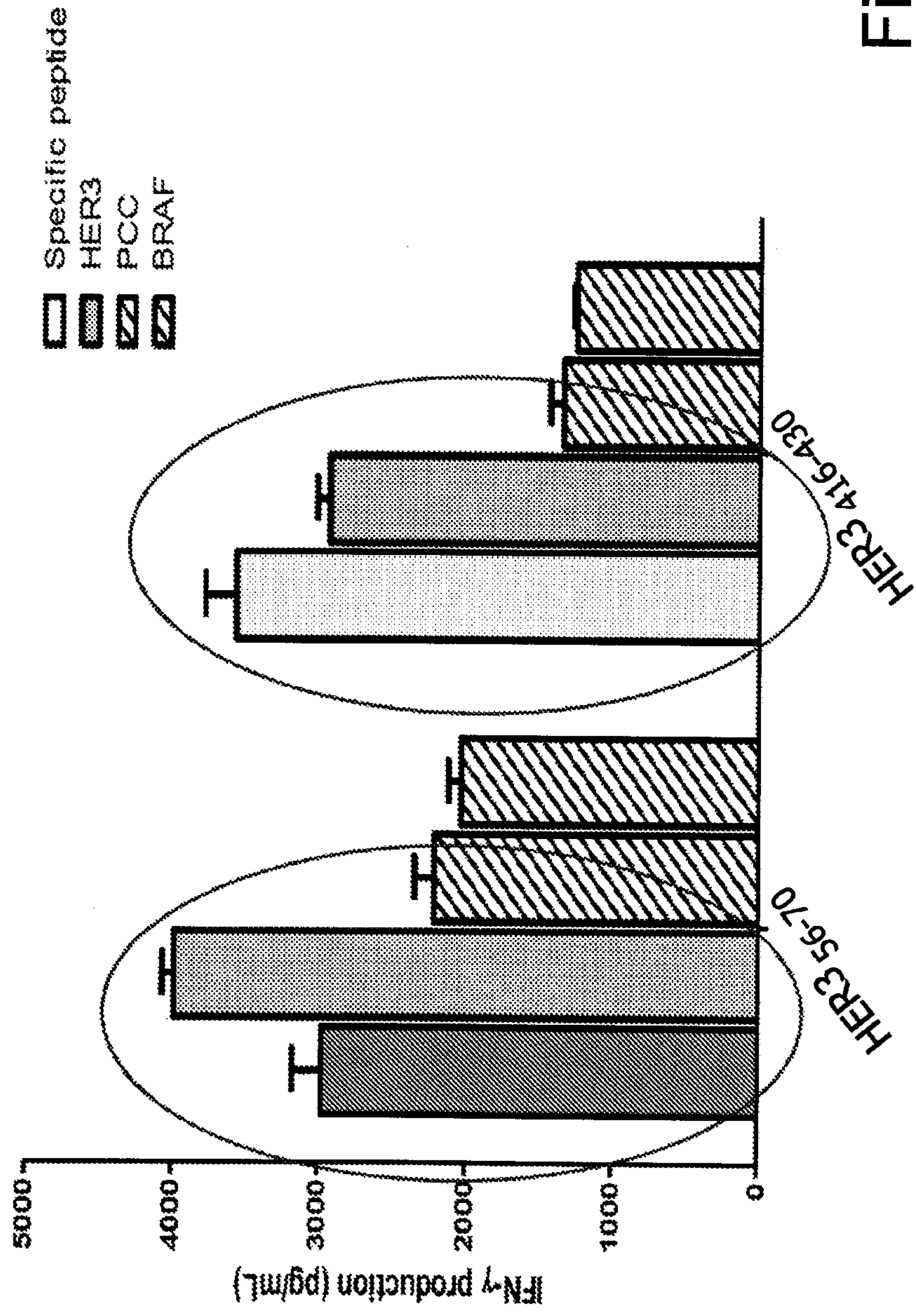
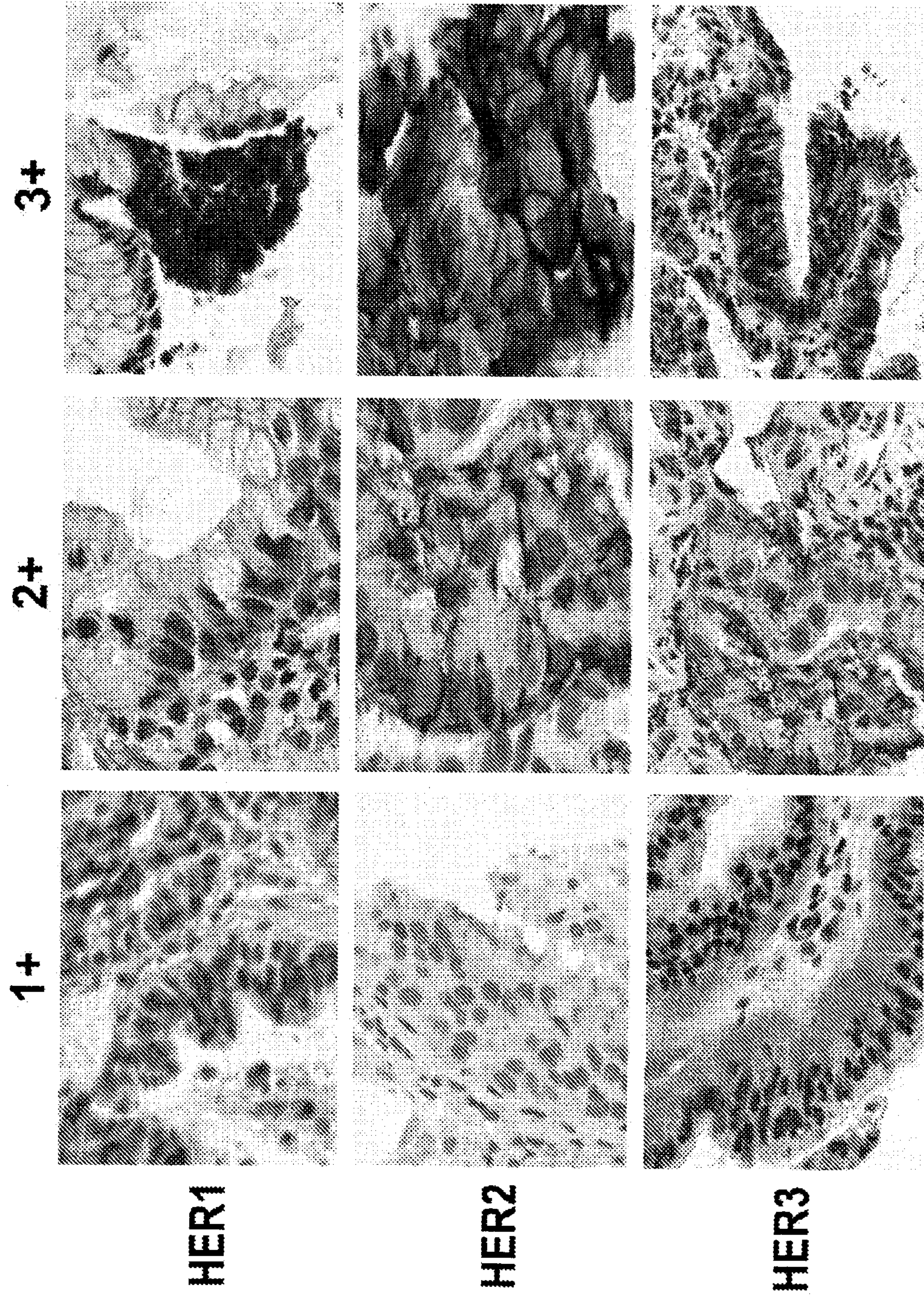
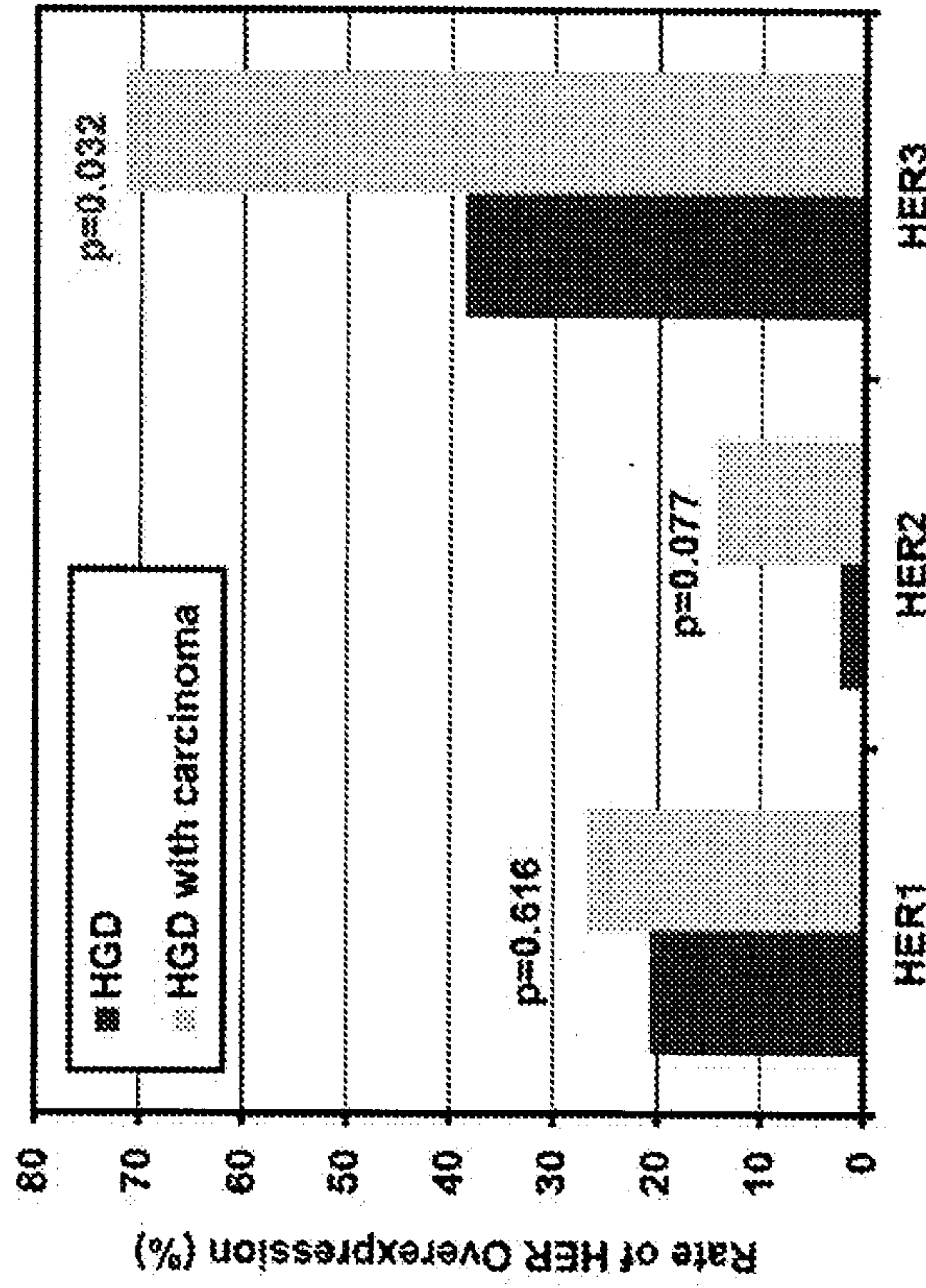


Figure 21

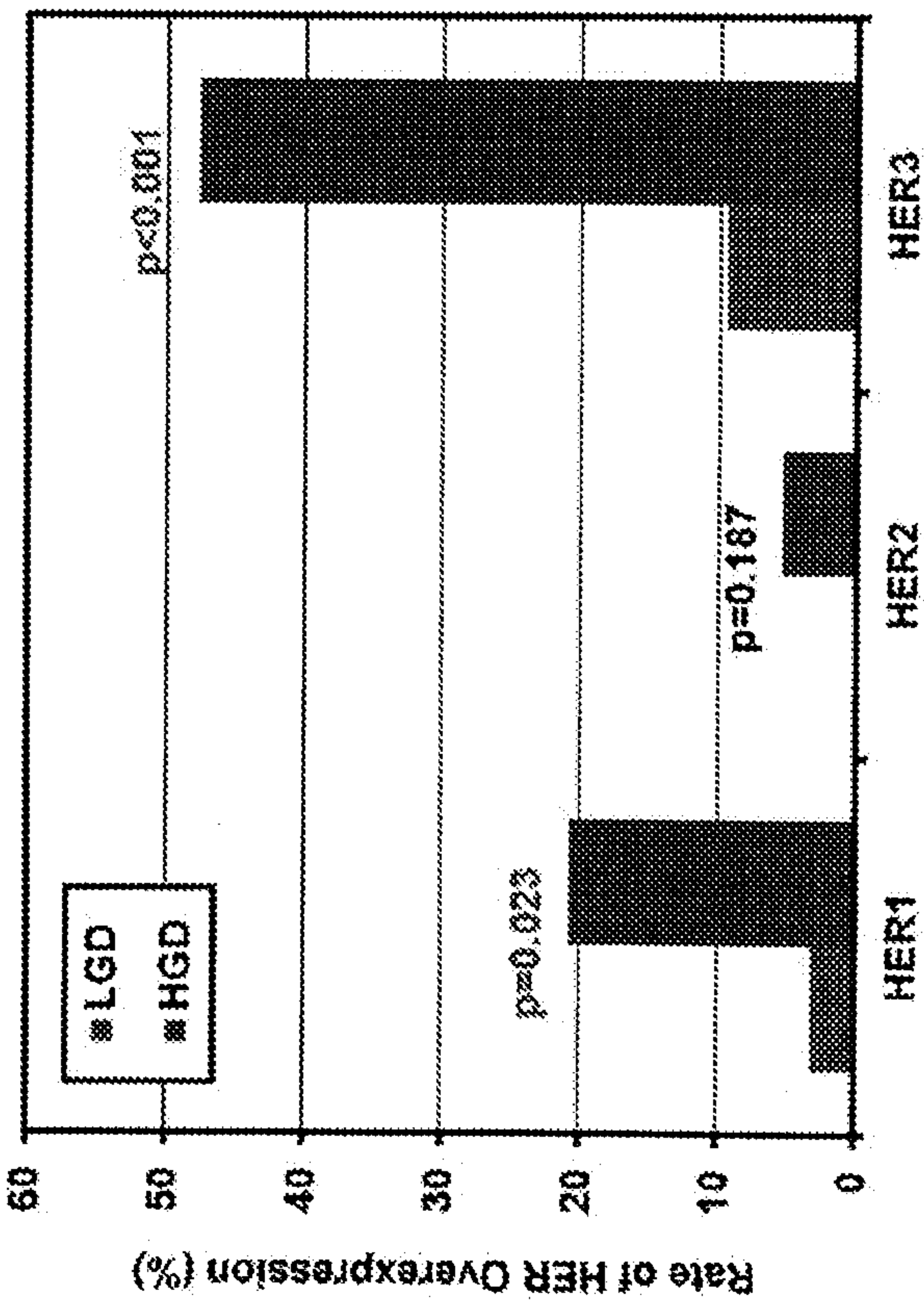
Figure 22 **Immunohistochemistry Scoring of HER Staining**



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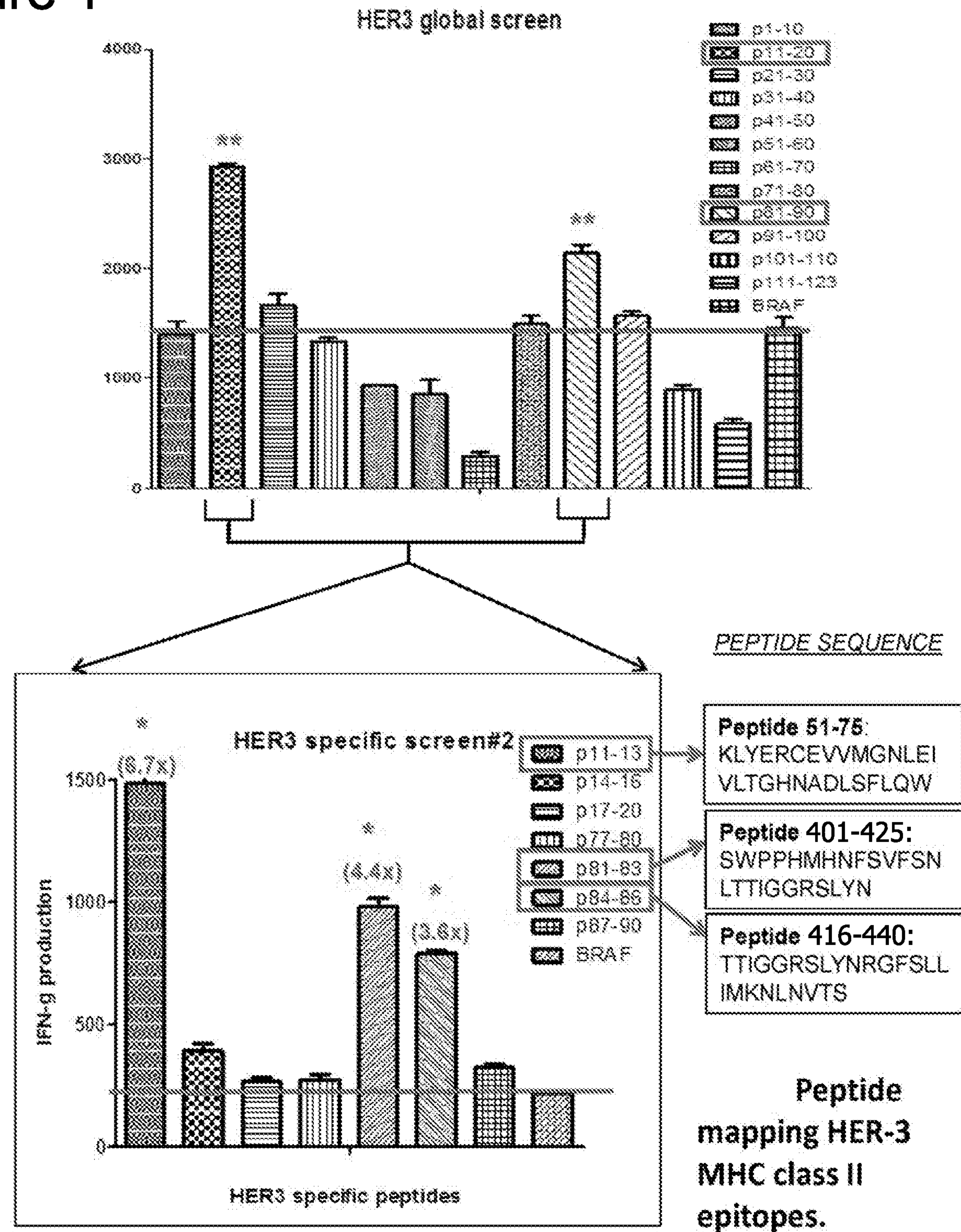
A



B

Figure 23

Figure 1



- p1-10
- p11-20
- p21-30
- p31-40
- p41-50
- p51-60
- p61-70
- p71-80
- p81-90
- p91-100
- p101-110
- p111-123
- BRAF

- p11-13
- p14-16
- p17-20
- p77-80
- p81-83
- p84-86
- p87-90
- BRAF

