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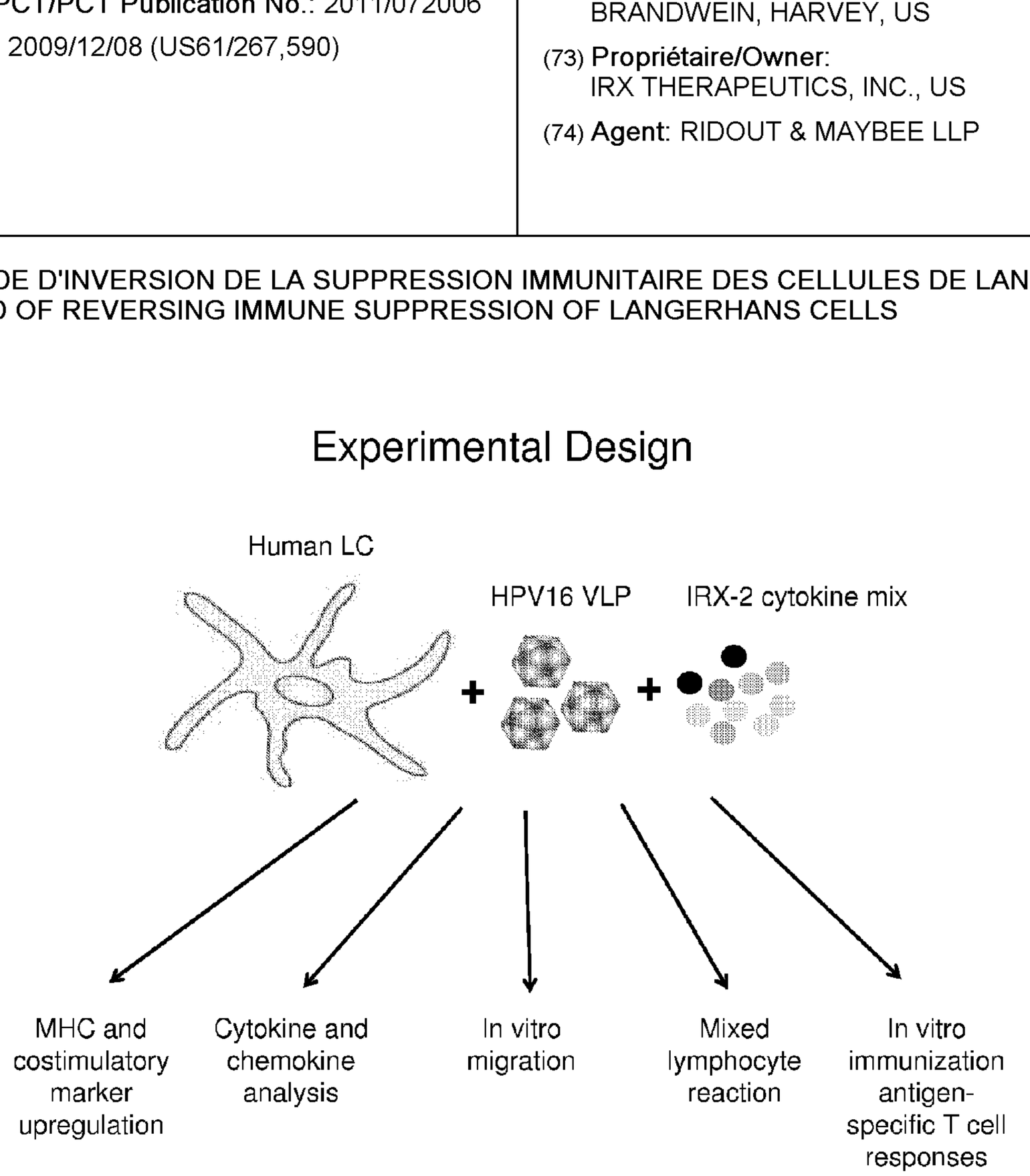
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(54) Title: METHOD OF REVERSING IMMUNE SUPPRESSION OF LANGERHANS CELLS



(57) Abrégé/Abstract:
A method of treating human papillomavirus (HPV), by administering a therapeutically effective amount of a primary cell-derived biologic to a patient infected with HPV, and inducing an immune response to HPV. A method of overcoming HPV-induced immune suppression of Langerhans cells (LC), by administering a therapeutically effective amount of a primary cell-derived biologic to a patient infected with HPV, and activating LC. A method of increasing LC migration towards lymph nodes, by administering a therapeutically effective amount of a primary cell-derived biologic to a patient infected with HPV, activating LC, and inducing LC migration towards lymph nodes. A method of generating immunity against HPV, by administering an effective amount of a primary cell derived biologic to a patient infected with HPV, generating immunity against HPV, and preventing new lesions from developing.

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(54) **Title**: METHOD OF REVERSING IMMUNE SUPPRESSION OF LANGERHANS CELLS

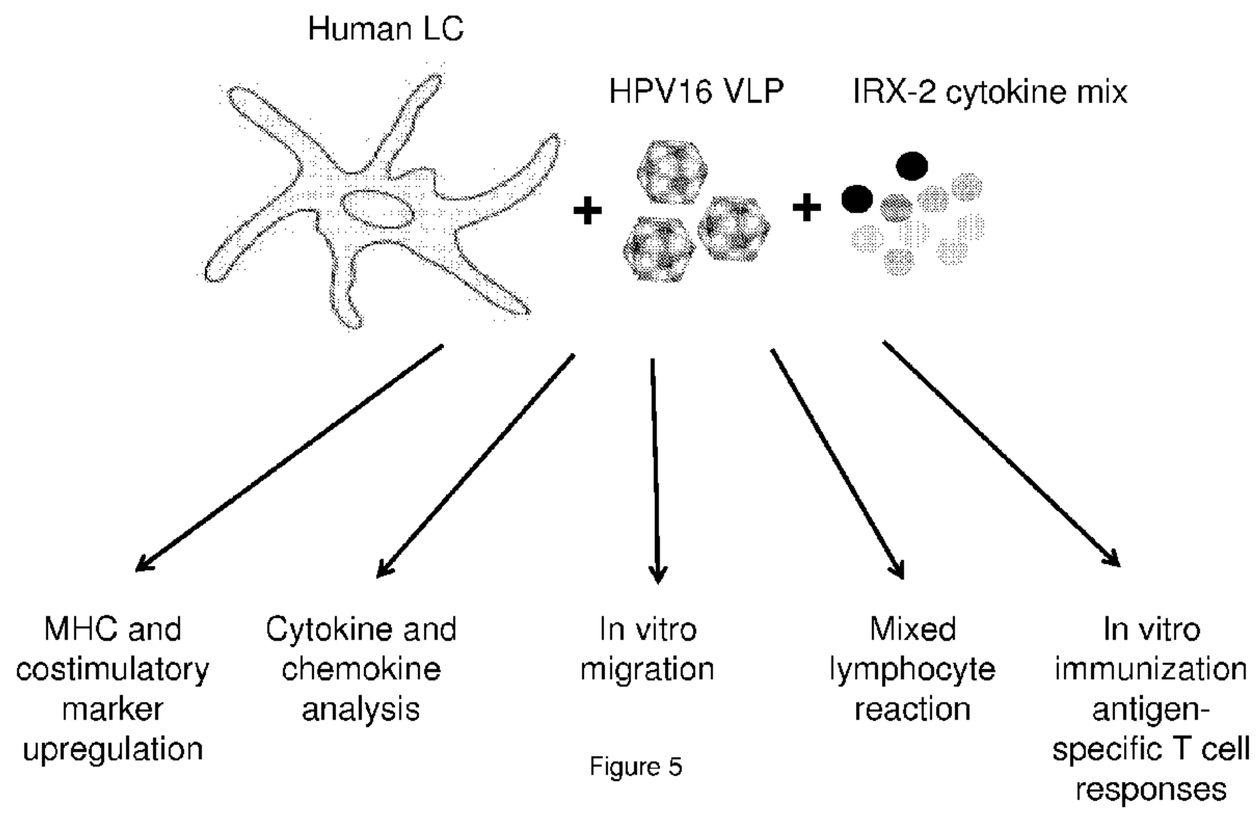


Figure 5

(57) **Abstract**: A method of treating human papillomavirus (HPV), by administering a therapeutically effective amount of a primary cell-derived biologic to a patient infected with HPV, and inducing an immune response to HPV. A method of overcoming HPV-induced immune suppression of Langerhans cells (LC), by administering a therapeutically effective amount of a primary cell-derived biologic to a patient infected with HPV, and activating LC. A method of increasing LC migration towards lymph nodes, by administering a therapeutically effective amount of a primary cell-derived biologic to a patient infected with HPV, activating LC, and inducing LC migration towards lymph nodes. A method of generating immunity against HPV, by administering an effective amount of a primary cell derived biologic to a patient infected with HPV, generating immunity against HPV, and preventing new lesions from developing.

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METHOD OF REVERSING IMMUNE SUPPRESSION OF LANGERHANS CELLS

TECHNICAL FIELD

[0001] The present invention relates to methods of reversing immune suppression. In particular, the present invention relates to reversing immune suppression in human papillomavirus (HPV) and treating HPV infection.

BACKGROUND OF THE INVENTION

[0002] Human papillomaviruses (HPV) are a family of sexually transmitted DNA viruses with over 100 different genotypes. The genotypes are divided into the low-risk and high-risk categories based on the spectrum of lesions they induce. The low-risk types primarily induce benign genital condylomas and low-grade squamous intraepithelial lesions whereas the high-risk types are associated with the development of anogenital cancers and can be detected in >99% of cervical cancers, with HPV16 found in about 50% of cases. In the United States, an estimated 75% of the sexually active population acquires at least one genital HPV type during their lifetime.

[0003] While morbidity and mortality caused by cervical cancer can be reduced with effective Papanicolaou (Pap) smear screening, early detection, and treatment, none of these are readily available in developing countries, the origin of many immigrants to the U.S. In developing countries, cervical cancer remains the second leading cause of cancer-related deaths among women. Importantly, the burden of this disease is expected to increase dramatically in the next decades due to

changing demographics. Even in the U.S. where screening programs have reduced the overall rate of invasive cancer, a disparity exists in the incidence of cancer development between white non-Hispanic, black, Hispanic, and economically disadvantaged women . Penetrance of the current FDA-approved preventive vaccine for HPV, GARDASIL® (Merck), in the U.S. has been disappointing. The vaccine was administered to only 25% of girls ages 13-17, 10% of all females ages 18-26, and was given to only 1.1% of Hispanic women in 2007 . Moreover, the vaccine is ineffective in women that have already been infected with the virus, whether they have developed (pre-) cancerous cervical lesions or not. Given the lifetime risk of HPV infection and the fact that populations severely underrepresented in vaccine coverage will likely continue to develop cervical and other HPV-related diseases at an alarming rate, it is clear that there is an enormous need for therapeutic approaches that would mitigate carcinogenic effects after viral infection has occurred.

[0004] HPV are non-lytic, non-enveloped viruses. Their genome coding regions are denoted E and L for “early” and “late” proteins. The E proteins fulfill regulatory functions vital for genome replication, two of which (E6 and E7) play a significant role in oncogenesis, while the two L proteins (L1 and L2) are the self-assembling capsid proteins responsible for DNA packaging and virion assembly. Infection by papillomaviruses is unique in that its productive lifecycle is coupled to the cellular differentiation of proliferating host epidermal or mucosal basal epithelial cells. HPV remains suprabasal throughout its lifecycle and therefore only contacts cells in the epidermis such as basal cells and Langerhans cells (LC). Due to the coupling of its

lifecycle to cellular differentiation, it is difficult to produce HPV virions in vitro. As an alternative to HPV virions, HPV virus-like particles (VLP) and HPV pseudovirions, capable of carrying reporter plasmids, have been developed and are both technologies that are established in the laboratory of Applicants.

[0005] Persistence of a high-risk HPV infection is a major risk factor in the development of cervical cancer. While a majority of women infected with HPV clear the virus, the time taken to do so can range from many months to years. About 15% of women that have high-risk HPV infections do not initiate an effective immune response against HPV, allowing the virus to persist for decades. The slow clearance rate and lack of effective immunity indicates that HPV somehow escapes the immune response.

[0006] HPV has developed a variety of escape mechanisms that circumvent immediate elimination, allowing viral replication and persistence in the host. Applicants have shown that HPV manipulates LC as a mechanism of immune escape, shown in FIGURE 4. LC located in the epithelial layer of the skin and mucosa are the first and critical APC to come into contact with HPV. Consequently, LC are responsible for initiating an effective immune response against HPV infection. Upon recognition of a foreign antigen, LC undergo maturation, which consists of phenotypic and functional changes including up-regulation of co-stimulatory molecules CD80 and CD86, MHC class I and II, chemokine receptors such as CCR7, secretion of cytokines and chemokines, and migration to regional lymph nodes . Applicants have established that LC exposed to HPV16 L1L2 VLP do not up-regulate co-stimulatory molecules and chemokine receptors, do not secrete

cytokines and chemokines and do not initiate epitope-specific immune responses against HPV16 VLP-derived antigens. In contrast, myeloid DC are activated by HPV16 L1L2 VLP and once activated, stimulate HPV-specific T cells. Different intracellular signaling cascades are initiated in DC versus LC upon uptake of HPV16 L1L2 VLP. When stimulated with HPV16 L1L2 VLP, the mitogen-activated protein kinase (MAPK) pathway is activated in DC whereas it is inactivated in LC. However, the phosphoinositide 3-kinase (PI3K) pathway is activated in LC, leading to a signaling cascade that results in the inactivation of Akt. HPV16 E7-specific T cells can recognize and kill LC exposed to HPV16 L1L2-E7 chimeric VLP (cVLP), indicating that HPV peptides are presented by LC after cVLP internalization but that HPV16 L1L2 VLP inhibit LC from inducing an immune response. Taken together, the data suggest that LC present HPV-derived peptides in the absence of co-stimulation, thereby becoming tolerogenic and immune-suppressive. This in turn can lead to persistence of the HPV infection and an increased likelihood of cancer development.

[0007] Prophylactic vaccines for HPV induce high titers of HPV-neutralizing antibodies and have shown high efficacy up to 6.5 years of follow-up and sustained levels of antibodies. However, in women infected with HPV, a phase 3 trial found no evidence of accelerated viral clearance in the vaccinated group as compared to the control group, illustrating conclusively the lack of therapeutic efficacy in preventive VLP-based vaccines. Furthermore, with the long incubation time of HPV, several mechanisms of immune evasion and only a few years of observation, sustained efficacy of prophylactic vaccines on cancer prevention has yet to be determined. The fact that about one third of cervical cancer is caused by HPV types other than those

currently present in the vaccines increases the scope of the problem. Thus, it will take decades to be able to detect a quantifiable effect on cervical cancer rates. Meanwhile, the need for therapies to treat HPV infections and associated lesions remains for the hundreds of millions of women worldwide that are currently infected with high-risk HPV or will become infected in the coming years.

[0008] Surgery, the standard of care for patients with cervical intraepithelial neoplasia (CIN) lesions, is usually quoted as being up to 90% effective in removing CIN lesions when followed for one year. However, it is less effective when women are monitored over their lifetime. Greater than 80% of women that undergo surgical procedures will subsequently return in need for a second related procedure in cases where surgery does not remove the HPV infection or where elimination of one HPV type encourages re-activation of secondary HPV infections. The therapeutic vaccines that are in development aim to control malignancy by activating the patient's own cellular immune response and target antigens present in the (pre-) cancer cells. Several candidate vaccines have been developed over the last fifteen years; however, to date, there is not a single cancer vaccine that has been approved by the Food and Drug Administration.

[0009] Interventions that prevent HPV infections from reaching the stage of inducing carcinogenesis are needed. Such interventions are feasible, since HPV infection can be detected early on with a commercially available HPV detection kit (Digene Corp.).

[0010] Several lines of evidence support the importance of the cellular immune system in controlling the pathogenesis of HPV and associated cervical lesions.

Firstly, 25-40% of HPV positive, mildly dysplastic lesions resolve spontaneously or shortly after local biopsy suggesting that induction of local inflammation may be involved with regression. Secondly, immunodeficiency is associated with increased incidence of HPV infection. Regressing HPV-associated skin warts and genital warts often have T lymphocytes in the lesions, suggesting that active cell-mediated immune responses to HPV may be a component of regression of the disease. Taken together, these studies illustrate that therapeutic regimens for HPV infection and its associated diseases should aim to induce strong cellular immunity at the site of infection.

[0011] Therefore, there is a need for both an effective immunological treatment of HPV over the lifetime of women as well as a method of overcoming the HPV-induced immune suppression of LC that prevents effective treatment.

SUMMARY OF THE INVENTION

[0012] The present invention provides a method of treating human papillomavirus (HPV), including the steps of administering a therapeutically effective amount of a primary cell-derived biologic to a patient infected with HPV, and inducing an immune response to HPV infection.

[0013] The present invention also provides for a method of overcoming HPV-induced immune suppression of Langerhans cells (LC), including the steps of administering a therapeutically effective amount of a primary cell-derived biologic to a patient infected with HPV, and activating LC.

[0014] The present invention provides for a method of increasing LC migration towards lymph nodes, including the steps of administering a therapeutically effective amount of a primary cell-derived biologic to a patient infected with HPV, activating LC, and inducing LC migration towards lymph nodes.

[0015] The present invention further provides for a method of generating immunity against HPV, including the steps of administering an effective amount of a primary cell derived biologic to a patient infected with HPV, generating immunity against HPV, and preventing new lesions from developing.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein:

[0017] FIGURE 1 is a histogram of phenotypic markers expressed in Langerhans cells (LC) (prior art);

[0018] FIGURE 2 is a graph showing CD86 expression by LC (prior art);

[0019] FIGURE 3 is a diagram of unactivated LC versus activated LC;

[0020] FIGURE 4 is a diagram of HPV inducing tolerization of LC;

[0021] FIGURE 5 is a diagram of the experimental design of experiments on LC activation;

[0022] FIGURE 6 is a graph of upregulation of surface activation markers on

human LC exposed to HPV16 and IRX-2;

[0023] FIGURE 7 is a graph showing that IRX-2 induces human LC migration in the absence and presence of HPV16;

[0024] FIGURE 8 is a graph showing that human Langerhans cells exposed to IRX-2 are superior in stimulating allogeneic T cells in the presence of HPV; and

[0025] FIGURE 9 is a graph showing human Langerhans cells treated with IRX-2 after HPV exposure secrete high levels of IL-8 and IP-10.

DETAILED DESCRIPTION OF THE INVENTION

[0026] The present invention provides in general for methods of treating HPV and overcoming HPV-induced immune suppression of LC by the administration of a primary cell-derived biologic (IRX-2). The treatment of the present invention is effective in treating patients with persistent HPV and whose immune system is not able to produce an effective response against HPV.

[0027] As used herein, “effective amount” refers to an amount of primary cell derived biologic that is needed to achieve the desired result of the present invention, namely, producing a reversal of immune suppression of LC in HPV-infected patients. One skilled in the art can determine the effective amount of the primary cell derived biologic that should be given to a particular patient.

[0028] “IRX-2”, also known as “citoplurikin”, is a leukocyte-derived, natural primary cell-derived biologic produced under cGMP standards by purified human white blood cells (mononuclear cells) stimulated by phytohemagglutinin (PHA) and

ciprofloxacin (CIPRO). The major active components are interleukin 1β (IL-1β, also referred to herein as IL-1), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor α (TNF-α), and γ-interferon (IFN-γ). Preferably, the IRX-2 used in the present invention includes these six critical cytokines and can contain only these six critical cytokines. IRX-2 has also previously been referred to as an "NCM", a natural cytokine mixture, defined and set forth in United States Patent Nos. 6,977,072 and 7,153,499. The terms IRX-2, primary cell-derived biologic, and NCM are used interchangeably herein.

[0029] Briefly, IRX-2 is prepared in the continuous presence of a 4-aminoquinolone antibiotic and with the continuous or pulsed presence of a mitogen, which in the preferred embodiment is PHA. Other mitogens, however, can also be used. The IRX-2 produced for administration to patients contains a concentration of IL-1β that ranges from 60 - 6,000 pcg/mL, more preferably, from 150 - 1,800 pcg/mL; a concentration of IL-2 that ranges from 600-60,000 pcg/mL, more preferably, from 3,000-12,000 pcg/mL, and concentrations of IFN-γ and TNF-α that range from 200-20,000 pcg/mL, more preferably, from 1,000-4,000 pcg/mL.

[0030] IRX-2 can also contain a concentration of IL-6 that ranges from 60-6,000 pcg/mL, more preferably, from 300-2,000 pcg/mL; a concentration of IL-8 that ranges from 6000-600,000 pcg/mL, more preferably from 20,000-180,000 pcg/mL; a concentration of TNF-α that ranges from 200-20,000 pcg/ml, more preferably, from 1,000-4,000 pcg/mL. Recombinant, natural or pegylated cytokines can be used, or IRX-2 can include a mixture of recombinant, natural or pegylated cytokines. IRX-2 can contain only the above cytokines; however, other cytokines can be included.

The IRX-2 of the present invention can further include other recombinant, natural or pegylated cytokines such as IL-7, IL-12, IL-15, GM-CSF (at a concentration that ranges from 100-10,000 pcg/mL, more preferably from 500-2,000 pcg/mL), and G-CSF. The method of making IRX-2 is disclosed in the above-cited patents as well as in U.S. Patent Application No. 12/423,601.

[0031] Also encompassed by the present invention are derivatives, fragments and peptides related to the cytokines disclosed herein, wherein such derivatives, fragments and peptides retain the biological activity of their respective cytokines.

[0032] The multiple active cytokine components of IRX-2 act on multiple cell types of the immune system, including T cells and dendritic cells. In clinical trials of H&NSCC patients, IRX-2 was shown to be safe, tolerable and biologically active, resulting in both apparent disease-free survival and overall survival. The physiologic quantities of cytokines in IRX-2 can be administered locally, including topically, providing an opportunity to alter the microenvironment where LC encounter HPV. Natural cytokine mixtures from monocyte-conditioned medium or mixtures of recombinant inflammatory cytokines containing TNFα, IL-1β, IL-6, and PGE2 have traditionally been used to mature DC for *ex vivo* generated DC-based cancer vaccines. The physiologic cytokine levels in IRX-2 are much lower than concentrations of recombinant cytokines used in *ex vivo* DC maturation or in high dose systemic cytokine therapies. The low levels of cytokines in IRX-2 allow it to be injected directly into patients with no significant toxicity.

[0033] IRX-2 also contains several cytokines that are critical mediators of T cell activation and proliferation. The ability of IRX-2 to activate both DC and T cells

makes it especially attractive since it is this combination of immune cell subsets that coordinate the immune response against virus-infected cells.

[0034] Other compounds can also be administered along with IRX-2, such as chemical inhibitors, non-steroidal anti-inflammatory drugs (NSAIDS), zinc, and combinations thereof.

[0035] The chemical inhibitor can be any chemotherapeutic agent that is not immunosuppressive (preferably used at low doses) and that has immunomodulatory effects so as to increase immunity and/or an immune response, e.g., by inhibiting immune suppression or suppressor mechanisms in the body. According to a preferred embodiment, the chemical inhibitor is an anti-neoplastic agent, including but not limited to alkylating agents, antimetabolites and antibiotics. The chemical inhibitor can also be an immunomodulating agent such as thalidomide. The chemical inhibitor can also be in a salt or other complex form. Preferably, the chemical inhibitor is the alkylating agent cyclophosphamide (CY).

[0036] The NSAID is preferably indomethacin (INDO), which is both a CoxI and CoxII inhibitor. The NSAID can also be ibuprofen or CoxII inhibitors such as celecoxib and rofecoxib, or combinations thereof.

[0037] The four components used together (i.e. chemical inhibitor, NSAID, primary cell derived biologic, and zinc) are able to address the suppressive environment created by the immune target and restore the cellular immune response of the patient. More specifically, the chemical inhibitor inhibits T regulatory cells; the NSAID reverses local immune suppression by prostaglandins, the primary cell derived biologic activates dendritic cells, stimulates T cells, and protects T cells from

apoptosis; and zinc provides key nutrients for T cell function. This combined action encourages immune response to both endogenous and exogenous antigens.

[0038] More specifically, the present invention provides for a method of treating HPV, by administering a therapeutically effective amount of IRX-2 to a patient infected with HPV, and inducing an immune response to HPV. Preferably, IRX-2 includes the six critical cytokines of IL-1, IL-2, IL-6, IL-8, TNF-α, and IFN-γ as described above. Additional cytokines can also be included as described above. Additional compounds such as a chemical inhibitor, NSAID, and zinc can also be administered as described above. Preferably, the IRX-2 is administered in the epithelium by injection where HPV infection is present as well as where the LC are located that are needed to induce an immune response. Topical application to the cervix is also preferred. Topical application methods include, but are not limited to, the dispersal of IRX-2 in the liposome formulation Biphasix™ (Helix Biopharma Corp., Aurora, Ontario, CA), followed by application to cervical epithelium; use of the Cervical Drug Delivery System™ (Cytocore®, Inc. Chicago, IL), wherein IRX-2 is absorbed into a bioadhesive polymer patch, which is then affixed to cervical epithelium; and the infusion of IRX-2 solution into the cervix via a cervical isolation and delivery apparatus such as that disclosed in U.S. Patent No. 7,165,550 to Tracy et al.

[0039] In general, IRX-2 acts to effectively “turn on” the immune system by maturing immature dendritic cells, stimulate the production of naïve T cells, and effectively present antigen to the naïve T cells. IRX-2 is able to effectively treat HPV by inducing an immune response to HPV, something that is lacking in HPV patients

due to the immune suppressive actions of HPV on LC.

[0040] The immune response to HPV is induced by activating LC. Activated versus unactivated LC are shown in FIGURE 3. Essentially, IRX-2 is able to overcome the immune suppression of LC by HPV. Activated LC can then secrete T cell-activating and immune modulating cytokines in order to induce an immune response to HPV, thus eliminating all present lesions as well as protecting against the eruption of future lesions due to the ability of the immune system to effectively attack HPV. For example, IRX-2 increases secretion of IL-8 and ILIP-10 production by LC. The LC are able to activate HPV-specific CD8+ T cells. As described in the examples below, activation of LC is confirmed by an upregulation of CD1a, MHC class I, MHC class II, CD40, CD80, CD83, CD86, and CCR7.

[0041] The present invention also provides for a method of overcoming HPV-induced immune suppression of LC, by administering a therapeutically effective amount of IRX-2 to a patient infected with HPV, and activating LC. The IRX-2 is described above. By activating LC, the IRX-2 is able to overcome immune suppression of LC due to HPV. Thus, the LC can now effectively induce the activation of T cells to attack the HPV present in the patient.

[0042] The present invention provides for a method of increasing LC migration towards lymph nodes, by administering a therapeutically effective amount of IRX-2, activating LC, and inducing LC migration towards lymph nodes. The IRX-2 is described above. Active LC can effectively migrate towards a patient's lymph nodes after IRX-2 treatment and produce an immune response in a patient suffering from HPV. Due to immune suppression by HPV, LC are normally not able to migrate to

the lymph nodes. However, treatment with IRX-2 reverses this immune suppression so that LC are able to effectively function in the immune system.

[0043] The present invention provides for a method of generating immunity against HPV, by administering an effective amount of IRX-2 to a patient infected with HPV, generating immunity against HPV, and preventing new lesions from developing. IRX-2 is able to activate LC which have been suppressed by HPV. Activated LC can generate an immune response to HPV. Having an immune system that actively recognizes and is able to attack HPV allows for the prevention of any new lesions from developing. An activated immune system is able to effectively treat HPV and prevent future development of the disease.

[0044] There are several benefits to the present invention. Since lesions are primarily caused by persistence of HPV, interventions that induce immunological clearance of infections and prevent the transmission of HPV can have an enormous impact on public health. Elimination of HPV persistence contributes to reduced health care spending for each HPV-related cancer that would have occurred. This approach is a low-cost alternative to repeated screening or expensive surgical intervention with a high reasonable expectation of success, because it targets the cause of HPV-induced lesion development, namely HPV persistence and its related immune escape.

[0045] For any of the above embodiments, the following administration details and/or protocols for treatment are used:

[0046] Preferably, the cytokine composition is applied locally by injection to HPV-infected epithelium. Alternatively, the cytokine composition of the present invention

can be injected around lymphatics that drain into lymph nodes regional to a lesion or other virus infected area being treated. More specifically, local perilymphatic injections or other injections that are known to those of skill in the art are administered to provide sufficient localization of the immunotherapy preparation.

[0047] In the embodiment wherein an exogenous antigen is to be utilized, exogenously provided synthetic or extracted antigens such as tumor antigen and peptides (see Bellone, 1998) can be administered into the pre-primed or co-primed regional or distal lymph node, either in a separate preparation or as part of the cytokine composition of the invention.

[0048] Endogenous suppression of T cells, which can be caused by, e.g., cancer or other immunosuppressive diseases, can be blocked by the co-administration of low dose cyclophosphamide (CY) and a non-steroidal anti-inflammatory drug (NSAID) (i.e., in combination with the cytokine compositions of the invention). The NSAID is preferably indomethacin (INDO) but ibuprofen or CoxII inhibitors such as celecoxib (CELEBREX®) or rofecoxib (VIOXX®) or combinations thereof can also be used. Side effects of NSAIDS can be aggressively treated with proton inhibitors and prostaglandin E analogs. Zinc and multi-vitamins, possibly including the addition of selenium, can also be added as agents to help restore T cell immunity. Preferably, the dose of zinc is 15 to 75 mg. A standard multivitamin can be administered. The zinc can be an available gluconate.

[0049] The cytokine compositions of the invention can be administered prior to or after surgery, radiotherapy, chemotherapy, or combinations thereof. The compositions of the invention can be administered during the recurrence of tumors,

i.e., during a period where tumor growth is occurring again after a period where tumors were thought to have disappeared or were in remission.

[0050] The Mechanism of Action of IRX-2.

[0051] As defined above, the primary cell-derived biologic of the invention acts as an adjuvant, i.e., stimulates or enhances the immune response of a patient to a particular antigen. Moreover, the IRX-2 compositions and methods of the invention are particularly suited to stimulate T cell-mediated immune responses. Immune responses promoted by the compositions and methods of the invention include the induction or generation of naïve T cells, the differentiation and maturation of dendritic cells, allowing for proper presentation of antigen to T cells (e.g., in the lymph nodes), and the activation of monocytes and macrophages. Specifically, in cancer patients, immune responses promoted by the compositions and methods of the invention include tumor infiltration by lymphocytes, tumor fragmentation and regression as well as a reduction in sinus histiocytosis (when present). Essentially, the primary cell-derived biologic induces immune production and blocks immune destruction. The mechanism of action of the primary cell-derived biologic is further described in U.S. Patent Application No. 12/323,595 to Applicants.

[0052] More specifically, the compositions and methods of the present invention aid in overcoming immune depression/suppression in patients by inducing the production of naïve T cells. The term "naïve" T cells, as defined herein, denotes newly produced T cells, which T cells have not yet been exposed to antigen. Thus,

the compositions and methods of the invention replenish or generate new T cells.

[0053] Because dendritic cells are known to play such a key role in antigen presentation in the production of an appropriate immune response *in vivo*, an agent having a stimulatory effect on dendritic cell maturation will act as an adjuvant in eliciting a good immune response to an antigen. The cytokine compositions of the present invention promote dendritic cell maturation. The cytokine compositions of the invention also provide a further adjuvant effect by acting as potent activators of monocytes/macropha*ges*. *M*onocytes are precursors to both DCs and macrophages in the body and thus an agent that promotes monocyte/macrophage activation has an adjuvant effect on immune responses *in vivo*.

[0054] The primary cell-derived biologic also blocks immune destruction by protecting the activated T cells from apoptosis. Clinical and experimental data show that certain cytokines, especially survival cytokines using the common receptor γ chain, are able to protect activated T cells from tumor-induced death and enhance their anti-tumor activity.

[0055] More specifically, there are several ways in which the primary cell-derived biologic protects T cells from apoptosis. The expression of anti-apoptotic signaling molecules (i.e. JAK-3 and phosphor-Akt) is up-regulated and the expression of pro-apoptotic molecules (i.e. SOCS-2) is down-regulated. Activation of caspases in CD8+ and CD4+ T lymphocytes is decreased and cFLIP expression is increased. Inhibition of the PI3K/Akt survival pathway is counteracted by IRX-2. The T cells are protected from both extrinsic apoptosis (MV-induced and FasL-induced apoptosis) and intrinsic mitochondrial apoptosis.

[0056] The protection from extrinsic MV-induced apoptosis is further accomplished by preventing down-regulation of JAK3, CD3-ζ, and STAT5; inhibiting dephosphorylation of Akt-1/2; and maintaining balanced ratios of Bax/Bcl-2, Bax-Bcl-xL, and Bim/Mcl-1. The protection from MV-induced apoptosis is also accomplished by preventing induction of the activity of caspase-3 and caspase-7. More specifically, the induction of the active cleaved form of caspase-3 is blocked, as is the loss of mitochondrial membrane potential. Nuclear DNA fragmentation is inhibited. Protection from intrinsic apoptosis by the primary cell-derived biologic is shown by its protection of activated T cells from staurosporine-induced apoptosis.

[0057] Importantly, the cytokines of the primary cell-derived biologic protect the activated T cells from apoptosis in a synergistic manner. In other words, the combination of the cytokines in the primary cell-derived biologic produces a greater effect than is seen by administering individual cytokines alone.

[0058] In view of the above, the compositions and methods of the present invention stimulate the immune system via multiple effects, including the *in vivo* maturation of dendritic cells resulting in effective peptide antigen presentation as well as activation of monocytes and macrophages and the production of naïve uncommitted T cells. The proper presentation of antigen leads to T and B cell clonal expansion, creating immunity in the patient. In the case of cancer patients, the effects noted above result in the infiltration, e.g., of lymphocytes, into tumors (e.g., via hematogenous spread) and tumor reduction and/or destruction. The result is increased survival due to immunologic memory.

[0059] The cytokine compositions of the present invention are administered and

dosed to promote optimal immunization either to exogenous or endogenous antigen, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, and body weight. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to promote immunization, leading to, e.g., tumor reduction, tumor fragmentation and leukocyte infiltration, delayed recurrence or improved survival rate, or improvement or elimination of symptoms, including increased T cell counts.

[0060] In the methods of the present invention, the compositions of the present invention can be administered in various ways. It should be noted that the cytokines or exogenous antigens used in the compositions of the invention can be administered in their standard forms or as pharmaceutically acceptable derivatives and can be administered alone or as active ingredients in combination with pharmaceutically acceptable carriers, diluents, adjuvants and vehicles. Furthermore, the compositions of the invention can be administered intra- or subcutaneously, or peri- or intralymphatically, intranodally or intrasplenically or intramuscularly, intraperitoneally, and intrathoracically. The compositions of the invention can also be applied topically to HPV-infected epithelium, for example by infusion into the cervix of a patient. The patient being treated is a warm-blooded animal and, in particular, mammals including man. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

[0061] The doses can be single doses or multiple doses over a period of several days. When administering the compositions of the present invention, they are generally formulated in a unit dosage injectable form (e.g., solution, suspension, or emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, or vegetable oils.

[0062] Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such a cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, can also be used as solvent systems for the compositions of the invention. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it is desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however,

any vehicle, diluent or additive used would have to be compatible with the cytokines or exogenous antigens of the invention.

[0063] Sterile injectable solutions can be prepared by incorporating the cytokines or exogenous antigens utilized in practicing the present invention in the required amount of the appropriate solvent with several of the other ingredients, as desired.

[0064] A pharmacological formulation of the present invention can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicles, additives, and diluents; or the cytokines and/or exogenous antigens utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of delivery systems useful in the present invention include those disclosed in U.S. Pat. Nos. 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

[0065] It should be apparent that the compositions and methods of the invention are useful for the treatment of antigen-producing diseases such as cancer, infectious diseases or persistent lesions, as discussed above. The compositions and methods promote immunization against the antigens produced by these diseases by stimulating immune responses in patients in vivo, which immune responses help to alleviate or eliminate the symptoms and effects of the disease in the patient.

[0066] The invention is further described in detail by reference to the following

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

[0067] EXAMPLE 1

[0068] Generation of human LC

[0069] As HPV only infects human beings, human immune cells were used to study the interaction of HPV with LC. Primary LC that were isolated from human skin, becoming activated through the migration process and express high levels of MHC and costimulatory molecules. Therefore, it is not feasible to isolate primary unactivated skin-derived LC from human donors with which to conduct meaningful *in vitro* long term and reproducible functional studies. For the experiments, primary LC were generated from peripheral blood monocytes isolated from healthy donors using differentiating cytokines *ex vivo*. Circulating monocytes were direct precursors of epidermal LC *in vivo*. Applicants and others have shown that *ex vivo* derived LC express the same surface markers as epidermal LC (Langerin, E-cadherin, CD11c, CD1a, high MHC class II and intracellular Birbeck granules) (FIGURE 1) and can be used consistently for *in vitro* LC studies.

[0070] FIGURE 1 shows that human monocyte-derived Langerhans cells express similar phenotypic markers as skin-derived Langerhans cells. Immature Langerhans cells were differentiated from adherent monocytes in 1000 IU/mL GM-CSF, 1000

IU/mL IL-4 and 10 ng/mL TGFβ for 7 days. Cells were analyzed by flow cytometry for the expression of MHC class II (HLA-DP,DQ,DR), CD1a, CD11c, Langerin, or E-cadherin (gray shaded histograms). Isotype controls are shown as black unshaded histograms. Data are representative of LC derived from several healthy donors.

[0071] Reversal of HPV immune escape

[0072] Applicants have previously embarked on strategies to reverse HPV immune escape by targeting LC. LC express a variety of TLRs, including TLR3, 7, 8, and 9, which recognize pathogen-associated molecular patterns (PAMPs) and upon engaging their ligands activate the cell. Surprisingly, it was found that a TLR 7 agonist ALDARA® (Imiquimod, Graceway Pharmaceuticals, LLC), FDA-approved for external genital warts, had absolutely no effect on LC activation, as measured by expression of CD86 (Figure 2). This might explain the yet unpublished observations that the use of Imiquimod for treating cervical lesions is not effective. Interestingly, a TLR 8 activator (3M-002) and a TLR7/8 agonist (Resiquimod) fully activated HPV infected LC such that they started to induce HPV-specific T cell responses *in vitro*. These data indicate that suppression of LC function by HPV can be reversed by activating certain “danger signal” pathways.

[0073] Activation of human Langerhans cells with IRX-2

[0074] The ability of LC to efficiently stimulate T cells after migration from the epidermis to the draining lymph nodes after exposure to pathogens or other “danger signals” requires the expression of costimulatory molecules and chemokine

receptors on their cell surface. The effect of IRX-2 on the phenotypic maturation of human monocyte-derived LC *in vitro* was examined. IRX-2 treatment was found to induce upregulation of both MHC class I and MHC class II, and the costimulatory molecules CD40, CD80 and CD86, the maturation marker CD83, and . LC activation was performed as previously described. Briefly, LC were harvested, washed, and either left untreated or treated with HPV16L1L2 VLP at a concentration of 10µg/106 cells for 1 hour at 37℃. Following the incubation, the cells were placed at 37℃ for 6 hours in complete medium. Next, the cells were left untreated or treated with IRX-2 (1:2 dilution) or with 1µg/mL LPS as a positive control. The cells were incubated for an additional 48 hours post IRX-2 treatment. Cells were harvested, washed, and stained for flow cytometric analysis staining for CD1a, MHC class I, MHC class II, CD40, CD80, CD83, CD86, or isotype controls. The fold change of surface markers between treatment groups were calculated from MFI values. Increased MFI indicates upregulation of markers and activation of LC. Data are representative of three individual donors

[0075] The source of IRX-2 was supernatant collected from human peripheral blood mononuclear cells stimulated by phytohemagglutinin (PHA) for a 24-hour period. QC testing was used to test levels of cytokines in the mixture and standardize according to the levels of four key cytokines. The cGMP manufacturing process yielded a highly consistent product with very similar cytokine concentrations from lot to lot. To ensure consistency in LC activation by IRX-2, two different lots of IRX-2 were used to activate LC.

[0076] As shown in FIGURE 6, expression of all six LC markers was strongly

elevated by IRX_2 treatment, regardless of whether the LC had been exposed to HPV16 L1L2 VLP. Interestingly, elevation of the costimulatory molecule CD86 was even more effective in HPV16 LIL2 VLP exposed LC than in controls. Greater than 95% of cells expressed CD86 and greater than 80% of LC expressed the maturation marker CD83 (not shown).

[0077] The results indicate that that IRX-2 is a potent inducer of LC activation and maturation, and that its effectiveness is not impaired by prior exposure of LC to HPV.

[0078] EXAMPLE 2

[0079] Establishing whether IRX-2, which is currently available for clinical use, can activate LC previously exposed to HPV16 is necessary. The effect of IRX-2 on LC expression of activation markers was reported in Example 1, In the next experiments, the strength of IRX-2 was tested by measuring cytokine secretion, migration, and activation of alloantigen-specific T cells.

[0080] Activation of APC, like LC, is required for successful interaction with and activation of primary T lymphocytes. Inflammatory cytokines have the ability to activate APC, resulting in maturation and an increase in antigen-presenting function. IRX-2 is a promising immune modulator that has the potential to influence the immunostimulatory capacity of LC when applied locally to HPV-infected epithelium. In order to determine whether IRX-2 phenotypically and functionally activates LC exposed to HPV, cytokine/chemokine secretion and migration as well as the ability to stimulate alloantigen-specific T cell responses was performed. These studies defined IRX-2 as able to reverse the immune suppression by HPV, similar to TLR8

agonists.

[0081] Experimental Design:

[0082] LC expression levels of T cell co-stimulatory markers was determined when LC were exposed to HPV16 and then to IRX-2. LC were differentiated from peripheral blood monocytes isolated from healthy donors. LC were exposed to HPV16 virus-like particles (VLP) for 6 hours, and then IRX-2 added for an additional 48 hours, as shown in FIGURE 5. Cell surface molecules were measured by flow cytometry using fluorescent antibodies. Cell culture supernatants were tested for the presence of immune-stimulatory cytokines and chemokines to determine whether LC have become activated and are secreting T cell-activating or other immune modulating cytokines. LC migration after exposure to HPV VLP and IRX-2 was measured by *in vitro* migration through a transwell membrane. The capacity to stimulate T cells was evaluated by culturing LC with allogeneic T cells in a mixed lymphocyte reaction (MLR). Untreated LC, LC exposed to HPV VLP alone, and LC treated with IRX-2 alone serve as controls in each experiment. Each experiment was repeated at least 3 times using LC derived from individual healthy donors. Subjects were chosen who are HLA-A*0201 positive, so that well defined T cell immune responses were measured against known HPV-derived peptide antigens. These data show that IRX-2 enables HPV-exposed LC to gain back their capacity to stimulateT cells and to migrate - both functions needed for a productive anti-viral immune response.

[0083] Generation of human Langerhans cells:

[0084] Primary LC were generated from commercially available peripheral blood monocytes isolated by leukapheresis of anonymous healthy donors using differentiating cytokines *ex vivo*. Monocyte-derived LC were generated first through plastic adherence of cryopreserved PBMC to culture flasks. Adherent cells were cultured for 7 days in medium containing 1000 U/mL recombinant human (rhu)-GM-CSF, 1000 U/mL rhu-IL-4 and 10 ng/mL rhu-TGF-β1, replenished twice during the culture period.

[0085] LC migration:

[0086] Chemokine directed migration of LC was carried out using 24-well Transwell plates with 5 μm-pore-size polycarbonate filters (Corning Costar). Media was added to the lower chamber containing either 250 ng/ml rhu CCL21 (R&D Systems) or medium alone to control for spontaneous migration. LC untreated or treated as described above, were added to the upper chamber and incubated for 4 hours at 37°C. The cells that migrated to the lower chamber were counted using a hemacytometer or automated cell counter, and CCL21-dependent migration was calculated as the ratio of cells that migrated with CCL21 to cells that migrated without CCL21, termed migration index. Increased migration index indicated a functional activation of LC to move from the tissue towards draining lymph nodes (FIGURE 7).

[0087] IRX-2 treatment produced a three to four fold increase in migration in both

control and HPV L1L2 VLP. The results indicate that IRX-2 can promote the migration of LC to regional lymph nodes, even when the LC had been exposed to HPV.

[0088] Mixed lymphocyte reaction assay:

[0089] LC were treated with HPV16 L1L2 VLP and IRX-2 as described above. LC were co-cultured with untouched allogeneic T cells isolated using a MACS negative selection human pan T cell isolation kit. Responder T cells and stimulator LC were cultured at R:S ratios of 10:1 and 5:1 for 5 days. T cells cultured alone, LC cultured alone, T cells cultured with autologous PBMC and T cells cultured with the T cell mitogen PHA served as controls for the assay. Radioactive ^{3}H-thymidine-pulsed cells were harvested and radioactivity counted on a scintillation plate counter. Radioactive cpm were determined and compared between treatments. Increased thymidine incorporation was indicative of greater T cell proliferation. Increased T cell proliferation after HPV16 L1L2 VLP exposure and IRX-2 stimulation confirmed that HPV16 L1L2 VLP-exposed LC were gaining back their immunostimulatory capacity in the presence of HPV (FIGURE 8).

[0090] Cytokine and chemokine analysis:

[0091] Supernatants were collected from LC stimulated with IRX-2 and tested for secreted cytokine and chemokines. LC were treated as described above. LC were washed 36 hours post IRX-2 treatment and cultured for an additional 36 hours, prior to collection of supernatants. This eliminated measurement of cytokines present in

IRX-2 mixture. The assays were completed using the Bio-Plex Suspension Array System which allowed for several inflammation and T cell stimulating and chemoattracting analytes to be assayed at once. The assayed cytokines and chemokines included IL-8, IFN-γ Inducible Protein 10 (IP-10),), Monocyte Chemoattractant Protein (MCP)-1, Macrophage Inflammatory Protein (MIP)-1α, MIP-1β, and RANTES. Data were analyzed by comparing the cytokine and chemokine concentrations in the supernatants of treatment groups. Increased Th1-associated cytokine and chemokine secretion indicates functional activation of LC that would support the induction of CD8+ T cell responses, while increased suppressive cytokines suggests a tolerizing or suppressive function of LC.

[0092] Treatment of LC with IRX-2 significantly increased the secretion of two Th1 associated chemokines, IP-10 and IP10 (FIGURE 9). Both chemokines are pro-inflammatory, and IP-10 is known to attract T cells, NK cells, and monocytes to sites of inflammation. The results show that, under IRX-2 stimulation, L1L2 VLP-exposed LC were gaining back their immunostimulatory capacity in the presence of HPV.

[0093] Conclusion:

[0094] IRX-2 phenotypically and functionally activated human LC from healthy donors, even after they were exposed to HPV L1L2 VLP. . Increased levels of MHC and surface activation molecules, increased secretion of inflammatory and T cell activating cytokines and chemokines, and an increased ability to perform chemokine-directed migration after HPV and IRX-2 treatment (FIGURES 6-9), These results all support the effectiveness of the present invention in overcoming HPV-

induced tolerization of LC and providing treatment for HPV infections. The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

[0095] Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

CLAIMS

1. A formulation comprising:
a primary cell-derived biologic which includes the cytokines IL-1, IL-2, IL-6, IL-8, IFN-gamma and TNF-alpha,
for use in immunotherapy of a patient infected with HPV.

2. The formulation of claim 1, wherein the primary cell-derived biologic includes a concentration of IL-1 from 60 - 6,000 pcg/mL, a concentration of IL-2 from 600-60,000 pcg/mL, a concentration of IL-6 from 60-6,000 pcg/mL, a concentration of IL-8 from 6000-600,000 pcg/mL, a concentration of TNF-α from 200-20,000 pcg/mL, and a concentration of IFN-γ from 200-20,000 pcg/mL.

3. The formulation of claim 1 or 2, wherein the primary cell-derived biologic further includes IL-7, IL-12, IL-15, GM-CSF, and G-CSF.

4. The formulation of any one of claims 1 to 3, further comprising an agent that inhibits T regulatory cells, wherein the agent is a chemical inhibitor selected from an alkylating agent, an antimetabolite, an antibiotic and an immunomodulating agent.

5. The formulation of claim 4, wherein the alkylating agent is cyclophosphamide.

6. The formulation of any one of claims 1 to 5, further comprising an NSAID selected from indomethacin, ibuprofen, celecoxib, rofecoxib, and combinations thereof.

7. The formulation of claim 6, wherein the NSAID is indomethacin.

8. The formulation of any one of claims 1 to 7, further comprising zinc to provide nutrients for T cell function.

9. The formulation of any one of claims 1 to 8, wherein the formulation is a sterile, injectable solution.

10. The formulation of claim 9, wherein the sterile, injectable solution is for injection in the patient's epithelium.

11. The formulation of any one of claims 1 to 8, wherein the formulation is for topical application to the patient's epithelium.

12. The formulation of any one of claims 1 to 11, for the further use of preventing new lesions from developing.

13. The formulation of any one of claims 1 to 12, wherein the patient has not received prior radiation treatment.

14. Use of a composition comprising:
a primary cell-derived biologic which includes the cytokines IL-1, IL-2, IL-6, IL-8, IFN-gamma and TNF-alpha,
in the manufacture of a medicament for use in immunotherapy of a patient infected with HPV.

15. The use of claim 14, wherein the primary cell-derived biologic includes a concentration of IL-1 from 60 - 6,000 pcg/mL, a concentration of IL-2 from 600-60,000 pcg/mL, a concentration of IL-6 from 60-6,000 pcg/mL, a concentration of IL-8 from 6000-600,000 pcg/mL, a concentration of TNF-α from 200-20,000 pcg/mL, and a concentration of IFN-γ from 200-20,000 pcg/mL.

16. The use of claim 14 or 15, wherein the primary cell-derived biologic further includes IL-7, IL-12, IL-15, GM-CSF, and G-CSF.

17. The use of any one of claims 14 to 16, wherein the composition further comprises an agent that inhibits T regulatory cells, wherein the agent is a chemical inhibitor selected from an alkylating agent, an antimetabolite, an antibiotic and an immunomodulating agent.

18. The use of claim 17, wherein the alkylating agent is cyclophosphamide.

19. The use of any one of claims 14 to 18, wherein the composition further comprises an NSAID selected from indomethacin, ibuprofen, celecoxib, rofecoxib, and combinations thereof.

20. The use of claim 19, wherein the NSAID is indomethacin.

21. The use of any one of claims 14 to 20, wherein the composition further comprises zinc to provide nutrients for T cell function.

22. The use of any one of claims 14 to 21, wherein the composition is formulated as a sterile, injectable solution.

23. The use of claim 22, wherein the sterile, injectable solution is for injection in the patient's epithelium.

24. The use of any one of claims 14 to 21, wherein the composition is formulated for topical application to the patient's epithelium.

25. The use of any one of claims 14 to 24, for the further prevention new lesions from developing.

26. The use of any one of claims 14 to 25, wherein the patient has not received prior radiation treatment.

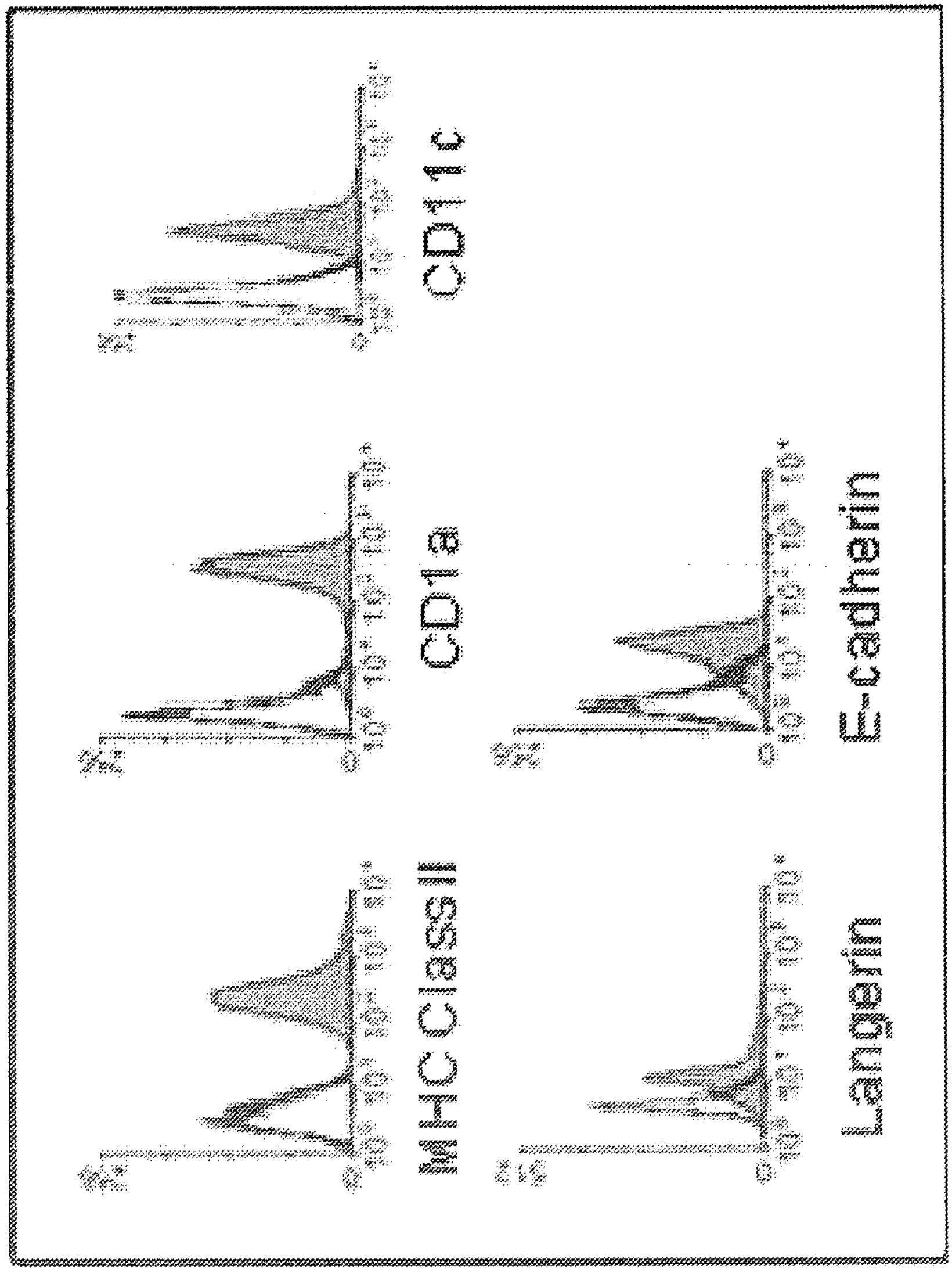


Figure 1 – Prior Art

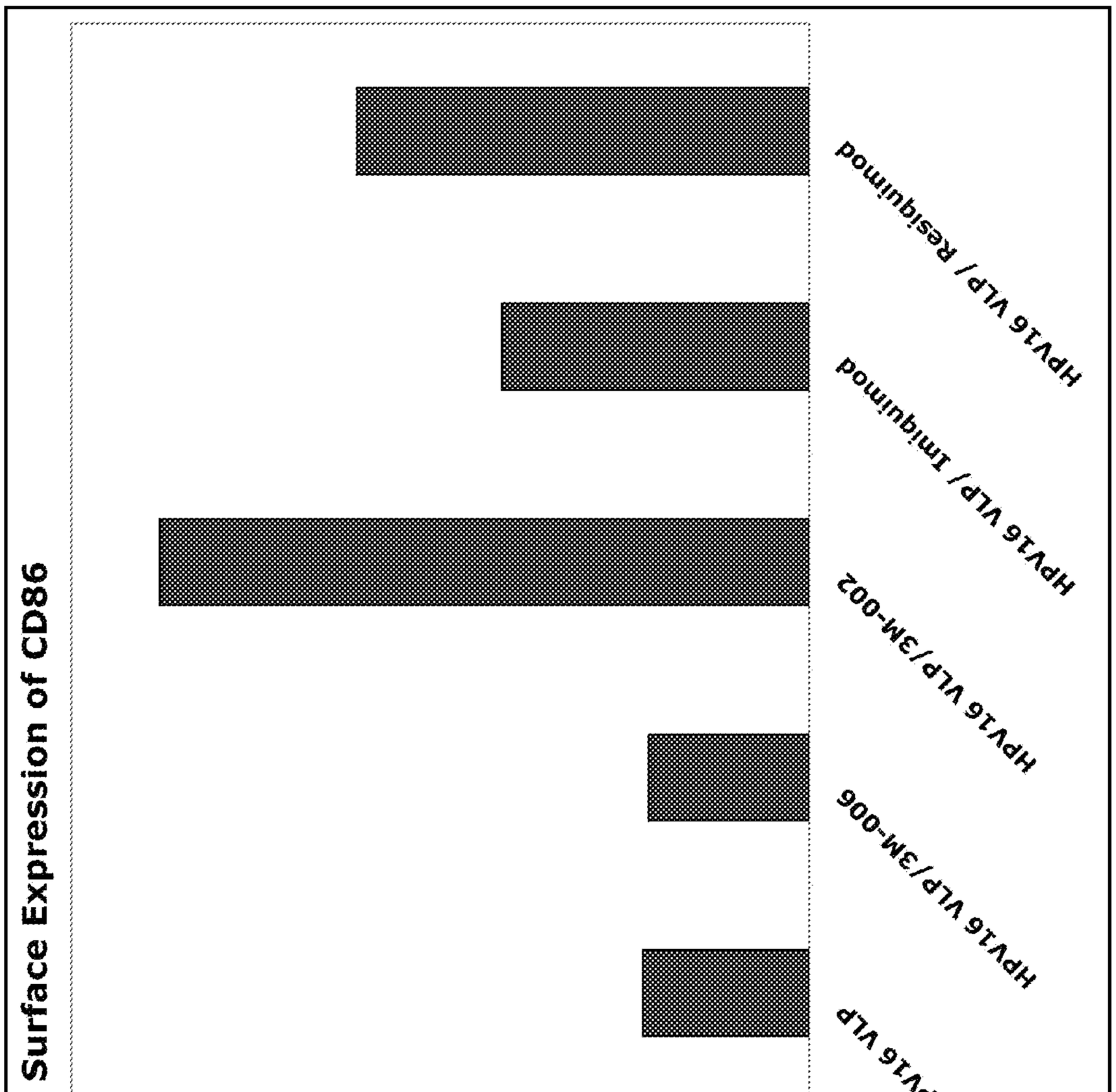


Figure 2 – Prior Art

Langerhans cells: Antigen Presenting Cells of the skin and mucosa

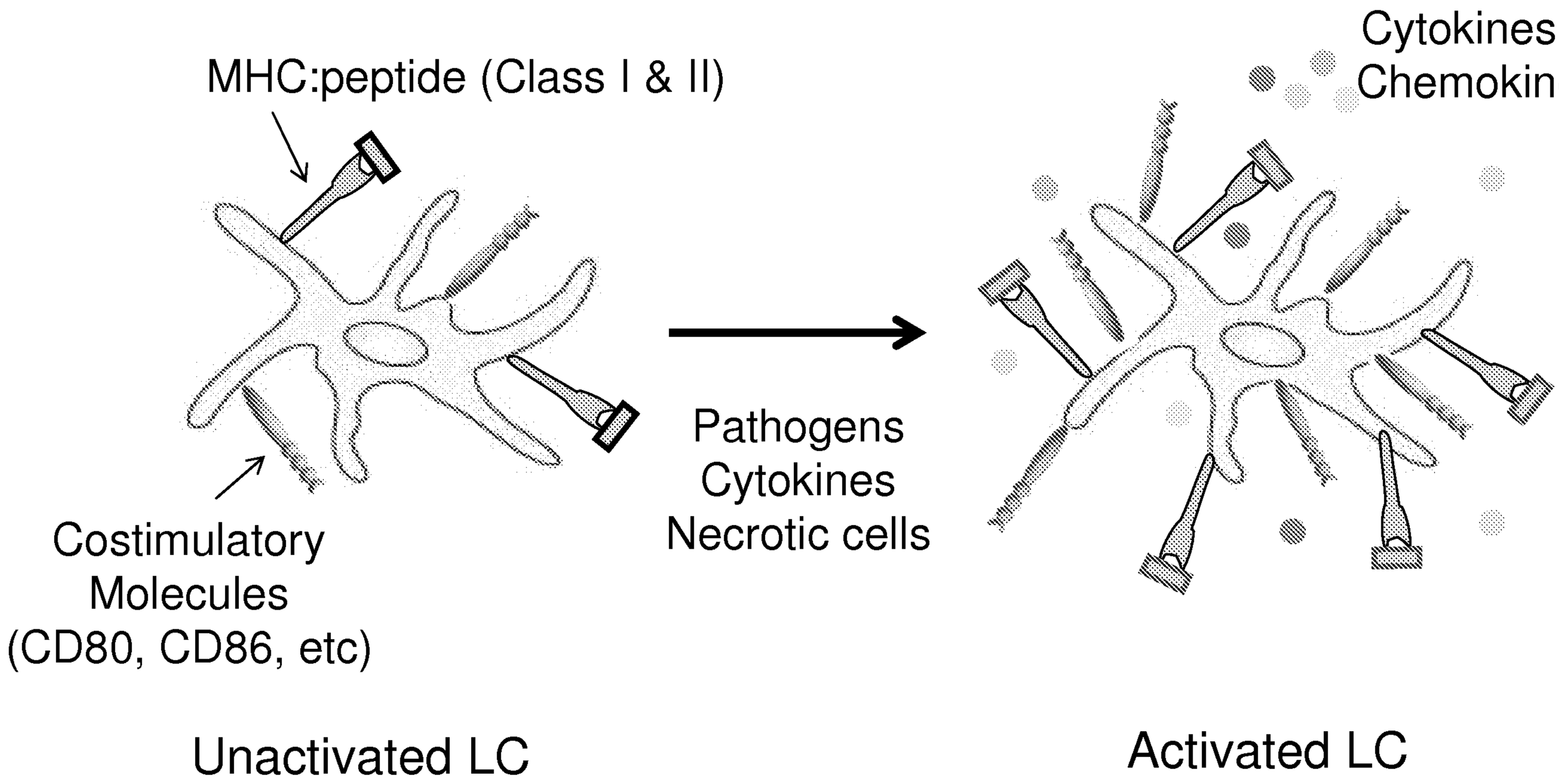


Figure 3

HPV Immune Escape: Antigen presentation by human Langerhans cells in the absence of costimulation/maturation

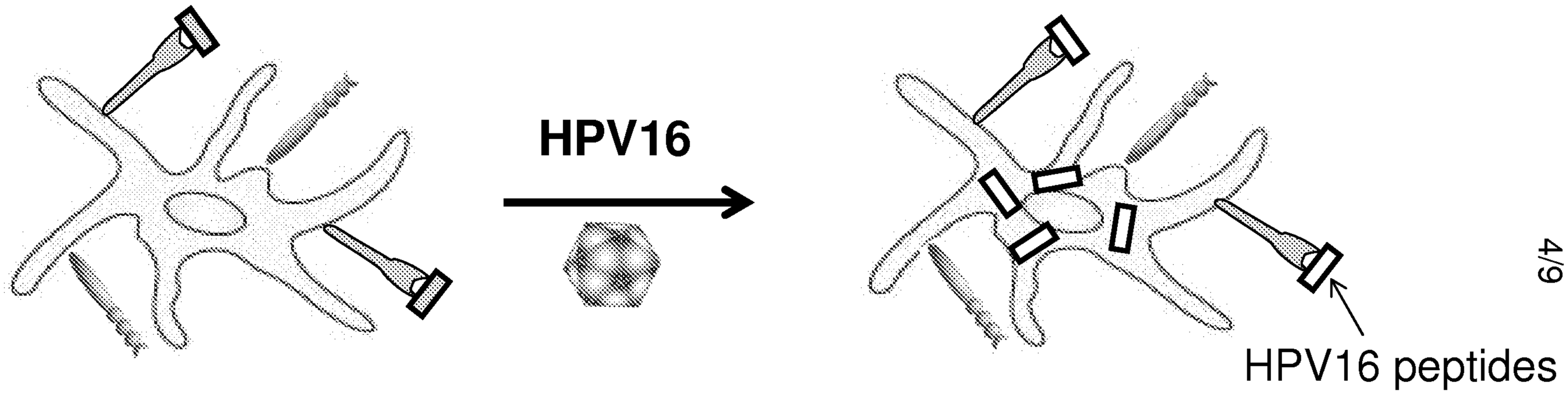


Figure 4

CA 02820202 2013-06-05

Experimental Design

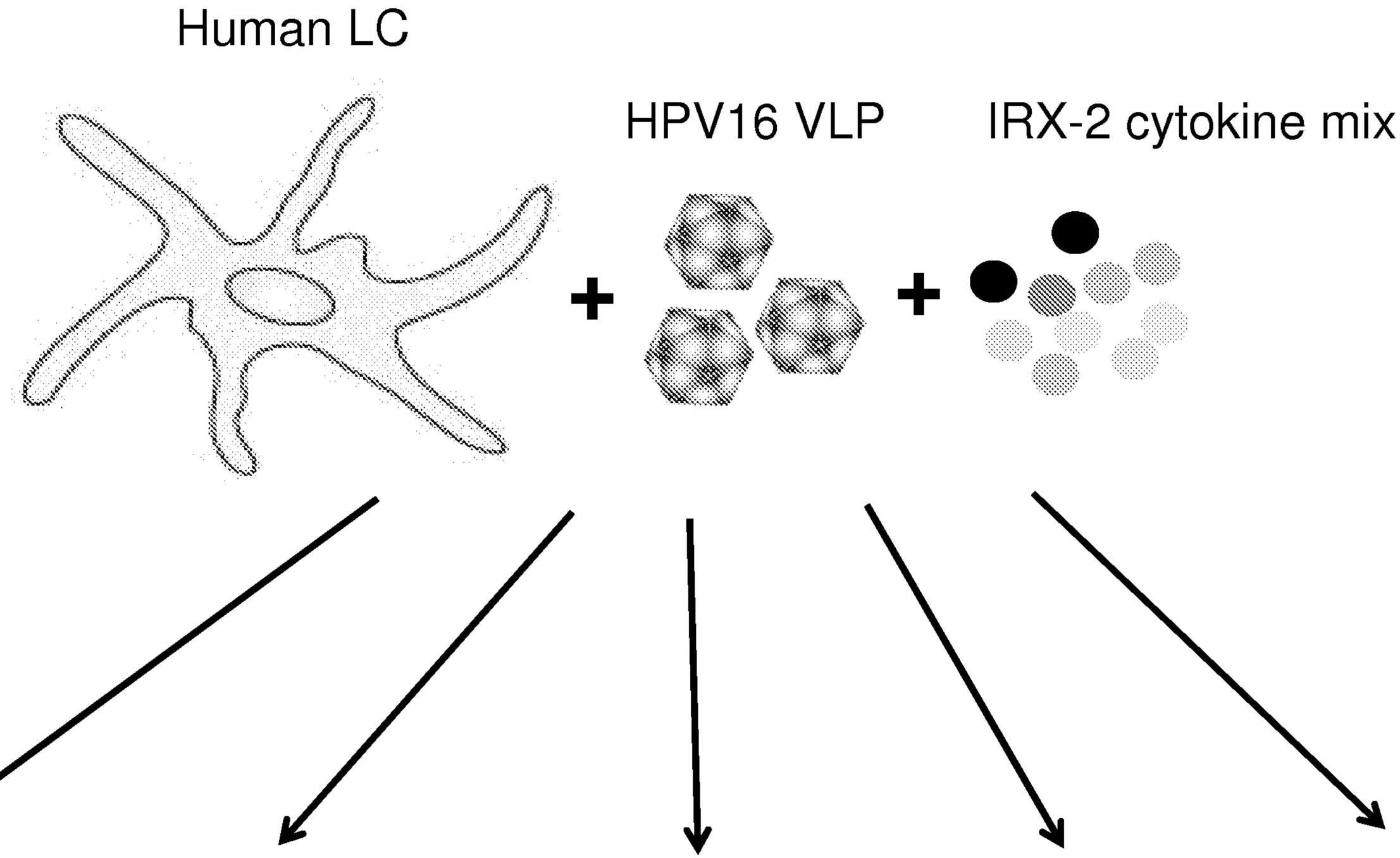


Figure 5

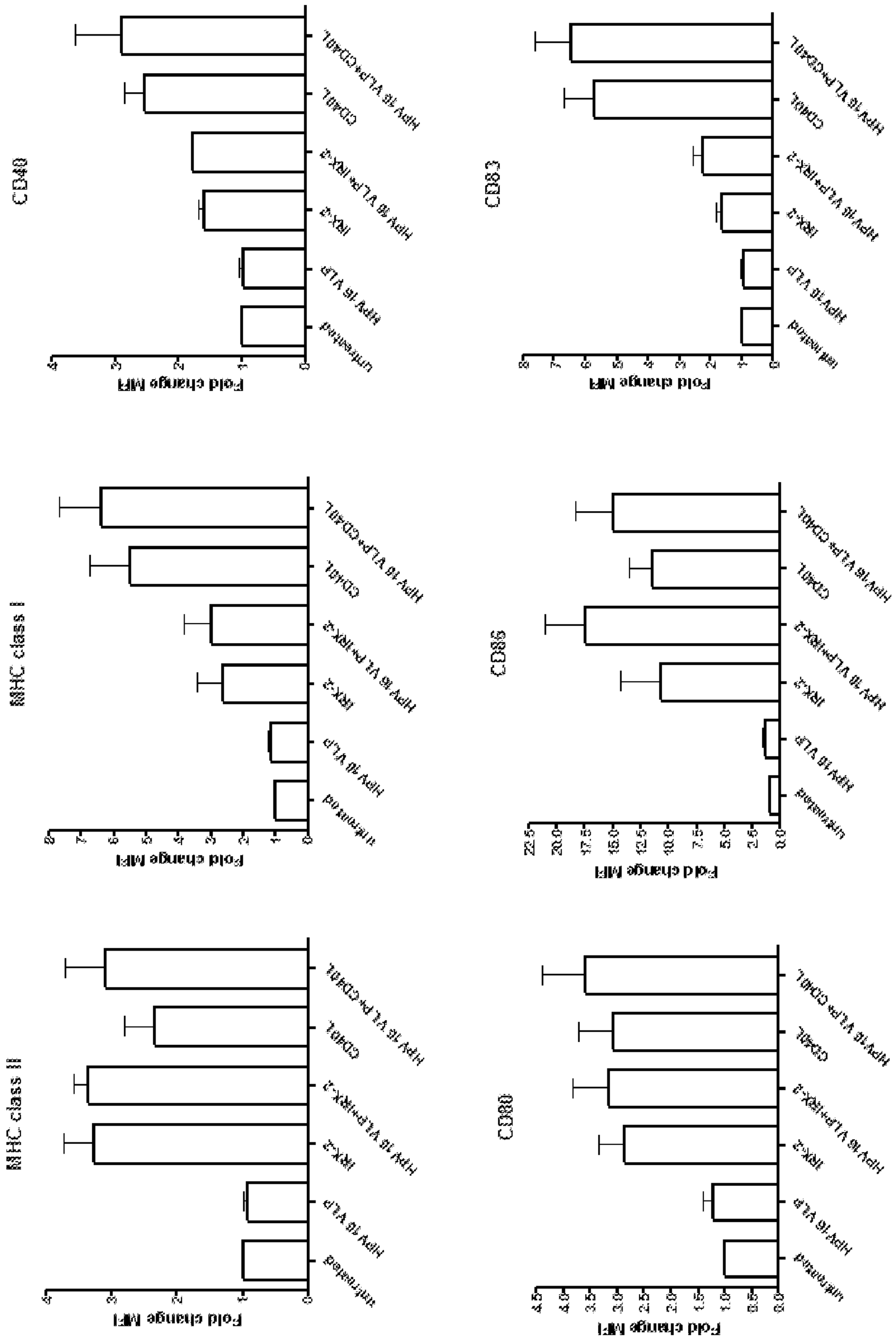

CD40
Fold change MFI
unstained
HPV 16 VLP
HPV 18 VLP
HPV 16 VLP+CD40L
CD40L
HPV 18 VLP+IRX-2
IRX-2
HPV 16 VLP+IRX-2
HPV 18 VLP+CD40L
CD83
MHC class I
CD86
MHC class II
CD80

Figure 6

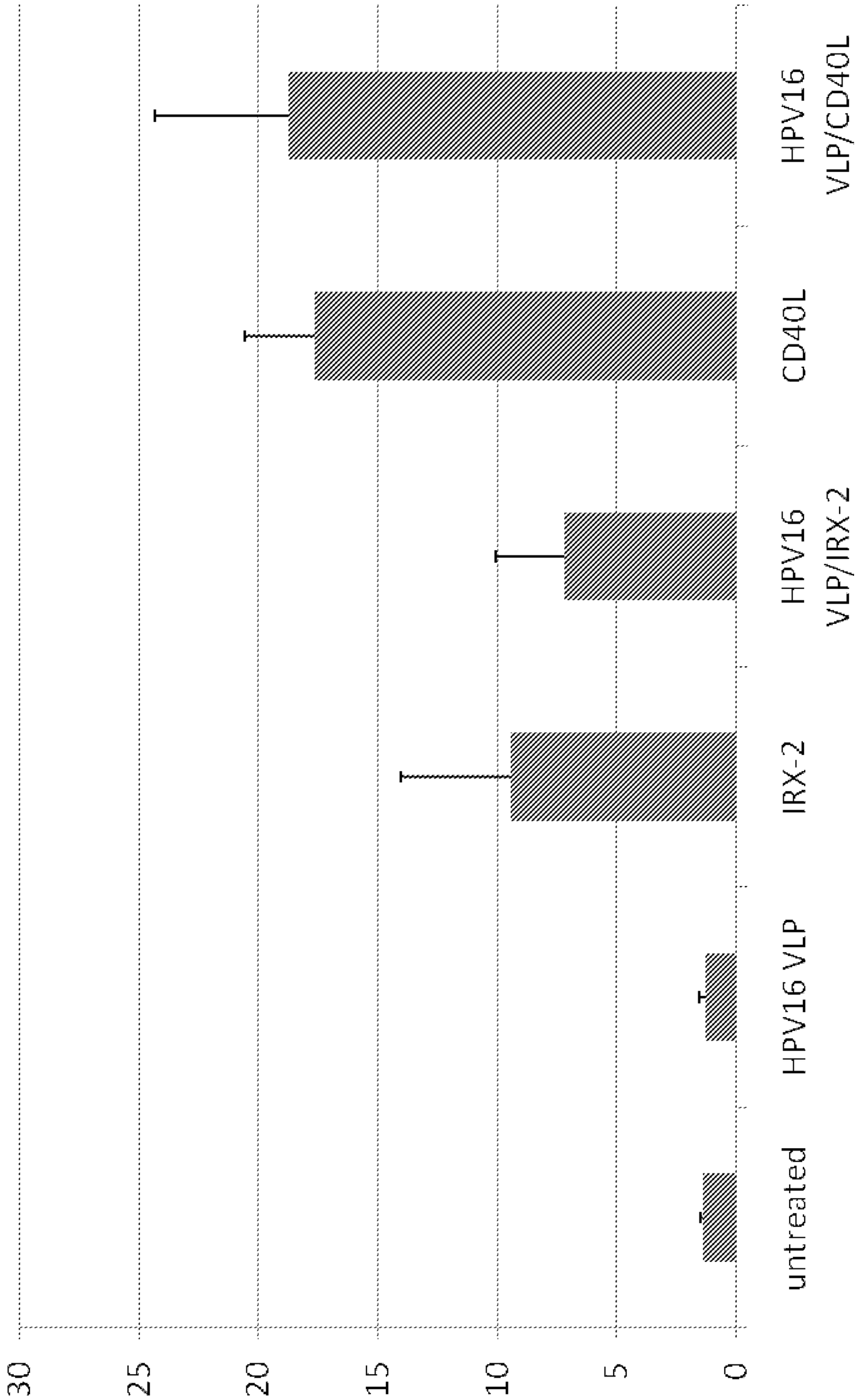

Migration Index
30
25
20
15
10
5
0
untreated
HPV16 VLP
IRX-2
HPV16 VLP/IRX-2
CD40L
HPV16 VLP/CD40L

Figure 7

Human LC exposed to IRX-2 are superior in stimulating allogeneic T cells even in the presence of HPV

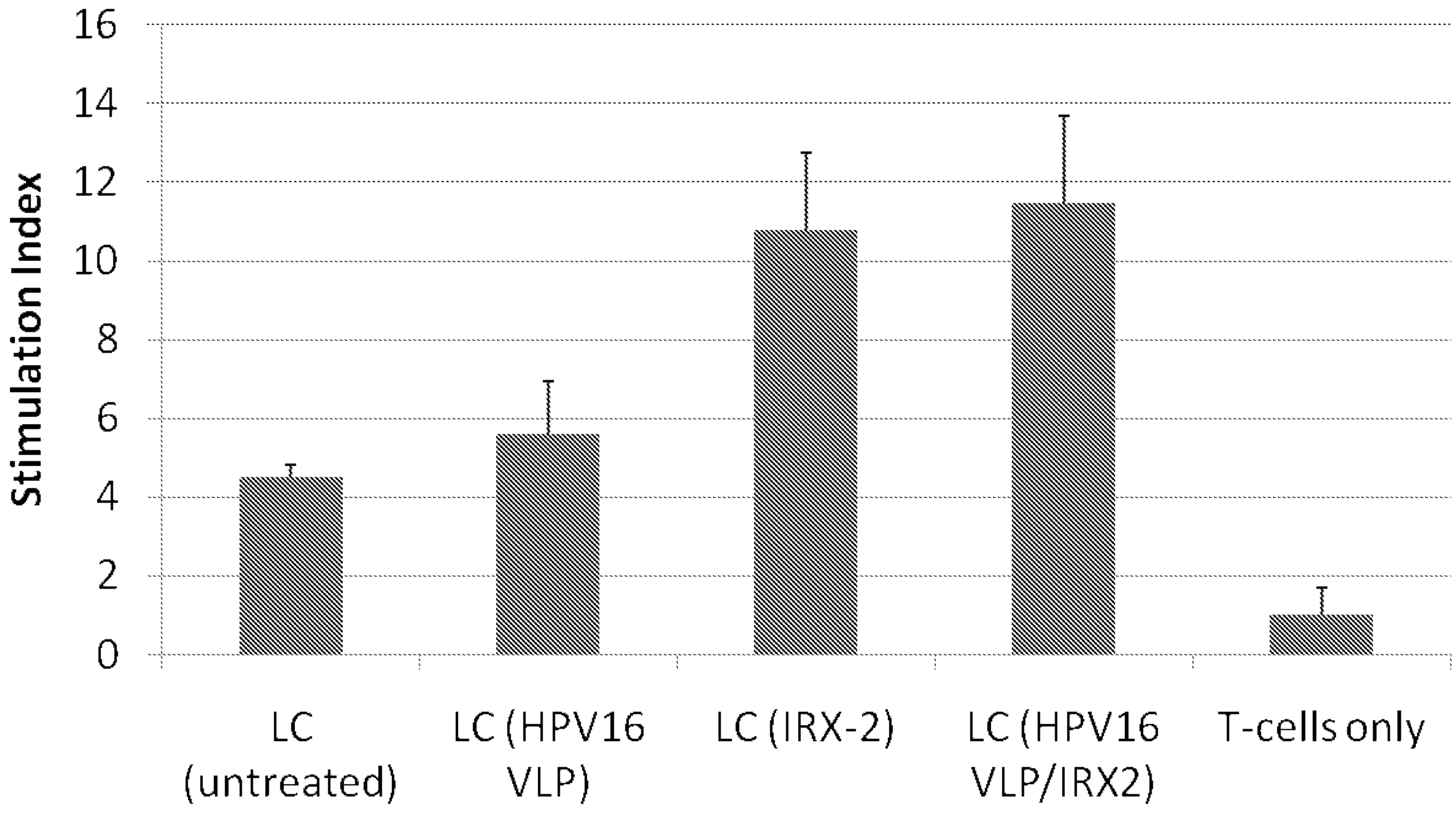


Figure 8

Human Langerhans cells treated with IRX-2 after HPV16 exposure secrete high levels of IL-8 and IP-10

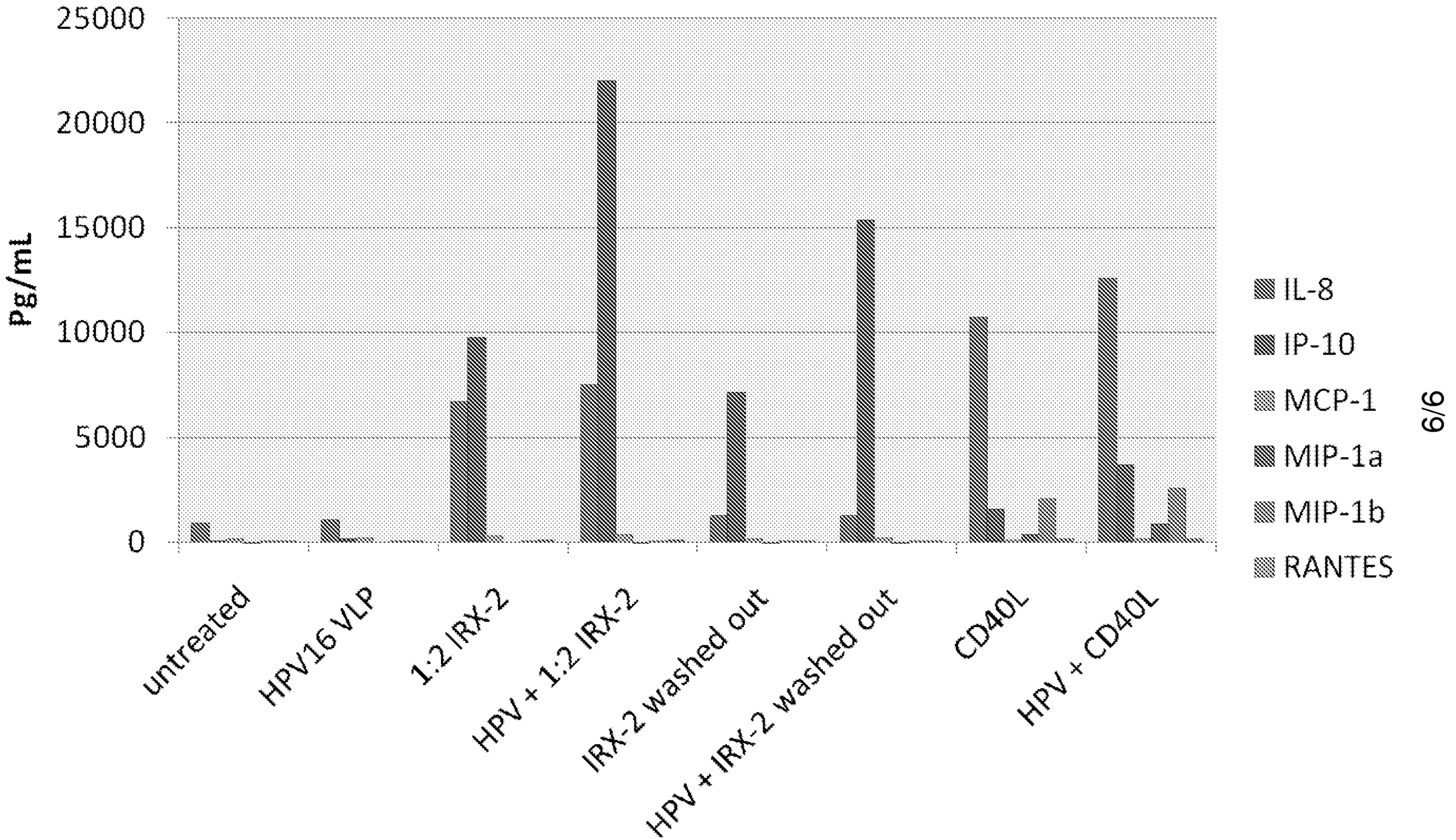


Figure 9

Experimental Design

