Abstract:
The present invention relates to an in vivo method for monitoring cytotoxic T lymphocyte (CTL) responses by inducing a delayed-type hypersensitivity (DTH) reaction using defined CTL epitopes. It further relates to the epitopes and the kits them.
METHOD FOR MONITORING CYTOTOXIC T LYMPHOCYTE (CTL) RESPONSES BY A DELAYED-TYPE HYPERSENSITIVITY REACTION USING DEFINED CTL VIRAL EPITOPES

Technical Description of the Invention

The present invention relates to an in vivo method for monitoring cytotoxic T lymphocyte (CTL) responses by inducing a delayed-type hypersensitivity (DTH) reaction using defined CTL epitopes. The invention also relates to a kit containing said defined CTL epitopes.

Background of the Invention

Simple and accurate immune monitoring assays that measure physiologically relevant effector function(s) of vaccine induced T and B cell responses are a prerequisite for effective HIV vaccine development. However, the design of such assays is hampered by the absence of well-defined immune correlates of viral control in HIV subjects and other issues. See Prado J, et al, Curr. Med. Chem. 2011; 18(26):3963-3970. The successful development of such immune monitoring approaches would benefit also from an improved understanding about how the reactive cells mediate their in vivo effects and which of their many effector functions can be most tightly linked to their in vivo effectiveness. In current vaccine trials, labor-intensive ex-vivo and in vitro assays are being employed to measure vaccine-induced T cell immunity. These include flow-cytometric analyses that assess cytokine production and degranulation capacity, the cells' potential to inhibit viral replication in vitro, and ELISpot assays that allow for the testing of large numbers of antigens, albeit with a very limited focus. See Koup R, et al, PLoS One 2010; 5(2):e9015 and Yang O, et al, Trends Immunol. 2003; 24(2):67-72. None of these assays has unequivocally defined markers of a durable immune control in HIV chronically infected subjects. While several assays (e.g. single-cytokine ELISpot, flow-cytometric detection of intracellular cytokine production) can be used to assess the magnitude and breadth of vaccine-induced responses, they are unlikely to show the effector functions that are crucial for an effective in vivo response.
Thus, alternative assays, measuring anti-viral effects more directly or assessing the functionality of effector cells, need to be developed. For large vaccine efficacy trials, such assays also need to be applicable in low-resource settings, without extensive infrastructure, and maintain their sensitivity and specificity.

Immune responses to some vaccines are tested by measuring cutaneous reactions to intradermal antigen exposure, which allows to assess the immunocompetence to infectious agents following previous infections or immunizations. See Birx D, et al, J. Acquir. Immune Defic. Syndr. 1993; 6(1):1248-1257, Palmer D, et al, J. Infect. Dis. 1974; 130(2):138-143, and Palmer D, et al, J. Infect. Dis. 1974; 130(2):132-137. Delayed type hypersensitivity (DTH) reactions manifest themselves clinically as an area of induration and erythema around the antigen injection site and become visible generally within 24-72h after antigen injection. The local cellular infiltrate is dominated by macrophages and T lymphocytes, commonly associated with Th1 cytokine profile. As the induction of DTH reactions is generally geared towards the detection of CD4+ T cell mediated immunity, no similarly widely-used and simple test exists to specifically assess the presence of antigen specific CD8 cytotoxic T cells. See Hladik F, et al, J. Immunol. 2001; 166(5):3580-3588. However, when well characterized peptide MHC class I restricted epitopes have been injected in mice, intradermal infiltrates of CD4+ and CD8+ T cells have been observed, suggesting that short, optimally defined CD8+ T cell epitopes could also elicit such DTH responses on their own. See Kundig T, et al, Proc. Natl. Acad. Sci. USA 1992; 89(16):7757-7761. Indeed, the injection of cancer-derived epitopes into the skin of cancer-vaccine recipients showed the presence of CD8+ antigen-specific CD8+ T cells, indicating that at least vaccine-induced responses to self-antigen can be detected by means of DTH reactions.

See Chen Q, et al, Cancer Immun. 2005; 5:5. In addition, allergic reactions to penicillin and its derivatives are considered to be mediated largely by CD8+ T cells that react to the haptenized class I epitopes and which also elicit a DTH reaction upon intradermal administration of penicillin. See Brander C, et al, J. Immunol. 1995; 155(5):2670-2678. However, there is no data in humans where defined CTL epitopes from different viruses have been tested for their potential to induce DTH reactions and that would have linked DTH reactivity to relative in vivo viral control.
Diagrams

Figure 1. A) Representative example of DTH response 72h after intradermal delivery of PBS, Candida, influenza-GL9 peptide, HIV-SL9 peptide and tetanus toxoid (TT). B) Extend of DTH (determined as the diameter of induration (mm)) is shown for all HLA-A2 expressing subjects included in the study. Black symbols indicate HIV uninfected subjects, white symbols represent HIV infected patients.

Figure 2. A) Representative example of gating strategy with dextramer positive (middle) and negative (right) staining for SL9 and GL9 specific T cells populations. B) Percentages of CD3CD8SL9 and CD3CD8GL9 positive cells expressing the maturation markers CCR7 and CD45RA. Naive cells are defined as CCR7+CD45RA\textsuperscript{high}, central memory cells (CMs) as CCR7+CD45RA\textsuperscript{low}, effector memory cell (EMs) as CCR7\textsuperscript{high} and terminally differentiated effector memory cells (EMRs) as CCR7-CD45RA\textsuperscript{high}. The right hand panel shows the direct comparison of the frequency of central memory cells among SL9 and GL9 positive cells. C) Level of CD27 expression expressed on T cell subsets specific for SL9 and GL9. The mean fluorescence intensity (MFI) is indicated, with special focus on the terminally differentiated effector memory cells in the right hand.

Figure 3. A) CLA expression differences among epitope specific populations, shown as percentages of all epitope-specific cells (left panels) and MFI levels (right panels Y) for both, the pre-epitope injection and 72h post injection time points. B) Level of CD103 expression on epitope specific cells shown as mean fluorescence intensity (MFI) at pre-epitope injection and 72h post injection time points. The percentage of positive cells is not shown as it did not differ between epitope specific CTL (p-value: 0.7622 Mann-Whitney). C) Expression of BLT1 (left panels) and CXCR1 (right panels) among epitope-specific CTL shown as percentage of cells with detectable surface expression levels, both at the pre-epitope-injection and 72h post injection time points.
**Brief Summary of the Invention**

The present invention refers to an in vivo method for monitoring the cytotoxic T lymphocyte (CTL) response against a virus in a subject. The method comprises the steps of i) administering a HLA-A2 restricted epitope of a viral antigen to the subject, and ii) determining if a cutaneous DTH reaction occurs, wherein a positive DTH reaction is indicative that the subject can mount an effective CTL response against the virus. The method involves administering the antigen epitope intradermally to the subject and observing if induration and erythema ensues after 48-72 hours post-injection. A positive response means that the subject has been exposed to the antigen at least 4 to 6 weeks before the administration and that his cell-mediated immunity mechanism is effective in recognizing and responding to the antigen. The lack of a DTH response to the antigen may be regarded as an evidence of anergy. In the absence of underlying diseases, anergy may indicate primary or secondary T cell immunodeficiency.

In second aspect, the invention relates to kit or composition comprising a HLA-A2 restricted epitope of the invention for assaying the CTL immune response in subject.

**Detailed Description of the Invention**

The induction CTLs is believed to be an important defense mechanism against viral infections. However, their in vivo effectiveness may vary in different infections. In addition, their in vitro, functional assessment often relies on surrogate markers, such as single cytokine release, that may be physiologically quite irrelevant. The availability of simple, sensitive, specific and physiologically informative in vivo tests, applicable to humans, would greatly facilitate immune monitoring in large vaccine trials.

The present invention demonstrates that local DTH reactions can be elicited by short CTL epitopes alone and that differences in dermal migration markers between HIV and influenza-specific CTL could reflect the impaired in vivo functionality of HIV-specific CTL. These data may help to establish "CTL-DTH" as a simple, cheap and sensitive immune monitoring tool for large-scale vaccine trials for HIV and other viral pathogens.
More specifically, the present invention relates to an in vivo method for monitoring the CTL response against virus by administering to a subject a HLA-A2 restricted CTL epitope of a viral antigen and observing if a DTH response is induced. Two GMP optimally defined HLA-A2 restricted CTL epitopes from influenza (GL9, GILGFVFTL) and HIV (SL9, SLYNTVATL) were prepared to elicit a cutaneous DTH reaction. The epitopes were administered to the subject intradermally and the appearance of induration and erythema was observed. The GL9 was able induce such a DTH reaction exclusively in HLA-A2 expressing individuals. Neither magnitude of the epitope-specific responses, HIV infection status, HIV viral loads or CD4 counts were predictive of the extent of DTH reactions. However, a markedly reduced expression of skin homing markers CD103 and cutaneous leukocyte antigen (CLA) on epitope specific populations was associated with a lack of SL9 DTH reactivity. The data demonstrates that DTH reactions can be elicited by optimally defined CTL epitopes per se. This fact may provide the basis for the development of novel approaches for immune efficacy of large scale (HIV)-vaccine trials.

1. Definitions of general terms and expressions

The term "AIDS", as used herein, refers to the symptomatic phase of HIV infection, and includes both Acquired Immune Deficiency Syndrome (commonly known as AIDS) and "ARC," or AIDS-Related Complex. See Adler M, et al, Brit. Med. J. 1987; 294: 1145-1 147. The immunological and clinical manifestations of AIDS are known in the art and include, for example, opportunistic infections and cancers resulting from immune deficiency.

The term "adjuvant" refers to a substance that enhances, augments or potentiates the host's immune response to a vaccine antigen either by contributing to presenting the vaccine antigen to the immune system or by non-specifically stimulating directly different components of the immune system.

The term "B cell", as used herein, refers to any member of a diverse population of morphologically similar cell types that develop in the bone marrow and that mediate the humoral immune response of the adaptive immune system. B cells are characterized by the presence of a B cell receptor able to bind specifically an antigen. Their principal
functions are to make antibodies against antigens, perform the role of antigen-presenting cells (APCs) and eventually develop into memory B cells after activation by antigen interaction. See Alberts B, et al., "Molecular Biology of the Cell" (Garland Publishing Inc., New York, NY, US, 2008, pp. 1363-1391).

The term "BLT1", as used herein, refers to the leukotriene B4 receptor 1, a protein encoded by the LTB4R gene in humans (UniProt accession no. Q15722).

The term "CCR7", as used herein, refers to the cluster of differentiation 197, the C-C chemokine receptor type 7 protein. CCR7 is expressed in various lymphoid tissues and activates B and T cells. It has been shown to control the migration of memory T cells home to secondary lymphatic organs, such as the lymphatic, as well as stimulate dendritic cell maturation (UniProt accession no. P32248).

The term "CD3", as used herein, refers to the cluster of differentiation 3, a protein complex composed of four distinct chains. In mammals, the complex contains a CD3y chain, a CD35 chain, and two CD3s chains. These chains associate with a molecule known as the T cell receptor (TCR) and the δ-chain to generate an activation signal in T lymphocytes. The TCR, δ-chain, and CD3 molecules together comprise the TCR complex (UniProt accession no. P07766).

The term "CD8", as used herein, refers to the cluster of differentiation 8, a transmembrane glycoprotein that serves as a co-receptor for the T cell receptor (TCR) expressed in the cytotoxic T cells (CTL). CTLs are implicated in the rejection of transplants and the destruction of tumor and virally infected cells (UniProt accession no. P10966).

The term "CD27", as used herein, refers to cluster of differentiation 27, a tumor necrosis factor receptor member of the TNF-receptor superfamily. This receptor is required for generation and long-term maintenance of T cell immunity. It binds to ligand CD70, and plays a key role in regulating B cell activation and immunoglobulin synthesis. The receptor transduces signals that lead to the activation of NF-κB and MAPK8/JNK. Adaptor proteins TRAF2 and TRAF5 have been shown to mediate the signaling process of this receptor (HGNC identification no. 11922).

The term "CD45RA", as used herein, refers to isoform RA of the cluster of differentiation 45, or protein tyrosine phosphatase, receptor type, C (PTPRC). CD45RA is expressed by naïve T cells (UniProt accession no. P08575).
The term "CD103", as used herein, refers to cluster of differentiation 103, or integrin αE (ITGAE). CD103 binds integrin β7 to form the complete heterodimeric integrin molecule αEβ7 (UniProt accession no. P38570).

The term "CLA", as used herein, refers to the cutaneous lymphocyte-associated antigen, a skin-homing receptor that facilitates the targeting of T cells to inflamed skin. CLA is defined by its reactivity towards the HECA-452 monoclonal antibody and its activity as a ligand for E-selectin. See Fuhlbrigge R, et al., Nature 1997; 389(6654):978-981.

The term "comprising" or "comprises", as used herein, discloses also "consisting of according to the generally accepted patent practice.

The terms "Cytotoxic T Lymphocyte" or "CTL" refers to lymphocytes which induce apoptosis in targeted cells. CTLs form antigen-specific conjugates with target cells via interaction of TCRs with processed antigen (Ag) on target cell surfaces, resulting in apoptosis of the targeted cell. Apoptotic bodies are eliminated by macrophages.

The term "cytotoxic T lymphocyte (CTL) response", as used herein is used to refer to the primary immune response mediated by CTL cells. This specific immune response can be e.g. the production of specific cytokines such as IFN-gamma (IFN-γ) (measured e.g. by ELISPOT or intracellular FACS), degranulation (measured e.g. by a granzyme-b specific ELISPOT), or cytolytic activity (e.g. measured by a ⁵¹Cr-release assay). Alternatively the antigen specific CD8+ cell can be detected directly by e.g. the use of tetramers.

The term "CXCR1" or "CD181", as used herein, refers to cluster of differentiation 181, also known as interleukin 8 receptor a (ILRA). CXCR1 is a chemokine receptor with high affinity for interleukin 8 (IL8). The protein encoded by the CXCR1 gene is a member of the G-protein-coupled receptor family (UniProt accession no. P25024).

The term "delayed-typed hypersensitivity" or "DTH", as used herein, refers to a cell-mediated response that develops two to three days after exposure to an antigen. In a DTH response CD4+ helper T cells recognize the antigen in a complex with class 2 HLA complex. The antigen-presenting cells in this case are macrophages that secrete IL-12, which further stimulates the proliferation of CD4+ Th1 cells. CD4+ T cells
secrete IL-2 and interferon gamma, inducing the release of other Th1 cytokines, thus mediating the immune response. Activated CD8+ T cells destroy target cells on contact, whereas activated macrophages produce hydrolytic enzymes and, on presentation with certain intracellular pathogens, transform into multinucleated giant cells.

The term "diagnosis" is used herein to refer to the identification of a molecular or pathological state, disease or condition or to refer to identification of a patient who may benefit from a particular treatment regimen. As will be understood by those skilled in the art, such an assessment is usually not intended to be correct for all (i.e. 100 percent) of the subjects to be identified. The term, however, requires that a statistically significant portion of subjects can be identified (e.g. a cohort in a cohort study). Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools, e.g., determination of confidence intervals, p-value determination, Student's t-test, Mann-Whitney test etc. Details are found in Dowdy and Wearden, Statistics for Research, John Wiley and Sons, New York 1983. Preferred confidence intervals are at least 90 percent, at least 95 percent, at least 97 percent, at least 98 percent or at least 99 percent. The p-values are, preferably, 0.1, 0.05, 0.01, 0.005, or 0.0001. More preferably, at least 60 percent, at least 70 percent, at least 80 percent or at least 90 percent of the subjects of a population can be properly identified by the method of the present invention.

The terms "HLA-A2" refers to the HLA-A2 type containing the subtypes, examples of which include, but are not limited to, HLA-A*0201, HLA-A*0202, HLA-A*0203, HLA-A*0204, HLA-A*0205, HLA-A*0206, HLA-A*0207, HLA-A*0210, HLA-A*0211, HLA-A*0213, HLA-A*0216, HLA-A*0218, HLA-A*0219, HLA-A*0228 and HLA-A*0250.

The term "HLA-A2 restricted peptide", as used herein, shall refer to any polypeptide comprising an epitope that is capable of, or predicted to be capable of, being bound by a human leukocyte antigen molecule of HLA class II. Preferably, the binding of the HLA-A2 restricted peptide to the HLA class II molecule occurs with an IC₅₀ lower than 1000 nM. The "HLA-A2 restricted peptide is about 8 to about 13 amino acids, or about 8, 9, 10 or 11 amino acids in length. In certain embodiments, one or more of said HLA-A2 restricted peptides are provided together in the form of a polypeptide peptide or construct. The polypeptide construct of the present invention
preferably includes 2 or more, 5 or more, 10 or more, 13 or more, 15 or more, 20 or more, or 25 or more CTL epitopes. More specific, the polypeptide construct comprises at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60 or more CTL epitopes. The "HLA-A2 restricted peptide may be of any size, including but not limited to having a size of less than 100,000 Dalton in molecular weight, less than 50,000 Dalton in molecular weight, less than 10,000 Dalton in molecular weight, less than 5,000 Dalton in molecular weight, less than 2,500 Dalton in molecular weight, or from about 1000 to 2000 Dalton in molecular weight. In certain embodiments, the HLA-A2 restricted peptide derives from an influenza virus antigen. A suitable influenza antigen can be a surface antigen, such as hemagglutinin (HA), neuraminidase (NA), M2, or a fragment thereof (e.g., one or more HTL or CTL epitopes). Other suitable influenza antigens include M1, NP, NS1, NS2, PA, PB1, and PB2, or fragments thereof. Suitable HLA-A2 restricted peptides derived from M1 include GILGFVFTL (SEQ ID NO:1), LLTEVETYV (SEQ ID NO:2), ILGFVFITLT (SEQ ID NO:3) and RMTTVTTEV (SEQ ID NO:4). Suitable HLA-A2 restricted peptide derived from HA include, without limitation GLFGAIAGFI (SEQ ID NO:5), FLDELTYNA (SEQ ID NO:6), ALSTLCLLI (SEQ ID NO:7) and HLCRFTFFL (SEQ ID NO:8). Suitable HLA-A2 restricted peptides derived from NP include KSCLPACVY (SEQ ID NO:9), CLPACVYGL (SEQ ID NO:10), LQNSQVFSV (SEQ ID NO:11) and FQGRGFEL (SEQ ID NO:12). Suitable HLA-A2 restricted peptide derived from NS1 include, without limitation, FQVDCFLWHV (SEQ ID NO:13), QVDCFLWHV (SEQ ID NO:14), FLWHVRKSV (SEQ ID NO:15), and IILKANFSL (SEQ ID NO:16). Suitable HLA-A2 restricted peptide derived from NS2 include, without limitation, FMQALQLLL (SEQ ID NO:17), MQALQLLLEV (SEQ ID NO:18) and MITQFESLK (SEQ ID NO:19). Suitable HLA-A2 restricted peptide derived from PA include, without limitation, FMYSDFHFI (SEQ ID NO:20), ALLKRFSEI (SEQ ID NO:21), MAWTVVNSI (SEQ ID NO:22), LLMDALKLSI (SEQ ID NO:23), LLAWQKVL (SEQ ID NO:24) and YINTALLNA (SEQ ID NO:25). Suitable HLA-A2 restricted peptide derived from PA include, without limitation, AQTDCVEA (SEQ ID NO:26), CVLEAMAFL (SEQ ID NO:27), RLIDFLKDV (SEQ ID NO:28), QIRGTVFYFV (SEQ ID NO:29), FVYFETLA (SEQ ID NO:30), RMFLAMITY (SEQ ID NO:31),
LLIDGTASL (SEQ ID NO:32), NMLSTVLGV (SEQ ID NO:33), FVANFSMEL (SEQ ID NO:34), AQMALQLFI (SEQ ID NO:35), and RLCNPLNPVFV (SEQ ID NO:36).

Suitable HLA-A2 restricted peptide derived from PA include, without limitation, LQDCKIAPL (SEQ ID NO:37), FQNWGIEHI (SEQ ID NO:38), FQNWGIEPI (SEQ ID NO:39) and RMQFSSLTV (SEQ ID NO:40).

The term "HIV", as used herein, include HIV-1 and HIV-2 and SIV. "HIV-1" means the human immunodeficiency virus type-1. HIV-1 includes, but is not limited to, extracellular virus particles and the forms of HIV-1 associated with HIV-1 infected cells. The HIV-1 virus may represent any of the known major subtypes (Classes A, B, C, D, E, F, G and H) or outlying subtype (Group O) including laboratory strains and primary isolates. "HIV-2" means the human immunodeficiency virus type-2. HIV-2 includes, but is not limited to, extracellular virus particles and the forms of HIV-2 associated with HIV-2 infected cells. The term "SIV" refers to simian immunodeficiency virus which is an HIV-like virus that infects monkeys, chimpanzees, and other nonhuman primates. SIV includes, but is not limited to, extracellular virus particles and the forms of SIV associated with SIV infected cells.

A/cat/Thailand/KU-02/04, A/Hong Kong/213/03, A/chicken/Guangdong/174/04, and/or
A/HK/159/97), H6N1 strains (e.g., A/teal/Hong Kong/1073/99), H6N2 strains (e.g.,
A/chicken/California/0 139/2001, and/or A/guilemot/Sweden/3/2000), H6N9 strains
(e.g., A/goose/Hong Kong/W2 17/97), H7N1 strains (e.g., A/FPV/Rostock/34), H7N3
strains (e.g., A/chicken/British Columbia/04, and/or A/bird/Italy/220 158/2002),
H7N7 strains (e.g., A/chicken/Netherlands/1/2003, A/Netherlands/2 19/03,
A/FPV/Dobson/27, and/or A/chicken/FPV/Weybridge), H9N2 strains (e.g.,
A/shorebird/Delaware/9/96, A/swine/Korea/S452/2004, A/duck/Hong Kong/Y439/97,
A/Hong Kong/1073/99, A/HK/2 108/2003, A/quail/Hong Kong/G1/97, A/duck/Hong
Kong/Y280/97, A/chicken HK/FY23/03, and/or A/chicken HK/G9/97), and B influenza
strains (e.g., B/Brisbane/60/2008).

The term "kit", as used herein, refers to a product containing the different
reagents necessary for carrying out the methods of the invention packed so as to allow
their transport and storage. Materials suitable for packing the components of the kit
include crystal, plastic (e.g. polyethylene, polypropylene, polycarbonate), bottles,
vials, paper, or envelopes.

The term "subject", as used herein, refers to an individual, plant or animal, such
as a human beings, a non-human primate (e.g. chimpanzees and other apes and monkey
species), a farm animal (e.g. birds, fish, cattle, sheep, pigs, goats and horses), a domestic
mammal (e.g. dogs and cats), or a laboratory animal (e.g. rodents, such as mice, rats and
guinea pigs. The term does not denote a particular age or sex. The term "subject"
embraces an embryo and a fetus.

The term "T cell", as used herein, refers to any member of a diverse population
of morphologically similar lymphocytes types that develop in the thymus and that
mediate the cellular immune response of the adaptive immune system. They are
characterized by the presence of a T cell receptor on the cell surface. There are several
subsets of T cells, each with a distinct function (i.e. helper, memory, regulatory, natural

The term "Treg" or "regulatory T cell", as used herein, refers to a T cell that
expresses the CD4 or CD25 marker at least and which is capable of reducing or
suppressing the activity of a T cell. This term includes T cells producing low levels of IL-2, IL-4, IL-5, and IL-1, and which suppress the activation of the immune system. Regulatory T cells suppress actively the proliferation and cytokine production of TH1, T\(^4\), or naïve T cells which have been stimulated in culture with an activating signal (e.g. antigen and antigen presenting cells or with a signal that mimics antigen in the context of HLA, such as, for instance, an anti-CD3 antibody plus an anti-CD28 antibody). Treg cells may express the FoxP3 marker.

2. Method of monitoring CTL response

In a first aspect, the invention relates to an in vivo method for determining whether a subject is capable of mounting an effective cytotoxic T lymphocyte (CTL) response against a virus comprising:

i) administering a peptide comprising an HLA-A2 restricted epitope of an antigen from said virus to the subject, and

ii) determining if a delayed-type hypersensitivity (DTH) reaction occurs,

wherein a positive DTH reaction is indicative that the subject can mount an effective CTL response against the virus.

In stage i) of the method for monitoring the CTL response of the invention, a peptide comprising an HLA-A2 restricted epitope of an antigen from said virus is administered to the subject. In one embodiment the peptide is administered intradermally, typically in a similar manner to the Mantoux test. The peptide can be administered epidermally. The peptide is typically administered by needle, such as by injection, but can be administered by other methods such as ballistics, for example the ballistics techniques which have been used to deliver nucleic acids. Published EPC Application No. EP-A-0693119 describes techniques which can typically be used to administer the peptide. In several examples, from 0.001 to 1000 \(\mu\)g, for example from 0.01 to 100 \(\mu\)g or 0.1 to 10 \(\mu\)g of peptide is administered. Alternatively an agent can be administered which is capable of providing the peptides in vivo. Thus a polynucleotide capable of expressing the polypeptide can be administered. The polynucleotide typically has any of the characteristics of the polynucleotide which is discussed below. Polypeptide is expressed from the polynucleotide in vivo and recognition of the peptide in vivo may be
measured. Typically from 0.001 to 1000 µg, for example from 0.01 to 100 µg or 0.1 to
10 µg of polynucleotide is administered. In a preferred embodiment, the peptide is
administered without adjuvant. In a preferred embodiment, the HLA-A2 restricted
epitope is derived from influenza. In a more preferred embodiment, the HLA-A2
restricted epitope is derived from the influenza matrix protein 1. In a still more preferred
embodiment, the HLA-A2 restricted epitope is GILGFVFTL (SEQ ID NO: 1).
Preferably, the subject is human. In a more preferred embodiment, the subject has been
previously infected by the virus and/or immunized with an immunogen comprising the
viral antigen.

In stage ii) of the method for monitoring the CTL response of the invention, the
method comprises determining if a delayed-type hypersensitivity (DTH) reaction
occurs. In a preferred embodiment, the determination of the delayed-type
hypersensitivity (DTH) reaction comprises determining the appearance of a cutaneous
DTH reaction. In a still more preferred embodiment, the cutaneous DTH reaction is
determined by determining the presence of induration and/or erythema of an area
surrounding a site of antigen exposure.

In another embodiment, the determination of the delayed-type hypersensitivity
(DTH) reaction comprises determining the increase in the number of CLA+ epitope-
specific CTL and/or the increase in the number of CD103+ epitope-specific CTL. The
increase in the number of CLA+ epitope-specific CTL and/or the increase in the number
of CD103+ epitope-specific CTL is usually determined with respect to a reference
value. In a preferred embodiment, the reference value correspond to the number of
number of CLA+ epitope-specific CTL or the number of CD103+ epitope-specific CTL
in a subject who has not been treated or in a subject who has been treated with a
peptide which does not comprise an HLA-A2 restricted epitope of an antigen from the
virus.

3. Methods for diagnosis

In another aspect, the invention relates to a method for the diagnosis of an
infection by a virus in a subject comprising
i) administering a peptide comprising a HLA-A2 restricted epitope of an antigen from said virus to the subject, and

ii) determining if a delayed-type hypersensitivity (DTH) reaction occurs, wherein a positive DTH reaction is indicative that the subject is infected by the virus.

In stage i) of the method for diagnosis of a viral infection, a peptide comprising an HLA-A2 restricted epitope of an antigen from said virus is administered to the subject. In one embodiment the peptide is administered intradermally, typically in a similar manner to the Mantoux test. The peptide can be administered epidermally. The peptide is typically administered by needle, such as by injection, but can be administered by other methods such as ballistics, for example the ballistics techniques which have been used to deliver nucleic acids. Published EPC Application No. EP-A-0693119 describes techniques which can typically be used to administer the peptide. In several examples, from 0.001 to 1000 µg, for example from 0.01 to 100 µg or 0.1 to 10 µg of peptide is administered. Alternatively an agent can be administered which is capable of providing the peptides in vivo. Thus a polynucleotide capable of expressing the polypeptide can be administered. The polynucleotide typically has any of the characteristics of the polynucleotide which is discussed below. Polypeptide is expressed from the polynucleotide in vivo and recognition of the peptide in vivo may be measured. Typically from 0.001 to 1000 µg, for example from 0.01 to 100 µg or 0.1 to 10 µg of polynucleotide is administered. In a preferred embodiment, the HLA-A2 restricted epitope is derived from influenza.

In a more preferred embodiment, the HLA-A2 restricted epitope is derived from the influenza matrix protein I. In a still more preferred embodiment, the HLA-A2 restricted epitope is GILGFVFTL (SEQ ID NO: 1). HLA-A2 restricted epitope is derived from influenza include, without limitation, the epitopes GILGFVFTL (SEQ ID NO: 1), LLTEVETYV (SEQ ID NO: 2), ILGFVFTLTV (SEQ ID NO: 3) and RMGTVTTEV (SEQ ID NO:4).

Preferably, the subject is human. In a more preferred embodiment, the subject has been previously infected by the virus and/or immunized with an immunogen comprising the viral antigen.
In stage ii) of the method for diagnosis of an infection by a virus, the method comprises determining if a delayed-type hypersensitivity (DTH) reaction occurs. In a preferred embodiment, the determination of the delayed-type hypersensitivity (DTH) reaction comprises determining the appearance of a cutaneous DTH reaction. In a still more preferred embodiment, the cutaneous DTH reaction is determined by determining the presence of induration and/or erythema of an area surrounding a site of antigen exposure.

In another embodiment, the determination of the delayed-type hypersensitivity (DTH) reaction comprises determining the increase in the number of CLA+ epitope-specific CTL and/or the increase in the number of CD103+ epitope-specific CTL. The increase in the number of CLA+ epitope-specific CTL and/or the increase in the number of CD103+ epitope-specific CTL is usually determined with respect to a reference value. In a preferred embodiment, the reference value correspond to the number of number of CLA+ epitope-specific CTL or the number of CD103+ epitope-specific CTL in a subject who has not been treated or in a subject who has been treated with a peptide which does not comprise an HLA-A2 restricted epitope of an antigen from the virus.

4. Peptide comprising at least one HLA-A2 restricted epitope and kits

In another aspect, the invention relates to a peptide comprising at least one HLA-A2 restricted epitope of the invention. In another embodiment, the invention relates to a kit comprising a peptide according to the invention.

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All publications mentioned herein are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.
General Procedures

1. Subjects

10 HIV infected (9 HLA-A*0201+) and 10 HIV uninfected (8 HLA-A*0201+) individuals were injected with soluble epitopes derived from influenza (matrix protein, SEQ ID NO. 1: GILGFVFTL, "GL9") and HIV (Gag p17, SEQ ID NO. 2: SLYNTVATL, "SL9") viruses. The purpose of this approach was to assess their potential to induce DTH reactions and elicit specific responses to HLA-A*0201. PBS, Tetanus toxoid and Candida antigens were also included as controls. See http://www.cdc.gov/mmwr/preview/mmwrhtml/0003075.htm, September 2012, Figure 1. The viral epitopes were injected in 200 µl containing 100 µg of peptides.

Control injections included standard intradermal injections of PBS, tetanus toxoid and Candida antigens. Cutaneous reaction at sites of injections was measured 72h after injection by palpating skin indurations and by measuring the diameter (mm) of erythema extension. Blood samples were obtained before injection and 72h thereafter, T cells isolated by density gradient centrifugation (Lymphoprep®, Axis-Shield pic, Dundee, Scotland, GB) and cryopreserved until use.

2. Flow cytometry analysis

For dextramer assay and CD8 T cells analysis, thawed cells (1 million of PBMCs) were stained with 10 µL of PE-couple HLA-A2/GL9 dextramer (Immudex AS, Copenhagen, DK) and 10 µL APC-couple HLA-A2/SL9 dextramer (Immudex AS, Copenhagen, DK) at room temperature for 20 minutes. Specific antibodies for activation/differentiation and homing panels were diluted in phosphate buffer saline (PBS) solution plus 1% of fetal bovine serum (FBS) and added for other 20 minutes incubation at room temperature. See Table 2. Cells were then washed twice and resuspended in 200 µl of PBS plus 1% FBS and analyzed on a LSRII flow cytometer with a 8 color parameter acquisition setting (BD Biosciences Corp., Franklin Lakes, NJ, US). Results were analyzed by FACSDiva software (BD Biosciences Corp., Franklin Lakes, NJ, US).
3. Statistical analysis

Statistical analyses were performed using Prism Version 4, GraphPad Prism. One Way ANOVA with Tukey's Multiple Comparison Test correction for DTH measurements was applied. A Mann-Whitney test was used for MFI and population percentages comparisons. For the analysis of population percentages and phenotype/homing markers expression in different time points, Wilcoxon matched pairs test was applied. In all analysis, p-values < 0.05 were considered statistically significant.

Example I

Induction of DTH reaction with HIV and influenza antigens

To determine if CTL epitopes per se, administered intradermally and without adjuvant, can elicit a DTH reaction, 2 well-defined, HLA-A*0201 restricted epitopes derived from HIV (SL9, SLYNTVATL) and influenza (GL9, GILGFVFTL) were tested in 10 HIV infected (9 HLA-A*0201+) and 10 HIV uninfected (8 HLA-A*0201+) individuals. As controls, DTH responses to PBS alone, tetanus toxoid (TT) and Candida antigens were monitored as well. See Figure 1A. No dermal reactions were observed after SL9 epitope injection in any of the 9 HLA-A*0201 expressing HIV-infected individuals, regardless of their CD4 count and HIV viral loads. As expected, HIV uninfected subjects showed no reaction to SL9 either. In contrast, 8 of the 8 HIV negative (100 %) and 4 of the 9 HIV positive (44%), HLA-A*0201 expressing individuals showed reaction to GL9 injection (induration diameter reaction ranging from 3-15mm). See Table 1.

While control injections provoked a response in 100% of the HIV uninfected individuals, the HIV-infected patients had a reduced response rate to these antigens as well (TT: 100% HIV- vs 90% HIV+; Candida: 100% HIV- vs 80% HIV+). However, none of these differences, including the GL9 response rate, reached statistical significance ($\chi^2$: 0.6209 df: 2 and p-value: 0.7331). In addition, the extent of induration and erythema at the viral antigen injection sites did not correlate with HIV status, CD4
counts or HIV viral loads and was not associated with the extent of DTH reactivity to the control antigens. See Figure 1B. These data demonstrated that at least the influenza-derived CTL epitope GL9, against which no CD4 T cell activities have been documented, triggers robust DTH reactions on its own. The absence of GL9 DTH reactivity in individuals without the restricting HLA class I allele indicated that the in vivo responses were epitope specific and further document that responses were mediated by HLA class I restricted CD8 T cells. See Chen, 2005, supra. The reduced extent of GL9 DTH reactions in HIV infected individuals compared to HIV uninfected subjects (induration diameters in HIV uninfected patients ranging from 3-15 mm, median 9 mm and in infected patient from 0 to 15 , median 4.5 mm p-value: 0.0927) is in line with the diminished DTH activities in HIV infected subjects to antigens such as BCG. See Johnson M, et al, J. Infect. Dis. 1992; 166(1): 194-198 and Costa N, et al, Rev. Soc. Bras. Med. Trop. 2011; 44(5):542-545. However, the complete absence of DTH reactions to SL9 in the HLA-A2 expressing HIV-infected subjects was somewhat surprising as the ex-vivo analyses by flow cytometry showed strong epitope-specific responses (peripheral blood SL9-specific CTL percentage range: 0.2-7.5% of CD8 T cells and median: 1.3%). Evidently, several reasons could explain this observation, including an SL9 epitope-specific effect that would not be observed for other HIV-derived CTL epitope. Alternatively, the time needed to observe a DTH reaction may be longer for HIV antigens than for Influenza specific responses, leading us to miss potential reactivities after 72h. See Hladik, 2001, supra. These and other qualitative differences in the CD8 T cell response to these two infections may exist as their transmission routes and ability to establish chronic infections differ drastically. Finally, the absence of SL9 DTH reactions may reflect a generally reduced functionality of HIV-specific CTL responses in chronically infected individuals, a phenomenon which would likely affect most HIV-specific CTL populations.

Example 2

Effect of CTL maturation and differentiation over DTH reaction

In order to assess whether the different capacity of SL9- and GL9-specific CTL to induce DTH reactions were due to differences in their relative maturation and
differentiation stages, epitope-specific populations in the peripheral blood were analyzed by flow cytometry. Parallel studies on biopsied DTH sites in three individuals were not feasible as this yielded insufficient cell material. Specific CTL were stained using SL9 and GL9 containing dextramers, respectively, together with antibodies against CD45RA, CCR7 and CD27. Cells were assessed in blood samples taken right before antigen injections and 72h later when DTH reactions were measured. The magnitude of the SL9 response was generally higher than the GL9 response, both at the time of antigen injection (median SL9 0.9% vs. GL9 0.6%, p=0.0938) as well as the 72h time point (median SL9 1.9% vs. GL9 0.5%, p=0.0313). However, there was no statistically significant change in either population over time (SL9 p=0.6875, GL9 p=1.000, Wilcoxon matched pairs test) that could possibly have been triggered by the antigen injections. The higher magnitude of the HIV-SL9 response compared to the Influenza GL9 response is in line with reports that have found HIV specific responses to be generally stronger than the relatively low magnitude GL9 response. See Brander C, et al, Eur. J. Immunol. 1993; 23(12):3217-3223 and Brander C, et al, J. Clin. Invest. 1998; 101(1):2559-2566.

The analysis of the GL9 and SL9 specific CD8 T cell subsets differentiation stages showed comparable frequencies of naive, effector memory and terminally differentiated effector memory cells for both subsets. The one statistically significant difference was observed for the central memory (CM) cell population, with higher proportions of GL9 specific cells showing this phenotype (CCR7+/CD45RA-) than seen among the SL9 specific cells (GL9 median: 7.45% vs SL9 median: 1.6%; p-value: 0.0002 Mann-Whitney). See Figure 2B. This data is in line with more comprehensive comparisons of CTL populations against different viral pathogens, which have as well shown a CM dominance in CTL responses to influenza infection and a predominantly EM and EMRA phenotype for HIV specific CTL. See Appay V, et al, Cytometry A 2008; 73(1):975-983. These discrepancies are likely to reflect the differences in chronic antigen stimulation in HIV infection, which has been described to drive differentiation of virus specific CTL populations to the terminally differentiated stages. See Appay V. et al, Nat. Med. 2002; 8(4):379-385. This interpretation is further supported by the significantly increased expression of the CD27 activation maker on to the surface of terminally differentiated SL9 T cells compared to Influenza GL9 specific
CTL in our study population (CD27 mean fluorescence intensity (MFI) for GL9 median = 289.5 vs. SL9 median = 474 MFI; p-value: 0.0002 Mann-Whitney). See Figure 2C.

**Example 3**

*Effect of specific dermal migration markers over DTH reaction*

To determine if DTH reactivity of GL9 and SL9 specific CTL depended on the expression of specific dermal migration markers, the expression of previously described homing receptors on epitope specific CD8 T cells was assessed. The tested markers included the cutaneous lymphocyte associated antigen (CLA), the αEβ7 integrin (CD103), leukotriene B4 receptor (BLT1) and the IL8 receptor alpha, also known as CXCR1.

CLA has been shown to be present on more than 80% of T cells infiltrating inflamed skin lesions, while only 5-20% of total T cells in peripheral blood are CLA+. CLA expression is typically increased in individuals with diverse inflammatory and skin diseases. See Ogg G, et al, J. Exp. Med. 1998; 188(6):1203-1208, Dworzak M, et al, J. Allergy Clin. Immunol. 1999; 103(5Pt1):901-906, Borowitz M, et al, Leukemia 1993; 7(6):859-863, Seneviratne S, et al, QJM 2007; 100(1):19-27, Antelo D, et al, Photodermatol. Photoimmunol. Photomed. 2011; 27(1):40-44, Sigmundsdottir H, et al, Clin. Exp. Immunol. 2001; 126(2):365-369, Koelle D, et al, J. Clin. Invest. 2002; 110(4):537-548, and Koelle D, et al, J. Virol. 2006; 80(6):2863-2872. In this assay CLA expression was selectively increased in the GL9 specific CTL (both, by >% positive cells as well as by the intensity of expression per cell; SL9 median 23.32% > and GL9 85.77% p-value <0.0001, Mann-Whitney; SL9 median 976 MFI and GL9 3537.5 MFI, p-value <0.0001, Mann-Whitney). This difference was consistent for both, at the time when the antigen was injected and at the 72h follow-up point. See Figure 3A. The higher levels of CLA in the influenza specific CD8 cells suggest that this marker could be responsible, or at least required for the observed DTH reactions seen against GL9 but not SL9.

The αEβ7 integrin (CD103) was initially described in intraepithelial T cells residing in the gut and other epithelial compartments such as skin and lung as well as the tonsils and, with its expression biased towards CD8+ T cells. CD103 is upregulated
by exposure to TGFp, IL4 and with streptococcal pyrogenic superantigen stimulation partly co-expressed with CLA. See Woodberry T. et al, J. Immunol. 2005; 175(7):4355-4362, Sigmundsdottir H, et al, Clin. Immunol. 2004; 111(1): 119-125, and Seneviratne S, et al, Clin Exp Immunol 2005; 141(1): 107-115. The HIV infected subjects showed comparable results (GL9 median: 100% vs SL9 median: 99.95%; p-value: 0.7622 Mann-Whitney), but the expression level was significantly higher on GL9-specific CTL (GL9 median: 71.165 MFI vs SL9 median: 2388 MFI; p-value <0.0001 Mann-Whitney). See Figure 3B. While CD103 is not necessarily considered a homing marker, its increased expression on GL9 specific cells may allow to retain these cells within epithelial layers, facilitating a rapid DTH reaction.


Based on the previous considerations and the earlier experimental data, the BLT1 and CXCR1 markers were also assessed on HIV SL9 and influenza GL9 specific CTL. In contrast to CLA and CD103 expression, both markers were found to be elevated on the HIV SL9-specific cells compared to the GL9 specific CTL population (for BLT1 expression: GL9 median: 56.9% vs SL9 median: 88.45%; p-value: 0.0001; for CXCR1: GL9 median: 12.97 % vs SL9 median: 32.27%; p-value<0.0001, Mann-Whitney). See Figure 3C. As for CLA and CD103, the expression of these inflammatory
tissue homing markers was not modulated between time of antigen injection and the 72h post-injection time point, suggesting that the large fraction of PBMC-derived GL9 and SL9 were either not stimulated by the intradermal antigen injection or that the SL9 specific cells express constitutively more BLT1 and CXCR1 than the GL9 specific CTL. These data thus suggest that both, CLA and CD103, but not BLT1 or CXCR1 may be involved in promoting the migration to or retention of GL9 specific CTL at the dermal antigen injection site. The absence of both of these markers on HIV SL9 specific cells is consistent with the lack of DTH reactivity to this antigen and may indicate differential effector function profiles of CTL directed against these viral infections.
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n.a: not applicable
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Sources: Immudex AS (Copenhagen, DK), BD Biosciences Corp (Franklin Lakes, NJ, US)
Claims

1. An in vivo method for determining whether a subject is capable of mounting an effective cytotoxic T lymphocyte (CTL) response against a virus comprising:
   i) administering an HLA-A2 restricted peptide of an antigen from said virus to the subject, and
   ii) determining if a delayed-type hypersensitivity (DTH) reaction occurs, wherein a positive DTH reaction is indicative that the subject can mount an effective CTL response against the virus.

2. The method of claim 1, wherein the HLA-A2 restricted peptide is an influenza virus peptide.

3. The method of claim 2, wherein the HLA-A2 restricted peptide is SEQ ID NO. 1.

4. The method of anyone of claims 1 to 3 wherein the peptide is administered without adjuvant.

5. The method of anyone of claims 1 to 4 wherein the peptide is administered intradermally.

6. The method of anyone of claims 1 to 5 wherein the subject has been previously infected by the virus and/or immunized with an immunogen comprising the viral antigen.

7. The method of any one of claims 1 to 6 wherein the determination of the delayed-type hypersensitivity (DTH) reaction comprises determining the appearance of a cutaneous DTH reaction.

8. The method of claim 7 wherein the cutaneous DTH reaction is determined by determining the presence of induration and/or erythema of an area surrounding a site of antigen exposure.
9. The method of any one of claims 1 to 6 wherein the determination of the delayed-type hypersensitivity (DTH) reaction comprises determining the increase in the number of CLA+ epitope-specific CTL and/or the increase in the number of CD103+ epitope-specific CTL.

10. The method as in any one of claim 1 to 9, wherein the subject is human.

11. A method for the diagnosis of an infection by a virus in a subject comprising
   i) administering a HLA-A2 restricted peptide of an antigen from said virus to the subject, and
   ii) determining if a delayed-type hypersensitivity (DTH) reaction occurs, wherein a positive DTH reaction is indicative that the subject is infected by the virus.

12. The method of claim 11 wherein the HLA-A2 restricted peptide is an influenza epitope and the infection to be diagnosed is an infection by the influenza virus.

13. The method of claim 12 wherein the HLA-A2 restricted peptide is SEQ ID NO. 1.

14. The method of anyone of claims 11 to 13 wherein the peptide is administered without adjuvant.

15. The method of anyone of claims 11 to 14 wherein the peptide is administered intradermally.

16. The method of any one of claims 11 to 15 wherein the determination of the delayed-type hypersensitivity (DTH) reaction comprises determining the appearance of a cutaneous DTH reaction.
17. The method of claim 16 wherein the cutaneous DTH reaction is determined by the presence of induration and/or erythema of an area surrounding a site of antigen exposure.

18. The method of any one of claims 11 to 15 wherein the determination of the delayed-type hypersensitivity (DTH) reaction comprises determining the increase in the number of CLA+ epitope-specific CTL and/or the increase in the number of CD103+ epitope-specific CTL.

19. The method as in any one of claim 11 to 18, wherein the subject is human.

20. A peptide comprising the HLA-A2 restricted epitope of SEQ ID NO. 1.

Fig. 1
Fig. 1 (cont.)
Fig. 2 (cont.)
Fig. 2 (cont.)
Fig. 3
Fig. 3 (cont.)
Fig. 3 (cont.)
1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
   a. (means)
      - [ ] on paper
      - [x] in electronic form
   b. (time)
      - [x] in the international application as filed
      - [ ] together with the international application in electronic form
      - [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
### INTERNATIONAL SEARCH REPORT

**International application No**

PCT/EP2012/067794

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**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K49/00
ADD. A61K39/145 A61K39/21 C07K4/02

According to International Patent Classification (IPC) or to both national classification and IPC

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**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K

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**DOCUMENTS CONSIDERED TO BE RELEVANT**

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**Date of the actual completion of the international search**

20 November 2012

**Date of mailing of the international search report**

28/11/2012

**Name and mailing address of the ISA**

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Viillard, Anne-Laure

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*Special categories of cited documents:*

- **A** document defining the general state of the art which is not considered to be of particular relevance
- **E** earlier application or patent both published or not published later than the international filing date
- **L** document published prior to the international filing date but later than the priority date claimed

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*Further documents are listed in the continuation of Box C.*
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