(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 October 2009 (08.10.2009)

PCT

(10) International Publication Number
WO 2009/121845 A2

(51) International Patent Classification:
A61K 39/21 (2006.01) *G01N 33/50* (2006.01)

(21) International Application Number:
PCT/EP2009/053750

(22) International Filing Date:
30 March 2009 (30.03.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
08103387.0 4 April 2008 (04.04.2008) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2009/121845 A2

(54) Title: OVERLAPPING PEPTIDES FROM VARIABLE ANTIGENS, T CELL POPULATIONS AND USES THEREOF

(57) Abstract: The present invention relates to set overlapping immunogenic peptides of a variable antigen, and uses thereof, in particular for diagnostic and therapeutic purposes. The present invention relates also to a sub-population of CD8 T cells associated with the non-progressive status in HIV-infected subjects.

Overlapping peptides from variable antigens, T cell populations and uses thereof

FIELD OF THE INVENTION

The present invention relates to a set of overlapping immunogenic peptides of a variable antigen and uses thereof, in particular for diagnostic and therapeutic purposes. The present invention relates also to a sub-population of CD8 T cells and its use for the diagnostic of HIV infected subjects.

BACKGROUND OF THE INVENTION

The growing knowledge of how the human immune system deals with different types of pathogens is allowing to better define the immune correlates that leads to the protective effects exerted by efficacious vaccines. Therefore, delineation of the immune correlates of protection occurring during natural infection and after vaccination has become a fundamental step in the development of new vaccines. Important steps toward the identification of the immune correlates relevant for protection from HIV have been made mimicking HIV-1 infection in animal models and studying the natural course of the HIV-mediated disease in different cohorts of HIV-seropositive individuals. Indeed, studies of passive immunization and immune depletion in animal models strongly suggest that both humoral (neutralizing antibodies) and cell-mediated immune responses may provide effective protection from infection and disease progression in HIV-1 infection (3, 24).

Indeed, induction of strong and durable T lymphocytes responses against HIV-1-infected cells has become an important immunogenicity outcome in vaccine trials, given the increasing evidence that HIV-1-specific cell-mediated immune responses partially protect macaques experimentally infected with SHIV (4, 24) and contribute substantially to control HIV-1 disease in humans (16, 18, 20, 22). Therefore, increasing efforts are directed towards the development of effective vaccines through induction of virus-specific T cell responses, which play a significant role in determining viral set point in HIV infection.

In recent years, methods for detecting both CD4 and CD8 T cell responses have been significantly improved by the introduction of cytokine enzyme-linked immonospot assays (particularly IFN-γ Elispot) and intracellular cytokine staining (ICS) assays (6, 23). This has led to more sensitive detection and quantification of the cellular immune response but, despite greater ease and rapidity of assessment these approaches have left a number of issues unresolved and there is not yet a consensus on the best methods for assaying the magnitude and the breadth of these responses. In this regard, two mayor issues are still debated among

scientists: the choice of the HIV-1 sequence/s to use for the generation of the screening peptides since HIV is a highly variable virus, and the best type of approach for defining cytotoxic T lymphocytes (CTL) and T-helper epitopes. Indeed, differences in sequence between two HIV-1 isolates within the same clade range between 7-15% and between viruses belonging to different clades can be as high as 30%. Constructing a consensus sequence may represent a compromise for this problem (13), although it has to be kept in mind that it is very difficult to generate artificial sequences at the hyper-variable regions and that viral escape from immune detection has been consistently linked to amino acid substitutions in HIV-1 T cell epitopes (10, 19). Regarding the second issue, two are the strategies currently used to define T cell epitopes: bioinformatics approach which utilizes predictive algorithms containing peptide-binding 'motifs' and 'supermotifs' for the major histo-compatibility complex (MHC) class I and II molecules to predict epitopes (9) or via overlapping peptides that span the viral proteins of interest (2, 6, 10). In this latter case the length and degree of overlap of peptides are further motif of scientific debate (6, 10, 11). The use of computer-assisted bioinformatics approaches, based on HLA peptide binding motifs and available viral sequences, has been shown to be a useful tool for identifying CTL epitopes derived from HIV-1(9). However, since binding motifs have not yet been defined for all HLA alleles, HLA profiling of population belonging to developing countries is scanty and peptide binding is not the sole determinant of epitope dominance, not all CTL epitopes will be identified by HLA motif-screening algorithms. On the other hand, the methods based on a comprehensive screening of HIV-1 encoded antigens via overlapping peptide pools are attractive because makes no assumptions about peptides that do not conform exactly to the peptide-binding motif, although they still do not detect all the T cell responses present in HIV-infected individuals (5). The main limitation to this type of screening assays is that a consensus on the best design with regards to peptide length and overlap has not been reached yet. Indeed, the length and degree of peptide overlap not only influence the detection of T cell responses but also has a major impact on cost, labor intensiveness and amount of blood needed to perform such studies.

WO0116163 discloses a peptide mixture, a pharmaceutical composition and a vaccine against a chronic infection caused by a virus comprising a mixture of 10 to 30 amino acids (aa) long peptides each with a 5 to 25 aa overlap of the adjacent overlapping peptide spanning the amino acid sequence of a viral protein of said virus, e.g. Hepatitis B, Hepatitis C, GBvirus-C, HIV and Herpes viruses. Hepatitis B has been used as an example and it is demonstrated that a peptide mixture composed of seventeen 20 to 23 aa long peptides spanning the amino acids

1 to 183 of the hepatitis B core antigen (HBcAg) could activate specific T cells regardless of the host MHC/HLA genotype that recognize the native protein processed by professional antigen presenting cells (APCs). Further, a method is described for the treatment of a viral infection, particularly a non-resolving chronic viral infection, making use of the novel peptide mixture immunogen.

WO2004031345 provides compositions containing HIV epitopes, which are recognized by cytotoxic T lymphocytes (CTL). Such polypeptides are used in vaccines and immunotherapies. HIV-1 epitopes represent early targets in a naturally-occurring response against HIV-1 infection. In particular, an immunogenic composition comprising an HLA class I restricted HIV-1 polypeptide, wherein said polypeptide consists essentially of 8-12 amino acids and wherein said peptide is characterized as an early CTL target in HIV-1 infection is claimed.

WO2005015207 discloses a method and diagnostic tests based on flow cytometric analysis of antigen-specific T lymphocytes. However the document does not define a specific population of T-cells and the method does not allow the diagnostic of the non progressive status in an HIV-1 infected subject.

Vardas et al. (26) discloses a method for designing HIV Tat specific overlapping peptides for HIV vaccine trials.

There is the need for identifying an optimal peptide design strategy. Optimal epitopes are protein fragments that are best recognized by T lymphocytes due to their efficient binding. Optimal CTL epitopes are 8–11 amino acid residues in length, and, therefore, an overlap of at least 10 residues between adjacent overlapping peptides is required to ensure that no 11-mer is missed in the screening process. Increasing the number of overlapped amino acids (11 vs 10 amino acids) has not produced a clear advantage in detecting CTL responses (10), beside a substantial increase in the number of peptide necessary to perform a complete HIV-1 peptide-scan (746 vs 600). This leads to a 20% increase in the cost of the assay and on the amount of cells needed, thus reducing the feasibility of such approach. A documented disadvantage of overlapping peptides is that epitopes situated in the middle of longer peptides may not be always detectable because of suboptimal epitope presentation. This implies that longer peptide sets (20 mers) may be less sensitive in detecting CD8 T cell responses (6, 10), whereas they seems to be more efficient in resolving Class II restricted CD4 responses (10). Indeed, a CD8 T-cell response to an epitope present within a longer peptide is best detected when the epitope is situated at the peptide C-terminus, both when peptides were designed to include the optimal epitope at every possible position and when responses towards optimal

epitopes and corresponding overlapping peptides were compared in a larger group of subjects (10). Furthermore, even overlapping 15-mers are less efficient than optimized epitopes in detecting low frequency CD8 responses (200 SFU/million PBMC), when the epitope is located centrally in the peptide (5).

Taking advantage of the well establish notion that only a minority of the 20 possible amino acidic residues at the peptide C-terminus, a primary anchor position that contributes substantially to binding of the peptide to the MHC class I molecule will be present in CTL epitopes (only 9 of them served as the C-terminal anchor position in 96% of described optimal epitopes and in 95% of peptide-binding motifs described for over 60 HLA class I alleles), a novel strategy that combines database information with the systematic experimental procedure has been recently experimented (10). The direct comparison between gold standard 15/11 and 15/10 overlapping residue peptide sets has demonstrated the efficacy of this approach that, allowing the use of longer peptide (18mers) without losing in detection of magnitude and breath of the immune response, permits a conspicuous reduction in the number of peptide needed (410 vs 746 for a complete HIV-1 genome scanning). Although an optimal overlapping set has still to be obtained (13% of the CTL responses were not shared between different peptide sets), this result suggests that more rational approaches to overlapping peptides design are feasible though, in some instances, the number of peptide needed is greatly increased (17).

SUMMARY OF INVENTION

In the present invention, the authors present a new strategy of T-cell epitope screening that, without compromising the detection of the full-range of CTL and T-helper responses, limits both the amount of human material and assay cost.

Departing from the teaching of Vardas et al. (26) the authors optimized a method of peptide designing and were able to set up different set of peptides. These peptides were able to identify a new T-cell population exclusively associated with the control of the HIV-infection in long term non progressors (LTNP).

The method was used to generate two sets of overlapping peptides derived by the HIV-1 regulatory antigens Tat and Nef that are superior in detecting CD8 CTL responses without loosing the ability to uncover the CD4 T-helper responses. Moreover, the new peptides pools were used together with an immunocytofluorimetric assay to investigate the T-cell immune response of a selected group of HIV-infected individuals. By analyzing the response of subjects able to control HIV replication, a specific recognition pattern of tat encoding peptides

was found. This approach allowed the identification of a new T-cell population exclusively associated with the control of the HIV-infection in long term non progressors (LTNP).

Therefore, the authors propose the new peptide strategy for designing a feasible strategy for the screening of a significant number of HIV candidate vaccine antigens in large cohorts of European and African individuals, and the use of the new peptide pools combined with the immunocytofluorimetric technique for the identification of the peculiar T-cell population that act as a marker to monitor efficacious anti–HIV T-cell responses.

Authors utilized a new method (called Variable Overlapping Peptide Scanning Design, VOPSD) to design overlapping peptides (10 to 19 aa long). Such peptides may be used for the detection of both CD4 and CD8 T-cell responses derived from any type of well defined microbial or tumor derived antigen. Given the superior ability of the VOPSD strategy to detect T-cell responses in humans the peptides sets generated following this method possess several relevant industrial applications. As a matter of facts, they can be used as a complete set, covering the complete sequence of a known antigen, for screening system to individuate the human T-cell mediated immune responses relevant for the development of T-cell based vaccines. The peptides may be used as vaccine components and they can be the major components of diagnostic kits for the detection of T-cell based immune responses. In particular the sets of peptides generated using the HIV-1 encoded Tat and Nef antigens belonging either to clade B or C can be used for monitoring the ability of HIV-1 infected individuals to naturally control HIV-1 replication..

The present invention discloses also a novel CD8 T-cell population exclusively present in HIV-infected subjects with a non-progressive course of the HIV disease. The detection of such a population will allow the monitoring of HIV-infected individuals for the presence of an efficacious anti HIV-1 T-cell response. A kit able to detect this population will be useful to evaluate, in HIV-seronegative individuals subjected to preventative anti HIV-1 vaccination programs, the elicitation of anti HIV-specific protective CD8 T-cell responses and, in HIV-infected individuals subjected to therapeutic vaccination protocols with HIV-encoded antigens, the appearance/increase of anti HIV-specific protective CD8 T-cell responses.

In addition, a kit able to measure such population will be useful to evaluate in patients naïve for antiretroviral therapy (ART) the timing for the therapeutic intervention.

Therefore it is an object of the present a set of overlapping peptides of an antigen, said antigen being a variable antigen, comprising :

a) a first sub-set of peptides longer than 10 amino acids consisting of conserved sequence regions between an amino acid sequence of a variant antigen and the consensus amino acid sequence of said variable antigen, and

b) a second sub-set of peptides, each peptide being between 10 and 19 amino acids long and containing an amino acid sequence of said variant antigen that differs for at least one amino acid residue from the consensus amino acid sequence of said variable antigen, and optionally one or more immunodominant region and/or a optimal epitope region or portions thereof at its N or C terminus, wherein each of said peptides does not have as the last C-terminal amino acid any of the following amino acids: Asn, Asp, Gln, Glu, Gly, His, Ser;

wherein each peptide of the first sub-set and of the second sub-set of peptides has a gap not longer than 8 amino acids with its closest overlapping peptide.

The wording "gap" refers to the fact that the following peptide starts between 0-8 aa. downstream of the aa. protein sequence. It is a common terminology for people experienced in the field.

The set of peptides of the invention are obtainable by a modification of the method disclosed by Vardas et al. (26). As a matter of fact, the length of peptides was optimized (between 10 and 19 amino acids) and it was found that each of the set of overlapping peptides should have a gap not longer than 8 amino acids with its closest overlapping peptide. Moreover, overlapping peptides must contain at their termini previously characterized T-cell epitopes or immunodominant fragments. The choice for positioning such epitopes in the N- terminus or in the C-terminus of the longer scanning peptides is based upon the evaluation of the HLA binding motifs proper of the epitope under investigation. Furthermore, it is also new with respect to Vardas et al (26) that, when different optimal epitopes are partially overlapped in the protein sequence, the choice for the best placement in the C-terminus of the epitopes is dictated by the frequency in the human population of the HLA allele/s restricting the epitope recognition and by the possibility to place it, as an alternative, to the N-terminus of a different scanning peptide.

Preferably, the variable antigen is an HIV antigen. More preferably the HIV antigen is an HIV-1 antigen. Still preferably the HIV antigen is an HIV-1 Tat antigen or an HIV-1 Nef antigen.

In a preferred embodiment of the invention, the set of overlapping peptides consists of peptides belonging to any of the following groups:

a) peptides of SEQ ID No. 1 to SEQ ID No. 11;

b) peptides of SEQ ID No. 42 to SEQ ID No. 51 and SEQ ID No. 7.

In a most preferred embodiment, the set of overlapping peptides comprises peptides of SEQ ID No. 2, 5 and 6.

In a preferred embodiment of the invention, the set of overlapping peptides consists of peptides belonging to any of the following groups:

a) peptides of SEQ ID No. 12 to SEQ ID No. 26;

b) peptides of SEQ ID No. 27 to SEQ ID No. 41;

c) peptides of SEQ ID No. 12 to SEQ ID No. 41;

d) peptides of SEQ ID No. 52 to SEQ ID No.63 and SEQ ID No. 20, SEQ ID No. 23 and SEQ ID No. 26;

e) peptides of SEQ ID No. 64 to SEQ ID No. 76 and SEQ ID No. 30, SEQ ID No. 31 and SEQ ID No.32;

f) peptides of SEQ ID No. 52 to SEQ ID No. 76 and SEQ ID No. 20, SEQ ID No. 23, SEQ ID No. 26, SEQ ID No. 30, SEQ ID No. 31 and SEQ ID No.32;

g) peptides of SEQ ID No. 77 to SEQ ID No. 86.

In a most preferred embodiment, the set of overlapping peptides comprises the peptide of SEQ ID No. 40.

It is a further object of the invention the set of overlapping peptides, or a mixture thereof, as defined above for use as a medicament, a vaccine or a vaccine adjuvant.

The set of overlapping peptides are used for monitoring CD4 and/or CD8 T cell responses in HIV patients ex vivo, in particular, for the development of immunological diagnostic test or kits. The peptides of the invention can be used for the development of any immunological test that it is based on measuring either the amount of T-cells that are specific for a given antigen or the function of the same cells.

The set of overlapping peptides, especially those having sequences SEQ ID No. 2, 5 and 6, and SEQ ID No. 40, are preferentially used for an *ex vivo* monitoring of T cell responses associated with the natural control of HIV-1 replication.

The set of overlapping peptides of the invention are used for identifying and/or selecting and/or monitoring a HIV-1 negative population for anti HIV-1 vaccine development; for the detection, in a biological sample, of a population of T cells belonging to any of the following groups:

a) CD4 T cells;

b) CD8 T cells;

c) $CD45RA^{+}IFN\gamma^{-}$ $MIP1\beta^{+}$ CD8 T cells.

The set of overlapping peptides of the invention can be used also for monitoring T-cell responses in HIV-negative individuals, subjected to preventative anti-HIV vaccine experimentations or in HIV seropositive individual subjected to therapeutic vaccination trials, independently from the need for the identification of the CD45 RA^+ $IFN\gamma^-$ $MIP1\beta^+$ cells.

It is a further object of the invention an isolated population of $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cells.

Such specific cell population can be advantageously used as a correlate of protection against HIV infection and/or HIV disease.

Correlates of protection to a virus or other infectious pathogen are *measurable* signs that a person (or other potential host) is immune, in the sense of being protected against becoming infected and/or developing disease. For many viruses, antibodies serve as a correlate of immunity. For HIV, the simple presence of antibodies is clearly not a correlate of protection since infected individuals develop antibodies without being protected against disease.

Moreover such specific cell population can be advantageously used as a marker for the non-progressive status of HIV-infected subjects, or as immune therapy. As a matter of facts, after sorting and expansion, expanded cells can then be re-infused in HIV infected patients.

The HIV-1 infected subject may be a subject not assuming antiretroviral therapy in which the capacity to control HIV disease and the timing of introduction of antiretroviral treatment is to be determined. More preferably the $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population is Nef specific.

The $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population may be used for measuring the efficacy of an HIV derived antigen vaccine in a vaccinated subject. The vaccinated subject can be HIV seropositive or not.

$CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cells are preferably detected using the VOSPD overlapping peptides of the invention. However, the detection of the $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell may be performed also using the standard 20mers pools or any other antigenic formulation known to those skilled in the art.

It is a further object of the invention a kit to detect a $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population in a biological sample comprising:

i) a set of peptides belonging to any of the following groups:

a) a set of overlapping peptides according to claims 1 to 9;

b) standard pools of overlapping peptides;

c) pools of optimal CD8 T-cell epitopes;

ii) a set of antibodies able to detect the following markers on CD8 T cells: $CD45RA^+$, $IFN\gamma^-$, and $MIP1\beta^+$.

A further aspect refers to a method for detecting a $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population in a biological sample comprising:

i) incubating said sample with a set of peptides belonging to any of the following groups:

a) a set of overlapping peptides according to the invention;

b) standard pools of overlapping peptides;

c) pools of optimal CD8 T-cell epitopes;

ii) staining said sample with a set of labelled antibodies able to detect the following markers on CD8 T cells: $CD45RA^+$, $IFN\gamma^-$, and $MIP1\beta^+$.

The kit is used to detect $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cells if found to be specific for any pathological status either upon viral infection (i.e. HIV, HCV, HBV) or tumoral status.

Another object of the invention refers to a method for the diagnostic of a long-term non-progressive (LTNP) status, or for monitoring the anti-HIV-specific protective CD8 T cell response, in a biological sample from a HIV-1 infected subject, comprising the steps of:

a) detecting the $CD45RA^+IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population as above described, and

b) measuring the % thereof with respect to the total CD8 T cells or to the total responding CD8 T cells.

Another object of the invention refers to a method for measuring the efficacy of an HIV derived antigen vaccine in a biological sample from a vaccinated HIV-1 infected subject, comprising the steps of:

a) detecting the $CD45RA^+IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population as above described, and

b) measuring the % thereof with respect to the total CD8 T cells or to the total responding CD8 T cells.

Another object of the invention refers to a method for monitoring the capacity of the immune system of an human to control HIV infection, comprising the steps of:

a) detecting the $CD45RA^+IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population as above described in a biological sample from a HIV-1 infected subject, and

b) measuring the % thereof with respect to the total CD8 T cells or to the total responding CD8 T cells.

The detection of the $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population can be performed with assays known to those skilled in the art like, for example, Bioplex, ELISPOT and ELISA. For example, using the ELISPOT technology it is possible to pre-select CD8+CD45RA+ cells

directly on the plate, perform the antigenic stimulation and detect simultaneously the production of MIP-1β and IFN-γ.

Preferably, the detection of the $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population is performed with an intracellular cytokine staining and the VOSPD overlapping peptides of the invention..

The detection of the $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population can be performed also with a secretion assay. In this way $CD45RA^+$ $IFN\gamma^-$ $MIP1\beta^+$ CD8 T cells are still alive after the staining allowing a successive sorting of such a population.

It is another object of the invention a method for the diagnostic of a long-term non-progressive (LTNP) status, or for monitoring the anti-HIV-specific protective CD8 T cell response, in a biological sample from an HIV-1 infected subject, comprising the steps of:

a) detecting the $CD45RA^+IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population, and

b) measuring the % thereof with respect to the total CD8 T cells or to the total responding CD8 T cells.

It is another object of the invention a method for measuring the efficacy of an HIV derived antigen vaccine in a biological sample from a vaccinated HIV-1 infected subject, comprising the steps of:

a) detecting the $CD45RA^+IFN\gamma^-$ $MIP1\beta^+$ CD8 T cell population, and

b) measuring the % thereof with respect to the total CD8 T cells or to the total responding CD8 T cells.

In the present invention the standard pool Tat 15mer comprises:

TC27	1-15	MEPVDPRLEPWKHPG	SEQ ID 87
TC28	6-20	PRLEPWKHPGS**Q**PKT	SEQ ID 88
TC29	11-25	WKHPGS**Q**PKTACTNC	SEQ ID 89
TC30	16-30	S**Q**PKTACTNCYCKKC	SEQ ID 90
TC31	21-35	ACTNCYCKKCCFHCQ	SEQ ID 91
TC32	26-40	YCKKCCFHCQVCF**I**T	SEQ ID 92
TC33	31-45	CFHCQVCF**I**TKALGI	SEQ ID 93
TC34	36-50	VCF**I**TKALGISYGRK	SEQ ID 94
TC35	41-55	KALGISYGRKKRRQR	SEQ ID 95
TC36	46-60	SYGRKKRRQRRR**PP**Q	SEQ ID 96
TC37	51-65	KRRQRRR**PP**Q**G**SQTH	SEQ ID 97
TC38	56-70	RR**PP**Q**G**SQTHQ**V**SLS	SEQ ID 98
TC39	61-75	**G**SQTHQ**V**SLSKQP**T**S	SEQ ID 99

TC40	66-80	Q**V**SLSKQP**T**SQ**S**RGD	SEQ ID 100
TC41	71-85	KQP**T**SQ**S**RGDPTGPK	SEQ ID 101
TC42	76-90	Q**S**RGDPTGPKEQKKK	SEQ ID 102.

The pool or set Tat-VOPSD (HIV-1B Tat Bh-10) comprises,

Peptide N°1	MEPVDPRLEPW	11 mer	SEQ ID 1
Peptide N°2	LEPWKHPGSQPKTACTNCY	19 mer (7aa gap)	SEQ ID 2
Peptide N°3	GSQPKTACTNCYCKKCCF	18 mer (7aa gap)	SEQ ID 3
Peptide N°4	ACTNCYCKKCCFHCQVCF	18 mer (6aa gap)	SEQ ID 4
Peptide N°5	KKCCFHCQVCFITKALGI	18 mer (7aa gap)	SEQ ID 5
Peptide N°6	QVCFITKALGISYGR	15 mer (7aa gap)	SEQ ID 6
Peptide N°7	LGISYGRKKRRQRR	14 mer (8aa gap)	SEQ ID 7
Peptide N°8	KKRRQRRRPPQGSQTHQV	18 mer (7aa gap)	SEQ ID 8
Peptide N°9	RPPQGSQTHQVSLSKQPT	18 mer (7aa gap)	SEQ ID 9
Peptide N°10	HQVSLSKQPTSQSRGDPT	18 mer (8aa gap)	SEQ ID 10
Peptide N°11	KQPTSQSRGDPTGPK	15 mer (6aa gap)	SEQ ID 11;

The standard pool Tat 20mer is described in http://www.nibsc.ac.uk/spotlight/aidsreagent/index.html under the reference number EV779.1 to EVA779.8 at page 123, as follows:

ARP779.1	EPVDPRLEPWKHPGSQPKTA	SEQ ID 123
ARP779.2	KHPGSQPKTACTTCYCKKCC	SEQ ID 124
ARP779.3	CTTCYCKKCCFHCQVCFTTK	SEQ ID 125
ARP779.4	FHCQVCFTTKALGISYGRKK	SEQ ID 126
ARP779.5	ALGISYGRKKRRQRRRPPQG	SEQ ID 127
ARP779.6	RRQRRRPPQGSQTHQVSLSK	SEQ ID 128
ARP779.7	SQTHQVSLSKQPTSQPRGD	SEQ ID 129
ARP779.8	QPTSQPRGDPTGPKE	SEQ ID 130.

The pool Nef N-ter VOPSD (HIV-1B Nef Bru) comprises,

Peptide N°1	MGGKWSKSSVVGWPTVR	17 mer	SEQ ID 12
Peptide N°2	SVVGWPTVRERMRRAEPAA	19 mer (8aa gap)	SEQ ID 13
Peptide N°3	VRERMRRAEPAADGVGAA	18 mer (7aa gap)	SEQ ID 14

Peptide N°4	AEPAADGVGAASRDLEK	17 mer	(7aa gap)	SEQ ID 15
Peptide N°5	GVGAASRDLEKHGAIT	16 mer	(6aa gap)	SEQ ID 16
Peptide N°6	DLEKHGAITSSNTAATNA	18 mer	(7aa gap)	SEQ ID 17
Peptide N°7	ITSSNTAATNAACAWL	16 mer	(7aa gap)	SEQ ID 18
Peptide N°8	ATNAACAWLEAQEEEEV	17 mer	(7aa gap)	SEQ ID 19
Peptide N°9	CAWLEAQEEEE	11 mer	(5aa gap)	SEQ ID 20
Peptide N°10	QEEEEVGFPVTPQVPLR	17 mer	(5aa gap)	SEQ ID 21
Peptide N°11	VGFPVTPQVPLRPMTYK	17 mer	(5aa gap)	SEQ ID 22
Peptide N°12	PQVPLRPMTY	10 mer	(6aa gap)	SEQ ID 23
Peptide N°13	RPMTYKAAVDLSHFLK	16 mer	(5aa gap)	SEQ ID 24
Peptide N°14	AVDLSHFLKEKGGLEGL	17 mer	(7aa gap)	SEQ ID 25
Peptide N°15	FLKEKGGLEGLI	12 mer	(6aa gap)	SEQ ID 26.

The pool Nef C-ter VOPSD (HIV-1B Nef Bru) comprises,

Peptide N°16	LEGLIHSQRRQDILDLWIY	19 mer	(7aa gap)	SEQ ID 27
Peptide N°17	QRRQDILDLWIYHTQGY	17 mer	(7aa gap)	SEQ ID 28
Peptide N°18	LDLWIYHTQGYFPDWQNY	18 mer	(6aa gap)	SEQ ID 29
Peptide N°19	YHTQGYFPDWQNYT	14 mer	(6aa gap)	SEQ ID 30
Peptide N°20	YFPDWQNYTPGPGVRY	16 mer	(5aa gap)	SEQ ID 31
Peptide N°21	QNYTPGPGVRYPLTFGW	17 mer	(5aa gap)	SEQ ID 32
Peptide N°22	GPGVRYPLTFGWCYKL	16 mer	(5aa gap)	SEQ ID 33
Peptide N°23	TFGWCYKLVPVEPDKVEEA	19 mer	(8aa gap)	SEQ ID 34
Peptide N°24	VPVEPDKVEEANKGENTSL	19 mer	(8aa gap)	SEQ ID 35
Peptide N°25	KVEEANKGENTSLLHPV	17 mer	(6aa gap)	SEQ ID 36
Peptide N°26	ENTSLLHPVSLHGMDDPER	19 mer	(8aa gap)	SEQ ID 37
Peptide N°27	PVSLHGMDDPEREVLEWR	18 mer	(7aa gap)	SEQ ID 38
Peptide N°28	DDPEREVLEWRFDSRLAF	18 mer	(7aa gap)	SEQ ID 39
Peptide N°29	VLEWRFDSRLAFHHVAREL	19 mer	(6aa gap)	SEQ ID 40
Peptide N°30	DSRLAFHHVARELHPEYF	18 mer	(6aa gap)	SEQ ID 41.

The standard pool Nef N-ter 20mers is described in http://www.nibsc.ac.uk/spotlight/aidsreagent/index.html under the reference number ARP7074.1 to ARP7074.10 at page 121, as follows:

ARP7074.1	GGKWSKSSVVGWPTVRERMR	SEQ ID 103
ARP7074.2	GWPTVRERMRRAEPAADGVG	SEQ ID 104
ARP7074.3	RAEPAADGVGAASRDLEKHG	SEQ ID 105
ARP7074.4	AASRDLEKHGAITSSNTAAT	SEQ ID 106
ARP7074.5	AITSSNTAATNAACAWLEAQ	SEQ ID 107
ARP7074.6	NAACAWLEAQEEEEVGFPVT	SEQ ID 108
ARP7074.7	EEEEVGFPVTPQVPLRPMTY	SEQ ID 109
ARP7074.8	PQVPLRPMTYKAAVDLSHFL	SEQ ID 110
ARP7074.9	KAAVDLSHFLKEKGGLEGLI	SEQ ID 111
ARP7074.10	KEKGGLEGLIHSQRRQDILD	SEQ ID 112

The standard pool Nef C-ter 20mers is described in http://www.nibsc.ac.uk/spotlight/aidsreagent/index.html under the reference number ARP7074.11 to ARP7074.20 at page 121 , as follows:

ARP7074.11	HSQRRQDILDLWIYHTQGYF	SEQ ID 113
ARP7074.12	LWIYHTQGYFPDWQNYTPGP	SEQ ID 114
ARP7074.13	PDWQNYTPGPGVRYPLTFGW	SEQ ID 115
ARP7074.14	GVRYPLTFGWCYKLVPVEPD	SEQ ID 116
ARP7074.15	CYKLVPVEPDKVEEANKGEN	SEQ ID 117
ARP7074.16	KVEEANKGENTSLLHPVSLH	SEQ ID 118
ARP7074.17	TSLLHPVSLHGMDDPEREVL	SEQ ID 119
ARP7074.18	GMDDPEREVLEWRFDSRLAF	SEQ ID 120
ARP7074.19	EWRFDSRLAFHHVARELHPE	SEQ ID 121
ARP7074.20	HHVARELHPEYFKNC	SEQ ID 122

The pool Tat C VOPSD (HIV-1B Tat consensus C) comprises:

Peptide N°1	MEPVDPNLEPW	11 mer	SEQ ID 42
Peptide N°2	LEPWNHPGSQPKTACNTCY	19 mer (7aa gap)	SEQ ID 43
Peptide N°3	GSQPKTACNTCYCKKCSY	18 mer (7aa gap)	SEQ ID 44
Peptide N°4	ACNTCYCKKCSYHCLVCF	18 mer (6aa gap)	SEQ ID 45

Peptide N°5 KKCSYHCLVCFQTKGLGI 18 mer (7aa gap) SEQ ID 46
Peptide N°6 LVCFITKGLGISYGR 15 mer (7aa gap) SEQ ID 47
Peptide N°7 KKRRQRRSAPPSSEDHQN 18 mer (7aa gap) SEQ ID 48
Peptide N°8 SAPPSSEDHQNPISKQPL 18 mer (7aa gap) SEQ ID 49
Peptide N°9 HQNPISKQPLPRTLGDPT 18 mer (8aa gap) SEQ ID 50
Peptide N°10 KQPLPRTLGDPTGSE 15 mer (6aa gap) SEQ ID 51
and the Peptide LGISYGRKKRRQRR , SEQ ID 7;

The pool Nef C N-ter VOPSD (HIV-1 Nef Consensus C) comprises:

Peptide N°1 MGGKWSKCSIVGWPAVR 17 mer SEQ ID 52
Peptide N°2 SIVGWPAVRERMRRTEPAA 19 mer (8aa gap) SEQ ID 53
Peptide N°3 VRERMRRTEPAAEGVGAA 18 mer (7aa gap) SEQ ID 54
Peptide N°4 TEPAAEGVGAASQDLDK 17 mer (7aa gap) SEQ ID 55
Peptide N°5 GVGAASQDLDKHGALT 16 mer (6aa gap) SEQ ID 56
Peptide N°6 DLDKHGALTSSNTAANNA 18 mer (7aa gap) SEQ ID 57
Peptide N°7 LTSSNTAANNADCAWL 16 mer (7aa gap) SEQ ID 58
Peptide N°8 ANNADCAWLEAQEEEEEV 18 mer (7aa gap) SEQ ID 59
Peptide N°9 CAWLEAQEEEE 11 mer (5aa gap) SEQ ID 20
Peptide N°10 QEEEEEVGFPVRPQVPLR 18 mer (6aa gap) SEQ ID 60
Peptide N°11 VGFPVRPQVPLRPMTYK 17 mer (5aa gap) SEQ ID 61
Peptide N°12 PQVPLRPMTY 10 mer (6aa gap) SEQ ID 23
Peptide N°13 RPMTYKAAFDLSFFLK 16 mer (5aa gap) SEQ ID 62
Peptide N°14 AFDLSFFLKEKGGLEGL 17 mer (7aa gap) SEQ ID 63
Peptide N°15 FLKEKGGLEGLI 12 mer (6aa gap) SEQ ID 26.

The pool Nef C C-ter VOPSD (HIV-1 Nef Consensus C) comprises:

Peptide N°16 LEGLIYSKKRQEILDLWVY 19 mer (7aa gap) SEQ ID 64
Peptide N°17 KKRQEILDLWVYHTQGY 17 mer (7aa gap) SEQ ID 65
Peptide N°18 LDLWVYHTQGYFPDWQNY 18 mer (6aa gap) SEQ ID 66
Peptide N°19 YHTQGYFPDWQNYT 14 mer (6aa gap) SEQ ID 30
Peptide N°20 YFPDWQNYTPGPGVRY 16 mer (5aa gap) SEQ ID 31
Peptide N°21 QNYTPGPGVRYPLTFGW 17 mer (5aa gap) SEQ ID 32

Peptide N°22 GPGVRYPLTFGWCFKL 16 mer (5aa gap) SEQ ID 67
Peptide N°23 TFGWCFKLVPVDPREVEEA 19 mer (8aa gap) SEQ ID 68
Peptide N°24 VPVDPREVEEANEGENNCL 19 mer (8aa gap) SEQ ID 69
Peptide N°25 EVEEANEGENNCLLHPM 17 mer (6aa gap) SEQ ID 70
Peptide N°26 ENNCLLHPMSQHGMEDEHR 19 mer (8aa gap) SEQ ID 71
Peptide N°27 PMSQHGMEDEHREVLKWK 18 mer (7aa gap) SEQ ID 72
Peptide N°28 EDEHREVLKWKFDSHLAR 18 mer (7aa gap) SEQ ID 73
Peptide N°29 VLKWKFDSHLARRHMAREL 19 mer (6aa gap) SEQ ID 74
Peptide N°30 DSHLARRHMARELHPEYY 18 mer (6aa gap) SEQ ID 75
Peptide N°31 RHMARELHPEYYKDC; 15 mer (6aa gap) SEQ ID 76;

the pool Nef C SA VOPSD (HIV-1C South African Nef Consensus) comprises,

Peptide N°1 YHTQGFFPDWQNYT 14 mer SEQ ID 77
Peptide N°2 FFPDWQNYTPGPGVRY 16 mer SEQ ID 78
Peptide N°3 DLDKHGALTSSNTAHNNA 18 mer SEQ ID 79
Peptide N°4 LTSSNTAHNNADCAWL 16 mer SEQ ID 80
Peptide N°5 HNNADCAWLEAQEEEEEV 18 mer SEQ ID 81
Peptide N°6 LDLWVYHTQGFFPDWQNY 18 mer SEQ ID 82
Peptide N°7 PMSQHGMEDEDREVLKWK 18 mer SEQ ID 83
Peptide N°8 EDEDREVLKWKFDSSLAR 18 mer SEQ ID 84
Peptide N°9 VLKWKFDSSLARRHMAREL 19 mer SEQ ID 85
Peptide N°10 DSSLARRHMARELHPEYY 18 mer SEQ ID 86.

The present invention shall be disclosed in detail also by means of non limiting examples referring to the following figures.

Figure 1. Comparison between peptide pools encoding the Tat antigen.

Comparison between Tat VOPSD and Tat 15mers responses (Figure 1A) and between Tat VOPSD and Tat 20mers (Figure 1B). The circle individuates the two responses not detected by the Tat VOPSD pool. Ellipses individuate the responses detected only using the Tat VOPSD peptide pools. The data reported on the x and y axes are expressed as SFU/1,000,000 cells.

Figure 2. Comparison between peptide pools encoding the Nef antigen.

Comparison between VOPSD peptide pools and standard pools. (2A) Nef N-terminus VOPSD encoding the protein N-terminus vs Nef N-ter 20mers. (2B) Nef C-terminus VOPSD encoding the protein C-terminus vs. Nef C-ter 20mers C-terminus. Hexagons individuate the responses detected only using the VOPSD peptide pools. The data reported on the x and y axes are expressed as SFU/1,000,000 cells.

Figure 3. Comparison between CD8 and CD4 T cell responses obtained using different peptide pools encoding the Nef antigen.

Comparison between the CD8 responses (A and B) and the CD4 responses (C and D) obtained using the peptide pools (VOPSD N-terminus and Nef N-terminus 20mers) encompassing the protein N-terminus (A and C) or C-terminus (VOPSD C-terminus and Nef N-terminus 20mers) (B and D).

Figure 4. Comparison between VOPSD and 20mers peptide pools encoding the HIV-1 antigens Tat and Nef .

Panel 4A: Recognition of the Tat VOPSD (grey line with filled diamond) and its 20mer standard homolog (Tat 20mer) (black line with filled circle) by PBMC derived from patient HSR-006.

Panel 4B: Recognition of VOPSD Nef N-terminus peptide pool and its standard 20mer homolog (NefN-terminus 20mers) by patient HSR-018. Symbols and line colours are reported as above described.

The dotted line individuates the negative cut-off of the assay in both panels.

Figure 5. single peptide Recognition of homologs peptides belonging to the Tat VOPSD and the Tat 20mers peptide sets .

Recognition of the VOPSD Tat peptide 1 (greyline with filled diamond) and its standar Tat 20mer homolog (black line with filled circle) by PBMC derived from patient HSR-028.

The dotted line individuates the negative cut-off of the assay.

Figure 6. CD8 T cells recognition of the minimal epitope FLKEKGGL present in the Nef specific overlapping set of peptides.

Panel 6A: recognition of the minimal epitope Class I B 08 restricted FLKEKGGL (SEQ ID 131) by CD8 T-cell belonging to patient V4 PBMC from subject V4 were stimulated with the FLKEKGGL peptide (grey diamond) or with an equivalent volume of DMSO (black circles), before staining for the detection of intracellular IFN-γ.

Panel 6B: recognition of the overlapping peptides containing the FLKEKGGL epitope. PBMC were stimulated with two Nef derived peptide from the Nef N-ter VOPSD set

(FLKEKGGLEGLI, SEQ ID No. 26, black squares; AVDLSHFLKEKGGLEGL, SEQ ID No. 25 black triangles) and one peptide from the classical Nef 20mer set (KAAVDLSHFLKEKGGLEGLI, SEQ ID No. 111, open squares). As negative control, PBMCs were stimulated with medium alone containing an equal volume of DMSO (black circles).

Figure 7. Gating strategy. The pseudo-colour dot plots show an example of gating strategy applied on CD8+ CD3+ living lymphocytes. A mock stimulated (none) and Nef C-termVOPSD (Nef 5) stimulated samples are shown.

Figure 8. Single data representation for the interesting populations and representative dot plots. Frequencies of CD8 T-cell responses (A, B and C) and % of the total response (D, E and F) are shown for the population discussed in the text: CD45RA+ IFN-γ- IL-2- MIP-1β+ (A and D), CD45RA+ IFN-γ+ IL-2- MIP-1β+ (B and E) and CD45RA- IFN-γ- IL-2- MIP-1β+ (C and F). Each point represents one patient. Medians are shown for each cohort. In (G) representative dot plots from a long term non progressor (LTNP), a late progressor (LP) and a chronically infected individual (CHI) are shown. Depicted events were previously gated on MIP-1β+ CD8 T-cells. The vertical line in each dot plot separates CD45RA positive and negative cells. IFN-γ+ cells are depicted in blue.

Figure 9. Single data representation for the percentage of the Tat-specific CD45RA+ IFN-γ- IL-2- MIP-1β+ responding CD8 T-cells. Each point represents one patient. Medians are shown for each cohort.

Figure 10. Characterization of the Nef-specific CD8 T-cell responses before and after therapy interruption. Absolute and relative frequencies of the Nef-specific response patterns for CD8 T-cells. The histogram plot shows the median frequency of the responding T-cells. Responses to the pools Nef N-term VOPSD and Nef C-term VOPSD are summed together to obtain the response to the entire Nef antigen. Asterisks indicate significant differences. The grey bars represent values before therapy interruption, the black bars represent the values after therapy interruption.

Figure 11. Comparison between VOPSD, a pool of Nef derived optimal CD8 T-cell epitopes and the standard 20 mer in detecting the CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cell population. Absolute frequencies of Nef-specific response patterns for CD8 T-cells are shown. The histogram plot shows the frequency of the responding T-cells. Responses detected with the pools Nef N-term VOPSD and Nef C-term VOPSD (white bars), the optimal pool (grey bar) and with the standard 20mer (black bars) are shown.

Figure 12. Magnitude of Tat-specific T-cell response in selected cohorts of HIV-1 infected individuals. The frequency of Tat-specific IFN-γ producing T-cell is significantly increased (p=0.0075) in HIV-1 infected individuals who naturally control HIV-1 viremia (EC) as compared to HIV-1 chronically infected patients in whom HIV-1 replication is maintained below the threshold of detection by ART regimens (CHI w T). Each point represents one patient. Medians are shown for each cohort. Significant differences are reported above the graph: (**) p <0.01.

Figure 13. The breadth of Tat-specific cellular response is significantly increased in EC. EC recognized a significantly higher number of Tat-specific peptides than CHI w T. Each point represents one patient. Medians are shown for each cohort. Significant differences are reported above the graph: (*) p <0.05.

Figure 14. EC selectively recognize a distinctive set of Tat-specific VOPSD peptides. Mapping of the Tat-specific T-cell responses in selected cohorts of HIV-1 infected individuals. Each Tat VOPSD peptide is reported on the x axes (corresponding to the sequences identification number from 1-11) whereas the number of individuals responding to each peptide is reported on the y axes. Each cohort is identified by a different bar pattern as described in the inset legend.

METHODS

Patients

Four cohorts of HIV-1 seropositive individuals and two cohorts of HIV-seronegative healthy blood donors were enrolled for the study (Table I).

Table I: Summary of parameters for HIV-1 seropositive cohorts enrolled for the study

Cohort	CD4 T cell count		HIV plasma load		Years of known seropositivity	ART
	median	1° -3°quartile	median	1° - 3°quartile		
1 (n = 75)	549	464-838	10685	860-28423	Weeks-22 yrs	
2 (n = 14)	417	375-593	50	50-472		
3 (n = 9)	502	441-775	1083	351-5377	> 13 yrs	
	395		50		> 20 yrs	

3 (n = 3) 3 (n = 23)	488	351-698	50		> 2 yrs	ART for > 2 yrs

In the largest cohort, used for the IFN-γ based ELISPOT, the authors enrolled 76 HIV-1 infected individuals with variable CD4 T cell counts (median 549; 1°-3° quartile 464 and 838 CD4/μl) and HIV-1 plasma load (median 10,685; 1°-3° quartile 860 and 28,423 genome equivalents/ml), as well as for length of the HIV-1 infection (from few weeks to over 22 years), route of infection and antiretroviral therapy (ART). Patients HSR 006, 018 and 028 belonged to this cohort. The smallest cohort (14 individuals), used for the immunocytofluorimetric assay, was composed by HIV-seropositive individuals with a CD4 T-cell count > 300 cells/μl (median 417; 1°-3° quartile 375 and 593) and low HIV-1 plasma load (median 50; 1°-3° quartile 50 and 472 genome equivalents/ml). Patient V4 belonged to this cohort. In the third one the authors enrolled a total of 35 HIV-1 infected individuals belonging to three different groups of patients. The first group was composed by 9 long term non progressors (LTNP) HIV-seropositive patients for more than 13 years, median CD4 T cell count of 502 cell/μl (1°-3° quartile 441 and 775) and median HIV-1 plasma load of 1083 genome equivalents/ml (1°-3° quartile 351 and 5377). The second group was composed by 3 late progressors (LP) patients that were HIV-seropositive for >20 years, They progressed to AIDS during the last year of observation and one (LP01) was not yet ART-treated when the blood sample was withdrawn. The third group included 23 chronically infected individuals (CHI) HIV-seropositive and under ART for >2 years. The median CD4 T-cell count was 488 cell/μl (1°-3° quartile 351 and 698) and viral load was undetectable in all but three subject (CHI-011 3,362 genome equivalents/ml; CHI-012: 36,600 genome equivalents/ml; CHI-3V: 13,965 genome equivalents/ml). Six CHI patients were tested again after an interruption of highly active antiretroviral therapy (HAART) for 2-4 weeks; the therapy was reintroduced when the viral load was higher than 100,000 genome equivalent /ml. In the fourth cohort the authors enrolled a total of 52 HIV-1 infected individuals divided in 5 groups. The first group was composed by 6 LTNP; the second one by 13 HIV-1 seropositive individuals with HIV-1 plasma load <50 copies/ml for > 6 months, ART naive and CD4 T-cell count > 500 cells/μl defined as Elite Controllers (EC); in the third group we enrolled 5 HIV-1 seropositive individuals who acquired undetectable HIV-1 plasma load under ART, maintaining the HIV-1 plasma load < 50 copies/ml after prolonged therapy interruption (>1 year) and termed

Controllers Upon Suspension (CUS); the fourth one was constituted by13 HIV-1 infected patients that are unable to maintain the HIV-1 plasma load <50 copies /ml in the absence of ART termed Chronically Infected without Therapy (CHIwo/T); in the fifth group we enrolled 15 HIV-seropositive individuals under successful ART for >2 years (HIV-plasma load <50 copies/ml).

Finally, to assess the background responses generated by cross-reactive T-cells a cohort of 75 HIV-seronegative healthy blood donors (HBD) was investigated using the ELISPOT technique, whereas a second small cohort of 5 HBD was used for the immunocytofluorimetric assay.

Antigens

Overlapping sets of peptides covering the HIV-1 encoded Tat (BH-10 strain; aa 1- 86 Gene bank accession number M15654) and Nef antigen (Bru strain; aa 1-205 gene bank accession number K02013) were used. For the Tat antigen both standard strategies of protein scanning design were utilized building–up two pools of peptides composed by either 15mers overlapped by 10 aa residues (designated Tat15mer) or 20mers overlapped by 10aa residues (designated Tat20mer). Only one peptide set formed by 20mers overlapped by 10aa residues was used for the Nef antigen (the set is composed by peptides Nef N-ter 20mers and Nef C-ter 20mers) to validate the new overlapping peptide antigen scanning strategy.

A peptide pool composed by 32 CD8 immunodominant restricted epitopes derived from Influenza, Cytomegalo and Epstein-Barr viruses (FEC pool) was used as HIV-1-unrelated positive control for monitoring the level of T-cell responses.

The Fec pool is described in http://www.nibsc.ac.uk/spotlight/aidsreagent/index.html under the reference number ARP7099 at page 135 .

Ex vivo ELISPOT assay for single–cell IFN-γ release

Peripheral blood mononuclear cells (PBMC) were seeded in duplicate at $2X10^5$ cells/well in 96-well plates (MAIPS4510; Millipore, Bedford, Mass.) precoated with anti-IFN-γ capture monoclonal antibody (B-B1; Diaclone, Besançon, France) and stimulated with the different peptide pools at the fixed concentration of 3 μg/ml of each peptide for 18 h at 37°C in air plus 5% CO2. Biotinylated anti-IFN-γ detection monoclonal antibody (B-G1; Diaclone) was added for 2 h, followed by the addition of streptavidin-alkaline phosphatase conjugate (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) for 1 h. A chromogenic substrate (nitroblue tetrazolium-BCIP [5-bromo-4-chloro-3-indolylphosphate]; Sigma, St. Louis, Mo.) was added for 10 min. The individual spots were counted by using an Automated ELISA-Spot

Assay Video Analysis System, Eli-Scan with the software Eli.Analyse V4.2 (A.EL.VIS, Hannover, Germany).

The responses were empirically scored as positive if the test wells contained a mean number of spot-forming cells (SFC) higher than the mean value plus two standard deviations in negative control wells, and if the number of SFC per million PBMC in stimulated wells (subtracted of the values of negative control wells) was ≥50. PBMC in medium alone were used to establish the negative control background, that had to be not higher than 50 SFC per million of cells (10 SFC/ well). PBMC stimulated with phytohemagglutinin (PHA-P; Sigma) at 5 mg/ml or the FEC peptide pool containing 1,5 μg/ml of each peptide were used as positive controls.

Intracellular cytokine staining

After thawing, PBMC were washed trice at room temperature in complete cell culture medium and plated in round bottom 96 wells/plates. All the incubations were carried out in round bottom 96 wells/plates to allow high throughput processing of the probes. 10^6 PBMC/well were incubated with 1.3 μg/ml of antibody to CD28 and with 1.3 μg/ml of antibody to CD49d (Becton Dickinson, Heidelberg, Germany) together with defined peptide pools. For each individual, a sample without peptides was included to calculate the background staining. Following one-hour incubation, 10 μg/ml of Brefeldin A were added to the cell suspension and the incubation carried out for additional 4 hours. Stimulated cells were then resuspended in Stain Buffer (0,2% BSA, 0,09% Na Azide in DPBS; Becton Dickinson) and incubated with the photoreactive fluorescent label ethidium monoazide (EMA; Molecular Probes, Leiden, The Netherlands) used as viability dye. After washing, cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ Kit, (Becton Dickinson). Then, the following fluorochrome-conjugated antibodies were added to the cell suspension: CD3-AmCyan, CD4-PerCP, CD45RA-PECy7, CD154-FITC, IFNγ-Al700, IL2-APC and MIP1β-PE from Becton Dickinson and CD8-PacB from DAKO (Hamburg, Germany). Incubation was carried out on ice for 30 min and after washing, cells were acquired using an LSRII flow cytometer (Becton Dickinson) equipped with a high throughput system. Sample analysis was performed using FlowJo version 8.5.3. Lymphocytes were gated on a forward scatter area versus side scatter area pseudo-colour dot plot and dead cells removed according to EMA staining. Events were gated on CD3+ events versus IFN-γ, IL-2, MIP-1β and CD154 to account for down-regulation. Then, CD3+ events were combined together using the Boolean operator "Or". The same procedure was used to successively gate CD8+ events. CD4+ events were excluded before creating a gate for each function or phenotype as shown in Figure 7.

Then, the authors created a series of Boolean gates that represent all the combination of the gate shown in Figure 7.

Data analysis

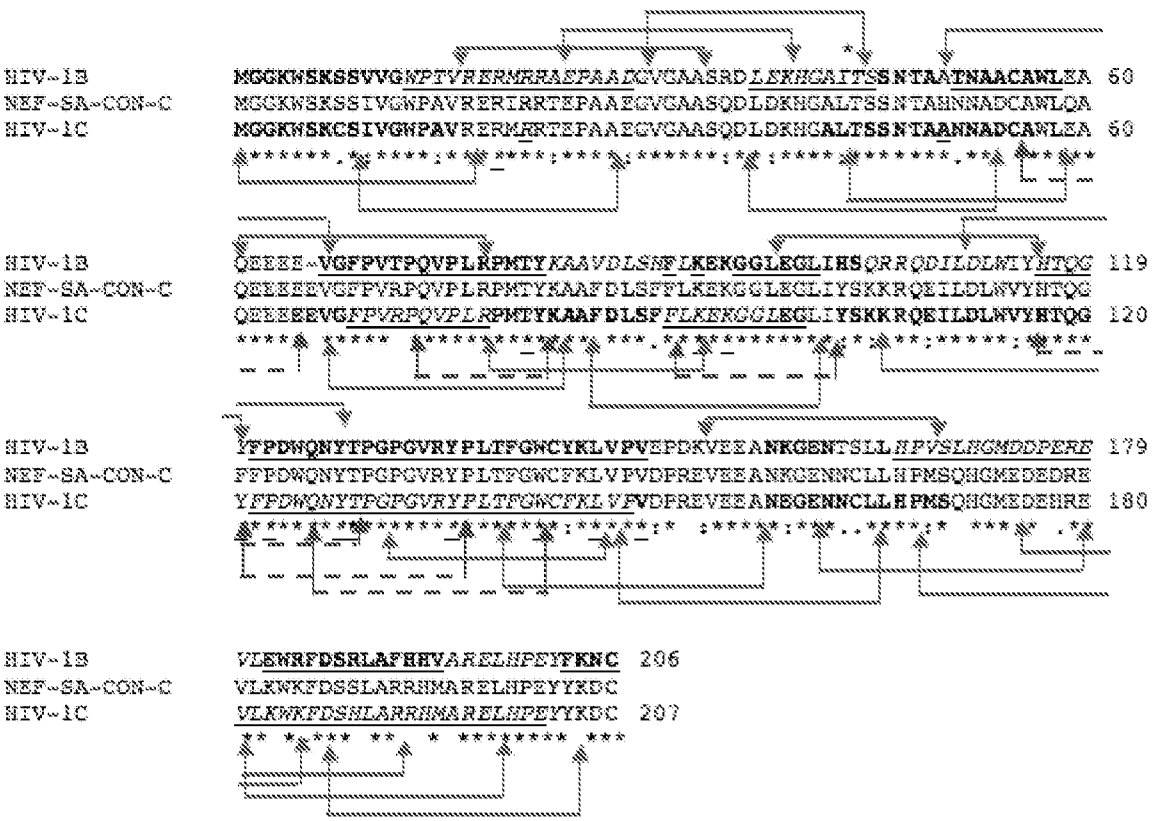
Since background levels varied between subpopulations, i.e. CD154 staining showed a higher background than IFN-γ and the combination of three or more functions had extremely low background, the authors calculated a threshold level for each subpopulation. Following background subtraction, the threshold level was calculated using the 90 percentile of the negative values. Then all the resulting negative values were set to zero and all values > 0 were considered as positive responses. SPICE version 4.1.5 was used for threshold calculation and graphical representation of the data. Statistical analysis was performed using SAS Version 9.1 and GraphPad Prism version 5.01. To account for multiple comparisons, the authors performed the Kruskal Wallis test before pair-wise comparisons by the Wilcoxon test. Statistical comparisons were considered moderately significant with a p value < 0.05 (*) significant with a p value <0.01 (**) and highly significant with a p value < 0.001 (***).

RESULTS

Peptide design

The new approach of peptide design combines the empirical-based approach with database information such as consensus antigen sequences belonging to different HIV-1 clades, immunodominat antigenic region and optimal epitope so far described.

As a first step the authors have aligned the protein sequence of candidate Tat (derived from the BH-10 strain belonging to clade B viruses) and Nef vaccine antigens (derived from the Bru strain belonging to clade B viruses) with the consensus clade C sequences of the homologous antigen, as presented below (Scheme 1, HIV-1B: SEQ ID No. 124, NEF-SA-CON-C: SEQ ID No. 125, HIV-1C: SEQ ID No. 126).



Scheme 1. Peptide scanning strategy of the HIV-1 encoded Nef antigen
Dotted arrows: peptides that share the same sequences between the Bru and the C consensus. Solid arrow: peptides that differ for one or more aa. Bold: Immunodominant regions; Bold underlined: MHC A restricted epitopes; Italics underlined: MHC B restricted epitopes; Italics: MHC C restricted epitopes

Secondly, the authors have mapped within these sequences all the reported immuno-dominant regions and optimal epitopes so far described. Thirdly, they have individuated conserved regions longer than 10 aa residues that were covered by a specific set of peptides (common peptides), completing the overlapping peptide scanning of both antigens with a set of peptides which contain all the protein fragment that differ for one or more amino acidic residues between the vaccine candidate and the consensus sequences. All the derived peptides were biased at the C-terminus avoiding seven amino acidic residues that were never found among optimal CTL epitopes and class I binding motifs (Asn, Asp, Gln, Glu, Gly, His, Ser). Moreover, the overlapping peptides were design placing the known optimal epitopes right in their N- or C-terminus of the longer scanning peptides.

In order to fit all of the above-mentioned design features, the derived peptides had variable length (from 10 to 19 aa residues) and degree of overlap (with a gap not larger than 8 aa).

Moreover, degree of overlap and overall peptide length were tuned accordingly to the frequency of known T-cell epitopes (higher overlap and shorter peptide length in epitope-rich regions and, conversely, wider not-overlapping fragment and maximal peptide length in epitope- poor regions). In addition, when multiple epitopes partially overlapped, the length of the peptide was chosen in order to fit either the higher number of epitopes or the epitope described for the most frequent Class I HLA allele. Moreover, we also considered, in the case of multiple partially overlapping epitopes, placing the ones that do not fit properly in the C-terminus of a given scanning peptides at the N-terminus of a different scanning peptide. Therefore, since variability in peptide length and degree of overlap is a distinctive novel feature of this peptide design strategy, the the newly generated HIV-1 derived peptide sets were denominated Tat VOPSD, Nef N-ter VOPSD, Nef C-ter VOPSD, TatC VOPSD, NefC N-ter VOPSD, NefC C-ter VOPSD.

Assessment of background responses on HIV-seronegative individuals

A cohort of 75 HIV-seronegative individuals with different ethnical and geographical origin was employed to measure the level of background T-cell responses generated by each peptide set. As shown in Table II, the IFN-γ elispot assay used showed an extremely low level of background signal (median 5 SFU/1,000,000 cells; 1-3 quartile: 2,5 and 10 SFU/1,000,000 cells) falling well below the maximum accepted threshold for negative controls (<50 SFU/1,000,000 cells; <10 SFU/well).

Table II. Background T-cell responses against HIV-1 peptide pools in HIV-seronegative HBD

	TCM	Tat 15mer	Tat VOPSD	Tat 20mers	Nef N-ter VOPSD	Nef C-ter VOPSD	Nef N-ter 20mers	Nef C-ter 20mers
Min Value	0	0	0	0	0	0	0	0
Max Value	25	47	51	60	55	78	48	42
Median	5	8	5	8	10	10	4	5
1° Quartile	2,5	4	2	2	3	3	2	2
3° Quartile	10,0	12	13	13	17	18	11	12
Mean	7	10	9	10	13	14	6	8
SD	6	11	11	11	13	15	9	8
Mean + 2SD	19	31	31	32	39	43	27	24

All the Tat specific peptide pools showed a negligible background of cross-reactive responses with both median and mean values not significantly different from the ones measured on negative controls (Table II). VOPSD Nef-specific peptide sets showed a higher background as compared to their homolog 20mers peptide sets or the negative control. However, also in this case both median, mean and mean + 2 deviation standard values of SFU were well below the accepted threshold for negative controls.

Comparison between distinct sets of Tat specific overlapping peptides for the detection of IFN-gamma secreting cells in HIV-infected individuals

Next, the authors evaluated in a cohorts of 76 HIV-infected patients heterogeneous for CD4 T cell counts, HIV-1–load, time and route of infection, the number of Tat specific T-cell responses detected by each set of overlapping peptides. Fifteen out of the seventy-six individuals recognized Tat specific peptides pools (20%). Interestingly, 13/15 responses were detected using the new peptide VOPSD set, whereas only 4/15 and 6/15 of the total responses were detected using the peptide pools composed by classical Tat 15mers and Tat 20mers peptide pools respectively (Figure 1 A-B). Moreover, this peptide set revealed 3/4 and 5/6 responses detected with the other two pools respectively, and in 2/3 and in 3/5 cases these responses were significantly higher when the VOPSD pool was used (Figure 1A-B).

Comparison between distinct sets of Nef specific overlapping peptides for the detection of IFN-gamma secreting cells in HIV-infected individuals

The same cohort of HIV-seropositive individuals was investigated to directly compare the ability of Nef specific VOPSD set against a peptide set composed by 20mers with a fix overlap of 10aa in detecting Nef-specific responses. Two pools for each peptide set were prepared: the first two covered the N-terminus and first half of the protein (aa 1-101 for the Nef N-ter VOPSD set and aa 1-110 for the Nef N-ter 20mers peptide set) whereas the other two covered the C-terminus and the other half of the protein (aa 96- 205 and aa 100-205 for Nef C-ter VOPSD and Nef C-ter 20mers, respectively).

In total 95 Nef specific T-cell responses were measured in 59/76 (78%) patients using the two VOPSD pools whereas only 81 responses in 51/76 (67%) patients were detected using the 20mers pools (Figure 2A-B). Interestingly, almost all the responses detected with the 20mers pools (80/81) were shared with the VOPSD pools. Overall, the analysis of the responses shared between the two sets of N-terminus (Figure 2A) and C-terminus pools (Figure 2B) showed that the vast majority of responses (66/80) were comparable, although in a significant fraction of cases (15/80) the SFU detected with the new peptide set were higher than the ones measured with the 20mer set (≥100% increase in the total spot number).

Detection of CD8 and CD4 T-cell responses using the VOPSD Nef peptides in HIV-infected individuals

Since the Elispot approach used (IFN-γ secretion of total PBMC) do not permit to recognize the phenotype of the responding T cell, the authors extended the analysis to a smaller independent cohort of HIV-infected patients (14 individuals), using a multiparameter cytofluorimetric approach that allows combining the recognition of the T cell phenotype

(CD4 or CD8) with the functional analysis (IFN-γ secretion) of the Nef specific T-cells. In this way, the authors could establish whether the higher number of individual responses measured with the new set of overlapping peptides was restricted to the CD8 compartment or interested also CD4 T-cell recognition. As shown in Figure 3, the majority of the HIV-infected patients recognized both Nef-specific peptide pools. As expected, since VOPSD has been particularly studied for the detection of MHC class I epitopes, CD8 responses measured by the new peptide set were brighter than the responses obtained with the classical 20mers pools (Figure 3A-B). Interestingly, when the CD4 T cell were considered, the new peptide set detected a higher number of responses of the pools prepared with 20mers overlapping peptides (8 and 4, respectively), a system that is considered more suited for monitoring CD4 T-cell responses.

Higher efficiency of T-cell epitope recognition is ensured by VOPSD peptide pools

Next, the authors investigated whether the small differences in molar concentration due to the different peptide length and peptide composition of the Tat Specific (1.4μM for the VOPS pool and 1.2μM for the 20mers) and Nef-specific peptide sets (1.5 μM and 1.2 μM for the N and C-terminus VOPS pools respectively, and 1.2 μM and 1.1 μM for the homolog 20mers pools respectively) were responsible for the observed differences in HIV-encoded antigens recognition. In this regard, two patients (patient HSR-006 and 018) showing a clear cut positive recognition of the sole VOPSD Tat or Nef pools when tested at the fixed single dose concentration in IFN-γ Elispot experiment (3μg/ml for each peptide) were selected for peptide-pool titration experiments. As shown, (Figure 4A) PBMC derived from HSR-006 recognized VOPS Tat peptide pool at concentration as low as 0,5 μM. On the contrary, the homolog 20mer pool was not yet detected at concentration eight times higher. Analogously, PBMC from patients HSR-018 showed an increasing ability to recognize the VOPSD N-terminus Nef peptide pool starting from the concentration of 2μM, but failed to recognize the 20mer Nef pool at the higher concentration tested.

The authors further tested the efficacy of the VOPSD peptide design at the level of recognition of individual peptide (Figure 5) as well as of a defined minimal T-cell epitope (Figure 6A,B).

PBMC derived from patient HSR-028 that in a previous experiment were shown to recognize only peptide n°1 of the VOPSD Tat pool, were tested against increasing concentrations of VOPSD Tat peptide 1 and its 20mer homolog. Maximal recognition of VOPSD peptide 1 was reached at peptide concentrations as low as 0,5 μM whereas its homolog was recognized only at the highest concentration tested (Figure 5).

To test the efficacy of VOPSD peptides in presenting T-cell minimal epitopes, PBMC obtained from patient V4, a subject whose Nef-specific T-cell responses were previously characterized (8), were tested against all the peptides belonging either to the VOPSD or the 20mer sets of Nef-specific peptides set containing the epitope over a range of different concentrations. Subject V4 demonstrated a strong CD8 T-cell response to the B8 restricted FLKEKGGL epitope (Figure 6A). Detection of the MHC class I minimal epitope FLKEKGGL was drastically improved by the new peptide design strategy as demonstrated by the marked reduction of peptide needed for the detection of the positive response (from 2 to 0,25μM) and by the higher number of peptide specific CD8 T cell measured with the two peptides belonging to the VOPSD set (Figure 6B).

Nef and Tat specific CD8 T-cells in LTNP produce MIP-1β but not IFN-γ and have a CD45RA+ phenotype

HIV-1 establishes a persistent chronic infection in human beings and long term survival of HIV-1 infected patients requires the administration of a special cocktail of antiretroviral drugs termed highly active antiretroviral therapy (HAART). Administration of HAART does not clear HIV-1 that, even under the strong pressure of the drugs, continues to replicate. In fact, interruption of HAART leads to a rise of the viral load that reaches pre-treatment levels in the majority of the patients. An exception to this scenario is represented by a cohort of HIV-1 infected individuals, named long-term non progressor (LTNP) that is able to naturally control viral replication and maintain a competent immune system without the help of HAART. Understanding why LTNP patients maintain an effective control of viral replication is of extreme relevance to the design of future vaccines and therapies against HIV-1.

The capacity to detect the complete repertoire of T-cells specific for a certain antigen is an important aspect for characterizing the adaptative immune response. In fact, in HIV-1 infected individuals multiple epitope recognition is associated with low steady-state viremia (14). Furthermore, functional and phenotypic heterogeneity between T-cells specific to different epitopes have been observed in several infectious diseases, such as EBV (7), HCMV (25), and HIV-1 (12). As a consequence, the characterization of the immune responses via the analysis of few T-cell specificities may be misleading.

Given the superior ability of both Tat and Nef–specific VOPSD sets in detecting T-cell specific immune responses, the authors decided to combine the new peptide sets with a highly sophisticated polychromatic flow cytometry approach to analyze PBMC derived from HIV+ patients after stimulation with Nef and Tat peptide pools. They coupled the simultaneous detection of four functional markers, IFN-γ, IL-2, CD154 and MIP-1β with the measurement

of the expression of CD45RA, a marker of T-cells maturation (Figure 7). The authors analysed the functional and differentiation phenotype of Nef and Tat specific CD8 T-cells in LTNP, late progressor (LP), chronically HIV-1 infected (CHI) individual under HAART and chronically HIV-1 infected individuals undergoing one cycle of treatment interruption. The simultaneous analysis of the immune response in these four cohorts allowed a clear discrimination between phenotypes associated with long term viral control and phenotypes that are the consequence of the exposure to the antigen.

The authors detected CD8 T-cell responses specific to Nef in the majority of the individuals. As expected, they did not find CD8 T-cells expressing CD154 and this marker was excluded from the analysis. Overall, LTNP showed higher frequencies of Nef-specific CD8 T-cells than LP and CHI, but differences resulted to be not statistically significant (data not shown). Nef-specific responses were composed of MIP-1β+ or MIP-1β+ IFN-γ+ cells while frequencies of IL-2 producing cells were generally low. Of particular interest, statistical significant differences between frequencies of CD8 T-cell responses in LTNP, LP and CHI individuals were found in CD45RA+ IFN-γ- IL-2- MIP-1β+ and CD45RA+ IFN-γ+ IL-2- MIP-1β+ CD8 T-cells. The frequency of CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells was significantly higher in LTNP than CHI with an extremely low p value ($p<0.0001$, Fig. 8a). A more accurate evaluation of the quality of the Nef specific response was assessed calculating the contribution of each population to the total CD8 T-cell response (Fig. 8 d, e and f). This data representation allows for the evaluation of the quality of the response independently from the magnitude. Statistical significant differences were again observed in CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells. The proportion of responding CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells was significantly higher in LTNP than CHI ($p<0.0001$; Fig. 8d).

A close inspection to the individual data values revealed that CD45RA+ IFN-γ- IL-2- MIP-1β+ responding CD8 T-cells were almost exclusively present in LTNP. In fact, they were observed in 7 out of 9 LTNP, 1 out of 3 LP and they were completely undetectable in all 23 CHI (Fig. 8 a and d).

Terminally differentiated CD8 T-cells, defined as CD45RA+ CCR7- that are able to express IFN-γ, have been found in LTNP (1) and in early infections with future control of HIV-1 (21). As shown in Figure 8b and e, the authors confirmed these findings. A significant higher frequency of CD45RA+ IFN-γ+ IL-2- MIP-1β+ CD8 T-cells was observed in LTNP when compared to CHI. However, the difference was not significant when the quality of the immune response was taken in consideration and CD45RA+ IFN-γ+ IL-2- MIP-1β+ CD8 T-

cells were present in the three cohorts analyzed. Thus, they not constitute an exclusive marker for the LTNP status.

The frequency and proportion CD45RA- IFN-γ- IL-2- MIP-1β+ CD8 T-cell in LTNP was higher than CHI (Fig. 8c and f), nevertheless differences did not reach statistical significance. A close inspection of the percentage of the total response relative to each individual revealed a high variability between individuals belonging to the same cohort and high percentages of CD45RA- IFN-γ- IL-2- MIP-1β+ CD8 T-cell were equally observed in LTNP, LP and CHI (Fig. 8c and f).

Since IL-2 producing cells were rarely detected, the authors reanalyzed the data considering only the combination of expression of CD45RA, IFN-γ and MIP-1β in the responding CD8 T-cells. The frequency of CD45RA+ IFN-γ- MIP-1β+ CD8 T-cells was significantly higher in LTNP than in CHI (p=0.0001), Fig. 8g. In addition, the proportion of responding CD45RA+ IFN-γ- MIP-1β+ CD8 T-cells was also significantly higher in LTNP than in CHI (p=0.0001), Fig. 8g. This analysis indicates that IL-2 expression does not represent an essential marker to define CD45RA+ IFN-γ- MIP-1β+ CD8 T-cells in LTNP.

Blood samples derived from study subjects LP02, LTNP13, LTNP14, LTNP15 and LTNP16 were drawn again nine months later and CD45RA+ IFN-γ- MIP-1β+ responding CD8 T-cells were still present; thus indicating a stability of the phenotype over the time (data not shown).

Tat specific immune responses were analyzed and as expected, Tat specific immune responses were detected only in a minority of subjects, 5/9 LTNPs, 2/3 LPs and 7/23 CHI (Fig 9a) Interestingly, CD45RA+ IFN-γ- IL-2- MIP-1β+ responding CD8 T-cells were found only in two LTNP (Fig. 9b). Overall, these data strongly support the idea that CD45RA+ IFN-γ- MIP-1β+ responding CD8 T-cells represent a positive marker of correlation for the long-term capacity of HIV-1 infected individuals to control viral replication.

MIP-1β+ IFN-γ- IL-2- CD45RA+ CD8 T-cells are not induced by exposure to antigen load

Antigen level and persistence contribute to the determination of the function and phenotype of the responding T-cells (15). The authors' cohorts of LTNP and CHI individuals are both characterized by a prolonged exposure to HIV-1 antigens. However, the plasma viral load in LTNP was significantly higher than in CHI individuals (see Materials and Methods section). To investigate the role of the antigen level in the generation of the MIP-1β+ IFN-γ- IL-2- CD45RA+ CD8 T-cells, the authors analysed Nef and Tat specific CD8 T-cell responses after one cycle of treatment interruption in six CHI patients. Following the interruption of the treatment, the viral loads became detectable in all the patients within day 5 and 21. Therapy

was resumed with viral loads higher than 100,000 RNA copies/ml. In general, Nef specific CD8 T-cell response followed the peak of the viral load. A significant increase in the frequency of CD45RA+ IFN-γ+ IL-2- MIP-1β+ (p=0.0313) and CD45RA- IFN-γ+ IL-2- MIP-1β+ CD8 T-cells (p=0.0313) was observed, while CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells remained undetectable (Fig. 10). Interestingly, the quality of the CD8 immune response remained unchanged despite the significant increase observed in the magnitude of the response. Immune response to Tat resulted to be almost undetectable before and after the interruption of the therapy (data not shown). These data demonstrate that MIP-1β+ IFN-γ- IL-2- CD45RA+ CD8 T-cells are not directly induced by the exposure to the antigen.

VOPSD peptides enhance detection of CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells

The authors have shown that VOPSD peptides are superior in detecting CD8 and CD4 responses using an IFN-γ-based ELISPOT and an IFN-γ-based ICS. The combination of the use of the VOPSD peptides and of the immunocytometric assay lead to the identification of the CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cell population almost exclusively present in LTNP. The question arises whether the use of the VOPSD peptides increases the capacity to detect not only IFN-γ+ CD8 T-cells but also CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells. To this aim, the authors compared Nef responses after stimulation with the VOPSD, a pool of Nef derived optimal CD8 T-cell epitopes (ref. 8) and the homolog 20mer set of Nef peptides in a subject with a strong Nef specific response (subject V4). The optimal pool was a set of peptides representing 16 optimally defined Nef CD8 epitopes (ref. 8; WPTVRERM : SEQ ID No. 135; ALTSSNTAA : SEQ ID No. 136; FPVTPQVPLR : SEQ ID No. 137; FPVRPQVPL : SEQ ID No. 138; RPQVPLRPMTY : SEQ ID No. 139; QVPLRPMTYK : SEQ ID No. 140; VPLRPMTY : SEQ ID No. 141; RPMTYKAAL : SEQ ID No. 142; AVDLSHFLK : SEQ ID No. 143; FLKEKGGL : SEQ ID No. 144; RRQDILDLWI : SEQ ID No. 145; TPGPGVRYPL : SEQ ID No. 146; YPLTFGWCY : SEQ ID No. 147; PLTFGWCFKL : SEQ ID No. 148; LTFGWCFKL : SEQ ID No. 149; VLEWRFDSRL : SEQ ID No. 150).

As shown in Figure 11, higher frequencies of responses were detected using the VOPDS peptides. The authors detected 0.83% CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells using the VOPSD peptides, 0.55% CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells using the optimal pool and 0.38% CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells using the 20mer set of peptides.

These data indicate that VOPSD peptides increase the probability to detect CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells. Of note, CD45RA+ IFN-γ- IL-2- MIP-1β+ CD8 T-cells are also detected using the 20mer set (Fig. 11) and using the B8 restricted FLKEKGGL epitope .

Analysis of the Tat –specific T-cell response in Elite Controllers

We finally tested the T-cell response in a particular group of HIV-1 infected individuals that are able to naturally control HIV-1 replication (EC) below the thresholds of detection of the routinary test for the measurement of HIV-1 RNA plasma viremia. The frequency of individuals recognizing Tat is higher in EC (9/13 subject) than in all the other group of patients studied (3/6 of the LTNP, 2/5 of the CUS, 4/13 of the CHI w/o T and 3/15 of the CHI w T). In addition EC individuals show a significantly more intense response than CHI w T patients in which RNA viremia is suppressed by ART (Fig. 12) (EC median = 299, 1° and 3° quartile [IQR]=18-717; CHI w T median= 0, 1° and 3° IQR= 0-21; $p<0.01$) and recognize a significantly higher number of Tat encoded peptides (Fig. 13) (EC median=2, 1°-3° IQR= 0-2; CHI w T median =0, 1°-3° IQR= 0-1; p= 0.03). Interestingly, EC patients showed, in addition, a peculiar pattern of Tat specific T-cell response that was concentrated mainly on three distinct VOPSD peptides (peptides 2.2, 2.5 and 2.6 corresponding respectively to Seq ID 2, 5 and 6), two being rarely recognized by other HIV-1 infected individuals (peptide 2.5: 4/13 EC, 1/6 LTNP, 1/5 CUS and 1/15 CHI w T; peptide 2.6: 7/13 EC, 1/5 CUS, 1/13 CHI w/o T and 1/15 CHI w T) and one (peptide 2.2) being solely recognized by 5/9 EC individuals (Fig. 14). Similarly, authors found that, among the VOPSD peptides covering the Nef antigen, peptide 5.29 (corresponding to Seq ID 40) encoded an immunodominant response only for EC individuals (7/13 patients) and not for any of the all the other groups of HIV-1 infected individuals.

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CLAIMS

1. A set of overlapping peptides of an antigen, said antigen being a variable antigen, comprising :

a) a first sub-set of peptides longer than 10 amino acids consisting of conserved sequence regions between an amino acid sequence of a variant antigen and the consensus amino acid sequence of said variable antigen, and

b) a second sub-set of peptides, each peptide being between 10 and 19 amino acids long and containing an amino acid sequence of said variant antigen that differs for at least one amino acid residue from the consensus amino acid sequence of said variable antigen, and optionally one or more immunodominant region and/or a optimal epitope region or portions thereof at its N or C terminus, wherein each of said peptides does not have as the last C-terminal amino acid any of the following amino acids: Asn, Asp, Gln, Glu, Gly, His, Ser;

wherein each peptide of the first sub-set and of the second sub-set of peptides has a gap not longer than 8 amino acids with its closest overlapping peptide.

2. The set of overlapping peptides according to claim 1 wherein the antigen is an HIV antigen.

3. The set of overlapping peptides according to claim 2 wherein the HIV antigen is an HIV-1 antigen.

4. The set of overlapping peptides according to claim 3 wherein the HIV-1 antigen is the Tat antigen.

5. The set of overlapping peptides according to claim 4 consisting of peptides belonging to any of the following groups:

a) peptides of SEQ ID No. 1 to SEQ ID No. 11;

b) peptides of SEQ ID No. 42 to SEQ ID No. 51 and SEQ ID No. 7.

6. The set of overlapping peptides according to claim 5 comprising peptides of SEQ ID No. 2, 5 and 6.

7. The set of overlapping peptides according to claim 3 wherein the HIV-1 antigen is the Nef antigen.

8. The set of overlapping peptides according to claim 7 consisting of peptides belonging to any of the following groups:

a) peptides of SEQ ID No. 12 to SEQ ID No. 26;

b) peptides of SEQ ID No. 27 to SEQ ID No. 41;

c) peptides of SEQ ID No. 12 to SEQ ID No. 41;

d) peptides of SEQ ID No. 52 to SEQ ID No.63 and SEQ ID No. 20, SEQ ID No. 23 and SEQ ID No. 26;

e) peptides of SEQ ID No. 64 to SEQ ID No. 76 and SEQ ID No. 30, SEQ ID No. 31 and SEQ ID No.32;

f) peptides of SEQ ID No. 52 to SEQ ID No. 76 and SEQ ID No. 20, SEQ ID No. 23, SEQ ID No. 26, SEQ ID No. 30, SEQ ID No. 31 and SEQ ID No.32;

g) peptides of SEQ ID No. 77 to SEQ ID No. 86.

9. The set of overlapping peptides according to claim 8 comprising the peptide of SEQ ID No. 40.

10. The set of overlapping peptides according to any of previous claims for use as a medicament, a vaccine or a vaccine adjuvant.

11. Use of the set of overlapping peptides according to claims 1 to 9 for *ex vivo* monitoring CD4 and/or CD8 T cell responses in HIV patients.

12. Use of a set of overlapping peptides according to claims 6 and 9 for an *ex vivo* monitoring of T cell responses associated with the natural control of HIV-1 replication.

13. Use of the set of overlapping peptides according to claims 1 to 9 for identifying and/or selecting and/or monitoring a HIV-1 negative population for anti HIV-1 vaccine development.

14. Use of the set of overlapping peptides according to claims 1 to 9 for the detection, in a biological sample, of a population of T cells belonging to any of the following groups:

a) CD4 T cells;

b) CD8 T cells;

c) $CD45RA^{+}IFN\gamma^{-}$ $MIP1\beta^{+}$ CD8 T cells.

15. An isolated population of $CD45RA^{+}IFN\gamma^{-}$ $MIP1\beta^{+}$ CD8 T cells.

16. Use of a $CD45RA^{+}$ $IFN\gamma^{-}$ $MIP1\beta^{+}$ CD8 T cell population as a correlate of protection against HIV infection and/or HIV disease.

17. Use of a $CD45RA^{+}$ $IFN\gamma^{-}$ $MIP1\beta^{+}$ CD8 T cell population as immune therapy.

18. A kit to detect a $CD45RA^{+}$ $IFN\gamma^{-}$ $MIP1\beta^{+}$ CD8 T cell population in a biological sample comprising:

i) a set of peptides belonging to any of the following groups:

a) a set of overlapping peptides according to claims 1 to 9;

b) standard pools of overlapping peptides;

c) pools of optimal CD8 T-cell epitopes;

ii) a set of antibodies able to detect the following markers on CD8 T cells: $CD45RA^{+}$, $IFN\gamma^{-}$, and $MIP1\beta^{+}$.

19. A method for detecting a $CD45RA^{+}$ $IFN\gamma^{-}$ $MIP1\beta^{+}$ CD8 T cell population in a biological sample comprising:

i) incubating said sample with a set of peptides belonging to any of the following groups:

a) a set of overlapping peptides according to claims 1 to 9;

b) standard pools of overlapping peptides;

c) pools of optimal CD8 T-cell epitopes;

ii) staining said sample with a set of labelled antibodies able to detect the following markers on CD8 T cells: $CD45RA^{+}$, $IFN\gamma^{-}$, and $MIP1\beta^{+}$.

20. A method for the diagnostic of a long-term non-progressive (LTNP) status, or for monitoring the anti-HIV-specific protective CD8 T cell response, in a biological sample from a HIV-1 infected subject, comprising the steps of:

a) detecting the $CD45RA^{+}IFN\gamma^{-}$ $MIP1\beta^{+}$ CD8 T cell population according to claim 17, and

b) measuring the % thereof with respect to the total CD8 T cells or to the total responding CD8 T cells.

21. A method for measuring the efficacy of an HIV derived antigen vaccine in a biological sample from a vaccinated HIV-1 infected subject, comprising the steps of:

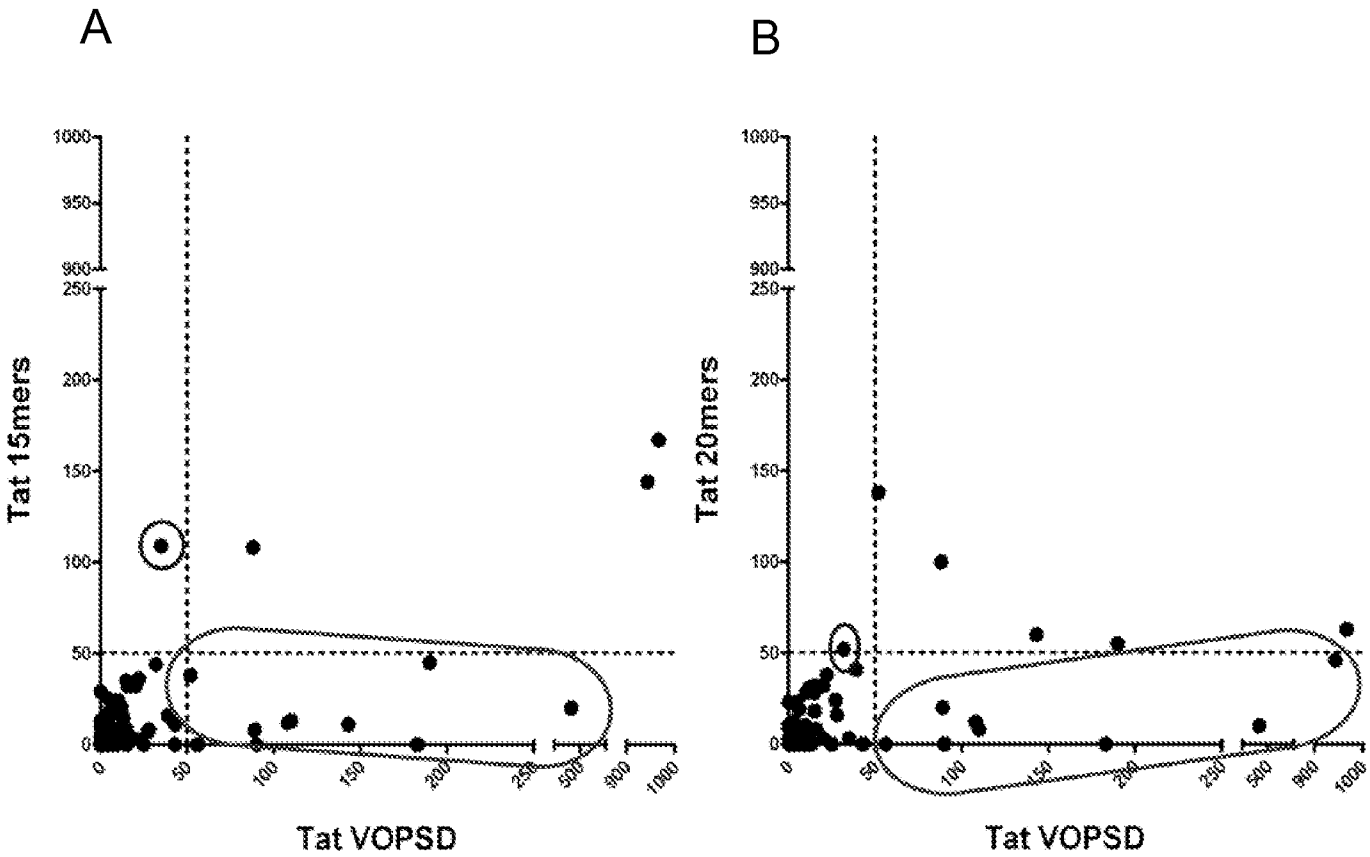
a) detecting the $CD45RA^{+}IFN\gamma^{-}$ $MIP1\beta^{+}$ CD8 T cell population according to claim 17, and

b) measuring the % thereof with respect to the total CD8 T cells or to the total responding CD8 T cells.

22. A method for monitoring the capacity of the immune system of an human to control HIV infection, comprising the steps of:

a) detecting the $CD45RA^{+}IFN\gamma^{-}$ $MIP1\beta^{+}$ CD8 T cell population according to claim 17 in a biological sample from a HIV-1 infected subject, and

b) measuring the % thereof with respect to the total CD8 T cells or to the total responding CD8 T cells.


A
B
Tat 15mers
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Tat VOPSD
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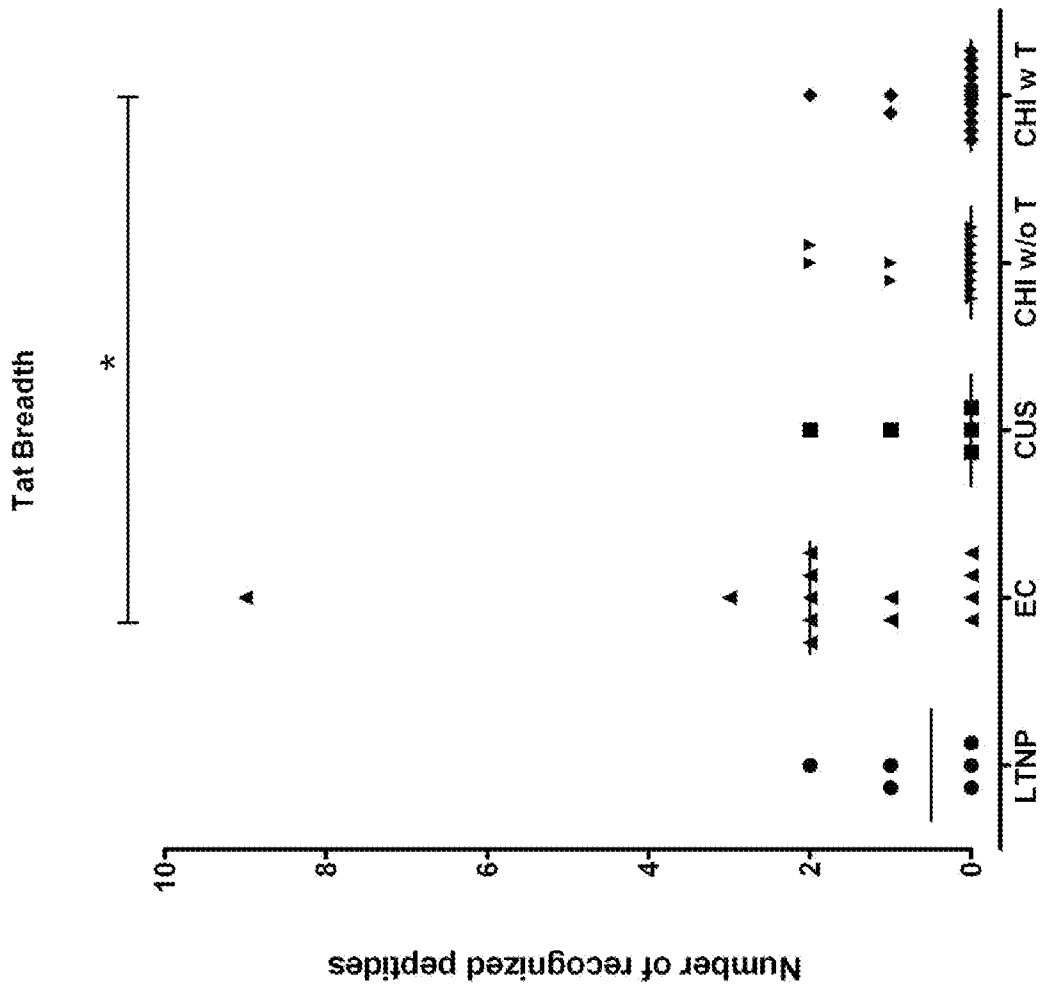
1000

Fig. 1

Fig. 13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Number of recognized peptides
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8
6
4
2
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LTNP
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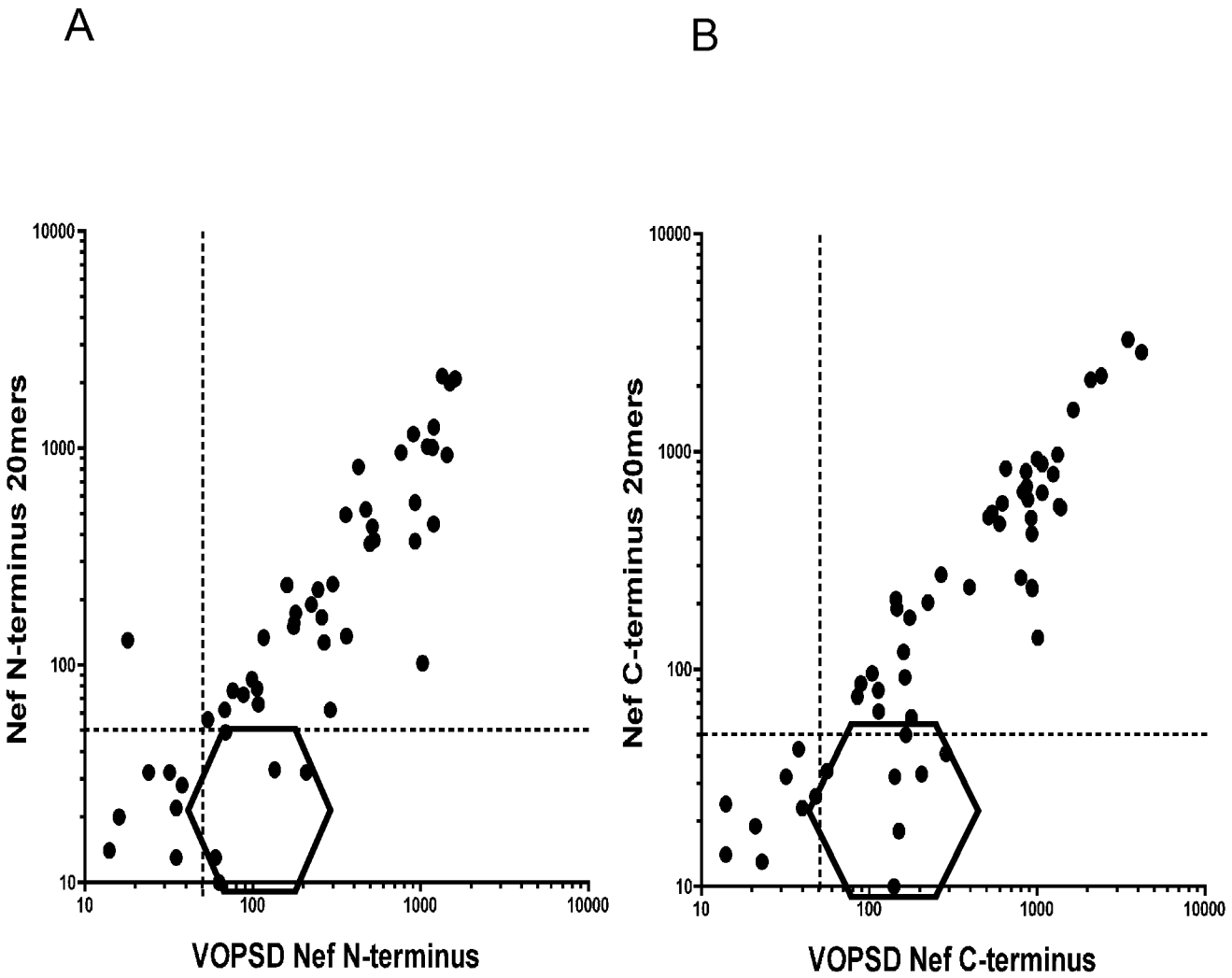
CHI w T


A
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Nef N-terminus 20mers
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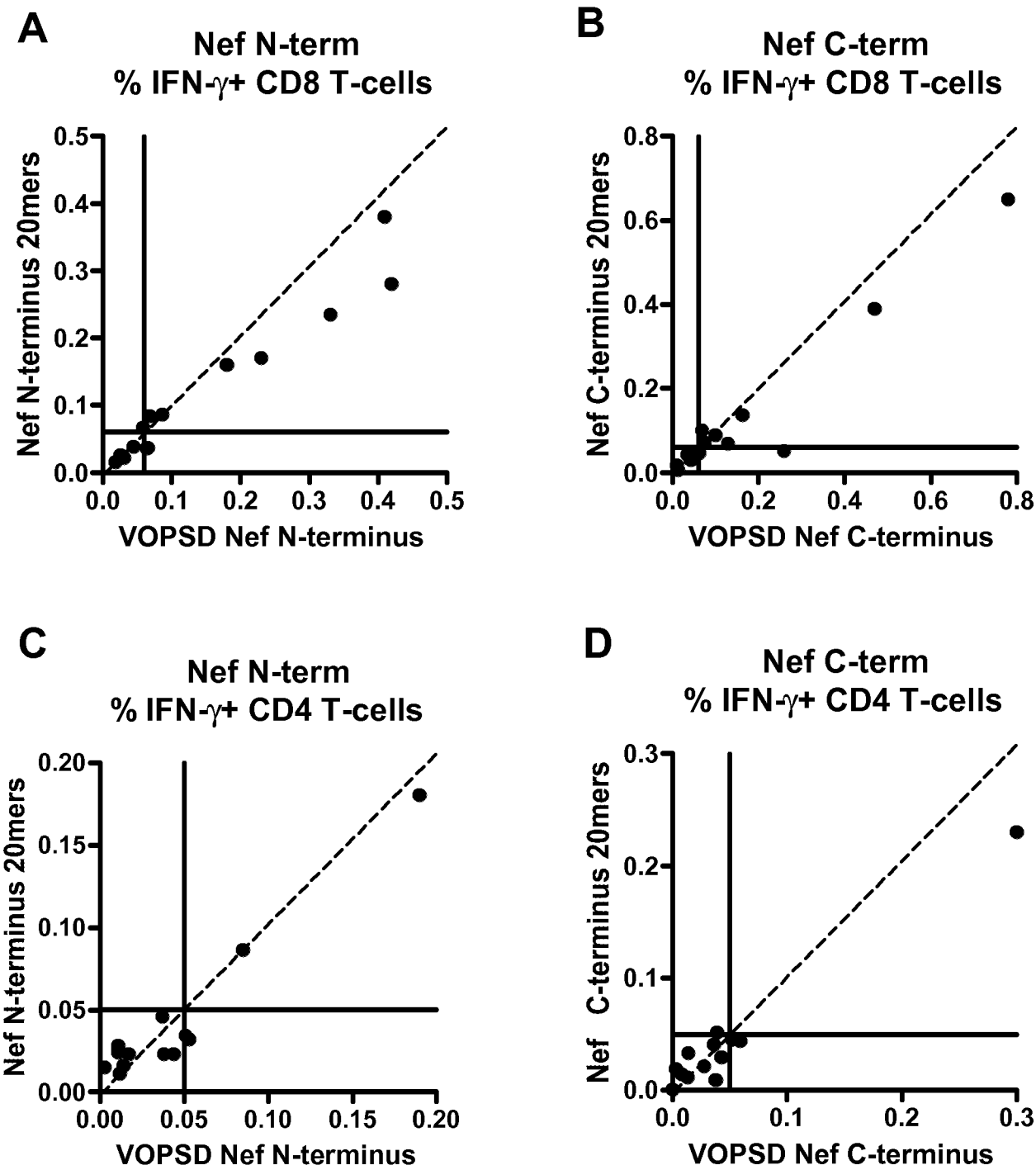
Fig. 2


A
Nef N-term
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Nef C-term
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VOPSD Nef C-terminus
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VOPSD Nef N-terminus
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Nef C-term
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Nef C-terminus 20mers
VOPSD Nef C-terminus

Fig. 3

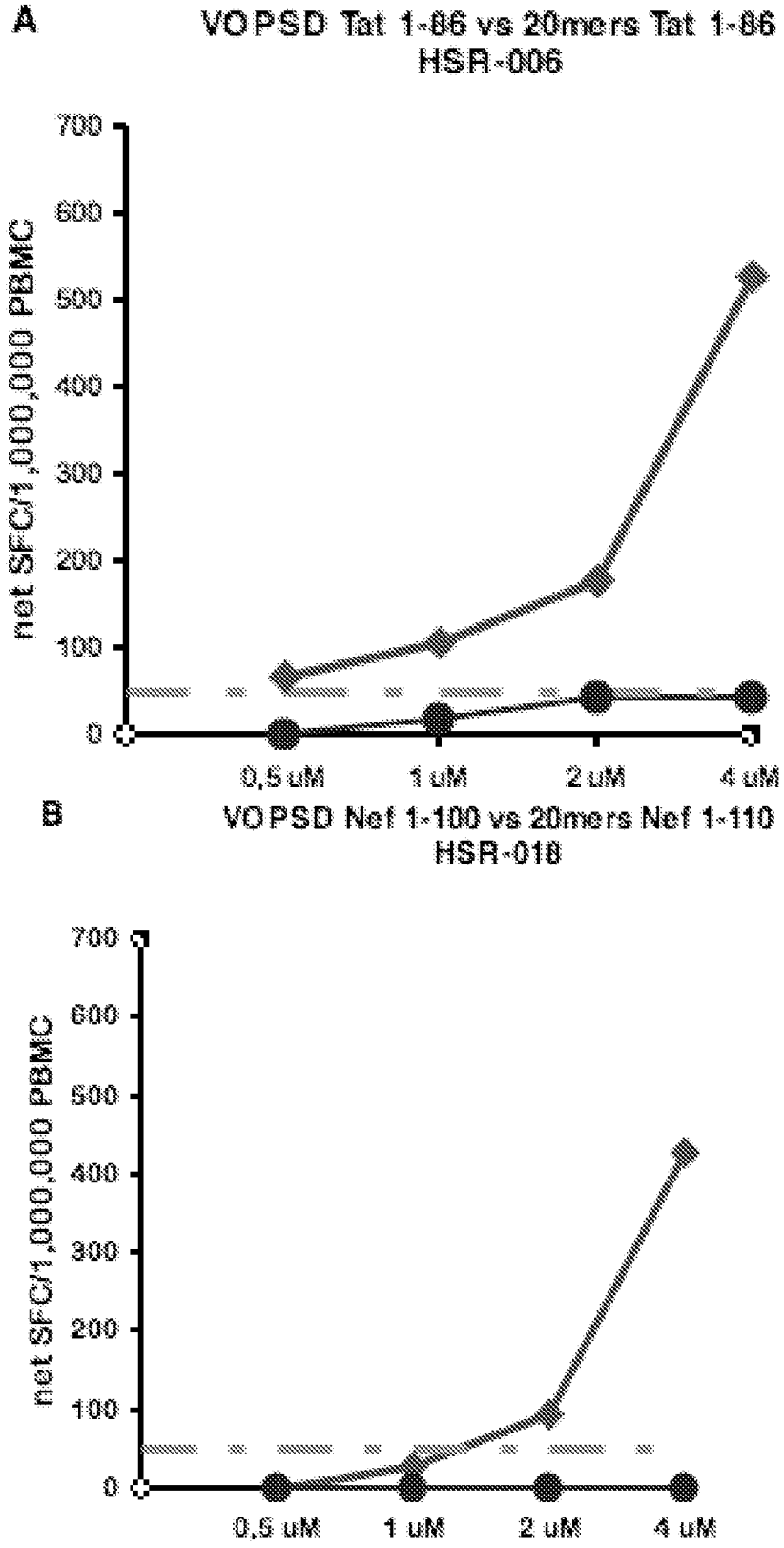

A
VOPSD Tat 1-86 vs 20mers Tat 1-86
HSR-006
net SFC/1,000,000 PBMC
700
600
500
400
300
200
100
0
0,5 uM
1 uM
2 uM
4 uM
B
VOPSD Nef 1-100 vs 20mers Nef 1-110
HSR-018
net SFC/1,000,000 PBMC
700
600
500
400
300
200
100
0
0,5 uM
1 uM
2 uM
4 uM
Peptide pools concentration

Fig. 4

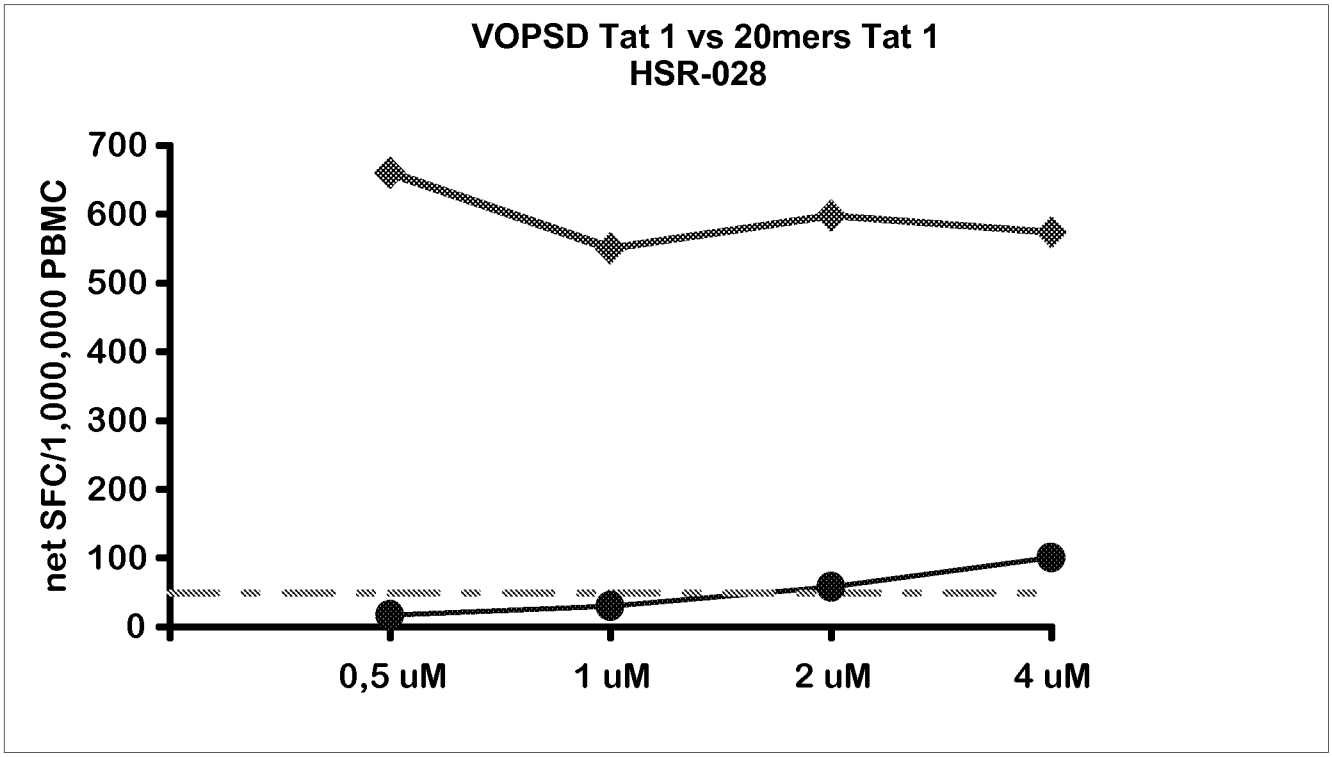

VOPSD Tat 1 vs 20mers Tat 1
HSR-028
net SFC/1,000,000 PBMC
700
600
500
400
300
200
100
0
0,5 uM
1 uM
2 uM
4 uM
Peptide concentration

Fig. 5

A

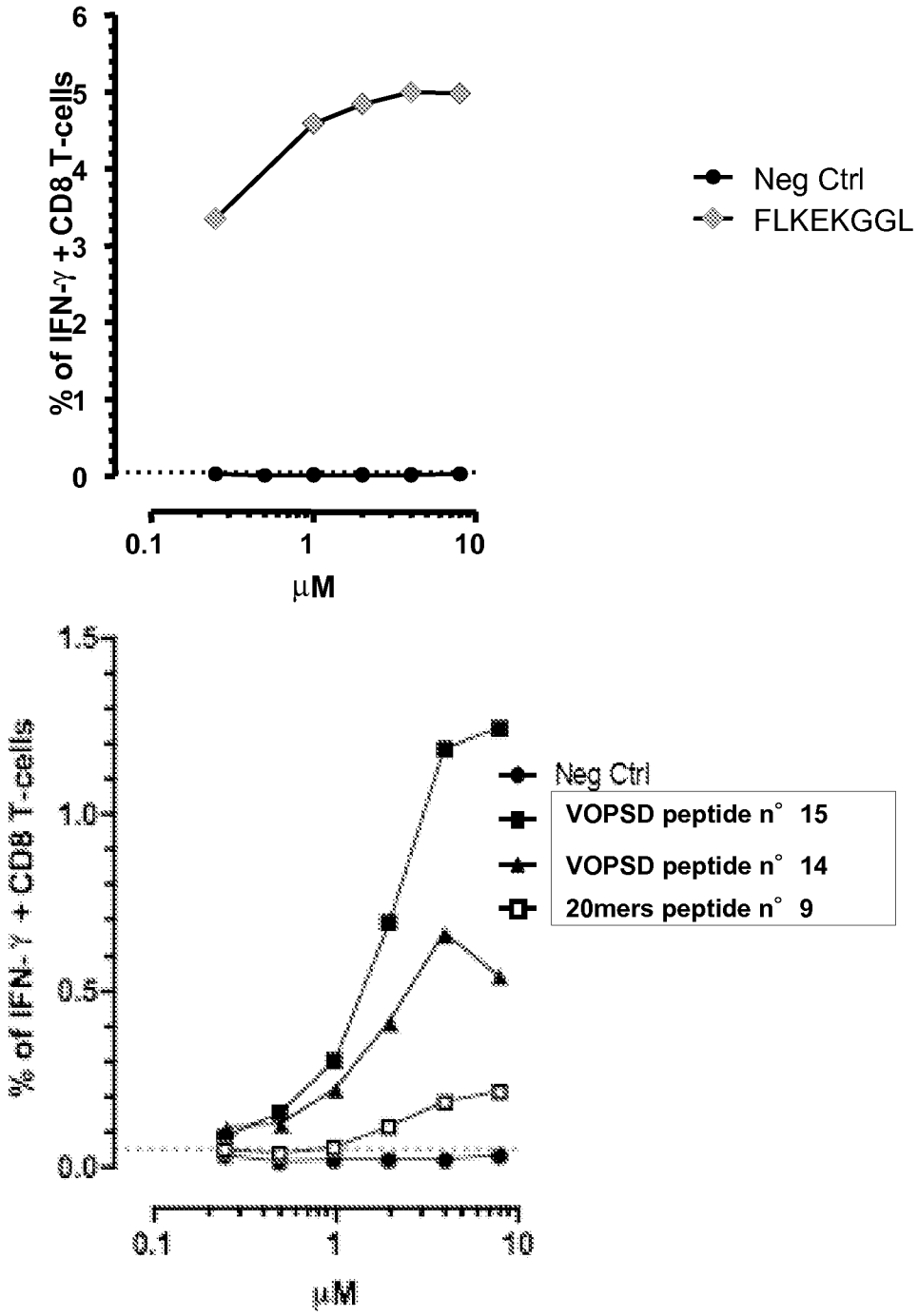

% of IFN-γ + CD8 T-cells
6
5
4
3
2
1
0
0.1
1
10
µM
Neg Ctrl
FLKEKGGL
% of IFN-γ + CD8 T-cells
1.5
1.0
0.5
0.0
0.1
1
10
µM
Neg Ctrl
VOPSD peptide n° 15
VOPSD peptide n° 14
20mers peptide n° 9

Fig. 6

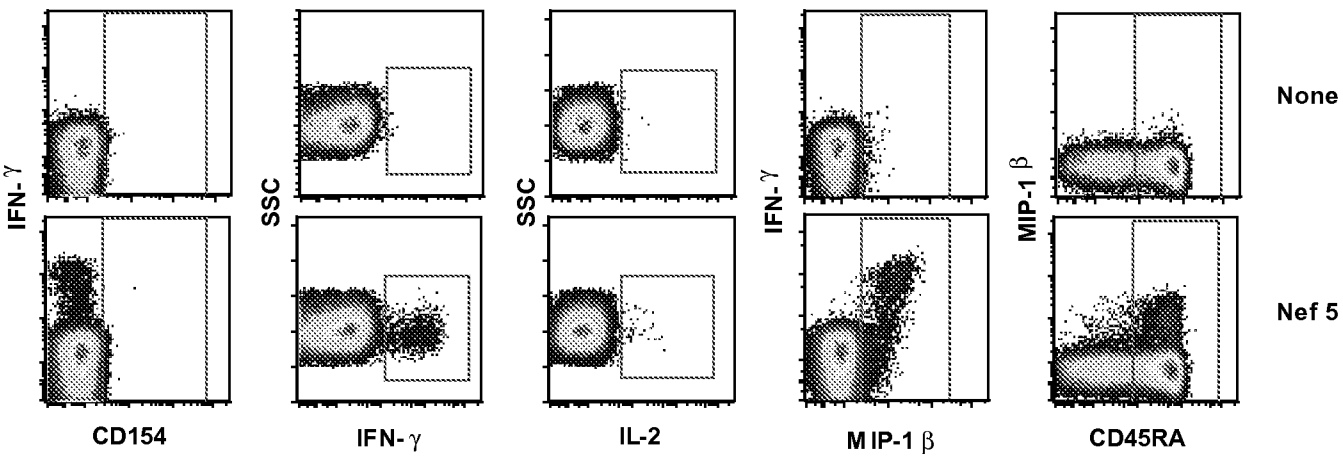

IFN-γ
SSC
SSC
IFN-γ
MIP-1β
None
Nef 5
CD154
IFN-γ
IL-2
MIP-1β
CD45RA

Fig. 7

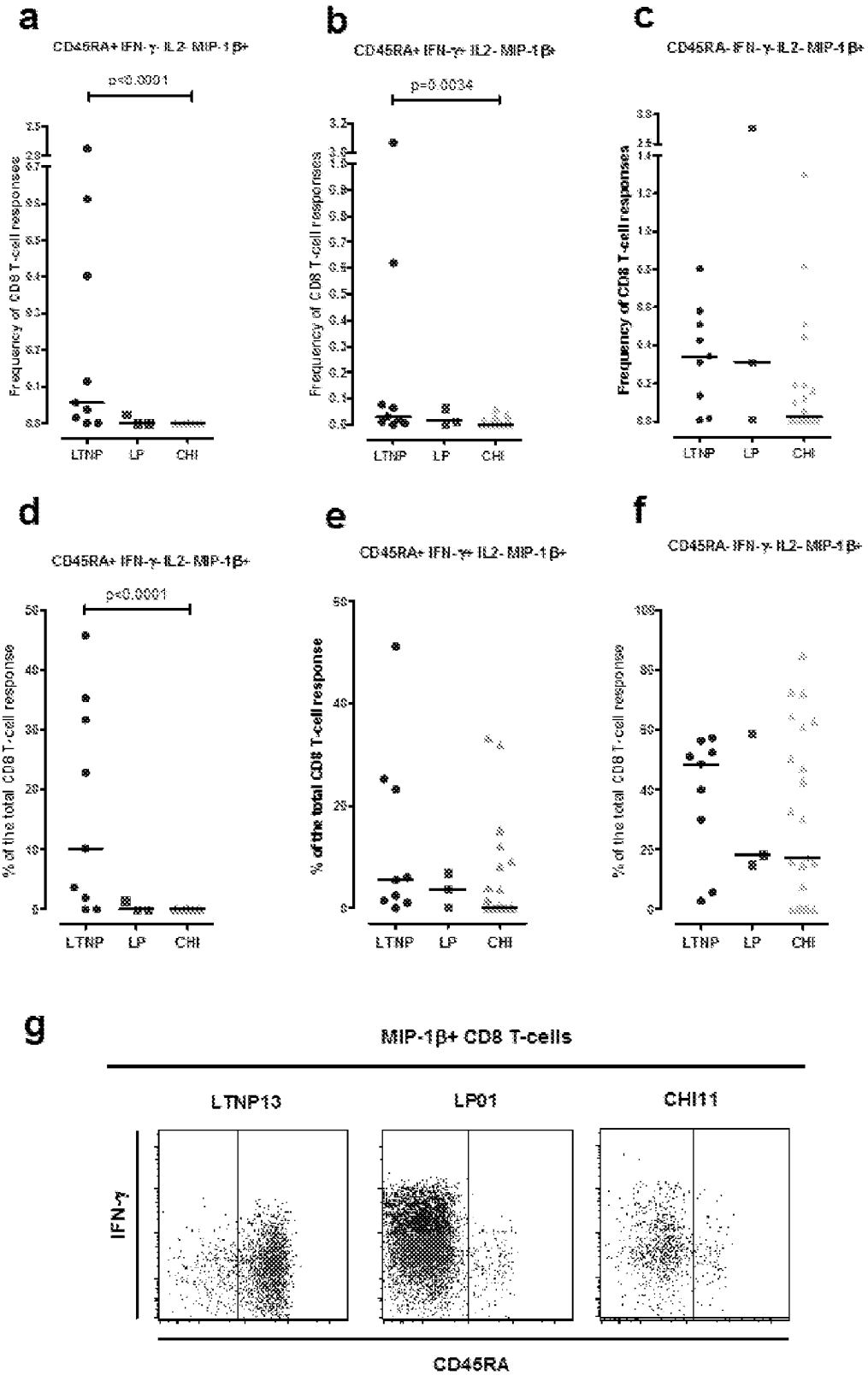

a
CD45RA+ IFN-γ- IL2- MIP-1β+
p<0.0001
Frequency of CD8 T-cell responses
LTNP
LP
CHI
b
CD45RA+ IFN-γ+ IL2- MIP-1β+
p=0.0034
Frequency of CD8 T-cell responses
LTNP
LP
CHI
c
CD45RA- IFN-γ- IL2- MIP-1β+
Frequency of CD8 T-cell responses
LTNP
LP
CHI
d
CD45RA+ IFN-γ- IL2- MIP-1β+
p<0.0001
% of the total CD8 T-cell response
LTNP
LP
CHI
e
CD45RA+ IFN-γ+ IL2- MIP-1β+
% of the total CD8 T-cell response
LTNP
LP
CHI
f
CD45RA- IFN-γ- IL2- MIP-1β+
% of the total CD8 T-cell response
LTNP
LP
CHI
g
MIP-1β+ CD8 T-cells
LTNP13
LP01
CHI11
IFN-γ
CD45RA

Fig. 8

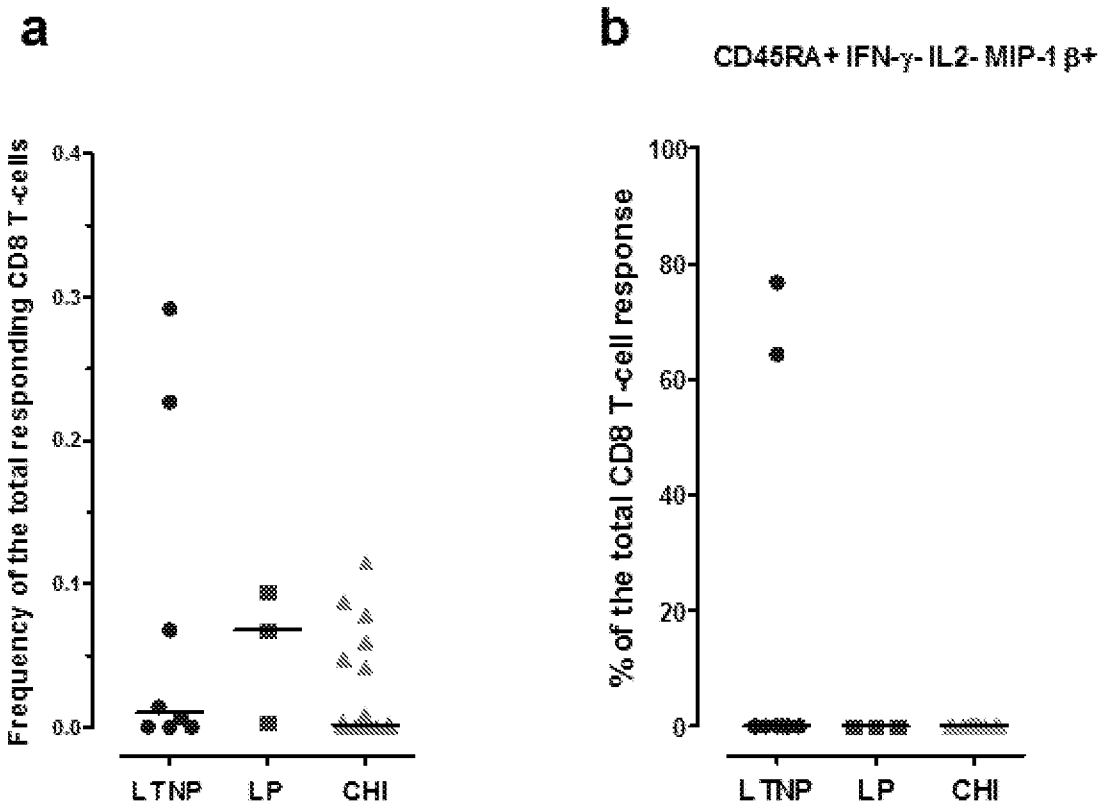

a
Frequency of the total responding CD8 T-cells
0.4
0.3
0.2
0.1
0.0
LTNP
LP
CHI
b
CD45RA+ IFN-γ- IL2- MIP-1 β+
% of the total CD8 T-cell response
100
80
60
40
20
0
LTNP
LP
CHI

Fig. 9

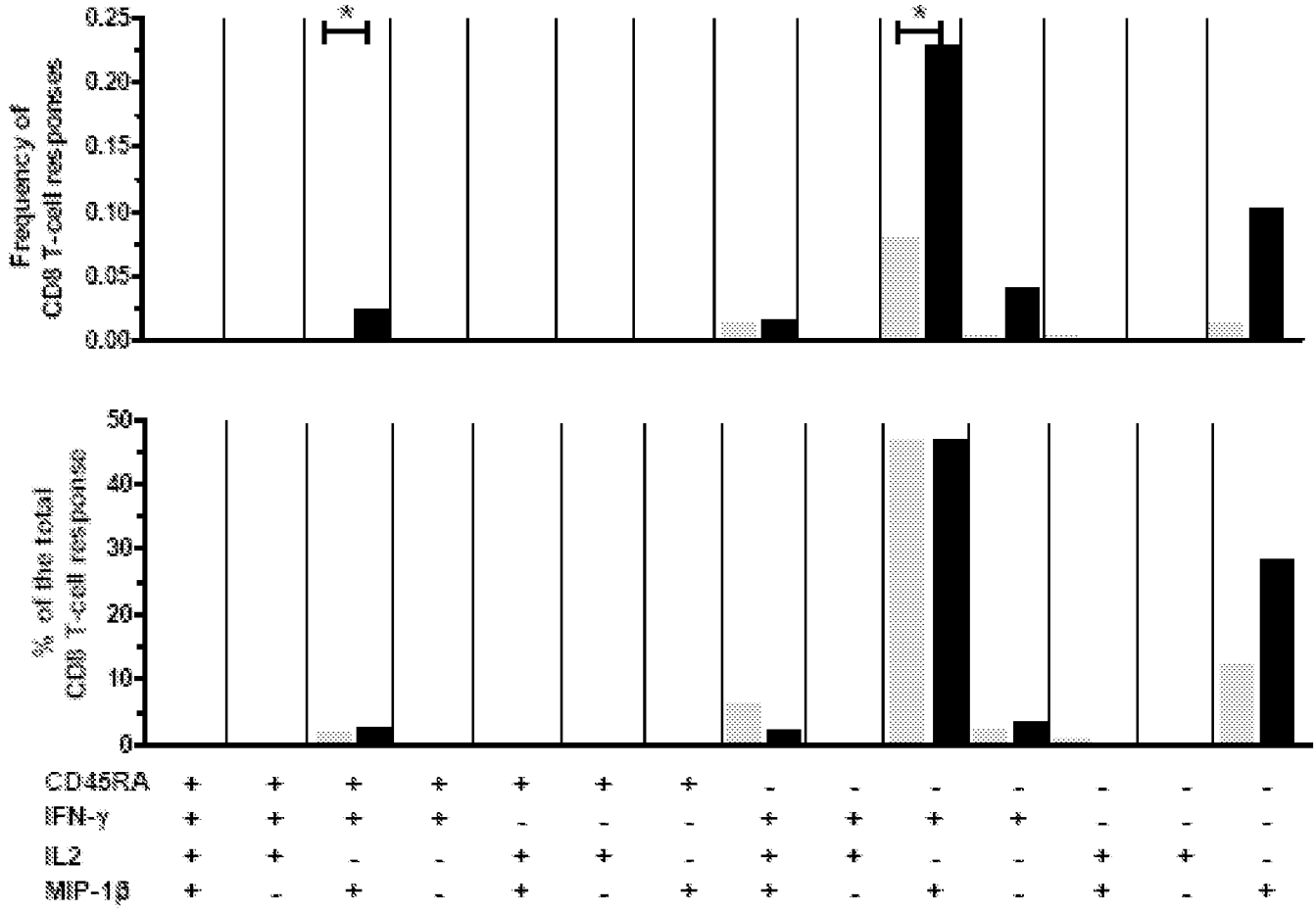

Frequency of CD8 T-cell responses
0.25
0.20
0.15
0.10
0.05
0.00
*
*
% of the total CD8 T-cell response
50
40
30
20
10
0
CD45RA + + + + + + + - - - - - - -
IFN-γ + + + + - - - + + + + - - -
IL2 + + - - + + - + + - - + + -
MIP-1β + - + - + - + + - + - + - +

Fig. 10

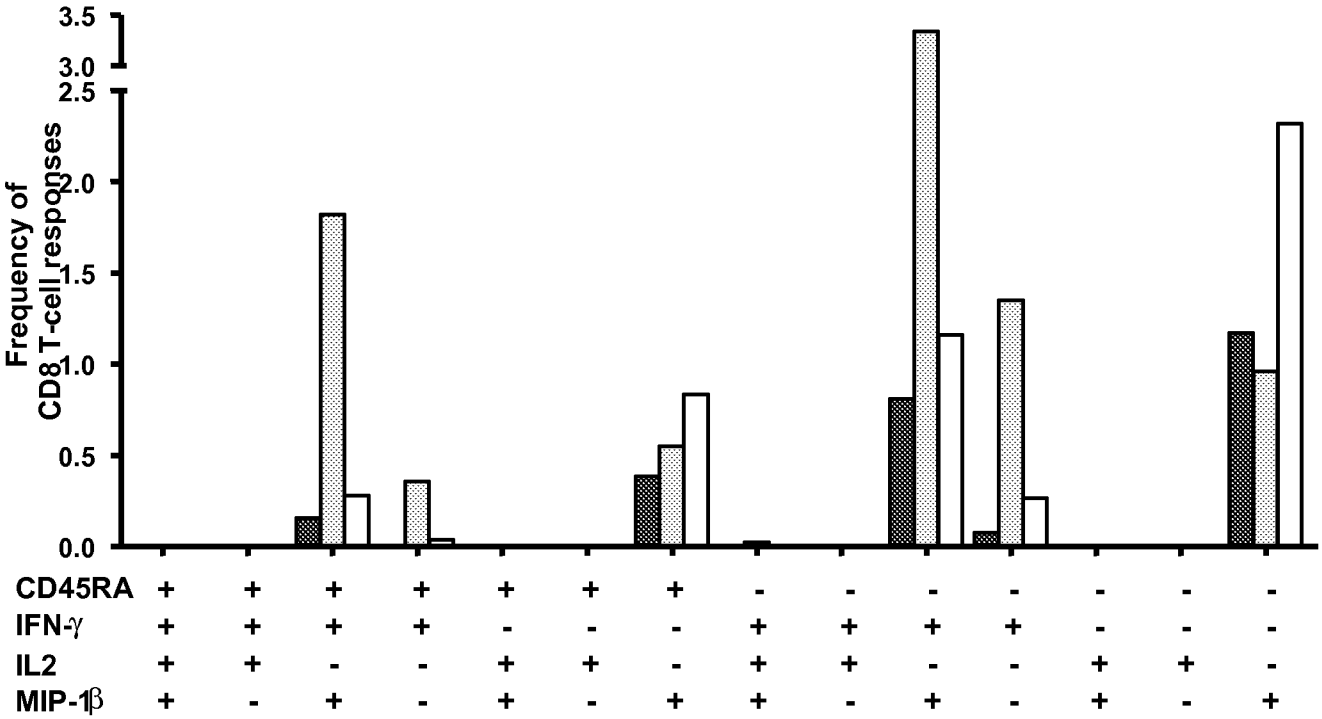

Frequency of CD8 T-cell responses
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CD45RA + + + + + + + - - - - - - -
IFN-γ + + + + - - - + + + + - - -
IL2 + + - - + + - + + - - + + -
MIP-1β + - + - + - + + - + - + - +

Fig. 11

Fig. 12

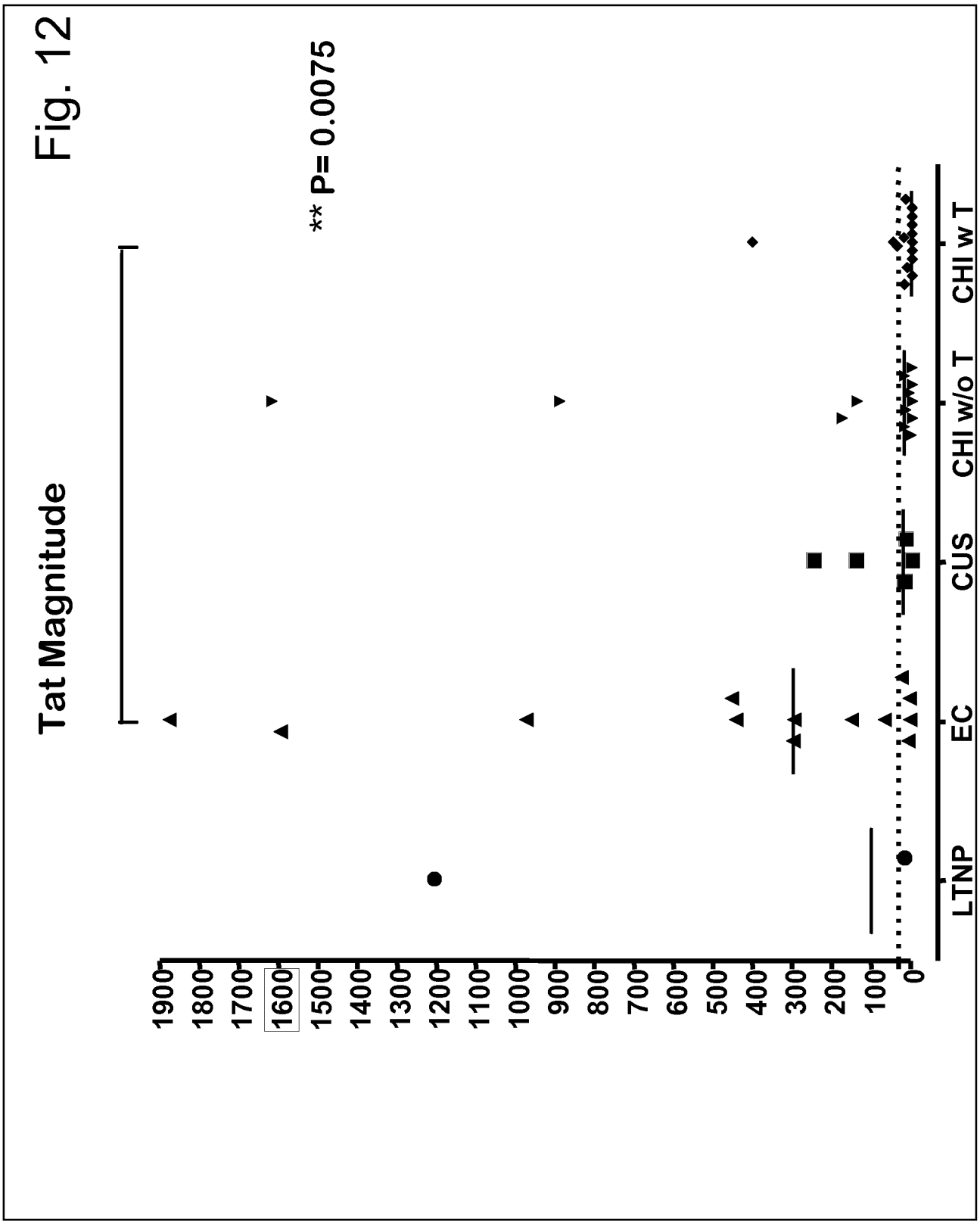
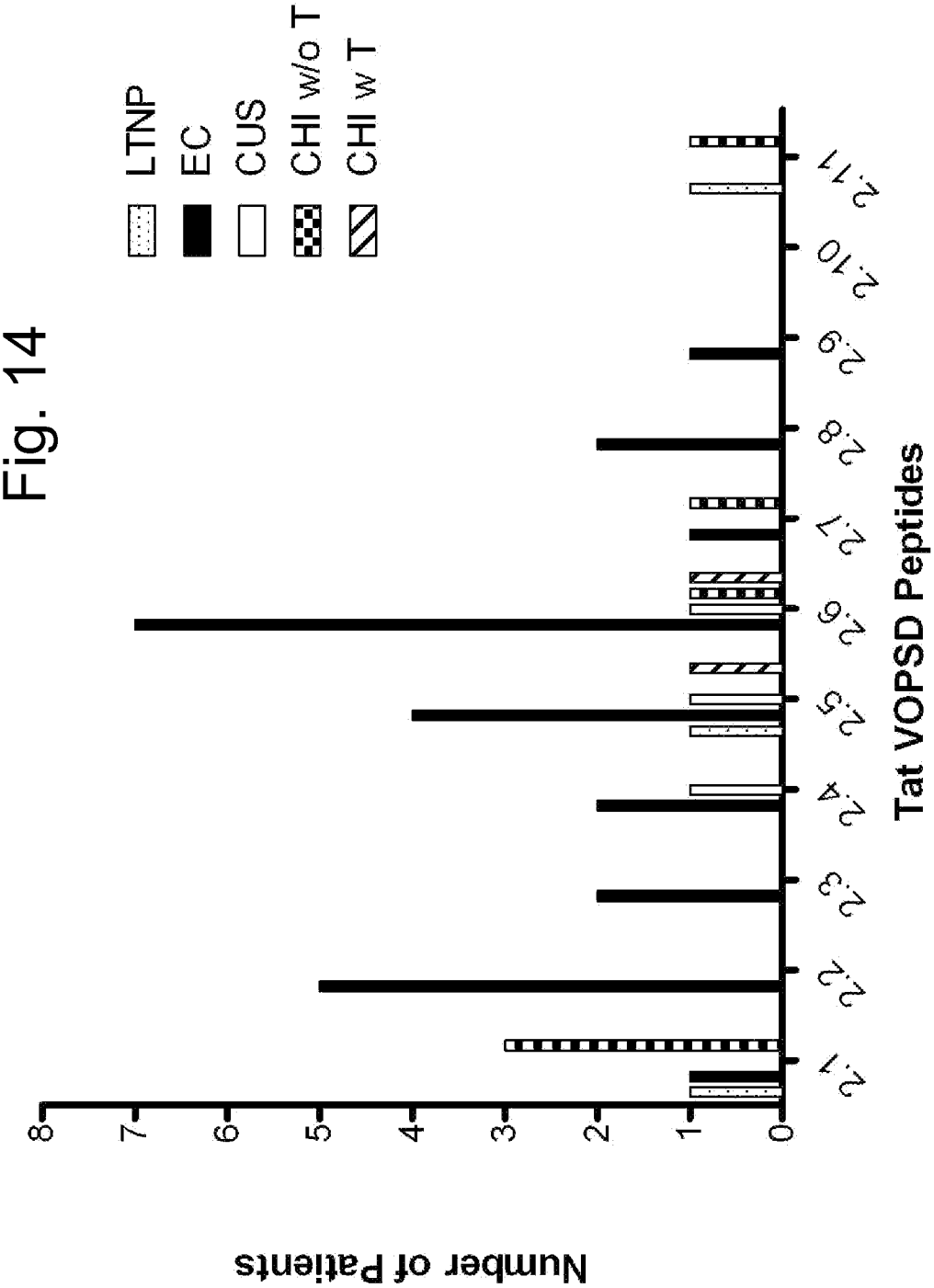

Tat Magnitude
** P= 0.0075
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
LTNP
EC
CUS
CHI w/o T
CHI w T

Fig. 14


LTNP
EC
CUS
CHI w/o T
CHI w T
Number of Patients
0
1
2
3
4
5
6
7
8
Tat VOPSD Peptides
2.1
2.2
2.3
2.4
2.5
2.6
2.7
2.8
2.9
2.10
2.11