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(71) **Applicants** *(for all designated States except US)*: **VIB VZW** [BE/BE]; Rijvisschestraat 120, B-9052 Gent (BE). **VRIJE UNIVERSITEIT BRUSSEL** [BE/BE]; Pleinlaan 2, B-1050 Brussel (BE). **INSTITUUT VOOR TROPISCHE GENEESKUNDE** [BE/BE]; Nationalestraat 155, B-2000 Antwerpen (BE).

(72) **Inventors; and**
(75) **Inventors/Applicants** *(for US only)*: **RAES, Geert** [BE/BE]; Heyenblok 15, B-1640 Sint-Genesius-Rode (BE). **DE BAETSELIER, Patrick** [BE/BE]; Mevrouw Courtmansstraat 9, B-2600 Berchem (BE). **VAN DEN BERGH, Rafaël** [BE/BE]; Dianalaan 45, B-2600 Berchem (BE). **VANHAM, Guido** [BE/BE]; Plashoevestraat 7, B-2500 Lier (BE).

(74) **Common Representative: VIB VZW**; Rijvisschestraat 120, B-9052 Gent (BE).

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(54) Title: THE NOVEL ADIPOCYTOKINE VISFATIN/PBEF1 IS AN APOPTOSIS ASSOCIATED FACTOR INDUCED IN MONOCYTES DURING IN VIVO HIV-1 INFECTION

(57) **Abstract:** The present invention relates to the use of monocyte markers for diagnostic, prognostic or theranostic applications during diseases and syndromes caused by HIV infection. More specifically, it relates to a method, comprising isolation of monocytes and determining gene expression, preferably PBEF1 gene expression. Said method is useful to determine the evolution of the disease or can be used to evaluate the efficacy of a treatment.

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ABSTRACT

The present invention relates to the use of monocyte markers for diagnostic, prognostic or theranostic applications during diseases and syndromes caused by HIV infection. More specifically, it relates to a method, comprising isolation of monocytes and determining gene expression, preferably PBEF1 gene expression. Said method is useful to determine the evolution of the disease or can be used to evaluate the efficacy of a treatment.

BACKGROUND

Monocytes, and macrophages as their more differentiated counterparts, play a fundamental role during HIV infection, since they act as both antigen-presenting cells and effector cells of cellular immunity. While monocytes can be infected by HIV, they do not enter apoptosis upon HIV infection. Hence, they can act as a reservoir for the virus, from where it can continue to replicate even during Highly Active Antiviral Therapy (HAART) (reviewed in e.g. Aquaro *et al.*, 2002). Infected monocytes display dysfunctional behaviour concerning the elimination of pathogens, which gives rise to a number of opportunistic infections (e.g. Kedzierska *et al.*, 2003). Additionally, they are capable of recruiting uninfected lymphocytes to sites of infection and rendering these cells susceptible for HIV infection in these cells (Swingler *et al.*, 1999; Swingler *et al.*, 2003). Furthermore, they have been shown to induce apoptosis in uninfected $CD4^+$ and $CD8^+$ T lymphocytes by a complex interplay of lymphocyte activation, membrane cross-linking (via the CD4 and CXCR4 receptors) and activation of apoptotic pathways, reviewed in Mahlknecht & Herbein, 2001. Conversely, they confer a form of protection against apoptosis on infected $CD4^+$ T lymphocytes (Mahlknecht & Herbein, 2001). In the context of HIV-associated disorders, inappropriate monocyte/macrophage activation in the central nervous system (CNS), leading to neuronal apoptosis, is the primary cause of HIV-associated dementia (HAD) (Anderson *et al.*, 2002), and we recently formulated the hypothesis that monocyte/macrophage hyperactivation during HIV infection could be involved in the novel Immune Reconstitution Disease (IRD) (Van den Bergh *et al.*, 2006).

In this study, we analysed the molecular basis of these monocyte/macrophage dysfunctions, using – amongst others – microarray technology. In this fashion, we aimed on the one hand to gain fundamental insight in monocyte/macrophage biology during HIV infection, and on the other hand to identify potential predictors/molecular markers of HIV- or HAART-associated disorders (such as HAD, IRD and lipodystrophy). Using both a commercially available genome-wide microarray platform and a custom designed ‘Macrophage Activation State’ (MAS) cDNA array containing 700 fragments of genes of interest, we identified monocyte gene expression patterns associated with *in vivo* HIV infection. We found that *in vivo* HIV-1 infection induces aberrant gene expression profiles within the circulating monocyte population, and that these gene expression patterns are sufficient to make a distinction between HIV-1-seropositive and -seronegative blood donors. Subsets of these differentially regulated genes can be clustered together in common pathways/processes. Through overrepresentational analysis we identified clusters of apoptosis-associated and lipid metabolism/insulin signaling-related genes which appear to be disrupted by HIV infection. Unexpectedly, the adipocytokine visfatin (also known as pre-B-cell colony enhancing factor 1 or PBEF1 or nicotinamide phosphoribosyltransferase or NAMPT) was found to be one of the genes of which the expression is induced in monocytes of HIV patients. PBEF1 expression correlated with the plasma viral load (rather than the CD4+ lymphocyte count) in these patients, suggesting that the presence of virus by itself can be responsible for changes in monocyte phenotype, rather than secondary effects mediated through dysfunction of the T lymphocyte population. As the infectivity of HIV for monocytes is limited, it is unlikely that PBEF1 induction is a result of direct infection of the cell. Moreover, our in vitro experiments have shown that treatment of monocytes from non-infected individuals with either infective or AT2-inactivated $\mathrm{HIV}_{\mathrm{BaL}}$ virus induces PBEF expression, possibly by the triggering of receptors on the monocyte membrane by intact virus or individual viral components, which was demonstrated previously to be sufficient to induce changes in monocyte/macrophage phenotype (e.g. Freedman *et al.*, 2003). We also found that PBEF1 expression was reduced again upon HAART, associated with the reduction in viral load.

PBEF1 is a relatively novel, illusive cytokine/adipokine (Samal *et al.*, 1994), which has rapidly been gaining interest the past years, especially in the context of obesitas- and diabetes-research as a result of its insulin-mimetic properties (reviewed in Stephens & Vidal-Puig, 2006). It is induced by inflammatory cytokines in epithelial cells (Ognjanovic *et al.*, 2005) and leukocytes (Jia *et al.*, 2004). In turn, it activates leukocytes and induces, amongst others, IL-1β, TNF-α and IL-6 in monocytes (Moschen *et al.*, 2007). Additionally, it is involved in intracellular regulation of nicotinamide adenine dinucleotide (NAD^+)-dependent reactions (van der Veer *et al.*, 2005). Schindler *et al.* (2006) recently described increased plasma levels of PBEF1 in HIV patients on HAART, but not in therapy-naïve HIV patients. They propose the interesting hypothesis that the increase of PBEF1 serves to compensate for HAART-induced insulin resistance. Our observation that PBEF1 expression in patients on HAART was reduced to the levels also found in healthy controls, suggests that circulating monocytes play no role in an increase in plasma PBEF1 upon HAART as described to occur by Schindler *et al.* On the other hand, our finding that expression of the insulin-mimetic (and insulin-sensitizing) PBEF1 is perturbed in the pre-therapy stage in circulating monocytes, provides a further suggestion that insulin resistance (and the associated metabolic syndrome) are heralded by pro-inflammatory events before therapy is initiated (e.g. Aboud *et al.*, 2007).

Of further interest, PBEF1 exerts an anti-apoptotic effect on neutrophils during inflammation and sepsis (Jia *et al.*, 2004), as well as on amniotic epithelial cells and fibroblasts (Ognjanovic *et al.*, 2005). As monocyte dysfunction during HIV infection is characterised by a persistent failure to enter apoptosis, as well as by anti-apoptotic effects mediated on HIV-infected T lymphocytes, PBEF1 may well be an important factor in this dysfunction. The immunogenic properties of PBEF1, on the other hand, and especially its activating properties on myeloid cells (Moschen *et al.*, 2007), suggest an involvement in the recruitment of, and induction of viral production in, host T lymphocytes, or a controlling function on HIV latency in cells of myeloid lineage.

DISCLOSURE OF THE INVENTION

Considering the expression of PBEF1 and the other genes disclosed in table 4 and table 5 in monocytes of HIV-infected patients, these genes and their gene products represent monocyte markers of value for diagnostic, prognostic or theragnostic applications during HIV infection. Moreover, considering the possible involvement of PBEF1 in monocyte dysfunction during HIV infection, PBEF1 represents a therapeutic target in monocytes/macrophages during HIV infection. PBEF1 genes have been described in humans (genbank accession number NM_005746 and NP_005737), mice (genbank accession number NM_021524 and NP_067499) and rats (genbank accession number NM_177928 and NP_808789). The Genbank numbers are cited as non-limiting examples of PBEF1 genes.

A first aspect of the invention is the use of the marker gene expression level for diagnosis, prognosis or theranosis of disease. Preferably, the use is for theranosis. Preferably, the disease is a disease caused by HIV infection. More preferably, the disease is a disease caused by HIV-1 infection. More preferably, the disease is selected from the group consisting of Acquired Immune Deficiency Syndrome (AIDS) or the HIV- or HAART-associated disorders HIV-associated dementia (HAD), Immune Reconstitution Disease (IRD) and lipodystrophy. Preferably, the marker is a gene selected from the genes mentioned in table 4. Even more preferably, the marker gene is a gene selected from the genes mentioned in table 5. More preferably, the marker is a gene mentioned in table 5. Most preferably, the marker is PBEF1.

Methods to measure the expression level of the marker are known to the person skilled in the art and include, but are not limited to, DNA-RNA hybridization and PCR-related methods, using primers specific for the marker messenger RNA. Alternatively, the expression level may be measured at the level of the protein, using, as a non-limiting example, antibody-based techniques such as ELISA. Still another way to measure the expression level is by the use of a reporter gene, operably linked to the marker promoter. "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A promoter sequence operably linked to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the promoter sequence. Alternatively, the reporter gene is fused to a coding sequence of the marker and expressed as a fusion protein, comprising a part of the marker amino acid sequence up to the total sequence. Suitable reporter genes are known to the person skilled in the art and include, but are not limited to, antibiotic resistance genes, genes encoding fluorescent proteins, or genes encoding surface markers.

Diagnosis or theranosis of the monocyte population can help to identify and treat the disease. "Theranosis" as used herein is a diagnostic method, wherein the results are used to follow the evolution of the disease, to evaluate the efficacy of the medication and/or to adapt the treatment in function of the result of the diagnosis. As the marker allows following the evolution of the monocyte population during the treatment, it allows theranosis in those diseases where there is an imbalance in macrophage/monocyte populations.

Another aspect of the invention is a method for diagnosis, prognosis or theranosis of HIV-related diseases, comprising (a) collection of a blood sample from a subject (b) isolation of the monocytes from this blood sample and (c) determination of gene expression in said monocytes. Preferably, said expression is the expression of a marker gene selected from the list of table 5. Even more preferably, said expression is the expression of a marker gene selected from the group consisting of ADORA1, PBEF1, TNFAIP3, STAT1 (α), STAT1 (β), DDIT3 and BNIP2 (table 4). Most preferably, said expression is the expression of PBEF1 mRNA, or the detection of its gene product. HIV- related diseases as mentioned here can be any HIV-related disease. Prefarably, said disease is selected from the group consisting of Acquired Immune Deficiency Syndrome (AIDS) or the HIV- or HAART-associated disorders HIV-associated dementia (HAD), Immune Reconstitution Disease (IRD) and lipodystrophy.

Preferably, said expression of PBEF1, or the detection of the gene product, is used as a marker for detection of coreceptor usage and/or coreceptor switch. The biomarker can be used to follow the switch from moderately virulent viruses to more aggressive strains, and is useful both in theranosis and in follow up of the effect of the treatment.

Another aspect of the invention is the use of PBEF1 as target for therapy aimed at repression or reduction of disease. Preferably, the target of therapy is PBEF1 expressed in myeloid cells. More preferably, the target of therapy is PBEF1 expressed in macrophages or monocytes. Even more preferably, the target of therapy is PBEF1 expressed in monocytes. Preferably, the disease is a disease caused by HIV infection. More preferably, the disease is a disease caused by HIV-1 infection. Most preferably, the disease is selected from the group consisting of Acquired Immune Deficiency Syndrome (AIDS) or the HIV- or HAART-associated disorders HIV-associated dementia (HAD), Immune Reconstitution Disease (IRD) and lipodystrophy. Said repression or reduction of the disease can be realized either by limiting the expression of PBEF1 in said cells or by impairing or inhibiting the binding capacity or enzymatic activity of

PBEF1 in said cells. Limitation of expression can be obtained, as a non-limiting example, by inactivating the PBEF1 encoding gene in said cells, by inactivation of the promoter of the PBEF1 encoding gene in said cells or by expressing PBEF1 RNAi in said cells. Methods to impair or inhibit the binding capacity or enzymatic activity of PBEF1 in said cells are known to the person skilled in the art and include, but are not limited to the targeting of anti-PBEF1 antibodies, anti-PBEF1 antibody fragments or inhibitors of PBEF1 enzyme activity to said cells. Antibodies and antibody fragments as used here include, but are not limited to classical antibodies, single chain antibodies, camelid antibodies and nanobodies. Specific inhibitors of PBEF1 enzymatic activity have been described and include, but are not limited to the anti-cancer agent FK866. Methods for targeting said antibodies, antibody fragments or inhibitors to said cells are known to the person skilled in the art and include, but are not limited to chemical or genetic coupling of said antibodies, antibody fragments or inhibitors to antibodies or antibody fragments recognizing surface markers on said cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Principal Components Analysis (PCA) on 'present' mean-normalised CodeLink datasets of HIV patient and healthy control samples. HIV patient samples are represented in light grey, healthy control samples in dark grey: a clear distinction can be made between the cluster of HIV patient samples and the healthy controls.

Figure 2. Expression values of PBEF1, as assessed by CodeLink HWG analysis, after mean normalisation of the dataset (A) and by the Macrophage Activation State (MAS) array (B). Expression of PBEF1 in HIV patient samples is significantly higher than in healthy control samples (uncorrected t-test, $p = 0.001$).

Figure 3. A) Expression of PBEF1, normalised to GAPDH expression, as assessed by RT-QPCR and plotted versus CD4+ T lymphocyte count. Upregulation is significant in patients with 200 < T4 < 500 cells/mm<3> (Mann-Whitney, $p < 0.05$). B) Expression of PBEF1, normalised to GAPDH expression, as assessed by RT-QPCR and plotted versus viral load. A positive correlation between viral load and PBEF1 expression was seen ($r^2=0.4881$; $p = 0.001$).

Figure 4. Expression of PBEF1, normalised to GAPDH expression, as assessed by RT-QPCR. PBEF1 is significantly upregulated in therapy-naive HIV patients, but not in patients on HAART (Mann-Whitney, $p < 0.05$).

Figure 5. Expression of PBEF1 in elutriation-purified monocytes after stimulation with mock- and AT2-inactivated HIV_{BaL}, normalised to GAPDH expression and expressed relative to non-treated controls.

Figure 6: Productive infection of PBMC cultures by HIV_{BaL}, as quantified by p24 secretion detected by ELISA, in presence and absence of visfatin.

Figure 7: Viral infectivity, expressed as TCID50, in monocyte-derived macrophages (A) and PBMC (B) after 14 days of culture in presence and absence of 200 ng/ml PBEF1. Representative results of 3 independent experiments shown.

Figure 8: Viral infectivity, expressed as TCID50, in PBMC after 14 days of culture in presence and absence of 100 μM nicotinamide mononucleotide.

Figure 9: Visfatin protein expression in monocytes of HIV patients (as assessed by ECL-Western Blot and normalised to β-actin expression), for patients with R5 or X4 virus (as assayed using infection of viral isolates in CCR5- or CXCR4-expressing U87 cells)

EXAMPLES

Materials and methods to the examples

Sample collection

50 ml blood samples were collected in EDTA-tubes from therapy-naïve HIV-1-seropositive patients from the HIV Clinic of the Institute of Tropical Medicine in Antwerp, Belgium. Patient details are shown in table 1 and table 6. Peripheral blood mononuclear cells (PBMC’s) were separated via a Ficoll gradient and plasma was concomitantly aspirated and stored at -80 °C. Monocytes were purified from the PBMC fraction using the negative selection-based Monocyte Isolation Kit II from Miltenyi-Biotec (Bergisch Gladbach, Germany), according to the manufacturer’s instructions. Yields were minimally 5 million monocytes with a purity > 85%, as verified through flow cytometry.

In vitro HIV treatment experiments

HIV_{BaL} was either inactivated with aldrithiol-2 (AT-2, 200 μM in DMSO at 37°C for 1h) or mock-inactivated with DMSO alone. Virus was subsequently enriched by filtration over a 100kDa cut-off membrane, aliquotted and stored at -80°C until use. Monocytes were purified via counterflow elutriation and subsequent E-rosetting from buffy coats of healthy blood donors from the Blood Transfusion Centre of Antwerp. Cells were cultured at 3x10<6> cells/ml in RPMI medium supplemented with 10% fetal bovine serum and were treated with infectious and AT-2-inactivated HIV_{BaL} for the indicated times at a concentration corresponding to 50 ng/ml of p24.

RNA isolation

For RNA extraction, monocytes isolated from patients or treated in vitro with virus were immediately lysed in Trizol (Invitrogen, Carlsbad, CA, USA), and Trizol pellets were stored at -80 °C. Total RNA was prepared from the Trizol pellets by chloroform extraction, as per the manufacturer’s recommendations. Ten randomly selected samples were checked for integrity on a BioAnalyzer (BioRad, Hercules, CA, USA). No protein contamination or degradation of RNA was detected.

CodeLink arrays

Selected RNA samples were prepared and hybridised to CodeLink HWG bioarrays according to the manufacturer's instructions (Amersham Biosciences, Freiberg, Germany). CodeLink datasets were analysed using the GeneMaths XT software package (Applied Maths, St.-Martens-Latem, Belgium): after background correction, genes which were called as 'absent' in more than four arrays were eliminated from the datasets. Subsequently array normalisation was performed: both quantile and simple mean normalisation were performed, without significant differences in the datasets. In this fashion, a normalised dataset containing only genes with a present call in minimum eight arrays was constructed.

Normalised 'present' datasets were further analysed for overrepresentation of specific processes/pathways. For this type of analysis two different software applications were used: the freeware program GenMAPP/MAPPFinder (Doniger *et al.*, 2003; http://www.genmapp.org) and the commercially available package GeneGo (http://www.genego.com). GenMAPP/MAPPFinder clusters genes together in common pathways/processes using the associated Gene Ontology (GO; Ashburner *et al.*, 2000) annotations; additionally, users can contribute pathways (MAPPs) for which overrepresentation can also be assessed. GeneGo uses a system of manually curated pathways, which are publicly available at http://www.invitrogen.com/ipath.

Macrophage Activation State arrays

The Macrophage Activation State (MAS) array was developed as a focussed and flexible tool for the analysis of gene expression patterns in monocytes/macrophages. A collection of 700 genes associated with different macrophage activation states was compiled, using a combination of literature data-mining and human 'translation' of murine models of macrophage activation available in our laboratory. Subsequently, gene specific primers were designed for the genes in this collection and fragments were amplified from total cDNA pools of monocytes under various *in vitro* and *in vivo* conditions. These fragments were applied in duplicate on 7x10 cm nylon membranes and were cross-linked to the membranes using UV-exposure.

RNA samples from all patients were selected for analysis on this MAS array. A reverse transcription was performed on 1 µg total RNA using oligo-dT and Superscript II reverse transcriptase (Invitrogen) in the presence of <33>P-dCTP (Amersham Biosciences), and the labelled cDNA was then hybridised to the membranes for 20h at 42 °C in NorthernMax hybridisation buffer (Ambion, Austin, TX, USA). Membranes were subsequently washed with SDS-containing buffer at 68 °C and were exposed to a phosphorscreen to reveal bound radioactivity. Phosphorscreens were then scanned in a phospho-imager (BioRad). Spot recognition and quantification, background correction and array normalisation were all performed using custom-designed software based on the program ImageJ (Image Processing and Analysis in Java, Sun Microsystems, Santa Clara, CA, USA).

Real-time semi-quantitative PCR

Expression of individual genes was examined using real-time semi-quantitative PCR (RT-QPCR). cDNA was prepared from 1 µg total RNA using oligo-dT and Superscript II reverse transcriptase (Invitrogen) and gene specific primers for the gene of interest (PBEF1) and a housekeeping gene (GAPDH) were designed:

PBEF1.F: 5'-GGCAAGGTGACAAAAAGCTA-3'
PBEF1.R: 5'-ATGAAAGGGCAGTATGTCCA-3'
GAPDH.F: 5'-AGCTCATTTCCTGGTATGACA-3'
GAPDH.R: 5'-TGGTTGAGCACAGGGTACTT-3'

PCR reactions were performed in duplicate in a BioRad MyCycler, with BioRad iQ SYBR Green Supermix; each PCR cycle consisted of 60 s denaturation at 94 °C, 45 s annealing at 55 °C and 60 s extension at 72 °C. Gene expression was normalised using the gene GAPDH, coding for the enzyme *glyceraldehyde-3-phosphate dehydrogenase*, as a housekeeping gene.

In vitro infection experiments

For *in vitro* infection experiments, monocytes were obtained from buffy coats of healthy donors of the Blood Transfusion Center of Antwerp (Rode Kruis Vlaanderen, Belgium) by counterflow elutriation, as described previously (Van Herrewege *et al.*, 2002). These cells were then differentiated to monocyte-derived macrophages (MDM) during 7 days in RPMI 1640 medium (Bio-Whittaker, Verviers, Belgium) supplemented with 10% bovine fetal calf serum (Biochrom, Berlin, Germany), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Roche Diagnostics, Mannheim, Germany) and 40 ng/ml M-CSF (PeproTech, London, United Kingdom) at 37°C and 5.0% CO_2. Half the medium was replaced after 4 days. Cells were then harvested and used for experiments in the same medium (without M-CSF).

Recombinant visfatin was obtained from PeproTech and Alexis (Zandhoven, Belgium). As both batches gave similar results in preliminary studies, all further experiments were performed using recombinant protein from PeproTech. The recombinant protein batches contained < 0.01 ng/µg LPS, as assessed by quantitative chromogenic limulus amoebocyte lysate assay (QLAL) (Bio-Whittaker).

For infection experiments, MDM were plated in 96-well plates at 7.5×10^5 cells/ml and pre-treated with recombinant visfatin (200 ng/ml) for 1 hour at 37°C and 5.0% CO_2. Then, virus was added in sixfold and incubated for 2 hours, again at 37°C and 5.0% CO_2. Cells were then washed 3x to remove unbound virus and incubated for 14 days. Productive infection was monitored via an in-house developed p24 antigen ELISA, as described elsewhere (Beirnaert *et al.*, 1998).

Viral isolation and coreceptor usage determination

Plasma separated from patient blood samples by Lymphoprep separation was stored at -80°C until use. 1 ml samples were added to $5x10^6$ phytohemagglutinin (PHA)/interleukin-2 (IL2) stimulated PBMC's obtained from buffy coats of healthy donors of the Blood Transfusion Center of Antwerp and were cultured in RPMI 1640 medium (Bio-Whittaker) supplemented with 10% bovine fetal calf serum (Biochrom), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Roche), PHA (0.5 µg/ml) (Murex Biotech Ltd., Dartford, United Kingdom) and IL2 (5 ng/ml) (Roche). Medium was refreshed twice weekly, and supernatants were monitored using p24 antigen ELISA. Additional PBMC were added on an *ad hoc* basis when cells became depleted. Cultures were followed until p24 levels in the supernatants reached overflow values in ELISA. Then viruses were harvested, aliquoted and stored at -80°C.

For coreceptor usage determination assays, plasma viruses were serially diluted and added to U87.R5 or U87.X4 cells in quadruplicate. Unbound virus was washed away after 2 hours incubation at 37°C and 5.0% CO_2, and productive infection was monitored by p24 ELISA after 7 and 14 days. Productive infection of either U87.CCR5 or U87.CXCR4, signifying respectively R5 and X4 usage, was clear-cut in all cases. HIV_{BaL} and HIV_{IIIB} viruses were assayed in parallel as positive controls for resp. R5 and X4 virus.

Statistical analysis

For CodeLink HWG bioarray data, an uncorrected t-test (*p*-value < 0.01 significant) and a Benjamini-Hochberg-corrected t-test (*p*-value < 0.05 significant) to control the false positive rate (Benjamini & Hochberg, 1995) were used; for the MAS data only an uncorrected t-test (*p*-value < 0.05 significant) was used. Significance of RT-QPCR data was assessed via a nonparametric Mann-Whitney test.

Example 1: CodeLink array hybridisations of monocyte samples from therapy-naïve HIV-infected patients.

Eight HIV patient samples with a broad range of $CD4^+$ T lymphocyte counts and four healthy control samples were selected for analysis on CodeLink HWG microarrays (P01-P08 & C01-C04; table 1) and were processed as described. A Principal Components Analysis (PCA) was performed on the normalised datasets of 'present' genes. This allowed a segmentation of the data into a cluster of HIV-samples, a cluster of control samples and one outlier control sample (fig. 1), suggesting that monocyte function is distinctly modulated during *in vivo* HIV infection.

Samples were grouped according to serostatus, i.e. no stratifications according to CD4$^+$ T lymphocyte count or viral load were performed, and gene expression values were compared between the HIV-positive and HIV-negative groups. Two different types of meaningful information can be extracted from datasets of this magnitude. On the one hand, by performing overrepresentational analysis on a broad group of genes for which expression is significantly different (i.e. p-value < 0.01 as only criterion), it is possible to identify specific pathways and/or functional groups of genes which are influenced as a whole. On the other hand, by using more stringent criteria (i.e. p-value < 0.01 and fold change > 1.5), individual genes which may play pivotal roles in the model at hand or which may be candidate molecular markers for certain conditions can be identified.

In the context of this study, overrepresentation analysis using the two described software applications (GeneGo and GenMAPP) revealed several cellular pathways/processes involved in apoptosis which were significantly modulated in monocytes of HIV patients (table 2), confirming our notion that the cellular apoptotic machinery is disturbed on a molecular level during HIV infection. Another class of processes which appears to be targeted in monocytes by HIV infection is a group of pathways involved in lipid metabolism and/or insulin signaling (table 3). To the best of our knowledge, no study to date has focused on these processes in monocytes during HIV infection.

An analysis of the datasets aimed at the identification of individual genes with an interesting expression pattern yields several candidates. One of the genes which passes the set criteria (p-value < 0.01, fold change > 1.5) is the novel cytokine/adipokine PBEF1 (pre-B-cell colony enhancing factor 1 or visfatin) (fig. 2), which possesses a documented involvement in both lipid metabolism and apoptosis.

Example 2: MAS array hybridisations of monocyte samples from therapy-naïve HIV-infected patients.

All HIV patient (n=29) and healthy control (n=8) samples (table 1) were analysed on our custom MAS array as described. Patients were grouped together according to their CD4$^+$ T lymphocyte count: T4 < 200 cells/mm<3> (group 1), 200 < T4 < 500 cells/mm<3> (group 2) and T4 > 500 cells/mm<3> (group 3). As this smaller-scaled array is less geared towards pathway analyses and more towards identification of individual genes of interest, overrepresentation/pathway analysis was not performed on this dataset. Statistical analysis, however, again with an additional fold change cut-off of 1.5, revealed a list of genes to be significantly up-regulated or to be significantly down-regulated in at least one of these patient groups (table 4). Within this list, a cluster of apoptosis-associated genes (n=6) could be compiled (table 5), using the Gene Ontology (GO) annotations in combination with a thorough screening of the available literature.

Several of these genes possess previously documented HIV-associated properties in cells of monocyte lineage. An example is STAT1, which was previously found to be induced in monocytes and monocyte derived macrophages by *in vitro* treatment with HIV-1 Nef or infectious HIV (Federico *et al.*, 2001) and in immature dendritic cells by HIV-1 Tat expression or *in vitro* HIV infection (Izmailova *et al.*, 2003). Additionally, as an interferon-γ-associated transcription factor, STAT1 is involved in many inflammatory pathways and has been implicated in HIV-associated pathogenesis in a multitude of studies (e.g. Abbate *et al.*, 2000; Asensio *et al.*, 2001; Roberts *et al.*, 2003). PBEF1 on the other hand was to the best of our knowledge never associated with monocyte dysfunction during HIV infection and was only very recently linked with HIV infection in general, in the context of HAART-treated HIV patients (Schindler *et al.*, 2006).

Example 3: RT-QPCR verification of PBEF1 expression in of monocyte samples from HIV-infected patients versus healthy controls.

Gene expression was analysed in selected patient samples (P03-P19 & C01-C06; table 1) using gene specific primers for PBEF1. Gene expression data were normalised using the housekeeping gene GAPDH and were compared between HIV patients and healthy controls (fig. 3). When patients were stratified according to $CD4^+$ T lymphocyte count, only group 2 (200 < T4 < 500 cells/mm<3>) appeared to display a significant (Mann-Whitney, $p < 0.05$) upregulation of PBEF1 (fig. 3A), and no significant correlation was found between lymphocyte counts and PBEF1 expression levels. However, this grouping of patients according to the lymphocyte counts may not be the best strategy, as the aberrant gene expression profile in monocytes may in the first place be a direct result of the circulating virus, and correlation between viral load and $CD4^+$ T lymphocyte count is not always strong. Therefore, the gene expression was also plotted *versus* the viral loads of the patients (fig. 3B). Linear regression analysis reveals that PBEF1 expression is significantly correlated with the viral load ($p = 0.001$).

Example 4. RT-QPCR analysis of the effect of HAART on PBEF1 expression in monocyte samples from HIV-infected patients.

Blood samples were collected from therapy-naïve and HAART-treated HIV patients (table 6). Body mass index was between 20 and 25 for all patients. Inclusion criteria for therapy-naïve patients were never to have received therapy and to have a viral load of more than 4 log copies/ml. For HAART patients, inclusion criteria were to have been on therapy for at least 1 year and to have an undetectable viral load. Samples from healthy seronegative donors with matching age and nationality were collected as negative controls. Monocytes were isolated from these blood samples and PBEF1 expression was analysed through RT-QPCR analysis using gene specific primers for PBEF1. Also in this analysis; therapy-naïve HIV patients displayed significantly higher PBEF1 expression than healthy controls. However, PBEF1 expression in patients on HAART was reduced to the levels also found in healthy controls (fig. 4). This observation confirms our previous findings and is in accordance with the role of visfatin as an inflammatory product (the viral load in patients on HAART is reduced to undetectable levels, resulting in a decreased inflammatory phenotype). However, it also suggests that circulating monocytes play no role in the increase in plasma visfatin described to occur by Schindler *et al* in patients undergoing HAART.

Example 5: RT-QPCR analysis of the effect of HIV treatment on PBEF1 expression in monocyte samples from non-infected individuals.

Monocytes were isolated from buffy coat from healthy control volunteers by counterflow elutriation. These cells were seeded in 6-well plates, and were treated with infective HIV_{BaL} virus or with virus that had been treated with aldrithiol-2 (AT2), which is reported to covalently modify essential zinc fingers in the nucleocapsid of HIV, rendering it incapable of productive infection while conserving its structure and binding properties (Rossio *et al*., J Virol 1998, **72**, 7992-8001). Both infective and inactivated viruses were added at a concentration corresponding to 50 ng/ml of p24. RT-QPCR analysis revealed that PBEF1 induction appeared to be an early event in monocyte cultures treated both with active and inactive virus (fig. 5), suggesting that simple interaction of the virus with the cell is enough to induce significant transcriptional changes in the monocyte.

Example 6: Addition of recombinant visfatin decreases the viral infectivity of the M-tropic R5 HIV labstrain BaL

As these expression analyses of visfatin showed interesting tendencies, we initiated a series of experiments aimed at elucidating the functional role of visfatin during HIV infection. Experiments concerning the effect on productive infection with HIV proved to be very promising. Addition of recombinant visfatin to activated PBMC or monocyte-derived macrophage (MDM) cultures prior to *in vitro* infection with the Mtropic R5 HIV lab-strain BaL decreases the viral infectivity for these cultures (fig. 6).

This viral infectivity can be quantified by calculating the TCID50 (Tissue Culture Infectious Dose 50%, i.e. the dose of virus which as a 50% chance of infecting a cell culture) values of the viral stocks. Addition of visfatin reduces the TCID50 values of BaL by approx. 1 log, in both PBMC and MDM cultures, signifying a 90% reduction in viral infectivity (fig. 7A).

Example 7: Visfatin promotes the HIV coreceptor switch

Interestingly, visfatin does not mediate this effect on the T-tropic X4 HIV lab-strain IIIB (=HxB2). This signifies that, by selectively inhibiting R5 and not X4 HIV, visfatin contributes to the HIV coreceptor switch seen in ca. 50% of all patients, in which the coreceptor usage of the dominant quasispecies within the patient switches from the moderately virulent R5 to the more aggressive X4, leading to increased progression to AIDS.

Because the BaL and IIIB strains differ significantly in their genetic background, and not only in their coreceptor usage, we repeated the same experiments using clinical isolates en lieu of lab strains. R5 and X4 strains of the 968 viral clone were isolated from one patient, and differ only in coreceptor usage. For the R5 strain of the 968 clone (968-3) the results were similar to those of the R5 lab strain BaL. For the X4 strain (968-1) visfatin even increased the viral titer in MDM (fig. 7B): while this strain is incapable of infecting untreated MDM, addition of visfatin allowed the 968-1 clone to establish a productive infection.

In order to rule out LPS contamination (which also mediates effects on viral infectivity in MDM and PBMC) as a possible contributing factor to this effect, the experiments concerning viral infectivity were reproduced using nicotinamide mononucleotide (NMN), the endproduct of the enzymatic function of visfatin. Simple addition of NMN to PBMC cultures also reduces the infectivity of HIVBaL (fig. 8) proving that it is in the first place the enzymatic function of visfatin which is mediating the described effects, rather than possible LPS contamination of the recombinant protein. Additionally, these results prove that it is specifically the enzymatic function of visfatin which is responsible for these effects, rather than e.g. the published cytokine-like or insulin-mimetic properties of this molecule.

In order to identify correlates for visfatin expression in HIV patients other than the viral load, several other parameters were examined. One such parameter was the coreceptor usage of primary viruses isolated from patient plasma samples. Coreceptor usage of these isolates was evaluated through infection experiments of CCR5- or CXCR4-expressing U87 cells (U87.R5 and U87.X4). When patients were grouped according to the coreceptor usage of their corresponding viral isolates (2 individuals with R5 virus and 3 with X4 virus), significant differences were found between the groups at the level of visfatin protein expression. High visfatin expression appeared to correlate with the presence of X4 virus in the clinical isolates, while low visfatin expression was associated with R5 viruses (fig. 9). This correlation, combined with our observation that visfatin is induced late in infection in patients with high viral loads, supports the application of visfatin as a biomarker for the coreceptor switch, and fits with the contribution of visfatin to said switch.

Table 1

Patient ID	Age (years)	Sex	Nationality	T4	VL	Time infected (months)
P01	30	M	Belgian	359	3.87	8
P02	61	M	Belgian	748	5.54	60
P03	43	M	Belgian	142	2.28	10
P04	33	M	Subsaharan Africa	446	3.91	32
P05	39	M	Cameroon	644	4.34	8
P06	39	M	Belgian	856	4.82	27
P07	36	M	Belgian	197	5.91	48
P08	21	M	Belgian	133	<2,70	39
P09	31	M	Belgian	1026	3.08	9
P10	45	M	Belgian	781	3.50	10
P11	51	F	Belgian	371	3.60	5
P12	43	M	European	436	4.28	48
P13	29	M	Belgian	532	4.78	9
P14	46	M	Argentinean	329	5.37	4
P15	32	M	Belgian	738	5.58	6
P16	44	M	Belgian	226	5.59	114
P17	45	M	Belgian	233	5.59	36
P18	47	M	Belgian	359	5.84	10
P19	?	?	?	382	ND	?
P20	39	M	Belgian	760	3.93	66
P21	35	M	Togo	462	4.06	17
P22	37	F	Burundi	374	4.24	21
P23	22	M	Belgian	503	4.32	16
P24	48	M	Belgian	540	4.36	27
P25	40	M	Polish	535	4.78	20
P26	39	M	Belgian	746	4.90	16
P27	47	M	Belgian	311	4.97	26
P28	39	M	Belgian	778	5.00	8
P29	65	M	Belgian	756	5.07	7
C01	31	F	Belgian	ND	NA	NA
C02	23	M	Belgian	ND	NA	NA
C03	23	F	Belgian	ND	NA	NA
C04	48	F	Belgian	ND	NA	NA
C05	25	M	Belgian	ND	NA	NA
C06	22	F	Belgian	ND	NA	NA
C07	24	M	Belgian	ND	NA	NA
C08	51	M	Belgian	ND	NA	NA

Table 1. Clinical details of included patients. T4: CD4+ T lymphocyte count (cells/mm<3>) – ND: not done; VL: viral load (log copies/ml) – NA: not applicable.

Table 2

PPF	**Software application**	***p*-val**	**% changed**	**Author**
Death receptor binding	GenMAPP (GO)	0.000	87.5%	GO
FAS signaling cascades. Part 2	GeneGo	0.001	34.9%	GO
TRAF proteins signaling network	GeneGo	0.001	37.1%	GO
Role SUMO in p53 regulation	GeneGo	0.002	42.9%	GO
AP1 activation by TRAF proteins signaling pathway	GeneGo	0.002	37.9%	GO
Cytoplasm/mitochondrial transport of proapoptotic proteins Bid, Bmf and Bim	GeneGo	0.003	34.3%	GO
BAD phosphorylation	GeneGo	0.004	26.2%	GO
TRADD interaction with MAPK cascade	GeneGo	0.004	34.4%	GO
Caspases activation via nuclear import	GeneGo	0.012	33.3%	GO
Hs Apoptosis	GenMAPP (contributed)	0.012	38.5%	Alexander C. Zambon and Beth Lawlor
TNFR1 signaling pathway	GeneGo	0.013	28.3%	GO
Hs MAPK signaling pathway KEGG	GenMAPP (contributed)	0.013	34.4%	Adapted from KEGG by Sebastien Burel
Caspases cascade	GeneGo	0.021	29.4%	GO
Apoptosis	GenMAPP (GO)	0.023	32.1%	GO
Antiapoptotic Function of TRADD/TRAF2 complex	GeneGo	0.036	28.1%	GO
Hs p38 MAPK signaling pathway	GenMAPP (contributed)	0.040	41.9%	Adapted from Biocarta by Sebastien Burel
Role of CARD-protein family in caspase cascade regulation and apoptosis	GeneGo	0.042	30.4%	GO
p38-MAPK cascade activation via FAS1 and TNFR1	GeneGo	0.043	28.6%	GO
Caspase cascade activation by FADD and RIPK	GeneGo	0.044	27.3%	GO

Table 2. Processes, pathways and molecular functions associated with apoptosis, identified by the software applications GenMAPP and GeneGo as overrepresented in CodeLink HWG datasets. PPF: name of the identified process, pathway or function; either the Gene Ontology (GO)/contributed term (GenMAPP) or the name of the curated pathway (GeneGO; http://www.invitrogen.com/ipath). P-val: p-value of overrepresented pathway, as calculated by

software application; % changed: percentage of the genes in the pathway which were called as significant; Author: author of contributed genMAPP (http://www.genmapp.org/).

Table 3

PPF	**Software application**	***p*-val**	**% changed**	**Author**
Hs Insulin Signaling	GenMAPP (contributed)	0.000	39.6%	Diabetes Genome Anatomy Project Investigators
Insulin receptor signaling pathway	GenMAPP (GO)	0.002	66.7%	GO
Phospholipid biosynthesis	GenMAPP (GO)	0.003	46.5%	GO
Lipid kinase activity	GenMAPP (GO)	0.014	50.0%	GO
Phospholipid metabolism	GenMAPP (GO)	0.016	39.7%	GO
Membrane lipid metabolism	GenMAPP (GO)	0.024	35.8%	GO
Insulin-like growth factor receptor binding	GenMAPP (GO)	0.041	66.7%	GO
Lipid binding	GenMAPP (GO)	0.041	32.7%	GO
Membrane lipid biosynthesis	GenMAPP (GO)	0.042	38.2%	GO

Table 3. Processes, pathways and molecular functions associated with lipid metabolism/insulin resistance, identified by the software applications GenMAPP and GeneGo as overrepresented in CodeLink HWG datasets. PPF: name of the identified process, pathway or function; either the Gene Ontology (GO)/contributed term (GenMAPP) or the name of the curated pathway (GeneGO; http://www.invitrogen.com/ipath). P-val: p-value of overrepresented pathway, as calculated by software application; % changed: percentage of the genes in the pathway which were called as significant; Author: author of contributed genMAPP (http://www.genmapp.org/).

Table 4

Gene symbol	Name	Gene Entrez ID	Diff Groups	TR
ADORA1	*adenosine A1 receptor*	134	T4 > 200	I
B2M	*beta-2-microglobulin*	567	T4 < 500	I
BCL6	*B-cell CLL/lymphoma 2*	604	T4 < 200	I
BNIP2	*BCL2/adenovirus interacting protein 2*	663	All	I
CAPG	*capping protein, gelsolin-like*	822	All	S
CCL18	*chemokine (C-C motif) ligand 18*	6362	All	I
CCL22	*chemokine (C-C motif) ligand 22*	6367	T4 < 500	S
CCL3L1	*chemokine (C-C motif) ligand 3-like 1*	374793	T4 < 200	S
CCR1	*chemokine (C-C motif) receptor 1*	1230	T4 < 200	I
CCR2_A	*chemokine (C-C motif) receptor 2, isoform A*	1231	T4 < 500	S
CD83	*CD83 antigen*	9308	T4 > 500	I
CLEC2D	*C-type lectin domain family 2, member D*	29121	T4 < 200	S
CNIH2	*cornichon homolog 2*	254263	T4 < 500	I
CTNNAL1	*catenin (cadherin-associated), alpha-like 1*	8727	T4 < 200	I
CX3CR1	*chemokine (C-X3-C motif) receptor 1*	1524	T4 < 500	S
CXCL2	*chemokine (C-X-C motif) ligand 2*	2920	All	S, I
DDIT3	*DNA-damage-inducible transcript 3*	1649	All	I
EN2	*engrailed homolog 2*	2020	T4 < 500	I
IL1A	*interleukin 1, alpha*	3552	All	I
IL1B	*interleukin 1, beta*	3553	T4 > 500	I
LAMP2_2B	*lysosomal-associated membrane protein 2, isoform 2B*	3920	All	I
LAT	*linker for activation of T cells*	27040	T4 > 200	S
LILRB4	*leukocyte immunoglobulin-like receptor, B4*	11006	All	S
LOC374794	onbekend	374794	All	S, I
LTB4DH	*leukotriene B4 12-hydroxydehydrogenase*	22949	T4 < 500	I
MAPK10	*mitogen-activated protein kinase 10*	5602	T4 > 500	I
MRC1	*mannose receptor, C type 1*	4360	T4 < 500	I
PBEF1	*pre-B-cell colony enhancing factor 1*	10135	All	I
PCDH7_b	*BH-protocadherin, isoform b*	5099	T4 > 500	I
PLA2G7	*phospholipase A2, group VII*	7941	All	S
PTGER2	*prostaglandin E receptor 2*	5732	T4 < 500	I
STAT1_a	*signal transducer and activator of transcription 1, isoform a*	6772	T4 < 500	I
STAT1_b	*signal transducer and activator of transcription 1, isoform b*	6772	T4 < 500	I
TEBP	*unactive progesterone receptor*	10728	T4 < 500	I
TIEG	*TGFB inducible early growth response*	7071	T4 < 500	I
TNFAIP3	*tumor necrosis factor alpha-induced protein 3*	7128	T4 > 500	I
XLKD1	*extracellular link domain containing 1*	10894	T4 < 200	I
YWHAZ	*tyrosine 3-monooxygenase activation protein*	7534	T4 < 200	I

Table 4 Genes identified from the MAS analysis as differentially expressed between samples from HIV-patients and controls. T4: CD4+ T lymphocyte count (cells/mm<3>). Diff Groups: Groups of patients (defined by T4 counts) in which the genes are differentially expressed. TR: type of regulation: I = induction, S = suppression.

Table 5

Gene name	**Symbol**	**Entrez ID**	**Group**	**Evidence**	**FC**	***P*-val**
adenosine A1 receptor	ADORA1	134	All	GO	-1.63	0.045
pre-B-cell colony enhancing factor 1	PBEF1	10135	All	Jia *et al.*, 2004	2.74	0.003
tumor necrosis factor alpha-induced protein 3	TNFAIP3	7128	> 500	GO	2.21	0.030
signal transducer and activator of transcription 1						
- isoform alpha	STAT1 (α)	6772	All	GO	1.88	0.007
- isoform beta	STAT1 (β)	6772	All	GO	1.57	0.006
DNA-damage-inducible transcript 3	DDIT3	1649	< 200	Oyadomari & Mori, 2004	1.67	0.018
BCL2/adenovirus E1B 19kDa interacting protein 2	BNIP2	663	> 500	GO	1.55	0.001

Table 5. Apoptosis-associated genes differentially expressed between monocytes of HIV patients and of healthy controls, as assessed by custom MAS array analysis. Group: patient group, based on CD4+ T lymphocyte count (cells/mm<3>); Evidence: evidence for assigning the gene to the cluster “Apoptosis-associated genes”; GO: Gene Ontology annotation; FC: fold change; P-val: p-value, determined via uncorrected student’s t-test.

Table 6

ID	Viral load (log copies/ml)	T4 count (cells/µl)	Age	Nationality	Months infected	Months on therapy
HAART001	0.00	352	33	Belgian	3	12
HAART002	0.00	430	71	Dutch	25	21
HAART003	0.00	468	33	Belgian	42	23
HAART004	0.00	328	37	Belgian	112	23
HAART005	0.00	416	52	Belgian	40	25
HAART006	0.00	781	38	Sub-Saharan Africa	73	64
HIV001	5.8	464	48	Belgian	8	N.A.
HIV002	5.08	874	26	Nigerian	13	N.A.
HIV003	5.60	775	41	Central American	35	N.A.
HIV004	4.97	365	43	Belgian	40	N.A.
HIV005	4.37	576	41	Belgian	41	N.A.
HIV006	5.49	312	34	Belgian	45	N.A.
HIV007	4.94	295	42	Belgian	73	N.A.
C001	N.A.	N.A.	52	Belgian	N.A.	N.A.
C002	N.A.	N.A.	28	Kenian	N.A.	N.A.
C003	N.A.	N.A.	34	Belgian	N.A.	N.A.
C004	N.A.	N.A.	31	Belgian	N.A.	N.A.
C005	N.A.	N.A.	54	Belgian	N.A.	N.A.

Table 6. Clinical details of patients enrolled in the study for PBEF1 levels in the monocytes of therapy-naïve patients and patients on HAART. T4: CD4+ T lymphocyte count (cells/mm<3>); NA: not applicable.

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CLAIMS

1. A method for diagnosis, prognosis or theranosis of HIV-related diseases, comprising (a) collection of a blood sample from a subject (b) isolation of the monocytes from this blood sample and (c) determination of gene expression in said monocytes.
2. The method according to claim 1, whereby said gene expression is PBEF1 mRNA expression.
3. The method according to claim 1, whereby said gene expression is the measurement of PBEF1 protein.
4. The method according to any of the claims 1-3, whereby said HIV-related disease is selected from the group consisting of Acquired Immune Deficiency Syndrome, HIV-associated dementia, Immune Reconstitution Disease and lipodystrophy.

Figure 1

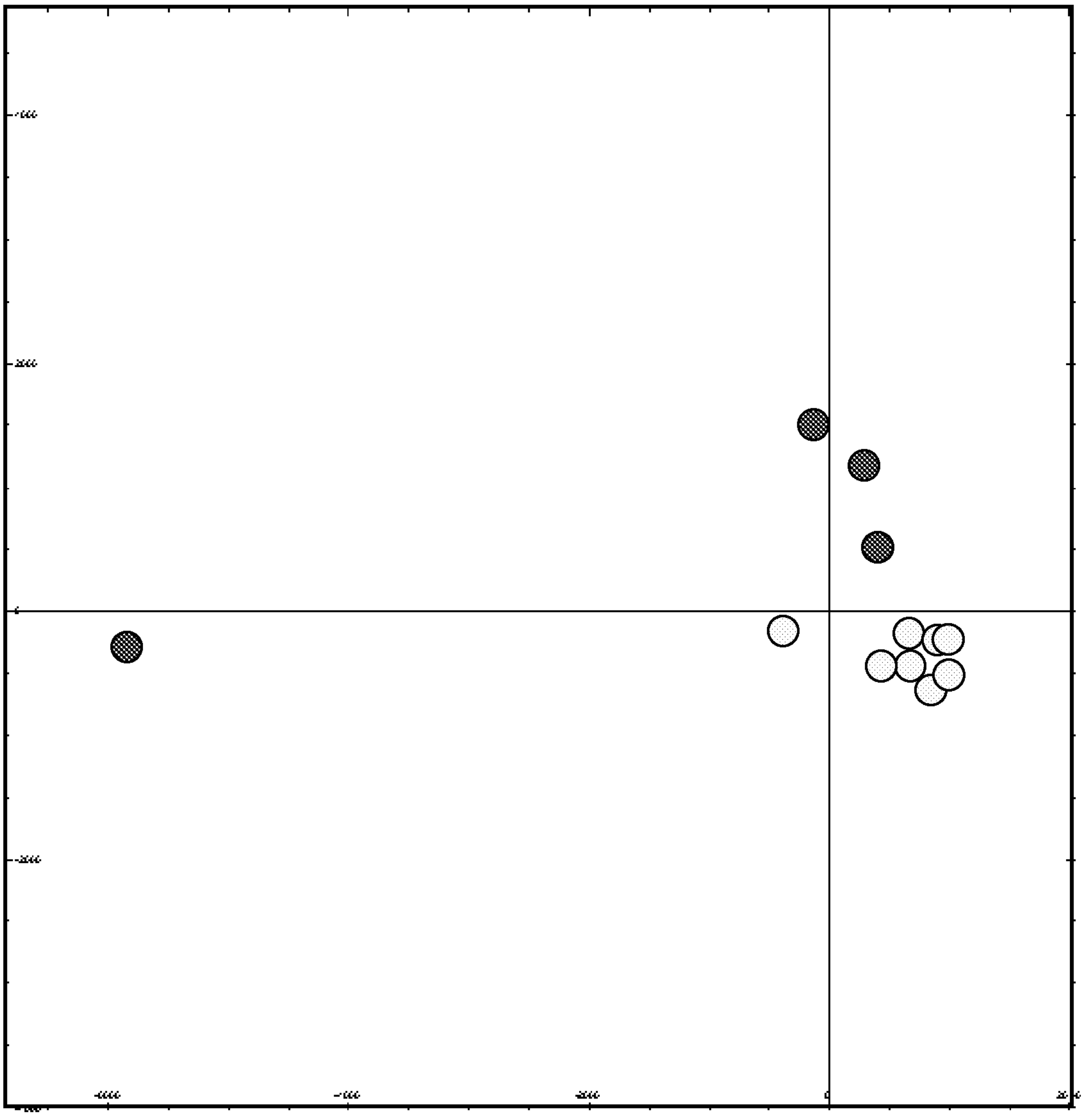
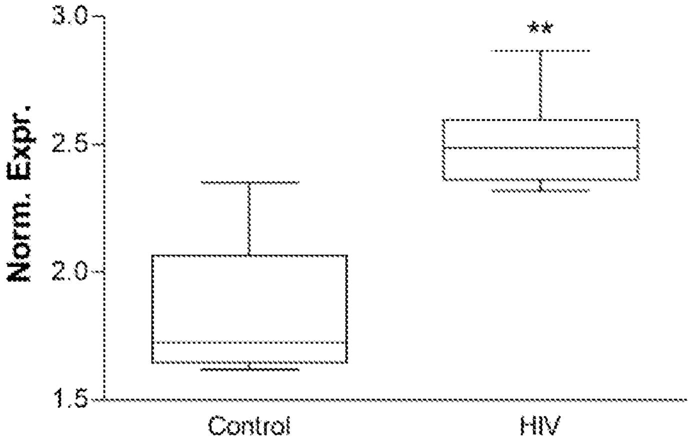


Figure 2

A


Norm. Expr.
3.0
2.5
2.0
1.5
**
Control
HIV

B

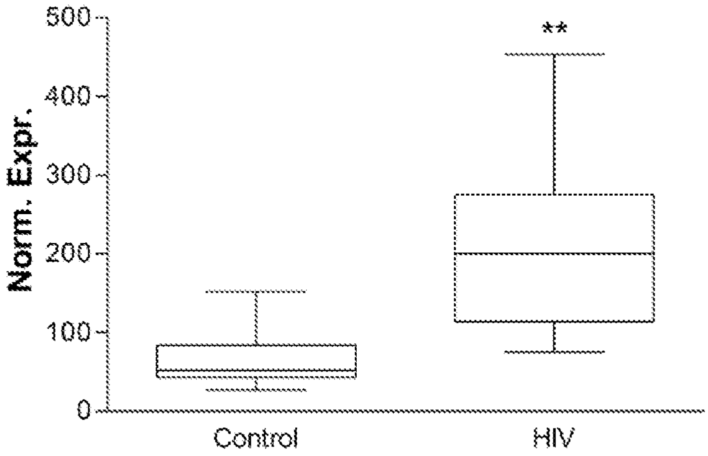
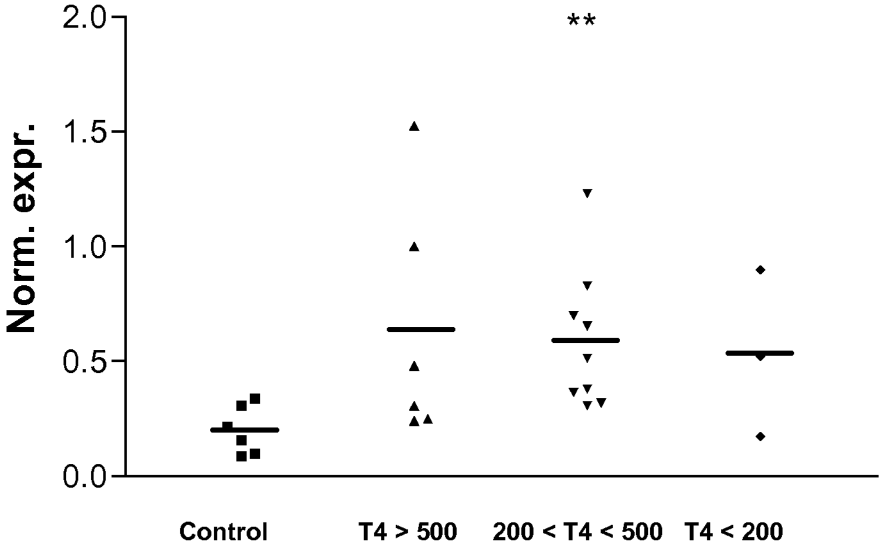

Norm. Expr.
500
400
300
200
100
0
**
Control
HIV

Figure 3

A


2.0
1.5
1.0
0.5
0.0
Norm. expr.
**
Control
T4 > 500
200 < T4 < 500
T4 < 200

B

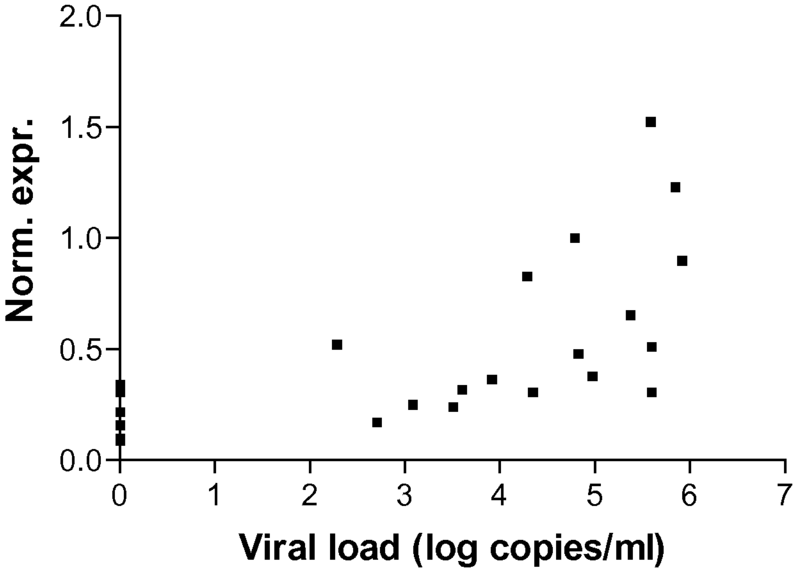

2.0
1.5
1.0
0.5
0.0
Norm. expr.
0
1
2
3
4
5
6
7
Viral load (log copies/ml)

Figure 4

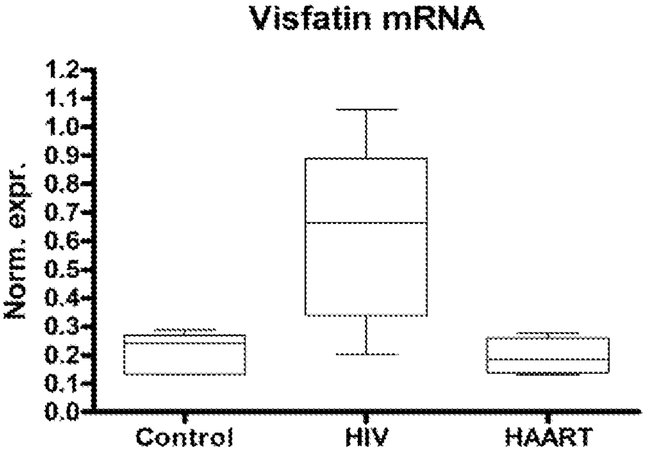


Figure 5

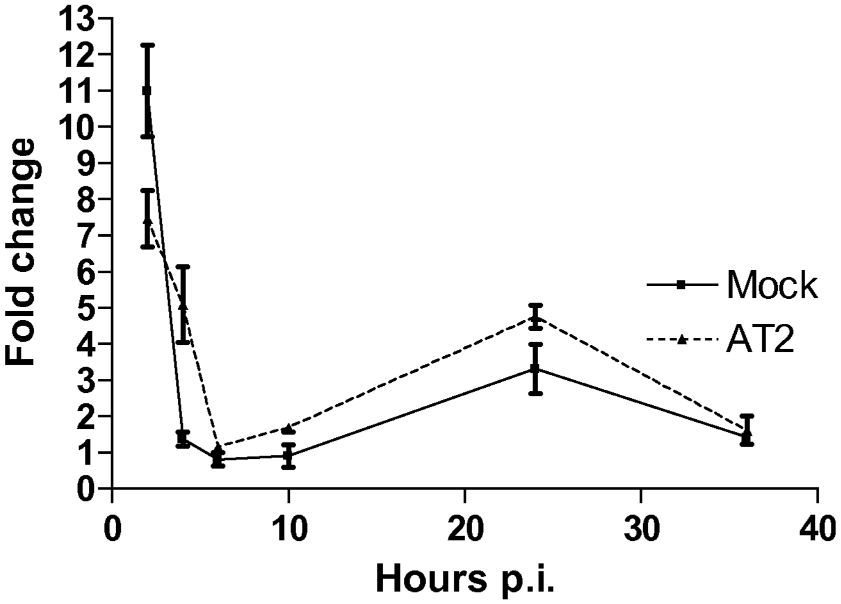


Figure 6

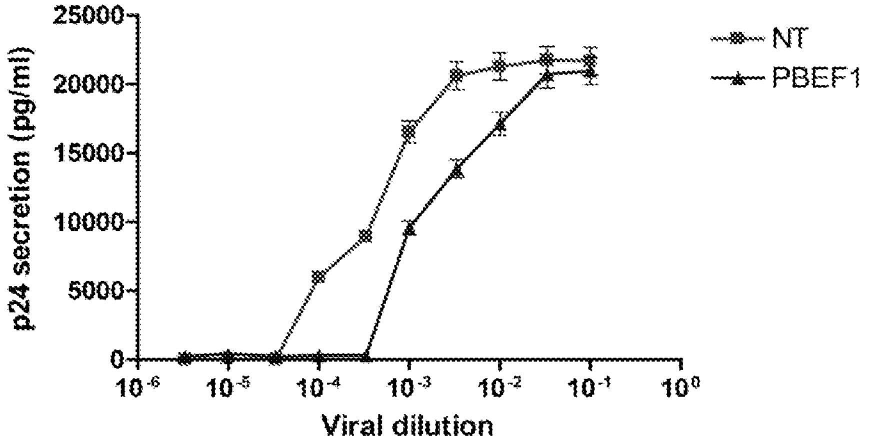
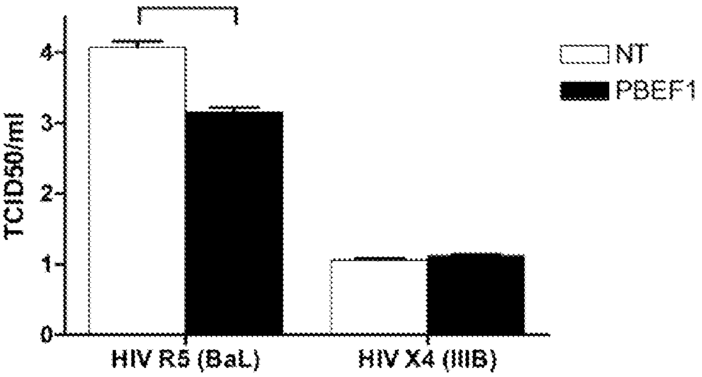


Figure 7

A



B

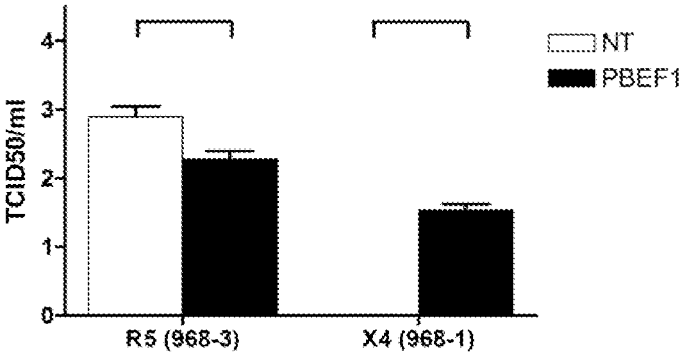


Figure 8

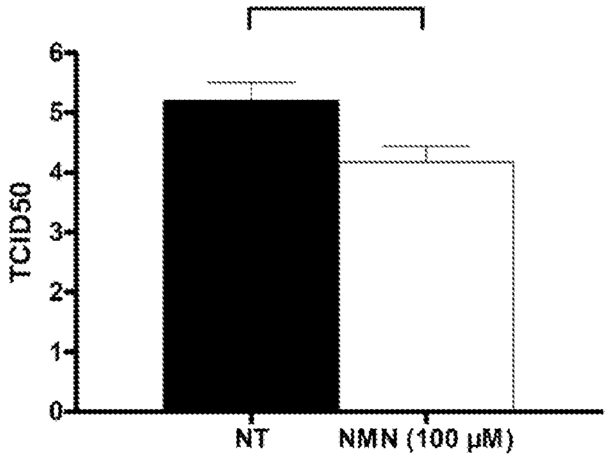
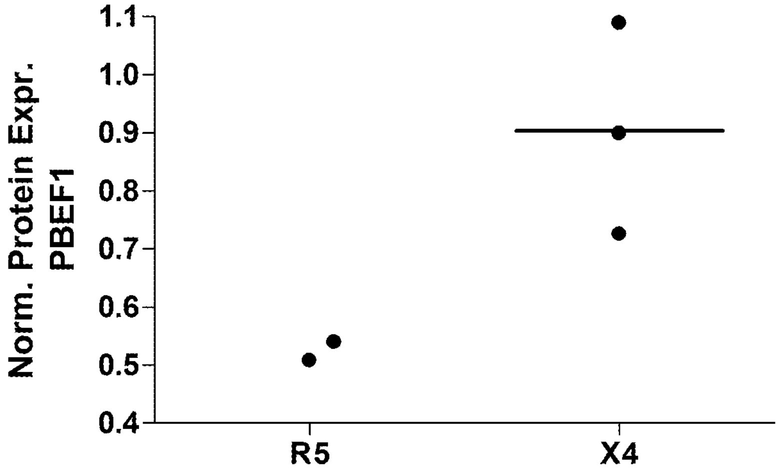


Figure 9



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/057025

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 G01N33/53

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/047408 A (PATHOLOGICA LLC [US]; HADLOCK KENNETH G [US]; DO HIEN KIM [US]; YU STE) 26 April 2007 (2007-04-26) paragraph [0032] paragraph [0046]; claims 15,16 paragraph [0091]	1-4

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

☒ See patent family annex.

* 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 document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search 10 September 2008	Date of mailing of the international search report 26/09/2008
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Authorized officer Reuter, Uwe

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAFAEL VAN DEN BERGH ET AL: "The Novel Adipocytokine Visfatin/PBEF1 is One of Several Apoptosis- and Lipid Metabolism-Associated Factors Induced in Monocytes during in vivo HIV-1 Infection" FIRST INTERNATIONAL SYMPOSIUM ONCGENETIC AND IMMUNE CORRELATES OF HIV INFECTION AND VACCINE-INDUCED IMMUNITY, [Online] 5 June 2007 (2007-06-05), XP002495322 Budapest Retrieved from the Internet: URL:http://www.diamond-congress.hu/hiv2007/binx/HIV2007_book_poster.pdf> [retrieved on 2008-09-10] webpage last modified on 05.06.2007 page 18 -----	1-4

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2008/057025

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007047408 A	26-04-2007	NONE	

Form PCT/ISA/210 (patent family annex) (April 2005) patsnap

专利名称(译)	新型脂肪细胞因子visfatin / PBEF1是VIVO HIV-1感染期间单核细胞诱导的凋亡相关因子		
公开(公告)号	EP2164983A1	公开(公告)日	2010-03-24
申请号	EP2008760600	申请日	2008-06-05
[标]申请(专利权)人(译)	根特大学 布鲁塞尔自由大学 INST VOOR TROPISCHE GENEESKUNDE		
申请(专利权)人(译)	VIB VZW 布鲁塞尔自由大学 INSTITUUT VOOR TROPISCHE GENEESKUNDE		
当前申请(专利权)人(译)	VIB VZW 布鲁塞尔自由大学 INSTITUUT VOOR TROPISCHE GENEESKUNDE		
[标]发明人	RAES GEERT DE BAETSELIER PATRICK VAN DEN BERGH RAFAEL VANHAM GUIDO		
发明人	RAES, GEERT DE BAETSELIER, PATRICK VAN DEN BERGH, RAFAËL VANHAM, GUIDO		
IPC分类号	C12Q1/68 G01N33/53		
CPC分类号	C12Q1/6883 C12Q2600/158 G01N33/56972 G01N33/56988		
优先权	60/933774 2007-06-08 US		
外部链接	Espacenet		

摘要(译)

本发明涉及单核细胞标志物在由HIV感染引起的疾病和综合征期间用于诊断，预后或治疗诊断应用的用途。更具体地，本发明涉及一种方法，包括分离单核细胞和确定基因表达，优选PBEF1基因表达。所述方法可用于确定疾病的进展或可用于评估治疗的功效。