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(74) Agent: **CABINET ORES**; 36 rue de St Pétersbourg,  
F-75008 Paris (FR).

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(71) Applicants (for all designated States except US): **INSTITUT PASTEUR** [FR/FR]; 28, Rue du Docteur Roux, F-75015 Paris (FR). **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE** [FR/FR]; 3, rue Michel Ange, F-75016 Paris (FR).

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(72) Inventors; and

(75) Inventors/Applicants (for US only): **ALCOVER, Andres** [ES/FR]; 65 avenue de la République, F-92120 Montrouge (FR). **PAIS CORREIA, Ana-Monica** [PT/FR]; 54 rue Saint Lambert, F-75015 Paris (FR). **THOULOZE, Marie-Isabelle, Bruna** [FR/FR]; 14 Square Adanson, F-75005 Paris (FR). **GESSAIN, Antoine** [FR/FR]; 116 avenue Felix Faure, F-75015 Paris (FR). **SACHSE, Martin** [DE/FR]; 98 avenue de Choisy, F-75013 Paris (FR). **GUADAGNINI, Stéphanie** [FR/FR]; 125 bis route de Guissey, F-91650 Breuillet (FR).

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(57) Abstract: The present invention relates to the field of virus transmission from cell to cell. More specifically, the present invention relates to the identification of a viral biofilm-like structure as a new mode of virus transmission and its use in methods of screening for a compound that affects formation and cohesion of such viral biofilm-like structure. The present invention also relates to compounds that affect virus transmission from cell to cell and use of such compounds in compositions and methods for the prevention and/or treatment of diseases or disorders associated with a virus inducing a biofilm-like structure.



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## **VIRALLY INDUCED BIOFILM-LIKE STRUCTURE AND USES THEREOF**

### **FIELD OF THE INVENTION**

The present invention relates to the field of virus transmission from cell to cell. More specifically, the present invention relates to the identification of a viral biofilm-like structure as a new mode of virus transmission and its use in methods of screening for a compound that affects formation and cohesion of such viral biofilm-like structure. The present invention also relates to compounds that affect virus transmission from cell to cell and use of such compounds in compositions and methods for the prevention and/or treatment of diseases or disorders associated with a virus inducing a biofilm-like structure.

### **BRIEF DESCRIPTION OF THE PRIOR ART**

Human T cell Lymphotropic Virus (HTLV) are a family of retroviruses including HTLV-1, HTLV-2, HTLV-3 and now HTLV-4. Human Immunodeficiency Virus (HIV) are a family of lentiviruses including HIV-1 and HIV-2.

HTLV-1 infects 15-20 millions people worldwide. Although most of the infected individuals are asymptomatic, 5-10 % develop T cell leukemia, or inflammatory syndromes such as HTLV-1 -associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Barmak, K., 2003) .

HTLV-1 transmission requires cell contacts (Okochi, K., 1984; Donegan, E., 1994). It was shown that HTLV-1 -infected lymphocytes polarized their microtubules and viral components upon contact with other T cells, forming virological synapses where HTLV-1 could spread from cell to cell (Igakura, T., 2003; Barnard, A.L., 2005; Nejmeddine, M., 2005; Majorovits, 2008).

Likewise, virological synapses mediate efficient cell-to-cell transmission of Human Immunodeficiency Virus (HIV-1) (Jolly, C., 2004; Sol-Foulon, N. 2007). Hence, HTLV-1 and HIV-1 appear to hijack the T cell

polarization machinery to direct virus budding to cell contacts, favoring virus transfer to target cells (Igakura, T., 2003; Majorovits, E., 2008, Sol-Foulon, N., 2007; Piguet, V., 2004) (**Figures 1,2**). Nonetheless, the formation of “polysynapses” by HIV-1 -infected T cells indicates that stable cell polarization is not absolutely required for HIV-1 transmission (Rudnicka, D., 2009). Unlike T cells, dendritic cells can be infected by cell-free HTLV-1 and transmit the virus to T lymphocytes (Jones, K.S., 2008).

### SUMMARY

The present invention concerns a method of screening for a compound that affects formation or cohesion of viral biofilm-like structure, comprising the steps of:

- contacting a cell with a virus naturally involved in viral biofilm-like structure and at least one candidate compound; and
- identifying the compound that affects the formation of said viral biofilm-like structure or the cohesion of said biofilm.

The present invention also concerns a method of screening for a compound that affects virus transmission from cell to cell, comprising the steps of:

- infecting a cell with a virus naturally involved in viral biofilm-like structure for a time sufficient to form a viral biofilm-like structure;
- contacting said virus infected cell with a non-infected cell and at least one candidate compound; and
- identifying the compound that affects transmission of said virus from the infected cell to the non-infected cell.

The present invention further concerns the method of the invention, wherein the viral biofilm-like structure consists in a structure comprising:

- carbohydrate rich matrix such as sialyl-Lewis X or sLeX, and/or
- adhesion molecules, such as collagen or agrin and/or cellular linker proteins such as tetherin, galectin-3 or CD62.

The present invention further concerns the method of the invention, wherein the virus is a lymphotropic virus.

The present invention further concerns the method of the invention, wherein the virus is HTLV or HIV.

The present invention further concerns the method of the invention, wherein the lymphotropic virus consists of HTLV-1, or HIV-1.

5 The present invention further concerns a compound that affects formation and/or cohesion of virus biofilm-like structure obtained by contacting a cell with a virus naturally involved in virus biofilm-like structure formation or cohesion.

The present invention further concerns a compound that affects virus transmission from cell to cell obtained by contacting a cell with a virus naturally  
10 involved in virus biofilm-like structure formation or cohesion.

The invention further provides a composition comprising the compound of the invention and a pharmaceutical acceptable carrier.

The present invention also concerns a method for preventing and/or treating diseases or disorders associated with a virus inducing a biofilm-like structure in a  
15 subject, the method comprising the step of administering to the subject a therapeutically effective amount of the composition of the invention.

The present invention also provides the use of a therapeutically effective amount of a composition of the invention for preventing and/or treating a subject against a biofilm-like structure forming associated disease. In one embodiment,  
20 the subject is a human. In another embodiment, the subject is immunocompromised. In another embodiment, the biofilm-like structure forming associated disease is caused by a lymphotropic virus. In another embodiment, the lymphotropic virus is HTLV or HIV. In another embodiment, the virus is HTLV-1 or HIV-1.

## 25 BRIEF DESCRIPTION OF THE FIGURES

**Figure 1:** HTLV-1 cell to cell transmission; Intracellular localization of Gag p19 complexes in HTLV-1-infected T-cell conjugates. Naturally infected T-cells (CD4-) purified from the PBMCs of HTLV-1-seropositive donors. It shows the superposition of staining with anti- $\beta$ -tubulin mAb (dark grey) and anti-Gag p19

mAb (light grey). (1a) shows unpolarized Gag protein in isolated T-cell. (1b) - Confocal images showing transfer of Gag p19 protein from HTLV-1-infected T cells to uninfected T cells. Conjugates were allowed to form for 120 min. HTLV-1-infected CD4<sup>+</sup> and normal CD4<sup>+</sup> T cell, Gag p19 (dark grey). HTLV-1-infected T cells were marked with carboxyfluorescein succinimidyl ester (CFSE) (light grey). The transmission picture is superimposed on a 0.4- $\mu$ m confocal fluorescence single section. Scale bar, 5  $\mu$ m. (1c)- The microtubule organizing centre (light grey) lies adjacent to the polarized HTLV-1 Gag protein at the cell-cell junction. CD4<sup>+</sup>-CD8<sup>+</sup> T cell conjugates were allowed to form for 40 min. Confocal images showing HAM autologous T cell conjugates stained with mAbs against tubulin-alpha (light grey) and HTLV-1 Gag p19 (dark grey). CD8<sup>+</sup> T-cells were counterstained with Cy5-conjugated anti-CD8 antibody (arrows). Scale bars represent 5 micrometers (Igakura, T., 2003).

**Figure 2:** HTLV-1 cell to cell transmission; a scheme of HTLV-1 cell to cell transmission showing a virological synapse, as commonly described on the basis of the seminal work by Igakura (Igakura, T., 2003).

**Figure 3:** HTLV-1 cell to cell transmission – (a) Scheme of a new mode of virus spread with HTLV-1<sup>+</sup> cell (left) reaching target cell (right) within 5 minutes through a viral biofilm containing an extracellular matrix containing infectious particles embedded in extracellular material (b and c)- Chronically infected MT2 cells were mixed with CFSE-labeled Jurkat cells (grey), incubated for 5 min, then fixed, permeabilized and stained with anti-Gag p19 mAb followed by Cy3-coupled 2<sup>nd</sup> Ab. Arrows point Gag clusters on the surface of the infected cells, arrowheads point to Gag<sup>+</sup> clusters on the surface of target cells. Scale bar 5 microns.

**Figure 4:** The infectious viral particles encased in an extracellular matrix “cocoon”, enable their efficient and protected transfer between cells. Extracellular matrix components and linker proteins ensure the cohesiveness and adhesiveness of these infectious structures. Disrupting these structures by competition would

expose encased viral particles to the existing immune response and thus, facilitate the clearance of the virus.

**Figure 5:** The viral biofilm-like structure ensures the protected transfer of infectious viral particles from one cell to another one either through tight junctions or through more distant contacts, such as filopodia or nanotubes, recently shown to support transfer of retroviruses between cells (Sherer, 2007; Sowinski, 2008).

**Figure 6:** The viral biofilm-like structure ensures the rapid and efficient transfer of infectious viral particles from one cell to another one. Scanning electron microscopy of chronically infected CD4<sup>+</sup> T cells forming multiples contacts with target cells (Jurkat CD4<sup>+</sup> T cells). Conjugates were allowed to form for 5 min. Secondary electron image detection (SEI) allows visualization of the morphology of cells as well as biofilm like structures already adhered to the surface of target cells.

**Figure 7:** The viral biofilm-like structure ensures the rapid and efficient transfer of infectious viral particles from one cell to another one. Another scanning electron microscopy of chronically infected CD4<sup>+</sup> T cells forming multiples contacts with target cells (Jurkat CD4<sup>+</sup> T cells). Conjugates were allowed to form for 5 min. Secondary electron image detection (SEI) allows visualization of the morphology of cells as well as biofilm like structures already adhered to the surface of target cells.

**Figure 8:** Extracellular HTLV-1 assemblies are carbohydrate-rich structures. Confocal microscopy of CD4<sup>+</sup> T cells from HAM/TSP individuals showing cell surface glycoproteins stained with Lens culinaris (LCA), and viral proteins stained with a HAM/TSP serum and with Gag-p19 antibody. Projections of three-dimensional reconstructions are shown. Bottom panels show the volume of co-localization of LCA and serum staining (green + red means LCA + TSPser). Examples of infected (a,b) and non-infected cells (c) are shown. Representative of three experiments were carried out with cells from two different patients.

**Figure 9:** Extracellular matrix and linker proteins are enriched in HTLV-1 viral assemblies. Confocal microscopy of CD4<sup>+</sup> T cells from HAM/TSP individual

immunostained for sialylLewisX (sLeX) (grey, column B), for the extracellular matrix components depicted within panels and for viral proteins (Gag-p19 antibody or HAM-TSP serum) (grey, column A). Infected (left) and uninfected cells (middle) are shown. Projections of three-dimensional reconstructions are shown. Right panels show quantification of the absence (–), the presence (+), or the accumulation (++) of each matrix component in cells from several HAM/TSP subjects (depicted by #) and in C91/PL and MT2 cell lines. Data represent means  $\pm$  S.D. of two independent observer's counts. Representative of three experiments for (a), two for (b) and (d) and five for (c) and (e).

**Figure 10:** Composition of viral biofilms – Expression of SLeX, Collagen, Galectin are induced by the virus; Expression of Agrin and Tetherin is induced by the infection.

**Figure 11:** HTLV-1 extracellular viral assemblies are carbohydrate-rich structures.

(Table) Listed plant lectins were used to stain cell surface glycoproteins of CD4<sup>+</sup> T cells from two HAM/TSP individuals, C91/PL and MT2 cell lines. The inventors ranked the general intensity of lectin cell staining (+++; ++; +; -), as well as the presence and accumulation (Acc.) of lectin labeling in viral assemblies. (a) CD4<sup>+</sup> T cells from a HAM/TSP individual surface stained with PNA and for viral proteins using HAM/TSP serum (column A) and Gag-p19 mAb (column A, arrows). (b) CD4<sup>+</sup> T lymphocytes from a healthy donor were transfected with HTLV-1 molecular clone (pCMV-HT1). Cells were surface stained with LCA (column B) and for viral proteins using HAM/TSP serum (column A) and Gag-p19 mAb (column A, arrow). A projection of three-dimensional image reconstructions and the volume of colocalization are shown (green + blue means LCA + Gag-p19 mAb). (c) CD4<sup>+</sup> T cells from HAM/TSP individuals were surface stained with ConA. Total fluorescence intensity in infected and non infected cells was quantified. Mann-Whitney test, ( $p < 0.0001$ ) was used for statistical analysis. A representative experiment is shown out of two experiments carried out.

**Figure 12:** Clusters of viral components are on the surface of infected cells. (a,b)

Confocal microscopy of primary CD4<sup>+</sup> T cells from HAM/TSP individuals

showing cell surface glycoproteins stained with Con A and viral proteins stained with HAM/TSP serum and with Gag-p19 antibody. Medial optical sections (Mid. Sect.), or projections of three-dimensional reconstructions are shown. (3D proj.). (c,d) Scanning electron microscopy of primary CD4<sup>+</sup> T cells from HAM/TSP individuals showing Env-gp46 stained by immunogold (15 nm). Secondary electron image detection (SEI) allows visualization of cell morphology, whereas backscattered detection (YAG) allows gold particle detection. Arrows point the areas enlarged in lower panels. Drawings schematize the shape of the cell and the area displaying putative Env<sup>+</sup> viral clusters. (a,b) Cells displaying on their surface one, or several, viral protein clusters (arrows). (c,d) Env<sup>+</sup> virus clusters on the cell surface, or on the edge of the membrane lamellipodium that adheres the cell to the coverslip (arrows). Representative of ten experiments were carried out for a and b and three for c and d with cells from different subjects.

**Figure 13:** HTLV-1 viral components cluster at the cell surface and in the uropod. (a-c) Confocal microscopy of CD4<sup>+</sup> T lymphocytes from an asymptomatic HTLV-1 carrier (a), C91/PL (b) and MT2 (c) cell lines showing staining of surface glycoproteins with ConA (grey) and HTLV-1 proteins with HAM/TSP serum (thin arrows) and Gag-p19 mAb (thick arrows). Medial confocal sections (Mid. Sect.), or projections of three dimensional reconstructions (3D proj.) are shown. (d,e) CD4<sup>+</sup> T lymphocytes from HAM/TSP individuals stained with the uropod markers ICAM-1 (d) and P-ERM antibodies (e), and Gag-p19 (d), (e) antibody (arrows).

**Figure 14:** Scanning electron microscopy of extracellular viral assemblies. C91/PL (a), MT2 (b) and Jurkat cells (Jkt) (c) were stained by immunogold with Env-gp46 antibody. SEI detector allows the visualization of cell morphology and YAG detector the visualization of gold particles. Jurkat cells were used as negative control (c).

**Figure 15:** Extracellular viral assemblies on the surface of HTLV-1 -infected cells. (a,b) Transmission electron microscopy of MT2 cells on coverslips displaying Env-gp46 stained by immunogold before Epon embedding. (b) Enlargement of

the area framed in (a). Insets show areas framed in (b) and marked 1, 2. Black arrowheads show mature virus particles (dense core surrounded by an envelope labeled by gold particles). The white arrowhead shows “empty vesicles” lacking dense cores and gold labeling. The arrow points to the electron dense mesh, putatively extracellular matrix. (c) Cryo-sections of MT2 cell pellets showing immunogold staining of Env-gp46 (15 nm gold) and Gag-p19 (10 nm gold) (P = plasma membrane). (d–f) CD4<sup>+</sup> T cells from HAM/TSP individuals showing Gag-p19 costaining by immunofluorescence and immunogold (10 nm). Infected cells identified by fluorescence microscopy (d) were processed for transmission electron microscopy (e, f). The framed area in (e) is enlarged in (f). Arrowheads show mature virus particles (dense core surrounded by an envelope labeled by gold particles). Representative of five experiments were performed for (a) and (b), and three for (c) and (d–f).

**Figure 16:** Selective accumulation of extracellular matrix proteins in HTLV-1 viral assemblies. (a–c) Confocal microscopy of CD4<sup>+</sup> T cells from HAM/TSP subjects showing staining of fibronectin,  $\alpha$ -dystroglycan ( $\alpha$ -dysr.), or neuropilin 1 (Nrp-1) and viral proteins using HAM/TSP serum or Gag-p19 antibody. (d,e) MT2 and C91/PL showing staining of agrin and Gag-p19. Projections of three-dimensional reconstructions are shown in a–c and medial optical sections in d,e. (f) Scanning electron microscopy of C91/PL cells showing immunogold labeling of agrin (15 nm). Arrows indicate zones corresponding to enlarged areas. A representative experiment is shown out of three experiments carried out for (a–e) and two for (f).

**Figure 17:** Treatment with Heparinase III or metalloproteinases fractionates HTLV-1 extracellular viral assemblies. Confocal microscopy showing CD4<sup>+</sup> T cells from HAM/TSP individuals (a), and C91/PL (left) and MT2 (right) cell lines treated with heparinase III or with a metalloproteinase cocktail and stained for surface glycoproteins with ConA, and for viral proteins with HAM/TSP serum and Gag-p19 antibody. Representative of three experiments carried out.

**Figure 18:** HTLV-1 extracellular viral assemblies spread at cell contacts. (a) Confocal microscopy of CD4<sup>+</sup> T cell conjugates from HAM/TSP individuals

showing cell surface glycoproteins stained with Con A (grey), and viral proteins stained with HAM/TSP serum (very light grey) and with Gag-p19 antibody (arrows). Conjugates between infected and uninfected cells were identified by the presence of cortical serum + Gag-p19 labeling, and stronger Con A staining of infected cells (i), compared to noninfected cells (ni) (see also **Figure 20**). Merge images of XY-medial optical sections, XY-projection of a three-dimensional reconstruction and XZ-medial optical section are shown. (b) CD4<sup>+</sup> T lymphocytes from HAM/TSP individuals co-cultured for 1 h with healthy donor CD4<sup>+</sup> T lymphocytes labeled with DDAO-SE dye (thick arrows) (Sol-Foulon, N., (2007)), stained with ConA (grey) and with Gag-p19 antibody (thin arrows). Projections of three-dimensional reconstructions are shown. Arrows point to Gag<sup>+</sup> clusters at the cell surface and at the side of the contact. (c) MT2 cells cocultured with CFSE-labeled Jurkat cells (Jkt, grey) incubated for 10 min, or 1 hr, then stained with Gag-p19 mAb. A medial optical section is shown. Gag<sup>+</sup> clusters on the surface of the infected cells (arrows) and of target cells (arrowheads) are shown. (d) Percentage of Jurkat target cells with Gag<sup>+</sup> clusters *versus* time of co-culture. (e) Percentage of cells conjugates displaying Gag clustered on the side or in the middle of the synapse *versus* time of co-culture. (f) T lymphocytes from HAM/TSP individuals immunogold labeled for Env gp46. (g) MT2 cells (chronically infected cells) in contact with Jurkat cells (Jkt) (target T cells) for 5 min. Right sub-panels are higher magnification of the framed areas. SEI and YAG detections reveal, respectively, cell morphology and gold particles. Representative of ten experiments were performed for (a), three for (b), (c), (f), five for (g) and two for (d), (e).

**Figure 19:** Confocal microscopy of CD4<sup>+</sup> T cell conjugates from HAM/TSP individuals showing cell surface glycoproteins stained with Con A (grey), and viral proteins stained with HAM/TSP serum (arrows) and with Gag-p19 antibody (arrows). Conjugates between infected and uninfected cells were identified by the presence of cortical serum + Gag-p19 labeling, and stronger Con A staining of infected cells, compared to noninfected cells. Extracellular

viral assemblies accumulate not only at the junction of the cells but also outside the contact zone.

**Figure 20:** Cell-to-cell transfer of extracellular viral assemblies. (a-c) Confocal microscopy of CD4<sup>+</sup> T cells from HAM/TSP individuals showing staining of surface glycoproteins with ConA and viral proteins with HAM/TSP serum and Gag-p19 antibody. The confocal microscope was set to observe Gag-p19 fluorescence in the cell cortex. Extracellular viral assemblies are therefore saturated. Infected cells in conjugates display cortical Gag-p19 staining, as well as higher ConA staining compared with non-infected cells from the same individual. (d-f) CD4<sup>+</sup> T cells from HAM/TSP individuals stained for tetherin, agrin or collagen and viral proteins with Gag-p19 antibodies. A representative experiment is shown out of five experiments carried out for (a-c) and two for (d-f).

**Figure 21:** Transfer of extracellular HTLV-1 assemblies at cell contacts. Transmission electron microscopy showing a MT2-Jurkat cell conjugate stained for Env-gp46 by immunogold. (a) Low magnification of a cell conjugate displaying extracellular viral assemblies spreading from the surface of the MT2 (chronically infected T cell) to the Jurkat (Jkt) cell (target T cell) at the side of the cell contact (white arrow). (b,c) High magnification of the center (b) and the lateral area (c) of the cell contact marked by frames in (a). Mature viral particles displaying dense cores and Env-gold labelling are pointed by arrowheads. The black arrow points to the mesh of electron dense material, putatively extracellular matrix. A representative experiment is shown out of five experiments carried out.

**Figure 22:** Relevance of extracellular HTLV-1 assemblies for cell-to-cell transmission. Viral assemblies can be removed by cell washing. C91/PL cells, or primary CD4<sup>+</sup> T cells from HAM/TSP patients were left unwashed, or washed by extensive pipetting, dilution and centrifugation in the absence (washed), or in the presence of heparin (washed + heparin). (a) Schematic representation of the experiment. (b) FACS on cells; Flow cytometry measurements of Gag-p19 that remained associated to C91/PL. (c): ELISA on supernatants; ELISA measurements of Gag-p19 released into the supernatant

of C9 1 /PL cells after the different wash treatments. (d–g) Luciferase activity of reporter Jurkat cells co-cultured for 24 h, with the following: (d) C9 1 /PL cells that underwent the different wash treatments; (e) C9 1/PL cells in the presence or absence of AZT; (f) 100x-concentrated supernatants from C91/PL cells that underwent the different wash treatments; (g) HAM/TSP primary CD4<sup>+</sup> T cells that underwent the different wash treatments. Data represent means  $\pm$  S.D. of triplicates. Representative experiment out of four experiments for (b–d), three for (e) and (f), and two for (g).

**Figure 23:** Cell washes do not perturb conjugate formation. Phase-contrast microscopy of C91/PL infected cells left unwashed (left), or washed mechanically, by pipetting, dilution and centrifugation, in the absence (middle), or in the presence (right) of heparin. After washing out the heparin, infected cells were co-cultured with the luciferase reporter cell line at a 1:2 ratio similarly to the experiment in **Figure 22**. A representative experiment is shown out of two experiments carried out.

**Figure 24:** Function of the viral biofilm in viral transmission. Infectious viral particles (a) are embedded in an extracellular matrix cocoon adhering to the cell surface that can be detached by specific enzymatic treatments, such as heparinase III (10 U/ml) (b).

**Figure 25:** Scheme summarizing a major pathway for HTLV-1 cell-to cell transmission. Extracellular adhesive structures are composed of infectious particles embedded in an extracellular matrix, forming protective carbohydrate-rich structures which rapidly adhere and infect other lymphocytes.

**Figure 26:** Comparison of viral biofilm-like structure (HTLV-1) (a) and bacterial biofilm (*Bordetella bronchiseptica*), (b) (Parise, 2007) both composed of infectious microbes embedded in an extracellular matrix, forming a structure that stocks, concentrates, protects and disseminates a microorganism.

**Figure 27:** Polarized budding of HIV-1 (T lymphocytes )- T cells were also apparently induced to form microvilli by adhering to plastic, as prominent microvilli were observed at the base of the cells. The most interesting aspect of adhesion to

plastic was polar secretion of HIV. This phenomenon was striking when cells were viewed with the SEM. In many cells, hundreds of virions were seen in the area immediately adjacent to the plastic, among microvilli, and on the plastic adjacent to the cell. However, virions were virtually absent from the rest of the cell surface (a)

5 SEM of a MOLT/HIV-1<sub>IIIB</sub> cell 40 min after being placed onto plastic in serum-free medium. The cell has an irregular shape, similar to cells with colchicine. (b) Higher magnification of the same cell. Microvilli emanate from the base of the cell. Numerous virions are seen on the base of the cell. Magnifications: (a) x 6,000;(b) x 17,000. (Pearce-Pratt, 1994)

10 **Figure 28:** Polarized budding of HIV-1 (T lymphocytes ); TEM through the base of a MOLT4/HIV-1<sub>IIIB</sub> cell 40 min after being placed onto plastic in serum-free medium. The matrix material (m) indicates that this section has transected the cell immediately above the plastic. Numerous mature and budding virions (arrows) are observed in association with the plasmalemma. Magnification, x 18,000.  
15 (Pearce-Pratt, 1994)

**Figure 29:** TEM of a MOLT4/HIV-1<sub>IIIB</sub> cell 40 min after the addition of colchicine. Virions are observed exclusively on the tip of the pseudopod (arrows). Magnification, x8,000.(Pearce-Pratt, 1994)

**Figure 30:** Polarized budding of HIV-1 (monocytes) (a)- Scanning electron  
20 micrograph of an HIV-infected (strain P1-2), activated monocyte. The surface of most of the monocytes is characterized by irregular microvilli. However, the monocyte's pseudopod (lower left) is covered with small spherical structures which appear to be HIV virions. Magnification, x4,650. (b) - Transmission electron  
micrograph of an activated monocyte showing a pseudopod with associated (strain  
25 O/S) virions (arrows). (c) Higher magnification of the cell shown in panel (b) showing mature HIV virions associated with the pseudopod. Magnification, 33,135  
(b); 338,950 (c).(Perotti, M-E,1996).

**Figure 31:** Use according to claim 10, wherein the biofilm-like structure forming associated disease is caused by Clusters of viral particles were localized on the  
30 surface of HIV-1-infected cells. Confocal microscopy of HIV-1 infected primary

CD4<sup>+</sup> T cells showing cell surface glycoproteins stained with Con A and viral proteins stained with Gag-p17 antibody. Medial optical sections are shown. Gag-p17 is the process form of the viral protein Gag only present in mature viral particles.

- 5 **Figure 32:** Extracellular HIV-1 assemblies are carbohydrate-rich structures. Confocal microscopy of HIV-1-infected CD4<sup>+</sup> T cells showing cell surface glycoproteins stained with *Glycine max* lectin, and viral proteins stained with Gag-p17 antibody. Projections of three-dimensional reconstructions are shown.

**Figure 33:** HIV-1 extracellular viral assemblies spread at cell contacts.

- 10 Confocal microscopy of HIV-1 infected CD4<sup>+</sup> T cell conjugated with non-infected T cell showing cell surface glycoproteins stained with Con A, and viral proteins stained with Gag antibody. Merge images of XY-medial optical sections and a XY-projection of a three-dimensional reconstruction are shown. Arrows point to Gag<sup>+</sup> clusters at the cell surface incubated for 30 min,  
15 then stained with Gag antibody.

#### DETAILED DESCRIPTION OF THE INVENTION

- The inventors have surprisingly found that virus cell-to-cell transmission (such as HTLV-1 or HIV-1 cell-to-cell transmission) does not necessarily occur directly through synaptic contacts. Rather, HTLV-1 virions  
20 bud at the plasma membrane and are transiently kept in adhesive extracellular structures rich in carbohydrates and composed of virally-induced extracellular matrix and linker proteins whose synthesis and/or rearrangement are virally induced. These are reminiscent of bacterial biofilms (Stewart, P.S. 2008). These extracellular viral assemblies rapidly adhere during cell contacts to other  
25 lymphocytes supporting their infection. The present findings uncover a novel and major mode of virus cell transmission which may serve as a model to develop new anti-viral agents to prevent or treat diseases or disorders associated with viruses capable of forming a viral biofilm-like structure (**Figures 3, 4, 5, 6, 7**).

As used herein, the expression "viral biofilm-like structure" refers to a virally induced extracellular structure comprising viral particles, extracellular matrix, linker proteins and/or adhesion molecules with carbohydrate moieties.

#### **1. Methods of screening/selection and compounds**

5 According to an aspect of the invention, there is provided a method of screening for a compound that affects formation of viral biofilm-like structure. The method comprises a step of contacting a cell with a virus naturally involved in viral biofilm-like structure and at least one candidate compound, and a step of identifying the compound that affects the formation of the viral biofilm-like  
10 structure or the cohesion of such biofilm.

As used herein, the expression "formation of virus biofilm-like structure" refers to either already formed biofilm or to a virus infected cell which is in conditions that induces or facilitates the formation of a viral biofilm.

According to a related aspect, the invention is thus concerned with a  
15 compound that affects formation and cohesion of viral biofilm-like structure obtained by the above detailed method.

One of the art will understand that the above mentioned contemplated compound of the invention may for instance inhibit the formation of the carbohydrate rich matrix, or interfere with the clustering of adhesion molecules in  
20 the viral biofilm-like structure. Such adhesion molecules may consist for instance of collagen, agrin or cellular linker proteins such as tetherin, galectin-3 and CD62.

According to another aspect of the invention, there is provided a method of screening for a compound that affects virus transmission from cell to cell. The method comprises a step of infecting a cell with a virus naturally involved in viral  
25 biofilm-like structure for a time sufficient to form a virus biofilm-like structure, a step of contacting the virus infected cell with a non-infected cell and at least one candidate compound, and a step of identifying the compound that affects transmission of said virus from the infected cell to the non-infected cell.

According to a related aspect, the invention is also concerned with a compound that affects virus transmission from cell to cell obtained by the previously mentioned method.

One of the art will understand that such a contemplated compound of the invention may for instance interfere with the adherence and/or the cohesion of the viral biofilm to other cells upon cell contact. More particularly, the contemplated compound may inhibit :

- the binding of the molecule (e.g. linker protein) forming the biofilm at the surface of the infected cell to the surface of the target cell and/or
- the binding and the interaction of the molecules of the biofilm among them.

It will be understood that, in accordance with the present invention, the term "cell" refers to any cell that is capable of being infected by a virus which naturally forms a viral biofilm-like structure. For instance, such a cell may be a lymphocyte.

It will be further understood that the virus used in accordance with the present invention may be for instance a lymphotropic virus, such as HTLV or HIV, and not limited to HTLV-1 or HIV-1.

As used herein, the terms "host", "subject", "patient" are synonymous.

## **2. Methods of use and compositions**

The compounds obtained by the screening/selecting methods of the invention may be used in many ways in the prevention and/or treatment of diseases or disorders associated with viruses inducing biofilm-like structure.

In this connection, another embodiment of the present invention relates to a composition for preventing and/or treating such diseases or disorders, and more particularly for preventing and/or impairing the formation and/or the cohesion of virally-induced biofilm-like structures. The composition of the present invention advantageously comprises a compound of the invention and an acceptable carrier.

As used herein, the term "impairing" refers to a process by which the formation or development of a virally-induced biofilm-like structure is affected or completely destroyed. As used herein, the term "preventing" when referring to

biofilm formation, refers to a process by which the formation, the cohesion, or the development of a virally-induced biofilm-like structure is obstructed or delayed.

When referring in general to diseases or disorders associated with a virus inducing a biofilm-like structure, the terms "preventing" or "prevention" refer to a process by which the symptoms of such diseases or disorders is obstructed or delayed. By the term "treating", it is intended, for the purposes of this invention, that the symptoms of such diseases or disorders be ameliorated or completely eliminated.

As used herein, the expression "an acceptable carrier" means a vehicle for containing the compounds obtained by the method of the invention that can be administered to a subject without adverse effects. Suitable carriers known in the art include, but are not limited to, gold particles, sterile water, saline, glucose, dextrose, or buffered solutions. Carriers may include auxiliary agents including, but not limited to, diluents, stabilizers (i. e., sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, viscosity enhancing additives, colors and the like.

Further agents can be added to the composition of the invention. For instance, the composition of the invention may also comprise other anti-viral agents well known in the art.

The amount of compounds obtained by the method for preventing and/or treating diseases or disorders associated with viruses inducing biofilm-like structure according to the invention is preferably a therapeutically effective amount. A therapeutically effective amount of compound obtained by the screening/selecting method of the invention is the amount necessary to allow the same to perform their inhibitor role in accordance with the present invention without causing overly negative effects in the host to which the composition is administered. The exact amount and/or dosage of compounds obtained by the method of the invention to be used and the composition to be administered will vary according to factors such as the mode of administration, the patient's weight, age, or condition, as well as the other ingredients in the composition.

The composition of the invention may be given to a subject through various routes of administration. For instance, the composition may be administered in the form of sterile injectable preparations, such as sterile injectable aqueous or oleaginous suspensions. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparations may also be sterile injectable solutions or suspensions in non-toxic parenterally-acceptable diluents or solvents. They may be given parenterally, for example intravenously, intramuscularly or sub-cutaneously by injection, by infusion or *per os*. Suitable dosages will vary, depending upon factors such as the amount of each of the components in the composition, the desired effect (short or long term), the route of administration, the age, the condition, and the weight of the subject to be treated. Any other methods well known in the art may be used for administering the composition of the invention particularly to avoid contamination via biologic fluids such as milk or sperm.

Yet, another embodiment of the invention is to provide a method for preventing and/or treating diseases or disorders associated with a virus inducing a biofilm-like structure in a subject, the method comprising the step of administering to the subject (e.g. a human which may be immunocompromised) a therapeutically effective amount of a composition as defined above.

The present invention will be more readily understood by referring to the following examples. These examples are illustrative of the wide range of applicability of the present invention and are not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred methods and materials are described. References cited throughout this description are incorporated by reference.

## **EXAMPLE 1: Biofilm-like extracellular viral assemblies mediate HTLV-1 cell-to-cell transmission at virological synapses**

### **Introduction**

Human T cell leukemia virus type 1 (HTLV-1) is a lymphotropic retrovirus whose cell to cell transmission requires cell contacts. HTLV-1-infected T lymphocytes form "virological synapses", but the mechanism of HTLV-1 transmission remains poorly understood. The inventors show here that HTLV-1-infected T lymphocytes transiently store viral particles in extracellular platforms that remain adhered to the cell surface. The viral particles are protected being poorly accessible to antibodies and proteases in the extracellular milieu. These carbohydrate rich extracellular assemblies are held together and attached to the cell surface by virally induced extracellular matrix components, including collagen and/or agrin, and/or cellular linker proteins, like tetherin, galectin-3 and/or CD 62. Extracellular viral assemblies rapidly adhere to other cells upon cell contacts allowing virus spread and infection of target cells. Their removal strongly reduces the ability of HTLV-1-producing cells to infect target cells. The present results unveil a novel virus transmission mechanism based on the generation of extracellular viral particle assemblies whose structure, composition and function resemble those of bacterial biofilms. HTLV-1 biofilm-like structures represent a major route for virus transmission from cell to cell.

### **MATERIALS and METHODS**

**Cells lines and reagents.** HTLV-1-infected cell lines MT2 and C91/PL, and HTLV-1 Env gp46 (0.5 $\alpha$ ) mAb were obtained from the NIH AIDS Research and Reference Reagent Program. Jurkat cells clone J77cl20 was previously described (Thoulouze, M.I., 2006). Rabbit Ab to Agrin (C95) was a gift from M. Rüegg (University of Fribourg, Switzerland). Mouse Env gp46 4D4 antibody (Grange, M.P., (1998)) was a gift of C. Pique (Institut Pasteur, Paris). Mouse mAb to Gag-p19 (TP-7) was obtained from Zeptometrix. Serum from a HAM/TSP individual (#1378) with strong reactivity against a

panel of viral proteins (HTLV-1 Blot Kit, Genelabs Diagnostics) was used (AG, unpublished). HTLV-1 protein labeling was not found neither in cells from healthy donors, nor in non-infected T cell lines, nor in a percentage of cells from HTLV-1 -infected patients, supporting the specificity of the inventor's antibodies to HTLV-1 proteins (**Figures 8, 9, 10**). CD1 5s (CSLEX1) and ICAM-1 (LB2) mAbs were obtained from Becton-Dickinson. Rabbit polyclonal Ab to human collagen I/II/III/IV/V was obtained from AbD Serotec. Mouse mAb to galectin-3 (clone A3A1 2) was obtained from Affinity BioReagents. Goat Abs to BST-2 (tetherin) (K- 15 and N-17) were obtained from Santa Cruz Biotechnology. Cyanine 3 (Cy3)-coupled Abs to mouse IgG2b, goat IgG, human Ig, mouse Ig and fluorescein-coupled mouse IgG2a, were obtained from Jackson ImmunoResearch. Fluorescein-coupled mouse IgG1 was obtained from Southern Biotechnology. Alexa<sup>488</sup>-coupled Ab to fluorescein was obtained from Molecular Probes. Colloidal gold protein A was obtained from the University Medical Center Utrecht, The Netherlands. Phycoerythrin-coupled Ab to mouse IgG was obtained from Beckman-Coulter. Metalloproteinases MMP Multipack- 1, was obtained from Biomol. Heparinase III was obtained from Sigma. Plant lectins (**Figure 11**), ConA, LCA, UEAI, SBA, PNA, WGA and PHA-E were obtained from Sigma and HHL was obtained from Vector Laboratories. HTLV-1 expression plasmid containing the wild-type proviral clone XMT, allowing the expression of the full-length HTLV-1 genome under the control of the cytomegalovirus immediate early promoter (Derse, D., (1997)) was a kind gift from Dr Frederic Dellebecque (Institut Pasteur, Paris).

**Isolation of primary CD4<sup>+</sup> T lymphocytes.** The inventors purified peripheral blood mononuclear cells through Ficoll-Hypaque centrifugation and isolated CD4<sup>+</sup> T cells by negative selection, using magnetic cell sorting (Miltenyi Biotec, MACS). The inventors placed the CD4<sup>+</sup>-enriched T cell population in culture for 18 hours as previously described (Igakura, T., 2003; Nejmeddine, M., 2005). The inventors analyzed cells from seventeen HAM/TSP subjects

and two asymptomatic carriers that contained 2-20 % of HTLV-1 -infected cells per sample, as assessed by immunofluorescence against viral proteins after 18 h of *ex vivo* culture.

**Heparinase and MMP treatments.** Cells were treated with heparinase III (10 U/ml) (Hep. III), or with a cocktail of metalloproteinases (10 nM) (MMP) for 1h at 37°C in serum-free medium.

**Immunofluorescence, confocal microscopy analysis and quantification of fluorescence intensity.** The inventors performed them as previously described (Thoulouze, M.I., (2006)). Cells were fixed with 4 % paraformaldehyde for 30 min at room temperature. Surface staining (e.g., lectin staining) was performed in the absence of detergent. Intracellular cellular proteins and Gag-p19 were stained incubating fixed cells in solutions containing 0.05 % saponin. Before staining, the inventors blocked nonspecific protein binding by incubating the coverslips for 15 min in 5% FCS 0.05% saponin in PBS. For collagen staining, the inventors treated cells after paraformaldehyde fixation with 100 % methanol, 40 min at -20°C. The inventors carried out confocal microscopy analysis on a Zeiss LSM5 10 using a x 63 objective. Z-series of optical sections at 0.2 µm increments were acquired. The inventors treated the images by deconvolution using Huygens software in order to reduce the fluorescence noise of the images. Three-dimensional image reconstructions of cells and the calculation of the volume of co-localization were done with Imaris software.

**Scanning electron microscopy.** Cell suspensions containing 10<sup>6</sup> cells/slide were put onto poly-L-lysine-coated coverslips, let to sediment for 3 min, centrifuged at 47 x g for 1 min and fixed for 20 min at room temperature in 4 % paraformaldehyde. Samples were washed in phosphate buffered saline (PBS) and incubated for 10 min with 50 mM NH<sub>4</sub>Cl in PBS. Samples were then washed in PBS 1 % BSA and incubated for 1 h with the anti-HTLV-1 Env gp46 (0.5 µg/ml) in PBS 1 % BSA. After washing in PBS 0.1 % BSA and in PBS 1 % BSA, samples were incubated for 15 min with

protein A coupled to 15 nm or 20 nm gold particles, washed in PBS and fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, overnight at 4°C. Cells were washed 3 times in 0.2 M cacodylate buffer (pH 7.2) for 5 min each, post-fixed for 1 h in 1 % (wt/vol) osmium tetroxide in 0.2 M cacodylate buffer, and then rinsed with distilled water. Samples were dehydrated through a graded series of 25, 50, 75 and 95 % ethanol solution for 5 min each time and 10 min in 100 % ethanol followed by critical point drying with CO<sub>2</sub> in a CPD Baltec apparatus. The dried specimens were mounted on stubs with carbon tape and ion sputtered with 15 nm carbon layer. Analysis of Secondary Electron Image (SEI) and backscattered images (YAG detector) was performed on a Jeol JSM 6700F microscope with a field emission gun operating at 5 kV.

**Transmission electron microscopy.** Before being contacted with HTLV-1 chronically infected MT2 cell suspensions, Jurkat cells were incubated for 1h with BSA coupled to 6 nm gold (Aurion, The Netherlands) with a final OD<sub>520</sub> of 2. Non-endocytosed BSA-gold was washed away with warm medium containing 10% FCS. MT2 cells were mixed with Jurkat cells at 1:1 ratio for different times and cell suspensions (10<sup>6</sup> cells/cover slip) were put onto poly-L-lysine-coated cover slips, let to sediment for 3 min, centrifuged at 47 x g for 1 min and fixed with 2% formaldehyde in 0.1 M phosphate buffer pH 7.4 for 5 min and then for 1h with 4 % formaldehyde in the same buffer. After fixation, remaining free aldehydes were quenched for 10 min in 50 mM NH<sub>4</sub>Cl. Cells were then labeled using saturating concentrations of the human anti-Env gp46 (0.5 mA) antibody and protein A coupled to 10 nm gold. The samples were then fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 before post-fixation with OsO<sub>4</sub>. During dehydration in a graded series of EtOH samples were stained with 1.2% uranyl acetate in 70% EtOH and after dehydration embedded in Epon and polymerized at 60°C for 48h. After polymerization, blocks were sectioned with a Leica ultramicrotome. After staining with uranyl acetate and lead citrate,

samples were observed at 80 kV with a Jeol 1010 microscope and images were acquired with a CCD camera (Keenview, SoftImaging). For immunolocalization on thawed cryo sections, cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. Processing of cells for ultrathin cryosectioning and double  
5 immunolabeling according to the protein A-gold method was done as described by Slot et al. (1991).

**Correlative immunofluorescence and transmission electron microscopy.**

Primary CD4<sup>+</sup> T cells from HAM/TSP patients cell suspensions, prepared as described above, were sedimented on MatTek glass bottom dishes, containing  
10 gridded cover slips to localize the cells of interest. Cells were fixed with 2% formaldehyde and 0.1% glutaraldehyde in 0.2 M Hepes buffer, pH 7.4 for 5 min, washed with 4% formaldehyde and fixation was continued for 30 min with 4% formaldehyde in 0.2 M Hepes, pH 7.4. After quenching of free aldehyde groups with NH<sub>4</sub>Cl (50 mM), cells were permeabilized with 0.05 % saponin in PBS and  
15 labeled with mouse anti-Gag p19 antibody, followed by goat anti-mouse coupled to Alexa-488 and 10 nm gold (Invitrogen). After acquisition of images in a fluorescence microscope (Leica SP5), samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 and embedded in EPON as described above. After polymerization, the cell of interested was localized and  
20 the area containing the cell was prepared for sectioning with a Leica ultramicrotome. After staining with uranyl acetate and lead citrate, all samples were observed at 80 kV with a Jeol 1010 microscope and images were acquired with a CCD camera (Keenview, SoftImaging).

**Cell transfection.** The inventors isolated peripheral blood CD4<sup>+</sup> T cells  
25 from healthy donors as described above, and then transfected the pCMV-XMT plasmid (gift of F. Delbecque (Institut Pasteur, Paris) using Amaxa according to the manufacturer's instructions. The inventors analyzed the transfected cells by fluorescence microscopy after 24 hrs of culture.

**Detachment of extracellular virus assemblies from infected cells.** The  
30 inventors left HTLV-1- infected cells unwashed, or washed three times in

RPML-1640 serum free medium and incubated 1 h at 37°C in the absence or in the presence of 50 µg.ml<sup>-1</sup> heparin (Sigma-Aldrich) in serum-free medium. After washing three times in RPML-1640 10 % FCS, the inventors processed cells for immunofluorescence analysis and flow cytometry, or to infect  
5 luciferase reporter gene target cells. The inventors detected viral antigens on cell culture supernatants by Gag-p19 ELISA according to the manufacturer's instructions (Zeptometrix).

**Luciferase reporter gene assay.** The inventors stably transfected Jurkat cells with a plasmid containing the luciferase gene under the control of HTLV-1 LTR  
10 (gift of R. Mahieux (Institut Pasteur, Paris)). The inventors co-cultured Luciferase reporter cells, in a 96-cell plate round bottom, with HTLV-1-infected cells at a cell ratio of 2:1, respectively. After 24 hours, the inventors assessed luciferase activity using a Promega luciferase kit assay and a TR717 Microplate Luminometer (Berthold Technologies). When indicated, reporter  
15 cells were incubated for 48 h with the reverse transcriptase inhibitor AZT at 50 µM prior to coculture with HTLV-1-infected cells. AZT was left in the co-culture.

## RESULTS

### **Viral components cluster on the surface of infected cells**

20 HTLV-1 -infected lymphocytes were shown to cluster Gag and Env viral proteins at the cell cortex, whereas Tax was localized at the microtubule-organizing center (MTOC) and in the nucleus (Igakura, T., 2003; Nejmeddine, M., 2005; Mazurov, D., 2006). Upon contact with other T cells, infected cells polarized their MTOC, and Gag, Env and Tax proteins towards the  
25 cell contact. Then, viral components appeared in non infected cells, indicating that transfer of virus to target cells had occurred. This supported the notion of virological synapses as being virus-induced organized cell contacts through which HTLV-1 was transmitted from cell to cell (Igakura, T., 2003; Nejmeddine, M., 2005; Majorovits, E., 2008). However, the precise localization of

viral components and the subcellular compartments involved remained ill defined.

The inventors therefore carried out confocal and electron microscopy analyses in primary CD4<sup>+</sup> T cells from HTLV-1 -infected individuals, and in the  
5 chronically infected cell lines C91 /PL and MT2. Gag-p19 was shown to cluster in discrete areas of the cell cortex (Igakura,, T., 2003; Nejmeddine, M., 2005; Mazurov, D., 2006) that could be intracellular compartments where viral particles assembled and/or accumulated ready to be transferred to target cells. To localize viral proteins with respect to the plasma membrane, the inventors  
10 used sera from HAM/TSP individuals reactive against various viral proteins, and Gag-p19-specific monoclonal antibody (mAb). The inventors then labeled cell surface glycoproteins with the lectin Concanavalin-A (ConA). To obtain maximal image definition, deconvolution of confocal images and three-dimensional reconstruction were performed.

15 Strikingly, cortical clusters of viral proteins were on the cell surface, appearing as single or multiple clusters, faintly labeled by ConA (**Figure 12a,b**). Similar results were found in samples from all the seventeen HAM/TSP subjects and two asymptomatic carriers studied (**Figure 13** and data not shown). The inventors did not detect viral protein clusters inside infected  
20 cells, although their plasma membrane appeared evenly and dimly stained by HAM/TSP sera and by Gag-p19 antibodies (**Figure 12b**). Notably, ConA staining was significantly higher in HTLV-1 positive cells (**Figure 11**). Finally, MT2 or C91 /PL cells also displayed viral protein clusters at the cell surface (**Figure 12b,c**). In polarized cells, viral proteins were often found on the  
25 uropod, as assessed by the enrichment in ICAM-1 and phosphorylated ERM (ezrin-radixin-moesin) (**Figure 13d,e**), suggesting that adhesion and cytoskeletal structures may maintain viral components polarized on the cell surface.

### **Viral protein clusters correspond to extracellular viral assemblies**

By scanning electron microscopy and immunogold staining of Env glycoprotein (gp46), cells from HAM/TSP individuals displayed putative Env<sup>+</sup> viral particle clusters embedded in a smooth material (**Figure 12c,d**). Virus clusters were of different sizes and could be observed at the edge of membrane extensions (**Figure 12d**). Similar Env<sup>+</sup> virus clusters were observed in C91/PL and MT2, but were absent from uninfected lymphocytes (**Figure 14a–c**). These data suggested that extracellular clusters of Env<sup>+</sup> viral particles were at the surface of infected cells. However, the inventors could not rule out that Env glycoprotein shed from viral particles could concentrate in heparan-sulfate-proteoglycan-enriched areas (Jones, K.S., 2005). The inventors therefore performed transmission electron microscopy (**Figure 15**). The inventors observed on the cell surface of MT2 cells mature viral particle assemblies, displaying electron-dense cores and Env gold-labeling (**Figure 15b**, black arrowheads), as well as empty vesicles (**Figure 15b**, white arrowhead). Moreover, the inventors observed a mesh of electron-dense material among the viral particles, putatively, extracellular matrix (**Figure 15b**, arrow). Moreover, double immunogold staining on thawed cryo-sections of MT2 cell pellets revealed extracellular assemblies of mature viral particles containing both Env and Gag-p19 (**Figure 15c**, arrowheads). Finally, cells from HAM/TSP individuals were observed using correlative immunofluorescence and transmission electron microscopy (**Figure 15d–f**). Positive cells by immunofluorescence (**Figure 15d**) displayed extracellular Gag-p19 immunogold-labeled mature viral particles with membrane and dense core (**Figure 15f**, arrowheads).

Altogether, the light and electron microscopy observations show the presence of extracellular viral assemblies on the surface of HTLV-1 -infected T lymphocytes.

**Extracellular viral assemblies are carbohydrate-rich structures**

Extracellular matrix components provide attachments for viruses, including HTLV-1, facilitating their entry (Jones, K.S., 2005; Pinon, J.D., 2003). They could also facilitate the attachment and concentration of budding viral particles into extracellular viral assemblies. To investigate this, the inventors used plant lectins recognizing glycans enriched in the extracellular matrix (Neu, T.R., 1999; McClure, S.F., 1997 (**Figure 11**, table). The inventors took advantage of the fact that HTLV-1 gp46 is poorly glycosylated if compared with glycoproteins of many other viruses, including HIV- 1 gp 120 (Le Blanc, I., 2001; Coffin, J.M, 1997). The inventors observed that, like ConA (**Figure 12**), most lectins evenly stained the surface of uninfected cells (**Figure 11**, table and data not shown). However, *Lens culinaris* (LCA) and *Arachis hypogaeae* (PNA) lectins, although stained evenly uninfected cells, they much strongly stained extracellular viral assemblies (**Figure 8**). The colocalization between lectin labeling and viral markers was however weak, indicating that lectins do not primarily bind viral Env glycoprotein, but glycoconjugates enriched in extracellular viral assemblies (**Figure 8a,b** and **Figure 11a**). Likewise, uninfected primary T cells transfected with expression vectors encoding the full length HTLV-1 genome exhibited LCA labeling on viral protein clusters (**Figure 11b**). Finally, the inventors observed that sialyl-LewisX (sLeX), a tetrasaccharide involved in lymphocyte adhesiveness, was overexpressed upon HTLV-1 infection (Hiraiwa, N., 2003; Kambara, C., 2002), and concentrated in extracellular viral assemblies (**Figure 9a**).

Therefore, extracellular viral particles are embedded in a carbohydrate-rich structure that is induced and spatially reorganized by viral infection.

**HTLV-1 assemblies contain extracellular matrix and linker proteins**

The inventors next searched for extracellular matrix components in HTLV-1 assemblies. Both collagen and fibronectin are overexpressed in HTLV-1 infected cells in a Tax-mediated manner (Munoz, E., 1995; Yi, T., 2000). The inventors found collagen enriched in viral assemblies, but

randomly distributed on uninfected cells (**Figure 9b**). In contrast, neither fibronectin (**Figure 16a**), nor laminin (data not shown) were coclustered with virions. Agrin, a heparan sulfate proteoglycan that cross-links cell surface receptors and is involved in neural, immunological and viral synapses (Dityatev, A., (2006); Khan, A.A., (2001); Alfsen, A., (2005)), was also concentrated in viral clusters (**Figure 9c**, **Figure 16d–f**). Interestingly,  $\alpha$ -dystroglycan, a heparan sulfate proteoglycan that clusters with agrin in synaptic structures (Zhang, J., (2006)), did not accumulate (**Figure 16b**), indicating preferential concentration of some proteoglycans in these structures.

Finally, neuropilin, a transmembrane protein involved in HTLV-1 entry, present in HTLV-1 virological synapses (Ghez, D., 2006) and in immunological synapses (Tordjman, R., 2002), was not concentrated in extracellular viral assemblies (**Figure 16c**).

Finally, treatment of cells with heparinases, or metalloproteinases, that hydrolyze heparan sulfate proteoglycans and various extracellular matrix proteins, respectively, lead to the detachment and fractionation of HTLV-1 assemblies from the cells (**Figure 17 a–d**). This further supports the involvement of extracellular matrix in the generation of these structures.

The inventors next hypothesized that galectins,  $\beta$ -galactoside-binding lectins that form lattices that condition extracellular matrix properties, could also be involved. Galectin-1 and galectin-3 are secreted by T lymphocytes (Rabinovich, G.A., (2009)), and upregulated by HTLV-1 infection (Hsu, D.K., 1996; Gauthier, S., 2008). The inventors found galectin-3 (**Figure 9d**), but not galectin-1, clustered in viral assemblies at the surface of cells from HAM/TSP individuals. However, in MT2 and C9 1 /PL cells, galectin-3 was poorly expressed and did not cluster with viral particles.

Finally, the inventors found tetherin concentrated in extracellular viral assemblies (**Figure 9e**). Tetherin (BST-2/CD3 17), is an interferon-inducible transmembrane protein whose degradation by the HIV-1 Vpu protein provokes an extracellular clustering of HIV-1 virions reminiscent of the extracellular HTLV-1

assemblies shown here (Neil, S.J., 2008; Van Damme, N., 2008). Since, HTLV-1 lacks *vpu*-like sequences, tetherin may facilitate virion attachment to the cell surface. Consistently, it was shown that Vpu expression enhanced HTLV-1 release (Jouvenet, N., 2009).

5 Altogether, these data strongly support the involvement of extracellular matrix and linker proteins in the cohesion and attachment of HTLV-1 assemblies to the surface of infected cells.

#### **Cell-to-cell transmission of extracellular viral assemblies**

10 Virological synapses were described as organized cell contacts allowing direct virus transfer through synaptic clefts (Igakura, T., 2003; Majorovits, E., 2008). Consistently, the inventors observed that HTLV-1 -infected T cells formed tight cell conjugates and transferred viral proteins to non-infected cells. However, three-dimensional views and xz-sections of cell conjugates surface-stained with ConA, showed extracellular viral assemblies overlapping  
15 cell contacts and bridging the gap between both cell surfaces, rather than filling contact sites (**Figure 18a**, right panel).

Extracellular viral assemblies forming the biofilm not only accumulate at the junction of the two cells, but also outside this contact zone, bridging the gap between both cell surfaces (**Figure 19**).

20 Infected cells were distinguished from non-infected cells by the presence of cortical viral proteins and higher ConA labeling (**Figure 20a–c**). Conjugates between CD4<sup>+</sup> T cells from HAM/TSP individuals and healthy subjects also displayed viral assemblies on the surface of infected and target cells and on the sides of cell contacts (**Figure 18b**). Extracellular matrix  
25 components were transfer together with viral components to target cells (**Figure 20d–f**). Similar results were found in MT2-Jurkat cell conjugates. At 10 min, ≈35 % of conjugates showed Gag-p19 labeling on target cells, increasing until 1h, (**Figure 18c,d** arrowheads). The large majority of cell conjugates displayed viral assemblies on the sides rather than in the center of  
30 the synapse (**Figure 18c,e** arrows).

Scanning electron microscopy showed Env<sup>+</sup> virus clusters very close to cell contact sites (**Figure 18f**). At 5 min of contact, the inventors already observed Env<sup>+</sup> viral particle clusters on the target cell surface (**Figure 18g**), indicating that viral assemblies are rapidly transferred to target cells during cell contacts.

5 Finally, transmission electron microscopy revealed Env<sup>+</sup> mature viral particle clustered on the sides of cell contacts together with a mesh of electron-dense material, putatively, extracellular matrix (**Figure 21a,c**, arrows). The inventors also observed Env<sup>-</sup> viral particles in potential synaptic clefts (**Figure 21b**, arrowheads), although in a much lesser amount.

10 Therefore, during cell contacts, extracellular HTLV-1 assemblies can be rapidly transferred outside the synaptic zone to the surface of other lymphocytes.

#### **Relevance of extracellular viral assemblies in HTLV-1 infection**

To quantify the relevance of extracellular viral assemblies in HTLV-1  
15 transmission, the inventors removed them by extensive pipetting or by competing the extracellular matrix with heparin. The inventors then measured virus transmission using reporter Jurkat T cells. (**Figure 22a**). The treatments progressively reduced cell-associated Gag-p19, increasing Gag-p19 in cell supernatants, (**Figure 22b,c**). Importantly, the capacity of heparin-washed cells  
20 to infect reporter cells (Jurkat cells) was strongly diminished ( $\approx 80\%$ ) and correlated with the amount of cell-associated Gag-p19 (**Figure 22d**). Luciferase activity was due to virus infection, since it was inhibited by the reverse transcriptase inhibitor AZT (**Figure 22e**). Cell supernatants from washed cells could infect reporter target cells, although much less efficiently,  
25 provided their previous concentration (**Figure 22f**). Heparin-containing wash supernatants could compete for virus attachment on target cells. Mechanical and heparin treatments did not alter the capacity of cells to form cell contacts (**Figure 23**). Finally, similar results were obtained when HTLV-1 transmission by T cells from HAM/TSP individuals was studied (**Figure 22g**).

30 In conclusion, extracellular viral assemblies account for over 80 % of the

infectious capacity of HTLV-1 infected cells.

## DISCUSSION

The inventors show that HTLV-1 infected cells produce and transiently store virions in extracellular adhesive structures rich in extracellular matrix components and linker proteins that are critical for HTLV-1 cell-to-cell transmission (**Figure 25**). The present results are in line with previously reported data showing extracellular clusters of HTLV-1 viral particles associated to electron dense material (Gessain, A., 1990; Poiesz, B.J., 1980; Zacharopoulos, V.R., 1992). Extracellular HTLV-1 assemblies are strikingly reminiscent of bacterial biofilms, which are composed of bacteria held together by a carbohydrate-rich extracellular matrix that ensures cohesion, protection, adhesiveness to a substrate and spreads upon fragmentation (Stewart, P.S., 2008) (**Figure 26**, Parise et al 2007). Bacterial biofilms are rich in exopolysaccharides produced by bacteria (Stewart, P.S., 2008) but extracellular matrix proteins like fibrinogen, or lectins like galectin-3, produced by host cells can also cooperate with bacterial proteins enhancing cohesion and adhesiveness (Fowler, M., (2006); Moran, A.P., 2008; Gupta, S.K., 1997; Bonifait, L., 2008). HTLV-1 hijacks similar host cell proteins enhancing their expression, modifying their carbohydrate composition, or their spatial organization to build extracellular assemblies. Interestingly, while collagen, fibronectin, galectin-1 and galectin-3 are overexpressed during HTLV-1 infection (Munoz, E., 1995; Yi, T., 2000; Hsu, D.K., 1996; Gauthier, S., 2008), the inventors found only collagen and galectin-3 accumulated in viral assemblies, indicating that preferential protein accumulation occurs to build these structures. Moreover, agrin, but not  $\alpha$ -dystroglycan, accumulates in viral assemblies, although these two proteoglycans cluster at immunological synapses (Khan, A.A., 2001; Zhang, J., 2006). Finally, HTLV-1 may utilize host cell adhesion molecules for attachment to the cell surface and for clustering at the uropod. It remains unknown, however, the site of virus budding into extracellular viral assemblies, and the contribution of cell-cell contacts to the formation or concentration of

these structures. Therefore, extracellular matrix components and cellular lectins might together generate cocoon-type structures able to concentrate virions in a confined protective environment. Interestingly, hyperglycosylation of envelope glycoproteins may help viruses, including HIV-1, to escape immune responses (Coffin, J.M., 1997; Humbert, M., 2006). Since HTLV-1 Env glycoprotein is poorly glycosylated, embedding viral particles in carbohydrate-rich supramolecular structures could help HTLV-1 to avoid immune recognition. Moreover, concentrating virions in these structures may locally increase "infectious titers" and help to convey the virus from cell to cell. Noteworthy, extracellular composition and structure of extracellular matrix, including collagen and galectin-3, is modified by the  $\gamma$ -irradiation of cell cultures (El Nabout, R., 1989; Joo, H.G., 2001). Therefore, irradiation could reshape the structure of extracellular viral assemblies enhancing viral transmission, explaining why irradiation of HTLV-1 producing cells increases their efficiency to infect other cells *in vitro*.

As reported (Igakura, T., 2003; Nejmeddine, M., 2005; Majorovits, E., 2008), the inventors observed HTLV-1 transmission *via* cell contacts. However, data do not support a model of contact-induced virus budding and direct transfer through synaptic clefts, but rather, the existence of preformed transient extracellular structures that rapidly adhere to the surface of target cells during cell contacts. Nevertheless, the inventors also observed Env<sup>-</sup> viral particles in the synaptic zone, but in a much lesser amount. The functional relevance of extracellular assemblies is definitely supported by the fact that removing these structures strongly inhibits HTLV-1 cell-to-cell transmission. Thus, although heparin washes did not completely remove extracellular viral assemblies, as assessed by fluorescence microscopy, the infectious capacity of cells was reduced by 80 %. Cell supernatants obtained after cell washes were infectious, although much less efficient,

indicating that the integrity of extracellular viral assemblies and their transfer at cell contacts are key for HTLV-1 transmission.

Extracellular viral assemblies were resistant to strong shear flow during extensive pipetting, suggesting that they can defy physiological fluid dynamics *in vivo*. Interestingly, irregular surfaces help the formation and stability of bacterial biofilms. Moreover, some bacteria induce host cell surface reorganization to resist shear flow (Mikaty, G., 2009). Similarly, HTLV-1-infected cells generate numerous ruffles and filopodia that could maintain viral assemblies adhered to the surface of virus producing cells, but facilitate their transfer to other cells during cell contacts. *In vivo*, viral assemblies might be rapidly transferred to other T lymphocytes or dendritic cells during cell contacts in secondary lymphoid organs. Dendritic cells, in turn, may get infected and/or transfer again HTLV-1 assemblies to T lymphocytes (Jones, K.S., 2008). In addition, dendritic cells could also process viral assemblies and present viral antigens to T lymphocytes, triggering and maintaining the anti-HTLV-1 immune response.

## **EXAMPLE 2: Biofilm-like extracellular viral assemblies mediate HIV-1 cell-to-cell transmission at virological synapses**

### **Introduction**

The inventors also performed experiments with HIV-1 infected lymphocytes in order to observe whether they form extracellular viral assemblies at their cell surface (**Figures 27, 28, 29 30**).

### **MATERIALS and METHODS**

#### **Reagents, cells and infection.**

The rabbit anti-HIV-1 Gag Ab was a gift from the NIH AIDS Research and Reference Reagent Program. Affinity purified anti-human CD62L mouse mAb to CD62L (clone DREG-56) was obtained from eBioscience. The other antibodies, lectins or reagents, have been described in Example 1. J.CaM1.6 (JCaM), a Lck-deficient line derived from Jurkat, was previously described (Straus, D. B., and A. Weiss. 1992). Peripheral blood mononuclear cells were

purified through Ficoll-Hypaque centrifugation and CD4<sup>+</sup> T cells were isolated by negative selection, using magnetic cell sorting (Miltenyi Biotec, MACS). Jurkat (parental or Lck-deficient) cell lines or primary CD4<sup>+</sup>T cells isolated from healthy donors were infected with HIV-1 X4-tropic HIV-1 strain NL4.3 (HIVwt) as previously described (Thoulouze et al. 2006). The inventors analyzed HIV-1-infected cells, as assessed by immunofluorescence against viral proteins after 72 h of infection.

**Immunofluorescence, confocal microscopy analysis and quantification of fluorescence intensity.**

The inventors performed them as previously described (Thoulouze, M.I., 2006). Cells were fixed with 4 % paraformaldehyde for 30 min at room temperature. Surface staining (e.g., lectin staining) was performed in the absence of detergent. Intracellular cellular proteins and Gag-p19 were stained incubating fixed cells in solutions containing 0.05 % saponin. Before staining, the inventors blocked nonspecific protein binding by incubating the coverslips for 15 min in 5% FCS 0.05% saponin in PBS. For collagen staining, the inventors treated cells after paraformaldehyde fixation with 100 % methanol, 40 min at -20°C. The inventors carried out confocal microscopy analysis on a Zeiss LSM5 10 using a x 63 objective. Z-series of optical sections at 0.2 µm increments were acquired. The inventors treated the images by deconvolution using Huygens software in order to reduce the fluorescence noise of the images. Three-dimensional image reconstructions of cells and the calculation of the volume of co-localization were done with Imaris software.

**Results**

Although the use of cell-to-cell interactions during transmission was originally believed to distinguish HTLV-1 from other retroviruses including HIV-1, later studies indicated that transmission of HIV-1 is two to three orders of magnitude more efficient when cells physically interact (Chen et al., 2007; Sourisseau et al., 2007; Mazurov et al., 2010). Noteworthy, HIV-1 transmission between infected and target cell through cell junctions recently appears to mostly

result from the lateral movement of viral particle on the surface of the cells (Yu et al., 2008). Finally, the inventors' current studies find HIV-1 clusters associated by the cell and composed by viral particles encased in a cocoon of modified cellular components, although the size and composition seems to differ from viral clusters characterized for HTLV-1.

Previous work on HIV-1 indicated that :

i) Some viral particles remain attached to the surface of infected cells.  
ii) Their spread in cell culture depends on or is enhanced by cell-to-cell contacts

iii) Viral dissemination occurs despite the production of high titers of functional neutralizing antibodies by the host,

iv) They were reported to induce the expression of extracellular matrix components, cellular lectins and modified glycosylation patterns of viral and cellular glycoproteins (Fogel et al., 1999; Krishnamoorthy et al., 2009).

The inventors carried out confocal microscopy analyses in primary CD4<sup>+</sup> T cells infected with HIV-1.

As previously observed in the case of HTLV-1, Gag was shown to cluster in discrete areas of the cell cortex that could be intracellular compartments where viral particles assembled and/or accumulated ready to be transferred to target cells. To localize viral proteins with respect to the plasma membrane the inventors used Gag-specific antibodies, and the inventors labeled cell surface glycoproteins with the lectin Concanavalin-A (ConA) on *in vitro* infected primary CD4<sup>+</sup> T cells from healthy donors, or on cells from the Jurkat T cell line. To obtain a maximal image definition, deconvolution of confocal images and three-dimensional reconstruction were performed.

Cortical clusters of Gag viral protein were observed on the surface of infected cells, appearing as single or multiple clusters, faintly labelled by ConA (**Figure 31**).

Therefore, light microscopy observations show the presence on the surface of HIV-1-infected T lymphocytes of extracellular Gag protein clusters

reminiscent extracellular viral assemblies previously observed by the inventors on HTLV-1-infected cells. HIV-1 clusters appeared as smaller multiple structures if compared with those of HTLV-1.

#### **Extracellular viral assemblies are carbohydrate-rich structures**

5       The inventors used a panel of plant lectins recognizing glycans enriched in the extracellular matrix. The inventors observed that, like ConA (**Figure 31**), most lectins evenly stained the surface of uninfected cells. However, some lectins, such as *Glycine Max*, stained much more strongly extracellular viral assemblies (**Figure 32**). The colocalization between lectin labeling and viral  
10       markers was however weak, indicating that lectins did not primarily bind viral Env glycoprotein, but glycoconjugates enriched in extracellular viral assemblies.

          Therefore, extracellular viral particles are embedded in a carbohydrate-rich structure, likely made of extracellular matrix, that is induced and spatially  
15       reorganized by viral infection.

#### **HIV-1 assemblies contain linker proteins**

          The inventors next searched for extracellular matrix components in HTLV-1 assemblies. The inventors found that the surface CD62L was enriched in viral assemblies, but randomly distributed on uninfected cells (data not  
20       shown). CD62L is an adhesion molecule whose surface expression and further cleavage and release are regulated by T cell activation.

          These data, together with those mentioned in the above paragraph, support the involvement of extracellular matrix and T cell surface linker proteins in the attachment and cohesion of HIV-1 assemblies to the surface  
25       of infected cells, similarly to what the inventors found in HTLV-1-infected cells. The carbohydrate moieties involved may be however distinct between HTLV-1 and HIV-1-infected T cells, since they were differentially labelled by plant lectins.

#### **HIV-1 extracellular viral assemblies spread at cell contacts**

          Confocal microscopy of HIV-1 infected CD4<sup>+</sup> T cell conjugated  
30       with non-infected T cell was realised showing cell surface glycoproteins stained

with Con A (green), and viral proteins stained with Gag antibody (red). Merge images of XY-medial optical sections and a XY-projection of a three-dimensional reconstruction are shown in **Figure 33**. Arrows point to Gag<sup>+</sup> clusters at the cell surface incubated for 30 min, then stained with Gag antibody.

### **T cell activation controls the formation of HIV-1 extracellular viral assemblies**

The inventors have observed in preliminary experiments that the size of HIV-1 extracellular clusters depend on a key T cell activation molecule, the protein tyrosine kinase Lck. Thus in T cells lacking this kinase (JCaM cells), the extracellular viral clusters are larger. Clusters became smaller again when the inventors corrected the levels of expression of this kinase by gene transfection.

Therefore, HIV-1 may control the formation of extracellular viral assemblies by controlling the activation machinery of infected cells.

### **Conclusion**

The inventors observed that HIV-1-infected T lymphocytes form extracellular viral assemblies at their cell surface, which are reminiscent to those that the inventors had observed on the surface of HTLV-1-infected lymphocytes. HIV-1 and HTLV-1 extracellular assemblies share some characteristics, like the presence of carbohydrate-rich structures and linker proteins, like CD62L. They seem however distinct in some respects, such as their size and the presence of particular carbohydrate moieties.

The evolutionary advantage of this transmission mechanism is at present unknown, but it might condition both virus spread and immune responses of HTLV-1 -infected subjects. It is likely that other viruses also developed transmission strategies based on similar biofilm-like viral assemblies. As extracellular structures of particular composition, biofilm-like viral assemblies might be potential targets for future anti-viral therapy.

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**CLAIMS:**

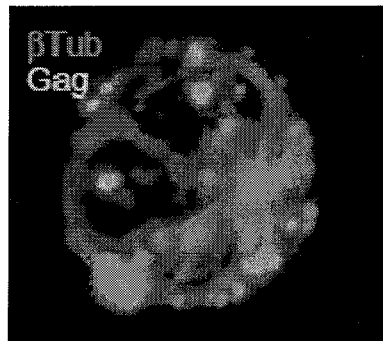
1. A method of screening for a compound that affects formation or cohesion of viral biofilm-like structure, comprising the steps of:
  - 5                   - contacting a cell with a virus naturally involved in viral biofilm-like structure and at least one candidate compound; and
  - identifying the compound that affects the formation of said viral biofilm-like structure or the cohesion of said biofilm.
2. A method of screening for a compound that affects virus transmission from  
10                   cell to cell, comprising the steps of:
  - infecting a cell with a virus naturally involved in viral biofilm-like structure for a time sufficient to form a viral biofilm-like structure;
  - contacting said virus infected cell with a non-infected cell and at least one candidate compound; and
  - 15               - identifying the compound that affects transmission of said virus from the infected cell to the non-infected cell.
3. The method of claim 1 or 2 , wherein the viral biofilm-like structure consists in a structure comprising:
  - carbohydrate rich matrix such as sialyl-Lewis X or sLeX, and/or
  - 20               - adhesion molecules, such as collagen or agrin and/or cellular linker proteins such as tetherin, galectin-3 or CD62.
4. The method of any one of claims 1 to 3, wherein the virus is a lymphotropic virus.

5. The method of claim 4, wherein the lymphotropic virus consists of HTLV, or HIV.
6. The method of claim 5, wherein the lymphotropic virus consists of HTLV-1 or HIV-1.
- 5 7. A compound that affects formation and/or cohesion of virus biofilm-like structure obtained by contacting a cell with a virus naturally involved in virus biofilm-like structure formation or cohesion.
8. A compound that affects virus transmission from cell to cell obtained by contacting a cell with a virus naturally involved in virus biofilm-like structure  
10 formation or cohesion.
9. A composition comprising a compound as defined in claim 7 or 8, and a pharmaceutically acceptable carrier.
10. A method for preventing and/or treating diseases or disorders associated with a virus inducing a biofilm-like structure in a subject, the method  
15 comprising the step of administering to the subject a therapeutically effective amount of a composition as defined in claim 9.
11. Use of a therapeutically effective amount of a composition as defined in claim 9 for preventing and/or treating a subject against a biofilm-like structure forming associated disease.
- 20 12. Use according to claim 11, wherein the subject is a human.
13. Use according to claim 12, wherein the subject is immunocompromised.
14. Use according to claim 11, wherein the biofilm-like structure forming associated disease is caused by a lymphotropic virus.

15. Use according to claim 11, wherein the biofilm-like structure forming associated disease is caused by HTLV or HIV.
16. Use according to claim 11, wherein the biofilm-like structure forming associated disease is caused by HTLV-1 or HIV-1.

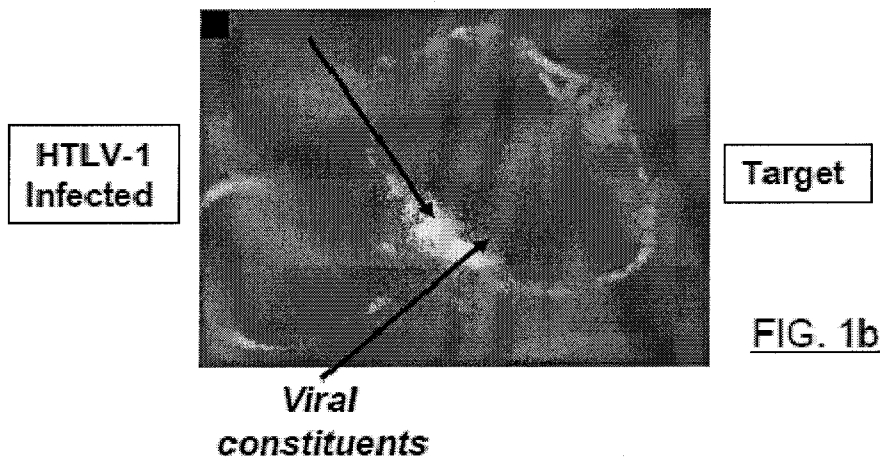
1/31

**Infected T lymphocyte**

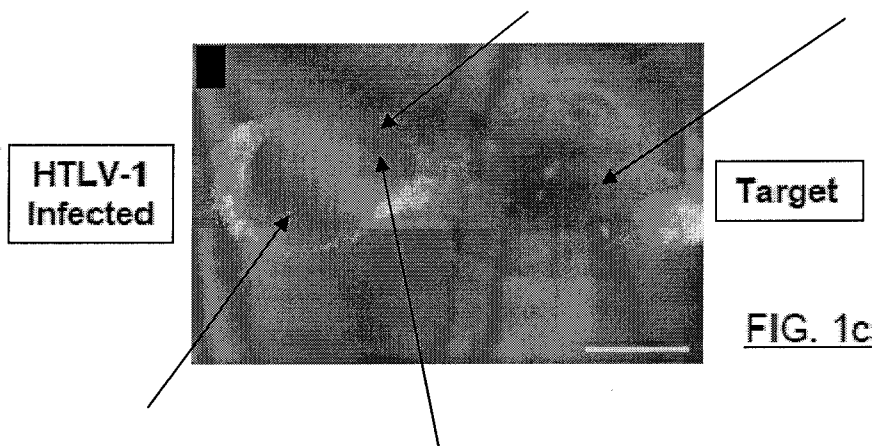


**FIG. 1a**

**MTOC**



**FIG. 1b**



**FIG. 1c**

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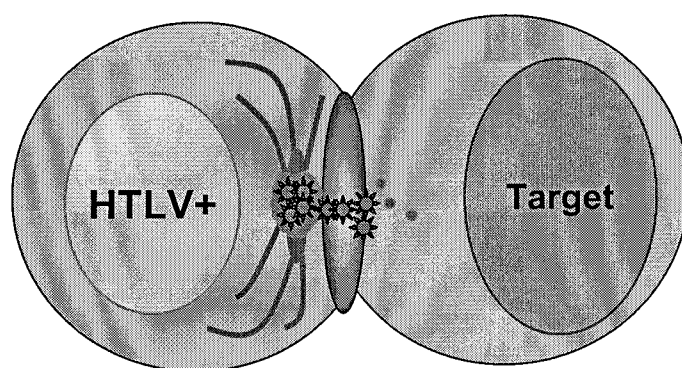


FIG. 2

3/31

**t = 5 min**

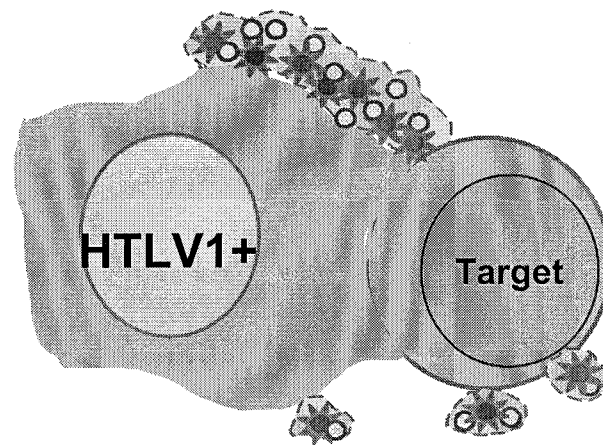
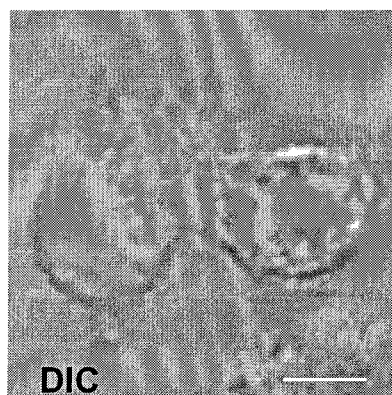


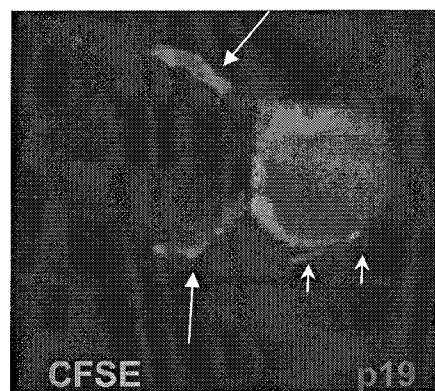
FIG. 3a



**HTLV-1  
Infected**

**Target**

FIG. 3b



**HTLV-1  
Infected**

**Target**

FIG. 3c

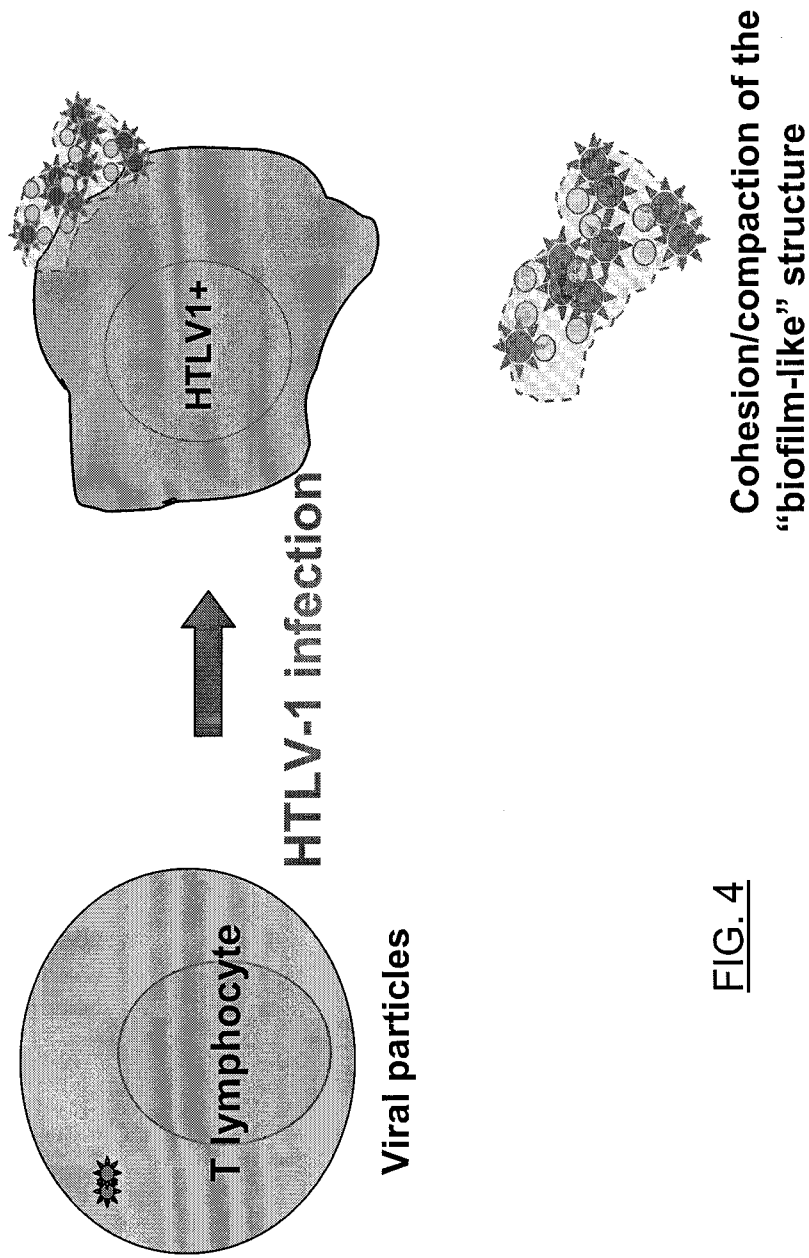
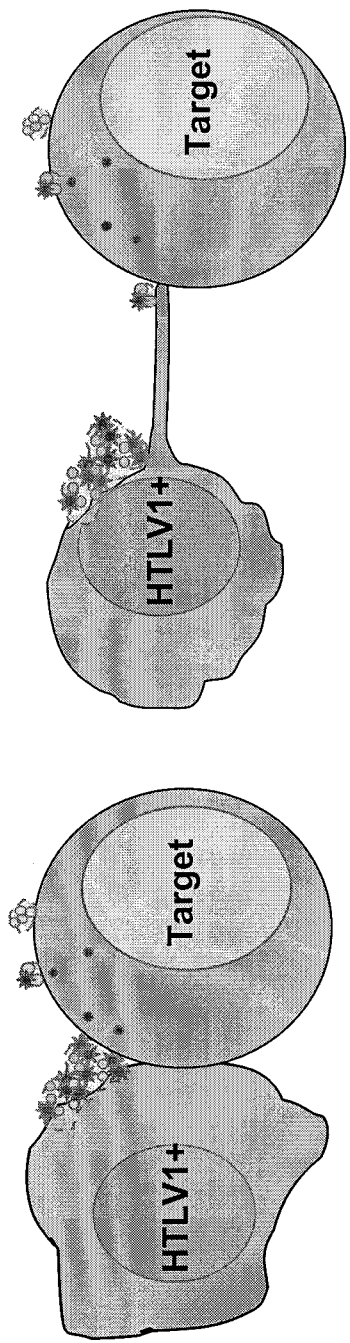


FIG. 4

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**Viral transference  
protected from immune system**

FIG. 5

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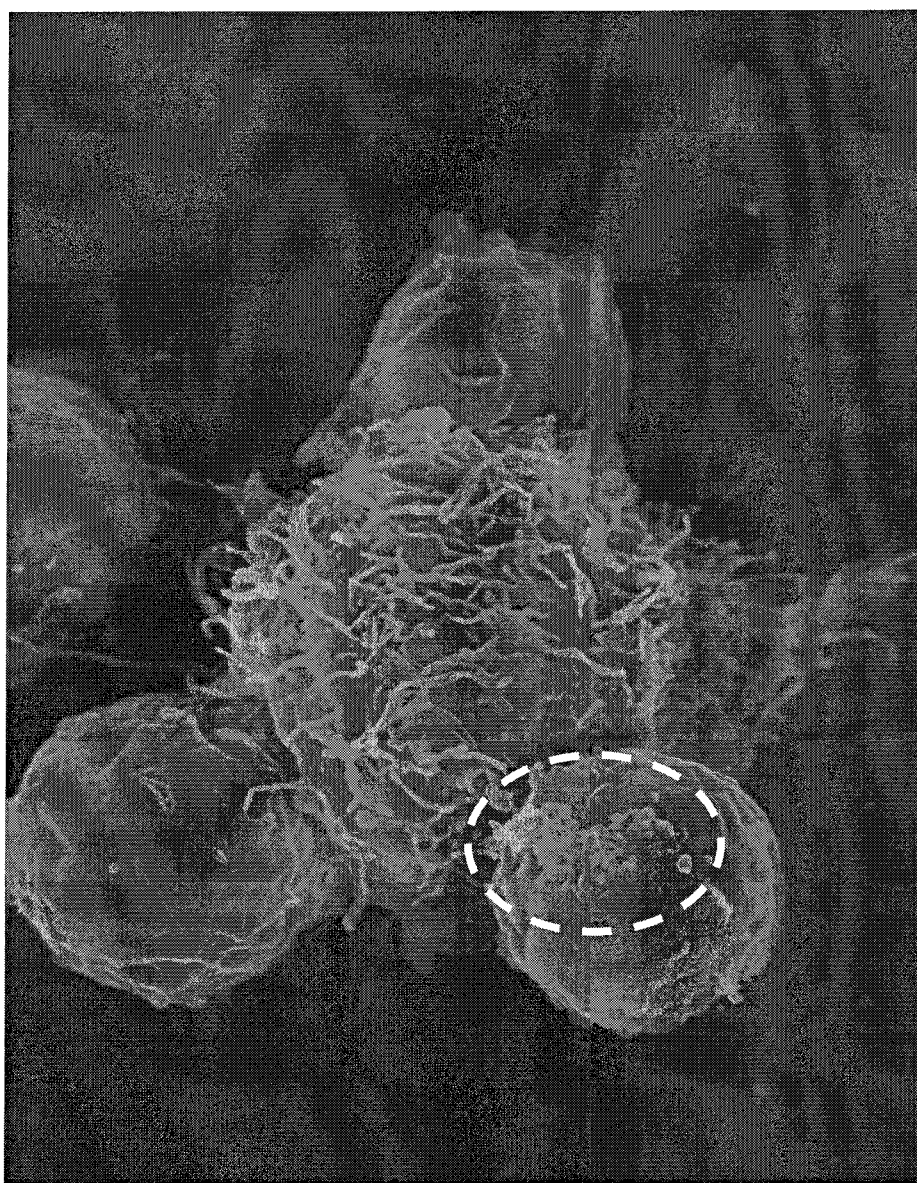


FIG. 6

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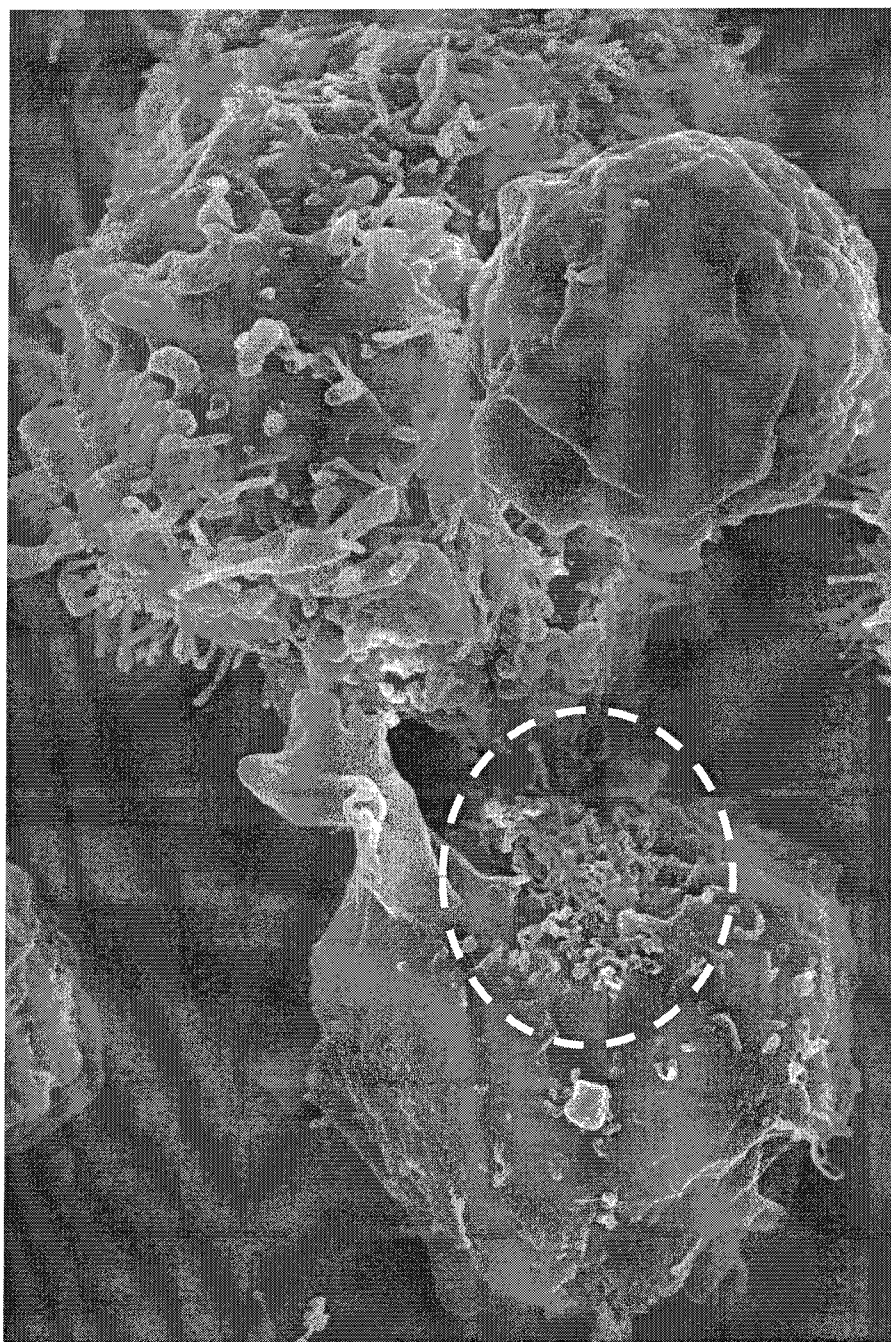


FIG. 7

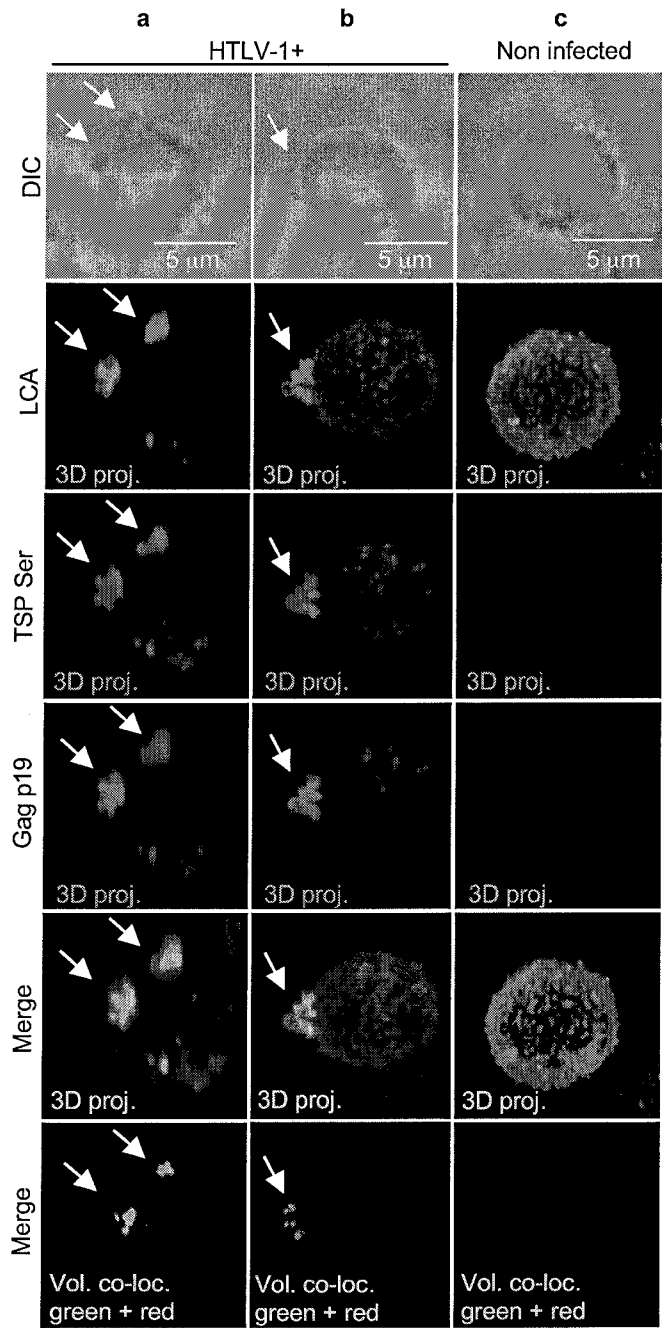
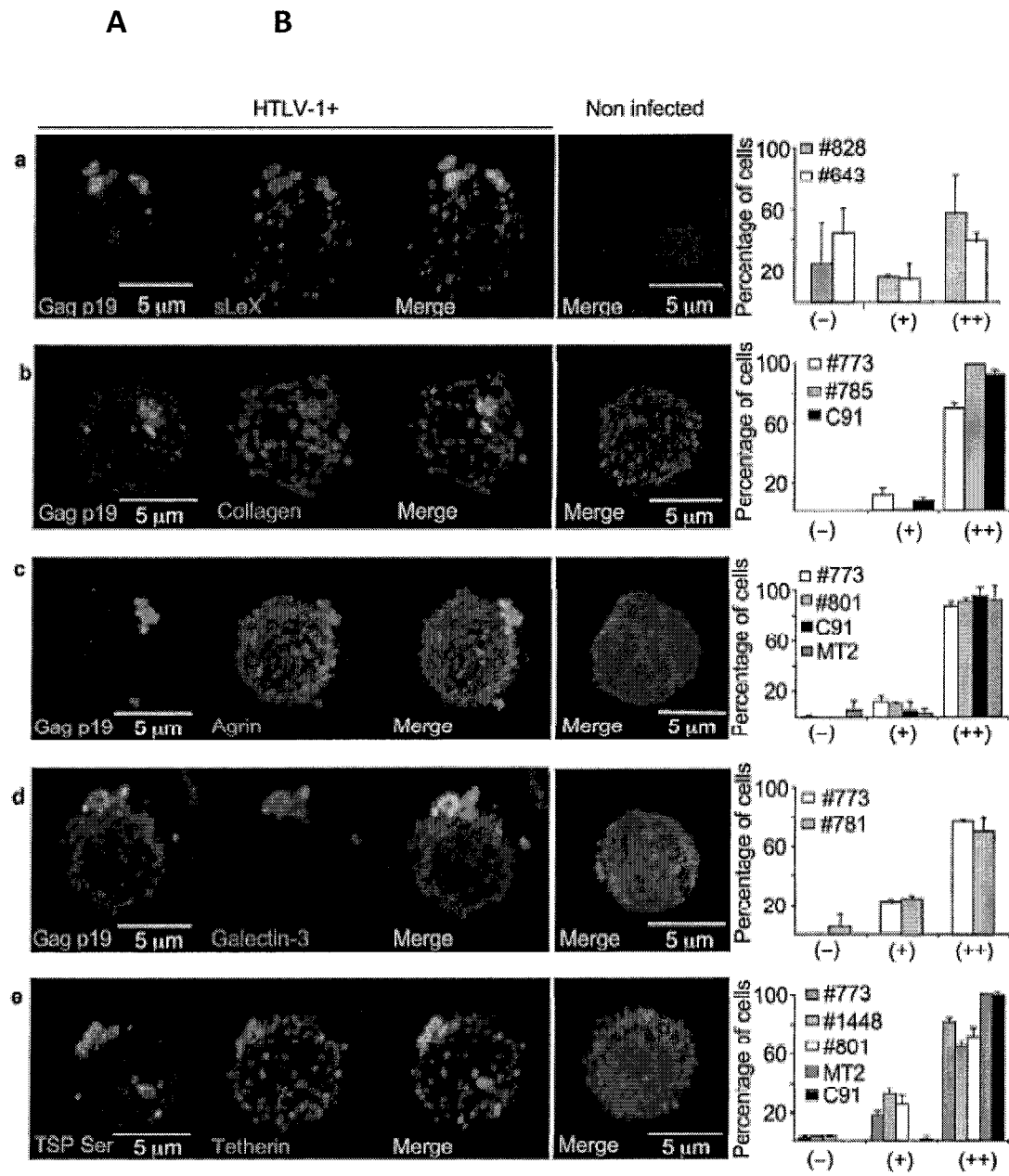


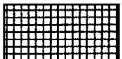
FIG. 8

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**FIG. 9**

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**Induced by the virus** SLeX Collagen Agrin Galectin-3 Tetherin **Induced by the infection** FIG. 10

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| Lectin                                          | Specificity                                                                                  | HAM/TSP #801 |          | HAM/TSP #1448 |          | C91/PL |          | MT2  |          |
|-------------------------------------------------|----------------------------------------------------------------------------------------------|--------------|----------|---------------|----------|--------|----------|------|----------|
|                                                 |                                                                                              | Cell         | Assembly | Cell          | Assembly | Cell   | Assembly | Cell | Assembly |
| <i>Canavalia</i><br><i>onsiformis</i><br>(ConA) | $\alpha$ -man, $\alpha$ -glc, $\alpha$ -N-acetylglucosamine, $\alpha$ -methylmannopyranoside | +++          | Presence | +++           | Presence | +++    | Presence | +++  | Presence |
| <i>Lens culinaris</i><br>(LCA)                  | $\alpha$ -man, $\alpha$ -glc, fuc $\alpha$ (1,6)-GlcNAc                                      | +++          | Acc.     | ++            | Acc.     | +++    | Acc.     | +++  | Acc.     |
| <i>Ulex europaeus</i><br>(UEA)                  | $\alpha$ -L-fuc                                                                              | -            | Absent   | +             | Absent   | +      | Absent   | -    | Absent   |
| <i>Glycine max</i><br>(SBA)                     | $\alpha$ and $\beta$ N-acetylgalactosamine                                                   | +            | Absent   | -             | Absent   | -      | Absent   | -    | Absent   |
| <i>Arachis hypogaeae</i><br>(PNA)               | Lactose, $\beta$ -D-galactose                                                                | ++           | Acc.     | -             | Absent   | ++     | Acc.     | -    | Absent   |
| <i>Triticum vulgaris</i><br>(WGA)               | (glcNAc) <sub>2</sub> , NeuNAc, GalNAc                                                       | +++          | Presence | +++           | Presence | +++    | Presence | ++   | Presence |
| <i>Hippeastrum</i><br><i>hybrid</i> (HHL)       | $\alpha$ -(1,3) and $\alpha$ -(1,6)-man                                                      | nd           | nd       | nd            | nd       | +      | Absent   | +    | Absent   |
| <i>Phaseolus vulgaris</i><br>(PHA-E)            | Oligosaccharides                                                                             | nd           | nd       | nd            | nd       | +      | Absent   | +    | Absent   |
| A                                               |                                                                                              | B            |          | C             |          | D      |          |      |          |

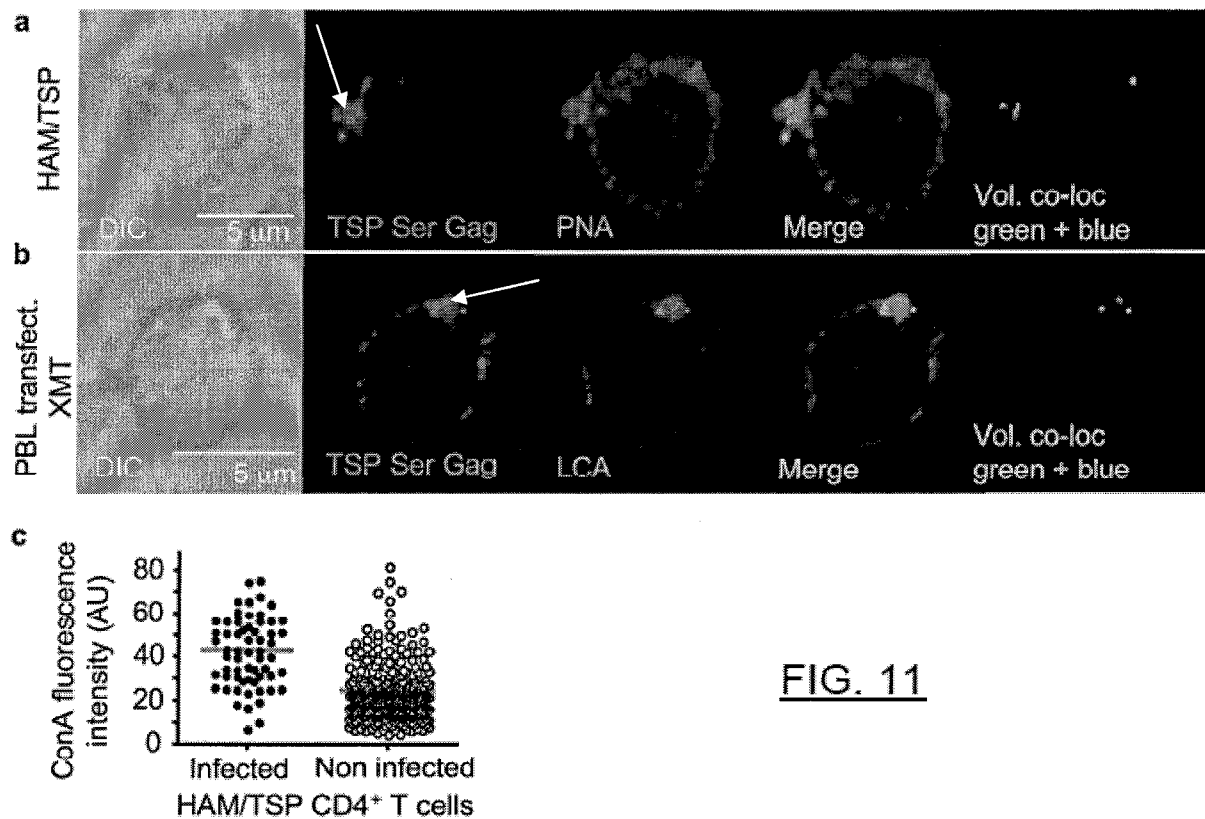


FIG. 11

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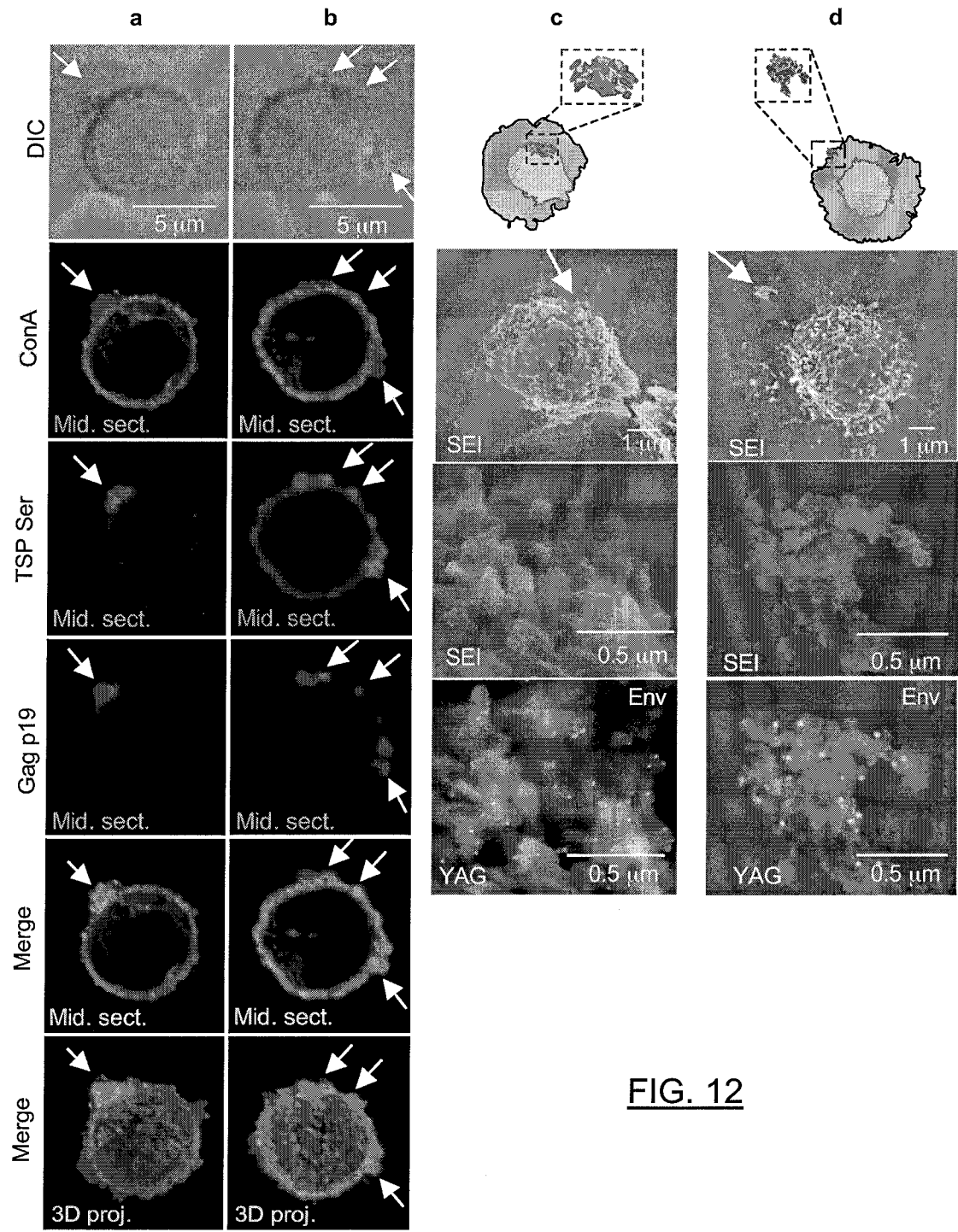


FIG. 12

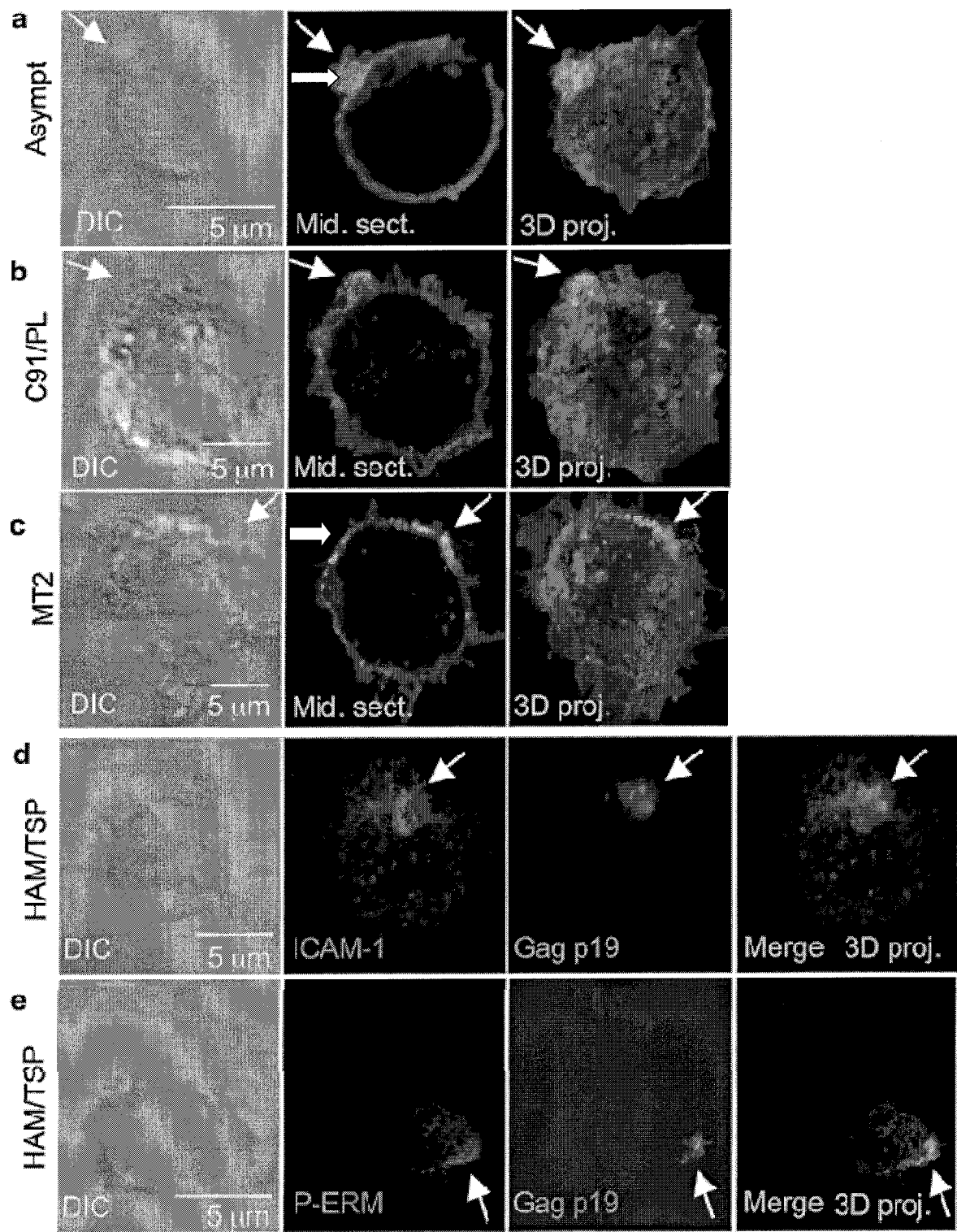


FIG. 13

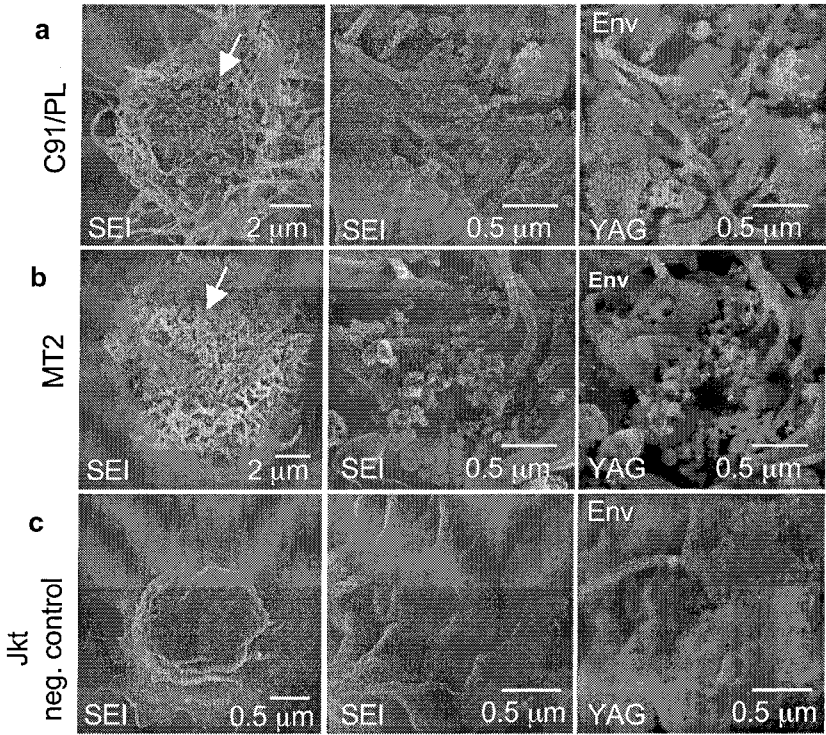


FIG. 14

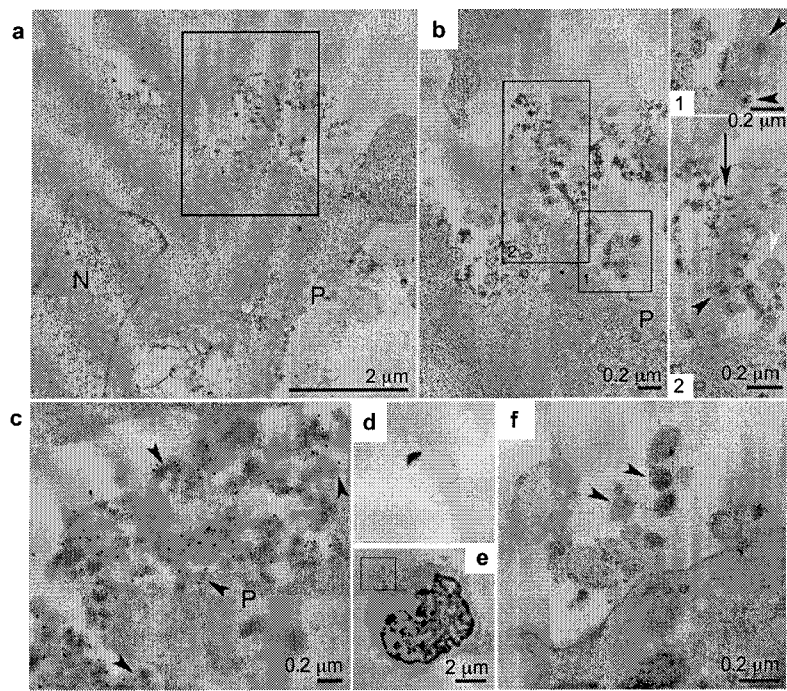
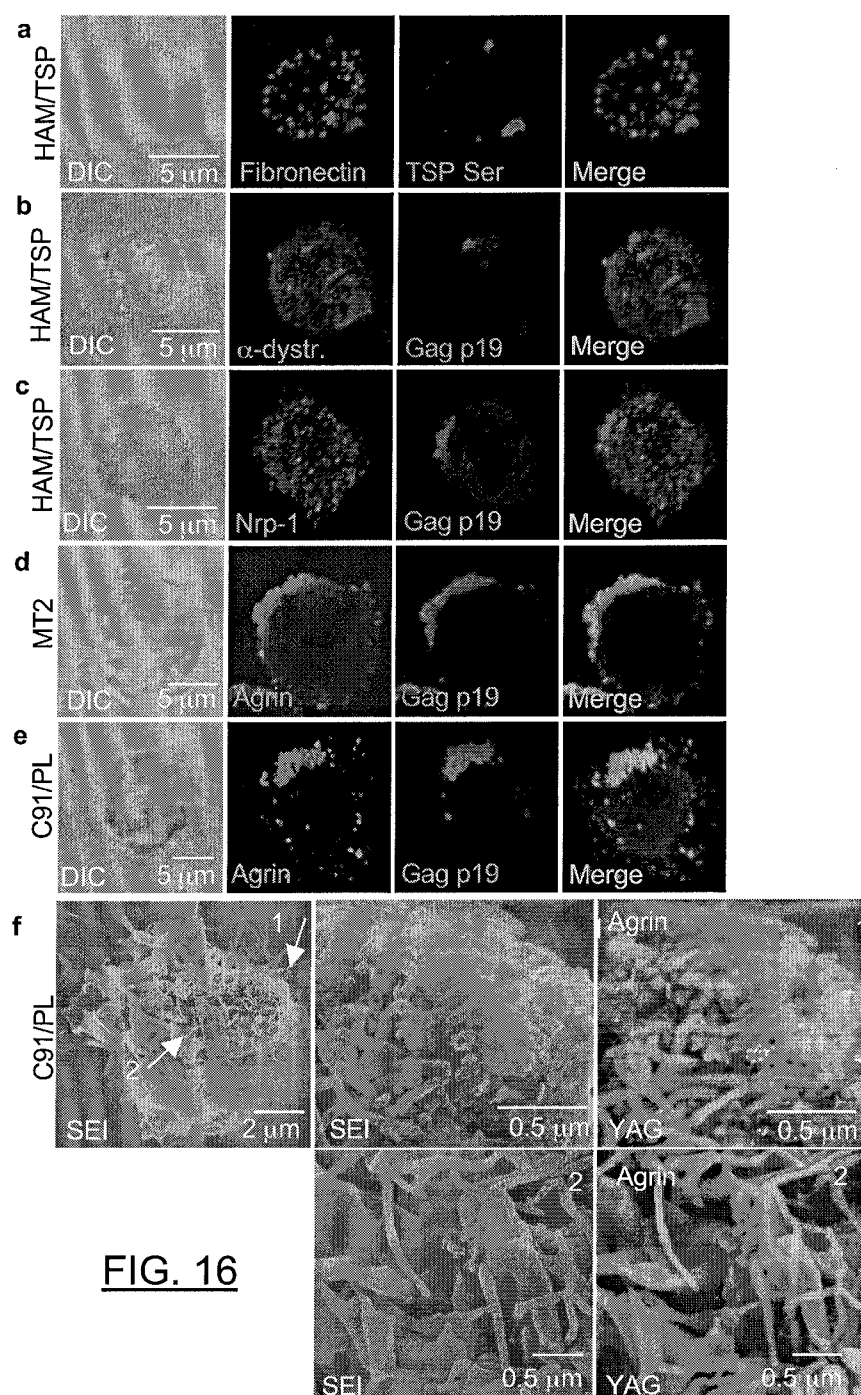


FIG. 15



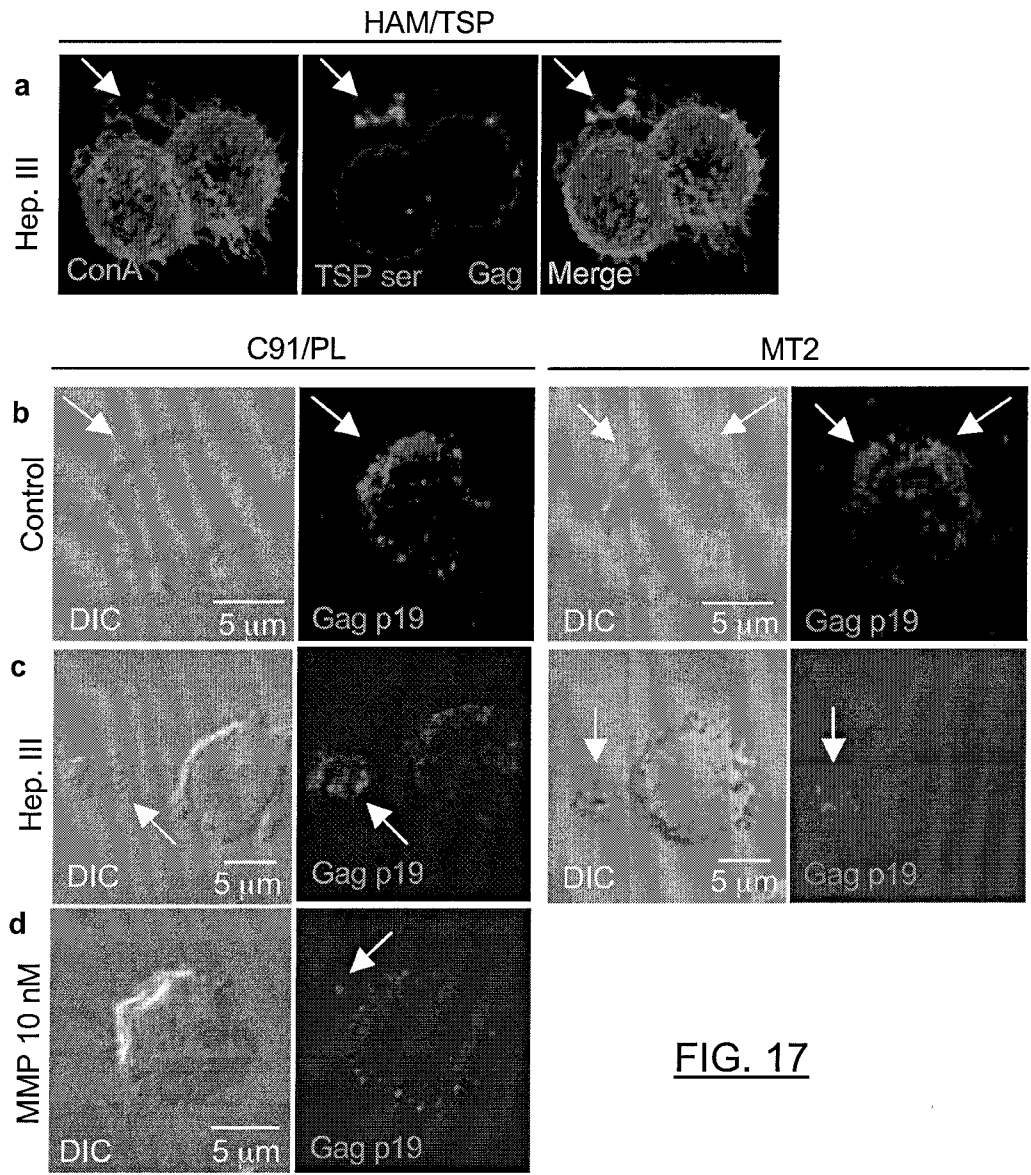


FIG. 17

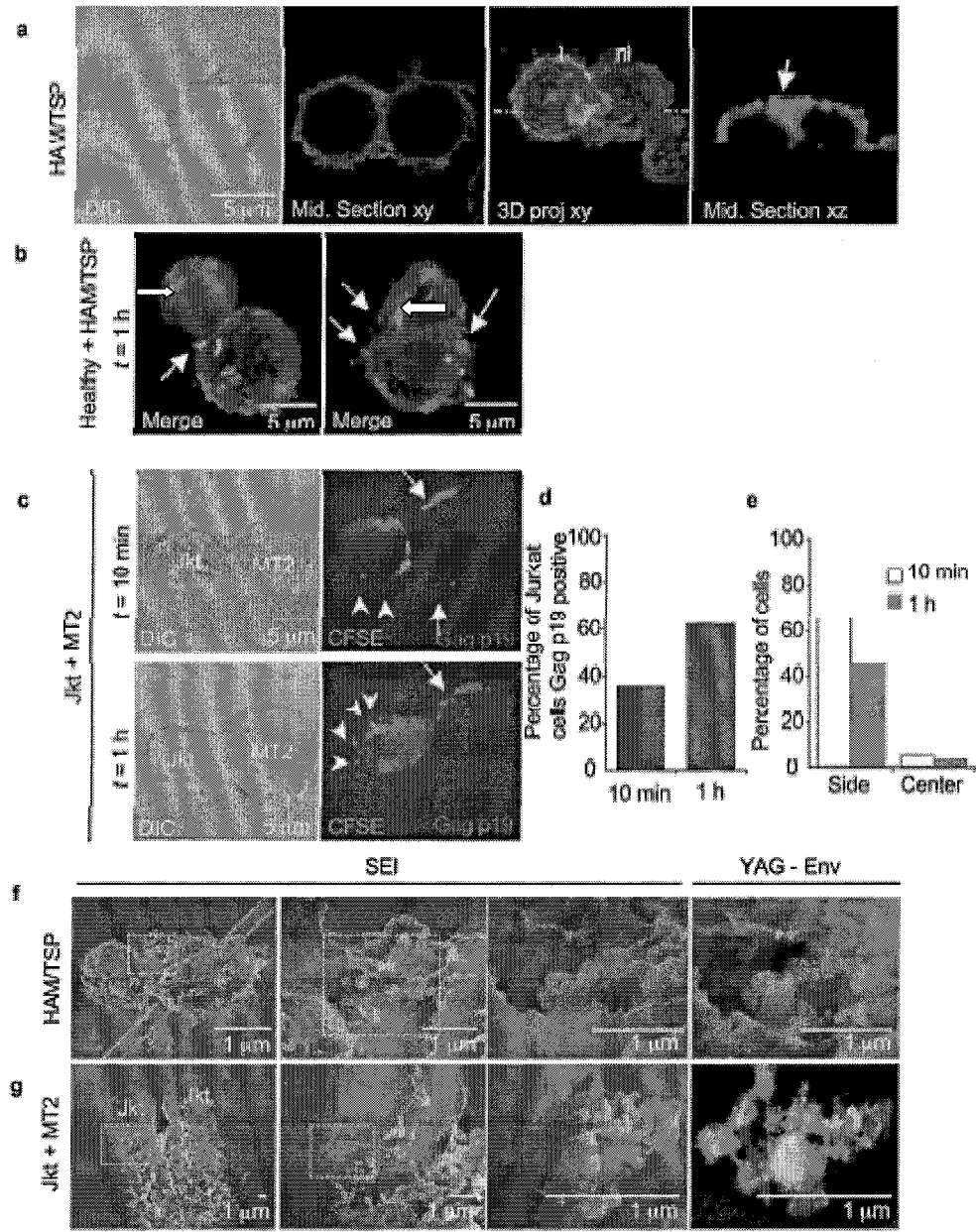


FIG. 18

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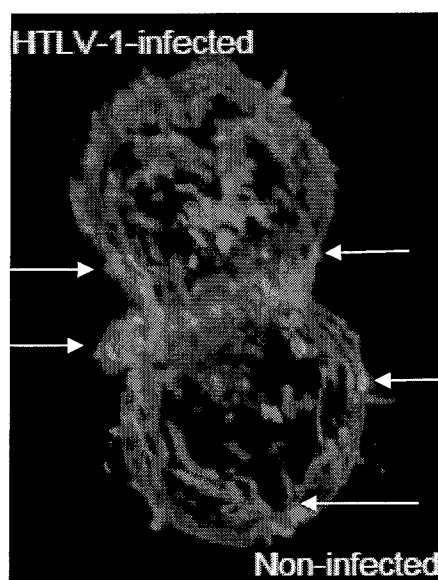


FIG. 19

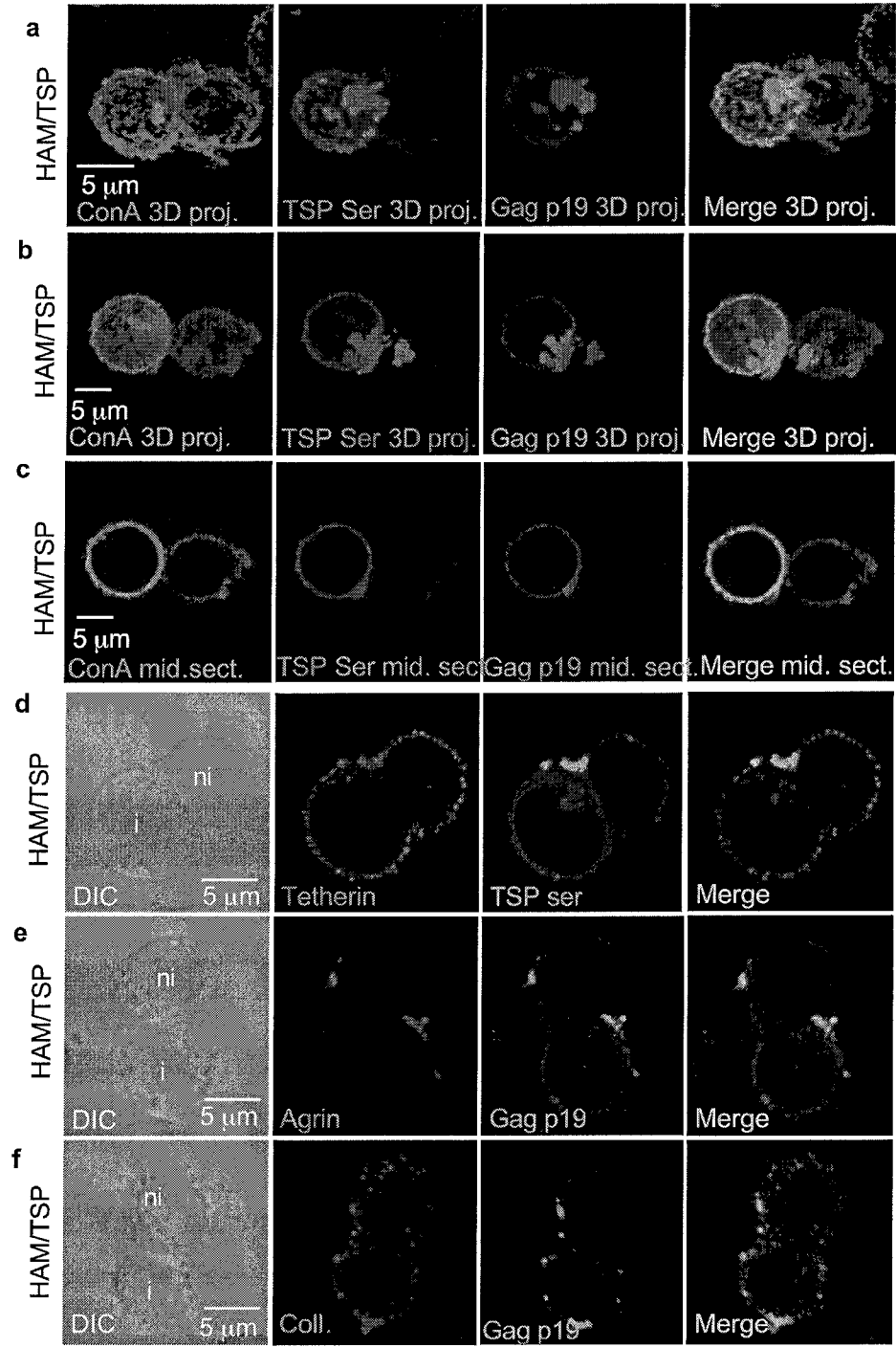


FIG. 20

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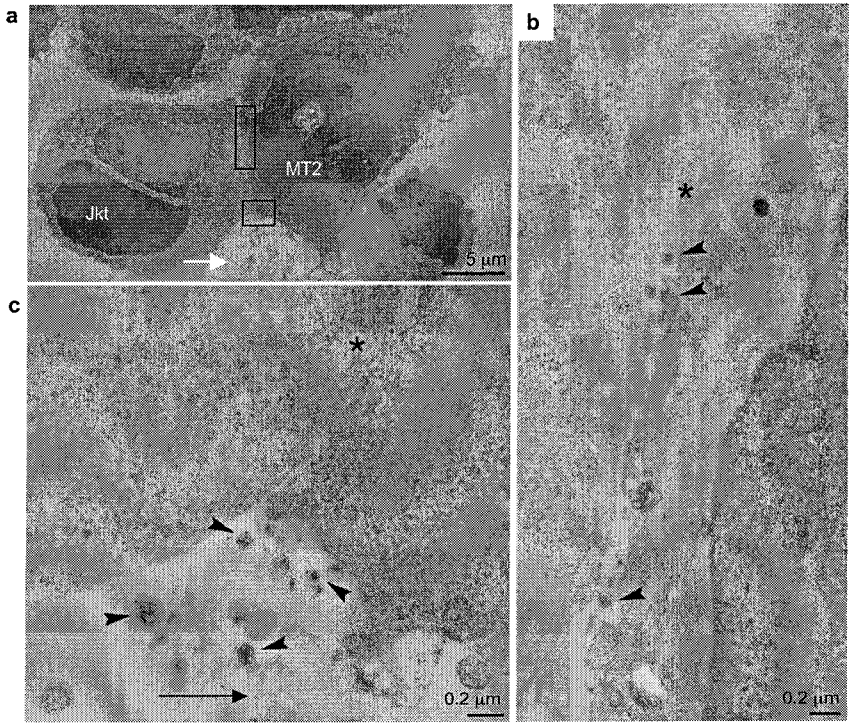
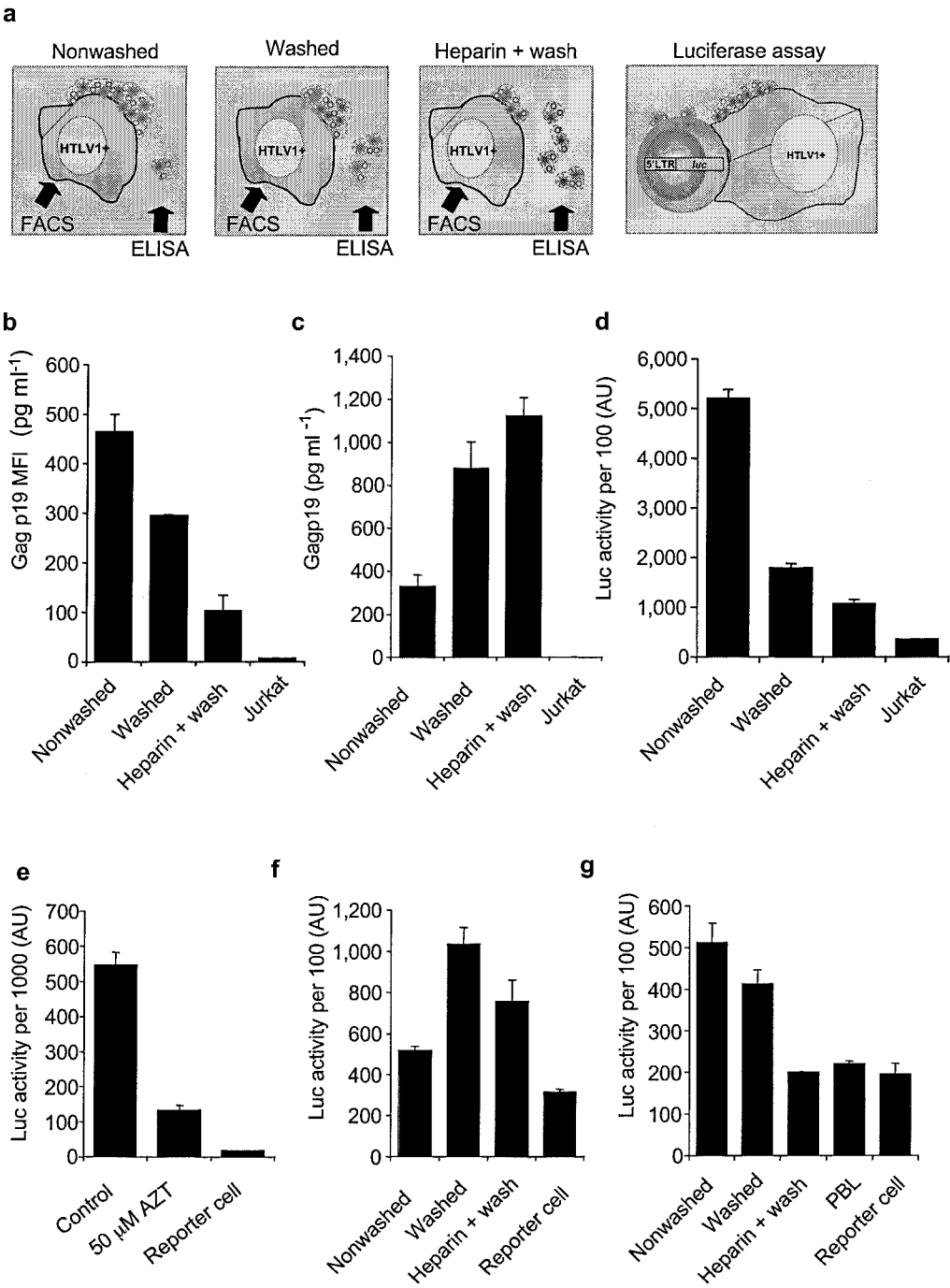


FIG. 21

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**FIG. 22**

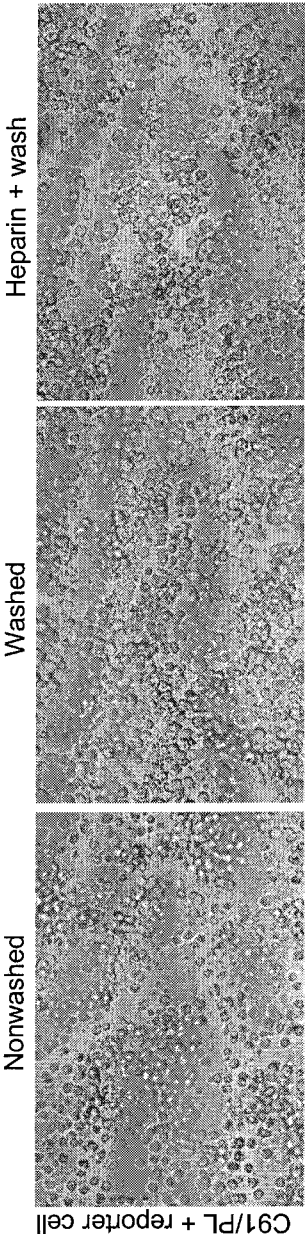


FIG. 23

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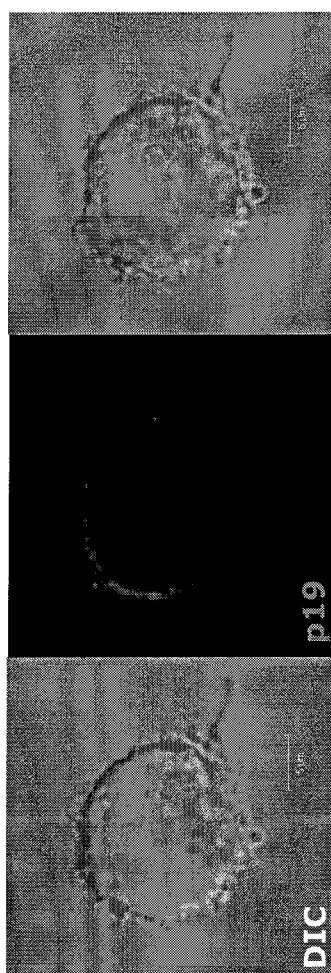


FIG. 24a

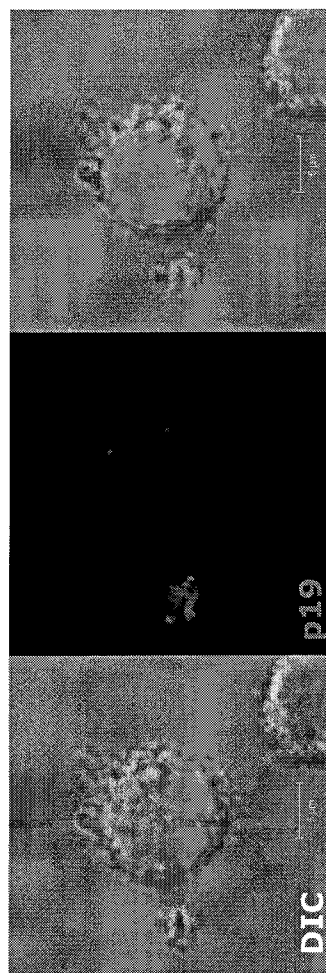
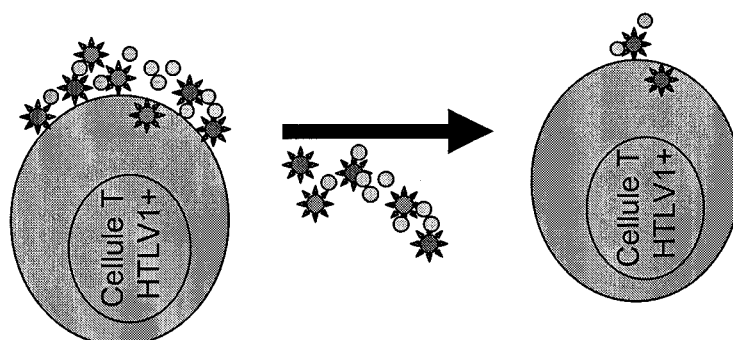


FIG. 24b



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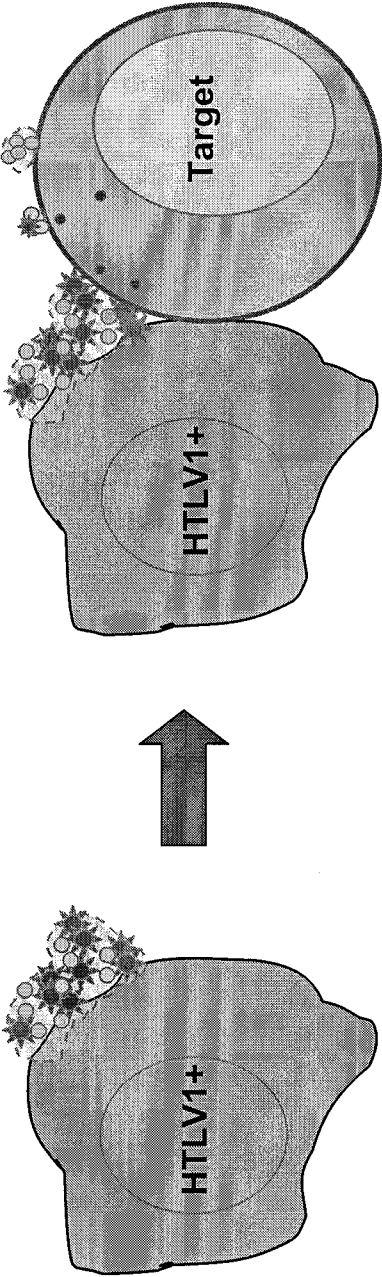


FIG. 25

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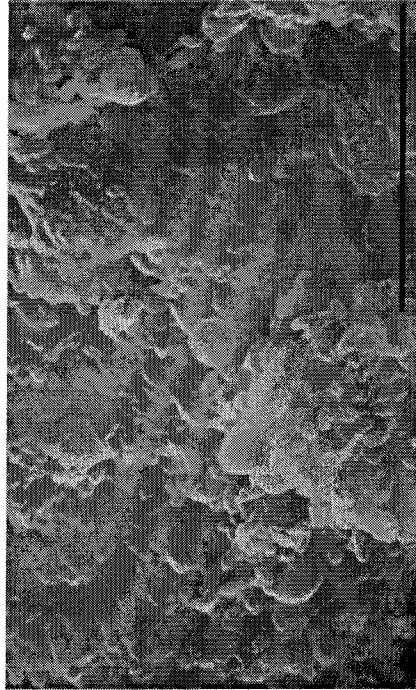
**Viral “biofilm-like” structure**



***HTLV-1***

**FIG. 26a**

**Bacterial biofilm**



***Bordetella bronchiseptica***

**FIG. 26b**

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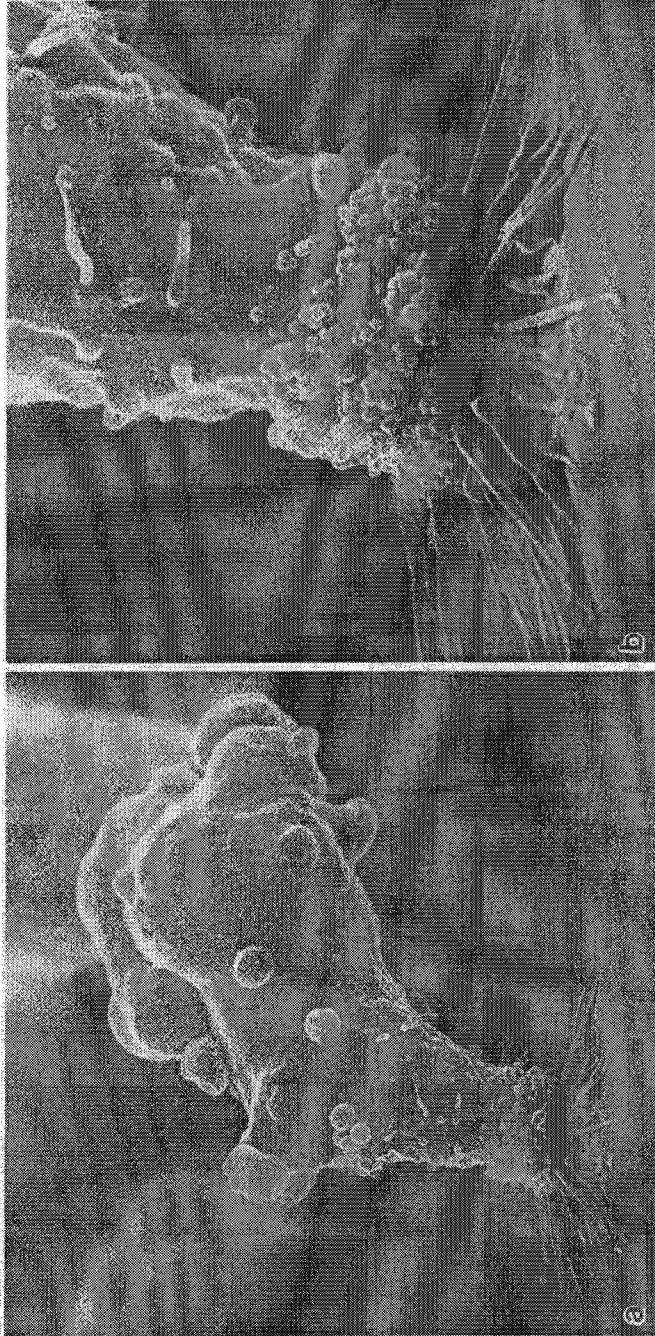


FIG. 27a

FIG. 27b

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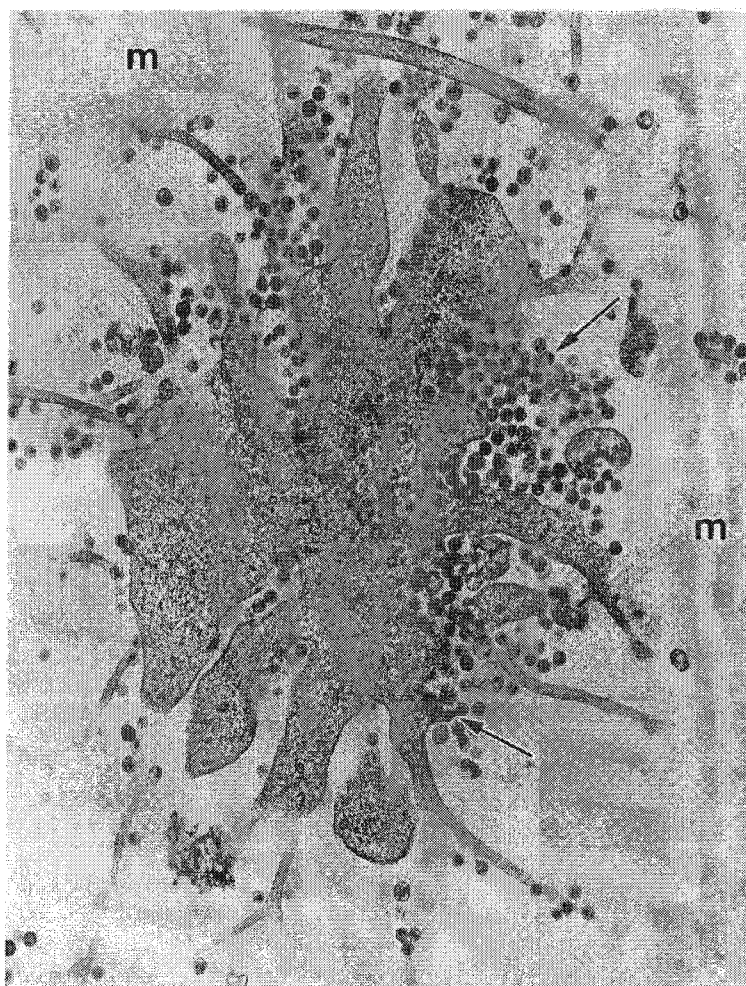


FIG. 28

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FIG. 29

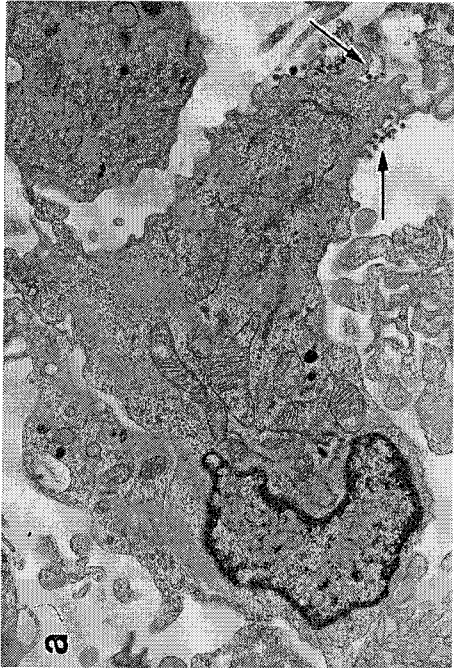


FIG. 30b



FIG. 30c

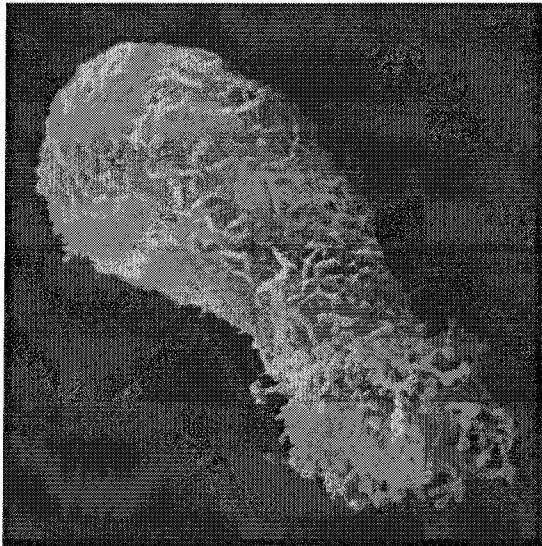


FIG. 30a

FIG. 31

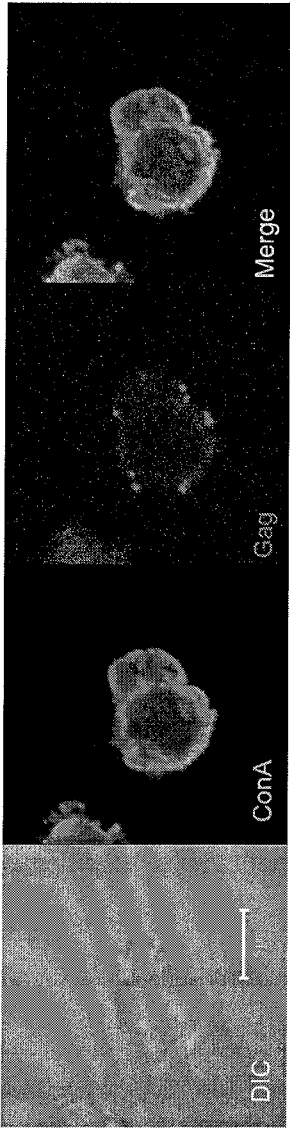


FIG. 32

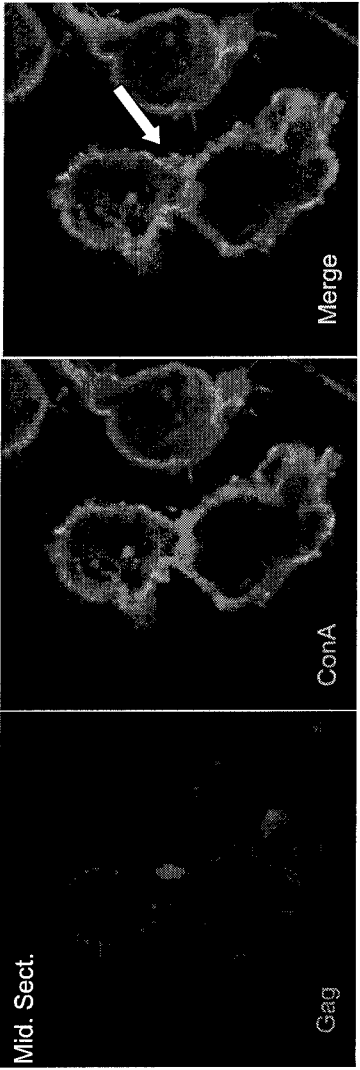
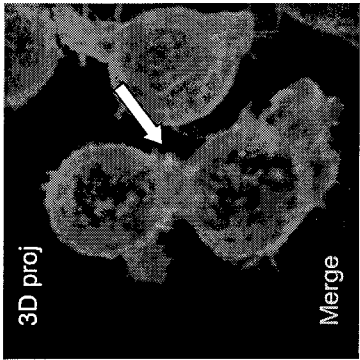
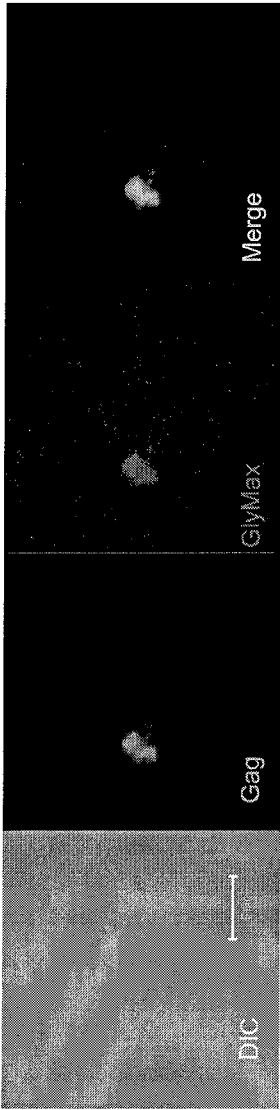


FIG. 33

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/IB2010/055747

A. CLASSIFICATION OF SUBJECT MATTER  
INV. G01N33/50  
ADD.

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)  
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, WPI Data, BIOSIS, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                          | Relevant to claim No. |
|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | <p>BALESTRIERI EMANUELA ET AL: "Inhibition of cell-to-cell transmission of human T-cell lymphotropic virus type 1 in vitro by carbohydrate-binding agents", August 2008 (2008-08), ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, VOL. 52, NR. 8, PAGE(S) 2771-2779, XP009147050, ISSN: 0066-4804<br/>abstract<br/>page 2771, column 2<br/>page 2772, column 1</p> <p style="text-align: center;">-----<br/>-/-</p> | 1-16                  |

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

☒ See patent family annex.

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"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

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Date of the actual completion of the international search

28 April 2011

Date of mailing of the international search report

09/05/2011

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

Authorized officer

Jones, Laura

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/IB2010/055747

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                         | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X,P       | PAIS-CORREIA ANA-MONICA ET AL:<br>"Biofilm-like extracellular viral<br>assemblies mediate HTLV-1 cell-to-cell<br>transmission at virological synapses",<br>January 2010 (2010-01), NATURE MEDICINE,<br>VOL. 16, NR. 1, PAGE(S) 83, XP009146981,<br>ISSN: 1078-8956<br>the whole document<br>-----                                                                          | 1-16                  |
| X,P       | VAN PROOYEN NANCY ET AL: "Human T-cell<br>leukemia virus type 1 p8 protein increases<br>cellular conduits and virus transmission",<br>30 November 2010 (2010-11-30), PROCEEDINGS<br>OF THE NATIONAL ACADEMY OF SCIENCES OF THE<br>UNITED STATES (PNAS), NATIONAL ACADEMY OF<br>SCIENCE, US, PAGE(S) 20738 - 20743,<br>XP009146982,<br>ISSN: 0027-8424<br>abstract<br>----- | 1-16                  |
| A         | PAIS-CORREIA ANA MONICA ET AL: "T cell<br>polarization and the formation of<br>immunological synapses: From antigen<br>recognition to virus spread",<br>1 August 2007 (2007-08-01), CURRENT<br>IMMUNOLOGY REVIEWS,, PAGE(S) 170 - 188,<br>XP001525737,<br>ISSN: 1573-3955<br>the whole document<br>-----                                                                   | 1-16                  |
| A         | RUDNICKA DOMINIKA ET AL: "Simultaneous<br>Cell-to-Cell Transmission of Human<br>Immunodeficiency Virus to Multiple Targets<br>through Polysynapses",<br>June 2009 (2009-06), JOURNAL OF VIROLOGY,<br>VOL. 83, NR. 12, PAGE(S) 6234-6246,<br>XP009146378,<br>ISSN: 0022-538X(print)<br>abstract<br>page 6243, column 2<br>-----                                             | 1-16                  |
| A         | MAJOROVITS ENDRE ET AL: "Human<br>T-Lymphotropic Virus-1 Visualized at the<br>Virological Synapse by Electron<br>Tomography",<br>1 May 2008 (2008-05-01), PLOS ONE, PUBLIC<br>LIBRARY OF SCIENCE, SAN FRANCISCO, CA, US,<br>PAGE(S) E2251, XP009147000,<br>ISSN: 1932-6203<br>the whole document<br>-----<br>-/--                                                          | 1-16                  |

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/IB2010/055747

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                    | Relevant to claim No. |
|-----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | ALFSEN ANNETTE ET AL: "HIV-1-infected blood mononuclear cells form an integrin- and agrin-dependent viral synapse to induce efficient HIV-1 transcytosis across epithelial cell monolayer", September 2005 (2005-09), MOLECULAR BIOLOGY OF THE CELL, VOL. 16, NR. 9, PAGE(S) 4267-4279, XP002633915, ISSN: 1059-1524<br>cited in the application<br>page 4267, column 1<br>page 4275, column 2<br>page 4276, column 2 | 1-16                  |
| A         | NEIL STUART J D ET AL: "Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu.", 24 January 2008 (2008-01-24), NATURE 24 JAN 2008 LNKD- PUBMED:18200009, VOL. 451, NR. 7177, PAGE(S) 425 - 430, XP009147477, ISSN: 1476-4687<br>abstract                                                                                                                                                               | 1-16                  |
| A         | HSU D K ET AL: "Human T lymphotropic virus-I infection of human T lymphocytes induces expression of the beta-galactoside-binding lectin, galectin-3.", May 1996 (1996-05), THE AMERICAN JOURNAL OF PATHOLOGY MAY 1996 LNKD- PUBMED:8623933, VOL. 148, NR. 5, PAGE(S) 1661 - 1670, XP009146143, ISSN: 0002-9440<br>abstract                                                                                            | 1-16                  |
| A         | WO 2004/019970 A2 (LEUVEN K U RES & DEV [BE]; BALZARINI JAN [BE]; PREOBRAZHENSKAYA MARIA) 11 March 2004 (2004-03-11)<br>claim 20                                                                                                                                                                                                                                                                                      | 1-16                  |
| A         | US 2005/276818 A1 (GODZIK ADAM [US] ET AL) 15 December 2005 (2005-12-15)<br>claims 1-4                                                                                                                                                                                                                                                                                                                                | 1-16                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2010/055747

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---------------------|----------------------------|---------------------|
| WO 2004019970 A2                          | 11-03-2004          | AU 2003260198 A1           | 19-03-2004          |
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|                                           |                     | JP 2006503015 T            | 26-01-2006          |
|                                           |                     | KR 20060091049 A           | 17-08-2006          |
|                                           |                     | US 2005250677 A1           | 10-11-2005          |
| -----                                     |                     |                            |                     |
| US 2005276818 A1                          | 15-12-2005          | NONE                       |                     |
| -----                                     |                     |                            |                     |