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Research paper

TRAIL protein localization in human primary T cells by 3D microscopy using 3D interactive surface plot: A new method to visualize plasma membrane

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ABSTRACT

The apoptotic ligand TNF-related apoptosis ligand (TRAIL) is expressed on the membrane of immune cells during HIV infection. The intracellular stockade of TRAIL in human primary $CD4^+$ T cells is not known.
Here we investigated whether primary $CD4^+$ T cells expressed TRAIL in their intracellular compartment and whether TRAIL is relocalized on the plasma membrane under HIV activation. We found that TRAIL protein was stocked in intracellular compartment in non activated $CD4^+$ T cells and that the total level of TRAIL protein was not increased under HIV-1 stimulation. However, TRAIL was massively relocalized on plasma membrane when cells were cultured with HIV. Using three dimensional (3D) microscopy we localized TRAIL protein in human T cells and developed a new method to visualize plasma membrane without the need of a membrane marker. This method used the 3D interactive surface plot and bright light acquired images.

1. Introduction

The TNF-related apoptosis ligand (TRAIL, Apo2L, TNFSF10, CD253), a TNF-α family member (Wiley et al., 1995), is an apoptotic ligand that induces cell death by binding to its two death receptors DR4 (TRAIL-R1, TNFRSF10A) and DR5 (TRAIL-R2, Apo2, TNFRSF10B, Trick2, TRANCE-R, CD262) (Sheridan et al., 1997; Wu et al., 1997). The two biologically active forms of TRAIL, membrane-bound (mTRAIL) and soluble TRAIL (sTRAIL), are regulated by type I interferon (Sato et al., 2001; Ehrlich et al., 2003; Tecchio et al., 2004). mTRAIL is expressed by leukocytes, including T lymphocytes (Kayagaki et al., 1999), natural killer cells (Smyth et al., 2001), dendritic cells (Vidalain et al., 2000), B cells, monocytes (Ehrlich et al., 2003) and macrophages (Herbeuval et al., 2003). TRAIL had been extensively studied in oncology (Ashkenazi and Herbst, 2008), due to its property to induce apoptosis of a wide range of tumor cells (Griffith and Lynch, 1998). However, TRAIL localization into immune cells remained poorly documented. We recently demonstrated that plasmacytoïd dendritic cells intracellularly stocked TRAIL. Under HTLV-1 stimulation, intracellular TRAIL is rapidly relocalized on plasma membrane transforming pDC into killer cells (IKpDC) (Colisson et al., 2010).

TRAIL may also play a role during HIV-1 infection and progression to AIDS. Indeed, HIV-1 infected patients exhibit higher serum levels of soluble TRAIL than non-infected healthy controls, and TRAIL levels correlate with HIV-1 viral load (Herbeuval et al., 2005a). TRAIL is one of

Abbreviations: 3D, three-dimensional; pDC, plasmacytoïd dendritic cell; $CD4^+$ T cells, $CD4^+$ T lymphocytes; TRAIL, TNF-related apoptosis-inducing ligand; DR5, death receptor 5

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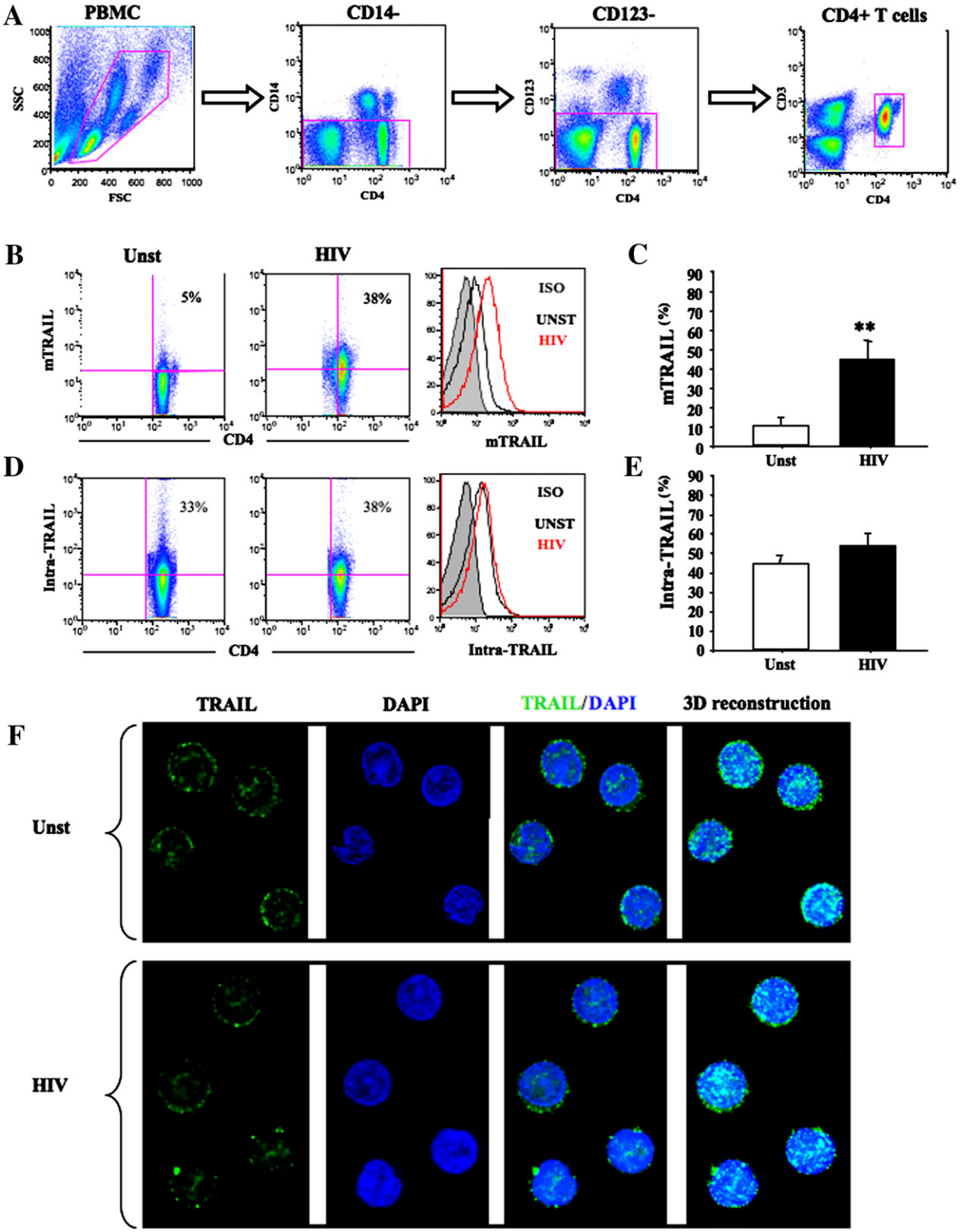
the first cytokines secreted during the acute phase of HIV infection (Gasper-Smith et al., 2008). TRAIL is expressed in lymphoid tissues of both HIV-1 infected individuals (Herbeuval et al., 2006) and SIV-infected macaques (Herbeuval et al., 2009). TRAIL selectively induces apoptosis of human HIV-1-exposed $CD4^+$ T cells *in vitro* (Lichtner



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et al., 2004) and participates *in vivo* in $CD4^+$ T cell depletion observed in HIV-1-infected hu-PBL-NOD-SCID mice (Miura et al., 2001). TRAIL-expressing killer pDC were demonstrated to be in close proximity to apoptotic $CD4^+$ T cells in tonsils from HIV-infected viremic patients (Stary et al., 2009).

Moreover, a recent study showed that the loss of memory B cells during chronic HIV-1 infection is driven by Foxo3a and TRAIL-mediated apoptosis (van Grevenynghe et al., 2011). We also reported that HIV-1 infection upregulates DR5 expression *in vivo* on primary $CD4^+$ T cells from infected patients, which were prone to TRAIL-mediated apoptosis (Herbeuval et al., 2005b). Although many studies demonstrated that HIV-1 induced membrane TRAIL expression on human $CD4^+$ T cells, TRAIL localization in human primary $CD4^+$ T cells remains unknown. Human primary T cells are characterized by a very large nucleus and a small cytoplasm. Thus, these characteristics make difficult the microscopy study of intracellular protein and membrane localization.

Here we investigated whether TRAIL is intracellularly stocked in human primary $CD4^+$ T cells and whether HIV-1 stimulation induces a membrane relocalization or not. Using three-dimensional (3D) microscopy we localized TRAIL in human T cells and developed a new method to visualize plasma membrane without the need of membrane marker. This method allowed us to precisely determine TRAIL membrane or intracellular localization of TRAIL protein in human primary $CD4^+$ T cells. The interest of the 3D microscopy is to visualize the entire cell, thus to observe each layer. We can then choose the best stack, meaning the one that represents what we want to show. We stain each protein of interest by a different color. When two proteins are close from each other the colors blend together, creating a new color. We can then deduce what we have a colocalization. We analyze the images with the ImageJ software, using the 3D interactive surface plot and 3D viewer. The interest of the 3D interactive surface plot is to allow us to visualize the membrane without the need of membrane markers. The 3D interactive surface plot is a plugin that creates interactive surface plots from all image types. The luminance of an image is interpreted as height for the plot. Internally the image is scaled to a square image using nearest neighbor sampling. We obtain different heights indicating the intensity of the color, thus the quantity of the stained protein. With the 3D interactive surface plot we observe one stack of the cell, which is a 2D picture image from a 3D acquisition. However it is also a 3D representation of the quantity of protein in the cell.

2. Material and methods

2.1. Blood samples

Blood from healthy HIV-1-seronegative blood bank donors was obtained from "Etablissement Français du Sang" (convention # 07/CABANEL/106), Paris, France. Experimental procedures with human blood have been approved by Necker Hospital Ethical Committees for human research and were done according to the European Union guidelines and the Declaration of Helsinki.

2.2. Isolation and culture of blood leukocytes

In vitro experiments were performed using peripheral blood mononuclear cells (PBMC) isolated by density centrifugation from peripheral blood leukocyte separation medium (Cambrex, Gaithersburg, MD). $CD4^+$ T cells were purified using the CD4 purification kit (Stem Cell, Grenoble, France). Cells were cultured in RPMI 1640 (Invitrogen, Gaithersburg, MD) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% Pen–Strep–Glut (Invitrogen).

2.3. Viral stimulation

PBMC or purified $CD4^+$ T cells were seeded at 10^6 cells per 1 mL and cultured overnight with HIV-1 (MN strand and AT2) at 60 ng/mL $p24^{CA}$ equivalent in RPMI 1640 (Invitrogen, Gaithersburg, MD) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% Pen–Strep–Glut (Invitrogen,). Cells were used for FACS and microscopic experiments.

2.4. Flow cytometry

Cultured cells were incubated for 20 min at 4 °C with fluorescein isothiocyanate (FITC)-conjugated anti-CD123 (BD Biosciences, San Jose, CA), phycoerythrin (PE)-conjugated TRAIL (eBioscience, San Diego, CA), allophycocyanin-Cy7 (APC-Cy7)-conjugated anti-CD14 (BD Biosciences), Vioblue-conjugated anti-CD4 (Miltenyi Biotech, Bergisch Gladbach, Germany), V500-conjugated anti CD3 or with appropriate isotype-matched control antibodies (at 5 mg/mL each) in PBS (Sigma, Saint Louis, MO) and Fc-receptor blockers (BD, Biosciences). Cells were washed twice in ice-cold PBS and FACS analysis was performed on a FACSCanto II 7 colors flow cytometer using FACSDiva software (BD Biosciences). Gated cells were then tested for the expression of surface markers using PE-labeled anti-TRAIL (eBioscience). FlowJo software

Fig. 1. Characterization of $CD4^+$ T cells in PBMCs and TRAIL expression. A: flow cytometry gating of $CD4^+$ T cells in PBMC. Live $CD4^+$ T cell population was gated using FCS/SSC, $CD4^+/CD14^-$, $CD4^+/CD123^-$, $CD4^+/CD3^+$. B: PBMC were cultured overnight in the absence or presence of HIV-1. Membrane TRAIL expression by unstimulated (Unst) or HIV-stimulated (HIV) $CD4^+$ T cells was quantified by flow cytometry. Results show the percentage of membrane TRAIL (mTRAIL) expression by $CD4^+$ T cells compared to isotype (ISO). C: statistical analysis of membrane TRAIL expression by unstimulated or HIV-1 activated $CD4^+$ T cells. D: total TRAIL expression by unstimulated (Unst) or HIV-1 stimulated (HIV) $CD4^+$ T cells using intracellular staining. Results represent the level of intracellular and membrane TRAIL expressed by cells. E: statistical analysis of membrane TRAIL expression by unstimulated or HIV-1 activated $CD4^+$ T cells. P values (p) were determined using a two-tailed Student's *t* test. $p<0.05$ was considered statistically significant. $p<0.05$ one star, $p<0.01$ two stars, $p<0.001$ three stars. F: study of TRAIL expression by three dimensional (3D) microscopy. Purified $CD4^+$ T cells were cultured overnight in the absence or presence of HIV-1. The first panel shows TRAIL staining (green), the second panel shows DAPI (blue), third panel shows merged images of TRAIL and DAPI. The fourth panel shows a merged compilation from each stack representing a view of total TRAIL staining by each cell. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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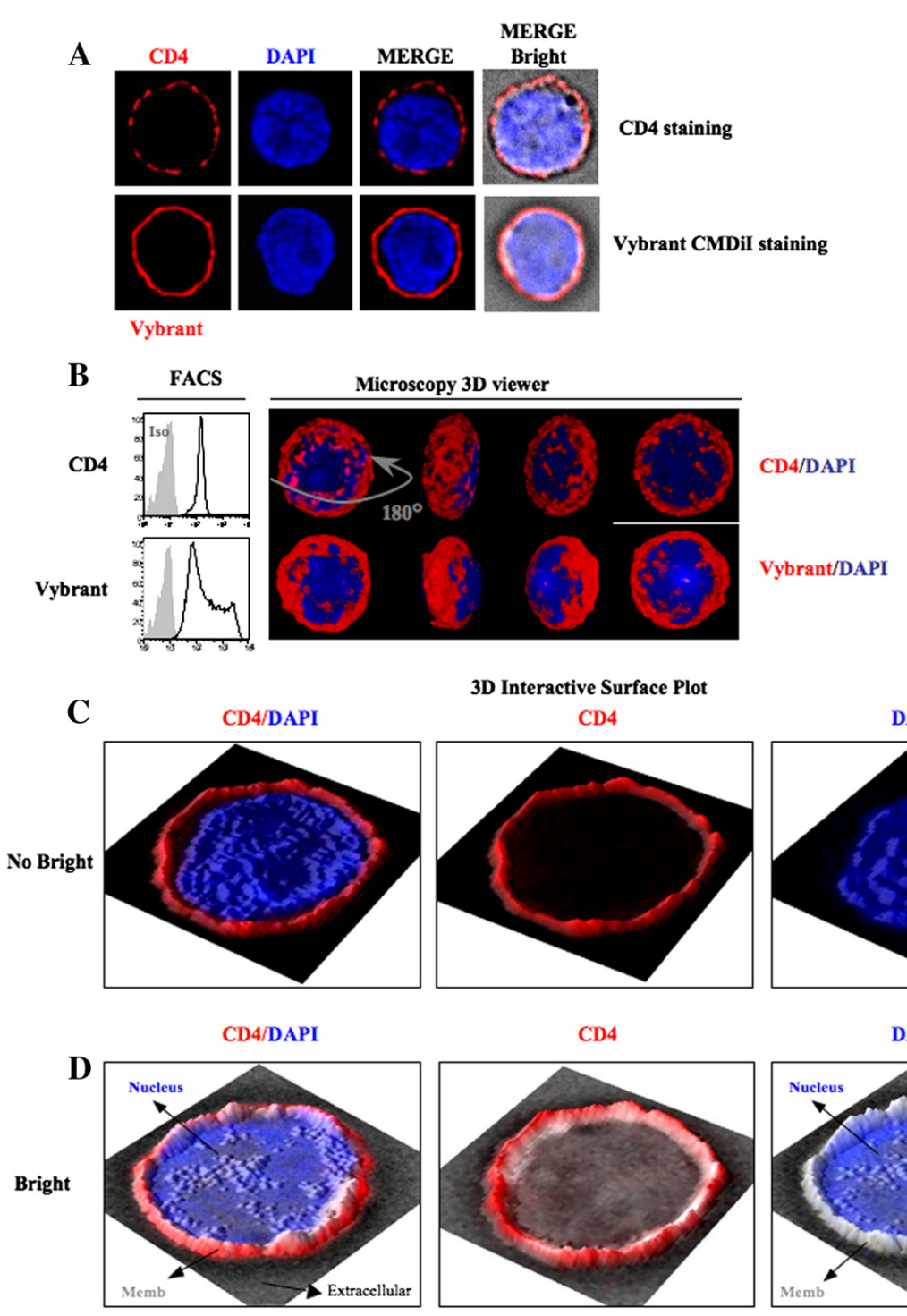
(Treestar, Ashland, OR) was used to analyze flow cytometry data.

2.5. Three dimensional microscopy

Purified CD4$^+$ T cells were cultured overnight in absence or presence of HIV-1. CD4$^+$ T cells were plated on poly-L-lysine (Sigma-Aldrich, St. Louis, MO)-coated slides and then fixed in 4% paraformaldehyde, quenched with 0.1 M glycine. Cells were incubated in permeabilizing buffer containing 1% saponin with monoclonal antibody anti-TRAIL (ebioscience) and with Alexa647 labeled anti-CD4 (BD Bioscience) or Vybrant CM-DiI (Invitrogen). TRAIL was revealed by a Donkey anti-mouse IgG-Alexa488 (Jackson ImmunoResearch, West Grove, PA).



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Nucleus was stained using DAPI (Molecular Probes, Paisley, UK). Mounted slides were scanned with a Nikon Eclipse 90i Upright microscope (Nikon Instruments Europe, Badhoevedorp, The Netherlands) using a 100× Plan Apo VC piezo objective (NA 1.4) and Chroma bloc filters (ET-DAPI, ET-GFP) and were subsequently deconvoluted (Sibarita, 2005) with a Meinel algorithm and 8 iterations and analyzed using Metamorph® (MDS Analytical Technologies, Winnersh, UK). TRAIL/CD4/DAPI/Overlay/Confocal plane: representative 2D focal plan. Overlay with bright: bright. Reconvolution overlays: 2D projections of the maximum intensity pixels along the Z axis. 3D: interactive surface plot, 3D reconstruction and 3D viewer analyses of purified $CD4^+$ T cells were performed using the ImageJ software (NIH, Bethesda, MD, USA).

3. Results

3.1. TRAIL expression by primary $CD4^+$ T cells

PBMC were isolated from healthy blood donors. $CD4^+$ T cells were characterized using a battery of immune cell markers (Fig. 1A). First, anti-CD14 antibodies were used to discriminate $CD4^+$ T cells between monocytes expressing CD4. Anti-CD123 antibodies were used to visualize APC that could potentially express CD4. Finally, anti-CD3 (T cell marker) and anti CD4 antibodies precisely identified $CD4^+$ T cells ($CD14^-$ $CD123^-$ $CD3^+$ $CD4^+$).

$CD4^+$ T cells were purified from PBMC and cultured with HIV-1 (MN). We tested HIV-1-mediated TRAIL expression on the cell surface of $CD4^+$ T cells. Membrane TRAIL (mTRAIL) was expressed by 15% of freshly purified $CD4^+$ T cells from HD when cultured in media overnight without any stimulation (Unst) (Fig. 1B). Thus, *in vitro* exposure to HIV-1 significantly increased the level of membrane TRAIL expression by $CD4^+$ T cells. The number of $CD4^+$ T cells expressing mTRAIL (Fig. 1B) was increased by HIV-1 (Fig. 1C) ($p = 0.0010$).

Intracellular staining of TRAIL revealed that unstimulated $CD4^+$ T cells expressed high levels of intracellular TRAIL (Fig. 1D). It should be noticed that when doing intracellular stainings, both intracellular and extracellular TRAIL are stained. Surprisingly, HIV did not significantly upregulate intracellular TRAIL (Fig. 1E). These results suggest that the increase of mTRAIL at cell surface by HIV is not due to a global increase of TRAIL protein but rather to a relocalization of TRAIL from intracellular compartment to plasma membrane. Here, we observe 33% of total TRAIL protein in unstimulated cells versus 38% in HIV stimulated cells. There is only a 5% difference in the quantity of TRAIL in and on the cells, which correspond to an increase of 15% of production of TRAIL with HIV.

To test whether TRAIL is relocalized from the intracellular compartment to plasma membrane in HIV-activated $CD4^+$ T cells, we performed 3D microscopy experiments. Purified $CD4^+$ T cells were cultured in media alone (unstimulated) or with HIV-1. Permeabilized $CD4^+$ T cells were stained with TRAIL-Alexa 488 (green) and nuclear staining DAPI (blue). Focal plane analysis revealed the presence of intracellular TRAIL expression in unstimulated $CD4^+$ T cells, confirming our cytometry data (Fig. 1F, upper panels). Images also revealed some 'peripheral' TRAIL expression that did not seem to be localized in the cytoplasm but rather on the membrane (Fig. 1F, lower panels). TRAIL expression profile in HIV-1-stimulated $CD4^+$ T cells did not seem to differ from unstimulated cells, even if TRAIL appeared to be decreased in the cytoplasm at the expense of "peripheral" TRAIL (Fig. 1F, lower panels). However, it remained hard to distinguish intracellular between membrane profile TRAIL expression in both conditions without the use of a membrane marker. Indeed, even if TRAIL expression profile is lightly different in unstimulated versus HIV-activated $CD4^+$ T cells, this method of representation is not sufficient to precisely localize TRAIL. Finally, we also used the 3D reconstruction analysis (construction of a 3D model of an object from several two-dimensional views of it) to characterize TRAIL localization in unstimulated and HIV-activated $CD4^+$ T cells.. The different 2D views are complied to create a 3D reconstruction. This representation allowed the visualization of the total staining of the different plans for each cell. TRAIL expression profiles were quite similar in unstimulated and HIV-stimulated $CD4^+$ T cells. Thus this 3D reconstruction analysis was not providing any further information on TRAIL localization.

3.2. Membrane visualization using markers and 3D interactive surface plot from ImageJ

To better characterize localization of proteins in $CD4^+$ T cells, we performed 3D experiments using membrane markers of $CD4^+$ T cells. Plasma membrane was visualized using anti-CD4 antibodies and the membrane marker Vybrant, and the nucleus was stained with DAPI. Image plane analysis showed that CD4 and/or Vybrant (red) was homogeneously expressed and precisely delineated T cell membrane (Fig. 2A). Overlay pictures also showed the very thin space between the nucleus (DAPI) and the membrane. Right panels showed CD4 and Vybrant using bright light.

Thus to better visualize membrane marker repartition, we showed CD4 expression on T cells using the ImageJ 3D viewer that allowed us to visualize cell surface in 3 dimensions (Fig. 2B). This 3D volume viewer plugin shows stacks as volume visualizations within a 3D XYZ-space. Stacks of the cells are taken from the top of the cell to the bottom. Those

Fig. 2. Visualization of plasma membrane using specific staining and interactive surface plot analysis. A: membrane visualization of purified $CD4^+$ T cells by 3D microscopy: first upper panel shows CD4 staining (red), second upper panel shows DAPI (blue), third panel shows merged images of CD4 and DAPI, and fourth panel shows a merge of third panel acquired using bright light (Bright). First lower panel shows Vybrant membrane marker staining (red, Vybrant), second panel is DAPI (blue), third panel shows merged images of Vybrant and DAPI, and fourth panel shows a merge of third panel acquired using bright light (Bright). B: 3D viewer analysis of $CD4^+$ T cells stained with CD4 or Vybrant: left plots show CD4 or Vybrant expression analyzed by flow cytometry. Right panels show different angles of CD4 (red) or Vybrant stainings (red) on $CD4^+$ T cells analyzed using 3D volume viewer. CD4 and Vybrant marker covers the cell surface of T cells. C: interactive 3D surface plot analysis of $CD4^+$ T cell staining (Vybrant, red) acquired without bright light. Left panel shows CD4 and DAPI staining, middle panel shows membrane marker Vybrant staining alone and right panel shows nucleus DAPI staining alone. D: interactive 3D surface plot analysis of $CD4^+$ T cell staining (Vybrant, red) acquired bright light. Cells were stained as previously with DAPI and Vybrant. Middle panel allows visualization of plasma membrane (red) that corresponds to grey color on the right panel. Thus bright light acquisition allows a clear visualization of plasma membrane (grey). Data showed are representative of 4 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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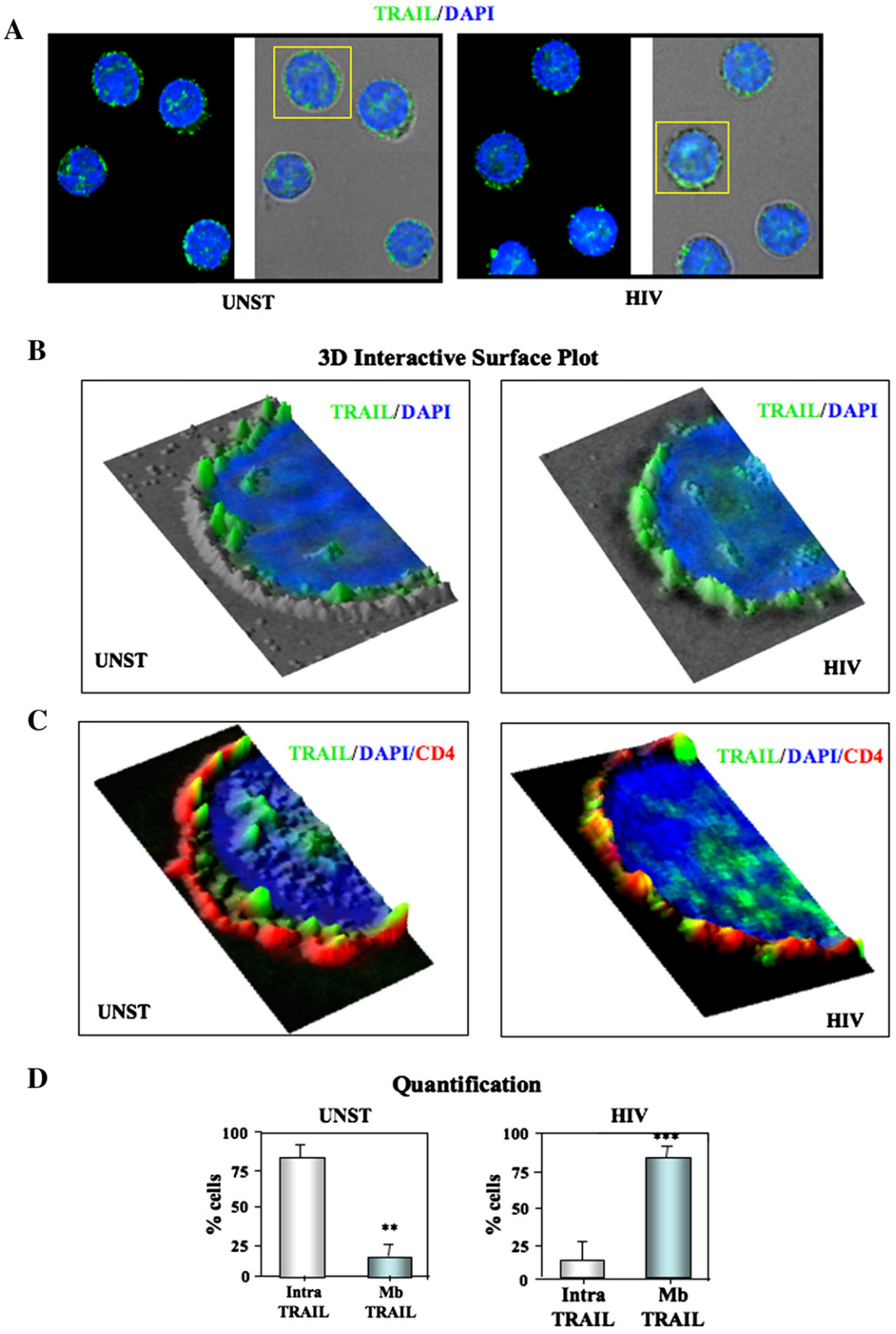

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stacks are then put together to get a 3D image. We found that CD4 and Vybrant marker covered the cell surface of T cells.

Thus we used the 3D interactive surface plot plugin of ImageJ software to analyze previous microscopy data. This plugin creates interactive surface plots from all kinds of 3D microscopy pictures. The luminance of each pixel in the image is interpreted as the height for the plot. An adjustment of the lightning condition improves the visibility of small differences. We developed here a new way to visualize the plasma membrane without the need of a marker.

Fig. 2C showed 3D surface plot of T cell where we can clearly see the plasma membrane stained with Vybrant (red) and the nucleus (blue). When Vybrant is removed (right panels) we still could distinguish the nucleus but not the membrane or the cytoplasm. Thus, we acquired the 3D samples with the bright light (Fig. 2D). We could observe the membrane in red, and the nucleus in blue, as observed in Fig. 2C. However, when the plasma membrane marker was removed, we still could observe the membrane, which appeared in light gray. This unique property is due to the different luminance between the plasma membrane (majority of lipids) and the cytoplasm. The interactive 3D surface plot analysis is based on the luminance of each pixel in the image, which is interpreted as the height for the plot. Thus, using bright light acquisition and 3D surface plot analysis, we could clearly visualize plasma membrane without the need of a membrane marker.

3.3. TRAIL localization in $CD4^+$ T cells using 3D surface plot

We next tested whether our method would permit to precisely localize TRAIL by unstimulated and HIV-activated $CD4^+$ T cells without any plasma membrane marker. Acquisitions from Fig. 1D were performed using bright light (Fig. 3A). Cells were stained with anti-TRAIL antibodies (green) and DAPI. One cell from each condition was selected (Fig. 3B) and interactive 3D surface plot was performed on the bright light acquisition. As shown in Fig. 3C, we could clearly visualize the plasma membrane that appeared in grey, confirming our findings in Fig. 2D. Furthermore, we thus observed that the majority of TRAIL protein was localized in the intracellular compartment. In contrast, when T cells were cultured overnight with HIV-1, 3D interactive surface plot analysis revealed that the vast majority of TRAIL protein was localized on the membrane, which thus appeared in green. These results were in accordance with the cytometry experiments that clearly showed that HIV-1-exposed $CD4^+$ T cells upregulated membrane TRAIL. Finally, to confirm our results, we used a plasma membrane marker to determine TRAIL expression. Unstimulated or HIV-exposed $CD4^+$ T cells were stained with anti-TRAIL antibodies (green), anti-CD4 antibodies (red) and DAPI (blue). As shown in Fig. 3C, TRAIL protein was revealed in the intracellular compartment and did not colocalize with CD4 in unstimulated cells. In contrast, HIV-activated $CD4^+$ T cells harbored both intracellular and plasma membrane TRAIL expression. We showed that TRAIL and CD4 colocalized (yellow spots) in HIV-activated cells.

Thus, we quantified the number of $CD4^+$ T cells ($n = 50$) in 3 independent experiments that expressed only intracellular TRAIL and intracellular and membrane TRAIL. As shown in Fig. 3D, 82% of unstimulated $CD4^+$ T cells only expressed intracellular TRAIL, and 18% of the cells expressed membrane TRAIL ($p = 0.002$). In contrast, 80% of HIV-exposed $CD4^+$ T cells expressed membrane (and intracellular) TRAIL and 20% only expressed intracellular TRAIL ($p = 0.0001$). It should be noted that all the $CD4^+$ T cells expressing membrane TRAIL also expressed intracellular TRAIL.

3.4. Quantification of membrane TRAIL by 3D interactive surface plot

Previous data of Fig. 3 demonstrated that HIV induced a relocalization of TRAIL from the intracellular compartment to the plasma membrane. Surprisingly, by analyzing more precisely TRAIL and CD4 colocalization, we observed that some TRAIL staining was localized on cell membrane but did not colocalize with CD4 (yellow arrow 2 and 3) (Fig. 4A). Thus, these staining dots of membrane TRAIL would appear as negative by using classic 3D microscopy colocalization software. We performed TRAIL expression quantification of 50 cells per condition by counting the number of intracellular and membrane TRAIL spots (Fig. 4B). Unstimulated $CD4^+$ T cells mainly expressed TRAIL in their intracellular compartment (89%, $p = 0.0009$) in contrast to HIV-stimulated $CD4^+$ T cells in which expressed 65% of TRAIL was localized on the membrane and 35% in the intracellular compartment ($p = 0.002$). Thus, HIV stimulation induced a changed of the TRAIL membrane/intracellular ratio, in favor of the membrane.

Finally, we quantified membrane TRAIL expressed by HIV-stimulated $CD4^+$ T cells using the 3D interactive surface plot and the CD4/TRAIL colocalization method (ImageJ software). As previously described in Fig. 4B, 65% of TRAIL was localized on plasma membrane when using 3D interactive surface plot method (Fig. 4C). In contrast, we statistically found less TRAIL protein on cell membrane when we quantified using the CD4/TRAIL colocalization method. Indeed, only 48% (versus 65%, $p = 0.0025$) of TRAIL was found to colocalize with membrane CD4. Intuitively this result could have been predicted, as we observed in Fig. 4A some "false negative" TRAIL staining. Yellow arrows 2 and 3 highlighted TRAIL dots localized on the plasma membrane but that do not colocalize with CD4.

Fig. 3. TRAIL expression study using 3D interactive surface plot in $CD4^+$ T cells. Purified $CD4^+$ T cells were cultured overnight in the absence or presence of HIV. TRAIL expression by $CD4^+$ T cells was analyzed by 3D microscopy and acquired using bright light. A: unstimulated (UNST) or HIV-1-activated (HIV) $CD4^+$ T cells were stained with anti-TRAIL (green) and acquired without (left panels) or with bright light (right panels). Yellow squares represent our selection of $CD4^+$ T cells that will be studied in detail. B: 3D interactive surface plot of $CD4^+$ cells selected in 3A using bright light. Left panel shows intracellular TRAIL (green) expression in unstimulated cells. The plasma membrane (grey) does not harbor TRAIL staining. Right panel shows membrane TRAIL expression (green) by HIV-1-stimulated $CD4^+$ T cells. C: 3D interactive surface plot of $CD4^+$ T cells using membrane marker CD4 (red). Left panel shows intracellular TRAIL staining (green) by unstimulated $CD4^+$ T cells. Colocalization between CD4 and TRAIL are not observed in unstimulated cells. Right panel shows membrane TRAIL expression (green), which colocalized (yellow) with membrane marker CD4 (red). D: quantification of the number of $CD4^+$ T cells (unstimulated and HIV-activated) expressing intracellular or membrane TRAIL using 3D interactive surface plot. P values (p) were determined using a two-tailed Student's *t* test. $p<0.05$ one star, $p<0.01$ two stars, $p<0.001$ three stars.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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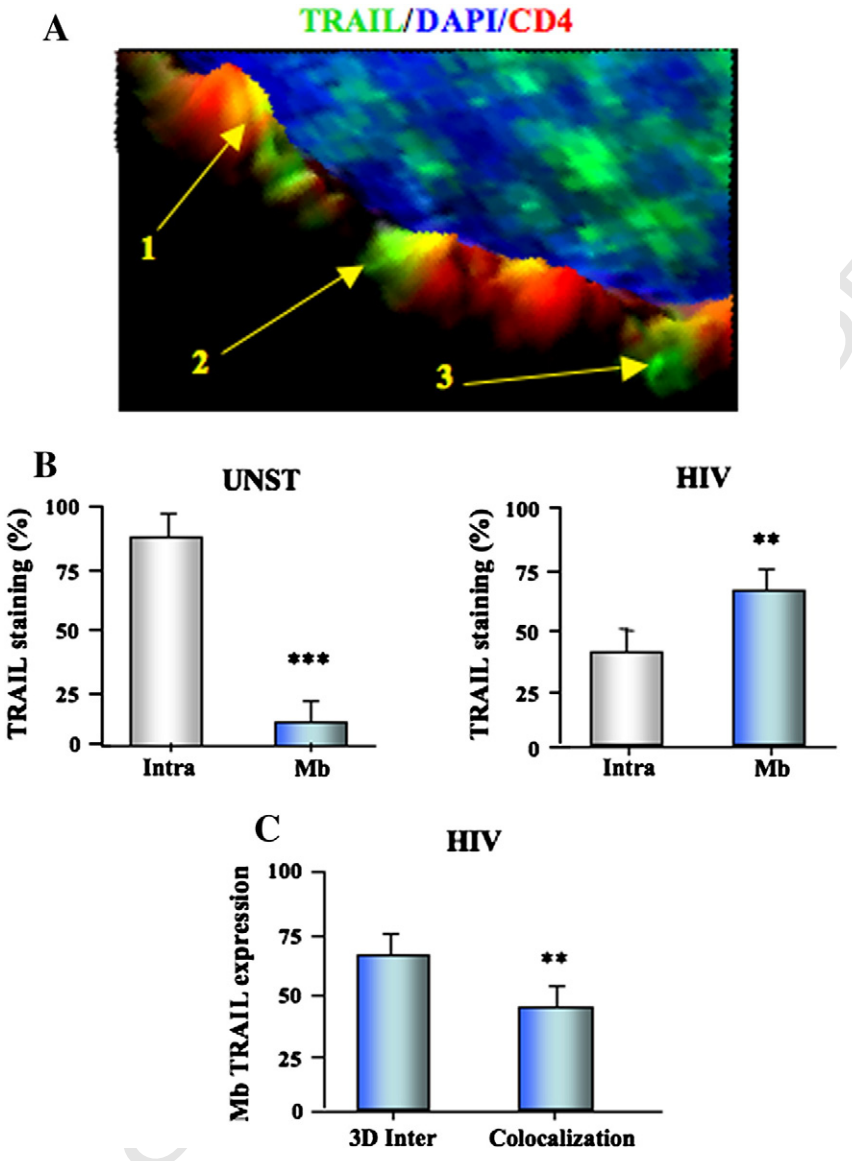

Fig. 4. TRAIL localization and quantification using 3D interactive surface plot. A: 3D interactive surface plot of an HIV-1-activated $CD4^+$ T cell stained with TRAIL (green), DAPI (blue) and CD4 (red). Yellow arrow 1 shows a colocalization dot between CD4 and TRAIL staining. Arrows 2 and 3 show that TRAIL localized on the membrane but that do not colocalize with CD4. B: TRAIL expression quantification of HIV-1-activated $CD4^+$ T cells ($n=50$) by counting the number of intracellular and membrane TRAIL spots. C: Quantification of membrane TRAIL expressed by HIV-1-stimulated $CD4^+$ T cells ($n=50$) using the 3D interactive surface plot and the CD4/TRAIL colocalization method (ImageJ software). The colocalization method was performed by counting the number of yellow spots (colocalization plugin for ImageJ. 3D interactive surface plot method quantified membrane TRAIL spots (yellow and green). P values (p) were determined using a two-tailed Student's *t* test. $p<0.05$ one star, $p<0.01$ two stars, $p<0.001$ three stars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Thus, membrane visualization by 3D interactive surface plot provides a new tool to visualize protein localization avoiding false negative results and thus could constitute a helpful support to classical methods especially in human primary cells.

4. Conclusion

The pro-apoptotic ligand TRAIL is expressed by many immune cells during HIV-1 infection including monocytes (Herbeuval et al., 2005a), plasmacytoid dendritic cells (Hardy et al., 2007; Stary et al., 2009), NK (Melki et al., 2009) and T cells (Herbeuval et al., 2005c; Lum et al., 2005). The release of TRAIL during HIV-1 transmission occurs very early at the onset of plasma viremia (Gasper-Smith et al., 2008), and TRAIL is expressed in lymphoid tissues where the massive $CD4^+$ T cell depletion occurs (Guadalupe et al., 2003; Stary et al., 2009). Tonsils from patients under antiretroviral therapy (ART) showed reduced expression of TRAIL compared to untreated HIV-positive patients (Herbeuval et al., 2009), and

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poor CD4$^+$ T cell recovery in response to ART has been associated with higher TRAIL receptor expression (Hansjee et al., 2004). These *in vitro* and *in vivo* results establish a potential crucial role of TRAIL in HIV immunopathogenesis (Herbeuval and Shearer, 2006; Cummins and Badley, 2010). Thus, mechanism understanding TRAIL regulation and expression appeared to be central to better define its role during infection.

Human primary T cells are characterized by a voluminous nucleus and a relatively small cytoplasm making intracellular localization of proteins difficult. Flow cytometry data showed that HIV-1 induced membrane TRAIL expression on CD4$^+$ T cells, in accordance with previous studies (Herbeuval et al., 2005c; Lum et al., 2005). Surprisingly, intracellular staining revealed that HIV-1 did not statistically increase the number of TRAIL expressing cells. Approximately 40% of cells were positive for intracellular TRAIL, irrespective of the activation state, suggesting that unstimulated T cells stocked TRAIL protein in the cytoplasm. This stockade of TRAIL protein in resting cells was also in favor of a relocalization of TRAIL from the intracellular compartment to the plasma membrane under HIV stimulation.

Thus, to better characterize TRAIL expression in CD4$^+$ T cells, we performed 3D microscopy experiments using anti-TRAIL antibodies and a nucleus marker (DAPI). Confirming our flow cytometry results, we found TRAIL protein in HIV-1-exposed and also in resting CD4$^+$ T cells. The use of the nuclear marker DAPI allowed us to show that TRAIL protein was not intra-nuclear, due to the absence of DAPI and TRAIL colocalization, but was not sufficient to precisely determine whether TRAIL was at the membrane or in the cytoplasm. Indeed, TRAIL expression profile in HIV-1-stimulated CD4$^+$ T cells was very similar to unstimulated cells, even if TRAIL appeared to be decreased in the cytoplasm at the expense of "peripheral" TRAIL. However, it remained impossible to clearly characterize TRAIL expression without the use of a membrane marker.

Thus, we developed a new method to visualize plasma membrane from 3D microscopy pictures using ImageJ software. Our method is based on the visualization of the plasma membrane by doing microscopic cell acquisition using bright light. Thus using a plugin of the ImageJ software, the 3D interactive surface plot, we performed analysis of microscopy data. 3D interactive surface plot allowed interpretation of the luminance of each pixel as the height for the plot. An adjustment of the lightning condition improves the visibility of small differences. The analysis of 3D microscopy data acquired with bright light using 3D interactive surface plot allowed us to visualize the plasma membrane in 3 dimensions due to its differential light reflection properties compared to extra- and intra-cellular compartments. Consequently, we were able to visualize plasma membrane proteins. Using this new method, we found that TRAIL was mainly stocked in the intracellular compartment of CD4$^+$ T cells. In contrast, when cells were exposed to HIV-1, CD4$^+$ T cells expressed TRAIL on their membrane. These results were confirmed by the use of plasma membrane markers (CD4, Vybrant), which colocalized with TRAIL only in HIV-1-activated cells. Our method of membrane visualization by 3D interactive surface plot offers several advantages. First, it saves the use of a plasma membrane marker in favor of intracellular markers. This remains very useful especially in human T cells that harbor very small cytoplasm. Second, this 3D representation of microscopic images avoid "false negative" counting. Indeed, we observed that some TRAIL protein localized on the plasma membrane but that did not colocalize with CD4. This TRAIL staining would not be counted by classical colocalization quantification method.

However, there are a few limitations of current imaging technologies. Currently, the closest microscope to the 3D one is the confocal microscope. With this technique we can observe different stacks of cells using fluorescence and identify colocalized spots. But the step between each stack is greater. Indeed, with the confocal microscope, we obtain a dozen stacks, which reduces precision with 3D reconstruction whereas with the 3D microscopy, we obtain around 40 stacks per cell. Each acquisition for each color takes up to several minutes whereas we obtain instantaneous pictures with the 3D microscope.

Thus, 3D interactive surface plot membrane visualization provides a new tool that could be used in addition to classical methods to improve precise protein localization.

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Author contributions

C.G. performed and analyzed the research. J.P.H designed and analyzed the research and wrote the paper. L. S, C. K. and M. G. provided new technologies. The authors declare no conflict of interest.

References

Ashkenazi, A., Herbst, R.S., 2008. To kill a tumor cell: the potential of proapoptotic receptor agonists. J. Clin. Invest. 118, 1979.

Colisson, R., Barblu, L., Gras, C., Raynaud, F., Hadj-Slimane, R., Pique, C., Hermine, O., Lepelletier, Y., Herbeuval, J.P., 2010. Free HTLV-1 induces TLR7-dependent innate immune response and TRAIL relocalization in killer plasmacytoid dendritic cells. Blood. Q6

Cummins, N.W., Badley, A.D., 2010. Mechanisms of HIV-associated lymphocyte apoptosis: 2010. Cell Death Dis. 1, e99.

Ehrlich, S., Infante-Duarte, C., Seeger, B., Zipp, F., 2003. Regulation of soluble and surface-bound TRAIL in human T cells, B cells, and monocytes. Cytokine 24, 244.

Gasper-Smith, N., Crossman, D.M., Whitesides, J.F., Mensali, N., Ottinger, J.S., Plonk, S.G., Moody, M.A., Ferrari, G., Weinhold, K.J., Miller, S.E., Reich III, C.F., Qin, L., Self, S.G., Shaw, G.M., Denny, T.N., Jones, L.E., Pisetsky, D.S., Haynes, B.F., 2008. Induction of plasma (TRAIL), TNFR-2, Fas ligand, and plasma microparticles after human immunodeficiency virus type 1 (HIV-1) transmission: implications for HIV-1 vaccine design. J. Virol. 82, 7700.

Griffith, T.S., Lynch, D.H., 1998. TRAIL: a molecule with multiple receptors and control mechanisms. Curr. Opin. Immunol. 10, 559.

Guadalupe, M., Reay, E., Sankaran, S., Prindiville, T., Flamm, J., McNeil, A., Dandekar, S., 2003. Severe CD4$^+$ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. J. Virol. 77, 11708.

Hansjee, N., Kaufmann, G.R., Strub, C., Weber, R., Battegay, M., Erb, P., 2004. Persistent apoptosis in HIV-1-infected individuals receiving potent

antiretroviral therapy is associated with poor recovery of CD4 T lymphocytes. J. Acquir. Immune Defic. Syndr. 36, 671.

Hardy, A.W., Graham, D.R., Shearer, G.M., Herbeuval, J.P., 2007. HIV turns plasmacytoid dendritic cells (pDC) into TRAIL-expressing killer pDC and down-regulates HIV coreceptors by toll-like receptor 7-induced IFN-alpha. Proc. Natl. Acad. Sci. U. S. A. 104, 17453.

Herbeuval, J.P., Shearer, G.M., 2006. HIV-1 immunopathogenesis: how good interferon turns bad. Clin. Immunol.

Herbeuval, J.P., Lambert, C., Sabido, O., Cottier, M., Fournel, P., Dy, M., Genin, C., 2003. Macrophages from cancer patients: analysis of TRAIL, TRAIL receptors, and colon tumor cell apoptosis. J. Natl. Cancer Inst. 95, 611.

Herbeuval, J.P., Boasso, A., Grivel, J.C., Hardy, A.W., Anderson, S.A., Dolan, M.J., Chougnet, C., Lifson, J.D., Shearer, G.M., 2005a. TNF-related apoptosis-inducing ligand (TRAIL) in HIV-1-infected patients and its *in vitro* production by antigen-presenting cells. Blood 105, 2458.

Herbeuval, J.P., Grivel, J.C., Boasso, A., Hardy, A.W., Chougnet, C., Dolan, M.J., Yagita, H., Lifson, J.D., Shearer, G.M., 2005b. $CD4^+$ T-cell death induced by infectious and noninfectious HIV-1: role of type 1 interferon-dependent, TRAIL/DR5-mediated apoptosis. Blood 106, 3524.

Herbeuval, J.P., Hardy, A.W., Boasso, A., Anderson, S.A., Dolan, M.J., Dy, M., Shearer, G.M., 2005c. Regulation of TNF-related apoptosis-inducing ligand on primary $CD4^+$ T cells by HIV-1: role of type I IFN-producing plasmacytoid dendritic cells. Proc. Natl. Acad. Sci. U. S. A. 102, 13974.

Herbeuval, J.P., Nilsson, J., Boasso, A., Hardy, A.W., Kruhlak, M.J., Anderson, S.A., Dolan, M.J., Dy, M., Andersson, J., Shearer, G.M., 2006. Differential expression of IFN-alpha and TRAIL/DR5 in lymphoid tissue of progressor versus nonprogressor HIV-1-infected patients. Proc. Natl. Acad. Sci. U. S. A. 103, 7000.

Herbeuval, J.P., Nilsson, J., Boasso, A., Hardy, A.W., Vaccari, M., Cecchinato, V., Valeri, V., Franchini, G., Andersson, J., Shearer, G.M., 2009. HAART reduces death ligand but not death receptors in lymphoid tissue of HIV-infected patients and simian immunodeficiency virus-infected macaques. AIDS 23, 35.

Kayagaki, N., Yamaguchi, N., Nakayama, M., Eto, H., Okumura, K., Yagita, H., 1999. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: a novel mechanism for the antitumor effects of type I IFNs. J. Exp. Med. 189, 1451.

Lichtner, M., Maranon, C., Vidalain, P.O., Azocar, O., Hanau, D., Lebon, P., Burgard, M., Rouzioux, C., Vullo, V., Yagita, H., Rabourdin-Combe, C., Servet, C., Hosmalin, A., 2004. HIV type 1-infected dendritic cells induce apoptotic death in infected and uninfected primary CD4 T lymphocytes. AIDS Res. Hum. Retroviruses 20, 175.

Lum, J.J., Schnepple, D.J., Badley, A.D., 2005. Acquired T-cell sensitivity to TRAIL mediated killing during HIV infection is regulated by CXCR4–gp120 interactions. AIDS 19, 1125.

Melki, M.T., Saidi, H., Dufour, A., Olivo-Marin, J.C., Gougeon, M.L., 2009. Escape of HIV-1-infected dendritic cells from TRAIL-mediated NK cell cytotoxicity during NK-DC cross-talk—a pivotal role of HMGB1. PLoS Pathog. 6, e1000862.

Miura, Y., Misawa, N., Maeda, N., Inagaki, Y., Tanaka, Y., Ito, M., Kayagaki, N., Yamamoto, N., Yagita, H., Mizusawa, H., Koyanagi, Y., 2001. Critical contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to apoptosis of human CD4(+) T cells in HIV-1-infected hu-PBL-NOD–SCID Mice. J. Exp. Med. 193, 651.

Sato, K., Hida, S., Takayanagi, H., Yokochi, T., Kayagaki, N., Takeda, K., Yagita, H., Okumura, K., Tanaka, N., Taniguchi, T., Ogasawara, K., 2001. Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta. Eur. J. Immunol. 31, 3138.

Sheridan, J.P., Marsters, S.A., Pitti, R.M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C.L., Baker, K., Wood, W.I., Goddard, A.D., Godowski, P., Ashkenazi, A., 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 277, 818.

Sibarita, J.B., 2005. Deconvolution microscopy. Adv. Biochem. Eng. Biotechnol. 95, 201.

Smyth, M.J., Cretney, E., Takeda, K., Wiltrout, R.H., Sedger, L.M., Kayagaki, N., Yagita, H., Okumura, K., 2001. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. J. Exp. Med. 193, 661.

Stary, G., Klein, I., Kohlhofer, S., Koszik, F., Scherzer, T., Mullauer, L., Quendler, H., Kohrgruber, N., Stingl, G., 2009. Plasmacytoid dendritic cells express TRAIL and induce $CD4^+$ T-cell apoptosis in HIV-1 viremic patients. Blood 114, 3854.

Tecchio, C., Huber, V., Scapini, P., Calzetti, F., Margotto, D., Todeschini, G., Pilla, L., Martinelli, G., Pizzolo, G., Rivoltini, L., Cassatella, M.A., 2004. IFNalpha-stimulated neutrophils and monocytes release a soluble form of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) displaying apoptotic activity on leukemic cells. Blood 103, 3837.

van Grevenynghe, J., Cubas, R.A., Noto, A., DaFonseca, S., He, Z., Peretz, Y., Filali-Mouhim, A., Dupuy, F.P., Procopio, F.A., Chomont, N., Balderas, R.S., Said, E.A., Boulassel, M.R., Tremblay, C.L., Routy, J.P., Sekaly, R.P., Haddad, E.K., 2011. Loss of memory B cells during chronic HIV infection is driven by Foxo3a- and TRAIL-mediated apoptosis. J. Clin. Invest. 121, 3877.

Vidalain, P.O., Azocar, O., Lamouille, B., Astier, A., Rabourdin-Combe, C., Servet-Delprat, C., 2000. Measles virus induces functional TRAIL production by human dendritic cells. J. Virol. 74, 556.

Wiley, S.R., Schooley, K., Smolak, P.J., Din, W.S., Huang, C.P., Nicholl, J.K., Sutherland, G.R., Smith, T.D., Rauch, C., Smith, C.A., et al., 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3, 673.

Wu, G.S., Burns, T.F., McDonald III, E.R., Jiang, W., Meng, R., Krantz, I.D., Kao, G., Gan, D.D., Zhou, J.Y., Muschel, R., Hamilton, S.R., Spinner, N.B., Markowitz, S., Wu, G., el-Deiry, W.S., 1997. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat. Genet. 17, 141.

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