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dans le maintien des réservoirs du HIV.

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à mes filles,

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Résumé

La relation possible entre la voie de biogenèse des exosomes et la production de virus de l'immunodéficience humaine (HIV) dans des infections transcellulaires a été examinée. Nous nous sommes intéressés en particulier à des médiateurs lipidiques requis pour la biogenèse des exosomes et/ou l'assemblage du HIV.

Les exosomes sont des vésicules bioactives de 50-90 nm, générées dans le compartiment endosomal tardif, ou corps multivésiculaires, et sont sécrétés dans le milieu extracellulaire par les cellules vivantes. Les exosomes sont retrouvés dans les fluides biologiques du corps et semblent être impliqués dans la communication intercellulaire, transportant à la fois des lipides bioactifs, des protéines, et des acides ribonucleiques (mRNAs, microRNAs) pouvant moduler la réponse de la cellule receveuse. Les exosomes contiennent tous les constituants d'un rétrovirus mais ne peuvent pas se répliquer. La première partie de ce doctorat fut consacrée à la mise en évidence de lipides bioactifs et de protéines associées dans les exosomes produits par une lignée de cellules mastocytaires, les RBL-2H3. Dans ces travaux, les exosomes ont été considérés comme des particules non infectieuses dont l'analyse est informative sur le virus. Nous avons ainsi pu mettre en évidence que les exosomes contiennent l'ensemble des classes de phospholipases : une PLC, une PLD et des membres des trois types de PLA_2 ($cPLA_2$, $iPLA_2$, $sPLA_2$) ainsi que des protéines associées. Tous les membres de la famille des Ras GTPase étant retrouvés dans les exosomes, nous avons pu observer une augmentation de l'activité PLD_2 et PLA_2 en présence de GTP. Des analyses lipidomiques des exosomes ont révélé la présence d'acides gras libres tels que l'acide arachidonique et plusieurs prostaglandines, médiateurs lipidiques qui peuvent être libérés à la suite de leur internalisation dans la cellule réceptrice. Par conséquent, l'accumulation de ces vésicules dans le compartiment endosomal a été remarquée, alors qu'un inhibiteur de cPLA2, le Methyl Arachidonyl Fluoro Phosphonate (MAFP) prévient cette accumulation.

L'implication du compartiment endosomal dans le stockage et la dissémination de pathogènes tels que le HIV, en particulier dans les cellules dites réservoirs comme les monocytes et macrophages, a été soulignée et pourrait rendre compte de l'impossibilité d'éradiquer le virus, même sous thérapie antirétrovirale efficace. Il a ainsi été suggéré que les coprs multivésiculaires pourraient être un site d'assemblage du HIV, permettant au virus de détourner la voie préexistante des exosomes pour sa sortie. De plus ce compartiment concentre à la fois le cholestérol et un phospholipide particulier : l'acide lysobisphosphatidique, dont la synthèse fait intervenir une $cPLA_2$ sensible au MAFP. Dans un second temps, nous avons donc évalué l'importance de l'homéostasie lipidique sur la production du HIV i) en modifiant la teneur et la distribution du cholestérol intracellulaire, ii) en inhibant l'activité PLA_2. Ces analyses ont été réalisées sur une lignée de cellules monocytaires humains (THP-1) infectées par le HIV et cultivées en cocultures avec ces mêmes cellules non infectées, et également sur des cocultures de macrophages dérivés de monocytes primaires humains infectés et non infectés. La progestérone, hormone stéroïde, et le MAFP, inhibiteur de PLA_2, ont permis d'inhiber la production de HIV dans nos conditions de culture et nous avons également pu observer l'absence de virions en périphérie de la cellule. Des traitements répétés ont renforcé ces résultats. Le LBPA pourrait être un élément commun à ces deux mécanismes et par voie de conséquence la PLA_2. Cibler

les PLA2s sensibles au MAFP représente donc une stratégie visant à altérer les réservoirs de HIV, et pourrait mener au développement de nouvelles molécules pour l’éradication du HIV.

Abstract

We have investigated the possible relationship between the exosome biogenesis pathway and the HIV production in transcellular infection. Studies were focused on lipid mediators required for exosomes and/or HIV assembly and bioactivities.

Exosomes are bioactive vesicles about 50-90nm generated in multivesicular bodies (late endosomes) and released in extracellular medium by intact cells. They can be found in body fluids and appear to be involved in intercellular signalling, carrying bioactive lipids, proteins, and ribonucleic acids (mRNAs, microRNAs) involved in the modulation of the recipient cell response. Therefore exosomes contain all the constituent of a retrovirus except they cannot replicate. The first part of this PhD aimed to investigate the presence of lipid-related proteins and bioactive lipids in exosomes secreted from a mast cell line, the RBL-2H3. Exosomes were taken as a non-infectious particle which could model HIV and be used to bring informations for further studies on the virus itself. In addition, mast cells are also HIV reservoirs. We could bring evidences that exosomes contained the whole set of phospholipases, i.e. PLC, PLD and members of the three phospholipase A2 class (cPLA2, iPLA2, sPLA2) together with phospholipase partners. Given that all members of the Ras GTPase family were detected, it is worth to note that GTP could enhance the PLD_2 and PLA_2 activity. Lipidomic analyses reveal the presence of free fatty acids such as arachidonic acid, and several prostaglandins, lipids mediators which can be released into a recipient cell following exosomes internalization. Consequently, we could observe an accumulation of these vesicles into the endosomal compartment. Noteworthy, exosome uptake by target cells could be inhibited by the cPLA2 inhibitor, Methyl Archachidonoyl Fluoro Phosphonate (MAFP).

It has been evidenced that endosomes could hide pathogens like HIV, particularly in monocytes and macrophages which represent an important reservoir and account for the failure of antiretroviral therapy. Despite conflicting results, it has been suggested that multivesicular bodies' membrane could be one site of HIV budding, allowing the virus to hijack the exosome pathway for its extracellular release. Moreover, this compartment concentrate both cholesterol and an unusual phospholipid : the lysobisphosphatidic acid (LBPA), whose synthesis implicate a MAFP sensitive $cPLA_2$. The second part of this work was to assess the importance of lipid homeostasis on HIV production by i) modifying cholesterol content and distribution and ii) inhibiting PLA_2 activity. This study was conducted in cocultures of HIV infected and uninfected human monocytic THP-1 cells, or cocultures of infected and uninfected human primary monocytes–derived macrophages from healthy donors. Our results reveal that the steroid progesterone and the phospholipase A2 inhibitor Methyl Arachidonyl Fluoro Phosphonate (MAFP) decreased HIV secretion, triggering the disappearance of the virus at the periphery of the cell. These observations were reinforced by a repetitive treatment with MAFP. A common mechanism could be the LBPA and subsequent PLA_2s. Therefore targeting MAFP-sensitive PLA_2s appears as a strategy to alter HIV reservoir content permitting the development of new molecules for HIV eradication.

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Abbreviation list

Ab : Antibody
Aβ : β-amyloid
AD : Alzheimer's disease
ADH : Antidiuretic hormone
Ag : Antigens
AFT3 :activating transcription factor 3
AICD : amyloid intracellular domain
APC : Antigen Presenting cells
APP : amyloid precursor protein
ART : antiretroviral therapies
BAT3 : HLA-B-associated transcript 3
BMDC : Bone Marrow Dendritic Cells
BMP (=LBPA): Bis(monoacylglycero)phosphate (= Lysobisphosphatidic acid)
CTFs : APP C-terminal fragments
CTL : Cytotoxic T Lymphocytes
DCs: Dendritic cells
DC-SIGN : DC-Specific Intercellular adhesion molecule-3-binding non integrin
Dex : exosomes derived from Dendritic cells.
EEA : Early Endosome Antigen
ESCRT : Endosomal sorting complex required for transport
FABP : fatty acid binding protein
FDCs : Follicular dendrtitic cells
HIV : Human Immunodeficiency Virus
HSP: Heat shock protein
HAART : highly active antiretroviral therapy
HUVEC : Human umbilical vein endothelial cells
ICAM-1: Intercellular adhesion molecule 1
IECs :Intestinal Epithelial Cells
Ig : Immunoglobulins
ILVs : Intralumenal vesicules
iNOS : inducible nitric oxide synthase
GM-CSF : Granulocyte-macrophage colony-stimulating factor
Gpi :glycosylphosphatidylinositol
LBPA (= BMP): Lysobisphosphatidic acid (=Bis(monoacylglycero)phosphate)
LFA-1 : leukocyte function – associated antigen-1
MDSCs: Myeloid-derived suppressor cells
MHC : Major Histocompatibility Complex
MIC A/B : MHC class I chain-related (MIC) proteins A and B
MVBs : Multivesicular Bodies
NKG2D : Natural Killer Group 2 member D
PAI-1 : Plasminogen Activator Inhibitor type 1
PAMPs : pathogen-associated molecular patterns
PrP : prion protein
PrPsc : prion protein scrapie

PS : Phosphatidylserin
RANTES (CCL5) : Regulated upon Activation, Normal T cell Expressed and Secreted
RBL-2H3 : Rat Basophil Leukemia 2H3 mast cell line
RGD-containing sequence : Arg-Gly-Asp-containing sequence.
Rh-PE : n-lissamine rhodamine b sulfonyl phosphatidylethanolamine
RNA : ribonucleic acid
SM : Sphingomyelin
Tex : Tumor derived exosomes
TfR : Transferrin Receptor
TIM-1 or -4 :T-cell immunoglobulin – and mucin domain containing molecules 1 or 4
TLR : Toll like receptor
TNFα : Tumor Necrosis factor -α
TRAIL : TNF-related apoptosis-inducing ligand
TSAP6 : Tumor suppressor-activated pathway 6
Tsg101:Tumour susceptibility gene 101
ULBP : UL-16 binding proteins
WT-1 : Wilms Tumor 1

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Introduction Générale

Introduction générale

A l'heure actuelle, presque 40 millions de personnes vivent avec le Virus de l'immunodéficience humaine -1 (HIV-1). Depuis le début de l'épidémie qui a débutée au début des années 80, le virus HIV-1 a causé plus de 25 millions de décès (Gavegnano & Schinazi, 2009). En dépit des efforts réalisés, le nombre de personnes vivant avec le HIV continue d'augmenter, de même que le nombre de décès liés au HIV.

Ce pathogène majeur humain est devenu l'un des virus le plus étudié. Le virus de l'immunodéficience humaine (HIV) est un rétrovirus enveloppé, appartenant à la famille des lentivirus, et comporte un génome sous la forme d'un simple brin d'acide ribonucléique (ARN) en double exemplaire.

En dépit de thérapies efficaces permettant d'obtenir l'indétectabilité du virus dans le plasma de patients infectés, le virus HIV n'est cependant pas éradiqué, entraînant un rebond de la charge virale en cas d'arrêt des traitements. Plusieurs travaux démontrent l'existence de cellules hématopoïétiques dites réservoirs qui pourraient rendre compte de cet échappement du virus aux thérapies actuelles. Ces cellules réservoirs sont constituées principalement de lymphocytes T CD4^{+} mémoires quiescents mais également de monocytes, macrophages, mastocytes, cellules dendritiques et cellules folliculaires dendritiques (Aquaro *et al.*, 2002; Blankson *et al.*, 2002; Zhu *et al.*, 2002; Keele *et al.*, 2008; Chomont *et al.*, 2009; Mavigner *et al.*, 2009; Sahu *et al.*, 2009). Dans ces cellules, le virus se trouve soit à l'état de provirus latent, dont le génome est intégré à l'ADN cellulaire et permettant une réplication virale faible, soit conservé sous forme de stocks viraux dans des compartiments d'origine endosomale (Sharova *et al.*, 2005; Benaroch *et al.*, 2010). Cette dernière observation a conforté plusieurs scientifiques à suggérer l'hypothèse du cheval de Troie, selon laquelle le virus pourrait emprunter une voie de trafic vésiculaire préexistante au niveau endosomal, celle des exosomes, pour transiter et bourgeonner et être sécrété dans le milieu extracellulaire sans lyse cellulaire (Gould *et al.*, 2003; Nguyen *et al.*, 2003; Pelchen-Matthews *et al.*, 2004; Izquierdo-Useros *et al.*, 2010). Les endosomes tardifs sont un compartiment clé dans le trafic intracellulaire et constituent un carrefour entre les voies d'endocytose et d'exocytose. Ils sont également le siège de biogenèse de vésicules dites vésicules intraluminales, générées par bourgeonnement interne de leur membrane d'où le nom de corps mutlivésiculaires ou MultiVesicular Bodies (MVB). Bien que considérés comme une étape précédent les lysosomes dans la voie d'endocytose, les MVBs peuvent également être dirigés vers la membrane plasmique avec laquelle ils fusionnent, libérant ainsi dans le milieu extracellulaire leur contenu vésiculaire alors appelé exosomes.

Le principal objectif de ce doctorat résidait dans l'analyse de la relation possible entre la voie de biogenèse des exosomes et la production du virus de l'immunodéficience humaine (HIV) dans des conditions de trans-infection. Nous nous sommes intéressés en particulier à des médiateurs lipidiques requis pour la biogenèse des exosomes et/ou l'assemblage du HIV.

De nombreux travaux fondés sur cette hypothèse du cheval de Troie, bien que contestée par ailleurs, ont permis de mettre en évidence plusieurs similitudes entre HIV et exosomes. Outre une taille similaire (exosomes : 50-100nm ; et particules virales : environ 100 nm), des protéines exosomales ont été retrouvées sur le HIV (Nguyen *et al.*, 2003; Chertova *et al.*, 2006). Ces deux types de particules partagent également une composition lipidique proche (Laulagnier *et al.*, 2004b; Brugger *et al.*, 2006). Certains composants de la machinerie cellulaire connus pour intervenir dans la biogenèse d'exosomes sont également recrutés par le virus pour son propre bourgeonnement (Pornillos *et al.*, 2003; Bieniasz, 2009; Benaroch *et al.*, 2010). Plusieurs travaux soulignent l'importance de domaines membranaires particuliers enrichis en certains lipides et protéines, les radeaux lipidiques (ou 'raft microdomains') dans le phénomène de vésiculation des corps multivésiculaires, ainsi que du bourgeonnement du HIV (Campbell *et al.*, 2001; Ono & Freed, 2001; Waheed & Freed, 2009). Alors que le bourgeonnement du HIV était admis avoir lieu au niveau de la membrane plasmique des cellules infectées, le rôle du compartiment endosomal (MVB) dans ce processus a pris de plus en plus d'importance ces dernières années (Nguyen *et al.*, 2003; Joshi *et al.*, 2009). Enfin, de récentes études mettent en évidence la présence de petits ARNs fonctionnels au sein des exosomes pouvant être transférés et traduits en protéines dans une cellule receveuse (Valadi *et al.*, 2007). Il a également été démontré que l'internalisation d'exosomes pouvait rentrer en compétition avec l'internalisation du virus HIV, indépendamment des glycoprotéines d'enveloppe, les deux types de particules se retrouvant au sein du même compartiment endosomal dans la cellule cible (Izquierdo-Useros *et al.*, 2009). Par conséquent, dans leur ensemble, ces données mettent en évidence des caractéristiques semblables entre les exosomes et le HIV, et laissent supposer que les rétrovirus pourraient être considérés d'un point de vue fondamental comme des exosomes.

La première partie de mes travaux expérimentaux s'est donc appuyée sur le savoir-faire acquis par l'équipe d'accueil dans le domaine des exosomes dérivés d'une lignée de cellules mastocytaires, nous permettant d'acquérir des données fondamentales transposables par la suite au HIV. Mon étude a dans un premier temps concerné l'analyse de médiateurs lipidiques potentiels, véhiculés par les exosomes. Les résultats obtenus démontrent la présence de phospholipases, activables par le GTP. De façon complémentaire, une analyse du protéome de ces vésicules a été réalisée et indique la présence de protéines partenaires de phospholipases. L'activité enzymatique des phospholipases conduit à la génération d'acides gras libres. Grâce à des analyses lipidomiques, nous avons pu mettre en évidence un large éventail d'acides gras libres associés aux exosomes et notamment d'acide arachidonique, précurseur de prostaglandines également identifiées. Parmi l'ensemble des prostaglandines révélées, la 15ΔPGJ2, agoniste du récepteur nucléaire PPARγ a été mise en évidence.

La sécrétion d'exosomes par une cellule constitue un moyen d'échange et de communication entre cellules avoisinantes mais aussi à distance ; en effet les exosomes sont présents dans

les liquides biologiques, dont le sang. Les médiateurs lipidiques portés par les exosomes pourraient ainsi être libérés dans une cellule réceptrice. Nous avons en effet observé l'internalisation de ces vésicules dans les endosomes (MVBs) de cellules cibles. Nous émettons l'hypothèse que ces vésicules internalisées et localisées au niveau des corps multivésiculaires pourraient, par un processus de fusion avec la membrane du MVB amener leur contenu dans le cytoplasme de la cellule, libérant des lipides bioactifs capables de moduler la réponse de la cellule cible.

Les corps multivésiculaires ont été décrits pour être le lieu de biogenèse et/ou de stockage de virions HIV (Joshi *et al.*, 2009). Par ailleurs les compartiments de la voie d'endocytose concentrent une partie importante du cholestérol cellulaire qui est enrichi au niveau des vésicules intraluminales des corps multivésiculaires (Mobius *et al.*, 2003). Associé au cholestérol, un lipide particulier influence sa distribution : l'acide lysobisphosphatidique (LBPA ou encore BMP pour Bis(monoacylglycero)Phosphate. Le LBPA se trouve également enrichi au niveau des membranes des corps multivésiculaires et sa présence semble nécessaire à la genèse des vésicules intraluminales (Kobayashi *et al.*, 1998a; Matsuo *et al.*, 2004). A l'aide d'un inhibiteur de phospholipase A_2 (PLA_2), le Methyl Arachidonyl Fluoro Phosphonate (MAFP), qui inhibe les acides aminés serines du site catalytique de la $cPLA_2$ et à un moindre degré de la $iPLA_2$, j'ai pu montrer le rôle de cet enzyme dans la biosynthèse du LBPA.

Dans la deuxième partie expérimentale de ce travail de doctorat, j'ai évalué l'importance de certains constituants lipidiques sur la production de virions HIV à partir de monocytes humains, dans des conditions de co-culture entre cellules infectées et non-infectées.
La distribution du cholestérol et l'effet du MAFP ont donc été testés sur la production de HIV à partir de cellules réservoirs (lignée monocytaire humaine THP1, macrophages humains dérivés de monocytes). Deux stratégies ont été menées en parallèle. Nous avons d'une part modifié la teneur et la distribution du cholestérol intracellulaire au moyen de molécules telles que l'U18666A, connu pour induire une accumulation du cholestérol dans les MVBs, et la progestérone qui permet de bloquer le cholestérol libre en périphérie cellulaire. D'autre part, le rôle de phospholipases A_2 type $cPLA_2$ a été évalué à l'aide d'inhibiteurs pharmacologiques comme le MAFP. Il a ainsi été démontré que l'inhibition de PLA_2 et la présence de progestérone diminuaient significativement la quantité de HIV libérés dans le milieu extracellulaire. L'élément clé, commun entre ces deux traitements pourrait être le taux de LBPA (BMP) contenu dans les MVB.

L'ensemble de ces travaux de thèse a donc permis d'étayer la présence de prostaglandines comme la 15ΔPGJ2 dans les exosomes, ce qui ouvre des perspectives d'investigation sur le rôle de ce type de médiateur lipidique dans la régulation de la sécrétion de particules du HIV à partir de cellules réservoirs. Par ailleurs le rôle de constituants lipidiques du compartiment MVB comme le LBPA (BMP) dans la production de

virions HIV, mis en évidence dans ce travail, souligne l'importance de ce compartiment cellulaire dans le contrôle de l'infection HIV. La modulation de la composition lipidique du compartiment MVB pourrait en effet déboucher sur de nouvelles cibles thérapeutiques visant à éliminer les réservoirs de HIV.

Ce mémoire est organisé dans une première partie autour d'un rappel bibliographique des connaissances actuelles sur les exosomes et leur relation avec le HIV, ainsi que du rôle d'un phospholipide spécifique au compartiment MVB l'acide lysobisphosphatidique (LBPA) ou BMP. **Après une deuxième partie** du manuscrit consacrée aux méthodes utilisées, l'ensemble des résultats expérimentaux sont exposés dans **une troisième partie.** Nous présenterons tout d'abord les exosomes comme modèle d'études de particules non infectieuses et convoyeurs de phospholipases et de prostaglandines. Par la suite, la relation entre PLA_2 et stérols dans la production de particules virales sera developpée.
Enfin, le dernier chapitre concernera une discussion générale et les perspectives de ce travail.

Introduction Bibliographique

Part 1. HIV : a replicative exosome ?

I. Exosomes

A. Exosome biogenesis.

Endosomal system consists in primary endocytic vesicles, early endosomes, late endosomes and lysosomes.

The early endosomes represent the major entry site for endocytosed material and display characteristics of a sorting area, with specific markers like Early Endosome Antigen (EEA) and endocytosed recycling receptors. They display a tubular appearance and are located at the outer margin of the cell. They do not contain intralumenal vesicles.

Late endosomes are spherical in shape and are located close to the nucleus. They derive from early endosomes through a maturation process that allow changes in contents and size. The key step in the formation of multivesicular bodies (MVBs) from late endosomes is inward budding (Keller *et al.*, 2006)(Figure 1)

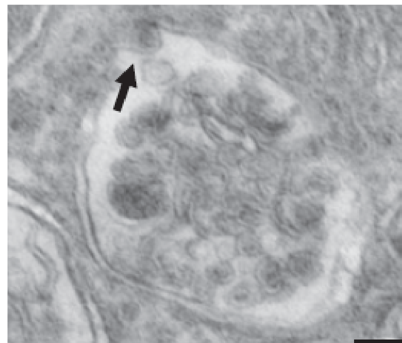


Figure 1 : Inward budding of the limiting membrane of an Multivesicular Body (arrow) leading to intraluminal vesicles generation. Electron Micrograph image showing intraluminal vesicules in Multivesicular body from EBV-infected lymphoblasts (LCL). Adapted from (Pegtel *et al.*, 2010).

Thus, late endosomes accumulate up to hundred intraluminal vesicles (ILVs) (Stoorvogel *et al.*, 2002). Hence, late endosomes are often referred to as multivesicular bodies (MVBs). ILVs are formed by inward budding and scission of vesicles from the limiting membrane of the MVB. The resulting vesicles contain cytosol and expose internal membrane of the MVB at their surface. This corresponds to a similar transmembrane orientation as the plasma membrane. They progressively accumulate during endosomes maturation. The endosomal sorting complex required for transport (ESCRT) play a role in the ILV formation. Tgs101 is a component of ESCRT I, Alix recruits ESCRT II and III. These components are thought to allow inward budding and scission of the ILVs but dissociate from the endosomal membrane

through the action of VPS4 ATPase. (Katzmann *et al.*, 2002; Raiborg *et al.*, 2003; Fevrier & Raposo, 2004) (Figure 2).

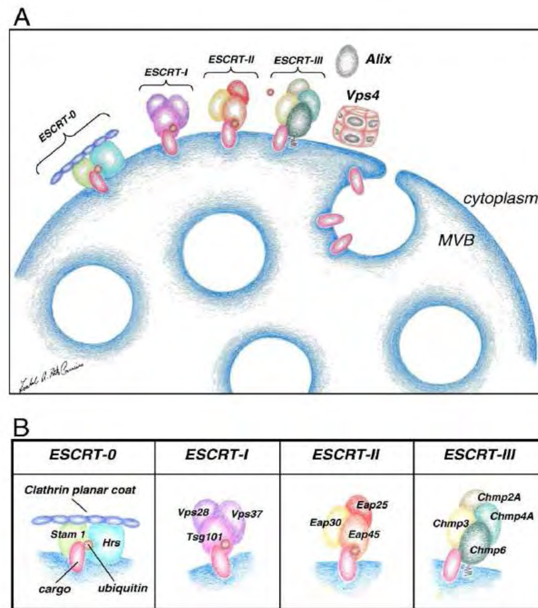


Figure 2 : The ESCRT machinery. **(A)** Organization of the ESCRT complexes at the limiting membrane of multivesicular bodies (MVBs). **(B)** Molecular composition of each ESCRT complex. The protein complexes – ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III- are involved in the protein sorting to the intraluminal vesicles of MVB. Ubiquitinated cargo proteins are recognized by the ubiquitin-binding proteins of the Hrs complex (Hrs, STAM and Eps15, called ESCRT-0). Hrs recruits ESCRT-I by interacting with Tsg101, which is also an ubiquitin-binding protein. Tsg101 then recruits ESCRT-III via ESCRT-II or AIP1/Alix, and these complexes function together to position cargo proteins into the inward-budding vesicles of the MVB. ESCRT-III interacts with the AAA-ATPase Vps4, which dissociates the ESCRT machinery and releases the class E proteins. Adapted from (Porto-Carreiro *et al.*, 2005).

Multivesicular bodies and their intraluminal vesicles potentially have 3 distinct fates. The earlier acknowledged function is their involvement in the sequestration of proteins and lipids destinated for degradation in lysosomes. However, when MVBs do not fuse with lysosomes, they can serve as storage compartment. It is known, for instance, that in immature dendritic cells (DCs), specialized late endocytic compartments serve as a storage site for major histocompatibility complex (MHC) II molecules in transit to plasma membrane, the so-called MIICs compartments (Raposo *et al.*, 1996). In pathogen-stimulated DCs, the luminal vesicles of MVBs fuse with the MVBs limiting membrane, from where MHC class II can be transferred subsequently to plasma membrane. An alternative destiny of MVBs is their possible fusion with plasma membrane, allowing the release of their intralumenal vesicles content in the extracellular medium (Figure 3).

Once secreted, these intralumenal vesicles are referred as exosomes (Figure 4). They should not be confused with the ribonuclease complex that has also been named exosomes and which will not be addressed in this work. This exocytosis represents another pathway for protein export.

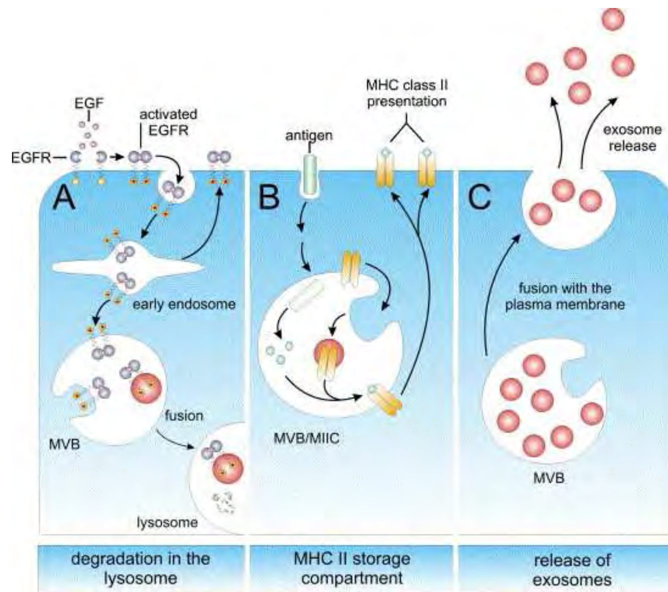


Figure 3 : Different fates of internalized vesicles. (A) Lysosomal degradation to down regulate receptor signaling: some cell surface receptors such as the EGFR are internalized following ligand binding and activation, traffic to lysosomes for degradation. **(B)** MHC class II storage compartment: antigens taken up into vesicles are processed into shorter peptides which bind to MHC class II molecules in the MHC class II storage compartment (MIIC). The MHC-peptides complexes are delivered to the cell surface and can be recognized by CD4+T cells. **(C)** Release of exosomes: multivesicular bodies can fuse with the plasma membrane and release internal vesicles named exosomes into the extracellular environment. Adapted from (Keller *et al.*, 2006).

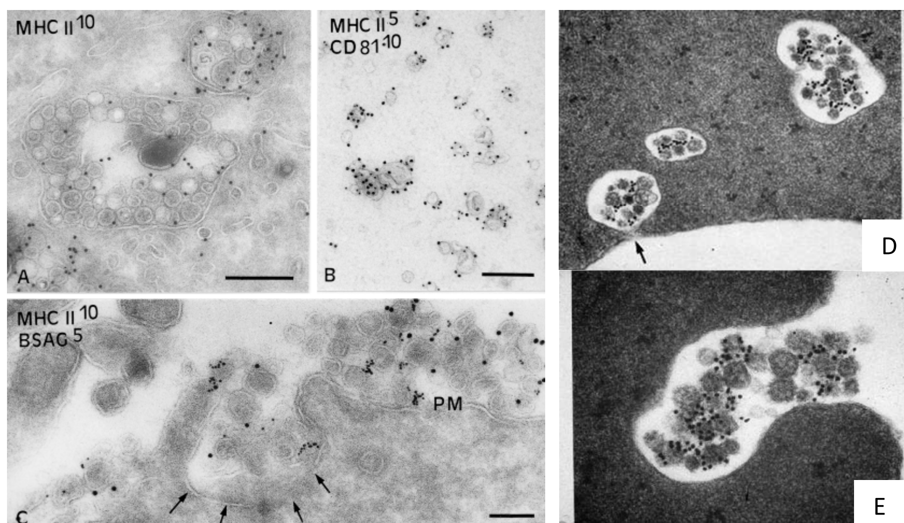


Figure 4 Fusion of multivesicular bodies with the cell surface and exosome release. A. Electron micrograph showing two multivesicular bodies in a dendritic cell labeled for MHC class II. Bar, 200 nm. **B.** Exosomes purified from the cell culture supernatants of dendritic cells. Exosomes are positive for both MHC class II and CD81 immunogold labelling. Bar, 200 nm. **C.** Electron micrograph showing a fusion profile of MVB with the plasma membrane (PM) (arrows) of a B lymphocyte. BSA-coupled to 5 nm gold particles were previously internalized and followed by fixation and MHC II immunogold labeling. Bar, 100 nm. Adapted from (Stoorvogel *et al.*, 2002). **D.** colloidal gold electron micrographs of maturing sheep reticulocyte exosomes; after 18h incubation with transferring receptor mAb localized to intracellular compartment (MVBs) associating with the surface of the internal exosomes – black arrow reveals a fusion profile of the internal compartment with the plasma membrane. **E**. After 36h of incubation the fusion is complete releasing exosomes in the extracellular medium. Adapted from (Simpson *et al.*, 2008).

The release of ILVs in the extracellular space requires the transport of the formed MVB towards the cell periphery and its docking and fusion with the plasma membrane which the mechanisms involved are unknown. Works have demonstrated that docking and fusion of MVBs to plasma membrane is dependent on Rab 11 in interrelation with calcium (Savina *et al.*, 2005). Moreover, there is an association between Rab proteins and lipids present in MVBs. For example, Rab7 associates with late endosomes membrane structures and its association raises with enrichment in MVB cholesterol content which inhibits MVBs motility (Lebrand *et al.*, 2002). Likewise; MVB docking and fusion with plasma membrane is likely to be mediated by SNAREs proteins and synaptotagmin family members (Vrljic *et al.*). These events are known to be regulated and seem to require Phospholipase D_2 (Laulagnier *et al.*, 2004a), calcium and Rab11 (Fader *et al.*, 2005).

Some researchers have however, recently described that exosomes might originate from endosome-like plasma membrane patches (Booth *et al.*, 2006), the 'endosome-like domains', enriched for exosomal and endosomal proteins such as CD81 and CD63. Those domains could also originate from exosomes having fused with peripheral cell membrane (Parolini *et al.*, 2009)

B. Exosome Characteristics and Composition

Exosomes are homogeneous vesicles in size and shape, about 40-100nm, with a lipid bilayer, originating from endocytic compartment and secreted in external medium by most cell types *in vitro*. They contain sorted sets of proteins involved in different cellular process, are able to transmit antigenic information and can be easily recovered from body fluids. All these characteristics have made exosomes very interesting tools to develop diagnostic protocols, and clinical assays for antitumoral immunotherapy are under development.

They have been described for the first time by Jonhstone and coll. in 1987 during the reticulocytes maturation process. Initially, exosomes were thought to be implicated in removing unneeded membrane proteins (Figure 4), for cells that have poor capacities to degrade proteins with a lysosomal system, or cells who are located towards a drainage system such as the tubule kidney or gut (Simons & Raposo, 2009). Since then, several cell types have been described to release exosomes in extracellular medium in vitro as hematopoietic cells (B cells, T cells, Dendritic cells, mast cells, platelets), intestinal epithelial cells, Schwann cells, neuronal cells, adipocytes, fibroblast (NIH3T3), tumor cells… Exosomes are found in vivo in several biological fluids such as urine, plasma, epididymal fluid, amniotic liquid, malignant and pleural effusions of ascites, bronchoalveolar lavage fluid, synovial fluid and breast milk. There are evidences that exosomes are also presents at the surface of follicular Dendritic cells (FDCs) in germinal centers (Denzer *et al.*, 2000).

They feature a density ranging from 1.13 g/ml for B cell exosomes (Raposo *et al.*, 1996) to 1.19 g/ml for epithelial intestinal cell exosomes (Trams *et al.*, 1981). We will see in the

section dedicated to the Human deficiency virus (HIV) that this density range is close to this infectious agent.

1. Molecular composition of exosomes

a) Protein content

Exosomes protein composition analyses have revealed both an ubiquitary composition and a cell type-specific protein composition. Most of the exosomal proteins that have been identified are found in the cytosol, in the membrane of endocytic compartment or at the plasma membrane of the parent cells. Exosomes contain both typical membrane proteins like GPI-anchored proteins, CD63 and Tumor Necrosis Factor Receptor 1 (TNFR1), and typical luminal proteins as annexin 2 and cytokines. Among proteins, exosomes contain also molecules involved in antigen-presentation (MHC-I, MHC-II) an co-presentation molecules (CD86), in cell adhesion (lactadherin, Integrins, MFGE8…), cell structure and motility (actins, myosin, tubulin…), Heat shock proteins and chaperones (HSP70, HSP90), Metabolic enzymes (α-Enolase, fatty acid synthase, Glyceraldehyde-3-phosphate dehydrogenase, peroxidases, pyruvate and lipid kinases ….), proteins referring to exosomes biogenesis or MVB Biogenesis (proteins of ESCRT machinery as Tsg101, Lysobisphosphatidic acid (LBPA) binding protein: Alix,), lysosomal markers (LAMP-1/2), signalling proteins (kinases, 14-3-3, GTPase Hras, RhoA, RAP1B, Guanine nucleotide-binding protein subunits -G proteins-…), tetraspanins (CD9, CD63, CD81, CD82), proteins involved in transcription and protein synthesis (histones, ribosomal proteins, ubiquitin…), and proteins involved in trafficking and membrane fusion (Annexins, Rab protein family, ARF..) (Figure 5). The functional significance of certain proteins is not completely understood. It is worth to note that markers commonly used to characterize a composition of exosomes may have varying distribution between cell types. For instance, the Transferring Receptor (TfR) is absent in exosomes derived from B cells (Raposo *et al.*, 1996) whereas exosomes from monocytes derived DCs or Bone Marrow derived DCs are enriched in this receptor (Zitvogel *et al.*, 1998).

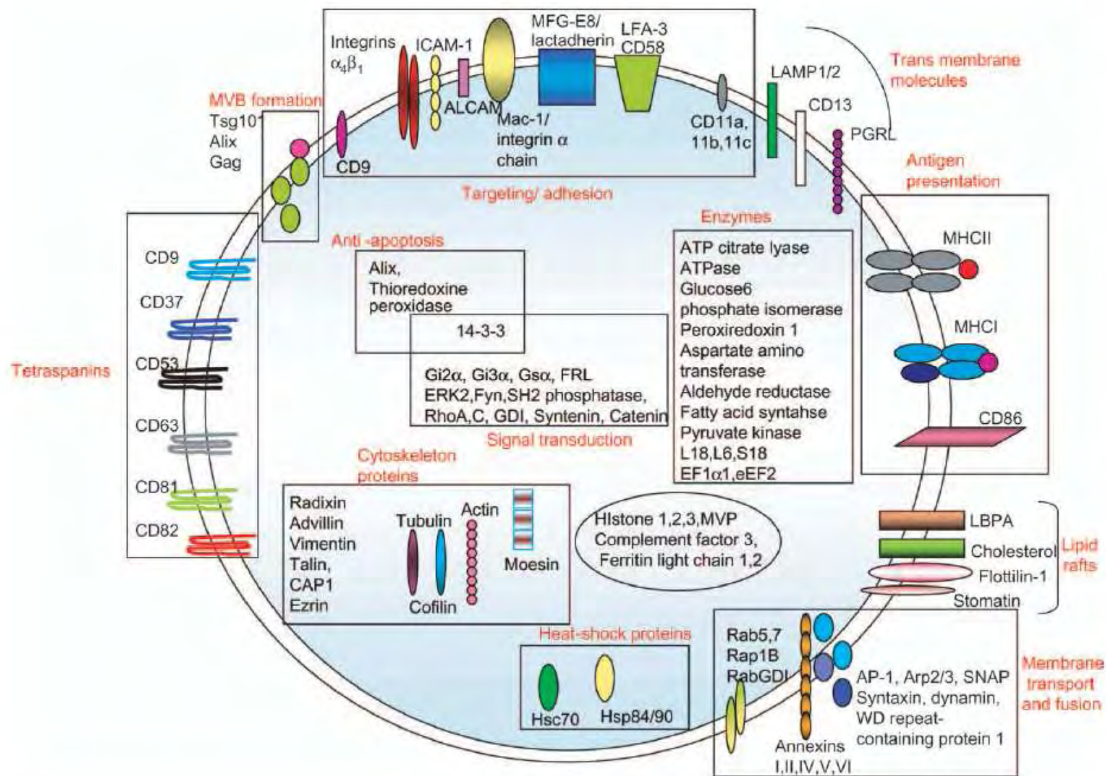


Figure 5 : Protein composition of exosomes indicating. GDI, GTP dissociation Inhibitor; ICAM1, intercellular adhesion molecule-1; CAP-1, adenylyl cyclase-associated protein; LAMP, lysosomal associated membrane protein-1; PGRL, PG regulatory-like protein. Adapted from (Schorey & Bhatnagar, 2008).

In addition to a common set of lipids and proteins, exosomes harbour particular characteristics inherited from the cell type of origin like CD3 from exosomes derived from T lymphocytes, perforin and granzyme for exosomes from cytotoxic T cells. Intestinal epithelial cells are able to secrete 2 types of exosomes : from apical and basolateral sides which have different proteic compositions for instance (van Niel *et al.*, 2001). Numbers of these molecules are shared by HIV.

The presence of CD55 and CD59 (glycosylphosphatidylinositol(GPI)-anchored membrane regulators of complement) makes antigen presenting cells (APCs)-derived exosomes (B cells and Dendritic cells lines) resistant to complement lysis, suggesting them to be stable in vivo (Rabesandratana *et al.*, 1998; Clayton *et al.*, 2003). As detailed in the next section, the particular lipid composition and membrane organization of exosomes may also contribute to their stability (Laulagnier *et al.*, 2004b). Moreover, It has been recently evidenced that in vivo*,* a prolonged Antigens (Ag) presentation during 14 days was mediated by exosomes, whereas the respective peptide –pulsed Dendritic cells injected in immunized mice were undetectable at 7 days (Luketic *et al.*, 2007). It has been demonstrated so, that exosomes were not susceptible to cytotoxic T Lymphocytes (CTL)-mediated elimination on the contrary of injected Dendritic cells following T cells activation.

b) Lipid content

It has been proposed that exosomes display a particular lipid organization and composition.
Some works have suggested the presence of lipid raft- like domains in exosome membranes. It has been proposed that lipid micro domains could be involved in the generation of the ILVs since proteins contained in raft-domains (flotilin and stomatin) are incorporated in reticulocytes and B lymphocytes exosomes (de Gassart *et al.*, 2003; Fevrier & Raposo, 2004), or in concert with tetraspanins (de Gassart *et al.*, 2003; Wubbolts *et al.*, 2003). Tetraspanins are highly enriched in exosomes, and have been localized to raft like micro domains. Indeed, lipid composition analyses have demonstrated that purified exosomes from B cells and Mast cells are enriched in cholesterol at least for B cells exosomes, sphingomyelin (SM) and sphingolipids : ceramide, glycolipid GM3 (Wubbolts *et al.*, 2003; Fevrier & Raposo, 2004; Subra *et al.*, 2007), glycerophospholipids with long and saturated fatty acyl chains, i.e. lipids that have been found in membrane rafts. Vidal et al. demonstrated that an exogenously administrated n-lissamine rhodamine b sulfonyl phosphatidylethanolamine (Rh-PE), can be also efficiently incorporated and secreted in association with exosomes (Vidal *et al.*, 1997).

Another important particularity is that exosomes seem to feature an unconventional asymmetrical membrane organization which has been thoroughly studied in Rat basophil leukemia 2H3 mast cells (RBL-2H3) (Laulagnier *et al.*, 2004b) conferring them a certain membrane rigidity and a subsequent high stability. Lipid analyses membrane revealed that exosomes express phosphatidylserine (PS) at their outer membrane leaflet which appears to be an essential requirement for exosomes budding within the late endosome. Moreover, former proteomic analyses in our lab have revealed the presence of a phospholipid scramblase which is involved in mixing the phospholipids between the two membrane leaflets (Pisitkun *et al.*, 2004)(unpublished data). Others works have demonstrated that a particular phospholipid, the lysobisphosphatidic acid (LBPA or BMP for bis(monoacylglycero)phosphate) enriched at the level of late endosome membranes (accounting for 15 mole percent of total organelle phospholipids and not detected elsewhere in the cell (Kobayashi *et al.*, 1998b), were efficiently incorporated into the internal vesicles of MVBs. Moreover, Matsuo and coll. found that the LBPA could induce in vitro the formation of multivesicular liposomes in large unilamellar liposomes submitted to the same pH gradient as that present in vivo in MVBs (acid inner pH, neutral outer pH) (Matsuo *et al.*, 2004).

A recent study gave importance to another lipid: the ceramide. Trajkovic and coll. demonstrated that purified exosomes from Oli-neu cells (mouse oligodendroglial cell line, myelinating cells of the central nervous system) are enriched in ceramide, and the release of exosomes was reduced after the inhibition of the neutral sphingomyelin (Trajkovic *et al.*, 2008).Compared to cellular membrane, the lipid composition of exosomes was similar to

lipid rafts, i.e. enriched in cholesterol, containing high amount of sphingolipids (sphingolmyelin and hexocylsceramide) and lower amount of phosphatidylcholine, an increase in saturated PC at the expense of polyunsaturated species, and a marked important enrichment in ceramide. The latter was thought to induce aggregation of lipid microdomains into larger (Gulbins & Kolesnick, 2003). Its cone-shape structure might induce the spontaneous membrane curvature by creating an area difference between the membrane leaflets. On the contrary to the LBPA which is not enriched into ILVs, ceramide seem to be used for generation of ILVs that are not destinated to the lysosomes but secreted as exosomes.

2. Sorting proteins in exosomes :

Enrichment of a particular set of proteins in exosomes gives evidence of a sorting process of proteins. For example, in B lymphocytes and Dendritic cells, MHC class II, MHC class I, and tetraspanins proteins are confined to the internal vesicles of the MVB whereas others as Lamp-1 or HLA-DM reside primarily at the limiting MVB membrane.

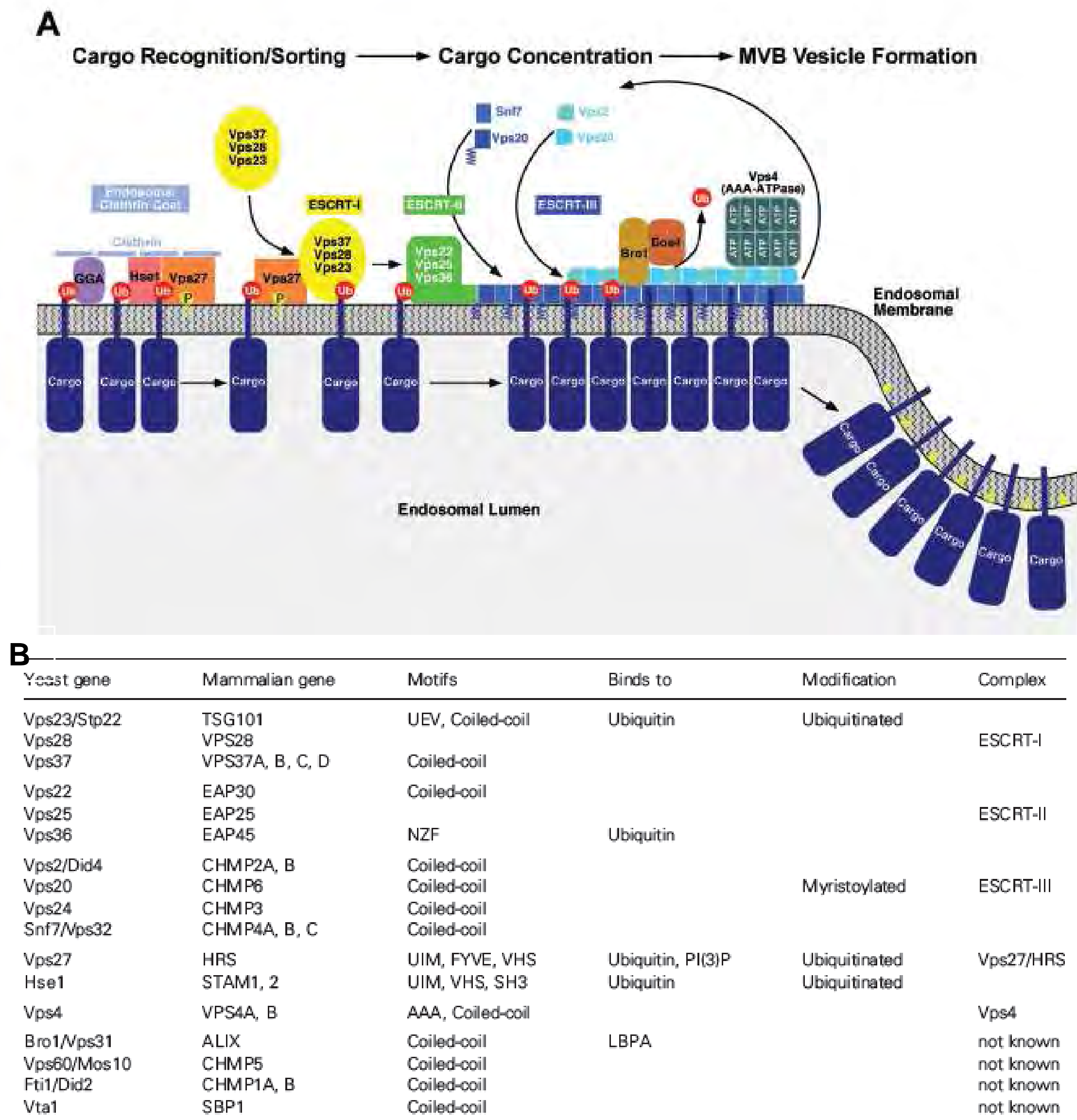
- *Sorting via the Endosomal Sorting Complex Required for Transport (ESCR)T machinery.* The sorting of protein complexes into MVB vesicles is a highly regulated process and a variety of studies indicate that monoubiquitination serves as a signal that directs protein complexes into the MVB pathway (Babst, 2005).

  ESCRT functions are to (1) select transmembrane protein cargos (generally those tagged by ubiquitination) and sort them into specified areas of endosomal membranes, (2) induce membrane invagination away from the cytoplasm and toward the endosomal lumen, and (3) fuse the neck of the induced membrane invagination to generate a vesicle within the endosomal lumen.

  ESCRT machinery is constituted of three separate protein complexes called ESCRT I, ESCRT II, ESCRT III. The ESCRT machinery is transiently recruited to the cytosolic side of the endosomal membrane for sorting of selected protein to ILVs and for ILV formation (Porto-Carreiro *et al.*, 2005). The ESCRT 0 complex represent the ubiquitin binding proteins of the HRS complex (Hrs, STAM, and Eps15). Vps27/Hrs proteins forms a complex with Hse1/STAM, Eps15 and clathrin, bind monoubiquitinated transmenbrane proteins and are important for the sorting of the MVB cargo. Vps27/Hrs recruits Tsg101 and its complex the ESCRT I which binds the ubiquitinated cargo protein and activates ESCRT II (Figure 6). Tsg101 then recruits ESCRT III via ESCRT II or AIP1(Alix). ESCRT II initiates the oligomerization and the formation of the ESCRT III complex that seems to function in the concentration of MVB proteins and sequesters proteins into the inward- budding vesicles of the MVB. Then ESCRT III recruits the deubiquitinating enzyme Doa4 which removes the ubiquitin tag from the

cargo protein prior to sorting into the MVB vesicles. Finally, a multimeric AAA-type (ATPase associated with various cellular activities) ATPase Vps4 binds to ESCRT III and disassembles the ESCRT III complex in an ATP-dependent manner.

We will develop in the second section of the introduction that several components of the ESCRT machinery are diverted by HIV for its own particle formation.



Yeast gene	Mammalian gene	Motifs	Binds to	Modification	Complex
Vps23/Stp22	TSG101	UEV, Coiled-coil	Ubiquitin	Ubiquitinated	
Vps28	VPS28				ESCRT-I
Vps37	VPS37A, B, C, D	Coiled-coil			
Vps22	EAP30	Coiled-coil			
Vps25	EAP25				ESCRT-II
Vps36	EAP45	NZF	Ubiquitin		
Vps2/Did4	CHMP2A, B	Coiled-coil			
Vps20	CHMP6	Coiled-coil		Myristoylated	ESCRT-III
Vps24	CHMP3	Coiled-coil			
Snf7/Vps32	CHMP4A, B, C	Coiled-coil			
Vps27	HRS	UIM, FYVE, VHS	Ubiquitin, PI(3)P	Ubiquitinated	Vps27/HRS
Hse1	STAM1, 2	UIM, VHS, SH3	Ubiquitin	Ubiquitinated	
Vps4	VPS4A, B	AAA, Coiled-coil			Vps4
Bro1/Vps31	ALIX	Coiled-coil	LBPA		not known
Vps60/Mos10	CHMP5	Coiled-coil			not known
Fti1/Did2	CHMP1A, B	Coiled-coil			not known
Vta1	SBP1	Coiled-coil			not known

Figure 6. Ubiquitin-dependent sorting of transmembrane proteins at the endosomes (ESCRT) in yeast. A) Ubiquitin-binding proteins (for example GGAs) and the Vps27/Hse1 complex with clathrin form an endosomal clathrin coat which assemble proteins into the ESCRT machinery. Vps27, localized to endosome through PtIns(3)P binding, recruits ESCRT-I from the cytoplasm, where it interacts with monoubiquitinated protein. Consequently, ESCRT-I activates ESCRT-II, which results in the formation of ESCRT-III. The ESCRT-III complex concentrates the MVB cargo and recruits additional factors such as Bro1 and the AAA-type ATPase Vps4. Bro1 functions in the recruitment of the deubiquitinating enzyme Doa4 that removes the ubiquitin tag from proteins.Vps4 dissociates the ESCRT machinery.. **(B)** Proteins of the ESCRT machinery in yeast and mammalian cells. Adapted from Adapted from (Babst, 2005)

The ESCRT-associated protein AIP1/Alix has also been implicated in intraluminal vesicle formation. It has been demonstrated that Alix associates with LBPA in reconstituted liposomes with an inner low pH and that Alix inhibits vesicles budding triggered by LBPA in these conditions and perhaps a certain asymmetry between the two leaflets of the bilayer (Matsuo *et al.*, 2004) whereas LBPA is enriched in late endosomes and ILVs (Kobayashi *et al.*, 1998b). Some of the ESCRT proteins and ESCRT-associated proteins such as Tsg101 and Alix can be recovered from exosomes.

However, in immature Dendritic cells, an alternative MVB sorting mechanism for MHC class II in antigens loaded DCs in response to cognate interactions with T cells was evidenced (Buschow *et al.*, 2009). This pathway is characterized by the incorporation of MHC class II in detergent-resistant protein complexes. MHC class II is sorted in MVB to ILVs for lysosomal targeting in a process that is driven by ubiquitination of the cytoplasmic domain of MHC class II β. When DC interacts with a cognate T cell, ubiquitination is blocked and MHC class II is sorted together with CD9 into ILVs of the MVB resulting in secretion via exosomes (Buschow *et al.*, 2009). Two distinct MVB pathways were evidenced: one for lysosomal targeting and the other for exosome secretion.

Some proteins without ubiquitination interact with component of ESCRT complex and are engulfed in exosomes too. TfR seems to associate with Alix for instance, suggesting that different protein interactions can provide access to the final steps of ESCRT processing. (Babst, 2005; Marsh & van Meer, 2008).

- *Sorting via an ESCRT independent sorting process*. The responsible signals for this passive mechanism are in some cases the presence of tetraspanin enriched or cholesterol enriched (lipid rafts) membrane micro domains (Keller *et al.*, 2006).
    - Co-sorting by lipid affinity (GPI-anchored). Tetraspanins have a high affinity for cholesterol and sphingolipids such as ceramides which may create domains as it occurs in membrane model. Tetraspanins and others proteins may partition into these domains because of their intrinsic physical properties and therefore into the inwardly budding vesicles. (Hemler, 2001; Trajkovic *et al.*, 2008)

      It has been evidenced for instance, that in Oli-neu cells (mouse oligodendroglial cell line, myelinating cells of the central nervous system) the proteolipid protein PLP (a major component of myelin) is released in association with exosomes. PLP is segregated into distinct subdomains on the MVB membrane and their transfer in exosomes is independent of the ESCRT machinery but require the sphingolipid ceramide. They provide an alternative pathway for sorting cargo into MVB, that seems to depend on raft-based microdomains (Hemler, 2001; Trajkovic *et al.*, 2008).

- Co-sorting by protein affinity (tetraspanin network) (Chaperones): Transient association with proteins. Tetraspanins are known to form oligomers with others tetraspanins or with other proteins (transmembrane or cytosolic) and may participate to proteins segregation (clustering proteins). For example, both HSP70 and Hsc70, found in exosomes of mostly cell type, interact with TfR. The interaction of TfR an Hsc70 regulates its release via exosomes since the lack of interaction of TfR receptor with Hsc70 increases its aggregation and association with exosomes. (Geminard *et al.*, 2001; Geminard *et al.*, 2004). Tetraspanins associate with proteins such as integrins, growth factor receptors, MHC class II and the costimulator CD86. They may provide a mean for proteins to go into MVB independently of the ubiquitin system.

- Higher-order oligomerization : recently, it has been described a novel exosomal protein-sorting pathway in Jurkat T cells that selectively incorporates proteins into exosomes (Fang *et al.*, 2007; Vella *et al.*, 2008) Proteins could be directed into exosomes either by exposing cell surface proteins to exogenous cross-linking agents, appending plasma membrane anchors to highly oligomeric cytoplasmic proteins, or adding multiple homo-oligomerization domains to intracellular acylated proteins. Either one of these treatment was sufficient to target a protein previously not associated with exosomes, to the exosomes pathway and consequently into exosomes.

- Some cytosolic proteins are probably nonspecifically, randomly, engulfed in exosomes during the inward budding.

3. Regulated Secretion

The secretion of exosomes can be constitutive or inducible depending on the cell type (Skokos *et al.*, 2001; Thery *et al.*, 2009).

Proteins destinated to the cell surface or to be secreted into the extracellular medium can be routed from the Trans Golgi Network (TGN) by an ubiquitary constitutive pathway that does not require a specific stimulus albeit controlled according to cell activity (intracellular signalling, cell growth, differentiation...). Structures involved in this anterograde pathway are pleiomorphic secretory vesicules and tubules ranging from 50nm-200nm up to 2µm in length and leave the TGN, move through the cytoplasm along cytoskeletal tracks and fuse with plasma membrane (Ponnambalam & Baldwin, 2003). Some Rab GTPases have been identified in the regulation of the traffic from TGN to plasma membrane such as Rab8, rab13 and Rab11. In the same manner, heterotrimeric G-protein

and protein kinase D (PDK1-2) are thought to be involved (Ponnambalam & Baldwin, 2003) as well as P2X7R (see below). These compartments contain only one or two exosomes.

Concerning the inducible secretion, several agents are listed hereafter known to modulate the exosomes production.

1. Exosome release is known to be sensitive to changes in intracellular calcium in mast cells and in human erythroleukemia cell line (Savina *et al.*, 2003).
Depolarisation induced by K^+ appears to increase the secretion of neuronal exosomes (Leenders *et al.*, 2002).

2. It has been shown that cross linking of CD3 in T cells or cross linking IgE R in mast cells (Raposo *et al.*, 1997) induces exosomes release.

3. Metalloprotease activators (4-aminophenylmercuric acetate) and agents which stimulate cholesterol extraction from the plasma membrane (methyl β cyclodextrin) are also stimulators of exosomes release (Stoeck *et al.*, 2006). In addition, inhibitors of ADAMs metalloproteases block the exosome formation. (ADAM10 and 17 are presents on exosomes).

4. The small GTPase Rab11 and the citron kinase, a rhoA effector, may participate in the exocytic event (Savina *et al.*, 2005; Loomis *et al.*, 2006).

5. Exosomes secretion from immature dendritic cells (imDCs) can be transiently increased by T cell clones interaction or cognate $CD4^+$ T cells (Buschow *et al.*, 2009; Thery *et al.*, 2009). On the contrary, another type of stimulus such as LPS have an opposite effect on the release of exosomes from DCs activated which is reduced (Thery *et al.*, 1999). Exosome secretion might not directly coupled to DC activation but rather depends on the type of signal. Moreover, their observation seems to be consistent with a recent observation that engagement with Ag specific $CD4^+$T cells stimulates exosome release by activated B cells (Muntasell *et al.*, 2007).

6. A recent study aiming to investigate the physiological function of Tumor Suppressor-Activated Pathway 6 (TSAP6), a glycosylated protein, p53 target gene, present in the trans-Golgi network, endosomal vesicular compartment and cytoplasmic membrane, demonstrated its crucial implication by means of TSAP6 knockout mice in exosomes production. Exosomes production appears as a tightly controlled biological process dependent of TSAP6 (Lespagnol *et al.*, 2008) which regulates the p53 dependent nonclassical exosomal protein secretion pathway. Thus, TSAP6 has been shown to increase the exosome secretion, downstream p53 activation following stress events which damage the DNA molecule (senescence, radiations ...) (Lespagnol *et al.*, 2008). This work corroborates findings

from others groups who previously highlighted the role of p53 in the secretion of exosomes (Yu *et al.*, 2006).

7. Others molecules such as diacylglycerol kinase and a brefeldin A-inhibited guanine-nucleotide exchange protein 2 (BIG2 or ARFGEF2) have been proposed to have a role in the secretion of exosomes or exosome-like vesicles (Thery *et al.*, 2009).

8. Saunderson et al. provide evidences that primary leukocytes release exosomes following signals such as cytokines or mitogen stimulation, and particularly when murine splenic B cells were stimulated via CD40 and the IL-4 receptor. Exosomes synthesis was similarly induced in human B cells by EBV infection and most importantly with a CD40/IL-4 stimulation. Immortalization of primary B cells by viral infection also provides signals for the induction of exosomes release. CD40 and IL-4 has previously been shown to induce B cells proliferation. IL-4 and CD40 stimulated human B cells who are potent APC, with similar potential for immunotherapy as DC, and a similar exosomes secretory mechanism (Saunderson *et al.*, 2008).

9. It has been proposed that P2X7 receptors, adenosine triphosphate (ATP)-gated ion channels susceptible to rapid changes in ionic fluxes, regulates the release of exosomes from multivesicular bodies among other membrane trafficking responses and non-classical secretion in immune and inflammatory effector cells (Qu & Dubyak, 2009). The authors have previously reported an involvement of exosomes as another non classical pathway for the P2X7R-stimulated secretion of IL-1β (Qu *et al.*, 2007). Other data suggest that P2X7R activation in murine macrophages and Dendritic cells triggers the rapid extracellular release of plasma membrane derived microvesicles and exosomes lacking P2X7R.

10. Using short hairpin RNA (shRNA) based screen targeting human Rab genes, Ostrowski and coll. observed that knocking down several Rab GTPase proteins among them Rab 27a and Rab 27b allowed to reduce exosomes secretion on Hela B6H4 cells tumour cell line (which secreted exosomes and soluble OVA through the classical secretory pathway) (Ostrowski *et al.*). Rab27a and Rab27b are encoded by two different genes and associated with lysosome-related organelles. A role of these two proteins was confirmed in the intracellular trafficking of multivesicular endosomes, their docking to the plasma membrane leading to exosomes secretion most likely through the slp4 and Slac2b effectors proteins. Other Rab GTPases associated with the endocytic system, Rab 7 and Rab 11, did not modify exosome secretion, suggesting that a specific subset of multivesicular endosomes participates in the generation of exosomes.

Once released into the extracellular medium, exosomes have to attach to their recipient cells.

4. Attachment mechanisms to recipient cells

Several mechanisms have been suggested to explain the exosomes/recipient cell interactions: i/ receptor-ligand (Raposo *et al.*, 1996), ii/ integrins attachment and fusion with plasma membrane (Rieu *et al.*, 2000; Clayton *et al.*, 2004) iii/ internalization by endocytosis (Morelli *et al.*, 2004).

Morelli et al. investigated the role of several surface molecules born either by exosomes or bone marrow DCs, in the endocytosis of exosomes (Morelli *et al.*, 2004). Their study reveals a diminution of the exosome uptake caused by a simultaneous inhibition of αv and β3 integrins, CD11a and its ligands CD54, Antibodies (Ab) directed against the tetraspanins CD9 and CD81, or a soluble analogue of Phosphatidylserin (PS). Indeed, exosomes adhesion to the recipient cell membrane could be mediated through lipids (PS) or ligand-receptor interactions. An implication of Milk fat globule-Epidermal Growth factor VIII protein (MFGE8or lactadherin) was also underlined (Morelli *et al.*, 2004). (Figure 7)

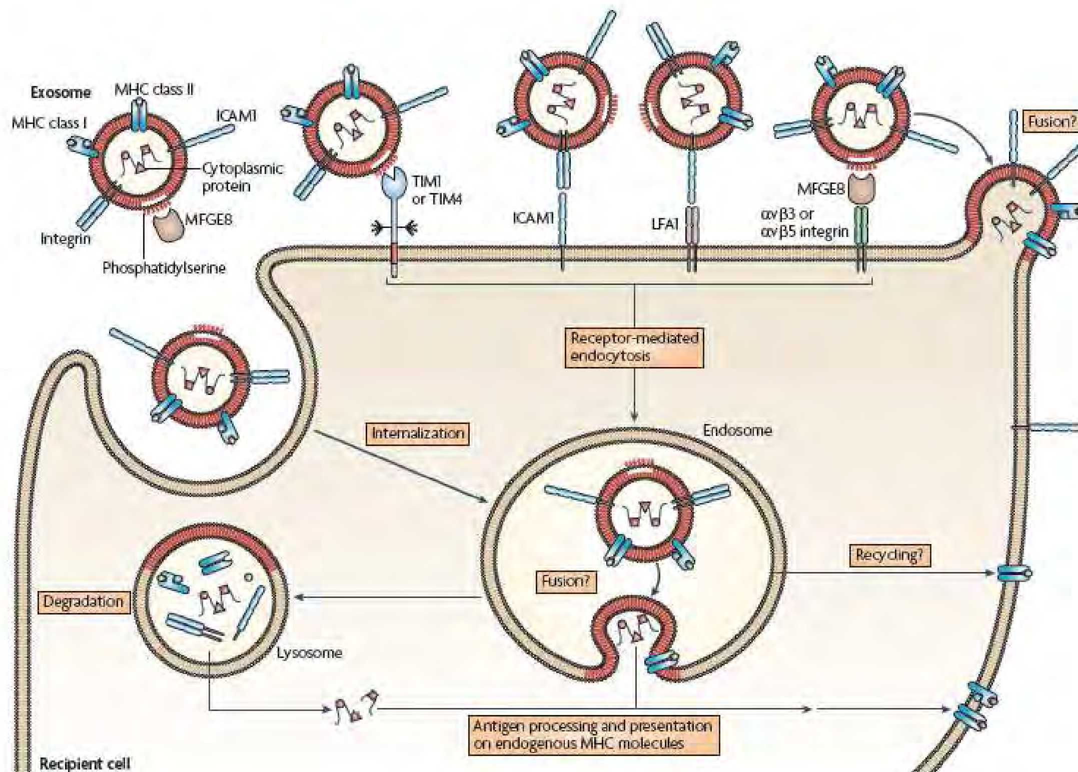


Figure 7 : Exosomes Interaction with recipient cells. Exosomes might bind recipient cell surface molecules listed in the text. The molecules involved are : intercellular adhesion molecule 1 (ICAM1) which binds to lymphocyte function-associated antigen e(LFA-1); Phosphatidylserine (PS) which binds to T cell immunoglobulin domain and mucin domain protein 1 (TIM1) or TIM4, and milk fat globule EGF factor 8 protein (MFGE8) which binds to αvβ3 or αvβ5 integrins. The fate of exosomes is unknown. It is supposed that after interaction with cell surface molecules, exosomes might directly fuse with plasma membrane leading to the release of exosome contents into the cytoplasm of the recipient cell. Alternatively, exosomes could be endocytosed and fuse with the limiting membrane of the endosome and release their contents in the cytoplasm. Finally, endocytosed exosomes could be degraded in the endocytic pathway. This degradation can lead to the production of antigenic peptides for loading onto MHC class II and class I molecule. Aadapted from (Thery *et al.*, 2009).

MGE8 is most likely involved in addressing exosomes to target cells and may bind to PS exposed on the surface of apoptotic cells and exosomes through its carboxy-terminal factor V/VIII-like domain. The amino-terminal domain of MFGE8, containing two Epidermal growth factor (EGF)- like domains with RGD-containing sequence, that is exposed on the surface of MFGE8-bearing apoptotic bodies binds to $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, and promotes their phagocytosis by macrophages. It is possible that MFGE8 is also involved in the capture of membrane vesicles by $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins expression on phagocytes. MFGE8 may also bind $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins on the DC side. In experiments conducted by Hanayama and coll, a MFGE8 agonist Ab raised the capture of exosomes by BMDC, as already described in macrophages where this Ab augmented endocytosis of apoptotic cells (Hanayama *et al.*, 2002) whereas a RGD-sequence reduced the uptake of exosomes by DCs. However, Clayton et al. suggested that this molecule expressed by DC derived exosomes and implicated with DC interaction is not necessarily involved in tumor exosomes uptake by DC. (Morelli *et al.*, 2004; Clayton & Mason, 2009).

CD91 has been identified as the common receptor for Hsp (including Hsp90, Hsp70, calreticulin, Hsp60...) and as an endocytic receptor that internalizes antigenic proteins or peptides chaperoned by Hsp (Binder *et al.*, 2000). Skokos and all (Skokos *et al.*, 2003) addressed the question about CD91 receptor and its implication in the presentation of Ag associated with exosomes by DC. They revealed an impairment of IL-2 production by Ag specific T cells when DCs were preincubated with an anti-CD91 antibody or the RAP peptide (a ligand for CD91 receptor) before the addition of mast cells exosomes followed by T cells.

Previous studies (Skokos *et al.*, 2001) suggested that the action on B and T cells of exosomes from mast cells bearing the leukocyte function – associated antigen-1 (LFA-1) and the intercellular adhesion molecule 1 (ICAM1) molecules were blocked by Ab against these two molecules. Indeed, ICAM-1 and LFA-1 have been suggested for the capture of the exosomes at the cell surface (Hwang *et al.*, 2003; Segura *et al.*, 2005b; Segura *et al.*, 2007; Nolte-'t Hoen *et al.*, 2009; Simons & Raposo, 2009). It has been shown that ICAM1 bearing exosomes derived from mature DC are captured by binding to LFA1 (a ligand for ICAM1), on the surface of $CD8^+$ DCs (Segura *et al.*, 2007) and activated T cells (Hwang *et al.*, 2003; Segura *et al.*, 2007; Nolte-'t Hoen *et al.*, 2009). It is worth to note that co-incubation of exosomes and DCs with blocking antibodies specific for various integrins, adhesion molecules or tetraspanins reduced exosome capture by DC by 5-30% (Morelli *et al.*, 2004; Nolte-'t Hoen *et al.*, 2009; Thery *et al.*, 2009): Indeed, Nolte'-t and coll. recently demonstrated that DC exosomes secreted in the extracellular medium during cognate T-cell-DC interactions are targeted to T cells activated. During T cell activation, LFA-1 undergoes a transient conformational change, inducing a high-affinity conformation of LFA-1 against ICAM-1, born by exosomes. Moreover, they maintain that exosomes are found on the T cell plasma membrane and that the majority of these exosomes neither fuse with the plasma membrane nor are efficiently internalized. In their study, anti-LFA-1 antibody interfered dose-

dependently with the recruitment of DC exosomes by activated T cells, suggesting that LFA-1 on activated T cells is involved in DC exosomes binding. When the high affinity of LFA-1 is induced and stabilized by divalent cation such as Mn^{2+}, resting T cells are capable of binding DC exosomes, process blocked by anti-LFA-1.

A common feature of exosomes is their expression of adhesion molecules in addition to ICAM-1. These often comprise members of the IgG superfamily and members of the integrin family, such as VLA-4 (reticulocytes and B cell exosomes), αM integrin, β2 integrin (Dendritic cell exosomes), which potentially mediate adhesive interactions to a multitude of ligands, including cell surfaces and/or to extracellular matrix components.
Researchers investigated the presence of integrins molecules on exosomes of B lymphocytes and demonstrated their functionality in anchoring exosomes to extracellular matrix components (collagen type I, fibronectin) and to activated fibroblasts. β1 and β2 integrins expressed on B cells exosomes might bind to ICAM1-expressing cells and extracellular matrix proteins such as fibronectin (Rieu *et al.*, 2000; Clayton *et al.*, 2004). The involvement of exosome VLA-4 (β1-α4) was also emphasized.

Phosphatidylserine (PS) is normally exposed on the surface of apoptotic cells. PS exposed on the outer leaflet of the plasma membrane is often used as a recognition signal for phagocytes to engulf apoptotic cells. It is also expressed on the surface of exosomes. Thus, exposure of phosphatidylserine can trigger the phagocytosis of apoptotic lymphocytes by macrophages. Thus, multiple PS binding proteins are susceptible to link exosomes on recipient cell including several classes of scavenger receptors, integrins, complement receptors and CD14 (Zakharova *et al.*, 2007) and T-cell immunoglobulin – and mucin domain containing molecules 4 (TIM-4). TIM-4 recognizes PS via its immunoglobulin domain and binds apoptotic cells and exosomes and mediates their engulfment. TIM-1 bind also PS. TIM-1 is expressed on the surface of activated lymphocytes (Th2 cells) and TIM-4 is expressed in phagocytes (macrophages) and various tissues such as the spleen, thymus, lymph nodes and salivary glands. They are thought to be implicated in the engulfment of apoptotic cells and exosomes which harbour PS at their surface. (Miyanishi *et al.*, 2007; Simons & Raposo, 2009).Works of Masanori Miyanishi et al. bring the evidence that Tim-1 and -4 expressing Ba/F3 B cells were bound by exosomes via PS. Moreover, exosomes derived from Ba/F3 cells stimulate the interaction between Tim-1 and Tim-4 (Miyanishi *et al.*, 2007).
Zakharova et al. reported that exosomes isolated from the supernatants of activated $CD4^{+}$ T cells enhance cholesterol accumulation in cultures human monocytes and THP-1 cells (Zakharova *et al.*, 2007). Anti PS receptor antibodies recognize surface protein on the monocytes plasma membrane and prevent exosomes induced cholesterol accumulation, indicating that exosomes internalization is mediated via endogenous PS receptor.

The fate of exosomes after binding to the surface of recipient cells is not known. Whether exosome fusion occurs on the surface of the recipient cell with the plasma

membrane, or after endocytosis in internal compartments is still unclear. Exosomes are thought to deliver their content into the cytoplasm.

C. Exosomes functions

Exosomes trigger positive and negative responses. They have been established to mediate communication between cells, to permit cellular material exchange, to eliminate unsuitable proteins like Transferin receptor (TfR) in reticulocytes, and to have an immunological role. A number of studies indicates that exosomes may be important protagonists stimulating immunocompetent cells and participating in the signalling events that contribute to antigen presentation to T cells, or deprivating it.

1. Positive effects of exosomes

a) Stimulation of immunocompetent cells.

Given the fact that Dendritic cell (DC) derived exosomes (Dex) bear molecules implicated in direct T cell activation or DC targeting, incuding MHC class I and II complex conferring them a role in Ag presentation, costimulatory molecules such as CD40, CD80, CD86, Heat Shock Proteins (HSPs), and adhesion molecules as ICAM-I, prompted many researchers to assess exosomes potency to modulate immune response.

a.1. Dendritic cell exosomes : Direct stimulation

Pioneer studies aimed to reveal the exosomes implication in modulating the immune system, shown for example, that Epstein-Barr virus transformed human B cell derived exosomes carrying MHC-mycobacterial heat shock protein 65 (hsp65) complexes that were demonstrated to stimulate $CD4^{+}$T cells *in vitro* in an antigen specific manner (Raposo *et al.*, 1996; Thery *et al.*, 2009). They demonstrated that exosomes, generated in the vesicular MIICs, carry MHC class II molecules, whereas TfR are excluded from exosomes, illustrating the selective incorporation of MHC class II in exosomes. Their works addressed the question of a direct T cell activation by exosomes in vitro, and in their hand, peptide bound to MHC class II on exosomes produced a strong peptide specific and MHC class II restricted stimulation of $CD4^{+}$ T cells clones, concluding that the MHC molecules associated with exosomes were functional. Another work shown that exosomes derived from human MART-1/melanA^{+} peptide pulsed DCs were able to stimulate IFNγ production and proliferation of peptide- specific restricted CTL clones. (Dufour *et al.*, 1997; Zitvogel *et al.*, 1998) Sustaining the Ag presentation role of exosomes, Kleijmeer et al. estimated for instance, that in

immature DCs, once endocytosed, MHC class II is efficiently sorted at MVBs. At the equilibrium, about 50% of total cellular MHC II and about 80% of MVB associated MHC II has been detected at the level of the ILVs (Kleijmeer *et al.*, 2001).

Their implication in modulating immune system has gained importance from Zitvogel and colleagues' works (Zitvogel *et al.*, 1998). Their study emphasized the ability of exosomes derived from murine Bone Marrow-DCs loaded with tumor peptides, to display antitumor activity in vivo, both in the murine mastocytoma P815 and the murine mammary carcinoma TS/A tumor models. These exosomes were able to trigger the eradication of established tumor or growth delay in mice, implying a long lasting immune response since the reject of a lethal challenge of the tumor was observed in a syngeneic manner. They also emphasized that T cells were primed by exosomes in an antigen specific manner and an antitumor CTL activity was revealed in cured animals (Zitvogel *et al.*, 1998).

Kovar et al. shown in a study that aimed to address a direct stimulation of $CD8^{+}$T cells by membrane vesicles from antigen presenting cells (APC), that DC exosomes loaded with a specific peptide, were able to stimulate proliferation of T cells albeit weakly compared to membrane vesicles coming from sonicated DCs (Kovar *et al.*, 2006).They also demonstrated that in vitro, mDex could induce potent stimulation of naïve $CD8^{+}$T cells (proliferation, cytokine synthesis and differentiation into CTL), suggesting that inducing DC maturation may improve the efficiency of Dex for induction of adaptative responses(Sprent, 2005; Chaput *et al.*, 2006a)

Several studies have revealed that only high rates of exosomes expressing both MHC class I and costimulatory molecules were able to directly stimulate T cell clones, but weakly T cell lines. On the contrary, they failed to stimulate naïve T cells (Vincent-Schneider *et al.*, 2002). However their capacity to stimulate T cells is increased in presence of DCs as already mentioned.

Many works also underlined that exosomes derived from DCs are able to directly activate T Cells in the absence of recipients APCs(Hwang *et al.*, 2003; Segura *et al.*, 2005a; Segura *et al.*, 2005b; Admyre *et al.*, 2006; Kovar *et al.*, 2006). Segura et al. showed that mature Dex (mDEx) released from BM-DC or D1 cells, are less abundant than immature Dex (imDex) produced by DCs but are much more efficient (-50- to 100 fold) for inducing proliferation and with 10-20 fold less proteins for priming $CD4^{+}$ T cell in vivo than imDex because they are enriched in molecules involved in mDex mediated $CD4^{+}$T cell priming such as ICAM-1 (Chaput *et al.*, 2006a). However, in the presence of APCs, T cell activation was strong with much less exosomes suggesting that physiologically, exosomes are transferred to recipient APCs, thus activating T cells. In addition to T cell stimulation exosomes could be able to support T cell survival with a mechanism implying NFkappaB (Matsumoto *et al.*, 2004).

The recent study of Qazi and co-workers demonstrated the efficiency of exosomes derived from Bone Marrow DCs, directly or indirectly loaded with OVA derived-peptide to induce specific T cell proliferation in vitro whereas only the latter elicit T cell proliferation in vivo and induce a specific antibody response. They confirmed that exosomes from mature or immature DC are able in vitro to stimulate expansion of Ag specific T cells. However, they

could observed an immune response when directly loaded exosomes where injected in combination with whole protein OVA and revealed a collaboration of B cells and T cells for exosomes mediated specific immune activation in vivo, suggesting that B cell are needed for exosomal T cells activation (Qazi *et al.*, 2009)(Figure 8).

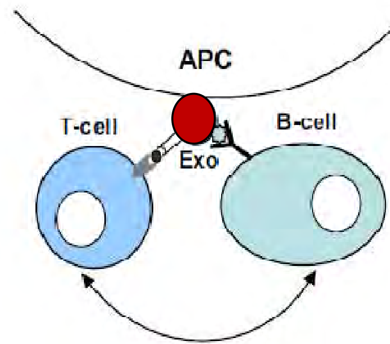


Figure 8 : Proposed mechanism showing indirectly loaded exosomes (-Exo- :red) facilitating interaction between B and T cells for efficient activation of the immune response. APC : antigen-presenting cell. Adapted from (Qazi *et al.*, 2009).

While some studies indicate that exosomes from DCs appear to be able to stimulate directly T cells, some others demonstrate that exosomes need the presence of APC to exert their function.

a.2. Dex : Indirect stimulation – cross presentation

Indeed, several studies have shown that Dex can transfer functional peptide loaded MHC class I and II complexes to DCs (Thery *et al.*, 2002; Andre *et al.*, 2004)

Works realized in 2002 by Thery and al. aimed to analyze the mechanism of antigen specific T cell stimulation by exosomes (Thery *et al.*, 2002). They found an indirect activation and proliferation of CMH II -restricted naïve $CD4^+$ T cells in response to DC derived exosomes bearing the specific H-Y Ag, in vitro and in vivo. The presence of mature DCs was underlined in lymph nodes even if deficients in MHC class II molecules, whereas no response was evidenced with the peptide alone in the last case. They also tested the requirement of mature DCs to stimulate T cells *in vitro* and concluded that H-Y exosomes activated $CD4^+$ T cells only in the presence of mature DC. Their data suggested that exosomes could become competent for naïve T cell activation after DCs uptake, which reprocessed the antigen contained in exosomes, or exosomes transfer the peptide-MHC class II complexes to neighboring DCs cells or between different DC populations, to stimulate $CD4^+$ T cells in an antigen specific manner.

Later on, Andre F. and colleagues demonstrated that Dendritic cells derived exosomes could transfer functional peptide - MHC class I to other Dendritic cells, that represents an important findings for peptide specific $CD8^+$ T cell priming and for the development of exosomes based vaccine against cancer (Andre *et al.*, 2004; Chaput *et al.*, 2004). Their data also brought the evidence of the need of mature DC to efficiently promote the activation and expansion of specific CTL in vitro and in vivo. Moreover, they suggested that exosomes can be viewed as an amplification process for DC mediated CTL responses, based on the fact that, in their experimental conditions, 2.10^9 exosomal MHC class I molecules can be released by 10^4-10^5 immature DCs in vitro in 24h (Andre *et al.*, 2004). APCs provide costimulatory molecules and cytokines requested for the priming of $CD4^+$T cells, helpers of CTL effectors. In vivo, positive effects of exosomes can be explained either by their proteic composition (presence of MHC molecules and enrichment in costimulatory molecules) than their lipid composition which can have adjuvant qualities. Consequently, in the second part of this study, the authors looked for a synthetic adjuvant that would trigger DC maturation in vivo, boosting immunogenicity of exosomes in vivo. Indeed, they could prime $CD8^+$ T cells with exosomes and the use of CpG DNA oligomeric sequences or dsRNA (which recognizes TLR3 expressed specifically on DC) as adjuvant. In this study, adjuvant was required for exosomes presenting gp100 to mediate tumor rejection in a B16 melanoma tumor model coexpressing human HLA-A2 and gp100 peptides (Chaput *et al.*, 2004).

Morelli et al. addressed the question of the internalization of exosomes from Bone Marrow Dendritic Cells (BMDCs) by DCs (Morelli *et al.*, 2004). They revealed that administrated exosomes following an intravenous injection in mice, were captured and processed likely partially in MVB compartments by specialized macrophages and immature DC of the spleen and also by hepatic kupffer cells for presentation to $CD4^+$ T cells. A higher internalization of allogenic exosomes by immature BMDCs than mature BMDCs was shown, agreeing with the fact that immature DCs exhibit higher endocytic capacity than the mature ones. They feature the ability, enhanced once activated, to present to $CD4^+$ T cells exosomes derived Ag at their surface, and subsequently to induce a T cell response. Thus, immature BMDC internalize and process allogeneic exosomes and increase their capability to present allopeptides derived from exosome on MHC class II molecules to $CD4^+$ T cells following their activation.
In the same way, it has been demonstrated that OVA-loaded Dex could be taken up by active, nonspecific $CD4^+$T lymphocytes, which were able to present peptide-MHC class I complexes such as APCs and stimulate an efficient $CD8^+$T cells response. These cells were also efficient in inducing long term immunity, since they could prevent from OVA transfected melanoma cells challenge in immunized mice (Hao *et al.*, 2007b).

Exosomes from dendritic cells are thus able to modulate immune response, either directly by exposing MHC and costimulatory molecules (Raposo *et al.*, 1996; Hwang *et al.*, 2003; Kovar *et al.*, 2006) or indirectly by conveying internal components to surrounding cells.

(Zitvogel *et al.*, 1998; Thery *et al.*, 2002; Vincent-Schneider *et al.*, 2002; Segura *et al.*, 2005b; Thery *et al.*, 2009). These conflicting observations might be a result of differences in the phenotype of the responder cells and/or the exosomes (expression level of MHC and costimulatory molecules), the exosome dose, or the affinity for the antigen used.

a.3. NK cell activation by Dex

The first clinical trials used immature DC derived exosomes as cell free vaccine in advanced melanoma and lung cancer bearing patients (developed in the next section). They failed to detect vaccine specific T cell responses while observing potent Dex-related NK cell activation (Viaud *et al.*), suggesting that Dex can either stimulate the innate immune response. Consequently, Viaud and colleagues recently shown that Dex, able to directly trigger NK cell activation in mice and cancer patients, secreted from bone marrow-derived or monocytes derived DC harbour functional membrane bound NKG2D ligands such as UL16 binding protein 1, or MIC A and MIC B (MHC class I polypeptide-related sequence A, B) and interleukin 15Rα (Viaud *et al.*, 2009; Viaud *et al.*). Dendritic cells derived exosomes were also shown to regulate NK cells functions, such as inflammatory cytokines (TNFα and IFNγ) production, via the protein HLA-B associated transcript-3 (BAT3) expressed on their surface (Simhadri *et al.*, 2008). This intracellular protein which is involved in DNA damage-induced apoptosis, represents the ligand for a natural cytotoxicity receptor expressed on NK cell, the NKp30, involved in direct cytotoxicity against tumor cells and directs maturation and selective killing of Dendritic cells.

a.4. Mast cell exosomes

Skokos et al. took interest in exosomes secreted from mouse Bone Marrow Mast Cells under IL-4 treatment or constitutively from Mast cell line (P815 and MC/9). They demonstrated that mast cell exosomes have the capacity to stimulate B and T cell proliferation and cytokines production (IL-2 and IFN γ) (Skokos *et al.*, 2001). Later on, the authors brought evidence that peptides-loaded exosomes from mast cells were also shown to elicit an efficient antibodies production in vivo following a subcutaneous injection in mice. Their data suggested that mast cells exosomes were able to induce phenotypic and functional maturation of DC (Skokos *et al.*, 2003). In their conditions, this maturation effect seemed to be unique to Mast Cells derived exosomes since those from B cells and macrophages were not able to convert DCs to the mature phenotype (probably due to a different accumulation of biological molecules as Hsps from different cell type). This might represents a mechanism by which exosomes efficiently prime the immune system in vivo. So, MHC-peptide exosomes transfer also appears to occur between different cell types, since

B cell and T cells are able to present antigens delivered by mast cell exosomes. Moreover, peptides loaded on MHC in exosomes can be loaded on MHC in the recipient cell (Skokos *et al.*, 2003).

a.5. B cell exosomes

Exosomes derived from B cells, in addition to the study of Raposo in 1996 (above-mentioned) were also shown to present MHC class II-allergen peptide complexes and induce a T cell proliferation and cytokines production in vitro suggesting that exosomes from B lymphocytes are immunostimulatory factors in allergic immune response (Admyre *et al.*, 2007a; Muntasell *et al.*, 2007). It has been found that exosomes release from B cells can be stimulated by the encounter of antigen--loaded B cells with antigen-specific CD4 T cells. Indeed, their data demonstrate that B cell derived exosomes can directly provide the specific peptide –MHC class II complexes required for proliferation and cytokine secretion from primed CD4 T cells, suggesting a role for B cell derived exosomes as modulators of an ongoing immune response or maintaining antigen-specific memory T cells (Muntasell *et al.*, 2007) Moreover, Papp et al. have recently shown that antigen loaded -B cell or macrophages secrete exosomes containing complement C3 derived fragments which enhance the antigen specific T lymphocytes response in the presence of APC compared with exosomes without the complement fragments (Papp *et al.*, 2008).

a.6. Tumor cell exosomes

Concerning tumor cell derived exosomes, it has been shown that exosomes purified from cultures cell lines or from ascites of patients with tumours, contain MHC class I molecules, and tumour antigens, and can induce the activation of antigen-specific T cells in vitro in the presence of recipient DCs (Wolfers *et al.*, 2001; Andre *et al.*, 2002; Hao *et al.*, 2007a). In these conditions, tumor antigens born by exosomes might provide a source of Ag for cross-presentation by DCs : they have to be transferred and processed by recipient DCs in an haplotype restricted manner and mediate $CD8^+$ T cell dependent anti tumor effects (Wolfers *et al.*, 2001; Andre *et al.*, 2002; Thery *et al.*, 2009). In vitro Tumor derived exosomes (TEX) enhance NK cell function through Heat Shock protein 70 surface expression (Gastpar *et al.*, 2005; Viaud *et al.*, 2009); they synergize with NKG2D ligands on the surface of tumor cell leading to the reduction of tumor growth and suppression of metastatic tumor (Elsner *et al.*, 2007). In the same manner, activating properties of tumor derived exosomes were reported when tumor cells have undergone stress such as heat shock which lead to an increase of Heat shock protein (HSP70) and stimulate NK cells. (Dai *et al.*, 2005; Zhang *et al.*, 2006).

On the contrary, there is evidence suggesting that Tex may be a kind of tumor escape mechanism and can induce tolerance of the host antitumor antigens (see the next section) (Clayton *et al.*, 2007; Valenti *et al.*, 2007; Dai *et al.*, 2008).

a.7. Intestinal epithelial cells exosomes

Exosomes derived from intestinal epithelial cells (IECs) are secreted from the apical and the basolateral sides of the polarized monolayer cells and both populations contain common set of proteins and specific molecules. Exosomes derived from these cells, despite their lack of costimulatory molecules, have also been shown to induce humoral immune response (Van Niel *et al.*, 2003). These exosomes bring luminal antigens and appear to be sensors of the antigen information present in the intestinal lumen.

b) Others Functions :

Follicular Dendritic Cells are essential cells for affinity maturation and immunoglobulin isotype switching of B cell clones in the germinal centers where they present Ags to B and T lymphocytes. They do not secrete exosomes themselves but abundantly display exosomes at their surface as demonstrated by (Denzer *et al.*, 2000). B cell-derived MHC class II^{+} exosomes specifically bind the surface of Follicular Dendritic Cells (FDCs) from human tonsil, with a possible role in development of high-affinity effector/memory B cells consequently. These cells do not synthesize MHC class II themselves but passively acquire peptide loaded MHC II complex from donor cells likely via exosomes. K Qazi proposed that exosomes might facilitate interaction between specific B and T cells leading to isotype switching and differentiation into plasma and memory B cells (Qazi *et al.*, 2009). Another study shown that membrane bound peptide-MHC complexes appear to be seeded in secondary lymphoid organs via exosomes (Muntasell *et al.*, 2007).

It has been recently evidenced that *in vivo,* a prolonged Ag presentation during 14 days was mediated by exosomes, whereas the respective peptide –pulsed dendritic cells injected in immunized mice were undetectable at 7 days (Luketic *et al.*, 2007). These authors demonstrated that exosomes derived from antigen pulsed DC were able to present Ag and activate Ag specific CD8^{+} T cells in vitro and in vivo, and that exosomes were not susceptible to CTL-mediated elimination. Their data support the hypothesis that exosomes secreted by DCs may mediate direct Ag presentation to naive T cells after peptide-bearing DCs are cleared from the system. Using exosomes as vaccine carrier may provide a novel platform for booster immunizations, knowing that the efficacy of readministered DCs is severely reduced by the presence of established CTL on the contrary to exosomes.

During the interaction of DC with cognate T cell, it has been proposed that MHCII carrying DC exosomes with the T cell plasma Membrane might allow continuation of T cell receptor (TCR) signaling even after dissociation of T cell receptor from DCs (Buschow *et al.*, 2009).

Several studies underlined that exosomes derived from macrophages infected with pathogens (such as M. tuberculosis, M. bovis, Salmonella enteric, Toxoplasma gondii) contain determinants that induce the secretion of proinflammatory cytokines by recipient macrophages as well as mycoplasma species which can induce B cell polyclonal activation. (Quah & O'Neill, 2007; O'Neill & Quah, 2008; Thery *et al.*, 2009). Mycoplasmal glycolipids were shown to represent powerful immunoregulators that induce a mitogenic response in B lymphocytes and activate macrophages (O'Neill & Quah, 2008). Indeed, pathogens like Mycobacterium tuberculosis or mycobacterium bovis that resides in macrophages endosomal compartments can constitute a source of antigens that are found on exosomes derived from. Exosomes derived from *M. bovis* BCG-infected macrophages activate antigen specific $CD4^+$ and $CD8^+$ T cells in vitro and in vivo (Giri & Schorey, 2008). Studies by Bhatnagar et al. reported that exosomes that are produced by bacterially infected macrophages are proinflammatory. They could stimulate neighbouring macrophages and neutrophils to release inflammatory mediators because of the presence of bacterially derived components such as glycolipids and glycopeptidolipids incorpored into exosome membrane (Bhatnagar & Schorey, 2007; Bhatnagar *et al.*, 2007). The further section concerning the spreading of pathogens will developed the cases of infections with Mycobacterium and Toxoplasma gondii. Another way in which exosomes could contribute to immune surveillance was illustrated by Jason D. Walker and coworkers (Walker *et al.*, 2009). Their studies revealed a specific $CD4^+$ T cells proliferation when cocultured with CMV-infected allogeneic endothelial cells (HUVEC) that do not express Class II MHC molecules, by means of exosomes. CMV-infected endothelial cells released CMV Ags associated with exosomes. The latters are able to transfer virus-derived antigens to DCs for activation of $CD4^+$T cells and stimulate allogeneic $CD4^+$ memory T cells. Their observations clarified how host CMV specific T cells may respond locally to infected graft cells throughout graft rejection process.

It has been shown on the contrary; that exosomes secreted by DCs infected with lymphocytic choriomeningitis virus (LCMV) do not bear LCMV antigens and are not involved in the induction of the immune response against this virus and no significant CTL cross-priming by dendritic cell-derived exosomes during murine lymphocytic choriomeningitis virus infection can be detected (O'Neill & Quah, 2008; Coppieters *et al.*, 2009; Thery *et al.*, 2009).

In addition to transfer of membrane component between cells; exosomes from mast cells were identified to contain nucleic acid (mRNA and microRNA) which can be translated after entering another cell, and was hypothesized to modulate of RNA content of the recipient cell (developed in a further section) (Valadi *et al.*, 2007).

Exosomes from reticulocytes contribute to membrane remodeling during the final stages of the erythroid differentiation (Blanc & Vidal, 2010), can serve to the clearance of specific proteins such as the transferring receptor (TfR), which disappear from the cell membrane and LAMP2 which disappear from the endo-lysosomal compartment. Knowing the amount of red blood cells (RBC) in the blood (about $2.5x10^{13}$ RBCs) in the body, and their lifespan in the peripheral circulation, it has been estimated that 10^{14} exosomes were released by reticulocytes in the bloodstream every day (Blanc *et al.*, 2005).We established that 10^5 exosomes was equivalent to 1 mg protein (Subra *et al.*, 2010). Therefore an efficient clearance system is necessary to avoid exosome accumulation in the blood stream. It has been shown that a calcium independent phospholipase A_2 ($iPLA_2$) was involved in the clearance of erythrocyte-derived exosomes (Blanc *et al.*, 2007).

Despite their clear implication in activating the immune system in response to nonself or tumor antigens, another role of exosomes has been hypothesized about their potent implication in the development of tolerance. This is the case for oral antigens that lead to tolerosomes (Karlsson *et al.*, 2001; van Niel *et al.*, 2001; Van Niel *et al.*, 2003; Thery *et al.*, 2009) and during pregnancy where placental exosomes lead to a suppression of T cell activation and contribute to the establishment of a tolerance state against the foetus (Keller *et al.*, 2006; Taylor *et al.*, 2006). Induction of regulatory T cells have been underlined in exosomes derived from breast milk (Foxp3, $CD4^+$, $CD25^+$ cells) (Admyre *et al.*, 2007b).Exosomes like particles found in thymus, has been suggested to induce formation of T lymphocytes with regulatory properties (T reg, CD4+ Foxp3+) via a TGFβ mechanism (Wang *et al.*, 2008).

2. Negative effects of Exosomes.

It is worth noting that Dendritic cells, monocytes, macrophages and lymphocytes can produce exosomes even if they are infected with pathogens like HIV. In this case, the exosome pathway displays not only positive effects since it can be hijacked by the virus. As described in the next section, HIV is able to divert the exosome pathway for its own budding.

Independently of HIV, others negatives functions are reported.

a) T cell exosomes

Few informations are available on T cells derived exosomes.

Pioneer studies reported that T cells could release exosomes following TCR/CD3 complex engagement. After activation, TCR/CD3 complexes with CD3ζ in its phosphorylated form were found associated with exosomes, suggesting a possible way to target to late endosomes and down regulate CD3 (Blanchard *et al.*, 2002). However, in return, this process allow to exosomes bearing TCR/CD3 complexes to exert potent at distance simulatory effects when they encounter the corresponding combination of MHC-petide.

FasL and APOE2/TRAIL death ligands found in T cell derived exosomes have been shown to suppress the immune response in vitro (Martinez & Krams, 1999; Monleon *et al.*, 2001), inducing Fas-dependant apoptosis. In the same time Monleon et al. indicated that activated T cells secrete exosome bearing FasL, which induces apoptosis of bystander T cells, participing in activation –induced cell death (AICD) (Monleon *et al.*, 2001).

Activated peripheral blood derived $CD4^+$T cells secrete exosomes which are internalized by monocyte/immature macrophages (PBMC and THP-1 cells) via phosphatidylserine receptor (PSR). Following their internalization, it has been demonstrated an enhancement of cholesterol accumulation, in esterified form or free, and production of TNF-α. Free cholesterol accumulation is a potent inducer of inflammatory cytokine production in macrophages. TNF-α is proinflammatory cytokine abundantly expressed in atherosclerotic lesions. Given that $CD4^+$T cells are known to infiltrate atherosclerotic lesions characterized by esterified cholesterol deposits in monocytes/macrophages , one can suppose that T cell exosomes may promote atherogenesis (Zakharova *et al.*, 2007).

b) Tumor cells derived exosomes

Despite their potent immune stimulatory properties because of tumor Ag harboring, many studies reveal that tumor cells derived exosomes mostly have detrimental effects on antitumor immune response, leading to suppressive pathways hampering immune defenses in patients (Wolfers *et al.*, 2001; Andre *et al.*, 2002). Consequently, tumor exosomes may contribute to more favorable conditions of in vivo tumor growth and participate in tumor evasion (Valenti *et al.*, 2007)

b.1. Inhibition of Effectors cells activation

While tumor exosomes (Tex) can prime the immune system in mice to delay the tumor development, Abusamra et al. studied Tex derived from a human prostate cancer cell line and observed that they could suppress T cell responses, both inhibiting T cell proliferation

and inducing apoptosis in a dose dependent manner (Abusamra *et al.*, 2005). Thus, the hypothesis of exosomes implication in tumor evasion emerged.
Moreover, pretreatment of mice with exosomes derived from murine mammary carcinomas (TS/A or 4T.1) before tumor implantation have led to an inhibition of NK cell proliferation and blockade of IL-2 mediated activation (Clayton *et al.*, 2007), and inhibition of cytotoxicity (down modulation of perforine expression), promoting an accelerated growth of tumors (Liu *et al.*, 2006).

Inhibitory effects of Tex may be related to some inhibitory molecules such as NKG2D, TGFβ1, FasL ligands and TRAIL, and humans leukocyte antigen (HLA)-G, harbored on the Tex.

Role of NKG2D

Exosomes from tumors cells harbor NKG2D ligand such as MICA*008 or ULBP3 proteins, which suppress Natural Killer cell toxicity (Ashiru *et al.*, 2010) NKG2D is an activating receptor with a C type lectin-like domain, expressed on all natural killer (NK) cells, TCR $\gamma\delta^+$ and $CD8^+$ $TCR\alpha\beta^+$ T cells. NKG2D can either costimulate the activation of naïve T cells or trigger cytotoxicity in the absence of T cell receptor (TCR) ligation. In humans, NKG2D binds to MHC class I related chain (MIC) A, MICB, and ULB16-binding proteins whose expression are restricted or absent on normal tissues but induced in situations of stress and disease. NKG2D is an important tumour-recognition molecule for Nk cells, $CD8^+$T cells, γδ-T cells, NK-T cells. Ligation of NKG2D with it ligand led to a down regulation of NKG2D expression. Thus, tumor exosomes bearing NKG2D ligands can acts on effector cells to down regulate NKG2D, impairing the immune response. Clayton et al. also brought evidences that Tex harbor NKG2D ligands (MICA/B, ULBP1-3 proteins) molecules leading to down regulation of NKG2D on PBL in vitro associated with an inhibition of T cell cytotoxicity (Clayton & Tabi, 2005; Clayton *et al.*, 2008).

Role of TGFβ1

In addition to NKG2D ligands, Clayton et al. have brought the evidenced that tumor exosomes carry and express growth factors such as TGFβ1. Membrane-bound transforming growth factor-β 1 (TGFβ1) on these vesicles displays the same function as NKG2D ligands i.e. the down regulation of surface NKG2D expression by NK cells and $CD8^+$T cells following a direct interaction between exosomes and cells. The mechanism involving TGFβ1 appears predominant (Clayton *et al.*, 2008). This prevents NK cells and T cell activation.
IL-2 promotes the survival, proliferation, and functional differentiation of several lymphocytres subset such as NK cells, $CD8^+$T cells, and $CD4^+CD25^+$ regulatory T cells. Clayton et al. revealed impairment in the $CD4^+$ lymphocyte proliferation and weakly in the NK cells, in response to IL-2, in the presence of tumor exosomes derived from mesothelioma cell lines, via membrane bound TGFβ1. It is worth to note that IL-10 and TGFβ1 are produced by many tumor types and may mediate antiproliferative and antocytotoxic effects. They also demonstrated that tumor exosomes combined with IL-2 enhanced the inhibitory and

suppressive functions of Treg cells while impairing cytotoxic NK cell functions (Clayton *et al.*, 2007).

Role of death Ligands

Exosomes secreted from tumor cell lines or tumor cells coming from patients can induce T cell apoptosis in vitro, through the expression of death ligands such as CD95 ligand (FASL) and TRAIL (Clayton & Tabi, 2005; Thery *et al.*, 2009).

FasL is an integral membrane protein, belonging to the TNF family. It is expressed in a variety of tissues and cells, in particular activated lymphocytes expressed FasL. Following interaction with its receptor named Fas (CD95/APO1), it induces apoptotic cell death. It was hypothesized that an activation of the T cell receptor by tumor exosomes promotes Fas upregulating in T lymphocytes. Consequently, the FasL molecule on the exosomes induces T cell apoptosis (Andreola *et al.*, 2002; Abusamra *et al.*, 2005). In that respect, Andreola et al. proved that microvesicles isolated from melanoma cells carried FasL capable of inducing Fas-mediated apoptosis of lymphoid cells (Andreola *et al.*, 2002). In the same way, Abusamra et al. bring evidences that exosomes purified from a human prostate cancer cell line could induce$CD8^+$T cells apoptosis via FasL (Abusamra *et al.*, 2005). A direct interaction between T cell and tumor exosomes may be required, or tumor exosomes binding Dendritic cells, using their adhesion/costimulatory molecules to form a stable interaction with the T cell and induce apoptosis (Ichim *et al.*, 2008).

Tumor necrosis (TNF)-related apoptosis-inducing ligand (TRAIL) is also a death ligand belonging too to the TNF family and promotes apoptosis via DR4 and DR5 receptors through a signaling pathway similar to that of FasL, in a wide variety of tumor cells but not in normal cells. Its presence and its implication in inducing lymphocytes apoptosis have been studied.
(Huber *et al.*, 2008). Some works have demonstrated that human melanoma as well as colon carcinoma cells (Huber *et al.*, 2005) constitutively routed FasL and TRAIL to exosomes while depleted from the plasma membrane. These vesicles are secreted and induce death by apoptosis in activated T cells. Indeed, they are thought to counterattack against activated antitumoral effector immune cells. (Martinez-Lorenzo *et al.*, 1999; Andreola *et al.*, 2002; Martinez-Lorenzo *et al.*, 2004). This observation was confirmed later in oral carcinoma (Kim *et al.*, 2005) (Figure 9).

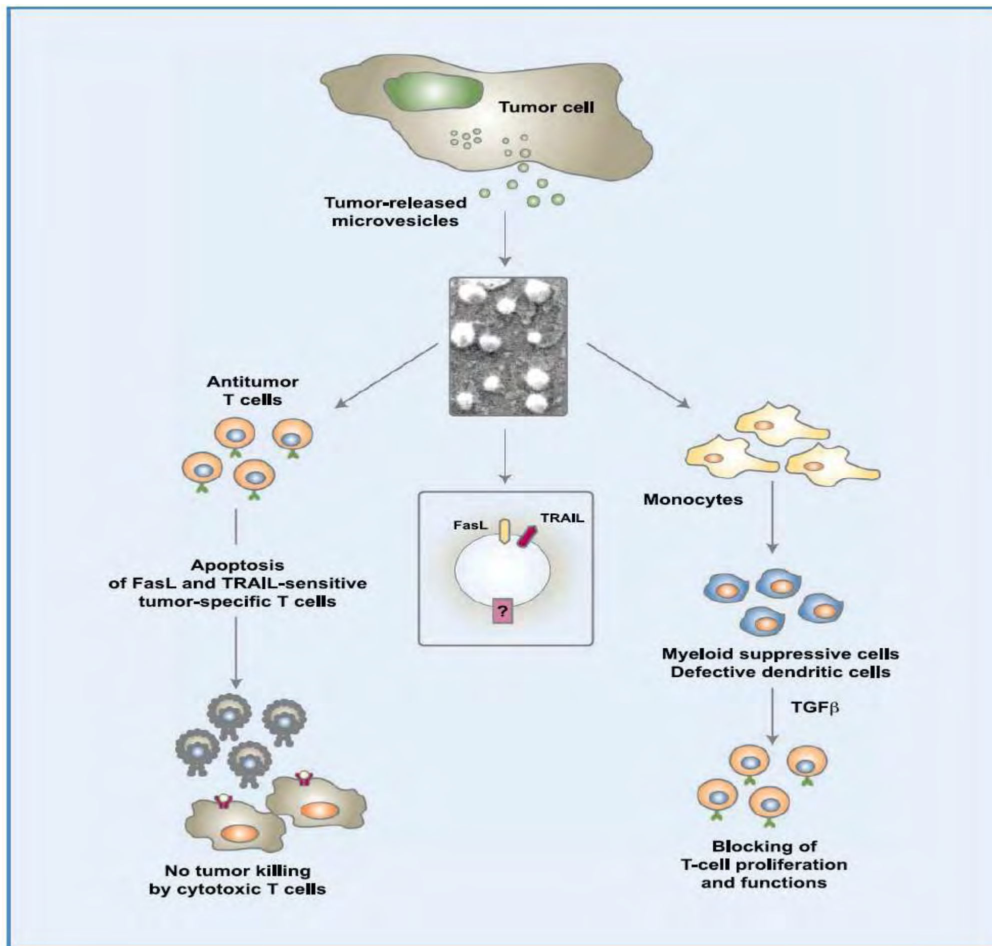


Figure 9 : Immunosuppressive effects of Tumor cells derived exosomes (Tex). Through FasL and TRAIL expression, these vesicles can induce apoptosis in activated antitumor T cells. They can alter monocyte differentiation into myeloid suppressive cells which exert inhibitory activity on T cell proliferation and functions through TGFβ secretion. Adapted from (Valenti *et al.*, 2007).

Down expression of CD3ζ

The group of Taylor and coll. demonstrated that tumor exosomes from ascites of ovarian cancer patients, in presence of T cell lines, resulted in the down regulation of CD3ζ and JAK3 which are components of the signaling pathway downstream TCR. This effect impaired the T cell response and led to T cells apoptosis. They unraveled the presence of FasL onto exosomes, responsible of apoptosis (Taylor & Gercel-Taylor, 2005). Kim et al. sustained the down regulation of CD3ζ of T lymphocytes, induced by FasL expression on oral carcinoma cells (Kim *et al.*, 2005).

Role of HLA-G

Another mechanism allowing tumor cells to escape from immune surveillance is the expression of the nonclassical human leucocyte antigen (HLA) class I molecule HLA-G which absent from others tissues except in trophoblasts and thymus. HLA-G display immunomodulatory properties such as inhibition of NK cell cytolysis and CTL responses.

The group of Riteau et al. highlighted the presence of HLA –G in exosomes isolated from patient bearing carcinoma cells, constitutively expressing high level of cell surface HLA-G. It has been hypothesized that HLA-G exposed by exosomes could deliver inhibitory signal via its receptors (CD85d, CD85j and CD158d) in immunocompetent cells and could be involved in the sustaining of a tolerogenic response against the tumor (Riteau *et al.*, 2003)

b.2. Induction of myeloid suppressive cells

Myeloid suppressive cells are found in large number in lymphoid organs, blood and tumor tissues in cancer patients and are immature myeloid cells, thought to promote tumor growth and metastasis, acting by inhibiting T cells functions in antitumor responses.

The first studies of Valenti et al. showed that melanoma and colorectal carcinoma-derived exosomes altered the monocyte differentiation into dendritic cells, leading to myeloid suppressive cells. These cells express myeloid markers (CD14 and CD11b) and are devoid of co-stimulatory molecules (HLA DR, CD80, CD86). They spontaneously secret TGFβ and have suppressive activity on activated T lymphocytes since they are able to inhibit T cells proliferation and cytolytic functions (Valenti *et al.*, 2006). Following this finding, the authors realized a comparison of peripheral blood of cancer patients with those of healthy donors, showing that cancer patients' blood contains cells harboring an immunosuppressive phenotype. Myeloid suppressive cells may contribute to suppressive pathways in cancer patients and contribute to tumor progression (Valenti *et al.*, 2007) (Figure 9).

Others studies also demonstrated the impairment of the differentiation of murine DCs from bone marrow precursors or from human monocytes were severely in the presence of murine mammary tumors or breast tumor exosomes respectively. (Yu *et al.*, 2007), Tumor exosomes were able to induce a blockade in the differentiation of myeloid precursor, leading to the accumulation of Myeloid-derived suppressor cells (MDSCs). Following these works, the authors confirmed their role in promoting tumor growth mediated in part by PGE2 and TGFβ carried by tumor exosomes, and induced cytokine productions such as IL-6, in favor of tumor growth (Xiang *et al.*, 2009). Contrary to this latter finding, Chalmin et al. have recently evidenced that membrane bound Hsp72 born by Tex isolated from murine EL4 thymona, TS/A mammary carcinoma and CT26 colon carcinoma, were responsible for the transcriptional factor STAT3 activation through the receptor TLR2 and its adaptator MyD88 and IL-6 production, in MSDCs, leading to their suppressive properties whereas no PGE2 was detected onto Tex (Chalmin *et al.*, 2010). This mechanism appears relevant in human. The role of MyD88 was confirmed by Liu Y in mediating the suppressive effects of MSDCs through IL-6 and TNFα secretion following cancer exosomes encounter (Liu *et al.*, 2010).

b.3. Angiogenic and Metastatic properties

Some works illustrate the angiogenic properties displayed by tumor exosomes. In a model of endothelial spheroid generated in matrigel with a an endothelial cell line capable of spontaneously aggregates in spheroids, Hood et al. demonstrated that melanoma (B16) exosomes could promote angiogenesis raising the communication network between cells, depending on the dose. In line with this observation, cytokine production analysis reported a panel of proangiogenic ones (Hood *et al.*, 2009). Given that hypoxic cells are thought to acquire a metastatic character, a quantitative proteomic of carcinoma exosomes under hypoxia have been performed and revealed the presence of proteins important in metastasis and angiogenic factors released in culture medium which 50% of the proteins found are associated with exosomes (Park *et al.*, 2010). Nazarenko et al. also reported a role of rat carcinoma derived exosomes in angiogenesis, mediated by the exosomal tetraspanin Tspan8 (Nazarenko *et al.*, 2010). Both colorectal cancer and glioblastoma derived exosomes were analyzed for their mRNA content (Skog *et al.*, 2008; Hong *et al.*, 2009). Transcripts related to cell cycle were revealed in colorectal exosomes that was concordant with their capacity to modulate endothelial cells cycle and proliferation, underlying their potent implication in angiogenesis (Hong *et al.*, 2009). Skog et al. revealed that mRNA found in glioblastoma could be translated in recipient cells such as endothelial cells and revealed the presence of proteins with angiogenic properties, stimulating tubule formation (Skog *et al.*, 2008).

As aforementioned, Liu et al evidenced the role of the cytoplasmic adaptator MyD88 downstream the Toll like receptor family, in mediating tumor exosomes effects on myeloid-derived suppressor cells. They could observe an augmentation of lung metastasis thanks to B16 melanoma derived exosomes treatment, while this effect was abolished on MyD88 KO mice (Liu *et al.*, 2010). Hao et al. assessed the metastatic potential of exosomes purified from a highly metastatic B16 melanoma cell line (BL6-10) bearing the metastasis marker Met72. They found Met72 expressed on exosomes which were able to confer this glycoprotein and metastatic properties to a poorly metastatic F1 tumor cells following exosome uptake (Hao *et al.*, 2006).

In many ways, tumor exosomes can reduce the function of T cells and contribute to the maintenance of an immune tolerance state in cancer patients.
Moreover, in the case of a HIV infection that leads to deficiency in the immune system reducing the level of effector cells, opportunistic diseases develop such as cancer since patients become more susceptible.

c) Exosomes and pathogens

Although exosomes may have a crucial role in tumor cells invasion and metastasis, they have also been implicated in the replication and propagation of transmissible pathogens such as prion, mycobacteria and HIV transmission.

Thus, recent data suggest that multivesicular endosomes are important cellular compartments in neurodegenerative disorders.

c.1. Prion

The prion is the infectious particle responsible for the transmissible neurodegenerative diseases such as Creutzfeldt-Jacob disease (CJD) of humans or bovine spongiform encephalopathy (BSE) of cattle. Prion diseases are linked to the accumulation in the central nervous system of the abnormally folded prion protein (PrP) scrapie (PrPsc), sought to be the infectious agent according to the protein hypothesis.
The normal cellular form of the prion protein (Prpc) is encoded by the PRNP gene and is expressed in all tissues of the human body .Although highest expression levels are observed in tissues of the central nervous system and brain, PrP is also present in some non-neuronal tissues and in cells from the immune system (Vella *et al.*, 2008). Its expression could be associated with a number of cell functions, including copper and/or zinc ion transport or metabolism, protection from oxidative stress, cellular signaling, membrane excitability and synaptic transmission, apoptosis and neurite outgrowth (Porto-Carreiro *et al.*, 2005).
PrPc and PrPsc are glycosylphosphatidylinositol-anchored proteins found at the plasma membrane associated with lipid rafts, microdomains of sphingolipids and cholesterol, and transit through the multivesicular bodies (Fevrier *et al.*, 2005; Vella *et al.*, 2008). PrPsc differs from its normal isoform by its high content in β sheet structure, its insolubility in mild detergents and its partial resistance to proteinase K treatment (Leblanc *et al.*, 2006). Visualization of PrPsc revealed late endosomes and lysosomes of infected mouse neuroblastoma cells N2a, as possible sites of accumulation suggesting that they could associate with the ILVs of MVB and consequently to exosomes (Porto-Carreiro *et al.*, 2005; Vella *et al.*, 2008; Veith *et al.*, 2009). Using this same cellular model of neuronal cells, Alais et al. previously found prion protein associated with exosomes-like structures (Alais *et al.*, 2008). Moreover, It has been evidence that in cultured human erythroblasts, membrane PrPc is rapidly internalized and routed in the endosomal pathway as attests colocalization with tetraspanin CD63. These works underlined the tetraspanins enriched domains implication in the trafficking of PrPc (Griffiths *et al.*, 2007).

The pioneer studies realized in 2004 by Fevrier and coll. demonstrated that PrPc and PrPsc were released into the extracellular environment of two distinct non neuronal cellular model in association with exosomes (Rov : RK13 cells overexpressing the ovine PrP and the Mov :

neuroglial cells derived from transgenic mice overexpressing ovine PrP) (Fevrier *et al.*, 2004). They found that both cell lines released PrPc and PrPsc associated with exosomes, before and after infection with sheep prions respectively. They brought evidence that exosomes bearing PrPsc were infectious. Indeed, exosomes–associated PrPsc inoculated mice died as a consequence of acute, typical neurological disorders. Exosomes–associated PrPsc released by the infected cells elicited conversion of endogenous PrPc to PrPsc when incubated with uninfected recipient cells. Exosomes could thus constitute vehicles for transmission of the infectious prion protein (Fevrier *et al.*, 2005). Since conversion of PrPc into PrPsc has been proposed to occur at a low pH, it is likely that infectious exosomes are internalized and fuse with endosomal limiting membranes, similar to the process of back fusion of the internal vesicles of MVBs.

Recent findings have shown that endogenous PrPc is associated with exosomes from epididymal fluid, platelets, and primary cultured cortical neurons suggesting that exosomes may contribute to spreading prions through the lymphoreticular system and potentially in the brain (Vella *et al.*, 2007). Vella and coll. demonstrate that in addition to a cell line that overexpresses mouse PrP, a mouse neuronal cells (GT1-7) endogenously expressing mouse PrP, released PrPc and PrPsc in association with exosomes when infected with prions. They provided a mechanism for the interneuronal dissemination of endogenous prions within the Central Nervous System. Their works also emphasized the role of exosomes from either non neuronal (MoRK13 : rabbit kidney epithelial cells transfected with a mouse PrP) and neuronal cells in the trafficking of infectious prions to different tissue types in vivo . They showed that PrP associated with exosomes is differently processed (N-terminally distinct) to that found in whole cell extracts, suggesting a novel processing pathway. They provided a possible mechanism for the movement of prions between distinct host tissues and around the brain. PrPsc once secreted in exosomes, may be endocytosed or interact with membrane bound PrPc on the recipient cell, initiating PrP conversion (Vella *et al.*, 2008). Following transfer of exosomal infection to the recipient cell, cell to cell contact would be the predominant means of transferring infectivity to surrounding cells in a particular tissue (Kanu *et al.*, 2002). In this study, a scrapie prion-infected cell (SMB cell : (scrapie mouse brain)) and derivatives cell lines are able to convert a neighboring uninfected cell by a process that is dependent upon cell contact whereas conditioned medium has no activity.

Since prion proteins and assembling retroviral particles colocalize to the same intracellular compartments, whether retroviruses could recruit prion proteins during assembly and budding was explored. Leblanc and coll. shown that retroviral infection by the moloney murine leukemia virus of a fibroblast cell line (NIH3T3) enhances the extracellular release of PrPc and PrPsc and prion infectivity by coinfected cells, mediated by viral particles and exosomes (Leblanc *et al.*, 2006). They demonstrated that both MoMulv virions and HIV-1 virions (on a lymphoblastoid cell line) recruited PrPc. In conclusion, it was proposed that retroviruses were cofactors to enhance exosome production and in the spreading of the

pathological prion agent. Moreover, retroviral gag is important in this mechanism, stimulating the exosome liberation.
Recent investigations have shown that a substantial amount of cell-associated PrPc in blood resides in platelets. Platelets activation leads to up-regulation of Prpc on the platelet surface and its release on exosomes and microparticles (Brouckova & Holada, 2009).

Altogether, these findings propose exosomes as a mean to carry and spread prion proteins.

Another protein involved in neurodegenerative disease and exploiting this common pathway previously unknown for proteins associated with neurodegenerative diseases, is the amyloid precursor protein (APP) which is associated with the Alzheimer's disease (AD)

c.2. Alzheimer APP protein

Alzheimer's disease (AD) is characterized by a continuous loss of neurons that are not replaces and the extracellular accumulation of insoluble amyloid fibrils as amyloid plaques in the brain. The main component of amyloid is polymerized β-amyloid peptide (Aβ), a 39-43 amino acid residue peptide produced by proteolytic cleavage thanks to the β- and γ-secretases, from the amyloid precursor protein (APP) (Rajendran *et al.*, 2006; Vella *et al.*, 2008). APP is produced in RE and passes through the Golgi to be finely transported to the plasma membrane via secretory vesicles, where it can undergo proteolytic cleavages. The molecule and its proteolytic products can undergo re-internalization and can be found almost other locations, in the endosomal/lysosomal system (Vella *et al.*, 2008).

Rajendra and coll. shown that β secretases, associated with lipid raft, operate in the early endosomes. The produced β–amyloid (Aβ) peptides are then routed to mulivesicular bodies in Hela and N2a cells. Subsequently, a minute fraction (<1%) of Aβ peptides can be secreted from the cells in exosomes. Although most of the extracellular Aβ is known to be soluble, the authors identified Aβ as a component of the exosomal membrane and found that other exosomal proteins such as Alix or flotilin-1 accumulated in the plaques of AD patients' brains. Exosomes vehiculating Aβ and APP-CTFs, may contribute to the amyloid deposition in the brain (Rajendran *et al.*, 2006).

β-amyloid peptides are thus intracellularly generated and were shown to be released to the extracellular space in association with exosomes. Roberta Ghidoni et al. proposed the exosomes as the Trojan horses of neurodegeneration, exosomes being the key player of neuronal degeneration. Neurodegeneration in AD would be triggered by proteins spread, cell by cell throughout brain areas mediated via exosomes ('infected shuttles') (Ghidoni *et al.*, 2008).

Vingtdeux et al. found in previous studies that the amyloid intracellular domain (AICD) which is released by γ secretase cleavage of APP C-terminal fragments (CTFs), was strongly increased upon treatment with alkalizing drugs, suggesting that the endosomal/lysosomal pathway regulates AICD degradation. AICD and CTFs were found in MVBs and secreted in exosomes in differentiated neuroblastoma (SY5Y stably overexpressing human APP) and rat primary neuronal culture cells. Under alkalizing drug treatments ($BafA_1$),. APP and derivatives accumulates in MVB underlying the role of exosomes as essential organelles for APP metabolism (Vingtdeux *et al.*, 2007).

Thus, APP proteolytic fragments were found in association with exosomes (Rajendran *et al.*, 2006; Vingtdeux *et al.*, 2007). Indeed, APP, APP-CTF and AICD – amyloid intracellular domain- have been reported integrated and secreted within exosomes from differentiated neuroblastoma and primary neuronal cortical cells (Vella *et al.*, 2008).

Exosomes from a stably transfected CHO-APP_{695} (wild type APP), contain both APP full length and APP fragments i.e. APP-CTFs as well as Aβ. Several members of the secretase family of proteases were also contained in exosomes : β secretase (BACE: β-site APP-cleaving enzyme-1); members of γ-secretase (a multimeric protein complex: presenilin (PS) 1,and PS2)), and ADAM10, a member of the α secretase. This suggests that exosomes could be a site of APP cleavage (Sharples *et al.*, 2008). Their data revealed that γ secretase inhibition allow an inhibition of Aβ production while an increase in sAPPα and α and β-CTFs increase in association with exosomes. Because full lenght-APP, Aβ, and other processing products of APP were found to be associated with exosomes, γ secretase inhibitors demonstrated a specific effect on the CTF associated with exosomes, they may provide a possible minor site for active processing of APP.

It is known that cystatin C have a neuroprotective role in alzeimer's disease. This protein is also targeted and secreted in exosomes. Ghidoni et al., have shown that reduced level of all cystatin C forms and APP metabolites were found in exosomes from mouse primary neuron over expressing a familial AD- associated presenilin 2 mutations (Ghidoni *et al.*, 2009).

Recently, Brouckova A and Holada K., bring evidences proving that PrPc found at the platelet surface and resulting on its release on exosomes following activation, are associated with lipid rafts in specific membrane domain and the platelet cytoskeleton, and that PrPc are found in intracellular alpha granules (Brouckova & Holada, 2009).

Thus, all these works indicate that proteins associated with neurodegenerative disorders can be selectively incorporated into intraluminal vesicles of MVB and subsequently released within exosomes (Figure 10).

c.3. PAMP : pathogen associated molecular patterns

Proinflammatory role of exosomes bearing pathogen–associated molecular patterns (PAMPs) of mycobacterium, salmonella and T. gondii have been emphasized.

Mycobacterium avium is a major opportunistic pathogen in HIV-positive individuals and is responsible for increased morbidity and mortality in AIDS patients which can be found both in the water and in soil in the environment. M avium is an intracellular pathogen that resides in the phagosome of host macrophages. Upon phagocytosis, it inhibits phagosome maturation limiting their exposure to hydrolytic enzymes. M. avium express glycopeptidolipids (GLPs) as a major cell wall constituent. GLPs consist of a tripeptide amino alcohol core modified with an amide-linked fatty acid, a methyl rhamnose and a deoxytalose. Previous studies postulated that GLPs could promote mycobacteria survival by interfering with membrane-mediated functions of the host cells, and that GLPs accumulate inside infected cells.

M avium-infected macrophages (J774) release exosomes containing GLPs. Thus, they permit the transfer of GLPs from infected to uninfected macrophages, and exosomes from infected macrophages can stimulate a proinflammatory response (TNF-α and RANTES secretion) in resting macrophages (BMM) (Bhatnagar & Schorey, 2007). Mycobacterial components such as GPL, PIM2 and other glycolipids, also are able to induce cytokines production. Studies have shown that mycobacterial lipids, such as Phosphoinisitolmannoside (PIM) and LAM which can enter compartments in the endocytic pathway are present on exosomes (Beatty *et al.*, 2000). GLPs containing exosomes interact with uninfected macrophages leading to retention of GLPs in these 'bystander' cells, and inducing a proinflammatory response in exosomes treated macrophages. Thus, exosomes carrying bacterial components can induce a proinflammatory response : stimulator molecules present on intracellular pathogens can be released from infected cells to promote an immune response.

Bhatnagar et al. also studied macrophages infected with mycobacterium tuberculosis, M. bovis BCG, salmonella typhimurium and T. gondii. Exosomes released from contained PAMPs and were able to stimulate cytokines production and proinflammatory response. (Bhatnagar *et al.*, 2007). This depends on Toll-like receptors TLR2, TLR4, and the TLR associated adaptor molecule MyD88, suggesting that these exosomes may contain TLR ligands (Bhatnagar & Schorey, 2007; Bhatnagar *et al.*, 2007). Thus, mycobacteria-infected macrophages can induce a proinflammatory response hypothesizing that exosomes play an important role in immune surveillance during intracellular bacterial infections.

Another group confirmed that infected macrophages release exosomes that incorporate bacterial molecules PAMPs which acts as TLR ligands and lead to uninfected macrophages activation (production of TNFα and RANTES, iNOS) and release of proinflammatory factors following TLRs binding (O'Neill & Quah, 2008) (Figure 10).

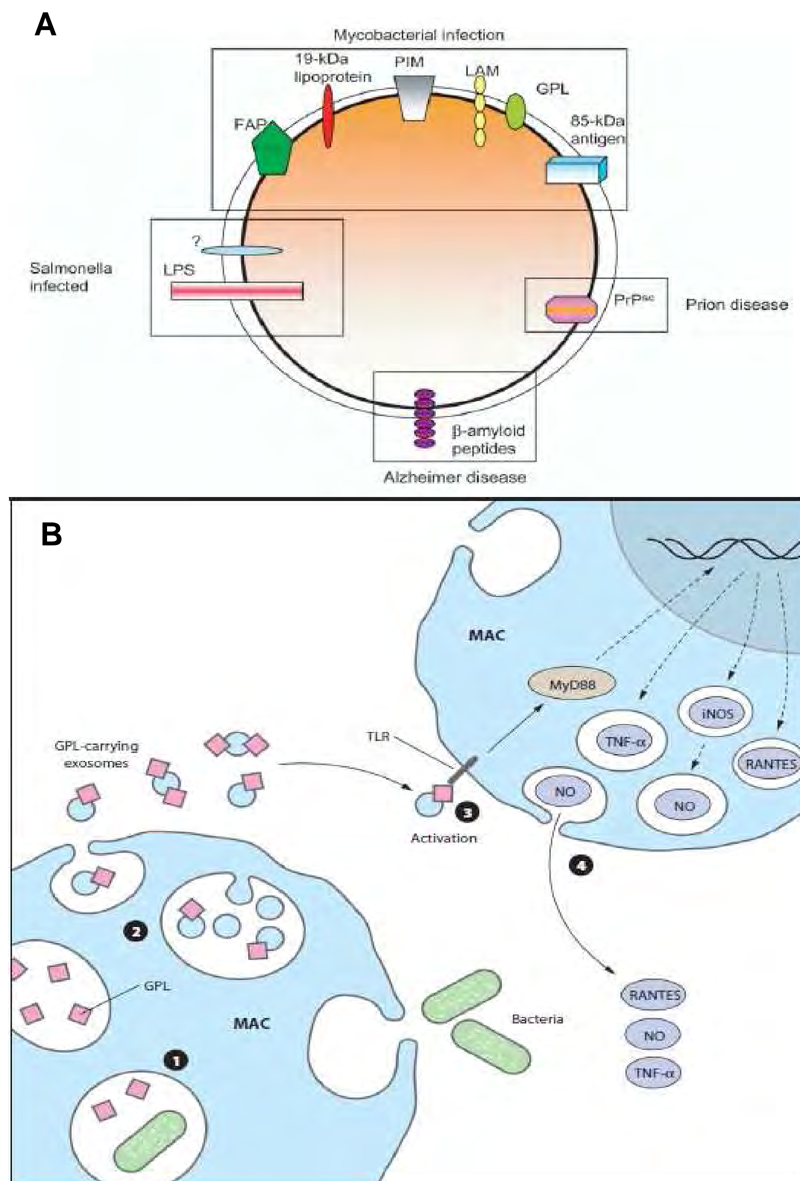


Figure 10 : Proinflammatory exosomes

(A) Exosomes from infected macrophages with microbial or infectious agents. Pathogen components found on exosomes. Adapted from (Schorey & Bhatnagar, 2008)

(B) Production of proinflammatory exosomes by bacteria infected macrophages. Bacteria taken up by macrophages (MAC) are found in endosomes. They carry glycopeptidolipids (GLPs) that associate with endosome membranes (1). Intraluminal vesicles formed by invagination of the limiting membrane within these endosomes would then carry the bacterial GLPs on their surface (2). Following MVBs fusion with the plasma membrane, GLPs-carrying exosomes are released extracellularly. They bind to PAMP recognition receptors (PRR, for example, TLRs) on nearby non infected macrophages, leading to MyD88-dependent macrophage activation (3). To the production of proinflammatory mediators, which include TNF-α, RANTES, and iNOS which results in production of NO (4). iNOS: inducible nitric oxide synthase Adapted from (O'Neill & Quah, 2008)

A further study reported that mycobacterial antigens were carried by exosomes released from M. bovis BCG infected macrophages and that they could stimulate a T cell response isolated from BCG-infected mice although APC presence seemed required, and trigger DC activation and maturation in vitro (Giri & Schorey, 2008).

3. **Exosomes are carrier of mRNA and miRNA.**

The first evidences demonstrating that exosomes could contain mRNA and microRNA were brought by Valadi and coll. in 2007 (Valadi *et al.*, 2007). Actually, 1300 different mRNA were identified in a mouse mast cell line MC/9, as many as in BMMC and human mast cell HMC-1 that was estimated to represent approximatively 8% of the mRNA detected in the donor cells. No less than 270 gene transcripts were exclusively found present in exosomes whereas they were indetectable in the donor cell, and they were related to cellular development, protein synthesis, RNA post-translational modification. Exosomes were also enriched in about 121 microRNA (miRNA, 19-22 nucleotides). Their function are stem cell differentiation control (let-7), differentiation and organogenesis (miR-1), hematopoiesis (miR-181) and exocytosis and tumorigenesis (miR-17, -18, -19, -20, -19b and -92-1).
An important finding was that these mRNA and microRNA were functional since they could be in vitro translated into mouse protein (from mast cells exosomes MC/9) in human mast cells HMC-1. Moerover, the major part is not present in the cytoplasm of the donor cells and can be delivered to another cell. Thus, exosomes appear as vehicle for RNA probably intervening in both in the microenvironment and in the systemic circulation, able to deliver their content and modulate recipient-cell protein production. The RNA that can be shuttled between cells via exosomes is suggested to be called 'exosomal shuttle' RNA (esRNA).

Later on, some others researchers have performed analyses to strengthen the hypothesis that exosomes are carriers of functional mRNA/microRNA and could be a mean to deliver genetic information and repress mRNA targets in recipient cells.
In 2008, Luo et al. reported that placental specific miRNAs, released via exosomes, can be exported from the placental syncytiotrophoblast into maternal circulation where they can be found abundantly albeit rapidly cleared after delivery (Luo *et al.*, 2009). Skog J et al. demonstrated that glioblastoma tumour cells release exosomes containing mRNA and microRNA, translated into brain microvascular endothelial recipient cells, after internalization. Exosomes also contain ESCRT-II subunits able to specifically bind mRNA (Skog *et al.*, 2008). Michael A et al. also discovered the presence of microRNAs within exosomes isolated from fresh and frozen glandular and whole human saliva (Michael *et al.*, 2010) Recently, exosomes from human saliva have been characterized for their mRNA content and 509 mRNA were found. In vitro, exosomes were able to transfer their genetic information to human oral keratinocytes modifying gene expression (Palanisamy *et al.*, 2010). In EBV-transformed lymphoblastoid B cells (LCL), the presence of miRNA has been evidenced in purified exosomes among which BHRF1 and BART EBV-miRNAs that are abundantly represented in EBV-associated tumors (Pegtel *et al.*, 2010). LCL-exosomes were capable of transfer EBV-miRNA in recipient monocyte-derived dendric cell and the EBV-miRNA BHRF1 or EBV-miRNA BART, permitted the repression of their specific target mRNAs : CXCL11 or LMP1 respectively. An interesting finding was the discovery of EBV-miRNA in non B cell populations of PBMC recovered from asymptomatic HIV patients, whereas, EBV-DNA was

exclusively restricted to B cell population, suggesting miRNA transfer between these cells in vivo.
Either, mitochondrial DNA have been evidenced carried by exosomes released from astrocytes and glioblastoma cells (Guescini *et al.*, 2010)
All these studies agree with the fact that specific microRNA content present in exosomes could represent a basis on which one can be develop future biomarkers of the diagnosis and prognosis of diverse pathology

Importantly, given that exosomes seem to be able to transfer nucleic acid into recipient cells, it has been recently suggested by Simon M and Raposo G (Simons & Raposo, 2009), that these vesicles display virus-like properties. This would implicate an exosome fusion with MVB membrane following their endocytosis. We will discuss this point in the second part of the bibliographic section.

The observation that exosomes carry mRNA made them a type of vesicles very similar to retroviruses.

	Exosomes		HIV	
Size	50-100nm		+/-100nm	
Density	1.14-1.21 (a)		1.15-1.18 (b)	
Total Proteins	282 (c)		266 (d,e)	
Common Proteins	37 (e)		37 (e)	
	Tetraspanins **HSPs**	Tsg101 Alix CD81,CD9 Hsc70,Hsp90 Integrines	Tsg101 Alix CD81,CD9 Hsc70,Hsp90 Integrines	**Tetraspanins** **HSPs**
Protein/Lipid Ratio				
Exos/cells or HIV/cells	4 ; 5.9 (c)		3.6 (f)	
Lipid ratios (c)				
Exos/cells or HIV/cells				
SM/SM	2.5		3.2	
Chol/PL	3		2.1	
Di-saturated PC/Di-saturated PC	2.8		3.5	
RNA Content	1300 mRNA 121 miRNA	8%of cell mRNA (g)	Cell mRNA =50% virions mRNA (h) Ribosomal RNA tRNA U6 spliceosome 7SL RNA HIV specific RNA dimer (9.8 Kb)	

Table 1 : Comparison between Exosomes and Human Immunodeficiency virus (HIV).
Data obtained from : (a) (Thery *et al.*, 2009); (b) (Wang *et al.*, 1999); (c) (Subra *et al.*, 2007); (d) (Trubey *et al.*, 2003); (e) (Chertova *et al.*, 2006); (f)(Aloia *et al.*, 1988); (g) (Valadi *et al.*, 2007); (h)(Houzet *et al.*, 2007).

II. HIV

A. The Human Immunodeficiency Virus (HIV)

Acquired immune deficiency syndrome (AIDS) is caused by a chronic infection with the HIV. Nowadays, 1.2 millions of Americans are currently living with HIV/AIDS although more than 500 000 people have died until 1981, date of the official starts of the epidemic in United States (Klimas *et al.*, 2008). In 2008 worldwide, the number of adults and children living with HIV was estimated at 33.4 million, including 2.1 million of children under 15 years (UNAIDS). HIV is among the leading causes of death worldwide and it causes more death than any other infectious diseases. Sub-saharan Africa comprises over two thirds (22.4 million) of the people living with HIV/AIDS worldwide and 76% of the AIDS death whereas Western and Central Europe regroup 850 000 persons estimated to be living with HIV in 2008 (UNAIDS).

1. AIDS definition

The first symptoms of infection during the first weeks corresponding to the acute HIV-1 infection syndrome are assimilated to a flu with rash (Levy, 2006). This initial phase of HIV infection is followed by a gradual deterioration of the immune system (Figure 11). A healthy person has 800 to 1200 $CD4^{+}$T cells per mm^3 of blood. Once the $CD4^{+}$ count falls <500 mm3, minor infections may occurs as cold sores (herpes simplex), warts (condyloma) and fungical infections and candidiases. The patient becomes vulnerable to the serious opportunistic infections and cancer that typify AIDS when the $CD4^{+}$ count falls under 200 cells/mm^3, the end stage of HIV disease. Thus, AIDS is defined as a $CD4^{+}$ count <200 cells/mm^3 or the presence of a serious infection, such as Pneumocystis carinii pneumonia, toxoplasmosis, cytomegalovirus infections of the eye or intestine, weight loss, diarrhea, HIV dementia and cancers, Kaposi's carcoma and lymphoma (Klimas *et al.*, 2008).

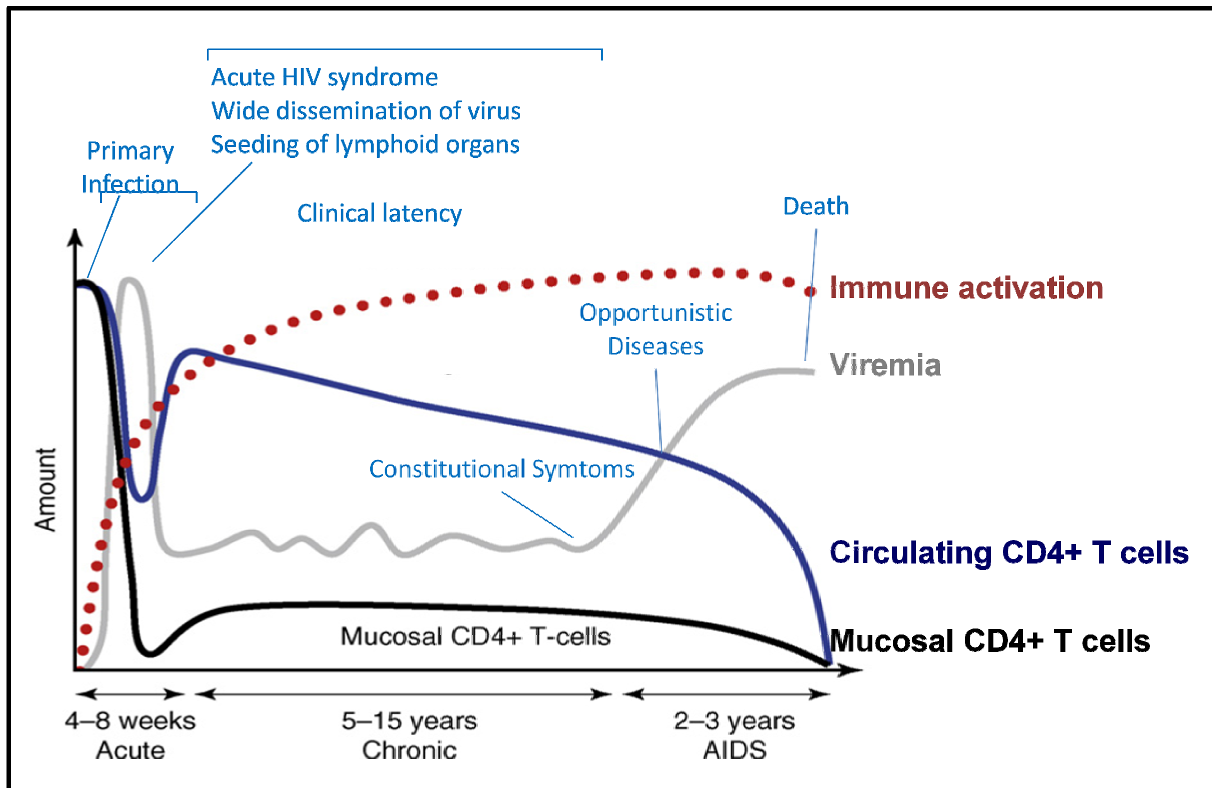


Figure 11 : Typical course of HIV infection. During the period following primary infection, HIV disseminates widely in the body; an abrupt decrease in CD4+ T cells in the peripheral circulation is often seen. An immune response to HIV ensures, with a decrease in detectable viremia. A period of clinical latency follows, during which CD4+ T cells counts continue to decrease, until they fall to a critical level below which there is a substantial risk of opportunistic infections. Adapted from (Forsman & Weiss, 2008).

2. HIV subtypes

HIV is a retrovirus which belongs to the subgroup of the lentiviruses or 'slow' viruses. The course of infection with these viruses is characterized by a long interval between initial infection and the onset of serious symptoms (Alcami, 2008).

Two genetically distinct viral types have been identified (Butler *et al.*, 2007; Klimas *et al.*, 2008). HIV-1 is the virus type associated with disease in United States, Europe, central Africa, and most others part of the world. HIV-2 has been found mainly in infected individuals in western Africa. HIV-1 and HIV-2 have the same tropism for cells of the immune system and cause illness that results from immune deficiency.

Genetic variation for HIV-1 is especially high. Three major groups of variants of HIV-1 exists : group M (main), group O (outlier), and group N (non-M/non-O). Group M is responsible for the majority of infections in the world and comprise 10 subtypes (A to K). Moreover, sub-subtypes or circulating recombinant forms have been identified, which among them, more than 20 are contained in the group M. HIV-1 subtypes C and A account for the majority of HIV cases while HIV-1 subtype B is predominant in North America, Western Europe and Australia.

3. HIV : the virus

Virions are vesicles about 100 nm of diameter and are composed by a lipidoproteic envelope and a central nucleocapsid called the core. The genetic material and enzymes required for the viral cycle which are not present in the cell such as the reverse transcriptase, integrase and protease (Figure 12) are contained insight the particles. Its genome is constituted by a simple strand RNA, like all retroviruses, about 9800 pair of bases, and it is formed by 3 genes of structure and 6 genes regulators.

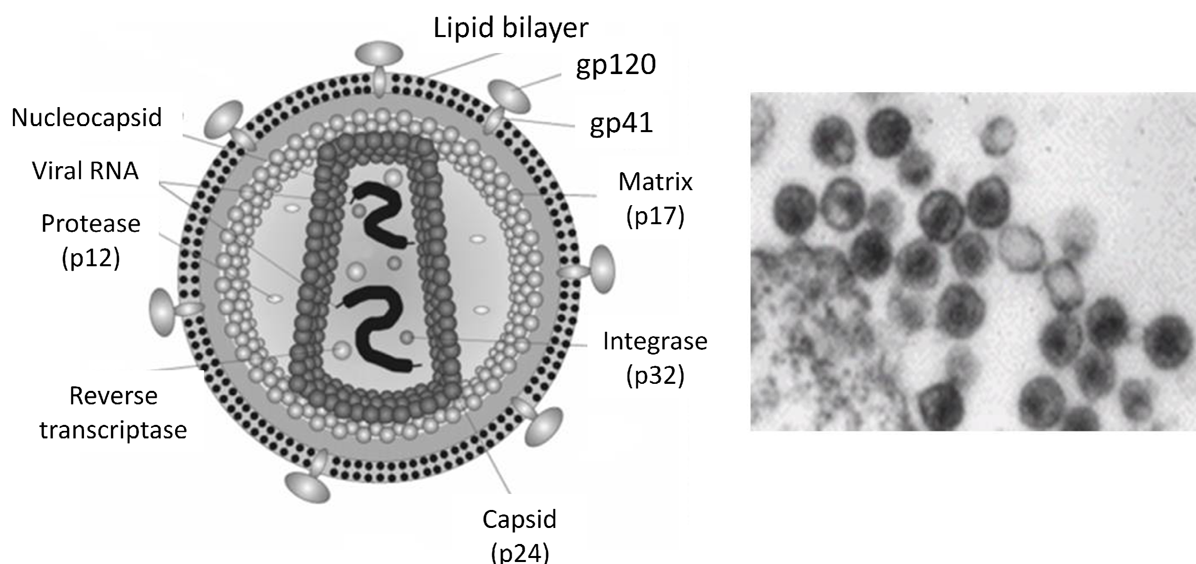


Figure 12 HIV particle. (Left) : HIV particule scheme. (Right) Electron micrograph by Edward C. Klatt, M.D., Department of Pathology, University of Utah. From AIDS Pathology.

a) Lipid content

HIV- morphogenesis is driven by the protein gag which has been shown to partially localize to detergent-resistant membranes in infected cells (see below). HIV-1 contains putative raft proteins, suggesting that HIV buds in lipid raft domains.

Consequently, initial works suggested that cholesterol and glycosphingolipids were present at a higher level in HIV-1 particles compared to plasma membrane of the parent cells (Aloia *et al.*, 1988; Aloia *et al.*, 1993; Nguyen & Hildreth, 2000; Mobius *et al.*, 2002; Chazal & Gerlier, 2003; Gould *et al.*, 2003; Brugger *et al.*, 2006; Trajkovic *et al.*, 2008; Izquierdo-Useros *et al.*)

Brugger et al. realized an extensive HIV lipid constituent analyses and confirmed that HIV lipid composition is similar to rafts but with unusual composition. They demonstrated for instance, a substantial enrichment of sphingomyelin and dihydrosphingomyelin, and a loss of viral infectivity upon inhibition of sphingolipid biosynthesis in host cells (Brugger *et al.*,

2006). It has been underlined an enrichment in plasmalogen PE, PS and cholesterol in the virus, likewise glucosylceramide whereas ceramide is reduced.

Furthermore, cholesterol appears to be essential for virus release and infectivity (Campbell *et al.*, 2002).
Moreover, ceramides are known to trigger exosomes genesis into the MVBs (Trajkovic *et al.*, 2008).
Like exosomes, phosphatidylserine are reported to be elevated in HIV envelope and may enhance infection of macrophages by binding to a PS receptor on monocytic cells (Callahan *et al.*, 2003; Waheed & Freed, 2009)

b) Protein content

Retroviruses as exosomes incorporate common molecules of the host cells during assembly and budding. Incorporation of MHC II by both exosomes and HIV has been described (Raposo *et al.*, 2002; Vincent-Schneider *et al.*, 2002), along with several cell surface molecules as integrins, costimulatory molecules (CD28, CD54), CD55 and CD59 neutralizing complement molecules (Thery *et al.*, 1999; Nguyen *et al.*, 2003; Cantin *et al.*, 2005). They display close buoyant density : 1.13-1.21 g/l (Thery *et al.*, 2001) for exosomes and 1.16-1.18 g/l for HIV particles (Wang *et al.*, 1999). Membranes bound proteins like tetraspanins (CD63, CD81), GPI proteins, Lamps, integrins, MHC proteins, and cytosolic proteins as actin, tsg101, heat shock proteins have been reported (Gould *et al.*, 2003; Nguyen *et al.*, 2003). HIV and exosomes display physical and biochemical properties that led authors to suggest that HIV is a viral exosome.

Despite common proteins, some allow to distinguish exosomes from HIV particules. Indeed, Cantin et al. suggested the use of acetylcholinesterase as exosome marker to purify exosomes from HIV particules (Cantin *et al.*, 2008).
Another exemple is CD45 which is abundantly expressed on the surface of hematopoietic cells and their vesicles. It has been shown that several exosome preparations derived from mostly hematopoïetic cells among them macrophages, contain the marker CD45 on the contrary to HIV particles (Nguyen *et al.*, 2003). Thus, CD45 protein is excluded from virions. This feature have led scientist to develop a CD45 immunoaffinity depletion method to purify HIV preparation and remove vesicles by a CD45 depletion and eliminate cellular protein contaminant. (Chertova *et al.*, 2006; Coren *et al.*, 2008).
CD45 is known to be associated with rafts. Another raft marker which seems to be excluded from the viral envelope from HIV prodeuced by macrophages is CD14 (Benaroch *et al.*, 2010).

Chertova et al. analyzed purified HIV from monocyte-derived macrophages using a CD45 immunaffinity depletion and found that HIV particles contained 26 of 37 proteins previously

described in exosomes derived from Dendritic cells. Many of the proteins identified are related to the MVBs compartment (Chertova *et al.*, 2006). They identified CD63, CD9, CD81, CD82, annexin 2 (known to promote fusion events in the endosomal/exosomal pathway,) Tsg101, Alix, integrins and many proteins related to the actin cytoskeleton, microtubules, ubiquitin/proteasomes, translation, protein chaperones, signal transduction, proteins belonging to endosomes/exosomes, metabolism and surface proteins associated to cell signaling, adhesion and antigen presentation. Annexin 2 was identified and it is known that annexin 2 binds Gag whereas small interefing RNA directed against annexin 2A promote its depletion and prevents viral budding from cells (Ryzhova *et al.*, 2006). Interestingly, NPC1 protein that transports cholesterol through the late endosomal system was observed.

Among the viral proteins incorpored both by exosomes and virions, APOBEC3G has been shown to be encapsulated in these particules almost in Vif-deficient strains. This protein displays a cytidine deaminase activity and constitutes a part of cellular defenses against HIV-1, restricting virus infection, and being vehiculated by exosomes. However, in wild type HIV, the viral protein Vif targets APOBEC3G for proteasome degradation. Few cells produce APOBEC3G and consequently few exosomes packaging APOBEC3G in vivo, and the major ones are activated T lymphocytes, those precisely targeted by HIV which reduce APOBEC3G rates driving it to the degradative pathway (Khatua *et al.*, 2009)

4. HIV Cycle

HIV infects $CD4^+$ T lymphocytes and a variety of other cells including monocytes, macrophages and thymocytes. The virus enter target cell via cell surface molecules : CD4 and chemokines coreceptor (CXCR4 and CCR5).

a) Attachment to the host cell

Glycoprotein-dependant

The first step in the virus cycle is its attachment to the host cell.

The major pathway allowing entry of the virus into the cell is mediated by interactions with two types of receptors : the molecule CD4 and the chemokin receptors as co-receptors including CXCR4 and CCR5 which are the most importants. CCR5 link naturally, among others molecules, RANTES, MIP-1a and MIP1-b and it is the principal receptor for the HIV subtype R5. The receptor CXCR4 has SDF1/CXCL12 for natural ligand and it is the principal receptor for the X4 subtype. The HIV tropism is defined by the aminoacid sequence and conformation of distinct regions of gp120.

This attachment of a viral particle to the CD4 receptor and co-receptor (CCR5 or CXCR4) leads to virion and cell membrane fusion. The binding requires the viral envelop glycoproteins gp120 and gp41. The gp120 links to CD4 on the target cell surface and induces conformational changes in the viral glycoproteins that induce its interaction with coreceptor. In turn gp120 conformational changes are induced together within gp41 leading to the fusion of viral and host cell membrane (Waheed & Freed, 2009) (Figure 13).

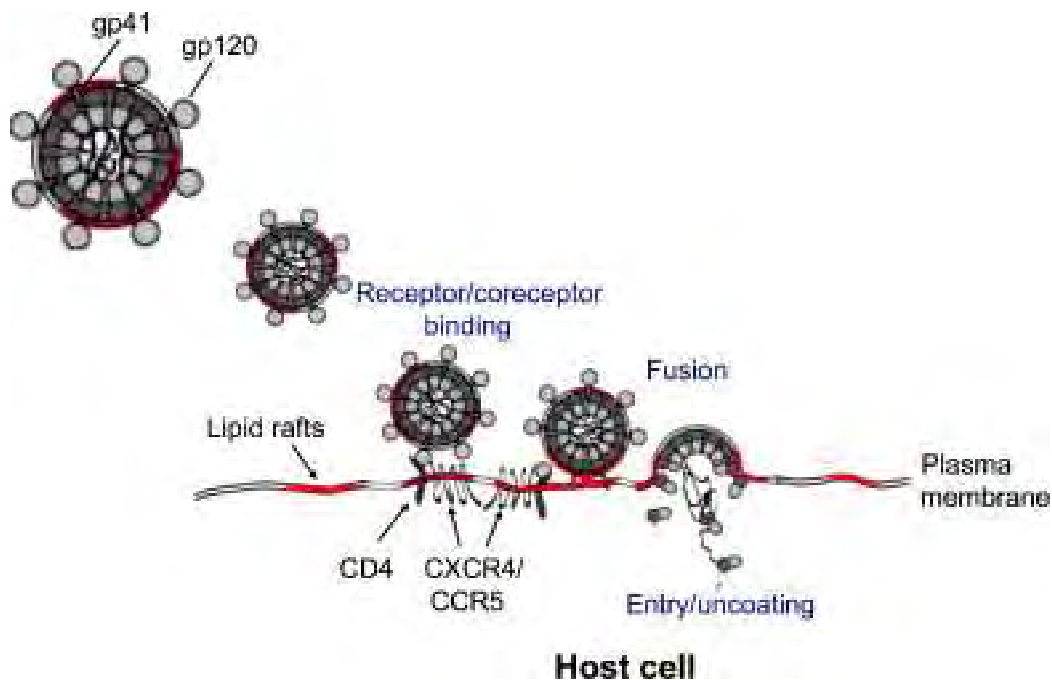


Figure 13. Classical HIV-1 entry. gp120 on HIV-1 virion binds to CD4 and coreceptor (CCR5 or CXCR4) in lipid rafts (red) of the plasma membrane. The transmembrane Env glycoprotein gp41 allows viral membrane fusion with plasma membrane leading to viral entry and uncoating**. Adapted from (Waheed & Freed, 2009)**

Sequence localized into the gp41 bind specifically cholesterol, while mutations in this domain prevent HIV infection (Salzwedel *et al.*, 1999; Vincent *et al.*, 2002; Waheed & Freed, 2009). Sphingomyelin along with cholesterol may promote surface aggregation of the gp41 in an ordered manner, enhancing fusion mediated by gp41.

Glycoprotein independent

Env-deleted HIV viruses conserve their ability to infect cells (Gould *et al.*, 2003). Moreover, HIV is able to infect many cell types including cells that lack viral receptors such as fibroblasts, hepatocytes and epithelial cells. This suggests that HIV can be taken up through another pathway preexisting for exosomal exchange (Nguyen *et al.*, 2003).

Non specific interactions between the viral envelop and molecules of the membranes of the cell can lead to the anchorage of virions at the cell surface. Such interactions such as with glucosaminoglucan structures are of low affinity. Interactions with lectines : DC-SIGN or

L-SIGN expressed by dendritic cells (Gringhuis *et al.*, 2010), and the virus , as others viruses such as hepatitis C, or mycobacteria, occur with a high affinity via carbohydrate moieties. Lectins allow the phagocytosis of linked-pathogens and the interaction with ICAM-3 onto lymphocyte membrane. These interactions permit and augment the infection of lymphocytes by HIV, while particles concentrate into a 'virological synapse', activating lymphocytes, spreading the virus from infected dendritic cells to lymphocytes $CD4^+$. Tightly bindings of HIV mediated by TLR-8 has been implicated too (Gringhuis *et al.*, 2010).

Some works suggest that additional unknown binding molecules could be implicated (Izquierdo-Useros *et al.*, 2007).
Izquierdo-useros et al. have reported that the use of de novo synthesis of sphingolipids inhibitors, impair glycosphingolipids formation (Izquierdo-Useros *et al.*, 2009). HIV released from treated cells was less taken up by DCs whereas release was unchanged. Sphingolipids on HIV and exosomes might be implicated in the initial particle attachment to the surface of maturs DCs. Moreover, Brugger et al. Reported that ceramides inhibitors led to a reduce infectivity and HIV release after treatment of the virus producer cells (Brugger *et al.*, 2006)
Host surface proteins integrated in the HIV envelope such as ICAM-1 which binds LFA-1 and HLA-DR, may participate in viruses binding to cell host. It has been described that ICAM-1 incorporated in HIV envelope could enhance the level of HIV infectivity (Fortin *et al.*, 1997; Cantin *et al.*, 2005)

Gummuluru et al. shown that HIV binding to host cell is independent on DC-SIGN or mannose binding C type lectin receptor and rather is mediated by a cholesterol dependent pathway. They observed that a β-cyclodextrine treatment of DCs aiming to sequester the cholesterol, abolished DC viral capture by affecting lipid raft endocytic pathway (Gummuluru *et al.*, 2003).

Most of these proteins are shared by exosomes for the binding to recipient cells. Moreover, these latter are able to compete which HIV uptake by Dendritic cells as above mentioned.

b) Inside the host cell

The viral particle release its content once inside the cell : internalization of the viral nucleocapsid occurs, followed by the decapsidation process. The genetic information of retroviruses is contained in ribonucleic acid (RNA) molecules. Once in the cytoplasm of the host cell, the enzyme reverse transcriptase Pol converts RNA into deoxyribonucleic acid (DNA) which can be incorporated into human cell's DNA thanks to the HIV integrases. This integrated form of the virus is called "provirus". In this form, the viral genome is flanked by 2 repeated sequences (long terminal repeat or LTR) which allow its integration into the cellular genome and permit the regulation of the initiation of the transcription. (Figure 14) The viral protein Vpr and the protein of the matrix p17 play a role in the transport process. Nef seems to raise the retrotranscription efficacy. HIV replication will be achieved using the cell

machinery, once the cell is activated. If the CD4+ cell is not activated, it is possible for the virus to persist in a latent stage for many years. This ability of the virus to remain in latently infected cells has greatly complicated attempts to eradicate HIV. This is the reason why infected patients must stay on antiviral therapy indefinitely. Cellular factors are implicated to pass from a silent form to an activated form of replication. Between others, NFκB represents the major element regulator for the transcription, acting in enhancer sequences and promoter, localized in the LTR regions.

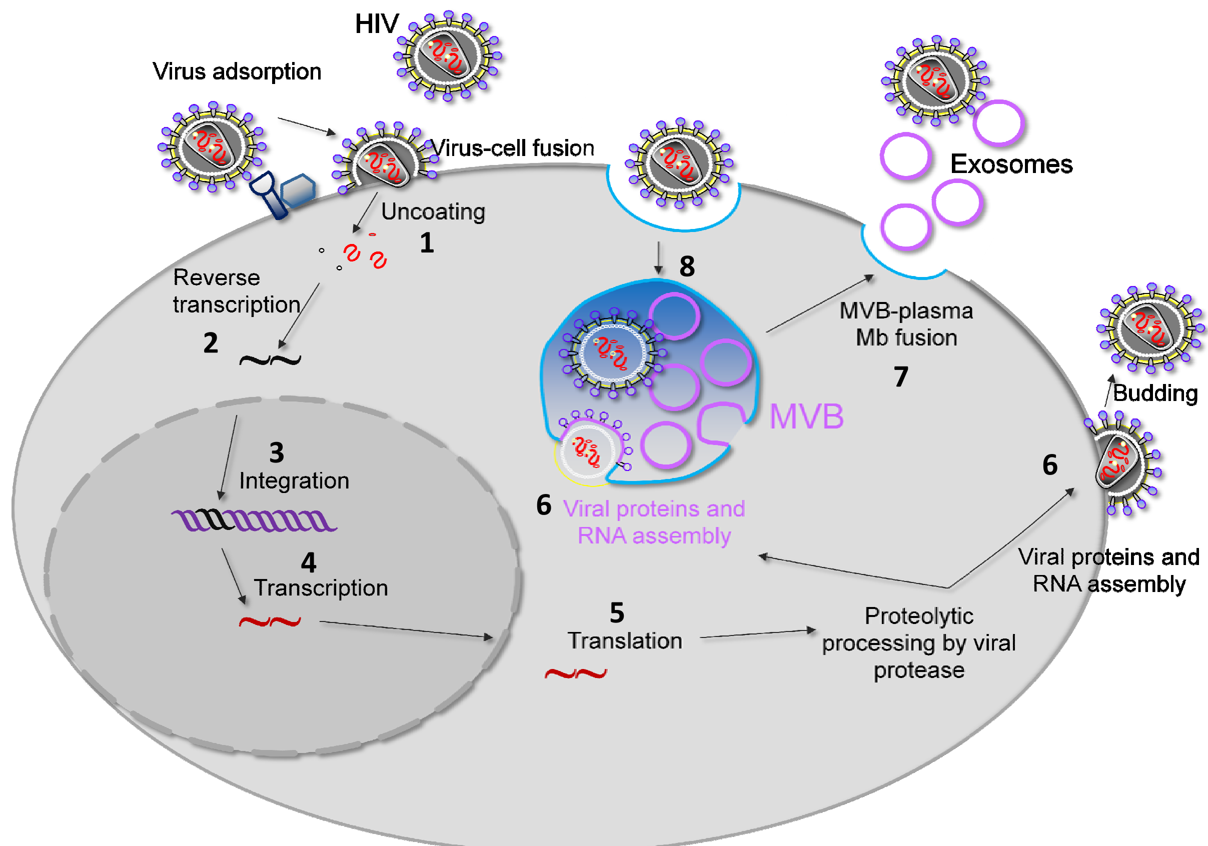


Figure 14 : HIV cycle. Following HIV fusion with plasma membrane, entry, and uncaoting (1), the viral RNA (red) is converted into viral DNA (black) by the enzyme reverse transcriptase (grey sphere)(2). The viral genome is delivered to the nucleus and integrated with the host cell DNA (3). The viral DNA then directs the production of viral RNA (4). Some viral RNA goes to form a new viral genome while other viral RNA directs the production of new viral proteins via ribosomes (5). The new viral components (genome + proteins) assemble at the cell surface or at the level of multivesicular bodies (6) and a new virus particles bud from the cell or into the MVB which fuse with plasma membrane oh the cell to release its intraluminal vesicles and virions (7). A potent endocytic pathway has been described for HIV entry (8).

Viral proteins are made by the transcription and translation machineries of the host cell from the viral integrated DNA. Transcription is controlled by both the host cell and the viral genes. Among viral proteins, Tat allows the total elongation of the RNA viral, acting as enhancer for the transcription and as transactivator in cooperation with cellular factors such as NF-κB and Sp1. Thus, RNA are translated following splicing and cutting down to size (Alcami, 2008; Klimas *et al.*, 2008). The protein Rev is implicated both in the transport out of the nucleus of the transcript RNA, in the processing of the RNA in smaller size. Rev participates to the assembly with the synthesis machinery and accelerates the synthesis of viral proteins by the

polysome. The viral proteins Vif, Vpu and the viral proteases intervene in the post-translational process of the new viral proteins synthesized. The protein Vif interacts with a cellular protein named APOBEC3G who has the function to augment the mutation rate during replication, raising the level of error of the reverse transcriptase. Vif promotes APOBEC3G degradation preventing its incorporation in produced virions and therefore its interference during the retrotranscription. A striking finding is that APOBEC3G can be packaged in exosomes (Khatua *et al.*, 2009). Vpu is a regulator protein which participates in the maturation and budding of the particules.

The precursors of gag $Pr55^{Gag}$ and gag-pol are processed by the viral protease to form proteins of the nucleocapsid, the reverse transcriptase and the viral protease.

The viral proteins, the core proteins, RNA and enzymes come at the host cell's membrane and the virus buds. The virus can replicate in a controlled manner or intensively leading to the cell death. It is worth to note that a single cell can make thousands of infectious particles of HIV, either chronically, over weeks, or as a single burst resulting in cell death (Alcami, 2008; Klimas *et al.*, 2008).

Gp160, precursor of the proteins of the envelope : gp120 and gp41 is processed by a cellular protease during the final maturation of the virions at the end of the cycle of infection. (Figure 15)

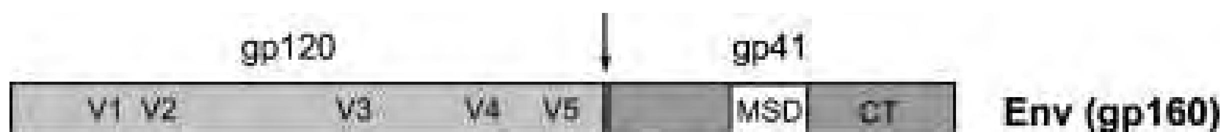


Figure 15 : Schematic representation of HIV-1 Env proteins. The HIV-1 Env glycoprotein is synthesized as a precursor (gp160). A proteolytical process splits gp160 (arrow) into the surface glycoprotein gp120 and the transmembrane glycoprotein gp41. The viral envelope (Env) glycoproteins mediate HIV entry into target cells by direct fusion of the viral envelope lipid bilayer with the target cell plasma membrane. **Adapted from (Waheed & Freed, 2009).**

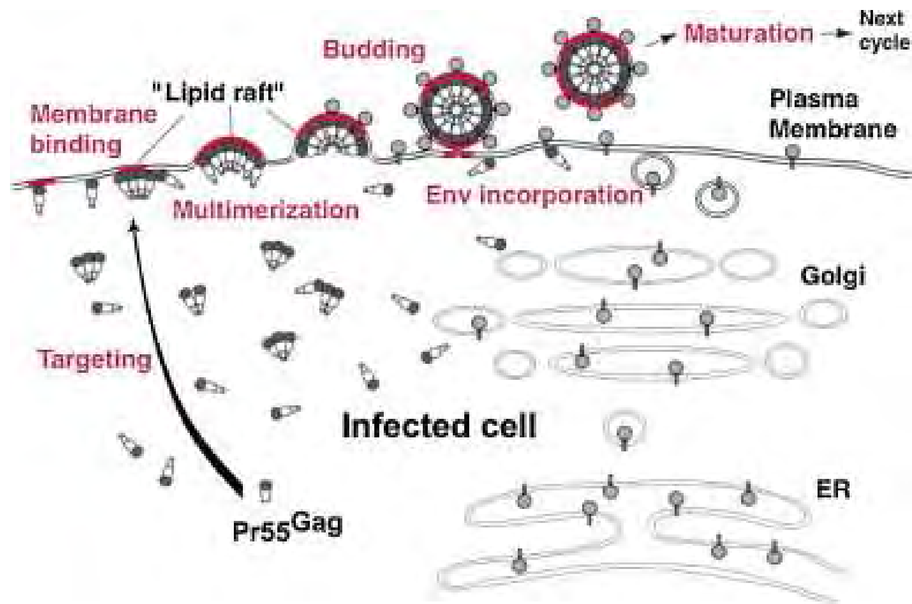


Figure 16 : Schematic representation of HIV assembly and release at the plasma membrane. $Pr55^{Gag}$ is directed to the plasma membrane where it associates via its Myristoylated domains with lipid raft microdomains (red). Gag–Gag multimerization lead to the assembly of viral particle and its budding from the plasma membrane. The viral Env glycoproteins are incorporated during the assembly process and virions ultimately pinch off from the cell surface. **Adapted from (Waheed & Freed, 2009).**

5. Transmission of HIV.

HIV is a sexually transmitted agent. First, HIV enters in contact with dendritic cells which carry the virus across the mucosa, and release the infectious virus into the lymph node. Bound to a $CD4^+$ cell, HIV travels to the lymphatic tissue and begins the first cycle of infection. HIV is also spread by contact with infected blood, most often through the practice of reusing or sharing syringes and needles with drugs. The risk of infection during blood transfusion is remote nowadays as since 1985, the blood supply has been screened for HIV.
There is a risk of transmitting HIV from a pregnant mother to the fetus or newborn either during pregnancy, delivery and breast feeding. In developed countries, ART, cesarean section and formula feeding have reduced the rate of mother to child transmission from about 25 % to 1%-2% (Anderson & Cu-Uvin, 2009).
Finely, occupational risk to healthcare workers through accidental needle stick or mucosal splash with contaminated blood exists (Alcami, 2008).

6. Viral load and disease progression

HIV replicates very rapidly once entered into the body. Viral load in this early infection can reach millions of virions per ml of blood. They are many factors that can influence the viral load and the progression of the disease such as type of immune response, coinfection with other sexually transmitted diseases, age, and behavior (Klimas *et al.*, 2008).

For example, it has been observed that infection with hepatitis C virus (HCV) was associated with a lower $CD4^+$ cell count (Cheng *et al.*, 2007; Klimas *et al.*, 2008). Moreover, in absence of antiretroviral therapy (ART), older individuals have higher rates of progression than younger adults as they may have fewer naïve cells and more $CD4^+$ cells and memory cells are depleted more rapidly in older population.

HIV infection ultimately associates with opportunistic co-infections and the progression of AIDS. Despite highly active antiretroviral therapy (HAART), AIDS associated malignancies continue to be a major clinical manifestation of HIV-infected individuals, with the increase of the longevity of the treated individuals and the prolonged effects of immunosuppression (Angeletti *et al.*, 2008). The acquired immunodeficiency syndrome (AIDS) is characterized by the development of a number of cancers such as Kaposi's sarcoma (KS), Hodgkin's and high-grade B cell non-Hodgkin's lymphomas (NHL), and primary central nervous system lymphoma (PNCL), and invasive cervical carcinoma. Before the introduction of antiretroviral treatment, the prognoses of patients were poor, mainly due to the aggressiveness of the tumors on immunosuppression, an increase in hematological toxicity due to the treatment, and complications, due to opportunistic infections. The three major types of cancers included as part of the AIDS-defining illnesses (such as KS, NHL and cervical cancers) have been linked to infectious viral agents such as HHV-8 or KSHV for KS, EBV for NHL, PCNSL, Hodgkin's disease…. HPV for cervical carcinoma. A number of mechanisms and viral genes were found to have transforming activities, and they may play a direct or indirect role in tumorigenesis (Angeletti *et al.*, 2008).

7. Antiretroviral therapies (ART)

There are currently 20 antiretroviral drugs for the treatment of HIV (Panel of antiretroviral guidelines for adult and adolescents, department of health and human services, January 29, 2008, pp1-128; http://aidsinfo.nih.gov/contentfiles/adultandadolescentGL.pdf)(Klimas *et al.*, 2008)

Six classes of ART that interrupt viral replication are available:

1) **Nucleoside/nucleotide reverse transcriptase inhibitors** (among them zidovudine – AZT- a nucleoside analog). They act as competitor for the natural nucleotides and as terminators of the helix during the viral DNA synthesis.
2) **Non nucleoside reverse transcriptase inhibitors.** They interfere with the reverse transcriptase by linking a domain near the catalytic site.
3) **Protease inhibitors.** They interact with the catalytic site, blocking the process of the precursor gag/pol, leading to the production of noninfectious viral particles.
4) **Fusion inhibitors.** There are synthetic peptides homologous to the HR2 region of the glycoprotein gp41 of the HIV-1, allowing to block the fusion process.

5) **CCR5 antagonists.** These molecules act following an alosteric mechanism, non competitive fixing the CCR5 receptor in a conformation, preventing its interaction with gp120 of the HIV.
6) **Integrase inhibitors**.

Current treatments consist of highly active antiretroviral therapy (HAART), which is composed of at least, three drugs belonging to two classes of antiretroviral agents. These treatment have toxicities and side effects such as immune reconstitution inflammatory syndrome which results from the rebound in immune response to a variety of occult infections (high fever, abdominal pain, inflammatory mass); hypersensitivity drug reactions, cardiovascular complications, exacerbation of underlying liver disease, and the development of the lipodystrophy syndrome characterized by fat redistribution and peripheral fat loss, and complex metabolic alterations including dyslipidemia and insulin resistance.

Because the virus is highly mutable, the goals of treatments are to suppress plasma viremia for as long as possible, to delay the selection of drug resistance mutations, and to preserve immune function and maintain immunocompetence.
Significant resistance-associated mutations are in the reverse transcriptase, protease, and envelope genes. The emergence of drug resistance in treated populations and the transmission of drug-resistant strains to newly infected individuals are important public health concerns in the prevention and control of HIV. Poor adherences to the drug regimens translate into multiple drug resistant strains of HIV and rapid progression.
Vaccine development has progressed slowly due to the capacity of this virus to escape immune pressure as well as the number of strain variations. A number of vaccine concepts are being pursued including live attenuated vaccines, subunit vaccines, and live recombinant vaccines. The first trial of a vaccine designed to elicit strong cellular immunity has shown no protection against infection. Instead, the vaccine seemed to increase the rate of HIV infection in individuals with prior immunity against the adenovirus vector used in the vaccine (Sekaly, 2008).

Future compounds which are currently in clinical or preclinical phases are news inhibitors of the viral entry such as molecules targeting the interaction of the viral envelop with CD4 or the coreceptors CXCR4 or CCR5, new inhibitors of the integrase, of the viral morphogenesis (targeting Vpu or gag interfering with the assembly process) and compounds reactivators of the latent reservoirs. Because of antiretroviral drug penetration failure and active efflux, the persistence of a reservoir of virus which is not accessible to anti-retrovirus treatments represents a major goal for HIV eradication.

8. HIV in monocytes/ macrophages reservoir cells

In spite of treatment allowing to decrease the plasma HIV-1 RNA load to below the detection limit of standard assays (<50 copies/ml), HIV is not eradicated by current regimens and low levels of HIV-1 RNA in the plasma can be detected with extremely sensitive reverse transcriptase assays (Delobel *et al.*, 2005; Mavigner *et al.*, 2009).

The two major cellular reservoirs are latently resting $CD4^+$ T cells and monnocytes/macrophages. It has been demonstrated that plasma and monocytes contain different viruses suggesting the existence of several sources of residual viruses and thus a compartmentalization of the viruses (Delobel *et al.*, 2005; Mavigner *et al.*, 2009).

Although the HIV follows the same virus cycle in T lymphocytes than in macrophages, several aspects are unique to macrophages infection (Carter & Ehrlich, 2008; Benaroch *et al.*, 2010). Macrophages are the long term persistence of productive infection, accumulating infectious viral particles in large vacuoles, sustained by the absence of cell death. In these cells, HIV can bud in MVBs without the need of cell activation and division, and macrophages produce and harbor the virus for a long period even in patients receiving highly acute antiretroviral treatment (HAART). Moreover, these cells display a greater survival than T cells following infection and have the capacity to cross the blood-tissue barrier and can potently deliver HIV to all tissues and organs including the central nervous system. They are the most productively HIV-infected cells in the CNS of infected patients. Viral particles are localized intracellularly that is thought to protect the virus from the immune system and from the action of antiviral drugs. Macrophages and primary lymphocytes express the two co-receptors CCR5 and CXCR4 while viral entry seems to depend too on the viral strains specificity determined by distinct characteristics of Env (Goodenow & Collman, 2006).

B. HIV Budding pathway.

While HIV budding has been initially depicted at the plasma membrane, growing evidences suggest that it could take place in intracellular compartments.

1. The Trojan hypothesis

First described in 2003 by Gould et al. the Trojan hypothesis attempted to bring explanations of the presence of viruses in multivesicular bodies and infections independently on receptors and envelop proteins (Gould *et al.*, 2003). Thus, it states that retroviruses use the preexisting exosome biogenesis pathway for i) the formation of infectious particles and ii) extracellular retroviruses internalization in a receptor and Env protein-independent manner. Using the same protein targeting and vesicles biogenesis pathway, this suggestion emphasized that exosomes and HIV display similar host cell proteins and lipids.

Consistent with this, Wiley et al. reported that DCs could capture HIV-1 particles that were driven in multivesicular bodies. They demonstrated that these virions were released outside the cell in association with exosomes. These endocytosed HIV-1 particules from DCs were 10-fold more infectious towards T cells than cell free virus particles (Wiley & Gummuluru, 2006).

Izquierdo-Useros et al. recently observed that HIV and other retroviruses could exploit this pathway in DCs in the absence of lytic degradation, allowing the final trans-infection of T CD4+T cells (Izquierdo-Useros *et al.*, 2010). Indeed, DCs in lymph node, key site of HIV replication, would be able to transmit and spread the virus without being infected. This mechanism involves gp120-independent virus binding, internalization without viral fusion, storing in endocytic compartments and release. It is named trans-infection (Figure 17). Authors previously demonstrated the existence of a common entry mechanism in mature DCs, by competitions between, HIV and exosomes (Izquierdo-Useros *et al.*, 2009).
Their works supported the Trojan exosomes hypothesis proposing that retroviruses take advantage of an intracellular vesicle traffic and exosomes exchange pathway, moving between cells in the absence of fusion events. They brought evidences that endocytosis of retroviruses are enhanced in mature DCs and that HIV captured by mDCs converges and accumulates with the same intracellular compartment of exosomes which is stained by CD81 and is negative for Lamp-1. They also provided a new implication of sphingolipids in the common uptake of both HIV and exosomes since their capture was abolished when parent cells were treated with inhibitors reducing glycosphingolipids level (Izquierdo-Useros *et al.*, 2009; Izquierdo-Useros *et al.*, 2010). The authors underlined the important potential of mDCs to spread HIV since they can randomly contact about a thousands of T cells per hour and transmit virus stored. These findings together with others similarities such as size, lipidic and proteic compositions, budding from lipids raft-like domains rich in cholesterol and tetraspanins (see below) have led the authors to suggest that retroviruses are, at their most fundamental level, exosomes which have derived by becoming replicatives.

Cell to cell transfer has been described to be more efficient and rapid means of viral propagation than infection by cell free virus, and in culture it represents the main HIV transfer process. Experience conducted with infected cells are a much more efficient mode of viral spread than viral infections initiated with cell-free virions (Vendrame *et al.*, 2009).
To comfort this idea, Miyauchi et al. recently demonstrated that HIV-1 enter into the recipient cell via an endocytic process before fusion, endosomal entry being the pathway that lead to a productive infection on the contrary to the fusion at the plasma membrane (Miyauchi *et al.*, 2009). Consistent with this, Hübner et al., regarding the transmission of HIV-1 through the virological synapse in cell-cell contact between T cells, observed viruses inside multivesicular bodies in the target cell in favor of an endocytic route (Hubner *et al.*, 2009). The virological synapses have been described in both trans-infection process between

Dendritic cells and lymphocytes (McDonald *et al.*, 2003), and cell-to-cell HIV-1 transmission between lymphocytes (Jolly & Sattentau, 2004). Ruggiero et al. also brought evidences of hiv-1 endocytosis during cell-cell contact T cells, together with a second mechanism of HIV transfer by cell fusion (Ruggiero *et al.*, 2008) not detected in primary macrophages. These two later studies underlined the requirement of Env-CD4 interactions.

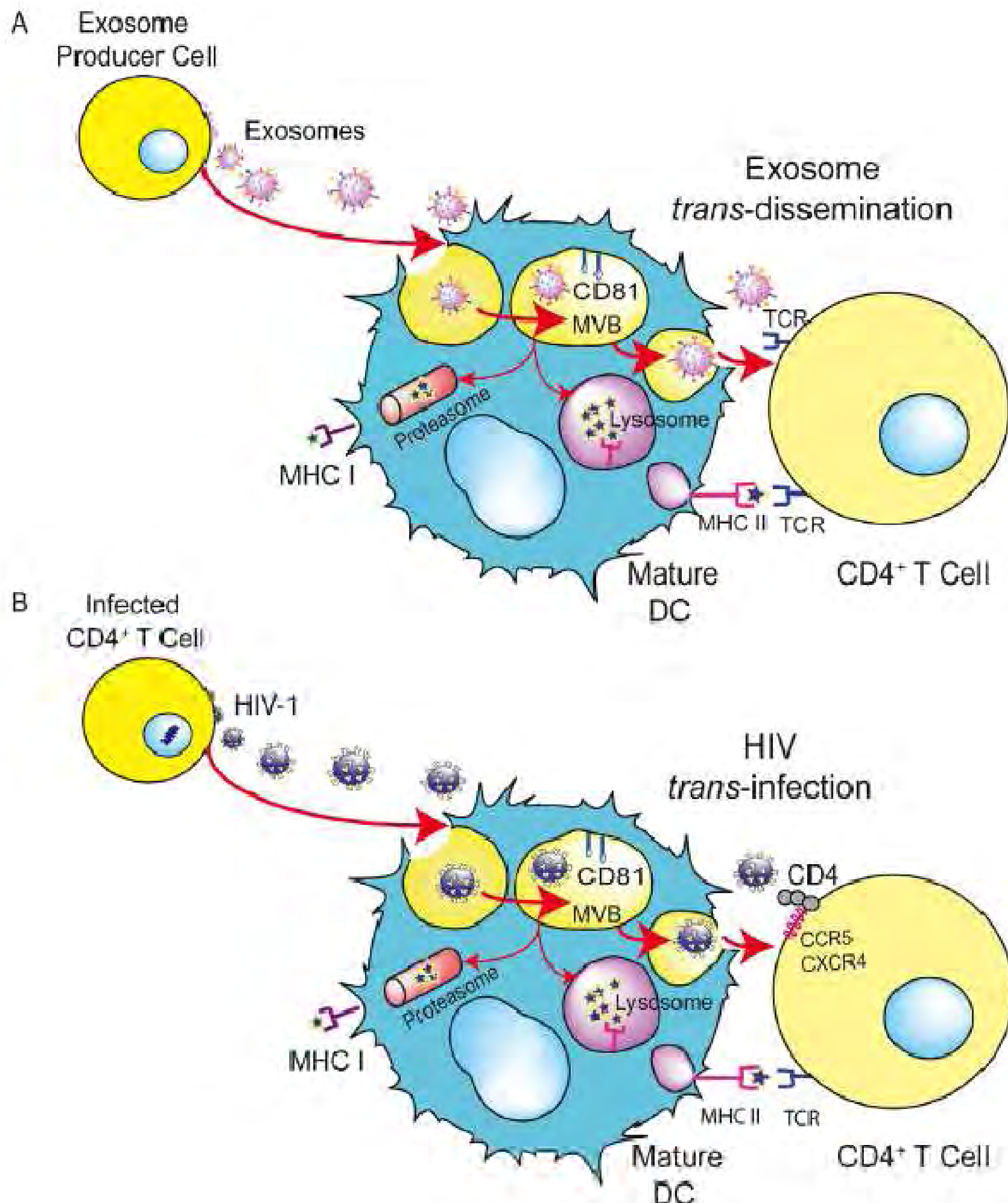


Figure 17 : Trans-dissemination pathway in mature DCs allowing trans-infection of CD4+T cells. (A) Exosomes uptake by Dendritic cells leading to cross presentation of exosomal antigen in mature Dendritic cells or exosomes delivery to effector cells by trans-dissemination. (B) HIV can exploit the preexisting exosome trans-dissemination allowing the final trans-infection of CD4+T cells. Adapted from (Izquierdo-Useros *et al.*, 2009)

In macrophages, HIV-1 assembly in endosomes appeared prominent in macrophages in which very large numbers of virions particles are found within compartments containing late endosomal markers (Pelchen-Matthews *et al.*, 2003). Similar studies reported localization of HIV-1 virions in endosomal compartments in fibroblast, epithelial and T lymphocyte cell line (Nydegger *et al.*, 2003; Sherer *et al.*, 2003; Bieniasz, 2009). However, several works sustain the idea that appearance of gag and or viral particles in endosomes can occur as a result of endocytosis or adherence of the virions to the infected cell surface (Bieniasz, 2009).
Indeed, newly formed virions secretion in HIV-infected macrophages and DCs resembles to that of exosomes. In these cells, HIV buds in MVB like compartments that are generally not continuous with plasma membrane, do not acidify due to the lack of recruitment of the proton pump vacuolar ATPase and are highly enriched in CD9 and several other members of the tetraspanin family. (Buschow *et al.*, 2009).

Given HIV component association to cellular markers of MVBs at the plasma membrane, the Trojan hypothesis was further extended and some works suggested that HIV might also bud from MVBs derived microdomains at the cell surface (Nydegger *et al.*, 2006; Carter & Ehrlich, 2008). In accordance with these works, Deneka et al. suggested using membrane-impermeant tracer, that HIV associates in macrophages in internally sequestered plasma membrane domains containing markers of the MVB compartments such as CD81, CD9, CD63 (Deneka *et al.*, 2007) In the same way, Welsch S found HIV in intracellular compartments marked by a membrane-impermeant tracer and that were devoid of endocytosed bovine serum albumin-gold (Welsch *et al.*, 2007). On the contrary Beneroch et al. recently indicated that their tracer (ruthenium red dye) access to the cytoplasm and mitochondria indicating that it is not totally membrane-impermeant in macrophages (Benaroch *et al.*, 2010).

However, recent works confirmed by real time observations that Gag accumulate both at the plasma membrane and in internal compartments carrying MVBs markers in infected macrophages (Gousset *et al.*, 2008) To strengthen this idea, Joshi et al. using Gag-matrix mutant (29/31KE), brought evidences that intracellular compartments such as MVBs can serve as productive site for HIV assembly in certain cell type such as Monocytes Derived Macrophages (MDM) and T cells. Other works bring evidences that Gag and viral RNAs can use at least two trafficking pathways to produce virions, one going directly from the plasma membrane and another one passing through endosomes (Molle *et al.*, 2009). Authors assure that late endosomes were not deep invaginations of plasma membrane as proposed in other systems, since they were negative for the marker of plasma membrane CD44 and that their specific labeling was not due to an endocytosis process. They demonstrated that endosomal Gag contributed to viral production and that their release could occur at specific site such as cell contacts (Molle *et al.*, 2009).

Owing to the electronic microscopy observations that expose particles at various stages of budding and comprising immature ones in many independent studies and because the viral Vpu, not only promotes virus release but also inhibits virus uptake by endocytosis, Benaroch et al. argue in favor of de novo produced virions in intracellular compartment rather than endocytosed particles. They suggested the existence of an intracellular compartment separated from the endocytic pathway, possessing a neutral pH and transiently connected via microchannels to the plasma membrane (Benaroch *et al.*, 2010) (Figure 18).

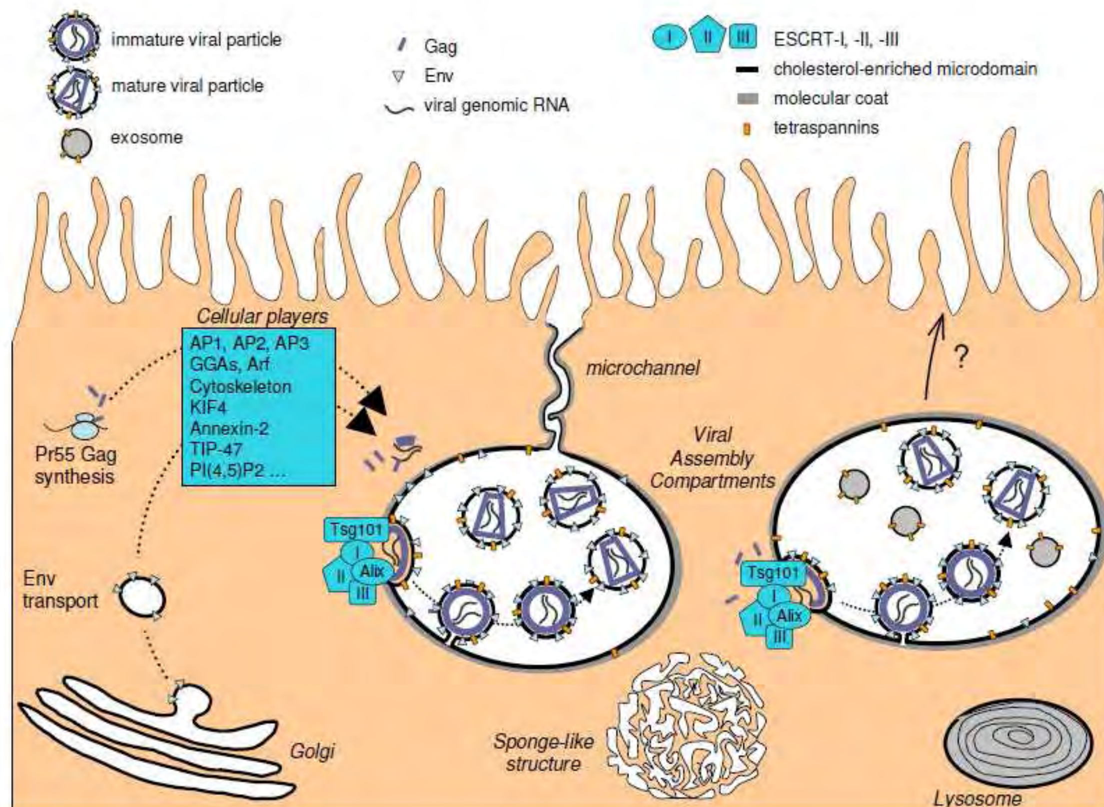


Figure 18 : HIV assembly in macrophages. Once synthesized, Gag and env proteins reached the budding sites with the help of putative indicated cellular factors. The intracellular assembly compartment could be transiently connected to the plasma membrane through thin microchannels (20nm) that do not allow virion passage. Adapted from (Benaroch *et al.*, 2010).

Another work sustains a similar hypothesis in a model of transinfection between Dendritic cells and T cells. Indeed, it has been demonstrated that mature DCs could store viruses into intracellular compartments distincts from endocytic vesicles and accessibles to the extracellular medium. This specialized compartment could allow particles to be delivered to T cells during trans-infection (Yu *et al.*, 2008).

2. Raft microdomains and specific lipids.

Lipid rafts are specialized regions, detergent-resistant membrane microdomains, composed of clusters of sphingolipids and cholesterol of the plasma membrane about (10-

200nm) and are characterized by a high content of cholesterol, sphingolipids and acylated- or glycosylphosphatidylinositol (GPI)-anchored proteins linked covalently to the lipid bilayer (Brown & London, 1998; Waheed & Freed, 2009). Abundance of signaling molecules in lipid rafts suggest that they are important in signal transduction.
Lipids raft are important at various stages of the human HIV-1 replication cycle (Campbell *et al.*, 2001; Guyader *et al.*, 2002; Viard *et al.*, 2002; Viard *et al.*, 2004) and several studies suggest that HIV-1 use lipid rafts as platforms for assembly and budding (Lindwasser & Resh, 2001; Ono & Freed, 2001).

Exosomes and retroviruses are enriched in cholesterol, glycosphingolipids phosphatidylethanolamine. (Gould *et al.*, 2003; Nguyen *et al.*, 2003). Therefore, lipid rafts are presents on HIV particles and depletion of viral membrane cholesterol blocks HIV infection. (Liao *et al.*, 2001; Graham *et al.*, 2003; Liao *et al.*, 2003; Waheed & Freed, 2009).
While HIV appears to be enriched in lipids raft, it carries protein markers of lipid-raft domains such as cyclophilin and GPI-anchored proteins (Ono & Freed, 2001). Noteworthy, lipid rafts were also observed in exosomes which carry another typical raft-associates protein, the flotilin (de Gassart *et al.*, 2003)

PI4,5P_2 was shown to bind to the matrix (MA) domain of Gag and this interactions seem to be involved in HIV budding from lipid rafts (Waheed & Freed, 2009). HIV has been shown to be enriched in PI4,5P_2 along with cholesterol, ceramides and GM3 (Chan *et al.*, 2008). A role of lipids rafts in HIV-1 entry has been reported (Carter *et al.*, 2009) and particularly for PI4,5P_2 (Barrero-Villar *et al.*, 2008). Reduction at the plasma membrane of PI4,5P_2 by overexpressing a specific phosphatase (polyphosphoinositide 5-phosphatase iV), lead to a relocalization of gag to $CD63^+$ compartments (Ono *et al.*, 2004; Waheed & Freed, 2009) in the same manner than overexpressing the small G protein ADP-ribosylation factor 6 (Arf6) which regulates the phosphatase responsible for the synthesis of PI4,5P_2 from PI4P (Ono *et al.*, 2004; Waheed & Freed, 2009). Moreover, Gag binds preferentially to plasma membrane acidic phosphoinosides such as PI(4,5)P_2 and liposomes containing PI(4,5)P_2 (Chukkapalli *et al.*, 2008; Chukkapalli *et al.*)

Nef, a viral protein of HIV, associates within rafts. Nef is involved in several functions, among them, the regulation of cholesterol trafficking in infected cells (Foster & Garcia, 2008). Nef impairs the cholesterol efflux, associating with ATP-binding cassette transporter A1 (ABCA1) in macrophages, and increases the cholesterol of virions ((Zheng *et al.*, 2003), enhancing particle infectivity (Mujawar *et al.*, 2006), when produced in macrophages. Contrary to these study, Brugger et al. didn't detect neither cholesterol increase in HIV when produced in MT-4 T cell line (Brugger *et al.*, 2007) nor a cholesterol association of Nef.

It has been demonstrated that an efficient HIV dissemintion takes place in a cell-cell transmission that occurs through a virological synpases formed at the site of contact

between infected cells and non infected ones. The formation of virological synapses takes place in lipid raft microdomains, enriched in tetraspanins (CD81 and CD9), facilitated by adhesion molecules recruitment. It has been described that CD4 and coreceptors are recruited into the virological synapses from the target cells together with Gag, Env and GM1 in lipid raft like patches on effector cells (Jolly & Sattentau, 2004, 2005; Jolly *et al.*, 2007; Jolly & Sattentau, 2007). Thus, HIV selectively subverts components of the intracellular trafficking machinery to achieve efficient exit.

3. ESCRT complex implication and role of HIV Gag

As shown in the first part of the manuscript related to exosomes, ESCRTcomplex functions to select and sort proteins to the endosomal membranes, triggers membrane inward budding to generate vesicles inside the MVBs(Bieniasz, 2009).

ESCRT components can also be recruited by enveloped viruses to sites of viral assembly where they have been proposed to mediate viral egress.

HIV-1 assembly is driven by the Gag precursor named $Pr55^{Gag}$ protein whose expression is sufficient for the assembly of viral-like particles.

Figure 19 : Schematic representation of HIV-1 gag precursors $Pr55^{Gag}$ proteins. The major domains MA, CA, NC and p6 are indicated. The N-terminal myristate (Myr) and L (late budding) domains are shown. **Adapted from (Calistri *et al.*, 2009; Waheed & Freed, 2009).**

Gag contains several domains that are essential for viral assembly : a membrane binding domain (M) in MA which represent docking site for Gag to the AP-3 complex and for annexin 2 that result in the targeting of Gag to internal membrane compartments (Carter & Ehrlich, 2008), a Gag-Gag interaction domain in CA; an assembly domain in NC allowing its multimerization, capture RNA genomes and couples RNA binding with assembly, and a late domain (L) in p6 which recruits the cellular budding machinery ESCRT and proteins associated (Bieniasz, 2009; Calistri *et al.*, 2009; Molle *et al.*, 2009).

Moreover, Gag displays properties known to induce the protein sorting into exosomes such as protein aggregation. (Katzmann *et al.*, 2002; Pornillos *et al.*, 2002; Gould *et al.*, 2003;

Pornillos *et al.*, 2003). It is worth to note that HIV Gag is monoubiquitinated at multiples sites (Calistri *et al.*, 2009) and HIV incorporate high level of ubiquitin into their particle.
Thus, Gag binds membrane, at the level of raft lipids domains (Ono & Freed, 2001), multimerizes and engages host cell factors to promote virus pinching off from the membrane (Waheed & Freed, 2009).

Gag mediates recruitment of the cellular ESCRTs-I, -III, AIP1/Alix and Vps4 proteins (Pornillos *et al.*, 2002; Strack *et al.*, 2003; Babst, 2005; Usami *et al.*, 2009; Roxrud *et al.*, 2010). Mutations that inhibit the function of these class E Vps proteins block the virus formation at a late stage. These proteins complexes have an essential function in final membrane closure and the budding step in the formation of both MVB and viral particles (Valiathan & Resh, 2008).
In HIV-1 infected macrophages where HIV-1 accumulates intracellularly at MVBs, virus assembly is dependent on Tsg101 which is essential for MVB biogenesis. Gag associates with Tsg101 via PTAP motif in L domain (Roxrud *et al.*, 2010). Studies suggest that Vps4 is required for HIV-1 budding too (Pornillos *et al.*, 2002; Gould *et al.*, 2003). Vps4 which disassembles the ESCRT components, is essential for the virus egress since mutants of Vps4 inhibits release of HIV-1. Gag interacts with Alix too (Strack *et al.*, 2003).

The protein Alix plays a central role among the set of proteins involved in exosome formation as well as in HIV budding since as cited above, HIV Gag binds Alix. However, this protein has been functionally linked to a peculiar phospholipid called lysobisphosphatidic acid (LBPA) which has been described to be particularly enriched in internal membrane of late endosomes (Kobayashi *et al.*, 1998b), the exosome generation site and potently, the HIV budding site in reservoir cells. Alix has been described to regulate LBPA effects in intraluminal vesicles generation (Matsuo *et al.*, 2004). Moreover, it has been demonstrated that LBPA is able to trigger vesicles formation by its own (Matsuo *et al.*, 2004). The following paragraph develops the current knowledge on LBPA and most generally lipids potently interesting in therapy against the HIV.

Part 2. Lipids in research for a new HIV therapy

I. Exosomes lipidomics

The following section constitutes a review published in the journal Biochimie which aimed to summarize knowledge on lipidic composition and function of exosomes and HIV with a special focus on bioactives lipids, cholesterol and lysobisphosphatidic acid (LBPA) also called Bis(monoacylglycero)phosphate (BMP).

(Subra *et al.*, 2007)

Subra C, Laulagnier K, Perret B & ***Record M***. (2007). Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. *Biochimie* **89,** 205-212.

Review:

A. Exosome Lipidomics unravels lipid sorting at the level of multivesicular bodies

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Keywords: *Exosomes ; lipidomics ; LBPA (BMP) ; HIV ; lipolytic enzymes ; MVB ; LPC ;cancer*

1. Summary:

Exosomes are part of the family of “bioactive vesicles” and appear to be involved in distal communications between cells. They vehiculate bioactive lipids and lipolytic enzymes and their biogenesis requires specific lipids and a membrane reorganisation. Their biogenesis pathway could be a way to secrete enzymes involved in lipid signalling and to generate “particulate agonists”. However this pathway seems also to be used by pathogens such as HIV. This review will consider several aspects of lipidomic studies which might help to understand the fate and role of these fascinating vesicles.

2. Introduction:

Bioactive vesicles are receiving increasing interest since they are an important process to enhance life diversity. Exosomes are the only type of bioactive vesicles originating from an intracellular compartment, namely MultiVesicular Bodies (MVB, or Late Endosomes) and released out of the cell. Several reviews describing « the exosome pathway » are now available (Fevrier & Raposo, 2004; Chaput *et al.*, 2006a). First observed in 1987 during reticulocyte maturation (Johnstone *et al.*, 1987) and extensively investigated in that field (Blanc *et al.*, 2005), the interest towards these vesicles was triggered again ten years later, when it was observed they were enriched in MHC II and released out of B lymphocytes following cell activation (Raposo *et al.*, 1996). The strong potency of exosomes loaded with a specific antigen to eradicate tumors in mice was demonstrated two years later (Zitvogel *et al.*, 1998). Phase I clinical trials were completed a year ago in humans, and exosomes appear as a promising tool for autologous treatments in cancer (Escudier *et al.*, 2005).
In that context exosome lipidomics appears as a requirement for the elaboration of « reconstituted exosomes », with the purpose of elaborating a tool in cancer treatment independent of any biological source. Vehiculating therapeutic drugs by encapsulation inside a liposome has been the dream of lipidologists for decades and many attempts are currently undergoing (Ignatius *et al.*, 2000). In all cases, lipid composition of liposomes has been empirical, based on the knowledge acquired for years on lipid organisation in membranes and subsequent physicochemical properties. Instead, exosomes offer the first opportunity of a « therapeutic vesicle » whose lipid composition and organisation could be a starting point to devise efficient liposomes, vehiculating tumor antigens to boost the immune response towards tumors.

During these last years, lipidomics has appeared as an emerging field, since genomics and proteomics could not bring all the responses scientists were expecting in the perspective to cure a panel of pathologies (Lagarde *et al.*, 2003). The lack of powerful tools to analyse the complexity of lipids has been overcome by novel analytical approaches, including liquid chromatography coupled to powerful mass spectrometry equipments (Wenk, 2005) easier to use than in the past, allowing for example the elaboration of « lipid arrays » (Ivanova *et al.*, 2004). This review will summarize the present knowledge on exosome lipidomics, in order to better understand how to regulate their biogenesis which undoubtly requires appropriated lipid mediators.

a) Analytical Lipidomics :

This part will consider the lipid composition and membrane organisation of exosomes.

a.1. Phospholipid composition.

Three major works in this field have been performed so far, on exosomes derived either from guinea-pig reticulocytes (1989) (Vidal *et al.*, 1989), or from a B lymphocyte cell line

(2003) (Wubbolts *et al.*, 2003), a rat mast cell line or from human dendritic cells (2004) (Laulagnier *et al.*, 2004b).

The phospholipid composition of reticulocyte-derived exosomes appears quite similar to that of plasma membrane (see Table 2), which is identical to the phospholipid composition of erythrocytes. No increase in cholesterol / phospholipid ratio was observed in exosomes as compared with the parent cells, at the opposite of what was reported for B cell-derived exosomes whose ratio was increased by 3 times (Table 2). In the latter case however, only MHCII-enriched exosomes were considered (Table 2) since a complete phospholipid composition of the other subpopulations was not reported. We have shown that mast cell derived exosomes contain at least three subpopulations according to the type of protein and lipid content (Laulagnier *et al.*, 2005). Considering the bulk of mast cells (RBL-2H3)-derived exosomes, we have observed no increase in the cholesterol / phospholipid ratio compared with parent cells. However this ratio is fairly low in mast cells when comparing to other analysis reported in reticulocytes and B lymphocyte cell line, but this observation does not exclude the clustering of cholesterol molecules that was detected by immunoelectron microscopy in B cell-derived exosomes (Wubbolts *et al.*, 2003). In that respect exosomes released by reticulocytes contain typical rafts (de Gassart *et al.*, 2003). Exosomes are distinct from rafts, as we reported from the lipidic composition of mast cell-derived exosomes which feature a sphingomyelin / cholesterol / glycerophospholipids molar ratio of 1/1/4 respectively, whereas the corresponding molar ratio in rafts would be 1/2.2/1.3 (Laulagnier *et al.*, 2004b). In addition rafts exhibit a low protein-to-lipid ratio and float on the top of a density gradient (average density of 1.07 (Chen *et al.*, 2006)), whereas it is quite the opposite for exosomes which feature a high protein-to-lipid ratio (Table 1) and a buoyant density of 1.12-1.15 (Thery *et al.*, 2001) from cells or 1.21-1.28 from blood (Caby *et al.*, 2005).

	SM	PC	PE	PS + PI	LPC	CHOL/Plip	Plip/Prot
Erythrocytes [12]							
Exosomes	15.9	44.4	23.9	15.8		0.89[a]	0.25
Parent cells	12.1	43.2	28.3	16.4		0.86	
Ratio	*1.31*	*1.03*	*0.84*	*0.96*		*1.03*	
Mast Cells [14]							
Exosomes	14	33	27	18	7	0.18[b]	0.17
Parent cells	5	50	25	15	5	0.18	0.35
Ratio	*2.8*	*0.66*	*1.08*	*1.2*	*1.4*	*1*	*0.48*
Dendritic cells [14]							
Exosomes	20	26	26	19	9		
Parent cells	9	43	23	12	13		
Ratio	*2.2*	*0.6*	*1.13*	*1.6*	*0.7*		
	SM + GM3	PC + PS + PI	PE + EthLip	LPC	CHOL/Plip	Plip/Prot	
B lymphocytes [13]							
MHC II exosomes	39.7	35.1	25.2		0.73[c]		
Parent cells	17	45.9	37.2		0.24		
Ratio	*2.3*	*0.76*	*0.7*		*3.04*		
	SM	PC	PE	PS	LPC	CHOL/Plip	
T lymphocyte and HIV [21]							
HIV	33.1	16	35.2	15.5		0.83	
MT4	10.4	43	33	7.4		0.39	
Ratio	*3.18*	*0.37*	*1.06*	*2.09*		*2.13*	

Table 2 Comparative phospholipid composition of exosomes and their parent cells. Results are expressed in moles percent of total phospholipids. Cholesterol/phospholipid values are expressed as molar ratio (**b**) [it is not known whether values ***a*** and ***c*** represent mass or molar ratios]. Phospholipid/Protein are mass ratios (w/w). Values in bold and italics represent ratios between exosomes over parent cells phospholid contents.
Note the decrease in Plip / Protein ratio in RBL-2H3 mast cells exosomes (last column).
EthLi : ether lipids. CHOL: cholesterol; PLIP : phospholipids ; Prot : proteins. SM :sphingomyelins ; PC : phosphatidylcholines ; PE : phosphatidylethanolamines ; PS : phosphatidylserines ; PI : phosphatidylinositols ; LPC : lysophosphatidylcholines.

The exosome biogenesis pathway appears to be “hijacked” by pathogens such as HIV, viruses budding inside the MVB similarly as exosomes (Grigorov *et al.*, 2006). The comparison between HIV lipid composition and the parent T cell line (Brugger *et al.*, 2006) exhibit similarities with exosome lipid composition reported in Table 2, specifically with B cell–derived exosomes which are enriched in cholesterol.
Cholesterol enrichment has been reported to occur in MVB intralumenal vesicles, which are precursors of exosomes, in the RN B-cell line. They appear to concentrate 63% of the cholesterol located within the endocytic track (Mobius *et al.*, 2003). LBPA, which has been claimed to associate with cholesterol (Kobayashi *et al.*, 1999), starts to accumulate in MVBs (14% of total phospholipids in late endosomes [MVB] versus 1.3 % in early endosomes of BHK cells), and could be prerequisite for intralumenal MVB vesicle formation. No enrichment in this phospholipid was noticed in biochemical analysis of mast cell (RBL-2H3)-derived exosomes as well as in B cell exosomes (Wubbolts *et al.*, 2003; Laulagnier *et al.*, 2004b). It seems conceivable that LBPA remains on the MVB limiting membrane once the intralumenal vesicle has been pinched off.

Detail composition of RBL and DC-derived exosomes is presented in Table 3. We observed a noticeable amount of disaturated molecular species in phosphatidylethanolamine and phosphatidylcholine classes. Disaturated PC and PE increase

by 2.6 and 3.7 times respectively as compare to parent cells, similarly to the increase in disaturated PC reported for HIV (Figure 20) (Brugger *et al.*, 2006). This observation strengthens the “Trojan horse hypothesis” (Gould *et al.*, 2003) suggesting that HIV would be an “exosome-like“ particle.

Noteworthy we observed that the amount of diglycerides was two times less in mast cell (RBL-2H3)-derived exosomes than in parent cells (Laulagnier *et al.*, 2004b). Diglycerides have been shown to modulate phospholipid membrane packing, and their limited amount in exosome membrane together with a high content in disaturated species suggested an elevated membrane rigidity.

Lyso-phosphatidylcholine	5.6
Sphingomyelin	12
Disaturated phosphatidylcholine	5.2
Phosphatidylcholine (other species)	21
Disaturated phosphatidylethanolamine	3.5
Phosphatidylethanolamine (other species)	18
Phosphatidylserine + phosphatidylinositol	14.4
Lyso-bis phosphatidic acid (BMP)	0.6
Cholesterol	14.1
Diglyceride	5.6

Table 3 Typical lipid composition of mast cell (RBL-2H3)-derived exosomes
Values are in mole percent of total. Note the presence of saturated phospholipids (8.7% of total), the almost 1/1 ratio between sphingomyelin and cholesterol, and the low amount of LBPA (<1%).

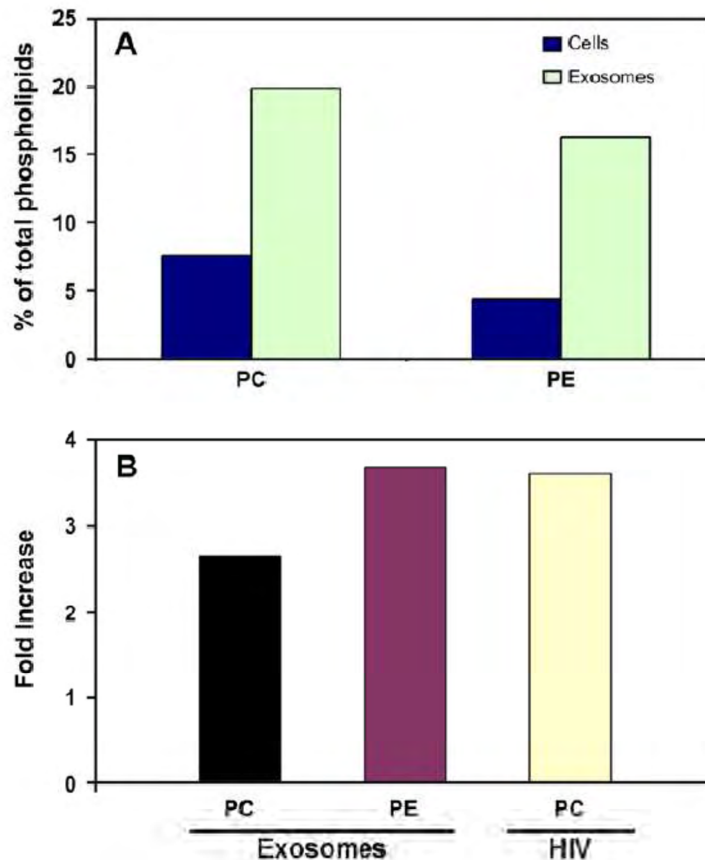

Figure 20 : Exosomes and HIV are enriched in saturated phospholipids.
(A) Respective content of disaturated species (16:/16:0 and 16:0/18:0, [14]) in PC and PE from mast cells (RBL-2H3) and their exosomes. **(B)** Fold increase of disaturated species in exosomes (calculated from **" A")** and HIV (value reported in [21]) comparatively to parent cells.

a.2. Membrane organisation.

Remarkably membrane rigidity of RBL-derived exosomes increases between acidic to neutral pH (Laulagnier *et al.*, 2004b), suggesting that some membrane reorganisation occurs when exosomes are released out of the MVB in the extracellular medium. Exosomes in a neutral pH environment thus feature a tight lipid packing on their surface but an elevated transmembrane movement of lipids [14]. Such a rapid flip-flop is not in favour of an asymmetrical distribution of phospholipids between the two membrane leaflets. Effectively, we have observed an equal distribution of PE between the two leaflets of exosome membrane (Laulagnier *et al.*, 2004b), whereas it is well-known that they are enriched in the plasma membrane inner leaflet in cells (Zwaal & Schroit, 1997).

Interestingly, proteomic analysis of RBL-derived exosomes unravelled the presence of a phospholipid scramblase (C.S, M.R., *unpublished data*), which is involved in the mixing of phospholipids between membrane leaflets (Zwaal & Schroit, 1997). No translocase, involved in the active maintain of PE and PS (Zwaal & Schroit, 1997) in the inner leaflet was detected. These results indicate that not only PE, as we reported, but also all the other phospholipids, are probably randomly distributed between the two membrane leaflets of exosomes.

Noteworthy, the absence of translocase was also reported in reticulocyte-derived exosomes, even though PE displayed an asymmetrical distribution in these exosomes (Vidal *et al.*, 1989), similar to what occurs in reticulocyte plasma membrane. It was concluded that PE were maintained in the inner leaflet because of the high curvature of membrane exosome, or *via* interaction with proteins present in the lumen of the vesicles. Because of their phospholipid composition which ressembles that of plasma membrane (Table 2) and the presence of a phospholipid asymmetry, reticulocyte-derived exosomes appear quite different from those released from immunocompetent cells, and more specifically those derived from the mast cell line RBL-2H3 cells.
However when making a ratio between the proportion of the same phospholipid present in exosomes and their parent cells, sphingomyelin is the only one to increase in reticulocyte as well as in immunocompetent exosomes (Table 2). Exosomes might be a new type of sphingomyelin domain. It has been shown that sphingomyelin only-rich domains are present in Jurkat T cells plasma membrane and are distinct from SM/Cholesterol/GM1 enriched domains called « rafts » (Kiyokawa *et al.*, 2005).

b) Cellular lipidomics

This part of the review will consider the cellular origin of lipids recovered in exosome composition.

b.1. Sphingomyelin :

Cross-linking of plasma membrane sphingomyelin (SM) by an appropriated probe (lysenin) triggers calcium influx and ERK phosphorylation, but not tyrosine phosphorylation, indicating that these domains could be specific signalling platforms (Kiyokawa *et al.*, 2005). Since plasma membrane are the cellular compartment with the higher SM content, exosome SM enrichment could originate from plasma membrane, because plasma membrane lipid rafts (SM/Cholesterol/GM1domains) can be endocytosed and reach the late endosomes (Simons & Toomre, 2000), corresponding to MVBs.

We have approached the analysis of lipid flux from parent cell compartments towards exosomes by means of fluorescent lipids [15]. In our conditions NBD-PC labelled only the plasma membrane of RBL cells and was weakly recovered in exosomes upon ionomycin-induced degranulation (Laulagnier *et al.*, 2005). Instead lipid fluxes from the Golgi and from the MVB limiting membrane appear as the main pathways to supply lipids in exosome biogenesis. This is in favour of a direct flux of SM from Golgi to exosomes via the MVB compartment.
In addition, the increase of SM in exosomes appears to occur at the expense of PC (Table 2). In that respect, sphingomyelin synthase (SMS) can potentially regulate in opposite direction the levels of ceramides and diacylglycerols (Luberto & Hannun, 1998) which can be used by the CDP-choline phosphotransferase to give sphingomyelins or phosphatidylcholines respectively. By driving the phosphocholine pool towards catalytic reaction with ceramide

instead of diglycerides, the SMS could account for the high level of SM in exosomes, at the expense of PC. Precisely, the SMS has been located in the Golgi (Schweizer *et al.*, 1994).

b.2. Lyso-bis Phosphatidic acid (Bis(monoacylglycero)phosphate):

The biogenesis of exosomes occurring at the level of a cell compartment enriched in LBPA (Kobayashi *et al.*, 1998b), namely the MVBs, attention has been focused of this fascinating lysolipid which seems to arrive from nowhere between early and late endosomes. In addition this compound tends to accumulate in organelles derived from MVBs, i.e multilamellar bodies (Mobius *et al.*, 2003). LBPA, also called BMP for bis(monoacylglycero)phosphate, rises from 1% to 15% of total phospholipids between early and late endosomes of BHK cells (Kobayashi *et al.*, 1998b). What metabolically occurs between these two compartments ? Comparison of their phospholipid composition shows a decrease in SM content (-5.8%), PE (-4.7%) and PS (-4.6%) in late endosomes, thus giving no special clue on how this lysolipid could be formed. Phosphatidylglycerol (PG), which has been shown to be the BMP (LBPA) precursor (Waite *et al.*, 1987), has not been measured in endosomes from BHK cells. Fatty acids (mainly oleic acid and docosahexaenoic acid) (Luquain *et al.*, 2000) which esterify the glycerol backbones appear to be brought by poorly characterized transacylases, directly at the sn-2 position. Degradation pathways in cells are also unknown since this compound appears to be refractory to *in-vitro* lipolytic enzyme attack (Kobayashi *et al.*, 2002). However conformation of the fatty acids might hide the polarity of the phosphate headgroup since LBPA migrates close to neutral lipids on thin layer chromatography (Kobayashi *et al.*, 1998b). Such a conformation could impair some lipolytic enzyme attacks. In addition, LBPA has been claimed as the only phospholipid in cells with an « unnatural » configuration (Joutti, 1979), being assigned to be in a sn1-sn1’ configuration when « natural » phospholipids exhibit a sn-3 configuration. Analysis of the stereoconfiguration of this lipid has to be revisited since the rationale on investigating that point by several authors a few decades ago is not clear (Joutti, 1979). In addition, an isomerase activity is required to obtain a 1-1’ LBPA compound from natural PG, and such isomerase has never been characterized. In any case a 1-1’ compound and a 3-3’ one should display a similar three-dimensional shape since both molecules exhibit the same symmetrical axis. In that respect, although the most efficient LBPA molecule able to trigger intraliposomal vesicle formation at acidic pH (pH 5.5, similar to pH of MVBs) has been reported as being a 1-1’ LBPA esterified at the sn-2 position (Matsuo *et al.*, 2004), a 3-3’ LBPA esterified also at the sn-2 position would certainly have been as efficient[a].

Since this peculiar phospholid appears at the step of MVB formation along the endocytic pathway, it certainly plays a key role in the dynamic of that compartment (Le Blanc *et al.*,

[a] For stereoconfiguration, we refer to the nomenclature based on the position of the phosphate moiety linked to the position 3 or 1 of the glycerol backbone. The works reported in references 33 and 35 refer to the stereoconfiguation of the carbon 2 of the glycerol.

2005). However the efficiency of such compound to trigger exosome biogenesis in cells remains to be established.

b.3. Cholesterol:

LBPA has been proposed to interact with cholesterol (Kobayashi *et al.*, 1999) and the effect of this neutral lipid in exosome biogenesis should also require attention. In the « RN » B cell line, MVB displays a high content in cholesterol and a low content in LBPA, the respective proportions of the two lipids becoming opposite in Multilamellar Bodies (Mobius *et al.*, 2003). Except that cell line, the cholesterol/phospholipid ratio is similar between parent cells and exosomes derived from either mast cells, dendritic cells or reticulocytes (Vidal *et al.*, 1989; Laulagnier *et al.*, 2004b). In that case also, cholesterol might remain on the MVB limiting membrane once the intralumenal vesicle has been pinched off.

b.4. Lysophosphatidylcholine:

On the reverse, Lysophosphatidylcholine (LPC) present in cells is also recovered on exosomes from dendritic cells or RBL mast cells, and accounts for about 10 % of phospholipids (Laulagnier *et al.*, 2004b). As depicted in Figure 21, the generation of LPC on MVB outer layer accounts for the membrane curvature required to make the neck of the vesicle, since the planar surface occupied by the LPC polar head group is higher than the surface of the fatty acid. Exosome thus appears as a new vector for LPC. Concentrations between 10-40 µM of LPC bound to serum albumin have been reported to trigger cell response (Kabarowski *et al.*, 2001). A recent work has established the presence of circulating exosomes in blood, with an average amount of 60µg protein for 300 ml of blood (Caby *et al.*, 2005). In our hands this amount of exosome represents 20 nmoles phospholipids, which would carry around 2 nmoles of LPC (Table 2). Such an amount of LPC for 300 ml of blood is equivalent to a concentration of about 7 nM. Therefore exosomes would represent a minor source of circulating LPC.

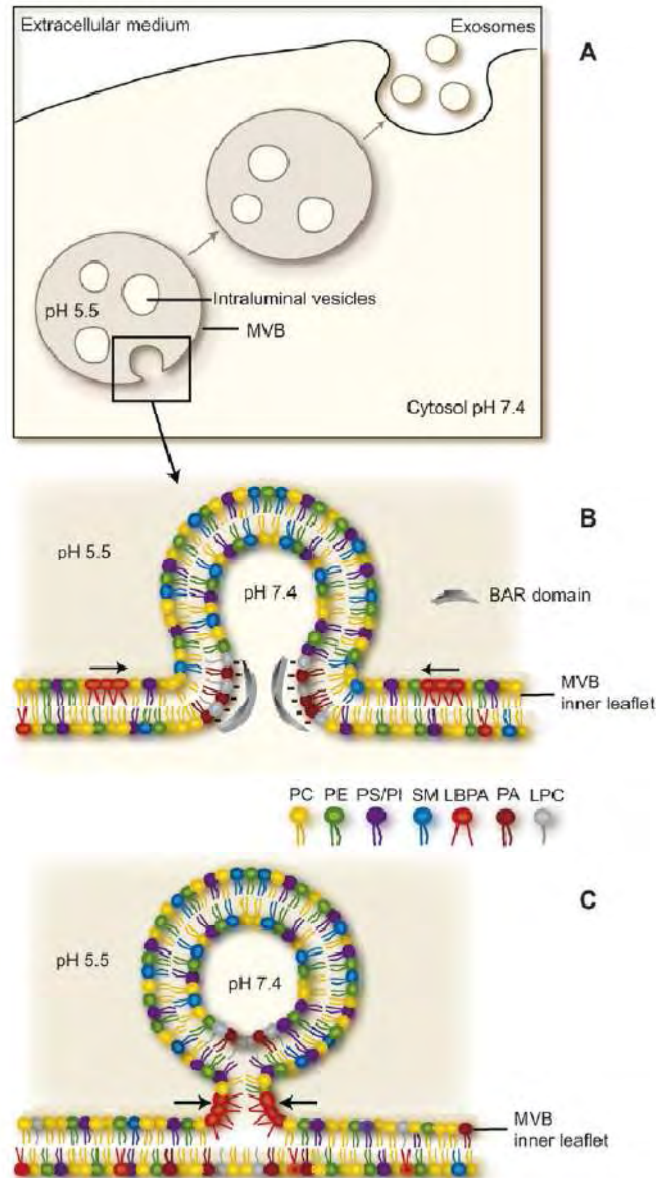

Figure 21 : **Exosome biogenesis in cell and at the molecular level inside the MultiVesicular Body (MVB) compartment.**

(A). Intralumenal vesicles, precursors of exosomes, are generated inside the MVB compartment (late endosomes), corresponding to secretory granules in hematopoïetic cells. Details of the biogenesis of the intralumenal vesicles are depicted in B and C. The budding vesicle membrane is based on the exosome lipid composition and the absence of phospholipid transmembrane asymmetry reported for exosomes derived from rat mast cells (RBL-2H3) and human dendritic cells (DC) [14].

(B).The donor membrane for the budding vesicle is the MVB membrane, whose composition is based on data reported in BHK cells [30]. We postulated that no phospholipid asymmetry was present in this membrane. The initial event triggering vesicle budding would be a higher molecular area of LBPA molecules which organize in clusters at acidic pH (see arrows), creating an excess of lipids on the MVB inner leaflet. Lipid sorting during that process would select more SM, less PC and about the same amount of PE and PS+PI, whereas negatively charged lipids (LPC, PA) with the help of positively charged BAR domains from appropriated proteins, trigger MVB outer leaflet bending at the neck of the vesicle.

(C). Fission of the vesicle occurs by the mixing of each leaflet induced by fusogenic lipids, PA and LPC on one leaflet, and by LBPA accumulated on the other leaflet at the neck of the vesicle. LBPA would remain on the MVB membrane after separation of the vesicle since no enrichment of this lipid has been found on immunocompetent-derived exosomes [14].

c) Functional lipidomics

This part will consider the dynamic of lipids involved in exosome biogenesis and the role of some lipolytic enzymes on their biological functions.

However exosome-bound LPC might act efficiently in target cells such as dendritic cells (DC). Exosomes appear to be endocytosed by immature dendritic cells and to enhance both cell maturation and antigen presentation (Thery *et al.*, 2002). Mature DC cells will then release new exosomes more efficient than those originating from immature cells (Segura *et al.*, 2005a). It has been shown that LPC can trigger DC maturation (Perrin-Cocon *et al.*, 2006), and it is conceivable that exosome-bound LPC could participate in that process. Also exosome-bound LPC could trigger lymphocyte chemotaxis via the G protein coupled receptor named « G2A » (Kabarowski *et al.*, 2001), and this could also account for exosome-mediated activity of T cells in lymph nodes. The membrane-bound LPC on exosomes, although representing a limited amount, could display a « bioactivity » more efficient than the « soluble » LPC: exosomes could thus be considered as « vehicules of particulate agonists ».

These « vehicles » appear very efficient when injected in a whole organism and it has been suspected that MFG-E8/lactadherin which binds strongly phosphatidylserine and which is highly enriched in exosomes (Veron *et al.*, 2005) could drive them towards target cells. However, invalidation of lactadherin gene has not evidenced a key role of PS/lactadherin in attracting exosomes to appropriated sites (Veron *et al.*, 2005).

Since the recent work of Caby et al. (Caby *et al.*, 2005) it is clear there is a circulating network of exosomes in the organism, organizing distal communication between cells. Positive aspects of this network are for example the enhancement of immune response or the maturation of reticulocytes (Geminard *et al.*, 2004), one negative aspect being that the « exosome pathway » is « hijacked » by pathogens, infectious (HIV (Gould *et al.*, 2003), mycobacterias (Beatty *et al.*, 2001)) or not (prion (Fevrier *et al.*, 2004)). Therefore regulating the exosome biogenesis appears critical to either enhance their production in order to boost the immune response towards tumors for example, or to block their production in order to stop the spreading of infectious or prion diseases. The knowledge in the protein machinery involved in exosome biogenesis (Gruenberg & Stenmark, 2004) is by far much more advanced than the lipids participating to that process. However all protein interactions lead to lipid modifications of the bilayer, in order to promote both the bending of the membrane and the biogenesis of fusogenic lipids. Subsequent fusion membrane of the opposite bilayers will occur at the level of the neck of the vesicle to trigger its removal from the parent membrane. A possible process of pre-exosome vesicle biogenesis, i.e the intralumenal vesicles generated inside the MVB, is represented in Figure 21.

The initiation event for the vesicle formation relies on the observation of Matsuo et al (Matsuo *et al.*, 2004), who showed that in a giant liposome with a similar size (600nm) and composition as the MVB (i.e containing 15 moles per cent of LBPA), adjusting the intraliposomal pH down to 5.5 (pH of the MVB) induces spontaneously the formation of small vesicles inside the giant liposome. With a neutral intraliposomal pH, nothing occurs. An hypothesis would be that LBPA molecular area increases at acidic pH and the lipid become

organized in clusters, inducing an excess of lipids on the inner leaflet of the liposome, with subsequent budding of this inner leaflet. This budding will then drive the liposome outer leaflet to form a vesicle. This process will eliminate the excess of lipids on the inner leaflet of the donor membrane and reequilibrate the lipid content of the two leaflets. Effectively in a vesicle of 60nm diameter (the average size of an intralumenal vesicle or an exosome), two thirds of the lipids are present on the outer layer [14]. It should be borne in mind that the inner leaflet of the MVB membrane becomes the outer leaflet of the exosomes membrane (Figure 21) In MVB like in giant liposomes, internal vesicle budding will then remove two times more lipids from the inner leaflet than from the outer one. When considering the three dimensional aspect of this process, inner leaflet LBPA has to be self-organized in such a way it triggers a tubule formation of 60 nm wide, which will be further « cut » by constriction to form a vesicle released by fission from the parent membrane. Tubule generation on liposomes has been shown to be triggered by proteins such as kinesin (Roux *et al.*, 2002) or endophilin (Huttner & Schmidt, 2002); thus the effect of LBPA alone would be quite unique. Since this lipid spontaneously triggers vesicle formation in a lipid phase, this process has to be controlled in cells. Effectively various LBPA interacting proteins have been described. The protein Alix (Matsuo *et al.*, 2004), present in cytosol of BHK cells inhibits the formation of vesicles when added to the outside of LBPA-containing liposomes. Similarly another LBPA-interacting protein, Saposin C (Chu *et al.*, 2005), inhibits MVB formation in human fibroblasts. Therefore, Alix and Saposin C interaction with LBPA appear to regulate the intralumenal vesicle formation in MVBs.

The second step is the constriction of the neck of the vesicle and its release. Separate events occur on each leaflet. In cells, mixing of the MVB outer leaflet at the level of the neck of the vesicle (Figure 21B) might involve lipid mediators such as phosphatidic acid (PA), and lyso-PC (LPC). These negatively charged lipids will allow BAR domain-containing proteins to bind and to bend the membrane in order to accelerate the process of vesicle invagination (Huttner & Schmidt, 2002). Because PA is a fusogenic lipid in presence of calcium (Harsh & Blackwood, 2001) it can help the mixing of opposite bilayers. As a consequence, the LBPA clusters in the MVB inner leaflet would accumulate at the neck of the vesicle (Figure 21C) thus reaching the critical concentration allowing its fusogenic properties. LBPA has been shown to be fusogenic at pH 5.5 in membranes containing 20-30% of the lipid (Kobayashi *et al.*, 2002). Consequently, the vesicle will then be released out of the parent membrane, and the transmembrane distribution of lipids will be re-organised to equilibrate with two-thirds of them on the outer layer of the vesicle.

Intralumenal vesicle formation thus requires enzymatic modification of lipids on the MVB membrane. Interplay between PLA2 and PLD has been observed in cells (Harsh & Blackwood, 2001). The presence of a calcium-independent PLA2 has been reported in reticulocyte-derived exosomes (Bette-Bobillo & Vidal, 1995), and we also observed a PLA2 activity (Laulagnier *et al.*, 2004a) in mast cell (RBL-2H3)-derived exosomes. The role of PA appears to depend upon the type of enzyme generating this mediator. Inhibition of Diacylglycerol (DG) kinase is required to enhance Jurkat T cell-derived exosomes (Alonso *et al.*, 2005), whereas

we have reported that overexpression of an active PLD2 multiply by two the amount of exosomes produced by the mast cell line (Laulagnier *et al.*, 2004a). This differential effect of PA producing-enzymes might depend upon the molecular species of PA generated. Interestingly, some of the lipolytic enzymes involved in intralumenal vesicle formation will be trapped inside exosomes, then released out of the cell and later on in the blood stream. Exosomes thus appear as vehicles for lipolytic enzymes.

Beside lipolytic enzymes, exosomes contain proteins participating in vesicle formation and trafficking. As an example they contain small G proteins and the LBPA-binding protein Alix [18]. GTPase might activate lipolytic enzymes, and LBPA concentration might control the sorting function of Alix. The lipid flux during exosomes biogenesis regulates the type and the amount of protein which can be inserted in the nascent exosome bilayer. Therefore regulating lipid exosomes biosynthesis appears as a key to either stimulate exosome production in order to boost the immune response, or on the opposite block their production in the case of HIV infection.

3. Conclusion:

Exosomes are a new type of bioactive vesicles which are suspected to play a key role in many biological responses in the body (Couzin, 2005). On model membranes, LBPA (BMP) seems to be essential for their biogenesis, but this requires confirmation in cells. In any cases, a better understanding on the metabolism of this lipid is necessary. Differences in exosomes lipid composition and membrane organisation have appeared between reticulocyte and immunocompetent cell-derived exosomes. Lipid composition of exosomes might in fact condition their fate and function in the body. An exhaustive lipidomics study of exosomes from various sources appears necessary for a better understanding on how these vesicles operate in the organism and how their biogenesis pathway can be « hijacked » by pathogens.

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Abreviations:

BAR : Bin/Amphiphysin/Rvs ; CHOL : cholesterol ; DC : dendritic cells ; LBPA (BMP) : Lysobis-phosphatidic acid (Bis(monoacylglycero)phosphate) ; LPC : lysophosphatidylcholines ; MHC II : Major Histocompatibilty Complex II ; MVB: MultiVesicular Bodies; EthLi : ether lipids ; PLIP : phospholipids ; PA : phosphatidic acid ; PC : phosphatidylcholines ; PE : phosphatidylethanolamines ; PG : phosphatidylglycerol ; PS : phosphatidylserines ; PI : phosphatidylinositols ; SM :sphingomyelins.

II. LBPA and Cholesterol

As reported previously in our review on exosome lipidomics, the lysobisphosphatidic acid or bis(monoacylglycero)phosphate is an essential component of the late endosomes, a cellular compartment involved both in exosome formation and HIV biogenesis.

A. LBPA

1. Structure and composition

Lysobisphosphatidic acid (LBPA), also commonly referred as bis(monoacylglycero)phosphate (BMP) is a structural isomer of its precursor, the lysophosphatidylglycerol, and exhibits an unusual sn1-glycerophospho-sn-1'-glycerol (sn:sn1') stereoconfiguration regarding the phosphate moiety on its two glycerol units. That differs from the typical sn-3 glycerophospahte configuration found in other glycerophospholipids. (Hullin-Matsuda *et al.*, 2009a) (Figure 22)

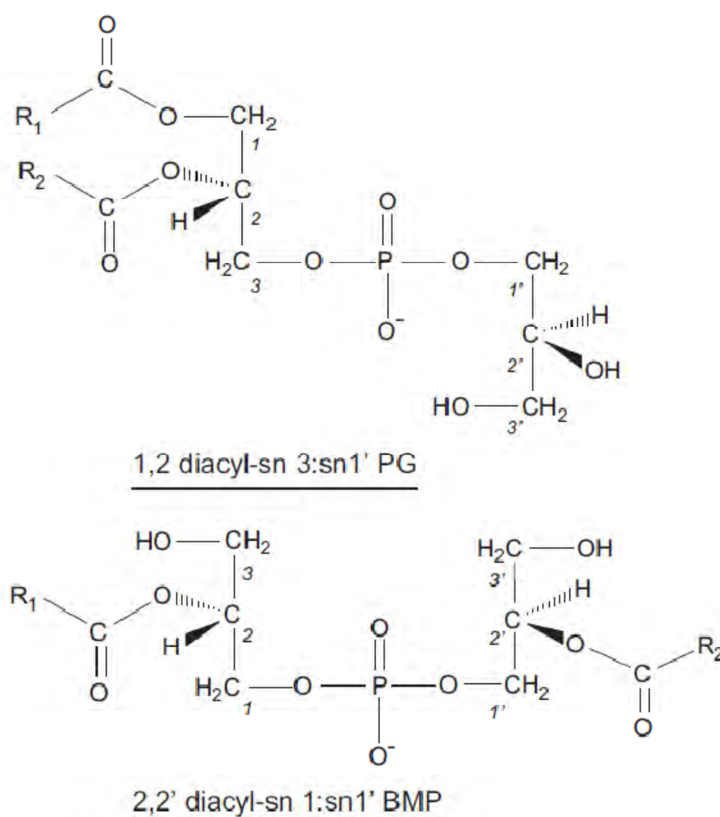


Figure 22 : Structure of Phosphatidylglycerol (PG) and Bis(monoacylglycero)phosphate (BMP). PG is converted into lyso-PG by a phospholipase A2. Complete biosynthesis pathway is reported later. Adapted from (Hullin-Matsuda *et al.*, 2009a)

This phospholipid is composed of a high proportion of oleic acid and among polyunsaturated fatty acids, it incorporates selectively docosahexaenoic acid (22:6n-3 DHA) (Luquain *et al.*, 2000; Besson *et al.*, 2006; Hullin-Matsuda *et al.*, 2009a)

2. Cellular distribution

This unconventional phospholipid represents about 1% of the total phospholipids in cells. Several studies have described LBPA localization mainly in the endocytic track. The group of Gruenberg, using a specific monoclonal antibody (mAb 6C4), found it particularly enriched in the internal membranes of late endosomes in baby hamster kidney (BHK) cells since it account for 15% of the phospholipids of this organelle (Kobayashi *et al.*, 1998b), although the mAb 6C4 recognizes only the lipids from the lumen side of the endosomes (Kobayashi *et al.*, 2001). Late endosomes contain raft lipids domains in which LBPA can be recovered (Sobo *et al.*, 2007). While Besson et al. later confirmed its presence in late endosomes in the monocytic cell line THP-1 (Besson *et al.*, 2006), others found LBPA localized rather at the level of lysosomes (Mobius *et al.*, 2003; Schulze *et al.*, 2009) (Figure 23). However, its precursor, the PG is absent in this compartment.

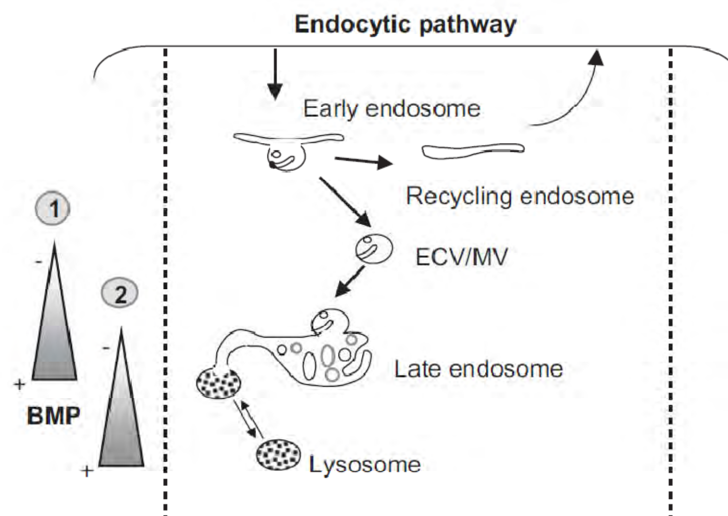


Figure 23 : Representation of the endocytic pathway and LBPA distribution. Following endocytosis, proteins and lipids undergo an efficient sorting in the first compartment, the early endosome. The fate of sorted proteins is i) to be recycled and the recycling endosome allow them to reach the plasma membrane or ii) are delivered to late endosome via multivesicular bodies (MV or ECV for endosomal carrier vesicle) where they are sorted for cellular distribution or for being ultimately degradated into lysosome. Two research groups (Kobayashi *et al.*, 1998b) and (Schulze *et al.*, 2009) claim either an enrichment of LBPA from ECV/MV to late endosomes (1), or from late endosomes to lysosomes (2). Adapted from (Hullin-Matsuda *et al.*, 2009a)

Some variety of subpopulation of MVB may exist, containing or not LBPA (White *et al.*, 2006), leading to the formation of intralumenal vesicles (ILVs) which contained or not LBPA. Subsequently, exosomes might contain or not this compound. These two situations were reported in the literature (Stoorvogel *et al.*, 2004; Laulagnier *et al.*, 2005).

LBPApresent in ILVs is implicated in the control of the back-fusion of ILV with the limiting endosomal membrane and in the regulation of degradative processes within the lysosomal pathway (Kolter & Sandhoff, 2005; van der Goot & Gruenberg, 2006).
It has been underlined that long-time serum starvation induces autophagy and allow to augment LBPA content (Luquain *et al.*, 2000).
It has been demonstrated that LBPA can organize in a highly structured and clustered manner. At acidic pH it forms small lamellar vesicles structures that make LBPA potently involved in the budding of vesicles in endosomes. Also the morphology and size of hydrated lipid dispersion of LBPA mixed with gangliosides GM1 (a glycosphingolipid present in the cell membrane and engulfed in the endocytic track for degradation) has been assessed. Given a precise percentage of GM1 mixed with BMP (20-30%), it has been observed interactions between the two lipids which spontaneously form spherical vesicles about 100nm, homogenous in size in a pH of 5.5 similar to that of late endosomes. These findings sustain a role in LBPA in intravesicles formation in multivesicular bodies. (Chebukati *et al.*, 2010).
A role of LBPA has been underlined in the glycosphingolipids metabolism since it enhances the degradation of lysosomal sphingomyelin by activating the acid sphingomyelinase. Recently, a binding of LBPA with Hsp70 has been depicted to facilitate LBPA effects on acid sphingomyelinase (Kirkegaard *et al.*, 2010). Also, it has been reported to enhance the activity of lysosomal acid lipase in vitro (Makino *et al.*, 2006).

3. LBPA biosynthesis and phospholipases involvement

Phosphatidylglycerol has been established as one of the endogenous precursor for the de novo synthesis of LBPA (Hullin-Matsuda *et al.*, 2007).

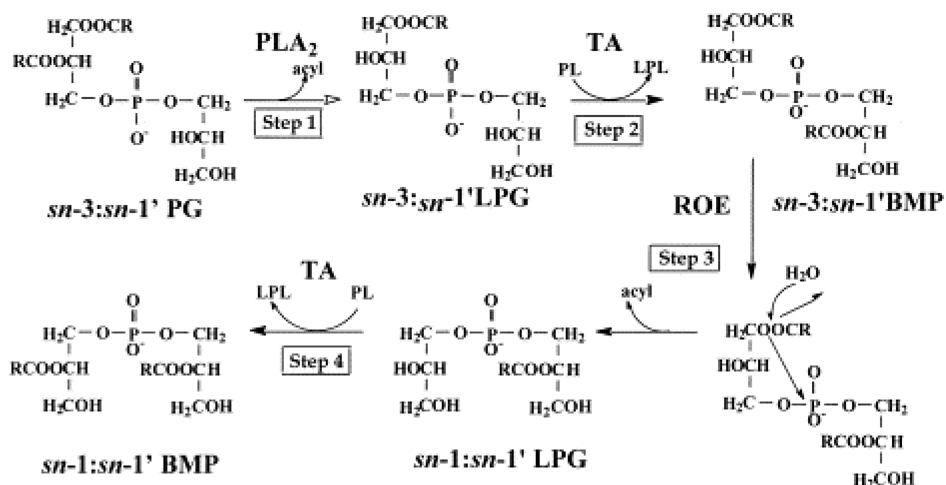


Figure 24 : Bis(monoacylglycero)phosphate (BMP/LBPA) biosynthetic pathway. Adapted from (Heravi & Waite, 1999)

Previous studies from Heravi et al. proposed a biosynthetic pathway of BMP involving a lysophosphatidylglycerol (LPG) phosphate as intermediate and suggested that biosynthesis occur at the endosomal level (Figure 24). They described a transacylase isolated from a macrophage cell line, acting at acidic pH which catalyses the acylation of 2-LPG leading to the formation of the sn3-sn1' bis(monoacyglycero)phosphate (BMP) (Heravi & Waite, 1999).
In this scheme (Figure 24), the first step involves a phospholipase A_2 (PLA_2) to form 1-acyl-sn-glycero-3-phospho-*rac*-glycerol (LPG). The second step consists in acylation of the glycerol of one sn3:sn1'LPG with an acyl group from a donor phospholipid. The next steps allow re-orientation of the phosphorylester from the sn-3 to the sn-1 position and the removal of one acyl chain. The final step involves a transacylase (TA) which adds an acyl chain to form the 2,2'diacyl sn-1:sn1'BMP. Later on, the authors characterized from a macrophage cell line, a lysosomal PLA_2 responsible for the first step of LBPA biosynthesis involving a cleavage of the 2-acyl group from PG. This enzyme was PG-specific, acting in an optimum acidic pH and displays also intrinsic PLA_1 activity. It is worth noting that this PLA2 was strongly inhibited by the cytosolic PLA2 inhibtor MAFP (Shinozaki & Waite, 1999).

More recently, Hullin et al. confirmed that phosphatidylglycerol is the endogenous precursor generated by the mitochondrial enzyme phosphatidylglycerophosphate (PGP) synthase (Hullin-Matsuda *et al.*, 2007). In knock-out mice for PGP synthase, compared to wild type mice, PG synthesis was lowered, inducing a decrease in BMP cell content. However only half of the BMP content was abolished, indicating that another biosynthesis pathway was present. Transfection of a PGP synthase in these mutant cells, restored and induced a 2-fold increase of the BMP synthesis. In the same way, it has been depicted that an exogenous PG supplementation led to LBPA accumulation in a macrophage cell line (Bouvier *et al.*, 2009) and that the fatty acid composition of LBPA relies on fatty acid composition of its PG precursor.

One can propose that LBPA could be formed from two others putative precursors : the Bis(diacylglycero)phosphate (BDP) and the hemi(BMP) (Figure 25).
The Bis(diacylglycero)phosphate (BDP) can be obtained by the transphosphatidylation activity of a Phospholipase D (van Blitterswijk & Hilkmann, 1993)(see below). Hemi(BMP) can be formed by a phospholipase A_2 activity from BDP, or following acylation of PG.

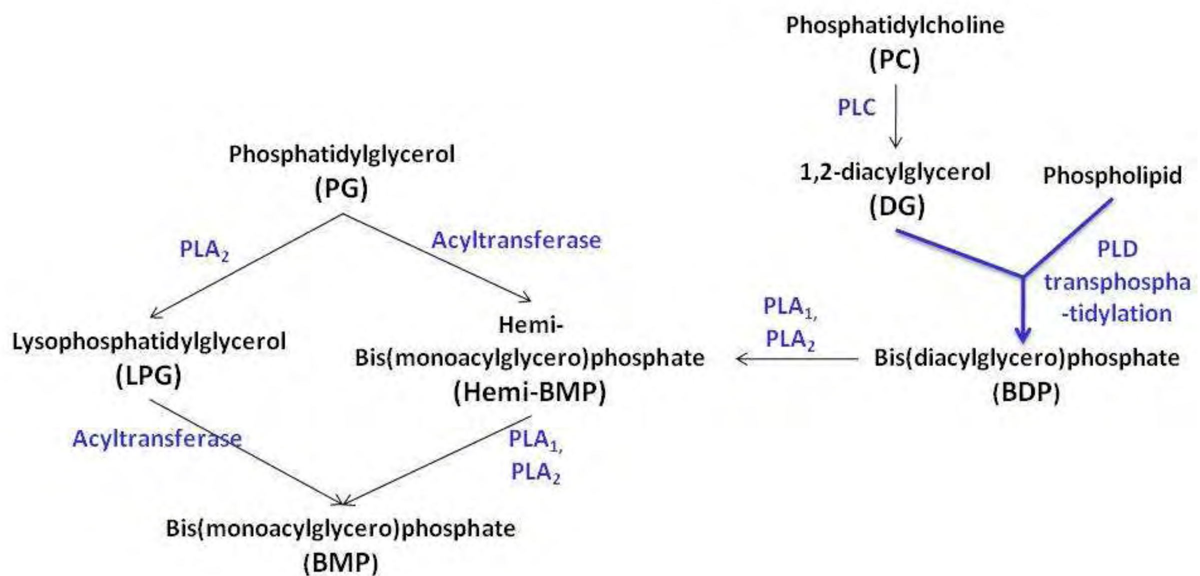


Figure 25 : putatives Bis(monoacylglycero)phosphate Biosynthesis Pathway

It has been recently highlighted acyltransferase (ALCAT1) catalyzing acylation of bismonoacylphosphatidylglycerol and other polyglycerophospholipids such as lysophosphatidyglycerol and cardiolipin (Cao *et al.*, 2009) suggesting an involvement of this enzyme in BMP metabolism.

Because of its unusual configuration, BMP appears resistant to phospholipases degradation albeit it has to be degraded since in normal conditions there is no accumulation of the compound (Matsuzawa & Hostetler, 1979; Schulze *et al.*, 2009).
However, It has been shown that a lysosomal phospholipase could weakly degraded LBPA (Matsuzawa & Hostetler, 1979; Hullin-Matsuda *et al.*, 2009a) into Lysophosphatidylglycerol, and into lysophosphatitic acid and monoglyceride by a lysosomal phosphodiesterase. Phospholipases A_2 able to degrade LBPA have been highlighted with an acidic optimum pH (Ito *et al.*, 2002; Abe & Shayman, 2009)
Bouvier et al. shown that increase incorporation of docosahexaenoic acid (DHA) in LBPA led to its degradation in oxidants conditions most likely due the formation of diDHA molecular species and its subsequent susceptibility to be peroxidized suggesting a protective antioxydant role of BMP for the neighboring lipids (Bouvier *et al.*, 2009).

4. Functions

Chevallier et al. recently reported the requirement of LBPA in the endosome biogenesis under the control of Alix/AIP1. Alix is a well-known participant into the multivesicular bodies sorting and interacts with LBPA (Chevallier *et al.*, 2008). Alix and LBPA play a role in

intraluminal vesicle fission from and fusion with endosome limiting membrane (Abrami *et al.*, 2004; Matsuo *et al.*, 2004; Le Blanc *et al.*, 2005)
It was shown that knocking down Alix induces a decrease of the BMP (LBPA) level and reduces the intraluminal vesicles number in late endosomes, concomitant with a decrease of cellular cholesterol rate (Chevallier *et al.*, 2008) without affecting LDL endocytosis and cholesterol synthesis. Authors explained the decrease of cellular cholesterol by an incapacity of MVB to store and retain cholesterol given the reduced amount internal membranes, suggesting that LBPA controls the cholesterol capacity of endosomes. The exogenously addition of LBPA in Alix Knocked down cells restores the internal vesicles and cellular cholesterol level. The Niemann-Pick type C (NPC) disease is characterized by a cholesterol storage disorder due to mutations in NPC1 or NPC2 gene. In tissues of patients, the cholesterol accumulation is concomitant with LBPA. However, Chevallier et al. brought the evidence that LBPA is limiting in NPC cells since exogenous LBPA addition can reverse the cholesterol accumulation in late endosomes (Chevallier *et al.*, 2008).

It was demonstrated that LBPA can distribute in a highly structured and clustered manner. At acidic pH it forms small lamellar vesicles structures that make LBPA potently involved in the budding of vesicles in endosomes. The morphology and size of hydrated lipid dispersion of LBPA mixed with gangliosides GM1 has been assessed. Given a precise percentage of GM1 mixed with BMP (20-30%), it has been observed interactions between the two lipids which form spherical vesicles about 100nm, homogenous in size in a pH of 5.5. These findings sustained a role of LBPA in intravesicles formation in multivesicular bodies (Chebukati *et al.*, 2010). A link with the saposin C was reported underlining the role of LBPA in the MVB formation (Chu *et al.*, 2005). This hypothesis was previously underlined by Matsuo et al. that shown the ability of LBPA to induce the intraluminal vesicles formation into liposomes with an acidic lumen (Matsuo *et al.*, 2004).

A role of LBPA has been underlined in the glycosphingolipids metabolism since it enhances the degradation of lysosomal sphingomyelin by activating the acid sphingomyelinase. Recently, a binding of LBPA with Hsp70 has been depicted to facilitate LBPA effects on acid sphingomyelinase (Kirkegaard *et al.*, 2010). Also, it has been reported to enhance the activity of lysosomal acid lipase in vitro (Makino *et al.*, 2006).

B. Cholesterol

In the organism, cholesterol is provided by the food and by its biosynthesis by the various organs, mostly the liver. Cholesterol is a steroid transported in the blood and which participates in cell membrane architecture. Cholesterol plays a major role in membrane structure and fluidity and it is the precursor of many biologically active molecules such as steroid hormones, bile acids, vitamin D (Charlton-Menys & Durrington, 2007; Storch & Xu,

2009). In membrane, cholesterol associates preferentially to phosphatidylcholine and phospholipids bearing saturated chains creating sterol-phospholipids complexes. This association allows to condense and to order bilayers (Lange & Steck, 2008).

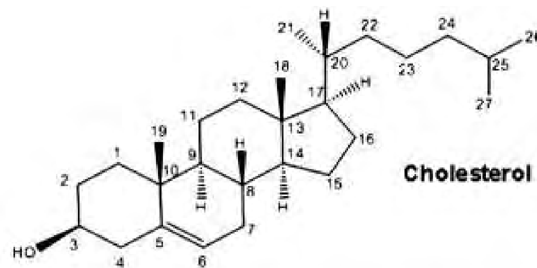
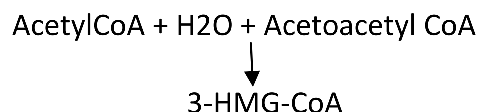


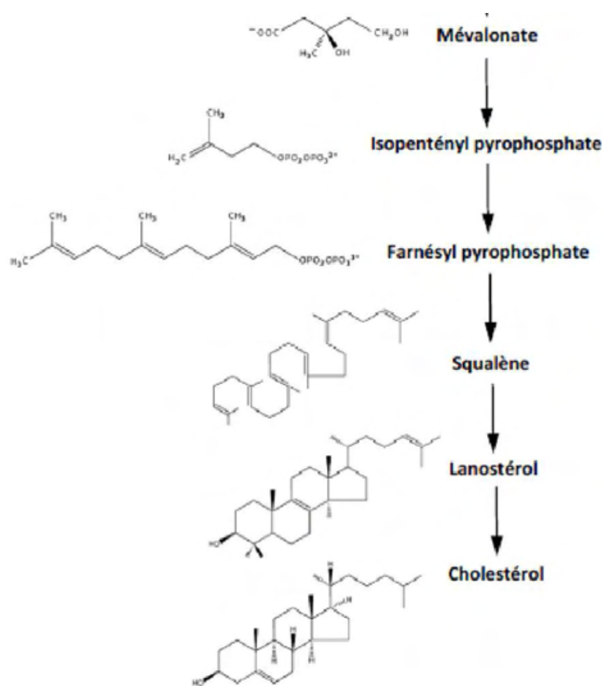
Figure 26 : The chemical structure of cholesterol.

1. Metabolism

Cholesterol can be endogenously synthesized or exogenously taken up via the endocytic pathway and subsequently delivered to endosomal/ lysosomal sites, plasma membrane and the endoplasmic reticulum.

De novo cholesterol synthesis takes place in the endoplasmic reticulum (ER) and then transfer to the plasma membrane. Synthesis starts with one molecule of acylCoA and one molecule of acetoacetyl-CoA which are deshydrated to form 3-hydroxy-3methylglutaryl CoA (HMG CoA). L'HMG CoA reductase is located on the endoplasmic reticulum and catalyses the conversion of HMG-CoA to mevalonic acid





Mevalonate is then converted to 3-isopentenyl pyrophosphate in 3 reactions. 3 molecules of this new compound condense to form farnesyl pyrophosphate through the action of geranyl transferase. Then 2 molecules of farnesyl pyrophosphate condense to form squalene by the squalene synthase. This compound is then cyclized to form lanosterol by the squalene cyclase. Lanosterol is then converted to cholesterol by six successive enzymatic steps.

The exogenously sources of cholesterol are low density lipoproteins (LDL) which are captured by cells by receptor mediated endocytosis of LDL (Storch & Xu, 2009). Exogenous cholesterol borne by LDL enters the cell by the LDL receptor pathway and is transported in the endocytic pathway (the early endosomes, late endosomes and lysosomes) in the form of cholesteryl esters and free cholesterol (Figure 27). The esters are hydrolyzed by an acidic cholesteryl ester hydrolaze activity. Unesterified cholesterol is transferred by the NPC2 protein from the lumen of endosomes the NPC1 protein for further cholesterol transport out of late endosomes towards the plasma membrane and to the Endoplasmic Reticulum (ER). via vesicular systems or specific lipid binding protein named StARS for steroid acute regulatory protein (StAR)-related lipid transfer (Schmitz & Grandl, 2009; Storch & Xu, 2009). Free cholesterol is esterified with fatty acids in the endoplasmic reticulum by the action of the acylcoenzyme A cholesterol acyltransferase (ACAT1), and the cholesteryl esters stored primarily in the cytoplasmic lipid droplets. The cholesteryl ester hydrolase can hydrolyzed cholesterylesters and release free cholesterol (Storch & Xu, 2009).

Excess of free cholesterol (unesterified and phospholipid complexes) can be removed from the cell by effluxes at the plasma membrane by the ATP-binding cassette transporter [(ABCs) ABCA1 and ABCG1] to ApoA-I, ApoE and HDL as extracellular acceptors Figure 27. Cholesterol is then transported to the liver for excretion : this is the reverse cholesterol transport. ABCA1 facilitates cellular cholesterol and phospholipid release to ApoA-I

containing HDL precursors. (Chang *et al.*, 2006; Lange & Steck, 2008; Schmitz & Grandl, 2009). In HDL, free cholesterol is re-esterified by the LCAT (lecithin acyltransferase).
SR-B1 is the receptor for HDL, mediating the transport of cholesterol and cholesteryl esters from HDL particles, expressed in liver and steroidogenic tissues involved in selective uptake of cholesteryl esters from HDL. Lipid transfer binding protein including CETP (cholesterol ester transfer protein) regulate cholesterol trafficking. For instance, CETP mediates the transfer of cholesterylesters from HDL to triglycerides-rich lipoproteins and LDL and inversely.
Synthesis and uptake of cholesterol are regulated via feedback mechanisms mediated by sterol-dependent intracellular localization of SREBP2 (Sterol Regulatory Element Binding Protein). SREBPs are transcription factors that bind to a sterol regulatory element DNA sequence (Schmitz & Grandl, 2009).

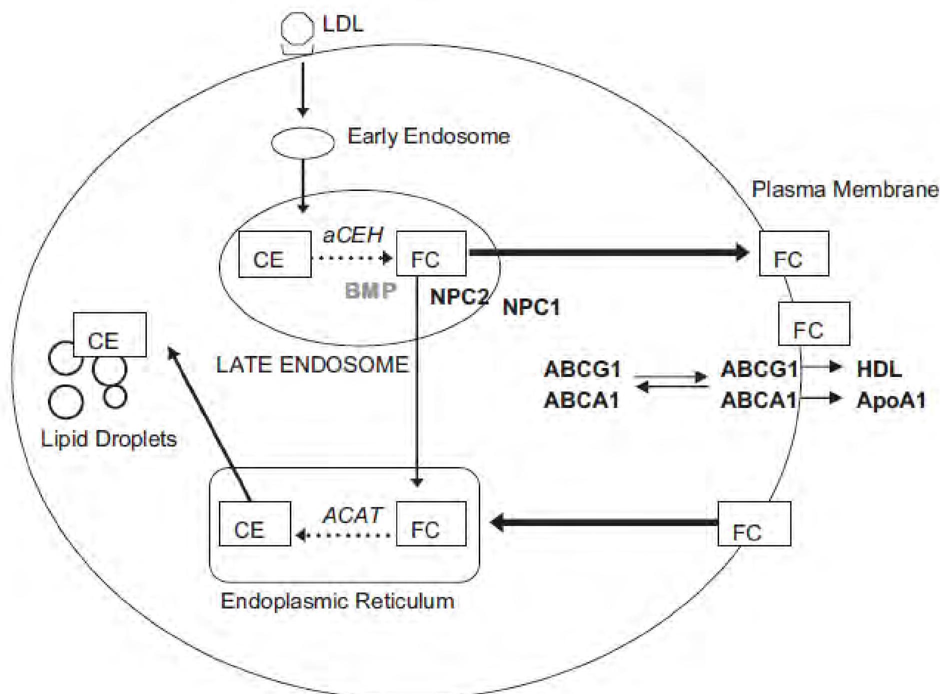


Figure 27 : Intracellular transport of exogenous cholesterol derived from LDL. Following internalization, Cholesteryl esters (CE) are converted into free cholesterol (FC) by an acidic cholesterol ester hydrolase (aCEH). Free cholesterol exits from late endosomes via NPC proteins and are routed to membranes or to endoplasmic reticulum where it undergoes re-esterification by Acyl-coenzyme A cholesterol acyl transferase (ACAT) to be stored in lipids droplets. Excess of free cholesterol can egress the cell through ABC transporters involving extracellular acceptors (HDL and ApoA1) that cycle between plasma membrane and intracellular compartment. Adapted from (Hullin-Matsuda *et al.*, 2009a).

2. Cellular distribution

Cholesterol is abundant in plasma membrane and in the endomembrane pathways i.e. endosomes, lysosomes, the endocytic recycling compartment and distal Golgi elements. (Hao *et al.*, 2002; Maxfield & Menon, 2006; Storch & Xu, 2009).The majority of cholesterol is

found at the plasma membrane although intracellular membranes contain appreciable amounts. Among endosomal membranes, internal vesicles of multivesicular bodies are enriched in cholesterol (Mobius *et al.*, 2003).

In membrane, cholesterol can associate with sphingolipids to form dynamic nanoscale assemblies about 10-20nm named lipid rafts microdomains that associate and dissociate to reorganize and stabilize bioactivity in cell membranes (Lingwood *et al.*, 2009). Rafts can coalesce by specific lipid-lipid, protein-lipid or protein-protein interactions, in activated conditions such as T cell activation in lymphocytes. T cells restructure their cell surface to form membrane domain at T cell receptor signaling site and immunological synapses. Following to a selective lipid sorting (accumulation of cholesterol, sphingomyelin and saturated phosphocholine), rafts appear as an “ordered” membrane. Protein localization into these membrane domains is mediated by a GPI (glycosylphosphatidylinositol) anchor, acylation or certain transmembrane domains. Rafts appear to form dynamic platforms in the bilayer, involved in membrane trafficking, signal transduction and cell polarization.

Endosomes contain variable amounts of cholesterol which is sorted during vesicles membrane formation in the endocytic pathway (Lange & Steck, 2008). Lysobisphosphatidic acid (LBPA/BMP) regulated endosomal cholesterol levels via the protein Alix as developed hereafter.

It has been reported that disrupting rafts by a cyclodextrin treatment,which removes cholesterol from membranes, inhibit HIV production (Ono & Freed, 2001). Moreover, raft have been described to be essential for the assembly process (Lindwasser & Resh, 2001) (Campbell *et al.*, 2001; Guyader *et al.*, 2002; Viard *et al.*, 2002; Viard *et al.*, 2004). Since, growing evidences tend to demonstrate that HIV can bud at the endosomal level, Lindwasser et al. shown that gag localization is regulated by the distribution of the cholesterol. Following U18666A treatment, they could observe an accumulation of Gag in multivesicular bodies (Lindwasser & Resh, 2004).

3. Niemann-Pick C disease.

Niemann-Pick C disease is an autosomal recessive endolysosomal storage lipid disorder that exhibits impaired cell trafficking of exogenously-derived cholesterol and other lipids including sphingolipids and glycolipids, result in their accumulation in late endosomes and lysosomes. 95% of disease cases are caused by mutation of the NPC1 gene. The second gene is NPC2. Cells bearing mutated NPCs proteins provide a tool to investigate the cellular distribution of both cholesterol and LBPA (Chevallier 2008).

Theproteins NPC1 and NPC2 are localized in the late endosomal and lysosomal compartments respectively, bind cholesterol and other sterols and are involved in the egress of cholesterol from these organelles (Storch & Xu, 2009). Transmembrane NPC1 is targeted to late endosomes/lysosomes while the soluble NPC2 is targeted to lysosomes. NPC2 role in cholesterol transport was reported underlying an implication of LBPA in the acceptor membrane. High LBPA content increased the cholesterol transfer rates, which was conversely reduced by an anti LBPA antibody (Babalola *et al.*, 2007; Xu *et al.*, 2008). Recently, the group of Sandhoff extended this observation, showing that LBPA strongly enhance the function of NPC2 in the cholesterol transfer potentiated by ceramide, and brought evidences that NPC2 in presence of LBPA could mediate membrane fusions (Abdul-Hammed *et al.*, 2010).

Treatment of cells with an anti-LBPA antibody results in a NPC-like phenotype characterized by substantial cholesterol accumulation (Kobayashi *et al.*, 1999). LBPA is known to accumulate along with cholesterol in NPC cells (Holtta-Vuori *et al.*, 2000)

4. Cholesterol synthesis and trafficking inhibitor

Among various pharmacological tools available to investigate cholesterol synthesis and trafficking, U18666A and progesterone have been used in the experimental part of this manuscript. Their effect is briefly described hereafter.

a) U18666A

U18666A is an amphiphatic cationic amine that displays multiples actions, inhibiting cholesterol synthesis, and disturbing intracellular cholesterol trafficking, since it inhibits the egress of cholesterol from late endosomes and lysosomes. This later properties make it an interesting agent mimicking the NPC disease.

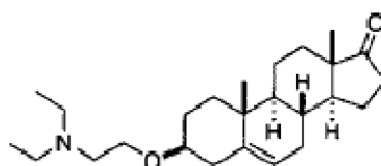


Figure 28 : The chemical structure of U18666A.

It has been reported that inhibition by U18666A of cholesterol synthesis occurs via at least three enzymes involved in sterol synthesis : the oxidosqualen open cyclase, the desmosterol reductase, and the sterol $-\Delta^8$-Δ^7 isomerase, which share 30% homology with sigma receptor (Cenedella, 2009). U18666A also Inhibits re-esterification of free cholesterol by the ACAT.

U18666a was shown to affect intracellular cholesterol movements. Effects of the molecule on the cholesterol transport at the level of endosomes were reported to be due to interference with NPC and its binding to membranes, altering their organization (Cenedella,

2009). Consequently, U18666A cell treatment induces a massive accumulation of cholesterol in endosomes. Also, the compound appears to affect cholesterol distribution from lysosome towards the ER or to the plasma membrane as well as between the plasma membrane to the ER.

Growing evidences tend to demonstrate that HIV can bud at the endosomal level.Lindwasser et al. showed that Gag localization is regulated by the distribution of the cholesterol. Following U18666A treatment, they could observe an accumulation of Gag in multivesicular bodies (Lindwasser & Resh, 2004). However several reports indicated that U18666A had no effect on HIV infection level in macrophages (Jouvenet *et al.*, 2006; Gousset *et al.*, 2008), indicating that cholesterol accumulation in endosomes (MVB) is not sufficient to inhibit HIV formation.

b) Progesterone

Whereas U18666A leads to sterol storage into endosome, progesterone has been shown to trigger cholesterol accumulation in the plasma membrane.

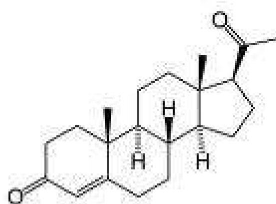


Figure 29 : The chemical structure of progesterone

Progesterone is a steroid hormone derived from cholesterol. Lange et al. demonstrated that cholesterol movements from plasma membrane in rat hepatoma cells to ER were inhibited by progesterone without inhibiting the neither the biosynthesis pathway of cholesterol nor esterification by ACAT (Lange, 1994). Regarding intracellular trafficking of free cholesterol, it has been shown that in macrophages foam cells progesterone t allowed sequestration of free cholesterol at the plasma membrane. Conversely, Butler et al. in a previous study on fibroblasts cultured with LDL, described a translocation blockage of cholesterol in lysosomes.

Recently, the effect of endogenous level of progesterone has been assessed in peripheral blood mononuclear cells. High concentrations of progesterone (10^{-7}M) decreased the activation of the LTR whereas low concentrations (10^{-9}M) increased it, indicating that progesterone could act at the transcription level (Asin *et al.*, 2008). However it was reported that high concentrations of progesterone (10mM) inhibited HIV replication in a placental cell line via inhibition of autocrine TNFα secretion (Munoz *et al.*, 2007). This is an interesting observation because progesterone concentration reaches high levels in the placental barrier (Munoz *et al.*, 2007).

Méthodes

The following section concerns an HPLC method to study phospholipase activities on exosomes and HIV and to identify derivatives. This corresponds to an article currently submitted.

Article :

Lipidomic Analysis of Bioactive Vesicles on Silica Diol With Efficient Resolution of Polyglycerophospholipids

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Abstract. An HPLC separation method based on silica diol support was used to analyze lipolytic activities borne by bioactive vesicles (exosomes) and to perform their lipidic composition. Fourteen different lipolytic activities were potentially monitored by separation of products generated from fluorescent BODIPY-containing lipids, allowing a detection in the picomolar range. The same procedure was applied to the separation of natural lipids, monitored by enhanced light scattering detection (ELSD), and showed an efficient resolution of ten different phospholipids including polyglycerophospholids such as bis(monoacylglycero)phosphate (BMP), hemi-bis(monoacylglycero)phosphate (hemi-BMP), or bis(diacylglycero)phosphate (BDP). BDP was separated using a solvent system for neutral lipids. As low as nanomole amounts of phospholipids were detected. Both procedures were applied to investigate lipolytic activities and lipid composition of bioactive vesicles (exosomes). These two analytical methods provide complementary information for lipidomic analyses.

Supplementary key words: BODIPY-PC, BMP (LBPA), BDP (bisPA), silica diol, ELSD, phospholipases, phosphatases, exosomes, HIV, lipidomics.

A. Introduction

Lipidomic analyses have been generally performed on cells or tissue extracts, but are more difficult to conduct on bioactive vesicles because of limited amounts of material. Bioactive vesicles, such as nanovesicles or "exosomes," have recently been the subject of increase interest because they are involved in intercellular signalling and participate in many pathophysiological processes (Schorey & Bhatnagar, 2008). Exosomes are vesicles released from intact cells. Tumor-derived exosomes promote tumor development whereas immunocomptent ones appear as a tool for cancer immunotherapy. Therefore differential lipidomic analysis would bring some insights in the role of bioactive lipids in the functional effects of these vesicles. Among bioactive lipids, phosphatidic acid (PA) and lysophosphatidic acid (LPA) are known to trigger cell proliferation, whereas lysophosphatidylcholine (LPC) induces cell differentiation.

We sought to identify appropriate analytical methods that would allow investigation of lipid composition and characterization of the lipolytic activities of exosomes (Allal *et al.*, 2004). To accomplish this goal, we have modified a method previously described for phospholipase D activity (Kemken *et al.*, 2000) and extended it to the characterization of the activities of other lipolytic enzymes, e. g., phospholipases A2, lipases, lipid phosphatases, and acyl-transferases. A total of fourteen different lipolytic activities were included in the new approach. The method is based on separation on a silica diol support, which can be used for either normal phase or reverse phase chromatographic separations. Silica diol is a well-known stationary phase that is less polar than silica and allows a better separation of both nonpolar and polar lipids within the same chromatogram. In the present work, we used silica diol supports to separate a variety of fluorescent and natural lipids including the polyglycerophospholipids.

Polyglycerophospholipids include bis(monoacylglycero)phosphate (BMP, also known as LBPA for lysobisphosphatidic acid), a phospholipid that plays a key role in exosome biogenesis [reviewed in (Subra *et al.*, 2007)], but which also regulates the intracellular cholesterol distribution from late endosomes (Hullin-Matsuda *et al.*, 2009b) and appears critical in the pathological lipidosis known as Niemann-Pick disease (Chevallier *et al.*, 2008). The native BMP contains primarily oleic acid (Hullin-Matsuda *et al.*, 2007). The technique described herein allows a single-step separation of BMP from other phospholipids and polyglycerophospholipids. The utilization of both fluorescence and ELSD detection offers complementary information to enhance data useful in both analytical and functional lipidomics.

B. Material and Methods

Materials:

a) Lipids:

Fluorescent lipids: 1-O-alkyl BODIPY-PC (#D-3771) [2-decanoyl-1-(O-(11-(4,4-difluoro-(5,7-dimethyl-4bora-3a,4a-diaza-s-indacene-3-propionyl)amino)undecyl)-sn-glycero-3-phosphocholine] ; BODIPY FL C_5-ceramide (#D-3521)[N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl) sphingosine] ; BODIPY FL C_{12}-sphingomyelin (#D-7711) [N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosyl phosphocholine] ; BODIPY FL-PE (#D3800) [N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt] were obtained from Invitrogen (Molecular Probes, Eugene, OR).

Lipolytic enzymes used to prepare standards were obtained from Sigma (St Louis, MO).
Natural oleoyl lipid standards were purchased from Avanti Polar Lipids, Birmingham, Alabama, USA. These included: mono, di and triglycerides (MG, DG, TG), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) , phosphatidylglycerol (PG), phosphatidylethanol (PET), phosphatidic acid (PA), lysophosphatidic acid (LPA), cyclic phosphatidic acid (cPA), bis(monoacylglycero)phosphate [*1S,R*-BMP (commonly named LBPA)], and hemi-bis(monoacylglycero)phosphate [*S,R*-hemi-BMP (commonly named semi-LBPA)]. The phospholipid bis(diacylglycero)Phosphate [*S,R*-BDP (commonly named bis-PA, or BPA)], containing 4 oleoyl groups, was prepared as described in the Supplementary Information, and followed earlier protocols (Jiang *et al.*, 2005). The *S,S*-octadecenyl BMP (also named BMP-ether, in which an 18:1 aliphatic chain ether was present at the *sn*-2 position of the glycerol) was also prepared as previously described (Jiang *et al.*, 2005). New BMP derivatives are now available from Echelon Biosciences (Salt Lake City, UT, USA). The compounds were stored dried under nitrogen at –20 °C.

b) Equipment:

The HPLC equipment (gradient former, pump, autosampler) was from Kontron Instruments and was interfaced with a computer allowing chromatrogram processing by the Kroma 3000 software (Bio-Tek Instruments, Milan, Italy). Fuorescence detection was performed by a SFM 25 fluorimeter (Kontron Instruments, USA). Natural lipid detection was monitored by an Enhanced Light Scattering Detector (ELSD 2000ES) from Alltech, USA (Serlabo Technologies, Entraigues, France). The ELSD 2000ES detector featured detection enhancement with gains 1,2,4,8,16,32. Silica diol columns (LiChrosphere 100 Diol (5µm)

250x4-mm) were supplied by Cluzeau Info Labo (CIL) (Sainte-Foy-la-Grande, France) or by AIT (Houilles,France). Solvents HPLC grade were from Merck (VWR).

C. Results and discussion

1. Separation procedure of fluorescent BODIPY-PC derived lipids

a) Preparation of standards

Non-commercially available fluorescent phospholipid standards were prepared from 1-O-alkyl BODIPY-phosphatidylcholine (PC) (Figure 30) dissolved in 1ml Tris-HCl pH 7.4 containing 1mM Ca^{++} and Mg^{++}. Commercial enzymes were added and reaction proceeded for one hour. Phospholipase D from *Streptomyces Chromofucus,* was used to generate BODIPY-PA (PA), and BODIPY-PEt (PEt) in presence of 5% v/v ethanol. BODIPY-lysophosphatidylcholine (LPC) and BODIPY-lysoPEt (LPET) were obtained by incubation of PC and PET respectively with *Naja-naja* phospholipase A2. BODIPY--lysophosphatidic acid (LPA) was obtained by treatment of PA by pig pancreatic phospholipase A2. BODIPY-diglycerides (DG) were prepared with *Bacillus cereus* phospholipase C and further processed with pancreatic lipase to obtain monoglycerides (MG). C_{12} BODIPY-ceramides were obtained from parental sphingomyelins using *Staphylococcus aureus* sphingomyelinase. Enzymatic reaction products were separated from remaining substrates by a short separation (10 cm) on silica plates with chloroform/methanol/ether 45/45/10 v/v as solvents to obtain pure standards. Lipids were extracted with 1-butanol, evaporated to dryness under nitrogen, and reconstituted into HPLC injection solvent (hexane/isopropanol/acetic acid 50/50/1 v/v/v including triethylamine 0.08 % v/v).

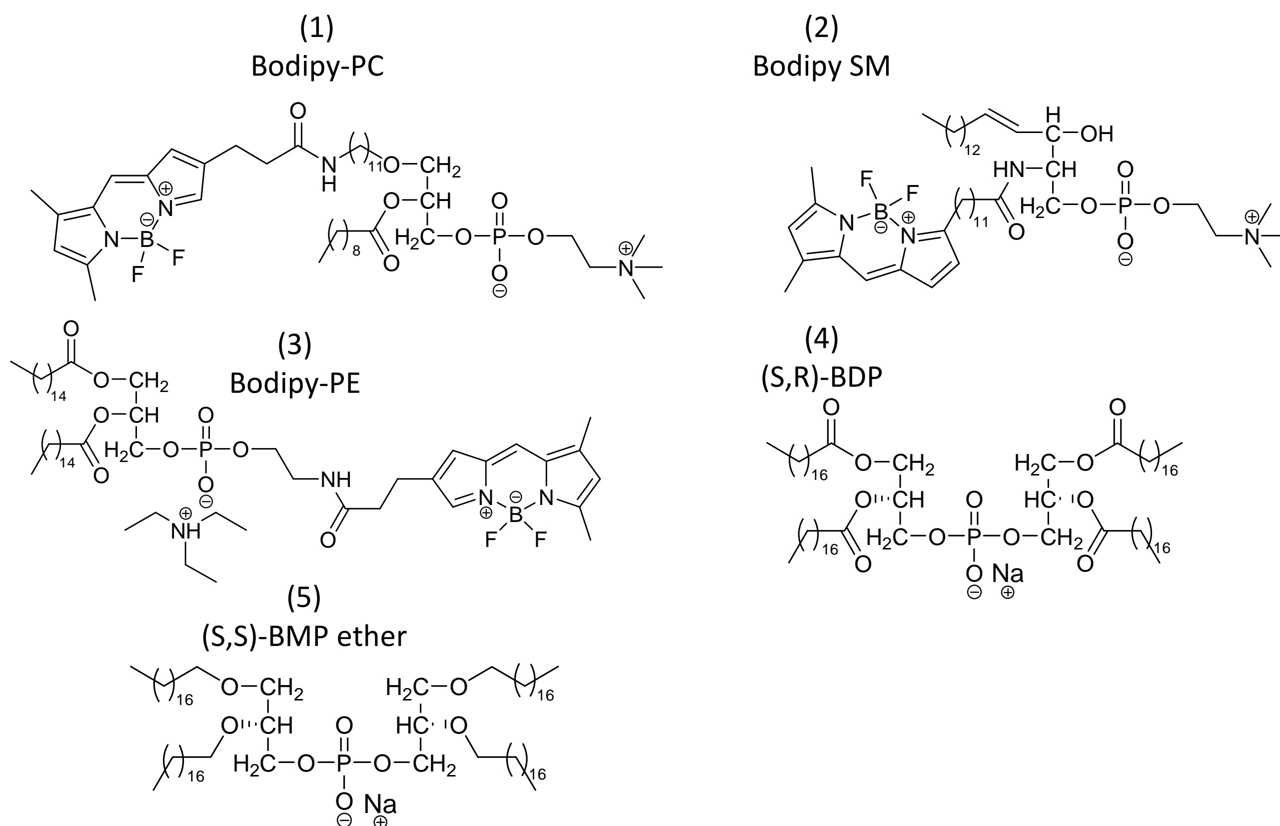

Figure 30 : **Some of the molecules separated by the chromatographic procedures reported.**
Fuorescent phosphatidylcholine (1), sphingomyelin (2), and phosphatidylethanolamine (3). Natural oleoyl Bis(Diacylglycero)Phosphate (4), and octadecenyl Bis(Monoacylglycero)Phosphate (5).

b) Incubation with bioactive vesicles

Exosomes prepared as previously reported (Laulagnier *et al.*, 2004a) were incubated with BODIPY-PC (2.4µM final) for 1 hour at 37°C in Ca^{++} Mg^{++}-containing PBS. Standards were added at the end of incubation time. Fluorescent lipids were extracted twice with 0.5 ml of 1-butanol, evaporated to dryness under nitrogen, and dissolved in the injection solvent.

c) Separation procedure

Lipid separation was realized by normal-phase on a LiChrosphere 100 Diol (5 µm) 250 x 4 mm column. An aliquot (20 µl) of each lipid sample was injected into the column, which was maintained at 30 °C. Binary gradient elution was performed by mixing mobile-phase solvents A [hexane-isopropanol-acetic acid (82:17:1, v/v/v)], and B [isopropanol-water-acetic acid (85:14:1, v/v/v)] at a flow rate of 0.4 ml/min. Triethylamine (0.08% v/v) was added extemporary to the solvents. The gradient profile was realized by increasing proportions of solvent B from 5% (at t = 0 min) to 40% in 25 min, then increasing to 100% for the next 5 min. The HPLC column was regenerated within 8 min by a return to the initial mobile phase containing 5% solvent B, and equilibrated at 5% solvent B for another 12 min. The HPLC system was coupled to a fluorescence detector (SFM25, Kontron Instruments) set at excitation and emission wavelengths of 475 nm and 515 nm, respectively. The signal from detector (µV) was recorded as function of time and processed with a chromatography data

software program (Kroma System 3000). A correlation between peak areas as function of the amount of BODIPY-PC gave a calibration curve linear up to 30 pmol and was represented by the equation **Y**(µV.min) = 31770**X** (pmol).

For analysis, dried lipid extracts or standards were dissolved into 300 µl of injection solvent. Half that volume was transferred into autosampler vials and 20 µl aliquots were injected onto the column.

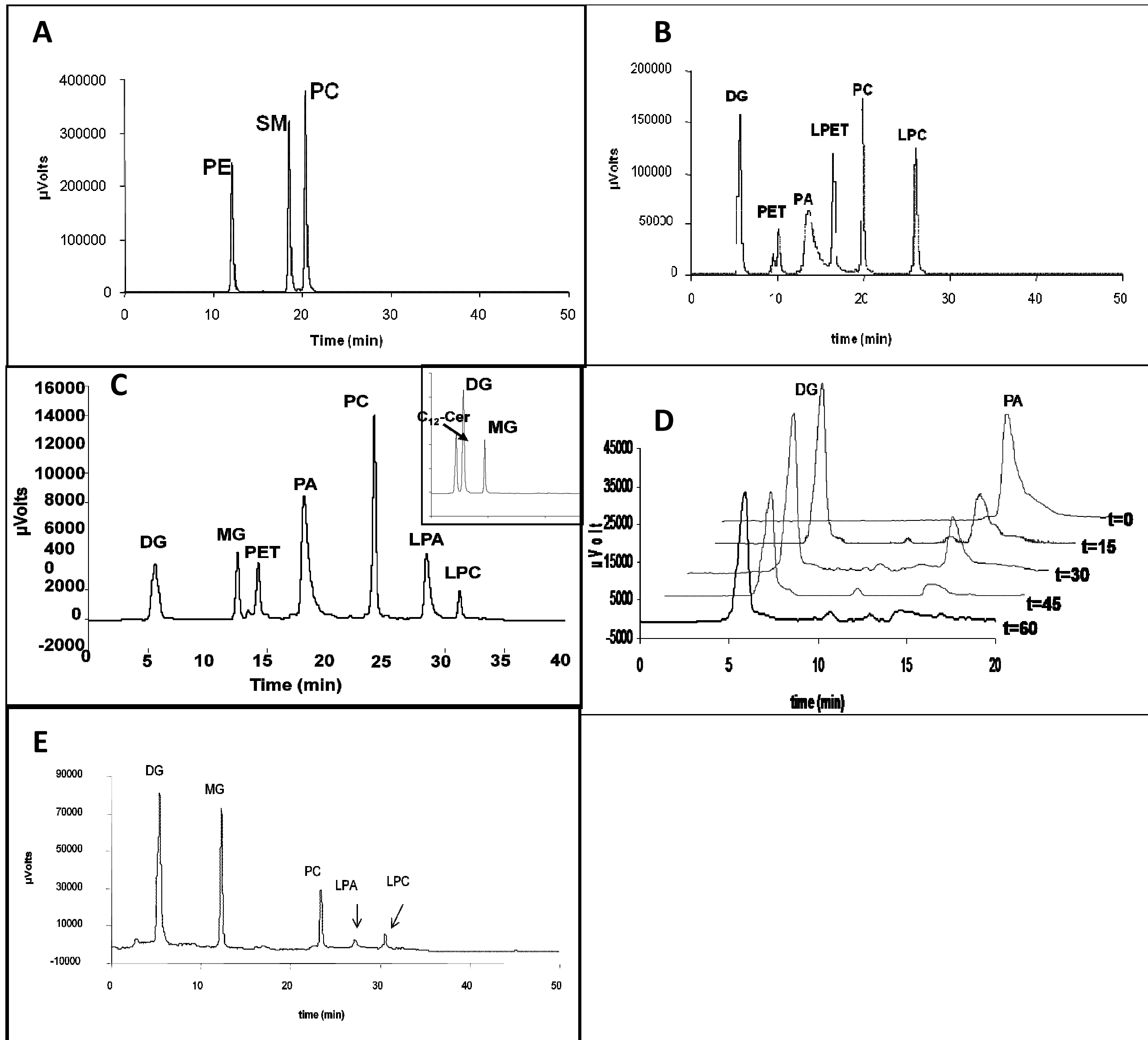

Figure 31 : **Typical separation of fluorescent BODIPY-containing lipids through silica diol column.** Detection by SFM25 fluorimeter.

(A) Separation of the major phospholipid classes. **(B)** Separation of **BODIPY-PC**-derived lipids. LPEt=lyso-phosphatidylethanol. **(C)** Similar separation as in (B) including LPA and MG as additional standards (column was from a different supplier as compared to A and B). Insert: migration of C_{12}-ceramide before DG. **(D)** Separation of PA-derived products upon incubation with exosomes. **(E)** Separation of LPA-derived products upon incubation with exosomes. Small amounts of PC and LPC were added for the HPLC run as references for migration. In (D) and (E), 80 pmol of PA or LPA were incubated for 1 h with 5 µg exosomes.

One pmole corresponded to 31770 +/- 142µV.min. Detection signals were similar between the various BODIPY-containing molecules(Kemken *et al.*, 2000)

2. Results

A typical separation of various polar and neutral fluorescent lipids was reported in (Figure 31). The procedure allowed to separate the major BODIPY-containing phospholipid classes Figure 31A. Separation of BODIPY -containing PA, PEt and DG from BODIPY -PC was already documented, (Kemken *et al.*, 2000) but we determined that the more polar lysophospholipids were efficiently resolved by the procedure.

SUBSTRATE	DG	MG	PA	PC	LPA	LPC
	a	*b*	*c*	*d*	*e*	*f*
PRODUCT						
DG (1)		Acyl-transferase	Phosphatidate Phosphatase (PAP)	Phospholipase C (PLC)		
MG (2)	Lipase				Lyso-phospholipid phosphase (LPP)	Lyso-PLC
PET (3)				Phospholipase D (PLD)		
PA (4)	DG kinase			Phospholipase D (PLD)	Acyltransferase	
PC (5)	DG Choline phosphotransferase					Acyltransferase Transacylase
LPA (6)		MG kinase	PA-specific phospholipase A2			Lyso-PLD (autotaxin)
LPC (7)				Phospholipase A2 (PLA2)		

Table 4 : Lipolytic activities monitored by fluorescent lipids. Lipolytic activities transform the substrates plotted in columns "**a-f**" into the products listed in lines" **1-7"**. In this table all the substrates were considered to be obtained from alkyl-Bodipy PC (see molecule #1 in Fig 30), i.e they all contained an ether-linkage at the sn1-position of the glycerol (see Materials), ruling out the possibility to monitor phospholipase A1 and sn1-lipase activities.

This allowed monitoring phospholipase A2 activity, lysophospholipase C activity (LPC to MG), and autotaxin, a lysophospholipase D activity (LPC to LPA) (Billon-Denis *et al.*, 2008) (Figure 31B and Figure 30C). In addition, PLA2 activity (PA to LPA) (Snitko *et al.*, 1997), could be monitored by this method. Acylation of LPA or LPC into PA or PC by LPA or LPC acyltransferases, respectively (Gluck *et al.*, 2008) could also be analyzed. Since MG was resolved from DG, lipase activities could also be investigated. A monoacylglycerol kinase activity, converting MG to LPA (Bektas *et al.*, 2005), or DG kinase transforming DG to PA, could be monitored by this procedure (Wakelam *et al.*, 2007). The CDP-choline

phosphotransferase activity could also be monitored; this enzyme of the *de novo* biosynthesis pathway of PC (Hunt *et al.*, 2004) catalyzed the condensation of DG with CDP-choline. Taken together, this method allowed the characterization of fourteen different types of lipid metabolic activities (**Table 4**). Lipolytic activities acting on choline-containing phospholipids could be monitored simultaneously, since sphingomyelin (SM) migrated distinctly from PC (Figure 31A) or any of its hydrolytic products (Figure 31B). Moreover, SM-derived ceramides (C_{12}-ceramides) were separated from DG (Figure 31C insert), in contrast to with C_5-ceramides (not shown). Ceramides are linked to exosome biogenesis (Trajkovic *et al.*, 2008). In addition, Figure 31 indicated that lipolytic enzymes specific for PE could also be investigated with the same procedure reported for PC.

Quantification of PC-derived neutral lipids was possible by adding ceramides as internal standards to the lipid extract since C_{12}Ceramides migrated before DG (Figure 31C, insert). For polar lipids, PEt can be used as internal standard. In case of PLD activity analysis, phosphatidylmethanol, which is slightly more polar than PEt, could alternatively be used.

An illustration of the interest of this procedure to measure lipolytic activities on biological membranes is illustrated in Figure 30D and E. Incubation of small amounts of exosomes with picomoles of fluorescent PA or LPA indicated that these vesicles contained a PA phosphatase (PAP) that generated DG (Figure 31D). A lysophospholipid phosphatase (LPP) was also detected since LPA was converted into MG (Figure 31E). Interestingly, DG were also observed, Figure 31, indicating that exosomes contained an acyltransferase activity that converted LPA-derived MG into DG (Figure 31E).

The HPLC procedure reported above was highly sensitive to characterize lipolytic enzymes borne by exosomes, but it appeared necessary to investigate also the lipid composition of these bioactive vesicles. The efficiency of the technique was then evaluated for the separation of natural lipids.

3. Separation procedure of natural lipids

a) Procedure

For polar lipid separation, the same procedure as that reported above was used. For neutral lipid separation an established solvent system (Silversand & Haux, 1997) was used at a flow rate of 0.4 ml/min, with solvents A: hexane/acetic acid (99/1 v/v), and solvents B: hexane/isopropanol/acetic acid (85/15/1 v/v/v) as eluants. Detection was performed by Enhanced Light-scattering Detection (ELSD). The temperature of the ELSD detector was set at 70 °C, with a pressure of 1.6 bar for the nitrogen flow. The signal from detector (µV) was recorded as function of time and processed with the chromatography data software program Kroma System 3000. The correlation between peak areas and the amount of PET as standard was linear up to 15 nmol and followed the equation: **Y**(µV.min)= 176870**X** (nmol).

b) **Chemical Synthesis of Bis(Diacylglycero)Phosphate.**

In order to evaluate the chromatographic behavior of BDP, one of the polyglycerophospholipids, the Bis(diC18:1)Phosphate was synthesized.

Following the reported procedures, 3-*O*-p-methyoxybenzyl (PMB)-*sn*-(2*S*)-glycerol was esterified with oleic acid using DCC/DMAP to give compounds in high yields (Chen *et al.*, 1996).

Oxidative removal of the PMB protective group with dichloro diycyanoquinon (DDQ) gave the corresponding 1,2-diacyl-*sn*-(2*R*)-glycerols in good yields without significant acyl migration. Although the 1,2-diacyl-*sn*-(2*R*)-glycerol was reasonably stable, slow purification on silica gel facilitated acyl diacyl- chain migration. Therefore, rapid flash chromatography was essential to obtain homogeneous 1,2-*sn*-(2*R*)-glycerols without acyl migration. Coupling of two molecules of this ester glyceryl intermediate with 2-cyanoethyl-bis-*N,N*-diisopropylphosphordiamidite in the presence of 1H-tetrazole followed by t-BuOOH oxidation gave the fully protected oleoyl BDP precursor in good yield (Jiang *et al.*, 2005; Jiang *et al.*, 2006). Under basic aprotic conditions in the presence of *N,O*-bis(trimethylsilyl)trifluoroacetamide, deprotection of cyanoethyl ester occurred at room temperature and without any side reactions to yield the final desired oleoyl BDP derivative.

4. Results

Data from Figure 32A showed a good resolution between phospholipids, except that SM and PS were not separated. Lipidic second messengers such as DG, PA, and LPC were well individualized. Two other bioactive phospholipids, LPA and cyclic PA (cPA), the second messengers produced by activation of lysophospholipase D and phospholipase D respectively, were also considered. LPA co-migrated with PC, but cPA was well isolated (Figure 32B).

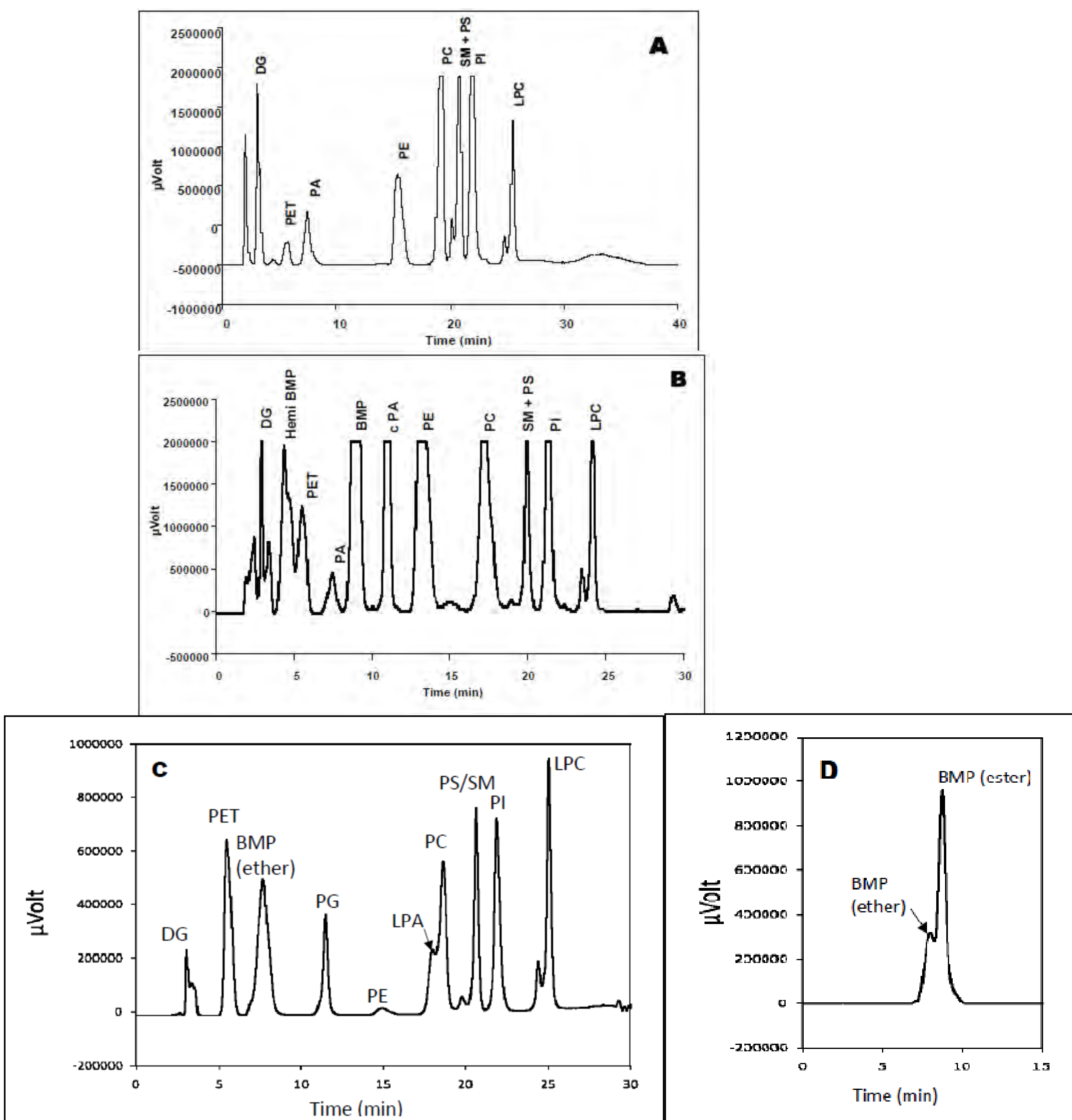

Figure 32 : **Typical separation profiles of natural 18:1-containing phospholipids.** Detection of products by ELSD using the same chromatographic conditions as in Fig 30.

(A) Separation of major phospholipids. **(B)** Separation of major phospholipids together with BMP and hemi-BMP. **(C)** Migration of PG, BMP (ether) and LPA comparatively to major phospholipids. **(D)** Comparative migration of BMP-ether (octadecenyl-BMP) and BMP-ester (18:1-BMP).

One nmol of phosphatidylethanol (PEt) corresponded to 176870 +/- 884 µV.min. Detection signals intensity can vary in a ratio from 1 to 2 depending upon the class of phospholipids (Silversand & Haux, 1997). Amount of each standard injected ranged from 1 to 3 nmol. Chromatograms were recorded with the ELSD at gain 1 except for Fig B (gain 2).

We next considered whether the polyglycerophospholipids BMP and related products (hemi-BMP and BDP) would be separable on silica diol support. Actually, the hemi-BMP (also known as semi-lysobisphosphatidic acid) migrated between DG and PA, and BMP was well resolved between PA and PE (Figure 32B). PG, the metabolic precursor of BMP (Hullin-Matsuda *et al.*, 2009b) eluted 4 min later (Figure 32C). DPG (cardiolipin), more polar than PG, migrated between PG and PE, close to PE (not shown).

Interestingly, when the ester linkage of the 18:1 fatty acid in natural BMP was changed into an ether-linkage, a shift in retention time between BMP-ether and BMP-ester was detectable (Figure 32D) and processable by the computer, allowing the use of octadecenyl-BMP as internal standard. Alternatively phosphatidylethanol (PEt) could be used also as internal standard. Therefore, the HPLC method reported herein allowed a one-step separation of BMP, which shortens the time required for its quantification as compared to currently employed three-step procedure, which requires a two-dimension thin-layer chromatography followed by an HPLC purification (Bouvier *et al.*, 2009). In addition, the method allows for quantification of the total pool of BMP in cells as compared to the use of immunodetection with the anti-LBPA 6C4 antibody, which monitors only MVB-associated BMP, a phospholipid also present in lysosomes (Schulze *et al.*, 2009).

Separation of another polyglycerophospholipid, namely BDP (also known as bisphosphatidic acid [bisPA]), was also considered, since the metabolic relationships between BMP, hemi-BMP and BDP are unclear (Hullin-Matsuda *et al.*, 2009b). Whereas it is well-established that BMP can be formed from phosphatidylglycerol (Hullin-Matsuda *et al.*, 2007), whether BDP and hemi-BMP are precursors or transformation products of BMP is not known. We found that BDP co-migrated with neutral lipids, indicating that the four fatty acids present in the molecule counterbalanced the charge of the phosphate moiety. Instead BDP could be isolated from TG and DG with a separation procedure designed for neutral lipid separation (Figure 33).

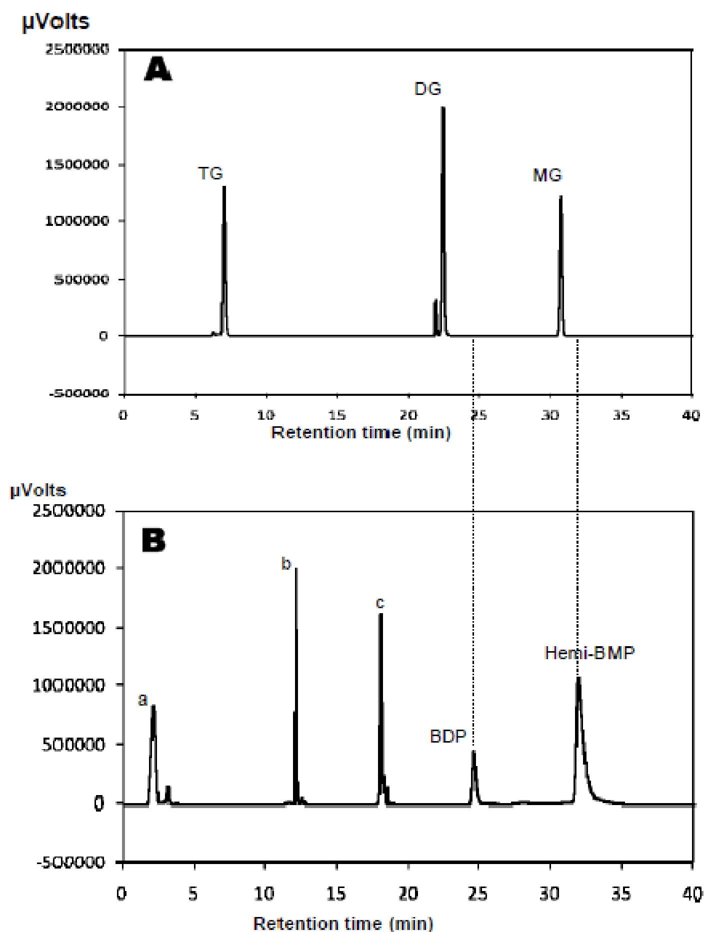

Figure 33 Separation profile of polyglycerophospholipids using a solvent mixture for neutral lipids. ELSD detection (gain 1). Isolation of 18:1-containing bis(diacylglycero)phosphate (BDP) and hemi-BMP (B) from 18:1-containing MG,DG and TG (A), using the neutral lipid solvent system reported in the text. Amount of each standard injected ranged from 2 to 6 nmol. Peaks "a" to "c" were not characterized.

The separation of lipids from exosomes was next considered. Lipids extracted from only 20 µg protein of exosomes (corresponding to 5 nmol phospholipids) gave detectable signals at gain 16 (over 32) with the ELSD detector (Figure 34). The separation profile reported the presence of the major phospholipids, and the presence of phosphatidic acid (PA), a fusogenic lipid in presence of calcium, which might participate in the fusion process recently reported between exosomes and recipient cells (Parolini *et al.*, 2009). BMP was not detectable (retention time 9 min) since it accounted for about 1% of total phospholipids (Laulagnier *et al.*, 2004b) representing 0.05 nmol, an amount below the detection limit of the detector in conditions of Figure 34. Other lipids including neutral lipids, poorly investigated in exosomes, appeared abundant (Figure 34) and will require further identification using chromatographic conditions of Figure 33. In this respect it will be worth to analyse whether polyglycerophospholids such as hemi-BMP or BDP would be present among the "other lipids" pool, taking advantage of their separation as reported in Figure 33, since preliminary data from mass spectra analysis indicated their presence in exosomes derived from melanoma cells (not shown). Data reported herein demonstrated the feasibility

of exosome lipid analysis by the HPLC procedures described, which require small amounts of material, and will allow lipidomics analysis of exosomes from various origins.

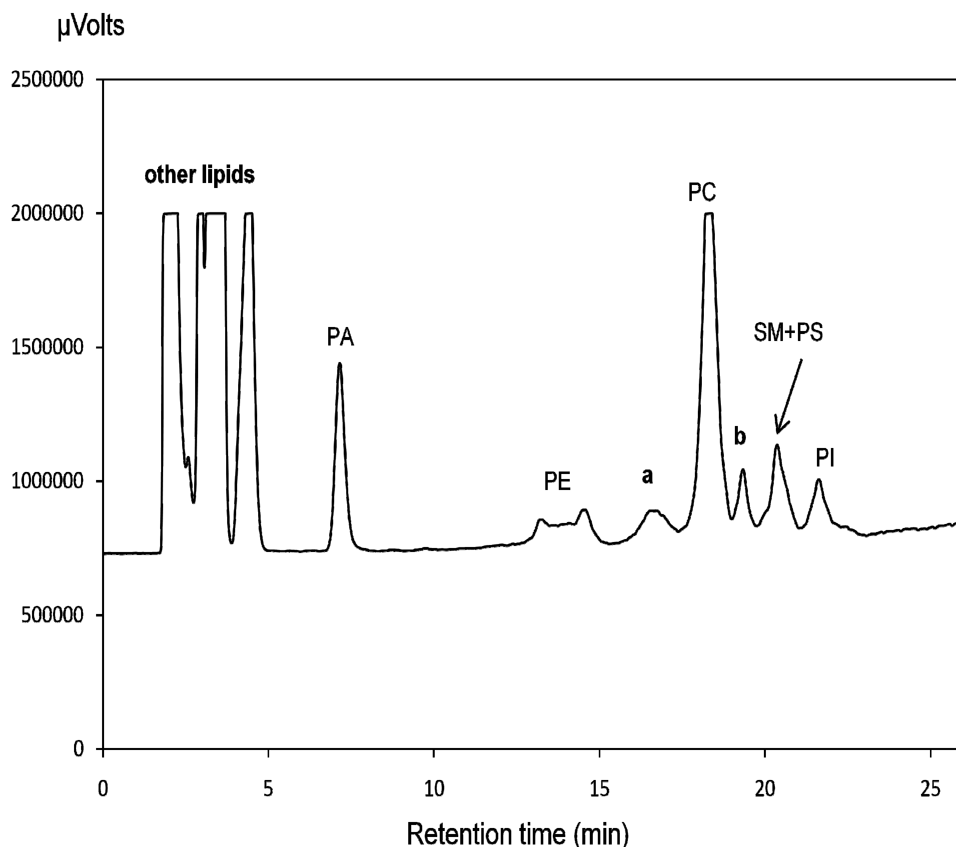

Figure 34 : Analysis of exosome lipids with ELSD detection using chromatographic conditions of Fig 30. Total amount of phospholipids injected corresponded to 5 nmol. Peaks "a" and "b" were not characterized. "Other lipids" contained neutral lipids. Detection signal intensity for neutral lipids can be 2 times higher than for phospholipids (Silversand & Haux, 1997). ELSD detection at gain 16 is reported.

Such investigations can be extended to viruses such as HIV, which features a similar size as exosomes and whose biogenesis takes place in part along the "exosome pathway" (Chertova *et al.*, 2006). We showed earlier that another enveloped virus, the human cytomegalovirus, requires a phospholipase A2 activity (Allal *et al.*, 2004) for infectivity. The characterization of this enzymatic activity as an infectivity index of viruses will benefit of the procedures reported herein.

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GDP thanks the University of Utah for support of the Chemical Synthesis Facility.

Résultats

As reported in the bibliographic section of this manuscript, the first part of my researches concerned fundamental analyses of mast cells derived exosomes, considered as non-infectious particles secreted from HIV reservoir cells. These works presented hereafter aimed to highlight the presence of lipidic mediators carried by exosomes which might be internalized by recipient cells. GTP-activatable phospholipase activities have been revealed along with resulting free fatty acids such as arachidonic acid (AA). We could identify some AA derived lipids as prostaglandines and among them, a PPARγ agonist: 15δ–PGJ2.

All these findings were recently submitted for publication at the Journal of Lipids Research. The article is presented hereafter.

Article :

I. Exosomes account for vesicle-mediated transcellular transport of activatable phospholipases and prostaglandins

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Abstract. Exosomes are bioactive vesicles released from multivesicular bodies by intact cells and participate in intercellular signalling. We investigated the presence of lipid-related proteins and bioactive lipids in RBL-2H3 exosomes. Besides a phospholipid scramblase and a Fatty Acid Binding Protein, the exosomes contained the whole set of phospholipases (A2, C and D) together with interacting proteins such as aldolase A and Hsp 70. They also contained the PLD / PAP1 pathway leading to the formation of diglycerides. RBL-2H3 exosomes also carried members of the three phospholipase A2 classes, i.e. the calcium-dependent cPLA$_2$-IVA, the calcium-independent iPLA$_2$-VIA and the secreted sPLA$_2$-IIA and V. Remarkably, almost all members of the Ras GTPase superfamily were present, and incubation of exosomes with GTPγS triggered activation of PLA$_2$s and PLD$_2$. A large panel of free fatty acids, including arachidonic acid, and derivatives such as prostaglandin E2 and 15-deoxy-Δ12,14-prostaglandinJ2 were detected. We observed that the exosomes were internalized by resting and activated RBLcells, and that they accumulated in an endosomal compartment. Endosomal concentrations were in the micromolar range for prostaglandins, i.e. concentrations able to trigger prostaglandin-dependent biological responses. Therefore exosomes are carriers of GTP-activatable phospholipases and lipid mediators from cell to cell.

Supplementary key words: exosomes, phospholipases (A2, C, D), phosphatidate phosphatase (PAP1), arachidonic acid, DHA, prostaglandins (PGE$_2$, 15d-PGJ$_2$)

Short title: Exosomes as vectors of phospholipases and prostaglandins

Abbreviations: PLA$_2$: phospholipase A$_2$; **PLD$_2$:** phospholipase D$_2$; **PLC**: phospholipase C; **PAP**: phosphatidate phosphatase; **PPAR**: peroxysome proliferator activated receptor; **MAFP**: methyl arachidonyl fluorophosphonate; **BEL**: bromo-eno-lactone; **DG**: diglycerides; **PA**: phosphatidic acid; **LPC**: lysophosphatidylcholine; **PEt**: phosphatidylethanol; **PC**: phosphatidylcholine; **PGE$_2$:** prostaglandin E2; **PGF2α**: prostaglandin F2α; **15-d PGJ$_2$**: 15-deoxy-Δ12,14-prostaglandinJ2; **Me-indoxam**: methyl-indoxam.

A. Introduction

Exosomes are nanovesicles (50-100 nm) released from viable cells, either constitutively or upon activation of cell secretion but not from lysed or apoptotic cells (Thery *et al.*, 2006). They are secreted from an intracellular compartment, the MultiVesicular Bodies (MVB), or late endosomes (Raposo *et al.*, 1996). The "TfR/tetraspanin/Heat-Shock Protein"-containing exosomes originating from MVB differ from the "CD73 (5'-nucleotidase)/ glycophorin/ CD45"-containing microvesicles produced by plasma membrane shedding (Hess *et al.*, 1999) and from "CD31/Annexin V"-containing apoptotic microparticles (Werner *et al.*, 2006).

Exosomes were first characterized as a pathway for elimination of obsolete proteins during erythrocyte maturation, then as being involved in an essential process in the immune response, and recently as enabling a mechanism to modulate the translational activity of target cells by transfer of selected micro RNA between cells (Valadi *et al.*, 2007). Whether exosomes participate dynamically in lipid metabolism is not known.

Exosomes appear to be involved in additional intercellular signalling beside soluble agonists. They interact with cell peripheral receptors, such as CD91 (Skokos *et al.*, 2003), a member of the LRP receptors, Tim4 (Zakharova *et al.*, 2007), the phosphatidylserine receptor (Miyanishi *et al.*, 2007), a GPCR member. Other nanovesicles similar to exosomes trigger the Notch signalling pathway (Ristorcelli *et al.*, 2009).

The exosome biogenesis pathway can be "hijacked" by pathogens like the human immunodeficiency virus (HIV), and also by proteins like prions involved in Creutzfeld-Jacob disease (Alais *et al.*, 2008), and the amyloid precursor protein (APP) of Alzheimer's disease (Schorey & Bhatnagar, 2008; Sharples *et al.*, 2008).

Mast cell derived exosomes trigger functional maturation of dendritic cells (DC) (Skokos *et al.*, 2003). The DC maturation process has been shown to involve lysophosphatidylcholine and secreted PLA_2s (Perrin-Cocon *et al.*, 2004), and prostaglandins (Thurnher, 2007). We previously reported that exosomes from RBL-2H3 cells contain a high amount of lysophosphatidylcholine (LPC) (Laulagnier *et al.*, 2004b) and that phospholipase D was involved in exosome release(Laulagnier *et al.*, 2004a).

We therefore undertook a large analysis of RBL-2H3 exosome content by proteomic high-throughput analysis together with immunodetection and determination of lipolytic activities. We showed that exosomes can behave as "signalosomes" by transporting GTP-activable phospholipases D_2 and PLA_2s, but also by carrying the whole set of prostaglandins, including PGE_2 and the PPARγ agonist 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ_2). We observed that exosomes could traffic between resting or activated RBL-2H3 cells, therefore being able to modulate RBL-2H3 cell activation by means of the lipid messengers they carry. In addition exosomes could constitute a mechanism of entry for 15d-PGJ_2, since the way this prostaglandin enters cells is as yet unknown (Scher & Pillinger, 2005; Gandarillas *et al.*, 2009; Scher & Pillinger, 2009).

B. Experimental Procedures

1. Materials

For cell cultures, RPMI 1640, PBS, penicillin, streptomycin, L-glutamine and FCS were purchased from Invitrogen.

4,4-difluoro-4-bora-3a,4a-diaza-s-incadene (BODIPY)-PC as phospholipase substrate, and BODIPY-Ceramide for exosome labeling and uptake detection by immunofluorescence, were obtained from Invitrogen-Molecular Probes and stored in ethanol at –20°C after dilution. GTPγS was from Sigma. Methyl arachidonyl fluorophosphonate (MAFP) was from Calbiochem. Bromoenol lactone (BEL, or HaloEnol Lactone Suicide Substrate) was from Biomol International. Pyrrolidine-1 and Me-Indoxam were generous gifts from Pr M.H.Gelb (University of Washington, Seattle, USA).

The $cPLA_2$ monoclonal antibody (recognizing type IVA) and $iPLA_2$ polyclonal antibody (recognizing type VIA) were from Santa Cruz Biotechnology. Mouse $sPLA_2$-IIA and -V recombinant proteins, and $sPLA_2$ antibodies raised against type IIA and V $sPLA_2$ were produced as described (Rouault *et al.*, 2007). Polyclonal antibodies against COX-1 and COX-2 were from Santa Cruz Biotechnology. Rabbit polyclonal anti-PLD antibody (N-PLD4) was from Johnson Pharmaceutical Research Institute (Raritan, NJ, USA) and was kindly supplied by Dr. D. Uhlinger. The HA.11 monoclonal mouse anti-HA antibody (clone 16B12) was from Eurogentec. Anti-CD63 antibody was from Santa Cruz Biotechnology. The anti-LBPA antibody (6C4) was kindly supplied by Dr T.Kobayashi, The Riken Institute, Tokyo, Japan (Kobayashi *et al.*, 1998b). Secondary antibodies labeled with horseradish peroxidase were from Santa-Cruz Biotechnology and PhycoErythrine-labeled antibodies from BD-Bioscience. FITC-labelled cholera toxin subunit was from Sigma. Isotype antibodies for flow cytometry were from Santa-Cruz Biotechnology,

Rhodamine-phosphatidylethanolamine (Rh-PE) was from Avanti Polar Lipids, Birmingham, AL, USA.

Protease inhibitor cocktail (P8340) was provided by Sigma. Chemical solvents were purchased from Sigma-Aldrich or from Merck for HPLC grade.

2. Methods

Cell lines

RBL-2H3 (**also referred to as RBLwt for RBL wild-type cells**) were grown in RPMI 1640 supplemented with 10 % (v/v) FCS, 4 mM L-glutamine, 140 units/ml penicillin and 140 µg/ml streptomycin, in a 5% CO_2 humidified atmosphere at 37°C.

Cells overexpressing the human HA-tagged PLD_2 (**also referred to as RBLpld2 cells**) were obtained by electroporation (250 V, 500 µF) of RBL-2H3 cells with linearized pcDNA3.1 vector containing the HA-tagged cDNA of human PLD_2. PLD_2 overexpressing cells were selected with G418 (500µg/ml). Clones grown within one week were recovered in PBS-EDTA,

mixed, expanded, and stored in liquid nitrogen. The characteristics of the RBLpld2 cell line are reported in Supplementary Material.

RBL cell degranulation

Cell secretion was monitored by the amount of ^{14}C-serotonin released from the MVB compartment. Adherent cells were loaded overnight with ^{14}C-serotonin, washed, and incubated for 4 hours with saturating concentrations of IgE directed against dinitrophenol conjugated to serum albumin (DNP-HSA, Sigma). Cell activation was triggered by FcεRI cross-linking with DNP-HSA and the radioactivity released measured by scintillation counting.

Exosome preparation

For exosome preparation 1.5 X 10^7 adherent cells were harvested with PBS-EDTA and added into 250 ml complete RPMI medium in a spinner bottle for cell culture in suspension. Culture volume was doubled every day in the spinner bottles to maintain a cell density of around 0.25 X 10^6 cells /ml for good cell viability, until about 10^9 cells were produced overall.

The cells were spun down, washed with DMEM medium, and concentrated to 10^8 cells in 10ml of DMEM without FCS to avoid contamination by any microvesicles that may be present in the fetal calf serum. Exosomes were recovered following 20 min cell stimulation by ionomycin (1µM final), and purified by differential centrifugations as we reported previously (Laulagnier *et al.*, 2004a). Correct exosome preparation required viable cells which was checked by trypan blue exclusion. Briefly, viable activated cells were eliminated by centrifugation at 300g for 5 min. To get rid of possible cell debris, the supernatant underwent two consecutive centrifugations at 2000g for 20 min at 4°C and 10 000g for 30 min at 4°C. Exosomes were isolated from the 10 000g supernatant by ultracentrifugation at 110 000g for 70 min at 4°C. The pellet was resuspended in PBS and centrifuged again at 110 000g for 70 min at 4°C. The final pellet referred to as exosomes was resuspended in PBS for analysis. The quality of the preparations was checked by D_2O/sucrose discontinous gradient (Chaput *et al.*, 2006b) and by electron microscopy (D.Lankar, Institut Curie Paris; B. Payre, CMEAB, UPS Toulouse III, France). We also checked the size homogeneity of vesicles obtained using a Zetasizer Nano ZS90 (see below). Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) in the presence of 0.1% w/v SDS final.

Size distribution and zeta potential analysis of RBL-2H3 derived exosomes

The Zetasizer Nano ZS 90 (Malvern Instruments, Orsay, France), allowed the analysis of particles with sizes ranging from 1 nm to 3 µm. Exosomes (50 µg from 2 pooled preparations) derived from RBLwt or RBLpld$_2$ cells were diluted in 1 ml PBS, and parameters such as zeta potential (electronegativity) and size distribution were analyzed at 37°C according to the manufacturer's instructions *(See Supplemental Material, Fig S2)*.

Quantification of exosome vesicles

The correlation between exosome protein content and the number of vesicles was established by FACS analysis on the basis of the method used to quantify the number of circulating microparticles (Werner *et al.*, 2006). Exosomes were diluted in PBS-EDTA and the number of vesicles was taken as the number of events in the SSC/FSC quadrant.

Quantification of exosome internalisation.

Exosomes were labeled with the fluorescent lipid probe Bodipy-Ceramide so that fluorescence monitored the amount of vesicles directly (Laulagnier *et al.*, 2004a). Fluorescent exosomes (25 µg proteins) were incubated with 10^6 adherent cells. At appropriate times, the excess of added exosomes removed, the cells washed, and cell-associated fluorescence monitoring internalized exosomes extracted with butanol and quantified. The fluorescence was converted into µg exosome protein using a calibration curve as previously reported (16).

Confocal microscopy

1- Internalization of fluorescent exosomes was monitored under a Zeiss LSM 510 confocal microscope on live cells using LSM 510 software. Cells ($3X10^4$ in RPMI medium buffered with 25 mM Hepes) were seeded in LabTek chambers and kept overnight in an incubator. Then, medium was removed and 0.5ml of the same fresh medium was added. The Lab Tek chambers were placed into a microscope chamber adaptor warmed to 37°C and with CO_2 flow. Exosomes (20 µg), previously made fluorescent by a 1hour incubation at 37°C with 1.2µM Bodipy-ceramide (Laulagnier *et al.*, 2005) and washed, were added in a small volume (20µl) into the cell medium and data acquisition started.

2- Characterization of the endosomal compartment in target cells

The compartment of exosome internalization in target cells was characterized by antibodies directed against late endosome markers. $2X10^5$ cells were seeded on coverglass in 1 ml RPMI culture medium and incubated for 24 hours with 75 µl anti-LBPA antibody (hybridoma supernatant) or 50µl (10µg) anti-CD63 antibody. Cells were washed with PBS, then overlayed with 0.5 ml culture medium and 10µg fluorescent (Bodipy-ceramide labeled)-exosomes were added. Incubation proceeded for 4 hours at 37°C. Cells were washed with PBS and fixed with 3.7 % PFA for 20 min and washed again. The remaining PFA was quenched with 50mM NH_4Cl for 10 min. The cells were washed with PBS, then maintained for 30 min in PBS 3% BSA. Permeabilisation was performed with 0.05% saponin in PBS 3% BSA for 10 min. The cells were washed and incubated 30 min with appropriate secondary antibodies (anti-mouse PE for LBPA and anti-goat FITC for CD63). Cover-slips were mounted with Mowiol and samples examined under a LSM 510 confocal microscope.

To label the late endosome compartment with Rhodamine-PE, cells were incubated in suspension at 4°C with 3µM final of the probe, washed with PBS 3% BSA, and incubated for 3 hours more at 37°C. Cells were seeded on cover-slips and pulsed for 4 hours with fluorescent exosomes. After washing, cells were fixed with PFA and examined with the LSM 510.

3- PLD distribution in RBLpld2 cells

Plasma membrane labeling was first performed on live RBLpld2 cells with fluorescent cholera toxin added in PBS containing 10 % BSA at 4°C for 30 min. The cells were washed with PBS, fixed with 3% PFA for 20 min at 4°C, and permeabilized for 15 min at room temperature with 0.05% saponin in PBS-BSA. HA-PLD_2 location was then detected by incubation with anti-HA antibody diluted at 1/50, followed by incubation with a secondary antibody (45 min each antibody) at room temperature. Acquisition was performed with a Zeiss LSM 510 confocal microscope.

Measurement of phospholipase activities

Identification of the various phospholipase activities in exosomes was performed by HPLC using a fluorescent phosphatidylcholine (BODIPY-PC) as substrate. Intact or sonicated (2x10 sec output 4-5 Micro Tip, Branson Sonifier) exosomes (50µg protein) were preincubated 10 min at room temperature in a total volume of 500 µl PBS containing 2 mM Ca^{++}/Mg^{++} with 5µl protease inhibitor cocktail, and as required 50µM PLA_2 inhibitors (MAFP for cPLA>iPLA_2, pyrrolidine for cPLA_2, Me-Indoxam for sPLA_2 or BEL for iPLA_2). When calcium was not required, Ca^{++}/Mg^{++} free-PBS was used. Concentrations of inhibitors and their specificity have already been documented (Allal *et al.*, 2004; Gubern *et al.*, 2008). When GTP dependency was checked, the non-hydrolysable analog of GTP (GTPγS) was added 10 min before monitoring phospholipase activity. Substrate (1µl BODIPY-PC, 2.34 µM final) was supplied in ethanol (0.2-2%v/v final). The reaction was performed for 1 hour at 37°C. Fluorescent lipids were extracted with 2 x 500 µl 1-n-butanol and were resolved by HPLC (*see below*).

Measurement of PA phosphatase activity (PAP1)

Fluorescent phosphatidic acid (Bodipy-PA) was prepared from Bodipy-PC by *in-vitro* hydrolysis with commercial phospholipase D. For enzymatic PAP activity measurement, 50 µg of exosomes were preincubated for 10 min at room temperature in a total volume of 500 µl of PBS with 2 mM Ca^{++}/Mg^{++}, with or without 100 µM GTPγS, in the presence of 5 µl protease inhibitor cocktail. The reaction was started by addition of 1µl Bodipy-PA (1µM final) supplied in ethanol and incubation proceeded at 37°C. Fluorescent lipids were extracted with 2x 500 µl 1-n-butanol and resolved by HPLC.

HPLC analysis

HPLC separation and quantification of BodipyPC-derived products were performed as already reported (Gayral *et al.*, 2006) on a silica diol column with a solvent flow rate of 0.4 ml/min**.** Fluorescent standards of lysophosphatidylcholine (LPC), phosphatidic acid (PA), diglycerides (DG) and phosphatidylethanol (PEt) were prepared by in-vitro incubations of Bodipy-PC with appropriate lipolytic enzymes.

Calibration curves for quantification were plotted with Bodipy-PC as standard. HPLC peaks across chromatograms were identified by fluorescent standards injected in the middle of each series of samples to overcome variations in retention times.

Phospholipase immunodetection

For phospholipases A2, cells and exosomes were lysed in Laemmli sample buffer at 95°C for 10min and sonicated. 10mM EDTA was added for phospholipase D detection. 40 µg of proteins were run on 7.5 % SDS-PAGE and transferred onto PVDF membrane. The membranes were saturated with 5% non-fat milk in TBS 0.1% Tween 20 for 1 h at room temperature and blotted at 4°C overnight with mouse or rabbit primary antibodies, supplied in blotting buffer. Membranes were then washed and incubated in TBS 0.1% Tween 20 with HRP-labeled anti-mouse IgG or anti-rabbit IgG secondary antibodies for 1 h at room temperature.

For $sPLA_2$ detection, 40µg of exosome proteins were separated on a 15 % SDS-polyacrylamide gel comparatively to 50 ng of group IIa and V recombinant $sPLA_2$ proteins as standards, transferred onto PVDF membrane. Membranes were saturated in NETG buffer (150mM NaCl, 5mM EDTA, 50mM Tris pH 7.4, 0.05% Triton X100, 0.25% gelatin), washed in PBS 0.05% Tween 20 and incubated in NETG buffer with HRP-labeled anti-rabbit IgG secondary antibodies.

In all cases, the signal was detected by the enhanced chemiluminescence system from GE-Healthcare/ Amersham.

Cyclooxygenase detection

Exosomes (5µg proteins) were incubated with anti-COX1 or anti-COX2 primary antibodies for 1 hour at room temperature and for 30 min with secondary FITC-labeled antibody, with respect to appropriate control isotype. COX expression was analysed by flow cytometry on the FITC chanel (FL-1).

Protein analysis :

High-throughput protein analysis was performed on 100µg protein of purified exosome, first separated by one-dimensional SDS-PAGE. The protein gel lane was cut into 16 pieces which were digested by trypsin. Tryptic peptides were analysed by nanoLC-MSMS with a Qstar XL spectrometer (Applied Biosystems). Data were searched against mouse entries in Sprot-Trembl with the Mascot software. Analyses were performed at the IPBS, CNRS, Toulouse, France (Bouyssie *et al.*, 2007).

Lipid analysis :

1-Determination of free fatty acids in exosomes was performed at the Lipidomics facility of IFR-150 BMT, Toulouse, France. RBL-2H3 derived exosomes (100 µg) were incubated in PBS with 2 mM Ca^{++}/Mg^{++} at 37°C for 4 h. The lipids were extracted by the Bligh and Dyer method (Bligh & Dyer, 1959) in the presence of EGTA in water. Fatty acids were methylated, and further analyzed by gas chromatography with an HP5890 instrument (Payre *et al.*, 2008).

2- Prostaglandins were quantified by GC/MS at the Lipidomics facility of IMBL/INSA-Lyon, Villeurbanne, France. Lipids from RBL-2H3 derived exosomes (70 µg) were extracted with ethylacetate, derivatized into pentafluorobenzyl esters and subsequently purified by

silicagel TLC using chloroform/ethanol (93:7, v/v), then modified into trimethylsilyl ethers before analysis by GC/MS. Samples were spiked with 10 ng of deuterated prostaglandin standards (Cayman) and GC-MS was carried out with a Hewlett Packard quadrupole mass spectrometer interfaced with a Hewlett Packard gas chromatograph (Soares *et al.*, 2005)**.**

Data presentation:

HPLC profiles and phospholipase determinations representative of at least two independent experiments were plotted. Pooled exosome preparations from two to three experiments were used for protein and lipid analysis, which fulfilled the quality control stipulations of the respective facilities (IPBS and IFR-BMT, Toulouse, France; IMBL/INSA-Lyon, Villeurbanne, France). Confocal pictures were representative of at least two experiments performed by distinct operators. Errors bars corresponded to Standard Errors to the Mean (SEM) from 3 determinations.

C. Results

1. Lipid-related proteins in exosomes

In order to determine which of the diverse lipid-related proteins were present on the exosomes, we first performed an exhaustive protein analysis of the vesicles. The proteins found in the present study are reported in Table 5. Typical exosome markers such as the transferring receptor, tetraspanins (CD63, CD81, CD82), and heat shock proteins were detected (Table 5A), assessing the quality of the preparation. Exosomes were also characterized by their size and their electronegativity (Supplementary Data 2).

Regarding lipid-related proteins, we found a phospholipid scramblase, a protein that transports phospholipids between the two membrane leaflets in both directions. The presence of this protein was consistent with the lack of membrane phospholipid asymmetry we reported earlier in RBL-derived exosomes (Laulagnier *et al.*, 2004a). Also a member of the Fatty Acid Binding Proteins (E-FABP) was detected. FABPs constitute a multigene family of structurally homologous cytosolic proteins which bind and transport polyunsaturated fatty acids such as arachidonic acid (Kitanaka *et al.*, 2006). Another type of protein was a prostaglandin F2 receptor negative regulator, also called FPRP(Stipp *et al.*, 2001). FPRP associates with the PGF2α receptor, thereby reducing ligand binding (Orlicky *et al.*, 1996). However, the PGF_2 receptor was not found in exosomes and the presence of the FPRP protein might be better related to its ability to form a tight complex with the tetraspan molecule CD81(Stipp *et al.*, 2001).

PROTEIN CATEGORY	ACCESSION NUMBER	PROTEIN TYPE	OBSERVATIONS
A/ Exosome markers			
	Q62351	Transferrin receptor	
	P41731	CD63 antigen	Tetraspanin
	P40237	CD82 antigen	Tetraspanin
	P35762	CD81 antigen	Tetraspanin
	P11499	Heat-shock protein 90-beta	
	P63017	Heat-shock cognate 70kDa (Hsc70)	Interacts with phospholipase A2 (iPLA2)
B/ Lipid-related proteins			
	Q8K4S1	Phosphoinositide-specific phospholipase C ε	Involved in phosphoinositide signalling
	Q9JJ00	Phospholipid scramblase	Mix phospholipids between membrane leaflets
	Q05816	Fatty acid binding protein (E-FABP)	Free fatty acid transporter
	Q5SRA8	Prostaglandin F2 receptor negative regulator	Interacts with CD81
C/ Phospholipase partners			
	P05064	Fructose- bisphosphate aldolase	Interacts with phospholipase D (PLD2)
	P63017	Heat Shock protein 70kDa	Interacts with phospholipase A2 (iPLA2)
	P67871	Casein kinase II beta subunit	Interacts with phospholipase A2 (sPLA2)
D/ GTP binding proteins			
Heterotrimeric	P08752	G(i)alpha-2 subunit	
	P62874	G(i)G(s) beta subunit 1	
	P62880	G(i)G(s) beta subunit 2	
Monomeric GTPases	P35278	Ras-related prorein Rab	
	Q9QUI0	Transforming protein RhoA	Interacts and activates phospholipase D (PLD2)
	P63835	Ras-related protein Rap-1A	
	Q61411	Transforming protein P21/H-Ras-1(c-H-Ras)	
	Q61820	GTP-binding nuclear protein Ran	
	P62331	ADP-ribosylation factor 6	Interacts and activates phospholipase D (PLD2)
	Q8BGX0	GTP-binding protein ARD-1	
		(ADP-ribosylation factor domain protein 1)	

Table 5 : Partial protein content of RBL-2H3wt exosomes
Only proteins related to exosome markers, lipid metabolism and G proteins are reported. The overall analysis identified 382 different proteins. Observations are detailed in the text.

Among the phospholipases, only phospholipase Cε hydrolyzing phosphoinositides was detected (Table 5B). It is noteworthy that, proteins known to interact with phospholipases D and A2 were present (Table 5C). Fructose bisphosphate aldolase interacts directly with phospholipase D isoform PLD_2 and inhibits its activity (Kim *et al.*, 2002). The exosomes contained casein kinase II (cK2) that can phosphorylate PLD_2 (Ganley *et al.*, 2001) and can also interact with $sPLA_2$-IIA (Shimoyama *et al.*, 2001), precisely one of the $sPLA_2$ isoforms we detected in this work (Figure 36). Hsp 70, one of the typical exosome markers (Table 5A), has also been shown to interact with the $iPLA_2$ (Mancuso *et al.*, 2000).

Phospholipase Cε has been shown to be regulated by G proteins, either the subunits of heterotrimeric G proteins or monomeric GTPases (Gandarillas *et al.*, 2009), both being recovered in the exosomes (Table 5D). This prompted us to consider that GTPases could participate to the regulation of exosome lipolytic enzymes. It is noteworthy that exosomes contained almost all members of the Ras superfamily GTPases : ARF, Rho, Rap, Rab, p21Ras, Ran (Table 5D) except Cdc42 (Konstantinopoulos *et al.*, 2007). Possible pathways connecting the Ras superfamily GTPases and phospholipases have been reported. The GTPases RhoA and Arf 6 (Table 5D) are direct activators of PLD_2 (Bae *et al.*, 1998; Hiroyama & Exton, 2005), from rat or human origin (Le Stunff *et al.*, 2000).

2. Exosomes contain the PLD/PAP pathway

Figure 35A reports that the presence of diglycerides (DG) phosphatidylethanol (PEt) and lysophophatidylcholine (LPC) when RBLwt exosomes were incubated with the fluorescent and membrane-diffusible phosphatidylcholine. We investigated whether an autonomous regulation of the lipolytic enzymes involved in the production of these lipid mediators could occur in exosomes. Addition of GTPγS up to 300µM in RBLwt exosomes had no effect on the PLD activity (Figure 35B curve “a”). The activity was not increased by exosome sonication. Immunodetection showed the selective sorting of the PLD_2 isoform in exosomes (Figure 35C lane 2) as compared to the parental cells, containing mainly PLD1 (Fig 35C lane 1). PLD_2 activation in RBLwt exosomes could be repressed by the Aldolase A reported by the protein analysis (Table 5[P05064] fructose bis-phosphate aldolase) and which has been established as a direct inhibitor of PLD_2 by acting on its PH domain (Kim *et al.*, 2002). Therefore the occupation of the PH domain might prevent activation of the phospholipase.

We expected to modidy the natural stoichiometry between the putative inhibitor Aldolase A and PLD_2 by overexpressing the human HA-PLD_2 in RBL cells (see Supplemental Figure S2), the hHA-PLD_2 being targeted to exosomes (Figure 35C, lanes 3,4). Indeed, the basal PLD activity in RBLpld2 exosomes was twice as high as that of RBLwt exosomes (Figure 35B; GTPγS=0). When increasing amounts of GTPγS were added, a clear GTP dependency of the PLD activity in RBLpld2 exosomes was then observed (Figure 35B curve”b”).

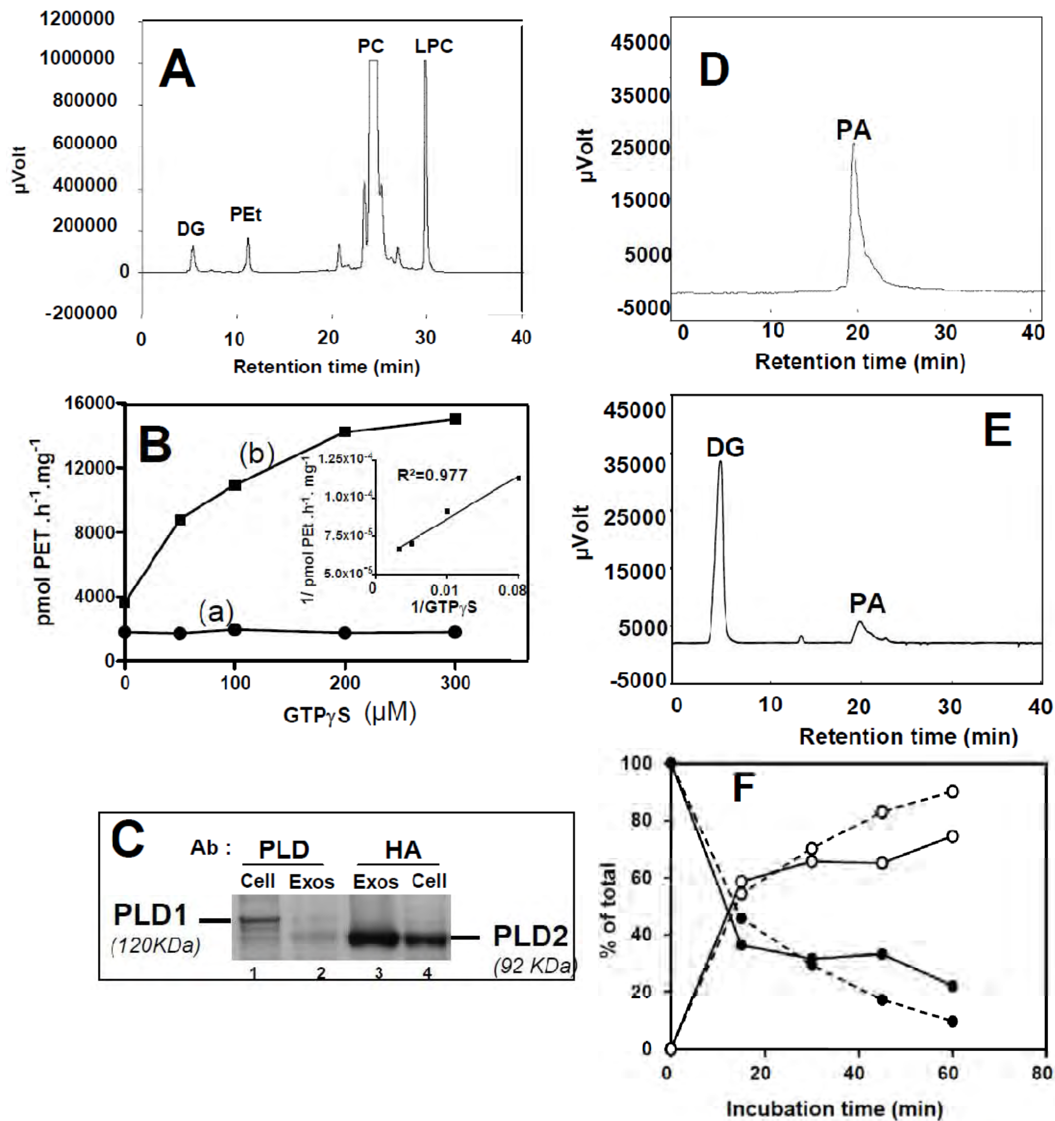

Figure 35 : **Exosomes contain the phospholipase D/ phosphatidate phosphatase pathway**

A. Presence of phospholipase-mediated second messengers on exosomes. Exosomes from RBLwt cells were incubated for 1 hour at 37°C with Bodipy-phosphatidylcholine (Bodipy-PC) as substrate. DG, diglycerides; PEt, phosphatidylethanol; PC, phosphatidylcholine; LPC, lysophosphatidylcholine. DG indicates the presence of a DG producing enzyme (phospholipase C or phosphatidate phosphatase); PEt was indicative of a phospholipase D activity, whereas LPC accounted for phospholipase A_2 activity.

B. Comparative regulation by GTPγs of PLD activity in RBLwt or RBLpld2 exosomes. Exosomes prepared from RBLwt cells (a)(●) or from RBLpld2 cells (b)(■) were incubated with Bodipy-PC for 1h at 37°C in the presence of increasing concentrations of GTPγS. Activity of PLD is expressed as pmol PET(phosphatidylethanol) / hr / mg protein. **Inset**-Double-reciprocal plot: 1/(activity) versus 1/[GTPγS].

C. The overexpressed human HA-PLD$_2$ is targeted to exosomes. Cell lysate and RBLpld2 exosome samples were blotted either with the anti-PLD antibody (N-PLD4, see Materials), recognizing both PLD1 and PLD$_2$ isoforms, or with the anti-HA antibody.

D.Typical HPLC profile of Bodipy-labeled phosphatidic acid (Bodipy-PA) prepared from in-vitro assay with phospholipase D (see Methods).The presence of positively charged triethylamine in the solvents delayed the elution of PA leading to a characteristic asymmetrical peak.

E. PA processing to yield diglyceride by RBLwt exosomes. Incubation of Bodipy-PA with exosomes proceeded for up to 1 hour at 37°C and the products were separated as in "D".

F. Kinetics of PA hydrolysis. RBLwt exosomes were incubated with or without 100 µM GTP�S for various period of time. The results are expressed as a percentage of the total products DG (○) + PA (●), with (------) or without (——) GTPγS.

Phospholipase D activity generates the transphosphatidylation product phosphatidylethanol (PEt) in the presence of ethanol (Figure 35A) which competes with the water required to form phosphatidic acid (PA).
However, even in the absence of ethanol, PA was not detected across the chromatograms, suggesting the presence of a phosphatidate phosphatase (PAP1) on exosomes. When purified Bodipy-PA was injected into the HPLC system, the resulting peak exhibited a typical asymmetrical shape (Figure 35D) which was not observed in any chromatograms obtained from exosome incubations with Bodipy-PC.

Indeed, upon incubation of Bodipy-PA with exosomes, a strong conversion into diglycerides was observed (Figure 35E). Kinetics analysis demonstrated that 60% of the PA was hydrolyzed into diglycerides within 15 min (Figure 35F) indicating the presence of a very active PAP1 in intact exosomes. The kinetics of PA hydrolysis were similar in the presence or absence of GTPγS (Figure 35F).

3. Exosomes from RBL cells carry GTP–activatable PLA_2s and contain the 3 classes of PLA_2s.

Figure 35A reported the presence of a PLA_2 activity as evidenced by the high LPC content. During the course of the studies on PLD activation (Figure 35) we noticed a GTP-dependent enhancement of the LPC peak, both on RBLwt and on RBLpld2 exosomes.
We then investigated whether a dynamic regulation of PLA_2 activity might occur in RBLwt exosomes (Figure 36). It can be noted that, GTPγS was able to reveal a PLA_2 activity on intact exosomes incubated in calcium-free PBS and in the presence of the inhibitor MAFP (Figure 36A). GTPγS dose-dependency PLA_2 activation (Figure 36B) fits a hyperbolic curve as shown by the linearity of the double-reciprocal plot (Figure 36B, insert).

Exosomes exhibited a higher PLA_2 activity following sonication, indicating that the PLA_2s were partly located in the exosome lumen. The relative part played by each PLA_2 class (cytosolic calcium dependent cPLA2, cytosolic calcium independent iPLA2, secreted $sPLA_2$s) was next investigated in the presence of GTPγS on sonicated exosomes. We observed that MAFP decreased the total PLA_2 activity by 60% (Figure 36C). MAFP inhibits both the $cPLA_2$ and to a lesser extent the $iPLA_2$ (Gubern *et al.*, 2008), and we next checked specific inhibitors. The specific $cPLA_2$ inhibitor (pyrrolidine-1) reduced the total PLA_2 activity by 37% (Figure 36C), whereas bromoenolactone (BEL), the specific $iPLA_2$ inhibitor, abolished 39% of the overall PLA_2 activity (Figure 36C). The concentration of inhibitors was 50µM, i.e above that used to inhibit the various PLA_2s in cells (Allal *et al.*, 2004) (Gubern *et al.*, 2008). Making the sum of the inhibitions triggered by pyrrolidine-1 and BEL in exosomes (Figure 36C) led to the reduction of the global PLA_2 activity by 76 %, indicating that another type of PLA_2 activity was present. Me-Indoxam, a specific inhibitor of secreted phospholipases (Lambeau & Gelb, 2008) was checked, and decreased the total PLA_2 activity by 19% (Figure 36C). Together, the cumulative effect of the three inhibitors diminished the total PLA_2 activity by 95 +/- 6.5%.

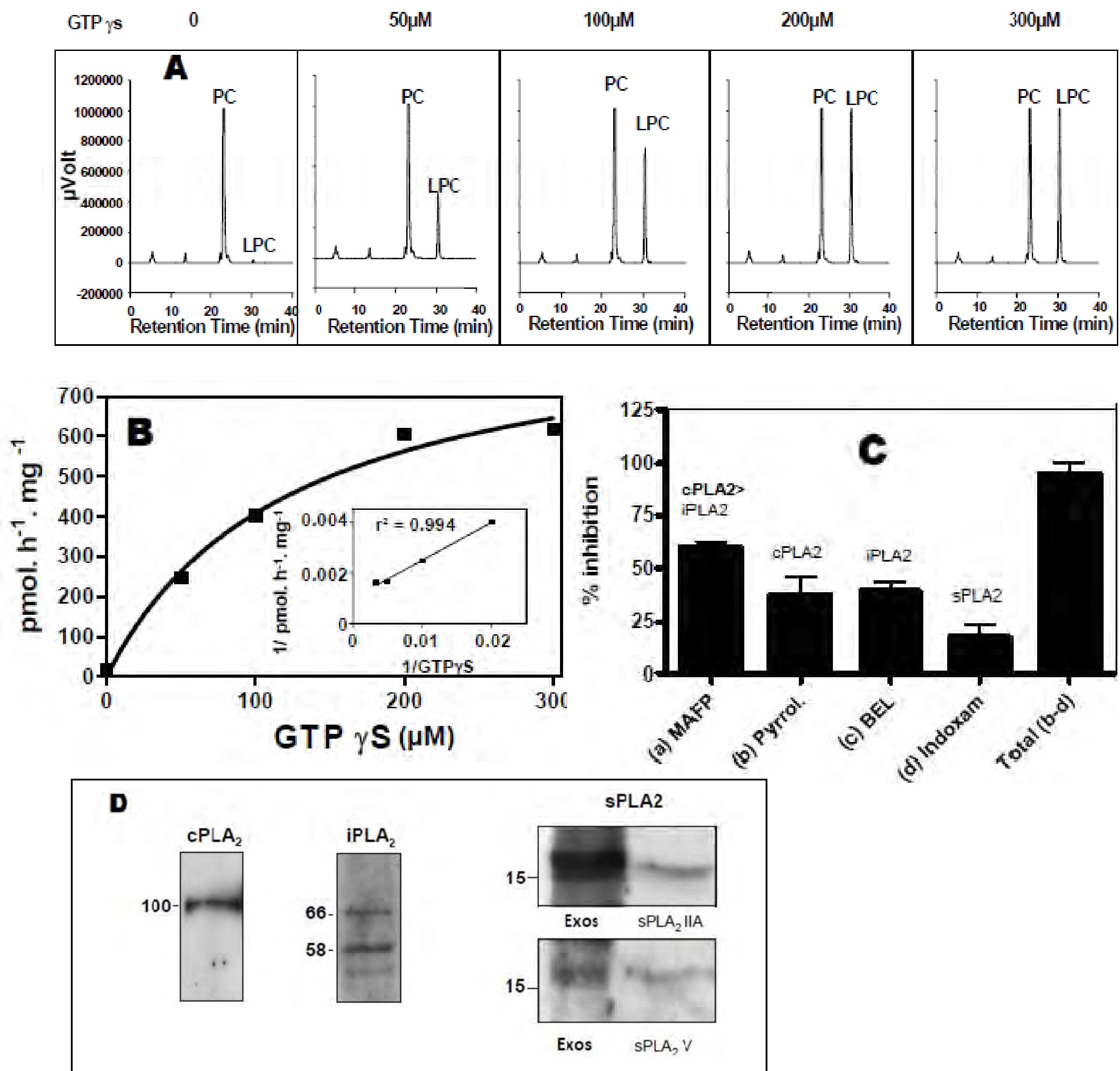

Figure 36 : **Exosomes are carriers of GTPγS-activatable phospholipases A$_2$.**

A. HPLC profiles of exosome phospholipase A2 activity in the presence of GTPγS. Intact RBLwt exosomes were preincubated with MAFP for 10 min at room temperature, and the reaction was started by adding Bodipy-PC in the presence of GTPγS at the indicated concentrations. Incubations were for 1 hour at 37°C in Ca^{++}/Mg^{++} free-PBS.

B. Concentration-dependent effect of GTPγS on exosome MAFP-insensitive PLA$_2$.
Activity is expressed in pmol/ hr/ mg of protein from HPLC profiles obtained in "**A**"
Inset- Double reciprocal plot: 1/(activity) versus 1/[GTPγS].

C. Effect of class-specific PLA$_2$ inhibitors on total exosome PLA$_2$ activity. Sonicated exosomes from RBLwt cells were preincubated for 10 min at room temperature with 50µM of the various class inhibitors: MAFP (cPLA$_2$ > iPLA$_2$ inhibitor), Pyrrolidine (cPLA$_2$ inhibitor), BEL (iPLA$_2$ inhibitor), Me-Indoxam (sPLA$_2$ inhibitor), and in the presence of 100µM GTPγS. The reaction was started by addition of Bodipy-PC. The respective inhibitions are expressed as percentages of the total activity measured without inhibitor. The sum of inhibitions triggered by Pyrolidine, BEL and Indoxam is indicated by the right bar (Total b-d). Results are means of 4 independent experiments +/- SEM for Pyrolidine, BEL and Indoxam treatments, and 6 independent experiments +/- SEM for MAFP treatment.

E. Immunodetection of PLA$_2$ class members in exosomes. The calcium dependent cPLA$_2$IVA, calcium independent iPLA$_2$-VIA, and secreted sPLA$_2$-IIA and V were detected in RBLwt-derived exosomes by western-blotting. "Exos"= exosomes; sPLA$_2$ IIA = recombinant sPLA$_2$-IIA ; sPLA$_2$ V = recombinant sPLA$_2$-V.

The residual 5 % activity might be related to PLA_2s insensitive to the inhibitors such as some secreted $sPLA_2$ (Singer *et al.*, 2002). Therefore, the three classes of PLA_2s participated to the global PLA_2 activity detected in the exosomes.

We next assessed the presence of members of the three PLA_2 classes by using specific antibodies (Figure 36D). $cPLA_2$-IVA was detected as a single band, whereas $iPLA_2$-VIA was present as processed forms (Blanc *et al.*, 2007) The iPLA2-VIA can be cleaved at 3 different sites by caspase 3 which generates various processed forms depending upon the combination of the sites effectively cleaved (Lauber *et al.*, 2003). Similar fragmented forms of iPLA2 as those we reported in Figure 35D were observed in erythrocyte-derived exosomes (Blanc *et al.*, 2007). Proteolytic processing has been shown to enhance the iPLA2-VIA activity (Lauber *et al.*, 2003). Among the third class of PLA_2s, namely secreted $sPLA_2$, the presence of $sPLA_2$-IIA and $sPLA_2$-V groups was observed (Figure 36D). Therefore, RBL exosomes concentrated members of each of the 3 classes of PLA_2s and are thus a unique cell compartment.

4. Exosomes as carriers of bioactive lipids.

A large panel of free fatty acids was recovered from exosomes (Figure 37A). Fatty acids could be carried from the parental cells or directly generated within the exosomes by the phospholipase A2 activities. We previously established that 1 mg of exosome protein contained 230 nmoles phospholipid [*see ref* (Laulagnier *et al.*, 2004a)]. Therefore 230 nmoles free fatty acid could be potentially released by the respective PLA_2 activities considering they displayed 100% efficiency, with an additional amount of free fatty acid originating from the lysophospholipase activity borne by the $cPLA_2$ and $iPLA_2$. However, a total amount of 1420 nmoles free fatty acid / mg exosome protein was measured (Figure 37A), indicating that most of the exosome free fatty acid content was already present at the time of exosome membrane biogenesis. The chain length of the saturated fatty acids ranged from 14 to 24 carbons (Figure 37B) with palmitic and stearic acids being the major ones. Monounsaturated fatty acids were essentially from the omega-9 series (Figure 37C) oleic acid being the most abundant. Polyunsaturated fatty acids almost exclusively contained members of the omega-6 series (Figure 37D). Only one member of the omega-3 series, namely docosahexaenoic acid (DHA) was detected. Interestingly, arachidonic acid accounted for about 30% of the total polyunsaturated fatty acids.

We next investigated whether bioactive lipids derived from arachidonic acid could be found in exosomes. Quantification of prostaglandins was performed by GC/MS and demonstrated the presence of mainly PGE_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 [15d-PGJ_2] (Figure 37E). The 15d-PGJ_2 was slightly enriched in the vesicles as compared to the parent cells. The respective amounts of the various prostaglandins ($PGF_2\alpha$, PGE_2, PGD_2 , 15d-PGJ_2) in exosomes was not enhanced by incubation with GTPγS (Figure 37E), indicating that the prostaglandins originated either from the basal exosome PLA_2s activities, or were loaded in

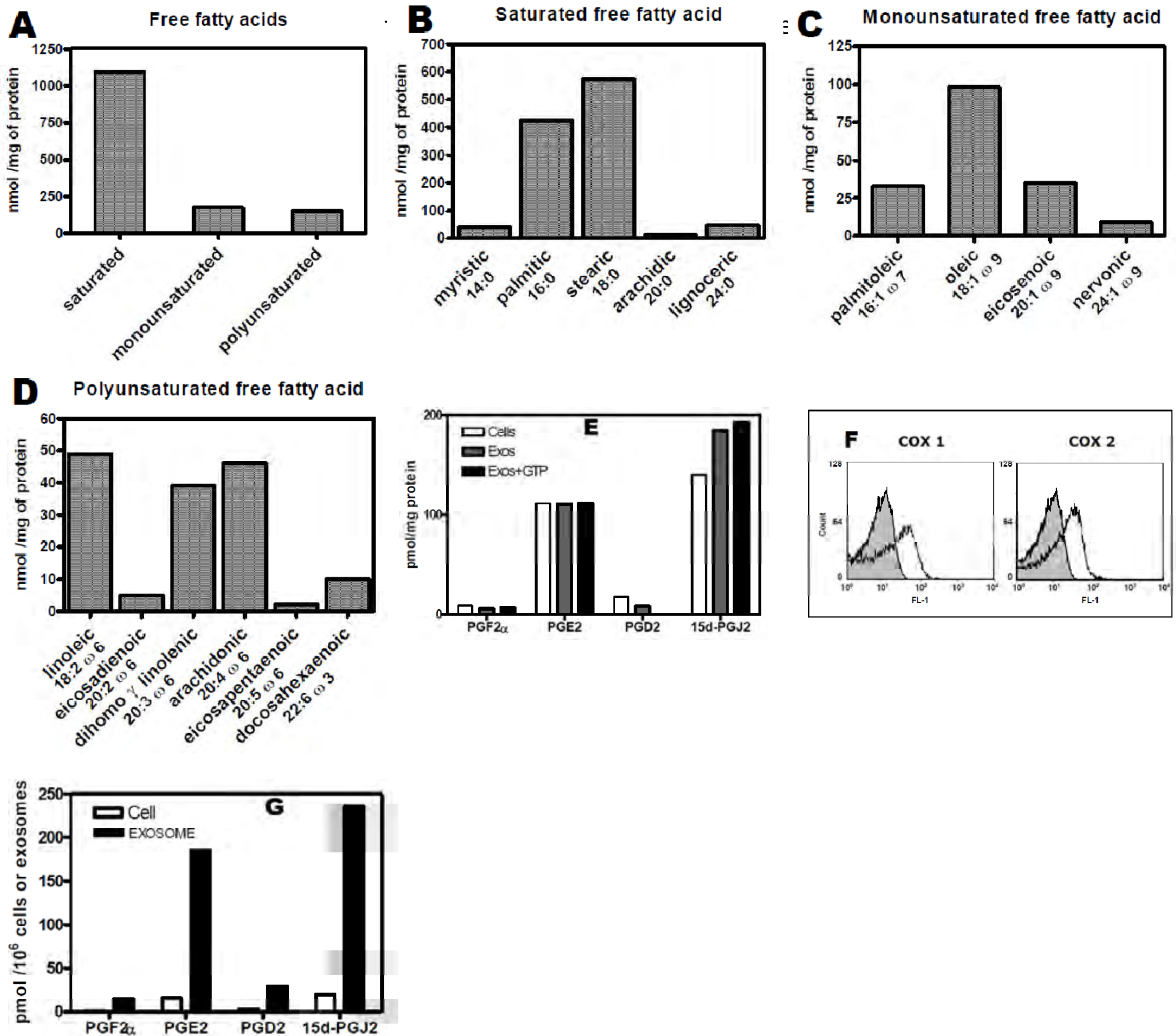

Figure 37 : Exosomes carry free fatty acids and arachidonic acid-derived bioactive lipids.
A-D. Fatty acid distribution in exosomes.
Details for analysis are reported in Methods.
E. Prostaglandin content of exosomes ("Exos") and parent cells ("Cells"). Prostaglandins were quantified by GC/MS in untreated ("Exos") or GTPγS-treated exosomes ("Exos +GTP") and in parent cells (RBLwt). GTPγS treatment was performed for 1 hour at 37°C with 200 µM of the nucleotide.
15d-PGJ_2: 15-deoxy-$\Delta^{12,14}$-PGJ_2. $PGF_2\alpha$, PGE_2, PGD_2: prostaglandins $F_2\alpha$, E_2 and D_2 respectively.
F. Exosomes contain cyclooxygenases 1 and 2.
Analysis of exosome cyclooxygenase expression by flow cytometry, comparatively to control isotype (grey shaded curves).
G.Comparative prostaglandin content of exosomes and parent cells.
Prostaglandin content per mg protein plotted in Fig E was converted into cell-equivalents or vesicle(exosome)-equivalents, and normalised to 10^6 cells or 10^6 exosome vesicles respectively. 1mg protein corresponded to (71.4 +/- 0.54)10^5 cells and (5.96+/-0.13)10^5 exosome vesicles.

steps of prostaglandin biosynthesis were expressed in exosomes (Figure 37F), indicating that exosomes could be autonomous biological structures for the biosynthesis of the various prostaglandins. In that respect the arachidonic acid concentration in exosome membrane

(45 nmoles/mg protein; Figure 37D) was in large excess compared with the total membrane prostaglandin concentration (0.31 nmoles / mg protein; Figure 37E).

The number of exosome vesicles per unit protein was established and used to calculate the amount of prostaglandin associated with a defined number of vesicles. As compared to the same number of parental cells, exosomes carried between 12 to 15 times more prostaglandins (Figure 37G). To our knowledge, this is the first report of vesicle-associated release of prostaglandins from cells.

In order to evaluate the potential of exosomes as vehicles of bioactive lipids, we investigated whether exosomes could traffic between RBL-2H3 cells.

5. Exosomes are internalized by resting and activated RBL-2H3 cells and concentrate into endosomes.

Confocal microscopy performed on living cells showed an accumulation of exosomes on the cell periphery detectable as soon as 5min, with subsequent internalization leading to the formation of intracellular aggregates indicating storage in an endosomal compartment, as observed after 1h and 4h (Figure 38A). Exosome uptake was an active process since cross-linking of peripheral protein on target cells by paraformaldehyde impaired intracellular exosome accumulation (Figure 38A, bottom panels). Only faint diffuse cell labeling was observed in this case and was attributed to some exchange of the lipidic fluorescent probe between exosomes and the target cell during the step of exosome interaction with the peripheral cell membrane. We investigated whether activated cells, which release exosomes, were also able to internalize them. Figure 38B reports that RBL-2H3 cells activated by Fcε-RI cross-linking appeared even more efficient at internalizing exosomes as compared to resting ones (Figure 38B).

Uptake was linear at least up to 1 hour of incubation as shown by flow cytometry monitoring of exosome internalization (Figure 38C). Similar data were obtained by measuring the Bodipy-ceramide content of target cells following organic extraction of the probe after exosome internalization. This procedure allowed us to quantify the amount of internalized exosomes. It was found that one microgram exosome was internalized in one hour in 10^6 resting cells (Figure 38D).

Late endosomes in RBL-2H3 cells feature a dual function: being able to release their contents upon stimulation or being recipients of an endocytosis activity (Barbu & Pecht, 2005). We investigated whether exosomes could be co-localized with endosome markers following their incubation with target cells. Three markers were checked, *i)* CD63, a general marker of late endosomes in RBL cells (Amano *et al.*, 2001), *ii/* the lysolipid lysobisphospatidic acid (LBPA, also called BMP for Bis[Monoacylglycero]Phosphate) which accumulates in MVB (Kobayashi *et al.*, 1998b), *i.e.* late endosomes containing intralumenal vesicles (as illustrated in *Supplementary Data 2A*), and *iii)* Rhodamine-Phosphatidylethanolamine a fluorescent lipidic probe which accumulates specifically in late endosomes. Figure 39(A-C) shows that exogenously-added exosomes were labeled inside the

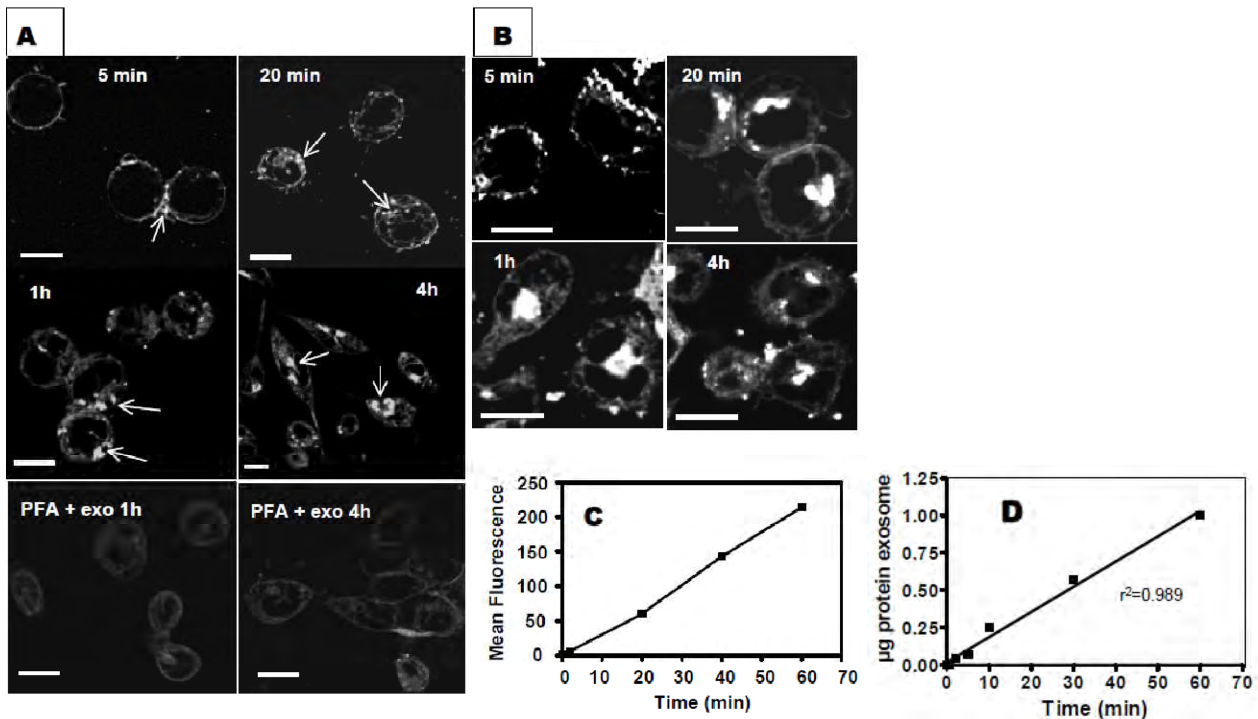

Figure 38 : Monitoring of exosome uptake by RBLwt cells

A. Time-lapse monitoring of fluorescent (Bodipy-ceramide)-labeled exosome uptake by confocal microscopy. Uptake of exosomes labeled with Bodipy-ceramide (FITC-type probe) was monitored in a temperature-controlled CO_2 chamber with Zeiss LSM 510 software, and pictures were taken at the indicated times. Controls were performed by treating cells with paraformaldehyde (PFA) then washing prior to the addition of exosomes; uptake was then recorded for 1 to 4 hours (bottom panels). Bars = 5µm.

B. Time-lapse monitoring of fluorescent (Bodipy-ceramide)-labeled exosome by activated RBLwt cells following Fc⍰⍰⍰cross-linking. The cells were incubated overnight with IgE directed against DNP-HSA and Fc⍰ cross-linking was triggered by adding DNP-HSA at the same time as fluorescent exosomes. Uptake was monitored as in (A). Bars= 5µm.

C. Time course of exosome internalisation recorded by flow cytometry. Mean fluorescence of cells incubated with Bodipy-Ceramide-labeled exosomes and plotted versus time. After each incubation time, non-internalized exosomes were washed away prior to FACS analysis.

D. Quantification of exosome internalisation. Fluorescent exosomes were incubated as function of time with resting cells. After washing at appropriate times, cell-associated fluorescence monitoring internalized exosomes was extracted with butanol and converted into µg exosome protein. Average of two determinations.

cells by anti-CD63 antibody internalized by fluid-phase endocytosis. Localisation of exosomes inside the endocytic track was more precisely investigated by monitoring the MVB distribution with anti-LBPA labeling (Figure 39 D-F). Remarkably, co-localisation of exosomes was observed inside MVBs located close to the nucleus (Figure 39F, circles and arrows). With Rh-PE as MVB-specific probe, supplementary evidence was obtained that exogenous exosomes joined the MVB compartment located close to the nucleus (Figure 39G-I).

We estimated the concentration that could be reached by exosome-transported lipid mediators accumulated inside the endosomal compartment of a target cell. Considering an average diameter of 600nm for an RBL-2H3 cell endosome (Grimberg *et al.*, 2003) with an average number of 30 endosomes per RBL-2H3 cell (Tadokoro *et al.*, 2007), the resulting volume of the entire endosomal compartment is about 500 times lower than the total cell volume (Table 6). Therefore exosome-transported lipid mediators accumulated inside

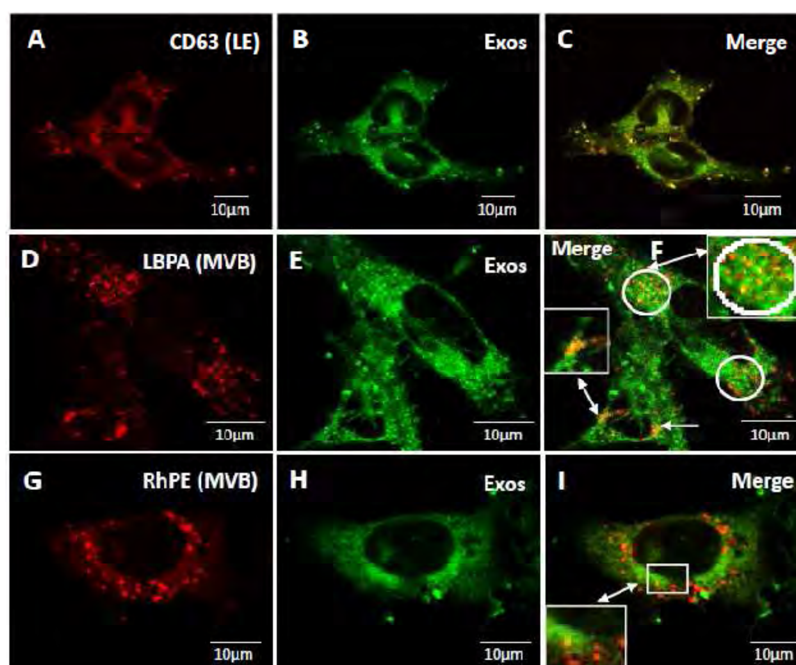

Figure 39 : Characterization of the intracellular compartment accumulating exosomes.
Antibodies against CD63 (late endosomes; LE) or LBPA (Multivesicular Bodies; MVB) and the MVB probe Rhodamine-PE (RhPE) were pre-internalized in the cellular endocytic track. Cells were washed, and then fluorescent Bodipy-Ceramide-labeled exosomes were pulsed for 4 hours into cells. Excess of exosomes was washed away and cells were fixed, permeabilized, and CD63 or LBPA labelling revealed by the appropriate secondary antibody.
A-C. Endosome anti-CD63 labelling (A), exosome labelling (Bodipy-Ceramide FITC) (B), and merge (C) showing the colocalisation between exosomes and endosomes (orange and yellow spots represent different amounts of exosomes internalized) .
D-F. MVB anti-LBPA labelling (D), exosome labelling (Bodipy-Ceramide FITC) (E), and merge (F) revealing various amounts of exosomes colocalized with MVBs (monitored by orange and yellow spots), occurring close to the nucleus (circles and arrows)
G-I. **MVB Rhodamine-PE labelling (G), exosome labelling (Bodipy-Ceramide FITC) (H), and merge (I) indicating different amounts of exosomes colocalizing with MVBs (monitored by orange or yellow spots) detected around the nucleus (square)**

endosomes (Figure 38Figure 39) were 500 times more concentrated than if they were diluted in the whole cell volume (Table 6). As a consequence, the resulting PGJ_2 endosomal concentration reached 52 µM (Table 6). Other endosomal concentrations were 33µM, 2.4 µM and 1.7 µM for PGE_2, PGD_2 and $PGF_2\alpha$ respectively, whereas fatty acids such as arachidonic acid or DHA reached millimolar concentrations (4mM and 0.9mM respectively). Thus, target cell endosomes behave as a “concentrator compartment” of lipid mediators transported by exosomes, allowing micromolar concentrations of prostaglandins to be reached, *i.e.* concentrations able to trigger further biological responses such as PGJ_2-mediated PPARγ activation. It can be noted that the extent of exosome internalization by cells (1µg exosomes /10^6 cells /hr; Figure 38D) was similar to the amount of exosomes released by 10^6 cells upon stimulation (1. 44+/-0.47 µg), suggesting an efficient cell-to-cell communication process.

	A Mean diameter	B Mean volume of 10^6 cells or their endosomes *ml*	C Exosomes internalized in 10^6 cells in 1 hr *µg*	D PGJ2 transported by 1µg exosomes *pmoles*	E Final PGJ2 concentration in cells or endosomes *µM*	F Concentration ratio
(1) EXOSOMES			**1**	**0.193**		
(2) CELL	**15 µm**	**1.76X10-3**			**0.11**	**1**
(3) ENDOSOMES	**600 nm**					
(4) TOTAL ENDOSOME COMPARTMENT (30 Endosomes per cell)		**3.38X10-6**			**57.1**	**519**

Table 6 : Estimated concentration of exosome-transported PGJ_2 in endosomes of target cells

The amount of PGJ2 (column D) transported by the amount of exosomes plotted in column C was converted into µM concentrations (column E) either by considering the total volume of the recipient cells (column E line 2), or the total volume of late endosomes present in cells (column E line 4). The resulting values indicated that PGJ2 was about 500 times more concentrated in the total endosome compartment than in the total cell volume (column F).

Values in column A were obtained from literature data (*see Results section)* and correspond to RBL-2H3 cells.

Values in column B. The total volume of 10^6 cells (line 2) and the corresponding volume of their endosome compartment (line 4) was calculated from data in column A (1ml= 10^{12}µm^3).

Values in column C. Net amount of exosome internalized in 10^6 cells in 1 hour (obtained from Figure 38D)

Values in column D. Amount of PGJ_2 transported by the amount of exosome in column C; calculated from data in Fig 31E (average of 193 pmol./mg protein in exosomes [Figure 37E right bars]).

Values in column E. PGJ_2 concentrations obtained by dividing the amount of PGJ_2 (column D) by the volume of cells (column B line 2) or the volume of their total endosome compartment, considering an average of 30 endosomes per cell (column B line 4).

Values in column F. Ratio between PGJ_2 concentrations in endosomal compartment versus total cell.

The same calculation procedure was applied to the other prostaglandins and two typical fatty acids (arachidonic acid and DHA) from data in Figure 37 and reported in the "Results" section of the text.

D. Discussion

Exosomes are nanovesicles released from intact viable cells and they participate in cell-to-cell communication in various physiological and pathological situations, such as the immune response (Simons & Raposo, 2009), inflammation (Bhatnagar & Schorey, 2007), or atherogenesis (Zakharova *et al.*, 2007). Mast cell derived exosomes trigger functional maturation of dendritic cells (Skokos *et al.*, 2003). This maturation process involves secreted

PLA_2s (Perrin-Cocon *et al.*, 2004) and prostaglandins (Thurnher, 2007). We therefore investigated the presence of lipid-related proteins and lipid mediators on exosomes derived from the mast cell line RBL-2H3.

High-throughput protein analysis reported the presence of only four proteins related to lipid metabolism. However we revealed the presence of other lipolytic proteins by their activity and by immunodetection. The presence of a high content in monomeric G proteins led us to hypothesize specific regulation of these phospholipases in exosomes. The subfamilies of Ras GTPases reported in Table 1 are cytosolic proteins likely to be located inside the exosomes. Therefore, GTP must cross the exosome membrane to activate GTPases and subsequently phospholipases. GTP transporters might be present in exosomes, since they have been reported in synaptic vesicles (Kondo *et al.*, 2006; Santos *et al.*, 2006), a type of vesicle very similar to exosomes.

The difference in activation of the PLD by GTP between RBLpld2 and RBLwt exosomes appeared related to the stoichiometry between PLD_2 and aldolase A. Among the proteins recovered by protein analysis and reported in Table 5, aldolase (P05064) exhibited one of the highest expression scores whereas PLDs were not even detected by high-throughput analysis. Purified aldolase dose-dependently inhibits the PLD_2 activity (Kim *et al.*, 2002) and inhibitor interaction occurs at the PH domain of $PLD_{2.}$ This interaction might impair GTPases such as RhoA or Arf6 to activate PLD_2, or might prevent another domain of the PLD_2, the phox homology domain (PX) which exhibits GAP activity, to activate the GTPases themselves (Lee *et al.*, 2006).

Interaction domains on Arf 6 and RhoA with PLD_2 have been mapped (Bae *et al.*, 1998; Hiroyama & Exton, 2005), whereas no direct interaction between any of the small G-proteins reported in the present study and PLA_2s have been reported so far. In whole cells, the $cPLA_2$ and the $sPLA_2$-IIA can be activated downstream of RhoA GTPases with subsequent effects on PGE_2 formation (Petry *et al.*, 2004). Similarly, $iPLA_2$ activation downstream of RhoA has been suggested (Maeda *et al.*, 2006). However, in a cell-free system such as exosomes, the signaling network between RhoA and the phospholipases might be different as compared to whole cells, and exosomes might reveal a specific regulation of PLA_2 activities.

Regarding the functional role of exosome phospolipases A_2, the calcium-independent $iPLA_2$ has been shown to allow the elimination of erythrocyte-derived exosomes by apoptotic cells (Blanc *et al.*, 2007). Concerning $sPLA_2$, exosomes transporting $sPLA_2$ IIA and V (Figure 37) might account for the transcellular activity of these phospholipases reported to occur from activated RBL-2H3 cells (Wijewickrama *et al.*, 2006). The group $sPLA_2$-V was reported to be secreted from RBL-2H3 cells and to trigger eicosanoid biosynthesis in neighboring target granulocytic cells (Wijewickrama *et al.*, 2006). The $sPLA_2$-V activity appears related to IgE-dependent PGD_2 formation and to enhanced exocytosis in RBL-2H3 cells (Sawada *et al.*, 1999). Vesicular secretion of $cPLA_2$ has not been reported so far.

The set of prostaglandins transported by exosomes (Figure 37) are derived from PGH2, and metabolic conversion of PGH2 has been shown to occur through a transcellular

mechanism beween two different types of cells, containing either the Cox 1 and 2 or the terminal prostaglandin synthases (Folco & Murphy, 2006; Salvado *et al.*, 2009). By transporting from cell-to-cell metabolic precursors such as PGD_2 (Figure 37G) and enzymes accounting for the early steps of prostaglandin biosynthesis such as COXs (Figure 37F), exosomes could account for transcellular metabolism of prostanoids reported to occur between normal and tumor cells (Folco & Murphy, 2006). Arachidonic acid present in exosomes (Figure 37D) would serve as transcellular biosynthetic precursor. Although eicosanoid transcellular metabolism has been reported to occur at inflammation sites between different cell types (Zarini *et al.*, 2009), one can conceive that RBL-derived exosomes are a mixed population bearing either the COX1 and 2 or the terminal prostaglandin synthases, so exosome exchange between RBL-2H3 cells would be required for completion of the entire prostanoid biosynthesis pathway. In this respect, we showed earlier that RBL-2H3 cells release three distinct subpopulations of exosomes (Laulagnier *et al.*, 2005). The present work opens further investigations to understand the mechanisms underlying the transcellular metabolism of eicosanoids.

This transcellular metabolism requires exosome trafficking between cells. We have indeed shown that exosomes added to target cells are rapidly internalized (Figure 38) into the endocytic track and join the MVB network located close to the nucleus (Figure 39). It is likely that the GTP-dependent activation of PLD and PLA_2 we observed in exosomes could occur inside the endocytic track of target cells. Many GTPases are present within the endocytosis track, some of them being maintained in an active state even in unstimulated cells (Kondo *et al.*, 2006). GTP-activated phospholipases could participate in exosome fusion with the limiting membrane of the endosome, a process called "back-fusion" (van der Goot & Gruenberg, 2006) (Falguieres *et al.*, 2009). This process allows the lumen content of the exosomes to be released into the cytosol. Back-fusion molecular mechanisms require the lipid lysobisphosphatidic acid (LBPA), also called Bis(Monoacylglycero)Phosphate (BMP), whose biosynthesis involves a $cPLA_2$-type activity (Shinozaki & Waite, 1999; Hullin-Matsuda *et al.*, 2007), as well as a combination of PLA_2 and PLD activities (van Blitterswijk & Hilkmann, 1993). A previous report describes GTP-dependent $cPLA_2$-mediated fusion of secretory granules(Sattar & Haque, 2007). Also phosphatidic acid resulting from PLD activity is a fusogenic compound in presence of calcium (Blackwood *et al.*, 1997). Diglycerides generated by the PI-PLCε (Table 5) or the PLD/PA phosphatase pathway (Figure 35), could participate in exosome-endosome fusion processes by lowering the surface pressure of the phospholipids (van Rossum *et al.*, 2008). More DG can be expected in RBLpld2 exosomes and could account for the modification of the biophysical parameters (size and electronegativity) reported in Supplementary Data 2. In addition, phospholipid mixing between exosome and endosome membranes triggered by the scramblase we reported in Table 5 would facilitate membrane fusion.

We established in this work that exosomes transport prostaglandins from the parent cells. RBL-2H3 cells feature a mast cell phenotype and eicosanoids play an essential role in mast cell physiology by regulating their function in host defense and disease (Boyce, 2007).

PGE_2 can block FcεRI-mediated exocytosis of mast cells (Boyce, 2007). Exosomes, during at least the first 5 to 20 minutes (Figure 39), provide a vehicle for PGE_2 to interact with its respective GPCRs on the periphery of target cells. Thereafter, exosome internalization provides the first mechanism described for 15deoxy$\Delta^{12,14}$-PGJ_2 to enter the cells and possibly reach its intracellular targets. Actually, no specific peripheral receptors or mechanisms of entry have been identified to date for this prostaglandin (Scher & Pillinger, 2005, 2009). The exosome as a vehicle would allow the plasma membrane to be bypassed and 15d-PGJ_2 to accumulate in the endosomes of target cells, from where the prostaglandin would be released into the cytosol after fusion between exosome and endosome membranes. A possible mechanism is summarized in Figure 40.

Exosomes could also supply the 15d-PGJ_2 already bound to its receptor since a recent report indicates the presence of the PPARγ receptor among proteins found in exosomes isolated from human serum (Looze *et al.*, 2009). Interestingly, the exosome FABP we report in Table 5 could bind the arachidonic acid (AA) present in exosomes (Figure 37), and then interact directly with the PPARγ receptor, the resulting FABP-AA-PPARγ complex being subsequently addressed to the nucleus of target cells to regulate transcription (Figure 40C) (Tan *et al.*, 2002). In line with the possible modulation of nuclear receptors by exosome-carried mediators, it should be noted that the diglyceride-generating enzyme we reported in Figure 35, PAP1, has recently been characterized as a transcriptional coactivator of the PPARα receptor (Reue & Brindley, 2008).

Further experiments are required to support the functional role of exosomes in RBL-2H3 cells. As a first step, we evaluated whether exosomes could carry sufficient amounts of the prostaglandin 15d-PGJ_2 to possibly trigger PPARγ activation in target cells. When added to cells, 15d-PGJ_2 has been reported to trigger biological effects in the 10-40µM range (Cerbone *et al.*, 2007). Exosome accumulation in endosomes allowed to reach values >50µM (Table 6), *i.e.* bioactive 15d-PGJ_2 concentrations.

Because of the dynamic regulation of their phospholipases by GTP, exosomes appear to behave as "signalosomes" (Figure 40A). The "signalosomes" would circulate between cells and might regulate their functions, whether cells are resting or activated (Figure 40B). Stimulated RBL-2H3 cells feature enhanced endocytosis (Barbu & Pecht, 2005) and could internalize exosomes they had just released. Preliminary data we obtained indicate that exosomes inhibited Fcε-mediated degranulation of RBL-2H3 cells. Whether this effect involves PGE_2, known to inhibit FcεRI-mediated exocytosis of mast cells (Boyce, 2007) appears conceivable on the basis of data reported herein. Also, by possibly providing 15d-PGJ_2 to PPARγ of target cells, exosomes can repress the transcription of pro-inflammatory mRNAs (Szanto & Nagy, 2008). By circulating simultaneously with allergens which activate cells *via* FcεRI receptors, exosomes appear as a signaling device able to modulate the FcεRI-mediated mast cell response by means of activatable phospholipases and lipid mediators.

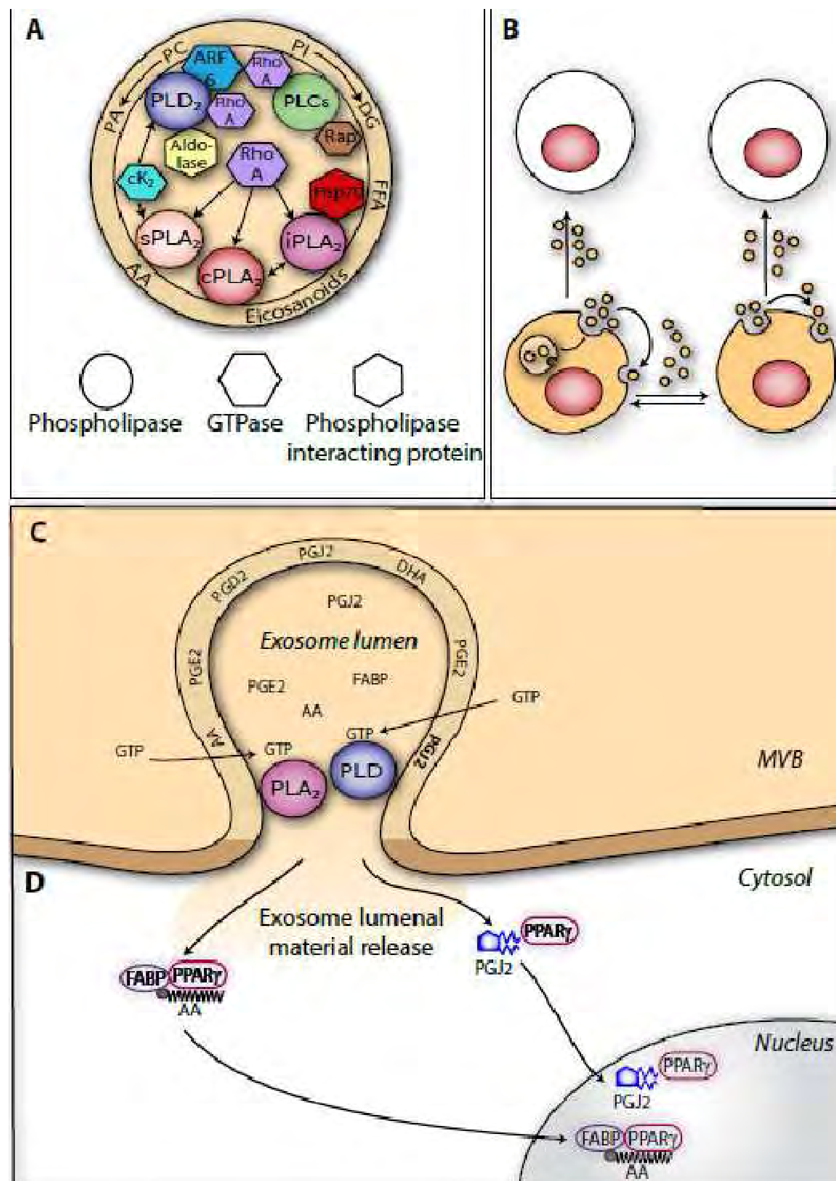

Figure 40 : Exosomes as intercellular signalosomes carrying GTP-activable phospholipases and prostaglandins
A. Exosomes carry GTP-activatable phospholipases. Proteins detected in RBLwt-derived exosomes related to phospholipase activation and reported in Table 5 were represented together with the phospholipases detected in the present work. Arrows indicate possible activation pathways based on literature data *(see Discussion)*. Note the presence of lipid mediators in the exosome membrane.
B. Exosomes as intercellular "signalosomes". Exosomes released upon cell activation can traffic between resting cells (represented in white) and activated cells (in colour). Exosomes could trigger autocrine and paracrine-type signals.
C and D. Possible mechanism of exosome-mediated bioactive lipid delivery from endosomes in target cells. Inside the intracellular compartment accumulating exosomes in target cells (endosomes, Figure 39), phospholipases borne by exosomes could participate upon activation by GTP in the fusion between exosomal and endosomal membranes, allowing delivery of exosome content into the cytosol. Exosomes carry prostaglandins such as the PPARγ agonist 15d-PGJ$_2$ (Figure 37) and a FABP (Table 5) which can bind arachidonic acid (Figure 37) and interact with PPARγ ***(C).*** The 15d-PGJ$_2$/PPARγ and FABP/Arachidonic Acid/PPARγ complexes would be further addressed to the nucleus ***(D)***.

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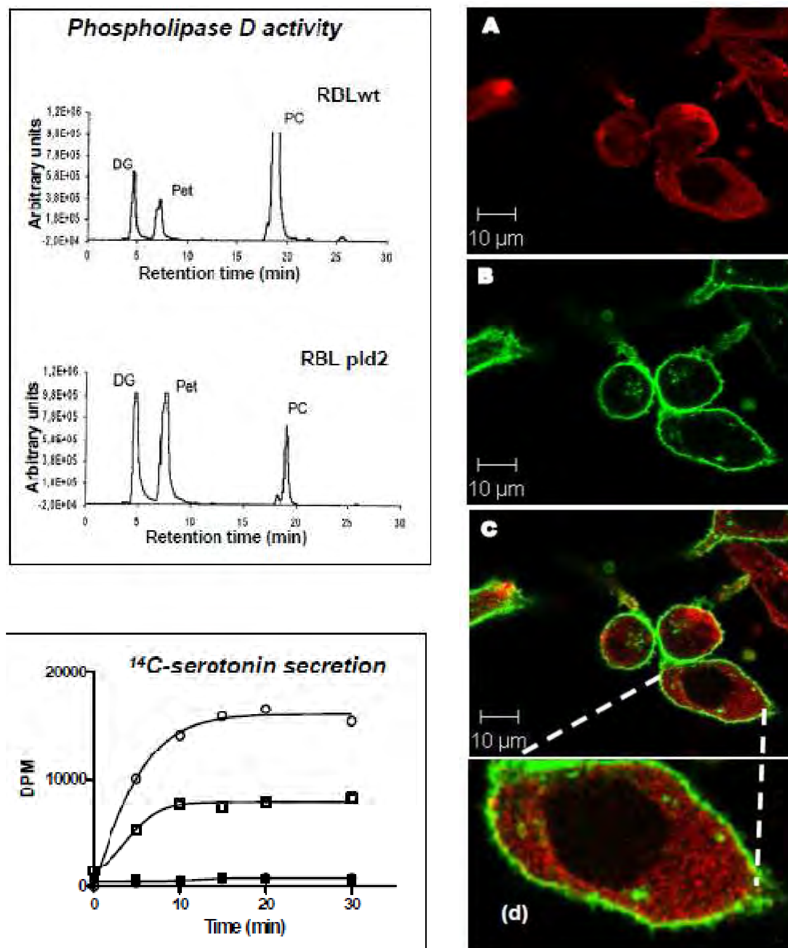
Supplementary Data

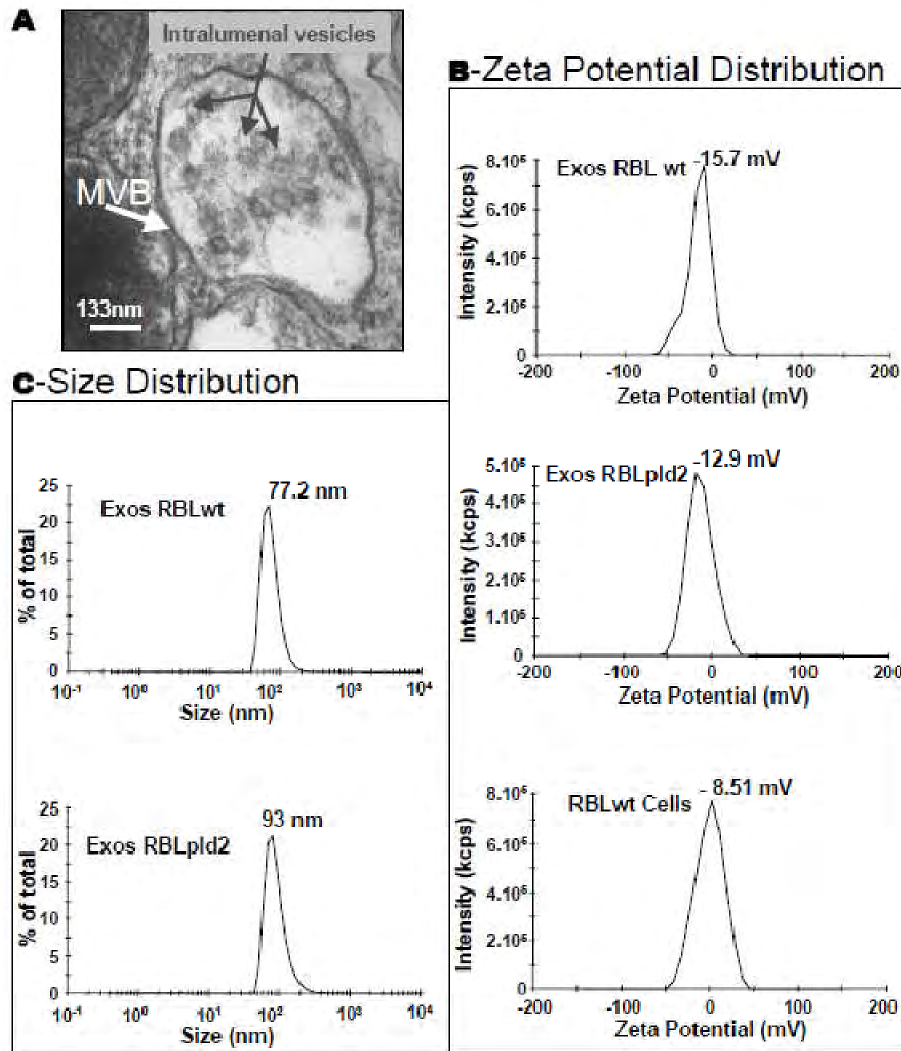


Supplementary Data 1 : Characterization of the stable RBL line overexpressing the human HA- PLD2

Upper left panel. HPLC profiles of phospholipase D activity in RBLwt and RBLpld2 cell lines. Cell lysates were incubated for 2 hours with the fluorescent substrate. DG=diglycerides; Pet= phosphatidylethanol, monitoring PLD activity; PC= phosphatidylcholine (fluorescent Bodipy-labeled substrate). Arbitrary units= fluorescence values.

Lower left panel. Kinetics of degranulation from the two cell lines . ^{14}C-serotonin release from RBLwt (□) and RBLpld2 cells (○) activated by Fcε-RI cross-linking with DNP-HSA, compared to resting cells (■).

Right panels. **Confocal imaging of HA-hPLD2 with antibody directed against the HA tag** (a)**, compared to plasma membrane labeling with cholera toxin** (b)**. Merge between (a) and (b) fluorescence in** (c)**. Enlargement of selected cell** (d)**. Bar= 10µm**



Supplementary Data 2 : Characterization of exosomes by nanosizer equipment.

A. Electron microscopy of intralumenal vesicles inside the MultiVesicular Body (MVB) of a RBLwt cell (X40 000). Once released out of the cells, intralumenal vesicles are called "exosomes".

B. Electronegativity (Zeta potentials) of exosomes ("Exos") derived from RBLwt and from $RBLpld_2$ cells, compared to parent cells (lower right panel). Data are expressed as number of particles (kcps= kilo counts per second) versus their zeta potential expressed in mVolts. Values at the peak were automatically generated by the apparatus (Nano ZS 90, Malvern Instruments). Note that exosomes are about two times more negatively charged than the parent cells.

C. Size distribution of exosomes ("Exos"), derived from RBLwt and $RBLpld_2$ cells. Data represent the distribution (in percent of total) of the vesicle sizes (in nm). Values at the peak were automatically given by the apparatus (Nano ZS 90, Malvern Instruments).

Given that multivesicular bodies which concentrate cholesterol and Bis(monoacylglycero)phosphate (BMP), are depicted as potent budding site for infectious particles such as the Human Immunodefiency Virus (HIV) in reservoir cells like monocytes and macrophages, we assessed the role of cholesterol biosynthesis and cellular repartition on HIV virions production. We explored the involvement of PLA_2 enzymes by pharmacological approaches.

The results are detailed hereafter and will constitute a second article.

Article :

II. Cell to cell HIV infection in Human monocytes and macrophages is regulated by lipid homoeostasis of the endosome compartment.

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Key words: macrophages, HIV, MVB, LBPA, cholesterol, progesterone, cPLA2

Abstract : HIV spreading in reservoir cells was investigated by cocultures between human monocytic THP1 cells with ratios of infected over uninfected cells ranging from 1 over 1000 to 1 over 2 cells, leading to infection levels from 10^6 to 10^9 virions per million cells respectively.
Cellular lipid homeostasy was modified by altering cholesterol content and distribution and by inhibiting phospholipase A_2 activity. Also the cationic amphiphile U18666A, known to accumulate cholesterol into the MVB, had no effect on infection level. Instead the steroid progesterone decreased by one log the infection, with disappearance of peripheral cell virus as shown by electron microscopy.

The phospholipase A_2 inhibitor Methyl Arachidonyl Fluoro Phosphonate (MAFP) also decreased by 1 log the HIV infection in THP1 cells and triggered the disappearance of peripheral cell virus. Repetitive treatment with MAFP displayed higher efficiency and appeared related to the alteration of the endosome compartment. Identical results were obtained on human monocyte-derived macrophages. Therefore targeting MAFP-sensitive PLA_2s appeared as a strategy to alter HIV reservoir content.

A. Introduction

The persistence of an HIV reservoir accounts for the failure of its eradication despite potent antiretroviral therapy. Whereas long life-span quiescent T CD4+ memory cells containing the latent provirus are the major part of this reservoir (Chomont *et al.*, 2009), a residual viral population in peripheral blood cells is also evidenced. We showed that this one is compartmented, arguing for a persistent low-level replication (Delobel *et al.*, 2005). Monocyte-macrophages are an important viral compartment, as they are able to stock infectious viral particles during a long time (Sharova *et al.*, 2005). The mechanisms governing the biogenesis of these intracellular stores in monocytes-macrophages still need clarification concerning its subcellular localisation, as conflicting results argue for HIV assembly either at the plasma membrane (Jouvenet *et al.*, 2006; Deneka *et al.*, 2007; Welsch *et al.*, 2007; Gousset *et al.*, 2008), or in the endosomal compartment (Raposo *et al.*, 2002; Nguyen *et al.*, 2003; Pelchen-Matthews *et al.*, 2003; Chertova *et al.*, 2006; Joshi *et al.*, 2009). These intracellular stores can efficiently infect new cells by cell-to-cell contact (Gousset *et al.*, 2008; Groot *et al.*, 2008), a process recently shown to implicate the endocytosis of infecting viral particles by the target cell (Ruggiero *et al.*, 2008; Hubner *et al.*, 2009; Miyauchi *et al.*, 2009).

Whatever the subcellular localisation of HIV assembly is, its budding involves membrane curvature out of the cytoplasm (either within the endosomal lumen or the extracellular medium). This process is physiologically observed in the cell at a step where multivesicular bodies (MVB) are formed by intraluminal vesicle budding, a process allowing either internalisation and degradation of membrane proteins, or back-fusion and cytoplasmic delivery of the intraluminal vesicle content, or exosome delivery in the extracellular medium. Lipid sorting is a key step in this intracellular vesicle trafficking (for review,(van der Goot & Gruenberg, 2006)). The lipid lysobisphosphatidic acid (LBPA, also named BMP for bis(monoacylglycero)phosphate) is specifically sorted into the MVB (Kobayashi *et al.*, 1999) and is necessary for endosome internal budding (Matsuo *et al.*, 2004). Moreover, LBPA interacts with Alix, an ESCRT-associated protein that also binds HIV (Strack *et al.*, 2003; Fisher *et al.*, 2007). LBPA precursor biosynthesis involves a methylarachidonoyl fluorophosphonate (MAFP)-sensitive phospholipase A_2 (PLA_2) activity (Shinozaki & Waite, 1999). MAFP also inhibits the cytosolic calcium-dependent phospholipase A_2 ($cPLA_2$) that

plays a major role in signal transduction and is involved in vesicle trafficking between membranes. In macrophages, this enzyme translocates to membranes of vesicles involved in the endocytosis pathway (for review, (Brown *et al.*, 2003)). This enzyme is also involved in cholesterol sorting between intracellular compartments (Grimmer *et al.*, 2005) and we previously showed that it is necessary for the optimal infectivity of another enveloped virus, the human cytomegalovirus (hCMV) (Allal *et al.*, 2004).

Interestingly, LBPA is associated to cholesterol and influences cellular cholesterol distribution (Delton-Vandenbroucke *et al.*, 2007; Chevallier *et al.*, 2008). Cholesterol is essential to HIV biogenesis and infectivity (Raulin, 2002). The viral envelope shares similar lipid content with lipid rafts (Brugger *et al.*, 2006) and cholesterol depletion with methyl-β-cyclodextrine triggers a loss of HIV infectiosity (Campbell *et al.*, 2002). HIV itself, by its viral accessory protein Nef, increases the cholesterol content of the infected cell (van 't Wout *et al.*, 2005; Mujawar *et al.*, 2006) and the ratio of sphingomyelin to phosphatidylcholine of the budding viral particles (Brugger *et al.*, 2007). Cholesterol in the cell has to be rapidly provided when necessary and this is achieved either by LDL internalization or de novo synthesis. Besides, compounds may perturb the intracellular cholesterol distribution, particularly the hormone progesterone that, at high concentrations, retains cholesterol to the plasma membrane and inhibits distribution to intracellular compartments (Lange, 1994). Also, U18666A, a sterol analog, is widely used to modify both cholesterol synthesis and distribution leading to its accumulation in the MVB compartment (Delton-Vandenbroucke *et al.*, 2007).

Here, we used a pharmacological approach to investigate the role of these lipids in HIV biogenesis. We screened drugs targeting key steps of cholesterol synthesis and modifying its intracellular distribution whose mechanism of action were previously characterized in the laboratory (Kedjouar *et al.*, 2004). Because of previous observations demonstrating the key role of a PLA_2 in the infectivity of the hCMV, an inhibitor of this enzyme activity was checked. HIV production was investigated either in the human promonocytic cell line THP1 or in human monocyte derived macrophages (MDM), and the amount of virus was quantified by RT-PCR, as performed in clinical monitoring of the pathology. We showed that progesterone and the PLA_2 activity controlled HIV egress both involving the MVB compartment.

B. Material and Methods

1. Materials

Rhodamine–PE [(1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)] were purchased at Avanti Polar Lipids, Birmingham, Alabama, USA.

Progesterone was purchased at Steraloids. Filipine was supplied by Sigma. MAFP was obtained from Biomol International. QIAamp Viral mini kits were purchased at Qiagen. RT-PCR kits were bought at Roche Diagnostics. Culture media were supplied by Invitrogen.

2. Methods

Cell culture

The promonocytic THP-1 cell line was purchased from ATCC (ATCC number TIB-202™) and maintained at a density of 5.10^5 cells/ml in RPMI 1640 supplemented with 10 % of decomplemented FCS.

Peripheral blood mononuclear cells were prepared from buffy-coats from healthy donors (Etablissement français du sang, Toulouse), plated in 6-well plates (10^7 cells/well) and incubated 2 hours at 37°C. Adherent monocytes were washed three times with PBS and cultured during 7 days in RPMI 1640 FCS10% containing 25 ng/ml M-CSF for differentiation into macrophages (MDM, monocyte-derived macrophages). Monocyte to macrophage differentiation was checked by FACS quantification of CD206 as previously described (Clavel *et al.*, 2008).

The viability of THP-1 cells was analysed by counting after Trypan Blue staining and that of MDM using the luminescent assay Cell Titer Glo kit® (Promega).

Infection

Infection from viral suspensions. HIV_{LAI} and $HIV_{Ba\text{-}L}$ strains were used to infect THP-1 cells and MDM, respectively, as follows: a pretitrated viral suspension was incubated with the cells during 3 hours at 37°C, in a ratio of 10 and 100 copies of viral RNA per cell, for THP-1 cells and MDM, respectively, and under gentle shaking for THP-1; then, the cells were washed 5 times successively in RPMI and maintained in RPMI FCS10%. In order to maintain the infected THP-1 cell line over time, 3 ml of uninfected THP-1 cells were added to 20 ml of the infected cell suspension once weekly.

Infection by coculture of infected and uninfected cells. At day 0, infected THP-1 cells were pelleted and added to uninfected THP-1 in variable ratios of infected versus uninfected cells. Similarly for MDM, infected MDM were scraped, pelleted and added to uninfected MDM in a ratio of 1:100.

Cell treatments

All the molecules used were supplied in ethanol as vehicle (0.1% final) .*THP1-cell treatments at day 0*. Molecules were added at day 0 after mixing infected and uninfected cells; they were added at the indicated final concentration in the culture medium containing FCS 1%.

Repetitive treatments of THP1 cells. Molecules were added at day 0 at the indicated final concentration in the culture medium containing FCS 1%. Daily, a small fraction of the culture medium was removed and replaced by fresh medium containing the same amount of

molecules as in day 0. In such treatments, assuming that molecules were not completely degraded, their concentrations gradually increased.

HIV quantification

RNA was extracted from 140 µl of culture supernatants using Qiamp viral RNA mini kit (Qiagen). Real time RT-PCR targeting the LTR gene was used as described by Mavigner et al (Mavigner *et al.*, 2009), the run being programmed for 45 cycles and the standard curve established from an infected PBMC culture supernatant previously quantified by a routine automated method (Roche Diagnostics).

Immunofluorescence :

Filipin labeling: 10^6 cells were pelleted by centrifugation 1200 rpm 5 min, washed in PBS and were then fixed with PFA 3.7¨% 20mn at room temperature. After rinsing with PBS, they were labelled with filipin at 50µg/ml in PBS 1h at room temperature. After washing, 3.10^5 cells were forced to adhere onto L-polylysine -coated microscope slides using a cytospin at 6000 rpm 10 min and finally mounted between sealed glass coverslips and microscope slides with Fluorsave®.

RhodaminePE labeling: Appropiate amounts of *RhodaminePE* stored in a in chloroform:methanol 2:1 were brought to dryness and resuspended in absolute ethanol. This ethanolic solution was diluted in PBS at the final concentration of 3µM and vortexed vigorously. 1ml of this dilution was then added to 2.10^6 cells per conditions, previously pelleted, at day 4 of treatments, and incubated during 60min at 4°C. The medium was removed, cells were extensively washed with PBS and re-ensemenced in RPMI FCS 1% during 3 hours at 37°C. At the end of the labeling, 3.10^5 cells were washed in PBS, coated onto L-polylysine -coated microscope slides by mean of centrifugation by cytospin at 6000 rpm 10 min, fixed with PFA 3.7% 20 min at room temperature and mounted and sealed with glass coverslips using Fluorsave® as mounting medium.

Wield field microscopy: labeled cells were observed on a Zeiss wield field microscope on the RIO cellular platform of IFR 150, Toulouse. For filipin labeling, deconvolution was applied using the Metamorph® software (Molecular Devices).

Transmission electron microscopy (TEM).

As previously reported (Payre *et al.*, 2008), cells were fixed with 2% glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH = 7.4) for 1 hours, washed with the Sorensen phosphate buffer (0.1 M) for 12 hours. Then were postfixed with 1% OsO_4 in Sorensen phosphate buffer (Sorensen phosphate 0.05 M, glucose 0.25 M, OsO_4 1%) for 1 hour, washed twice with distilled water and prestained with 2% uranyl acetate aqueous solution for 12 hours. Samples were dehydrated in an ascending ethanol series and embedded in epoxy resin (Epon 812, Electron Microscopy Sciences). After 24 hours of polymerisation at 60°C, ultrathin sections (70nm thick) were mounted on 150 mesh collodion-coated copper grids and

poststained with 3% uranyl acetate in 50% ethanol and with 8.5% lead citrate before being examined on a HU12A Hitachi electron microscope at an accelerating voltage 75 KV.

C. Results

1. HIV infection between monocytic THP1 cells

Given the ability of HIV to spread between cells by direct cell-to-cell transmission (Sattentau, 2008), we chose a model of coculture of infected and non infected cells. Infected monocytic THP1 cells were mixed with uninfected ones in various ratios, and the amount of virus released out of the cells was quantified as a function of time. Ratios between cells varied from 1 infected cell for 2 uninfected ones ("high infection ratio"), to 1 infected cell for 1000 uninfected ones ("low infection ratio") (Figure 41A,B), in conditions where cell proliferation was at a steady-state (Figure 41-C). At a ratio of 1:2, a very high titer of 10^9 viral particles / ml of cell culture supernatant was obtained (Figure 41A) and it was necessary to dilute infected cells by a thousand times to obtain a viral titer of 10^6 virus / ml (Figure 41A). At day 4 the plateau of viral replication was reached (Figure 41A) and a correlation was observed between the viral titer and the ratio of infected versus uninfected cells (Figure 41D). Even at high infection ratio (1:2), AZT inhibited the infection by 1 log (Figure 41D), which is the efficiency reported in *in vitro* experiments (Aquaro *et al.*, 2002).

Infection was visualized by electron microscopy after 4 days of co-culture i.e when infection reached a plateau (see Figure 41A). As compared to control uninfected cells (Figure 41F), infected ones showed accumulation of high amounts of virus which appeared agglutinated around the cells (Figure 41G). Budding of the virus from the plasma membrane was evidenced in Figure 41H. The virus was also present inside intracellular vesicles (Figure 41I-K), from where it could be released by exocytosis (Figure 41K).

2. Modification of cholesterol distribution and viral infection

We next investigated the incidence of the cellular distribution of cholesterol on HIV production in experimental conditions reported above in Figure 41. The U18666A, a cationic amphiphilic compound reported to trigger cholesterol accumulation into intracellular vesicles (Delton-Vandenbroucke *et al.*, 2007), exhibited no effect on the amount of virus produced (Figure 42A) even at low ratio of infected /uninfected cells, and whether a single amount or cumulative amounts of U18666A were added. We next analyzed the effect of

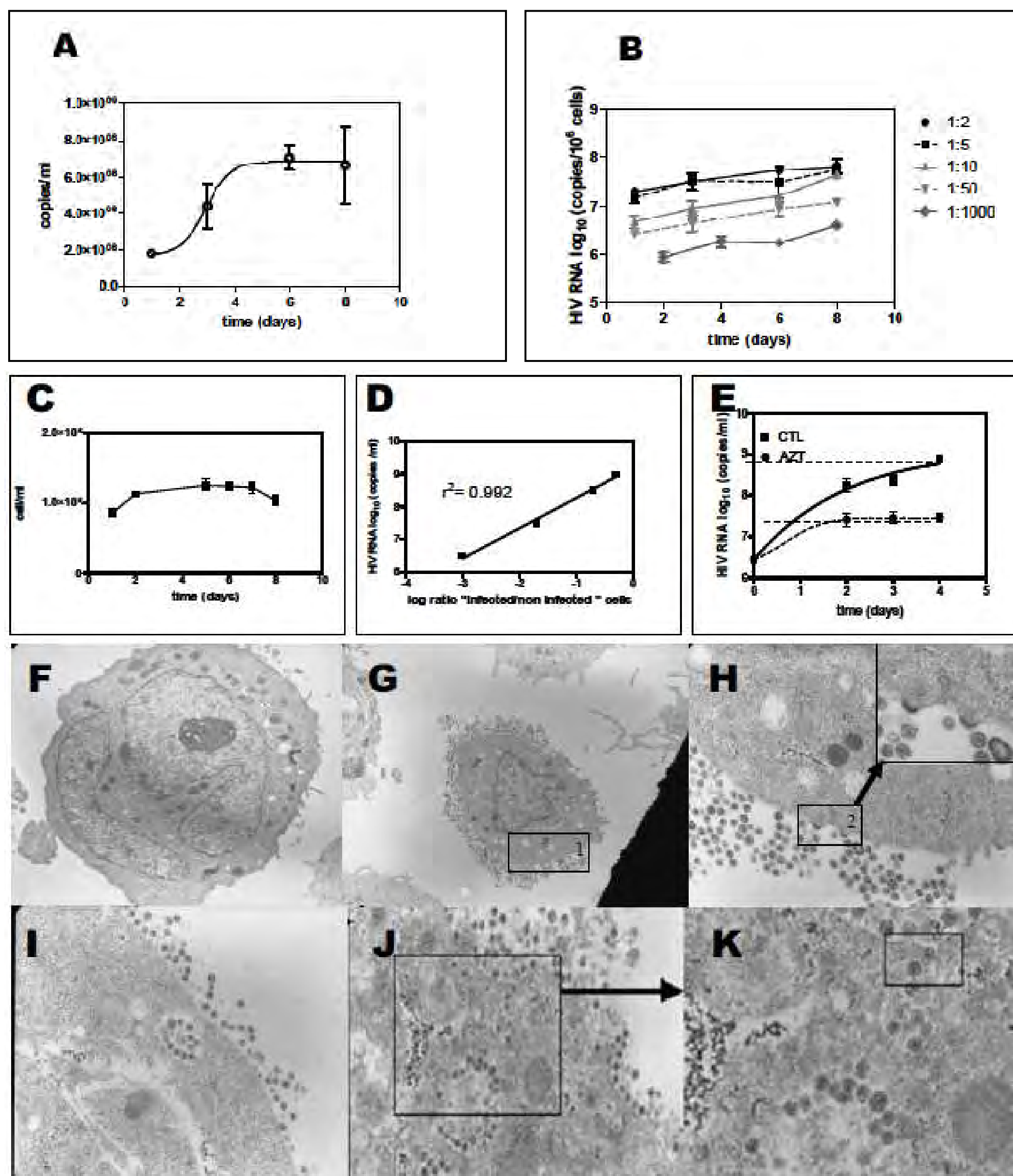

Figure 41 : HIV production according to ratios of infected versus uninfected cells
Infected THP1 cells were mixed with uninfected ones and the amount of virus released out of the cells was quantified by RT-PCR for the next 8 days.
(A) Typical evolution of viral titer as a function of time. The ratio of infected versus uninfected cells of 1:2 is shown, but all the other ratio checked gave a similar sigmoïdal curve with a plateau at day 4.
(B). Evolution of the viral titer as a function of the ratio between infected versus uninfected cells .
(C) Cell viability across infection period
(D) Correlation between viral titer at day 4 and the ratio of infected versus uninfected cells.
(E) Effect of AZT on the infection between THP1 cells.

Electron microscopy :
Cells infected at high ratio (1:2) were visualized at day 4 of coculture, i.e when infection reached a plateau.
(F) Control uninfected cells [X 6000]. **(G)** Typical infected cell with peripheral virus accumulation [X 5000]. **(H)** High magnification [X20000] of area 1 showing plasma membrane budding of viral particles and electronic enlargement of area in square. **(I,J)** Infected cell [X 20000] with intracellular vesicle containing viral particles. **(K)** Computer-imaging enlargement of the area in I (square) showing viral particles channeled out of the cell.

progesterone which has been reported to trigger cholesterol accumulation at the cell periphery (Lange, 1994). At low infection ratio of THP1 cells (1:1000), 10 µM progesterone added at day 0 inhibited by one log the level of HIV production (Figure 42B1), and by about 0.5 log at high ratio (1:2) of infected/uninfected cells (Figure 42B2). Daily addition of progesterone on cells infected at high ratio (1:2) was no more efficient (Figure 42B3) than a single addition at day 0.

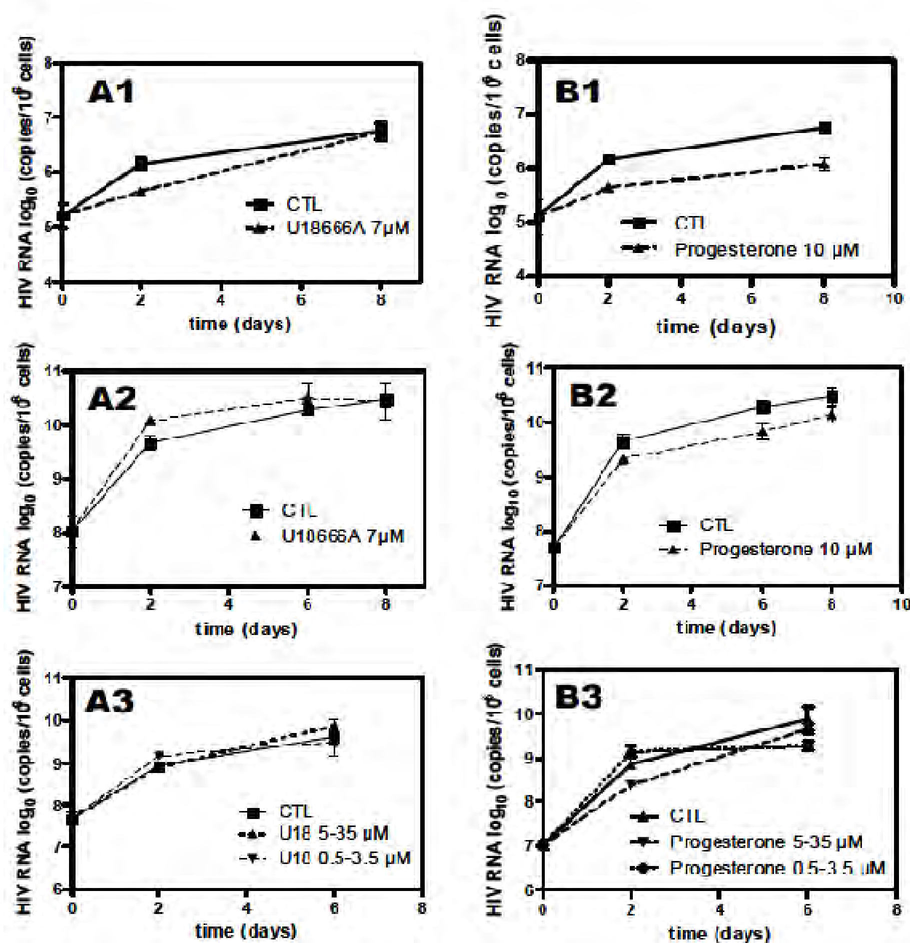

Figure 42 : **Effect of U18666A and progesterone on cell-to-cell HIV infection of THP1 cells.**
Data are expressed as log HIV-RNA copies / million of viable cells.
(A) U18666A (7 µM) was added at the day 0 on cells with low (1:1000, A1) or high (1:2, A2) infection ratios. Also repetitive treatment by daily additions of 0.5 µM or 5 µM of the compound in high infection ratio (1:2) conditions was checked (A3).
(B) Identical experimental approach with progesterone added at 10 µM final at day 0, on infected cells (1:2 and 1:1000 infection ratios in B1 and B2 respectively) or added daily at 0.5 µM or 5 µM (Fig B3) on cells at high infection ratio (1:2).

We checked by filipin staining the cholesterol distribution in infected cells under various treatments. As compared with ethanol as a vehicle of the molecules (Figure 43A1A2), showing a diffuse cholesterol distribution in the cytosol (Figure 43 A3-A4)), the U18666A increased cholesterol accumulation into intracellular vesicles (Figure 43C 3-4). Instead progesterone treatment triggered localization of cholesterol into punctus structures at the cell periphery (Figure 43A5-A6).

Electron microscopy performed at day four, i.e at the plateau phase of infection, showed in cells treated with U18666A, the presence of virus mainly around the cell (Figure 43B1-B3). Instead, under progesterone treatment, external accumulation of virus around the cell disappeared whereas HIV was present inside intracellular compartments (Figure 43 C1-C3). Therefore peripheral cholesterol accumulation triggered by progesterone could prevent virus budding at the plasma membrane level.

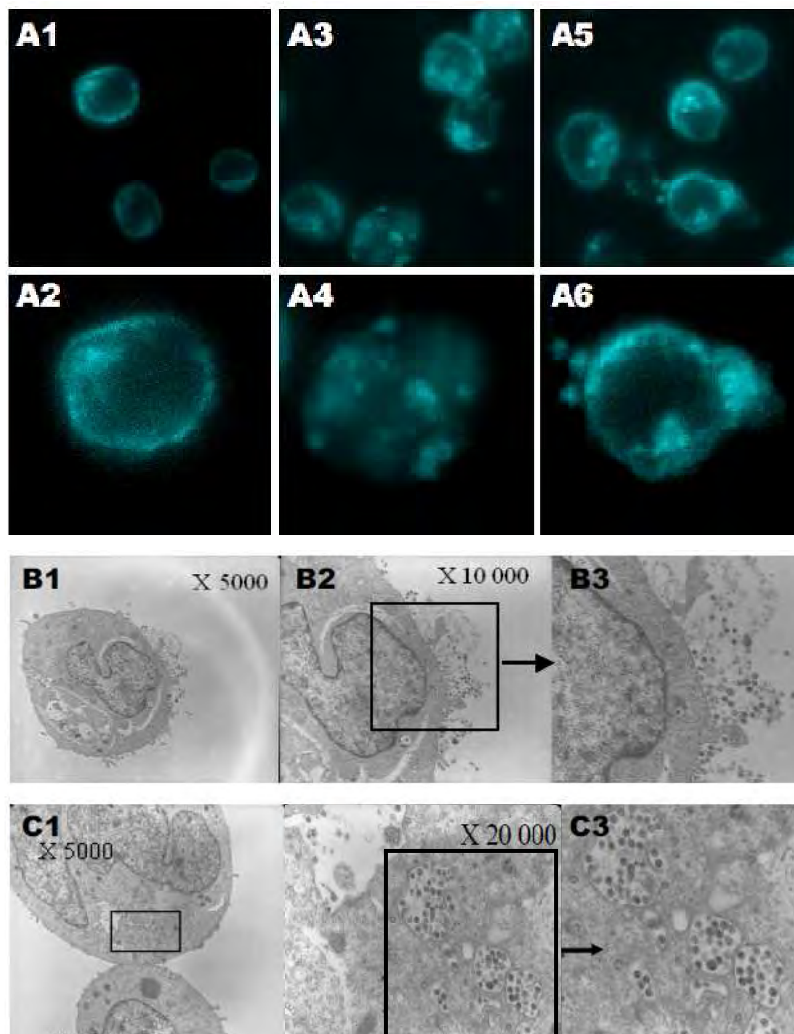

Figure 43 : (A) Filipin staining of cholesterol cellular distribution following cell treatment with ethanol (vehicle, A1-2), U18666A (7µM, A3-4), progesterone (10µM, A5-6). Immunofluorescence was performed with a Zeiss wield-field microscope (63X ocular) and pictures were processed for deconvolution.
(B) Electron microscopy at day 4 of cells at high infection ratio (1:2) and treated with 7 µM of U18666A added at day 0. The right panel is a computer enlargement of the central one.
(C) Electron microscopy at day 4 of infected cells (1:2 ratio) treated with 10 µM progesterone added at day 0. The right panel is a computer enlargement of the central one.

3. Effect of a cPLA2 inhibitor on HIV infection in THP1 cells

Cholesterol also regulates the $cPLA_2$ activity (Grimmer *et al.*, 2005). This prompted us to consider the role of the PLA_2 inhibition on HIV production. Therefore we investigated the effect of a PLA_2 inhibitor, the Methyl Arachidonoyl Fluoro Phosphonate (MAFP) on cell-to-cell HIV infection. This compound inhibited by 0.5 log the HIV production at low ratio of infected cells (1:1000, Figure 44A) and to a lesser extend at high infection ratio (Figure 44B) when added at day 0. Electron microscopy performed at day 4 of infection showed the almost total disappearance of peripheral viral aggregates (Figure 44C-43E) whereas viruses were present into large intracellular compartments, which appeared to contain more viral particles as compared to untreated control infected cells (Figure 41F). These observations indicated a blockade of virus inside intracellular vesicles by the PLA2 inhibitor.

Interestingly, repetitive additions of MAFP efficiently inhibited by 1 log the HIV production at high ratio of infected cells (Figure 44F), the most efficient concentrations being in the range of 5-35 µM, assuming MAFP was not degraded in the meantime.

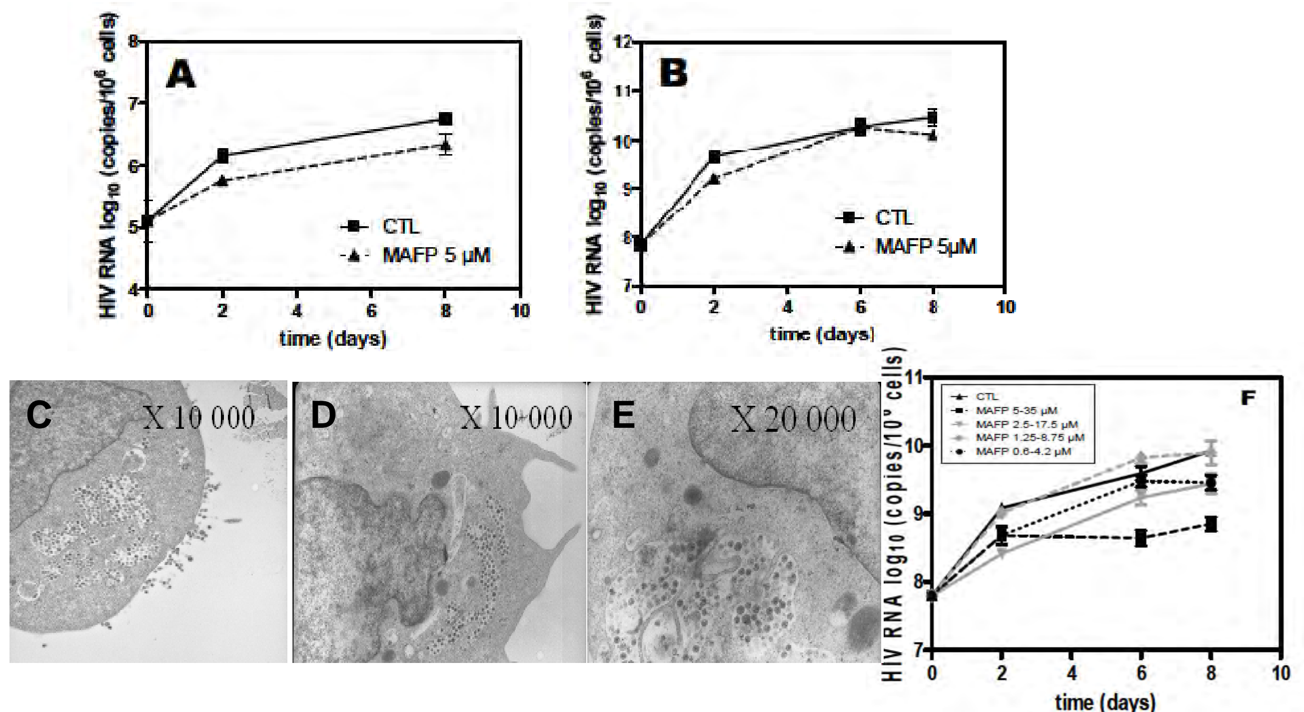

Figure 44 : Effect of Methyl Arachidonyl Fluorophosphonate (MAFP), a cytosolic phospholipase A2 inhibitor, on HIV production by THP1 cells.

Data are expressed as log HIV-RNA copies / million of viable cells.

Cells infected at low **(A)** or high **(B)** ratio of infection (1:1000 and 1:2 respectively) were treated by 5 µM MAFP added at day 0, Electron microscopy at day 4 of cells infected at 1:2 ratio and treated by 5 µM MAFP at day 0 showed accumulation of the virus into larged intracellular vesicles (**C-E)**, assumed to be large MVBs as shown by Rh-PE labeling (see Fig 44 E,), whereas plasma membrane was cleaned of most peripheral virus. Pictures are different magnifications of a typical cell. For control uninfected and infected cells refer to Fig 41F an 41G respectively.

(F). Effect of repeated daily addition of the same amount of MAFP (0.6 µM, 1.25 µM, 2.5 µM, 5 µM respectively) allowing to cover concentration ranges from 0.6 µM to 35 µM.

4. Effect of the lipidic molecules on the MVB compartment of THP1 cells.

HIV budding into the Multivesicular Bodies (MVB) is part of the morphogenesis process (Joshi *et al.*, 2009). We have therefore evaluated the impact of the various lipidic molecules we used in this work on the morphology of the MVB compartment, by means of Rhodamine-PE (Rh-PE) labelling. Rh-PE is a fluorescent probe which accumulates specifically into the MVB compartment when added externally to cells.

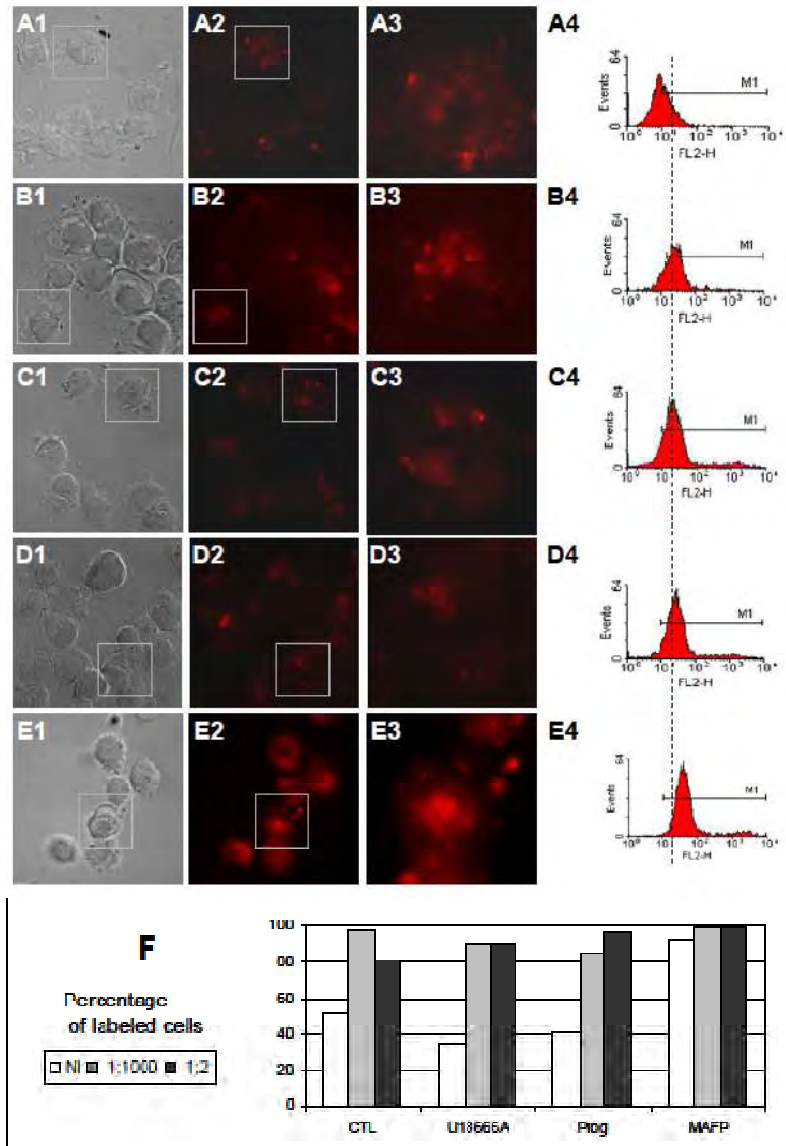

Figure 45 : Effect of HIV infection, U18666A, progesterone and the PLA2 inhibitor on the MVB compartment of THP1 cells

The integrity of the overall MVB compartment was evaluated either qualitatively by immunofluorescence (A1-E3) or quantitatively by FACS measurement (A4-E4 and F,G) by incubating cells with Rh-PE, a fluorescent lipid specifically targeted to the MVBs. Immunofluorescence was performed with a Zeiss microscope (63X ocular). Cells were examined at day 4 of the respective treatments

(A) non infected THP-1 cells; (B-E) infected (1:2 ratio) THP-1 cells treated with vehicle (B) or repetitive addition of U18666A 7 µM (C), progesterone 10 µM (D) or MAFP 5 µM (E).

(F) quantitative analysis of a representative experiment

Rh-PE in MVBs can be quantified by FACS, allowing to estimate the total volume of the set of MVBs present in cells. In comparison, immunofluorescence microscopy of Rh-PE labeled cells will monitor the morphology of MVBs. Both quantification and morphology were reported in Figure 45.
HIV infection itself increased the number of MVB per cell (Figure 45B vs 44A) as quantified by the increase of the mean fluorescence intensity (MFI) of infected versus uninfected cells (Figure 45G). Furthermore, the percentage of labeled cells doubled when cells were infected (Figure 45F). As compared to untreated infected cells (Figure 45B), neither U18666A nor progesterone modified the total volume of the MVB compartment as monitored by the RhPE labeling (Figure 45C-D). Treatment with MAFP triggered the formation of large MVBs (Figure 45E) and led to a strong increase in the mean fluorescence intensity (MFI from 28 to 52 at ratio 1:2 and to 128 at ratio 1:1000) (Figure 45G). Interestingly, MAFP highly increased the percentage of labeled cells independently of infection (Figure 45F).

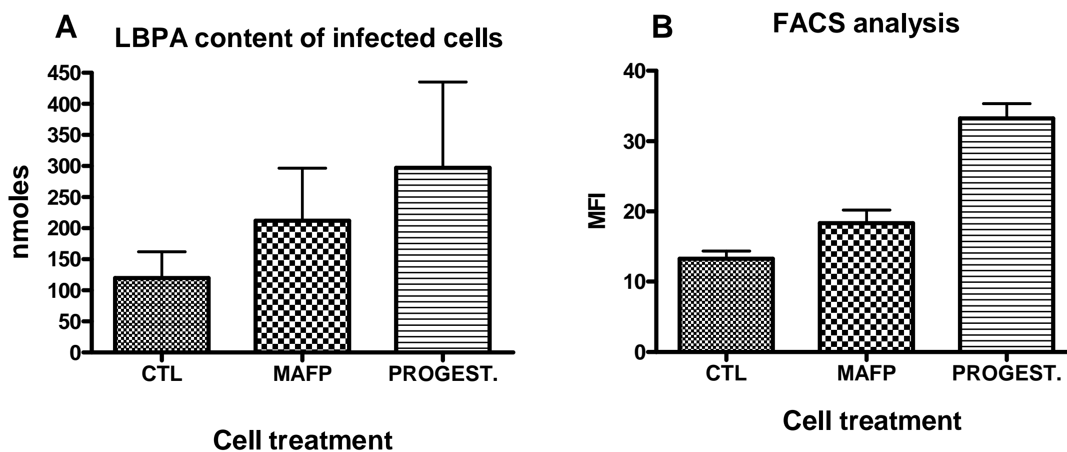

Figure 46 : Effect of MAFP and progesterone on LBPA content. (A) The cells content of LBPA was quantified by mass spectrometry in cocultures of HIV infected THP-1 cells at the ratio 1:2 either in cells treated with the vehicle(CTL) or at J4 of repetitive additions of MAFP 5µM or progesterone 10µM. (B) LBPA quantification by FACS analyses using the 6C4 antibody in the same conditions than in (A)

Comparison of FACS profiles (Figure 45 panels A4 to E4) further assessed that infection increased the total MVB volume (panel B4 compared to A4) and that MAFP treatment, by triggering formation of large MVB (Figure 44E3) increased the total MVB volume in cells (panel E4 compared to B4). MAFP thus provided large storage compartments (see Figure 44C-E) allowing to block the virus inside the cells and avoiding its release out of the cells. Progesterone appeared to act distinctly since no modification of the MVB compartment was detected on the basis of RhPE labeling (Figure 45, panel D4 compared to B4), whereas morphological modifications of the MVB compartment similar to those triggered by MAFP were observed by electron microscopy (Figure 43C1-C3). In any cases, MAFP and progesterone trapped the virus into large intracellular compartments which impaired its release out of the cells, and thus appear as new tools to control HIV spreading.
LBPA content was assessed in these conditions of culture either by mass spectrometry than by FACS analyses using a specific antibody (Figure 46). MAFP and progesterone treatments

led to an increase of LBPA content, consistent to morphologic modifications observed by electron microscopy.

5. Effect of cholesterol distribution, progesterone and PLA2 inhibition on MDM cell infection

The previous observations related to U18666A, progesterone and MAFP on THP1 cells were further checked on human monocyte-derived macrophages, comparatively to AZT (Figure 47). Compounds were added every day (repetitive treatment). Similarly to THP1, progesterone at high concentrations and MAFP decreased HIV proliferation in MDM (Figure 47)Therefore both molecules were efficient to decrease HIV production both in monocytes and macrophages.

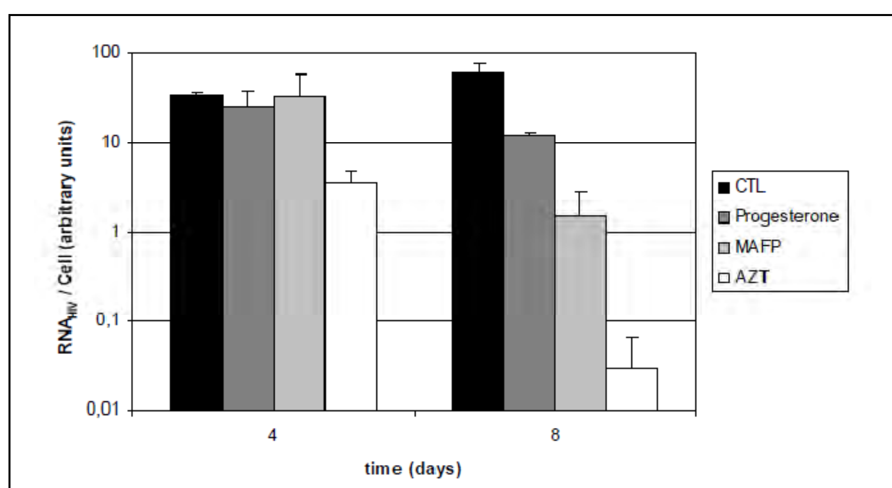

Figure 47 : Effect of U18666A, progesterone and the PLA2 inhibitor MAFP on HIV production by cell-to-cell infection of human MDM.
Data are expressed as log HIV-RNA copies / million of viable cells.
MDM were prepared as described in Material and Methods section, infected by coculture in a 1:100 ratio and treated by repetitive addition of U18666A 7 µM, progesterone 10 µM, or MAFP 5 µM. HiV RNA was normalized to the signal obtained from viable cells with the Cell Titer Glo ® Promega kit. Results at 4 days and 8 days infection times are reported.

D. Discussion

Our study shows the inhibition of HIV production by progesterone and MAFP in cocultures of infected and non infected cells of the monocyte-macrophage lineage. Infection by cell-to-cell contact through virological synapses is known to be highly efficient, either from MDM or DC to T-cell, T-cell to T-cell (McDonald *et al.*, 2003; Jolly & Sattentau, 2004) or from MDM to MDM (Gousset *et al.*, 2008; Groot *et al.*, 2008). Regarding monocytes, the efficiency of THP1 infection in coculture of infected and non infected cells was illustrated in Figure 41, since only one infected cell mixed with 1000 uninfected ones leads to a viral titre comparable to those observed in the circulation of non-treated patients (10^6 viral particles /ml).

Interestingly, in control cells, virus accumulated at the cell surface, reminiscent to the biofilm-like extracellular structure recently observed in HTLV-1 cell-to-cell transfer (Pais-Correia *et al.*).
Concerning the entry counterpart of HIV infection, HIV endocytosis via clathrin coated pits has recently been shown as a major mechanism (Ruggiero *et al.*, 2008; Hubner *et al.*, 2009; Miyauchi *et al.*, 2009). This endocytosis pathway culminates in the formation of MVB where incoming HIV could be stored. Indeed, in Nef transfected HeLa cells, Nef induces an intracellular accumulation of MVB (Stumptner-Cuvelette *et al.*, 2003). In MDM, this MVB accumulation is described as a consequence of a partial inhibition of autophagy by Nef (Kyei *et al.*, 2009). In this work, using the Rh-PE probe which accumulates into MVB, we confirm the MVB accumulation in infected cells. However, since the ability of the virus to spread from one cell to the other is not altered when Nef is deleted (Sharova *et al.*, 2005), other factors than Nef are implied in this MVB accumulation.
HIV storage in intracellular vesicles could also result from intracellular budding of HIV. Whereas the plasma membrane has been considered for long as the site of viral morphogenesis (Sattentau, 2008), evidence that productive HIV assembly can also occur in late endosomes in MDM is documented (Raposo *et al.*, 2002; Nguyen *et al.*, 2003; Pelchen-Matthews *et al.*, 2003; Joshi *et al.*, 2009). Whatever the subcellular localisation of HIV budding is, cholesterol is an essential lipid for HIV biogenesis. U18666A, a sterol derived cationic amphiphilic compound classically used to accumulate cholesterol in cells, has actually pleiotropic effects both on cholesterol distribution and synthesis (Kedjouar *et al.*, 2004). At the concentration commonly used (7µM), we verified the accumulation of cholesterol into MVB but we noticed no effect on HIV infection level (see Figure 43A). Previous observations indicated the absence of effect of U18666A treatment on viral particle production in MDM, indicating that cholesterol distribution to MVB is not sufficient to alter HIV biogenesis (Jouvenet *et al.*, 2006; Gousset *et al.*, 2008). Considering Gag distribution in U18666A-treated versus untreated cells, these authors concluded that HIV buds at the plasma membrane. However, in Niemann-Pick disease cells infected with HIV, the blockade of cholesterol redistribution from MVB induces a decrease of HIV production (Tang *et al.*, 2009). In these cells, it has been shown that cholesterol is associated to LBPA and this lipid can therefore be the limiting factor in cholesterol distribution (Chevallier *et al.*, 2008). Although cholesterol could regulate the $cPLA_2$ activity (Grimmer *et al.*, 2005), PLA_2 was a candidate in the control of HIV infection because we previously reported its key role in the human CMV infectiosity, another enveloped virus (Allal *et al.*, 2004). Effectively inhibiting this enzyme was highly efficient in THP1 cells, and daily addition of the compound allowed to inhibit by one log the titer obtained at high level of infection ratio (1:2). Moreover, the PLA_2 inhibitor MAFP decreased significantly the HIV production in primary human macrophages. This inhibition of virus production was associated to a marked increase in the MVB size, as shown by the Rh-PE labelling and LBPA content. Correspondingly, large intracellular vesicles containing numerous viral particles were observed by electron microscopy. Moreover, a higher percentage of these MAFP-treated cells contained MVB as compared to control. It has

been shown that inhibition of PLA_2 resulted in an inhibition of the multiple endocytic membrane trafficking pathway (Doody *et al.*, 2009). By blocking MVB fate, MAFP could retain HIV in large intracellular compartments and slow HIV egress. It has been suggested a role of a MAFP-sensitive PLA_2 in the degradation pathway of LBPA (Ito *et al.*, 2002; Hullin-Matsuda *et al.*, 2009a). Indeed, inhibiting the $cPLA_2$ activity could lead to a LBPA increase in the cell, consistent with our observations.
Moreover, PLA_2, which releases arachidonic acid from phospholipids, plays an important role in signal transduction. Arachidonic acid release is involved in the regulation of genes controlling cholesterol cell content and distribution (Grimmer *et al.*, 2005; Zhou *et al.*, 2009). Also, PLA_2 is activated downstrean of the sigma receptors (Starr & Werling, 1994). Interestingly, agonists of these receptors, including cocain, lead to an increase in HIV production (Roth *et al.*, 2005) whereas progesterone is an antagonist of these receptor (Maurice & Su, 2009). The role of these receptors could account for the effect of progesterone on HIV retention in cells reported in the present work, in addition to the effect of cholesterol redistribution to the cell periphery. Whether sigma receptors link the effect of progesterone and the $cPLA_2$ activity remains to be established. Noteworthy, $cPLA_2$ knock-out is not deleterious for mice survival (Chaminade *et al.*, 1999), and therefore $cPLA_2$ inhibitors might become new therapeutic agents distinct of the reverse transcriptase and protease inhibitors used so far in HAART treatments. Altogether, the present work has unravelled the $cPLA_2$ -type as a new therapeutic target to control HIV infection by cell-to-cell communication, a major pathway for HIV egress from reservoir cells.

Discussion et Perspectives

L'ensemble de ces travaux de doctorat s'inscrit dans une dynamique visant à développer de nouveaux axes de recherche dans la lutte contre l'infection par le virus de l'immunodéficience humaine (HIV).

L'analyse protéomique et lipidomique d'exosomes dérivés d'une lignée de cellules mastocytaires, les RBL- 2H3, ont permis d'étayer leur composition et la présence de phospholipases a été démontrée. Ces trois types de phospholipases que sont les PLA_2, PLC et PLD portées par les exosomes se sont révélées être fonctionnelles et activables par un analogue non hydrolysable du GTP.

Les phospholipases portées par les exosomes pourraient, en partie, rendre compte de la présence d'acides gras libres identifiés dans ces vésicules. En particulier, l'acide arachidonique, un médiateur lipidique important a été mis en évidence ainsi que certaines prostaglandines dérivées. La présence de prostaglandines pourrait donc contribuer au rôle des exosomes sur le système immunitaire et dans l'inflammation. De plus, la 15dPGJ2 est l'agoniste du récepteur nucléaire PPARγ. Cependant, le rôle de la 15dPGJ2 portée par les exosomes sur des cellules réceptrices reste à être confirmé. Les exosomes sont-ils capables de fournir une concentration suffisante de prostaglandines pour engendrer des effets intracellulaires ? Comme nous l'avons calculé, leur accumulation dans les endosomes permet d'obtenir des concentrations importantes de molécule actives. Il reste à démontrer leur efficacité.
Plusieurs travaux concernant l'effet de la15dPGJ2 sur le virus HIV ont été reportés. Par exemple, il a été montré que des agonistes de PPARγ inhibent la réplication virale sur des monocytes sanguins (Skolnik *et al.*, 2002) et que la 15dPGJ2 inhibe la transcription dépendante de la protéine transactivatrice Tat, et la réplication du virus HIV (Kalantari *et al.*, 2009). L'infectivité du virus est diminuée dans des cellules épithéliales traitées par la 15dPGJ2 qui inhibe la transcription du virus par un mécanisme lié à NFκB (Boisvert *et al.*, 2008). Des exosomes enrichis en 15dPGJ2 pourraient potentialiser ces effets et participer à diminuer la charge virale circulante.
Les exosomes de mastocytes que nous avons analysés comportent les cyclooxygénases 1 et 2 responsables de la génération de prostaglandines. Compte tenue d'une grande similarité entre les exosomes et les particules virales du rétrovirus HIV, il est envisageable que le HIV véhicule également des lipides bioactifs tels que les prostaglandines et la machinerie nécessaire à leur production. Par conséquent rechercher la présence de ce type de composé approfondirait nos connaissances sur ce virus et pourrait mener au développement de cibles potentielles

Le cholestérol est un autre lipide important, enrichi sur les particules virales et dont le taux au niveau de la cellule hôte est critique à la fois pour l'infection par le HIV mais aussi pour l'assemblage du virus. Dans un deuxième temps, nous avons donc évalué l'homéostasie lipidique sur la production de particules virales par des cellules dites 'réservoirs' (monocytes,

macrophages). Ces cellules renferment et sécrètent, sans lyse, des particules virales qui peuvent être stockées dans des compartiments intracellulaires. Nous avons pu montrer en modifiant la biosynthèse et/ou la distribution du cholestérol que, en dépit de l'absence d'effet du composé u18666A qui accumule le cholestérol au niveau des endosomes tardifs, un traitement des cellules par la progestérone permet une redistribution du cholestérol plus en périphérie cellulaire et diminue d'un log la production virale. Cet effet s'accompagne de la disparition de particules virales en périphérie de la cellule et semble modifier la morphologie des corps multivésiculaires qui accumulent le virus. Aux concentrations utilisées dans notre étude, la progestérone a une activité antagoniste des récepteurs intracellulaires appelés « récepteurs sigma » (Maurice *et al.*, 2001). Ces récepteurs jouent un rôle important dans la répartition cellulaire du cholestérol. Il existe par ailleurs un recouvrement entre certains ligands de ces récepteurs et le site AEBS (Anti-Estrogen-Binding Site) impliqué dans la biosynthèse du cholestérol (Kedjouar *et al.*, 2004). Le rôle des récepteurs sigma reste donc à démontrer dans l'effet de la progestérone. Dans cet effet, il serait intéressant de rechercher l'implication du site AEBS.

La présence d'une phospholipase A_2 (PLA_2) a été démontrée dans les exosomes de mastocytes. Basé sur leur similarité avec le HIV, il serait intéressant de rechercher la présence de PLA_2 dans les particules virales. Précédemment, il avait été démontré que l'activité de la PLA_2 cytosolique ($cPLA_2$) dans les cellules était nécessaire à l'infectivité et l'infectiosité d'un autre virus enveloppé qu'est le cytomégalovirus. Alors qu'un lien a été établi entre le polymorphisme du gène codant pour les PLA_2s chez des patients infectés par le HIV et la progression du syndrome de l'immunodéficience acquise (SIDA) résultant de l'infection par le HIV (Limou *et al.*, 2008), une revue récente met en avant,in vitro, le rôle de PLA_2s de venins de serpent ou d'insecte contre le virus (Meenakshisundaram *et al.*, 2009). Il a également été démontré qu'une PLA_2s sécrétoire pouvait inhiber la réplication du virus in vitro (Kim *et al.*, 2007). Au vue de ces données, il apparait nécessaire d'approfondir le rôle des PLA_2s dans l'infection par le HIV. Nos premiers résultats ont souligné l'importance d'une PLA_2 cytosoliques ($cPLA_2$) et/ou d'une PLA_2 indépendante du calcium ($iPLA_2$) dans la diminution de la production virale. En parallèle, nous avons observé l'apparition de larges compartiments intracellulaires, type Multivésicular Bodies (MVBs) ou corps multivésiculaires retenant le virus.

Ces observations s'accompagnent d'une augmentation d'un lysophospholipide particulier présent dans endosomes tardifs : l'acide lysobisphosphatidique ou Bis(monoacylglycero)Phosphate. Bien que le métabolisme de ce lipide soit encore mal connu, il a été proposé que des phospholipases de types A1 ou A2 soient impliquées tant dans sa biosynthèse que dans sa dégradation. A la vue de nos résultats, il semblerait que l'inhibiteur de PLA_2 utilisé (MAFP) inhibe plutôt une étape de dégradation, conduisant à l'accumulation de BMP dans les endosomes tardifs. Une PLA2 sensible au MAFP dans la voie

de dégradation du LBPA a d'ailleurs été suggérée, bien que l'association de cette enzyme avec une lipase ne soit pas exclue dans ces travaux (Ito *et al.*, 2002). Fonctionnellement lié au cholestérol, le BMP (LBPA) apparait essentiel dans le bourgeonnement de vésicules intraluminales (Matsuo *et al.*, 2004) et probablement dans le bourgeonnement du virus HIV. Par conséquent, cibler des phospholipases A_2 impliquées dans la constitution lipidique des endosomes pourrait être une stratégie visant à diminuer le réservoir de HIV. Egalement, une activité lipase présente dans des granules de sécrétions pourrait être impliquée dans la dégradation du BMP (LBPA) et son implication sur la sécrétion du HIV pourrait être envisagée.

Ainsi ce travail de doctorat souligne un rôle clé de médiateurs lipidiques dans la production du HIV et cibler les enzymes impliquées dans la biosynthèse de ces médiateurs apparaît constituer une stratégie thérapeutique pouvant donner une alternative aux thérapies actuellement utilisées.

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HIV et Exosomes : Rôle de l'homéostasie lipidique du compartiment endosomal dans le maintien des réservoirs du HIV.

Les exosomes sont des vésicules de 50-90 nm, générées dans le compartiment endosomal tardif, ou corps multivésiculaires, et sécrétés dans le milieu extracellulaire par les cellules vivantes. Les exosomes sont impliqués dans la communication intercellulaire, transportant à la fois des lipides bioactifs, des protéines, et des acides ribonucleiques (mRNAs, microRNAs) pouvant moduler la réponse de la cellule receveuse. Nous avons montré que les exosomes dérivés de RBL-2H3 contiennent l'ensemble des différentes phospholipases : une PLC, une PLD et des membres des trois classes de PLA_2 ($cPLA_2$, $iPLA_2$, $sPLA_2$) ainsi que tous les membres de la famille des Ras GTPase. Nous avons pu observer une augmentation de l'activité PLD_2 et PLA_2 en présence de GTP. Des analyses lipidomiques des exosomes ont révélé la présence d'acides gras libres tels que l'acide arachidonique et plusieurs prostaglandines. L'accumulation de ces vésicules dans le compartiment endosomal de cellules cibles a été observée.

L'implication du compartiment endosomal dans le stockage et la dissémination du HIV, dans les cellules réservoirs comme les monocytes/macrophages, pourrait rendre compte de l'impossibilité d'éradiquer le virus, même sous thérapie antirétrovirale efficace. Les corps multivésiculaires pourraient être un site d'assemblage du HIV permettant au virus de détourner, pour sa sortie, la voie préexistante des exosomes. Ce compartiment concentre à la fois le cholestérol et l'acide lysobisphosphatidique (LBPA), dont le métabolisme fait intervenir une $cPLA_2$ sensible au MAFP. Dans un second temps, nous avons évalué l'importance de l'homéostasie lipidique sur la production du HIV i) en modifiant la teneur et la distribution du cholestérol intracellulaire, ii) en inhibant l'activité PLA_2. Ces analyses ont été réalisées sur une coculture de cellules monocytaires humains (THP-1) infectées par le HIV et non infectées, et également sur des cocultures de macrophages dérivés de monocytes primaires humains infectés et non infectés. La progestérone, hormone stéroïde, et le MAFP, inhibiteur de PLA_2, ont permis d'inhiber la production de HIV dans nos conditions de culture et nous avons également pu observer l'absence de virions en périphérie de la cellule. Le LBPA pourrait être un élément commun à l'effet de la progestérone et du MAFP. Cibler les PLA_2s sensibles au MAFP représente donc une stratégie visant à altérer les réservoirs de HIV, et pourrait mener au développement de nouvelles molécules pour l'éradication du HIV, complémentaires des thérapies actuelles.

MOTS-CLES : HIV, Exosomes, Phospholipases, Prostaglandines, Endosomes, Réservoirs

HIV and exosomes : lipid homeostasis of endosomal compartment controls HIV reservoirs

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Exosomes are vesicles about 50-90nm, generated in the late endosome compartment or multivesicular bodies, and released in the extracellular medium. We have considered them as non infectious particles and found that mast cells -derived exosomes contain phospholipases : a phospholipase C (PLC), a PLD, 3 classes of PLA_2 ($cPLA_2$, $iPLA_2$, $sPLA_2$) which are activatables by GTP. They also contain free fatty acids such as arachidonic acid, and prostaglandins. Following internalization, they accumulate in late endosome compartments which are a site for storage and assembly for HIV. This compartment concentrates lipids such as cholesterol and lysobisphosphatidic (LBPA) acid for which a PLA_2 intervenes in metabolism. We assessed the role of lipidic homeostasis on HIV production using human monocytes coculture, by i) modifying the rate and distribution of intracellular cholesterol,and ii) inhibiting PLA_2 activity. We found that the steroid hormone progesterone, and the PLA_2 inhibitor, (MAFP), allowed to lower HIV production and to reduce the release of particles from cell periphery. The LBPA could be a common element to the effect of progesterone and MAFP. Targeting MAFP sensitive PLA_2s represents a strategy to alter HIV reservoir and could lead to the development of new molecules in HIV eradication.