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THE ROLE OF TETRASPANIN PROTEIN CD63 IN HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION AND REPLICATION

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THE ROLE OF TETRASPANIN PROTEIN CD63 IN HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION AND REPLICATION

by
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THE ROLE OF TETRASPANIN PROTEIN CD63 IN HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION AND REPLICATION

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Human Immunodeficiency Virus (HIV) infection typically involves interaction of Env with CD4 and a chemokine coreceptor, either chemokine (C-C motif) receptor 5 (CCR5) or chemokine (C-X-C motif) receptor (CXCR4). Other cellular factors supporting HIV replication have recently been characterized. Previous works demonstrated a role for CD63 in early HIV infection events in macrophages via inhibition by pretreatment of an anti-CD63 antibody, which did not inhibit HIV replication in peripheral blood lymphocytes or in two CD4+ cell lines tested. To confirm the requirement for CD63 in HIV replication, CD63 expression in cells was decreased by RNA interference using short interfering RNAs (siRNA). Inhibition of HIV replication was demonstrated in macrophages following CD63-specific siRNA treatment. The inhibition effect was also shown when the anti-CD63 antibody treatment was delayed 12 hours after HIV infection, or by CD63 siRNA treatment 72 hours post infection, suggesting that late HIV replication events may also be affected. In U373-MAGI cells engineered to stably express either CCR5 or CXCR4, CD63-specific siRNA treatment resulted in over 90% reduction in CD63, which was associated with decreased HIV replication, even though these cells were refractory to HIV inhibition by the anti-CD63 antibody treatment. Using an R5/X4 HIV-89.6, CD63 downregulation was shown to decrease HIV replication in the U373-MAGI cells expressing either CCR5 or CXCR4. Although anti-CD63 antibody was previously shown to inhibit early HIV infection events only in macrophages, current progress shows a role for CD63 in HIV replication in CD4+ cell lines. Further delineation of the role of CD63 in HIV replication may lead to development of novel therapeutic compounds.

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LIST OF ABBREVIATIONS

ABS	antibody binding sites
AIDS	acquired immunodeficiency syndrome
CCR5	chemokine (C-C motif) receptor 5
CXCR4	chemokine (C-X-C motif) receptor 4
CTx	cholera toxin
ELISA	Enzyme-linked immunosorbent assay
Env	viral envelope proteins
HAART	highly active antiretroviral therapy
HCV	hepatitis C virus
HIV	human immunodeficiency virus
LTR	long terminal repeats
MCF	mean channel florescence
MCSF	macrophage survival factor
MIIC	MHC class II compartment
MVB	multi-vesicular body
RISC	RNA induced silencing complex
RT	reverse transcriptase
siRNA	short interfering RNAs
SIV	simian immunodeficiency viruse
SV40	simian vacuolating virus 40
TEM	tetraspanin enriched microdomains
TX100	Triton X-100
UEV	ubiquitin E2 variant
VSV	vesicular stomatitis virus

CHAPTER 1: BACKGROUND

INTRODUCTION

Human immunodeficiency virus (HIV) infection was recognized 25 years ago (Gartner et al., 1986; CDC, 1981), rapidly became a global pandemic affecting both Western regions and underdeveloped countries, and is expected to continue to spread. Infection results in a gradual decline in the number of CD4+ T cells and impaired cellular immune function, leading to the development of the clinical disease state known as acquired immunodeficiency syndrome (AIDS) (CDC, 1985). Advanced AIDS is characterized by infection with a number of opportunistic pathogens as well as certain malignancies (Ackah et al., 1995; Alcabes et al., 1993a; Alcabes et al., 1993b). According to the Joint United Nations Program of HIV/AIDS, it is estimated that 42 million people have been infected worldwide and that 3.1 million people died of the disease in 2002 alone (Zanakis et al., 2007). Hence, the study of HIV is important and necessary in order to foster the development of new and better therapies to control infection and reduce further spread of this virus.

Although therapies approved by the Food and Drug Administration prior to 2003 all target the viral reverse transcriptase or protease, additional agents have recently been developed which target viral entry (Rusconi et al. 2007). The entry process begins with interaction of the trimetric forms of the viral envelope proteins (Env) gp120 with the cell surface receptor CD4 on macrophages or T cells. This binding causes a conformational change in gp120 that facilitates interaction with the chemokine receptors CXCR4 or CCR5. A second conformational change then occurs which allows for insertion of a hydrophobic portion of gp41 (the fusion peptide) into the target cell membrane, which facilitates fusion between viral and cellular membranes and entry of the viral core into the cytoplasm of the cell (Rusconi et al. 2007).

The existing knowledge of viral entry is incomplete. In addition to CD4 and chemokine receptors, other cell surface molecules may also be involved in HIV entry (Arthur et al., 1992). In this study, the hypothesis was tested that the tetraspan

transmembrane glycoprotein CD63 plays a role in HIV-1 infection and replication of macrophages and T cells. Identification and characterization of this potential role is the major focus of this study.

MOLECULAR BIOLOGY OF HIV-1

Virion and Genome Structure

Human immunodeficiency virus (HIV-1) is a member of the *Lentivirus* genus of the family Retroviridae, which are commonly referred to as the retroviruses (Coffin et al., 1986). This family of viruses received its name based on the unusual manner in which the genome is replicated. Initially identified in 1983 as the virus that causes AIDS, HIV-1 was the first human retrovirus discovered to cause cellular depletion instead of proliferation (CDC 1985; Blattner et al., 1988; Coffin et al., 1986). Although in the early 1980's, the reported AIDS cases in the U.S.A. were from homosexual men, nowadays, HIV infection is worldwide pandemic, affecting men, women, and children of all races and sexual orientation (Friedland et al., 1989; Ammann et al., 1983a; Ammann et al., 1983b). A precise origin for HIV-1 is uncertain, however, the virus is closely related to the simian immunodeficiency viruses (SIVs) and is likely to have originated in Africa through a cross-species infection between humans and the chimpanzee subspecies *Pan troglodytes troglodytes* (Ammann et al., 1983a; Ammann et al., 1983b; Ammann et at., 1991; Andes et al., 1989; Andiman et al., 1990; Aoki-Sei et al, 1992).

Broadly, HIV-1 strains are divided into different groups: M (major), N (non-M-non-O), and O (outlier) (Louwagie et al., 1993; Myers et al., 1994). Group M can be further divided into different clades/subtypes or circulating recombinant forms (CRFs) based on phylogenetic analyses of viral *env* and *gag* genes, and more recently on full length viral genomes. Currently, there are 9 genetic subtypes of HIV-1 (A, B, C, D, F, G, H, J and K) and 4 CRFs (AE, AG, AG and AB—the pure parental subtype E virus has not been found) that are thought to play a role in AIDS epidemics. Additional subtype recombinants have been identified, which do not play a role in the spread of HIV-1 epidemically. Different subtypes are responsible for epidemics in different geographical

regions, with subtype B predominating in the U.S. and subtype C the most widespread globally (Louwagie et al., 1993).

HIV virus particle contains two main parts, the inner core and viral membranes. The inner core of a mature HIV particle is a pear-shaped 'capsid' made by core protein p24, which contains HIV's genome. Two identical strands of HIV RNA are found in the p24 capsid. The full HIV genome is encoded on one long strand of RNA (Fig. 1).

HIV genome includes**:** *gag*; *pol*; *env*; and regulatory genes: *tat, rev, nef, vif, vpr,* and *vpu* (Fig. 2). The HIV genome also has a "Long Terminal Repeat" (LTR) at each end of its genome. Two HIV RNA molecules form dimer. Beside two RNA strands, there are also some enzymes in viral capsid, including protease, reverse transcriptase and integrase. Protease is vital to HIV function; it helps to process the gag and pol proteins into functional forms at the same time as, or shortly after the virus buds from the cell surface. Protease inhibitors, such as lopinavir and amprenavir, are suggested to be potential components of many highly active antiretroviral therapy (HAART) treatments.

HIV-1 Proteins

Gag

Along with *env* and *pol*, *gag* is one of the three main genes found in all retroviruses. It contains around 1500 nucleotides, encoding four separated proteins, which form the viral core after processed by proteases: capsid protein (CA and p24), matrix protein (MA and p17), nucleocapsid protein (NC and p9), and p6. Among them, p17 is not actually part of the viral core but is the "matrix" which anchors the core to the viral envelope. p9 binds to HIV's genomic RNA and holds it in place in the virus core. p6 helps Vpr to be incorporated into newly-made virus particles (Freed et al., 1998).

Pol

It encodes four proteins: reverse transcriptase (RT), protease, RNAse H, and integrase.

Unique to retroviruses, RT copies virus RNA into DNA after HIV particle fuses with a cell, and ready to be integrated into the cell's DNA genome. Viral RT has low fidelity. It has been estimated that during each round of HIV-1 replication, 1-10 mistakes

are occurred. This poor fidelity in the transcription makes the pattern of HIV genes and proteins changes quite rapidly, and escapes the surveillance of immune system.

The major function of protease is to process proteins made from HIV's genome into parts of fully-functioning HIV particles. RNAse H is responsible for the degradation of DNA-bound RNA after reverse transcription. Integrase integrates DNA copy of HIV's genome into the host DNA. It catalyses the 'cut-and-paste' operation by snipping the host DNA and attaching the proviral genome to the snipped ends. There are three domains of integrase, including a 'Zinc finger', a central catalytic domain, and a domain binds to DNA. Due to the unique feature of integrase, it is speculated that inhibition of integrase will be a potential pharmaceutical target in HIV treatment.

The process of reverse transcription and integration are actually overlapped in HIV infection. The RNA, reverse transcriptase, integrase, and other HIV proteins form a 'pre-integration complex', which moves into the cell's nucleus. The reverse transcription begins and the integration begins at roughly the same time.

In HIV genome, *gag* and *pol* have overlapping reading frames, and can be expressed in one long strand call '*gag-pol*'. When the proviral genome is being transcribed from DNA into fresh RNA, sometimes the cell machinery makes a 'mistake'. Instead of finishing copying out the *gag* gene, it hops onto *pol* and produces one longer code for gag-pol. The combined gag-pol transcript encodes some of HIV's most important proteins, such as reverse transcriptase and integrase. Gag-pol gets produced around 1 in every 20 occasions, and this keeps its resultant proteins at around one twentieth of the concentration of gag proteins, which maintains the normal percentage for viral particle production.

Env

It encodes a single protein, gp160. When gp160 is synthesized in the cell, cellular enzymes add complex carbohydrates to it and turn it from a protein into a glycoprotein. gp160 travels to the cell surface, where cellular enzymes again attach to it, and chopping it into two pieces, gp120 and gp41. When new virus buds off from the host cell, these two pieces lie on opposite sides of the virus membrane. gp120 sits on the outside of the

virus particles, forming the virus's spikes, while gp41 sits on the insides of the membrane. Each gp41 being anchored to a gp120 through the member and three set of these form a trimer (Willey et al., 1988).

Tat (transactivator)

As a regulatory gene, *Tat* is responsible for the acceleration of the production of HIV virus. Without it, HIV completely fails to replicate itself. Tat works by binding to 5' regulatory elements of a new HIV RNA strand, in a region called 'Transactivator Active Region' or TAR. The TAR is the first 59 nucleotides of the HIV genome. This interaction stabilizes the mRNA and enhances translation of viral proteins, including the early protein Tat. Tat is essential for replication. Besides Tat, HIV also has proteins that can serve as negative regulators, such as nef and vif. These complex functions are only seen in human and simian lentiviruses, not in other retroviruses (Dayton et al, 1986; Fisher et al., 1986).

Rev

As another regulator gene, *Rev* stimulates the production of HIV proteins, but suppresses the expression of HIV's regulatory genes. The mRNAs of HIV can either be sent to the protein-producing part of the cell intact, or they can have bits cut out of them first (splicing). The intact mRNA tends to encode HIV proteins, such as envelope and capsid proteins, while the spliced mRNA encodes regulatory genes such as *tat* and *nef*. What rev does is to help intact mRNA to be exported from the cell nucleus. It binds to the mRNA at a specific point (Rev-Responsive Element), and this complex of RNA and rev is sent out of the nucleus. A molecule of rev can 'shuttle' in and out of the nucleus, potentially taking a new set of RNA out each time it leaves the nucleus. The Rev-Responsive Element is chopped out from the completely-spliced HIV mRNA. This spliced mRNA is sent out of the nucleus by the ordinary cell machinery (Nakielny et al., 1997).

Nef (negative replication factor)

Nef encodes a protein that may have functions both at the cell membrane and in protein trafficking in the cytoplasm, it has been shown to retard HIV replication. It does

this possibly by modifying cellular proteins that regulate the initiation of transcription, which tell the cell whether or not to make mRNA.

Vif (virion infectivity factor)

Vif encodes 'virion infectivity factor', a protein that promotes the infectivity of the HIV particles. The protein is found inside HIV-infected cells, and it works by interfering with one or the immune system's defenses, a cellular protein called APOBEC3G. APOBEC3G sneaks into newly-produced viral particles and makes them less infective by interfering with the replication of the viral genome by base misincorporation when the particles infect new target cells. Vif protein specifically binds to APOBEC3G and causes its degration (Argyris et al. 2004; Navarro et al., 2004; Rose et al., 2004).

Vpr (viral protein R)

It helps to accelerate the production of HIV proteins and facilitate the nuclear localization of the pre-integration complex. Vpr carries 'nuclear localization signals', which encodes sequences that are recognized by cellular machinery to be transported into the nucleus. Vpr also has a role to stop the host cell in G2 phase of a cell cycle, which will be a nicer environment for HIV replication. There are around 100 copies of vpr in every HIV virion (Lu et al., 1993).

Vpu (viral protein U)

Vpu helps the assembly of new virus particles and help them to bud from the host cell. Without Vpu, HIV still buds, but only about 10% or 20% as many new virus particles are produced. Vpu also enhance the degradation of CD4 proteins within the infected cells, and reduce superinfection. A secondary effect of vpu is to delay the cytopathic effects of virus infection, keeping the cell alive slightly longer for virus replication. (Ernest et al., 1989; Bour, et al., 2003;)

ROLE OF CELLULAR FACTORS

During the life cycle of HIV, CCR5 or CXCR4 are required for the attachment and entry of HIV (Feng et al., 1996; Oberlin et al., 1996). After entry, initial steps of HIV

replication is independent of cellular proteins; cellular proteins are required for transportation of HIV genome and integration into host DNA; cellular factors are heavily utilized for late HIV replication events.

Receptor and Co-receptor

HIV infection typically requires viral interactions with both CD4 and a chemokine receptor. As the primary HIV receptor for both macrophages and CD4+ T-cells, CD4 is necessary but not sufficient for entry, the α-chemokine receptor CXCR4 (Feng et al., 1996; Oberlin et al., 1996) and the β-chemokine receptor CCR5 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996) were shown to be involved in post-CD4 binding interactions required for efficient HIV entry. Based on the usage of coreceptors, HIV strains are divided into three groups: T-cell line-adapted virus strains predominately use CXCR4 and are called X4 strains; macrophage-tropic strains use CCR5 as a major coreceptor and are called R5 strains; and those could use both CXCR4 and CCR5 are termed dual tropic (R5X4) virus. Macrophages express CD4 and both coreceptors on their surface, but abundance is significantly different from CD4+ T cells. CD4 expression is over 1000-fold lower in macrophages, CCR5, and especially CXCR4 is also less abundant. Although CXCR4 is expressed on macrophages and some atypical HIV strains can utilize this coreceptor along with CD4 for entry, viruses that use CD4 and CCR5 (R5 strains) typically enter macrophages far more efficiently than those using CD4 and CXCR4 (X4 strains) (Bazan et al., 1998). It has been proposed that aspects such as receptor/coreceptor density levels (Platt, et al., 1998; Tokunaga et al., 2001), inadequate cell surface associations between CD4 and CXCR4 (Dimitrov, et al., 1993, Lee et al., 2000), and CCR5 signaling (Wang et al., 1999) may be important for macrophages tropism. There have been few studies, however, evaluating whether there may be unique factors, in addition to CD4 and CCR5, are involved HIV infection of macrophages.

Besides receptor-mediated entry, HIV can also use internalization pathway to entry. Cells have evolved to use many routes to internalize molecules from their surface; these include macropinocytosis, phagocytosis, clathrin-mediated endocytosis, caveolae-

mediated endocytosis, and clathrin- and caveolae- independent endocytosis. Because of the number of entry routes, endocytic recycling pathways play an essential role in maintaining the correct composition of various organelles and for returning essential molecules that perform specific functions to the appropriate compartments (Maxfield et al., 2004). These pathways are regulated by small GTPases, phosphatidylinositol lipids, and other secondary effectors (Perret et al., 2005). Viruses and toxins can enter the cell through numerous routes. Many, including SV40 and Cholera toxin, use whatever endocytic pathway is available (Perret et al., 2005). Although there are multiple internalization pathways in macrophages, the mode of internalization of extracellular particles the cells use depends on which receptors are stimulated and the size of the particles being internalized (Tse et al., 2003). Phagocytosis is the uptake of large particles (>500 nm) by pseudopodia extensions (Marechal et al., 2001). It is triggered by cell surface phagocyte receptors and is dependent on phosphatidylinositol 3-kinases (PI3K) and syk tyrosine kinases which lead to actin polymerization and pseudopodia extension (Lefkir et al., 2004). To accommodate this pseudopodia extension, membranes from internal stores, such as the endoplasmic reticulum, recycling endosome, and EE (early endosome), are recruited to the sites of extensions (Niedergang et al., 2004). Receptors for phagocytosis include receptors for mannose residues, phosphatidylserine, C-reactive protein, and opsonins such as complement and immunoglobulins (Niedergang et al., 2004). Clathrin also seems to be involved in phagocytosis, but its role is not well understood (Tse et al., 2003). These phagosomes can then fuse with lysosomes for degradation (Perret et al., 2005). Clathrin-mediated endocytosis is the best-studied pathway. It includes transmembrane receptors that are organized into clathrin-coated pits (Marechal et al., 2001), and in macrophages, approximately 10-20% of the membrane surface area can be coated with clathrin (Kaplan et al., 1990). Macrophages use clathrin-mediated endocytosis to internalize nutrients, antigens, growth factors, and pathogens (Takei et al., 2001). These clathrin-derived vesicles (<150 nm) can fuse with early endosomes in the endocytic pathway. It has been shown that HIV-1 can use clathrin-mediated endocytosis for entry into cells (Daecke et al., 2005). Another mode of entry is

caveolae-mediated endocytosis. Caveolins are associated with lipid rafts in the plasma membrane (Gargalovic et al., 2003), and they form small vesicles (50-80 nm) that are rich in caveolin, cholesterol, and sphingolipids (Marechal et al. 2001). Caveolae-mediated endocytosis has been shown to be important in signal transduction, lipid homeostasis, and subcellular trafficking of molecules (Gargalovic et al., 2003). It also participates in the internalization of macromolecules, GPI-linked proteins, toxins, and viruses (Marechal et al., 2001). These caveolae-derived vesicles can fuse with the SE (Perret et al., 2005). Finally, macropinocytosis involves trapping large amounts of macromolecules and fluids into large vesicles (0.2-3 um) (Marechal et al., 2001). Macropinocytosis also plays an important role in antigen uptake for presentation by MHC I and II molecules. These macropinocytotic vesicles resemble an "empty" phagosome, and can either fuse with lysosomes or re-fuse with the plasma membrane to send its contents back into the extracellular space (Amyere et al., 2002). Like phagocytosis, macropinocytosis is also dependant on the actin cytoskeleton.

APOBEC System

Mobile genetic elements, such as retrotransposons, have profoundly shaped the genomes of all living organisms. In human and murine genomic DNA, there is >45% mobile elements. (Boeke et al., 1997; Lander et al., 2001; Waterston et al., 2002; Deininger et al., 2003; Bannert et al., 2004; Kazazian et al., 2004;) They can be grouped into two groups; around 30% are well-characterized long interspersed nuclear elements (LINE); and the long terminal repeat (LTR) retrotransposons, which are closely related to infectious retroviruses. Most of these endogenous retroviruses have lost the replication capacity, but several still mobile. (Dewannieux et al., 2004; Ribet et al., 2004) The requirement for genome stability has led eukaryotes to adopt several strategies to restrict the proliferation of transposable elements. One strategy is mutational inactivation of the elements in the course of their retrotransposition cycle, by cellular cytosine deaminases (Esnault et al., 2005; Dutko et al., 2005; Schumacher et al., 2005).

In human, the cytosine deaminase family comprises numerous members including AID(activation-induced deaminase), APOBEC1 (Apoliprotein B mRNA-editing enzyme), APOBEC2, the APOBEC3A-G group and APOBEC4 (Jarmuz et al., 2002; Wedekind et al., 2003; Rogozin et al., 2005). These enzymes can deaminate cytosine to uracil within DNA and/or RNA molecules, among which, APOBEC3G is most extensively studied in APOBEC family. When it is present in adequate amounts, APOBEC3G is incorporated into virus particles and attack newly made HIV RNA or DNA by converting cytosine (C) residues to uracil (U) residues in viral RNA, or guanosine (G) to adenosine (A) residues in viral DNA, thus yielding a nonactive form of the HIV genome (Sheehy et al., 2002; Mangeat et al., 2003; Lecossier et al., 2003; Bishop et al., 2004; Liddament et al., 2004). HIV counteracts APOBEC3G's anti-retroviral activity by expressing 'Vif' protein that prevents it incorporation into virions, primarily by inducing its degradation via the proteasome (Conticello et al., 2003; Kao et al.,2003; Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003; Mehle et al., 2004).

TSG101

During the process of HIV assembly, maturation, and budding, several steps must occur. First, Env (gp160) must be translated in the endoplastic reticulum (ER) and then processed in the Golgi complex. While in the Golgi complex, Env is glycosylated and is also cleaved into gp120 and gp41, which are then transferred and inserted into the plasma membrane (Freed, et al., 2004). In T-cells, these proteins remain in the plasma membrane, while in macrophages these proteins are internalized into vesicles and end up in the MHC class II compartment (MIIC) compartment, an MVB (Pelchen-Matthews et al., 2003). The process of viral assembly is very similar between T-cells and macrophages; however, in T-cells, viral assembly occurs on the plasma membrane, while assembly in macrophages occurs on the membrane of the MIIC compartment (Raposo et al., 2002; Pelchen-Matthews et al., 2003). The next step is Gag multimerization. These Gag-Gag complexes target the membrane where virus assembles. Gag binds to viral genomic RNA and brings the RNA to the site of assembly (Bukrinskaya et al., 2004). These Gag

complexes also associate with viral proteins Vpr and Vif, and with cellular host proteins CyPA and HP68. All of these complexes are then directed to the membrane of viral assembly. Gag-pol complexes are also formed and are trafficked to the site of viral assembly. Another important function of Gag is the binding to TSG101, which is part of the ESCRT pathway (Katzmann et al., 2001). TSG101 is a cellular endosomal sorting protein, which is required for biogenesis of vesicles that bud into the lumen of late endosomal compartments call multivesicular bodies (MVBs). Gag recruits TSG101 to facilitate the final stages of virus budding. A conserved P(S/T)AP tetrapeptide motif within Gag (the 'late domain/L domain') binds directly to the NH_2-terminal ubiquitin E2 variant (UEV) domain of TSG101. This binding allows Gag to hijack this pathway to aid in viral assembly (Garrus et al., 2001; Martin-Serrano et al., 2001; Myers and Allen 2002, Timmins et al., 2003; Dong et al., 2005)

Once all the parts are brought together, the virus is assembled and the process of pinching off begins. Viral maturation occurs during this process as well. Maturation mainly consists of proteolytic cleavage and RNA dimerization and is needed for viral stability and increased infectivity (Bukrinskaya et al., 2004). During maturation, Gag is cleaved into matrix protein (MA), capsid protein (CA), nucleocapsid (NC), p6, and spacer proteins. Pol is cleaved into protease (PR), reverse transcriptase (RT), and integrase (IN). The final step is release from the host cell. In T-cells, this involves direct budding from the plasma membrane, while in macrophages, the budding occurs into the MIIC compartment vesicle and the vesicle then fuses with the plasma membrane to release the virus into the environment (Raposo et al., 2002).

IMPORTANCE OF MACROPHAGES IN HIV PATHOGENESIS

HIV predominantly infects CD4+ T lymphocytes and macrophages. Macrophages are of particular importance for the pathogenesis of HIV, as these cells are likely to be the major cell type involved in mucosal transmission of the virus, serve as a long-lived viral reservoir, contribute to viral dissemination and may be responsible for many of the debilitating clinical manifestations of AIDS, including AIDS-related dementia,

pulmonary disease, and encephalopathy (Shaw et al., 1985; Eiley et al., 1986;Gartner et al., 1986; Koenig et al., 1986; Tschachler et al., 1987; Eilbott et al., 1989; Meltzer et al., 1990; Zhu et al., 1993; Orenstein et al., 1997; Zhang et al., 1998; Igarashi et al., 2001; Lambotte et al., 2003). Furthermore, virus infected macrophages have been found to be less susceptible to the cytopathic effects of HIV and more resistant to HIV-induced apoptosis. Compared to T cells, not so much work was done on HIV infected macrophages. Development of cell-specific therapies may be possible through the identification of molecular determinants of macrophages infection.

The HIV Nef protein can activate the CD40 signal pathway in macrophage. Through the activation of CD40 signaling, HIV promotes the release of chemoattractants and of soluble factors which increase susceptibility of CD4+ T-lymphocytes to infection. HIV can activate a macrophage survival factor (MCSF), a major macrophage maintenance factor, and help to sustain virus production in macrophage. In this way, macrophage serves as a reservoir for virus.

Compared with the plasma assembly of HIV in lymphocytes and T cell lines, more and more experimental data show that HIV assembly happens in the endosome / late endosome membrane of macrophages. HIV virions have been proved to contain many cellular proteins besides gp120 and gp41. These proteins must be obtained during the process of viral budding. Variant studies show that HIV budding on T cells membrane happens on the specific membrane domains, called lipid rafts, which is detergent resistant and enriched in cholesterol and cell membrane signal proteins. By budding through lipid rafts in T cells, HIV selectively incorporates raft markers and excludes non-raft proteins. This process is well studied in T cells, but it is unknown whether lipid rafts serve as budding sites for HIV in macrophages.

Study on HIV replication in macrophage showing that, besides the plasma membrane budding, HIV can bud intracellular into the lumen of endosome, similar to the formation of exosome in multivesicles bodies (MVBs). Viral particles are then released by the fusion of MVBs with the plasma membrane. (Arthur et al., 1992; Orentas et al.,

1993; Ott 1997; Escola et al., 1998; Sakalian et al., 1998; Nguyen et al., 2000; Thery et al., 2001).

Due to the incorporate of endosome proteins in virion surface from macrophages, HIV may fuse with the receptor cell in a receptor-independent and Env-independent way. Removal of the Env gene does not eliminate HIV's ability to infect cells and Env-deleted HIV particles infect CD4- cells as efficiently as WT HIV particles. (Ott 1997; Pang et al., 2000; Chow et al., 2002)

Intracellular formation of HIV particles in macrophages may be important in HIV pathogenesis. The impact includes, first, the adaptive immune system fails to control HIV pathogenesis and the ineffectiveness of viral antigen-based vaccines, although B and T cell responses are generated efficiently to viral antigens (Sellon 1993; Rimmelzwaan et al., 1994; Connor et al., 1998; Addo et al., 2003), since virus may use exosome pathway to spread. The second, neutralized antibodies can't block the infection completely, since virus may use other proteins that used by exosome to fuse with cell membrane, both through plasma membrane and endosome membrane. There is another possibility that HIV infected cells make many exosomes that containing Env proteins of HIV, which can bind to the antibodies. The third, T cell depletion in HIV patient may cause by HIV particles through exosome pathway, since exosomes are actively exchanged in APCs and T cells. The proteins that incorporated in the virions surface can also activate receptor cells and provide an environment ready for viral replication; also the existence of MHC molecule loaded with viral peptides on virus surface will help these viral particles directly bind to the specific T cells that can response to virus infection, speeding the disease progress.

TETRASPAN PROTEINS AND THEIR GENERAL ROLES

The tetraspanins, first discovered on the surface of human leucocytes (Hotta et al., 1988; Oren et al., 1990; Wright et al., 1990), are a large superfamily of cell surface membrane proteins. They are best characterized by conserved four transmembrane domains, which span the membrane four times, forming two extracellular domains. They

are thus also known as the transmembrane 4 (TM4) superfamily or the TM4SF, or tetraspan family (Fig. 3).

Based on expressed sequence tag database, 32 tetraspanins has been discovered in human and mouse. Tetraspanins are medium-sized proteins, containing 204- to 355-amino acids. With cytoplasmic NH_2 and COOH termini, four transmembrane domains form two extracytoplasmic regions of unequal sizes, a small extracellular loop (EC1) containing 20-28 amino acids and a large extracellular loop (EC2) containing 76-131 amino acids (Wright et al., 1994, Maecker et al., 1997). There are 4 to 6 conserved extracellular cystines in EC2. Four of these cystines are absolutely conserved in all tetraspanins analyzed to date, including 2 cysteines in a CCG motif, and another cysteine in a PXSC motif (Wright et al., 1994; Berditchevski et al., 2001). Tetraspanins also typically contain conserved polar residues within transmembrane domains, as well as several other conserved residues (Todres et al., 2000; Hemler 2001; Stipp et al., 2003). The mammalian tetraspanin family contains 32 members, whereas there are at least 35 members in *Drosophila melanogaster*, and 20 in the *Caenorhabditis elegans* genome (Todres et al., 2000). Despite the size of the tetraspanin family and their abundance and wide distribution on cells and tissues, often expressed at 30000 -100000 or more copies per cell (Fradkin et al., 2002; Sincock et al., 1997; Higashihara et al., 1990), the functions of majority of tetraspanins have been minimally studied or not at all. Currently, the tetraspanins include leucocyte differentiation antigens CD9, CD37, CD53, CD63, CD81/TAPA-1, CD82 and CD151; antigens first identified on tumors, TALLA-1, Co-029 or SAS (Szala et al., 1990; Jankowski 1994; Takagi et al., 1995) and uroplakins UP1a and UP1b (Sun et al., 1996) are mostly characterized. This protein family has been implicated in regulating multiple biological processes and the general roles of it include antigen presentation, cell adhesion and migration, cell-cell fusion, cell activation, and proliferation.

Tetraspanins and Fertilization

The role of tetraspanins in fertilization is described by studying gene knock-out mice. Mice that have deletion of the CD9 gene grew and developed without obvious

abnormalities, but oocytes from $CD9^{-/-}$ mice showed severely impaired sperm-egg fusion (Kaji et al., 2000; Le Naour et al., 2000). CD9 is concentrated in the oocyte microvilli surrounding the sperm head, but is not function in attachment or penetration, as sperm penetration through the zona pellucida and binding to egg plasma membrane were normal. Recent research has revealed CD9 as a molecular facilitator that controls the redistribution of some membrane proteins including the alpha 6 beta1 integrin, a partner of CD9, into clusters that may be necessary for gamete fusion (Ziyyat et al., 2006). Tetraspanin CD81 is also a required molecule, as fertilization is totally blocked when lacking both tetraspanins CD9 and CD81 (Deng et al., 2000; Kaji et al., 2002; Rubinstein et al., 2006).

Tetraspanins and Immune Cells

Around 20 tetraspanins can be expressed on leukocytes surface. Specific tetraspanins has been revealed to interact with a wide range of leukocyte receptors, including CD2, CD4, CD5, CD8, CD19, CD21, Fc receptors and MHC class I and class II molecules. Although immunological implications of many of these interactions remain to be established, tetraspanins have important role in immune response and signal transduction (Olweus et al., 1993; Szollosi et al., 1996; Lagaudriere-Gesbert et al., 1997; Tai et al., 1997; Hammond et al., 1998; Maecker et al., 1998; Toyo-oka et al, 1999; Knobeloch et al., 2000; Kaji et al., 2001; Engering et al., 2001).

In leukocytes, cross-linking tetraspanins at the cell surface can result in tyrosine phosphorylation, calcium fluxes and inositolphosphate turnover. These signals ultimately regulate cell proliferation, motility and adhesion. As tetraspanins don't have enzymatic activity, the molecular mechanism by which tetraspanins transducer signals is likely rely on the presence of signaling molecules in tetraspanin microdomains (Carmo et al., 1995; Yauch et al., 2000).

Roles of tetraspanins CD37 and CD81 have been studied in B cells of mice. Depletion of CD37, a major B lymphocyte molecule in mice, results in reduced IgG1 production in unchallenged mice and impaired stimulation when challenged by antigens (Knobeloch et al., 2000). CD81 belongs to a mutimolecular complex called

CD19/CD21/CD81/Leu-13, which respond to antigen stimulation at the surface of B Lymphocytes (Tedder et al., 1997). The colligation of CD19 with the B cell receptor decreases the threshold of response to the antigen, thus B lymphocytes can respond to low antigen concentractions despite the weak affinity of the antigen receptors. Deletion of CD81 will reduce the expression of CD19 on cell surface, and impair acquired immunity (Fearon et al., 1995).

Ligation of CD9, CD53, CD81 or CD82 provides a co-stimulatory signal in T cells (Lebel-Binay et al., 1995; Lagaudriere-Gesbert et al., 1997; Tai et al., 1997; Witherden et al., 2000). *In vitro* proliferation of T lymphocytes from CD81-null mice in response to stimulation was enhanced. Additional in vitro studies showed that during T cell response to antigen presentation by B cells, CD81 was required on the T cells but not on the B cells (Deng et al., 2000). CD81 also played a key role in driving helper T cells toward the polarized Th2 differentiation state in vivo (Maecker et al., 1997; Deng et al., 2000). Associations of CD81 with molecules such as CD4 and CD8 on T cells and a subset of MHC class II on antigen-presenting cells are also relevant to the mechanism by which CD81 affects T cell proliferation and polarization (Levy et al., 1998; Kropshofer et al., 2002). During adapted immune response, in immune synapse between T cells and antigen-presenting cells, CD81 in both T cells and antigen-presenting B cells is rapidly recruited to the central of the synapse (Bromley et al., 2001; Mittelbrunn et al., 2002). Hence, CD81 plays a critical role in antigen presentation.

Tetraspanins is also likely to be involved in antigen presentation. CD9, CD53 and CD81 are associated with MHC class II receptors on the surface of dendritic cells (DCs), and CD9 and CD63 in cytoplasmic compartments (Engering et al., 2001). These tetraspanin-MHC class II complexes can form microdomains, which could regulate MHC class II clustering, a process necessary to effectively engage clustered TCRs on the T-cell surface in adapted immune response (Kropshofer et al., 2002). Tetraspanins (CD37, CD53, CD63 CD81 and CD82) are also enriched on subset of cytoplasmic MIIC, named exosomes. Exosomes are thought to present antigen and exchange membrane proteins, which can deliver peptide-loaded MHC class II molecules for long-range stimulation of

T lymphocytes (Thery et al., 2002, Wolfers et al. 2001). Alternatively, tetraspanins could function in the endocytosis and/or trafficking of MHC class II molecules (Simonsen et al., 2001).

Interaction between Tetraspanins

Tetraspanins are associated with a wide range of other proteins including integrins, various Ig superfamily members (e.g., CD2, CD3, CD4, CD8, MHC class I, MHC class II, etc), proteoglycans (syndecan, CD44), complement-regulatory proteins (CD21, CD46), growth factor receptors and ligands (EGFR, c-kit, proTGFα, proHB-EGF) and miscellaneous other molecules (CD19, ADAM10, etc.) and other tetraspanins. Numerous biochemical analyses and functional studies predicted the existence of tetraspanin-enriched microdomains (TEMs) that together form the tetraspanin web (Charrin et al., 2003, Levy et al., 2005). Tetrasapanin associations can be divided into primary, secondary, and tertiary levels based on their strength, usually determined by different detergents.

Primary interactions are direct interactions of tetraspanins and other proteins, which is resisted to strong detergents, like Triton X-100. Tetraspanins uroplakin 1a (UP1a) and UP1b directly interact with Uroplakin II and Uroplakin III (Wu et al., 1995); CD151 interact with integrin α3β1 (Serru et al., 1999) and α7β1 (Sterk et al., 2002); and CD9/CD81 with transmembrane IgSF proteins EWI-2 and EWI-F (Charrin et al,. 2001; Clark et al., 2001; Stipp et al., 2001). These interactions can be captured by covalent crosslinking and occur at high stoichiometry. A relatively week but still belong to primary interactions are interactions of CD81 with CD19, and CD9 with HB-EGF.

Secondary interactions are typically involved the association between tetraspanins. The tendency of tetraspanins to interact with each other provides the means to link together primary complexes into a network, or TEMs. These microdomains can be isolated by medium detergent, like Brij 96/97 (Stipp et al., 2001). Tertiary interactions are defined as those tetraspanin interactions that are disrupted in detergents such as Brij 96/97 and Triton X-100 but are retained in less hydrophobic detergents such as Brij 58, Brij 99, Brij 35 and CHAPS.

TEMs have some properties that are similar to lipid-rafts (Yashiro-Ohtani et al., 2000, Claas et al., 2001, Mairhofer et al., 2002). Lipid rafts are cell surface microdomains that are enriched in sphingolipids, cholesterol, and phospholipids with long, saturated acylside chains. Despite a few similarities to lipid rafts, TEMs have many distinct functions. TEMs can modulate growth factor signaling, which will affect tumor malignancy (Bienstock et al., 2001; Boucheix et al., 2001; Kawashima et al., 2002). Tetraspanins are associating with different integrins, which are major extracellular matrix receptors that form a bridge between the extracellular matrix and the cellular cytoskeleton, and these associations can affect integrin-dependent migration, spreading, and/or cell morphology (Berditchevski et al., 1997; Berditchevski et al., 1999; Radford et al., 1997; Kazarov et al., 2002). TEMs might also function as receptors for secreted proteins, and modulate signal transduction by forming some complex with transmembrane receptor tyrosine kinase (Anzai et al., 2002), or by recruiting signaling enzymes such as phosphatases (Carmo et al., 1995; Kurita-Taniguchi et al., 2002) and tyrosine kinases (Skubitz et al., 1996).

Tetraspanins and Infectious Diseases

Tetraspanins may also play a role in virus-induced syncytium formation and virus replication. The association of CD9 with HB-EGF increases the number of sites for diphtheria toxin with no change in affinity (Iwamoto et al., 1994; Cha et al., 2000). In addition, CD81 is an attachment receptor for the hepatitis C virus (HCV). The hepatitis C virus-encoded envelope, glycoprotein E2, can bind specifically and tightly to CD81 (Higginbottom et al., 2000). Besides serves as possible virus entry site, tetraspanins web may also be the budding sites for virus. HIV-1 particles produced from infected cells specifically incorporate CD63 (Escola et al., 1998; Ott et al., 2002). Using confocal microscopy, HIV-1 envelop glycoproteins and Gag protein, which directs viral assembly and release, are found to accumulate at surface TEMs. This suggests HIV-1 egree can be gated through TEMs (Sascha et al. 2006).

CD63 AS A POTENTIAL TARGET

In order to identify cell-associated molecules that may be important for macrophages tropism, our lab screened a myeloid-specific mAb library (N=120) to identify molecules that were upregulated over one week in culture, since macrophages show increased permissiveness to HIV infection after 6-7d adherence to plastic (Rich et al., 1992). Of the 16 mAbs that exhibited >30% increase in mean channel florescence (MCF) at day 6, as compared with day 3 values, five mAb showed inhibition of HIV replication in macrophages, four of which were directed toward one molecule, CD63, a tetraspan membrane glycoprotein (Metzelaar et al., 1991; Radford et al., 1995).

Previously published work (von Lindern et al., 2003) demonstrated the effectiveness of anti-CD63 antibody in macrophages, but not T-cells or other cell lines with high CD4 expression that we have tested. Cells were pretreated with anti-CD63 antibody or controls for 30 min prior to infection, and p24 production was assessed at day 7. Inhibition was also achieved with dual tropic (R5X4) strains, but the two primary X4 isolates we tested were resistant to anti-CD63 antibody.

CD63 antigen is closely associated with β-1 integrins, MHC antigens and with other tetraspan integral membrane proteins (Mannion et al., 1996; Rubinstein et al., 1996; Hammond et al., 1998; Schaeffer et al. 2001), but the biological function of CD63 is not well defined. In general, tetraspanins seem to act as facilitators in the cellular adhesion process, together with β-1 integrins (Skubitz et al., 1996). Tetraspanins are associated with β-1 integrins within tetraspanin-containing adhesion structures, and this appears to be important for modulating integrin signaling (Berditchevski et al., 1999). CD63 mAb binding appears to trigger a transient activation signal that requires extracellular calcium. The associated protein tyrosine kinase activity may play a role in signal transduction by CD63 to regulate other cell functions (Skubitz et al., 1996), perhaps including HIV entry. Collman et al., (1992) demonstrated that HIV use of CCR5 and CXCR4 as coreceptors was associated with rapid phosphorylation of the focal adhesion-related tyrosine kinase Pyk2 in macrophages. This signal was also induced by MIP-1β and stromal cell-derived factor-1α (SDF-1α), which are ligands for CCR5 and CXCR4, respectively. Implication

of CD63 in this signaling pathway may be an important link toward HIV gp120-induced signaling and virus uptake (Del Corno 2001). The recent studies indicate a role for CD63 in later stages of HIV replication post-entry, similar to the apparent role suggested for CD9 in FIV infection, where anti-CD9 did not affect entry or integration, but did inhibit extracellular virus production (deParseval et al., 2001). Therefore, the role for tetraspan membrane glycoproteins in retroviral infection is indicated.

CHAPTER 2: GENERAL METHODS

CELL CULTURES

Primary monocyte-derived macrophages were isolated from healthy HIV-1-negative blood donors by Ficoll-Hypaque centrifugation followed by adherence for 7 days to plastic petri dishes coated with human AB serum (Rich et al., 1992). By use of this methodology, the macrophage purity (CD14+ cells) is found to be >98%. Nonadherent cells will be treated with phytohemagglutinin (PHA) for 72 h prior to propagation in RPMI 1640 supplemented with 20% fetal calf serum and 20 U of interleukin-2 per ml. During differentiation, macrophages will be cultured in Iscove's modified Dulbecco's medium supplemented with 20% fetal calf serum (O'Brien et al., 1994). All cell lines were maintained in DMEM with 10% FBS.

ANTIBODIES

The following antibodies will be used in immunoprecipitation, Western blotting, and confocal microscopy: mouse monoclonal anti-CD48 (4H9); rabbit polyclonal anti CD55 (H-319); rabbit polyclonal anti-CD46 (H-294); rabbit polyclonal anti-LAT (FL-233); rabbit polyclonal anti-CD71 (H-300); rabbit polyclonal anti-β-integrin (M-106); mouse monoclonal anti-β-integrin (4B7R); rabbit polyclonal anti-CD4 (H-370); mouse monoclonal anti-CCR5 (D-6); goat polyclonal anti CCR5 (C-20); rabbit polyclonal anti-CCR5 (H-185); mouse monoclonal anti-CD63 antibody (MX-49.129.5); goat polyclonal anti-CD63 antibody (K-19); rabbit polyclonal anti-CD63 antibody (FL-238); rabbit polyclonal anti-CXCR4 (H-118) and goat polyclonal anti-CXCR4 (G-19)(Santa Cruz Biotechnology, Santa Cruz, CA). Monoclonal mouse anti-CD45; mouse anti-CD63 antibody (H5C6); mouse anti-CXCR4 (12G5); mouse anti-CCR5 (3A9); mouse anti-CCR5 (2D7) and mouse anti-HLA-DR,P,Q are purchased from BD Pharmingen (San Diego, CA).

Secondary antibodies for western blotting: horseradish peroxidase-conjugated donkey anti-mouse-IgG; donkey anti-rabbit IgG; and donkey anti-goat IgG (Santa Cruz

Biotechnology). Control antibodies: normal mouse IgG; normal goat IgG and normal rabbit IgG (Santa Cruz Biotechnology). Secondary antibodies for confocal microscopy: FITC-donkey-anti-mouse and Cy3-rabbit-anti-goat whole antibody and Fab fragments.The CXCR4 inhibitor AMD3100 has been generously provided by Anormed (Langley, British Columbia, Canada). Enzyme-linked immunosorbent assays (ELISAs) for p24 were performed according to the manufacturer's instructions.

VIRUSES

R5 HIV strains SF162 (Shioda et al., 1991) and ADA (Westervelt et al., 1992), R5X4 HIV strain 89.6 (Collman et al., 1992) and X4 HIV strain 92 HT599 (Moarefi et al., 1997) were obtained from the NIH AIDS Research and Reference Reagent Program.

INFECTION ASSAYS

Infections are performed in 96 well trays at a moi of 0.005 or 0.01. Cells will be pretreated for 30 minutes at 37^{o}C with media alone or increasing concentrations (1, 10, and 25 ug/ml) of anti-CD63 antibody (CLB-gran12, Invitrogen Corporation), 25 ug/ml of mouse IgG1 isotype control antibody (MOPC21, Invitrogen Corporation), or 25 ug/ml anti-CD4 antibody (RPA-T4, BD Sciences) as a positive inhibition control. Thereafter, virus will be added and incubated for 2 hours at 37 degrees, followed by washing. Cells will then be cultured in appropriate medium for seven days at which time supernatants will be collected and assayed by ELISA for p24 production.

IMMUNOPRECIPITATION AND WESTERN BLOTTING

After 7 days differentiation, 1×10^{7} macrophages will be scraped from the plates and lysed in 4 ml 1% Brij97 (Sigma) TNE lysis buffer (50mM Tris, pH7.5, 150mM NaCl, 5mM EDTA) supplemented with protease inhibitor mixture (complete; Roche Diagnostics). After 30min incubation on ice, insoluble materials will be removed by centrifugation at 1000×g at 4 ^{o}C and the cell lysate will be pre-cleared for 2h by addition 1/200 volume heat inactivate goat serum and 1/50 volume protein G-Sepharose beads (Sigma). Proteins will be immunoprecipitated by adding 2µg mAb (goat anti-CD4, goat

anti-CCR5, goat anti-CD63 antibody and isotype control antibody) and 20µl protein G-Sepharose beads to 1ml of the lysate. After 4-5h incubation at 4^{o}C under constant agitation, the beads were washed 5× in lysis buffer. The immunoprecipitates will be separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and transferred to a nitrocellulose membrane (Amersham-pharmacia). For Western blotting, membranes will be blocked using 5% non-fat dried milk in TBS-T (20mM Tris pH7.6, 130mM NaCl, 0.05%Tween-20) and incubated for 1h. Blots will then be incubated with specific antibodies, followed by the appropriate horseradish peroxidase (HRP)-coupled secondary antibodies. To avoid cross-reaction with the antibodies used in immunoprecipitation, different species of antibodies will be used in Western blotting. After detection of one protein, membranes will be reused for another protein detection by stripped in stripping buffer (25 mM glycine-HCl, pH 2, 1% SDS) for 30 min. at 50℃, and washed 3× for 5min each in 1×TBS-T. Blotting for different proteins (CD4, CCR5 or CD63) will be performed in the same membrane.

COIMMUNOPRECIPITATIONS

To assess cellular associations of CD63 with CD4 or chemokine receptors, primary macrophages, PBLs, and cell lines mentioned in Experimental Design will be compared. Cells will be lysed in mild detergent (10% w/v CHAPS in 0.15M NaCl/0.01M Tris, pH 7.4) and precipitated with anti-CD4, anti-CCR5, or anti-CXCR4 antibody and protein G sepharose. Precipitates will be resolved on SDS-PAGE gels and immunoblotted for CD63.

FLOW CYTOMETRY

Anti-CD63 antibody effects on CD4 or CCR5 in macrophages and effects of transfected CD63 on CD4 or chemokine receptors in QT6 cells will be assessed by quantitative FACS analysis using a standardized microbeads kit (Sigma, St. Louis, MO). This has the advantage over traditional flow cytometry in that it can be used to measure the actual number of antibody binding sites (ABS) per cell rather than the relative

fluorescence intensities of the entire cell population. The microbeads kit consists of five uniformly-sized microbead populations that are coated with differing amounts of goat anti-mouse antibodies and therefore bind defined amounts of mouse antibodies (a population which does not bind mouse IgG is used as a control). The beads and cells will be processed identically using fluorochrome-conjugated anti-CD4, anti-CCR5, or anti-CXCR4 mouse IgG1 antibodies and then analyzed by FACS. The known binding capacities of the stained microbeads will be regressed against the corresponding MCF of the bead populations to generate a regression curve and then the MCFs of experimental samples will be compared against this curve to get the number of ABS per cell. The MCF of the isotype control for each antibody is also converted to ABS and then subtracted from the ABS value obtained with the experimental sample.

LIPID RAFTS ISOLATION AND WESTERN BLOTTING

Macrophages (1×10^7) will be scraped into 50ml tubes and washed 1× with PBS and lysed on ice for 30min with 1ml of 1% Triton X-100 or Brij97 TNE lysis buffer supplemented with protease inhibitors (Complete; Roche). The cell lysates will be homogenized with 10 strokes of a Dounce homogenizer and centrifuged for 5min at 1000×g at 4 ^{o}C to remove insoluble material and nuclei. The supernatant will be mixed with 1ml of 80% sucrose in lysis buffer, placed at the bottoms of ultracentrifuge tubes and overlaid with 5.5ml of 38% and 3ml of 5% sucrose in lysis buffer. Lysates will be ultracentrifuged at 4 ^{o}C in a SW41 rotor (Beckman) for 16 to 18h at 38,000rpm. After centrifugation, low-density material containing rafts, or TEMs, will exist on the boundary between 5 and 38% sucrose. Samples will be collected from the bottom of the tubes with 0.5ml fractions per sample and analyzed by Western blotting or stored at -80 ^{o}C. Aliquots of 30ul of individual sucrose gradient fractions will be analyzed by SDS-PAGE under non-reducing condition and transferred to nitrocellulose membranes for Western blot to CD4, CCR5, CD63, CXCR4, CD46 and LAT. CD46 serves as an out-of-raft control and LAT serves as an in-the-raft control.

DOWNREGULATION OF CD4, CCR5 AND CD63 IN U373 AND MACROPHAGES BY SIRNA

5×10^4 U373 or 4×10^5 macrophages will be seeded on 24-well plates one day before siRNA transfection. For each transfection well, dilute 2µl of a 20µM stock siRNA (CD4, CCR5, CD63 and scrambled control) and 0.5µl RNase inhibitor (Ambion) in 40µl serum free RPMI1640 (for U373) or Isocove's modified dulbecco medium (for macrophages), mix gently. Dilute 2µl Oligofectamine in 5.5µl serum free PRMI1640, or Isocove's modified dulbecco medium to give a final volume of 7.5µl. Mix gently and incubate for 5-10min at room temperature. After incubation, combine the diluted siRNA with the diluted Oligofectamine to reach a total volume of 50µl. Mix gently and incubate for 15-20min at room temperature to allow the siRNA-Oligofectamine complexes to form. While complexes are forming, remove the growth medium from the cells, and add 200µl of serum free RPMI1640, or Isocove's modified dulbecco medium to each well. Add 50µl of siRNA-Oligofectamine complexes to each well and incubate at 37 ^{o}C in a 5%CO2 incubator for 4h. After incubation, 125µl RPMI1640 containing 30% serum, or Isocove's modified dulbecco medium containing 60% serum is added to each well without removing the transfection mixture. Cells will be lysed in the wells by 1% Triton X-100 in PBS for 30min at 24-72h post-transfection. Downregulation of CD4, CCR5 or CD63 will be assessed by SDS-PAGE and Western blotting. Downregulation will also be measured by flow cytometry.

INFECTION OF U373 AND MACROPHAGES AFTER CD4, CCR5, OR CD63 DOWNREGULATION

1×10^4 U373 or 5×10^4 macrophages will be seeded on 96-well plates one day before siRNA transfection. The amount of siRNA and Oligofectamine is proportion to 24-well plates. As general, for each transfection well, dilute 0.5µl of a 20µM stock siRNA (CD4, CCR5, CD63 and scrambled control) and 0.5µl RNase inhibitor in 16µl serum free medium, mix gently. Dilute 0.5µl Oligofectamine in 2.5µl serum free medium to give a final volume of 3µl. Mix gently and incubate for 5-10min at room temperature. After incubation, combine the diluted siRNA with the diluted Oligofectamine to reach a

total volume of 20µl. Mix gently and incubate for 15-20min at room temperature to allow the siRNA-Oligofectamine complexes to form. While complexes are forming, remove the growth medium from the cells, and add 80µl of serum free medium to each well. Add the 20µl of siRNA-Oligofectamine complexes to each well and incubate at 37 ^{o}C in a 5%CO2 incubator for 4-5h. After incubation, 50µl medium containing 3× serum is added to each well without removing the transfection mixture. After about 30h (for U373), or 48h (for macrophages) downregulation of CD4, CCR5 and CD63, cell medium is removed and two different amount of SF162, 0.1ng and 1ng, will be added to each well in quadruplicate. After 2h incubation at 37 ^{o}C, 100µl fresh medium will be added. For U373, after 48h post-infection, cell medium is removed and 50µl medium with 50µl β-galactosidase assay reagent is added to each well, after 30min incubation, cell lysates will be transferred to a white plate and read in luminometer. For macrophages, p24 ELISA will be used to measure infection at 7 days post-infection.

Flow cytometry: Cells (U373 or Macrophages) will be removed from wells with 1×trypsin and washed with cold PBS containing 5% human serum for 1×. Cells will be counted and adjust to a concentration of 1×10^{6} cells/ml, and 1ml will be used for each labeling. Cells will be labeled with PE or FITC conjugated antibodies for 30min on ice and wash with cold PBS with 5% human serum for 3×. After wash, cells is resuspended in 300µl cold PBS and 300µl 4% paraformaldehyde (PFA) in PBS is added to reach a final concentration of 2% PFA. After fixing for 30min, cells are ready for flow cytometry detection.

CONFOCAL MICROSCOPY

Differentiated human macrophages or U373-MAGI-CCR5 cells are per-incubated with non-specific control antibodies and then incubated with anti-CD63 antibody (Caltag) and/or anti-CD4 (Becton Dickinson). Antibodies are labeled with Alexa 568 and 488 respectively, using the Zenon Alexa Fluor labeling kit (Molecular Probes). For time 0 min, cells are fixed with 4% paraformaldehyde and then stained with Alexa-labeled antibodies for 30 min at RT, and then fixed for additional 20 min. For all other samples,

cells are incubated with anti-CD63 antibody and anti-CD4 for the times indicated in the figure, and then washed twice with cold PBS and fixed with 4% paraformaldehyde. Fluorescence is analyzed using a Zeus LSM 510 confocal microscope.

PSEUDOTYPE VIRUS

Pseudotype viruses containing luciferase gene provide an efficient way to measure infection after CD4, CCR5 and CD63 downregulation. Co-transfection of 293T or 293FT cells with a pcDNAIII expression vector (Stratagene) containing different Env gene of retroviruses, together with the Env-deleted, NL4-3 proviral clone with luciferase in place of *nef* (Env^-Luc) by calcium phosphate. Vesicular stomatitis virus (VSV) pseudotype virus is made by co-transfection of 293T cells with a pcDNAIII expression vector for VSV-Env together with Env-deleted, luciferase NLs-3 proviral clone (Env^- Luc). Transfection in 293FT cells is performed under this condition: Cells are grown in DMEM high glucose medium (containing 10% FCS, 1% Pen/Strp, 1% L-Glu and 1% G418), split a confluent plate of cells into 5 plates (1:5) 3 days before transfection; 2h before transfection, split confluent plates 2:3, final cells will be 70-80% confluence. Transfection mixture is made in a 15ml conical tube. 15ug CsCl purified DNA is diluted in 500ul with 0.1X TE buffer, after adding 500ul of 2X BES buffer, mix throughly by vortex. 62ul of 2M $CaCl_2$ is adding dropwisely while mixing by vortex; then incubate at RT for 20min. Mix the solution again and added drop wisely to cells on plate, while swirling. On the second day, cells are rinsed with 5ml of medium containing FCS, and replaced with 7-8ml of fresh medium. Supernatant is collected 48h after transfection. In order to get high concentration of pseudotype viruses, supernatant is concentrated through a 20% sucrose cushion. 5ml 20% sucrose in PBS is added into a 30ml ultracentrifuge tube, and the culture medium is carefully layered on the top of sucrose. Tubes are subjected to 3.5h untra-centrifigation at 25000rpm in SW28 rotor. Pellet is re-suspended in cell medium (DMEM with 10% FCS).

CHAPTER 3: RESULTS

ASSOCIATION OF CD63 WITH CD4 AND CHEMOKINE RECEPTORS

Association of CD4 with CD63 by Immunoprecipitation

Since anti-CD63 antibody does not affect expression of CD4 on primary macrophages (von Lindern et al. 2003), it is more likely that CD63 is helping to facilitate HIV binding in infection through interaction between receptor and co-receptor, which must form a tripartite interaction, involving three molecules of gp120 and three molecules of CD4 and co-receptor (Nguyen et al, 2000; Liao et al, 2003; Graham et al, 2003). To assess this possibility, immunoprecipitation studies were preformed using precipitation with antibody to CD63, CD4 or controls, subsequently probed with antibody to CD4. In Fig. 4 (Lane 1), a weak association between CD4 and CD63 was observed, suggesting that CD4 expression may be dependent on CD63. There is no band in control antibody lane (Lane 2) and a strong band in anti-CD4 lane (Lane 3).

Association of CD4 and CD63 in Lipid Rafts

Besides direct interaction, CD63 may also help to facilitate HIV binding during infection through colocalization of receptor and co-receptor. To assess this possibility, fractionation studies were performed in primary macrophages. A membrane domain well defined in a variety of cells, and shown to be important for HIV replication, is lipid rafts. These are cholesterol rich regions that are preferentially used for virus budding, presumably because virus core assembles below and is able to bud through the lipid rafts where HIV envelope is also expressed (Nguyen et al., 2000; Graham et al., 2003). To localize receptor, co-receptor and CD63 in lipid rafts, cell lysates were treated with detergent (TritonX-100), layered over sucrose gradient and centrifuged to delineate fractions based on buoyancy. Different fractions after centrifugation were collected from the bottom the tub. Soluble fractions are located at the bottom and insoluble at the top. As shown in Fig. 5, CD4 can be seen both within the lipid rafts (insoluble fractions) as

well as outside the lipid rafts (soluble fractions), but CD63 and CCR5 are mostly outside the rafts.

Association of CD4 and CD63 in Tetraspanin Webs

Tetraspanin proteins will form tetraspanin enriched microdomains (TEMs), and may be important for HIV infection of macropahges as well. These domains can be recovered by treatment of macrophages with weak detergent (Brij 58). In this case, different from the separation of lipid rafts, CD63 and CCR5, as well as CD4, are found to some extent within TEMs (Fig. 6). Although both CD4 and CD63 were shown in the insoluble fraction, it is not clear whether these two molecules are close to each other in the same membrane microdomains. To assess this, cells were labeled with specific antibodies and examined under confocal microscope.

Colocalization and Internalization of CD63 and CD4 in Macrophages

Macrophages, pre-blocked by non-specific antibodies, were treated with FITC-labeled anti-CD63 antibody mAb and examined by confocal microscopy in order to determine whether mAb treatment affected the cell surface expression of CD63. In Fig. 7**,** antibody was found associated with CD63 on the surface of the cell at T=0, but at 30 minutes and 60 minutes there was rapid downregulation of CD63 with clustering of CD63 mAb in intracellular clusters. Labeled anti-CD4 also showed surface distribution of CD4 with anti-CD4 mAb, and there was no change in distribution at 30 or 60 minutes. When both antibodies were added at the same time, co-localization of CD4 and CD63 was shown, and both CD4 and CD63 were taken up together and sequestered in intracellular clusters.

Colocalization and Internalization of CD63 and CD4 in Cell Lines

The effect of treatment with anti-CD4, anti-CD63 antibody or both was also assessed in U373-MAGI cells (Fig. 8). U373 cells, obtained through the NIH AIDS Research and Reference Reagent Program (contributed by Dr. Michael Emerman and Dr. Adam Geballe), are a cell line derived from a glioblastoma that has been modified by stable transfection of LTR-β-galactosidase (U373-MAGI) (Harrington et al., 1993;

Vodicka et al., 1997). U373-MAGI cells were stably transfected with either CCR5 or CXCR4 to enable infection by HIV(Vodicka et al, 1997). Cells were propagated in DMEM and 10% fetal calf serum. Treatment with anti-CD4 alone did not result in CD4 downregulation; most signals associated with FITC-anti-CD4 were found on the cell surface. As seen with macrophages, labeled anti-CD63 antibody resulted in rapid downregulation and sequestration intracellularly. When labeled anti-CD63 antibody and anti-CD4 were applied to U373-MAGI cells at the same time, they colocalized; some of the CD4 signal was found inside the cell, but some still remained on the surface. This may reflect the greater abundance of CD4 expressed on the surface of U373-MAGI, as compared with primary macrophages, or a different pattern of association between CD4 and CD63.

Post-entry anti-CD63 Antibody Treatment in Macrophages

In order to assess whether CD63 may be involved in later, post-entry events of HIV replication in macrophages, a time course of mAb treatment was also performed (Fig. 9). HIV replication was evaluated following anti-CD63 antibody mAb treatment prior to and during infection, or mAb treatment 12 hours or 3 days post infection. Maximal inhibition was seen with anti-CD63 antibody pretreatment, followed by infection for 7 days, and then assessed for HIV p24 production. Inhibition was shown with mAb pretreatment continued until 12 hours post infection, and also when antibody treatment was not initiated until 12 hours post infection. Less inhibition was seen if anti-CD63 antibody treatment began 3 days post infection (Fig. 9A). Like anti-CD63 antibody treatment, maximal inhibition with anti-CD4 was seen with treatment that began before infection and continued up to the time of extracellular p24 production at day 7 (Fig. 9B). Inhibition was also greater if anti-CD4 treatment began before infection and continued until 12 hours after infection, as compared with anti-CD4 treatment delayed until 12 hours after infection and removed after 3 days. This was expected since the absence of early anti-CD4 treatment allowed virus entry, and withdrawal of anti-CD4 after three days allowed reinfection and spread after that time. Partial inhibition was seen when anti-

CD4 treatment was initiated 12 hours after infection and continued up to the time of harvest, since reinfection was effectively inhibited.

Post-entry anti-CD63 Antibody Treatment in Cell Lines

Effect of anti-CD63 antibody treatment at various times during infection in U373-MAGI-CCR5 cells was also assessed (Fig. 10). Anti-CD4 treatment before and especially during the time of infection (0-12h) inhibited HIV replication, as compared with untreated controls. Anti-CD4 initiated 12h after infection at the time of media change had much less effect on virus replication, as predicted, since anti-CD4 mAb acts on virus binding and entry, and treatment after infection would have less effect in cells in which infection has been established. Anti-CD63 antibody treatment before, during, or after infection of U373-MAGI-CCR5 cells had no effect on HIV replication, as measured by β-galactosidase production 3 days post-infection (Fig. 10A), or by extracellular HIV p24 production at 7 days post-infection (Fig. 10B). Anti-CD4 treatment prior to or during infection of U373-MAGI-CCR5 cells effectively inhibited virus production, with a pattern similar to that seen in macrophages, but was less effective if added 12 hours after infection.

DOWNREGULATION OF CD63 IN MACROPHAGES AND CELL LINES

Previous work shown anti-CD63 antibody can block HIV infection in macrophages, but not PBL or cell lines (von Lindern et al., 2003). In order to study the role of CD63, RNA inference (RNAi) with small interfering RNAs (siRNA) was used to transiently downregulate CD63 in cell lines and macrophages.

RNA Interference (RNAi)

RNAi is a technique that inhibits gene expression at the stage of translation or by facilitating degredation of specific mRNAs. It is an RNA-dependent gene silencing process that is controlled by the RNA-induced silencing complex (RISC) and is initiated by short double-stranded RNA molecules in cell's cytoplasm (Fig. 11). Small interfering RNA strands (siRNA) are key to the RNAi process, and have complementary nucleotide sequences to the targeted RNA strand. After been transfected into target cell cytoplasm

by oligofectamine (Invitrogen), specific siRNAs are incorporated into RISC and pairs with complementary message RNA sequences. This binding will induce the degradation of specific mRNAs by argonaute, the catalytic component of the RISC complex, and cause post-transcriptional gene silencing.

In this project, CD63 specific siRNAs were designed and made by Dharmacon, Inc and used to knock out CD63 in cell lines and macrophages. CD4, CCR5, CXCR4 and a non-specific scrambled siRNA were also purchased from Dharmacon for experiments.

Downregulation of CD4 and CD63 in Cell Lines

Downregulation of CD63, CD4 or CCR5 by RNAi was assessed in the U373-MAGI-CCR5 cell line. Both CD4 and CD63 protein levels were markedly reduced 48h following transfection of CD4 and CD63-specific siRNA, as shown by Western blot (Fig. 12). In 24h, notable reduction can be observed, and in 48h, CD4 and CD63 are reduced to undetectable. In each experiment, cells were also transfected with scramble control siRNA. To ensure the same amount of proteins were loaded in each lane, actin level was also measured on the same membrane after blotting for CD4 or CD63.

Measurement of Downregulation by Flow Cytometry in Cell Lines

To assess surface expression of CD4, CD63 and CCR5, effects of siRNA treatment were also determined by flow cytometry. 24h after siRNA transfection, cells were removed from plates and labeled with specific antibodies on ice then fix by 4% PFA. CD4-specific siRNA resulted in modest reduction of CD4 expression (Fig. 13A), but specific expression was markedly reduced by both CCR5- and CD63-specific siRNA treatment (Fig. 13B and 13C). There is a non-specific effect for different siRNA, which will decrease the protein level, as show in control siRNA transfected cells. Notably, CD63 siRNA treatment did not affect expression of either CD4 or CCR5 (Fig. 13D), (von Lindern et al., 2003).

Infection of Cell Lines after Downregulation of CD63 and other Genes

U373-MAGI-CCR5/CXCR4 cells are stable transfected with LTR-β-galactosidase, and can be used to measure infection. Replication of HIV-SF162 (R5) or

HIV 89.6 (R5X4), as determined by β-gal activity three days after infection, was reduced following CD63 or CCR5 downregulation in U373-MAGI-CCR5 cells, although CD4 downregulation resulted in nearly complete suppression of virus replication (Fig. 14A). Using a related indicator cell line, U373-MAGI-CXCR4, this time expressing the chemokine coreceptor CXCR4 and not CCR5, CD4, CD63 or CXCR4 downregulation significantly reduced virus replication, as compared with controls transfected with a control siRNA, or transfection negative controls (Fig. 14B). A similar pattern of inhibition was seen with the X4 strain HIV-92HT599 (measuring extracellular p24 level in day 3 after infection) or the R5/X4 strain HIV-89.6 in U373-MAGI-CXCR4 cells, measuring by p24 ELISA. As expected, CCR5-specific siRNA treatment did not affect virus replication in these cells, since U373-MAGI-CXCR4 cells do not express CCR5 (Fig. 14C and 14D).

Downregulation of CD4 and CD63 in Macrophages

To confirm the importance of CD63 in primary macrophages, transfection condition of siRNA in these cells was optimized. Using oligofectamine, siRNA transfection efficiency in macrophages was approximately 50% (data not shown). Day 7 differentiated macrophages were transfected with CD63 and control siRNA and collected at 24 or 48h. As shown by Western blot, there was little effect on levels of CD63 after 24h, but reduction was significant at 48h (Fig. 15).

Infection of Macrophages after Downregulation

To assess the effect of CD63 downregulation on HIV replication, macrophages were transfected with CD4, CD63 CCR5, CXCR4 specific siRNAs or scrambled control siRNA after 5 days of adherence. Cells were infected 48h later with the R5 strain HIV-SF162 and virus production was assessed in culture supernatant by p24 ELISA after 7 days. Virus production was significantly reduced following transfection with siRNA specific for CD4, CCR5 or CD63 ($p<0.05$, as compared to control siRNA transfected cells), but there was no effect on virus production following CXCR4-specific siRNA transfection or that of scrambled control siRNA (Fig. 16).

In order to assess whether CD63 may play a role in later events in HIV replication, CD63-specific siRNA, or siRNA directed against HIV receptors or a control siRNA were introduced into macrophages three days after HIV infection (Fig. 17). In contrast to results seen with siRNA-induced CD4 downregulation prior to infection, treatment with CD4-specific siRNA 3d after infection did not significantly affect virus production, similar to results seen with CXCR4-specific siRNA treatment and controls. However, CD63-specific siRNA treatment 3 days after infection significantly ($p<0.05$, as compared to control siRNA transfected cells) reduced virus production at one week, suggesting an important role for CD63 in macrophages during HIV replication events occurring after proviral integration, which occurs 48 hours post infection (O'Brien et al., 1994).

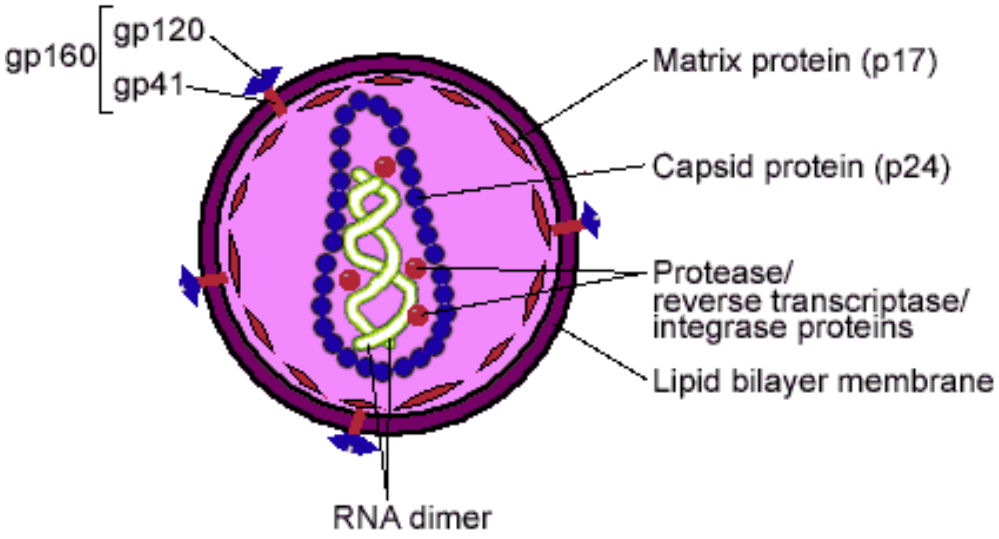


Figure 1. Structure of HIV virion**.** HIV virion contains two main parts, the inner core and viral membranes. Inner core is made by p24, containing two identical HIV genome. HIV gp160 exists on the viral membranes. (Adapted from Fundamental Virology, 4th Edition)

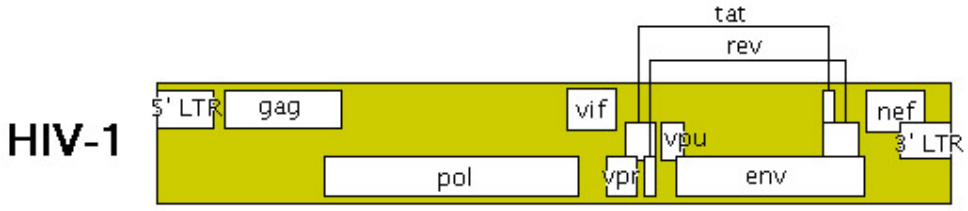


Figure 2. Structure of HIV-1 genome. Gag, pol and env are the three main genes in HIV genome. There are also other regulatory genes. (Adapted from Fundamental Virology, 4th Edition)

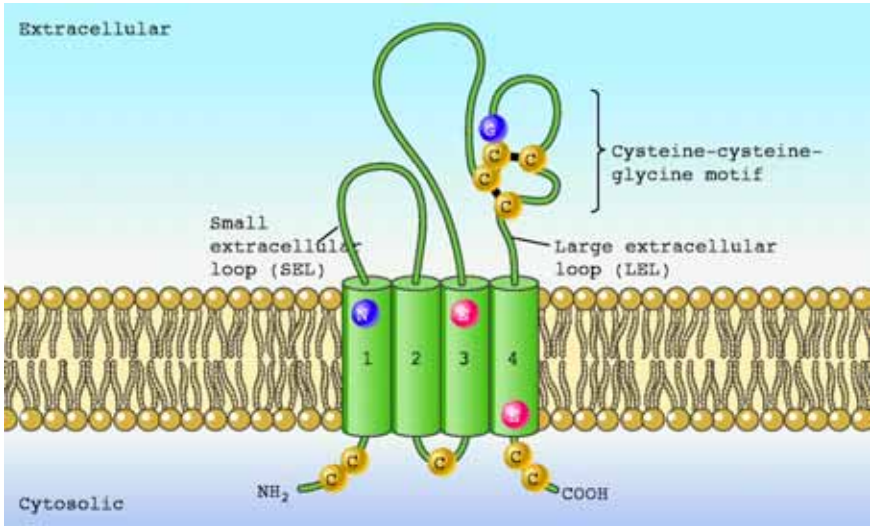


Figure 3. Structural features of tetraspanins. Tetraspanins are composed of 4 transmembrane domains (TM, green cylinders) containing conserved residues. The number of disulfide bridges varies from 2-4 among family members (Maecker et al., 1997; Hemler et al., 2001; Seigneuret et al., 2001) .

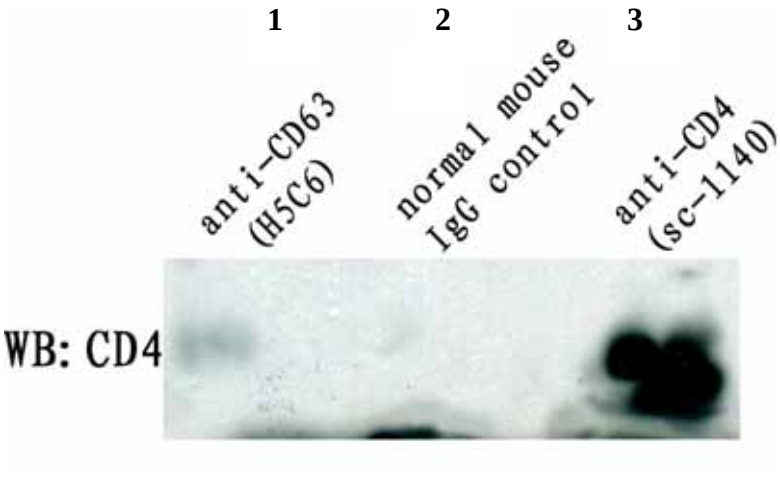


Figure 4. Co-immunoprecipitation of CD4 and CD63 in Macrophages. Differentiated macropahges were scraped and lysed in 1% Brij58 for 30 min on ice. Insoluble materials were spun down at 1500 rpm for 5 min. Lysate was separated into different tubes. Immunoprecipitation was performed by adding different antibodies to the lysate with incubation on rotator for 5h at 4°C after reducing the non-specific binding by adding protein G bead for overnight. Beads were spun down at 2000 rpm for 5 min. and washed with PBS 4 times. Precipitated proteins were detected by SDS-PAGE/ Western blot.

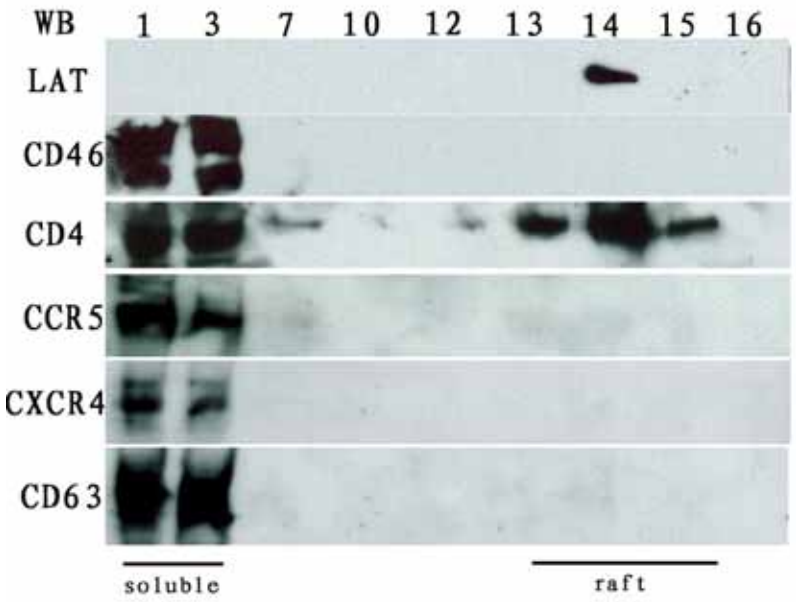


Figure 5. Separation of lipid rafts in Macrophages. Sucrose-gradient ultra- centrifugation separation of lipid rafts in primary macrophages was achieved by lysing in TNE buffer containing 1% Triton X-100 for 30 min on ice. After pelleting large cell debris, lysates were brought to 40% sucrose in TNE, overlaid with 5.5ml 35% sucrose TNE, and 3.5ml 5% sucrose in TNE to create a discontinuous sucrose gradient, and subjected to equilibrium centrifugation. 22 0.5ml fractions are collected from the bottom and subjected to immuno-blotting after SDS-PAGE. Fractions 1-4 correspond to soluble fractions, confirmed by the location of non-raft marker CD46. Lipid raft fractions (13-15) are determined by the location of raft marker LAT.

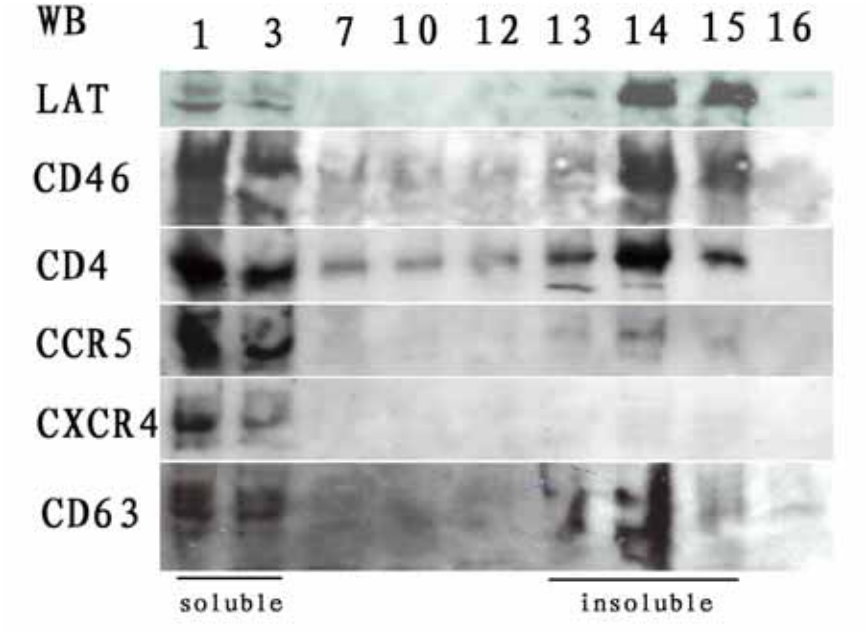


Figure 6. Separation of TEMs in Macrophages. Sucrose-gradient ultracentrifugation separation of tetraspan webs in primary macropahges was achieved by lysis in TNE buffer containing 1% Brij58 for 30 min. on ice. After pelleting large cell debris, lysates were brought to 40% sucrose in TNE overlaid with 5.5ml 35% sucrose TNE and 3.5ml 5% sucrose in TNE to create a discontinuous sucrose gradient, and subjected to equilibrium centrifugation. The same membrane proteins as in lipid raft separation are tested by Western blot.

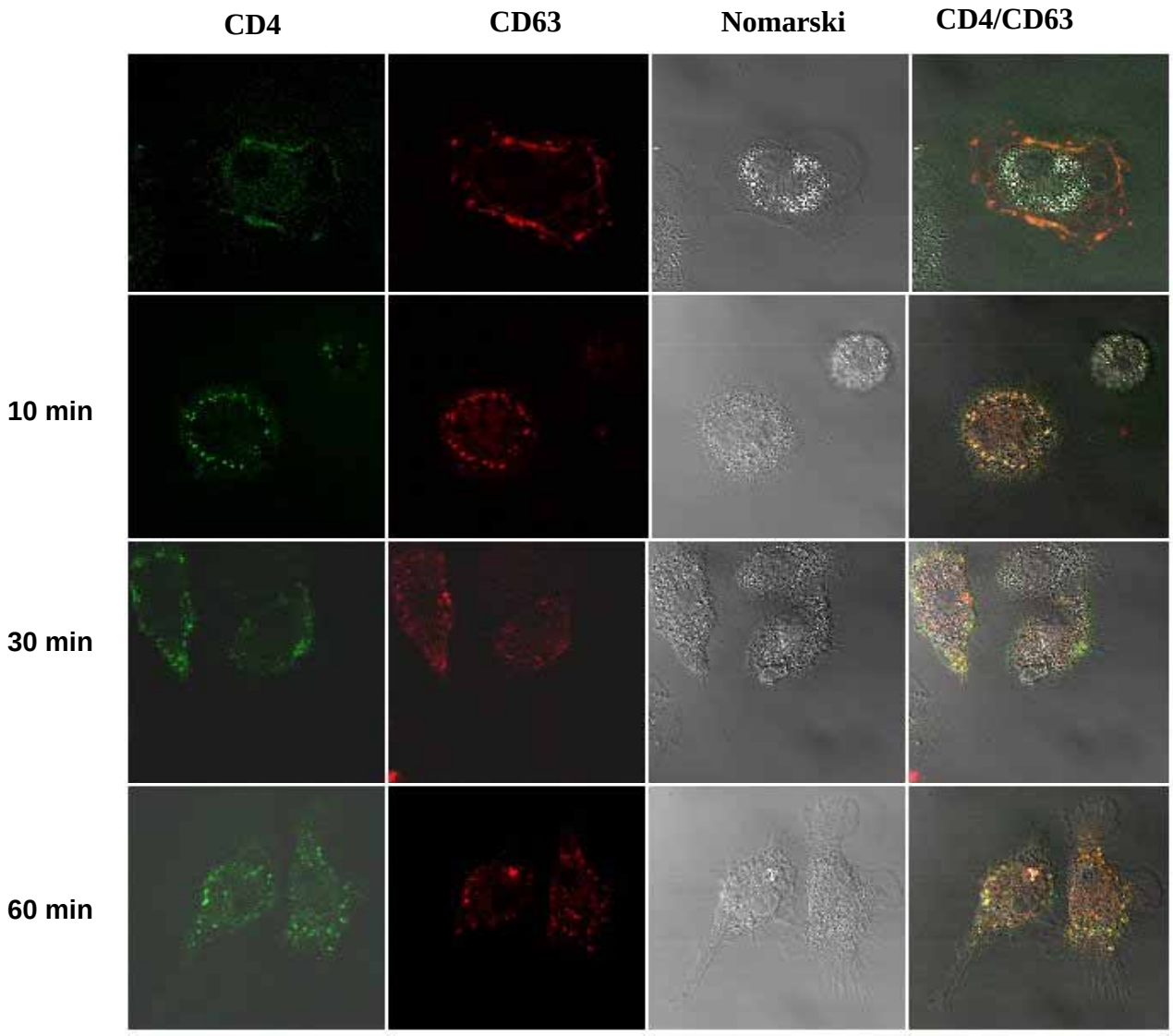


Figure 7. Colocalization and internalization of CD63 and CD4 in Macrophages. After blocking with non-specific antibodies, differentiated human macrophages were incubated with anti-CD63 antibody (Caltag, labeled by Alexa 568) and/or anti-CD4 (Becton-Dickinson, labeled by Alexa 488). For time 0 min, cells were fixed with 4% paraformaldehyde and then stained with Alexa-labeled antibodies for 30 min at RT, and then fixed for an additional 20 min. For all other samples, cells were incubated with antibody for the indicated times at 37^{o}C, and then washed twice in cold PBS and fixed with 4% paraformaldehyde.

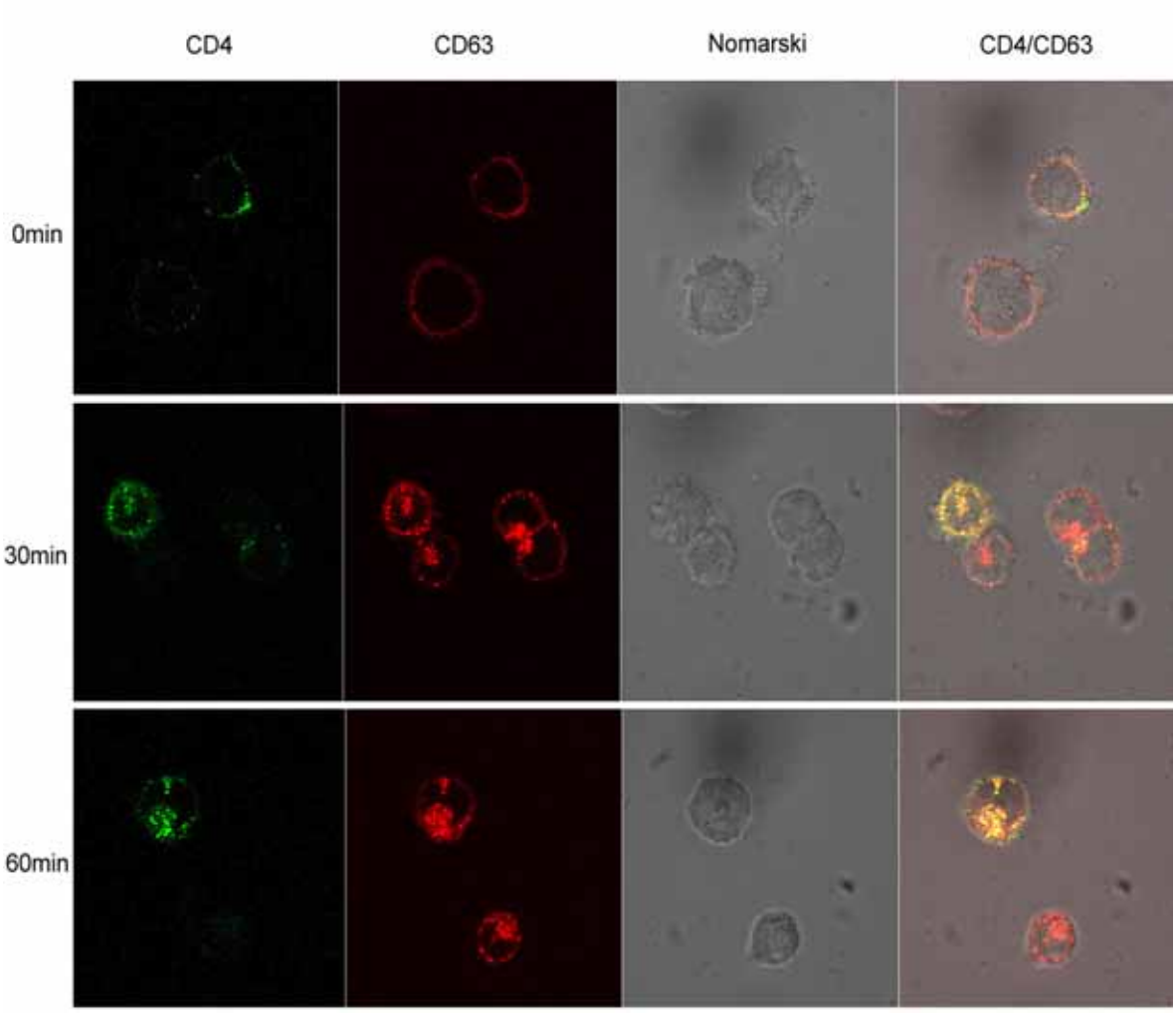
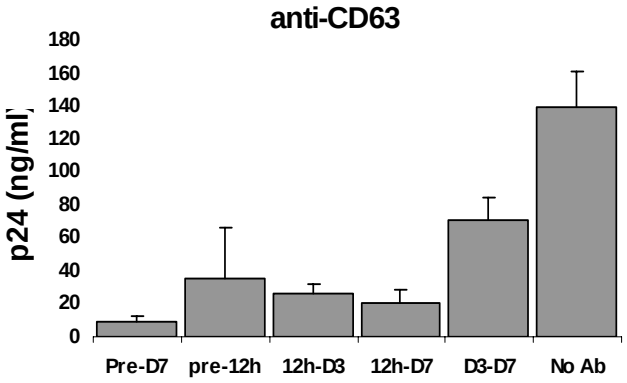
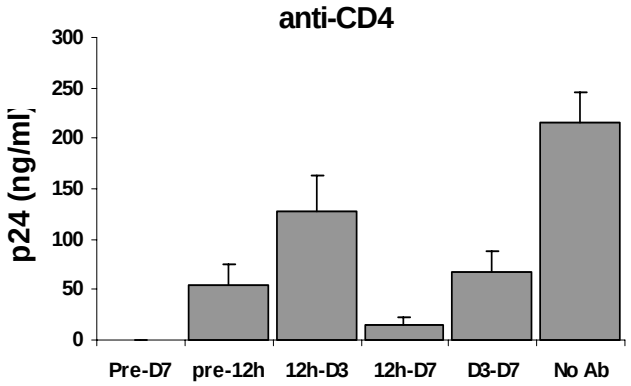


Figure 8. Colocalization and internalization of CD63 and CD4 in U373-MAGI-CCR5 cells. After blocking with non-specific antibodies, U373-MAGI-CCR5 cells were incubated with anti-CD63 antibody (Caltag, labeled by Alexa 568) and/or anti-CD4 (Becton-Dickinson, labeled by Alexa 488). For time 0 min, cells were fixed with 4% paraformaldehyde and then stained with Alexa-labeled antibodies for 30 min at RT, and then fixed for an additional 20 min. For all other samples, cells were incubated with antibody for the indicated times at 37^{o}C, and then washed twice in cold PBS and fixed with 4% paraformaldehyde.

A.


anti-CD63
p24 (ng/ml)
180
160
140
120
100
80
60
40
20
0
Pre-D7
pre-12h
12h-D3
12h-D7
D3-D7
No Ab

B.


anti-CD4
p24 (ng/ml)
300
250
200
150
100
50
0
Pre-D7
pre-12h
12h-D3
12h-D7
D3-D7
No Ab

C.

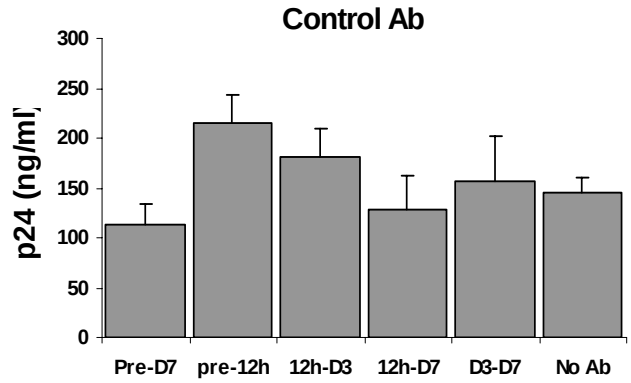
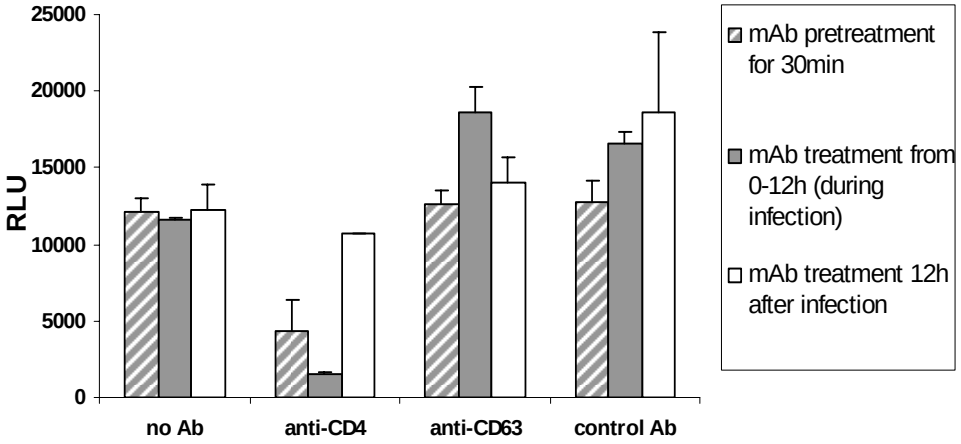

Control Ab
p24 (ng/ml)
300
250
200
150
100
50
0
Pre-D7
pre-12h
12h-D3
12h-D7
D3-D7
No Ab

Figure 9. Time course of antibody inhibition in macrophages. Antibody treatment was applied at various times beginning one hour prior to infection (Pre) and continued up to 7 days following infection (D7). Antibody treatment conditions of macrophages are shown with the time of initial application and the duration of treatment shown below each lane for (A) anti-CD63 antibody, (B) anti-CD4 or (C) isotype control antibody. Assessment of viral production after infection with HIV-SF162 (R5) was assessed by p24 (ELISA) at day 7.

A.


25000
20000
15000
10000
5000
0
RLU
no Ab
anti-CD4
anti-CD63
control Ab
mAb pretreatment for 30min
mAb treatment from 0-12h (during infection)
mAb treatment 12h after infection

B.

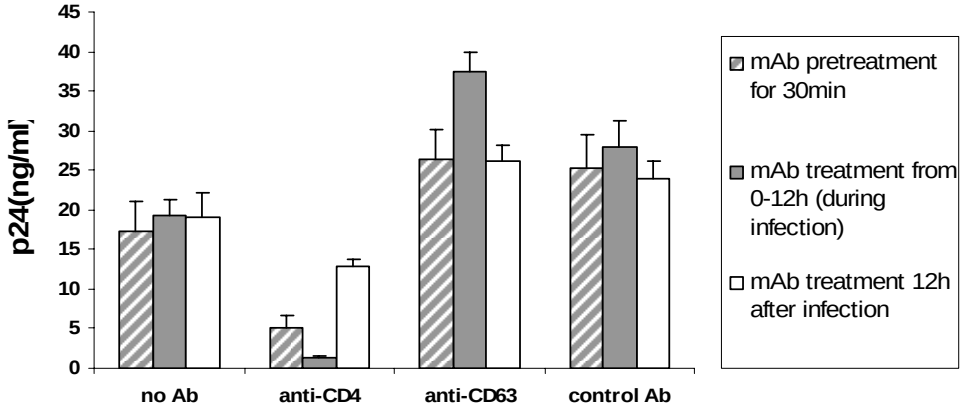

45
40
35
30
25
20
15
10
5
0
p24(ng/ml)
no Ab
anti-CD4
anti-CD63
control Ab
mAb pretreatment for 30min
mAb treatment from 0-12h (during infection)
mAb treatment 12h after infection

Figure 10. Time course of antibody inhibition in U373-MAGI-CCR5 cells. Anti-CD63 antibody pretreatment, or treatment during (0–12h) or starting 12h after infection did not affect HIV replication, as measured by β-gal activity at day 3 (A, RLU) or by extracellular virus production (B, p24 ng/ml).

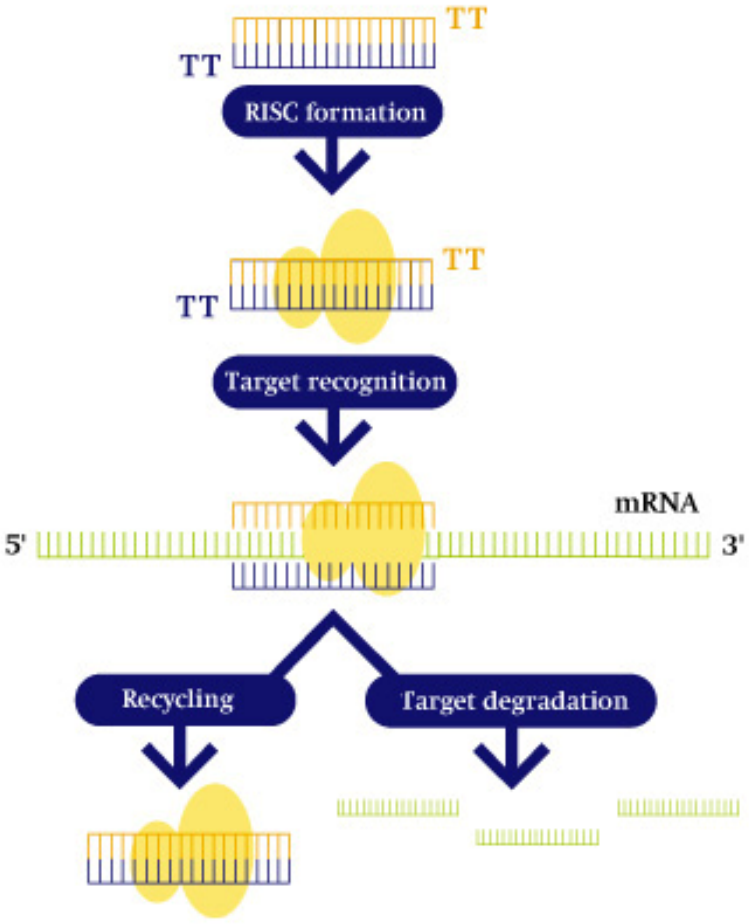


Figure 11. Downregulation of specific cellular protein by RNAi. Specific siRNA was transfected into cells and recognized by specific cellular proteins and form RISC. This complex pairs with complementary message RNA sequences and degrades mRNAs (Adapted from: http://www.biolegio.com/products/sirna/)

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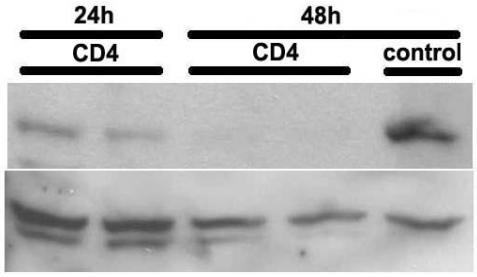
A.



B.

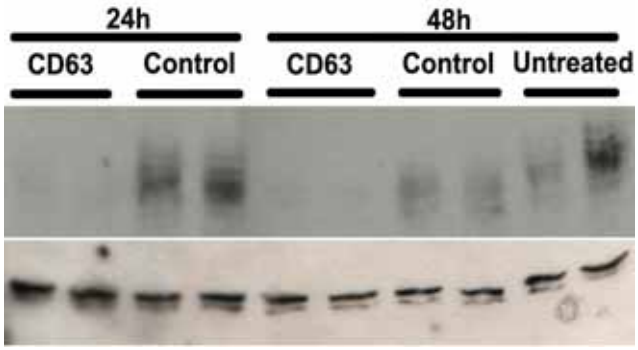


Figure 12. Downregulation of CD4 and CD63 in U373-MAGI-CCR5 by siRNA. U373-MAGI-CCR5 cells were transfected 24h or 48h with (A) CD4-specific siRNA or (B) CD63-specific siRNA. Scrambled control siRNA was also included. Cells were lysed in 1% TritonX-100 in PBS and resolved by SDS-PAGE, followed by Western blotting for CD4 (A) and CD63 (B). Actin expression was assessed as a control for cellular protein.

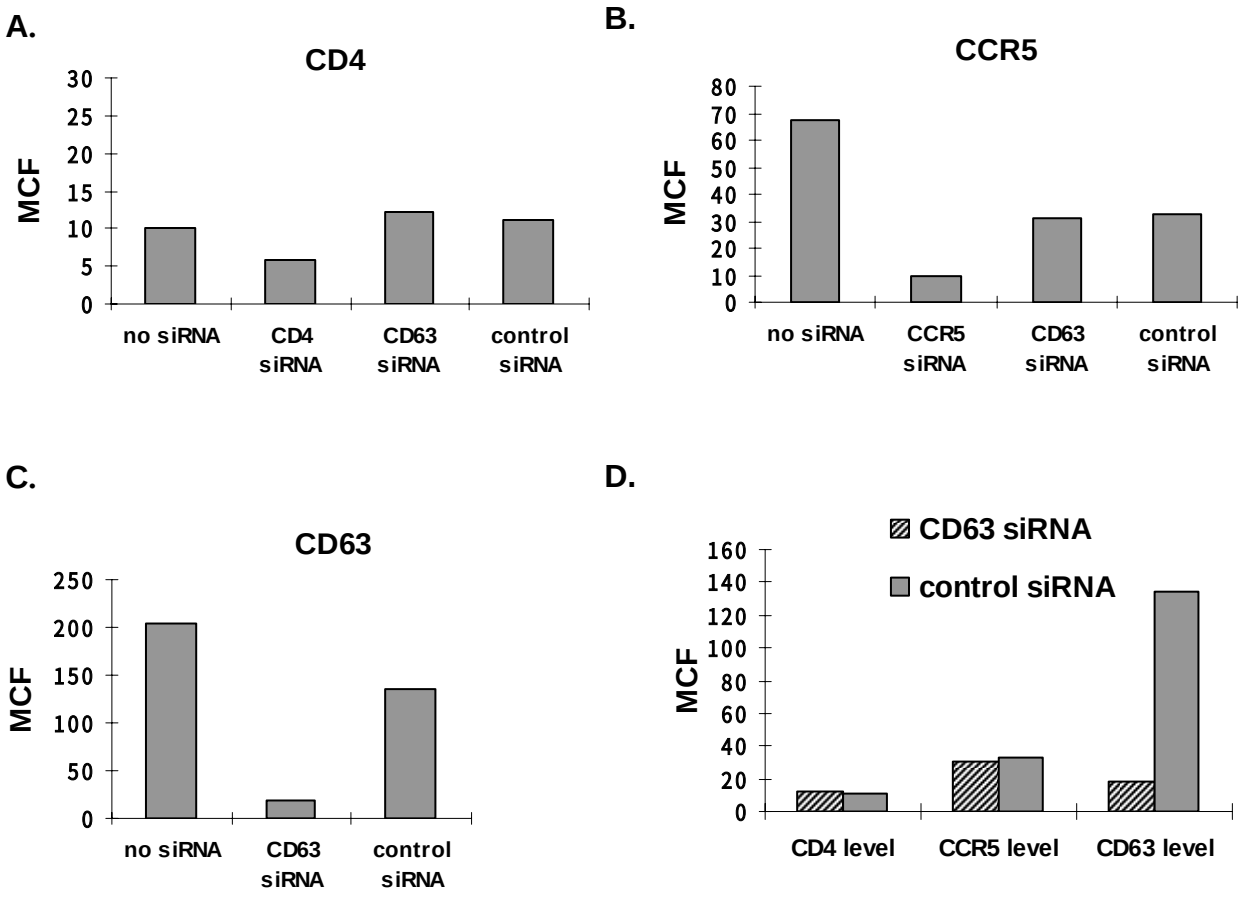


Figure 13. CD4, CCR5 and CD63 levels on U373-MAGI-CCR5 24h after siRNA transfection. U373-MAGI-CCR5 cells (5×10^4) were seeded in 24-well plates and transfected with CD4, CCR5, CD63 or scrambled control siRNAs. At 24h post-transfection, cells were detached using 1x trypsin and stained with (A) anti-CD4-PE, (B) anti-CCR5-PE, or (C) anti-CD63 antibody-PE and measured by flow cytometry. Levels of CD4, CCR5 and CD63 in control or CD63 siRNA transfectants are compared in panel (D)

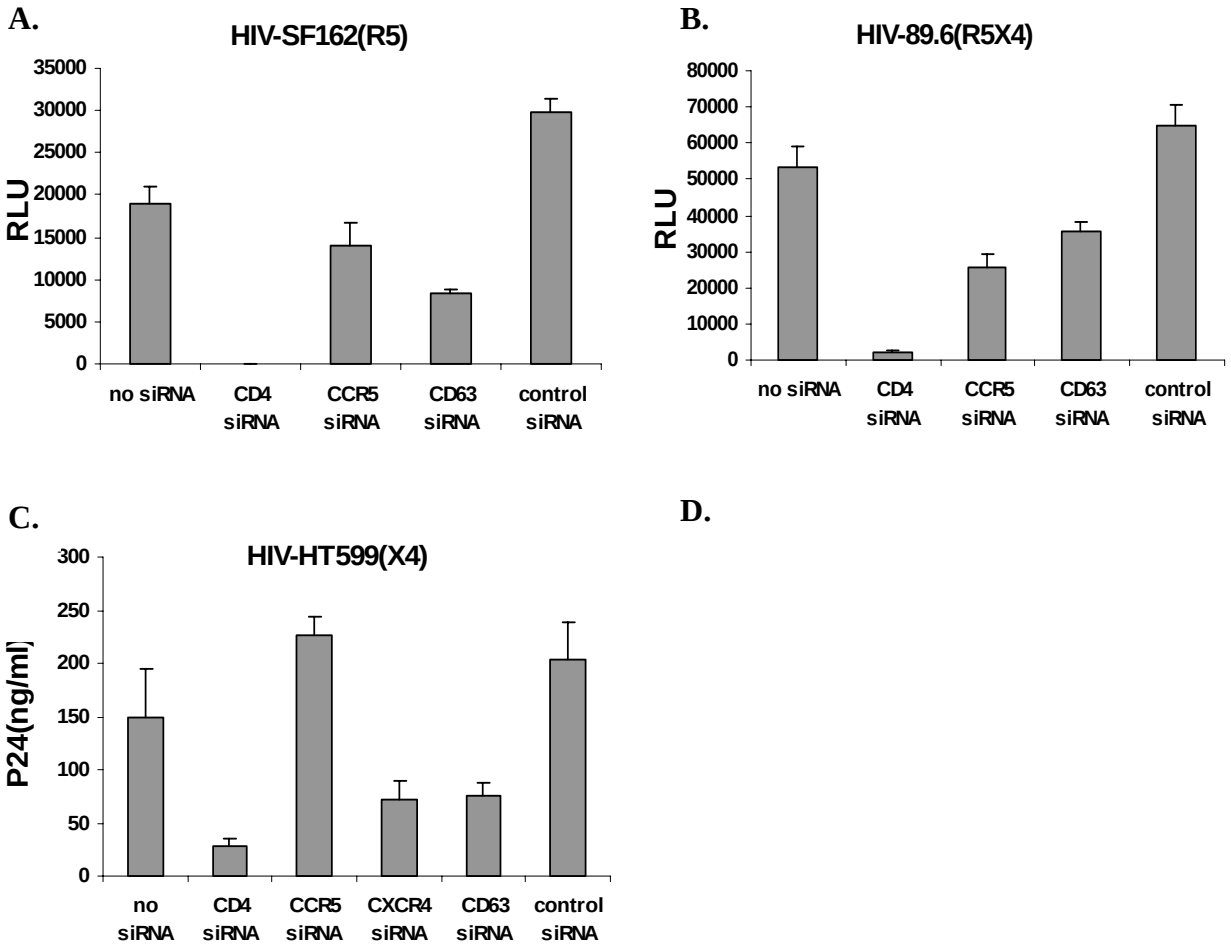


Figure 14. Infection of U373-MAGI-CCR5 or U373-MAGI-CXCR4 30h post-transfection of siRNA. U373-MAGI-CCR5 cells (10^4) were seeded in 96-well plates the day before transfection. Cells were transfected with CD4 siRNA, CCR5 siRNA, CD63 siRNA or scrambled control siRNA in quadruplicate. Transfection reagent was included in the no siRNA transfection control. After about 30h, cells were infected with an m.o.i. of approximately 0.02 with HIV-SF162 (R5) (A), or with HIV-89.6 (R5X4) (B). U373-MAGI-CXCR4 cells treated the same way were infected with HIV-92HT599 (X4) (C) or HIV-89.6 (R5X4) (D). p24 was measure by ELISA and β-galactosidase activity was measured by Beta-Glo™ Assay System (Promega) 48h post-infection, with subtraction of signal from uninfected U373-MAGI cells.

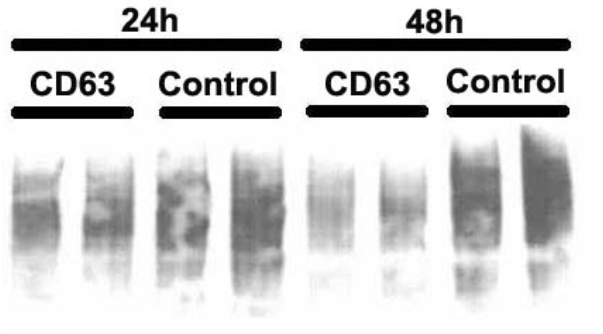


Figure 15. Downregulation of CD63 in macrophages by RNAi. Following adherence for 6 days, macrophages ($4x10^5$ per well in 24-well plates) were transfected with 100 nM siRNA using oligofectamine. Cells were removed from plastic dishes at 24h or 48h post transfection and duplicate samples were subjected to SDS-PAGE with anti-CD63 antibody.

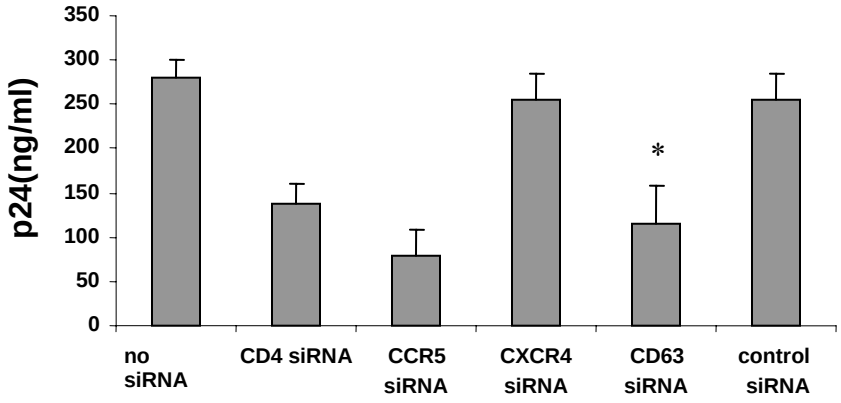


Figure 16. Inhibition of HIV-SF162 (R5) infection after CD63 downregulation in macrophages. Macrophages were purified by adherence to plastic, and were treated with siRNA on day 5. Cells ($4x10^5$) were infected with HIV-SF162 (R5) on day 7 using an MOI of 0.02 and extracellular virus was measured in the cultured supernatant 7d post-infection by p24 ELISA. (*, $p<0.05$ as compared to no siRNA or control siRNA transfected cells)

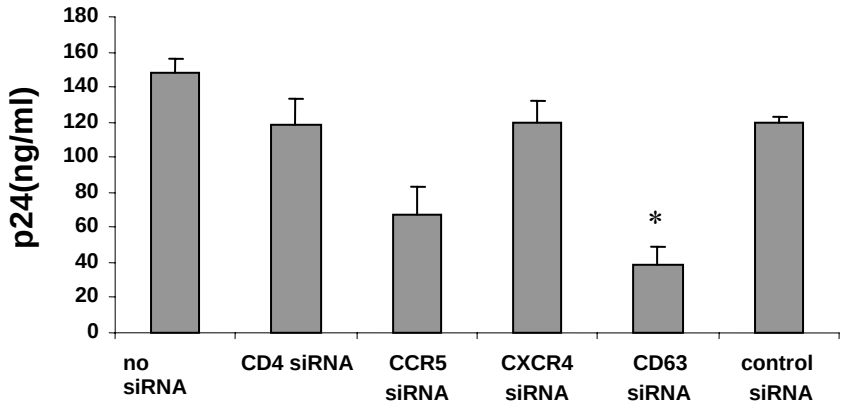


Figure 17. Inhibition of HIV-SF162 (R5) production after CD63 downregulation in macrophages. Macrophages were purified by adherence to plastic. $4x10^5$ cells were infected with HIV-SF162 (R5) on day 7 using an MOI of 0.02. On day 10, cells were transfected with siRNA. Extracellular virus production was measured in the cultured supernatant 7d post-infection by p24 ELISA. (*, $p<0.05$ as compared to no siRNA or control siRNA transfected cells)

CHAPTER 4: DISCUSSION

ASSOCIATION OF CD63 WITH CD4 IN TETRASPAN

CD63, a tetraspanin membrane glycoprotein found in nearly all human cells on the plasma membrane and especially abundant in endosomes, was previously shown to be involved in early events of HIV replication in macrophages (von Lindern et al., 2003). In this study, an expanded role of CD63 in HIV replication was presented. The function of CD63 is not only limited to early events in primary macrophages, but also likely involving later HIV replication events in cell lines as well as in macrophages.

CD4 is the primary receptor for HIV-1, and previous work shows CD63 may also be involved in the infection process. In order to look for the relation between CD63 and CD4, a co-immunoprecipitation experiment was performed (Fig. 4). There is no strong association between CD4 and CD63, only a weak interaction can be observed. CD63 was reported to directly interact with beta-1 integrin, no other natural ligand has been reported. Thus, it is not possible CD4 is a direct ligand for CD63. Another possibility is the colocalization of CD4 and CD63 in specific membrane microdomains, TEMs. Formation of TEMs is a property of tetraspanin proteins and the role of these microdomains is still not clearly defined. They contain not only tetraspanin proteins, but also many cellular receptors and seem to have multiple functions in signal transduction and protein traffic. Separation of TEMs was based on the property of insoluble of these domains in weak detergent (Brij97, Brij58) but soluble in strong detergent (TX100). In figure 7 and 8, both lipid rafts, which resistant to TX100, and TEMs were separated in a sucrose gradient ultracentrifugation. The presence of CD4 in lipid rafts has been reported in multiple papers, and this localization does not seem to be important for HIV infection. For example, a mutant CD4 without lipid raft localization signal still showed infection when incubate with HIV (Popik et. al., 2004); this indicates lipid rafts may not be important in HIV infection. When TEMs were separated using weak detergent, both CD63 and CD4 were seen in the insoluble fractions. Interestingly, some CCR5 molecules were also present in these fractions (Fig. 6). However, it is not clear whether CD4 and

CD63 co-existed in the same TEM microdomain or in different regions. Confocal microscopy was used to examine the colocalization. In Fig. 7, when cells were labeled on ice, only weak colocalization can be observed between CD63 and CD4 on macrophage surface. If after labeling on ice, cells were incubate at 37^{o}C for 30min or 60min, strong colocalization was observed between CD63 and CD4 and both of these two molecules were internalized into intracellular compartments. If the cells were treated with only anti-CD4, there was no internalization following incubation. Interestingly, the same effect happened in cell line U373-MAGI-CCR5/CXCR4 (Fig. 8). All cells were blocked with non-specific control antibodies previous to specific antibody labeling, which will exclude the affect of FC receptor on cell surface. These experiments indicate CD63 has some relation to CD4 and this may affect CD4 function during HIV infection. Although both macrophage and cell line (U373-MAGI) showed similar patterns when incubated with anti-CD63 antibody, HIV infection was blocked in macrophages but not in cell lines (Fig. 9 and 10). There are several possibilities to explain this difference.

DIFFERENCE OF HIV INFECTION IN MACROPHAGES AND CELL LINES

There are several potential reasons that might explain why HIV infection of macrophages was inhibited by anti-CD63 antibody treatment, whereas entry into primary T-cells or cell lines was not. Although it was appealing to consider that anti-CD63 antibody may downregulate both CD63 and CD4 in macrophages but not U373-MAGI cells, this was not shown to be the case by confocal microscopy. In both macrophages and U373-MAGI cells, anti-CD63 antibody caused rapid and extensive downregulation of CD63, and showed colocalization and downregulation of CD4 when anti-CD63 antibody and anti-CD4 mAb treatments were applied at the same time.

There are important cell-specific differences between the entry process in macrophages and cell lines or T cells that could explain the lack of anti-CD63 antibody inhibition of HIV replication in U373-MAGI cells or PBL. For example, a previous study showed that macrophages take up viral particles bound to the cell surface through macropinocytosis (Marechal et al., 2001). Shortly after exposure of macrophages to HIV

(independent of viral envelope-receptor interactions), viral particles were visualized in intracellular vesicles. While most X4 and all Env-deleted virions were subsequently degraded, virions with R5-tropic envelopes achieve virus fusion, leading to capsular release into the cytoplasm and productive infection (Marechal et al., 2001). Although early studies indicated that HIV infection is pH-independent and does not require endocytosis of the CD4 receptor (Stein et al., 1987; Gauduin et al., 1988; McClure et al., 1988), HIV can enter through clathrin-coated vesicles which fuse with endosomal membranes (Bourinbaiar et al., 1991; Grewe et al., 1990; Pauza et al., 1988). CD4+ HeLa cell line endocytosis was shown to contribute to HIV entry using *trans* dominant-negative mutants of dynamin and Eps15, which are required for endocytosis. Inhibition was shown by analysis of reverse transcription products by real-time PCR and by entry by delivery of virion-associated Vpr-β-galactosidase fusion protein, (Daecke et al., 2005), whereas dynamin is essential for both clathrin- and caveolar-dependent transport. The *trans* dominant-negative mutants decreased HIV entry up to 95%, confirming a role for endocytosis in productive infection. Expression of dominant-negative variants Eps15 is specific for inhibition of clathrin-dependent endocytosis. It is possible that this mode of entry may be more important in phagocytic cells such as macrophages. CD63, which is present, but less abundant in lysosomal membranes, may be involved in preventing degradation of the virus through lysosomal pathways, by routing the virus core through late endosomes, which can give rise to productive infection.

Another important difference between macrophages or trophoblasts and T-cells or cell lines is the density of CD4 expression. As calculated by quantitative flow cytometry (QFACS), CD4 binding sites were estimated to be approximately 200/cell on monocytes / macrophages, as compared with 5000/cell on primary T cells and T cell lines (Lee et al., 1999). The relatively low density of CD4 may limit infectibility of primary macrophages, as well as effects of anti-CD63 antibody. Since anti-CD63 antibody can cause downregulation of both CD63 and CD4, it is possible that there may be more complete depletion of cell surface CD4 expression in macrophages with a critical level of CD4 maintained on the surface of U373-MAGI cells and PBL, rendering these cells resistant

to inhibition of HIV infection by anti-CD63 antibody treatment. It is therefore possible that a requirement for CD63 may exist, either structurally or through direct viral interaction involving CD63, which is necessary for HIV infection of cells with relatively low CD4 expression.

A FUNDAMENTAL ROLE OF CD63 IN HIV INFECTION

The role of CD63 was further studied when using RNAi technique to knockout the CD63 in cell line and primary macrophages post-transcriptionally. Specific designed siRNA to CD63, as well as CD4, CCR5, CXCR4, and scramble control siRNA were transfected into cell line U373-MAGI and infected by R5, X4 and R5X4 viruses after 24h (in U373) or 48h (in macrophages). The infection data shown that, although PBL and cell lines were resistant to inhibition of HIV infection by anti-CD63 antibody, HIV replication in cell lines was inhibited following downregulation of CD63 (Fig. 14).

Inhibition of infection of U373-MAGI cells by CD63 siRNA treatment (Fig. 14) suggests a more fundamental role for CD63 in HIV replication in addition to the previously described role at the level of virus entry into macrophages. In the cell line U373-MAGI-CCR5 or U373-MAGI-CXCR4, cells are stably transfected with an LTR-β-galactosidase construct that allows quantitative assessment of HIV replication based on Tat production. Tat is an early protein, expressed prior to expression of structural genes, and therefore, decreased β-galactosidase production in siRNA- treated cells indicates a block to HIV replication in a step prior to initial HIV translation events. Using a similar detection system, in this case using cells stably transfected with LTR-*luciferase*, downregulation of Rab9, known to be necessary for transport of cargo proteins from the late endosome to the *trans*-Golgi, no effects are seen since this intracellular protein trafficking occurs after expression of Tat (Murray et al., 2005). Therefore, effects of CD63 appear to occur during very early viral translation, prior to production of the structural genes. The same results were acquired when using pseudotype virus packing with different HIV envelops.

The role of CD63 in post-entry HIV replication events is reinforced by data in primary macrophages, in which extracellular virus production is inhibited in cells in which RNAi via CD63-specific siRNA transfection 3 days after infection still inhibits HIV replication (Fig. 17). In macrophages, HIV genome will insert into cell genome within 48h (O'Brien et al., 1994). There are important differences in late replication events in macrophages, as compared with other HIV target cells. Although virus production in T cells and cultured cell lines is thought to occur as result of budding through the plasma cell membrane in T cells (Garrus et al., 2001), virus assembly in macrophages appears to occur in a subset of endocytic organelles that carry markers found in late endosomes of MVBs, most notably CD63 (Pelchen-Matthews et al., 2003). Electron microscopy and immunoprecipitation experiments suggest that virus release from macrophages involves initial budding into endosomal organelles, which are then released by fusion of these organelles with the plasma membrane (Raposo et al., 2002; Pelchen-Matthews et al., 2003; Kramer et al., 2005). Nonetheless, despite different modes of egress between macrophages and T cells, events prior to virus assembly and budding into MVBs require CD63, and are affected by CD63 downregulation (Fig. 17).

Although the virus maturation and extracellular release mechanisms appear to differ between macrophages and T cells or cell lines, CD63 appears to be involved in late replication events in all cell types tested. It is possible that there is a fundamental process at the level of initial virus protein production and assembly that requires participation of CD63, and that other factors are also involved in the process by which virus is released in the extracellular environment. To understand the detailed role of CD63, pseudotype viruses will be used in further experiment. Pseudotype virus was made by co-transfecting HIV or VSV-G envelop with envelop defective HIV backbone. The HIV backbone also encodes luciferase which will be activated by Tat during HIV life cycle, and can be used to measure the infection level. Pseudotype viruses can be used for single round infection in macrophages or cell lines. VSV-G pseudotype virus doesn't need to use CD4 to enter the cells, and it will serve as the control for HIV infection. An entry assay based on

pseudotype viruses will also be used to study the role of CD63 on HIV entry. The viruses used in entry assay incorporated with a luciferase enzyme into the viral particles, by linking to virus protein Nef, and the entry can be measured based on the luciferase activity in two hours after infection (Andrey et al., 2004). The assembly of HIV will be studied on a HIV-infected monocytic cell line U1, which will exclude the infection part of HIV life cycle. Using these techniques, the role of CD63 can be studied in different steps of HIV life cycle.

REFERENCES

Ackah AN, Coulibaly D, Digbeu H, Diallo K, et al. Response to treatment, mortality, and CD4 lymphocyote counts in HIV-infected persons with tuberculosis in Abidjan, Cote d'Ivoire. Lancet 1995; 345(8950):607-10.

Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, Strick D, Johnston MN, Corcoran C, Wurcel AG, Fitzpatrick CA, Feeney ME, Rodriguez WR, Basgoz N, Draenert R, Stone DR, Brander C, Goulder PJ, Rosenberg ES, Altfeld M, Walker BD (2003) Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. Journal of Virology 77:2081-2092.

Alcabes P, Munoz A, Vlahov D, Friedland GH. Incubation period of human immunodeficiency virus. Epidemiol Rev 1993a; 15(2):303-18.

Alcabes P, Schoenbaum EE, Klein RS. Correlates of the rate of decline of CD4+ lymphocytes among injection drug users infected with the human immunodeficiency virus. Am J Epidemiol 1993b; 137(9):989-1000.

Alkhatib G., Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger E A (1996) CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 272:1955-1958.

Ammann AJ, Abrams D, Conant M, Chudwin D, et al. Acquired immune dysfunction in homosexual men: immunologic profiles. Clin Immunol Immunopathol 1983a;27(3):315-25.

Ammann AJ, Cowan MJ, Wara DW, Weintrub P, et al. Acquired immunodeficiency in an infant: possible transmission by means of blood products. Lancet 1983b;1(8331):956-8.

Ammann A. T-cell immunodeficiency disorders. In: Stites D, Terr A, eds. Basic and Clinical Immunology; 7th ed. Norwalk: Appleton and Lange, 1991, pp. 335-40.

Amyere M, Mettlen PM, Van Der Smissen A, Platek B, Payrastre A, Veithen C, Courtoy PJ (2002) Origin, originality, functions, subversions and molecular signalling of macropinocytosis. International Journal of Medical Microbiology 291:487-494.

Andes WA, Rangan SR, Wulff KM. Exposure of heterosexuals to human immunodeficiency virus and viremia: evidence for continuing risks in spouses of hemophiliacs. Sex Transm Dis 1989;16(2):68-73.

Andiman WA, Simpson BJ, Olson B, Dember L, et al. Rate of transmission of human immunodeficiency virus type 1 infection from mother to child and short-term outcome of neonatal infection. Am J Dis Child 1990;144:758-66.

Anzai N, Lee Y, Youn BS, Fukuda S, Kim YJ, Mantel C, Akashi M, Broxmeyer HE (2002) C-kit associated with the transmembrane 4 superfamily proteins constitutes a functionally distinct subunit in human hematopoietic progenitors. Blood 99:4413-4421.

Aoki-Sei S, Yarchoan R, Kageyama S, Hoekzema DT, et al. Plasma HIV-1 viremia in HIV-1 infected individuals assessed by polymerase chain reaction. AIDS Res Hum Retroviruses 1992;8(7):1263-70.

Arthur LO, Bess JW Jr, Sowder RC 2nd, Benveniste RE, Mann DL, Chermann JC, Henderson LE (1992) Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. Science 258:1935-1938.

Bannert N, Kurth R (2004) Retroelements and the human genome: new perspectives on an old relation. Proceedings of the National Academy of Sciences of the United States of America 13:14572–14579.

Bazan HA, Alkhatib G, Broder CC, Berger EA (1998). Patterns of CCR5, CXCR4, and CCR3 usage by envelope glycoproteins from human immunodeficiency virus type 1 primary isolates. Journal of Virology 72:4485-4491.

Berditchevski F, Tolias KF, Wong K, Carpenter CL, Hemler ME (1997) A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. The Journal of Biological Chemistry 272:2595-2598.

Berditchevski F, Odintsova E (1999) Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. The Journal of Cell Biology 146:477-492.

Berditchevski F (2001) Complexes of tetraspanins with integrins: more than meets the eye. Journal of Cell Science 114:4143-4151.

Bienstock RJ, Barrett JC (2001) KAI1, a prostate metastasis suppressor: prediction of solvated structure and interactions with binding partners; integrins, cadherins, and cell-surface receptor proteins. Molecular Carcinogenesis 32:139-153.

Bishop KN, Holmes RK, Sheehy AM, Davidson NO, Cho SJ, Malim MH (2004) Cytidine deamination of retroviral DNA by diverse APOBEC proteins. Current Biology 14:1392–1396.

Blattner W, Gallo RC, Temin HM. HIV causes AIDS. Science 1988; 241(4865):515-6.

Boeke, JD; Stoye JP (1997) In: *Retroviruses.* Coffin J.M., Hughes S.H., Varmus H.E. , editors. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1997. pp. 343–435.

Boucheix C, Rubinstein E (2001) Tetraspanins. Cellular and Molecular Life Science 58:1189-1205.

Bour S, Strebel K (2003) The HIV-1 Vpu protein: a multifunctional enhancer of viral particle release. Microbes and Infection 11:1029-1039.

Bourinbaiar AS, Phillips DM (1991) Transmission of human immunodeficiency virus from monocytes to epithelia. Journal of Acquired Immune Deficiency Syndromes 4:56-63.

Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML (2001) The Immunological Synapse. Annual Review of Immunology 19:375-396.

Bukrinskaya AG (2004) HIV-1 assembly and maturation. Archives of Virology 149:1067-1082.

Carmo AM, Wright MD (1995) Association of the transmembrane 4 superfamily molecule CD53 with a tyrosine phosphatase activity. European Journal of Immunology 25:2090-2095.

CDC. *Pneumocystis pneumonia* – Los Angeles. MMWR Morb Mortal Wkly Rep. 1981; 30: 250-252

CDC. Revision of the case definition of acquired immunodeficiency syndrome for national reporting - United States. MMWR 1985; 34:373-5.

Cha JH, Brooke JS, Ivey KN, Eidels L (2000) Cell surface monkey CD9 antigen is a coreceptor that increases diphtheria toxin sensitivity and diphtheria toxin receptor affinity. The Journal of Biological Chemistry 275:6901-6907.

Charrin S, Le Naour F, Oualid M, Billard M, Faure G, Hanash SM, Boucheix C, Rubinstein E (2001) The major CD9 and CD81 molecular partner. Identification and characterization of the complexes. The Journal of Biological Chemistry 276:14329-14337.

Charrin S, Manié S, Thiele C, Billard M, Gerlier D, Boucheix C, Rubinstein E (2003) A physical and functional link between cholesterol and tetraspanins. European Journal of Immunology 33:2479-2489.

Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G., Newman W, Gerard N, Gerard C, Sodroski J (1996) The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 85:1135-1148.

Chow YH, Yu D, Zhang JY, Xie Y, Wei OL, Chiu C, Foroohar M, Yang OO, Park NH, Chen IS, Pang S (2002) gp120-Independent infection of CD4(-) epithelial cells and CD4(+) T-cells by HIV-1. Journal of Acquired Immune Deficiency Syndromes 30:1-8.

Claas C, Stipp CS, Hemler ME (2001) Evaluation of prototype transmembrane 4 superfamily protein complexes and their relation to lipid rafts. The Journal of Biological Chemistry 276:7974-7984.

Clark KL, Zeng Z, Langford AL, Bowen SM, Todd SC (2001) PGRL is a major CD81-associated protein on lymphocytes and distinguishes a new family of cell surface proteins. Journal of Immunology 167:5115-521.

Coffin J, Haase A, Levy JA. Human immunodeficiency viruses. Science 1986; 232-697

Collman R, Balliet JW, Gregory SA, Friedman H, Kolson DL, Nathanson N, Srinivasan A (1992) An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1. Journal of Virology 66:7517-7521.

Connor RI, Korber BT, Graham BS, Hahn BH, Ho DD, Walker BD, Neumann AU, Vermund SH, Mestecky J, Jackson S, Fenamore E, Cao Y, Gao F, Kalams S, Kunstman KJ, McDonald D, McWilliams N, Trkola A, Moore JP, Wolinsky SM (1998) Immunological and virological analyses of persons infected by human immunodeficiency virus type 1 while participating in trials of recombinant gp120 subunit vaccines. Journal of Virology 72:1552-1576.

Conticello SG, Harris RS, Neuberger MS (2003) The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. Current Biology 13:2009–2013.

Daecke J, Fackler OT, Dittmar MT, Krausslich HG (2005) Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry. Journal of Virology 79:1581-1594.

Dayton AI, Sodroski JG, Rosen CA (1986) The trans-activator gene of the human T cell lymphotropic virus type II is required for replication. Cell; 44:941-947

Deininger PL, Moran JV, Batzer MA, Kazazian HH. Jr (2003) Mobile elements and mammalian genome evolution. Current Opinion in Genetics and Development 13:651–658.

Del Corno MLQ (2001) HIV-1 gp120 and chemokine activation of Pyk2 and mitogen-activated protein kinases in primary macrophages mediated by calcium-dependent, pertussis toxin-insensitive chemokine receptor signaling. Blood 98:2909-2916.

Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR,. Landau NR (1996) Identification of a major co-receptor for primary isolates of HIV-1. Nature 381:661-666.

Deng J, Yeung VP, Tsitoura D, DeKruyff RH, Umetsu DT, Levy S (2000) Allergen-induced airway hyperreactivity is diminished in CD81-deficient mice. Journal of Immunology 165:5054-5061.

deParseval A, Lerner DL, Borrow P, Willett BJ, Elder JH (2001) Blocking of feline immunodeficiency virus infection by a monoclonal antibody to CD9 is via inhibition of virus release rather than interference with receptor binding. Journal of Virology 71:5742-5749.

Dewannieux M, Dupressoir A, Harper F, Pierron G, Heidmann T (2004) Identification of autonomous IAP LTR retrotransposons mobile in mammalian cells. Nature Genetics 36:534–539.

Dimitrov DS, Broder CC, Berger EA, Blumenthal R (1993) Calcium ions are required for cell fusion mediated by the CD4-human immunodeficiency virus type 1 envelope glycoprotein interaction. Journal of Virology 67:1647-1652.

Dong X, Li H, Derdowski A, Ding L, Burnett A, Chen X, Peters TR, Dermody TS, Woodruff E, Wang JJ, Spearman P (2005) AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly. Cell 120:663-674.

Dragic T, Litwin V, Allaway G.P, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 381:667-673.

Dutko JA, Schafer A, Kenny AE, Cullen BR, Curcio MJ (2005) Inhibition of a yeast LTR retrotransposon by human APOBEC3 cytidine deaminases. Current Biology 15:661–666.

Eilbott DJ, Peress N, Burger H, LaNeve D, Orenstein J, Gendelman HE, Seidman R, Weiser B (1989) Human immunodeficiency virus type 1 in spinal cords of acquired immunodeficiency syndrome patients with myelopathy: expression and replication in macrophages. Proceedings of the National Academy of Sciences of the United States of America 86:3337-3341.

Eiley S, Mendelson J, Clecner B (1986) Effect of recombinant interferon alpha 2 on clinical course of first episode genital herpes infection and subsequent recurrences. Genitourinary Medicine 62:97-101.

Engering A, Pieters J (2001) Association of distinct tetraspanins with MHC class II molecules at different subcellular locations in human immature dendritic cells. International Immunology 13:127-134.

Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ (1998) Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. The Journal of Biological Chemistry 273:20121-20127.

Esnault C, Heidmann O, Delebecque F, Dewannieux M, Ribet D, Hance AJ, Heidmann T, Schwartz O (2005) APOBEC3G cytidine deaminase inhibits retrotransposition of endogenous retroviruses. Nature 433:430–433.

Fearon DT, Carter RH (1995) The CD19/CR2/TAPA-1 complex of B lymphocytes: linking natural to acquired immunity. Annual Review of Immunology 13:127-149.

Feng Y, Broder CC, Kennedy PE, Berger EA (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272:872-877.

Fisher AG, Feinberg MB, Josephs. (1986). The trans-activator gene of HTLV-III is essential for virus replication. Nature 320: 367-371

Fradkin LG, Kamphorst JT, DiAntonio A, Goodman CS, Noordermeer JN (2002) Genomewide analysis of the Drosophila tetraspanins reveals a subset with similar function in the formation of the embryonic synapse. Proceedings of the National Academy of Sciences of the United States of America 99:13663-13668.

Freed EO. (1998) HIV-1 Gag proteins: Diverse functions in the virus life cycle. Virology; 251: 1-15

Freed EO (2004) HIV-1 and the host cell: an intimate association. Trends in Microbiology 12:170-177.

Friedland G. Parenteral drug users. In: Kaslow RA, Francis DP, eds. (1989) The Epidemiology of AIDS. New York: Oxford University Press, pp. 153-78.

Gargalovic P, Dory L (2003) Caveolins and macrophage lipid metabolism. Journal of Lipid Research 44:11-21.

Garrus JE, von Schwedler UK, Pornillos OW, Morham SG, Zavitz KH, Wang HE, Wettstein DA, Stray KM, Côté M, Rich RL, Myszka DG, Sundquist WI (2001) Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell 107:55-65.

Gartner S, Markovits P, Markovitz DM, Kaplan MH, Gallo RC, Popovic M (1986) The role of mononuclear phagocytes in HTLV-III/LAV infection. Science 233:215-219.

Gauduin MC, Allaway GP, Olson WC, Weir R, Maddon PJ, Koup RA (1998) CD4-immunoglobulin G2 protects Hu-PBL-SCID mice against challenge by primary human immunodeficiency virus type 1 isolates. Journal of Virology 72:3475-3478.

Graham DR, Chertova E, Hilburn JM, Arthur LO, Hildreth JE (2003) Cholesterol depletion of human immunodeficiency virus type 1 and simian immunodeficiency virus with beta-cyclodextrin inactivates and permeabilizes the virions: evidence for virion-associated lipid rafts. Journal of Virology 77:8237-8248.

Grewe C, Beck A, Gelderblom HR (1990) HIV: early virus-cell interactions. Journal of Acquired Immune Deficiency Syndrome 3:965-974.

Hammond C, Denzin LK, Pan M, Griffith JM, Geuze HJ, Cresswell P (1998) The tetraspan protein CD82 is a resident of MHC class II compartments where it associates with HLA-DR, -DM, and -DO molecules. Journal of Immunology 161:3282-3291.

Harrington RD,. Geballe AP (1993) Cofactor requirement for human immunodeficiency virus type 1 entry into a CD4-expressing human cell line. Journal of Virology 67:5939-5947.

Hemler ME (2001) Specific tetraspanin functions. The Journal of Cell Biology 155:1103-1107.

Higashihara M, Takahata K, Yatomi Y, Nakahara K, Kurokawa K (1990) Purification and partial characterization of CD9 antigen of human platelets. FEBS Letters 264:270-274.

Higginbottom A, Wilkinson I, McCullough B, Lanza F, Azorsa DO, Partridge LJ, Monk PN (2000) Antibody cross-linking of human CD9 and the high-affinity immunoglobulin E receptor stimulates secretion from transfected rat basophilic leukaemia cells. Immunology 99:546-552.

Hotta H, Ross AH, Huebner K, Isobe M, Wendeborn S, Chao MV, Ricciardi RP, Tsujimoto Y, Croce CM, Koprowski H (1998) Molecular cloning and characterization of an antigen associated with early stages of melanoma tumor progression. Cancer Research 48:2955-2962.

Hotta T, Utsumi M (1998) Chronic myelomonocytic leukemia (CMML)--characterization of clinical features and prognosis. Nippon Ketsueki Gakkai Zasshi 51:1441-1447.

Igarashi T, Brown CR, Endo Y, Buckler-White A, Plishka R, Bischofberger N, Hirsch V, Martin MA (2001) Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): Implications for HIV-1 infections of humans. Proceedings of the National Academy of Sciences of the United States of America 98:658-663.

Iwamoto R, Higashiyama S, Mitamura T, Taniguchi N, Klagsbrun M, Mekada E (1994) Heparin-binding EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. The EMBO Journal 13:2322-2330.

Jankowski SA, Mitchell DS, Smith SH, Trent JM, Meltzer PS (1994) SAS, a gene amplified in human sarcomas, encodes a new member of the transmembrane 4 superfamily of proteins. Oncogene 9:1205-1211.

Jarmuz A, Chester A, Bayliss J, Gisbourne J, Dunham I Scott J, Navaratnam N (2002) An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. Genomics 79:285–296.

Kaji K, Oda S, Shikano T, Ohnuki T, Uematsu Y, Sakagami J, Tada N, Miyazaki S, Kudo A (2000) The gamete fusion process is defective in eggs of Cd9-deficient mice. Nature Genetics 24:279-282.

Kaji K, Takeshita S, Miyake K, Takai T, Kudo A (2001) Functional association of CD9 with the Fc gamma receptors in macrophages. Journal of Immunology 166:3256-3265.

Kaji K, Oda S, Miyazaki S, Kudo A (2002) Infertility of CD9-deficient mouse eggs is reversed by mouse CD9, human CD9, or mouse CD81; polyadenylated mRNA injection

developed for molecular analysis of sperm-egg fusion. Developmental Biology 247:327-334.

Kao S, Khan MA, Miyagi E, Plishka R, Buckler-White A, Strebel K (2003) The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15), a cellular inhibitor of virus infectivity. Journal of Virology 77:11398–11407.

Kaplan J, Ward DM (1990) Movement of receptors and ligands through the endocytic apparatus in alveolar macrophages. American Journal of Physiology 258:L263-270.

Katzmann DJ, Babst M, Emr SD (2001) Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell 106:145-155.

Kawashima M, Doh-ura K, Mekada E, Fukui M, Iwaki T (2002) CD9 expression in solid non-neuroepithelial tumors and infiltrative astrocytic tumors. The Journal of Histochemistry and Cytochemistry 50:1195-1203.

Kazarov AR, Yang X, Stipp CS, Sehgal B, Hemler ME (2002) An extracellular site on tetraspanin CD151 determines alpha 3 and alpha 6 integrin-dependent cellular morphology. The Journal of Cell Biology 158:1299-1309.

Kazazian HH Jr (2004) Mobile elements: drivers of genome evolution. Science 303:1626–1632.

Knobeloch KP, Wright MD, Ochsenbein AF, Liesenfeld O, Löhler J, Zinkernagel RM, Horak I, Orinska Z (2000) Targeted inactivation of the tetraspanin CD37 impairs T-cell-dependent B-cell response under suboptimal costimulatory conditions. Molecular and Cellular Biology 20:5363-5369.

Koenig S, Gendelman HE, Orenstein JM, Dal Canto MC, Pezeshkpour GH, Yungbluth M, Janotta F, Aksamit A, Martin MA, Fauci AS (1986) Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. Journal of Acquired Immune Deficiency Syndrome 5:1089-1093.

Kolokoltsov AA, Davey RA (2004) Rapid and sensitive detection of retrovirus entry by using a novel luciferase-based content-mixing assay. Journal of Virology 78:5124-5132.

Kramer B, Pelchen-Matthews A, Deneka M, Garcia E, Piguet V, Marsh M (2005) HIV interaction with endosomes in macrophages and dendritic cells. Blood Cells, Molecules and Diseases 35:136-142.

Kropshofer H, Spindeldreher S, Röhn TA, Platania N, Grygar C, Daniel N, Wölpl A, Langen H, Horejsi V, Vogt AB (2002) Tetraspan microdomains distinct from lipid rafts enrich select peptide-MHC class II complexes. Nature Immunology 3:61-68.

Kurita-Taniguchi M, Hazeki K, Murabayashi N, Fukui A, Tsuji S, Matsumoto M, Toyoshima K, Seya T (2002) Molecular assembly of CD46 with CD9, alpha3-beta1 integrin and protein tyrosine phosphatase SHP-1 in human macrophages through differentiation by GM-CSF. Molecular Immunology 38:689-700.

Lagaudrière-Gesbert C, Le Naour F, Lebel-Binay S, Billard M, Lemichez E, Boquet P, Boucheix C, Conjeaud H, Rubinstein E (1997) Functional analysis of four tetraspans, CD9, CD53, CD81, and CD82, suggests a common role in costimulation, cell adhesion, and migration: only CD9 upregulates HB-EGF activity. Cellular Immunology 182:105-112.

Lambotte O, Cacoub P, Costedoat N, Le Moel G, Amoura Z, Piette JC (2003) High ferritin and low glycosylated ferritin may also be a marker of excessive macrophage activation. Journal of Rheumatology 30:1027-1028.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W (2001) Initial sequencing and analysis of the human genome. Nature 409:860–921.

Le Naour F, Rubinstein E, Jasmin C, Prenant M, Boucheix C (2000) Severely reduced female fertility in CD9-deficient mice. Science 287:319-321.

Lebel-Binay S, Lagaudrière C, Fradelizi D, Conjeaud H (1995) CD82, member of the tetra-span-transmembrane protein family, is a costimulatory protein for T cell activation. Journal of Immunology 155:101-110.

Lecossier D, Bouchonnet F, Clavel F, Hance AJ (2003) Hypermutation of HIV-1 DNA in the absence of the Vif protein. Science 300:1112.

Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW (1999) Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. Proceedings of the National Academy of Sciences of the United States of America 96:5215-5220.

Lee S, Lapham CK, Chen H, King L, Manischewitz J, Romantseva T, Mostowski H, Stantchev TS, Broder CC, Golding H (2000) Coreceptor competition for association with CD4 may change the susceptibility of human cells to infection with T-tropic and macrophagetropic isolates of human immunodeficiency virus type 1. Journal of Virology 74:5016-5023.

Lefkir Y, Malbouyres M, Gotthardt D, Ozinsky A, Cornillon S, Bruckert F, Aderem A A, Soldati T, Cosson P, Letourneur F (2004) Involvement of the AP-1 adaptor complex in early steps of phagocytosis and macropinocytosis. Molecular Biology of the Cell 15:861-869.

Levy S, Todd SC, Maecker HT (1998) CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. Annual Review of Immunology 16:89-109.

Levy S, Shoham T (2005) The tetraspanin web modulates immune-signalling complexes. Nature Review of Immunology 5:136-148.

Liao Z, Graham DR, Hildreth JE (2003) Lipid rafts and HIV pathogenesis: virion-associated cholesterol is required for fusion and infection of susceptible cells. AIDS Research and Human Retroviruses 19:675-687.

Liddament MT, Brown WL, Schumacher AJ, Harris RS (2004) APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo. Current Biology 14:1385–1391.

Louwagie J, McCutchan FE, Peeters M, et al.(1993) Phylogenetic analysis of gag genes from 70 international HIV-1 isolates provides evidence for multiple genotypes. AIDS: 7: 769-780

Lu YL, Spearman P, Ratner L. (1993) Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. Journal of Virology 67: 6542-6550.

Maecker HT, Todd SC, Levy S (1997) The tetraspanin superfamily: molecular facilitators. The FASEB Journal 11:428-442.

Maecker HT, Do MS, Levy S (1998) CD81 on B cells promotes interleukin 4 secretion and antibody production during T helper type 2 immune responses. Proceedings of the National Academy of Sciences of the United States of America 95:2458-2462.

Mairhofer M, Steiner M, Mosgoeller W, Prohaska R, Salzer U (2002) Stomatin is a major lipid-raft component of platelet alpha granules. Blood 100:897-904.

Mangeat B., Turelli P., Caron G., Friedli M., Perrin L., Trono D. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature.* 2003;424:99–103.

Mannion BA, Berditchevski F, Kraeft SK, Chen LB, Hemler ME (1996) Transmembrane-4 superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associated with integrin alpha 4 beta 1 (CD49d/CD29). Journal of Immunology 157:2039-2047.

Maréchal V, Prevost MC, Petit C, Perret E, Heard JM, Schwartz O (2001) Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis. Journal of Virology 75:11166-11177.

Mariani R, Chen D, Schrofelbauer B, Navarro F, Konig R, Bollman B, Munk C, Nymark-McMahon H, Landau NR (2003) Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. Cell 114:21–31.

Marin M, Rose KM, Kozak SL, Kabat D (2003) HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. Nature Medicine 9:1398–1403.

Martin-Serrano J, Zang T, Bieniasz PD (2001) HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. Nature Medicine 7:1313-1319.

Maxfield FR, McGraw TE (2004) Endocytic recycling. Nature Reviews. Molecular Cell Biology 5:121-132.

McClure MO, Sattentau QJ, Beverley PC, Hearn JP, Fitzgerald AK, Zuckerman AJ, Weiss RA (1987) HIV infection of primate lymphocytes and conservation of the CD4 receptor. Nature 330:487-489.

Mehle A, Strack B, Ancuta P, Zhang C, McPike M, Gabuzda D (2004) Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. The Journal of Biological Chemistry 279:7792–7798.

Meltzer MS, Skillman DR, Hoover DL, Hanson BD, Turpin JA, Kalter DC, Gendelman HE (1990) Macrophages and the human immunodeficiency virus. Immunology Today 11:217-223.

Metzelaar MJ,. Wijngaard PLJ, Peters PJ, Sixma JJ, Nieuwenhuis HK, Clevers HC (1991) CD63 antigen. The Journal of Biological Chemistry 266:3239.

Mittelbrunn M, Yáñez-Mó M, Sancho D, Ursa A, Sánchez-Madrid F (2002) Cutting edge: dynamic redistribution of tetraspanin CD81 at the central zone of the immune synapse in both T lymphocytes and APC. Journal of Immunology 169:6691-6695.

Moarefi I, LaFevre-Bernt M, Sicheri F, Huse M, Lee CH, Kuriyan J, Miller WT (1997) Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. Nature 385:650-653.

Murray JL, Mavrakis M, McDonald NJ, Yilla M, Sheng J, Bellini WJ, Zhao L, Le Doux JM, Shaw MW, Luo CC, Lippincott-Schwartz J, Sanchez A, Rubin DH, Hodge TW (2005) Rab9 GTPase is required for replication of human immunodeficiency virus type 1, filoviruses, and measles virus. Journal of Virology 79:11742-11751.

Myers EL, Allen JF (2002) Tsg101, an inactive homologue of ubiquitin ligase e2, interacts specifically with human immunodeficiency virus type 2 gag polyprotein and results in increased levels of ubiquitinated gag. Journal of Virology 76:11226-1135.

Myers G. (1994) Tenth anniversary perspectives on AIDS. HIV: Between past and future. AIDS Res Hum Retroviruses 10: 1317-1324
Nguyen DH, Hildreth JE (2000) Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. Journal of Virology 74:3264-3272.

Nakielny S, Fischer U, Michael WM, et al. (1997) RNA transport. Annual Review Neurosciences 20: 269-301

Niedergang F, Chavrier P (2004) Signaling and membrane dynamics during phagocytosis: many roads lead to the phagos(R)ome. Current Opinion in Cellular Biology 16:422-428.

Oberlin E, Amara A, Bachelerie F, Bessia C, Virelizier JL, Arenzana-Seisdedos F, Schwartz O, Heard JM, Clark-Lewis I, Legler DF, Loetscher M, Baggiolini M, Moser B (1996) The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevent infection by T-cell line-adapted HIV-1. Nature 382:833-835.

Olweus J, Lund-Johansen F, Horejsi V (1993) CD53, a protein with four membrane-spanning domains, mediates signal transduction in human monocytes and B cells. Journal of Immunology 151:707-716.

Oren R, Takahashi S, Doss C, Levy R, Levy S (1990) TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. Molecular and Cellular Biology 10:4007-4015.

Orenstein JM, Fox C, Wahl SM (1997) Macrophages as a source of HIV during opportunistic infections. Science 276:1857-1861.

Orentas RJ, Hildreth JE (1993) Association of host cell surface adhesion receptors and other membrane proteins with HIV and SIV. AIDS Research and Human Retroviruses 9:1157-1165.

Ott DE (1997) Cellular proteins in HIV virions. Reviews in Medical Virology 7:167-180.

Ott DE (2002) Potential roles of cellular proteins in HIV-1. Reviews in Medical Virology 12:359-374.

Pang S, Yu D, An DS, Baldwin GC, Xie Y, Poon B, Chow YH, Park NH, Chen IS (2000) Human immunodeficiency virus Env-independent infection of human CD4(-) cells. Journal of Virology 74:10994-1000.

Pauza CD, Galindo J, Richman DD (1988) Human immunodeficiency virus infection of monoblastoid cells: cellular differentiation determines the pattern of virus replication. Journal of Virology 62:3558-3564.

Pelchen-Matthews A, Kramer B, Marsh M (2003) Infectious HIV-1 assembles in late endosomes in primary macrophages. The Journal of Cell Biology 162:443-455.

Perret E, Lakkaraju A, Deborde S, Schreiner R, Rodriguez-Boulan E (2005) Evolving endosomes: how many varieties and why? Current Opinion in Cellular Biology 17:423-434.

Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D (1998) Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. Journal of Virology 72:2855-2864.

Radford KJ, Mallesch J, Hersey P (1995) Suppression of human melanoma cell growth and metastasis by the melanoma-associated antigen CD63 (ME491). International Journal of Cancer 62:631-635.

Radford KJ, Thorne RF, Hersey P (1997) Regulation of tumor cell motility and migration by CD63 in a human melanoma cell line. Journal of Immunology 158:3353-3358.

Raposo G, Moore M, Innes D, Leijendekker R, Leigh-Brown A, Benaroch P, Geuze H (2002) Human macrophages accumulate HIV-1 particles in MHC II compartments. Traffic 3:718-729.

Ribet D, Dewannieux M, Heidmann T (2004) An active murine transposon family pair: retrotransposition of ‘master’ MusD copies and ETn trans-mobilization. Genome Research 14:2261–2267.

Rich EA, Chen IS, Zack JA, Leonard ML, O'Brien WA (1992) Increased susceptibility of differentiated mononuclear phagocytes to productive infection with human immunodeficiency virus-1 (HIV-1). Journal of Clinical Investigation 89:176-183.

Rimmelzwaan GF, Siebelink KH, Huisman RC, Moss B, Francis MJ, Osterhaus AD (1994) Removal of the cleavage site of recombinant feline immunodeficiency virus envelope protein facilitates incorporation of the surface glycoprotein in immune-stimulating complexes. The Journal of General Virology 75:2097-2102.

Rogozin IB, Basu MK, Jordan IK, Pavlov YI, Koonin EV (2005) APOBEC4, a new member of the AID/APOBEC family of polynucleotide (Deoxy)cytidine deaminases predicted by computational analysis. Cell Cycle 4:9.

Rubinstein E, Le Naour F, Lagaudriere-Gesbert C, Billard M, Conjeaud H, Boucheix C (1996) CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. European Journal of Immunology 26:2657-2665.

Rubinstein E, Ziyyat A, Wolf JP, Le Naour F, Boucheix C (2006) The molecular players of sperm-egg fusion in mammals. Seminars in Cell and Developmental Biology 17:254-263.

Rusconi S, Scozzafava A, Mastrolorenzo A, Supuran CT. (2007) An update in the development of HIV entry inhibitors. Current topics in medicinal chemistry 2007; 7(13):1273-89.

Sakalian M, Hunter E (1998) Molecular events in the assembly of retrovirus particles. Advances in Experimental Medicine and Biology 440:329-339.

Schaeffer E, Geleziunas R, Greene WC (2001) Human immunodeficiency virus type 1 Nef functions at the level of virus entry by enhancing cytoplasmic delivery of virions. Journal of Virology 75:2993-3000.

Schumacher AJ, Nissley DV, Harris RS (2005) APOBEC3G hypermutates genomic DNA and inhibits Ty1 retrotransposition in yeast. Proceedings of the National Academy of Sciences of the United States of America 6:6.

Seigneuret M, Delaguillaumie A, Lagaudrière-Gesbert C, Conjeaud H (2001) Structure of the tetraspanin main extracellular domain. A partially conserved fold with a structurally variable domain insertion. The Journal of Biological Chemistry 276:40055-40064.

Sellon DC (1993) Equine infectious anemia. The Veterinary clinics of North America. Equine Practice 9:321-336.

Serru V, Le Naour F, Billard M, Azorsa DO, Lanza F, Boucheix C, Rubinstein E (1999) Selective tetraspan-integrin complexes (CD81/alpha4beta1, CD151/alpha3beta1, CD151/alpha6beta1) under conditions disrupting tetraspan interactions. The Biochem Journal 340:103-111.

Shaw GM, Harper ME, Hahn BH, Epstein LG, Gajdusek DC, Price RW, Navia BA, Petito CK, O'Hara CJ, Groopman JE, Cho ES, Oleske JM, Wong-Stall F, Gallo RC (1985) HTLV-III infection in brains of children and adults with AIDS encephalophathy. Science 227:177-181.

Sheehy AM, Gaddis NC, Choi JD, Malim MH (2002) Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature 418:646–650.

Sheehy AM, Gaddis NC, Malim MH (2003) The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. Nature Medicine 9:1404–1407.

Shioda T, Levy JA, Cheng-Mayer C (1991) Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature (London) 349:167-169.

Simonsen A, Wurmser AE, Emr SD, Stenmark H (2001) The role of phosphoinositides in membrane transport. Current Opinion in Cell Biology 13:485-492.

Sincock PM, Mayrhofer G, Ashman LK (1997) Localization of the transmembrane 4 superfamily (TM4SF) member PETA-3 (CD151) in normal human tissues: comparison with CD9, CD63, and alpha5beta1 integrin. The journal of Histochemistry and Cytochemistry 45:515-525.

Skubitz KM, Campbell KD, Iida J, Skubitz AP (1996) CD63 associates with tyrosine kinase activity and CD11/CD18, and transmits an activation signal in neutrophils. Journal of Immunology 157:3617-3626.

Stein BS, Gowda SD, Lifson JD, Penhallow RC, Bensch KG, Engleman EG (1987) pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell 49:659-668.

Sterk LM, Geuijen CA, van den Berg JG, Claessen N, Weening JJ, Sonnenberg A (2002) Association of the tetraspanin CD151 with the laminin-binding integrins alpha3beta1, alpha6beta1, alpha6beta4 and alpha7beta1 in cells in culture and in vivo. Journal of Cell Science 115:1161-1173.

Stipp CS, Kolesnikova TV, Hemler ME (2001) EWI-2 is a major CD9 and CD81 partner and member of a novel Ig protein subfamily. The Journal of Biological Chemistry 276:40545-40554.

Stipp CS, Kolesnikova TV, Hemler ME (2003) Functional domains in tetraspanin proteins. Trends in Biochemical Sciences 28:106-112.

Sun TT, Zhao H, Provet J, Aebi U, Wu XR (1996) Formation of asymmetric unit membrane during urothelial differentiation. Molecular Biology Reports 23:3-11.

Szala S, Kasai Y, Steplewski Z, Rodeck U, Koprowski H, Linnenbach AJ (1990) Molecular cloning of cDNA for the human tumor-associated antigen CO-029 and identification of related transmembrane antigens. Proceedings of the National Academy of Sciences of the United States of America 87:6833-6837.

Szöllósi J, Horejsí V, Bene L, Angelisová P, Damjanovich S (1996) Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. Journal of Immunology 157:2939-2946.

Tai XG, Toyooka K, Yashiro Y, Abe R, Park CS, Hamaoka T, Kobayashi M, Neben S, Fujiwara H (1997) CD9-mediated costimulation of TCR-triggered naive T cells leads to activation followed by apoptosis. Journal of Immunology 159:3799-3807.

Takagi S, Fujikawa K, Imai T, Fukuhara N, Fukudome K, Minegishi M, Tsuchiya S, Konno T, Hinuma Y, Yoshie O (1995) Identification of a highly specific surface marker of T-cell acute lymphoblastic leukemia and neuroblastoma as a new member of the transmembrane 4 superfamily. International Journal of Cancer 61:706-715.

Takei K, Haucke V (2001) Clathrin-mediated endocytosis: membrane factors pull the trigger. Trends in Cellular Biology 11:385-391.

Tedder TF, Inaoki M, Sato S (1997) The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. Immunity 6:107-118.

Terwilliger EF, Cohen EA, Lu Y, Sodroski JG, Haseltine WA (1989) Functional Role of Human Immunodeficiency Virus Type 1 vpu. Proceedings of the National Academy of Sciences of the United States of America 86:5163-5167.

Timmins J, Schoehn G, Ricard-Blum S, Scianimanico S, Vernet T, Ruigrok RW, Weissenhorn W (2003) Ebola virus matrix protein VP40 interaction with human cellular factors Tsg101 and Nedd4. Journal of Molecular Biology 326:493-502.

Théry C, Boussac M, Véron P, Ricciardi-Castagnoli P, Raposo G, Garin J, Amigorena S (2001) Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. Journal of Immunology 166:7309-7318.

Théry C, Zitvogel L, Amigorena S (2002) Exosomes: composition, biogenesis and function. Nature Reviews Immunology 2:569-579.

Todres E, Nardi JB, Robertson HM (2000) The tetraspanin superfamily in insects. Insect Molecular Biology 9:581-590.

Tokunaga K, Greenberg ML, Morse MA, Cumming RI, Lyerly HK, Cullen BR (2001) Molecular basis for cell tropism of CXCR4-dependent human immunodeficiency virus type 1 isolates. Journal of Virology 75:6776-6785.

Toyo-oka K, Yashiro-Ohtani Y, Park CS, Tai XG, Miyake K, Hamaoka T, Fujiwara H (1999) Association of a tetraspanin CD9 with CD5 on the T cell surface: role of particular transmembrane domains in the association. International Immunology 11:2043-2052.

Tschachler E, Groh V, Popovic M, Mann DL, Konrad K, Safai B, Eron L, diMarzo Veronese F, Wolff K, Stingl G. (1987) Epidermal Langerhans cells - A target for HTLV-III/LAV infection. The Journal of Investigative Dermatology. 88:233-237.

Tse SM, Furuya W, Gold E, Schreiber AD, Sandvig K,. Inman RD, Grinstein S (2003) Differential role of actin, clathrin, and dynamin in Fc gamma receptor-mediated endocytosis and phagocytosis. The Journal of Biological Chemistry 278:3331-3338.

Vodicka MA, Goh WC, Wu LI, Rogel ME, Bartz SR, Schweickart VL, Raport CJ, Emerman M (1997) Indicator cell lines for detection of primary strains of human and simian immunodeficiency viruses. Virology 233:193-198

von Lindern JJ, Rojo D, Grovit-Ferbas K, Yeramian C, Deng C, Herbein G, Ferguson MR, Pappas TC, Decker JM, Singh A, Collman RG, O'Brien WA (2003). Potential role for CD63 in CCR5-mediated human immunodeficiency virus type 1 infection of macrophages. Journal of Virology 77:3624-3633.

Wang Z, Lee B, Murray JL, Bonneau F, Sun Y, Schweickart V, Zhang T, Peiper SC (1999) CCR5 HIV-1 coreceptor activity. Role of cooperativity between residues in N-terminal extracellular and intracellular domains. The Journal of Biological Chemistry 274:28413-28419.

Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P (2002) Initial sequencing and comparative analysis of the mouse genome. Nature 420:520–562.

Wedekind JE, Dance GS, Sowden MP, Smith HC (2003) Messenger RNA editing in mammals: new members of the APOBEC family seeking roles in the family business. Trends in Genetics 19:207–216.

Westervelt P, Henkel T, Trowbridge DB, Orenstein J, Heuser J, Gendelman HE, Ratner L (1992) Dual regulation of silent and productive infection in monocytes by distinct human immunodeficiency virus type 1 determinants. Journal of Virology 66:3925-3931.

Willey RL, Bonifacino JS, Potts BJ (1988) Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. Proc Natl Acad Sci USA, 85: 9580-9584

Witherden DA, Boismenu R, Havran WL (2000) CD81 and CD28 costimulate T cells through distinct pathways. Journal of Immunology 165:1902-1909.

Wolfers J, Lozier A, Raposo G, Regnault A, Théry C, Masurier C, Flament C, Pouzieux S, Faure F, Tursz T, Angevin E, Amigorena S, Zitvogel L (2001) Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nature Medicine 7:297-303.

Wright MD, Henkle KJ, Mitchell GF (1990) An immunogenic Mr 23,000 integral membrane protein of Schistosoma mansoni worms that closely resembles a human tumor-associated antigen. Journal of Immunology 144:3195-3200.

Wright MD, Tomlinson MG (1994) The ins and outs of the transmembrane 4 superfamily. Immunology Today 15:588-594.

Wu XR, Medina JJ, Sun TT (1995) Selective interactions of UPIa and UPIb, two members of the transmembrane 4 superfamily, with distinct single transmembrane-domained proteins in differentiated urothelial cells. The Journal of Biological Chemistry 270:29752-29759.

Yashiro-Ohtani Y, Zhou XY, Toyo-Oka K, Tai XG, Park CS, Hamaoka T, Abe R, Miyake K, Fujiwara H (2000) Non-CD28 costimulatory molecules present in T cell rafts induce T cell costimulation by enhancing the association of TCR with rafts. Journal of Immunology 164:1251-1259.

Yauch RL, Hemler ME (2000) Specific interactions among transmembrane 4 superfamily (TM4SF) proteins and phosphoinositide 4-kinase. The Biochemical Journal 351:629-637.

Zanakis SH, Alvarez C, Li V (2007) European Journal of Operational Research: 176: 1811-1838.

Zhang H, Dornaldula G, Beumont M., Livornese L, VanUitert B Jr, Henning K, Pomerantz RJ (1998) Human immunodeficiency virus type 1 in the seme of men receiving highly active antietroviral therapy. New England Journal of Medicine 17:1803-1816.

Zhu T, Mo H, Wang N, Nam D, Cao Y, Koup R, Ho D (1993) Genotypic and phenotypic characterization of HIV-1 in patients with primary infection. Science 261:1179-1181.

Ziyyat A, Rubinstein E, Monier-Gavelle F, Barraud V, Kulski O, Prenant M, Boucheix C, Bomsel M, Wolf JP (2006) CD9 controls the formation of clusters that contain tetraspanins and the integrin alpha 6 beta 1, which are involved in human and mouse gamete fusion. Journal of Cell Science 119:416-424.

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Publications:

Chen H，von Lindern JJ，O'Brien WA. Possible role for CD63 in stabilization of HIV receptor/coreceptor organization in macrophages. FASEB Summer Research Conferences. Pine Mountain, GA, USA 2004

Chen H, Rojo D, von Lindern JJ, Liburd N, Ferguson MR, O'Brien WA (2005) Mechanism of cellular resistance to anti-CD63 inhibition of HIV infection (abstract 71).Antiviral Therapy 10:S78.

O'Brien WA, **Chen H**. A Role for the Tetraspanin CD63 in HIV Entry. The 3rd IAS Conference on HIV Pathogenesis and Treatment. Rio de Janiero, Brazil 2005

Chen H, Friedrich B, Rojo D, O'Brien WA. Complex role for the tetraspanin CD63 in HIV replication. HIV DART. Cancun, Mexico 2006

Friedrich B, **Chen H**, Rojo D, Chen Z, O'Brien WA. A complex role for the tetraspanin CD63 in HIV replication. XVI International AIDS Conference. Toronto, Canada 2006

Hasan S, **Chen H**, D Rojo, J Von Lindern, N Liburd, M Ferguson, and W O'Brien. A Critical Role for CD63 in HIV Infection of Macrophages. 13th Conference on Retroviruses and Opportunistic infections. Denver, CO, USA 2006

Chen H, Friedrich B, Rojo D, O'Brien WA. A complex role for the tetraspanin CD63 in HIV replication. 4th IAS Conference on HIV Pathogenesis, Treatment and Prevention. Sydney, Australia 2007

Li YH, **Chen H**, Guo K, Xie YM, Ren DM (2001) Prophylaxis of tumor through oral administration of IL-12 GM-CSF gene carried by live attenuated *Salmonella.* Chinese Science Bulletin 46:317-321.

Li YH, Xie YM, Guo KY, **Chen H,** Ren DM (2001) Treatment of tumor in mice by oral administration of cytosine deaminase gene carried in live attenuated *Salmonella.* ACTA Biochimica et Biophysica Sinica 33:233-237.

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