Strategies for Targeting Tetraspanin Proteins
Potential Therapeutic Applications in Microbial Infections

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Abstract

The identification of novel targets and strategies for therapy of microbial infections is an area of intensive research due to the failure of conventional vaccines or antibiotics to combat both newly emerging diseases (e.g. viruses such as severe acute respiratory syndrome (SARS) and new influenza strains, and antibiotic-resistant bacteria) and entrenched, pandemic diseases exemplified by HIV. One clear approach to this problem is to target processes of the host organism rather than the microbe. Recent data have indicated that members of the tetraspanin superfamily, proteins with a widespread distribution in eukaryotic organisms and 33 members in humans, may provide such an approach.

Tetraspanins traverse the membrane four times, but are distinguished from other four-pass membrane proteins by the presence of conserved charged residues in the transmembrane domains and a defining 'signature' motif in the larger of the two extracellular domains (the EC2). They characteristically form promiscuous associations with one another and with other membrane proteins and lipids to generate a specialized type of microdomain: the tetraspanin-enriched microdomain (TEM). TEMs are integral to the main role of tetraspanins as 'molecular organizers' involved in functions such as membrane trafficking, cell–cell fusion, motility, and signaling. Increasing evidence demonstrates that tetraspanins are used by intracellular pathogens as a means of entering and replicating within human cells. Although previous investigations focused mainly on viruses such as hepatitis C and HIV, it is now becoming clear that other microbes associate with tetraspanins, using TEMs as a 'gateway' to infection.

In this article we review the properties and functions of tetraspanins/TEMs that are relevant to infective processes and discuss the accumulating evidence that shows how different pathogens exploit these properties in infection and in the pathogenesis of disease. We then investigate the novel and exciting possibilities of targeting tetraspanins for the treatment of infectious disease, using specific antibodies, recombinant EC2 domains, small-molecule mimetics, and small interfering RNA. Such therapies, directed at host-cell molecules, may provide alternative options for combating fast-mutating or newly emerging pathogens, where conventional approaches face difficulties.

1. Tetraspanin Functions

The tetraspanins constitute a diverse superfamily of transmembrane protein, with 33 members in mammalian cells.[1] The family is ancient, with the first member having appeared some 570 million years ago, and is widespread amongst Eukaryota.[2] In accordance with the conservation of the family during evolution, the tetraspanins are involved in basic cell functions such as motility, fusion, and membrane trafficking,[3,4] and play roles in a range of physiological processes including sperm–egg fusion, antigen presentation, and tissue differentiation.[4-6] Tetraspanins are widely expressed with most cells having several members on their surface and/or on internal membranes. Whilst some tetraspanins (e.g. CD9 [TSPAN29], CD63 [TSPAN30], CD81 [TSPAN28], CD82 [TSPAN27], and CD151 [TSPAN24]) have a broad tissue distribution, others show restricted expression and are involved in more specialized functions (e.g. peripherin/rds [TSPAN22] and ROM-1 [TSPAN23] in photoreceptor rod cells[7]). Broadly, tetraspanins appear to act as molecular organizers by virtue of their propensity to interact as homo- or heterodimers and form a novel type of microdomain known as tetraspanin-enriched microdomains (TEMs).[8] As components of TEMs, tetraspanins maintain a network of interactions with other membrane proteins (e.g. integrins, signaling molecules), regulating the assembly of multi-molecular signaling platforms.

Tetraspanins are relatively small membrane proteins (200–350 a.a.) and share common structural features: four transmembrane domains, two extracellular loops (EC1 and EC2), a small intracellular loop and two short intracytoplasmic termini.[9-12] Conserved charged amino acids within the transmembrane domains distinguish tetraspanins from other four-pass membrane proteins. In addition, the large extracellular domain (EC2, 70–140 amino acids) contains 'signature motifs' that are conserved amongst all tetraspanins, in particular Cys-Cys-Gly (figure 1). Disulfide bonding between the conserved cysteines in the EC2 produces a sub-loop structure; this region shows greatest variability between family members and between individual tetraspanin species homologues.[9] Some tetraspanins have additional two-to-four cysteines within this sub-loop that may also participate in disulfide bonding. The remainder of the EC2 region, comprising three alpha helices, shows greater sequence conservation. Based on x-ray crystallographic data for CD81, the tetraspanin EC2s are predicted to have a mushroom-like structure, where the relatively conserved 'stalk' supports a 'head' domain, which includes the variable sub-loop structure.[14] Unsurprisingly, the EC2 appears to represent the main site of functional specificity in tetraspanins[9] and most tetraspanin-specific monoclonal antibodies (mAb) bind to this region.[4]

There is increasing evidence that tetraspanins are involved in intracellular microbial infections. The tetraspanin CD151, also a blood group antigen, shows a high degree of pathogen-driven selection indicative of a role in host defence or vulnerability to infection.[15] Several infectious agents appear to have evolved to exploit tetraspanins at the level of pathogen entry, subsequent intracellular trafficking, replication or egress. Whilst associations of tetraspanins with the major human pathogens hepatitis C virus (HCV) and HIV are well documented,[16,17] the past few years have produced increasing evidence for their involvement in other types of infections, including those by *Plasmodium* species that cause malaria,[18] certain types of bacteria,[19] and even prions.[20]
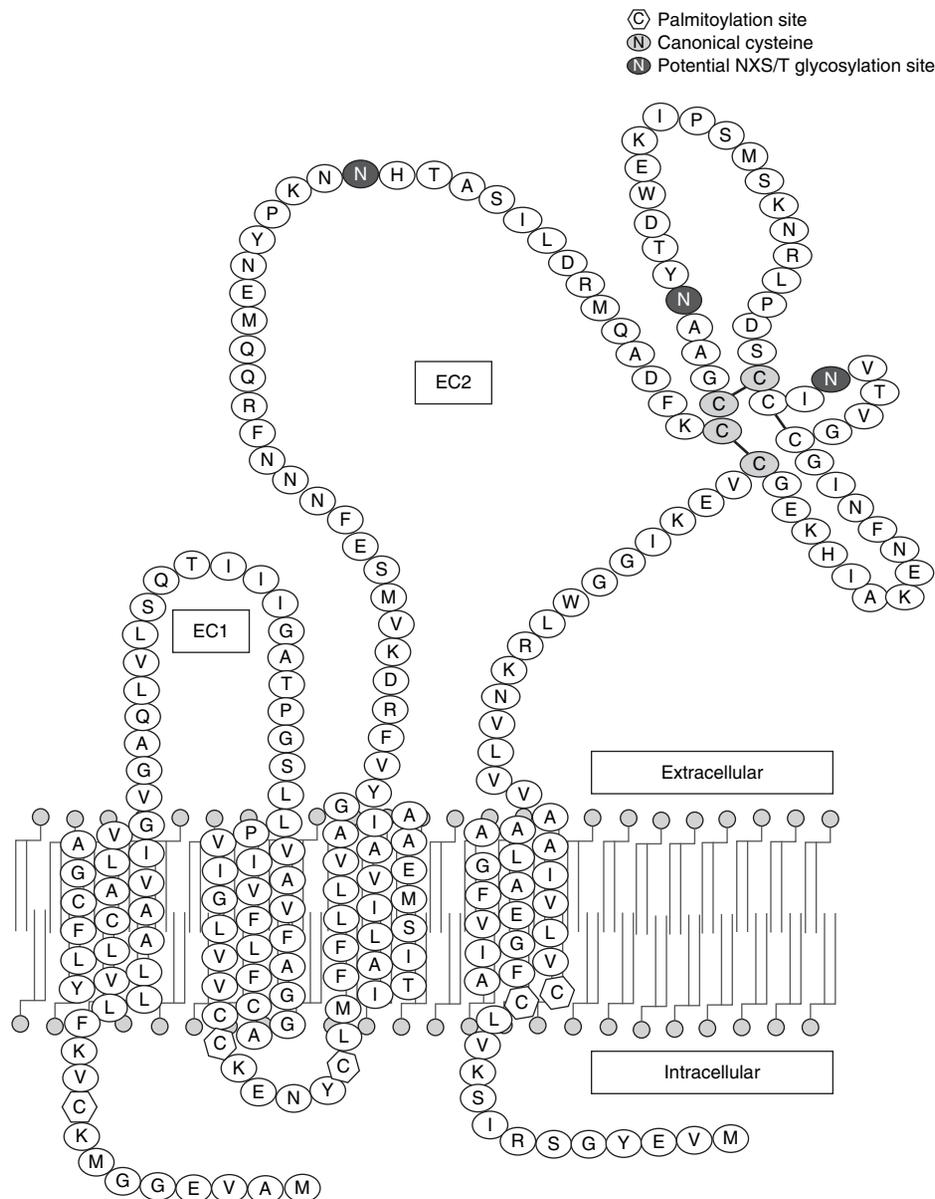
Fig. 1. The tetraspanin CD63. The primary sequence of CD63 (single-letter amino acid code) is shown in the context of its membrane architecture. Key residues that are conserved in the tetraspanin superfamily are highlighted, including charged transmembrane residues and canonical cysteines. The sites of potential modification by *N*-glycosylation and palmitoylation are highlighted. The C-terminal residues GYEVM constitute a lysosomal targeting/internalization motif.[13] **EC1** = extracellular loop 1; **EC2** = extracellular loop 2; **N** = asparagine; **X** = any amino acid except proline; **S** = serine; **T** = threonine.

This has lead to interest in the possibility of targeting tetraspanins for the treatment of infectious disease. Here we will review recent advances in our understanding of the roles of tetraspanins in microbial disease, with emphasis on the effects of modulating tetraspanins/TEMs on pathogen infectivity and the potential application to therapies. Such therapies offer an alternative to antiviral drugs, antibiotics or vaccination, since they are directed at host-cell processes upon which the microbe is dependent (such as cell–cell fusion and intracellular trafficking) rather than the microbe itself. Targeting of tetraspanins may therefore provide new or complementary treatments for pathogens that are refractory to conventional approaches.

1.1 Tetraspanin Enriched Microdomains (TEMs)

In a few cases, tetraspanins have been shown to act as receptors, but most of the functions ascribed to them do not appear to require the binding of specific ligands. Instead, tetraspanins

have been described as 'molecular organizers', forming structures called TEMs by the lateral association of tetraspanins with other tetraspanin and non-tetraspanin membrane proteins.[8] This network is also referred to as the tetraspanin web.[21] Examples of the >30 non-tetraspanin membrane proteins so far found in TEMs are members of the immunoglobulin and integrin superfamilies, MHC proteins, growth factors such as heparin-binding epidermal growth factor-like growth factor (HB-EGF), structural proteins such as claudins, intracellular adaptor molecules such as syntenin, and signaling molecules, such as phosphatidylinositol (PI)-4-kinase. The ability to form these associations helps explain the involvement of tetraspanins in so many inter- and intracellular functions.[22] The nature of the lateral interactions has been probed using panels of detergents to dissociate the TEM components. On this basis, different levels of interactions have been observed, ranging from strong associations (e.g. between CD151 and α3β1 integrin, stable even in 1% Triton X-100[23]) to much weaker associations (e.g. between CD9 and α3β1 integrin) stable only in less hydrophobic detergents such as Brij97.[24] TEMs are recoverable in low-density sucrose gradient fractions and are enriched in certain lipids (e.g. cholesterol and ganglioside GM3).[24] The palmitoylation of juxtamembrane cysteine residues of tetraspanins (figure 1) is critical for the assembly of TEM[25] and is necessary for tetraspanin/tetraspanin interactions, probably stabilized by membrane cholesterol.[26] Some integrins are also palmitoylated and this appears to promote their association with TEMs.[25] By comparison, lipid rafts have different biophysical properties, are more sensitive to cholesterol depletion and contain different arrays of membrane proteins.[27] Visualization of TEMs by fluorescence and electron microscopy[28] have suggested that cells contain many hundreds of TEMs, each ~0.2 μm^2 in area and containing multiple tetraspanins. Single-molecule analysis of CD9 in living cells has also recently shed light on tetraspanin dynamics in TEMs and the plasma membrane.[29] These studies indicate that tetraspanins can form stable interaction platforms that are distinct from lipid rafts and in permanent exchange with the rest of the membrane. CD9 mobility and partitioning into these platforms were shown to be palmitoylation and cholesterol dependent.

The functions of TEMs are becoming clearer. A subset of MHC II molecules have been shown to partition into TEMs rather than lipid rafts; this subset binds a distinct set of peptides that are preferentially displayed for T-cell recognition.[30] In T cells themselves, CD82-containing TEMs promote actin cytoskeleton associations that may lead to more efficient signaling.[31] CD81 is involved in the formation of the immune synapse in both partner cells and has been suggested to facilitate antigen presentation or signaling.[32] EGF receptor signaling can be attenuated by tetraspanin-mediated inhibition of receptor dimerization.[33] Leukocyte adhesion to endothelial cells is partly dependent on the clustering of adhesion molecules in tetraspanin-containing structures termed adhesion platforms.[34] The selective enrichment of tetraspanins in B cell intracellular structures and exosomes suggests a role for TEMs in the formation and trafficking of vesicles.[35] Thus, it seems that a major role for TEMs is the organization of membrane proteins into functional units.

The localization of tetraspanins in TEMs has paradoxically made it difficult to define the functions of individual family members. Reagents such as antibodies that cross-link specific tetraspanins may also perturb the organization and functions of interacting molecules, giving misleading information.[36] Some investigators, including ourselves, have tried to address this by using soluble recombinant EC2 domains as alternative tools for investigating the role of specific tetraspanins. Recombinant EC2s appear to fold correctly and have been shown to have biological activity in a number of systems.[6,37-39] The interactions of tetraspanins with one another in TEMs may also explain the apparent 'functional redundancy' of the family. Of those reported to date, tetraspanin knockout mice are viable and show relatively mild defects, suggesting a capacity for different members of the family to compensate for one another.[4,40] Nonetheless, knockouts clearly demonstrate that particular cellular functions (e.g. T-cell proliferation,[41] sperm–egg fusion[6]) can be affected by the modulation of a specific tetraspanin. Thus, targeting of individual tetraspanins may provide a therapeutic means to modulate cellular processes without a global detrimental effect on the organism.

1.2 Role of Tetraspanins in Trafficking

Although tetraspanins were first identified as cell surface markers, it is now clear that some are also associated with the endosomal pathway (i.e. early and late endosomes, multivesicular bodies [MVBs] and lyzosomes) and with various types of secretory vesicles.[3,42] Tetraspanins on the plasma membrane can be internalized via endocytosis and traffic to intracellular vesicles; conversely, on cell stimulation, secretory vesicles containing tetraspanins may fuse with the plasma membrane. Late endosomes and MVBs can also fuse with the plasma membrane and release 'exosomes' that are enriched in certain tetraspanin proteins. Exosomes released from cells infected with intracellular pathogens, including mycobacteria, are potent stimulators of inflammation in uninfected cells (reviewed in Schorey and Bhatnagar[43]) and can present antigens to T cells.[44] Thirteen of the 33 mammalian tetraspanins contain potential tyrosine-based

sorting motifs.[3] These are based on the sequence YXXØ (where X is any amino acid and Ø is a bulky hydrophobic residue), which is recognized by a family of adaptor protein (AP) complexes that determine the cellular localization of proteins they interact with.[13] Other tetraspanins (CD231 [TSPAN7], oculospanin [TSPAN10] and TSSC6 [TSPAN32]) carry potential dileucine sorting motifs.[3] However, in most cases, the functionality of these motifs in tetraspanins is not known.

The intracellular trafficking of tetraspanin CD63 has been best characterized.[3,42] It has a conserved GYEVM motif at its C-terminus, which interacts primarily with AP-2 complexes, linking the protein to clathrin-mediated endocytosis pathways, and with AP-3 complexes for trafficking from endososmes to lysosomes, although the protein may also be targeted to lysosomes directly from the trans Golgi network.[45] CD63 can interact directly through its C-terminus with syntenin,[46] which may compete with AP-2 and AP-3 to alter the trafficking of this protein. It has also been recently suggested that CD63 may internalize from the plasma membrane by endocytosis of caveolae.[3,42]

One of the roles of CD63 related to trafficking appears to be in regulating the functions of molecules it associates with on the plasma membrane by inducing their internalization. Experimental evidence for this comes from investigations on its association with, and effects on, members of the H$^+$, K$^+$-adenosine triphosphatase family of proton pumps,[47] membrane-type 1 matrix metalloproteinase,[48] and integrins.[49] Other tetraspanins that possess internalization motifs may display similar activities, although these have been less extensively studied.[3] A role for CD63 in intracellular targeting was recently shown by the demonstration that it cooperates in the delivery of the enzyme elastase to secretory vesicles of neutrophils.[50]

1.3 Role of Tetraspanins in Fusion

Tetraspanins have been implicated in various cell–cell fusion processes. Most notably, studies using knockout mice have shown that CD9 and CD81 are required for sperm–egg fusion, since oocytes from CD9–/– or CD9–/–, CD81–/– mice are unable to fuse with sperm, resulting in partial or complete infertility, respectively (reviewed in Sutovsky[51]). Studies using antibodies and tetraspanin over-expression have also implicated CD9 and CD81 in muscle cell fusion.[52] Investigations using knockout mice and antibodies have identified a role for CD9 and CD81 in the fusion of monocytes/macrophages to form multinucleated giant cells (MGCs).[53] Using soluble recombinant EC2 domains, we confirmed roles for CD9 and CD81 in MGC formation and

additionally demonstrated the involvement of CD63.[39] In contrast to their requirement for sperm–egg fusion, the evidence suggests that CD9 and CD81 act as negative regulators of MGC formation, whereas CD63 appears to be positively involved.[39,53] MGCs are a feature of granulomatous inflammation, formed in response to chronic infections with hard-to-clear pathogens (e.g. tubercle bacilli, schistosomes).[54] Some viruses (e.g. HIV, human T-cell leukemia virus [HTLV]) have the capacity to induce cell–cell fusion resulting in the formation of giant cells or virus syncitia that contribute to spread of infection. Various tetraspanins including CD9, CD81, and CD82 have been linked with virus-induced syncitium formation (reviewed in Martin et al.[16]) and discussed in this review.

2. Role of Tetraspanins in Infections

2.1 Viral Infections

Viruses are obligate intracellular parasites, relying on host-cell functions for most aspects of their life-cycle. To initiate this cycle, viruses must first form a stable attachment to the host-cell surface and then gain access to the cytoplasm. In the case of enveloped viruses, this involves fusion of the lipid envelope with the host plasma membrane, or after endocytosis and trafficking, the envelope may fuse with the membranes of intracellular vesicles. Following virus uncoating, the viral genome that is released into the cytosol must gain access to an appropriate site where replication can proceed, using the host-cell biosynthetic machinery. Assembled mature virus particles then exit the cell to spread the infection. Whilst this can be mediated by cell lysis, enveloped viruses can also bud out through the host-cell plasma membrane and increasing evidence suggests that they may also exploit vesicular trafficking to be released via exosomes.[55] Some viruses are also transmitted directly from infected cells to uninfected cells via an 'infective' or 'virological synapse'[56] or through fusion of infected cells with uninfected cells to form viral syncitia.[16] Tetraspanins, particularly in the context of TEMs, are involved in many of these functions and unsurprisingly have been increasingly linked to crucial events in the life-cycle of a variety of viruses.[40]

2.1.1 Family Flaviviridae: Hepatitis C Virus (HCV)

The best characterized physical interaction of a tetraspanin with a virus is that of CD81 with HCV. The flaviviridae are enveloped, positive single-stranded RNA viruses. HCV infects approximately 180 million patients worldwide.[57] It affects liver tissue almost exclusively, resulting in hepatitis, which may become chronic, progressing to cirrhosis and hepatocellular

carcinoma. As well as showing marked tropism for liver, HCV shows strict species specificity, infecting only humans and chimpanzees.[58] Two glycoproteins, E1 and E2, that assemble as a heterodimer, are embedded in the HCV envelope and are essential for virus binding and entry into host cells.[59] CD81 was the first protein ligand identified for HCV[60] and interacts directly with the E2 protein as demonstrated by inhibition of binding by anti-CD81 mAbs and soluble CD81 EC2 domains.[60,61] However, whilst expression of human CD81 is sufficient for E2 binding, accumulated evidence has shown that it is not in itself sufficient for infection by HCV.[37] CD81 has thus been identified as a required co-receptor for HCV.[62]

Role of CD81 in HCV Entry

In addition to CD81, three other host-cell molecules have now been shown to be essential for HCV entry: scavenger receptor BI (SR-B1),[63] the tight junction protein claudin-1 (CLDN1)[64] and most recently, human occludin.[65] Entry of HCV into host cells is complex and involves multiple steps; the exact role of the co-receptors in this process remains to be determined. Following binding to host cells, HCV is internalized by clathrin-dependent endocytosis and acid-dependent fusion probably occurs in early endosomes[66] (reviewed in Helle and Dubuisson[17]). Antibodies to CD81 that inhibit infection appear to act post-attachment and CD81 is not essential for virus binding.[67] SR-B1 also binds to HCV E2 proteins[68] and there is evidence that it may act co-operatively with CD81 in HCV entry at a post-binding stage.[69] Studies on the kinetics of antibody inhibition suggest that SR-B1 and CD81 act concomitantly (half maximal inhibition ~17 minutes[70,71]). Claudin, by contrast, is required for a later stage in the entry of the virus (half maximal inhibition of entry by antibody ~73 minutes[64]). Interestingly, fluorescence resonance energy transfer (FRET) analysis has shown that CD81 can interact with claudin on the surface of susceptible hepatocytes.[72] Claudin and occludin are both components of tight junctions, structures that form seals between adjacent cells and allow cells to become polarized.[65] Other viruses have been shown to exploit tight junctions as a means of entering cells, e.g. coxsackie virus B, which also requires occludin for entry.[73] Recent work has shown that engagement of CD81 by antibodies or HCV proteins results in guanosine triphosphatase (GTPase)-dependent actin rearrangement allowing lateral movement of ligated CD81 to tight junctions where claudin and occludin are localized.[74] This has suggested a model where following binding to the apical surface of hepatocytes, HCV associates with SR-B1 then CD81, which then traffics HCV to tight junctions.[65,74] Consistent with this, disruption of tight junctions and/or polarization of hepatocytes appeared to reduce HCV infectivity.[75]

Other Roles of CD81 in HCV Infection

CD81 engagement activates the Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling cascade; inhibitors of this pathway significantly reduced HCV infection efficiency, indicating that this pathway affects post-entry events of the virus life-cycle.[74] There is also evidence that CD81 may affect trafficking of HCV envelope proteins, directing their incorporation into exosomes where it associates with them.[76] Exosomes containing HCV RNA have been isolated from the plasma of HCV patients and it is suggested that this may represent a route of infection. As discussed in section 2.1.2 below, exosomes have also been suggested as a route of infection for HIV.

At least *in vitro*, it has been demonstrated that HCV infection can also occur via cell–cell contact and it is speculated that this may contribute to its spread *in vivo*. However, CD81 does not appear to be required for this mode of infection.[77]

CD81 is widely expressed, particularly on cells of the immune system. It is still uncertain whether HCV infects these cells, but binding of HCV proteins to CD81 may have pleiotropic effects, including downregulation of natural killer cell functions,[78] priming and activation of T cells,[79,80] and hypermutation of the immunoglobulin genes in B cells.[81] In addition, E2 binding to CD81, on hepatic stellate cells, upregulates matrix metalloproteinase-2 possibly increasing inflammation and liver damage *in vivo*.[82]

2.1.2 Family Retroviridae

HIV-1

HIV-1 is an enveloped lentivirus, the main causative agent of AIDS. Whilst combinations of drugs have helped control the disease in developed countries, the expense of this treatment has hindered its use worldwide. So far, an effective vaccine has not been developed to prevent AIDS, in large part because of the high mutation rate of the virus.

Tetraspanins have been implicated in various stages of the HIV life-cycle in different cell types. The virus is known to enter and multiply in macrophages, as well as CD4+ T lymphocytes, although it replicates more slowly in the former, which may act as a 'reservoir' of infection.[83] Dendritic cells are also involved in the pathogenesis of HIV[84] by either passing the virus into CD4+ T cells without themselves becoming infected (trans-infection)[85] or, more rarely, by acting as a host for virus replication (cis-infection).[86] Infection is initiated when the viral envelope glycoprotein gp120 binds to the CD4 receptor[87] on target cells, which induces conformational changes leading to binding of gp120 to the CCR5 or CXCR4 co-receptors.

In T cells, a fusion event between host plasma membrane and the viral envelope mediated by viral gp41 follows, culminating in the delivery of the virus into the cell. In macrophages and cis-infected dendritic cells, it has been suggested that HIV virus may be endocytosed prior to fusion.

Role of Tetraspanins in the HIV Virion

Early reports that CD63 is selectively incorporated into the envelope of the HIV-1 virion[88-90] suggested a role for this tetraspanin in infection. Perhaps surprisingly, however, virus particles derived from macrophages where CD63 expression has been knocked down showed no decrease in infectivity.[91] Interestingly, another recent report shows that increased incorporation of CD63 into the viral envelope correlates with decreased viral infectivity.[92] This effect is strain-dependent, appearing to relate to differences in the Env protein, and affects a post-attachment step, which results in decreased fusion with T cells. A similar decrease in infectivity was observed with increased viral envelope incorporation of other tetraspanin proteins (CD9, CD81, CD82, and CD231) but not L6, a non-tetraspanin four-span membrane protein. As well as modulating HIV-1 infectivity, these findings suggest a role for tetraspanins in HIV-1 entry.

Role of Tetraspanins in Early HIV Infection

A role for CD63 in early stages of HIV-1 infection was also indicated by its inhibition by anti-CD63 antibodies,[93] although the effect here was CCR5 dependent, specific for macrophages and not observed with antibodies to other cell surface tetraspanins CD9, CD81, and CD82. The authors speculated that the effects were post-fusion; however, this was based on lack of effects of CD63 overexpression or anti-CD63 antibodies on a cell-line model of macrophage syncitium formation, rather than virus–cell fusion. Our group demonstrated that macrophage HIV-1 infection could also be inhibited by soluble recombinant tetraspanin EC2 domains.[38] Although the CD63 EC2 was most potent, other tetraspanins (CD9, CD81, and CD151) were also inhibitory for both CCR5- and CXCR4-dependent infection of macrophages. At higher doses, these tetraspanin EC2s also inhibited CCR5-, but not CXCR4-dependent T-cell infection. Since effects were observed with a range of tetraspanin EC2s, we speculated that they disrupted cell surface TEMS, interfering with binding to TEM-dependent complexes or with TEM-dependent membrane fusion events.

As mentioned in section 1.3, tetraspanins CD9, CD63 and CD81 have known roles in fusion events. Gordon-Alonso and co-workers[94] showed that mAbs against CD81 enhanced HIV-1 infection of T lymphoblasts and mAbs to CD9 and CD81 enhanced HIV-1 envelope-induced syncytia formation. The same effect was obtained by knockdown of the two proteins, while their over-expression decreased both entry and syncytia formation.[94] The effects observed here, indicating a negative role for CD9 and CD81 in syncitium formation, has parallels with the effects of antibodies and CD9 and CD81 knockout on macrophage fusion leading to the formation of giant cells.[39,53]

Role of Tetraspanins in HIV-1 Trafficking and Egress

In trans-infection of dendritic cells, it has been reported that infectious particles are taken up into a non-conventional, non-lysosomal, tetraspanin-rich compartment, similar to MVBs, and may then be transferred to T cells via the 'infective synapse'[56] or by exocytosis and capture.[55] This has clear analogies with the tetraspanin-enriched compartments involved in antigen presentation[30,32] and the role of tetraspanins in formation/organization of the immunological synapse.[32,95] Replicating virus seems to reside in a similar compartment in cis-infection of dendritic cells.[96]

In macrophages, HIV-1 has also been reported to accumulate in compartments resembling multivesicular endosomal bodies that are enriched for tetraspanins.[97-100] It was speculated that the virus budded into this compartment and was then released via the exosomal pathway. However, more recent data indicate that this compartment is, in fact, a deep invagination of the plasma membrane, also observed in non-infected cells, that may be related to patocytotic compartments involved in the uptake of aggregated cholesterol.[101] Such structures have also been observed in non-infected dendritic cells and they resemble the compartments in dendritic cells where HIV accumulates. In uninfected macrophages, this compartment contains tetraspanins CD81, CD9, and CD53 (TSPAN25), but not CD63; CD63 was recruited into the compartment in infected cells and subsequently incorporated into the viral envelope.[101]

Experiments designed to examine the role of CD63 in HIV-1 infection of macrophages using small interfering RNA (siRNA) knockdown have produced contradictory results. Whilst one group found no effect of CD63 knockdown on virus assembly or infectivity of macrophage-derived virus,[91] another reported that this treatment inhibited viral replication in both primary macrophages and a macrophage cell line.[102] The reasons for this apparent discrepancy are unclear, but the inhibitory effects of anti-CD63 antibodies[93] and CD63 EC2 proteins[38] argue that this tetraspanin is involved in at least the initial stages of macrophage infection by HIV-1.

HIV-1 buds from the plasma membrane of CD4+ T cells. As mentioned above, CD63 is specifically enriched in HIV particles, despite its low abundance at the surface of T lymphocytes.[89] To address this apparent contradiction, Nydegger and

co-workers[28] used fluorescence and immunoelectron microscopy to examine the distribution of tetraspanins in HeLa cells transiently transfected with HIV-1 provirus or with plasmids expressing Gag or Env proteins. In untransfected HeLa cells, they observed that CD63 clustered at discrete sites in the plasma membrane with CD9, CD81 and CD82 to form TEMs. In transfected cells, these CD63-enriched TEMs co-localized in the plasma membrane with Gag, the major structural protein that directs viral assembly and release, together with Env. Components of the host-cell extra-vesiculation system utilized by HIV-1 for budding were also recruited to CD63-enriched TEMs in these cells, suggesting that TEMs may act as 'gateways' for HIV-1 exit. HIV-1 also co-localized with CD63 and CD9 in Jurkat T cells, indicating that TEMs are used for virus egress by T lymphocytes. Other groups have also found that HIV-1 buds from the plasma membrane of T cells in exosomes or microdomains that are enriched for tetraspanins.[103,104] It was also reported that CD63, CD81, and, to a lesser extent, CD9 were recruited to the infective/virological synapse formed between infected and non-infected T cells and that antibodies to these tetraspanins inhibited synapse formation.[103] In addition to co-localizing with TEMs, co-immunoprecipitation of CD81 and HIV-1 Gag from a chronically infected T cell line indicated direct or indirect interactions of these proteins in TEMs.[105] Furthermore, anti-CD81 antibodies and siRNA knockdown of CD81 inhibited HIV-1 release and impaired virus infectivity, correlating with redistribution of Gag at the cell surface on CD81 silencing. Taken together, this suggests a critical role for TEMs, and CD81 in particular, in the late stages of HIV-1 infection of T cells.

Other Roles of Tetraspanins in HIV Infection

A role for CD81 ligation in increasing HIV-1 transcription in infected T cells has been reported.[106] This might have significance where HIV-positive patients are co-infected with HCV, the only known direct 'ligand' for CD81.

A possible role for CD63 in trafficking of the CXCR4 co-receptor for HIV-1 in T cells is indicated by recent work.[107] A mutated version of CD63, lacking the last 81 N-terminal residues, caused mislocalization of CXCR4 to late endosomes/lysosomes, blocking HIV-1 entry. Wild-type CD63 also has some effects on CXCR4 trafficking, but was much less potent.

Finally, a role for tetraspanin-enriched exosomes derived from CD8+ cytotoxic T cells in defence against HIV-1 infection has recently been reported.[108] These exosomes were shown to suppress CCR5-tropic and CXCR4-tropic replication of HIV through a noncytotoxic mechanism involving an as yet unidentified protein moiety. This finding may have parallels with inhibitory effects of envelope-embedded tetraspanins referred to above.[92]

Human T-Cell Leukemia Virus

HTLV-1 or primate T-lymphotropic virus is the infectious agent associated with adult human T-cell leukemia and HTLV-1-associated myelopathy/tropical spastic paraparesis.[109] It belongs to the Deltaretrovirus genus and resembles other retroviruses in having an envelope that plays an important role in viral adhesion and fusion. The virus infects CD4+ and CD8+ T cells; free virus particles are not highly infective and virus spread is mainly due to cell-to-cell transmission.[110,111] The tetraspanin CD82 is abundant on the surface of T cells, increases after T-cell activation[112] and its engagement leads to increased cell–cell adhesion mediated by lymphocyte function-associated antigen-1 (LFA-1). CD82 can also couple cell surface complexes to the cytoskeleton.[95] CD82 was originally identified as the protein target of mAbs that inhibited T-cell syncytium formation induced on co-culture with HTLV-1 *in vitro*,[113,114] and so was suggested to have a negative regulatory role in the HTLV-1 life cycle. In addition, co-expression of CD82 with HTLV-1 envelope proteins in COS-1 cells decreased virus-induced syncytium formation as well as cell–cell transmission.[115] Co-immunoprecipitation and co-capping experiments showed that CD82 and HTLV-1 envelope proteins were associated, both inside cells and at the cell surface. Gag, which is essential for viral assembly and release,[116] is targeted to the plasma membrane by its matrix (MA) domain. Gag protein was shown to co-localize with CD82 and other tetraspanins (CD53, CD81, and CD231) on the plasma membrane of T leukemia cell-line cells and co-segregated with CD82 on immune synapse formation with B cells. When expressed alone, the MA domain also co-immunoprecipitated with CD82 from cells lysed using mild detergent.[117] Further studies using site-specific mutagenesis have revealed that CD82 and CD81 inner loops are essential for their interaction with HTLV-I Gags.[118] Taken together, this suggests an essential role for CD82-enriched TEMs in the transmission of the budding virus to intercellular adhesion loci that form the infectious synapse. This has clear analogies with the reported involvement of TEMS in HIV-1 release.[28] Finally, tetraspanin CD151 associates with α5β1 in HTLV-1 infected T cells and enhanced the α5β1-mediated adhesion of infected cells to fibronectin, further supporting a role for tetraspanins in HTLV1 infection.[119]

Feline Immunodeficiency Virus

Tetraspanin CD9 also appears to be involved in infection by feline immunodeficiency virus (FIV), which, like HIV, is a lentivirus and is responsible for an AIDS-like disease in cats.[120] An antibody that blocked FIV infection of target cells was shown to recognize CD9, suggesting it as a possible receptor for

FIV.[121] However, cells that are negative for CD9 can be infected by FIV[122] and the anti-CD9 antibody was subsequently shown to act after viral entry, affecting virus assembly or release.[123,124] Cells ectopically expressing CD9 showed enhanced infectivity for FIV, suggesting a direct role for this protein in virus trafficking or assembly that was disrupted by the anti-CD9 antibody.[123]

2.1.3 Paramyxoviridae: Canine Distemper Virus

Canine distemper virus (CDV) is an enveloped, negative-stranded RNA morbillivirus causing a measles-like infection in carnivores with a high incidence of encephalitis.[125] A relationship with tetraspanins was first demonstrated by the finding that a mAb against CD9 bound to the surface of target cells inhibiting CDV infection, whereas ectopic expression of CD9 rendered cells susceptible to infection and increased virus production.[126] There was no direct interaction between CD9 and CDV and the anti-CD9 mAb did not affect virus entry, but inhibited virus release and syncytium formation, indicating an effect on trafficking and cell–cell fusion.[126,127] Susceptibility to CD9 antibodies was shown to be specifically dependent on the extracellular domain of CDV haemagglutinin protein.[128] This suggests that CD9 is involved in regulating the activity or membrane configuration of an unknown receptor for CDV haemagglutin that is involved in cell–cell fusion. Most recently, it was shown that specific anti-CD9 antibodies induced CD9 clustering and the formation of CD9-enriched microvilli-like protrusions at areas of cell–cell contact.[129] Clustering was not induced by all anti-CD9 antibodies or by Fab fragments and this correlated with an inability to inhibit CDV-induced cell fusion, implying dependence on cross-linking and/or epitope specificity. The clusters also contained other tetraspanins (CD63, CD81, and CD82) indicating that anti-CD9 antibodies can alter the overall surface organization of TEMs. CDV proteins, but not related measles virus (MV) proteins, were specifically excluded from these clusters, suggesting that anti-CD9 antibodies act in this case by effectively removing the viral fusion machinery from cell contact areas.

Intriguingly, CD9 is reported to associate with CD46, the human receptor for MV,[130] and forms a complex with α3β1 integrin and the tyrosine phosphatase SHP-1 during induced differentiation of monocytes to macrophages.[131] Formation of the complex correlates with increased permissiveness for MV infection; however, no direct role for CD9 in MV infection has been reported.[128-130]

2.1.4 Porcine Reproductive and Respiratory Syndrome Virus

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped single-stranded positive-sense RNA virus belonging to the Arteriviridae family, which causes respiratory and reproduction disease in swine. The virus primarily targets alveolar macrophages. Porcine CD151 was recently shown to interact specifically with the 3′ untranslated region (UTR) of PRRSV RNA.[132] Ectopic expression of CD151 rendered cells sensitive to PRRSV infection and siRNA knockdown of CD151 reduced viral replication, whereas an antibody to CD151 completely blocked infection. The significance of the interaction of CD151 with the 3′-UTR of PRRSV RNA is not clear. However, CD151 is known to be involved in intracellular trafficking[3] and the authors speculate that it may be involved in the localization of ribonucleoprotein complexes to the site of viral replication.[132]

2.1.5 Papillomaviridae

Human papillomaviruses (HPVs) are non-enveloped DNA viruses associated mainly with benign warts on infection of epithelia.[133] However, some types, notably HPV16, are directly related to the development of cancers of the lower female genital tract, especially cervical carcinoma.[134] HPVs bind to modified heparin sulphate proteoglycans on host cells and are taken up following a conformational change in capsid proteins.[135] Transfer to transient 'receptors' including α6 integrin and laminin 5 is thought to occur prior to uptake.[136] Clathrin-mediated endocytosis was previously implicated in HPV16 uptake.[137] However, a recent report using specific inhibitors indicated that uptake of this virus is clathrin-, caveolae- and lipid-raft independent, and is mediated by a mechanism involving TEMs.[138] HPV16 pseudovirions were found to co-localize with CD63 and CD151 on the surface of HeLa cells and mAbs against CD63, CD81, and CD151 inhibited virus uptake and infection, with anti-CD151 antibodies showing greatest potency. CD151 siRNA knockdown also inhibited HPV16 uptake.[138] CD151 is highly expressed on epithelial cells and the authors propose that TEMs enriched for CD151 may act as entry platforms for HPV16. Antibodies may cause internalization of TEM components and knockdown may disrupt TEMs, inhibiting viral uptake. CD151 is known to interact with α4β6 integrin, but siRNA knockdown of CD151 did not affect α6 expression on HeLa cells. This suggests that binding of the virus to this integrin alone is not sufficient for viral entry, although CD151 silencing may affect the interaction of α4β6 integrin with TEMs. The subsequent mechanism of viral uptake is unknown, but, as mentioned previously, CD63 can associate with syntenin and could thereby mediate clathrin-independent endocytosis.[3] There is also evidence that CD151 may internalize by alternative mechanisms.[3] TEM-dependent internalization might serve as a general alternative

entry route for other viruses e.g. HIV-1 and possibly other pathogens.

2.2 Protozoal Infection: *Plasmodium* Species

Malaria remains one of the most common parasitic diseases causing millions of fatalities annually.[139] Infection with *Plasmodium* spp. is initiated by transfer of sporozoites following a bite by an infected female *Anopheles* mosquito. The sporozoites quickly invade hepatocytes and reside in a parasitophorous vacuole where they differentiate into the merozoites that invade erythrocytes causing the symptoms of malaria.[140] CD81 has been shown to be necessary for infection of human and rodent hepatocytes, but not erythrocytes, with *Plasmodium falciparum* and *Plasmodium yoelii*, respectively.[18] Sporozoites were unable to infect hepatocytes from CD81 knockout mice *in vivo* and *in vitro* and anti-CD81 antibodies inhibited sporozoite development in both rodent and human hepatocytes. By contrast, CD81$^{-/-}$ hepatocytes were infected with another rodent parasite, *Plasmodium berghei*, demonstrating that it can use CD81-independent pathways of invasion. No inhibition of infection of susceptible hepatocytes was observed with soluble CD81 EC2s, indicating that the tetraspanin does not act as a direct receptor for sporozoites but may be involved in their internalization or in the mechanism of parasitophorous vacuole formation.

A role for membrane cholesterol in the CD81-dependent infection of hepatocytes has been indicated, as cholesterol depletion inhibited *Plasmodium yoelii* and *Plasmodium falciparum* infection of hepatocytes whereas *Plasmodium berghei* infection was unaffected.[141] Certain tetraspanins have been shown to associate with cholesterol,[142] and cholesterol depletion reduces the binding of an anti-CD81 antibody that specifically recognizes CD81 when it is associated with other tetraspanins. These results suggest that cholesterol depletion disrupts the organization of CD81 within TEMs, which is key to its role in facilitating *Plasmodium* spp. entry. Cholesterol depletion has also been reported to affect HCV infectivity, in this case by increasing CD81 internalization;[69] however, in the hepatocyte/ malaria studies, Silvie and co-workers[141] reported no effects on CD81 cell surface expression.

Plasmodium infection is independent of CD9, but CD9 is thought to have a similar overall folding pattern to CD81 and has 45% identity at the amino acid level. Attempts have been made to define the region on CD81 that is critical for *Plasmodium* spp. infection using CD9/CD81 chimeras.[143] These studies highlighted a stretch of 21 amino acids in the structurally conserved sub-domain of the EC2 of CD81 as being important

for infectivity. This corresponds to the junction of A and B alpha helices in the intact protein, which is quite distinct from the site in the variable sub-domain of the EC2 involved in HCV E2 binding.[14] Mutagenesis confirmed the importance of this region and of residue D137 in particular. Surprisingly, an antibody recognizing this region did not block infection with *Plasmodium* spp.,[143] in contrast to other antibodies, e.g. 1D6, which is known to bind to a peptide corresponding to residues 179–193 in the variable sub-domain of the EC2 region.[144] This suggests that inhibitory antibodies may perturb the interaction of CD81 with partner molecule(s) or otherwise disrupt its organization within TEMS, whereas the non-inhibitory antibodies may fail to recognize certain functionally important TEM-associated pools of CD81. Mutations in the EC1 also affected *Plasmodium* infectivity, but this is thought to reflect its role in stabilizing the conformation of the EC2 region.[143]

It is clear that CD81 is not the only molecule involved in the CD81-dependent entry of *Plasmodium* spp. into hepatocytes. Expression of human CD81 in CD81-knockout mouse hepatocytes confers susceptibility to *P. yoelii* but not *P. falciparum* sporozoite infection. Also, expression of CD81 in a human hepatocarcinoma cell line is sufficient to promote the formation of parasitophorous vacuoles by *P. yoelii* but not *P. falciparum* sporozoites.[145] Intriguingly, host scavenger receptor SR-BI, which together with CD81 is required for HCV infection of hepatocytes, has also been shown to be important in infection by *Plasmodium* spp., affecting both sporozoite invasion and intracellular parasite development.[146]

2.3 Bacterial Infections

Tetraspanins have been implicated in the pathogenesis of numerous bacterial species, including direct roles in bacterial growth/ infection and in the pathogenesis caused by bacterial products.

2.3.1 Diphtheria

Diphtheria is an acute illness caused by the bacterium *Corynebacterium diptheriae* releasing diphtheria toxin (DT), an inhibitor of cellular protein synthesis, at the site of infection and in the circulation.[147] CD9 was identified as a protein that associates with the diphtheria toxin receptor (DTR),[148] which is identical to the membrane anchored form of the HB-EGF.[149] Overexpression of CD9 increases DT binding to the surface of cells rendering them more susceptible to DT. A co-receptor function for CD9 has been suggested by the finding that it increases the affinity of the DTR for DT and interacts with the receptor through its EC2.[150,151]

2.3.2 Uropathogenic Escherichia coli

Urinary tract infections are amongst the most common infectious diseases and >80% are caused by *Escherichia coli* that bind to the bladder epithelia through adhesive pili, also known as type I fimbriae.[152] Of the four types of uroplakins that are the major protein constituents of the plaques that cover the apical surface of the urothelium, two (uroplakin 1a [TSPAN21] and uroplakin 1b [TSPAN20]) are tetraspanins.[153] Following a demonstration that fimbriated *E. coli* bound to uroplakin 1a and/or uroplakin 1b *in vitro*,[154] uroplakin 1a was confirmed as a receptor for Fim H, the lectin found at the tip of type I fimbriae.[155] Binding has been attributed to high levels of terminally exposed mannose residues that are present in uroplakin 1a, but not uroplakin 1b.[156] Uroplakin 1a is associated in bladder epithelial cells with lipid rafts, and *E. coli* have recently been shown to invade these cells in a caveolin lipid raft-dependent manner.[157] Internalized *E. coli* may cause quiescent infections that are resistant to antibiotic treatment, host immune defences and release during bladder voiding.[158] A recent report has shown that internalized *E. coli* reside in CD63+ vacuoles within the bladder epithelium. Treatment with agents that induced exocytosis reduced the number of internalized bacteria.[159]

2.3.3 Chlamydiae

Chlamydia cause a number of human diseases, including the common sexually transmitted infection caused by *Chlamydia trachomatis*. *C. trachomatis* is also the main cause of infectious blindness worldwide.[160] *Chlamydiae* are obligate intracellular pathogens that can only replicate within a vacuole known as the inclusion body. Within the inclusion, the bacterium acquires the host-cell derived components required for its survival. A recent study investigating the interaction between the chlamydial inclusion body and MVBs showed that CD63, which is strongly expressed in MVBs, was selectively delivered to the inclusion. Exogenously applied antibodies to CD63 trafficked to the inclusion and appeared to disrupt chlamydial development and reduced the number of infective progeny produced.[19] However, a more recent study showed that siRNA-mediated CD63 knockdown had no effect on the interaction of MVB with the inclusion, cholesterol accumulation or chlamydial development. An effect of whole antibodies, but not Fab fragments was seen, indicating that CD63 itself is not involved in these processes, but the binding of divalent antibody may affect the function of adjacent membrane components.[161]

2.4 Prions

There have been recent reports that tetraspanins are involved in the interactions and trafficking of the normal version of the prion protein, PrP(c). An altered form of the prion protein, PrP(Sc), is the infective agent associated with the transmissible spongiform encephalopathies (e.g. Creutzfeldt-Jakob disease [CJD]). PrP(c) associates with lipid rafts and in some cells is endocytosed through caveolae, whereas in others, such as neurons, it translocates out of the rafts and is endocytosed via clathrin-coated pits.[162] In human erythroblasts, PrP(c) has been shown to co-localize in the plasma membrane with CD81.[20] Cross-linking with antibodies to PrP(c) or CD81 causes clustering of the two proteins, suggesting that they reside in the same microdomain, possibly TEMs. PrPc is rapidly internalized by the endocytic pathway in erythroblasts, where it co-localizes with CD63. The authors speculated that release of PrP(Sc) in exosomes formed via the endocytic recycling pathway could contribute to the spread of infective prions in blood. Furthermore, using a yeast two-hybrid system, bovine CD231 (TSPAN7) was identified as a partner for PrP(c) and shown to co-localize with the prion protein.[163] CD9 has also been reported to show elevated expression in the brains of prion-infected mice and in CJD patients,[164] although no discernible difference in prion replication was observed between wild type and CD9–/– mice.[165]

2.5 Tetraspanins Expressed by Vectors/Pathogens

Since tetraspanins are widely expressed in eukaryotic organisms, they are also expressed by some pathogens or pathogen vectors, where they may provide further therapeutic targets.

2.5.1 Dengue Fever

Dengue virus is transmitted between humans by mosquito vectors, mainly *Aedes aegypti* and *Aedes albopictus* and causes Dengue fever, an acute self-limited disease that may progress to Dengue haemorrhagic fever. Dengue fever is the most common arthropod-borne viral infection in humans[166] with >50 million cases per year. Whilst mammalian cells usually undergo apoptotic cell death on infection,[167] the Dengue virus may result in permissive infection in mosquito cells, although the infected cells may form syncytia.[168] Using subtractive hybridization techniques, C189 was recently identified as a mosquito tetraspanin that is upregulated ~4-fold after Dengue virus infection.[169] Although knockdown of C189 expression using RNA interference (RNAi) had no apparent effect on virus growth, the tetraspanin was shown to co-localize with viral proteins in the intracellular membranes and especially the plasma membrane of infected cells. It would be interesting to determine if C189 plays any role in cell–cell spread or virus release, as appears to be the case for

mammalian tetraspanins.[56] However, C189 was not identified as an insect host factor in a recent genome-wide RNAi screen.[170]

2.5.2 Tetraspanins in Schistosomes

One of the first members of the tetraspanin family to be identified was the antigen Sm23, expressed by the blood-dwelling helminth, *Schistosoma mansoni*.[171] Although Schistosomes are not microbes, these pathogens infect 200 million people worldwide and schistosomiasis kills hundreds of thousands annually. Tetraspanins are expressed abundantly on the tegument of Schistosomes and recombinant versions of *S. mansoni* tetraspanins TSP-1 and TSP-2 have been shown to be protective in a mouse model.[172] Infestation of water buffalo by *S. japonicum*, a helminth transmitted to humans mainly from infected animals, was also reduced by a DNA vaccine based on the tetraspanin SjC23.[173]

3. Targeting Tetraspanins

From the preceding sections it is clear that tetraspanins are involved in infections caused by diverse pathogens (table I) and that their roles can be broadly divided into two groups. First, there are effects via direct interactions of tetraspanins with the pathogen/specific pathogen-expressed proteins (e.g. CD81 with HCV E2 and uroplakin Ia with *E.coli* Fim H). Secondly, there are indirect effects by host-cell functions that are dependent on TEMs. These may include the organization of host-cell receptor complexes or processes such as endocytosis, trafficking, fusion, or exocytosis (table I). These groups overlap, as it is likely that direct pathogen–tetraspanin interaction reflects a requirement of the pathogen to interface with TEMs. Many pathogens are thought to have evolved strategies to exploit or 'highjack' lipid rafts.[174] TEMs represent a more specialized microdomain, which may similarly have been targeted by certain microbes. In this way, TEMs may provide various pathogens with 'gateways' to enter, as well as to leave, host cells.[28]

Instances where tetraspanins interact directly with pathogen proteins provide clear strategies for therapeutic targeting of specific diseases (i.e. by designing antagonists of host tetraspanin-microbe interactions). A more intriguing possibility, however, is an approach that modulates TEM-dependent host-cell functions to provide a more broadly active type of therapy. This is of interest for several reasons:

1. It would target fundamental cellular processes in pathogen pathology that the microbe would not be able to surmount by mutagenesis of its own genome.

2. By targeting cellular processes (e.g. fusion, trafficking) rather than the antigenic/mechanistic idiosyncrasies of a specific pathogen, tetraspanin therapies may be able to provide inhibition with broader specificity.

3. Importantly, the viability of tetraspanin knockout mice indicates that modulation of one or two tetraspanins can affect specific cellular functions without affecting viability. Thus, the targeting of tetraspanins may provide a viable approach to inhibit the pathogenicity of infectious organisms with minimal adverse effects on the host.

The necessity for the development of such approaches is becoming increasingly clear with the failure of traditional approaches to viral infection (vaccination) for diseases such as HIV, and their limited usefulness for influenza, of which the emergence of novel strains (e.g. avian and swine flu) in human populations are of great concern.[175] Furthermore, it has become evident that the emergence of zoonotic viruses in new animal hosts/human populations is one of the greatest potential threats to human health, as highlighted by the recent outbreaks of the bat viruses Severe Acute Respiratory Syndrome (SARS) and Nipah/Hendra in human populations via agriculturally/economically important livestock.[176,177] Such outbreaks, accompanied by panic in the human population and mass culling of livestock, also highlight the potential of viral application to bioterrorism.[178] In cases encountering novel viruses, vaccination is impractical, and a broad specificity rapid-response strategy is required. Therapies targeting host-cell functions may provide such an approach. In addition, although there is currently less evidence for a role for tetraspanins in bacterial infections, with increasing concerns over antibiotic resistance, alternative tetraspanin-based therapies may provide valuable antibacterial strategies in the future.

3.1 Interfering with Tetraspanin Binding by Pathogens

There are relatively few examples of direct interactions of pathogen molecules with specific tetraspanins and the best characterized is that of HCV E2 with CD81. As mentioned in section 2.1.1, recombinant CD81 EC2 domains inhibit HCV,[179] and such proteins, or smaller representative derivatives thereof, might prove useful for treatment of HCV infection. The binding site of HCV E2 on CD81 EC2 has been mapped to a hydrophobic patch in the variable sub-loop region of the tetraspanin[14,180] with residues Leu 162, Ile 182, Asn 184 and Phe 186 highlighted as important by mutagenesis. A small peptide analogue comprising residues 176–189 can inhibit E2 binding[181] and small-molecule inhibitors of the interaction between CD81 and HCV E2 have also been designed.[182]

However, recent work has indicated that recombinant CD81 EC2s are unable to prevent infection by serum-derived HCV, whereas anti-CD81 antibodies or siRNA downregulation of

Table I. Pathogens that exploit tetraspanins during infective processes

Pathogen	Tetraspanin[a]	Process	References
HCV	CD81 (TSPAN28, TM4SF10, Tapa-1)	Co-receptor required for entry. Interacts directly with HCV E2 protein	36,59-61,67
		Signaling involved in viral replication	74
		Possible role in trafficking	76
HIV-1	CD9 (TSPAN29, TM4SF2, DRAP-27, MRP)	Virus-induced syncitium formation	94
	CD63 (TSPAN30, TM4SF1,ME491, Lamp3, granulopyhsin)	Infection of macrophages	37,93,94
		Selectively incorporated into virion	88-91
		Virus protein trafficking	107
	CD81	Virus-induced syncitium formation	94
		Interacts with Gag, involved in stages T-cell infection	105
	TEMs (CD9, CD63, CD81, CD151)	Infection of macrophages	37
	TEMs (CD9, CD53, CD81)	In compartment where virus replicates	96-101
	TEMs (CD9, CD63, CD81, CD82)	Virus egress from T cells	27,103-105
HTLV	CD82 (TSPAN27, TM4SF11, C33, KAI1, R2 Ag)	Infective synapse formation	115
		Associates with Gag protein	116-118
	CD151 (TSPAN24, TM4SF32, PETA3)	Enhances adhesion of infected T cells	119
	TEMs (CD53, CD81, CD231)	Co-localizes with Gag; infective synapse formation?	116
FIV	CD9	Virus trafficking/assembly or release	122-124
CDV	CD9, TEMs (CD9, CD63, CD81, CD82)	Virus trafficking/syncitium formation	126-129
PRRSV	CD151	Interacts with 3′-UTR PRRSV RNA. Role in trafficking?	132
HPV	CD151 (with CD63, CD81 in TEMs)	Component of viral entry 'platform'	138
Dengue virus	C189 (mosquito tetraspanin)	Upregulated on infection, function unknown	169
Plasmodium spp.	CD81	Co-receptor for internalization/parasitophorous vacuole formation	17,141-143
Corynebacterium diphtherithiae	CD9	Increases cell sensitivity to diphtheria toxin	148-151
Uropathogenic *Escherichia coli*	Uroplakin 1a (TSPAN21, UP1a)	Receptor for Fim1 on adhesive pilus	154-157
	CD63	Expressed in bacteria-containing intracellular compartment	159
Chlamydia spp.	CD63	Expressed in bacteria-containing intracellular compartment	19,161
Normal PrP(c)	CD81	Co-localizes with PrP(c)	20
	CD231 (TSPAN 7, A15,TALLA-1)	Bovine form interacts with PrP9(c)	163

a The commonly used CD (cluster of differentiation) nomenclature for tetraspanins is given where appropriate, with the official gene nomenclature in parentheses. Alternative, previous names of the tetraspanins are given subsequently. Where TEMs are thought to be involved, the principle tetraspanins that have been identified in these are given. The main processes that the pathogen is thought to target are listed – for details see text.

CDV = canine distemper virus; **FIV** = feline immunodeficiency virus; **HCV** = hepatitis C virus; **HPV** = human papilloma virus; **HTLV** = human T-cell leukemia virus; **PrP(c)** = prion protein; **PRRSV** = porcine reproductive and respiratory syndrome virus; **TEM** = tetraspanin-enriched microdomain; **UTR** = untranslated terminal repeats.

CD81 were very effective.[183] Antibodies to CD81 have recently been shown to be protective *in vivo* in a SCID (severe combined immunodeficiency) mouse model.[184] This suggests that anti-CD81 may be useful prophylactically, for example, in preventing reinfection of allografts after liver transplantation in HCV-infected patients. Mouse mAbs would obviously need to be humanized. Interestingly, we recently probed the human combinatorial library HuCal[185] with recombinant CD81 EC2 regions and obtained a panel of human bivalent minibodies that bound to native CD81 and inhibited HCV infection (unpublished observations).

Another CD81-dependent inhibitor of HCV infection has recently been identified as EWI-2wint, which is expressed by several cell lines but not hepatocytes.[186] EWI-2wint is a cleavage product of the ectodomain of the transmembrane protein EWI-2, a direct interaction partner of CD81 belonging to the immunoglobulin superfamily.[187] Ectopically expressed EWI-2wint blocked HCV infection of a hepatocarcinoma cell line, most likely by inhibiting the interaction between CD81 and the E2 envelope protein.

3.2 Strategies to Disrupt TEMs

Apart from direct interference of HCV E2 –CD81 binding, most of the biological activities of soluble tetraspanin EC2 domains are likely to be mediated by disruption of lateral interactions between membrane-embedded tetraspanins and their interacting partners in TEMS. This likely explains why EC2s representing several different tetraspanins all inhibited HIV uptake by macrophages.[38] However, CD63 EC2 showed the strongest effect here, and this, together with the finding that only antibodies to CD63 inhibited HIV macrophage uptake, suggests a more direct role for this tetraspanin. Soluble tetraspanin EC2 regions may therefore have a broad range of direct and indirect effects.

In many infections it appears that antibodies also act by disrupting TEMS. Interestingly, even for HCV, antibodies that inhibit E2 binding vary considerably in their efficacy to prevent HCV infection, indicating they may act in different ways,[70] with some additionally affecting lateral CD81 interactions with other membrane proteins. Mutagenesis has also highlighted the importance of residues in the transmembrane and cytoplasmic regions of CD81 in HCV entry, which presumably relate to the intramolecular interactions mediated by this tetraspanin that are crucial to entry.[70] Also, in HIV, antibodies to multiple tetraspanins seem to have similar effects at various stages of infection (e.g. syncytium formation, virus release) indicating the involvement of TEMs in these processes. Papilloma virus

entry into epithelial cells also involves multiple tetraspanins, although CD151 appears to have a more direct role. It is therefore possible that these infective processes could be targeted not just by antibodies, but by reagents based on soluble tetraspanin EC2 or other agents that disrupt TEMs.

The palmitoylation of tetraspanins is known to be important in TEM assembly,[188] and an acyl transferase, which promotes tetraspanin palmitoylation,[189] could conceivably provide another target for microbial infections.[40]

3.3 Downregulation of Tetraspanins

Where they are involved in pathogen entry, downregulation of the tetraspanins by antibodies that induce internalization (e.g. antibodies to CD63 or CD151) may have therapeutic benefit. However, the most efficient method of downregulating tetraspanin expression is by using siRNA-mediated knockdown. This is most likely to be useful where a particular tetraspanin has been shown to have a more direct role in infection e.g. CD81 knockdown in HCV or malaria. An efficient method of targeting siRNA *in vivo* is still needed. Encouragingly, a very recent paper has demonstrated the efficient uptake of siRNA by macrophages *in vivo*.[190] This might be useful for achieving knockdown of CD63 in macrophages in HIV patients, although the controversy over the effects of CD63 modulation (see section 2.1.2) on viral infection must obviously first be resolved.

3.4 Using Tetraspanins to Target Drugs

Finally, exogenously applied antibodies to tetraspanins that internalize could be used to target antibiotics or other drugs to intracellular pathogens that reside in vesicles. CD63 has previously been identified as a target for this type of approach in cancer treatment.[191] Hope that this might also be effective in treating microbial infections comes from the studies on chlamydial infections[19] where CD63 antibodies were shown to traffic inclusions.

4. Conclusions

Tetraspanins are being increasingly linked to infectious disease caused by a variety of intracellular pathogens. Such pathogens utilize the normal cellular processes that involve tetraspanins/TEMs, such as fusion, endocytosis, intracellular trafficking, and exocytosis, to enter host cells, replicate and spread infection. Tetraspanins therefore constitute an attractive novel target for alternative, host-cell-based antimicrobial

therapies. The tetraspanin superfamily has, however, received little attention from the scientific community compared with other membrane proteins, e.g. integrins. For the full potential of tetraspanins for therapeutics to be realized, more research tools are required, such as specific antibodies and recombinant, soluble EC2 domains targeting all 33 mammalian tetraspanins. More information on the 3-dimensional structure of tetraspanins is also required, along with further investigations on the effects of knockdown or knockout in cells and model organisms, to determine the functions of individual tetraspanins. Finally, there is a need for sophisticated live-cell imaging techniques to investigate the contribution of TEMs to the fundamental cellular activities that pathogens exploit.

Acknowledgments

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