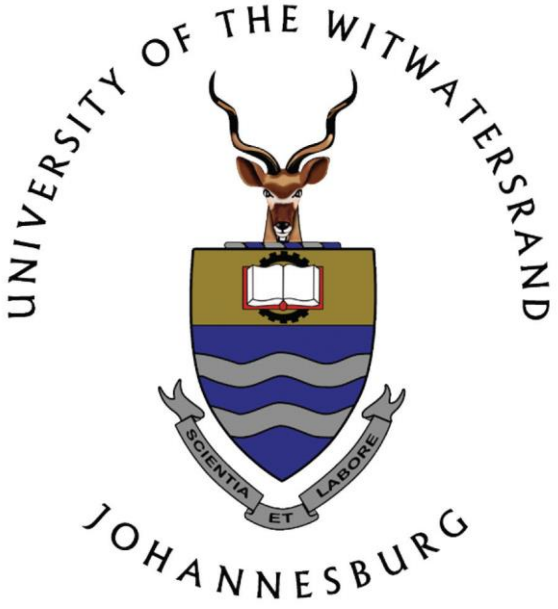
The role of redox-dependent CD4 isomerization and membrane re-distribution in HIV-1 infection



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Doctor of Philosophy

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Declaration

I, Naazneen Moolla, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other institution.

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Publications

The results of this study have been published in the following journal articles:

1. **Cebula, M., N. Moolla, A. Capovilla and E. S. Arner** (2013). "The rare TXNRD1_v3 ("v3") splice variant of human thioredoxin reductase 1 protein is targeted to membrane rafts by N-acylation and induces filopodia independently of its redox active site integrity." J Biol Chem **288**(14): 10002-10011. (Chapter 2).

2. **Moolla N, Killick M, Papathanasopoulos M, Capovilla A** (2016). "Thioredoxin (Trx1) regulates CD4 membrane domain localization and is required for efficient CD4-dependent HIV-1 entry. " Biochimica et Biophysica Acta. May 24;1860(9):1854-63. PubMed PMID: 27233453. Epub 2016/05/29. (Chapter 3).

Abstract

CD4, a key molecule of the immune system, is expressed on the surface of certain T lymphocytes (T cells) and participates in MHC class II driven lymphocyte activation. It is also the essential primary receptor for Human Immunodeficiency Virus (HIV) cell entry. Reactive oxygen species (ROS) and other redox-active molecules are important components of the immunological response. They initiate cytocidal responses of the pathogen defence scheme, and redox-activated signalling events ensure appropriate induction of adaptive immunological responses. A redox imbalance can result in failure of essential regulatory mechanisms and the development of pathological immune conditions. An increasing amount of evidence suggests that redox active enzymes such as thioredoxin (Trx) are implicated in CD4 immunological function and in HIV entry at the cell surface, and the dynamic localization of CD4 in specific plasma membrane microdomains, like detergent resistant membrane microdomains (DRM) or lipid rafts, has been shown to play a key role in these regards. However, the biological utility of both the microdomain distribution and the disulphide reduction of CD4, together with the interplay between these processes and the role of cellular oxidoreductases therein remain poorly understood. In this study, we investigated a cell surface-based Trx redox system, and asked whether a relationship exists between these two fundamental aspects of CD4 function by analysing how manipulating cell surface redox conditions affects CD4 membrane domain localization and HIV entry into host CD4-positive ($CD4^+$) cells.

Our investigation of the role of a cell surface redox system in regulating CD4 function was prefaced by research into the membrane association of a variant of Thioredoxin reductase 1 (TrxR1), the enzyme responsible for reducing (and thereby recharging) the active site cysteines of Trx. These studies, carried out in the laboratory of Prof. E Arner (Medical Biochemistry and Biophysics, Karolinska Institutet), were the first to show that a TrxR1 variant called TXNRD1_v3 (henceforth v3) is targeted to DRM domains via N-terminal acylation. Although the role(s) of v3 in this context remains poorly understood, the evidence suggesting that TrxR can associate with the plasma membrane under certain circumstances alludes to the importance of redox capacity at the cell surface, which increasingly suggests it is essential for the function of CD4.

To this end, using a transgenic cell line that has been extensively used to model HIV entry and various HIV pseudoviruses, we then analysed the effects of manipulating cell surface redox conditions on CD4 membrane domain distribution and HIV entry. Our results showed that under normal cell growth conditions, the majority of CD4 is associated with detergent soluble regions of the plasma membrane (non-raft regions). Intriguingly, we found that the inhibition of cellular oxidoreductases, and specifically Trx1, results in a redistribution of CD4 into DRMs. CD4 DRM redistribution appears to be targeted, as other cell surface molecules (such as the HIV co-receptor, CCR5) remain unaffected. Furthermore, the redistribution of CD4 to the DRM's correlates with reduced CD4-dependent HIV infection.

Overall, these findings provide evidence for the presence of cell surface-acting redox systems and demonstrate how redox exchanges influence CD4 localization and function. In the context of HIV, our data support previous findings that the thioredoxin system plays an important role in regulating viral entry, which may be related to uncoupled trafficking of CD4 and the HIV co-receptor. Trx-mediated regulation of CD4 membrane domain trafficking may represent a redox switch for functional CD4 clustering during T cell activation.

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"Guidance is not attained except with knowledge & correct direction is not attained except with patience"

~ Ibn Taymiyyah

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List of Abbreviations:

The abbreviations used in this thesis describe the following unless otherwise indicated.

AIDS	Acquired Immunodeficiency Syndrome
Arg	Arginine
Asp	Aspartic Acid
CA	Capsid Protein
CCR5	Chemokine Receptor Type-5
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
$CD4^+$	Cluster of Differentiation 4 positive
CD45	Cluster of Differentiation 45
CD4bs	CD4 binding site
CD8	Cluster of Differentiation 8
$CD8^+$	Cluster of Differentiation 8 Positive
CDR	Complementarity Determining Region
CXCR4	Chemokine (C-X-C) Motif Receptor 4
Cys	Cysteine
D1-D4	Domain 1- Domain 4
DC-SIGN	Dendritic Cell-Specific Intracellular Adhesion Molecule-3 –Grabbing Non-Integrin
DNA	Deoxyribonucleic Acid
DRLM	Detergent Resistant Lipid Membrane
DRM	Detergent Resistant Membrane
DSM	Detergent Soluble Membrane
ELISA	Enzyme Linked Immuno-Sorbent Assay
Env	Envelope protein of HIV
ER	Endoplasmic Reticulum
Glu	Glutamic Acid
Gly	Glycine

GM	Membrane Ganglioside
Grx	Glutaredoxin
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidised)
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus Type-1
HIV-2	Human Immunodeficiency Virus Type-2
Ig	Immunoglobulin
IN	Integrase
ITAM	Immunoreceptor Tyrosine-based Activation Motifs
LTR	Long Terminal Repeat
MA	Matrix Protein
MHC-II	Major Histocompatibility class II
mRNA	Messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NC	Nucleocapsid Protein
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PDI	Protein Disulphide Isomerase
Phe	Phenylalanine
PI	Protease Inhibitors
Pol	Polymerase
PR	Protease
Redox	Reducing/Oxidising
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Reverse Transcriptase
RTK	Receptor Tyrosine Kinases
sCD4	Soluble CD4; Extracellular domains of CD4

SDS	Sodium Dodecyl Sulphate
SH	Sulfhydryl Groups
SpTrx	Thioredoxin expressed in Spermatozoa
T cell	T lymphocyte
TCR	T cell Receptor
Thr	Threonine
Trp	Tryptophan
Trx	Thioredoxin
Trx1	Thioredoxin1
Trx2	Thioredoxin2
TrxR	Thioredoxin Reductase
TSP-1	Thrombospondin-1
TXNIP	Thioredoxin Interacting Protein
TXNRD1	Thioredoxin reductase 1 encoding gene
V3 (GRx)	GRx domain of V3
V3	TXNRD1_Variant 3
Zap70	Zeta Chain Associated Protein 70

The IUPAC-IUBMB three and one letter codes for amino acids are used

Chapter 1:
Introduction

1.1 CD4

Cluster of Differentiation 4, CD4, a central molecule in the immune system, is a transmembrane glycoprotein widely expressed on the surface of a subset T lymphocytes (T cells) and to a lesser extent on B lymphocytes, granulocytes and other cells of monocyte/macrophage lineage (1, 2). CD4 expressing ($CD4^+$) lymphocytes have both regulatory roles in lymphocyte development and differentiation over and above their vital function in enhancing antigen recognition by Major Histocompatibility class II (MHC-II) molecules (3, 4). Importantly, CD4 was also identified in the 1980's as the primary receptor for Human Immunodeficiency Virus-1 (HIV-1), facilitating initial viral attachment to the host cell (5, 6).

1.1.1 CD4 function in antigen presentation

Biologically, one of the key roles of CD4 is in antigen presentation to MHC-II molecules and facilitation of the formation a functional immunological synapse (3). To this end, $CD4^+$ T cells recognize peptide-loaded MHC-II complexes through their T cell receptors (TCRs) (7). TCRs are optimized for binding MHC-II but are varied enough to recognize a multitude of peptides with which they then form complexes. These complexes can then be encountered by $CD4^+$ T cells (7). The direct binding of CD4 to non-polymorphic regions of MHC-II molecules, is thought to stabilize the interaction between the T cell receptor (TCR) and the peptide with MHC-II (8, 9). In binding to the TCR-MHC-II complex, CD4 also delivers the Src tyrosine kinase p56Lck (Lck) (non-covalently linked to the cytoplasmic tail of CD4) into the

area of the TCR-MHC-II complex. This allows immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3, in the TCR complex, to be phosphorylated (10-12). Other downstream signalling molecules (such as the Zeta chain associated protein kinase 70 [Zap-70]) are then recruited to the TCR complex, triggering a signalling cascade that culminates in the production of cytokines and T cell activation (12). CD4 recruitment appears to be regulated by the half-life of the initial TCR-MHC-II complex (12) and the localized co-engagement of CD4 and the TCR to MHC-II is required for an antigen specific response (13). Interestingly, in the absence of the MHC-II-CD4 interactions, binding of the TCR to MHC-II results in an unresponsive T cell, a phenomenon known as " T cell anergy" (9, 13). Of note too, is that both the T cell activation and lymphocyte development functions of CD4 need not involve signalling through Lck (14, 15).

1.1.2 CD4 structure

Belonging to the immunoglobulin superfamily of molecules, CD4 is a typical Type I integral membrane protein, consisting of an N-terminal extracellular portion, a transmembrane portion and a short cytoplasmic tail (16-18). The four extracellular N-terminal domains display sequence similarity with immunoglobulin (Ig) variable regions, and have been described as a concatemer of four Ig-like domains, designated domains 1, 2, 3, and 4 (D1-D4) (19, 20). All four Ig domains contain seven β strands, which form two apposed β sheets (21). (Figure 1)

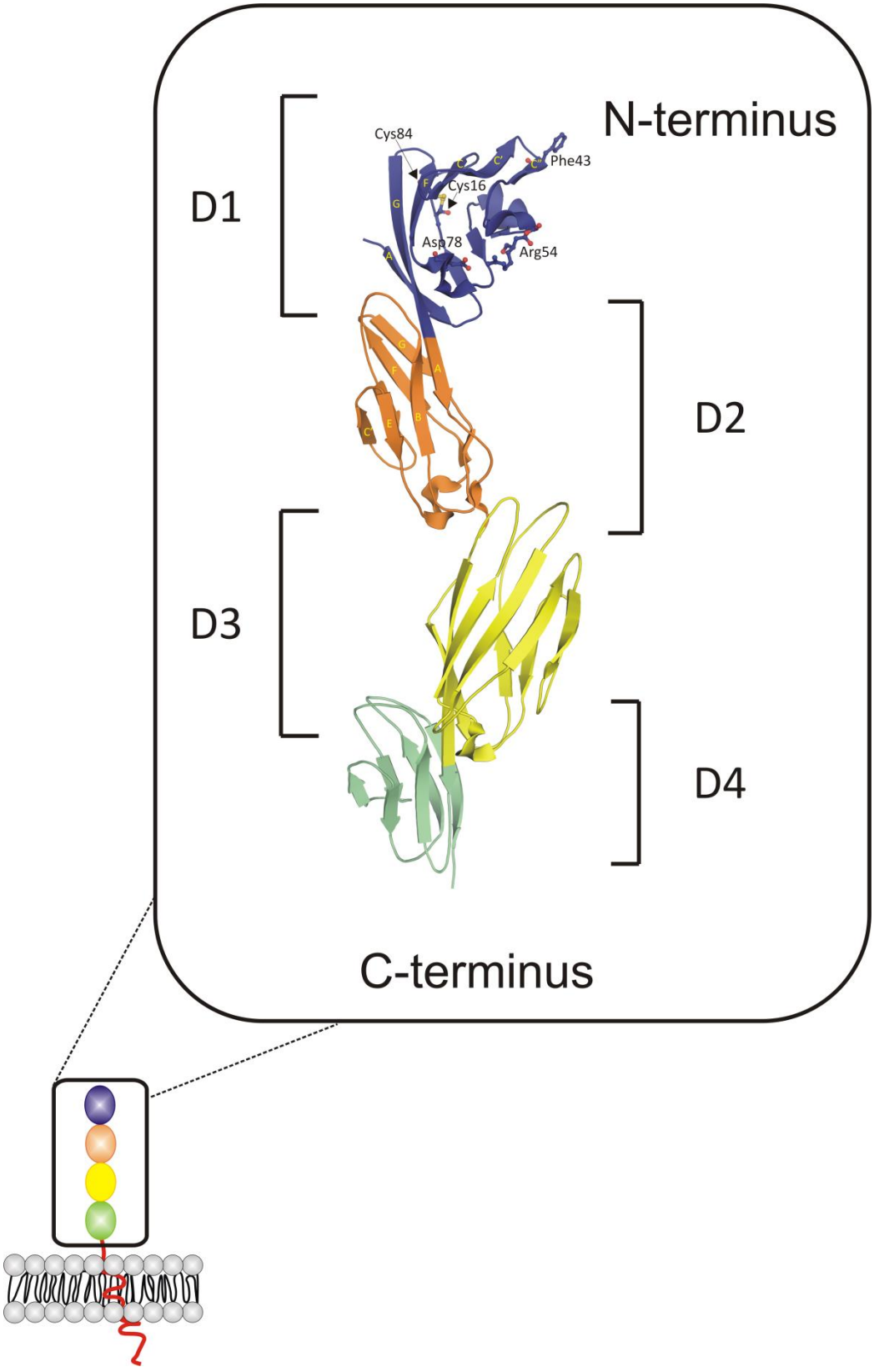

Figure 1: Ribbon representation of the Extracellular domains of CD4
The extracellular portion of CD4 containing domains 1 (blue), 2 (orange), 3 (yellow) and 4 (green) [D1-D4] is shown as a ribbon diagram. Amino acids in the D1 disulphide (Cys16-Cys84) and salt (Asp78-Arg54) bridges are shown in stick and ball representation and labelled. Select strands in D1 and D2 have been annotated as by Harrison *et* al(18). The figure was generated using PDB ID 1WIQ (22) and PyMOL.

1.1.2.1 The extracellular domains

The D1 domain is most characteristic of an Ig variable domain and has nine strands in two β-sheets (20). The core of the domain has several hallmarks typical of an Ig variable light chain, including several conserved hydrophobic residues, a pair of inter-sheet disulphide-bridged cysteines (Cys16-Cys84) and an arginine (Arg54)-aspartic acid (Asp78) salt bridge, that links strand D with the EF loop, amongst other features (18-20, 23). It differs significantly though from an Ig variable domain in that it lacks features responsible for dimerization, and has a lengthened C'C" loop (18, 20), which presents a phenylalanine (Phe43) at its apex that is essential for the HIV gp120 interaction (19, 23). CD4 also engages class II MHC via D1 (24); however, while there is overlap between the MHC-II and gp120 binding sites on CD4, these sites are distinct and can be separated (24, 25). D1 and D2 form a rod-like structure and are linked by a continuous β-strand with several consecutive non-polar residues, thought to stabilize the rod-like unit, at the domain 1/2 interface (18).

The second domain of CD4 resembles a miniaturized Ig constant domain with shortened strand lengths (20). D2 has the very distinctive feature of a disulphide bond between strands in the same sheet rather than between sheets as is typical of an Ig domain, while a hydrophobic core, common in all Ig domains, is maintained (20). This atypical disulphide is susceptible to changes in redox potential, resulting in facile reduction that is purported to have a functionally significant allosteric effect on CD4 structure (21, 26, 27). X-ray crystallographic data of soluble CD4 (D1-4) suggests a flexible juncture between domains 2 and 3, comparable with the hinge region of Ig molecules (23).

Low diffractive resolution and the presence of highly polymorphic crystals in human sCD4 have resulted in inferences of structural features of domains 3 and 4 from rat CD4 (18, 23).

Thus the inferred structure of domain 3 maintains the fundamental Ig variable domain organization, although it has a noteworthy lack of a disulphide linkage and key amino acid substitutions when compared to the archetypical Ig framework (18). The link between domains 3 and 4 mimics that between domains 1 and 2 (18). D4 structure is similar to that of D2 with the same seven β-strand topology however, the membrane proximal tip of the domain is shortened making it more compact (18).

1.1.2.2 The transmembrane and cytoplasmic domains

Structural characterization of the transmembrane and cytoplasmic domains of CD4 indicates the presence of an α-helix in each region (28). The transmembrane α-helix spans residues 372-395 of CD4 and is stable and inflexible (29). Recently, sequence analysis of the CD4 transmembrane domain of select mammals identified a highly conserved Gly-Gly-X-X-Gly sequence motif that appears to function in the Lck-independent role of CD4 in T-cell activation (30). The cytoplasmic α–helix is the shorter of the two (residues 403-413) but nevertheless is still stable (29). The C-terminus extremity of the cytoplasmic tail is unstructured and this domain contains the two cysteine residues (Cys420 and Cys422) , known as a cysteine clasp, that are important for activation of the Src kinase, Lck, and thus CD4's signal transduction function (10, 29, 31). These two cysteine residues have also been implicated in the homodimerization of CD4 (32). Cysteine residues 394 and 397 of the cytoplasmic domain are post translationally palmitoylated (33), a feature that has been suggested to be important for CD4 aggregation and translocation to lipid rafts (34).

1.1.3 CD4 oligomerization

Many cell surface receptors dimerize or oligomerize in order to acquire full functionality (35). Indeed, several receptor tyrosine kinases (RTKs) have been shown to be activated in this way (35). For example, epidermal growth factor, an RTK, is activated by dimerization and/or higher order oligomerization, which is induced by ligand binding (36-38). (36-38). In addition to the established lateral association of CD4 with other molecules (39), early analysis of the crystal structure of CD4 showed that it has the potential for self-dimerization or oligomerization (23). In 1999 Lynch *et al*, demonstrated that native CD4 dimers and oligomers are present on the surface of lymphoid cells (40). More recently, it has been shown that CD4 homodimers can vary from 32% to 42% of total CD4 depending on the cell line analysed (32). Several studies have shown that CD4 dimers or oligomers are in fact the functional form, required for stable binding to MHC-II and proper T-cell activation (41-44). Interestingly, it appears that a balance between the monomeric and dimeric forms of CD4 could adjust the threshold for T cell activation (41). However, some have proposed that the formation of homodimers is proportional to the amount of CD4 expressed (32).

There is however much ambiguity about the mechanisms underlying CD4 dimer and oligomer formation and particularly which domains of CD4 are involved in the process. All four of the extracellular domains of CD4 have been implicated in CD4 dimerization or oligomerization. Initially, the crystal structure of the extracellular domains of CD4 (sCD4) showed that CD4 can form dimers through its D4 domain (22). In support of this, Moldovan *et al* demonstrated that maintaining the two residues, highly conserved among mammals in D4 domain of CD4 (K318 & Q344), is an absolute requirement for CD4 dimer formation (42). However, more recent insights based on modelling this type of dimer formation from the

crystal structure of the TCR-MHC-II-CD4 complex, indicates that CD4 dimer formation through D4 is unlikely to occur because of stereochemical constraints (13). In D1, the Complementarity Determining Region (CDR)-3 loop was proposed as a major dimerization site (45). Some evidence pointed to a role for D3/D4 in mediating CD4 oligomerization (44). Recently, compelling evidence from experimental and molecular modelling approaches has suggested that the cleavage of the D2 disulphides on the surface of cells leads to the formation of CD4 dimers, implying that D2 could facilitate the formation of CD4 dimers and oligomers through redox changes (27, 46). In fact, a D2 disulphide bond mutant is not able to form dimers (27).

In another study, Fragoso *et al* proposed that the two palmitoylation sites near the transmembrane domain drove dimerization (34). More recently, the two cysteines in the cytoplasmic domain of CD4 (the same two that mediate zinc dependent Lck binding) have been shown to be indispensable for CD4 dimerization (32) while the transmembrane glycine patch (Gly-Gly-X-X-Gly) appears to have no role in CD4 dimerization (30). Given this uncertainty, it is possible that several domains of CD4 are involved in oligomerization, with certain domains potentiating the formation of oligomers and others stabilizing them. It is likely that factors such as liganded state, plasma membrane microenvironment and other environmental factors all play critical roles in CD4 oligomerization with multi-domain involvement (47).

1.1.4 CD4 plasma membrane compartmentalization

Together with increasing knowledge about the structural dynamics of CD4, the recent findings that CD4 undergoes functionally significant lateral movement between different plasma membrane microdomains are augmenting our understanding of the complex

physiological and molecular determinants of CD4 function. The spatial distribution of receptors and signalling molecules in the plasma membrane combined with their re-arrangement during ligand engagement is fundamentally important to the efficiency of most cell signalling pathways (48). During MHC-II-based antigen presentation, specific, efficient and well coordinated cell signalling must occur on the T cell following successful engagement of the TCR by peptide-loaded MHC-II (48, 49). This, in part at least, is achieved by spatially restricting and regulating the physical segregation of signalling molecules by compartmentalization (50). Broadly speaking, this entails ligand binding to immune receptors, the movement of critical elements to key contact zones and the nucleation of active signalling complexes (50). This, in turn, requires two fundamental cellular processes: mobilization of the supporting cellular architecture such as cytoskeletal networks, and lateral segregation of certain membrane-bound proteins into distinct microdomains (50).

1.1.4.1 Plasma membrane structure and lipid rafts

The Singer-Nicolson fluid mosaic model of cellular membranes proposed in the early 1970s described the plasma membrane as a bilayer of phospholipids with itinerant integral membrane proteins and glycoproteins intercalated into this lipid platform (51). Since then, the basic model has been refined to include additional layers of complexity which accommodate the existence of specialized membrane microdomains, controversially termed 'lipid rafts' (see below), and protein/glycoprotein complexes that are critical for many cellular functions (52-55).

Lipid rafts are biochemically distinct microdomains of the plasma membrane in which the lipid components exist in a liquid-ordered state that differs significantly from the liquid-disordered regions of the bilayer (56) (Figure2). These ordered regions, are predominantly

enriched in cholesterol, sphingomyelin, and glycosphingolipids, but may also contain other saturated acyl lipids such as ethanolamine glycophospholipids, phosphotidylserine, arachidonic acid, phosphatidylglucloside, ceramide and lactosylceramide (53, 57). This composition results in lipid rafts having a low density and resistance to disruption by non-ionic detergents (53). Consequently, these domains are commonly, and more correctly perhaps, referred to as detergent resistant lipid membrane domains (DRLM's or DRM's)[1] and may be isolated based on these properties. Shortly after the 'lipid raft' hypothesis was conceptualized, it became evident that the use of detergents and certain conditions under which these membrane domains are frequently isolated (low temperatures in particular) can produce artefactual clustering of raft lipids with certain proteins, and this raised questions regarding the physiological relevance of the raft hypothesis. Since then, various methodologies, including the use of milder detergents/ no detergents, as well as morphological approaches like super-resolution microscopy (53, 58) have provided compelling evidence for the physiological existence of these unique membrane microdomains, and it is now widely accepted that they play fundamentally important biological roles. More recently, DRM's have been further segregated into high density or heavy DRMs and light DRM's which are distinguished based on their differential separation by sucrose density gradient centrifugation following solubilisation with polyoxyethylene type detergents (50). Membrane rafts have been linked to an ever-rising number of biologically important functions including signalling events and intracellular and membrane trafficking of proteins and lipids (59, 60). For instance, the recruitment of the T-cell receptor to lipid rafts during TCR activation, coincides with the aggregation of rafts and the triggering of signalling cascades (61). Lipid rafts are often viewed as moving platforms that carry

[1] In this work, the terms, lipid raft, DRM and DRLM are used interchangeably, although we acknowledge growing evidence that distinct types of lipid rafts exist.

specific proteins, since they maintain the ability to move laterally and rotate in the plasma membrane (59). They have also been implicated as being sites for host-pathogen interactions and participate in cell morphogenesis (55, 59, 62).

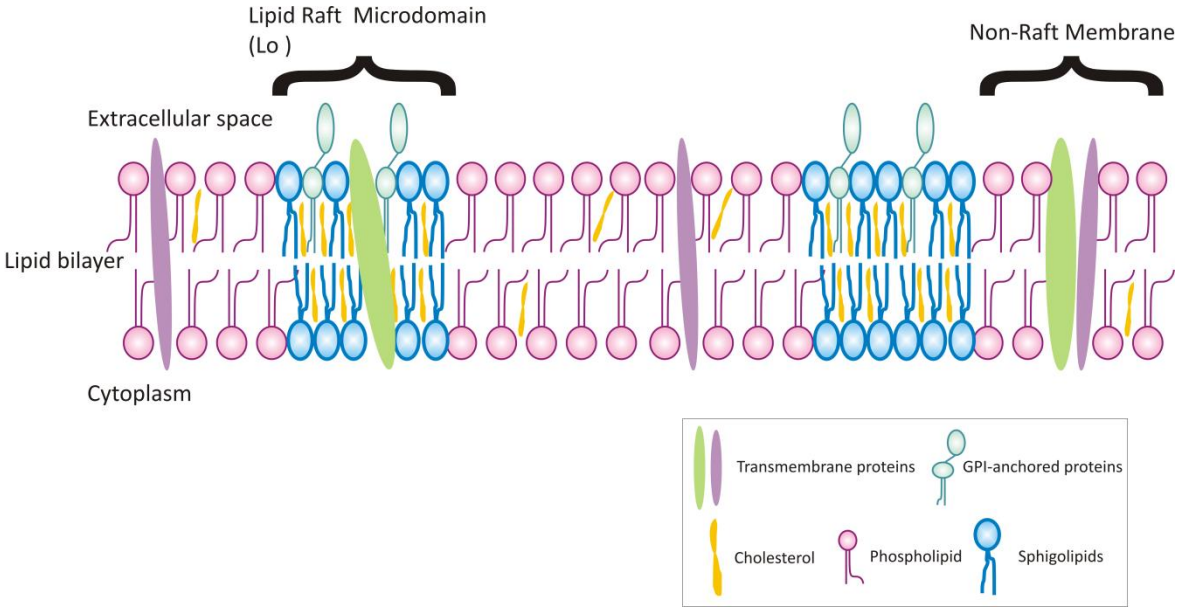

Figure 2: Schematic representation of the plasma membrane with lipid raft (Lo) microdomains
The plasma membrane lipid bilayer is segregated into raft and non-raft microdomains. Lipid rafts are more ordered (Lo) and tightly packed than the surrounding lipid bilayer. Lipid rafts represent specialized microdomains of dynamic, nanoscopic assemblies of lipids and proteins, often rich in cholesterol that facilitate the functional compartmentalization of membrane molecules. Figure adapted from Simons *et al*. (63).

1.1.4.2 CD4-Lipid Raft association

By compartmentalization in membrane microdomains under normal conditions, the spatial separation of signalling proteins is thought to prevent the spontaneous formation of signal transducing activation complexes (50). In this model, control of activation is regulated by microdomain-restricted negative feedback loops and the biophysical constraints imposed by these ordered lipid structures. In the case of T cell activation, the model proposes that presentation of an appropriate antigenic stimulus results in the juxtaposition of

microdomains such as those containing the TCR complex and CD4, which facilitates efficient and co-ordinated interaction of the molecules required for signal transduction amplification (50).

To this end, recent studies have suggested that several molecules involved in T cell activation such as CD4 and CD8, the Src family kinase members Lck and Fyn, CD3 of the TCR complex and the transmembrane adaptor, Linker for Activation of T cells (LAT), are lipid raft associated (34, 50, 64), and that TCR complex signalling depends largely on the integrity of lipid rafts (50). Importantly, major subsets of CD4 associated with Lck have been shown to localize to lipid rafts (65). In addition, it has been shown that CD4 is required for both TCR association with lipid rafts and the TCR/Protein Kinase C θ subunit clustering at the site of the immunological synapse (64). It has also been observed that the lipid raft-based membrane order is important for the clonal expansion of $CD4^+$ T cells following antigen stimulation (49). Perhaps the most compelling evidence for the importance of CD4 and lipid raft involvement in T cell signalling, is from the work of Nagafuku *et al,* who demonstrated that knockout animals lacking the membrane ganglioside, GM3, a major component of lipid rafts, have severely compromised $CD4^+$ dependent T cell signalling but not $CD8^+$ T cell signalling (66).

Despite the strong evidence for the importance of raft localized CD4 in T cell activation, there is considerable ambivalence in the literature with regards to the actual localization of CD4 in the plasma membrane of normal, resting cells. While some studies have shown that CD4 is enriched in raft microdomains (67, 68) others indicate that only a small percentage of CD4 (10%) is present in these detergent resistant microdomains and that the rest can be found in the detergent soluble membrane (DSM) (65). Image tracking of CD4 on live cells

provides evidence that CD4 exhibits two distinct behaviours on a cell. In one instance, CD4 is able to freely diffuse, consistent with its association with the soluble membrane portion, while at other times, CD4 receptors were restricted (either transiently or permanently) to certain domains, arguing for raft microdomain association (69). Intriguingly, there is evidence that CD4 dimers and CD4 monomers segregate to different microdomains of the plasma membrane (32). Thus, it seems most plausible that pools of CD4 are present in both the soluble and raft portions of the plasma membrane. Interestingly, a few studies have shown that even raft associated CD4 may reside in different types of DRM domains. Millan *et al* showed that CD4 can be found in either microdomains with GPI-anchored proteins and high glycolipid associated kinase activity or in DRMs lacking glycolipid-associated kinases and GPI –anchored proteins (65). Similarly, work by Filipp *et al* proposes two pools of CD4 DRMs exist - one of DRM associated kinase inactive CD4-Lck complexes, that is largely lacking in CD45, and another of active Lck associated CD4 that also contains TCR/CD3 and CD45 (50). Based on this data, they posit that there are functional differences between different types of DRMs in the context of their roles in T cell activation (50). Consequently, it seems that the disparity in the literature with regards to CD4 plasma membrane distribution is likely reflective of functional differences of CD4 localized to different microdomains and underscores the need to better understand CD4 localization and its role in activated and resting lymphoid cells.

1.2 CD4 redox biology

1.2.1 Disulphide bonds and protein function

CD4 like many cell surface proteins that function in the extracellular space, has disulphide bonds- covalent links between cysteine residues (20, 23). Historically, it was generally

believed that disulphide bonds in mature proteins are inert and function principally as stabilizers of protein structure (70). Of late however, there is growing interest in the role of reversible oxidation of cysteine residues on proteins and the consequence of these changes on protein function (71). These disulphide bonds are referred to as being "redox-labile" or "allosteric" due to their propensity for easier reduction and accessibility to reducing agents (71-76). Indeed, several cellular proteins exploit the inherent efficacy of electron transfer via redox active disulphides in their activity and in so doing, initiate signalling pathways (74, 77). In such instances, the catalytic disulphides which mediate thiol/disulphide exchange in other proteins are located at the active sites of oxidoreductases and thiol isomerases (78). This demonstrates the aptness of rapid, readily reversible thiol/disulphide exchange reactions for directing protein function, by changing the redox state of structural or catalytic sulfhydryl (SH) groups (73). In general, allosteric disulphides are cleaved by either oxidoreductases or by thiol/disulphide exchange within the protein containing the allosteric disulphide and in certain instances both cleavage mechanisms can take place (74). An example of disulphide bond-controlled switches in protein function is that of the thiol-disulphide oxidoreductase, protein disulphide isomerase (PDI) mediated disulphide interchange of thrombospondin-1 (TSP-1). TSP-1 functions in the growth and differentiation of tissues, and different disulphide bonded isoforms of TSP-1 have been found *in vivo*. Each of these isoforms appears to have different cell adhesive activities, suggesting functional significance for the different disulphide bonded isoforms (74).

1.2.2 CD4 redox regulation

Several studies have shown that CD4 itself is a target for cellular oxidoreductases, and that CD4 reduction is a fundamentally important component of its biological activity. In

particular, the pioneering work of Hogg and colleagues has shown that the atypical disulphide bond in D2 is redox active and can exist in both the reduced dithiol form or oxidised form on the surface of cells (27, 79). Biochemical analysis has implicated this disulphide bond in the redox-dependent formation of naturally occurring oligomers and isoforms of CD4 (22, 27, 40, 80). These redox dependent isoforms appear to be important both for CD4-MHC-II interaction and CD4 interaction with HIV-1 gp120 (26, 41, 42, 44, 81, 82). For instance, the redox state of CD4 is linked to the activation state of T cells as an increase in the dithiol form of CD4 was noted when T cells were activated (80). Activated T cells also upregulate their constitutive secretion of the cellular oxidoreductase, thioredoxin (Trx), and Trx can reduce CD4 (27, 83). More recently, our group has shown that an isoform of Trx, Trx1 is capable of highly efficient reduction and isomerization of CD4 (83). This results in the formation of distinct monomeric CD4 isoforms and a disulphide linked dimer, and the formation of these requires the participation of both D1 and D2 disulphides (83). Interestingly, Trx1 (84) and the oxidoreductase Glutaredoxin 1 (Grx1), (85) but not PDI (86) can mediate cleavage of disulphide bonds in D1, D2 and D4 domains of CD4. The finding that D2 CD4 mutants are dimerization defective, and T cells expressing such CD4 molecules are not activated through MHC-II based mechanisms (27, 42) demonstrates the functional importance of oxidoreductive isomerisation of CD4 in the context of antigen presentation.

1.2.3 Cellular antioxidant systems

Since the reversible oxidation of cysteine residues can impact protein function, homeostasis of the reducing/oxidising (redox) environment is critical for maintaining normal cellular functions. Under physiological conditions, the redox balance of the cellular environment is largely maintained by the disulphide/dithiol-reducing activity of the two major thiol

antioxidants, the glutathione (GSH) and Trx systems (87, 88). These systems, with overlapping and distinctive roles, are responsive to oxidative stress and regulate a diversity of cellular events through distinct redox pathways which impact on redox signalling (88). The GSH system comprises GSH, glutathione reductase and glutaredoxins which catalyse the nicotinamide adenine dinucleotide phosphate (NADPH) dependent reduction of oxidised glutathione (GSSG) (78, 88). Likewise, the Trx system has thioredoxin, which is reduced by electrons from NADPH via thioredoxin reductase (TrxR) (78, 88). Other enzyme antioxidant systems include superoxide dismutases, superoxide reductases, catalases, and peroxiredoxins, while non-enzymatic antioxidant compounds comprise low molecular weight compounds such as vitamins C and E and selenium containing compounds like selenite (89).

1.2.4 Oxidoreductases

Thiol-disulfide oxidoreductase enzymes facilitate protein folding and repair. They act by reducing disulphides and forming a catalytic site disulphide which is then reduced by an external electron donor. Cellular oxidoreductases are characterized by a Cys-X-X-Cys catalytic sequence within a thioredoxin-like domain that is required for enzymatic activity of all the thioredoxin superfamily protein members (78). This family includes the oxidoreductases Trx, other protein disulphide isomerases (such as PDI) and functionally dissimilar proteins such as the glutathione *S*-transferases and glutathione peroxidase (90). These enzymes occur in different cellular compartments and also have varied protein substrates and mechanisms of reactivation. For example, glutaredoxins have thiol reductase and S-glutathionylation activity, important regulatory mechanisms of many biological

processes, while protein disulfide isomerases are generally located in the endoplasmic reticulum (ER) where they function as chaperones in protein folding (78, 88, 91).

1.2.4.1 The Thioredoxin (Trx) system

Thioredoxins are small (12kDa) reductases, with catalytic protein disulphide/dithiol exchange activity mediated by the conserved active site motif Cys-Gly-Proline-Cys (78). In mammalian cells, there are two isoforms of Trx, thioredoxin 1 (Trx1) and thioredoxin 2 (Trx2) which localize to different cellular compartments. Trx1 is mainly cytosolic, but can move to the nucleus and be secreted at the plasma membrane (89, 92) while Trx2 is restricted to the mitochondria (89, 93). A third variant, spTrx, is highly expressed in spermatozoa (89, 94). Trx is essential for cellular and organism survival with Trx1 knockout showing embryonic lethality in mice (78, 95). Thioredoxins function as general and potent oxidoreductases by facilitating the reduction of many proteins through cysteine thiol-disulfide exchange (89, 90, 96). In so doing, they can regulate enzyme activity, protect proteins from oxidative damage and facilitate protein folding (89, 96). Known targets of Trx include, ribonucleotide reductase, PDI, and the transcription factors p53 and NF-κB, amongst others (89, 97-99). Trx is also known to function in an immunological capacity, where it functions as a cytokine (100, 101) and co-cytokine (92, 102) and has growth-factor like effects that stimulate lymphocyte proliferation and recruitment (89, 100). Generally, Trx expression is induced under conditions of oxidative stress and under such conditions, Trx increases the reduction of intracellular proteins and maintains redox homeostasis (78, 89, 90). In addition, Trx can carry out its antioxidant activities by regulating the signal transduction properties of Reactive Oxygen Species (ROS) (103), by the reduction of intracellular protein disulphides (104) and by the direct lowering of ROS levels (89). Trx

activity can be regulated endogenously by the protein thioredoxin interacting protein (TXNIP/TBP2/VDUP1) (78, 95).

The second oxidoreductase of the Trx system is thioredoxin reductase (TrxR) which catalyses the reduction of the active site disulphide in oxidised Trx as well as other protein disulphides, low molecular weight disulphide compounds and non-disulphide compounds, giving it a wide substrate specificity (89, 105). As TrxR is responsible for reducing the active site disulphide of Trx to a dithiol, it is essential for all downstream Trx regulated activities (90, 101, 106). In higher eukaryotes, a high molecular weight (55-65kDa) homodimeric form of this protein is found (78, 89, 101, 107). In parallel with its substrate Trx, three types TrxR have been identified, cytosolic TrxR1, mitochondrial TrxR2 and a testis-specific thioredoxin glutathione reductase (TGR), which in addition to Trx can also directly reduce GSSG (78, 108). Each of these isoenzymes is encoded by a separate gene and interestingly, all three human TrxR genes, undergo extensive splicing, predominantly at the 5' end resulting in numerous transcripts which encode different protein isoforms (109, 110). A unique feature of TrxR is that it is a selenoprotein, containing selenocysteine (Sec), the selenium analogue of cysteine, and that this Sec residue is essential for its enzymatic activities (89, 111, 112). Functional impairment of TrxR results in pro-oxidant effects characterized by reduced activity of Trx and many other TrxR substrates and increased ROS accumulation, ultimately decreasing the total cellular antioxidant capacity (89, 113, 114).

1.2.5 Redox regulation at the cell surface and in the extracellular microenvironment

Despite the high oxidizing potential of the extracellular environment, it is now well-established that proteins containing redox active reduced thiol groups, exist on the cell surface and carry out important biological functions (115, 116). As referenced above, evidence suggests that many cell surface/membrane proteins have redox-labile, non-structural disulphide bonds that regulate molecular function upon reduction to cysteine (73, 115-117). For example, a previous study identified 30 proteins on the leukocyte cell surface that have labile disulphide bonds, these included receptors, integrins, transport and cell–cell recognition proteins (72). In addition, the demonstration that Trx and PDI are present and active on cell surface (118, 119) provides a mechanism for oxidative regulation of membrane protein function, and further support for the biological importance of such activity.

Examining the secreted form of Trx, which has a well-established role as an autocrine growth-like and cytokine-like factor (92, 120), Bertini and colleagues suggested that it catalyses oxidoreduction of thiols in one or more membrane proteins with chemotactic functions (121). Since disulphide bond reduction by Trx is a catalytic process and one molecule may reduce a number of disulphide bonds, it would be necessary for the active site disulphide of Trx to be reduced to a dithiol (122, 123). The presence of this functional capacity, poses the question as to how Trx (or other oxidoreductases) at the cell surface are kept reduced? A high likelihood possibility is the presence and activity of the enzyme thioredoxin reductase 1 (TrxR1). TrxR1 with its broad substrate specificity could, using NADPH, supply electrons to Trx / other Trx superfamily members, to allow continuous

turnover keeping the enzymes reduced and active. In support of this, extracellular TrxR1 has been shown to be secreted by cells and present in plasma (124). Nevertheless, the presence and activity of pro- and anti-oxidant systems including the Trx system at the plasma membrane is not well studied and the possible association of Trx and TrxR with lipid rafts is largely unknown. Of interest, is the observation that a rare alternative transcript derived from the thioredoxin reductase 1 encoding gene, TXNRD1, that expresses an atypical N-terminal Grx domain fused TrxR1 module and encoded by alternative exons located upstream of the core promoter, known as TXNRD1_v3 (v3), was shown to have the capacity to induce cell membrane protrusions (122). GFP- fusion variants of v3 were found located along the length and growing tips of these protrusions leading to cell membrane restructuring with the promotion of actin polymerization (122). Since actin has been described as stabilizer of membrane rafts, (125) this argues for a possible raft association of this TrxR1 variant. In support of this notion, Volonte and Galbiati showed that TrxR1 localized with specialized lipid rafts structures called caveolae, and that the key protein of caveolae, caveolin-1, can bind to and modulate TrxR1 activity via this interaction (126).

Interestingly, it has been shown that lymphocytes require a reducing environment for activation and proliferation (127-129). For example, the activation of T cells results in up regulation of thioredoxin resulting in the augmentation of thiols at the T cell surface (129-131). It is thought that this influences the persistence of T cells and consequently immune outcomes as cells bearing high levels of surface thiols, appear to have enhanced survival in oxidative microenvironments (132). In addition, T cell activation is associated with an increase in ROS (132) and enhanced production levels of reversible cysteine sulfenic acid formation, important reactive oxygen intermediate that is required for disulphide bond

formation and S-glutathionylation, implicating it as an important molecule in regulating redox-related T cell activation (133). Michalek, *et al* showed that certain proteins, including actin, undergo increased sulfenic acid modification after TCR stimulation, and that the reversible formation of sulfenic acid was indispensable for the proliferation of naive $CD8^+$ and $CD4^+$ T cells (133). Dendritic cells have also been shown to convert cystine to cysteine and release thioredoxin during antigen presentation, creating the reducing environment for T lymphocyte activation (127). This suggests that decreases in redox potential of the extracellular space in the immunological synapse can occur during antigen presentation to T cells, potentially creating a microenvironment that favours the reduction of labile disulphide bonds at the immune-cell surface (73, 130).

These insights into the role of CD4 in antigen presentation, its membrane compartmentalization and redox biology at the membrane surface raises questions as to how these phenomena might be integrated. The conformational changes in CD4, from its oxidised to reduced isoforms, require redox active enzymes and CD4 is a known substrate of cellular oxidoreductases (79, 84, 85). In addition, CD4's membrane spatial distribution and its association with lipid rafts is known to influence its activity (64) both with respect to its immune- and HIV receptor function. $CD4^+$ T cell function is also subject to redox regulation by ROS, a feature that can influence activation states and thus the outcome of immune responses (132). However, how redox events regulate the distribution of CD4 between different membrane microdomains, and the nature of functional oxidoreductase systems that might control these events at the cell surface are incompletely understood.

1.3 The CD4-HIV-redox biology paradigm

In addition to being an important component of the adaptive immune response, CD4 is also the primary receptor for HIV entry into cells.

1.3.1 HIV entry

1.3.1.1 Gp120 interaction with CD4

HIV-1 gp120 is known to bind to CD4 through a high-affinity interaction involving a large contact surface on gp120 known as the CD4 binding site (CD4bs) (134). This site incorporates two regions on gp120, the larger of which is a shallow cavity with residues that do not form direct contact with CD4, a feature that affords flexibility in the otherwise well conserved CD4bs (134) The smaller rounded, hydrophobic pocket is lined with highly conserved residues and is deeply concealed within gp120 where it becomes 'plugged' with the Phenylalanine 43 (Phe43) residue of CD4 upon CD4 binding (134). The conserved residues important for CD4 binding include Trp432, Trp427, Thr257, Asp368, Asp457, and Glu370 (134, 135). The most important contacts are those between Phe43 of CD4 and Glu370 and Trp427 of gp120, together with electrostatic interactions between Arg59 of CD4 and Asp368 of gp120 (19, 134) (Figure 3). Interestingly, this interaction seems to mimic the interaction observed between CD4 and MHC-II, in which both Phe43 and Arg59 (of CD4) are important residues (19). Additionally, five of gp120's nine disulphide bonds are all in close proximity to amino acids in gp120 that make contact with CD4 (134). Recent evidence has suggested that complex rearrangements of disulphide bonds in gp120 are required before, during and after the binding event, and that dynamic redox regulation of these bonds is

important for virus entry (136, 137). The binding of gp120 to CD4 induces structural changes in both proteins, with the conformational changes induced in gp120 facilitating further interaction with the cellular co-receptors, CCR5 or CXCR4.

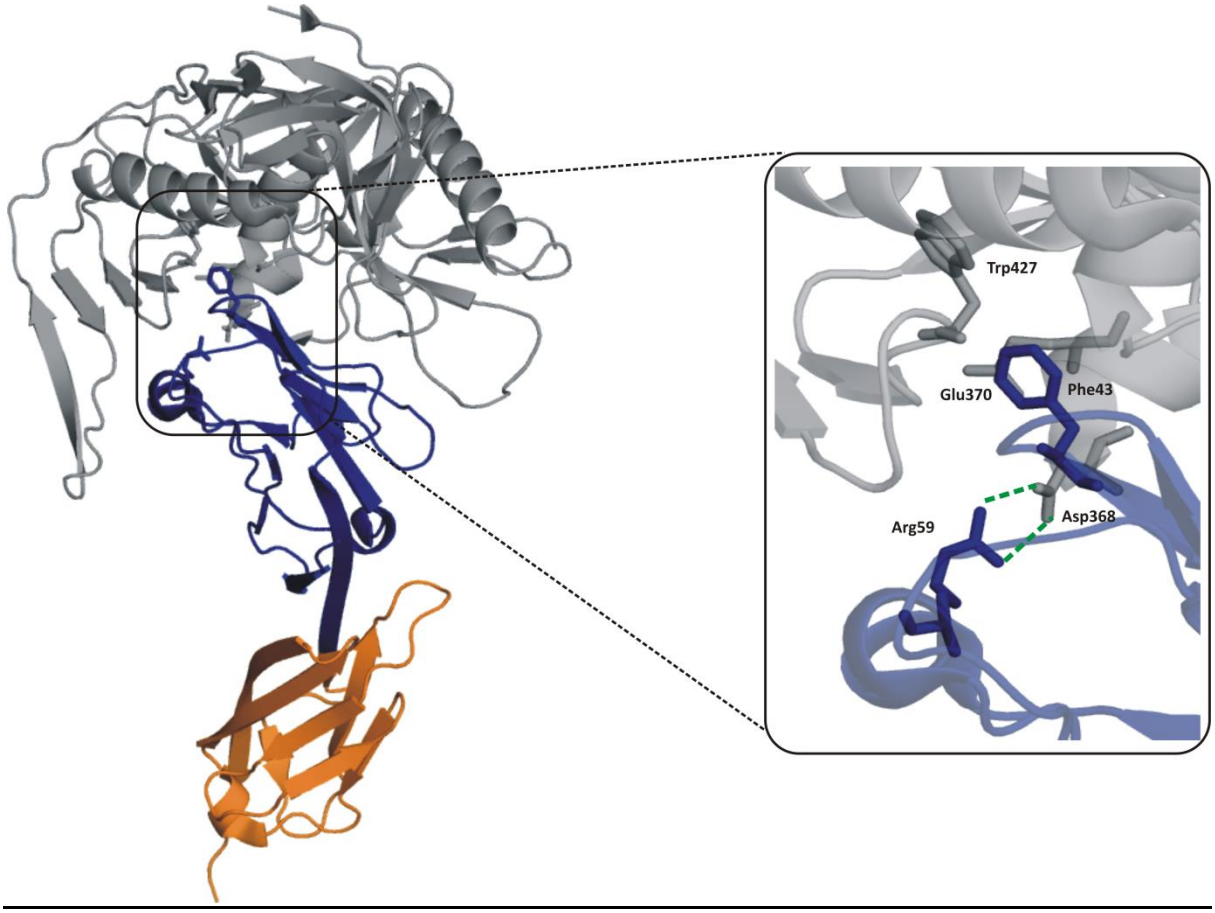

Figure 3: Ribbon representation of the CD4-gp120 interaction
A ribbon diagram of gp120 (grey) binding to CD4 domain (D1, blue) and domain 2 (D2, orange). The inset shows the gp120-CD4 interface with important interacting residues between gp120 (Asp368, Glu370 and Trp427) and CD4 (Phe43 and Arg59) shown in stick representation and labelled. The binding pocket in which the Phe43 residue of CD4 is inserted is indicated. The electrostatic interaction between Arg59 of CD4 and Asp368 of gp120 is shown as green dashed lines. Hydrophobic interactions are established between Phe43 of CD4 and Trp427 and Glu370 of gp120. Generated using PDB ID 1G9M (134) and PyMOL.

1.3.1.2 HIV–host cell membrane fusion

The sequential binding of gp120 to CD4 and the co-receptor leads to re-arrangements in the HIV-1 transmembrane glycoprotein subunit, gp41, resulting in its transformation into a "fusion-active" state and triggering of the membrane fusion cascade (138). At this stage, the formation of a triple-stranded coiled-coil enables the hydrophobic N-terminal fusion peptide of gp41 to insert into the target membrane and gp41 adopts a pre-hairpin intermediate conformation that bridges the viral and target membranes (139, 140) (Figure 4). Subsequent bending back of the coiled-coil on itself leads to the formation of the six-helix bundle which brings the viral and target membranes into close enough proximity for fusion to occur, culminating in the internalization of the virus capsid (138), (141).

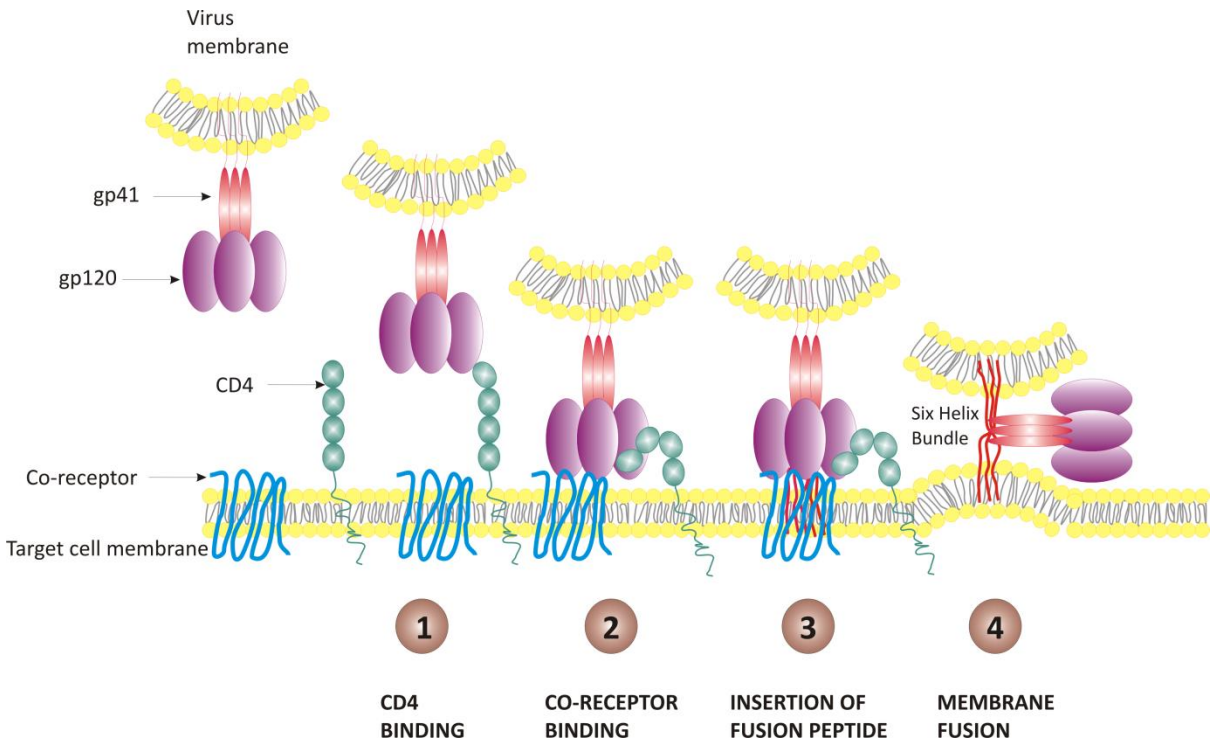

Figure 4: Schematic representation of the key steps leading to HIV-host cell membrane fusion
(1) HIV-1 gp120 binds to CD4 (CD4 binding) exposing the conserved co-receptor binding site on gp120. **(2)** The newly exposed co-receptor binding site on gp120 recognizes and binds either CCR5 or CXCR4 (co-receptor binding). **(3)** Engagement of the co-receptor exposes the gp41 fusion peptide and results in its insertion into the target membrane. **(4)** The formation of a helical hairpin structure (six-helix bundle) culminates in membrane fusion. Figure adapted from Delhalle *et al*. (142)

1.3.2 The redox biology of HIV-1 entry

Several studies have signified an important role for thiol/disulphide exchange at the cell surface in the entry mechanisms of a number of viruses (73, 143, 144). An example of this is the entry of Newcastle disease virus, in which free thiols in the Fusion (F) glycoprotein (required for virus entry into the cell) are detected only after binding of the virus to the target cell surface, suggesting that the reduction of disulphide bonds occurs (143). This results in conformational changes in the F protein leading to membrane fusion (143, 145). Similarly, changes in the topology of disulphide bonds appear to be important in the entry and pathogenesis of other viruses, such as Hepatitis C virus and Sindbis alphavirus (73, 143, 146).

The accepted HIV entry model attributes the structural changes within gp120 largely to intrinsic properties of the viral envelope, but the molecular mechanism(s) by which these changes are effected is poorly understood. An increasing amount of evidence suggests that these changes occur in the context of redox active enzymes present at the cell surface (147), implying that associated host factors may play an important role in the membrane fusion process. Both CD4 and gp120 have disulphide bonds that could be reduced under such physiological conditions, and there is a growing body of evidence that the cleavage of disulphide bonds in both gp120 and CD4 may be necessary for envelope-mediated cell-cell fusion and HIV entry (147-149). Further support for this is provided by the observations that compounds that block the cleavage of disulphide bonds also inhibit HIV-1 entry (148, 150).

1.3.3 CD4 oligomerization and compartmentalization: role in HIV infection

Several lines of evidence suggest that the oligomerization status of CD4 as well as its membrane microdomain localization influence virus entry and pathogenesis. As discussed in section 1.1.3, CD4 dimers and oligomers are required for T cell activation, with the monomeric form of CD4 displaying weak affinity for its natural ligand, MHC-II (42-44, 151). Conversely, it has been shown that HIV-1 preferentially binds to the monomeric reduced form of CD4 (82) (although the potential of gp120 to bind dimeric CD4 has not been unequivocally excluded) and that when CD4 dimerization is disrupted, enhanced viral entry is observed (81).

There is also data to suggest that HIV can alter the steady-state quotient of CD4 monomers and dimers to favour monomers (32, 152). Whether this is a mechanism for delaying the native immune response and facilitating immune surveillance evasion or if it is a requirement for the entry process is not known. CD4 oligomerization may be important for downstream events in the virus replication cycle although it is not necessary for viral entry (45). For these reasons, there is still some uncertainty around whether HIV-1 is capable of using CD4 dimers for entry or if CD4 dimers are disrupted by interaction with HIV-1 gp120.

Likewise, the membrane microdomain distribution of CD4 in the context of HIV-1 infection remains a subject of debate. Lipid rafts are used by a host of pathogens including viruses, parasites and bacteria as portals of entry into cells (59). In the case of HIV-1, certain studies support the notion that HIV-1 uses raft- associated CD4 as a site of entry (153-155). For example, in macrophages, it seems that CD4 raft localization is a requirement for productive infection (153, 155). In contrast, other studies have shown that CD4 located in non-raft plasma membrane microdomains supports entry of the virus into cells (156, 157). In

addition, the membrane microdomain distribution of HIV-1 co-receptors is also unclear. The co-receptors for HIV-1 may reside in microdomains different to that in which CD4 is found (158-160). Consequently, it may be necessary for co-mobilization of CD4 and/or co-receptor into or out of raft regions for efficient HIV-1 entry (161, 162). Dumas *et al*, proposed that these contradictory observations may be accounted for by considering the possibility that different steps of the entry process were being assessed in each study (161). Hence, raft localization of CD4 may not be required for virus binding, but post-binding fusion/entry steps may require intact lipid rafts (156, 161). There is an ever growing body of evidence that glycosphingolipids and cholesterol, both components of lipid rafts, play a vital, perhaps diverse role in HIV-1 pathogenesis, from viral entry to assembly of progeny virions (162-164). Taken together, these insights underscore the complexity of the structural and membrane localization dynamics of CD4 that are at play during the formation of immune signalling complexes and during HIV infection, and the factors involved in the regulation thereof.

1.3.4 CD4, gp120 and cellular oxidoreductases

Agents that interfere with thiol-disulphide exchange following the interaction of CD4 and gp120 have been shown to inhibit HIV infection (149, 150, 165). This effect is attributed to the inhibition of cell surface oxidoreductases such as PDI, although the precise molecular mechanism for this inhibition has not been elucidated (150). In the context of the gp120-CD4 interaction, Auwerx and colleagues provided evidence that the interaction between CD4 and HIV-1 gp120 can be regulated by Grx1 and Trx1 (85). Taken together, the data showing that HIV-1 gp120 preferentially binds to reduced isoforms of CD4, and that manipulating the activities of cell surface active oxidoreductases such as PDI, Grx and Trx

has significant effects on HIV replication *in vitro*(150, 166, 167), suggests that redox exchanges play a critical role in the mechanism of HIV-1 entry. However, elucidating the role of these oxidoreductases on CD4 is complicated by the observations that gp120, itself containing nine disulphide bonds, is also a substrate for PDI-, Trx- and Grx1-mediated reduction (167).

Accordingly, despite the increasing amount of evidence alluding to the importance of dynamic oxidoreductive isomerisation and the plasma membrane microdomain localization of CD4 in both T cell activation and HIV entry, our understanding of the molecular mechanisms thereof and consequences of oxidoreductase activity on CD4 in the cellular context is limited. Expanding our knowledge on these intricate aspects of CD4 biology is essential for gaining a complete understanding of both CD4-dependent cell signalling and HIV entry, and has relevance for the conceptualization of novel antiviral and immunomodulatory therapies.

1.4 Thesis outline and main objectives

In this thesis, we report the findings of studies that provide further insights into the biological significance of cell surface redox systems, particularly in the context of immune- and HIV receptor functions of CD4. These are presented in the form of two journal publications. In Chapter 2 we show the results of collaborative research lead by Prof Elias Arner (Karolinska Institutet), which investigated the membrane association of the v3 variant of Thioredoxin reductase 1 (TXNRD1_v3) (v3). These studies show that v3 is targeted to lipid rafts via N-terminal acylation, where it may be involved in regulating changes in the underlying cytoskeleton. While the physiological relevance of a membrane-associated Trx system remains unclear, the findings allude to the importance of redox capacity at the cell

surface, which increasingly suggests is essential for the function of CD4. With this in mind, we then investigated the effects of manipulating cell surface redox activity on CD4 membrane domain trafficking and function as HIV receptor. We describe experiments on the membrane microdomain distribution of CD4 under conditions that alter the extracellular redox environment by general and specific inhibition of cellular oxidoreductases, and discuss how this segregation relates to known viral inhibition by agents that interfere with thiol-disulphide exchange. To assess this, we used a combination of modern and classical methodologies for analyzing the plasma membrane microdomain contents, and were able to obtain quantifiable data on the microdomain localization of CD4 from a flow cytometric detergent solubility assay. In parallel, we assessed the effect of these altered conditions of HIV infectivity of viruses with differing CD4 dependencies. These results are presented in Chapter 3 as a published research article. In conclusion to this thesis, Chapter 4 summarizes our key observations and provides perspective on the role of oxidoreductases, particularly those of the Trx system, in HIV entry.

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The Rare TXNRD1_v3 ("v3") Splice Variant of Human Thioredoxin Reductase 1 Protein Is Targeted to Membrane Rafts by *N*-Acylation and Induces Filopodia Independently of Its Redox Active Site Integrity*

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Background: The TXNRD1_v3 ("v3") protein is a rare variant of human thioredoxin reductase 1.
Results: Membrane targeting of v3 occurs by N-terminal myristoylation and palmitoylation, and its overexpression triggers induction of filopodia independently of its redox active site integrity.
Conclusion: The v3 protein is targeted to membrane rafts.
Significance: These results imply that v3, shown to be targeted to membrane rafts, may be involved in signaling events.

The human selenoprotein thioredoxin reductase 1 (TrxR1), encoded by the *TXNRD1* gene, is a key player in redox regulation. Alternative splicing generates several TrxR1 variants, one of which is v3 that carries an atypical N-terminal glutaredoxin domain. When overexpressed, v3 associates with membranes and triggers formation of filopodia. Here we found that membrane targeting of v3 is mediated by myristoylation and palmitoylation of its N-terminal MGC motif, through which v3 specifically targets membrane rafts. This was suggested by its localization in cholera toxin subunit B-stained membrane areas and also shown using lipid fractionation experiments. Utilizing site-directed mutant variants, we also found that v3-mediated generation of filopodia is independent of the Cys residues in its redox active site, but dependent upon its membrane raft targeting. These results identify v3 as an intricately regulated protein that expands *TXNRD1*-derived protein functions to the membrane raft compartment.

Thioredoxin reductase (TrxR)[2] and thioredoxin (Trx) together with NADPH comprise the Trx system, which is involved in a wide range of cellular processes, including cell proliferation and differentiation, antioxidant defense, maintenance of deoxyribonucleotide synthesis, signaling of apoptosis, redox control of protein function, transcription factor activity, and cancer development (1–7). The Trx system orchestrates its many functions mainly through redox reactions, whereby Trx reduces disulfides in target proteins for the support or modulation of their activities, whereas Trx in turn is kept reduced and active by TrxR using NADPH.

Human cells carry three genes encoding three distinct TrxR isoenzymes. The *TXNRD1* gene encodes the classical and most abundant, predominantly cytosolic, TrxR1, which is expressed in most human cells and uses Trx1 as its prime substrate (3, 4, 8, 9). The predominantly mitochondrial TrxR2 enzyme is encoded by *TXNRD2* and mainly reduces mitochondrial Trx2 (10–12). The *TXNRD3* gene encodes a thioredoxin glutathione reductase isoenzyme that contains a monothiol glutaredoxin (Grx) domain as an N-terminal addition to the TrxR module, which otherwise is similar in domain structure to TrxR1 and TrxR2. The thioredoxin glutathione reductase isoenzyme was found to be involved in the maturation of sperm cells and is mainly expressed in early spermatids in testis (13–15). Both the cytoplasmic and the mitochondrial Trx systems are essential for mammals, as demonstrated by the embryonically lethal phenotype of knock-out mice for any one of the enzymes TrxR1, Trx1, TrxR2, or Trx2 (16–19).

The human *TXNRD1* gene on chromosome 12 (12q23-q24.1) displays a complex genomic organization. It gives rise to numerous transcripts that can undergo extensive splicing, in particular at the 5′-end, producing several different protein isoforms (8, 9, 20–22). One of these isoforms, TXNRD1_v3 ("v3"), is peculiar by utilizing three additional exons encoding an atypical dithiol active site Grx domain, which is expressed in N-terminal fusion to the classical TrxR1 module (8, 20, 23, 24). These three exons, termed β_{-VIII}, β_{-VI}, and β_{-V}, are unique to v3 and are encoded by a genomic region upstream of the more commonly transcribed *TXNRD1* exons. Therefore, transcription of v3 must initiate upstream of the previously characterized core promoter of TrxR1 (8, 21, 22, 25, 26) and must thus be regulated by an alternative promoter, which hitherto has remained uncharacterized. Intriguingly, humans, chimpanzees, and dogs

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[2] The abbreviations used are: TrxR, thioredoxin reductase; Trx, thioredoxin; TXNRD1_v3, splice variant of human TrxR1 carrying an additional N-terminal glutaredoxin domain; *TXNRD1*, human gene encoding TrxR1; CT-B, cholera toxin subunit-B; Grx, glutaredoxin; v3, short notation for the TXNRD1_v3 splice variant; v3(Grx), glutaredoxin domain of v3; 2-HMA, 2-hydroxymyristic acid; 2-BPA, 2-bromopalmitic acid; GAP, growth-associated protein.

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express v3, but mice or rats do not (20). Endogenous expression of v3 has been demonstrated in human testis by Northern blot analyses as well as using immunohistochemistry, with the latter displaying particularly strong staining in Leydig cells (23). Immunoblotting and mass spectrometry also indicated v3 protein expression in a human mesothelioma cell line (24), and v3 could furthermore be detected in extracts of bovine and dog testis (20). In addition, several human cancer cell lines show expression of v3-encoding transcripts, as detected by first-strand reverse transcription-polymerase chain reaction (PCR), with v3 expression also found to be induced by estradiol or testosterone treatment (23). However, transcripts for v3 are rarely found in the form of expressed sequence tag clones with only few such clones currently found in the National Center for Biotechnology Information (NCBI) databases (including five from testis, accession numbers BG772375, AY057105, BG717223, DC401599, and DC400412; four from trachea, accession numbers AK304241, DC417264, DB230289, and DB233566; two from glioblastoma, accession numbers BF342747 and AW027910; one from squamous cell carcinoma, accession number BP355955; and one from astrocytes, accession number DA033928). This should be compared with more than 1,700 expressed sequence tag clones found to encode the other forms of TrxR1. It should be noted, however, that some of those other sequences could also be derived from v3-encoding transcripts, although they will not be discovered as such if they have incomplete 5′-ends, which is the case with many expressed sequence tag clones.

The Grx domain of v3 has an atypical CTRC redox active site motif (8, 20) and lacks activity in any of the classical Grx assays (20). However, when mutated to CPYC, the motif commonly found in Grx proteins (27), the altered v3 protein also gained classical Grx activity (20). The v3 isoform, when overexpressed in human cells either as the isolated Grx domain or in fusion with the TrxR1 module as its C-terminal partner, triggers rapid changes in cell shape and a dynamic formation of cell membrane protrusions (23). GFP fusion variants of v3 were found to locate along the length and growing tips of these protrusions, in close proximity to actin. Furthermore, v3 seemed to lead actin into these protrusions followed by β-tubulin (23). These cell membrane protrusions were later characterized as having all features typical of filopodia (28). In the present study, we wished to further characterize the features of v3 that trigger these changes of the cellular phenotype and to understand how the protein is targeted to the membrane compartment. Because it was previously found that expression of either the complete TXNRD1_v3 protein or only the isolated v3(Grx) domain was sufficient for membrane targeting and induction of filopodia (23), we focused here on this property as held by the v3(Grx) domain.

By mutating the two v3(Grx) active site Cys residues to Ser and thus converting its CTRC motif to STRS, thereby incapacitating any potential redox activity of this motif, we show herein that association of v3(Grx) with actin polymerization as well as its membrane targeting is independent upon the integrity of its active site. Instead we found that *N*-acylation of the N terminus of v3(Grx) is both required and sufficient to target the protein to the plasma membrane. We furthermore found that it is specifically targeted to membrane rafts. These membrane structures have commonly also been called "lipid rafts," but are in the present study named and defined according to the 2006 consensus of the Keystone Symposium on Lipid Rafts and Cell Function (29).

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All regular chemicals or reagents were of high purity and obtained from Sigma-Aldrich, unless otherwise specified.

Cell Lines—Human A549 lung carcinoma (A549 cells) (CCL-185; ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 4.5 g/liter glucose at 37 °C in a humidified atmosphere with 5% CO_2. Human embryonic kidney cells (HEK293) (CRL-1573; ATCC) were cultured in Eagle's minimum essential medium (ATCC), and human ovarian SKOV3 cells (HTB-77) were cultured in McCoy's 5A medium, modified with L-glutamine and sodium bicarbonate (Sigma-Aldrich) at 37 °C in a humidified atmosphere with 5% CO_2. Cell culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin (all from PAA Laboratories).

Vectors for Expression of the Glutaredoxin Domain of v3 in Fusion with GFP and Mutants Thereof—The construct expressing the wild type Grx domain of v3 with GFP as a C-terminal fusion partner (here called "v3(Grx)") was kindly provided by Dr. Anastasios E. Damdimopoulos (Karolinska Institutet, Stockholm, Sweden) and was previously described in detail (23). Using standard cloning techniques with that plasmid as template, we created vectors expressing the active site double mutant C76S/C79S and the G2A and C3S mutants, as well as a variant encompassing only the first 14 amino acids of v3 in fusion with GFP (1–14), as further described under "Results." We also used a pure GFP control. Primers were purchased from Thermo Scientific, and all constructs were sequenced by GATC Biotech to confirm the desired mutations.

Transfection and Immunocytochemistry—Cells were grown on glass chamber slides (Lab-Tek II chamber slide system, Nalge Nunc International) and transiently transfected using Lipofectamine 2000 (Invitrogen) or TurboFect transfection reagent (Thermo Scientific) according to the manufacturer's instructions. About 18 h after transfection, the slides were washed with PBS or treated with 2-hydroxymyristic or 2-bromopalmitic acid (see below) before fixation in 4% paraformaldehyde solution for 15 min. For experiments that involved subsequent actin staining, the slides were washed two times with PBS and cells were permeabilized using PBS containing 0.5% Triton X-100 and 2% BSA for 20 min at ~20 °C whereupon they were washed two times in PBS and incubated with rhodamine-conjugated phalloidin (1:500; Molecular Probes/Invitrogen) in PBS for 1 h at ~20 °C. For experiments involving subsequent staining of membrane rafts/caveolae, slides were then washed two times with PBS and subsequently incubated with Alexa Fluor 555-conjugated cholera toxin subunit-B (CT-B; Molecular Probes/Invitrogen) (1 mg/ml) for 20 min in chilled complete growth medium on ice. Finally, all slides were washed two times with PBS and mounted with glass coverslips (Menzel-Gläser/Thermo Scientific) using ProLong Gold antifade reagent with

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DAPI (4′,6-diamidino-2-phenylindole, Invitrogen). Confocal imaging was carried out on a LSM700 (Zeiss). In all cases, multicolor imaging was performed sequentially to minimize cross-talk between the channels.

Treatment with 2-Hydroxymyristic Acid (2-HMA) and 2-Bromopalmitic Acid (2-BPA)—2-Hydroxymyristic acid and 2-bromopalmitic acid (Santa Cruz Biotechnology) were stored as 100 mM stock solutions in ethanol and delivered to cells as complexes with BSA. To prepare each complex, the fatty acid compound was incubated at a concentration of 2 mM in serum-free medium containing 2 mM fatty acid-free BSA for 2 h at 37 °C. Subsequently, transfected cells were treated with the fatty acid-BSA solution for 2 h before adding serum containing full medium to final concentrations of 1 mM fatty acid, 1% (v/v) ethanol, and 5% (w/v) serum. The cells were thereupon incubated for 24 h with 2-bromopalmitic acid or for 48 h with 2-hydroxymyristic acid and subsequently prepared for immunocytochemistry as described above.

Isolation of Membrane Rafts—HEK293 cells transfected with the wild type v3(Grx), G2A, or C3S expression plasmids were harvested for membrane extractions 48–64 h after transfection. Membrane rafts were extracted by "flotation" ultracentrifugation according to methods described by Alexander *et al.* (30), with minor modifications. Briefly, ~6×10^6 transfected cells were harvested and resuspended in 400 μl of flotation buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM β-glycerol phosphate disodium salt pentahydrate, 30 mM sodium pyrophosphate, and 1% Triton X-100). The suspension was centrifuged at 2,000 $\times$ *g* for 5 min, the pellet was washed with 100 μl of fresh flotation buffer, and sucrose was added to the combined supernatants to a concentration of 45% (final volume of 2 ml in flotation buffer). The 2 ml of sucrose-containing lysate was transferred to the bottom of a 12.5-ml polycarbonate centrifuge tube (Beckman), over which 30% (5.5-ml) and then 5% (4.5-ml) sucrose solutions in flotation buffer were gently and sequentially layered. The tubes containing the sucrose cushions were then centrifuged at 200,000 $\times$ *g* for 18 h at 4 °C. 1-ml fractions (12 in total) were gently collected from the top of the gradient, snap-frozen in liquid nitrogen, and stored at −80 °C for further analysis.

Biochemical Analysis of Grx-GFP Association with Detergent-resistant Membrane Fractions—The level of expression and distribution of v3(Grx)-GFP variants in transfected HEK293 cells was assessed by Western and slot blotting approaches, respectively. Firstly, to confirm that all three variants were uniformly expressed, total cell lysates for each transfected culture were generated by pooling equal quantities of each fraction (1–12) from the membrane raft preparations. The pooled samples were diluted in PBS, reconcentrated, and analyzed by SDS-PAGE and Western blot using an HRP-conjugated monoclonal anti-GFP antibody (Rockland Immunochemicals) and standard procedures. In the experiments designed to gain insights into the oligomeric state of the raft-associated Grx-GFP proteins, only fractions 4–6 and 10–12 from each sample were pooled, and the samples were then either subjected to conventional SDS/DTT/heat treatment or treated with a DTT-free loading buffer and not heated, before loading onto gels. To control for the specificity of GFP detection, mock-transfected cells were processed and analyzed identically. To evaluate the extent of localization of the Grx-GFP variants in detergent-resistant membrane fractions, all of the fractions 1–12 from each preparation were analyzed individually by a slot blot procedure. Briefly, a 50-μl sample of each fraction was diluted 5-fold in Tris-buffered saline (TBS, pH 7.4) containing 10% methanol, with 100 μl subsequently adsorbed onto a nitrocellulose membrane using a Minifold II slot blot apparatus (Schleicher & Schuell). Detection of GFP fusion protein was carried out using the anti-GFP antibody as described above. To confirm consistency of raft enrichment for each sample, the same fractions were immobilized and probed in parallel with an HRP-conjugated CT-B (Life Technologies), which binds the exclusively membrane raft-residing GM1 ganglioside with high affinity.

RESULTS

Membrane Targeting of v3 by Myristoylation and Palmitoylation at Its N-terminal MGC Motif—When overexpressed in cancer cells, the glutaredoxin domain of v3 (v3(Grx)) in fusion with GFP at its C-terminal end displays a distinct localization pattern that is characterized by strong staining of the perinuclear region and cytosolic speckles as well as accumulation of the protein at the plasma membrane (23, 28). An N-terminal myristoylation motif of v3 was suggested using ExPASy Prosite and NMT – The MYR Predictor, as reported earlier (8, 28, 31, 32), but this has not yet been experimentally studied. The Grx domain of v3 also carries an atypical dithiol active site motif, as discussed above. To characterize the importance of these motifs for v3 targeting to cell membrane regions, we expressed a number of v3(Grx)-derived mutant variants in fusion with GFP (see Fig. 1*A* for a scheme of the constructs) using three different human cell lines (A549, HEK293, and SKOV3) (Fig. 1*B*). This revealed that a variant with the two Cys moieties of the redox active site changed to redox inactive Ser residues (C76S/C79S) yielded an identical phenotype of membrane association as seen with wild type v3(Grx), which was highly reminiscent of that reported earlier for the wild type protein (23, 28). Both variants strongly accumulated in the perinuclear area and showed distinct cytosolic structures in a dotted pattern, as well as a pronounced plasma membrane association in all three cell types (Fig. 1*B*). Interestingly, however, a single substitution of the Gly residue at position 2 with Ala (G2A) completely abolished the membrane association of the protein. This G2A mutant, destroying the myristoylation consensus motif (33–35), showed a diffuse cytosolic and nuclear distribution similar to that of pure GFP (Fig. 1*B*). Thus, the prominent features of plasma membrane association, cytosolic speckles and strong perinuclear accumulation, were all impeded by this single amino acid substitution. In contrast, substituting solely the Cys residue at position 3 with Ser, yielding the (C3S) construct that is expected to eliminate the possibility of palmitoylation (35, 36) while maintaining the myristoylation site at the Gly-2 residue (35, 37, 38), lowered the extent of plasma membrane association and the amount of cytosolic speckles, but maintained a strong compartmentalization of the protein, with mainly perinuclear localization (Fig. 1*B*). To study whether the membrane targeting could indeed be guided solely by acylation of the N-terminal motif of v3, we also analyzed a construct

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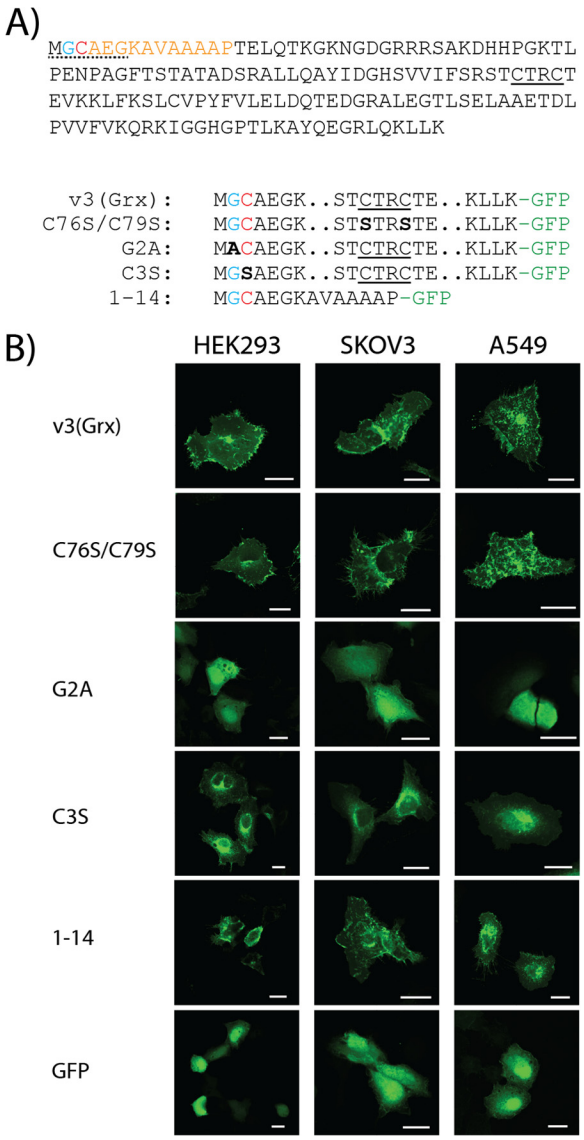

FIGURE 1. **The redox active site dithiol motif of v3 is dispensable, whereas its N-terminal acylation motif is required for membrane association of the v3(Grx) domain.** *A*, the Grx domain of v3 is here shown with the proposed consensus sequence for myristoylation *underlined* (*dotted*; presumed myristoylated Gly residue shown in *blue*) at the beginning of a 14-amino acid stretch at the N terminus of the protein (*orange*). A Cys-3 residue may potentially be palmitoylated (*red*). The dithiol active site motif of v3(Grx) is *underlined* (*solid*). Final amino acid sequences of the v3(Grx)-derived constructs studied herein are schematically indicated below. Mutated residues are marked *bold* and in *black* with the names of the variants to the *left*. *Two dots* indicate omitted residues (for full sequence, see *top panel*), and *-GFP* in *green* indicates a C-terminal GFP fusion partner. *B*, A549, HEK293, and SKOV3 cells were transfected using either of the v3(Grx)-derived GFP fusion constructs: wild type v3(Grx), the active site mutant C76S/C79S, the N-terminal G2A or C3S mutants, or a truncated variant solely containing the first 14 amino acids of v3 (*1–14*). Control transfections were performed using a construct expressing GFP alone (*GFP*). Fluorescence was recorded 18 h after transfection using confocal microscopy. *Scale bar* = 20 μm.

encompassing only the first 14 amino acids of v3(Grx) in fusion with GFP. Cells expressing this protein displayed the same phenotype as seen with wild type v3(Grx) (Fig. 1*B*). Together, these results revealed that the N-terminal MGC motif of v3(Grx) is both required and sufficient for the targeting of this protein to specific membrane structures of the transfected cells.

To further study the dependence of the v3 membrane targeting upon myristic and palmitic acid, we incubated A549, HEK293, or SKOV3 cells expressing v3(Grx) with 2-hydroxymyristic acid (2-HMA) and 2-bromopalmitic acid (2-BPA), two competitive inhibitors of myristoylation and palmitoylation, respectively (39–41). After treatment with 2-BPA, the subcellular localization of the protein changed to a mainly perinuclear localization with significantly reduced plasma membrane staining and fewer cytosolic speckles (Fig. 2). This pattern coincides with the expression profile of the palmitoylation-impeded C3S variant (compare Fig. 1*B* and Fig. 2). The 2-HMA treatment, on the other hand, nearly completely abolished compartmentalization of the protein and gave a diffuse cytosolic distribution closely reminiscent of pure GFP or the G2A variant of v3 (compare Fig. 1*B* and Fig. 2).

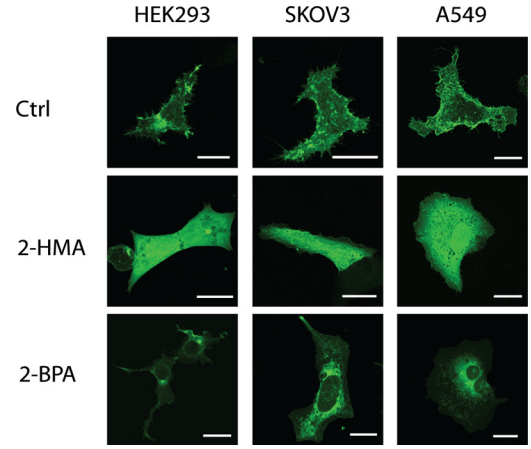

FIGURE 2. **Inhibition of myristoylation and palmitoylation by 2-HMA and 2-BPA.** A549, HEK293, and SKOV3 cells were transfected using the wild type v3(Grx)-GFP fusion construct. 18 h after transfection, cells were treated with either 1% EtOH or 2-hydroxymyristate for 48 h or with 2-bromopalmitate for 24 h. GFP fluorescence was recorded using confocal microscopy. *Scale bar* = 20 μm. *Ctrl*, control.

Co-localization of v3(Grx) with the Membrane Raft Marker CT-B—Next we investigated the subcellular localization of v3(Grx) in relation to membrane structures binding CT-B, a marker often used for membrane rafts (42–44). For this, A549 cells were transfected with the GFP fusion constructs expressing wild type v3(Grx), the active site mutant C76S/C79S, or the N-terminal mutants G2A and C3S. To subsequently assess localization in relation to the CT-B marker for membrane rafts, the cells were fixed 24 h after transfection and incubated with Alexa Fluor 555-conjugated CT-B. Incubation with CT-B did not affect the overall cellular appearance nor the subcellular GFP fluorescence patterns obtained with the various v3(Grx) variants. CT-B showed a similar pattern in all of the cells, with plasma membrane staining in selected localized areas, as well as dotted cytosolic and perinuclear distribution. The latter compartments were previously identified as being early endosomes and Golgi apparatus or endoplasmic reticulum, respectively (45, 46). The GFP signal of the v3(Grx) and C76S/C79S variants closely overlapped with that of CT-B staining, although the overlap was not exclusive, and fractions of the cells also showed staining for only one of the fluorophores (Fig. 3, *arrows* and *magnified lower panel*). In contrast, the G2A variant of v3(Grx), which displayed a diffuse cytosolic distribution, lacked subcellular proximity with CT-B in all cellular compartments (Fig. 3). Devoid of the palmitoylation site (Cys-3) but maintaining an

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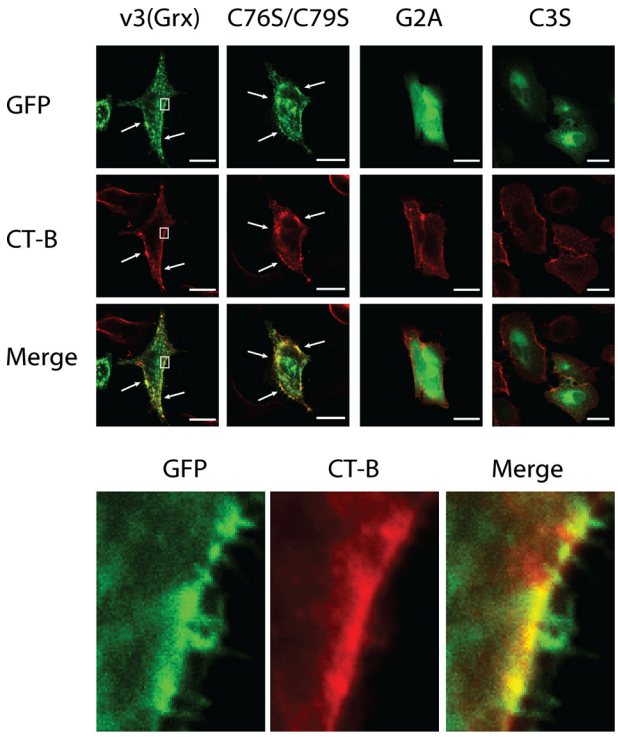

FIGURE 3. **Co-localization of v3(Grx)-GFP fusion variants with the membrane raft marker CT-B.** A549 cells were transfected with constructs expressing either the wild type v3(Grx) domain or mutants thereof (C76S/C79S, G2A, or C3S) fused to GFP, as indicated. 18 h after transfection, the cells were fixated and stained using Alexa Fluor 555-conjugated CT-B for 20 min on ice to visualize membrane rafts. Fluorescence was acquired using confocal microscopy. The *lower panel* shows a higher magnification of the selected area in the cells transfected with the wild type v3(Grx) (*white rectangle*). Regions with a high degree of co-localization are indicated by *arrows*. *Scale bar* = 20 μm.

intact myristoylation site at Gly-2, the C3S variant displayed mainly its perinuclear distribution, where it showed some overlap with CT-B, but appeared only minimally at the plasma membrane (Fig. 3) as also shown above.

These results strongly suggested that overexpressed v3 in transfected cells becomes targeted to cell membranes through myristoylation and palmitoylation, where it furthermore closely associates with the membrane raft marker CT-B. We next wished to confirm the localization of v3(Grx) in membrane rafts by the alternative method of membrane fractionation.

Appearance of v3(Grx) in CT-B-positive Purified Membrane Raft Fractions—The proposed sizes of membrane raft microdomains are below 100 nm and are thus not resolvable by conventional confocal microscopy, thereby limiting the interpretation of co-localization studies by microscopy. To further validate co-localization of v3(Grx) with CT-B, we therefore purified membrane rafts from HEK293 cells expressing either the wild type v3(Grx) or the G2A or C3S variants and analyzed these by slot and Western blot approaches. The membrane rafts were purified by conventional flotation methods, which involve separation of intact, detergent-resistant rafts from Triton X-100 solubilized membranes and cytosolic proteins, based on their unique "buoyancy" in sucrose-containing media subjected to high speed centrifugation (Fig. 4*A*, *left*). The v3(Grx), G2A, and C3S variants were all expressed as similar levels and recovered to the same extent in this centrifugation, as visualized using immunoblotting with antibodies directed against GFP, which were used to probe pooled fractions (Fig. 4*B*). In agreement with the co-localization data suggested by fluorescent microscopy, only the wild type v3(Grx) variant showed association with membrane rafts (fractions 4–6), whereas the G2A and C3S variants were not detected in these membrane microdomain fractions (Fig. 4*C*, *right panel*). This effect was specific and directly related to the amino acid sequences of the N-terminal raft-localization domain of the proteins because the amounts of purified membrane rafts, measured by enrichment of CT-B-binding lipids in fractions 4–6, were comparable between the samples (Fig. 4*C*, *left panel*).

To additionally confirm the findings and gain further insights into the nature of the raft-associated v3(Grx)-GFP, we next analyzed fractions 4–6 (rafts) and fractions 11–12 (soluble) by conventional and "mildly denaturing, nonreducing" SDS-PAGE/Western blot protocols. For this, we pooled fractions 4–6 and 11–12 and then treated them with SDS without or with DTT and heat, before separation on SDS-PAGE and subsequent Western blot analyses. Although all v3(Grx) variants displayed a clear and equally strong signal at the expected size of ~48 kDa in the soluble fractions, again confirming comparable total expression levels, only the wild type v3(Grx) variant was seen in the membrane raft fractions. This membrane raft-associated protein appeared partly in the form of a dimer in the absence of DTT and heat, whereas reducing and denaturing conditions resolved the dimeric band into a solely monomeric protein (Fig. 4*D*).

Induction of Filopodia, Changes of Cell Morphology, and Effects on Actin Polymerization—Concomitant with its compartmentalized membrane targeting, v3(Grx) overexpression was previously found to be correlated with actin polymerization and induction of cell membrane protrusions, identified as filopodia (23, 28). Here we analyzed how these features compared between wild type v3(Grx) and the C76S/C79S, G2A, or C3S mutants, as visualized using detection of the GFP fusion partner and co-staining of actin with rhodamine-conjugated phalloidin. Both v3(Grx) and actin appeared localized in close proximity with each other at the cell membrane, as found earlier (28). This was also seen with the redox active site mutant C76S/C79S (Fig. 5). Particularly strong accumulation could be seen at cell-to-cell contact sites (Fig. 5, see C76S/C79S variant). As also found above, cell membrane accumulation required the Gly-2 and Cys-3 residues (Fig. 5), *i.e.* uncompromised N-terminal myristoylation and palmitoylation motifs, respectively (Fig. 1*A*). Expression of v3(Grx) as well as C76S/C79S triggered high increases in the number of filopodia-like membrane protrusions, and at higher magnification, both proteins could be seen directly associated with these protrusions (Fig. 5). In contrast, the mainly cytosolic G2A and preferentially perinuclear C3S variants did not induce this filopodia-enriched cellular phenotype, although a few membrane protrusions were also seen in cells expressing these proteins (Fig. 5).

DISCUSSION

Here we found that the association of v3 with cell membranes was independent of its dithiol redox active site motif and fully governed by targeting to membrane rafts through its N-termi-

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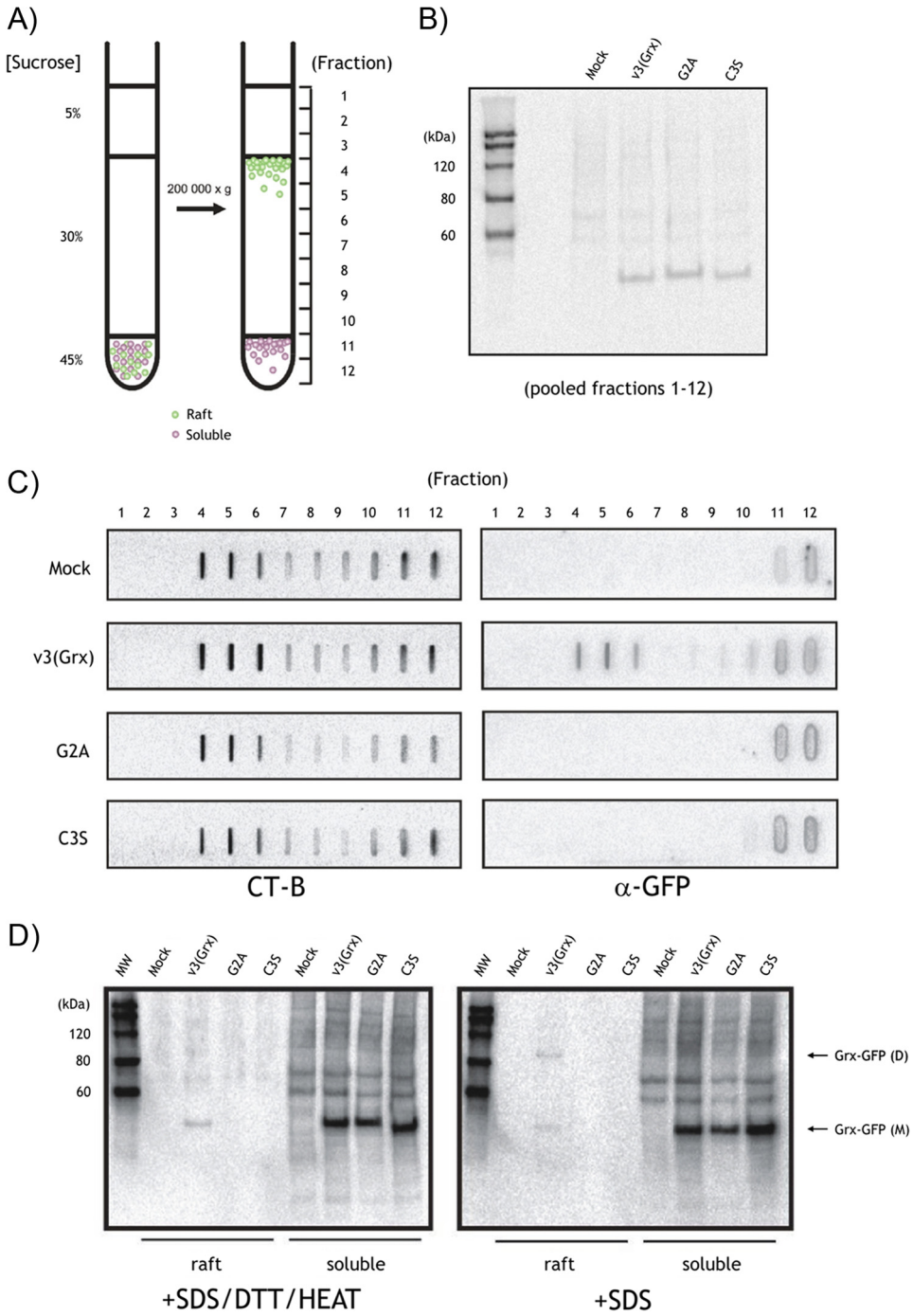

FIGURE 4. **Localization of wild type v3(Grx) in purified, CT-B-positive membrane raft fractions.** *A*, principle of membrane raft purification by centrifugation and flotation. HEK293 cells expressing the v3(Grx)-GFP fusion variants were harvested and lysed 48 h upon transfection. The Triton X-100-solubilized cell lysate was overlaid sequentially with the indicated sucrose cushions, and the samples were centrifuged at 200,000 × *g* for 18 h. Consecutive 1-ml fractions (*fractions 1–12*) were collected from the top of the centrifuge tubes. *B*, Western blot analysis of pooled fractions (*fractions 1–12*) showing uniform expression of the ~48-kDa v3(Grx)-GFP variants in all transfected cultures. *C*, slot blot analysis of individual fractions 1–12, probed with an HRP-conjugated CT-B (*left panel*) or GFP antibody (α-GFP, *right panel*). *MOCK*, mock-transfected. *D*, fractions 4–6 (*raft*) or fractions 11–12 (*soluble*) of each sample were pooled and analyzed by Western blot for the presence of v3(Grx)-GFP fusion variants using α-GFP. The samples were either treated with SDS, DTT, and heat or treated only with SDS. The monomeric v3(Grx)-GFP variants (*Grx-GFP (M)*) as well as the dimeric form of the wild type variant v3(Grx) (*Grx-GFP (D)*) are indicated by *arrows*. *MW*, molecular size markers.

nal MGC myristoylation and palmitoylation motif. This targeting of the protein to membrane rafts was also sufficient and required to support v3-stimulated increases in the number of cell membrane filopodia.

The N-terminal MGCAEG sequence of v3 meets the general consensus motif for myristoylation, even if the second Gly residue at position 6 deviates from more commonly seen Ser or Thr residues in myristoylated proteins (8, 31, 32). Upon further inspection of this sequence, we noted that the Cys-3 residue might potentially be palmitoylated, which led us to the construction and characterization of the C3S variant. The results presented herein indeed strongly suggest that the Cys-3 residue

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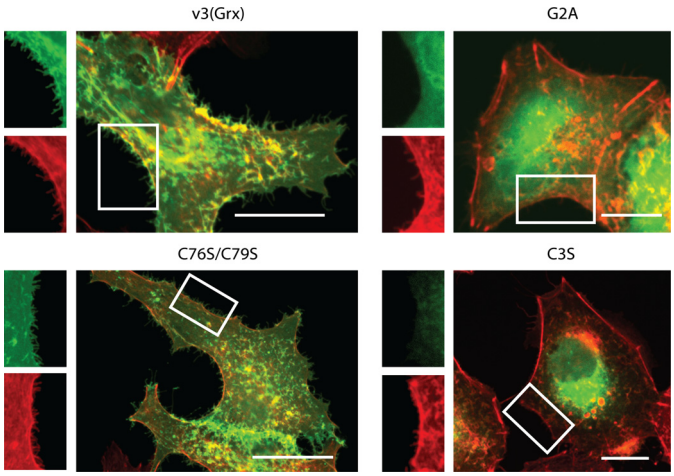

FIGURE 5. **The relation of v3(Grx) to cell morphology and actin polymerization is independent of the active site but requires a functional *N*-acylation motif.** A549 cells were transfected using the indicated v3(Grx)-GFP fusion constructs. Cells were additionally stained for actin using rhodamine-conjugated phalloidin, and fluorescence was recorded using confocal microscopy. The pictures show merged fluorescent signals of GFP (*green*) and rhodamine (*red*) after excitation at 488 and 555 nm, respectively. Higher magnifications of selected areas, showing only GFP or rhodamine signal, are displayed for all variants as indicated. The intensity and contrast of the magnified areas were optimized for visualization of filopodia. *Scale bar* = 20 μm.

of v3 is palmitoylated, as judged from the typical subcellular targeting patterns of the different fusion variant proteins and their changes in localization upon treatment with the palmitoylation inhibitor 2-BPA. *N*-Acylation of proteins is a rather well characterized process. It occurs predominantly co-translationally with myristic acid (C14:0) linked via an amide bond to the N-terminal Gly residue in *N*-acylation motifs of targeted proteins. This myristoylation will, however, not provide stable membrane attachment but serves to increase the hydrophobicity of the N-terminal end of the modified protein to facilitate transient membrane association (47). Myristoylated membrane-associated proteins thus gain spatial access to membrane-bound DHHC (Asp-His-His-Cys) domain proteins that may subsequently catalyze the addition of palmitic acid (C16:0) to Cys residues located adjacent to the myristoylation site, which further increases the hydrophobicity of a target protein. In this manner, palmitoylation yields stable binding or association to membranes of *N*-acylated proteins (48). Our substitution of Gly-2 in v3(Grx) with Ala as well as the treatment with 2-HMA resulted in diffuse cellular distribution, similar to sole expression of GFP. We suggest that myristoylation of v3 at the Gly-2 residue is the only explanation for the observed protein localization phenotypes. Importantly, all of the unique subcellular targeting features of v3(Grx)-GFP, including plasma membrane association, formation of cytosolic speckles, and strong perinuclear accumulation, were completely impeded by this single Gly-for-Ala amino acid substitution or by use of the inhibitor of myristoylation, hence strongly suggesting that v3 is indeed *N*-acylated at its MGC motif. The C3S mutant also showed a clear reduction of plasma membrane association and cytosolic speckles, whereas maintaining strong compartmentalization with mainly perinuclear localization. This phenotype was also highly similar to the change in subcellular targeting of the wild type v3(Grx) if the cells were treated with the palmitoylation inhibitor 2-BPA and also exactly mimicked the phenotype of other *N*-acylated proteins, having eliminated *N*-palmitoylation but maintained *N*-myristoylation (35, 37, 38). We thereby suggest that v3 is myristoylated at its Gly-2 residue and palmitoylated at its Cys-3 residue.

The permanently strong perinuclear staining of wild type v3(Grx) and the C76S/C79S variant should be the result of highly regulated cellular palmitoylation processes (49). The dynamics of palmitoylation and depalmitoylation have been studied for several *N*-acylated proteins, including Ras, endothelial nitric-oxide synthase (eNos), GAP43, and $G_i\alpha_1$, or a number of model peptides, which all display subcellular localization patterns highly similar to those found here for v3 (49–51). As an example, H- and N-Ras need to acquire palmitoylation to achieve stable membrane association and trafficking between the Golgi and plasma membrane, thereby yielding patterns of localization highly reminiscent of those seen here for v3 (51). Also, expressing a synthetic protein with a consensus myristoylation motif including a palmitoylable Cys residue (MGCTLS-), Navarro-Lérida *et al.* (35) found very similar distribution of that model protein as that seen here with v3; a G2A mutant completely impeded membrane association, whereas a C3S mutant showed perinuclear distribution representing accumulation in the Golgi apparatus. Thus, here we found that v3 displays typical compartmentalization properties as previously shown for other proteins that are myristoylated and palmitoylated at their *N*-acylation motifs.

The close overlap of v3(Grx) with CT-B-stained specific substructures of the plasma membrane and in perinuclear areas, but not in the intracellular vesicles, was a striking finding. With CT-B being a well recognized probe for membrane rafts, the overlap in signal with wild type v3(Grx) strongly suggested to us that the protein was targeted to membrane rafts and the Golgi, where raft-specific gangliosides to which CT-B bind are known to accumulate (45, 46). The intracellular vesicles that solely showed CT-B-coupled fluorescence were likely endosomes (45, 46), and it should therefore not be surprising that they lacked the v3(Grx)-GFP signal. Because the v3-derived fusion proteins were intracellularly expressed, they would not co-localize with CT-B in endosomes, carrying extracellular proteins as taken up from the medium. The resolution of conventional confocal microscopy, however, cannot resolve membrane rafts as these are thought to be dynamic membrane domains with sizes of less than 100 nm in length (29, 52). We therefore purified CT-B-binding membrane raft fractions from HEK293 cells expressing v3(Grx) and probed for co-localization using immunoblotting. It was thereby notable that we could not only validate membrane raft association of wild type v3(Grx), but additionally detected a loosely associated dimeric form of the protein, which was specifically seen in the membrane raft fractions. We propose that this dimeric variant could have been formed by the GFP domain, which has a tendency to dimerize at high concentrations (53). Overexpression of v3(Grx)-GFP in combination with the partitioning into membrane rafts should likely create a high local concentration of the protein, thereby triggering dimerization. Such effects have previously been reported by Zacharias *et al.* (37) who studied partitioning of GFP variants into membrane microdomains by FRET. Expressing myristoy-

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lated and palmitoylated 13-amino acid NH_2-terminal fragments of the Lyn protein in fusion with either CFP or YFP, they could detect strong clustering at the plasma membrane (37). They also showed that hydrophobic residues of the GFP dimer interface contributed to dimer formation specifically at the membrane microdomains (37).

Partitioning of v3 into membrane rafts may also give further insights to the close association of v3 with actin polymerization and the stimulated generation of filopodia. With regards to signaling via glycosylphosphatidylinositol-anchored proteins, it is well known that actin plays a central role in organization of sphingolipid- and cholesterol-rich membrane domains and is tightly linked to these structures (43, 54–56). Actin has indeed been described as a stabilizer of membrane rafts (57), with proteins targeted to these structures being able to affect the organization of the cytoskeleton (58, 59). Additionally, several studies link induction of filopodia to stimulation or patching of membrane raft microdomains (43, 60–63). An example that is reminiscent of expression of v3(Grx) with induction of filopodia was given by Gauthier-Campbell and co-workers (61, 62), studying phenotypes of Cos-7 cells as induced by expression of diverse palmitoylated peptides. In particular, a 14-amino acid doubly palmitoylated peptide derived from GAP43 (GAP-1–14) induced formation of filopodia when expressed in fusion with GFP (61, 62). Another example reminiscent of our findings herein is the neuronal glycoprotein M6a, which also associates with membrane rafts and induces formation of filopodia (63). Thus, our findings open the possibility that v3 is first targeted to membrane rafts through *N*-acylation, whereupon the protein can interact with actin in a compartmentalized manner, directly or indirectly, which may trigger the generation of filopodia as observed.

The role(s) of endogenous v3 in membrane rafts are still unknown, but its targeting to these structures by *N*-acylation clearly expands the possible spectrum of *TXNRD1*-derived protein functions. The Trx and Grx systems are generally not well studied in terms of signal regulation within membrane rafts, although these structures are known as key players in redox signaling events (64). However, a few studies have reported upon Trx1 association with membrane rafts, in leukocyte-endothelial cell interaction during inflammation (65) or when internalized through endocytosis (66). Interestingly, Volonte and Galbiati (67) showed that caveolin 1, a key protein of caveolae, is an inhibitor of TrxR1 through direct binding via a proposed caveolin-binding motif of TrxR1 (amino acids 454–463). They also showed that a constitutively active variant of TrxR1, lacking the caveolin-binding motif, could inhibit oxidative-stress-mediated activation of $p53/p21^{waf1/Cip1}$ and induction of premature senescence (67). It is not yet clear whether or how those findings relate to targeting of v3 to membrane rafts. A large number of proteins important for cellular signaling events are myristoylated and located to membrane rafts, including several Src family tyrosine kinases and other protein kinases, phosphatases, Ca^{2+}-binding proteins, cytoskeleton-binding proteins, viral proteins, and specific redox-related proteins such as NO synthases (31). The v3 protein should hereby also be considered in the context of cellular signaling through membrane rafts, and its possible relation to cellular signaling events clearly deserves further study.

In conclusion, we have herein identified the mechanisms for targeting of v3 to membrane rafts to be dependent upon its N-terminal motif and likely to involve myristoylation at Gly-2 and palmitoylation at its Cys-3 residue. We also showed that the induction of filopodia triggered by overexpression of v3(Grx) was independent of its redox active site motif, but required its ability to associate with membrane microdomains.

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Thioredoxin (Trx1) regulates CD4 membrane domain localization and is required for efficient CD4-dependent HIV-1 entry

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ABSTRACT

Background: CD4 is a glycoprotein expressed on the surfaces of certain immune cells. On lymphocytes, an important function of CD4 is to co-engage Major Histocompatibility Complex (MHC) molecules with the T Cell Receptor (TCR), a process that is essential for antigen-specific activation of T cells. CD4 localizes dynamically into distinct membrane microdomains, an important feature of its immunoregulatory function that has also been shown to influence the efficiency of HIV replication. However, the mechanism by which CD4 localization is regulated and the biological significance of this is incompletely understood.
Methods: In this study, we used confocal microscopy, density-gradient centrifugation and flow cytometry to analyze dynamic redox-dependent effects on CD4 membrane domain localization.
Results: Blocking cell surface redox exchanges with both a membrane-impermeable sulfhydryl blocker (DTNB) and specific antibody inhibitors of Thioredoxin-1 (Trx1) induces translocation of CD4 into detergent-resistant membrane domains (DRM). In contrast, Trx1 inactivation does not change the localization of the chemokine receptor CCR5, suggesting that this effect is targeted. Moreover, DTNB treatment and Trx1 depletion coincide with strong inhibition of CD4-dependent HIV entry, but only moderate reductions in the infectivity of a CD4-independent HIV pseudovirion.
Conclusions: Changes in the extracellular redox environment, potentially mediated by allosteric consequences of functional disulfide bond oxidoreduction, may represent a signal for translocation of CD4 into DRM clusters, and this sequestration, another potential mechanism by which the anti-HIV effects of cell surface oxidoreductase inhibition are exerted.
General significance: Extracellular redox conditions may regulate CD4 function by potentiating changes in its membrane domain localization.

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1. Introduction

Human CD4 is a 55 kDa type I integral membrane glycoprotein found on the surfaces of certain cells of the innate and adaptive immune system, and comprises 4 immunoglobulin-like ectodomains (D1–D4) tethered to the cell membrane by short transmembrane- and cytosolic sequences [1]. One of the most important biological functions of CD4 is to potentiate the transduction of immunostimulatory signals conveyed to T-lymphocytes by antigen presenting cells (APCs), which present antigenic peptides associated with the class II Major Histocompatibility Complex (pMHC) to cognate T-Cell Receptors (TCRs) [2,3]. CD4 is also the primary cellular receptor for Human Immunodeficiency Virus 1 (HIV-1) [4–6], which attaches to host $CD4^+$-cells *via* the envelope surface glycoprotein gp120. While the identity and structures of many of the proteins that underlie these 2 processes have been defined, our understanding of the complex dynamics that regulate their interactions is incomplete.

To this end, it is now known that CD4 is able to move laterally on the cell surface into 'lipid rafts' [7], detergent-resistant membrane (DRM)[1] micro-domains rich in cholesterol and sphingolipids that serve as platforms for functional compartmentalization of specific membrane receptors and signaling molecules [8,9]. CD4 is recruited into DRM 'patches' that are assembled on the T-Cell around pMHC-TCR signaling complexes during antigen presentation [10,11], and several lines of evidence have suggested a role for DRMs in HIV infection [12–14], although their significance in this regard remains unclear. Indeed, certain studies have shown that DRM localization of CD4 is essential

Abbreviations: APC, antigen presenting cell; DRM, detergent-resistant membrane; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; HIV, Human Immunodeficiency Virus; MBCD, Methyl-β-Cyclodextrin; MHC, Major Histocompatibility Complex; TCR, T Cell Receptor; TFR, transferrin; Trx1, Thioredoxin-1; Grx1, Glutaredoxin-1.

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[1] In this article we will refer to 'lipid-raft' and 'detergent-resistant membrane/DRM' interchangeably, while acknowledging the historical controversy surrounding the nomenclature.

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for HIV entry [15,16], while others have shown that cells with CD4 retained in detergent-soluble (DSM)/'non-raft' membranes support robust HIV replication [17,18]. A more recent study has enriched the debate about the involvement of membrane microdomains in HIV entry by showing that the virus-host fusion reaction occurs at the 'edges' of cholesterol-rich lipid domains [19]. The reasons for these apparently discrepant findings are unclear, but they clearly highlight important effects of CD4 shuttling between membrane microdomains on the cell surface, a precise definition of which has been challenged by variations in experimental design, models of HIV entry employed and physiological context.

Adding another level complexity to models describing the functional significance of CD4 dynamics at the cellular level are recent insights into how the interactions of CD4 with cognate immune and viral receptors may be regulated at the structural level. Of particular salience in this regard, revealed through several computational, cell-based and biophysical studies, is the finding that CD4 can exist in a number of different, naturally occurring isoforms [20], transitions between which may be controlled by an allosteric effect of reduction of the atypical CD4 domain 2 (D2) disulfide bond [21]. These isomers appear to have distinct ligand-binding properties [22], with evidence suggesting that an oxidized, probably domain-swapped [23], CD4 dimer is required for functional MHCII engagement and TCR signaling [24], while a reduced, monomer is the preferred target of HIV-1 gp120 [25,26]. The latter is consistent with the observations that agents which interfere with thiol-disulfide exchange such as 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [27], and specific inhibitors of Glutaredoxin (Grx-1) [28] and Thioredoxin (Trx) [29] - cellular oxidoreductases that are present on the surface of cells [30,31] and known to catalyze the reduction of CD4 disulfides *in vitro* - result in significant impairment of HIV replication in cell culture.

Despite these insights, the mechanisms and functional significance of spatiotemporal changes in CD4 localization and structure remain poorly understood. Together with evidence which suggests that CD4 isoforms segregate into different membrane microdomains [32], however, they now advance the intriguing question as to whether there is any causality or integration between these 2 processes that could be reconciled with the central role played by CD4 in HIV entry and T-Cell signaling. CD4 undergoes C-terminal S-palmitoylation [33], a reversible acylation reaction catalyzed by palmitoyl acyltransferases that regulates the shuttling of many proteins between membrane microdomains [34–36], and a number of studies have suggested this to be important for the mechanism by which CD4 clusters into rafts and enhances immune receptor signaling [10,37]. In contrast, others have shown that palmitoylation *per se* is not sufficient for association of CD4 with DRM [13], demonstrating instead that a linear sequence of C-terminal amino acids (RHRRR) in its cytosolic domain is a dominant determinant of raft localization [18]. Thus, while the mobilization of CD4 into rafts is a well-defined feature of immune synapse formation and TCR signaling, and has a demonstrable, albeit incompletely characterized, effect on HIV replication, the mechanism(s) of CD4 shuttling, whether this is mechanistically associated with oxidoreductive isomerization, and how these events are important for HIV replication are not entirely clear and require further investigation.

With this in mind, in this study we set out to analyze the effect of manipulating cell surface redox conditions on CD4 membrane domain localization and HIV entry. Our results show that movement of CD4 into DRM domains is induced by the membrane-impermeable sulfhydryl blocker, DTNB, which, consistent with previous studies, mediates significant of impairment of HIV entry. Remarkably, these effects are reproduced by treatment of cells with purified anti-Trx1 antibodies, suggesting that changes in the activity or level of this oxidoreductase on the cell surface may represent a physiological trigger for CD4 membrane relocalization. We posit that redox-dependent CD4 allostericity may thus be a fundamental component of its membrane trafficking potential, and its influence on HIV infection may be related to both the membrane context and structure required for virus receptor engagement and host cell membrane fusion.

2. Materials and methods

2.1. Antibodies and reagents

Methyl-β-Cyclodextrin (MBCD) and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Aldrich (St Louis, USA). HIV-1 pseudoviruses SF162.LS [38] and ZM53M.PB12 (HIV-1 Clade C) [39] were produced by transient transfection using the following reagents obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (contributor in parenthesis): the HIV Env-deficient backbone, pSG3Δenv (Dr. John C. Kappes and Xiaoyun Wu), and complementing Env (gp160)-expressing plasmids ZM53M.PB12 (Drs E. Hunter and C. Derdeyn) and SF162.LS (Dr. D. Montefiori). The following primary antibodies were used (all monoclonal and raised in mice unless otherwise indicated): goat, purified polyclonal anti-human thioredoxin (α-Trx1) (IMCO Corporation, Stockholm, Sweden); anti-CD4 (α-CD4) clones MT310 (for Western blotting) (Santa Cruz Biotechnology, Santa Cruz, USA), RPA-T4 (for immunohistochemistry) (BD Biosciences, San Jose, USA) and RFT4 PE conjugate (α-$CD4^{PE}$ for flow cytometry) (Dr. D Glencross, National Health Laboratory Services and University of the Witwatersrand, South Africa in collaboration with Royal Free College, United Kingdom); anti-β-adaptin (α-adaptin) and anti-Flotillin-1 (α-Flotillin) (both Santa Cruz Biotechnology); anti-transferrin (CD71) FITC conjugate (α-TFR^{FITC}) (Thermofisher, Pleasanton, USA); anti-CCR5 (CD195) clone 2D7/CCR5 PE conjugate (α-$CCR5^{PE}$) (BD Biosciences). Cholera toxin subunit B conjugated with Alexa Fluor®488 (CTB^{AF488}) and Alexa Fluor® 594 (CTB^{AF594}) (Thermofisher) were used for detection of GM1 ganglioside. The following secondary monoclonal antibodies were used (all raised in goats and obtained from Thermofisher): anti-human IgG HRP conjugate (α-$hIgG^{HRP}$); anti-mouse IgG HRP conjugate (α-$mIgG^{HRP}$), anti-mouse IgG FITC conjugate (α-$mIgG^{FITC}$) and anti-mouse IgG Alexa Fluor 594 conjugate (α-$mIgG^{AF594}$).

2.2. Cell lines and culture conditions

TZM-bl cells, a HeLa-derived cell clone engineered to express CD4, CCR5 and CXCR4 [40], were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. [41–44]. The cell line also has an integrated firefly luciferase (F-Luc) reporter gene under the control of an HIV-1 long terminal repeat sequence [44], which enables quantitative determination of viral infection efficiency by luminescence measurements. TZM-bl cells are known to be highly permissive to infection by most strains of HIV, including primary HIV-1 isolates and envelope-pseudotyped viruses, and are widely used in models of HIV entry. The cells are maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin, and 100 U streptomycin at 37 °C in a 5% CO_2 humidified incubator, and passaged using Trypsin/EDTA (Sigma Aldrich) according to standard procedures.

2.3. Cell treatments with MBCD, DTNB, α-Trx1 and HIV-1 pseudoviruses

For the treatment of TZM-bl cells with MBCD, DTNB and α-Trx1, medium from TZM-bl cultures at 80% confluence was decanted, cells were washed with phosphate buffered saline (PBS) and MBCD/DTNB/α-Trx1 added at the indicated concentrations in DMEM supplemented with 5% fetal calf serum and 2 mM L-glutamine. Cells were incubated at 37 °C for 40 min (MBCD), 10/30/120 min (DTNB) or 12 h (α-Trx1), unless otherwise stated. After washing with PBS, the cells were either processed further for immunohistochemistry, flow cytometry

or membrane/raft extraction, or they were infected with HIV-1 pseudoviruses SF162.LS or ZM53M.PB12 (see Section 2.7).

2.4. Immunohistochemistry

Washed, treated and untreated TZM-bl cells were blocked for 10 min at 4 °C using PBS containing 0.5% bovine serum albumin (BSA). Cells were then incubated with α-CD4 (RPA-T4) and either α-TFR^{FITC} or CTB^{AF594} for 30 min on ice, and then washed with cold PBS + 0.5% BSA. Unlabelled antibodies were detected by further incubation with a fluorophore-conjugated secondary antibody (α-mIgG^{FITC} or α-mIgG^{AF594}) for 30 min on ice. The washed cells were fixed using a freshly prepared 4% formaldehyde solution in cold PBS for 15 min at 4 °C. Cell nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) (Thermofisher) for 10 min at room temperature. The fixed, stained cells were washed and stored in PBS at 4 °C in the dark until visualization. Cells were visualized with a 63 × oil immersion objective and images were captured by scanning confocal microscopy on a Zeiss LSM 780 instrument (Carl Zeiss, Oberkochen, Germany). Colocalization analysis was performed on >5 cells/sample and each sample was analyzed in triplicate by Image J software (http://rsbweb.nih.gov/ij) using the colocalization plug-ins JACOP and Colocalization Finder according to published methods [45,46].

2.5. Membrane/lipid raft isolation

Total cell- and detergent-resistant (DRM) membrane microdomains were isolated by density-gradient centrifugation (flotation) according to published procedures [47] with minor modifications. The following protocol was applied to 1.5×10^7 cells, and can be adjusted proportionally for increases/decreases in cell number. Briefly, TZM-bl cells were rinsed with PBS, detached in PBS supplemented with 2 mM EDTA, collected using a cell scraper, centrifuged at 1000 ×*g* and washed once in PBS at room temperature. For total cell membrane isolation, cell pellets were resuspended in 400 μl of cold flotation buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM β-glycerol phosphate, 30 mM sodium pyrophosphate) and lysed by 3 cycles of freeze/thawing in a liquid nitrogen/37 °C water bath. Lysed cells were briefly sonicated (3 × 15 s cycles, 0.3 kJ per cycle), then centrifuged at 2000 ×*g* for 5 min, and the supernatant (S1) was collected and set aside on ice. The pellet was washed with 100 μl of cold flotation buffer, re-centrifuged, and the supernatant (S2) added to S1. Sucrose was added to the combined supernatants to a final concentration of 80% (w/v) in a total volume of 2 ml, and this was placed at the bottom of 14 ml round-bottom Ultra Clear™ ultracentrifuge tube (Beckman Coulter, Brea, USA). Layers of flotation buffer (5.5 ml, 65% sucrose followed by 4.5 ml, 10% sucrose) were gently added to the top of the cell lysate, taking care not to disturb the interfaces that formed between each layer. Centrifugation was performed at 220,000 ×*g* for 18 h, 4 °C. 1 ml fractions were collected from the top of the gradient, aliquoted and stored at −70 °C for later analysis. Detergent resistant membranes were prepared in the same way as total membranes, with the following adjustments: (i) 1% Triton X-100 (Sigma Aldrich) was added to the flotation buffer (including lysate and sucrose cushions); (ii) the final sucrose concentration in the cell lysate was adjusted to 45%; (iii) in the order of addition, the 'cushions' layered on top of the 2 ml cell lysate contained 30% and 5% sucrose. CD4 and control marker proteins (β-adaptin, Flotillin) were detected in cell- and detergent-resistant membrane fractions by standard Western blotting and chemiluminescence procedures using the indicated primary antibodies and HRP-conjugated secondary antibodies.

2.6. Flow cytometry

Treated and untreated TZM-bl cells were detached in PBS supplemented with 2 mM EDTA, collected using a cell scraper, centrifuged at 300 x g and washed once with PBS at 4 °C. The cells were then resuspended in 1 ml of PBS and 100 μl aliquots were left unlabelled or labelled with α-CD4^{PE} CTB^{AF488}, α-CCR5^{PE} or α-TFR^{FITC} for 30 min on ice. All antibodies were titrated and used at the optimal concentration for staining. Cells were washed with 1 ml of cold PBS by centrifugation and resuspended in 300 μl of PBS. The relative abundance of CD4 and controls (transferrin, GM1, CCR5) in DSM and DRM domains were then quantified by the method of Gombos et al. [48] with minor modifications. Briefly, the labelled and unlabelled cells were incubated with or without 0.1% Triton X-100 for 20 min on ice. Thereafter, the samples were acquired on a FACSCalibur flow cytometer which was calibrated using Calibrite beads and FACSComp software (BD Biosciences). Ten thousand events were acquired for all samples using CellQuest Pro software (BD Biosciences), and FlowJo (TreeStar Inc., Ashland USA) was used for all data processing and analyses. Cells were gated using their forward and side scatter properties and the same gate was used for untreated and detergent-treated cells. Data was analyzed as the mean fluorescence intensity (MFI) for (CTB/TRF) FITC and (CD4/CCR5) PE. Unlabelled cells were used to measure auto-fluorescence. In order to calculate the Flow Cytometry Detergent Resistance Index (FDRI), the mean fluorescence intensity (MFI) of the following conditions was obtained: labelled, detergent-treated cells (MFIdet); unlabelled detergent-treated cells (MFIAFdet); labelled, PBS-treated cells (MFImax); unlabelled PBS-treated cells (MFIAF). The extent of detergent resistance was calculated as follows:

$$\mathrm{FDRI} = (\mathrm{MFIdet} - \mathrm{MFIAFdet})/(\mathrm{MFImax} - \mathrm{MFIAF}).$$

Three independent results were generated for each treatment datapoint, and reported results reflect average FDRI values. Error bars represent standard deviations, and statistical significances were calculated by Student's *t*-test.

2.7. HIV-1 pseudovirion inhibition assay

Pseudoviruses were generated as previously described by Li et al. [49]. Briefly, HEK293T cells were cotransfected with the HIV-1 env-deficient backbone expression plasmid (pSG3Δenv) and either of the Env-expressing plasmids (pZM53M.PB12 or pSF162.LS) using Polyfect Transfection Reagent (Qiagen, Hilden, Germany) as per the manufacturer's instructions at an experimentally optimized ratio of envelope to backbone plasmid. Virus-containing supernatants were harvested 48 h later, centrifuged at 1000 ×*g*, adjusted to a final concentration of 20% fetal calf serum (FCS), and filtered through a 0.2 μm filter. Aliquots were stored at −70 °C. The median Tissue Culture Infectious Dosages (TCID50) of the produced HIV-1 pseudoviruses were titrated against the TZM-bl cell line according to methods previously described [49]. Importantly, DEAE dextran hydrochloride, a polycation frequently used in HIV pseudovirus assays to enhance virus adsorption onto target cells [50], was omitted in this protocol. The measured viral entry efficiencies thus represent the result of unenhanced, *bona fide* CD4-pseudovirus envelope interactions. Following treatment of cells with DTNB or α-Trx1, SF162 and ZM53 pseudoviruses were added at TCID50s of $200/1 \times 10^4$ cells, and the cultures were incubated for 24–48 h at 37 °C. Luciferase activity was then quantified by measuring luminescence using the Bright Glo Kit (Promega, Sunnyvale, USA) according to the manufacturer's instructions. Measurements were read on a Victor3, 1420 Multilabel Counter Luminometer (Perkin Elmer, Waltham, USA).

3. Results

3.1. CD4 localizes to non-raft membrane domains in TZM-bl cells

A number of studies have investigated the distribution of CD4 across different membrane microdomains in several different T- and non-T cell lines, as well as primary T cells [10,12,13,15–18,37]. These

have shown a large range of CD4 enrichment levels within raft- and non-raft membranes, with some studies showing that CD4 is predominantly associated with rafts, while others have detected significantly lower amounts. Taken together with experiments that have demonstrated large increases in the proportion of CD4 in the detergent-resistant membrane fraction upon mitogenic stimulation [10,15], these results illustrate that CD4 membrane microdomain distribution is substantially dependent on cell type and state of activation.

With this in mind, we set out to examine the pattern of CD4 distribution in detergent-sensitive (DSM) and detergent-resistant (DRM) microdomains in TZM-bl cells, a HeLa-derived line stably expressing human CD4 (and the HIV coreceptors CCR5 and CXCR4) that is widely used to model HIV infection in cell culture [40,51]. We first sought to gain qualitative insights in this regard by microscopic analysis of untreated TZM-bl cultures, which were grown under normal conditions until 80% confluent, and then probed with a primary mouse monoclonal

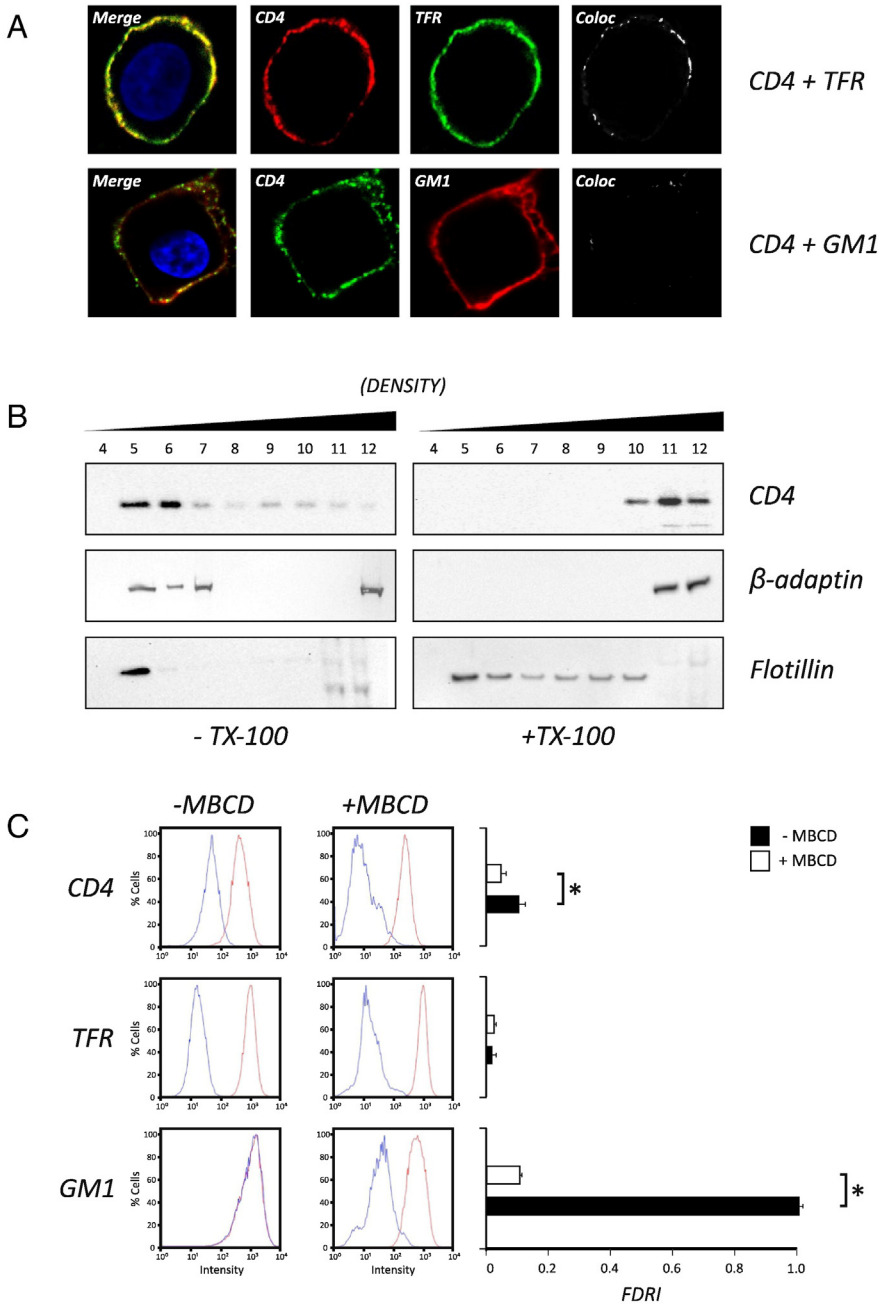

Fig. 1. Membrane microdomain localization of CD4 in TZM-bl cells. (A) TZM-bl cells were cultured in DMEM with 10% BSA until 80% confluent, washed with PBS, fixed and then probed with a mouse monoclonal anti-CD4 antibody (RPA-T4) and either a monoclonal anti-transferrin antibody (α-TFR^{FITC}, green) or Cholera Toxin subunit B (CTB^{AF594}, red). Bound CD4 primary antibodies were detected with secondary anti-mouse IgGs (AF594-conjugated when co-labelling with TFR, and FITC-conjugated when co-labelling with GM1). The labelled cells were visualized by scanning confocal microscopy, and co-localized protein detected by Image J software (http://rsbweb.nih.gov/ij) using the co-localization plug-ins JACOP and Colocalization Finder [45,46]. (B) Total- and detergent-resistant membrane (DRM) fractions were isolated from TZM-bl cells by density gradient centrifugation, and analyzed by Western blotting using monoclonal anti-CD4, anti-β-adaptin and anti-Flotillin antibodies. (C) Quantitative analysis of membrane microdomain localization by calculation of the Flow Cytometry Detergent Resistance Index (FDRI). Live, unfixed TZM-bl cells were treated or left untreated with Methyl-β-Cyclodextrin (MBCD, 10 mM), washed and then labelled with α-CD4^{PE}, α-TFR^{FITC} or CTB^{AF594}. The mean fluorescence intensities (MFI) of 10,000 cells was then analyzed by flow cytometry, and the FDRI calculated as the ratio of normalized MFI values in the presence:absence of detergent. The FDRI values represent averages from 3 independent experiments performed in triplicate, and error bars are standard deviations. Statistically significant differences were calculated by Student's *t*-test and are indicated with an asterisk ($P < 0.05$).

anti-CD4 antibody (RPA-T4) and either a FITC-conjugated mouse monoclonal anti-transferrin antibody (α-TFR^{FITC}) or Alexa Fluor 594-conjugated Cholera Toxin subunit B (CTB^{AF594}). Bound primary CD4 antibodies were detected with either secondary anti-mouse IgG Alexa Fluor594- or FITC-conjugated secondary antibodies (α-mIgG^{AF594}/α-mIgG^{FITC}). Excluded from DRM domains, TFR is commonly used as a target marker of non-raft membrane regions, while CTB binds GM1 ganglioside, a glycosphingolipid constituent of DRMs frequently used to mark lipid rafts. Using scanning confocal microscopy, we performed co-localization analyses on 5 randomly selected CD4/GM1- and CD4/TFR-labelled cells from 3 independent cultures, and representative images are shown in Fig. 1A. These results suggested that cell surface CD4 is located primarily in non-raft membrane domains in TZM-bl cells cultured under normal growth conditions.

Because of the technical complexity involved in generating accurate quantitative co-localization data by conventional microscopy, we then sought to confirm these findings by isolating total- and DRM fractions from normal TZM-bl cells by flotation centrifugation, and analyzing these by Western blot. In these experiments, we used antibodies against a different pair of raft- and non-raft marker proteins (Flotillin-1 and β-adaptin respectively), which we reasoned would provide additional confidence in the pattern of localization that had been suggested by microscopy. Consistent with this, we found that CD4 was located exclusively in the same detergent-sensitive membrane regions of TZM-bl cells occupied by the non-raft protein β-adaptin (Fig. 1B, left top and middle panels), with none detected (Fig. 1B, right top and middle panels) in detergent-resistant membranes enriched with the raft marker Flotillin (Fig. 1B, bottom right panel).

Since quantitative, high-throughput, multi-condition analysis of membrane localization dynamics is technically difficult to perform by conventional microscopy or immunoblotting of fractionated membranes, we then set up an assay based on the method of Gombos et al. [48] to examine the membrane distribution of CD4 (and controls) in a defined population and number of live TZM-bl cells. In this approach, cells were labelled with α-CD4^{PE}, α-TFR^{FITC} or CTB^{FITC}, and the mean fluorescence intensities (MFI) of cells that had been subjected to mild detergent treatment with TX-100, or left untreated, were determined by flow cytometry. Since detergent solubilization of DSM-localized proteins effects a reduction in MFI, which is reflected graphically by a leftward shift (reduction) in peak fluorescence, the normalized MFI(detergent-treated cells):MFI(detergent-untreated cells) ratio – defined as the FDRI value – is indicative of the proportion of protein located in DRM [48]. Consistent with this, and the exclusive residence of the GM1 ganglioside in DRM domains, TX-100 treatment had no effect on the MFI of CTB-labelled cells (Fig. 1C, bottom left panel), which have an FDRI value of approximately 1. In contrast, detergent treatment results in dramatic reduction in MFI of cells labelled with α-TFR^{FITC} relative to untreated cells (Fig. 1C, middle left), and a typically low FDRI (<0.1). Similarly, TX-100 treatment also resulted in significant reduction in MFI of anti-CD4 labelled cells (Fig. 1C, top left panel), although the FDRI value was slightly, but significantly higher at 0.12, suggesting that a small amount of CD4 (not readily detectable by microscopy or WB) is present in DRM in these cells. In order to confirm that the FDRI assay approach legitimately reflected changes in membrane domain localization, we then labelled cells with the α-CD4^{PE}, α-TFR^{FITC} and CTB^{FITC} probes and performed FDRI analysis following depletion of membrane cholesterol, another integral component of DRM, with Methyl-β-Cyclodextrin (MBCD). As expected, MBCD treatment resulted in dramatic reductions in MFI and FDRI of CTB-labelled cells (Fig. 1C, bottom right panel), consistent with ablation of GM1-containing rafts that is effected by cholesterol extraction. Furthermore, incubation with MBCD decreased the FDRI value of α-CD4-labelled cells to levels seen for α-TFR-labelled counterparts (Fig. 1C, top right panel), providing further support to the notion that a small, but measureable quantity of CD4 is located in DRM in TZM-bl cells. Taken together, these results provide further evidence that under normal growth conditions, CD4 is based predominantly in non-raft membranes on the surface of TZM-bl cells.

3.2. Inhibition of cell surface Thioredoxin induces translocation of CD4 to lipid rafts

We next sought to investigate the effects of manipulating cell surface redox conditions on CD4 domain distribution. First we compared CD4/GM1 co-localization in cells that had been treated or untreated with the non-specific, membrane-impermeable sulfhydryl oxidant, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), by confocal microscopy. TZM-bl cells were incubated with 5 mM DTNB in growth media or an equivalent volume of growth media only for 2 h, washed with PBS, and then probed with α-CD4 RPA T4 and CTB^{AF594}. Intriguingly, DTNB treatment induced the formation of several clusters of CD4 that had co-localized with CTB-stained membranes (Fig. 2A, bottom right image). This phenomenon was not related to any toxic effect of DTNB treatment that may have compromised cell viability or caused gross perturbation in membrane structure, since treated and untreated cells were equally viable (data not shown), and had qualitatively similar distributions of the raft- and non-raft marker proteins Flotillin and β-adaptin in total- and DRM membrane fractions (Fig. 2B). These data provided us with the first tentative suggestions that redox events on the cell surface are capable of influencing CD4 localization dynamics.

In order to gain further insights in this regard, we then investigated changes in CD4 distribution on DTNB-treated cells by FDRI analysis. TZM-bl cells were incubated with 1.0 and 5.0 mM of DTNB (or dilution control) for 2 h, and 1.0 mM of DTNB for 0, 30 and 120 min, then washed and labelled with α-CD4^{PE} and CTB^{FITC}, and treated with TX-100 before analyzing by flow cytometry. Consistent with results from microscopy, these results confirmed that DTNB induced the movement of CD4 into DRMs, effecting progressive increases in the FDRI values of α-CD4-labelled cells that were both concentration (Fig. 3C) and time-dependent (Fig. 3D).

Since DTNB is a synthetic, non-specific blocker of sulfhydryls, a logical question could be asked regarding the biological relevance and mechanism of redox-dependent effects on CD4 membrane domain localization. Considering that several cellular oxidoreductases are now known to be secreted onto the extracellular surfaces of cells [30,31,52], and have been shown to reduce CD4 disulfide bonds *in vitro* [21,28], we hypothesized that one or several of these enzymes, or an alternative yet to be characterized in this context, may play an important role in this regard. Among these, Thioredoxin-1 (Trx1) has gained prominence as a putative physiological reductant of CD4, since the calculated redox potential of its active site dithiol is of sufficient magnitude to effect thermodynamically favorable reduction of the CD4 D2 disulfide (130C-C169) [25], and Trx1 has been shown to react with CD4 on the surfaces of lymphocytes [21,29]. Moreover, we recently showed that Trx1 catalyzes robust oxidoreductive isomerization of recombinant 2-domain CD4 (2dCD4) [26].

With this in mind, we next investigated whether direct inhibition of Trx1 by treatment of TZM-bl cells with purified inhibitory Trx1 antibodies (α-Trx1) resulted in any detectable change in CD4 localization. Cells were incubated with different doses of α-Trx1 (IMCO Ltd., Sweden), and then analyzed by confocal microscopy and FDRI analysis as described above. Remarkably, Trx1 inhibition resulted in the same visible co-clustering of CD4 with CTB-labelled membranes induced by DTNB (Fig. 3A), the quantitative effect of which was a considerable increase in the MFI of TX-100-treated cells (Fig. 3C), and a corresponding 5-fold increase in FDRI (Fig. 3B). In contrast, α-Trx1 treatment had no significant effect on the MFI (Fig. 3C) or FDRI (Fig. 3B) of CTB-labelled cells. We again disqualified the possibility that this effect could have been related to any toxic effect of the Trx1 antibodies, since the viabilities of control TZM-bl cells and α-Trx1-treated cells were equivalent (data not shown).

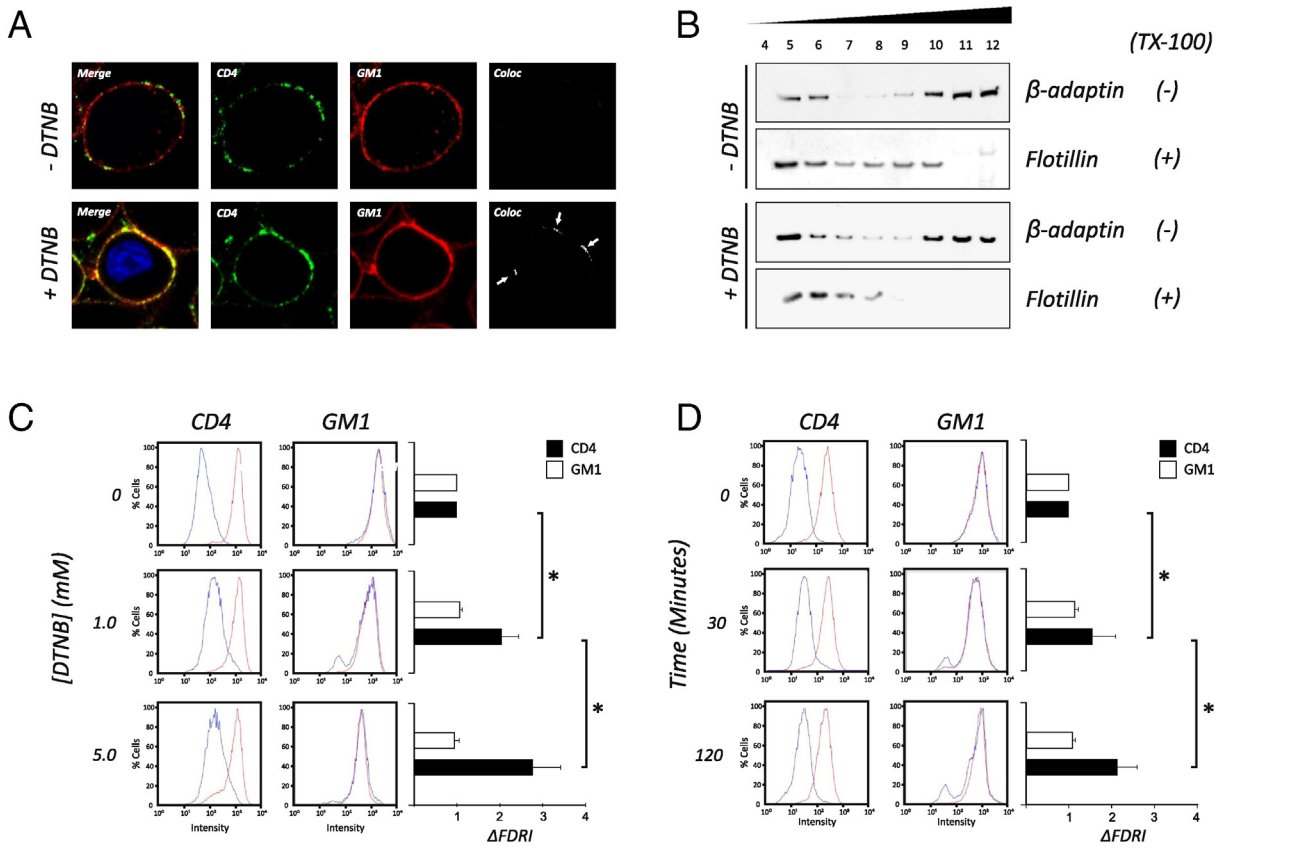

Fig. 2. Analysis of the effect of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) treatment on the membrane domain localization of CD4. (A) TZM-bl cells were treated or left untreated with DTNB (1 mM) for 1 h, washed with PBS, fixed, labelled with α-CD4^{FITC} and CTB^{AF594} and then analyzed microscopically as described. (B) Total- and DRM membrane fractions were isolated from DTNB-treated and control cells, which were then analyzed by Western blotting. Monoclonal antibodies against β-adaptin and Flotillin were used, respectively, to compare total- and DRM membrane integrity under each condition. (C and D) The extent of change in microdomain localization of CD4 as a function of DTNB concentration (C) and incubation time (D) was analyzed by FDRI. TZM-bl cells were incubated with the indicated concentration of DTNB for 1 h (left) or with 1 mM of DTNB for the indicated time (right), washed with PBS, labelled with α-CD4^{PE} or CTB ^{AF594} and the FDRI values for each marker calculated by flow cytometry as described. Data for each data point (DTNB concentration or incubation time) was generated independently 3 times in triplicate, and are represented as average fold-changes in FDRI relative to corresponding untreated controls. Error bars represent standard deviations and statistically significant differences are indicated with an asterisk ($P < 0.05$).

We then assessed whether the observed translocation of CD4 might be caused by an 'off-target' effect of exposing cells to immunoglobulin, by checking to see if an irrelevant antibody of the same species (goat) and at the same (highest) concentration as α-Trx1 induced movement of CD4 into DRM. Indeed this was not the case, since cells treated with an anti-human IgG antibody raised in goats had the same FDRI values as untreated controls (Fig. 3D). That the changes induced by Trx1 inhibition represented *bona fide* localization of CD4 into rafts was additionally confirmed by incubating cells with MBCD following treatment with α-Trx1. Consistent with this, the FDRI value of the MBCD-treated α-CD4-labelled cells reverted to the same value as those seen in untreated cells (Fig. 3D).

Collectively, these results provide compelling evidence that extracellular redox events, mediated through the enzymatic activity of Thioredoxin-1 (and potentially other oxidoreductases active on the cell surface), play a role in regulating CD4 membrane localization dynamics.

3.3. Inhibition of Thioredoxin-1 impairs CD4-dependent HIV entry

Previous studies have demonstrated that blocking redox exchanges on the cell surface compromises HIV replication in cell culture [25,27–29,53], although the molecular basis for this is poorly understood. Considering this, we next checked whether these antiviral effects were faithfully reproduced under the same conditions that induce CD4 translocation, which we reasoned might provide additional insights into the mechanism(s) by which Trx1 is able to regulate HIV replication. TZM-bl cells were treated with the indicated concentrations of DTNB, α-Trx1, control IgG or corresponding dilution buffers, washed with PBS, and then infected with the HIV pseudovirus ZM53. Consistent with published data, both DTNB and α-Trx1 effected robust inhibition HIV ZM53 (Fig. 4A). The inhibitory effect of α-Trx1 was specifically related to Trx1 inhibition, since control goat IgGs at 20 μg/ml had no effect on HIV entry. Interestingly, while a 5 mM dose of DTNB resulted in complete blockage of ZM53 infection, α-Trx1 was only able to achieve a maximum of 80% inhibition at its highest concentration (20 μg/ml) and further increases (up to over 2-fold at 50 μg/ml) did not enhance this antiviral effect further (Fig. 4B). This suggests, in line with the findings of other studies, that other sources of oxidoreductase activity may be located on the cell surface that have overlapping substrate reduction capabilities. Related to this, it should also be noted that because of structural conservation of the Thioredoxin-fold among different oxidoreductases [54,55], some cross reactivity may occur between α-Trx1 and enzymes other than Trx1, which may be present on the cell surface and capable of catalyzing redox exchanges with CD4. It is possible, therefore, that such enzymes, not alluded to in this study, are also involved in the redox-dependent mechanism by which CD4 membrane domain localization is regulated.

We then tested the effects of DTNB and α-Trx1 on the HIV pseudovirus, SF162. ZM53 and SF162 are distinguishable only by the identities of their surface envelope glycoproteins: ZM53 is derived from a primary viral isolate and is dependent on both CD4 and the chemokine co-receptor CCR5 for entry into host cells [56], while SF162 displays an envelope cloned from a laboratory-adapted virus that is able to

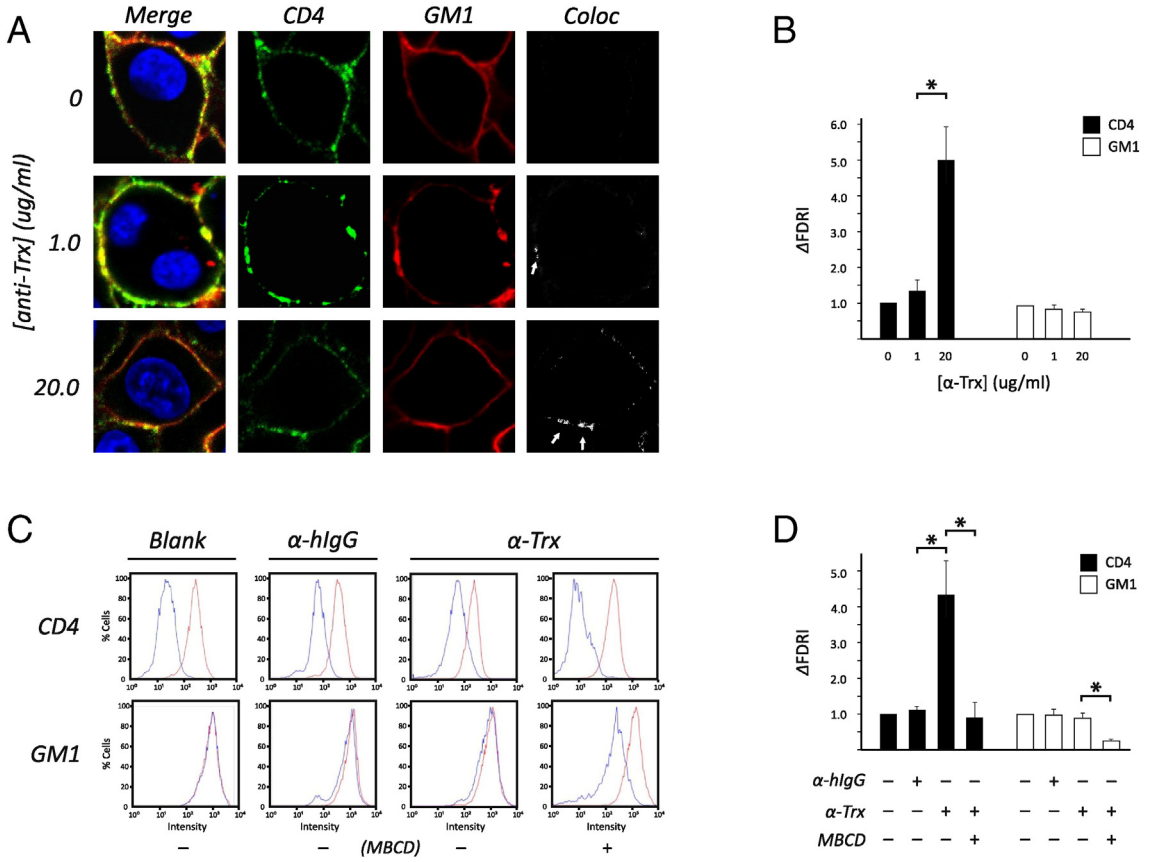

Fig. 3. Analysis of the effect of Thioredoxin (Trx1) depletion on the membrane domain localization of CD4. (A) TZM-bl cells were treated or left untreated with anti-thioredoxin antibodies (α-Trx1) at the concentration shown for 12 h, and then analyzed microscopically as described. (B) TZM-bl cells were incubated with the indicated concentrations of α-Trx1 for 12 h, washed, labelled with α-CD4^{FITC} or CTB^{AF594}, and the fold-changes in FDRI of α-Trx1-treated (relative to untreated controls) were calculated for CD4 and for GM1 by flow cytometry. (C and D) The specificity of the DRM-localizing effect of Trx depletion was confirmed by analyzing changes in the localization of CD4 in TZM-bl cells treated in the same way with non-specific goat anti-human antibodies. Cells were incubated with 20 μg/ml of either α-Trx1 or gα-hIgG, washed and then analyzed either directly – or after further treatment with MBCD for 1 h – by flow cytometry to calculate (C) Mean Fluorescence Intensities and (D) fold-change in FDRI values of CD4- and GM1-labelled cells relative to untreated controls. Statistical parameters and significance testing were as described in Fig. 2.

infect HIV permissive cells expressing CCR5 but lacking CD4 [57,58]. In contrast to ZM53, which was effectively inhibited by DTNB and α-Trx1, the same treatments exerted only mild inhibition (20–40%) of the CD4-independent pseudovirus, SF162 (Fig. 4C), suggesting that efficient HIV entry requires the catalytic activity of Trx1 at a point in the entry process preceding engagement of CCR5 (or CXCR4 potentially) by gp120.

With this in mind, we reasoned that the dramatic translocation of CD4 induced by Trx1 inactivation - in addition to the redox-dependent changes in the CD4-gp120 complex structure that have been suggested to be necessary for exposing the gp120 co-receptor binding sites immediately after CD4 binding [59–61] - could also compromise viral entry by disrupting the spatiotemporal co-ordination of CD4 and co-receptor that is required for efficient assembly of virus-host pre-fusion complexes. To test the feasibility of this hypothesis, we investigated how Trx1 inhibition affected the co-localization dynamics of CD4 and CCR5 by FDRI analysis. The FDRI value calculated for CCR5-labelled cells was 0.11 (data not shown), almost identical to that of the CD4-labelled counterparts (FDRI = 0.12), suggesting that CCR5 is found predominantly in non-raft membranes in normal, untreated TZM-bl cells. Unlike CD4-labelled cells, however, α-Trx1 treatment did not result in a significant change in the FDRI of CCR5-labelled cells (Fig. 4D), implying that blocking Trx1 does not induce synchronous translocation of CCR5 to DRM.

Taken together, while our data does not account for the antiviral effect of Trx inhibition that results from structural changes induced in CD4 (and possibly gp120), our results suggest that differential sequestration of surface receptor molecules into membrane microdomains may represent an additional hypothetical mechanism by which oxidoreductases regulate HIV entry that warrants further investigation.

4. Discussion and conclusion

T cells are activated when their antigen-specific T Cell Receptors (TCR) stably engage cognate MHC proteins displayed on the surfaces of antigen-presenting cells (APC). Among the earliest events precipitated by MHCII-TCR binding is the mobilization of the submembranal cytoskeletal (actin/myosin) apparatus and the condensation of CD4 and other cell adhesion and signaling molecules into clusters – so called supramolecular activation complexes (SMACs) [62] – that coalesce around the TCR on the T cell membrane (reviewed in [3]). Here, CD4 binds MHCII and conveys its non-covalently associated tyrosine kinase Lck to activation motifs (ITAMs) located within cytosolic subunits of the TCR-CD3 complex [63], the phosphorylation of which initiates the signaling cascade that culminates in T cell proliferation and inflammatory cytokine production. Failure of CD4 to engage TCR-bound MHCII leads to T cell anergy and apoptosis [64], a finding that alluded to the essential role played by CD4 in ensuring that T cells are activated efficiently, but only under conditions that simultaneously encourage its association with TCR at the TC-APC contact site.

Many studies that have investigated CD4 localization - with respect to both its membrane microdomain context and proximity to TCR (and other important components of the T cell signaling apparatus) – have provided detailed insights into lateral trafficking dynamics that are an

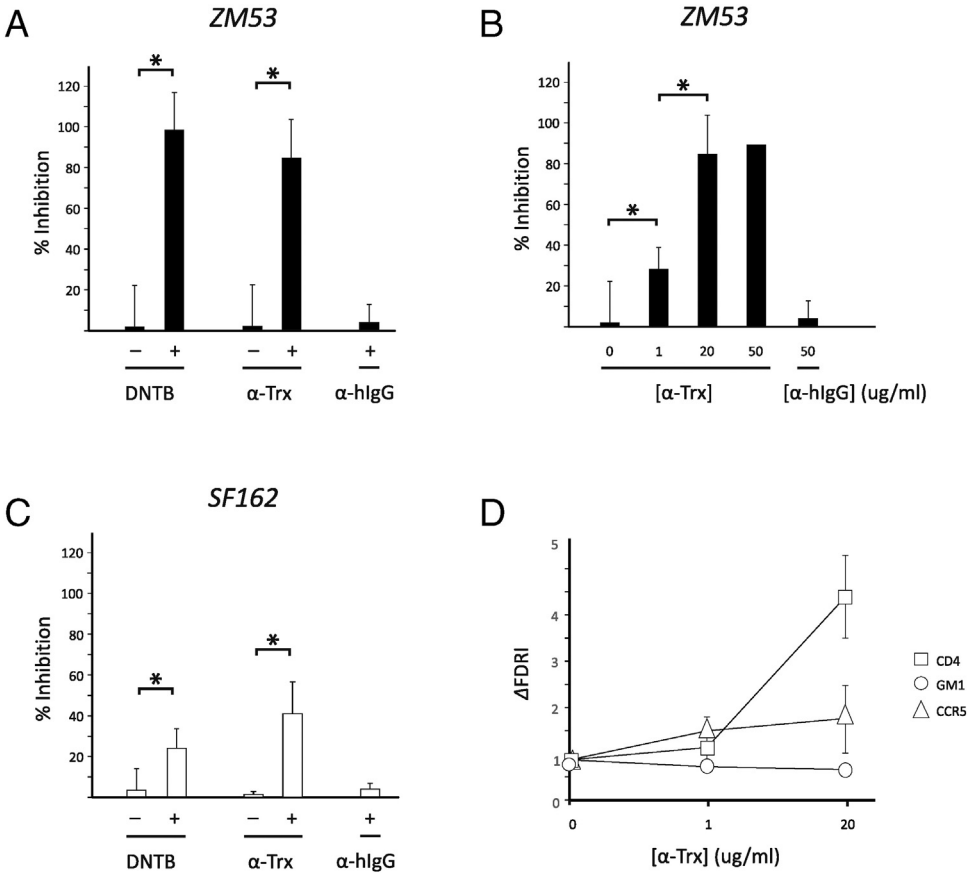

Fig. 4. Depletion of cell surface Trx inhibits CD4-dependent HIV entry. (A and C) TZM-bl cells were treated with DTNB (5 mM for 2 h), α-Trx1 or control α-hIgG (both 20 μg/ml for 12 h) prior to infection with HIV pseudoviruses ZM53 (A) or SF162 (C). α-Trx1-mediated entry inhibition was evaluated by measuring HIV Tat-induced firefly luciferase reporter gene expression levels. (B) Dose-dependent inhibition of HIV by α-Trx1 by was assessed by treating TZM-bl cells with the indicated concentrations of antibodies as described above, followed by infection with pseudovirus ZM53. In all cases, infections were performed in triplicate, and the inhibitory efficiency of DTNB/α-Trx1 treatment expressed as average percentage decreases in luminescence relative to untreated controls. Error bars are standard deviations, and statistically significant differences were calculated by *t*-test. (D) Relative changes in dynamic localization of CCR5 were investigated by calculating FDRI values for CD4-, CCR5-, and GM1-labelled TZM-bl cells that had been treated with the indicated concentrations of α-Trx1 according to the procedures outlined in Fig. 3.

important feature of CD4's biological function. However, our understanding of the immediate molecular consequences of TCR-pMHCII binding that trigger translocation of CD4, and the physiological and biophysical factors that regulate this, is limited. Sites for S-palmitoylation, a process known to play a fundamental role in regulating the association of many proteins with lipid rafts, were identified in the C-terminus of CD4 almost 25 years ago, and several studies have firmly established the presence of CD4 in different membrane microdomains, most recently in the form of nanoclusters separated from those containing TCR [65,66]. Yet the mechanism by which CD4 traffics on the cell membrane remains unclear, with no clear consensus on the roles played by S-palmitoylation, Lck association and putative C-terminal raft-localizing signal sequences. How these factors combine to coordinate membrane compartmentalization dynamically, a clearly important component of the immunoregulatory function of CD4, remains an open question, therefore. The central finding of this study that manipulation of cell surface redox activity - and Thioredoxin-1 inhibition in particular - induces profound changes in CD4 localization makes an important contribution in this regard, and is integrable with several features of CD4 biology, T cell activation and HIV entry.

Firstly, it is now clear that enzymatic oxidoreduction of 'allosteric' disulfides represents an important mechanism by which the activities of many proteins are regulated (reviewed in [67]), and since the first seminal studies by Hogg and colleagues demonstrated functional effects of CD4 disulfide bond reduction [21], it has become generally accepted that thiol-disulfide exchange is important for normal CD4 functioning, although the basis for this has remained poorly understood. Secondly, and consistent with this, recent studies conducted in our laboratory have revealed how an intact domain 2 (D2) disulfide (130C-C159) contributes to CD4 metastability, and its reduction results in stabilizing structural changes that appear to be propagated beyond D2 [68]. Although these studies were performed only on single- and 2-domain N-terminal variants of CD4, they indirectly suggest that redox exchanges in the ectodomain of CD4 have distal allosteric effects, which may interface with the proposed mediators of CD4 microdomain localization described above. Thirdly, reactive oxygen species (ROS) have been shown to promote lipid raft formation in lymphocytes [69], and it is well-established that ROS regulate the activation of T Cells (reviewed in [70,71]). While their precise role(s) in this regard remains controversial, having been shown to potentiate both proliferative and apoptotic effects that are dependent on their source, type, concentration and localization, extracellular ROS produced by phagocytic APCs presenting antigen in the immunological synapse would correlate with localized Trx depletion, providing a hypothetical mechanism by which TCR-MHC engagement could be linked to redox-dependent CD4 translocation.

Finally, several experiments have now demonstrated unequivocally that inhibiting cell surface oxidoreductase activity impairs HIV entry. Until recently, this effect was thought to be related principally to redox-dependent structural changes induced in CD4 that influence its ligand-binding specificity. While our results do not directly imply an additional mechanism in this regard, considering the many studies that have described correlations between the membrane microdomain contexts of the HIV receptors and their ability to support efficient HIV

entry, and our observation that the movements of CD4 and coreceptor in response to Trx inactivation are not coupled, we reason that strong antiviral effects of oxidoreductase inhibition are potentially also mediated through dramatic changes induced in CD4 localization.

As with all studies that employ cell culture surrogates to model physiological processes, our data is presented with the acknowledgement that important differences may exist between TZM-bl cells and CD4-expressing cells *in vivo* (*e.g.* plasma membrane lipid and protein composition, extracellular redox environment) that need to be considered when interpreting this data. For example, it has been shown that different oxidoreductases appear to be active on the surfaces of different primary $CD4^+$ cell lineages, at least insofar as their requirement for HIV infection is concerned [29]. To this end, ongoing studies in primary T cells and other models of T cell activation should provide further insights into the physiological importance, potentiators and mechanism of redox-dependent CD4 membrane trafficking. However, the profound effect that Trx depletion has on CD4 localization in a cell line that has been used extensively to model HIV entry, and must reasonably be presumed to reflect the dynamics of the receptor and co-receptor in this context accurately, therefore, encourages us to propose the following intriguing hypothesis for further consideration: that oxidoreductase-dependent CD4 allostericity plays an important role in regulating CD4 membrane microdomain localization, and this may represent an important mechanistic basis for triggering functional CD4 clustering, and an additional potential mechanism by which changes in redox conditions at the cell surface impair HIV entry.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

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Chapter 4:
Concluding Remarks

Antigen engagement by the T cell receptor triggers a sophisticated signalling network leading to the activation, proliferation and differentiation of T cells (168). It has long been recognized that reactive oxygen species (ROS) play an important role in the regulation of T cell signalling (169, 170). ROS production by immunological cells forms part of the host response to pathogens and the release of ROS during inflammatory responses are important for balancing T-cell activation/anergy, leading to the regulation of immune outcomes (170). One consequence of the change in the redox potential during inflammation and immune activation is the reversible oxidation of cysteine residues that result in altered protein function, which is now known to represent an important mechanism by which signal transduction is regulated (71, 171). Indeed, a variety of signalling molecules involved in T cell activation have been shown to possess cysteine residues that are sensitive to oxidation, including Lck, Zap-70 and LAT (171-173). In addition, ROS have been shown to participate in the formation of lipid rafts and the inhibition of ROS is linked with decreased TCR-mediated raft signalling platforms (174). Furthermore, the spatiotemporal distribution of molecules (such as CD4, CD28 and TCR) impacts on the efficiency of T cell signalling and is recognized as a modulatory mechanism for T cell activation (48).

Over the last two decades it has also been recognized that redox events play a fundamental role in the HIV entry mechanism. The key viral and host entry proteins, gp120 and CD4 both require oxidoreduction of allosteric disulphides to induce the conformational changes necessary for productive entry to occur (79, 83, 136). Implicated in several studies as the key facilitators in these processes are cellular oxidoreductases belonging to the thioredoxin

superfamily (84, 136, 148, 150, 167, 175, 176). Disulphide bond reduction by oxidoreductases is a catalytic process, thus a single enzyme molecule can reduce several disulphide bonds and there is an obvious requirement for co-enzymes to ensure a continuous turnover. Although evidence of the activity and the importance of redox mechanisms within the plasma membrane environment continues to mount, conclusive identification of physiologically relevant molecules is still lacking and is thus needed for the full elucidation of these redox active membrane systems, so that we can understand how they work and what their roles are in pathophysiological processes.

In this study, we have described an important link between redox homeostasis at the cell membrane and the spatial-temporal distribution of CD4 in the plasma membrane, which expands our understanding of the relationship between oxidative stress and HIV entry, and puts forward an intriguing hypothesis regarding how redox events may be involved in regulating CD4-dependent T cell signalling processes.

First, we presented the results of our collaborative efforts with colleagues at the Karolinska Institutet, who analyzed the association of an isoenzyme of TrxR1 with lipid raft membrane microdomains. Significantly, this study demonstrated that TrxR1 enzymes are targeted to lipid raft membrane microdomains, providing strong additional support to an emerging picture of the importance of redox systems in regulating protein function at the cell surface.

In the second part of this study, we present our findings on the membrane microdomain distribution of CD4 and how altering the extracellular redox environment (by the inhibition of cellular oxidoreductases) affects CD4 membrane distribution. Importantly, we demonstrated enhanced CD4 lipid raft association in the presence of inhibitors of the cellular oxidoreductase, Trx1. Our data provide compelling evidence that oxidoreductases

play an important role in CD4's spatial distribution in plasma membrane microdomains. We posit that in the physiological context, the enhanced redox-mediated CD4 raft association may be related to the generation of ROS (by the inhibition of thioredoxin, a critical antioxidant), the accumulation of which has been implicated in enhanced lipid raft formation and clustering (177, 178). Lipid rafts can be the direct targets for oxidative stress and raft clustering mediated by S-S-bonded or S-X-S bonded crosslinking of cell-surface proteins (179). Indeed, it has been shown that ROS promote raft assembly and entry of the TCR complex into lipid rafts leading to T cell activation (174). Hence, CD4 movement to the raft, in the presence of elevated ROS may be an important component of this process.

There is also evidence that the removal of ROS impairs the ability of Lck, the CD4-associated Src family protein tyrosine kinase that is essential for MHCII-dependent T Cell activation, to stay in lipid rafts (174). Lck, a key molecule in TCR signalling localizes to lipid rafts upon TCR/CD4 activation, and impairing its association with lipid rafts impacts negatively on T cell signalling (180, 181). These observations have interesting implications in the context of HIV infection and appear to support yet another role for the involvement of thioredoxin and/or other antioxidants in HIV entry. Oxidoreductases are likely to play a pivotal role in maintaining redox balance at the cell surface, by preventing the accumulation of ROS and reducing the association of CD4 (and by implication Lck), with DRMs/rafts. This would lead to conditions that impact negatively on T cell activation, which may, by delaying the immune response, be more favourable for HIV-1 entry and consistent with the concept that HIV-1 utilizes non-raft associated CD4 for entry (156).

To verify this theory and elucidate the mechanism underlying the inhibition of HIV-1 infection in the presence of Trx1 inhibitors, we have initiated work to extend our analysis of

the potential changes in CD4 microdomain localization, on cells exposed to HIV pseudovirions (Appendix A). Our preliminary observations indicate that HIV *per se* does not induce any changes in CD4 localization in TZM-bl cells (Appendix A- Figure 1A). Interestingly, however, when cells that had been treated with inhibitors of Trx1 were infected with an HIV-1 pseudovirus, we observed a reversal of the Trx1 inhibitor-induced CD4 DRM association (Appendix A, Figures 1B and 1C), an effect that was more pronounced in cells infected with the CD4-dependent virus, ZM53 (Appendix A, Figure 2). Notably, these observations are consistent with the findings of previous studies that have shown HIV-mediated destabilization of rafts. Together with the results of this study which show that CD4 is localized almost exclusively in non-raft membrane microdomains in TZM-bl cells, and that the induced localization of CD4 into DRM is associated with impaired HIV entry, these data provide further support for a model in which CD4-dependent HIV entry proceeds through non-raft membrane microdomains. Moreover, they indirectly suggest that while HIV particles are able to interact with raft-localized CD4 - a notion supported by our observation that recombinant gp120 colocalizes robustly with DRM following treatment of cells with DTNB (Appendix A, Figure 3), as well as previous studies that have shown that an anti-CD4 antibody exerts inhibitory effects on HIV by trapping CD4 in DRMs post CD4-gp120 binding (182) - such viruses are blocked at a critical point in the maturation of the virus-host membrane fusion complex.

Integrating these preliminary observations with our published findings on redox-dependent effects on CD4 localization and HIV entry, we propose that although HIV is capable of binding raft-localized CD4, productive viral entry must proceed through detergent-sensitive membrane domain compartments. In this regard, we hypothesize that potent inhibitory

effects on HIV entry may result from two aggregated consequences of blocking redox activity at the cell surface: 1) inhibition of the oxidoreductase-dependent conformational changes that must be effected in the virus-host receptor complex after CD4-gp120 binding (70, 79, 83, 136), and 2) uncoupling of CD4 and co-receptor localization, shown in this study to result from cell surface oxidoreductase inhibition.

Collectively, our data affirm the role of cellular redox–active proteins, specifically oxidoreductases of the thioredoxin system, in the redox biology of HIV entry and add credence to the recent targeting of these cellular antioxidant systems in the search for novel HIV treatments (176, 183). Our results support the evolving concept that redox conditions in the extracellular space potentiate dynamic changes in CD4 membrane domain localization, which influence the efficiency of virus entry and are likely to play an important role in regulating CD4-dependent signalling events. Our studies suggest that oxidoreductases may play a role in maintaining CD4 dissociation from lipid rafts, which may reflect the requirement for the virus-host receptor complex to be localized in an appropriate (non-raft) membrane domain context to effect efficient fusion, a concept supported by preliminary experiments conducted during the course of this study. The potential involvement of redox events in the mechanism of HIV entry is proposed in the model illustrated in Chapter 4's, Figure 1. Herein, we propose that initially, by maintaining the membrane surface redox balance and preventing the accumulation of ROS, Trx minimizes the raft association of CD4 and in so doing, creates conditions that are more favourable for HIV entry. Secondly, Trx keeps CD4 reduced, the preferred isoform for HIV gp120 interaction (184) and finally, Trx catalyses the conformational changes in CD4 and/or gp120 that are essential for virus entry (27, 166).

In conclusion, this work describes important aspects of the involvement of the extracellular redox system in regulating spatiotemporal changes in CD4 membrane localization, and provides evidence that redox systems exist at the appropriate locations to support this process. It also suggests a further functional role for cellular oxidoreductases in regulating HIV entry. Through ongoing studies, we aim to investigate the membrane contexts required for efficient HIV entry, the roles played by cellular oxidoreductases in establishing these, and the effects of CD4 oxidoreduction in T cell lines and human peripheral blood mononuclear cells (PBMCs) that will provide more detailed insights into the effects of redox events on CD4 function.

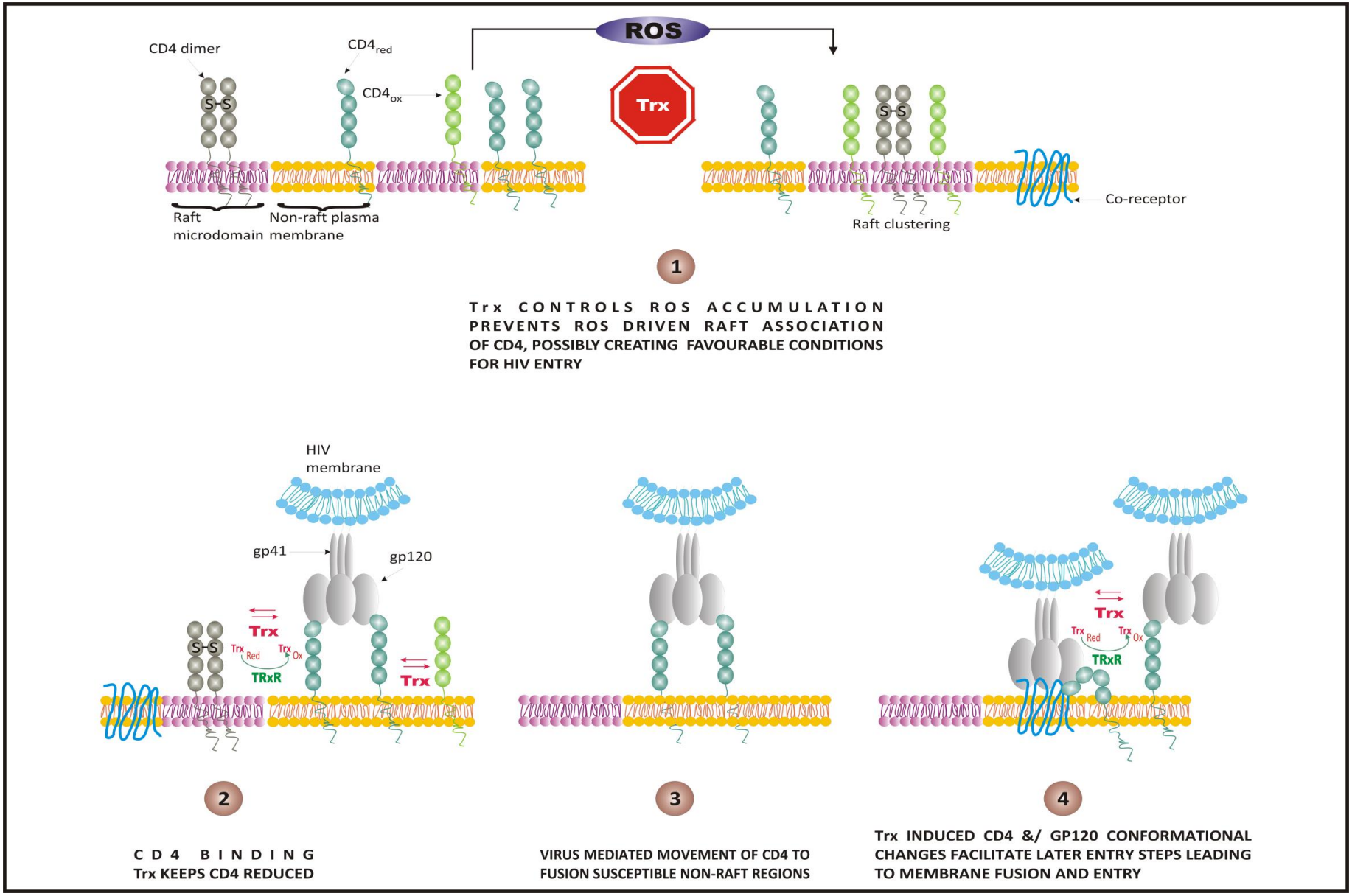

Figure 14: Proposed model for the involvement of redox events in the mechanism of HIV entry.(1) More favourable conditions for HIV entry are created when Trx prevents ROS accumulation at the cell surface preventing CD4 association with rafts. **(2)** CD4 is kept reduced by Trx, the preferred gp120 binding isoform. **(3)** Possible HIV-mediated movement of CD4 to non-raft membrane regions, more conducive to virus entry. **(4)** Trx mediated conformational changes in both CD4 and gp120 are essential for post-binding entry steps.

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Appendices

Appendix A: CD4 distribution in cells treated with HIV gp120 protein and HIV-1 pseudo-typed virus, in the absence and/ or presence of Trx1 inhibitors

The data presented in Chapter 3 established that CD4 membrane microdomain distribution is influenced by the extracellular redox environment. Coupling this information with the existing knowledge that inhibition of thiol active oxidoreductases results in HIV entry inhibition, we conducted preliminary analyses on the impact of HIV-1 envelope pseudo-typed viruses, in the presence or absence of inhibitors of cellular oxidoreductases on CD4 distribution. This data is presented below.

1. Materials and Methods

1.1 Cell treatments with recombinant HIV-1 $gp120_{BAL}$, HIV-1 Env pseudo-typed virus, in the presence of Trx1 inhibitors

For the treatment of TZM-bl cells with HIV-1 gp120 and Env pseudo-typed virus, growth media was decanted, cells were washed with phosphate buffered saline (PBS) and an excess of recombinant HIV-1 $gp120_{BAL}$ (1μg/1x10^5 cells) or HIV-1 pseudovirus SF162.LS (SF162) or ZM53M.PB12 (ZM53) (200 $TCID_{50}$ /1x10^4 cells) (185) was added in DMEM supplemented with 5% foetal calf serum and 2 mM glutamine. The cells were incubated at 37 °C for 2 hours, unless otherwise stated. For the treatment of cells with the thiol modifying agent DTNB in combination with recombinant $gp120_{BAL}$ protein or the pseudovirus SF162.LS or ZM53M.PB12, cells were treated for 1.5 hours with 5mM DTNB followed by the addition of protein or Env pseudo-typed virus in the presence of DTNB and incubated for a further 1.5

hours. For the treatment with the anti thioredoxin antibody, cells were treated overnight (approximately 18 hours) with either antibody only, or with a combination of antibody and recombinant $gp120_{BAL}$ protein or the pseudovirus ZM53M.PB12 added simultaneously to the cells. Following treatments, cells were processed for confocal microscopy or the flow cytometric detergent solubility assay as outlined in method section of chapter 3.

1.2 Expression and purification of recombinant HIV-1 $gp120_{BAL}$

The nucleotide sequence encoding a codon optimized (mammalian expression) sequence for amino acids 30-500 of HIV-1 gp120 Envelope, BaL isolate (Genebank No. M68893) was synthesized with a C-terminal Histidine tag [6 x His] (Geneart; Germany) and sub-cloned into the *Xho1* and *EcoR1* sites of the pCI-neo expression vector (Promega, USA). Following sequence confirmation, 293-F cells were stably transfected with the construct using Lipofectamine 2000 (Invitrogen, USA) as per the manufacturer's recommendations. A clone expressing high levels of secreted recombinant HIV-1 $gp120_{BAL}$ protein (HIV-1 $gp120_{BAL}$) was selected using G418 sulphate (Gibco, Life Technologies, USA) and propagated. Collected culture supernatants from this clone, were purified by an optimized Lectin affinity chromatography protocol. Briefly, four milliliters of *Galanthus (G.) nivalis* lectin cross-linked to 4% beaded agarose (Sigma Aldrich, USA) was incubated with approximately 500 ml of harvested culture supernatant containing the expressed protein, with stirring, overnight at 4˚C. The resin was packed into a liquid chromatography column and the resin bed was washed with 20 column bed volumes (80 ml) of 0.65 M sodium chloride (NaCl) in PBS, followed by 20 column bed volumes of 1.0 M NaCl in PBS, and a final wash of 20 column bed volumes of PBS. Bound $gp120_{BAL}$ was eluted with 7.5 bed volumes of PBS containing 1M methyl α-D-mannopyranoside [MMP] (Sigma Aldrich, USA). Washing and elution procedures

were carried out using the Biologic LP Chromatography System (BioRad Laboratories, USA). Eluted recombinant $gp120_{BAL}$ was filtered through a 0.45 µm, low-protein binding filter (PALL Corporation, USA) and then concentrated to approximately 1 ml using a 50kDa molecular weight cut-off Amicon® Ultra Centrifugation Filter Unit (Merck Millipore, Germany). The purified protein was quantified using the Bradford Concentration Assay [BCA] kit (Pierce, ThermoFisher Scientific, USA) and aliquots were stored at −70 ^{o}C for later use. Initial verification of the expression and purification steps of recombinant $gp120_{BAL}$ was evaluated by SDS-PAGE followed by Western blotting or Coomassie blue staining. HisProbe™-HRP (ThermoFisher Scientific, USA) was used for the detection of recombinant HIV-1 $gp120_{BAL}$ by Western blot.

The functional integrity of the purified gp120 was evaluated by checking CD4-binding activity using an Enzyme Linked Immuno Sorbent Assay (ELISA). In brief, 96-well microtiter plates (Maxisorp, Nunc, Denmark) were coated overnight at 4°C with 100 µl of the monoclonal anti-gp120 antibody D7324 (1µg/ml, Aalto Bio Reagents Ltd., Ireland). The plates were then blocked with PBS containing 0.05% Tween 20 and 1% BSA (250µl per well) for 1 hour at room temperature. A dilution series ranging from 1– 8ng/ml of purified $gp120_{BAL}$ was prepared and each dilution was incubated with an equal volume of 2µg/ml of soluble CD4 (sCD4) (Progenics Pharmaceuticals Inc., USA) at room temperature for 1 hour. Thus, a range of 0.5 – 4ng/ml of $gp120_{BAL}$ was tested. Blocked plates were washed thrice with 300µl /well of PBS-T (PBS containing 0.05% [v/v] Tween 20), using an automated plate washer (BioTek, USA). 100 µl of each dilution of $gp120_{BAL}$/sCD4 or $gp120_{BAL}$ only was added to the plate and incubated at room temperature for 2 hours. Dilutions were assayed in duplicate and wells to which no $gp120_{BAL}$ was added were included as a control. Unbound

protein was removed by washing each well 5 times with 300µl/well of PBS-T and a 1:2000 dilution of the antibodies 2G12 (wells containing $gp120_{BAL}$ only) or 17b (wells with $gp120_{BAL}$/sCD4), prepared in PBS-T with 1% BSA, was added to the wells for 2 hours at room temperature. 17b binds to a conserved, conformation-dependent epitope of HIV-1 gp120 that is exposed only when gp120 is bound to CD4; efficient 17b binding is therefore diagnostic of functional CD4-gp120 engagement (186-188). The binding epitope for 2G12 comprises glycans on the gp120 surface, and is also conformation-dependent (189). Plates were washed as before and bound antibody was detected using 100µl of HRP-conjugated monoclonal anti-human Fc antibody (Amersham Biosciences, United Kingdom) diluted 1:2000 in PBS-T for 1 hour at room temperature. Following washing, plates were developed using the chromogenic substrate Sure BlueTM TMB peroxide substrate (KPL, USA) and TMB stop solution (KPL, USA) as per the manufacturer's instructions. Absorbance values were quantified spectrophotometrically at 450 nm using a BioRad Model 680 microplate reader (BioRad, USA).

2. Results

2.1 CD4 distribution in cells treated with HIV-1 virus

To investigate whether HIV-1 causes changes in the plasma membrane distribution of CD4 we treated cells with HIV-1 Env pseudo-typed virus and assessed changes in CD4 localization by flow cytometry. We observed no significant changes in the DSM or DRM distributions of CD4 in cells treated with Env pseudo-typed virus. The FDRI of CD4 for cells treated with HIV-1 Env pseudo-typed virus was comparable to that of untreated cells (Figure 1A).

Given that CD4 is mostly associated with DSM in TZM-bl cells, and that HIV *per se* does not induce changes in CD4 localization, this data appears to support a model in which CD4-dependent HIV entry proceeds through non-raft membrane microdomains. This model would be consistent with our previous observations that enhanced CD4 DRM association results in the inhibition of virus entry.

2.2 CD4 distribution in cells treated with HIV-1 virus, in the presence of DTNB or anti Trx1 antibodies

Next we sought to assess whether the addition of HIV-1 Env pseudo-typed virus to cells treated with DTNB or anti Trx1 antibodies (α–Trx1) - shown previously to mediate the movement of CD4 into DRMs (Chapter 3) - had any effect on the localization of CD4. Interestingly, when we added HIV-1 Env pseudo-typed virus to cells that had been treated with DTNB/α–Trx1, we observed a reversal of the Trx1 inhibitor-induced CD4 DRM association (Figures 1B and 1C), an effect that is more pronounced by the CD4-dependent virus (ZM53) (Figure 2). These differences are not as a result of gp120/virus binding and interfering with CD4 detection during the flow cytometric assay, as the binding sites for gp120 and the epitope of the CD4-detecting antibody do not overlap on CD4 (190). We verified this using flow cytometry and confocal microscopy (Figure 3) by treating the cells in the presence of DTNB with both pseudo-typed virus and soluble gp120 and analyzing CD4 staining thereafter.

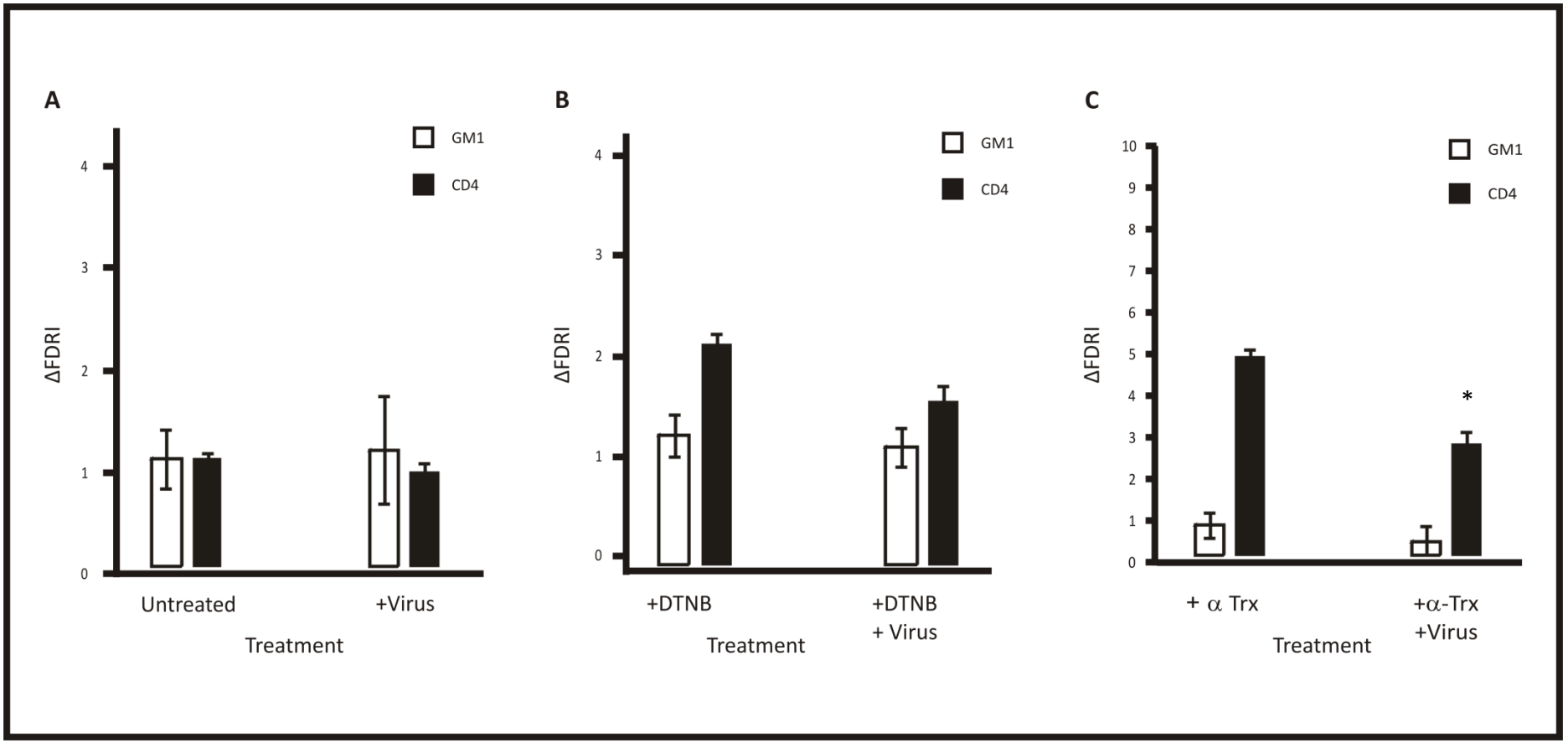

Figure 1: Changes in CD4/GM1 raft association in the presence of HIV Env pseudo-typed virus and Trx1 inhibitors.
The histogram plots show the change in FDRI (△FDRI) after the treatment of cells with Env pseudo-typed virus only (A); treatment of cells with Env pseudo-typed virus in the presence of DTNB (B); treatment of cells with Env pseudo-typed virus in the presence of anti-Trx1 antibodies (α-Trx) (C). Reduced CD4 raft association is observed for cells treated with Env pseudo-typed viruses in the presence of Trx1 inhibitors. Results presented are representative of 3 or more independent experiments; error bars represent standard deviations and statistically significant differences are indicated with an asterisk (*) ($P<0.05$).

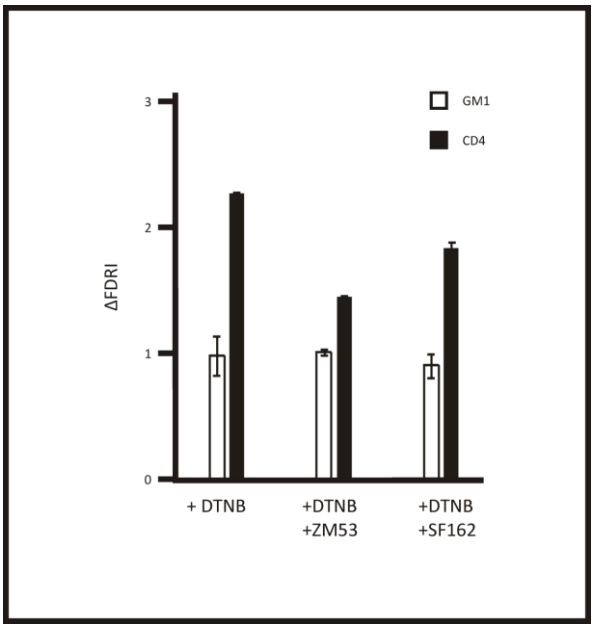

Figure 2: HIV Env pseudo-typed virus induced changes in CD4 DRM association in the presence of DTNB.
DTNB induced CD4 DRM association is reversed/inhibited by the addition of Env pseudo-typed viruses. The CD4 dependent virus ZM53, has a more marked effect than the less CD4 dependent virus SF162. Results are represented as fold change (ΔFDRI) compared to untreated cells. Results presented are representative of 3 or more independent experiments; error bars represent standard deviations.

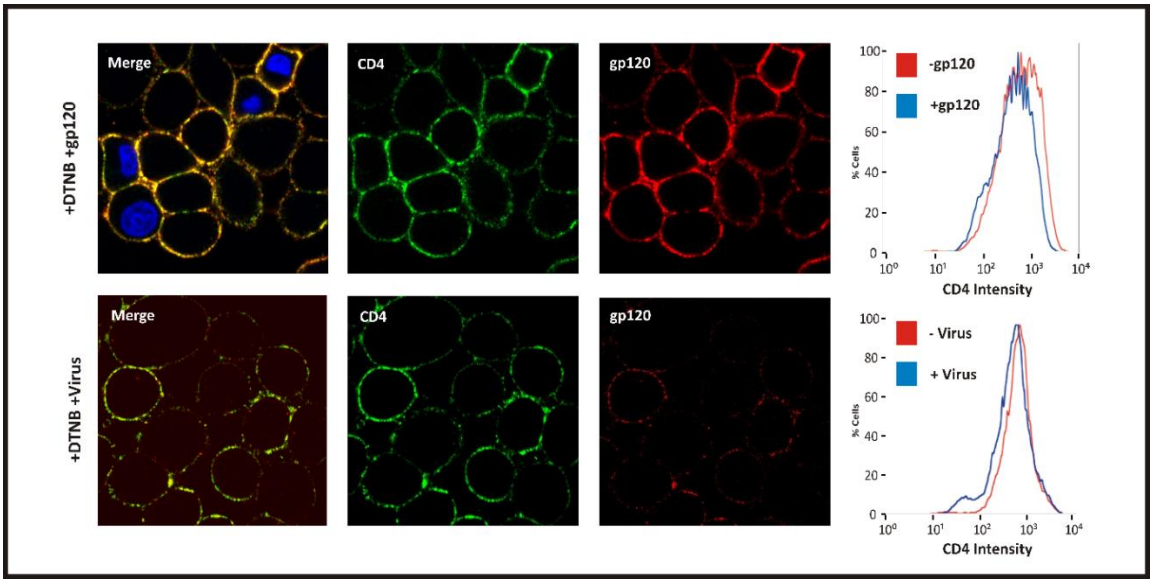

Figure 3: Detection of CD4 and gp120 on DTNB treated cells.
DTNB treatment does not affect the binding of gp120/virus particles to CD4, implying HIV is able to interact with raft associated CD4. CD4 staining intensity is not affected by the addition of gp120/virus particles, confirming that observed differences are not artifacts of the methodology employed. Methods used are outlined in the chapter 3.

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