CHAPTER SIX

TRIM21-Dependent Intracellular Antibody Neutralization of Virus Infection

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Abstract

The ability of antibodies to prevent viral infection has long been recognized. I*n vitro* neutralization assays, which take place in the absence of professional immune effector mechanisms, have demonstrated that the process of neutralization can occur by a variety of molecular mechanisms. Most known mechanisms involve the blocking of an event essential for infection, for instance, the steric inhibition of attachment to entry receptors. As such, neutralization is often thought of as a passive process that can occur without the need for host effector machinery. In contrast to this view, it has recently been demonstrated that neutralization can depend on the widely expressed cytosolic Fc binding protein TRIM21. This unique and novel Ig receptor directs the ubiquitin and proteasome-dependent degradation of intracellular antibody-bound viral particles and prevents infection. It has been further demonstrated that detection of cytosolic antibody by TRIM21 activates inflammatory signaling pathways and promotes the production of cytokines and chemokines. Studies in a TRIM21-null mouse demonstrate the

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importance of these activities: homozygous knockouts suffer fatal viral infection where wild-type mice survive. Though there is much to be learned about the role of TRIM21 in immunity, it is clear that there is a hitherto unappreciated role for antibodies in the intracellular environment.

1. INTRODUCTION

The adaptive immune system can mount specific responses to a near limitless range of antigens. The relative role of antibodies versus cell-mediated and other forms of immunity in overcoming viral infection has been a source of investigation over the past several decades. However, determining the true correlates of immunity for any particular pathogen is complicated by the redundant nature of the immune system and the potential for misleading co-correlation.[1] The natural experiment of transcytosis of IgG from mother to infant provides evidence that antibody-mediated immunity can be protective against a range of viruses including poliovirus, influenza virus, hepatitis A, and hepatitis B viruses. Furthermore, animal studies strongly support a role for antibodies in antiviral protection. For instance, mice unable to produce antibody are acutely susceptible to adenovirus infection while passive transfer of immune serum to naïve animals is sufficient to restore protection.[2]

Since the very first experiments studying the effect of antibodies on animal viruses, it has been clear that antibodies are capable of reducing viral titre during *in vitro* infections of permissive cells. This process, termed neutralization, applies specifically to such *in vitro* activity, thus excluding many alternative mechanisms by which antibodies may exert antiviral activity *in vivo*. It is due to the belief that the production of neutralizing antibodies is a correlate of (or causative of) antiviral protection that it is routinely measured as marker for pathogen-specific immunity. Nonetheless, the mechanisms by which antibodies exert their neutralization effect are often not known and have been a source of controversy over previous decades.

In the past few years, our laboratory has shown that IgG can be carried into the cell during infection by human adenovirus. Once within the cytosol, antibody is bound by the atypical Fc receptor TRIM21, which mediates the proteasome-dependent destruction of virus particles. As such we have demonstrated that neutralization can be an active process that depends on host cell machinery. This review summarizes these findings and discusses them in the context of both antibody neutralization and other intracellular pathogen restriction factors.

2. THE TRIPARTITE MOTIF FAMILY

TRIM21, also known as Ro52 and SS-A, belongs to the large family of tripartite motif proteins of which there are approximately 100 members in the human genome.[3,4] Members of the TRIM family share a RING, one or two B Box domains, and a coiled-coil domain, known collectively as the RBCC. Approximately half of TRIM proteins, including TRIM21 and the antiretroviral factor TRIM5α, possess an additional C-terminal PRYSPRY domain that mediates interactions with target proteins (Fig. 1A). The RING and B Box are Zn^{2+}-coordinating alpha-helical domains with the RING domain capable of E3 ubiquitin ligase activity. The coiled-coil mediates homo-dimerization of TRIM molecules in an antiparallel orientation.[5] The PRYSPRY is a fusion of two more ancient

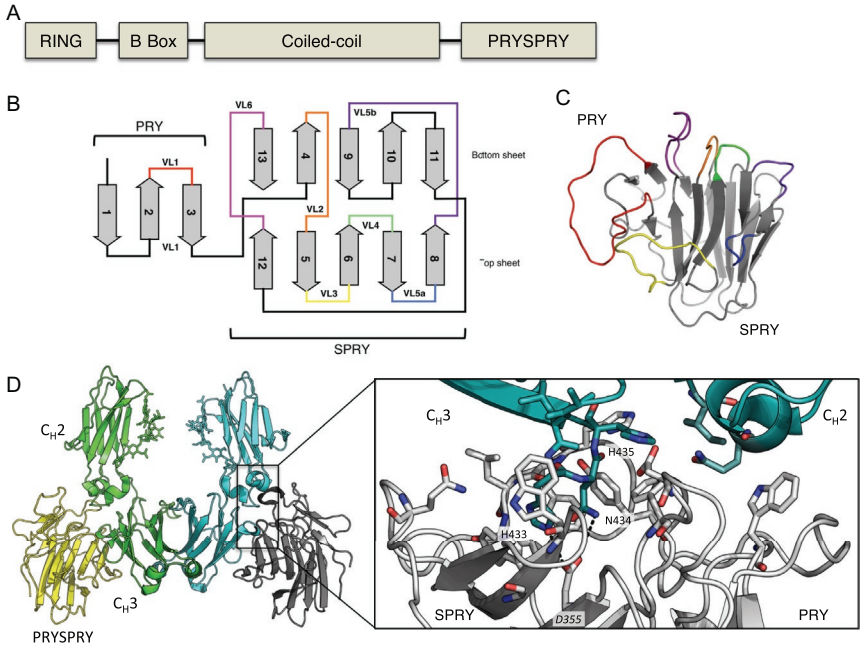

Figure 1 TRIM21 structure and antibody binding mechanism. (A) TRIM21 domain structure. (B) Schematic diagram of TRIM21 PRYSPRY secondary structure in which the β-sheets and flexible binding site loops (VLs) are numbered. (C) Structure of TRIM21 PRYSPRY domain with VLs depicted in the same color scheme as in (B). (D) Structure of TRIM21 PRYSPRY:IgG Fc based on PDB 2IWG. The Fc is indicated in green and cyan and the PRYSPRY in yellow and gray. The boxed region is a close-up view of the interface. The "HNH" motif from Fc is marked.

subdomains, the PRY and the SPRY comprising approximately 60 and 150 amino acids, respectively. Crystal structures of the PRYSPRY reveal a twisted beta-sheet with variable loop (VL) regions that, between TRIM members, vary in both primary sequence and length, and are the major determinants of ligand specificity (Fig. 1B and C).[6] The TRIM family is evolutionarily ancient with homologues of TRIM37 found in diverse taxa including fungi, plants, and various protozoans. However, TRIM proteins with PRYSPRY domains are uniquely present in vertebrates, with a large expansion of such genes in mammals resulting from gene duplication events.[7] As a member of a recently expanded cluster of *TRIM* genes on human chromosome 11, *TRIM21* is therefore likely to have arisen in the same epoch as IgG, which also only occurs in mammals.[8]

3. TRIM21 IS A HIGH-AFFINITY CYTOSOLIC Fc RECEPTOR

A crystal structure of the TRIM21 PRYSPRY:Fc complex has been solved, which shows that the PRYSPRY domain forms a deep binding pocket surrounded by flexible loops (VL2–6) and supported at the base by a concave beta-sheet (Fig. 1D). This binding pocket accommodates the Fc $H_{433}N_{434}H_{435}$ motif that protrudes from the C_H3 domain of IgG. This HNH motif has been found to be important for TRIM21-mediated neutralization, since its mutation to alanine reduces the ability of a monoclonal antibody to prevent adenovirus infection.[9] Hydrogen bonds are also made between Fc H_{433} and N_{434} to PRYSPRY D_{355}, which is buried deep within the PRYSPRY binding pocket, an interaction also found to be important for neutralization (Fig. 1D). Additional interactions are mediated by residues in the PRYSPRY VL1 and VL6, which form contacts with the IgG C_H2 domain. The TRIM21 binding site on Fc overlaps with that of the superantigen *Staphylococcus aureus* protein A, and has been shown to compete for binding,[6] but is distinct from the FcγRI binding site. Isothermal titration calorimetry (ITC) studies reveal that TRIM21 PRYSPRY binds with a 2:1 stoichiometric ratio to Fc, meaning that a dimer of TRIM21 is able to interact with a single antibody molecule. TRIM21 therefore does not cross-link antibody, with the possible exception of the case where an antibody is directed against TRIM21 itself. This is of potential medical significance given that TRIM21 is a major target for antibodies in the autoimmune diseases Sjogren's syndrome and systemic lupus erythematosus.[10,11] These diseases are characterized by circulating and deposited immune complexes that contribute to their pathophysiology. An involvement of TRIM21 in bipolar

bridging of antibody suggests a potential mechanism by which immune complexes may form and be stabilized.

The dissociation constant of monomeric PRYSPRY and Fc has been determined by ITC to be 37 nM[6] and the avidity effects of TRIM21 dimerization increase affinity further to approximately 600 pM.[12] Alongside IgεR, TRIM21 is therefore the highest affinity antibody receptor in the human body and the highest affinity receptor for IgG. The TRIM21:Fc interaction is widely conserved among mammals: mouse antibodies bind to mouse TRIM21 PRYSPRY with a similar affinity as the cognate human TRIM21:human Fc interaction.[13] The interaction is also species cross-reactive: human and mouse TRIM21 PRYSPRY domains can bind IgG from diverse mammalian host species. These findings imply that evolution acts to maintain the high-affinity binding of IgG to TRIM21. Finally, in addition to binding IgG, TRIM21 has been shown to bind directly to IgM, albeit with a reduced affinity.

4. TRIM21 MEDIATES ANTIBODY-DEPENDENT INTRACELLULAR NEUTRALIZATION

TRIM21 has been shown to mediate the neutralization of a replication-deficient human adenovirus type 5 vector (Fig. 2). When TRIM21 was depleted in HeLa cells, a cell line used frequently for such studies over the past half century, neutralization by pooled human serum IgG was diminished by approximately 10-fold.[12] In agreement with molecular interaction data, human TRIM21 was shown to provide the majority of neutralization for anti-adenovirus antibodies raised in goats and mice.[12,14] Moreover, TRIM21 was found to be crucial for efficient neutralization in mouse cells as fibroblasts from TRIM21$^{-/-}$ mice permitted only low levels of neutralization when compared to wild-type fibroblasts.[9] This activity of TRIM21 is referred to as "antibody-dependent intracellular neutralization" or ADIN.[15]

TRIM21 mediates neutralization by targeting antibody-coated viruses for degradation in the proteasome. The intracellular adenovirus:antibody complex is degraded within 2 h of infection and degradation can be prevented by depletion of TRIM21.[12] Furthermore, chemical inhibitors of the proteasome prevent degradation and restore infectivity of antibody-coated virus. TRIM21 is an interferon-stimulated gene and its expression is substantially upregulated by type I interferons. Under conditions of IFN stimulation, antibodies are capable of mediating more potent

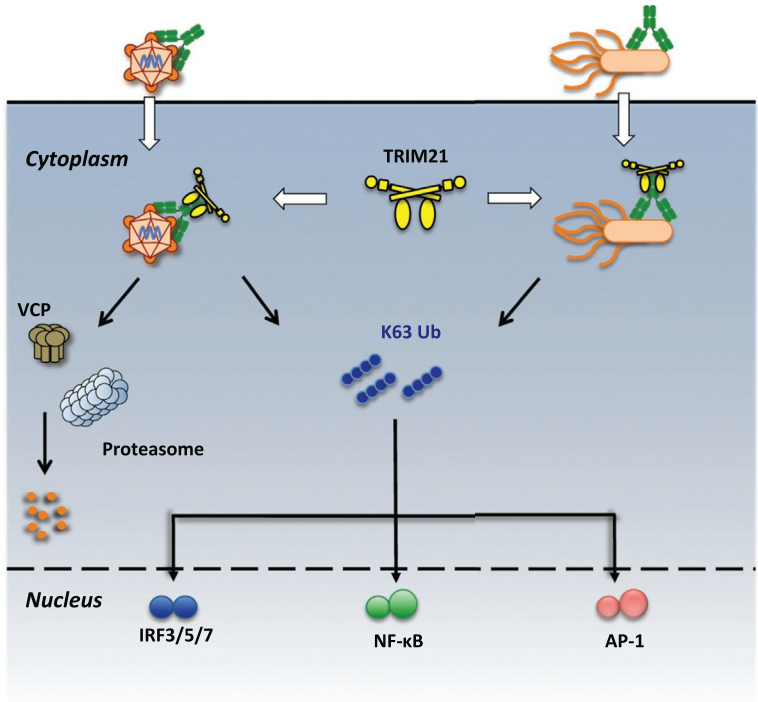

Figure 2 TRIM21 detects antibody-coated pathogens in the cytosol and mediates signaling and neutralization pathways.

neutralization and TRIM21 depletion reverses this effect. Such observations are not unprecedented: potentiation of neutralization by interferon has been previously demonstrated for adenovirus type 3 as well as other nonenveloped viruses.[16] The involvement of the proteasome in neutralization raises the question of how a challenging substrate such as an adenovirus particle can be processed: the proteasomal pore is no greater than 2 nm in diameter[17] yet the adenovirus capsid is approximately 90 nm.[18] Indeed it has been demonstrated that TRIM21 neutralization requires the AAA ATPase valosin-containing protein (VCP, also known as p97 or Cdc48 in yeast), a homo-hexameric barrel-shaped enzyme involved in the disassembly of protein complexes and their proteasomal degradation.[19,20,21] Inhibition of VCP by the chemical inhibitor DBeQ[22] or its depletion by RNAi severely diminishes neutralization.[23] Interestingly, degradation of IgG heavy chain expressed in the cytoplasm of cells is prevented by proteasome inhibition but not VCP inhibition. This suggests that it is the type of substrate that stimulates the recruitment of VCP rather than VCP being a constitutive auxiliary

in TRIM21-mediated degradation. This hypothesis is supported by findings that VCP is recruited to stalled proteasomes.[20]

5. TRIM21 IS A SENSOR FOR CYTOPLASMIC ANTIBODY

Infection can be sensed via the detection of pathogen-associated molecular patterns by germline-encoded molecules of the innate immune system.[24] Alternatively, infection can be sensed by the mislocalization of host molecules or danger-associated molecular patterns (DAMPs).[25] The transfer of antibodies from the extracellular milieu to the cytoplasm is detected by TRIM21, which initiates a signaling cascade activating NF-κB, AP-1, and IRF transcription factors (Fig. 2).[14] These findings demonstrate that antibodies in the cytosolic environment act as a DAMP, signaling a breach of membrane integrity. Stimulation of TRIM21 signaling with adenovirus:antibody complexes resulted in the production of proinflammatory chemokines and cytokines including IL-6, tumor necrosis factor and IFN-β. When supernatant from cells stimulated in this manner was transferred to fresh cells, an antiviral state was induced. This state included the upregulation of TRIM21 itself, a mechanism ensuring that bystander cells are primed to exhibit high levels of virus neutralization.

The ability to signal in response to intracellular antibody is not limited to professional immune cells but also occurs in cells that are not of myeloid or lymphoid lineage such as fibroblasts, airway epithelium, and several commonly used cell lines.[14] Indeed TRIM21 exhibits widespread tissue expression, and may therefore represent a prevalent sensor for intracellular antibody. Experiments using the replication competent mouse adenovirus-1 (MAV-1) demonstrated that neutralization and signaling occur simultaneously to reduce viral titers.[26] This ability to both neutralize and sense infection makes TRIM21 a dual effector-sensor receptor, a class of molecules to which the antiretroviral factors TRIM5α and tetherin also belong.[27,28] It is likely that other pathogen-specific "restriction factors" will also be found to display such dual capabilities. In a similar fashion, multiple serum proteins are known to attach to pathogens in circulation and may act alongside antibody as DAMPs during infection.

6. TRIM21 FUNCTIONS ARE UBIQUITIN DEPENDENT

In addition to binding antibodies, TRIM21 is an active E3 ubiquitin ligase and autoubiquitination can be observed *in vitro*. TRIM21-mediated

ubiquitination is required for both neutralization and signaling as inhibition of the proteasome or deletion of the RING and B Box domains abolishes these activities. TRIM21 has been shown to catalyze the formation of ubiquitin polymers with different chain linkages and function with different E2 enzymes *in vitro*. In the presence of the promiscuous E2 enzyme UbcH5, TRIM21 undergoes autoubiquitination with K48-ubiquitin chains, while using the E2 enzyme pair Ubc13/UEV1A TRIM21 synthesizes the formation of unanchored K63 chains. These different chain types appear to be important for TRIM21 activity. K63-linked ubiquitin chains can promote NF-κB signaling via the TAK1 complex[28,29] and IKKα/β/NEMO complex.[30,31] Inhibition of these pathways by (5Z)-7-oxozeaenol, IKK VII or panepoxydone—inhibitors of TAK1, IKKα, and IκB, respectively—is sufficient to prevent TRIM21-mediated signaling in response to antibody-coated virus. From these data, it is clear that ubiquitination is crucial to TRIM21 function; however, the specifics are still unclear, including the complete range of ubiquitin chains that are synthesized and the order in which they are made and the substrates that undergo modification. Moreover, there is a clear need for these ubiquitination activities to be strictly regulated in order to act as a trigger for effector and sensor pathways. How this is accomplished is not currently understood but intuitively must be related to the binding of TRIM21 to antibody-coated virus.

7. *IN VIVO* RELEVANCE

Neutralizing antibodies, which inhibit infection *in vitro*, are a principal correlate of protection for infectious disease (for review, see Refs. 15,32). Their elicitation is used to measure vaccine efficacy and to determine prior pathogen exposure. Antibodies that are not neutralizing *in vitro* can also be protective *in vivo*, either because they require effector mechanisms (such as ADCC, complement, or phagocytosis) or because they require epitopes or specific physiological conditions that are only present *in vivo* (for discussion, see Ref. 33). The importance of antibody protection can be studied *in vivo* by genetic ablation of genes required for antibody production and maturation or that encode Fc receptors and by passively transferring antibodies to naïve animals. The importance of TRIM21 in antiviral immunity has been studied using knockout animals challenged with the model nonenveloped virus (MAV-1). In these studies, it was shown that naïve mice lacking TRIM21 had a higher viral load and increased mortality, with 25% of knockout animals succumbing to virally induced encephalomyelitis within

the first week of infection. As these experiments were carried out in naïve animals, this protection was presumably a result of the use of IgM by TRIM21. Passive transfer experiments in TRIM21 knockout animals have also been carried out, in which the ability of antisera against MAV-1 to protect against a high viral dose was determined. Antiserum was shown to fully protect wild-type animals given a lethal virus dose; however, the same sera failed to protect most TRIM21 knockout mice. As with the naïve experiments, the observed protection by TRIM21 correlated with a reduction in viremia. Importantly, no difference in mortality was observed between genotypes when neutralizing F(ab′)2 fragments were administered. These results illustrate the importance of both antibodies and TRIM21 in antiviral protection. Furthermore they show that, in the absence of TRIM21, antibodies are still protective but with reduced efficacy while in the absence of antibody, TRIM21 gives no antiviral protection. The latter finding is consistent with the role of TRIM21 being entirely dependent upon antibody.

8. VIRAL DETERMINANTS OF TRIM21-MEDIATED NEUTRALIZATION

The recent identification of TRIM21 neutralization means that the spectrum of pathogens that are susceptible to ADIN is still being determined. Susceptibility is likely to be dependent upon the particulars of the pathogen replication cycle and whether the pathogen has evolved a mechanism of antagonism. However, the basic requirement for ADIN sensitivity can be defined as an infection event in which the pathogen enters the cytosol with an attached antibody. Fulfilling this criterion is sufficient to provoke an immune response, as demonstrated in experiments in which antibody-coated latex beads transfected into the cell are rapidly bound by TRIM21 and activate immune signaling.[12,14] Thus, any cytoplasmic antibody-bound particle may, in principle, be subject to the activities of TRIM21. Immunofluorescent microscopy has demonstrated that adenovirus particles are capable of entering the cell with antibody attached and are bound by TRIM21 within 30 min of infection.[12] Similar findings were demonstrated for the facultatively intracellular bacterium *Salmonella enterica typhimurium*, which may reside either within vesicles or free in the cytoplasm.[34,35] However, the capability of different pathogens to traffic antibodies into the cytoplasm will depend on their mechanism of entry and replication.

Nonenveloped viruses are presumed to be the major target of ADIN because antibodies against them bind directly to their capsid. This means that

unless the virus divests itself of its capsid before entering the cytosol it will be visible to TRIM21. For adenovirus, entry occurs after binding of viral fiber protein to the cell surface receptors coxsackie and adenovirus receptor or CD46.[36,37] Subsequent infectious entry is via clathrin-mediated endocytosis though macropinocytosis is also stimulated.[38,39,40] Once within the endosome, adenovirus escapes into the cytosol through breakage of the endosomal membrane. This process relies on the partial uncoating of the virus particle and the release of the interior capsid protein VI, which induces membrane damage.[40] The subviral particle is then free in the cytosol and uses microtubule-associated motor proteins to access the nuclear pore complex where the viral DNA genome and its associated proteins are released from the capsid and enter the nucleus.[41] This method of entry to the cell allows antibodies directed to the major capsid protein hexon to remain associated with the subviral particle, permitting direct contact with the cytosol. Adenovirus may therefore represent a virus that is particularly susceptible to TRIM21.

Feline calicivirus was shown to elicit TRIM21 signaling when incubated with feline IgG.[14] Comparatively little is known about the entry of this virus into the cytosol, though it has been demonstrated that infection by FCV results in the permeabilization of the cell membrane to the 17 kDa toxin α-sarcin.[42] Given this finding, it is likely that some exposure of endosomal contents to the cytosol occurs, which may explain the ability of TRIM21 to detect antibody. Other positive-sense RNA viruses such as the picornaviruses are thought to translocate their genomes through pores in the viral capsid while remaining in endosomes. For instance, human rhinovirus 2 is triggered at low pH to insert amphipathic peptides contained within proteins VP1 and VP4 into the endosomal membrane.[43] A pore approximately 10 Å in diameter is then formed at the pseudo threefold axis of the capsid which permits translocation of viral RNA into the cytosol.[44] Capsid proteins remain within endosomes and are subsequently degraded within lysosomes[45] and only low molecular weight dyes are able to penetrate to the cytosol.[46] Direct contact between capsid and cytosol is therefore prevented. HRV-2 and other viruses such as poliovirus that use a similar strategy may therefore evade detection by TRIM21. However, rhinoviruses belonging to the major group such as HRV-14 have been shown to enter the cell by endosomal lysis with subviral particles detectable in cytoplasmic fractions.[47] Future experiments are required to determine exactly how different entry pathways affect the ability of TRIM21 to detect viral infection.

Enveloped viruses are not believed to be susceptible to ADIN because the antibodies that target them bind not to their capsid but to antigens embedded in the lipid membrane that surrounds them. When enveloped viruses infect cells they do so through a fusion event between their envelope and either the plasma membrane or, if they have entered through endocytosis, an endosomal membrane. This has the result of leaving any attached antibodies on the outside of the cell or inside an endosome and therefore not accessible to TRIM21 in the cytosol. As an example of this, the plasma-membrane-fusing respiratory syncytial virus has been shown to evade TRIM21 signaling.[14] However, there are reports that cellular membranes become permeable to macromolecules even during enveloped virus entry[48] and this could potentially permit the entry of antibody and facilitate TRIM21 sensing. Future work will determine whether this is the case.

9. TRIM21 EXERTS HIGHLY EFFICIENT INCREMENTAL NEUTRALIZATION

The number of antibodies required for virus neutralization has been a source of considerable investigation and controversy since some of the earliest experiments on virus neutralization.[49] Controversy has centered on the single-hit and multi-hit hypotheses, under which viruses are neutralized by a single antibody or a threshold number (greater than 1) of antibodies, respectively. Such models assume "all-or-nothing" neutralization where the remaining level of infectivity is governed solely by the proportion of viruses with subthreshold numbers of antibodies attached. During single-hit neutralization only viruses without any antibody molecules bound will be infectious. Remaining infectivity of virus will decrease linearly when plotted on a logarithmic scale against the number of antibodies per virus. The curve will pass through e^{-1}, or 37% remaining infectivity, when a single antibody is bound and e^{-2}, 14% remaining infectivity, when two antibodies are bound (Fig. 3A). For multi-hit neutralization, an initial "lag" is predicted that becomes more pronounced as the threshold number of antibodies increases.

The observation that poliovirus is neutralized with first-order kinetics was interpreted under the assumptions of all-or-nothing neutralization and has been proposed as evidence in support of single-hit neutralization.[49] Such assertions appeared to be at least partially substantiated by experimental data when it was shown that both poliovirus and adenovirus can be neutralized by relatively few (about 5 and 1.4, respectively) antibodies per

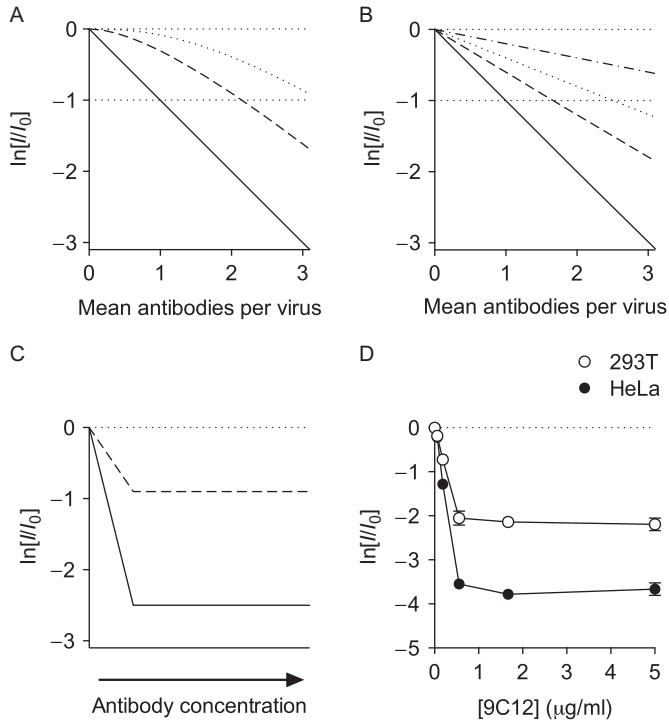

Figure 3 Features of neutralization curves that are influenced by TRIM21. (A) All-or-nothing neutralization curves. If it is assumed that antibodies are distributed among viruses according to a Poisson distribution, then the proportion of viruses that remain infectious will decline as the number of antibody molecules per virus (r) increases above a threshold number (n) of antibodies required for neutralization. Remaining infectivity (I/I_0) is thus equal to $I/I_0 = \sum_{r=0}^{n-1}(m^r e^{-m})/r!$ where m is the average number of antibodies per virus. The remaining infectivity of virus is plotted against a natural logarithmic scale (solid line). Under a single-hit model ($n=1$), a straight-line curve is observed, whereas under a multi-hit model ($n=2$, dashed and $n=3$, dotted), a lag is observed. (B) Incremental neutralization. Neutralization by TRIM21 results in straight-line curves of differing slopes. This suggests that antibodies contribute incrementally to neutralization, rather than providing all-or-nothing neutralization. As TRIM21 levels increase, so the efficiency of neutralization increases toward the theoretical maximal efficiency of neutralization where a single antibody is sufficient for neutralization, depicted by the solid line. (C) Experimental observations over the past several decades have often demonstrated the existence of a proportion of viruses that is resistant to neutralization, the persistent fraction (PF), marked by a leveling off in neutralization curves. Under conditions where TRIM21 levels are high, the PF is low (solid line). However, depletion of TRIM21 or inhibition of VCP or the proteasome increases the PF (dashed line). (D) It has previously been demonstrated that the PF varies between cell lines in a similar manner to changing TRIM21 levels (see text). This observation is also true for adenovirus, which has a markedly different PF on two commonly used human cell lines when treated with mouse monoclonal antibody 9C12.

virus particle.[50,51] However, these findings have proven controversial and potentially flawed.[52] More recently it has been argued that apparent first-order kinetics cannot be interpreted as evidence for single-antibody neutralization.[53]

The mouse monoclonal antibody 9C12, which binds the major capsid protein, hexon,[54] does not block entry of adenovirus 5 and acts in concert with TRIM21 to neutralize infection.[15] 9C12 was found by immunogold electron microscopy to bind adenovirus particles approximating a Poisson distribution.[9] No evidence for a lag was observed and neutralization curves were found to be approximately linear. The gradient of the slope was determined by TRIM21 levels, with a steep gradient observed when TRIM21 levels were high. Parallel infection and quantification experiments, where virus was simultaneously examined for infectivity and stoichiometry of binding, demonstrated that few antibodies were sufficient for neutralization. Under conditions of high TRIM21 expression (after IFN stimulation) in mouse cells, neutralization by 9C12 approached its theoretical maximal efficiency, requiring 1.6 antibody molecules per virus. Under conditions of low TRIM21 expression (obtained by shRNA), approximately fivefold greater levels of antibody were required to obtain the same level of neutralization. These findings argue against an all-or-nothing model of neutralization, given that multiple straight-line curves were observed with differing slopes. Rather the findings suggest that TRIM21 exerts incremental neutralization whereby each antibody that binds contributes to neutralization and that the magnitude of this contribution is in turn determined by the levels of TRIM21 (Fig. 3B). When experiments were performed using polyclonal anti-adenovirus sera or pooled human IgG, where antibodies that neutralize via multiple mechanisms are likely to be present, neutralization was found to shift from straight-line curves to multi-hit curves as TRIM21 was depleted.[9] The shape of a neutralization curve, in particular the presence or absence of a lag, may therefore provide useful information, not perhaps as originally envisaged as a way of illustrating that few antibodies can neutralize and that neutralization is all-or-nothing, but concerning the mechanism by which neutralization takes place (e.g., entry blocking or TRIM21 mediated).

The finding that neutralization by TRIM21 can be exceedingly efficient suggests that antibodies may exert a protective role even when present at very low concentrations or low affinities. Such low stoichiometric complexes are likely to fail to elicit effector responses that rely on cross-linking Fc receptors such as phagocytosis, but may nonetheless contribute to

clearance of viral infection via TRIM21. This may be particularly important in situations where pathogen-specific antibodies may be at low concentrations, for example, in the initial stages of a primary humoral response or during secondary challenge before activation of memory B cells.

10. THE PERSISTENT FRACTION

The addition of high concentrations of antibody, or prolonged incubation with excess concentrations of antibody, frequently results in a fraction of viruses that remain resistant to neutralization. These "non-neutralizable" viruses are referred to as the persistent fraction (PF; Fig. 3C). Mechanisms proposed to underpin the PF include the cross-linking of virus particles, dissociation of virus antibody complex upon dilution and the slow attainment of steady-state conditions.[52,55,56] Addition of 9C12 to adenovirus resulted in PF occurring at stoichiometries of approximately 50 antibodies per virion, a level well below saturation binding, which occurred at stoichiometries of approximately 200.[9] This implies that factors other than antibody become limiting, resulting in the PF. Depletion of TRIM21 was found to increase the PF from 5% of remaining infectivity to 30% demonstrating that limiting levels of TRIM21 can be a cause of the PF. Similarly, depletion or chemical inhibition of TRIM21 co-factor VCP also increased the PF.[23] These findings indicate that the PF can be influenced by the intracellular environment. It is interesting to note that the PF of enterovirus 71 was found to vary with cell type.[57] In the human rhabdomyosarcoma cell line RD, 1% of viruses were resistant to neutralization but on green monkey kidney cells 10% of viruses treated with the same neutralizing serum were resistant. It is tempting to speculate that saturation of intracellular neutralization machinery was the underlying cause of the PF in these experiments and that altered levels of PF between the cell lines are a result of cell type-specific variations in such machinery. Though the role of TRIM21 in the neutralization of picornaviruses has yet to be investigated, the variable PF observed by Kjellen *et al.* is observed during adenovirus infection of commonly used human cell lines (Fig. 3D).

11. COMPARISON OF TRIM21 WITH TRIM5α

While many of the cofactors and basic principles necessary for TRIM21-mediated signaling and neutralization have been identified, the

detailed molecular mechanisms of these processes remain to be elucidated. In attempting to understand these mechanisms it is useful to compare TRIM21 to other related antiviral proteins. In particular, TRIM21 shares many properties with the antiretroviral restriction factor TRIM5α. Both proteins have dual-antiviral activities, mediating both effector and sensor pathways. They are also similar in their mechanism of function. TRIM5α and TRIM21 have the same domain architecture and both are E3 ubiquitin ligases. In each case, their ability to activate innate immunity is thought to be dependent upon their catalysis of free K63-ubiquitin chains.

TRIM5α was identified as a host factor that prevents the infection of rhesus macaque cells by HIV-1.[58] While the human homologue is inactive against HIV, it retains activity against other retroviruses. In contrast to TRIM21, the block to infection provided by TRIM5α is not reversed by proteasome inhibitors (though such compounds do relieve the block to reverse transcription).[59] While deletion of the TRIM5α RING domain does not prevent HIV restriction,[60] deletion, or mutation of the B Box abolishes TRIM5α inhibition of HIV infection.[61] Together this suggests that TRIM5α restriction of HIV is independent of ubiquitination but dependent upon the B Box. The B Box domain of TRIM5α has been shown to mediate higher order oligomerisation and this activity correlates with its ability to bind to *in vitro* assembled capsid protein.[62] The current model by which TRIM5α is believed to inhibit HIV-1 replication is by forming a lattice that encases the viral capsid, thereby preventing proper disassembly. Formation of this TRIM5α lattice is hypothesized to require initial binding of the PRYSPRY domain to the viral capsid followed by intermolecular B Box interactions. In support of this, in cells where the proteasome has been inhibited, TRIM5α forms large stable cytoplasmic bodies localized to viral particles.[63] Meanwhile, cryo-EM experiments have demonstrated that TRIM5α forms a lattice on two-dimensional arrays of hexagonal capsid protein.[64]

Whether TRIM21 utilizes a mechanism like TRIM5α to bind and/or capture antibody-coated virus is unknown, however, current evidence suggests that this is not the case. In domain swap experiments it was found that the RING, B Box and coiled-coil domains of TRIM5α could be substituted, and restriction maintained, with those from TRIM6 and TRIM34 but not TRIM21.[65] However, the RING, B Box and coiled-coil domains of TRIM21 could be substituted for those from TRIMCyp, a variant of TRIM5α that also restricts HIV-1.[66] The transplanted RING and B Box domains in TRIM21Cyp were shown to be active as inhibition of

the proteasome rescued the block to reverse transcription. However, deletion of the RING and B Box domains from TRIMCyp does not rescue infection, unlike with TRIM5α.[67] Thus, the ability of the B Box to mediate higher order assembly is not required for TRIMCyp restriction. The fact that the TRIM21 B Box can substitute for TRIMCyp but not TRIM5α suggests that it does not perform this function and only has a role in recruiting the proteasome.

The above analysis suggests that despite the obvious similarities between TRIM5α and TRIM21 they utilize different mechanisms of restriction. Experiments into the relative stoichiometric requirements of each protein also support this conclusion. As discussed earlier, TRIM21 can restrict viruses that are bound by <2 antibody molecules. The necessary stoichiometry for TRIM5α and TRIMCyp activity has also been estimated, by testing chimeric viruses that have different ratios of restriction-sensitive capsid protein. In these studies, most of the block to infection was lost once the ratio of sensitive protein reached ≤25%,[68] approximately 375 capsid monomers per virion. This contrast in the necessary stoichiometries between TRIM5α and TRIM21 further supports the notion that restriction by the former is a direct consequence of binding, whereas the latter requires a catalytic step and the involvement of the proteasome.

If it is assumed that TRIM5α forms a more-or-less complete lattice around incoming viral capsids, whereas TRIM21 is more sparsely recruited then there are also implications for their respective mechanisms of regulation. As both proteins have a role in innate immune signaling via the production of K63-ubiquitin chains, there must be a switch that triggers or upregulates this activity upon viral infection. In the case of TRIM5α, the template-dependent assembly of higher order oligomers on the capsid surface provides an attractive model of regulation. Indeed, it has been shown that the addition of virus greatly increases K63-chain formation by TRIM5α.[28] However, the cross-linking of multiple molecules as a mechanism of regulation is harder to imagine in the case of TRIM21 given that only a few copies per virus are sufficient for activity. Cross-linking or higher order assembly of TRIM21 is also not observed either when unliganded or in the presence of IgG Fc, with which it forms a stable 1:1 complex. Finally, the different propensity of TRIM5α and TRIM21 to form higher order multimers is suggested by the fact that while TRIM5α overexpression is sufficient to activate innate immune pathways, TRIM21 overexpression either does not,[69] or appears to be inhibited at higher levels of expression.[70]

12. CONCLUSIONS

In addition to being the highest affinity Fc receptor yet identified, TRIM21 represents a very different type of antibody receptor to those previously described. It is structurally unrelated to other mammalian receptors, which all belong to the immunoglobulin superfamily, widely expressed rather than confined to professional immune cells, cytosolically located rather than membrane-associated, neither subtype nor isotype specific, being able to bind both IgM and IgG, and in possession of its own intrinsic enzymatic activity. TRIM21 does not fit into conventional immunological classifications. It is an immune-stimulated gene and therefore part of innate immunity but it uses antibodies to target pathogens, which are part of adaptive immunity. TRIM21 possess dual signaling and effector functions and these events appear to occur simultaneously, as opposed to a signaling event proceeded by an effector response as is the typical paradigm in immunology. The discovery of TRIM21 also challenges widespread assumptions specifically concerning humoral immunity. It illustrates that neutralization is not necessarily a receptor-independent event and that antibodies mediate important immune functions inside cells, within the cytoplasm, as well as in the extracellular space. It remains to be seen whether TRIM21 is the only protein that possesses the many unexpected properties summarized here or whether other conceptually related receptors exist.

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