Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21)

Donna L. Mallery[a,1], William A. McEwan[a,1], Susanna R. Bidgood[a,1], Greg J. Towers[b], Chris M. Johnson[a], and Leo C. James[a,2]

[a]Protein and Nucleic Acid Chemistry Division, Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 0QH, United Kingdom; and [b]Division of Infection and Immunity, Medical Research Council Centre for Medical Molecular Virology, University College London, London W1T4JF, United Kingdom

Edited* by Douglas T. Fearon, University of Cambridge School of Clinical Medicine, Cambridge, United Kingdom, and approved October 12, 2010 (received for review September 19, 2010)

Antibodies provide effective antiviral immunity despite the fact that viruses escape into cells when they infect. Here we show that antibodies remain attached to viruses after cell infection and mediate an intracellular immune response that disables virions in the cytosol. We have discovered that cells possess a cytosolic IgG receptor, tripartite motif-containing 21 (TRIM21), which binds to antibodies with a higher affinity than any other IgG receptor in the human body. TRIM21 rapidly recruits to incoming antibody-bound virus and targets it to the proteasome via its E3 ubiquitin ligase activity. Proteasomal targeting leads to rapid degradation of virions in the cytosol before translation of virally encoded genes. Infection experiments demonstrate that at physiological antibody concentrations TRIM21 neutralizes viral infection. These results reveal an intracellular arm of adaptive immunity in which the protection mediated by antibodies does not end at the cell membrane but continues inside the cell to provide a last line of defense against infection.

Viruses and their hosts have been coevolving for millions of years, and this has given rise to a complex system of immunity traditionally divided into innate and adaptive responses. Innate immunity comprises germ-line encoded receptors and effector mechanisms that recognize pathogen-associated molecular patterns (PAMPs) (1). The advantage of innate immunity is that it is fast and generic; however, viruses are adept at avoiding recognition by inhibiting innate immunity or by changing their molecular patterns. In contrast, adaptive immunity can clear a host of infection and provide protection against future infection. Unlike the PAMP receptors of innate immunity, adaptive immunity uses proteins such as antibodies to target pathogens. Antibodies are unique in the human body in that they evolve during the lifetime of an individual and can continue to target evolving pathogens (2). The weakness of adaptive immunity is that it can take 1 to 2 wk to reach full effectiveness. Moreover, the dogma of antibody immunity for the last 100 y has been that antibodies only provide extracellular protection (3). It is thought that once a virus has entered the cytosol of a cell, antibodies are helpless to prevent its infection.

Recently we described an intracellular cytosolic protein called tripartite motif-containing 21 (TRIM21) that is capable of binding to an invariant region of antibody molecules via its PRYSPRY domain (4). We found this activity to be structurally, thermodynamically, and kinetically conserved across mammals (5). However, antibodies are extracellular proteins, as are all known mammalian IgG receptors (with the exception of FcRn, which is intracellular but not cytosolic). It therefore seemed incongruous to us that TRIM21 should be a universally conserved intracellular protein and yet be a high-affinity, highly specific IgG receptor. We hypothesized that there might be an antibody effector mechanism inside cells, mediated by TRIM21. Here we demonstrate the existence of this mechanism and its operation in preventing infection by adenovirus. Our data reveal that humoral immunity provides intracellular protection, whereby antibodies function inside cells by engaging with a potent effector mechanism that targets virus for degradation.

Results

TRIM21 Mediates Intracellular Antibody Neutralization. It is assumed that antibodies do not routinely enter the cytosol during viral infection. To test this, we preincubated adenovirus (a model virus that causes respiratory disease) with different antibodies and added the virions to cultured HeLa cells. Adenovirus was chosen because it is a nonenveloped virus, and its capsid is naturally exposed to serum antibody before cellular infection. This is in contrast to enveloped viruses, in which the capsid is protected from antibody. After 30 min of infection the cells were fixed, and a fluorescent anti-IgG antibody was added to detect antibody-coated virions. As can be seen in Fig. 1*A*, antibody-coated virions successfully enter cells. Similar results were obtained using different polyclonal antihexon antibodies and human serum IgG. Adenovirus enters the cell by binding to the coxsackie and adenovirus receptor and becoming endocytosed. We found that addition of antibody does not prevent this process and that antibody remains attached to virus after entry. To address whether antibody-coated virus is accessible to cytosolic TRIM21, we costained for TRIM21. As shown in Fig. 1*A*, TRIM21 is efficiently recruited to antibody-coated viral particles.

Next we tested the effect of TRIM21 recruitment to virions by quantifying the levels of adenovirus infection. We used a virus that carries a GFP gene so that infection efficiency could be determined by flow cytometry analysis. A standard viral neutralization assay was performed on HeLa cells pretreated with control siRNA, TRIM21 siRNA, IFN-α , or IFN-α and TRIM21 siRNA (Fig. 1*B*). To take account of toxicity and variable cell death between these different conditions, we measured the decrease in infection due to the addition of antibody. In the absence of antibody, adenovirus infected ≈50% of cells. The percentage of infected cells decreased rapidly with increasing antibody concentration, such that at 400 ng/mL antibody, infection was reduced by ≈60-fold (Fig. 1*B*). However, in cells depleted of TRIM21, addition of 400 ng/mL antibody had a minimal effect on infection (≈3-fold).

During an immune response, IFN-α activates the transcription of antiviral genes. TRIM21 is IFN-α regulated, and the modest levels of endogenous TRIM21 protein are substantially increased

Author contributions: D.L.M., W.A.M., S.R.B., and L.C.J. designed research; D.L.M., W.A.M., S.R.B., C.M.J., and L.C.J. performed research; G.J.T. contributed new reagents/analytic tools; D.L.M., W.A.M., S.R.B., and L.C.J. analyzed data; and D.L.M., W.A.M., S.R.B., and L.C.J. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

[1]D.L.M., W.A.M, and S.R.B. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: lcj@mrc-lmb.cam.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1014074107/-/DCSupplemental.

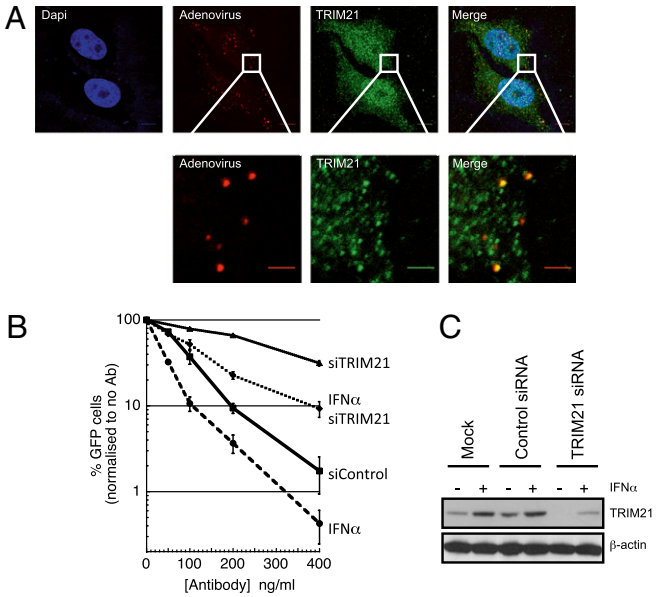

Fig. 1. TRIM21 mediates intracellular antibody neutralization. (*A*) Confocal microscopy images of adenovirus-infected HeLa cells. Adenovirus precoated in antibody and detected after infection with an Alexa Fluor 546 secondary (red) can be seen inside cells. The images are a Z-projection. (Scale bars, 10 μm; 2 μm in *Insets*.) (*B*) Cells treated with IFN-α, TRIM21 siRNA, siRNA control, or IFN-α and TRIM21 siRNA were infected with GFP adenovirus at different polyclonal antibody concentrations. Adenovirus infection is reduced by 2 logs in cells expressing the highest levels of TRIM21. (*C*) Western blot of TRIM21 protein levels in each condition.

by IFN-α (Fig. 1*C*). Consistent with this regulation, preincubation of cells with IFN-α markedly increased the effect of antibody neutralization. IFN-α has pleiotropic effects, but without addition of antibody we observed little impact on adenovirus infection. To test whether IFN-α/antibody neutralization synergy is TRIM21 dependent, we specifically depleted the TRIM21 levels that are up-regulated by IFN-α, leading to >20-fold recovery of infectivity (Fig. 1*B*). In all experiments, antibody neutralization of viral infection directly correlated with TRIM21 levels (Fig. 1*C*). For example, cells expressing the most TRIM21 were almost 2 orders of magnitude more resistant to adenovirus infection than those expressing the least.

We confirmed the robustness of this phenotype by examining the effect of different siRNA sequences, cell types, and types of antibody. Different TRIM21 siRNAs with different target sequences reversed antibody neutralization of adenovirus infection by depleting TRIM21 levels (Fig. S1*A*). TRIM21 neutralization occurred in a range of cell lines including HeLa, HT1080, and TE671 (Fig. S1*B*). A stable TRIM21 knockdown line was established in each case using an shRNA vector based on the sequence of siRNA 2. In all cells, TRIM21 mediated antibody neutralization of adenovirus (Fig. S1*B*). Finally, we tested the effect on adenovirus neutralization of two different anti-Ad5 polyclonal antibodies (Abd Serotec and Millipore) and an anti-Ad5 hexon monoclonal. In every case, neutralization of adenovirus was enhanced by TRIM21 up-regulation and reversed by TRIM21 depletion (Fig. S1*C*).

Unlike entry neutralization, antibody effector mechanisms are reliant on interaction with the Fc domain. To test whether TRIM21 neutralization was Fc dependent, we treated IgG with pepsin to remove the Fc and generate Fab2 fragments. Fab2 fragments are bivalent, bind antigen with the same affinity as IgG, and can cross-link viral epitopes. However, we found that Fab2 fragments were not able to mediate efficient TRIM21 neutralization (Fig. 2*A*). Unlike with IgG, when using Fab2, IFN-α treatment or TRIM21 depletion no longer dramatically affected adenovirus infection. Thus in order for antibodies to mediate intracellular viral neutralization they must contain an Fc domain and TRIM21 must be present. These results reveal a unique effector mechanism in humoral immunity that extends protection into cells.

During the early stages of infection, in which innate immunity is critical, IgM rather than IgG antibodies dominate the antibody repertoire. We tested whether TRIM21 interacts with IgM and if so the importance of TRIM21 in IgM viral neutralization. To investigate TRIM21:IgM binding, we labeled the TRIM21 PRYSPRY domain with an Alexa 488 fluorophore and measured its fluorescence anisotropy upon titration of IgM (Fig. 2*B*). The resulting titration curve was fit (*Materials and Methods*) to give an affinity (K_D) of 16.8 ± 1.5 μM. This is weaker than the affinity with which TRIM21 binds IgG Fc [0.2 μM under physiological conditions (4)]. However, the affinity of TRIM21 to IgM is likely to be significantly higher in vivo because full-length TRIM21 is a multimer. Complement C1q, which binds IgM with nanomolar affinity, has undetectable affinity when measured as a monomer (6).

Next we tested the effect of serum IgM on adenovirus infection. We found that pooled human serum IgM and TRIM21 operate synergistically to neutralize adenovirus infection (Fig. 2*C*). Furthermore, as with IgG, the neutralization of virus by IgM requires TRIM21. This suggests that TRIM21-mediated immunity could be effective in the early stages of an infection. Furthermore, it demonstrates that TRIM21 utilizes a broader usage of isotypes than classical Fc receptors, which are usually both isotype and subtype specific.

Mechanism of TRIM21 Immunity. The previous experiments demonstrate that there is an intracellular immune response mediated by TRIM21 and antibodies that is capable of preventing viral infection. Next we examined the mechanism by which this intracellular neutralization occurs. We investigated the mechanism in three ways. First, we determined how TRIM21 targets antibody and the thermodynamics of interaction. Second, we examined what events subsequent to targeting are required for neutralization. Third, we asked how virus is neutralized.

TRIM21 is a multidomain protein consisting of RING, B Box, coiled-coil, and PRYSPRY domains. We tested the role of these domains in IgG binding using multiangle light scattering (MALS) and fluorescence titration spectroscopy. Analysis of the MALS data reveals that recombinant full-length TRIM21 forms a stable dimer and not a trimer as previously reported (7) (Fig. 3*A*). There are conflicting data on the oligomeric state of other TRIM proteins, which have been shown to be both trimers (8) and dimers (9). Our results support the view that a dimer is the preferred state for TRIM proteins. When mixed with IgG, TRIM21 forms a stoichiometric complex consisting of one antibody and one TRIM21 (Fig. 3*A*). Deletion of the RING domain alone resulted in a destabilized recombinant protein; however, deletion of both RING and B Box did not affect TRIM21 stability or its ability to dimerize. Fluorescence titration spectroscopy revealed that full-length TRIM21 and ΔRING-Box bound to IgG with a similar dissociation constant (K_D) of <1 nM (Fig. 3 *B* and *C*). Because the monomeric PRYSPRY domain binds with ≈150 nM affinity (4, 5), this indicates that the coiled-coil domain is required for TRIM21 dimerization and the simultaneous engagement of both IgG heavy chains. The sub-nM affinity of TRIM21 for IgG makes TRIM21 the highest-affinity antibody receptor in the human body. The evolution of such a high-affinity interaction explains how TRIM21 efficiently targets virus.

Next we looked at what happens to virus after TRIM21 is recruited and the role of the RING and B Box domains. Because RING domains often display E3 ubiquitin ligase activity, we

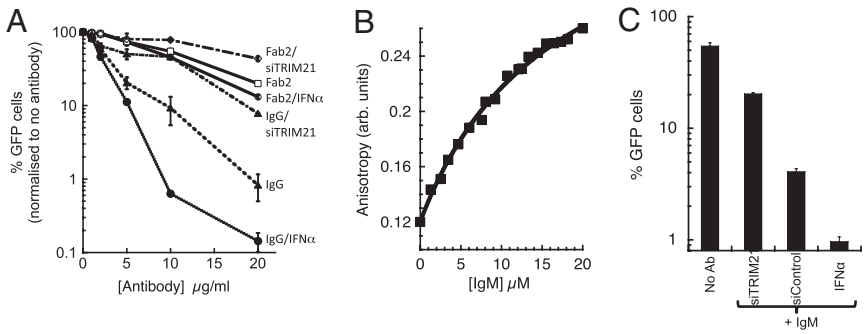

Fig. 2. TRIM21 mediates neutralization using serum IgG and IgM but not Fab2 fragments. (*A*) Antibody-dependent TRIM21 neutralization of virus requires the Fc domain. (*B*) TRIM21 binds IgM with an affinity of 16.8 ± 1.5 μM. (*C*) Serum IgM antibodies can be used by TRIM21 to neutralize virus. Depletion of TRIM21 reverses this effect, and IFN-α increases it. Error bars in all panels were calculated from triplicate experiments.

hypothesized that TRIM21 may target bound virus for degradation via ubiquitination. Cells possess two pathways for degradation of ubiquitinated material: the proteasome and autophagy. To explore the role of these pathways in TRIM21 neutralization of virus we performed viral infection experiments in the presence of MG132 (a proteasome inhibitor) and 3-methyladenine (3-MA; an autophagy inhibitor). The autophagy inhibitor had no effect on infectivity; however, MG132 significantly reversed TRIM21 neutralization of infectivity (Fig. 3*D*). Titration experiments showed a direct correlation between increasing levels of MG132 and reduced neutralization (Fig. 3*E*). The ability of MG132 to reverse neutralization was dependent upon the presence of antibody and TRIM21. Moreover, addition of MG132 could not recover infection in cells depleted of TRIM21, showing that TRIM21 and proteasome function are essential components in the same neutralization pathway (Fig. 3*F*).

To determine whether ubiquitination is essential to target virus to the proteasome, we tested the ability of full-length TRIM21 and ΔRING-Box recombinant proteins to neutralize infection. We incubated protein with antibody-coated virions and allowed the virus to infect cells depleted of TRIM21. As can be seen in Fig. 4*A*, deletion of the RING and B Box domains prevents TRIM21 neutralization of virus. We attempted to repeat these experiments in cells overexpressing TRIM21; however, this led to loss of function. Loss of function could be partially reversed with IFN, suggesting that overexpression titrates essential cofactors rather than generates inactive protein. To confirm that the neutralization we observe with recombinant proteins correlates with ubiquitin ligase activity, we compared the ability of full-length and ΔRING-Box proteins to autoubiquitinate. Although deletion of the RING and B Box domains has no effect on IgG binding, it abolishes autoubiquitination (Fig. 4*B*). Thus both TRIM21 ubiquitination activity and proteasomal function are required for

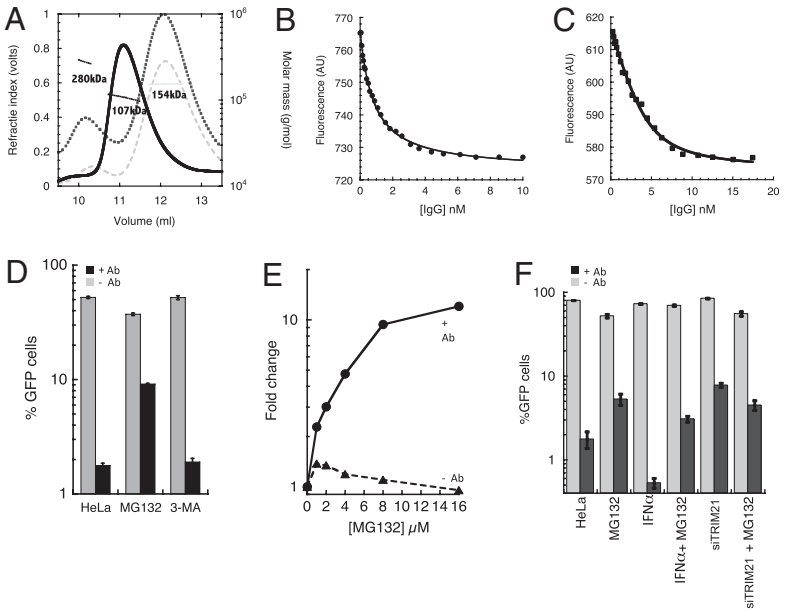

Fig. 3. Mechanism of TRIM21 neutralization. (*A*) SEC MALS chromatograms of TRIM21 (black), IgG (light gray), and TRIM21 in complex with IgG (dark gray). The continuous traces represent the refractive index signal (left-hand axis), and the short horizontal lines represent the calculated mass at each sampling interval (1 s) within each peak (right-hand axis). Analysis indicates that TRIM21 is dimeric with a mass of 107 kDa, that IgG has a mass of 154 kDa, and that TRIM21:IgG complex yields a peak corresponding to free IgG and a 1:1 complex with mass ≈280 kDa. (*B* and *C*) Steady-state fluorescence titration of IgG with full-length TRIM21 (*B*) and ΔRING-Box TRIM21 (*C*) reveals an affinity of 0.6 ± 0.1 nM and 0.9 ± 0.2 nM, respectively. (*D*) TRIM21 neutralization is reversed by the proteasome inhibitor MG132 but not the autophagy inhibitor 3-MA. Error bars were calculated from triplicate experiments. (*E*) Direct correlation between MG132 concentration and reversal of TRIM21 neutralization. MG132 only reverses neutralization in the presence of antibody. (*F*) Proteasomal degradation, TRIM21, and antibody are necessary factors in the same pathway of viral neutralization.

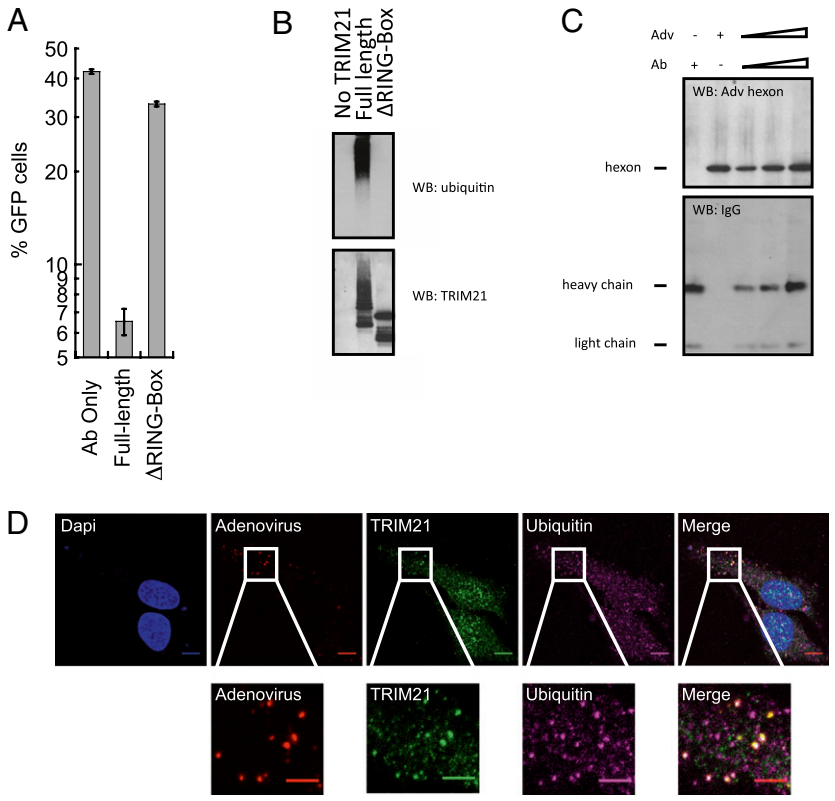

Fig. 4. TRIM21 E3 ubiquitin ligase function is essential for viral neutralization. (*A*) Recombinant full-length TRIM21 neutralizes virus, but TRIM21 lacking the RING and B Box domains does not. (*B*) TRIM21 is an active E3 ligase, but deletion of RING and B Box domains prevents autoubiquitination. (*C*) TRIM21 does not ubiquitinate viral capsid (hexon) or bound antibody. (*D*) Confocal microscopy Z-projection showing HeLa cells infected with antibody-coated adenovirus. TRIM21-colocalized virions are positive for ubiquitin.

viral neutralization. However, although the in vitro assay indicates that TRIM21 is an active ligase, it cannot be assumed that it is autoubiquitination that drives proteasomal targeting in vivo.

Although E3 ubiquitin ligases are known to autoubiquitinate, it is the transfer of ubiquitin to substrate that is thought to be important for proteasomal targeting. However, we hypothesized that proteasomal targeting via autoubiquitination would allow TRIM21 to neutralize any virus and prevent evolution of viral mutants that escape ubiquitin conjugation. Consistent with this mechanism, although we found that TRIM21 efficiently forms ubiquitin chains on itself, we found no detectable ubiquitination of either IgG or virus in our in vitro ubiquitination assay (Fig. 4*C*). This suggests that recruitment to the proteasome is not dependent upon direct ubiquitination of either the antibody or virus but rather autoubiquitination of TRIM21 or a recruited cellular cofactor. Proteasomal recruitment through TRIM21 autoubiquitination is consistent with the extremely high affinity with which TRIM21 has evolved to bind antibody. If TRIM21 were transferring ubiquitin to antibody or virus through normal enzymatic turnover, then a high affinity would translate as a highly inefficient K_M. However, we cannot rule out that a capsid protein other than hexon is ubiquitinated or that although undetected in vitro, ubiquitination of hexon or antibody occurs in vivo. Finally, to demonstrate that the intracellular TRIM21-associated viral particle is ubiquitinated, we examined infected cells by confocal microscopy and stained for ubiquitin. As can be seen in Fig. 4*D*, virions colocalized with TRIM21 are also positive for ubiquitin.

To determine what happens to virus after TRIM21-mediated targeting to the proteasome, we performed a fate-of-capsid time-course experiment. In this experiment we infected cells with virus preincubated with antibody and compared the levels of hexon protein (viral capsid) in infected HeLa cells with those in cells depleted of TRIM21. By 2 h after infection there was markedly less hexon in HeLa compared with TRIM21-depleted cells (Fig. 5). This indicates both that TRIM21 mediates degradation of virus and that it is a rapid process. Addition of MG132 slowed the decline in hexon levels, confirming that virus is being physically degraded in a proteasome-dependent manner. Because proteasomal targeting by TRIM21 requires virus to be antibody-bound, we also looked at the antibody levels in infected cells. We found that destruction of virus is paralleled by disposal of antibody (Fig. 5). In contrast to hexon and antibody, we saw little change in TRIM21 levels after 2 h. Treatment with MG132 did not increase TRIM21 levels, suggesting either that TRIM21 is not degraded by the proteasome or that ubiquitination and degradation are coupled to IgG binding. In the latter case, only a fraction of the total pool of cellular TRIM21 would be degraded. We also cannot rule out the possibility of a feedback loop that maintains TRIM21 levels.

The combination of antibody targeting and TRIM21 autoubiquitination implies that no direct viral interactions are required for neutralization. To test this, we transfected cells with streptavidin latex beads coated in antistreptavidin antibody. We found that TRIM21 is efficiently recruited to the antibody-bound beads (Fig. 6). Furthermore, TRIM21-associated beads are positive for ubiquitin. This result suggests that direct interaction with an invading viral particle may not be required to recruit TRIM21 and that virus may not be the target of ubiquitination. We believe that TRIM21-mediated neutralization may be effective against other nonenveloped viruses.

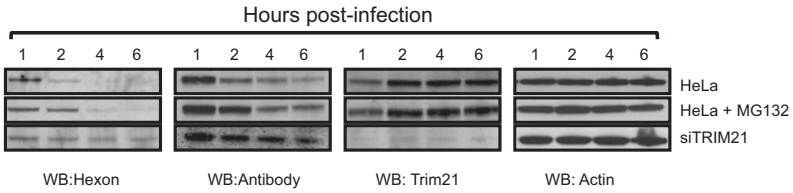

Fig. 5. TRIM21 mediates rapid degradation of virus. Western blots of hexon, antibody, and TRIM21 protein levels 1–6 h after infection. Adenovirus hexon protein and antibody are rapidly degraded in a TRIM21-dependent manner. Addition of MG132 partially rescues degradation.

Discussion

We have discovered that there is a system of intracellular immunity through which antibodies mediate the neutralization of virus inside the cytosol of infected cells. This intracellular system combines features traditionally associated exclusively with either adaptive or innate immunity. Pathogen targeting is provided by adaptive immunity in the form of antibodies, whereas neutralization is provided by an intracellular receptor (TRIM21) and an innate degradation pathway. This combination of features may make it difficult for viruses to evolve and escape.

Most importantly, our results show that humoral immunity is not limited to extracellular protection but can neutralize a virus even after it has entered a cell. Indeed, the antibody effector function provided by TRIM21 offers a different kind of protection from known extracellular mechanisms. Extracellular effector functions are mainly provided by professional cells and are based on immune surveillance. In contrast, TRIM21 is expressed in most tissues and not just professional cells. Crucially, encapsulating immunity within host cells means that every infection event is an opportunity for neutralization. This may provide more effective protection against viral spread than surveillance. Moreover, TRIM21 provides a noncytotoxic mechanism of immunity, in contrast to T cell killing or antibody-dependent cellular cytotoxicity that target an infected cell rather than a virus. Because TRIM21 neutralization occurs rapidly, before transcription of viral genes, this offers the possibility of "curing" rather than killing an infected cell. Finally, because TRIM21 utilizes both IgM and IgG, it is capable of operating alongside both innate immunity during the early stages of infection and adaptive immunity to provide long-term protection.

It is conceivable that in addition to making use of adaptive and innate immunity to neutralize viruses, TRIM21 also helps to activate these pathways. Because TRIM21 detects virus early in cellular infection it is well placed to stimulate immune signaling, for example by stimulating NF-κB. Similarly, because TRIM21 uses the proteasome to dispose of virus, this may provide a way for signaling directly to adaptive immunity through MHC presentation.

It is possible that TRIM21 has been contributing to many antibody neutralization experiments over the last 100 y. Indeed, because we see that TRIM21 mediates potent antibody neutralization of adenovirus, it will be important to reassess whether the antibody neutralization of other viruses is caused by a block to entry or is TRIM21 dependent. This may be an important consideration in vaccine design, because effective vaccines may need to stimulate TRIM21 immunity. We suggest that a good predictor of TRIM21 involvement in the antibody neutralization of other viruses is a synergistic relationship between IFN and antibody. Unexplained synergy between IFN and antibody has been reported for enterovirus 70 (10) and coxsackie virus (10). TRIM21 may also contribute to viral neutralization in experiments in which no antibody is added because the calf serum used in routine tissue culture contains a repertoire of antibodies of potentially cross-reactive specificity.

The existence of a TRIM21/antibody intracellular immune response may help to resolve crucial unexplained observations in viral infection. For instance, early antibody neutralization experiments on the newly identified adenovirus revealed that there is a linear-log relationship between antibody dilution and neutralization and that prolonged antibody incubation does not increase neutralization (11). It has since been shown that antibody neutralization of poliovirus occurs even when viruses are allowed to preadhere to target cells (12). It has also been observed that a single IgG is sufficient to mediate neutralization of poliovirus (13, 14) and adenovirus (15) and that only five to six IgG molecules are required for rhinovirus (16). Finally, intact antibodies have long been known to be far more effective than their proteolysed fragments. In 1943 Kalmanson and Bronfenbrenner showed that digestion with papain reactivated poliovirus infectivity but not through dissociation of binding (17). Intriguingly, several groups later showed that virus could be reneutralized by addition of anti-Ig (18, 19). These findings could be explained through a cross-linking or conformational model of neutralization, but they are also consistent with an Fc effector mechanism.

We have demonstrated TRIM21-mediated restriction of adenovirus; however, we believe that TRIM21 should be effective against other nonenveloped viruses. The streptavidin bead experiments suggest that any intracellular antibody-coated particle is capable of being targeted by TRIM21.

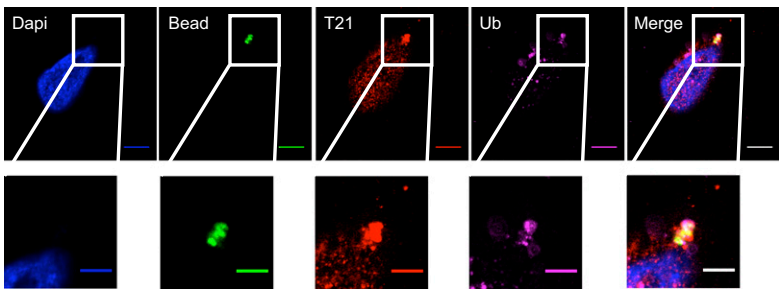

Fig. 6. Intracellular antibody-coated beads recruit TRIM21 and are ubiquitinated. Streptavidin-conjugated latex beads coated with antistreptavidin antibody and transfected into cells recruit TRIM21 and colocalize with ubiquitin. (Scale bars, 10 μm; 5 μm in *Insets*.)

Materials and Methods

For full details, see *SI Materials and Methods*.

Cells Lines and Viral Production. HEK293T, HeLa, TE671, and HT1080 were maintained in standard conditions. 293F cells (Invitrogen) were grown in serum-free Freestyle medium (Invitrogen). Adenovirus Ad5-GFP (20) was grown in transcomplementation cell line 293F for 72 h and purified to 10^8 to 10^9 IU/mL. Transient siRNA knockdowns were performed in six-well plates with 150 pmol of oligo per 1×10^5 cells.

Virus Neutralization Assays. HeLa cells in six-well plates were infected with 5×10^4 infectious units (IU) AdV5-GFP for 30 min and then incubated for 48 h before fixing. Goat antiadenovirus polyclonal antibody was used in all virus neutralization assay experiments unless otherwise stated (AB1056; Millipore).

Immunofluorescence. HeLa cells were seeded onto coverslips in 24-well plates and infected essentially as described above, then fixed and stained with appropriate primary and secondary antibodies. Confocal images were taken using a Zeiss 63× lens on a Jena LSM 710 microscope (Carl Zeiss MicroImaging).

Fluorescence Titration. Full-length and ΔRING-Box recombinant TRIM21 was expressed as maltose binding protein-fusion proteins in *Escherichia coli* and purified using amylose resin and size-exclusion chromatography. Fluorescence experiments were performed by titrating IgG using a Cary Eclipse fluorescence spectrophotometer.

Fluorescence Anisotropy. The PRYSPRY domain of TRIM21 was expressed and purified as previously described (4, 5). The protein was labeled with Alexa Fluor 488 5-SDP ester (Invitrogen) and its polarized fluorescence measured at 530 nm using a Cary Eclipse fluorescence spectrophotometer. IgM (Athens Research and Technology) was titrated into 50 nM PRYSPRY and the change in anisotropy averaged over 5 s.

SEC MALS. Size-exclusion chromatography (SEC) MALS was performed using a Wyatt Heleos II 18-angle light-scattering instrument coupled to a Wyatt Optilab rEX online refractive index detector. Samples were prepared as described above and passed through the light-scattering and refractive index detectors in a standard SEC MALS format.

In Vitro Ubiquitination Assays. In vitro assays were carried out largely as previously described (21). Reaction mixtures were incubated at 37 °C for 1 h then visualized by Western blot for TRIM21 (1:500, sc-25351; Santa Cruz Biotechnology), Ad5 hexon (donkey anti-goat IgG HRP 1:5,000 sc-2056; Santa Cruz Biotechnology), or ubiquitin (1:1,000, FK-2; Enzo Life Sciences) as indicated.

ACKNOWLEDGMENTS. An initial stock of adenovirus Ad5-GFP was kindly provided by Dr. Olivier Danos (Cancer Biology, University College London Cancer Institute, London, United Kingdom).

1. Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* 124:783–801.
2. Brenner S, Milstein C (1966) Origin of antibody variation. *Nature* 211:242–243.
3. Murphy KTP, Walport M (2008) *Immunobiology* (Garland Science, New York), 7th Ed, p 887.
4. James LC, Keeble AH, Khan Z, Rhodes DA, Trowsdale J (2007) Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function. *Proc Natl Acad Sci USA* 104:6200–6205.
5. Keeble AH, Khan Z, Forster A, James LC (2008) TRIM21 is an IgG receptor that is structurally, thermodynamically, and kinetically conserved. *Proc Natl Acad Sci USA* 105:6045–6050.
6. Chen FH, et al. (1997) Domain-switched mouse IgM/IgG2b hybrids indicate individual roles for C mu 2, C mu 3, and C mu 4 domains in the regulation of the interaction of IgM with complement C1q. *J Immunol* 159:3354–3363.
7. Rhodes DA, Trowsdale J (2007) TRIM21 is a trimeric protein that binds IgG Fc via the B30.2 domain. *Mol Immunol* 44:2406–2414.
8. Mische CC, et al. (2005) Retroviral restriction factor TRIM5alpha is a trimer. *J Virol* 79: 14446–14450.
9. Langelier CR, et al. (2008) Biochemical characterization of a recombinant TRIM5alpha protein that restricts human immunodeficiency virus type 1 replication. *J Virol* 82: 11682–11694.
10. Langford MP, Villarreal AL, Stanton GJ (1983) Antibody and interferon act synergistically to inhibit enterovirus, adenovirus, and herpes simplex virus infection. *Infect Immun* 41:214–218.
11. Ginsberg HS (1956) Characteristics of the new respiratory viruses (adenoviruses). I. Qualitative and quantitative aspects of the neutralization reaction. *J Immunol* 77:271–278.
12. Vrijsen R, Mosser A, Boeyé A (1993) Postabsorption neutralization of poliovirus. *J Virol* 67:3126–3133.
13. Wetz K, Willingmann P, Zeichhardt H, Habermehl KO (1986) Neutralization of poliovirus by polyclonal antibodies requires binding of a single IgG molecule per virion. *Arch Virol* 91:207–220.
14. Icenogle J, et al. (1983) Neutralization of poliovirus by a monoclonal antibody: Kinetics and stoichiometry. *Virology* 127:412–425.
15. Wohlfart C (1988) Neutralization of adenoviruses: Kinetics, stoichiometry, and mechanisms. *J Virol* 62:2321–2328.
16. Smith TJ, et al. (1993) Structure of human rhinovirus complexed with Fab fragments from a neutralizing antibody. *J Virol* 67:1148–1158.
17. Kalmanson GMB, J. (1943) Restoration of activity of neutralized biologic agents by removal of the antibody with papain. *J Immunol* 47:387–407.
18. Keller R (1968) Studies on the mechanism of the enzymatic reactivation of antibody-neutralized poliovirus. *J Immunol* 100:1071–1099.
19. Emini EA, Ostapchuk P, Wimmer E (1983) Bivalent attachment of antibody onto poliovirus leads to conformational alteration and neutralization. *J Virol* 48:547–550.
20. de Martin R, Raidl M, Hofer E, Binder BR (1997) Adenovirus-mediated expression of green fluorescent protein. *Gene Ther* 4:493–495.
21. Mallery DL, Vandenberg CJ, Hiom K (2002) Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. *EMBO J* 21:6755–6762.