minimal role, if any, in the observed inhibitory effect on HIV replication by Lenti shNef366.

Lentivirus-based nef shRNA inhibits HIV-1 replication and affects chemokine production in MDMs

Swingler and coworkers reported that HIV-1 Nef expression in macrophages mediated lymphocyte chemotaxis and activation through the induction of MIP-1α and MIP-1β expresion.[8] To determine the effect of Nef expression during HIV-1 infection in MDMs, we infected MDMs with wild-type HIV-1_{NF462} or the corresponding *nef* gene-deletion mutant, HIV-1_{NF462}dNef, and assessed the kinetics of virus replication by p24-specific ELISA. Representative results from 2 donors are shown in Figure 4A. We consistently observed that the level of HIV-1_{NF462} replication was 2- to 6-fold higher than that of HIV-1_{NF462}dNef in MDMs. These results were consistent with those reported by Swingler et al.[9] Although no apparent T-cell damage was observed during cultivation for 3 weeks following HIV-1 infection, the amount of virus production gradually decreased. We analyzed chemokine production in MDMs infected with HIV-1 wild-type and *nef*-deleted HIV-1 at days 10, 14, and 17 after infection. The level of chemokine production in uninfected MDMs varied depending on the donor, but both donors produced a high level of IL-8 and monocyte chemotactic protein-1 (MCP-1), and a low level of MIP-1α and MIP-1β (data not shown). HIV infection per se, independent of the presence or absence of Nef, did not affect this trend, in that the levels of these chemokines, with the exception of MIP-1β, were only slightly affected by HIV infection. Notably, virus replication resulted in an increased production of MIP-1β, which peaked at 14 days after infection, in parallel with the peak of viral replication. Figure 4B shows the results of the analysis of the levels of MIP-1β and MIP-1α in the 2 donors. HIV-1 infection induced a 2-fold increase in the level of MIP-1β compared with mock-infected MDMs. In contrast, infection with Nef-deleted HIV-1 caused a reduction in the level of MIP-1β in the MDMs from both donors, indicating that Nef is responsible for the up-regulation of MIP-1β, but does not affect MIP-1α, MCP-1, or IL-8 production.

To examine whether shRNAs against the U3-overlapping region of *nef* were able to block HIV-1 replication in MDMs, we infected MDMs with Lenti control or Lenti shNef366, at an MOI of 10 or 2 (Figure 5A left and right panels, respectively). After 2 hours of incubation, cells were extensively washed and cultivated overnight, and the following day, they were infected with HIV-1_{NF462}. Culture supernatants were collected every 3 or 4 days and the level of p24 antigen was measured by ELISA. Of note, despite the extensive washing after lentivirus infection, the level of p24 was quite high up to 7 days after HIV-1 infection. We detected a second peak of virus production, which we interpreted as true HIV-1 replication in MDMs transduced with lentiviral vectors expressing shRNAs. In addition, presumably because of the toxic effect of infection by lentivirus pseudotyped with VSV, the level of p24 antigen was lower than that in MDMs infected with HIV-1 virus. Nevertheless, we observed a similar level of inhibition of HIV-1 replication in MDMs by Lenti shNef366 at 2 different doses of infection (Figure 5A), and the inhibition was maintained for at least 3 weeks after HIV-1 infection.

Macrophages can mediate efficient infection of lymphocytes *in trans*,[9,24] suggesting that macrophages serve as a major reservoir and vehicle for HIV-1 dissemination. We were interested in whether the progeny virus produced from MDMs harboring Nef366 shRNA maintained their ability to infect T cells. Supernatants from MDM cells transduced with Lenti control or Lenti shNef366 were collected 10 days after HIV infection, and the level of p24 antigen was measured and used to quantitate the amount of HIV present. These sources of HIV were designated as HIV/Lenti cont or HIV/Lenti shNef366. Using CEMx174 CCR5/LTR-EGFP cells as indicator cells, we estimated the infectivity of HIV/Lenti cont or HIV/Lenti shNef366 by analyzing the number of $EGFP^+$ T cells following infection (Figure 5B). Compared with HIV-1/Lenti cont, HIV-1/Lenti shNef366 had a significant loss of infectivity in $CCR5^+$ T cells. Our results suggested that Lenti shNef366 has the potential to protect HIV-1 dissemination to T cells by HIV-1–infected MDMs

We also examined the level of chemokine production following HIV infection of MDMs transduced with shRNA lentivirus vectors. Although the basal level of MIP-1α and MIP-1β production was slightly increased following lentivirus infection, the level of MIP-1β decreased in Lenti shNef366 cells compared with Lenti control (Figure 5C). The levels of MCP-1 and IL-8 were either unaffected or somewhat restored by Lenti shNef366 (data not shown).

Lentivirus-based nef shRNA protects progression from latent HIV-1 infection to productive infection

Latent HIV-1 infection can be established following provirus integration into the host genome.[25-27] A small number of infected cells re-enter the resting stage, harboring an integrated copy of the HIV-1 genome. These latent HIV-infected cells represent a barrier to successful virus eradication because subsequent cytokine or

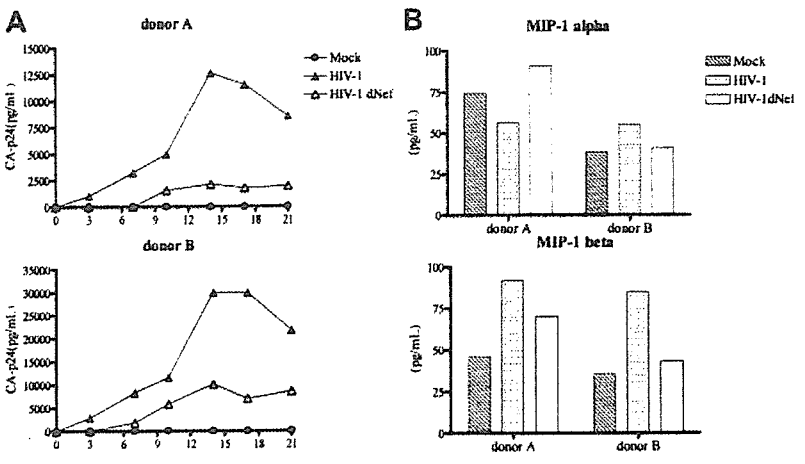

Figure 4. The effect of Nef expression during HIV-1 infection in MDMs. (A) MDMs (2×10^5/well) of 2 donors were infected either with wild-type HIV-1_{NF462} or HIV-$1_{NF462dNef}$. The supernatants of these wells were harvested at 3- or 4-day intervals after infection, and viral production was monitored by sequential quantitation of p24 by ELISA. (B) The CBA kit was used to measure the level of chemokines (MIP-1α and MIP-1β) in cell supernatants 14 days after HIV infection.

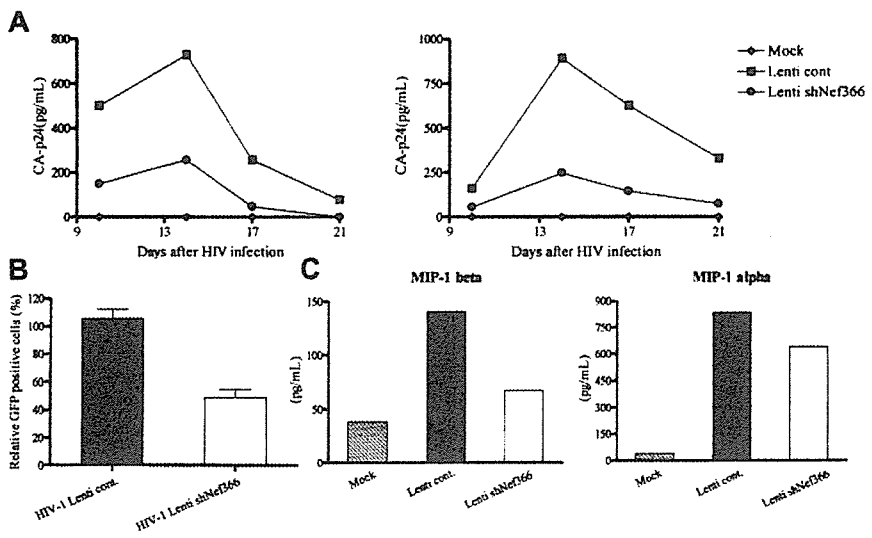

Figure 5. Lentivirus-expressed nef shRNA inhibits HIV-1 replication and affects chemokine production in MDMs. (A) MDMs were transduced with Lenti cont or Lenti shNef366 at an MOI of 2. At 2 hours after infection, cells were washed twice, then cultured for another 24 hours, at which point the cells were infected with HIV-1_{NF462}. The culture supernatants were collected at 3- or 4-day intervals after HIV infection, and the level of p24 antigen was measured by ELISA. (B) MDMs transduced either with Lenti control or Lenti shNef366 were infected with HIV-1 and supernatants were collected 10 days after infection and designated as HIV-1 Lenti cont and HIV-1 Lenti shNef366, respectively. CEMx174 CCR5/LTR-EGFP cells were infected either with HIV-1 Lenti cont or HIV-1 Lenti shNef366 and GFP$^+$, HIV-1–infected T cells were analyzed by FACS 48 hours later. The data represent the average ± SD of 3 independent experiments. (C) The culture supernatants of MDMs transduced with lentivirus vectors were collected 14 days after infection and the levels of the chemokines MIP-1α and MIP-1β were measured.

other stimuli can reactivate viral gene expression, and reinitiate HIV-1 replication.[28-31] We were interested in whether Lenti shNef366 was able to regulate the progression of latent HIV-1 infection to productive infection in U1 cells.[17] U1 cells are U937 cells in which a latent HIV-infection has been established, and HIV-1 replication can be induced in these cells on appropriate activation. We transduced U1 cells with Lenti control or Lenti shNef366 at an MOI of 1. After 2 hours of infection, cells were extensively washed and maintained in culture. Two weeks after transduction, the cells were sorted by FACSaria, and the EGFP$^+$ cell population was stimulated with 1 ng/mL recombinant GM-CSF. Culture supernatants were collected at different time points (days 2 and 5) and the level of p24 antigen was measured by ELISA. As shown in Figure 6, the levels of p24 antigen were dramatically decreased in U1 cells harboring Lenti shNef366 at all time points examined.

Discussion

In this study, we constructed an shRNA expression system that targeted HIV *nef* gene sequences that overlap the 3′ LTR U3 (Nef366) and showed that Nef366 shRNA had a strong inhibitory effect on *nef* gene expression in Nef-expressing HeLa-CD4 cells. Furthermore, expression of shNef366 in monocytic cell lines strongly inhibited the replication of HIV-1 at an early stage of HIV infection. The rational for using shNef366 to target HIV *nef* was several-fold. Because the U3 region is required during reverse transcription for first template transfer and integration of the viral genome into the host genome, siRNA targeting of the U3 region may induce not only specific degradation of *nef* mRNA, but also inhibit HIV-1 reverse transcription. Furthermore, although others have observed escape mutations in RNAi experiments targeting *nef* or *tat*,[32,33] the nef/U3 sequence we targeted is highly conserved as discussed in the paragraph after the next one. If a mutation were to occur in the U3 region, it would affect the overall transcription efficiency of HIV-1 after integration because the U3 region of the HIV-1 LTR contains the transcription initiation or promoter/enhancer sites that are essential for efficient HIV transcription. Of note, the strategy used Jacque et al[20] using siRNA targeting of the 5′ region of *nef* turned out to induce an escape mutant.[33] Although we did not extensively test for the emergence of escape mutants, targeting the 3′ LTR U3-overlapping region of *nef* (Nef366) represented a potentially potent strategy for controlling HIV-1 replication.

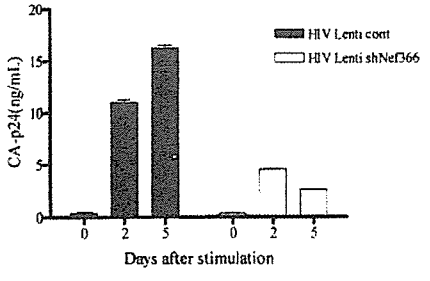

Figure 6. The effect of Lenti shNef366 on latent HIV-1 infection. Latent HIV-1–infected U1 cells were transduced with Lenti control or Lenti shNef366 at an MOI of 1. Two weeks after infection, EGFP$^+$ cells were sorted by FACSaria, and EGFP$^+$ cells were stimulated with 1 ng/mL recombinant GM-CSF. Cell-culture supernatants were collected 0, 2, and 5 days after stimulation, and the level of p24 antigen was measured by ELISA. The data represent the averages ± SDs of 3 independent experiments.

Macrophages are one of the major target cell populations in the early phase of HIV-1 infection, when R5 viruses predominate.[34] HIV-1 replication in macrophages is usually slow and less cytopathic compared with that in activated T cells, allowing the virus to survive long after infection. Thus, macrophages serve as one of the reservoirs for HIV in an infected individual.[35] Therefore, therapeutic strategies that target macrophages are promising approaches to the control of persistent HIV-1 infection in vivo. Taking advantage of the lentivirus expression system, which is an efficient way to introduce a desired gene into primary cells, we were able to show that expression of Nef366 shRNAs in primary MDMs inhibited HIV-1 replication in these cells.

In this context, several groups have demonstrated that RNAi, mediated by the introduction of HIV-specific siRNA duplexes, can inhibit viral replication in T cells, although the effect was transient.[20,36-38] Das et al were able to show a stable inhibitory effect on viral replication using a murine retrovirus vector expressing Nef-specific siRNAs in T-cell lines. However, the block in virus replication was not absolute and escape mutants emerged.[33] These previous results prompted us to develop a novel strategy of RNAi-mediated inhibition of HIV infection that did not induce a type 1 interferon and had a stable, long-term effect. We chose to

transduce Nef366 shRNA into low or nondividing primary macrophages, as opposed to actively proliferating T cells, using a lentivirus expression vector, and were able to demonstrate RNAi effect during macrophage cultivation for 3 weeks. Using an alignment of 200 HIV-1 sequences obtained by BLAST search analysis, only one base mismatch in the Nef366 region was detected in a subtype A virus (GenBank no. AB098332 and no. AB098333, HIV-1 UG029). Further study will be required to determine whether this subtype A virus is resistant to shRNA Nef366. Because Nef/LTR is in a completely conserved region, at least among subtype B viruses, this region might have quite an important function for HIV-1 replication. We speculate that if escape mutants were to emerge in the presence of lentiviral-shRNA Nef366, the compensatory mutation would occur outside of this region.

Importantly, using this system, we were also able to demonstrate a decrease in the infectivity of HIV-1 produced from infected MDMs. This attenuation effect is potentially significant because it implies that lentivirus-mediated RNAi may also reduce transmissibility of HIV-1 overall. However, in light of the significant problem of viral escape during chronic HIV infection, it may become necessary to combine multiple sites of siRNAs targeting the *nef-U3* region in the future.

Control of the latent phase of HIV infection is a key issue for effective therapeutic intervention. We demonstrated here that Lenti shNef366 was able to suppress the reactivation of HIV from latently infected cells. The expression of integrated HIV-1 in latently infected cells is controlled at the level of transcription by cellular factors and the viral transactivator Tat, both of which act through the HIV-1 LTR.[39] Transcription of integrated viral RNA is initiated at the R region of the 5′ LTR. The fact that shNef366, which targeted the U3-overlapping region of Nef, was effective in latently infected cells, suggests that shNef366 can directly target cleavage of *nef* mRNAs or total viral RNAs at the 3′ end. Therefore, our lentivirus-based shRNA expression system appears to be able to control both early and latent HIV-1 infection.

MIP-1α and MIP-1β are ligands of the HIV-1 coreceptor, CCR5. Through interaction with the CCR5 receptor, they promote the maturation of Th1 cells.[40,41] Swingler et al reported that MIP-1α and MIP-1β were induced by Nef in macrophages during HIV infection and that culture supernatants derived from Nef-expressing macrophages induced both chemotaxis and activation of resting T lymphocytes, enabling productive HIV-1 infection of those T cells.[8] These and other results have led to a model of HIV infection in which expression of Nef in HIV-infected MDMs enhances the secretion of MIP-1β, which recruits mainly $CCR5^+$ Th1 cells, resulting in the expansion of R5 tropic HIV-1 during macrophage–T-cell interactions. Our results were partially consistent with this model because the degradation of *nef* mRNA expression resulted in the decreased MIP-1β production. Of note, the production of MIP-1α in our system appeared to be unaffected by Nef expression but was induced by lentivirus infection. Because the production of MIP-1α in HIV-infected MDMs was similar to that in uninfected MDMs, it seems likely that MIP-1α production was enhanced by a non–HIV-specific component of the lentivirus expression system, perhaps VSV-G protein. Although the levels of MCP-1 and IL-8 varied depending on the donor and were independent of Nef expression, we cannot rule out the possibility that other unknown chemokines are induced by Nef. Any such dysregulated chemokine production by Nef expression in macrophages might provide an appropriate environment for HIV to establish an efficient infection and dissemination.

In summary, we demonstrated the feasibility of using lentiviral expression vectors to express shRNAs against the U3-overlapping region of *nef* in primary MDMs, as a type of intracellular immunization and potential gene therapy approach against HIV-1. Future development of an AIDS vaccine based on the specific inhibition of viral gene expression combined with existing therapeutic strategies may provide keys to help eradicate HIV.

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Negative regulation of interferon-regulatory factor 3–dependent innate antiviral response by the prolyl isomerase Pin1

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Recognition of double-stranded RNA activates interferon-regulatory factor 3 (IRF3)–dependent expression of antiviral factors. Although the molecular mechanisms underlying the activation of IRF3 have been studied, the mechanisms by which IRF3 activity is reduced have not. Here we report that activation of IRF3 is negatively regulated by the peptidyl-prolyl isomerase Pin1. After stimulation by double-stranded RNA, induced phosphorylation of the Ser339–Pro340 motif of IRF3 led to its interaction with Pin1 and finally polyubiquitination and then proteasome-dependent degradation of IRF3. Suppression of Pin1 by RNA interference or genetic deletion resulted in enhanced IRF-3-dependent production of interferon-β, with consequent reduction of virus replication. These results elucidate a previously unknown mechanism for controlling innate antiviral responses by negatively regulating IRF3 activity via Pin1.

The innate immune response is an important, evolutionarily conserved mechanism that protects the host from both viral and microbial infections[1–3]. Increasing evidence has shown the importance of pattern-recognition receptors in immune responses after viral and microbial infection by invading pathogens[3]. Toll-like receptor 3 (TLR3) detects extracellular viral double-stranded RNA (dsRNA) internalized into the endosomes, whereas retinoic acid–inducible gene I (RIG-I), a DExD/H box RNA helicase containing a caspase-recruitment domain, detects intracellular viral dsRNA[3–5]. TLR4, in contrast, recognizes microbial components such as bacterial lipopolysaccharide (LPS)[6]. Engagement of any of those receptors triggers rapid production of type I interferon (IFN-αβ) and thus establishes the innate immune status against infectious agents[3,5,7].

Interferon-regulatory factor 3 (IRF3), a ubiquitously expressed transcription factor, is responsible for the primary induction of IFN-β and is important in the establishment of innate immunity in response to either viral or microbial infection[1–3]. After the detection of pathogens, IRF3 is phosphorylated on multiple phosphorylation acceptor (phospho-acceptor) sites, forms homodimers and then translocates to the nucleus, where it binds to the interferon stimulation–response elements of target genes, as well as the positive regulatory domain III-I in the IFN-β promoter[1–3]. The mechanisms underlying the phosphorylation-induced activation of IRF3 have been the subject of many extensive studies[8–16]. The substitution of alanine for either the Ser385 or Ser386 residue of IRF3 abolishes its activation[8,9]. Additionally, phosphorylation of Ser386 on IRF3 is induced by TLR3 engagement and by viral infection and only for IRF3 dimers[9]. The importance of five critical serine or threonine residues of IRF3 (Ser396, Ser398, Ser402, Thr404 and Ser405) for its activation has been demonstrated[10,11]. Notably, the substitution of alanine for all five amino acids abrogates the function of IRF3 to activate transcription, whereas aspartic acid substitutions result in a constitutively active protein. Those published data demonstrate that phosphorylation of both C-terminal phospho-accepter clusters (Ser385-Ser386 and Ser396-Ser398-Ser402-Thr404-Ser405) is important for the activation of IRF3. Other studies have also shown that two IκB kinase (IKK)–like kinases, TBK1-NAK and IKK-*i*–IKKε, are required for the activation of IRF3 by inducing the phosphorylation of its two C-terminal phospho-acceptor clusters and thus are essential in the expression of type I interferon[14–17]. Phosphorylation-dependent post-translational modifications of IRF3 are therefore crucial for regulating the function of IRF3.

Pin1 is a peptidyl-prolyl isomerase that via its WW domain (with two conserved tryptophan residues) specifically recognizes phosphorylated serine or threonine residues followed by proline and then catalyzes a conformational change of the bound substrate in a

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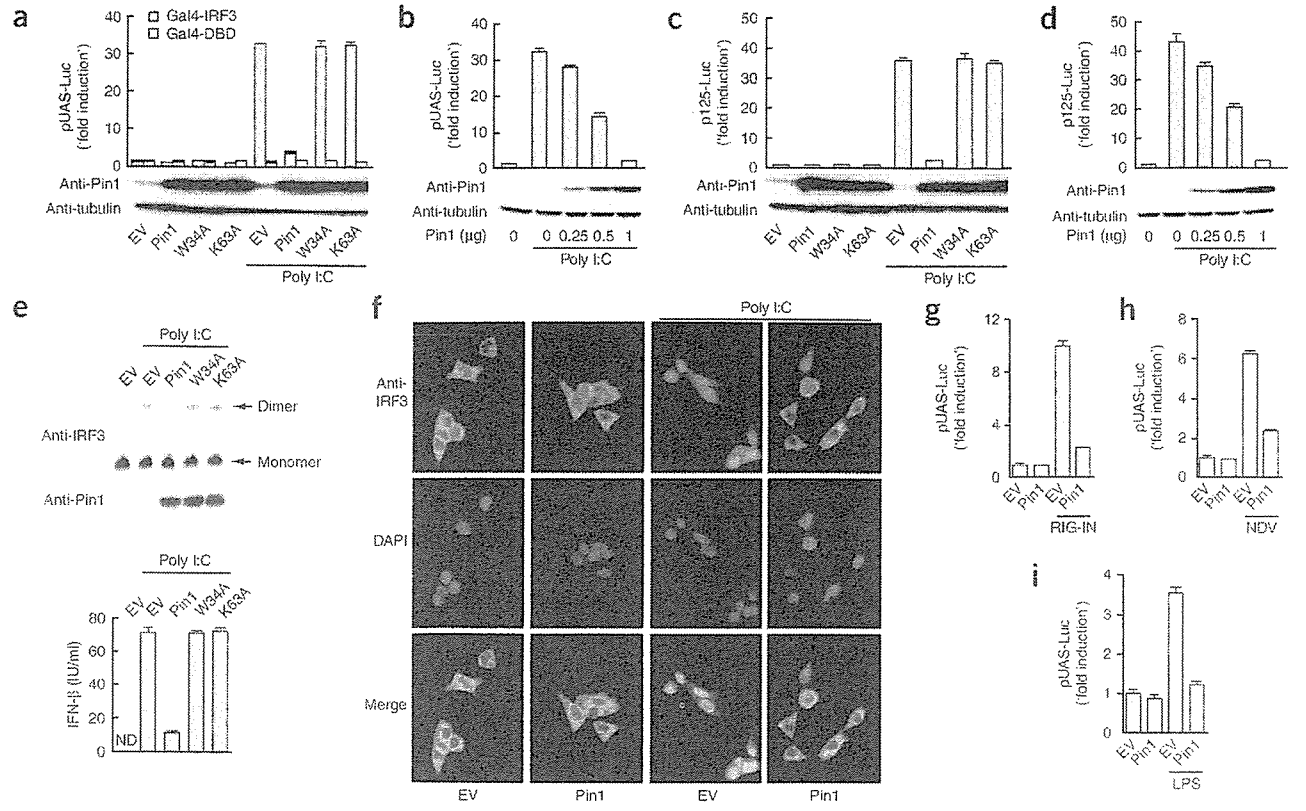

Figure 1 Pin1 suppresses IRF3-dependent transcriptional activation. (**a–d**) Luciferase assay (above) and immunoblot (below) of lysates from 293-TLR3 cells transfected with pEF1-lacZ and either the Gal4-site luciferase reporter pUAS-Luc (**a**,**b**) or the IFN-β promoter reporter p125-Luc (**c**,**d**), transiently expressing empty vector (EV), wild-type Pin1 or Pin1 mutants and either Gal4-IRF3 or Gal4-DBD (control; **a**,**c**) or transiently expressing various amounts of wild-type Pin1 (below lanes; **b**,**d**), and then stimulated with poly(I)•poly(C) (poly I:C). Luciferase activity is normalized to β-galactosidase activity; results are means ± s.d. from three separate transfections. (**e**) Immunoblots of endogenous IRF3 dimer detected by native PAGE (top) and Pin1 expression detected by SDS-PAGE (middle) in 293-TLR3 cells transiently expressing empty vector, wild-type Pin1 or Pin1 mutants and left unstimulated (far left) or stimulated for 3 h with poly(I)•poly(C). Bottom, ELISA of IFN-β secreted into the culture supernatant. ND, not detected. (**f**) Immunohistochemistry to detect endogenous IRF3 (green) in 293-TLR3neo^r cells stably expressing Pin1 with or without poly(I)•poly(C) stimulation. Cell nuclei are blue (DAPI, 4,6-diamidino-2-phenylindole). Original magnification, ×40. (**g**) Luciferase assay of lysates prepared from 293 cells at 36 h after transfection with pEF1-lacZ and pUAS-Luc and expressing Pin1 and Gal4-IRF3 with or without the RIG-I N-terminal caspase-recruitment domain (RIG-IN). (**h**,**i**) Luciferase assay of lysates from 293 cells (**h**) or U373-CD14 cells (**i**) transfected with pEF1-lacZ and pUAS-Luc, transiently expressing Pin1 and Gal4-IRF3, and then infected with NDV (**h**) or treated with LPS (**i**). Data are representative of two independent experiments.

phosphorylation-dependent way[18,19]. By that mechanism, Pin1 has been shown to regulate the stability and or localization of its substrates during transcriptional activation, cell cycle progression and cell death, and deregulated expression or loss of function of Pin1 leads to the progression of important human diseases such as cancer and Alzheimer disease[18,20–28]. However, a regulatory function for Pin1 in the host defense against infectious agents and associated signal transduction pathways has not been reported before to our knowledge. Published findings showing that post-translational modification of IRF3 by phosphorylation controls the IRF3 activity prompted us to assess the involvement of Pin1 in regulating IRF3 signaling.

RESULTS

Pin1 suppresses IRF3-dependent transcriptional activation

A well characterized stimulation for IRF3 activation is TLR3 engagement with the synthetic dsRNA poly(I)•poly(C)[7,14,29,30]. We therefore used that mode of activating IRF3 to investigate possible involvement of Pin1 in regulating the activity of IRF3. Reporter gene assays using a yeast transcription factor Gal4–IRF3 fusion protein showed that exogenous expression of wild-type Pin1 inhibited poly(I)•poly(C)-induced IRF3-dependent transcriptional activation in a dose-dependent way, but expression of its WW domain mutant (W34A) or peptidyl-prolyl isomerase domain mutant (K63A) did not (**Fig. 1a,b**). That result indicated that functional WW and peptidyl-prolyl isomerase domains of Pin1 are required for the regulation of IRF3 signaling. Similarly, exogenous expression of wild-type Pin1 suppressed TLR3-mediated, IRF3-dependent activation of the IFN-β promoter and reduced IFN-β secretion in culture supernatants (**Fig. 1c–e**). We next addressed whether Pin1 affects the dimerization and nuclear localization of endogenous IRF3 that normally occurs after poly(I)•poly(C) stimulation. Native gel electrophoresis and immunoblot of cell lysates after poly(I)•poly(C) stimulation showed that exogenous Pin1 expression produced a considerable reduction in the activated dimer form of IRF3 (**Fig. 1e**). Immunocytochemical analyses confirmed that Pin1 overexpression decreased the amount of nuclear IRF3 (**Fig. 1f**).

We next assessed if exogenous Pin1 expression would inhibit IRF3 signaling induced by RIG-I-mediated detection of RNA virus infection or by LPS stimulation of TLR4. In agreement with published results[5], we found that IRF3-dependent transcriptional activation by the N-terminal caspase-recruitment domain of RIG-I was inhibited by exogenously expressed Pin1 (**Fig. 1g**). In addition, expression of Pin1 inhibited Newcastle disease virus (NDV)–induced IRF3-dependent

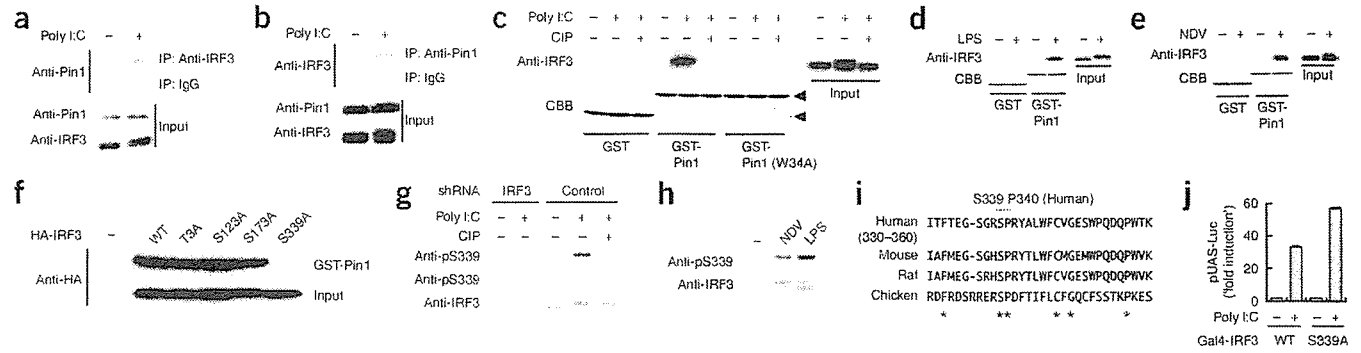

Figure 2 Pin1 interacts with IRF3 through its phosphorylated Ser339–Pro340 motif. (**a**,**b**) Immunoprecipitation (IP) and immunoblot of lysates from 293-TLR3 cells stimulated for 2 h with poly(I)•poly(C), analyzed with either polyclonal anti-IRF3 and anti-Pin1 (**a**) or anti-Pin1 and anti-IRF3 (**b**) or control immunoglobulin G (IgG). (**c**) PAGE and immunoblot with anti-IRF3 of affinity-purified lysates from 293-TLR3 cells stimulated for 2 h with poly(I)•poly(C) or left untreated. Lysates were left untreated or were treated with calf intestinal alkaline phosphatase (CIP), followed by incubation with GST, GST-Pin1 or GST-Pin1 W34A. Recombinant GST proteins (arrowheads) are visualized by Coomassie brilliant blue staining (CBB). (**d**,**e**) GST-Pin1 affinity assay of lysates from U373-CD14 cells stimulated for 2 h with LPS (**d**) or from 293 cells infected for 12 h with NDV or left untreated (**e**). (**f**) GST-Pin1 affinity assay of lysates from 293-TLR3 cells transfected with hemagglutinin-tagged wild-type IRF3 (WT) or various IRF3 mutants, stimulated with poly(I)•poly(C). (**g**) Immunoblot of lysates from U373-CD14 cells infected with a retroviral IRF3-specific or control shRNA construct and selected with puromycin. Cell pools were stimulated with poly(I)•poly(C), then lysates were treated with calf intestinal alkaline phosphatase and were analyzed by PAGE and immunoblot with anti–phospho-Ser339 (Anti-pS339), preincubated with phosphorylated (middle) or unphosphorylated (top) Ser339 peptide. Bottom, immunoblot with anti-IRF3. (**h**) PAGE and immunoblot of lysates from U373-CD14 cells treated with NDV or LPS, analyzed with anti–phospho-Ser339. (**i**) Amino acid alignment of the C-terminal regions of human, mouse, rat and chicken IRF3. *, amino acid residues conserved among the four species; boxed amino acid residues correspond to human Ser339 and Pro340. (**j**) Luciferase assay of lysates from 293-TLR3 cells transfected with pEF1-lacZ and pUAS-Luc and expressing either wild-type Gal4-IRF3 (WT) or the Gal4-IRF3 S339A mutant (S339A), stimulated for 9 h with poly(I)•poly(C). Data are representative of two independent experiments.

transcriptional activation, which is mediated by RIG-I (ref. 5; **Fig. 1h**). Finally, expression of Pin1 also suppressed TLR4-mediated IRF3-dependent transcriptional activation (**Fig. 1i**). These results strongly suggested that Pin1 is a negative regulator of a transcriptional activator of IFN-β and acts on a mediator shared by three independent pathways of IRF3 activation, indicating direct negative regulation of IRF3 itself.

Pin1 interacts with IRF3 in a phosphorylation-dependent way

Because Pin1 has been reported to regulate a subset of transcription factors[18], we determined if it physically interacts with IRF3. Immunoprecipitation followed by immunoblot analysis showed that poly(I)•poly(C) stimulation induced interaction of endogenous Pin1 with endogenous IRF3 *in vivo* (**Fig. 2a**,**b**). Glutathione *S*-transferase (GST) affinity assays further demonstrated an *in vitro* interaction between purified GST-Pin1 and endogenous IRF3 (**Fig. 2c**), and pretreatment of cell lysates with calf intestinal alkaline phosphatase abolished the interaction, indicating that phosphorylation is a prerequisite for the Pin1-IRF3 interaction. Consistent with that, the WW domain mutant of Pin1 (W34A) failed to interact with IRF3. LPS stimulation and NDV infection also induced the interaction of Pin1 with IRF3 (**Fig. 2d**,**e**).

We next sought to identify the specific Ser-Pro or Thr-Pro residues of IRF3 targeted by Pin1. The phospho-accepter residues required for IRF3 activation do not contain the Ser-Pro or Thr-Pro motif required for interaction with Pin1 (refs. 8–16). There are five Ser-Pro or Thr-Pro sites in human IRF3, and four of those (Thr3, Ser 123, Ser 173 and Ser339) are conserved between human and mouse. We analyzed mutants with site-directed substitution of each potential Pin1-binding site of IRF3 (substitution of alanine for serine or threonine) for Pin1 binding after poly(I)•poly(C) treatment; only the S339A substitution substantially disrupted the interaction between IRF3 and Pin1 (**Fig. 2f**). Immunoblot analysis with antibodies raised against an IRF3 peptide phosphorylated at Ser339 (anti–phospho-Ser339) demonstrated that this Ser residue of endogenous IRF3 was phosphorylated after stimulation with poly(I)•poly(C), LPS or NDV (**Fig. 2g**,**h**). Preincubation of anti–phospho-Ser339 with the phosphorylated peptide blocked detection of phosphorylated IRF3, but preincubation with the unphosphorylated peptide did not, establishing the specificity of the antibody (**Fig. 2g**). Suppression of endogenous IRF3 expression by RNA interference or by treatment of cell lysates with alkaline phosphatase diminished the immunoreactivity of anti–phospho-Ser339, indicating that it recognizes phosphorylated IRF3 (**Fig. 2g**). Notably, the amino acid residues corresponding to Ser339-Pro340 in human IRF3 are highly conserved among other species, such as mouse, rat and chicken (**Fig. 2i**), suggesting the importance of this Ser-Pro motif. At present, the kinase responsible for Ser339 phosphorylation remains unknown. Although phosphorylation at Ser396, which is reported to be mediated by TBK1 and IKK-*i*[9,15,16], reached a peak 1 h after stimulation with poly(I)•poly(C) or LPS, phosphorylation at Ser339 was detected several hours later (**Supplementary Fig. 1** online). Loss-of-function experiments using an RNA-interference strategy demonstrated involvement of TBK1 and IKK-*i* in the phosphorylation of Ser339 as well as Ser396 after poly(I)•poly(C) stimulation (**Supplementary Fig. 2** online). However, we do not have evidence for direct phosphorylation of Ser339 by these IKK-related kinases and cannot exclude the possibility that phosphorylation of the C-terminal phospho-accepter clusters by TBK1 and IKK-*i* somehow facilitates subsequent phosphorylation of Ser339 by another kinase. We confirmed the biological consequences of Ser339 phosphorylation by reporter gene assay. The S339A substitution augmented the activation of the reporter gene by poly(I)•poly(C) stimulation (**Fig. 2j**). These results collectively suggest that phosphorylation of IRF3 Ser339 and subsequent interaction with Pin1 are important for the negative regulation of IRF3 signaling.

Pin1 destabilizes activated IRF3

The interaction of Pin1 and IRF3 leading to suppression of IRF3 activity prompted us to examine whether Pin1 regulates IRF3

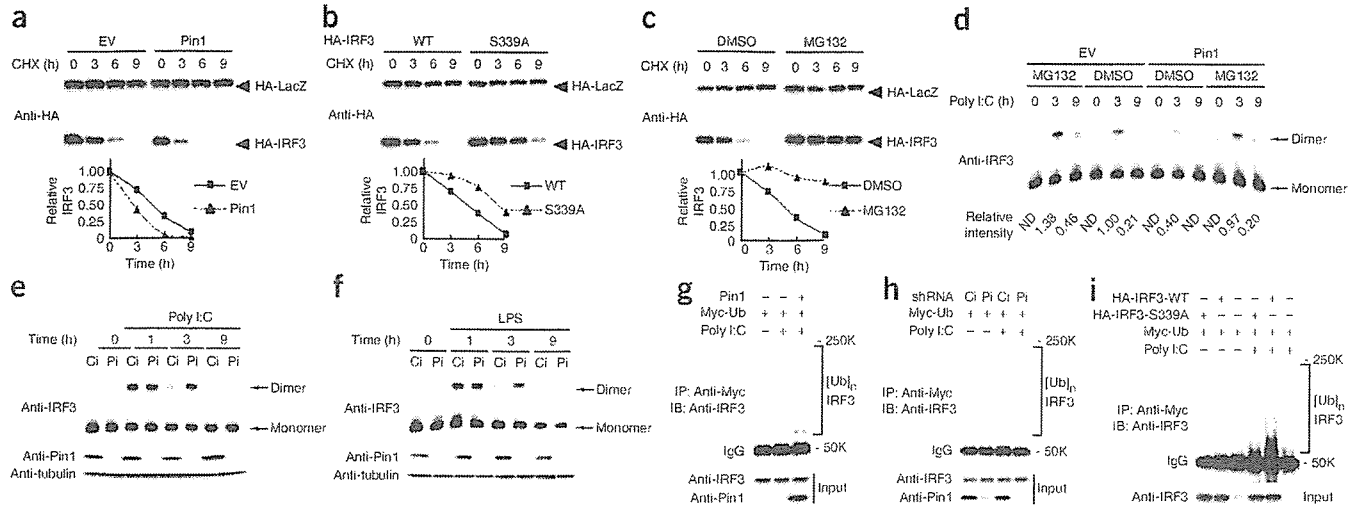

Figure 3 Pin1 regulates the stability of IRF3. (**a**) Immunoblot of lysates from 293-TLR3 cells transiently expressing Pin1, HA-lacZ and HA-IRF3, stimulated with poly(I)•poly(C) and then cultured in the presence of cycloheximide (CHX; time, above lanes). Expression of HA-IRF3 is normalized to that of HA-lacZ (graphed below). (**b**) Immunoblot of lysates from 293-TLR3 cells transiently expressing wild-type HA-IRF3 (WT) and HA-lacZ or HA-IRF3 S339A (S339A) and HA-lacZ and then treated as described in **a**. (**c**) Immunoblot of lysates from 293-TLR3 cells transiently expressing HA-IRF3 and HA-lacZ and then stimulated with poly(I)•poly(C) in the presence of cycloheximide and either DMSO (dimethyl sulfoxide) or MG132 (10 μM). (**d**) Native PAGE and immunoblot with anti-IRF3 of lysates from 293-TLR3 cells transiently expressing Pin1 and stimulated with poly(I)•poly(C) (time, above lanes) in the presence or absence (DMSO) of 10 μM MG132. Bottom, relative amount of IRF3 dimer. (**e**,**f**) Native PAGE and immunoblot with anti-IRF3 of lysates from U373-CD14 cells infected with a retroviral control (Ci) or Pin1-specific (Pi) shRNA construct and stimulated (time, above lanes) with poly(I)•poly(C) (**e**) or LPS (**f**). (**g**) Immunoprecipitation with anti-Myc and immunoblot (IB) with anti-IRF3 of whole-cell lysates from 293-TLR3 cells expressing Myc-tagged ubiquitin (Myc-Ub) and stimulated with poly(I)•poly(C) in the presence of MG132. $[Ub]_n$, polyubiquitination. (**h**) Immunoprecipitation with anti-Myc and immunoblot with anti-IRF3 of lysates from 293 cells infected with retroviral control or Pin1-specific shRNA, transiently expressing human TLR3 and Myc-tagged ubiquitin and stimulated with poly(I)•poly(C) in the presence of MG132. (**i**) Immunoprecipitation with anti-Myc and immunoblot with anti-IRF3 of whole-cell lysates from 293-TLR3 cells transiently expressing various proteins (above lanes) and stimulated with poly(I)•poly(C) in the presence of MG132. Data are representative of two independent experiments.

protein stability. Because IRF3 is highly stable in the steady state[31] (data not shown), we induced IRF3 activation by stimulation with poly(I)•poly(C). After activation with poly(I)•poly(C), IRF3 degraded more rapidly in 293 cell cultures stably expressing human TLR3 ('293-TLR3' cells) expressing exogenous Pin1 (**Fig. 3a**), and the IRF3 S339A mutant, which was defective in Pin1 binding, had a slower turnover than that of wild-type IRF3 (**Fig. 3b**). In the presence of the proteasome inhibitor MG132, IRF3 demonstrated considerably increased stability (**Fig. 3c**), suggesting that a proteasome-dependent mechanism underlies the negative regulation of IRF3. Our finding that Pin1 reduced the homodimer of IRF3 (**Fig. 1e**), in combination with these data demonstrating that turnover of IRF3 may involve a proteasome-dependent process, led us to determine how MG132 and/or Pin1 influences the status of the activated dimer form of IRF3 induced by poly(I)•poly(C) stimulation. Treatment with MG132 substantially increased the amount of IRF3 dimer, whereas the addition of exogenous Pin1 expression reduced the amount of IRF3 dimer (**Fig. 3d**). The addition of MG132 reversed the suppressive effects of Pin1 on IRF3 (**Fig. 3d**).

To verify the function of endogenous Pin1 in IRF3 signaling, we generated a short hairpin RNA (shRNA) expression construct capable of 'knocking down' Pin1 expression. Stable expression of Pin1-specific shRNA effectively reduced the amount of endogenous Pin1 but did not alter the expression of α-tubulin (**Fig. 3e,f**). Native PAGE coupled with immunoblot analysis showed that the suppression of endogenous Pin1 expression greatly increased IRF3 dimers induced by poly(I)•poly(C) or LPS at later time points but not at 1 h after stimulation (**Fig. 3e,f**). These results indicated that Pin1 'preferentially' promotes proteasome-dependent degradation of activated IRF3.

Ubiquitination-dependent degradation of transcription factors is an important mechanism for the termination of transcriptional activation[32]. Because we had found that proteasome inhibition delayed the degradation of IRF3 (**Fig. 3c**), we determined whether IRF3 is ubiquitinated. Immunoprecipitation of ubiquitin followed by immunoblot analysis for IRF3 demonstrated that polyubiquitination of IRF3 was induced by poly(I)•poly(C) stimulation (**Fig. 3g**) and that polyubiquitination was augmented by Pin1 expression and abrogated by expression of Pin1-specific shRNA (**Fig. 3g,h**). Consistent with the finding that Pin1 facilitated IRF3 degradation after poly(I)•poly(C) stimulation (**Fig. 3a**), the S339A mutant of IRF3 was less susceptible to polyubiquitination than was wild-type protein (**Fig. 3i**). These results indicated that Pin1 regulates ubiquitination and proteasome-mediated proteolysis of IRF3.

Endogenous Pin1 regulates TLR3-mediated IFN-β production

We next investigated the function of endogenous Pin1 in dsRNA-induced, IRF3-dependent transcriptional activation. As anticipated, expression of Pin1-specific shRNA enhanced IRF3-dependent transcriptional activation and thus enhanced activation of the IFN-β promoter triggered by the engagement of TLR3 (**Fig. 4a,b**). In contrast, expression of Pin1-specific shRNA did not affect TLR3-mediated activation of NF-κB, a transcription factor also involved in IFN-β production (**Supplementary Fig. 3** online). Moreover, expression of a second Pin1-specific shRNA also augmented IRF3-dependent reporter gene activation, and complementation with mouse Pin1, which does not contain the human Pin1 shRNA target sequence, reversed the effects on IRF3-dependent transcriptional activation (**Supplementary Fig. 4** online). To investigate the temporal regulation of IRF3-dependent transcription by Pin1, we used a

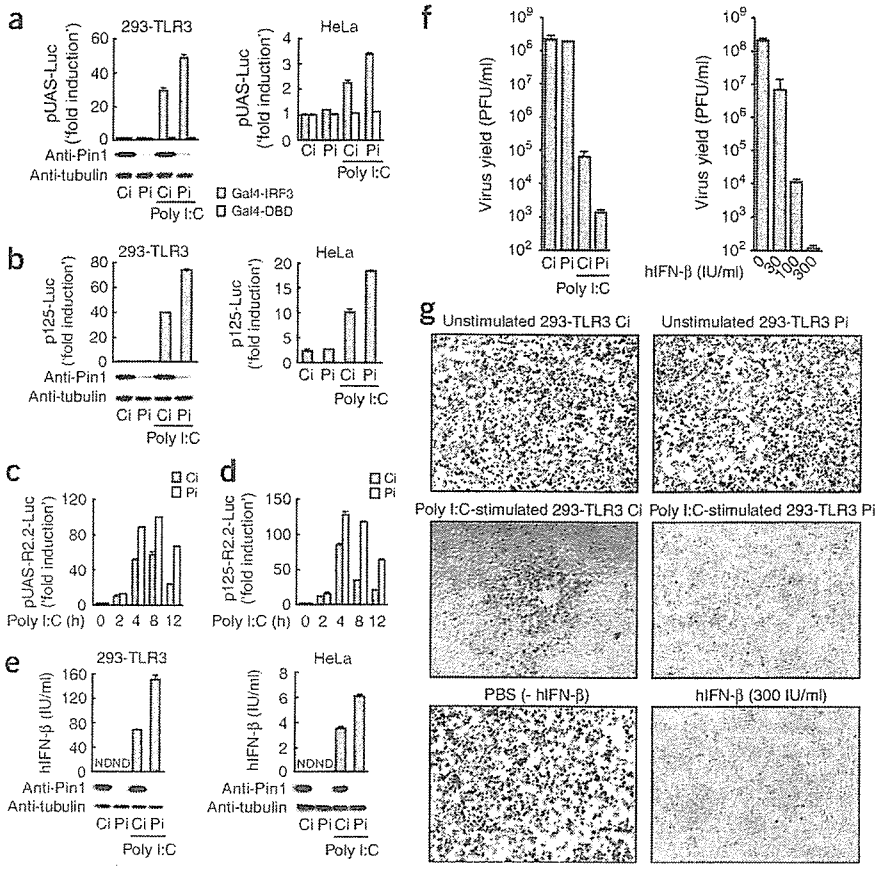

Figure 4 Endogenous Pin1 negatively regulates TLR3-mediated IRF3-dependent transcriptional activation and IFN-β production. (**a–d**) Luciferase assay of lysates from 293-TLR3 cells (**a–d**) and HeLa cells (**a,b**, right) transfected with pEF1-lacZ and pUAS-Luc (**a**), p125-Luc (**b**), pUAS-R2.2-Luc (**c**) or p125-R2.2-Luc (**d**) and transiently expressing control or Pin1-specific shRNA (**a–d**) and Gal4-IRF3 (**a,c**). Cells were stimulated with poly(I)•poly(C) for 9 h (**a,b**) or for various times (horizontal axes; **c,d**); relative luciferase activities are presented as in **Figure 1**. Expression of Pin1 in 293-TLR3 cells was verified by immunoblot (**a**, **b**, bottom). (**e**) ELISA of human IFN-β (hIFN-β) secretion from either 293-TLR3 cells transiently expressing control or Pin1 shRNA (left) or HeLa cells infected with retroviruses expressing either control or Pin1 shRNA (right), analyzed after stimulation for 12 h with poly(I)•poly(C). Data are means ± s.d. from three separate samples. (**f**) VSV production (in plaque-forming units (PFU)/ml) 24 h after infection of Vero cells previously treated with supernatants from 293-TLR3 cells transiently expressing control or Pin1 shRNA and stimulated for 12 h with poly(I)•poly(C) (left) or with various concentrations of human IFN-β (right). (**g**) Phase-contrast micrographs of VSV-infected Vero cells. Dead cells (round shape) are detached from the culture dish. Original magnification, ×20. Data are representative of two independent experiments.

rapid-response luciferase reporter gene. Real-time reporter gene assays showed that suppression of endogenous Pin1 expression substantially prolonged both IRF3-dependent transcription and IFN-β promoter activation after poly(I)•poly(C) stimulation (**Fig. 4c,d**). Consistent with the inhibitory effects of Pin1 on the IFN-β promoter, expression of Pin1-specific shRNA but not that of control shRNA increased the production of IFN-β induced by poly(I)•poly(C) (**Fig. 4e**). Because exogenous expression of Pin1 greatly reduced the production of a potent antiviral secreted factor (IFN-β) from poly(I)•poly(C)-stimulated 293-TLR3 cells (**Supplementary Fig. 5** online), we assessed if endogenous Pin1 was sufficient to induce the same regulation. Culture supernatants of poly(I)•poly(C)-stimulated 293-TLR3 cells expressing Pin1-specific shRNA had approximately 50 times more antiviral activity than that of supernatant of cells expressing control shRNA (**Fig. 4f**); moreover, this amount of secreted antiviral factor was sufficient to fully protect fresh cells from productive infection by vesicular stomatitis virus (VSV; **Fig. 4g**). These results indicated that Pin1 is involved in terminating IRF3 signaling triggered after TLR3 engagement with activating ligand and thus Pin1 regulates the expression of IRF3 target genes relevant to the innate antiviral response. However, unlike other negative regulators of TLR signaling whose expression is upregulated by stimulation[3], the expression of Pin1 protein was not substantially different after stimulation with poly(I)•poly(C) or IFN-β (**Supplementary Fig. 6** online).

Pin1 deficiency enhances IFN-β production induced by dsRNA

To assess the involvement of Pin1 in dsRNA-induced innate antiviral response in immune competent cells, we prepared bone marrow–derived macrophages from *Pin1*$^{-/-}$ or *Pin1*$^{+/+}$ mice. After being stimulated with poly(I)•poly(C), macrophages from *Pin1*$^{-/-}$ mice secreted more IFN-β than did wild-type cells (**Fig. 5a**). Those findings prompted us to determine if Pin1 also limits IFN-β induction *in vivo*. The amount of IFN-β produced after intraperitoneal injection of poly(I)•poly(C) was much greater in sera of *Pin1*$^{-/-}$ mice than in sera of similarly treated wild-type mice (**Fig. 5b**). These results provided

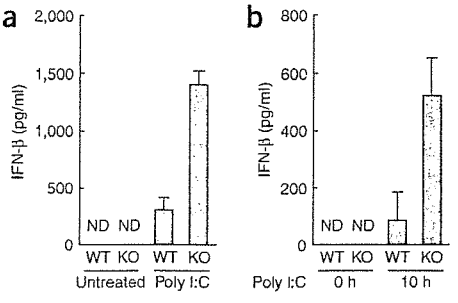

Figure 5 Pin1 deficiency enhances IFN-β production in response to dsRNA. (**a**) ELISA of IFN-β production from bone marrow–derived macrophages prepared from *Pin1*$^{+/+}$ (WT) or *Pin1*$^{-/-}$ (KO) mice; cells were left untreated or were stimulated for 8 h with poly(I)•poly(C) (25 μg/ml). Data are means ± s.d. of three independent experiments. (**b**) ELISA of IFN-β production in sera from *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ mice ($n = 3$ per group) injected intraperitoneally with 5 μg poly(I)•poly(C) per gram body weight. Sera were collected from each mouse before or 10 h after treatment. Error bars represent s.d.

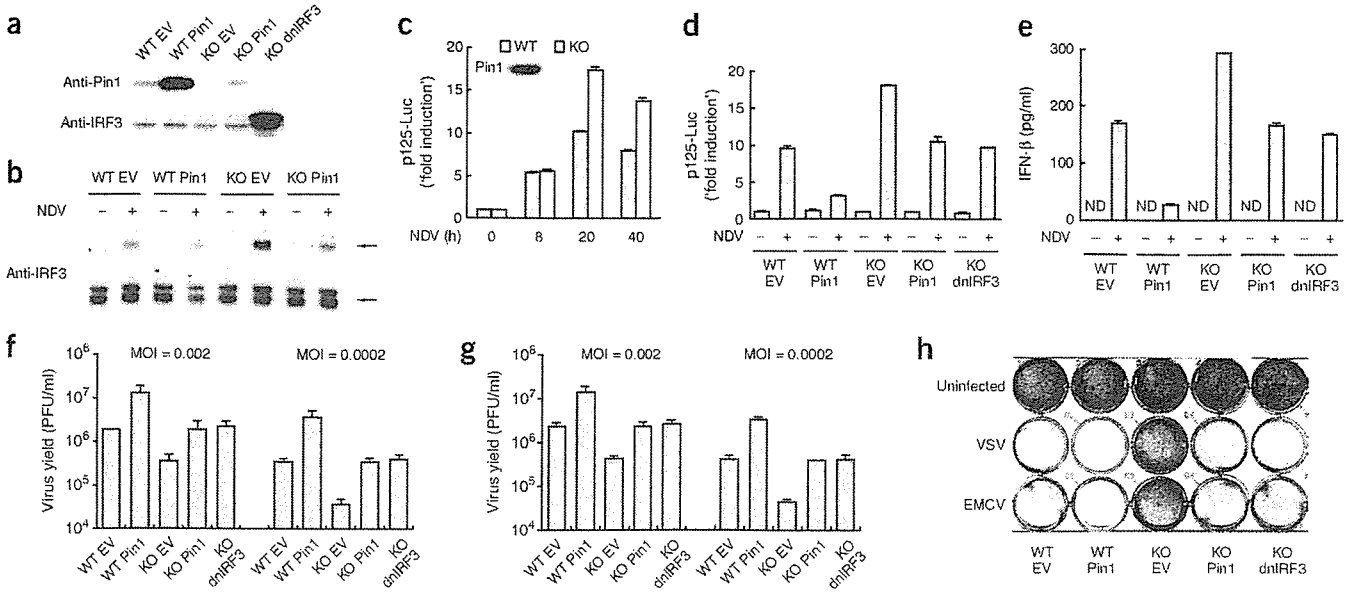

Figure 6 Pin1 regulates RIG-I-mediated IRF3 signaling and innate antiviral response. (**a**) Immunoblot with anti-Pin1 or anti-IRF3 of lysates from MEFs isolated from *Pin1*$^{+/+}$ (WT) or *Pin1*$^{-/-}$ (KO) mice infected with retroviruses expressing mouse Pin1 (WT-Pin1 or KO-Pin1) or hemagglutinin-tagged dominant negative IRF3 (KO dnIRF3) and selected with blasticidin S. (**b**) Native PAGE and immunoblot with anti-IRF3 of lysates from the MEFs described in **a**, infected for 30 h with NDV. Arrows (right margin) indicate IRF3 dimer (top) and monomer (bottom). (**c**) Luciferase assay of lysates from *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ MEFs transfected with p125-Luc and pEF1-lacZ and then infected with NDV (times, horizontal axis). Inset, immunoblot for Pin1. (**d**) Luciferase assay of lysates from the MEFs in **a**, transfected with p125-Luc and pEF1-lacZ and then infected for 20 h with NDV. (**e**) ELISA of IFN-β produced from the MEFs in **a**, infected for 40 h with NDV. (**f**,**g**) Virus production from the MEFs in **a**, infected with VSV (**f**) or EMCV (**g**) at a multiplicity of infection (MOI) of 0.002 (left) or 0.0002 (right). Virus yield in the supernatants at 20 h after infection was determined by plaque assay. (**h**) Amido black staining of the MEFs in **a**, infected for 20 h with VSV (MOI, 0.002) or EMCV (MOI, 0.002) but not killed; cells were fixed and then stained. Data are representative of two independent experiments.

genetic evidence that Pin1 regulates dsRNA-induced IFN-β production both *in vivo* and *in vitro*.

Pin1 regulates RIG-I-dependent antiviral cellular responses

To assess the involvement of Pin1 in the RIG-I-dependent antiviral cellular response, we used mouse embryonic fibroblasts (MEFs) isolated from *Pin1*$^{-/-}$ mice (**Fig. 6a**). Native PAGE coupled with immunoblot analysis showed that loss of Pin1 expression considerably increased the amount of IRF3 dimer (**Fig. 6b**). Complementation of *Pin1*$^{-/-}$ MEFs with expression of wild-type Pin1 normalized the IRF3 response to NDV infection, confirming that deregulated IRF3 activation in *Pin1*$^{-/-}$ MEFs was due to the loss of Pin1 expression. Reporter gene assays showed that the NDV-induced activation of the IFN-β promoter was enhanced at later time points in *Pin1*$^{-/-}$ MEFs compared with that of wild-type MEFs, an effect that was reversed by complementation with either wild-type Pin1 or a dominant negative mutant of IRF3 (**Fig. 6c,d**). Consistent with that finding, *Pin1*$^{-/-}$ MEFs demonstrated increased production of IFN-β after NDV infection (**Fig. 6e**). To demonstrate the specificity of Pin1 on IRF3 activation, we also evaluated NF-κB activation in *Pin1*$^{-/-}$ MEFs after NDV infection and found that it was only marginally affected by the absence of Pin1 (**Supplementary Fig. 3**). Finally, we noted that NDV infection weakly and transiently increased Pin1 protein expression (**Supplementary Fig. 6**).

We next sought to determine if Pin1 regulated replication of VSV and encephalomyocarditis virus (EMCV), as RIG-I-mediated IRF3 signaling is critical in restricting replication of these RNA viruses[5]. Consistent with the suppressive effects of Pin1 on RIG-I-mediated IRF3 signaling (**Fig. 1g,h**), exogenous expression of Pin1 increased the production of infectious VSV or EMCV in the culture supernatants of infected MEFs, whereas loss of Pin1 expression decreased it (**Fig. 6f,g**). Furthermore, the reduction of virus yield in *Pin1*$^{-/-}$ MEFs was reversed by the expression of a dominant negative form of IRF3. In agreement with those results, increased expression of Pin1 augmented virus-mediated cell killing, whereas loss of Pin1 expression protected cells (**Fig. 6h**). These results provide biological evidence that Pin1 acts as a negative regulator of RIG-I-mediated IRF3 signaling and thereby modulates the innate antiviral cellular response.

DISCUSSION

Studies have identified functions for Pin1 in a variety of pathological conditions[18]. Pin1 prevents the accumulation of hyperphosphorylated tau protein in the brain and thus protects neuronal cells from age-dependent degeneration[18,25]. However, increased expression of Pin1 has often been detected in many different neoplasms, such as breast cancer, and is also involved in the malignant transformation of cancer cells[18,24,33]. Here we have demonstrated that Pin1 is involved in the termination of IRF3-dependent transcriptional activation, which thereby provides negative regulation of the innate antiviral response against infection by RNA viruses. The results presented here suggest that increased expression of Pin1 suppresses the innate antiviral response and thus may allow the replication and persistence of infectious agents. If true, such a possibility could partly explain why cancer cells are more susceptible to lytic infection by VSV[34].

We have also demonstrated that the genetic absence or RNA interference-mediated suppression of Pin1 expression enhanced poly(I)•poly(C)-induced activation of IRF3 and consequent production of IFN-β. Because activation of IRF3 and production of IFN-β are known to mediate TLR-mediated toxicity[35,36] and because ectopic expression of a constitutively active IRF3 mutant induces cell death[37],

IRF3 activation and 'deactivation' must be strictly controlled. Pin1-mediated post-translational regulation of IRF3, therefore, is likely to be important in determining the exact nature of the final immune responses against viruses or microbes.

TBK1-NAK and IKK-*i*–IKKε have been identified as IRF3 kinases responsible for the phosphorylation of the two C-terminal phospho-accepter clusters (Ser385-Ser386 and Ser396-Ser398-Ser402-Thr404-Ser405) and are indispensable for IRF3 activation[14–17]. Our results here have demonstrated that Ser339 is another important phosphorylation target for IRF3 regulation that governs the termination of IRF3-dependent transcriptional activation. Of note, the Pin1 target motif of human IRF3 is conserved in human, monkey, mouse and rat IRF7, another member of the IRF family. As IRF7 is a 'master regulator' for type I interferon expression induced by TLR7 and TLR9 stimulation[38], Pin1 might act as a critical regulator of type I interferon production by modulating both IRF3 and IRF7 signaling. Further studies are needed to fully elucidate the functions of Pin1 in the regulation of type I interferon production.

Here we have demonstrated activation-induced destabilization of IRF3 through direct engagement with Pin1. In general, degradation of transcription factors is one of the principal mechanisms that reduce or terminate transcriptional activation. This type of mechanism occurs not only during immune responses but also in many other biological phenomena. An example of this appeared in a study demonstrating that the turnover of c-Myc is under strict control by phosphorylation-dependent post-translational modification and that c-Myc deregulation results in the induction of oncogenic transformation of fibroblasts[28,39]. Phosphorylation at Ser62 of c-Myc is required for its stabilization, a phosphorylation event that is also a prerequisite for subsequent phosphorylation at Thr58 by glycogen synthase kinase-3β, which in turn leads to ubiquitination and proteasome-dependent degradation of the protein. Thus, sequential phosphorylation events are critical for regulation of the c-Myc-induced proliferation signals[28,39]. Consistent with that, substitution of Thr58 with alanine renders c-Myc more stable and oncogenic[28]. Notably, Pin1 is recruited to the phosphorylated Thr58-Pro59 motif of c-Myc and is critical in the phosphorylation-induced destabilization of c-Myc[28]. Because Pin1 is recruited to phosphorylated IRF3 after stimulation, it would be useful to determine if phosphorylation of the two clusters essential for activation of IRF3, but which do not mediate Pin1 binding, alters IRF3 stability and the efficiency of IRF3 interaction via the Pin1-binding motif on IRF3. Phosphorylation of the C-terminal phospho-accepter cluster of IRF3 is a prerequisite for MG132-sensitive reduction of IRF3 in a human cell line after infection with Sendai virus[10], although the underlying molecular mechanism was not clarified in that study. Although Pin1 regulates the protein stability of many transcription factors, it does not directly catalyze the ubiquitination or subsequent proteasome-dependent degradation of its substrates. Therefore, the identification of an IRF3-specific ubiquitin ligase or conjugating enzyme would facilitate fuller understanding of the regulation of IRF3 signaling.

METHODS

Reagents. Monoclonal anti-α-tubulin and anti-hemagglutinin (HA-7) were purchased from Sigma. Polyclonal anti-Pin1 and monoclonal anti-Myc were purchased from Cell Signaling Technology. Monoclonal anti-Pin1 was purchased from R&D Systems. Polyclonal anti-IRF3 (FL-425) was purchased from Santa Cruz Biotechnology. Polyclonal antibody to human IRF3 phosphorylated at Ser339 was elicited by immunization of a rabbit with the phosphorylated peptide N-CEGSGR(pS)PRY-C. Poly(I)•poly(C) was purchased from Amersham. Calf intestinal alkaline phosphatase was purchased from Takara Shuzo. MG132 was purchased from the Peptide Institute. Human IFN-β and the human IFN-β enzyme-linked immunosorbent assay (ELISA) kit were purchased from FujiRebio. The mouse IFN-β ELISA kit was purchased from PBL Biomedical Laboratories. All other reagents were purchased from Sigma unless indicated otherwise. Polyclonal anti-IRF3 has been described[8]. NDV, EMCV and VSV were prepared as described[5].

Plasmids. The expression constructs pFLAG-CMV1-hTLR3, pMX-puro, pEF1-lacZ and p125-Luc (IFN-β promoter with a luciferase (Luc) reporter) were donated by K. Fitzgerald (University of Massachusetts, Worcester, Massachusetts), T. Kitamura (University of Tokyo, Tokyo, Japan), S. Memet (Institut Pasteur, Paris, France) and T. Taniguchi (University of Tokyo, Tokyo, Japan), respectively. The pcDNA3-Pin1, pcDNA3-Pin1 W34A, pcDNA3-Pin1 K63A, pGEX-Pin1, pCMV-Myc-Ub, pEF-BOS hemagglutinin-tagged IRF3 (HA-IRF3), pEF-BOS dominant negative HA-IRF3 (58-427), pGal4-DBD, pGal4-IRF3, pUAS-Luc, pHCMV-VSV-G, pMRX-IRES-puro and pMRX-IRES-bsr constructs have been described[5,8,20,26,40,41]. The pEF-myc-cyto and pcDNA3 plasmids were purchased from Invitrogen. Construction details for the other plasmids are in the **Supplementary Methods** online.

Cells and mice. Neomycin-resistant 293-TLR3neo^r, Plat-E and U373-CD14 cells have been described[9,29,42]. The 293-TLR3 cultures were established after infection with retrovirus produced from pMX-Flag-hTLR3-puro. *Pin1*$^{-/-}$ mice have been described[20,25,26,43], as have MEFs isolated from *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ mice[26].

Reporter assays and retrovirus preparations. Cells were transfected using the FuGene6 transfection reagent (Roche) according to the manufacturer's instructions. The culture supernatant of the packaging cell line Plat-E, cotransfected with a retroviral vector and pHCMV-VSV-G, was filtered and used for infection[40]. Reporter assays were done as described[30]. Firefly luciferase activity was normalized to β-galactosidase activity.

Preparation of bone marrow–derived macrophages. Bone marrow–derived macrophages isolated from 12-week-old *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ mice were differentiated *in vitro* for 6 d. Macrophages were then left unstimulated or stimulated with poly(I)•poly(C). Secreted IFN-β was measured with the mouse IFN-β ELISA kit according to the manufacturer's guidelines.

Injection of poly(I)•poly(C) into mice. Poly(I)•poly(C) (5 μg per gram of body weight) was injected into the peritonea of 12-week-old *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ mice. Blood was drawn from the tail vein before and 10 h after treatment. Collected sera were immediately frozen. IFN-β in sera was measured with the mouse IFN-β ELISA kit according to the manufacturer's guidelines.

Immunocytochemistry and nuclear staining. Immunocytochemistry and nuclear staining with fluorescent dyes were done as described[8,26].

Immunoblot, immunoprecipitation and GST affinity assays. Immunoprecipitation and GST affinity assays were done as described[26,30]. For the ubiquitination assay, cells either treated or left untreated were suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (volume/volume) glycerol and 1% (volume/volume) Nonidet-P40) supplemented with 0.5 mM phenyl methyl sulfonylfluoride, 3 μg/ml of leupeptin, 10 μM MG132, 10 μM MG115, 5 mM NaF and 1 mM Na_3VO_4. Cell lysates were incubated for 2 h with anti-Myc and then were incubated for 1 h with protein G–Sepharose. Beads were washed four times with lysis buffer. SDS-PAGE and native PAGE coupled with immunoblot analysis were done as described[26,30].

Protein stability assay. Protein stability was analyzed as described[26]. After 293-TLR3 cells were transfected with the HA-IRF3 expression plasmid together with pcDNA3-HA-lacZ, they were subjected to various treatments. After the addition of cycloheximide, cells were collected at various time points. Hemagglutinin-tagged proteins were detected by immunoblot and were semiquantified with NIH image software.

Virus yield titration. MEFs or Vero cells were infected with VSV or EMCV. Virus yield in culture supernatants was determined by plaque assay as described[5].

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Anti-V3 Humanized Antibody KD-247 Effectively Suppresses Ex Vivo Generation of Human Immunodeficiency Virus Type 1 and Affords Sterile Protection of Monkeys against a Heterologous Simian/Human Immunodeficiency Virus Infection

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In an accompanying report (Y. Eda, M. Takizawa, T. Murakami, H. Maeda, K. Kimachi, H. Yonemura, S. Koyanagi, K. Shiosaki, H. Higuchi, K. Makizumi, T. Nakashima, K. Osatomi, S. Tokiyoshi, S. Matsushita, N. Yamamoto, and M. Honda, J. Virol. 80:5552-5562, 2006), we discuss our production of a high-affinity humanized monoclonal antibody, KD-247, by sequential immunization with V3 peptides derived from human immunodeficiency virus type 1 (HIV-1) clade B primary isolates. Epitope mapping revealed that KD-247 recognized the Pro-Gly-Arg V3 tip sequence conserved in HIV-1 clade B isolates. In this study, we further demonstrate that in vitro, KD-247 efficiently neutralizes CXCR4- and CCR5-tropic primary HIV-1 clade B and clade B′ with matching neutralization sequence motifs but does not neutralize sequence-mismatched clade B and clade E isolates. Monkeys were provided sterile protection against heterologous simian/human immunodeficiency virus challenge by the passive transfer of a single high dose (45 mg per kg of body weight) of KD-247 and afforded partial protection by lower antibody doses (30 and 15 mg per kg). Protective neutralization endpoint titers in plasma at the time of virus challenge were 1:160 in animals passively transferred with a high dose of the antibody. The antiviral efficacy of the antibody was further confirmed by its suppression of the ex vivo generation of primary HIV-1 quasispecies in peripheral blood mononuclear cell cultures from HIV-infected individuals. Therefore, KD-247 promises to be a valuable tool not only as a passive immunization antibody for the prevention of HIV infection but also as an immunotherapy for the suppression of HIV in phenotype-matched HIV-infected individuals.

Because most primary strains of human immunodeficiency virus type 1 (HIV-1) are relatively resistant to neutralization, the specificities of antibodies that confer protective immunity against it are still not understood (22). Previously, we and others (9, 31) have reported that chimpanzees can be protected against infection with the T-cell-line-adapted strain HIV-1_{IIIB} by passive transfer of either HIV immunoglobulin (Ig) (HIVIG) or anti-HIV-1_{IIIB} V3 monoclonal antibodies (MAbs). Passive administration of the anti-HIV-1 gp41 human MAb 2F5 (24) to two chimpanzees prior to challenge with primary HIV-1_{5016} resulted in a delay in plasma viremia and reduced viral load. Since the chimpanzee model is limited by the failure of HIV-1 to induce disease in these animals, a pathogenic model was developed in monkeys using a simian/human immunodeficiency virus (SHIV) strain that is capable of inducing high plasma viremia, $CD4^+$-T-cell loss, and simian AIDS (11, 14, 15, 37). Following pathogenic SHIV 89.6P challenge, Mascola and colleagues (20) previously noted a synergistic effect with the passively transferred antibody HIVIG, a MAb against membrane-proximal external region 2F5 (27), and 2G12, a glycan-dependent MAb (41). Monkeys were afforded protective immunity against pathogenic SHIV DH12 by chimpanzee HIVIG and provided sterile protection against the challenge virus when given high-dose inoculations (27, 36). However, sterile protection was strain specific, and the antiserum did not bind a V3 loop peptide or block the interaction of gp120 with CD4. In several passive immunization studies using MAbs, the antibodies 2G12 and 2F5 as well as 4410, a MAb against membrane-proximal external region 4E10 (4), have been shown to inhibit SHIV in monkeys (2, 20, 21). Furthermore, human MAb b12, targeting the CD4-binding domain of gp120, has been reported to elicit complete protection against viral challenge (29) and partial protection against MAb 2G12 (22) in monkeys. Recently, passively transferred antibodies with 2G12, 2F5, and 4E10 were shown to delay the rebound of HIV-1 after the cessation of antiretroviral therapy, with that delay especially pronounced in acutely infected individuals.

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The in vivo effect of the neutralizing antibody cocktail was found to depend on 2G12 activity by escape mutant analysis (42).

It has been established that anti-V3 antibodies, induced by brief immunization protocols in animals, are capable of neutralizing HIV-1 in cell cultures and in animal challenge studies (13, 16, 27, 28). However, that capability has not been fully exploited because the V3 sequence is extremely diverse, and so the anti-V3 antibodies are extremely type specific and displayed little cross-reactivity. In the accompanying paper (8a), we describe how we sequentially immunized mice with V3 peptides derived from several different HIV-1 clade B field isolates. The antibody response could be traced to a tip sequence of the HIV-1 gp120 V3 domain, a relatively conserved motif (11, 18, 45). We reshaped anti-V3 MAb C25 into KD-247, a humanized MAb directed against the V3 tip motif Pro-Gly-Arg of the V3 domain. KD-247 cross-neutralized primary isolates with a matching neutralization sequence motif, suggesting that it could be used to overcome the previous limitations surrounding anti-V3 neutralizing antibody production by active immunization strategies.

In this study, we show that the humanized MAb KD-247 is suitable not only for use as a passive immunization antibody for the prevention of immunodeficiency virus infection but also to passively transfer antibodies for immunotherapy. Using 18 primary HIV-1 isolates, we evaluate the neutralizing capacity of KD-247. We also assess its efficacy against ex vivo generation of HIV from the peripheral blood mononuclear cells (PBMCs) of four HIV-infected individuals. Finally, we examine whether KD-247 can suppress HIV-1 replication in monkeys.

MATERIALS AND METHODS

Passive transfer of KD-247 to monkeys followed by pathogenic virus challenge. All animals used in this study were mature, cycling, male cynomolgus monkeys (*Macaca fascicularis*) from the Tsukuba Primate Center, National Institute of Infectious Diseases (NIID), Japan. They were free of known simian retroviruses, herpesviruses, bacteria, and parasites. They were housed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science under the Japanese Law Concerning the Protection and Management of Animals (1, 38) and were maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of NIID, Japan. Once approved by an institutional committee for biosafety level 3 experiments, these studies were conducted at the Tsukuba Primate Center, NIID, Japan, in accordance with the requirements specifically stated in the laboratory biosafety manual of the World Health Organization (44a).

The pathogenic SHIV strain C2/1 is an SHIV strain 89.6 variant isolated by in vivo passage in cynomolgus monkeys (37). The original SHIV 89.6 strain was kindly provided by Y. Lu at the Harvard AIDS Institute (Boston, MA) (19, 32). Virus stocks of SHIV C2/1 were stored at −125°C and thawed just prior to use. The challenge stock was provided by K. Shinohara of the National Institute of Infectious Diseases, Tokyo, Japan. Cynomolgus monkeys injected intravenously with SHIV C2/1 showed high levels of viremia and marked $CD4^+$-T-cell depletion within 2 weeks after inoculation (1, 34, 35, 37). Naïve monkeys were intravenously administered 0, 15, 30, or 45 mg/kg of KD-247 along with either 45 mg/kg of purified normal human immunoglobulin (Nihon Pharmaceutical Co., Tokyo, Japan) or saline. Twenty-four hours after antibody transfer, the animals were intravenously challenged with 20 50% tissue culture infective doses ($TCID_{50}$s) of SHIV C2/1.

In vitro virus neutralization assays. The primary clinical isolate $HIV\text{-}1_{MNp}$ was kindly provided by J. Sullivan of the University of Massachusetts Medical School, Worcester, MA. The virus was confirmed to be neutralization resistant (5). Laboratory-adapted $HIV\text{-}1_{89.6}$ and $HIV\text{-}1_{MN}$ were obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD. GHOST cell neutralization assays were performed as described previously (5, 38). Briefly, GHOST cells expressing either CXCR4 or CCR5 coreceptors were used as targets of HIV-1 infection. The cells were then analyzed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA). The same concentration of either purified normal human immunoglobulin consisting primarily of the IgG1 subclass (Nihon Pharmaceutical Co.) or saline was used as control.

Neutralization activities in monkey plasma were assayed by detecting the neutralizing titers in the assay measuring 100% neutralization against the challenge virus as described previously by Nishimura et al. (26). In brief, plasma samples were serially diluted and incubated with 100 $TCID_{50}$s of challenge virus, and M8166 cells were then incubated as previously described (26). The neutralization was expressed as the percent inhibition of simian immunodeficiency virus p27 antigen production in the culture supernatants (38, 39). Normal monkey plasma was used as a control.

PBMC-based virus neutralization assay. $HIV\text{-}1_{MN}$ (H9/HTLV-III MN) was kindly provided by the AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD (45). The WHO primary isolates 92TH002, 92TH022, 92TH023 (all clade E), and 92TH014 (clade B′) were used as virus stocks (12). The primary isolates $HIV\text{-}1_{JR\text{-}CSF}$ and the CS and JCI series of HIV-1 isolates were provided by Y. Koyanagi (40) and Y. Okamoto (27). In vitro virus neutralization assays were performed as previously described (7, 12). Neutralization titers are expressed as either the concentration of serum IgG antibody or the reciprocal of the serum dilution that yielded a 50% (50% inhibitory concentration [IC_{50}]) or 90% (IC_{90}) reduction in HIV-1 p24 production over that seen in controls using purified serum IgG from healthy individuals or preimmune mouse sera.

Ex vivo virus neutralization assays. The PBMCs of patients infected with HIV-1 were depleted of $CD8^+$ cells by magnetic separation using polystyrene beads coated with anti-CD8 MAb (Dynabeads M-450 CD8; Dynal, Oslo, Norway). The negatively selected cells were stimulated with OKT3 antibody (1 μg/ml; Jannsen-Kyowa, Tokyo, Japan) and subsequently cultured in the presence of interleukin-2 (20 U/ml; Boehringer, Mannheim, Germany) together with KD-247 (60 and 240 μg/ml). The amount of HIV-1 p24 antigen in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA) (Dainabot, Tokyo, Japan). Approval by the ethical committee and written informed consent from all the human subjects were obtained according to the guidelines of the Ministry of Health, Labor, and Welfare, Japan, and to those of the Kumamoto University Medical School, Kumamoto, Japan.

Competitive PCR quantitation of SHIV RNA in plasma. Quantitative competitive reverse transcription-PCR was performed as described previously by Piatak et al. (30), with both the substitution of a different competitor RNA and a different DNA template (35). The detection limit of this assay was 500 RNA copies/ml in monkey plasma.

Flow cytometric evaluation of cell surface antigen expression and absolute cell count. Mouse MAbs conjugated with either fluorescein isothiocyanate, phycoerythrin (PE), PE-Cy5, or peridinin chlorophyll protein were used in flow cytometric analyses to detect cellular expression of monkey CD3 (NF-18; BioSource International Inc., Camarillo, CA), human CD4 (Nu-TH/I; Nichirei Co., Tokyo, Japan), CD8 (SK-1; Becton Dickinson & Co., San Jose, CA), and CD95 (CH11 and 7C11; Becton Dickinson) (30). To determine absolute cell counts, samples of whole blood were analyzed following the addition of fluorescein isothiocyanate-conjugated anti-CD3 (BioSource), PE-conjugated anti-CD4 (Becton Dickinson), and peridinin chlorophyll protein-conjugated anti-CD8 (Becton Dickinson) MAbs as previously described (35).

Plasma concentration of KD-247. HIV-1 V3 peptide-based ELISA was used for quantification of KD-247 antibody. In brief, 96-well ELISA plates (Maxisorp; Nunc A/S, Roskilde, Denmark) were coated with 100 μl of a KD-247 antigen peptide (SP1 [YNKRKRIHIGPGRAFYTTKNC]) per well in 50 mM carbonate buffer (pH 9.3) at 1 μg/ml overnight at 4°C. KD-247 was diluted to concentrations ranging from 2.5 to 40 ng/ml as a reference. Bound KD-247 was detected with a peroxidase-conjugated anti-human IgG MAb (in-house preparation; The Chemo-Sero-Therapeutic Research Institute). The concentrations of KD-247 in the plasma of monkeys were determined using a calibration curve (SOFTmax; Molecular Devices Co., Menlo Park, CA).

Statistical analysis. The plasma concentrations at various data points postdose were applied to a two-compartment model using an automatic pharmacokinetic analysis program (nonlinear least-squares method), and pharmacokinetic parameters were calculated.

RESULTS

Neutralization ability of the humanized antibody KD-247 against a panel of primary isolates as determined by a PBMC-based study. In the initial series of the study, we showed that

TABLE 1. PBMC-based neutralization of primary and laboratory isolates by KD-247[a]

Isolate	Env V3 sequence[b]		GHOST cell	KD-247		447-52D IC_{50}[c]
				IC_{90}	IC_{50}	
Laboratory isolates, clade B						
HIV-1_{MN}	CTRPNYNKRKRIHI	GPGRAFYTTKNIIGTIRQAHC	X4	1	0.1	0.1
HIV-1_{SF2}	-----N-T--C---	-------A-EK-V-D------	X4	5	1.0	1.0
HIV-$1_{89.6}$	-----N-T-R-LS-	-------ARR----D------	R5/X4	2.5	0.2	>10
Primary isolates, clade B						
HIV-1_{JR-CSF}	----SN-K--S---	---------GE---D------	R5	5	0.4	>10
HIV-1_{CS2-2}	-----N-T--S--M	---K-----GD---N----Y-	R5	>50	>50	ND
HIV-1_{CS3-5}	---I-N-T--S---	-------A-GE---N-K----	R5	10	1.4	ND
HIV-1_{CS4-4}	-I---N-T--G---	-L-- WK--A-G--N------	R5/X4	>50	>50	ND
HIV-1_{CS6-6}	--G--N-T--S-R-QR------V-IGK --NM-----		R5	>50	>50	ND
HIV-1_{CS6-8}	-I---N-T--G---	-------A-D----N------	R5	8	1.2	ND
HIV-1_{JC1-1}	----HKTI------	----------Q-E-N-----	X4	5	0.4	ND
HIV-1_{JC1-2}	----SN-T-R----	---------RQ-R-D-----	X4	4	0.2	ND
HIV-1_{JC1-3}	-----N-I--H---	---------RG -RD--K---	R5	10	0.6	ND
HIV-1_{JC1-5}	-------T--G---	------V---G -RD--K---	X4	4	0.2	ND
HIV-1_{JC1-6}	----SN-T-R----	-----S--A-Q-RGD------	X4	6	0.7	ND
HIV-1_{JC1-9}	-------T--G---	------V---G -RD--K---	R5	21	1.6	ND
HIV-1_{JC1-11}	-------TS-G-R-	-------ASER -RD--K---	R5	34	3.2	ND
HIV-1_{JC1-22}	-----N-I--H---	---------RG -RD--K---	R5	12	1.2	ND
Primary isolates, clade B′						
HIV-$1_{92TH014}$	-----N-T--S-PL	-----W---GQ---D------	R5	8	0.9	>1.5
Primary isolates, clade E						
HIV-$1_{92TH002}$	----SN-T-TS-T-	---QV--R-GD---D--K-Y-	R5	>50	>50	ND
HIV-$1_{92TH022}$	----SN-T-TS-T-	---QV--R-GD---D--K-Y-	R5	>50	>50	>10
HIV-$1_{92TH025}$	----SN-T-TS-N-	---QV--R-GD---D--K-Y-	R5	>50	>50	ND
SHIV-B						
SHIV 89.6PD	-----N-T-R-LS-	-------ARR----D------	R5/X4	5	0.5	ND
SHIV C2/1	-----N-T-E-LS-	-------ARR----D------	R5/X4	5	0.5	ND

[a] The HIV-1 sequences were confirmed by proviral DNA sequencing of virus-infected cells.
[b] Dashes indicate sequence homology to HIV-1_{MN}, and spaces represent the presence of a deletion.
[c] ND, not done.

sequential immunization with synthetic V3 peptides from representatives of primary HIV-1 clade B isolates generated cross-reactive antisera and produced a high-affinity humanized MAb, KD-247, directed against the tip of the HIV-1 V3 domain, PGR. Furthermore, the humanized antibody more effectively neutralized several primary isolates of HIV-1 clade B than did previously reported neutralization antibodies (8a, 10, 23, 27). To further analyze the divergence of the cross-neutralization ability of the antibody by a PBMC-based HIV-1 neutralization assay, we used a panel of a total of 23 immunodeficiency viruses: 18 primary isolates of HIV-1 clade B, clade B′, and clade E viruses; 3 laboratory HIV-1 clade B viruses; and 2 highly pathogenic SHIVs (Table 1). The KD-247 antibody effectively neutralized HIV-1_{MN}, HIV-1_{SF2}, and HIV-$1_{89.6}$, containing the consensus V3 sequence of HIV-1 clade B, IGPGR AFY, with an IC_{90} and IC_{50} from 1 to 5 and from 0.1 to 1.0 μg/ml, respectively (Table 1, laboratory isolates, clade B). We next sought to assess whether the neutralization of primary isolates by KD-247 required a matching neutralization sequence motif. As expected, KD-247 effectively neutralized primary CCR5-tropic clade B and B′ isolates (IC_{90} and IC_{50} from 5 to 34 and from 0.4 to 3.2 μg/ml, respectively) and all four of the CXCR4-tropic clade B isolates (IC_{90} and IC_{50} from 4 to 6 and from 0.2 to 0.7 μg/ml, respectively) with matching IGPGR or V3 tip sequences. Thus, CCR5-tropic isolates with an IC_{90} of a mean concentration of neutralization antibody of 13.5 μg/ml were more than 2.8 times less sensitive to the neutralization by KD-247 than primary CXCR4-tropic isolates with a mean IC_{90} of 4.8 μg/ml. In contrast, the neutralization-resistant virus CS2-2 did not match the neutralization sequence motif, and the CS6-6 virus showed a QR insertion in the V3 tip sequence. The HIV-1 isolates containing a glutamine (Q) residue at position 20 in the V3 region, such as those of subtype E, were also resistant to neutralization by KD-247. Therefore, KD-247 effectively neutralizes both the CCR5- and CXCR4-tropic primary isolates with matching neutralization motifs.

Ex vivo suppressive effects of KD-247 on the generation of HIV-1 quasispecies from PBMCs of HIV-infected individuals. To fully assess the antiviral efficacy of KD-247, we next sought to determine whether it would suppress the generation of HIV-1 from PBMCs of HIV-infected individuals and whether it would do so as efficiently as an established anti-V3 humanized antibody, Cβ1 (23). As shown in Table 2, we investigated the effect of KD-247 at concentrations of 60 and 240 μg/ml on the ex vivo generation of HIV-1 using $CD8^+$-T-cell-depleted PBMC cultures from four Japanese individuals infected with HIV-1 clade B (Env V3 sequence in Table 2). In the presence of KD-247 at concentrations of 60 and 240 μg/ml, the gener-

TABLE 2. Ex vivo neutralizing activity of KD-247 against HIV-1 present in PBMC cultures established using cells from HIV-infected individuals[a]

Patient	HIV-1 Env V3 sequence (no. of clones)	PBMCs, (no. of cells/well)	KD-247 (μg/ml)	p24 (log_{10} pg/ml)
KU008	CTRPHNNTRKSIHIGPGRAFYATGDIIGNIRQAHC (3)	6.5×10^5	0	3.93
	----------E---D--R-- (2)		60	0.37
	----------E---D----- (1)		240	0.08
	----------D----- (1)			
KU045	CTRPNNNTRKGIHIGPGRAFYGTDIVGDIRQAHC (5)	7.3×10^5	0	3.70
	----------E-T-N----Y- (2)		60	0.88
	----------N------ (1)		240	0.56
KU037	CTRPNNNTRKSIPIGPGRAFYATGDIIGDIRKAHC (3)	1.3×10^6	0	3.81
	-------I---------- (1)		60	3.86
	-I------G---------- (1)		240	0.25
KU040	CTRPNNNTRKSVHIGPGRAWYATGEIIGNIRQAHC (2)	8.0×10^5	0	4.12
	----------A----F---------- (1)		60	2.34
	----------I--------H---------- (1)		240	2.62
	---H-------I-L---G--H---D--------- (1)			

[a] Ex vivo neutralization activity was directly detected by using $CD8^+$ cell-depleted PBMCs from HIV-infected individuals as described in Materials and Methods.
[b] The number of analyzed DNA clones from each patient is indicated in parentheses. Dashes indicate sequences identical to those of the upper major clone from each patient.

ation of viruses from PBMCs of KU008 was reduced in a dose-dependent manner, with 3.56- and 3.85-log reductions in the culture supernatants, respectively; reductions of 2.82 and 3.14 logs of virus generation from PBMCs of KU045 were also detected in the presence of 60 and 240 μg/ml of KD-247, respectively, KU037 showed a reduction of 3.56 logs at only 240 μg/ml. However, KU040 showed no dose-dependent suppressive effects of virus generation by KD-247. When the irrelevant antibodies of Cβ1 and normal serum IgG were added to cell cultures, they showed no suppressive effects on virus generation (data not shown). These results demonstrate that KD-247 effectively neutralizes nonpassage viruses generated in the primary culture of PBMCs from individuals infected with HIV-1 clade B with neutralization sequence motifs matching that of the quasispecies, IGPGR.

Induction of complete protection of monkeys against a highly pathogenic SHIV strain by a single passive transfer of a high dose of KD-247. PBMCs from 12 juvenile male cynomolgus monkeys were first evaluated in vitro to establish their susceptibility to infection with the SHIV C2/1 challenge stock in standard viral infectivity assays (35, 37) (data not shown). Challenge virus SHIV C2/1 originated from SHIV 89.6 but did share an identical envelope sequence with the parental strain, $HIV\text{-}1_{89.6}$, and showed 17 nucleotide mutations with amino acid changes (1, 34). The neutralization sensitivity of SHIV C2/1 to KD-247 was found to be similar to that of $HIV\text{-}1_{89.6}$, with an IC_{90} and IC_{50} of 5 and 0.5 μg/ml in human PBMC-based neutralization assays, respectively (Table 1, laboratory isolates, clade B and SHIV-B), suggesting that the neutralization potency of KD-247 in vitro might be sufficient to warrant passive transfer experiments.

Of the 12 monkeys, 5 were inoculated with KD-247, 2 were inoculated with control normal human IgG (NHIgG) (45 mg/kg), and the remaining 5 were given saline alone. Of the five animals receiving KD-247, two were given a dose of 45 mg/kg, two received 30 mg/kg, and one received 15 mg/kg. Twenty-four hours after antibody transfer, all 12 monkeys were given an intravenous challenge of 20 $TCID_{50}$s/ml SHIV (Fig. 1). At the time of viral challenge, the plasma concentrations of KD-247 were 151, 443, 496, 866, and 678 μg/ml of the antibody in immune sera from monkeys 3968, 3969, 3972, 4092, and 4099, respectively (Fig. 1a). The area under the plasma concentration time curve (AUC) values for monkeys 3968, 3969, 3972, 4092, and 4099 were calculated from the antibody concentration data to be 1.8, 3.5, 5.0, 6.5, and 5.6 mg · day/ml, respectively.

The percentage of $CD4^+$ T cells and the levels of plasma viremia were also monitored after SHIV challenge (Fig. 1b and c). All monkeys that were intravenously inoculated with normal human IgG or saline showed a loss of $CD4^+$ T cells within 7 days of viral challenge, accompanied by plasma viremia reaching 10^7 to 10^8 viral RNA copies/ml (data from the five control monkeys that received saline alone are not shown). Of the two control monkeys that received 45 mg/kg of NHIgG, both seroconverted against SHIV p27 antigen (monkeys 3967 and 3974) (Fig. 1d). At autopsy, all control monkeys showed $CD4^+$-T-cell depletion in lymphoid organs, a finding consistent with our previous observations using this model (35, 37).

Both monkeys that received a single high dose of 45 mg of KD-247 per kg of body weight prior to SHIV challenge were completely protected from viral challenge, maintaining stable $CD4^+$-T-cell counts and not seroconverting or exhibiting plasma viremia (Fig. 1b to e, monkeys 4092 and 4099, indicated by red lines and red characters). When evaluated at autopsy using PCR for SHIV *gag* proviral DNA, their tissues showed no sign of infection (data not shown). The titers in plasma resulting from 100% in vitro neutralization against 100 $TCID_{50}$s of the challenge virus at the time of virus challenge were 1:160 in both monkeys 4092 and 4099. The titers in partially protected monkeys 3969 and 3972 were 1:40 and 1:80, respectively. No neutralization activity of less than 1:10 was measured in the animals receiving 45 mg/kg of NHIgG (monkeys 3967 and 3974). Thus, although the highest titers of neutralization activities were detected in plasma from protected animals, the neutralization activity was high even in animals with only partial protection.

Administration of lower doses of KD-247, 30 mg/kg to two monkeys (monkeys 3969 and 3972, indicated by blue lines and

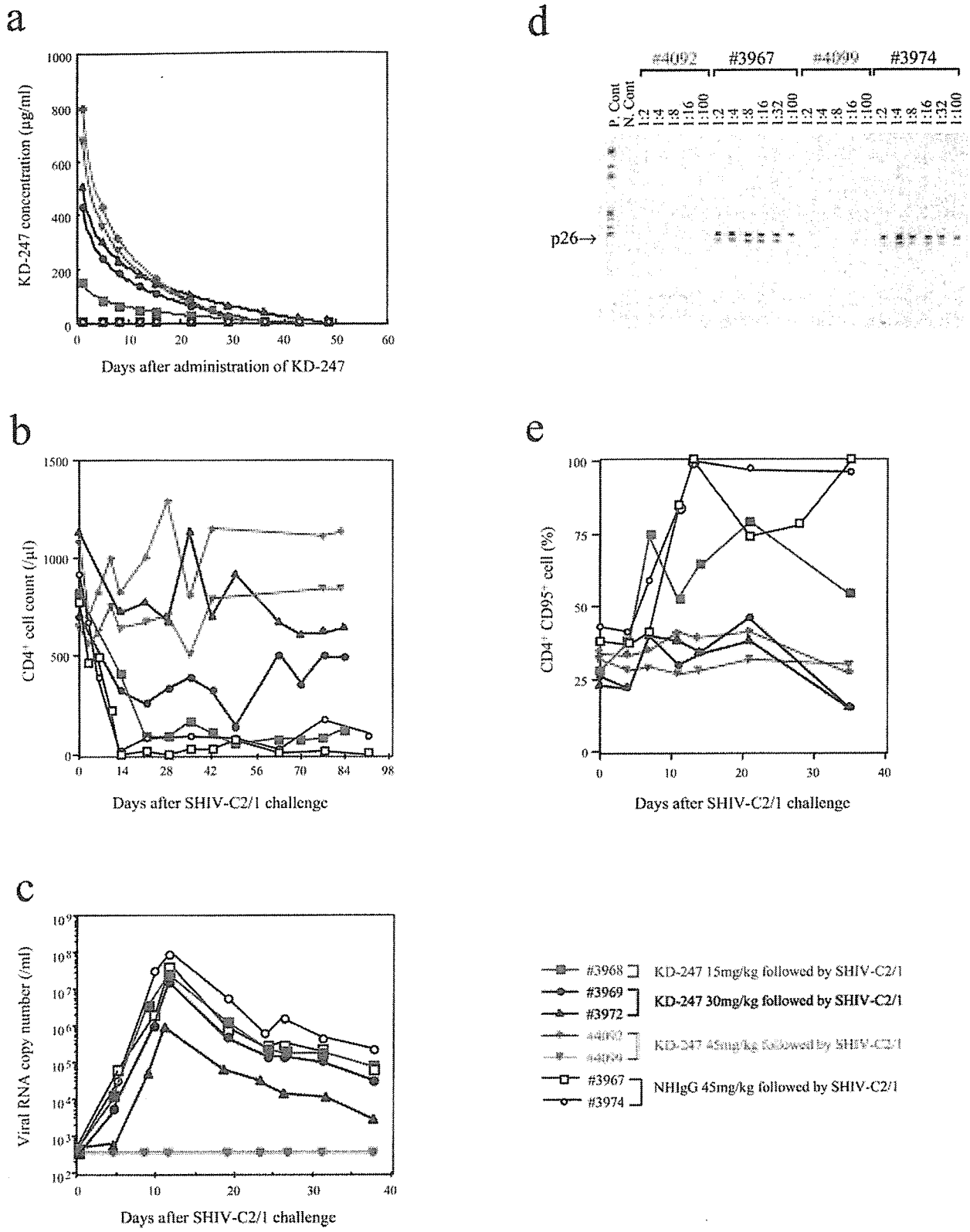

FIG. 1. KD-247 efficiently protects monkeys from pathogenic virus challenge. A total of 12 cynomolgus monkeys were used for virus challenge studies with SHIV C2/1. In the first group, five monkeys were intravenously inoculated with various doses of KD-247, followed by 20 $TCID_{50}$s of SHIV C2/1 challenge 24 h after antibody transfer. Monkeys in the second and third groups were injected prior to virus challenge with either 45 mg/kg of normal human immunoglobulin (two monkeys) or saline alone (five monkeys). The following parameters were measured in monkeys given KD-247: (a) concentration of KD-247 in plasma following passive transfer, (b) $CD4^+$-T-cell counts, (c) plasma viremia, (d) Western blot analysis using an HIV-2 Western blot kit (Diagnostics Pasteur, Marnes-La-Coquette, France) (6) of serum samples obtained at autopsy from monkeys given a single high dose (45 mg/kg) of KD-247 (monkeys 4092 and 4099) or NHIgG controls (monkeys 3967 and 3974), and (e) CD95 antigen expression on PBMCs from monkeys challenged with SHIV.

blue characters in Fig. 1) and 15 mg/kg to one monkey (monkey 3968, indicated by green lines and green characters in Fig. 1), afforded partial protection from SHIV infection. Monkey 3972 (Fig. 1, closed triangle with blue line) showed better partial protection than monkey 3969, which received 30 mg/kg of antibody. That superior degree of partial protection may be related to better blood concentration of the antibody and to better AUC values. All three monkeys described above seroconverted against SHIV p27 antigen (data not shown), but their loss of $CD4^+$ T cells seemed to be inversely proportional to the plasma concentration of KD-247 (Fig. 1a and b). Although the $CD4^+$-T-cell decline indicated minimal protection in the monkey given 15 mg/kg of KD-247 (monkey 3968) (Fig. 1b), CD95 antigen expression, a marker for cell stimulation, was significantly lowered in this animal and completely inhibited in the other four monkeys receiving KD-247 (Fig. 1e), suggesting that KD-247 significantly suppressed PBMC stimulation by the virus challenge in these animals (monkeys 3969, 3972, 4092, and 4099).

These results therefore demonstrate that KD-247 efficiently neutralizes primary HIV isolates regardless of cell tropism. Furthermore, passive immunization with a single dose of 45 mg of antibodies per kg of body weight 24 h prior to viral challenge completely protected animals from viral challenge, showing that at high concentrations, KD-247 lowers the viral load and induces sterilizing immunity in the monkey model.

DISCUSSION

In this study, KD-247 proved an effective antiviral agent for the targeting of phenotype-matched viruses, one capable of both in vitro neutralization of primary isolates and in vivo passive transfer of the antibody as well as of suppressive effects against ex vivo generation of HIV from HIV-infected individuals. Although it has already been established that brief immunizations with a V3 peptide can elicit neutralizing antibodies to homologues of the CXCR4-tropic virus, the limitations of anti-V3 antibodies have been known for over a decade (8, 13, 16, 28). Also, at reasonable IC_{50}s, the anti-V3 antibodies did not neutralize CCR5-tropic strains. In the accompanying paper (8a), we described the derivation of a humanized MAb, KD-247, that was produced by sequential immunization using six different HIV-1 Env V3 peptides derived from HIV-1 clade B field isolates. We suggested that KD-247 could potentially overcome the previous limitations to immunologically exploiting the anti-V3 antibody induced by brief immunization protocols, i.e., its extraordinary sequence variability and the associated isolate specificity of anti-V3 antibodies (27, 38). The findings of our current study suggest that KD-247 may curb the spread of viral infection and reduce viral loads in HIV-infected individuals who have been determined to share the V3 tip sequence of the virus by virus neutralization phenotype-matching analysis.

In vitro, KD-247 has potent neutralizing activity against a variety of primary HIV-1 clade B isolates, including CCR5-tropic viruses, at low concentrations. We found that KD-247 neutralized a variety of clade B primary viruses containing IGPGR V3 sequences, although its neutralization ability was affected by some of the surrounding amino acids of the V3 tip region, as discussed in the accompanying paper (8a). Based upon these results, we should be able to predict the neutralization ability of KD-247 by prior sequencing of the HIV-1 Env V3 region of the target virus. Using the previously published sequences found in the Los Alamos HIV-1 sequence database, we determined that the IGPGRA sequence is present in the majority of HIV-1 clade B isolates (45) to which KD-247 would be expected to have cross-neutralization activity. Moreover, KD-247 significantly curbed the generation of primary HIV-1 quasispecies in ex vivo cultures of $CD8^+$-T-cell-depleted PBMCs from seropositive individuals. However, as described above, the major limitation of KD-247 as an antiviral agent is its inability to neutralize variants expressing amino acid alterations in the binding site PGR motif and additional amino acids.

What are the properties that make KD-247 an effective neutralizer of CCR5-tropic viruses? First, the site-specific binding of KD-247 to epitopes on the virus envelope glycoprotein seems to be key to its virus neutralization ability. Indeed, the results of the Pepscan analysis reported in the accompanying paper suggest that KD-247 can react with core V3 sequences from various HIV-1 clade B isolates (8a). The shortest peptide that was reactive with KD-247 was IGPGR, but that epitope was stabilized by the addition of one or more amino acids. Furthermore, IGPGRA and GPGRAF sequences occur in the majority of HIV-1 isolates from donors in the United States (17). The results of Pepscan with replacement peptides also suggest that KD-247 has broad binding activity to HIV-1. While the number of amino acid substitutions tolerated in the central PGR sequence of the V3 tip peptide was small, replacement of amino acids in the flanking region was relatively permissible. Second, ex vivo neutralization assays using patient-derived isolates containing APGR and GPGG sequences in the V3 tip showed incomplete neutralization (Table 2, KU040). Thus, KD-247 would be expected to bind with HIV-1 quasispecies having a recognition sequence similar to the neutralization phenotype. Third, as the accompanying paper demonstrates, high-affinity antibody binding is apparently required for neutralization, because the kinetic parameters of KD-247 were identified to be fast on and slow off rates, similar to those of a type-specific MAb, Rμ5.5, although the equilibrium dissociation constant value of KD-247 for binding to a control SP1 peptide was higher than that of Rμ5.5 (8a). This is a reasonable assumption, since the epitope of KD-247 (IGPGR) is shorter than that of Rμ5.5 (IHIGPGRAFYT). The high association rate of KD-247 might be responsible for exerting the observed cross-neutralization activity against various primary isolates. These results are consistent with the hypothesis that virus neutralization can be explained by the kinetic parameters of antibody binding.

Most recent passive transfer studies with monoclonal antibodies used common combinations of broadly cross-reactive human MAbs capable of neutralizing primary HIV-1 isolates. In monkeys, human MAbs b12 (29) and 2G12 (20) were shown to induce complete and partial protection, respectively, against viral challenges. In contrast, the MAb chosen for this study, KD-247, is a humanized antibody indueed by sequential immunization with a set of V3 peptides from primary isolates. Because the KD-247 IC_{90} value from an in vitro neutralization assay in our study, 5.0 μg/ml of the antibody, approximates that obtained by a single antibody, b12 (3), and a combination of

the two MAbs 2F5 and 2G12 or a triple combination of HIVIG, 2F5, and 2G12, as previously reported (41, 43), we postulated that KD-247 was sufficiently potent to achieve protection of monkeys against a pathogenic SHIV challenge. Since our previous experience (9) has taught us to expect approximately 500 to 1,000 μg/ml in sera from monkeys passively immunized with 30 to 45 mg of antibody per kg of body weight, the potency of KD-247 should prove sufficient for passive transfer experiments of effective antibodies in animals in vivo. We also expected that a single passive transfer of KD-247 via inoculation with 15 and 30 mg of antibody would result in approximately 150 to 500 μg/ml of plasma concentration at the time of viral challenge. As expected, we found an AUC value of 1.8 to 5.0 mg · day/ml. Consequently, we found that animals passively immunized with 45 mg/kg of KD-247 showed 678 and 866 μg/ml of KD-247 in plasma at the time of viral challenge and an AUC value of 5.6 and 6.5 mg · day/ml. Those animals were provided sterile protection against intravenous challenge with the pathogenic virus SHIV C2/1. The protective endpoint titers of neutralization antibodies in plasma at the time of virus inoculation were 1:160 in both animals that elicited sterile immunity, and a high titer of neutralization activity in plasma was similarly detected in completely protected monkeys, as described previously by Nishimura et al. (26) and Parren et al. (29). Thus, the high titers of neutralization activity in plasma confer sterile protection against viral challenge in the passively immunized animals with neutralizing antibodies. Furthermore, the pharmacokinetic information consisting of the plasma concentration of the neutralizing antibodies at the time of viral challenge and the AUC value may be closely related to the ability of the antibody to provide sterile protection against viral challenge. Since those protected macaques demonstrated the inhibition of $CD4^+$ cell loss, the pharmacokinetic properties of KD-247 may also be closely associated with the inhibition of $CD4^+$ cell decline in the peripheral circulation of the challenged monkeys.

In this study, we also detected lower viremia with lesser $CD4^+$ cell decline in animals that were inoculated with intermediate doses of antibody. However, we noted that the lesser doses of the antibody provided complete protection against enhanced rates of the $CD4^+$ $CD95^+$ cell subpopulation in the peripheral circulation of the challenged animals, suggesting that the reshaping MAb might be able to control the activation of peripheral $CD4^+$ T cells in animals by its passive transfer. Although the number of monkeys enrolled in this study was limited, it remains noteworthy that a single inoculation with KD-247, even at a suboptimal dose for viral protection, appeared to be effective for maintaining $CD4^+$ T cells in monkeys inoculated with virus. Since it has been previously reported that the limited effect of neutralizing antibody may be related to the rapid appearance of an escape mutant in infected individuals, high titers of neutralization activity should be generated in the passively immunized animals (25, 33, 44). In our preliminary study, we isolated the escape mutant from the neutralization resistance virus $HIV\text{-}1_{JR\text{-}FL}$ in the presence of KD-247: at passage 8 of the culture in the presence of 1,000 μg/ml KD-247, one amino acid substitution, GPGR to GPER, was identified in the V3 tip (K. Yoshimura et al., unpublished results). Collectively, these results suggest that KD-247 shows clinical promise both for passive immunization and as a strategy for preventing viral spread in phenotype-matched HIV-infected individuals.

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