61. Burastero SE, Gaffi D, Lopalco L, Tambussi G, Borgonovo B, De Santis C, *et al.* **Autoantibodies to CD4 in HIV type 1-exposed seronegative individuals.** *AIDS Res Hum Retrovir* 1996; **12**:273–280.
62. Lopalco L, Pastori C, Cosma A, Burastero SE, Capiluppi B, Boeri E, *et al.* **Anticell antibodies in exposed seronegative individuals with HIV type 1-neutralizing activity.** *AIDS Res Hum Retrovir* 2000; **16**:109–115.
63. Lopalco L, Magnani Z, Confetti C, Brianza M, Saracco A, Ferraris G, *et al.* **Anti-CD4 antibodies in exposed seronegative adults and in newborns of HIV type 1-seropositive mothers: a follow-up study.** *AIDS Res Hum Retrovir* 1999; **15**:1079–1085.
64. Callahan LN, Roderiquez G, Mallinson M, Norcross MA. **Analysis of HIV-induced autoantibodies to cryptic epitopes on human CD4.** *J Immunol* 1992; **149**:2194–2202.
65. Furci L, Beretta A, Siccardi A, Lazzarin A, Confetti C, Magnani Z, *et al.* **Human immunodeficiency virus type 1 glycoprotein 120-specific T lymphocytes provide intermolecular help for anti-CD4 autoantibody production in exposed uninfected subjects.** *AIDS Res Hum Retrovir* 1997; **13**:1461–1469.
66. Lopalco L, Barassi C, Pastori C, Longhi R, Burastero SE, Tambussi G, *et al.* **CCR5-reactive antibodies in seronegative partners of HIV-seropositive individuals down-modulate surface CCR5 in vivo and neutralize the infectivity of R5 strains of HIV-1 in vitro.** *J Immunol* 2000; **164**:3426–3433.
67. Pastori C, Weiser B, Barassi C, Uberti-Foppa C, Ghezzi S, Longhi R, *et al.* **CCR5 internalization by antibodies in a subset of long-term non progressors: a possible protective effect against disease progression.** *Blood* 2006; **107**:4825–4833.
68. Margolis L, Shattock R. **Selective transmission of CCR5-utilizing HIV-1: the 'gatekeeper' problem resolved?** *Nat Rev Microbiol* 2006; **4**:313–318.
69. Lopalco L, Barassi C, Paolucci C, Breda D, Brunelli D, Nguyen M, *et al.* **Predictive value of anticell and antihuman immunodeficiency virus (HIV) humoral responses in HIV-1-exposed seronegative cohorts of European and Asian origin.** *J Gen Virol* 2005; **86**:339–348.
70. Rizzardini G, Piconi S, Ruzzante S, Fusi ML, Lukwiya M, Declich S, *et al.* **Immunological activation markers in the serum of African and European HIV-seropositive and seronegative individuals.** *AIDS* 1996; **10**:1535–1542.
71. Rizzardini G, Trabattoni D, Saresella M, Piconi S, Lukwiya M, Declich S, *et al.* **Immune activation in HIV-infected African individuals. Italian–Ugandan AIDS cooperation program.** *AIDS* 1998; **12**:2387–2396.
72. Clerici M, Butto S, Lukwiya M, Saresella M, Declich S, Trabattoni D, *et al.* **Immune activation in Africa is environmentally-driven and is associated with upregulation of CCR5.** *AIDS* 2000; **14**:2083–2092.
73. Ditzel HJ, Rosenkilde MM, Garred P, Wang M, Koefoed K, Pedersen C, *et al.* **The CCR5 receptor acts as an alloantigen in CCR5 Delta32 homozygous individuals: identification of chemokine and HIV-1-blocking human antibodies.** *Proc Natl Acad Sci U S A* 1998; **95**:5241–5245.
74. Bouhlal H, Hocini H, Quillent-Gregoire C, Donkova V, Rose S, Amara A, *et al.* **Antibodies to C-C chemokine receptor 5 in normal human IgG block infection of macrophages and lymphocytes with primary R5-tropic strains of HIV-1.** *J Immunol* 2001; **166**:7606–7611.
75. Bouhlal H, Latry V, Requena M, Aubry S, Kaveri SV, Kazatchkine MD, *et al.* **Natural antibodies to CCR5 from breast milk block infection of macrophages and dendritic cells with primary R5-tropic HIV-1.** *J Immunol* 2005; **174**:7202–7209.
76. Eslahpazir J, Jenabian M, Bouhlal H, Hocini H, Carbonneil C, Gresenguet G, *et al.* **Infection of macrophages and dendritic cells with primary R5-tropic human immunodeficiency virus type 1 inhibited by natural polyreactive anti-CCR5 antibodies purified from cervicovaginal secretions.** *Clin Vacc Immunol* 2008; **15**:872–874.
77. Russo S, Lopalco L. **Is autoimmunity a component of natural immunity to HIV?** *Curr HIV Res* 2006; **4**:177–190.
78. Zandman-Goddard G, Shoenfeld Y. **HIV and autoimmunity.** *Autoimmun Rev* 2002; **1**:329–337.
79. de Santis C, Lopalco L, Robbioni P, Longhi R, Rappocciolo G, Siccardi AG, Beretta A. **Human antibodies to immunodominant C5 region of HIV-1 gp120 cross-react with HLA class I on activated cells.** *AIDS Res Hum Retrovir* 1994; **10**:157–162.
80. Haynes BF, Fleming J, St Clair EW, Katinger H, Stiegler G, Kunert R, *et al.* **Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies.** *Science* 2005; **308**: 1906–1908.
81. Alam SM, McAdams M, Boren D, Rak M, Scearce RM, Gao F, *et al.* **The role of antibody polyspecificity and lipid reactivity in binding of broadly neutralizing anti-HIV-1 envelope human monoclonal antibodies 2F5 and 4E10 to glycoprotein 41 membrane proximal envelope epitopes.** *J Immunol* 2007; **178**:4424–4435.
82. Haynes BF, Moody MA, Verkoczy L, Kelsoe G, Alam SM. **Antibody polyspecificity and neutralization of HIV-1: an hypothesis.** *Hum Antibodies* 2005; **14**:59–67.
83. Vcelar B, Stiegler G, Wolf HM, Muntean W, Leschnik B, Mehandru S, *et al.* **Reassessment of autoreactivity of the broadly neutralizing HIV antibodies 4E10 and 2F5 and retrospective analysis of clinical safety data.** *AIDS* 2007; **21**:2161–2170.
84. Scherer EM, Zwick MB, Teyton L, Burton DR. **Difficulties in eliciting broadly neutralizing anti-HIV antibodies are not explained by cardiolipin autoreactivity.** *AIDS* 2007; **21**:2131–2139.
85. Kozlowski PA, Neutra MR. **The role of mucosal immunity in prevention of HIV transmission.** *Curr Mol Med* 2003; **3**:217–228.
86. Outlaw MC, Armstrong SJ, Dimmock NJ. **Mechanisms of neutralization of influenza virus in tracheal epithelial and BHK cells vary according to IgG concentration.** *Virology* 1990; **178**:478–485.
87. Robert-Guroff M. **IgG surfaces as an important component in mucosal protection.** *Nat Med* 2000; **6**:129–130.
88. Burns JW, Siadat-Pajouh M, Krishnaney AA, Greenberg HB. **Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity.** *Science* 1996; **272**:104–107.
89. Krause RM, Dimmock NJ, Morens DM. **Summary of antibody workshop: the role of humoral immunity in the treatment and prevention of emerging and extant infectious diseases.** *J Infect Dis* 1997; **176**:549–559.
90. van Egmond M, Damen CA, van Spriel AB, Vidarsson G, van Garderen E, van de Winkel JG. **IgA and the IgA Fc receptor.** *Trends Immunol* 2001; **22**:205–211.
91. Belec L, Ghys PD, Hocini H, Nkengasong JN, Tranchot-Diallo J, Diallo MO, *et al.* **Cervicovaginal secretory antibodies to HIV type 1 that block viral transcytosis through epithelial barriers in highly exposed HIV-1- seronegative African women.** *J Infect Dis* 2001; **184**:1412–1422.
92. Bomsel M, Heyman M, Hocini H, Lagaye S, Belec L, Dupont C, Desgranges C. **Intracellular neutralization of HIV transcytosis across tight epithelial barriers by anti HIV envelope protein dIgA or IgM.** *Immunity* 1998; **9**:277–287.
93. Barassi C, Lazzarin A, Lopalco L. **CCR5-specific mucosal IgA in saliva and genital fluids of HIV-exposed seronegative subjects.** *Blood* 2004; **104**:2205–2206.
94. Wanchu A, Sachdev R, Bagga R. **HIV-1 gag-specific mucosal antibodies in a cohort of exposed but uninfected heterosexual partners of HIV-1-infected Individuals in north India.** *CROI* 2008; Boston, MA, USA; 3–6 February 2008, abstract 430.
95. Kaul R, Trabattoni D, Bwayo JJ, Arienti D, Zagliana A, Mwangi FM, *et al.* **HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers.** *AIDS* 1999; **13**:23–29.
96. Hirbod T, Kaul R, Reichard C, Kimani J, Ngugi E, Bwayo JJ, *et al.* **HIV-neutralizing IgA and HIV-specific proliferation are independently associated with reduced HIV acquisition in Kenyan sex workers.** *AIDS* 2008; **22**:727–735.
97. Beyrer C, Artenstein AW, Rugpao S, Stephens H, VanCott TC, Robb ML, *et al.* **Epidemiologic and biologic characterization of a cohort of human immunodeficiency virus type 1 highly exposed, persistently seronegative female sex workers in northern Thailand.** *J Infect Dis* 1999; **179**:59–67.
98. Nguyen M, Pean P, Lopalco L, Nouhin J, Phoung V, Ing R, *et al.* **HIV specific antibodies but not T cell responses are associated to protection in seronegative partners of HIV infected individuals in Cambodia.** *J AIDS* 2006; **42**:412–419.
99. Ghys PD, Belec L, Diallo MO, Ettiegne-Traore V, Becquart P, Maurice C, *et al.* **Cervicovaginal anti-HIV antibodies in HIV-seronegative female sex workers in Abidjan, Cote d'Ivoire.** *AIDS* 2000; **14**:2603–2608.

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100. Buchacz K, Parekh BS, Padian NS, Van Der Straten A, Phillips S, Jonte J, Holmberg SD. **HIV-specific IgG in cervicovaginal secretions of exposed HIV-uninfected female sexual partners of HIV infected men.** *AIDS Res Hum Retrovir* 2001; **17**:1689–1693.
101. Farquhar C, VanCott T, Bosire R, Bermudez C, Mbori-Ngacha D, Lohman-Payne B, *et al.* **Salivary human HIV-specific immunoglobulin A in HIV-1-exposed infants in Kenya.** *Clin Exp Immunol* 2008; **153**:37–43.
102. Sangster MY, Riberdy JM, Gonzalez M, Topham DJ, Baumgarth N, *et al.* **An early CD4+ T cell-dependent immunoglobulin A response to influenza infection in the absence of key cognate T–B interactions.** *J Exp Med* 2003; **198**:1011–1021.
103. Gärdby E, Wrammert J, Schön K, Ekman L, Leanderson T, *et al.* **Strong differential regulation of serum and mucosal IgA responses as revealed in CD28-deficient mice using cholera toxin adjuvant.** *J Immunol* 2003; **170**:55–63.
104. Fayette J, Dubois B, Vandenabeele S, Bridon JM, Vanbervliet B, *et al.* **Human dendritic cells skew isotype switching of CD40-activated naive B cells towards IgA1 and IgA2.** *J Exp Med* 1997; **185**:1909–1918.
105. Clerici M, Barassi C, Devito C, Pastori C, Piconi S, Trabattoni D, *et al.* **Serum IgA of HIV-exposed uninfected individuals inhibit HIV through recognition of a region within the alpha-helix of gp41.** *AIDS* 2002; **16**:1731–1741.
106. Lopalco L, Longhi R, Ciccomascolo F, De Rossi A, Pelagi M, Andronico F, *et al.* **Identification of human immunodeficiency virus type 1 glycoprotein gp120/gp41 interacting sites by the idiotypic mimicry of two monoclonal antibodies.** *AIDS Res Hum Retrovir* 1993; **9**:33–39.
107. Devito C, Hinkula J, Kaul R, Lopalco L, Bwayo JJ, Plummer FA, *et al.* **Mucosal IgA from HIV-exposed seronegative individuals neutralize a primary HIV-1 isolate.** *AIDS* 2000; **14**:1917–1920.
108. Devito C, Hinkula J, Kaul R, Kimani J, Kiama P, Lopalco L, *et al.* **Cross-clade HIV-1-specific neutralizing IgA in mucosal and systemic compartments of HIV-1-exposed, persistently seronegative subjects.** *J AIDS* 2002; **30**:413–420.
109. Devito C, Broliden K, Kaul R, Svensson L, Johansen K, Kiama P, *et al.* **Mucosal and plasma IgA from HIV-1-exposed uninfected individuals inhibit HIV-1 transcytosis across human epithelial cells.** *J Immunol* 2000; **165**:5170–5176.
110. Alfsen A, Iniguez P, Bouguyon E, Bomsel M. **Secretory IgA specific for a conserved epitope on gp41 envelope glycoprotein inhibits epithelial transcytosis of HIV-1.** *J Immunol* 2001; **166**:6257–6626.
111. Lazarus NH, Kunkel EJ, Johnston B, Wilson E, Youngman KR, Butcher EC. **A common mucosal chemokine (mucosae-associated epithelial chemokine/CCL28) selectively attracts IgA plasmablasts.** *J Immunol* 2003; **170**:3799–3805.
112. Hieshima K, Kawasaki Y, Hanamoto H, Nakayama T, Nagakubo D, Kanamaru A, *et al.* **CC chemokine ligands 25 and 28 play essential roles in intestinal extravasation of IgA antibody-secreting cells.** *J Immunol* 2004; **173**:3668–3675.
113. Castelletti E, Lo Caputo S, Kuhn L, Borelli M, Gajardo J, Sinkala M, *et al.* **The mucosae-associated epithelial chemokine (MEC/CCL28) modulates immunity in HIV infection.** *PLoS ONE* 2007; **2**:e969; doi: 10.1371/journal.pone.0000969.
114. Buchacz K, Parekh BS, Padian NS, Van der Straten A, Phillips S, Jonte J, Holmberg SD. **HIV-specific IgG in cervicovaginal secretions of exposed HIV-uninfected female sexual partners of HIV-infected men.** *AIDS Res Hum Retrovir* 2001; **17**:1689–1693.
115. Dorrell L, Hessell AJ, Wang M, Whittle H, Sabally S, Rowland-Jones S, *et al.* **Absence of specific mucosal antibody responses in HIV-exposed uninfected sex workers from the Gambia.** *AIDS* 2000; **14**:1117–1122.
116. Jackson S, Prince S, Kulhavy R, Mestecky J. **False positivity of enzyme-linked immunosorbent assay for measurement of secretory IgA antibodies directed at HIV type 1 antigens.** *AIDS Res Hum Retrovir* 2000; **16**:595–602.
117. Wright PF, Kozlowski PA, Rybczyk GK, Goepfert P, Staats HF, VanCott TC, *et al.* **Detection of mucosal antibodies in HIV type 1-infected individuals.** *AIDS Res Hum Retrovir* 2002; **18**:1291–1300.
118. Kutteh WH, Prince SJ, Hammond KR, Kutteh CC, Mestecky J. **Variations in immunoglobulins and IgA subclasses of human uterine cervical secretions around the time of ovulation.** *Clin Exp Immunol* 1996; **104**:538–542.
119. Hirbod T, Broliden K. **Mucosal immune responses in the genital tract of HIV-1-exposed uninfected women.** *J Intern Med* 2007; **262**:44–58.
120. Becquart P, Hocini H, Levy M, Sepou A, Kazatchkine D, Belec L. **Secretory antihuman immunodeficiency virus (HIV) antibodies in colostrums and breast milk are not a major determinant of the protection of early postnatal transmission of HIV.** *J Infect Dis* 2000; **181**:532–539.
121. Kuhn L, Trabattoni D, Kankasa C, Sinkala M, Lissoni F, Ghosh M, *et al.* **HIV-specific secretory IgA in breast milk of HIV-positive mothers is not associated with protection against HIV transmission among breast-fed infants.** *J Pediatr* 2006; **149**: 611–616.
122. Giavendoni LD, Velasquillo MC, Parodi LM, Hubbard GB, Hodora VL. **Cytokine expression, natural killer cell activation, and phenotypic changes in lymphoid cells from rhesus macaques during acute infection with pathogenic simian immunodeficiency virus.** *J Virol* 2000; **74**:1648–1657.
123. Kittilil S, Chun T-W, Mori S, Liu M, McLaughlin C, *et al.* **Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function.** *J Infect Dis* 2003; **187**:1038–1045.
124. Bruunsgaart H, Pedersen C, Skinhøj P, Pedersen BK. **Clinical progression of HIV infection: role of NK cells.** *Scad J Immunol* 1997; **46**:91–95.
125. Jennes W, Verheyden S, Demanet C, Adje-Toure CA, Vuylsteke B, *et al.* **Resistance to HIV-1 infection among African female sex workers is associated with inhibitory KIR in the absence of their HLA ligands.** *J Immunol* 2006; **177**:6588–6592.
126. Boulet S, Sharafi S, Simic N, Bruneau J, Routy J-P, *et al.* **Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals.** *AIDS* 2008; **22**:595–599.
127. Ravet S, Scott-Algara D, Bonnet E, Tran HK, Tran T, Nguyen N, *et al.* **Distinctive NK-cell receptor repertoires sustain high-level constitutive NK-cell activation in HIV-exposed uninfected individuals.** *Blood* 2007; **109**:4296–4305.
128. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ, *et al.* **Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS.** *Nat Genet* 2002; **31**:429–434.
129. Boulet S, Kleyman M, Kim JY, Kamya P, Sharafi S, Simic N, *et al.* **A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection.** *AIDS* 2008; **22**:1487–1491.
130. Chiu YL, Greene WC. **Multifaceted antiviral actions of APOBEC3 cytidine deaminases.** *Trends Immunol* 2006; **27**:291–297.
131. Biasin M, Piacentini L, Lo Caputo S, Kanari Y, Magri G, Trabattoni D, *et al.* **Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G: a possible role in the resistance to HIV of HIV-exposed seronegative individuals.** *J Infect Dis* 2007; **195**:960–964.
132. Oliva H, Montserrat P, Lopez A, Martinez-Navio J, Franco R, Miro' J, *et al.* **APOBEC3G protein expression in blood resting and activated CD4+ and CD8+ T cells. Its effect on susceptibility to HIV infection and progression.** *CROI* 2008; Boston, MA, USA; 3–6 February 2008, abstract 212.
133. Misse D, Yssel H, Trabattoni D, Oblet C, Lo Caputo S, Mazzotta F, *et al.* **IL-22 participates in an innate anti-HIV-1 host-resistance network through acute-phase protein induction.** *J Immunol* 2007; **178**:407–415.
134. Yang C, Li M, Limpakarnjanarat K, Young NL, Hodge T, Butera ST, *et al.* **Polymorphisms in the *CCR5* coding and noncoding regions among HIV type 1-exposed, persistently seronegative female sex-workers from Thailand.** *AIDS Res Hum Retrovir* 2003; **19**:661–665.
135. Sriwanthana B, Hodge T, Mastro TD, Dezzutti CS, Bond K, Stephens HA, *et al.* **HIV-specific cytotoxic T lymphocytes, HLA-A11, and chemokine-related factors may act synergistically to determine HIV resistance in *CCR5Δ32*-negative female sex workers in Chiang Rai, northern Thailand.** *AIDS Res Hum Retrovir* 2001; **17**:719–734.
136. Smith MW, Dean M, Carrington M, Winkler C, Huttley GA, Lomb DA, *et al.* **Contrasting genetic influence of *CCR2* and *CCR5* variants on HIV-1 infection and disease progression.** *Science* 1997; **277**:959–965.

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137. Louisirirotchanakul S, Liu H, Roongpisuthipong A, Nakayama EE, Takebe Y, Shioda T, Wasi C. **Genetic analysis of HIV-1 discordant couples in Thailand: association of *CCR2-64I* homozygosity with HIV-1-negative status.** *J Acquir Immune Defic Syndr* 2002; **29**:314–315.
138. Liu H, Hwangbo Y, Holte S, Lee J, Wang C, Kaupp N, *et al.* **Analysis of genetic polymorphisms in CCR5, CCR2, stromal cell-derived factor-1, RANTES, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in seronegative individuals repeatedly exposed to HIV-1.** *J Infect Dis* 2004; **190**:1055–1058.
139. Winkler C, Modi W, Smith MW, Nelson GW, Wu X, Carrington M, *et al.* **Genetic restriction of AIDS pathogenesis by an SDF-1 chemokine gene variant.** *Science* 1998; **279**:389–393.
140. Garred P, Madsen HO, Balslev U, Hofmann B, Pedersen C, Gerstoft J, *et al.* **Susceptibility to HIV infection and progression of AIDS in relation to variant alleles of mannose-binding lectin.** *Lancet* 1997; **349**:236–240.
141. Mombo LE, Lu CY, Ossari S, Bedjabaga I, Sica L, Krishnamoorthy R, *et al.* **Mannose-binding lectin alleles in sub-Saharan Africans and relation with susceptibility to infections.** *Genes Immun* 2003; **4**:362–367.
142. Wichukchinda N, Kitamura Y, Rojanawiwat A, Nakayama EE, Song H, Pathipvanich P, *et al.* **The polymorphisms in DC-SIGNR affect susceptibility to HIV type 1 infection.** *AIDS Res Hum Retrovir* 2007; **23**:686–692.
143. de la Torre MS, Torres C, Nieto G, Vergara S, Carrero AJ, Macias J, *et al.* **Vitamin D receptor gene haplotypes and susceptibility to HIV-1 infection in injection drug users.** *J Infect Dis* 2008; **197**:405–410.
144. Ball TB, Ji HZ, Kimani J, McLaren P, Marlin C, Hill AVS, *et al.* **Polymorphisms in IRF-1 associated with resistance to HIV-1 infection in highly exposed uninfected Kenyan sex workers.** *AIDS* 2007; **21**:1091–1101.
145. Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G, *et al.* **The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility.** *Science* 2005; **307**:1434–1440.
146. Dolan MJ, Kulkarni H, Camargo JF, He W, Smith A, Anaya JM, *et al.* **CCL3L1 and CCR5 influence cell-mediated immunity and affect HIV-AIDS pathogenesis via viral entry-independent mechanisms.** *Nat Med* 2007; **8**:1324–1336.
147. Kuhn L, Schramm DB, Donninger S, Meddows-Taylor S, Coovadia AH, Sherman GG, *et al.* **African infants' CCL3 gene copies influence perinatal HIV transmission in the absence of maternal nevirapine.** *AIDS* 2007; **21**:1753–1761.
148. Pilotti E, Elviri L, Vicenzi E, Bertazzoni U, Re MC, Allibardi S, *et al.* **Postgenomic up-regulation of CCL3L1 expression in HTLV-2-infected persons curtails HIV-1 replication.** *Blood* 2007; **109**:1850–1856.
149. Hardie RA, Luo M, Bruneau B, Knight E, Nagelkerke NJ, Kimani J, *et al.* **Human leukocyte antigen-DQ alleles and haplotypes and their associations with resistance and susceptibility to HIV-1 infection.** *AIDS* 2008; **22**:807–816.
150. MacDonald KS, Embree J, Njenga S, Nagelkerke NJD, Ngatia I, Mohammed Z, *et al.* **Mother-child class I HLA concordance increases perinatal human immunodeficiency virus type 1 transmission.** *J Infect Dis* 1998; **177**:551–556.
151. Kanari Y, Clerici M, Abe H, Kawabata H, Trabattoni D, Lo Caputo S, *et al.* **Genotypes at chromosome 22q12–13 are associated with HIV-1-exposed but uninfected status in Italians.** *AIDS* 2005; **19**:1015–1024.
152. Cole AM, Cole AL. **Antimicrobial polypeptides are key anti-HIV-1 effector molecules of cervicovaginal host defense.** *Am J Reprod Immunol* 2008; **59**:27–34.
153. Mackewicz CE, Yuan J, Tran P, Diaz L, Mack E, Selsted ME, Levy JA. **α-Defensins can have anti-HIV activity but are not CD8+ cell anti-HIV factors.** *AIDS* 2003; **17**:F23–F32.
154. Kulkarni PS, Butera ST, Duerr AC. **Resistance to HIV-1 infection: lessons learnt from studies of highly exposed persistently serogenative (HEPS) individuals.** *AIDS Rev* 2003; **5**:87–103.
155. Soriano A, Martinez C, Garcia F, Plana M, Palou E, Lejeune M, *et al.* **Plasma stromal cell-derived factor (SDF)-1 levels, *SDF1-3'A* genotype, and expression of CXCR4 on T lymphocytes: their impact on resistance to human immunodeficiency virus type 1 infection and its progression.** *J Infect Dis* 2002; **186**:922–931.
156. Malik S, Arias M, Di Flumeri C, Garcia LF, Schurr E. **Absence of association between mannose-binding lectin gene polymorphisms and HIV-1 infection in a Colombian population.** *Immunogenetics* 2003; **55**:49–52.
157. Marquet S, Sanchez FO, Arias M, Rodriguez J, Paris SC, Skamene E, *et al.* **Variants of the human *NRAMP1* gene and altered human immunodeficiency virus infection susceptibility.** *J Infect Dis* 1999; **180**:1521–1525.
158. Chesebro B, Miyazawa M, Britt WJ. **Host genetic control of spontaneous and induced immunity to Friend murine retrovirus infection.** *Annu Rev Immunol* 1990; **8**:477–499.
159. Miyazawa M. **Host genes that influence immune and non-immune resistance mechanisms against retrovrial infections.** *Recent Res Dev Virol* 2004; **6**:105–118; 2004.
160. Miyazawa M, Tsuji-Kawahara S, Kanari Y. **Host genetic factors that control immune responses to retrovirus infections.** *Vaccine* 2008. doi: 10.1016/j.vaccine.2008.01.004.
161. Carrington M, O'Brien SJ. **The influence of HLA genotype on AIDS.** *Annu Rev Med* 2003; **54**:535–551.
162. Carrington M, Bontrop RE. **Effects of MHC class I on HIV/SIV disease in primates.** *AIDS* 2002; **16 (Suppl 4)**:S105–S114.
163. Miyazawa M, Fujisawa R, Ishihara C, Takei YA, Shimizu T, Uenishi H, *et al.* **Immunization with a single T helper cell epitope abrogates Friend virus-induced early erythroid proliferation and prevents late leukemia development.** *J Immunol* 1995; **155**:748–758.
164. Kawabata H, Niwa A, Tsuji-Kawahara S, Uenishi H, Iwanami N, Matsukuma H, *et al.* **Peptide-induced immune protection of $CD8^{+}$ T cell-deficient mice against Friend retrovirus-induced disease.** *Int Immunol* 2006; **18**:183–198.
165. Iwanami N, Niwa A, Yasutomi Y, Tabata N, Miyazawa M. **Role of natural killer cells in resistance against Friend retrovirus-induced leukemia.** *J Virol* 2001; **75**:3152–3163.
166. Takeda E, Tsuji-Kawahara S, Sakamoto1 M, Langlois M-A, Neuberger MS, Rada C, *et al.* **Mouse APOBEC3 restricts Friend leukemia virus infection and pathogenesis *in vivo*.** *J Virol* 2008. [Epub ahead of print].
167. Santiago ML, Montano M, Benitez R, Messer RJ, Yonemoto W, Chesebro B, *et al.* ***Apobec3* encodes *Rfv3*, a gene influencing neutralizing antibody control of retrovirus infection.** *Science* 2008; **321**:1343–1346.
168. Clerici M, Shearer GM. **Is HIV infection associated with a TH1→ TH2 switch?** *Immunol Today* 1993; **14**:107–111.
169. Salk J, Bretscher PA, Salk PL, Clerici M, Shearer GM. **A strategy for prophylactic vaccination against HIV.** *Science* 1993; **260**:1270–1272.
170. Lehner T, Wang YF, Cranage M, Bergmeier LA, Mitchell E, *et al.* **Protective mucosal immunity elicited by targeted iliac lymph node immunization with a subunit SIV envelope and core vaccine in macaques.** *Nat Med* 1996; **2**:767–775.

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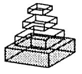

Mammalian microRNAs: post-transcriptional gene regulation in RNA virus infection and therapeutic applications

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RNA silencing mediated by microRNAs (miRNAs) is a recently discovered gene regulatory mechanism involved in various aspects of biology, such as development, cell differentiation and proliferation, and innate immunity against viral infections. miRNAs, which are a class of small (21–25 nucleotides) RNAs, target messenger RNA (mRNA) through incomplete base-pairing with their target sequences resulting in mRNA degradation or translational repression. Although studies of miRNAs have led to numerous sensational discoveries in biology, many fundamental questions about their expression and function still remain. In this review, we discuss the dynamics of the mammalian miRNA machinery and the biological function of miRNAs, focusing on RNA viruses and the various therapeutic applications of miRNAs against viral infections.

Keywords: mammalian microRNAs, RNA virus infection, host–virus interaction, gene therapy

INTRODUCTION

The human genome is about 3,300 Mb in size and comprises 1% exons and 24% introns (the remainder being transposons (45%) and repetitive sequences). It is assumed that less than 40,000 protein-coding genes are present (Lewin, 2004), so why do we have such a large genome containing so many non-coding regions? Historically, the idea that RNA regulates the gene expression profile of each cell was proposed by Britten and Davidson (1969). This hypothesis was realized in the 1990s, especially after the first key finding (in *Caenorhabditis elegans*) that double-stranded RNA is cleaved by the ribonuclease (RNase), Dicer, into small, ~22 nucleotide (nt) RNAs, and induces gene silencing (RNA silencing) (Fire et al., 1998). Subsequent extensive studies and computer-based bioinformatics approaches shed light on "the world of small RNAs", which consist of small RNAs (~20–30 nt) with a variable origin, including exogenous and endogenous short interfering RNAs (siRNA), Piwi (P-element-induced whimpy testes)-interacting (pi)RNAs and microRNAs (miRNAs) (see review in Zamore and Haley, 2005; Choudhuri, 2009).

The post-transcriptional gene silencing (PTGS) mechanism mediated by these small RNAs plays a fundamental role in development, differentiation, proliferation, and transposon mobility (Bartel, 2004; Zamore and Haley, 2005; Berkhout and Jeang, 2007). Currently, nearly 1,000 mature human miRNAs are registered in the miRNA data base (http://www.mirbase.org/). Although our knowledge of small RNAs is expanding rapidly, the expression profiles and function of many of these miRNAs remains unclear. In this expanding field of science, it seems impossible to cover all the aspects of miRNA research and there are already numerous reviews of miRNA focusing, for example, on the immune system (Baltimore et al., 2008; Lodish et al., 2008; Xiao and Rajewsky, 2009; O'Connell et al., 2010), cancer (Croce, 2009), and virus-encoded miRNAs (Gottwein and Cullen, 2008; Boss et al., 2009; Cullen, 2009). Here, we discuss recent topics regarding miRNAs (mostly human) with a focus on RNA virus infection and the development of novel technologies that take advantage of the particular characteristics of miRNA for therapeutic purposes.

BASIC KNOWLEDGE OF MAMMALIAN miRNA

STRUCTURE OF miRNA PROCESSING MACHINERY

At present, siRNAs, which are exogenously induced double-stranded or short hairpin RNAs, are utilized as an essential tool for gene-specific silencing. In contrast, miRNAs play an important role in both post-transcriptional and translational regulation (Bartel, 2004). To obtain insights into the molecular mechanisms underlying RNA silencing pathways, the three-dimensional structures of proteins participating in miRNA biosynthesis have been deduced (see review in Jinek and Doudna, 2009). The protein structure of the human miRNA processing machinery is illustrated in **Figure 1**.

Drosha, a nuclear RNaseIII enzyme, has two RNaseIII catalytic sites with a double strand RNA binding domain (dsRBD) at the C terminus (Lee, 2003) and a proline-rich domain and arginine/serine-rich domains at the N terminus. DGCR8 has two dsRBDs and a WW domain containing two conserved tryptophan (W) residues. DGCR8 binds to the base of the long primary transcript (pri-)miRNA hairpin, positioning Drosha to cleave the pri-miRNA stem at a distance of 11 base pairs from the junction between the dsRNA stem and the flanking ssRNA regions (Han et al., 2006). The core region of human DGCR8 has been crystallized (Sohn et al., 2007).

Dicer, cytoplasmic RNaseIII, has ATPase and helicase domains at the N-terminus, followed by DUF283 (unknown function) and PAZ (PIWI/Argonaute/Zwille) domains, in addition to two RNaseIII domains and one dsRBD (Bernstein et al., 2001; Jinek and Doudna, 2009). The PAZ domain binds specifically to the 3′ end of single-stranded RNA. The crystal structure of *Giardia* Dicer suggests that it functions as a molecular ruler by anchoring the 3′ dinucleotide of the dsRNA bound to the PAZ domain, cleaving it at

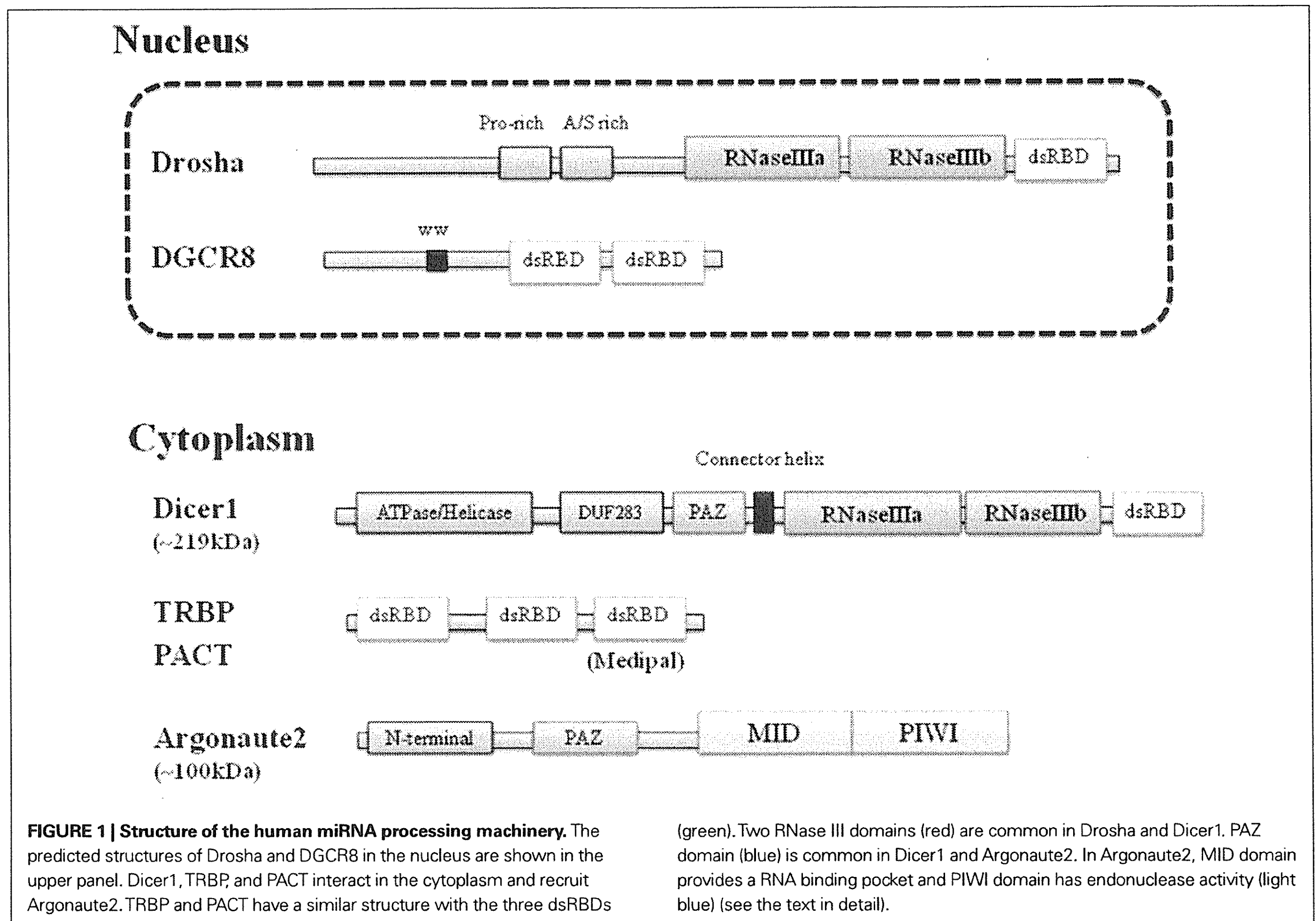

FIGURE 1 | Structure of the human miRNA processing machinery. The predicted structures of Drosha and DGCR8 in the nucleus are shown in the upper panel. Dicer1, TRBP, and PACT interact in the cytoplasm and recruit Argonaute2. TRBP and PACT have a similar structure with the three dsRBDs (green). Two RNase III domains (red) are common in Drosha and Dicer1. PAZ domain (blue) is common in Dicer1 and Argonaute2. In Argonaute2, MID domain provides a RNA binding pocket and PIWI domain has endonuclease activity (light blue) (see the text in detail).

a fixed distance from that end, and generating products of defined length (Macrae et al., 2006). A connector helix may be the main determinant of product size.

TAR RNA-binding protein (TRBP) and PACT have similar structures, with three dsRBDs that directly interact with each other and with Dicer (Kok et al., 2007). Interestingly, PACT is an activator of protein kinase R (PKR), whereas TRBP is an inhibitor. One dsRBD known as Medipal in TRBP is a protein–protein interaction domain that binds Merlin, Dicer, and PACT. The TRBP C-terminus of Madipal interacts with Dicer via its ATPase/Helicase domain (Daniels et al., 2009).

Like Dicer, the Argonaute protein also has a PAZ domain, which binds to the 3′ end of guide RNA (Wang et al., 2008). The MID domain provides a binding pocket for the 5′-phosphate of guide RNA and the PIWI domain adopts an RNaseH fold and has endonuclease activity (Boland et al., 2010). In humans, there are four genes encoding Argonaute and only one of these, Ago2, has endonuclease activity. The mechanism by which the Ago protein mediates translational repression remains controversial and may be different in different cell types (for details, see review in Peters and Meister, 2007).

BIOGENESIS OF miRNA

Although there are considerable variations in miRNA biogenesis and regulatory pathways (Winter et al., 2009), a representative pathway is shown in **Figure 2**.

Human miRNAs are present within the introns of coding genes and the introns and exons of non-coding transcripts (Berkhout and Jeang, 2007). pri-miRNA, which is transcribed by RNA polymerase II, forms a distinctive hairpin structure with a 5′-capped poly(A) tail. This characteristic hairpin structure is recognized by Drosha (Bartel, 2004). Drosha forms a complex with DGCR8 and cleaves pri-miRNA at the unpaired flanking regions, leaving a monophosphate group at the 5′ ends and a two-nt overhang at the 3′ ends (Han et al., 2006). The products of these processed pri-miRNAs are ~70-nt stem-loop RNA intermediates (pre-miRNA). The pre-miRNA is exported from the nucleus to the cytoplasm by exportin 5 in a Ran guanosine triphosphate-dependent manner (Lund et al., 2004), where it is processed by Dicer.

Human Dicer works together with its dsRNA-binding partner proteins, TRBP and PACT, to cleave pre-miRNA into ~22-nt long dsRNAs, though TRBP and PACT are not essential for cleavage (Haase et al., 2005; Lee et al., 2006). The complex formed by Dicer, dsRNA, TRBP, and PACT then recruits Argonaute to form the RNA-induced silencing complex (RISC) loading complex (Chendrimada et al., 2005). Finally, Argonaute delivers mature miRNA to the processing bodies (PBs) or stress granules (SGs), where the target messenger RNA (mRNA) is located (Peters and Meister, 2007).

Mature miRNA, carried by Argonaute, can destroy target mRNA with complementary sequences or repress protein synthesis by partially binding to the 3′ untranslated region (UTR) of target

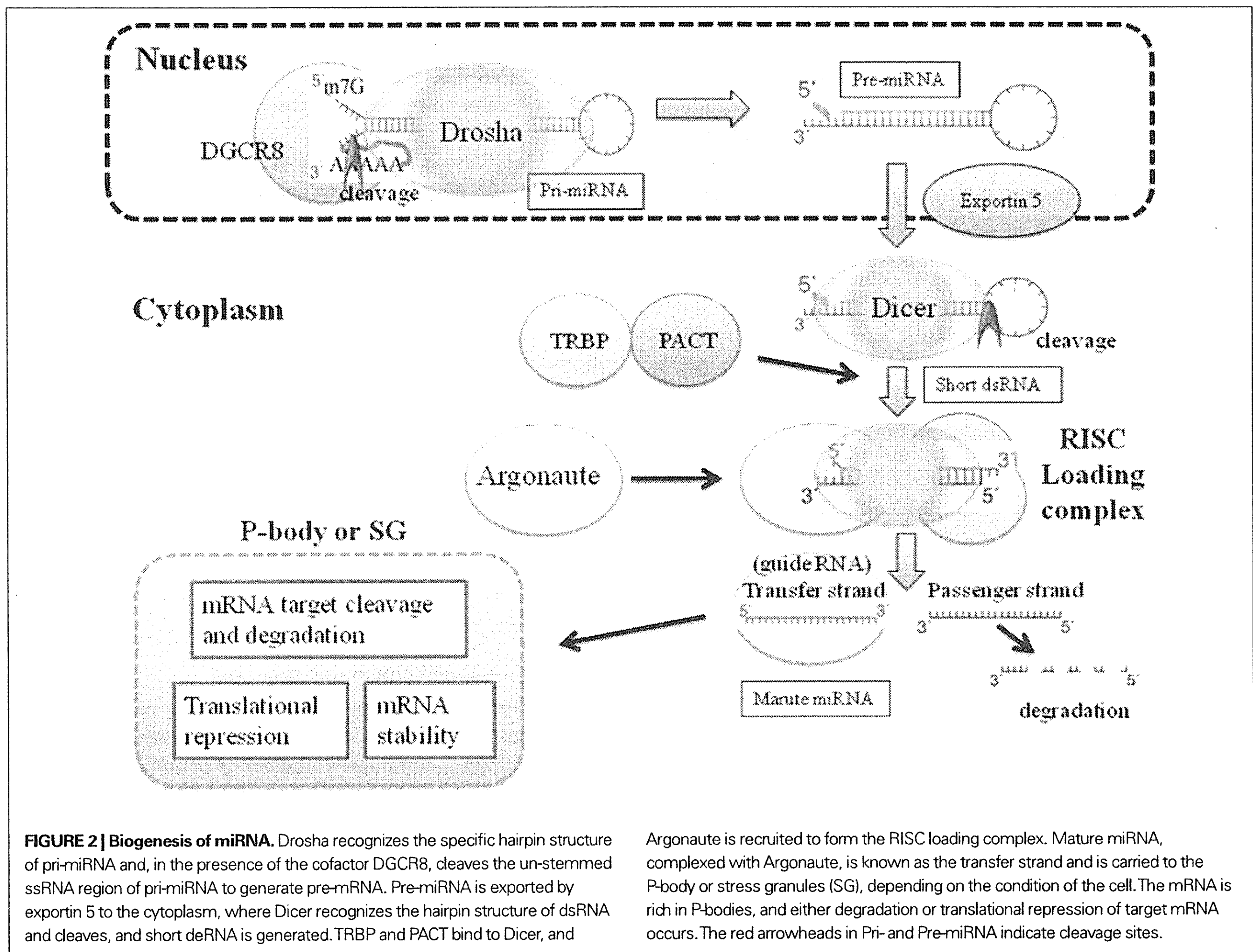

FIGURE 2 | Biogenesis of miRNA. Drosha recognizes the specific hairpin structure of pri-miRNA and, in the presence of the cofactor DGCR8, cleaves the un-stemmed ssRNA region of pri-miRNA to generate pre-mRNA. Pre-miRNA is exported by exportin 5 to the cytoplasm, where Dicer recognizes the hairpin structure of dsRNA and cleaves, and short deRNA is generated. TRBP and PACT bind to Dicer, and Argonaute is recruited to form the RISC loading complex. Mature miRNA, complexed with Argonaute, is known as the transfer strand and is carried to the P-body or stress granules (SG), depending on the condition of the cell. The mRNA is rich in P-bodies, and either degradation or translational repression of target mRNA occurs. The red arrowheads in Pri- and Pre-miRNA indicate cleavage sites.

mRNA. Because seed sequences of only 7–8 nt are required to loosely base-pair with the target sequence, a single miRNA is estimated, on average, to broadly target ~200 species of RNA (Lewis et al., 2005; Bartel, 2009).

HIGH THROUGHPUT PROFILING ANALYSIS

Expression of miRNAs may change temporally, in a spatial and tissue- or cell-type-specific manner. Large scale cloning and expression analyses of mammalian miRNAs were carried out by Landgraf et al. (2007) and the expression profiles of distinct human and mouse mature miRNAs were characterized. Comparison of miRNA expression between the hematopoietic system and all other organ systems indicated that only five miRNAs are highly specific to cells of the hematopoietic lineage (miR-142, miR-144, miR-150, miR-155, and miR-223). It is worth noting that species-specificity is also demonstrated by the data; miR-150 and miR-155 are highly hematopoietic-specific in mice, but not in humans. They also observed that the miRNA expression profiles of different sorted mature T cell types were similar and clustered together, and that no striking differences in the miRNA expression profiles of the different myeloid sub-lineages existed, except in granulocyte/monocyte-specific miR-223 expression. Thus, exclusive expression of miRNA by particular tissues or cell types is rare.

To identify the functionally important targets of miRNA, Selbach et al. (2008) employed a novel isotope pulse-labeling method of proteins (pulsed stable isotype labeling with amino acids in cell culture, pSILAC) in combination with mass-spectrometry-based proteomics. They evaluated changes in production of ~5000 proteins associated with the overexpression of several miRNAs in HeLa cells, which included tissue-specific miRNAs not expressed in HeLa cells (miR-1 and miR-155) and those expressed ubiquitously (miR-16, miR-30a, and let-7b). This pSILAC technology was quite useful in identifying functionally important targets of miRNAs at the protein level. They demonstrated that a single miRNA can directly downregulate the production of hundreds of proteins, and identified the seed sequence in the 3′ UTR as the primary motif for miRNA-mediated regulation of protein synthesis, probably acting via both mRNA degradation and translational repression. Notably, the repressive effect on individual proteins was relatively small, which may reflect the paucity (less than 3) of seed sites for each miRNA in individual target 3′ UTRs. Importantly, the results obtained from miRNA overexpression systems are equivalent to those obtained from knock down systems, confirming that their conclusions were not experimentally biased.

Baek et al. (2008) demonstrated the proteomic impact of over-expressed miRNAs (miR-1, miR-181, and miR-124) using the same SILAC technology. They also analyzed the relationship between

seed sequence matches and protein repression in HeLa cells and found that mRNAs with single 7-mer or 8-mer site matches, but not 6-mers, were significantly downregulated by miRNA. The seed sites within the 3′ UTRs were generally more effective than those in coding regions. Again, they demonstrated that the targeting principles elucidated from overexpressed exogenous miRNAs also apply to endogenous miRNA targeting at the level of protein downregulation, as argued by Selbach et al. (2008). We assume that coordinated post-transcriptional gene regulation by miRNAs contributes to the fine tuning of protein expression by cells in a tissue-, lineage-, differentiation-, proliferation-, or cytokine signal-specific manner. Accumulating knowledge about miRNA expression profiles and their biological functions will be used to build sophisticated bioinformatics programs to clarify various aspects of miRNA biology. Such computer-based tools for predicting the target genes of miRNAs are available at http://www.targetscan.org/, http://www.microrna.org/ and other sites (Bartel, 2009), and will prove helpful for the identification of targets with physiological relevance.

VIRUS–HOST INTERACTIONS AND miRNA

Recently, miRNAs were broadly implicated in viral infection of mammalian cells, having either positive, or negative effects on virus replication and host immune responses. Herpes-family viruses, which have a large DNA genome, are known to encode viral miRNA (v-miRNA), probably for the regulation of their own replication and latency and to control cellular defense mechanisms. However, the existence of v-miRNA in smaller DNA and RNA viruses remains controversial. It is argued that small viruses have size constraints that mean a greater proportion of their genome is required for coding purposes and is, therefore, less likely to retain non-coding RNAs (Berkhout and Jeang, 2007). In contrast, long-term, latently infected viruses such as herpes viruses derive a greater survival benefit from utilizing the cellular miRNA machinery (Cullen, 2010).

Because DNA virus-encoded v-miRNAs have been reviewed in detail elsewhere (see review in Gottwein and Cullen, 2008; Boss et al., 2009; Cullen, 2009), we will focus on the interaction of cellular miRNAs with RNA viruses as a host defense mechanism against viral infection.

IMPLICATIONS OF miRNA CONTRIBUTIONS TO ANTIVIRAL ACTIVITY

In plants, worms and flies, RNA silencing suppresses the mobilization of endogenous retroviruses. Therefore, it is expected that the miRNA silencing machinery may be involved in defense against viral infections in vertebrates. Triboulet et al. (2007) demonstrated that, by silencing RNase III, Dicer, or Drosha, HIV-1 replication is inhibited in PBMCs from HIV-infected donors and in latently infected U1 cells in which HIV-1 is not efficiently transcribed through its promoter (the long terminal repeat, LTR) due to a Tat mutation. They analyzed the level of miRNA in Jurkat cells using microarray analysis and found that a polycistronic miRNA cluster, miR-17/92, was markedly decreased upon HIV-1 infection. Although miR-17/92 does not directly target the viral genome, the histone acetylase, PCAF, which is a cofactor for Tat transactivation, has four potential target sequences for miR-17-5p and 20a in its 3′ UTR. Triboulet et al. clearly demonstrated the relationship between HIV-1 replication and the expression of miRNA and PCAF/Tat. Thus, the miRNA biogenesis machinery contributes to host defense against HIV-1. However, additional cellular miRNAs, or other inhibitory mechanisms, may also be involved in virus–host interactions.

Mammals have a single Dicer gene, Dicer1. Inactivation of Dicer1 in mice causes embryonic lethality or severe damage to tissue morphogenesis, cell differentiation and development (Bernstein et al., 2003). Otsuka et al. (2007) managed to produce such Dicer1-deficient mice expressing low levels of Dicer in some tissues and almost none in peritoneal macrophages. Using these mice, they demonstrated for the first time *in vivo* that miRNAs target the RNA genome of vesicular stomatitis virus (VSV). Interestingly, these Dicer1-deficient mice were susceptible to VSV, but not to other RNA viruses, including encephalomyocarditis virus (EMCV), lymphocytic choriomeningitis virus (LCMV), and Influenza A virus. Computer prediction models allowed them to select 24 candidate miRNAs to potentially target the positive-strand sequence of VSV. They demonstrated that miR-93 and miR-24 suppress VSV replication by targeting the P (polymerase cofactor)- and L (RNA-dependent RNA polymerase)-encoding regions of VSV, respectively.

CELLULAR miRNAs DIRECTLY TARGET VIRAL RNA

For the inhibition of virus replication, the seed sequences of miRNA must be complementary to the viral targets for silencing (Gottwein and Cullen, 2008). Although a number of computer-based programs that search for homology between human miRNAs and viral genomes can predict complimentarity to various regions of viral sequences, the effects of RNAi need to be demonstrated in infected cells. The following are examples of successful approaches used to identify cellular miRNAs that target viral RNA.

Lecellier et al. (2005) found no evidence of virus-derived siRNAs within primate foamy virus type 1 (PFV-1), which is a complex retrovirus closely related to HIV and encodes two accessory factors, Bet and Tas. Instead, by fusing viral fragments to the UTR of a GFP-tagged reporter gene, they found that the 3′ end fragment of the PVF-1 genome, which encodes Bet and Env/Bet proteins (and is also within the 3′ UTR), reduced GFP levels and was a target for miR-32. Thus, miR-32 exerts a direct, sequence-specific effect against PFV-1. Moreover, they demonstrated that the viral Tas protein interacts with cellular miRNAs and acts as a broadly effective silencing suppressor. Similar, virally coded, RNA silencing suppressor (RSS) activity was shown for HIV-1 Tat (Bennasser et al., 2005), vaccinia virus E3L, influenza A virus NS1 and Ebola virus VP35 proteins (Haasnoot et al., 2007).

Huang et al. (2007) inserted DNA fragments from the 3′ UTR of HIV-1 RNA into the 3′ UTR of the pEGFP vector and identified the region targeted by a potential miRNA-mediated mechanism in resting primary CD4⁺ T cells. They found that miRNAs, such as miR-28, miR-125b, miR-150, miR-223, and miR-382, are abundant in resting T cells, but not in activated T cells, and the replication of latent viruses in patients on HAART was prevented by inhibiting these miRNAs. However, there are more than 100 cellular mRNA targets for each miRNA (Lewis et al., 2005) and the consequences of modulating the expression of these miRNAs may differ between T-cell subpopulations at distinct differentiation stages (see below).

Furthermore, because transfection of RNA or DNA into resting T cells is a very inefficient procedure, applying miRNA expression in a therapeutic context may be very difficult.

Using microarray technology, Pedersen et al. (2007) analyzed RNA derived from interferon (IFN)-stimulated cells and found that eight miRNAs induced by IFN had seed sequences that were 100% complementary with hepatitis C virus (HCV). Of these putative miRNAs, five (miR-196, miR-296, miR-351, mir-431, and miR-448) were able to attenuate HCV replication in Huh7 cells. miR-196 and miR-448 did not prevent the replication of HCV containing mutant target sequences, indicating that the antiviral effect is not induced by non-specific alterations in cellular gene expression. Furthermore, miR-122, which is essential for HCV replication (Jopling et al., 2005), was downregulated in response to IFN-β. Thus, these results suggest that the modulation of the expression levels of these miRNAs has an important role in the antiviral effects of IFN-β against HCV. However, a recent analysis of liver biopsy samples from chronic hepatitis C patients revealed no correlation between miR-122 expression and viral load (Sarasin-Filipowicz et al., 2009). Moreover, most of the miRNAs that mediate the effects of IFN-β on HCV replication were present only at very low levels, even after IFN treatment, arguing against a protective role for these miRNAs. Nevertheless, the finding that IFN-treatment significantly reduces miR-122 expression in non-responders, as opposed to complete responders, indicates the usefulness of miRNA profiling as a prognostic marker for IFN therapy.

ALTERED EXPRESSION OF CELLULAR miRNA UPON VIRAL INFECTION: PATHOGENICITY

The involvement of RNAi in influenza virus infection was identified by knocking down Dicer in Vero cells, which lack type I IFN genes (Matskevich and Moelling, 2007). Any acute virus infection may alter the expression pattern of both mRNA and miRNA in infected cells. Li et al. (2010) analyzed miRNA expression profiles in the lungs of mice infected with a highly pathogenic 1918 influenza A virus and compared them with those of mice infected with a non-lethal seasonal influenza A virus. By analyzing the inverse correlation between altered miRNA expression and the expression of predicted mRNA targets, they demonstrated that the target mRNAs of one altered miRNA, miR-200a, were associated with the type I IFN signaling pathway, which plays an important role in the pathogenesis in 1918 influenza A virus infection in mice (Kash et al., 2006). They also found that miR-223 indirectly downregulated CREB activity, which is required for the maintenance of cell survival and growth.

Another example can be found in HIV-1 infection. Cells of the monocyte/macrophage lineage are susceptible to HIV-1 infection; however, only macrophages can support massive virus production. This differentiation-dependent restriction mechanism has been attributed to the distinct expression of the APOBEC3 subfamily by monocytes (Peng et al., 2007). Recently, Wang et al. (2009) reported that high expression of miR-28, miR-150, miR-223, and miR-382 was correlated with low susceptibility of monocytes to HIV-1 infection. Although the target mRNAs of these miRNAs were not identified, the results suggest that monocyte differentiation and HIV-1 susceptibility are linked by a common set of miRNAs. In relation to monocyte/macrophage differentiation, the expression of Cyclin T1, required for transactivation by HIV-1 Tat, increases during macrophage differentiation and enhances HIV-1 replication within macrophages (Liou et al., 2002). A recent report indicates that miR-198-mediated repression of Cyclin T1 may contribute to HIV-1 replication during monocyte differentiation into macrophages (Sung and Rice, 2009).

These results indicate that RNA viruses utilize cellular miRNAs to manipulate the expression of cellular genes to enhance their own survival and expansion. The known interactions between cellular miRNA and RNA viruses are summarized in **Figure 3**.

EFFECTS OF miRNAs ON IMMUNE CELL DIFFERENTIATION AND VIRAL INFECTION

The conditional knockdown of Dicer in murine T or B lymphocytes causes abnormal lymphocyte differentiation (Cobb et al., 2005; Muljo et al., 2005). Antigen-induced immune cell differentiation is a key feature of host defense and elicits coordinated immune responses to protect the host from pathogens (Baltimore et al., 2008). For example, profound changes in gene expression occur during antigen-induced $CD8^+$ T cell differentiation in mice (Wu et al., 2007). Wu et al. identified seven miRNAs (miR-16, miR-21, miR-142-3, miR-142-5, miR-150, miR-15b, and let-7f) frequently expressed in all T cells and observed alterations in the miR-21 expression signature as cells differentiated from naïve to effector cells. We have also shown the existence of similar, but not identical, differentiation-associated miRNA expression profiles in human $CD8^+$ T lymphocytes. The function of these miRNAs requires further investigation.

Recent microarray analysis studies show that monocyte differentiation into dendritic cells is regulated and coordinated by miR-34a and miR-21 (Hashimi et al., 2009). JAG1 and WNT1 were identified as targets for these miRNAs using a bioinformatic target ranking system. Considering that monocyte-derived dendritic cells (MDDCs) are poor producers of HIV-1 compared with monocyte-derived macrophages (Tsunetsugu-Yokota, 2008), it is highly likely that miRNA expression associated with cell differentiation from monocytes to either macrophages or MDDCs may also regulate HIV replication in monocytes (Wang et al., 2009). Although such differentiation-associated miRNAs have any impact on virus replication within infected cells remains to be addressed, the results of extensive miRNA profiling analyses of viral infections both *in vitro* and *in vivo* will be highly informative for the diagnosis and treatment of viral infections in humans.

THERAPEUTIC APPLICATION OF miRNAs TO VIRAL INFECTIONS

Knowledge regarding the basic biology and function of mammalian miRNAs has increased greatly in recent times. The idea of utilizing RNA interference by miRNA for genetic manipulation is already being realized. Taking advantage of the tissue-specificity of miRNAs, Kelly et al. inserted the target sequences of muscle-specific miRNAs (miR-133a and miR-206) into the 3′ UTR of Coxsackievirus A21 (CVA21), a pathogenic picornavirus that causes lethal myositis and oncolysis in tumor-baring mice (Kelly et al., 2008). The result was tissue-specific attenuation that allowed the virus to replicate well in muscle cells without being pathogenic. Likewise, by incorporating target sites for the neuron-specific miR-124a into the 3′ end of

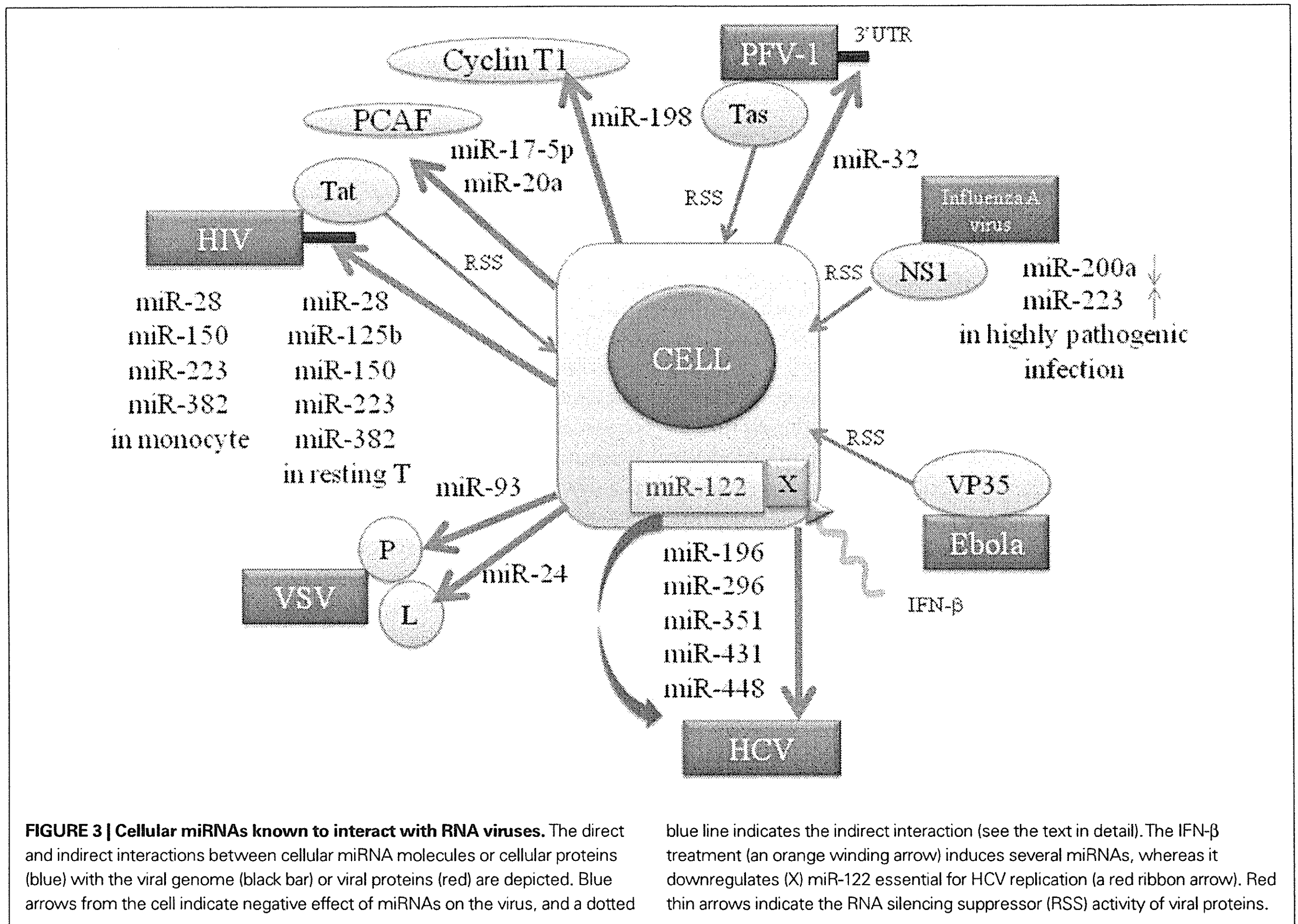

FIGURE 3 | Cellular miRNAs known to interact with RNA viruses. The direct and indirect interactions between cellular miRNA molecules or cellular proteins (blue) with the viral genome (black bar) or viral proteins (red) are depicted. Blue arrows from the cell indicate negative effect of miRNAs on the virus, and a dotted blue line indicates the indirect interaction (see the text in detail). The IFN-β treatment (an orange winding arrow) induces several miRNAs, whereas it downregulates (X) miR-122 essential for HCV replication (a red ribbon arrow). Red thin arrows indicate the RNA silencing suppressor (RSS) activity of viral proteins.

the 5′ UTR and between the structural and non-structural genes within the coding region of the poliovirus, an attenuated poliovirus vaccine lacking any neurovirulence was developed (Barnes et al., 2008). Similar engineering strategies were also applied to the VSV by inserting neurotropic miR-125 target sequences into the 3′ UTR of the viral polymerase gene (Kelly et al., 2010) and to adenoviruses by inserting a hepatocyte-selective miR-122 target site into the 3′ UTR of the E1A transcription cassette (Cawood et al., 2009). The structures of these attenuated viruses are illustrated in **Figure 4**. In this context, Perez et al. successfully attenuated the influenza A virus by incorporating species-specific, non-avian microRNA (miR-93) targets into nucleoproteins. The result was attenuated viral activity in mice, but not in eggs as expected (Perez et al., 2009). Thus, miRNA-mediated control of viral replication is a promising technology that can be used to develop safe attenuated viruses for cancer therapy and vaccines.

The RNAi-based gene delivery systems using lentivirus vectors have been developed for the control of HIV-1 infection, and such lentivirus can be utilized as potential AIDS vaccine candidates (Morris and Rossi, 2006; Rossi et al., 2007; Yamamoto and Tsunetsugu-Yokota, 2008). Likewise, considering the therapeutic applications of miRNA, efficient *in vivo* miRNA delivery systems by lentivirus vectors are a promising gene-transfer medium. However, because the VSV envelope glycoprotein used to encapsulate the transfer gene is pantropic, transgene expression in all cells could be problematic. To overcome the problem of non-specific gene transfer, Brown et al. (2007) systemically treated a mouse model of hemophilia B with a lentivirus vector expressing clotting factor IX (F.IX) under the control of a hepatocyte-specific promoter. Although F.IX is only expressed in the liver, an anti-F.IX immune response was elicited, probably due to off-target expression by the hepatocyte-specific promoter in hematopoietic cells. By inserting four tandem-repeat target sequences of the hematopoietic-specific miR-142-3p into the 3′ UTR of the transgene expression cassette, they were able to achieve sustained F.IX production in hemophilia B mice. In these mice, miR-142-3p levels were 50-fold higher in the spleen than in the liver. Importantly, the introduction of lentivirus containing miR-142-3p target sequences did not affect the normal *in vivo* level of miRNA, including that of miR-142-3p. Thus, by combining a highly cell lineage-specific promoter with PTGS using cell state- or lineage-specific miRNAs, the engineered lentivirus delivery system should make it possible to achieve specific expression of transgenes within the desired cells or tissues (Brown et al., 2007).

As described in the previous section (4.2), the abundantly expressed, liver-specific miRNA, miR-122, binds to two closely spaced target sites within the 5′ UTR of the HCV genome and is required to maintain high HCV RNA abundance in liver cells (Jopling et al., 2005, 2008). Interestingly, the same miR-122 binding site placed in the 3′ UTR of a reporter mRNA downregulated

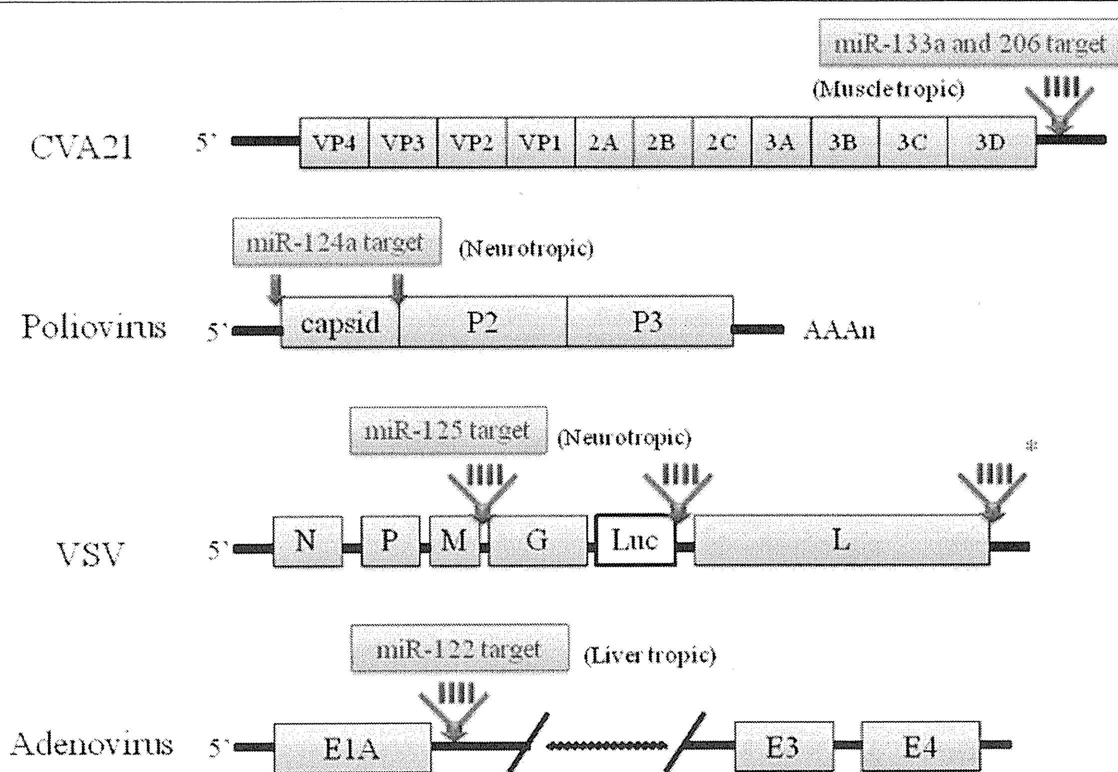

FIGURE 4 | Schematic genome structures of attenuated viruses. The localization of miRNA target insertion site in each virus is depicted (red arrow). In the case of CVA21, VSV and adenovirus, 4-tandem repeat target sequences (red bars) were inserted. Asterisk indicates the most effective site for attenuation of VSV.

mRNA expression, which clearly demonstrates location-dependent gene regulation by miRNA (Jopling et al., 2008). Based on these findings, Lanford et al. (2010) utilized a locked nucleic acid (LNA)-modified phosphorothioate oligonucleotide (SPC3649) complementary to miR-122 to treat chimpanzees chronically infected with HCV. Four animals (two each) received a high or low dose (5 or 1 mg/kg) of SPC3649 intravenously once a week for 12 weeks and were observed over a 17-week treatment-free period. They showed long-standing suppression of HCV viremia without obvious disease, side effects, virus mutation, or virus rebound, which are all consistent with potent and sustained suppression of miR-122 in the liver. Their results clearly show the feasibility and future potential of LNA drug therapy to antagonize specific miRNA functions.

All these studies indicate a promising future for antiviral therapy via the regulation of cellular miRNA expression pathways. By further understanding the biological functions of miRNAs and applying them for diagnostic and therapeutic purposes, we may yet achieve unexpected and exciting discoveries.

CONCLUSION

In the post-human genome era, our research efforts have shifted more toward the regulation of gene expression and to understanding the function of non-coding regions. Now, accumulating evidence indicates that the coordinated expression of distinct sets of miRNAs regulate many aspects of biological events occurring in cells, such as differentiation, cell-cycle progression, and proliferation, in a cell- or tissue-specific manner. We are just beginning to understand the physiological and pathological roles played by miRNAs in viral infections. We expect that, as we learn more about the targets of miRNAs and their regulatory function in cell physiology, we may be able to develop more sophisticated technologies to treat infectious diseases in humans.

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REFERENCES

Baek, D., Villen, J., Shin, C., Camargo, F. D., Gygi, S. P., and Bartel, D. P. (2008). The impact of microRNAs on protein output. *Nature* 455, 64–71.

Baltimore, D., Boldin, M. P., O'Connell, R. M., Rao, D. S., and Taganov, K. D. (2008). MicroRNAs: new regulators of immune cell development and function. *Nat. Immunol.* 9, 839–845.

Barnes, D., Kunitomi, M., Vignuzzi, M., Saksela, K., and Andino, R. (2008). Harnessing endogenous miRNAs to control virus tissue tropism as a strategy for developing attenuated virus vaccines. *Cell Host Microbe* 4, 239–248.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233.

Bennasser, Y., Le, S. Y., Benkirane, M., and Jeang, K. T. (2005). Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. *Immunity* 22, 607–619.

Berkhout, B., and Jeang, K. T. (2007). RISCy business: microRNAs, pathogenesis, and viruses. *J. Biol. Chem.* 282, 26641–26645.

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363–366.

Bernstein, E., Kim, S. Y., Carmell, M. A., Murchison, E. P., Alcorn, H., Li, M. Z., Mills, A. A., Elledge, S. J., Anderson, K. V., and Hannon, G. J. (2003). Dicer is essential for mouse development. *Nat. Genet.* 35, 215–217.

Boland, A., Tritschler, F., Heimstadt, S., Izaurralde, E., and Weichenrieder, O. (2010). Crystal structure and ligand binding of the MID domain of a eukaryotic Argonaute protein. *EMBO Rep.* 11, 522–527.

Boss, I. W., Plaisance, K. B., and Renne, R. (2009). Role of virus-encoded microRNAs in herpesvirus biology. *Trends Microbiol.* 17, 544–553.

Britten, R. J., and Davidson, E. H. (1969). Gene regulation for higher cells: a theory. *Science* 165, 349–357.

Brown, B. D., Cantore, A., Annoni, A., Sergi, L. S., Lombardo, A., Della Valle, P. D'Angelo, A., and Naldini, L. (2007). A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. *Blood* 110, 4144–4152.

Brown, B. D., Gentner, B., Cantore, A., Colleoni, S., Amendola, M., Zingale, A. Baccarini, A., Lazzari, G. Galli, C., and Naldini, L. (2007). Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat. Biotechnol.* 25, 1457–1467.

Cawood, R., Chen, H., Carroll, F., Bazan-Peregrino, M., van Rooijen, N., and Seymour, L. W. (2009). Use of tissue-specific microRNA to control pathology of wild-type adenovirus without attenuation of its ability to kill cancer cells. *PLoS Pathog.* 5, e1000440. doi:10.1371/journal.ppat.1000440.

Chendrimada, T. P., Gregory, R. I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., and Shiekhattar, R. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* 436, 740–744.

Choudhuri, S. (2009). Lesser known relatives of miRNA. *Biochem. Biophys. Res. Commun.* 388, 177–180.

Cobb, B. S., Nesterova, T. B., Thompson, E., Hertweck, A., O'Connor, E., Godwin, J., Wilson, C. B., Brockdorff, N., Fisher, A. G., Smale, S. T., and Merkenschlager, M. (2005). T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J. Exp. Med.* 201, 1367–1373.

Croce, C. M. (2009). Causes and consequences of microRNA dysregulation in cancer. *Nat. Rev. Genet.* 10, 704–714.

Cullen, B. R. (2009). Viral and cellular messenger RNA targets of viral microRNAs. *Nature* 457, 421–425.

Cullen, B. R. (2010). Five questions about viruses and microRNAs. *PLoS Pathog.* 6, e1000787. doi:10.1371/journal.ppat.1000787.

Daniels, S. M., Melendez-Pena, C. E., Scarborough, R. J., Daher, A., Christensen, H. S., El Far, M., Purcell, D. F., Laine, S., and Gatignol, A. (2009). Characterization of the TRBP domain required for dicer interaction and function in RNA interference. *BMC Mol. Biol.* 10, 38.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811.

Gottwein, E., and Cullen, B. R. (2008). Viral and cellular microRNAs as determinants of viral pathogenesis and immunity. *Cell Host Microbe* 3, 375–387.

Haase, A. D., Jaskiewicz, L., Zhang, H., Laine, S., Sack, R., Gatignol, A., and Filipowicz, W. (2005). TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. *EMBO Rep.* 6, 961–967.

Haasnoot, J., de Vries, W., Geutjes, E. J., Prins, M., de Haan, P., and Berkhout, B. (2007). The Ebola virus VP35 protein is a suppressor of RNA silencing. *PLoS Pathog.* 3, e86. doi:10.1371/journal.ppat.0030086.

Han, J., Lee, Y., Yeom, K. H., Nam, J. W., Heo, I., Rhee, J. K., Sohn, S. Y., Cho, Y., Zhang, B. T., and Kim, V. N. (2006). Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* 125, 887–901.

Hashimi, S. T., Fulcher, J. A.; Chang, M. H., Gov, L., Wang, S., and Lee, B. (2009). MicroRNA profiling identifies miR-34a and miR-21 and their target genes JAG1 and WNT1 in the coordinate regulation of dendritic cell differentiation. *Blood* 114, 404–414.

Huang, J., Wang, F., Argyris, E., Chen, K., Liang, Z., Tian, H., Huang, W., Squires, K. Verlinghieri, G., and Zhang, H. (2007). Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. *Nat. Med.* 13, 1241–1247.

Jinek, M., and Doudna, J. A. (2009). A three-dimensional view of the molecular machinery of RNA interference. *Nature* 457, 405–412.

Jopling, C. L., Schutz, S., and Sarnow, P. (2008). Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. *Cell Host Microbe* 4, 77–85.

Jopling, C. L., Yi, M., Lancaster, A. M., Lemon, S. M., and Sarnow, P. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 309, 1577–1581.

Kash, J. C., Tumpey, T. M., Proll, S. C., Carter, V., Perwitasari, O., Thomas, M. J., Basler, C. F., Palese, P., Taubenberger, J. K., Garcia-Sastre, A., Swayne, D. E., and Katze, M. G. (2006). Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* 443, 578–581.

Kelly, E. J., Hadac, E. M., Greiner, S., and Russell, S. J. (2008). Engineering microRNA responsiveness to decrease virus pathogenicity. *Nat. Med.* 14, 1278–1283.

Kelly, E. J., Nace, R., Barber, G. N., and Russell, S. J. (2010). Attenuation of vesicular stomatitis virus encephalitis through microRNA targeting. *J. Virol.* 84, 1550–1562.

Kok, K. H., Ng, M. H. Ching, Y. P., and Jin, D. Y. (2007). Human TRBP and PACT directly interact with each other and associate with dicer to facilitate the production of small interfering RNA. *J. Biol. Chem.* 282, 17649–17657.

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A. O., Landthaler, M., Lin, C., Socci, N. D., Hermida, L., Fulci, V., Chiaretti, S., Foa, R., Schliwka, J., Fuchs, U., Novosel, A., Muller, R. U., Schermer, B., Bissels, U., Inman, J., Phan, Q., Chien, M., Weir, D. B., Choksi, R., De Vita, G., Frezzetti, D., Trompeter, H. I., Hornung, V., Teng, G., Hartmann, G., Palkovits, M., Di Lauro, R., Wernet, P., Macino, G., Rogler, C. E., Nagle, J. W., Ju, J., Papavasiliou, F. N., Benzing, T., Lichter, P., Tam, W. Brownstein, M. J. Bosio, A. Borkhardt, A. Russo, J. J. Sander, C. Zavolan, M., and Tuschl, T. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 129, 1401–1414.

Lanford, R. E., Hildebrandt-Eriksen, E. S., Petri, A., Persson, R., Lindow, M., Munk, M. E., Kauppinen, S., and Orum, H. (2010). Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 327, 198–201.

Lecellier, C. H., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., Saib, A., and Voinnet, O. (2005). A cellular microRNA mediates antiviral defense in human cells. *Science* 308, 557–560.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim S., and Kim, V. N. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425, 415–419.

Lee, Y., Hur, I., Park, S. Y., Kim, Y. K., Suh, M. R., and Kim, V. N. (2006). The role of PACT in the RNA silencing pathway. *EMBO J.* 25, 522–532.

Lewin, B. (2004). *Genes.* Upper Saddle River, NJ: Pearson Education, Inc.

Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15–20.

Li, Y., Chan, E. Y., Li, J., Ni, C., Peng, X., Rosenzweig, E., Tumpey, T. M., and Katze, M. G. (2010). MicroRNA expression and virulence in pandemic influenza virus-infected mice. *J. Virol.* 84, 3023–3032.

Liou, L. Y., Herrmann, C. H., and Rice, A. P. (2002). Transient induction of cyclin T1 during human macrophage differentiation regulates human immunodeficiency virus type 1 Tat transactivation function. *J. Virol.* 76, 10579–10587.

Lodish, H. F., Zhou, B. Liu, G., and Chen, C. Z. (2008). Micromanagement of the immune system by microRNAs. *Nat. Rev. Immunol.* 8, 120–130.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E., and Kutay, U. (2004). Nuclear export of microRNA precursors. *Science* 303, 95–98.

Macrae, I. J., Zhou, K. Li, F., Repic, A., Brooks, A. N., Cande, W. Z., Adams, P. D., and Doudna, J. A. (2006). Structural basis for double-stranded RNA processing by Dicer. *Science* 311, 195–198.

Matskevich, A. A., and Moelling, K. (2007). Dicer is involved in protection against influenza A virus infection. *J. Gen. Virol.* 88, 2627–2635.

Morris, K. V., and Rossi, J. J. (2006). Lentiviral-mediated delivery of siRNAs for antiviral therapy. *Gene Ther.* 13, 553–558.

Muljo, S. A., Ansel, K. M., Kanellopoulou, C., Livingston, D. M., Rao, A., and Rajewsky, K. (2005). Aberrant T cell differentiation in the absence of Dicer. *J. Exp. Med.* 202, 261–269.

O'Connell, R. M., Rao, D. S., Chaudhuri, A. A., and Baltimore, D. (2010). Physiological and pathological roles for microRNAs in the immune system. *Nat. Rev. Immunol.* 10, 111–122.

Otsuka, M., Jing, Q. Georgel, P., New, L., Chen, J., Mols, J., Kang, Y. J., Jiang, Z., Du, X., Cook, R., Das, S. C. Pattnaik, A. K., Beutler, B., and Han, J. (2007). Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and

miR93 expression. *Immunity* 27, 123–134.

Pedersen, I. M., Cheng, G., Wieland, S., Volinia, S., Croce, C. M., Chisari, F. V., and David, M. (2007). Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature* 449, 919–922.

Peng, G., Greenwell-Wild, T., Nares, S., Jin, W., Lei, K. J., Rangel, Z. G., Munson, P. J., and Wahl, S. M. (2007). Myeloid differentiation and susceptibility to HIV-1 are linked to APOBEC3 expression. *Blood* 110, 393–400.

Perez, J. T., Pham, A. M., Lorini, M. H., Chua, M. A., Steel, J., and tenOever, B. R. (2009). MicroRNA-mediated species-specific attenuation of influenza A virus. *Nat. Biotechnol.* 27, 572–576.

Peters, L., and Meister, G. (2007). Argonaute proteins: mediators of RNA silencing. *Mol. Cell* 26, 611–623.

Rossi, J. J., June, C. H., and Kohn, D. B. (2007). Genetic therapies against HIV. *Nat. Biotechnol.* 25, 1444–1454.

Sarasin-Filipowicz, M., Krol, J., Markiewicz, I., Heim, M. H., and Filipowicz, W. (2009). Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. *Nat. Med.* 15, 31–33.

Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. *Nature* 455, 58–63.

Sohn, S. Y., Bae, W. J., Kim, J. J., Yeom, K. H., Kim, V. N., and Cho, Y. (2007). Crystal structure of human DGCR8 core. *Nat. Struct. Mol. Biol.* 14, 847–853.

Sung, T. L., and Rice, A. P. (2009). miR-198 inhibits HIV-1 gene expression and replication in monocytes and its mechanism of action appears to involve repression of cyclin T1. *PLoS Pathog.* 5, e1000263. doi:10.1371/journal.ppat.1000263.

Triboulet, R., Mari, B., Lin, Y. L., Chable-Bessia, C., Bennasser, Y., Lebrigand, K., Cardinaud, B., Maurin, T., Barbry, P., Baillat, V., Reynes, J., Corbeau, P., Jeang, K. T., and Benkirane, M. (2007). Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* 315, 1579–1582.

Tsunetsugu-Yokota, Y. (2008). "Transmission of HIV from dendritic cells to CD4+ T cells: a promising target for vaccination and therapeutic intervention," in *AIDS Vaccines, HIV Receptors and AIDS Research*, ed. L. B. Kendow (New York: Nova Science Publishers, Inc.), 117–128.

Wang, X., Ye, L., Hou, W., Zhou, Y., Wang, Y. J., Metzger, D. S., and Ho, W. Z. (2009). Cellular microRNA expression correlates with susceptibility of monocytes/macrophages to HIV-1 infection. *Blood* 113, 671–674.

Wang, Y., Sheng, G., Juranek, S., Tuschl, T., and Patel, D. J. (2008). Structure of the guide-strand-containing argonaute silencing complex. *Nature* 456, 209–213.

Winter, J., Jung, S., Keller, S., Gregory, R. I., and Diederichs, S. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat. Cell Biol.* 11, 228–234.

Wu, H., Neilson, J. R., Kumar, P., Manocha, M., Shankar, P., Sharp, P. A., and Manjunath, N. (2007). miRNA profiling of naive, effector and memory CD8 T cells. *PLoS One* 2, e1020. doi:10.1371/journal.pone.0001020.

Xiao, C., and Rajewsky, K. (2009). MicroRNA control in the immune system: basic principles. *Cell* 136, 26–36.

Yamamoto, T., and Tsunetsugu-Yokota, Y. (2008). Prospects for the therapeutic application of lentivirus-based gene therapy to HIV-1 infection. *Curr. Gene Ther.* 8, 1–8.

Zamore, P. D., and Haley, B. (2005). Ribo-gnome: the big world of small RNAs. *Science* 309, 1519–1524.

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Differential Anti-APOBEC3G Activity of HIV-1 Vif Proteins Derived from Different Subtypes*[S]

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Antiretroviral cytidine deaminase APOBEC3G, which is abundantly expressed in peripheral blood lymphocytes and macrophages, strongly protects these cells against HIV-1 infection. The HIV-1 Vif protein overcomes this antiviral effect by enhancing proteasome-mediated APOBEC3G degradation and is key for maintaining viral infectivity. The 579-bp-long *vif* gene displays high genetic diversity among HIV-1 subtypes. Therefore, it is intriguing to address whether Vif proteins derived from different subtypes differ in their viral defense activity against APOBEC3G. Expression plasmids encoding Vif proteins derived from subtypes A, B, C, CRF01_AE, and CRF02_AG isolates were created, and their anti-APOBEC3G activities were compared. Viruses produced from cells expressing APOBEC3G and Vif proteins from different subtypes showed relatively different viral infectivities. Notably, subtype C-derived Vif proteins tested had the highest activity against APOBEC3G that was ascribed to its increased binding activity, for which the N-terminal domain of the Vif protein sequences was responsible. These results suggest that the biological differences of Vif proteins belonging to different subtypes might affect viral fitness and quasispecies *in vivo*.

Among the seven human APOBEC3[3] cytidine deaminase proteins (from A to H) that act as intrinsic restriction factors against endogenous and exogenous retroviruses (1–9), APOBEC3G provides the most potent retroviral restriction *in vitro* and *in vivo* (10–12). This host protein is abundantly expressed in peripheral blood mononuclear cells (PBMCs) and macrophages. APOBEC3G deaminates deoxycytidine to deoxyuridine in nascent viral minus-strand cDNA, thereby inducing G-to-A hypermutations during reverse transcription (13–16). It also partially restricts viral replication in a deamination-independent fashion, mainly by blocking DNA synthesis (17–20). Human immunodeficiency virus type 1 (HIV-1) is armed with Vif[3] protein, which induces proteasome-mediated APOBEC3G degradation (21–24) via a mechanism involving the Cullin5 (Cul5)-containing E3 ubiquitin ligase (25–28). As a result, Vif protein reduces virion incorporation of APOBEC3G in virus-producer cells (29–32), leading to efficient reverse transcription in the target cells. By the same mechanism, Vif protein can inactivate APOBEC3F and APOBEC3DE, which are expressed in PBMCs and suppress Vif-deficient HIV-1 infection to a lesser extent than does APOBEC3G (33–35).

Several *in vivo* studies have demonstrated that APOBEC3G-induced G-to-A hypermutation is frequently observed in patient-derived proviral DNA (36–42) even in the presence of full-length but polymorphic *vif* genes (43). The *vif* genes also have high *in vivo* genetic variability (11, 44–46) and subtype-dependent amino acid substitutions (47). These findings imply that the sequence diversity of *vif* genes (possibly in a subtype-dependent manner) might harbor differential levels of anti-APOBEC3G activity. Among the strains tested in the present study, Vif protein derived from subtype C strains harbored the most robust anti-APOBEC3G activity. This activity was determined by the N-terminal region of the protein, which bound APOBEC3G more efficiently than subtype B-derived Vif. Consistent with this, subtype B-based viruses carrying subtype C-derived Vif proteins were rarely deaminated in primary lymphocytes endogenously expressing APOBEC3G. These results indicate that the sequence variability of Vif proteins dependent on HIV-1 subtypes leads to differential anti-APOBEC3G activity, presumably resulting in differential levels of HIV-1 fitness and viral progeny diversity.

EXPERIMENTAL PROCEDURES

Viruses—The HIV-1 isolates utilized in this study were registered in GenBank™ and included: subtype A, UG029-A3 (#AB098332), UG031-A1 (#AB098330), UG031-A2 (#AB098331), 92RW025A (#AB287376), and 92UG037 (#AB253428); subtype B, NL4-3 (48), JRFL (49), SF2 (50), BaL (51), and 01JPDR3884 (#AB289589); subtype C, 02ZMJCC05 (#AB254155), 02ZMJMC18 (#AB254156), 02ZM109C31

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.
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[3] The abbreviations used are: APOBEC3, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like protein 3; PBMC, peripheral blood mononuclear cell; Vif, virion infectivity factor; VSV-G, vesicular stomatitis virus glycoprotein; RRE, Rev-responsive element; Cul5, Cullin5.

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(#AB573087), 02ZM112C23 (#AB254145), 02ZMDBC33 (#AB254153), and 02ZMGNC46 (#AB573088); CRF01_AE, 93TH051 (#AB220944), 93TH057AE18 (#AB253424), 93TH060 (#AB220946), 93TH062 (#AB220947), and 93TH065 (#AB220948); CRF02_AG, 03GH178AG1 (#AB572922), 03GH180AG13 (#AB572923), GH184AG25 (#AB286860), GHNJ188 (#AB231896), and 97GH_AG2 (#AB052867). Amino acid alignments of these Vif proteins are shown in supplemental Fig. S1.

DNA Construction—The HIV-1 proviral constructs pNL4-3 (48), Vif-deficient HIV-1 proviral indicator construct pNL-Luc-F(−)E(−) (34), Rev expression plasmid pCA-Rev (52), vesicular stomatitis virus glycoprotein (VSV-G) expression vector pHIT/G (53), and HA-tagged human APOBEC3G expression plasmid pCA-hA3G-HA (8) were previously described. To create a C-terminal FLAG-tagged expression plasmid, a synthetic double-stranded oligonucleotide NotI linker harboring an MscI site upstream of the FLAG epitope (sense, 5′-GGC CTA TGG CCA CGA TTA TAA AGA CGA TGA CGA CAA GTA GAG C-3′; antisense, 5′-GGC CGC TCT ACT TGT CGT CAT CGT CTT TAT AAT CGT GGC CAT A-3′) was inserted into the NotI site of the mammalian expression vector pCAGGS (54), in which the preexisting MscI site was disrupted for further cloning. To confer Rev-dependent expression on the expression plasmids, the pNL4-3-derived Rev-responsive element (RRE; nucleotide 7759–7992) was PCR-amplified, digested with NotI, and inserted into the FLAG-tagged expression plasmid, resulting in pCAGGS-FLAG-RRE. HIV-1 *vif* genes derived from the different subtypes described above were PCR-amplified, digested with KpnI and MscI, and cloned into pCAGGS-FLAG-RRE.

To create chimeric constructs between the Vif proteins of NL4-3 and 02ZMDBC33 (representative of subtypes B and C, respectively), the KpnI-PflMI fragment (corresponding to Vif residues 1–87) of pC-NLvif-FLAG-RRE (NL-Vif) or pC-DBvif-FLAG-RRE (DB-Vif) was replaced with that of DB-Vif or NL-Vif, respectively, resulting in NL/DB-Vif or DB/NL-Vif, respectively. DB(38–87)-Vif consisting of the N-terminal NL-Vif region (residues 1–37), the middle DB-Vif region (residues 38–87), and the C-terminal NL-Vif region downstream of the PflMI site was created with overlapping PCR-based cloning using NL-Vif and DB/NL-Vif as templates. Similarly, chimeric constructs between the N-terminal DB-Vif region (residues 1–37, 1–34, 1–31, and 1–23) and the C-terminal NL-Vif region, which were designated DB(1–37)-Vif, DB(1–34)-Vif, DB(1–31)-Vif, and DB(1–23)-Vif, were created using DB-Vif and NL-Vif as templates, respectively. DB(9–37)-Vif consisting of the DB-Vif region of residues 9–37 with the NL-Vif backbone was created with the QuikChange site-directed mutagenesis kit (Stratagene) using DB(1–37)-Vif as a template.

To introduce the Lys-17 → Arg or Lys-19 → Arg mutations into DB(1–31)-Vif to create DB(1–31)K17R-Vif or DB(1–31)K19R-Vif, respectively, QuikChange site-directed mutagenesis was performed using DB(1–31)-Vif as a template. The Vif-chimeric HIV-1 proviral constructs pNL-DBvif and pNL-DB(1–31)vif were created with overlapping PCR-based cloning using pNL4-3 and DB-Vif as templates, respectively. A Vif-deficient HIV-1 proviral construct pNL-Δvif was generated by introducing a stop codon linker at the PflMI site of pNL4-3. The T7 epitope-tagged (55) APOBEC3G plasmid pCA-hA3G-T7E was generated by inserting an APOBEC3G fragment amplified from pCA-hA3G-HA (8) into a modified pCAGGS expression vector carrying a C-terminal T7 epitope tag.

To create the Elongin C expression plasmid pC-EloC-HA, total RNA from H9 cells was subjected to reverse transcription followed by amplification with specific oligonucleotides. An amplified Elongin C fragment was cloned into modified pCAGGS carrying a C-terminal HA tag. To generate the Cul5 expression plasmid pC-Cul5, total RNA isolated from MOLT-4 cells was subjected to RT-PCR amplification of the Cul5 gene using specific oligonucleotides, and an amplified Cul5 fragment was cloned into pCAGGS. All constructs were verified using an ABI model 3130 Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis—Patient-derived *vif* genes and the *vif* sequences of reference strains representing the different genetic subtypes were aligned using the ClustalW program (56). A phylogenetic tree, constructed by the neighbor-joining method with branching order reliability determined by the bootstrap approach, was implemented with the ClustalW program. Genetic distances were estimated by the Kimura 2-parameter method (57).

Cell Maintenance, Transfections, and Protein Analyses—To confirm Vif protein expression, the 293T cells, maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, were cotransfected with 200 ng of pCA-Rev, 200 ng of FLAG-tagged Vif expression plasmids, and empty vector up to 1 μg of total DNA by using the FuGENE 6 transfection reagent (Roche Applied Science). Cell extracts from the transfected cells were subjected to Western blot analysis using the anti-FLAG mouse monoclonal antibody M2 (Sigma).

Virion Production, APOBEC3G Degradation, and Viral Infectivity Assay—To prepare VSV-G-pseudotyped HIV-1 luciferase reporter viruses, 3.5×10^5 293T cells were cotransfected with 35 ng of pCA-hA3G-HA, 0.1 μg of the VSV-G expression plasmid pHIT/G, 8 ng of the Vif expression plasmids, and 0.87 μg of an empty vector together with 1 μg of pNL-Luc-F(-)E(-) using FuGENE 6. Sixteen hours later cells were washed with phosphate-buffered saline, and 1 ml of fresh complete medium was added. After 24 h, supernatants were treated with 37.5 units/ml DNase I (Roche Applied Science) for 37 °C for 30 min and then harvested.

To analyze the level of Vif-degraded APOBEC3G, cells were lysed and subjected to Western blot analysis using the anti-HA mouse monoclonal antibody HA-7 (Sigma). The p24 antigen levels in viral supernatants were measured by an HIV-1 p24-antigen capture enzyme-linked immunosorbent assay (Advanced BioScience Laboratories). To determine the viral infectivity, 3.5×10^4 293T cells were incubated with 1 ng of p24 antigen of the HIV-1 supernatants. After 48 h, cells were lysed in 100 μl of lysis buffer. The firefly luciferase activity was determined with a Centro LB960 (Berthold) luminometer.

Immunoprecipitation—The 293T cells (7×10^5) were cotransfected using FuGENE 6 with 200 ng of pCA-hA3G-T7E, 64 ng of pC-Vif-FLAG-RRE, 64 ng of pCA-Rev, and empty vector up to 2 μg of total DNA. After 36 h, transfected cells were treated with 20 μM of MG-132 (Calbiochem) for 9 h and sus-

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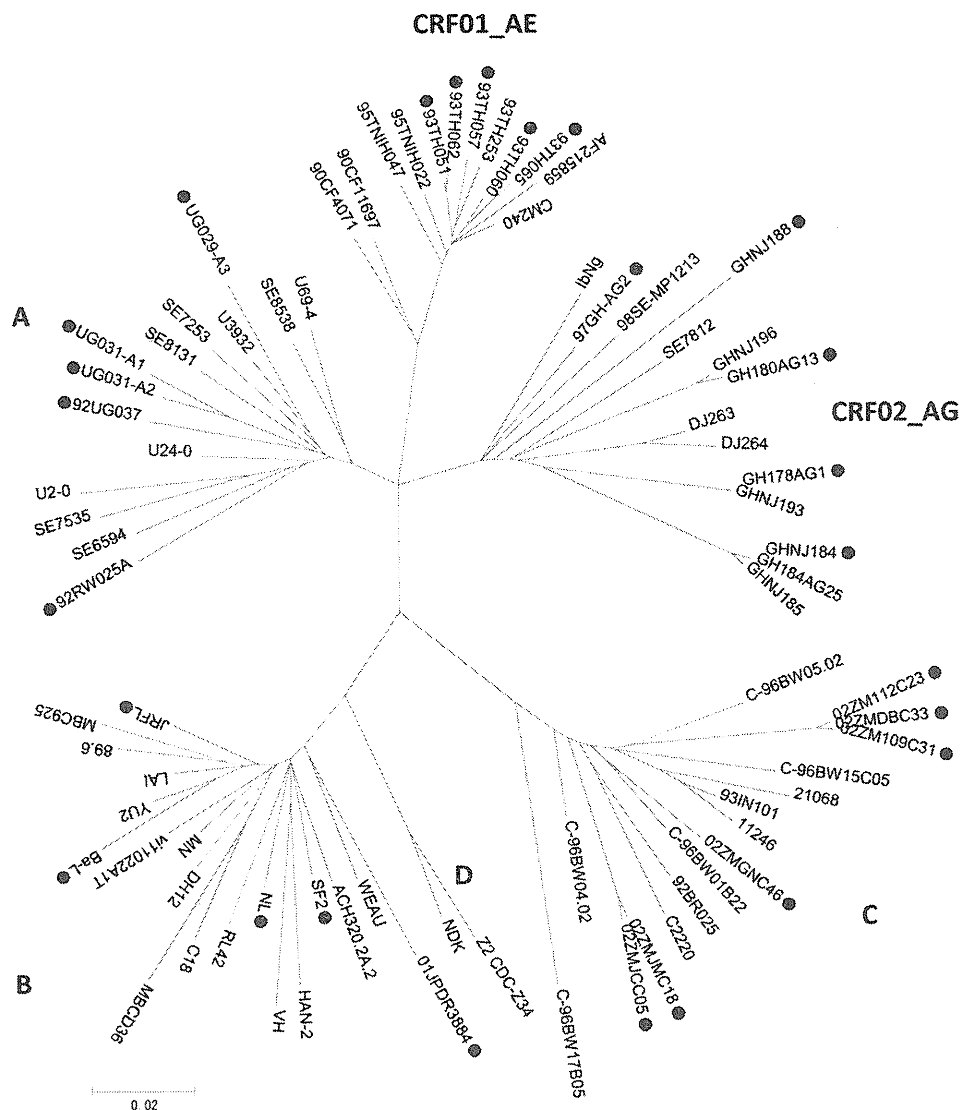

FIGURE 1. **Sequence variation of HIV-1 accessory gene *vif*.** An unrooted phylogenetic tree based on HIV-1 *vif* sequences from group M reference strains was generated by the neighbor-joining method. The *vif* sequences from the major subtypes (A, B, C, D, CRF01_AE, and CRF02_AG) containing reference strains, and isolates tested in this study are included. Different subtype-derived *vif* sequences selected for functional testing are indicated by ●.

pended in 500 μl of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 20 μM MG-132, and complete protease inhibitor mixture (Roche Applied Science)). The resultant lysates were clarified by brief centrifugation, pre-cleared with 30 μl of rProtein A-Sepharose Fast Flow (GE Healthcare) for 1 h at 4 °C, and then incubated with an anti-FLAG M2 Affinity Gel (Sigma). After 1 h at 4 °C, the immune complexes were extensively washed with radioimmunoprecipitation assay buffer. Equal aliquots of the total and bound fractions were subjected to gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were probed with an anti-T7 tag mouse monoclonal antibody (Novagen), an anti-FLAG rabbit polyclonal antibody (Sigma), or an anti-β-actin mouse monoclonal antibody AC-74 (Sigma). The signal intensity of the immunoprecipitated APOBEC3G protein on Western blots was quantified using the LAS-3000 imaging system (Fujifilm).

G-to-A Mutation Assay—Freshly isolated PBMCs (1×10^6) were stimulated with phytohemagglutinin (3 μg/ml) for 72 h. After 293T transfection with the proviral DNA clones pNL4-3, pNL-DBvif, NL-DB(1–31)vif, and pNL-Δvif, PBMCs were infected overnight with 50 ng of the resulting four viruses. Cells were cultured in the presence of interleukin-2 (10 units/ml). After 12 days, the total cellular DNAs from infected cells were isolated using DNeasy (Qiagen). Fragments of the 3′ region of the envelope gene (nucleotides 8127–8756, which has a high G-to-A mutational frequency (58)) were PCR-amplified with High Fidelity DNA Polymerase (Roche Applied Science) and cloned into the TOPO TA-cloning vector pCR4 (Invitrogen). The nucleotide sequence was determined to compare the G-to-A mutational frequency using ABI3130 (ABI).

RESULTS

Phylogenetic Analysis of the HIV-1 Accessory Gene vif Derived from Different Subtypes—HIV-1 Vif expression plasmids were generated by PCR-amplifying known and recently isolated *vif* genes derived from the worldwide prevalent subtypes A, B, C, CRF01_AE, and CRF_02AG. Five subtype A (UG029-A3, UG031-A1, UG031-A2, 92RW025A, and 92UG037), five subtype B (NL, SF2, JRFL, BaL, and DR3884), six subtype C (02ZMJCC05, 02ZMJMC18, 02ZM109C31, 02ZM112C23, 02ZMDBC33, and 02ZMGN46), five subtype CRF01_AE (93TH051, 93TH057, 93TH060, 93TH062, and 93TH065), and five subtype CRF_02AG strains (03GH178, 03GH180C13, 03GH184AG25, GHNJ188, and 97GH_AG2) were used. The genes were cloned into FLAG-tagged mammalian expression plasmids, sequenced, and genetically compared. Fig. 1 shows the phylogenetic analysis results for the HIV-1 *vif* genes utilized in this study. This tree clearly classifies each subtype based on difference between the *vif* sequences.

Anti-APOBEC3G Activities of HIV-1 Vif Proteins Differ in a Subtype-dependent Manner—Because the *vif* sequences appear to be variable (11, 44–47), we examined whether Vif proteins derived from different subtypes with the sequence diversity shown here would show differential levels of anti-APOBEC3G activity. To do this we first determined the optimal doses of the APOBEC3G and Vif expression plasmids. The mRNA levels of APOBEC3G endogenously expressed in three donor-derived

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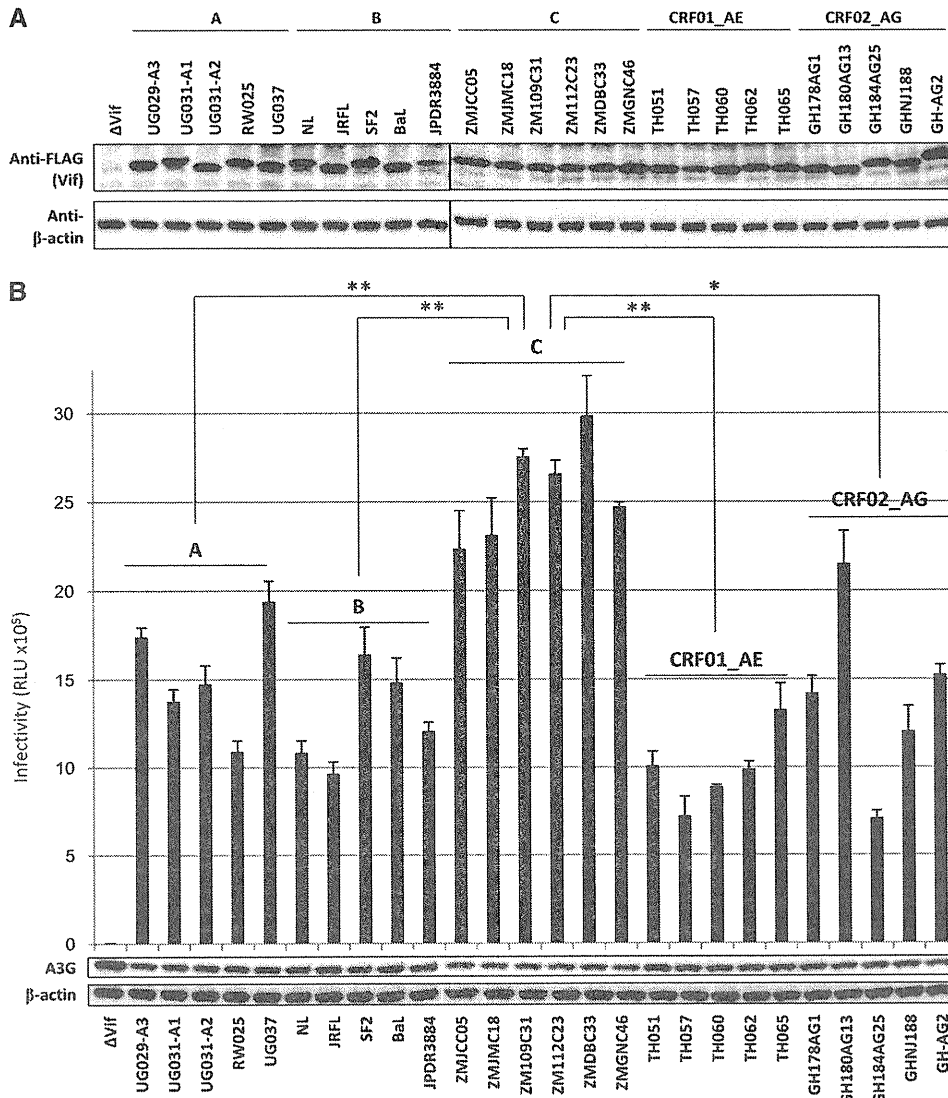

FIGURE 2. **Functional testing to compare the anti-APOBEC3G activity of Vif proteins from different subtypes.** *A*, Western blot analysis was performed using extracts from 293T cells transfected with combinations of plasmids encoding Rev and RRE-carrying/FLAG-tagged Vif derived from five major subtypes. Antibodies specific for FLAG and β-actin were used. Note that a larger amount (200 ng) of Vif expression plasmids was used to confirm the expression of Vif proteins, whereas the optimal amount (8 ng) was used for virus preparation in B. *B*, 293T cells were cotransfected with a luciferase-based Vif/Env-deficient HIV-1, VSV-G, and HA-tagged APOBEC3G expression plasmids together with plasmids encoding FLAG-tagged Vif derived from different subtypes. After 40 h, cell lysate and viral supernatant were harvested. To examine the proteasomal degradation of APOBEC3G in the virus-producing cells, the lysate was subjected to Western blot analysis (shown below the *bar graph*) using monoclonal antibodies against HA or β-actin. Supernatants normalized by the p24 antigen of VSV-G-pseudotyped luciferase viruses were incubated with 293T cells for an additional 48 h. Cells were then lysed and subjected to a luciferase assay. Results are representative of at least five independent triplicate experiments. *RLU*, relative light units. Data shown are the mean ± S.D.; *, $p < 0.005$, **, $p < 0.001$, *t* test.

PBMCs were compared with levels expressed in cells transfected with the serially diluted APOBEC3G expression plasmid. Levels of Vif protein physiologically expressed from the Vif-positive NL4-3 proviral construct were compared with levels expressed from the serially diluted NL-Vif expression plasmid. Real-time RT-PCR revealed that introduction of 25 ng of APOBEC3G plasmid into 293T cells reproduced the endogenous expression level of APOBEC3G in PBMCs (supplemental Fig. S2*A*).

Infectivity enhancement by the Vif protein was evaluated from the proviral construct or from the expression plasmid in the presence of a fixed amount of the APOBEC3G plasmid (as determined above). The optimal dose of the Vif plasmid that reflected its physiological expression was 8 ng (supplemental Fig. S2*E*). Using the same approach, the endogenous expression level of APOBEC3F in PBMCs was also determined (supplemental Fig. S2*B*). The corresponding dose (~0.8 ng) of the APOBEC3F expression plasmid showed only a ~30% reduction of Δvif virus infectivity, whereas that of APOBEC3G showed an ~400-fold reduction (supplemental Fig. S2, *D* and *C*, respectively). These findings suggest that the endogenous APOBEC3F level is significantly less potent than that of APOBEC3G in inhibiting HIV-1 infection, as previously and very recently described (12, 59, 60).

Vif protein expression in the plasmid-transfected cells was confirmed by immunoblotting using anti-FLAG antibodies (Fig. 2*A*). Vif functional testing was performed using viruses from 293T cells cotransfected with a Vif-Env-double-defective HIV-1 construct and a VSV-G expression plasmid together with optimal doses of APOBEC3G and various subtype-derived Vif expression plasmids. Most Vif proteins derived from subtypes A, B, CRF01_AE, and CRF_02AG showed non-significant but somewhat differential activity levels based on infectivity profiles (which represent the anti-APOBEC3G activity) (Fig. 2*B*). In contrast, subtype C-derived Vif proteins showed exclusively high anti-APOBEC3G activity levels. The Vif-mediated infectivity enhancements generally correlated with the proteasomal degradation levels of APOBEC3G protein (Fig. 2*B*). Compared with Vif from other subtypes, subtype C-derived Vif proteins seemed to harbor higher activity against the antiviral APOBEC3G protein.

The N-terminal Domain of Subtype C-derived Vif Confers a Robust Anti-APOBEC3G Activity—To identify the smallest segment of subtype C-derived Vif protein needed to control anti-APOBEC3G activity, chimeric Vif constructs were created between subtypes B (derived from NL4-3) and C (derived from 02ZMDBC33) utilizing a preexisting restriction enzyme site PflMI commonly located in the middle of Vif. The chimeric Vif construct carrying N-terminal residues 1–87 of subtype C-de-

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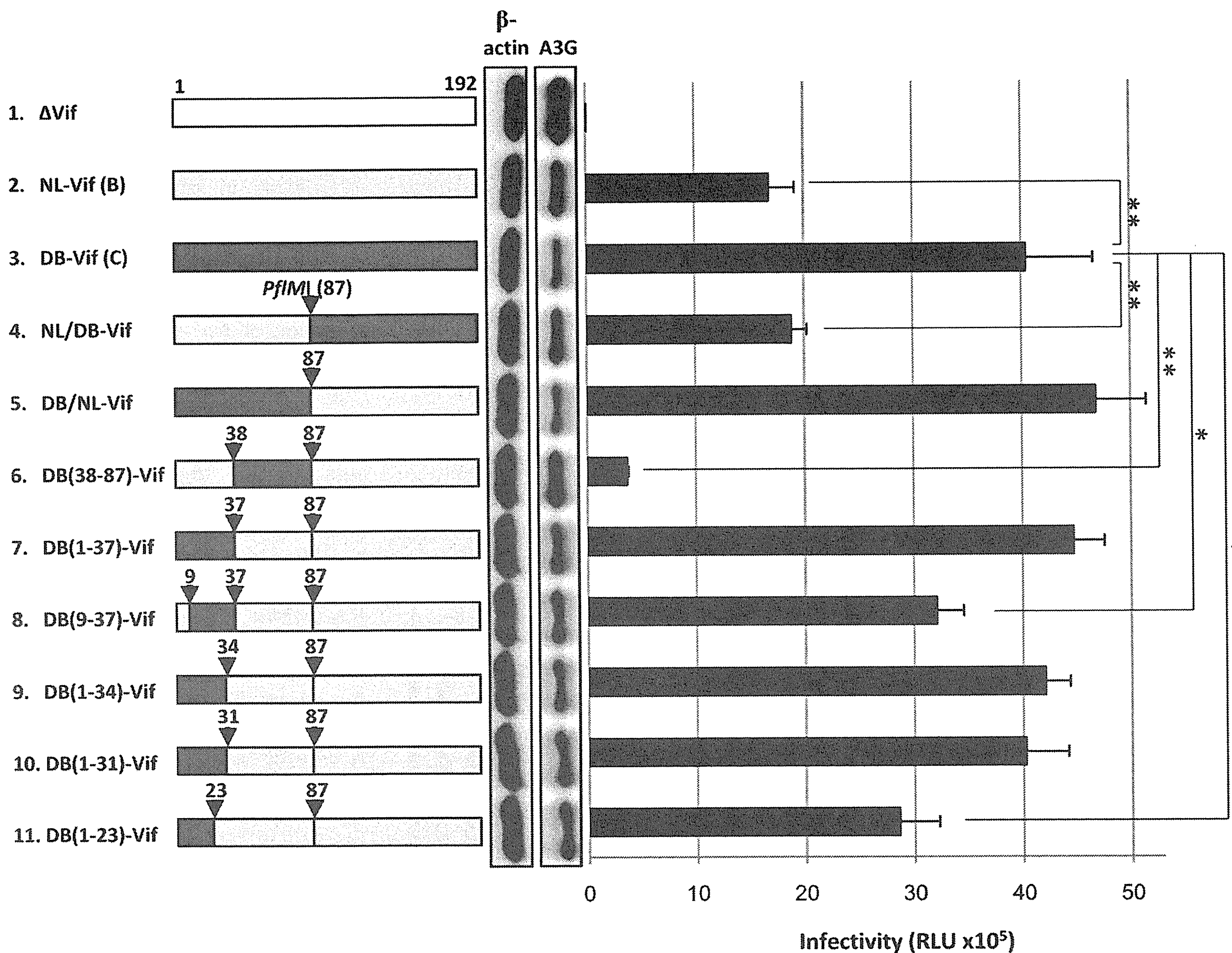

FIGURE 3. **Mapping the determinant of the enhanced anti-APOBEC3G activity of subtype C-derived Vif protein.** Vif chimeras between subtypes B and C (depicted on the *left*) were first tested for the ability of Vif proteins to induce proteasomal degradation of APOBEC3G (as shown on the *left side of the bar graph*) by performing Western blot analysis as described in Fig. 2*B*. Functional testing for the anti-APOBEC3G activity of the chimeric Vif proteins in the single-round replication assay is also described in Fig. 2*B*. Results are representative of at least four independent triplicate experiments. *RLU*, relative light units. Data shown are the mean ± S.D.; *, $p < 0.05$; **, $p < 0.01$, *t* test.

rived Vif (DB/NL-Vif) displayed an even higher activity than the construct carrying C-terminal residues 88–189 of subtype C-derived Vif (NL/DB-Vif) (Fig. 3, *lanes 4* and *5*). Therefore, we dissected the N-terminal half of subtype C-derived Vif by substituting the upstream (residues 1–37) or downstream (residues 38–87) half of this fragment for the corresponding region of subtype B-Vif using the overlapping PCR method (resulting in DB(1–37)-Vif and DB(38–87)-Vif). N-terminal residues 1–37 appeared to determine the subtype C-derived Vif activity against APOBEC3G (Fig. 3, *lanes 6* and *7*). Narrowing the N-terminal region of subtype C-derived Vif protein from residues 1 to 37 to residues 9–37, 1–34, 1–31, or 1–23 revealed that the N-terminal region up to position 31 was crucial for the full anti-APOBEC3G activity seen in subtype C-derived Vif proteins (Fig. 3, *lanes 8–11*). The viral infectivity levels were consistent with those of APOBEC3G degradation (Fig. 3), as observed in Fig. 2*B*.

Among the cluster of amino acids residues 1–31 of subtype C-derived Vif, 8L and 31V were found to be critical based on the chimeric experiments described above (Fig. 3; see also supplemental Fig. S3*A*). Although 8L is highly specific for the subtype C-derived Vif sequences available from the Los Alamos HIV sequence data base (www.hiv.lanl.gov), 31V is not subtype C-specific but is conserved in ~40% of subtype C-derived Vif sequences in the data base. To address whether the critical amino acids would include 17K (which is not found in other subtypes but is conserved in ~50% of subtype C-derived Vif sequences, the other half of which carry 17R; supplemental Fig. S3*A*) and 19K (which is rather rare in subtype C-derived Vif sequences in the data base, most of which normally carry 19R; supplemental Fig. S3*A*), the lysine at position 17 or 19 was replaced with arginine. The results indicated that 17K was important, whereas19K was replaceable with arginine (supplemental Fig. S3, *B* and *C*). Thus, the critical amino acid cluster not present in NL is the N-terminal cluster of 8L, 17K, 19K/R, 22N (highly conserved in non-B subtypes), 23S (conserved in all subtypes but NL), and 31V.

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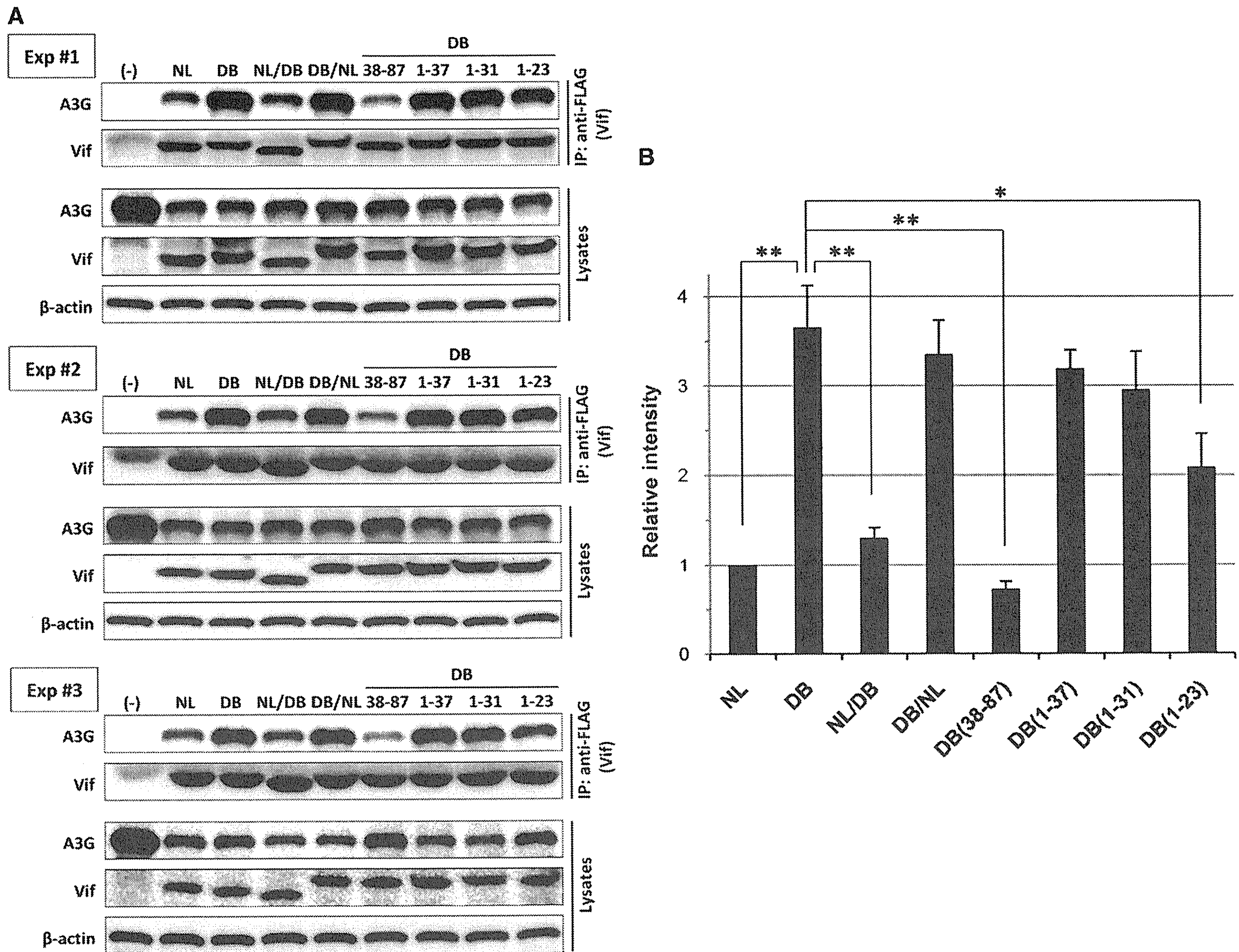

FIGURE 4. **Binding activity of chimeric Vif proteins to APOBEC3G.** *A*, 293T cells were cotransfected with the T7 epitope-tagged APOBEC3G expression plasmid, Rev expression plasmid, and RRE-carrying/FLAG-tagged chimeric Vif expression plasmids were immunoprecipitated (*IP*) with an anti-FLAG monoclonal antibody. The resulting complexes were analyzed by immunoblotting with monoclonal antibodies to the T7 epitope or with polyclonal antibodies to FLAG to detect APOBEC3G and Vif proteins, respectively (*upper two panels in each experiment*). Cell lysate aliquots were also analyzed by immunoblotting in parallel for T7 epitope and FLAG together with β-actin (*lower three panels*). Results of three independent experiments are individually shown as Experiments 1, 2, and 3. *B*, binding activity of chimeric Vif proteins to APOBEC3G was quantified based on the band intensity of the immunoprecipitated APOBEC3G protein. Results are the mean ± S.D. of three experiments. *, $p < 0.01$; **, $p < 0.001$, *t* test.

N-terminal Region of Subtype C-derived Vif Harbors Higher APOBEC3G Binding Activity—To examine whether subtype C-derived Vif or B-Vif carrying the N-terminal 1–31 fragment of subtype C-derived Vif could bind to APOBEC3G more efficiently than subtype B-Vif, we analyzed the interaction between APOBEC3G and Vif by coimmunoprecipitation. In three independent experiments (Figs. 4, *A* and *B*), the chimeric B-Vif proteins carrying the N-terminal half up to residues 1–31 of subtype C-derived Vif (DB/NL, DB(1–37), DB(1–34), and DB(1–31)) and DB-Vif showed higher levels of APOBEC3G binding than did the B-Vif protein. The binding levels of Vif proteins to APOBEC3G were consistent with the infectivity enhancement levels. Thus, the enhanced anti-APOBEC3G activity of subtype C-derived Vif protein is determined by its higher APOBEC3G binding activity at N-terminal amino acids 1–31. As expected, interactions with components of the E3 ubiquitin ligase complexes Cul5 and Elongin C were equivalent among all Vif proteins tested. This is consistent with the conservation of highly conserved SOCS box and the upstream cysteines (Cys-114 and Cys-133) required for E3 ligase complex assembly (supplemental Fig. S4).

Subtype C-derived Vif Strongly Protects Viral Genomes from G-to-A Mutations Caused by Endogenous Levels of APOBEC3G Expression—Finally, we addressed whether the anti-APOBEC3G activity of the Vif protein would correlate with the frequency of viral G-to-A mutations caused by endogenous APOBEC3G in PBMCs multiply infected by replication-competent viruses. We created the pNL4-3-based full-length proviral DNA harboring the entire or N-terminal (residues 1–31) domain of the subtype C-derived Vif sequence to compare their viral G-to-A mutation rate of the resulting viruses with those of NL4-3 wild-type (WT) (used as a subtype B reference) and with Vif-deficient viruses. Freshly isolated PBMCs were stimulated for 72 h with phytohemagglutinin and interleukin-2 and were infected with NL, NL-DBvif, NL-DB(1–31)vif, or NL-Δvif viruses produced from 293T cells transfected with their provi-

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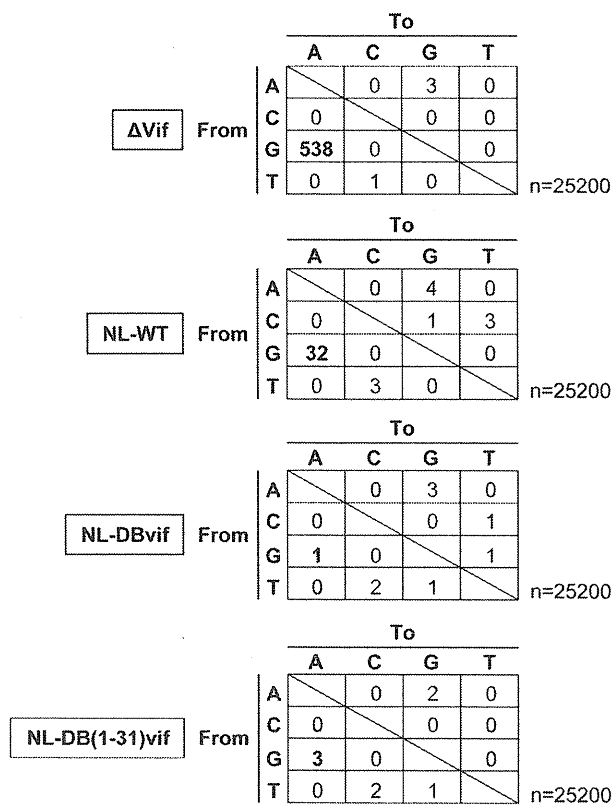

FIGURE 5. **Analysis of G-to-A mutational frequency of proviral DNAs in PBMCs.** Viruses from pNL4-3-based full-length proviral DNA clones carrying *vif*-deficient (Δ*vif*), WT, subtype C-derived (*DBvif*) or B/C-chimeric (*DB(1–31)vif*) *vif* genes were used to infect freshly isolated and phytohemagglutinin-stimulated PBMCs. Infected cells were harvested and lysed for total DNA extraction at 12 days after infection. An amplified HIV-1 *env* fragment was cloned and analyzed for the G-to-A mutational frequency by sequencing. Numbers of substitutions are depicted for each viral infection of PBMCs.

ral DNA clones. At 12 days post-infection, the total cellular DNAs were extracted from the infected cells. The DNAs were subjected to PCR amplification targeting the envelope region followed by TA-cloning. Forty clones each from four different infected cells were sequenced.

The endogenous level of APOBEC3G expression in the PBMCs efficiently induced the G-to-A mutation of Vif-deficient viruses. As expected, envelope sequences (nucleotides 8137–8766) derived from viruses carrying the entire or N-terminal domain of subtype C-derived Vif displayed lower G-to-A mutation rates than those from NL4-3 WT viruses (Fig. 5). However, viruses with subtype C-derived Vif sequences did not show distinct growth kinetics from NL4-3 WT viruses over the time course of the experiment (supplemental Fig. S5). Thus, in primary lymphocytes expressing endogenous APOBEC3G levels, viruses harboring the subtype C-derived Vif protein can maintain the viral nucleotide sequences with less frequent G-to-A mutations than those harboring the subtype B-derived Vif.

DISCUSSION

To date most functional analyses of HIV-1 Vif proteins against APOBEC3G have been based on subtype B-derived Vif, and none has focused on the effect of subtypic differences on anti-APOBEC3G activity. The present study demonstrated that Vif proteins derived from subtype C strains had the most enhanced activity against APOBEC3G among the subtypes tested. Based on experiments using chimeric Vif constructs between subtypes B and C, the enhanced anti-APOBEC3G activity observed in subtype C-derived Vif was determined by residues 1–31, which positively regulated the binding activity for APOBEC3G. Consistent with this observation, these residues were also related to resistance to the G-to-A mutation at an endogenous level of APOBEC3G expression.

The worldwide prevalence of subtype C viruses, including recombinant forms, is >50% among HIV-1-infected individuals (see the UNAIDS website). This finding implies that the viruses *per se* might display characteristics that are distinct from other subtypes. *In vitro* functional testing confirmed our hypothesis that subtype C-derived Vif proteins display different activity against APOBEC3G than those derived from other subtypes. Several lines of evidence suggest the uniqueness of subtype C viruses, such as their extra copy of the NF-κB enhancer element in the long terminal repeat region (61, 62), their low replication level in macrophages and CD4-postitive lymphocytes (63–65) with reduced pathogenic fitness (66), their predominant CCR5 tropism (63, 67–69), and the fact that autologous neutralizing antibody induction is associated with shorter V1-to-V5 envelope lengths (70, 71).

Our new finding of the robust anti-APOBEC3G activity of subtype C-derived Vif might be restricted to certain regional populations of subtype C strains. Among the identified cluster of residues 1–31 critical for robust anti-APOBEC3G activity, 31V is not subtype C-specific but is conserved in ~40% of subtype C-derived Vif proteins. This conservation is particularly high in Indian (80%, $n = 15$) and Zambian (77%, $n = 18$) subtype C-derived Vif sequences (available as interpatient samples from the data base). Conservation of 17K, which is specific for and conserved in ~50% of all subtype C-derived Vif sequences, also depends on the sampling country (*e.g.* Botswana, 62% ($n = 45$); India, 93% ($n = 15$); Tanzania, 50% ($n = 20$); South Africa, 48% ($n = 269$); Zambia, 61% ($n = 18$)). Based on the data base and chimeric/mutational analyses shown in Fig. 3, residues 19K/R, 23S (conserved in all subtypes but NL), 22N (conserved in non-B subtypes), and 8L (highly specific for subtype C), completely conserved in subtype C-derived Vif sequences, were not sufficient for the full activity of subtype C-derived Vif proteins. This full activity likely requires the existence of 31V and 17K described above, both of which are conserved in all of our Zambian subtype C samples, and in 44% of Zambian ($n = 18$) or 73% of Indian ($n = 15$) subtype C-derived Vif sequences in the data base. It is likely that the observed subtype C viruses carrying fully active Vif are regionally circulating strains.

Still, the central findings of the present study are consistent with the previous results reported by Janini *et al.* (38), who have provided some evidence in favor of the hypothesis that subtype C HIV-1 is relatively protected from APOBEC3G effects *in vivo*. In the study they evaluated the presence of hypermutated HIV-1 sequences in proviral DNA from the PBMCs of 53 patients using a screening method that identified AT-rich sequences. They showed that the hypermutation occurred in 57% of subtype A, 67% of subtype D, 44% of subtype B, and 21% of subtype C infections and that subtype C hypermutated

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sequences (in 3 of 14 patients) also had lower levels of the G-to-A mutation than other subtype sequences, although the differences did not reach statistical significance because of the small sample size. To confirm these *in vivo* observations, further analysis involving large-scale sequencing of patient derived-subtype C proviral DNA will be necessary (note that the G-to-A hypermutation is rarely detectable in plasma RNA sequences (40–42)).

Several groups have identified distinct APOBEC3G binding domains of Vif (11, 72–81). It should be noted that the N-terminal amino acid cluster identified here that underlies the increased APOBEC3G binding activity of subtype C-derived Vif partially overlaps with previously identified domains, *e.g.* the N-terminal ^{21}W*x*SLVK^{26} (76) or ^{23}SLV*x*4Y*x*9Y^{40} (77) motif. It seems likely that additional changes of the N-terminal amino acids of Vif protein surrounding these reported motifs might result in a previously unknown gain of enhanced binding activity of this protein to APOBEC3G, as observed in the subtype C-derived Vif proteins tested. This needs to be further elucidated by structure-based analyses of Vif proteins.

Consistent with our observation that subtype C-derived Vif proteins showed higher anti-APOBEC3G activity than subtype B-derived Vif proteins, a much lower G-to-A mutational rate was observed in viruses carrying subtype C-derived Vif than in NL4-3 WT viruses at endogenous APOBEC3G expression levels in PBMCs. Sadler *et al.* (82) recently reported that sublethal G-to-A mutation levels induced by APOBEC3G even in the presence of Vif allowed the viruses to yield sufficiently replication-proficient viral progeny with a highly diverse pool of quasispecies. In this context it would be intriguing to conjecture that subtype C viruses might not be as flexible in modulating HIV-1 fitness or in increasing diversity as subtype B viruses, which have moderate but not robust Vif activity in APOBEC3G neutralization. Further studies are required to test this hypothesis.

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REFERENCES

1. Langlois, M. A., and Neuberger, M. S. (2008) *J. Virol.* **82,** 4660–4664
2. Sheehy, A. M., Gaddis, N. C., Choi, J. D., and Malim, M. H. (2002) *Nature* **418,** 646–650
3. Yu, Q., Chen, D., König, R., Mariani, R., Unutmaz, D., and Landau, N. R. (2004) *J. Biol. Chem.* **279,** 53379–53386
4. Delebecque, F., Suspène, R., Calattini, S., Casartelli, N., Saïb, A., Froment, A., Wain-Hobson, S., Gessain, A., Vartanian, J. P., and Schwartz, O. (2006) *J. Virol.* **80,** 605–614
5. Stenglein, M. D., and Harris, R. S. (2006) *J. Biol. Chem.* **281,** 16837–16841
6. Bogerd, H. P., Wiegand, H. L., Hulme, A. E., Garcia-Perez, J. L., O'Shea, K. S., Moran, J. V., and Cullen, B. R. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 8780–8785
7. Muckenfuss, H., Hamdorf, M., Held, U., Perkovic, M., Löwer, J., Cichutek, K., Flory, E., Schumann, G. G., and Münk, C. (2006) *J. Biol. Chem.* **281,** 22161–22172
8. Kinomoto, M., Kanno, T., Shimura, M., Ishizaka, Y., Kojima, A., Kurata, T., Sata, T., and Tokunaga, K. (2007) *Nucleic Acids Res.* **35,** 2955–2964
9. Sasada, A., Takaori-Kondo, A., Shirakawa, K., Kobayashi, M., Abudu, A., Hishizawa, M., Imada, K., Tanaka, Y., and Uchiyama, T. (2005) *Retrovirology* **2,** 32
10. Bishop, K. N., Holmes, R. K., Sheehy, A. M., Davidson, N. O., Cho, S. J., and Malim, M. H. (2004) *Curr. Biol.* **14,** 1392–1396
11. Simon, V., Zennou, V., Murray, D., Huang, Y., Ho, D. D., and Bieniasz, P. D. (2005) *PLoS Pathog.* **1,** e6
12. Zennou, V., and Bieniasz, P. D. (2006) *Virology* **349,** 31–40
13. Zhang, H., Yang, B., Pomerantz, R. J., Zhang, C., Arunachalam, S. C., and Gao, L. (2003) *Nature* **424,** 94–98
14. Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., and Trono, D. (2003) *Nature* **424,** 99–103
15. Harris, R. S., Bishop, K. N., Sheehy, A. M., Craig, H. M., Petersen-Mahrt, S. K., Watt, I. N., Neuberger, M. S., and Malim, M. H. (2003) *Cell* **113,** 803–809
16. Shindo, K., Takaori-Kondo, A., Kobayashi, M., Abudu, A., Fukunaga, K., and Uchiyama, T. (2003) *J. Biol. Chem.* **278,** 44412–44416
17. Newman, E. N., Holmes, R. K., Craig, H. M., Klein, K. C., Lingappa, J. R., Malim, M. H., and Sheehy, A. M. (2005) *Curr. Biol.* **15,** 166–170
18. Guo, F., Cen, S., Niu, M., Saadatmand, J., and Kleiman, L. (2006) *J. Virol.* **80,** 11710–11722
19. Luo, K., Wang, T., Liu, B., Tian, C., Xiao, Z., Kappes, J., and Yu, X. F. (2007) *J. Virol.* **81,** 7238–7248
20. Mbisa, J. L., Barr, R., Thomas, J. A., Vandegraaff, N., Dorweiler, I. J., Svarovskaia, E. S., Brown, W. L., Mansky, L. M., Gorelick, R. J., Harris, R. S., Engelman, A., and Pathak, V. K. (2007) *J. Virol.* **81,** 7099–7110
21. Sheehy, A. M., Gaddis, N. C., and Malim, M. H. (2003) *Nat. Med.* **9,** 1404–1407
22. Marin, M., Rose, K. M., Kozak, S. L., and Kabat, D. (2003) *Nat. Med.* **9,** 1398–1403
23. Stopak, K., de Noronha, C., Yonemoto, W., and Greene, W. C. (2003) *Mol. Cell* **12,** 591–601
24. Mehle, A., Strack, B., Ancuta, P., Zhang, C., McPike, M., and Gabuzda, D. (2004) *J. Biol. Chem.* **279,** 7792–7798
25. Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P., and Yu, X. F. (2003) *Science* **302,** 1056–1060
26. Mehle, A., Goncalves, J., Santa-Marta, M., McPike, M., and Gabuzda, D. (2004) *Genes Dev.* **18,** 2861–2866
27. Yu, Y., Xiao, Z., Ehrlich, E. S., Yu, X., and Yu, X. F. (2004) *Genes Dev.* **18,** 2867–2872
28. Kobayashi, M., Takaori-Kondo, A., Miyauchi, Y., Iwai, K., and Uchiyama, T. (2005) *J. Biol. Chem.* **280,** 18573–18578
29. Alce, T. M., and Popik, W. (2004) *J. Biol. Chem.* **279,** 34083–34086
30. Svarovskaia, E. S., Xu, H., Mbisa, J. L., Barr, R., Gorelick, R. J., Ono, A., Freed, E. O., Hu, W. S., and Pathak, V. K. (2004) *J. Biol. Chem.* **279,** 35822–35828
31. Zennou, V., Perez-Caballero, D., Göttlinger, H., and Bieniasz, P. D. (2004) *J. Virol.* **78,** 12058–12061
32. Schäfer, A., Bogerd, H. P., and Cullen, B. R. (2004) *Virology* **328,** 163–168
33. Wiegand, H. L., Doehle, B. P., Bogerd, H. P., and Cullen, B. R. (2004) *EMBO J.* **23,** 2451–2458
34. Zheng, Y. H., Irwin, D., Kurosu, T., Tokunaga, K., Sata, T., and Peterlin, B. M. (2004) *J. Virol.* **78,** 6073–6076
35. Dang, Y., Wang, X., Esselman, W. J., and Zheng, Y. H. (2006) *J. Virol.* **80,** 10522–10533
36. Fitzgibbon, J. E., Mazar, S., and Dubin, D. T. (1993) *AIDS Res. Hum. Retroviruses* **9,** 833–838
37. Borman, A. M., Quillent, C., Charneau, P., Kean, K. M., and Clavel, F. (1995) *Virology* **208,** 601–609
38. Janini, M., Rogers, M., Birx, D. R., and McCutchan, F. E. (2001) *J. Virol.* **75,** 7973–7986
39. Koulinska, I. N., Chaplin, B., Mwakagile, D., Essex, M., and Renjifo, B. (2003) *AIDS Res. Hum. Retroviruses* **19,** 1115–1123
40. Russell, R. A., Moore, M. D., Hu, W. S., and Pathak, V. K. (2009) *Retrovirology* **6,** 16
41. Bailey, J. R., Williams, T. M., Siliciano, R. F., and Blankson, J. N. (2006) *J. Exp. Med.* **203,** 1357–1369
42. Kieffer, T. L., Kwon, P., Nettles, R. E., Han, Y., Ray, S. C., and Siliciano, R. F. (2005) *J. Virol.* **79,** 1975–1980
43. Pace, C., Keller, J., Nolan, D., James, I., Gaudieri, S., Moore, C., and Mallal, S. (2006) *J. Virol.* **80,** 9259–9269

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