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IFN-γ–Producing Effector CD8 T Lymphocytes Cause Immune Glomerular Injury by Recognizing Antigen Presented as Immune Complex on Target Tissue

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We investigated the role of effector CD8 T cells in the pathogenesis of immune glomerular injury. BALB/c mice are not prone to autoimmune disease, but after 12 immunizations with OVA they developed a variety of autoantibodies and glomerulonephritis accompanied by immune complex (IC) deposition. In these mice, IFN-γ–producing effector CD8 T cells were significantly increased concomitantly with glomerulonephritis. In contrast, after 12 immunizations with keyhole limpet hemocyanin, although autoantibodies appeared, IFN-γ–producing effector CD8 T cells did not develop, and glomerular injury was not induced. In β_2-microglobulin–deficient mice lacking CD8 T cells, glomerular injury was not induced after 12 immunizations with OVA, despite massive deposition of IC in the glomeruli. In mice containing a targeted disruption of the exon encoding the membrane-spanning region of the Ig μ-chain (μMT mice), 12 immunizations with OVA induced IFN-γ–producing effector CD8 T cells but not IC deposition or glomerular injury. When CD8 T cells from mice immunized 12 times with OVA were transferred into naive recipients, glomerular injury could be induced, but only when a single injection of OVA was also given simultaneously. Importantly, injection of OVA could be replaced by one injection of the sera from mice that had been fully immunized with OVA. This indicates that deposition of IC is required for effector CD8 T cells to cause immune tissue injury. Thus, in a mouse model of systemic lupus erythematosus, glomerular injury is caused by effector CD8 T cells that recognize Ag presented as IC on the target renal tissue. *The Journal of Immunology*, **2013, 191: 000–000.**

Glomerular injury is a major clinical feature of systemic lupus erythematosus (SLE) and is found in up to 50% of SLE patients. This injury has been attributed to the induction of an immunopathology resulting from immune complex (IC) deposition (1–7). However, it is also clear that IC by itself is not sufficient for the development of glomerular injury (8–11) and that CD4 T cells also contribute to the glomerular injury seen in SLE. Wofsy et al. (12–14) and Jabs et al. (15) showed that anti-CD4 T cell Ab therapy could significantly reduce the frequency and the extent of glomerulonephritis in both NZB/W F_1 and MRL/*lpr* mouse models of lupus. Jevnikar et al. (16) showed that deficiency of MHC class II results in the amelioration of autoimmune renal disease in MRL/*lpr* mice. In contrast, Chesnutt et al. (17) showed that nephritis is not abolished in CD4-deficient MRL/*lpr* mice, whereas Christianson et al. (18) and Chan et al. (19) showed that glomerular injury is prevented in MHC class I–deficient MRL/*lpr* mice, which lack CD8 T cells. D'Agati et al. (20) showed that CD8, rather than CD4, T cells predominate in most of the biopsied kidney samples from patients with SLE. Couzi et al. (21) showed that CD8 T cells predominantly infiltrate to the periglomerular region of lupus kidney and are significantly associated with the prognosis of lupus nephritis. Together, these findings reinforce the importance of CD8 T cells in the pathogenesis of lupus nephritis. Nevertheless, discrepancies still exist in assessing the contribution of CD4 versus CD8 T cells to glomerular injury and IC deposition (22–25). Thus, we wished to better clarify the role of CD8 T cells in this pathology.

We previously succeeded in inducing glomerular injury with characteristics almost identical to human SLE in mice normally not prone to autoimmune disease (26). In that study, we showed that both CD4 T cell help and Ag cross-presentation are fundamental for activating CD8 T cells to become fully matured CTLs and, subsequently, to induce lupus kidney disease. If this finding is correct, then discrepancies in the literature describing differing contributions of CD4 or CD8 T cells to lupus nephritis (12–21) might be reconciled. Therefore, in the current study, we used β_2-microglobulin (β_2m)-deficient mice, which lack functional effector CTLs, and mice harboring a targeted disruption of the exon encoding the membrane-spanning region of the Ig μ-chain (μMT), which lack B cells, to examine the relationships among IC deposition, effector CD8 T cells, and the nephritis induced in mice by repeated immunization with OVA.

Materials and Methods

Animal studies

Animal studies were performed with the approval of the Institutional Animal Care And Use Committee and according to animal experimental regulations at Kobe University and Kyushu University. Eight-week-old

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Abbreviations used in this article: AU, arbitrary unit; IC, immune complex; KLH, keyhole limpet hemocyanin; β_2m, β_2-microglobulin; μMT, targeted disruption of the exon encoding the membrane-spanning region of the Ig μ-chain; RF, rheumatoid factor; SLE, systemic lupus erythematosus; Treg, regulatory T cell; WT, wild-type.

female BALB/c mice (Japan SLC, Hamamatsu, Japan), β_2m-deficient mice (BALB/c background) (27), and μMT mice (BALB/c background) (28) were immunized with 500 μg OVA (grade V; Sigma, St. Louis, MO), 100 μg keyhole limpet hemocyanin (KLH; Sigma), or PBS by i.p. injection every 5 d. Nine days after the final immunization, proteinuria was measured semiquantitatively using urine dipsticks (Albstix; Siemens Healthcare Diagnostics, Tarrytown, NY), and B, T, CD4 T, and CD8 T cells were isolated from spleen to >90% purity using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated cells were adoptively transferred i.v. into naive BALB/c mice (2.5×10^7/mouse), and an additional booster i.p. injection of 500 μg OVA or 500 μl sera from mice immunized 12 times with OVA was given at 24 h after transfer. Sera, urine, and organs were collected 2 wk later. CD4 and CD8 T cells in the kidney were analyzed by flow cytometry 9 d after the final immunization with allophycocyanin-conjugated anti-CD4 Abs (RM4-5; BioLegend, San Diego, CA) and PerCP-conjugated anti-CD8 Abs (53-6.7; BD Pharmingen, San Diego, CA). Glomerular injury in mice was evaluated by studying 30 glomeruli/mouse.

Immunofluorescent staining

Frozen kidney sections were stained for C3 and IgG using goat anti-C3 Abs (Bethyl Laboratories, Montgomery, TX), Alexa Fluor 488–conjugated anti-goat IgG Abs, or Alexa Fluor 594–conjugated anti-mouse IgG Abs (both from Molecular Probes, Eugene, OR).

Intracellular IFN-γ staining

Spleen cells (1×10^6/ml) were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of brefeldin A (10 μg/ml; all from Sigma) for 4 h and stained with PerCP-conjugated anti-CD8 Ab, followed by fixation in 2% formaldehyde, permeabilization with 0.5% saponin (Sigma), and staining with PE-conjugated anti–IFN-γ Ab (XMG1.2; BD Pharmingen).

ELISA

Sera were assayed for rheumatoid factor (RF) by ELISA (Shibayagi, Gunma, Japan), for anti-Sm Ab using plates coated with Sm Ag (ImmunoVision, Springdale, AR), and for anti-dsDNA Ab using plates coated with dsDNA (Worthington Biochemical, Lakewood, NJ) that had been digested using S1 nuclease (Promega, Madison, WI). Serum IgG, IgG1, and IgG2a were measured by ELISA (Bethyl Laboratories). Serum IC was measured using anti-C3 Ab (Bethyl Laboratories) and HRP-conjugated anti-mouse IgG Ab (Kirkegaard & Perry Laboratories, Gaithersburg, MD), followed by reaction with *o*-phenylenediamine (Sigma). An arbitrary unit (AU) of 1.0 is the equivalent of the titer found in sera of 25-wk-old MRL/*lpr* mice. Anti-OVA Ab in sera was quantified as a reference using mouse anti-OVA mAb (OVA-14; Sigma).

Statistical analysis

Statistical analyses were performed using the Student *t* test, and the data are expressed as the mean ± SD.

Results

Requirement of activated CD8 T cells for glomerular injury

Wild-type (WT) BALB/c mice, which are normally not prone to autoimmune disease, were repeatedly immunized with OVA every 5 d. After 12 immunizations, we observed an increase in autoantibodies, including RF, anti-Sm, and anti-dsDNA Abs, and an increase in serum IC and glomerular injury (Fig. 1A, 1B). Glomerular injury was assessed by proteinuria and glomerulonephritis, according to the International Society of Nephrology/Renal Pathology Society classification. Glomerular injury consisted of the following: class II mesangial proliferative glomerulonephritis in 26.29 ± 7.53% of the specimens (n = 9, mean ± SD) (Fig. 1Ca), class III focal glomerulonephritis in 13.70 ± 7.53% of the specimens (Fig. 1Cb), class IV diffuse glomerulonephritis in 38.14 ± 7.28% of the specimens (Fig. 1Cc), class V membranous glomerulonephritis in 3.70 ± 3.51% of the specimens (Fig. 1Cd), and class VI advanced sclerosing glomerulonephritis in 10.37 ± 3.09% of the glomerular specimens (n = 9, mean ± SD) (Fig. 1Ce). We also immunized mice 12 times with KLH and observed a similar induction of RF and anti-Sm Ab; however, there was no anti-dsDNA Ab induction, proteinuria, or glomerular injury (Fig. 1A, 1B). We also observed an increase in serum IC and the massive deposition of IC in the glomeruli of mice. Serum IgG1 and IgG2a, which were reported to increase concomitantly with autoimmune renal diseases (29, 30), were also increased after immunization with either OVA or KLH (Fig. 1D). Importantly, however, we noted that IFN-γ–producing activated CD8 T cells were increased in OVA-immunized, but not KLH-immunized, mice (Fig. 1E). These IFN-γ–producing CD8 T cells infiltrated into the sites of OVA deposition in the glomeruli of the mice immunized 12 times with OVA (26). Compared with KLH-immunized and control mice, there were increased numbers of CD8 T cells, but not CD4 T cells, in the kidneys of OVA-immunized mice (Fig. 1F). This indicates that mice immunized 12 times with KLH do not induce effector CD8 T cells, which suggests that, despite the massive deposition of IC in the kidneys, the lack of glomerular injury in these mice is due to the fact that KLH was not cross-presented to T cells.

Glomerular injury induced by repeated OVA immunization could be adoptively transferred into naive recipients via $CD8^+$ T cell transfer (Fig. 2A). This adoptive transfer of glomerular injury was not accompanied by the generation of autoantibodies (26) or by any significant increase in IC or anti-OVA Ab in sera of the recipient mice (Fig. 2B). IC was only minimally deposited in the glomeruli of recipients, because they were boosted only once with OVA after cell transfer. Thus, these findings suggest that CD8 T cells are required for the generation of glomerular injury, whereas massive IC deposition is not required, although low amounts of IC deposition may still be necessary.

To prove that effector CD8 T cells are required for immune glomerular injury, we immunized β_2m-deficient mice, which lack functional effector CTLs (27). Following 12 immunizations of these mice with OVA, there was a marked increase in serum autoantibodies, including anti-dsDNA Ab (26), IC, and anti-OVA Ab, and there was massive deposition of IC in the kidney (Fig. 3B, 3C). However, glomerular injury was minimal, as demonstrated by low proteinuria (Fig. 3A) and the absence of glomerulonephritis by histopathologic analysis (Fig. 3B). This finding indicates that functional effector CD8 T cells are required for the induction of immune glomerular injury.

Requirement of IC

We next tested whether the presence of Ag in the form of IC is required for immune glomerular injury. For this, we used μMT mice, which lack B cells, do not induce Ag-specific Ab responses (28), and do not generate detectable IC in sera even after 12 immunizations with OVA (data not shown). In these mice, IC deposition and renal disease were both absent (Fig. 4A, 4B). However, IFN-γ–producing CD8 T cells developed to levels comparable to those seen in WT mice (Fig. 4C). This indicates that deposition of Ag in the form of IC is required before effector CD8 T cells can cause glomerular injury. To verify this further, we performed serum-transfer experiments. When CD8 T cells from mice immunized 12 times with OVA were transferred into naive recipients, they could reproducibly induce glomerular injury, but only when a single injection of OVA was given simultaneously (Fig. 2A). We subsequently tested whether one injection of sera from mice immunized 12 times with OVA could substitute for the single booster injection of OVA. We found that IC was indeed deposited in the recipients' glomeruli and was accompanied by glomerular injury at 2 wk after the transfer of CD8 T cells (Fig. 5). This demonstrates that deposition of at least some amount of Ag

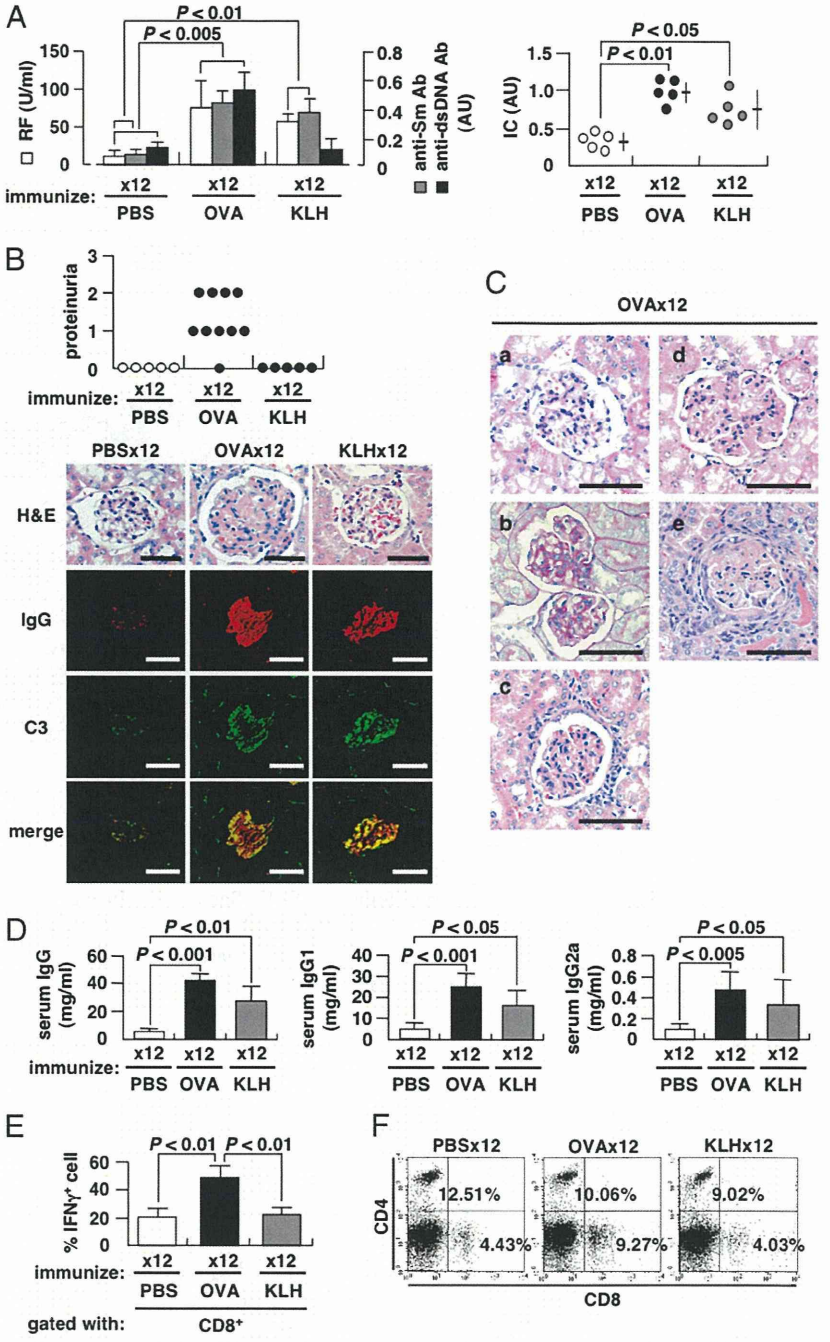

FIGURE 1. Renal disease is induced by repeated immunization with OVA but not with KLH. BALB/c mice were repeatedly injected i.p. with 500 μg of OVA, 100 μg of KLH, or PBS every 5 d. (**A**) Serum RF, anti-Sm, and anti-dsDNA Abs (*left panel*) and IC (*right panel*) were quantified by ELISA 2 d after final immunization (mean ± SD; 5 mice/group). AU refers to the value obtained with the sera of MRL/*lpr* mice. (**B**) Proteinuria was assessed 9 d after the final immunization and graded with a score of 0 (<30 mg/dl); 1 (30–99 mg/dl); 2 (100–299 mg/dl); or 3 (300–999 mg/dl). Kidney histopathology (H&E; scale bar, 50 μm; original magnification ×400; *top row*) and deposition of IC, IgG, and C3 in the glomeruli of the mice immunized 12 times with PBS, OVA, or KLH (scale bar, 50 μm; original magnification ×300; *second, third,* and *fourth rows*). (**C**) Representative kidney histopathology stained with H&E or periodic acid-Schiff and classified according to the International Society of Nephrology/Renal Pathology Society (scale bar, 50 μm; original magnification ×400). (**a**) Class II mesangial proliferative glomerulonephritis. (**b**) Class III focal glomerulonephritis. (**c**) Class IV diffuse glomerulonephritis. (**d**) Class V membranous glomerulonephritis. (**e**) Class VI advanced sclerosing glomerulonephritis. (**D**) Serum IgG, IgG1, and IgG2a assayed by ELISA 2 d after final immunization (mean ± SD, 5 mice/group). (**E**) Spleen cells stimulated with 50 ng/ml of PMA and 500 ng/ml of ionomycin for 4 h in the presence of brefeldin A (10 μg/ml) and stained for intracellular IFN-γ (mean ± SD, 5 mice/group). (**F**) Infiltrating CD4 and CD8 T cells from the kidneys of mice immunized 12 times with OVA or KLH were extracted and detected by flow cytometry. Each experiment was performed three times independently.

in the form of IC is required for effector CD8 T cells to exert their cytotoxicity and induce immune glomerular injury.

Discussion

The results in this study show that IFN-γ–producing effector CD8 T cells, which can recognize Ag presented as IC on target tissue, are required for the induction of glomerular injury in this mouse model of SLE. This finding is consistent with results showing that CD8 T cells are the dominant T cell population in renal biopsy specimens from lupus patients who presented with class III and IV glomerulonephritis (21). Heymann et al. (11) showed that glomerular Ag-specific CTLs induce renal immunopathology with the help of CD4 T cells. Such cooperation between CD8 and CD4 cells is indeed likely and may also explain several somewhat contradictory reports describing the contributions of CD8 and/or CD4 T cells to the pathogenesis of immune glomerular disease (12–21). In a previous study of experimentally induced SLE, we found that both Ag cross-presentation and CD4 T cell help were essential for generating effector CD8 CTLs, leading to glomerular injury (26). We proposed the "self-organized criticality theory," which explains that systemic autoimmunity, or SLE, necessarily takes place when a host's immune system is overstimulated by repeated exposure to Ag, achieving levels that surpass the immune system's stability limit (i.e., self-organized criticality). This theory proposes that autoreactive lymphocyte clones are newly generated via de novo TCR revision from nonautoreactive clones at the periphery (26, 31). We named this novel T cell type an autoantibody-inducing CD4 T cell and postulated that these cells stimulate B cells to generate various autoantibodies, as well as to promote the final differentiation of CD8 T cells into CTLs via Ag cross-

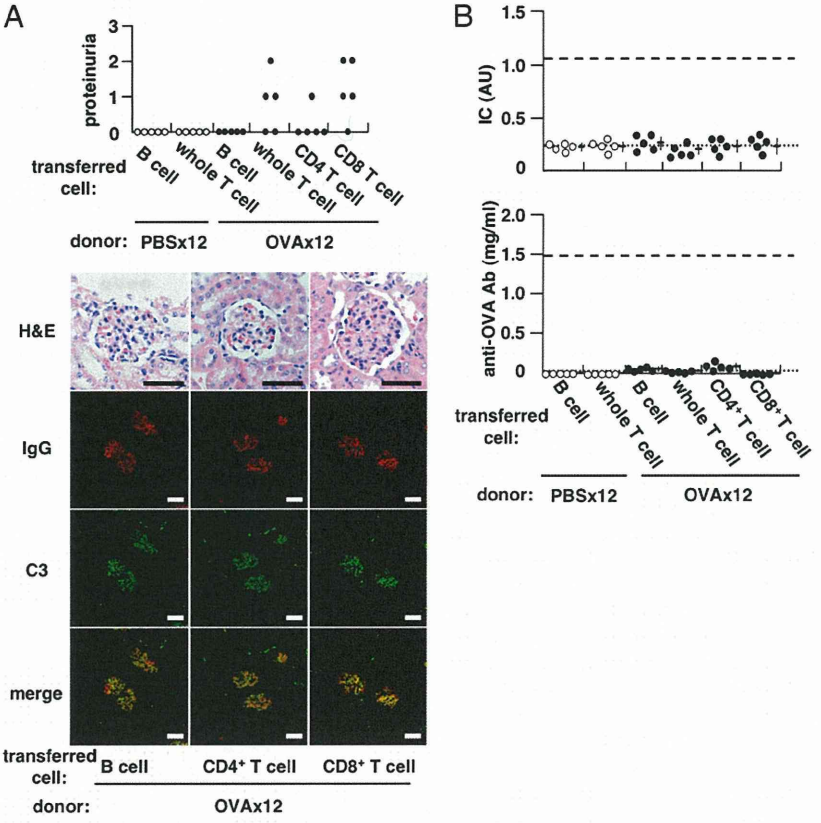

FIGURE 2. Glomerular injury is transferrable via fully matured effector CD8 T cells. Splenocytes of OVA-immunized BALB/c mice were adoptively transferred to naive recipients, and the recipients were injected with 500 μg of OVA 24 h after transfer. (**A**) Proteinuria, histopathology (H&E; scale bar, 50 μm; original magnification ×400) and the deposition of IC, IgG, and C3 in the glomeruli of recipient mice (scale bar, 50 μm; original magnification ×200) 2 wk after cell transfer. (**B**) Serum IC and anti-OVA Ab in recipients as measured by ELISA 2 wk after cell transfer (mean ± SD). Thin or bold dotted lines represent the averaged value in the donor mice immunized 12 times with PBS or OVA, respectively. AU refers to the value obtained with sera of MRL/*lpr* mice. Each experiment was performed twice independently.

presentation, leading to the tissue injuries found in SLE. Such a scenario is consistent with the previously demonstrated roles of CD8 and/or CD4 T cells in the pathogenesis of kidney disease (12–21) (i.e., CD8 T cells must mature into effector CD8 T cells with the help of CD4 T cells, primarily in the induction phase of glomerulonephritis).

In the current study, with regard to the role of effector CD8 T cells in the effector phase of glomerulonephritis, we focused on whether effector CD8 cells were directly responsible for the induction of glomerular injury. First, we found that effector CD8 T cells recognized Ag presented as IC on target renal tissue and consequently exerted immune glomerular injury. Glomerulone-

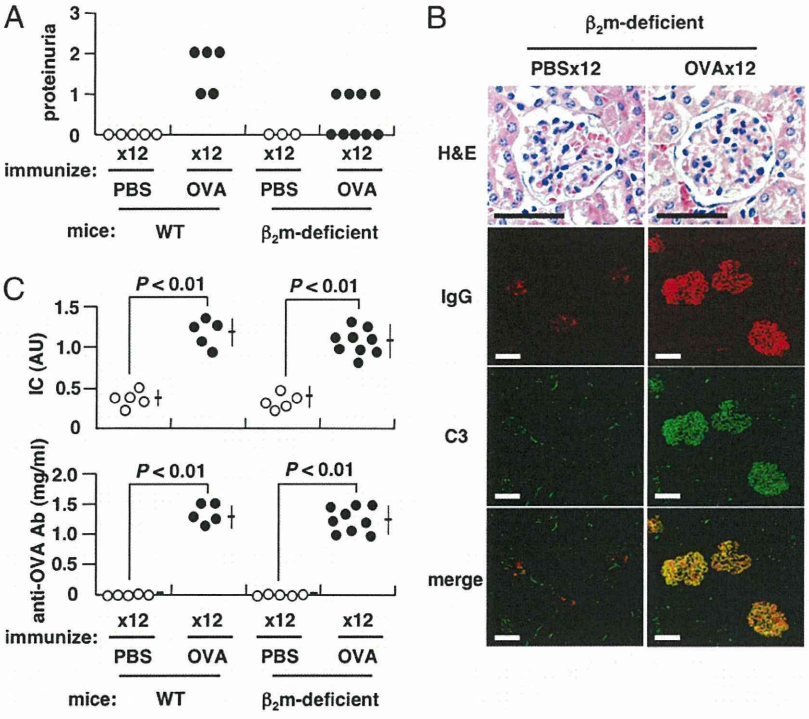

FIGURE 3. Glomerular injury is minimal in β_2m-deficient mice. β_2m-deficient mice were repeatedly injected i.p. with 500 μg OVA or PBS every 5 d. (**A**) Proteinuria in WT or β_2m-deficient mice assayed 9 d after 12 immunizations with OVA. (**B**) Histopathology (H&E; scale bar, 50 μm; original magnification ×400) and the deposition of IC, IgG, and C3 in the glomeruli of β_2m-deficient mice immunized 12 times with PBS or OVA (scale bar, 50 μm; original magnification ×200). (**C**) Serum IC and anti-OVA Ab measured by ELISA 2 d after 12 immunizations with OVA (mean ± SD). AU refers to value obtained with sera of MRL/*lpr* mice. Each experiment was performed three times independently.

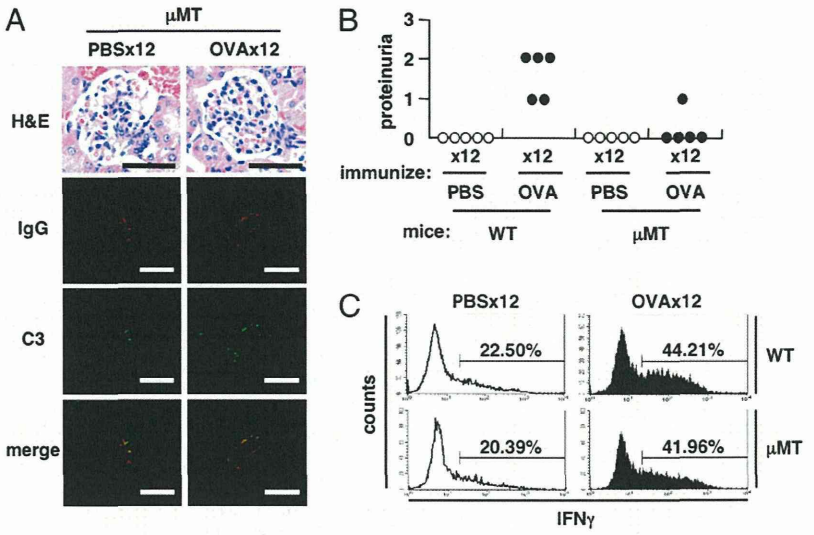

FIGURE 4. IC deposition and effector CD8 T cell–induced glomerular injury. μMT mice were repeatedly injected i.p. with 500 μg OVA or PBS every 5 d. (**A**) Histopathology (scale bar, 50 μm; original magnification ×400) and the deposition of IC, IgG, and C3 in the glomeruli of μMT mice immunized 12 times with PBS or OVA (scale bar, 50 μm; original magnification ×300). (**B**) Proteinuria in WT or μMT mice assayed 9 d after 12 immunizations with OVA. (**C**) IFN-γ–producing CD8 T cells in spleen of μMT mice immunized 12 times with OVA. Each experiment was performed three times independently.

phritis was not observed in the absence of effector CD8 T cells. Second, there must be at least some minimal amount of IC deposited on the target renal tissues for effector CD8 T cells to cause immune injury.

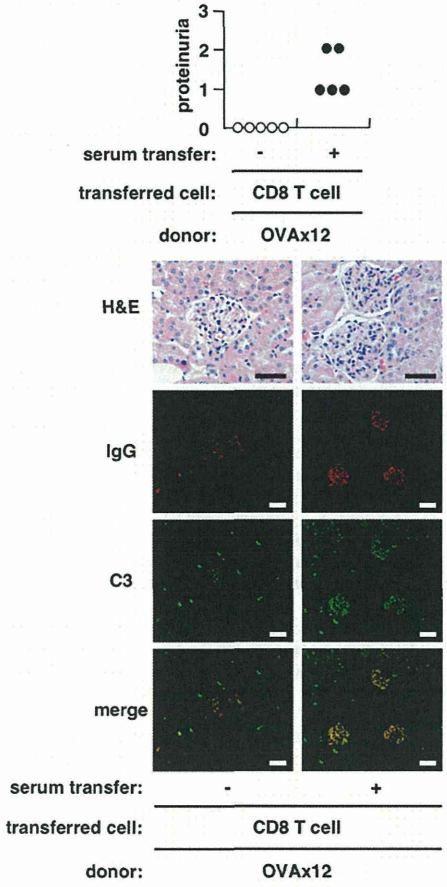

FIGURE 5. Requirement of IC deposition for effector CD8 T cell and subsequent glomerular injury. CD8 T cells from OVA-immunized BALB/c mice were adoptively transferred to naive recipients. The recipients were also injected once with 500 μl of sera from mice immunized 12 times with OVA 24 h after cell transfer. Shown are proteinuria, histopathology (H&E, scale bar, 50 μm; original magnification ×400), and the deposition of IC, IgG, and C3 in the glomeruli of recipient mice (scale bar, 50 μm; original magnification ×200) 2 wk after cell transfer.

Previous studies showed that β_2m is required for the surface expression of MHC class I, as well as CD1d and Qa-1. Although lack of CD1d expression can lead to NKT cell deficiency (32), we found that repeated OVA immunization of CD1d knockout mice led to both the production of various autoantibodies and the development of proteinuria to the same degree observed in WT mice (K. Tsumiyama and S. Shiozawa, manuscript in preparation). Qa-1 plays important roles in the suppression of CD4 T cells and CD8 regulatory T cell (Treg) functions (33, 34). Deficiency of Qa-1 causes exaggerated secondary CD4 responses against virus or self-peptide and impairs CD8 Treg function, ultimately leading to autoimmunity (35, 36). However, we found that the levels of autoantibodies generated after 12 repeated immunizations with OVA were similar between β_2m-deficient mice and WT mice (Supplemental Fig. 3 in Ref. 26). In addition, tissue injuries, including glomerulonephritis, were clearly not induced in the β_2m-deficient mice (Fig. 3), even in mice that had received CD8 T cells transferred from OVA-stimulated WT mice (figure 1C of in Ref. 26). In previous studies, the phenotype of inhibitory CD8 Tregs was shown to fluctuate (37–40). Inhibitory CD8 Treg function was reported to be defective in human SLE patients and in animal models of SLE (41–43). However, it was also observed that lupus nephritis can be suppressed by anti-CD8 Ab treatment or by MHC class I deficiency (18, 19, 44). Further, recent studies show that Tregs are actually required for the final differentiation of CD8 T cells (45). Thus, CD1d, Qa-1, NKT cells, or CD8 Tregs do not appear to play a causative role in immune-mediated glomerular disease.

In summary, immune tissue injury requires, first, that CD8 T cells mature into effector cells with the help of CD4 T cells, primarily in the induction phase. Second, in the effector phase, effector CD8 T cells recognize Ag presented as IC on target tissue, and this recognition is required for their cytotoxic actions.

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Disclosures

The authors have no financial conflicts of interest.

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Defining HIV-1 Vif residues that interact with CBFβ by site-directed mutagenesis

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ABSTRACT

Vif is essential for HIV-1 replication in T cells and macrophages. Vif recruits a host ubiquitin ligase complex to promote proteasomal degradation of the APOBEC3 restriction factors by poly-ubiquitination. The cellular transcription cofactor CBFβ is required for Vif function by stabilizing the Vif protein and promoting recruitment of a cellular Cullin5-RING ubiquitin ligase complex. Interaction between Vif and CBFβ is a promising therapeutic target, but little is known about the interfacial residues. We now demonstrate that Vif conserved residues E88/W89 are crucial for CBFβ binding. Substitution of E88/W89 to alanines impaired binding to CBFβ, degradation of APOBEC3, and virus infectivity in the presence of APOBEC3 in single-cycle infection. In spreading infection, NL4-3 with Vif E88A/W89A mutation replicated comparably to wild-type virus in permissive CEM-SS cells, but not in multiple APOBEC3 expressing non-permissive CEM cells. These results support a model in which HIV-1 Vif residues E88/W89 may participate in binding CBFβ.

Introduction

HIV-1 Vif is one of six viral accessory proteins and it is essential for the viral replication in T cells and macrophages (Gabuzda et al., 1992, 1994). Vif recruits host proteins, cullin 5 (CUL5), RING-box protein 2 (RBX2), elongin C (ELOC) and elongin B (ELOB), and forms a ubiquitin ligase complex that promotes poly-ubiquitination and proteasomal degradation of the APOBEC3 (A3) retrovirus restriction factors (Jäger et al., 2011; Marin et al., 2003; Sheehy et al., 2003; Shirakawa et al., 2006; Stopak et al., 2003; Yu et al., 2003). A3s are DNA cytosine deaminases that convert cytosines to uracils in single-stranded DNA (Chelico et al., 2006; Harris et al., 2003). In the absence of the Vif protein, at least two A3 family members, A3F and A3G, are efficiently incorporated into budding virions, where they deaminate cytosines in newly reverse-transcribed minus-strand virus DNA in target cells, leading to guanine to adenine hypermutation of the virus genome (Harris et al., 2003; Hultquist et al., 2011b; Sheehy et al., 2002; Zhang et al., 2003).

Amino acid sequences of HIV-1 Vif vary among viral strains, but more than ten regions of residues are conserved (Dang et al., 2010), and several conserved motifs have been shown to interact with host proteins. The BC-box motif ^{144}SLQYLA^{149} binds to ELOC (Mehle et al., 2004; Yu et al., 2004), and the HCCH motif ^{108}Hx$_5$Cx$_{17-18}$Cx$_{3-5}$H^{139} binds to CUL5 (Luo et al., 2005; Mehle et al., 2006). Furthermore, the N-terminal half of Vif contains distinct regions involved in the Vif-A3 protein-protein interactions; ^{11}WQxDRMR^{17} and ^{76}ExxW^{79} motifs are involved in neutralization of A3F (He et al., 2008; Russell and Pathak, 2007), whereas ^{40}YRHHY^{44} motif is involved in neutralization of A3G (Russell and Pathak, 2007); ^{22}KSLVK^{26} and ^{55}VxIPLx4-5Lxφx2YWxL^{72} motifs were reported to be involved in neutralization of both A3F and A3G (Chen et al., 2009; Dang et al., 2009; He et al., 2008; Pery et al., 2009), although there exists a report that K26 is required for neutralization of A3G, but not of A3F (Albin et al., 2010).

Recently, the transcription factor core binding factor-β (CBFβ) has been shown to be involved in the Vif ubiquitin ligase complex (Jäger et al., 2011; Zhang et al., 2011), and critical for its function by stabilizing the Vif protein in cells (Jäger et al., 2011), and enabling the recruitment of CUL5 (Zhang et al., 2011). There are two isoforms of CBFβ, and both isoforms stabilize Vif protein, enhance A3 degradation, and increase virion infectivity (Hultquist et al., 2011a). Thus, the interaction between Vif and CBFβ is a promising

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therapeutic target, but little is known about the interfacial amino acids. Hultquist et al. reported that surface F68 residue of CBFβ is involved in binding and stabilizing Vif (Hultquist et al., 2012). Zhang et al. reported that W21 and W38 residues of Vif are required for binding to CBFβ by co-immunoprecipitation experiments (Zhang et al., 2011; Kim et al., 2013). Furthermore, Kim et al. recently suggested that L64 and I66 residues are involved in binding to CBFβ by co-purification in *Escherichia coli* (Kim et al., 2013).

Because a previous study indicated that a hydrophilic region ^{88}EWRKKR^{93} is essential for Vif expression and HIV-1 replication (Fujita et al., 2003), we hypothesized that conserved residues E88 and W89 in this region may be required for CBFβ binding. In this study, we generated amino acid substitution mutants of these residues as well as W21 and W38, and simultaneously analyzed both binding to CBFβ and Vif-mediated degradation of A3F and A3G. We show that the conserved residues E88 and W89 of HIV-1 Vif are directly involved in CBFβ binding and Vif-mediated degradation of A3F and A3G.

Results

The conserved residue W89 of HIV-1 Vif is required for the interaction with CBFβ

To test our hypothesis that conserved residues E88 and W89 may be required for CBFβ binding, we generated nine Vif amino acid substitution mutants, D14A/R15A, W21A, W38A, Y40A, Y69A, G84D, E88A, W89A, and E88A/W89A (Fig. 1A). W21 and W38 were reported to be involved in CBFβ binding (Zhang et al., 2011), D14 and R15 for APOBEC3F binding (Russell and Pathak, 2007), Y40 for A3G binding (Russell and Pathak, 2007), Y69 and G84 for both A3F and A3G binding (Dang et al., 2010; Pery et al., 2009). All of these residues are highly conserved, suggesting that they could be involved in CBFβ binding region of Vif. We first performed co-immunoprecipitation experiments in 293T cells by over-expression of Vif with C-terminal myc tag. Vif is a relatively unstable protein with a short half-life and it is degraded by the cellular proteasome (Dussart et al., 2004; Fujita et al., 2004; Mehle et al., 2004). Mouse double minute 2 homolog (MDM2) is the E3 ubiquitin ligase which targets Vif for degradation (Izumi et al., 2009). Because it has been reported that Vif degradation is accelerated in the absence of CBFβ, and that treatment with the proteasome inhibitor MG132 reverses this effect (Jäger et al., 2011), we used MG132 to minimize proteasomal proteolysis of Vif in cell culture and immunoprecipitation experiments. Although we transfected with the same amount of plasmid DNA, expression levels of E88A, W38A, D14/R15AA, Y40A, Y69A and G84D were comparable to wild-type Vif, but those of W89A, E88/W89A and W21A mutants were obviously impaired (Fig. S1, 2nd top panel). These modest expression levels of these mutants can be simply explained by misfolding, but an alternative explanation is due to loss of CBFβ binding. The amounts of immunoprecipitated Vif protein showed a smaller variation, compared to expression levels (Fig. S1, bottom panel). More importantly, endogenous CBFβ co-precipitated with wild-type Vif, D14A/R15A, Y40A, G84D, and E88A, but not with W21A, W89A, or E88A/W89A (Fig. S1, 3rd top panel). W38A and Y69A showed intermediate results (Fig. S1, lanes 7 and 10, 3rd top panel). To exclude the possibility that low expression of W21A, W89A and E88A/W89A mutants caused the low amount of co-immunoprecipitated CBFβ, we compensated by increasing the amount of transfected plasmid DNA, and performed an additional round of co-immunoprecipitation experiments. Although expression levels of W21A, W89A and E88A/W89A mutants were now higher than wild-type Vif, endogenous CBFβ did not co-precipitate with these mutants (Fig. 1B). We next examined whether CUL5 co-precipitated with Vif mutants by immunoblotting with some of the samples of Fig. 1B, because CBFβ binding has been reported to be required for Vif to interact with CUL5 (Zhang et al., 2011). Endogenous CUL5 appeared to co-precipitate with wild-type Vif, but not with W21A, W38A, W89A, or E88A/W89A (Fig. 1C, 2nd bottom panel). All

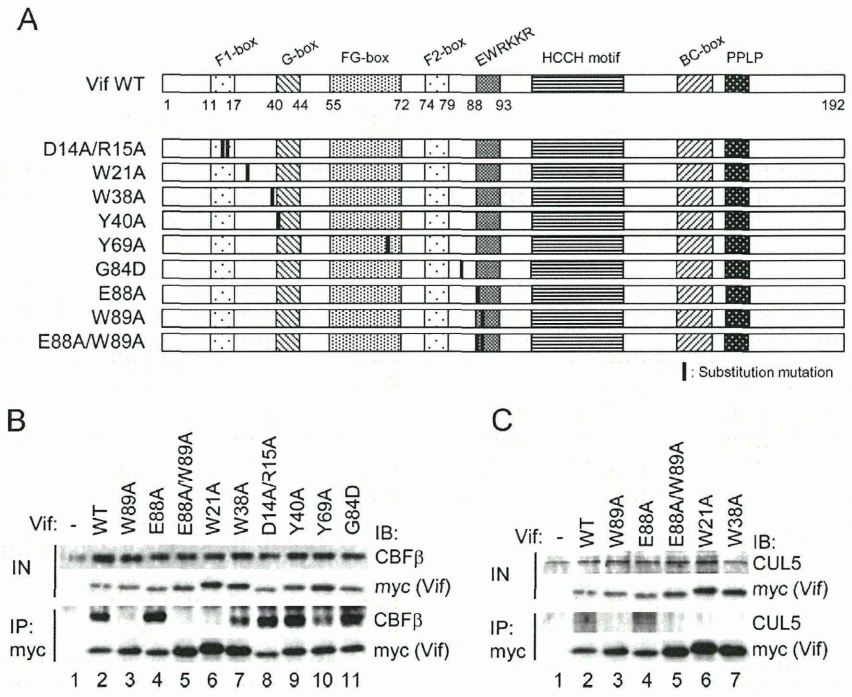

Fig. 1. Vif substitution mutants and binding capacity to CBFβ. (A) Schematic of Vif conserved motifs and nine substitution mutants generated. The residues substituted are indicated by bold lines. (B) Co-immunoprecipitation of endogenous CBFβ with Vif. Lysates of 293T cells transiently expressing myc-tagged Vif wild-type or mutant were immunoprecipitated by anti-myc serum. Samples were analyzed by immunoblotting with anti-CBFβ and anti-myc sera. (C) Co-immunoprecipitation of endogenous CUL5 with Vif. Samples from (B) were also analyzed with anti-CUL5 serum. Panels for Vif were re-produced from (B).

of these mutants of Vif bound to ELOB (Fig. S2), suggesting that they are not entirely misfolded proteins, although previous reports indicated that fragments of the Vif BC box is sufficient for binding to ELOB and ELOC (Bergeron et al., 2010; Wolfe et al., 2010). Altogether, these results suggest that W89 is involved in CBFβ binding, as well as W21 and W38.

The substitution of E88/W89 to alanines impairs Vif-mediated degradation of both A3F and A3G proteins

To examine whether the loss of binding to CBFβ causes impaired Vif-mediated degradation of A3 proteins, we next performed a series of co-transfection and immunoblot experiments. 293T cells were co-transfected with expression vectors for A3G with myc tag and Vif wild-type or mutant, and protein levels of A3G were analyzed by immunoblotting. To obtain comparable expression levels of each of the Vif mutants, we again adjusted the amount of plasmid DNA transfected. Co-transfection of wild-type Vif reduced APOBEC3G levels, but E88A/W89A, W21A or W38A did not (Fig. 2A, lanes 1, 2 and 7–12). E88A or W89A alone reduced A3G levels close to wild-type Vif (Fig. 2A, lanes 3–6). Because W21 and W38 are close to A3G binding residues of Vif, ^{40}YRHHY^{44}, the loss of A3G degradation by W21A or W38A might be caused by loss of A3G binding. To exclude this possibility, we next performed co-transfection experiments with Vif and A3F expression vectors and similar results were obtained (Fig. 2B). These results suggest that the substitution of E88/W89 as well as W21 and W38 to alanines leads to an impairment of Vif-mediated degradation of both A3F and A3G due to the loss of binding to CBFβ, not to A3F or A3G.

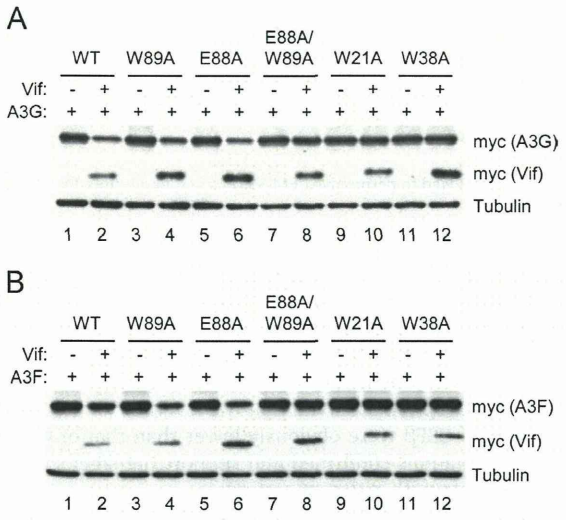

Fig. 2. Degradation of A3F and A3G by Vif. (A) Degradation of A3G. HEK293T cells were co-transfected with expression vectors for myc-tagged A3G and Vif wild-type or mutant, and protein levels of A3G and Vif were analyzed by immunoblotting with anti-myc serum and anti-tubulin antibody for loading control. The same amount of the A3G expression plasmid was transfected in each sample. (B) Degradation of A3F. Similar experiments to (A), but an A3F expression vector was used instead of that of A3G.

The substitution of E88/W89 to alanines impairs the ability of Vif to counteract the restriction by both A3F and A3G

To examine the ability of Vif mutants to counteract the restriction of HIV-1 by A3 proteins, we next performed single cycle infection experiments using luciferase-reporter viruses. The Vif mutations were introduced into pNL4-3 ΔEnv-Luc, and transfected into 293T cells with co-transfection of VSV-G expression plasmid in the presence or absence of co-transfection of A3G expression plasmid. Virus-containing supernatant was harvested and infectivity was measured by challenging to fresh 293T cells and assaying luciferase activity. Viruses with Vif mutations showed comparable infectivity in the absence of A3G, as expected (Fig. 3A). In the presence of A3G, virus with E88A mutation showed comparable infectivity to wild-type, but virus with W21A, W38A or E88A/W89A showed deeply impaired infectivity close to that of ΔVif (Fig. 3A). Virus with W89A showed intermediate and obviously impaired infectivity in the presence of A3G (Fig. 3A). We also performed these assays with A3F instead of A3G, and obtained similar results (Fig. 3B). These data indicate that E88/W89 residues as well as W21 and W38 are involved in Vif activity to counteract both A3F and A3G, suggesting these residues are involved in CBFβ binding, not A3F or A3G binding.

The substitution of E88/W89 to alanines impairs HIV-1 replication in non-permissive CEM cells

Finally, we performed spreading infection experiments in both permissive CEM-SS and multiple A3-expressing CEM cells. We introduced Vif mutations into NL4-3, a replication-competent

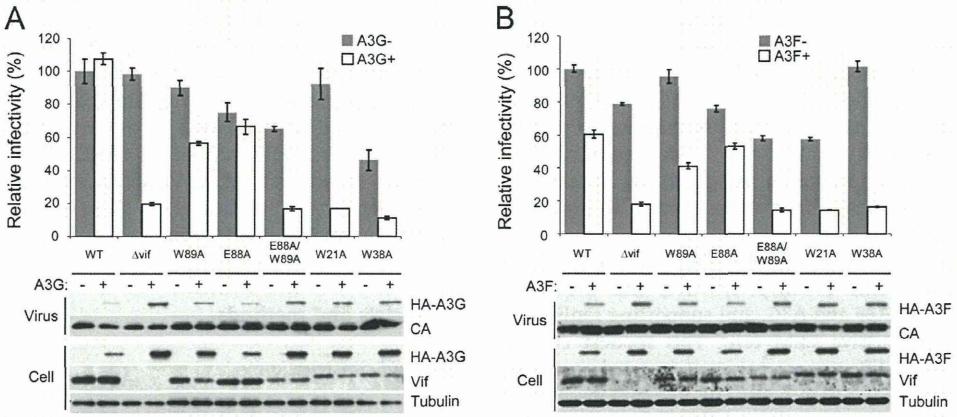

Fig. 3. Single-cycle infection experiments with VSV-G pseudo-typed viruses with vif mutations. (A) Counteracting abilities of Vif mutants against the restriction by A3G. 293T cells were transfected with pNL43/ΔEnv-Luc with vif mutation, together with pVSV-G, in the presence or absence of pcDNA3/HA-A3G. pNL43/ΔEnv-Luc without vif mutation and pNL43/ΔEnvΔvif-Luc were also used for control. Virus-containing supernatant was challenged to fresh 293T cells and infectivity was determined by luminometer. Values were normalized to that of the virus without vif mutation in the absence of A3G. Average and standard errors of 3 independent experiments are shown. Levels of A3G in cells and virions and Vif in cells were also analyzed by immunoblotting. (B) Counteracting abilities of Vif mutants against the restriction by A3F. Similar experiments to (A), but pcDNA3/HA-A3F was used instead of the A3G plasmid.

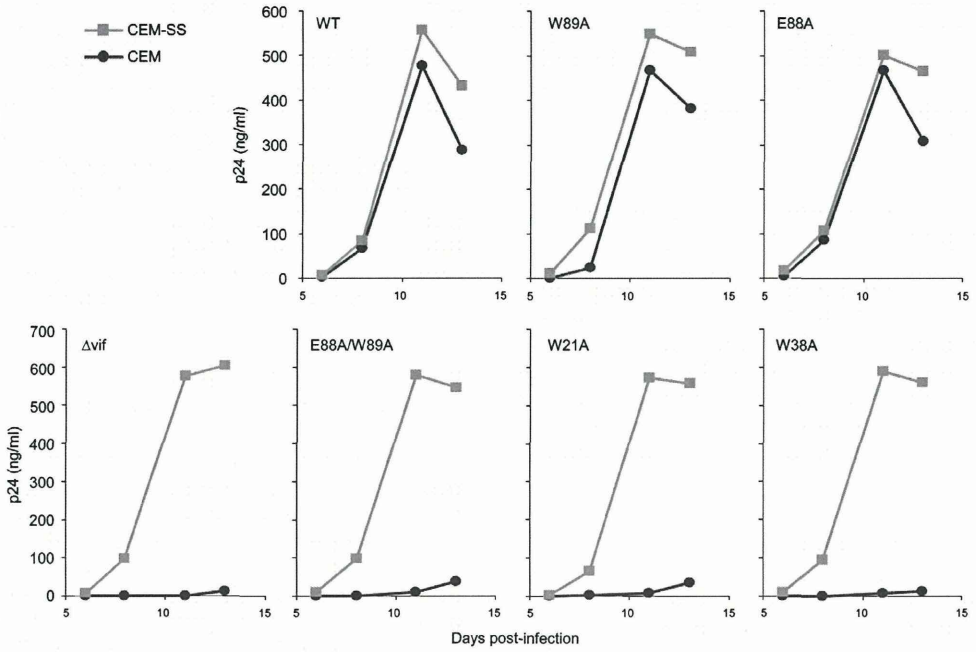

Fig. 4. Spreading infection of NL4-3 with vif mutations. The viruses were produced in 293T cells and challenged to permissive CEM-SS cells and nonpermissive CEM cells at MOI of 0.005. Culture supernatant was collected periodically and p24 levels were determined by ELISA.

molecular clone, and analyzed whether the substitution mutation impairs virus replication with spreading infection assays. Virus with E88A or W89A showed comparable replication profiles to wild-type virus in both CEM-SS and CEM cells. Virus with ΔVif, E88A/W89A, W21A, or W38A showed indistinguishable replication profiles to wild type in CEM-SS cells, as expected, whereas in CEM cells, showed deeply impaired replication profiles (Fig. 4). These results indicate that the residues E88/W89 as well as W21 and W38 are critical for Vif to counteract multiple A3s, suggesting that these residues are directly involved in CBFβ binding.

Discussion

In this study, we report that HIV-1 Vif residues E88 and W89 are involved in CBFβ binding, therefore in rendering Vif capable of stable expression and inducing ubiquitination of both A3F and A3G proteins. Fujita et al. reported that deletion or substitution of these residues impairs steady-state levels of Vif protein and virus replication in nonpermissive H9 cells and monocyte-derived macrophages (Fujita et al., 2003). We confirmed lower expression levels of substitution mutants using both Vif expression vectors and molecular clones of HIV-1. We also confirmed inefficient replication of virus with the mutation using another nonpermissive T cell line, CEM cells. These observations may be all explained by the loss of CBFβ binding.

Our results suggest that the single amino acid substitution mutant W89A does not bind to CBFβ, however this mutant is capable of degradation of A3F and A3G, and supporting replication of the virus in non-permissive CEM cells. This modest conflict may be due to the differences in experimental settings; co-immunoprecipitation experiments test the interactions *in vitro* in complex total cell lysates, and degradation experiments and infectivity experiments test Vif functionality in living cells.

Zhang et al. reported that Vif residues W21 and W38 are involved in binding to CBFβ, but functional correlation of these residues was not described (Zhang et al., 2011). We confirmed that these residues are critical for CBFβ binding, and further demonstrated that these residues are critical for degradation of both A3F and A3G, counteracting restriction by both A3F and A3G, and replication in nonpermissive, multiple A3-expressing cells.

Jäger et al. reported that CBFβ functions to up-regulate steady-state level of Vif protein by using cells with CBFβ knock-down (Jäger et al., 2011). We observed that the levels of Vif mutants that do not bind to CBFβ were obviously lower than that of wild-type Vif. Our observations confirmed and support the Jäger's report by the experiments with different settings.

Several E3 ligases including CUL5, NEDD4, and AIP4, have been reported to induce Vif ubiquitination, although biological implications have not been well defined (Dussart et al., 2004; Mehle et al., 2004). We previously reported that MDM2 targets for Vif as an E3 ligase to induce its ubiquitination and proteasomal degradation, and that the N-terminal region of Vif (residues 4–22) is required for MDM2 binding (Izumi et al., 2009). Because one of the CBFβ binding residues of Vif, W21, is located close to MDM2 binding region, the loss of CBFβ binding might facilitate ubiquitination and degradation of Vif by MDM2. Further investigations will be required to test this possibility.

We provide here the evidence indicating that residues W21, W38, E88 and W89 of HIV-1 Vif are involved in binding surface to CBFβ. Further studies on the Vif-CBFβ co-crystal structure will be the key to understanding Vif-CBFβ interaction surfaces, and to pharmaceutical applications of these pieces of information for patients with HIV-1 infection.

Materials and methods

Plasmid construction

C-terminally myc-tagged Vif expression plasmid, pDON-Vif-myc, was generated by amplifying NL4-3 vif coding sequence with primers ATA GGA TCC ATG GAA AAC AGA TG G CAG GTG GCA GGT GAT G and CGC GTC GAC CTA CAG ATC CTC TTC AGA GAT GAG TTT CTG CTC GTA GTG TCC ATT CAT TGT ATG GCT CCC, and inserting it into pDON-AI (Takara) at BamH I/Sal I sites. Expression plasmids for