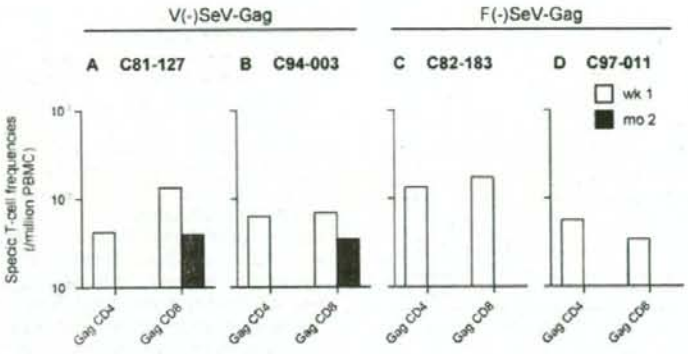

Fig. 2. Gag-specific T-cell frequencies in PBMCs two months after SeV boost. Gag-specific CD4⁺ T-cell and CD8⁺ T-cell frequencies one week (open boxes) or two months (closed boxes) after an intranasal boost with V(-)SeV-Gag (macaques C81-127 (A) and C94-003 (B)) or F(-)SeV-Gag (macaques C82-183 (C) and C97-011 (D)) are shown.

all (Fig. 1B). Thus, replication-competent V(-)SeV-Gag boost and replication-defective F(-)SeV-Gag boost both elicited robust Gag-specific and SeV-specific T-cell responses.

3.2. Replication-competent V(-)SeV-Gag elicited more durable Gag-specific T-cell responses than replication-defective F(-)SeV-Gag

We followed up two of the V(-)SeV-Gag-boosted macaques and two of the F(-)SeV-Gag-boosted macaques, and examined Gag-specific T-cell responses in these macaques two months after the boost. Gag-specific CD4⁺ T-cell responses became undetectable in both groups. However, Gag-specific CD8⁺ T-cell responses were still detectable in both of the V(-)SeV-Gag-boosted macaques but became undetectable in both of the F(-)SeV-Gag-boosted macaques (Fig. 2).

We then combined this result with our previous data obtained from four V(-)SeV-Gag-boosted macaques and four F(-)SeV-Gag-boosted macaques [11], and compared Gag-specific CD8⁺ T-cell levels between the V(-)SeV-Gag-boosted and the F(-)SeV-Gag-boosted groups by statistical analysis (Fig. 3). No significant difference in Gag-specific CD8⁺ T-cell levels one week after the boost was observed between the two groups ($p = 0.5112$ by unpaired two-tailed t test). However, a few months after the boost, the V(-)SeV-Gag-boosted macaques showed significantly higher levels of Gag-specific CD8⁺ T-cell responses than the F(-)SeV-Gag-boosted ($p = 0.0169$ by unpaired two-tailed t test). Indeed, Gag-specific CD8⁺ T-cell responses were still detectable in all six V(-)SeV-Gag-boosted macaques but became undetectable in five of six F(-)SeV-Gag-boosted macaques. These results indicate that the V(-)SeV-Gag boost elicited more durable Gag-specific CD8⁺ T-cell responses compared to the F(-)SeV-Gag boost.

3.3. Gag-specific T-cell responses were maintained in the lymph nodes around the nasal mucosa

The V(-)SeV-Gag-boosted and F(-)SeV-Gag-boosted macaques in the present study were euthanized one week, two weeks, or two months after the boost. We then obtained the axillary LN and the retropharyngeal LN by autopsy from two of four V(-)SeV-Gag-boosted and all seven F(-)SeV-Gag-boosted macaques. We examined Gag-specific CD8⁺ T-cell frequencies in these LNs (Fig. 4), although we failed to obtain autopsy samples from V(-)SeV-Gag-boosted C86-095 and C94-003. The retropharyngeal LN is known to receive the primary lymphocyte drainage from the nasal cavity [22]. In all animals, Gag-specific CD8⁺ T-cell frequencies in the retropharyngeal LNs were no less than those in the axillary LNs. Both V(-)SeV-Gag-boosted animals showed high levels of Gag-specific CD8⁺ T-cell responses. Remarkably, even in F(-)SeV-Gag-boosted macaques C82-183 and C97-011 that were euthanized two months after the boost, Gag-specific CD8⁺ T-cell responses were clearly

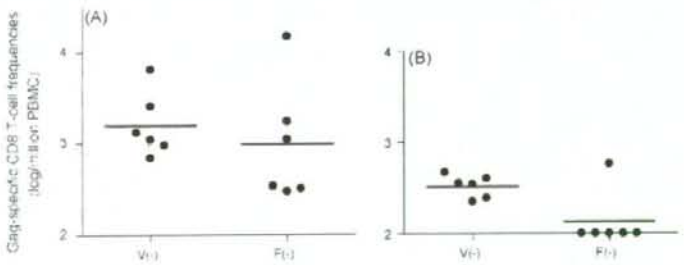

Fig. 3. Comparison of Gag-specific CD8⁺ T-cell frequencies between the V(-)SeV-Gag-boosted and the F(-)SeV-Gag-boosted macaques. The replication-competent V(-)SeV-Gag-boosted group (V[-], $n = 6$) is consisting of macaques C81-127 and C94-003 and previously-reported macaques V1, V2, V3, and V4 [11]. The replication-defective F(-)SeV-Gag-boosted group (F[-], $n = 6$) is consisting of macaques C82-183 and C97-011 and previously-reported macaques V5, V6, V7, and V8 [11]. Gag-specific CD8⁺ T-cell frequencies one week (A) or two or three months (B) after the boost were log-transformed and compared between the two groups by unpaired two-tailed t test. Geometric means of Gag-specific CD8⁺ T-cell frequencies one week after the boost were 1.6×10^3 cells/million PBMCs in the V(-)SeV-Gag-boosted group and 1.0×10^3 cells/million PBMCs in the F(-)SeV-Gag-boosted group. Geometric means of Gag-specific CD8⁺ T-cell frequencies two or three months after the boost were 3.3×10^2 cells/million PBMCs in the V(-)SeV-Gag-boosted group and 1.3×10^2 cells/million PBMCs in the F(-)SeV-Gag-boosted group.

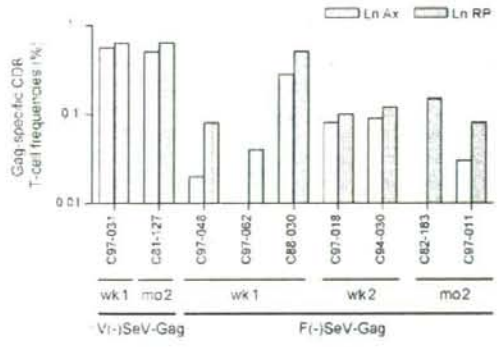

Fig. 4. Gag-specific CD8⁺ T-cell frequencies in LNs after SeV boost. Macaques were euthanized one week (macaques C97-031, C97-048, C97-062, and C88-030), two weeks (macaques C97-018 and C94-030), or two months (macaques C81-127, C82-183 and C97-011) after boost. Gag-specific CD8⁺ T-cell frequencies (%) in CD8⁺ T lymphocytes in axillary LNs (Ln Ax, open boxes) and retropharyngeal LNs (Ln RP, closed boxes) obtained at autopsy are shown.

detected in the retropharyngeal LNs although not in the axillary LNs.

4. Discussion

Recombinant viral vectors are promising vaccine tools for eliciting antigen-specific T-cell responses. Some of them are derived from nonpathogenic parental viruses but others are from pathogenic ones. For their clinical uses in safe, especially in the latter cases, viral vectors are prepared from attenuated viruses but not from pathogenic ones themselves. Alternatively, viral vectors are constructed by deleting or revising viral genomes for attenuation; some are constructed to lose their replication competency without losing their ability to express antigens.

SeV is pathogenic for mice but is considered nonpathogenic for primates including humans [10,12,13]. Thus, replication-competent SeV vectors including V(-)SeV may be used for humans, but we also have a replication-defective F(-)SeV vector that has the ability to efficiently express antigens. We previously showed that both V(-)SeV-Gag and F(-)SeV-Gag vector vaccines can elicit Gag-specific T-cell responses in macaques [9,11,18], but their immunogenicity has not been evaluated precisely. Indeed, our previous analyses showed that infectious V(-)SeV in the nasal swab became undetectable in a week after its intranasal inoculation, indicating that V(-)SeV replication is not so efficient in macaques [10]. Thus, it has remained unclear whether this short period of SeV replication can significantly affect T-cell responses.

The present study showed that both V(-)SeV-Gag boost and F(-)SeV-Gag boost elicited robust systemic Gag-specific T-cell responses, whereas the responses induced by the former were more durable than those by the latter. Indeed, systemic Gag-specific CD8⁺ T-cell responses were maintained detectable in all the V(-)SeV-Gag-boosted macaques but became undetectable in most of the F(-)SeV-Gag-boosted macaques in a few months. This durability of Gag-specific CD8⁺ T-cell responses may be due to the short period of V(-)SeV-Gag replication. Both groups showed similar levels of Gag-specific and SeV-specific CD4⁺ T-cell responses, and we found no evidence indicating involvement of CD4⁺ T-cell responses in induction or maintenance of Gag-specific CD8⁺ T-cell responses. Interestingly, our results suggested that, even after a few months after the F(-)SeV-Gag boost, localized Gag-specific CD8⁺ T-cell responses were maintained detectable in the retropharyngeal LNs around the nasal mucosa despite the absence of detectable systemic Gag-specific CD8⁺ T-cell responses. We obtained the tonsils at autopsy from five F(-)SeV-Gag-boosted macaques and found efficient Gag-specific CD8⁺ T-cell responses in four of them (C97-062: 0.02%; C88-030: 0.74%; C97-018: 0.25%, C94-030: 0.22%, and C97-011: 0.12%). These results imply possible Gag-specific CD8⁺ T-cell responses in mucosal tissues by intranasal SeV-Gag immunization, which may be effective against HIV-1/SIV transmission.

In development of a prophylactic T cell-based AIDS vaccine, what to be induced by vaccination is not effector but memory T cells that can efficiently respond to viral exposure. In the previous experiment of SIVmac239 challenge three months after boost [11], we found no significant difference in protective efficacy between V(-)SeV-Gag-boosted and F(-)SeV-Gag-boosted macaques; two of four V(-)SeV-Gag-boosted and three of four F(-)SeV-Gag-boosted macaques controlled SIV replication. Even the F(-)SeV-Gag-boosted macaques that had no detectable Gag-specific CD8⁺ T cells at SIV challenge showed rapid secondary responses and controlled SIV replication. The present study suggested a possibility that Gag-specific CD8⁺ T-cell responses induced by F(-)SeV-Gag boost can be maintained in a local, retropharyngeal LN, and these localized, persistent Gag-specific CD8⁺ T-cell responses may contribute to the rapid secondary responses after SIV challenge. Thus, replication-defective F(-)SeV-Gag-induced Gag-specific CD8⁺ T-cell responses that are less durable compared to those induced by replication-competent V(-)SeV-Gag may be sufficient for SIV control.

In summary, we showed that both replication-competent V(-)SeV-Gag and replication-defective F(-)SeV-Gag vectors have the potential to elicit robust Gag-specific CD8⁺ T-cell responses in macaques, whereas the responses induced by the former are more durable than those by the latter in this case. However, our results indicated that even the latter-induced Gag-specific CD8⁺ T-cell responses can persist in local LNs around the nasal mucosa, suggesting a possibility of HIV-1/SIV control even in the absence of detectable vaccine-induced persistent virus-specific CD8⁺ T-cell responses at the virus exposure. Although the vaccine-induced virus-specific CD8⁺ T-cell levels that are sufficient or adequate for HIV-1/SIV control remain unclear, these findings provide important data for establishment of a vaccine protocol using SeV vectors.

Acknowledgements

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology and grants from the Ministry of Health, Labor, and Welfare in Japan.

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center, National Institute of Biomedical Innovation with the help of the Corporation for Production and Research of Laboratory Primates. We thank A. Kato, M. Kano, H. Nakamura, F. Ono, A. Hiyaoka, K. Oto, K. Komatsuzaki, H. Ogawa, H. Akari, Y. Yasutomi, N. Yamamoto, T. Kurata, A. Nomoto, and Y. Nagai for their help.

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Genetic factors that confer sensitivity to HAART in HIV-infected subjects: implication of a benefit of an earlier initiation of HAART

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Evaluation of: Ahuja SK, Kulkarni H, Catano G *et al.*: *CCL3L1-CCR5* genotype influences durability of immune recovery during antiretroviral therapy of HIV-1-infected individuals. *Nat. Med.* 14(4), 413–420 (2008). It is widely accepted that the effect of highly active antiretroviral therapy (HAART) varies widely among HIV-infected individuals. Host genetic factors are thought to be linked to the sensitivity to HAART in HIV-infected individuals. Ahuja *et al.* attempted to identify the genes that determine the sensitivity to HAART in HIV-infected subjects. Based on the hypothesis that CD4$^+$ depletion and the recovery process in HIV-infected subjects are under the control of specific common genetic pathways, they evaluated the associations of genetic variations, such as *CCR5* genotype, *CCL3L1* copy number variation and *HLA* alleles, with the sensitivity to HAART in two cohorts from the USA. They found that the *CCL3L1-CCR5* genetic risk status, but not *HLA-B*57*, is apparently a good predictor of the recovery rate of CD4$^+$ T cells during HAART. In particular, the recovery rate of CD4$^+$ T cells during HAART has the most sensitive association with the copy number of *CCL3L1*. Furthermore, Ahuja *et al.* studied the impact of *CCL3L1-CCR5* genetic risks in HIV-infected individuals initiating HAART during acute or early infection. They suggested that *CCL3L1-CCR5* genetic risk status may be a useful guide in deciding whether to initiate HAART in HIV-infected subjects with a level of 350 CD4$^+$ T cells/mm^3 or more. This study has provided a critical breakthrough in predicting the response to HAART in HIV-infected subjects.

The combination of antiretroviral drugs known as highly active antiretroviral therapy (HAART) has drastically improved the prognosis for great numbers of HIV-infected patients in a very short period of time. Ahuja *et al.* have identified a new genetic mechanism controlling susceptibility to HAART [1]. The AIDS death rate in the USA declined by more than two-thirds within 2 years of the appearance of the protease inhibitors [2]. However, it is widely reported that the effect of HAART, such as the level of viral replication suppression and the recovery rate of CD4$^+$ T-cell counts, varies widely among HIV-infected individuals. Host genetic factors in all likelihood are linked to the sensitivity to HAART in HIV-infected individuals. In fact, there have been several studies performed to evaluate the impact of genetic factors on the sensitivity to HAART. The main features and outcomes of these studies are summarized in Table 1. The majority of these studies have been focused on sequence variations in *HLA-B* [3,4] and *CCR5* [5–9].

Ahuja *et al.* sought to identify the genes that determine the sensitivity to HAART in chronic HIV-infected subjects. They hypothesized that CD4$^+$ depletion and the recovery process are under the control of specific common genetic pathways, and that genetic factors determining HIV-1/AIDS susceptibility are tightly linked to the sensitivity to HAART. In the study, they focused on three genetic variations, *CCR5* genotypes, *CCL3L1* copy number variations and *HLA-B*57*. These three genes have been reported to be tightly linked to HIV/AIDS susceptibility. Sequence variations in *CCR5*, which result in reduced or absent cell-surface expression of the HIV coreceptor CCR5, decrease the susceptibility to HIV-1 infection [10–13]. CCL3L1, a natural ligand for CCR5, is a potent HIV-1-suppressive chemokine. *CCL3L1* copy number variation is tightly linked to HIV/AIDS susceptibility, and a lower copy number is associated with both an enhanced risk for acquiring HIV-1 and also progressing more rapidly to AIDS and death [14,15]. *HLA-B*57* has also been reported to be associated with a better prognosis in HIV-infected individuals [10,11,16,17].

Furthermore, the report has shed new light on the benefits of an earlier initiation of HAART. In the current guidelines for the starting of HAART [101], the earlier initiation of HAART, especially in HIV-infected individuals with a level of 350 CD4$^+$ T cells/mm^3 or more, remain controversial. The authors studied the impacts of

Keywords: CCL3L1, CCR5, HAART, HIV-1, HLA

10.2217/14622416.9.9.1347 © 2008 Future Medicine Ltd ISSN 1462-2416

Table 1. The main features and outcomes of seven studies performed to evaluate the effects of HLA genotypes and *CCR5Δ32* on the clinical course after HAART.

Gene	Study	Sample size	Cohort and ethnic backgrounds	Effects on the clinical course after HAART	Ref.
HLA-B	Brumme *et al.* (2007)	n = 765	HOMER cohort, British Colombia, Canada	Impaired CD4+ T-cell recovery in HLA class I homozygosity Slower virologic suppression in subjects with uncommon HLA alleles	[3]
	Rauch *et al.* (2008)	n = 265	Swiss HIV cohort study and Western Australian HIV cohort study	Impaired CD4+ T-cell recovery in HLA-Bw4 homozygosity	[4]
CCR5Δ32	Bratt *et al.* (1998)	n = 147	Subjects from Sweden	No significant effect on the efficacy of HAART	[5]
	Valdez *et al.* (1999)	n = 113	White subjects from Cleveland, OH, USA	A better response (viral load and CD4+ T cells) in CCR5 wt/Δ32 heterozygotes in comparison with CCR5 wt/wt homozygotes	[6]
	O'Brien *et al.* (2000)	n = 273	AIDS Clinical Trial Group 343 study, Caucasians with CD4+ T-cell ≥200/mm^3 and plasma HIV RNA ≥1000 copies/ml	No significant effect on the efficacy of HAART	[7]
	Kasten *et al.* (2000)	n = 107	Subjects from Germany	A better response (viral load and CD4+ T cells) in CCR5 wt/Δ32 heterozygotes in comparison with CCR5 wt/wt homozygotes	[8]
	Laurichesse *et al.* (2007)	n = 565	French Agence Nationale de Recherrche SERCO/HEMOCO cohort	A better virological response in CCR5 wt/Δ32 heterozygotes in comparison with CCR5 wt/wt homozygotes No significant effect on survival or AIDS-free survival	[9]

HAART: Highly active antiretroviral therapy; HEMOCO: Hemophiliacs HIV-1 infection; HLA: Human leukocyte antigen; HOMER: HAART Observational Medical Evaluation and Research SERCO: Seroconversion; wt: Wild-type.

CCL3L1-CCR5 genetic risks in HIV-infected individuals initiating HAART during acute or early infection.

Genes responsible for HAART sensitivity in chronic HIV-infected subjects

In the study by Ahuja *et al.*, the Wilford Hall Medical Center (WHMC; TX, USA) cohort was studied to evaluate the associations of genetic variations with the sensitivity to HAART in chronic HIV-infected subjects. The WHMC cohort is a component of the USA Military Tri-Service AIDS Clinical Consortium Natural History Study, which is one of the largest cohorts of HIV-positive patients followed prospectively at a single medical center. In the WHMC cohort, 502 HIV-infected subjects received HAART.

Ahuja *et al.* classified a large cohort of HIV-1-infected subjects into three *CCL3L1-CCR5* genetic risk groups on the basis of the copy number of *CCL3L1* and *CCR5* genotype [1]. The high-risk group possessed both a population-specific low *CCL3L1* copy number (*CCL3L1^{low}*) and detrimental *CCR5* variations (*CCR5^{del}*). The low-risk group had both a population-specific high *CCL3L1* copy number (*CCL3L1^{high}*) and non-detrimental *CCR5* variations (*CCR5^{nondel}*). The moderate risk group harbored either one or the other of the two risk factors, *CCL3L1^{low} or CCR5^{del}*. Their cohort was also categorized based on the levels of viral load suppression after HAART to investigate whether, among subjects with a similar viral load suppression, the rate and extent of CD4+ T-cell recovery differed according to the *CCL3L1-CCR5* genetic risk status. They also categorized the cohort based on the levels of CD4+ T cells with which HAART was started. They found that the *CCL3L1-CCR5* genetic risk status is apparently a good predictor of the recovery rate of CD4+ T cells during HAART, especially in those who attained viral load suppression after HAART and those who started

HAART with levels of less than 350 $CD4^+$ T cells/mm^3. They also reported that the recovery rate of $CD4^+$ T cells during HAART has the most sensitive association with the copy number of *CCL3L1*.

On the other hand, possession of *HLA-B*57* had no impact on the recovery rate of $CD4^+$ T cells in HIV-infected subjects receiving HAART. Surprisingly, individuals possessing *HLA-B*57*, which was associated with a delayed disease progress, had an impaired $CD4^+$ T-cell recovery during the first 2–3 years after starting HAART. Similar findings have also been reported by others. Rauch *et al.* have reported that *HLA-Bw4* homozygosity, which has been reported to be linked to a better prognosis in HIV-infected subjects, was associated with an impaired $CD4^+$ T-cell recovery after HAART [4]. They also reported that the possession of *HLA-B*57* was likely to be associated with an impaired $CD4^+$ T-cell recovery. The basis for *HLA-B*57* protection seems to be related, in part, to a highly conserved immunodominant epitope in Gag, a response to which seems to confer early protection during acute infection [18]. HIV-1 specific $CD8^+$ T-cell response restricted by *HLA-B*57* provides a potential mechanism for epidemiological protection, even though it might have no beneficial effects on $CD4^+$ T-cell recovery during HAART.

Interestingly, *HLA-B*57* has been reported to be associated with a hypersensitivity reaction to adacavir, a nucleotide reverse-transcriptase inhibitor [19,20]. The current treatment guidelines recommend screening for *HLA-B*5701* before starting patients on an abacavir-containing regimen, to reduce the risk of a hypersensitivity reaction [101]. *HLA-B*57*-related mechanisms for HAART resistance and hypersensitivity to abacavir are not clear; however, we must pay extra attention to HIV-infected subjects possessing *HLA-B*57* during the course of HIV treatment.

Genetic risk status & earlier initiation of HAART in acute or early HIV-infected subjects

Despite the possible benefits of HAART in HIV-infected subjects with $CD4^+$ T-cell counts over 350 cells/mm^3, there are a couple of reasons which mitigate against the earlier initiation of HAART [101]. However, the earlier initiation of HAART in HIV-infected subjects with a high risk of disease progression appears to be indicated.

Ahuja *et al.* studied the Acute Infection and Early Disease Research Program (AIEDRP) cohort to evaluate the impacts of *CCL3L1-CCR5* genetic risks on the sensitivity to HAART in HIV-infected individuals initiating HAART during acute or early infection [1]. In this prospective study, 315 HIV-infected subjects with signs or symptoms of an acute retroviral syndrome or evidence of recent HIV infection were enrolled. Among the subjects who received HAART during acute infection and who attained viral load suppression, a low genetic risk of *CCL3L1-CCR5* was associated with a greater recovery rate of $CD4^+$ T cells in comparison with a moderate or high genetic risk. They suggested that *CCL3L1-CCR5* genetic risk status may be a useful guide in deciding whether to initiate HAART in HIV-infected subjects with a level of 350 $CD4^+$ T cells/mm^3 or more.

Future perspective

Ahuja *et al.* provided critically important information regarding the genes that determine the sensitivity to HAART in HIV-infected subjects. However, further studies are required to assess the following four issues. First, the association between genetic variations and the HAART sensitivity should be replicated by using other population samples. The authors evaluated two cohorts, WHMC and AIEDRP, both of which were from the USA. Since subtype B of HIV-1 is the most prevalent in the USA [2], it remains possible that the genotype–phenotype interactions observed in this study might not be identified in the context of other HIV-1 subtypes.

Second, additional genes might be linked to the HAART sensitivity in HIV-infected subjects. Other HIV/AIDS susceptible genes [10,11,15] and/or genes linked to drug metabolism might be good candidates. Recently, genome-wide association studies have proven to be a powerful approach to identify the genes responsible for human common diseases [21,22]. Scanning the entire human genome by a genome-wide association study could serve an alternative approach to identifying the genes tightly linked to the HAART sensitivity in HIV-infected subjects.

Third, what is the mechanism by which the *CCL3L1-CCR5* genetic risk status influences the sensitivity to HAART? The HIV-1 entry-dependent effect through the interaction among HIV-1, *CCR5* and *CCL3L1* has been widely accepted as a key mechanism of inhibiting HIV-1 infection [10,11], because CCR5 is a HIV co-receptor and *CCL3L1* is a natural ligand of *CCR5*. Alternatively, viral entry-independent mechanisms have been suggested as the major protective

mechanisms acting through the *CCL3L1* and *CCR5* axis in their previous study [23]. These two mechanisms might have synergistic and/or additive effects with HAART-induced viral load suppression. However, the HIV-1 entry-independent mechanisms remain to be elucidated. Shalekoff *et al.* reported that *CCL3L1* copy numbers were tightly linked to $CD4^+$ and $CD8^+$ T-cell responses to the HIV-1 Gag protein [24]. *CCL3L1-CCR5* genetic risk status might influence cell-mediated immunity such as HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses. Understanding the HIV-1 entry-independent mechanisms would be useful for establishing new strategies of HIV treatment and to develop new types of HIV drugs.

Finally, the long-term effect of earlier initiation of HAART on survival and/or AIDS-free survival should be evaluated. Several studies have demonstrated that an earlier initiation of HAART is associated with a greater recovery rate of $CD4^+$ T cells, but not with a significant reduction in the risk of non-AIDS events/morbidity [10]. Long-term and prospective studies in HIV-infected subjects with high genetic risk are needed to definitively evaluate the benefit of the earlier initiation of HAART.

It is widely accepted that genetic variations are tightly linked to the sensitivity to HAART in HIV-infected subjects. Ahuja *et al.* provided a critical breakthrough to predict the response to HAART in HIV-infected subjects. They opened the door of 'personalized medicine' in HIV therapy. It is to be hoped that further progress will provide great insights into an understanding of the contribution of genetic factors to the response to HIV treatment.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Study design

- The Wilford Hall Medical Center (TX, USA) cohort was studied to evaluate the associations of genetic variations with the sensitivity to highly active antiretroviral therapy (HAART) in chronic HIV-infected subjects.
- The Acute Infection and Early Disease Research Program cohort was studied to evaluate the impacts of genetic risks on the sensitivity to HAART in HIV-infected subjects initiating HAART during acute or early HIV infection.

Analyses performed

- The associations of *CCR5* genotypes, *CCL3L1* copy number variations and *HLA* alleles with HAART sensitivity (viral load suppression and $CD4^+$ T-cell recovery rate) were evaluated.
- HIV-1 infected subjects were classified into three *CCL3L1-CCR5* genetic risk groups – low, moderate and high – on the basis of the copy number of *CCL3L1*, as well as *CCR5* genotype.

Results

- *CCL3L1-CCR5* genetic risk status, but not *HLA-B*57*, were associated with the recovery rate of $CD4^+$ T cells during HAART.
- The recovery rate of $CD4^+$ T cells during HAART is the most sensitive activity associated with the copy number of *CCL3L1*.
- Among the subjects who received HAART during acute infection and who attained viral load suppression, a low genetic risk of *CCL3L1-CCR5* was associated with a greater recovery rate of $CD4^+$ T cells in comparison with a moderate or high genetic risk.

Conclusion

- *CCL3L1-CCR5* genetic risk status, but not *HLA-B*57*, is apparently a good predictor of the recovery rate of $CD4^+$ T cells during HAART.
- *CCL3L1-CCR5* genetic risk status may be a useful guide in deciding whether to initiate HAART during acute or early HIV infection.

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Immunogenetics
DOI 10.1007/s00251-008-0332-0

ORIGINAL PAPER

Natural selection in the *TLR*-related genes in the course of primate evolution

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Received: 4 June 2008 / Accepted: 4 September 2008

Abstract The innate immune system constitutes the front line of host defense against pathogens. Toll-like receptors (TLRs) recognize molecules derived from pathogens and play crucial roles in the innate immune system. Here, we provide evidence that the TLR-related genes have come under natural selection pressure in the course of primate evolution. We compared the nucleotide sequences of 16 TLR-related genes, including *TLRs* (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14*, among seven primate species. Analysis of the non-synonymous/synonymous substitution ratio revealed the presence of both strictly conserved and rapidly evolving regions in the TLR-related genes. The genomic segments encoding the intracellular Toll/interleukin 1 receptor domains, which exhibited lower rates of non-synonymous substitution, have undergone purifying selection. In contrast, *TLR4*, which carried a high proportion of non-synonymous substitutions in the part of extracellular domain spanning 200 amino acids, was found to have been the suggestive target of positive Darwinian selection in primate evolution. However, sequence analyses from 25 primate species, including eight hominoids, six Old World monkeys, eight New World monkeys, and three prosimians, showed no evidence that the pressure of positive Darwinian selection has shaped the pattern of sequence variations in *TLR4* among New World monkeys and prosimians.

Keywords Toll-like receptor · Natural selection · Primate evolution

Electronic supplementary material The online version of this article (doi:10.1007/s00251-008-0332-0) contains supplementary material, which is available to authorized users.

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Introduction

Toll-like receptors (TLRs) recognize molecules derived from pathogens and play crucial roles in the innate immune system. TLRs are type I integral membrane glycoproteins characterized by the extracellular domains with varying numbers of leucine-rich-repeat motifs (LRR) and a cytoplasmic signaling domain termed the Toll/interleukin 1 receptor (TIR) domain (Akira et al. 2006; Bowie and O'Neill 2000). Different TLRs recognize a variety of

pathogen-associated molecules, including lipids and nucleic acids, and all TLRs transduce signals through TIR domains to activate immune cells (Akira et al. 2006; Bowie and O'Neill 2000). Stimulation with TLR ligands recruits adaptor proteins such as Myeloid differentiation factor 88 (MYD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor molecule 1 (TICAM1), and TICAM2, all of which also have a TIR domain, to the cytoplasmic portion of the TLRs and activate signaling cascades to produce proinflammatory cytokines and chemokines (Akira et al. 2006; Bowie and O'Neill 2000).

Viral, bacterial, and parasitic infections have been postulated to be among the strongest selective pressures on primate evolution. It is also widely accepted that the susceptibility to infectious pathogens, such as *Mycobacterium tuberculosis* bacilli and HIV-1, are different among primate species (Isaza 2003; Stremlau et al. 2004). Given that TLRs play crucial roles in the innate immune system, the intriguing hypothesis that TLRs have emerged under the intense pressure of natural selection in the course of primate evolution is rising. Actually, it is suggested that *TLR1*, *TLR6*, and *TLR10* have come under particular natural selection pressures in the human population, because the sequence variations of these three genes display considerable geographical diversity in the British population (Wellcome Trust Case Control Consortium 2007). Moreover, it has been reported that natural selection has acted on *TLR4* in humans, since excess of rare non-synonymous polymorphisms in *TLR4* are observed in humans (Smirnova et al. 2001).

To investigate the natural selection hypothesis, we analyzed the nucleotide sequences of 16 TLR-related genes, including ten *TLRs* (TLR1–10), four genes linked to signal transduction (*MYD88*, *TILAP*, *TICAM1*, and *TICAM2*), and two genes linked to *TLR4* (*MD2* and *CD14*) in primates. MD2 and CD14 are key molecules of the LPS signaling through TLR4 (Poltorak et al. 1998; Shimazu et al. 1999; Nagai et al. 2002). Our study shows that the genomic segments encoding the intracellular TIR domains have undergone purifying selection and that the extracellular domain of *TLR4* has been the suggestive target of positive Darwinian selection in the course of primate evolution. We concluded that natural selection has indeed shaped the sequence patterns of TLR-related genes in primate evolution.

Materials and methods

DNA sequences

DNA samples from 25 primates, including human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), black gibbon (*Hylobates concolor*), white-handed gibbon (*Hylobates lar*), siamang (*Hylobates syndactylus*), crab-eating macaque (*Macaca fascicularis*), rhesus macaque (*Macaca mulatta*), hamadryas baboon (*Papio hamadryas*), black and white colobus (*Colobus guereza*), silvered lutong (*Trachypithecus cristatus*), dusky lutong (*Trachypithecus obscurus*), common marmoset (*Callithrix jacchus*), cotton-top tamarin (*Saguinus oedipus*), red-handed tamarin (*Saguinus midas*), lion tamarin (*Leontopithecus rosalia*), common squirrel monkey (*Saimiri sciureus*), tufted capuchin (*Cebus apella*), long-haired spider monkey (*Ateles belzebuth*), and Central American spider monkey (*Ateles geoffroyi*), tarsiers (*Tarsius* spp.), lesser galago (*Galago senegalensis*), and ring-tailed lemur (*Lemur catta*) were analyzed. Overlapping primer sets covering all coding exons of 16 genes including the *TLRs* (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14* were designed on the basis of size and overlap of PCR amplicons. Genomic DNA was subjected to PCR amplification followed by sequencing using the BigDye Terminator cycling system. Sequencing analysis was performed in an ABI3130x automated DNA sequencer (Applied Biosystems).

Statistical analysis

Sequence alignments were performed by the Clustal X program (Thompson et al. 1997). All values for Ka, Ks, Ka/Ks, %GC, and Codon Bias Index (CBI) were evaluated by DnaSP (Rozas et al. 2003). The Bn–Bs program (Zhang et al. 1998) was applied to evaluate the Ka/Ks ratio in individual branches of the primate phylogenetic tree. We studied positive Darwinian selection for the target region of TLR4 by using the MEGA version 4.0 program (Tamura et al. 2007). Ancestral amino acid sequence was estimated by a parsimony method using PROTPARS program in PHYLIP (Felsenstein 1989).

Results

The nucleotide sequences of tn *TLRs* (TLR1–10), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14* were determined among seven primates, including human, chimpanzee, bonobo, gorilla, orangutan, crab-eating macaque, and rhesus macaque. All sequences were newly determined in the study, and all accession numbers were shown in Table S1. The lengths of the deduced coding sequences for each gene differed among the seven primates, as summarized in Table 1.

To evaluate the non-synonymous/synonymous substitution ratio, we applied the Bn–Bs program (Zhang et al. 1998). This program uses a modified Nei–Gojobori method (Nei and Gojobori 1986) to estimate pairwise synonymous

Table 1 The non-synonymous and synonymous substitution ratio for 16 TLR-related genes among seven primates

Gene	Chromosome (Human)	Size of coding region (bp)	Entire coding sequence			TIR domain			Non-TIR region		
			Σbn	Σbs	Σbn/Σbs	Σbn	Σbs	Σbn/Σbs	Σbn	Σbs	Σbn/Σbs
TLR1	4p14	2,358	0.041	0.095	0.429	0.019	0.103	0.164	0.046	0.093	0.491
TLR2	4q32	2,352, 2,349[Orangutan]	0.025	0.086	0.290	0.009	0.058	0.157	0.028	0.091	0.311
TLR3	4q35	2,712	0.032	0.121	0.267	0.018	0.070	0.259	0.035	0.130	0.270
TLR4	9q32–33	2,517, 2,490[Orangutan] 2,478[Crab-eating, Rhesus]	0.038	0.085	0.447	0.006	0.118	0.053	0.045	0.079	0.566
TLR5	1q41–42	2,574	0.030	0.108	0.282	0.018	0.173	0.105	0.032	0.097	0.339
TLR6	4p14	2,388, 2,385[Bonobo]	0.030	0.120	0.240	0.021	0.199	0.105	0.031	0.105	0.293
TLR7	Xp22.3–p22.2	3,147	0.014	0.069	0.202	0.003	0.087	0.034	0.016	0.066	0.236
TLR8	Xp22.3–p22.2	3,123, 3,120[Crab-eating, Rhesus]	0.020	0.095	0.209	0.003	0.056	0.054	0.023	0.101	0.224
TLR9	3p21.3	3,096	0.029	0.153	0.187	0.003	0.202	0.016	0.032	0.145	0.224
TLR10	4p14	2,433	0.024	0.106	0.228	0.018	0.045	0.402	0.026	0.118	0.216
MYD88	3p22–p21.3	888	0.009	0.096	0.094	0.000	0.087	0.000	0.017	0.102	0.165
TIRAP	11q23–q24	663, 660[Crab-eating, Rhesus]	0.035	0.164	0.216	0.032	0.254	0.126	0.037	0.110	0.341
TICAM1	19p13.3	2,139[a]	0.039	0.171	0.227	0.007	0.163	0.041	0.046	0.172	0.269
TICAM2	5q23.1	705	0.020	0.119	0.167	0.000	0.132	0.000	0.033	0.110	0.300
MD2	8q21.11	480	0.015	0.054	0.269	–	–	–	–	–	–
CD14	5q31.1	1,125	0.013	0.040	0.332	–	–	–	–	–	–

[a] *TICAM1* has a CCT(Pro)-repeat variation

and non-synonymous distances among the sequences and then estimates the branch lengths in terms of synonymous (bs) and non-synonymous substitutions (bn) per site by using the ordinary least-squares method, while the tree topology is given. Σbn and Σbs indicate the value summing up bn and bs, respectively, in the lineages. When the value of Σbn and Σbs and the ratio of Σbn/Σbs were evaluated for the entire coding sequences from each gene, there was no evidence to support that these genes have come under the pressure of positive natural selection. All of the values of the Σbn/Σbs ratio from the analyzed genes were much lower than 1.0, which suggested that these genes have been under the pressure of negative selection (Table 1).

To identify the genomic segments, which have undergone natural selection, a sliding window plot analysis (600-bp window with 30-bp steps) was performed throughout these genes. Analysis of the Σbn/Σbs ratio revealed the presence of both strictly conserved and rapidly evolving regions in the TLR-related genes. Three candidate segments, where the pressure of negative or positive natural selection might have operated, were identified in *TLR7*, *MYD88*, and *TLR4* (Fig. 1a).

Two target segments showed little non-synonymous nucleotide difference among the seven primates (Fig. 1a and Supplementary material, Fig. S1). One was located at the coding segment encoding the C-terminal of TLR7 and the other at the segment encoding the C-terminal of MYD88, both of which encode the TIR domain (Fig. 1a). Phylogenetic comparisons from 14 human sequences encoding TIR domains reveal no obvious similarity between *TLR7* and *MYD88* (Fig. 2a). We then evaluated the Σbn/Σbs ratios for the TIR domains for 14 genes with TIR domains. The sizes of the genomic segments encoding TIR domains [average 393 bp (249–426 bp)] were smaller than the window size (600 bp) used in our analysis so that our window analysis would underestimate the Σbn/Σbs ratios for TIR domains. The values of Σbn and Σbn/Σbs ratio for TIR domains displayed lower values when compared with those of the non-TIR coding sequences except for Σbn/Σbs ratio from *TLR10* (Table 1 and Fig. 2b). In particular, *TLR7*, *TLR8*, *TLR9*, *MYD88*, and *TICAM2* have much lower values for Σbn at the TIR domains. Taken together, it is suggested that the TIR domains have been under the control of negative/purifying selection.

On the other hand, sequence comparisons among the seven primates support the positive Darwinian selection at the extracellular domain of TLR4, for which the Σbn/Σbs ratios were much higher than 1.0 (the highest value in the 600-bp window is 2.37, with a statistical significance in *Z* test; Zhang et al. 1998, Tamura et al. 2007; *Z* score 2.16; *p* value <0.01; Fig. 1). Among analyzed windows fro TLR-related genes, *TLR4* and *TICAM1* have extreme high values of Σbn. However, the windows of *TICAM1* harboring high values of Σbn also have the high value of Σbs. The high values of %GC seem to be associated with the high-nucleotide substitution rate in *TICAM1* (Fig. 1a and b). A lower value of CBI was also correlated with a lower synonymous nucleotide substitution rate (data not shown).

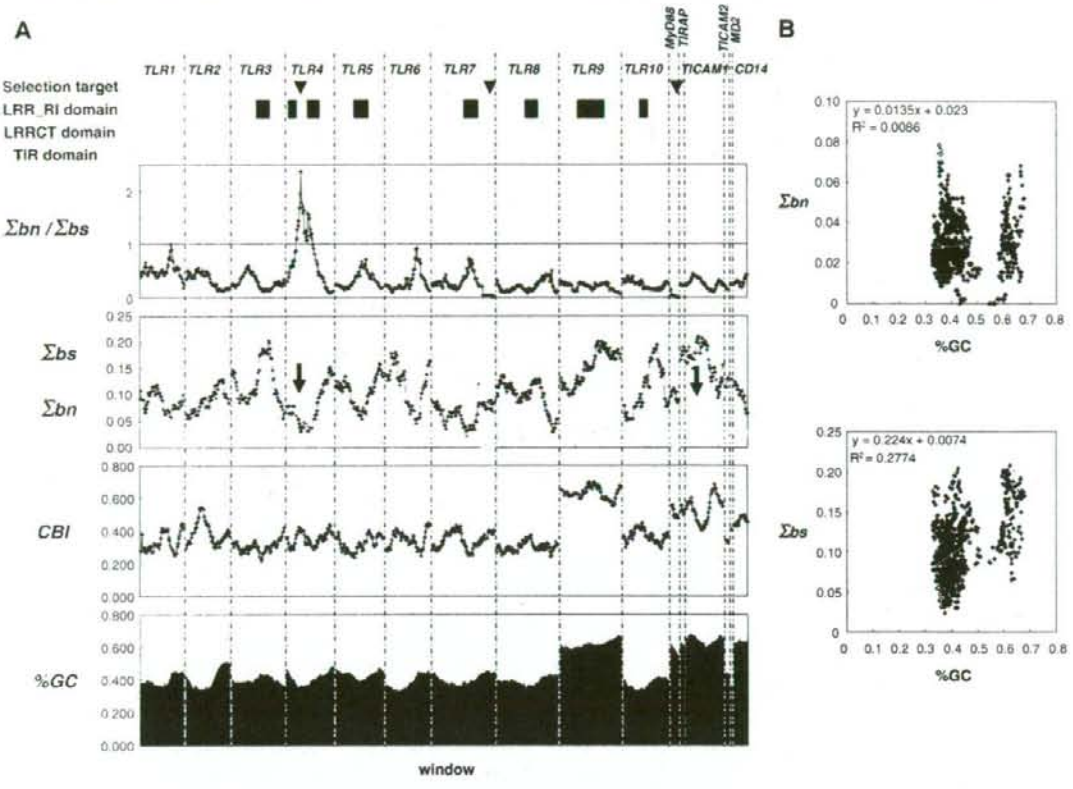

Fig. 1 **a** The values of Σbn/Σbs, Σbn, Σbs, CBI, and %GC based on the sliding window plot analysis for the TLR-related gene (600-bp window with 30-bp steps). The *arrow heads* indicate the candidate segments for the pressure of positive or negative natural selection. The *arrows* indicate that *TLR4* and *TICAM1* have extreme high values of Σbn among analyzed windows. CBI is a measure of the deviation from the equal use of synonymous codons, which indicates the extent to which a gene uses a subset of optimal codons (Bennetzen and Hall 1982). Three conserved domain structures, *LRR_RI* (leucine-rich repeats, ribonuclease inhibitor-like subfamily), *LRRCT* (leucine-rich repeat C-terminal domain), and *TIR* (Toll/interleukin-1 receptor homology domain), are referred from CD-search (Marchler-Bauer and Bryant 2004). **b** Pairwise comparisons between Σbn and %GC and between Σbs and %GC. All values of Σbn, Σbs, and %GC were based on the sliding window plot analysis for the TLR-related gene. *TLR4* (*white lozenge*) and *TICAM1* (*gray lozenge*) have several windows with extreme high values of Σbn

In the window of *TLR4* harboring the highest value of Σbn, its level of CBI (0.398) was almost equivalent to the average level among analyzed windows (0.387±0.114).

The estimated values of bn and bs of each lineage at *TLR4* target region were shown in Fig. 3. The values of bn in three lineages since the emergence of great apes were significantly higher than those of bs. These lineages have a relatively low value of bs; however, the values of bn were much larger than the estimated value of bs for entire TLR4 coding sequences in each lineage except for orangutan lineage (Supplementary material, Fig. S1). These lines of evidence suggested that the extracellular domain of TLR4 has been the possible target of positive Darwinian selection in the course of primate evolution.

To evaluate this finding further, we determined the sequences of a ~600 bp *TLR4* target region from additional 18 primates, including three gibbons (black gibbon, white-handed gibbon, and siamang), four Old World monkeys (hamadryas baboon, black and white colobus, silvered lutong, and dusky lutong), eight New World monkeys (common marmoset, cotton-top tamarin, red-handed tamarin, lion tamarin, common squirrel monkey, tufted capuchin, long-haired spider monkey, and Central American spider monkey), and three prosimians (tarsiers, lesser galago, ring-tailed lemur). Each of target sequences from three gibbons, two lutongs, and two tamarins is identical so that a total of 21 sequences were advanced to further analyses.

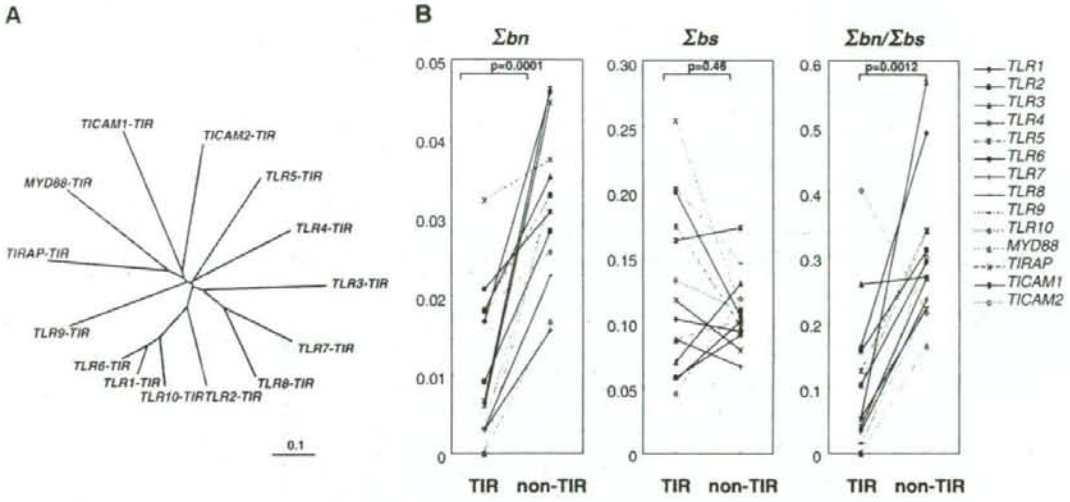

Fig. 2 **a** A neighbor-joining phylogenetic tree for 14 human sequences encoding TIR domains. Alignment and neighbor-joining tree for sequences was inferred using the Clustal X (Thompson et al. 1997) and TreeView programs. **b** The values of Σbn, Σbs, and Σbn/Σbs for the TIR domains and non-TIR coding regions of 14 genes, including *TLRs* (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, and *TICAM2*. Statistical significance was tested with the Wilcoxon matched-pairs signed-ranks test

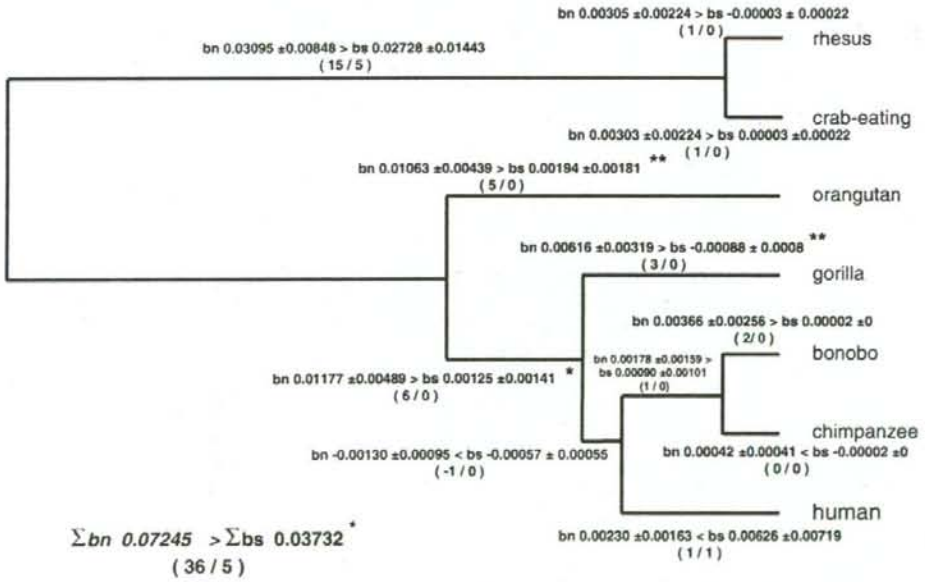

Fig. 3 Phylogenetic tree and the value of bn and bs for the selection target in the extracellular domain of TLR4 among seven primate species. We applied the Bn-Bs program (Zhang et al. 1998) to evaluate the value of bn and bs in individual branches of the primate phylogenetic tree (the values were shown with standard errors). Numbers of non-synonymous/synonymous substitutions for each branch are shown in *parentheses*. The values of bn in three lineages since the emergence of great apes were significantly higher than those of bs (*$p<0.05$ and **$p<0.01$ in Z test)

The estimated values of bn are larger than those of bs in several lineages among hominoids and Old World monkey, which is much evident in the lineages among hominoids. These lineages have a relatively low value of bs. On the other hand, the most estimated value of bn in the lineages among New World monkeys and prosimians are lower than those of bs (Fig. 3a, Supplementary material, Table S2; Fig. 4). Similar findings were shown in the analyses of synonymous (Ks) and non-synonymous nucleotide substitution rate (Ka) for the pairing of the 21 primate sequences at the target region of *TLR4* are shown in Fig. 3b and Supplementary material, Table S1. The Ka/Ks ratios from the comparisons among hominoids and Old World monkeys were much higher than 1.0; however, all values from the comparisons among New World monkeys and prosimians were under 1.0.

Ancestral amino acid sequence for 21 primates was estimated by a parsimony method using PROTPARS program in PHYLIP (Felsenstein 1989; Fig. 5). Among 210 amino acids from TLR4 target region, less than 50% of

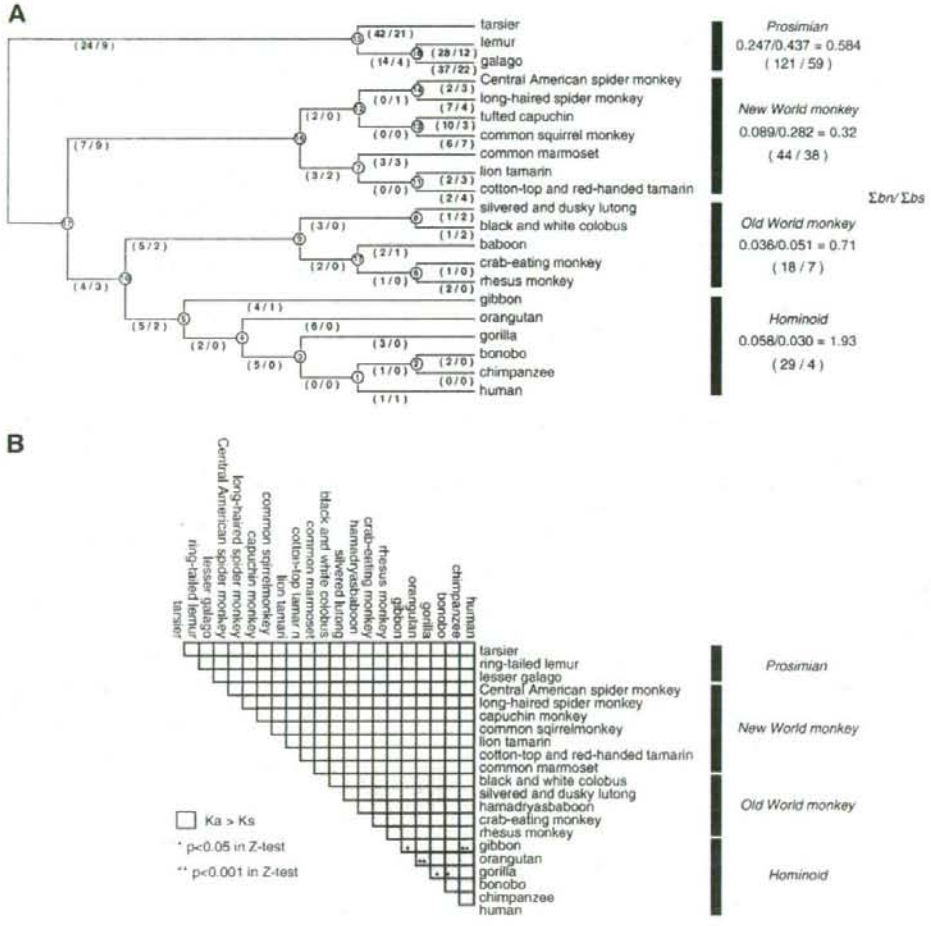

Fig. 4 **a** Phylogenetic tree and the nucleotide substitutions for the extracellular domain of TLR4 from 21 sequences. We applied the Bn–Bs program (Zhang et al. 1998) to evaluate the values of bn and bs in individual branches of the primate phylogenetic tree. *Numbers in parentheses* indicate the number of nucleotide substitutions at non-synonymous/synonymous sites. In several lineages, the value of bn is larger than that of bs (Table S3). Σbn and Σbs indicate the value summing up bn and bs, respectively, in the lineages from hominoids, Old World monkeys, and New World monkeys. Branches are not scaled. *Numbered circles* are used to specify individual branches and are referenced in Supplementary material, Table S3. **b** Ka/Ks ratios for all comparisons among 21 TLR4 target sequences. The values of Ka/Ks for 627-bp target sequences were evaluated, because the comparisons among 21 sequences showed that a 3-bp deletion were observed in sequences from lesser galago (*G. senegalensis*) and ring-tailed lemur (*L. catta*)

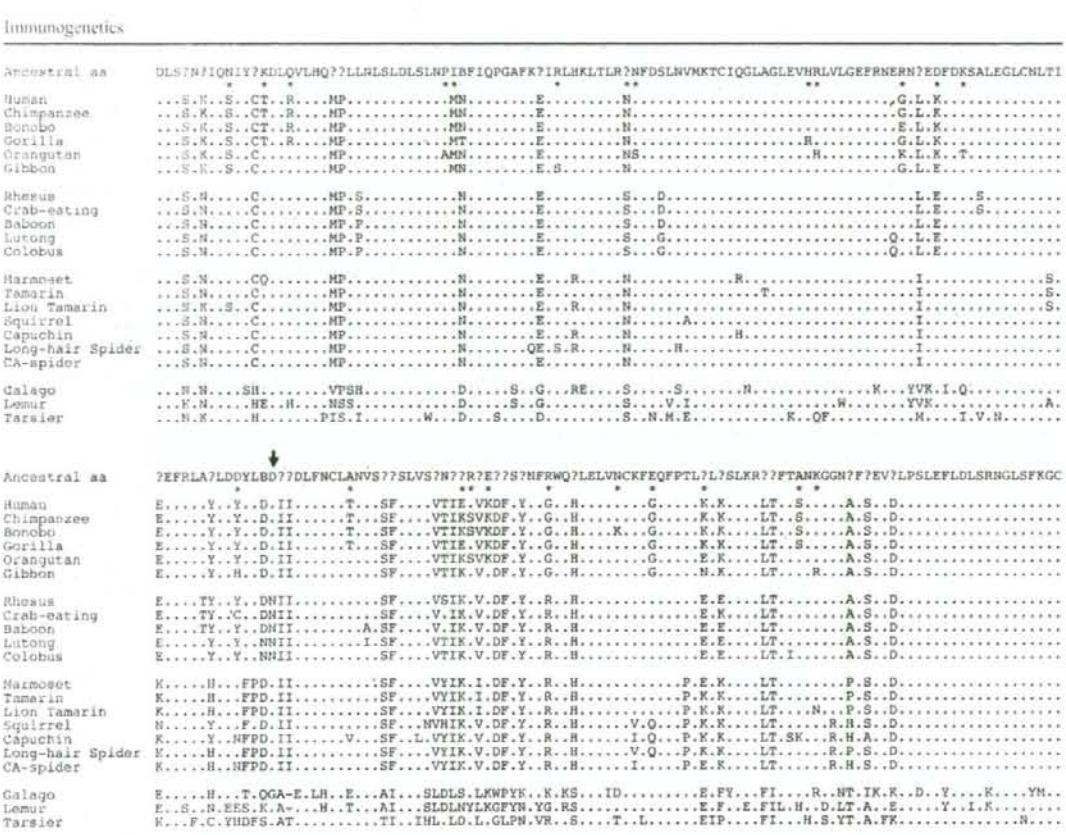

Fig. 5 Alignment of TLR4 target amino acid sequences of 21 primate species. *Dots* indicate identity to the ancestral sequence predicted by PROTPARS program in PHYLIP (Tamura et al. 2007), and *dashes* indicate a gap. *Asterisks* indicate amino acids which had been substituted since the emergence of hominoids. *Arrow* indicates the position corresponding to human mutation, D299G. The protein sequences are given by the *one-letter code* and consistent with the IUB standard abbreviations. *Question marks* in the ancestral amino acid sequence indicate the amino acid of which ancestral sequence was not determined by PROTPARS program (Felsenstein 1989)

amino acids [42.9% (90/210)] were conserved among 21 primate species, and at least 24 amino acids were replaced since the emergence of hominoids.

Discussion

Several lines of evidence suggested that genes linked to immune and defense systems are good candidate genes under the pressure of positive natural selection. However, most of the TLR-related genes have come under negative natural selection in the course of primate evolution. Our study suggested that the genomic segments encoding the intracellular TIR domains have undergone strong purifying selection and that the extracellular domain of TLR4 has been a single target of positive Darwinian selection among the TLR-related genes. We have no strong evidence that the extracellular domains of other TLRs, which recognize a variety of pathogen-associated molecules, has been under the pressure of natural selection in the course of primate evolution.

The TIR domains are key components in TLR signal transduction. In particular, an adaptor protein, MYD88, is tightly linked to all of the TLR signaling pathways except for TLR3 (Akira et al. 2006; Bowie and O'Neill 2000). This may be the reason for that the amino acid sequences have not been altered in the course of primate evolution. Most of mutations arising at the TIR domains would in all likelihood have been deleterious and thus reduced the fitness in the primates who harbored such changes.

Among the TLRs, *TLR7*, *TLR8*, and *TLR9* have much lower values of Σbn at the TIR domains. These three genes recognize nucleic acids in the innate immune system and are categorized into highly related subfamilies based on phylogenetic comparison (Akira et al. 2006). TLR7 and TLR8 are known to recognize single-strand RNA molecules

and the natural ligand of TLR9 is CpG motif-containing DNA (Akira et al. 2006; Heil et al. 2004). *TLR7* and *TLR8* are also mapped on chromosome X in the human, chimpanzee, and rhesus macaque genome, which might be linked to the reduced Σbn for these two genes in primate evolution, because reduced divergence has been observed in chromosome X when a comparison was performed on the human and rhesus macaque genome (Rhesus Macaque Genome Sequencing and Analysis Consortium 2007).

On the other hand, it is likely that the part of the extracellular domain of TLR4 has been under the pressure of positive natural selection in the several lineages since the emergence of Catarrhina. The values of bn were higher than those of bs in the several lineages. In particular, the values of bn in the lineages of gorilla and the lineage just after the split of ancestors of four great apes (gorilla, bonobo, chimpanzee, and human) were much larger than the estimated value of bs for entire TLR4 coding sequences. However, we cannot rule out the possibility that chance variation in the value of bs, rather than positive selection, accounted for the observed pattern (Hughes and Friedman 2008), because these lineages have a relatively low value of bs.

This suggestive *TLR4* target region, which encodes the extracellular domain next to the domain with LRR, has been reported to be hypervariable and to contribute to species-specific recognition of several molecules such as taxol, a lipid IVa, and LPS (Smirnova et al. 2000; Hajjar et al. 2002; Lien et al. 2000). Our study showed that the positions of amino acid replacements since the emergence of Catarrhina, which might be linked to species-specific recognition of LPS, are widely distributed in the TLR4 selection target. This target region also has been reported to be linked to the susceptibility to LPS in humans (Arbour et al. 2000). A missense human mutation D299G, in which an aspartic acid is replaced by a glycine at the 299 amino acid position of human TLR4, is associated with a blunted response to LPS and increased susceptibility to Gram-negative bacterial infections. An aspartic acid at the 299 amino acid position of human TLR4 is highly conserved among great apes and gibbon, whereas an aspartic acid has been replaced by a glycine in the lineage of Old World Monkeys (Fig. 5). These results indicated that the sensitivity to a certain type of LPS might differ between great apes and Old World Monkeys.

Given that TLR4 recognizes a wide variety of ligands such as LPS and viral envelope proteins, the differences in the species-specific susceptibility to infectious disease might have been linked to natural selection pressure. It is widely accepted that the susceptibility to infectious pathogens differ among primates. For example, Asian Old World monkeys are highly susceptible to infection with *M. tuberculosis* bacilli, while New World monkeys appear to be fairly resistant (Isaza 2003). Furthermore, the species-specific restrictions operating on HIV-1 infection are well known. Humans as well as chimpanzees but not New and Old World monkeys are susceptible to HIV-1 (Stremlau et al. 2004). Though we are not aware of the exact pathogens, it is highly possible that wide-spread pathogens might have been linked to the selective pressure for TLR4.

In this study, we evaluated the molecular evolution of TLR-related genes in primates and concluded that natural selection has indeed shaped the sequence patterns of TLR-related genes in the course of primate evolution, but positive selection pressure has been limited across the TLR family.

Acknowledgments This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and research grants from the Ministry of Health, Labor and Welfare, Japan, the Japan Health Science Foundation, and the program of Founding Research Centers for Emerging and Reemerging Infection Disease supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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MDM2 is a novel E3 ligase for HIV-1 Vif

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Published: 7 January 2009

Retrovirology 2009, **6**:1 doi:10.1186/1742-4690-6-1

Received: 16 September 2008
Accepted: 7 January 2009

This article is available from: http://www.retrovirology.com/content/6/1/1

Abstract

The human immunodeficiency virus type 1 (HIV-1) Vif plays a crucial role in the viral life cycle by antagonizing a host restriction factor APOBEC3G (A3G). Vif interacts with A3G and induces its polyubiquitination and subsequent degradation via the formation of active ubiquitin ligase (E3) complex with Cullin5-ElonginB/C. Although Vif itself is also ubiquitinated and degraded rapidly in infected cells, precise roles and mechanisms of Vif ubiquitination are largely unknown. Here we report that MDM2, known as an E3 ligase for p53, is a novel E3 ligase for Vif and induces polyubiquitination and degradation of Vif. We also show the mechanisms by which MDM2 only targets Vif, but not A3G that binds to Vif. MDM2 reduces cellular Vif levels and reversely increases A3G levels, because the interaction between MDM2 and Vif precludes A3G from binding to Vif. Furthermore, we demonstrate that MDM2 negatively regulates HIV-1 replication in non-permissive target cells through Vif degradation. These data suggest that MDM2 is a regulator of HIV-1 replication and might be a novel therapeutic target for anti-HIV-1 drug.

Background

Host restriction factors protect hosts from viruses, whereas viruses evade these proteins to replicate more efficiently in host cells. The interplay between the host restriction factors and viral proteins is therefore very important for regulating viral replication [1,2]. A3G (Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G) is a newly identified anti-HIV-1 host factor [3], which belongs to the APOBEC superfamily of cytidine deaminases, consisting of APOBEC1, APOBEC2, AID (activation-induced cytidine deaminase), APOBEC3(A-H), and APOBEC4 [4]. A3G is incorporated into HIV-1 virions and inhibits HIV-1 replication by inducing G-to-A hypermutation in viral cDNA during reverse transcription [5-8]. HIV-1 Vif counteracts A3G by targeting it for proteasomal degradation, thus supporting HIV-1 replication in non-permissive target cells [9-11]. Vif forms a ubiquitin ligase (E3) complex with Cullin5 (Cul5), Elongin B, and Elongin C and functions as a substrate recognition subunit of this complex to induce ubiquitination and subsequent degradation of A3G [12,13]. Vif also counteracts several APOBEC3 proteins including APOBEC3F (A3F) [14,15]. These observations reconcile the long-standing mystery of why Vif function is necessary for HIV-1 to infect non-permissive cells. On the other hand, it has been shown that intracellular levels of Vif are maintained relatively low by ubiquitination in virus-producing cells [16-18]. Although several groups have reported E3 ligases important for Vif ubiquitination [17,18], the precise roles and mechanisms of Vif ubiquitination remain unclear. Here we demonstrate that MDM2 is a novel E3 ligase for Vif and that it induces ubiquitination and degradation of Vif, thereby regulating HIV-1 replication.

Results

MDM2 downregulates cellular Vif levels by inducing its degradation in a proteasome-dependent manner

To investigate the biological roles and molecular mechanisms of Vif ubiquitination, we tried to identify a novel E3 ligase that may be involved in the ubiquitination of Vif. During a search for Vif-interacting proteins in the HIV, Human Protein Interaction Database of National Institute for Allergy & Infectious Diseases http://www.ncbi.nlm.nih.gov/RefSeq/HIVInteractions/, we were struck by a protein called Gankyrin (proteasome 26S subunit, non-ATPase, 10 (PSMD10)). We first examined the biological effects of Gankyrin, but could not detect a downregulation of Vif (data not shown). As we previously reported that Gankyrin itself doesn't have an enzymatic activity and that it rather enhances the E3 ligase activity of MDM2 on p53 ubiquitination and degradation as a co-factor [19], we tested the possibility that MDM2 plays an important role in Vif ubiquitination as a novel E3 ligase. We examined the effect of several E3 ligases including MDM2 (a RING finger type E3 that mediates p53 ubiquitination and degradation [20]), Cul5 (another RING finger type E3 that forms a complex with Vif and is reported to induce Vif ubiquitination [17,21]), and Parkin (another RING finger type E3) on cellular Vif levels (Fig. 1A). HEK293T cells were transfected with a subgenomic expression vector pNL-A1 that expressed all HIV-1 proteins except for *gag* and *pol* products [22], together with the expression plasmids for these E3 ligases. We found that the ectopic expression of MDM2 downregulated the cellular levels of Vif as well as p53 in transfected cells in a dose-dependent manner (Fig. 1A, lanes 8–10), whereas Parkin and Cul5 did not affect their cellular levels (lanes 2–4 and 5–7, respectively), even though the latter proteins were expressed more than MDM2. Our results are discrepant with previous reports that demonstrated Cul5 induced Vif ubiquitination and degradation [17,23]. We assume that overexpression of Cul5 alone is insufficient to induce Vif degradation, because other E3 components are not overexpressed. Ectopic expression of MDM2 did not affect cellular levels of another viral protein such as Nef, suggesting that MDM2 specifically downregulated Vif levels; this result also excluded the possibility that MDM2 affected the transcriptional activity of the HIV-1 LTR.

Because it is well known that MDM2 regulates p53 levels by modulating its protein stability, we next examined the protein stability of Vif with the ectopic expression of MDM2. HEK293T cells were transfected with pNL-A1 with or without a MDM2 expression vector and treated with cycloheximide 21 hrs after transfection. After cycloheximide treatment, cellular levels of Vif decreased by 60% in MDM2-transfected cells and by 20% in control cells, respectively (Fig. 1B &1C), indicating that Vif decayed much faster when MDM2 was overexpressed. The stability profile of Vif protein was similar to that of p53 (Fig. 1B). However, in our hands, the half-life of Vif protein was longer than those shown in previous studies from several laboratories. We interpret that this difference is attributable to divergent methods used in the studies which employed radioisotopes or cycloheximide. Thus, our findings suggest that MDM2 affects the stability of Vif protein similar to its effect on p53. We also examined the stability of Vif in MDM2-/- MEF cells. Vif decayed much faster in p53-/- MEF cells than in p53-/-MDM2-/- double knock-out (DKO) MEF cells (Additional file 1), suggesting that endogenous MDM2 can also influence the stability of Vif. We then tested a RING finger domain-deleted MDM2 mutant, ΔRF, which is inactive for the ubiquitination activity of MDM2 [24]. Ectopic expression of MDM2 suppressed cellular Vif levels, but the expression of ΔRF did not (Fig. 1D). This result suggests that ubiquitination of Vif by MDM2 is involved in the downregulation of cellular Vif levels. We further treated transfected cells with a proteasome inhibitor MG132 to see whether the down-

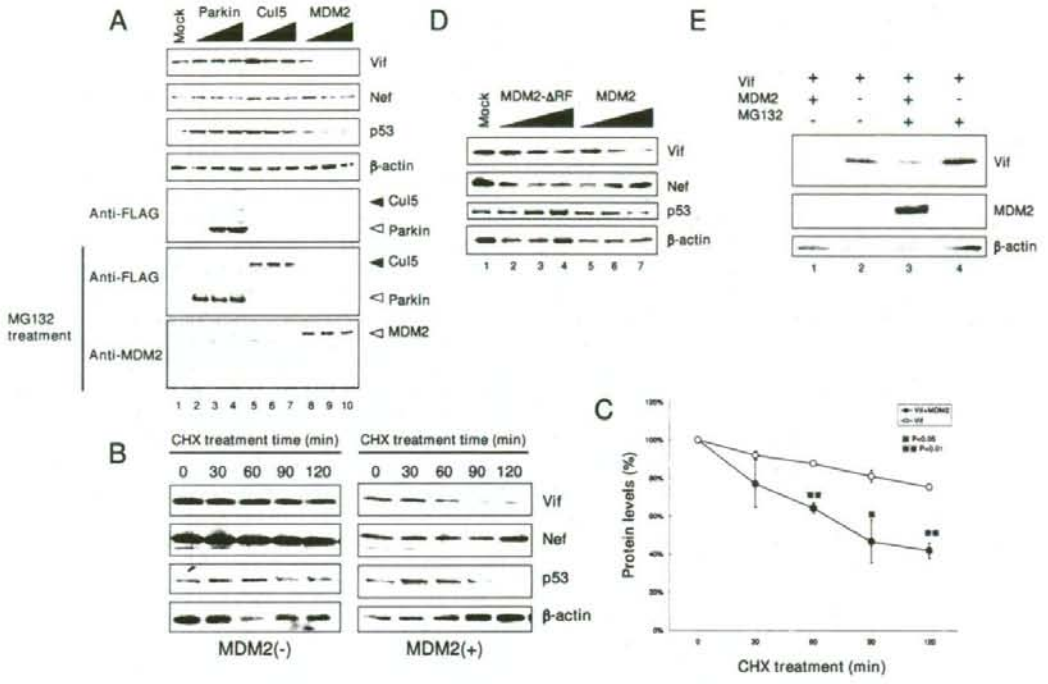

Figure 1

MDM2 downregulated cellular Vif levels in a proteasome dependent manner. (A) MDM2 reduced cellular levels of Vif as well as p53, but not that of Nef. HEK293T cells were cotransfected with expression vectors for the indicated E3 ligases and a subgenomic HIV-1 expression vector pNL-A1. Cell lysates were subjected to immunoblotting with the indicated Abs. We could not detect the expression of FLAG-MDM2 without MG132 treatment, because of a rapid degradation of MDM2. MG132 treatment enabled us to detect expression of MDM2 only with anti-MDM2 Ab, but not with anti-FLAG mAb. (B) Twenty-two hours after transfection, the cells were treated with cycloheximide (CHX)(80 μg/ml) for the indicated times, and cell lysates were subjected to immunoblotting with the indicated Abs. (C) The amounts of Vif and Nef were quantified by densitometry, and Vif protein levels were calculated using Nef protein levels as normalizing loading controls and presented as percentage values relative to that without CHX treatment set as 100%. Values are presented as averages of three independent experiments. (D) MDM2 downregulated Vif, but a ΔRF mutant did not. HEK293T cells were cotransfected with expression vectors for MDM2 and the mutant together with pNL-A1, and cell lysates were subjected to immunoblotting with the indicated Abs. (E) p53$^{-/-}$MDM2$^{-/-}$ DKO-MEF cells were cotransfected with expression vectors for MDM2 and Vif, and treated with 10 μM MG132 for 6 hrs, and cell lysates were subjected to immunoblotting with the indicated Abs.

regulation of Vif by MDM2 was proteasome-dependent. Treatment with MG132 clearly restored the cellular Vif level that was downregulated by MDM2 (Fig. 1E, top panel, lane 3 as compared with lane 1), supporting that the MDM2-mediated downregulation of Vif was proteasome-dependent. Taken together, we concluded that MDM2 downregulates cellular Vif level by inducing its degradation in a proteasome-dependent manner.

MDM2 specifically binds and downregulates Vif

To further investigate the molecular link between MDM2 and Vif, we next examined the physical interaction of MDM2 with Vif. Immunoprecipitation assays showed that Vif was co-precipitated with MDM2 (Fig. 2A). Glutathione S-transferase (GST) pull-down assays showed that MDM2 was found in GST-Vif-bound, but not GST-bound, material (data not shown). Using a series of MDM2 deletion mutants, we determined that the central region of MDM2 (amino acids 168–320) was necessary for Vif binding (Fig. 2B, left panel &2C). To more precisely