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).

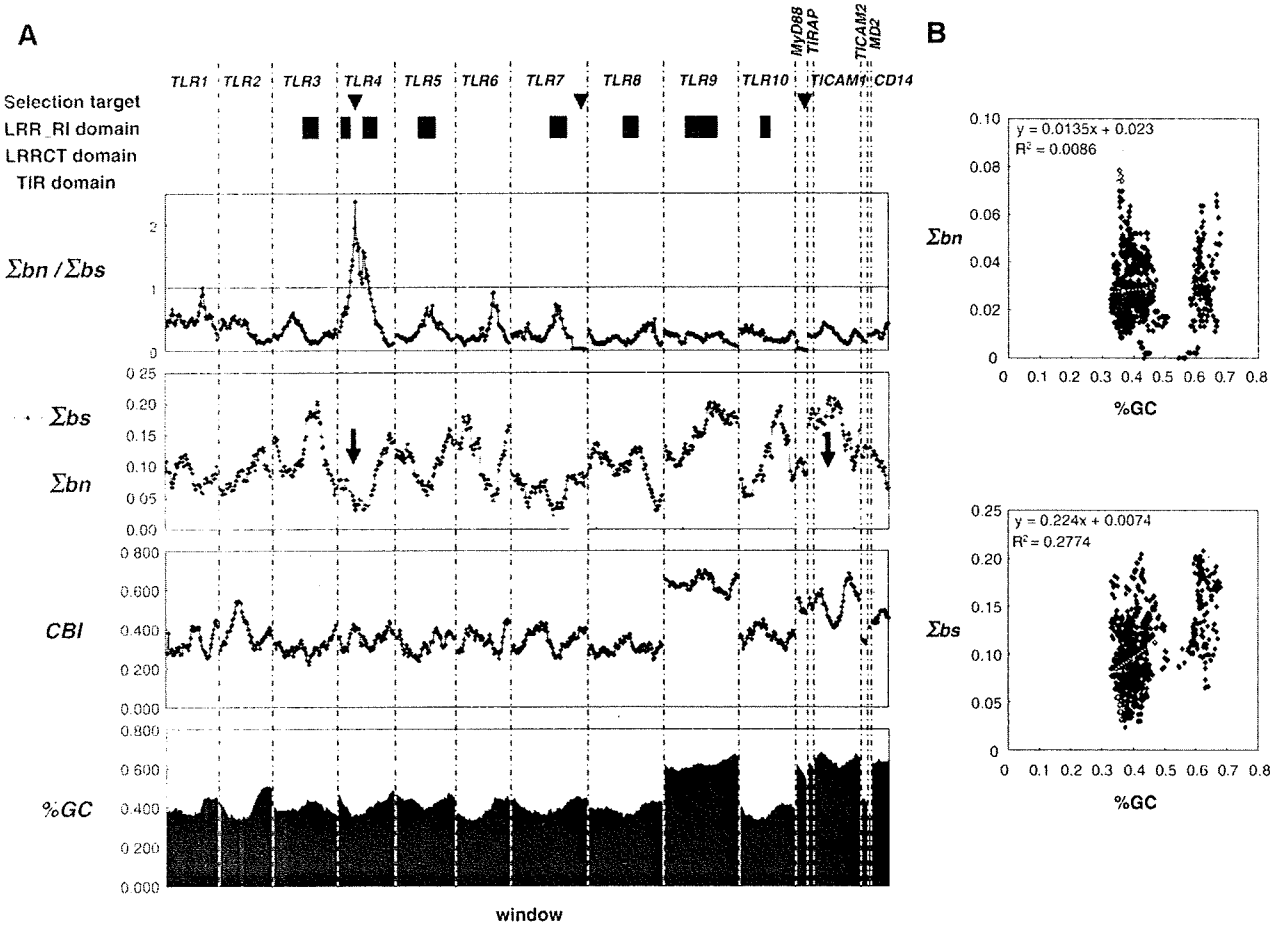

Fig. 1 **a** The values of Σbn/Σbs, Σbn, Σbs, CBI, and %GC based on the sliding window plot analysis for the TLR-related gene (600-bp window with 30-bp steps). The *arrow heads* indicate the candidate segments for the pressure of positive or negative natural selection. The *arrows* indicate that *TLR4* 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 [illegible]). 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.

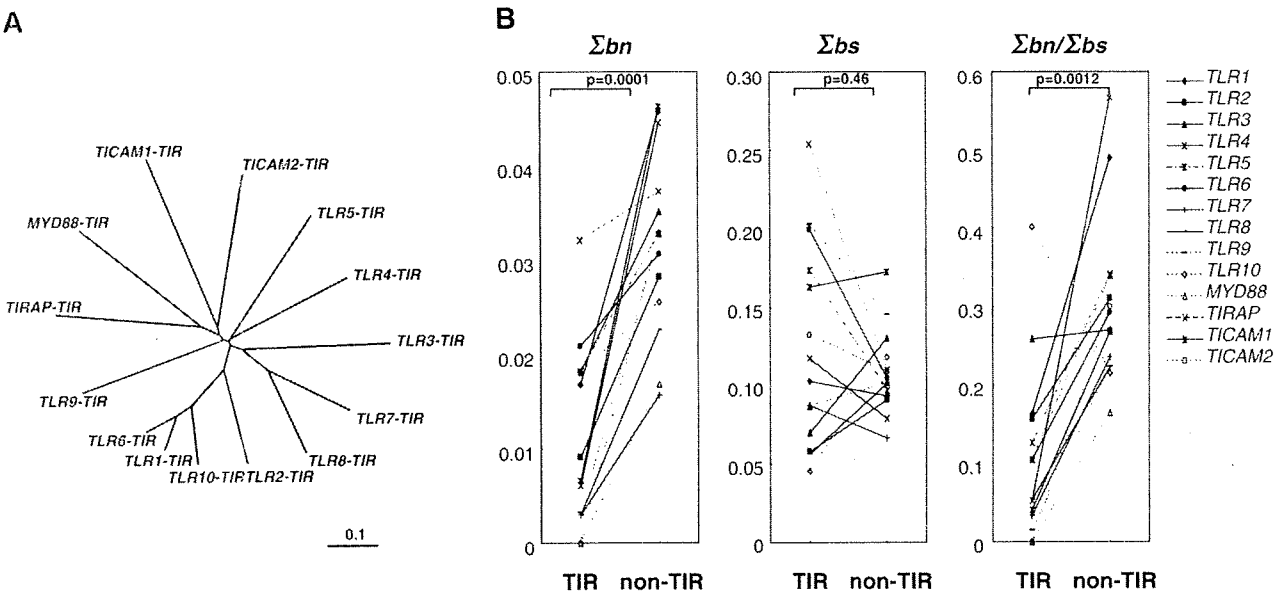

Fig. 2 **a** A neighbor-joining phylogenetic tree for 14 human sequences encoding TIR domains. Alignment and neighbor-joining tree for sequences was inferred using the Clustal X (Thompson et al. [illegible]) and TreeView programs. **b** The values of Σbn, Σbs, and Σbn/Σbs for the TIR domains and non-TIR 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

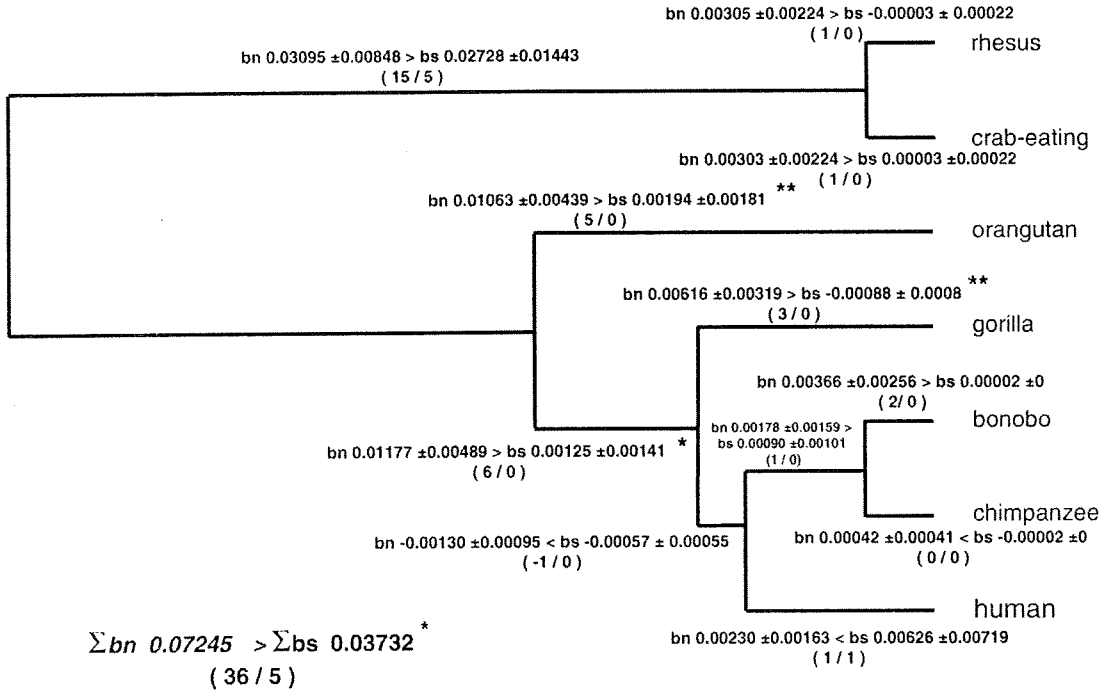

Fig. 3 Phylogenetic tree and the value of bn and bs for the selection target in the extracellular domain of TLR4 among seven primate species. We applied the Bn Bs program (Zhang et al. 1998) to evaluate the value of bn and bs in individual branches of the primate phylogenetic tree (the values were shown 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

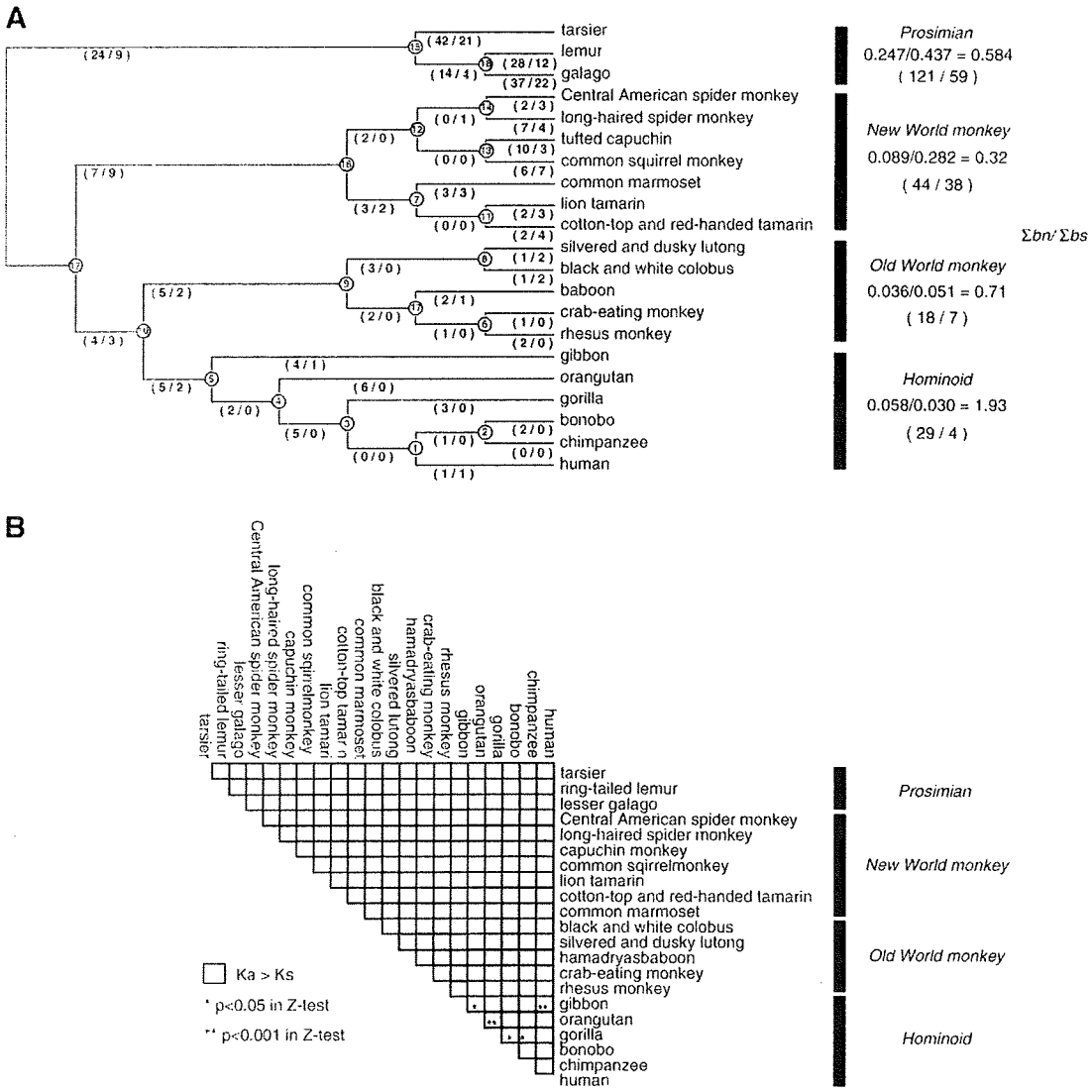

Fig. 4 a Phylogenetic tree and the nucleotide substitutions for the extracellular domain of TLR4 from 21 sequences. We applied the Bn–Bs program (Zhang et al. 1998) to evaluate the values of bn and bs in individual branches of the primate phylogenetic tree. *Numbers in parentheses* indicate the number 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*)

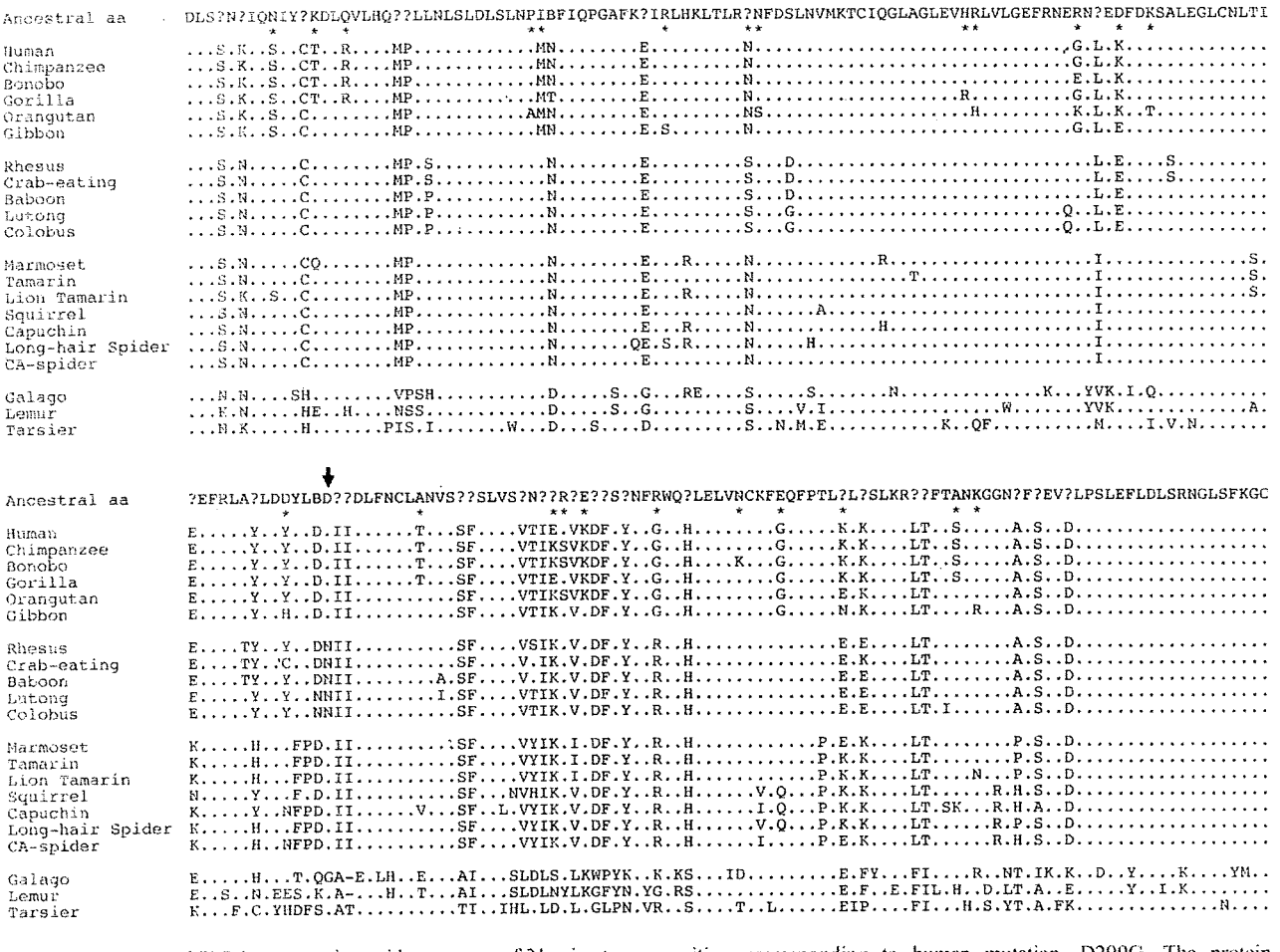

Fig. 5 Alignment of TLR4 target amino acid sequences of 21 primate species. *Dots* indicate identity to the ancestral sequence predicted by PROTPARS program in PHYLIP (Tamura et al. 2007), and *dashes* indicate a gap. *Asterisks* indicate amino acids which had been substituted since the emergence of 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 1980)

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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Cytogenet Genome Res 123:156–160 (2008)
DOI: 10.1159/000184703

Cytogenetic and Genome Research

HIV-1/AIDS susceptibility and copy number variation in *CCL3L1*, a gene encoding a natural ligand for HIV-1 co-receptor CCR5

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Accepted in revised form for publication by H. Kehrer-Sawatzki and D.N. Cooper, 27 May 2008.

Abstract. Variations of gene copy number in the human genome are increasingly recognized as a genetic factor in phenotypic variation. Human CC chemokine ligand 3-like 1 gene *(CCL3L1)*, which is located on human chromosome 17q11.2, is highly variable in copy number owing to having a hot spot for segmental duplications. CCL3L1, a natural ligand for HIV-1 co-receptor CCR5, is a potent HIV-1–suppressive chemokine. *CCL3L1* copy number variation (CNV) is tightly linked to HIV-1/AIDS susceptibility, and a lower copy number is associated with an enhanced risk for acquiring HIV-1 and also progressing more rapidly to AIDS and death. In this article we review recent studies to evaluate the association between the *CCL3L1* copy number and HIV-1/AIDS susceptibility.

It is estimated that more than 50 million people worldwide have become infected with HIV-1 in the past 25 years, and a third of them have died (UNAIDS, 2006). It is generally accepted that there are several different genetic factors which influence HIV-1 susceptibility and/or acquired immunodeficiency syndrome (AIDS) progression (O'Brien and Nelson, 2004; Kaslow et al., 2005). The clinical course of HIV-1 infection varies considerably from person to person. Some patients rapidly progress to AIDS, while others spontaneously achieve viral control and relative immunologic stability. Thus, it is urgent to identify the genes responsible for HIV-1/AIDS susceptibility.

This work was supported in part by research grants from the Ministry of Health, Labor and Welfare, Japan, the Japan Health Science Foundation, the program of Founding Research Centers for Emerging and Reemerging Infection Disease, Grant-in-Aids for Scientific research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, and a grant from the Department of Biotechnology (DBT), Ministry of Science and Technology, Govt of India.

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Genetic association analyses of AIDS cohorts have implicated certain genes to be responsible for HIV-1/AIDS susceptibility (O'Brien and Nelson, 2004; Kaslow et al., 2005). These genes regulate the mechanisms of HIV-1 cell entry and the immune response to HIV-1, and their impact on individual and population sensitivity to HIV-1/AIDS is considerable. Insights into the genetic determinants of HIV-1/AIDS susceptibility have emerged from studies on HIV-1 resistant people, the so called 'long-term nonprogressors (LTNPs)'. One of these findings includes genetic variations in the HIV-1 co-receptor chemokine receptor 5 *(CCR5)*, which has provided a great advance in genetic analyses for HIV-1/AIDS susceptibility (Liu et al., 1996; Samson et al., 1996). Sequence variations in *CCR5*, which result in reduced or absent cell-surface expression of CCR5, decrease the susceptibility to HIV-1 infection.

Recently, copy number variations (CNVs) in *CCL3L1* have been reported to be linked to the susceptibility to HIV-1 infection (Gonzalez et al., 2005). CCL3L1, also known as macrophage-inflammatory protein 1α (MIP-1αP) or LD78β, is a natural ligand for the HIV-1 co-receptor CCR5 (Menten et al., 2002). *CCL3L1* exhibits CNVs on chromosome 17q11.2 (Hirashima et al., 1992; Townson et al., 2002), and the possession of a lower copy number of *CCL3L1* is as-

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sociated with markedly enhanced HIV-1/AIDS susceptibility. In this article we review the recent studies to evaluate the association between *CCL3L1* CNV and HIV-1/AIDS susceptibility and also review the mechanisms by which the interaction of CCL3L1 with the HIV-1 co-receptor CCR5 may build up resistance to HIV-1 infection.

CCL3L1 is a natural ligand of HIV-1 co-receptor CCR5 and a potent HIV-1 suppressive chemokine

The initial entry of HIV-1 into a cell requires the expression of co-receptors on the cell surface as well as the HIV-1 cellular receptor CD4. Several chemokine receptors have been identified as HIV-1 co-receptors and two chemokine receptors, CCR5 and CXCR4, have been reported to play crucial roles in the entry of HIV-1 into CD4+ T-cells (O'Brien and Nelson, 2004; Arenzana-Seisdedos and Parmentier, 2006). The CD4 molecule first interacts with the HIV-1 surface envelope protein and retains the virus on the cell surface. A cell-surface co-receptor then comes into contact with HIV-1 bound to CD4. The interaction between the bound HIV-1 and the co-receptor causes the HIV-1 gp41 protein to undergo a conformational change and penetrate the cell membrane. The HIV-1 particle then fuses with the cell membrane and infects the cell. Based on the type of co-receptor usage, HIV-1 viruses are categorized into two major types (O'Brien and Nelson, 2004; Arenzana-Seisdedos and Parmentier, 2006). One is the R5 strain, which uses CCR5 as a co-receptor, and the other is the X4 strain, which uses CXCR4. In most infected people, a mutational shift occurs in *env*, which alters the viral co-receptor preference from CCR5 to CXCR4, usually coincident with a depletion of CD4+ T-cells.

The importance of CCR5 in the pathogenesis of AIDS was highlighted by the identification of the *CCR5* variant with a 32-bp deletion in its coding region, the so called *CCR5Δ32* (Liu et al., 1996; Samson et al., 1996). Homozygotes for *CCR5Δ32* were suggested to be relatively resistant to infection with HIV-1. In addition, sequence variations in the promoter region of *CCR5* were associated with HIV-1/AIDS susceptibility (Martin et al., 1998). Subsequent studies confirmed that these variations, which cause reduced or absent cell-surface expression of CCR5, lead to diminished susceptibility to HIV-1 infection.

A number of chemokines, such as CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES), have been identified as ligands for CCR5. They may physically block the entry of HIV-1 by occupying the requisite co-receptors. Among the chemokine ligands for the CCR5 co-receptor, CCL3L1, particularly the CD26-cleaved form lacking the first two amino acids of the mature protein, is the most potent natural chemokine inhibitor of HIV-1 binding and entry through CCR5 (Arenzana-Seisdedos and Parmentier, 2006). Thus, genetic differences that alter the expression and/or function of CCL3L1 might have an impact on HIV-1/AIDS susceptibility.

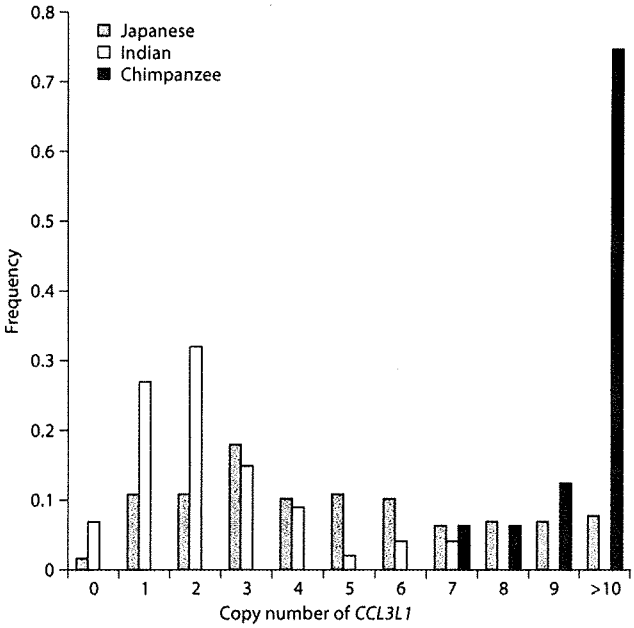

Fig. 1. The distribution of the *CCL3L1* copy number among an Indian population (n = 100), a Japanese population (n = 205), and chimpanzees (n = 16).

CNVs in *CCL3L1*

Redon et al. (2006) have reported that genes having CNVs are very common in the human genome. There are at least 1,447 copy number variable regions covering 12% of the entire genome which contain hundreds of genes. Variations in the gene copy number are increasingly recognized as a genetic determinant of phenotypic variation. Several pieces of evidence that common disease phenotypes are associated with such variations have been steadily accumulating and continue to be reported.

CCL3L1, which is located on human chromosome 17q11.2, is highly variable in its copy number owing to having a hot spot for segmental duplications in the human genome (Hirashima et al., 1992; Townson et al., 2002). Segmental duplications of the genomic region covering *CCL3L1* are also observed in the chimpanzee (Gonzalez et al., 2005; Shao et al., 2007) and macaque genome (Rhesus Macaque Genome Sequencing and Analysis Consortium, 2007). *CCL3*, which displays >90% sequence identity with *CCL3L1*, is also located on chromosome 17q11.2, but it is a single-copy gene with two copies per diploid genome. The average copy number of *CCL3L1* differs among ethnic groups. Briefly, African populations have significantly larger copy numbers of *CCL3L1* than non-Africans (Gonzalez et al., 2005). We compared the distribution of the *CCL3L1* copy number between Japanese and Indian populations and also evaluated the copy number of *CCL3L1* orthologs in 16 chimpanzees. As shown in Fig. 1, the distribution of the *CCL3L1* copy number was significantly different between the Japanese

Table 1. The main features and outcomes of four studies performed to evaluate the association between *CCL3L1* copy number and HIV/AIDS susceptibility

Reference	Subjects	Sample size	Ethnic background	Association of *CCL3L1* copy number				Comment
				HIV-1 acquisition	Viral load	Decline of CD4	AIDS progression	
Gonzalez et al. (2005)		A total of 4,308 subjects including HIV-1(+) and HIV-1(−)	Diverse ethnic populations including African-American, European-American, Hispanic-American, etc.	Yes	Yes	Yes	Yes	First study with the largest cohort
Shao et al. (2007)	Adolescents (mostly female) in the early chronic phase	227 HIV-1(+) and 184 HIV-1(−) (population control)	African-American and others	No	No	No	No data	REACH Study of the Adolescent Medicine and HIV/AIDS Research Network
Nakajima et al. (2007)	Long term survivors with hemophilia	95 HIV-1(+) including 48 non-progressors and 47 slow progressors and 205 population controls	Japanese	Yes	No	No	No data	Study for long-term prognosis of chronic HIV-1 infection
Kuhn et al. (2007)		79 pairs of mother and infant with perinatal HIV-1 transmission and 235 non-transmitting pairs	African from Johannesburg, South African	No data	Yes in mother	Yes in mother	No data	Study for perinatal HIV-1 transmission. Infants with a higher copy number have a reduced risk of HIV-1 transmission from infected mothers.

and Indian population. The average copy number of *CCL3L1* was 5.00 ± 0.22 in Japanese, while it was 2.34 ± 0.17 in Indians. Chimpanzees have much larger copy numbers of *CCL3L1*. Similar findings have been reported by others (Gonzalez et al., 2005; Shao et al., 2007).

Given that individuals harboring lower copy numbers of *CCL3L1* among populations have an increased risk of HIV-1 infection and also predispose to rapid progression of AIDS, the intriguing hypothesis that *CCL3L1* copy number might contribute to the ethnic differences in HIV-1/AIDS susceptibility has been put forward. Based on the average copy number of *CCL3L1*, African populations appear more resistant to HIV-1 infection. Moreover, it is widely accepted that chimpanzees are naturally resistant to the development of AIDS from HIV infection (de Groot et al., 2002; Bontrop and Watkins, 2005), and their higher copy number of *CCL3L1* might be linked to this. Further investigation is needed to clarify the impacts of *CCL3L1* CNV on primate and human evolution.

CNV in *CCL3L1* and susceptibility to HIV-1/AIDS

There have been several studies of varying size performed to evaluate the association between the *CCL3L1* copy number and HIV-1/AIDS susceptibility (Gonzalez et al., 2005; Kuhn et al., 2007; Nakajima et al., 2007; Shao et al., 2007). The main features and outcomes of these studies are summarized in Table 1.

Gonzalez et al. (2005) first evaluated more than 4,000 HIV-1 infected subjects to determine the association between the *CCL3L1* copy number and HIV-1/AIDS susceptibility. They compared the distribution of the *CCL3L1* copy number in HIV-1-infected and non-infected subjects from diverse ethnic backgrounds and reported that possession of a lower *CCL3L1* copy number than the population average is associated with an enhanced risk for acquiring HIV-1. Moreover, a lower copy number was associated with an increasing risk of progression to AIDS and death, as well as the viral set point and a decline of CD4+ T-cells.

In a previous study (Nakajima et al., 2007), we investigated the impact of the *CCL3L1* dose on the long-term prognosis for chronic HIV-1 infection. We examined 95 HIV-1 infected long-term survivors with hemophilia, including 48 non-progressors and 47 subjects receiving anti-retroviral treatment. The average copy number of *CCL3L1* in the 95 HIV-1-infected subjects was significantly lower than in 205 controls. Moreover, subjects possessing two or fewer copies of *CCL3L1* had a significantly higher risk of acquiring HIV-1. However, the *CCL3L1* copy number had no significant effect on disease progression among the long-term survivors who had been afflicted with chronic HIV-1 infection for more than 15 years. Furthermore, CNV in *CCL3L1* had little effect on the level of HIV-1 load. We thus concluded that variation in the *CCL3L1* copy number is apparently not a factor that determines the prognosis of chronic HIV-1 infection, even though it is linked to HIV-1 susceptibility.

Shao et al. (2007) investigated the *CCL3L1* copy number in an HIV-1 infected adolescent population in the early chronic phase without AIDS. However, they found no difference in the distribution of the *CCL3L1* copy number be-

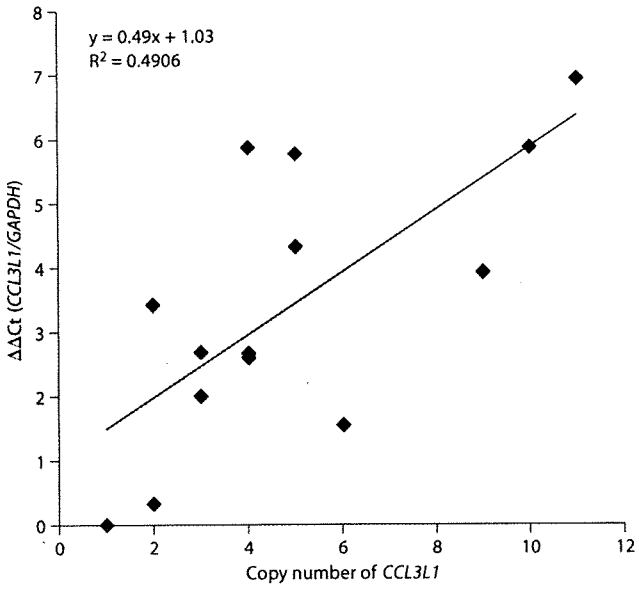

Fig. 2. The association between the *CCL3L1* copy number and the expression level of *CCL3L1* mRNA in B lymphoblastoid cell lines. The expression levels of *CCL3L1* are shown as ΔΔCt values calculated from quantitative real-time PCR evaluation of *CCL3L1* and *GAPDH* transcripts.

tween HIV-1 infected and control subjects. Furthermore, *CCL3L1* failed to exert a major impact on early outcome after HIV-1 infection based on the analyses of HIV-1 viral load and CD4+ T-cell count. In our unpublished findings, we could not replicate the association of the *CCL3L1* copy number with acquiring HIV-1 in the Indian population either (2.34 ± 0.17 in control vs. 2.13 ± 0.15 in HIV-1-infected subjects; not statistically significant). The Indian subjects had a relative low average *CCL3L1* copy number, which might be a possible explanation for the less effect of *CCL3L1* CNV. These results suggested that further analyses are required to clarify the association of the *CCL3L1* copy number with HIV-1/AIDS susceptibility.

Kuhn et al. (2007) investigated whether the maternal or infant *CCL3L1* copy number is associated with perinatal HIV-1 transmission and compared the *CCL3L1* copy number between HIV-1 transmitting and non-transmitting mother-infant pairs. Infant, but not maternal, *CCL3L1* copy number was found to be a determinant of enhanced perinatal HIV-1 transmission. Infants with a higher copy number had a reduced risk of HIV-1 transmission from infected mothers.

CCL3L1 copy number is linked to the expression of CCL3L1 and CCR5

It has been reported that an increased *CCL3L1* copy number is positively associated with *CCL3L1* mRNA expression and CCL3L1 secretion. Townson et al. (2002) have reported that monocytes from individuals with a high *CCL3L1* copy number exhibited an increased ratio of *CCL3L1* versus *CCL3* mRNA and also an increased functional chemokine production after the stimulation of LPS. Gonzales et al. (2005) and Pilotti et al. (2007) reported similar findings. We also evaluated the association between the *CCL3L1* dose and the expression levels of *CCL3L1* mRNA in B lymphoblastoid cell lines. The amounts of *CCL3L1* mRNA were standardized by *GAPDH* mRNA among 14 different cell lines. As shown in Fig. 2, the *CCL3L1* gene dose was linked to the expression level of the *CCL3L1* transcripts.

In addition, the *CCL3L1* copy number is tightly linked to the expression of the HIV-1 co-receptor CCR5 on the cell surface. Gonzalez et al. (2005) showed that an increasing *CCL3L1* copy number was inversely associated with the proportion of CD4+ T-cells that express CCR5. The interaction between CCL3L1 and CCR5 might induce the down-modulation of CCR5. The internalization of chemokine receptors in response to ligand binding is a well-established feature of these receptors (Arenzana-Seisdedos and Parmentier, 2006). The down-modulation of CCR5 in turn could enhance the protective effects of CCL3L1 against HIV-1 infection.

HIV-1 entry-independent effect by the interaction between CCL3L1 and CCR5 and the susceptibility to HIV-1/AIDS

Since CCL3L1 is a natural ligand of CCR5, it is evident that CCL3L1 efficiently inhibits infection by receptor blockade and down-modulation. As described above, the HIV-1 entry-dependent effect through the interaction between CCL3L1 and CCR5 has been widely accepted as a key mechanism to inhibit HIV-1 infection. However, Dolan et al. (2007) suggested that viral entry-independent mechanisms and their effects on cell-mediated immunity were the major protective mechanisms acting through the CCL3L1 and CCR5 axis. They 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* as well as the *CCR5* genotype. The high risk group possesses both a population-specific low *CCL3L1* copy number ($CCL3L1^{low}$) and detrimental *CCR5* variations ($CCR5^{del}$). The low risk group has a population-specific high *CCL3L1* copy number ($CCL3L1^{high}$) and non-detrimental *CCR5* variations ($CCR5^{nondel}$). The moderate risk group harbors either one or the other of the two risk factors, $CCL3L1^{low}$ or $CCR5^{del}$. The cohort was also categorized based on the levels of viral load to investigate whether, among subjects with a similar viral load, the rate and extent of CD4+ T-cell loss and disease progression differed according to the *CCL3L1-CCR5* genetic risk status. In the study, 'cumulative CD4+ T-cell number (cCD4)' was applied to adapt epidemiological parameters. They found that the *CCL3L1-CCR5* genetic risk status and viral load were independent predictors of cCD4 as well as the rate of AIDS progression, and they concluded that the *CCL3L1-CCR5* genetic risk status influenced cell-mediated immunity in in-

dividuals, which was in part dependent on viral entry-independent mechanisms. Shalekoff et al. (2008) reported that individuals with higher *CCL3L1* copy numbers have greater CD4+ and CD8+ T-cell responses to the HIV-1 gag protein. These results also supported that viral entry-independent mechanisms were one of the major protective mechanisms of CCL3L1.

It is widely accepted that variations in gene copy number are tightly linked to phenotypic variation. Gonzalez et al. (2005), who first reported the association of *CCL3L1* CNV with HIV-1/AIDS susceptibility, provided a great breakthrough which enabled studies of the association between CNVs and phenotypic variations. They highlighted how CNVs in the human genome can function as a susceptibility allele that is involved in a complex human phenotype. It is to be hoped that further progress will provide great insights into understanding the contribution of CNVs to human phenotypic diversity and disease susceptibility.

Acknowledgements

We are grateful for sample collection in Japan to Drs. Jun-ich Mimaya (Shizuoka Children's Hospital), Hiroshi Terunuma (Biotherapy Institute of Japan), H. Hanabusa (Ogikubo Hospital), J. Matsuda (Teikyo University School of Medicine), M. Sakai (University of Occupational and Environmental Health), S. Ikeda (Sasebo Municipal Hospital) and T. Fujii (Hiroshima University School of Medicine), and for sample collection in India to Dr. SK Sharma (All India Institute of Medical Sciences). We would like to thank Dr. Taeko Naruse (Tokyo Medical and Dental University) for kindly providing human B lymphoblastoid cell lines and Gaurav Sharma for sample preparation.

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Retrovirology

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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-

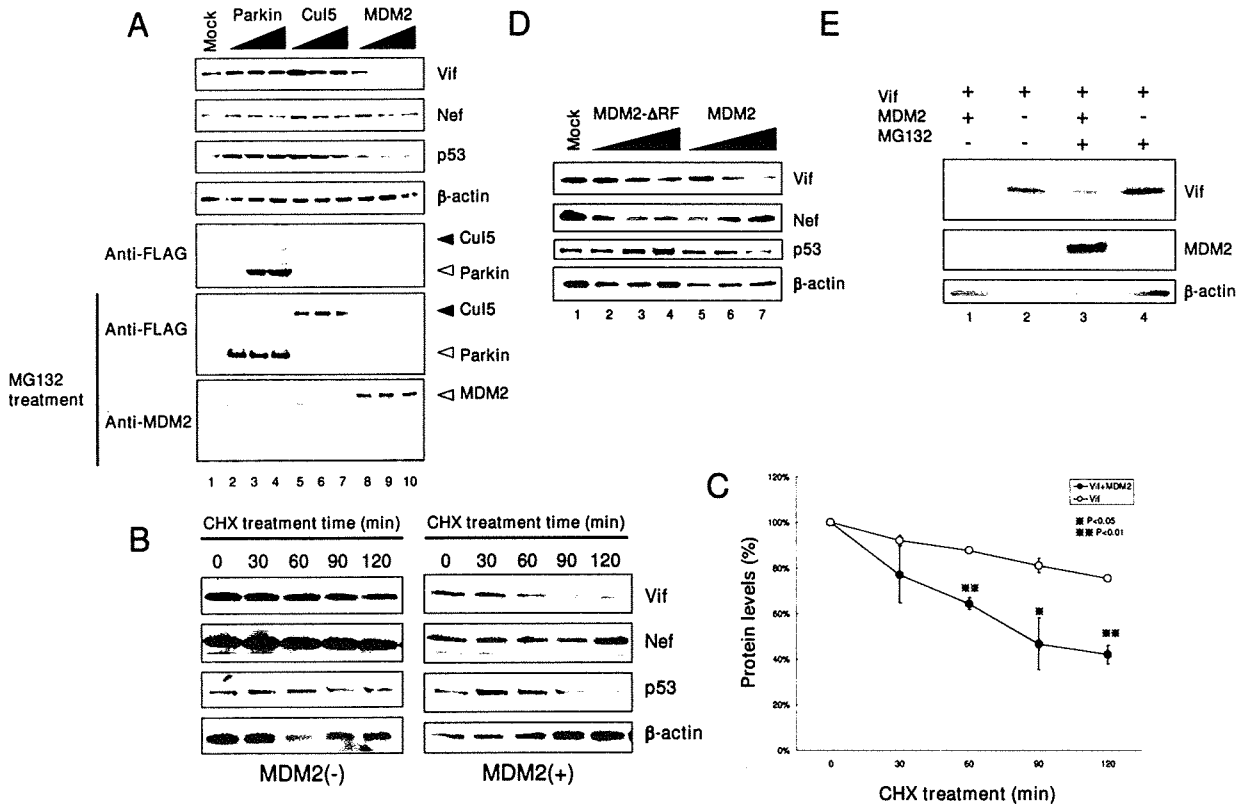

Figure 1

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

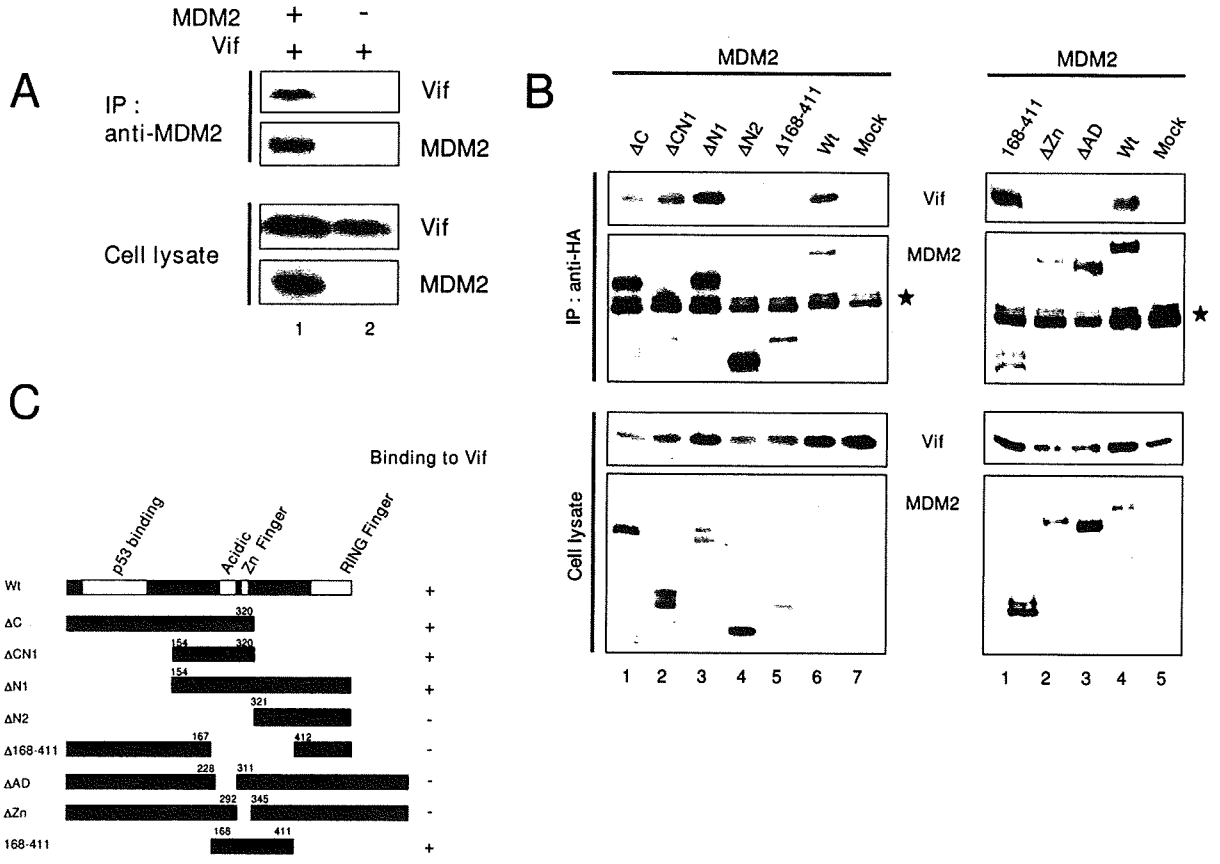

Figure 2
MDM2 bound Vif in its central domain. (A) Immunoprecipitation assays revealed the interaction of MDM2 with Vif *in vivo*. HEK293T cells were cotransfected with expression vectors for MDM2 and Vif and treated with MG132 for 6 hrs prior to harvest. Cell lysates were immunoprecipitated with anti-MDM2 mAb followed by immunoblotting with the indicated Abs (upper two panels). Cell lysates were also subjected to immunoblotting with the indicated Abs (lower two panels). (B) The interaction domain of MDM2 with Vif. HEK293T cells were cotransfected with expression vectors for HA-tagged MDM2 wild type (Wt) and mutants together with pNL-A1, and cell lysates were immunoprecipitated with anti-HA mAb followed by immunoblotting with the indicated Abs. Asterisk indicates immunoglobulin heavy chains from thenimmunoprecipitation. (C) Schematics of MDM2 mutants binding to Vif are shown.

determine a Vif-binding domain, we further tested mutants deleted in a Zn Finger domain (ΔZn) or in an acidic domain (ΔAD). Neither mutant could bind Vif, whereas the mutant containing amino acids 168–411 was able to bind Vif, suggesting that both domains are necessary and that the central domain is sufficient for Vif binding (Fig. 2B, right panel &2C). Additionally, using a series of Vif deletion mutants, we also found that the N-terminal region of Vif (amino acids 4–22) is needed for MDM2 binding (Fig. 3A &3C). Furthermore, we examined the MDM2-mediated downregulation of Vif mutants. MDM2 was able to efficiently downregulate cellular levels of the MDM2-binding Vif mutants but not that of an MDM2-non binding mutant, Δ4–45 (Fig. 3B). Collectively, these results indicated that the Vif-MDM2 interaction is required for MDM2-mediated downregulation of Vif (Fig. 3C).

MDM2 induces ubiqutination of Vif

Since we found that MDM2 bound Vif and promoted its degradation via a proteasomal pathway, we next examined whether MDM2 is involved in the polyubiquitination of Vif. *In vitro* ubiquitination assays revealed that bacterially expressed GST-MDM2 was able to induce the

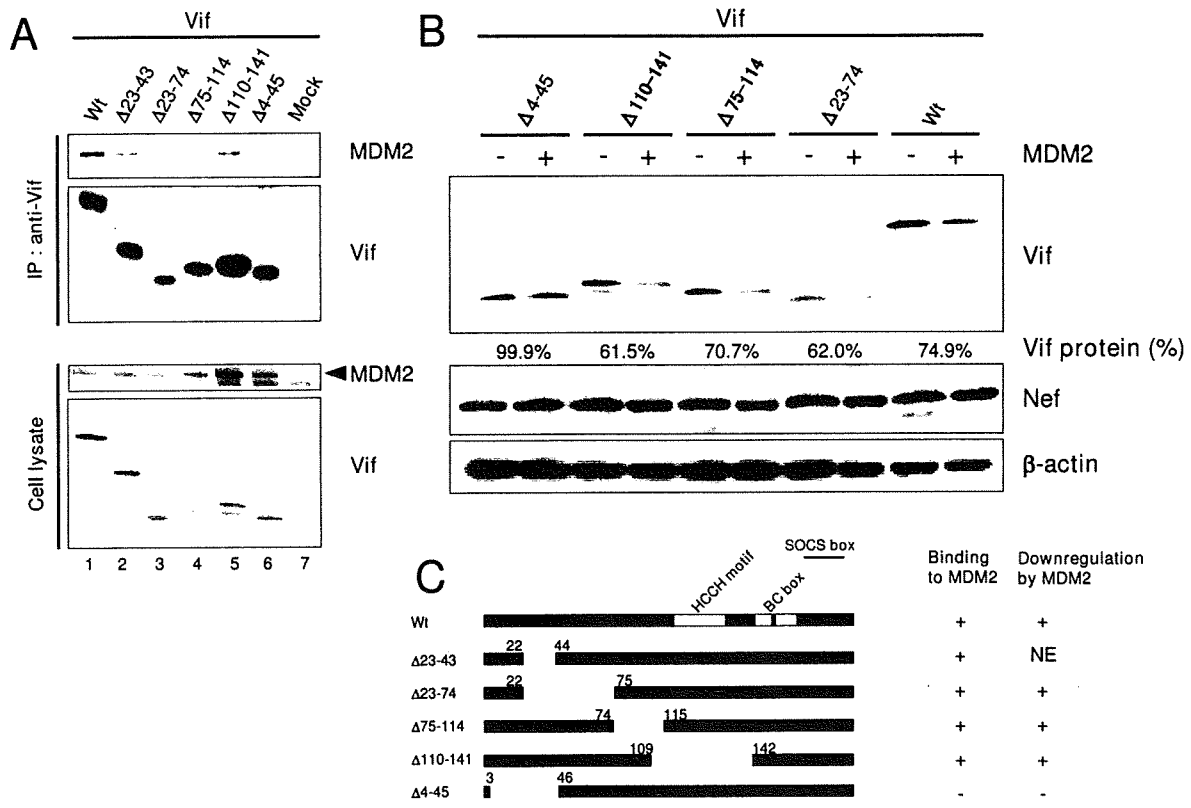

Figure 3
MDM2 specifically bound and downregulated Vif. (A) The interaction domain of Vif with MDM2. HEK293T cells were cotransfected with expression vectors for Vif and mutants together with pCMV/HA-MDM2, and cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblotting with the indicated Abs. Arrowhead indicates MDM2. (B) The downregulation of Vif protein by MDM2. HEK293T cells were cotransfected with expression vectors for Vif and mutants with or without pCMV/HA-MDM2, and cell lysates were subjected to immunoblotting with the indicated Abs. The amounts of Vif were quantified by densitometry and shown as the protein ratio relative to that without expression of MDM2. (C) Schematics of Vif mutants bound by and downregulated by MDM2. NE: not examined.

polyubiquitination of purified GST-Vif protein *in vitro* (Fig. 4A). The ubiquitination of Vif by MDM2 was specific, as the omission of ubiqutin, E1, E2, or MDM2 prevented Vif-ubiquitination as shown in our previous experiments [13]. We also performed *in vitro* ubiquitination assays using immunopurified MDM2 and Cul5. Immunopurified MDM2 was able to induce ubiquitination of Vif *in vitro* to the same extent as Cul5 (Additional file 2, part A), while it could not ubiquitinate the N-terminal Vif deletion mutant Δ22 that was defective for binding MDM2 (Additional file 2, part B). These findings suggest that the interaction with MDM2 is important for Vif ubiquitination. We performed *in vivo* ubiquitination assays to further investigate the importance of MDM2 in Vif ubiquitination. Lysates of cells co-expressing Vif, either with an MDM2 wild type (Wt) or a ΔRF mutant, and His-tagged Ubiquitin (His-Ub) were analyzed for the presence of ubiquitinated Vif conjugates (Fig. 4B). Unfortunately, we detected a Vif band that non-specifically bound to Ni-NTA agarose (arrowhead) due to its nature as a sticky protein. Overexpression of MDM2 induced a ladder detected by anti-Vif Ab, even in the absence of His-Ub (lane 2), suggesting that this ladder represented Vif protein polyubiquitinated with endogenous Ub (arrows with asterisk). Furthermore, in the presence of His-Ub, we detected a doublet of ladder which presumably represented Vif protein polyubiquitinated with endogenous and His-tagged Ub (arrows with asterisk and arrows, respectively). We also obtained similar results using a UbiQapture™-Q Kit (data not shown). We thus concluded that the overexpres-

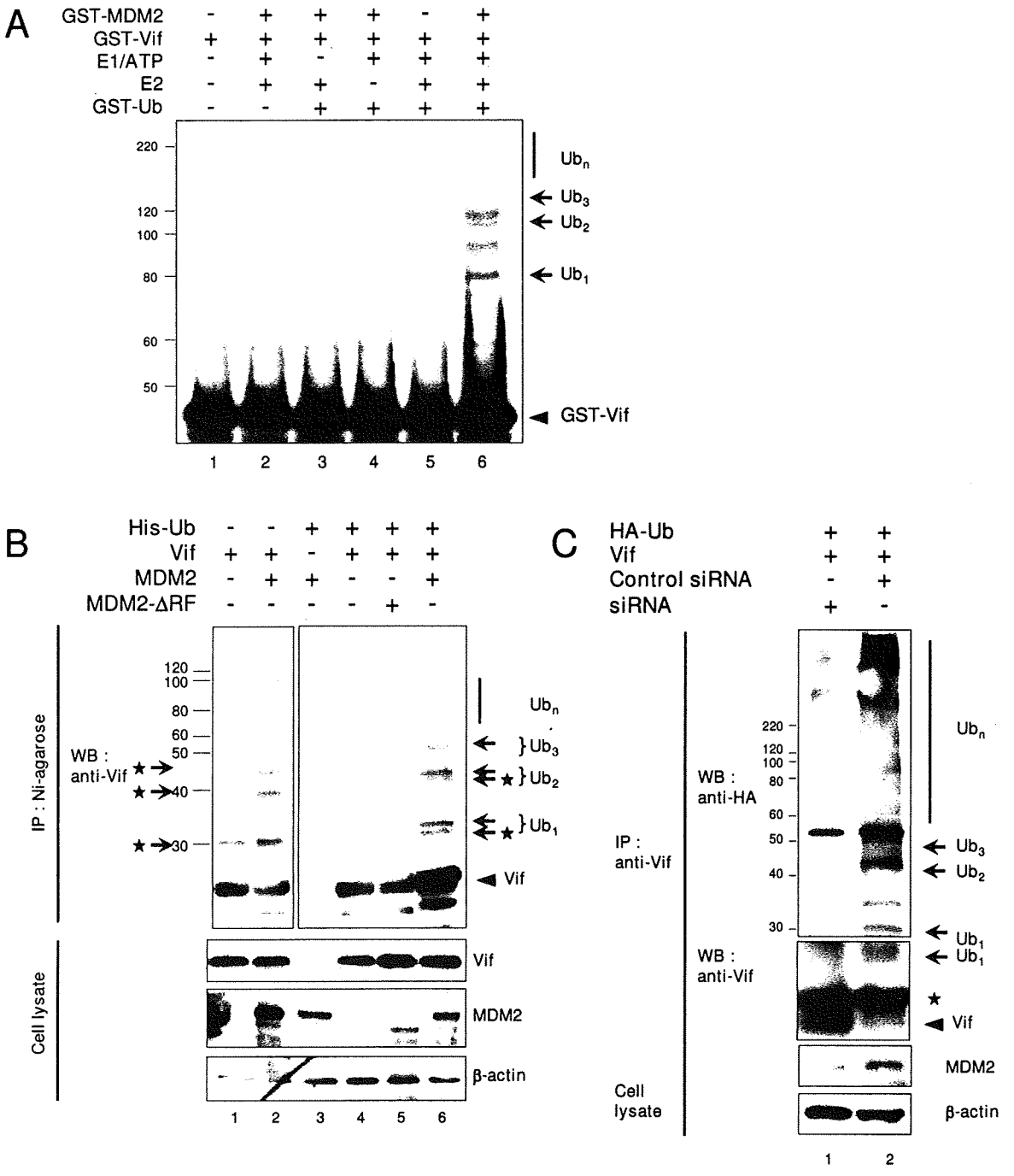

Figure 4 *(see legend on next page)*

Figure 4 *(see previous page)*
MDM2 induced the polyubiquitination of Vif *in vitro* and *in vivo*. (A) GST-MDM2 induced the polyubiquitination of Vif *in vitro*. Bacterially expressed GST-Vif was subjected to *in vitro* ubiquitination assays. The reaction was performed in the presence or absence of E1, E2, GST-MDM2, and GST-Ubiquitin as indicated. Reactions were subjected to immunoblotting with anti-Vif mAb. Arrows indicate GST-ubiquitin-conjugated Vif. (B) Overexpressed MDM2 induced the polyubiquitination of Vif *in vivo*. HEK293T cells were cotransfected with expression vectors for MDM2 Wt and a ΔRF mutant together with expression vectors for Vif and His-Ubiquitin (His-Ub) as indicated. Cells were treated with MG132 for 6 hrs, and cell lysates were precipitated with Ni-NTA agarose beads followed by immunoblotting with the indicated Abs. Since Vif naturally bound to Ni-NTA agarose, we detected a Vif band itself (arrowhead), whereas no signal was detected in cells lacking Vif (lane 3). Arrows indicate His-Ub-conjugated Vif. Arrows with asterisk indicate Vif conjugated with endogenous ubiquitin. (C) Transduction of siRNA reduced cellular levels of endogenous MDM2 and polyubiquitination of Vif. HEK293T cells were cotransfected with expression vectors for MDM2 siRNA and control siRNA together with expression vectors for Vif and HA-Ubiquitin (HA-Ub). Cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblotting with the indicated Abs. Asterisk indicates immunoglobulin light chains from the immunoprecipitation.

sion of exogenous MDM2 efficiently induced polyubiquitination of Vif *in vivo*. Furthermore, the knock-down of endogenous MDM2 expression by introduction of MDM2-specific short interfering RNA (siRNA) resulted in a significant reduction in the amount of polyubiquitinated Vif, commensurate with the extent of reduced MDM2 expression (Fig. 4C). Collectively, these data indicated that MDM2 mediates polyubiquitination of Vif both *in vitro* and *in vivo*.

MDM2 negatively regulates HIV-1 replication in non-permissive cells through ubiqutination and degradation of Vif

Next, we examined the effect of MDM2 on HIV-1 replication. In a single round infection assay (Fig. 5A), in the absence of A3G, viral replication was not affected by expression of MDM2 and/or Vif (lanes 1–6). In contrast, in the presence of A3G in a non-permissive cell setting, without the expression of MDM2, the wild type virus could replicate but the ΔVif virus could not, as previously reported (lanes 7 & 8) [3,8]. Co-expression of MDM2 reduced the cellular level of Vif (Fig. 5B, upper panel, lanes 5 & 11), resulting in the increased virion incorporation of A3G (Fig. 5B, 2nd lower panel, lane 11 as compared with lanes 7) and the greater suppression of viral replication (Fig. 5A, lane 11 as compared with lane 7).

We also tested the effect of MDM2 on HIV-1 replication in the presence of A3F. MDM2 suppressed viral replication in the presence of A3F, similar to results shown for A3G (Additional file 3). These data indicated that the MDM2-mediated Vif downregulation led to upregulated cellular A3G and A3F levels in producer cells, resulting in less infectious HIV-1 virions produced. Since MDM2 was previously reported to upregulate HIV-1 transcription by ubiquitination of Tat, we further examined HIV-1 replication in macrophages knocked down for MDM2 (Fig. 5C). We chose terminally differentiated macrophages as the target, because the knockdown of MDM2 is lethal for proliferating cells. HIV-1 replicated more efficiently in macrophages transfected with MDM2 siRNA than in control siRNA-transfected macrophages. These data indicated that MDM2 negatively regulated HIV-1 replication in non-permissive target cells through the ubiquitination and degradation of Vif.

To obtain further insights into the mechanisms why our MDM2 system did not induce the ubiquitination of A3G which was bound to Vif, we tested the expression levels and the binding affinity of A3G to Vif in transfected cells. Co-expression of MDM2 reduced the cellular levels of Vif and inversely increased the A3G levels in a dose dependent manner (Fig. 5D). Immunoprecipitation assays revealed that the co-expression of MDM2 blocked the binding of A3G to Vif in a dose dependent manner (Fig. 5E). These data suggest that the interaction between MDM2 and Vif precludes A3G from binding to Vif.

Discussion

In this study, we report that MDM2 is a novel E3 ligase for HIV-1 Vif. MDM2 physically interacts with Vif and functions as an E3 ligase for Vif to induce its polyubiquitination and proteasomal degradation. Several E3 ligases including Cul5 [17], Nedd4, and AIP4 [18], have been reported to induce Vif ubiquitination, and the roles of Cul5 for Vif ubiquitination and degradation are especially well documented. Dang et al. have recently reported that Cul5 induces A3G degradation not by direct ubiquination of A3G but indirectly through Vif ubiqutination and that polyubiquitinated Vif might serve as a vehicle to transport A3G into proteasomes for degradation [23]. In this manuscript, we show that MDM2 only targets Vif for degradation but not A3G, although MDM2 and Cul5 both induce Vif ubiquitination (Additional file 2, part A). MDM2 reduced cellular Vif levels and inversely increased A3G levels (Fig. 5B &5D), unlike Cul5. One possible explanation is that the binding of MDM2 to Vif precluded A3G from binding Vif (Fig. 5E), whereas a Cul5-Vif complex

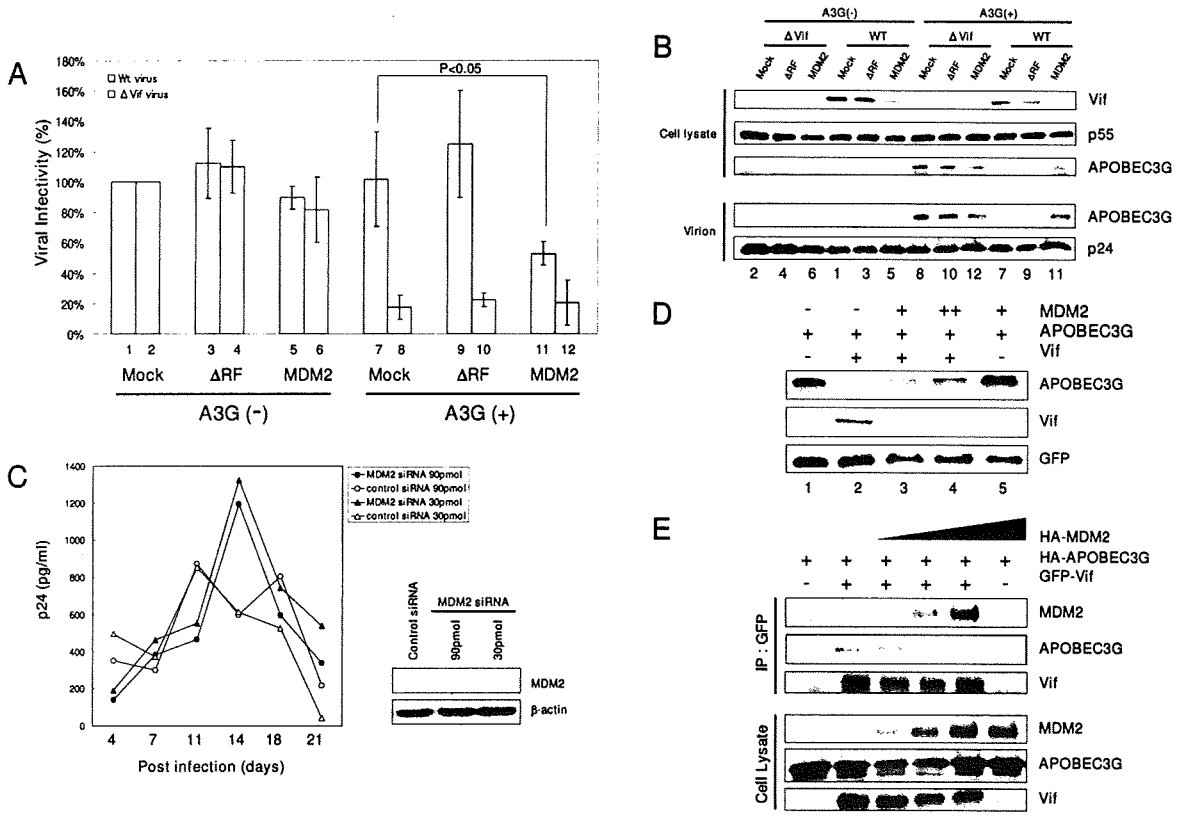

Figure 5

MDM2 negatively regulated HIV-1 replication in non-permissive cells through the degradation of Vif. (A) The overexpression of MDM2 inhibited HIV-1 replication in the presence of A3G. NL-43 Wt and ΔVif viruses were produced from HEK293T cells transfected with expression vectors for MDM2 Wt and a ΔRF mutant in the presence or absence of A3G. The viral infectivity was examined using M8166 cells. Values are presented as averages of more than 3 independent experiments. (B) MDM2 reduced cellular levels of Vif, resulting in more incorporation of A3G into HIV-1 virions. Immunoblotting for cell lysates (upper 3 panels) and precipitated virions (lower 2 panels) was performed with the indicated Abs. Lane numbers correspond to those in Fig. 4A. (C) HIV-1 replication in macrophages transfected with MDM2- and control-siRNA. MDM were transfected with MDM2- and control-siRNA and challenged with R5 HIV-1_{JR-FL} (left panel). Cell lysates were subjected to immunoblotting with the indicated antibodies (right panels). (D) Coexpression of MDM2 reduced cellular levels of Vif and inversely increased A3G levels in a dose dependent manner. HEK293T cells were cotransfected with expression vectors for A3G, Vif, GFP, and MDM2 as indicated. Cell lysates were subjected to immunoblotting with the indicated Abs. (E) Immunoprecipitation assays revealed that the coexpression of MDM2 blocked the binding of A3G to Vif in a dose dependent manner. HEK293T cells were cotransfected with expression vectors for A3G, GFP-Vif, and MDM2 as indicated. Cell lysates were immunoprecipitated with anti-GFP mAb followed by immunoblotting with the indicated Abs.

can bind A3G to form a ternary complex. MDM2 binds the N-terminal region of Vif which does not overlap with, but is close to the A3G/A3F binding domain [25]. This binding might affect the interaction of Vif with A3G and/or A3F. Furthermore, the evidence that an MDM2 ΔRF mutant failed to protect A3G indicated that the ubiquitination and degradation of Vif is necessary to protect A3G and A3F from Vif. These findings suggest that different E3 ligases might play different roles in Vif ubiquitination. Further studies on the different roles of Vif ubiquitination by different E3 ligases and their virological significance should be investigated.

We demonstrate that MDM2 negatively regulated HIV-1 replication through Vif degradation. Through the degradation of target proteins (p53, pRB, etc), MDM2 can exert profound physiological effects on the regulation of cell cycle, cell proliferation, DNA repairs and other processes. To our knowledge, this is the first report to show that MDM2 plays an important role in viral replication