研究成果の刊行に関する一覧表

平成18年度

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Ⅲ.　研究成果の刊行物・別刷

Vaccine-based, long-term, stable control of simian/human immunodeficiency virus 89.6PD replication in rhesus macaques

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The X4-tropic simian/human immunodeficiency virus (SHIV) 89.6P (or 89.6PD) causes rapid CD4$^+$ T-cell depletion leading to an acute crash of the host immune system, whereas pathogenic R5-tropic simian immunodeficiency virus (SIV) infection, like HIV-1 infection in humans, results in chronic disease progression in macaques. Recent pre-clinical vaccine trials inducing cytotoxic T lymphocyte (CTL) responses have succeeded in controlling replication of the former but shown difficulty in control of the latter. Analysis of the immune responses involved in consistent control of SHIV would contribute to elucidation of the mechanism for consistent control of SIV replication. This study followed up rhesus macaques that showed vaccine-based control of primary SHIV89.6PD replication and found that all of these controllers maintained viraemia control for more than 2 years. SHIV89.6PD control was observed in vaccinees of diverse major histocompatibility complex (MHC) haplotypes and was maintained without rapid selection of CTL escape mutations, a sign of particular CTL pressure. Despite the vaccine regimen not targeting Env, all of the SHIV controllers showed efficient elicitation of *de novo* neutralizing antibodies by 6 weeks post-challenge. These results contrast with our previous observation of particular MHC-associated control of SIV replication without involvement of neutralizing antibodies and suggest that vaccine-based control of SHIV89.6PD replication can be stably maintained in the presence of multiple functional immune effectors.

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INTRODUCTION

The well-established importance of cytotoxic T lymphocyte (CTL) responses in the control of immunodeficiency virus replication has led the way to development of prophylactic AIDS vaccine regimens that augment virus-specific CTL responses (Borrow *et al.*, 1994; Koup *et al.*, 1994; Matano *et al.*, 1998; Ogg *et al.*, 1998; Jin *et al.*, 1999; Schmitz *et al.*, 1999; McMichael & Hanke, 2003; Goulder & Watkins, 2004). In a model of X4-tropic simian/human immunodeficiency virus (SHIV) 89.6P or 89.6PD infection (Reimann *et al.*, 1996; Lu *et al.*, 1998), which causes rapid CD4$^+$ T-cell depletion leading to an acute crash of the host immune system in macaques, several pre-clinical trials of prophylactic AIDS vaccines have successfully shown that efficient CTL induction results in control of virus replication and prevention of acute AIDS progression (Barouch *et al.*, 2000; Amara *et al.*, 2001; Matano *et al.*, 2001; Rose *et al.*, 2001; Shiver *et al.*, 2002; Willey *et al.*, 2003). In contrast, most trials of such CTL-based vaccines have failed to show viraemia control in models of R5-tropic simian immunodeficiency virus (SIV) infection, which result in chronic

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disease progression in macaques as in human immunodeficiency virus type 1 (HIV-1) infection in humans (Feinberg & Moore, 2002; Horton *et al.*, 2002; Casimiro *et al.*, 2005). Comparison of vaccine effects on virus replication in the acute AIDS model of X4-tropic SHIV infection with those in the chronic model of R5-tropic SIV infection could contribute to the development of an effective prophylactic AIDS vaccine for control of persistent HIV-1 replication.

We have developed a prophylactic AIDS vaccine using a DNA-prime/Gag-expressing Sendai virus (SeV-Gag) vector boost system and have shown its potential for efficient induction of Gag-specific CTL responses in Burmese rhesus macaques (Kano *et al.*, 2002; Matano *et al.*, 2004). In preclinical trials in an acute AIDS model, all of the macaques vaccinated with the DNA-prime/SeV-Gag vector boost system controlled SHIV89.6PD replication after challenge (Matano *et al.*, 2001; Takeda *et al.*, 2003). Furthermore, a trial of the prophylactic DNA-prime/SeV-Gag boost vaccine showed control of SIVmac239 replication leading to undetectable set-point plasma viraemia in five out of eight vaccinees (referred to as SIV controllers), despite failure of virus control in the other three vaccinees (referred to as SIV non-controllers) (Matano *et al.*, 2004). All of the SIV controllers showed rapid selection of viral CTL escape mutations, and analysis of the rhesus major histocompatibility complex (MHC) suggested that SIV control was associated with particular MHC haplotypes such as *90-120-Ia* and 'elite' CTL responses specific for the MHC-restricted epitopes (Matano *et al.*, 2004). Follow up of these SIV controllers revealed that some lost this control with accumulation of multiple viral CTL escape mutations (Kawada *et al.*, 2006).

In this study, we followed up, for more than 2 years, rhesus macaques that showed vaccine-based control of SHIV89.6PD replication (referred to as SHIV controllers). Our results showed durable and stable virus control in the SHIV controllers, contrasting with our previous observation in SIV controllers.

METHODS

Animal experiments. Ten vaccinated macaques used in our previous SHIV89.6PD challenge experiments (Matano *et al.*, 2001; Takeda *et al.*, 2003) were analysed in this study. The animal list is shown in Table 1. All were Burmese rhesus macaques (*Macaca mulatta*) and were maintained in accordance with the Guidelines for Laboratory Animals of the National Institute of Infectious Diseases and National Institute of Biomedical Innovation.

The immunization and challenge protocols have been described previously (Matano *et al.*, 2001; Takeda *et al.*, 2003). Of the ten macaques in the SHIV89.6PD challenge experiment, three (R00-013, R00-015 and R00-017) received a single intranasal immunization with replication-competent SeV expressing SIVmac239 Gag (SeV-Gag) (Kato *et al.*, 1996; Kano *et al.*, 2002) before challenge. Two (R99-007 and R99-011) received four immunizations with FMSIV DNA followed by a single SeV-Gag booster. The FMSIV plasmid DNA used in this DNA vaccination protocol (DNAv1) was constructed from an $SHIV_{MD14YE}$ molecular clone DNA (Shibata *et al.*, 1997a) by replacing SHIV *env* with ecotropic Friend murine leukemia virus (FMLV) *env*

Table 1. Summary of the vaccinees challenged with SHIV89.6PD

Animal	MHC I haplotype*	Vaccine protocol	Set-point virus load†	Virus load around year 2‡	Gag mutations around month 2§	Gag mutations after month 6‖
R00-013	ND	SeV-Gag	10^4–10^6	10^4–10^6 (at wk 52)	ND	ND
R00-015	*90-120-Ib*	SeV-Gag	<400	<400	None	None at wk 60
R00-017	*90-030-Ih*	SeV-Gag	<400	<400	None	None at wk 58
R99-007	ND	DNAv1/SeV-Gag	<400	<400 (at wk 28)	None	ND
R99-011	*90-010-Ie*	DNAv1/SeV-Gag	<400	<400	None	None at wk 51
R99-005	*90-010-Ie*	DNAv2/SeV-Gag	<400	<400	None	None at wk 49
R99-012	*90-030-Ih*	DNAv2/SeV-Gag	<400	<400	None	None at wk 51
R00-020	*90-122-Ie*	DNAv3/F^-SeV-Gag	<400	<400	None	None at wk 52
R00-023	ND	DNAv3/F^-SeV-Gag	<400	<400	None	None at wk 52
R00-024	*90-120-Ib*	DNAv3/F^-SeV-Gag	<400	<400	None	None at wk 52

*MHC I haplotype was determined by reference strand-mediated conformation analysis, as described previously (Arguello *et al.*, 1998; Matano *et al.*, 2004). MHC I haplotype *90-120-Ib* is derived from breeder R90-120, *90-010-Ie* from R90-010, *90-122-Ie* from R90-122 and *90-030-Ih* from R90-030. MHC I haplotypes *90-010-Ie* and *90-122-Ie* are identical.

†Plasma viral loads [RNA copies (ml plasma)$^{-1}$] around week 20.

‡Macaque R00-013 developed AIDS and was euthanized at week 53. Macaque R99-007 was euthanized at week 29 because of the limitation of available cage numbers.

§A *gag* gene fragment was amplified from plasma RNA at week 5 or from PBMC-derived DNA at week 7 or 8 and subjected to sequencing to determine predominant mutations leading to Gag amino acid changes. The results are shown in Table 2.

‖A *gag* gene fragment was amplified from PBMC-derived DNA and subjected to sequencing to determine predominant mutations leading to Gag amino acid changes.

ND, Not determined.

(Matano *et al.*, 2000). Two macaques (R99-005 and R99-012) received four immunizations with both the FMSIV DNA and an FMLV receptor (mCAT1)-expression plasmid DNA (Albritton *et al.*, 1989) followed by a single SeV-Gag booster. This second DNA vaccination protocol (DNAv2) has been shown to elicit efficient CTL responses by confined mCAT1-dependent FMSIV replication (Matano *et al.*, 2000). Three macaques (R00-020, R00-023 and R00-024) received a single immunization with CMV-SHIVdEN DNA (DNAv3) followed by a single boost with an F-deleted replication-defective SeV-Gag (F^-SeV-Gag) (Li *et al.*, 2000; Takeda *et al.*, 2003). This CMV-SHIVdEN plasmid DNA was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA and had the genes encoding SIVmac239 Gag, Pol, Vif and Vpx, SIVmac239/HIV-1_{DH12} chimeric Vpr and HIV-1_{DH12} Tat and Rev (Matano *et al.*, 2004). All ten animals were challenged intravenously with 10 $TCID_{50}$ SHIV89.6PD (Lu *et al.*, 1998) approximately 3 months after the last immunization. Four unvaccinated animals were also challenged with SHIV89.6PD and all failed to control virus replication.

Quantification of plasma viral loads. Plasma RNA was extracted using a High Pure Viral RNA kit (Roche Diagnostics). Serial fivefold dilutions of RNA samples were amplified in quadruplicate by nested RT-PCR using SIV *gag*-specific primers to determine the end point. Plasma SIV RNA levels were calculated according to the Reed–Muench method, as described previously (Matano *et al.*, 2004). The lower limit of detection was approximately 4×10^2 RNA copies ml^{-1}.

Sequencing of viral and proviral genomes. Plasma RNA was extracted as described above and genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using a DNeasy kit (Qiagen). A fragment corresponding to nt 458–2185 (containing the entire *gag* region) in the SHIV89.6P genome (GenBank accession no. U89134) was amplified from plasma RNA by nested RT-PCR. Alternatively, fragments corresponding to nt 458–2185, 2019–3187, 3038–4197, 4056–5213, 5079–6250, 6065–7225, 7047–8176 and 7998–9172 in the SHIV89.6P genome were amplified from proviral DNA by nested PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems). Alternatively, PCR products were subcloned into plasmids using a TOPO cloning system (Invitrogen) and sequenced.

Measurement of virus-specific T-cell levels by intracellular cytokine staining. We measured virus-specific T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (Matano *et al.*, 2001, 2004). In brief, PBMCs were co-cultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) infected with a vesicular stomatitis virus G (VSV-G)-pseudotyped SIVGP1 for SHIV-specific stimulation. The pseudotyped virus was obtained by co-transfection of COS-1 cells with a VSV-G-expression plasmid and the SIVGP1 DNA, an *env*- and *nef*-deleted SHIV molecular clone DNA, constructed by removing the whole FMLV *env* region from the FMSIV DNA. Alternatively, PBMCs were co-cultured with B-LCLs pulsed with peptide mixture (final concentration of each peptide, 0.5–2 μM) for peptide-specific stimulation. A panel of 117 overlapping peptides (15–17 aa in length and overlapping by 10–12 aa) spanning the entire SIVmac239 Gag sequence (Sigma-Aldrich) were divided into ten pools (1–10) each consisting of 11 or 12 peptides. Intracellular IFN-γ staining was performed using a Cytofix/Cytoperm kit (Becton Dickinson) according to the manufacturer's instructions. Fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3 and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting non-specific IFN-γ^+ T-cell frequencies from those after SHIV-specific or peptide-specific stimulation. Specific T-cell levels of < 100 cells per 10^6 PBMCs were considered negative.

Measurement of virus-specific neutralizing titres. We performed a neutralizing assay for the measurement of virus-specific neutralizing titres in plasma, as described previously (Shibata *et al.*, 1997b). Serial twofold dilutions of heat-inactivated plasma were prepared in duplicate and mixed with 10 $TCID_{50}$ SHIV89.6PD. In each mixture, 5 μl diluted plasma was incubated with 5 μl virus. After a 45 min incubation at room temperature, each 10 μl mixture was added to 5×10^4 MT4 cells in a well of a 96-well plate. After 12 days of culture, supernatants were harvested. Progeny virus production in the supernatants was examined by ELISA for detection of SIV p27 core antigen (Beckman Coulter) to determine the 100 % neutralizing end point. The lower limit of detection was a titre of 1 : 2.

RESULTS

MHC haplotypes of the SHIV controllers

In our previous SHIV89.6PD challenge experiment (Matano *et al.*, 2001; Takeda *et al.*, 2003), three animals received a single SeV-Gag vaccination alone, whilst the remaining seven animals were immunized with a DNA-prime/SeV-Gag boost vaccine before challenge (Table 1). The seven animals vaccinated with the prime–boost vaccine (R99-007, R99-011, R99-005, R99-012, R00-020, R00-023 and R00-024) were able to control virus replication, with undetectable set-point plasma viraemia. Two (R00-015 and R00-017) of the three animals vaccinated with SeV-Gag alone were also able to control viraemia, but the remaining one (R00-013) failed to control virus replication and showed acute $CD4^+$ T-cell depletion. This animal R00-013 developed AIDS and was euthanized at week 53.

In the present study, we determined the MHC class I (MHC I) haplotypes of the SHIV controllers and their viral genome sequences at around 1 or 2 months after challenge to examine whether SHIV controllers showed rapid selection of CTL escape mutations as observed in our previous analysis, in particular MHC-associated control of SIV replication. Importantly, control of SHIV89.6PD replication was observed in vaccinees with diverse MHC haplotypes (Table 1). Analysis of the proviral *gag* region in PBMCs at around week 8 showed a predominance of the wild-type sequence in all nine SHIV controllers (Table 2). Sequencing of the plasma viral *gag* region at week 5 in three of them confirmed the lack of dominant mutations (Table 2). Thus, the SHIV controllers controlled virus replication without rapid selection of CTL escape mutations.

Follow-up of the SHIV controllers

We followed up eight of the nine SHIV controllers except for one animal, R99-007, which was euthanized at week 29 because of a limitation on available cage numbers (Table 1). All eight SHIV controllers maintained control of virus replication for more than 2 years (Fig. 1). Viraemia was undetectable and peripheral $CD4^+$ T-cell counts were maintained during the observation period. Analysis of the *gag* region in PBMC-derived proviral DNA revealed that the wild-type sequence was still dominant around 1 year after challenge in all eight (Table 1). Additionally, we succeeded

Table 2. Mutations in SHIV *gag* at 1 or 2 months post-challenge

A *gag* gene fragment was amplified by nested PCR from PBMC-derived DNA at week 7 (in R00-017) or week 8 (in others) or by nested RT-PCR from plasma RNA at week 5. The viral *gag* fragment was amplified from plasma RNA in only three of the nine SHIV controllers (R00-017, R00-023 and R00-024); this was due to lower viral loads at week 5 in the remaining SHIV controllers.

Animal	Frequency*	Position of Gag changes (aa)†
Proviral DNA at week 7 or 8		
–R00-015	8/9	None
	1/9	373
–R00-017	9/10	None
	1/10	384
–R99-007	8/9	None
	1/9	485
–R99-011	9/10	None
	1/10	141
–R99-005	8/9	None
	1/9	495
–R99-012	8/10	None
	1/10	210
	1/10	372, 456
–R00-020	9/9	None
–R00-023	7/10	None
	3/10	385
–R00-024	7/7	None
Plasma RNA at week 5		
–R00-017	2/9	None
	1/9	49
	1/9	208
	1/9	443
	1/9	49, 103
	1/9	270, 448
	1/9	59, 232, 293
	1/9	391, 481, 499
–R00-023	2/11	None
	2/11	218
	1/11	27
	1/11	434
	1/11	444, 493
	1/11	76, 182, 379
	1/11	118, 272, 380
	1/11	5, 140, 312, 434
	1/11	6, 17, 112, 205
–R00-024	2/9	None
	1/9	227
	1/9	42, 301
	1/9	272, 434
	1/9	9, 48, 367
	1/9	50, 176, 247
	1/9	103, 364, 386
	1/9	108, 137, 364, 386, 411

*Number of clones with change(s)/total number of clones.
†Amplified *gag* fragments were subcloned into plasmids for sequencing and the positions of amino acid changes in SHIV Gag in each clone are shown.

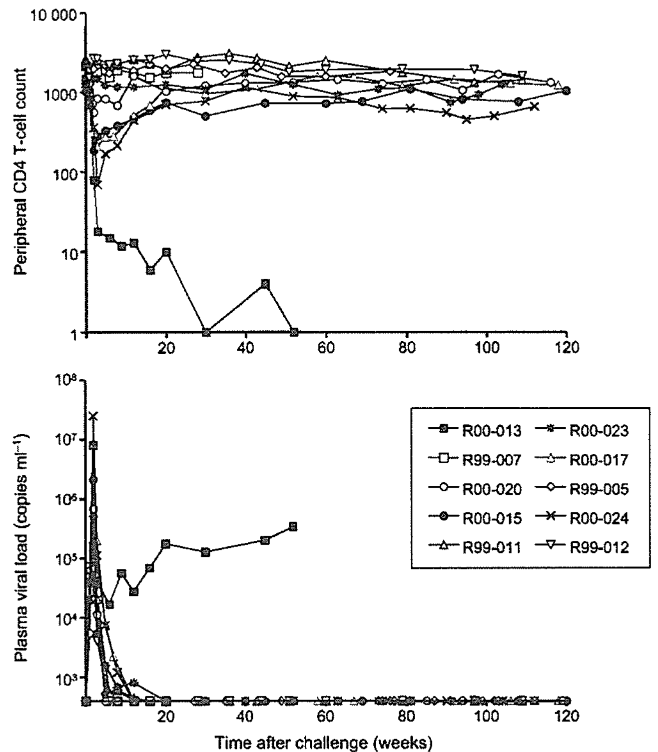

Fig. 1. Follow-up of vaccinated macaques after SHIV89.6PD challenge. Macaque R00-013 was a non-controller that failed to control virus replication, with acute $CD4^+$ T-cell depletion, whereas the other nine animals were SHIV controllers. (a) Peripheral $CD4^+$ T-cell counts μl^{-1}. (b) Plasma viral loads [viral RNA copies (ml plasma)$^{-1}$].

in amplifying almost the entire coding region of the proviral genomes from three (R00-015, R00-017 and R00-023) of the eight controllers at around 1 year for sequencing and found no dominant non-synonymous mutations except for one leading to a change in aa 401 in Env in macaque R00-015, suggesting inefficient virus replication during the period of SHIV control.

Virus-specific T-cell responses

We next examined changes in virus-specific T-cell frequencies during the period of SHIV89.6PD control. The SHIV controllers did not rapidly lose SHIV-specific T cells but most showed a gradual decrease in SHIV-specific T-cell levels, except for macaque R99-011, which maintained constant SHIV-specific $CD8^+$ T-cell levels (Fig. 2). Thus, none of the SHIV controllers showed a significant increase in SHIV-specific T-cell levels, suggesting stable virus control without any sign of a virus replication burst in the chronic phase.

In addition to virus-specific T-cell levels, we examined epitopes that were recognized by CTLs. We focused on two SHIV controllers, R00-015 and R00-017, that were vaccinated with SeV-Gag alone and examined CTL responses

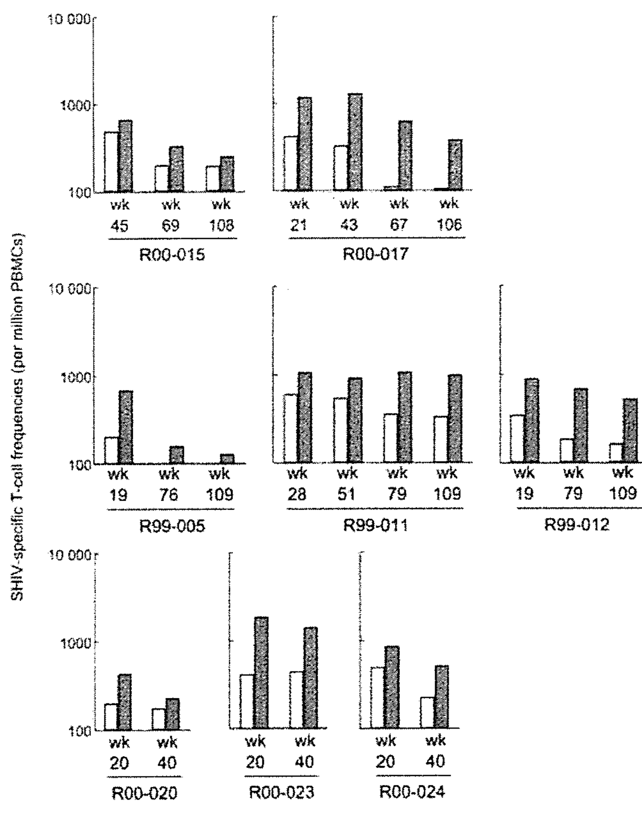

Fig. 2. SHIV-specific T-cell levels in SHIV controllers at various time points after challenge. The frequencies of SHIV-specific CD4$^+$ T cells (open bars) and CD8$^+$ T cells (shaded bars) in PBMCs are shown.

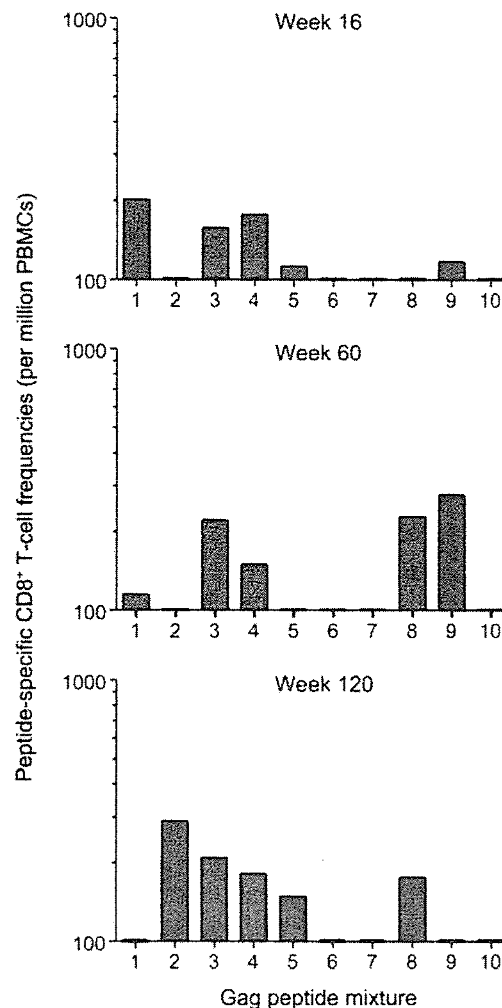

Fig. 3. Changes in frequencies of CD8$^+$ T cells specific for pools of Gag peptides in PBMCs of macaque R00-015 during the period of virus control. The frequencies at week 16 (top panel), week 60 (middle panel) and week 120 (bottom panel) after SHIV89.6PD challenge are shown. A panel of overlapping peptides spanning the entire SIV Gag sequence was divided into ten pools: 1 (aa 1–65), 2 (aa 55–114), 3 (aa 104–165), 4 (aa 155–213), 5 (aa 202–265), 6 (aa 255–316), 7 (aa 306–364), 8 (aa 354–416), 9 (aa 406–464) and 10 (aa 453–510). Each pool was used for stimulation to detect peptide-pool-specific CD8$^+$ T cells.

specific for ten pools of Gag-overlapping peptides. In macaque R00-015, significant Gag peptide pool 1-specific CD8$^+$ T-cell responses were detected at week 16 but became undetectable by week 120, whereas pool 8- and 2-specific CD8$^+$ T-cell responses that were undetectable at week 16 appeared at week 60 or 120, respectively, and pool 3- and 4-specific CD8$^+$ T-cell responses were detectable throughout the observation period (Fig. 3). A similar pattern of disappearance (pool 10-specific), appearance (pool 3- and 9-specific) and maintenance (pool 6- and 8-specific) of CD8$^+$ T-cell responses during the period of SHIV control was also observed in macaque R00-017 (Fig. 4). These results suggested that SHIV89.6PD replication was not completely contained in these macaques.

Virus-specific neutralizing antibody responses

We next examined virus-specific neutralizing antibody responses by determining the end-point plasma titres required to neutralize the replication of 10 $TCID_{50}$ of virus on MT4 cells. Our vaccine regimens did not utilize Env as an immunogen and no neutralizing antibody responses were induced before challenge in any of the vaccinees, as expected. Remarkably, however, SHIV89.6PD-specific neutralizing antibodies appeared rapidly between weeks 3 and 6 post-challenge and were maintained during the observation period in all of the SHIV controllers (Fig. 5). In contrast to such efficient induction of neutralizing antibodies in SHIV controllers, macaque R00-013, which failed to control SHIV replication, showed no neutralizing antibody induction after challenge.

DISCUSSION

Long-term control of X4-tropic pathogenic SHIV has been reported in follow-up studies of several pre-clinical AIDS

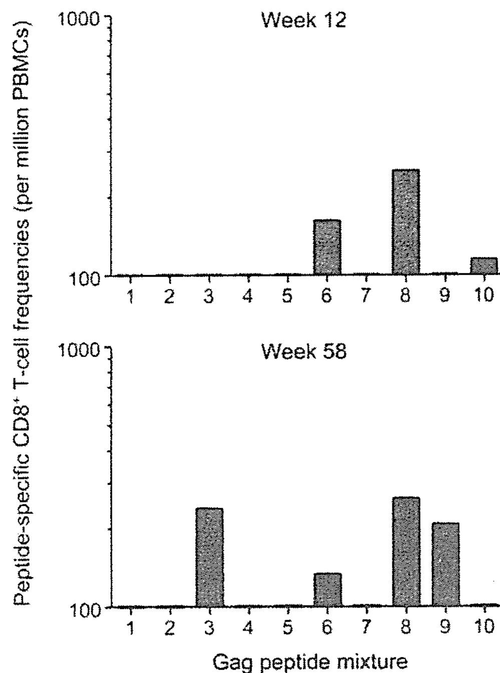

Fig. 4. Changes in frequency of $CD8^+$ T cells specific for pools of Gag peptides in PBMCs of macaque R00-017 during the period of virus control. The frequencies at week 12 (top panel) and week 58 (bottom panel) after SHIV89.6PD challenge are shown. Ten pools of Gag peptides were used for stimulation to detect peptide pool-specific $CD8^+$ T cells, as described in the legend to Fig. 3.

vaccine trials (Willey *et al.*, 2003; Sadagopal *et al.*, 2005). Whilst these vaccine regimens utilized Env as an immunogen (Amara *et al.*, 2002), we have developed vaccine regimens not targeting Env and demonstrated their efficacies leading to control of SHIV89.6PD replication in rhesus macaques (Matano *et al.*, 2001; Takeda *et al.*, 2003). In the present study, we followed up these SHIV controllers for more than 2 years after challenge. All maintained this control with undetectable plasma viraemia, indicating that efficient CTL induction by a prophylactic AIDS vaccine not targeting Env can result in sustained control of virus replication and protection from AIDS progression in a model of X4-tropic SHIV infection.

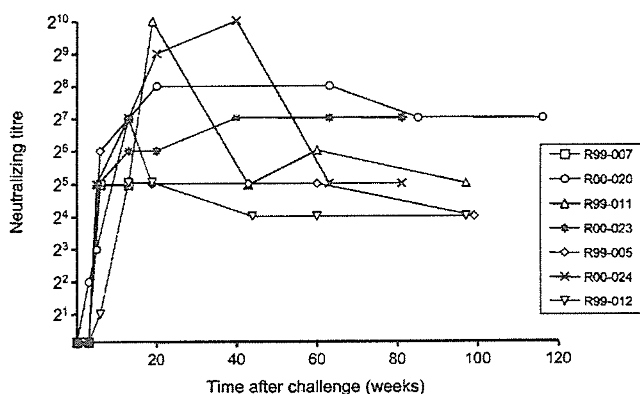

Fig. 5. SHIV89.6PD-specific neutralizing antibody levels in plasma of SHIV controllers. Plasma titres that neutralized replication of 10 $TCID_{50}$ SHIV89.6PD in seven out of nine SHIV controllers are shown. In the remaining SHIV controllers, R00-015 and R00-017, we confirmed induction of neutralizing antibodies at weeks 5, 12 and 20, but their titres were not determined.

X4-tropic SHIV and R5-tropic SIV target different $CD4^+$ T-cell subsets in rhesus macaques and this difference has been indicated as resulting in their divergent clinical courses (Nishimura *et al.*, 2004). Indeed, it has been shown that X4-tropic SHIV targets $CXCR4^+$ naive $CD4^+$ T cells for depletion, whereas R5-tropic SIV, like HIV-1 infection in humans, eliminates $CCR5^+$ effector memory $CD4^+$ T cells in rhesus macaques during the acute phase of infection (Picker *et al.*, 2004; Li *et al.*, 2005; Mattapallil *et al.*, 2005; Nishimura *et al.*, 2005; Picker & Watkins, 2005). In the latter chronic AIDS model, several CTL vaccine trials have recently shown partial reductions in viral loads with amelioration of acute memory $CD4^+$ T-cell loss, but this partial control was transient and unstable (Letvin *et al.*, 2006; Mattapallil *et al.*, 2006; Wilson *et al.*, 2006). In our previous study (Matano *et al.*, 2004), SIV control was observed consistently in the three vaccinees possessing MHC I haplotype *90-120-Ia*, but this control was not stable and two of them lost viraemia control around week 60 after challenge. In the present study showing long-term, stable SHIV control, we found several differences between X4-tropic SHIV controllers and R5-tropic SIV controllers.

First, patterns of *de novo* neutralizing antibody induction were completely different between the two. Although the vaccine regimens did not target Env, SHIV-specific neutralizing antibodies appeared rapidly and became detectable by week 6 post-challenge in the SHIV controllers, whereas no neutralizing antibody induction was observed in the SHIV non-controllers. Thus, SHIV-specific neutralizing antibodies can be rapidly induced if animals are protected by CTLs from complete $CD4^+$ T-cell depletion in the acute phase and may be involved in viraemia control at the set point and after (Rasmussen *et al.*, 2002). In contrast, SIV-specific neutralizing antibody induction in the SIV controllers was poor and less efficient than the SIV non-controllers (data not shown), indicating that neutralizing antibody responses are not involved in SIV control.

Secondly, all of the SIV controllers showed rapid selection of viral CTL escape mutations, whereas this sign of particular CTL pressure (Borrow *et al.*, 1997; Goulder *et al.*, 1997; Price *et al.*, 1997; Goulder & Watkins, 2004; Matano *et al.*, 2004) was not observed in any of the SHIV controllers. Additionally, SIV control was associated with some MHC haplotypes such as *90-120-Ia*, but SHIV control was observed in vaccinees with diverse MHC haplotypes. Indeed, none of the SHIV controllers had the MHC

haplotype *90-120-Ia* associated with SIV control. Although the involvement of functional virus-specific $CD4^+$ T-cell responses remains unclear, these results support the notion that multiple target-specific CTL effectors are involved in SHIV control, whereas relatively limited regions of viral antigens are targeted by effectors responsible for SIV control.

All of the SHIV controllers maintained virus control for more than 2 years. Sequencing of viral genomes revealed a predominance of the wild-type sequence around 1 year after SHIV89.6PD challenge, and analysis of SHIV-specific T-cell levels showed no signs of a burst of virus replication during the chronic phase. These results indicated stable virus control in the chronic phase in the SHIV controllers. Interestingly, however, analysis of Gag peptide-specific $CD8^+$ T-cell responses in some of the SHIV controllers showed a shift of targeting epitopes during the period of virus control, suggesting that virus replication was inefficient but not completely contained, even in the SHIV controllers.

In summary, the present study revealed several differences in vaccine-based virus control in a model of X4-tropic SHIV compared with R5-tropic SIV infections. Our results suggest that, compared with virus control with limited effectors in SIV controllers, the control of X4-tropic SHIV89.6PD replication may be maintained more stably in the presence of multiple functional immune effectors.

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ELSEVIER

Direct Determination of Single Nucleotide Polymorphism Haplotype of *NFKBIL1* Promoter Polymorphism by DNA Conformation Analysis and Its Application to Association Study of Chronic Inflammatory Diseases

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ABSTRACT: We previously revealed that one of the human leukocyte antigen-linked susceptibility genes for Takayasu's arteritis (TA) was mapped between *TNFA* and *MICB* loci and that −63T allele of *NFKBIL1*, which is between *TNFA* and *MICB* loci, was associated with rheumatoid arthritis (RA) in the Japanese population. We have developed a novel typing method based on reference strand-mediated conformation analysis for the upstream sequence of the *NFKBIL1* gene, where −422 $(T)_8/(T)_9$, −325 C/G, −263 A/G, and −63 T/A polymorphisms were found. Upon the analysis of the patients with TA (n = 84), those with RA (n = 120), and healthy control subjects (n = 217), five common haplotypes named IKBLp*01 through IKBLp*05 were found in the Japanese population. The frequency of IKBLp*03 was significantly increased in the patient with TA (57.1% vs 35.0%, giving an odds ratio of 2.47). In addition, the frequency of IKBLp*01, but not that of other −63T-bearing alleles, was increased in the patients with RA (73.3% vs 58.1%, giving an odds ratio of 1.99), suggesting that the susceptibility to RA was conferred not by −63T alone but by combination of single nucleotide polymorphisms in the *NFKBIL1* promoter. A higher promoter activity associated with IKBLp*03 and a lower activity associated with IKBLp*01 may contribute to the susceptibility to TA and RA, respectively. *Human Immunology 67, 363–373 (2006).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: HLA class III region; SNP haplotype; DNA conformation analysis; association study

ABBREVIATIONS

TA	Takayasu's arteritis
RA	rheumatoid arthritis
LD	linkage disequilibrium
RSCA	reference strand-mediated conformation analysis
SNP	single nucleotide polymorphism
HLA	human leukocyte antigen
SSCP	single strand conformation polymorphism

INTRODUCTION

The HLA class III region of the short arm of human chromosome 6 contains multiple genes involved in the host defense mechanism against pathogens and inflammatory processes. Polymorphisms in the genes in this region were associated with several infectious diseases and autoimmunity. In addition to the association with HLA-B alleles, B*5201 and B*3902 [1], we previously reported that an allele of microsatellite polymorphic locus *C1_2_A* and certain alleles of other markers in the *TNFA-MICB* interval of the HLA class III region [2] were associated with Takayasu's arteritis (TA), also known as aortitis syndrome or pulseless disease, which is a chronic inflammatory disease mainly affecting the aorta

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and its major branches accompanying sustained cellular infiltration in the vessel walls including gamma-delta T cells [3, 4].

Rheumatoid arthritis (RA) is another well-known HLA-associated disease characterized by autoimmunity against synovial membrane and other body components, in which primary association with HLA class II polymorphisms was reported in several different ethnic groups [5]. An additional susceptibility gene for RA was mapped within the same *TNFA-MICB* interval as TA [6]. These observations prompted us to seek a gene or gene polymorphism that may confer the susceptibility to TA and RA within the *TNFA-MICB* interval. The *NFKBIL1* locus is one of several candidates for the susceptible gene near the *C1_2_A* locus, because it encodes for the I kappa B-like (IKBL) protein which has a putative ankyrin repeat sequence and may interact with members of the NF-kappa B/Rel family [7]. As a result, we observed the association of one of the *NFKBIL1* polymorphisms with susceptibility to RA [8]. The association of genetic polymorphism and disease susceptibility is attributed either to direct contribution of the observed genetic alteration to the susceptibility or to linkage disequilibrium (LD) between detected polymorphism and hidden causative genetic alteration(s) of the real disease-susceptibility locus. Accordingly, detailed analysis of haplotype of disease-associated polymorphisms is required.

A genotyping method known as reference strand-mediated conformation analysis (RSCA) has been developed to identify the polymorphisms of several genetic loci including HLA class I [9]. RSCA is based on the double-strand DNA conformation polymorphism of a heteroduplex between a fluorescent dye-labeled "reference" DNA strand and the DNA to be examined. The heteroduplex DNA principally exhibits a unique retardation in mobility upon native polyacrylamide gel electrophoresis according to the number and distribution of mismatched basepairs within the duplex molecule. Therefore, the method is sufficiently robust to detect combinatory difference in DNA sequence such as haplotype of single nucleotide polymorphism (SNP), where the discrimination of genotype is largely dependent on the sequence of the reference DNA strand.

In the present study, we sought candidate polymorphisms for the genetic predisposition to two chronic inflammatory diseases, namely TA and RA, in *NFKBIL1*. As a result, four SNPs in the upstream sequence of the gene were found; -422 $(T)_8/(T)_9$ (eight or nine consecutive thymidines), -325 C/G, -263 A/G, and -63 T/A. Then, we used a method to determine the diplotype, *i.e.,* the combination of haplotypes of these four SNPs for each individual, without family samples by means of RSCA. Consequently, five common SNP haplotypes, or *NFKBIL1* alleles, were observed in the Japanese population, one of which showed a significant association to TA. The TA-associated *NFKBIL1* allele, IKBLp*03, was in strong linkage disequilibrium with C1_2_A*238, which confirmed our previous observation of a risk factor for TA in the HLA region in addition to the HLA-B-linked susceptibility locus. In addition, our analysis revealed that the susceptibility to RA was associated with a different *NFKBIL1* allele, IKBLp*01. To explore the functional relevance of these disease-associated *NFKBIL1* alleles, we examined transcriptional activity of the promoter. We report here that the TA-associated IKBLp*03 showed higher promoter activity than IKBLp*01, suggesting that expressivity of IKBL protein may be differently involved in the predisposition to TA and RA.

MATERIALS AND METHODS

DNA Samples

All study protocols were approved by the Ethics Reviewing Committee of the Medical Research Institute, Tokyo Medical and Dental University. The diagnosis of TA was based on the criteria proposed by the Aortitis Research Group of the Ministry of Health and Welfare, Japan as described previously [10]. The patients with RA were a subset of the patient group enrolled in our previous studies [6, 11] who met the 1987-revised diagnostic criteria of the American Rheumatism Association. Controls were randomly chosen from healthy volunteers representing the Japanese general population. A total of 84 patients with TA [2], 120 patients with RA [6], and 217 healthy control individuals were enrolled in the study. All the patients and control individuals were Japanese and genetically unrelated to each other. DNA was prepared from a blood sample which was obtained from each subject according to given informed consent. HLA and HLA-linked microsatellite polymorphisms were typed as described [2, 6].

Searching for Polymorphisms

PCR–single-strand DNA conformation polymorphism (SSCP) analysis was performed to search for polymorphisms in the promoter and coding region of the *NFKBIL1* gene among 96 individual DNA samples randomly picked up from the controls in the present study, according to the standard method previously described [12]. Primers for the amplification of genomic DNA are listed in Table 1A. Some representative PCR products that manifested different mobility characteristics in the SSCP gel were chosen to be sequenced directly after ExoSAP-IT (Amersham, NJ, USA) treatment. The primer for the direct sequencing reaction was either forward or reverse primer used for amplification.

TABLE 1 Oligonucleotides used in this study

A. Primers for amplification of NFKBIL1 gene

Amplicon	Forward primer	Reverse primer
P3	P3F 5′-TTCCAAACTCCTAAGGGAGG-3′	P3R 5′-TTGTAAGCCCGCAGCTTTGG-3′
P2	P2F 5′-GCCTGGGAGCAGCAGAGACC-3′	P2R 5′-AGACAAAAGACGGAAGAAGAC-3′
P1	P1F 5′-AAATTTTTGCATCTCACTTGCC-3′	P1R 5′-GTTCTTGGCCAGATCTCCC-3′
E1	E1F 5′-CAGACGGCCCCTTTAATTTAAG-3′	E1R 5′-GTCACAGATAATCTCCAATAATG-3′
E2	E2F 5′-CAAGGCTGAAGTCCTGACTG-3′	E2R 5′-GTCAGCTGCTTATGACCTTG-3′
E3	E3F 5′-CTAACTTCTGCTCCCTGCTC-3′	E3R 5′-GGGGAAGGGCAGCTGTGG-3′
E4A	E4AF 5′-ATCACCTTCTCACAGCCTC-3′	E4AR 5′-GGCACATCACCAAATCGCC-3′
E4P	E4PF 5′-AAGAGCACCCCAGAGGAGCG-3′	E4PR 5′-GAGGCTGCAGCCCCGAAGTT-3′

B. Primer for RSCA analysis of NFKBIL1 gene upstream sequence

Forward primer	Reverse primer
P1F 5′-AAATTTTTGCATCTCACTTGCC-3′	E1R 5′-GTCACAGATAATCTCCAATAATG-3′

C. Mutation oligonucleotides[a]

−263mtF	5′-GGCGGGGGAAAAACCTCCA-3′
−263mtR	5′-AGGTTTTTCCCCCGCCTCC-3′
−325mtF	5′-TTCTCTGTGGTTCTCATCTTTTC-3′
−325mtR	5′-GATGAGAACCACAGAGAAAATAGAGG-3′
−63mtF	5′-CTCCACCAAGCGTCTCTGCT-3′
−63mtR	5′-GAGACGCTTGGTGGAGGAC-3′

D. Oligonucleotides for enhancer test constructs[b]

01x2_F	5′-TCGGGGAGGCGGGAAAAAACCTCCGTGGAGGCGGGAAAAAACCTCC-3′
01x2_R	5′-CCGAGGAGGTTTTTTCCCGCCTCCACGGAGGTTTTTTCCCGCCTCC-3′
03x2_F	5′-TCGGGGAGGCGGGGAAAAACCTCCGTGGAGGCGGGGAAAAACCTCC-3′
03x2_R	5′-CCGAGGAGGTTTTTCCCCGCCTCCACGGAGGTTTTTCCCCGCCTCC-3′

E. Primers for real time quantitative PCR

Amplicon	Forward primer	Reverse primer
IKBL	qIF 5′-CGTCGCTTTCGTCGTTACTT-3′	qIR 5′-CCTTGGAGGCATCATCTTCT-3′
GAPDH	qGF 5′-CTTCACCACCATGGAGAAGGC-3′	qGR 5′-GGCATGGACTGTGGTCATGAG-3′

[a] The modified nucleotide is underlined.

[b] Sequences of the 20-bp repeat are underlined.

Cloning and Sequencing of PCR Products

PCR products were ligated to pGEM T-Easy plasmid vector (Promega, WI, USA) and introduced to *Escherichia coli* DH10B competent cells (Invitrogen, CA, USA) according to the manufacturer's instructions. Plasmid DNA was extracted by alkali lysis and subjected to sequencing using the BigDye terminator cycle sequencing kit and ABI 310 automated fluorescent sequencer system (Applied Biosystems, CA, USA).

RSCA of *NFKBIL1* Promoter Polymorphism

A 669-bp *NFKBIL1* gene fragment containing four SNP loci was amplified by PCR under the standard conditions using primers P1F and E1R (Table 1B). The reference strand was prepared by PCR as the amplicon of the 669-bp genomic DNA fragment except for the use of 5′-Cy5-labeled P1F primer instead of ordinary P1F primer. For the modification of the sequence of reference strand, a site-directed mutagenesis by overlap extension PCR was employed [13]. In brief, a pair of mutagenic oligonucleotides for the substitution of G for A at position −262 (relative to the 5′ end of GenBank entry of *NFKBIL1* cDNA, Accession No. X77909) for a plasmid clone derived from PCR of IKBLp*03 was −263mtF/−263mtR (Table 1C). The PCR product of the plasmid DNA template with P1F and −263mtR primers and that with −263mtF and E1R primers were combined and subjected to ligation-PCR using flanking primers, P1F and E1R. Similarly, an insertion of G at −325 and

an insertion of T at −63 were introduced into a plasmid clone of IKBLp*04 using two pairs of mutagenic oligonucleotides; −325mtF/−325mtR and −63mtF/−63mtR, respectively (Table 1C). Then, these two mutant DNA clones were cut with restriction enzyme *Bgl*II and ligated to combine both modified sites.

Heteroduplexes were formed by the addition of 1 μl of reference PCR product (200–300 ng/μl) to 3 μl of test PCR product (200–300 ng/μl) followed by denaturating at 95°C for 4 minutes, annealing at 55°C for 5 minutes, and cooling down to 15°C for 5 minutes; 6x Ficoll loading dye (15% Ficoll, 0.25% bromophenol blue) and internal standards for the mobility (519-bp and 870-bp Cy5-labeled DNAs) were added after duplex formation. Samples were separated by electrophoresis in a nondenaturing 6% Long Ranger polyacrylamide gel (Biowhittaker Molecular Applications, ME, USA)/1×TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.0) on ALFexpress Sequencer/Fragment Analyzer (Pharmacia Biotech, Uppsala, Sweden) at 30 W constant power. The gel temperature was maintained at 40°C during electrophoresis. The mobility of each fluorescent fragment was analyzed by Fragment Manager Software (Pharmacia Biotech).

Statistical Analysis

The frequencies of each allele, each genotype, and carrier for each allele in the patient group were compared with those in the control group. The strength of the association was expressed by the odds ratio (OR) and statistical significance was examined by Fisher's exact test using R statistical package. Interval estimate for OR was obtained according to the literature [14]. The p values of the OR were further corrected for multiple statistical tests by multiplying with the number of *NFKBIL1* promoter alleles ($n = 5$) found in the Japanese population. When the corrected p value (p_c) was less than 0.05, the association was considered to be significant.

Analysis of Linkage Disequilibrium

LD between *NFKBIL1* promoter allele and HLA polymorphism was assessed by calculating normalized LD coefficient (D′) [15] and correlation coefficient (r^2) [16] following the estimation of haplotype frequency of the control population from individual data set by means of EH program obtained from Web resource at the Rockefeller University (http://linkage.rockefeller.edu/ott/eh.htm). Significance of LD was evaluated using the value of minus twice the logarithm of likelihood ratio under assumptions of presence and absence of LD which is known to approximately follow a χ^2 distribution with one degree of freedom [17].

Stratification Analysis

To examine the interaction between IKBLp polymorphism and HLA-B allele, the risk conferred by IKBLp polymorphism was evaluated by the value of OR when the patients and controls were classified into subgroups by the presence or absence of a certain HLA-B allele and vice versa [18].

Functional Analysis of *NFKBIL1*-263 Polymorphism

To explore whether −263G/A polymorphism can contribute to the difference in transcriptional enhancer activity, we constructed a series of oligomeric tandem repeats of the 20-bp-long sequence around the SNP (−272 to −253) ligated with a basal promoter derived from adenovirus E1B gene [19]. In brief, a double-strand DNA containing two copies of the repeats corresponding either to −263G or to −263A allele was made by annealing of oligonucleotides 01×2_F and 01×2_R or 03×2_F and 03×2_R (nucleotide sequences are listed in Table 1D). Then it was inserted into the nonsymmetrical cohesive ends of pUC00CAT cleaved with *Ava*I restriction enzyme as a monomer or head-to-tail ordered oligomers [20]. The repeats were cut *en bloc* after transformation of *dcm* mutant *E. coli* host (BL21) with *Apa*I and *Bam*HI and inserted at an upstream position of E1B TATA box cloned in the pGL3-basic firefly luciferase reporter plasmid (Promega). The enhancer activity was measured by relative luciferase activity upon the transfection. Raji cells (1×10^6) were transfected with a mixture of the luciferase reporter and a Renilla luciferase reporter plasmid pRL-tk by using Trancfectin (Bio-Rad). The cell lysate was collected at 24 hours after transfection. The promoter activity was measured as relative light intensity of reporters with Dual Luciferase assay kit (Promega) by using Luminous CT-9000 luminometer (DIA-Iatron, Germany).

Promoter Assay

The 1204 bp-long *NFKBIL1* gene fragment spanning from −1182 to +82 was amplified by PCR using primers P3F and E1R. The resultant DNA fragment corresponding to each of five alleles (IKBLp*01 through *05) was ligated to pGL3-basic plasmid in which was the firefly luciferase reporter to be driven by the inserted DNA upon the transfection to Raji cells, as described above. The assays were performed with six different preparations of reporter plasmid DNA for each of five constructs to take into account a possible variation in quality among different preparations of plasmid DNA. The data were analyzed with one-way ANOVA.