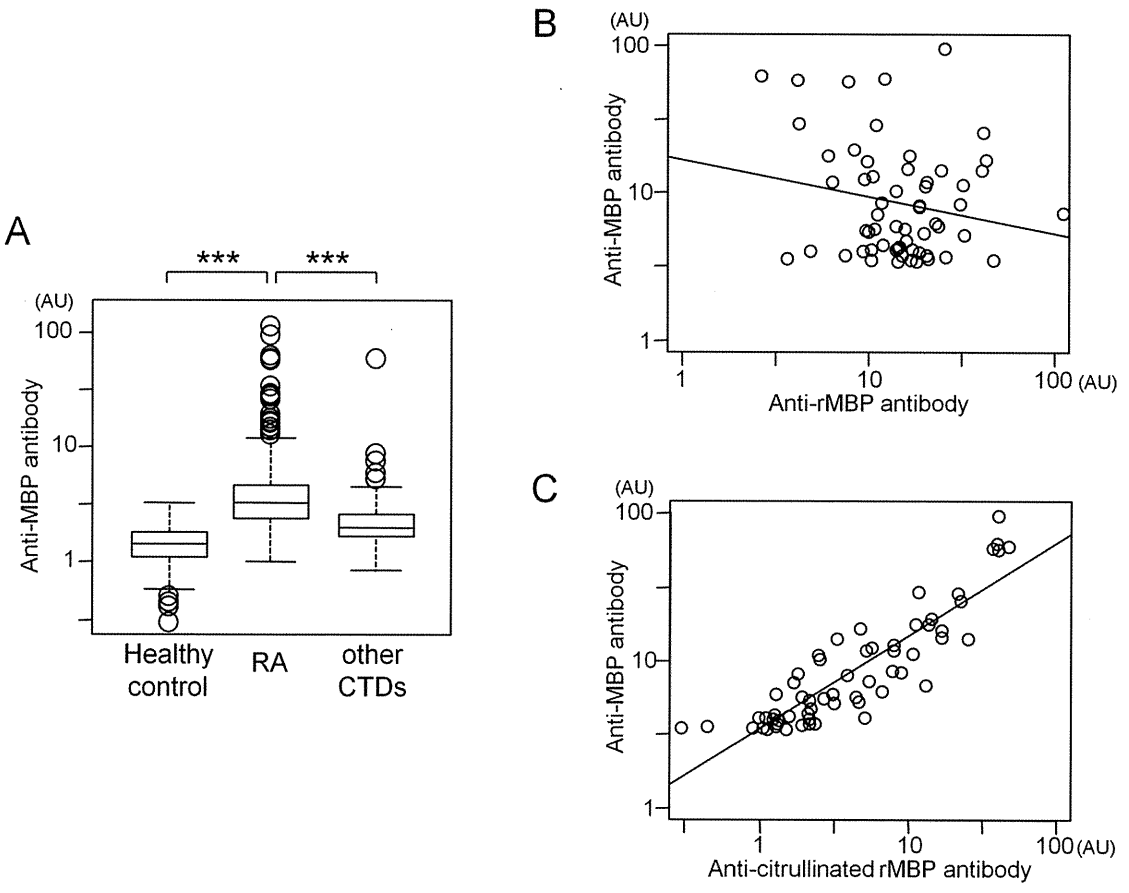

Figure 4. Quantification of circulating antibodies against MBP protein. A. Each boxplot indicates distribution of anti-MBP antibody titers in healthy controls, in RA patients, and in other connective tissue diseases (CTDs) (for detailed composition of the disease patients, see materials and methods). Results were representative of the two independent experiments. *** indicates statistical *p*-value smaller than 0.001. B. Correlation of autoantibody levels between human brain-derived MBP and recombinant MBP (rMBP). C. Correlation of autoantibody levels between human brain-derived MBP and citrullinated rMBP. Sixty RA patients who were positive for anti-MBP antibody were used for the analysis. In each figure, antibody titers were shown as arbitrary unit (AU).
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DNA collections of RA patients followed by replication in two additional collections led to identification of a novel risk variant, rs2000811 ($p = 2.7 \times 10^{-8}$, OR 1.23, 95% CI: 1.14–1.32), in the second intron of the *MBP* gene at chromosome 18q23. This is the second largest genome-wide association study to date in the Japanese population, and the first to have identified chromosome 18q23 as a potential susceptibility locus for RA. 18q23 was not reported as a candidate genetic locus in recent GWA study of RA in Japanese [17]. As summarized in Table S4, we confirmed in the current study the association of three out of seven genes reported to date whereas there was no evidence of association for the other four genes. A comprehensive genetic study by a meta-analysis using the results of different genome scans followed by the validation study with a large number of patients and controls will clarify relative contribution of each genetic locus to RA in the Japanese population. There are no GWA studies to date that indicate chromosome 18q23 as a potential genetic locus related to the susceptibility to RA in European populations [5–12]. By taking into account the detection power of the meta-analysis by Stahl *et al.*, it is natural to consider that the association of *MBP* with RA is limited to Japanese (and possibly other Asian populations).

The disease-associated marker rs2000811 is isolated in an 18-kb segment of the intron, and does not show evidence of significant LD with known markers in the region or neighboring genomic regions (Figure 1). The LD structure of the human *MBP* locus was very similar between Europeans and Japanese, and there was no clear LD block in the region encompassing rs2000811 in both populations (Figure S2). Sequencing of the DNAs of 84 healthy controls failed to identify other polymorphisms that were in strong LD with rs2000811 in the exons or the promoter region of the *MBP* gene. While bioinformatics analysis failed to identify any *cis*-acting elements encompassing rs2000811, allele-specific expression analysis demonstrated the elevated transcription level of *MBP* with the risk allele. This raises the possibility that *cis*-acting regulatory elements that encompass unknown genetic variations in strong LD with rs2000811.

A strong expression of the MBP protein was observed in synoviocytes of RA patients while expression was weaker in those of non-inflammatory controls. The fact that the expression of MBP was strongest in the synovial lining layer suggests the role of anti-MBP antibody as a trigger of inflammatory reactions through attacking synoviocytes. Although a study investigating citrullinated proteins in synoviums did not clearly identify MBP [30], MBP is possibly one of the unidentified subset of the citrullinated proteins. MBP expression was weaker in synoviocytes adjacent to the follicules of infiltrated lymphocyte. It would be of interest to examine whether MBP in synovium is

citrullinated and such phenomena are correlated with the disease activity, in particular, at different stages of proliferative synovitis but data for such an investigation are not available in our study. In the absence of a quantitative assay of expression in synoviocytes, it was not possible to discern a correlation between the levels of *MBP* expression with the risk genotype in our experiments.

MBP is a well-known target autoantigen in multiple sclerosis (MS), a human neurodegenerative disease with an active destruction of myelin sheath [29]. The MBP protein has six different isoforms of which isoforms 1, 2, 3 and 4 with shorter open reading frames are expressed preferentially in the central nervous system (CNS). Isoforms 7 and 8 with higher molecular weights (also called Golli-MBP) are known to be expressed relatively ubiquitously including cells of the hematopoietic lineage [31]. The fact that RA patients rarely present CNS symptoms may suggest the involvement of Golli-MBP protein in the generation of anti-MBP antibody and expression in the synovium. An immunological study using relatively small numbers of plasma samples showed that anti-MBP antibody is present in 60% (or six out of ten) of RA patients [32]. To our knowledge, however, there is no study which quantitatively characterized anti-MBP antibody in RA patients as well as in patients of other connective tissue diseases and in healthy controls. On the other hand, ACPA which recognizes citrullinated proteins by peptidylarginine deiminase is considered as a specific and predictive marker for RA [13,33–34]. Indeed, several studies showed the existence of antibodies to citrullinated MBP in RA patients [35–36]. However, the role of MBP and anti-MBP antibody in the pathogenesis of RA is yet to be elucidated. In the current study, we found that a part of brain-derived MBP was citrullinated in physiological conditions and that higher levels of anti-MBP antibody in RA patients can be attributed to MBP citrullination, although anti-MBP antibody is not in complete cross-reactivity with ACPA. We examined whether or not rs2000811 was predominantly associated with patients who were positive for ACPA. However, there was no statistical difference in allele frequency of rs2000811 between patients with and without ACPA ($p = 0.40$, data not shown).

We observed no correlation between the levels of anti-MBP antibody and genotypes at the risk locus. The repertoire of autoantibody is likely to depend on numerous factors such as the immunogenicity and extent of citrullination of the MBP protein. Also, a series of immunological reactions including antigen presentation in thymus and peripheral organs, activation of dendritic cells, T- and B-lymphocytes, and balance of Th1/Th2 and effector/regulatory T-lymphocytes affect antibody production. A possible explanation for the lack of correlation is that the disease-associated genotypes affect these factors in a way that impacts risk without directly influencing the quantitative values of anti-MBP antibody, which we find to be highly variable between RA patients that we have studied. Therefore, it will be of interest to compare the expression levels of *MBP* transcripts and MBP epitopes in synovial tissue, and between different genotypes and disease activity.

Taken together, this is the first genetic study which identified *MBP*, an autoantigen gene, to be associated with RA in Japanese. Transcription of *MBP* was increased with the risk allele of the associated SNP, rs2000811. Strong expression of the MBP protein was observed in the synovial tissues of the patients. Furthermore, significant increase of circulating autoantibodies against MBP protein was demonstrated in RA patients as compared to those with other connective tissue diseases, implicating its role as a disease-associated biomaker.

Materials and Methods

Ethics Statement

Written informed consent was obtained from all the participants at the institute of sample collection after being approved for genetic studies by the local ethical committee, namely, Kyoto University Graduate School and Faculty of Medicine, Ethics Committee, The Ethic Committee, Sagamihara National Hospital, NHO, Dohgo Spa Hospital Ethical Committee, University of Tokyo Medical Research Center Ethics Committee, Tokyo Women's Medical University Genome Ethics Committee, Ethics Committee of Tokyo Women's Medical University and Aichi Cancer Center Ethical Committee for human genome research.

Study subjects

RA collections 1 to 4 consisted of 658 affected individuals and 934 controls, 332 and 302, 874 and 855, and 1,264 and 948, respectively (summarized in Table S1). The case subjects of collections 1, 2 and 3 were recruited at the rheumatology departments of Kyoto University Hospital, Dohgo Spa Hospital, Sagamihara National Hospital, and Tokyo University Hospital, and those for collection4 were from Tokyo Women's Medical University. All cases fulfilled the revised criteria (1987) of the American College of Rheumatology (ACR) for rheumatoid arthritis [37]. Genotype count data of the Japanese Single Nucleotide Polymorphism (JSNP) database [38] were used as controls for collection1. The control subjects for collection2 were from the Department of Ophthalmology and Visual Science at Kyoto University Hospital [39]. DNA samples of healthy Japanese volunteers in Pharma SNP Consortium [40] and in Aichi Cancer Center Hospital and Research Institute [41] were used for collections 3 and 4, respectively. Plasma of 323 RA patients and 162 patients of other connective tissue diseases (38 of SLE, 25 each of Sjögren's syndrome and systemic sclerosis, 20 each of Behçet's disease and mixed connective tissue disease, 19 of polymyositis/dermatomyositis and 15 of vasculitis) were obtained at Kyoto University Hospital, and those of 131 healthy controls were from Dohgo Spa Hospital.

Genome-wide association analysis

Genome scan for collections 1 and 2 was performed using Infinium Technology (Illumina Inc., San Diego, CA). Case subjects of collection1 were genotyped with Human-Hap300 (version 1.0, 302,627 SNPs) or Human CNV370-Duo (version 1.0, 332,270 SNPs). For collection2, case and control subjects were genotyped on Human610-Quad (version 1.0, 577,348 SNPs), and HumanHap550 (version 3.0, 547,163 SNPs), respectively. Validation studies using collections 3 and 4 were performed using Taqman technology (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's specifications.

Quality control and statistical tests for case-control association

277,420 SNPs that were common among the four arrays described above were chosen for association study. Publicly available genotype counts from the JSNP project were used as control collection1. For this collection, detailed information such as individual genotypes and cluster plots are not disclosed. DNA samples with a call rate smaller than 0.90 (three in collection1 cases), showing high degree of kinship (PI_HAT>0.10 by PLINK [42], eleven in collection1 cases, one in collection2 cases, and four in collection2 controls) and with evidence of possible contamination (one in collection2 controls) were removed from statistical analyses. PCA was performed using the genome scan results of the remaining 644 cases of collection1, in addition to 331 cases and 297 controls of collection2. At this stage, five DNA samples (one in

collection1 cases, and four in collection2 cases) that did not fall into the Japanese cluster were removed. Regarding the SNP markers, a total of 225,079 SNPs with call rate greater than 0.95 for both cases and controls and minor allele frequency greater than 0.05 either in case or in control of each collection were used for analysis. The *p*-value for rs2000811 corrected by PCA was calculated using the remaining 970 cases and 297 controls and compared with the *p*-value without correction.

The case-control association was examined independently for collections 1 and 2 with Cochran-Armitage trend test, followed by meta-analysis with Cochran-Mantel-Haenszel (CMH) test by combining the two collections. Population stratification in collections 1 and 2 was examined and corrected with Genomic Control [43]. The SNPs that showed $p<0.005$ in both collections and meta-analysis *p*-value smaller than 10^{-4} were selected as candidates for further evaluation. Among multiple SNPs in the same region that fulfilled the above criteria, the SNP with the smallest *p*-value in the meta-analysis was chosen for validation with collections 3 and 4. To be more specific, among the four SNPs in the *PLEKHK1* region on chromosome 10q21, namely rs3910172 ($p=4.4\times10^{-5}$), rs6479805 ($p=5.4\times10^{-5}$), rs10733769 ($p=5.8\times10^{-5}$), and rs4147233 ($p=6.6\times10^{-5}$), rs3910172 was selected for the replication study. Likewise, in the region of chromosome 10p14, rs2026628 ($p=1.6\times10^{-5}$) was chosen over rs11253857 ($p=5.3\times10^{-5}$), and rs687848 ($p=5.1\times10^{-5}$) was chosen over rs587404 ($p=7.1\times10^{-5}$) in the *MACF1* region on chromosome 1p31–32. SNPs in the *HLA* and *PADI4* loci were not included in validation studies. Haploview version 4.1 software [44] was used for LD evaluation, and MapViewer (build 36.3) [45] was used to identify the location and structure of the genes in the region.

Quantification of allelic difference in gene expression in MBP transcription

Allele specific gene expression analysis was performed as previously described [16]. Briefly, human B-lymphoblastoid cell lines transformed by EBV were obtained from the Health Science Research Resources Bank of Japan (Osaka). Genomic DNA and total RNA were extracted by standard procedures from 22 cell lines heterozygous for rs2000811 alleles. The ratio of MBP primary transcripts (hnRNA) was quantified between the risk and wild-type alleles by TaqMan assay with SNP genotyping probes. Genomic DNA of a cell line homozygous for the wild-type allele (C) were mixed with DNA homozygous for the risk allele (T) at eight different molar ratios (2:1, 3:2, 1:1, 2:3, 1:2, 1:3, 1:4, 1:6) to draw a standard curve for the evaluation of RNA quantity.

Immunohistochemistry

Synovial tissue specimens of 23 RA patients and five non-inflammatory controls were obtained from Department of Diagnostic Pathology and Department of Orthopaedic Surgery in Kyoto University Hospital. Paraffin-embedded tissues were prepared in a standard method and sectioned at a thickness of 3 μm. The section was mounted on a glass slide coated with 2% 3-aminopropyl triethoxy silane (Tokyo Kasei, Tokyo, Japan). Immunohistochemical staining of MBP was performed by using the standard avidin-biotin-peroxidase complex (ABC) method, as previously described [46]. The sections were incubated overnight at 4°C with an affinity-purified murine anti-human MBP monoclonal antibody (Leica Microsystems, Wetzlar, Germany) diluted at 1:100 in PBS. The sections were then incubated with biotinylated horse anti-mouse IgG antibody (Vector Lab, Burlingame, CA) for 40 minutes, followed by incubation with peroxidase-conjugated streptavidin (Vector Lab, Burlingame, CA) at room temperature for 50 minutes. The coloring reaction was performed with 0.3 mg/ml diaminobenzidine and 0.003% H_2O_2 in 50 mM Tris-HCl (pH 7.6). Each section was counterstained with haematoxylin. Evaluation of MBP expression was performed by a blind test by two rheumatologists as well as a pathologist, and statistical significance in positivity was calculated with Fisher's exact test.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Nalge Nunc International K.K., Tokyo, Japan) were coated with 50 μl of MBP protein at 5 μg/ml extracted from human brain (Sigma, St. Louis, MO) or with recombinant human MBP (Genscript, Piscataway, NJ.) in 50 mM bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. Citrullination of recombinant human MBP was done *in-vitro* by rabbit skeletal PAD (Sigma, St. Louis, MO) for 3 hours as previously described [47]. After wells were washed and coated with phosphate-buffered-saline (PBS) containing 2% bovine serum albumin (BSA), 50 μl of plasma samples diluted to 1:150 with 2% BSA in PBS containing 5 U/ml heparin (Mochida Pharmaceutical Co., Ltd. Tokyo, Japan) were added and incubated at room temperature for 2 hours. 50 μl each of purified goat anti-human IgG polyclonal antibody conjugated to alkaline phosphate (Millipore. Billerica, MA) diluted to 1:2000 was added and incubated at room temperature for 1.5 hours. 50 μl of BCIP/NBT substrate (Sigma, St. Louis, MO) in 2 μM $MgCl_2$ was then added and incubated for 45 minutes in the dark. The optical density (OD) value at 405 nm was measured by a SpectraMax Plus^{384} Microplate Reader (Molecular Devices, Sunnyvale, CA). A standard curve was generated by serial dilution (1:50, 1:150, 1:500, 1:1500 and 1:5000) of a plasma sample with a high titer of anti-MBP antibody. The titer of diluted standard sample in 1:50 was set as 100 U for human brain-derived MBP. All samples were examined in duplicate except for negative control in which plasma was replaced by PBS with 2% BSA and was measured in quadruplicate. The specificity of ELISA results was confirmed by standard immunoblotting analysis using sera of 10 each of RA patients and controls.

Statistical analysis of ELISA results

The titer of anti-MBP antibody between RA or its subgroups and controls was compared with Wilcoxon rank-sum test. The correlation between the titers of autoantibody was estimated with Pearson's correlation coefficient in logarithm scale. These statistics were performed in the R statistical system (http://www.R-project.org) and SPSS(ver18).

Supporting Information

Figure S1 QQ plot to show the observed and expected *p*-values of the combined genome scan results. Vertical and horizontal axes indicate observed and expected *p*-values, respectively (A) and in logarithmic scale (B). The analysis using genomic control method showed no significant effect of population stratification ($\lambda_{GC}=1.03$) between the case and control groups.
(TIF)

Figure S2 Imputation analysis and LD structure of the human *MBP* locus. Imputation was performed using the case genotypes of collections 1 and 2 and control genotypes of collection2. Individual genotypes of control population of collection1 (JSNP) were not available. Determination of LD structure was performed by using the GWAS results in this study and the HapMap results of Japanese and Caucasians (Hap JPT and Hap CEU, respectively).
(TIF)

Figure S3 LD structure of the 156-kb region spanning the *MBP* gene. LD plot was generated with Haploview using polymorphisms with reference allele frequencies between 0.05 and 0.95.
(TIF)

Figure S4 Quantification of allelic difference in *MBP* transcription. Human B-lymphoblastoid cell lines transformed by EBV were obtained from the Health Science Research Resources Bank of Japan (Osaka, Japan). Total RNA was extracted by standard procedures from the cell lines that were either homozygous for the wild-type allele (50 cell lines), heterozygous (50 cell lines) or homozygous for the risk allele (49 cell lines) of rs2000811. The amount of *MBP* cDNA in each cell line was measured and normalized to that of β-glucronidase using Taqman Gene Expression Assay (for MBP; Hs00921943-m1, for β-glucronidase; Hs99999908_m1, Applied Biosystems Inc., Foster City, CA) in GeneAmp 7500 Sequence Detection System. The comparative ΔΔCT method and Jonckheere-Terpstra test were used for the analysis.
(TIF)

Figure S5 Immunoblotting of anti-MBP antibody. Immunoblotting analysis was performed to confirm specific binding of circulating anti-MBP antibody. Lane 1, 2 to 6 and 7 were incubated with control plasma, plasma of patients, and rabbit polyclonal anti-human MBP antibody, respectively. The intensity was variable between RA patients whereas no signal was obtained in controls. Similar results were obtained using plasma of the other five RA patients and nine controls.
(TIF)

Figure S6 Comparison of circulating anti-MBP antibody and IgG-RF. We investigated whether the presence of RF in sera enhances the signal in ELISA through binding of IgG-RF to the constant region of anti-MBP antibody or through non-specific binding of IgM-RF to secondary antibody used in the experiments. Initially, correlation between IgG-RF and anti-MBP antibody titers was assessed to examine whether IgG-RF had any influence on anti-MBP antibody titers. 114 patients with RA and 13 other connective tissue disease patients for whom IgG-RF measurement was available were enrolled in this evaluation. As a result, no correlation was observed between the titers of anti-MBP antibody and IgG-RF (Spearman's rank-sum coefficient being 0.145 with p-value of 0.103). Subsequently, ELISA experiments using human IgM or IgG as target antigens were undertaken. Non-specific binding of secondary antibodies to human IgM compared to IgG was less than 1%.
(TIF)

Table S1 Summary of the study populations used for the association analysis. Abbreviations were as follows; ACPA: antibodies to citrullinated peptide antigens, RF: rheumatoid factor, SD: standard deviation, N/A, not available.
(DOC)

Table S2 Summary of quality control for genome scan results.
(DOC)

Table S3 Association of *HLA* and *PADI4* loci with rheumatoid arthritis in the Japanese population. *risk allele for the disease, **risk allele frequency, and ***p-value in meta-analysis using Cochran-Mantel-Haenszel test.
(DOC)

Table S4 Association of previously reported non-*HLA* genes in the current study. *p-value in meta-analysis using Cochran-Mantel-Haenszel test.
(DOC)

Table S5 Replication results of the four regions. The order of SNPs is in accordance with *mhp*-value. Chromosome and dbSNPID refer to NCBI build 36.3. *P*-values are calculated using the Cochran-Armitage trend test. *risk allele for the disease, **risk allele frequency, and ***OR, odds ratio with 95% confidence interval. ***p-value in meta-analysis using Cochran-Mantel-Haenszel test.
(DOC)

Table S6 Oligonucleotide primers used for sequencing of the exons and the promoter region of the *MBP* gene.
(DOC)

Table S7 The list of genetic polymorphisms discovered by sequencing the exons and the promoter region of the *MBP* gene. *Positions of polymorphisms are according to NCBI Refseq Build 36.3. Polymorphisms are located between position 1 and position 2.
(DOC)

Method S1 Sequencing of the exons and the promoter region of the MBP gene.
(DOC)

Method S2 Bioinformatics analysis.
(DOC)

Method S3 Immunoblotting of anti-MBP antibody.
(DOC)

Method S4 Evaluation of non-specific binding of secondary antibodies.
(DOC)

Method S5 Amino acid analysis.
(DOC)

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The RA Clinical and Genetic Study Consortium includes Naoichiro Yukawa, Daisuke Kawabata, Takaki Nojima, Takashi Usui, Takao Fujii at Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto, Japan, Kota Shimada, Hiroshi Furukawa, Shigeto Tohma at Department of Rheumatology, National Hospital Organization Sagamihara Hospital, Sagamihara, Kanagawa, Japan, Kenichiro Matoba, Kiyoshi Takasugi at Department of Internal Medicine, Center for Rheumatic Diseases, Dohgo Spa Hospital, Matsuyama, Ehime, Japan, Kazuyuki Nakagome at Department of Allergy and Rheumatology, The University of Tokyo Hospital, Tokyo, Japan, Atsuo Taniguchi, Hisashi Yamanaka, Naoyuki Kamatani at Institute of

Rheumatology, Tokyo Women's Medical University, Tokyo, Japan, Hiromu Ito, Hiroyuki Yoshitomi, Takashi Nakamura at Department of Orthopaedic Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan, Ryoko Ito at Laboratory of Diagnostic Pathology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

Author Contributions

Conceived and designed the experiments: CT KO T. Mimori IM ML RS RY FM. Performed the experiments: CT M. Kokubo NY GD MS M. Katayama MT. Analyzed the data: CT RY SH IG ML YT T. Manabe HDGWG. Contributed reagents/materials/analysis tools: HDGWG RCGSC. Wrote the paper: CT KO MT T. Mimori ML FM.

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Predictive Value of Early Viral Dynamics During Peginterferon and Ribavirin Combination Therapy Based on Genetic Polymorphisms Near the *IL28B* Gene in Patients Infected With HCV Genotype 1b

Hidenori Toyoda,[1]* Takashi Kumada,[1] Toshifumi Tada,[1] Kazuhiko Hayashi,[2] Takashi Honda,[2] Yoshiaki Katano,[2] Hidemi Goto,[2] Takahisa Kawaguchi,[3] Yoshiki Murakami,[3] and Fumihiko Matsuda[3]

[1]*Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan*
[2]*Department of Gastroenterology, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[3]*Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan*

A study was carried out to determine whether early viral dynamics retain prediction of the outcome of peginterferon (PEG-IFN) and ribavirin combination therapy based on different genetic polymorphisms near the *IL28B* gene, the strongest baseline predictor of response to this therapy. A total of 272 patients infected with hepatitis C virus (HCV) genotype 1b were grouped according to genetic polymorphisms near the *IL28B* gene (rs8099917). The ability of reduced HCV RNA levels at 4 and 12 weeks after starting therapy to predict a sustained virologic response was evaluated based on these genotypes. Among patients with the TT genotype for rs8099917 (associated with a favorable response), the rates of sustained virologic response were higher in patients with a $\geq$3 $\log_{10}$ reduction in serum HCV RNA levels at 4 weeks after starting therapy ($P < 0.0001$). In contrast, among patients with the TG/GG genotype (associated with an unfavorable response), there were no differences in this rate based on the reduction in HCV RNA levels at 4 weeks. Early viral dynamics at 4 weeks after starting therapy retains its predictive value for sustained virologic response in patients with the TT genotype for rs8099917, but not in patients with the TG/GG genotype. Patients who are likely to achieve sustained virologic response despite unfavorable TG/GG genotype cannot be identified based on early viral dynamics during therapy. In contrast, lack of early virologic response at 12 weeks retains a strong predictive value for the failure of sustained virologic response regardless of *IL28B* polymorphisms, which remains useful as a factor to stop therapy. ***J. Med. Virol. 84:61–70, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: chronic hepatitis C; early viral dynamics; genetic polymorphisms near the *IL28B* gene; peginterferon; response-guided therapy; ribavirin

INTRODUCTION

The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although this treatment regimen has increased markedly the number of patients with a sustained virologic response, i.e., the eradication of hepatitis C virus (HCV), only 50% of patients infected with HCV genotype 1 achieved a sustained virologic response approximately.

Many investigators have examined factors that predict the treatment outcome of PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1. In addition to the baseline factors, the response of HCV during combination therapy, i.e., the changes in serum HCV RNA levels after starting therapy, has been shown to be an important predictor of the treatment outcome [Zeuzem et al., 2001; Buti

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*Correspondence to: Hidenori Toyoda, MD, PhD, Department of Gastroenterology, Ogaki Municipal Hospital 4-86, Minaminokawa, Ogaki, Gifu, 503-8502, Japan.
E-mail: hmtoyoda@spice.ocn.ne.jp

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et al., 2002; Berg et al., 2003], with the emphasis on "response-guided therapy" [Lee and Ferenci, 2008; Marcellin and Rizzetto, 2008]. Recent reports have emphasized the importance of evaluating the viral dynamics at 4 weeks after starting therapy to predict a sustained virologic response. A rapid virologic response, in which serum HCV RNA is undetectable at 4 weeks after starting therapy, has been the strongest predictive factor of a sustained virologic response reportedly [Martinez-Bauer et al., 2006; Poordad et al., 2008; de Segadas-Soares et al., 2009; Martinot-Peignoux et al., 2009]. In addition, the predictive value of reduced serum HCV RNA levels at 4 weeks after starting therapy has been clarified further, and a ≥ 3 $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy has high predictive value that a patient will achieve a sustained virologic response as a final outcome, even in the absence of a rapid virologic response [Toyoda et al., 2011].

In contrast, the lack of an early virologic response, defined as either undetectable serum HCV RNA or HCV RNA levels decreased by >2.0 $\log_{10}$ from the pretreatment level at 12 weeks after starting therapy, has been the most important predictor for the failure of a sustained virologic response in patients infected with HCV genotype 1 reportedly [Fried et al., 2002; Davis et al., 2003]. Therefore, treatment may be discontinued in patients without an early virologic response at 12 weeks of treatment, according to the recommendation in the AASLD guidelines [Ghany et al., 2009].

More recently, several studies reported that genetic polymorphisms near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19 affect the virologic response to PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. Furthermore, genetic polymorphisms near the *IL28B* gene are the strongest baseline predictive factor of the final outcome of combination therapy. An additional report showed the effects of genetic polymorphisms near the *IL28B* gene on HCV viral dynamics during PEG-IFN and ribavirin combination therapy [Thompson et al., 2010].

Although early HCV viral dynamics during therapy was shown originally to have a high predictive value for a sustained virologic response in HCV genotype 1-infected patients before genetic polymorphisms near the *IL28B* gene were linked to a therapeutic response, it is not clear whether early viral dynamics retain their predictive value in light of this additional information. The purpose of the present study was to investigate whether response-guided therapy based on viral dynamics at 4 or 12 weeks after initiating therapy retains its ability to predict the final outcome of PEG-IFN and ribavirin combination therapy after accounting for genetic polymorphisms near the *IL28B* gene.

MATERIALS AND METHODS

Patients and Treatment

Between January 2007 and June 2008, a total of 402 patients with chronic hepatitis C received antiviral combination therapy with PEG-IFN and ribavirin for HCV infection at the Ogaki Municipal Hospital or the Nagoya University Hospital. Among these patients, 272 were infected with HCV genotype 1b and had pretreatment HCV RNA levels >5.0 $\log_{10}$ IU/ml based on a quantitative real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Molecular Systems, Pleasanton, CA; Lower limit of quantification, 1.7 $\log_{10}$ IU/ml: Lower limit of detection, 1.0 $\log_{10}$ IU/ml) [Colucci et al., 2007; Pittaluga et al., 2008]. This study did not include any patients infected with HCV genotype 1a because this genotype is not found in the general Japanese population.

All patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough, Kenilworth, NJ) daily. The PEG-IFN and ribavirin doses were adjusted based on the patient's body weight. Patients weighing $\leq$45 kg were given 60 μg of PEG-IFN alpha-2b once a week, those weighing >45 and $\leq$60 kg were given 80 μg, those weighing >60 and $\leq$75 kg were given 100 μg, those weighing >75 and $\leq$90 kg were given 120 μg, and those weighing >90 kg were given 150 μg. Patients weighing $\leq$60 kg were administered 600 mg of ribavirin per day, those weighing >60 and $\leq$80 kg were given 800 mg per day, and those weighing >80 kg were administered 1000 mg per day. The PEG-IFN and ribavirin doses were modified based on the manufacturer's recommendations. All patients were scheduled to undergo 48 weeks of treatment. The treatment duration was extended up to 72 weeks in some patients. In addition, treatment was discontinued before 48 weeks in some patients who had a low likelihood of achieving an eradication of HCV due to the presence of serum HCV RNA at 24 weeks after starting therapy.

A sustained virologic response was defined as undetectable serum HCV RNA at 24 weeks after ending the therapy. A patient was considered to have relapsed when serum HCV RNA was detectable between the end of treatment and 24 weeks after completing treatment, although serum HCV RNA was undetectable during and at the end of therapy. Patients were considered to have non-response if serum HCV RNA was detectable at 24 weeks after initiating therapy (i.e., null response or partial response according to the American guidelines [Ghany et al., 2009]). Patients were considered to have a rapid virologic response if they had undetectable serum HCV RNA at 4 weeks after starting therapy. An early virologic response was defined as the disappearance or decrease in serum HCV RNA levels by at least 2 $\log_{10}$ at 12 weeks after starting therapy. Patients were considered to have a complete early virologic response if serum HCV RNA was undetectable at 12 weeks after starting therapy and a partial early virologic response if the serum

HCV RNA levels had decreased by at least 2 $\log_{10}$ at 12 weeks after initiating therapy. Patients were considered not to have an early virologic response if their HCV RNA levels did not decrease by more than 2 $\log_{10}$ at 12 weeks compared to the pretreatment levels. Patients were considered to have a slow virologic response if the serum HCV RNA became undetectable between 12 and 24 weeks.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of the Ogaki Municipal Hospital and the Nagoya University School of Medicine. Prior to initiating the study, each patient provided written informed consent to use the laboratory data, analyze genetic polymorphisms near the *IL28B* gene, and test stored serum samples.

Assessments of Serum HCV RNA Levels and Genetic Polymorphisms Near the *IL28B* Gene

After a patient provided informed consent, serum samples were obtained at the patient's regular hospital visits, just prior to initiating treatment, every 4 weeks during the treatment period, and during the 24-week follow-up period after treatment. Serum samples were stored at −80°C until further use. The HCV RNA levels were measured using a quantitative real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System).

Genotyping of rs 8099917 polymorphisms near the *IL28B* gene was performed using the TaqMan SNP assay (Applied Biosystems, Foster City, California) according to the manufacturer's guidelines. A predesigned and functionally tested probe was used for rs8099917 (C__11710096_10, Applied Biosystems).

Statistical analyses. Quantitative values are reported as the mean ± SD. In between-group differences were analyzed by the chi-square test. Univariate and multivariate analyses using a logistic regression model were performed to identify factors that predict a sustained virologic response, including age, sex, body weight, serum alanine aminotransferase activity, serum aspartate aminotransferase activity, serum gamma-glutamyl transpeptidase levels, serum alkaline phosphatase values, serum albumin levels, total serum bilirubin values, white blood cell counts, hemoglobin, platelet counts, hepatitis activity grade (A0 and A1 vs. A2 and A3), liver fibrosis grade (F0 and F1 vs. F2 and F3), pretreatment HCV RNA levels ($\geq$6.5 $\log_{10}$ vs. <6.5 $\log_{10}$), reduction in peginterferon dose and ribavirin dose, reduction in HCV RNA levels at 4 weeks after starting therapy ($\geq$3 $\log_{10}$ vs. <3 $\log_{10}$), and the type of an early virologic response. All *P*-values are two-tailed, and $P < 0.05$ was considered significant statistically.

RESULTS

The characteristics of the patients examined in this study are shown in Table I. Liver histology was evaluated according to the METAVIR score [The French METAVIR Cooperative Study Group, 1994]. Although some patients had a reduction in their PEG-IFN and ribavirin doses during therapy, respectively, all patients except for those who discontinued the therapy had more than 80% adhesion to both the PEG-IFN and ribavirin regimens. No patients discontinued the therapy because of adverse effects. The treatment duration was extended up to 72 weeks in 51 of 71 patients (71.8%) who exhibited a slow virologic response. As a final outcome, 118 patients (43.4%) achieved a sustained virologic response, 84 patients (30.9%) relapsed, and the remaining 70 patients (25.7%) had no response.

TABLE I. Characteristics of all Study Patients (n = 272)

Age (years)	56.0 ± 10.9
Sex (female/male)	139 (51.1)/133 (48.9)
Body weight (kg)	57.8 ± 10.5
Alanine aminotransferase (IU/L)	64.6 ± 56.4
Aspartate aminotransferase (IU/L)	53.9 ± 42.7
Gamma-glutamyl transpeptidase (IU)	48.5 ± 43.9
Alkaline phosphatase (IU/L)	267.9 ± 101.3
Albumin (g/dl)	4.04 ± 0.37
Total bilirubin (mg/dl)	0.79 ± 0.30
White blood cell count (/μl)	4892 ± 1333
Hemoglobin (g/dl)	14.0 ± 1.3
Platelet count ($\times 10^3$/μl)	163 ± 51
Liver histology-activity (A0/A1/A2/A3)*	3 (1.2)/136 (55.3)/ 92 (37.4)/15 (6.1)
Liver histology-fibrosis (F0/F1/F2/F3)*	27 (11.0)/114 (46.3)/ 70 (28.5)/35 (14.2)
Pretreatment HCV RNA concentration ($\log_{10}$ IU/ml)	6.35 ± 0.79
Reduction in the peginterferon dose	81 (29.8)
Reduction in the ribavirin dose	130 (47.8)
Final outcomes (sustained virologic response /relapse/ no response)	118 (43.4)/ 84 (30.9)/70 (25.7)

HCV, hepatitis C virus.
Percentages are shown in parentheses.
*Liver biopsy was not performed in 26 patients.

Reduction in Serum HCV RNA Levels at 4 Weeks after Starting Therapy and Treatment Outcome According to Genetic Polymorphisms Near the *IL28B* Gene

An analysis of genetic polymorphisms at rs8099917 near the *IL28B* gene indicated that 207 patients (76.1%) had a TT genotype, 3 patients had a GG genotype (1.1%), and the remaining 62 patients were TG heterozygote (22.8%). Table II shows the comparison of the background characteristics between patients with the favorable TT genotype and those with the unfavorable TG/GG genotype. As reported previously [Abe et al., 2010], gamma-glutamyl transpeptidase level was higher significantly in patients with the TG/GG genotype. As a final outcome, the rate of a sustained virologic response was higher significantly in patients with the TT genotype. Among 207 patients with the TT genotype, serum HCV RNA became undetectable in 19 patients (9.2%) at 4 weeks after starting therapy (a rapid virologic response). In the remaining 188 patients, the decrease in serum HCV RNA levels at 4 weeks after starting therapy ranged from 0.12

TABLE II. Characteristics of Study Patients According to the Genetic Polymorphisms Near the *IL28B* Gene

	Patients with TT genotype of rs8099917 (n = 207)	Patients with TG/GG genotype of rs8099917 (n = 65)	*P*-value
Age (years)	56.5 ± 10.4	54.4 ± 12.4	0.4112
Sex (female/male)	107 (51.7)/100 (48.3)	32 (49.2)/33 (50.8)	0.8384
Body weight (kg)	57.8 ± 10.9	57.8 ± 9.4	0.8361
Alanine aminotransferase (IU/L)	65.1 ± 53.3	62.8 ± 65.6	0.2548
Aspartate aminotransferase (IU/L)	53.6 ± 34.8	54.7 ± 62.0	0.3339
Gamma-glutamyl transpeptidase (IU)	44.2 ± 37.1	62.3 ± 59.0	0.0003
Alkaline phosphatase (IU/L)	263.1 ± 90.3	282.8 ± 129.9	0.3875
Albumin (g/dl)	4.04 ± 0.36	4.05 ± 0.43	0.8020
Total bilirubin (mg/dl)	0.79 ± 0.30	0.76 ± 0.32	0.3010
White blood cell count (/μl)	4826 ± 1333	5100 ± 1320	0.1608
Hemoglobin (g/dl)	13.9 ± 1.3	14.1 ± 1.4	0.3339
Platelet count ($\times 10^3$/μl)	161 ± 49	169 ± 57	0.3871
Liver histology-activity (A0/A1/A2/A3)*	2 (1.1)/98 (52.4)/ 74 (39.6)/13 (6.9)	1 (1.7)/38 (64.4)/ 18 (30.5)/2 (3.4)	0.3241
Liver histology-fibrosis (F0/F1/F2/F3)*	21 (11.2)/83 (44.4)/ 57 (30.5)/26 (13.9)	6 (10.2)/31 (52.5)/ 13 (22.0)/9 (15.3)	0.6401
Pretreatment HCV RNA concentration ($\log_{10}$ IU/ml)	6.37 ± 0.85	6.29 ± 0.55	0.0582
Reduction in the peginterferon dose	61 (29.5)	20 (30.8)	0.9644
Reduction in the ribavirin dose	101 (48.8)	29 (44.6)	0.5565
Final outcomes (sustained virologic response /relapse/ no response)	106 (51.2)/ 69 (33.3)/32 (15.5)	12 (18.4)/15 (23.1)/ 38 (58.5)	<0.0001

HCV, hepatitis C virus.
Percentages are shown in parentheses.
*Liver biopsy was not performed in 26 patients.

$\log_{10}$ to 5.71 $\log_{10}$ (mean, 3.12 $\log_{10}$). The reduction in serum HCV RNA levels was ≥3 $\log_{10}$ in 98 patients (47.3%), <3 $\log_{10}$ and ≥2 $\log_{10}$ in 52 patients (25.1%), <2 $\log_{10}$ and ≥1 $\log_{10}$ in 23 patients (11.1%), and <1 $\log_{10}$ in 15 patients (7.3%). Figure 1A shows the rate of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy in patients with the TT genotype. The rates were higher significantly in patients who achieved a rapid virologic response or had a ≥3 $\log_{10}$ decrease in

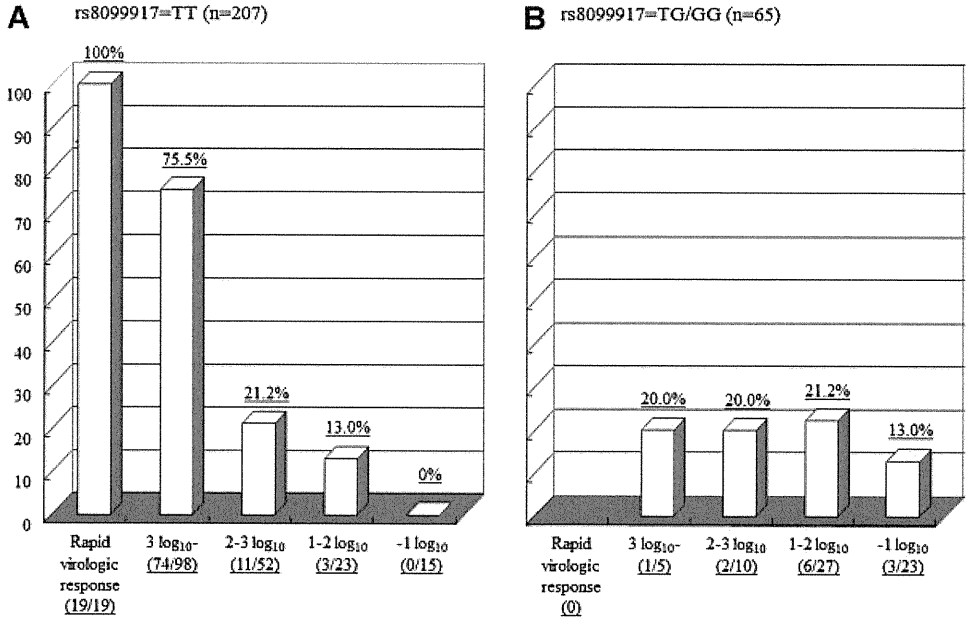

Fig. 1. The rate of sustained virologic responses (%) based on the reduction in serum HCV RNA levels at 4 weeks after starting therapy. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

serum HCV RNA levels at 4 weeks compared to those with a <3 $\log_{10}$ decrease in serum HCV RNA levels ($P < 0.0001$). When a 3 $\log_{10}$ decrease in serum HCV RNA levels was defined as the cut-off point, 56.5% of patients were considered to have a ≥3 $\log_{10}$ decrease in serum HCV RNA levels. The sensitivity, specificity, positive predictive value, and negative predictive value for a sustained virologic response were 86.8, 75.2, 78.6, and 84.4%, respectively.

Among the 65 patients who had the TG/GG genotype, no patient achieved a rapid virologic response at 4 weeks after initiating therapy. The decrease in serum HCV RNA levels at 4 weeks after starting therapy ranged from 0.11 $\log_{10}$ to 4.75 $\log_{10}$ (mean, 1.66 $\log_{10}$). The reduction in serum HCV RNA levels at 4 weeks after starting the therapy were smaller in patients with the TG/GG genotype than those with the TT genotype (1.66 ± 1.02 $\log_{10}$ in patients with the TG/GG genotype vs. 3.12 ± 1.37 $\log_{10}$ in patients with TT genotype excluding RVR, $P < 0.0001$). The reduction in serum HCV RNA levels was ≥3 $\log_{10}$ in five patients (7.7%), <3 $\log_{10}$ and ≥2 $\log_{10}$ in 10 patients (15.4%), <2 $\log_{10}$ and ≥1 $\log_{10}$ in 27 patients (41.5%), and <1 $\log_{10}$ in 23 patients (35.4%). Figure 1B shows the rates of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy in patients with the TG/GG genotype. There were no differences in the rate of a sustained virologic response based on the reduction in HCV RNA levels at 4 weeks after starting therapy; the rate of a sustained virologic response remained at 20% approximately regardless of the reduction in HCV RNA levels in 42 patients with a ≥1 $\log_{10}$ reduction in serum HCV RNA levels.

Association Between an Early Virologic Response at 12 Weeks and Treatment Outcome Based on Genetic Polymorphisms Near the *IL28B* Gene

Figure 2 shows the rate of patients with the TT genotype or TG/GG genotype for rs8099917 who achieved a complete early virologic response, a partial early virologic response, and those who did not achieve early virologic response at 12 weeks after starting therapy based on the reduction in serum HCV RNA level at 4 weeks after initiating therapy. Nearly 75% of patients with the TT genotype whose HCV RNA levels were reduced by ≥3 $\log_{10}$ at 4 weeks after starting the therapy achieved a complete early virologic response. In contrast, 80% of patients with the TG/GG genotype whose HCV RNA levels were reduced by ≥3 $\log_{10}$ at 4 weeks after starting the therapy showed a partial early virologic response. The majority of patients with the TT or TG/GG genotypes achieved a partial early virologic response when their reduction in HCV RNA levels was <3 $\log_{10}$ and ≥2 $\log_{10}$ or <2 $\log_{10}$ and ≥1 $\log_{10}$.

Figure 3 shows the rates of a sustained virologic response according to the type of early virologic response in patients with the TT genotype (Fig. 3A) and TG/GG genotype (Fig. 3B). Among patients with the TT genotype, the rate of sustained virologic response was significantly higher in patients with a complete early virologic response than in those with a partial early virologic response ($P < 0.0001$). In contrast, there was no difference in the rate of a sustained virologic response between patients with a complete early virologic response and those with a partial early virologic response ($P = 0.8917$) among patients with

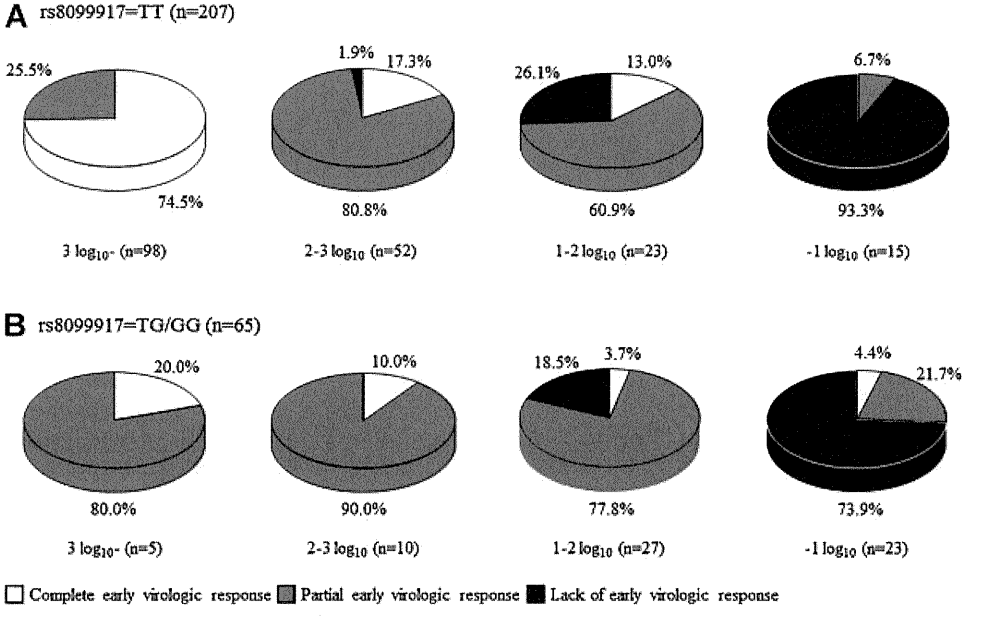

Fig. 2. The association between the virologic responses at 12 weeks after starting therapy and the reduction in serum HCV RNA levels at 4 weeks after starting therapy. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

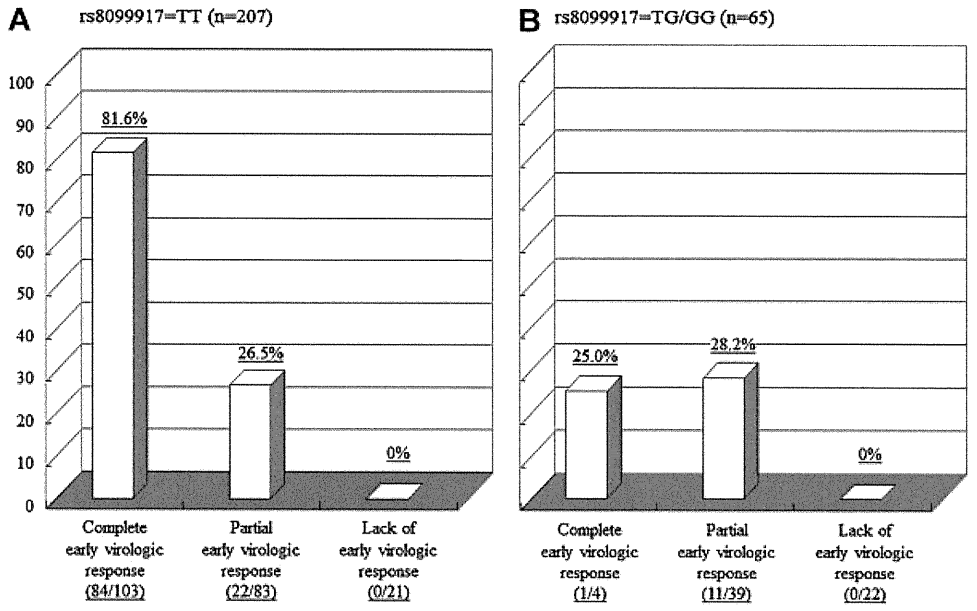

Fig. 3. The rate of sustained virologic responses based on the type of early virologic response. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

the TG/GG genotype. None of the patients with the TT genotype or TG/GG genotype who yielded a lack of an early virologic response reached a sustained virologic response.

Univariate and Multivariate Analyses for Factors Associated With a Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients With the TT and the TG/GG Genotype for the rs8099917

Univariate and multivariate analyses were conducted for factors associated with a sustained virologic response based on different genetic polymorphisms near the *IL28B* gene. In patients with the TT genotype, the factors that were associated with a sustained virologic response included serum alkaline phosphatase levels, serum albumin, platelet counts, hepatitis activity grade, liver fibrosis grade, reduction in HCV RNA levels at 4 weeks after starting therapy, and a complete early virologic response based on a univariate analysis (Table IIIA). In a multivariate analysis, the serum albumin levels, reduction in HCV RNA levels 4 weeks after starting therapy, and a complete early virologic response were independent factors that were significantly associated with a sustained virologic response (Table IIIB). A reduction in HCV RNA levels 4 weeks after starting therapy was the strongest factor that affected a sustained virologic response. In patients with the TG/GG genotype, the factors that were associated with a sustained virologic response included patient age, platelet counts, and pretreatment HCV RNA levels based on a univariate analysis (Table IIIA). A reduction in the HCV RNA levels at 4 weeks after starting therapy was not associated with a sustained virologic response. In a multivariate analysis, patient age and pretreatment HCV RNA levels were independent factors that were significantly associated with a sustained virologic response (Table IIIC).

Characteristics of Patients who Achieved a Sustained Virologic Response to the Combination Therapy Despite the Unfavorable TG/GG Genotype Near the *IL28B* Gene

Table IV shows the characteristics of 12 patients who achieved a sustained virologic response despite having the unfavorable TG/GG genotype for rs8099917 near the *IL28B* gene. All but one patient was under 60 years old and had liver fibrosis not more than grade 2 (one patient did not undergo a liver biopsy). Except for one patient, the reduction in the serum HCV RNA levels at 4 weeks after starting therapy was less than 3 $\log_{10}$ and all but one patient showed a partial early virologic response at 12 weeks after starting the therapy. In all 11 patients with a partial early virologic response, the serum HCV RNA was undetectable up to 24 weeks after starting the therapy. All but one patient extended the treatment duration from 48 to 72 weeks (two patients discontinued therapy at 60 weeks during the extended treatment period). When the characteristics of patients who achieved a sustained virologic response were compared between those with the unfavorable TG/GG genotype and those with the favorable TT genotype, patients with the TG/GG genotype were younger (41.8 ± 14.4 years vs. 55.1 ± 10.4 years, $P = 0.0023$) and had lower pretreatment HCV RNA levels (5.91 ± 0.44 $\log_{10}$ IU/ml vs. 6.21 ± 1.05 $\log_{10}$ IU/ml, $P = 0.0199$).

TABLE III. Univariate and Multivariate Analyses for Factors Associated With a Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients With the TT and the TG/GG Genotype for the rs8099917

(A) Univariate analyses	*P*-value	
	Patients with TT genotype of rs8099917 (n = 207)	Patients with TG/GG genotype of rs8099917 (n = 65)
Age (years)	0.0505	0.0007
Sex (female/male)	0.1830	0.2296
Body weight (kg)	0.6891	0.2456
Alanine aminotransferase (IU/L)	0.7988	0.4032
Aspartate aminotransferase (IU/L)	0.5021	0.1705
Gamma-glutamyl transpeptidase (IU)	0.6340	0.6648
Alkaline phosphatase (IU/L)	0.0315	0.0599
Albumin (g/dl)	0.0002	0.6594
Total bilirubin (mg/dl)	0.2929	0.7130
White blood cell count (/μl)	0.2508	0.5549
Hemoglobin (g/dl)	0.0847	0.2289
Platelet count ($\times 10^3/\mu l$)	0.0454	0.0411
Liver histology-activity (A0–1/A2–3)	0.0445	0.1117
Liver histology-fibrosis (F0–1/F2–3)	0.0002	0.2283
Pretreatment HCV RNA concentration (≥ 6.5 $\log_{10}$ vs. <6.5 $\log_{10}$)	0.5279	0.0379
Reduction in the peginterferon dose	0.4316	0.5563
Reduction in the ribavirin dose	0.1823	0.4272
Reduction in HCV RNA levels at 4 weeks after starting the therapy (≥ 3 $\log_{10}$ vs. < 3 $\log_{10}$)	<0.0001	0.9265
Early virologic response (complete vs. partial)	<0.0001	0.9777
Early virologic response (partial vs. non)	0.8632	0.0686

(B) Multivariate analyses: Patients with TT genotype of rs8099917	*P*-value	Odds ratio (95% confidence interval)
Alkaline phosphatase (IU/L)	0.2617	
Albumin (g/dl)	0.0365	28.287 (1.4107–755.41)
Platelet count ($\times 10^3/\mu l$)	0.2599	
Liver histology-activity (A0–1/A2–3)	0.6678	
Liver histology-fibrosis (F0–1/F2–3)	0.2307	
Reduction in HCV RNA levels at 4 weeks after starting the therapy (≥ 3 $\log_{10}$ vs. <3 $\log_{10}$)	<0.0001	16.029 (6.8593–40.406)
Early virologic response (complete vs. partial)	0.0224	0.3685 (0.1557–0.8749)

(C) Multivariate analyses: Patients with TG/GG genotype of rs8099917	*P*-value	Odds ratio (95% confidence interval)
Age (years)	0.0022	0.0034 (0.0000–0.0840)
Platelet count ($\times 10^3/\mu l$)	0.3344	
Pretreatment HCV RNA concentration (≥ 6.5 $\log_{10}$ vs. <6.5 $\log_{10}$)	0.0304	0.0548 (0.0020–0.4950)

HCV, hepatitis C virus.

DISCUSSION

Several previous studies reported that patients who achieved a rapid virologic response, in which serum HCV RNA become undetectable at 4 weeks after starting therapy, had a high likelihood of achieving a sustained virologic response [Martinez-Bauer et al., 2006; Poordad et al., 2008; de Segadas-Soares et al., 2009; Martinot-Peignoux et al., 2009]. In addition, several recent studies reported the predictive value of the degree of reduction in serum HCV RNA levels at 4 weeks after starting therapy [Yu et al., 2007; Huang et al., 2010; Toyoda et al., 2011]. Therefore, the viral dynamics of HCV at 4 as well as 12 weeks after starting therapy is important for response-guided therapy.

Genetic polymorphisms near the *IL28B* gene have emerged as the strongest predictive factor of a sustained virologic response in patients infected with HCV genotype 1 [Hayes et al., 2011; Kurosaki et al., 2011]. In addition, Thompson et al. [2010 reported that genetic polymorphisms near the *IL28B* gene were associated strongly with early viral dynamics during PEG-IFN and ribavirin combination therapy. These findings raised an important issue of whether response-guided therapy, based on the reduction in serum HCV RNA levels at 4 or 12 weeks after starting

TABLE IV. Patients who Achieved a Sustained Virologic Response Despite the TG/GG Genotype for the rs8099917

	Age (years)	Sex	Liver histology	Pretreatment HCV RNA level (log_{10} IU/ml)	HCV RNA reduction at 4 weeks	Response at 12 weeks	HCV RNA became undetectable (weeks)	Treatment duration (weeks)
1.	31	Female	A1/F1	6.13	2.19	partial EVR	20	48
2.	55	Male	A1/F1	5.80	1.77	partial EVR	16	72
3.	57	Female	A1/F1	5.58	3.01	partial EVR	16	72
4.	57	Female	A1/F1	6.21	1.81	partial EVR	20	72
5.	62	Male	N.D.	6.23	1.13	partial EVR	24	72
6.	21	Male	A1/F2	6.04	1.83	partial EVR	24	72
7.	42	Male	A1/F1	6.27	0.57	partial EVR	24	72
8.	29	Female	A1/F2	5.83	1.83	partial EVR	20	60
9.	52	Male	A1/F0	5.91	2.12	complete EVR	12	48
10.	40	Male	A2/F1	5.84	1.34	partial EVR	20	72
11.	27	Male	N.D.	5.63	0.42	partial EVR	24	72
12.	28	Male	A1/F0	6.59	0.76	partial EVR	20	60

N.D., not done; HCV, hepatitis C virus; EVR, early virologic response.

therapy, retains a predictive value when considering genetic polymorphisms near the *IL28B* gene.

In the present study, the predictive value of the decrease in serum HCV RNA levels was evaluated at 4 and 12 weeks after starting therapy in Japanese patients infected with HCV genotype 1b based on genetic polymorphisms near the *IL28B* gene. Consistent with previous reports, patients with the TG/GG genotype for rs8099917 had a smaller reduction in serum HCV RNA levels at 4 weeks after starting treatment ($P < 0.0001$), which indicates an unfavorable response to the combination therapy. Patients with the TT genotype for rs8099917, which is associated with a favorable response to the combination therapy, exhibited a significant difference in the rate of a sustained virologic response based on the reduction in serum HCV RNA levels at 4 weeks after initiating the therapy. Patients with a rapid virologic response or with a ≥ 3 log_{10} reduction in HCV RNA levels had a higher likelihood of achieving a sustained virologic response.

In contrast, these factors did not have any predictive value in patients with the TG/GG genotype. Only 18.5% of patients achieved a sustained virologic response (12 of 65 patients), and it was difficult to identify these patients based on the reduction in HCV RNA levels at 4 weeks or the type of an early virologic response at 12 weeks after starting therapy. Patients who achieved a sustained virologic response, despite the TG/GG genotype for rs8099917, were identified among those with a <2 log_{10} and ≥ 1 log_{10} or even <1 log_{10} reduction in HCV RNA levels at 4 weeks after starting therapy. Interestingly and paradoxically, the possibility of a sustained virologic response can be expected in patients with a <1 log_{10} reduction in HCV RNA levels at 4 weeks after starting therapy only when they have the unfavorable TG/GG genotype.

In the evaluation at 12 weeks after starting therapy, patients with the TT genotype who achieved a complete early virologic response had a higher rate of a sustained virologic response significantly than patients who achieved a partial early virologic response, whereas this difference was not found in patients with the TG/GG genotype. No patients who failed to achieve an early virologic response achieved a sustained virologic response regardless of the genetic polymorphisms near the *IL28B* gene. Thus, the lack of an early virologic response retained a strong predictive value for the failure of achieving a sustained virologic response. This result supports the recommendation in the AASLD guidelines, in which treatment may be discontinued in patients without an early virologic response at 12 weeks of treatment.

The characteristics of patients who achieved a sustained virologic response despite the unfavorable TG/GG genotype were younger in age and lower pretreatment HCV RNA levels. Most patients with the TG/GG genotype who achieved a sustained virologic response showed a partial early virologic response and extended the treatment duration. It was difficult to identify these patients according to viral dynamics at 4 or 12 weeks after starting therapy.

There are several limitations in this study. Some patients with a slow virologic response did not have their treatment period extended from 48 to 72 weeks. This is because the effectiveness of a 72-week combination therapy regimen in patients with HCV genotype 1 with a slow virologic response [Berg et al., 2006; Pearlman et al., 2007] had not been established in Japan in the earlier part of this study. This fact might have influenced the treatment outcome especially in patients with the unfavorable TG/GG genotype. Another limitation is a smaller sample size of patients with the TG/GG genotype in comparison to that of patients with the TT genotype. This sample size could have caused the lack of statistical significance in the rate of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy or according to the type of an early virologic response in patients with the TG/GG genotype. In addition, the data were based on Japanese patients infected with HCV genotype 1b. Therefore, these results should be confirmed in other ethnicities and patients infected with HCV genotype 1a.

In conclusion, among patients infected with HCV genotype 1b with the TT genotype for rs8099917, a rapid virologic response or a ≥ 3 $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy, or a complete early virologic response indicate strongly that these patients will achieve a sustained virologic response as a final outcome for PEG-IFN and ribavirin combination therapy. Early viral dynamics retain the predictive value in this patient subpopulation. A reduction in HCV RNA levels at 4 weeks after starting therapy or the type of an early virologic response does not predict the likelihood that patients with the TG/GG genotype will achieve a sustained virologic response. In contrast, the lack of an early virologic response retains a strong predictive value for the failure to achieve a sustained virologic response regardless of *IL28B* polymorphisms, which remains useful as a factor to stop therapy.

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研究成果の刊行に関する一覧表

研究分担者　琉球大学大学院医学研究科 准教授　齊藤峰輝

雑誌

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RETROVIROLOGY

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Identification of an unique CXCR4 epitope whose ligation inhibits infection by both CXCR4 and CCR5 tropic human immunodeficiency type-I viruses

Tetsuya Adachi[1], Reiko Tanaka[1], Akira Kodama[1], Mineki Saito[1], Yoshiaki Takahashi[2], Aftab A Ansari[2] and Yuetsu Tanaka[1*]

Abstract

Background: Small chemical compounds which target chemokine receptors have been developed against human immunodeficiency virus type 1 (HIV-1) and are under investigation for use as anti-HIV-1 microbicides. In addition, monoclonal antibodies (mAbs) against chemokine receptors have also been shown to have anti-HIV-1 activities. The objective of the present study was to screen a panel of three anti-CXCR4 specific monoclonal antibodies (mAbs) for their ability to block the HIV-1 infection using *in vitro* activated primary peripheral blood mononuclear cells (PBMCs).

Results: PBMCs from normal donors were pre-activated with anti-CD3 and anti-CD28 mAbs for 1 day, and aliquots were infected with a low dose of CCR5-tropic (R5), CXCR4 tropic (X4) or dual tropic (X4R5) HIV-1 isolates and cultured in the presence of a panel of anti-CXCR4 mAbs. The panel included clones A145 mAb against the N-terminus, A120 mAb against a conformational epitope consisting of extracellular loops (ECL)1 and ECL2, and A80 mAb against ECL3 of CXCR4. Among these mAbs, the A120 mAb showed the most potent inhibition of infection, by not only X4 but surprisingly also R5 and X4R5 HIV-1. The inhibition of R5 HIV-1 was postulated to result from the novel ability of the A120 mAb to induce the levels of the CCR5-binding β-chemokines MIP-1α, MIP-1β and/or RANTES, and the down modulation of CCR5 expression on activated $CD4^+$ T cells. Neutralizing anti-MIP-1α mAb significantly reversed the inhibitory effect of the A120 mAb on R5 HIV-1 infection.

Conclusions: The **d**ata described herein have identified a unique epitope of CXCR4 whose ligation not only directly inhibits X4 HIV-1, but also indirectly inhibits R5 HIV-1 infection by inducing higher levels of natural CCR5 ligands.

Background

CXCR4 and CCR5 belonging to the family of G-protein coupled receptors (GPCR) serve as receptors for the CXC-chemokine stromal derived factor 1 (SDF-1) and the CC-chemokines MIP-1α, MIP-1β and RANTES, respectively. The ligation of these chemokine receptors transmits a number of intracellular signals, and the receptors also serve as co-receptors for HIV-1 [1-5]. Under normal physiological conditions, CXCR4 molecules form closely linked dimers [6] and heterodimers with other chemokine receptors including CCR5 [7]. CXCR4 is expressed extracellularly, consisting of an N-terminal (NT) region and extracellular loops (ECL) 1, ECL2 and ECL3. Several lines of evidence indicate that the interaction between CXCR4 and SDF-1 or HIV-1 involves multiple domains of the receptor. For example, while the NT and the ECL2 domains appear to be critical for SDF-1 binding and signaling, the regions contiguous to the ECL2 and ECL3 have been implicated in HIV-1 co-receptor activity and homologous cell adhesion [8-11]. Studies with CXCR4 mutants have revealed that the HIV-1 co-receptor activity of CXCR4 is

* Correspondence: yuetsu@s4.dion.ne.jp
[1]Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan
Full list of author information is available at the end of the article

independent of its ability to function as a chemokine receptor and/or transduce intracellular signaling [11,12].

Current and prospective anti-HIV-1 therapy includes the use of small chemical compounds which target chemokine receptors that are termed viral occupancy inhibitors (VIROC) [13]. In addition, mAbs against chemokine receptors have also been shown to have a potential for HIV-1 inhibition. For example, an anti-human CCR2 mAb that is neither an agonist nor an antagonist blocks both X4 and R5 HIV-1, due to oligomerization of CCR2 with CCR5 and CXCR4, but not receptor down-modulation [14]. In addition, an unique mAb with specificity for the N-terminus region of CCR5 that does not block the interaction between HIV-1 gp120 and CCR5, blocks R5 HIV-1 infection by inducing CCR5 dimerization [15].

Herein, we examined a series of three rat IgG anti-human CXCR4 mAbs made by our laboratory [16], and we demonstrate that clone A120, that recognizes a conformational epitope encompassing the ECL1 and ECL2 domains of CXCR4, has a unique functional property. Thus, the interaction of the A120 mAb with CXCR4 inhibits not only X4, but also R5 HIV-1 infection of in vitro activated PBMCs, via mechanisms detailed herein. The novel anti-CXCR4 mAb function described in this study potentially provides a unique adjunct to conventional anti-HIV-1 chemotherapy with activity against not only CXCR4 but also CCR5 and dual tropic HIV-1.

Results

Suppressive effects of anti-CXCR4 mAbs on HIV-1 infection in primary activated PBMCs

We first tested our 3 different anti-CXCR4 mAb clones (A145, A120 and A80) for their potential to inhibit the infection of the prototype X4 HIV-1_{NL4-3} and for purposes of controlling the prototype R5 HIV-1_{JR-FL} in *in vitro* activated primary PBMC cultures. None of these anti-human CXCR4 mAbs cross-reacts with human CCR5, and only the A120 mAb can block the SDF-1-mediated Ca^{2+} influx [16]. Thus, the PBMCs infected with low levels of HIV-1 (at a multiplicity of infection of lower than 0.01) were cultured for 5 days in the presence or absence of 10 μg/ml of either anti-CXCR4 mAb or isotype control. As shown in Figure 1a, while the A145 mAb had minimal inhibitory effect, the A120 and A80 mAbs markedly inhibited the infection of the

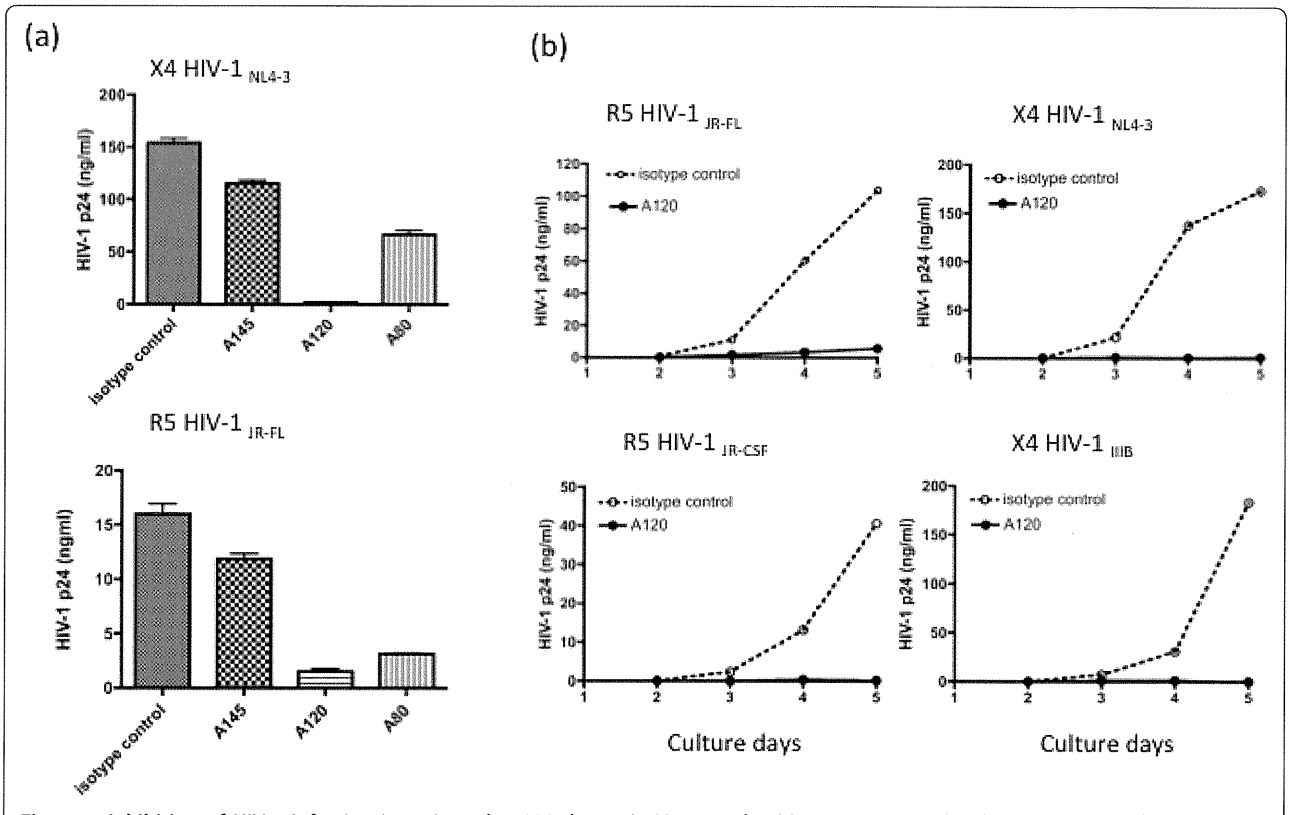

Figure 1 Inhibition of HIV-1 infection in activated PBMCs by anti-CXCR4 mAbs. (a) PBMCs activated with anti-CD3/CD28 for 1 day were infected with either R5 HIV-1_{JR-FL} or X4 HIV-1_{NL4-3} for 2 hours, washed and then cultured in the presence of 10 μg/ml of the A145, A120, A80 rat IgG mAbs or isotype control rat IgG mAb mixture. After 5 days, virus production in the culture supernatants was determined by p24 ELISA. (b) Activated PBMCs infected with R5 HIV-1_{JR-FL}, R5 HIV-1_{JR-CSF}, X4 HIV-1_{NL4-3} or X4 HIV-1_{IIIB} were aliquoted and cultured in the presence of 10 μg/ml of the A120 mAb or isotype control mAb. The p24 levels in the culture supernatants were monitored daily by ELISA. Data shown for both (a) and (b) are representative of 3 independent experiments using PBMCs from different donors.

X4, but to our surprise, also the R5 HIV-1 strain. Since the inhibitory potential of the A120 mAb was the highest among these mAbs, we selected the A120 mAb for further characterization. Although the production of HIV-1 from activated PBMCs was influenced by culture conditions, mostly cell concentration at time of infection and cultivation steps, as shown in Figure 1b, the inhibitory effect of A120 mAb was further confirmed using an additional R5 (JR-CSF) and X4 (IIIB) HIV-1 strains.

To examine tPBMC donor variabilities, the ability of the A120 mAb to inhibit R5 HIV-1_{JR-FL} and X4 HIV-1_{NL4-3} in activated PBMCs from 6 different unrelated donors was also studied. Viral production was quantitated by measuring both the levels of p24 and the frequency of infected cells using flow cytometry as outlined in the methods section. As seen in Figure 2a, whereas there was indeed considerable variability in the relative susceptibility of *in vitro* activated PBMCs from different donors to support R5 and X4 HIV-1 infection, the addition of the A120 mAb to the cultures showed variable levels of moderate to significant inhibition in each case (differences in the ability of PBMCs from different donors to support R5 versus X4 HIV-1 is an interesting subject that is currently under study). In addition, the fact that the addition of the A120 mAb also inhibited the increase in the frequency of infected

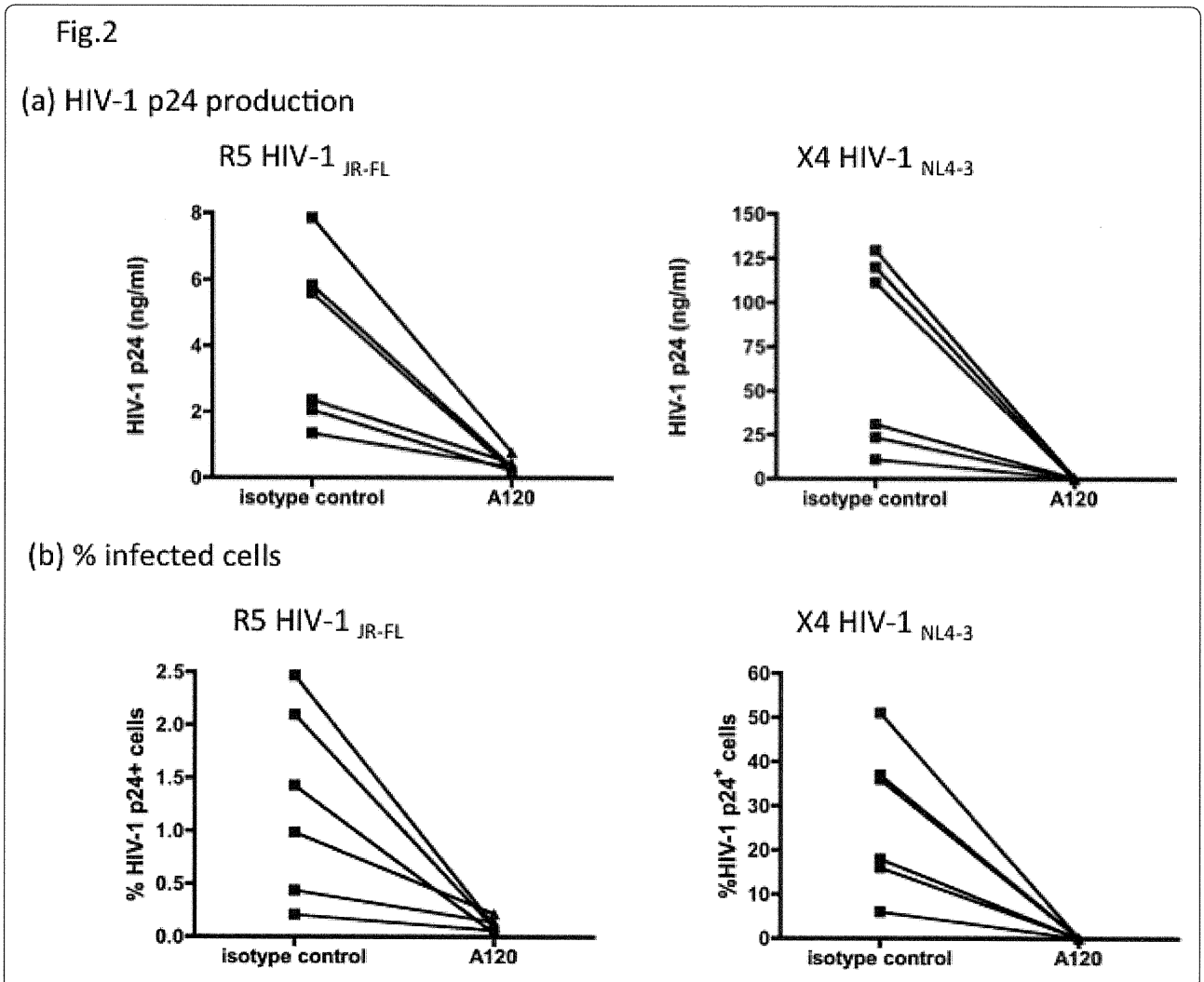

Figure 2 The A120 mAb-mediated inhibition of HIV-1 infection in activated PBMCs from different donors. Activated PBMCs from 6 different donors were infected with either R5 HIV-1_{JR-FL} or X4 HIV-1_{NL4-3} for 2 hours. After extensive washing, the PBMCs were aliquoted and cultured in the presence of A120 or isotype control IgG at 10 μg/ml. (a) After 3~5 days, virus production was determined by p24 ELISA in the culture supernatants, and values obtained on day 4 are shown as representative. P values were 0.007 and 0.032 for R5 HIV-1 and X4 HIV-1, respectively. (b) The PBMC samples obtained on day 4 after infection were fixed and permeabilized, and then stained with anti-HIV-1 p24 mAb labeled with Alexa Fluor 488 and examined by flow cytometry. The frequencies (percentages) of $p24^+$ cells were plotted. P values were 0.026 and 0.031 for R5 HIV-1 and X4 HIV-1, respectively. Representative data from 3 independent experiments are shown.