

**Figure 1. (continued)** (C) Histology and cytology of B-LBL cells with dual integration. Immunostaining of Vpreb, and hematoxylin and eosin stainings are shown. Original magnification was  $\times 1000$ . Vpreb is a component of the pre-BCR, a surface marker of the pre-B cells. (D) IgH gene rearrangement in FACS-purified BM pre-B cells and lymphomas. Lane 1, BM pre-B cells from SL/Kh mice at 3 weeks of age; lanes 2–4, SL/Kh, lymphoma cells with MLV integrations into *Stat5a* and *Fiz1*; lanes 5 and 6, SL/Kh, lymphoma cells with integrations into *Stat5a* and *Hiphk2*; lane 7, SL/Kh lymphoma cells with MLV integrations into *Stat5a* and *Hiphk2* but not monoclonal. In lanes 1 and 7, polyclonally or oligoclonally rearranged bands were observed. (E) RT-PCR assay. In double-integration lymphomas, neither Igκ nor Igλ was detected.

sequences were: 5B4, 5'-GAGGGCTTGGACCTCTCGTCTCCTA-AAAAACCAGG-3', and 5F1, 5'-GTCTCTCCCAAAGTCTGCCCTCTC-CAACC-3', in the first step and 5F2, 5'-CCTCCTCTGACGGAGATGGC-GACAGAGAAGAGG-3', and 5B1, 5'-GAGGGCTTGGACCTCTCGTCTC-CTAAAAGAACCACG-3', in the second step of cycles for nested PCR. Sequence analysis of the amplification products was performed with a Thermosequenase kit (GE Healthcare, Buckinghamshire, UK).

### Southern blot of lymphoma DNA

The detailed protocol for detection of provirus integration into the host gene has been reported previously [9]. In brief, lymphoma DNA was digested with *NcoI* and *ScaI* (New England Biolabs, Ipswich, MA, USA). Probes for *Fiz1* and *Hiphk2* were synthesized by PCR from the genomic DNA of SL/Kh mice and labeled with the Megaprime DNA-labeling system (GE Healthcare). The sequences were: 5'-CTTCCGTCATGGAAGACTCC-3' and 5'-AGCAGGCCAGTG-GAGTCTCG-3' for *Fiz1* (exon 2) and 5'-TACCTTACGAGCAGACCATC-3' and 5'-CAAAGACTAAGCAGGTGTGG-3' for *Hiphk2* (exon 2). When the integration was completely clonal, the density ratio of the digested fragment, including MLV and the host gene DNA from the integrated allele, to the native *Stat5a* gene from the intact allele was nearly 1.0.

### Colony growth assay

Prior to stimulation, lymphoma cells were grown for 6 h in RPMI. Lymphoma cells were washed once with PBS. After washing, they were suspended in 10 ml of a semi-solid medium.

Methocult (Stemcell Technologies, Vancouver, Canada), containing IL-7, was used as a basic semisolid medium in the colony assay. To detect early growth before 48 h, we defined the colony as a cluster of greater than five cells but after 48 h, only those of  $> 20$  cells. The cells were incubated in the

medium with 10% FCS, 50 mM  $\beta$ -ME, and 100 mg/ml penicillin-streptomycin. Cultures were established in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>.

### Ig gene recombination in the genome of lymphoma tissue

We confirmed the clonality by detection of a single IgH D<sub>H1</sub>J<sub>H1</sub> recombination [8]. In brief, the lymphoma genomic DNA was subjected to PCR with primers designed to amplify four possible junctions between the D-Q52 and J<sub>H1</sub> regions. The primers for the first round were: 5'-CACAGAGAATTCATAGTGTGAT-AGCTCAG-3' (D<sub>H1</sub>Q52-1; sense) and 5'-AGGCTCTGAGATCCCTAGACAG-3' (J<sub>H1</sub>4-1; antisense). PCR conditions were as follows: denaturation for 1 min at 95°C, annealing for 1 min at 60°C, and extension for 2.5 min at 72°C (28 cycles). Two microliters of each reaction was subjected to a second round of PCR using a pair of internal primers: 5'-GCCTCAGAATTCCTGTGCTCTCT-GACTGGT-3' (D<sub>H1</sub>Q52-2; sense) and 5'-GGGTCTAGACTCTCAGCCGGCTC-CCTCAGGG-3' (J<sub>H1</sub>4-2; antisense). PCR was repeated as above. The PCR products were electrophoresed in an agarose gel, blotted onto a nylon membrane (GE Healthcare), and probed with a J<sub>H1</sub>4 probe for confirmation of the clonality of the cells by detection of IgH rearrangement [20]. RT-PCR assay for IgL will be described below. IgH J<sub>H1</sub> probe was the *EcoRI-HindIII* fragment encoding J<sub>H1</sub>4 [5, 24].

### Flow cytometry

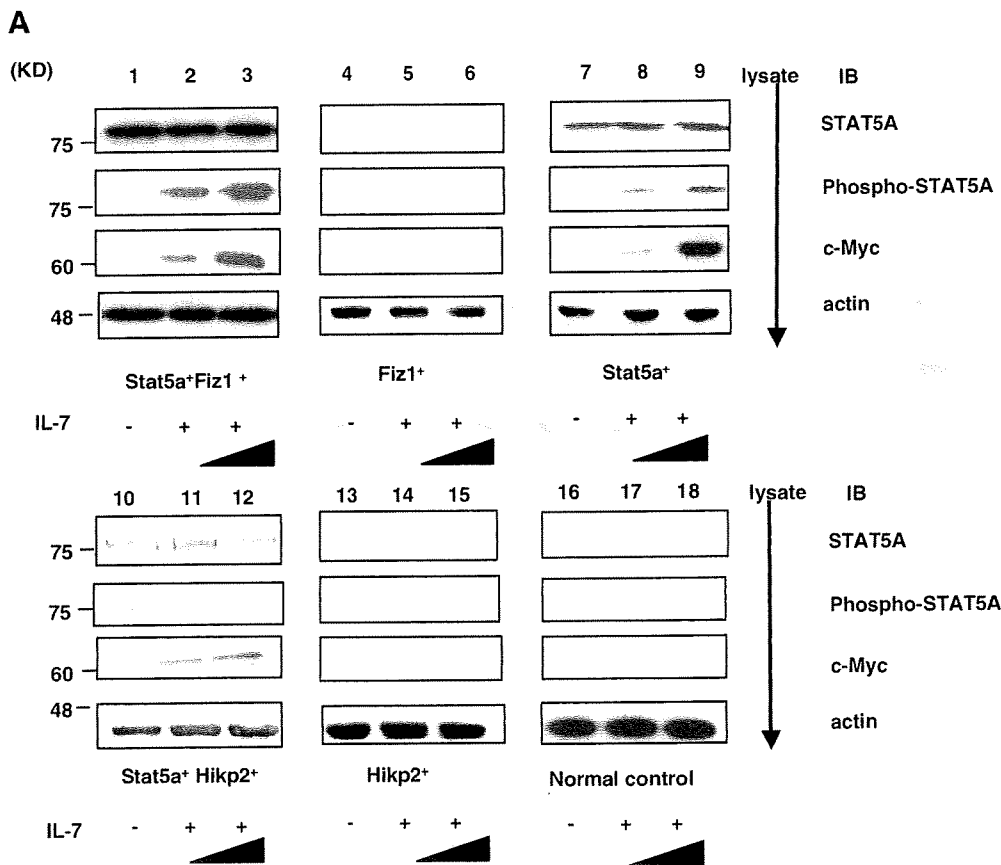
We used a protocol that has been described previously [8]. In brief, a single-cell suspension was prepared from femur BM plugs and axillary lymphoma tissue. The cell density was adjusted to 106 cells/ml, and the cells were analyzed in a FACScan apparatus (Becton Dickinson, Mountain View, CA, USA). The following antibodies were purchased commercially: FITC-labeled anti-BP-1 (clone 6C3), PE-labeled anti-B220 (clone RA3-6B2), anti-IL-7R (clone A7R34), and anti-CD24 (clone 30-F1;

eBioscience, San Diego, CA, USA) and anti-CD43 (clone S7), FITCanti mouse IgG1, and PE-anti mouse IgG2 (BD Biosciences, San Diego, CA, USA). We stained BM early B cells and their fate in SL/Kh, NFS.SL/Kh-Bomb1, and NFS. It was contained with 1:10,000-diluted mAb for 15 min.

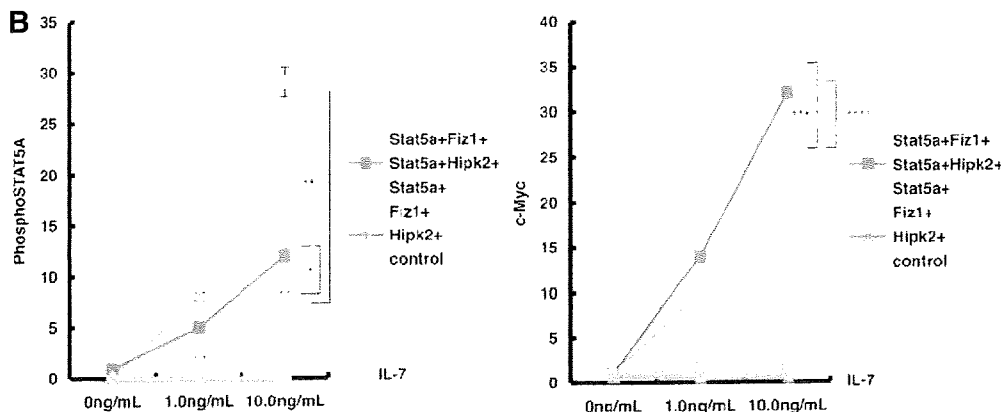
**Western blots and immunohistochemistry**

The extract of sorted BP-1<sup>+</sup> B220<sup>+</sup> pre-B cells from the SL/Kh BM were used as a control. Antibodies to Fiz1 (sc-101955), Flt3 (sc-1496-1), Hipk2 (sc-10294), and Vpreb (sc-33128) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Western blotting protocol has been described previously [5]. Phospho-specific antibodies against STAT5A (Tyr694) were obtained from Cell Signaling Technology (Beverly, MA, USA). Total cell lysate was precleared using protein L-Sepharose (Pierce Biotechnology, Rockford, IL, USA) or pro-

tein G-Sepharose (Sigma-Aldrich, St. Louis, MO, USA) at 4°C with 1 h of agitation. For each immunoprecipitation reaction, 400 microliters of the cleared lysate was incubated with 50 microliters MEM59 at 4°C overnight, and then 50 microliters protein L-Sepharose or protein G-Sepharose was added and incubated for an additional 1 h. Immunocomplexes were precipitated by centrifugation, washed three times with a radioimmunoprecipitation assay buffer [25 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Nonidet P-40, a protease inhibitor mixture (Complete Mini EDTA-free, Roche Diagnostics, Mannheim, Germany)], and finally, resuspended in the sample buffer and boiled for 5 min. The released proteins were examined by Western blotting. Antibody against mouse Ig polyclonal antibody was used for control of the blot of the immunoprecipitate (Dako, Gostруп, Denmark). The intensity of the blot was measured by using LumiVision (Aishin, Nagoya, Japan).



**Figure 2. IL-7 induced phosphorylation of STAT5A and expression of c-Myc.** (A) A representative blot of LBL cells with dual integration. Lanes 1, 4, 7, 10, 13, and 16, No IL-7 in the medium; lanes 2, 5, 8, 11, 14, and 17, 1.0 ng/mL IL-7; lanes 3, 6, 9, 12, 15, and 18, 10 ng/mL IL-7. IB, Immunoblot; Phospho, phosphorylated. (B) Intensity of phosphorylated STAT5A and c-Myc. The measurement was performed independently three times following IL-7R stimulation of the clone (the mean ± SD; n=5).



## RT-PCR

Total RNA was extracted from tissues using the ISOGEN reagent (Nippon Gene, Toyama, Japan). First-strand cDNA synthesis was performed using a Ready-To-Go T-primed first-strand kit (Amersham, Piscataway, NJ, USA). RT-PCR was performed as described previously [5]. The primers for  $\beta$ -actin were also prepared as reported previously. The primers for other genes were as follows: Igk, 5'-GGCTGCAGSTCCAGTGGCAGTGGRTCWGGR-3' and 5'-CAT-TCTGTGAAGCTCTTGACAATGGGIG-3'; Ig $\lambda$ , 5'-GCCTTCTACACTG-CAGTGGGTATGCAACAAT-3' and 5'-AGCCACTYACCTAGGACAGTSASYTT-GGTTCC-3' [25, 26]. A control  $\beta$ -actin probe was amplified by using RT-PCRmer™  $\beta$ -actin (Gene Link, Hawthorne, NY, USA). For real-time RT-PCR, total RNA was reverse-transcribed using the Omniscript RT kit (Qiagen, Valencia, CA, USA). For relative quantification with RT-PCR, 20 ng each cDNA was analyzed using a FastStart DNA Master SYBR Green kit (Roche Diagnostics) with software, Version 3.5. For each primer pair, a standard curve was developed.

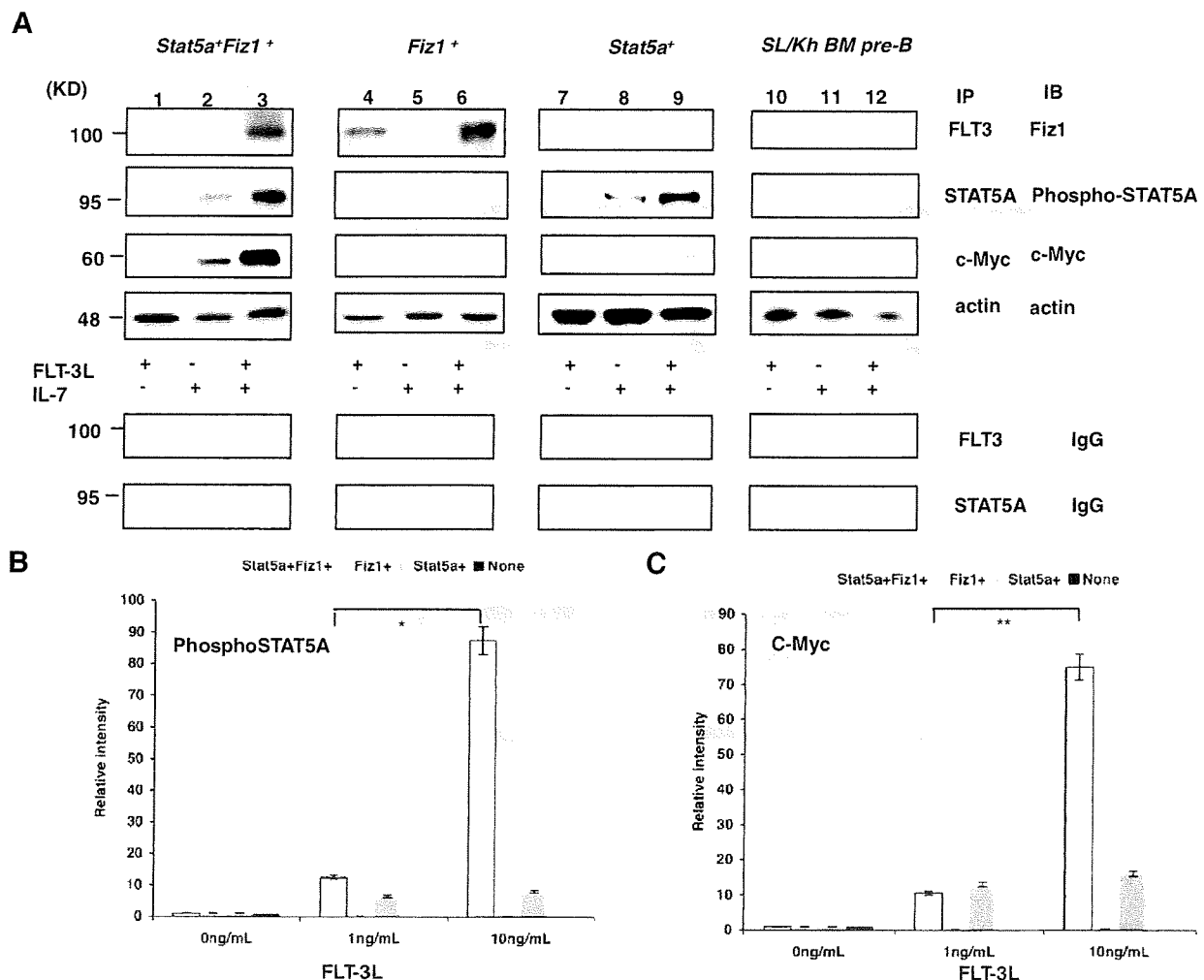
## Statistical analysis

Analysis was done with the unpaired *t*-test. A *P* value <0.05 was considered to be statistically significant.

## RESULTS

### Identification of *Emv11* that is crucial for lymphomagenesis in SL/Kh mouse genome

In the current study, we found that studied SL/Kh mice share the *Emv11* that is mapped to 15.14cM on chromosome 7. Provirus *Emv11* was identified previously in strain AKR/J [27]. To study the susceptibility to pro-B cell lymphomagenesis by this proviral integration, we observed (SL/Kh × NFS/N)F1, back-crossed them to NFS/N, which did not possess pathogenic



**Figure 3. FLT3L induces phosphorylation of STAT5A and expression of c-Myc.** (A) Blots of lymphoma cells with dual integration (*Stat5a<sup>+</sup>Fiz1<sup>+</sup>*) and single integration (*Stat5a<sup>+</sup>*). Lanes 1, 4, 7, and 10, 1.0 ng/mL FLT3L and no IL-7; lanes 2, 5, 8, and 11, no FLT3L and 10 ng/mL IL-7; lanes 3, 6, 9, and 12, 1.0 ng/mL FLT3L and 10 ng/mL IL-7. IP, Immunoprecipitation. Normal control (no MLV integration) was BP-1<sup>+</sup>B-220<sup>+</sup> BM pro-B cells from SL/Kh. (B) Relative intensity of phospho-STAT5A in immunoprecipitates in using an antibody recognizing STAT5A (the mean ± SD; n=5). (C) Relative intensity of c-Myc in immunoprecipitates in using an antibody recognizing c-Myc (the mean ± SD; n=5).

MLV, and analyzed spontaneous tumors. In F1, 32 of 62 (51%) individual mice developed pro-B cell lymphomas. In the backcross of the F1 to NFS/N, 38 of 102 (37.2%) mice developed lymphomas. Significantly, none of the backcross mice without the *Emv11* developed them. This evident cosegregation of lymphoma development with the *Emv11* plays a pivotal role in lymphomagenesis.

### B-LBL clones with dual MLV integration

From primary B-LBL lymphomas of SL/Kh mice, we isolated several B-LBL clones with the *Emv11* integration(s) (Table 1). Southern blot analysis of *NcoI*- or *ScaI*-digested DNA with probes hybridizing to *Stat5a*, *Fiz1*, or *Hipk2* (Fig. 1A) revealed clonal rearrangements of these genes induced by the *Emv11* integration. FACS analysis revealed that these clones were composed homogeneously of BP-1<sup>+</sup> CD43<sup>+</sup> sIgM-B220<sup>+</sup> cells, indicating that the lymphoma cells were pro- to early pre-B cells (Fig. 1B). Histology of the lymphomas showed positive for Vpreb, one of the surrogate light chain component, and lymphoblastic morphology (Fig. 1C). PCR-Southern blot analysis of IgH rearrangement demonstrated monoclonal DQ52-J<sub>H</sub>1 or -J<sub>H</sub>2 rearrangement, indicating that the lymphoma cells used in this study were monoclonal (Fig. 1D, lanes 2–6). In addition, neither Igκ nor Igλ was detected in the lymphomas by the RT-PCR assay (Fig. 1E). The lymphoma clones used in this study are listed in Table 1.

### Stimulation with IL-7R of B-LBL clones with double integration

Using these B-LBL clones, we studied the effect of IL-7R stimulation on STAT5A phosphorylation and induction of c-Myc, one of the downstream targets of STAT5A. Sorted BM pro-B cells were used as the control. It was found that the *Stat5a* target genes were commonly up-regulated in concomitance with the *Emv11* integration (Fig. 2A). After incubation in IL-7-free medium for 6 h, the B-LBL cells were added with 1.0 or 10.0 ng/ml IL-7 for 1 h. In B-LBL cells, with clonal *Stat5a* integration, the phosphorylation of STAT5A and the expression of c-Myc increased in a dose-dependent manner (Fig. 2A, lanes 2, 3, 8, 9, 11, and 12). In the absence of IL-7, phosphorylation of STAT5A and expression of c-Myc were nearly undetectable. Therefore, IL-7R stimulation was followed by STAT5a phos-

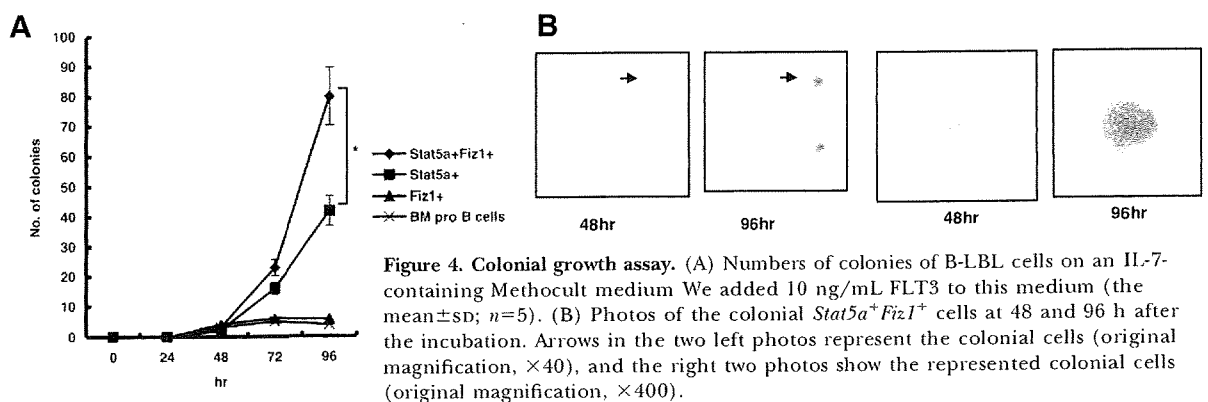
phorylation in B-LBL cells, with constitutive expression of STAT5A driven by the *Emv11* integration. Individual visualized blots of phosphorylated STAT5A and c-Myc were measured quantitatively (Fig. 2B). The results revealed that increases in STAT5A phosphorylation and c-Myc expression in the clones with the integration into the *Stat5a* and *Fiz1* or *Hipk2* were more than in the clones with the single integration into the *Stat5a* (\*,  $P=0.003$ ; \*\*,  $P=0.004$ ; \*\*\*,  $P=0.003$ ; \*\*\*\*,  $P=0.012$ ; Fig. 2B).

### STAT5A phosphorylation was induced by stimulation of FLT3 with FLT3L in *Fiz1*<sup>+</sup> *Stat5a*<sup>+</sup> B-LBL cells

Next, we examined the effect of FLT3 stimulation with FLT3L on STAT5A phosphorylation. After 6 h in IL-7-free culture medium, B-LBL cells with the *Stat5a* and *Fiz1* integrations (*Stat5a*<sup>+</sup>*Fiz1*<sup>+</sup>) or with only *Stat5a* integration (*Stat5a*<sup>+</sup>) were stimulated with 1.0 ng/mL FLT3L (Fig. 3A). IL-7 (1.0 ng/mL) was supplied again or not. The result revealed that in the *Stat5a*<sup>+</sup>*Fiz1*<sup>+</sup> and *Fiz1*<sup>+</sup> B-LBL cells, *Fiz1* was found to be associated with FLT3 when stimulated by FLT3L (Fig. 3A, lanes 1, 3, 4, and 6), and more phosphorylated STAT5A was observed following the supply of FLT3L (Fig. 3A, lanes 2 vs. 3 and 8 vs. 9). Moreover, we changed concentration of FLT3L from 1.0 ng/mL to 10 ng/mL and investigated the effects on phosphorylation of the STAT5A and c-Myc expression in the presence of 10 ng/mL IL-7. The result revealed that phosphorylation of STAT5A was increased in the *Stat5a*<sup>+</sup>*Fiz1*<sup>+</sup> B-LBL cells following stimulation with 10 ng/mL FLT3L (Fig. 3B, lanes 2 vs. 3; \*,  $P=0.008$ ) but not in the *Stat5a*<sup>+</sup> clone (Fig. 3B,  $P=0.07$ ). c-Myc expression was also up-regulated in the *Stat5a*<sup>+</sup>*Fiz1*<sup>+</sup> clone following stimulation with 10 ng/mL FLT3L (Fig. 3B, \*,  $P=0.012$ ), but this up-regulation was not seen in the *Stat5a*<sup>+</sup> clone (Fig. 3B,  $P=0.051$ ).

### Colony growth of B-LBL cells on a semisolid medium

Next, we examined the effect of increased expression of these genes in the *Emv11* integration sites by culturing the cells on Methocult medium, semisolid methylcellulose medium containing 1.0 ng/mL IL-7, which was optimized for the culture of pre-B cells. We added 10 ng/mL FLT3L to the medium and observed the growth of *Stat5a*<sup>+</sup>*Fiz1*<sup>+</sup> and *Stat5a*<sup>+</sup> clones, which began to grow after 48 h, forming col-



onies in the semisolid medium. Colonies were grown on an average of six dishes that each contained  $1.3 \times 10^6$  cells. Following the initial incubation, *Stat5a*<sup>+</sup>*Fiz1*<sup>+</sup> clones grew faster than *Stat5a*<sup>+</sup> clones (Fig. 4, A and B). This result indicates that *Fiz1* up-regulation in expression promotes the growth of B-LBL cells with a supplement of FLT3L and IL-7 (\*,  $P=0.003$ ; Fig. 4A).

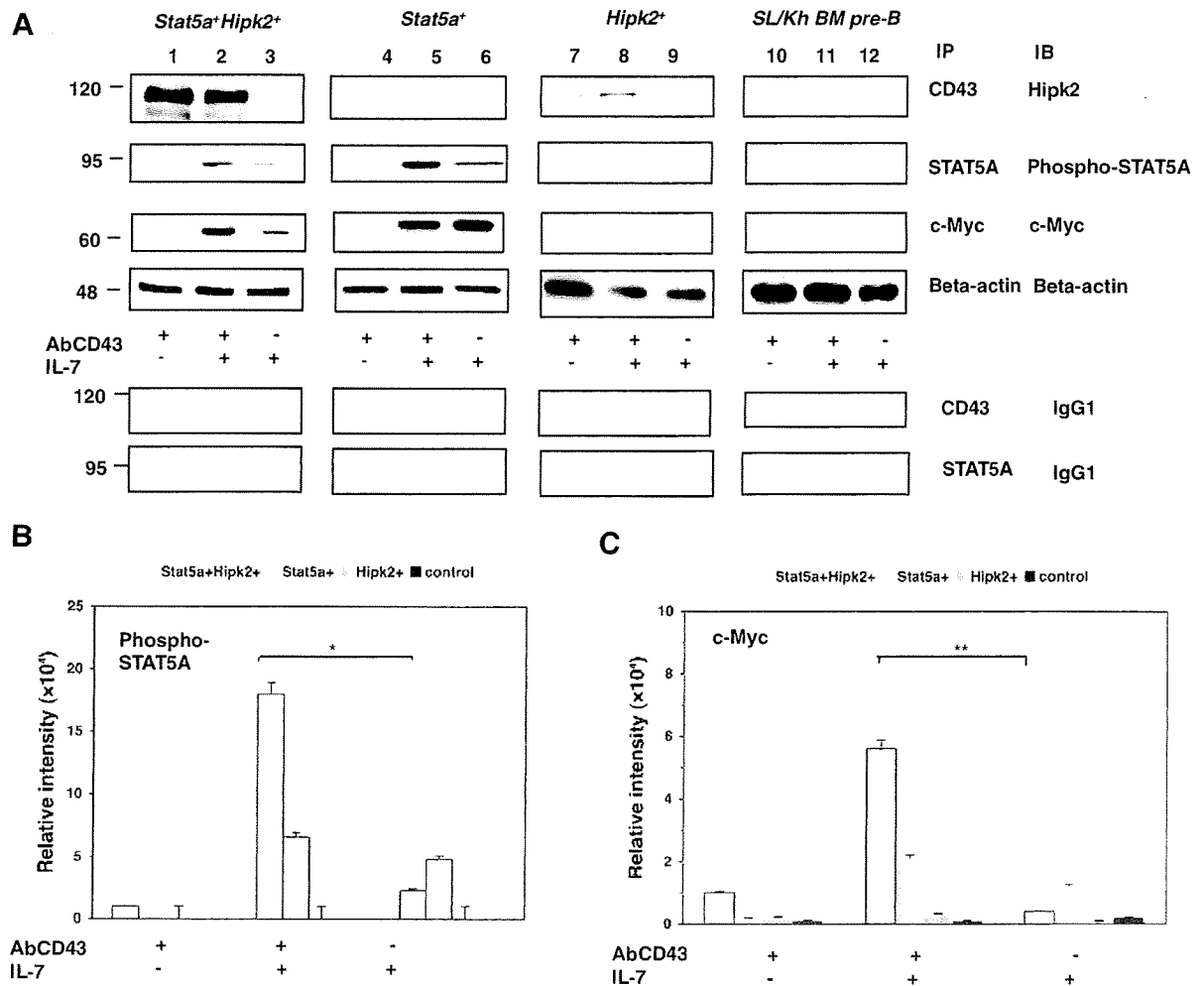
### STAT5A phosphorylation was induced by stimulation with anti-CD43 in *Stat5a*<sup>+</sup>*Hipk2*<sup>+</sup> B-LBL cells

Next, we examined the effect of CD43 stimulation by cross-linking in B-LBLs. After 6 h in IL-7-free medium, CD43 was cross-linked using an anti-CD43 antibody. The result revealed that *Hipk2* was found to be associated with CD43 following the cross-linking (Fig. 5A, lanes 1, 2, 7, and 8) in the *Stat5a*<sup>+</sup>*Hipk2*<sup>+</sup> and *Hipk2*<sup>+</sup> clones. In the *Stat5a*<sup>+</sup>*Hipk2*<sup>+</sup> clone, phosphorylated

STAT5A was increased significantly by CD43 cross-linking in the *Stat5a*<sup>+</sup>*Hipk2*<sup>+</sup> B-LBL cells (Fig. 5A, lanes 2 vs. 3; \*,  $P=0.008$ ; Fig. 5B) but not significantly in the *Stat5a* single integration clone (Fig. 5A, lanes 5 vs. 6;  $P=0.07$ ; Fig. 5B). In close correlation with this, the significant increase in c-Myc expression in the *Stat5a*<sup>+</sup>*Hipk2*<sup>+</sup> clones was observed following the cross-link (Fig. 5A; lanes 2 vs. 3; \*\*,  $P=0.006$ ; Fig. 5C), but it was not observed in the *Stat5a*<sup>+</sup> clones (Fig. 5A, lanes 5 vs. 6;  $P=0.09$ ; Fig. 5C).

### DISCUSSION

Constitutive activation of *Stat5a* by retrovirus integration contributes to lymphomagenesis via activation of the IL-7R signaling pathway [9]. In this study, we identified *Fiz1* and *Hipk2* as novel targets for integration. FLT3 is one of the markers and



**Figure 5.** Cross-linking of CD43 induces phosphorylation of STAT5A and expression of c-Myc. (A) Lanes 1, 4, 7, and 10, No IL-7 with cross-linking; lanes 2, 5, 8, and 11, 1.0 ng/mL IL-7 with cross-linking; lanes 3, 6, 9, and 12, 1.0 ng/mL IL-7 without cross-linking. Anti-CD43 (AbCD43) was used at 20 ng/mL (lanes 1, 2, 4, 5, 7, 8, 10, and 11). (B) Relative intensity of phospho-STAT5A in blots of immunoprecipitates using an antibody recognizing phosphorylated STAT5A (the mean  $\pm$  SD;  $n=5$ ). (C) Relative intensity of c-Myc in immunoblots of immunoprecipitates using an antibody recognizing c-Myc (the mean  $\pm$  SD;  $n=5$ ).

functional molecules expressed in early B lymphoid progenitor cells [28–32]. As these pathways act selectively in the pro-B cell stage of B cell development, constitutive activation of these molecules may stabilize the immature host cellular phenotype and prevent further differentiation. Choudhary et al. [30] reported that STAT5A is involved in the FLT3 pathway. Mutations in the receptor tyrosine kinase FLT3 are a commonly occurring genetic lesion in acute myeloid leukemia [31]. ITD mutations clustered in the juxta-membrane domain are the most frequent and best-characterized type of mutation found in FLT3. FLT3-ITD-induced STAT5 activation is independent of Src, JAKs, and FLT3. The juxta-membrane domain of FLT3 is critical for ligand-dependent activation of FLT3 and for the transforming potential of oncogenic FLT3 mutants. In addition, phosphorylated STAT5 in the nucleus was detectable in 20% of B-lymphoblastic leukemia cells in the reported cases, and FLT3 is involved in B-acute lymphocytic leukemia with karyotype t(13;13)(q12;q22) [32]. Thus, the FLT3 pathway can contribute to lymphomagenesis via STAT5A activation. We showed that the activation of the FLT3 pathway by increasing *Fiz1* expression may also contribute to STAT5 activation and B-LBL pathogenesis. As aforementioned, this pathway is enhanced by the supply of IL-7, suggesting the interaction between the IL-7R pathway and the FLT3 pathway.

In addition, we demonstrated a possibility that the CD43 pathway involved *Hipk2* and STAT5A. In T cell development, TCR-dependent cell response is modulated by CD43 engagement [33]. Lee et al. [34] demonstrated that *Hipk2* promotes Wnt/Wg signaling by stabilizing  $\beta$ -catenin/Armadillo and stimulating target gene expression. This group also showed that *Hipk2* interacts with lymphoid-enhancing factor 1, which acts as a transcriptional factor, promoting *c-Myc* and cyclin D1 expression. In the erythroleukemic cell line TF-1, STAT5A is not associated with CD43-mediated cell proliferation [35]. Recently, it was found that CD43 functions as an E-selectin counter-receptor in human pre-B cell leukemia NALL-1 cell line [36]. Thus, CD43 signaling pathway is one of the candidate pathways that actively operates in the B-LBLs. In our study, CD43 cross-linking resulted in an increase in STAT5A phosphorylation and *c-Myc* expression in *Stat5a*<sup>+</sup>*Hipk2*<sup>+</sup> B-LBL cells when IL-7 was supplied. These data suggest that CD43 enhances the IL-7R signal pathway, which normally enhances *c-Myc* expression via STAT5A phosphorylation. Taken together, we propose a scheme of interactions among the IL-7, CD43, and FLT3 signaling pathways (Fig. 6). Thus, we hypothesize that these three pathways form an interacting network and affect B-LBL development. SL/Kh mice serve as one of the appropriate models for studying the signaling pathways leading to lymphomagenesis, using MLV integration as a tool. The data we report here will provide new insights into the aberrant signaling pathways leading to lymphomagenesis.

## AUTHORSHIP

Tatsuaki Tsuruyama planned all of the experiments. Tatsuaki Tsuruyama, Yukiko Imai, Haruya Takeuchi, Takuya Hiratsuka, Yasuhiro Maruyama, Kazuya Kanaya, Richard Kaszynski, and

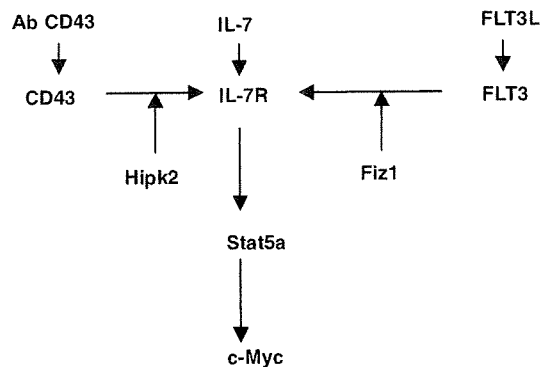


Figure 6. Plausible signaling pathways in B-LBL cells.

Guang Jin conducted all of the experiments. Tatsuaki Tsuruyama, Tomoko Okuno, Munetaka Ozeki, Takuro Nakamura, Tetsuya Takakuwa, Toshiaki Manabe, and Hiroshi Hiai critically advised on the signaling pathway and morphology of the lymphoma tissue. Tatsuaki Tsuruyama and Keiji Tamaki analyzed the data. Tatsuaki Tsuruyama and Richard Kaszynski wrote the paper.

## ACKNOWLEDGMENTS

This work was supported by a grant-in-aid for cancer research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and a grant for strategic research on cancer from the Ministry of Health, Labor, and Welfare, Japan. We are grateful for the generous financial support from the Kyoto Shimizu Immunology Foundation.

## REFERENCES

- Coffin, J. M., Hughes, S. H., Varmus, H. E. (1997) *Retroviruses*. New York, NY, USA, Cold Spring Harbor Laboratory.
- Suzuki, T., Shen, H., Akagi, K., Morse, H. C., Malley, J. D., Naiman, D. Q., Jenkins, N. A., Copeland, N. G. (2002) New genes involved in cancer identified by retroviral tagging. *Nat. Genet.* **32**, 166–174.
- Abujiang, P., Yamada, Y., Haller, O., Kobayashi, H., Kamoto, T., Lu, L. M., Ogawa, M., Ishimoto, A., Katoh, H., Kanchira, K., Ikegami, S., Fukumoto, M., Hiai, H. (1996) The origin of SL family mice. *Lab. Anim. Sci.* **46**, 410–417.
- Hiai, H., Yamada, Y., Abujiang, P., Lu, L., Kamoto, T., Tsuruyama, T. (1999) Genetic and epigenetic susceptibility to endogenous retrovirus-induced lymphomas in SL mice. *Prog. Exp. Tumor Res.* **35**, 64–77.
- Shimada, M. O., Yamada, Y., Nakakuki, Y., Okamoto, K., Fukumoto, M., Honjo, T., Hiai, H. (1993) SL/KH strain of mice: a model of spontaneous pre-B-lymphomas. *Leuk. Res.* **17**, 573–578.
- Lu, L.-M., Shimada, M., Higashi, S., Zeng, Z.-Z., Hiai, H. (1999) Bone marrow pre-B-1 (Bombl): a quantitative trait locus inducing bone marrow pre-B cell expansion in lymphoma-prone SL/Kh mice. *Cancer Res.* **59**, 2593–2595.
- Moll, B., Hartley, J. W., Rowe, W. P. (1979) Induction of B-tropic and N-tropic murine leukemia virus from B10.BR/SgLi mouse embryo cell lines by 5-iodo-2'-deoxyuridine. *J. Natl. Cancer Inst.* **63**, 213–217.
- Hiratsuka, T., Tsuruyama, T., Kaszynski, R., Kometani, K., Minato, N., Nakamura, T., Tamaki, K., Hiai, H. (2008) Bone marrow pre-B expansion by SL/Kh-Bombl locus: not sufficient for lymphomagenesis. *Leuk. Res.* **32**, 309–314.
- Tsuruyama, T., Nakamura, T., Jin, G., Ozeki, M., Yamada, Y., Hiai, H. (2002) Constitutive activation of *Stat5a* by retrovirus integration in early pre-B lymphomas of SL/Kh strain mice. *Proc. Natl. Acad. Sci. USA* **99**, 8253–8258.
- Jin, G., Tsuruyama, T., Yamada, Y., Hiai, H. (2003) *Svi3*: a provirus common integration site in *c-myc* in SL/Kh pre-B lymphomas. *Cancer Sci.* **94**, 791–795.

11. Wu, X., Li, Y., Crise, B., Burgess, S. M. (2003) Transcription start regions in the human genome are favored targets for MLV integration. *Science* **300**, 1749–1751.
12. Ihle, J. N. (2001) The Stat family in cytokine signaling. *Curr. Opin. Cell Biol.* **13**, 211–217.
13. Matsumura, I., Kitamura, T., Wakao, H., Tanaka, H., Hashimoto, K., Albanese, C., Downward, J., Pestell, R. G., Kanakura, Y. (1999) Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. *EMBO J.* **18**, 1367–1377.
14. Nosaka, T., Kawashima, T., Misawa, K., Ikuta, K., Mui, A. L., Kitamura, T. (1999) STAT5 as a molecular regulator of proliferation, differentiation, and apoptosis in hematopoietic cells. *EMBO J.* **18**, 4754–4763.
15. Lanvin, O., Gouilleux, F., Mullié, C., Mazière, C., Fuentes, V., Bissac, E., Dantin, F., Mazière, J. C., Régnier, A., Lassoued, K., Gouilleux-Gruart, V. (2004) Interleukin-7 induces apoptosis of 697 pre-B cells expressing dominant-negative forms of STAT5: evidence for caspase-dependent and -independent mechanisms. *Oncogene* **23**, 3040–3047.
16. Sexl, V., Piekorz, R., Moriggi, R., Rohrer, J., Brown, M. P., Bunting, K. D., Rothhammer, K., Rousset, M. F., Ihle, J. N. (2000) *Stat5a/b* contribute to interleukin 7-induced B-cell precursor expansion, but *abl*- and *bcr/abl*-induced transformation are independent of *stat5*. *Blood* **96**, 2277–2283.
17. Dai, X., Chen, Y., Di, L., Podd, A., Li, G., Bunting, K. D., Hennighausen, L., Wen, R., Wang, D. (2007) *Stat5* is essential for early B cell development but not for B cell maturation and function. *J. Immunol.* **179**, 1068–1079.
18. Sudo, T., Ito, M., Ogawa, Y., Iizuka, M., Kodama, H., Kunisada, T., Hayashi, S., Ogawa, M., Sakai, K., Nishikawa, S. (1989) Interleukin 7 production and function in stromal cell-dependent B cell development. *J. Exp. Med.* **170**, 333–338.
19. Funk, P. E., Stephan, R. P., Witte, P. L. (1995) Vascular cell adhesion molecule 1-positive reticular cells express interleukin-7 and stem cell factor in the bone marrow. *Blood* **86**, 2661–2671.
20. Malin, S., McManus, S., Cobaleda, C., Novatchkova, M., Delogu, A., Bouillet, P., Strasser, A., Busslinger, M. (2010) Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development. *Nat. Immunol.* **11**, 171–179.
21. Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D., Hasegawa, K. (1991) Resolution and characterization of pro-B and pre-pro-B cells stage in normal mouse bone marrow. *J. Exp. Med.* **173**, 1213–1225.
22. Wolf, I., Rohrschneider, L. R. (1999) *Fiz1*, a novel zinc finger protein interacting with the receptor tyrosine kinase *Flt3*. *J. Biol. Chem.* **274**, 21478–21484.
23. Wang, W., Link, V., Green, J. M. (2000) Identification and cloning of a CD43-associated serine/threonine kinase. *Cell. Immunol.* **205**, 34–39.
24. Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., Tonegawa, S. (1980) Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy-chain genes. *Nature* **286**, 676–683.
25. Sakano, H., Huppi, K., Heinrich, G., Tonegawa, S. (1979) Sequences at the somatic recombination sites of immunoglobulin light-chain genes. *Nature* **280**, 288–294.
26. Da Silva, T. R., Fong, I. C., Cunningham, L. A., Wu, G. E. (2007) RAG1/2 re-expression causes receptor revision in a model B cell line. *Mol. Immunol.* **44**, 889–899.
27. Lenz, J., Crowther, R., Straceski, A., Haseltine, W. (1982) Nucleotide sequence of the *Akv* env gene. *J. Virol.* **42**, 519–529.
28. Rappold, I., Ziegler, B. L., Köhler, I., Marchetto, S., Rosnet, O., Birnbaum, D., Simmons, P. J., Zannettino, A. C., Hill, B., Neu, S., Knapp, W., Alitalo, R., Alitalo, K., Ullrich, A., Kanz, L., Bühring, H. J. (1997) Functional and phenotypic characterization of cord blood and bone marrow subsets expressing FLT3 (CD135) receptor tyrosine kinase. *Blood* **90**, 111–125.
29. Ogawa, M., Sugawara, S., Kunisada, T., Sudo, T., Hayashi, S., Nishikawa, S., Kodama, H., Nishikawa, S. (1998) *Flt3/Flk-2* and *c-Kit* are not essential for the proliferation of B lymphoid progenitor cells in the bone marrow of the adult mouse. *Exp. Hematol.* **26**, 478–488.
30. Choudhary, C., Brandts, C., Schwable, J., Tickenbrock, L., Sargin, B., Ueker, A., Böhrer, F. D., Berdel, W. E., Müller-Tidow, C., Serve, H. (2007) Activation mechanisms of STAT5 by oncogenic *Flt3-ITD*. *Blood* **110**, 370–374.
31. Vempati, S., Reindl, C., Wolf, U., Kern, R., Petropoulos, K., Naidu, V. M., Buske, C., Hiddemann, W., Kohl, T. M., Spiekermann, K. (2008) Transformation by oncogenic mutants and ligand-dependent activation of FLT3 wild-type requires the tyrosine residues 589 and 591. *Clin. Cancer Res.* **14**, 4437–4445.
32. Tzankov, A., Sotlar, K., Muhlematter, D., Theocharides, A., Went, P., Jotterand, M., Horny, H. P., Dirnhöfer, S. (2008) Systemic mastocytosis with associated myeloproliferative disease and precursor B lymphoblastic leukemia with t(13)13)(q12)q22) involving FLT3. *J. Clin. Pathol.* **61**, 958–961.
33. Fierro, N. A., Pedraza-Alva, G., Rosenstein, Y. (2006) TCR-dependent cell response is modulated by the timing of CD43 engagement. *J. Immunol.* **176**, 7346–7353.
34. Lee, W., Swarup, S., Chen, J., Ishitani, T., Verheyen, E. M. (2009) Homeodomain-interacting protein kinases (Hipks) promote Wnt/Wg signaling through stabilization of  $\beta$ -catenin/Arm and stimulation of target gene expression. *Development* **136**, 241–251.
35. Miura, Y., Mizutani, C., Nishihara, T., Hishita, T., Yanagi, S., Tohyama, Y., Ichiyama, S., Yamamura, H., Uchiyama, T., Tohyama, K. (2001) Adhesion via CD43 induces Syk activation and cell proliferation in TF-1 cells. *Biochem. Biophys. Res. Commun.* **288**, 80–86.
36. Nonomura, C., Kikuchi, J., Kiyokawa, N., Ozaki, H., Mitsunaga, K., Ando, H., Kanamori, A., Kannagi, R., Fujimoto, J., Muroi, K., Furukawa, Y., Nakamura, M. (2008) CD43, but not P-selectin glycoprotein ligand-1, functions as an E-selectin counter-receptor in human pre-B-cell leukemia NALL-1. *Cancer Res.* **68**, 790–799.

KEY WORDS:

murine leukemia · *Stat5a* · B-LBL · *Flt3* · CD43

## Small intestine CD4<sup>+</sup> cell reduction and enteropathy in simian/human immunodeficiency virus KS661-infected rhesus macaques in the presence of low viral load

Katsuhisa Inaba,<sup>1</sup> Yoshinori Fukazawa,<sup>1</sup> Kenta Matsuda,<sup>1</sup> Ai Himeno,<sup>1</sup> Megumi Matsuyama,<sup>1</sup> Kentaro Ibuki,<sup>1</sup> Yoshiharu Miura,<sup>2</sup> Yoshio Koyanagi,<sup>2</sup> Atsushi Nakajima,<sup>3</sup> Richard S. Blumberg,<sup>4</sup> Hidemi Takahashi,<sup>5</sup> Masanori Hayami,<sup>1</sup> Tatsuhiko Igarashi<sup>1</sup> and Tomoyuki Miura<sup>1</sup>

Correspondence  
Tomoyuki Miura  
tmiura@virus.kyoto-u.ac.jp

<sup>1</sup>Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, 53 Shogoinkawaramachi, Sakyo-ku, Kyoto 606-8507, Japan

<sup>2</sup>Laboratory of Viral Pathogenesis, Institute for Virus Research, Kyoto University, 53 Shogoinkawaramachi, Sakyo-ku, Kyoto 606-8507, Japan

<sup>3</sup>Division of Gastroenterology, Yokohama City University Graduate School of Medicine, Yokohama, Japan

<sup>4</sup>Division of Gastroenterology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

<sup>5</sup>Department of Microbiology and Immunology, Nippon Medical School, Tokyo, Japan

Human immunodeficiency virus type 1, simian immunodeficiency virus and simian/human immunodeficiency virus (SHIV) infection generally lead to death of the host accompanied by high viraemia and profound CD4<sup>+</sup> T-cell depletion. SHIV clone KS661-infected rhesus macaques with a high viral load set point (HVL) ultimately experience diarrhoea and wasting at 6–12 months after infection. In contrast, infected macaques with a low viral load set point (LVL) usually live asymptotically throughout the observation period, and are therefore referred to as asymptomatic LVL (Asym LVL) macaques. Interestingly, some LVL macaques exhibit diarrhoea and wasting similar to the symptoms of HVL macaques and are termed symptomatic LVL (Sym LVL) macaques. This study tested the hypothesis that Sym LVL macaques have the same degree of intestinal abnormalities as HVL macaques. The proviral DNA loads in lymphoid tissue and the intestines of Sym LVL and Asym LVL macaques were comparable and all infected monkeys showed villous atrophy. Notably, the CD4<sup>+</sup> cell frequencies of lymphoid tissues and intestines in Sym LVL macaques were remarkably lower than those in Asym LVL and uninfected macaques. Furthermore, Sym LVL and HVL macaques exhibited an increased number of activated macrophages. In conclusion, intestinal disorders including CD4<sup>+</sup> cell reduction and abnormal immune activation can be observed in SHIV-KS661-infected macaques independent of virus replication levels.

Received 5 October 2009  
Accepted 3 November 2009

### INTRODUCTION

The intestinal tract, which is the largest mucosal and lymphoid organ and which contains the majority of the total lymphocytes in the body, is an important port of entry for human immunodeficiency virus type 1 (HIV-1) infection in vertical and homosexual transmission (Smith *et al.*, 2003). Additionally, the intestinal tract is a central site in the interaction between HIV-1 and its host, and suffers profound pathological changes as a result of HIV-1

infection. HIV-1 infection of the intestinal tract is characterized by virus replication (Fackler *et al.*, 1998), CD4<sup>+</sup> T-cell depletion (Brenchley *et al.*, 2004), opportunistic infection and HIV enteropathy, which is an idiopathic intestinal disorder observed in infected patients with diarrhoea (Kotler, 2005). In particular, CD4<sup>+</sup> T-cell depletion, which is the immunological hallmark in the development of AIDS, preferentially takes place in the intestinal tract rather than in the peripheral blood throughout the infection (Brenchley *et al.*, 2004). This



observation is based on the following findings: (i) most naturally transmitted HIV-1 strains are chemokine receptor 5 (CCR5)-tropic; and (ii) the intestinal tract, especially the lamina propria, contains a large number of activated memory CCR5<sup>+</sup> CD4<sup>+</sup> T cells, which indicates a high susceptibility for HIV-1 infection, whereas the peripheral blood has a relatively small population of these cells (Anton *et al.*, 2000; Lapenta *et al.*, 1999). CD4<sup>+</sup> T-cell depletion from the intestinal tract by HIV-1 infection is thought to lead to progressive dysfunction of mucosal immunity, which triggers immunodeficiency (Paiardini *et al.*, 2008). In addition to CD4<sup>+</sup> T-cell depletion in the intestinal tract, HIV-1 infection causes histopathological changes in the intestine, including villous atrophy, crypt hyperplasia and acute/chronic inflammation (Batman *et al.*, 1989).

Chronic disease of the intestinal tract generally manifests as inflammation (Kahn, 1997). Diarrhoea is a major intestinal symptom caused by various stimuli to the intestinal tract such as pathogens, toxins and dysfunction of the immune system (Gibbons & Fuchs, 2007). Because HIV-1 infection weakens the host immune system, AIDS is one of the primary causes of chronic diarrhoea (Sestak, 2005). In developing countries, diarrhoea was a major symptom in advanced HIV-1 infection prior to the establishment of highly active antiretroviral therapy (HAART) (Wilcox & Saag, 2008). Dehydration and malabsorption as a result of chronic diarrhoea can lead to progressive weight loss and can contribute to morbidity and mortality in HIV-1-infected patients (Sharpstone & Gazzard, 1996). Therefore, chronic diarrhoea is one of the most important clinical signs in AIDS patients.

AIDS models using non-human primates have provided many important observations on AIDS pathogenesis. The first finding of early CD4<sup>+</sup> T-cell depletion from the intestinal tract was reported in a study using simian immunodeficiency virus (SIV)-infected macaques (Veazey *et al.*, 1998). Intestinal CD4<sup>+</sup> T cells of rhesus macaques predominantly exhibit a CCR5<sup>+</sup> activated memory phenotype, and CD4<sup>+</sup> T cells of this phenotype are selectively eliminated in SIV-infected macaques, indicating that the majority of intestinal CD4<sup>+</sup> T cells are primary targets of SIV infection (Veazey *et al.*, 2000a, b). Accordingly, detailed analysis of the intestinal tract using animal models is essential for an understanding of AIDS pathogenesis.

Simian/human immunodeficiency virus (SHIV)-KS661 is a molecular clone and a pathogenic virus in rhesus macaques. SHIV-KS661 systemically depletes CD4<sup>+</sup> T cells of rhesus macaques within 4 weeks of infection (Miyake *et al.*, 2006). Based on our observations over a number of years, intravenous infection of rhesus macaques with SHIV-KS661 consistently results in high viraemia and CD4<sup>+</sup> T-cell depletion, followed by malignant morbidity as a result of severe chronic diarrhoea and wasting after 6–18 months. Generally, the time to clinical morbidity in rhesus macaques infected with pathogenic SHIVs, such as SHIV-89.6P and SHIV-KS661, is considerably shorter than

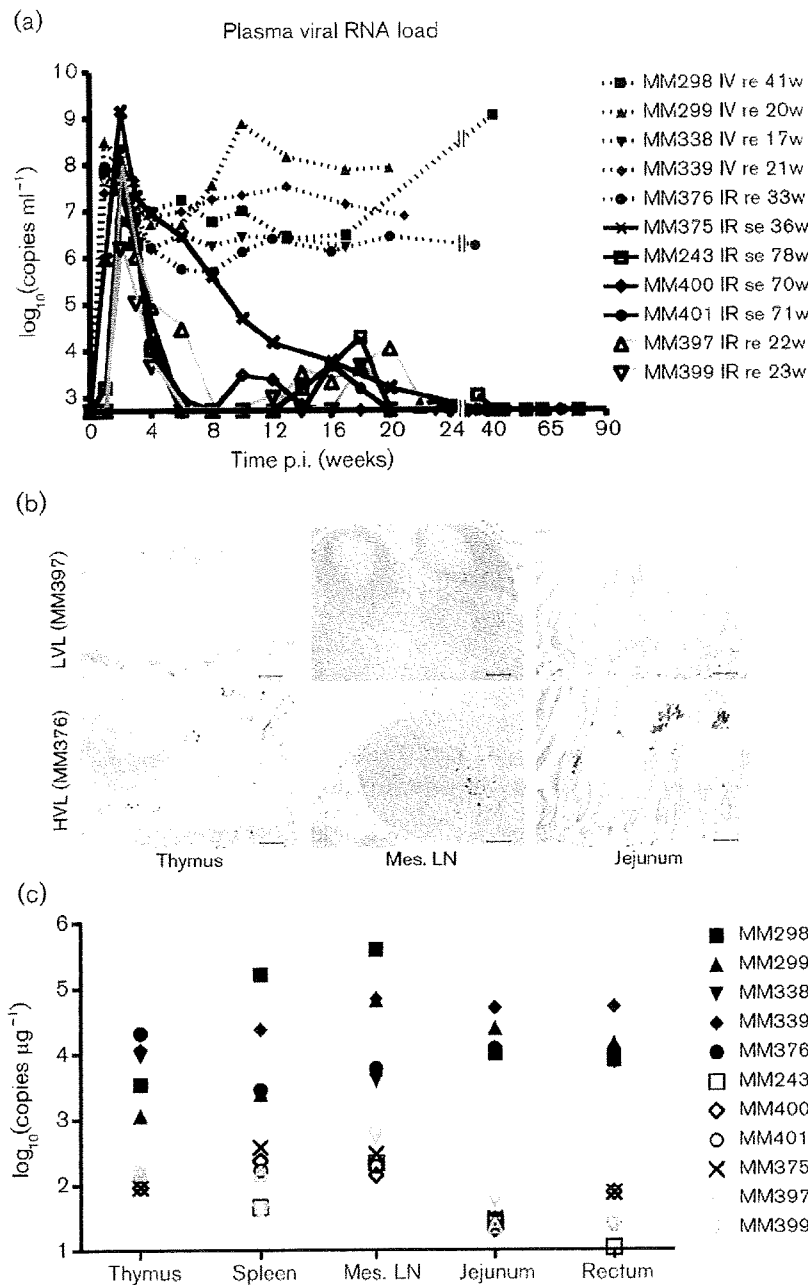
in HIV-1-infected humans, who take an average of 10 years to progress to AIDS. In addition, all subsets of CD4<sup>+</sup> T cells including memory and naïve T cells are thoroughly depleted in pathogenic SHIV-infected macaques. However, in the SHIV-KS661 macaque model, diarrhoea and wasting, which are major symptoms in advanced HIV-1 infection, can clearly be recognized and defined in association with disease progression.

Recently, we observed that, in many rhesus macaques infected intrarectally with SHIV-KS661, plasma viral RNA loads decreased gradually to undetectable levels in the chronic phase, which is quite different from the case with intravenous infection. It is well known that pathogenic SIV and SHIV infections in monkeys, like HIV-1 infections in humans, generally lead to high viraemia, profound CD4<sup>+</sup> T-cell depletion and death. Interestingly, in this study, two out of six intrarectally inoculated macaques with a low plasma viral load experienced malignant morbidity manifest as severe diarrhoea and wasting, similar to what we observed in infected macaques with high viraemia. The purpose of this study was to elucidate why macaques with a low plasma viral load experienced diarrhoea and wasting. As an explanation for this morbidity, we hypothesized that, even if the viral load set-point is suppressed, SHIV-KS661-infected macaques would have the same degree of intestinal abnormalities as infected macaques with high viraemia. To test this hypothesis, we analysed CD4<sup>+</sup> cell frequencies in lymphoid and intestinal tissues and damage to the intestinal mucosa in infected macaques with high and low viral load set points (HVL and LVL, respectively). Here, we have provided evidence for the development of intestinal disorders in SHIV-KS661-infected macaques irrespective of the plasma viral RNA load.

## RESULTS

### Diarrhoea and wasting in two macaques despite low viral load

All macaques inoculated intravenously with SHIV-KS661 and one out of seven macaques inoculated intrarectally with SHIV-KS661 exhibited high set points of plasma viral RNA loads, persisting at over 10<sup>6</sup> copies ml<sup>-1</sup> until they needed to be euthanized as a result of diarrhoea and wasting (Fig. 1a). In contrast, in the remaining six macaques inoculated intrarectally with SHIV-KS661, the set points of plasma viral RNA load gradually decreased to undetectable levels (Fig. 1a). We called these macaques showing high and low set points of viral RNA load HVL and LVL macaques, respectively. During an observation period of approximately 1.4 years, two LVL macaques (MM397 and MM399) experienced severe diarrhoea and wasting and required euthanasia at approximately 22 weeks post-infection (p.i.), similar to HVL macaques, whereas the remaining four LVL macaques were asymptomatic (Fig. 1a). We termed the healthy LVL macaques asymptomatic LVL macaques (Asym LVL) and the LVL



**Fig. 1.** Distribution of virus in various tissues of SHIV-KS661-infected rhesus macaques. (a) Time course of plasma viral RNA loads as measured by quantitative RT-PCR. The detection limit of plasma viral RNA loads was 500 copies  $\text{ml}^{-1}$ . The animal ID numbers, infection route and when and how they were euthanized are indicated on the figure. IV, Intravenous inoculation; IR, intrarectal inoculation; re, required euthanasia; se, scheduled euthanasia; w, number of weeks after infection when euthanasia was performed. (b) Immunohistochemical detection of Nef antigen in thymus, mesenteric lymph nodes (Mes. LN) and jejunum. Brown staining indicates Nef<sup>+</sup> cells. The upper panels show representative tissue sections from a Sym LVL macaque (MM397) and the lower panels show representative tissue sections from an HVL macaque (MM376). Bars, 100  $\mu\text{m}$ . (c) Proviral DNA loads in different tissues of SHIV-KS661-infected macaques, as measured by quantitative PCR. The detection limit of proviral DNA loads was 10 copies  $\mu\text{g}^{-1}$ . Filled black symbols indicate HVL macaques, open black symbols indicate Asym LVL macaques and open grey symbols indicate Sym LVL macaques.

macaques with diarrhoea and wasting symptomatic LVL macaques (Sym LVL).

#### Antibody response against SHIV in infected macaques

The LVL macaques showed antibody responses to SHIV-KS661 at 3–4 weeks p.i. and then developed strong antibody responses that persisted up to 18 weeks p.i. (Table 1). In contrast, two of the HVL macaques (MM298 and MM299) showed no antibody response, whilst the remaining two (MM338 and MM339) showed very low

antibody responses. Among the HVL macaques, only MM376 showed a strong antibody response: the titre reached 1:2048 at 6 weeks p.i., but then decreased to a much lower value. These results showed that LVL macaques succeeded in maintaining a strong antibody response, whilst HVL macaques failed to do so.

#### Viral levels in tissues from Sym LVL and Asym LVL macaques are not significantly different

To investigate whether the infected macaques had different viral levels in their lymphoid and intestinal tissues, we used

**Table 1.** Anti-HIV antibody titres in infected monkeys

– indicates a titre of &lt;32.

Time (weeks)	Intrarectal inoculation						Intravenous inoculation				
	LVL						HVL				
	MM243	MM397	MM399	MM400	MM401	MM375	MM376	MM298	MM299	MM338	MM339
0	–	–	–	–	–	–	–	–	–	–	–
1	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	64	64
3	32	–	32	–	–	128	–	–	–	32	32
4	32	16 384	32	64	32	512	512	–	–	–	–
6	8 192	16 384	256	64	4 096	1 024	2 048	–	–	–	–
8	4 096	16 384	1 024	128	1 024	16 384	512	–	–	–	–
10	16 384	16 384	2 048	512	512	16 384	512	–	–	–	–
12	16 384	16 384	256	512	4 096	16 384	512	–	–	–	–
13	–	–	–	–	–	–	–	–	–	–	–
14	16 384	16 384	1 024	512	2 048	–	–	–	–	–	–
16	4 096	8 192	1 024	1 024	1 024	16 384	64	–	–	–	–
17	–	–	–	–	–	–	–	–	–	–	–
18	8 192	16 384	2 048	8 192	4 096	–	–	–	–	–	–

the Nef antigen as a marker of virus infection using immunohistochemistry and quantitative analysis of proviral DNA in lymphoid and intestinal tissues. Nef<sup>+</sup> cells were detected in large numbers in the tissues of HVL macaques, but were undetectable in both Sym LVL (Fig. 1b) and Asym LVL (data not shown) macaques.

In the HVL macaques, high proviral DNA loads (>1000 copies  $\mu\text{g}^{-1}$ ) were found in all of the tissues examined (Fig. 1c). In contrast, the proviral DNA loads in the tissues of the LVL macaques were only several tens to several hundreds of copies  $\mu\text{g}^{-1}$  (Fig. 1c). Furthermore, Sym LVL and Asym LVL macaques exhibited comparably low proviral DNA loads in these tissues (Fig. 1c). The low viral levels in lymphoid and intestinal tissues in the LVL macaques were consistent with their set points of plasma viral RNA loads. The viral levels in lymphoid and intestinal tissues were not significantly different between Sym LVL and Asym LVL macaques.

#### Diarrhoea and wasting in LVL macaques correlate with CD4<sup>+</sup> cell frequency in lymphoid and intestinal tissues, but not in peripheral blood

Because CD4<sup>+</sup> T-cell depletion is the hallmark of AIDS, we first examined CD4<sup>+</sup> T-cell counts in peripheral blood. Whilst peripheral CD4<sup>+</sup> T cells were completely and irreversibly depleted in HVL macaques throughout the infection, they displayed various kinetics in LVL macaques (Fig. 2a). MM397 (Sym LVL) and MM401 (Asym LVL) had very low CD4<sup>+</sup> T-cell counts (<150 cells  $\text{ml}^{-1}$ ) at all times at which they were examined after infection, whereas MM399 (Sym LVL) and MM400 (Asym LVL) maintained

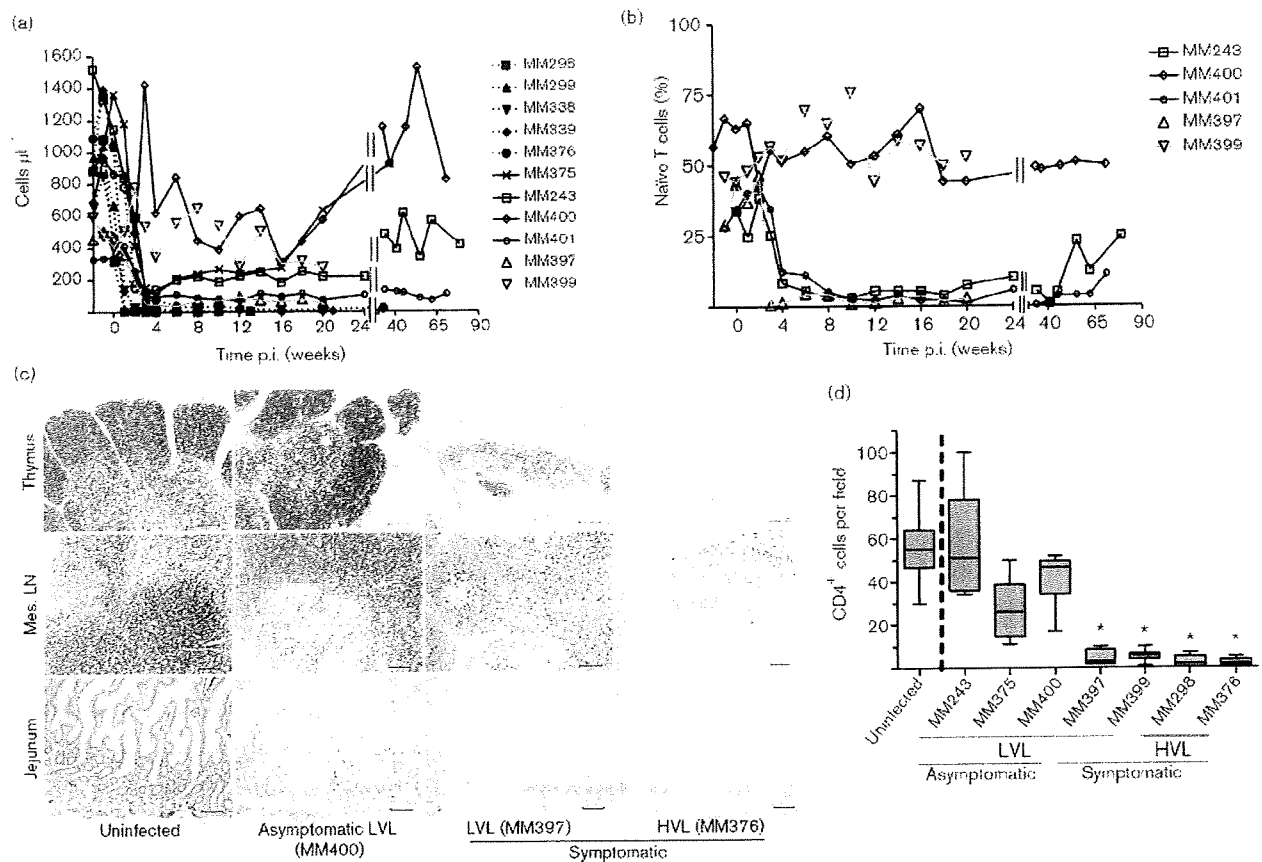
moderate CD4<sup>+</sup> T-cell counts (>300 cells  $\text{ml}^{-1}$ ) throughout the experiment (Fig. 2a).

Naïve CD4<sup>+</sup> T cells of MM397 (Sym LVL), MM243 (Asym LVL) and MM401 (Asym LVL) were depleted as early as 4 weeks p.i., whereas those of MM399 (Sym LVL) and MM400 (Asym LVL) remained at moderate levels (Fig. 2b). The HVL macaques were not examined because their peripheral CD4<sup>+</sup> T cells were depleted.

In addition to evaluating CD4<sup>+</sup> T cells in the blood, we evaluated CD4<sup>+</sup> cells in lymphoid and intestinal tissues using CD4 staining. The HVL macaques showed severe depletion of CD4<sup>+</sup> cells in all lymphoid tissues and intestine compared with the uninfected macaques (Fig. 2c, d). Interestingly, the CD4<sup>+</sup> cell frequencies in the tissues were clearly lower in Sym LVL macaques than in uninfected macaques (Fig. 2c, d). However, the CD4<sup>+</sup> cell frequencies in the tissues of Asym LVL macaques were comparable to those in uninfected macaques. These findings indicated that the emergence of diarrhoea and wasting in LVL macaques correlated with the low CD4<sup>+</sup> cell frequency in lymphoid tissues and the intestines, but not with the counts of peripheral CD4<sup>+</sup> T-cell subsets.

#### Infected animals exhibit significantly shorter villi

Symptomatic animals (Sym LVL and HVL macaques) exhibited diarrhoea. To examine whether the jejunum of symptomatic animals exhibited the histopathological changes that suggest AIDS-related enteropathy, we measured villous length on haematoxylin and eosin (H&E)-stained samples of jejunum in uninfected and infected macaques. Surprisingly, villous length was significantly



**Fig. 2.** Counts of circulating CD4<sup>+</sup> T-cell subsets and CD4<sup>+</sup> cell frequency in lymphoid and intestinal tissues at the time of euthanasia in SHIV-KS661-infected rhesus macaques. Counts of circulating CD4<sup>+</sup> T-cell subsets were analysed by flow cytometry and whole-blood counts. (a) Circulating CD4<sup>+</sup> T-cell counts. The ID numbers of the macaques are indicated on the figure. (b) Proportion of CD95<sup>+</sup> naive cells in circulating CD4<sup>+</sup> T cells of LVL macaques. Solid black lines indicate Asym LVL macaques and solid grey lines indicate Sym LVL macaques. (c) CD4<sup>+</sup> cell frequencies in thymus, mesenteric lymph nodes (Mes. LN) and jejunum of representative uninfected, Asym LVL, Sym LVL and HVL macaques. Bars, 100 µm. (d) Quantification of jejunum CD4<sup>+</sup> cells in uninfected and infected macaques. The numbers of CD4<sup>+</sup> cells were enumerated in at least ten fields of the tissues at a magnification of 200×. Statistical analysis was performed using Student's *t*-test for the data from five uninfected and each infected macaque (\*, *P* < 0.0001). Data for MM299, MM338, MM339 and MM401 were not available.

shorter in all of the infected animals than in uninfected animals (*P* < 0.0001) (Fig. 3a, b). This suggested that SHIV-infected animals develop villous atrophy, irrespective of viral load.

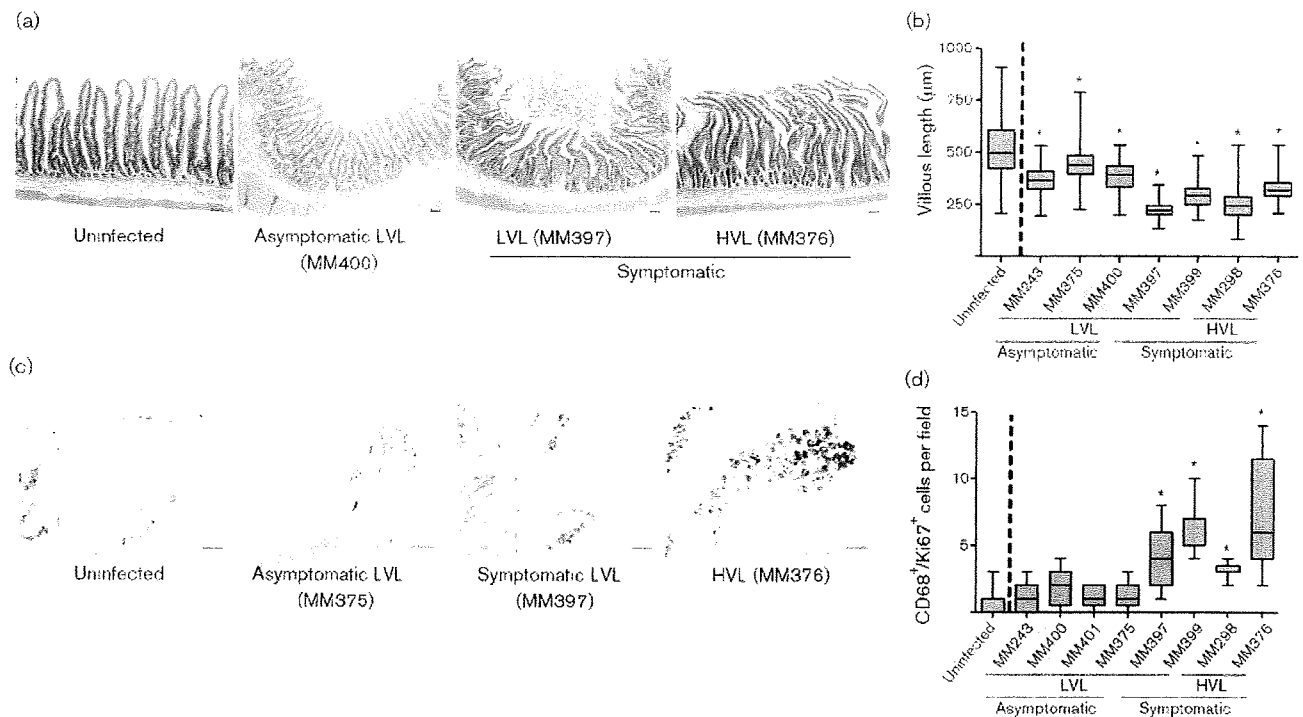
#### Increased number of activated macrophages in the jejunum of symptomatic animals

Macrophages appeared to be more abundant in H&E-stained jejunal sections in symptomatic animals. This was confirmed by CD68 staining: the frequency of CD68<sup>+</sup> macrophages in the jejunum was considerably higher in symptomatic animals than in uninfected animals, but was not significantly different between uninfected animals and Asym LVL macaques (data not shown). Furthermore, CD68<sup>+</sup> macrophages in the small intestine of Sym LVL and HVL macaques appeared to be

activated because their size was increased. To examine whether the number of activated CD68<sup>+</sup> macrophages increased in the small intestine, we double stained for CD68 and Ki67 in the small intestine sections by immunohistochemistry. The frequency of CD68<sup>+</sup> Ki67<sup>+</sup> macrophages in the jejunum of all symptomatic animals examined was significantly higher than that of uninfected animals (*P* < 0.0001) (Fig. 3c, d). This suggested that abnormal activation of intestinal macrophages occurred in symptomatic animals irrespective of viral load.

## DISCUSSION

It is important to discuss initially why some SHIV-infected macaques had an HVL at the late stage, whilst others had



**Fig. 3.** Villous length in jejunum and counts of activated macrophages in the small intestine at the time of euthanasia in SHIV-KS661-infected rhesus macaques. (a) H&E-stained sections of jejunum of representative uninfected, Asym LVL, Sym LVL and HVL macaques. Bars, 200 µm. (b) Comparison of villous length in uninfected and infected macaques. The lengths of at least 100 villi were measured in each macaque. Statistical analysis was performed using Student's *t*-test for the data from four uninfected and each infected macaque (\*,  $P < 0.0001$ ). Data for MM299, MM338, MM339 and MM401 were not available. (c) Ki67 and CD68 staining in the small intestine of representative uninfected, Asym LVL, Sym LVL and HVL macaques. Brown staining indicates Ki67<sup>+</sup> cells and blue staining indicates CD68<sup>+</sup> cells. Bar, 50 µm. (d) Comparison of CD68<sup>+</sup> Ki67<sup>+</sup> cell counts in uninfected and infected macaques. The numbers of CD68<sup>+</sup> Ki67<sup>+</sup> cells were enumerated in at least ten fields of the tissues at a magnification of 200×. Statistical analysis was performed using Student's *t*-test for the data from seven uninfected and each infected macaque (\*,  $P < 0.0001$ ). Data for MM299, MM338 and MM339 were not available.

an LVL. The LVL macaques had much stronger antibody responses than the HVL macaques (Table 1). SHIV-89.6P is easily controlled by the antibody response (Montefiori *et al.*, 1998). SHIV-KS661, which shares its genetic origin with SHIV-89.6P, might be strongly affected by the antibody response. Virus replication during the primary phase clearly occurred later in the intrarectally inoculated macaques than in the intravenously inoculated macaques. Therefore, this delay might contribute to the continuous and strong antibody response in the intrarectally inoculated macaques, consequently resulting in a low viral load in most of the intrarectally inoculated macaques.

The purpose of this study was to elucidate why LVL macaques experience diarrhoea and wasting. A comparison of circulating CD4<sup>+</sup> T-cell counts (Fig. 2a) and relative levels of naïve T-cells (Fig. 2b) in LVL macaques did not reveal a substantial difference between Sym LVL (which showed diarrhoea and wasting) and Asym LVL (which were healthy) macaques. The villous length in the intestine

also did not affect the level of malignancy of the disease condition, as all infected monkeys showed significant villous atrophy, suggesting a high sensitivity to infection itself. However, Sym LVL and HVL macaques exhibited two findings that Asym LVL macaques did not: (i) CD4<sup>+</sup> cell reduction in intestinal and lymphoid tissues (Fig. 2c, d), a hallmark of AIDS; and (ii) abnormal innate immune activation, which was reflected by an increased number of activated macrophages within the intestines (Fig. 3c, d). Ki67 serves as a proliferation marker and proliferation of macrophages may seem unlikely. However, there are some reports about local macrophage proliferation in inflammation sites, indicating the infiltration of activated macrophages associated with tissue damage (Isbel *et al.*, 2001; Norton, 1999). These observations indicated the existence of immunopathological disorders in the intestines not only in HVL macaques but also in Sym LVL macaques.

Many studies have shown positive correlations between the development of AIDS and some characteristic features in

the intestinal tracts of HIV-1-infected humans and pathogenic SIV- or SHIV-infected monkeys: continuous CD4<sup>+</sup> T-cell depletion (Brenchley *et al.*, 2004; Ling *et al.*, 2007), abnormal and chronic immune activation (Brenchley *et al.*, 2006; Hazenberg *et al.*, 2003) and enteropathy (Kotler, 2005). Immune activation (as shown by an increased number of intestinal activated macrophages) and intestinal CD4<sup>+</sup> cell depletion in Sym LVL macaques strongly suggest the presence of an AIDS-like disease in this subset of animals. Hence, these results suggest that an AIDS-like intestinal disease can occur in LVL macaques despite their low viral load, as well as in HVL macaques.

Some HIV-1-infected patients experience poor recovery of circulating CD4<sup>+</sup> T cells, even when their plasma HIV-1 RNA load is suppressed by HAART (Kaufmann *et al.*, 2003; Marchetti *et al.*, 2006; Piketty *et al.*, 1998). These individuals are called immunological non-responders (Marchetti *et al.*, 2006), and have been found to have increased plasma lipopolysaccharide levels, suggesting that bacteria had been translocated from the intestines into the circulation with concomitant activation of T-cell compartments (Marchetti *et al.*, 2006, 2008). Furthermore, some patients who maintain an undetectable or nearly undetectable plasma viral RNA load in the absence of HAART also develop AIDS disease progression (Madec *et al.*, 2005) and have abnormal immune activation and increased plasma lipopolysaccharide levels (Hunt *et al.*, 2008). These observations may indicate that disease progression in a subset of HIV-1-infected individuals is independent of viraemia. Accordingly, the disease progression under conditions of low viral load that we observed in SHIV-KS661-infected macaques can also occur in HIV-1-infected individuals.

Consistent with the fact that intestinal CD4<sup>+</sup> cell depletion triggers mucosal immune dysfunction, a notable difference observed between Sym LVL and Asym LVL macaques was the low CD4<sup>+</sup> cell frequency in the intestines of the Sym LVL macaques. We propose that the intestinal CD4<sup>+</sup> cells in Sym LVL macaques were not able to recover after intestinal CD4<sup>+</sup> cell reduction during the early phases of infection. We reported previously that SHIV-KS661 infection of rhesus macaques caused early intestinal CD4<sup>+</sup> T-cell depletion (Fukazawa *et al.*, 2008; Miyake *et al.*, 2006). Although we did not examine the macaques during the early phases of infection, the intestinal CD4<sup>+</sup> T cells of both Sym LVL and Asym LVL macaques should have been depleted at this time, as even moderately pathogenic SHIV can cause intestinal CD4<sup>+</sup> cell reduction during the early phase of infection (Fukazawa *et al.*, 2008). Therefore, the near-normal frequency of intestinal CD4<sup>+</sup> cells in Asym LVL macaques would be the result of CD4<sup>+</sup> cell recovery after intestinal CD4<sup>+</sup> cell reduction during the early phase of infection. In contrast, intestinal CD4<sup>+</sup> cells in Sym LVL macaques may be unable to recover, even though virus replication has been controlled. Similarly, intestinal CD4<sup>+</sup> cell recovery was found to be important for halting disease progression in SIVmac239-infected

rhesus macaques (Ling *et al.*, 2007). Accordingly, one of the important determinants for disease progression in SHIV-KS661-infected macaques may be CD4<sup>+</sup> cell recovery in the intestines.

We further hypothesize that this inappropriately low level of CD4<sup>+</sup> cells within the intestines of the SHIV-KS661-infected animals (and phenotypically similar humans) is permissive to the excessive activation of resident tissue macrophages. One implication of these studies is that regulatory T-cell subsets of CD4<sup>+</sup> cells may be especially vulnerable to this depletion, thus allowing this macrophage activation in view of the well-known role of regulatory T cells in inhibiting innate immune responses (Maloy *et al.*, 2003). This hypothesis will be important to assess in future studies to understand the pathophysiology in the intestines during the chronic phase of HIV-1 infection.

Taken together, the present results suggest that CD4<sup>+</sup> cell reduction and enteropathy can occur in SHIV-KS661-infected rhesus macaques even when the viral load is low. The ability or inability to restore intestinal CD4<sup>+</sup> cells may be a key factor determining disease progression, irrespective of virus replication levels in the chronic phase of SHIV-KS661 infection. The reason that the recovery of intestinal CD4<sup>+</sup> cells is impeded is unknown, although we can speculate on some possibilities such as the co-existence of other infectious microbial agents or impaired T-cell reconstitution caused by damage during thymopoiesis at an early phase of SHIV infection (Motohara *et al.*, 2006). We demonstrated comparable proviral DNA loads in the examined tissues between Sym and Asym LVL macaques, although the CD4<sup>+</sup> cell frequencies in the tissues were clearly reduced in Sym LVL macaques. Therefore, the quantity of provirus per CD4 cell in the tissues of Sym LVL macaques is considered to be relatively higher than that of Asym LVL macaques, and low-level replication that may be undetectable in the plasma viral load might be maintained in Sym LVL but not in Asym LVL macaques. Identifying the mechanisms of poor recovery of intestinal CD4<sup>+</sup> cells is needed to understand AIDS pathogenesis, because, as stated above, some HIV-1-infected patients have low CD4<sup>+</sup> T-cell counts even when viraemia is controlled. One useful approach is comparative and periodical analysis, including cellular immunology data, of the intestinal tract of the same animals from the early to the chronic phases using Sym LVL and Asym LVL macaques in this SHIV infection macaque model.

## METHODS

**Virus, animals and sample collection.** Highly pathogenic SHIV-KS661 is a molecular clone of SHIV-C2/1 (GenBank accession no. AF217181), which was derived through *in vivo* passages of SHIV-89.6 (Shinohara *et al.*, 1999). The virus stock was prepared from the supernatant of virus-infected CEMx174 and M8166 human lymphoid cell lines.

All rhesus macaques used in this study were treated in accordance with the institutional regulations approved by the Committee for

Experimental Use of Non-human Primates in the Institute for Virus Research, Kyoto University, Japan. All macaques were inoculated with  $2 \times 10^3$  50% tissue culture infectious dose of SHIV-KS661 measured with CEMx174. The animal ID numbers, infection route and when they were euthanized are provided in Fig. 1(a).

Blood was collected periodically using sodium citrate as an anti-coagulant and examined by flow cytometry and for quantification of plasma viral RNA load. Tissue samples were obtained at the time of euthanasia and were used for quantification of proviral DNA and histopathology.

**Determination of plasma viral RNA and proviral DNA loads.** The viral loads in plasma and proviral DNA loads in lymphoid and intestinal tissues were determined by quantitative RT-PCR and quantitative PCR, respectively, as described previously (Motohara *et al.*, 2006). DNA samples were extracted directly from frozen tissue sections of each monkey using a DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol.

**Determination of antibody titres.** Anti-HIV antibody titres were determined using a commercial particle agglutination kit (Serodia-HIV1/2; Fujirebio). Isolated plasma samples were serially diluted and assayed. The end point of the highest dilution giving a positive result was determined as the titre.

**Flow cytometry.** Flow cytometry was performed as described previously (Motohara *et al.*, 2006). Briefly, CD4<sup>+</sup> T cells were analysed by a combination of fluorescein isothiocyanate (FITC)-conjugated anti-monkey CD3 (clone FN-18; BioSource) and phycoerythrin-conjugated anti-human CD4 (clone NU-TH1; Nichirei), and subsets of naive and memory CD4<sup>+</sup> cells were analysed by a combination of FITC-conjugated anti-human CD95 (clone DX2; BD Pharmingen) and allophycocyanin-conjugated anti-human CD4 (clone L200; BD Pharmingen). CD95<sup>-</sup> CD4<sup>+</sup> cells were defined as naive CD4<sup>+</sup> T cells and CD95<sup>+</sup> CD4<sup>+</sup> cells were defined as memory CD4<sup>+</sup> T cells. Labelled lymphocytes were examined on a FACSCalibur analyser using CellQuest software (BD Biosciences).

**Histology and immunohistochemistry.** Tissue samples were fixed in 4% paraformaldehyde in PBS at 4 °C overnight and embedded in paraffin wax. Sections (4 µm) were dewaxed using xylene, rehydrated through an alcohol gradient, and stained with H&E. The villous length of the jejunum was measured with a micrometer. At least 40 villi from each section were measured.

For immunohistochemistry, sections were rehydrated and processed for 10 min in an autoclave in 10 mM citrate buffer (pH 6.0) to unmask the antigens, sequentially treated with TBS/Tween 20 (TBST) and aqueous hydrogen peroxide, left at 4 °C overnight or at room temperature for 30 min or 1 h for primary antibody reactions, washed with TBST, incubated at room temperature for 1 h with an Envision + kit (a horseradish peroxidase-labelled anti-mouse immunoglobulin polymer; Dako), visualized using diaminobenzidine (DAB) substrate (Dako) as a chromogen, rinsed in distilled water, counterstained with haematoxylin and analysed by light microscopy (Biozero BZ-8000; Keyence).

For double staining (CD68 and Ki67) of sections, appropriately processed sections were incubated at room temperature for 1 h with unlabelled anti-Ki67 antibody at a dilution of 1:2000, the highly sensitive tyramide amplification step (CSAIL; Dako) was performed, the slides were reacted with DAB to visualize the results and incubated with unlabelled anti-CD68 antibody at 4 °C overnight followed by incubation at room temperature for 1 h with Histofine Simple Stain AP (an alkaline phosphatase-labelled anti-mouse immunoglobulin polymer (Nichirei), and the results were visualized with a Blue Alkaline Phosphatase Substrate kit III (Vector Laboratories).

Measurements of CD68<sup>+</sup> Ki67<sup>+</sup> cell counts were performed in ten fields at a magnification of 200× by light microscopy.

Primary antibodies used in immunohistochemistry were anti-human CD4 (diluted 1:30; clone NCL-CD4; Novocastra Laboratories), anti-SIV Nef (diluted 1:500; FIT Biotech), anti-human CD68 (diluted 1:50; clone KP-1; Dako) and anti-human Ki67 (Ki-S5; Dako).

**Statistical analysis.** The significance of CD4<sup>+</sup> or CD68<sup>+</sup> Ki67<sup>+</sup> cell frequency measurements and villous length in the jejunum of infected monkeys compared with uninfected monkeys was analysed using an unpaired Student's *t*-test (two-tailed) using GraphPad Prism 4.0E software (Varsity Wave).

## ACKNOWLEDGEMENTS

We are grateful to Dr James Raymond for editing the English of this manuscript; to Takahito Kazama, Reii Horiuchi, Noriko Nakajima and Tetsutaro Sata for technical support; to Dr Michael A. Eckhaus for histopathological interpretation; and to Takeshi Kobayashi for critical reading. This work was supported, in part, by Research on HIV/AIDS in Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare, Japan; a Grant-in-Aid for Scientific Research from the Ministry of Education and Science, Japan; a Research Grant for AIDS on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation; and a Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan.

## REFERENCES

- Anton, P. A., Elliott, J., Poles, M. A., McGowan, I. M., Matud, J., Hultin, L. E., Grovit-Ferbas, K., Mackay, C. R., Chen, I. S. Y. & Giorgi, J. V. (2000). Enhanced levels of functional HIV-1 co-receptors on human mucosal T cells demonstrated using intestinal biopsy tissue. *AIDS* 14, 1761–1765.
- Batman, P. A., Miller, A. R., Forster, S. M., Harris, J. R., Pinching, A. J. & Griffin, G. E. (1989). Jejunal enteropathy associated with human immunodeficiency virus infection: quantitative histology. *J Clin Pathol* 42, 275–281.
- Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., Nguyen, P. L., Khoruts, A., Larson, M. & other authors (2004). CD4<sup>+</sup> T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* 200, 749–759.
- Brenchley, J. M., Price, D. A., Schacker, T. W., Asher, T. E., Silvestri, G., Rao, S., Kazzaz, Z., Bornstein, E., Lambotte, O. & other authors (2006). Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med* 12, 1365–1371.
- Fackler, O. T., Schafer, M., Schmidt, W., Zippel, T., Heise, W., Schneider, T., Zeitz, M., Riecken, E. O., Mueller-Lantzsch, N. & Ullrich, R. (1998). HIV-1 p24 but not proviral load is increased in the intestinal mucosa compared with the peripheral blood in HIV-infected patients. *AIDS* 12, 139–146.
- Fukazawa, Y., Miyake, A., Ibuki, K., Inaba, K., Saito, N., Motohara, M., Horiuchi, R., Himeno, A., Matsuda, K. & other authors (2008). Small intestine CD4<sup>+</sup> T cells are profoundly depleted during acute simian-human immunodeficiency virus infection, regardless of viral pathogenicity. *J Virol* 82, 6039–6044.
- Gibbons, T. & Fuchs, G. J. (2007). Chronic enteropathy: clinical aspects. *Nestle Nutr Workshop Ser Pediatr Program* 59, 89–101.
- Hazenbergh, M. D., Otto, S. A., van Benthem, B. H., Roos, M. T., Coutinho, R. A., Lange, J. M., Hamann, D., Prins, M. & Miedema, F.

- (2003). Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS* 17, 1881–1888.
- Hunt, P. W., Brenchley, J., Sinclair, E., McCune, J. M., Roland, M., Page-Shafer, K., Hsue, P., Emu, B., Krone, M. & other authors (2008). Relationship between T cell activation and CD4<sup>+</sup> T cell count in HIV-seropositive individuals with undetectable plasma HIV RNA levels in the absence of therapy. *J Infect Dis* 197, 126–133.
- Isbel, N. M., Nikolic-Paterson, D. J., Hill, P. A., Dowling, J. & Atkins, R. C. (2001). Local macrophage proliferation correlates with increased renal M-CSF expression in human glomerulonephritis. *Nephrol Dial Transplant* 16, 1638–1647.
- Kahn, E. (1997). Gastrointestinal manifestations in pediatric AIDS. *Pediatr Pathol Lab Med* 17, 171–208.
- Kaufmann, G. R., Perrin, L., Pantaleo, G., Opravil, M., Furrer, H., Telenti, A., Hirschel, B., Ledergerber, B., Vernazza, P. & other authors (2003). CD4 T-lymphocyte recovery in individuals with advanced HIV-1 infection receiving potent antiretroviral therapy for 4 years: the Swiss HIV Cohort Study. *Arch Intern Med* 163, 2187–2195.
- Kotler, D. P. (2005). HIV infection and the gastrointestinal tract. *AIDS* 19, 107–117.
- Lapenta, C., Boirivant, M., Marini, M., Santini, S. M., Logozzi, M., Viora, M., Belardelli, F. & Fais, S. (1999). Human intestinal lamina propria lymphocytes are naturally permissive to HIV-1 infection. *Eur J Immunol* 29, 1202–1208.
- Ling, B., Veazey, R. S., Hart, M., Lackner, A. A., Kuroda, M., Pahar, B. & Marx, P. A. (2007). Early restoration of mucosal CD4 memory CCR5 T cells in the gut of SIV-infected rhesus predicts long term non-progression. *AIDS* 21, 2377–2385.
- Madec, Y., Boufassa, F., Porter, K. & Meyer, L. (2005). Spontaneous control of viral load and CD4 cell count progression among HIV-1 seroconverters. *AIDS* 19, 2001–2007.
- Maloy, K. J., Salaun, L., Cahill, R., Dougan, G., Saunders, N. J. & Powrie, F. (2003). CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med* 197, 111–119.
- Marchetti, G., Gori, A., Casabianca, A., Magnani, M., Franzetti, F., Clerici, M., Perno, C. F., Monforte, A., Galli, M. & Meroni, L. (2006). Comparative analysis of T-cell turnover and homeostatic parameters in HIV-infected patients with discordant immune-virological responses to HAART. *AIDS* 20, 1727–1736.
- Marchetti, G., Bellistri, G. M., Borghi, E., Tincati, C., Ferramosca, S., La Francesca, M., Morace, G., Gori, A. & Monforte, A. D. (2008). Microbial translocation is associated with sustained failure in CD4<sup>+</sup> T-cell reconstitution in HIV-infected patients on long-term highly active antiretroviral therapy. *AIDS* 22, 2035–2038.
- Miyake, A., Ibuki, K., Enose, Y., Suzuki, H., Horiuchi, R., Motohara, M., Saito, N., Nakasone, T., Honda, M. & other authors (2006). Rapid dissemination of a pathogenic simian/human immunodeficiency virus to systemic organs and active replication in lymphoid tissues following intrarectal infection. *J Gen Virol* 87, 1311–1320.
- Montefiori, D. C., Reimann, K. A., Wyand, M. S., Manson, K., Lewis, M. G., Collman, R. G., Sodroski, J. G., Bolognesi, D. P. & Letvin, N. L. (1998). Neutralizing antibodies in sera from macaques infected with chimeric simian–human immunodeficiency virus containing the envelope glycoproteins of either a laboratory-adapted variant or a primary isolate of human immunodeficiency virus type 1. *J Virol* 72, 3427–3431.
- Motohara, M., Ibuki, K., Miyake, A., Fukazawa, Y., Inaba, K., Suzuki, H., Masuda, K., Minato, N., Kawamoto, H. & other authors (2006). Impaired T-cell differentiation in the thymus at the early stages of acute pathogenic chimeric simian–human immunodeficiency virus (SHIV) infection in contrast to less pathogenic SHIV infection. *Microbes Infect* 8, 1539–1549.
- Norton, W. T. (1999). Cell reactions following acute brain injury: a review. *Neurochem Res* 24, 213–218.
- Paiardini, M., Frank, I., Pandrea, I., Apetrei, C. & Silvestri, G. (2008). Mucosal immune dysfunction in AIDS pathogenesis. *AIDS Rev* 10, 36–46.
- Piketty, C., Castiel, P., Belec, L., Batisse, D., Si Mohamed, A., Gilquin, J., Gonzalez-Canali, G., Jayle, D., Karmochkine, M. & other authors (1998). Discrepant responses to triple combination antiretroviral therapy in advanced HIV disease. *AIDS* 12, 745–750.
- Sestak, K. (2005). Chronic diarrhea and AIDS: insights into studies with non-human primates. *Curr HIV Res* 3, 199–205.
- Sharpstone, D. & Gazzard, B. (1996). Gastrointestinal manifestations of HIV infection. *Lancet* 348, 379–383.
- Shinohara, K., Sakai, K., Ando, S., Ami, Y., Yoshino, N., Takahashi, E., Someya, K., Suzuki, Y., Nakasone, T. & other authors (1999). A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. *J Gen Virol* 80, 1231–1240.
- Smith, P. D., Meng, G., Salazar-Gonzalez, J. F. & Shaw, G. M. (2003). Macrophage HIV-1 infection and the gastrointestinal tract reservoir. *J Leukoc Biol* 74, 642–649.
- Veazey, R. S., DeMaria, M., Chalifoux, L. V., Shvetz, D. E., Pauley, D. R., Knight, H. L., Rosenzweig, M., Johnson, R. P., Desrosiers, R. C. & Lackner, A. A. (1998). Gastrointestinal tract as a major site of CD4<sup>+</sup> T cell depletion and viral replication in SIV infection. *Science* 280, 427–431.
- Veazey, R. S., Mansfield, K. G., Tham, I. C., Carville, A. C., Shvetz, D. E., Forand, A. E. & Lackner, A. A. (2000a). Dynamics of CCR5 expression by CD4<sup>+</sup> T cells in lymphoid tissues during simian immunodeficiency virus infection. *J Virol* 74, 11001–11007.
- Veazey, R. S., Tham, I. C., Mansfield, K. G., DeMaria, M., Forand, A. E., Shvetz, D. E., Chalifoux, L. V., Sehgal, P. K. & Lackner, A. A. (2000b). Identifying the target cell in primary simian immunodeficiency virus (SIV) infection: highly activated memory CD4<sup>+</sup> T cells are rapidly eliminated in early SIV infection in vivo. *J Virol* 74, 57–64.
- Wilcox, C. M. & Saag, M. S. (2008). Gastrointestinal complications of HIV infection: changing priorities in the HAART era. *Gut* 57, 861–870.



