responses to T-dependent HIV-1 antigens might be stimulated without inducing serum IgG production, and putative human homolog of the mouse gene influencing the T cell-dependent production of FV-neutralizing antibodies might be involved in the above activation of mucosal IgA-production in EUI individuals. In fact, the segment of mouse chromosome 15 between the D15Mit1 and D15Mit118 loci and the corresponding segment of human chromosome 22 harbor several genes that are known to be involved in T- and B-cell growth and activation. Expression analyses of these candidate genes both in the mouse model and in humans are currently underway.

None of the previously reported human genes that affect the risk of HIV acquisition are located in chromosome 22, *CCR5* and *CCR2* being located at 3p21, *SDF1* and *MBL2* at 10q11.1 and 10q11.2, respectively, *HLA* including the polymorphic *TNF* and *MIC* loci at 6p21.3, *KIR*s at 19q13.4, *IL10* at 1q31-32, and *SLC11A1 (NRAMP1)* at 2q35 [3,40–52]. In addition, the homozygous *CCR5-Δ32* mutation, which results in the lack of the HIV coreceptor [3,40–42], is known to be rare among the EUI individuals in Italy and Thailand [4,9,49], and was not found in the enrolees of the present study, although three of the 42 EUI individuals were heterozygous for this mutation (data not shown). In a very recent analysis of a separate cohort of repeatedly exposed but HIV-1-seronegative individuals in the USA, Liu *et al.* [53] demonstrated the lack of association between genotypes at the *CCR2*, *SDF1*, and *RANTES* loci and the uninfected status. The homozygous *CCR5-Δ32* mutation was also rare (3.2%) among the seronegative individuals. The same authors also noted a significant difference in the frequencies of heterozygosity at the polymorphic *DC-SIGN (CD209)* locus at 19p13.2 between the exposed but seronegative and HIV-1-infected groups: however, the observed frequency of heterozygotes was 3.2% (3/94), and thus, this genetic skewing could not explain the possible mechanisms that confer HIV resistance to the majority of the seronegative individuals. Altogether, our results have indicated the possible presence in human chromosome 22 of a novel genetic factor that is associated with strong T-cell and mucosal immune responses to HIV-1 antigens.

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Appendix

The possible presence of a dominant allele having different frequencies between the phenotypic groups was examined as follows: Define x_{ij} as the number of individuals having the genotype i/j $(i \leq j)$ for the EUI group, where $n = \sum_{i \leq j} x_{ij}$ is the total number of individuals belonging to this group. Assume that $x = (x_{ij})_{i \leq j}$ has a multinomial distribution with the parameter $a = (a_{ij})_{i \leq j}$, where $\sum_{i \leq j} a_{ij} = 1$. For convenience, let $a_{ji} = a_{ij}$. Similarly, define the notations y, b and z, c for the HIV-1-infected and healthy control groups, respectively. The frequency of the individuals having the allele i for the EUI group is expressed as $a_i = \sum_k a_{ik}$. Similarly, define b_i and c_i. The hypothesis where the frequencies of the individuals having the allele i for the EUI and HIV-infected groups is the same is expressed

as H_i: $a_i = b_i$. Similarly, consider the hypothesis $a_i = c_i$ to compare the EUI with healthy control groups.

We tested whether or not the frequency of individuals possessing a certain dominant allele in the EUI group was different from those in other groups. Since there are multiple candidate alleles at each locus, we must take multiple comparisons into consideration. Let t_i be the test statistic for allele i. t_i and t_j can be strongly correlated, especially when most of the individuals having allele i or j are of the genotype i/j. Therefore, the typically used Bonferroni correction can be too conservative. A universally applicable method for overcoming this problem is a closed testing procedure [29], where t_i is based on a well-acquainted variance stabilizing transformation and the test statistic for a common hypothesis is based on the maximization of t_i values.

Let $\overline{H}$ be the closed set consisting of all the intersections of the hypotheses' H_i values. Assume that we can make the reject region with common significance level α for any hypothesis $H \in \overline{H}$. The closed testing procedure says that we can reject $H \in \overline{H}$ only after we reject all the hypotheses including H, using the corresponding reject region. Let t_i be the standardized test statistic for the hypothesis H_i. The corresponding reject region becomes $W_i = \{|t_i| > e_i\}$. Consider a common hypothesis H. For example, let H be the intersection of $H_1, \ldots, H_I$. The corresponding reject region can be defined by $W = \{\max_{i=1,\ldots,I}|t_i| > e\}$. We used the following variance stabilizing type as the standardized test statistic:

$$t_i = \frac{\left(\sin^{-1}\sqrt{x_i/n_x} - \sin^{-1}\sqrt{y_i/n_y}\right)}{\sqrt{1/4n_x + 1/4n_y}}$$

where $n_x = \sum_{i \leq j} x_{ij}$ and $n_y = \sum_{i \leq j} y_{ij}$. As an advantage over the commonly used likelihood ratio and Pearson's χ^2 tests, the above type enables us to infer that the smaller a P value is the stronger the rejection of the corresponding null hypothesis, because the variances of the arcsine are constant independent of the samples. In view of the closed testing procedure, if the maximal intersection hypothesis $H \in \overline{H}$ is rejected, the individual hypothesis corresponding to the minimum P value can automatically be rejected. In addition, if the hypothesis corresponding to the minimum P value alone is rejected among the individual hypotheses, it is the only rejected hypothesis.

The joint distribution of t_i values can be approximated by the multivariate normal distribution under the null hypothesis, and the corresponding approximated P values can easily be calculated for the individual hypotheses. The approximated P values for a common hypothesis can be calculated by using the central limit theorem and the parametric bootstrap [30] based on the asymptotic null distribution of t_i values. To avoid unnecessary disturbances, we tested only the hypotheses having the estimated frequency ≥ 0.1 when considering the common hypotheses, because alleles with a frequency < 0.1 cannot explain the phenotype of the whole group. Calculations were performed by drawing 100 000 random samples from the approximated multivariate normal distribution for each hypothesis.

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Peptide-induced immune protection of CD8$^+$ T cell-deficient mice against Friend retrovirus-induced disease

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Abstract

CD8$^+$ CTLs and virus-neutralizing antibodies have been associated with spontaneous and vaccine-induced immune control of retroviral infections. We previously showed that a single immunization with an *env* gene-encoded CD4$^+$ T cell epitope protected mice against fatal Friend retrovirus infection. Here, we analyzed immune cell components required for the peptide-induced anti-retroviral protection. Mice lacking CD8$^+$ T cells were nevertheless protected against Friend virus infection, while mice lacking B cells were not. Virus-producing cells both in the spleen and bone marrow decreased rapidly in their number and became undetectable by 4 weeks after infection in the majority of the peptide-immunized animals even in the absence of CD8$^+$ T cells. In the vaccinated animals the production and class switching of virus-neutralizing and anti-leukemia cell antibodies were facilitated; however, virus-induced erythroid cell expansion was suppressed before neutralizing antibodies became detectable in the serum. Further, the numbers of virus-producing cells in the spleen and bone marrow in the early stage of the infection were smaller in the peptide-immunized than in unimmunized control mice in the absence of B cells. Thus, peptide immunization facilitates both early cellular and late humoral immune responses that lead to the effective control of the retrovirus-induced disease, but CD8$^+$ T cells are not crucial for the elimination of virus-infected cells in the peptide-primed animals.

Introduction

Understanding the types of immune responses associated with and responsible for effective control of viral infection is pivotal for the development of antiviral vaccines. We, along with other researchers, have been studying the requirements of different immune cell components and their regulation by host genetic factors utilizing the mouse model of Friend retrovirus infection. Friend retrovirus complex (FV) is composed of replication-competent Friend murine leukemia virus (F-MuLV) and defective spleen focus-forming virus (SFFV), the latter of which induces rapid growth and terminal differentiation of infected erythroid progenitor cells (1, 2). FV is known to induce fatal erythroleukemia associated with severe immunosuppression when inoculated into immunocompetent adult mice of susceptible strains (1, 3). Genotypes at both MHC class I and class II loci, along with those at a non-MHC locus located on chromosome 15, affect spontaneous immune resistance against FV-induced disease development which is phenotypically manifested by the regression of early splenomegaly and clearance of viremia (1, 4–8). As predicted, requirements of both CD4$^+$ and CD8$^+$ T cells for the above

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spontaneous resistance have been demonstrated through antibody-mediated depletion of T cell subsets and through the blocking of T cell responses by administration of anti-MHC class II antibodies (9, 10). Further, different roles of $CD4^+$ and $CD8^+$ T cells and of virus-neutralizing antibodies have been demonstrated for immune protection against FV infection induced with a live attenuated vaccine (11, 12).

We previously showed that a single immunization with an 18-mer peptide that contains a single $CD4^+$ T cell epitope identified within the *env* gene product SU of F-MuLV induces strong protective immunity against fatal FV infection in susceptible strains of mice (13, 14). In peptide-immunized (B10.A × A.BY)F_1 mice, the vast majority of virus-producing cells were eliminated from the spleen between 8 and 12 days after FV challenge, and the SFFV-induced early splenomegaly regressed rapidly. Production and class switching of virus-neutralizing antibodies roughly coincided with the above reduction in the number of virus-producing cells in the spleen (13), suggesting the possible importance of virus-neutralizing antibodies in the vaccine-induced confinement of FV infection. However, since the activation of both $CD4^+$ and $CD8^+$ cytotoxic effector cells and of NK cells was detectable prior to the decrease of virus-producing cells in peptide-immunized (BALB/c × C57BL/6)F_1 ($CB6F_1$) mice (14), it was also possible that the cellular responses, rather than the antibodies, were mainly responsible for the control of FV-induced disease development conceivably through the destruction of virus-producing cells. Moreover, since $CD8^+$ CTLs and NK cells were activated in comparable degrees both in peptide-immunized and unimmunized animals after FV infection (14), their actual extents of contribution to the peptide-induced immune protection remained unclear.

To directly evaluate the role of each separate immune cell component in peptide-induced protection against FV infection and to compare the effector mechanisms induced by the peptide immunization with those induced by previously described live attenuated vaccines (11, 15), we performed the protection experiments on the highly susceptible strain of mice that lacked either $CD8^+$ T or B lymphocytes.

Methods

Mice

BALB/c-AJcl and $CB6F_1$ mice were purchased from Japan SLC, Inc., Hamamatsu, Japan. (B10.A × A.BY)F_1 mice were those described previously (13). Breeding pairs of BALB/c-J-*B2m*^{tm1Unc} and C57BL/6J (B6)-*B2m*^{tm1Unc} mice carrying homozygous disruption of the β_2-microglobulin gene ($\beta_2m^{-/-}$) were purchased from the Jackson Laboratory, Bar Harbor, ME, USA, and F_1 crosses were produced at the Animal Facilities, Kinki University School of Medicine. Phenotypic lack of $CD8^+$ T cells in the produced F_1 crosses was confirmed by bleeding each mouse from the tail vein and staining peripheral blood with a mixture of fluorescence-labeled anti-CD4 and anti-CD8 mAbs as described in the following section.

B6-*Igh-6*^{tm1Cgm} mice carrying homozygous disruption of the membrane exon of the Ig μ-chain gene (μ-chain membrane exon-targeted: *μMT/μMT*) and thus lacking B cells (16) were also purchased from the Jackson Laboratory. To introduce the μ-chain disruption into BALB/c background, a cross-intercross production of a congenic strain was performed as follows: the B cell-deficient B6 male mice were mated with BALB/c female mice and F_1 crosses carrying heterozygous disruption of the μ-chain membrane exon were obtained. These heterozygous F_1 crosses were cross-mated, and F_2 mice carrying the homozygous μ gene disruption were selected by performing both genetic and phenotypic analyses as described below. The homozygous disruption of the μ-chain membrane exon in the resulting F_2 crosses was confirmed by PCR analyses as follows: genomic DNA was prepared from the tail tip of each mouse using DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Oligo-DNA primers (5′ primer: 5′-TCTATCGCCTTCTTGACGAG-3′, 3′ primer: 5′-TACAGCTCAGCTGTCTGTGG-3′) were prepared based on the sequence information on the knockout cassette (16) and were used for PCR amplification of genomic DNA fragments. PCR products were separated by electrophoresis in a 4% agarose gel and were visualized under a UV light after ethidium bromide staining. In addition to the above genetic analyses, peripheral blood was stained with a mixture of fluorescence-labeled anti-CD3 and anti-CD19 mAb, and multicolor flow cytometric analyses were performed as described in the following section. Male F_2 mice carrying homozygous disruption of the μ-chain membrane exon and thus lacking B cells were mated with BALB/c female mice again, and this cross-intercross mating procedure was repeated seven times. After the seventh cycle of crossing and intercrossing, the resultant BALB/c-background mice possessing homozygous disruption of the μ-chain gene were maintained by sister–bother mating, and $CB6F_1$ mice lacking B cells were produced by crossing the B6-*Igh-6*^{tm1Cgm} and the above-established BALB/c-*μMT/μMT* mice.

For immune protection experiments, both male and female mice aged 8–11 weeks at the time of immunization were used throughout the present study. All the animal experiments were approved by the Animal Experiment Committee and performed under the guidelines of Kinki University.

Viruses and their inoculation

A stock of B-tropic FV complex was originally given by Bruce Chesebro, Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases, Hamilton, MT, USA. The stock used in the present study has been described (14, 17). SFFV and F-MuLV titers of the FV stock were determined as described previously (13, 18). For inoculation into $CB6F_1$ mice, a dilution of the virus stock prepared with phosphate-buffered balanced salt solution (PBBS) containing 2% fetal bovine serum (FBS) was injected intravenously into the tail vein. Infected mice were observed at least twice a day and the number of surviving mice was determined. The development of splenomegaly was monitored by palpation as described (5, 17, 19). In some experiments, moribund mice were killed by cervical dislocation and spleen weights were measured to compare the results of palpation with actual spleen weights. Spleens weighing >0.5 g were consistently marked as palpable splenomegaly. Mice found dead were

dissected, and their spleen weight was measured to confirm leukemic death.

Peptide synthesis and immunization

The peptides used for detailed mapping of the $CD4^+$ T cell epitope were synthesized by Fmoc chemistry and purified, and their molecular weight confirmed by quad-polar mass spectrometry as described previously (20–22). Peptides used for immune protection experiments were ordered from Qiagen K. K. (Tokyo, Japan). For immunization each peptide was dissolved in PBBS and emulsified with an equal volume of CFA (Difco Laboratories, Detroit, MI, USA). Mice were injected intradermally with 100 µl of the emulsion given as multiple split doses into the abdominal wall. Control mice were given an emulsion of PBBS and CFA that did not contain any peptide.

T cell proliferation assays

Two T cell clones, F5-5 and FP7-11 (20, 23), specific for the $E^{b/d}$-restricted C-terminal epitope of F-MuLV *env* gene product were maintained as described previously (20). For examination of proliferative responses, 2×10^5 spleen cells irradiated with 40 Gy γ-ray were mixed with 2×10^5 T cells and various concentrations of a peptide in a well of 96-well microculture plates. After 48 h of incubation at 37°C, each culture was pulsed with 18.5 kBq [^{3}H]thymidine (Du Pont NEN, Boston, MA, USA) for the final 18 h. Cells were harvested onto a glass fiber filter, and incorporated radioactivity was measured with a microplate scintillation counter (TopCount, Packard Instrument Co., Meriden, CT, USA). For the calculation of relative stimulatory effect of each peptide, the concentration of peptide i (µM) required to induce 50% of the maximum proliferative response (ED_{50}) was divided with ED_{50} (µM) of the peptide in question (22, 23). In the present study proliferative responses were measured for a range of peptide concentrations between 0.01 and 20 µM in 2-fold dilutions, and peak responses (>30 000 counts per minute) were observed by stimulation with 1 µM of peptide i. ED_{50} of peptide i was 0.2 µM.

Assays for virus-neutralizing antibodies

The *in vitro* assays for quantitative measurement of F-MuLV-neutralizing antibodies have been described elsewhere in detail (5, 13, 17, 19). Mice were bled from the tail vein under ether anesthesia and sera separated were stored at −30°C until use. Stock of an infectious molecular clone of F-MuLV, FB29 (24), was prepared from a high-producer clone of chronically infected *Mus dunni* cells. Serial 2-fold dilutions of sera were made with PBBS containing 1% FBS and mixed with an appropriate dilution of the F-MuLV stock and inoculated to *Mus dunni* cells in 24-well plates. Control wells were inoculated with the virus dilution admixed with the diluent alone. Two days later, foci of F-MuLV-infected cells were visualized with mAb 720 (18) and counted under a dissecting microscope. Neutralizing titers were determined by the reciprocals of maximum dilutions that gave a reduction in the number of F-MuLV-infected cell foci to <25% of those in the control wells. IgG titers were determined by treating each serum sample with 0.05 M 2-mercaptoethanol whereas IgM titers were calculated by dividing the neutralizing titers of the untreated sera by the corresponding IgG titers (6).

Infectious center assays

These assays were performed as described previously (13, 17). Briefly, spleen and bone marrow cell suspensions prepared from mice challenged with FV were serially diluted with PBBS containing 2% FBS, plated in triplicate at concentrations between 30 and 3×10^6 cells per well onto monolayers of *Mus dunni* cells that had been seeded at 1.0×10^4 cells ml^{-1} per well on the previous day and then co-cultured for 2 days. After washing with PBBS and fixation with methanol, F-MuLV-infected cell foci were stained with mAb 720 (18), visualized by using the avidin-biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA, USA) and counted under a magnifier. The numbers of detected foci are in linear correlation with the numbers of spleen cells inoculated in the range between 30 and 3×10^6 cells per well. For the plating of the whole spleen cells from each mouse, 5×10^6 spleen cells per well were added similarly to the previously started culture of *Mus dunni* cells in 20 wells of a 24-well plate, and the remaining spleen cells were diluted and plated at 5×10^5 and 5×10^4 cells per well into separate wells.

Flow cytometry

Flow cytometric analyses of cell-surface markers were performed as described elsewhere (14, 17, 25). Spleen and bone marrow tissues were dissociated in PBBS containing 2% FBS, and a single-cell suspension was prepared by passing each dissociated tissue through a nylon mesh. Cells were stained with a combination of the following mAbs, washed three times with PBBS containing 2% FBS and 0.05% NaN_3 and stained with 20 µg ml^{-1} 7-aminoactinomycin D (7-AAD). 7-AAD was used to exclude dead cells (26). The mAbs and their final concentrations used in the present study were: cychrome-conjugated anti-mouse CD3 (hamster IgG, PharMingen, San Diego, CA, USA) at 0.5 µg per 10^6 cells, FITC-conjugated anti-mouse CD4 (rat IgG2b, Seikagaku Corporation, Tokyo, Japan) at 0.5 µg per 10^6 cells, R-PE-conjugated anti-mouse CD8 (rat IgG2a, Caltag Laboratories, Burlingame, CA, USA) at 1 µg per 10^6 cells, PE-conjugated anti-mouse CD19 (rat IgG2a, PharMingen) at 1 µg per 10^6 cells, FITC-conjugated anti-mouse CD69 (hamster IgG, PharMingen) at 1 µg per 10^6 cells and allophycocyanin-conjugated anti-mouse TER-119 (PharMingen) at 0.2 µg per 10^6 cells. TER-119 reacts with a molecule associated with glycophorin A, and marks the late erythroblasts and mature erythrocytes, but not burst-forming and colony-forming units of erythroid cells (27). Biotinylated mAb 720 (IgG1) and 514 (IgM) used for the detection of F-MuLV gp70 and SFFV gp55, respectively, on infected cell surfaces has been described (13, 17). mAb 34 (IgG2b) reactive with the p15 (MA) protein (28) was similarly purified and biotinylated to detect cell-surface expression of the *gag* gene products (19). All staining reactions were performed in the presence of 0.25 µg per 10^6 cells anti-mouse CD16/CD32 (PharMingen) as described previously (25) to prevent the binding of mAb to FcR-expressing cells. Isotype-matched control antibodies were either purchased from the same suppliers or prepared as purified and biotinylated Ig of an irrelevant specificity as described (25), and staining patterns obtained with the negative-control antibodies were used to draw demarcation lines between cells positively stained and

those not stained. Multicolor flow cytometric analyses were performed with a Becton Dickinson FACSCalibur and CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Mature erythrocytes and dead cells were excluded from the analyses by setting a polygonal gate in the dot plots showing intensities of forward scatter and fluorescence for 7-AAD.

Titration of serum antibody reactive to the surface of FV-induced leukemia cells

Sera were serially diluted between 1/4 and 1/256 with PBBS and 100 μl of each dilution was incubated with 10^6 FV-induced leukemia cells Y57-2C ($H2^{b/b}$). Characteristics of the leukemia cell line used in the present study have been described (14). After washing twice with PBBS containing 2% FBS, bound IgM and IgG were differentially detected by incubating the cells either with FITC-conjugated anti-mouse IgM (μ-chain specific, Southern Biotechnology Associates, Inc., Birmingham, AL, USA) at 5 μg per 10^6 cells or with FITC-conjugated anti-mouse IgG (γ-chain specific, Zymed Laboratories, Inc., South San Francisco, CA, USA) at 1.5 μg per 10^6 cells, respectively, for 20 min. Stained cells were washed three times before being examined by flow cytometry as described above.

Purification of T cells and their transfer

Purification of T cell subsets from the spleen of naive, immunized and/or FV-infected mice was performed by using mAb-conjugated magnetic microbeads and a magnetic cell sorter I (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Spleen cells were first treated with Tris-buffered ammonium chloride solution to lyse erythrocytes, and incubated with anti-B220 mAb-conjugated magnetic beads to remove B cells by passing through a negatively selecting CS column. To purify $CD4^+$ T cells, $B220^-$ cells were then incubated with anti-CD8 mAb-conjugated magnetic beads, passed through a CS column to remove $CD8^+$ cells and then incubated with anti-CD4 mAb-conjugated microbeads to positively select $CD4^+$ cells by passing through a VS column. Multicolor flow cytometric analyses revealed that the resultant cell preparation was >99% $CD4^+$. $CD8^+$ cells were similarly purified from $B200^-$ cells by removing $CD4^+$ cells and positively selecting $CD8^+$ cells. This preparation was 97–98% $CD8^+$ in repeated experiments. Percentages of $CD4^+$ and $CD8^+$ T cells in the spleen after FV inoculation were determined by flow cytometry in $CB6F_1$ mice immunized with peptide i. To reconstitute the full number of T cells that belonged to each subset in the immunized mice, unimmunized recipient mice were injected intravenously with 2×10^7 to 2.5×10^7 $CD4^+$ or $CD8^+$ T cells per mouse.

Depletion of $CD4^+$ T cells

Anti-mouse CD4 mAbs were purified from culture supernatant of the hybridoma cell GK 1.5 (29) as described previously (13, 25). Control rat myeloma IgG was purchased from Zymed Laboratories, Inc. The amount of the mAbs required for complete depletion of $CD4^+$ T cells from $CB6F_1$ mice was determined by intravenously administering the purified mAb and monitoring the number of $CD4^+$ and $CD8^+$ cells in the spleen by flow cytometry. The schedule of mAb administration finally adopted was as follows: $CB6F_1$ mice were immunized with 3 μg per mouse of peptide i emulsified in CFA. Three weeks later, mice were intravenously given 125 μg per mouse purified anti-CD4 mAb. Five additional intravenous doses of 125 μg per mouse anti-CD4 mAb were given 2, 4, 6, 9 and 18 days after the first administration. The negative-control rat IgG was given to a separate group of mice on the same schedule. Mice were inoculated with 150 spleen focus-forming units (SFFU) FV 7 days after the beginning of the mAb administration. Peripheral blood was collected from three representative animals at each time point through the tail vein at post-infection day (PID) 3, 6, 10 and 13, and flow cytometric analyses were performed to confirm the absence of $CD4^+$ T cells.

Statistical analyses

Differences in survival curves expressed by the Kaplan–Meier method were compared by a Mantel–Haenszel logrank test using GraphPad Prism 3 (GraphPad Software, Inc., San Diego, CA, USA). The numbers of mice that developed or lacked splenomegaly were compared between the immunized and unimmunized groups by Fisher's exact test. Average numbers of infectious centers between experimental groups and anti-leukemia cell antibody titers were compared by Mann–Whitney's *U*-test because these values were not expected to follow a Gaussian distribution. Differences in IgM and IgG titers of virus-neutralizing antibodies were compared by paired *t*-test. Spleen weights and percentages of $TER\text{-}119^+$, $gp70^+$ cells in the spleen and bone marrow between the immunized and unimmunized groups of mice were compared by Student's or Welch's *t*-test depending on whether the variances of the compared samples were estimated to be equal or not.

Results

Suppression of the early growth of FV-infected erythroid cells and prevention of leukemic death in highly susceptible $CB6F_1$ mice by immunization with a single-epitope $CD4^+$ T cell vaccine

Mice of BALB/c background are extremely susceptible to FV-induced disease, and $CB6F_1$ mice all died within 60 days after infection with only 15 SFFU of FV without showing any signs of spontaneous recovery (Fig. 1a). This was striking because even $(B10.A \times A/WySn)F_1$ mice that have been used as a strain typically susceptible to FV infection have shown mortality rates of 70–80% at 90–100 days after inoculation with 15 SFFU FV (1, 5). Therefore, the following immune protection experiments were performed in $CB6F_1$ mice with 150 SFFU of FV to ensure that peptide-induced immune responses protect this highly susceptible strain of mice from doses of FV large enough to kill all unimmunized animals.

The efficacy of peptide i in priming $CD4^+$ T cells *in vivo* has been demonstrated by the establishment of $CD4^+$ T cell clones reactive to this peptide from the peptide-immunized $CB6F_1$ mice (23), and by more pronounced expansion of $CD4^+$ T cells in the spleen after FV challenge in the peptide-immunized than in the unimmunized control $CB6F_1$ mice (14). To directly demonstrate the priming of $CD4^+$ T cells in peptide-immunized

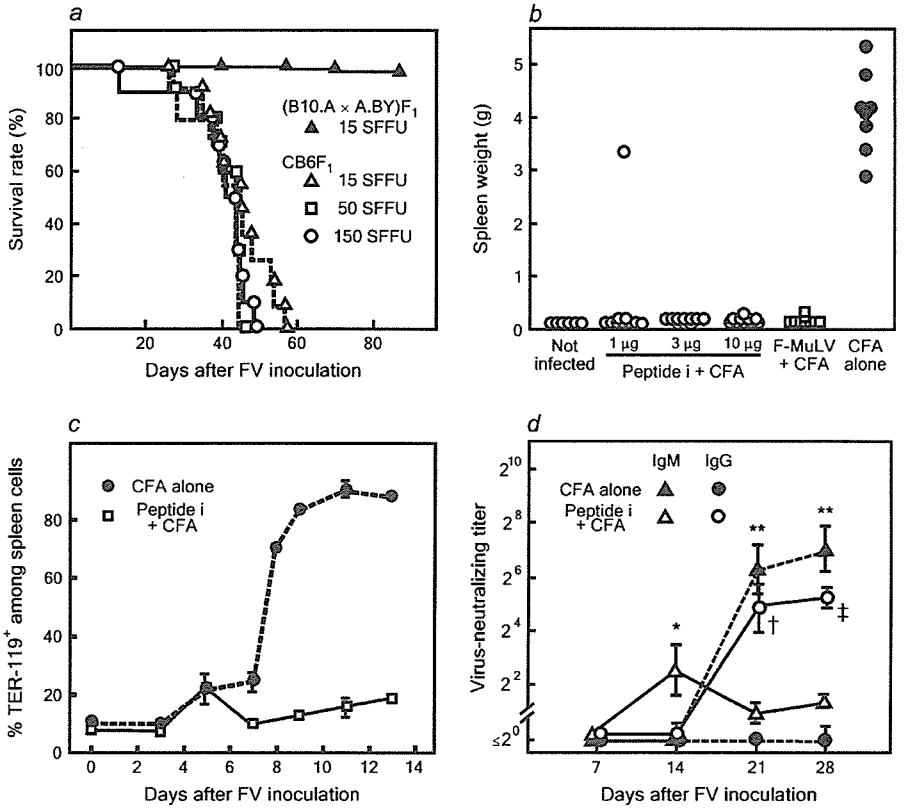

Fig. 1. Development of FV-induced disease in $CB6F_1$ mice and its prevention by immunization with the single-epitope peptide i. (a) $CB6F_1$ mice were inoculated intravenously with 15 ($n = 11$), 50 ($n = 10$) or 150 SFFU ($n = 10$) of FV and the survival of infected animals was examined. Similar curves were obtained in three repeated experiments. For a comparison, $(B10.A \times A.BY)F_1$ ($n = 40$) mice were also infected with 15 SFFU of FV and followed for their survival until PID 90. (b) The effect of different doses of peptide i on protective immunity against FV. $CB6F_1$ mice were immunized once with 1, 3 or 10 μg per mouse of peptide i in CFA, or given a CFA emulsion of purified F-MuLV particles at 40 μg per mouse. The F-MuLV particles used had been inactivated by UV irradiation as described (19). Control mice were given CFA without any peptide (CFA alone). Mice were challenged with 150 SFFU FV 4 weeks after immunization, and their spleen weight was measured as soon as they died (CFA alone group) or were killed at PID 45. Significant differences in spleen weights were only observed between the CFA alone group and five other groups ($P < 0.006$). (c) Changes in the percentages of TER-119⁺ erythroid cells among nucleated spleen cells of FV-infected $CB6F_1$ mice. Mice were immunized once with 10 μg (5 nmol) of peptide i in CFA or given CFA alone and inoculated with FV 4 weeks later. Each data point shows mean ± SEM calculated by using four to five individual mice per group. (d) Changes in serum titers of virus-neutralizing IgM and IgG antibodies after FV infection. $CB6F_1$ mice were either immunized once with 10 μg per mouse peptide i in CFA or given CFA alone, and challenged with 150 SFFU FV 4 weeks later. Each data point shows mean ± SEM calculated by using seven to eight individual mice per group. Serum titers of F-MuLV-neutralizing IgM and IgG were compared by paired *t*-test: *IgM titers are significantly higher than IgG titers at $P < 0.05$; **$P < 0.0001$. †, IgG titers are significantly higher than IgM titers at $P < 0.05$; ‡, $P < 0.01$.

$CB6F_1$ mice, $CD4^+$ and $CD8^+$ T cells were purified from $CB6F_1$ mice at 3 weeks after a single immunization with peptide i, and re-stimulated *in vitro* in the presence of syngeneic, γ-irradiated spleen cells as antigen-presenting cells (APCs). As shown in Fig. 2(a), $CD4^+$ T cells purified from the immunized $CB6F_1$ mice proliferated vigorously when stimulated with 1 μM of peptide i, while the proliferative responses of $CD4^+$ T cells purified from the control mice given CFA alone were below the background level even when stimulated with 20 μM of the same peptide. As controls, $CD8^+$ T cells purified either from the peptide-immunized or unimmunized control mice showed no significant proliferative responses even when stimulated with 20 μM peptide i.

To determine the minimal amount of the peptide that is required for the effective induction of protective immunity against FV infection, three different amounts of peptide i were given as a single intradermal immunization to $CB6F_1$ mice, and immunized mice were challenged with 150 SFFU FV. Since most of the unimmunized $CB6F_1$ mice died by PID 45 (Fig. 1a), infected mice were either dissected soon after their death or killed at PID 45, and their spleen weight was measured. As shown in Fig. 1(b), a single immunization with 3 μg (1.7 nmol) per mouse of peptide i was as effective as 10 μg per mouse of the same peptide, and only one mouse among the ten that were given 1 μg peptide i developed splenomegaly after FV infection. Thus, in the following experiments, 3–10 μg per mouse of peptide i was used as a sufficiently large protective dose.

FV-induced early splenomegaly is caused by the rapid growth and differentiation of SFFV-infected erythroid progenitor

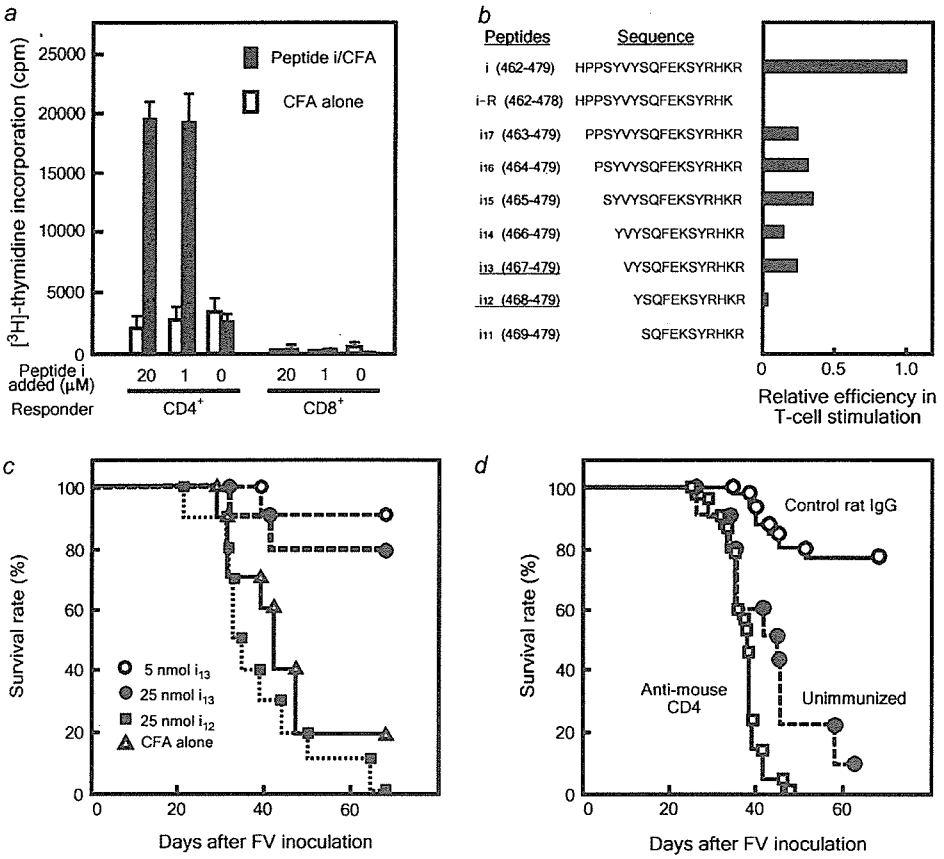

Fig. 2. Priming of CD4⁺ T cells by peptide immunization and efficacies in CD4⁺ T cell stimulation *in vitro* and immune protection *in vivo* of peptide i and its truncated derivatives. (a) Both CD4⁺ and CD8⁺ T cells were purified from the spleen of $CB6F_1$ mice at 3 weeks after a single immunization with 10 μg per mouse peptide i emulsified in CFA. Proliferative responses were measured 2 days after stimulation with the indicated concentration of peptide i along with syngeneic, γ-irradiated spleen cells as APCs. CD4⁺ and CD8⁺ T cells purified from $CB6F_1$ mice given CFA without a peptide (CFA alone) were used as controls. Data shown are averages + SEM of triplicate cultures, and the experiments were performed three times with essentially the same results. (b) Sequences of peptide i and its truncated derivatives, and their relative efficiency to stimulate FV-specific T cell clones. Representative data obtained with clone F5-5 are shown, while the data obtained with clone FP7-11 were consistent with those presented here. ED_{50} of the full-length i was 0.2 μM. (c) Protection of $CB6F_1$ mice against FV infection with the truncated peptide. $CB6F_1$ mice ($n = 10$ per group) were immunized once with 5 nmol per mouse of peptide i_{13}, 25 nmol per mouse of i_{13} or 25 nmol per mouse of i_{12}. Control mice were given CFA emulsion containing no peptide. Four weeks later, they were inoculated with 150 SFFU FV and followed for their survival. (d) $CB6F_1$ mice ($n = 22$ per group) were immunized once with 3 μg per mouse of peptide i and repeatedly injected with the anti-CD4 mAb (□) or control rat IgG (○). Four weeks after immunization, these mice and a group of unimmunized control mice (●) were inoculated with 150 SFFU FV and followed for their survival.

cells, and the resultant erythroblasts and maturing red cells are marked by mAb TER-119 (27). Thus, bursting of the TER-119⁺ erythroid cells was observed in the unimmunized control mice starting from PID 7, following the slow initial increase of the same cell population (Fig. 1c). On the other hand, the number of TER-119⁺ erythroid cells in the spleen started to decrease between PID 5 and 7 in the $CB6F_1$ mice that had been immunized once with peptide i. Virus-neutralizing antibodies in the serum were not detectable at PID 7 in FV-infected $CB6F_1$ mice regardless of whether they had been immunized with peptide i or not (Fig. 1d). In the $CB6F_1$ mice immunized with peptide i, virus-neutralizing IgM became detectable by PID 14, and the antibodies switched to IgG between PID 14 and 21. In the unimmunized mice, however, virus-neutralizing antibodies became detectable at PID 21, a week later than in the peptide-immunized mice, and they did not switch to IgG even at PID 28. These results indicated that the FV-induced expansion of erythroid cells was prevented in the peptide-immunized mice before virus-neutralizing antibodies became detectable in the serum.

Protection against FV disease correlates with CD4⁺ T cell stimulation

To identify the minimal effective sequence of the peptide vaccine, a series of truncated peptides were compared for their *in vitro* T cell-stimulating and *in vivo* protection efficacies (Fig. 2b and c). It was clear that the C-terminal Arg residue was indispensable for the recognition of this epitope by T cells. In fact, a 17-mer peptide, i-R, that lacked only the C-terminal

Arg totally lost the ability to stimulate $CD4^+$ T cell proliferation *in vitro*, while another 17-mer, i_{17}, that retained the Arg residue but lacked the N-terminal His kept the ability, albeit less efficiently than peptide i, to stimulate the T cells. When N-terminal residues were further removed from the 18-mer i and their efficacy to stimulate the $CD4^+$ T cells was examined through the range of concentrations between 0.01 and 20 μM, the 13-mer (i_{13}) retained the T cell-stimulating activity and showed a stimulatory effect comparable to peptide i_{17}, while the 12-mer (i_{12}) showed a stimulatory effect <1/24 of that of the full-length peptide i. Peptide i_{11} did not induce significant proliferation even when as much as 20 μM was added to the culture. In line with this result, the 13-mer retained the ability to induce protection against FV challenge in immunized $CB6F_1$ mice, while the 12-mer did not protect the same strain of mice against FV-induced disease even when five times more molecules were administered (Fig. 2c).

The requirement of $CD4^+$ T cells for the peptide-induced immune protection was further confirmed by depleting $CD4^+$ T cells from vaccinated $CB6F_1$ mice. The adopted schedule of the antibody administration resulted in undetectable $CD4^+$ T cells in the spleen in separately examined uninfected animals for a period equivalent to PID 0–14, and lack of $CD4^+$ T cells in the peripheral blood was confirmed in the vaccinated and infected group on PID 3–13 (data not shown). Antibody-induced depletion of $CD4^+$ T cells abrogated the efficacy of peptide immunization, and $CD4^+$ T cell-depleted animals died even more rapidly than the unimmunized control mice ($P < 0.05$). Injection of the control rat IgG did not affect the protective efficacy of the peptide vaccine, and ~80% of the peptide-immunized $CB6F_1$ mice that had been given the control antibody survived past PID 60 (Fig. 2d).

Peptide-induced immune protection against FV-induced disease in $CB6F_1$ mice genetically lacking a single component of the immune system

To examine possible effectiveness of the peptide immunization in mice genetically lacking either $CD8^+$ T or B cell components of the immune system, we produced $CB6F_1$ mice with a homozygous disruption of the β_2m gene or of the Ig μ-chain gene. The absence of $CD8^+$ T or B lymphocytes, respectively, was confirmed by flow cytometric analyses of the spleen and PBMCs (data not shown). In accordance with the prior experiments (Figs 1 and 2), ≥80% of the wild-type $CB6F_1$ mice were protected against FV infection when immunized with peptide i. Protective efficacy of the 13-mer peptide, i_{13}, was further confirmed, and the development of early splenomegaly was prevented in 70% of the $CB6F_1$ mice given i_{13} (Fig. 3a). Surprisingly, when $CB6F_1$-$\beta_2m^{-/-}$ mice lacking $CD8^+$ T cells were immunized with peptide i, only <30% of the immunized mice developed splenomegaly and >70% survived until PID 100 in repeated experiments (Fig. 3). The observed survival curves were not significantly different between the peptide-immunized wild-type and $\beta_2m^{-/-}$ groups ($P > 0.4$), indicating a similar effectiveness of the peptide vaccine both in the presence and absence of $CD8^+$ T cells. On the other hand, when the mice of the same susceptible $CB6F_1$ background that lacked B cells due to the homozygous *μMT* mutation were immunized with peptide i, they developed splenomegaly and all died by PID 100, indicating crucial roles of B cells for the peptide-induced immune protection. Interestingly, however, the temporal changes in the incidences of splenomegaly and leukemic death delayed significantly in repeated experiments in the peptide-immunized, B cell-deficient mice compared with those in the unimmunized control mice of the same deficiency (Fig. 3c and f). The delay in the development of splenomegaly in the peptide-immunized *μMT/μMT* mice was also substantiated by flow cytometric enumerations of FV-infected erythroid cells: at PID 7, 28.2 ± 3.7% ($n = 5$) of the nucleated spleen cells were positive for both TER-119 and F-MuLV gp70 in the unimmunized control mice, while the proportion of the TER-119^+, $gp70^+$ cells in the spleen was significantly smaller ($P < 0.05$) 12.1 ± 8.2% ($n = 5$) in the peptide-immunized *μMT/μMT* mice. The effect of peptide immunization was more striking in the bone marrow where the percentage of TER-119^+, $gp70^+$ cells in the unimmunized mice was 11.1 ± 4.4%, while that of the peptide-immunized mice was 0.72 ± 0.22% ($P < 0.03$) at PID 7. These results indicate some functions of non-B cells in delaying the FV-induced disease development.

Elimination of FV-producing cells from the spleen and bone marrow in the $\beta_2m^{-/-}$ mice immunized with peptide i

We next compared the numbers of FV-infected cells between peptide-immunized and unimmunized control mice using infectious center assays. The relative ratio in the number of FV-producing cells in the spleen between the peptide-immunized and unimmunized mice started to decrease at PID 8 as observed in the previous experiments (13, 14), and FV infectious centers became undetectable by our assays by PID 28 in peptide-immunized wild-type mice (Fig. 4a). The lack of detectable FV-producing cells in the spleen of all the tested, peptide-immunized $CB6F_1$ mice was confirmed by seeding the cells prepared from the entire spleen ($>10^8$) of each animal as infectious centers at PID 28. The number of FV-producing cells in the bone marrow was also significantly lower in the peptide-immunized wild-type mice than in the unimmunized control mice at PID 8, 14 and 21, and became undetectable at PID 28 (Fig. 4d). At PID 28, 2.1×10^7 bone marrow cells were tested from each mouse and no infectious centers were detectable by our assays in any of the examined animals. In the $CB6F_1$-$\beta_2m^{-/-}$ mice, the numbers of FV-producing cells in the spleen and bone marrow were significantly lower in the peptide-immunized than in unimmunized control mice at PID 14 and 28 (Fig. 4b and e), in accordance with the observed effectiveness of the peptide immunization in preventing the FV-induced disease development in the absence of $CD8^+$ T cells (Fig. 3). It should be noted that in seven of the nine immunized animals tested at PID 28 no infectious centers were detectable even when the cells of the entire spleen were inoculated into the culture. However, there were also individuals among the peptide-immunized $\beta_2m^{-/-}$ mice in which FV-producing cells were still detectable in the spleen or bone marrow at PID 28 (Fig. 4b and e), while such cells were not detectable in any of the immunized wild-type mice tested at the same time point. These results imply that $CD8^+$ T cells were not necessarily required but may play some roles in the elimination of virus-infected cells in peptide-immunized $CB6F_1$ mice.

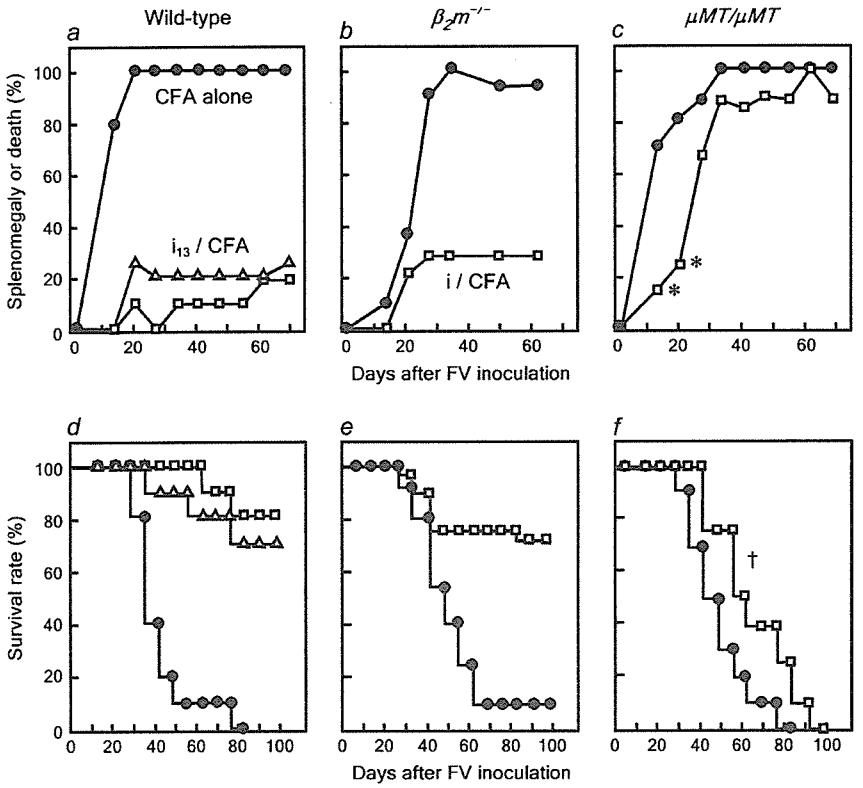

Fig. 3. Effects of immunization with peptide i on the development of FV-induced disease in $CB6F_1$ mice lacking $CD8^+$ T or B cells. Wild-type $CB6F_1$ mice (a and d), $CB6F_1$ mice lacking $CD8^+$ T cells due to homozygous targeting of the β_2m gene (b and e) and $CB6F_1$ mice lacking B cells due to homozygous targeting of the membrane exon of Ig μ-chain gene (c and f) were either immunized with 10 μg per mouse of peptide i in CFA (□) or given CFA alone (●). Another group of the wild-type mice were immunized with 10 μg per mouse of peptide i_{13} in CFA (Δ). Four weeks later, they were inoculated with 150 SFFV FV and followed for the development of splenomegaly and leukemic death. In (c), * indicates significant differences in the frequency of splenomegaly between the immunized and control groups ($P < 0.001$), and in (f), † indicates significant difference between the two survival curves ($P = 0.041$). The number of animals in each group were: (a) and (d), □, 10; Δ, 10; ●, 10; (b) and (e), □, 23; ●, 20 and (c) and (f), □, 12; ●, 16. The experiments were performed twice with essentially identical results.

In accordance with the lack of protection against FV-induced disease development, virus-producing cells constantly increased between PID 5 and 21 in the spleen and bone marrow of the $CB6F_1$-*μMT/μMT* mice, regardless of whether the hosts were immunized with peptide i or not. Interestingly, however, the numbers of virus-producing cells both in the spleen and bone marrow were significantly lower in the peptide-immunized, B cell-deficient mice than those in the unimmunized control mice of the same deficiency at PID 8 (Fig. 4f). This observation is consistent with the significant delay in the development of early splenomegaly and leukemic death (Fig. 3), and smaller numbers of TER-119⁺ and viral $gp70^+$ FV-infected erythroid cells in the spleen and bone marrow in peptide-immunized, B cell-deficient $CB6F_1$ mice.

Priming and re-activation of CD4⁺ T cells in the peptide-immunized μMT/μMT mice

To examine the possibility that the observed inefficiency in anti-FV protection of the immunization with peptide i in $CB6F_1$-*μMT/μMT* mice might be due to the lack of APC activity, rather than antibody-producing function, of B lymphocytes, peptide-specific proliferative responses were compared between the wild-type and *μMT/μMT* animals. When $CD4^+$ T cells purified from the wild-type $CB6F_1$ mice previously immunized with peptide i were used as responders, irradiated spleen cells both from the wild-type and from the *μMT/μMT* animals induced strong proliferative responses, although the peptide-specific proliferation was significantly weaker when *μMT/μMT* instead of the wild-type spleen cells were used as APC (Fig. 5a). FV infection significantly affected the APC function of wild-type spleen cells, but that of *μMT/μMT* spleen cells was not significantly reduced when used at PID 10. Similar results were observed when $CD4^+$ T cells purified from immunized *μMT/μMT* animals were used as responders. Thus, $CD4^+$ T cells were primed with peptide i in the absence of B cells, and spleen cells from *μMT/μMT* animals could present the peptide antigen to primed $CD4^+$ T cells, albeit less efficiently than the wild-type spleen cells, even after FV infection. Successful priming of $CD4^+$ T cells and their re-activation upon FV infection in *μMT/μMT* animals were further confirmed *in vivo* by analyzing the expression of an early activation marker, CD69, on T cells. Upon FV infection of peptide-immunized $CB6F_1$-*μMT/μMT*

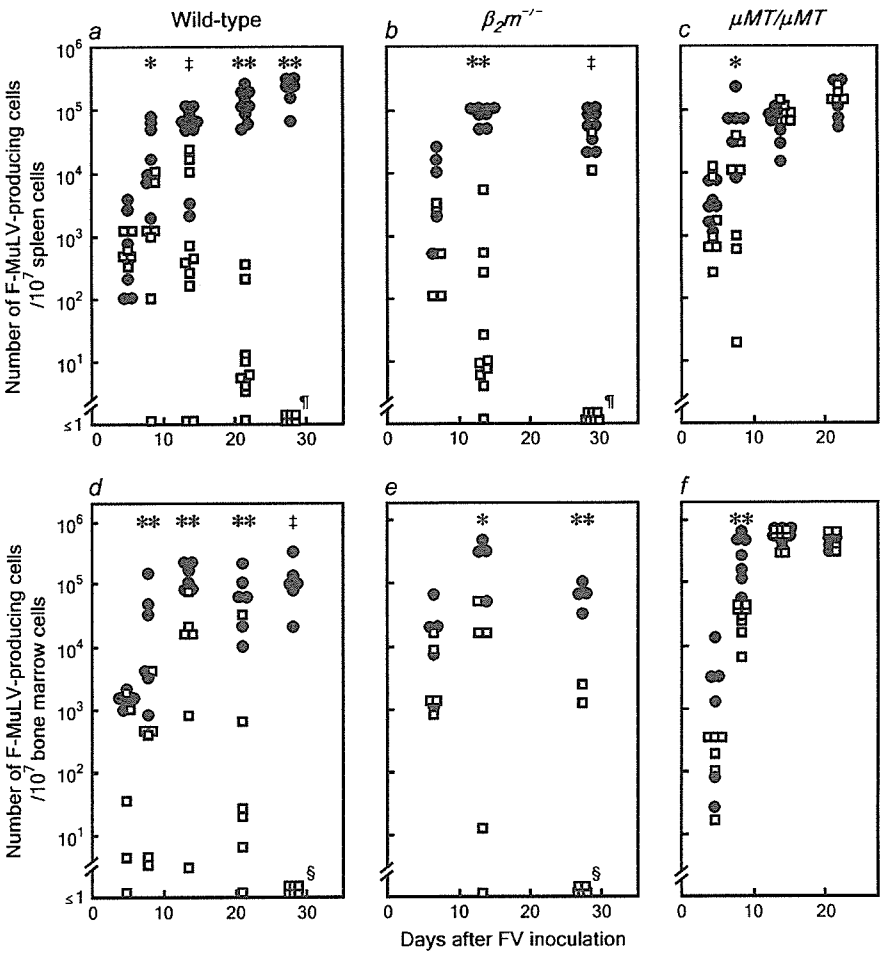

Fig. 4. Effects of immunization with peptide i on the number of FV-producing cells in $CB6F_1$ mice lacking $CD8^+$ T or B cells. Wild-type $CB6F_1$ mice (a and d), the $CB6F_1$ mice lacking $CD8^+$ T cells (b and e) and the $CB6F_1$ mice lacking B cells (c and f) were either immunized with 10 μg per mouse of peptide i in CFA (□) or given CFA alone (●). Four weeks later, they were inoculated with 150 SFFV FV and FV-producing infectious centers were enumerated in the spleen (a–c) and bone marrow (d–f). Each data point shows the actual number of infectious centers detected from each individual mouse. At least 10^7 spleen and bone marrow cells were tested from each animal. At PID 28, the cells prepared from the entire spleen ($>10^8$, ¶) and 2.1×10^7 (from wild-type mice) or 3.1×10^7 (from $\beta_2m^{-/-}$ mice) bone marrow cells (§) were inoculated as infectious centers to ensure the lack of detectable virus-producing cells. Infectious centers were undetectable from any of the tested animals indicated with ¶ or § at PID 28. Statistical significance of the difference between the immunized and unimmunized groups at each time point was examined: $*P < 0.04$; $**0.001 < P < 0.01$; ‡, $0.0002 < P < 0.001$.

mice, an increase in the proportion of $CD69^+$ cells among $CD4^+$ T cells was readily detectable (Fig. 5b). The percentages of $CD69^+$ cells among $CD4^+$ T cells in the spleen at PID 7 were significantly higher in the peptide-immunized than in the unimmunized animals, indicating re-activation of peptide-primed T cells upon FV infection (Fig. 5c). The effect of peptide immunization on the induction of CD69 expression was even more pronounced when bone marrow cells were tested (Fig. 5d). Interestingly, the $CD69^+$ population among $CD8^+$ T cells also showed a significant increase when peptide-immunized and unimmunized *μMT/μMT* mice were compared at PID 7, confirming the previously demonstrated activation of $CD8^+$ cytotoxic cells at PID 7 (14).

Production and class switching of serum antibodies reactive to the surface of FV-induced leukemia cells in the peptide-immunized mice

Although virus-neutralizing antibodies were not detectable in FV-infected animals until PID 14 (Fig. 1d), non-neutralizing anti-FV antibodies might have been produced at earlier time points, and might have contributed to the observed decrease in the number of FV-infected cells in the vaccinated animals, which was evident at as early as PID 7 (Figs 1c and 4). Thus, the possible presence of anti-FV antibody in the serum was examined using FV-induced leukemia cells as indicators. The hemisyngeneic ($H2^{b/b}$), FV-induced leukemia cells Y57-2C expressed both the F-MuLV *gag* and *env* gene products as

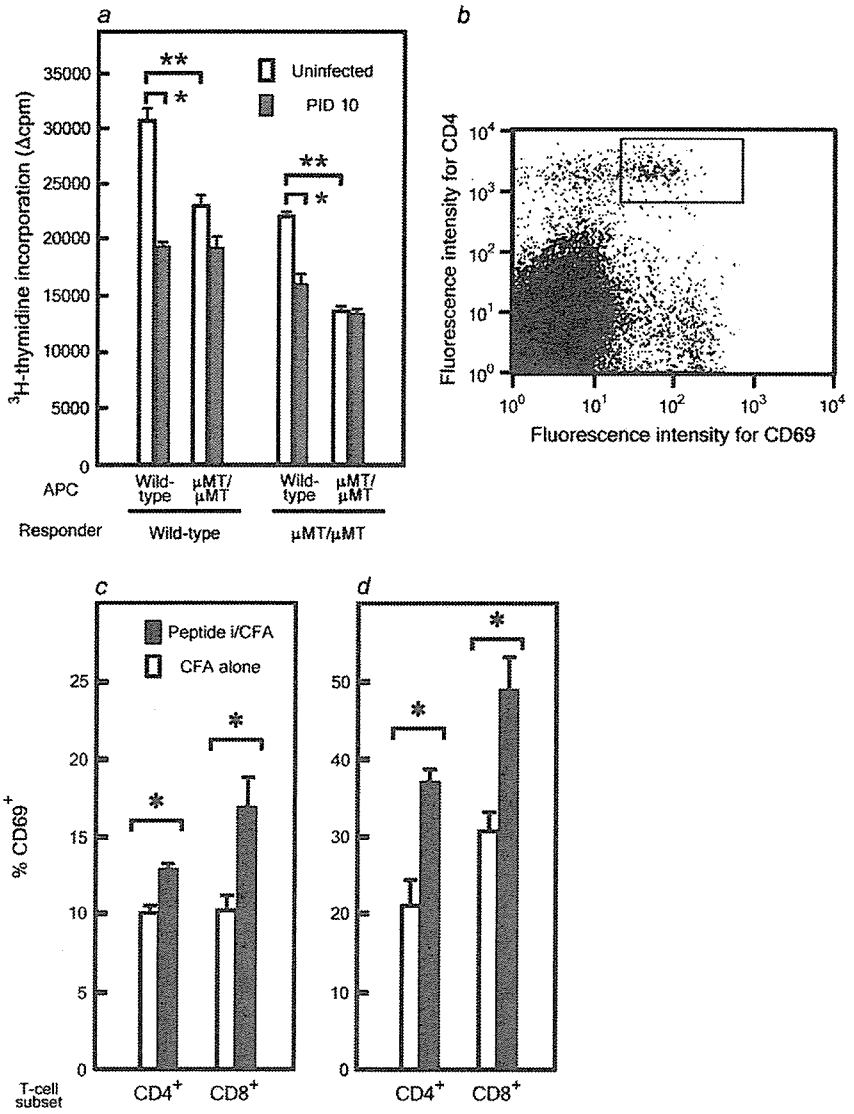

Fig. 5. Priming and re-activation of CD4$^+$ T cells with peptide i in the B cell-deficient mice. (a) CD4$^+$ T cells were purified from the spleen of wild-type and homozygous *μMT/μMT* $CB6F_1$ mice at 3 weeks after a single immunization with 10 μg per mouse peptide i emulsified in CFA. Proliferative responses were measured at 2 days after stimulation with 1 μM peptide i along with the indicated APC. Spleen cells as APC were prepared from the wild-type and *μMT/μMT* $CB6F_1$ mice either without FV inoculation or at PID 10, and γ-irradiated. The magnitude of antigen-specific proliferation is shown by Δ counts per minute (c.p.m.) in this chart by subtracting the average [^{3}H]thymidine ([^{3}H]TdR) incorporation into the cultures containing no peptide from that in the peptide-containing cultures. Levels of [^{3}H]TdR incorporation into the cultures without a peptide were <120 c.p.m. Data shown are averages + SEM of triplicate cultures, and the experiments were performed twice with essentially the same results. *, significantly different at $P < 0.01$; **$P < 0.001$. (b) A representative pattern of CD69 expression on CD4$^+$ T cells in the bone marrow of the *μMT/μMT* mice previously immunized with peptide i. The small rectangle indicates the gate used to calculate the percentage of CD69$^+$ cells among CD4$^+$ T cells. (c) and (d) Comparison of the percentages of CD69$^+$ activated cells among CD4$^+$ and CD8$^+$ T cells in the spleen (c) and bone marrow (d) at 7 days after FV inoculation between the peptide-immunized and unimmunized *μMT/μMT* mice. Data are averages + SEM calculated with five mice per group. *, the percentage of CD69$^+$ population is significantly higher in the immunized than in the unimmunized mice at $P < 0.005$.

well as SFFV gp55 on their surfaces (Fig. 6a). Sera from FV-infected $CB6F_1$ mice bound onto the surface of Y57-2C cells, and geometric means of the fluorescence intensities decreased in proportion to serum dilutions (Fig. 6b). Therefore, at each time point titers of serum antibodies reactive to the surface of the FV-induced leukemia cells, designated hereinafter anti-leukemia cell antibody titers, were determined by dividing geometric means of fluorescence intensities obtained by incubating the indicator cells with a 1/16 dilution of serum samples by the geometric mean of fluorescence intensities obtained with the same dilution of pooled control serum collected from uninfected $CB6F_1$ mice. Interestingly,

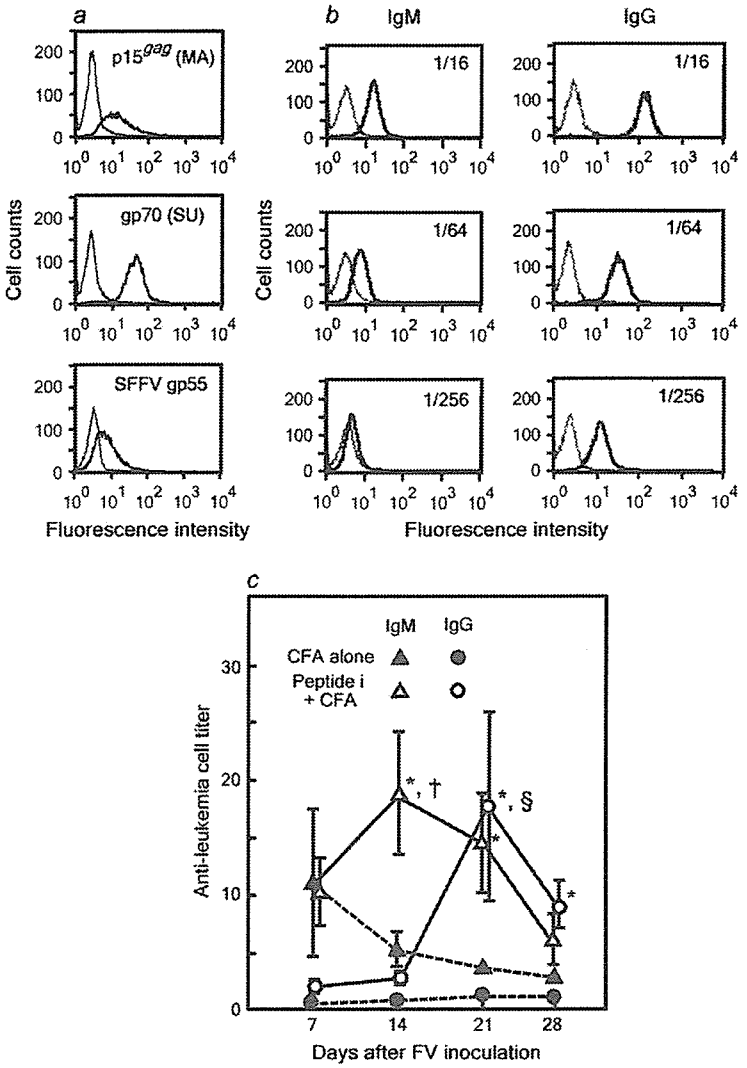

Fig. 6. Detection of serum antibody reactive to the surfaces of FV-induced leukemia cells in FV-infected $CB6F_1$ mice. (a) Expression of FV antigens on the surfaces of Y57-2C cells used as the indicators for FACS analyses. Thick lines represent the binding of each mAb reactive to the indicated FV gene product, while thin lines represent the binding of each isotype-matched control antibody. (b) Representative patterns showing the binding of serum IgM and IgG onto Y57-2C cells. A serum sample obtained from a peptide-immunized $CB6F_1$ mouse at PID 21 was diluted serially and incubated with Y57-2C cells. FACS patterns of IgM and IgG binding at the indicated serum dilution are shown, with thin lines representing the binding of the pooled control serum prepared from uninfected $CB6F_1$ mice at the same indicated dilution. Similar results were obtained when $H2^{a/a}$ AA-41 leukemia cells were used as indicator cells. (c) Changes in the titers of IgM and IgG anti-leukemia cell antibodies detectable in the sera of $CB6F_1$ mice after FV infection. $CB6F_1$ mice were either immunized once with 10 μg per mouse peptide i in CFA or given CFA alone and challenged with 150 SFFU FV 4 weeks later. Each data point shows mean ± SEM calculated by using five to six mice per group. *, titers in the immunized mice are significantly higher than those in unimmunized mice at $P < 0.05$; †, the IgM titer is significantly higher than IgG titer at $P < 0.05$; §, the average IgG titer at PID 21 is significantly higher than that at PID 14, $P < 0.01$.

serum anti-leukemia cell antibodies were detectable at as early as PID 7, but average titers of these antibodies decreased in the following 3 weeks of infection, and no class switching to IgG was observed in the unimmunized animals (Fig. 6c). In contrast, anti-leukemia cell IgM titers were significantly higher in the peptide-immunized than in the unimmunized control mice at PID 14, and IgG class of anti-leukemia cell antibodies were detectable at PID 21 and 28 in the peptide-immunized animals.

Role of CD8⁺ T cells in the induction of Ig class switching of virus-neutralizing and anti-leukemia cell antibodies

Kinetics of the production and class switching of virus-neutralizing and anti-leukemia cell antibodies in the genetically

modified animals were analyzed between PID 7 and 28. Serum titers of FV-neutralizing IgM and IgG in unimmunized $\beta_2m^{-/-}$ mice were not significantly different from those in the unimmunized wild-type mice (compare Figs 1d and 7a). As in the case of peptide-immunized wild-type mice, production of virus-neutralizing IgM was detected at PID 14 in the serum of peptide-immunized $\beta_2m^{-/-}$ mice genetically lacking $CD8^+$ T cells. However, in contrast to the peptide-immunized wild-type mice, neutralizing IgG titers in the peptide-immunized $\beta_2m^{-/-}$ animals were not significantly higher than their IgM titers even at PID 28, suggesting some roles of $CD8^+$ T cells in facilitating class switching of virus-neutralizing antibodies in FV-infected mice. The role of $CD8^+$ T cells in the induction of IgG class virus-neutralizing antibodies was further confirmed by transferring purified $CD8^+$ T cells from peptide-immunized to unimmunized mice. Unimmunized mice did not possess detectable levels of virus-neutralizing antibodies in their serum at PID 10, and the antibodies were IgM-dominant at PID 20 (Fig. 7b), confirming the results of the kinetic analyses shown in Fig. 1(d). As expected, the recipients of $CD4^+$ T cells from the peptide-immunized and FV-infected mice showed the production of neutralizing IgG at PID 20. Interestingly, the recipients of highly purified $CD8^+$ T cells from peptide-immunized and challenged mice also showed the production of virus-neutralizing IgG, the level of which was comparable to that in the recipients of the $CD4^+$ T cell transfer. As controls, transfer of purified $CD4^+$ or $CD8^+$ T cells from unimmunized control mice into FV-infected $CB6F_1$ mice did not induce significant class switching of virus-neutralizing antibodies even at PID 20 (data not shown). No neutralizing antibodies were detectable in FV-infected $\mu MT/\mu MT$ mice regardless of whether they were immunized with peptide i or not.

When anti-leukemia cell antibodies in the sera were tested, unimmunized $\beta_2m^{-/-}$ mice possessed anti-leukemia cell IgM at PID 7 and their titers decreased toward PID 14 as observed in the umimmunized wild-type mice (Fig. 7c). High titers of anti-leukemia cell IgM were also detectable in the peptide-immunized $\beta_2m^{-/-}$ mice at PID 7; however, in contrast to the peptide-immunized wild-type mice, $\beta_2m^{-/-}$ mice did not show a significant increase in the IgG titers between PID 14 and 21, confirming inefficient class switching of both neutralizing and anti-leukemia cell antibodies in the $\beta_2m^{-/-}$ animals.

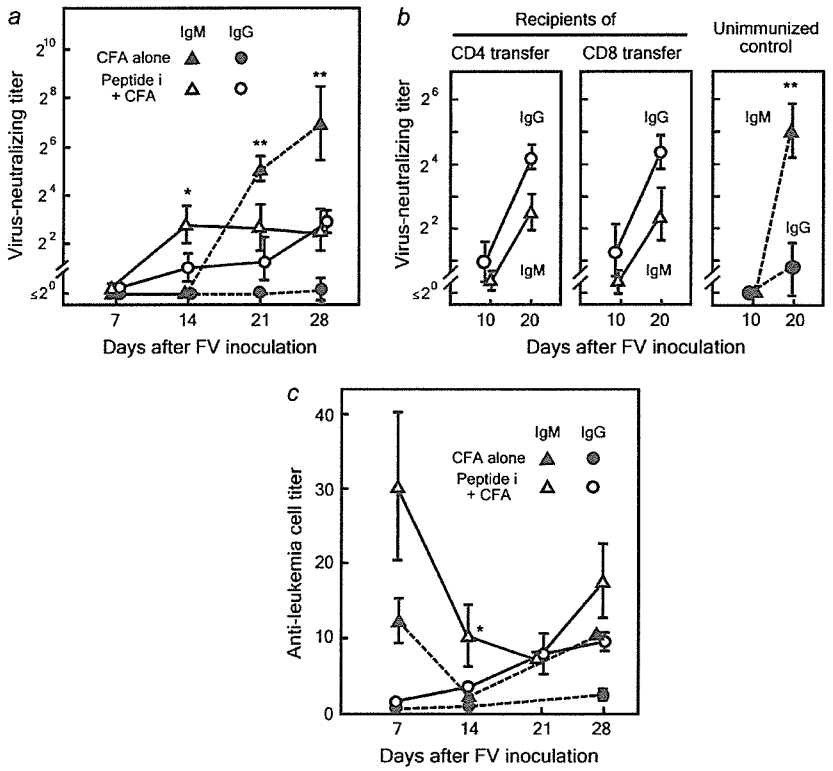

Fig. 7. Titers of virus-neutralizing and anti-leukemia cell IgM and IgG antibodies in sera from the $CD8^+$ T cell-deficient $CB6F_1$ mice and the effect of immune T cell transfer on the production of virus-neutralizing antibody. (a) $CB6F_1$-$\beta 2m^{-/-}$ mice were either immunized with peptide i or given CFA alone and challenged with FV as described. Sera were collected at the indicated time points and their neutralizing IgM and IgG titers were determined. Each data shows mean ± SEM calculated from five to nine serum samples, with statistically significant differences between the paired IgG and IgM titers indicated with $*P < 0.03$; $**P < 0.001$. (b) $CD4^+$ and $CD8^+$ T cells were purified from the spleen of peptide-immunized $CB6F_1$ mice at PID 7 and separately transferred into unimmunized control mice at PID 7. Sera were collected 3 (PID 10) and 13 days (PID 20) after cell transfer. Data shown here are mean ± SEM calculated from 4 to 5 serum samples at each time point. Differences between neutralizing IgM and IgG titers were compared by paired *t*-test: $**P < 0.005$. (c) Changes in the titers of IgM and IgG anti-leukemia cell antibodies detectable in the sera after FV infection. Each data point shows mean ± SEM calculated by using four to seven mice per group. *, the indicated titer in the immunized mice is significantly higher than that in unimmunized mice at $P < 0.05$.

Discussion

In the present study, we attempted to unravel the different roles of $CD8^+$ T and B cells in peptide-induced immune protection against FV-induced disease development by using genetically modified animals of the highly susceptible $CB6F_1$ background. $CB6F_1$ mice immunized only once with the peptide that contained a single $CD4^+$ T cell epitope were protected against fatal FV disease, and the development of early erythroid cell proliferation was prevented by the peptide immunization. The minimum sequence of the peptide required for *in vivo* protection against FV challenge, VYSQFEKSYRHKR, was the same as that required for $CD4^+$ T cell stimulation *in vitro*, indicating a close correlation between the peptide's ability to stimulate $CD4^+$ T cells and its efficacy in inducing protective immunity against FV-induced disease development. The requirement of $CD4^+$ T cells for the peptide-induced immune protection against FV infection was further confirmed by the lack of protection in the vaccinated animals after antibody-induced depletion of $CD4^+$ T cells.

In contrast to the bursting of TER-119^+ erythroid cells in the spleen of the unimmunized mice starting from PID 7, the reduction in the number of erythroid cells was observed between PID 5 and 7 in the peptide-immunized $CB6F_1$ mice, before virus-neutralizing antibodies became detectable in the serum (Fig. 1), and the numbers of spleen and bone marrow infectious centers were significantly smaller in the immunized than in the unimmunized control mice at PID 8 (Fig. 4). Although IgM antibodies reactive to the surfaces of FV-induced leukemia cells were detectable at as early as PID 7 (Figs 6 and 7), these antibodies are unlikely to play major roles in the observed suppression of the early growth of FV-infected cells in the vaccinated animals because anti-leukemia cell titers were not significantly different between the immunized and unimmunized groups at PID 7 (Fig. 6). This notion is consistent with the significantly reduced numbers, in comparison with those in unimmunized animals, of infectious centers in the spleen and bone marrow of the vaccinated mice at PID 8 even in the absence of B cells (Fig. 4c and f), and suggests the possibility that cellular immune responses, rather than antibodies, are involved in the regulation of SFFV-induced erythroid cell proliferation. In this regard, significantly larger populations of both $CD4^+$ and $CD8^+$ T cells were activated in the peptide-immunized, B cell-deficient mice in comparison with those in the unimmunized control mice at PID 7 (Fig. 5), and cytotoxic effector functions exerted by $CD4^+$ and $CD8^+$ T cells and those exerted more efficiently by NK cells have been demonstrated at as early as PID 7 in FV-infected $CB6F_1$ mice (14). These non-B effector cells might be involved in the control of SFFV-induced erythroid cell proliferation through direct killing of infected target cells. On the other hand, the numbers of virus-producing cells detected after PID 14 in the spleen and bone marrow were not different between the immunized and unimmunized groups of the B cell-deficient mice (Fig. 4c and f). This is again consistent with the detection of significantly higher titers of virus-neutralizing and anti-leukemia cell antibodies in the serum of vaccinated than in the unimmunized wild-type and $\beta_2m^{-/-}$ animals starting from PID 14 (Figs 1d, 6 and 7). These data suggest that the observed elimination of FV-producing cells in the peptide-immunized mice after PID 14 may depend mainly on the production of antibodies. Thus, early prevention of erythroid cell proliferation apparently depends mainly on cellular immune responses, but humoral responses seem to play crucial roles in the elimination of virus-producing cells in the later stage.

As to the relationship and relative importance between virus-neutralizing and anti-leukemia cell antibodies, tempos of the production and class switching of these antibodies after PID 14 were similar in the vaccinated wild-type animals (Figs 1d and 6c). However, in unimmunized animals, significant production of virus-neutralizing IgM was detectable after PID 21 when anti-leukemia cell titers were low and diminishing. Similarly, paradoxical production of virus-neutralizing antibodies in the presence of low anti-leukemia cell antibody titers has been observed in $H2^{a/b}$ (A.BY × A/WySn)F_1 mice at 20 days after FV infection (Miyazawa, M., Ishihara, C., and Takei, Y. A., unpublished results). It should be noted that although the anti-leukemia cell IgM titers at PID 21 and 28 were low, mean fluorescence intensities were four to five times higher than that obtained with the control serum, which reflects significant shifting of the peaks of fluorescence (Fig. 6b). Thus, it is conceivable that only a small proportion of serum antibodies detectable as anti-leukemia cell antibodies exhibit virus-neutralizing capability especially in the early stage of FV infection, and the proportion of neutralizing antibodies among anti-FV antibodies increases in the later stage. This interpretation also suggests that the production of antibodies reactive to virus-neutralizing epitopes, but not just any anti-FV antibody, depends on T cells. Further studies are required to elucidate the molecular and epitope specificities of neutralizing and anti-leukemia cell antibodies. It can be pointed out that the presence of virus-neutralizing IgM at PID 21 is not effective to reduce the number of FV-infected cells as clearly shown in the case of unimmunized, wild-type $CB6F_1$ mice (Figs 1d and 4). Further, class switching to IgG of virus-neutralizing and anti-leukemia cell antibodies may not be a requisite because the number of FV infectious centers were reduced in the absence of efficient class switching in the peptide-immunized $\beta_2m^{-/-}$ mice (Figs 4 and 7). Thus, the presence of virus-neutralizing and/or anti-leukemia cell IgM at around PID 14 might be crucial in preventing the spread of FV infection to a large enough number of target cells to support progressive infection.

Using partially FV-resistant (B10.A × A.BY)F_1 mice and an N-tropic F-MuLV as an attenuated vaccine, Dittmer, Brooks, and Hasenkrug (11) dissected different roles of the immune cell components in protection against FV-induced disease development. Their demonstration of the effectiveness in inducing the recovery from initial splenomegaly of cell transfer from vaccinated to naive animals after depletion of immune $CD8^+$ T cells is in agreement with our results showing the peptide-induced reduction of FV-producing cells in the spleen and bone marrow in the absence of $CD8^+$ T cells (Fig. 4). However, the development of splenomegaly was prevented only when the whole spleen cells or all three sub-populations ($CD4^+$, $CD8^+$ and $CD19^+$) of lymphocytes were transferred from the vaccinated to naive animals in the above live vaccine experiments, while in the present study the development of early splenomegaly was prevented in >70% of the $CB6F_1$-$\beta_2m^{-/-}$ mice after immunization with peptide i. This apparent discrepancy may be due to the difference in challenge dose of

FV (10 000 versus 150 SFFU), or might also be explained by the exaggerated and earlier production of IFN-γ from $CD4^+$ T cells and persistent activation of NK cells in $\beta_2m^{-/-}$ mice in comparison with those in their wild-type counterparts (30), as discussed below.

Dittmer, Brooks, and Hasenkrug (11) also showed that passive immunization with a virus-neutralizing mAb prior to FV challenge resulted in a significant reduction in the number of virus-producing cells at PID 10 and recovery from the initial development of splenomegaly, although 2.9×10^6 infectious centers on average were still detectable in the spleen. This result is partly consistent with our demonstration that B cells are required for the elimination of virus-producing cells from the spleen and bone marrow, especially after PID 14. However, the reported lack of protection after the transfer of B cells from vaccinated to naive mice contrasts with our demonstration of the crucial role of B cells. As they discussed later (12), transferred B cells might not have produced a sufficient level of FV-reactive antibodies until they were re-stimulated upon FV challenge of the recipients. In this regard, the production of virus-neutralizing antibodies in FV-infected mice is dependent on $CD4^+$ T cells (31). Further, we have shown in our previous (13, 17) and the present experiments that peptide-induced priming of $CD4^+$ T cells facilitates both production and class switching of virus-neutralizing and anti-leukemia cell antibodies upon FV infection. Thus, the lack of protection by the transfer of purified B cells alone from vaccinated to naive mice does not necessarily contradict our demonstration of the requirement of B cells for peptide-induced immune protection, especially because the titers of anti-FV antibody had not been determined in the above-reported B cell-transferred animals. The same authors (32) have shown that the presence of virus-neutralizing antibodies at the time of infection is crucial for a vaccine-induced protection of naturally resistant C57BL/6 mice against FV infection.

The observed lack of protection in the *μMT/μMT* mice might also be caused by the lack of or inefficient priming and/or re-activation of $CD4^+$ T cells due to the absence of B cells as APC. Although successful priming of $CD4^+$ and $CD8^+$ T cells with protein as well as cellular antigens and effective induction of CTL responses have been reported in the *μMT/μMT* mice (33, 34), the effect of the homozygous *μMT* mutation on T cell priming can be variable (35). However, since we were using as immunogen the 18-mer peptide which has been shown to directly bind onto the MHC class II $E^{b/d}$ molecule (23), the uptake and processing of the given antigen by B cells were not required. In such cases where already processed peptide is used as an immunogen, B cells are regarded as unnecessary for the priming of T cells (35). In fact, $CD4^+$ cells purified from the peptide-immunized *μMT/μMT* mice showed potent proliferative responses upon re-stimulation with peptide i, and larger numbers of $CD4^+$ T cells were activated upon FV infection in the peptide-immunized than in the unimmunized B cell-deficient mice (Fig. 6). Thus, the *μMT/μMT* mice were not protected most conceivably because they lacked antibody production.

In previous reports, $CD8^+$ T cells have always been associated with spontaneous and vaccine-induced immune resistance against FV infection: antibody-induced depletion of $CD8^+$ T cells abrogated spontaneous recovery from FV-induced splenomegaly in highly resistant (C57BL/10 × A.BY)F_1 mice (9), and recovery from splenomegaly was induced by transferring purified $CD8^+$ T cells from (B10.A × A.BY)F_1 mice previously vaccinated with the live N-tropic F-MuLV into naive animals (11). These results apparently contradict the present results showing successful protection of the $\beta_2m^{-/-}$ mice with the peptide vaccine. However, in the case of spontaneous recovery observed in the strain (C57BL/10 × A.BY)F_1, $CD4^+$ T cells were not primed prior to FV infection, and mice were infected with 1500 SFFU of B-tropic FV, a ten times higher dose than we used in the present study. Thus, infection-induced priming of $CD4^+$ T cells might have been inefficient or too slow in inducing effector mechanisms other than $CD8^+$ T cells, which might be required to confine the rapid spread of inoculated FV. In this regard, $CD8^+$ T cells are required not solely as cytotoxic effector cells but are also involved in the generation of T helper type 1 cells in FV-infected mice (36, 37). Thus, antibody-induced depletion of $CD8^+$ T cells quite likely had also affected $CD4^+$ T cell functions in the experiment reported by Robertson *et al.* (9), and adoptive transfer of immune $CD8^+$ cells must have induced the activation of $CD4^+$ effector cells in the recipients in the experiment performed by Dittmer, Brooks, and Hasenkrug (11). The role of $CD8^+$ T cells in inducing class switching of virus-neutralizing and anti-leukemia cell antibodies (Fig. 7) might reflect the reported influence of $CD8^+$ T cells on helper functions of $CD4^+$ T cells. In fact, the effect of $CD8^+$ T cell transfer from vaccinated to naive animals along with passive immunization with the neutralizing mAb was dependent on the presence of endogenous $CD4^+$ T cells in the recipients (11). Thus, the reported requirement of $CD8^+$ T cells for spontaneous resistance and vaccine-induced protection against FV infection might be compensated, at least partly, by the peptide-induced priming of $CD4^+$ T cells. It has been shown that the effect of $CD8^+$ T cells on the induction of protective $CD4^+$ T cell responses is blocked by neutralizing anti-IFN-γ antibody (37). Likewise, intravenous administration of neutralizing anti-IFN-γ antibody on the day of FV challenge and at PID 7 abrogated the effect of peptide vaccine in six of seven injected $CB6F_1$ mice in our preliminary experiment (data not shown). Thus, all these data indicate that the apparent requirement of $CD8^+$ T cells for spontaneous resistance and observed effectiveness of $CD8^+$ T cell transfer in vaccine-induced protection against FV infection might have been actually mediated through the effect of $CD8^+$ T cells on $CD4^+$ T cell functions, and peptide-induced priming of $CD4^+$ T cells may have bypassed the $CD8^+$ T cell functions and induced protection in the $\beta_2m^{-/-}$ mice.

Actual effector mechanisms involved in the observed elimination of FV-infected erythroid cells in the absence of $CD8^+$ T cells (Fig. 4) may include the previously described $CD4^+$ CTLs and NK cells (14), as well as FV-reactive, cytotoxic antibody (38). However, anti-leukemia cell IgMs were detectable in both immunized and unimmunized animals at PID 7 (Figs 6 and 7), excluding the role of these antibodies in controlling the growth of FV-infected cells in the early stage. It should be emphasized that killing activities of $CD4^+$ cytotoxic and NK cells were detectable in the peptide-immunized $CB6F_1$ mice at as early as PID 7, and NK cells were much more efficient than $CD8^+$ CTLs in killing FV-induced leukemia

cells (14). Further, it has been shown that activation of NK cells after lymphocytic choriomeningitis virus infection is prolonged in $\beta_2m^{-/-}$ mice than in the wild-type mice (30). Thus, similarly enhanced activation of NK cells, along with the exaggerated production of IFN-γ from vaccine-primed $CD4^+$ T cells, might have compensated otherwise indispensable $CD8^+$ T cell functions in the peptide-immunized $CB6F_1$-$\beta_2m^{-/-}$ mice. In this regard, the incidences of splenomegaly in unimmunized $CB6F_1$-$\beta_2m^{-/-}$ mice at PID 14 and 21 were significantly lower than those in the unimmunized wild-type mice ($P < 0.001$), although their survival curves were not significantly different ($P > 0.05$) (Fig. 4). These observations are consistent with the previously observed activation and killing efficacy of NK cells in FV-infected $CB6F_1$ mice without prior immunization (14), and with the reported enhancement of virus-induced NK cell activity in $\beta_2m^{-/-}$ mice (30).

Taken together, the present study has demonstrated that for efficient immune protection against FV infection with the single-epitope peptide, B cells are more important than $CD8^+$ T cells, but B cell-independent responses, probably exerted by previously demonstrated $CD4^+$ CTLs and NK cells, do play some roles in the earlier stage of FV-induced disease development in suppressing the expansion of FV-infected erythroid cells. Careful comparison of these results and other reports may suggest the possibility that priming of $CD4^+$ T cells with the peptide vaccine might allow the bypassing of the $CD8^+$ T cell functions that have been reported to induce $CD4^+$ T_h1 effector cells. These observations may contribute to the development of efficient vaccine strategies against other virus infections.

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Abbreviations

7-AAD	7-aminoactinomycin D
APC	antigen-presenting cell
B6	C57BL/6
$CB6F_1$	(BALB/c × C56BL/6)F_1
ED_{50}	dose required to induce 50% of the maximum response
FBS	fetal bovine serum
F-MuLV	Friend murine leukemia virus
FV	Friend retrovirus complex
β_2m	β_2-microglobulin
μMT	Ig μ-chain membrane exon-targeted
PBBS	phosphate-buffered balanced salt solution
PID	post-infection day
SFFU	spleen focus-forming unit
SFFV	spleen focus-forming virus

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Mycobacterial Codon Optimization Enhances Antigen Expression and Virus-Specific Immune Responses in Recombinant *Mycobacterium bovis* Bacille Calmette-Guérin Expressing Human Immunodeficiency Virus Type 1 Gag†

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Although its potential for vaccine development is already known, the introduction of recombinant human immunodeficiency virus (HIV) genes to *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has thus far elicited only limited responses. In order to improve the expression levels, we optimized the codon usage of the HIV type 1 (HIV-1) p24 antigen gene of *gag* (p24 *gag*) and established a codon-optimized recombinant BCG (rBCG)-p24 Gag which expressed a 40-fold-higher level of p24 Gag than did that of nonoptimized rBCG-p24 Gag. Inoculation of mice with the codon-optimized rBCG-p24 Gag elicited effective immunity, as evidenced by virus-specific lymphocyte proliferation, gamma interferon ELISPOT cell induction, and antibody production. In contrast, inoculation of animals with the nonoptimized rBCG-p24 Gag induced only low levels of immune responses. Furthermore, a dose as small as 0.01 mg of the codon-optimized rBCG per animal proved capable of eliciting immune responses, suggesting that even low doses of a codon-optimized rBCG-based vaccine could effectively elicit HIV-1-specific immune responses.

The *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has been widely used as a live bacterial vaccine against *Mycobacterium tuberculosis* infection. Its recombinant form, rBCG, which has been used successfully to express foreign antigens and to induce immune responses, has been proposed as a vaccine candidate against a number of diseases (26, 32, 33), especially human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) (11, 13, 30). Moreover, mucosal immunization of rBCG has been found to elicit a long-term virus-specific immunity in animals (10, 14, 15), even in Th1- and Th2-deficient conditions (10). In short, an rBCG-based vaccine offers several clear advantages over other types of recombinant vector-based approaches in that it (i) induces cellular immune responses that are maintained for at least 1 to 2 years; (ii) is easy to administer, usually requiring only one or two immunizations; (iii) and is affordable because it can be easily and cheaply produced. These findings suggest that rBCG could be a potent vaccine against HIV-1 infection, one that is likewise capable of inducing safe, virus-specific immunity.

However, the results described above were obtained with high doses of rBCG, doses 10- to 100-fold larger than that needed for a practical BCG vaccination dose against tuberculosis in humans (7, 11). Therefore, the low immunogenicity seen in rBCG-inoculated animals is likely due to their inoculation with only a "normal," not a high, vaccination dose (15). Moreover, high doses of BCG administration in vivo may also act as the driving force for the replication of the immunodeficiency virus and its dissemination by hyperactivating T cells (6, 41).

We sought here to produce an rBCG vaccine that would be efficacious even in the low doses required for human vaccination. Because low-dose immunization of rBCG has been suggested to act as a prophylactic vaccination against HIV-1 (15, 28), we adopted the preferred codon of BCG to enhance the expression of the foreign HIV gene. In recombinant protein production, the potency of codon-optimized gene expression systems was demonstrated in *Escherichia coli* (39) and in mammalian cells (42). These results clearly show that codon-optimized recombinant genes induce vigorous expression by foreign genes in the host. Since 1998, many groups have reported that a sequence-modified DNA vaccine confers high immunogenicity against various foreign antigens, e.g., listeriolysin O of *Listeria monocytogenes* (37), HIV-1 Gag (43), Env (3), tetanus toxin (34), L1 protein of human papillomavirus (18), and merozoite surface protein 1 of *Plasmodium falciparum* (25). Most of these studies focused on demonstrating how mammalian codon usage bias efficiently enhanced the expression and immunogenicity of foreign antigens in DNA vaccination. However, although the effect of codon optimization in mammalian cells has been well documented, its effect in recombinant BCG vector-based vaccines has never been fully elucidated.

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