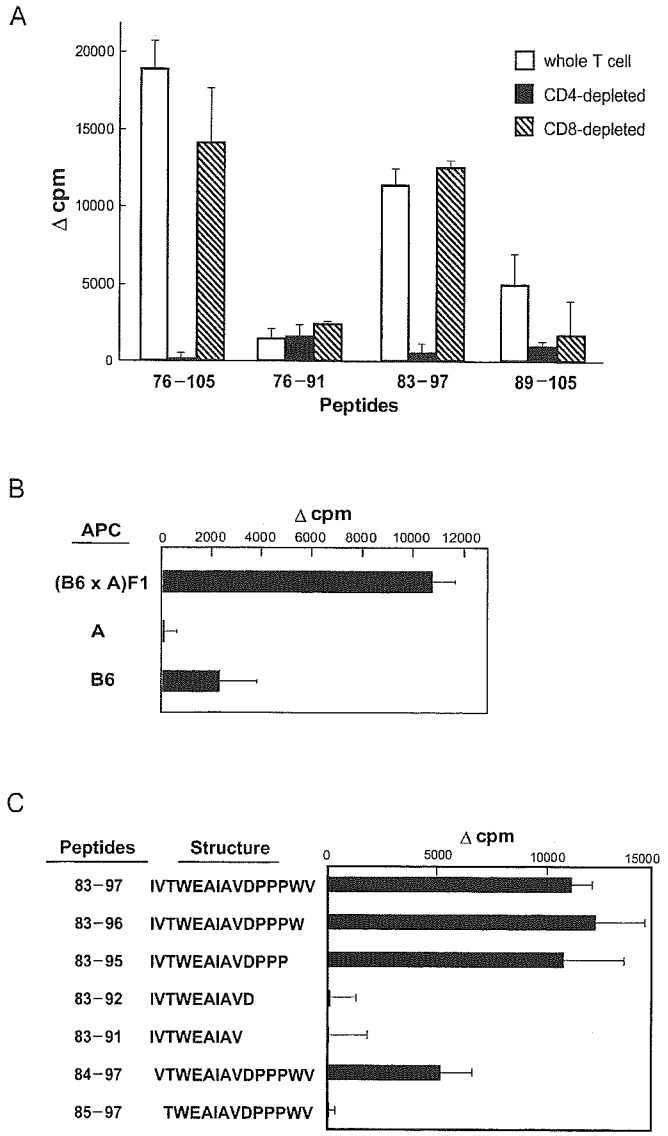

FIG. 7. Fine specificities and MHC restriction of primed $CD4^+$ T cells prepared from vMA-immunized mice. (A) Spleen T cells prepared from vMA-immunized mice were depleted of either $CD4^+$ or $CD8^+$ T cells by the magnetic cell sorting system. Unseparated T cells or purified T-cell subsets were incubated with each indicated peptide and with syngeneic spleen cells as APC, and antigen-specific proliferative responses were measured. Amino acid sequences of the peptides used here are shown in Fig. 6A. In this experiment, T cells from six immunized mice were pooled before the depletion of each subset. (B) T cells pooled from two to three vMA-immunized (B6 × A)F_1 mice were stimulated with peptide 83-97 and irradiated spleen cells prepared from F_1, B6, or A mice, and proliferative responses were analyzed as above. (C) T cells pooled from two vMA-immunized mice were stimulated with each indicated peptide and the syngeneic APC, and proliferative responses of T cells were measured. All data shown here are representative of two to four independent experiments with essentially identical results.

ments might have an influence on their immunogenicity. To prove this assumption, we first examined the intracellular localization of the mutant MA proteins by staining them with an anti-His Ab. The native MA protein expressed by vMA infection was present throughout the cells in a diffuse distribution and also localized at the plasma membrane (Fig. 9), as expected. In contrast, the mutant form of full-length MA, which lacked the site of myristylation, expressed by vMAmu infection, was localized more prominently in the nucleus than in the cytoplasm. In addition, the fluorescent intensity in vMAmu-infected cells was relatively low at the edges of the cells in comparison with that in vMA-infected cells. Interestingly, the MA protein from which the N-terminal 24 residues had been deleted, expressed by vMAC2 infection, was localized largely in the nucleus, which implies that, in addition to the glycine residue, the N-terminal short region of F-MuLV MA (as shown for HIV-1 MA [61, 63]) is necessary for its localization at the plasma membrane. The mutant MA proteins expressed by vMAmu and vMAC2 were observed by fluorescence at levels comparable to that of the native MA protein expressed by vMA infection, and a quite similar result showing the almost equal level of their expression was also obtained by Western blot analysis (data not shown).

To confirm that these changes in intracellular localization of the MA protein do affect its immunogenicity, we next compared the abilities of these MA mutants to prime the antigen-specific $CD4^+$ T cells (Fig. 10). T cells from vMAmu-immunized mice showed moderate proliferative responses when stimulated with the epitope-harboring peptide 76-105, but the response level was significantly lower than that of T cells taken from vMA-immunized mice. T cells prepared from vMAC2-immunized mice proliferated only marginally in response to stimulation with the Th peptide, showing no significant difference from the response of T cells from vHS1-immunized control mice. These results indicated that targeting of the MA protein to the plasma membrane might be critical for induction of efficient priming of MA-specific $CD4^+$ T cells.

DISCUSSION

We have demonstrated here, for the first time, efficient protection against a pathogenic retrovirus infection through the priming of Gag-specific $CD4^+$ T cells with an MA peptide. Immunization with the rVV expressing native MA alone was sufficient to protect mice from FV-induced disease development, and a protective epitope was present within residues 83 to 97 of MA. T cells primed in vivo with the native MA protein proliferated in vitro when stimulated with the minimal peptide 83-95 (IVTWEAIAVDPPP), and reactivity was completely abolished when $CD4^+$ T cells were depleted, indicating that the protective epitope is recognized by $CD4^+$ T cells. A hybrid class II molecule expressed on $H\text{-}2^{b/a}$ cells is required for the presentation of this epitope. Of note, this peptide is highly immunogenic, since a single vaccination with the epitope-harboring peptide was sufficient to protect mice against FV-induced disease. As in the previously demonstrated case of protection against FV infection induced by priming $CD4^+$ T cells with an Env-derived single-epitope peptide (16, 31), multiple effector mechanisms might have been activated upon FV infection in mice immunized with the MA-derived peptide. In agreement with our results, it has been shown that HIV-specific $CD4^+$ T cells in long-term nonprogressors are mainly directed against p17, the N-terminal HIV Gag protein analogous to F-MuLV MA (42). Thus, the high immunogenicity of

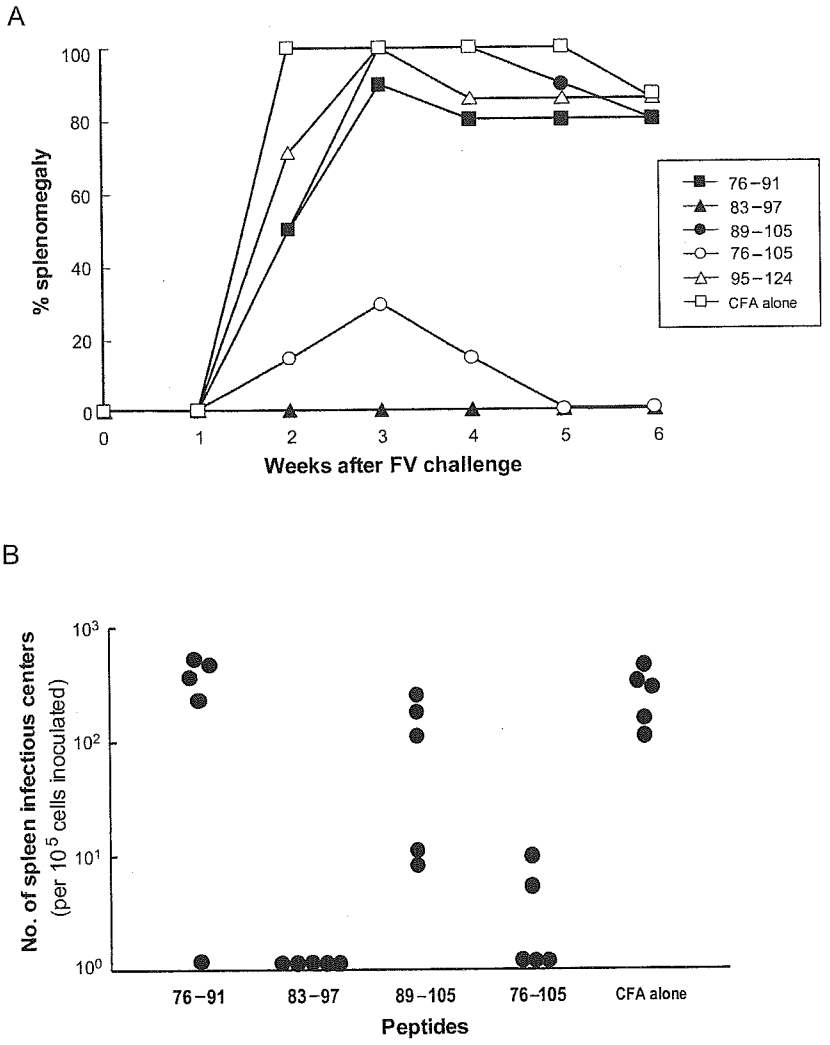

FIG. 8. Protection against FV infection induced by immunization with the Th epitope-harboring peptide alone. Mice (eight per group) were immunized once with 50 μg of one of the peptides shown or with CFA alone, followed by challenge with 1,500 SFFU of FV. (A) Splenomegaly over a 6-week period after challenge was observed as an indicator of FV-induced disease. (B) Frequencies of spleen infectious centers were determined at 4 weeks after FV challenge (five mice per group). The differences between CFA-injected and peptide 83-97- or 76-105-immunized mice were significant ($P < 0.01$ by Student's t test).

MA might be a common feature among diverse retroviruses. In support of this, broadly reactive Th epitopes were also identified in the MA region by using macaques chronically infected with SIV (48). Taken together, these data suggest that MA might be a potential target for the development of effective antiretrovirus vaccines.

In all the mice immunized with native MA expressed from the rVV or with the Th epitope-harboring peptide alone, virus-producing cells became undetectable by 4 weeks after FV challenge. The immune effector functions exerted by the virus-specific $CD4^+$ T cells might be very diverse (36), including helper functions provided for B- and $CD8^+$ T-cell responses, production of antiviral cytokines, and direct cytolysis. Our results here have shown that the class switching of virus-neutralizing Abs from IgM to IgG after FV challenge is remarkably accelerated in vMA-immunized mice. MA-specific Abs were detected by Western blotting before FV challenge in the sera of mice immunized with the rVV expressing native MA, but they were incapable of neutralizing F-MuLV (Fig. 4). Thus, the presence of a dominant neutralizing epitope(s) within MA is unlikely. Since the neutralizing Abs were produced in vMA-immunized mice only after FV challenge, it is more likely that the MA-primed $CD4^+$ T cells may have provided a helper function to Env-reactive B-cell responses. Alternatively, the production of Abs directed against the cell surface $gPr80^{gag}$ might have been facilitated after FV challenge by help from the MA-primed $CD4^+$ T cells. Prevention of cell-to-cell transmission of retroviruses by anti-$gPr80^{gag}$ Abs has been demonstrated (41).

The virus-specific $CD4^+$ T cells might also have been required for the maintenance of functional virus-specific CTL responses, as has been shown in other reports (1, 28, 40, 53). However, the mechanisms by which Gag-specific $CD4^+$ T cells may fulfill this role is poorly understood. In HIV-1-infected

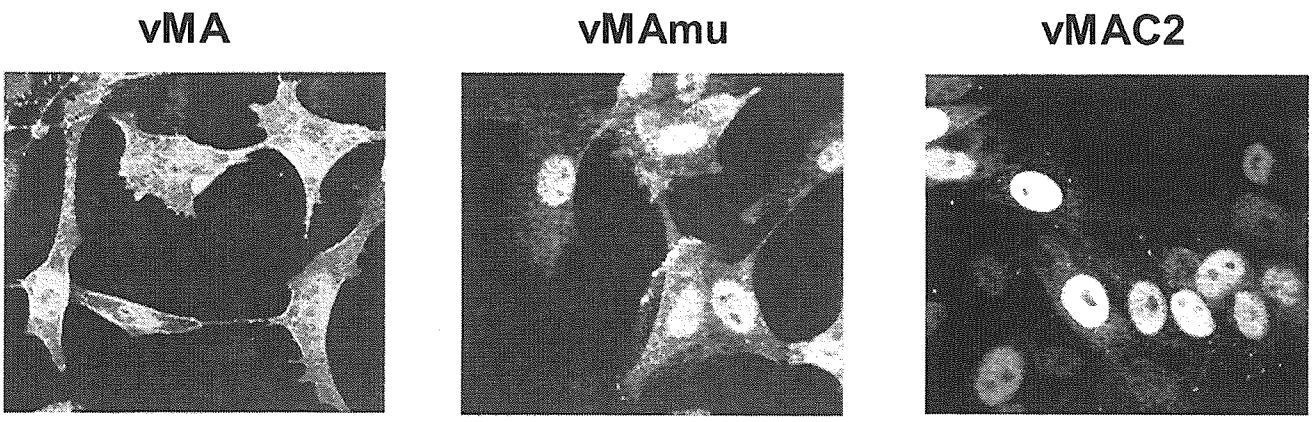

FIG. 9. Intracellular localization of mutant MA proteins. CV-1 cells were infected with vMA, vMAmu, or vMAC2; 24 h later, they were fixed and permeabilized. His tag-conjugated MA proteins were visualized with an anti-His tag Ab. The experiments were performed three times with essentially identical results.

individuals, enhanced $CD4^+$ T-cell responses have been associated with a higher level of virus-specific CTL responses and lower viral loads (22, 46). In this regard, we and others (7, 25, 54) previously found the presence of overlapping CTL epitopes in the leader sequence upstream of the ATG start codon for $Pr65^{gag}$, but not in the MA region, although it has not been determined whether these epitopes are protective or not. However, our previous work clearly demonstrated that the rVV expressing $Pr65^{gag}$ without the leader sequence was as effective as the rVV that expressed $gPr80^{gag}$, indicating that the CTL epitope in the leader sequence is not a requisite for protection against FV infection. Thus, the $CD4^+$ T cells primed with MA might have induced rapid responses of Gag-specific CTL, as well as Env-specific B cells, but the possible importance of Gag-specific CTL responses, if any, has yet to be identified.

Gag-specific $CD4^+$ T cells may also have direct roles in the control of FV infection through their possible cytotoxic activities and production of antiviral cytokines. Direct cytotoxic activities of $CD4^+$ T cells have been described in a number of viral infections (2, 19, 36, 59). A previous study with the FV-infected mouse model, in which direct cytotoxic activities of Env-specific $CD4^+$ T cells were detected, also supported those observations (16). Among $CD4^+$ T-cell clones established from HIV-1-infected individuals with vigorous Gag-specific responses, some displayed virus-specific cytotoxic activities (35, 55). Furthermore, $CD4^+$ T cells have been shown to directly control virus replication by production of gamma interferon in FV infection (10, 18). Thus, $CD4^+$ T cells primed with the MA protein might have contributed to the observed protection against FV infection through multiple effector functions.

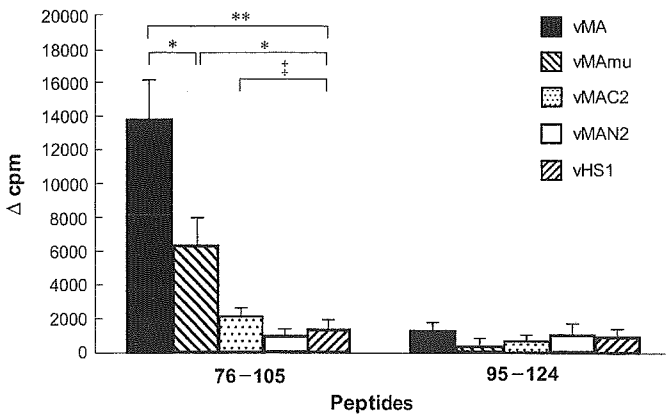

FIG. 10. Different abilities of mutant and truncated MA proteins at priming $CD4^+$ T cells. Proliferative responses of T cells obtained from mice immunized with each rVV were tested by stimulation with peptide 76-105. Each result is expressed as the mean Δcpm for data obtained from four to five mice. Error bars, standard errors of the means. The experiments were performed twice with essentially identical results. Statistically significant differences were observed by Student's t test (*, $P < 0.05$; **, $P < 0.001$). ‡, no significant difference ($P > 0.05$).

The present study has also provided useful information on the structural requirements for effective priming of virus-specific $CD4^+$ T-cell responses by the MA protein. T cells primed in vivo with native MA (vMA) proliferated when stimulated with the Th epitope-harboring peptide 76-105. In contrast, full-length MA lacking the N-terminal myristylation site (vMAmu) and the MA from which the N-terminal 24 residues had been deleted (vMAC2), despite carrying the whole Th epitope, induced only moderate or marginal T-cell responses, respectively, when used to prime T cells in vivo. Of note, their different abilities to elicit the $CD4^+$ T-cell response were well correlated with their efficacies in inducing protection against FV infection in vivo. There was also a correlation between the observed degree of localization of the MA protein at the plasma membrane and its ability to elicit T-cell proliferation and immune protection: By the destruction of the myristylation site, the degree of localization of the MA protein at the plasma membrane was diminished, and the MA lacking the N-terminal 24 residues localized predominantly in the nucleus. These results indicate that the N-terminal region of F-MuLV MA, not just the myristylation site, is responsible for its subcellular localization. A highly basic domain between MA residues 17 and 31 in HIV-1, besides the myristylation signal, has been implicated in membrane binding of the Gag polyprotein (61, 63), and there is a corresponding basic region present between MA residues 17 and 34 in F-MuLV.

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Both T and non-T cells with proliferating potentials are effective in inducing suppression of allograft responses by alloantigen-specific intravenous presensitization combined with suboptimal doses of 15-deoxyspergualin

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Received 10 September 2003; accepted 21 January 2004

Abstract

In an MHC class I-disparate combination of mouse strains, a single intravenous injection of donor spleen cells combined with 10 suboptimal doses of 15-deoxyspergualin (DSG) administration was effective in inducing donor-specific suppression of cytotoxic T-lymphocyte (CTL) responses and prolonged survival of the relevant skin allograft. Proliferative potentials of the donor spleen cells were requirement for the induction of suppressed allospecific responses, but both highly purified T cells and non-T cells were equally effective to induce the suppression of CTL responses by intravenous injection. These results have shown that, although working on different mechanisms, DSG is as effective as FK506 or rapamycin in inducing allograft tolerance when used at suboptimal doses along with the donor-specific intravenous presensitization, and an immune mechanism other than well-characterized veto T cells is working in this model in suppressing alloreactive CTL precursors.

Keywords: Donor-specific intravenous presensitization; 15-deoxysupergualin; Skin graft; Tolerance; Veto cell

1. Introduction

The favorable effects of pretransplantation transfusion of donor blood, often referred to as donor-specific transfusion (DST), on allograft survival has been well recognized (for recent reviews see [1,2]). Mechanisms of the effects of DST and influences of different transfusion protocols on the prolongation of graft survival and on recipient immune functions have been studied using various rodent models [3–11]. However, contrasting outcomes can still be observed depending on the degree of disparity at different loci in major histocompatibility complex (MHC) [1,2,11]. In mice, highly reproducible suppression of allograft responses can be induced by a single or repeated intravenous injection(s) of donor spleen cells (donor-specific intravenous presensitization: DSP) in combination with or without suboptimal doses of an immunosuppressive reagent [12–18]. Compelling evidence has indicated that DSP alone suppresses donor antigen-specific proliferative and cytokine-producing responses of recipient T-cells, but cytotoxic T-lymphocyte (CTL) induction is suppressed only by a combination of DSP with antibody-mediated $CD8^+$ T-cell depletion or administration of suboptimal doses of FK506 or rapamycin [12–17]. Cells required

Abbreviations: B6, C57BL/6; Con A, concanavalin A; CTL, cytotoxic T-lymphocyte; DSG, 15-deonxysupergualin; DSP, donor-specific intravenous presensitization; DST, donor-specific transfusion; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MMC, mytomycin C; PBBS, phosphate-buffered balanced salt solution; R-PE, R-phycoerythrin.

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doi:10.1016/j.trim.2004.01.004

for the induction of the suppressed allograft responses by DSP are shown to be radio-sensitive [12] and belong to T-cell populations [12,15], indicating possible roles of veto cells. In the present study, we investigated the efficacy of DSP combined with suboptimal doses of 15-deoxysupergualin (DSG), an immunosuppressive reagent that has mechanisms of action quite different from those of FK506 or rapamycin, in suppressing allograft responses. In addition, we further analyzed cell populations effective in inducing the suppression of allospecific cellular immune responses by highly purifying T and non-T cells using magnetically labeled antibodies.

2. Objectives

The objectives of the present study were (1) to examine if DSG that has mechanisms of action quite different from those of previously tested FK506 and rapamycin is effective in inducing the suppression of allograft responses when used at suboptimal doses in combination with DSP; and (2) to identify a donor cell type(s) and its antigenic and proliferative characteristics required for the effective suppression of allospecific cellular immune responses when used for DSP.

3. Materials and methods

3.1. Mice

C57BL/6 (B6, $H2^b$), B10.QBR ($H2^{bq4}$), and BALB/c ($H2^d$) mice were purchased from Japan SLC, Hamamatsu, Japan, and kept and maintained in animal facilities at Kinki University School of Medicine under specific pathogen-free conditions. B6 and B10.QBR mice differ at the class I D locus, B6 possessing the D^b and B10.QBR the D^q allele, but share the same alleles at other MHC loci. The following experiments were performed under relevant guidelines of the Japanese government and the University, and were approved by the Animal Experiment Committee of the School of Medicine.

3.2. Recipient manipulation and skin grafting

(−)-15-deoxyspegualin was kindly provided by Nippon Kayaku Co., Ltd., Tokyo, Japan as Gusperimus trihydrochloride. A suboptimal dose of 1 mg/kg was given intraperitoneally to recipient B6 mice 10 times at 2-day intervals starting from 8 days before transplantation (Fig. la). For donor-specific presensitization, spleen cells were prepared aseptically from B10.QBR mice and red cells were removed as described in Refs. [14–17,19]. Single-cell suspension was prepared by passing it through sterile nylon mesh, and 1×10^7 cells were injected intravenously into each B6 recipient mouse at 7 days before skin grafting. Skin grafts were prepared by removing 7×7-mm sheets from the tail of B10.QBR mice under ether anesthesia, and transplanted onto the back of each recipient B6 mouse according to the techniques described in Ref. [20]. Bandages were removed on day 7 after transplantation, and graft tissue was observed daily. Skin grafts were determined to be rejected when >80% of the tissue became necrotic as described previously [14–17]. Some recipient mice were killed at 14 days after grafting by cervical dislocation and the spleen was removed for CTL and mixed lymphocyte reaction (MLR) assays as described previously [14–17,19].

3.3. Tissue culture media and reagents

For preparation of spleen cells phosphate-buffered balanced salt solution (PBBS) supplemented with 2% fetal bovine serum (FBS) was used as described in Ref. [19]. To remove erythrocytes, spleen cells were treated with the Tris-buffered ammonium chloride solution, and rinsed three times with PBBS [19]. For tissue culture RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol and 10% heat-inactivated FBS selected for low background stimulation was used. Concanavalin A(Con A), lipopolysaccharide from *Escherichia coli* 0111:B4(LPS), and mytomycin C (MMC) were purchased from Sigma Chemical Co., St. Louis, Missouri.

3.4. CTL and MLR assays

To generate CTL effector cells, 5×10^6 B6 spleen cells were mixed with 1.5×10^6 X-ray (4000 rad)-irradiated B10.QBR or BALB/c stimulator cells, and cultured for 5 days in each well of 24-well tissue culture plates with 2 ml culture medium as described above. Target cells were prepared by stimulating B10.QBR or BALB/c spleen cells with 5 μg/ml Con A for 48 h, and labeling them with 3.7 MBq ^{51}Cr (NEN Life Science Products, Inc., Boston, Massachusetts) per 10^6 cells as described previously [19]. The resultant effector and target cells were mixed at indicated ratios, and the release of ^{51}Cr into the supernate from the 1×10^4 target cells per well of 96-well culture plates in 4 h was measured by using a gamma counter. Levels of antigen-specific target cell lysis were calculated by using a standard formula [19], with maximum release determined by adding 1% Triton X-100 into wells of labeled target cells. For MLR assays, 5×10^5 responder B6 spleen cells were mixed with 4×10^5 4000-rad-irradiated stimulator spleen cells in each well of 96-well tissue culture plates. After culturing for the indicated period, [^{3}H]thymidine (Amersham Life Science, Buckinghamshire, England) was added at 18.5 kBq/well, and the culture was continued for an additional 8 h. Incorporation of [^{3}H]thymidine was measured by using a microplate scintillation counter (TopCount, Packard

Instruments, Meriden, Connecticut) as described previously [19]. The magnitude of antigen-specific proliferation was shown in Δcpm calculated by using a standard formula [19].

3.5. Modification of donor spleen cells and analyses of their antigenicity

B10.QBR spleen cells were prepared as described above, and either irradiated at 1500 or 4500 rad in an X-irradiator, or incubated with 500 μg/ml MMC for 60 min at 37 °C. The treated cells were washed three times with PBBS containing 2% FBS and used as donor cells for DSP. To analyze possible changes in their antigenicity, the above-treated B10.QBR spleen cells, along with untreated ones, were used as stimulator cells and mixed with naive B6 spleen cells in MLR assays as described in Section 3.4. Mixed cultures were incubated for 3, 5 and 7 days, and [^{3}H]thymidine incorporation was measured as described above. Proliferating potentials of the above-treated spleen cells were tested by stimulating them with 5 μg/ml Con A or 10 μg/ml LPS, and [^{3}H]thymidine incorporation was measured at 1, 2, 3, 5 and 7 days after the beginning of culture as described above. Cell surface expression of class I D^q molecules on treated and untreated B10.QBR spleen cells was also analyzed by flow cytometry using an anti-D^q monoclonal antibody (clone KH117, PharMingen, San Diego, California) as described previously [19].

3.6. Purification of T and non-T cells from the spleen

Donor spleen cells were separated into CD90$^+$ T and CD90$^-$, CD45R$^+$ non-T cell populations by using a magnetic cell sorting system. Magnetic microbeads-conjugated anti-mouse CD90 (Thy 1.2) and anti-mouse CD45R (B220) antibodies and CS depletion and VS+ separation columns were purchased from Miltenyi Biotec, Berigische Gladbach, Germany and used according to the manufacturer's instructions. To purify CD90$^+$ T cells, spleen cells were first depleted of B220$^+$ cells and CD90$^+$ cells were positively selected from the B220-depleted population. To purify B220$^+$ non-T cells, spleen cells were first depleted of CD90$^+$ T cells, and B220$^+$ cells were selected from the CD90$^-$ population. Purities of each cell population were confirmed by flow cytometric analyses by using a FACSCaliblur (Becton-Dickinson Immunocytometry Systems, San Jose, California). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD3 and R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD19 antibodies and their isotype-matched controls were purchased from PharMingen.

3.7. Statistical analyses

Averages of graft survival periods and magnitudes of proliferative responses between groups of mice were compared by Student's t test. Average percentages of specific lysis at five different effector-to-target ratios were compared between groups as curves by using Hotelling's T^2 test.

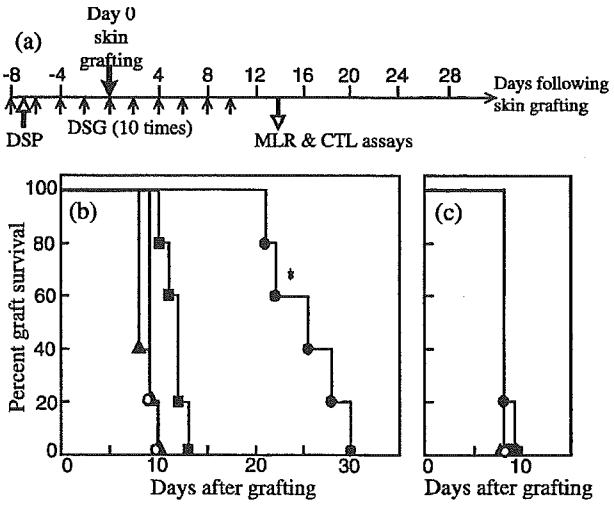

Fig. 1. Experimental protocols and time-courses of graft rejection. (a) Mice were injected with DSG and donor spleen cells as indicated, and received a skin graft. (b) Time-courses of the rejection of B10.QBR skin graft in untreated B6 mice (○), B6 mice treated with DSP alone (▲), those treated with DSG administration alone (■), or those treated with the combination of DSP and DSG administration (●). *, $P<0.001$. (c) Time-courses of the rejection of BALB/c skin graft in untreated and treated B6 mice. Symbols used are the same as those in b.

4. Results

4.1. Prolongation of skin graft survival by DSP combined with suboptimal doses of DGS in the MHC class I-disparate combination

When B6 mice were treated either by a single DSP or 10 suboptimal doses of DSG injection alone, no significant prolongation of graft survival was observed (Fig. 1b). However, in B6 mice treated with the combination of an intravenous injection of B10.QBR spleen cells and 10 suboptimal dose of DSG injection, skin grafts from the MHC class I-incompatible B10.QBR mice survived significantly longer than those transplanted onto untreated B6 mice. Skin grafts transplanted from the third party BALB/c mice were rejected within 9 days regardless of the treatment of recipient B6 mice (Fig. 1c). These results showed that DSG is as effective as FK506 and rapamycin in inducing the elongation of allograft survival when used at suboptimal doses in combination with DSP.

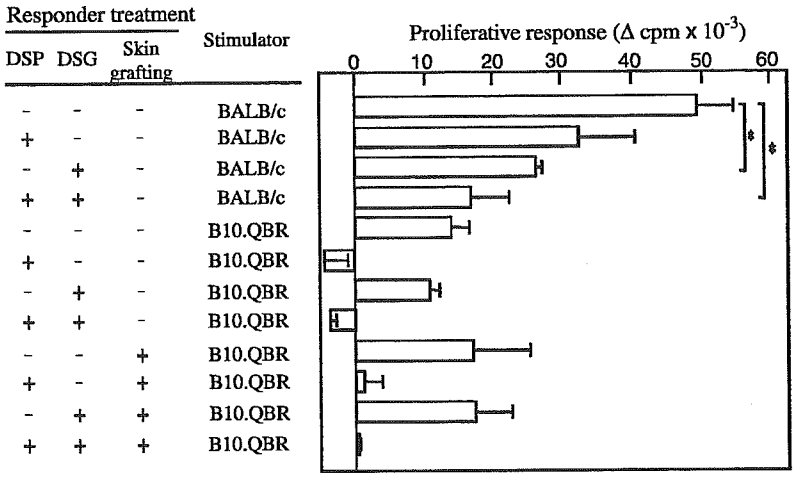

Fig. 2. Proliferative responses of untreated and treated B6 spleen cells to irradiated BALB/c or B10.QBR spleen cells. [^{3}H]thymidine incorporations were measured on days 3, 5 and 7 after the beginning of the MLR responses, and peak responses were always observed on day 5. Thus, average Δcpm values ($n=3$) at day 5 are shown with bars representing S.E.M. *, $P<0.04$.

4.2. Suppression of MLR and CTL responses in B6 mice treated with DSP and/or DSG

When spleen cells from treated and untreated B6 mice with or without skin grafting were tested for allospecific proliferative responses, administration of 10 doses of DSG alone significantly weakened MLR responses to the third party BALB/c stimulator cells, but vigorous proliferative responses were still observed even after DSP with B10.QBR spleen cells plus DSG administration (Fig. 2). In accordance with the previous observations, DSP alone, but not DSG administration alone, completely abolished the ability of B6 spleen cells to respond in MLR assays to MHC class I-disparate B10.QBR simulator cells, and this unresponsiveness was retained in the DSP-treated mice even after being grafted

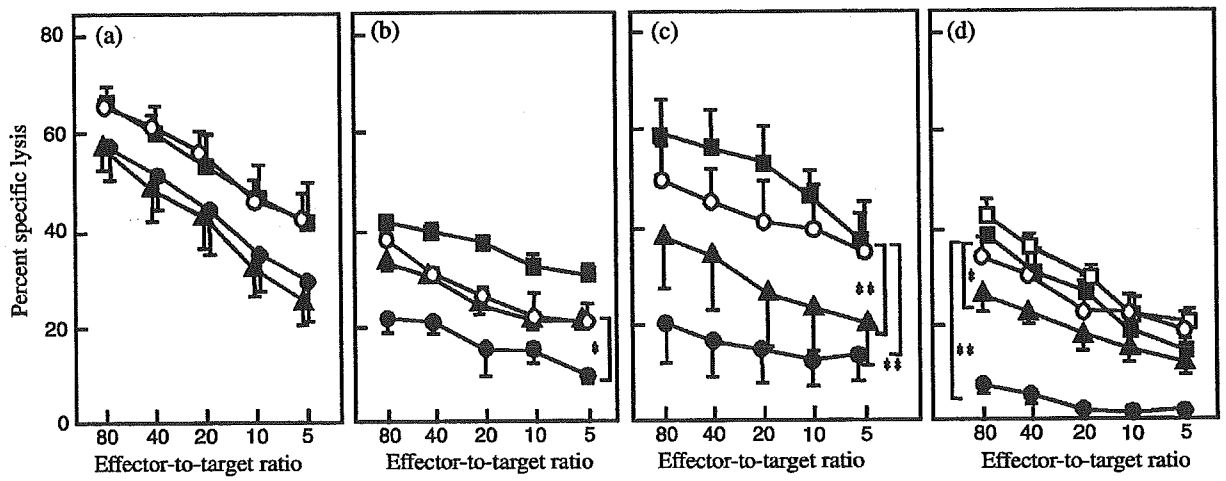

Fig. 3. CTL responses exerted by in vitro-stimulated effector cells derived from untreated and treated B6 mice. (a) Effector cells were induced by in vitro stimulation with irradiated BALB/c spleen cells, and target cells used were labeled BALB/c blast cells. (b–d) Effector cells were induced by in vitro stimulation with irradiated B10.QBR spleen cells, and target cells used were labeled B10.QBR blast cells. In a and b effector cells were prepared from untreated B6 mice (○), B6 mice treated with DSP alone (▲), those treated with DSG administration alone (■), or those treated with the combination of DSP and DSG administration (●). In c B6 mice were treated as above (shown with the same symbols), and received a B10.QBR skin graft. In d effector cells were prepared from untreated B6 mice (○), B6 mice injected with MMC-treated B10.QBR spleen cells plus DSG (▲), those injected with 1500 rad-irradiated B10.QBR spleen cells plus DSG (■), those injected with 4500 rad-irradiated BIO.QBR spleen cells plus DSG (□), or those injected with the combination of untreated BIO.QBR spleen cells and DSG administration (●). Each data point represents the mean from 3 to 6 repeated experiments, and bars represent S.E.M. *, $P<0.03$ by Hotelling's T2 test; **, $P<0.001$ by the same test.

with B10.QBR skin. However, CTL responses against B10.QBR target cells were not significantly affected by DSP alone (Fig. 3b). Only in the group of mice treated with the combination of a single DSP and suboptimal doses of DSG administration, CTL responses to B10.QBR target cells were significantly reduced. Unchanged killing activity of the in vitro-stimulated spleen cells prepared from the treated mice against BALB/c target cells (Fig. 3a) confirmed the alloantigen-specificity of the suppression of CTL activities. Furthermore, significant suppression of CTL activities against B10.QBR target cells was also observed in the B6 mice treated with the combination of DSP and DSG administration even after they received a B10.QBR skin graft (Fig. 3c). CTL activities detected in the untreated control mice and those of the mice treated only with DSG administration were apparently enhanced after skin grafting (Fig. 3b,c), reflecting in vivo priming of CTL precursor cells through alloantigen presentation. However, this priming effect was not observed in mice treated with the combination of DSP and DSG administration. Interestingly, CTL activities detected from the mice treated with a single DSP alone were also significantly lower than those in the control mice when tested after skin grafting (Fig. 3c), probably reflecting the contribution of $CD4^+$ T helper cell functions in the induction of CTL effector cells, which is reduced in the DSP-treated mice (Fig. 2). These data indicated that a significant proportion of CTL precursors were unable to be primed in vivo with the relevant alloantigen when treated with DSP, especially in combination with suboptimal doses of DSG administration.

4.3. Characterization of cellular requirements for the suppression of allograft responses by DSP

To characterize cell properties required for the induction of suppressed allograft responses by DSP, donor spleen cells were treated with several different procedures that modulated their proliferating potentials and antigenicities before intravenous injection. Irradiation (either 1500 or 4500 rad) or MMC treatment of donor spleen cells almost completely abolished their proliferative potentials after Con A or LPS stimulation: no significant incorporation of [^{3}H]thymidine into the treated cells was observed at any one of the four time-points at which the measurements were performed between 1 and 7 days after the beginning of cell cultures. When used as stimulator cells to induce proliferative responses of naive B6 spleen cells in vitro, B10.QBR spleen cells irradiated with 1500 or 4500 rad of X-ray showed significantly reduced antigenicity. They induced only 25% and 15% of peak Δcpm values at day 5 after the begin-ning of the MLR assays, respectively, in comparison with untreated B10.QBR cells, while the stimulatory potential of MMC-treated spleen cells was not significantly reduced. No significant changes in the levels of cell surface expression of D^q molecules between untreated, irradiated, and MMC-treated B10.QBR spleen cells were observed by flow cytometric analyses (data not shown). Interestingly, when used for DSP along with suboptimal doses of DSG, 1500 rad-irradiated spleen cells induced statistically significant but only marginal suppression of MLR responses, and 4500 rad-irradiated spleen cells were not effective at all in inducing the suppression (Table 1). However, MMC-treated B10.QBR spleen cells induced significant suppression of MLR responses when injected intravenously along with DSG administration. When CTL responses of the in vitro-stimulated spleen cells prepared from the untreated and treated B6 mice were compared, irradiated and MMC-treated B10.QBR spleen cells did not induce the suppressive effect that was observed when untreated B10.QBR spleen cells were injected (Fig. 3d), although the injection of MMC-treated B10.QBR spleen cells resulted in slightly reduced CTL activities in comparison with those exerted by the stimulated spleen cells of control untreated mice. These results, especially those with MMC-treated donor cells, indicated that proliferating potentials of donor cells, not just the presence of the relevant alloantigen on their surfaces, are required for the suppression of CTL responses by DSP plus DSG administration.

4.4. Both T and non-T cells were effective in inducing the suppressed allospecific cellular immune responses

To further characterize the donor cells that are involved in the induction of the suppression of allograft responses by DSP and DSG administration, spleen cells used for DSP were separately purified into $B220^-$, $CD90^+$ T and $CD90^+$, $B220^-$ non-T cell populations using a magnetic cell sorter (Fig. 4a). The obtained T-cell population was >98% $CD3^+$ and almost completely devoid of $CD19^+$ B cells, while the non-T cell population was almost completely free from contaminating T cells and comprised of 94% B cells. A single injection of both T and non-T cell populations in combination with suboptimal doses of DSG administration completely abolished the donor antigen-specific proliferative responses in the treated B6 mice (Fig. 4b). Further, CTL responses against B10.QBR target cells were significantly reduced in mice injected with the donor T or non-T cell population (Fig. 4c). As measured by the induction of suppressed alloantigen-specific CTL responses, the purified T cell population was as effective as the whole spleen cells, and purified non-T cells were even more effective and induced almost total suppression of the CTL responses.

5. Discussion

Several different mechanisms have been proposed for the induction of allograft tolerance by DST or DSP in

Table 1
Effects of donor cell irradiation or MMC treatment on the suppression of MLR responses after DSP plus DSG administration

Donor cell treatment	Proliferative responses of recipient spleen cells (Δcpm)	Significance in difference from the control
1500 rad irradiated	7820±528	$P=0.007$
4500 rad irradiated	18 714±2520	NS
MMC treated	2384±468	$P=0.004$
None	−1031±528	$P=0.003$
Control (No DSP)	13 947±2190	

Δcpm values are shown as mean±S.E.M.
NS, not significant.

rodent models. In rats tolerized by DST for heterotopic heart transplantation, recipient immune cells still infiltrated into graft tissues, but cytokine production from the immune cell infiltrated was deeply impaired [1]. Generation of an inhibitory signal from DST-primed host immune cells upon secondary presentation of donor antigens has been reported. In a model using class I L^d-specific T-cell receptor transgenic mice, a single dose of DSP with L^d-expressing spleen cells induced a significant deletion of the donor antigen-reactive T cells, and activated a regulatory subset of T cells in the recipient, which resulted in permanent survival of skin allografts [10]. The above regulatory T cells have been recently characterized as a novel CD4- and CD8- double negative population that exerts Fas-mediated killing of $CD8^+$ effector cells [18]. DST combined with anti-CD154 (CD40 ligand) antibody administration is shown to be effective in tolerance induction for islet grafts, and this model also is apparently mediated by the induction of $CD4^+$ regulatory T cells [2]. For the induction of the above tolerance by DST plus anti-CD154 antibody administration, small resting B cells are sufficient, and T cells are not required in the DST preparation.

In the MHC class I-incompatible model similar to the present study, however, radio-sensitive T cells in the donor cell inoculum is shown to be required for the induction of prolonged skin graft survival associated with reduced CTL precursor frequencies [12,15], suggesting that donor T cells in the DSP preparation may function as veto cells. In the H2 class I-disparate model

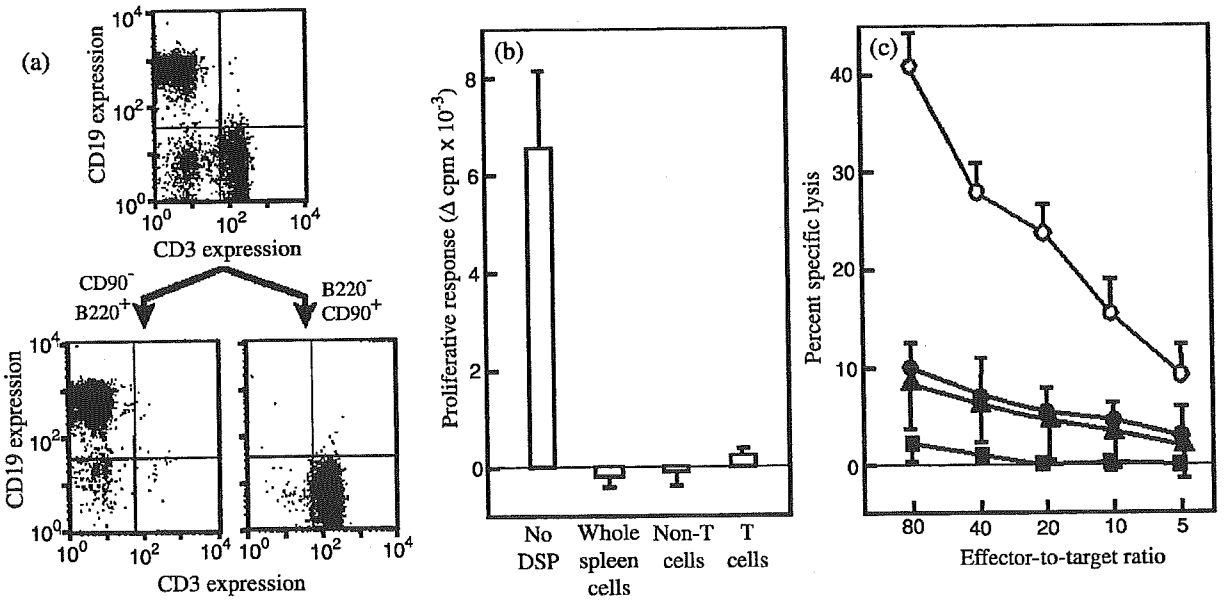

Fig. 4. Identification of cell types in DSP preparation effective in suppressing allospecific cellular immune responses. (a) Flow cytometric analyses confirming purity of injected T and non-T cell populations. (b) Proliferative responses of untreated and treated B6 spleen cells upon stimulation with irradiated B10.QBR spleen cells. Each data show mean±S.E.M. calculated with data obtained from 3 to 6 individuals per group. (c) CTL responses exerted by in vitro-stimulated effector cells prepared from untreated B6 mice (○), B6 mice injected with the purified T cells plus DSG (▲), those injected with the purified non-T cells plus DSG (■), or those injected with the whole B10.QBR spleen cells plus DSG (●). Each data point represents the mean from 3 to 6 repeated experiments, and bars represent S.E.M.

identical to the present combination of B6 recipient and B10.QBR donor mice, it has been shown that (1) a single dose of DSP alone induces a significant reduction in MLR and donor antigen-specific IL-2 production responses, but fails to suppress CTL responses [14]; and (2) DSP combined with either antibody-mediated depletion of $CD8^+$ T cells [14], suboptimal doses or FK506 [16], or suboptimal doses of rapamysin administration [17] results in significant suppression of CTL induction and prolonged skin graft survival. Similar suppression of MLR but not CTL responses was also is observed in rat models of kidney transplantation after DSP [9].

In addition to the previously demonstrated efficacy of FK506 and rapamysin, DSG is now shown to be similarly effective in inducing CTL suppression and prolonged allograft survival when used at suboptimal doses along with a single DSP. FK506 and rapamycin share intracellular receptor molecules, FK-binding proteins, and block the transcriptional activation of interleukin-2 gene in response to T-cell receptor cross-linking or abolish the cell-cycle progression of cytokine-stimulated T cells from G_1 to the S phase, respectively [21]. Mechanisms of action of DSG are quite different from those of FK506 and rapamycin: it suppresses macrophage proliferation [22], blocks pre-T and pre-B cell differentiation [23], and inhibits dendritic cell maturation and antigen presentation [24]. In the present study, suboptimal doses of DSG alone did not affect MLR responsiveness of B6 spleen cells to class I-disparate B10.QBR cells, and CTL induction was significantly suppressed only when DSG was given in combination with DSP. Since DSG is not directly involved in the uncoupling of T-cell receptor signaling and clonal activation, unlike FK506 and rapamycin, effectiveness of DSG in suppressing CTL induction, when combined with DSP, might suggest possible roles of inappropriate antigen presentation and resultant T cell ignorance or anergy [25].

If the induction of T-cell ignorance or anergy is the main mechanism, the expression of relevant alloantigens on the injected cell surfaces, but not functional activities of the injected donor cells, should be sufficient in inducing the suppression of CTL responses and prolonged graft survival. In fact, previous literatures have indicated that cells used for DST can be non-proliferative erythrocytes [7] or even heated blood [8]. However, in the present study, both irradiation and MMC treatment of the donor spleen cells abolished the effect of DSP in suppressing CTL induction. Since T-cell stimulating antigenicity was preserved, albeit reduced, on MMC-treated spleen cells, and MLR responses were indeed largely suppressed in B6 recipient mice injected with MMC-treated B10.QBR spleen cells (Table 1), possible induction of alloantigen-specific regulatory T cells, if any, is also unlikely to be affected by this treatment of donor cells. Rather, a proliferating potential seems to be directly required for the injected donor cells to suppress CTL induction. One possible mechanism that can be affected by irradiation or MMC treatment of the DSP preparation is active involvement of injected donor cells, perhaps as veto cells. Veto phenomenon was originally proposed as a form of antigen-specific suppression of T cells by other lymphoid cells that results in the functional elimination of self-reactive peripheral effector cells [26]. The concept of veto function was later expanded to the inactivation of alloreactive CTL precursor cells upon introduction of allogeneic lymphoid cells [26,27]. Although several different donor cell types are known to exert the veto cell activity when injected intravenously, T cells, especially $CD8^+$ cells, are commonly shown to be the most potent veto cells [26,28,29]. In fact, in the class I-disparate model similar to the present study, the suppression of donor-specific CTL activity by DSP was dependent on the presence of radiosensitive T cells in the injected donor cell preparation [12,15]. Interestingly, however, not only purified T cells but also T cell-depleted $CD90^-$, $B220^+$ cells were effective in inducing almost complete suppression of the donor-specific CTL responses in the present study (Fig. 4). Thus, the results may indicate that the previously described veto T cells are unlikely to be involved in the suppression of CTL induction in the present model. They are rather consistent with the previous finding that as long as relevant MHC molecules are expressed, even transfected fibroblasts can induce immunological unresponsiveness and prolonged graft survival upon intravenous injection [30]. Further studies are required to identify the precise mechanisms by which allospecific CTL responses are suppressed by DSP combined with DSG administration.

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MHCテトラマーによる抗原特異的T細胞の解析

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・抗原特異的T細胞の細胞数や頻度をダイレクトに定量する．
・抗原特異的T細胞の機能や成熟過程をフローサイトメトリーで解析する．

研究への応用のヒント

CD8陽性の細胞傷害性T細胞（cytotoxic T lymphocyte：CTL）は，主要組織適合性抗原（major histocompatibility complex：MHC）に提示されたペプチドを抗原として認識する．これまでCTLの抗原特異性は細胞傷害活性により評価されてきたが，この方法では抗原特異的CTLの細胞数（頻度）を直接的に測定することはできなかった．

抗原ペプチド・MHC複合体（pMHC）を四量体化したMHCテトラマーは，そのpMHCに特異性をもつT細胞を直接検出する．これにより，生体内での抗原特異的CTLの頻度の定量的解析が可能となったばかりでなく，抗原特異的CTLの成熟過程や機能分子の発現をフローサイトメトリーを用いて同時に評価できるようになった．例えば，ヒト免疫不全ウイルス（human immunodeficiency virus：HIV）に特異的なCTLとサイトメガロウイルス（cytomegalovirus：CMV）に特異的なCTLとの成熟状態や機能の違いを，個々の細胞レベルで知ることができる．しかしながら，MHCテトラマーを用いるには，病原体由来の抗原ペプチドとそれを提示するMHC拘束分子が同定されている必要がある．また，可溶型pMHC複合体の調製方法が単純ではない．さらに，MHCテトラマーの性質上，個々の抗原ペプチドへの特異性に優れるため，逆に病原体に特異的なT細胞の全体量を解析することはできない．

一方，T細胞は抗原特異的にサイトカインを産生する．この性質を利用して，抗原刺激後にサイトカインを産生したT細胞を測定することにより，抗原特異的なT細胞の頻度を定量することができる．検出手段としては，細胞内染色によりサイトカインを産生したT細胞をフローサイトメトリーで解析する方法と，プレート上でサイトカインを産生した細胞をスポットとして検出する方法（ELISpot）がよく用いられている．また，検出するサイトカインとしては，インターフェロンガンマ（interferon-γ：IFN-γ）が最も一般的である．これらの方法はいずれも，T細胞エピトープの構造が決められていなくてもよい．刺激に用いる抗原は，いくつかの異なった配列からなる合成ペプチドや，ウイルス感染細胞あるいは腫瘍細胞などを用いることが

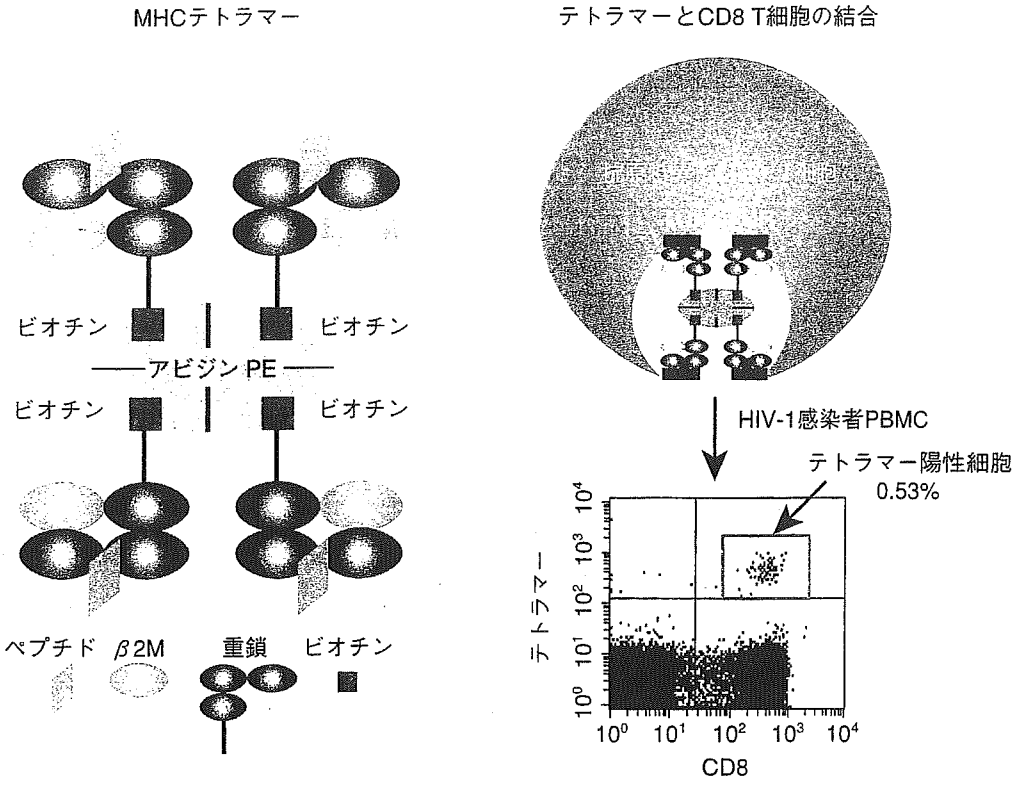


図1 MHC テトラマーによる抗原特異的 CD8 T 細胞の検出
HIV 感染者の末梢血リンパ球を抗 CD8 抗体と HIV 特異的な MHC テトラマーで染色後，フローサイトメトリーで解析した．CD8 陽性画分にテトラマー陽性細胞が検出された．その頻度は全 CD8 T 細胞中の 0.53 %であった

できる．

どの方法も一長一短があるため，研究目的に応じた使い分けが必要である．なお，本稿では MHC テトラマーを用いた方法について詳しく述べる．

原理

T 細胞の抗原特異性は，T 細胞レセプター（T cell receptor ： TCR）が担っている．TCR は，ターゲット細胞（あるいは抗原提示細胞）上に発現している MHC 分子とそこに提示されたペプチドとの複合体（pMHC）と結合し，T 細胞に活性化シグナルを伝達する．したがって，pMHC を用いれば抗原特異的 T 細胞を検出することが原理的に可能である．しかしながら，TCR-pMHC の結合親和性は弱く（抗原抗体反応の 100 ～ 1,000 分の 1 程度），実験操作中に離れてしまうため，フローサイトメトリーでの検出は不可能であった．

Altman らは[1]，可溶型 pMHC（MHC 分子の細胞外ドメインで構成される）をアビジン・ビオチン結合を利用して四量体（すなわちテトラマー）にすると（図 1 A），TCR を介して T 細胞上により安定に結合することを発見した．抗原ペプチドを含む pMHC と蛍光標識されたアビジンを用いることにより，抗原特異的な T 細胞をフローサイトメトリーや蛍光顕微鏡で検出することが可能となった（図 1 B）．

準備するもの

1）試薬

□ ビオチン化したpMHC複合体

□ 蛍光標識されたアビジン
PE（phycoerythrin）あるいはAPC（allophycocyanin）標識されたものが望ましい

□ 10％NCS（new born calf serum）を添加したRPMI-1640（R-10）またはPBS

□ 蛍光標識された抗ヒトCD8抗体

□ 1％パラフォルムアルデヒド・PBS溶液

2）細胞

□ ヒト末梢血リンパ球

□ 抗原ペプチドで刺激後，10日間程度培養したリンパ球

3）実験器具および測定機器

□ 冷却遠心器

□ フローサイトメトリー

プロトコール

1．テトラマーの作製

□ ビオチン化したpMHC複合体のタンパク量を測定

▼

□ pMHC複合体のモル濃度を計算し，添加する蛍光標識アビジンの量を見積もる
（アビジンとpMHCのモル比＝1：4）

▼

□ ビオチン化したpMHC溶液に蛍光標識アビジンを添加❶

▼

❶ プロテアーゼの混入を避けるために，使用するチューブ，チップなどはすべてオートクレーブ滅菌したものを用いる．フリーのビオチンはプラスチックに吸着しやすいので，❶の操作にはガラスのチューブを用いる．アビジンは最終的に加える量の10分の1ずつを1時間ごとに分けて添加する．操作はすべて4℃で行い，チューブは常にローテーターでゆっくり回転させてよく混合する．アビジンの量が過剰で，四量体が形成されない

（次ページに続く）

memo

われわれは研究室内でpMHCの調製とビオチン化を行っている．最近では，受託製造するメーカが出てきたので，下記ホームページを参照されたい．

・Proimmune：http://www.proimmune.com

・ベックマン・コールター：http://www.immunomic-sonline.com/

また，アメリカNIAID（national institute of allergy and infectious disease）が運営しているAIDS reagent program（http://www.niaid.nih.gov/reposit/tetramer/index.html）に，MHCテトラマーの合成を依頼することも可能である．このサイトでは，MHCテトラマーに関するプロトコールなど詳細な情報を入手できる．

□ 全量を添加後，ローテーターを用いて24時間撹拌する（4℃）

▽

4．MHCテトラマーを用いたT細胞の染色

□ 細胞数をカウントし，1～4×10^6 ㉑の細胞を50 μlのR-10に懸濁する
（1.5 ml容量のマイクロチューブを使うと便利）

▽

□ MHCテトラマー溶液を添加する ㉒

▽

□ 37℃，30分間放置

▽

□ 37℃に暖めたR-10で2回遠心（5,500 rpm，3分）洗浄 ㉓

▽

□ 洗浄液を完全に除去し，細胞をR-10で懸濁する ㉔

▽

□ 蛍光標識された抗CD8抗体を添加 ㉕

▽

□ 4℃，20分放置

▽

□ R-10で2回遠心（5,500 rpm，3分）洗浄 ㉗

▽

□ 1％パラフォルムアルデヒド・PBS溶液あるいは1％NCS-PBS溶液を添加 ㉘

▽

□ 細胞溶液をフローサイトメトリー測定用のチューブに移す

▽

□ フローサイトメトリーで測定

と，抗原特異的なT細胞が検出されない．例えば，調製したpMHCの純度によっては，実際のモル量よりも多く見積もってしまうために起こる．このようなときには，アビジンとpMHCのモル量比を1：8などにするとよい．

㉑ 実験に用いる細胞数は，期待される抗原特異的T細胞の頻度によって異なる．例えば，抗原刺激を加えていない末梢血リンパ球の場合はテトラマー陽性細胞の頻度が非常に少ないことが予想されるため，多くの細胞を用いた方がよい．細胞の懸濁に用いるR-10はあらかじめ37℃に暖めておく．

㉒ テトラマーによって最適な濃度が異なるため，われわれはCTLラインなどを用いて最適濃度を決めている．

㉓㉔ 遠心操作は室温（20～25℃）で行う．

㉕ R-10はあらかじめ4℃に冷却しておく．

㉖ 他の表面抗原の解析を同時に行う場合は，この段階で蛍光標識抗体を添加する．

㉗㉘ R-10および遠心器は前もって4℃に冷却しておく．

㉘ 感染検体やすぐに測定を行わない場合は必ずパラフォルムアルデヒドで固定を行う．

実験条件を最適化するコツ

㉙ 保存条件

MHCテトラマーは4℃で保存しているが，テトラマーの種類や調製方法によっては，数カ月で劣化する．そこで長期に渡って同一のテトラマーを使うような場合は，われわれはビオチン化したpMHC複合体を少量ずつ小分けして－80℃で冷凍保存し，必要に応じてアビジンを結合させている．この方法だと，1年以上にわたって安定である．

テトラマーやpMHCの不安定性は，ペプチドが解離してしまうことや，調製中に混入した（あるいは除ききれなかった）微量のプロテアーゼが原因と考えられる．したがって，ペプチドやMHCの種類と調製方法によって不安定性が大きく異なる．

㉚ 蛍光色素の種類とアビジンの品質

われわれは，テトラマー化する際に用いるアビジンについて，これまでに数種類の異なった蛍光色素を試みたが，PE（phycoerythrin），APC（allophycocyanin），APC-Cy7でいい結果を得ている．その他の蛍光色素，例えばFITCではあまりよい結果を得ていない．また，アビジンの品質

が適切でないと，MHCテトラマーの比活性が下がるうえ，バックグラウンドが上昇するため，特異性も感度も大きく低下する．われわれは現在Molecular Probes社のアビジンを使用している．

◎ テトラマーの濃度

リンパ球をMHCテトラマーで染色する際，濃度が濃すぎてもよい結果が得られない．バックグラウンドの上昇という理由だけでなく，MHCテトラマーによりT細胞に抗原刺激が入るためである．T細胞によっては，細胞死を起こしてしまう．したがって，MHCテトラマーを最初に用いる際には，染色に用いる最適濃度を実験的に定めることをすすめる．抗原特異的なCTL株やクローン（抗原特異的な細胞傷害活性やサイトカイン産生を示す）をあらかじめ作っておき，MHCテトラマーによる陽性コントロールとするとよい．

トラブルシューティング

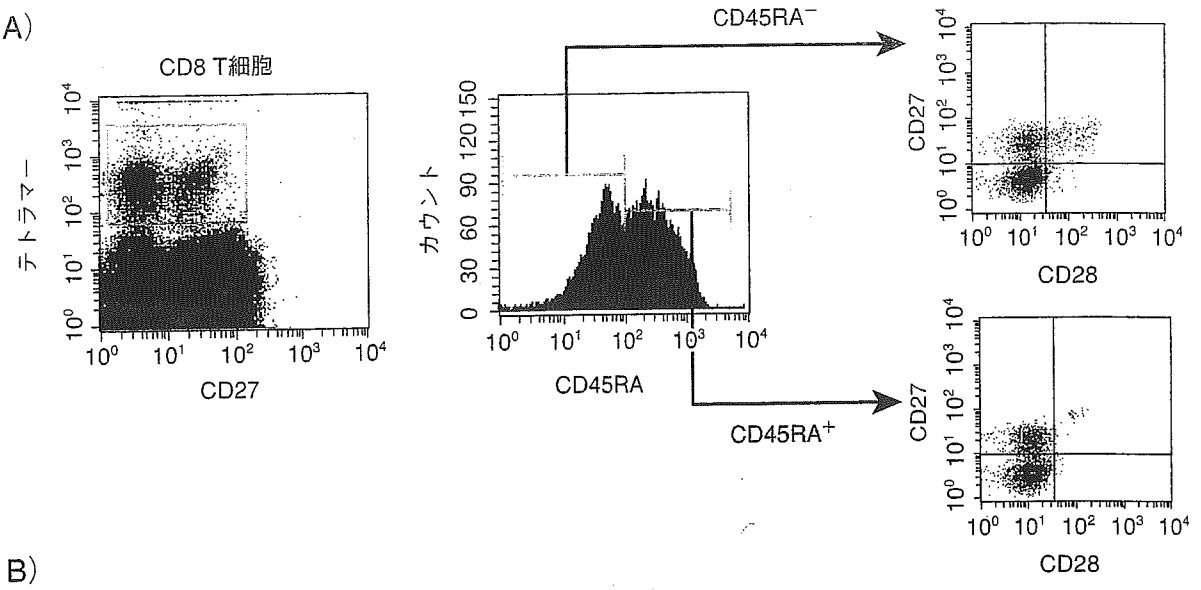
トラブル	考えられる原因	解決のための処置
以前テトラマー陽性細胞が検出された試料で，陽性細胞が検出されなくなった	テトラマーが劣化している．	凍結保存したビオチン化pMHCを用いてテトラマーを調製する．
陰性コントロールに対してバックグラウンドが高い	テトラマーによってはCD8分子との相互作用が強いことがある．	抗CD8抗体で染色してからテトラマーを染色する．
テトラマー陽性細胞のドットプロットの集積が悪い	アビジンの品質が悪いと非特異的な結合が大きくなる．	アビジンを変えてテトラマーを再調製する．

実験例

まずはじめに，MHCテトラマーと表面抗原に対する抗体を用いて，ウイルスに特異的なCD8 T細胞をフローサイトメトリーで解析した例を示す．図2は，ヒトサイトメガロウイルス（HCMV）に特異的なCD8 T細胞の解析結果である．解析した検体はすべて，HCMVに感染しているが，健康な人の末梢から採取したリンパ球を用いている．末梢のCD8 T細胞を，APCで標識されたMHCテトラマー（抗原ペプチド：HCMV pp65，NLPMVATV／MHC拘束分子：HLA-A＊0201）とヒトCD8 T細胞の分化マーカーであるCD27，CD28，CD45RA抗原に対する抗体で四重染色し，フローサイトメトリーで解析した．HCMV特異的CD8 T細胞は$CD27^+CD28^+CD45RA^-$，$CD27^+CD28^-CD45RA^-$，$CD27^-CD28^-CD45RA^-$，と$CD27^-CD28^-CD45RA^+$の4つのサブセットで頻度が高かった（図2 B）．

次にMHCテトラマーと細胞内染色技術を組合せて，抗原に特異的なCD8 T細胞内の機能分子の発現を解析した結果を示す．図3は，細胞傷害性分子であるパーフォリンの発現量とその頻度を解析した結果である．テトラマー陽性サブセット中の$CD27^+CD28^-CD45RA^-$および$CD27^-CD28^-CD45RA^-$細胞では，パーフォリンの発現量が高いため，これらの細胞群が細胞傷害活性をもつことを示唆する．実際この両サブセットをセルソーターを用いてソーティングして，それぞれの細胞傷害活性

A)



B)

各サブセットの頻度（%）

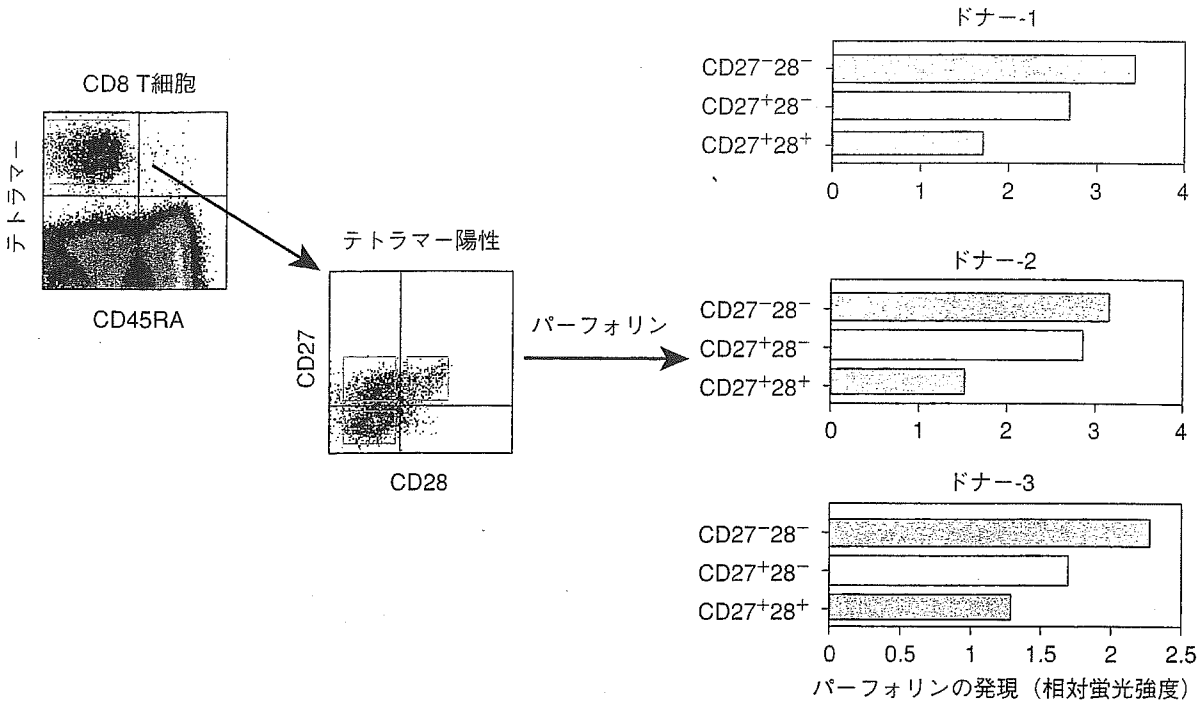
	CD45RA⁻				CD45RA⁺				
Donor	CD27⁺CD28⁺	CD27⁺CD28⁻	CD27⁻CD28⁻	CD27⁻CD28⁺	CD27⁺CD28⁺	CD27⁺CD28⁻	CD27⁻CD28⁻	CD27⁻CD28⁺	Tetramer⁺/CD8⁺
U-2	3.94	9.19	19.70	1.53	0.66	7.63	56.69	0.66	1.61
A-7	10.29	7.85	42.19	2.19	2.16	4.40	30.15	0.78	0.40
A-16	20.54	20.67	34.54	2.00	5.25	6.38	10.50	0.14	0.90
M-14	43.44	24.55	12.15	1.28	1.19	6.92	10.48	0.00	1.62
M-20	3.38	12.87	37.64	0.64	0.56	13.04	31.68	0.18	1.44
M-22	35.95	23.41	10.87	0.84	4.23	11.99	12.70	0.00	0.14
M-26	19.06	6.14	3.17	0.49	23.20	30.09	16.47	1.38	1.47
M-30	10.30	31.72	53.93	2.30					1.60
	18.36±14.7	17.05±9.3	26.77±17.8	1.41±0.7	5.32±8.1	11.49±8.8	24.10±16.9	0.45±0.5	

図2 HCMV-特異的 CD8 T 細胞の分化
詳細は本文を参照

を測定したところ，HCMV ペプチドをパルスした標的細胞に対してほぼ同等の細胞傷害活性を示した（図 4）．

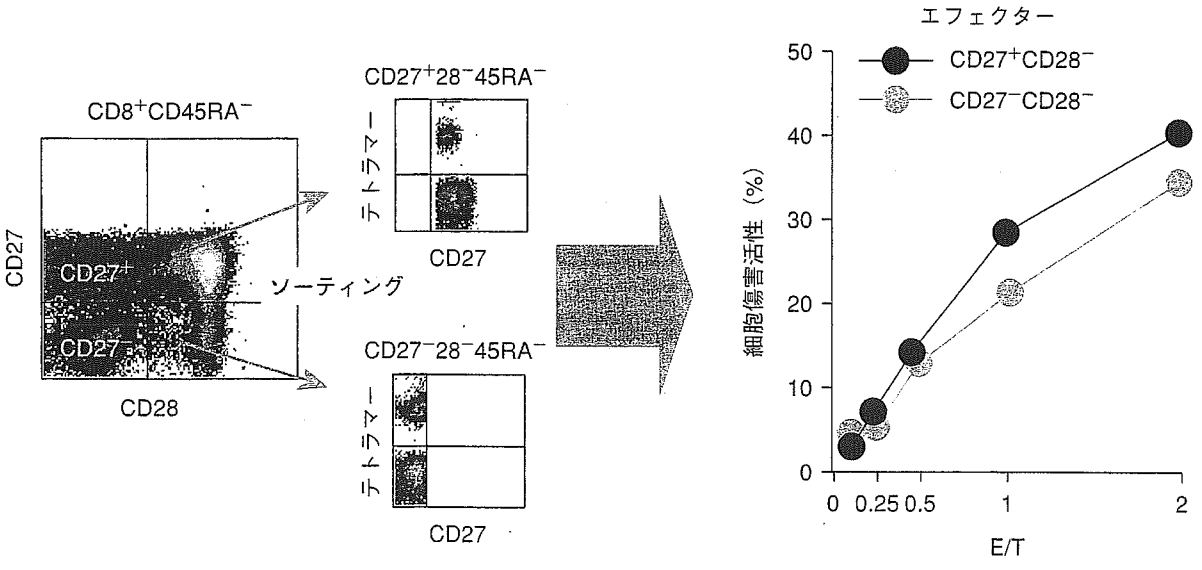
おわりに

MHC テトラマーは，1996 年に Altman らにより，抗原特異的 T 細胞を直接的に定量できる画期的な方法として報告された[1]．今では当初の予想をはるかに超え，さまざまな応用が試みられている．例えば，抗原特異的 T 細胞をセルソーターを用いて分離したり，TCR に対する抗体と組合せて TCR レパートリーの変化を調べることも可能である．あるいは，*in situ* 染色により，抗原特異的 T 細胞の組織での局在を調べることもできる．また，実験例で示したようにテトラマーと機能性分子に対する抗体を組合せることで，これまで知られていなかった抗原特異的 T 細胞の性質が明らかになってきた．われわれは，CMV 特異的な CD8 T 細胞と EB ウイルス特異的な CD8 T 細胞では，末梢での分化状態が異なることを明らかとした[2]．このように MHC テトラマーは抗原特異的な T 細胞に直接結合するため，特異性，感度，定量性



抗原特異的 CD8 T 細胞のパーフォリンの発現

HCMV 抗体陽性者の CD8 T 細胞を APC 標識したテトラマー，抗ヒト CD27-FITC，抗ヒト CD28-CyChrome，抗ヒトパーフォリン -PE 抗体で染色した．CD27，CD28 抗原の発現で分類した 3 つの分画のパーフォリンの発現をフローサイトメトリーで解析した．その結果，抗原特異的 CD8 T 細胞のパーフォリン発現量は $CD27^+28^+45RA^-$ < $CD27^+28^-45RA^-$ < $CD27^-28^-45RA^-$ の順で高かった



抗原特異的なメモリー CD8 T 細胞の細胞傷害活性

HCMV 抗体陽性者の CD8 T 細胞を，抗ヒト CD27-FITC，抗ヒト CD28-PE，抗ヒト CD45RA-CyChrome 抗体で重染色して，$CD8^+CD28^-CD45RA^-$ 分画をさらに，CD27 抗原陽性と陰性分画に分け，HCMV 由来ペプチドに対する細胞傷害活性を測定した．同時に，同一検体をテトラマーで染色して，各分画に含まれるテトラマー陽性細胞の頻度を測定して，E/T 比（effector/target）を決定した

ともに優れているが，ウイルス感染などに伴う免疫応答の全容を捉えるといった研究には不向きかもしれない．また，MHCテトラマーを作製する技術は，タンパク質を専門としていない研究者にはかなり困難である．しかしながら最近では，昆虫細胞や培養細胞を用いた新たな発現系が開発されてきているうえ，委託合成を行うメーカーも現われてきたため，こうした技術的な壁は近いうちに取り除かれるであろう．

最後に，四量体化することでレセプター（あるいはリガンド）との結合親和性を高めてフローサイトメトリーで検出するというコンセプトは，MHCクラスⅠ分子にとどまらずさまざまに応用されてきている．例えば，MHCクラスⅡ，TCR，NKレセプター，CD1分子群などである．こうしたアプローチにより，まだ明らかにされていない免疫担当細胞の認識機構がさらに進むものと期待される．

参考文献

1）Altman, J. D. et al. : Science, 274 : 94-96, 1996
2）Tomiyama, H. et al. : J. Immunol., 168 : 5538-5550, 2002