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Advances in Brief

The Immunogenicity of Experimental Tumors Is Strongly Biased by the Expression of Dominant Viral Cytotoxic T-Lymphocyte Epitopes[1]

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Abstract

The immunogenic Friend-Moloney-Rauscher (FMR) virus-induced tumors have been used extensively to clarify the cellular and molecular mechanisms responsible for tumor rejection and to develop immunotherapeutic strategies. We characterize here the trimolecular complex MHC class I-antigenic determinant-T cell receptor involved in the induction of a protective CTL response against the RMA thymoma. This complex is mainly composed by the D^b molecule interacting with a Rauscher virus antigen (Ag) determinant and the $V\beta5^+$ T cell receptor. We also show that the chemically induced EL-4 thymoma acquires the susceptibility to recognition by anti-RMA CTLs and the ability to elicit a protective anti-RMA CTL response only upon infection by a virus of the FMR family and that RMA and FMR virus infected EL-4 cells share tumor-associated Ag. The data strongly support the hypothesis that the high immunogenicity of virus-induced or infected tumors is determined by the expression of immunodominant virus-encoded Ag. The demonstration of a different outcome in the immune responses elicited in the presence or in the absence of viral Ag further open the contention of the molecular requirements for immunogenicity and should stimulate a more careful revision of unexpected cross-reactivity among tumors.

Introduction

Highly immunogenic tumors induced by MuLV[3] have been used to clarify the cellular mechanisms responsible for tumor rejection (1) and to develop more effective immunotherapeutic strategies (2). Retrovirus-encoded proteins have been demonstrated to be targets of antitumor immune responses (3). MuLV-like sequences have been demonstrated in the human genome and are expressed in human tumors (see, *e.g.*, Ref. 4), although it is still to be elucidated whether retroviral encoded proteins represent Ag targets for T cells also in humans.

The oncogenic MuLVs can be divided in two major groups: the endogenous AKV/MCF viruses and the exogenous FMR viruses (1). In both groups of MuLV-induced lymphomas, tumor rejection and prolonged survival of the animals result from an effective CTL-mediated immune response (5). FMR virus-induced tumors share common Ags (6). CTLs specific for tumors induced by one of the FMR viruses cross-react with and are responsible for the rejection of tumors induced by any other virus of the FMR group (7). Similarly, tumors induced by viruses of the AKV/MCF type share common Ags, which allow cross-reactivity of antivirus CTLs and cross-protection against tumors induced by viruses within the AKV/MCF family (8). However, the Ags expressed by FMR virus-infected cells are different from the ones expressed by AKV/MCF-type virus-infected cells, and neither cross-reactivity of CTL nor cross-protection between the two groups occurs (8, 9).

The Ag specificity of CTLs directed against FMR virus-induced neoplasms is still under investigation. Both gag and envelope virus-encoded proteins seem to contain CTL epitopes, although the recognition is profoundly biased by the genetic background of the animals (8, 10–12). Major Ags of the Rauscher virus-induced RMA thymoma were identified in a glycoprotein of M_r 175,000 plus a protein of M_r 50,000 (13). It is still debated, however, whether the immunodominant Ag determinant(s) of RMA is represented by RNA virus-encoded proteins (13, 14).

We recently characterized a $CD3^+$ $CD8^+$ T-cell line (named 7-4-94) obtained from spleen cells of a C57BL/6 mouse challenged *in vivo* and repeatedly stimulated *in vitro* with B7.1-transfected RMA cells (15). RMA lysis by 7-4-94 cells is CD8 dependent and MHC-I D^b restricted, as demonstrated by the lack of killing of the MHC-I-negative RMA-derived RMA-S cells and by the inhibition of the lysis of RMA cells in the presence of anti-D^b and not of anti-K^b mAbs (15). We took advantage of the 7-4-94 CTL line and of a panel of retrovirus-induced or -infected tumors to verify whether (*a*) the protective CTL response against RMA is dominated by virus Ag determinants recognized by TCR characterized by a restricted $V\beta$ gene usage and (*b*) the presentation of viral Ag influences the immunogenicity and tumorigenicity of a neoplasm.

Materials and Methods

Tumor Cell Lines. The H-2^b T-cell lymphoma RMA is a Rauscher virus-induced tumor (16). It has a MiTD of 1×10^3 cells when injected s.c. (17). However, a single injection of nonreplicating RMA cells protects all or part of syngeneic mice from challenge with up to 100 × MiTD of RMA cells (17). RMA-S cells, derived by chemical mutagenesis from RMA cells, are a MHC class I-negative (*i.e.*, <10% of expression of MHC class I molecules than RMA) variant (18). The procedures for cDNA cloning and transfection of the human B7.1 molecule in RMA cells have been described elsewhere (19). EL-4^- is a 9,10-dimethyl-1,2-benzanthracene-induced thymoma (H-2^b; Ref. 20). EL-4^+ is a variant of EL-4^-, known to be infected by and to express surface Ags of FMR-type viruses (21). L1210 is a methylcholantrene-induced lymphocytic mouse leukemia (H-2^d). MBL-2 is a Moloney Leukemia virus-induced lymphoma (H-2^b; Ref. 7). All cell lines were cultured in RPMI 1640 supplemented with penicillin-streptomycin and 10% FCS. For detection of the surface expression of the env-gp70 proteins, tumor cells were stained with saturating mAb from hybridoma 273 (IgG2a) obtained from the NIH AIDS Research and Reference Reagent Program (22) and a FITC-labeled rabbit antimouse second-step reagent (DAKO A/S, Glostrup, Denmark) and were analyzed by FACS (Becton Dickinson, Sunnyvale, CA), as described in Ref. 23. Expression of the env-gp70 proteins was calculated as Δ − MFI (*i.e.*, MFI

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[3] The abbreviations used are: MuLV, murine leukemia virus; Ag, antigen; MiTD, minimal tumorigenic dose; mit-c, mitomycin c; NWSC, nylon wool spleen cell; MFI, mode fluorescence intensity; FMR, Friends-Moloney-Rauscher; MCF, mink cell focus-inducing; TAA, tumor-associated Ag; env-gp70, envelope-gp70; TCR, T-cell receptor; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorting; PE, phycoerythrin.

of the sample treated with the two-step reagents minus the MFI of the sample treated with the second reagent only).

Mice and Immunization Procedures. C57BL/6 (H-2^b) female mice, 8–10 weeks old, were purchased from Charles River Breeding Laboratories (Calco, Italy), housed in a pathogen-free animal facility, and treated in accordance with the European Community guidelines. The *in vivo* experiments were approved by the Ethical Committee of the Istituto Scientifico H San Raffaele. The animals were immunized by a single s.c. injection of 1×10^6 mit-c-treated (15) RMA, RMA expressing the human B7.1 molecule, or EL-4^- or EL-4^+ cells; 2 weeks later, mice were challenged in the opposite flank with 10× MiTD of RMA (*i.e.*, 1×10^4) or EL-4^- (*i.e.*, 5×10^5) replicating cells. Tumor size was evaluated by measuring two perpendicular diameters by a caliper twice a week. Animals were scored positive when the mean tumor diameter was >2 mm. Mice with no visible or palpable tumor 12 weeks after tumor challenge were scored negative.

***In Vitro* CTL Induction.** Spleen cells from mice that had rejected the neoplasm were used either unfractionated or enriched in T cells by passage on a nylon wool column (24). Spleen cells and the eluted nonadherent cells (NWSCs) were resuspended in RPMI 1640 containing 10% heat-inactivated FCS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (culture medium). Thirty $\times 10^6$ unfractionated spleen cells or NWSCs were mixed with 3×10^6 mit-c treated tumor cells in 10 ml of culture medium. Five days later, blasts were isolated on a lympholyte-M gradient (Cedarlane, Hornby, Ontario) and analyzed by FACS using anti-CD3-FITC, anti-CD4-PE, anti-CD8-FITC, anti-NK-PE, and antimouse immunoglobulin-FITC mAbs (PharMingen, San Diego, CA) or resuspended in culture medium supplemented with 20 IU/ml human recombinant interleukin 2. CTL lines were generated from the blasts obtained from the spleen cells or the NWSCs of mice challenged with RMA or EL-4^+ cells by repeated cycles of restimulation and expansion in 20 IU/ml recombinant interleukin 2, as described in Ref. 15. The anti-Moloney virus-induced leukemia CTL clone CHM-14 was obtained from mice injected with the virus (10), and it is described in Ref. 11. Its lytic activity is D^b restricted (10) and directed against Ag determinants of the gag proteins (11). Phytohemagglutinin, concanavalin A, and lipopolysaccharide blasts were obtained from C57BL/6 spleen cells stimulated with 10 μg/ml of mitogen for 3 days. The characteristics of the CD4^+ T cell line specific for the sequence peptide 152–171 of the human heat shock protein M_r 70,000 are described in Ref. 25. Thymocytes were collected from naive C57BL/6 mice by filtering the disrupted thymus through a nylon cell strainer. Cells were washed, resuspended in RPMI 1640, and used as targets. FACS analysis showed that thymocytes were mostly CD4^+CD8^+ (*i.e.*, ~95%).

Assay of the TCR Vβ Region Usage. FACS analysis for TCR Vβ usage was performed using the following mAbs: biotinylated anti-Vβ (5.1 + 5.2), anti-Vβ6, anti-Vβ(8.1 + 8.2), anti-Vβ9, anti-Vβ13 (PharMingen), and FITC-conjugated avidin (Becton Dickinson, San Jose, CA); anti-Vβ2, anti-Vβ7, anti-Vβ10, anti-Vβ11, and anti-Vβ14 from culture supernatants of the B20–6-5, TR3–10, B21–5, RR3–15, and MR14–2 hybridoma, respectively; and FITC-conjugated goat antirat serum (Southern Biotechnology, Birmingham, AL). The cells were costained with anti-CD8-PE (Sigma Chemical Co., St. Louis, MO) and the percentage of each single Vβ was calculated for CD8^+ cells only.

Cytotoxicity Assays. Blasts were tested for cytolytic activity in a standard 4-h ^{51}Cr release assay, as described previously (23). The percentage of specific ^{51}Cr release of triplicates was calculated as [(average experimental cpm − average spontaneous cpm)/(average maximum cpm − average spontaneous cpm)] × 100. In cold target inhibition assays, unlabeled inhibitor tumor cells were seeded together with labeled RMA (1000/well) cells at ratios of 3:1 to 100:1. 7-4-94 or 24-5-96 CTLs were then added at effector:hot target ratios of 5:1 and 10:1, respectively. The percentage of inhibition by cold targets was calculated as [(1 − % lysis of CTL with cold targets)/(% of lysis of CTL in the absence of cold targets)] × 100.

Results and Discussion

The CTL Response against RMA Is Dominated by Virus Ag Determinants. The surface expression of virus RNA-encoded env-gp70 proteins is an unambiguous demonstration of infection by FMR viruses. To verify whether the protective CTL response against RMA is dominated by virus Ag determinants, 7-4-94 cells were challenged in a standard cytotoxicity assay against the env-gp70^+ targets RMA and MBL-2 as well as against the immunogenic, chemically induced env-gp70^- EL-4 thymoma (EL-4^-) and its variant EL-4^+, which is known to be infected by a FMR virus (21) and is env-gp70^+ (Fig. 1*F*). 7-4-94 cells killed to a similar extent RMA, MBL-2, and EL-4^+ targets and not EL-4^-, the chemically induced H-2^d L1210 leukemia and the MHC-I negative RMA-S thymoma (Fig. 1*A*), therefore suggesting that anti-RMA CTL recognizes viral epitopes on their targets. Moreover, no lytic activity could be measured when untransformed syngeneic thymocytes, lipopolysaccharide, phytohemagglutinin, and concanavalin A blasts and a CD4^+ T cell line were used as targets (data not shown), thus ruling out the recognition of tissue-specific Ags common to the two thymomas RMA and EL-4^+.

The reasons that 7-4-94 CTLs kill both RMA and EL-4^+ cells may be that: (*a*) these two neoplasms share TAA recognized by the 7-4-94 cells or (*b*) the 7-4-94 line contains at least two populations, each one specific for one of the two thymomas. Cold target inhibition experiments were undertaken to discriminate between the two possibilities. When RMA cells were used as hot targets, the lysis was inhibited by the addition of either unlabeled RMA or EL-4^+ cells but not of unlabeled RMA-S cells (Fig. 1*D*), therefore supporting the hypothesis that RMA and EL-4^+ share TAA.

Previous reports demonstrated the absence of cross-protection between virus-induced and chemical-induced tumors. In particular, it has been reported that CTLs induced against RMA do not lyse and cross-protect against EL-4 (7, 8). Johnston *et al.* (26) and Dudley and Roopenian (27) reported that either B7-CD28 costimulation or loss of an immunodominant TAA allowed the spreading of the antitumor CTL response specific for subdominant epitopes, which may be expressed by tumors of different histotypes as well as by untransformed tissues. Because anti-RMA 7-4-94 cells were induced by stimulation with B7.1^+ RMA cells, we wanted to verify whether in our case as well B7.1 costimulation determined the spreading of the antitumor CTL response (26) against subdominant TAA shared by the two thymomas. Hence, an anti-RMA CTL response was induced by *in vivo* immunization and *in vitro* restimulation of spleen cells with wild-type RMA cells. A lytic activity was obtained with characteristics of specificity and MHC-I restriction comparable to the one exerted by the 7-4-94 line (data not shown). Also, in this case, anti-RMA CTLs equally cross-reacted against EL-4^+ cells and not against EL-4^- cells (data not shown).

To verify whether CTLs directed against EL-4^+ also kill RMA cells, a CTL line (named 24-5-96) was induced by *in vivo* immunization and *in vitro* restimulation with EL-4^+ cells. 24-5-96 CTLs killed both EL-4^+ and RMA cells and not EL-4^- or L1210 targets (Fig. 1*B*). In cold target inhibition experiments, we further proved that this cross-reactivity is due to the presence in the anti-EL-4^+ line of a population of CTLs specific for TAA shared by RMA and EL-4^+ (Fig. 1*E*).

To further sustain the hypothesis that the CTL response against RMA is dominated by virus Ag determinants, we took advantage of the CHM-14 CTL clone, which has been induced in H-2^b mice by injection with Moloney virus (11). The CHM-14 CTL clone specifically kills the Moloney virus-induced MBL-2 cells and cross-reacts with FMR virus-induced tumors (11). As expected, the CHM-14 clone lysed not only MBL-2 and RMA targets but also EL-4^+ and not EL-4^- or L1210 cells (Fig. 1*C*).

Vβ TCR Repertoire of Anti-RMA CTL. The identification of TCR involved in the anti-RMA CTL response is essential to the characterization of the molecular interactions between CTLs and tumor cells and may have direct applications in antitumor immunotherapy (*i.e., in vivo* activation of tumor-specific CTLs bearing a

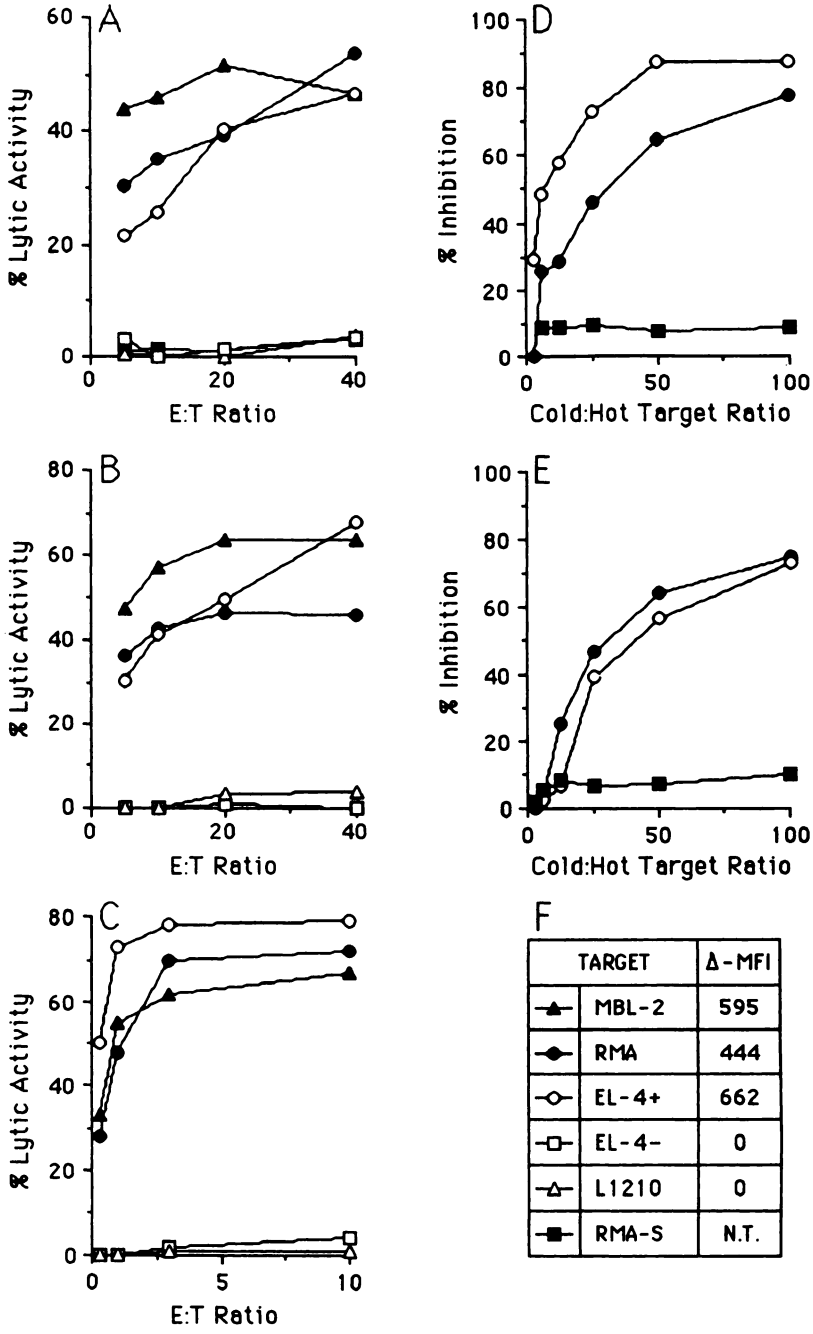

Fig. 1. CTLs induced against RMA and EL-4$^+$ cross-react against EL-4$^+$ and RMA and recognize Ag determinants shared by the two thymomas. 7-4-94 anti-RMA CTLs, 24-5-96 anti-EL-4$^+$ CTLs, and anti-Moloney virus clone CHM-14 were tested for their specific cytolytic activity against a panel of env-gp70$^+$ and env-gp70$^-$ targets in a standard 4-h ^{51}Cr release assay. 7-4-94 (*A*), 24-5-96 (*B*), and CHM-14 (*C*) CTLs efficiently lysed the env-gp70$^+$ RMA (●), MBL-2 (▲), and EL-4$^+$ (○) cells and not EL-4$^-$ (□) and L1210 (△) targets. Values are expressed as percentage of specific ^{51}Cr release at E:T ratios ranging from 40:1 to 0.6:1. Decreasing numbers of cold RMA (●), EL-4$^+$ ○), and RMA-S (■) were admixed to hot RMA cells (at cold:hot target ratios ranging from 100:1 to 3:1) and challenged in a standard 4-h ^{51}Cr release assay against 7-4-94 (*D*) or 24-5-96 (*E*) CTLs. Values are expressed as percentage of inhibition of the lysis of RMA at effector:hot target ratios of 5:1 and 10:1, respectively. In *F*, the results of surface expression of the env-gp70 molecules on the targets used are reported, as obtained by FACS analysis, using an anti-gp70 mAb and a FITC-conjugated antimouse second-step reagent. The Δ − MFI was calculated as the difference in MFI between sample and negative control and is expressed in linear arbitrary units. *N.T.*, not tested. See "Materials and Methods" for experimental details.

defined Vβ-TCR by specific mAbs). To this aim, we studied the Vβ TCR repertoire of the 7-4-94 line by immunofluorescence with a panel of anti-Vβ TCR mAbs. As expected for a T-cell line propagated in culture for more than 2 years, 7-4-94 cells homogeneously expressed a single Vβ family, *i.e.*, the Vβ(5.1 + 5.2) (Table 1), therefore demonstrating the restricted *TCR Vβ* gene usage of the CTLs.

The expression of the Vβ TCR was evaluated also for the anti-EL-4$^+$ CTL line 24-5-96. Immunofluorescence staining was performed after the second, sixth, and eighth *in vitro* restimulation (Table 1). Vβ(5.1 + 5.2)$^+$ CD8$^+$ cells were selected by repetitive contact with the Ag, and after eight restimulations (approximately 12 weeks of culture), only Vβ5$^+$ CD8$^+$ cells constituted the 24-5-96 line. It is very likely that priming with RMA or RMA/B7.1 cells activates a CTL response that is not only Vβ5 restricted. However, our results demonstrate that the immunodominant Ag determinant(s) strongly biases the TCR repertoire toward the *Vβ5* gene usage.

C57BL/6 Mice Vaccinated with RMA or EL-4$^+$ Cells Are Protected against a Challenge with the Reciprocal Lymphoma. To verify whether the TAAs shared by RMA and EL-4$^+$ cells were recognized also *in vivo*, C57BL/6 mice were injected s.c. in the right flank with nonreplicating RMA or EL-4$^+$ cells. Two weeks later, the mice were challenged in the opposite flank with RMA cells. All animals vaccinated with RMA cells or EL-4$^+$ cells were protected from tumor growth, whereas all control mice injected with PBS and challenged with RMA cells developed tumors within 10 days (Table 2). We also performed the reciprocal experiments, vaccinating with either EL-4$^+$ or RMA cells and challenging the animals with EL-4$^+$ cells. However, protection could not be evaluated, because EL-4$^+$ tumors, once they appeared, spontaneously regressed also in the nonvaccinated control animals (data not shown). On the other hand, vaccination with nonreplicating EL-4$^-$ or RMA cells did not protect against a challenge with the reciprocal thymoma (Table 2). To defin-

itively rule out the possibility of shared TAA between RMA and EL-4$^-$ cells, we also performed protection experiments with nonreplicating RMA/B7.1$^+$ cells as vaccine. Mice vaccinated with RMA/B7.1 cells were not protected against a challenge with EL-4$^-$ cells (Table 2). Therefore, even under more permissive conditions, *i.e.*, immunization with tumor cells expressing the B7.1 molecule (26), a sharing of Ag determinants between RMA and EL-4$^-$ thymomas could not be unveiled.

The phenomenon of spontaneous regression of EL-4$^+$ tumors is reminiscent of Moloney retrovirus-induced tumors (28). The fact that EL-4$^-$ tumors, although immunogenic, do not regress spontaneously (17, 20) is worth of note, because it demonstrates that also in immunogenic tumors, *i.e.*, tumors able to elicit a strong CTL response *in vivo* (17), virus infection drastically modifies not only the hierarchy of Ag determinant recognized by CTLs but also the tumorigenicity of the neoplasm.

Concluding Remarks. The results herein reported may allow us to draw the following conclusions. The CD3$^+$ CD8$^+$ CTL response that characterizes the anti-RMA immune response both *in vivo* and *in vitro* is dominated by the trimolecular complex D^b-Rauscher virus Ag determinant(s)-Vβ5$^+$ TCR. The data strongly support also the hypothesis that the high immunogenicity of virus-induced or infected tumors is determined by the expression of virus-encoded Ag.

Vaccination with nonreplicating RMA or EL-4$^-$ thymomas did not protect against a challenge with EL-4$^-$ or RMA cells, respectively. Even the B7.1 costimulatory molecule did not unveil subdominant epitopes, which, in other mouse models, have been demonstrated to be shared by tumors of different lineages (26, 27). The appearance of a viral Ag in EL-4 cells elicited a full cross-reactivity and cross-protection between EL-4$^+$ and RMA and transformed the former into such a highly immunogenic neoplasm to be spontaneously rejected, even in the absence of any modification in the costimulatory molecule repertoire expressed by the tumor cells. The demonstration of such a different outcome in the immune responses elicited in the presence or in the absence of viral Ag further opens the contention of the molecular requirements for immunogenicity and should stimulate a more careful revision of unexpected cross-reactivity among tumors.

All together, these results seem to point to a "qualitative" difference in the first Ag signal delivered by the tumor cell that apparently determines its *in vivo* immunogenicity: the ability to trigger an effective immune response in the absence of any "danger" signal (29) was strictly related to the presentation of exogenous viral epitopes. This bears important consequences, because most spontaneous human tumors do not express "strong" viral Ag and may not physiologically provide Ag determinants in association with danger signals to ensure the development of a tumor-specific immune response.

Table 1 *Vβ5$^+$ TCR skew toward RMA/EL-4$^+$ recognition*

MAb[a]	7-4-94 CTL line[b]	24-5-96 CTL line[c] II[d]	VI[d]	VIII[d]
Vβ2	<1	<1	<1	NT
Vβ5	100	21	44	100
Vβ6	<1	9	2	NT
Vβ7	<1	2	2	NT
Vβ8	<1	18	13	<1
Vβ9	<1	<1	6	NT
Vβ10	<1	3	2	NT
Vβ11	<1	16	<1	NT
Vβ13	<1	7	12	<1
Vβ14	<1	<1	<1	NT

[a] The cells were costained with anti-CD8 and anti-Vβ TCR mAbs of different specificities and analyzed by FACS. The percentage of each single Vβ was calculated for CD8$^+$ cells only. Values represent the percentage of positive cells.

[b] Anti-RMA CTLs were obtained by repetitive *in vitro* stimulations of *in vivo* sensitized spleen cells with B7.1$^+$ RMA cells. FACS analysis for Vβ TCR expression was performed after at least 1 year of *in vitro* culture. At that time, all the cells were CD8$^+$.

[c] Anti-EL-4$^+$ CTLs were obtained by repetitive *in vitro* stimulations of *in vivo* sensitized spleen cells with EL-4$^+$ cells and irradiated syngeneic spleen cells as APC.

[d] FACS analysis for Vβ TCR expression was performed after the second, sixth and eight *in vitro* restimulations, as indicated. After the sixth *in vitro* stimulation, all the cells were CD8$^+$. NT, not tested.

Table 2 *Cross-protection in vivo*

Tumor vaccine[a]	Tumor challenge[b]	Tumor take/challenged mice[c]	Appearance of the tumor (Days)[d]
PBS	RMA	5/5 (0)	10.5 ± 1.20
RMA	RMA	0/5 (100)	
EL-4$^+$	RMA	0/9 (100)	
PBS	EL-4$^-$	5/5 (0)	9.4 ± 2.50
EL-4$^-$	RMA	5/5 (0)	11.4 ± 1.30
RMA	EL-4$^-$	5/5 (0)	12.4 ± 1.34
RMA/B7.1	EL-4$^-$	5/5 (0)	11.4 ± 1.94

[a] Mice were injected in the right flank with PBS or 1×10^6 mit-c-treated tumor cells as indicated.

[b] Two weeks later, mice were challenged in the left flank with 1×10^4 RMA or 5×10^5 EL-4$^-$ live cells. Tumor size was evaluated by measuring two perpendicular diameters with a caliper twice a week. Animals were scored positive when the mean diameter was >2 mm.

[c] The percentage of protected mice is reported in parentheses.

[d] Numbers indicate the arithmetic average ± SD of the time of appearance of the tumor.

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