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A Large Number of T Lymphocytes Recognize Moloney-Murine Leukemia Virus-Induced Antigens, but a Few Mediate Long-Lasting Tumor Immunosurveillance

Antonella Facchinetti,* Silvia Dalla Santa,* Silvio Mezzalira,* Antonio Rosato,* and Giovanni Biasi2†

The CD8+ T cell response to Moloney-murine leukemia virus (M-MuLV)-induced Ags is almost entirely dominated by the exclusive expansion of lymphocytes that use preferential TCRVβ chain rearrangements. In mice lacking T cells expressing these TCRVβ, we demonstrate that alternative TCRVβ can substitute for the lack of the dominant TCRVβ in the H-2-restricted M-MuLV Ag recognition. We show that, at least for the H-2d-restricted response, the shift of TCR usage is not related to a variation of the immunomodinant M-MuLV epitope recognition. After virus immunization, all the potentially M-MuLV-reactive lymphocytes are primed, but only the deletion of dominant Vβ rescues the alternative Vβ response. The mechanism of clonal T cell “immunodomination” that guides the preferential Vβ expansion is likely the result of a proliferative advantage of T cells expressing dominant Vβ, due to differences in TCR affinity and/or cosignal requirements. In this regard, a CD8 involvement is strictly required for the virus-specific cytotoxic activity of CTL expressing alternative, but not dominant, Vβ gene rearrangements. The ability of T cells expressing alternative TCRVβ rearrangements to mediate tumor protection was evaluated by a challenge with M-MuLV tumor cells. Although T cells expressing alternative Vβ chains were activated and expanded, they were not able to control tumor growth in a long-lasting manner due to their incapacity of conversion and accumulation in the T central memory pool. The Journal of Immunology, 2005, 174: 5398–5406.

Pathogen infection is followed by the expansion of activated CD8+ CTLs that recognize antigenic peptides bound to self-MHC class I molecules. Single cell assays of MHC/peptide tetramer staining or of peptide-induced cytokine release have also shown that the response to virus infection is much greater than previously estimated and may involve a large proportion of peripheral CD8+ T cells (1, 2).

Although a large number of virus-specific CD8 T cells are detectable during both primary and secondary infection, only few virus gene products are recognized in an MHC-restricted fashion (3, 4). Furthermore, T cell responses often appear limited to a single encoded immunodominant peptide which generally stimulates a large number of specific CTL, while subdominant epitopes elicit T cell response that do not offer a high protection level against pathogens (5, 6). Clonotypic analysis of CD8 T cell response in many cases provides evidence that immunodominant determinants are recognized by a restricted T cell repertoire that uses a limited number of TCRVβ and/or Vα domains (7–11).

Immunodominance and TCRVαβ restriction seem to represent a general characteristic of T cell responses and have been previously reported for the CD4 T cell response to complex proteins (12, 13). However, the MHC class II-restricted response remains in the absence of the dominant TCRVβ elements because a shift of TCRVβ usage permits maintenance of the fine specificity pattern of response (14–16). TCR flexibility seems to occur also in the CD8+ T cell response to Moloney-murine leukemia virus (M-MuLV)1-induced Ags; while C57BL/6 (B6) mice preferentially use TCRVβ5 expressing T cells to reject M-MuLV-infected tumor cells (17), congenic B6.Vβ8 mice, in which the dominant Vβ chain is not expressed, use alternative Vβ chains to recognize the same immunodominant epitope (18). However, previous observations speak against a class I-restricted TCR repertoire flexibility because the elimination of T cells bearing the specific Vβ region for virus- or chemically induced tumor Ag(s), through endogenous superantigen expression, abrogates tumor immunosurveillance (19, 20).

The present study investigates the flexibility of MHC class I-restricted TCR usage after injection of Moloney-murine sarcoma virus (M-MSV)/M-MuLV complex (M-MSV/MuLV) in mice in which the preferential Vβ responses were either naturally or intentionally abrogated. The injection of transforming M-MSV and its natural MuLV helper gives rise to sarcomas that rapidly undergo spontaneous regression within a few days, mostly due to CD8 response (21). Different MHC class II and I Ag presentation pathways are used in generating virus-specific CTL (22), and a preferential use of specific Vβ chain rearrangements in the H-2d- and H-2k-restricted CD8 recognition of M-MuLV-infected Ags has been shown (17). To date, only peptides recognized in the H-2d context have been identified: the immunodominant CCLCLTVFL epitope shared by gag-encoded protein of Friend/Moloney/Rauscher

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3 Abbreviations used in this paper: M-MuLV, Moloney-murine leukemia virus; M-MSV, Moloney-murine sarcoma virus; MLTC, mixed leukocyte tumor cell culture; MLPC, mixed leukocyte peptide culture; TIL, tumor-infiltrating lymphocyte; LUM, Lytic unit; FMR, Friend/Moloney/Rauscher; Tc50, T central memory.
(FMR) retrovirus group (23), recognized by CD8<sup>+</sup>Vβ<sup>+</sup>Vα3.2<sup>+</sup> (17), and a subdominant en-encoded SSWSFITV epitope (24) that is less efficient in inducing a protective immune response against M-MuLV tumor cell challenge (25).

The results presented in this paper show that in recognizing M-MuLV-induced tumor Ags, alternative TCRVβ chain usage can hierarchically substitute for the lacking dominant TCRVβ elements. Moreover, we observed that H-<sup>D</sup>D<sup>-</sup>-restricted virus-specific CTL, which express dominant Vβ3 or any alternative Vβ chain, recognize the same immunodominant gag-encoded epitope. However, while CD8<sup>+</sup>Vβ<sup>+</sup> effector cells confer full protection against a M-MuLV leukemic tumor cell challenge, the alternative Vβ usage cannot supply in vivo the same long-lasting functional activity.

Materials and Methods

Mice

BALB/c and C57BL/6 (B6) mice originally obtained from Harlan Italy were bred in our specific pathogen-free facility for one to two generations and then crossed to obtain (BALB/c × B6)F<sub>1</sub> hybrids. Procedures involving animals and their care conformed to institutional guidelines that comply with all national and international regulatory laws and policies.

Virus-immune and carrier mice

Adult (7- to 8-week-old) mice were inoculated i.m. in the thigh region with 0.1 ml of a cell-free preparation of M-MSV/MuLV complex that had an in vitro M-MSV titer of 1.2 × 10<sup>5</sup> focus-forming U/ml on ST3/FL cells, and M-MuLV titer of 2 × 10<sup>6</sup> PFU/ml on SC-1 XC cells. These mice developed sarcomas at the site of injection that, in all instances, regressed within 14 days and were thus used as immune mice. To obtain virus carriers, mice were injected s.c., within 48 h after birth, with 0.05 ml of 0.1 gEq of a primary leukemia cell-free M-MuLV extract which had a titer of 5 × 10<sup>6</sup> PFU/ml on SC-1 XC cells. When these mice were 8–10 wk old, they served as virus carrier spleen cell donors.

Tumor cell lines

M-MuLV-infected MBL-2 (H-<sup>D</sup>d), LSTRA (H-<sup>D</sup>d), A6/ATL (H-<sup>D</sup>d) (26), and chemically induced thymoma EL-4 (H-<sup>D</sup>d) cell lines were maintained in vitro or by in vivo i.p. passage in syngeneic recipients. Experimental groups of mice received 2 × 10<sup>6</sup> MBL-2 or 1 × 10<sup>6</sup> LSTRA viable leukemic cells s.c. in the flank region and were then examined daily for any sign of tumor growth. Previous experiments showed this minimal cell dose induced tumor growth in >95% of naive mice (data not shown). Tumor-bearing mice were sacrificed within 2 wk from tumor onset.

In vivo mAb treatments

BALB/c mice were depleted of lymphocytes expressing specific TCRVβ (>95% cell depletion as evidenced by indirect immunofluorescence PBL staining) by i.p. injection of 0.5 mg of anti-Vβ4 (KT4) and anti-Vβ8 (F23.1) 3 and 2 days before, and then weekly, after virus immunization, until sacrifice. Only relative increases in the percentages of remaining TCRVβ families, to compensate the specific Vβ deletion, were detected by direct PBL flow cytometry analysis.

Cell cultures and CTL lines

Virus-specific CTL were generated in vitro in a 5-day mixed leukocyte tumor cell culture (MLTC) (27) or mixed leukocyte peptide culture (MLPC) (25). Usually, 25 × 10<sup>6</sup> responder spleen or 20–25 × 10<sup>6</sup> lymph node cells from virus-immune mice and 5 × 10<sup>6</sup> irradiated leukemic (MBL-2/LSTRA) or 2 × 10<sup>6</sup> stimulator spleen cells from carrier mice were cocultured in MLTC in 15 ml of DMEM (Invitrogen Life Technologies) supplemented with 2 mM l-glutamine, 10 mM HEPES, 20 mM 2-ME, antibiotics, and 10% heat-inactivated FBS (Invitrogen Life Technologies). In the MLPC, responder cells were stimulated in the presence of the peptide corresponding to aa 85–93 of the FMR gag-encoded protein (CCLCLTVFL) at a final concentration of 1 μM.

In some experiments, responder B6 spleen cells were partially depleted of Vβ<sup>+</sup> T cells (>95% as evidenced by flow cytometry analysis) by panning on anti-mouse plus anti-Vβ5 (MR9-4) mAb precoated petri dishes (28) before culture. Cells recovered from mixed cultures were washed and maintained for an additional 3 days in culture (2 × 10<sup>6</sup> cells/ml) in complete medium supplemented with 10 U/ml rIL-2 (a generous gift of Sanoz) to allow for flow cytometry analysis of TCRVβ expression (17). In some experiments, spleen cells from virus-immune mice were stimulated repeatedly for multiple cycles (MLTC-n) in the presence of syngeneic irradiated leukemic cells and spleen cells as a source of APC in 10 U/ml rHL-2-supplemented complete medium.

Cytotoxic assays

Cytotoxic activity of CTL generated in MLTC was determined by a 4-h incubation in <sup>51</sup>Cr release assay in which 2 × 10<sup>5</sup> labeled target cells and different numbers of effector cells were plated in 96-well microplates. Target cells were either M-MuLV infected LSTRA (H-<sup>D</sup>d), MBL-2-H-<sup>D</sup>d, A6/ATL (H-<sup>D</sup>d), or EL-4 (FMR-uninfected H-<sup>D</sup>b thymoma) cells. Cytotoxic activity was expressed as the percentage of specific lysis or in terms of lytic units (LU) where 1 LU was the number of cells producing 30% specific lysis of 5 × 10<sup>5</sup> targets (27).

For peptide pulsing, 10<sup>5</sup> Cr-labeled EL-4 cells per milliliter were incubated for 30 min at 37°C with 10 μM final concentration of the FMR gag (CCLCLTVFL)- or env (SSWSFITV)-encoded peptide (25) and then washed three times before use. In some experiments, effector cells were preincubated for 20 min at 37°C in microplates with variable concentrations of anti-CD8 (53-6.72) mAb or with an anti-K<sup>+</sup>, K<sup>+</sup> cross-reacting (20-8-4s) mAb (10 μg/ml) before target cells addition for Ab blocking. Finally, to evaluate the peptide/MHC requirements of effector cells, EL-4 target cells were pulsed with variable concentrations of CCLCLTVFL peptide in the presence or absence of blocking anti-CD8 mAb (10 μg/ml).

Flow cytometry

Cells recovered from in vitro cultures were double stained with anti-CD8 PE-conjugated (CT-CD8A; Caltag Laboratories) and a panel of anti-Vβ FITC-conjugated mAbs including Vβ2 (B20.6), Vβ4 (KT4), Vβ5 (MR9-4), Vβ6 (RR4-7), Vβ7 (TR310), Vβ8 (1B3.3), Vβ9 (MR10-2), Vβ10 (B21.5), Vβ13 (MR12-3), Vβ14 (14-2) (BD Pharmingen), and Vβ12.1 (KJ16; Caltag Laboratories). In some experiments, 50-μl aliquots of PBL from retro-orbital sinus, collected from virus-immune mice after viable MBL-2 leukemic cell challenge, were triple stained with anti-CD8 PE, anti-Vβ FITC, and biotin-conjugated anti-CD62L (Mel-14; BD Pharmingen) mAbs, followed by streptavidin SpectraRed (SPRD)-conjugated (Southern Biotechnology Associates). The erythrocytes were lysed by incubation with isotonic ammonium chloride buffer (155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA). To examine TCRVβ expression in CD8<sup>+</sup>-gated tumor-infiltrating lymphocytes (TIL), the MBL-2 tumor mass was removed and forced through a multilayer nylon sieve to obtain cell suspension before labeling. All the samples were gated on viable cells on the basis of forward and side scatter parameters and run on EPICS ELITE (Coulter) or FACSCalibur (BD Biosciences).

Results

Identification of the CD3TCRVβ repertoire specific for the H-<sup>D</sup>d-restricted anti-M-MuLV immune response

In the immune response to M-MuLV-induced Ags, BALB/c (H-<sup>D</sup>d) mice preferentially use CTL expressing the TCRVβ4 region as shown in vivo by PBL analysis and, more markedly, in vitro after secondary stimulation of their spleen cells in MLTC (17). Cytometric analysis of the TCRVβ repertoire expressed by the CD8<sup>+</sup> cells using a panel of anti-Vβ mAbs revealed an increase in the TCRVβ4 cell percentage (Vβ<sub>4</sub> CD8<sup>+</sup>/CD8<sup>+</sup> = 35.1–39.8%) in five MLTC as compared with the baseline levels in immune (13.2 ± 1.3 SD) and in six naive spleen cells (8.0 ± 0.3 SD).

To investigate whether the expansion of Vβ4<sup>+</sup> T cells can mask the involvement of other Vβ families in the M-MuLV-specific H-<sup>D</sup>d-restricted response, BALB/c mice were treated with a Vβ4<sup>+</sup> cell-depleting mAb before virus priming. These mice, like mAb-untreated control mice, developed sarcomas that regressed in a few days, suggesting the involvement of Vβ families different from Vβ4. To identify the vicarious Vβ specificity used by the virus-specific effectors, spleen cells from anti-Vβ4 mAb treated mice were used as responders in syngeneic MLTC 2 wk after M-MSV/MuLV infection. The 51Cr release assay performed against syngeneic leukemic target cells (LSTRA) evidenced the generation of a strong cytotoxic activity (Fig. 1A) although lower than that generated in control cultures conducted with spleen cells from mAb-untreated immune mice (149 ± 82 vs 286 ± 91 LU/culture,
mean ± SD in 12 MLTC). The cytotoxic activity was virus-specific and H-2-restricted, as CTLs were unable to kill either BALB/c Con A blast spleen cells, antigenically unrelated L1210 (H-2d) or the H-2b M-MuLV-induced MBL-2 leukemic target cells (data not shown). Cytometric analysis of the TCRVβ repertoire in five MLTC always revealed a substantial increase in the Vβ8.3 cell percentage (Vβ8.3+/CD8+ = 23–32% from 6.8 ± 0.7% SD of immune spleen cells) with an additional increase in the Vβ 6% (16%) in only one MLTC. Representative results of 51Cr release assay (Fig 1A) and Vβ expression (Fig. 1B) of above described experiments are reported in Fig. 1.

Additionally, further Vβ families appear to participate in the H-2d-restricted M-MuLV recognition. BALB/c mice regressed M-MSV/MuLV-induced tumors even when depleted by mAb treatment of both Vβ4 and Vβ8+ cell subsets, and their spleen cells, as demonstrated by the representative results reported in Fig. 1, generated in vitro virus-specific CTL capable of killing LSTRA (151 ± 74 LU/culture in five MLTC) but not BALB/c Con A blast nor L1210 target cells (data not shown). Analysis of TCRVβ expression in CD8+ cells (results of one of five experiments reported in Fig. 1) revealed a selective increase in the Vβ6+ (Vβ6+/CD8+/CD8+ = 30–51% from 12.9 ± 0.6% SD of immune spleen cells) in three MLTC, in the Vβ13+ (52%) of another, and in the Vβ6 (26%), Vβ13 (19%), and Vβ9 (16%) percentages of the last. On the whole, the fact that different Vβ can compensate for the lack of the Vβ4+ cells indicates the existence of a hierarchy of TCRVβ usage in the H-2d-restricted response to M-MuLV.

Even though the M-MuLV dominant epitope recognized by Vβ4+CD8+ cells in H-2d mice remains unknown, it has long been established that the CTL response in these mice is restricted by Kd class I molecules (29). Therefore, we studied whether the effectors that emerge after mAb treatments use the same H-2d class I restriction molecule for viral epitope/s recognition. As shown in Table I, regardless of Vβ expression, all CTL generated in MLTC (following one or multiple cycles of stimulation with LSTRA) recognized M-MuLV-induced Ags associated with the Kd molecule, because they were neither able to lyse the M-MuLV induced A6.ATL (Kd+) leukemic cell line nor recognize LSTRA leukemic cells in the presence of anti-Kd cross-reacting (20-8-4s) mAb.

Identification of the CD8+TCRVβ repertoire specific for the H-2d-restricted anti-M-MuLV immune response

Previous studies have shown the preferential usage of the TCRVβ rearrangements in the H-2d-restricted response of B6 mice to M-MuLV-induced Ags (17). To investigate the possible involvement of different TCRVβ families in the H-2d-restricted virus recognition, we used (BALB/c x B6)F1 (H-2d/b) mice in which the BALB/c background leads to the clonal deletion of Vβ5 T cells due to Mls Ag tolerance. Two weeks after M-MSV/MuLV injection, spleen cells were stimulated in different MLTC with parental H-2d (LSTRA) or H-2b (MBL-2) leukemic cells, or spleen cells from H-2b syngeneic M-MuLV carrier mice, or a mixture of LSTRA and MBL-2.

Table I. Kd-restricted recognition of M-MuLV-induced Ags by anti-Vβ mAb-treated BALB/c mice

<table>
<thead>
<tr>
<th>Effector Cells from mAb-Treated Mice</th>
<th>Predominant Vβ</th>
<th>Percentage of Specific 51Cr Release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LSTRA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E:T cell ratio</td>
</tr>
<tr>
<td>Anti-Vβ4 mAb</td>
<td></td>
<td>20:1</td>
</tr>
<tr>
<td>MLTC-3</td>
<td>8.3</td>
<td>65</td>
</tr>
<tr>
<td>T cell line</td>
<td>8.3</td>
<td>74</td>
</tr>
<tr>
<td>Anti-Vβ4-Vβ8 mAb</td>
<td></td>
<td>6-13-9</td>
</tr>
<tr>
<td>MLTC-1</td>
<td>6</td>
<td>46</td>
</tr>
<tr>
<td>MLTC-2</td>
<td></td>
<td>20:1</td>
</tr>
</tbody>
</table>

1 Spleen cells from M-MuLV immune mice depleted of cells expressing specific TCRVβ by mAb treatments were stimulated in syngeneic MLTC for 1–3 cycles. Vβ8.3+ T cell line (96% Vβ8.3+ among CD8+) was derived after multiple cycles of MLTC with irradiated LSTRA plus syngeneic spleen cells as a source of APC. Effector cells were used in 51Cr release assay at different E:T cell ratios.

2 The Vβ percentages among CD8+ cells were: MLTC-3 = 59% Vβ8.3; MLTC-1 = 26% Vβ6—19% Vβ13—16% Vβ9; MLTC-2 = 37% Vβ6.

3 Anti-Kd (20-8-4s) mAb was added at 10 µg/ml final concentration in culture during 51Cr release assay.
As shown in the results of one representative experiment reported in Table II, the (BALB/c × B6)F1 effectors generated in response to parental H-2\(^d\) M-MuLV leukemic cells exerted a strong H-2-restricted cytotoxic activity against LSTRA target cells. As expected, analysis of the CD8Vβ repertoire disclosed that CD8\(^{+}\) Vβ4\(^{+}\) cells were overrepresented in these cultures (data not shown). Furthermore, high H-2\(^d\)-restricted CTL generation was seen following stimulation in MLTC with syngeneic M-MuLV infected H-2\(^{ab}\) cells (Table II, line 2; 188 ± 82 SD LU/culture in nine MLTCs). In contrast, a low H-2\(^b\)-restricted cytotoxic activity (18 ± 14 SD LU/culture) was generated in the same MLTCs in response to M-MuLV-infected H-2\(^{ab}\) target cells. However, the same spleen cells could recognize the M-MuLV-induced Ags in the H-2\(^b\) context because they were able to generate H-2\(^b\)-restricted effectors (126 ± 83 LU/culture in nine MLTCs) when stimulated in MLTC by MBL-2 cells (Table II, line 2). Moreover, in four different experiments, a substantial H-2\(^d\) (119 ± 50 SD)-plus H-2\(^b\)-restricted (116 ± 51 SD) response was detected in response to a mixture of LSTRA and MBL-2 leukemic cells (Table II, line 4).

To investigate the TCRVβ usage of Vβ\(^{5+}\) (BALB/c × B6)F1 mice for MBL-2 recognition, we screened the CD8\(^{+}\) TCR repertoire in 26 of 35 MLTCs in which virus-specific CTL generation was evaluated by \(^{51}\)Cr release assay. Flow cytometry analysis disclosed substantial increases in the Vβ7\(^{+}\) among CD8\(^{+}\) cells in six MLTC (Vβ\(^7\) CD8\(^{+}\)/CD8\(^{+}\) = 12–44% from 7.0 ± 0.3% SD of immune spleen cells), the Vβ8.1,2 (28–41% from 24.8 ± 1.8) in eight, and the Vβ13 (13–42% from 5.1 ± 0.4) in four. In five other MLTC, a simultaneous increase in two or three of these families was detected, while no variations over the mean values ± 2SD of Vβ\(^{5+}\) T cells among CD8 spleen cells of nonimmune mice (Vβ\(^7\) = 6.9 ± 0.3, Vβ8.1,2 = 25.1 ± 0.5, Vβ13 = 4.9 ± 0.3 mean ± SD in five mice) were detectable in the three remaining MLTC. No important variation of the residual undeleted Vβ\(^{5+}\) was observed among CD8 cells after stimulation in MLTC. These results evidence a flexibility in TCR usage for the H-2\(^b\)-restricted anti-M-MuLV response that seems to compensate for the natural deletion of the dominant Vβ7 family.

Previous studies in B6 mice have show that M-MuLV-specific H-2\(^b\)-restricted CD8\(^{+}\)Vβ\(^{5+}\) cells recognize the gag-encoded peptide CCLCLTVFL (23). We then evaluated whether (BALB/c × B6)F1 effector cells recovered from MLTC after repeated cycles of restimulation with MBL-2 leukemic cells were able to recognize the same gag-encoded peptide. The results of one representative experiment show that CTL from three different MLTC (Fig. 2), in which the respective increase of either Vβ7, Vβ8.1,2, or Vβ13 among CD8\(^{+}\) cells are reported, were indifferently able to specifically lyse EL-4 lymphoma cells pulsed with the CCLCLTVFL peptide. In addition, analysis during consecutive MLTC restimulation showed that cytotoxic activity expressed in terms of LU/ recovered cells paralleled the increase in the percent of Vβ bias, and in one MLTC-2, selected for a simultaneous increase of Vβ7, 8.1,2, and 13 CD8\(^{+}\) subsets, these effectors almost exclusively mediated the lytic activity. In fact, the depletion by cytometric cell sorting of these Vβ subsets, but not of a unrelated Vβ5, 6, 8, 14 expressing counterparts, produced a quite complete loss of specific cytotoxic activity measured at a E:T cell ratio of 60:1 against either MBL-2 or EL-4 gag-peptide pulsed target cells (data not shown).

Finally, the preferential increase of either Vβ7, Vβ8.1,2, or Vβ13 among CD8 cells was also detected when spleen cells from (BALB/c × B6)F1 virus-immune mice were stimulated in MLPC with CCLCLTVFL peptide (data not shown). These findings indicate that the same immunodominant epitope is recognized by Vβ7\(^{+}\) T cells and T cells expressing alternative Vβ.

**Immunodominance of the TCRVβ repertoire in the anti-M-MuLV immune response**

Further studies were addressed to determine why the alternative M-MuLV-specific TCRVβ effectors do not expand, either in vivo or in vitro, in the presence of the main TCRVβ responses. We reasoned that, at least for the H-2\(^b\)-restricted response, the predominance of one Vβ cannot be due to differences in the immunogenicity of the recognized epitope, nor likely due to the Vβ pool size of M-MuLV-specific CTL precursors, because mice lacking the main Vβ are competent in generating a substantial cytotoxic T cell response in MLTC.

![Figure 2](http://www.jimmunol.org/)

**TABLE II.** H-2-restricted M-MuLV-specific CTL generation by (BALB/c × B6)F1 mice

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>Percentage of Specific (^{51})Cr Release</th>
<th>MBL-2 (H-2(^{b}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E:T cell ratio</td>
<td>E:T cell ratio</td>
</tr>
<tr>
<td></td>
<td>60:1</td>
<td>20:1</td>
</tr>
<tr>
<td>LSTRA (H-2(^d))</td>
<td>69</td>
<td>54</td>
</tr>
<tr>
<td>MBL-2</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>H-2(^{ab}) M-MuLV+</td>
<td>58</td>
<td>43</td>
</tr>
<tr>
<td>LSTRA + MBL-2</td>
<td>50</td>
<td>34</td>
</tr>
</tbody>
</table>

* Splenocytes from H-2\(^{ab}\) immune mice were cultured in MLTC with parental H-2\(^{d}\) (LSTRA) or H-2\(^{b}\) (MBL-2) leukemic cells, or spleen cells from syngeneic M-MuLV carrier mice, and used as effectors in \(^{51}\)Cr release assay at different E:T cell ratios.
We first ascertained whether the priming of the alternative M-MuLV-specific TCRVβ repertoire had occurred in the presence of Vβ5+ CTL precursors. Spleen cells from immune B6 mice, after partial (>95%) TCRVβ5 cell depletion by panning on anti-mouse plus anti-Vβ5 mAbs, were stimulated in MLTC to evaluate the possible alternative M-MuLV-specific TCRVβ repertoire expansion. Flow cytometry analysis of seven different primary MLTC (MLTC-1) evidenced that among CD8+, Vβ13+ cells were enriched by about 5-fold (30%) in one, or 3-fold (23%) together with a slight increase of Vβ5 in another MLTC. In the other three MLTC, an expansion of Vβ5 alone was observed, while in the remaining two Vβ expansion was not evidenced using the mAb we used despite a moderate, virus-specific CTL generation was detected. The cells recovered from these latter two MLTC-1 were then repeatedly stimulated in vitro until a bias in Vβ13 (33%) or Vβ7 (28%) was clearly observed in MLTC-4.

The results provide evidence that a wide potentially M-MuLV-reactive repertoire was primed by in vivo sensitization. However, the fact that detectable alternative Vβ expansion is not easily reached in B6 mice, even after the elimination of Vβ5+ cells, possibly indicates that M-MuLV-specific precursors different from Vβ5 are numerically limited and/or their differentiation into the memory pool is shaped by the numerical expansion of Vβ5+ cells.

It is possible that the hierarchical emergence of Vβ usage could reflect differences in TCRVβ affinity/avidity for the MHC/Ag complex. In this regard, it was recently observed that the wide range of TCR affinities among different EBV-reactive clonotypes segregates with Vβ usage, and it was proposed that the variable contribution of the CD8 coreceptor to their functional activity may govern in vivo clonotype persistence (30). Pioneering studies reported that the virus-specific activity of CTL generated in MLTC by spleen cells from M-MSV/MuLV primed B6 mice were not significantly inhibited by anti-CD8 mAb (31); therefore, we evaluated the effect of anti-CD8 mAb on H-2b-restricted cytotoxic activity of CTL generated in MLTC by Vβ5+ (BALB/c × B6)F1 mice. The results of three experiments comparing the effect of different anti-CD8 mAb dilutions on the activity of CTL generated in MLTC by (BALB/c × B6)F1, and B6 immune mice against MBL-2 cells are reported in Fig. 3A. They show that the cytotoxic activity of effectors from Vβ5+ B6 strain was not significantly inhibited in the presence of high concentrations (10 μg/ml) of anti-CD8 mAb, while the activity from Vβ5+ (BALB/c × B6)F1 mice was reduced in a dose-dependent fashion. Furthermore, we observed a comparable inhibition of (BALB/c × B6)F1 CTL activity independently of the TCRVβ7, 8.1,2,3 expression (data not shown).

In a different approach, we compared the peptide/MHC requirements of B6 and (BALB/c × B6)F1 effectors for cytotoxicity against EL-4 target cells sensitized with decreasing doses of CCLCLTVFL peptide in the absence/presence of saturating amounts of anti-CD8 mAb. The cytotoxic response curves of representative experiments (see Fig. 3B) show that to give the half-maximal lysis the (BALB/c × B6)F1 effectors cells, in comparison to B6, ~8-fold increase of peptide dose (132 nM/16 nM) in the absence, and ~30-fold increase (2200 nM/72 nM) in the presence of saturating amounts of anti-CD8 mAb. Thus, only a low difference in the TCR complex avidity characterizes the effectors generated in bulk cultures from B6 and (BALB/c × B6)F1 spleen cells. However, blocking the CD8 coreceptor resulted in a more evident shift of the (BALB/c × B6)F1 cytotoxic response curve, clearly seen by decreasing the density of peptide/MHC complex at the surface of the EL-4 target cells. Particularly, at 1 μM peptide concentration the CD8 blocking produces ~80% decrease of cytotoxic response of (BALB/c × B6)F1 and ~5% of B6 effectors, values overlapping the inhibition of cytotoxic responses detected against MBL-2 target cells (Fig. 3A). All together these findings indicate that, in T cells expressing alternative Vβ, the CD8/MHC interaction is required for optimal CTL activity. In this light, if the different CD8 dependency between dominant and alternative Vβ indirectly estimates differences in TCR affinity, it might explain the Vβ5 overwhelming in B6 mice.

**Lack of anti-M-MuLV protection by alternative Vβ repertoire**

Other studies were undertaken to ascertain whether the alternative Vβ usage can provide in vivo a functional anti-M-MuLV immune response in (BALB/c × B6)F1 mice. The presence of alternative Vβ bias was evaluated among activated CD62L+ CD8+ cells in the PBL of mice challenged with 2 × 106 MBL-2 cells 4 wk after M-MSV/MuLV injection, as previously described to detect the dominant Vβ5 bias in B6 mice (17). The results of one representative experiment reported in Fig. 4A show the expansion of the alternative Vβ repertoire in the CD62L+ CD8+ PBL subset detected 10 days after tumor cell boosting. PBL, triple stained with mAbs against CD8, CD62L, and VβT, show a strong down-regulation of CD8 coreceptor, a common feature of Ag-specific activation of CD8+ cells in vivo, and a high bias of Vβ8.1,2, but not Vβ8.3, in the CD62L− when compared with the CD62L+ subset, immune and normal counterparts.
However, even though an expansion of the alternative Vβ repertoire was invariably detected (eight of eight mice), more than two-thirds of (BALB/c × B6)F1 virus-immune mice challenged with 2 × 10⁵ MBL-2 were unable to control MBL-2 tumor cell growth (Table III); similar results were confirmed in groups of mice receiving half or double MBL-2 cell dose (data not shown). The lack of anti-M-MuLV protection appears linked to the inefficiency of H-2b-restricted response alone, because (BALB/c × B6)F1 mice were fully competent in rejecting a challenge of 10⁶ LSTRA cells (Table III). It is noteworthy that the alternative Vβ repertoire bias among CD62L⁺ lymphocytes was still detected in PBL during tumor progression until animal sacrifice (Fig. 4B) and also into lymphocytes that infiltrate MBL-2 growing tumor (TIL) as shown by one of four representative experiments reported in Fig. 4C. In contrast, alternative Vβ bias was undetected at lymph node level different than the strong Vβ⁵ expansion observed in B6 lymph node (Fig. 4C).

To better assess the lack of anti-M-MuLV protection by the alternative Vβ repertoire in (BALB/c × B6)F1 mice, we subsequently studied the kinetics of the CD8⁺ T cell response in these mice by evaluating, at 2, 4, and 7 wk following virus injection, the ability of their spleen and lymph node cells to generate virus-specific CTL in MLTC. The results reported in Fig. 5 show that different from B6, which is capable of generating virus-specific effectors in MLTC for long time following in vivo priming, (BALB/c × B6)F1 spleen cells gradually decrease their ability in CTL generation until complete failure at 7 wk postimmunization. Therefore, following in vivo priming the dominant Vβ⁵ and alternative Vβ CTL precursors appear to differ in their persistence in the spleen, thus in their capacity to convert and accumulate in T central memory (TCM) (32) pool. Even more striking differences were detected at a lymph node level: (BALB/c × B6)F1 mice, despite the virus-specific H-2d-restricted response persistence (data not shown), were in fact unable to generate H-2b-restricted CTL in MLTC at any time after immunization (Fig. 5). Thus, the impairment in the control of MBL-2 tumor growth appears to correlate with an inefficient long-lasting virus-specific effector cell generation.

**Discussion**

This study was performed to establish whether TCRVβ gene usage can affect tumor resistance in mice which preferentially use CD8⁺

### Table III. The dominant Vβ repertoire is required for full protection against M-MuLV tumor growth

<table>
<thead>
<tr>
<th>Recipients</th>
<th>M-MSV/MuLV</th>
<th>Tumor Cell Challenge</th>
<th>Tumor Incidence</th>
<th>Tumor Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>–</td>
<td>MBL-2</td>
<td>6/6</td>
<td>8–10</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>MBL-2</td>
<td>0/12</td>
<td></td>
</tr>
<tr>
<td>(BALB/c × B6)F₁</td>
<td>–</td>
<td>MBL-2</td>
<td>8/8</td>
<td>7–12</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>MBL-2</td>
<td>23/33</td>
<td>7–13</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>LSTRA</td>
<td>4/4</td>
<td>8–11</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>LSTRA</td>
<td>0/8</td>
<td></td>
</tr>
</tbody>
</table>

⁻ Mice were challenged with 2 × 10⁵ MBL-2 or 10⁶ LSTRA s.c. 4 wk after M-MSV/MuLV injection.

⁻ Number of mice dead with tumor/total number of injected mice.

⁻ Range of tumor onset in days.
B6)F1 mice were fully capable of regressing sarcomas induced by compensating for the lack of the dominant V\textsubscript{Ags}. Thus, in both instances a shift of TCR gene usage appears to be the mean ± SD of percent of \textsuperscript{51}Cr release (four different MLTC) in two experiments.

To this end, we used mice in which the dominant V\textbeta responses were either intentionally abrogated before Ag priming by depleting mAb treatment or clonally deleted by natural tolerance. In both instances, anti-V\textbeta mAb-treated BALB/c or V\textbeta\textsuperscript{5} (BALB/c × B6)F1 mice were fully capable of regressing sarcomas induced by the injection of the M-MSV/MuLV complex. In addition, spleen cells from the same mice were able to generate a substantial H-2\textsuperscript{d}-restricted cytotoxic activity when stimulated in MLTC with LSTRA (H-2\textsuperscript{d}) or MBL-2 (H-2\textsuperscript{b}) leukemic cells, respectively. Flow cytometry analysis of CTL thus generated suggests that different V\textbeta gene segments (V\textbeta8.3 and 6 for H-2\textsuperscript{d}, and V\textbeta7, 8, and 13 for H-2\textsuperscript{b}) can be used for the recognition of M-MuLV-induced Ags. Thus, in both instances a shift of TCR gene usage appears to compensate for the lack of the dominant V\textbeta response.

However, it is important to note that tumor regression in (BALB/c × B6)F1 mice could mainly be due to the H-2\textsuperscript{d}-restricted recognition of M-MuLV-induced Ags. In fact, spleen cells from these mice give rise almost exclusively to an H-2\textsuperscript{d}-restricted CTL response, following in vitro stimulation with syngeneic M-MuLV-infected H-2\textsuperscript{d/b} cells. The low H-2\textsuperscript{b}-restricted CTL generation cannot be due to a lack of epitope recognition because a substantial CTL generation was induced in the same responder cells following MBL-2 stimulation. Moreover, suppressive effects possibly exerted by H-2\textsuperscript{d} on the H-2\textsuperscript{b}-restricted CTL differentiation can be ruled out because responses against both the parental haplotypes were elicited by the simultaneous stimulation with LSTRA and MBL-2 leukemic cells, i.e., by class I/epitope presentation on separate cells.

On the whole, the results indicate that the “immunodomination” by CTL sharing defined V\textbeta gene segments is a central feature of the response to M-MuLV-induced Ags. The term immunodomination has mostly been used to describe the inhibition of a specific immune response to a subdominant epitope during a concomitant response to a dominant epitope of the same molecule (33), thus indicating that the determinants can be ordered in a hierarchy of domination. Interestingly, for the H-2\textsuperscript{d}-restricted response to M-MuLV-induced Ags, we show that the same immunodominant gag-encoded peptide (23) is recognized by different TCR\textbeta specificities: the dominant V\textbeta and alternative V\textbeta. This situation provides a unique opportunity to demonstrate that hierarchical response is dominated by the expansion of lymphocytes that use preferential V\textbeta chain rearrangements; indeed, in both anti-M-MuLV H-2\textsuperscript{d}- and H-2\textsuperscript{b}-restricted responses, only the deletion of dominant V\textbeta rescues the nondominant V\textbeta response.

In this light, if the V\textbeta hierarchy of domination equally governs the concomitant Ag recognition on different class I molecules, then the immunodomination of V\textbeta4 (H-2\textsuperscript{d}-restricted) on the nondominant H-2\textsuperscript{d}-restricted (V\textbeta7, 8, 1.2, 13) CTL counterpart would be expected following in vitro stimulation with syngeneic M-MuLV-infected H-2\textsuperscript{d/b} cells. The H-2\textsuperscript{d}-restricted immunodomination detected in (BALB/c × B6)F1 mice parallels previous results on in vitro CTL generation against minor histocompatibility Ags showing that subdominant epitope recognition is inhibited by the response to the dominant epitope when presented on the same APC, but not when subdominant and dominant moieties are recognized on separate cells (34). Therefore, the preferential clonal V\textbeta expansion, that indifferently occurred following the recognition of epitope(s) associated with the same or different class I alloantigen(s), very likely could be explained by a phenomenon of T cell competition around a same APC (1, 35).

When questioning whether priming of the alternative V\textbeta repertoire also occurs in the presence of the V\textbeta dominant counterpart, we reasoned that the subdominant H-2\textsuperscript{b}-restricted repertoire in (BALB/c × B6)F1 mice must be sensitized by virus injection. This because, despite the presence of H-2\textsuperscript{d}-restricted dominant V\textbeta4 CTL precursors, the H-2\textsuperscript{b}-restricted CTL would not be as easily evidenced in MLTC in secondary response to MBL-2 (21). The demonstration that spleen cells from M-MSV/MuLV immune B6 mice, after depletion of the V\textbeta\textsuperscript{5} subset, were able to differentiate the virus-specific alternative V\textbeta repertoire in MLTC directly demonstrated that the priming of all the potentially M-MuLV-reactive lymphocytes had occurred. However, despite alternative V\textbeta T cell priming, the immune response to M-MuLV-induced Ags is dominated by the almost exclusive expansion of T cell clones sharing V\textbeta5 gene segments (17). These findings confirm the observations that the massive CD8\textsuperscript{+} T cell expansion during viral infection (1, 2) in many cases is accompanied by the selection of a V\textbeta restricted T cell repertoire (7–11).

It has been proposed that the emergence of a preferential V\textbeta usage in the immune response to virus-induced Ags could be a related requirement in narrowing the memory T cell pool during infectious recall (36). However, the rules and mechanisms that govern the shaping (expansion and/or narrowing) of the activated Ag-specific T cells remain to be investigated, even to a better understanding as to how and to what extent the naive and memory T cell repertoires differ (37).

The selective V\textbeta immunodomination observed in our study cannot be due exclusively to a different frequency of virus-specific CTL precursors, because a conspicuous expansion of alternative V\textbeta elements was detected, in the absence of the V\textbeta immunodominant precursors, in both in vitro and in vivo response to M-MuLV. It is, instead, more likely that the V\textbeta-driven expansion could result from a proliferative advantage due to differences in the TCR affinity for the recognized MHC/peptide complex. In agreement, by evaluating the CD8 dependency of H-2\textsuperscript{d}-restricted CTL activity, as an indirect estimate of TCR affinity, we observed that the cytotoxic activity of alternative V\textbeta\textsuperscript{5} , but not dominant V\textbeta\textsuperscript{5} CTL, against MBL-2 and EL-4 pulsed with appropriate amounts of peptide was strongly inhibited by anti-CD8 mAb. In the opposite situation when no single TCR\textbeta dominated the others as in the response to HBV, it was observed that T cell clones, expressing different TCR\textbeta, recognize the same dominant epitope with similar affinity (38).

By analyzing the functional activity of the H-2\textsuperscript{b}-restricted T cell repertoire involved in the virus-specific response, we observed that...
in (BALB/c × B6)F1 mice the alternative Vβ T cell repertoire was unable to confer full immune protection against the MBL-2 tumor cell challenge even if it was expanded, retained the activated phenotype during tumor progression and infiltrated growing tumors. Therefore, despite the evident TCR flexibility in the recognition of M-MuLV Ags, in these mice the TCR repertoire capable of counteracting tumor growth appears in great part negatively selected. This does not necessarily exclude that different genetic-encoded backgrounds favor the expression of a protective H-2a-restricted TCR flexibility, like that observed in the congenic B6.Vpr mice (18).

Our in vivo findings are in line with previous observations that activated virus-specific CD8 T cells can persist without effector function in LCMV chronically infected mice (39) and circulating CD8+ T cells specific for melanoma Ags are unable to contain tumor progression in humans (40–42).

When the ability of (BALB/c × B6)F1 mice to generate an anti-M-MuLV immune response was investigated, we evidenced in their spleen a strong decrease in the H-2a-restricted CTL generation in vitro few weeks postimmunization. In some viral systems it was suggested that functional immune response declines rapidly because T cell help, required to sustain and expand an initial Th-independent response, is not available (43). This does not apply to our study as the H-2a-restricted anti-M-MuLV response is strictly CD4-dependent in B6 (27) as well as in (BALB/c × B6)F1 mice (data not shown). The declining of CTL generation we detected in (BALB/c × B6)F1 mice is likely due to the absence of TCM that has the intrinsic capacity to proliferate following Ag recall. In this line, in the lymph node, where TCM subset persists long-term (32), the alternative TCRVβ repertoire was not expanded at any time after virus immunization. Whether TCM and effector memory cells represent separate or related lineages is still unclear (32, 44). In line with the hypothesis that TCM represents a separate lineage, the lack of CTL generation we described in (BALB/c × B6)F1 mice might simply derived from the fact that the virus-specific alternative TCRVβ repertoire represents a subset incapable to generate TCM and so, functionally different from the dominant TCRVβ subset. Conversely, whether effector memory cells and TCM are part of a continuum in a linear differentiation pathway (32), then in B6 mice the dominant TCRVβ5 repertoire would rapidly seed and convert in the lymph node as TCM in <2 wk postimmunization. Even though it is unclear as to why (BALB/c × B6)F1 mice become unresponsive, on the whole our findings indicate that a high proportion of CD8+ T cells is involved in Ag-specific recognition, but only a restricted subset is able to generate TCM and mediate long-lasting functions. Because T cells have the potential to recognize self Ags as well (45), the narrowing of the immune response, as previously suggested (6), could indeed be advantageous to limit cross-reactivity and autoimmunity. However, in some circumstances the selected T cell repertoire would be unable to recognize immunodominant peptides or, even with recognition, as in our study, may not be fully efficient in countering tumor growth.

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Disclosures

The authors have no financial conflict of interest.

References


