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Clonal dynamics of tumor-infiltrating lymphocytes

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The presence of tumor-infiltrating lymphocytes (TIL) provides important evidence of anti-tumor immunity *in vivo*. However, TIL are usually not sufficient for inhibiting tumor growth. We explored the spatial and temporal aspects of clonal accumulation of TIL using RT-PCR/single-strand conformation polymorphism analysis. In CMS5 fibrosarcomas in BALB/c mice, accumulated T cell clones were specific in that dominant TIL were identical between distant tumors. Moreover, dominant TIL in the first tumor appeared consistently in the second tumor inoculated after formation of the first tumor. These results suggest that TIL show a certain level of specific tumor surveillance. When we characterized $CD4^+$ and $CD8^+$ TIL separately, $CD8^+$ TIL were highly concentrated and persistently localized at the tumor site, while most $CD4^+$ TIL clones were less concentrated and less persistent. A functional analysis showed that TIL had a certain degree of anti-tumor activity when $CD4^+$ and $CD8^+$ TIL were co-transferred. Co-transfer of $CD4^+$ and $CD8^+$ TIL exhibited equivalent anti-tumor activity, irrespective of tumor stage. However, the numbers of TIL did not increase after the early phase of tumor progression. These data suggest that TIL are specific to the tumor and potentially retain anti-tumor activity, although their accumulation in mice is impaired.

Key words: Tumor-infiltrating lymphocyte · T cell receptor · T cell clonality · RT-PCR · SSCP

Introduction

It has long been known that $CD8^+$ cytotoxic T lymphocytes play an important role in the immune responses against tumors. Previous studies have investigated tumor-reactive $CD8^+$ T lymphocytes with regard to their function and Ag specificity [1, 2]. Further investigations have revealed the frequency of T cell repertoires [3, 4] and have restricted the use of TCR-Vβ [5–7]. Tumor-specific T cell lines can be generated from tumor-infiltrating lymphocytes (TIL) in patients with ovarian carcinoma [8]. Studies of murine and human tumors have shown that TIL are able to recognize tumor antigens and to mediate anti-tumor immunity associated with long-term survival [7, 9]. Our knowledge of anti-tumor T cell immunity has been greatly facilitated both by studies using T cell lines and those using TCR transgenic T cells [10–12]. However, it is unknown whether these *ex vivo* cultured cells or transgenic T cells represent real physiological conditions in the tumor microenvironment. Despite the presence of TIL and T cell recognition, most tumors are not rejected efficiently. Various trials have also indicated that TIL are inefficient for controlling tumor growth [5, 6]. Thus, to explain the discrepancy between the *in vitro* and *in vivo* function of such TIL, we here focused on the TIL dynamics in a natural tumor progression model.

T cell clonality analysis by clonotype mapping, cloning of TCR sequences, and spectra typing have demonstrated highly clonal expansion of TIL [13], skewed usage of some Vβ subsets [14], and the diversity of the TCR repertoire [15]. However, the specificity, surveillance, and local accumulation of the overall panel of TCR V regions in tumors have been poorly investigated. Moreover, the kinetics of $CD4^+$ and $CD8^+$ TIL in growing tumors has not been elucidated.

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Abbreviations: TIL: Tumor-infiltrating lymphocyte · **SSCP:** Single-strand conformation polymorphism

We have previously established a method for analyzing T cell clonality by RT-PCR/single-strand conformation polymorphism (SSCP) [16–18]. This method offers a means of detecting nucleotide differences of the CDR3 regions, providing a comprehensive image of clonally expanded T cells both *in vitro* and *in vivo* [16]. Using this method, we have demonstrated that in patients with solid tumors, T cell oligoclonal expansion extended from the original tumor to the draining lymph nodes and to PBL during the progression of the metastasis [17].

Here, we demonstrated that TIL clonotypes showed a certain level of specific tumor surveillance, similar to that of conventional memory T cells. Although $CD8^+$ and $CD4^+$ TIL clones were characterized by different concentrations and degrees of persistency, both exhibited equivalent anti-tumor activity, irrespective of tumor stage. However, the accumulation of TIL in the tumors of mice was impaired.

Results

CMS5 TIL infiltrate the tumor from the early stages in an unrestricted pattern

CMS5 is a methylcholanthrene-induced fibrosarcoma of BALB/c origin ($H\text{-}2^d$) [19]. Measurable tumors first appeared on day 5 after inoculation with 1×10^6 CMS5 cells (Fig. 1A). We performed RT-PCR/SSCP analysis of the tumors, the spleen, and the peripheral lymph nodes from 7-day tumor-bearing mice. SSCP analysis revealed the full range of the TCR Vβ repertoire, which showed oligoclonal expansion in the Vβ repertoire in TIL populations, covering Vβ1–19 (Fig. 1B). In contrast, the T cell populations in the spleen (Fig. 1C) exhibited a smear pattern, which indicates the appearance of polyclonal T cells.

Kinetics of multiple TIL clonotypes *in vivo*

To determine whether the distribution of TIL clonotypes depends on the local sites of the tumor, we compared two specimens from different sites of a single tumor mass, which we designated as Tumor1 (T1) and Tumor2 (T2). For most TCR Vβ subfamilies, especially Vβ2, 4, 6,

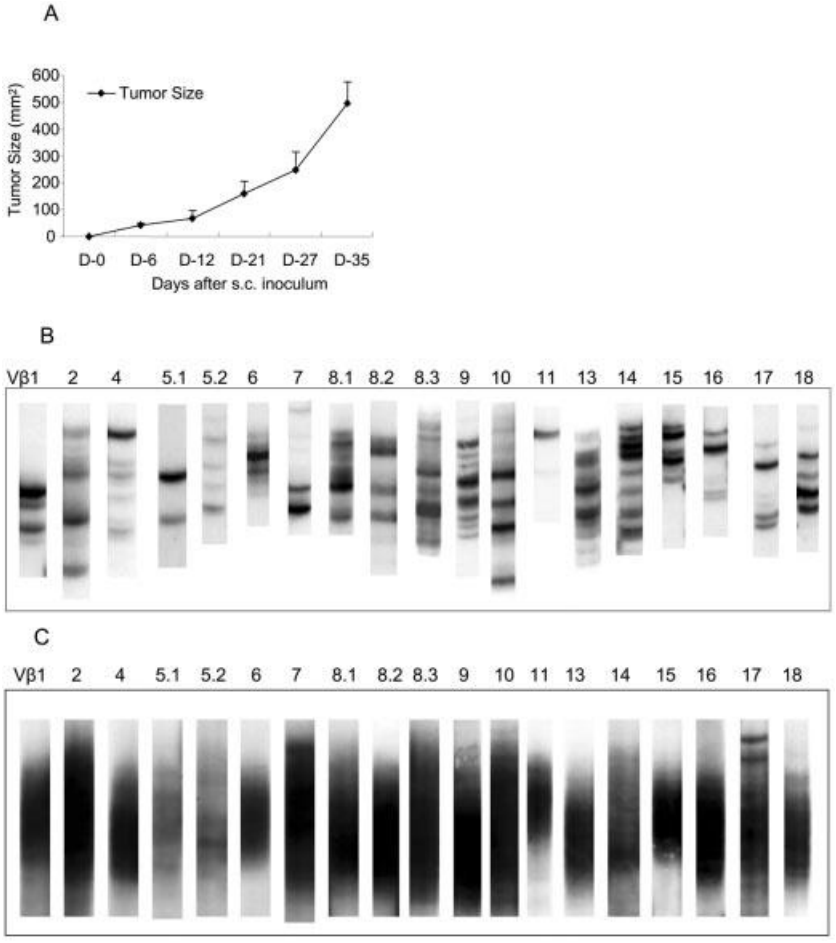

Fig. 1. Accumulation of unrestricted oligoclonal TIL in CMS5 tumor-bearing mice. (A) Mice were inoculated with 1×10^6 CMS5 cells, and tumor growth was determined by measuring changes over time of the means of two perpendicular diameters of tumor mass. Results are presented as the means ± SEM of nine mice. (B) Analysis of the clonality of TIL or (C) splenic T cells at the early stages (day 7) of tumor growth by RT-PCR/SSCP. The numbers represent the diverse TCR Vβ subsets. Four individual mice were analyzed, and all mice yielded essentially the same results.

7, 8.1, 9, 10, 13, 14, and 17, multiple identical bands were demonstrated in T1 and T2 (Fig. 2A). These results indicate that the Ag-specific immune responses initiated by the tumor were substantially homogenous throughout the entire tumor at the early stages (days 5–7).

To investigate the persistence of expanded TIL, we performed RT-PCR/SSCP analysis of TIL and splenic T cells in 10-day-, 14-day-, 18-day-, and 25-day-old tumors after the analysis of 7-day-old tumors. The results of the SSCP demonstrated oligoclonal accumulation of TIL from day 10 to day 25 of tumor development, while no oligoclonal accumulation of splenic T cells could be detected at any time point (data not shown). For nearly all the observed Vβ subfamilies in each group, the numbers of dominant bands of the TIL were not significantly changed from the early to the late stages (Fig. 2B). These results suggest that no significant clonal skewing or expansion of TIL occurred as a general tendency during tumor progression.

Surveillance of TIL *in vivo*

To investigate the systemic specificity of TIL, we compared the TIL clonotypes after simultaneous injections of CMS5 cells into two separate sites (the left and right flanks). As shown in Fig. 3A, the TIL migration patterns derived from the two different tumor sites were identical.

To examine the clonal specificity of these TIL to the CMS5 tumor, we compared the SSCP patterns of CMS5 and several other tumor lines. CMS5 cells were inoculated on the left flank, and a control tumor was simultaneously inoculated on the right flank. TIL were analyzed at 10 days after inoculation. When Ba/F3 was used as a control tumor, the TCR-SSCP profiles of the CMS5 TIL were significantly different from those of Ba/F3 (Fig. 3B), and TCR-SSCP of the spleen contained no band identical to these TIL. When the A-20 tumor was used as a control, the results were essentially the same as those for the Ba/F3 tumor (Fig. 3B). Therefore, it can be concluded that the TIL of CMS5 are specific to the CMS5 tumor in terms of clonality.

We next compared the TIL clonotypes after delayed injections of CMS5 cells into two separate sites. We first inoculated mice with CMS5 cells on the right flank to form CMS5 tumors (T 1^{st}). After 12 days, a second inoculation was performed on the left flank. The second tumor (T 2^{nd}) appeared as the first tumor (T 1^{st}) kept

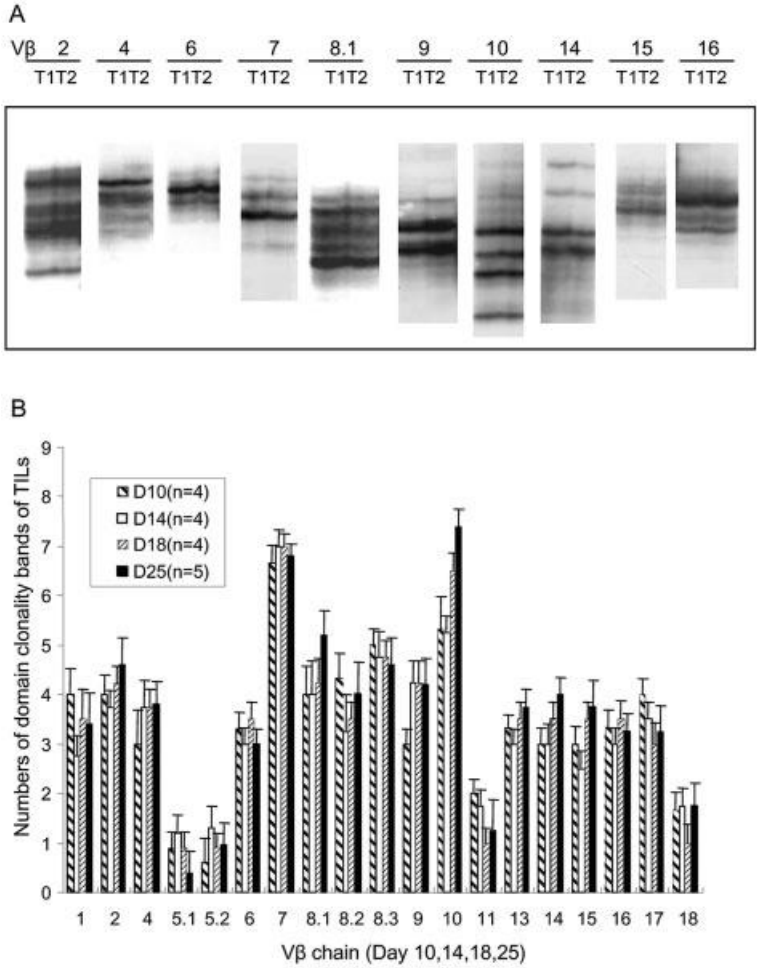

Fig. 2. Analysis of the distribution and accumulation of TIL clonotypes *in vivo*. (A) The TIL clonality in two different sites of a day 7 tumor (T1 and T2) was compared by RT-PCR/SSCP. Analysis of day 10, 14, 18, and 25 tumors yielded similar results. (B) Continuous oligoclonal accumulation of TIL was detected during the progression of tumors in the TCR Vβ repertoire. The bars represent the numbers of dominant bands in each Vβ subset based on the SSCP images of day 10, 14, 18 and 25 tumors. Error bars represent ± SD. At least four individual mice were analyzed in each group.

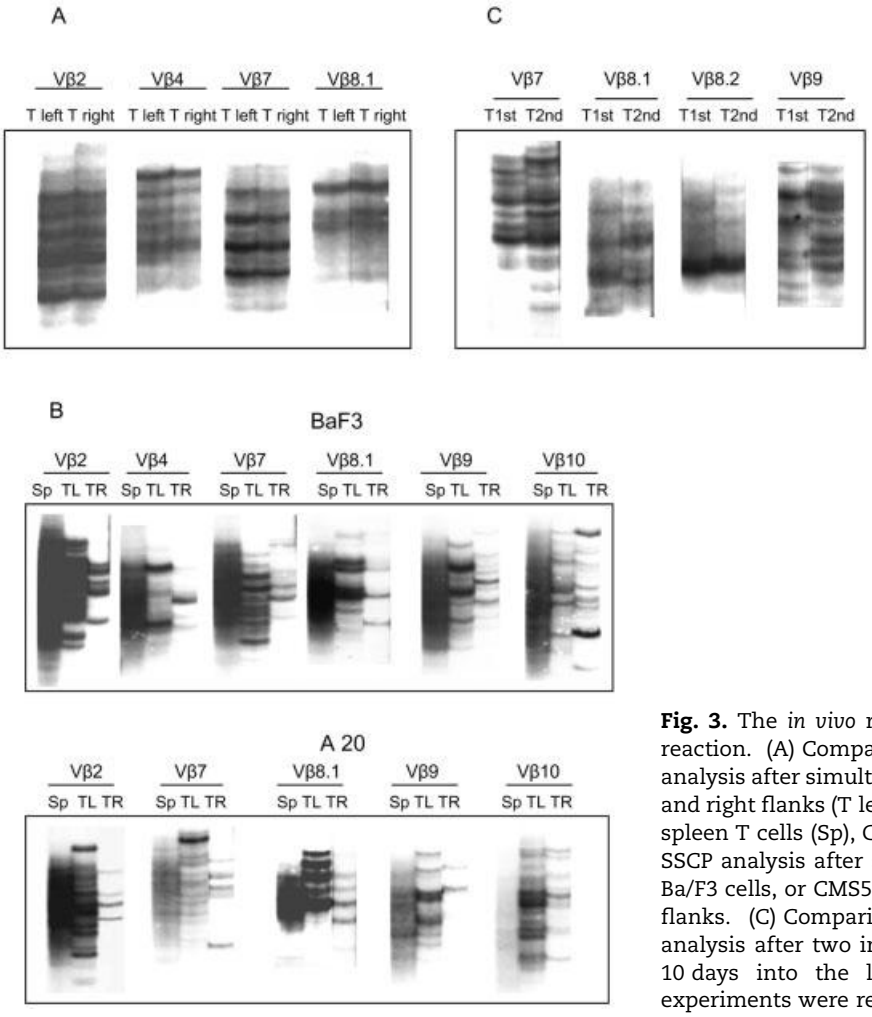

Fig. 3. The *in vivo* response of TIL is a specific and systemic reaction. (A) Comparison of TIL clonotypes by RT-PCR/SSCP analysis after simultaneous injections of CMS5 cells into the left and right flanks (T left, T right). (B) Comparison of clonotypes of spleen T cells (Sp), CMS5 TIL (TL) and other TIL (TR) by RT-PCR/SSCP analysis after simultaneous injections of CMS5 cells and Ba/F3 cells, or CMS5 cells and A-20 cells, into the left and right flanks. (C) Comparison of TIL clonotypes by RT-PCR/SSCP analysis after two injections of CMS5 cells with an interval of 10 days into the left and right flanks (T 1^{st}, T 2^{nd}). The experiments were repeated four times with similar results.

growing. Then, 25 days after the first inoculation, the tumors from both flanks were removed and their TIL clonotypes were analyzed (Fig. 3C). Although the second tumor grew faster and had a smaller proportion of TIL than the first tumor, both TIL expressed the same high level of the CD69 phenotype (data not shown). Moreover, the clonotypes of the TIL were identical to those of the first tumor in most Vβ subfamilies, as shown in Fig. 3C. These results indicate that the initial T cells were capable of surveying, migrating, and infiltrating into the newly appearing isogeneic tumor *in vivo*.

TIL *in vivo* display a predominantly effector/memory phenotype compared to the splenic T cells of tumor-bearing mice

On phenotypic analysis, both fresh $CD4^+$ and $CD8^+$ TIL (>95% CD3 positive) displayed a combination of significantly higher expression of effector and memory phenotypes than their counterparts in the spleen, as defined by low expression of CD62L and high expression of CD69 and ICOS (Fig. 4). The expression of these phenotypes was not significantly altered throughout the tumor progression (data not shown). The difference between the two subsets was that $CD8^+$ TIL had a higher proportion of $CD62L^{low}$ T cells compared to $CD4^+$ TIL.

$CD8^+$ T cells play a more dominant role in the TIL repertoire than $CD4^+$ T cells

To directly examine the compositions of the $CD4^+$ and $CD8^+$ TIL in CMS5 fibrosarcoma, $CD4^+$ and $CD8^+$ TIL were simultaneously isolated from the fresh tumors and analyzed directly by FACS at the same four time points as those used for the kinetic analysis of TIL. There was no significant difference in the CD4/CD8 ratio between naive splenic T cells (2.47 ± 0.53:1) and splenic T cells from tumor-bearing mice (2.25 ± 0.67:1). In contrast, the CD4/CD8 ratios of TIL were decreased in CMS5 tumors (1.33:1 ~ 1:1) throughout the period of tumor progression (Fig. 5A). As shown in Fig. 5B, the absolute number of $CD8^+$ TIL ceased to increase at around 1×10^4 cells per tumor, even when the overall number of tumor cells reached 5×10^7 at day 25 (data not shown). Kinetics analysis showed that before day 12, $CD8^+$ TIL expanded relatively quickly and reached 7×10^3–8×10^3 cells.

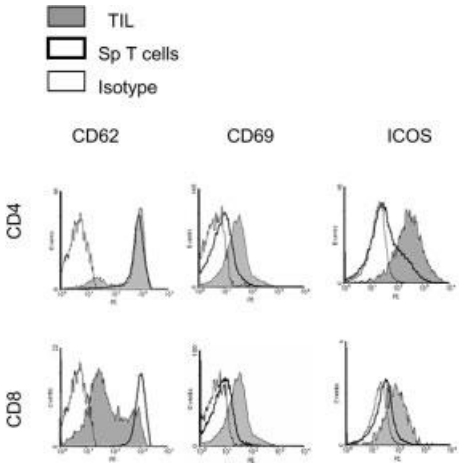

Fig. 4. Flow cytometric analysis of the fresh TIL and splenic T cells in tumor-bearing mice. $CD4^+$ and $CD8^+$ TIL were purified from day 14 tumors by MACS and stained with FITC-conjugated anti-CD8 Ab or anti-CD4 Ab and the indicated PE-conjugated second reagent. Thin-lined histogram, isotype control; both $CD4^+$ and $CD8^+$ TIL (shaded histogram) expressed a combination of effector/memory phenotypes compared with the splenic T cells (thick-lined histogram): $CD62L^{low}$, CD69, ICOS. Similar results were obtained from day 10, 14, 18, and 25 tumors. Figures exhibit representative data from three separate experiments.

However, in contrast to the unrestricted proliferation of tumor cells, the expansion of $CD8^+$ TIL stopped after day 12. Finally, no notable increase in infiltration was observed.

We next compared the $CD4^+$ and $CD8^+$ TIL using SSCP images. The oligoclonal banding patterns of the $V\beta6^+CD8^+$, $V\beta7^+CD8^+$, and $V\beta10^+CD8^+$ TIL from a 14-day-old tumor are shown in Fig. 5C. Strikingly, they match well with the total TIL of the original Vβ subsets. In contrast, $CD4^+$ TIL had migration patterns that were clearly distinct from those of the original total TIL (Fig. 5C). A similar phenomenon was observed in most Vβ subsets at various time points of tumor growth. These findings suggest that although the $CD4^+$ and $CD8^+$ subsets were in a nearly 1:1 ratio in the tumor milieu, the $CD8^+$ clones were the dominant component of clonally expanded TIL.

To address the involvement of the $CD4^+$ and $CD8^+$ TIL separately, we sequenced the CDR3 regions of Vβ7 from total TIL, $CD4^+$ TIL and $CD8^+$ TIL (Fig. 5D). Among 90 clones per group bearing each motif, approximately 56% of the $V\beta7^+$ TIL maintained a highly conserved amino acid sequence motif in the TCR CDR3 region. This major motif was synchronized precisely in the $V\beta7^+CD8^+$ TIL up to 73%. In contrast to the highly skewed $CD8^+$ TIL, only 26% of the $V\beta7^+CD4^+$ TIL shared a major motif that belongs to the smallest minor group (3.3%) of the total $V\beta7^+$ TIL. The remainder of the $CD4^+$ TIL showed multiple clonotypic sequences of CDR3 with numbers greater than 40 in 68 clones (data not shown) as well as multiple Jβ, including Jβ1.2, 1.4, and 1.6, which had never been observed in the $V\beta7^+CD8^+$ TIL. The appearance of a large number of unrepeatable minor $CD4^+$ clones suggests that a wide diversity of clones may exist in the $CD4^+$ TIL population.

We next compared the clonality of sorted $CD8^+CD69^+$ TIL from a day 18 tumor with whole TIL from the same tumor site. In several Vβ subfamilies, we observed identical bands (Vβ7 and 17; Fig. 5E). These data demonstrated that at least certain selected Vβ subfamilies, especially those with high-frequency Vβ, harbor strongly stimulated clones in the tumor site.

Persistence of $CD8^+$ TIL and fluctuations of $CD4^+$ TIL *in vivo*

To detail these clonal expansions of TIL *in vivo*, we performed a time-course RT-PCR/SSCP analysis of an "individual mouse", to track the kinetics of the TIL clonotypes. At day 10 post inoculation, an evenly spaced, approximately 9-mm^2 area of the tumor was resected as a source of early-stage $CD4^+$ and $CD8^+$ TIL. At day 22, the TIL from the same tumor were isolated as later-stage TIL. In SSCP, although a few new bands appeared in addition to the major bands of some Vβ such as Vβ8.1 at day 22, the major bands of $CD8^+$ TIL were virtually identical between day 10 and 22 in nearly all of the detected Vβ subfamilies (Fig. 6A). These results suggest that once these $CD8^+$ TIL have infiltrated, the major clones are stably maintained in the environment of tumor progression. In comparison, distinct bands were demonstrated between the two phases in $CD4^+$ TIL (Fig. 6B). In most Vβ subfamilies, the electrophoresis patterns were found to be essentially different between day 10 and day 20 $CD4^+$ TIL. The parallel sequence analysis of $CD4^+$ Vβ8.1 TIL obtained on day 10 and day 22 is also consistent with this observation (data not shown). This phenomenon indicates that, in the growing tumor milieu, most $CD4^+$ clones in the early phase are replaced by newly evolving clones that become dominant in the late stage.

Protective properties of $CD4^+$ and $CD8^+$ TIL in BALB/c nu/nu mice

To directly examine whether these systemically surveying TIL have anti-tumor effects *in vivo*, BALB/c nu/nu recipient mice were injected i.v. with $CD4^+$, $CD8^+$ or whole TIL derived from a day 10 CMS5 tumor-bearing mouse. Transferred cells were allowed to expand for 14 days and then challenged with 1×10^6 CMS5 cells. Groups receiving either 1×10^3 naive splenic T cells or no T cells were used as controls. A population of up to

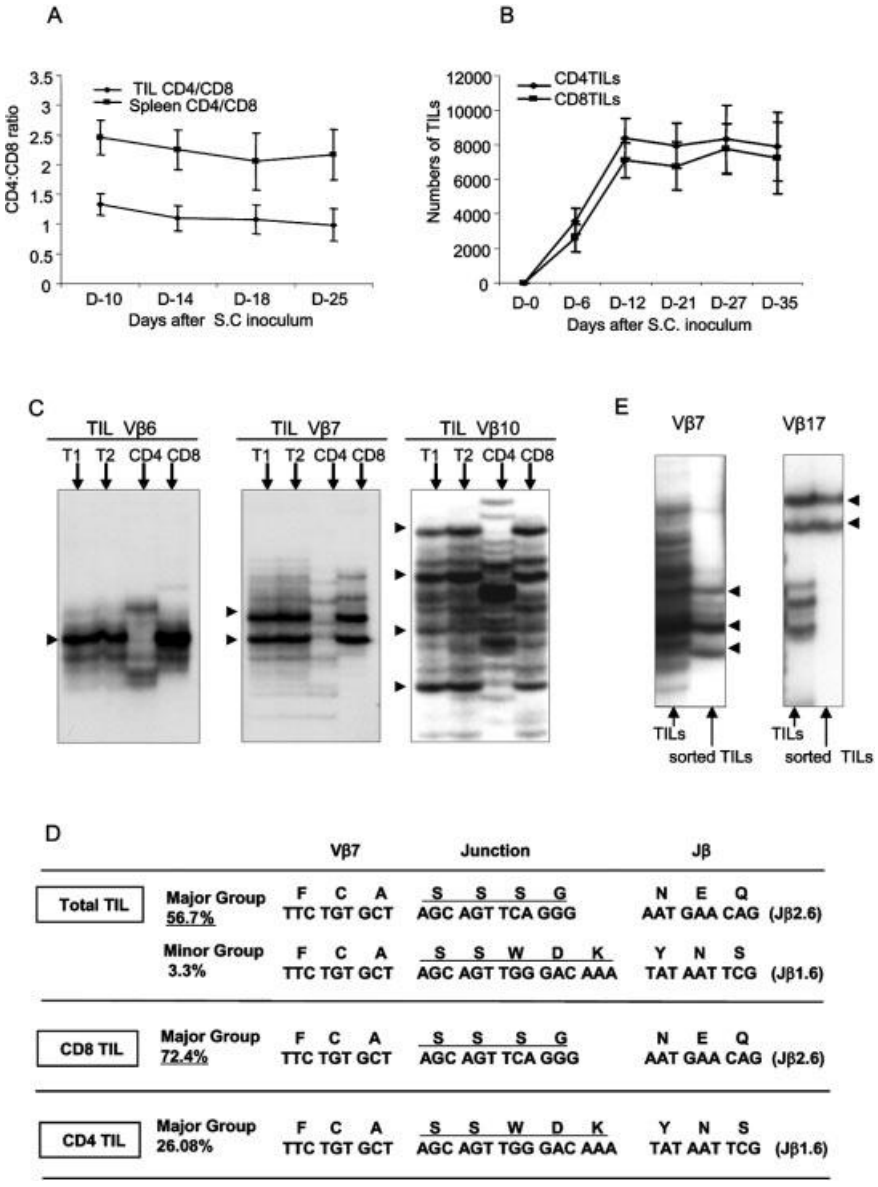

Fig. 5. $CD8^+$ TIL dominate the immune response compared to $CD4^+$ TIL. (A) Fresh TIL and splenic T cells were stained with FITC-conjugated anti-CD8 Ab + PE-conjugated anti-CD4 Ab simultaneously at the indicated time points, and the ratio of CD4/CD8 was analyzed by flow cytometry. Four individual mice were analyzed in each group. (B) The absolute numbers of $CD4^+$ and $CD8^+$ TIL at the indicated time points were quantified. Four individual mice were analyzed in each group. Error bars represent $\pm$ SD. (C) Comparison of T cell clonality in $CD4^+$, $CD8^+$ TIL, and whole TIL (T1, T2) from day 14 tumors by RT-PCR/SSCP. Black triangles indicate the identical TCR Vβ clonotypes expanded in $CD8^+$ TIL and whole TIL. Four individual mice were analyzed in each group, and all mice yielded essentially the same results. (D) Comparison of the CDR3 regions used by CMS5 $CD4^+$, $CD8^+$, and total TIL Vβ7 clones. Amino acid sequences are displayed in a single-letter code above the nucleotide sequences. The common amino acid motifs found in these clonotypes from the different subsets of TIL are underlined. The percentages indicated under the major or minor groups reveal the frequency of the clonotypes bearing each motif. (E) Comparison of T cell clonality in FACS-sorted $CD8^+CD69^+$ TIL and whole TIL derived from the same tumor by RT-PCR/SSCP. Black triangles indicate the identical expanded bands shared by $CD8^+CD69^+$ TIL and whole TIL.

2×10^5 T cells was detected in the spleen of each T cell recipient mouse 2 weeks after the transfer. As shown in Fig. 7A, the group receiving no T cells developed the largest CMS5 tumors. In contrast, the nude mice treated with either $CD4^+$ or $CD8^+$ TIL showed less tumor growth than the control or naive T cell groups. In particular, the mice that received whole TIL grew the smallest tumors of the four groups ($p<0.01$). SSCP analysis demonstrated that the tumors of recipients were infiltrated with T cells containing clones identical to the original TIL (Fig. 7B).

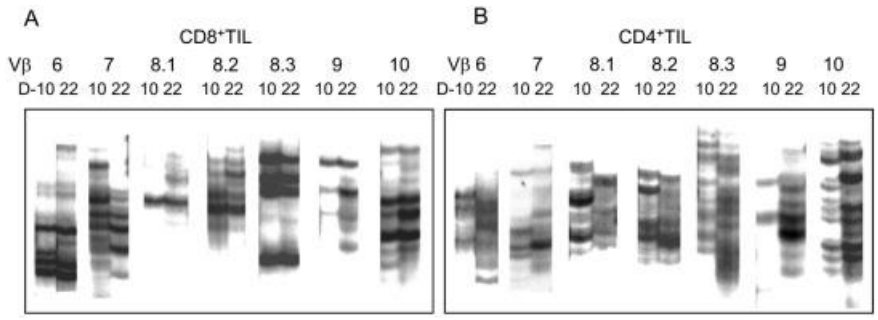

Fig. 6. Persistence of $CD8^+$ TIL clonotypes and fluctuations of $CD4^+$ TIL clonotypes *in vivo*. SSCP analyses of day 10 (d10) and day 22 (d22) $CD8^+$ TIL (A) and $CD4^+$ TIL (B) obtained from the same mouse were performed on the same background.

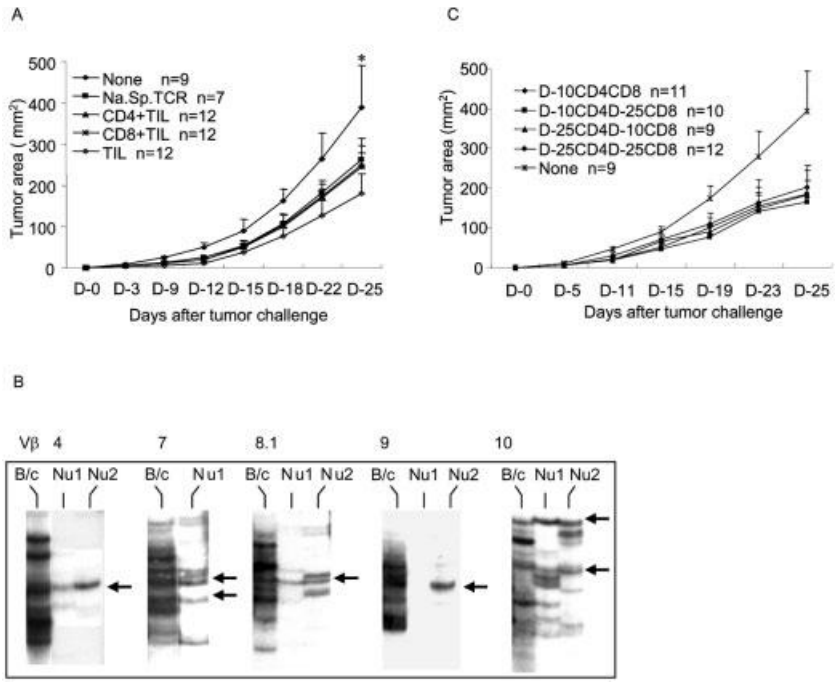

Fig. 7. TIL transferred to lymphopenic mice can reaccumulate at the newly developed CMS5 tumor lesions and mediate an anti-tumor response. (A) Naive splenic T cells or TIL were isolated from naive BALB/c or day 10 CMS5 tumor-bearing mice by a MACS magnetic column. Naive splenic T cells, $CD4^+$, $CD8^+$ or whole TIL (1×10^3) were transferred i.v. into BALB/c nu/nu mice. A no-transfer group (black diamond) was used as a control. Each group contained seven to twelve mice. Each mouse was inoculated s.c with 1×10^6 CMS5 cells 14 days post transfer. Statistical analysis revealed that a significant difference ($^*p<0.01$) existed in tumor size between the group receiving whole TIL (open diamond) and all other groups. (B) Comparison of TIL clonotypes by RT-PCR/SSCP analysis. B/c indicates host TIL derived from tumor-bearing BALB/c mice; Nu1 and Nu2 indicate reaccumulated TIL at the newly developed CMS5 tumors of two nude mice. Black arrows indicate the identical bands shared by host TIL and recipient TIL. (C) Comparison of the anti-tumor response of TIL at different phases in the adoptive transfer experiment. Four groups of TIL (day 11 $CD4^+$ and $CD8^+$ TIL, day 25 $CD4^+$ and $CD8^+$ TIL, day 11 $CD4^+$ and day 25 $CD8^+$ TIL, and day 25 $CD4^+$ and day 11 $CD8^+$ TIL; 1×10^3 cells per group) were transferred into the four groups of recipients, as described in (A). A no-transfer group (black diamond) was used as control. Error bars represent ± SD. There was no significant difference among the four groups ($p>0.05$).

Alteration of the $CD4^+$ TIL clonotypes in the different phases of tumor development had the possibility of influencing the anti-tumor response *in vivo*. We performed a comparative *in vivo* analysis. Four groups of TIL (each combination of 1×10^3 day 11 $CD4^+$ and $CD8^+$ TIL, day 25 $CD4^+$ and $CD8^+$ TIL, day 11 $CD4^+$ and day 25 $CD8^+$ TIL, and day 25 $CD4^+$ and day 11 $CD8^+$ TIL) were injected i.v. into the four groups of recipients. Two weeks later, these mice were inoculated s.c. with 1×10^6 CMS5 tumor cells. A comparison of the two phases of $CD4^+$ TIL transferred into recipients showed a similar anti-tumor effect (Fig. 7C). These results indicate that alterations in $CD4^+$ TIL clonality may not directly account for the profound tumor-specific suppression during tumor growth.

Discussion

Our previous study showed that human ovarian tumor-infiltrating T cells exhibit clonal expansion and are specific for autologous tumors [20]. In this model, the clonally expanded TIL are essentially homogenous (Fig. 2) and specific to CMS5 tumors (Fig. 3B). This observation is consistent with our recent observations on the TIL of a P815 solid tumor [21]. Because there is a very high diversity of TCR in mice (2×10^6 different clonotypes/body) [22], the demonstration of identical dominant TIL clones between distant tumors in several Vβ subfamilies suggests that these dominant TIL are specific for CMS5 tumor cells (Fig. 3). There is the possibility that the similarity of the clonotypes observed in the two tumors is caused by activation of independent naive T cells with the same Vβ-CDR3 usage. However, if the probability of activation of independent T cells with the same Vβ-CDR3 usage was so high, we would have been able to identify many identical Vβ-CDR3 sequences in different individual mice. In fact, we could detect no identical sequence among major clones in Vβ7 and Vβ10 from more than ten mice (data not shown). We therefore believe it reasonable to speculate that the similarity of the clonotypes observed in the two tumors (more than ten clonotypes per mouse) reflects a migration of T cells from the initial tumor.

Our finding that clonally expanded TIL were detected throughout tumor progression suggests that the overall immune response in the tumor might be attributable to the cooperation of multiple homogenous tumor-specific T cell clonotypes. Moreover, the identical clonotypes suggest a significant immunosurveillance by tumor-specific TIL in the hosts, although the effect is not sufficient to suppress tumor growth.

In contrast to the unrestricted tumor growth shown in Fig. 1A, the expansion of $CD8^+$ TIL peaked at approximately 1×10^4 cells and then decreased (Fig. 5B). Altered antigen presentation to TIL is one possible explanation for this decrease. The site of tumor-antigen presentation could be either the lymphoid organs [23] or the tumor itself [24]. It has become evident that the stroma constituted by growing solid tumors may impede the migration of TIL [25] or prevent tumor cells from draining to the lymph nodes [23]. It is possible that the stroma prevents CMS5 tumor cells from reaching the lymphoid organs after the early phase. A limited number of precursor memory T cells from the lymphoid organs might migrate and respond to the new isogeneic tumor cells (T 2^{nd}) before the new stroma is consolidated. While T cells can infiltrate the newly developed CMS5 tumor in a tumor-bearing state (Fig. 3C), the infiltration may be inhibited after the early phase.

Our study revealed the dominance of the $CD8^+$ TCR repertoire in TIL (Fig. 5D). Recent assays have revealed qualitative and quantitative differences between $CD4^+$ and $CD8^+$ T cells in antiviral infection: $CD8^+$ T cells divide faster than $CD4^+$ T cells during LCMV infection, and the magnitude of the $CD8^+$ response vastly exceeds that of the $CD4^+$ response [26]. The different life-spans of $CD4^+$ and $CD8^+$ TIL in the tumor milieu could also be attributable to the different clonal kinetics. Bcl-2 can prevent the loss of cells by apoptosis [27]. We observed decreased expression of Bcl-2 in $CD4^+$ TIL compared to $CD8^+$ TIL in CMS5 (data not shown). This decrease may be associated with the shorter clonal duration in $CD4^+$ TIL.

Although $CD8^+$ T cells are dominant in the TIL repertoire, infiltration of $CD4^+$ cells is indispensable to the partial inhibition of CMS5 tumor progression (Fig. 7A). Previous studies have indicated that the $CD8^+$ T cells require help from $CD4^+$ T cells [28]. In a murine sarcoma and leukemia virus complex model, the depletion of $CD4^+$ T cells results in a decreased infiltration of macrophages and a significant reduction of $CD8^+$ T cells [11]. It is notable that in the case of the CMS5 tumor, not only did the late-phase $CD8^+$ TIL function as well as early-phase $CD8^+$ TIL, but the late-phase $CD4^+$ TIL also exhibited an anti-tumor response similar to that of the early-phase $CD4^+$ TIL (Fig. 7C), despite the fluctuation of early and late $CD4^+$ TIL in one individual. In all scenarios, both $CD4^+$ and $CD8^+$ TIL were found to be specific to CMS5 tumors to a certain degree.

We focused on CMS5 as a model system of anti-tumor immunity. There are many kinds of tumors with different characteristics. The immune response to a tumor can be determined by many parameters such as the origin of the tumor, the rate of tumor growth, and mutated oncogenes. To construct a generalized view of the TIL response, further investigation of TIL in different tumors is required.

In summary, our data have revealed an instructive picture of an ongoing clonal TIL response to CMS5 tumors within the normal T cell repertoire. We clearly demonstrated that $CD8^+$ TIL exist in high concentrations and could persistently localize at the tumor site with multiple highly specific clonotypes. While most $CD4^+$ clones exist in low concentrations and show fluctuations at the repertoire level, both early and late $CD4^+$ TIL were found to be functionally tumor specific to some degree. These TIL can be used for tumor cell surveillance, even in a tumor-bearing state, but they lose their capacity for efficient infiltration during tumor progression.

Materials and methods

Mice and tumor cells

BALB/c and BALB/c nu/nu mice were obtained from Japan SLC (Shizuoka, Japan). The mice were female and were used at 7–10 weeks of age. CMS5 cells were cultured in complete medium consisting of RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For *in vivo* inoculation, 1×10^6 tumor cells were injected s.c. into the flank. In some experiments, mice received a second challenge with CMS5 cells on contralateral sites at disparate times or simultaneously, as indicated. For specificity studies of TIL, CMS5 cells and Ba/F3 cells, or CMS5 cells and A-20 cells were injected s.c. into the left and right flanks of BALB/c mice. Ba/F3, a murine pro-B cell line that expresses both MHC class I and class II [29], was cultured in complete medium consisting of RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 ng/ml murine IL-3. A-20, a murine B cell lymphoma cell line [30], was cultured in complete medium consisting of RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Tumor growth was measured using a digimatic caliper (Mitsutoyo, Japan) and was recorded as an elliptical shape [longest surface length (a) and width (b), $\pi(a\times b)/4$]. All animal experiments were carried out in accordance with institutional and national guidelines.

Antibodies

The following mAb were used to detect the surface molecules: biotinylated anti-CD3, FITC- or PE-conjugated anti-CD4 (GK1.5), FITC- or PE-conjugated anti-CD8 (53–6.7), PE-

conjugated anti-CD62L (MEL-14), and PE-conjugated anti-CD69 (H1 2F3) mAb (all from PharMingen, San Diego, CA); anti-ICOS and anti-PD-1 mAb (eBioscience, CA). Before staining with the desired antibodies, cells were treated with Fc receptor block, anti-CD16/32 Ab (PharMingen).

Isolation of TIL and flow cytometric analysis

Tumors were dissected and divided into two pieces. One piece was minced to yield 1–2-mm pieces. To release the tumor cells and TIL, the tumor pieces were incubated in a mixture of 1 mg/ml type IV collagenase (Sigma, St. Louis, MO) and 20 μg/ml DNase (Sigma) in complete medium for 90 min at 37°C. The cell suspension was strained through nylon mesh and washed with PBS. The $CD4^+$ and $CD8^+$ TIL were isolated from the cell suspension by FITC-labeled anti-CD4 mAb and anti-CD8 mAb, followed by collection with anti-FITC Ab-conjugated paramagnetic beads and a MACS LS separation column according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were then stained with the mAb. Samples were analyzed for fluorescence using an EPICS XL flow cytometer (Coulter, Palo Alto, CA).

RNA extraction and cDNA synthesis

Total RNA from the tumor pieces was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method [10] using ISOGEN™ (WAKO, Tokyo, Japan). Total RNA (10 μg) was converted into cDNA with random primers (Gibco BRL) and Superscript II™ reverse transcriptase.

SSCP

The SSCP study was performed as described [16–18]. In brief, the synthesized cDNA was amplified by PCR with a set of Vβ1 to Vβ19 primers and a Cβ common primer. Aliquots of PCR products were electrophoresed on a non-denaturing 4% polyacrylamide gel. After transfer onto a nylon membrane, the cDNA was hybridized with a biotinylated internal common Cβ oligonucleotide probe and visualized by subsequent incubations with streptavidin, biotinylated alkaline phosphatase, and a chemiluminescence substrate system (Phototope-Star Chemiluminescence Detection Kit; New England Biolabs, Beverley, MA). The bands that were clearly distinct from the background on the films were counted [16].

Adoptive transfer of TIL

TIL and spleen cells from BALB/c mice were pooled and stained with anti-CD8, anti-CD4, or anti-TCRβ Ab on ice in PBS containing 1% BSA. $CD8^+$ T cells, $CD4^+$ T cells, and $TCR\beta^+$ cells were separated using MACS magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. Isolated cells in the final population were examined by flow cytometry, and equal numbers (2×10^3) of $CD4^+$ T cells, $CD8^+$ T cells, or $TCR\beta^+$ cells were injected i.v. into nude BALB/c recipients in a volume of 0.2 ml PBS.

Statistical analysis

The significance of observed differences was calculated using the Mann-Whitney U test. Values of $p<0.05$ were considered to indicate statistical significance.

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References

1 **Sadelain, M., Riviere, I. and Brentjens, R.,** Targeting tumours with genetically enhanced T lymphocytes. *Nat. Rev. Cancer* 2003. **3:** 35–45.

2 **Yee, C., Thompson, J. A., Roche, P., Byrd, D. R., Lee, P. P., Piepkorn, M., Kenyon, K., Davis, M. M., Riddell, S. R. and Greenberg, P. D.,** Melanocyte destruction after antigen-specific immunotherapy of melanoma: direct evidence of T cell-mediated vitiligo. *J. Exp. Med.* 2000. **192:** 1637–1644.

3 **Dudley, M. E., Wunderlich, J. R., Robbins, P. F., Yang, J. C., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., Sherry, R., Restifo, N. P. et al.,** Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002. **298:** 850–854.

4 **Dhodapkar, M. V., Krasovsky, J. and Olson, K.,** T cells from the tumor microenvironment of patients with progressive myeloma can generate strong, tumor-specific cytolytic responses to autologous, tumor-loaded dendritic cells. *Proc. Natl. Acad. Sci. USA* 2002. **99:** 13009–13013.

5 **Prevost-Blondel, A., Zimmermann, C., Stemmer, C., Kulmburg, P., Rosenthal, F. M. and Pircher, H.,** Tumor-infiltrating lymphocytes exhibiting high *ex vivo* cytolytic activity fail to prevent murine melanoma tumor growth *in vivo*. *J. Immunol.* 1998. **161:** 2187–2194.

6 **Blohm, U., Roth, E., Brommer, K., Dumrese, T., Rosenthal, F. M. and Pircher, H.,** Lack of effector cell function and altered tetramer binding of tumor-infiltrating lymphocytes. *J. Immunol.* 2002. **169:** 5522–5530.

7 **Hoppe, R. T., Medeiros, L. J., Warnke, R. A. and Wood, G. S.,** CD8-positive tumor-infiltrating lymphocytes influence the long-term survival of patients with mycosis fungoides. *J. Am. Acad. Dermatol.* 1995. **32:** 448–453.

8 **Freedman, R. S. and Platsoucas, C. D.,** Immunotherapy for peritoneal ovarian carcinoma metastasis using *ex vivo* expanded tumor infiltrating lymphocytes. Cancer *Treat. Res.* 1996. **82:** 115–146.

9 **Rosenberg, S. A., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. H. and White, D.E.,** Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J. Natl. Cancer Inst.* 1994. **86:**1159–1166.

10 **Lustgarten, J., Dominguez, A. L. and Cuadros, C.,** The $CD8^+$ T cell repertoire against Her-2/neu antigens in neu transgenic mice is of low avidity with antitumor activity. *Eur. J. Immunol.* 2004. **34:** 752–761.

11 **Schepers, K., Toebes, M., Sotthewes, G., Vyth-Dreese, F. A., Dellemijn, T. A., Melief, C. J., Ossendorp, F. and Schumacher, T. N.,** Differential kinetics of antigen-specific $CD4^+$ and $CD8^+$ T cell responses in the regression of retrovirus-induced sarcomas. *J. Immunol.* 2002. **169:** 3191–3199.

12 **Helmich, B. K. and Dutton, R. W.,** The role of adoptively transferred CD8 T cells and host cells in the control of the growth of the EG7 thymoma: factors that determine the relative effectiveness and homing properties of Tc1 and Tc2 effectors. *J. Immunol.* 2001. **166:** 6500–6508.

13 **Thor, S. P., Guldberg, P., Gronbaek, K., Hansen, M. R., Kirkin, A. F., Seremet, T., Zeuthen, J. and Becker, J. C.,** *In situ* T cell responses against melanoma comprise high numbers of locally expanded T cell clonotypes. *J. Immunol.* 1999. **163:** 443–447.

14 **Sensi, M. and Parmiani, G.,** Analysis of TCR usage in human tumors: a new tool for assessing tumor-specific immune responses. *Immunol. Today* 1995. **16:** 588–595.

15 **Pannetier, C., Even, J. and Kourilsky, P.,** T cell repertoire diversity and clonal expansions in normal and clinical samples. *Immunol. Today* 1995. **16:** 176–181.

16 **Masuko, K., Kato, T., Ikeda, Y., Okubo, M., Mizushima, Y., Nishioka, K. and Yamamoto, K.,** Dynamic changes of accumulated T cell clonotypes during antigenic stimulation *in vivo* and *in vitro*. *Int. Immunol.* 1994. **6:** 1959–1966.

17 **Yamamoto, K., Masuko, K., Takahashi, S., Ikeda, Y., Kato, T., Mizushima, Y., Hayashi, K. and Nishioka, K.,** Accumulation of distinct T cell clonotypes in human solid tumors. *J. Immunol.* 1995. **154:** 1804–1809.

18 **Zhou, G., Fujio, K., Sadakata, A., Okamoto, A., Yu, R. and Yamamoto, K.,** Identification of systemically expanded activated T cell clones in MRL/lpr and NZB/W F1 lupus model mice. *Clin. Exp. Immunol.* 2004. **136:** 448–455.

19 **Matsui, K., O'Mara, L. A. and Allen, P. M.,** Successful elimination of large established tumors and avoidance of antigen-loss variants by aggressive adoptive T cell immunotherapy. *Int. Immunol.* 2003. **15:** 797–805.

20 **Hayashi, K., Yonamine, K., Masuko-Hongo, K., Iida, T., Yamamoto, K., Nishioka, K. and Kato, T.,** Clonal expansion of T cells that are specific for autologous ovarian tumor among tumor-infiltrating T cells in humans. *Gynecol. Oncol.* 1999. **74:** 86–92.

21 **Tahara, H., Fujio, K., Araki, Y., Setoguchi, K., Misaki, Y., Kitamura, T. and Yamamoto, K.,** Reconstitution of CD8(+) T cells by retroviral transfer of the TCR alpha beta-chain genes isolated from a clonally expanded P815-infiltrating lymphocyte. *J. Immunol.* 2003. **171:** 2154–2160.

22 **Casrouge, A., Beaudoing, E., Dalle, S., Pannetier, C., Kanellopoulos, J. and Kourilsky, P.,** Size estimate of the alpha beta TCR repertoire of naive mouse splenocytes. *J. Immunol.* 2000. **164:** 5782–5787.

23 **Ochsenbein, A. F., Klenerman, P., Karrer, U., Ludewig, B., Pericin, M., Hengartner, H. and Zinkernagel, R. M.,** Immune surveillance against a solid tumor fails because of immunological ignorance. *Proc. Natl. Acad. Sci. USA* 1999. **96:** 2233–2238.

24 **Kedl, R. M. and Mescher, M. F.,** Migration and activation of antigen-specific $CD8^+$ T cells upon *in vivo* stimulation with allogeneic tumor. *J. Immunol.* 1997. **159:** 650–663.

25 **Yu, P., Lee, Y., Liu, W., Chin, R. K., Wang, J., Wang, Y., Schietinger, A., Philip, M., Schreiber, H. and Fu, Y. X.,** Priming of naive T cells inside tumors leads to eradication of established tumors. *Nat. Immunol.* 2004. **5:** 141–149.

26 **Homann, D., Teyton, L. and Oldstone, M. B.,** Differential regulation of antiviral T cell immunity results in stable $CD8^+$ but declining $CD4^+$ T cell memory. *Nat. Med.* 2001. **7:** 913–919.

27 **Bouillet, P., Metcalf, D., Huang, D. C., Tarlinton, D. M., Kay, T. W., Kontgen, F., Adams, J. M. and Strasser, A.,** Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999. **286:** 1735–1778.

28 **Toes, R. E., Ossendorp, F., Offringa, R. and Melief, C. J.,** CD4 T cells and their role in antitumor immune responses. *J. Exp. Med.* 1999. **189:** 753–756.

29 **Deng, M. and Daley, G.Q.,** Expression of interferon consensus sequence binding protein induces potent immunity against BCR/ABL-induced leukemia. *Blood* 2001. **97:** 3491–3497.

30 **Pizzoferrato E.,** B7–2 expression above a threshold elicits anti-tumor immunity as effective as interleukin-12 and prolongs survival in murine B cell lymphoma. *Int. J. Cancer* 2004. **110:** 61–69.

