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Comparative delineation of T cell clonotypes in coexisting syngeneic B16 melanoma

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Abstract B16 is a murine melanoma of C57Bl/6 origin, which rapidly develops as a tumor when inoculated into syngeneic immunocompetent hosts. Nevertheless, B16 tumors are considered to be immunogenic since tumor regression can be induced by means of immunotherapeutic intervention. Furthermore, B16 melanoma cells express several melanoma-associated antigens that may serve as targets for autologous T cells. To study the in vivo T cell response against B16, with particular emphasis on diversity and systemic involvement, we examined the spectra of T cell clonotypes in coexisting B16 melanoma lesions in C57Bl/6 mice. Three tumors from each animal ($n = 8$) were examined for the presence of clonotypic T cells using the highly sensitive T cell receptor (TCR) clonotype mapping technology. Systematic analysis of the TCRB variable regions 1–16 revealed from 19 to more than 30 clonotypic TCR transcripts in each tumor. To study intra- and inter-individual variations in the T cell response further, more than 600 clono-typic TCR transcripts were compared for sequence identity. Overall, approximately 2% of the T cell clonotypes were detected in more than one tumor from the same animal. Furthermore, none of the detected clonotypes was present in more than one animal, arguing against recurrent or "public" T cell responses against B16 melanoma. Our data strongly suggest that anti-melanoma T cell responses in this murine model encompass mainly localized T cells, and that systemic involvement is limited.

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Introduction

The past decade has revealed new insights into the role of T lymphocytes in the host's immune response to cancer. Thus, it is well established that tumors of various origins are immunogenic and subject to immunological growth control. The capability of the immune system to intervene in tumor growth is clearly illustrated by clinical trials in which immunostimulatory treatment modalities have induced prolonged survival of the patients [15, 19, 20]. However, in most cases the immunological response is inadequate to prevent tumor progression.

Recently, a large number of proteins serving as targets for the immunological response against melanoma cells have been characterized, and the peptide epitopes have been defined [6, 35]. Encouraged by the identification of these T cell targets, as well as by the presence of tumor-specific T cells in the blood of melanoma patients, several different immunotherapeutic approaches have been pursued in different animal models as well as in a clinical setting [3, 13, 15, 16]. Although some of the clinical trials have induced long-lasting tumor regressions in a fraction of the patients, it has become obvious that immunological induction of tumor regression is far more readily generated in animal models than in humans. The molecular background for this phenomenon is largely unknown, emphasizing the need to study anti-melanoma responses in humans and murine models, as a means to dissect and compare the mechanisms involved in anti-tumor immune responses in the two species.

The effective action of T cells is dependent on the activation, clonal expansion and correct localization of T cell clones [1]. We and others have demonstrated the presence of clonotypic T cells in human melanoma lesions, indicating that these T cells recognize peptides associated with melanoma-associated antigens in the

context of HLA molecules [14, 24, 29]. However, only limited data are available on in vivo T cell responses to murine tumors [13, 32]. A detailed analysis of the T cell response to the murine mastocytoma P815 revealed the presence of two T cell clonotypes that reproducibly emerged in most animals [13]. Whether this characteristic of the T cell response to P815 tumors is a general feature of antitumor responses in murine systems is, however, unknown. Antitumor T cell responses in humans have been studied in detail and available data strongly suggest that such recurrent or "public" T cell clonotypes are not involved in the human system [18, 28, 29]. It is well established that mouse tumors commonly elicit immunity against unique antigens whereas most defined human antigens are shared. In this regard, it could be speculated that unique antigens are recognized by "public" clonotypes in animal models. Thus, the presence of public T cell clones responding against syngeneic tumors could potentially represent a species-specific characteristic of murine antitumor T cell responses.

Several lines of data on human in situ anti-melanoma T cell responses have demonstrated a limited systemic involvement. Thus, the detection of identical T cell clonotypes in several lesions from the same patient is rare [29, 34], and a functional dissociation between systemic and local immune responses has been suggested [12]. However, it remains unresolved whether this characteristic of anti-melanoma T cell responses is also representative of anti-melanoma responses in mice. In the present study we aimed to address these issues by a detailed analysis of the clonotypic T cell responses in several coexisting B16 melanoma lesions in C57Bl/6 mice. This approach was chosen in order to delineate the T cell clonotypes in all lesions and conduct intra- and inter-individual comparisons. Our data demonstrate that tumor-infiltrating lymphocytes (TIL) of B16 melanoma comprise a large number of clonotypic T cells. However, no recurrent or public T cell clonotypes were detected. Furthermore, the vast majority of the T cell clonotypes detected were confined to a single lesion, indicating a limited systemic involvement of the T cell response against B16 melanoma.

Materials and methods

Animals

C57Bl/6 mice were obtained from Jackson Laboratory at the age of 6 weeks. These animals were housed under specific-pathogen-free conditions and all experiments were performed according to National Institute of Health guidelines for care and use of laboratory animals.

Cell lines

The murine melanoma cell line B16G 3.12, which is a non-metastasizing and homogeneous B16 variant, has been described [26]. Cells were cultured as monolayers in RPMI-1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine.

Subcutaneous tumors

Tumors were induced by subcutaneous injection of 5×10^4 B16G 3.12 tumor cells in RPMI-1640 medium resulting in tumors of approximately 40 µl volume within 10 days. The three tumors were induced on the back, and on the left and right sides of the animal respectively. In mice numbered 1 to 4 (group 1), three tumors (A, B, and C) were all induced on the same day. In animals 5–8 (group 2), the second tumor (B) was induced 3 days after the first tumor (A), and tumor C was induced 5 days after tumor B. All tumors were excised 10 days after induction.

RNA extraction and reverse transcription/polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Purescript Isolation Kit (Gentra Systems Inc. N.C.). Synthesis of cDNA was done with 1–3 µg total RNA using oligo (dT) and SuperScript II reverse transcriptase (Gibco-BRL, Life Technologies Inc., Gaithersburg, Md., USA) in a total volume of 50 µl 1X buffer (Gibco-BRL, Life Technologies Inc.) containing 10 mM dithiothreitol. Incubations were performed at 42 °C for 60 min, then 72 °C for 5 min. Primers used for the analysis of murine T cell receptor B variable (TCRBV) regions include 16 primers specific for BV families 1–16 and a constant-region primer, BC, as described [32]. cDNA concentrations were adjusted using primers specific for the TCRBC region (5′-GCTACCTTCTGGCACAATCCTC-3′ and 5′-TAGGCATTTTCCAGGTCACAAG-3′). The conditions used were 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s. Polymerase and nucleotides were added at an 80 °C step after the denaturation step of the first cycle (hot-start). Amplifications of the variable TCR regions were performed by 25 cycles followed by a 32-cycle reamplification of a 1-µl sample of the first PCR product in a Perkin Elmer GeneAmp PCR System 9600 (Perkin Elmer Cetus Corporation, USA), using previously described conditions [32]. Negative controls were samples without cDNA.

TCR clonotype mapping by denaturing gradient gel electrophoresis (DGGE)

DGGE analysis for clonotype mapping of the murine TCRBV regions 1–16 has been described [32]. In brief, "GC-clamped" PCR products were loaded onto a 6% polyacrylamide gel containing a 20%–80% gradient of urea and formamide [32]. Electrophoresis was performed at 160 V for 4.5 h in $1 \times$ TAE buffer (Tris/Ammoniate/EDTA) at a constant temperature of 54 °C. After electrophoresis, the gels were stained with ethidium bromide and photographed under UV transillumination. For all variable regions, the amplified fragments have been functionally analyzed for their ability to resolve in denaturing gradient gels by the use of cloned transcript [32]. The analysis of a polyclonal T cell population (peripheral blood lymphocytes, PBL) was shown to result in a smear in the denaturing gradient gel [32].

Sequencing reaction

PCR products were subjected to sequence analysis using the Thermo Sequenase cycle sequencing kit (Amersham, Life Science, Cleveland, USA) following the manufacturer's instructions. Briefly, bands were excised from the denaturing gradient gel, and DNA was eluted in H_2O and reamplified. A 0.2-µl aliquot of the PCR product was used as template in a 40-cycle sequencing reaction, using the BC primer labeled with ^{33}P as sequencing primer. Gels were dried under vacuum and exposed to a Phosphor Screen.

Results

TCR clonotype repertoire in B16 tumors

To study the repertoire of TIL in coexisting syngeneic melanoma lesions, eight C57Bl/6 mice were subcutaneously injected with 5×10^4 B16 melanoma cells at three different sites (tumors A, B and C). In one group of animals (numbers 1–4), the three tumors were induced on the same day. In the other group (animals 5–8), the tumors were induced at 3-day intervals. Tumor lesions were removed after 10 days, at which point they had reached a size of approximately 40 μl.

Each of the 24 tumors was examined for the presence of clonally expanded T cells by TCR clonotype mapping of BV regions 1–16 [32]. Individual TCR transcripts were subjected to reverse transcription, amplified by PCR using primers specific for each BV region, and resolved according to nucleotide sequence in a gel containing an increasing gradient of denaturants. By this approach, a clonal transcript is revealed as a band on a background smear if it constitutes more than 2.5% of the total number of transcripts for that particular BV region [27]. An example of a complete TCR clonotype map is depicted in Fig. 1. A total of more than 600 clonotypic transcripts were identified in the 24 tumors, with the number of T cell clonotypes in individual tumors ranging from 19 to more than 30 (Table 1). T cell clonotypes were detected in all BV regions, although more rarely in some regions, e.g. regions 6 and 11 (Table 1).

To test the hypothesis that the presence of a tumor may influence the immunological response to subsequent tumor challenge, we compared the TCR clonotype repertoire in the tumors of group 1 (inoculated at the same time; animals 1–4) to those of group 2 (inoculated sequentially; animals 5–8). The total numbers of T cell clonotypes detected in the subsequent tumors (B and C) did not substantially differ from the numbers detected in the initial tumors (A) in either group (Table 1).

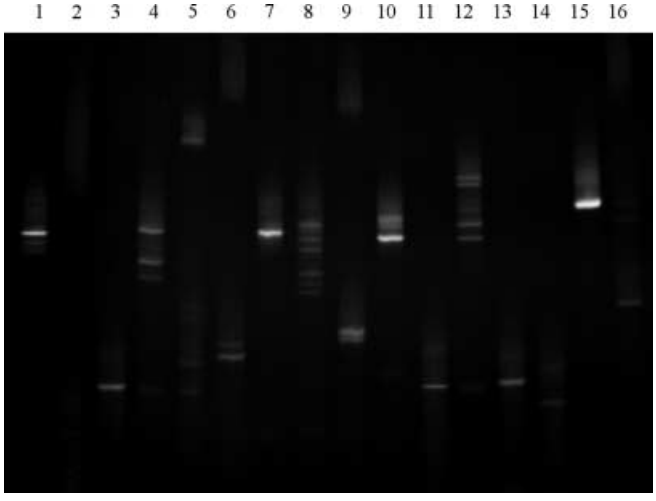

Fig. 1 T cell receptor (TCR) clonotype mapping of the T cell infiltrate in tumor 2C, covering TCRB variable regions 1–16. Polymerase chain reaction products were loaded onto a 20%–80% denaturing gradient gel and run for 4.5 h at 160 V at a constant temperature of 56 °C. DNA was stained with ethidium bromide and photographed under UV light

Comparative delineation of T cell clonotypes

The oligoclonal patterns of T cells identified by TCR clonotype mapping of individual B16 tumors suggested a T cell response with a high degree of intra- and inter individual variation, similar to what has previously been observed in humans [29, 34]. To examine specifically whether the T cell response may contain a systemic component and/or a public component, we exploited the RT-PCR/DGGE method to compare individual T cell clonotypes systematically for sequence identity.

For each animal, the clonotypic TCR transcripts detected in the three tumors were grouped according to BV region and examined in adjacent lanes in a denaturing gradient gel. A representative example is given in Fig. 2 depicting the comparative analysis of TCRBV9 T cell clonotypes. Bands migrating to the same positions in the gel were further examined by sequence analysis to exclude or confirm sequence identity. This comparative DGGE analysis demonstrated the presence of identical T cell clones in separate tumors in six of the animals (2, 3, 4, 5, 6 and 8; Table 2). Eight of these 13 T-cell clones were limited to two of the three tumors. In the five remaining cases, clonotypic transcripts were detected in all three tumors from the same animal, including two different BV10 transcripts in animal 4, two different BV8 transcripts in animal 6, and one BV11 transcript in mouse 8. Only one or two identical T cell clonotypes were detected in each animal, with mouse 4 being a clear exception. In this animal, six clonotypes were detected in more than one tumor and two of these were detected in all three lesions. Prompted by this finding we also analyzed PBL, skin and spleen from animal 4 by RT-PCR/DGGE. One of the BV10 clones present in all three tumors from this animal was detected in PBL and skin, but was not detectable in the spleen (Fig. 3). Surprisingly, the analysis of PBL from this animal revealed the presence of more than 20 clonotypic TCR transcripts (data not shown). The numbers of T cell clones in PBL from tumor-bearing as well as healthy animals normally range from 0 to 5 [32].

To examine whether public T cell clones participate in the response against B16 melanoma, we extended our analyses to the interindividual level by comparing the clonotypic transcripts among TIL from all 24 tumors. Multiple rounds of comparative DGGE revealed a small fraction of the more than 600 clonotypic transcripts that resolved at similar positions in the gel. However, in all cases sequence analysis established that these potentially identical clones were, in fact, different. These data suggest that public T cell clonotypes are not involved in the response to B16 melanoma.

Table 1 The numbers of T cell clones and the variable region expression of these in each tumor. Bold type indicates detection of identical T cell receptor. *TCRBV* T cell receptor B variable region

TCRBV	Mouse 1 lesions			Mouse 2 lesions			Mouse 3 lesions			Mouse 4 lesions			Mouse 5 lesions			Mouse 6 lesions			Mouse 7 lesions			Mouse 8 lesions		
	1A	1B	1C	2A	2B	2C	3A	3B	3C	4A	4B	4C	5A	5B	5C	6A	6B	6C	7A	7B	7C	8A	8B	8C
1	1	1	1	1	1	3	1	3	2	2	2	1	0	2	1	1	2	1	1	1	1	1	1	1
2	1	2	1	1	2	0	1	3	1	0	**2**	**1**	0	**1**	**1**	0	2	1	1	2	1	3	0	1
3	1	2	1	1	2	1	1	0	1	**1**	1	**1**	1	2	1	1	1	1	1	0	1	0	0	0
4	1	2	2	1	1	3	2	3	0	2	**1**	**1**	2	1	0	2	1	1	1	1	1	2	2	2
5	0	0	1	1	2	3	3	1	3	3	4	1	3	2	2	2	1	2	1	2	3	1	0	1
6	0	0	0	1	0	2	1	0	0	0	0	0	0	0	1	0	1	0	1	2	2	0	1	0
7	1	3	2	0	0	1	1	1	2	1	1	1	2	2	0	1	1	2	1	0	3	2	1	3
8	1	2	0	2	2	5	2	2	3	0	0	1	0	1	1	**2**	**3**	**2**	2	0	3	1	0	0
9	1	3	2	3	1	2	**1**	**3**	1	0	2	0	1	1	1	1	2	1	1	1	2	2	2	2
10	0	0	0	0	1	1	0	0	0	**2**	**2**	**2**	1	2	0	1	1	1	1	2	1	1	1	2
11	0	1	1	2	1	1	1	1	1	0	0	0	**1**	**2**	1	0	1	0	1	0	0	**1**	**1**	**1**
12	1	1	0	1	0	4	1	2	0	0	1	0	1	1	1	2	1	1	1	2	1	1	1	0
13	3	3	2	2	1	1	1	3	1	2	2	1	3	5	3	2	3	3	3	2	1	3	4	5
14	2	1	5	2	1	1	3	4	2	**1**	**1**	0	4	1	0	2	2	2	1	1	1	1	4	3
15	2	3	0	1	**1**	**1**	1	1	1	2	3	3	3	3	2	1	3	3	3	1	3	2	2	0
16	3	3	2	1	5	2	1	2	2	1	2	5	4	4	3	4	2	4	3	4	3	2	2	2

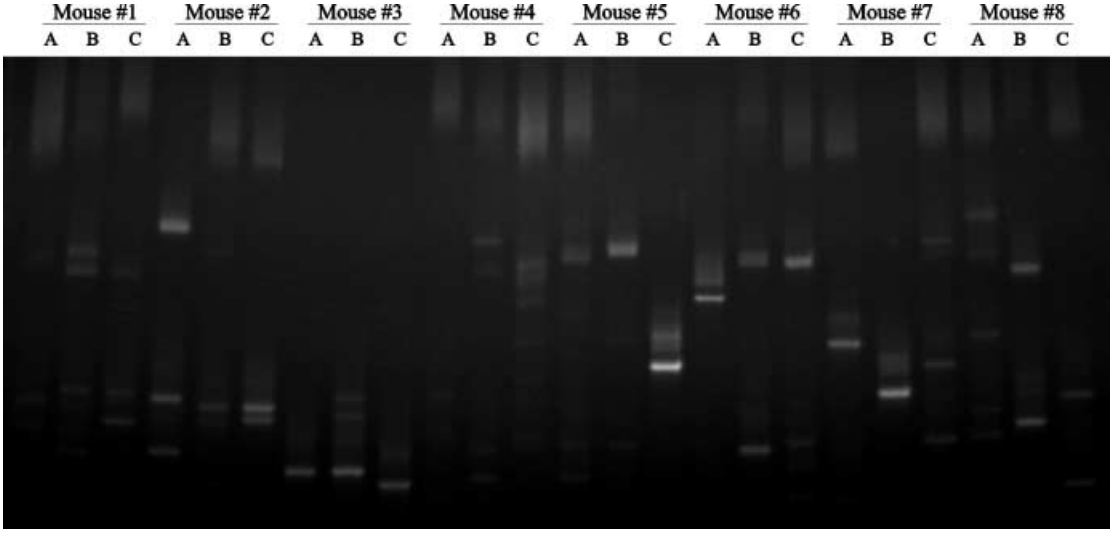

Fig. 2 TCR clonotype mapping used for a comparative analysis of TCR clonotypes detected in several tumors. The present example shows the comparison of all BV9 clonotypes detected in the three tumors (*A*, *B*, *C*) in each of the eight animals. Identical TCR transcripts were detected in animal 3, tumors A and B. Although the third tumor from this animal also showed the presence of a T cell clonotype (animal 3, tumor C) this transcript is clearly different from the transcripts in tumors A and B

Discussion

The B16 melanoma model has been used in a number of studies to investigate the feasibility of immunological intervention in the control of tumor growth. Human melanoma and B16 melanoma are comparable in a number of ways. Thus, B16 melanoma is a rapidly growing tumor of spontaneous origin and it expresses several human melanoma-associated antigen counterparts (tyrosinase, gp100, MART, and TRP-1 and TRP-2) [5, 7, 36]. In both B16 and in humans these melanoma-associated antigens have been shown to comprise epitopes recognized by autologous T cells [11, 25]. However, strategies to implement successful immunotherapy have proved to be much easier in mice than in humans. For example, rejection of B16 tumors can be induced by treatment with immunological modulators such as interleukin-2 (IL-2) or IL-12 [2, 9], whereas most clinical trials pursuing similar immunological strategies for treatment of human metastatic melanoma have yielded more or less disappointing results. The molecular background for these differences remains largely unknown.

T cells are believed to be the main effector cells in anti-melanoma immune responses in both human and murine systems, and several studies have been performed with the aim of analyzing in situ T cell responses in the human system [10, 18, 21–23, 29]. Collectively, the data from these studies suggest that the T cell response to human melanoma is highly heterogeneous and

Table 2 T cell clones detected in more than one lesion and the BV region expression of the clones

Mouse no.	BV region expressed
2	15
3	9
4	2
	3
	4
	10[a]
	10[a]
	14
5	2
	11
6	8[a]
	8[a]
8	11[a]

[a] This T cell clone was detected in all three tumors from the corresponding animal, whereas the remaining clones were found in 2 of the 3 tumors (see Table 1)

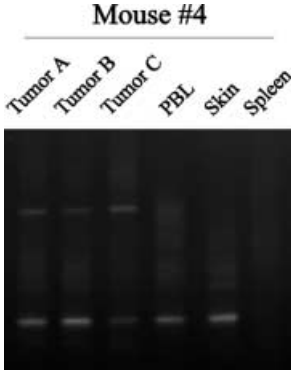

Fig. 3 Comparative analysis of the BV10 clones in peripheral blood lymphocytes (*PBL*), skin, spleen and tumors of mouse 4. The *upper band* represents a T cell clone detected in all tumors of animal 4 but not detected in PBL, skin or spleen. Conversely, the *lower band* represents the BV10 T cell clone detected in all tumors in addition to PBL and skin. In the spleen no T cell clonotypes were detected

distinct for each patient. Although several murine models have been used to study the efficacy of various immunotherapeutic approaches, only a limited number of studies have included detailed analyses of the T cell response. In one extensive study, the T cell response against syngeneic P815 tumors in DBA/2 mice was characterized at the molecular level [4, 13]. An intriguing finding in this system was the detection of recurrent or public T cell clones that emerged independently in tumors from different animals. Apart from these public T cell clones, the T cell repertoire differed between the animals and thus accounted for the "private" component [13]. In the present study, more than 600 T cell clonotypes detected in 24 B16 melanomas induced in eight individual C57Bl/6 mice were systematically compared. However, none of the clonotypic TCR transcripts was detected in more than one animal. Thus, public T cell clones do not appear to participate in the response against B16 tumor cells. The reason for the discrepancy between the two models remains unknown. It could be speculated that public T cell clones would emerge at a later time. However, compared to the P815 system, the tumors reside in the animals for approximately the same period of time (10 days compared to 14 days). In the present study, we chose to sacrifice the animals after 10 days in order to analyze the early phases of the T cell response, as it is well established that heavy tumor burdens as well as long-lasting ongoing immune responses may induce changes in the T cell population [17]. Still, the sensitivity of the TCR clonotype mapping methodology and the fact that a large number of clones were detected strongly indicate that sufficient time was allowed for the response to develop.

One of the public T cell clonotypes in the P815 system recognized a peptide epitope encoded by a ubiquitously expressed protein harboring a point mutation [4], whereas the other public T cell clone recognized a peptide belonging to the group of cancer/testis-specific antigens [13]. The latter group of antigens is expressed by a variety of human tumor cells, but in most cases the murine counterparts have not been characterized. Whether epitopes derived from this group of antigens are expressed and presented by B16 cells remains to be established, but probably this group of antigens is more likely to select high-affinity TCR, than are the antigens from the group of differentiation antigens. The group of cancer/testis antigens has been demonstrated to be important rejection antigens in the human system [15].

Using TCR clonotype mapping we recently analyzed human melanoma lesions from two patients each presenting with three subcutaneous metastases. The results from these studies demonstrated that the systemic involvement is limited and that the in situ T cell response against human melanoma is mainly comprised of locally expanded T cells [29, 31]. These results have been strengthened by similar findings of Strohal and colleagues [34]. A major issue in the present study was to investigate whether re-circulating T cell clones participate in the response against coexisting tumor lesions in the murine B16 model. Among the more than 600 T cell clonotypes, only 13 were detected in more than one tumor from the same animal, and only five clones were detected in all three tumors. Interestingly, of the two clones detected in all three lesions of animal 4, one was also detected in PBL and skin. Furthermore, an exceedingly high number of expanded T cells was detected in the PBL of this animal. There were no signs of infections, inflammation or wounds, and the background for these findings thus remains unknown. Nevertheless, our data suggest that locally expanded T cells execute the T cell response against B16 melanoma, and that the majority of these T cell clonotypes do not recirculate and home to other metastatic sites.

Possibly, a high proportion of the T cell clonotypes involved in a cellular response are effector cells that are predestined to die by apoptosis, whereas only a minority of the cells possess the capacity to persist as long-term memory cells. The generation of memory T cells, and the

factors that influence the differentiation between effector and memory cells are poorly understood. However, an important difference between memory T cells and effector T cells is related to the different growth capacities of these cells. Possibly, the in situ T cell response against B16 tumors does not support the generation of memory T cells. Thus, the majority of the clonotypic T cells in TIL are effector T cells with a limited proliferative potential, which, in turn, implies that T cells leaving the local environment possess a finite capacity for further expansion. Accordingly, these cells will not expand to a detectable level upon recognition of antigen in distant metastases. This would also explain the repeatedly observed inability to grow in vivo expanded T cell clonotypes in vitro [8, 33].

The sequential induction of tumors did not appear to influence the T cell response to subsequent tumor challenge. However, the high growth rate of B16 cells implies that such a sequential induction must be conducted within a very limited period. Possibly this period is too short to have an effect on the immunological response. At least as judged by the numbers of TCR clonotypes in the lesions, no differences in the magnitude of T cell response were observed.

In summary, our data demonstrate a lack of public T cell clones in the T cell response to syngeneic B16 melanomas, and that the T cell clonotypes among TIL of B16 melanoma mainly comprise localized T cell clones. Furthermore, the systemic involvement of the response is limited, a characteristic shared with in situ T cell responses against human melanoma. Nevertheless, a few T cell clones could be detected in three tumors from the same animal, providing evidence for migration of clonally expanded T cells via the bloodstream.

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