

# $\alpha/\beta$ - and $\gamma/\delta$ TCR<sup>+</sup> lymphocyte infiltration in necrotising choroidal melanomas

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## Abstract

**Aim**—To detect specific tumour infiltrating T cells (TIL) carrying antigen specific MHC-I restricted receptor genes on necrotising and non-necrotising malignant melanomas and to correlate the findings with clinical data.

**Methods**— $\alpha/\beta$ - and  $\gamma/\delta$ - TIL were determined by immunohistochemical staining in melanomas of patients with known follow up of more than 10 years. An antigen retrieval method was used to determine variable genes  $\delta 1$  and  $\gamma 1$  on TCR<sup>+</sup> cells by an anti-TCR V $\delta 1$  and anti-C $\gamma$ M1, and of V $\alpha$  and V $\beta$  TCR<sup>+</sup> by an anti-pan-TCR<sup>+</sup>  $\alpha/\beta$  antibody.

**Results**—Intratumoral TIL were present in 86 of 113 (76.1%) necrotising melanomas (NMM) v 21 of 100 (21%) in non-necrotising melanomas (MM); of these, V $\alpha/\beta$ - TCR<sup>+</sup> cells were present in 52 of 74 (70.3%) TIL harbouring NMM v four of 21 (19%) MM; V $\gamma 1$  in 29 of 74 (39.2%) NMM v two of 21 (10%) MM; and V $\delta 1$  in 39 of 74 (52.7%) NMM v three of 21 (14%) MM. Extratumoral lymphocytic infiltration was seen in 86 (76.1%) NMM including V $\alpha/\beta$  TCR<sup>+</sup> cells in 10 (11.6%) cases, v five (5%) MM cases with no V $\alpha/\beta$  TCR<sup>+</sup> cells detected. V $\gamma 1$  and V $\delta 1$  TCR<sup>+</sup> cells were not found in extratumoral infiltrates.

**Conclusions**—In NMM, the median survival was 69.3 (range 6–237) months, 19 of 74 patients (25.7%) survived 5 years, and mortality was associated with advanced stage ( $p < 0.001$ ), patient age ( $p < 0.023$ ), and extent of necrosis ( $p < 0.048$ ). Survival was increased with evidence of V $\gamma 1$  and V $\delta 1$  TCR<sup>+</sup> cells ( $p < 0.026$ ).

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T cells can play a central role in the local immune response to cancer with tumour specific T lymphocyte reactivity provided by the TCR variable genes V $\alpha$ /V $\beta$  chain heterodimer or the V $\gamma$  or V $\delta$  chains. The identification and functional characterisation of MHC class I restricted antigen specific TCR expressing tumour infiltrating lymphocytes (TIL) has been the subject of intense research in the recent years, since tumour reactivity by these genes may provide novel applications for immunotherapy including the redirection of TIL specificity, and bone marrow stem cell therapy.<sup>1–5</sup>

$\gamma$  and  $\delta$  TCR<sup>+</sup> cells have been detected to selectively accumulate or to be amplified in non-ocular solid melanomas and are thought to indicate a unique type of host reaction

against the tumour.<sup>6</sup> Furthermore, tumour infiltrating cells expressing the  $\gamma$  and  $\delta$  TCR<sup>+</sup> CD3<sup>+</sup> complex have been found crucial for spontaneous regression of inheritable melanomas in the Sinclair miniature swine.<sup>7</sup>

Since the intraocular space contains a specific immunological microenvironment, the identification of the local T cell immune response with respect to the TCR gene repertoire seems warranted in the study of choroidal melanomas. In this retrospective study necrotising malignant melanomas of the choroid were (immuno)histochemically compared with melanomas without evidence of necrosis considering the existence of defined TIL. Factors predicting survival in patients with necrotising malignant melanomas were sought from clinical and histochemical data.

## Materials and methods

### SPECIMENS

In all, 701 paraffinised globes from the eye pathology laboratory at the University Eye Hospital Eppendorf, which had been enucleated from 1967 to 1998 and contained choroidal melanomas, were evaluated. One hundred and thirteen cases exhibited areas of necrosis, and were selected for further immunohistochemical comparison with 100 age matched specimens exhibiting no necrosis.

### SIZE AND PREPARATION

The tumours were more than 5 mm in diameter in most cases leaving only 11 (10.6%) necrotising melanomas (NMM) and three (3%) non-necrotising melanomas (MM) with a diffuse growth, and some difficulty of evaluation at different sites. Exclusion criteria for all specimens included prior radiation, brachytherapy, photocoagulation, or cryotherapy. Patient variables monitored were sex, interval from first diagnosis to enucleation, age at, and survival after, enucleation. Clinical data retrieval was complete in 74 patients.

### HISTOLOGY AND TUMOUR INFILTRATING LYMPHOCYTES (TIL)

The tumours were TNM classified. Necrotising malignant melanomas were grouped according to the extent of necrotic areas covering >95%, 60–95%, 30–50%, and <30% of 10 high power fields (hpf) at 40 $\times$ 10 magnification.

Lymphocytic infiltration was evaluated at the tumour base, the perivascular areas, in the parenchyme, and in a merging tumour free choroidal area according to Davidorf and Lang.<sup>8</sup> Lymphocyte infiltration was determined by light microscopy (hpf, magnification 40 $\times$ 10) in 10–15 different areas in a hpf, and

evaluated for 0, <30, 30–100, and >100 TIL. A strong infiltration was defined with two areas harbouring >100 TIL, medium one area >100 TIL or some areas 30–110 TIL, and slight infiltration one area 30–100 TIL or some areas <30 TIL.

#### DETERMINATION OF $\alpha/\beta$ AND $\gamma/\delta$ T CELL RECEPTOR TIL

Immunohistochemical staining was performed in 74 globes of 74 patients suffering from a NMM with a known further course of disease of more than 10 years. Formalin fixated and paraffinised specimens were prepared by an antigen retrieval method.<sup>9</sup> In brief after deparaffinisation and dehydration samples were flushed with distilled water for 5 minutes, incubated in diluted Antigen Retrieval Glyca solution in a microwave oven for 3 minutes at 600 W. Sections were cooled for 15 minutes at room temperature, flushed with distilled water, and put into phosphate buffered saline (PBS) at pH 7.4. For staining, anti-TCR V $\delta$ 1 and anti-C $\gamma$ M1 to determine variable genes  $\delta$ 1 and  $\gamma$ 1 on TCR<sup>+</sup> cells, and an anti-pan TCR<sup>+</sup>  $\alpha/\beta$  for identification of V $\alpha$  and V $\beta$  TCR<sup>+</sup> cells (T Cell Diagnostics, Inc, Cambridge, MA, USA) were done in addition to haematoxylin and eosin staining of specimens.

The primary antibodies pan  $\alpha/\beta$ , C $\gamma$ M1, and V $\delta$ 1 were incubated for 30 minutes at room temperature, and the sections were flushed in PBS 3  $\times$  5 minutes when biotinylated link antibody (Super Sensitive System, BioGenex, San Ramon, CA, USA) was added, and incubated for 20 minutes at room temperature. Sections were washed 3  $\times$  5 minutes in PBS, and the substrate alkaline phosphatase and fast red added, and incubated for 7 minutes at 37°C, then washed for 5 minutes in PBS. Counterstaining was performed with Mayer's haematoxylin and water soluble Aquatex was used. Light microscopy was performed for 10 different areas in a hpf (magnification 40 $\times$ 10).

Negative controls were non-immune serum of mice, and primary antibody substituted for a negative control serum. The sections were evaluated prospectively—for example, the course of the patient disease was not known at the time of histological evaluation.

#### STATISTICS

The study (NMM) and the control populations (MM) were analysed statistically by descriptive protocols. Evaluation included patient age, elapsed time between first diagnosis until enucleation, localisation of the tumour, stage, cell types, and area of necrosis.

Statistical evaluation was done at the institute for mathematics and biomedical data at the University of Hamburg. Significance of data disparities of the two populations was evaluated by  $\chi^2$  tests when single variables were compared. In ranking statistics of TIL and tumour growth the U test of Wilcoxon, Mann and Whitney or the H test by Kruskal and Wallis was used when two or more independent samples were compared. Patient and tumour

variables were estimated by the Cox proportional hazards model as relating to patient survival.

## Results

### PATIENTS

In 74 of 113 cases of NMM complete patient data could be evaluated for a median of 121.4 months (14–340 months) after enucleation.

The age of the patients with NMM was 28–92 years, median 66 years with no statistically different sex differences (64 v 67 years). The median age of patients with MM was 59 years (26–87 years), also without statistically significant sex differences (59 v 58 years).

Patients presenting with NMM were significantly older than those with MM ( $p < 0.005$ ): 31 (27.4%) patients with NMM presented at <60 years, 82 (72.6%) at >60 years, whereas 46 (46%) patients with MM presented at <60, 54 (54%) at >60 years.

Elapsed time between first diagnosis and enucleation for NMM was significantly longer compared with MM ( $p < 0.001$ ). Intervals for NMM exhibiting >95% necrotic areas were 12–24 months (40%), for NMM exhibiting 60–95% necrotic areas 25–48 months, and for NMM exhibiting <30% necrotic areas 1–10 months; for MM the interval was 1–12 months. In 17 patients (15.04%) with NMM an enucleation was performed only more than 2 years after presentation to an ophthalmologist owing to misleading diagnoses: in seven patients intraocular inflammations, in five patients vitreous haemorrhage, in another five patients secondary glaucomas in blind eyes had been diagnosed and not resulted in an adequate examination and treatment.

### NECROSIS

Areas covered by necrotic tumour tissue were evaluated for differences in NMM according to the grouping of <30%, 30–60%, 60–95%, and >95%: significances read  $p < 0.001$  in the <30% v >95% groups;  $p < 0.001$  in the 60–95% v >95% groups, and non-significant ( $p < 0.02$ ) in the <30% v 60–95% groups (Table 1).

### PATTERNS

NMM showed significantly more diffuse, rarely discoid patterns compared with MM ( $c_2$  8.68, v3,  $p < 0.05$ ). Most of the NMM were of the mixed and epithelioid cell types (54.9%), and most of the MM harboured spindle cells (51%) ( $c_2$  4.68,  $p < 0.05$ ). There was no statistically significant difference in the localisation of melanomas ( $c_2$  11.86,  $p > 0.25$ ) (Table 2).

### TUMOUR CHARACTERISTICS

Advanced tumour stages were found significantly more often in NMM. NMM at stages T3 and T4 comprised 45.14% and 36.28%

Table 1 Incidence of necrotic areas in 113 necrotising malignant melanomas of the choroid

Necrotic area (10 hpf, mag 40 $\times$ 10)	Cases (%)
>95%	25 (22%)
60–95%	18 (16%)
30–60%	18 (16%)
<30%	52 (46%)

Table 2 Localisation of extratumoral lymphocytic infiltrates in eyes harbouring a malignant melanoma of the choroid

Area of necrosis (n)	Localisation				
	Tumour	Choroid	Iris + CB	Sclera	Episclera
>95% (25)	25 (100%)	24 (96.0%)	24 (96.0%)	10 (40.0%)	18 (72.0%)
60–95% (18)	16 (88.9%)	15 (83.3%)	13 (72.2%)	8 (44.4%)	7 (38.9%)
30–60% (18)	17 (94.4%)	13 (72.2%)	12 (66.7%)	8 (44.4%)	6 (33.3%)
<30% (52)	28 (53.9%)	34 (65.4%)	23 (44.2%)	14 (26.9%)	6 (11.5%)
None	21 (21%)	14 (14%)	4 (4%)	3 (3%)	1 (1%)

respectively, T1 and T2 4.42% and 14.16% respectively compared with MM with 37% and 16% at T3 and T4 respectively and 15% and 32% at T1 and T2 respectively. Differences between MM and NMM with respect to the above stages were statistically significant ( $c_2$  21.79,  $v_3$ ,  $p < 0.005$ ).

Scleral invasion was significantly more frequent in NMM (76 cases, 67.3%) compared with MM (37 cases, 37%) (H8.66,  $v_2$ ,  $p < 0.025$ ). The incidence of extrascleral tumour growth (TNM stage S2) was significantly increased in NMM (43 cases, 38.1%) compared with MM (16 cases, 16%) ( $t_{2.91}$ ,  $p < 0.005$ ). Perivascular infiltration in NMM occurred more frequently (33 cases, 29.2%) than in MM (21 cases, 21%); however, statistical significance was low ( $p < 0.05$ ). Differences in the grouped data for scleral invasion, perivascular infiltration, and extrascleral tumour growth when comparing NMM and MM were highly significant ( $c_2$  21.1,  $v_2$ ,  $p < 0.005$ ).

#### TUMOUR INFILTRATING LYMPHOCYTES

Specimens of patients <60 years exhibited significantly more TIL than specimens of patients >60 years of age ( $u_{2.3}$ ,  $0.02 < p < 0.5$ ). NMM showed TIL in 86 cases (76.11%) and MM in 21 cases (21%). This difference was highly significant ( $c_2$  64.44,  $v_1$ ,  $p < 0.005$ ). The density of lymphocytic infiltration in NMM increased with increasing areas of necrosis ( $c_{235}$ ,  $v_9$ ,  $p < 0.005$ ).

Different tumour cell types were not associated with significant differences in TIL. However, in 62 NMM designated "more malignant" and bearing more than three mitotic figures per hpf, 43 (69.4%) had differing amounts of TIL, and 19 (30.6%) had no TIL. The mitotic activity of tumour cells correlated significantly with TIL ( $p < 0.01$ ).

Tumour diameter and prominence, as well as stages correlated positively with increased findings of TIL. Differences of TIL with respect to stages T1–3 and T4 (H6.91,  $v_2$ ,  $p < 0.05$ ) and stages T1–2 *v* T4 ( $t_{2.59}$ ,  $p < 0.02$ ) were statistically significant.

Table 3 Univariate analysis of factors of survival and mortality according to the Cox proportional hazards model

Risk factor	$\chi^2$ test	Probability (p value)
Tumour stage	20.23	<0.001
Patient age	9.54	0.023
No $V\gamma 1$ and $V\delta 1$ TCR <sup>+</sup> cells	9.25	0.026
Tumour necrosis	7.89	0.048
Lymphocytic infiltration	3.44	0.33
$V\alpha/V\beta$ -TCR <sup>+</sup> lymphocytes	2.84	0.2
Tumour cell type	2.28	0.52
Secondary glaucoma	0.045	0.83

#### INTRATUMORAL TCR<sup>+</sup> TIL

Immunohistochemical analysis identified the major proportion of TIL to exhibit characteristics of  $V\alpha/V\beta$  and  $V\gamma 1$  or  $V\delta 1$  TCR<sup>+</sup> cells in the 74 NMM specimens evaluated.

Thus, 52 of 74 (70.3%) evaluated specimens showed an infiltration with  $\alpha/\beta$  TCR<sup>+</sup> cells, and 29 of 74 (39.2%) an infiltration with  $V\gamma 1$  TCR<sup>+</sup> cells, and in 39 of 74 (52.7%) an infiltration with  $V\delta 1$  TCR<sup>+</sup> cells. The intratumoral infiltration with  $V\gamma 1$  and  $V\delta 1$  TCR<sup>+</sup> cells was significantly more frequent in the age group of less than 60 years ( $V\gamma 1$  TCR<sup>+</sup> cells: 18 62.1%,  $V\delta 1$  TCR<sup>+</sup> cells: 25 64.1%) ( $p < 0.01$ ).

In four of 21 (19%) MM with TIL, an infiltration with  $\alpha/\beta$  TCR<sup>+</sup> cells, in two of 21 (10%) an infiltration with  $V\gamma 1$  TCR<sup>+</sup> cells, and in three of 21 (14%) an infiltration with  $V\delta 1$  TCR<sup>+</sup> cells could be substantiated. The differences in incidences were non-significant.

#### EXTRATUMORAL TCR<sup>+</sup> LYMPHOCYTES

Extratumoral inflammation and necrosis was found in 86 specimens (76.1%) of NMM, and bystander cells in these areas were identified as lymphocytes, polymorphonuclear leucocytes, plasma cells, and macrophages correlating positively with increased tumour necrosis. The lymphocytes exhibited features of  $\alpha/\beta$  TCR<sup>+</sup> cells in 10 (11.6%) specimens, and but no  $V\gamma 1$  or  $V\delta 1$  TCR<sup>+</sup> cells were found. In our specimens, the staining of the latter cells was restricted to the tumours only.

The appearance of extratumoral necrosis was significantly correlated with the NMM; none of the specimens without necrotic areas in the melanomas exhibited extratumoral necrosis.

#### PATIENT SURVIVAL IN NMM

Complete postoperative data could be obtained in 74 of 113 specimens (65.5%) of patients with NMM, covering 121.4 months (31 years 14 months). Median survival was 69.3 months (6–237 months); 55 patients (74.3%) died of metastasising disease, four (5.41%) still survive with metastases. Three patients (4.05%) died for unrelated diseases, and in two (2.7%) the reason for death was unknown.

Only 10 patients (13.52%) survived without metastasis or tumour recurrence. Mortality was highest in the second year after enucleation (eight patients 14.5%); 31 (41.9%) died from metastatic disease up to 5 years.

Median survival in NMM was 4.69 years; 44 of 55 (81.8%) patients had died of hepatic, three (5.5%) intracranial, five (9%) bronchial, one (1.8%) osseous, and two (3.6%) of generalised metastases.

In the univariate analysis of all variables tested, the survival plots according to Kaplan–Meyer and the Cox proportional hazards model showed the strongest positive correlations with tumour stage, and patient age (Table 3, Figs 1–3).

No correlation was found for the detection of overall TIL and survival in the Cox model. If, however, data were split for  $\alpha/\beta$  TCR<sup>+</sup> cells, no significant correlations were found for  $\alpha/\beta$

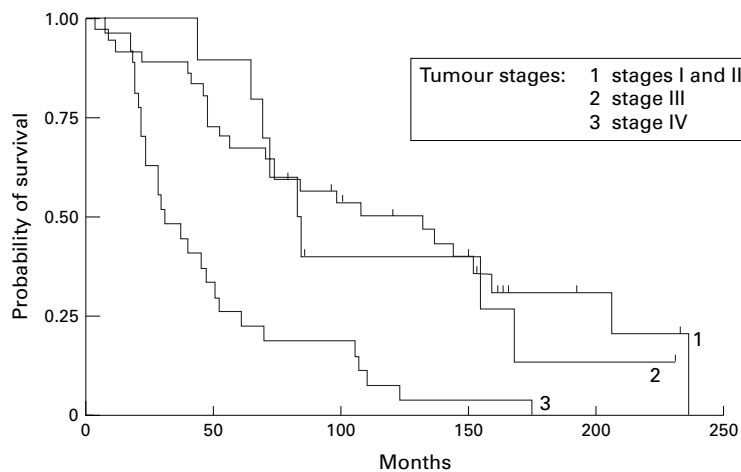


Figure 1 Kaplan-Meier plot showing significant negative correlation of tumour stage and patient survival ( $p<0.001$ ).

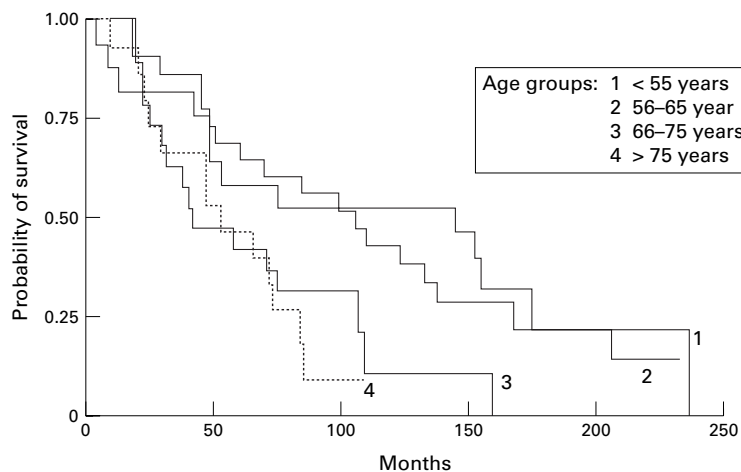


Figure 2 Kaplan-Meier plot showing significant negative correlation of patient age and patient survival ( $p=0.023$ ).

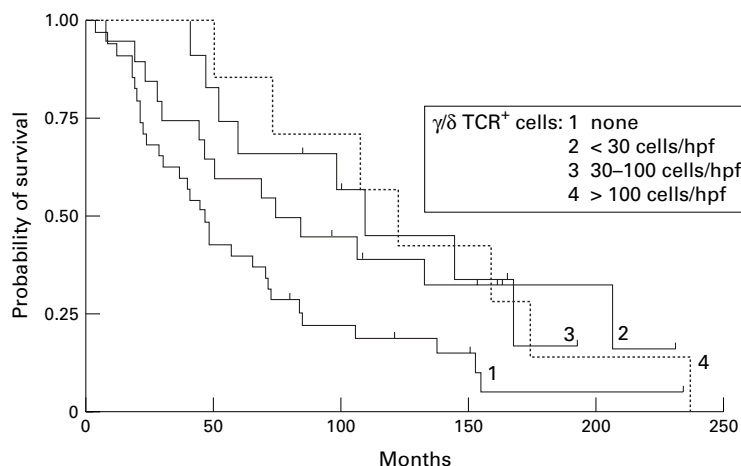


Figure 3 Kaplan-Meier plot showing significant positive correlation of  $V\gamma$  and  $V\delta$  TCR<sup>+</sup> cell infiltration and patient survival ( $p=0.026$ ).

TCR<sup>+</sup> cells, but a negative highly significant correlation was validated when  $V\gamma 1$  and  $V\delta 1$  TCR<sup>+</sup> cells had been observed in the tumours. Thus, infiltration with  $V\gamma 1$  and  $V\delta 1$  TCR<sup>+</sup> cells did represent a prognostically beneficial variable of high significance (Fig 3).

The extent of necrotic areas in tumours was highly significantly associated with mortality,

no significant correlation could be substantiated for tumour cell types.

A multivariate analysis showed that only the tumour stage and the infiltration by  $V\gamma 1$  and  $V\delta 1$  TCR<sup>+</sup> cells represented the most significant variables for patient mortality survival.

## Discussion

Spontaneous regression of necrotising melanomas has been documented in humans<sup>10 11</sup> and in animals.<sup>7</sup> Significant interest has focused on the precise definition of tumour infiltrating lymphocytes, since knowledge about these cells targeted by the bodily immune response may be a valuable tool for the development of new therapeutic strategies. Most of the research has been projected on MHC class I restricted  $V\alpha$  and  $V\beta$  TCR<sup>+</sup> cells.

Patient age at the time of diagnosis of every type of MM has been reported to be 55 years<sup>12 13</sup> correlating with the MM population in our study; however, most (72.4%) patients with NMM are significantly older (65.7 years). The history of symptoms and/or diagnosis was significantly longer in NMM, tumours are larger, and secondary lesions were more frequent.

In contrast with the study of Reese *et al*<sup>10</sup> this study has found a correlation of necrotic areas with tumour volume and extraocular growth. These differences may have been caused by the small number of cases investigated by Reese *et al* in 1970.

Survival has been reported to depend on tumour volume,<sup>14</sup> cell type,<sup>14 15</sup> extrascleral spread,<sup>16</sup> and elderly age.<sup>17</sup> The current study has confirmed these variables as most important for estimating patient survival.

Tumour infiltrating lymphocytes have been found at variable frequencies in 12.4–44.2% of cases, which had not considered NMM specifically.<sup>16 18 19</sup> Our results confirm earlier reports by Faber and Kock<sup>20</sup> on the frequent occasion of intratumoural TIL in NMM with an overall prevalence of 76% in the current, and 93% in the cited, study. In the current study, patients less than 60 years exhibited significantly more TIL than patients >60 years ( $p<0.05$ ); the density of TIL correlated with tumour volume, scleral invasion, and extraocular spread, a finding already mentioned by Davidorf and Lang.<sup>8</sup> Mitotic figures were more frequent in NMM with TIL, and 63% were of “more malignant” mixed or epithelioid cell types.

Intraocular cytotoxic lymphocytes have already been described to infiltrate large ocular melanomas and have been found to correlate with tumour mass.<sup>18–21</sup> Most TIL in choroidal melanomas have been described as CD2<sup>+</sup>CD3<sup>+</sup> T cells, CD8<sup>+</sup>, and few CD4<sup>+</sup> T cells and few B and CD16<sup>+</sup> NK cells<sup>21–26</sup>; however, TCR gene analysis of TIL has not yet been reported to our knowledge.

In the current evaluation 76.11% of NMM harboured intratumoural TIL, and 88.4% a peritumoural lymphocytic infiltrate. The area of necrosis and the extent of inflammation correlate positively ( $p<0.005$ ).

In most—for example, 52 of 74 cases (70.3%) of NMM reported in this study infiltration with  $\alpha/\beta$  TCR<sup>+</sup> cells could be verified. This observation is consistent with findings in solid melanomas, in which the TCR  $\beta$  chain was reportedly overexpressed in TIL compared with peripheral blood lymphocytes.<sup>27</sup> A clonal expansion or accumulation of melanocyte lineage specific and MHC class I restricted T cell clone probably occurs at the site of tumour growth. Functional aspects including affinity by surface resonance remain to be elucidated.

In this report, we have observed a substantial amount of TIL being V $\gamma$ 1 (29–39.2%) and V $\delta$ 1 (39–52.7%) TCR<sup>+</sup> cells. The exact role of V $\gamma$ 1 and V $\delta$ 1 TCR<sup>+</sup> cells in tumour immunology has still to be defined; however,  $\gamma/\delta$  TCR<sup>+</sup> cells may be stimulated to secrete lymphokines and may act as activated killer cells and exert some cytolytic activity on several tumours.<sup>1</sup> Extending earlier porcine<sup>7</sup> and human<sup>6, 28</sup> reports on the existence of these cells in melanomas and the occurrence of spontaneous regression of melanomas,<sup>11</sup> patient survival was analysed with respect to the infiltration by these cells. Interestingly, a highly significant positive correlation for survival could be defined. Considering also the observation that this infiltration was more evident in the younger patient group, studies on the antigen specificity of TCR<sup>+</sup> TIL in ocular melanomas seems warranted. Furthermore, it has to be clarified, if V $\gamma$ 1 and V $\delta$ 1 TCR<sup>+</sup> cells just accumulate in the tumours or if they are amplified in response to the expression of tumour specific ocular melanoma antigens.

In conclusion, this study has demonstrated the existence of tumour infiltrating  $\alpha/\beta$  and V $\gamma$ 1 and V $\delta$ 1 TCR<sup>+</sup> lymphocytes in a major proportion of necrotising choroidal melanomas, which were distributed in focal or diffuse patterns. The results have indicated that a selective repertoire of TCR genes was used in T cell mediated antinecrotising melanoma responses known to be MHC restricted and antigen specific. Since paraffin embedded specimens do not allow for a functional assessment of TIL, further studies have to focus on this aspect in fresh or frozen tissues. The precise characterisation of tumour infiltrating lymphocytes and their MHC restricted antigen specific function may result in novel immunotherapeutic approaches in the treatment of this tumour.

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