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ORIGINAL ARTICLE

Peripheral CD8$^+$ T cell proliferation is prognostic for patients with advanced thoracic malignancies

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Abstract There is a complex interplay between the immune system and a developing tumor that is manifest in the way that the balance of T cell subsets in the local tumor environment reflects clinical outcome. Tumor infiltration by CD8$^+$ T cells and regulatory T cells (Treg) is associated with improved and reduced survival, respectively, in many cancer types. However, little is known of the prognostic value of immunological parameters measured in peripheral blood. In this study, peripheral CD8$^+$ T cells and Treg from 43 patients with malignant mesothelioma or advanced non-small-cell lung cancer scheduled to commence palliative chemotherapy were assessed by flow cytometry and evaluated for association with patient survival. Patients had a higher proportion of peripheral Treg, proliferating CD8$^+$ T cells and CD8$^+$ T cells with an activated effector phenotype compared with age-matched healthy controls. Higher proportions of Treg and proliferating CD8$^+$ T cells were both associated with poor survival in univariate analyses (hazard ratio [HR] 3.81, 95 % CI 1.69–8.57; $p < 0.01$ and HR 2.86, 95 % CI 1.26–6.50; $p < 0.05$, respectively). CD8$^+$ T cell proliferation was independently predictive of reduced survival in multivariate analysis (HR 2.58, 95 % CI 1.01–6.61; $p < 0.05$). These findings suggest that peripheral CD8$^+$ T cell proliferation can be a useful prognostic marker in patients with thoracic malignancies planned for palliative chemotherapy.

Keywords Mesothelioma · Non-small-cell lung cancer · T cells · Prognosis

Abbreviations

CTL	Cytotoxic lymphocyte
ECOG	Eastern Cooperative Oncology Group
HR	Hazard ratio
MM	Malignant mesothelioma
NSCLC	Non-small-cell lung cancer
TAA	Tumor-associated antigen
TTP	Time to progression

Introduction

Malignant mesothelioma (MM) and advanced non-small-cell lung cancer (NSCLC) have an equally poor prognosis with a median survival of 8–12 months for patients beginning standard palliative platinum-doublet chemotherapy regimens [1–4]. Individual survival times, however, vary widely. Prognostic information is important for patients and their families, for clinicians when deciding on the most appropriate treatment option, and for the stratification of patients in clinical trials. Physical performance

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status, tumor stage and/or histology and presence of symptoms (chest pain, dysphagia and weight loss) are consistently associated with a poorer prognosis [5–9]. Some hematological parameters also predict shorter survival including high white blood cell and platelet counts, and low hemoglobin [7, 8]. Tumor metabolic activity, measured by fluorodeoxyglucose positron emission tomography (FDG-PET), also has prognostic value in subgroups of patients with MM and NSCLC [9, 10]. However, further prognostic markers are needed to more accurately predict outcome and help design appropriate therapies.

It is well documented that spontaneous anti-tumor immune responses occur, even in the context of progressive disease. In melanoma, for example, T cells specific for tumor-associated antigens (TAA) are detectable in around 50 % of patients [11, 12], and the presence of circulating TAA-specific T cells shows a strong positive correlation with survival in patients with metastatic disease [12]. The frequent observation of lymphocytic infiltrates within tumors provides further evidence of an interaction between the immune system and the developing tumor. Intratumoral $CD8^+$ T cell infiltration is associated with an improved prognosis in ovarian [13], colorectal [14] and esophageal cancer [15], while conversely, tumor infiltration by regulatory T cells (Treg), a subset of $CD4^+$ T cells that suppress immune responses, can predict a worse outcome [16–18]. Tumor-associated antigen (TAA)-specific cellular immune responses in MM and NSCLC have not been well characterized. However, tumor-infiltrating $CD8^+$ T cells have been shown to predict improved survival in early stage NSCLC following surgical resection and in MM following extra pleural pneumonectomy [19, 20]. Antibodies to mesothelin, a mesothelial cell differentiation antigen over-expressed in many cancer types, have also been detected in the sera of patients with MM [21].

Immunological parameters measured in peripheral blood potentially represent simple, relatively non-invasive prognostic markers. This is of particular importance in MM and advanced NSCLC where surgical interventions are not standard and tumor tissue samples are therefore often unavailable. Furthermore, peripheral blood is continuously accessible to determine longitudinal changes in response to therapy. The potential of peripheral biomarkers to predict clinical outcome following immunotherapy has recently been highlighted in renal cell cancer [22].

In this study, we assessed the proportion, activation status and proliferation of peripheral T cell subsets in 43 patients with MM or advanced NSCLC prior to platinum-based chemotherapy. Based on their respective roles as effectors and negative regulators of anti-tumor immunity, we hypothesized that increased $CD8^+$ T cell activation/proliferation and fewer Treg would predict improved outcome. We observed an increase in $CD8^+$ T cell proliferation and activation and a higher proportion of Treg in patients compared with a group of healthy controls and found no evidence of impaired $CD8^+$ T cell function in patient samples. Contrary to our hypothesis, a higher level of $CD8^+$ T cell proliferation and a greater proportion of Treg pre-chemotherapy were both associated with shorter survival, with $CD8^+$ T cell proliferation independently predictive of poorer prognosis. We propose that peripheral T cell proliferation may reflect more advanced disease and could prove a useful prognostic marker in MM and advanced NSCLC.

Patients and methods

Donors

Blood samples were collected from 43 patients diagnosed with MM or NSCLC, prior to commencement of standard platinum-doublet chemotherapy for advanced or subtotally resected disease. All patients were ≥18 years of age, had not received chemotherapy within the previous 3 months or oral/intravenous steroids within the previous 72 h, were not undergoing concurrent radiotherapy and had no known autoimmune disease. Patients were followed for disease progression and survival until 1 year after enrollment of the last patient. Overall survival was defined as the time from study enrollment to death. Time to progression (TTP) was defined as the time between study enrollment and the date of first observation of radiological progression, unequivocal clinical progression, or death without observed radiological progression. Patient characteristics are summarized in Table 1. Details of histological subtypes are provided in Supplementary Table 1 (Online Resource). Control blood samples were obtained from two cohorts: 14 volunteers from the University of Western Australia and 13 spouses of patients enrolled on the study. All patients and controls gave written informed consent to participate. The study was approved by the institutional Human Research Ethics Committee and was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonisation (ICH) Good Clinical Practice guidelines.

Cell preparation

PBMC were isolated by density gradient centrifugation from blood collected into BD Vacutainer® CPT™ Cell Preparation Tubes (BD Diagnostics, Australia) according to the manufacturer's instructions. Cells were resuspended at 2×10^6 cells/ml in RPMI-1640 (Invitrogen, Australia) supplemented with 10 % heat-inactivated FCS (Invitrogen,

Table 1 Patient Characteristics

	All patients ($n = 43$)	MM ($n = 27$)	NSCLC ($n = 16$)
Age (years), mean (range)	65 (42–81)	64 (45–78)	67 (42–81)
Female, *n* (%)	9 (21)	5 (19)	4 (25)
ECOG performance status, *n* (%)			
0	12 (28)	9 (33)	3 (19)
1	27 (63)	17 (63)	10 (63)
2–3	4 (9)	1 (4)	3 (19)
Tumor stage[a], *n* (%)			
I	3 (7)	3 (11)	0 (0)
II	2 (5)	2 (7)	0 (0)
III	13 (30)	9 (33)	4 (25)
IV	24 (56)	12 (44)	12 (75)
Unknown	1 (2)	1 (4)	0 (0)
Weeks since diagnosis, median (IQR)	6 (3–12)	4 (3–12)	6 (4–12)
Prior chemotherapy, *n* (%)	1 (2)	0 (0)	1 (6)
Prior surgical resection, *n* (%)	3 (7)	1 (4)	2 (13)
Overall survival (months), median (IQR)	10.7 (6.8–19.3)	10.7 (7.1–17.3)	9.5 (4.5–21.4)
TTP (months), median (IQR)	6.3 (3.5–8.6)	6.3 (5.1–8.3)	4.6 (2.6–11.9)

ECOG Eastern Cooperative Oncology Group, *IQR* interquartile range, *TTP* time to progression

[a] Tumors were staged according to the 1997 UICC International Union Against Cancer TNM classification system

Australia) and 10 % DMSO (Sigma-Aldrich, Australia) and cryopreserved until analysis.

Viral antigen-specific T cell expansion and restimulation

$CD8^+$ T cell responses to common viral antigens were assessed using the CEF Class I Peptide Pool Plus (Cellular Technology Ltd, Cleveland, OH, USA). This pool contains 32 peptides corresponding to HLA Class I–restricted epitopes from Cytomegalovirus, Epstein–Barr virus and Influenza virus, covering 15 HLA Class I alleles. Peptides were reconstituted and used according to the manufacturer's instructions. PBMC [2–5 $\times$ 10^6/ well in 24-well flat-bottom plates (Falcon, BD Biosciences)] were pulsed with CEF peptides for 1 h, then washed and cultured for 9 days in RPMI-1640 supplemented with 10 % non heat–inactivated FCS, 100 U/ml penicillin/streptomycin, 2 mM glutamax (all Invitrogen, Australia), 10 mM HEPES (Sigma-Aldrich, Australia), 50 uM 2-ME (Merck, West Point, PA, USA) and 25 IU/ml recombinant human interleukin 2 (rIL-2) (Roche Diagnostics, Australia). Expanded PBMC were seeded at 0.5 $\times$ 10^6 cells/well in 96-well U-bottom plates and incubated for 5 h with CEF peptides in RPM-1640 (Invitrogen, Australia) supplemented with 5 % heat-inactivated FCS (Invitrogen, Australia), 48 μg/ml gentamycin (Pharmacia and Upjohn, WA, Australia) and 60 mg/ml benzylpenicillin (CSL, VIC, Australia). Brefeldin A (BFA) (Sigma-Aldrich, Australia) at 10 μg/ml was added after the first hour. 2 μg/ml Leukocyte Activation Cocktail (BD Biosciences, Australia) was added to positive control wells and DMSO (Sigma-Aldrich, Australia) at a final concentration of 0.1 % (equivalent to that present in CEF-restimulated samples) was added to negative control wells. CEF-specific cells were identified by production of IFNγ by flow cytometric analysis. The proportion of CEF-specific $CD8^+$ T cells was calculated by subtracting the proportion of $IFN\gamma^+$ cells present after 5 h in negative control wells.

Flow cytometry

PBMC (0.5 $\times$ 10^6/well in 96-well U-bottom plates) were stained for expression of surface markers using specific anti-human monoclonal antibodies (mAb) against the following molecules: CD3 (SK7 or HIT3a), CD4 (RPA-T4), CD8 (SK3), CD38 (HIT2), CD127 (eBioRDR5) and HLA-DR (L243). Staining was performed on ice for 20 min, protected from exposure to light, in PBS with 2 % heat–inactivated FCS (Invitrogen, Australia), 1 % BSA (Sigma-Aldrich, Australia) and 0.01 % sodium azide (Sigma-Aldrich, Australia); PBS-FCS-BSA, following a 15 min incubation at 4 °C with human FcR blocking reagent (Miltenyi Biotec, Australia) to prevent non-specific antibody binding. After washing, cells were fixed and permeabilized using FACS lysing solution (BD Biosciences,

Australia) and permeabilization solution (eBioscience, Australia), then stained for intracellular marker expression using mAb against the following molecules: Bcl-2 (100), Ki67 (B56), Foxp3 (206D), IFNγ (4S.B3) and TNFα (MAb11) at RT for 30 min, protected from exposure to light in PBS–FCS–BSA. All antibodies were purchased from BioLegend, BD Biosciences or eBioscience and were directly conjugated to FITC, PE, PECy5.5, PECy7, APC, Alexa Fluor® 647 or APC-H7. Samples were stored in stabilizing fixative (BD Biosciences, Australia) and analyzed within 48 h. Data were acquired using Diva software on a FACSCanto II flow cytometer (both BD Biosciences) and analyzed using FlowJo software (Treestar Inc, Ashland, OR, USA). Compensation was performed post-acquisition using anti-mouse Ig and anti-rabbit/hamster Ig compensation particles (BD Biosciences, Australia) as positive and negative staining controls.

Statistical analysis

Statistical analysis was performed using SAS version 9.3 (SAS Institute Inc, Cary, NC, USA) and GraphPad Prism version 5.0 (GraphPad software Inc, San Diego, CA, USA). Differences between groups were determined using the student's *t*-test. Linear regression models were used to assess relationships between dependent and independent variables. Median overall survival and TTP were estimated using the Kaplan–Meier method with groups compared using the logrank test. Hazard ratios were determined using the Cox proportional hazards model. Differences and associations were considered significant where $p < 0.05$. Three investigators (MJM, RAL, AKN) formed structured hypotheses by consensus before data were collected, in order to investigate the relationships between immunological endpoints and clinical outcomes. Analyses were performed according to these pre-determined hypotheses only; therefore, correction for multiple comparisons was not conducted.

Results

Increased proliferating and effector $CD8^+$ T cells in patients compared with healthy controls

Proportions of $CD8^+$ and $CD4^+$ T cells, proliferating/effector $CD8^+$ T cells, Treg and proliferating Treg were compared to 27 healthy controls. Proliferating T cells were identified by intracellular staining for the nuclear protein Ki67, expressed by cycling and recently divided lymphocytes, but not naïve or resting cells [23] (Fig. 1b). T cells with an activated effector phenotype were identified by surface expression of the HLA class II molecule HLA-DR and the type II transmembrane glycoprotein CD38 (Fig. 1d). Both HLA-DR and CD38 are upregulated upon activation, and in combination, have been shown to accurately identify antigen-specific effector T cells following yellow fever and small pox vaccination [24]. Within the control group, whose mean age was significantly younger than the patient cohort (52.6 years, range 23–76 vs. 65.0 years, range 42–81; student's *t*-test $p < 0.0001$), the proportion of proliferating $CD8^+$ T cells was higher in those $\geq$50 years than in those <50 years ($p = 0.02$; data not shown). Therefore, only the $\geq$50 control group ($n = 15$) was used for analyses comparing proliferating/effector T cell populations. The mean age of these two groups did not differ significantly (63.1 ± 1.5 vs. 65.0 ± 2.3).

No difference in $CD8^+$ or $CD4^+$ T cells as a proportion of total lymphocytes was observed between patients and controls (data not shown). However, patients had significantly more proliferating ($Ki67^+$) and effector ($HLA\text{-}DR^+CD38^+$) $CD8^+$ T cells (Fig. 1a, c). In both patients and controls $HLA\text{-}DR^+CD38^+$ cells displayed clear downregulation of the anti-apoptotic protein Bcl-2, which is constitutively expressed by resting T cells, but downregulated upon antigen-driven activation [24, 25], providing further evidence that this cell population has an activated phenotype (Supplementary Figure 1; Online Resource). Proportions of proliferating and effector $CD8^+$ T cells were independent of platelet count or total globulin levels, suggesting that this is not simply a reflection of non-specific systemic inflammation (Supplementary Figure 2a–d; Online Resource). As expected, there was a strong correlation between platelet count and globulin levels (Supplementary Figure 2e; Online Resource). Patients also had a significantly larger population of $CD4^+Foxp3^+CD127^{lo}$ Treg than that of healthy controls (Fig. 1e), consistent with previous reports of increased peripheral Treg in cancer patients [26–28]. No difference in Treg proliferation was observed between patients and controls (data not shown).

Recall responses to common viral antigens are not impaired

Several previous studies have found peripheral T cells from patients with advanced malignancies to be deficient in their capacity to produce Th1 cytokines [29–31]. Where cell numbers permitted, we assessed IFNγ production following a 5-h incubation with PMA/ionomycin and following expansion and restimulation with a pool of peptides corresponding to HLA class I–restricted epitopes from common viral antigens (cytomegalovirus, Epstein–Barr virus and influenza virus; CEF peptide pool). All patient PBMC tested ($n = 18$) responded to PMA/

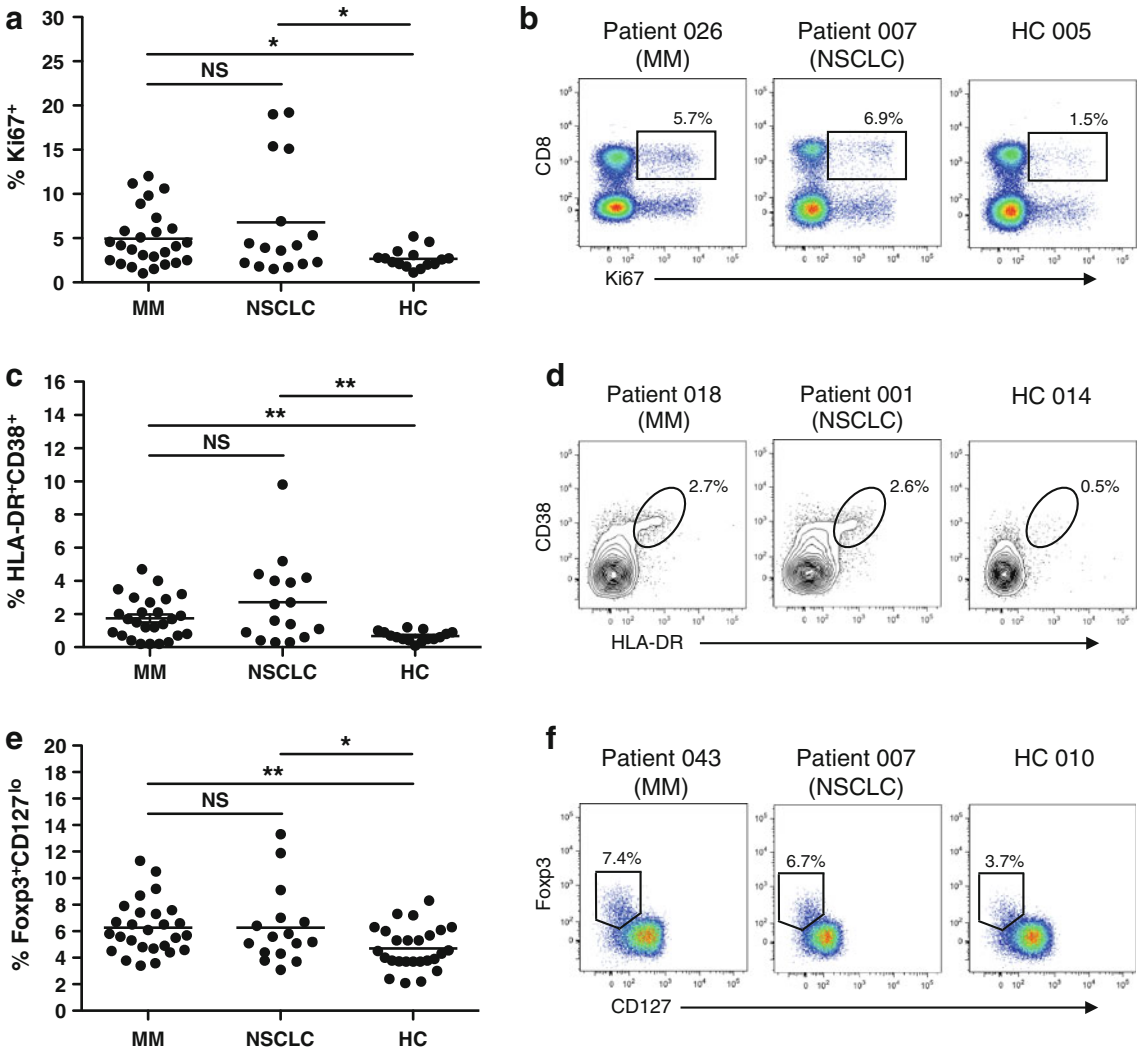
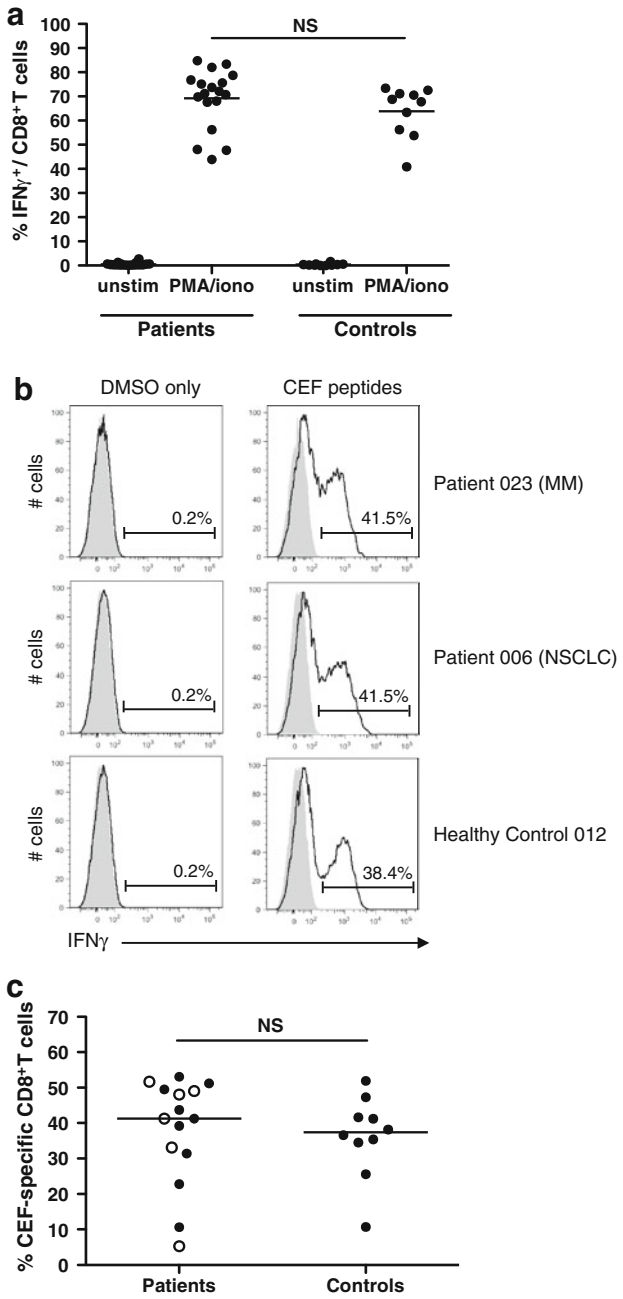


Fig. 1 Peripheral T cell populations in patients and healthy controls. Cryopreserved PBMC were thawed and stained for expression of surface and intracellular molecules to identify proliferating ($Ki67^+$) $CD8^+$ T cells (**a**, **b**), effector ($HLA\text{-}DR^+CD38^+$) $CD8^+$ cells (**c**, **d**) and Treg (**e**, **f**). **a**, **c** and **e** *Dots* represent individual patients/controls; *line* at mean. **b**, **d** and **f** Representative *plots* from two patients and a healthy control gated on $CD3^+$ lymphocytes (**b**), $CD3^+$-$CD8^+$ lymphocytes (**d**) and $CD4^+$ lymphocytes (**f**). Values represent percentage of $CD8^+$ T cells (**a**–**d**) or $CD4^+$ T cells (**e**, **f**). Groups were compared using the student's *t*-test. *NS*, not significant; *HC*, healthy controls. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

ionomycin with 44–85 % (mean 69 %) of $CD8^+$ T cells producing IFNγ. This was not significantly different from healthy controls ≥50 years of age (41–73 %, mean 64 %), demonstrating no deficiency in cytokine production capacity in the patient group (Fig. 2a). Responses to the viral CEF peptides were detected in 15/18 patients (83 %). The proportion of CEF-specific $CD8^+$ T cells identified in responding patient samples ranged from 5 to 53 % (mean 38 %), which was again comparable to the control group (11–52 %, mean 36 %) indicating that $CD8^+$ T cells from patients with MM, and NSCLC are not impaired in their ability to respond to recall antigens (Fig. 2b, c).

Baseline immunological parameters predict survival following chemotherapy

Cox proportional hazards regression analyses were performed to determine whether immunological parameters were associated with clinical outcome. Baseline clinical variables previously shown to have prognostic value in MM and NSCLC [6–8] and immunological variables selected according to pre-determined hypotheses were included in the regression models. Pre-determined hypotheses for selection of immunological variables were formed according to the respective roles of $CD8^+$ T cells and Treg in anti-tumor immunity, that is, effectors and



◀ **Fig. 2** Assessment of cytokine production capacity and ability to respond to recall antigens. **a** PBMC from 18 patients (11 MM and 7 NSCLC) and 10 healthy controls were incubated for 5 h with PMA and ionomycin in the presence of BFA, or with BFA only (unstim), and then, IFNγ production assessed by flow cytometry. *Dots* represent individual patients/controls; *line* at mean. Groups were compared using the student's *t*-test. *NS*, not significant. **b** and **c** Patient and healthy control PMBC were expanded and restimulated with CEF peptides (see Patients and Methods). Negative control samples (DMSO only) were expanded with the CEF peptides and then incubated with an equivalent volume of DMSO to that present in peptide-restimulated samples. **b** Representative *plots gated* on $CD8^+$ T cells; *shaded area* corresponds to the isotype control. **c** Percentage of CEF-specific $CD8^+$ T cells in patient versus healthy control samples; *line* at mean; groups compared using the student's *t*-test. In the patient group, *closed circles* represent patients with MM and *open circles* represent patients with NSCLC. % CEF-specific cells = % $IFN\gamma^+$ cells in CEF-restimulated sample—% $IFN\gamma^+$ cells in DMSO only control

suppressors of the response. These were that a greater proportion of proliferating or effector $CD8^+$ T cells and/or a lower proportion of Treg or proliferating Treg would be associated with improved outcome. Dichotomization of immunological variables was performed at the most significant cut-off point (proliferating $CD8^+$ T cells and total Treg), or the median value if no significant point of division could be found (effector $CD8^+$ T cells and proliferating Treg). As we observed no significance difference in proportions of these T cell subsets according to diagnosis, patients with MM and NSCLC were combined for survival analyses.

Contrary to our hypothesis, greater proportions of both Treg and proliferating $CD8^+$ T cells were predictive of poorer survival in univariate regression (hazard ratio [HR] 3.81, 95 % CI 1.69–8.57; $p < 0.01$ and HR 2.86; 95 % CI 1.26–6.50; $p < 0.05$, respectively; Table 2). The median survival of patients with a Treg population representing $\geq$7.5 % of total $CD4^+$ T cells was 5.3 versus 10.8 months in those with fewer Treg; $p < 0.001$; Fig. 3a. Patients with $\geq$2.9 % of $CD8^+$ T cells actively proliferating had a median survival of 7.9 months compared to 20.9 months in those with <2.9 % proliferating $CD8^+$ T cells; $p < 0.01$; Fig. 3b. Proportions of Treg and proliferating $CD8^+$ T cells were also predictive of survival when analyzed as continuous variables using univariate regression (Supplementary Table 2; Online Resource). Proliferating Treg and effector $CD8^+$ T cells did not impact significantly on survival (Table 2 and Fig. 3c, d). Of known prognostic factors, ECOG performance status and white blood cell count were the significant predictors of outcome (Table 2 and Fig. 4). No immunological or clinical variables were predictive of TTP or best radiological response determined according to the RECIST [32] or modified RECIST [33] criteria for patients with NSCLC and MM, respectively, (data not shown).

$CD8^+$ T cell proliferation is an independent prognostic factor

Multivariate analysis was performed using immunological variables significant in univariate analysis (proliferating $CD8^+$ T cells and Treg), together with clinical and demographic variables. The proportion of proliferating $CD8^+$ T cells was independently predictive of survival in multivariate regression (HR 2.58, 95 % CI 1.01–6.61; $p < 0.05$; Table 2). No clinical variables remained significant in the multivariate model. Again, analysis of continuous rather than dichotomized variables yielded similar results (Supplementary Table 2; Online Resource).

Table 2 Cox proportional hazards regression analysis for overall survival from enrollment

	Univariate			Multivariate		
	HR	95 % CI	*p*	HR	95 % CI	*p*
Proliferating $CD8^+$ T cells (%), high/low	**2.86**	**1.26–6.50**	**0.012**	**2.58**	**1.01–6.61**	**0.049**
Effector $CD8^+$ T cells (%), high/low	1.69	0.83–3.42	0.146	–	–	–
Treg (%), high/low	**3.81**	**1.69–8.57**	**0.001**	2.40	0.94–6.17	0.069
Proliferating Treg (%), high/low	0.73	0.36–1.48	0.377	–	–	–
Gender, male/female	0.48	0.18–1.25	0.132	0.70	0.18–2.65	0.594
Age (years), above/below median	1.22	0.60–2.47	0.586	0.84	0.38–1.89	0.662
ECOG status (0, 1 or 2–3)	**2.24**	**1.14–4.43**	**0.020**	1.69	0.76–3.78	0.200
WBC ($x10^9$/L), high/low	**2.09**	**1.02–4.29**	**0.045**	2.41	0.92–6.32	0.075
Hemoglobin (g/L), high/low	0.7	0.35–1.40	0.309	0.62	0.26–1.48	0.284
Platelets ($x10^9$/L), high/low	1.04	0.51–2.11	0.912	0.73	0.31–1.69	0.457
Diagnosis, MM/NSCLC	0.85	0.41–1.78	0.673	1.55	0.63–3.85	0.344

All categorical covariates were transformed into numeric codes before entering into the model. Immunological variables were divided into high and low at the point demonstrating the strongest association with survival in univariate analysis (data-driven dichotomization) or the median if no significant association was found. Only those significant in univariate analysis were included in the multivariate model. Clinical variables were dichotomized at the median

Bold values denote significant associations ($p < 0.05$)

CI confidence interval, *ECOG* Eastern Cooperative Oncology Group, *HR* hazard ratio, *WBC* white blood cell count

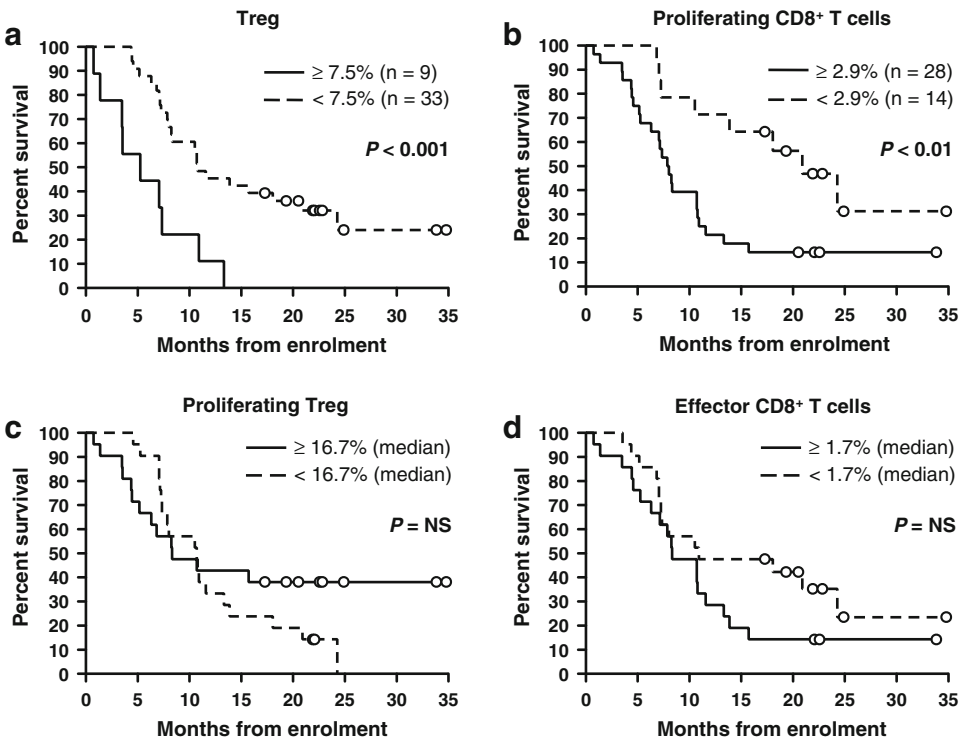


Fig. 3 Kaplan–Meier plots of overall survival by proportion of Treg (**a**), proliferating ($Ki67^+$) $CD8^+$ T cells (**b**), proliferating Treg (**c**) and effector $CD8^+$ T cells (**d**). Data were dichotomized at the point demonstrating the strongest association with survival in univariate regression analysis (**a**, **b**) or the median value if no statistically significant cut-off point could be found (**c**, **d**). For comparison, median and 95th percentile values for healthy controls were as follows: Treg 4.4, 7.3 %. Proliferating $CD8^+$ T cells 2.6, 5.2 %. Proliferating Treg 18.5, 30.2 %. Effector $CD8^+$ T cells 0.6, 1.4 %. Groups were compared using the logrank test. *Circles* represent censored observations

Discussion

It is now widely recognized that there is a dynamic interaction between the adaptive immune system and a developing tumor. $CD8^+$ cytotoxic T cells are the key effectors of an anti-tumor immune response, Treg down modulate this response. While tumor infiltration by $CD8^+$ T cells and Treg is associated with an improved and poor prognosis, respectively, in many cancer types [13–18, 20], little is known about the relationship between immunological parameters measured in peripheral blood and clinical outcome. In the current study, we assessed whether the

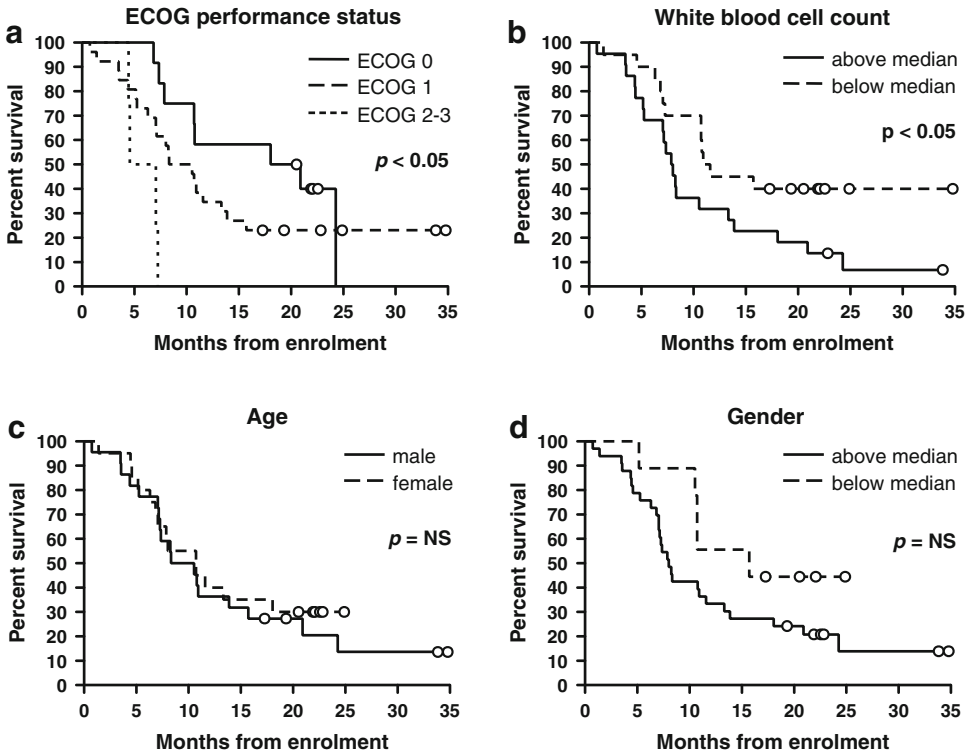


Fig. 4 Kaplan–Meier plots of overall survival by ECOG performance status **a**, white blood cell count **b**, age **c** and gender **d**. Groups were compared using the logrank test. *Circles* represent censored observations

proportion, activation status and extent of proliferation in peripheral T cell subsets provided any evidence for an underlying anti-tumor immune response in a cohort of patients with MM and NSCLC, and whether such parameters could be useful in predicting survival following chemotherapy.

Patients were found to have a significantly increased proportion of proliferating ($Ki67^+$) $CD8^+$ T cells, effector ($HLA\text{-}DR^+CD38^+$) $CD8^+$ T cells and $CD4^+Foxp3^+$ $CD127^{lo}$ Treg compared with healthy controls. Consistent with these data, there have been several previous reports of increased peripheral Treg in cancer patients, including those with NSCLC [26–28]. Increased peripheral T cell activation and proliferation relative to healthy donors has been observed in B-cell lymphoma [34, 35], and higher proportions of apoptotic T cells have been found in the blood of patients with head and neck cancer and melanoma [36], suggested by the authors to reflect increased T cell activation. This is, however, to the best of our knowledge, the first report of increased proliferating and effector T cells in patients with thoracic malignancies. Interestingly, proportions of proliferating and effector T cells did not correlate with markers of non-specific inflammation (platelet count and globulin levels). These results could be explained by the presence of an underlying anti-tumor immune response blocked by the concurrent expansion of Treg, and at least suggest that cancer is associated with increased peripheral T cell activity. The investigation of tumor-specific immune responses in MM and NSCLC, however, is hampered by the paucity of defined TAA CTL epitopes, and we were therefore unable to draw any conclusions regarding the specificity of these cells. We also acknowledge the potential dissociation between activated effector phenotype and effector function. While we found no evidence for a detrimental effect of malignancy on Th1 cytokine production capacity or on the ability of $CD8^+$ T cells to respond to antigenic stimulation in this patient cohort, limited cell numbers precluded the use of co-culture experiments to assess cytolytic or suppressor function. In a future similar study, it would be useful to evaluate the expression of additional molecules associated with $CD8^+$ T cell activation and/or effector function such as CD137 (4-1BB), which is upregulated upon activation and its ligation known to enhance effector function and promote anti-tumor immunity [37, 38], and programmed death-1 (PD-1), which is upregulated following chronic antigen exposure and negatively regulates T cell function and anti-tumor immune responses [39–41].

Few studies to date have investigated the relationship between immunological parameters measured in peripheral blood and clinical outcome. In the current study, higher proportions of both Treg and proliferating $CD8^+$ T cells at baseline were associated with poorer survival in patients who subsequently received chemotherapy. Baseline $CD8^+$ T cell proliferation was independently predictive of survival in multivariate analysis. The association between increased peripheral Treg and poorer survival is consistent with the suppressive role of this population in anti-tumor

immunity. Tumor infiltration by Treg has been shown to predict reduced survival in several cancer types including NSCLC [16–18] and the proportion of peripheral Treg at baseline was negatively associated with survival in a cohort of patients with various metastatic cancers receiving immunotherapy with DC-activated lymphocytes and/or tumor-pulsed DC [42]. Our finding that higher baseline $CD8^+$ T cell proliferation also predicts reduced survival seems counter intuitive, but is not without precedent; tumor infiltration by proliferating $CD8^+$ T cells was independently predictive of reduced survival following surgical resection in patients with stage I-IIIa NSCLC [43] and activation marker expression by peripheral T cells has been associated with poor prognosis in head and neck cancer and melanoma [44, 45]. We propose that increased immune activity in cancer patients may reflect more advanced disease, representing a continuing attempt by the immune system to mount an anti-tumor response, blocked by the concurrent expansion of Treg. As staging information is not comparable between patients with MM and those with NSCLC, and no other measure of tumor burden was available, no direct measure of the extent of disease could be included in the multivariate analyses to disprove this theory. Patient numbers precluded performing survival analyses by disease group. However, the presence of tumor infiltration by both $CD8^+$ T cells and Treg has been associated with poor prognostic factors in breast cancer, including negative hormone receptor status, high tumor grade and lymph node involvement [46]. The proportion of melanoma patients mounting a spontaneous NY-ESO-1-specific antibody response also increases with disease stage [47], and NY-ESO-1-specific T cell responses are known to develop alongside antibody-mediated responses [48, 49]. In the current study, we chose to focus on the role of $CD8^+$ T cells and Treg as effectors and negative regulators of the anti-tumor immune response, respectively. Investigation of multiple additional parameters would have undermined the statistical power with a cohort of this size. However, this is not to say that other cell types such as $CD4^+$ T helper cell subsets and myeloid-derived suppressor cells (MDSC) do not play an important role in regulating anti-tumor immune responses in MM and NSCLC. These populations and other immunological parameters including serum cytokine levels may have prognostic or predictive value, as has been shown in other cancers [22, 50, 51].

We acknowledge a number of potential weaknesses in this study. Firstly, the patient group was heterogenous in disease type, including both MM and NSCLC. However, both are thoracic malignancies treated with platinum-based chemotherapy regimens, and as there is substantial molecular and clinical heterogeneity within both MM and NSCLC, neither could be considered 'one disease'. Furthermore, since only NSCLC patients with advanced, unrespectable disease were included, disease trajectory and survival are similar. No significant differences in T cell subsets were observed according to diagnosis. The vast majority of our patient group (93 %) underwent subsequent chemotherapy; hence, it is possible that our findings are predictive rather than prognostic, until validated in a group not undergoing treatment or treated surgically. Finally, patients underwent diverse subsequent chemotherapy regimens ($n = 19$ (MM) pemetrexed with cisplatin, $n = 6$ (MM) pemetrexed with carboplatin, $n = 12$ (NSCLC) gemcitabine with carboplatin and $n = 3$ (NSCLC) paclitaxel with carboplatin), although each has shown similar survival benefits in clinical trials, and there were no detectable survival differences between treatment groups in our study.

The results of this study suggest that thoracic malignancies are associated with increased peripheral Treg, proliferating $CD8^+$ T cells and $CD8^+$ T cells with an activated effector phenotype. Our finding that peripheral $CD8^+$ T cell proliferation is prognostic for poorer survival is novel and, if validated in an independent study, this may represent a useful prognostic marker in patients with MM and advanced NSCLC receiving palliative chemotherapy.

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Conflict of interest The authors declare that they have no conflict of interest.

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