# Marrow infiltrating regulatory T cells correlate with the presence of dysfunctional CD4+PD-1+ cells and inferior survival in patients with newly diagnosed multiple myeloma

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## Translational Relevance (149 words)

Multiple myeloma(MM) is the second commonest haematological malignancy and remains incurable. Beyond tumour biology and genomic features driving disease resistance, host factors including impaired immunity and frailty also contribute to poor outcomes. Despite reports of immune dysfunction in this cancer, clear evidence for the contribution to clinical outcomes remains lacking.

We show, for the first time, that high abundance of Treg and PD-1+CD4 effector cells in bone marrow of newly diagnosed patients are independent predictors of early relapse. This work supports growing literature on the importance of CD4 effector cells in MM, and confirms a role for the PD-1/PD-L1 axis to MM pathobiology.

Our work identifies Tregs and PD-1+CD4 effectors as potential therapeutic targets, and opens up avenues for further mechanistic studies into early relapse. Pending confirmation in future patient cohorts, such immune parameters may refine existing risk models, facilitating patient stratification for therapeutic strategies targeting key CD4 populations.

**Abstract (250)**

**Purpose:** Immune dysregulation is described in multiple myeloma(MM). While preclinical models suggest a role for altered T cell immunity in disease progression, the contribution of immune dysfunction to clinical outcomes remains unclear. We aimed to characterise marrow infiltrating T cells in newly diagnosed patients and explore associations with outcomes of first line therapy.

**Experimental Design:** We undertook detailed characterisation of T cells from bone marrow(BM) samples, focusing on immune checkpoints and features of immune dysfunction, correlating with clinical features and progression free survival.

**Results:** We found that patients with MM had greater abundance of BM regulatory T cells (Tregs) which, in turn, expressed higher levels of the activation marker CD25 compared to healthy donors. Patients with a higher frequencies of Tregs ($Treg^{hi}$) had shorter PFS, and a distinct Treg immune checkpoint profile (increased PD-1, LAG-3) compared to $Treg^{lo}$ patients. Analysis of CD4 and CD8 effectors revealed that low CD4effector:Treg ratio, and increased frequency of PD-1 expressing $CD4^{eff}$ cells were independent predictors of early relapse over and above conventional risk factors such as genetic risk and depth of response. Ex-vivo functional analysis and RNA sequencing revealed that CD4 and CD8 cells from patients with greater abundance of $CD4^{eff}$PD-1+ cells displayed transcriptional and secretory features of dysfunction.

**Conclusions:** BM infiltrating T cell subsets, specifically Treg and PD-1 expressing CD4 effectors, negatively influence clinical outcomes in newly diagnosed patients. Pending confirmation in larger cohorts and further mechanistic work, these immune parameters may inform new risk models, and present potential targets for immunotherapeutic strategies.

## INTRODUCTION

Multiple myeloma (MM) is a common cancer of plasma cells (PC) which is responsible for 2% of cancer deaths(1). Despite significant progress seen with the inclusion of proteasome inhibitors (PIs) and immunomodulatory drugs (IMiDs) into the mainstay of treatment regimens(2), myeloma remains almost universally incurable. Along with intrinsic drug sensitivities of tumour cells, and genomic drivers of clonal evolution, host factors, including immunological fitness and function, likely also influence clinical outcomes of treatment.

Accumulating evidence points towards a global immune dysregulation in MM including impaired antigen presentation(3), impaired T cell effector function(4) with accumulation of suppressive cell types(5,6). These mechanisms appear to converge on disabling T cell driven anti tumour immunity(7) and accordingly alterations in T cell phenotype and function have been consistently reported in models of MM. Firstly, T regulatory cells (Tregs) suppress T cell cytotoxicity and have been reported to be an important driver of disease progression(8). Secondly, there appears to be a relative reduction in cytotoxic T cells relative to Tregs(8). Thirdly, checkpoint proteins, such as the co-inhibitory receptor, PD-1, are reported to be expressed on T cells from MM patients (9,10) with increased expression of its ligand PD-L1 on tumour cells (11). Despite these reports, the influence of these alterations to T cell phenotype on patient outcomes remains to be clarified. Data regarding Treg numbers and relationship to clinical outcomes are conflicting (12–14) and reports of increased PD-1 on T cells from MM patients have not been generally corroborated or correlated to outcome(15). Reasons for these discrepancies include different assay systems, examination of peripheral blood versus marrow or the use of heterogenous patient cohorts. Many studies included relapsed refractory patients, where the host immune system is likely to be affected by prior therapies, repeated infection and advanced disease.

In order to resolve some of these issues, we investigated the marrow infiltrating T cell populations in untreated MM patients, with focus on Tregs and co-inhibitory receptors seeking to understand the influence of these recognized suppressive T cell populations on the clinical outcomes of first line treatment.

## METHODS

### *Patients and controls*

BM aspirates were obtained from newly diagnosed (ND) MM patients with written informed consent (Research ethics committee reference: 07/Q0502/17). Control BM aspirates (n=15) were collected from healthy volunteers undergoing BM harvesting with Anthony Nolan, and subjects undergoing bone marrow sampling who had no haematological diagnosis (Supp. Table 1)(REC reference: 15/YH/0311). All BM samples were collected in ethylenediamine-tetraacetic acid (EDTA) and processed within 24 hours. Patients were considered to have adverse risk disease if fluorescent-in-situ-hybridisation (FISH) demonstrated one of: t(4,14), t(14,16), t(14,20), and del(17p).

### *Isolation of mononuclear cells from bone marrow aspirates*

BM mononuclear cells (MNCs) were isolated by Ficoll Paque (GE Healthcare) centrifugation and cryopreserved in foetal bovine serum (FBS) (Gibco) containing 10% DMSO (Sigma Aldrich). Aliquots were subsequently thawed for antibody staining and flow cytometry, functional studies or RNA sequencing.

### *Flow cytometry analysis*

Surface antigen staining was performed using the fluorochrome conjugated antibodies CD3, CD4, PD-1, ICOS, CD25, CD33, CD11b, CD8, LAG-3, CD4, CD14, CD45RA, CCR7, and fixable viability dye-e780. For intracellular staining, cells were fixed/permeabilized using the FoxP3 Transcription Factor Staining Buffer Set (eBioscience), then stained with Foxp3, CTLA-4, Ki-67 and GzmB. Details of all antibodies are in Supp. Table 2. Data acquisition was on a BD LSR II Fortessa (BD Biosciences).

### *Cytokine stimulation experiments*

Cryopreserved BM MNCs were thawed and cultured at 0.5x16^cells/mL in RPMI (Lonza), 20%FBS (Gibco), and 1%Penicillin/Streptomycin (Gibco) (complete medium), at $37^0$C with soluble anti-CD3 (OKT3) and anti-CD28 (0.5 µg/ml, 15E8; Miltenyi Biotec). GolgiPlug (1 µl/ml, BD Biosciences) was added for last 4 hours of incubation. Cells were then stained for surface markers, CD4, CD8, CD69, and

fixable viability dye, washed and fixed/permeabilised for staining for intracellular TNF-alfa, IFN-gamma, IL-2, and FoxP3 (Supp. Table 2).

### *RNA sequencing and analysis*

RNA was extracted from flow sorted CD3+CD4+ and CD3+CD8+ cells from BM MNCs using ReliaPrep™ RNA Cell Miniprep System (promega). cDNA libraries were prepared using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech Laboratories, Inc.). Samples were sequenced on two lanes of the HiSeq 3000 instrument (Illumina, San Diego, US) using a 75bp paired end run at UCL Institute of Child Health.

RNAseq data were processed with a modified version of the nextflow nf-core RNAseq pipeline (https://github.com/nf-core/rnaseq). Reads were trimmed with TrimGalore v0.4.1, aligned against hg19 with STAR v2.5.2a, and duplicated reads were marked with Picard v2.18.9. Read counts per gene were generated with featureCounts v1.6.2 and used for differential gene expression analysis. Gene set enrichment analysis (GSEA) was run using Gene Ontology (GO) pathways and previously reported sets of genes differentially expressed by dysfunctional CD4(16–18) and CD8 T cells(19,20). Human orthologues of mouse genes were identified using Ensembl and NCBI HomoloGene databases.

### *Statistical analysis*

Progression free survival (PFS) was defined as time from start of first line therapy to first progression or death (as per International Myeloma Working Group criteria(21)). Flow cytometric data were analysed with FlowJo version 10 (Tree Star Inc). The percentage of a cell population expressing any given marker is designated as “frequency” (of that marker) within the relevant Treg, CD4 effector, or CD8 populations. Statistical analyses were performed with GraphPad Prism software (Prism 7). P values were calculated using Mann-Whitney U test. PFS was estimated using Kaplan-Meier methods with log-rank test. A multivariate Cox regression model was used to evaluate the independent contribution of variables. All tests of significance were 2-sided and p values ≤0.05 considered statistically significant.

## RESULTS

### *Patient characteristics and treatment outcomes*

Seventy-eight NDMM patients were identified, with median age 59 years (35-86), 64.1% were male (Supp Table 3). FISH defined genetic risk was available in 74 patients, of whom (19.2%) were adverse risk. All patients commenced active treatment, most (68, 87.18%) with proteasome inhibitor regimens, and 25 (31.25%) underwent autologous stem cell transplant (ASCT). Overall response rate (ORR) was 87%, and 53.8% achieved complete response/very good partial response (CR/VGPR). With median follow up of 22 months (1-43), median PFS was not reached (NR). There was a trend for improved PFS with standard risk genetics (p=0.075 cf high risk), ASCT (p=0.06), and in patients with deeper response (CR/VGPR vs. rest, p=0.09) (Supp Fig. 1).

### *BM of newly diagnosed MM patients contains high frequency of Treg cells*

We first examined the relative frequencies of T cell subsets in the BM of MM patients (gating strategy in Fig. 1A). While the frequencies of CD3, CD4 and CD8 cells were comparable to healthy donors (HD, Supp Fig. 2A), the frequency of Treg cells (CD4+FoxP3+) was significantly higher in BM of MM patients (0.51% of live MNCs, vs 0.07% in HD; p=<0.0001, 3.33% of CD4+ cells vs 1.13%; p=0.0006) (Fig. 1B). This was also the case when Treg cells were identified as CD4+CD25+FoxP3+ (3.41% of CD4 cells in MM BM vs 1.27% in HD; p=0.001) (Fig. 1B).

The balance between Tregs and effector T cells shapes the anti-tumour immune response (22). We defined CD4 effectors ($CD4^{eff}$) as CD4+FoxP3- cells, and observed that the $CD4^{eff}$:Treg ratio in MM patients was significantly lower when compared with HD (20.83 vs 140.2; p=<0.0001), this was also the case for the CD8:Treg ratio (36.34 vs 170.4; p=<0.0001) (Fig. 1C). We found no correlation between Treg cells, $CD4^{eff}$:Treg ratio or CD8:Treg ratio with percentage of plasma cells in BM (Supp Fig. 3A). Neither did we find any correlation of CD4:CD8 ratio with plasma cell infiltration.

### *Higher frequency of Treg cells is associated with a shorter progression free survival*

We sought to determine whether the presence of Treg cells in the BM of newly diagnosed patients had any influence on clinical outcomes. We used PFS, a

common primary endpoint for studies in MM patients (23). Identifying Treg as CD4+FoxP3+ cells, we observed that MM patients with a high frequency of Tregs (>median, $Treg^{hi}$) had significantly shorter PFS when compared to MM patients with low frequency of Treg (≤median, $Treg^{lo}$) (HR:2.91; 95%CI 1.21-7.04; p=0.021) (Fig. 2A). Similar findings were also seen when Tregs were identified as CD4+FoxP3+CD25+ cells (p=0.022, Supp Fig. 2B). We used *surv_cutpoint* function from the 'survminer' R package (https://github.com/kassambara/survminer) to determine the optimal cut off value for Treg frequency, and ascertained this to be 3.31%, which is the median value.

Having noted that the ratios of effector cells to Treg in MM patients are low compared with HD, we next examined the association with PFS. We observed that patients with low $CD4^{eff}$:Treg ratio (≤median) had significantly shorter PFS compared to high $CD4^{eff}$:Treg ratio (>median) (HR:4.22; 95%CI 1.79-10.15; p=0.005) (Fig. 2B). There was a weaker association of CD8:Treg ratio with PFS (p=0.067) (Fig. 2B). Triple colour immuno-histochemistry (IHC) was performed on BM trephine biopsies to confirm presence of Tregs in representative $Treg^{hi}$ and $Treg^{lo}$ patients (Fig. 2C). There were no associations between CD4 effectors, CD8 cells or CD4:8 ratio with PFS (Supp Fig. 2B).

### *Activation status of Treg cells*

We next examined the phenotype of marrow infiltrating Tregs, to better understand their influence on clinical outcomes. We observed higher expression of CD25 on Tregs from MM patients compared to HD suggesting higher level of activation of MM Tregs (Fig. 3A), as CD25 expression is associated with Treg activity and suppressive function(24). In this cohort of MM patients, both the abundance of $CD25^{hi}$ cells and expression intensity of CD25(MFI) was greater amongst Tregs compared to CD4 effectors and CD8 T cells (Fig. 3B). While there were no significant differences in frequencies of PD-1, LAG-3, or CTLA-4 on Tregs from MM patients compared to HD (Fig. 3A), there was a greater frequency of PD-1 and LAG-3 on Tregs from $Treg^{hi}$ patients compared to $Treg^{lo}$ (Fig. 3C). These differences in checkpoint protein expression suggest that functional as well as quantitative features of marrow infiltrating Tregs in MM patients may be important (25,26). We further explored the differentiation status of BM Tregs in a separate cohort of newly diagnosed MM

patients, observing that the majority are CD45RA- indicating that marrow Tregs in these patients have an activated phenotype (Fig. 3D).

### *Expression of immune checkpoint proteins on CD4 and CD8 effector cells in MM patients*

Next we asked if altered Treg frequency and activation state was reflected in effector T cell function in MM BM. Examining co-inhibitory and co-activation receptors on $CD4^{eff}$ and CD8 T cells, we observed that frequencies of LAG-3 and Ki-67 were higher on both $CD4^{eff}$ and CD8 T cells from MM patients compared to HD (p=0.001, p=0.009, p=0.0001, p=0.0001 respectively) (Fig. 4A and 4B) with no significant differences in ICOS or CTLA-4 (Fig. 4A and 4B). In addition a higher percentage of CD8 T cells from MM patients expressed PD-1 (p=0.045) and the cytotoxic granule GzmB compared to HD (p=0.01)(Fig. 4B). There was no correlation between the frequency of any co-inhibitory or co-activation receptor on $CD4^{eff}$ or on CD8 T cells with disease burden in the BM, except for frequency of LAG-3 on CD8 T cells (r=0.27, p=0.028; supp Fig. 3B).

Notably, we observed a positive correlation between Treg frequency and the fraction of PD-1+ $CD4^{eff}$ and CD8 cells (Supp Fig. 4), but no correlation with the frequency of any other co-inhibitory or co-activation receptors. Accordingly, PD-1 expression on CD4 effectors also correlated with PD-1 on CD8 cells (Supp Fig.4), and a positive correlation was also noted between PD-1 expression on Treg and on CD4 effectors (Supp Fig.4D). To understand the relationship between PD-1 expression and differentiation status of marrow infiltrating effector cells, we further studied a similar cohort of newly diagnosed MM patients. Interestingly, while terminally differentiated effector memory cells re-expressing CD45RA (TEMRA) comprise a large proportion of CD8 cells, this subset comprises only a minority of CD4 effectors, with the effector memory (EM) subset being dominant in most patients(Supp Fig. 5A). PD-1+CD4 effectors were enriched for central memory (CM, CCR7+CD45RA-), and effector memory (EM, CCR7-CD45RA-) cells when compared with PD-1-CD4 effectors(Supp Fig. 5B).

Finally, the frequency of monocytic myeloid-derived suppressor cells (M-MDSCs) in the BM of MM patients was higher when compared to HD (p=0.006 , Supp Fig. 6A).

The frequency of M-MDSCs showed only a weak correlation with CD4$^{eff}$PD-1+ levels (Supp Fig. 6).

### *Frequency of CD4$^{eff}$PD-1+ T cells correlates with PFS*

Next we examined the association of co-inhibitory receptor expression on CD4 and CD8 effectors with clinical outcomes. When we divided patients into two groups based on the frequency of PD-1 on CD4$^{eff}$, we observed that MM patients with more CD4$^{eff}$PD-1+ cells (>median, termed CD4$^{eff}$PD-1$^{hi}$) had significantly shorter PFS compared to those with less CD4$^{eff}$PD-1+ cells (≤median, CD4$^{eff}$PD-1$^{\mathbf{lo}}$) (HR:3.98; 95%CI 1.66-9.55; p=0.007) (Fig. 4C). In contrast, there was no correlation between frequency of PD-1 on CD8 T cells and PFS (Fig. 4C). There was no correlation between frequency of LAG-3, ICOS or CTLA4 on either CD4$^{eff}$ or CD8 T cells and PFS (Supp Fig 7A-C). Similarly, no correlation was found between GzmB or Ki-67 or on either CD4$^{eff}$ or CD8 T cells and PFS (Supp Fig 7D-E).

### *Co-inhibitory and co-activation markers on effector T cells from CD4$^{eff}$PD$^{hi}$ patients*

Given the association with clinical outcomes, we examined the CD4$^{eff}$PD-1+ cell fraction in MM in more detail. This subset co-expressed the exhaustion markers LAG-3/ CTLA-4 and the terminal differentiation marker GzmB more frequently in CD4$^{eff}$PD-1$^{hi}$ compared with CD4$^{eff}$PD-1$^{lo}$ patients (p=0.0035, p=0.046, p=0.034 respectively) (Fig. 4D), suggesting this subset is characterised by a dysfunctional state that is more pronounced amongst CD4$^{eff}$PD-1$^{hi}$ patients.

### *CD4$^{eff}$:Treg ratio and CD4$^{eff}$PD-1+ cells are independent of known clinical and cytogenetic predictors of PFS*

Having identified immune features with prognostic value, we examined both CD4$^{eff}$:Treg ratio and CD$^{eff}$PD-1+ cell frequency for associations with known clinical prognostic parameters. We found no association between ISS, genetic risk, ASCT, or response depth with either CD4$^{eff}$:Treg ratio or CD$^{eff}$PD-1+ cells (Supp Fig. 8). A multivariate Cox regression model was built including genetic risk, ASCT, ISS and depth of response, and the immune features identified above. In this model, CD4$^{eff}$:Treg ratio retained independent prognostic value, along with CD4$^{eff}$PD-1+ cells, genetic risk, ASCT, and depth of response (Fig. 5A). A risk model was bulit

including $CD4^{eff}$:Treg ratio, $CD^{eff}$PD-1+ cells, and genetic risk, stratifying patients into 3 risk groups based on diagnostic features. Patients with 2 or more risk factors had significantly shorter PFS (Fig. 5B).

### *Effector T cells from $CD4^{eff}PD^{hi}$ patients display transcriptional and secretory features of dysfunction*

To gain mechanistic insight into the potential dysfunction of effector T cells from $CD4^{eff}$PD-$1^{hi}$ patients, we sorted CD4 and CD8 cells from $CD4^{eff}$PD-$1^{hi}$ and $CD4^{eff}$PD-$1^{lo}$ patients for RNA sequencing. Gene set enrichment analysis (GSEA) carried out using gene sets from previous studies of impaired CD4 function (16–18) revealed that CD4 cells from $CD4^{eff}$PD-$1^{hi}$ patients have transcriptional features of CD4 dysfunction. Amongst three gene sets tested, all were enriched amongst genes differentially expressed by CD4 cells from $CD4^{eff}$PD-$1^{hi}$ patients, although only the Tilstra et al. signature reached statistical significance ($p < 0.001$, Fig. 6A). Similarly, CD8 T cells from $CD4^{eff}$PD-$1^{hi}$ patients also displayed transcriptional features of dysfunction ( Fig. 6A). We then performed GSEA to identify pathways enriched inT cells from $CD4^{eff}$PD-$1^{hi}$ vs. $CD4^{eff}$PD-$1^{lo}$ patients. Pathways related to activation downstream of T cell receptor signalling, proliferation, and regulation of apoptosis were enriched in CD4 cells from $CD4^{eff}$PD-$1^{hi}$ patients (Supp Fig. 9A). Similar pathways of activation and proliferation were also upregulated in CD8 T cells from $CD4^{eff}$PD-$1^{hi}$ patients (Supp Fig. 9A), as previously described for dysfunctional CD8 T cells(27,28).

To further explore the notion that T cells from $CD4^{eff}$PD-$1^{hi}$ patients are functionally impaired, we next assessed cytokine secretion by stimulating whole BM MNCs with anti-CD3 and anti-CD28 antibodies. We found that after 6 hours stimulation, there was a trend towards higher TNF-alfa, IFN-gamma, and IL-2 production in activated CD4 effectors (CD4+FoxP3-CD69+) from $CD4^{eff}$PD-$1^{lo}$ patients compared to CD4 effectors from $CD4^{eff}$PD-$1^{hi}$ patients, however only the frequency and intensity (MFI) of TNF-alfa reached statistical significance ($p=0.0043$, Fig. 6B, $p=0.0411$, Supp Fig. 9B). A similar pattern was observed with activated CD8 T cells (CD8+CD69+) from patients with $CD4^{eff}$PD-$1^{lo}$; these effectors produced more TNF-alfa compared to those from $CD4^{eff}$PD-$1^{hi}$ patients ($p=0.026$, Fig. 6B), with a trend towards higher IFN-gamma and IL-2 production.

Collectively, these data suggest that CD4 effectors and CD8 T cells from CD4effPD-1$^{hi}$ patients display transcriptional and functional features of dysfunction that may contribute to poorer outcomes.

## DISCUSSION

We present data correlating the phenotype and function of BM CD4 T cell subsets at diagnosis to clinical outcomes of first line treatment in a large cohort of MM patients. Specifically we report for the first time that patients with a high frequency of marrow infiltrating Tregs at diagnosis have poorer clinical outcomes. Beyond numerical differences, high frequency of Tregs is accompanied by phenotypic changes (increased PD-1 and LAG-3) suggestive of increased suppressive capacity. Tregs contribute to cancer progression by directly suppressing the effector T cell activity and here we also report that CD4$^{eff}$:Treg ratio may be independently prognostic in MM. We are also the first to present data in MM correlating PD-1 expression on CD4 T cells to patient outcomes, and to impaired cytokine production as well as transcriptional signatures of dysfunctional CD4 and CD8 cells. This supports a growing body of evidence underpinning the role of CD4 T cells in the anti-tumour immune response (29), and suggests the independent importance of immune dysregulation on prognosis.

Myeloma cells have been shown to promote Treg expansion in vivo(8) and in vitro(30). In addition, Treg depletion improves survival in a syngeneic murine model of MM(8), indicating that this is a key immunosuppressive population that facilitates disease progression. Previous studies report higher levels of Tregs in PB in MM patients compared to age matched controls(6,12) and in BM compared to MGUS patients(14). One study reported that higher levels of Tregs in PB correlated with shorter time to progression(14), but no study has systematically examined Treg numbers and phenotype in the BM of newly diagnosed patients. Our is the first study to examine BM infiltrating Treg at diagnosis and significantly extends these earlier reports because we show for the first time that CD4$^{eff}$:Treg ratio in the tumour environment independently associates with clinical outcomes. We also observed that increased Treg numbers associated with greater frequencies of the checkpoint proteins, PD-1 and LAG-3 (on Tregs), consistent with murine models of MM(8). Previous work has confirmed the suppressive function of Tregs from BM of MM

patients(31,32), while expression levels of these checkpoint proteins is reported to associate with Treg suppressive function in other cancers (25, 26, 33). Further functional and molecular studies on PD-1 expressing Tregs from BM of MM patients are planned, to provide mechanistic insights.

Tregs actively suppress cytolytic T cell activity(8), and the ratio of Tregs to effector cells has been reported to correlate with survival outcomes(6). In this series of patients the high frequency of Tregs in the BM resulted in lower effector T cell: Treg ratios however only the $CD4^{eff}$:Treg ratio significantly correlated to PFS. In comparison, there was only a trend of CD8:Treg ratio to outcome (p=0.067) which challenges the prevailing view that CD8+ T cells are the dominant contributors to anti-tumour immunity(34). Indeed, the anti-tumour functions of the CD4 tumour compartment are increasingly recognised(29) which encompasses their helper function for cytotoxic CD8+ T cells(35) as well as the ability to directly eliminate tumour(36). In MM, CD4 mediated cytolysis of autologous tumour cells has been demonstrated in vitro(37) and in a syngeneic murine myeloma model, direct CD4 mediated cytotoxicity was demonstrated even in the absence of tumour MHC II expression(38). Moreover, in a recent in vivo autograft model, significant reduction in tumour control was observed on depletion of either CD4 or CD8 T cells(39).

PD-1 is an early marker of the T cell dysfunction observed in chronic infections and cancer characterised by a hierarchical loss of effector function and proliferation. Classically, analysis of this dysfunctional immune state has focused on CD8 T cells(40). Studies in small patient cohorts report increased PD-1 levels on CD8 cells in the PB and BM of MM patients(10,41), but we are the first to show that PD-1 on CD4 cells is prognostic of clinical outcomes. Despite a correlation between PD-1 on CD4 effectors and CD8 cells, we did not find any association of CD8 parameters with clinical outcomes. On the other hand, the CD8 compartment from $CD4^{eff}PD\text{-}1^{hi}$ patients also (as well as CD4 effectors) manifested reduced cytokine secretion and transcriptional features of dysfunction, suggesting that the presence of increased PD-1+CD4 effectors is indicative of a broader, pan-T cell dysfunctional phenotype.

Examining transcriptomic profiles of T cells from $CD4^{eff}PD\text{-}1^{hi}$ patients, we observed enrichment of pathways that are characteristic of T cell dysfunction. Amongst both

CD4 and CD8 T cells, we found enrichment of both T cell receptor (TCR) and non-classical NF-κB pathways indicative of ongoing antigen stimulation and activity of co-stimulatory pathways (42) respectively. In keeping with upregulated TCR signalling, we found enrichment of pathways related to transcription and cell cycle, suggestive of cell activation. Whilst initial reports of T cell dysfunction in murine models of chronic infection indicated a near total loss of T cell effector function (43), it is increasingly clear from studies of solid malignancy that the effector potential of dysfunctional T cells is reduced but not absent and active cell proliferation is a key feature of this state (28,44). Consistent with previous reports of T cell dysfunction (45,46) we additionally observed enrichment of metabolic pathways including oxidative phosphorylation amongst both subsets and an expression profile indicative of heightened sensitivity to apoptosis amongst CD4 but not CD8 T cells.

Impaired cytokine production by dysfunctional T cells has previously been reported (28) and we extend this finding to BM infiltrating T cells in MM. Here we tested T cell cytokine production and found this to be reduced in both CD4 and CD8 effectors from $CD4^{eff}PD\text{-}1^{hi}$ patients that reached statistical significance only for TNF-alfa. Larger studies that take into account several variables such as stimulus, duration of stimulation and cell population are required to confirm these observations. We observed increased numbers of MDSCs in MM but further work is needed to explore the contribution of the myeloid compartment to the immune dysfunction in untreated MM marrow.

In this work, we used patient BM as opposed to peripheral blood as we wished to examine the MM-driving, immune changes within the tumour microenvironment. Recent in vivo MM models report differences in the immune phenotype of circulating and BM infiltrating T cells(8) in disease, and indicate earlier changes within the BM immune microenvironment. Similarly, a study in patient samples also reported functional differences between BM and PB effector T cells(47). Additionally, we found the age of patients did not correlate with $CD4^{eff}$:Treg ratio or $CD4^{eff}$PD-1 cells. However, as our cohort of healthy donors were younger, comparisons with myeloma patients need to be interpreted with caution. Another point to note is that a minority of patients (10%) had >80% BM plasma cell infiltration, which may have amplified

differences in marker expression, thus our findings await confirmation in further patient cohorts.

Our study suggests that immune parameters in BM of untreated MM patients may inform risk of relapse, and that combining such immune features with genetic risk in a new risk model identifies patients likely to have very poor outcomes. In this patient cohort, we used the median frequency of Tregs (3.31%) as a cut-off value (confirmed using '*survminer*'). Pending confirmation in a larger validation cohort, this measure could be used to identify patients with inferior treatment outcomes who may benefit from adjunctive immune-directed therapies, eg.Treg depletion strategies. Promising agents include Interferon alpha/beta receptor antagonists and the use of CD25 antibodies optimised for depletion(22). Blockade of the PD-L1/PD-1 axis has already been explored in MM(48), but in the relapsed refractory setting, and it remains to be established if checkpoint blockade could overcome immune dysfunction in newly diagnosed patients, eg. with high CD4 effector levels of PD-1 either as a monotherapy or in combination with Treg depleting agents. The disappointing results of single agent checkpoint blockade in MM has been suggested to relate to T cell senescence rather than exhaustion(49). These authors however, only examined CD8+ T cells, thus the question of the effect of PD-1 blockade on CD4 effector function remains unanswered. Interestingly, only 3/78 patients in our cohort received the IMiD lenalidomide, which acts to enhance cytokine release, augmenting T cell co-stimulation signals(50). Thus, the prognostic impact of PD-1 expression on CD4 cells remains to be confirmed in the context of lenalidomide therapy.

In conclusion our work demonstrates that increased Treg in association with dysfunctional CD4 effectors identified by high PD-1 expression correlate with significantly shorter PFS in newly diagnosed MM patients. These data support the importance of CD4 T cells as mediators of anti-tumor immunity in myeloma and prompt further mechanistic studies to gain better understanding of the biology of CD4 dysfunction and Treg function, and open up therapeutic opportunities for these patients.

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## *FIGURE LEGENDS*

**Figure 1. T cell subsets in BM of newly diagnosed MM patients.**
(A) Dot plots display gating strategy for CD4 effectors (CD4+FoxP3-, B), and Tregs, as (CD4+FoxP3+, A) and as (FoxP3+CD25+, C).
(B) Frequency of Treg, identified as CD4+FoxP3+, as % of live MNCs (left), and % of live CD4+ cells (middle) and identified as FoxP3+CD25+ as % of live CD4 cells (right) in healthy donors (HD) and myeloma patients (MM).
(C) CD4eff:Treg ratio (left) CD8:Treg ratio (middle panel) and CD4:CD8 ratio (right). Medians indicated. **p < 0.01, ***p < 0.001, ****p <0.0001.

**Figure 2. Influence of Treg cells on PFS.**
(A) Frequency of Tregs (CD4+FoxP3+ cells as % of CD4) in $Treg^{lo}$ and $Treg^{hi}$ patients (left), PFS in $Treg^{lo}$ and $Treg^{hi}$ patients (middle panel), and representative FACS plot for patient with $Treg^{hi}$ (top) and $Treg^{lo}$ (bottom). ****p <0.0001.
(B) PFS in patients with high and low CD4eff:Treg ratio (left) and CD8:Treg ratio (right), defined as >median, and ≤ median.
(C) Immunohistochemical staining for CD138 (red), CD4 (brown), and FoxP3 (blue) from patient with $Treg^{hi}$ (left) and $Treg^{lo}$ (right). Magnification: ×400 .
$Treg^{hi}$ = patients with frequency of Treg >median
$Treg^{lo}$ = patients with frequency of Treg ≤median

**Figure 3. Expression of checkpoint proteins on Treg**
(A) Frequency of CD25, PD-1, LAG-3, and CTLA-4 on Treg cells (gated as CD4+FoxP3+) in HD and MM. (B) CD25 expression as frequency (left) and MFI (right) on CD4 effectors, CD8, and Treg cells. (C) Frequency of PD-1, LAG-3, CTLA-4, and CD25 on Treg (CD4+FoxP3+) in $Treg^{lo}$ (frequency of Treg ≤ median) and $Treg^{hi}$ patients (frequency of Treg >median). Mean ± SEM. *p < 0.05, **p < 0.01, ****p <0.0001, ns, not significant.
MM=Myeloma patients (n=78, A; n=43, B)
HD=Healthy donors (n=15, A; n=12, B)
(D) Resting (CD45RA+), and activated (CD45RA-) Tregs (CD4+FoxP3+) in a separate cohort of newly diagnosed MM patients (n=12)(left) and representative FACS plot (right) showing gating for resting and activated Tregs . ****p <0.0001

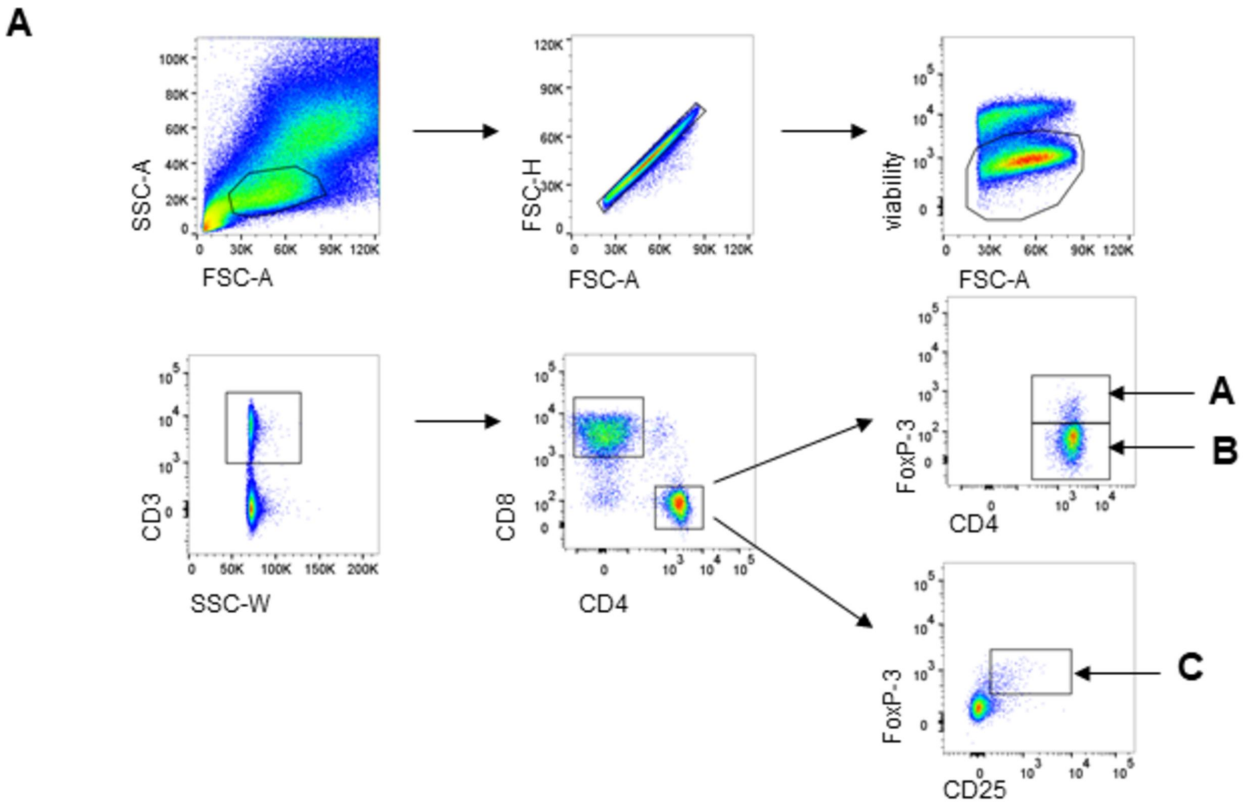
**Figure 4. Co-activation and co-inhibitory receptors on CD4 and CD8 effector T cells and correlation with PFS.**
PD-1, LAG-3, ICOS, CTLA-4, GzmB and Ki-67 expression (% positive) on (A) CD4 effectors and (B) CD8 T cells in HD and MM patients. .
(C) PFS in patients according to frequency of PD-1+ on CD4 effectors (left), and CD8 T cells (right). $PD\text{-}1^{hi}$ = >median, $PD\text{-}1^{lo}$ = ≤median (D) Expression of LAG-3, CTLA-4, and GzmB on PD-1+ CD4 effectors from $CD4^{eff}PD\text{-}1^{lo}$ and $CD4^{eff}PD\text{-}1^{hi}$

patients (mean±SEM), and representative FACS plots of $CD4^{eff}PD\text{-}1^{lo}$ (top) and $CD4^{eff}PD\text{-}1^{hi}$ patients (bottom).
*p < 0.05, **p < 0.01 , ***p < 0.001, ****p <0.0001

**Figure 5. Clinical and immune parameters influencing PFS in newly diagnosed MM.**
(A) Forest plot showing hazard ratios (HR) and 95% confidence intervals (CI) for each parameter, by multivariate Cox regression analysis. (B) risk model based on 3 baseline risk factors: CD4eff:Treg ratio (≤ median), CD4eff PD-1 (>median), and genetic risk (High).
Group 1 = 0 risk factors (n=20)
Group 2 = 1 risk factor (n=21)
Group 3 = 2 or more risk factors (n=33)

**Figure 6. Effectors in $CD4^{eff}PD\text{-}1^{hi}$ patients are transcriptionally and functionally distinct from those in $CD4^{eff}PD\text{-}1^{lo}$ patients**
(A) GSEA dot plots showing preferential expression of genes related to dysfunction in CD4+ effectors (left) and CD8 cells (right) from $CD4^{eff}PD\text{-}1^{hi}$ patients, insets refer to gene sets used, NES, normalized enrichment score (B) TNF-alpha (left), IFN-gamma (middle), and IL-2 (right) producing CD4 effectors (top) and CD8 effectors (bottom) following stimulation with anti-CD3 and anti-CD28 for 6 hours. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 1



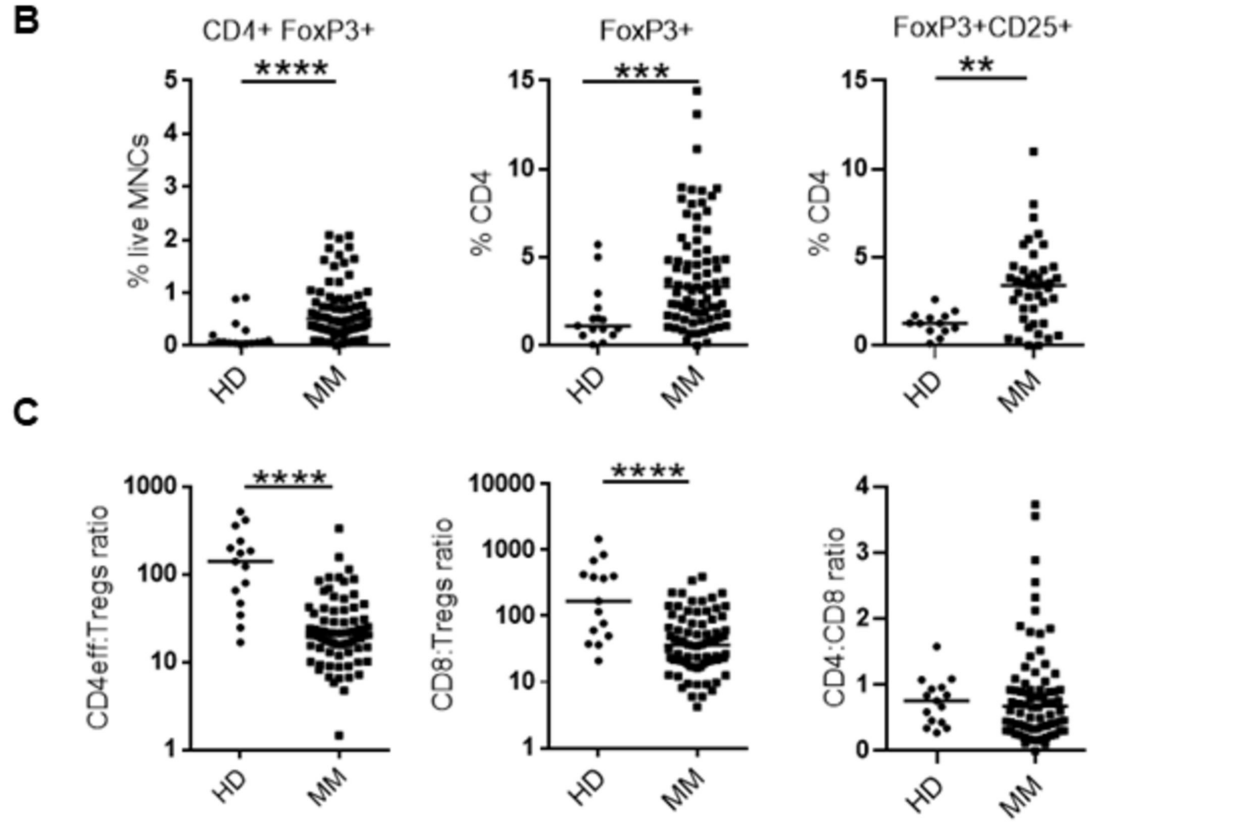
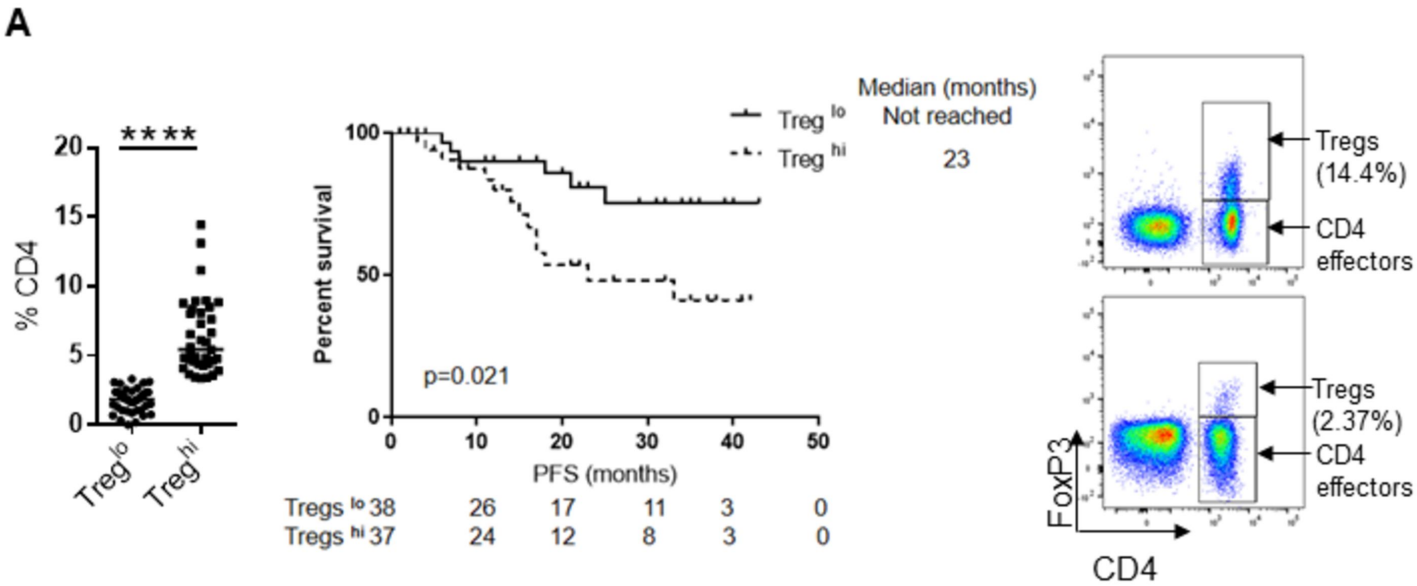
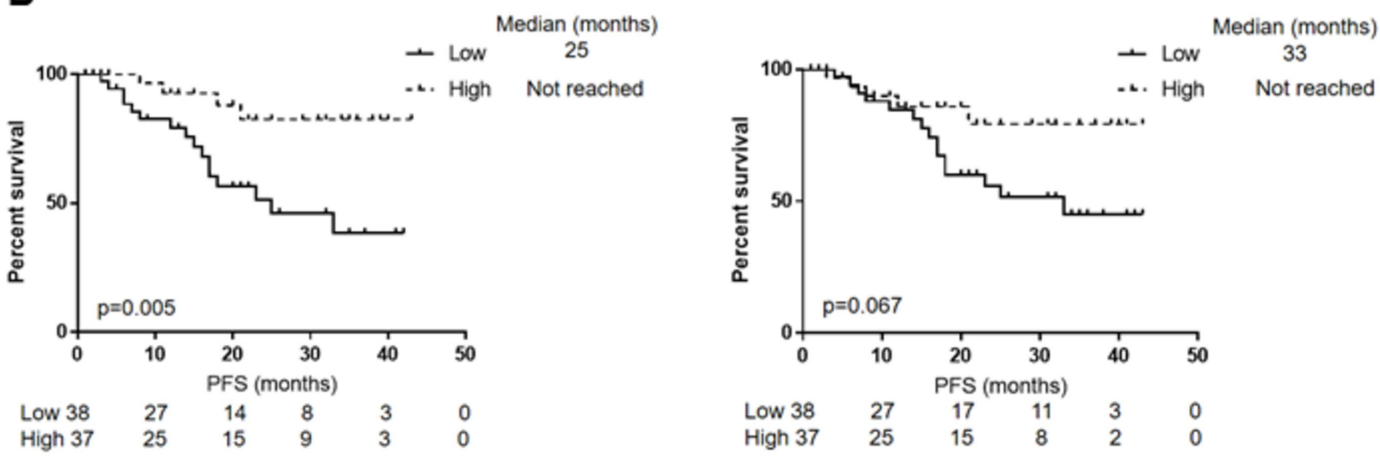



Figure 2



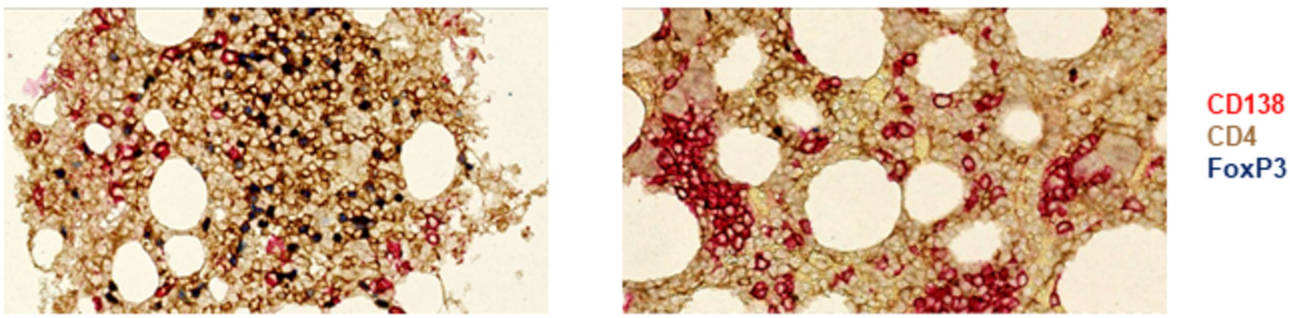



Figure 3

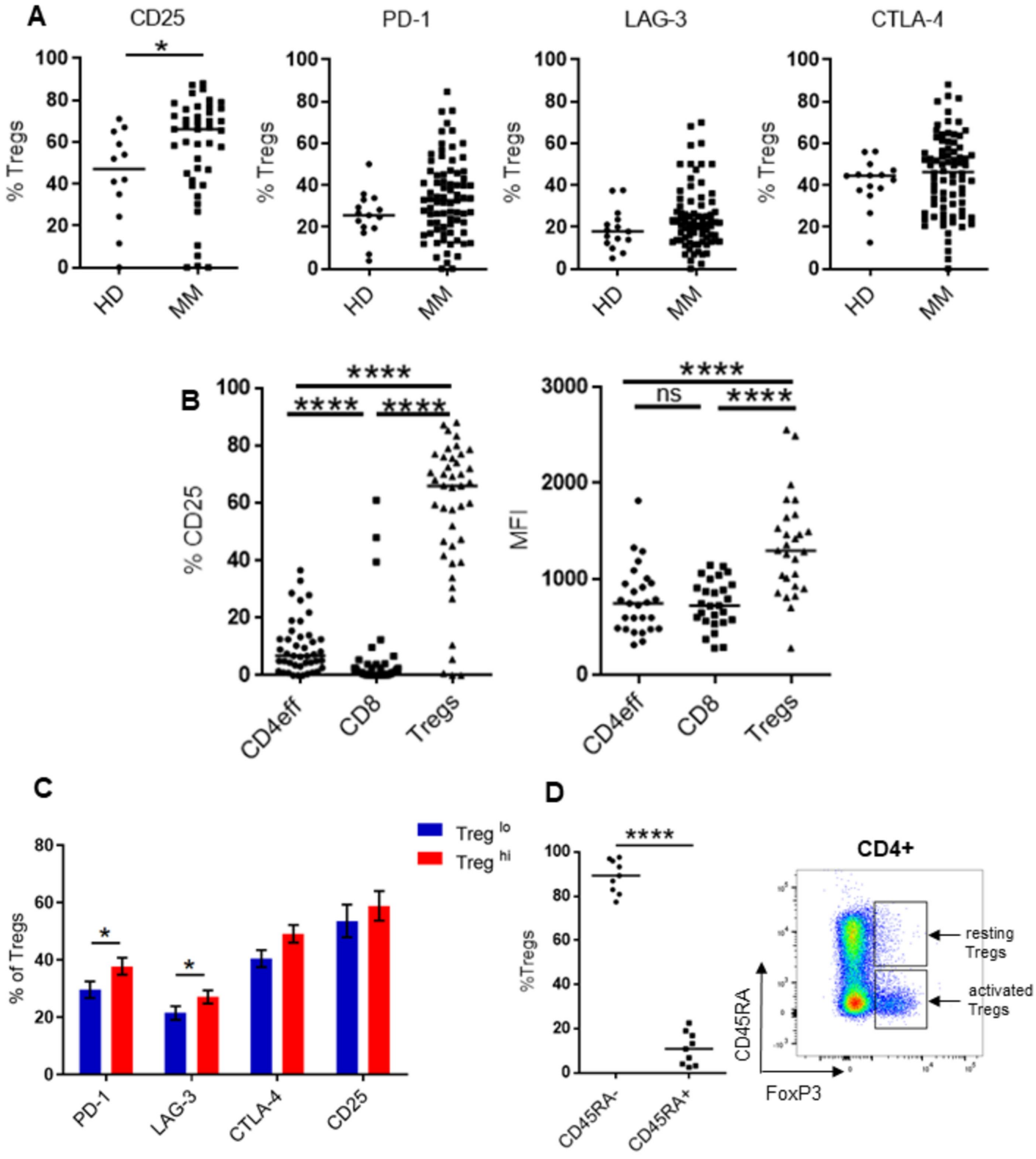


Figure 4

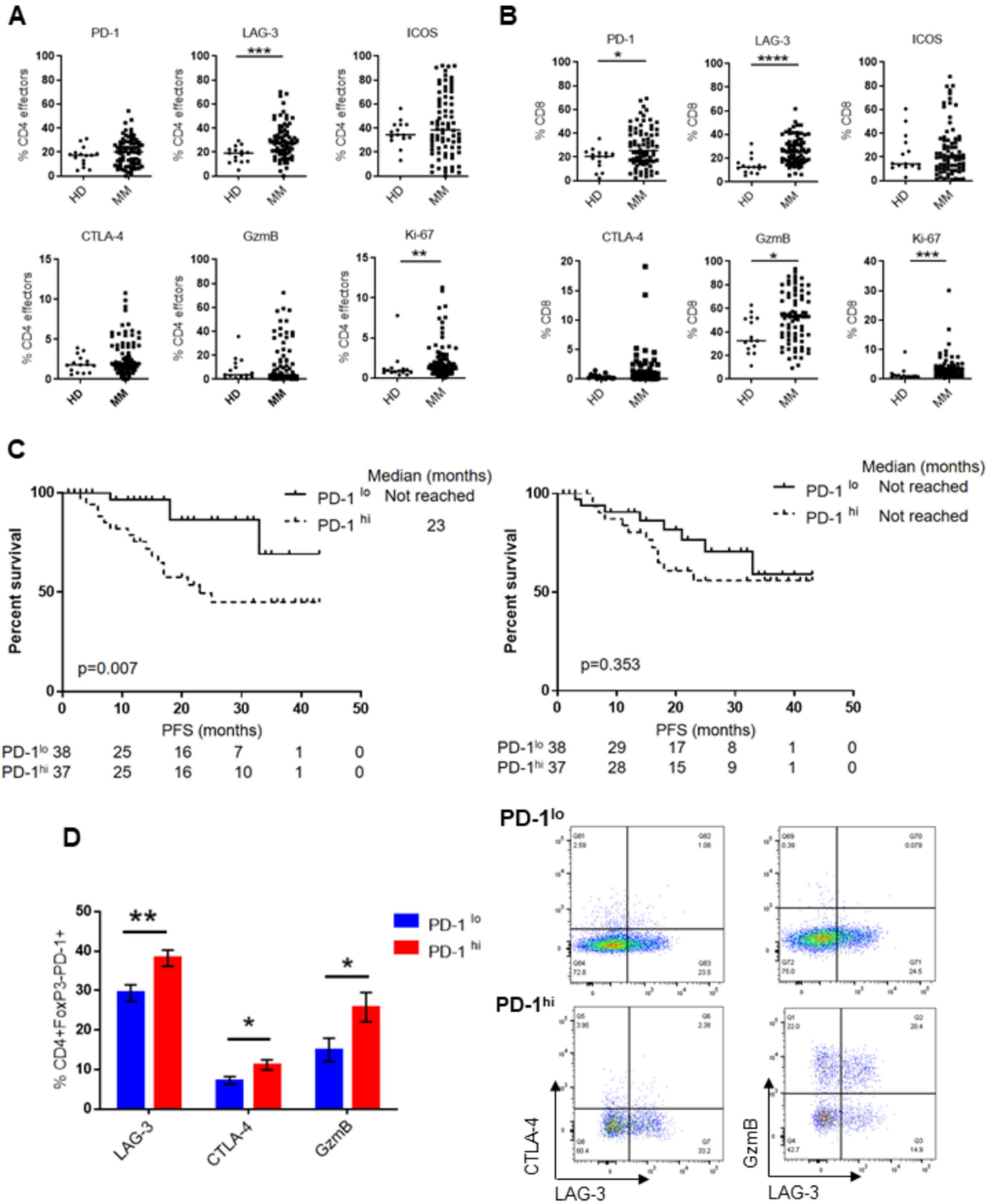


**Figure 5**

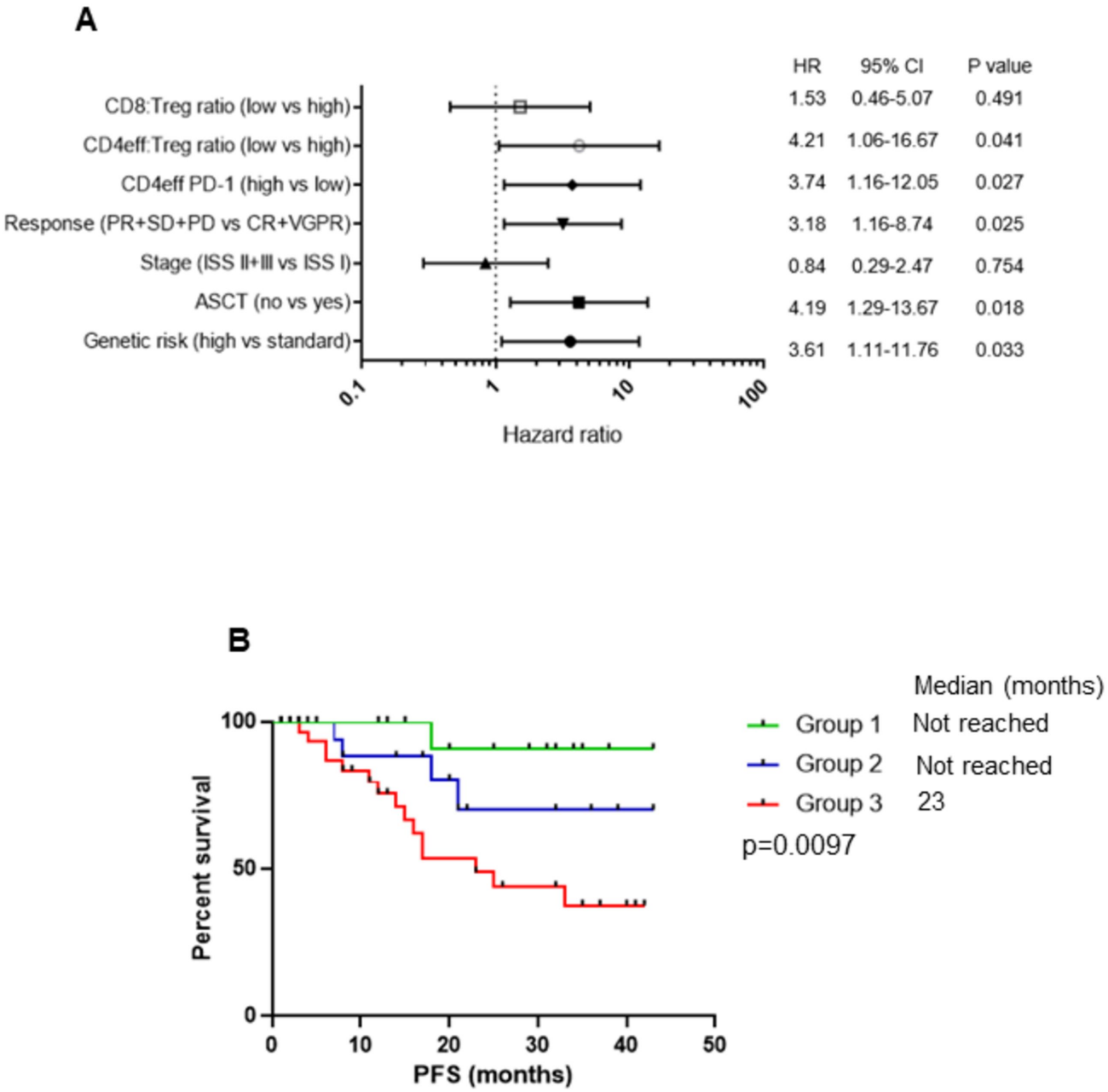
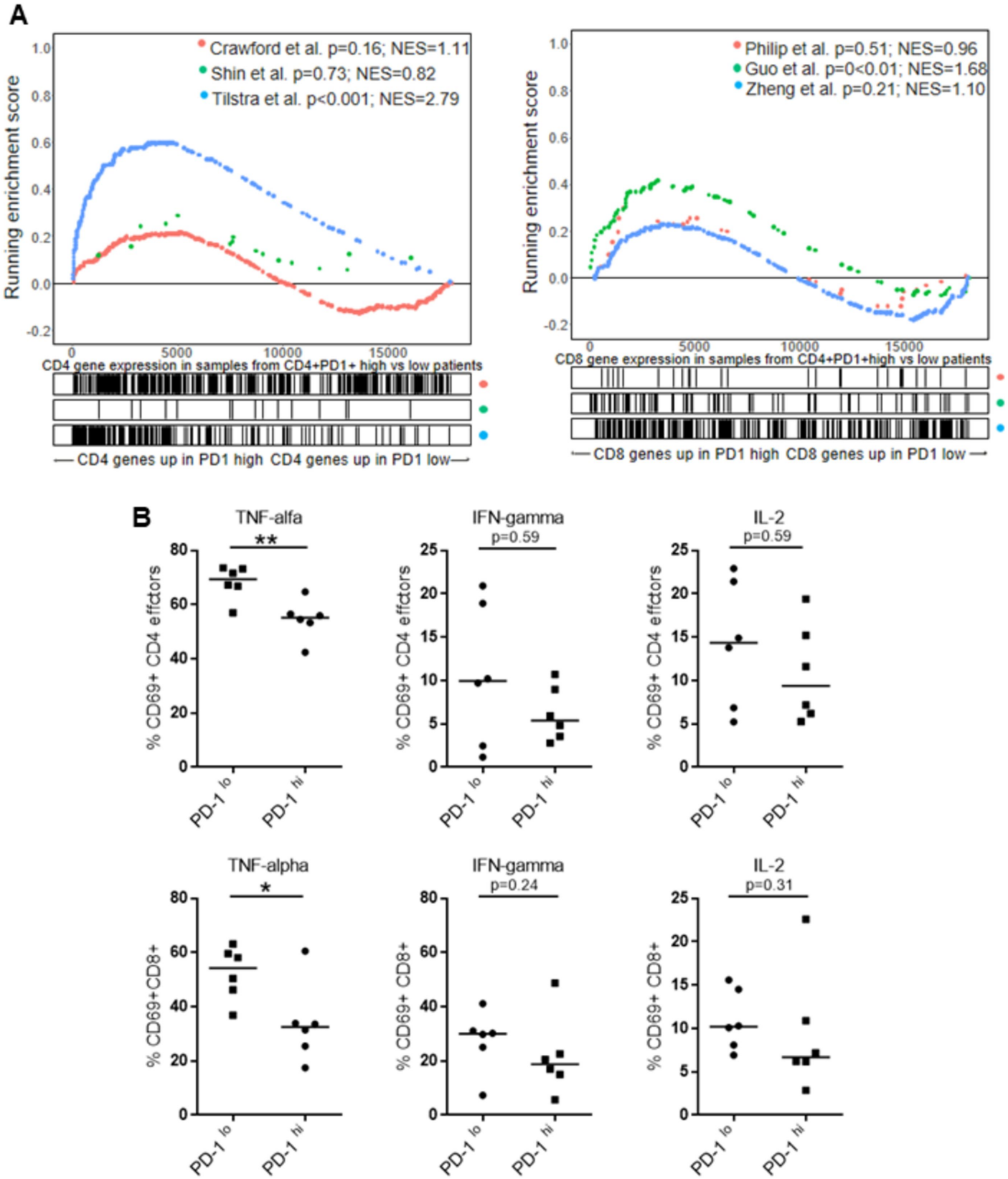


**Figure 6**



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# Marrow infiltrating regulatory T cells correlate with the presence of dysfunctional CD4+PD-1+ cells and inferior survival in patients with newly diagnosed multiple myeloma

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