

CD4⁺CD25⁺FoxP3⁺ regulatory T cells are increased whilst CD3⁺CD4⁻CD8⁻ $\alpha\beta$ TCR⁺ Double Negative T cells are decreased in the peripheral blood of patients with multiple myeloma which correlates with disease burden

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Summary

Increased levels of naturally occurring regulatory T cells (T_{Reg} cells) have been found in a variety of solid tumours and haematological malignancies. In multiple myeloma (MM), evidence suggests that T_{Reg} cells are increased though controversy exists with regards to their function and no relationship to disease stage and treatment has been demonstrated. Here, we demonstrate significantly elevated levels of functional CD4⁺CD25⁺FoxP3⁺ T_{Reg} cells in a large cohort of patients with MM as well as monoclonal gammopathy of uncertain significance (MGUS) in comparison to age-matched, healthy controls. The frequency of Double Negative T_{Reg} cells was also evaluated, demonstrating that these cells were reduced in patients with MM. Furthermore, a characteristic profile of immunomodulatory cytokines in the peripheral blood and bone marrow of patients with MM and MGUS was demonstrated, compared with healthy controls. This data adds further evidence to the understanding of the role of T_{Reg} cell subsets in tumour immunology and the fundamentals of the host/tumour immune conflict.

Keywords: myeloma, regulatory T cells, Double Negative T cells, Foxp3, MGUS.

Multiple myeloma (MM) is an incurable malignant plasma cell dyscrasia associated with both cellular and humoral immune deficiencies (Harrison & Cook, 2005). Attempts to harness the putative graft-versus-myeloma effect of allogeneic stem cell transplantation have faltered due to treatment-related toxicities. Induction of autologous tumour-specific immune responses using vaccination strategies have also had limited success due to poor host immune responses. Many potential mediators of the immunologically hostile microenvironment have been proposed including tumour-derived transforming growth factor (TGF)- β (Cook *et al*, 1999), prostaglandin E₂ (PGE₂) and interleukin-10 (IL-10) (Kim *et al*, 2006). In addition to soluble mediators, the role of regulatory T cell (T_{Reg} cell) subsets in mediating tumour-

related immune suppression has recently been investigated. Several different subsets of regulatory T cells have been identified including *naturally occurring* T_{Reg} cells (nT_{Reg} cells; CD4⁺CD25⁺FoxP3⁺), and inducible T_{r1} and T_{H3} CD4⁺T_{Reg} cells (Knutson *et al*, 2007) as well as CD8⁺ T_{Reg} cells (Filaci *et al*, 2007) and double-negative T_{Reg} cells (DN T_{Reg} cells) (Thomson *et al*, 2006) but their role in cancer immunity remains unclear. Increased levels of T_{Reg} cells have been found in a variety of solid tumours (Bates *et al*, 2006; Miller *et al*, 2006) as well as haematological malignancies (Beyer *et al*, 2006; Prabhala *et al*, 2006; Gjerdrum *et al*, 2007). In particular, in patients with MM, evidence suggests that T_{Reg} cells are increased though controversy exists with regards to their function and no relationship to disease stage and

treatment has been demonstrated (Beyer *et al*, 2006; Prabhala *et al*, 2006).

This study assessed the frequency and functionality of nT_{Reg} cells as well as DN T_{Reg} cells in patients at different stages of MM and compared those with age-matched healthy controls and patients with MGUS. We demonstrated that functional CD4⁺CD25⁺FoxP3⁺ nT_{Reg} cells were significantly increased in MGUS and MM compared to healthy age-matched controls and correlated with disease activity. Treatment with the immunomodulatory drug thalidomide significantly altered the number of nT_{Reg} cells whilst patients were taking the drug. We further demonstrated that the number of nT_{Reg} cells is higher in the peripheral blood (PB) than in the corresponding bone marrow (BM) of patients. Patients with MGUS and MM demonstrated a reduction in numbers of DN T_{Reg} cells, which express a unique cell surface marker and cytokine profile (Zhang *et al*, 2000).

Material and methods

Patients and normal donors

PB samples were collected from 128 patients with MM and 44 patients with MGUS for analysis of regulatory T-cell subsets (Table I). To determine if a differential effect occurred according to varying disease activity, samples were sub-classified into disease groups according to the level of disease burden: 42 samples taken at time of first diagnosis (New), 57 samples at a low disease burden at the end of treatment in plateau phase response (LD) and 29 taken when patients were deemed to have either relapsed or refractory disease (RR). Blood from patients in the RR group was taken prior to any treatment of relapse. PB was also collected from 36 age-matched, healthy volunteers for comparison. We were able to collect BM with the corresponding blood sample from a subgroup of patients for comparison of regulatory T-cell

numbers. The study was approved by the local ethics committee and written informed consent was obtained from all individuals in accordance with the Declaration of Helsinki.

Cell and serum preparation

All samples were collected in sterile EDTA containers. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Lymphoprep (Axis-Shield, Bicton, UK) and stored in fetal calf serum with 10% dimethyl sulphoxide in the vapour phase of liquid nitrogen at -270°C until the day of analysis. Sera for enzyme-linked immunosorbent assay (ELISA) were obtained at simultaneous time points by centrifugation of clotted blood at 400 g for 20 min and rapidly frozen and stored at -20°C until analysis.

Fluorescence-activated cell sorting (FACS) analysis

Four-colour flow cytometry was performed on a LSRII flow cytometer (BD Biosciences, Oxford, UK) and analysed with FACS DIVA software. Directly conjugated monoclonal antibodies (mAbs) against CD4-allophycocyanin (APC), CD8-APC, CD3-peridinin chlorophyll (PerCP), CD25-phycoerythrin (PE), $\alpha\beta$ T-cell receptor (TCR)-PE, $\gamma\delta$ TCR-fluorescein isothiocyanate (FITC) (all from BD Biosciences) and FoxP3-FITC (eBioscience, San Diego, CA, USA) were used according to the manufacturer's protocol with corresponding isotype-matched controls. 1×10^6 cells were stained and the fixation and dead cell discrimination kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) was used to exclude dead cells within the intracellular staining protocol. Using a sequential gating strategy, nT_{Reg} cells were identified as CD4⁺/CD25⁺/FoxP3⁺ T cells and expressed as a percentage of the CD4⁺T-cell population. We gated on the total CD25⁺ population, not just the CD25^{high} cells, because Foxp3 has been described as the most specific marker for regulatory T cells (Ahmadzadeh *et al*,

Table I. Patient characteristics.

	Control	MGUS	New	LD	RR	P
N	36	44	42	57	29	
Gender (M/F)	14/22	24/20	26/16	35/22	15/14	0.2
Age, years (range)	63 (47–89)	71 (38–89)	67 (34–86)	62 (34–87)	70 (43–84)	0.06
Paraprotein						
Type N (%)						
IgG		40 (91)	23 (55)	34 (60)	20 (69)	0.0001
IgA		2 (4.5)	11 (26)	9 (16)	7 (24)	
IgM		2 (4.5)	1 (2)	0	0	
IgD		0	1 (2)	1 (2)	0	
LC		0	6 (14)	12 (21)	2 (7)	
NS		0	0	1 (2)	0	
Paraprotein						
Level, g/l (range)		7 (0–31)	15 (0–60)	0 (0–43)	19 (0–60)	0.0001

MGUS, monoclonal gammopathy of uncertain significance; New, newly diagnosed patients; LD, low disease burden/plateau; RR, relapsed/refractory patients; N, number; LC, light chain myeloma; NS, non-secretory myeloma.

2008). The distinction of CD25^{high} versus CD25^{low} is very subjective, particularly in the resting state of PBMC, and CD4^{high}CD25⁺ regulatory T cells have been shown to have very high suppressive potential (Tu *et al*, 2008). DN T_{Reg} cells were identified as CD3⁺/CD4⁺/CD8[−]/αβTCR⁺/γδTCR[−] and expressed as a percentage of the CD3⁺ lymphocyte population as previously described (Zhang *et al*, 2000).

Magnetic purification of T-cell subsets from PBMNC

For suppression assays, CD4⁺CD25^{high} T cells were isolated using the CD4⁺CD25⁺ regulatory T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. First, CD4⁺ T cells were indirectly magnetically labelled with a biotin-conjugated AB-cocktail and depleted over a magnetic-activated cell sorting (MACS) column. The CD4 enriched fraction was then positively selected after labelling with a CD25 antibody.

Proliferation and suppression assays

PBMC from PB of patients and controls were sorted into CD4⁺CD25[−] effector cells and CD4⁺CD25⁺ T_{Reg} cells as described above. The CD4⁺CD25[−] responder cells were plated in 96-well round bottom plates (Nunc plates; Thermo Fisher Scientific, Roskilde, Denmark) in triplicates at a concentration of 1×10^5 cells per well in culture medium. Purified CD4⁺CD25⁺ T_{Reg} cells were added at different concentrations (4:1 and 8:1 responder to suppressor ratio). The suppressive capability of the T_{Reg} cell fraction was determined by ³H-Thymidine incorporation for 18 h at 37 kBq per well after 72 h stimulation with CD3/CD28-Antibiotin MACS beads (Miltenyi Biotec) at a 1 bead: 2 cell concentration. ³H-thymidine incorporation in the stimulated responder only wells was set as 100% and a stimulation index (SI) calculated.

ELISA for IL-10 and TGF-β

Capture and detection antibodies were used from BD Biosciences, Oxford, UK according to the manufacturers protocol. Briefly, high protein binding 96-well ELISA plates (MaxiSorp, Scientific Laboratory Supplies Ltd, Hesse, UK) were coated at 4°C overnight with IL-10 and TGF-β capture antibodies at 1:500 dilution in 1×1 mol/l NaHCO₃ pH 8.2 at 100 µl per well. For TGF-β ELISA, serum samples were diluted 1:5 with phosphate-buffered saline (PBS) and activated with 1N HCl at room temperature for 15 min, then neutralised with 1N NaOH. After blocking with PBS containing 10% fetal calf serum for 2 h at room temperature, samples and standards were loaded at 100 µl per well and incubated at 4°C overnight. 100 µl per well detection antibody was then added at 1:1000 dilution for IL-10 and 1:500 dilution for TGF-β and incubated for 2 h at room temperature followed by Extravidin-Avidin conjugate (100 µl per well at 1:500 dilution in PBS/Tween for 1 h) and substrate solution (Sigma, Dorset, UK) for approximately 30 min for development in the dark. Samples were

analysed in triplicate and measured spectrophotometrically at 405 nm.

Statistical analysis

Results were analysed with the Statistical Package for the Social Sciences (SPSS) software, version 14.0 for Windows. Values are given as means [$\pm$ standard error of the mean (SEM)]. Kruskal–Wallis test, Mann–Whitney test and Spearman's correlation were used to test for differences where appropriate. A *P*-value of <0.05 was considered statistically significant.

Results

Increased frequency of functional CD4⁺CD25⁺FoxP3⁺ T_{Reg} cells in the PB but not BM of patients with MM

Quantification of nT_{Reg} cells, identified as CD4⁺/CD25⁺/FoxP3⁺ T cells, in the PB of patients with MGUS and MM was compared with age-matched healthy controls. The number of nT_{Reg} cells was significantly increased in MGUS and MM compared to controls ($2.4\% \pm 0.2$, $3.6\% \pm 0.2$ and $1.9\% \pm 0.3$, respectively, $P < 0.0001$; Fig 1A and B). Next, the level of marrow-infiltrating CD4⁺/CD25⁺/FoxP3⁺ T cells (MIL_{Reg} cells) was determined in aspirated BM from 20 patients. Patients with MM had significantly higher numbers of nT_{Reg} cells in the PB compared with BM ($3.6\% \pm 0.2$ cf $2.0\% \pm 0.4$, $P = 0.003$; Fig 1C). This same trend was present in the MGUS group but was not significant, ($2.8\% \pm 0.5$ in PB vs. $1.5\% \pm 0.5$ in BM, $P = 0.34$). The mean fluorescent intensity (MFI) of FoxP3 was reduced in MGUS (827 ± 41) and MM (849 ± 23) compared to controls (970 ± 49 , $P = 0.03$; Fig 2A). Illustrative example histograms of FoxP3 expression are shown in Fig 2B. The functionality of CD4⁺CD25⁺FoxP3⁺ T_{Reg} cells was assessed in a tritiated thymidine incorporation assay. The results demonstrated that nT_{Reg} cells from MM patients could significantly suppress the proliferation of autologous CD4⁺CD25[−] effector T cells in a similar nature to the suppression induced by T_{Reg} cells from age-matched controls (Fig 2C).

Increased nT_{Reg} cell numbers correlate with disease burden

To date no relationship with disease or burden and T_{Reg} cells has been investigated in the setting of cancer. Therefore we sought to clarify the relationship in MM. In newly diagnosed patients with MM, there was a trend of increased nT_{Reg} cell numbers in the International Staging System stage II versus stage I (Greipp *et al*, 2005) with $2.3\% \pm 0.2$ vs. $3.4\% \pm 0.5$, $P = 0.049$. Too few patients presented with stage III disease to allow for statistically meaningful analysis. When the PB of MM patients with differing disease burden was examined by FACS, a significant difference in nT_{Reg} cell numbers in each category was identified; the highest levels of nT_{Reg} cell numbers were seen in the LD group (New $2.5\% \pm 0.2$, LD $4.3\% \pm 0.5$, RR

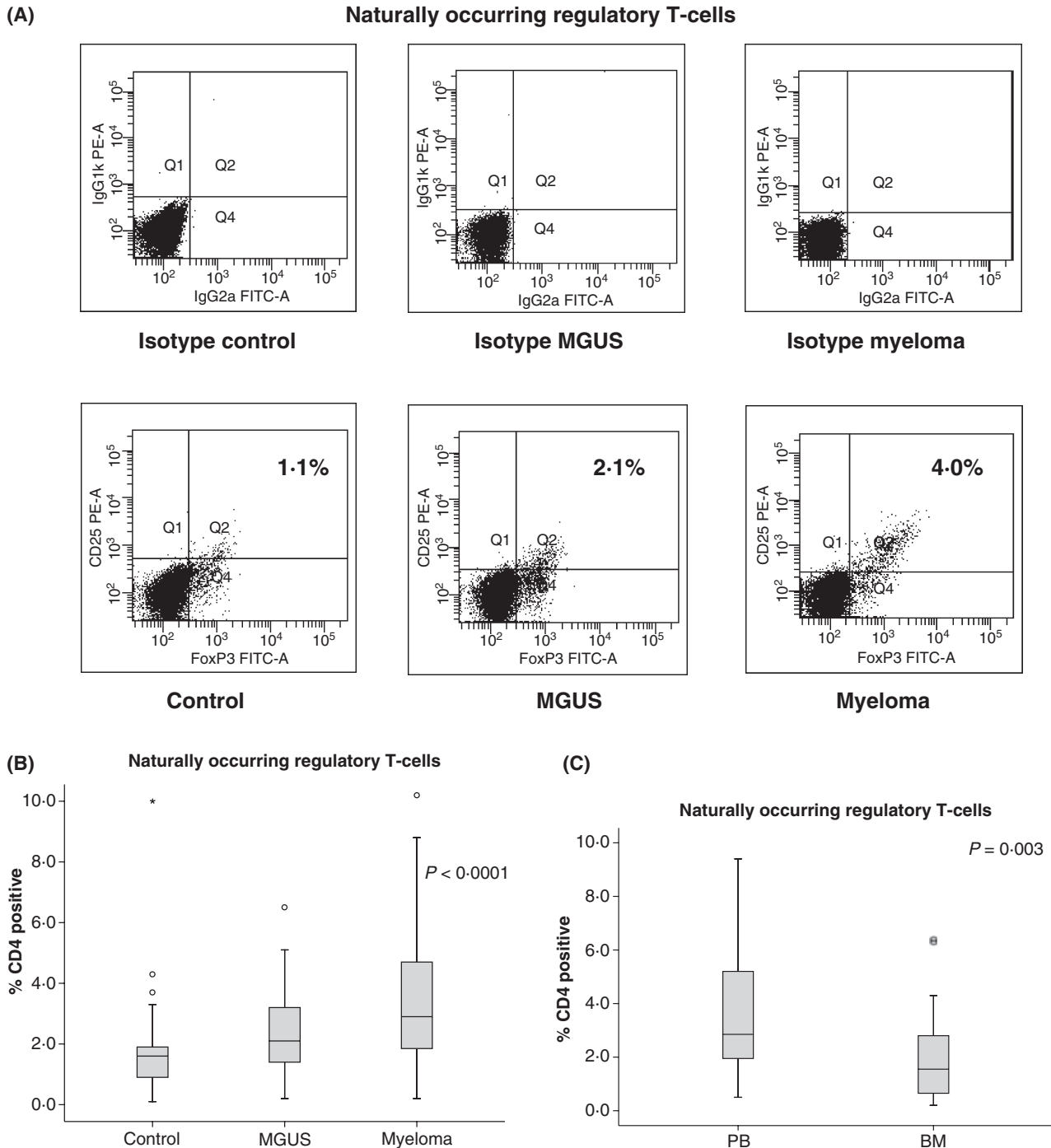


Fig 1. Frequencies of $CD4^+CD25^+FoxP3^+$ T_{Reg} cells. (A) Representative flow cytometric analysis plots of healthy, age-matched controls (left), MGUS (middle), multiple myeloma (right) with their corresponding isotypes. Samples are gated on $CD4^+$ cells and shown as $CD25^+FoxP3^+$ in the right upper quadrant, expressed as percentage of $CD4^+$ cells. (B) Frequency of $CD4^+CD25^+FoxP3^+$ T_{Reg} cells in healthy, age-matched controls ($n = 36$), patients with MGUS ($n = 44$) and myeloma patients ($n = 128$). Shown are median, 75th percentile (box), standard deviation (whiskers) and outliers (dots), $P < 0.0001$, Kruskal–Wallis test. (C) Frequency of $CD4^+CD25^+FoxP3^+$ T_{Reg} cells in peripheral blood (PB) and bone marrow (BM) of patients with MM ($n = 20$; $P < 0.01$, Mann–Whitney U -test).

$3.7\% \pm 0.4$, $P < 0.0001$; Fig 3A). T_{Reg} cell numbers positively correlated with the paraprotein level (Spearman's rho correlation $R = 0.175$, $P = 0.013$) further supporting a relationship

with disease activity. Next we sought to determine if there was a relationship with prior or concurrent therapy with the immunomodulatory drug, thalidomide. Patients who were

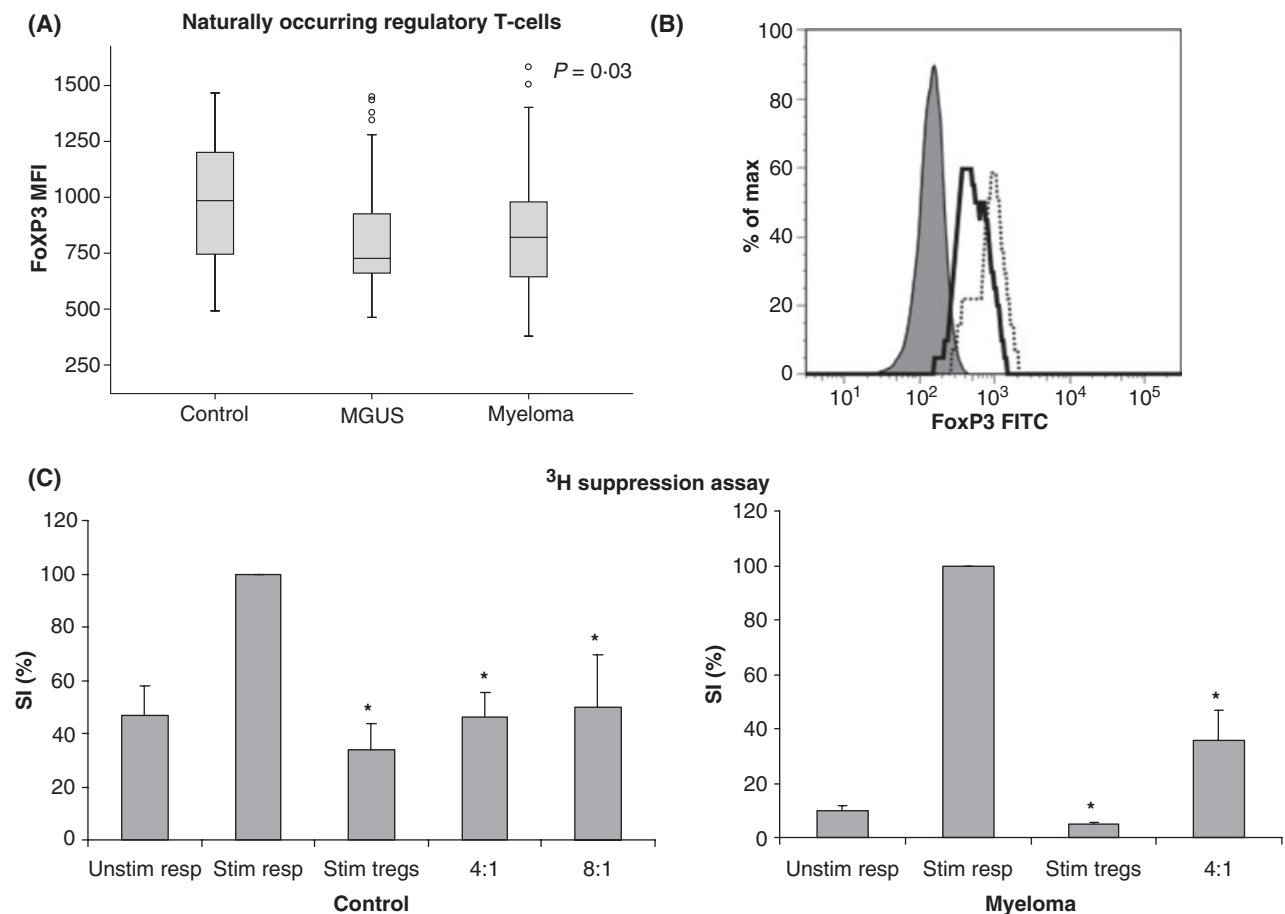


Fig 2. Functional analysis of CD4⁺CD25⁺FoxP3⁺ T_{Reg} cells. (A) FoxP3 mean fluorescence intensity (MFI) in gated CD4⁺CD25⁺ cells from the peripheral blood of healthy, age-matched controls ($n = 36$), MGUS patients ($n = 44$) and myeloma patients ($n = 128$). Shown are median, 75th percentile (box), standard deviation (whiskers) and outliers (dots), $P < 0.038$, Kruskal–Wallis test. (B) Illustrative example overlay histograms of FoxP3 MFI for isotype (grey filled), control (dotted) and MM (solid black line). (C) Tritiated thymidine suppression assay representing stimulation index (SI) of 3 healthy controls (left) and 3 myeloma (right).

receiving thalidomide treatment at the time of sampling ($n = 14$) had a significantly higher level of nT_{Reg} cell numbers ($7.5\% \pm 1.2$) compared with those patients who had previously received thalidomide ($3.7\% \pm 0.4$, $n = 29$) or patients who have never received thalidomide ($2.8\% \pm 0.2$, $n = 79$; $P < 0.0001$) as illustrated in Fig 3B.

Patients with MM demonstrate higher serum Interleukin-10 and TGF- β levels

We and others have previously shown altered levels of immune regulatory cytokines in the serum of patients with MM (Cook *et al*, 1999; Kim *et al*, 2006). Recent evidence has suggested that the regulatory subset, Tr1 cells, which predominantly produce IL-10, are increased in association with MM (Fiore *et al*, 2005). We therefore sought to determine the level of TGF- β and IL-10 in the PB and BM serum of patients with MM and MGUS, in relation to the levels of T_{Reg} cells and compare them to those detected in the PB and BM of control subjects. There were no significant differences in the mean PB

IL-10 levels detected by ELISA in each of the patient cohorts, although a trend to higher values in MGUS was suggested [controls ($n = 20$): 22 ± 6 pg/ml; MGUS ($n = 28$) 74 ± 28 pg/ml; New ($n = 32$) 31 ± 17 pg/ml; LD ($n = 52$) 29 ± 6 pg/ml; RR ($n = 22$) 82 ± 36 pg/ml]. Simultaneous PB and BM serum samples ($n = 20$) were analysed and a significant increase in the level of IL-10 was demonstrated both in control and newly diagnosed patients (299 ± 137 pg/ml and 330 ± 279 pg/ml, respectively; $P < 0.01$) with a trend in those patients with relapsed or refractory disease being evident (Fig 4A).

When the PB serum content of TGF- β was analysed, no significant difference in the level was detected between control subjects and patients at differing disease burden categories, though a trend to higher levels in newly diagnosed patients was suggested (controls: 3276 ± 286 ng/ml; MGUS: 3450 ± 284 ng/ml; New: 4650 ± 1669 ng/ml; LD: 2242 ± 164 ng/ml; RR: 2276 ± 314 ng/ml; $P = \text{NS}$). Analysis of simultaneous PB and BM sera did not demonstrate any significant difference, though again, a trend to higher levels in newly diagnosed

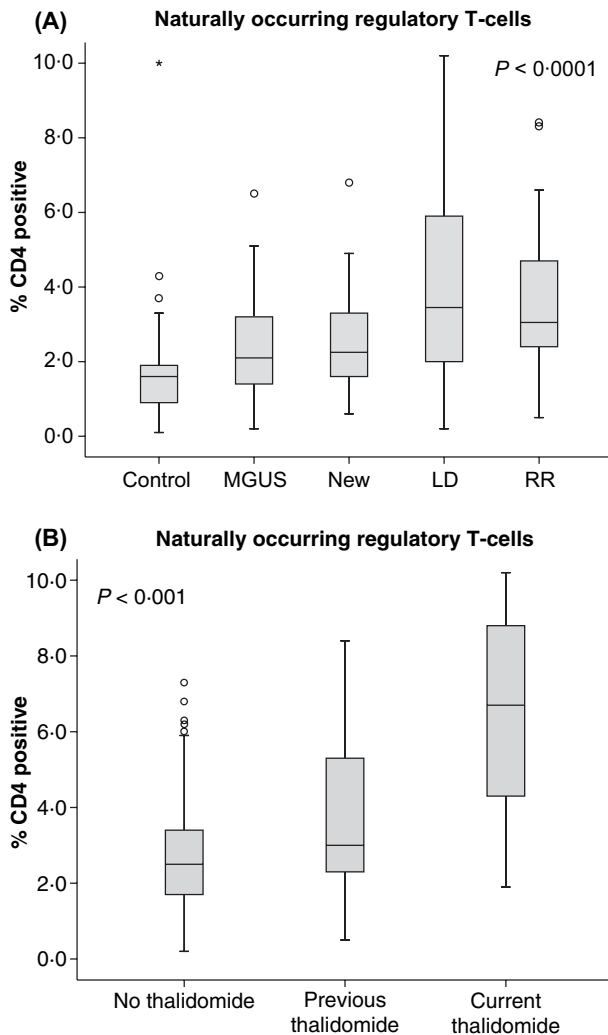


Fig 3. $CD4^+CD25^+FoxP3^+$ T_{Reg} cells correlate with disease stage. (A) Frequency of $CD4^+CD25^+FoxP3^+$ T_{Reg} cells in healthy, age-matched controls ($n = 36$), MGUS patients ($n = 44$), newly diagnosed patients (New; $n = 42$), patients with treated, low disease burden (LD; $n = 57$) and relapsed or refractory patients (RR; $n = 29$). Shown are median, 75th percentile (box), standard deviation (whiskers) and outliers (dots), $P < 0.0001$, Kruskal–Wallis test. (B) Frequency of $CD4^+CD25^+FoxP3^+$ T_{Reg} cells in patients with myeloma never treated with thalidomide ($n = 73$), patients treated previously with thalidomide ($n = 29$) and patients taking thalidomide at the time of sample collection ($n = 14$), $P < 0.0001$, Kruskal–Wallis test.

patients was suggested (controls: 1953 ± 351 ng/ml; MGUS 2592 ± 660 ng/ml; New: 4442 ± 2471 ng/ml; LD: 1747 ± 276 ng/ml; RR: 1637 ± 332 ng/ml; Fig 4B) There was no significant correlation between the PB and BM serum level of either IL-10 or TGF- β with nT_{Reg} cell numbers.

Double Negative ($CD3^+CD4^-CD8^- \alpha\beta TCR^+$) regulatory T cells are reduced in the PB but not BM of MM patients

To date, no reports have been published with regards to the analysis of DN T_{Reg} cell numbers in patients with MM. The

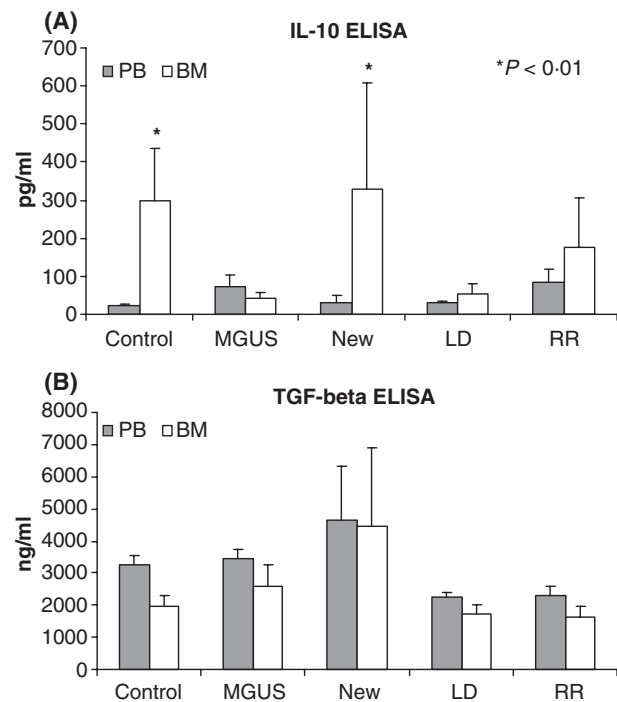


Fig 4. IL-10 and TGF- β ELISA on patients sera. (A) Mean and SEM IL-10 ELISA level (pg/ml) in peripheral blood (PB) serum (grey filled) and BM (white boxes). (B) Mean and SEM TGF- β ELISA (ng/ml) in PB serum (grey filled) and BM (white boxes).

PB of age-matched controls contained significantly higher DN T_{Reg} cell numbers compared with that of both MGUS and MM [controls ($n = 29$): $1.8\% \pm 0.3$; MGUS ($n = 35$): $1.3\% \pm 0.1$; MM ($n = 113$): $1.2\% \pm 0.1$; $P = 0.02$]. Representative FACS plots are shown in Fig 5A and the summary of data is illustrated in Fig 5B. When analysed for disease burden in the cohorts described above, a greater reduction in DN T_{Reg} cell numbers was demonstrated in both MGUS and patients with low disease burden after prior anti-myeloma therapy [New ($n = 37$): $1.2\% \pm 0.2$; LD ($n = 51$): $1.1\% \pm 0.1$; RR ($n = 25$): $1.3\% \pm 0.1$; $P < 0.01$, Fig 5C]. In a sub-group analysis where simultaneous PB and BM samples were available ($n = 20$), no significant difference in the numbers of DN T_{Reg} cell numbers was evident (BM: $0.95\% \pm 0.15$ cf PB at $1.16\% \pm 0.08$).

Discussion

The study presented here sought to characterise different regulatory T-cell subsets in a large cohort of patients with MGUS and MM and to compare those to healthy, age-matched controls. By utilising a sequential gating strategy to accurately determine the number of naturally occurring $CD4^+CD25^+FoxP3^+$ regulatory T cells, we were able to confirm and consolidate the data of Beyer *et al* (2006), who showed increased T_{Reg} cell numbers in MM, MGUS and controls not only of central/effector memory type but also of naïve

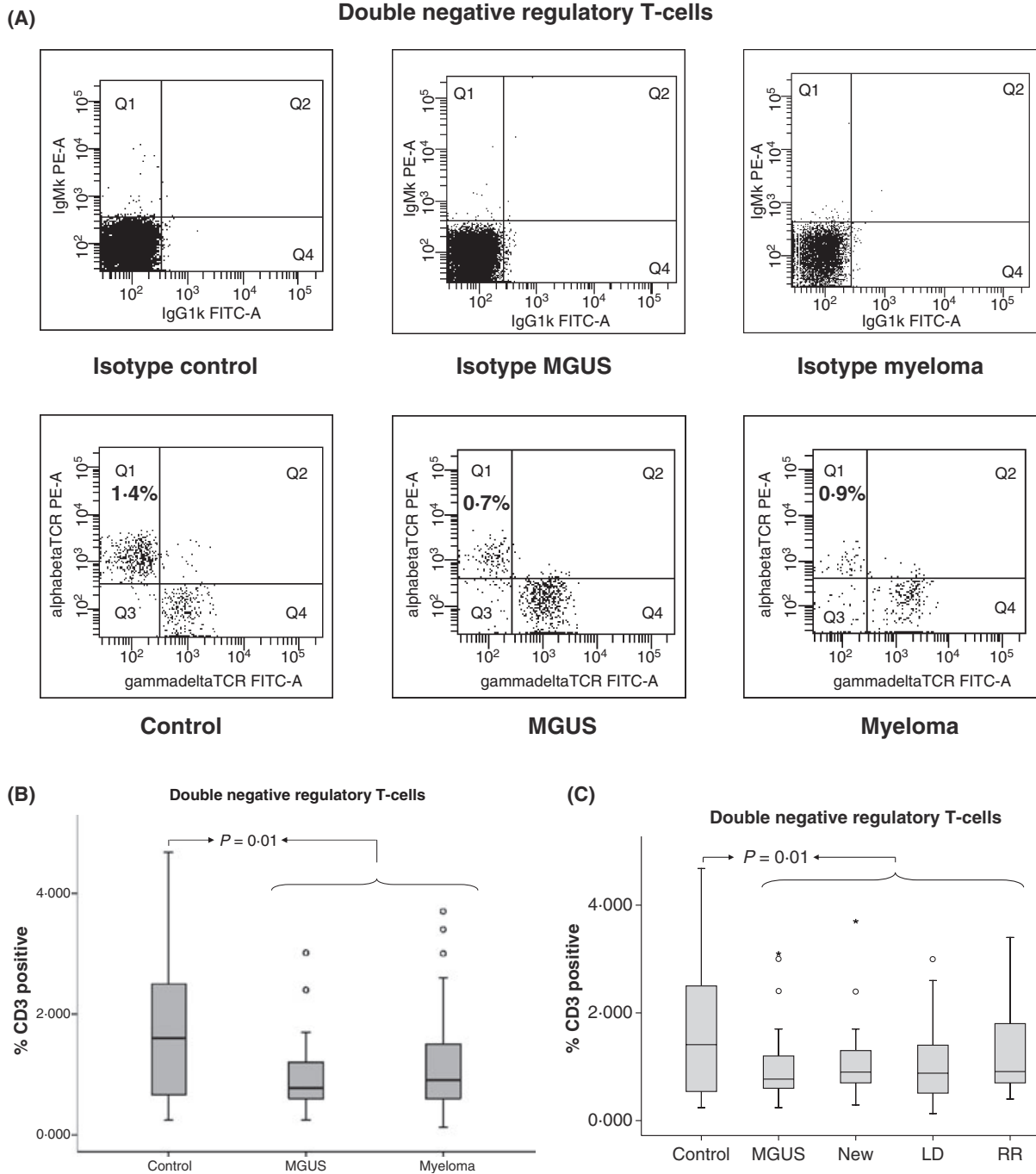


Fig 5. Frequencies of Double Negative ($CD3^+CD4^-CD8^-αβTCR^+$) regulatory T cells. (A) Representative flow cytometry plots and corresponding isotypes of age-matched, healthy control (left), and MGUS (middle) and MM (right) patients. Samples are gated on $CD4$ and $CD8$ negative but $CD3^+$, $αβTCR^+$, $γδTCR^-$ and expressed as a percentage of total $CD3^+$ cells. (B) Double Negative ($CD3^+CD4^-CD8^-αβTCR^+$) regulatory T cells in 29 healthy, age-matched controls, 35 with MGUS patients and 113 MM patients (myeloma), $P < 0.01$, Mann–Whitney U -test between controls and MGUS/MM. (C) Double Negative ($CD3^+CD4^-CD8^-TCRαβ^+$) regulatory T cells in healthy, age-matched controls ($n = 29$), patients with MGUS ($n = 35$), patients with newly diagnosed myeloma (New; $n = 37$), patients with low disease burden (LD, $n = 51$) and patients with relapsed or refractory disease (RR, $n = 25$), $P = 0.023$, Mann–Whitney U -test between controls and myeloma.

$CCR7^+CD45RA^+$ T_{Reg} cells. Furthermore, in our hands those nT_{Reg} cells equally maintained their suppressive function. Absolute percentages in our study are lower than described by Beyer *et al* (2006), which may be accounted for by the fact that

we incorporated FoxP3 directly into our gating strategy and therefore enumerated T_{Reg} cells more specifically. As described by Beyer *et al* (2006), the present study demonstrated that patients with treated MM had higher T_{Reg} cell

numbers in PB. This elevation was even more striking in patients treated with thalidomide at the time of sample collection. The effect of thalidomide on regulatory T cells is not known though recently it has been reported that a reduction in T_{Reg} cells is seen after thalidomide treatment in chronic lymphocytic leukaemia (Giannopoulos *et al*, 2007). However, as the anti-tumour effect of thalidomide is not fully elucidated, possibly related to its anti-tumour necrosis factor- α effect, the impact of thalidomide on the regulatory immune system remains obscure. Recent evidence has suggested that the interaction of thrombospondin with the T-cell receptor, CD47, can induce $FoxP3^+$ T_{Reg} cells and many anti-angiogenic drugs including thalidomide, can increase the production of thrombospondin (Grimbert *et al*, 2006). The biological effect of thalidomide, both directly and indirectly, on $FoxP3^+$ T_{Reg} cells generation is currently the focus of *in vitro* studies.

It is generally accepted that regulatory T cells are increased in solid tumours (Bates *et al*, 2006; Lau *et al*, 2007; Ling *et al*, 2007; Esendagli *et al*, 2008) as well as haematological malignancies (Barath *et al*, 2006) but contrary results have been published with regards to prognostic correlation. In ovarian cancer, increased T_{Reg} cell numbers are associated with reduced survival (Curiel *et al*, 2004), whereas increased tumour infiltration with $FoxP3$ positive T_{Reg} cells in cutaneous T-cell lymphomas, diffuse large B cell lymphomas, follicular and classical Hodgkin lymphoma seems to be associated with improved outcome (Gjerdrum *et al*, 2007; Tzankov *et al*, 2008). Those studies investigated infiltrating lymphocytes of the tumour tissue by immunohistochemical $FoxP3$ detection. The present study examined both PB and BM T_{Reg} cell numbers, the latter representing the tumour microenvironment, by FACS and found significantly reduced numbers in the BM of myeloma patients.

Regulatory cytokine networks in MM patients, including IL-10 and TGF- β , may play a role in the generation and function of inducible regulatory T-cell subsets ($Tr1$ cells and T_{H3} cells), contributing to the immune hostility in MM (Cook *et al*, 1999; Kim *et al*, 2006). One of these inducible regulatory T-cell subsets, $Tr1$ cells have been characterised as $CD4^+CD25^-IL4^-IL10^+$ cells capable of exerting immune suppression by production of IL-10 (Roncarolo *et al*, 2001). Recently, these cells have been shown to expand in co-cultures with tumour cell lines and their phenotype was shown to be $FoxP3^+$ (Bergmann *et al*, 2007). The present study evaluated IL-10 and TGF- β levels by ELISA in the serum of patients with MGUS and MM and compared them to healthy, age-matched controls. It is of interest that patients with *de novo* disease actually showed lower IL-10 levels than control subjects. However, IL-10 levels were significantly higher in the BM, suggestive of a local microenvironment effect rather than systemic one. This same trend was present in the TGF- β levels but, due to wide variation, the results were not significantly different. The direct evaluation of inducible regulatory T-cell subsets is still hampered by a lack of specific

surface markers, low cell numbers and suboptimal proliferation stimuli.

DN T_{Reg} cells, first characterised in mice in 2000 (Thomson *et al*, 2006), account for up to 5% of $\alpha\beta$ -TCR $^+$ T cells in the PB of humans and have a key role in mediating suppression of the immune response, especially the self/non-self response (Chen *et al*, 2004; Zhang *et al*, 2007). The role of DN T_{Reg} cells in mediating cancer-related immune suppression has not been studied, especially in MM. The data presented in our studies demonstrate for the first time that DN T_{Reg} cells are significantly reduced in patients with MGUS and MM, again confirming abnormal immunoregulatory properties in MGUS as an expression of the premalignant state. DN T_{Reg} cells have been shown to be generated from the peripheral lymphoid pool through $CD4$ gene silencing, resulting in a potent immunosuppressive population (Ford *et al*, 2006; Zhang *et al*, 2007). As the immune system is an adaptively homeostatic system, influencing the development of immune effector and regulatory cells (T_{H1} , T_{H2} , T_{Reg} and DN T_{Reg} cells) from naive $CD4^+CD25^-$ T cells under the influence of antigen concentration/dominance coupled with cytokine networks, the reduction in the number of DN T_{Reg} cells may represent a shift in the differentiation pathway of naive $CD4^+$ T cells in response to altered cytokine networks and/or chronic or tumour-related antigenic stimulation (Abbas *et al*, 1996; Chen *et al*, 2003; Park *et al*, 2005). Alternatively, the tumour clone, either directly or mediated through accessory immune cells, may silence DN T_{Reg} cells through apoptotic mechanisms. Clearly further work is required in this area.

In summary, we systematically analysed subsets of regulatory T cells in patients with MM and MGUS. We showed that naturally occurring T_{Reg} cells are increased in number and maintain their suppressive capacity. Such an increase is modulated by thalidomide with no discernable correlation with the circulating immunosuppressive cytokines, IL-10 and TGF- β . Furthermore, DN T_{Reg} cells were shown to be reduced in patients with MM, which has not previously been described. These data therefore demonstrate further, that the tumour clone in MM modulates the microenvironment to allow tumour escape of immune-surveillance. Future designs of effective immunotherapeutic interventions will need to be adapted to the influence of regulatory T cells if the potential of such therapies is to be achieved.

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