

ORIGINAL ARTICLE

Long-term survival in multiple myeloma is associated with a distinct immunological profile, which includes proliferative cytotoxic T-cell clones and a favourable Treg/Th17 balance

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Despite improved outcomes in multiple myeloma (MM), a cure remains elusive. However, even before the current therapeutic era, 5% of patients survived >10 years and we propose that immune factors contribute to this longer survival. We identified patients attending our clinic, who had survived >10 years ($n = 20$) and analysed their blood for the presence of T-cell clones, T-regulatory cells (Tregs) and T helper 17 (Th17) cells. These results were compared with MM patients with shorter follow-up and age-matched healthy control donors. The frequency of cytotoxic T-cell clonal expansions in patients with <10 years follow-up (MM patients) was 54% ($n = 144$), whereas it was 100% ($n = 19/19$) in the long-survivors (LTS-MM). T-cell clones from MM patients proliferated poorly *in vitro*, whereas those from LTS-MM patients proliferated readily (median proliferations 6.1% and 61.5%, respectively ($P < 0.0001$)). In addition, we found significantly higher Th17 cells and lower Tregs in the LTS-MM group when compared with the MM group. These results indicate that long-term survival in MM is associated with a distinct immunological profile, which is consistent with decreased immune suppression.

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INTRODUCTION

Before the introduction of immunomodulatory agents and proteasome inhibitors, less than 5% of patients with multiple myeloma (MM) survived for longer than 10 years. Attempts to identify factors associated with prolonged survival, arbitrarily set at 10 years, suggested younger age, lower tumour mass and response to therapy.^{1–4} The ability to achieve disease control with allogeneic transplantation⁵ and donor lymphocyte infusions⁶ indicates the potential for immune-mediated control in patients with MM. There is also indirect evidence of host anti-tumour immune activity through the identification of premalignancy-specific effector T cells in patients with monoclonal gammopathy,⁷ the phenomenon of 'plateau phase' where despite a significant residual tumour burden the disease does not progress⁸ and the association between the presence of expanded T-cell clones and an improved survival.^{9–11} We hypothesised that patients with myeloma who are long-term survivors (LTS-MM) have greater immunocompetence and that a study of immune biomarkers in these LTS-MM patients might provide a novel approach to understanding the mechanisms of immune dysfunction in MM.

Expanded T-cell clones have been reported to be present in the blood of patients with MM,^{9–11} Waldenstrom's macroglobulinemia,¹² chronic myeloid leukemia¹³ and myelodysplastic syndromes.^{14,15} The incidence in patients with MM was 48% in a national clinical trial ($n = 120$)¹⁰ and 54% in our single institution cohort ($n = 144$).⁹ These expanded T-cell clones have the immunophenotype of

effector memory T cells ($CD3^+ CD8^+ CD57^+$), have a restricted T-cell receptor (TCR) $V\beta$ expression and comprise up to 50% of the total T-cell population.^{9–11,16,17} As representative TCR $V\beta$ -restricted expansions from both patients with MM and Waldenstrom's macroglobulinemia have all been proven to be clonal by sequencing TCR CDR3 hypervariable regions, it can be considered that the detection of all expanded TCR $V\beta$ -restricted populations are associated with a T-cell clone.^{12,16,17} Clonal expansions of cells with an effector memory T-cell phenotype have been associated with chronic antigen stimulation in the setting of persistent viral infection.¹⁸ However, in the MM cohorts studied, there was a lack of association with any common viral serology or cytomegalovirus pp65 tetramer staining, suggesting that chronic stimulation by a non-viral antigen is involved.^{16,17} Furthermore, neither idiotype- nor cancer germline-specific tetramers have identified the specificity of these clones, as such tetramer-positive cells are rarely more than 0.1% of the T-cell population.^{16,19} Expanded T-cell clones are clinically relevant as they are strongly associated with improved outcomes in MM,^{10,16} their incidence increases with exposure to thalidomide and their presence is associated with prolonged remission in patients receiving maintenance thalidomide after autologous transplantation.¹⁰ Identification of the specificity of expanded T-cell clones has been restricted as they fail to respond in either proliferation or cytotoxicity assays, suggesting that these cells are predominantly anergic.¹²

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Although the presence of T-cell clones has prognostic significance, changes in other clinically significant immune biomarkers such as T-regulatory cells (Tregs) should also be considered. The balance between the suppressive Treg cells and pro-inflammatory T helper 17 (Th17) cells is a major factor in immunoregulatory control^{20–22} and although there is considerable variation between reports of the number of Treg cells in patients with MM, this can be attributed to technical differences in assay methodology and patient selection.^{23,24}

We hypothesise that the immune function of LTS-MM patients is different from other patients with MM and that an analysis of immune biomarkers may provide novel opportunities to identify good prognosis patients, further characterise the immune dysfunction associated with MM and reveal insights for immune-based therapies. We analysed the number of cytotoxic T-cell clones and their ability to proliferate and secrete cytokines, as well as the balance between suppressive Tregs and pro-inflammatory Th17 cells. These immune biomarkers were determined in LTS-MM patients, MM patients with shorter follow-up and a group of age-matched controls. Differences in all parameters were evident, demonstrating that long-term survival is associated with a distinct immunological profile suggestive of less immune suppression.

MATERIALS AND METHODS

Patients, controls and samples

All patients attending our clinic for > 10 years from diagnosis of MM and who had required treatment were identified and included in the study group ($n = 20$). These patients represent approximately 5% of the total patients attending the clinic. Sample collection and clinical record review were performed with informed consent in accordance with the Declaration of Helsinki. The frequency of T-cell clones was compared with two large cohorts of MM patients, one composed of patients collected sequentially at our clinic ($n = 144$)⁹ and another of patients ($n = 120$) enrolled in a national clinical trial investigating the role of maintenance thalidomide, with samples collected before randomisation.¹⁰ Results for the T-cell clone proliferation assay, Treg and Th17 cell analysis were compared with sequentially acquired MM patients attending our clinic and a group of age-matched controls with normal haematological parameters and no known medical abnormality.

Flow cytometry analysis

All studies were performed on EDTA-anticoagulated peripheral blood samples, which were prepared by Ficoll Paque (GE Healthcare, Uppsala, Sweden) centrifugation and cryopreserved for later analysis. All flow cytometry was performed on a BD FACS ARIA II (BD Biosciences, San Jose, CA, USA). T-cell clonal expansions were detected using TCRV β repertoire analysis using an IOTest Beta Mark TCRV β Repertoire Kit (Beckman Coulter, Brea, CA, USA) as previously described.^{10–12,15–17} TCRV β ⁺ clones were identified by the overexpression of one TCRV β family. This was defined as greater than the mean plus 3 standard deviations of the TCR V β analysis of 42 age-matched controls for each TCRV β family. We have previously confirmed the clonality of these expansions by sequencing CDR3 hypervariable regions.^{12,16,17} TCRV β panels included antibodies to CD3, CD8 and CD57 (BD Biosciences) and antibodies to specific TCR V β families (Beckman Coulter). The phenotype of T-cell clones is CD3⁺CD8⁺CD57⁺ in > 93% of cases. Tregs were defined as CD3⁺CD4⁺CD25⁺CD127^{lo} cells as previously described.²⁵ Th17 cells were detected as CD3⁺CD4⁺ cells secreting IL17 (anti-IL17; BD Biosciences) after incubation with phorbol myristate acetate, ionomycin and brefeldin according to manufacturer's instructions and interferon- γ expression was determined with anti-interferon- γ (BD Biosciences).

T-cell sorting and proliferation assay

T-cell clones were sorted on a BD FACS ARIA II (BD Biosciences) as shown (Figure 1c). The proliferation of sorted clonal and non-clonal T-cells was measured using carboxyfluorescein succinimidyl ester (CFSE) tracking. In brief, 1×10^5 T cells from each individual were labelled with 5 μ M CFSE using CellTrace CFSE Cell Proliferation Kit (Life Sciences, Carlsbad, CA, USA), cultured in RPMI 10 and stimulated with anti-CD3 and anti-CD28 beads (Miltenyi Biotec, Bergisch Gladbach, Germany) at a ratio of 1:1 for 4 days.

At this point, proliferation was measured as the proportion of cells that had passed through ≥ 1 division.

Statistical analysis

Statistical analysis involving the Mann–Whitney (U) and Student's t -test was performed as appropriate for parametric and non-parametric data using GraphPad PRISM v5.01 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Clinical characteristics of LTS-MM patients

There were 20 patients attending our clinic > 10 years after a diagnosis of symptomatic MM. Table 1 displays the clinical characteristics of the LTS-MM cohort and the cohorts of MM patients used to compare the proliferation of T-cell clones, Treg frequency and Th17 cell frequency. The frequency of clonal T-cell expansions was compared with patients tested in the context of a clinical trial ($n = 120$), with a similar median age and International Staging System (ISS) risk group at diagnosis,^{10,26} and a large local cohort of MM patients as previously published ($n = 144$).⁹ As all LTS-MM patients were diagnosed > 10 years ago, there is little fluorescence *in situ* hybridization data available, and treatment was predominantly with multi-agent chemotherapy as novel agents were not widely available at that time. The entire LTS-MM cohort is included even though samples were not available for all tests to be performed. Two patients have recently relapsed and died.

LTS-MM is associated with an increased frequency of T-cell clonal expansions

We have identified T-cell clones in two previous cohorts of MM patients and found their incidence to be 48% ($n = 120$) and 54% ($n = 144$).^{9,23,24} In comparison, T-cell clones were found in 100% ($n = 19/19$) of the LTS-MM patients tested, accounting for a median of 14.9% of CD3⁺ cells. There was no clear over-representation of any particular TCR V β subfamily. A representative analysis of a MM sample is shown in Figures 1a and b. In this case, there is an expanded TCRV β 13.1 population.

T-cell clonal expansions in LTS-MM lack the anergy found in patients with MM

T-cell clones of patients with MM had a low proliferative capacity *in vitro* in 4-day CFSE assays, whereas the non-clonal T cells in these patients were highly proliferative ($U = 0.0$, $P < 0.0001$; Figure 2a). In contrast, the T-cell clones in the LTS-MM patients had a high proliferative capacity, similar to autologous non-clonal T cells (Figure 2a). The median proliferation of the T-cell clones was 61.5% ($n = 15$) for the LTS-MM patients compared with 6.1% ($n = 10$) for MM patients ($U = 6.0$, $P < 0.0001$; Figure 2a). Interferon- γ production was not significantly different in the T-cell clones of the MM and LTS-MM patients (Figure 2b).

T-cell clones remain in the blood of LTS-MM patients for long periods

Eight of the LTS-MM patients had serial blood samples tested for T-cell clones over an interval of more than 10 years. For seven of these eight patients, the same expanded TCR V β family persisted for more than 10 years.

The Treg/Th17 balance is abnormal in MM and is more favourable in LTS-MM

Treg and Th17 cell numbers were analysed in the peripheral blood. Representative flow plots of the analysis of CD3⁺CD4⁺CD25⁺CD127[−] Tregs (Figure 3a) and CD3⁺CD4⁺IL-17⁺ Th17 cells (Figure 3b) are shown. MM patients had a decreased absolute CD4 count and there was no significant difference in the CD4

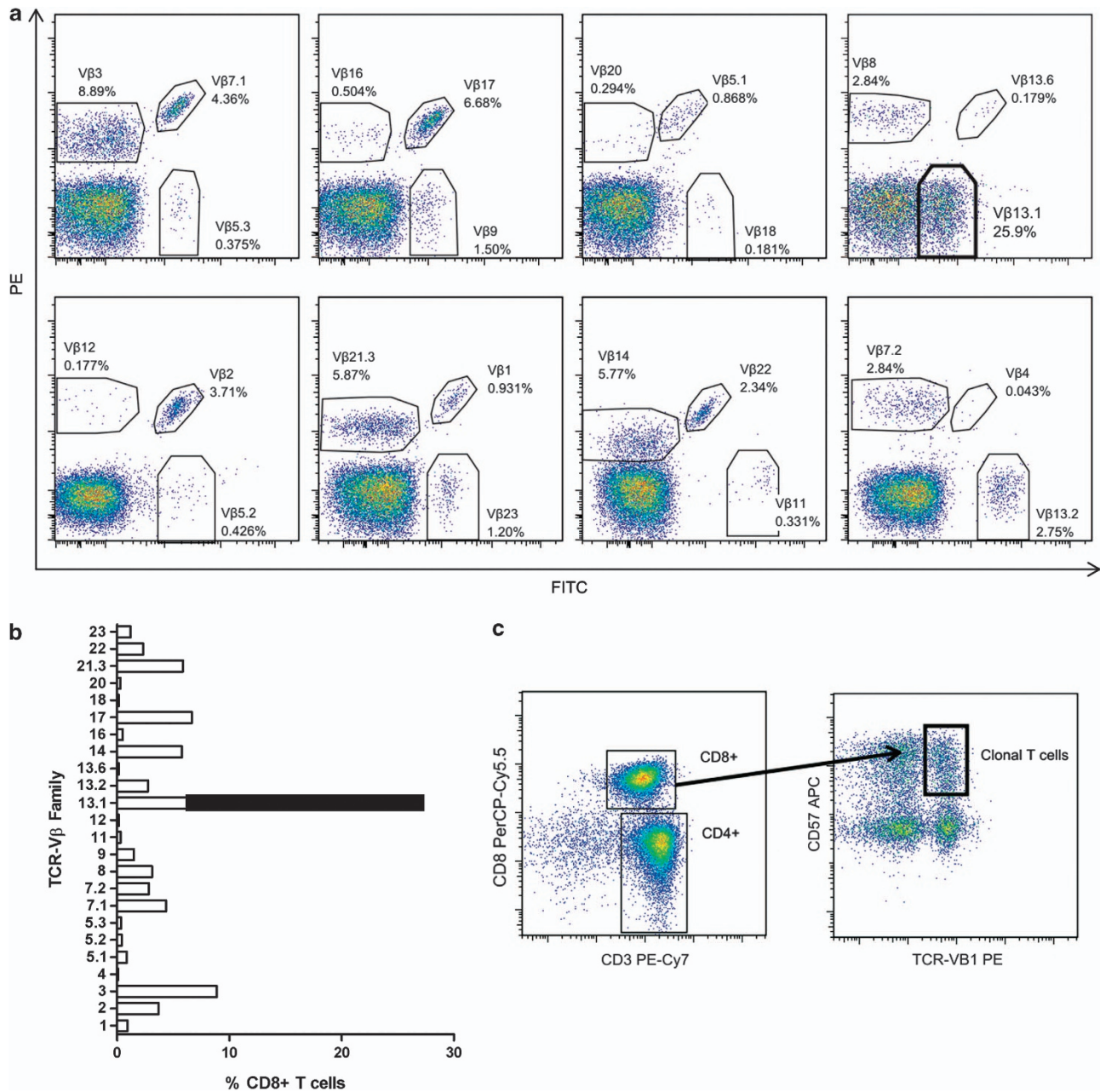


Figure 1. The detection of clonal expansions of T cells using flow cytometry to measure TCR Vβ subfamily overexpression. **(a)** Representative flow scattergrams and **(b)** bar graph demonstrating a clonal expansion of Vβ family 13.1 and **(c)** the gating strategy utilised for T-cell clone sorting. In this example, a TCR Vβ1 expansion was detected from CD3 + CD8 + region. The TCRV β1 expansion had both CD57⁺ and CD57⁻ subpopulations. PE, phycoerythrin.

count between the MM and LTS-MM patients (Figure 3c). Tregs were increased in the blood of MM patients compared with age-matched healthy control donors (Figure 3d), when expressed as a percentage of CD4⁺ T cells. Tregs were decreased in LTS-MM patients ($n=16$) when compared with MM patients ($n=30$), whether expressed as a percentage of CD4⁺ cells ($t=3.01$; $P<0.005$) or as an absolute number ($U=155$; $P<0.02$; Figures 3d–e). Th17 cells were decreased in MM patients ($n=12$) when expressed as an absolute number ($U=2.49$; $P<0.02$) but not significantly different from controls when expressed as a percentage of T cells. Th17 cells were, however, increased in LTS-MM patients when compared with MM patients whether expressed as a percentage ($U=19$; $P<0.005$) or an absolute number ($U=48$; $P<0.05$; Figures 3f–g).

The Treg/Th17 ratio (Figure 3h) was increased in MM patients, however, it was significantly lower in the LTS-MM patients and similar to the control group ($U=8.0$; $P<0.001$). The Treg/Th17 ratio may be the best discriminator of immunoregulatory control, as it indicates the balance of suppressive and pro-inflammatory regulatory cells.

DISCUSSION

We have identified a group of patients with symptomatic MM attending a single clinic who survived for >10 years. We have assayed a range of immunological biomarkers in their peripheral blood samples to assess whether specific immune features are associated with longer survival. We have provided evidence

Table 1. Demographic details for the LTS-MM cohort and the comparison MM cohorts for the proliferation of T-cell clones, analysis of Treg frequency and analysis of Th17 cell frequency

Cohort	n	Sex (% male)	Age, median (range) ^a , years	Months since diagnosis (range)	$\beta 2M$, median (range) ^a	Albumin, median (range) ^a	ISS, median ^a	Autologous transplants, proportion received ^b (%)	Allogeneic transplant, proportion received ^b (%)	Conventional chemotherapy, proportion received ^b (%)	Novel agents, proportion received ^b (%)
LTS-MM	20	46%	58 (32–73)	141 (98–252)	1.7 (1.2–3.7)	42 (32–47)	1	50	15	100	40
MM (Proliferation of T cell clones)	10	50%	53 (23–76)	36 (18–96)	2.1 (2–3.6)	40 (35–47)	1	60	10	100	100
MM (frequency of Tregs)	30	69%	64 (39–82)	45 (2–67)	3.2 (1.7–10.4)	38 (35–45)	1	39	9	87	70
MM (frequency of Th17 cells)	12	54%	67 (52–82)	38 (4–72)	3.8 (1.8–5.9)	38 (34–45)	1	27	0	73	64

Abbreviations: LTS, long-term survivor; MM, multiple myeloma; Tregs, T-regulatory cells. ^aAt diagnosis. ^bFrom time of diagnosis to analysis.

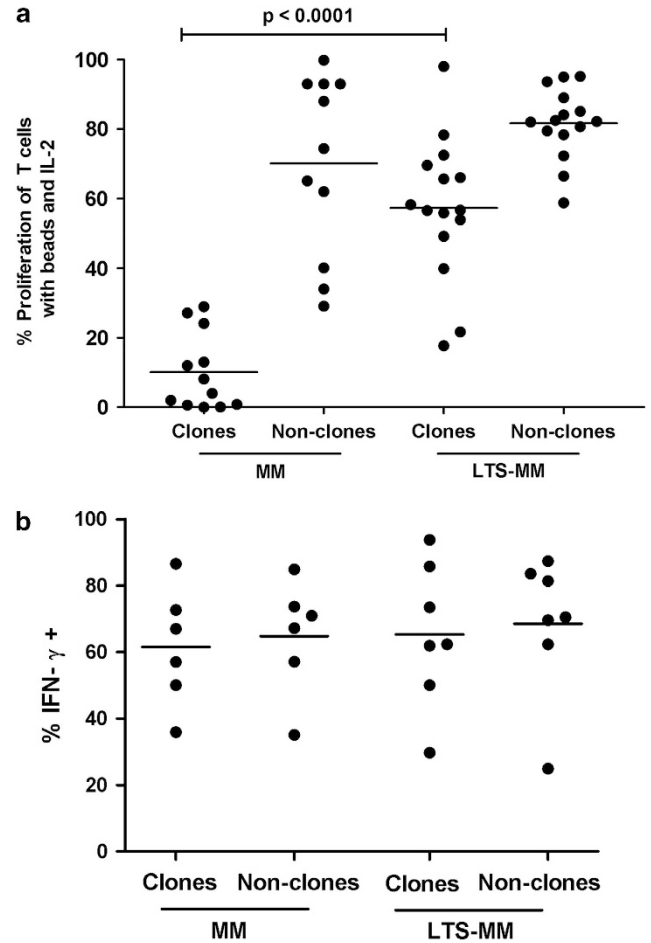


Figure 2. The proliferation and cytokine production of T-cell clones. Cells were tracked with CFSE after stimulation with anti-CD3 and anti-CD28 beads for 4 days. Proliferation was assessed as the % cells that progressed through at least one cell division. **(a)** Proliferation of clonal T cells from LTS-MM patients and MM patients ($P < 0.001$) and the respective non-clonal T cells. **(b)** Interferon- γ production in clonal and non-clonal T cells from LTS-MM patients and MM patients ($P =$ not significant).

that this group of patients have a range of immunological biomarkers, which are different from other patients with MM. The major findings reported here are: first, that all LTS-MM patients analysed had expanded cytotoxic T-cell clones present, and unlike the majority of patients with MM, the T-cell clones in LTS-MM patients respond to stimulation by proliferating and producing cytokines, and second, the Treg/Th17 ratio, which is increased in MM patients, is not increased in LTS-MM patients.

There are clear demographic differences between the LTS-MM group (Table 1) and the comparison cohorts. It is predictable that the LTS group will contain fewer males and have lower measures of tumour bulk at diagnosis, as these measures are associated with longer survival in MM. However, despite this, all groups contained patients predominantly in the lowest ISS risk category. Similarly, differences in therapy between the groups are understandable given the fact that most LTS-MM patients received treatment > 10 years ago, and the management of MM has changed considerably over that time. Possibly, the most significant difference is in treatment with novel agents, and this is predominantly accounted for by thalidomide, with low exposure in the LTS-MM group. As thalidomide enhances the proliferation of T cells *in vitro*,²⁷ this

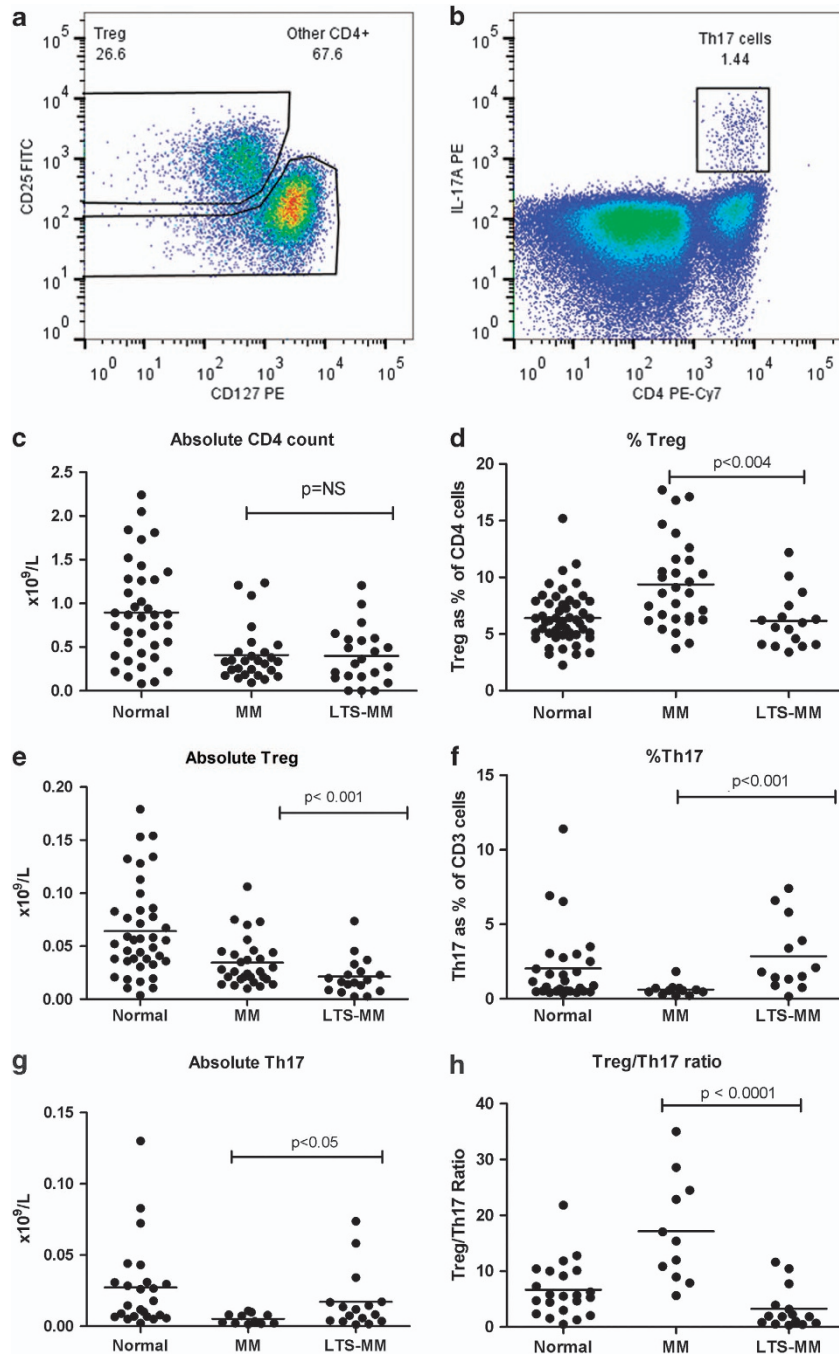


Figure 3. Analysis of Tregs and Th17 cells. Representative flow scattergrams demonstrating the identification of (a) Tregs as CD3+CD4+CD25++ and CD127- and (b) Th17 cells as CD4+IL-17+. The comparison of results from LTS-MM, MM and healthy donor controls for (c) absolute CD4 count. (d) Percentage Tregs. (e) Absolute Tregs. (f) Percentage Th17 cells. (g) Absolute Th17 cells. (h) Treg/Th17 ratio. NS, not significant.

does not explain the observed increase in proliferation of T-cell clones in LTS-MM patients. Thalidomide has also been shown to increase Treg number in MM. Although there was higher exposure in the MM group than the LTS-MM group (57% and 25%, respectively), few (17% and 6%, respectively) were taking it at the time of testing, and its propensity to increase Tregs is seen mainly during treatment.²⁸

We have attempted to expand T-cell clones from MM patients *in vitro* but have consistently found them to have low or no proliferative response despite normal proliferation of other T cells from the same patient (Figure 2a). As *ex vivo* expansion of

these cells may provide a potential therapeutic intervention, we have attempted to overcome their anergy through the use of a broad panel of stimulatory cytokines and antibodies, which enhance proliferation. However, this has not yet been possible (data not shown). As these T-cell clones retain the ability to secrete interferon- γ despite a lack of proliferation (Figure 2b), we suggest that T-cell clones in patients with MM exist in a state of split anergy.²⁹ This anergic state may be induced by regulatory T cells, which can suppress anti-tumour cytotoxic T-cell responses but not interferon- γ production through transforming growth factor β (TGF β) signalling.³⁰

We recently published observations that suggest that multiple abnormalities cause the anergy in T-cell clones of patients with MM and Waldenström's macroglobulinemia.¹² We performed gene set-enrichment analysis after microarray of flow-sorted clonal and non-clonal CD8⁺ T cells and demonstrated upregulation of a number of signalling pathways. This included the RAS pathway, and upregulated expression of *Ras*, *BCL2L1* (*BCL-XL*) and *CHUK* (*IKK*) could inhibit apoptosis in the expanded T cells, thus allowing their long-term persistence.³¹ An upregulated CSK pathway would recruit *CREBBP* to activate *CSK* and inhibit T-cell activation.³² An upregulated TOB pathway, in which *TGF β* could activate *SMADs* to interact with *Tob* and maintain an unstimulated T cell.³³ *TGF β* could also engage with *TGFBR*, *CDKN2A* (*p16*) within a G1/S transition pathway to downregulate *CCND2* and *CDK6*, leading to cell cycle G1/S transition arrest.³⁴ Finally, upregulation of *PTPN7* (*HePTP*) expression could lead to a suppression of T-cell proliferation by inactivating *ERK*.³⁵ Although this microarray data suggest that multiple mechanisms contribute to T-cell anergy, the neutralisation of TGF β may be a key factor in the dysfunction of both T cells.³⁶

Tregs are instrumental in the maintenance of tolerance to self-antigens. They suppress low-affinity self-reactive T-cells, which escape negative selection in the thymus, but in doing so may also impair tumour-specific immune responses. Tregs induce anergy in tumour-specific CD8 T-cells through multiple mechanisms³⁷ and increased Treg numbers are associated with worse outcomes in MM³⁸ and in solid organ malignancies.^{39–41} We and others have found Tregs to be increased in MM patients^{42,43} and we now report that they are significantly lower in LTS-MM patients⁴⁴ indicating a less tolerogenic immune environment.

Th17 cells have a well-established role in the promotion of auto-inflammation⁴⁵ and they promote the survival of tumour-reactive T-cells in the context of malignancy.⁴⁶ Although bone marrow Th17 cells support MM cell growth⁴⁷ and bone disease,⁴⁸ the balance of Treg/Th17 cells also regulates auto-reactive immune responses. The consideration of peripheral blood Treg and Th17 numbers may provide a better indication of the regulatory cell involvement than a determination of Treg cells alone. Th17 cell numbers were significantly increased in LTS-MM patients and interestingly, although the Treg/Th17 ratio was markedly increased in MM patients, it was decreased in LTS-MM patients when compared with MM and even controls. This suggests a fundamental difference in the T-cell helper environment in LTS-MM patients.

In conclusion, LTS-MM patients have a distinct immunological profile. This includes an improved Treg/Th17 balance and an increased incidence of persistent T-cell clonal expansions which, unlike those found in other MM patients, are proliferative. These features suggest that patients who survive MM long term have decreased immune suppression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

CB, HS and RB designed the experiments, analysed/interpreted data and wrote the paper; JF, SY and EA performed experiments and analysed the data; JG, PJH, HI and DEJ contributed the collection of patient samples, designed experiments and wrote the paper; PF, NW, NN and DH contributed to designing experiments and wrote the paper; all co-authors reviewed and discussed the manuscript.

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