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Multiple myeloma causes clonal T cell immunosenescence: Identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade

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Multiple myeloma causes clonal T cell immunosenescence: Identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade

Running title: Senescent T cells in multiple myeloma

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Conflict-of-interest disclosure

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ABSTRACT

Tumour-induced dysfunction of cytotoxic T cells in patients with multiple myeloma (MM) may contribute to immune escape and be responsible for the lack of therapeutic efficacy of immune checkpoint blockade. We therefore investigated dysfunctional clonal T cells in MM and demonstrated immunosenescence but not exhaustion as a predominant feature. T cell clones were detected in 75% of MM patients and their prognostic significance was re-validated in a new post-IMiD cohort. The cells exhibited a senescent secretory effector phenotype: KLRG-1+/CD57+/CD160+/CD28-. Normal-for-age telomere lengths indicate that senescence is telomere-independent and potentially reversible. p38-MAPK, p16 and p21 signaling pathways known to induce senescence were not elevated. Telomerase activity was found to be elevated and this may explain how normal telomere lengths are maintained in senescent cells. TCR signaling checkpoints were normal but elevated SMAD levels associated with T cell inactivation were detected and may provide a potential target for the reversal of clonal T cell dysfunction in MM. Low PD-1 and CTLA-4 expression detected on T cell clones infers that these cells are not exhausted but suggests that there would be a sub-optimal response to immune checkpoint blockade in MM. Our data suggest that other immunostimulatory strategies are required in MM.

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INTRODUCTION

The failure of immune surveillance in human cancer can be attributed to tumor-induced molecular and cellular mechanisms that incapacitate immune cells and effector responses, including tumour-specific cytotoxic T cells, which allows for tumor progression. Immune cells introduced as adoptive cellular immunotherapy can also be suppressed.[1,2] Thus, overcoming tumor-induced T cell hypo-responsiveness is an important consideration for immune therapies. However, the underlying mechanisms of dysfunction must first be characterised.

Immune dysfunction in patients with multiple myeloma (MM) is multi-factorial and includes TGF-β induced dendritic cell dysfunction,[3] regulatory T cell (Tregs)/Th17 cell imbalance,[4,5] generation of acquired Tregs by trogocytosis[6] and increased myeloid derived suppressor cells.[7] Tumor-induced immune dysfunction is also greater in patients with MM than other B cell malignancies.[5-9] T cell dysfunction appears to be restricted to the those cells affected by the MM cancer cells as patients with MM do not exhibit the classical pattern of infections associated with a T cell immunodeficiency state like fungal and mycobacterial infections.

Dysfunctional T cells observed in cancer can be classified as anergic, exhausted or senescent according to phenotypic characteristics (Figure 1 and Table 1).[10] Anergic T cells are hypo-responsive due to inadequate TCR co-stimulation through CD28 signaling or in the presence of high co-inhibitory signaling such as CTLA-4[11] and the cells fail to produce IL-2.[12] T cell exhaustion occurs after chronic antigen over-

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stimulation and results in the upregulation of multiple inhibitory receptors including PD-1, CTLA-4, CD160, Tim-3 and LAG-3 and an inability to express IFN-γ. [10,13-15] Senescent T cells are late differentiated memory T cells generally associated with aging. They lack CD28, express regulatory receptors, are in cell cycle arrest and have enhanced secretion of inflammatory cytokines[16]. There are two types: telomere-dependent senescence (replicative senescence), which results from telomere attrition triggered by DNA damage, p53 protein or cyclin-dependent kinase inhibitor (CDKI) p21CIP1/WAF1 (p21) pathways[17]; and telomere-independent senescence, which is induced by stress and an altered signal transduction pathway generally involving the CDKI p16INK4a (p16) and the retinoblastoma tumor suppressor (pRb)[18]. NF-κB signaling has also been implicated in the induction of a phenomenon known as senescence-associated secretory phenotype (SASP). These cells are senescent but retain their ability to secrete pro-inflammatory factors.[19]

Cytotoxic T cells are the predominant effector cell involved in cancer immune destruction and expanded clones of cytotoxic T cells exist in patients with MM[20-22], chronic myeloid leukemia,[23,24] Waldenström's macroglobulinemia (WM)[25] and myelodysplastic syndromes.[26,27] The T cell clones in MM patients can constitute up to 50% of all blood lymphocytes and are hypo-responsive *in vitro,*[4,25] but despite this, their presence, is related to better survival.[20-22,28,29] Overcoming the hypo-responsiveness of these cells, regardless of their specificity could provide a novel cell therapy based on the restoration of the host's immune response.

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In this study, a large cohort of MM patients was tested to verify the prognostic advantage of expanded T cell clones in patients receiving current therapy including novel agents. We identified MM T cell clones as telomere-independent senescent cells and attempted to identify agents that were responsible for inducing senescence. We also demonstrated normal TCR signaling and low PD-1 and CTLA-4 expression. This suggests that the response to immune checkpoint blockade therapy would be sub-optimal in MM. In addition, we have identified abnormalities in p-SMAD levels in the TGF-β pathway that could be responsible for dysfunction in these cells and suggest this may provide a novel target for immune recovery therapy.

METHODS

Patients

Peripheral blood (PB) and bone marrow (BM) samples were collected from 103 MM patients attending our clinic. Sample collection and clinical record review from patients and aged-matched normal controls were performed after patient informed consent and approval by the institutional Human Ethics Review Committee (X12-0205) in accordance with the Declaration of Helsinki. The clinical characteristics of the different patient cohorts are shown in Supplementary Table 1.

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Flow cytometric analysis

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll Paque (GE Healthcare, Uppsala, Sweden). Flow cytometric analysis was performed on a BD FACS Canto II (BD Biosciences, San Jose, CA, USA) and flow sorting was

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performed on a BD FACS ARIA II (BD Biosciences). Clonal T cells were classified as CD3+/CD8+/TCR-Vβ+/CD57+ (hereafter clones) and were compared to the internal biological control: non-clonal T cells which were CD3+/CD8+/TCR-Vβ-/CD57+ (hereafter non-clonal or Vβ-57+). Two other CD8+ subsets: Vβ+CD57- and Vβ-CD57- were also included.

TCRVβ repertoire analysis

Patients were screened for clonal T cell expansions by analysis of the TCRVβ (hereafter Vβ) repertoire (Supplementary Figure 1) using an IOTest® Beta Mark TCRVβ Repertoire Kit (Beckman Coulter, Brea, CA, USA) as previously described.[4,25,29] Vβ+ clones were identified by the overexpression of a Vβ family defined as greater than the mean plus 3 SD after Vβ analysis of 42 age-matched controls for each Vβ family.

CFSE proliferation assay

CFSE-labelled clonal and non-clonal T cells were stimulated with anti-CD3/CD28 MACS iBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) at a 1:1 ratio and 0.1μg/mL IL-2 (R&D Systems, Minneapolis, MN, USA) for 4 days, as previously described.[4] The effect of 0.1μg/mL IL-12 (R&D Systems), 20ng/mL IL-15 (R&D Systems), 50ng/mL IL-21 (Invitrogen Corporation, Camarillo, CA, USA), 50ng/mL anti-CD137 (BD Biosciences), 50ng/mL anti-CD134 (BD Biosciences) and 5 μM lenalidomide (Celgene Corporation, San Diego, CA, USA) on T cell proliferation was also tested.

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14 day *ex vivo* expansions

Purified T cell clones from MM patients were expanded using the Miltenyi T cell activation/expansion kit according to the manufacturer's protocol. In brief, T cells were stimulated at a ratio of 1:1 with anti-CD3/28 beads and cultured in RPMI supplemented with 10% AB serum and 20 units IL-2/mL (R&D) in a 96 well U bottom plate (BD Biosciences) for 14 days.

Phospho-flow analysis of signaling pathways

For phospho-flow cytometry (PFC) analysis of phosphorylated (p) proteins: p-SMAD2 (pS465/pS467)/ p-SMAD3 (pS423/pS425) (pSMAD), p-SHP-2 (pY542), p-p38-MAPK (pT180/pY182) (BD Biosciences), PBMC were labelled with anti-CD8 PE-Cy7 and PE or FITC conjugated anti-Vβ. Cells were fixed with BD Cytofix buffer (BD Biosciences) at 37°C then permeabilised with ice cold BD Perm III Buffer (BD Biosciences). Cells were labelled with anti-CD57 efluor-450 (BioLegend, San Diego, CA, USA), CD3 PerCP-Cy5.5 (BD Biosciences) and anti-phospho-antibody.

For p-ZAP-70 (pY142), CD3ζ, p16 (BD Biosciences), p21 (Genesearch, Arundel, QLD, Australia) and Bcl-xL (Abcam, Cambridge, UK), cells were stained with surface markers to identify clonal T cells and then fixed and permeabilised with BD Cytofix/Cytoperm (BD Biosciences) on ice. Cells were washed twice with BD Perm/Wash buffer before incubation with intracellular antibody. p-ERK (BD Biosciences) was studied using Beckman Coulter Perfix-P Kit (Beckman Coulter)

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according to manufacturer's instructions. Fluorescence minus one controls were used to establish positive expression limits and determine constitutive phosphorylated protein expression.

Phenotypic analysis of T cell clones

The surface phenotype was studied using flow cytometry (FC) to classify the T cell clones as either anergic, exhausted or senescent.[10] Three antibody panels were used: all with anti-CD3 V500, anti-CD8 APC-H7, anti-CD57 V450 (BD Biosciences) and anti-Vβ PE/FITC (Beckman Coulter) for the identification of T cell clones and then either 1) anti-PD1 PE-Cy7 and anti-PD-L1 FITC/PE (BD Biosciences); 2) anti-LAG-3 APC (R&D Systems) and anti-TIM-3 PE-Cy7 (BioLegend) or 3) CD27 FITC/PE and CD28 PerCP-Cy5.5 (BD Biosciences) . The senescent phenotype was studied by flow detection of KLRG-1 (Biolegend) and intracellular p16, p21 and p38-MAPK (BD Biosciences) and measurement of telomere length (TL) by qPCR and flow-FISH.

Telomere assessment by qPCR

DNA was extracted using the Maxwell® 16 cell low elution volume DNA purification kit (Promega, Wisconsin, USA) and cryopreserved for PCR. Assessment of TL was performed according to previous methods[30,31]. All PCR reactions were performed in triplicate on the Rotorgene Q (Qiagen, Hilden, Germany). Relative TL was calculated by dividing the telomeric DNA quantity by the single copy gene DNA quantity (T/S). T/S of MM patient T cell clones were compared to an age-matched

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normal range established by Dr. P. Barbaro, Children's Medical Research Institute, Westmead, NSW, Australia.

Telomere assessment by Flow-FISH

TL was also measured using the DAKO Telomere PNA kit/FITC (Elitech, VIC, Australia) according to manufacturer's protocol. Relative TL was calculated by comparing the TL of patient cells to a control cell line with long telomeres (CCRF-CEM; kindly provided by Dr. Tatjana Kilo).

Measurement of Telomerase by FC

Human telomerase reverse transcriptase (hTERT), the catalytic subunit of human telomerase was measured by FC. PBMC were fixed and permeabilised using the Leucoperm kit (AbD Serotec, Oxford, UK), labelled with purified anti-hTERT (1:200; Clone 2C4, Thermo Scientific, Victoria, Australia) or purified mouse anti-IgM κ isotype control (1:100, BD Biosciences). Cells were washed, labelled with secondary goat anti-mouse Ig-APC (1:100, BD Biosciences) and then washed twice before labelling with clonal T cell surface antibodies.

Statistical Analysis

Statistical analysis using the Student t test, Mann-Whitney test and Kaplan-Meier survival curves were performed using Prism (Version 6, GraphPad Software).

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RESULTS

Expanded T cell clones in patients with MM are associated with an improved survival and have an increased incidence post-IMiD therapy

Clonal T cell expansions were detected in 75% of MM patients and more than 90% were CD8+. Of the patients who had received therapy, 70% had received IMiDs. The presence of T cell clones at any stage of disease was associated with an improved survival ($\chi2$=21.01; p<0.0001; Figure 2A). There was no correlation between presence of T cell clones and disease status, as was shown previously,[20,28] nor with ISS stage, previous treatment or current treatment (Supplementary Table 2). Exposure to thalidomide induces additional T cell clones in patients, raising their frequency from approximately 50% to 76%,[28] which was similar to the incidence of 75% identified in this study (Table 2). There was no prognostic advantage for patients with a large clone size (>20% of CD3+ cells) compared with a moderate clone size (<20% of CD3 cells) ($\chi2$= 0.11; p=NS; Figure 2B). There was also no preferential usage of any one TCR Vβ family (Figure 2C).

Clonal T cells in MM are hypo-responsive and fail to respond to a panel of immune modulators

T cell clones from MM patients (n=15) failed to proliferate when stimulated with anti-CD3/28 beads, whereas non-clonal T cells showed active proliferation in 4 day cultures (p<0.0001; Figure 3B). The addition of potential immune modulators as well as anti-CD2/3/28 beads and IL-2 failed to increase the proliferation of the T cell

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clones *in vitro* (Figure 3C). We previously reported that MM patients surviving more than 10 years invariably demonstrate clonal expansions that have proliferative capacity[4]. This was re-examined using an expanded 10-year MM survivor group and the same correlation was found (n=32; Figure 3B). The lack of response of clonal and non-clonal T cells from a representative 10-year MM survivor to a variety of immune modulators is also shown in Supplementary Figure 2. In 14 day *ex vivo* expansions, flow sorted clonal T cells from 6 of 8 10-year MM survivors could be expanded whereas 6 of 7 non-10-year survivors did not expand (Figure 3D).

MM T cell clones have normal TCR signaling

Components of the TCR signaling pathway (Supplementary Figure 3) were assessed by PFC. Following TCR engagement, the tyrosine kinase, ZAP-70 is required for activation of downstream signaling pathways[32]. Both constitutive and stimulated p-ZAP-70 expression was present at similar levels in MM T cell clones compared to non-clonal T cells (n=18). SHP-2 is a tyrosine phosphatase that dephosphorylates signaling molecules and dampens T cell signaling[33]. There was a trend for both constitutive and stimulated p-SHP-2 levels to be higher in the T cell clones compared to non-clones, but this was not statistically significant (n=12). CD3-ζ, a TCR component[34], was present at significantly higher levels on clonal T cells compared to non-clonal T cells (n=19; $p<0.02$) suggesting that signal transduction was not impaired (Supplementary Figure 4).

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MM T cell clones have a defective p-SMAD pathway that blocks cell cycle progression

We previously identified several dysfunctional pathways in expanded T cell clones in WM by bioinformatic analysis of microarray expression data[25]. We selected key protein targets from these pathways (Supplementary Figure 3) and examined the expression levels by PFC in clonal and non-clonal T cells from 10-year and non-10-year survivors of MM.

In the TOB pathway, TGF-β stimulates p-SMAD, which can interact with Tob to maintain T cell inactivation. Tob can also interact with CDKI p27kip1 causing cell cycle arrest.[35] Expression of p-SMAD did not differ between the different CD8+ T cell subsets studied when all MM patients were analyzed together (n=11; Supplementary Figure 5B). However, significantly higher levels of this phosphorylated protein were found in non-proliferative T cell clones of non-10-year survivors compared to proliferative T cell clones of 10-year MM survivors (U=5.0; p=0.01; Supplementary Figure 5C). Treatment and disease status did not impact these findings (Supplementary Table 1).

No significant differences were found between different CD8+ T cell subsets studied or between T cell clones of 10-year and non-10-year survivors for p-ERK (related to proliferation[36]) or Bcl-xL (anti-apoptotic protein[37]) (Supplementary Figure 5).

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Low PD-1 and CTLA-4 expression suggests that immune checkpoints are not responsible for hypo-responsiveness and may not be appropriate therapeutic targets in patients with MM

For immune checkpoint blockade to be successful in MM, it is essential that antigen-specific effector CD8+ T cells are present and that these cells can recognise myeloma-specific targets. As clonal T cells in MM are possibly tumor-specific T cells, we examined CTLA-4 and PD-1 expression on PB and BM T cells from MM patients and age-matched controls. The T cell clones expressed significantly lower levels of PD-1 than the non-clonal T cells in both PB (n=12; p=0.0005; Figure 4B) and BM (n=11; p=0.02; Figure 4C) and also in comparison to CD8+ CD57+ T cells from age-matched normal controls. (U=11; p=0.01). Similarly, CTLA-4 expression was significantly lower on T cell clones found in the PB compared to non-clonal cells (n=19; p=0.005; Figure 4E) but this was not significantly different in the BM (n=4; Figure 4F).

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MM T cell clones have a phenotype which suggests a state of telomere-independent senescence rather than anergy or exhaustion

MM T cell clones expressed low levels of LAG-3, TIM-3 (n=9; Figure 5B,C), PD-1 and CTLA-4 (Figure 4B,E) and did not express CD28 (Figure 5E). The expression of these molecules did not differ from non-clonal T cells. Based on the exclusion of these phenotypic markers, T cell clones are not anergic or exhausted. T cell clones in MM patients are CD57+, express CD160 (n=9; Figure 5D) and are of the late

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differentiated phenotype as demonstrated by the lack of CD27 and CD28. KLRG-1 was expressed at significantly higher levels on T cell clones than non-clonal T cells (n=8; t=4.60; p<0.003; Figure 5F). Based on these phenotypic markers, T cell clones are senescent.

TL measurement by qPCR demonstrated that clonal T cells did not have shortened telomeres compared to non-clonal T cells (n=4; Figure 5G) and an age-matched normal range (data not shown). Likewise, the TL of clonal T cells as measured by Flow-FISH (Figure 5H) was not significantly different from non-clonal T cells or age-matched normal controls (n=5; Figure 5I). Telomerase activity (hTERT levels) was significantly higher in T cell clones than non-clonal cells (data not shown; t=2.35; p<0.04).

Table 1 summarises the phenotype of T cell clones from MM patients and compares this with the phenotype of anergic, exhausted and senescent T cells. The results suggest that the T cell clones of patients with MM are telomere-independent senescent cells. Treatment and disease status did not impact these findings (Supplementary Table 1).

Telomere-independent senescence cannot be attributed to involvement of p38 MAPK signaling or elevated p16 or p21 pathways

Blockade of p38-MAPK signaling has reversed telomere-independent senescence in CD4+CD45RA+CD27- T cells[38] and in CD8+ EMRA T cells.[39,40] We studied the p38 signaling pathway in T cell clones by PFC to determine if elevated p-p38-MAPK

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signaling was responsible for inducing senescence. Constitutive p-p38 expression in T cell clones did not differ from other T cell subsets (n=7) and PMA stimulation did not augment p38 levels (n=6; Supplementary Figure 6) indicating that this pathway was not responsible for inducing senescence.

The p16 and p21 proteins can maintain Rb in a hypophosphorylated, active state to initiate and sustain cell cycle arrest,[18] however the levels of these proteins were not elevated in T cell clones, compared to the other CD8 T cell subsets (n=13; n=12; Supplementary Figure 7). Thus, the telomere independent senescence of the MM T cell clones appears to relate to other mechanisms.

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DISCUSSION

Expanded CD8+ T cell clones present in the blood of patients with MM have previously been shown to be associated with a good prognosis.[20,28] Using a new cohort of patients, we have validated the association between the presence of T cell clones, at all stages of the disease, with a favourable prognosis and shown an increased incidence of T cell clones after IMiD therapy. However, these cells are hypo-responsive and fail to proliferate *in vitro*. Whether these potentially protective cytotoxic T cells are anergic, exhausted or senescent could help determine if their hypo-responsiveness is reversible, regardless of their specificity. We have made the important new observation that the T cell clones displayed features of senescent T cells: i.e. KLRG-1+, CD57+, CD160+ and CD28- cell surface phenotype. A key finding of this study is that these clonal cells have TLs that are identical to the non-clonal T cells of age-matched controls, which suggests that their senescence is telomere-independent and potentially reversible.

A plausible explanation for the presence of senescent T cells that do not have shortened telomeres, is the upregulation of telomerase to maintain TL during long term persistence. T cell clones had significantly higher levels of hTERT than non-clonal T cells. The T cell clones in MM are highly differentiated effector memory T cells that re-express CD45RA (EMRA) and studies have shown that whilst EMRA T cells do have shorter telomeres than naïve CD8 T cells, they have longer telomeres than effector memory T cells that express CD45RO (EM).[39]

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Immune dysfunction in cancer may be mediated by the downregulated expression of TCR related signaling proteins.[41] An earlier study of MM patients revealed slightly lower levels of CD3 ζ chain and ZAP-70 expression in resting CD4+ and CD8+ T cells when compared to healthy controls, and after *in vitro* activation with super antigen staphylococcal enterotoxin B, both proteins were significantly downregulated. This was more pronounced in patients with stage III MM.[42] In contrast, we found that these two TCR-signaling proteins were not significantly downregulated in T cell clones when compared to non-clonal T cells, or healthy controls (data not shown). This difference may be explained by the fact that we measured the more functionally relevant ZAP-70 in the phosphorylated state (pY292) whereas the previous MM study measured total ZAP-70 protein.[42] We also measured pZAP-70 after hydrogen peroxide stimulation but no significant difference between clonal and non-clonal T cells was found. The TCR signaling pathway can also be inhibited by PD-1 mediated recruitment of SHP-2 to inactivate T cells. It has been demonstrated that with aging T cells associated with replicative senescence, there may be an imbalance of positive and negative TCR signaling, with increased inhibitory signaling through SHP1/2.[43] However, after TCR ligation with anti-CD3/28 beads, both constitutive and stimulated pSHP-2 levels did not differ between clonal and non-clonal T cells, suggesting that SHP-2 does not have an inhibitory effect on T cell signaling. Furthermore, we have shown that PD-1 expression is low on these T cell clones, implying minimal SHP-2 recruitment. These data suggest that there is no abnormality in the TCR signaling in the MM T cell clones.

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TGF-β-dependent p-SMAD activation has been shown to inhibit CD3/28 mediated T cell proliferation in mouse models[44]. We detected significantly higher levels of this protein in the T cell clones of non-10-year survivors, which are non-proliferative after CD3/28 activation suggesting that this is a possible molecular target to reverse hypo-responsiveness.

To characterise the mechanism of senescence induction in T cell clones, we examined pathways that are associated with telomere-dependent and independent senescence. Telomere-independent senescence is induced in response to cellular stress or disruptions to normal cell signalling, such as upregulation of p16 and Rb, leading to an anti-proliferative state.[45] Senescence triggered by shortened telomeres (telomere-dependent senescence) is regulated by signaling through ATM to p53, leading to the upregulation of p21 and therefore cell-cycle arrest.[17] Elevated levels of p16 were not detected in the T cell clones of MM patients and they were not different from non-clonal T cells, indicating that the p16 pathway is not responsible for inducing clonal T cell senescence. Our data correlates with a study that also showed senescent cells with average TL had low levels of p16.[46] Cells with low levels of p16 at senescence are able to proliferate again after p53 inactivation, whereas cells with high p16 are unable to proliferate after p53 inactivation[45], suggesting that it may be possible to reactivate the MM T cell clones. MM T cell clones did not have upregulated p21 expression and therefore do not have telomere-dependent senescence.

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The p38-MAPK pathway has been implicated in senescence and blocking of this pathway has successfully reversed senescence in CD4+ CD27-CD28- T cells[38] and CD8+ EMRA T cells[39,40]. The T cells had either high constitutive p38-MAPK expression or upon PMA activation, were unable to upregulate p38-MAPK expression to the levels of non-senescent T cells. Our data indicate constitutive p38-MAPK levels are not elevated in clonal T cells and upon PMA activation, there was no impairment of p38-MAPK upregulation when compared to non-clonal T cells. These results suggest that this pathway does not play a role in inducing senescence in MM T cell clones.

Other cell signaling molecules may be responsible for inducing senescence in MM T cell clones. NF-κβ signaling is required for the maintenance of SASP[19] and inhibition of this pathway can bypass growth arrest and reverse senescence.[47] IL-6, a cytokine present at high levels in the MM microenvironment, has been described as a key player in oncogene-induced senescence[48] and can up-regulate NF-κβ signaling, whilst preventing apoptosis to maintain cell senescence.[19] MM T cell clones retain their ability to produce IFN-γ[4] and display the characteristics of SASP so the NF-κβ pathway should be investigated. Overexpression of c-myc can also cause cell senescence as part of a cancer defence mechanism.[49] Microarray data from WM T cell clones indicated that c-myc and NF-κβ were upregulated in T cell clones[25] and the relationship between these proteins and clonal T cell responses need to be explored in the MM setting. In mouse models, KLRG-1+/CD8+ T cell senescence was related to p15 INK4b expression. The human equivalent of this protein is P14

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ARF.[50] Further investigation of the levels of these other cell-cycle regulatory proteins is required to elucidate the mechanism of senescence induction.

The finding that these MM T cell clones are senescent rather than anergic or exhausted is significant. T cells in the cancer setting are usually considered to be exhausted, similar to the T cells observed in patients with chronic viral infection due to the constant exposure to high levels of tumor antigens and an immunosuppressive microenvironment.[51] Exhausted T cells commonly express the inhibitory receptors PD-1, LAG-3, TIM-3 and CTLA-4 and have dysregulated signaling pathways.[14,52] The MM T cell clones expressed low levels of these inhibitory receptors. They are also unlike the exhausted T cells found in melanoma patients, a highly antigenic tumor, which have high levels of PD-1 and therefore are susceptible to PD-1 blockade. Anergic T cells are induced during inadequate TCR co-stimulation or in the presence of high inhibitory signaling, which may be present in the cancer microenvironment. The hypo-responsiveness of the MM T cell clones however cannot be reversed with endogenous IL-2 and inhibitory receptors like CTLA-4 and PD-1 are not highly expressed. MM T cell clones are also unlike an aged T cell (replicative senescence) as they do not have shortened telomeres exhibited by T cells that have gone through multiple rounds of proliferation. The MM T cell clones are held in a suspended non-proliferative state, and persist for long periods of time due to inhibition of apoptosis. T cell clones in MM are also not expansions of large granular lymphocytes. They exhibit the same phenotype (CD3+CD8+CD57+) but do not have increased STAT3 expression (data not shown) that is associated with large

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granular lymphocytic leukaemia.[53] As MM T cell clone immunosenescence is not related to shortened telomeres, we believe that it is potentially reversible if the mechanisms of senescence induction can be elucidated. If it is related to dysfunctional signalling pathways, it may be possible to re-modulate the pathways with small molecule inhibitors or drugs such as IMiDs or HDAC inhibitors.

We have highlighted the low expression of immune checkpoint target PD-1 on T cell clones found in the BM in a previous report[54]. This study extends that observation, documenting low levels of PD-1 and CTLA-4 detected on T cell clones which may explain why Phase 1 studies of PD-1 inhibitor Nivolumab failed to provide a meaningful clinical response in 100% of MM patients.[55] It may be worthwhile to continue investigation of immune checkpoint blockade as part of combination therapy in multiple myeloma. It has recently been demonstrated that lenalidomide can enhance immune checkpoint blockade-induced immune responses in myeloma[56]. The reason for down-regulated PD-1 expression in T cell clones in MM is unknown and transcription factors that regulate PD-1 expression including T-bet or Blimp-1[57,58], are under investigation. Interestingly, it has also been reported that T-bet controls cytotoxic and cytokine secretory functions of CD8+ effector T cells and also the sensitivity of cells to senescence.[59]

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In conclusion, the T cell clones in MM have a distinct molecular signature of telomere-independent senescence rather than anergy or exhaustion. An improved understanding of the mechanisms involved in the induction of these senescent clonal T cells and how their cellular functions are affected has provided potential targets to

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restore clonal T cell function, including pSMAD. This may present a unique opportunity to enhance tumour immunity in MM.

Supplementary information is available at Leukemia's website.

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CONFLICT-OF-INTEREST DISCLOSURE

The authors declare no conflict of interest.

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REFERENCES

1. Kofler DM, Chmielewski M, Rappl G, Hombach A, Riet T, Schmidt A*, et al.* CD28 costimulation impairs the efficacy of a redirected T-cell antitumor attack in the presence of regulatory T cells which can be overcome by preventing Lck activation. *Mol Ther* 2011; **19:** 760-767.

2. Motz GT, Coukos G. Deciphering and reversing tumor immune suppression. *Immunity* 2013; **39:** 61-73.

3. Brown R, Pope B, Murray A, Esdale W, Sze DM, Gibson J*, et al.* Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-beta1 and interleukin-10. *Blood* 2001; **98:** 2992-2998.

4. Bryant C, Suen H, Brown R, Yang S, Favaloro J, Aklilu E*, et al.* 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. *Blood Cancer Journal* 2013; **3:** e148.

5. Favaloro J, Brown R, Aklilu E, Yang S, Suen H, Hart D*, et al.* Myeloma skews regulatory T and pro-inflammatory T helper 17 cell balance in favor of a suppressive state. *Leukemia & Lymphoma* 2014; **55:** 1090-1098.

6. Brown R, Kabani K, Favaloro J, Yang S, Ho PJ, Gibson J*, et al.* CD86+ or HLA-G+ can be transferred via trogocytosis from myeloma cells to T cells and are associated with poor prognosis. *Blood* 2012; **120:** 2055-2063.

7. Favaloro J, Liyadipitiya T, Brown R, Yang S, Suen H, Woodland N*, et al.* Myeloid derived suppressor cells are numerically, functionally and

Accepted manuscript

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phenotypically different in patients with multiple myeloma. *Leuk Lymphoma* 2014; **55:** 2893-2900.

8. Brown R, Suen H, Favaloro J, Yang S, Ho PJ, Gibson J*, et al.* Trogocytosis generates acquired regulatory T cells adding further complexity to the dysfunctional immune response in multiple myeloma. *Oncoimmunology* 2012; **1:** 1658-1660.

9. Brown RD, Joshua DE. T cell responses in myeloma. In: Munshi N, Anderson K (eds). *Advances in biology and therapy of multiple myeloma*, vol. 2. Springer 2012, pp 3-24.

10. Crespo J, Sun H, Welling TH, Tian Z, Zou W. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. *Current opinion in Immunology* 2013; **25:** 214-221.

11. Choi S, Schwartz RH. Molecular mechanisms for adaptive tolerance and other T cell anergy models. *Semin Immunol* 2007; **19:** 140-152.

12. Schwartz RH. T cell anergy. *Annual Review of Immunology* 2003; **21:** 305-334.

13. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A*, et al.* Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 2009; **10:** 29-37.

14. Fourcade J, Sun Z, Pagliano O, Guillaume P, Luescher IF, Sander C*, et al.* CD8(+) T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. *Cancer Res* 2012; **72:** 887-896.

Accepted manuscript

© 2016 Macmillan Publishers Limited. All rights reserved.

15. Woo S-R, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, *et al.* Immune Inhibitory Molecules LAG-3 and PD-1 Synergistically Regulate T-cell Function to Promote Tumoral Immune Escape. *Cancer research* 2012; **72:** 917-927.

16. Dock JN, Effros RB. Role of CD8 T Cell Replicative Senescence in Human Aging and in HIV-mediated Immunosenescence. *Aging and disease* 2011; **2:** 382-397.

17. Herbig U, Jobling WA, Chen BPC, Chen DJ, Sedivy JM. Telomere Shortening Triggers Senescence of Human Cells through a Pathway Involving ATM, p53, and p21CIP1, but Not p16INK4a. *Molecular Cell* 2004; **14:** 501-513.

18. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 2007; **8:** 729-740.

19. Salminen A, Kauppinen A, Kaarniranta K. Emerging role of NF-κB signaling in the induction of senescence-associated secretory phenotype (SASP). *Cellular Signalling* 2012; **24:** 835-845.

20. Brown R, Yuen E, Nelson M, Gibson J, Joshua D. The prognostic significance of T cell receptor beta gene rearrangements and idiotype-reactive T cells in multiple myeloma. *Leukemia* 1997; **11:** 1312-1317.

21. Raitakari M, Brown RD, Gibson J, Joshua DE. T cells in myeloma. *Hematological Oncology* 2003; **21:** 33-42.

Accepted manuscript

© 2016 Macmillan Publishers Limited. All rights reserved.

22. Joshua D, Brown R, Sze DM, Raitakari M, Gibson J, Ho PJ, *et al.* The role of T cells in myeloma. *The Hematology Journal* 2003; **4:** S26-28.

23. Li Y, Yang LJ, Chen SH, Zhang YP, Zhang XL, Luo GX. T cell receptor Vbeta repertoire usage and clonal expansion of T cells in chronic myelogenous leukemia. *Chinese Medical Journal* 2004; **117:** 840-843.

24. Mustjoki S, Ekblom M, Arstila TP, Dybedal I, Epling-Burnette PK, Guilhot F, *et al.* Clonal expansion of T/NK-cells during tyrosine kinase inhibitor dasatinib therapy. *Leukemia* 2009; **23:** 1398-1405.

25. Li J, Sze DM-Y, Brown RD, Cowley MJ, Kaplan W, Mo S-L, *et al.* Clonal expansions of cytotoxic T cells exist in the blood of patients with Waldenström macroglobulinemia but exhibit anergic properties and are eliminated by nucleoside analogue therapy. *Blood* 2010; **115:** 3580-3588.

26. Epling-Burnette P, Painter JS, Rollison DE, Ku E, Vendron D, Widen R, *et al.* Prevalence and clinical association of clonal T-cell expansions in Myelodysplastic Syndrome. *Leukemia* 2007; **21:** 659-667.

27. Vries Ad, Langerak AW, Verhaaf B, Niemeyer CM, Stary J, Schmiegelow K, *et al.* T-cell receptor Vbeta CDR3 oligoclonality frequently occurs in childhood refractory cytopenia (MDS-RC) and severe aplastic anemia. *Leukemia* 2008; **22:** 1170-1174.

28. Brown R, Spencer A, Ho PJ, Kennedy N, Kabani K, Yang S, *et al.* Prognostically significant cytotoxic T cell clones are stimulated after thalidomide therapy in patients with multiple myeloma. *Leukemia & Lymphoma* 2009; **50:** 1860-1864.

Accepted manuscript

© 2016 Macmillan Publishers Limited. All rights reserved.

29. Sze D, Giesajtis G, Brown RD, Raitakari M, Gibson J, Ho J, *et al.* Clonal cytotoxic T cells are expanded in myeloma and reside in the CD8(+)CD57(+)CD28(-) compartment. *Blood* 2001; **98:** 2817-2827.

30. Aviv A, Hunt SC, Lin J, Cao X, Kimura M, Blackburn E. Impartial comparative analysis of measurement of leukocyte telomere length/DNA content by Southern blots and qPCR. *Nucleic Acids Res* 2011; **39:** e134.

31. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Research* 2002; **30:** e47.

32. Au-Yeung BB, Deindl S, Hsu L-Y, Palacios EH, Levin SE, Kuriyan J, *et al.* The structure, regulation, and function of ZAP-70. *Immunological Reviews* 2009; **228:** 41-57.

33. Qu CK. The SHP-2 tyrosine phosphatase: Signaling mechanisms and biological functions. *Cell Research* 2000; **10:** 279-288.

34. Guy CS, Vignali DA. Organization of proximal signal initiation at the TCR:CD3 complex. *Immunol Rev* 2009; **232:** 7-21.

35. Tzachanis D, Freeman GJ, Hirano N, van Puijenbroek AA, Delfs MW, Berezovskaya A, *et al.* Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. *Nature Immunology* 2001; **2:** 1174-1182.

36. Pettiford SM, Herbst R. The protein tyrosine phosphatase HePTP regulates nuclear translocation of ERK2 and can modulate megakaryocytic differentiation of K562 cells. *Leukemia* 2003; **17:** 366-378.

Accepted manuscript

© 2016 Macmillan Publishers Limited. All rights reserved.

37. Boise LH, González-García M, Postema CE, Ding L, Lindsten T, Turka LA, *et al.* bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 1993; **74:** 597-608.

38. Di Mitri D, Azevedo RI, Henson SM, Libri V, Riddell NE, Macaulay R, *et al.* Reversible Senescence in Human CD4+CD45RA+CD27− Memory T Cells. *The Journal of Immunology* 2011; **187:** 2093-2100.

39. Henson SM, Lanna A, Riddell NE, Franzese O, Macaulay R, Griffiths SJ, *et al.* p38 signaling inhibits mTORC1-independent autophagy in senescent human CD8(+) T cells. *J Clin Invest* 2014; **124:** 4004-4016.

40. Henson SM, Macaulay R, Riddell NE, Nunn CJ, Akbar AN. Blockade of PD-1 or p38 MAP kinase signaling enhances senescent human CD8(+) T-cell proliferation by distinct pathways. *Eur J Immunol* 2015; **45:** 1441-1451.

41. Mizoguchi H, O'Shea JJ, Longo DL, Loeffler CM, McVicar DW, Ochoa AC. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* 1992; **258:** 1795-1798.

42. Mozaffari F, Hansson L, Kiaii S, Ju X, Rossmann ED, Rabbani H, *et al.* Signalling molecules and cytokine production in T cells of multiple myeloma-increased abnormalities with advancing stage. *British Journal of Haematology* 2004; **124:** 315-324.

43. Moro-Garcia MA, Alonso-Arias R, Lopez-Larrea C. Molecular mechanisms involved in the aging of the T-cell immune response. *Current genomics* 2012; **13:** 589-602.

Accepted manuscript

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ACCEPTED ARTICLE PREVIEW

44. McKarns SC, Schwartz RH, Kaminski NE. Smad3 Is Essential for TGF-β1 to Suppress IL-2 Production and TCR-Induced Proliferation, but Not IL-2-Induced Proliferation. *The Journal of Immunology* 2004; **172:** 4275-4284.

45. Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P*, et al.* Reversal of human cellular senescence: roles of the p53 and p16 pathways. *The EMBO Journal* 2003; **22:** 4212-4222.

46. Migliaccio M, Raj K, Menzel O, Rufer N. Mechanisms That Limit the In Vitro Proliferative Potential of Human CD8+ T Lymphocytes. *The Journal of Immunology* 2005; **174:** 3335-3343.

47. Rovillain E, Mansfield L, Caetano C, Alvarez-Fernandez M, Caballero OL, Medema RH*, et al.* Activation of nuclear factor-kappa B signalling promotes cellular senescence. *Oncogene* 2011; **30:** 2356-2366.

48. Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ*, et al.* Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* 2008; **133:** 1019-1031.

49. Grandori C, Wu KJ, Fernandez P, Ngouenet C, Grim J, Clurman BE*, et al.* Werner syndrome protein limits MYC-induced cellular senescence. *Genes & development* 2003; **17:** 1569-1574.

50. Grange M, Giordano M, Mas A, Roncagalli R, Firaguay G, Nunes JA*, et al.* Control of CD8 T cell proliferation and terminal differentiation by active STAT5 and CDKN2A/CDKN2B. *Immunology* 2015; **145:** 543-557.

51. Schietinger A, Greenberg PD. Tolerance and exhaustion: defining mechanisms of T cell dysfunction. *Trends Immunol* 2014; **35:** 51-60.

Accepted manuscript

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52. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ*, et al.* Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res* 2012; **72:** 917-927.

53. Teramo A, Gattazzo C, Passeri F, Lico A, Tasca G, Cabrelle A*, et al.* Intrinsic and extrinsic mechanisms contribute to maintain the JAK/STAT pathway aberrantly activated in T-type large granular lymphocyte leukemia. *Blood* 2013; **121:** 3843-3854.

54. Suen H, Brown R, Yang S, Ho PJ, Gibson J, Joshua D. The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase 1 study. *Leukemia* 2015; **29:** 1621-1622.

55. Lesokhin AM, Ansell SM, Armand P, Scott EC, Halwani A, Gutierrez M*, et al.* Preliminary Results of a Phase I Study of Nivolumab (BMS-936558) in Patients with Relapsed or Refractory Lymphoid Malignancies. *Blood* 2014; **124:** 291-291.

56. Gorgun G, Samur MK, Cowens KB, Paula S, Bianchi G, Anderson JE*, et al.* Lenalidomide Enhances Immune Checkpoint Blockade-Induced Immune Response in Multiple Myeloma. *Clin Cancer Res* 2015; **21:** 4607-4618.

57. Kao C, Oestreich KJ, Paley MA, Crawford A, Angelosanto JM, Ali MA*, et al.* Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nat Immunol* 2011; **12:** 663-671.

58. Lu P, Youngblood BA, Austin JW, Rasheed Mohammed AU, Butler R, Ahmed R*, et al.* Blimp-1 represses CD8 T cell expression of PD-1 using a

Accepted manuscript

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feed-forward transcriptional circuit during acute viral infection. *The Journal of Experimental Medicine* 2014; **211:** 515-527.

59. Intlekofer AM, Takemoto N, Kao C, Banerjee A, Schambach F, Northrop JK, *et al.* Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. *J Exp Med* 2007; **204:** 2015-2021.

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FIGURE LEGENDS

Figure 1. Different types of dysfunctional T cells in cancer.

Dysfunctional T cells can be classified as anergic, exhausted or senescent based on different phenotypic markers or activated signaling pathways. **Anergic T cells** are induced when there is incomplete co-stimulation provided by CD28 during TCR ligation or in the presence of high co-inhibitory signaling by CTLA-4. **Exhausted T cells** result from chronic-overstimulation and results in the overexpression of multiple inhibitory markers like PD-1, CTLA-4, CD160, Tim-3 and LAG-3. **Senescent T cells** are associated with aging and the natural life span of cells and express markers CD57, CD160, KLRG-1 and lack CD28. Telomere-dependent senescent cells are characterised by shortened telomeres and cell-cycle arrest and upregulated p16 and p21 pathways (replicative senescence). Telomere-independent senescent cells have normal TL, possibly maintained with upregulated telomerase activity and senescence is induced by an upregulated p38-MAPK pathway (Adapted from Crespo et al. 2013).[10]

Figure 2. Prognostic significance of T cell clones in MM

(a) Overall survival of 103 MM patients in this cohort according to the presence of T cell clones (χ^2=15.51; p<0.0001). **(b)** Overall survival of patients with large clone size (>20% of all CD3+ cells) and moderate clone size (<20% but >3SD of age-matched mean; χ^2= 0.11; p=NS). **(c)** Incidence of the TCR Vβ families amongst the T cell expansions.

Figure 3. Proliferation of T cell clones in MM

T cell clones were stimulated with anti-CD3/28 beads for 4 days and proliferation was tracked with CFSE. Proliferation was measured as % cells that had undergone more than 1 cell division. **(a)** Representative histogram measuring CD8+ T cell proliferation using CFSE. (shaded: unstimulated; unshaded: stimulated). **(b)**

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27 Proliferation of clonal and non-clonal T cells from 10-year survivors and non-10-
28 year survivor MM patients. (t=6.675; p<0.0001). **(c)** Effect of different immune
29 modulators on clonal T cell proliferation as compared to cells stimulated with anti-
30 CD3/28 beads and IL-2 only **(d)** 14 day ex vivo expansions of clonal T cells from
31 10-year survivors (mean fold expansion: 19.5) and non-10-year survivor (mean fold
32 expansion=1.0) MM patients.

33 **Figure 4. Immune checkpoints on MM T cell clones**

34 Representative histograms measuring **(a)** PD-1 and **(d)** CTLA-4 on PB clonal T cells
35 from a MM patient. PD-1 expression on clonal and non-clonal T cells in both **(b)** PB
36 (p=0.0005) and **(c)** BM (p=0.02) of MM patients. CTLA-4 expression on clonal and
37 non-clonal T cells in both **(e)** PB (p=0.005) and **(f)** BM (p=NS) of MM patients.

38 **Figure 5. Expression of anergic/exhausted/senescent phenotypic markers and**
39 **TL measurement of MM T cell clones**

40 **(a)** Representative histograms of phenotypic markers (LAG-3, TIM-3, CD160,
41 KLRG1) and expression of CD27 and CD28 on MM T cell clones for the
42 classification of T cell clones as anergic, exhausted or senescent. **(b)** LAG-3
43 expression on clonal T cells as compared to non-clonal T cells (p=NS) **(c)** TIM-3
44 expression on clonal T cells as compared to non-clonal T cells (p=NS) **(d)** CD160
45 expression on MM CD8+ T cells. **(e)** Classification of T cell clones into late stage
46 phenotype (CD27-CD28-) or intermediate (int) stage phenotype (CD27+CD28-) **(f)**
47 KLRG-1 expression on clonal T cells as compared to non-clonal T cells (t=4.60;
48 p<0.003). **(g)** TL measurement by qPCR (T/S: telomere:single copy gene ratio) of
49 clonal and non-clonal T cells (p=NS) **(h)** Representative analysis of TL by flow-
50 FISH (telomere:single copy gene according to fluorescence of fluorescein conjugated
51 telomeric probes in a MM patient. **(i)** TL measurement by flow-FISH of clonal T
52 cells and non-clonal T cells and CD8+CD57+ T cells from age-matched normal
53 controls (p=NS).

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Table 1. Identification and categorisation of dysfunctional T cells by phenotype

	Anergic	Exhausted	Senescent-telomere dependent	Senescent-telomere independent	References	MM T cell clones (current study)
CD28	+	+	-	-	10	-
CD57	+	-	+	+	10	+
PD-1	+	+	-	-	10	-
CTLA-4	+	+/-	-	-	10	-
LAG-3	+	+	-	-	10	-
Tim-3	-	+	+	-	10	-
CD160	-	+	+	+	10	+
KLRG-1	-	-	+	+	10	+
p16	?	?	↑	- /low	17, 18, 45	- /low
p21	?	?	↑	-	17	Normal
p38-MAPK	↑	?	↑	↑	39, 40	Normal
IFN-γ	+	-	+	+	10	+
Telomere length	Normal	Shortened	Shortened	Normal	17,18, 46	Normal

N: normal
-: negative
+: positive

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Table 2. Incidence of T cell clones in different historical cohorts of patients with MM and the effect of IMiDs

Dates	Cohort	Incidence of T cell clones	N	Detection method	Reference
1994-1997	Pre-IMiDs	32%	119	SB	21
1998-2004	Pre-IMiDs	54%	144	FC	22
2002-2005	MM6 Pre-transplant	48%	104	FC	28
2003-2005	MM6 Thal maintenance	76%	61	FC	
2003-2005	MM6 control maintenance	47%	57	FC	
2009-2014	All treated- 70% had previous Thal or Len	75%	103	FC	Current study

FC: Flow cytometry; IMiDs: Immunomodulatory drugs; Len: Lenalidomide; N:Number; SB: Southern Blot; Thal: Thalidomide

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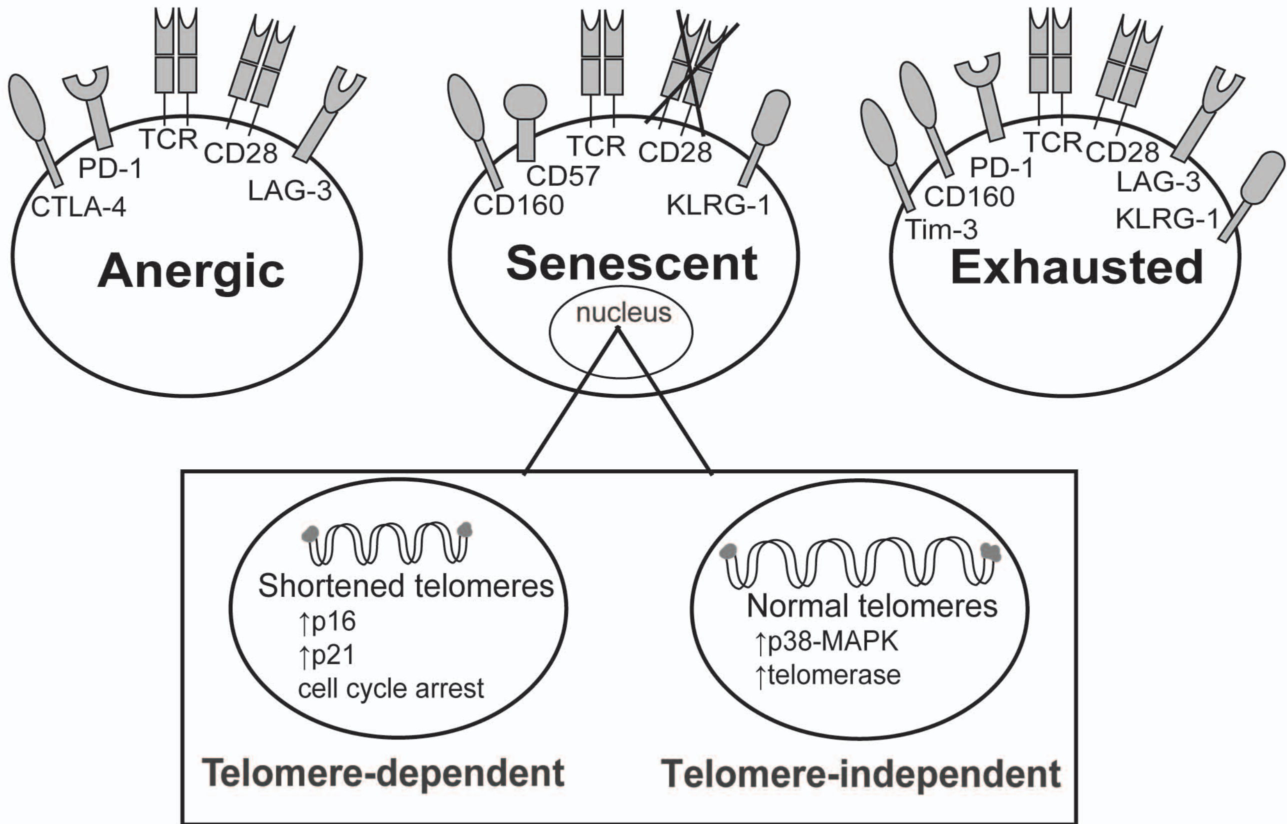

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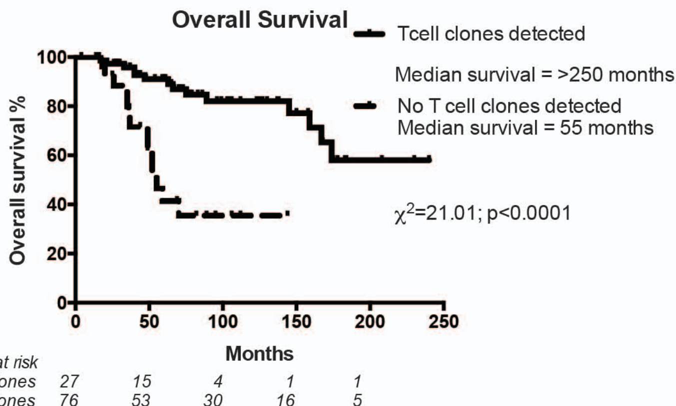
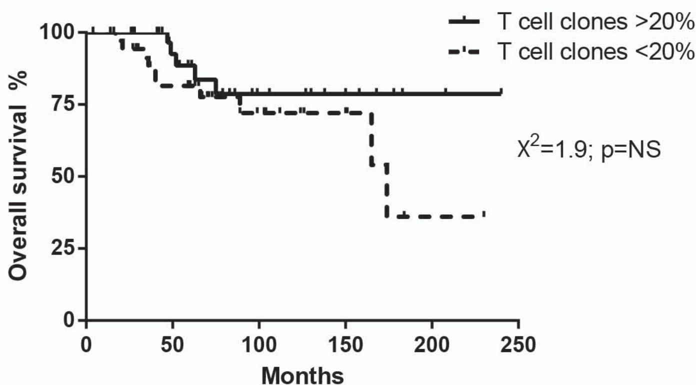


No of patients at risk

No Tcell clones	*27*	*15*	*4*	*1*	*1*
T cel clones	*76*	*53*	*30*	*16*	*5*

B



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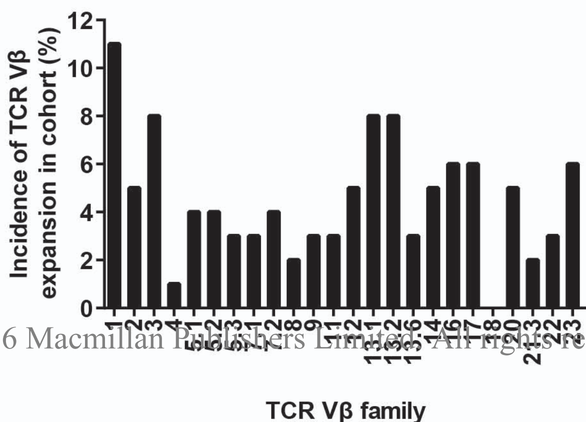

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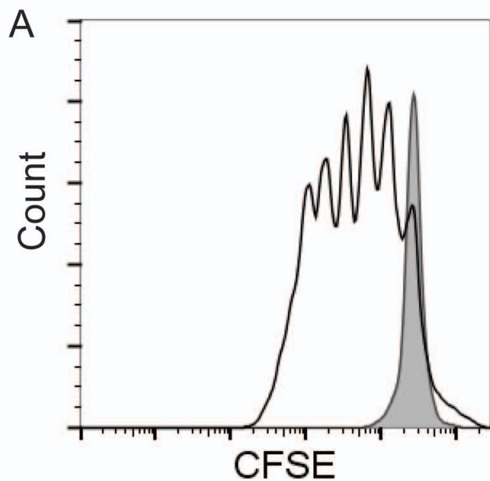

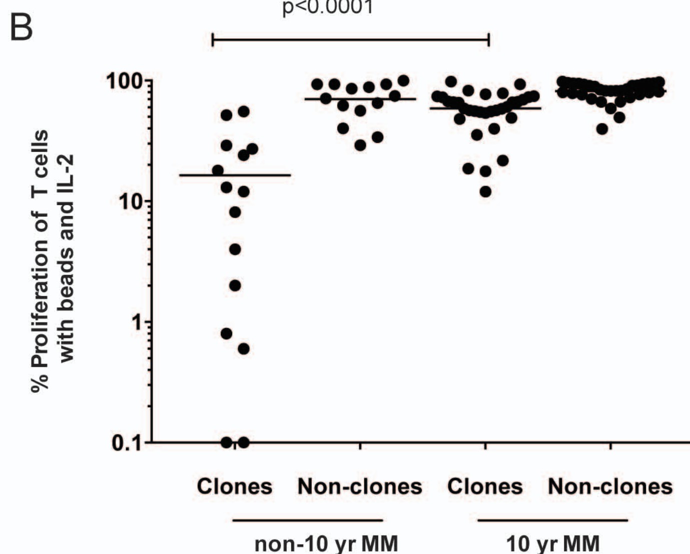

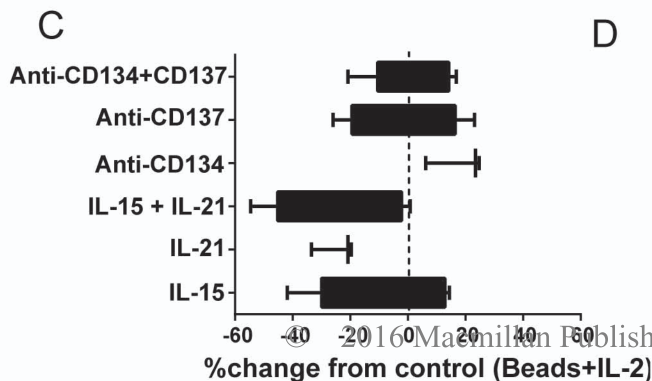

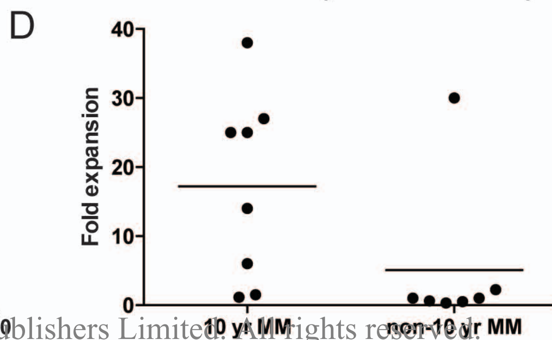

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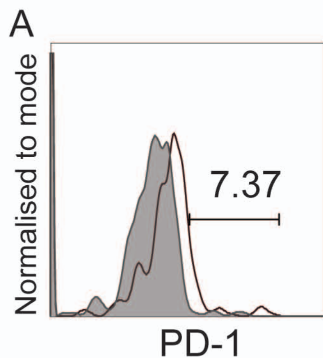

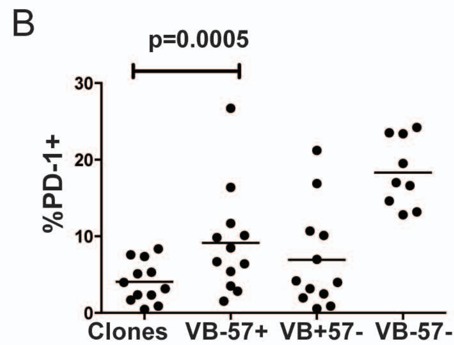

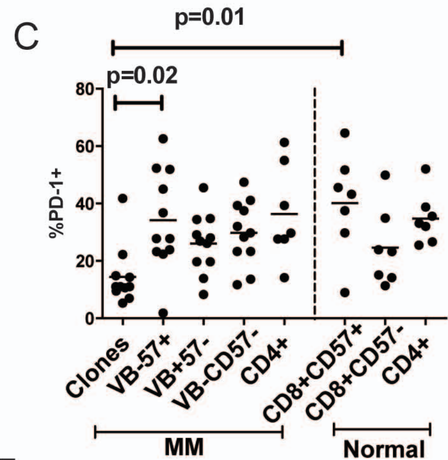

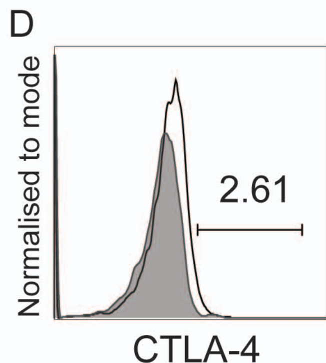

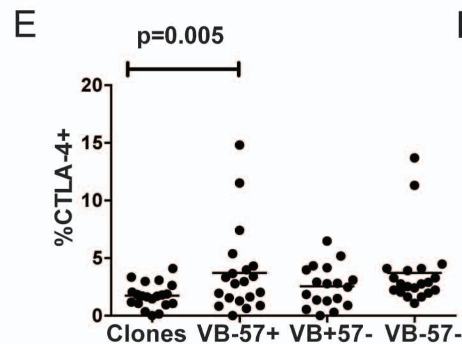

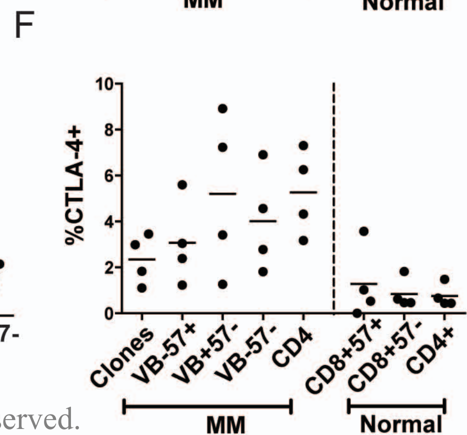

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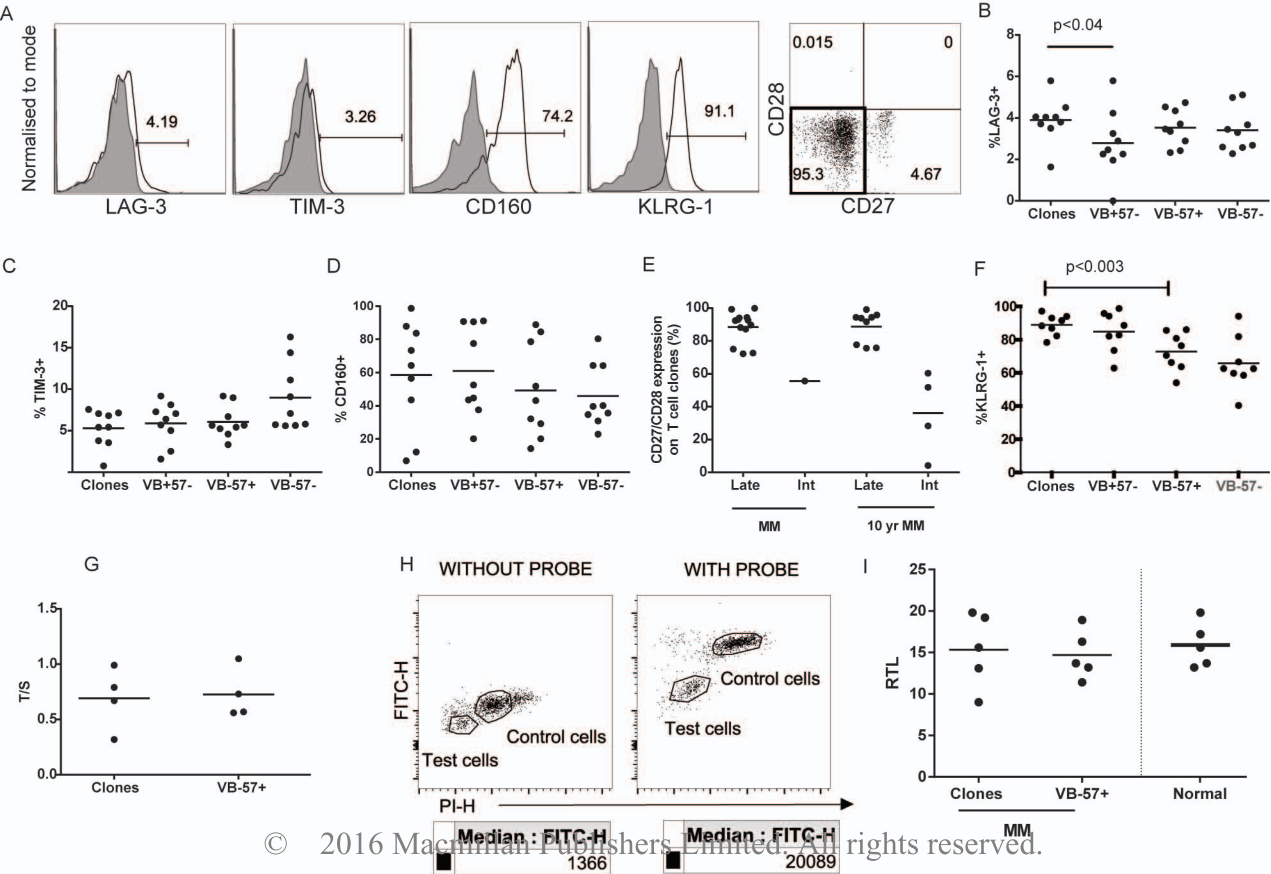

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