Tumour Cell Generation of Inducible Regulatory T-Cells in Multiple Myeloma Is Contact-Dependent and Antigen-Presenting Cell-Independent

Sylvia Feyler1,2,3, Gina B. Scott1,3, Christopher Parrish1, Sarah Jarmin1, Paul Evans3, Mike Short3, Katherine McKinley4, Peter J. Selby5, Gordon Cook1,6*

1 Transplant Immunology Group, Academic Department of Haematology and Oncology, University of Leeds, Leeds, United Kingdom, 2 Department of Haematology, Calderdale and Huddersfield NHS Trust, Huddersfield, United Kingdom, 3 Haematological Malignancy Diagnostic Service, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom, 4 Department of Clinical Immunology, Leeds Teaching Hospitals Trust, Leeds, United Kingdom, 5 Academic Department of Haematology and Oncology, University of Leeds, Leeds, United Kingdom, 6 Department of Haematology, St James’s Institute of Oncology, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom

Abstract

Regulatory T-cells (TReg cells) are increased in patients with multiple myeloma (MM). We investigated whether MM cells could generate and/or expand TReg cells as a method of immuno-surveillance avoidance. In an in vitro model, CD4+CD25+FoxP3+ T-cells co-cultured with malignant plasma cells (primary MM cells and cell lines) induced a significant generation of CD4+CD25+FoxP3+ inducible TReg cells (tTReg cells; p<0.0001), in a contact-dependent manner. tTReg cells were polyclonal, demonstrated a suppressive phenotype and phenotypically, demonstrated increased FoxP3 (p = 0.0001), increased GITR (p<0.0001), increased PD1 (p = 0.003) and decreased CD62L (p = 0.007) expression compared with naturally occurring TReg cells. FACS-sorted tTReg cells differentiated into FoxP3+IL-17+ and FoxP3+IL-17+CD4+ cells upon TCR-mediated stimulation. Blocking experiments with anti-ICOS-L MoAb resulted in a significant inhibition of tTReg cell generation whereas blockade did not. MM tumour cells can directly generate functional TReg cells in a contact-dependent manner, mediated by ICOS/ICOS-L. These features suggest that tumour generation of TReg cells may contribute to evasion of immune surveillance by the host.

Introduction

The paradoxical observation of tumor growth despite an attempt by the tumour-bearing host immune system to control and eliminate the malignant cells suggests that the anti-tumour immune response is being attenuated limiting competent immune surveillance [reviewed in (1)]. This has been extensively studied by tumour immune-biologists, with results pointing towards soluble factors and altered antigenicity as mechanistic explanations. More recently, with the discovery of a number of different immune-regulatory cell types, focus has shifted towards cellular mediated tumour-induced immune suppression and evasion. Several different subsets of regulatory T-cells have now been identified including naturally occurring TReg cells [nTReg cells; CD4+CD25+FoxP3+], and inducible TReg1 and TReg3 CD4+ FoxP3+ cells [2] as well as CD8+ TReg cells [3] and Double Negative TReg cells. Originally it was thought that TReg cells were centrally generated in the thymus, though more recently evidence suggests that peripheral generation is also possible, thereby providing a biological back-drop to investigating their role in the cancer-bearing host [4,5]. In fact, several studies have shown that increased levels of TReg cells can be found in a variety of solid tumours [6,7] and haematological malignancies [8,9,10].

Multiple Myeloma (MM), an incurable malignant plasma cell dyscrasia, is associated with both cellular and humoral immune deficiencies [11]. Many potential mediators of the immunologically hostile microenvironment have been proposed including tumour-derived TGFß [12], Prostaglandin E2 (PGE2) and Interleukin-10 (IL-10) [13]. In addition to soluble mediators, we and others have demonstrated that TReg cell subsets are functional and increased in the peripheral blood of patients with MM, associated with their disease burden [14]. In particular, we demonstrated a higher level in the “pre-myelomatous” condition, MGUS but to a lesser extent than when full disease is present though higher levels of IL-10 were seen in the PB of MGUS compared with patients with MM. In light of this recent evidence, it would now seem that the most promising and synergistic approaches for cancer immunotherapy will be strategies that augment specific anti-tumor immunity whilst simultaneously reducing the effect of tumour-induced immune-regulation. However, in order to perform this later component, a greater understanding of the in vivo mechanism of tumour-induced immune suppression is needed.
In this study, using an in vitro model system, we demonstrate that the tumour cells of MM are not only capable of expanding nT\textsubscript{Reg} cells but generating T\textsubscript{Reg} cells de novo, mediated through cell contact. Through our experimentation, we demonstrate that surface ICOS-L on the tumour cells mediates this phenomenon and that the tumour-induced T\textsubscript{Reg} cells whilst sharing some are phenotypic features also display phenotypic differences but are functionally similar to nT\textsubscript{Reg} cells. The data presented here offers further evidence of direct tumour manipulation of the immune system to augment immune evasion and propagation of the malignant cell clone.

Materials and Methods

Cell Lines, Culture Medium and Reagents

The human MM cell lines (HMCL) JJN3 and RPMI8226 (in house) were expanded in mycoplasma-free cultures, maintained in complete tissue culture medium (CM: RPMI 1640 medium, 10% FCS, 2 mM L-Glutamine, 100 iu/ml penicillin, 100 μg/ml streptomycin) in ventilated tissue culture flasks at 37°C in a 5% CO\textsubscript{2} humidified incubator [15,16,17,18,19]. The HMCL used in co-culture were HLA class II positive (HLA-DR\textsuperscript{+}), in particular expressing HLA-DR. HMCL were treated with 50% liquid nitrogen at -270°C until the day of analysis. The HMCL used in co-culture were HLA class II positive (HLA-DR\textsuperscript{+}), in particular expressing HLA-DR. HMCL were treated with 50% liquid nitrogen at -270°C until the day of analysis.

In vitro Modeling of Tumour Cell and T-cell Interactions

Mononuclear cell (MNC) preparations were made from leukocyte concentrates provided by the National Blood Service. MNC were isolated by density gradient centrifugation on Lymphoprep (Axis-Shift, UK) and stored in foetal calf serum with 10% DMSO in the vapour phase at -80°C until the day of analysis.

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Four-colour flow cytometry was performed on a LSRII (BD Biosciences, Oxford, UK) according to the manufacturers protocol. Four-colour flow cytometry was performed on a LSRII (BD Biosciences, Oxford, UK) according to the manufacturers protocol. In short, high protein binding 96 well ELISA plates (MaxiSorp, Scientific Laboratory Supplies Ltd., Hesse, UK) were coated at 4°C overnight with IL-10 and TGFβ capture antibodies at 1:500 dilution in 1x1 M NaHCO\textsubscript{3} pH 8.2 at 100 μl per well. For TGFβ ELISA, serum samples were diluted 1:5 with PBS and activated with 1N HCl at room temperature for 15 minutes and neutralized with 1N NaOH. After blocking with PBS containing 10% FCS for 2 hrs 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 TGFβ and incubated for 2 hrs at room temperature. 1N NaHCO\textsubscript{3} pH 8.2 at 100 μl per well. For TGFβ ELISA, serum samples were diluted 1:5 with PBS and activated with 1N HCl at room temperature for 15 minutes and neutralized with 1N NaOH. After blocking with PBS containing 10% FCS for 2 hrs 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 TGFβ and incubated for 2 hrs at room temperature.
temperature followed by Extravidin-Avidin conjugate (100 μl per well at 1:500 dilution in PBS/Tween for 1 hr) and substrate solution (Sigma, Dorset, UK) for approximately 30 minutes for development in the dark. Samples were analysed in triplicate and measured spectrophotometrically at 405 nm. For LUMINEX Extracellular assay, spectrally encoded antibody-conjugated 5.6 μm polystyrene beads were used according to the manufacturer's protocol. Plates were pre-wet and 25 μl antibody coated beads and 200 μl wash solution were added and washed once. Then, 50 μl incubation buffer was added to 100 μl standard or 50 μl sample/50 μl assay diluents. After a 2 hour incubation and washing, the plate was then incubated with 100 μl PBS with the cytokine specific biotinylated detector antibodies. The fluorescent streptavidin-RPE was added and after incubation was analysed with the Luminex IS software.

T-cell Receptor Clonality by PCR
T-cell receptor (TCR) clonality was determined by PCR analyses of TCRG rearrangements as previously described [20]. In brief, DNA was isolated from FACS sorted cells and subjected to PCR performed using the BIOMED-2 multiplex strategy (InVivoScribe Technologies, San Diego, CA). PCR products were labeled in the 6FAM, HEX and NED fluorochromes and Vβ usage was identified using ABI Fluorescence detection. Positive controls for clonal T-cell populations were derived from the peripheral blood of patients with T-cell lymphoproliferative disease.

Statistical Analysis
Results were analysed using SPSS version 14.0 for Windows software. Multiple independent variables were analysed with the
Kruskal-Wallis test for non-parametric samples and with the Mann-Whitney-U test for 2 independent samples. A p-value of <0.05 was considered statistically significant. Comparison of patient samples was expressed as median values and co-culture experiments as mean values.

Results
Malignant Plasma Cells Induce Regulatory T-cell Generation
We have previously shown an increase in functional T_{Reg} cells in the peripheral blood of patients with MM, relating to the stage of their disease [14]. To examine the relationship between myeloma tumour cells and T_{Reg} cells, we first determined the effect of co-culturing naturally-occurring T_{Reg} cells (nT_{Reg} cells) with mitomycin-C treated HMCL (U266B HLA-class II^{67,p}). When nT_{Reg} cells were sorted from the PB of healthy volunteers and cultured in CM alone, a significant reduction in the proportion of nT_{Reg} cells was observed (Day 0: 28.0% vs Day 7: 7.3% ± 5.3; n = 4, p = 0.013). However, if co-cultured with HMCL, nT_{Reg} cell expansion was evident (Day 0: 28.0% ± 6 vs Day 7: 43.5% ± 5; n = 4, p = 0.029; 1-way ANOVA p = 0.001; Figure 1A and 1C). We hypothesized that the MM tumour cells could directly induce T_{Reg} cells, in the absence of antigen presenting cells. Firstly we examined the starting cell population in this model, culturing MNC and purified CD4^{+}CD25^{+} effector T-cells (antigen-presenting cell free population) from healthy donor PB with mitomycin C-treated HMCLs. MNC from healthy controls contained a mean of 6.2% ± 0.4% CD4^{+} nT_{Reg} cells. nT_{Reg} cell depletion was very effective and achieved a CD4^{+}CD25^{+} T cell contamination of 0.08% ± 0.05%, representing a >97.5% depletion efficiency (n = 7; p = 0.0001, Kruskal-Wallis test). Co-culture of unselected MNCs demonstrated a non-significant increase in T_{Reg} cells compared with controls (Day 0: 4.8% ± 1.1 vs Day 7: 7.6% ± 1.5; p = 0.06) which was enhanced when CD4^{+}CD25^{+} T-cells only were seeded with cell lines (Day 0: 0.7% ± 0.3 vs Day 7: 31.6% ± 3.6; n = 10, p < 0.001; Figure 1C & D).

Next to determine if this was a MM-specific effect, we co-cultured CD4^{+}CD25^{+} T-cells with a selection of HMCL (U266, JJN3, JIM3 & KMS11), a myeloid-derived cell line (K562) and non-hematopoietic cell lines (Mel888 & HeLa). A clear induction of T_{Reg} cells was seen with each of the HMCL and K562, but not the non-hematopoietic cell lines Mel888 or HeLa cell lines (n = 6, 1-way ANOVA p = 0.0015; Figure 1E). When sorted primary bone marrow plasma cells taken from patients with myeloma (n = 7) were co-cultured with CD4^{+}CD25^{+} T-cells from healthy donors, a significant generation of T_{Reg} cells was observed (1.2% ± 0.31 vs 12.02 ± 4.4, n = 7; p = 0.004), similar to the HMCL, U266B (1.2% ± 0.31 vs 21.9±% ± 5.6, n = 7; p < 0.0001; Figure 1F).

Tumour-generated Regulatory T-cells are Phenotypically Different to Natural T_{Reg} Cells
Differences in phenotype between naturally occurring and inducible T_{Reg} cells have been reported [21,22]. We therefore sought to characterize the phenotype of tT_{Reg} cells generated in our in vitro assay compared with naturally occurring T_{Reg} cells selected from steady PB of healthy volunteers. Given the potential for heterogeneity of response between the different samples from healthy volunteers, we utilized the one HMCL to provide consistency in the in vitro model, though similar results were generated using other MM cell lines (JIM3, JJN3 & RPMI8226-data not shown). When CD4^{+}CD25^{+} T-cells were selected as the starting population, the level of FoxP3 expression was significantly greater than naturally occurring T_{Reg} cells either from the PB of healthy controls or patients with MM (1505 ± 101 vs 834 ± 67;p < 0.0001, Kruskal-Wallis test; Figure 2A). Next, using a sequential gating strategy, we examined the expression of key surface markers on CD4^{+}CD25^{+}FoxP3^{+} T-cells. tT_{Reg} cells demonstrated a similar level of CD127 (p = 0.413) and CD4 (p = 0.413) expression but demonstrated significantly higher levels CD69 of CD25 (43.492 ± 6800 vs 1896 ±137; p < 0.0001), GITR (70.2 ± 5 vs 10 ± 3; p < 0.001) and PD-1 (49.8 ± 2 vs 53 ± 0.8; p = 0.003), as illustrated in Figure 2B and C. With regards to CD62L, there was an overall lower mean fluorescence intensity (MFI) compared to naturally occurring T_{Reg} cells (88.9 ± 0.54 ± 97.3 ± 0.54, p = 0.008; Figure 2C), but a bi- phasic pattern of expression suggests two populations of cells, some of which demonstrated similar expression of CD62L as naturally occurring T_{Reg} cells (Figure 2C). To determine the clonality of tumour-induced T_{Reg} cells, CD4^{+}CD25^{+}CD127^{Dim} T-cells were FACS sorted after 7 days of co-culture with mitomycin-C treated HMCL and DNA prepared from sorted cell populations. TCRG PCR was performed on genomic DNA derived from the tT_{Reg} cells. The spectrophotogram indicates multiple “spikes” representative of a polyclonal population in respect to the TCRG rearrangements, compared to a single “spike” representative of a monoclonal population (Figure S1).

tT_{Reg} Cells though Functionally Similar to nT_{Reg} Cells
It has been reported that T_{Reg} cells from tumour-bearing hosts demonstrated altered suppressive capabilities [9,23] though our studies in myeloma patients demonstrate that T_{Reg} cells are functionally active in suppression of autologous T-cell responses to TCR stimulation [14]. First we sought to determine the proliferative response of tT_{Reg} cells to TCR-mediated stimulation. CD4^{+}CD25^{+} T-cells were isolated and co-cultured with HMCL for 7 days then CD4^{+}CD25^{+}CD127^{Dim} T-cells (tT_{Reg} cells) were FACS-sorted. tT_{Reg} cells were stimulated using CD3/CD28-coated beads for 5 days, determining their proliferative response by tritiated thymidine incorporation, comparing their response to sorted nT_{Reg} cells from healthy donors and patients with myeloma, similarly stimulated. tT_{Reg} cells demonstrated greater proliferative responses to TCR-mediated stimulation compared with nT_{Reg} cells from normal controls and MM patients, who demonstrated the weakest proliferative responses (16193 ± 1860 cpm vs 1510 ± 314 cpm vs 605 ± 73 cpm, p < 0.001; 1 way ANOVA). Next we examined their suppressive capabilities. tT_{Reg} cells generated in a 7 day co-culture were FACS-sorted and co-cultured with autologous T-cells stimulated with CD3/CD28-coated beads at the ratios described, for 5 days. The suppressive capacity of tT_{Reg} cells was compared with nT_{Reg} cells from healthy controls. We demonstrate that tT_{Reg} cells were able to suppress anti-CD3/anti-CD28-induced T-cell proliferation in a dose dependent fashion similar to naturally occurring T_{Reg} cells (Figure 3A). Next we sought to determine the cytokine production by tT_{Reg} cells in this culture system. When the supernatant was analysed for IL-10 on Day 7, the co-culture of T-cells with HMCL generated significantly higher levels of IL-10 compared to HMCLs or CD4^{+} CD25^{+} T-cells cultured alone (p<0.001; Figure 3B). However, when the production of IL-10 by tT_{Reg} cells was determined at the single-cell level by FACS, very few tT_{Reg} cells produced IL-10 (Figure 3C). When the culture supernatant was examined for the level of Interferon-γ (IFNγ), the co-culture of T-cells with HMCL generated significantly higher levels of IFNγ compared to either HMCLs or CD4^{+}CD25^{+} T-cells cultured alone (p<0.0006; Figure 3D). We sought to determine the cellular origin of IFNγ and demonstrated that IFNγ-producing tT_{Reg} cells could...
readily be identified, contributing to the production of IFN\(\gamma\) (Figure 3E). Analysis of nTReg cells from peripheral blood of healthy age-matched controls and patients with MM demonstrates a subset, albeit small subset, of nTReg cells that produce IFN\(\gamma\) (Figure 3F).

It is known that the effector T-cell lineage shows great plasticity and that human TReg cells can differentiate into IL-17-producing cells [24,25]. When tTReg cells were generated in our \textit{in vitro} culture model, a significant production of IL-17 was noted in the supernatant after 7 days of co-culture of CD4\(^+\)CD25\(^-\) T-cells with HMCL (30.7\(\pm\)6.1 pg/ml vs 0.2\(\pm\)0.1 pg/ml; \(p<0.001\); Figure 4A). Therefore, we wished to determine if Th17 cells could be generated directly from tTReg cells and thus, characterizing the plasticity of tTReg cells generated in our \textit{in vitro} model, compared to naturally occurring TReg cells. CD4\(^+\)CD25\(^-\) T-cells co-cultured with mitomycin-C-treated HMCL for 7 days were FACS-sorted and re-stimulated with CD3/CD28-coated beads with rhIL-20 U/ml for 5 days. For comparison, naturally occurring TReg cells were sorted using Miltenyi columns, co-cultured with mitomycin-C-treated HMCL for 7 days, then FACS-sorted and stimulated under identical conditions. After re-stimulation, a sub-population of IL-17-producing CD4\(^+\) T-cells was identified from the FACS-sorted tTReg cells, similar to nTReg cells (4.08\(\pm\)2.0 of nTReg cells vs 3.62\(\pm\)2.0 of nTReg cells, \(p=0.87\); Figure 4B). Closer analysis demonstrated that a smaller sub-population of FoxP3 and IL-17 double positive cells were generated, in similar quantities from both nTReg cells and tTReg cells (3.6\(\pm\)2.4 of nTReg cells vs 2.7\(\pm\)1.8 of tTReg cells, \(p=0.7\); Figure 4C).

Myeloma Induces Regulatory T-cells by Contact

Myeloma-generated Regulatory T-cells are Induced by Surface ICOS/ICOS-L Interactions not Tumour-derived TGF\(\beta\)

The mechanisms for controlling the induction and expansion of TReg cells remains to be fully clarified with some investigators demonstrating soluble factors as central to induction whilst others emphasize cell-to-cell contact, especially with dendritic cell contact, as key [26,27,28]. We adapted our antigen presenting cell-free \textit{in vitro} model to investigate the role of humoral factors versus contact mediation. CD25\(^+\)CD4\(^+\) T-cells were isolated from PB and co-cultured with mitomycin C-treated HMCL for 7 days with and without transwell separation. The generation of CD4\(^+\)CD25\(^-\)Foxp3\(^+\) TReg cells through co-culture with HMCL was significantly reduced by abolishing cell-to-cell contact (30.7\(\pm\)5 CD4\(^+\) TReg cells vs 0.11\(\pm\)0.04 CD4\(^+\) TReg cells, \(n=7\), \(p<0.001\); Figure 5A). The inhibition of tumour-generated CD4\(^+\) TReg cells through abolation of cell-to-cell contact was
Figure 3. Functional characteristics of tumour-induced regulatory T-cells. A. Suppression of anti-CD3/anti-CD28-induced autologous T-cell proliferation by tumour-generated and naturally occurring TReg cells (n = 3), as determined by tritiated thymine incorporation. Results expressed as counts per minute (cpm) ± SEM representing assays performed in triplicate. Key: Unstim – resting CD4+CD25− T cells, Stim – CD3/CD28 stimulated CD4+CD25+ T-cells, 4:1 etc – ratio of stimulated autologous T-cells to TReg cells. B. The generation of IL-10 in co-cultures of CD25+CD4+ sorted T-cells and HMCL, compared with HMCL alone and culture medium (n = 6, p = 0.0004). Results represent all experiments, expressed as mean ± SEM and analyzed using student t-test. C. IL-10 production by tTReg cells after 7 days of co-cultures of CD25+CD4+ sorted T-cells and HMCL. Results represent all experiments, expressed as mean ± SEM (n = 3) and analyzed using student t-test. D. IL-10 production by tTReg cells after 7 days of co-cultures of CD25+CD4+ sorted T-cells and HMCL. Results represent all experiments, expressed as mean ± SEM (n = 3) and analyzed using student t-test. E. Representative flow cytometry plots demonstrating the generation of IFNγ−FoxP3+CD25+CD4+ T-cells from CD4+CD25+ T-cells in a 7 day co-culture assay with mitomycin C-treated U266B cells. F. The proportion of IFNγ−producing FoxP3+CD25+CD4+ T-cells detectable in the peripheral blood of age-matched controls (n = 15), patients with MM (n = 15) and tTReg cells generated in vitro after 7 days of co-cultures of CD25+CD4+ sorted T-cells and HMCL (n = 3). Histograms represent IFNγ production by cells gated on FoxP3+CD25+CD4+ positive staining. Results expressed as mean ± SEM. doi:10.1371/journal.pone.0035981.g003
associated with a reduction in the production of IL-10 (69.8±33.6 pg/ml vs. 6.3±0.01 pg/ml, n = 3, p = 0.079) and IFNγ (12154±4174 pg/ml vs. 0.04±0.01 pg/ml, n = 3, p < 0.001).

The role that surface TGFβ plays in the induction and, in conjunction with IL-10, the propagation of T_{Reg} cells has been extensively studied in both human and murine systems [22]. We therefore wished to determine the role that TGFβ may play in the generation of tT_{Reg} cells in our model. Whilst HMCL produce soluble TGFβ (data not shown) they express the modulatory cytokine on their surface (Figure 5B) in addition to HLA class II (DR) and the negative co-stimulatory molecule (second signal) ICOSL (CD275). Therefore, CD4^+ CD25^- T-cells were isolated from PB and co-cultured with mitomycin C-treated HMCL for 7 days with and without the specific TGFβ antagonist, Latency Associated Peptide (LAP) and an anti-TGFβ monoclonal antibody (MoAb). Neither LAP nor anti-TGFβ MoAb demonstrated significant inhibition of tT_{Reg} cell generation in our in vitro model (Inhibition with anti-TGFβ MoAb: 10.5±6.3 at 10 μM and 18.4±23 at 100 μM, n = 3; Inhibition with LAP: 9.6±4.6 at 10 μM and 5.0±0.1 at 100 μM, n = 3; Figure 5C Data).

Similarly, the use of anti-IL-10 MoAb failed to demonstrate any significant inhibition of tT_{Reg} cell generation (7.5±2.5 at 10 μM and 4.3±4.1 at 100 μM, n = 3, Figure 5C).

The B7 family members, ICOS/ICOSL have previously been implicated in T_{Reg} cell generation. When examined, HMCL express surface ICOS-L (Figure 5B). We therefore determined the level of ICOS expression on newly generated tT_{Reg} cells and nT_{Reg} cells. A mean of 65.6±7% tT_{Reg} cells were isolated from PB and co-cultured with mitomycin C-treated HMCL for 7 days with and without the specific TGFβ antagonist, Latency Associated Peptide (LAP) and an anti-TGFβ monoclonal antibody (MoAb). Neither LAP nor anti-TGFβ MoAb demonstrated significant inhibition of tT_{Reg} cell generation in our in vitro model (Inhibition with anti-TGFβ MoAb: 10.5±6.3 at 10 μM and 18.4±23 at 100 μM, n = 3; Inhibition with LAP: 9.6±4.6 at 10 μM and 5.0±0.1 at 100 μM, n = 3; Figure 5C Data).

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Discussion

The efficient generation of an immune response is coupled with a regulatory system to limit that response, preventing the destruction of healthy cells and tissues [29]. However, transformed malignant tissue may adopt one or more mechanisms to interfere with either the effector immune response or the regulatory cell compartment in an attempt to evade immune surveillance [30,31,32,33,34]. In myeloma, both dysfunctional effector responses and augmented regulatory cell compartments have been described [9,12,35,36]. To date, the origin of the expanded regulatory T-cell population has remained elusive. We therefore sought to elucidate a causal relationship between the tumour cell in myeloma and T Reg cell generation. Our model system demonstrates, for the first time, a direct induction of T Reg cells (tTReg cells) by both fresh myeloma cells and cell lines that demonstrate the phenotype and functionality of TReg cells whilst inducing IL-10 production in non T Reg cells. However, phenotypic differences between tTReg cells induced and naturally occurring T Reg cells were noted. In particular tTReg cells demonstrated a CD25High FoxP3High GITR+PD-1+ phenotype, distinct from naturally occurring T Reg cells. IFNγ-producing regulatory T cells have been described in the setting of intestinal infection and allograft rejection and evidence suggests a central role of IFNγ in inducible TReg cell generation [37,38]. IFNγ cellular effects are mediated through STAT1 phosphorylation and it is known that there is a STAT1 binding site in the proximal region of the FoxP3 gene promoter in humans (but not mice) [39,40]. In addition to which, IFNγ has been shown to mediate FoxP3 gene induction in synergy with IL-27[41]. Here we demonstrate for the first time in a cancer setting, the generation of inducible TReg cells from CD4+CD25- T cells where a subset produce IFNγ in vitro. In addition, similar to naturally occurring T Reg cells, CD4+CD25CD127low FACS sorted tTReg cells demonstrate lineage plasticity by differentiating into IL-17-producing T-cells following further TCR-mediated stimulation [24,42]. Furthermore, data published to date, has demonstrated a central role for antigen presenting cells (APC) in the interactions of tumour cells and T Reg cells. However, in our in vitro system, myeloma tumour cells generate and expand tTReg cells in an APC-free manner, that is directly inducing CD4+CD25- T-cells. The importance of the generation of previously considered pro-inflammatory cytokines in the generation and or propagation of T Reg cells in cancer remain to be elucidated.

Figure 5. *In vitro* mechanisms of tumour regulatory T-cell induction. A. The generation of FoxP3+CD25+CD4+ tTReg cells from CD4+CD25- T-cells, expressed as a percentage of CD4+ T-cells, in a co-culture assay with mitomycin C-treated U266 cells with and without transwell inserts (n = 7). Results represent all experiments, expressed as mean ± SEM and analysed using a student t-Test. B. Surface expression of HLA-DR, ICOSL (CD275) and TGFβ by human myeloma cell lines. C. Inhibition of tTReg cells generation from CD4+CD25- T-cells by co-culture with HMCL (n = 3) through blockade of TGFβ and IL-10 using monoclonal antibodies and Latency-associated Peptide (LAP). Results represent all experiments, expressed as mean ± SEM. D. Surface expression of ICOS by tTReg cells, presented as both percentage expression of CD4+CD25+FoxP3+ cells and representative dot plots. Results represent all experiments, expressed as mean ± SEM (n = 3) and analysed using a student t-test. E. Inhibition of tTReg cells generation from CD4+CD25- T-cells by co-culture with HMCL through blockade of anti-ICOS-L (αICOS 1, 10, 100 μM) monoclonal antibody (n = 6), expressed as percentage of CD4+ T-cells and percent inhibition of tTReg cell generation. Results represent all experiments, illustrated as median with maximum and minimum values and analysed using a student t-test.

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Figure 5. In *vitro* mechanisms of tumour regulatory T-cell induction. A. The generation of FoxP3+CD25+CD4+ tTReg cells from CD4+CD25- T-cells, expressed as a percentage of CD4+ T-cells, in a co-culture assay with mitomycin C-treated U266 cells with and without transwell inserts (n = 7). Results represent all experiments, expressed as mean ± SEM and analysed using a student t-Test. B. Surface expression of HLA-DR, ICOSL (CD275) and TGFβ by human myeloma cell lines. C. Inhibition of tTReg cells generation from CD4+CD25- T-cells by co-culture with HMCL (n = 3) through blockade of TGFβ and IL-10 using monoclonal antibodies and Latency-associated Peptide (LAP). Results represent all experiments, expressed as mean ± SEM. D. Surface expression of ICOS by tTReg cells, presented as both percentage expression of CD4+CD25+FoxP3+ cells and representative dot plots. Results represent all experiments, expressed as mean ± SEM (n = 3) and analysed using a student t-test. E. Inhibition of tTReg cells generation from CD4+CD25- T-cells by co-culture with HMCL through blockade of anti-ICOS-L (αICOS 1, 10, 100 μM) monoclonal antibody (n = 6), expressed as percentage of CD4+ T-cells and percent inhibition of tTReg cell generation. Results represent all experiments, illustrated as median with maximum and minimum values and analysed using a student t-test.

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In patients with cancer, T_{Reg} cells are continuously exposed to tumour antigen (TAg), either directly or through the tumour microenvironment which in turn, results in high levels of ICOS expression as has been demonstrated in melanoma and prostate cancer [7,43]. T_{Reg} cells generated in this environment produce high levels of IL-10 which mediates their suppressive capabilities, especially dendritic cell function [44]. We demonstrate with our in vitro model that in myeloma, ICOS-L+ Tumour cells directly induce T_{Reg} cell generation mediated in a contact-dependent manner, in the absence of antigen-presenting cells, which is inhibited significantly though not totally using anti-ICOSL monoclonal antibodies. This model of induction T_{Reg} cell however, does not account for chronic antigen stimulation by the tumour-bearing host, nor does it this culture system take allowances of the effects of immunomodulatory drugs such as steroids and IMIDs (Thalidomide, Lenalidomide, Pomalidomide) which may account for differences in ICOS expression between T_{Reg} cell and T_{Reg} cells from MM patients, a significant level of IL-10 is produced though this contributes minimally to the generation of T_{Reg} cells (as evidenced by lack of inhibition through monoclonal antibody blockade). Furthermore, although IL-10 production by ICOS-induced T_{Reg} cells has been documented in both human and murine in vitro systems [45,46,47], the T_{Reg} cells induced in our system did not produce IL-10.

The role of TGFβ in both the generation of T_{Reg} cells and in the mediation of their suppressive effects has been the subject of conflicting reports and may relate to the experimental design of in vitro systems used to study this relationship. In murine model systems, TGFβ-mediated FoxP3 induction in naive T-cells augmented by IL-2, produce T_{Reg} cells with a suppressive phenotype which are rendered hypo-responsive to TCR-mediated stimulation [4,48,49]. In contrast, other investigators have demonstrated TGFβ independence in both the generation and mediation of suppression [50,51,52]. Murine prostatic and renal cell cancer cells have been shown in vitro to generate T_{Reg} cells mediated through TGFβ. We have previously shown TGFβ to have a central role in myeloma-mediated effector cell dysfunction and is detected at high level in peripheral blood and bone marrow [12,36]. However, the data from our in vitro model did not demonstrate a prominent role for TGFβ in the induction of T_{Reg} cell generation, despite the production of TGFβ in co-culture supernatant (data not shown) and expressed on the surface of tumour cells.

Recent studies have suggested a close relationship between CD4+CD25+FoxP3+ T_{Reg} cells and pro-inflammatory IL-17-producing T helper cells (Th17) [53]. In our studies, we demonstrate that T_{Reg} cells have a capacity, upon TCR-mediated stimulation to generate IL-17 producing T-cells, both CD4+FoxP3+ and CD4+FoxP3− cells, indicative of a plasticity of the T_{Reg} cells, similar to previous reports [53,54,55]. More recently, it has been shown that different myeloid-derived cellular subsets (CD14+HLA-DR^dim as CD14+HLA-DR^+) can induce both T_{Reg} cell and Th17 cells, with a recognized degree of plasticity [56]. However, our in vitro model system is APC-free and devoid of the proposed myeloid-derived cellular subsets. Though the T_{Reg} cells were generated by co-culture with HMCL, in the absence of additional TCR-mediated stimulation, the plasticity we observed with these T_{Reg} cells was purely upon TCR-mediated stimulation in the absence of HMCL and suggests an independent functional plasticity of T_{Reg} cells.

In summary, our in vitro studies demonstrate that the tumour cells of Myeloma are capable of inducing T-cells with the phenotypic and functional characteristics of T_{Reg} cells, associated with the production of IL-10 and IFNγ. The induction of T_{Reg} cells is mediated by cell-to-cell contact with the ICOS/ICOS-L system demonstrating a central role in the induction. The data presented here offers a better understanding of the immune evasion adopted by MM tumour cells offering a potential opportunity to manipulate the tumour-bearing host immune micro-environment pharmacologically. The pre-clinical data presented here offers a scientific basis for the development of suitable clinical research protocols to test this in vivo.

Supporting Information

Figure S1 DNA PCR analyses of TCRG rearrangements of FACS sorted T_{Reg} cells performed using the BIOMED-2 multiplex strategy. Representative example of 3 experiments. Positive control used was peripheral blood from a patient with T-cell lymphoproliferative disease.

Author Contributions
Conceived and designed the experiments: GC. Performed the experiments: SF GBS CP SJ PE MS KM. Analyzed the data: SF GBS PJS GC. Wrote the paper: GC. Reviewed and edited the manuscript: SF GBS CP SJ PE MS KM PJS GC. Gave final approval to the manuscript: SF GBS CP SJ PE MS KM PJS GC.

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