IMMUNE REGULATION IN PLASMA CELL MYELOMA

Esther Aklilu

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of Student:

Date:

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Bryant C, Suen H, Brown R, Yang S, Favaloro J, Aklilu E, Gibson J, Ho PJ, Iland H, Fromm P, Woodland N, Nassif N, Hart D, Joshua D (2013) 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 cell balance. *Blood Cancer journal 3:e148.*

Conference abstracts

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Bryant CE, Brown RD, Yang S, Suen H, Aklilu E, Favaloro J, Hart DNJ, Fromm P, Woodland N, Nassif N, Iland H, Gibson J, Ho PJ, Joshua DE. (2011) Immunological Biomarkers in 10 year Survivors of Multiple Myeloma. *Proceedings of the Haematology Society of Australia and New Zealand.* O/021.

Bryant CE, Brown RD, Yang S, Suen H, Aklilu E, Favaloro J, Hart DNJ, Fromm P, Woodland N, Nassif N, Iland H, Gibson J, Ho PJ, Joshua DE. (2011) Ten year survivors of multiple myeloma demonstrate a differential expression of immunological biomarkers including a high incidence of cytotoxic T-cell clones which have not acquired myeloma-associated anergy. *Blood.* 118:S38865.

Aklilu E, Brown R, Yang S, Kabani K, Woodland N, Nassif N, Ho P, Gibson J, Joshua D. (2010) Treg number, Treg function and Th17 cells in plasma cell dyscrasias. *Proceedings of the Haematology Society of Australia and New Zealand. A94.*

Aklilu E, Brown R, Yang S, Kabani K, Woodland N, Nassif N, Ho PJ, Gibson J, Joshua D (2010) Treg number, Treg function and Th17 cells in plasma cell dyscrasias. Australian Flow Cytometry group (AFCG) meeting. A87.

Aklilu E, Brown R, Yang S, Kabani K, Woodland N, Nassif N, Ho PJ, Gibson J, Joshua D (2010) Treg number, Treg function and Th17 cells in plasma cell dyscrasias, The XXVIIth Annual scientific research meeting. P42.

Abbreviations

Ab/ Abs	Antibody (-ies)
AF-647	Alexa Fluor 647
AML	Acute myeloid leukaemia
Anti-	Antibody against
APC	Allophycocyanin
APCs	Antigen presenting cells
ATLL	Adult T-cell leukaemia/lymphoma
B cell	B lymphocyte
β_2M	Beta-2 microglobulin
BM	Bone marrow
BMMC	Bone marrow mononuclear cells
Bort-	Bortezomib (Velcade)
˚C	Degrees Celsius
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLL	Chronic lymphocytic leukaemia
CLMF	Cytotoxic lymphocyte maturation factor
CRAB	Hypercalcaemia, renal failure, anaemia and bone lesion
CRP	C-reactive protein
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
CTLA-8	Cytotoxic T lymphocyte-associated antigen-8
DC	Dendritic cells
Dex	Dexamethasone
DMSO	Dimethyl sulfoxide
DVT	Deep vein thrombosis
EDTA	Ethylenediamine tetra-acetic acid
FACS	Fluorescence activated cell sorting
FISH	Fluorescent *in situ* hybridisation
FITC	Fluorescein isothiocyanate

FoxP3	Forkhead box P3
FSC	Forward scatter
g	Gravitational force
G-CSF	Granulocyte-colony stimulating factor
GITR	Glucocorticoid-induced tumour necrosis factor -receptor-related
GvHD	Graft versus host disease
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hi	High
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMiD/ IMiDs	Immunomodulatory drug (s)
iTreg	Induced regulatory T cell
ISS	International staging system
kDa-	Kilo Dalton
LAP	Latency associated peptide
Len	Lenalidomide
Len + Dex	Lenalidomide in combination with dexamethasone
mAB	Monoclonal Antibody
MFI	Mean fluorescence intensity
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
min	Minutes
MM	Plasma cell myeloma
MRI	Magnetic resonance imaging
NK	Natural killer
NKSF	Natural killer cell stimulatory factor
NS	Not significant (P>0.05)
nTreg	Natural regulatory T cell

PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCLI	Plasma cell labelling index
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PMA	Phorbol 12-myristate 13-acetate
Pom	Pomalidomide
Rh	Recombinant human
ROR	Retinoid-related orphan receptor
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SAM	Significance analysis of microarray
SSC	Side scatter
SCT	Stem cell transplantation
SD	Standard deviation
SEM	Standard error of mean
SM	Smouldering myeloma
SPE	Serum protein electrophoresis
STAT3	Signal transducer and activator of transcription 3
T cell	T lymphocyte
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper cell
Thal-	Thalidomide
TILs	Tumour infiltrating lymphocytes
TNF-α	Tumour necrosis factor -α
Treg	Regulatory T lymphocyte
WM	Waldenström macroglobulinaemia

Abstract

The pathogenesis of monoclonal gammopathies, particularly plasma cell myeloma (MM), is multifaceted and complex. In recent years, Treg and Th17 cells have emerged as key factors in the development and progression of malignancies including MM. However, there are still conflicting reports on whether regulatory T (Treg) cells and Th17 cells are increased or decreased in the peripheral blood (PB) of patients with MM. This is partly due to technical difficulties associated with the use of the transcriptional repressor, forkhead box P3 (FoxP3) to identify Treg cells. Studies have shown FoxP3 results to be dependent on the clones, fluorochromes attached and fixation/permeabilisation methods used. More recent studies have defined Treg cells as $CD4^{+}CD25^{hi}$ cells which do not express CD127, an IL-7 receptor. This methodology was used to determine Treg cell number and develop assays for the assessment of Treg cell function. The study also extends to exploring Th17 cell number in plasma cell dyscrasias and the overall effect of the Treg and Th17 cell equilibrium on the survival of patients with MM.

$CD4^{+}CD25^{hi}CD127^{-}$ expression was used to quantitate Treg cell numbers and an intracellular IL-17 assay on $CD3^{+}CD4^{+}$ cells was used for Th17 cell enumeration. Treg cell function was determined using carboxyfluorescein succinimidyl ester (CFSE) tracking of Treg depleted lymphocyte preparations stimulated by anti-CD3,CD2,CD28 beads at a 1:1 ratio for 4 days $\pm$ 1:1 fluorescence-activated cell sorted Treg cells. This functional assay was also used to investigate the effect of recombinant human (rh) TGF-β and rhIL-12 on Treg cells.

The mean proportion of Treg cells in the $CD4^{+}$ compartment of PB of patients with MM (n=32) was 8.9±0.6% and this was increased compared to the mean of the normal cohort (n=36) at 6.5±0.4% (p=0.009). However, no significant difference was observed between the frequency of PB Treg cells in the control group compared to patients with monoclonal gammopathy of undetermined significance (MGUS) (n=20) (mean=7.5±0.8%; P=0.24) and patients with Waldenström macroglobulinaemia (WM) (n=13) (mean=6.0±0.5%; P=0.48). Interestingly, a comparison of the absolute numbers exhibited different results. A significantly lower number of Treg cells was observed in patients with MM [(3.2±0.4) $x10^{7}$/L; P<0.01] and WM [(3.0±0.6) $x10^{7}$/L; P<0.01] compared to the control group [(6.4±0.7) $x10^{7}$/L]. However, no significant difference was observed when comparing patients with MGUS [(4.3±0.8) $x10^{7}$/L; P= 0.06] to the normal cohort. It was observed that a significantly

higher proportion of PB Treg cells in patients with MM (85.9±1.8%; P<0.01) and WM (86.4±2.1%; P=0.02) to be of the $CD45RO^+$ memory phenotype compared to the normal cohort (76.7±2.5%). However this was not observed in patients with MGUS (73.4±4.0%; P=0.47). In addition, the study revealed that PB Treg cell proportions were not influenced by MM stage. Thalidomide treated patients with MM appeared to have an increased PB Treg cell proportion, however only a small number of thalidomide treated patients were tested due to the use of thalidomide therapy being phased out and its replacement with lenalidomide. Treg cells from bone marrow (BM) were compared to matched PB samples from patients with MM, demonstrating a significantly greater proportion (p=0.02) of Treg cells in the $CD4^+$ compartment of the BM (9.7±1.2%) compared to PB (6.7±1.4%).

Regarding Th17 cells, a significant decrease (p=0.03) in the mean proportion and absolute number of Th17 cells was observed in the PB of patients with MM (n=22) compared with the controls (n=20) (0.7±0.1% and 2.0±0.6% respectively). However, the mean number of Th17 cells in patients with MGUS (2.2±0.6%; n=15) and WM (1.1±0.2%; n=12) was not significantly different from normal. No correlation was observed between Th17 cell number and MM staging or therapy.

Additionally, the study explored the Treg/Th17 cell ratio in PB of patients with monoclonal gammopathies with comparison made to normal subjects. The mean Treg/Th17 cell ratio of patients with MM (16.1±2.4) was significantly higher (p=0.0002) than the healthy control group (6.6±1.0). The Treg/Th17 cell ratio of WM and MGUS patients was 7.0±1.0 and 4.9±0.5 respectively, neither of which were statistically different to the ratio of the normal controls. Most interestingly, patients who have survived with MM for 10 or more years possessed a Treg/Th17 cell ratio similar to the normal controls (7.04±2.47; p=0.84) and this was shown to affect overall survival. The data demonstrated that patients with MM observed to have a high Treg/Th17 cell ratio had an overall shorter survival compared to those whose Treg/Th17 cell ratio was lower (p<0.025).

The suppressive capability of Treg cells from MM patients (n=15) was variable. The Treg cell function of patients treated with lenalidomide (n=5) was increased (mean=68%) compared to patients treated with thalidomide (n=5; mean=23%), Velcade (n=3; mean=12%), untreated patients (n=5; mean=36%) and normal controls (n=11; mean=31%). The suppression exerted upon the $CD4^+$ T cell subset in patients treated with bortezomib was

significantly lower when compared to the normal cohort. However, no significant difference in $CD8^+$ T cell suppression was found between patients with MM and the normal controls. rhTGF-β increased the suppressive capabilities and rhIL-12 reduced the function of Treg cells from both MM and normal PB samples.

In conclusion, immune regulation is dysfunctional in patients with MM as the proportion of PB Treg cells is increased and Th17 cells are reduced. Also, the cytokine microenvironment and treatment have a major impact on the function of Treg cells. The data clearly delineate the importance of the PB Treg/Th17 cell equilibrium, revealing a strong association between the Treg/Th17 cell homeostatic balance and disease progression and survival in MM, indicating an imbalance may cause either or both the innate and adaptive immune system to be dormant and incapacitate the anti-tumour response.

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