Clinical & Translational Immunology

Australian and New Zealand Society for Immunology Inc.

Clinical & Translational Immunology 2021; e1235. doi: 10.1002/cti2.1235
www.wileyonlinelibrary.com/journal/cti

SHORT COMMUNICATION

Successful identification of predictive profiles for infection utilising systems-level immune analysis: a pilot study in patients with relapsed and refractory multiple myeloma

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Received 17 September 2020;
Revised 18 November 2020;
Accepted 14 December 2020

doi: 10.1002/cti2.1235

Clinical & Translational Immunology 2021; **10**: e1235

Abstract

Objectives. Patients with multiple myeloma (MM) are at increased risk for infection. Clinical assessment of infection risk is increasingly challenging in the era of immune-based therapy. A pilot systems-level immune analysis study to identify predictive markers for infection was conducted. **Methods.** Patients with relapsed and/or refractory MM (RRMM) who participated in a treatment trial of lenalidomide and dexamethasone were evaluated. Data on patient demographics, disease and episodes of infection were extracted from clinical records. Peripheral blood mononuclear cells (PBMCs) collected at defined intervals were analysed, with or without mitogen re-stimulation, using RNA sequencing and mass cytometry (CyTOF). CyTOF-derived cell subsets and RNAseq gene expression profiles were compared between patients that did and did not develop infection to identify immune signatures that predict infection over a 3-month period. **Results.** Twenty-three patients participated in the original treatment trial, and we were able to access samples from 17 RRMM patients for further evaluation in our study. Nearly half the patients developed an infection (8/17) within 3 months of sample collection. Infections were mostly clinically diagnosed (62.5%), and the majority involved the respiratory tract (87.5%). We did not detect phenotypic or numerical differences in immune cell populations between patients that did and did not develop infections. Transcriptional profiling of stimulated PBMCs revealed distinct Th2 immune pathway signatures in patients that developed infection. **Conclusion.** Immune cell counts were not useful predictors of infection risk. Functional assessment of

stimulated PBMCs has identified potential immune profiles that may predict future infection risk in patients with RRMM.

Keywords: CyTOF, immune profiling, infection risk prediction, multiple myeloma, RNAseq

INTRODUCTION

Advances in immune-based therapies have transformed multiple myeloma (MM) into a chronic disease that is managed with multiple and protracted therapeutic interventions. These treatments have a cumulative impact on immunity.[1] Infections contribute significantly to morbidity and mortality in patients with MM.[2] In particular, patients with relapsed or refractory progressive disease have an increased risk of infections including blood stream infections.[3] The mechanisms or cell types responsible for the increased risk are not well defined. Consequently, assessment of infection risk in patients has become complex and unreliable.[1] Pre-emptive targeting of pathogens to prevent infection has become challenging and would be greatly aided if risk could be reliably assessed.

Immune profiling could assist with quantifying risk of infection. In a previous exploratory study of immune variables associated with increased risk of infection in patients with newly diagnosed MM, a Th2-dominant cytokine response, detected after *in vitro* mitogen stimulation, was associated with an increased risk of infection. In particular, the IL-5 response to PMA antigen stimulation was a key predictor of infection risk.[4] Other groups have utilised mass cytometry and detection of transcriptomes individually as a means of predicting infection outcomes in patients undergoing HSCT.[5,6] To date, there has not been a comprehensive approach incorporating several immune profiling platforms to identify future risks of infection in patients with haematological malignancies.

A more detailed understanding of the immune profile during treatment at a systems level could provide useful information to enable patient risk stratification and appropriate management. This study was conducted utilising a systems-level approach to profile immune characteristics that may be associated with risk of subsequent infection in patients with relapsed and refractory MM (RRMM) managed with immunomodulatory drug (IMiD) therapy.

RESULTS

Patient characteristics, episodes of infection and outcomes

Twenty-three patients with relapsed and refractory MM (RRMM) participated in the clinical trial at PMCC. Samples were available for 17 patients. The majority of patients (64.7%) were male with a median age of 67.8 years (range 60.4–77.4 years) and median Charlson co-morbidity score of 4 (3–7). Most had IgG MM (64.7%), for a median of 4.6 years (range 0.8–14.2 years). All patients received pneumocystis prophylaxis, mostly with trimethoprim/sulfamethoxazole (94.1%). Eight patients (47.1%) developed an infection within 3 months of sample collection. There were no significant demographic or treatment differences, including corticosteroid dosing, between patients who did or did not develop infection within 3 months of sample collection (Table 1). Eight infections were defined in 8 patients. Three infections (37.5%) were microbiologically diagnosed (MDI) whilst the remaining 5 episodes were clinically diagnosed (CDI) (62.5%). Of the three microbiologically diagnosed infections, all were respiratory viral infections (influenza $N = 2$, picornavirus $N = 1$). There were no proven episodes of bacterial or fungal infection. Both episodes of influenza infection were treated with oseltamivir. Of the CDIs, 80% (4/5) were upper respiratory tract infections and 20% (1/5) gastrointestinal. 80% of CDIs were treated with amoxycillin-clavanulate acid (Augmentin DF). Most episodes of infection were grade 2 in severity (75.0%) with the remainder, grade 3 (25.0%). The two grade 3 episodes that required hospital admission were due to MDI (influenza $N = 1$, picornavirus $N = 1$). There was no intensive care unit admission or mortality over the study period.

Immune cell phenotyping

Total white blood cell count (WCC), absolute neutrophil count (ANC), eosinophil and basophil

counts were quantified at time of sample collection (see Table 2). CyTOF (Helios Mass Cytometry) was used to define PMBC subsets in the patients at cycle 4 (for definition of treatment cycles, see methods section). No single immune cell parameter or activation phenotype amongst the identified cellular subsets was associated with increased risk for infection. Mean total cell numbers ($\times 10^6$ mL^{-1} of blood) are summarised in Table 2.

Transcriptional profiling

In order to characterise the transcriptional landscape in MM patients, whole genome RNAseq analysis was performed on *in vitro* stimulated or non-stimulated PBMCs. After filtering and normalising, 23 302 genes could be included in the differential expression analysis with a false discovery rate (FDR) of 10%. When evaluating

Table 1. Demographics of patients

	Infection within 3 months $N = 8$ (47.1%)	No infection within 3 months $N = 9$ (52.9%)	Overall $N = 17$	*P*-value
Age in years (Range)	70.0 (62.2–77.3)	67.0 (60.4–77.4)	67.8 (60.4–77.4)	0.29
Sex				
Male	5 (62.5)	6 (66.7)	11 (64.7)	1.00
Female	3 (37.5)	3 (33.3)	6	
Charlson co-morbidity index (Range)	5.0 (4.0–7.0)	4.0 (3.0–5.0)	4.0 (3.0–7.0)	0.06
Myeloma type				
IgG	6 (75.0)	5 (55.6)	11 (64.7)	0.62
IgA	2 (25.0)	2 (22.2)	4 (23.5)	
LC	0 (0.0)	2 (22.2)	2 (11.7)	
Time since diagnosis in years (Range)	4.5 (0.8–14.2)	4.6 (0.8–13.0)	4.6 (0.8–14.2)	0.60
Previous lines of therapy (Range)	2.0 (1.0–4.0)	2.0 (1.0–5.0)	2.0 (1.0–5.0)	0.88
30-day cumulative dose of corticosteroids[a] (Range)	1600 mg (965–1600)	1600 mg (965–1600)	1600 mg (965–1600)	0.77

[a]Prednisone equivalent dose.

Table 2. Total cell numbers of cell populations from CyTOF-based phenotyping

Immune cell type	No Infection $N = 9$ ($\times 10^6$ mL^{-1}) – mean	Infection $N = 8$ ($\times 10^6$ mL^{-1}) – mean	*P*-value
White blood cell count (WBC)	4.344	4.613	0.74
Total neutrophil count (ANC)	2.491	2.478	0.98
Eosinophils	0.077	0.094	0.64
Basophils	0.032	0.038	0.60
B cells – Naïve	0.071	0.069	0.97
B cells – Memory	0.007	0.08	0.80
B cells – Plasma	< 0.001	< 0.001	0.21
$CD8^+$ – Effector T killer cells	0.283	0.298	0.58
$CD8^+$ – Naïve T killer cells	0.105	0.123	0.60
$CD8^+$ – Memory T killer cells	0.027	0.036	0.60
$CD4^+$ – Effector T helper cells	0.042	0.036	0.58
$CD4^+$ – Naïve T helper cells	0.067	0.094	0.39
$CD4^+$ – Memory T helper cells	0.221	0.265	0.53
Monocytes – Classical	0.248	0.317	0.48
Monocytes – Non-classical	0.045	0.026	0.40
Dendritic cells – Myeloid	0.120	0.072	0.11
Dendritic cells – Plasmacytoid	0.003	0.006	0.16
NK cells	0.120	0.193	0.12

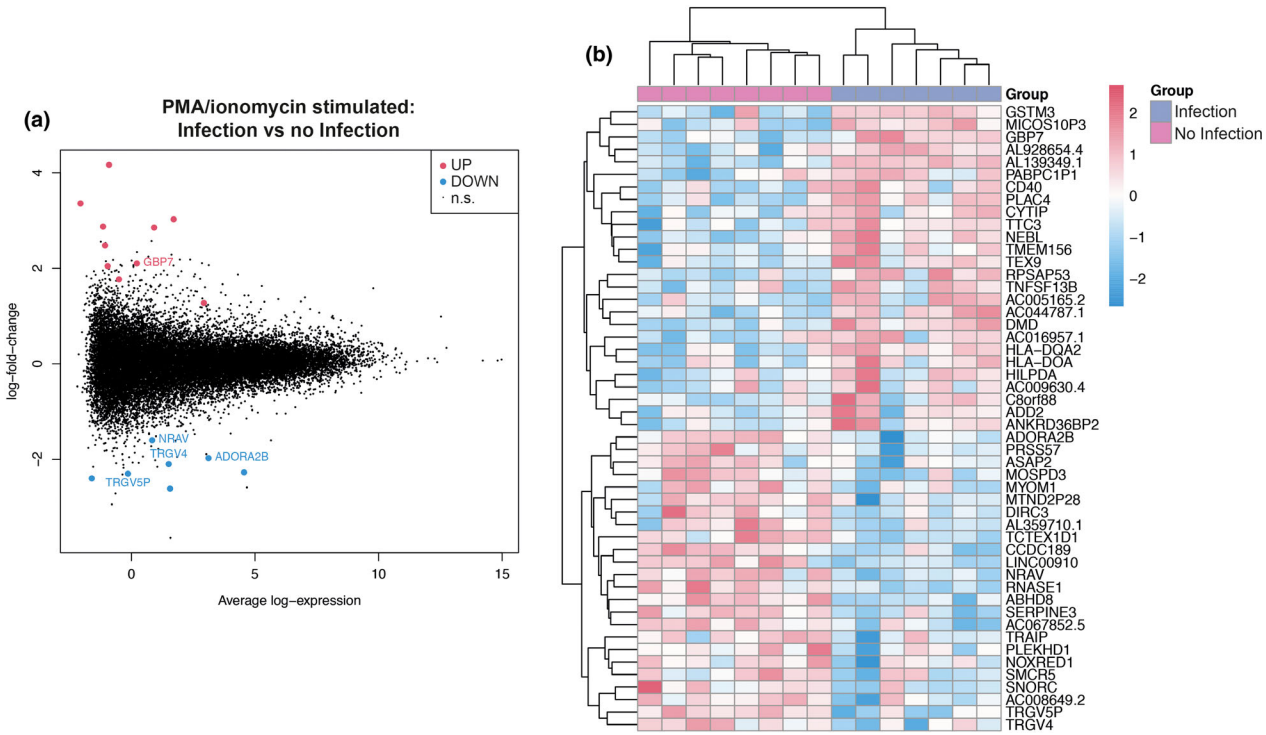

Figure 1. Transcriptome analysis in stimulated PBMCs comparing infection and no infection cases reveals 17 differentially expressed genes. **(a)** Schematic overview of differential gene expression comparison of PMA/ionomycin-stimulated samples, between patients with and without subsequent infection. **(b)** MD plot of differentially expressed genes between stimulated cells from patients with subsequent infection vs patients without subsequent infection. Seventeen genes were identified as differentially expressed with FDR 10% (see also Supplementary table 2). UP = upregulated, DOWN = downregulated, n.s. = not significant. **(c)** Heatmap of the top 50 genes in mitogen-stimulated samples. Red – maximum expression, blue – minimum expression (logCPM adjusted for patient cessation year).

non-stimulated cells, only the two pseudogenes RF00004 and RNY3 were identified to be differentially expressed between patients that developed infection compared to patients that were infection free during the 3-month period after sample collection (Supplementary figure 1a–c).

When evaluating mitogen-stimulated cells, 17 differentially expressed genes were identified that distinguished patients that developed infection from those that did not (Figure 1a, b, Supplementary table 2). Patterns of distinct overall gene expression between the outcome groups are indicated in the heatmap of the top 50 genes based on FDR (Figure 1c).

We next looked at differences in gene expression between cells that were stimulated compared to unstimulated cells within each outcome group. When we compared stimulated vs unstimulated immune cells from patients that did not develop an infection, a total of 14 847 genes were differentially expressed (Supplementary figure 2a–c). In contrast, when the same comparison was made in PBMCs from patients that developed infection, 13 741 genes were differentially expressed between stimulated and unstimulated immune cells (Supplementary figure 2d–f).

Amongst the genes that were differentially expressed after PMA/ionomycin stimulation, 11 500 were shared between patients that did and did not develop infections. However, 2241 differentially expressed genes were peculiar to patients who developed infection and 3347 were unique to patients who did not develop infection. Hallmark gene sets analysis of the transcriptional response to mitogen stimulation between infection and infection-free patients revealed differences and distinct patterns in immune regulation pathways (Figure 2b). Differentially expressed genes involving the IL-2-STAT-5 and the IL-6-STAT-3 pathways were highly represented in patients who subsequently developed infection whilst differentially expressed genes involved in

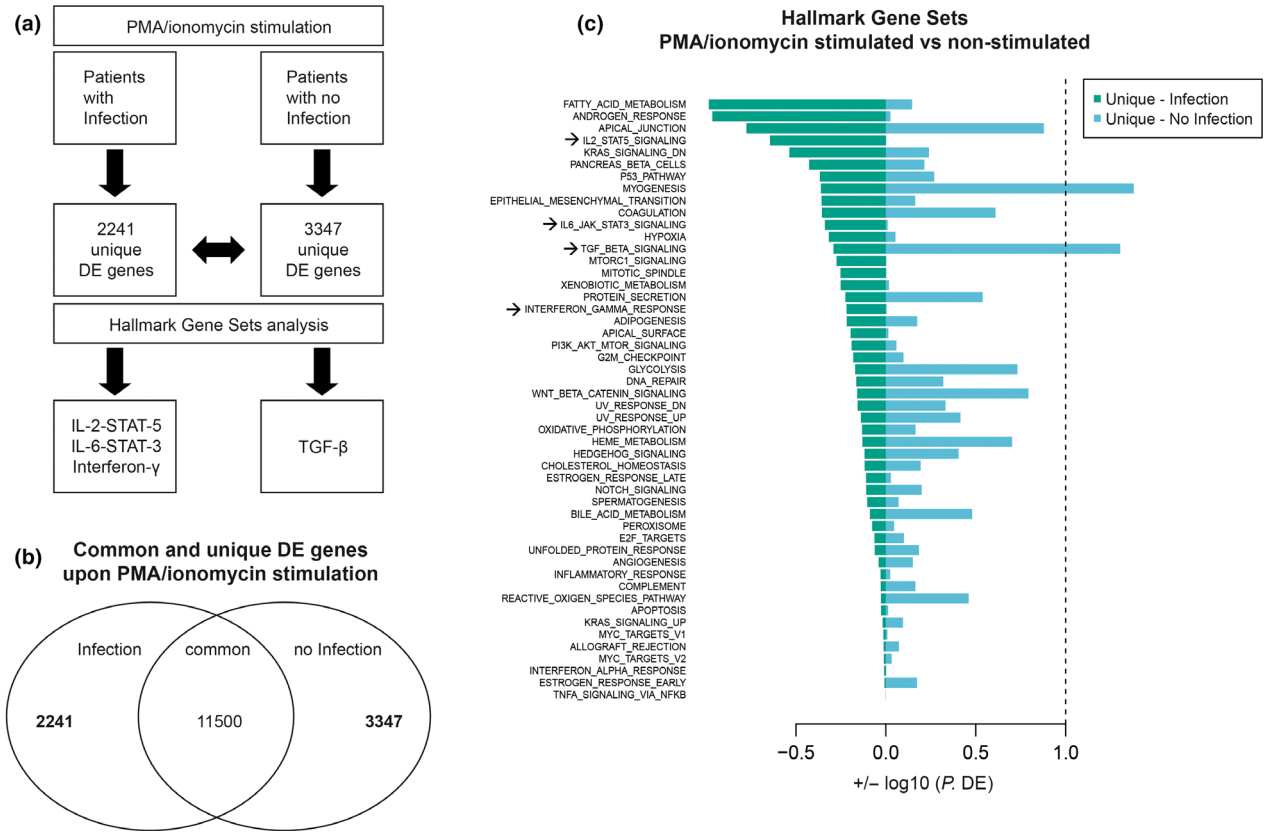

Figure 2. Pathway analysis of genes between patient groups. **(a)** Schematic overview of differential gene expression comparison in mitogen-stimulated PBMCs between patients who developed and did not develop an infection. **(b)** Venn diagram showing the number of common/unique genes between mitogen-stimulated cells from patients who developed and did not develop an infection. **(c)** Hallmark gene set waterfall plot generated from analysis of unique differentially expressed genes between stimulated cells from patients who developed an infection vs patients who did not develop an infection. Dotted line indicates FDR of 10%. Arrowheads indicate hallmark gene sets of particular interest.

TGF-ß pathway were overrepresented in patients who did not develop infection within 3 months (Figure 2b).

DISCUSSION

Patient co-morbidities (renal impairment), disease stage and status as well as treatment factors including type (high-intensity conventional chemotherapy) and burden (increasing lines of therapy) have all been independently associated with increased risk for infection in patients with MM.[2,7] Predicting infection risk in MM patients is becoming increasingly difficult because of the complexity and diversity of treatment regimens and their impact on disease state and immune health. This is especially true for patients with relapsed and refractory disease managed with multiple lines of therapy.[2,3] In a previous study, Th2 cytokine signatures, particularly IL-3 and IL-5 release from PBMCs in response to PMA stimulation, were associated with risk of infection within 3 months.[4] Measurement of the immune response to a pan-antigen (mitogen) such as PMA could be useful in predicting future risk of infection. However, the utility of this approach was only evaluated in newly diagnosed MM patients following autologous stem cell transplant and maintenance therapy. To expand on these findings, population-based immune cell phenotyping using CyTOF and systems-level transcriptional profiling using RNAseq was performed in a defined cohort of patients with RRMM.

In concordance with previous studies,[4] the main immune cell populations and their effector/memory status ($CD4^+$, $CD8^+$, NK, DC, B cells and monocytes) did not differ between patients who did or did not subsequently development infection. This highlights that overall immune cell

numbers per se did not predict infection risk. Interestingly, a reduction in circulating $CD3^+$ $CD4^+$ $CD161^+$ cells has recently been associated with severe infection in lenalidomide- and dexamethasone-treated patients.[8] We hypothesised that a more detailed interrogation of immune function in MM patients during treatment would allow for better prediction of infection risk.

Transcriptional profiling of immune cells, analysed in the absence of *in vitro* stimulation, identified significant differences in the expression of two pseudogenes between patients that developed infection compared to those that did not. We also detected clear patterns and differences in the regulation of the top 50 genes between patients who developed infection compared to those who did not. Profiling of immune cells that were stimulated with PMA/ionomycin showed an expanded array of differentially expressed genes between the patient groups, supporting the utility of this approach in identifying more subtle changes and differences. Amongst the differentially expressed genes, identified after stimulation, several have been implicated in host responses to infection. GBP7 is a guanylate-binding protein induced by interferon that has been reported to promote oxidative mediated killing of pathogens.[9] We identified upregulated GB7 gene expression in patients who subsequently developed infection. ADORA2B, a type of adenosine receptor, was found to be downregulated in these same patients. Interestingly, adenosine receptors have been implicated in inflammation and host response to infection[10] and were shown to be involved in host protection against influenza infection.[11] Furthermore, the long non-coding RNA (lnRNA) NRAV was also found to be downregulated in patients who developed infection. NRAV has been reported to be involved in interferon responses during influenza viral infection by regulating DNA histone modifications of several interferon-stimulated genes (ISGs).[12] This is particularly noteworthy given that the majority of infections in our study were respiratory tract infections including influenza infections. Gamma delta T cells have been implicated in host defence against influenza infection.[13] Strikingly, expression levels of TCR gamma variants TRGV5 and TRGV4 were also significantly decreased in patients that progressed to infection.

We identified distinct differences in the overall PBMC immune activation status, after mitogen stimulation, between the patient outcome groups. In all, 3347 genes were upregulated in PBMCs collected from patients that did not develop infections. In contrast, 2241 genes were upregulated in patients who subsequently developed infection. This difference, across more than 1000 genes, suggests that patients who subsequently develop infection lack the ability to activate certain immune pathways that may be important in immune defence.

Pathway analysis revealed a striking overrepresentation of genes involved in IL-2-STAT-5 and IL-6-STAT-3 signalling in stimulated PBMCs from patients that progressed to overt infection compared to PBMCs from patients that did not develop infection. Interestingly, STAT-5 activation has been shown to play an important role in driving Th2 differentiation[14] and IL-6-mediated activity has been reported to inhibit Th1 differentiation[15]. These transcriptional profiles correlate with previous findings that Th2-biased immune responses, after *in vitro* mitogen stimulation, appear to predict infection risk. Furthermore, genes involved in interferon gamma signalling were overrepresented in patients that progressed to infection. Recent reports have linked increased susceptibility to influenza infections with an aberrant IFN-γ response.[16,17] The use of corticosteroids in particular at doses used in this trial has been known to blunt the immunostimulatory effects of lenalidomide[18] and potentially impact gene expression signatures. However, patients in this study received standardised myeloma therapy which minimise the confounding effect of therapy (including corticosteroids) on observed immune profiles. There was no significant difference in the 30-day cumulative dose of corticosteroids received prior to sample collection between patients that did or did not develop subsequent infection.

TGF-ß signalling was strongly overrepresented in patients that did not develop subsequent infection. TGF-ß is a pleiotropic cytokine has been implicated as an important inhibitory master regulator of Th1 and Th2 cytokine responses. TGF-β has also been shown to inhibit Th2 responses, particularly airway Th2 cell-induced inflammation.[19] Given the important role of TGF-ß in progression of multiple myeloma, an overlapping or additive affect in controlling cancer growth and immunity could be considered.

Limitations of this study include the focus upon a small cohort of RRMM patients and the relatively short duration of follow-up utilised (3 months) for predicting infectious outcomes. These may limit generalisability of our findings across all MM stages and treatment groups and may not be predictive of longer term risks of infection. Nonetheless, the results are clinically relevant and may inform patient management. RNAseq was performed on the whole PBMC population, thus limiting attribution of observed gene expression signatures to particular cell types. However, overall detection of predictive profiles is hypothesis generating and represents a key step towards translating the use of immune profiling into clinical practice. To improve our understanding of the mechanisms that drive risk for infection in treated MM patients, future studies including single-cell RNA sequencing could be useful in attributing causation. It is possible that some of the signatures detected in our study represent the affect of covert infection rather than the cause of overt infection. Regardless, they still represent useful biomarkers to guide management. CyTOF-based immune phenotyping was restricted to main cellular subsets, but should be expanded in the future to include surface markers informed by both the results of our RNAseq transcriptome analysis as well as recently published data sets.[8,13]

CONCLUSIONS

The results of this study support previous findings that numerical differences in immune cell populations are not predictive of infection risk. Transcriptional profiling identified Th2-biased immune responses as potential immune signatures that define susceptibility of MM patients to infections, in the near term, and this finding requires further prospective evaluation and validation.

METHODS

Patient population and definitions

Patients with RRMM enrolled in a clinical treatment trial at Peter MacCallum Cancer Centre (PMCC) were evaluated.[20] Patients who had received one prior line of therapy were eligible to participate. In brief, patients received 15 mg of oral lenalidomide on days 1–21 of a 28-day cycle. All patients received dexamethasone 20 mg orally, days 1–4, 9–12 and 17–20 for the first four cycles, then dexamethasone 20 mg orally, days 1–4 only, from cycle five onwards. Blood samples were collected prospectively at multiple defined time points from patients who participated in this trial. Trimethoprim–sulfamethoxazole prophylaxis was used routinely for patients receiving more than 20 mg prednisolone equivalent for more than 4 weeks or in the setting of known intensive immunosuppression at PMCC.

Clinical and microbiology records were reviewed to capture patient demographics, MM characteristics, and characteristics of infective episodes. Episodes of infection were defined and classified as microbiologically (MDI), clinically defined infections (CDI) or fever of unknown focus (FUF) according to international definitions.[21] In brief, MDI consisted of infection episodes with pathogen(s) isolated on microbiological testing whilst CDI were infections in which site(s) of infection were identified but no pathogens were isolated on microbiological testing or due to absence of testing.[21] FUF was episodes of fever with no identifiable site of infection or pathogen.[21] Severity of infection was graded according to the common terminology criteria for adverse events.[22]

Sample collection and blood cell preparation for *in vitro* assays

For the purpose of this study, samples collected prior to cycle 4 of therapy were utilised. Total white blood cell count (WCC), total neutrophil count (ANC), eosinophil and basophil counts in patient samples were established at the time of sample collection using an automated cell counter (Cell Dyn Sapphire; Abbott Diagnostics, Abbott Park, IL, USA). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density separation and stored in RPMI/FBS/10% DMSO (foetal bovine serum, dimethyl sulfoxide) in liquid nitrogen prior to analysis. PBMCs were carefully thawed in pre-warmed thawing medium (RPMI + 10% FBS + Benzonase), washed twice and rested in RPMI supplemented with 10% (vol/vol) FBS for 2 h at 37°C. The cells were then split, and immune cell population data were collected using Helios™ Mass cytometry (CyTOF) or either re-stimulated with phorbol myristate acetate (PMA)/ionomycin or left untreated for 4 h for transcriptome analysis by RNA sequencing (RNAseq).

CyTOF immune phenotyping

PBMCs were labelled using an expanded MaxPar® Human Peripheral Blood Phenotyping Panel Kit (Cat# 201304; Fluidigm/DVS Science, Sunnyvale, CA, USA) (for CyTOF antibody panel, see supplementary table 1) and were analysed using Helios™ Mass cytometry. Briefly, 1×10^6 cells were resuspended in 300 μL in pre-warmed serum-free media and Cell-ID *Cisplatin 198* (Cat# 201198; Fluidigm/DVS Science) was added to final concentration of 1 μM (1000× dilution of 1 mM stock solution). The cells were mixed well and incubated at 37°C for 5 min before cisplatin staining was quenched by washing with pre-warmed serum-containing complete media using 5–10× the volume of the stained cells (~3 mL). The cells were pelleted and fixed using 500 μL Fix I buffer per sample for 15 min at room temperature, washed in Maxpar Cell Staining Buffer before

barcoding for 30 min using 20-plex Cell-ID Pd barcoding plex kit (Cat# 201060; Fluidigm/DVS Science). The cells were washed in Maxpar Cell Staining Buffer, and human TruStain FcX (Fc receptor blocking solution; Cat# 422302; Biolegend, San Diego, CA, USA) was added to each tube to incubate at RT for 10 min on a rotating shaker followed by adding the extracellular antibody cocktail for 30 min. After staining, cells were washed two times in Maxpar Cell Staining Buffer and iridium intercalator (Cat# 201192A; Fluidigm/DVS Science) was added in 100 μL 1.6% PFA to the tubes for overnight incubation (125 nM final concentration). The next day, the cells were washed twice using Maxpar Cell Staining Buffer followed by ddH_2O and the cells resuspended in normalisation bead solution (approximately 1 mL per 1×10^6 cells) to optimise the flow rate (350–400 cells per s) and acquire the samples. All major peripheral blood immune cell subsets including Effector $CD8^+$ T Killer Cells, Naïve $CD8^+$ T Killer Cells, Activated $CD8^+$ T Killer Cells, Effector $CD4^+$ T Helper Cells, Naïve $CD4^+$ T Helper Cells, Activated $CD4^+$ T Helper Cells, Memory $CD4^+$ T Helper Cells, Memory B Cells, Naïve B Cells, Non-Canonical Monocytes, Canonical Monocytes, Dendritic Cells and NK cells were identified based on surface marker staining using the gating hierarchy indicated in Supplementary figure 2. Gating was performed according to the Fluidigm Human Peripheral Blood Phenotyping Panel Kit data sheet (Cat# 201304; Fluidigm/DVS Science) and using the software Fluidigm. Cytobank.org.

Transcriptional profiling

PBMC RNA extraction after 4 h *in vitro* re-stimulation was performed using the Isolate II RNA mini kit (Cat# BIO-52072; Meridian Bioscience, Cincinnati, OH, USA). An input of 100 ng of total RNA were prepared and indexed separately for iIlumina sequencing using the TruSeq RNA sample Prep Kit (Cat# RS-122-2001; Illumina, San Diego, CA, USA) with RiboGlobin depletion as per manufacturer's instruction. Each library was quantified using the Agilent Tapestation (using RNA ScreenTape [Cat#5067-5576] on a 2200 TapeStation system (Cat# G2964AA; Agilent Technologies, Waldbrunn, Germany) and the Qubit™ DNA BR assay kit for Qubit 3.0® Fluorometer (Cat# Q32850; Thermo Fisher Scientific, Waltham, MA, USA). The indexed libraries were pooled for single end sequencing (1×75 cycles) on a NextSeq 500 instrument using the v2 150 cycle High Output kit (Cat# 20024906; Illumina, San Diego, CA, USA) as per manufacturer's instructions with a coverage of 30M reads per sample. The base calling and quality scoring were determined using Real-Time Analysis on board software v2.4.6, whilst the FASTQ file generation and demultiplexing utilised bcl2fastq conversion software v2.15.0.4.

All reads were aligned to the human genome, build hg38, using align from the Rsubread software package v2.0.1.[23] Over 90% of reads were successfully mapped for each sample. The number of reads overlapping genes was summarised into counts using featureCounts from Rsubread.[24] An average of 37% of reads were assigned to genes for each sample. Genes were identified using Gencode annotation for the human genome v31. Differential expression (DE) analyses were then undertaken using the edgeR[25] and limma[26] software packages v3.30.0 and v3.44.1, respectively.

Prior to analysis, all genes with no current symbol, ribosomal RNAs and non-protein coding immunoglobulin genes were removed. Gender-specific genes including XIST and those unique to the Y-chromosome were also removed to avoid gender biases. Expression-based filtering for lowly expressed genes was then performed using edgeR's filterByExpr function with default parameters. Following filtering, 23,308 genes remained. Library sizes were then normalised using the trimmed mean of M-values (TMM) method.[27]

Following filtering and normalisation, the data were transformed to $\log_2$-counts per million (CPM) and the correlation between samples from the same patient estimated using limma's duplicateCorrelation function.[28] Sample weights were also calculated using limma's arrayWeights function.[29] Differential expression was then assessed using linear models and robust empirical Bayes moderated *t*-statistics with a trended prior variance (the limma-trend pipeline).[26,30] To increase precision, the linear models included not only the patient correlation estimate and sample weights, but also an adjustment for the cessation year for each patient. The false discovery rate (FDR) was controlled below 0.1 using the method of Benjamini and Hochberg. Analyses of the Gene Ontology (GO) terms and KEGG pathways were performed using limma's goana and kegga functions, respectively. The analysis of the Hallmark gene sets from the Molecular Signatures Database was achieved using limma's fry function.

The mean-difference (MD) plots were drawn using limma's plotMD function and the heatmaps using the pheatmap CRAN software package v1.0.12.

Statistical analysis

Categorical variables were summarised as proportions whilst continuous variables were summarised with median and interquartile range (IQR). Episode of infection (MDI, CDI, FUF) within 3 months after sample collection was the outcome of interest for analysis. Categorical variables between patients with or without infection were compared utilising chi-square or Fisher's exact test whilst continuous variables were compared utilising the Mann–Whitney *U*-test with analyses performed on Stata version 13.1 (Statacorp, USA.) and $P < 0.05$ considered statistically significant. This study was approved by the PMCC human research ethics committee (HREC/17/PMCC/209).

ACKNOWLEDGMENTS

We thank Dr Stefanie Eggers and Maelle Le Moing from the MCRI Translational Genomics Unit for performing RNA sequencing runs. BWT is supported by a National Health and Medical Research Council Early Career Fellowship and Peter MacCallum Cancer Centre Foundation Partners Fellowship. MD has received support by the Jack Brockhoff Foundation, the CASS Foundation and the Scobie and Claire MacKinnon Trust. MP is supported by an NHMRC Investigator grant GNT1175011.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

BWT, MD and MP: Study conception; writing – original draft. MD: All PBMC stimulations for CyTOF and RNAseq analysis; analysis of CyTOF data sets and RNAseq data sets. ALG: Analysis of all RNAseq data sets. SF: Assisting the analysis of CyTOF and RNAseq data.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.