Single-cell analysis of the T-cell receptor repertoire in untreated myeloma patients suggests potential myeloma-reactive CD8+ T cells are shared between blood and marrow

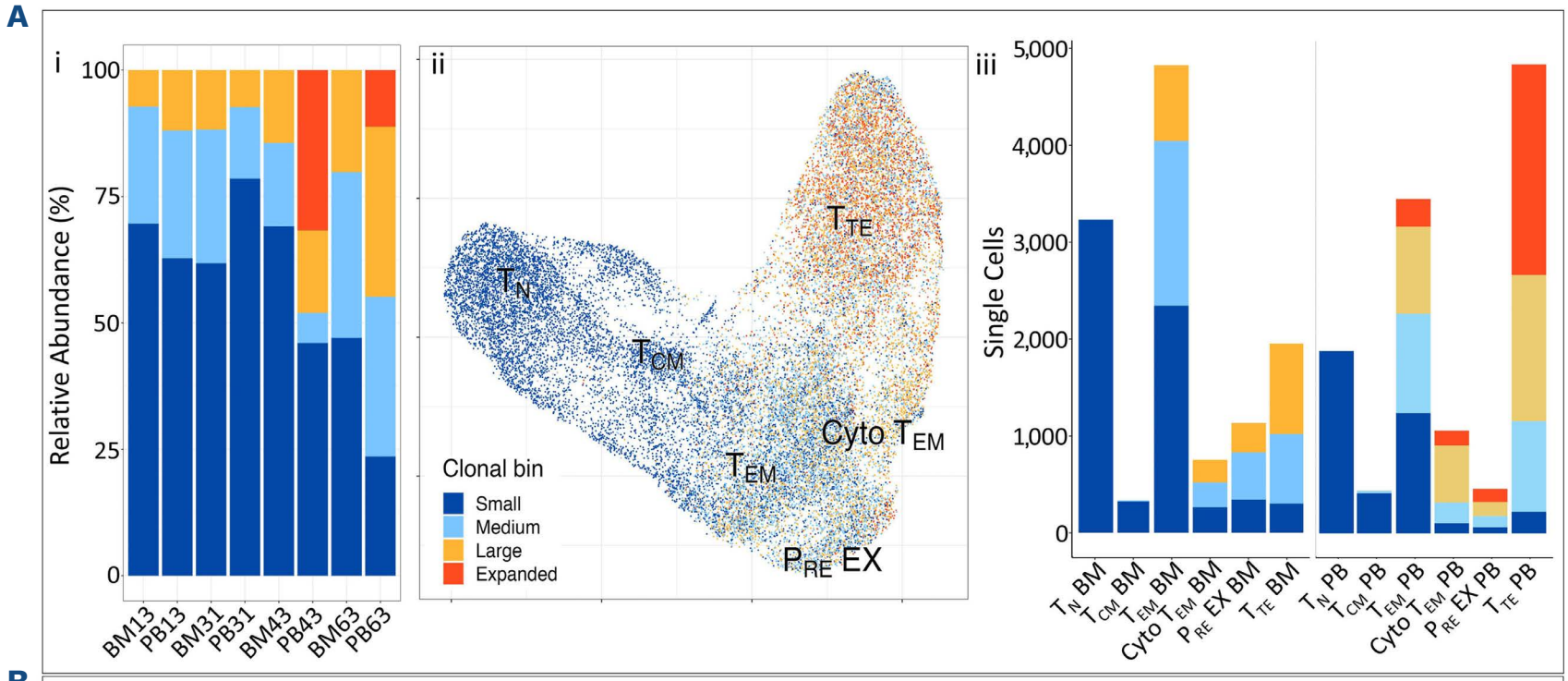
Substantial evidence supports the concept that CD8+ T cells contribute to the control of multiple myeloma (MM),[1] a malignancy characterized by the uncontrolled proliferation of neoplastic plasma cells in the bone marrow (BM), and an inflammatory tumor microenvironment.[2] We previously demonstrated that T-cell receptor (TCR) Vβ-restricted terminally differentiated CD8+ T-cell (T_{TE}) clonal expansions, capable of specific killing of autologous MM cells *in vitro*, exist within BM and peripheral blood (PB) of MM patients.[3] Although these cells appear to possess a senescent secretory effector phenotype,[4] their presence in PB correlates with improved patients' outcomes.[5] Recently, we presented an in-depth multi-omic single-cell analysis of paired CD8+ T-cell samples isolated from BM and PB of untreated, newly diagnosed (ND) MM patients,[6] demonstrating that potent cytotoxic effectors with low levels of co-inhibitory molecules reside within the tumor bed. Herein we present the results of a novel bioinformatics pipeline probing the effect of the tumor microenvironment on clonal CD8+ T cells and investigating the cognate antigens of these cells.

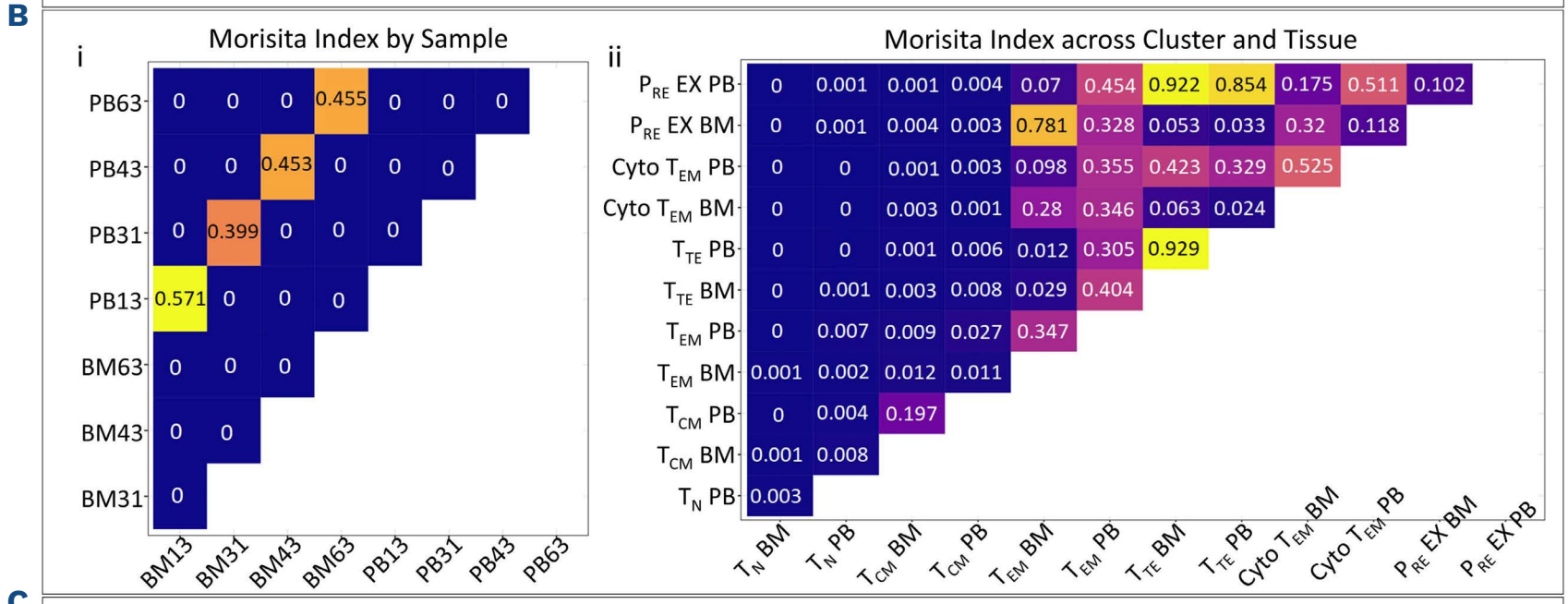
We analyzed CD8+ T cells in the BM and PB of untreated, NDMM patients using single-cell RNA sequencing, inclusive of paired TCR sequencing, via a novel bioinformatics workflow (detailed at *https://github.com/JFavaloro*). We demonstrate that dominant clones are evident in both BM and PB, expanded in both, and are composed of similar T-cell subsets. Dominant clones appear transcriptionally unaffected by the tumor microenvironment, suggesting exploitation of these cells in a disease which remains incurable[2] is of immunotherapeutic interest.

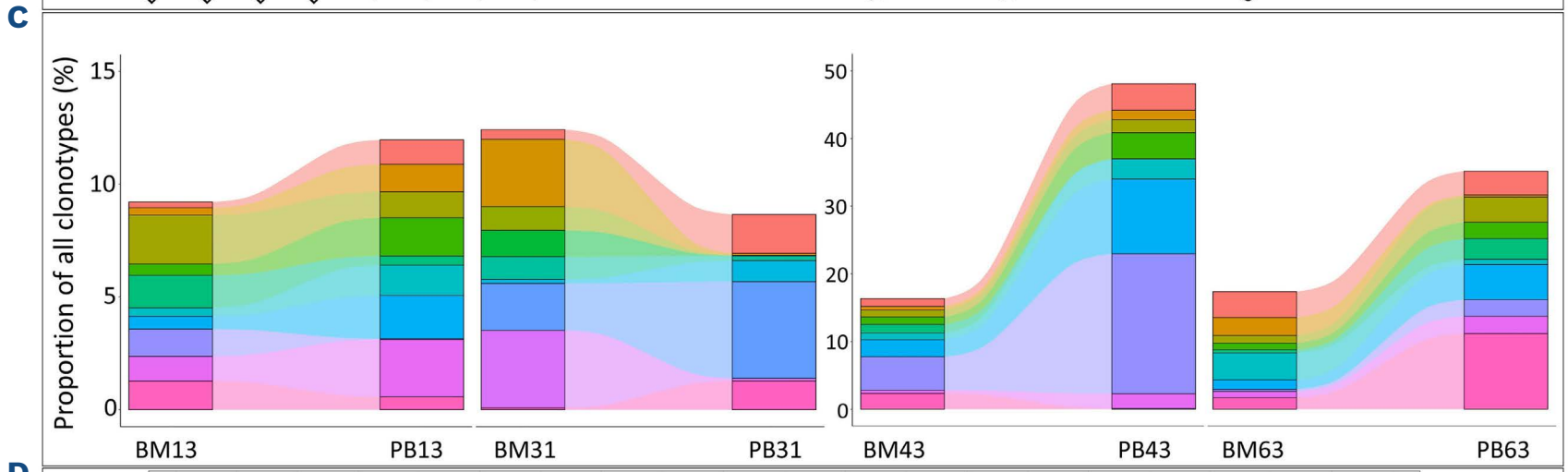
Purified CD8+ T cells isolated from BM and PB of four NDMM patients were subjected to the 10x workflow and processed as previously described (*Online Supplementary Table S1*).[6] The single-cell RNA-sequencing data were analyzed using the R package 'Seurat' (v. 4.04) with TCR clonality and diversity assessed with the R packages 'scRepertoire' (v. 1.7.2) and 'Immunarch' (v. 0.7.0), for data with and without paired gene-expression data available, respectively. Clonal bins were established at $\log_{10}$ intervals and clones were considered expanded if they accounted for >10% of a sample's repertoire, with additional categorization into large (between 1% and 10%), medium (between 0.1% and 1%) and small (<0.1%). Sequence similarity was determined using the web-based algorithms for predictive TCR-epitope binding, TCRex[7] and TCRMatch.[8] Predictive analysis of antigen-specificity of TCR clonotypes was performed against a list of myeloma antigens using a web-based algorithm. This utilized extended TCR-Peptide Binding Predictor (ERGO-II)[9] in conjunction with a list of 197 immunogenic peptides over-expressed on malignant plasma cells.[10] Briefly, identified clonotypes were assessed for reactivity using the web-based tool and results with a confidence score of ≥0.9 were selected and assessed for frequency, tissue distribution and, where possible, transcriptome. The study was approved by the Institutional Human Research Ethics Committee. All patients provided informed consent before sample collection, following the amended Declaration of Helsinki.

Assessment of clonal homeostasis demonstrated that small clonotypes dominated in most samples, with expanded clones present in two samples: PB43 and PB63 (Figure 1A, i). Transcriptional similarities with established clusters (as described in our recent publication)[6] were evident upon projecting data on Uniform Manifold Approximation and Projection (UMAP), with larger expansions demonstrating the expression of genes defining more mature cells and more diverse clonotypes clustering in the region of naïve-like clusters, matching the canonical understanding of T-cell ontogeny (Figure 1A, ii). To determine whether clonal cells exist within both the BM and PB in similar ontological states, assessment of cells across clusters and tissue compartment was performed by means of the Morisita index. This revealed a high degree of clonal sharing across tissue, primarily in the T_{TE} cluster, with the remaining overlap chiefly evident in the T_{EM} cluster of the PB and T_{EM}, Cyto-T_{EM} and P_{RE}-Ex clusters of the BM (Figure 1B). Focusing on the top ten dominant clones within an individual (*Online Supplementary Table S2*), analysis of distribution revealed these to be shared between BM and PB, expanded to a greater degree in the PB in three of four samples, and accounted for a highly variable proportion of the total TCR repertoire between patients (Figure 1C). To determine whether differences in the distribution of cell subsets between shared dominant clones were affected by existing within the tumour microenvironment relative to the PB, a ranked pie chart of cluster distribution across the top ten dominant clones was constructed. This revealed a high degree of compositional overlap, with shared clones appearing compositionally similar irrespective of tissue location (Figure 1D).

To query the potential reactivity of CD8+ T cells, several computational approaches were employed. Analysis of all clonotypes by TCRex for the MM-associated LLLGIGILV epitope of the HM21.4 antigen identified only a small number







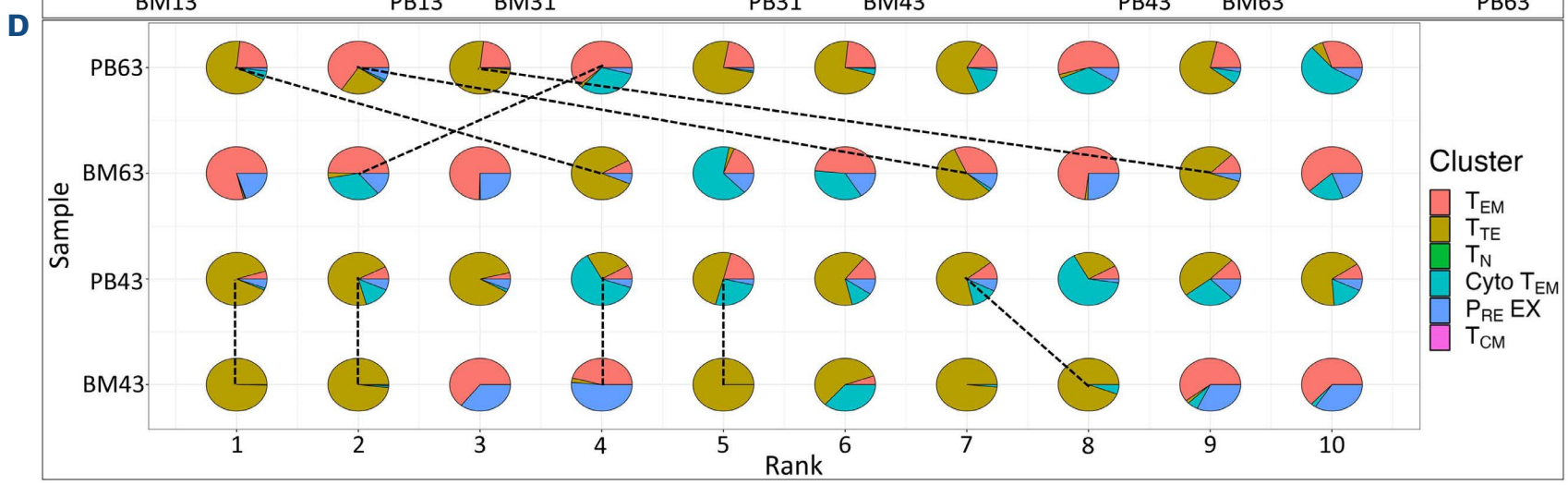


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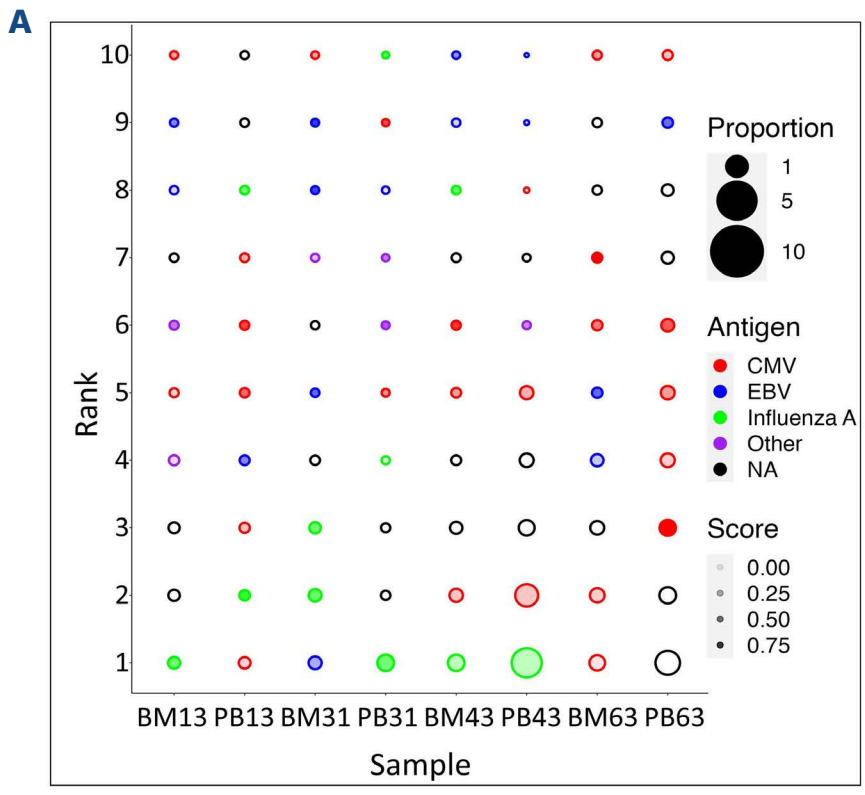
Figure 1. Analysis of T-cell receptor clonal overlap of CD8⁺ T cells between bone marrow and peripheral blood of newly diagnosed multiple myeloma patients shows inconsistency in frequency but compositional similarity. (A) i) Clonal space homeostasis of T-cell receptor (TCR) clonotypes in paired bone marrow (BM) and peripheral blood (PB) CD8⁺ T cells across small (<0.1% of the sample's TCR repertoire; dark blue), medium (0.1%-1.0%; light blue), large (1.0%-10.0%; yellow) and expanded (≥10%; red) clonal bins. ii) Uniform manifold approximation and projection of the combined single-cell object colored according to clonal bin. Single cell RNA-sequencing data without matching single-cell TCR-sequencing data are highlighted in gray. iii) Stacked bar plots of clonal bin distribution across identified clusters in the BM (left) and PB (right) of two patients with newly diagnosed multiple myeloma (NDMM) (#43 and #63). (B) Clonal overlap across tissue and cluster as measured by the Morisita index. Higher numbers (colored yellow) indicate a greater level of clonal sharing, low numbers (colored blue) indicate low/no overlap. (C) Alluvial graphs demonstrating proportions of the top ten observed TCR clonotypes between paired BM/PB samples of four NDMM patients (#13 far left, #31 left, #43 right and #63 far right). Each color represents a TCR clonotype observable in both tissues. The size of the bar represents the proportion (y axis) of the repertoire of either the BM (left) or PB (right). (D) Pie charts of the top ten dominant TCR clonotypes in paired BM and PB CD8⁺ T cells from NDMM patients (#043 and #063). Colors represent the cluster to which each clone belongs, ranked 1-10 (x axis) by level of expansion. The dashed line represents clones shared between BM and PB evident in the top ten of each sample. TCR analysis of NDMM patients demonstrates enriched BM diversity and evident clonal sharing. T_N: naïve T cells; T_{CM}: central memory T cells; T_{EM}: terminally differentiated T cells; Cyto T_{EM}: cytotoxic effector memory T cells; P_{RE} EX: pre-exhausted T cells.

of potentially reactive clones, none of which was within the top ten dominant clones (*data not shown*). Computational prediction of TCR specificity across the top ten dominant clones in all samples, undertaken using TCRMatch,[8] suggested potential reactivity against several viral antigens, chiefly cytomegalovirus, Epstein-Barr virus and influenza A, and a minority against non-viral antigens (e.g., gliadin and insulin) (Figure 2A). However, as TCRMatch is limited to reference databases that primarily consist of data on viral-specific T cells, we leveraged the deep-learning tool ERGO-II[9] to detect cells potentially reactive to a published list of 197 peptides from 58 proteins known to be presented by class I MHC on malignant plasma cells.[10] After restricting results to "hits" with confidence scores ≥0.9 and clones comprising ≥0.1% of a sample's repertoire, a total of 14 clonotypes were identified against six unique peptides from six proteins with a degree of gene-sharing evident in the overrepresentation of *TRBV27* (Table 1). Clones were generally observable within both BM and PB compartments but with greater representation in BM, with most clustered within the T_{EM} cluster, and some clustering in the P_{RE}-EX and Cyto-T_{EM} clusters (Figure 2B, i-vi). However, the most dominant clone in the dataset, accounting for 4.9% and 20.6% of the BM and PB repertoires of NDMM #43, respectively, and identified as potentially reactive against a peptide derived from influenza A in the previous analysis, and one derived from Cap methyltransferase 1 (CMTR1) by ERGO-II, demonstrated near exclusive clustering within the T_{TE} cluster (Figure 2B, vii). Differential expression testing of this (Figure 2C, *Online Supplementary Table S3*), and other (*data not shown*) dominant clones shared across the BM and PB demonstrated few differences.

Of the 197 peptides assessed by deep learning, three stood out: RPRKAFLLLL, RPFHGWTSL and IHILDVLVL, derived from the proteins disulphide isomerase precursor A 4 (PDIA4), mannosyl-oligosaccharide glucosidase (MOGS) and CMTR1, respectively. These were observed to be shared across multiple patients with evident restriction in TCR-Vβ gene



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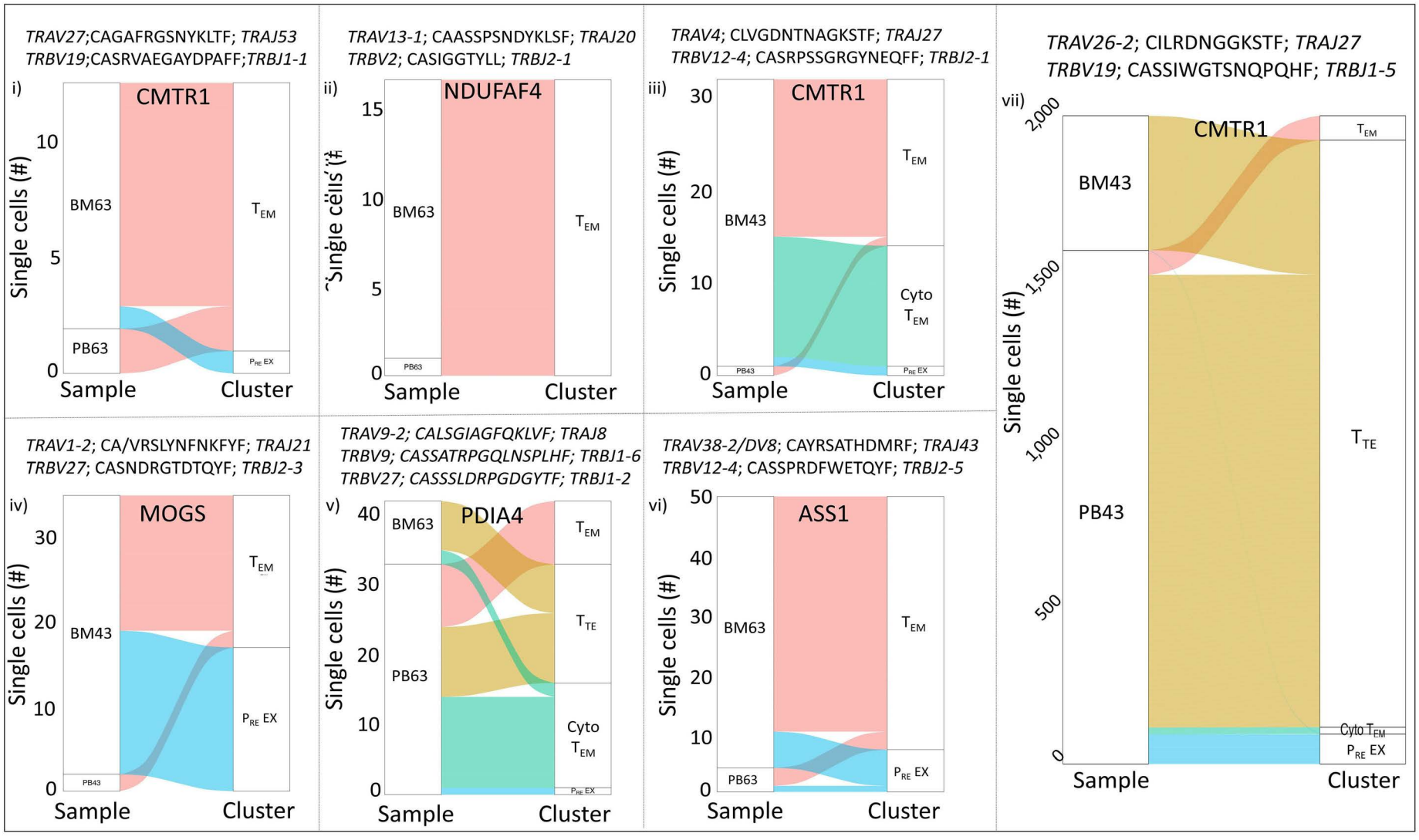

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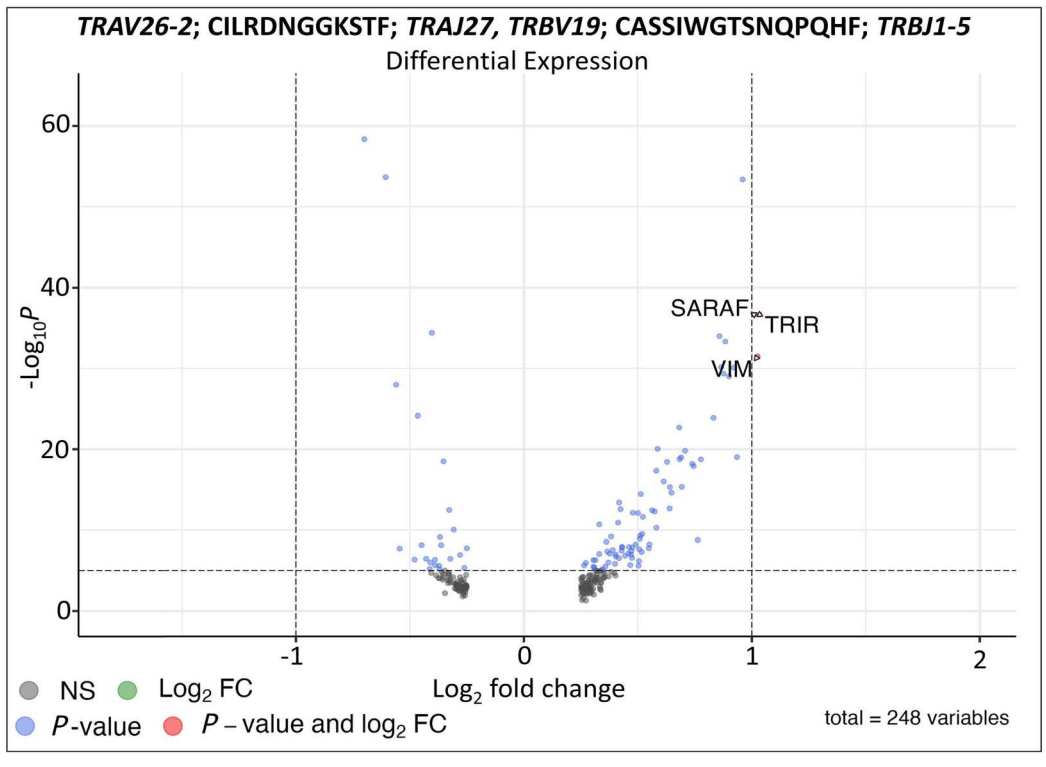

Figure 2. Results of computational predication of reactivity and transcriptional analysis of identified clones. (A) Bubble plot illustrating potential reactivity of the top ten dominant T-cell receptor (TCR) clonotypes in paired bone marrow (BM) and peripheral blood (PB) CD8⁺ T cells from newly diagnosed multiple myeloma (NDMM) patients (#013, #031, #043 and #063). The size of the dot represents the proportion (as a percentage) of all observed clonotypes within that individual sample, ranked 1-10 (y axis) based on level of expansion. The opacity of the dot represents the confidence (darker implies greater confidence) to which a particular clonotype is reactive to a particular antigen (cytomegalovirus, red; Epstein-Barr virus, blue; influenza A virus, green; other [e.g., yellow fever], purple). NA refers to clonotypes for which no data were recovered. (B) Alluvial graphs of clones identified by deep learning as being potentially myeloma reactive arranged by size (smallest to greatest; i-vii). Each line represents the tissue of origin of a single cell (left alluvium) colored by the cluster in which it resides (right alluvium). Each pair is listed with the identified TCR sequence along with the protein from which the peptide the clone is potentially reactive to is derived. (C) Volcano plot illustrating differential expression results comparing the most dominant clone in NDMM#43 across the BM and PB.

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Red dots represent genes that are both significantly upregulated and expressed at a log_2 fold change (FC) value of >1 in the BM (right) relative to the PB or <1 in the PB (left) relative to the BM. Blue dots represent genes that are significantly different but not expressed at a great enough level (i.e., >1 or <1 log_2 FC). Green dots represent genes that are highly expressed (i.e., >1 or <1 log_2 FC) but found to be not significantly differentially expressed between the two tissues. Black dots represent tested genes that are neither highly expressed nor significantly different between the two tissues. CMV: cytomegalovirus; EBV: Epstein-Barr virus; CMTR1: cap methyltransferase 1; NDUFAF4: NADH ubiquinone oxidoreductase complex assembly factor 4; MOGS: mannosyl oligosaccharide glucosidase; PDIA4: protein disulphide isomerase family A member 4; ASS1: argininosuccinate synthase 1.

Table 1. Results of ERGO-II peptide prediction against peptides demonstrated to be overexpressed on malignant plasma cells.

Patient ID	Tissue	Size (%)* repertoire	Clone	Confidence	Protein	Peptide	Dominant Cluster
NDMM#13	BM	0.396	*TRAV12-1*; CVVPWYSSASKIIF; *TRAJ3*	0.93	PDIA4	RPRKAFLLLL	N/A
	PB	0.012	°*TRBV27*; CASGTGQNQPQHF; *TRBJ1-5*				N/A
NDMM#13	BM	0.247	*TRAV38-2/DV8*; CAYTSGTYKYIF; *TRAJ40*	0.91	PDIA4	RPRKAFLLLL	N/A
	PB	0.299	°*TRBV27*; CASSLSPVNYGYTF; *TRBJ1-2*				N/A
NDMM#13	BM	0.04	*TRAV19*; CALHNAGKSTF; *TRAJ27*	0.91	MOGS	RPFHGWTSL	N/A
	PB	0.383	°*TRBV27*; CASSLQRNTEAFF; *TRBJ1-1*				N/A
NDMM#13	BM	0.025	*TRAV27*; CAGPSGNTGKLIF; *TRAJ37*	0.92	MOGS	RPFHGWTSL	N/A
	PB	0.23	°*TRBV27*; CASSLSRRVGSYGYTF; *TRBJ1-2*				N/A
NDMM#31	BM	0.128	*TRAV14/DV4*; CAMREPLNNAGNMLTF; *TRAJ39*	0.9	MOGS	RPFHGWTSL	N/A
	PB	N/A	°*TRBV27;* CASSLGGGWTEAFF; *TRBJ1-1*				N/A
NDMM#31	BM	0.176	*TRAV22;* CALTDSWGKLQF; *TRAJ24*	0.94	CMTR1	IHILDVLVL	N/A
	PB	N/A	*TRBV5-4*; CASLPYSGANVLTF; *TRBJ2-6*				N/A
NDMM#31	BM	0.064	*TRAV8-1*; CAVIGFQKLVF; *TRAJ8*	0.91	LAP3	DVNNIGKYR	N/A
	PB	0.815	°*TRBV27;* CASSLTASHYGYTF; *TRBJ1-2*				N/A
NDMM#43	BM	4.929	*TRAV26-2*; CILRDNGGKSTF; *TRAJ27*	0.94	CMTR1	IHILDVLVL	T_{TE}
	PB	20.567	*TRBV19*; CASSIWGTSNQPQHF; *TRBJ1-5*				T_{TE}
NDMM#43	BM	0.473	*TRAV1-2*; CAVRSLYNFNKFYF; *TRAJ21*	0.91	MOGS	RPFHGWTSL	T_{EM}
	PB	0.014	°*TRBV27*; CASNDRGTDTQYF; *TRBJ2-3*				T_{EM}
NDMM#43	BM	0.3	*TRAV4*; CLVGDNTNAGKSTF; *TRAJ27*	0.97	CMTR1	IHILDVLVL	T_{EM}
	PB	0.014	*TRBV12-4*; CASRPSSGRGYNEQFF; *TRBJ2-1*				T_{EM}
NDMM#63	BM	0.674	*TRAV38-2/DV8*; CAYRSATHDMRF; *TRAJ43*	0.96	ASS1	NIGQKEDFEEA	T_{EM}
	PB	0.059	*TRBV12-4*; CASSPRDFWETQYF; T*RBJ2-5*				T_{EM}
NDMM#63	BM	0.196	*TRAV13-1*; CAASSPSNDYKLSF; *TRAJ20*	0.94	NDUFAF4	APRHPSTNSL	T_{EM}
	PB	0.03	*TRBV2*; CASIGGTYLL; *TRBJ2-1*				T_{EM}
NDMM#63	BM	0.174	*TRAV27*; CAGAFRGSNYKLTF; T*RAJ53*	0.93	CMTR1	IHILDVLVL	T_{EM}
	PB	0.015	*TRBV19*; CASRVAEGAYDPAFF; T*RBJ1-1*				T_{EM}
NDMM#63	BM	0.196	*TRAV9-2*; CALSGIAGFQKLVF; *TRAJ8*	0.9	PDIA4	RPRKAFLLLL	T_{EM}
	PB	0.03	*TRBV9*; CASSATRPGQLNSPLHF; T*RBJ1-6* °*TRBV27*; CASSSLDRPGDGYTF; *TRBJ1-2*				T_{EM}

*The proportion of the repertoire is derived from Immunarch analysis of raw data with non-productive clones removed. °Clones with *TRBV27*. ID: identity; NDMM: newly diagnosed multiple myeloma; BM: bone marrow; PB: peripheral blood; T_{EM}: effector memory T cells; T_{TE}: terminally differentiated T cells; N/A: not applicable; PDIA4: protein disulphide isomerase family A member 4; MOGS: mannosyl oligosaccharide glucosidase; CMTR1: cap methyltransferase 1; LAP3: leucine aminopeptidase 3; ASS1: argininosuccinate synthase 1; NDUFAF4: NADH ubiquinone oxidoreductase complex assembly factor 4.

usage including an over-representation of *TRBV27* (Table 1). While PDIA4 has an established role in MM,[11] both MOGS and CMTR1 remain unexploited. MOGS is a transmembrane protein, found in the endoplasmic reticulum, which catalyzes a reaction essential for immunoglobulin production, while CMTR1 is essential for mRNA stability and is vital for the propagation of influenza A.[12] Although our analysis revealed that most potential myeloma-reactive $CD8^+$ T cells reside primarily within the dominant BM-T_{EM} cluster, the most highly expanded clonotype in our dataset, which both TCRMatch and ERGO-II suggested was associated with influenza A, revealed a highly consistent cytotoxic effector

transcriptional profile across both BM and PB. These findings highlight that shared public antigens may be implicated in T-cell immunity in MM, as has previously been suggested in melanoma;[13] however, further investigations, including an age-matched control cohort, are required.

While these *in-silico* results are not definitive evidence of specificity, our approach of using a disease-specific MHC-presented peptide library, a TCR-library derived from CD8$^+$ T cells within the tumor bed and machine-learning to look for high probability hits is novel, and has utility not only in MM, but also in other diseases, fast-tracking peptide selection for use in validation assays. Although speculative, it is possible that identified clonal cells residing primarily in memory clusters either once contributed to disease control until such a time that myeloma clonal evolution rendered these targets mute or represent potential auto-reactive clones that may be activated in the presence of immunomodulatory drugs. Future work should endeavor to assess the transcriptome/proteome of the myeloma tumor itself; although not proof of specificity, this would provide supportive evidence if it were determined that tumor does indeed express the epitope that dominant CD8$^+$ T-cell clones are reactive against. Further, analysis of paired tumor ligandome by mass spectrometry would grant further confidence in the *in-silico* results and guide functional studies, particularly were this method applied to a disease in which mutation is more commonly observed (e.g., melanoma).[13] Clinical trials have demonstrated that *ex-vivo* expanded T cells harvested from the BM of MM patients are able to extend the autologous graft-*versus*-myeloma effect attributable to transplant.[14] In support of this, our data suggest that most potential anti-myeloma CD8$^+$ T cells likely reside primarily (but not exclusively) within *CD69*-expressing clusters in the BM (i.e., the T_{EM}, Cyto T_{EM} and P_{RE}-Ex clusters). Taken together, these data suggest that the gene-expression profiles of dominant T-cell clones are not influenced to an appreciable degree by the tumor microenvironment. Nevertheless, this work highlights two peptides as currently unexploited immunotherapeutic targets in multiple myeloma, warranting further investigation.

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Disclosures

No conflicts of interest to disclose.

Contributions

JF and CEB designed and performed the research, analyzed the data, organized the human ethics and wrote the paper. EA and SY performed the research and analyzed the data. SG assisted with the bioinformatics and analyzed the data. NN, BAO'B and LMS assisted in research design and data analysis. CEB, EA, TK and PJH assisted in research design, reviewed patients, and assisted with the collection of patients' samples and clinical information. RB reviewed patients undergoing hip arthroplasty and designed research. DEJ and PJH were involved in research design, analysis of data and writing the paper.

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Data-sharing statement

Data may be made available by contacting the corresponding author.

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