

Mass Cytometry Analysis Shows That a Novel Memory Phenotype B Cell Is Expanded in Multiple Myeloma

Leo Hansmann¹, Lisa Blum², Chia-Hsin Ju², Michaela Liedtke³, William H. Robinson², and Mark M. Davis^{1,4,5}

Abstract

It would be very beneficial if the status of cancers could be determined from a blood specimen. However, peripheral blood leukocytes are very heterogeneous between individuals, and thus high-resolution technologies are likely required. We used cytometry by time-of-flight and next-generation sequencing to ask whether a plasma cell cancer (multiple myeloma) and related precancerous states had any consistent effect on the peripheral blood mononuclear cell phenotypes of patients. Analysis of peripheral blood samples from 13 cancer patients, 9 precancer patients, and 9 healthy individuals revealed significant differences in the frequencies of the T-cell, B-cell, and natural killer-cell compartments. Most strikingly, we identified a novel B-cell pop-

ulation that normally accounts for $4.0\% \pm 0.7\%$ (mean \pm SD) of total B cells and is up to 13-fold expanded in multiple myeloma patients with active disease. This population expressed markers previously associated with both memory ($CD27^+$) and naïve ($CD24^{lo}CD38^+$) phenotypes. Single-cell immunoglobulin gene sequencing showed polyclonality, indicating that these cells are not precursors to the myeloma, and somatic mutations, a characteristic of memory cells. SYK, ERK, and p38 phosphorylation responses, and the fact that most of these cells expressed isotypes other than IgM or IgD, confirmed the memory character of this population, defining it as a novel type of memory B cells. *Cancer Immunol Res*; 3(6); 650–60. ©2015 AACR.

Introduction

The human immune system has a unique role in cancer pathogenesis, modulating therapeutic responses, and disease outcome. Especially with emerging immune therapeutics and treatment regimens, the characterization of cancer-associated immunologic signatures has been of considerable interest. However, most studies of tumor "immunomes" rely on gene expression data and focus on the tumor microenvironment (1–3). To some extent, this may be due to the limited resolving power of standard cell analysis methods such as flow cytometry and the heterogeneity of peripheral blood cells. However, recent advances in cell phenotyping technology, particularly cytometry by time-of-flight (CyTOF), which supports 40-label analysis (4–6), and next-generation sequencing of immunoglobulin (Ig) genes (7, 8), have greatly increased the possible depth of analysis at the single-cell level and expanded the resolving power of immune phenotyping

tremendously. This suggests that it may be possible to detect disease-related immune signatures in human peripheral blood using these methods. As peripheral blood is readily and safely obtainable from almost any patient, finding useful signatures of disease in this type of specimen could be very valuable both clinically and in understanding the immune response to particular cancers at a systems biology level.

For this reason, we analyzed the peripheral blood of patients with hematopoietic cancer (multiple myeloma) and its corresponding precancer [asymptomatic myeloma and monoclonal gammopathy of undetermined significance (MGUS)]. Multiple myeloma, a malignancy of the B lineage, is characterized by the accumulation of clonal plasma cells in the bone marrow and the production of monoclonal Ig. The corresponding precancer diseases, MGUS and asymptomatic myeloma, are characterized by the production of monoclonal Ig and the lack of symptoms in often undiagnosed patients. They are not considered malignant because they do not always progress into multiple myeloma; however, patients have an approximate risk of 1% per year to develop multiple myeloma (9), and many, if not all, multiple myeloma cases develop from a preceding MGUS or asymptomatic myeloma (10, 11).

Multiple myeloma represents a prototypical disease model for tumor–microenvironment interactions (12, 13), and recent studies of the multiple myeloma microenvironment have underlined its role in promoting tumor growth and survival (14), as well as protection from T-cell responses (15) and chemotherapeutics (16, 17). The myeloma cells "reprogram" their environment, influencing many chemokine and cytokine levels, including IL6, IL1 α , IL1 β , MIP-1 α , TNF α , M-CSF, and VEGF (13). With this profound effect on the bone marrow—the organ in which hematopoiesis takes place in adults—we reasoned that multiple myeloma and

¹Department of Microbiology and Immunology, Stanford University, Stanford, California. ²Division of Immunology and Rheumatology, Stanford University, Stanford, California. ³Division of Hematology, Department of Medicine, Stanford University, Stanford, California. ⁴Institute for Immunity, Transplantation, and Infection, Stanford University, Stanford, California. ⁵The Howard Hughes Medical Institute, Stanford University, Stanford, California.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

Corresponding Author: Mark M. Davis, Stanford University, 279 Campus Drive, Beckman Center, Stanford, CA 94305. Phone: 650-725-4755; Fax: 650-725-7778; E-mail: mmdavis@stanford.edu

doi: 10.1158/2326-6066.CIR-14-0236-T

©2015 American Association for Cancer Research.

perhaps its precursor diseases might have a significant impact on immune cells in the peripheral blood that could be detectable with these new technologies.

Here, we report that we have found major effects of multiple myeloma, but not MGUS or asymptomatic myeloma, that are detectable in peripheral blood cells of patients with active disease, in particular significant changes in the T-cell, B-cell, and natural killer (NK)-cell compartments, and most notably, the expansion of a novel, polyclonal B-cell subset.

Materials and Methods

Subjects

Thirteen multiple myeloma, 9 MGUS/asymptomatic myeloma patients (either untreated or without cancer-specific treatment during the last 6 months; Table 1 and Supplementary Table S1), and 5 treatment-naïve colorectal cancer patients (Supplementary Fig. S10) were recruited in accordance with federal and local human subjects regulations (Institutional Review Board protocol ID 25310). Nine age- and sex-matched healthy individuals were recruited through the Stanford Blood Center. The median ages of the multiple myeloma, precancer, and healthy control groups were 67, 69, and 68 years, respectively. Male subjects accounted for 54% in the cancer cohort, 56% in the precancer cohort, and 56% in the healthy control group.

PBMC preparation

Peripheral blood mononuclear cells (PBMC) were isolated from up to 40 mL of freshly drawn heparin anticoagulated blood using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB) centrifugation and resuspended in cell culture medium (Supplemen-

tary Table S2). Freshly isolated PBMCs were immediately used for *in vitro* stimulation and CyTOF staining; remaining cells were frozen after the addition of an equal volume of FBS containing 20% DMSO (both Sigma Aldrich).

Cell stimulation

For CyTOF, PBMCs were stimulated in 1 mL of cell culture medium containing 20 ng/mL phorbol 12-myristate 13-acetate (PMA), 1 μ mol/L ionomycin (Sigma Aldrich), 5 μ g/mL R848, or 3 μ g/mL CpG ODN2216 (both InvivoGen) or left unstimulated for 6 hours. GolgiPlug (1 μ L) and 0.7 μ L GolgiStop (both BD Biosciences) were added at the beginning of the stimulation for PMA/ionomycin or to unstimulated samples, or added after 2 hours for R848 or after 3 hours for CpG ODN2216/DOTAP. DOTAP liposomal transfection reagent (Roche) was added at 1 μ L/mL for CpG ODN2216 stimulation. Stimulation was done at 37°C and 5% CO₂.

For phosphorylation analysis, stimulation was done in reverse time order in 250 μ L prewarmed cell culture medium containing 50 μ mol/L CpG ODN2006 (InvivoGen), or 10 μ g/mL goat anti-human IgM (Life Technologies) and 10 μ g/mL goat F(ab)₂ anti-human IgG (AbD Serotec). For B-cell receptor (BCR) stimulation, H₂O₂ (MP Biomedicals) was added within 10 seconds after addition of the stimulating antibodies to a final concentration of 3.3 mmol/L.

CyTOF antibody labeling and staining

Purified antibodies were labeled using MaxPar DN3 kits (Fluidigm) and stored at 4°C at 0.2 mg/mL in W buffer (Fluidigm) containing antibody stabilizer (Candor).

Table 1. Clinical characteristics of MM, AM, and MGUS patients

Subject	Disease	Age (years)	Sex	M-component type	Stage (ISS)	BM infiltration (%)	Cytogenetics	Last treatment
1	AM	56	F	IgA λ	n/a	15–20	n.d.	—
2	MGUS or AM	77	M	IgG λ	n/a	n.d.	n.d.	—
5	MGUS or AM	70	M	IgG κ	n/a	n.d.	n.d.	—
9	MGUS	70	M	IgA κ	n/a	9	Normal karyotype	—
23	AM	69	M	IgG λ	n/a	20	Extra copy of 1q21, partial deletion of chromosome 13, +1, dic(1;8), t(8;22), del13, and t(14;20)	—
24	MGUS or AM	69	F	IgG κ	n/a	n.d.	n.d.	—
26	MGUS or AM	62	M	IgG κ	n/a	5	n.d.	—
27	MGUS or AM	47	F	IgG λ	n/a	n.d.	n.d.	—
28	AM	51	F	IgG λ	n/a	17	n.d.	—
3	MM	69	F	IgG κ	n.d.	40	n.d.	Melphalan/BCNU, autologous transplant
4	MM	65	F	IgA κ	n.d.	30	Hyperdiploidy	RVD
6	MM	46	M	IgA λ	3	60	Normal karyotype	—
7	MM	63	F	n.k.	n.d.	n.d.	n.d.	TD, high-dose melphalan
8	MM	57	M	IgA κ	1	30	Normal karyotype	—
10	MM	56	F	κ light chain	3	50	Del13q, t(11;14)	—
11	MM	72	M	IgG λ	2	48	del Y	—
18	MM	81	M	IgG κ	1	30	Normal karyotype	—
19	MM	61	F	κ light chain	3	50	Normal karyotype	—
20	MM	77	M	IgA κ	3	15	t(1;16), gain of IgH	—
21	MM	67	M	IgG κ	3	25	Normal karyotype	—
25	MM	85	M	IgG κ	3	10–90	n/a	MP
32	MM	77	F	IgG κ	3	40	n/a	—

NOTE: None of the patients with indicated last treatment have been treated during the last 6 months before the study blood draw.

Abbreviations: AM, asymptomatic myeloma; BM, bone marrow; ISS, International Staging System; MM, multiple myeloma; MP, melphalan prednisone; n/a, not applicable; n.d., not determined; RVD, revlimide velcade dexamethasone; TD, thalidomide dexamethasone.

Hansmann et al.

For staining, 1 to 10×10^6 cells were washed in CyFACS buffer (Supplementary Table S2) and stained in $50 \mu\text{L}$ CyFACS buffer containing a surface antibody cocktail (Supplementary Table S3) for 30 minutes. The $\gamma\delta$ T-cell antibody stain was done separately, and the metal-labeled anti-PE antibody was added to the surface antibody cocktail. Cells were washed in CyPBS, PBS (Ambion), and stained with maleimide-DOTA loaded with ^{115}In for 20 minutes at room temperature. After washing in CyFACS and CyPBS, cells were fixed in $150 \mu\text{L}$ of 2% paraformaldehyde (PFA; Electron Microscopy Sciences) over night. Cells were washed twice in permeabilization buffer (eBioscience) and stained in $50 \mu\text{L}$ intracellular antibody cocktail (Supplementary Table S3) for 30 minutes on ice. After another wash in permeabilization buffer and CyPBS, cells were stained with iridium DNA intercalator (Fluidigm) for 20 minutes at room temperature, washed $2 \times$ in CyFACS, $2 \times$ in CyPBS, $2 \times$ in H_2O , and resuspended in H_2O for analysis on a CyTOF instrument (Fluidigm).

Cell signaling analysis

Cells were thawed, washed twice in prewarmed cell culture medium, and rested for 2 hours at 37°C with 5% CO_2 . Cells were washed in pure PBS and stained with zombie aqua (BioLegend), washed once in pure PBS and stained with CD24 and CD38 antibodies (Supplementary Table S4) for 15 minutes at 4°C . After washing in prewarmed cell culture medium, cells were resuspended in $250 \mu\text{L}$ of warm cell culture medium and immediately stimulated as described in the cell stimulation section. Stimulation was stopped by adding $150 \mu\text{L}$ of 4% PFA and incubated for 15 minutes at room temperature. Cells were washed with pure PBS and permeabilized in methanol at -80°C overnight. After washing twice in pure PBS, cells were stained with an intracellular staining cocktail of antibodies specific for phosphorylated signaling molecules and additional phenotyping markers (Supplementary Table S4), washed, and finally stained with AF488-labeled goat anti-rabbit antibody (Supplementary Table S4) before analysis on an LSRII flow cytometer (BD Biosciences).

Single-cell immunoglobulin sequencing

PBMCs were thawed and stained with fluorochromes according to Supplementary Table S4. Single B cells were identified by their forward scatter/side scatter (FSC/SSC) characteristics, CD19, and CD20 expression and sorted into RT-PCR buffer in 96-well plates according to the gating strategy in Fig. 4A and Supplementary Fig. S8. Ig genes were amplified and sequenced as previously described (7, 8).

FISH

Cells were stained as described for single-cell Ig sequencing and bulk sorted according to the gating scheme in Supplementary Fig. S8. Cells were stained with Vysis LSI CCND1 and Vysis LSI IGH probes (both Abbott Molecular) according to the standard protocols, and 200 cells per population (105 for $\text{CD}19^+ \text{CD}20^+ \text{CD}24^{\text{hi}} \text{CD}38^- \text{CD}27^- \text{IgD}^- \text{IgM}^-$ B cells) were analyzed.

Cytometry data analysis and statistics

All two-dimensional gating analysis was done using FlowJo (Treestar). For heatmaps (Figs. 1A and 5; Supplementary Figs. S2 and S9), data in FlowJo-exported tables were trimmed to the 95th and 5th percentiles per gated population and visualized using heatmap.2 (gplots version 2.11.0). Principal component analysis (PCA) was done using prcomp (stats version 2.15.3) and visual-

ized with ggplot2 (version 0.9.3.1) and scatterplot3d (version 0.3–34).

For statistics in scatterplots (Fig. 2B and Supplementary Fig. S3), we used ANOVA. *P* values were adjusted using the Benjamini–Hochberg correction, and all differences that reached *q* values below 0.05 were followed up with TukeyHSD, the results of which are indicated as *P* values in the respective plots. All statistics were done in R version 2.15.3 (18).

Citrus version 0.05 (<https://github.com/nolanlab/citrus>; ref. 19) was used on all healthy control and all multiple myeloma samples. Each file was pre-gated on live $\text{CD}45^+$ cells in FlowJo. The file sample size was set to 10,000 events and CD33, CD4, CD20, CD8, CD16, CD19, CD11c, CD24, CD10, CD1c, CD14, CCR7, CD7, CD28, CD5, CD38, CD45RA, CD123, CD45RO, CD56, HLA-DR, CD25, TCR- $\alpha\beta$, and CD127 were used as clustering markers. Median differences (median columns) were calculated for IgM, CD57, IgD, CD27, CCR7, CD28, TNF α , IL17A, and HLA-DR. The minimum cluster size was set to 0.02%, and Significance Analysis of Microarrays (SAM) was used as model type.

Results

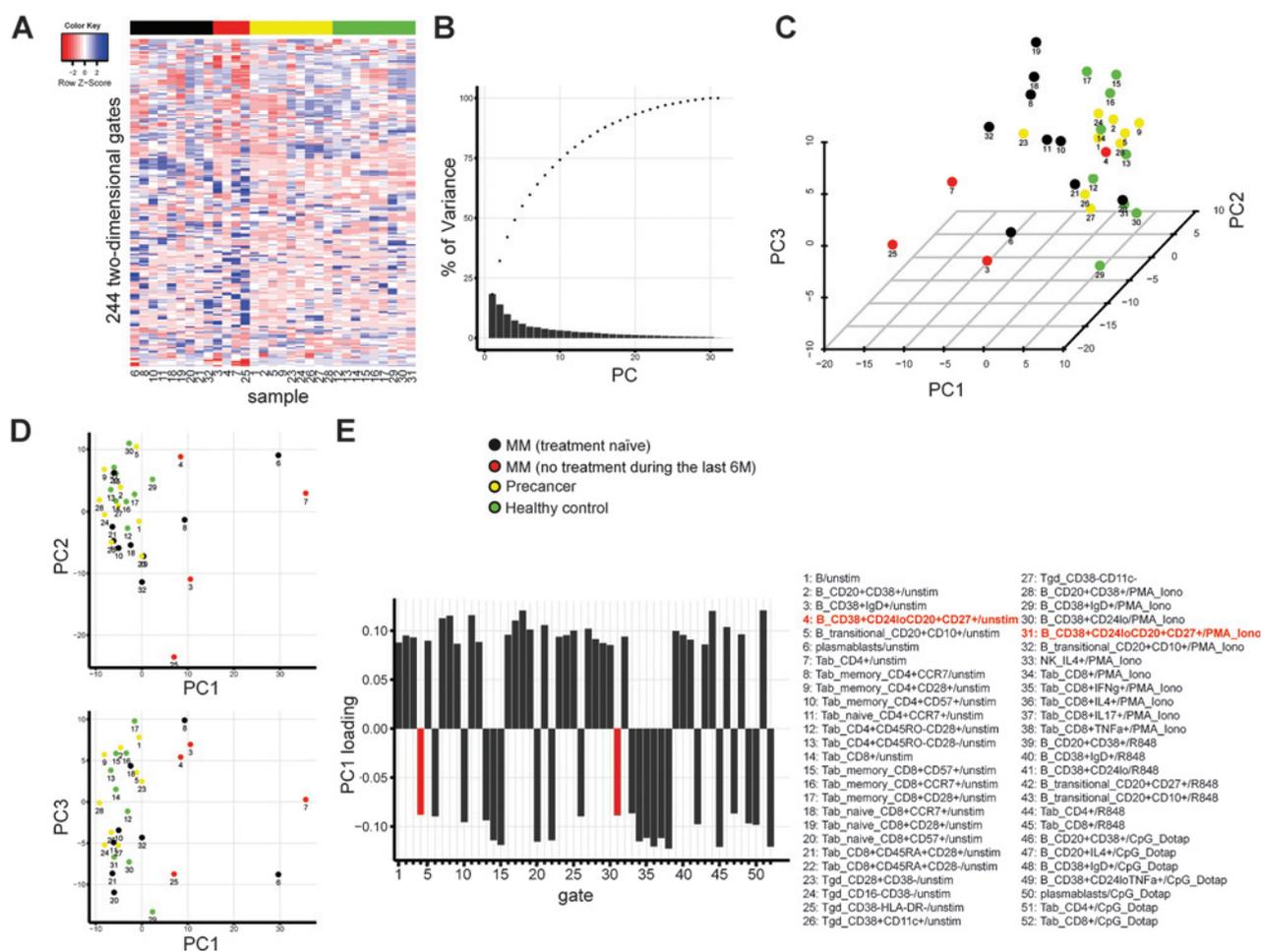
Phenotypic diversity of PBMC subsets in multiple myeloma

We used CyTOF with a 39-marker panel (Supplementary Table S3) to analyze the human immune system in freshly isolated PBMCs from multiple myeloma patients, from precancer MGUS and asymptomatic myeloma patients (Table 1 and Supplementary Table S1), and from healthy individuals. Frequencies of all major PBMC subsets and their cytokine expression patterns were determined by two-dimensional gating as percentages of the corresponding parent populations in four different stimulus conditions (see Supplementary Fig. S1 and Supplementary Table S5 for typical gating and a list of all applied gates).

We visualized cell frequencies in each of the 244 two-dimensional gates in a heatmap representing the immune phenotypic diversity in the peripheral blood of patients with multiple myeloma, precancer patients, and healthy controls (Fig. 1A). Samples were arranged by disease group, and no clustering was applied to the columns. Emphasizing the phenotypic heterogeneity of the dataset, sample clustering by Euclidian distances of relative cell frequencies did not lead to the formation of clear clusters for cancer patients, precancer patients, and healthy individuals (Supplementary Fig. S2).

The direct comparison of individual population frequencies between disease groups revealed significant differences in the T ($\text{CD}57^+ \text{CD}8^+ \text{CD}45\text{RA}^+ \alpha\beta$ and regulatory) and B lymphocyte (total, $\text{CD}27^+$, and transitional) compartments in different stimulus conditions as summarized in Supplementary Fig. S3.

To analyze whether individual markers or combinations of those could distinguish cancer samples from precancer or healthy samples, we applied PCA to cell frequencies in the same 244 two-dimensional gates. The first four principal components described close to 50% of the variance in our dataset (Fig. 1B), and plotting principal components one to three confirmed phenotypic heterogeneity in samples from the cancer group, whereas the precancer and healthy control samples tended to cluster (Fig. 1C and D). Nevertheless, PC1 separated a subset of cancer samples from precancer and healthy donor samples. The loadings for PC1 were dominated by $\alpha\beta$ T-cell gates, including costimulatory molecules

**Figure 1.**

Phenotypic diversity of multiple myeloma (MM), asymptomatic myeloma, and MGUS patients. Displayed are the CyTOF data from 13 multiple myeloma patients, 11 precancer patients, and 9 healthy individuals. The sample numbers in A, C, and D correspond to the subject numbers in Table 1. A, normalized cell frequencies (%) in 244 two-dimensional gates per sample are visualized as a heatmap. Gates are arranged in rows, samples in columns. No clustering was applied to the columns. B–E, PCA on the cell frequencies displayed in the gates in A. B, cumulative (dots) variance explained by the individual (bars) principal components (PC). C and D, three- and two-dimensional visualizations of the first three principal components. E, loadings of PC1 > 0.085. X axis labels and bar colors correspond to the numbered and colored gate names in the lower right panel of the figure.

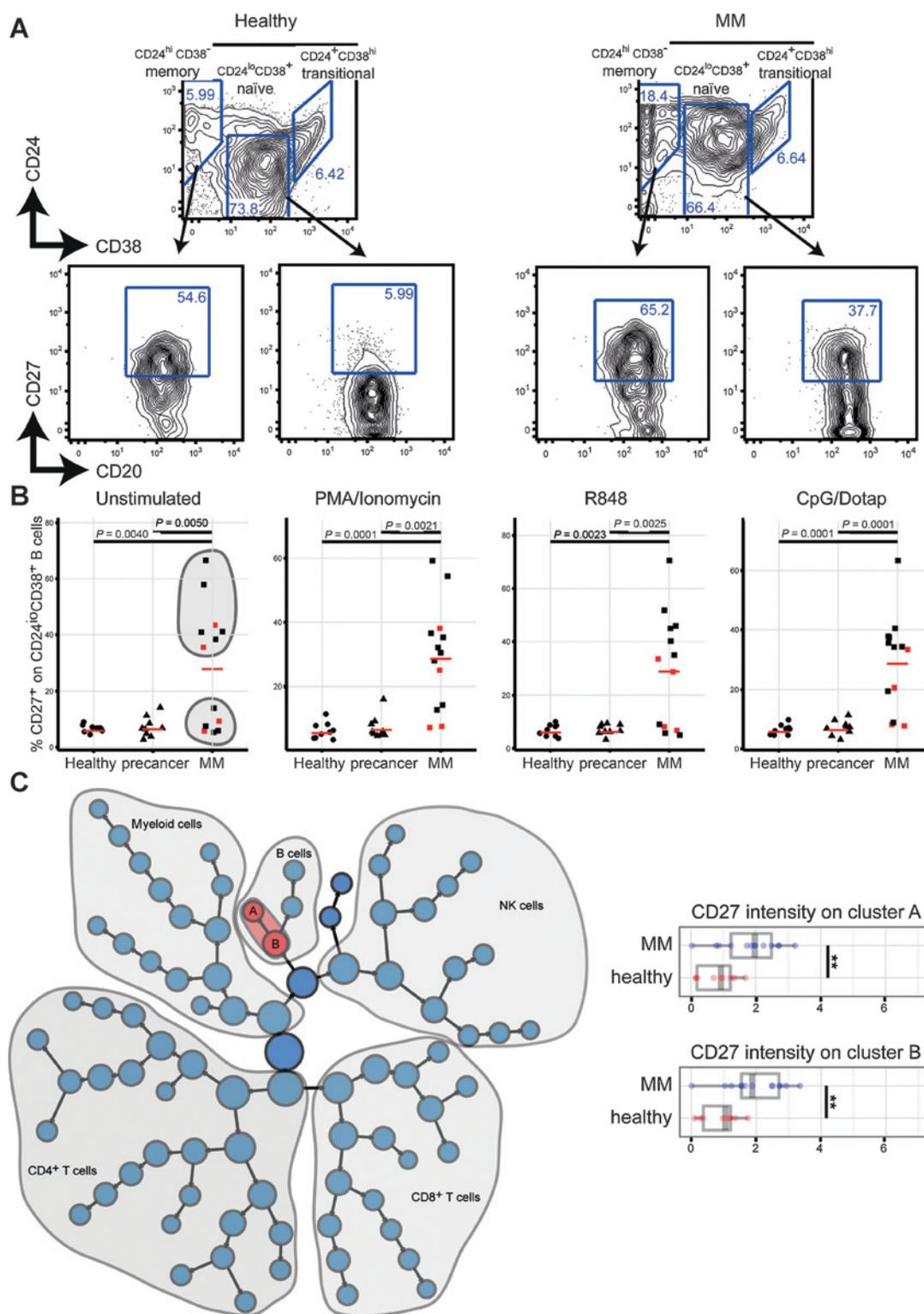
(CD28), and markers that have been associated with T-cell exhaustion (CD57), or homing (CCR7), both on CD4⁺ and CD8⁺ T cells (Fig. 1E). Surprisingly, CD19⁺CD20⁺ B cells with a distinct marker combination (CD24^{lo}CD38⁺) and expression of CD27 contributed to PC1 across different stimuli conditions (unstimulated and after PMA/ionomycin stimulation, Fig. 1E, in red). This population was particularly interesting because it combined two markers (CD24^{lo}CD38⁺) that have been associated with the naïve stage of B-cell differentiation (20), and CD27, which is associated with memory B cells (21). This is a novel phenotype that has not been reported previously.

CD24^{lo}CD38⁺CD27⁺ B cells are uniquely expanded in the peripheral blood of multiple myeloma patients and associated with active disease

On human peripheral blood B cells, CD24 and CD38 expression levels can be measured to identify memory (CD24⁺CD38⁻), naïve (CD24^{lo}CD38⁺), and transitional (CD24⁺CD38^{hi}) subsets (20).

As expected, the majority of CD24⁺CD38⁻ B cells expressed CD27 in the peripheral blood of all three groups (multiple myeloma, precancer, and healthy individuals), and we detected only minor amounts of CD24^{lo}CD38⁺ B cells expressing CD27 in precancer patients and healthy individuals (Fig. 2A and B). Strikingly, in the multiple myeloma samples, we identified an up to 13-fold expanded CD27⁺ population among the CD24^{lo}CD38⁺ B cells when compared with that in healthy controls (Fig. 2A and B). Notably, the expression of CD27 on CD24^{lo}CD38⁺ B cells in multiple myeloma samples showed a very broad range (5.3%–66.6%) with samples clustering at the high and low ends of this distribution, suggesting the existence of two patient populations (Fig. 2B, shaded in gray). This dichotomy was already noted in the PCA, where PC1 separated only a subset of multiple myeloma samples from precancer and healthy control samples (Fig. 1C). Nevertheless, the frequency of CD24^{lo}CD38⁺CD27⁺ B cells was significantly higher in multiple myeloma samples than in precancer samples ($P = 0.005$) and never exceeded 10% in

Hansmann et al.

**Figure 2.**

Expansion of CD24^{lo}CD38⁺CD27⁺ B cells in the peripheral blood of multiple myeloma (MM) patients. A, the two-dimensional B-cell phenotype characteristics in one multiple myeloma and one healthy control sample analyzed with CyTOF. The plots are pre-gated on CD19⁺ cells. CD27 gates were defined based on the CD27 expression in total CD19⁺ B cells. B, frequencies of CD19⁺CD20⁺CD24^{lo}CD38⁺CD27⁺ B cells in all samples included in the study analyzed with CyTOF. Red squares represent samples from patients who have received multiple myeloma-specific treatment in the past but not during the last 6 months. Red bars represent frequency means. Stimuli conditions are indicated at the top of the plot. C, citrus analysis comparing the expression of CD27 between 13 multiple myeloma and 9 healthy control samples of our CyTOF dataset. Highlighted in red are the nodes with significantly different CD27 expression (false discovery rate < 0.01), which is normalized and plotted for each of the two nodes (A and B) in the box plots in the right plot of the figure. **, $q < 0.01$.

healthy controls ($P = 0.004$; Fig. 2B, P values for unstimulated samples). *In vitro* stimulation showed only minor influences on CD27 expression on CD24^{lo}CD38⁺ B cells, suggesting that the observed phenotype is not due to transient activation. CD24^{lo}CD38⁺CD27⁺ B cells in multiple myeloma samples showed mixed characteristics of other B-cell development-associated markers: 15.3% \pm 9.5% expressed CD10; 27.5% \pm 18.3% were IgD⁺, and 63.1% \pm 27.3% expressed TNF α after PMA/ionomycin stimulation (all mean \pm SD; Supplementary Fig. S4). Notably, none of the patients were diagnosed with plasma cell leukemia or showed phenotypic characteristics of malignant or de-differentiated plasma cells in the CD24^{lo}CD38⁺ gate (Fig. 2A and Supplementary Fig. S5).

Given the 39-dimensional space of our CyTOF dataset, two-dimensional gating fails to capture the richness that comes from including all possible marker combinations. We therefore used a new analysis method, Citrus (19), for the identification of significantly different marker expressions on cell populations from high-dimensional data from multiple myeloma patients and healthy individuals (Fig. 2C and Supplementary Fig. S6). Among the different expression levels of CD57 on T-cell and NK-cell subsets (Supplementary Fig. S6B and S6C), Citrus detected significantly higher CD27 levels in two B-cell subsets (Fig. 2C). Cluster A (Fig. 2C) showed lower CD24 expression and therefore most likely represents the CD38⁺CD24^{lo} B-cell subset identified with two-dimensional gating and PCA.

Taken together, CyTOF data analyzed using two different methods enabled us to identify a uniquely expanded B-cell population with aberrant expression levels of CD24, CD38, and CD27 in the peripheral blood of many multiple myeloma patients (Fig. 2B). This population was not evident in MGUS or asymptomatic myeloma patients and healthy individuals.

We then verified these findings with flow cytometry, analyzing PBMCs from all multiple myeloma samples, which confirmed the expansion of the CD24^{lo}CD38⁺CD27⁺ B cells (Supplementary Fig. S7). In addition, we also analyzed IgA, IgM, and IgD expression levels on B-lineage cells, and serum levels of IgG, IgA, IgM, kappa, and lambda immunoglobulin light chains (Fig. 3 and Table 2).

We found that only a minority of this novel B-cell population expressed IgD (29.4% \pm 17.2% IgD⁺) or IgM (32.1% \pm 16.0% IgM⁺, both mean \pm SD), indicating that they largely represent a differentiation stage after class-switch recombination (Table 2). With 20.8% \pm 10.1% of this population expressing IgA, the majority can be assumed to be IgG⁺. Strikingly, the expansion of CD27⁺ B cells among CD24^{lo}CD38⁺ cells was associated with high serum levels of the patient's predominant Ig light chain and therefore might be useful as a measure of disease activity. Disease activity was also reflected in an either very high or very low K/ Λ ratio depending on the M protein type of the disease (Table 2).

Immunoglobulin genes of single CD24^{lo}CD38⁺CD27⁺ B cells from multiple myeloma patients are polyclonal and show somatic mutations

To analyze clonality and whether the CD24^{lo}CD38⁺CD27⁺ population is a memory or naive B-cell population, we sequenced the Ig genes from single CD24^{lo}CD38⁺CD27⁺IgD⁻IgM⁻IgA⁻ B cells from 2 multiple myeloma patients (sort gates in Fig. 4A).

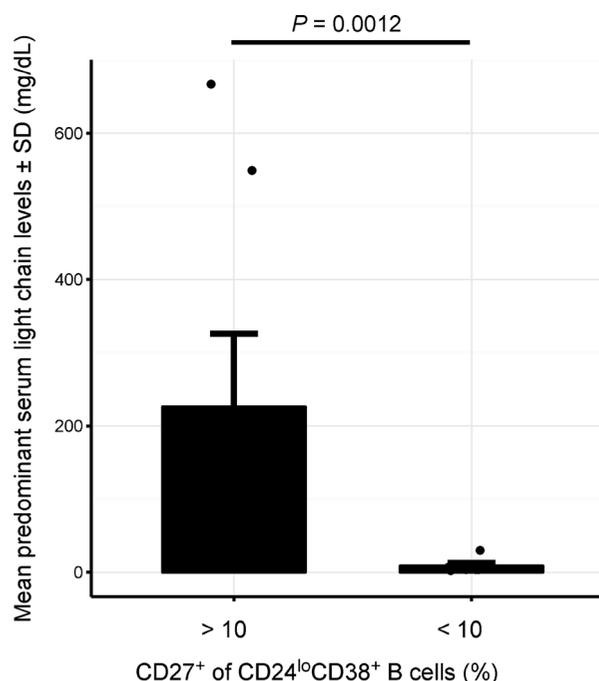


Figure 3. Association of CD24^{lo}CD38⁺CD27⁺ B-cell expansion with multiple myeloma activity. Elevated predominant serum light chain levels are associated with the expansion (>10%) of the CD27⁺ population within CD24^{lo}CD38⁺ B cells. The unpaired two-sided Wilcoxon rank-sum test was used to calculate the P value. Raw data for this figure are included in Table 2. The frequencies of CD24^{lo}CD38⁺CD27⁺ B cells and surface immunoglobulin expression were determined with FACS, and plots for each of the 13 multiple myeloma samples are shown in Supplementary Fig. S7.

In total, light chain sequences from 93 individual cells were sequenced and further analyzed. Both patients used a variety of different V region genes that partly overlapped between patients (Fig. 4B and C). The direct comparison of individual cells' Ig sequences within the same sample revealed 8 cells in subject 7 and 7 cells in subject 11 whose sequences occurred twice (Fig. 4D). Nevertheless, based on different Ig sequences and the variety of V regions used, the CD24^{lo}CD38⁺CD27⁺ B-cell compartment has to be considered polyclonal, although an underlying antigen-driven process responsible for the expansion of this B-cell subset is possible. Notably, there were no identical sequences across both patients.

We detected an average of 18 (subject 7) or 14 (subject 11) somatic mutations per V region, 5 (subject 7) and 3 (subject 11) of which were silent and 13 (subject 7) or 11 (subject 11) were nonsilent mutations. None of the V region sequences were germline in either patient (Fig. 4E), a further indication that this B-cell subset is a memory population.

To further strengthen the case that the investigated B-cell populations are not directly related to the malignant plasma-cell clone, we FACS-sorted bulk CD24^{lo}CD38⁺CD27⁺, CD24^{lo}CD38⁺CD27⁻IgM⁺, CD24^{hi}CD38⁻CD27⁺, and CD24^{hi}CD38⁻CD27⁻IgM⁻IgD⁻ B cells (Supplementary Fig. S8) from 1 patient whose malignant plasma-cell population contained the t(11;14). Sorted cells were analyzed with FISH, and the t(11;14) could not be detected in any of these populations (Supplementary Fig. S8).

Hansmann et al.

Table 2. Association of CD24^{lo}CD38⁺CD27⁺ B-cell expansion with multiple myeloma activity

Subject	% pos on CD24 ^{lo} CD38 ⁺ PB B cells			Serum levels (mg/dL)				% pos on CD24 ^{lo} CD38 ⁺ CD27 ⁺ PB B cells		
	CD27	K	Λ	K/Λ	IgG	IgA	IgM	IgA	IgM	IgD
3	5.2	3.9	1.2	1.9	1,410	58	23	3.0	41.9	41.4
4	2.2	3.7	1.0	3.7	688	61	37	8.2	56.1	56.5
6	48.5	1.9	667	0.0	1,160	4,800	96	17.1	13.6	12.4
7	5.4	1.6	2.2	0.9	1,250	526	121	20.2	32.4	36.1
8	6.1	7.3	0.6	12.1	342	3,690	15	26.7	34.7	33.3
10	32.7	549.0	0.2	2,745.0	528	24	<6	39.9	9.7	7.0
11	86.0	0.2	42.1	0.0	1,350	24	<6	34.7	28.5	14.9
18	56.3	85.7	1.0	85.7	3,580	20	25	21.7	20.6	19.3
19	68.0	98.1	0.2	490.5	397	16	10	18.2	30.3	25.2
20	4.7	30.3	2.5	12.1	992	1,060	22	23.8	44.9	45.5
21	5.6	3.6	1.1	3.3	3,060	82	39	10.9	57.1	56.5
25	78.2	41.6	1.9	21.9	1410	28	13	26.5	39.8	26.2
32	33.2	99.0	0.6	165	709	15	19	19.3	8.32	7.8

NOTE: Displayed are light chain and immunoglobulin serum concentrations and the percentages of CD24^{lo}CD38⁺CD27⁺ B cells that expressed the indicated immunoglobulin. FACS data for each individual sample are shown in Supplementary Fig. S7. Serum K and Λ levels of the predominant light chain are printed in bold. Clinical data for each subject can be found in Table 1.

CD24^{lo}CD38⁺CD27⁺ B cells show phosphorylation patterns similar to total CD27⁺ memory B cells after BCR and TLR9 stimulation

Aberrant intracellular signaling has already been demonstrated in cancer, especially in lymphoma B cells (22). We measured the phosphorylation of one BCR proximal (SYK) and two downstream (ERK and p38) signaling molecules in response to BCR stimulation or BCR-bypassing TLR9 stimulation. The BCR was stimulated with polyclonal anti-human IgG and IgM in the presence of H₂O₂. The addition of H₂O₂ can amplify early signaling events by inhibiting protein tyrosine phosphatase activity (23), thereby increasing signal strength. Phosphorylation levels in either unstimulated cells or after the addition of H₂O₂ will be referred to as baseline phosphorylation.

The quantification of signaling molecule phosphorylation with flow cytometry requires intracellular staining after PFA fixation and methanol permeabilization, which particularly affects CD38 PE-Cy7 staining. Because of this decrease in the CD38 signal, CD24^{lo}CD38⁺ and CD24^{hi}CD38⁻ B cells will be referred to as CD24^{lo} and CD24^{hi} B cells (Fig. 5A). CD24^{hi} and CD24^{lo} populations corresponded to CD24⁺CD38⁻ and CD24^{lo}CD38⁺ populations respectively after staining without fixation/permeabilization, as shown by the predominant expression of CD27 on CD24^{hi} (memory) B cells in healthy individuals after methanol permeabilization (Supplementary Fig. S9A). Phosphorylation responses to anti-human IgG/IgM or TLR9 ligand CpG ODN2006 stimulation were clearly distinguishable from baseline phosphorylation, as shown in Fig. 5B.

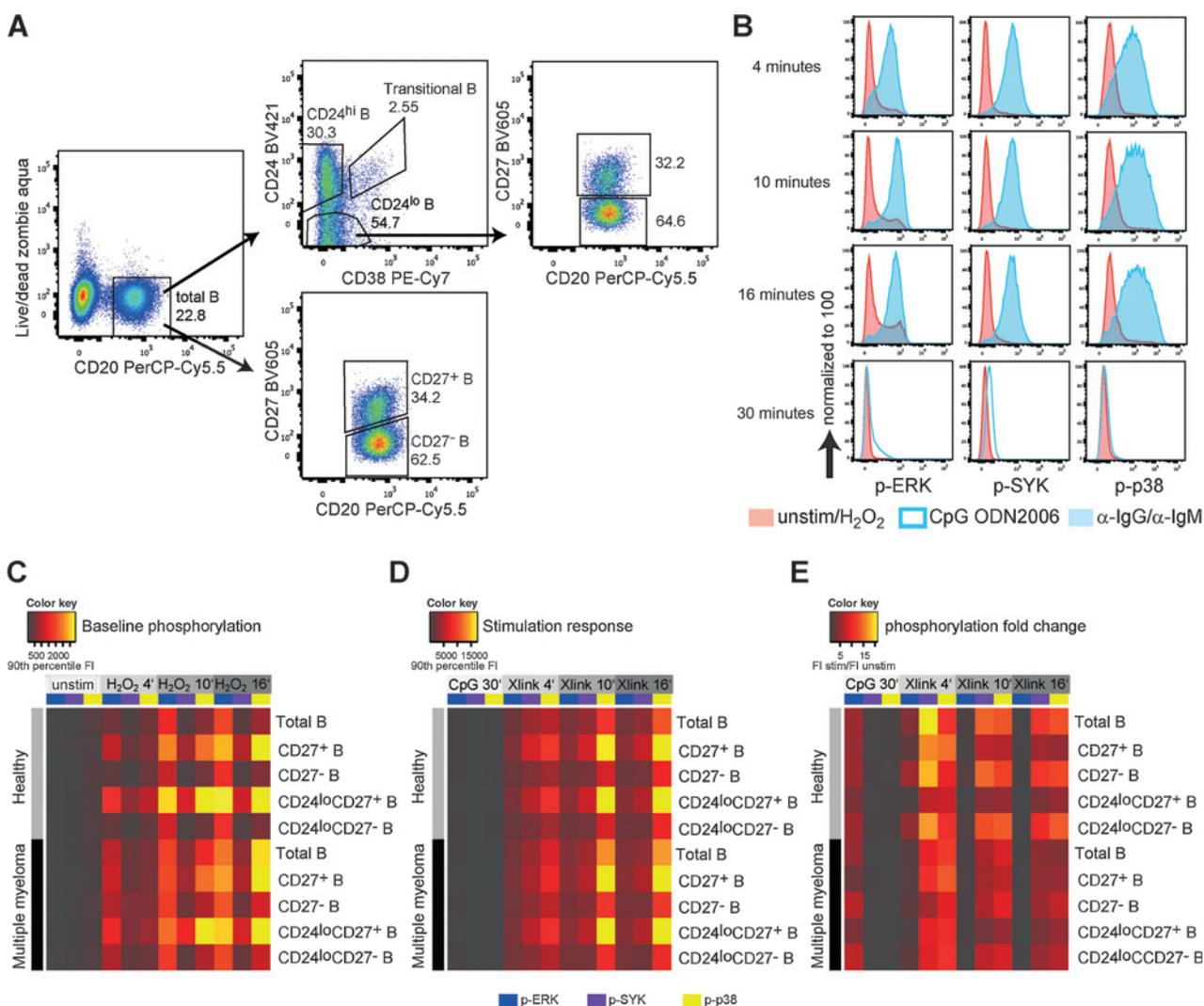
No significant phosphorylation was detectable at baseline in any subpopulations of unstimulated B cells. The addition of H₂O₂ resulted in slightly increased phosphorylation over time that was most pronounced in ERK and p38 (Fig. 5C). Interestingly, total CD27⁺ and CD24^{lo}CD27⁺ B cells showed very similar phosphorylation levels in both healthy individuals and PBMCs from multiple myeloma patients. Stimulation with CpG ODN2006 or anti-human IgG/IgM elicited up to 21-fold higher phosphorylation when compared with that of H₂O₂ controls (Fig. 5D and E). The unique phosphorylation patterns of CD27⁺, CD27⁻, and total B-cell populations were consistent at baseline and after stimulation, suggesting essentially different intracellular signaling responses in these subsets; however, significant differences

between healthy and multiple myeloma B-cell phosphorylation levels could be detected neither at baseline nor after stimulation. Overall, CpG ODN2006 stimulation caused mostly ERK phosphorylation, whereas BCR stimulation elicited predominantly SYK and p38 phosphorylation in the analyzed populations (Fig. 5E). The unique phosphorylation patterns of CD27⁺, CD27⁻, and total B-cell subsets at baseline and after BCR stimulation were confirmed in two additional sample pairs (Supplementary Fig. S9).

Discussion

In this study, we present high-dimensional cytometry data on the human immunologic landscape of peripheral blood cells across most of the known developmental stages of multiple myeloma (multiple myeloma, asymptomatic myeloma, MGUS, and healthy individuals). We manually determined the frequencies of all major PBMC subsets (αβ T, γδ T, B, NK, myeloid dendritic cells, plasmacytoid dendritic cells, and monocytes) and the expression of additional phenotypic and functional markers in four different stimulus conditions. With 434 two-dimensional initial gates per sample, to the best of our knowledge, this is the most detailed phenotypic and functional analysis of PBMC subsets in this disease. After removing mutually exclusive gates (e.g., from CD38⁺CD4⁺ and CD38⁻CD4⁺ T-cell gates, only the CD38⁺CD4⁺ was kept), 244 of these gates (listed in Supplementary Table S5) were used for statistical analyses and data visualization. Cell frequencies in each of these gates were compared between disease groups, one at a time, and using PCA. The manual comparisons allowed a very detailed view of particular cell subsets. Elevated numbers of CD57⁺CD45RA⁺CD8⁺ T cells (Supplementary Fig. S3A) could be expected given the previous work of Sze and colleagues (24); cells sharing this phenotype are considered to be clonally restricted and associated with a favorable disease outcome. In addition, we detected lower numbers of regulatory T cells (Treg) in the peripheral blood of multiple myeloma patients (Supplementary Fig. S3A). In multiple myeloma, the Treg/Th17 balance correlates with disease outcome (25, 26); however, differences in frequencies of IL17-producing cells between patients with multiple myeloma, precancer patients, and healthy individuals did not reach statistical significance, and Treg

Hansmann et al.

**Figure 5.**

Intracellular signaling in B-cell subsets of multiple myeloma patients and healthy individuals. A, FACS gating on different B-cell populations after stimulation, fixation, permeabilization, and intracellular staining. B, typical phosphorylation responses in total CD20⁺ B cells of one healthy individual measured with FACS. C–E, mean phosphorylation responses of multiple myeloma patients ($n = 3$) and sex- and age-matched healthy controls ($n = 3$). CpG, CpG ODN2006; FI, fluorescence intensity; Unstim, cells cultured without any additional additives for 30 minutes; Xlink, addition of anti-human IgG and anti-human IgM. For the calculation of fold changes, 90th percentile FI values of stimulated samples were divided by 90th percentile FI values of unstimulated or H₂O₂ samples.

low percentages in our study may therefore reflect an advanced stage of disease in our patients, most of whom were in International Staging System (ISS) stadium 3 (Table 1), and/or the limited sample size. Memory (CD20⁺CD27⁺) B-cell frequencies were also elevated (Supplementary Fig. S3A) as shown along with all other direct subset comparisons in Supplementary Fig. S3B.

The most striking finding was the selective expansion of CD24^{lo}CD38⁺CD27⁺ B cells in the peripheral blood of multiple myeloma patients with active disease as measured by serum immunoglobulin light chain levels. This B-cell phenotype has not been described before and attracted our attention for two reasons. First, because of a curiously mixed phenotype, in which, although CD24^{lo}CD38⁺ expression is a characteristic of naïve B cells, CD27 is currently one of the most well-established memory B cell markers and has been associated with the presence of somatic mutations in Ig genes (21, 29, 30). Second, this phenotype was

stable even after 6 hours of *in vitro* stimulation. CD24 expression on CD24^{lo}CD38⁺CD27⁺ B cells in multiple myeloma patients appeared to be higher than in their healthy counterparts, but was still lower than in CD24⁺CD38⁻ memory B cells (Fig. 2A). Even though this population shows variable CD24 expression (Supplementary Fig. S7), CD38 expression was clearly present while (CD27⁺) memory B cells have been shown to be CD38⁻ in various studies (20, 31).

Citrus is a new analysis tool that compares marker expression in individual multidimensional cell clusters between sample groups in a statistically rigorous fashion (19). Besides differences in CD27 expression on B-cell subsets (Fig. 2C), Citrus confirmed differences in CD57 expression on T-cell and NK-cell subsets (Supplementary Fig. S6B and S6C). Automated gating/clustering strategies confirmed our findings from data analyst-influenced manual gating with high statistical significance

(false discovery rate < 0.01), but did not add any categorically new findings. Thus, the two-dimensional analysis approach even in this 39-dimensional dataset was still able to reveal most of the significant differences between sample groups, although it could not address all marker combinations on every single cell. The advantages of Citrus are represented in the "unsupervised" nature of the approach and the enormous reduction of analysis time.

Multiple myeloma plasma cells are usually not highly proliferative, and the dividing multiple myeloma precursor pool is assumed to comprise post-germinal-center B cells (31–34), which could match the phenotype of CD24^{lo}CD38⁺CD27⁺ B cells (Supplementary Fig. S4). Yet, the existence and phenotype of clonotypic B cells in the peripheral blood of multiple myeloma patients is still a matter of debate (33, 35–37). Taking into account the phenotype of CD24^{lo}CD38⁺CD27⁺ B cells (Fig. 2A and Supplementary Fig. S5), the polyclonality of immunoglobulin genes, and the absence of multiple myeloma clone-specific translocations (Supplementary Fig. S8), this B-cell population is unlikely to be directly derived from or preceding the multiple myeloma plasma-cell population. The presence of somatic mutations in all single cells analyzed characterized this population as a memory-cell population, raising a question about the exclusive use of CD24 and CD38 for the identification of naïve and memory B cells. To determine whether CD24^{lo}CD38⁺CD27⁺ B cells can also be expanded in malignancies other than multiple myeloma, we analyzed peripheral blood samples from 5 colorectal cancer patients (Supplementary Fig. S10) and could not detect any significant expansion of this population. Therefore, we suggest that the polyclonal expansion of CD24^{lo}CD38⁺CD27⁺ B cells in multiple myeloma is most likely a phenomenon driven by active disease that results in some cues to which this B-cell type is responsive.

Earlier studies have shown aberrant intracellular signaling in different types of lymphoma B cells and that BCR signaling can be phenotype dependent (22, 38). Our intracellular signaling data indicated that the multiple myeloma B-cell compartments are not obviously different from their counterparts in healthy individuals on a functional level. In this context, CD24^{lo}CD38⁺CD27⁺ B cells behaved very similarly to "conventional" CD27⁺ B cells, further indicating that these cells are not in some unusual state. The

trigger for their expansion in multiple myeloma can be assumed in some cues exposed during active disease.

Disclosure of Potential Conflicts of Interest

W.H. Robinson is the founder, has ownership interest (including patents), and is a consultant/advisory board member for Atreca, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: L. Hansmann, C.-H. Ju, W.H. Robinson, M.M. Davis
Development of methodology: L. Hansmann, C.-H. Ju, W.H. Robinson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Hansmann, L. Blum, M. Liedtke
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Hansmann, L. Blum, W.H. Robinson, M.M. Davis
Writing, review, and/or revision of the manuscript: L. Hansmann, L. Blum, M. Liedtke, W.H. Robinson, M.M. Davis
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Hansmann, M.M. Davis
Study supervision: L. Hansmann, W.H. Robinson, M.M. Davis
Other (generation of figures): L. Hansmann

Acknowledgments

The authors thank Jonathan M. Irish (Vanderbilt University), William O'Gorman (now at Genentech), and Michael Leipold and Henrik Mei (at the Human Immune Monitoring Center, Stanford) for very helpful phosphoflow and CyTOF advice, Dana Bangs at Stanford Cytogenetics for help with FISH analyses, Cindy Kin for help with consenting patients, as well as Alessandra Aquilanti and Murad Mamedov for critically reading the article. CyTOF analysis was performed in the Human Immune Monitoring Center at Stanford, and single-cell sorting was performed in the Stanford Shared FACS Facility.

Grant Support

Single-cell sorting was performed in the Stanford Shared FACS Facility using NIH S10 Shared Instrument Grant (S10RR025518-01). L. Hansmann was supported by a research fellowship from the German Research Foundation (DFG, HA 6772/1-1). This research was funded by grants (to M.M. Davis) from the NIH (U19 AI00019) and the Howard Hughes Medical Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 12, 2014; revised February 9, 2015; accepted February 12, 2015; published OnlineFirst February 20, 2015.

References

1. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* 2013;39:782–95.
2. Olechnowicz SW, Edwards CM. Contributions of the host microenvironment to cancer-induced bone disease. *Cancer Res* 2014;74:1625–31.
3. Restifo NP. A "big data" view of the tumor "immunome". *Immunity* 2013;39:631–2.
4. Bendall SC, Simonds EF, Qiu P, Amir el AD, Krutzik PO, Finck R, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* 2011;332:687–96.
5. Newell EW, Sigal N, Bendall SC, Nolan GP, Davis MM. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8⁺ T cell phenotypes. *Immunity* 2012;36:142–52.
6. Newell EW, Sigal N, Nair N, Kidd BA, Greenberg HB, Davis MM. Combinatorial tetramer staining and mass cytometry analysis facilitate T-cell epitope mapping and characterization. *Nat Biotechnol* 2013;31:623–9.
7. Lu DR, Tan YC, Kongpachith S, Cai X, Stein EA, Lindstrom TM, et al. Identifying functional anti-Staphylococcus aureus antibodies by sequencing antibody repertoires of patient plasmablasts. *Clin Immunol* 2014; 152:77–89.
8. Tan YC, Blum LK, Kongpachith S, Ju CH, Cai X, Lindstrom TM, et al. High-throughput sequencing of natively paired antibody chains provides evidence for original antigenic sin shaping the antibody response to influenza vaccination. *Clin Immunol* 2014;151:55–65.
9. Zingone A, Kuehl WM. Pathogenesis of monoclonal gammopathy of undetermined significance and progression to multiple myeloma. *Semin Hematol* 2011;48:4–12.
10. Landgren O, Kyle RA, Pfeiffer RM, Katzmann JA, Caporaso NE, Hayes RB, et al. Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. *Blood* 2009;113:5412–7.
11. Weiss BM, Abadie J, Verma P, Howard RS, Kuehl WM. A monoclonal gammopathy precedes multiple myeloma in most patients. *Blood* 2009;113:5418–22.
12. Mitsiades CS, Mitsiades N, Munshi NC, Anderson KC. Focus on multiple myeloma. *Cancer Cell* 2004;6:439–44.
13. Mitsiades CS, Mitsiades NS, Richardson PG, Munshi NC, Anderson KC. Multiple myeloma: a prototypic disease model for the characterization and

Hansmann et al.

- therapeutic targeting of interactions between tumor cells and their local microenvironment. *J Cell Biochem* 2007;101:950–68.
14. Hideshima T, Nakamura N, Chauhan D, Anderson KC. Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. *Oncogene* 2001;20:5991–6000.
 15. de Haart SJ, van de Donk NW, Minnema MC, Huang JH, Aarts-Riemens T, Bovenschen N, et al. Accessory cells of the microenvironment protect multiple myeloma from T-cell cytotoxicity through cell adhesion-mediated immune resistance. *Clin Cancer Res* 2013;19:5591–601.
 16. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. *Blood* 2004;104:607–18.
 17. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Akiyama M, et al. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. *Cancer Cell* 2004;5:221–30.
 18. Team RC. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013.
 19. Bruggner RV, Bodenmiller B, Dill DL, Tibshirani RJ, Nolan GP. Automated identification of stratifying signatures in cellular subpopulations. *Proc Natl Acad Sci U S A* 2014;111:E2770–7.
 20. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. *Blood* 2005;105:4390–8.
 21. Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med* 1998;188:1679–89.
 22. Irish JM, Czerwinski DK, Nolan GP, Levy R. Altered B-cell receptor signaling kinetics distinguish human follicular lymphoma B cells from tumor-infiltrating nonmalignant B cells. *Blood* 2006;108:3135–42.
 23. Reth M. Hydrogen peroxide as second messenger in lymphocyte activation. *Nat Immunol* 2002;3:1129–34.
 24. Sze DM, Giesajtis G, Brown RD, Raitakari M, Gibson J, Ho J, et al. Clonal cytotoxic T cells are expanded in myeloma and reside in the CD8(+)CD57(+)CD28(–) compartment. *Blood* 2001;98:2817–27.
 25. Bryant C, Suen H, Brown R, Yang S, Favaloro J, Aklilu E, et al. Long-term survival in multiple myeloma is associated with a distinct immunological profile, which includes proliferative cytotoxic T-cell clones and a favourable Treg/Th17 balance. *Blood Cancer J* 2013;3:e148.
 26. Favaloro J, Brown R, Aklilu E, Yang S, Suen H, Hart D, et al. Myeloma skews regulatory T and pro-inflammatory T helper 17 cell balance in favor of a suppressive state. *Leuk Lymphoma* 2014;55:1090–8.
 27. Feng P, Yan R, Dai X, Xie X, Wen H, Yang S. The alteration and clinical significance of Th1/Th2/Th17/Treg cells in patients with multiple myeloma. *Inflammation* 2014;38:705–9.
 28. Kay NE, Leong T, Kyle RA, Greipp P, Billadeau D, Van Ness B, et al. Circulating blood B cells in multiple myeloma: analysis and relationship to circulating clonal cells and clinical parameters in a cohort of patients entered on the Eastern Cooperative Oncology Group phase III E9486 clinical trial. *Blood* 1997;90:340–5.
 29. Seifert M, Kuppers R. Molecular footprints of a germinal center derivation of human IgM+(IgD+)CD27+ B cells and the dynamics of memory B cell generation. *J Exp Med* 2009;206:2659–69.
 30. Agematsu K, Nagumo H, Yang FC, Nakazawa T, Fukushima K, Ito S, et al. B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. *Eur J Immunol* 1997;27:2073–9.
 31. Caraux A, Klein B, Paiva B, Bret C, Schmitz A, Fuhler GM, et al. Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138– and CD138+ plasma cells. *Haematologica* 2010;95:1016–20.
 32. Kukreja A, Hutchinson A, Dhodapkar K, Mazumder A, Vesole D, Angitapalli R, et al. Enhancement of clonogenicity of human multiple myeloma by dendritic cells. *J Exp Med* 2006;203:1859–65.
 33. Mahindra A, Hideshima T, Anderson KC. Multiple myeloma: biology of the disease. *Blood Rev* 2010;24 Suppl 1:S5–11.
 34. Matsui W, Wang Q, Barber JP, Brennan S, Smith BD, Borrello I, et al. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. *Cancer Res* 2008;68:190–7.
 35. Gonsalves WI, Rajkumar SV, Gupta V, Morice WG, Timm MM, Singh PP, et al. Quantification of clonal circulating plasma cells in newly diagnosed multiple myeloma: implications for redefining high-risk myeloma. *Leukemia* 2014;28:2060–5.
 36. Matsui W, Huff CA, Wang Q, Malehorn MT, Barber J, Tanhehco Y, et al. Characterization of clonogenic multiple myeloma cells. *Blood* 2004;103:2332–6.
 37. Thiago LS, Perez-Andres M, Balanzategui A, Sarasquete ME, Paiva B, Jara-Acevedo M, et al. Circulating clonotypic B-cells in multiple myeloma and monoclonal gammopathy of undetermined significance. *Haematologica* 2014;99:155–62.
 38. Irish JM, Czerwinski DK, Nolan GP, Levy R. Kinetics of B cell receptor signaling in human B cell subsets mapped by phosphospecific flow cytometry. *J Immunol* 2006;177:1581–9.

Cancer Immunology Research

Mass Cytometry Analysis Shows That a Novel Memory Phenotype B Cell Is Expanded in Multiple Myeloma

Leo Hansmann, Lisa Blum, Chia-Hsin Ju, et al.

Cancer Immunol Res 2015;3:650-660. Published OnlineFirst February 20, 2015.

Updated version Access the most recent version of this article at:
doi:[10.1158/2326-6066.CIR-14-0236-T](https://doi.org/10.1158/2326-6066.CIR-14-0236-T)

Supplementary Material Access the most recent supplemental material at:
<http://cancerimmunolres.aacrjournals.org/content/suppl/2015/02/20/2326-6066.CIR-14-0236-T.DC1.html>

Cited articles This article cites 37 articles, 19 of which you can access for free at:
<http://cancerimmunolres.aacrjournals.org/content/3/6/650.full.html#ref-list-1>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.