

1 **A targeted multi-omic analysis approach measures protein**
2 **expression and low abundance transcripts on the single cell level**

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24 human immunology

25 **Summary (150 words)**

26 High throughput single-cell RNA sequencing (sc-RNAseq) has become a frequently
27 used tool to assess immune cell function and heterogeneity. Recently, the combined
28 measurement of RNA and protein expression by sequencing was developed, which is
29 commonly known as CITE-Seq. Acquisition of protein expression data along with
30 transcriptome data resolves some of the limitations inherent to only assessing
31 transcript, but also nearly doubles the sequencing read depth required per single cell.
32 Furthermore, there is still a paucity of analysis tools to visualize combined transcript-
33 protein datasets.

34 Here, we describe a novel targeted transcriptomics approach that combines analysis
35 of over 400 genes with simultaneous measurement of over 40 proteins on more than
36 25,000 cells. This targeted approach requires only about 1/10 of the read depth
37 compared to a whole transcriptome approach while retaining high sensitivity for low
38 abundance transcripts. To analyze these multi-omic transcript-protein datasets, we
39 adapted One-SENSE for intuitive visualization of the relationship of proteins and
40 transcripts on a single-cell level.

41

42 **Introduction**

43 While pioneering work almost 20 years ago illustrated the ability to study the
44 transcriptome at the single-cell level (Chiang and Melton, 2003; Phillips and Eberwine,
45 1996), recent advances in microfluidics and reagents allow the high-throughput
46 analysis of transcripts of 10^4 single cells in one experiment (Jaitin et al., 2014; Klein et
47 al., 2015; Macosko et al., 2015). Although several methods have been developed for
48 this purpose, currently the most widely adopted platform is a droplet-based
49 microfluidic system commercialized by 10x Genomics (Zheng et al., 2017).

50 Though analysis of transcript expression on the single cell level is a powerful tool to
51 characterize the relationship and functional properties of cells, it is imperative to
52 consider the relationship between transcript and protein when trying to extrapolate
53 biology. Typically, transcripts are expressed at a much lower level than proteins – for
54 example, murine liver cells have a median copy number of 43,100 protein molecules
55 but only 3.7 RNA molecules per gene (Azimifar et al., 2014). Similarly, the dynamic
56 range of expression is much greater for proteins with copy numbers spanning about 6-
57 7 orders of magnitude while transcript copy numbers span about 2 orders of
58 magnitude (Schwanhausser et al., 2011). Finally, the correlation of gene expression and
59 protein expression has been estimated to have a Pearson correlation coefficient
60 between 0.4 (Schwanhausser et al., 2011) and 0.6 (Azimifar et al., 2014). These
61 discrepancies in transcript and protein expression patterns are relevant for the
62 biological interpretation of single cell transcriptome data, but also pose analytical
63 challenges. Suitable approaches are required to visualize the data despite the
64 pronounced differences in abundance and dynamic range of expression.

65 The parallel measurement of transcript and protein phenotype by sequencing has been
66 recently reported as cellular indexing of transcriptomes and epitopes (CITE-seq)
67 (Stoeckius et al., 2017) or RNA expression and protein sequencing (REAP-seq)
68 (Peterson et al., 2017). These technologies leverage existing sc-RNAseq platforms that
69 use an unbiased whole transcriptome (WTA) detection approach capturing cellular
70 mRNA via its poly-A tail, and utilize oligonucleotide-labelled antibodies (carrying unique
71 barcodes and also a poly-A tail) to interrogate surface protein abundance. Typically,
72 current droplet-based WTA approaches result in the detection of ~1000 unique
73 transcripts per single cell for the transcriptome (with a substantial fraction of these
74 transcripts encoding ribosomal proteins), while antibody panels of up to 80 targets
75 have been reported (Peterson et al., 2017).

76 Though proof-of-principle for this technology has been established, it remains unclear
77 how the sequencing-based antibody detection compares to established flow
78 cytometry-based assays in different experimental settings with regards to capturing the
79 dynamic range of protein expression and identifying low abundance protein
80 expression. In addition, the combined WTA plus protein approach can quickly become
81 resource intensive. Finally, droplet-based WTA pipelines may still miss specific
82 transcripts of interest if they are below the limit of detection, with current high
83 throughput chemistries capturing an estimated 10% of the total cellular mRNA (Zheng
84 et al., 2017).

85 Here, we report using a high throughput ($>10^4$ single cells) targeted transcriptomic
86 approach employing nanowells to capture single cells (Rhapsody platform,
87 commercialized by BD Biosciences) (Fan et al., 2015) in combination with

88 oligonucleotide-barcoded antibodies (termed AbSeq). Specifically, we simultaneously
89 interrogated over 400 immune-related genes and 41 surface proteins that are
90 commonly used for immunophenotyping. We found that this targeted approach was
91 efficient at detecting low-abundance transcripts while only requiring about 1/10 of the
92 sequencing read depth needed for WTA, indicating that targeted transcriptomics is a
93 sensitive and cost-efficient alternative when the focus is on interrogating defined
94 transcripts. Of note, this approach clearly separated different memory T cell subsets as
95 well as regulatory T cells (Tregs) solely based on transcript information, which is often
96 difficult due to the low amount of RNA recovered from T lymphocytes (Zheng et al.,
97 2017). Furthermore, we used 30-parameter fluorescent-based flow cytometry to
98 measure the same proteins targets as in the multi-omic assay. Our data indicate that
99 the validation of oligonucleotide-barcoded antibody panels is necessary for meaningful
100 interpretation of the multi-omic data.

101 To demonstrate the sensitivity and robustness of the system we analyzed T and NK
102 cells before and after one hour of stimulation, revealing an unexpected disconnect in
103 transcript and surface expression levels of the commonly used early activation marker
104 CD69. Analysis of chemokine expression showed distinct phenotypes within the CD8⁺
105 T cell population as early as 60 minutes after stimulation, suggesting significant
106 heterogeneity within this compartment.

107 Finally, to visualize protein and transcriptome data in an intuitive single plot, we
108 adapted One-SENSE, which was originally developed for visualization of mass
109 cytometry data (Cheng et al., 2016). This adaptation allows for effective visualization
110 and identification of cellular phenotypes that differ either by transcript or by protein.

111 Overall, we provide a methodological toolset for generating high throughput multi-omic
112 single cell data with a focus on maximizing target transcript sensitivity at minimal read
113 depth and an analytical tool to visualize these protein and transcript datasets.

114

115

116 **Results**

117 *Comparison of oligonucleotide-labelled antibody probes to high-dimensional flow* 118 *cytometry*

119 For our reference data set we obtained peripheral blood mononuclear cells (PBMCs)
120 from three healthy control subjects carrying the HLA-A*02:01 allele, which allowed
121 isolation of EBV-specific CD8⁺ T cells using an EBV-Tetramer reagent (Dunne et al.,
122 2002). To ensure sufficient cell numbers of these rare, antigen-specific T cells, we
123 enriched tetramer-positive T cells by fluorescence-activated cell sorting (FACS). In
124 parallel, we sorted CD45⁺ live leukocytes from PBMCs (Figure 1A). Moreover, to
125 minimize batch effects during subsequent staining with 41 oligo-nucleotide labelled
126 antibodies (Figure 1B), we utilized a multiplexing protocol using barcoded cell-hashing
127 antibodies (Stoeckius et al., 2018). All samples were processed simultaneously using
128 the Rhapsody platform, a nano-well based cartridge system (Fan et al., 2015) for
129 single-cell RNA sequencing with a targeted approach focusing on 490 immune-
130 relevant transcripts (all targets are listed in Suppl Table 1). Following quality control
131 and removal of multiplets, we recovered 27,258 cells from the sequencing data, which
132 were evenly distributed across the three different donors.

133 First, we wanted to assess whether the surface protein phenotypes as defined by
134 sequencing match known biology. For this, we designed two optimized 30-parameter
135 immunophenotyping panels (adapted from (Mair and Prlic, 2018)) covering the same 41
136 protein targets in an overlapping fashion. We used these panels to stain whole
137 unsorted PBMC samples from the same 3 donors, down-sampled the cytometry data
138 to 27,000 cells and used biaxial gating to identify the main immune lineages of the

139 myeloid compartment (Figure 1C) as well as the lymphoid compartment (Figure 1D). All
140 populations were present at comparable frequencies in the two different data sets
141 (Figure 1E and Figure 1F), with myeloid cells showing slightly lower abundance due to
142 the sorting procedure required to enrich EBV-Tetramer⁺ cells as well as CD45⁺ live
143 cells. Of note, even low-abundance cell populations such as CD1c⁺ conventional
144 dendritic cells (cDCs) and crosspresenting CD141⁺ cDCs were clearly identified by their
145 surface protein phenotype. Furthermore, the oligonucleotide-labelled antibodies
146 allowed to discriminate the CD45 splice variants CD45RO and CD45RA, which cannot
147 be distinguished by 3' transcriptomic analysis alone.
148 However, for the anti-TCR $\gamma\delta$ reagent we used, discordant patterns were observed
149 when comparing the expression within CD3⁺ T cells to conventional flow cytometry
150 (Supplementary Figure 1A). This was not immediately evident when visualizing the data
151 on a heatmap (Supplementary Figure 1B), emphasizing the need for careful reagent
152 validation for sequencing-based protein measurements. Thus, we did not analyze $\gamma\delta$ T
153 cells separately for the rest of our study. Furthermore, the CCR7 reagent delivered sub-
154 optimal but usable resolution (data not shown).

155
156 *Targeted transcriptomics faithfully captures cellular heterogeneity similar to whole*
157 *transcriptome approaches at lower read depths*

158 Next, we wanted to assess how well a targeted transcriptomics approach can identify
159 immune cell heterogeneity compared to a commonly used whole transcriptome (WTA)
160 pipeline. For this, we used a single donor and compared the resulting populations after
161 graph-based-clustering of the transcript data using the R package Seurat

162 implementation of PhenoGraph at standard resolution settings (Butler et al., 2018;
163 Levine et al., 2015) (Figure 2A and Suppl. Figure 2A and 2B). For visualization, we used
164 uniform manifold approximation and projection (UMAP), a dimensionality reduction
165 approach that has recently been adopted for single-cell data (Becht et al., 2018;
166 McInnes et al., 2018). Overall, the targeted transcriptomic approach utilizing 490 genes
167 revealed similar or even improved resolution of known immune subsets in the
168 peripheral blood. In particular, CD4⁺ T cells and CD8⁺ T cells separated well, and we
169 observed regulatory T cells (Tregs) expressing FOXP3 and CTLA4 as a separate cluster
170 (Figure 2B). For verification of this Treg cluster, we utilized the corresponding protein
171 signature, which showed high expression of CD25, and low expression of CD127
172 (Figure 2C). Next, we compared the gene expression for four phenotypically similar
173 clusters in the WTA and the targeted transcriptomics data set, showing very similar
174 patterns for the top differentially expressed genes (Suppl. Figure 2B). To obtain a
175 relative measure of detection efficiency, we calculated the average number of
176 transcripts per cell both for the targeted transcriptomics as well as the WTA data set
177 from the same donor. Around 75% of the assayed genes showed equal or slightly
178 superior detection efficiencies (Figure 2D), suggesting that targeted transcriptomics
179 can deliver valuable information at relatively low sequencing cost (i.e. approximately
180 2500 reads/cell).

181 Finally, to directly assess the effect of different read-depths on resolution of protein
182 and transcript signals, we analyzed a different donor to a total of approximately 27,000
183 reads/cell (approximately 18,000 reads/cell for the antibody library, 9,000 reads/cell for
184 the transcript library) and subsampled the number of reads used during processing of

185 the raw data to 20% (approximately 4000 reads/cell for antibody library, 2000
186 reads/cell for transcript library) and 10%. Visualization of the resulting clusters on a
187 UMAP plot as well as the top-differentially expressed genes on a heatmap revealed no
188 major differences between using 100% or 20% of the reads (Supplementary Figure
189 2C). For the protein signal, the same was observed, while using only 10% of the reads
190 resulted in noticeable loss of signal intensities (Supplementary Figure 2D). Overall, we
191 conclude that using at least 2000 reads/cell for the transcript portion of the library and
192 at least 200 reads/antibody/cell for the antibody portion of the antibody library delivers
193 sufficient resolution.

194

195 *Multi-omic analysis identifies canonical memory T cell populations and allows the study*
196 *of rare-antigen specific CD8⁺ T cells*

197 To test the value of multi-omic single cell analysis on a specific subset of the immune
198 compartment, we performed an in-depth analysis of the CD8⁺ T cell compartment.
199 First, we visualized protein and RNA data collected from total CD45⁺ live cells from
200 PBMCs from three patents on separate UMAP plots (Fig 1A). We found that cells from
201 different donors comingled and separated by cell type rather than by donor,
202 suggesting that batch effect across donors was minimal (Figure 3A). Of note, protein
203 information overlayed on the transcript-generated UMAP plot allowed accurate
204 identification of all main immune clusters (Figure 3B), which is not necessarily the case
205 when using transcript information for the corresponding lineage markers. This is
206 exemplified by biaxial plots showing protein signal on the y-axis and transcript signal
207 on the x-axis (Figure 3C): While for CD8A, transcript and protein are co-expressed in

208 most cells, only half of the CD4-protein⁺ (throughout the manuscript abbreviated as
209 CD4-P) cells contained detectable CD4-transcript. In turn, there were other molecules
210 of interest where the inverse was observed: CD69-RNA (plotted on the x-axis) was
211 detected across a large number of T cells, but as expected only few T cells in the
212 peripheral blood express CD69 protein (CD69-P, plotted on y-axis) on their surface.
213 For CD27, we observed a higher correlation between transcript and protein (Figure 3C).
214 Overall, these observations emphasize the importance of parallel measurement of
215 protein and transcript to faithfully study T cell biology.

216 Next, we continued our analysis of CD3⁺CD4⁻CD8⁺ T cells as defined by surface protein
217 expression using SCAMP (Selected Clustering Annotated using Modes of Projections)
218 (Greene et al., 2018). Unbiased graph-based clustering using transcript information
219 suggested the presence of 5 distinct cellular clusters (Figure 3D). Visualization of the
220 top differentially expressed genes such as SELL (encoding CD62L), CCR7 and GZMB
221 suggested that these 5 clusters reflect canonical naïve and memory T cell populations
222 (Sallusto et al., 1999) (Figure 3E). Additionally, our data allowed identification of CD8⁺
223 mucosal associated invariant T (MAIT) cells, which express high levels of IL18RAP and
224 TNF (Slichter et al., 2016) (Mori et al., 2016). We confirmed the resemblance of these
225 populations by surface protein expression (Figure 3F), with central memory CD8⁺ T
226 cells expressing low levels of CD45RA-protein, and high CD27- and CD28-protein
227 (Sallusto et al., 2004) (Hamann et al., 1997). Of note, the splice variants CD45RO and
228 CD45RA cannot be distinguished by analyzing transcript alone, highlighting the added
229 value of combined protein and transcript analysis.

230 To visualize the correspondence between transcript and protein expression in the
231 multi-omic data set, we adopted One-SENSE, which has originally been developed for
232 biologically meaningful visualization of mass cytometry data (Cheng et al., 2016). For
233 this, we mapped cells separately by proteins and transcripts each on to a single UMAP
234 dimension, similar to a recently published 1D t-stochastic neighbor embedding (t-SNE)
235 representation for sc-RNA sequencing data (Linderman et al., 2019). The combined
236 plot shows the overall distribution of protein expression profiles in the x-axis and the
237 top-differentially expressed gene profiles on the y-axis. Aligned heatmaps that
238 represent median expression with bins of cells are provided to annotate the one-
239 dimensional UMAP protein and gene expression profiles. This approach allows easy
240 identification of cellular clusters that are similar by transcript, but separated by protein,
241 and vice versa (Figure 3G). One example for this is highlighted in Figure 3G (red box
242 and arrow), where cluster 2 (light green, containing TEMRA cells) is relatively
243 homogenous by transcript, but can be separated by CD56 protein expression,
244 probably marking some NKT cells. In turn, a fraction of cells between cluster 1 (dark
245 blue, effector memory CD8⁺ T cells) and 2 (green, TEMRA) shares the same protein
246 signature, but can be distinguished by GNLY and GZMH expression (Fig. 3G, red box
247 and arrows). Varying degrees of concordance and ability to discriminate cellular
248 subsets from gene and protein expression profiles can be seen across this plot.

249 To determine if targeted transcriptomics is amenable for studying rare antigen-specific
250 T cell populations, we analyzed CD8⁺ T cells recognizing an EBV-epitope (Dunne et al.,
251 2002). Visualization on the UMAP plot revealed remarkable similarity of EBV-specific T
252 cells across all three donors (Figure 3H). As expected, most of the cells grouped within

253 the effector memory CD8⁺ T cell cluster. However, relative to the EBV-nonspecific
254 memory T cell cluster the EBV-Tet⁺ T cells showed a significant downregulation of the
255 effector molecule Granulysin, and an upregulation of YBX3, an RNA binding protein
256 whose function has not been defined in T cells, but has recently been shown to be a
257 critical regulator for the stability of specific mRNAs (Cooke et al., 2019).
258 Overall, this data show that combining targeted transcriptomics and protein
259 phenotyping by sequencing is a valuable approach for studying T cell subsets and
260 could be used a resource-efficient tool for studying T cell responses in human disease.

261

262 *Short-term stimulation of T and NK cells reveals chemokine heterogeneity and a*
263 *disconnect with the early activation marker CD69*

264 Cytokines and chemokines are the quintessential effector molecules of T cells, and the
265 existence of specific T cell subsets that are poised for the production of certain
266 cytokines has been the subject of intense research over the past decade (van den
267 Broek et al., 2018; Zhou et al., 2009). To test whether multi-omic single-cell analysis
268 can provide additional insight, we purified pan T cells together with NK cells and
269 stimulated them for one hour with Phorbol-Myristate-Acetate (PMA) and Ionomycin.
270 We probed early transcriptional changes with a T cell centric targeted transcriptomic
271 approach covering 259 genes. Transcripts encoding for IFNG, FASL and ICOS
272 exhibited robust upregulation in the stimulated versus unstimulated sample (Figure 4A),
273 as was the case for CD69, a commonly utilized protein marker for early T cell activation
274 (Figure 4B). Of note, when we analyzed cytokine expression relative to the surface
275 protein expression of CD69, we observed that both IFN γ as well as TNF transcript was

276 primarily expressed in CD69-transcript positive, but CD69-protein negative cells,
277 suggesting that during very early stages of activation, CD69 protein might not be an
278 ideal marker for T cell activation. However, FOSB, part of the transcription factor AP-1,
279 was co-expressed with CD69-protein (Figure 4B), suggesting a close relationship of
280 FOSB and CD69 expression.

281 We focused our further analysis on CD8⁺ T cells only, though our data set also contains
282 information on NK cells. Projection on a UMAP plot showed 8 discernable clusters that
283 were selected manually. Protein expression patterns for CD45RA and CD45RO
284 highlight the naïve and the memory T cells within this plot (Figure 4C). A heatmap
285 visualization of the most highly expressed transcripts show that these clusters are
286 defined by differential expression of CCL3, CCL4, IFNG, TNF, and various granzymes
287 (Figure 4D). Overall, this analysis reveals considerable functional diversity within the
288 CD8⁺ T cell compartment that is detectable as early as one hour after stimulation.

289

290 *Multi-omic analysis of the peripheral myeloid compartment reveals inflammatory*
291 *subsets not captured by surface protein phenotype*

292 Next, we wanted to assess whether the targeted transcriptomics approach can also be
293 used for other immune populations that are not as well studied as T cells. During the
294 past decade it has become evident that the myeloid cell compartment is complex in
295 terms of cellular heterogeneity (Guilliams et al., 2014; See et al., 2017; Villani et al.,
296 2017), and that commonly used bone-marrow derived differentiation protocols do not
297 faithfully capture the phenotype of myeloid cells *in vivo* (Guilliams and Malissen, 2016;
298 Helft et al., 2015). Thus, we tested how well targeted transcriptomics could dissect the

299 heterogeneity of the peripheral myeloid compartment. Unbiased clustering using
300 transcript suggested the presence of 5 different populations (Figure 5A), with clear
301 separation for CD14 and CD16 protein expression (Figure 5B). As expected,
302 visualization of the top differentially expressed genes (Figure 5C) as well as key surface
303 proteins (Figure 5D) mapped these clusters to CD123⁺ plasmacytoid dendritic cells
304 (pDCs), CD1c⁺ conventional DCs (cDC2s), CD16⁺ monocytes and CD14⁺ monocytes.
305 We used One-SENSE to further explore the relationship between cluster 0 and 1,
306 revealing that these two populations were very similar in terms of surface protein
307 profile (CD14⁺CD16⁻), but separated by a specific set of transcripts encoding for pro-
308 inflammatory cytokines and chemokines (Figure 5E). We confirmed that these
309 transcripts were part of differentially expressed genes as identified by MAST (Finak et
310 al., 2015), with higher expression in cluster 1 of CXCL3 and CCL4 (also known as MIP-
311 1b, a chemoattractant for natural killer cells) (Figure 5E). Thus, combining protein and
312 transcriptome data allowed us to observe multiple functional subsets within the
313 peripheral CD14⁺ myeloid population which were not apparent by surface marker
314 expression alone. In summary, this data highlights that targeted transcriptomics can be
315 used for exploratory studies of different immune compartments.

316

317

318 **Discussion**

319 Current efforts in the field of single cell analysis focus on the integrative measurement
320 of multiple modalities per cell. Ultimately, being able to analyze genome accessibility
321 status, transcript, regulatory RNAs and protein expression all together would allow a
322 holistic understanding of cellular function, but this has not been achieved yet (Stuart
323 and Satija, 2019). Arguably one of the most important steps on this trajectory has been
324 the ability to combine protein and transcript measurements by sequencing at the single
325 cell level using high-throughput methods (Peterson et al., 2017; Stoeckius et al., 2017).
326 However, with increased cell numbers, these combined measurements can quickly
327 become resource intensive, mostly due to the high number of sequencing reads that
328 are required per cell. Moreover, to fully leverage the advantage of multi-omic single-cell
329 analysis approaches, it is imperative to collect large cell numbers to adequately
330 represent low-abundance cellular populations such as antigen-specific T cells, or
331 antigen-presenting cells.

332 The targeted transcriptomic approach that we describe here provides an alternative
333 platform that significantly lowers the number of reads required for sequencing
334 saturation of transcript compared to whole transcriptome (WTA) approaches, but still
335 provides valuable information on up to 499 immune-centric genes. Though this
336 approach sacrifices the unbiased nature of WTA measurements, many immunological
337 applications center on a set of critical immune effector molecules, such as cytokines,
338 chemokines or transcription factors. Also, a targeted approach avoids the significant
339 number of reads used by transcripts encoding ribosomal proteins which are often also
340 captured using a poly-A based whole transcriptome workflow. Furthermore, as shown

341 here, in some cases, targeted analysis can permit higher sensitivity when it comes to
342 detecting relatively low abundance genes. Overall, in many experimental setups it
343 might be beneficial to combine both approaches: first utilize a WTA platform to identify
344 potentially unknown transcripts, and then use a targeted approach (which can be
345 tailored towards gene sets of interest) for profiling larger cell numbers or interrogating
346 cellular responses to specific stimuli. We provide proof-of-concept data that as early
347 as one hour after stimulation CD8⁺ T cells show heterogeneous patterns of chemokine
348 expression. Comprehensive chemokine and cytokine profiling of T cells after a very
349 short stimulus could be very valuable to gain additional insights into their function e.g.
350 in the context of cancer immunotherapy (Nagarsheth et al., 2017).

351 The decreased number of reads per cell required for targeted transcriptomics makes
352 the approach very suitable for combined profiling of transcript and protein for larger
353 number of cells. Doing so is particularly relevant in the context of T cell biology, where
354 well established T cell subsets, such as memory T cells and regulatory T cells (Tregs)
355 up to date have been difficult to resolve in some droplet-based sc-RNAseq studies
356 solely on the basis of transcript (Zheng et al., 2017). This has been attributed to the fact
357 that lymphocytes contain a relatively low amount of mRNA, which in combination with
358 the inherent drop-out rate of sc-RNAseq protocols fails to detect some low abundance
359 transcripts that are defining these cellular subsets (Stuart and Satija, 2019). This issue
360 can be alleviated by measuring surface protein markers such as the splice variants
361 CD45RA and CD45RO, which have been well studied in the context of naïve and
362 memory T cells, or the IL-2 receptor alpha chain (CD25) and IL-7 receptor (CD127) for
363 the distinction of Tregs. In addition, parallel measurement of surface protein

364 phenotypes allows to link novel cellular clusters (that are defined solely by transcript)
365 with a large body of literature that used to define cells by surface protein phenotype
366 only. Finally, the combined measurement approach can be useful to identify targets
367 with a significant disconnect between transcript and protein expression such as CD69,
368 probably indicative of active post-transcriptional modifications.

369 Of note, the development of novel technologies can sometimes outpace our ability to
370 validate platforms and reagents. Given that typical single cell sequencing experiments
371 require complex pre-processing steps and are often visualized using dimensionality
372 reduction techniques such as UMAP or t-SNE, there is a disconnect between the
373 actual raw data and the interpretation of final heatmaps. While this might be less of a
374 problem for transcript counts, antibody-based probes require careful validation. Here,
375 we have used high-dimensional cytometry, highlighting that not all reagents, even if the
376 same antibody clone is used, perform equally well in a multi-omic sequencing
377 experiment relative to conventional cytometry. Thus, with the more widespread
378 adoption of sequencing-based protein measurements, we argue that reagents need to
379 be carefully tested, preferably with parallel deposition in public databases.

380 Ultimately, to advance our understanding of biology the field relies on innovative
381 approaches to analyze and visualize complex high-dimensional data (Butler et al.,
382 2018; Cao et al., 2019; Stuart and Satija, 2019). Due to the different expression scales
383 this presents a challenge for combined protein-transcript data sets. To alleviate this
384 problem, we have adopted an analysis approach successfully used for high-
385 dimensional cytometry data, one-dimensional soli expression by nonlinear stochastic
386 embedding (One-SENSE) (Cheng et al., 2016). By visualizing the top-differentially

387 expressed genes in one dimension relative to the measured protein phenotypes this
388 method allows to easily dissect cells that are similar in transcript, but different in
389 surface phenotype, and vice versa. This will be a useful tool for biologists to explore
390 future multi-omic data sets to extract biological meaning from these complex multi-
391 dimensional data.

392

393

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403

404 **Author contributions**

405 F.M. and J.R.E. designed and performed experiments, analyzed data and wrote the
406 manuscript. V.V. analyzed data and provided critical input. Y.S. performed
407 experiments. T.B. analyzed data. A.J.T. and J.M. provided critical input. E.W.N., R.G.
408 and M.P. designed the study, analyzed data and co-wrote the manuscript.

409

410 **Conflict of interest**

411 A.J.T. and J.M. are employees of BD Biosciences (manuscript approval by BD
412 Biosciences was not required and BD Biosciences had no influence in regard to data
413 analysis, data interpretation and discussion). R.G. has received support from Juno
414 Therapeutics and Janssen Pharma, has consulted for Takeda Vaccines, Juno
415 Therapeutics and Infotech Soft, and has ownership interest in CellSpace Bio.

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418

419 **Figure Legends**

420

421 **Figure 1: Comparison of oligo-nucleotide antibody probes to high-dimensional**
422 **flow cytometry.**

423 (A) Schematic graph describing the workflow of the experiment. PBMC samples from
424 three donors were split in half, with one aliquot used for the multi-omic workflow, and
425 one aliquot used for flow cytometry phenotyping using two 30-parameter panels. (B)
426 Overview of antibody targets used in both the multi-omic and conventional flow
427 cytometry experiment. (C) Manual gating of main immune subsets using the combined
428 AbSeq data set (upper panel, red) and concatenated and down-sampled events
429 (27,000 cells) from the conventional (conv) flow cytometry data set (lower panel, blue).
430 (D) Manual gating of various T cell markers using the combined AbSeq data set (upper
431 panel, red) and concatenated, down-sampled events from the cytometry data set
432 (lower panel, blue). (E) Quantification of main immune subsets in the AbSeq and flow
433 cytometry data set across the three different donors. (F) Quantification of main T cell
434 populations and selected phenotyping markers in the AbSeq and flow cytometry data
435 set across the three different donors.

436

437 **Figure 2: Targeted transcriptomics faithfully captures cellular heterogeneity in**
438 **peripheral blood mononuclear cells.**

439 (A) Graph-based clustering of the transcript data from one representative donor is
440 shown on a UMAP (uniform manifold approximation projection) plot. Clusters have

441 been annotated by expression of key lineage genes. (B) The top 10-differentially
442 expressed genes for each cluster were identified using the Seurat implementation of
443 MAST (model-based analysis of single-cell transcriptomes) and visualized on a
444 heatmap after z-score normalization. Cluster names are shown in the same color
445 scheme as in (A). (C) Expression of the indicated transcripts and proteins on the three
446 different CD4⁺ T cell clusters, highlighting the CD25⁺ CD127^{low} Treg cluster. (D) Relative
447 detection ratio of all detected transcripts relative to a whole transcriptome data set
448 from the same donor. Genes are manually assigned into four different groups
449 according to their relative detection ratio.

450

451 **Figure 3: Multi-omic targeted transcriptomics identifies canonical memory T cell**
452 **populations and allows the study of rare-antigen specific CD8⁺ T cells**

453 (A) UMAP plots calculated on protein (left) or transcript (right) show that there is no
454 batch effect across the three donors analyzed. (B) Example UMAP plots (calculated on
455 transcript) representing the expression of the main immune lineage protein markers
456 which allow the unequivocal identification of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, and
457 CD14⁺ as well as CD16⁺ myeloid cells. (C) Example plots showing the poor correlation
458 of transcript and protein levels for CD4 and CD69, and good correlation for CD8 and
459 CD27. Protein signal is plotted on the y-axis, transcript on the x-axis. (D) UMAP plot
460 and graph-based clustering of the CD3⁺ CD8⁺CD4⁻ T cell compartment, revealing 5
461 distinct populations. (E) Examples of top differentially expressed genes identified by
462 MAST for each of the 5 clusters highlighted in (D). (F) Protein signatures of the 5
463 clusters identified canonical naive and memory CD8⁺ T cell subsets, including mucosal

464 associated invariant T cells (MAIT cells). (G) One-SENSE plot depicting protein
465 expression heatmap along the x-axis, and transcript expression heatmap of the top
466 differentially expressed genes along the y-axis. (H) Identification of EBV-specific CD8⁺
467 T cells relative to all CD8⁺ T cells, and expression pattern of two differentially
468 expressed genes between Tetramer-positive cells and Tetramer negative cells in the
469 effector memory cluster 1.

470

471 **Figure 4: Multi-omic analysis of the T and NK cell compartment 1 hour after**
472 **stimulation**

473 (A) Representative plots showing the upregulation of selected effector transcripts such
474 as IFNG, FASL and ICOS after stimulation (red) relative to unstimulated cells (blue). (B)
475 Disconnect between surface protein expression of the early activation marker CD69
476 and IFNG and TNF transcript within CD8-protein⁺ T cells. Blue overlay indicated
477 unstimulated cells, red stimulated cells. (C) UMAP plot of CD8-protein⁺ T cells with
478 manually identified clusters, and CD45RA and CD45RO protein expression. (D)
479 Heatmap showing the expression of key effector transcripts within the clusters
480 identified in (C).

481

482 **Figure 5: Combined protein and transcript phenotyping of the peripheral myeloid**
483 **compartment reveals inflammatory subsets not captured by surface protein**
484 **phenotype**

485 (A) UMAP plot and graph-based clustering of the peripheral non T/non NK/non B cell
486 compartment, revealing 5 distinct populations. (B) Heatmap overlay of CD14⁻ and

487 CD16-protein expression. (C) Heatmap of the top differentially expressed genes
488 identified by MAST for each of the 5 clusters highlighted in (A). (D) Protein signatures of
489 the 5 clusters identifies canonical CD123⁺ plasmacytoid DCs, CD1c⁺ conventional DCs
490 and CD16⁺ monocytes, but two of the clusters mapping to CD14⁺ monocytes. (E) One-
491 SENSE plot depicting protein expression heatmap along the x-axis, and transcript
492 expression heatmap of some of the top differentially expressed genes along the y-axis.
493 Red box and arrows are highlighting the differentially expressed genes between
494 cluster 0 and 1. (F) Violin plots showing key genes of the respective myeloid population
495 (upper panel) and differentially expressed genes between cluster 0 and 1, suggesting
496 the presence of an inflammatory subpopulation within CD14⁺ CD16⁻ monocytes that
497 expresses high levels of IL1B, TNF, CXCL3 and CCL4.

498

499 **Supplementary figure 1: Example for a poorly performing reagent**

500 (A) Manual gating of main immune subsets using the combined AbSeq data set (upper
501 panel, red) and concatenated and down-sampled events from the flow cytometry data
502 set (lower panel, blue), highlighting the population of $\gamma\delta$ T cells. (B) Heatmap overlay of
503 the TCRgd signal on a CD4 vs CD8 plot for the AbSeq data set (upper panel) and flow
504 cytometry data set (lower panel).

505

506 **Supplementary figure 2: Comparison of targeted transcriptomics to whole** 507 **transcriptome data (WTA) and assessment of required sequencing depth**

508 (A) Graph-based clustering of WTA data obtained from the same donor as in main
509 Figure 2. (B) Four of the clusters that matched most closely in terms of their expression

510 pattern were selected from both experiments and plotted using the top differentially
511 expressed genes obtained from the targeted transcriptomics approach. Heatmap
512 represents relative expression after z-score normalization. Left plot shows WTA data,
513 right plot shows targeted transcriptomic (cells obtained from the same donor). (C)
514 5,400 cells from a different donor were sequenced at a total depth of approximately
515 30,000 reads/cell. Upper panel depicts UMAP plot after graph-based clustering and a
516 heatmap of the top differentially expressed genes (z-score normalized expression) at
517 full read depth, lower panel using only 20% of the reads. Read depth per cell for the
518 transcript library is indicated on the right). Squared box on the UMAP plot indicates
519 one cluster that is separated as cluster 11 at full read depth, but pooled with cluster 8
520 at lower read-depth (D) Protein signals at the indicated read depths.

521

522 **STAR methods**

523

524 **Cells**

525 Peripheral blood mononuclear cells (PBMCs) were obtained as cryopreserved samples
526 from healthy controls (Seattle Area Control Cohort) via the HIV Vaccine Trial network
527 (HVTN). Vials with cryopreserved cells were thawed at 37°C until a tiny ice crystal was
528 left in the tube, and then carefully diluted in 1mL of pre-warmed RPMI with 10% FBS
529 and transferred to a new tube. An additional 13 mL of pre-warmed RPMI with 10%
530 FBS were added drop by drop, followed by centrifugation for 5 minutes at 400g and
531 resuspension in 1 mL of RPMI.

532

533 **Flow Cytometry and Cell sorting**

534 For flow cytometric analysis good practices were followed as outlined in the guidelines
535 for use of flow cytometry (Cossarizza et al., 2017). Following thawing, PBMCs were
536 incubated with Fc-blocking reagent (BioLegend Trustain FcX, #422302) and fixable UV
537 Blue Live/Dead reagent (ThermoFisher, #L34961) in PBS for 15 minutes at room
538 temperature. If required, cells were stained with an EBV-Tetramer reagent (peptide
539 YVLDHLIVV; Fred Hutch Immune Monitoring Core) diluted in FACS buffer (PBS with
540 2% FBS, Nucleus Biologics) for 30 minutes at room temperature, followed by two
541 washes. After this, cells were incubated for 20 minutes at room temperature with
542 antibody master mix freshly prepared in Brilliant staining buffer (BD Bioscience, #
543 563794), followed by two washes. All antibodies were titrated and used at optimal

544 dilution, and staining procedures were performed in 96-well round-bottom plates.
545 Stained cells were fixed with 4% PFA for 20 minutes at room temperature, washed,
546 resuspended in FACS buffer and stored at 4°C in the dark until acquisition.
547 All samples were acquired using a FACSymphony A5 (BD Biosciences), equipped with
548 30 detectors and 355nm, 405nm, 488nm, 532nm and 628nm lasers and FACSDiva (BD
549 Biosciences). Detector gains were optimized using a modified voltage titration
550 approach (Perfetto et al., 2012) and standardized from day to day using 6-peak Ultra
551 Rainbow Beads (Spherotec, # URCP-38-2K). Single-stained controls were prepared
552 with every experiment using antibody capture beads diluted in FACS buffer (BD
553 Biosciences anti-mouse, #552843 and anti-rat, #552844). After acquisition, data was
554 exported in FCS 3.1 format and analyzed using FlowJo (version 10.5.x, BD
555 Biosciences). Doublets were excluded by FSC-A vs FSC-H gating. For some of the
556 plots, the number of acquired cells was down-sampled using the appropriate FlowJo
557 plugin to match the number of cells analyzed by AbSeq.
558 All cell sorting was performed on a FACS Aria III (BD Biosciences), equipped with 20
559 detectors and 405nm, 488nm, 532nm and 628nm lasers. For all sorts, an 85 µm nozzle
560 operated at 45 psi sheath pressure was used. Cells were sorted into chilled Eppendorf
561 tubes containing 500 µL of RPMI, washed once in PBS and immediately used for
562 subsequent processing.

563

564 **Targeted Transcriptome and protein single-cell library preparation and**

565 **Sequencing**

566 CD45⁺ live PBMCs and EBV-tetramer⁺ CD8⁺ T cells were sequentially labeled using
567 Single Cell Labelling with the BD Single-Cell Multiplexing Kit and BD AbSeq Ab-Oligos
568 reagents strictly following the manufacturers protocol (BD Biosciences). Briefly, cells
569 from each donor or subtype of cells (after sorting) were labelled with sample tags
570 (Stoeckius et al., 2018). Each sample was washed twice with FACS buffer before
571 pooling all samples together. Pooled samples were washed one more time and then
572 stained with AbSeq Ab-Oligos (BD Biosciences). The pooled sample was then washed
573 twice, counted and resuspended in cold BD Sample Buffer (BD Biosciences) to achieve
574 approximately 20,000 cells in 620 μ l. Single cells from the pooled sample were isolated
575 using Single Cell Capture and cDNA Synthesis with the BD Rhapsody Express Single-
576 Cell Analysis System following the manufacturers protocol (BD Biosciences). After
577 priming the nanowell cartridges, the pooled sample was loaded onto two BD
578 Rhapsody cartridges and incubated at room temperature. Cell Capture Beads (BD
579 Biosciences) were prepared and then loaded onto the cartridge and incubated prior to
580 shaking at 1,000rpm at room temperature for 15 seconds on a ThermoMixer C
581 (Eppendorf). According to the manufacturers protocol, cartridges were washed, cells
582 were lysed, and Cell Capture Beads were retrieved and washed prior to performing
583 reverse transcription and treatment with Exonuclease I. cDNA Libraries were prepared
584 using mRNA Targeted, Sample Tag, and BD AbSeq Library Preparation with the BD
585 Rhapsody Targeted mRNA and AbSeq Amplification and BD Single-Cell Multiplexing
586 Kits and protocol (BD Biosciences). In brief, cDNA underwent targeted amplification
587 using the Human Immune Response Panel primers and a custom supplemental panel
588 (all targets are listed in Supplementary Table 1) via PCR (10 cycles). PCR products

589 were purified, and mRNA PCR products were separated from sample tag and AbSeq
590 products with double-sided size selection using SPRIselect magnetic beads (Beckman
591 Coulter). mRNA and Sample Tag products were further amplified using PCR (10
592 cycles). PCR products were then purified using SPRIselect magnetic beads. Quality
593 and quantity of PCR products were determined by using an Agilent 2200 TapeStation
594 with High Sensitivity D5000 ScreenTape (Agilent) in the Fred Hutch Genomics Shared
595 Resource laboratory. Targeted mRNA product was diluted to 2.5 ng/ μ L and sample tag
596 and AbSeq PCR products were diluted to 1 ng/ μ L to prepare final libraries. Final
597 libraries were indexed using PCR (6 cycles). Index PCR products were purified using
598 SPRIselect magnetic beads. Quality of final libraries was assessed by using Agilent
599 2200 TapeStation with High Sensitivity D5000 ScreenTape and quantified using a Qubit
600 Fluorometer using the Qubit dsDNA HS Kit (ThermoFisher). Final libraries were diluted
601 to 2nM and multiplexed for paired-end (150bp) sequencing on a HiSeq 2500 sequencer
602 (Illumina).

603

604 **Whole Transcriptome single-cell library preparation and sequencing**

605 cDNA libraries of CD45⁺ Live PBMCs were generated using the Chromium Single Cell
606 3' Reagent Kits v2 (10x Genomics) protocol targeting 5,000 cells in two separate wells.
607 Briefly, single cells were isolated into oil emulsion droplets with barcoded gel beads and
608 reverse transcriptase mix. cDNA was generated within these droplets, then the droplets
609 were dissociated. cDNA was purified using DynaBeads MyOne Silane magnetic beads
610 (ThermoFisher). cDNA amplification was performed by PCR (10 cycles) using reagents

611 within the Chromium Single Cell 3' Reagent Kit v2 (10x Genomics). Amplified cDNA
612 was purified using SPRIselect magnetic beads (Beckman Coulter). cDNA was
613 enzymatically fragmented and size selected prior to library construction. Libraries were
614 constructed by performing end repair, A-tailing, adaptor ligation, and PCR (12 cycles).
615 Quality of the libraries was assessed by using Agilent 2200 TapeStation with High
616 Sensitivity D5000 ScreenTape (Agilent). Quantity of libraries was assessed by
617 performing digital droplet PCR (ddPCR) with Library Quantification Kit for Illumina
618 TruSeq (BioRad). Libraries were diluted to 2nM and paired-end sequencing was
619 performed on a HiSeq 2500 sequencer (Illumina).

620

621 **Cell Ranger processing for WTA data**

622 Raw base call (BCL) files were demultiplexed to generate Fastq files using the
623 cellranger mkfastq pipeline within Cell Ranger 2.1.1 (10x Genomics). Targeted
624 transcriptome Fastqs were further analyzed via Seven Bridges (BD Biosciences). Whole
625 transcriptome Fastq files were processed using the standard cellranger pipeline (10x
626 genomics) within Cell Ranger 2.1.1. Briefly, cellranger count performs alignment,
627 filtering, barcode counting, and UMI counting. The cellranger count output was fed into
628 the cellranger aggr pipeline to normalize sequencing depth between samples. The final
629 output of cellranger (molecule per cell matrix) was then analyzed in R using the
630 package Seurat (version 2.3 and 3.0) as described below.

631

632 **Seven Bridges processing for targeted transcriptomics data**

633 Targeted transcriptomics Fastq files were processed via the standard Rhapsody
634 analysis pipeline (BD Biosciences) on Seven Bridges (www.sevenbridges.com). First,
635 R1 and R2 reads are filtered for high-quality reads, dropping reads that are too short
636 (less than 64 bases for R2) or have a base quality score of less than 20. Then, R1 reads
637 are annotated to identify cell label sequences and unique molecular identifiers (UMIs),
638 and R2 reads are mapped to the respective reference sequences using Bowtie2.
639 Finally, all valid R1 and R2 reads are combined and annotated to the respective
640 molecules. For all of our analysis, we utilized recursive substitution error correction
641 (RSEC) as well as distribution-based error correction (DBEC), which are manufacturer-
642 developed algorithms correcting for PCR and sequencing errors. For determining
643 putative cells (which will contain many more reads than noise cell labels), a filtering
644 algorithm takes the number of DBEC-corrected reads into account, calculating the
645 minimum second derivative along the cumulative reads as the cut-off point. Final
646 expression matrices contain DBEC-adjusted molecule counts in a CSV format. For
647 further analysis, these molecule count tables were read into the R package Seurat
648 (version 2.3 and 3.0) using customized scripts and analyzed as described below.

649

650 **Seurat workflow for targeted and WTA data**

651 The R package Seurat (Butler et al., 2018) was utilized for all downstream analysis. For
652 whole transcriptome data, cells that had at least 200 genes (with $\leq 20\%$ being
653 mitochondrial genes) were included in analysis. A natural log normalization using a
654 scale factor of 10,000 was performed across the library for each cell. UMIs and
655 mitochondrial genes (only for WTA data) were linearly scaled to remove these variables

656 as unwanted sources of variation. Dimensionality reduction using UMAP and clustering
657 was performed on a subset of variable genes. For targeted transcriptomics, no gene
658 per cell cutoffs were imposed, data were normalized with the same method. However,
659 when scaling data, UMI was the only regressed variable. Dimensionality reduction
660 using UMAP and clustering was based on either all genes or all proteins. For
661 differential gene expression analysis we utilized the Seurat implementation of MAST
662 (model-based analysis of single-cell transcriptomes) (Finak et al., 2015). For generation
663 of some FCS files the antibody molecule count tables were converted using the R
664 packages *premess* and *flowCore*. FCS-files with antibody molecule count signals
665 were analyzed in FlowJo 10.5.x (BD Biosciences) using either an arcsin transform or
666 biexponential transform. All the scripts used, listing the detailed parameters for each
667 step are available at https://github.com/MairFlo/Targeted_transcriptomics. Raw data
668 will be deposited on the NCBI gene expression Omnibus at
669 <https://www.ncbi.nlm.nih.gov/geo/>.

670

671 **Data processing for One-SENSE and generation of FCS files**

672 CSV files of raw counts were converted to FCS files using a script adapted
673 from <https://gist.github.com/yannabraham/c1f9de9b23fb94105ca5>. Raw counts were
674 normalized based on total counts per cell, then scaled to a value of 10,000 based on
675 the Seurat normalization algorithm. A natural log transformation was applied to gene
676 expression data, while protein expression data was randomized by adding a random
677 uniform distribution from 0 to 1, followed by transformation with the function
678 $\text{arcsinh}(x/5)$. Dimensionality reduction using UMAP was performed separately on all

679 genes and proteins to reduce them to one dimension before plotting. Cells were also
680 split into 500 bins of equivalent width based on one-dimensional UMAP data, then
681 used to generate heatmaps colored by median marker intensity per bin. All scripts
682 used for data processing and plot generation are available at
683 https://github.com/MairFlo/Targeted_transcriptomics.

684

685

686

687

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Figure 1

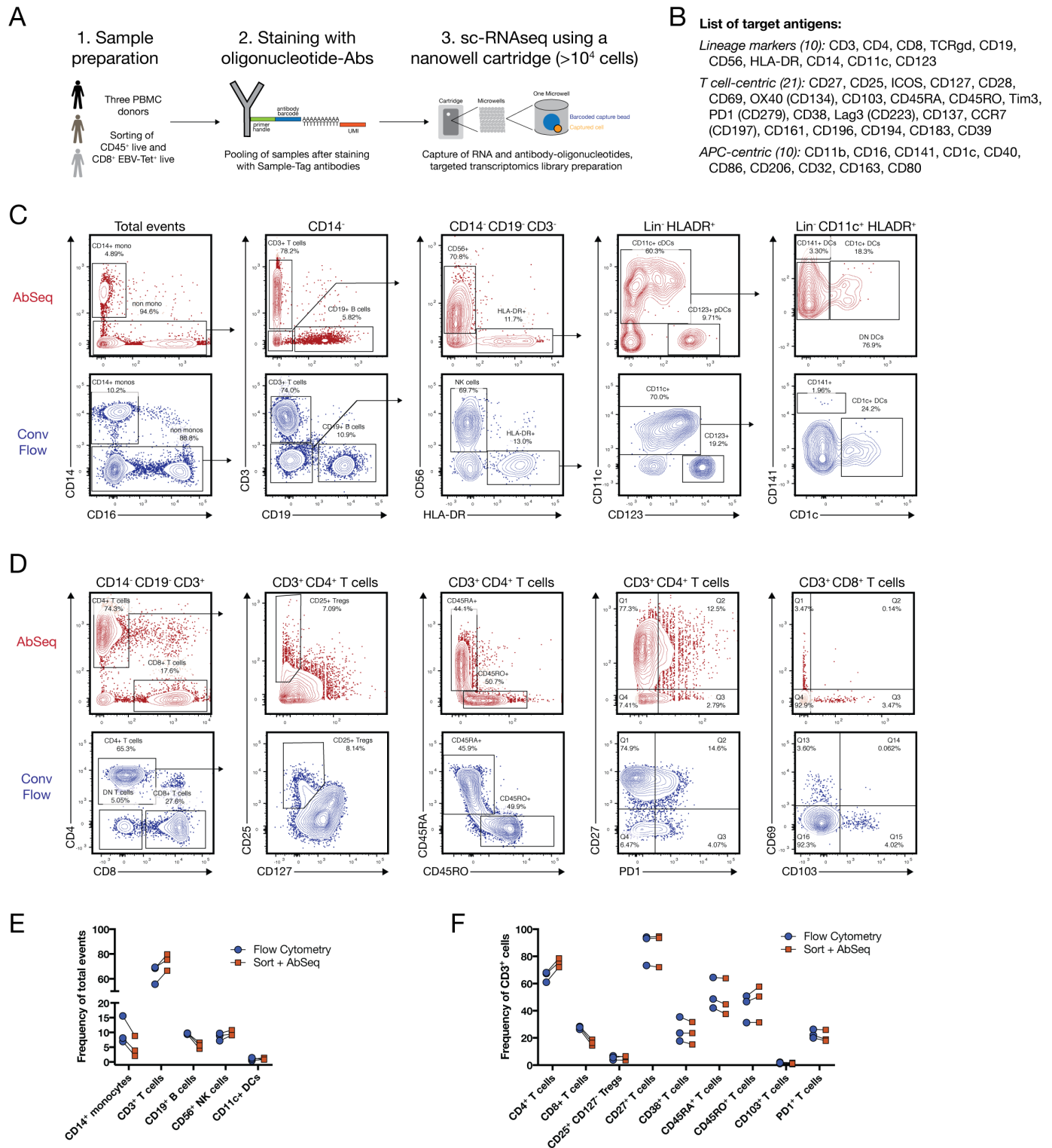


Figure 1: Comparison of oligo-nucleotide antibody probes to high-dimensional flow cytometry.

(A) Schematic graph describing the workflow of the experiment. PBMC samples from three donors were split in half, with one aliquot used for the multi-omic workflow, and one aliquot used for flow cytometry phenotyping using two 30-parameter panels.

(B) Overview of antibody targets used in both the multi-omic and conventional flow cytometry experiment.

(C) Manual gating of main immune subsets using the combined AbSeq data set (upper panel, red) and concatenated and down-sampled events (27,000 cells) from the conventional (conv) flow cytometry data set (lower panel, blue).

(D) Manual gating of various T cell markers using the combined AbSeq data set (upper panel, red) and concatenated, down-sampled events from the cytometry data set (lower panel, blue).

(E) Quantification of main immune subsets in the AbSeq and flow cytometry data set across the three different donors.

(F) Quantification of main T cell populations and selected phenotyping markers in the AbSeq and flow cytometry data set across the three different donors.

Figure 2

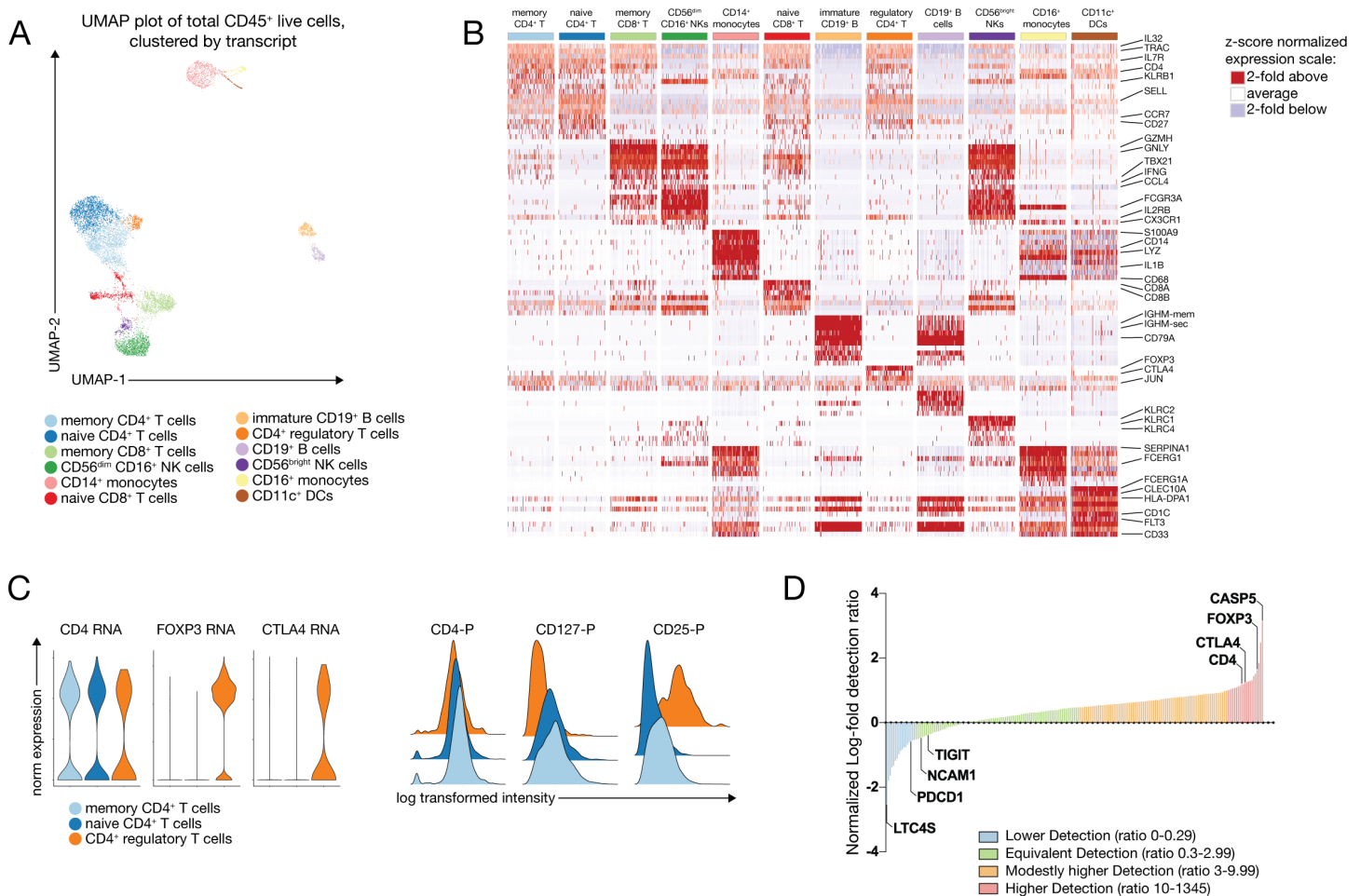


Figure 2: Targeted transcriptomics faithfully captures cellular heterogeneity in peripheral blood mononuclear cells.

(A) Graph-based clustering of the transcript data from one representative donor is shown on a UMAP (uniform manifold approximation projection) plot. Clusters have been annotated by expression of key lineage genes.

(B) The top 10-differentially expressed genes for each cluster were identified using the Seurat implementation of MAST (model-based analysis of single-cell transcriptomes) and visualized on a heatmap after z-score normalization. Cluster names are shown in the same color scheme as in (A).

(C) Expression of the indicated transcripts and proteins on the three different CD4⁺ T cell clusters, highlighting the CD25⁺ CD127^{low} Treg cluster.

(D) Relative detection ratio of all detected transcripts relative to a whole transcriptome data set from the same donor. Genes are manually assigned into four different groups according to their relative detection ratio.

Figure 3

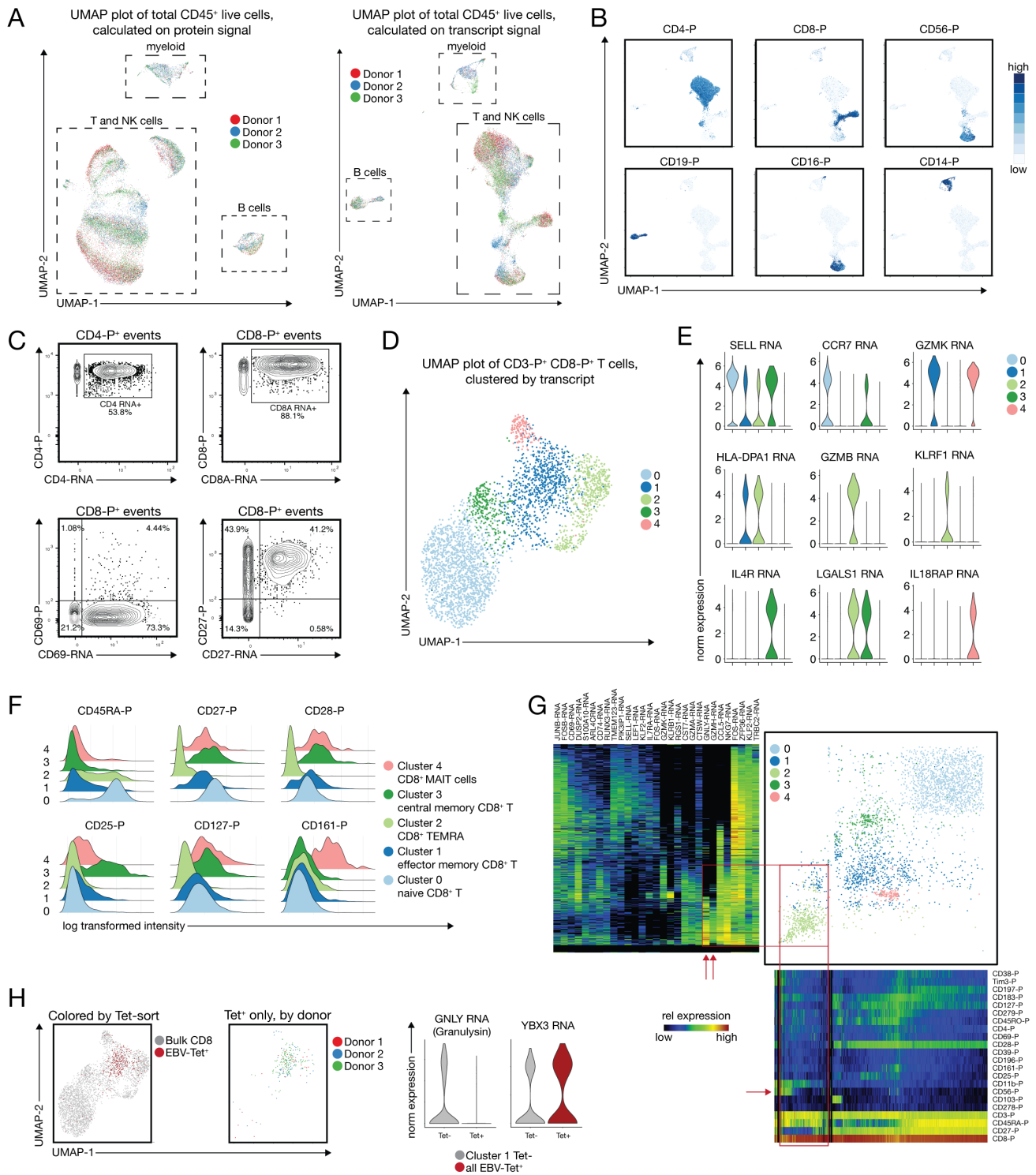


Figure 3: Multi-omic targeted transcriptomics identifies canonical memory T cell populations and allows the study of rare-antigen specific CD8⁺ T cells

(A) UMAP plots calculated on protein (left) or transcript (right) show that there is no batch effect across the three donors analyzed.

(B) Example UMAP plots (calculated on transcript) representing the expression of the main immune lineage protein markers which allow the unequivocal identification of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, and CD14⁺ as well as CD16⁺ myeloid cells.

(C) Example plots showing the poor correlation of transcript and protein levels for CD4 and CD69, and good correlation for CD8 and CD27. Protein signal is plotted on the y-axis, transcript on the x-axis.

(D) UMAP plot and graph-based clustering of the CD3⁺ CD8⁺ CD4⁻ T cell compartment, revealing 5 distinct populations. (E) Examples of top differentially expressed genes identified by MAST for each of the 5 clusters highlighted in (D).

(F) Protein signatures of the 5 clusters identified canonical naive and memory CD8⁺ T cell subsets, including mucosal associated invariant T cells (MAIT cells).

(G) One-SENSE plot depicting protein expression heatmap along the x-axis, and transcript expression heatmap of the top differentially expressed genes along the y-axis.

(H) Identification of EBV-specific CD8⁺ T cells relative to all CD8⁺ T cells, and expression pattern of two differentially expressed genes between Tetramer-positive cells and Tetramer negative cells in the effector memory cluster 1.

Figure 4

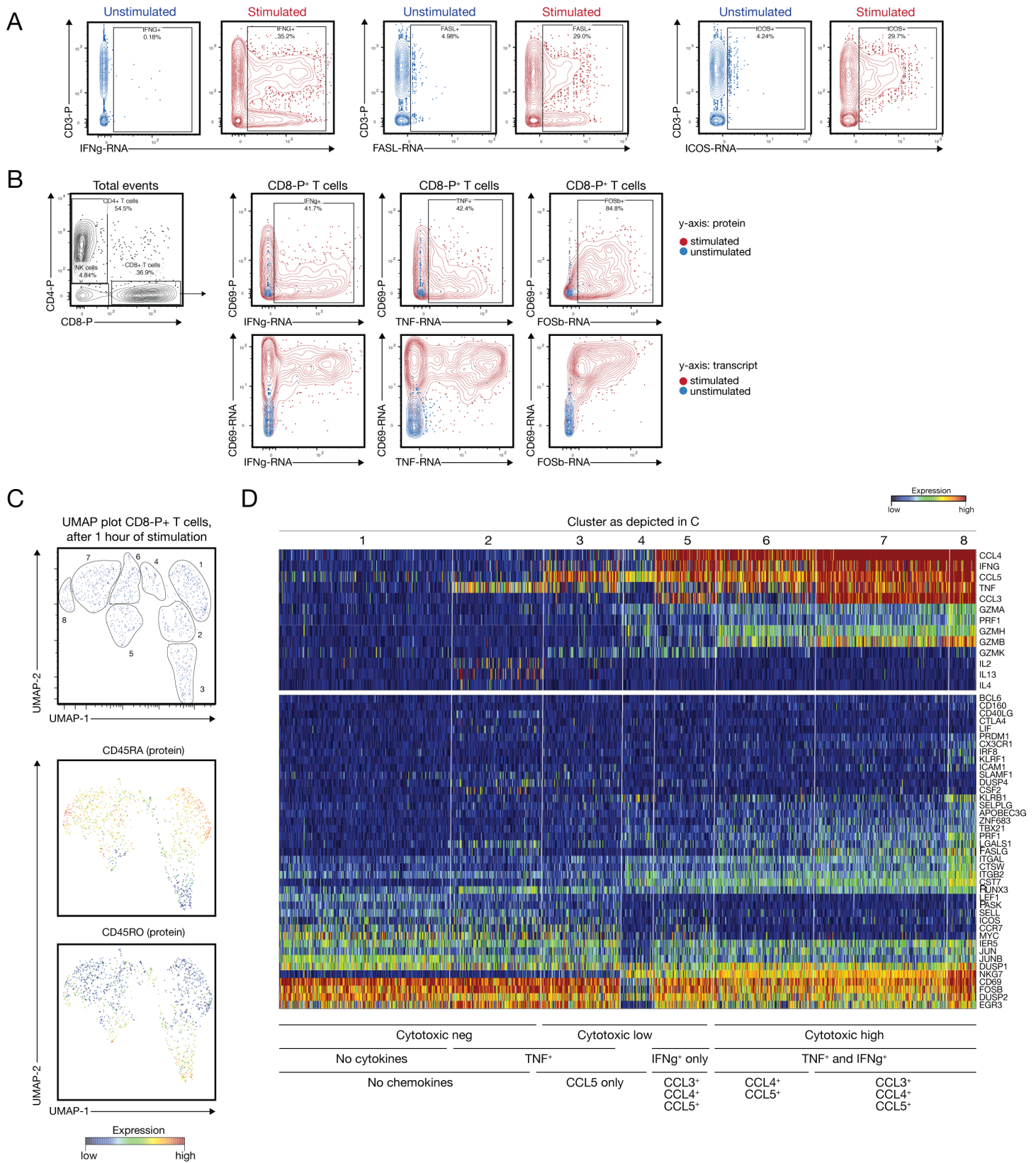


Figure 4: Multi-omic analysis of the T and NK cell compartment 1 hour after stimulation

(A) Representative plots showing the upregulation of selected effector transcripts such as IFNG, FASL and ICOS after stimulation (red) relative to unstimulated cells (blue).

(B) Disconnect between surface protein expression of the early activation marker CD69 and IFNG and TNF transcript within CD8-protein⁺ T cells. Blue overlay indicated unstimulated cells, red stimulated cells.

(C) UMAP plot of CD8-protein⁺ T cells with manually identified clusters, and CD45RA and CD45RO protein expression.

(D) Heatmap showing the expression of key effector transcripts within the clusters identified in (C).

Figure 5

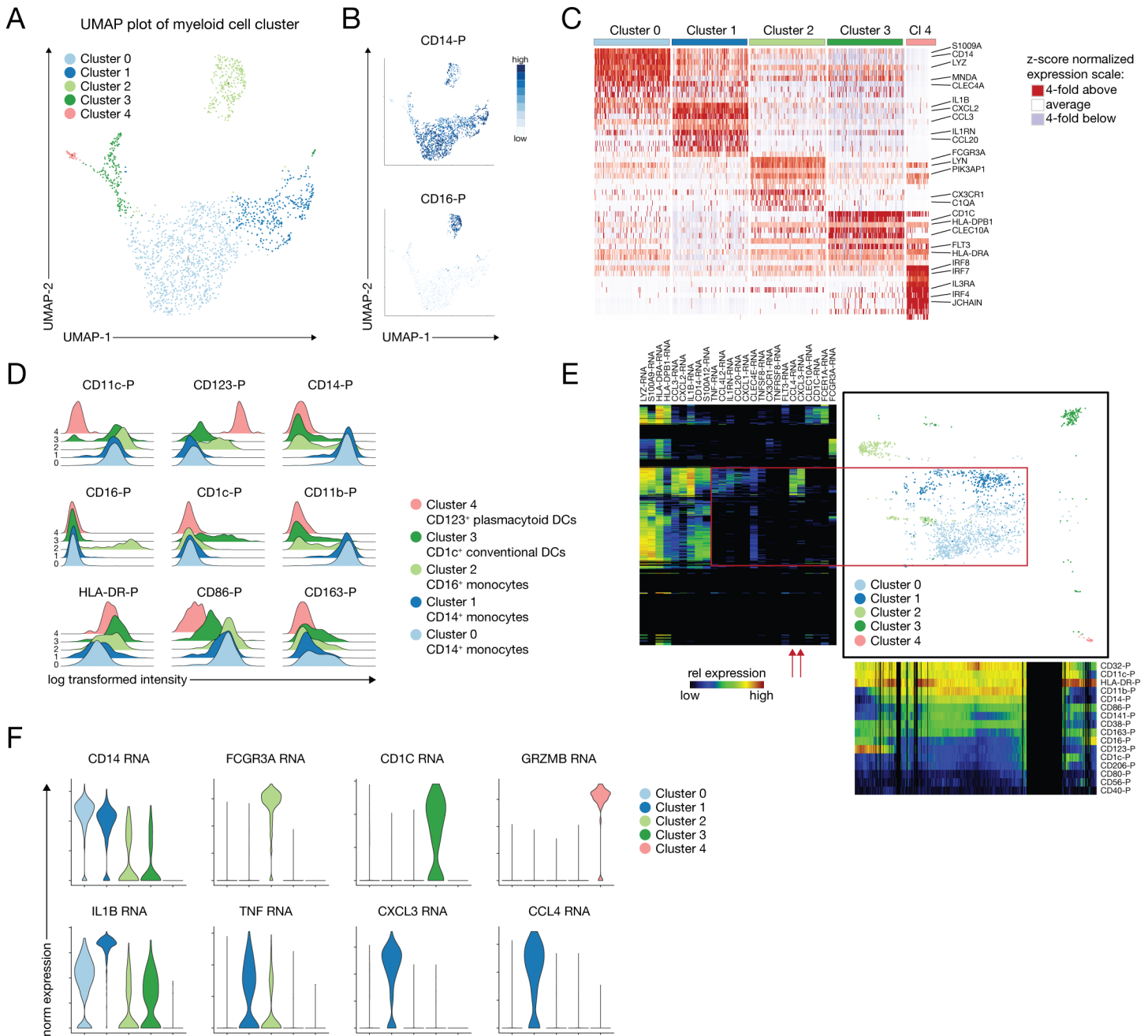
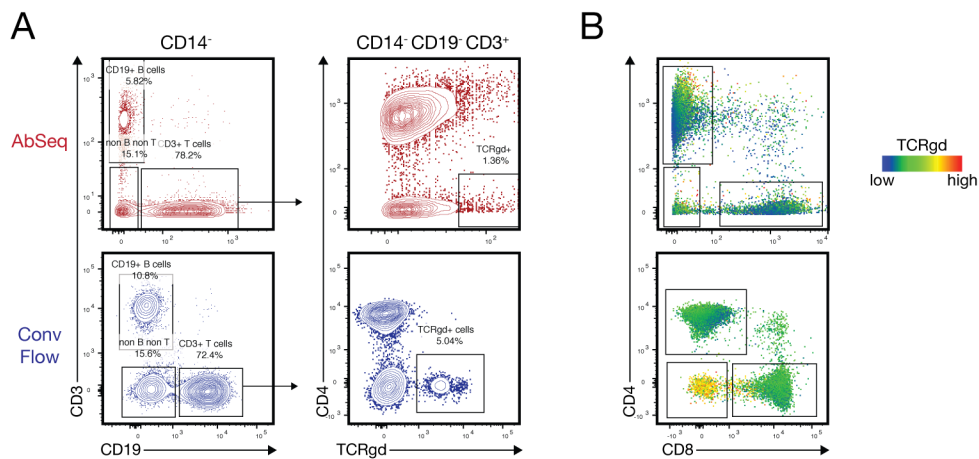


Figure 5: Combined protein and transcript phenotyping of the peripheral myeloid compartment reveals inflammatory subsets not captured by surface protein phenotype

- (A) UMAP plot and graph-based clustering of the peripheral non T/non NK/non B cell compartment, revealing 5 distinct populations.
- (B) Heatmap overlay of CD14- and CD16-protein expression.
- (C) Heatmap of the top differentially expressed genes identified by MAST for each of the 5 clusters highlighted in (A).
- (D) Protein signatures of the 5 clusters identifies canonical CD123⁺ plasmacytoid DCs, CD1c⁺ conventional DCs and CD16⁺ monocytes, but two of the clusters mapping to CD14⁺ monocytes.
- (E) One-SENSE plot depicting protein expression heatmap along the x-axis, and transcript expression heatmap of some of the top differentially expressed genes along the y-axis. Red box and arrows are highlighting the differentially expressed genes between cluster 0 and 1.
- (F) Violin plots showing key genes of the respective myeloid population (upper panel) and differentially expressed genes between cluster 0 and 1, suggesting the presence of an inflammatory subpopulation within CD14⁺ CD16⁻ monocytes that expresses high levels of IL1B, TNF, CXCL3 and CCL4.

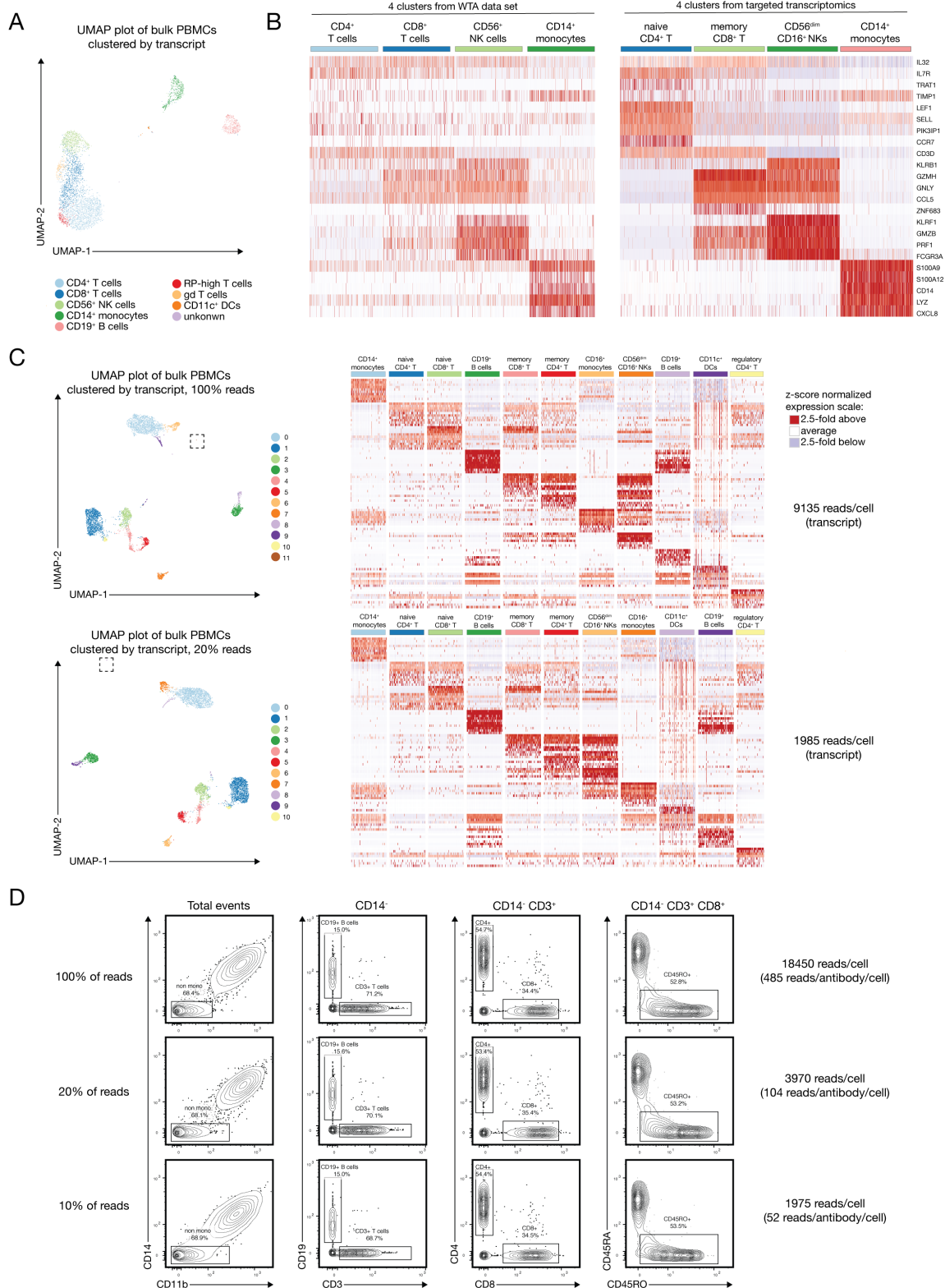
Suppl. Figure 1



Supplementary figure 1: Example for a poorly performing reagent

(A) Manual gating of main immune subsets using the combined AbSeq data set (upper panel, red) and concatenated and downsampled events from the flow cytometry data set (lower panel, blue), highlighting the population of $\gamma\delta$ T cells. **(B)** Heatmap overlay of the TCR $\gamma\delta$ signal on a CD4 vs CD8 plot for the AbSeq data set (upper panel) and flow cytometry data set (lower panel).

Suppl Figure 2



Supplementary figure 2: Comparison of targeted transcriptomics to whole transcriptome data (WTA) and assessment of required sequencing depth

(A) Graph-based clustering of WTA data obtained from the same donor as in main Figure 2.

(B) Four of the clusters that matched most closely in terms of their expression pattern were selected from both experiments and plotted using the top differentially expressed genes obtained from the targeted transcriptomics approach. Heatmap represents relative expression after z-score normalization. Left plot shows WTA data, right plot shows targeted transcriptomics (cells obtained from the same donor).

(C) 5,400 cells from a different donor were sequenced at a total depth of approximately 30,000 reads/cell. Upper panel depicts UMAP plot after graph-based clustering and a heatmap of the top differentially expressed genes (z-score normalized expression) at full read depth, lower panel using only 20% of the reads. Read depth per cell for the transcript library is indicated on the right. Squared box on the UMAP plot indicates one cluster that is separated as cluster 11 at full read depth, but pooled with cluster 8 at lower read-depth

(D) Protein signals at the indicated read depths.