Clinical grade adjuvants to mature CD141+ DCs for immunotherapy

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Abstract

Stimulation of dendritic cells (DC) is considered critical in cancer immunotherapy. BATF-3-dependent subsets, that express in humans CD141 (BDCA-3), promote CD8 T-cell cross-priming against tumor antigens. Here, we evaluate two clinical-grade stimuli for peripheral blood CD141+ myeloid dendritic cells (mDCs), a rare DC subset that is currently being explored for use in immunotherapy. In contrast to routine evaluation methods, which focus on predefined maturation markers on the surface or factors released from the activated cells, we applied an unbiased transcriptome-based method using both RNA-sequencing (RNA-seq) and microarrays. Specifically, we analyzed the mRNA of CD141+ cells, we applied an unbiased transcriptome-based method using both RNA-sequencing (RNA-seq) and microarrays. Specifically, we analyzed the mRNA of CD141+ mDCs from five human donors upon activation with two clinical-grade adjuvants, Hiltonol (poly-ICLC, a TLR3 ligand) and protamine RNA (pRNA, a TLR7/8 ligand), and compared these samples to unstimulated counterparts. Both methods, RNA-seq, and microarray showed that Hiltonol and pRNA lead to almost identical changes in the transcriptome of CD141+ mDCs. A gene ontology (GO) term analysis suggested that these changes were mainly related to activation and maturation pathways, including induction of type I IFN and IL-12 transcription, while pathways related to adverse effects or cell damage were not strongly affected. The combination of both reagents in the DC cultures gave a very similar result as compared to either stimulus alone, suggesting no synergistic effect. Furthermore, our analysis demonstrates that microarray and RNA-seq analysis gave similar conclusions about the activation status of these cells. Importantly, microarray analyses instead of the advantages of RNA sequencing may still be suitable for studying the activation of rare cell types that are minimally represented or in very low frequency in the organism. Together, our results indicate that both stimuli are potent clinical grade adjuvants with comparable effects to mature CD141+ mDCs in short-term cultures to be used in immunotherapy.

Keywords: Myeloid dendritic cells (mDC); Cross-presenting CD141+ (BDCA-3+); RNA-seq; Microarrays; Immunotherapy; Clinical trial

1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) and are found in most tissues of the human body [1]. Upon activation, DCs migrate to the lymph nodes and activate T cells [2]. Among the primary circulating blood DCs, there are two main subsets, the myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs). The mDC subset can be subdivided into CD1c+ (BDCA1+) mDCs and CD141+ (BDCA3+) mDCs. Each subset has been shown to have specific functions. Whereas pDCs are known to produce high amounts of type I interferons (IFNs) upon sensing viral pathogens via TLR7 or TLR9, CD1c+ mDCs can sense bacterial pathogens via TLR3, TLR4, or TLR8 and release IL-12 p70 and IL-1β. CD141+ mDCs show a high expression of TLR3, have been characterized as high IFN-λ and IL-12 producers, and are highly efficient cross-presenters to CD8 T cells, raising interest in them as a target for DC-based immunotherapies [3–10]. Yet, due to their low frequencies in the human blood, CD141+ mDCs have not been included in clinical trials so far and have not been studied as extensively as the more abundant CD1c+ mDCs and pDCs.

Efficient activation is a crucial step for DC-based cancer immunotherapy [11–17]. DC activation can be measured based on different parameters. Firstly, surface expression of maturation markers like CD40, CD80, CD83, CD86, HLA-DR, or PD-L1, as well as chemokine receptors like C-C chemokine receptor type 7 (CCR7), can be measured using flow cytometry [18]. Co-stimulatory molecules like CD80 or CD40, but also co-inhibitory molecules like PD-L1, do not only represent the maturation state of DCs but also play an important role in regulating T cell stimulation. Secondly, cytokines and chemokines released by the cells indicate their viability, functionality, and maturation state. Furthermore, as indicators of the DC activation state, their ability to prime T cells or other immune cells can be measured by co-culture experiments in which T cell proliferation or phenotypic changes are used as a read-out. All these methods are based on a pre-defined set of markers and targets. On one hand, this means that those experiments are focused and comparable. On the other hand, however, important effects that are not known or expected a priori, or do not belong to the most obvious targets, can be missed.

In clinical trials, we have used CD1c+ mDCs and pDCs for therapeutic vaccination to treat cancer patients and observed favorable clinical outcomes in a minority of...
Stimulation has strong effects on the transcriptomes of CD141+ mDCs. Principal Coordinates Analysis (PCoA) was performed to globally assess the differences between transcriptomes. Each point represents the transcriptome of one sample and the first and second principle coordinates are plotted. For both the pooled (A) RNA-seq and (B) microarray datasets, as well as for separated PCoA for each donor, and for (C) RNA-seq and (D) microarray datasets, the first principle coordinate aligned roughly with stimulation. The arrows in (A) and (B) highlight an outlier sample (donor 3) in which the stimulation appears to have failed. This sample was removed from subsequent analysis. S1 represents the sample stimulated with Hiltonol, S2 represents the sample stimulated with pRNA and S1 + S2 represents the combination of both stimuli.

We evaluated several clinical-grade reagents to induce DC maturation and determined pRNA as the most suitable stimulus [13,17]. Since CD141+ mDCs appear promising for future immunotherapies [20], we here aimed to identify an optimal stimulus for this DC subset. Because CD141+ mDCs express TLR8 and TLR3 to a high extent [21], we tested TLR8 ligand pRNA and Hiltonol, as a commercially available clinical-grade Poly I:C (PolyICLC) that can trigger TLR3 activation.

To compare the effects of these two adjuvants in an unbiased and global manner, we applied transcriptome analysis. We employed both RNA-seq and microarray measurements to interrogate the DC transcriptome and asked whether these techniques would lead to similar conclusions. RNA-seq is known to have a higher sensitivity and therefore increases the chance to observe changes in low-abundant genes, while the microarray approach has the advantage of requiring substantially less starting material, which can be a limiting factor for rare cell populations like peripheral blood CD141+ mDCs [22].

2. Materials and methods

2.1 Cell isolation and culture of CD141+ cells

For RNA-seq and microarray measurements, cells were obtained from aphaeresis of 5 different donors. Due to the limited cell numbers of donor 1, only 4 donors could
Fig. 2. Characterization of CD141⁺ cells selected. (A) Sorting strategy performed of CD141⁺ mDCs before RNA isolation. Gating cells selected: Lineage (CD3, CD16, CD19, CD20 and CD56) neg/HLA-DR pos/CD14 neg/CD141 pos. (B) Membrane cell markers (CD40, CD69, CD80, CD83, CD86, MHC-II, CCR7, PD-L1) of the recovered fraction. (C) Supernatant cytokine production of IL-1β, IL-6, IL-10, IL-12, and TNF-α using ELISA-sandwich.

Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll density centrifugation (Ficoll Paque Premium, GE Healthcare, Chicago, IL, USA). Anti-Human Lineage Cocktail 1 in FITC (LIN1) (BD Bioscience Pharmingen, San Jose, CA, USA) containing antibodies for CD3, CD16, CD19, CD20, CD56 receptors, together with the anti-FITC conjugated magnetic microbeads of Miltenyi Biotec (Bergisch-Gladbach, Germany) were used to deplete the LIN1⁺ cell fraction, by following manufacturer’s instructions. CD14 conjugated in PerCP (Miltenyi) was used to exclude the monocyte population. Next, CD141⁺ mDCs were further purified by
Fig. 3. RNA-seq and microarray analysis strongly agree on the effects of each stimulus. Microarray and RNA-seq fold change estimates for significantly up- or downregulated genes (applying a \( p \)-value cut-off of 0.05 after multiple testing correction) upon (A) Hiltonol and (B) pRNA were plotted against each other. The overall correlation was quantified using Spearman’s rho.

sorting (flowcytometry) using anti-CD141-APC combined with anti-HLA-DR-PE-Vio770 (Miltenyi Biotec) to a purity of 99.9%. DCs were cultured in X-VIVO-15 medium (Lonza, Basel, Switzerland) supplemented with 2% human AB serum (Corning). DCs were stimulated with pRNA (15 \( \mu \)g/mL) and/or Hiltonol (10 \( \mu \)g/mL) for 16 hours maintained at 37 ºC and 5% CO\(_2\) in a cell incubator.

2.2 Protamine-RNA (pRNA) complexes

pRNA complexes were made freshly before adding to the cells. Protamine (protamine hydrochloride MPH 5000 IE/mL; Meda Pharma BV Amstelveen, the Netherlands) was diluted to 0.5 mg/mL in RNase free water and mixed with 2-kbp-long single-stranded mRNA (coding for human gp100 protein) [14]. It was extensively mixed and incubated for 5–10 minutes at room temperature, before being added to the cells.

2.3 RNA sequencing and microarray analysis

CD141\(^+\) mDCs were isolated as described above and total RNA was extracted using Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the standard protocol. The quality control of the isolated RNA (concentration, RIN, 28S/18S, and size) was performed with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). RNA sequencing and read alignment were performed by BGI TECH SOLUTIONS (Tai Po, Hong Kong). Reads were aligned to human genome version 19. The microarray analysis of the RNA was performed by using the Clarion D assay (Thermo Fisher Scientific).

2.4 Hierarchical clustering

Data were transformed to log2 values for performing hierarchical clustering analysis (One minus Pearson correlation). Using the standard settings of the MORPHEUS - Versatile matrix visualization and analysis software version 0.0.2 (Broad Institute; Cambridge, MA, USA; https://clue.io/morpheus).

2.5 Statistical analysis

Data were analyzed using the R platform for statistical computing. Specifically, the package “edgeR”, version 3.16.5, in Bioconductor version 3.4 (http://bioconductor.org/, released on 31 October 2018) was used for whole-transcriptome principal coordinates analysis (using the “plotMDS” command), differential gene expression analysis, and GO term analysis. Differential expression was determined by fitting a generalized linear model using the “glmFit” command, and significance was determined using the likelihood ratio test provided by the “glmLRT” command. Data from the Clarion D assays were imported into R using the Bioconductor packages “affycoretools” [23] and “oligo” [24] and were analyzed using the empirical Bayes procedure [25] implemented in the “limma” package, version 3.30.13 (The Walter and Eliza Hall Institute of Medical Research, WEHI, Australia. Maintainer: Gordon Smyth (smyth@wehi.edu.au)). Throughout, \( p \)-values were corrected for multiplicity using the Benjamini-Hochberg method. For GO term analyses, a significance cutoff of 0.05 was used. All data analysis scripts are available as supporting information for this article.
Fig. 4. Hiltonol and pRNA stimulation have similar effects on the transcriptome of CD141⁺ mDCs. (A,B) Scatterplots depicting gene expression changes upon both stimuli as measured by RNA-seq (A) and microarrays (B). Each point represents a gene. (C,D) Volcano plots show the magnitude of transcript abundance changes (x-axis: log fold) together with the statistical significance (y-axis: −log₁₀ p-value). Both stimuli, (C) Hiltonol and (D) pRNA, were compared to the unstimulated sample. Genes with a log fold change of more than 8 were labeled with the gene name. Fold changes and p-values were determined using edgeR (see Material & Methods section). p-values were corrected for multiplicity using the Benjamini-Hochberg method.

3. Results
3.1 Microarray and RNA-seq measurements lead to similar results

We obtained CD141⁺ mDCs from five different donors, isolating between 0.7 and 1.5 × 10⁶ cells per donor. In an overnight (16 h) stimulation assay, we tested the two different stimuli separately and in combination. We used both RNA-seq and microarray-based mRNA analysis on the same sample. For 4 out of 5 donors (donors 2–5), we obtained sufficient material to perform both RNA-seq and microarray analysis. From donor 1, the amount of RNA was not sufficient for both analyses and therefore only microarray analysis was performed. As a first data exploration step, we applied Principle Coordinate Analysis (PCoA) to the combined data of all conditions and all donors for both the RNA-seq (Fig. 1A) and the microarray data (Fig. 1B).
In these plots, the first principle coordinates separated stimulated from unstimulated cells, whereas the second coordinate appeared to be related to donor differences and no clustering of the different stimuli was observed. Separate MDS plots per donor (Fig. 1C, RNA-seq, and Fig. 1D, microarray) also showed the first coordinate to align in each case with stimulation. The combined stimulation was located in between the two individual stimuli in all cases, except for donor 3. For this donor, in both the RNA-seq and the microarray data, the pRNA-stimulated sample clustered together with the unstimulated samples (highlighted with an arrow in Fig. 1A,B). As this outlier was present in both datasets, this indicates that the cells in this sample were not stimulated as expected, perhaps due to an experimental mistake or genuine variability in how well CD141+ mDCs respond to pRNA. To prevent biased conclusions, the best course of action appeared to be to exclude this outlier from the rest of the analysis presented in this paper.

Since CD141+ mDC is a newly identified subpopulation of DCs we have carried out a study of the cell markers used and the purity achieved after cell selection. Flow cytometry plots of strategy are presented in Fig. 2A.

Likewise, after stimulation, we have carried out experiments to verify the effectiveness of the stimuli (Hiltonol and pRNA) checking the maturation state of cells (Fig. 2B,C). For this purpose, we have analyzed dendritic cell markers (Fig. 2B) and the production of cytokines in supernatant (Fig. 2C) previously described for this type of stimuli in DC populations [4,9].

Significant increases in cell markers CD40, CD80, CD86, MHC-II and CCR7 were observed (Fig. 2B). It is consistent with a strong stimulation of an APC cell profile with an increase in co-stimulatory molecules, antigen presentation capacity and migration to lymph nodes. Also, cells cultured in vitro secrete IL-6, IL-12, and TNF-α into the medium (Fig. 2C), which is consistent with the cytokine-profile observed in CD141+ mDCs.

Next, we directly compared the RNA-seq and microarray results for single genes, to see if the conclusions from both analyses are similar. Focusing on the set of genes that showed significant up- or downregulation upon stimulation (Fig. 3A,B), we observed that the direction of the change was the same in all cases (i.e., they were either up- or down-regulated in both datasets) with the single exception being the E2F3P1 pseudogene (Fig. 3B). The correlation between the estimated fold changes in the RNA-seq versus microarray data for the selected gene set was 0.9 and 0.91, respectively (Fig. 3A,B), largely due to the consistency in the direction of the effect. The fold-change estimated from the RNA-seq datasets was often several orders of magnitude larger than that obtained from the microarray datasets, which was expected due to the higher dynamic range of RNA-seq [22]. However, this analysis does show that both datasets are expected to yield similar qualitative conclusions, especially in downstream analyses that focus more on the direction of a change (i.e., after applying a fold-change cutoff) than on its magnitude, such as gene ontology (GO) analyses.

![Fig. 5. Venn-diagram based on differentially expressed genes (DEGs) between different stimuli.](image)

**3.2 Both stimuli affect most genes similarly**

Aiming to identify similarities and differences between the two stimuli, we next correlated the transcript fold-change values upon each stimulus (pRNA or Hiltonol) to each other for the RNA-seq (Fig. 4A) versus the microarray (Fig. 4B) data. This showed that (a) both stimuli upregulated more genes in CD141+ DC than were downregulated and (b) both had a similar effect; indeed, a differential gene expression analysis based on both datatypes showed no significantly different genes (below a p-value of 0.05 after multiple testing correction) between the two different stimuli (not shown). Focusing on highly upregulated genes (log fold changes >5 for RNA-seq, >2 for microarray) showed, again for both methods, that both stimuli strongly upregulated more transcripts in CD141+ DC than were downregulated. In summary, both stimuli appear to have very similar effects on the transcriptome; as expected, RNA-seq and microarray data both led to this conclusion. It is noteworthy that those genes up- and down-regulated after stimulation with Hiltonol present higher values than those obtained after stimulation with pRNA.

For reasons of simplicity, we therefore focus on the RNA-seq data only in the remaining of this paper.

To detect the most dominant changes upon each stimulus, we generated volcano plots in which the genes with a log fold change of higher than 8 were labeled with the gene names (Fig. 4C,D). Upon both stimuli, genes for IFN-λ, IL-27, IL-12A, or CCL19 were among the most strongly
Fig. 6. Relative expression of genes belonging to chemokines, chemokine receptors, and cytokines (type I IFNs). Genes that are related to (A) chemokines, (B) chemokine receptor, (C) type I IFN, and (D) other cytokines are clustered regarding their similar expression pattern. Each column represents one condition and each row represents one gene.

upregulated, and DLL4, GBGT and ZBTB32 belong to the group of genes that behaved most consistently. As expected, due to the omission of the outlier sample, the p-values of the pRNA were much higher, but the overall log-fold changes were very similar.

To identify specific pathways that changed in CD141⁺ mDCs upon stimulation with Hiltonol or pRNA, we performed a GO term analysis. The more genes belonging to a certain gene cluster change their expression upon stimulation, the higher this gene cluster (i.e., GO term) is ranked. Comparing both stimuli based on the GO term analysis, three overlapping gene clusters among the ten most significant gene clusters of each stimulus were observed (Table 1). The top differentially expressed GO term upon Hiltonol stimulation was “response to stress”, whereas for pRNA it was “response to virus”. But overall, both stimuli led to an upregulation of similar GO terms, with most terms relating to immune responses or antiviral responses. We have compared the three treatments: Hiltonol, protamine-RNA complex, and combination to check if significant overlapping genes were found. We have not noticed any significant difference between them as can be seen by Venn-diagrams based on differentially expressed genes (DEGs) (Fig. 5).

Importantly, the top 100 affected GO terms contained no pathways related to cell damage or apoptosis (Supplementary Tables 1,2,3 show different pathways affected after stimulation).

3.3 Type I/III IFN upregulation upon stimulation

Chemokines, chemokine receptors, and interferons are among the most relevant groups of genes for the T cell activating function of DCs. Therefore, we investigated these gene groups directly (Fig. 6). Hierarchical clustering of samples based on a selected set of these genes mirrored the results of our earlier PCoA analysis: unstimulated conditions and stimulated conditions formed clear separate clusters. This confirms the clear effect of the stimuli on the phenotype and the function of the CD141⁺ mDCs concerning chemokines, their receptors, and interferons. Furthermore, consistent with the lack of a major difference between the different TLR stimuli or combinations thereof, the stimuli did not cluster separately. One big cluster of genes that was predominantly upregulated upon stimulation included chemokines like CCL3, CCL4, and CCL5 (Fig. 6A). Additionally, T and NK cells attracting chemokines like CXCL9, 10, and 11 appeared strongly upregulated upon both stimuli. By contrast, CXCL16, CXCL1, and CXCL6 were more highly expressed in the unstimulated samples than upon stimulation [26–28].

Comparing the chemokine receptor genes, again unstimulated samples cluster together whereas the samples of the different stimuli form a second, mixed cluster (Fig. 6B). This analysis showed a cluster of 3 chemokine receptors (CCR6, CXCR4, and CXCR6) that were all downregulated upon stimulation. Another cluster (lower part of Fig. 6B) contained genes that were upregulated upon stimulation, in-
Fig. 7. Both stimuli upregulate maturation markers on CD141\(^+\) mDCs. Normalized transcript abundance values, computed with the edgeR package (See Material &Methods section), are shown for the surface receptors (A) CD80, (B) CCR7, (C) HLA-DR, (D) CD40, and (E) CD86.

including the gene for the migration-related chemokine receptor CCR7, but also for CXCR3, CXCR5, and CCR4. Type I/III interferons were mainly present upon pRNA stimulation (Fig. 6C). In conclusion, a comparison of the cytokine genes revealed one big cluster containing IL-27, IL-36\(\gamma\), and IL-12p40, which were all upregulated upon stimulation and several smaller clusters with less clear patterns.

Finally, we were interested in the transcript levels of established CD141\(^+\) mDC maturation markers and therefore investigated the CD80, CD40, and CD86 transcripts (Fig. 7). Here, pRNA led to the strongest upregulation of CD80 and CD40, while CD86 was most upregulated upon the combination of both stimuli. However, these differences were very small and not statistically significant. Upon stimulation with Hiltonol and pRNA, the cells upregulated the C-C chemokine receptor type 7 (CCR7). However, the expression of the MHC class II receptor, HLA-DR, was lower in all stimulated conditions. The combination of the two stimuli had no additional effect on the transcript levels of the maturation markers and the chemokine receptor CCR7.

To investigate the similarities and differences of CD141\(^+\) mDCs to CD1c\(^+\) mDCs and pDCs, we pooled our RNA-seq data with the datasets of our previous study on the effects of different clinical stimuli on those more common DC subsets [29]. Performing a PCoA of all three stimulated and unstimulated DC subsets (Fig. 8), the three DC subsets clustered separately. Interestingly, the distance between the stimulated pDCs and CD1c\(^+\) mDCs increased compared to the unstimulated samples, while pDCs and CD141\(^+\) mDCs kept similar distances upon stimulation. No clear correspondence was visible between the CD1c\(^+\) mDCs and CD141\(^+\) mDCs. Also, the batch effect of different stimulants used has indeed been corrected, so the most plausible explanation is that the separation of the DC subpopulations is due to the batch effect. The CD1c\(^+\) mDCs and CD141\(^+\) mDCs showed the highest homogeneity suggesting once again a high similarity of the two stimuli tested in this study.

4. Discussion

We compared two clinical-grade TLR stimuli and their effect on CD141\(^+\) mDCs and demonstrated that both ad-


Table 1. Both stimuli affect similar regulatory pathways.

| Term                                      | Ontology | N genes | N up/down | –log_{10} p-value |
|-------------------------------------------|----------|---------|-----------|-------------------|
| Hiltonol                                  |          |         |           |                   |
| Response to stress                        | BP       | 3557    | 1028      | 28.7              |
| Cytoplasm                                 | CC       | 10,548  | 2601      | 28.2              |
| Immune system process                     | BP       | 2348    | 717       | 26.1              |
| Viral process                             | BP       | 942     | 344       | 25.8              |
| Regulation of response to stimulus        | BP       | 3503    | 1000      | 25.6              |
| Multi-organism cellular process           | BP       | 947     | 344       | 25.4              |
| Symbiosis, encompassing mutualism through parasitism | BP   | 973     | 351       | 25.3              |
| Interspecies interaction between organisms | BP       | 973     | 351       | 25.3              |
| Response to virus                         | BP       | 304     | 147       | 24.3              |
| Binding                                   | MF       | 13,201  | 3127      | 24.1              |

| Term                                      | Ontology | N genes | N up/down | –log_{10} p-value |
|-------------------------------------------|----------|---------|-----------|-------------------|
| pRNA                                      |          |         |           |                   |
| Response to virus                         | BP       | 304     | 70        | 23.5              |
| Defense response to virus                 | BP       | 226     | 59        | 22.9              |
| Defense response                          | BP       | 1461    | 169       | 18.8              |
| Defense response to other organisms       | BP       | 475     | 80        | 17.8              |
| Immune response                           | BP       | 1509    | 170       | 17.7              |
| Response to biotic stimulus               | BP       | 839     | 114       | 17.6              |
| Immune system process                     | BP       | 2348    | 232       | 17.3              |
| Response to stress                        | BP       | 3557    | 315       | 16.9              |
| Response to external biotic stimulus      | BP       | 801     | 109       | 16.9              |
| Response to other organisms               | BP       | 801     | 109       | 16.9              |

| Term                                      | Ontology | N genes | N up/down | –log_{10} p-value |
|-------------------------------------------|----------|---------|-----------|-------------------|
| Hiltonol + pRNA                           |          |         |           |                   |
| Immune system process                     | BP       | 2348    | 694       | 30.4              |
| Response to stress                        | BP       | 3557    | 975       | 30.2              |
| Cytoplasm                                 | CC       | 10,548  | 2428      | 27.3              |
| Regulation of response to stimulus        | BP       | 3503    | 943       | 26.0              |
| Cytoplasmic part                          | CC       | 7919    | 1890      | 25.8              |
| Regulation of immune system process       | BP       | 1302    | 416       | 24.6              |
| Cell surface receptor signaling pathway   | BP       | 2595    | 727       | 24.3              |
| Intracellular signal transduction         | BP       | 2588    | 719       | 22.9              |
| Immune response                           | BP       | 1509    | 461       | 22.7              |
| Response to cytokine                      | BP       | 782     | 275       | 22.7              |

Changes in clusters of transcripts were detected using a GO term analysis. The top 10 most differential expressed gene clusters upon each stimulus and the combination was selected. The description for each gene cluster is shown in the columns. The GO contains three sub-ontologies: molecular function (MF), cellular component (CC), and biological process (BP). N genes stand for the total amount of genes contained in a cluster and N up/down denotes the number of genes that changed their expression values upon the stimulus. The table is sorted on the –log_{10} p-value shown in the rightmost column.

Juvenants had similar effects at the transcriptional level on this subset even though different TLRs were targeted. Furthermore, we used two independent methods to analyze the transcriptome and similar conclusions could be drawn. Overall, our analysis suggests that both stimuli are potent adjuvants for CD141+ mDC immunotherapeutic applications.

We used RNA-seq to obtain unbiased and global overviews of the transcriptome. Previously, we studied the effect of pRNA and other adjuvants on CD1c+ mDCs and pDCs and pointed out the stronger adjuvant potential of pRNA as compared to FSME or GM-CSF [29]. Those results were in line with an initial study by Skold et al. [14] to characterize the effect of pRNA on DC subsets. Addition-
be observed by comparing the upregulation of type I/III IFN genes, which explains why the fold changes estimated from microarray data are often less prominent than those estimated from RNA-seq. Due to its larger dynamic range when measuring transcript abundance, RNA-seq can be a good and cheaper alternative. Nonetheless, for diagnostics and clinical applications, our data suggest that microarrays are also a suitable alternative.

Nonetheless, for diagnostics and clinical applications, our data suggest that microarrays are also a suitable alternative. Due to its larger dynamic range when measuring transcript abundance, RNA-seq can be a good and cheaper alternative. Nonetheless, for diagnostics and clinical applications, our data suggest that microarrays are also a suitable alternative. However, RNA-seq has decreased, the technique has become commercially available and the required amount of RNA has diminished, it has become a standard technique for measuring gene expression, including for some clinical applications [30–33]. A major advantage of RNA-seq is its ability to quantify and detect novel transcripts, unlike microarray techniques [34,35]; further, it directly yields RNA sequences, which simplifies data analysis. The usefulness of RNA-seq for immunotherapy was recently demonstrated by the use of this technique to detect mutations or to search for neo-antigens [36–38]. Another study performed by Van Allen et al. [39] investigated transcriptomic changes of melanoma cells upon treatment with ipilimumab, a CTLA-4 inhibitor. Such studies point to the potential for RNA-seq to become an integral component of personalized medicine.

In summary, we provide extensive and unbiased genome-wide data regarding how CD141+ mDCs react upon stimulation with two different clinical-grade stimuli targeting different TLRs. Both stimuli (Hiltonol and pRNA) have been widely characterized in different subpopulations of DCs, obtaining similar maturation results [9,11,14,18]. Until now, studies including this rare DC subset have been limited due to the low numbers of CD141+ mDCs, but we anticipate that the improved isolation methods and also techniques to increase CD141+ mDCs in vivo will draw more attention to this subset. We do not claim that RNA-seq will replace other commonly used protein-level methods like flow cytometry or western blotting. Instead, we suggest combining both approaches once interesting targets have been detected by unbiased RNA-seq. Additionally, with the microarray and the RNA-seq approach, we observed similar results, which point out the efficacy of the micro-array approach, since it requires a significantly lower amount of RNA. However, RNA-seq does have established advantages such as being more sensitive and precise, and being able to deliver additional information about differential splicing, detect new gene variants and new genes, which are highly relevant from the research-related point of view. Nonetheless, for diagnostics and clinical applications, our data suggest that microarrays are also a suitable alternative. From a methodological point of view, RNA-seq and microarray analyses rendered similar results.

5. Conclusions

We show different clinical-grade stimuli for an immune system cell called CD141+ myeloid dendritic cells (mDCs), a rare DC subset that is currently being explored...
Conflict of interest

The authors declare no conflict of interest. Given his/her role as Editor, Carlos Alfaro had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to any person of Editorial Board.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://www.impress.com/journal/FBE/14/1/10.31083/j.fbe1401002.

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