

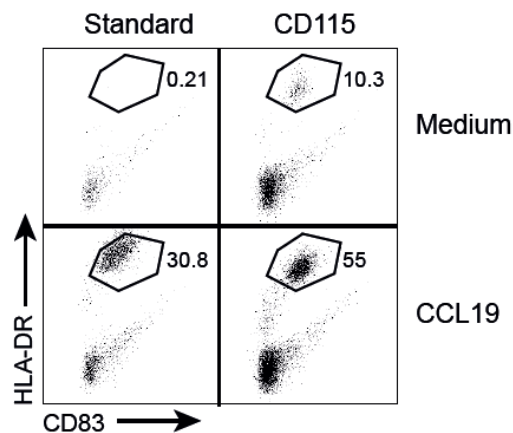
Reviewer 3:

General comments:

Plantinga et al., demonstrate that the conventional DCs are originated from a CD115+ precursor population and it is a novel information and they are able to demonstrate that the origin of cDCs from CD115+ cells and their similarity with pre-cDC. Considering the novelty the article should be considered for publication but it requires a major revision with reanalyzing the data as well as performing few additional experiments to unambiguously confirm the identity of DC subset in generated from the CD115+ cells. I think it is important to demonstrate the true identity of these cells subsets to extrapolate their application for cancer immunotherapy.

Major comments

Point 1: Line 119: Migration assay: The authors trying to compare two different systems with a different base lines. DCs generated from bulk culture have few CD11c+HLADR+ DCs or MoDCs (10-20%), whereas the CD115 derived cultures are almost 70-90 % of DCs after differentiation. So there is more mature DCs to start with CD115 cultures and authors are comparing that with mixed population of undifferentiated, differentiated and potential precursors of 115 DCs. Hence the authors should compare the migration of Sorted MoDCs from Bulk vs the CD115 DCs to make any solid conclusion. Even though the differences are not statistically significant with the current results, the MoDCs may have a better migration if you start with the purified fractions.



Answer point 1: We indeed compare an almost pure DC population with the mixed bulk population. We here try to confirm that the CD115-DCs indeed migrate to at least a similar extend as the bulk population (containing the CD115-DCs + undifferentiated, differentiated and potential precursors). As the reviewer suggested that stronger conclusion can be made when we compare sorted bulk DCs vs CD115-DCs we did a similar migration assay as described in the methods but used sorted CD83+ DCs from both cultures (shown above). For this experiment no clear difference is seen between the two cultures. We did not change the data in the paper.

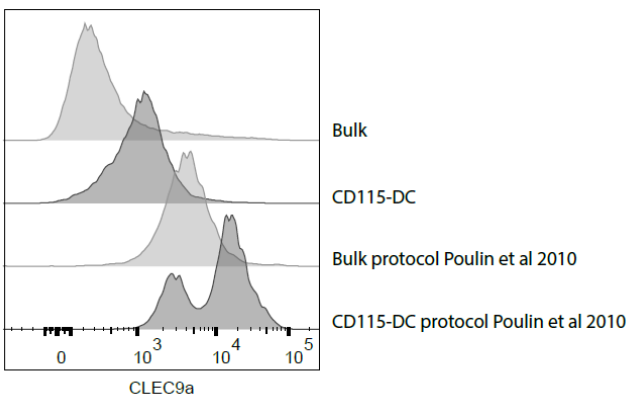
Point 2: Line 123: T cell activation: again authors are doing the same comparison with a bulk population to more homogenous DC subsets. The current set may not provide an equal probability to interact with the same number of DCs and T cells in two experimental conditions. So they should start with more homogenous or FACS sorted populations.

Answer point 2: For MLR co-stimulation plays a less important role and even precursors could activate T cells without proper co-stimulatory signals. But certainly for the WT-1 T cell activation the amount of mature DC per culture could play a role. Therefore DCs were sorted as suggested and loaded overnight with WT1 peptivator. The next day WT1-specific T cells were activated by CD83+DCs from both cultures. Again the DCs show their strong T cell stimulatory capacity, however no significant difference was observed between the two cultures, confirming that bulk DC originate from CD115+ precursors.

We changed figure 4 B with the data from 2 independent experiments for which we sorted the CD83+ DCs from both cultures.

Point 3: Authors FACS staining clearly shows that the CD115+ cells are clec9a+ and CD141+ (Fig 6A). It is a clear and specific signature of cDC1 phenotype, even though the author claims the expression of Clec9a is intermediate that is quiet normal with an in vitro generated cDC1.

Answer point 3:

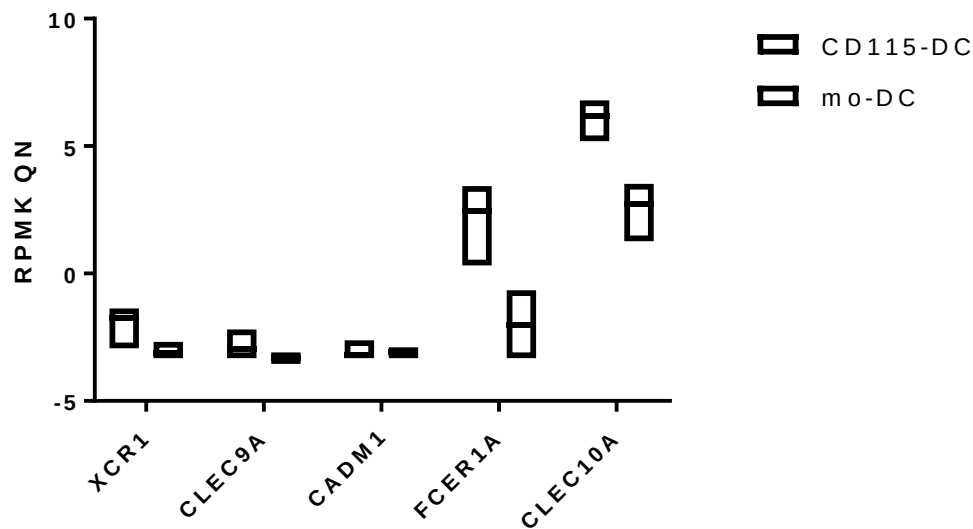


The claim that the expression of CLEC9a is intermediate was put forward because cDC1 cultured with the protocol published by Poulin et al 2010 (ref 19 in revised paper) to generate cDC1 showed high levels of CLEC9a, specifically using CD115+ precursors to differentiate. Those might represent the cDC1 (CLEC9A hi), which we do not see in our GMP-compliant culture protocol (CLEC9A int). This staining is added to supplementary figure 3.

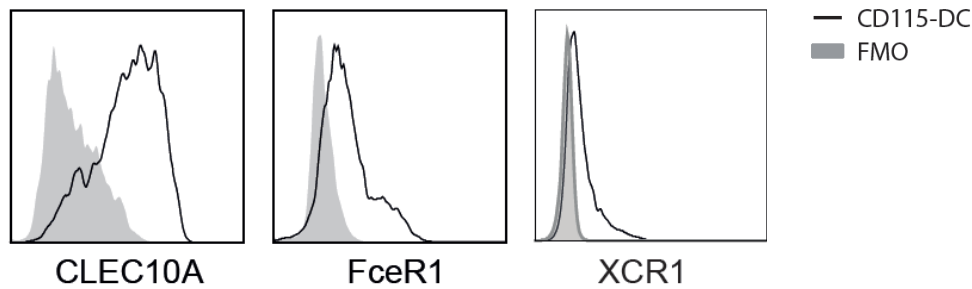
Point 4: Authors should additional FACS staining on markers including CADM1 (clone 3E1 from MBL), XCR1 (Bio legend), FCERIA, Clec10A to confirm the identity of these subsets.

It is critical to confirm the expression of these genes at protein level with FACS and also demonstrate the expression at transcriptome level from the RNA seq data.

Answer point 4:



Expression levels of cDC1 and cDC2 specific markers on RNA level.

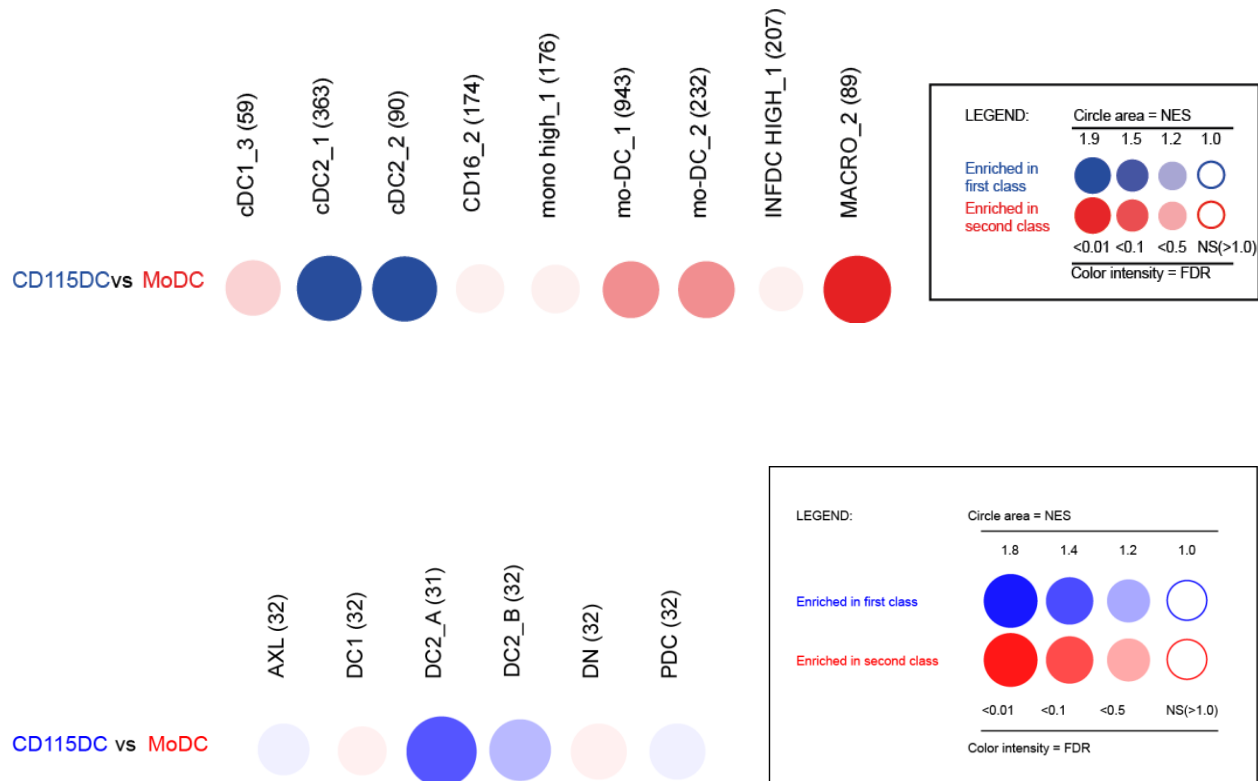


In addition to CLEC9a expression we measured CLEC10A, FceR1 and XCR1 expression on CD115-DCs. CD115-DC are CLEC10A positive on RNA and protein level, and partly positive for FceR1. XCR1 is hardly expressed on CD115-DC. Unfortunately we could not test CADM1 antibodies. XCR1, CLEC9A and CADM1 levels are low or even negative on RNA level.

If the cultures are a mixture of cDC1 and cDC2 it will be great approach to distinguish them and separate them for validating the nature of in vitro generated DCs.

Point 5: The data clearly shows the signature of cDC1 and unfortunately the authors are clearly overlooking those signatures in transcriptome analyses and perform a bubblegum analysis with cDC2 and MoDC signatures (Fig 6D and E). The data should be reanalyzed with cDC1 gene signature enrichment. (please kindly refer some mentioned papers performed similar detailed analysis on such in vitro generated subsets and refer the gene sets). The analysis should be performed in a non-biased way and why the authors specifically avoiding the gene set specific to the cDC1.

Answer point 5:



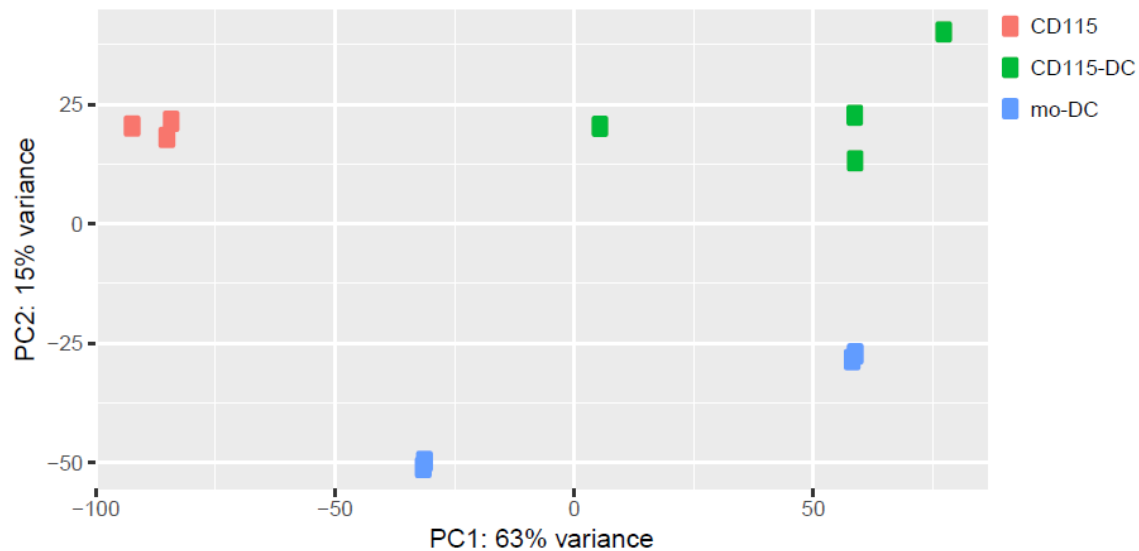
As suggested, we added reference gene set for cDC1 from the data described by Balan et al (ref 12 of the revised paper). _ (1) refers to Bakdash et al, (2) to Segura et al and (3) refers to the paper of Balan et al. In addition, we compared our transcriptome data to the 6 different types of DCs recently described by Villani et al (ref 15 in revised paper). Including a cDC1 signature in the unbiased bubblegum analysis showed a strong enrichment of CD115-DCs in cDC2.

Taken together the positive RNA levels and protein levels and the enrichment in the cDC2 gene sets and the very low levels of cDC1 genes for both RNA levels as well as enrichment, it seems that in our GMP-culture protocol CD115-progenitors generate cDC2. Although we cannot conclude that CD115-progenitors are committed cDC2 precursors, as is also shown by the upregulation of CLEC9A in the Poulin protocol. We agree that this additional information strengthen the paper and therefore added the FceR1 and CLEC10A staining as well as the additional bubble gum in the paper (figure 6).

Point 6: I think major revamp is required on reanalyzing the data and preparing new figures by combining figure 5 and 6 to present more precise and detailed analysis. The authors can prepare a PCA or unsupervised hierarchical clustering by pooling the RNA seq data from the pre and post differentiated subsets to show their difference and similarity.

Answer point 6: The experiments are performed at different timepoints, but fortunately we do have mo-DC at both timepoints. So we prepared a PCA plot from the two different experiments and concluded

that the batch effect is too large, since even the mo-DC (as internal control) from two experiments do not overlap. Therefore it is difficult to compare the two, which will not correctly explain the biological differences but rather technical differences.



We therefore re-analyzed the data with the suggestion of the reviewer to look at highly expressed genes and prepared a heatmap comparing CD115 with monocytes and CD115-DC with mo-DC for 54 genes previously described to be involved in DC biology. In addition, we plotted a heatmap of the significant genes between CD115-DC and mo-DC. From the top 500 significantly differential expressed genes we performed Gene Ontology analysis. Top panel right (fig 5D) is upregulated in CD115-DC, lower panel right is upregulated in mo-DC.

The authors should perform a GSEA or bubble gum analysis on more unbiased way and must include the cDC1 signature.

Point 7: Authors should present the list of highly expressed genes or differentially expressed in each subsets before and after differentiation and they can illustrate that in the form of heat map.

Also it will be interesting to see the heatmap with the top hits to describe the first 10-15 genes to explain the identity of the subsets. That can replace the figure 5B, 5C and 6C.

Answer point 7:

As pointed out by the reviewer we reanalyzed the data and prepared new figures to present more precise and detailed analysis. In the new figure 5 we show a detailed transcriptomic analysis to reveal the differences between monocytes and CD115 on one hand and CD115-DC and mo-DC on the other.

To have a closer look at the resemblance with conventional DCs, we show in figure 6 protein levels of well described markers for cDC progenitors (6A) and RNA levels of DC subset identification (6B) of which

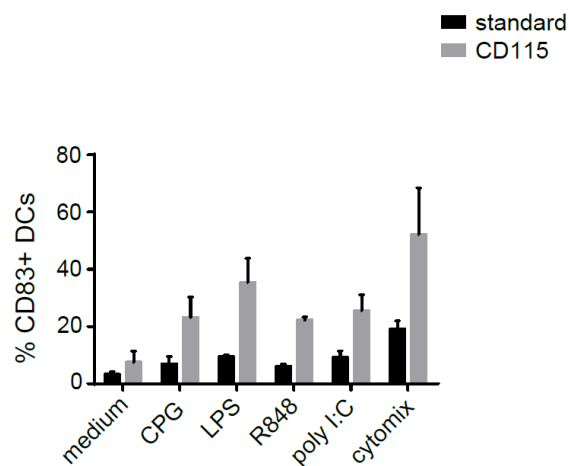
most confirmed by flow cytometry (6C). Thereafter we show geneset enrichment analysis using Bubble gum including cDC1, cDC2 and mo-cells profiles, where most enrichment of CD115-DC is seen with cDC2.

The changed text is highlighted in the word file using track changes. As well as the figures, legends and methods when necessary.

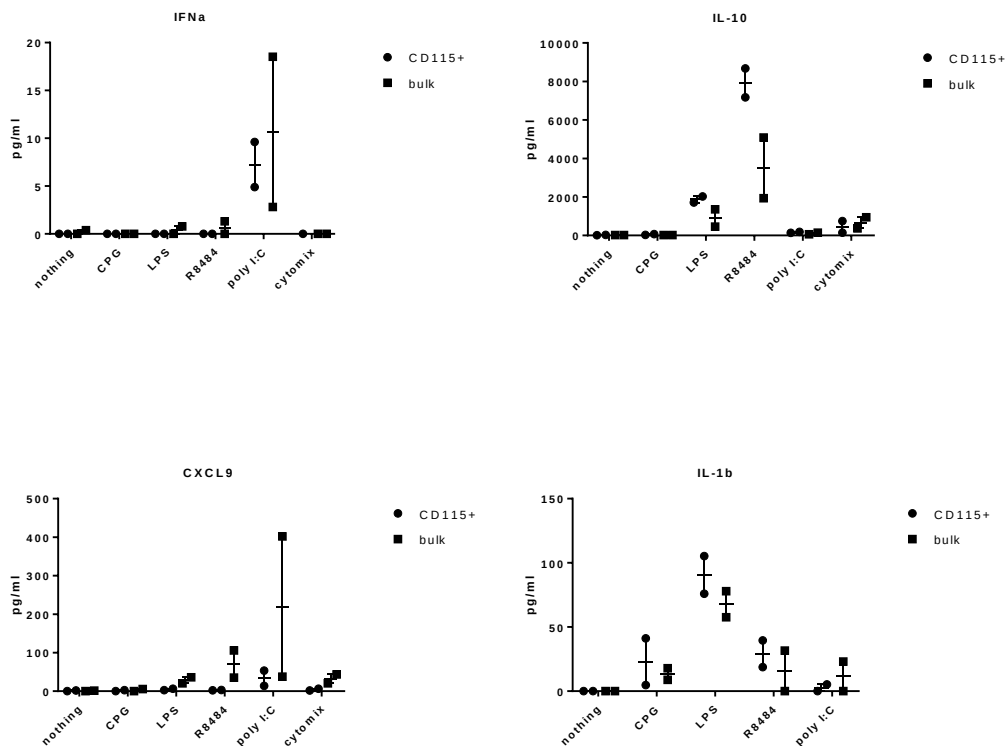
Additional suggestions

Point 8: It will be great to include a poly I:C (Poly I:C LC), R848 and LPS as maturation stimuli (both are clinically relevant) and check for the cytokine production. That can be another experiment to confirm the true identity of the subsets based on the cytokine profile including type II IFN production, IL-12, IL-10, CCL9 production etc. They have differential response to each TLR ligands and produce different cytokines (refer the suggest papers)

Answer point 8:



IFNg not detected in any case. IL-1b is present in the cytomix and is therefore excluded from this analysis. IL-12 is not detected after maturation, but we did measure IL-12p70 after additional stimulus with IFNg in combination with aCD40 [1]. However, these experiments are not performed with the CD115-DC.

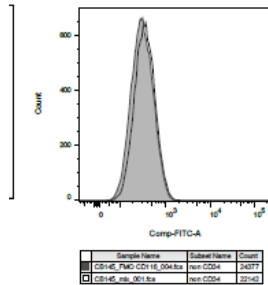


CXCL9 is produced by murine cDC1 in a tumor model [2] recruiting CD8 T-cells, within the tumor microenvironment. However, we hardly detect CXCL9, maybe due to the single TLR stimulus. R848 in addition to poly I:C has been shown to enhance CXCL9 production in cDC1 [3]. Taken together all the RNA and protein data, our protocol seems to generate mainly cDC2 and showed IL-1b production after LPS stimuli. R848 induced some IL-1b production together with IL-10. This data is added as supplementary figure 4.

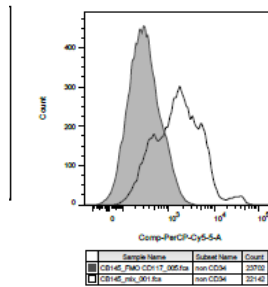
Minor comments

Point 9: 85-95: on different subsets: The CD116 and CD117 populations are not very distinct subsets from the FACS staining as compared to the CD115 vs CD34 or HLADR vs CD11c. It will be great include an FMO controls in FigS2 to make sure that they are a real population.

Answer point 9: Point well taken. I will add FMO staining Monday. If CD117/CD116 is then similar, will remove data from paper (it did not develop in sufficient DCs anyway)



CD116 + FMO



CD117 + FMO

So indeed CD116 not clear population (only some donors). CD117 clear distinction (although no DC formation).

Will remove text mentioning CD116 as separate population.

Page 2 Line 88 and line 93 removed CD116 population. And from supp figure 2.

Point 10: There are few instances the authors mention that the data not shown. I think most of the current journals do not support such claims and it will be better to include all those data as supplementary figures.

Answer point 10:

line 243; The expression of CD209 and the loss of CD209 expression one IL-4 is omitted in the differentiation protocol is added to the supplementary figures (supp fig 3). Together with the upregulation of CD209 if cultured with the Poulin protocol.

255; Protein levels of several markers after culturing CD115 or bulk according to the protocol described by Poulin et al. to generate cDC1 is added to supplementary figure 3.

line 348; controls for T cell assay are added as a supplementary figure (supp figure 4).

Other comments

It will be great to select references where the DC subsets are well characterized with multiple parameters than few ambiguous surface markers as well as redefining the published information based on the recent literatures. (Reference 8) The study identifies cDC2 as BDCA1+ CD14+ and there are well defined markers to identify the cDC2 including FCERIA, CLec10 A, BTLA and CD32b etc are more specific markers to identify the cDC2. References 10 and 11 clearly shows that the CD123+BDCA2+ are a mixed population of cells and they are not pure pDCs. They also demonstrate that pDCs are poor stimulators of T cells. So we are not sure the pDCs in this paper is true pDCs or mixture of cells and that is the reason they get a response and other paper fails to show pDCs elicit T cell response. The concern is also applicable for this manuscript and the authors claims the population is more cDC2 based on CD1c expression and they have to define them better by additional markers. There are publications already demonstrate that the in vitro generated cDC1 and even MoDCs can express high level of CD1c (PMID: 25009205). Some of these publications may be useful for better defining the observed DC subsets in the culture (PMID: 25009205, PMID: 30110645, PMID: 29344995, PMID: 29163495)

We thank the reviewer for pointing this out to us and added the references to our paper.

1. de Haar C, Plantinga M, Blokland NJ, van Til NP, Flinsenberg TW, Van Tendeloo VF, Smits EL, Boon L, Spel L, Boes M *et al*: **Generation of a cord blood-derived Wilms Tumor 1 dendritic cell vaccine for AML patients treated with allogeneic cord blood transplantation.** *Oncoimmunology* 2015, **4**(11):e1023973.
2. Spranger S, Dai D, Horton B, Gajewski TF: **Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy.** *Cancer Cell* 2017, **31**(5):711-723 e714.
3. Pearson FE, Chang K, Minoda Y, Rojas IML, Haigh OL, Daraj G, Tullett KM, Radford KJ: **Activation of human CD141(+) and CD1c(+) dendritic cells in vivo with combined TLR3 and TLR7/8 ligation.** *Immunology and cell biology* 2018, **96**(4):390-400.