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Response to "CD8 subunit expression by plasmacytoid dendritic cells is variable, and does not define stable subsets"

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To the Editor: We recently identified three subsets of plasmacytoid dendritic cells (pDCs) based on the expression of CD8α and -β subunits.[1] We showed that all subsets exist in the spleen, lungs, lymph nodes, blood, and thymus and that $CD8\alpha^+\beta^-$ and $CD8\alpha^+\beta^+$ but not $CD8\alpha^-\beta^-$ pDC subsets actively induce $FOXP3^+$ regulatory T cells *in vitro* and *in vivo* that protect mice from allergen-induced airway hyperreactivity and inflammation.[1] We are pleased that Brown *et al.*[2] confirmed the presence of reported pDC subsets in a recent correspondence. However based on only flow cytometry analysis, the authors claimed that these subsets may not be stable and might convert to each other after stimulation.

We persistently find that expression of CD8 subunits by pDCs is stable and defines subsets with distinct functions. pDC subsets have been extensively studied in our lab for their plasticity and ontogeny and we find that expression of CD8 subunits are not affected by toll-like receptor (TLR) stimulation. Similar to the figure shown by Brown *et al.*[2] we performed a series of experiments, where we purified $CD8\alpha^-\beta^-$ pDC and then cultured in either media alone or in the presence of TRL7 agonist, R848, or TLR9 agonist, CpG1668, for 16 h followed by the evaluation of CD8 subunits expression using flow cytometry (**Figure 1a**). As persistently observed, we did not find upregulation of either CD8 subunits in any of cultured conditions. To confirm flow cytometry findings, we repeated the same experiment and used real-time quantitative PCR to assess the relative gene expression of CD8α and -β in all sorted fresh pDCs and in sorted and cultured $CD8\alpha^-\beta^-$ pDCs (**Figure 1b**). Consistent with flow cytometry data we found no expression of CD8 subunits in either culture conditions.

Interestingly, we found that expression of CD8 subunits by pDCs defines stable subsets that respond differently to TLR stimulation. In a series of experiments where total mouse splenocytes were cultured in media alone or in the presence of R848 or CpG1668, we observed that $CD8\alpha^+\beta^+$ pDCs express higher level of TLR9 compared to $CD8\alpha^-\beta^-$ pDCs and preferentially proliferate (measured by Ki-67) in response to TLR9 stimulation (**Figure 1c–f**). This suggests that even low number of $CD\alpha^+\beta^+$ pDCs in purified $CD\alpha^-\beta^-$ pDCs can preferentially proliferate in response to TLR9 signaling, which leads to a similar observation as Brown *et al.*[2] reported. Thus purity of sorted cells is a crucial factor in determining the outcome of studies.

REFERENCES

1. Lombardi, V., Speak, A.O., Kerzerho, J., Szely, N. & Akbari, O. CD8alpha(+)beta(-) and CD8alpha(+)beta(+) plasmacytoid dendritic cells induce Foxp3(+) regulatory T cells and prevent the induction of airway hyper-reactivity. *Mucosal Immunol.* **5**, 432–443 (2012).
2. Brown, A.S., Bourges, D., Ang, D.K., Hartland, E.L. & van Driel, I.R. CD8 subunit expression by plasmacytoid dendritic cells is variable, and does not define stable subsets. *Mucosal Immunol.* **7**, 200–201 (2014).

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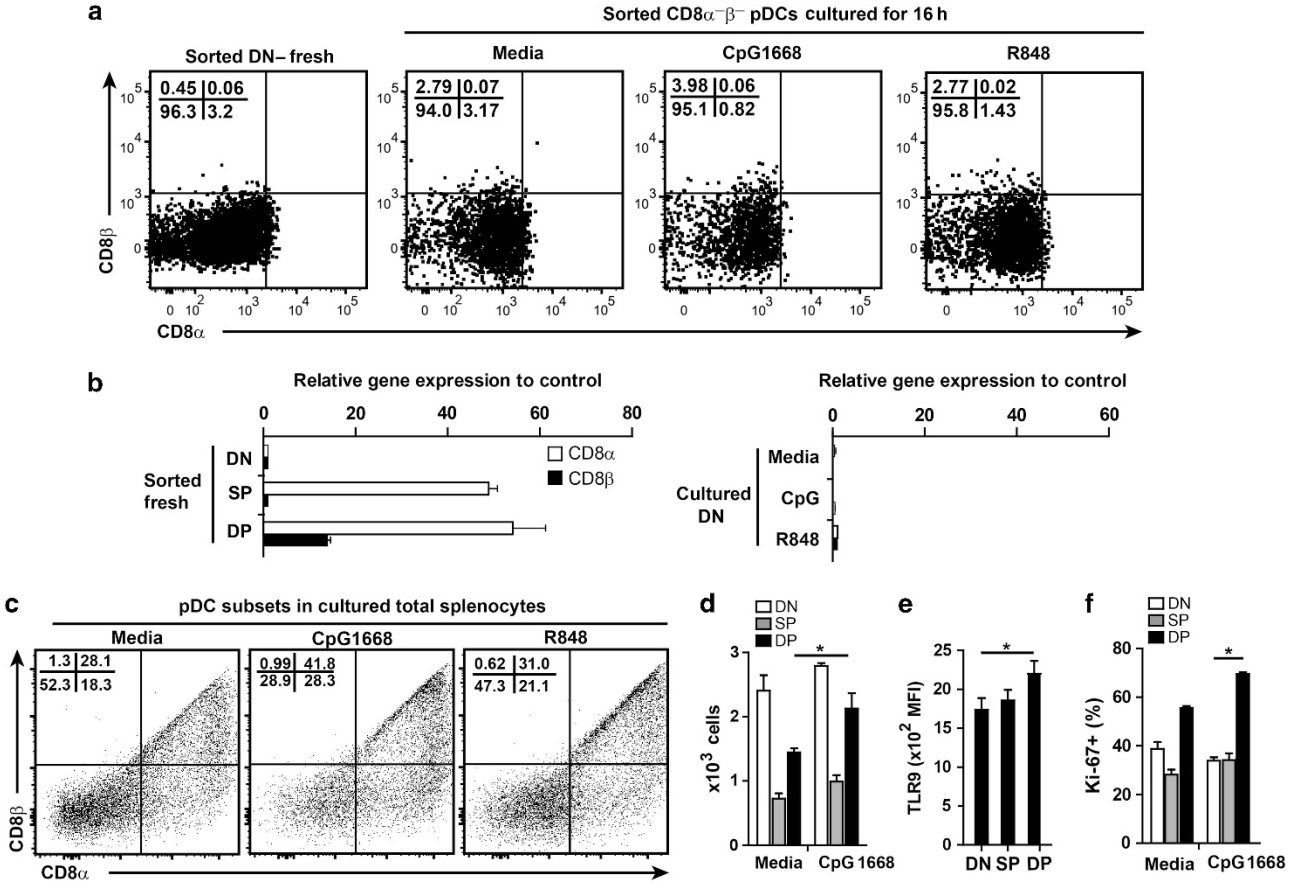

Figure 1 CD8α⁻β⁻ plasmacytoid dendritic cells (pDCs) do not express CD8α or CD8β upon TLR7 or TLR9 stimulation. (**a**) CD8α⁻β⁻ double negative (DN) splenic pDCs (CD11c^{intermediate}, mPDCA-1^{+}, SiglecH^{+}) were sorted, then cultured in the presence of TLR7 agonist, R848, TLR9 agonist, CpG1668, or media alone for 16 h followed by evaluating the expression of CD8α and CD8β using flow cytometry. (**b**) Relative gene expression of CD8α and CD8β was measured in freshly isolated DN, CD8α^{+}β^{-} single positive (SP), and CD8α^{+}β^{+} double positive (DP) pDCs by real-time quantitative PCR (left panel), and in cultured DN cells in the presence of R848, CpG1668 or only media (right panel). (**c**) Total splenocytes were cultured in the presence of R848, CpG1668 or media alone for 16 h followed by staining for pDCs (CD11c^{intermediate}, mPDCA-1^{+}, SiglecH^{+}), CD8α, and CD8β and evaluation by flow cytometry. Dot plots are gated on pDCs and show expression of CD8α and CD8β by cultured pDCs. (**d**) Bar-graph shows total number of pDC subsets after 16 h *in vitro* culture of total splenocyte in the presence or absence of CpG1668. (**e**) Intracellular expression of TLR9 by pDC subsets was evaluated by flow cytometry and shown as mean fluorescence intensity (MFI) of TLR9. (**f**) Bar-graph shows the percent of i-67+ cells of each pDC subset after 16 h *in vitro* culture of total splenocyte in the presence or absence of CpG1668 as evaluated by flow cytometry. Ki-67 is an indication of proliferation. Data are representative of at least three independent experiments ($N = 6$–12) and analyzed using Student's *t*-test confirmed by Mann–Whitney test. *A *P*-value of <0.05 was considered significant.