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**The activation trajectory of plasmacytoid dendritic cells in vivo during a viral infection.**

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1       **The activation trajectory of plasmacytoid dendritic cells in vivo during a**  
2       **viral infection**

3

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33

34 **Abstract**

35 Plasmacytoid dendritic cells (pDCs) are a major source of type I interferon (IFN-I).  
36 What other functions pDCs exert in vivo during viral infections is controversial and  
37 more studies are needed to understand their orchestration. Here, we characterize in-  
38 depth and link pDC activation states in animals infected by mouse cytomegalovirus,  
39 by combining *Ifnb1* reporter mice with flow cytometry, single-cell RNA sequencing,  
40 confocal microscopy and a cognate CD4 T cell activation assay. We show that IFN-I  
41 production and T cell activation were performed by the same pDC, but sequentially in  
42 time and in different micro-anatomical locations. In addition, we show that pDC  
43 commitment to IFN-I production was marked early on by their downregulation of LIFR  
44 and promoted by cell-intrinsic TNF signaling. We propose a novel model of how  
45 individual pDCs are endowed to exert different functions in vivo during a viral  
46 infection in a manner tightly orchestrated in time and space.

47

## 48 Introduction

49 Plasmacytoid dendritic cells (pDCs) produce massive amounts of type I  
50 interferons (IFN-I) in response to virus-type stimuli<sup>1</sup>, including in vivo during mouse  
51 cytomegalovirus (MCMV) infection<sup>2, 3</sup>. pDCs exert this function by engulfing material  
52 derived from viruses or infected cells, and routing it to dedicated endosomes for toll-  
53 like receptors 7/9 triggering and activation of the MyD88-to-IRF7 signaling pathway<sup>1</sup>.

54 Soon after their discovery, pDCs were proposed to first produce IFN-I and  
55 then acquire features of conventional dendritic cells (cDCs)<sup>4</sup>, including dendrites and  
56 the ability to present antigens<sup>4-7</sup>, upon specific stimulation conditions. However,  
57 adequate pDC purification methods and single-cell analysis tools were lacking to  
58 prove this hypothesis. Indeed, when defined by few surface markers, pDCs can be  
59 contaminated with cDC precursors<sup>8-10</sup>, pDC-like cells<sup>11</sup>, non-canonical CD8 $\alpha$ <sup>+</sup> cDCs<sup>12</sup>,  
60 <sup>13</sup> or transitional DCs (tDCs)<sup>14, 15</sup>. These cells harbor mixed phenotypic and functional  
61 characteristics of pDCs and cDCs and are more powerful than resting pDCs for T cell  
62 activation. Hence, T cell activation by pDCs was claimed to result from this  
63 contamination. However, discrepancies in the stimuli used to activate pDCs likely  
64 contributed to fuel the controversy on their T cell activation capacity<sup>16, 17</sup>. It is not  
65 clear to which extent pDC phenotypic and functional heterogeneity reflects their true  
66 developmental or stimulation-induced plasticity versus a lineage contamination by  
67 other cells<sup>18-21</sup>. pDC activation differs in response to synthetic TLR ligands, viral  
68 particles or infected cells<sup>22-24</sup>. Rigorously characterizing pDC cell fate and functional  
69 plasticity in vivo during viral infections thus remains a major challenge<sup>15, 19, 21</sup>.

70 Here, we characterized and linked pDC activation states in MCMV-infected  
71 animals, by combining *Irf1*-reporter mice<sup>25</sup> with flow cytometry, single-cell RNA  
72 sequencing (scRNAseq), confocal microscopy and a CD4 T cell priming assay.

73 Contrary to currently prevailing models of pDC plasticity<sup>19, 20</sup>, we demonstrated that  
74 IFN-I production and T cell activation were performed by the same pDC, but  
75 sequentially in time and in different microenvironments.

76

77 **Results**

78 **pDCs specifically express IFN-I during MCMV infection.**

79 We analyzed IFN-I production kinetics in splenic pDCs during MCMV infection  
80 (Fig. 1a-b). IFN-I-producing pDCs were detected at 33h post-infection, their  
81 percentage increased at 36h and 40h, diminished at 44h and became undetectable  
82 at 48h. Since pDC-like cells share *Zbtb46* expression with cDCs<sup>10, 11, 26</sup>, we  
83 harnessed *Zbtb46*<sup>Egfp</sup> mice to identify them and show that they do not express IFN-I at  
84 36h post-infection (Extended Data Fig. 1a). Thus, IFN-I expression occurred  
85 specifically in a minor fraction of splenic pDCs around 36h post-infection, consistent  
86 with previous work<sup>27</sup>.

87

88 **Microarray analysis of pDCs isolated from MCMV-infected *Ifnb1*<sup>Eyfp</sup> mice**  
89 **suggests their heterogeneity.**

90 We harnessed the *Ifnb1*<sup>Eyfp</sup> reporter mice<sup>25</sup> to characterize *Ifnb1*-producing  
91 pDCs and follow their fate in vivo during MCMV infection. The half-life of YFP in live  
92 cells exceeds 24h<sup>28, 29</sup>. Consistently, YFP<sup>+</sup> pDCs sorted from 36h infected *Ifnb1*<sup>Eyfp</sup>  
93 mice and cultured ex vivo for 8h harbored stable YFP expression (Extended Data Fig.  
94 1b). At 36h, 44h and 48h post-infection, both the percentage and MFI of YFP<sup>+</sup> cDCs  
95 were very low (Extended Data Fig. 1c-e), whereas around 80% of YFP<sup>+</sup> splenocytes  
96 were XCR1<sup>-</sup> CD11b<sup>-</sup> CD11c<sup>int</sup> Bst2<sup>hi</sup> (Extended Data Fig. 1f-g), therefore being bona  
97 fide CCR9<sup>+</sup> pDCs (Extended Data Fig. 1c-e). Thus, *Ifnb1*<sup>Eyfp</sup> mice allow reliable  
98 identification and fate-mapping of *Ifnb1*-producing pDCs in vivo.

99 YFP and CD86 co-expression at 36h post-infection identified three pDC  
100 subpopulations (Fig. 1c,d). In uninfected (UN) animals, most pDCs were CD86<sup>-</sup>YFP<sup>-</sup>

101 (DN cells), with the remainder being CD86<sup>+</sup>YFP<sup>-</sup> (SP cells). In infected (IN) mice, half  
102 of the pDCs expressed CD86 (IN\_SP cells), with a minority being YFP<sup>+</sup> (IN\_DP  
103 cells). pDC subpopulations were sorted by flow cytometry for gene expression  
104 profiling (Fig. 1e). Interferon-stimulated genes (ISG) were induced in all pDC  
105 subpopulations from infected mice (Fig. 1e, red names). *Cd86* induction was stronger  
106 in IN\_SP and IN\_DP pDCs (Fig. 1e), consistent with their purification as CD86<sup>+</sup>.  
107 Unexpectedly, IN\_DN and IN\_SP pDCs expressed IFN-I genes (Fig. 1e, green  
108 names), including *Ifnb1* (green arrow), and other MyD88-dependent genes (MSG)<sup>22</sup>  
109 (Fig. 1e, blue names), but at lower levels than IN\_DP cells. The IN\_DP cells  
110 specifically up-regulated genes known to be higher in steady state pDC-like cells<sup>11</sup> or  
111 tDCs<sup>13, 15</sup> (Fig. 1e, violet names), including genes involved in T cell activation by  
112 cDCs. The IN\_DP cells expressed lower *Tcf4* and higher *Id2* levels (Fig. 1e, black  
113 arrows), two transcription factors driving pDC versus cDC1 differentiation and identity  
114 maintenance respectively<sup>19, 30</sup>. All pDC subpopulations expressed high and  
115 comparable levels of genes known to be specific of steady state pDCs as compared  
116 to cDC-like cells, tDCs and cDCs<sup>10, 11, 13, 15</sup>, including *Ccr9*, *Ly6d*, *Klk1* and *Klk1b27*  
117 (Fig. 1e, bold black names). Thus, YFP<sup>-</sup> pDCs isolated from infected mice  
118 unexpectedly expressed IFN-I genes, while YFP<sup>+</sup> pDCs acquired a transcriptomic  
119 program shared with tDCs and cDCs, suggesting further heterogeneity within each  
120 pDC subpopulation.

121

## 122 **IFN-I and YFP co-expression reveals three consecutive pDC activation states.**

123 We analyzed YFP and IFN-I co-expression in splenic pDCs isolated from 36h  
124 MCMV-infected *Ifnb1*<sup>Eyfp</sup> mice (Fig. 1f). A small fraction of YFP<sup>-</sup> pDCs expressed  
125 IFN-I, albeit with a lower MFI than YFP<sup>+</sup> pDCs. Thus, IFN-I expression preceded YFP

126 detection in pDCs, likely explaining the moderate induction of IFN-I genes in IN\_DN  
127 and IN\_SP pDCs in microarray analyses. Half of YFP<sup>+</sup> pDCs did not express IFN-I,  
128 suggesting that they were in a late activation state, where they had already secreted  
129 IFN-I but retained YFP expression. Therefore, we examined IFN-I and YFP co-  
130 expression kinetically during infection (Fig. 1g-i). Whereas IFN-I expression peaked  
131 between 36h and 40h post-infection (Fig. 1g-h), the frequency of YFP<sup>+</sup> pDCs  
132 continued increasing to reach a plateau between 44h and 48h (Fig. 1g-h). Hence,  
133 within pDCs expressing IFN-I or YFP, the proportions of IFN-I<sup>+</sup>YFP<sup>-</sup> cells decreased  
134 after 33h, to become undetectable at 48h, whereas the proportions of IFN-I<sup>+</sup>YFP<sup>+</sup>  
135 cells peaked at 36h, and the proportions of IFN-I<sup>-</sup>YFP<sup>+</sup> cells increased steadily (Fig.  
136 1i). Thus, pDCs undergo a more complex activation than anticipated, encompassing  
137 at least three consecutive stages for IFN-I production. At an early stage, cytokine  
138 production starts at relatively low level as revealed by lack of YFP expression (IFN-  
139 I<sup>+</sup>YFP<sup>-</sup>). Then, high expression of both YFP and IFN-I marks pDCs at peak IFN-I  
140 production (IFN-I<sup>+</sup>YFP<sup>+</sup>). Finally, pDCs undergo a late activation stage, previously  
141 overlooked, where they have secreted IFN-I but remain fate-mapped for their  
142 expression (IFN-I<sup>-</sup>YFP<sup>+</sup>). Thus, pDCs survive more than 12h after ceasing IFN-I  
143 production, raising the question of their terminal activation state and functions.

144

145 **scRNAseq confirms the existence of different pDC activation states during**  
146 **infection.**

147 To better characterize pDC activation states without the confounding effect of  
148 cross-contamination between populations as can happen in bulk transcriptomics, we  
149 performed scRNAseq on splenic pDCs from one uninfected and one 36h MCMV-  
150 infected *Ifnb1*<sup>Eyfp</sup> mice (Fig. 2a), using Smart-seq2 (SS2)<sup>31</sup>. For the infected mouse,

151 94 pDCs were index sorted, with enrichment of YFP<sup>+</sup> pDCs to one third of all cells  
152 (SS2 dataset#1).

153 Principal component analysis (PCA) did not show heterogeneity in steady-  
154 state pDCs (Fig. 2b). In contrast, the pDCs from the infected mouse (Fig. 2c) clearly  
155 segregated along the first PCA axis (PC1) into YFP<sup>+</sup> (red dots) and YFP<sup>-</sup> (blue dots)  
156 subpopulations. Consistently, IFN-I genes and other MSG strongly contributed to  
157 PC1. Based on top PC1 genes, hierarchical clustering not only separated most YFP<sup>-</sup>  
158 from YFP<sup>+</sup> pDCs, but also split YFP<sup>+</sup> pDCs in two clusters (Fig. 2d). The first YFP<sup>+</sup>  
159 pDC cluster co-expressed *Eyfp* and IFN-I genes (Fig. 2d, green arrow and names),  
160 therefore corresponding to the peak IFN-I<sup>+</sup>YFP<sup>+</sup> activation state identified by flow  
161 cytometry. Most cells of the second YFP<sup>+</sup> pDC cluster expressed neither *Eyfp* nor  
162 IFN-I genes, or at very low levels (Fig. 2d). However, *Eyfp*<sup>-</sup>YFP<sup>+</sup> and *Eyfp*<sup>+</sup>YFP<sup>+</sup>  
163 pDCs shared the expression of other MSG, including *Il12b*, *Ccl5*, *Ccl3* and *Tnf* (Fig.  
164 2d, bold blue names). As compared to YFP<sup>-</sup> pDCs, YFP<sup>+</sup> pDCs expressed pDC-like  
165 cell/tDC/cDC genes, including *Cd83*, *Cd40* and *Ccr7* (Fig. 2d, bold violet names).  
166 Thus, the *Eyfp*<sup>-</sup>YFP<sup>+</sup> pDC cluster matched with the late IFN-I<sup>-</sup>YFP<sup>+</sup> activation state  
167 identified by flow cytometry, corresponding to pDCs that had secreted IFN-I and  
168 stopped transcribing IFN-I genes.

169 Out of the 65 YFP<sup>-</sup> pDCs, 4 segregated with YFP<sup>+</sup> pDCs, both in PCA and  
170 hierarchical clustering (Fig. 2c-d). These YFP<sup>-</sup> pDCs expressed high levels of *Eyfp*  
171 and IFN-I genes (Fig. 2d,e), confirming that detectable induction of IFN-I precedes  
172 that of YFP. Thus, *Eyfp*<sup>+</sup>YFP<sup>-</sup> pDCs corresponded to the early IFN-I<sup>+</sup>YFP<sup>-</sup> activation  
173 state identified by flow cytometry.

174 *Eyfp* and IFN-I gene expression were tightly correlated in single pDCs (Fig. 2d-  
175 e and Supplementary Table 1), consistent with the known synchronous induction of

176 all IFN-I<sup>32</sup> in a manner dependent on IRF7 but not IFN-I receptor signaling,  
177 specifically in pDCs<sup>22, 33-35</sup>. Thus, in *Ifnb1<sup>Eyfp</sup>* mice, *Eyfp* reliably reports expression of  
178 all IFN-I genes in pDCs.

179 In summary, combined expression of the *Eyfp* gene and of the YFP protein in  
180 *Ifnb1<sup>Eyfp</sup>* mice enabled accurate tracking in scRNAseq analyses of three consecutive  
181 activation states of the pDCs engaged in IFN-I production, corresponding to early  
182 *Eyfp<sup>+</sup>YFP<sup>-</sup>*, peak *Eyfp<sup>+</sup>YFP<sup>+</sup>* and late *Eyfp<sup>-</sup>YFP<sup>+</sup>* pDC clusters.

183

#### 184 **LIFR is selectively downregulated on IFN-I-expressing pDCs.**

185 The small number of *Eyfp<sup>+</sup>YFP<sup>-</sup>* pDCs limited the accuracy of the analysis of  
186 their gene expression program. Therefore, we sought for a cell surface marker to  
187 enrich them. *Cd83*, *Ccr7* and *Lifr* were differentially expressed between YFP<sup>+</sup> and  
188 YFP<sup>-</sup> pDCs. *Cd83* and *Ccr7* were induced on pDCs expressing *Ifnb1* or YFP.  
189 Reciprocally, *Lifr* was selectively downregulated in the vast majority of *Ifnb1<sup>+</sup>* pDCs  
190 regardless of their YFP expression (Fig. 2f-g). Flow cytometry analysis confirmed a  
191 stronger downregulation of LIFR on YFP<sup>+</sup> or IFN-I<sup>+</sup> pDCs (Fig. 2h-i), whereas CD83  
192 and CCR7 were induced only on a fraction of YFP<sup>+</sup> pDCs. Since IFN-I<sup>+</sup> pDCs express  
193 the highest levels of BST2 (Fig. 1a), we combined LIFR downregulation with YFP and  
194 BST2 expression for enriching *Eyfp<sup>+</sup>YFP<sup>-</sup>* pDCs (Extended Data Fig. 2a).

195 pDCs from one uninfected and one 36h MCMV-infected *Ifnb1<sup>Eyfp</sup>* mice were  
196 index-sorted with enrichment of LIFR<sup>lo</sup> cells (SS2 dataset#2). This led to a  
197 contamination of pDCs by limited numbers of pDC-like or B cells, none of which was  
198 YFP<sup>+</sup> (Supplementary Data 1, Extended Data Fig 2b-f). The expression of *Eyfp* and  
199 IFN-I genes were again tightly correlated (Supplementary Table 2). The remaining

200 cells were bona fide pDCs expressing high levels of key pDC signature genes,  
201 including *Klk1*, *Siglech*, *Pacsin1*, *Dntt* and *Irf8* (Extended Data Fig 2f, black arrows),  
202 but low levels of the genes selective of lymphocytes<sup>36</sup>, B cells, plasma cells<sup>37</sup> or pDC-  
203 like cells<sup>11</sup> (Extended Data Fig 2f).

204       Upon UMAP dimensionality reduction and graph-based cell clustering (Fig.  
205 3a), the pDCs from the uninfected mouse regrouped together (C0) remotely from the  
206 7 clusters derived from the pDCs of the infected mouse. Projection on the UMAP of  
207 the expression levels of YFP (Fig. 3b), *Eyfp* (Fig. 3c) and *Ifnb1* (Fig. 3d) identified the  
208 clusters corresponding to the three pDC activation states uncovered in our previous  
209 experiments: *Eyfp*<sup>+</sup>YFP<sup>-</sup> (C5), *Eyfp*<sup>+</sup>YFP<sup>+</sup> (C6) and *Eyfp*<sup>-</sup>YFP<sup>+</sup> (C7). *Eyfp*<sup>+</sup>YFP<sup>-</sup> C5  
210 cells expressed *Eyfp* and most IFN-I genes, although at lower levels than the  
211 *Eyfp*<sup>+</sup>YFP<sup>+</sup> C6 cells (Fig. 3e; green names on Fig. 3f). C5 represented 16% of the  
212 LIFR<sup>lo</sup>BST2<sup>hi</sup> pDCs from the infected mouse (Supplementary Table 3), and  
213 encompassed 88% of LIFR<sup>lo</sup>BST2<sup>hi</sup> sorted pDCs. LIFR expression was  
214 downregulated most strongly in clusters C5, C6 and C7 (Fig. 3g-h), thus helping to  
215 discriminate C5 *Eyfp*<sup>+</sup>YFP<sup>-</sup> cells from all other YFP<sup>-</sup> cells. In contrast to steady state  
216 pDCs (C0), all pDC clusters from the infected mouse (C1-C7) harbored high  
217 expression of *Oas1* (Fig. 3i) and other ISG (Fig. 3f, red names), confirming that all  
218 individual pDCs had responded to IFN-I at 36h post-infection. The expression of  
219 *IL12b*, *Ccr7* and several other genes occurred in C6 rather than C5 and was  
220 maintained in C7, thus being delayed and prolonged as compared to IFN-I genes  
221 (Fig. 3f, violet names; Fig. 3i).

222       In summary, our enrichment strategy of LIFR<sup>lo</sup> cells improved recovery of the  
223 rare *Eyfp*<sup>+</sup>YFP<sup>-</sup> pDCs, enabling the generation of a more robust scRNAseq dataset to  
224 characterize these cells including their relationships with other pDC activation states.

225

## 226 **Reconstruction of the pDC activation trajectory.**

227 We used Monocle to infer a pseudo-temporal sequence of pDC activation  
228 states during MCMV infection (Fig. 4a), which yielded a distinctive linear branch  
229 ordering pDCs clusters along pseudo-time in the following sequence:  $Eyfp^-YFP^-$  (C2)  
230  $\rightarrow Eyfp^+YFP^-$  (C5)  $\rightarrow Eyfp^+YFP^+$  (C6)  $\rightarrow Eyfp^-YFP^+$  (C7) (Fig. 3a and Fig. 4a). We  
231 defined an IFN-I meta-gene and plotted its expression along pseudo-time in  
232 comparison with that of other genes or of phenotypic markers. Transcription of the  
233 IFN-I meta-gene started around pseudo-time 10, preceding detectable expression of  
234 YFP around pseudo-time 12 (Fig. 4b, upper graph). At later pseudo-time, expression  
235 of the IFN-I meta-gene stopped whereas YFP expression remained high. LIFR  
236 expression decreased to reach its minimal plateau around pseudo-time 10,  
237 simultaneously to the initiation of IFN-I meta-gene expression (Fig. 4b, lower graph).  
238 Hence, pseudo-time analysis fits with delayed but prolonged expression of YFP, as  
239 compared to IFN-I transcription, and with LIFR downregulation upon pDC  
240 engagement into IFN-I production.

241 For independent trajectory inference, we used the Velocity software, which  
242 computes for each cell a vector predicting its future state by precisely defining and  
243 integrating the transcriptional states of all the cells from the dataset, based on their  
244 unspliced/spliced mRNA ratio for many genes<sup>38</sup>. The projection of Velocity vectors  
245 onto the UMAP space revealed the predicted pDC activation trajectory (Fig. 4c). YFP  
246 expression was not taken into account in this model, and neither were *Eyfp* and IFN-I  
247 genes since they are intron-less. Yet, Velocity predicted the same activation  
248 trajectory (Fig. 4c) as Monocle (Fig. 4a) and YFP/IFN-I co-expression analysis (Fig.  
249 1g-i).

250 RNA Velocity unraveled a new pDC activation state among C2, immediately  
251 preceding the initiation of IFN-I production corresponding to C5. We manually  
252 assigned these pDCs to “sub-cluster 8” (Fig. 4c, dotted black ellipse). Consistent with  
253 their location in the Velocyto activation trajectory, sub-cluster 8 pDCs presented a  
254 distribution of Monocle pseudo-time values higher than those of other C2 pDCs and  
255 just below those of C5 *Eyfp*<sup>+</sup>YFP<sup>-</sup> pDCs (Fig. 4d), further illustrating the convergence  
256 and complementarity between Velocyto and Monocle for trajectory inference.

257 Both Monocle (Fig. 4e, Extended Data Fig. 2g) and Velocyto (Fig. 4f,  
258 Extended Data Fig. 2h) predicted similar waves of gene expression along the pDC  
259 activation trajectory. *Tnf* and *Ccl3* induction preceded that of IFN-I genes, or of their  
260 most correlated intron-bearing genes such as *Kdm6b*. *Il12b* transcription started later,  
261 followed by that of *Ccr7*, both reaching their maximum at the latest pseudo-times. As  
262 compared to the other C2 pDCs, sub-cluster 8 pDCs specifically expressed *Egr1*,  
263 also detected in the C5 *Eyfp*<sup>+</sup>YFP<sup>-</sup> pDCs. Consistently, the pseudo-temporal analysis  
264 showed that *Egr1* expression pattern resembled and closely preceded that of the  
265 IFN-I meta-gene (Fig. 4g, top graph). *Egr1* is a transcription factor induced early in  
266 response to TLR triggering<sup>39, 40</sup>. Hence, *Egr1* expression is likely an early and  
267 transient marker of the initial sensing of the viral infection by individual pDCs, and  
268 might promote their IFN-I production. Finally, Monocle showed a late induction of *Id2*  
269 (Fig. 4g, bottom graph), which might have contributed to cDC gene induction in *Eyfp*<sup>-</sup>  
270 YFP<sup>+</sup> pDCs.

271 We analyzed the kinetic of IFN-I, YFP and IL-12 expression in pDCs during  
272 infection (Extended Data Fig. 3). IL-12 peak production was reached later than that of  
273 IFN-I (Extended Data Fig. 3a-b), and, at each time point analyzed, most IL-12<sup>+</sup> pDCs  
274 were YFP<sup>+</sup> (Extended Data Fig. 3c). Thus, these results confirm at the protein level

275 that pDC IL-12 production during infection occurred mostly in the cells that expressed  
276 IFN-I, but with a delay and for a longer time window.

277 We attempted characterizing by scRNAseq the activation trajectory of splenic  
278 pDCs from 36h MCMV-infected WT C57BL/6 mice, upon harnessing LIFR  
279 downregulation to enrich the pDCs engaged in IFN-I production. We used a different  
280 method for library preparation, FB5P-seq<sup>41</sup>, that overcomes the throughput limitations  
281 of SS2, while keeping a better sensitivity for low copy number gene detection than  
282 high-throughput droplet-based methods. We index-sorted and analyzed 188 pDCs  
283 from one uninfected mouse, and 468 pDCs from a 36h infected animal split in 2/3  
284 LIFR<sup>hi</sup> and 1/3 LIFR<sup>lo</sup> pDCs (FB5P C57BL/6 dataset, Extended Data Fig. 4). Monocle  
285 analysis resulted in a pDC activation trajectory bifurcating in two main branches, Y53  
286 and Y50. The Y53 branch ended with IFN-I-expressing pDCs and harbored a  
287 stronger downregulation of LIFR shortly after its bifurcation from the Y50 branch  
288 (Extended Data Fig. 4a-c). The gene expression pattern of the IFN-I-producing pDCs  
289 from WT C57BL/6 mice was very similar to that observed in *Ifnb1*<sup>Eyfp</sup> mice, with an  
290 early induction of *Ccl3* preceding that of the IFN-I genes, whereas *Il12b* and *Ccr7*  
291 expression were maximal at late pseudo-times (Extended Data Fig. 4b,d).

292 In conclusion, three independent methods based on distinct information and  
293 different methodologies, flow cytometry, Monocle and Velocyto, converged onto the  
294 same pDC activation trajectory during infection, both in *Ifnb1*<sup>Eyfp</sup> reporter and WT  
295 mice, with *Ccl3* and *Tnf* initial induction preceding the transient expression of IFN-I  
296 genes that was followed by prolonged *Il12* expression and late *Ccr7* induction.

297

298 **An independent kinetic scRNAseq dataset confirms and extends the pDC**  
299 **activation trajectory.**

300 To reinforce our results, we performed another scRNAseq experiment using  
301 pDCs harvested at different times after infection: 0h, 33h, 36h and 48h (FB5P kinetic  
302 dataset). We surmised that using LIFR downregulation on pDCs from WT animals  
303 may not guarantee sufficiently unbiased and efficient sampling of the different pDC  
304 activation states for robust inference of their activation trajectories. Therefore, we  
305 used *Ifnb1<sup>Eyfp</sup>* mice. Total pDCs were sorted at 0h. For the other time points, we  
306 sorted LIFR<sup>lo</sup> pDCs for enriching cells engaged in IFN-I production, as well as YFP<sup>+</sup>  
307 and bulk pDCs (Extended Data Fig. 5a-b). We removed contaminating pDC-like and  
308 B cells (Extended Data Fig. 5b-d).

309 Monocle identified 15 pDC clusters (Fig. 4h) and inferred an activation  
310 trajectory encompassing 4 branches (Fig. 4i). The pDCs from the uninfected mouse  
311 and most of those from the 48h infected animal were at opposite ends of the longest  
312 branch, whereas the pDCs from 33h and 36h infected animals were spread in-  
313 between (Fig. 4i-j). The pseudo-time analysis for this dataset confirmed the  
314 progression of pDC activation from “early” (cluster 15, *Eyfp*<sup>+</sup>YFP<sup>-</sup>) to “peak” (clusters  
315 4, *Eyfp*<sup>hi</sup>YFP<sup>+</sup> and 7, *Eyfp*<sup>hi</sup>YFP<sup>hi</sup>) to “late” (clusters 8, *Eyfp*<sup>lo</sup>YFP<sup>hi</sup> and 9, *Eyfp*<sup>-</sup>YFP<sup>hi</sup>)  
316 states (Fig. 4k). IFN-I transcription started around pseudo-time 25, preceding  
317 detectable YFP expression, and stopped around pseudo-time 35 (Fig. 4l) at the  
318 transition from C7 to C8 (Fig. 4k), whereas YFP remained expressed at high levels  
319 until late pseudo-times in C8 and C9 (Fig. 4k-l). The activation state inferred as the  
320 initiation phase of IFN-I production (C15) was more represented in pDCs harvested  
321 at the earliest time points after infection (33h and 36h) and had disappeared at 48h  
322 (Fig. 4m). Conversely, the late activation states corresponding to post-IFN-I

323 production (C8 and C9) were absent at 33h and increased at the latest time points  
324 (Fig. 4m).

325 Velocityto confirmed in part the trajectory inferred from Monocle (C15,  
326  $Eyfp^+YFP^- \rightarrow C4$ ,  $Eyfp^{hi}YFP^+ \rightarrow C7$ ,  $Eyfp^{hi}YFP^{hi}$ ), as well as C9 as one endpoint of  
327 pDC activation (Extended Data Fig. 5e). Results were less clear for the relationships  
328 between C7, C8 and C9. However, computing Jaccard indexes to determine  
329 equivalencies between the pDC clusters of the FB5P kinetic dataset and SS2  
330 dataset#2 showed good consistency (Fig. 4n). Consistent with its late pseudo-time  
331 and detection only at 48h post-infection, the FB5P C9  $Eyfp^-YFP^{hi}$  pDC cluster did not  
332 strongly overlap with a specific 36h SS2 cluster (Fig. 4m).

333 In summary, this kinetic analysis confirmed and extended the interpretation  
334 drawn from the 36h dataset, showing that, upon commitment to IFN-I production,  
335 individual pDCs sequentially underwent 5 distinct activate states:  $Eyfp^{-/lo}YFP^-$  (FB5P  
336 C15, SS2 C8)  $\rightarrow Eyfp^{hi}YFP^{-/int}$  (FB5P C4, SS2 C5)  $\rightarrow Eyfp^{hi}YFP^{hi}$  (FB5P C7, SS2 C6)  
337  $\rightarrow Eyfp^{lo/-}YFP^{hi}$  (FB5P C8, SS2 C7)  $\rightarrow Eyfp^-YFP^{hi}$  (FB5P C9).

338

### 339 **IFN<sup>-</sup>YFP<sup>+</sup> end-matured pDCs transcriptionally converge towards tDCs.**

340 We examined in the FB5P kinetic dataset the expression along pseudo-time of  
341 genes known to be expressed at steady state to higher levels in pDCs over tDCs  
342 (“pDC-versus-tDC\_UP” meta-gene) or conversely (“pDC-versus-tDC\_DN” meta-  
343 gene). The latest IFN<sup>-</sup>YFP<sup>+</sup> pDC activation state (FB5P C9, “end-matured”)  
344 transcriptionally converged towards tDCs (Fig. 5a). However, all pDCs expressed the  
345 pDC meta-gene at a higher level than the tDC meta-gene (Fig. 5a), and maintained a  
346 distinctive expression of pDC signature genes including *Ly6d*, *Ly6a*, *Klk1*, *Klk1b27*,

347 and *Ccr9* to some extent (Fig. 5b). Some pDC-like cell or tDC genes were induced to  
348 high levels in a sizeable fraction of end-matured pDCs (Fig. 5c), which also harbored  
349 a decreased *Tcf4* and increased *Id2* expression (Fig. 5d). Hence, the end-matured  
350 pDCs did not convert into pDC-like cells/tDCs. Rather, the pDCs engaged into IFN-I  
351 production progressively converged transcriptionally toward tDCs/cDCs, already  
352 starting at the time of their peak IFN-I production.

353

354 **GSEA-based inference of the molecular regulation and functional**  
355 **specialization of pDC activation states.**

356 We performed a Gene Set Enrichment Analysis (GSEA) using BubbleGUM<sup>42</sup>, to  
357 identify candidate biological processes or upstream regulators associated to gene  
358 modules induced or repressed in the FB5P kinetic dataset along the pDC activation  
359 trajectory (Fig. 6a). The expression of selected meta-genes (Fig 6b) and of one of  
360 their representative genes (Fig. 6c, e) were plotted along pseudo-time. *Egr1* was  
361 selectively expressed in C15, with a transient induction around pseudo-time 25 (Fig.  
362 6c), consistent with its pattern of expression in the SS2 dataset#2 (Fig. 4g). ISG were  
363 induced strongly early on, around pseudo-time 5 (cluster C1), followed by a further  
364 increase centered around pseudo-time 20 (C3), a decrease most pronounced around  
365 pseudo-time 35 (C7), and a late re-expression (C8 and C9), as illustrated with *Oas12*  
366 (Fig. 6c). A similar expression pattern was observed for the genes induced by IFN- $\gamma$   
367 (Fig. 6a-c, e). The genes associated with TNF and NF- $\kappa$ B signaling also followed an  
368 induction pattern in three waves, but with a delayed central wave reaching its peak  
369 immediately before maximal expression of IFN-I genes, around pseudo-time 25 (C15)  
370 (Fig. 6a-c, e). This suggested that TNF signaling through NF- $\kappa$ B might be one of the  
371 early signals that pDCs need to receive together with TLR9 triggering to become high

372 IFN-I producers. The last wave of TNF response encompassed genes associated  
373 with antigen processing and presentation, including *Cd83* (Fig. 6c), *Cd80*, *Il12b*,  
374 *Relb*, *Rel*, *Tap1* and *Tapbp* (Fig. 6e, bold violet names). This implied that TNF could  
375 be involved later in shutting off IFN-I production by pDCs and promoting their end  
376 maturation, as previously reported for human pDCs<sup>4, 17, 43</sup>.

377 The expression of gene modules associated with antigen processing and  
378 presentation decreased progressively from early pseudo-time, until minimal  
379 expression coinciding with peak expression of the IFN-I genes, followed by a sharp  
380 increase at late pseudo-time (Fig. 6a, d-e). This is consistent with the transcriptomic  
381 convergence of end-matured pDCs with tDCs/cDCs (Fig. 5). Genes contributing to  
382 oxidative phosphorylation followed the same expression pattern (Fig. 6a, e),  
383 suggesting different metabolic regulation of IFN-I production versus cognate  
384 interaction with T cells in pDCs.

385

### 386 **Cell-intrinsic TNF signaling promotes IFN-I production by pDCs.**

387 Because *Tnf* expression and transcriptomic responses to TNF were induced in  
388 pDCs earlier than IFN-I genes, we tested whether TNF activity promoted pDC IFN-I  
389 production. In vivo antibody blockade of the TNF receptor did significantly reduce the  
390 frequency of IFN-I<sup>+</sup> pDCs at 36h post-infection (Fig. 7a-b). To test whether this  
391 process was due to cell-intrinsic TNF responses in pDCs, we examined IFN-I  
392 production in CD45.1 versus CD45.2 pDCs from CD45.1<sup>+</sup> WT: CD45.2<sup>+</sup> WT versus  
393 CD45.1<sup>+</sup> WT: CD45.2<sup>+</sup> *Tnfr1*<sup>-/-</sup> *Tnfr2*<sup>-/-</sup> (TNFRDKO) mixed bone marrow chimera  
394 mice infected with MCMV (Fig. 7c-d). We observed a significant decrease in the  
395 CD45.2<sup>+</sup> to CD45.1<sup>+</sup> ratio within IFN-I<sup>+</sup> pDCs in the TNFRDKO MBMC mice as

396 compared to their CTRL counterparts (Fig. 7d). Thus, cell-intrinsic TNF signaling is  
397 necessary for optimal IFN-I production by pDCs.

398

### 399 **YFP<sup>+</sup>CCR7<sup>+</sup> pDCs harbor phenotypic and functional features of mature DCs.**

400 Our scRNAseq data suggested sequential induction in single pDCs first of  
401 IFN-I genes, transiently, and then of genes modules associated with antigen  
402 processing and presentation or transcriptional convergence towards tDCs. We thus  
403 analyzed the phenotype, functions and anatomical location of the early, peak and late  
404 IFN-I-producing pDC activation states, to investigate their abilities to activate T  
405 lymphocytes.

406 A fraction of YFP<sup>+</sup> pDCs transiently upregulated CCR7 between 36h and 44h  
407 after MCMV infection (Fig. 8a-b), corresponding mostly to cells that were terminating  
408 or had stopped IFN-I production (Fig. 8c). Since CCR7 in DCs promotes their  
409 migration to the T cell zones of secondary lymphoid organs for T cell activation, we  
410 compared the expression of T cell regulatory signals by steady state pDCs and by  
411 the three final activation states of the pDCs engaged in IFN-I production in 36h  
412 infected mice (Fig. 8d). CCR7 expression was significantly higher on IFN-I<sup>-</sup>YFP<sup>+</sup>  
413 pDCs than on IFN-I<sup>-</sup>YFP<sup>-</sup>, IFN-I<sup>+</sup>YFP<sup>-</sup> and IFN-I<sup>+</sup>YFP<sup>+</sup> pDCs. We sorted YFP<sup>-</sup>, YFP<sup>+</sup>  
414 CCR7<sup>-</sup>, and YFP<sup>+</sup> CCR7<sup>+</sup> pDCs from 36h infected *Ifnb1<sup>Eyfp</sup>* mice, cultured them for 8h  
415 ex vivo in feeder CD45.1 bone marrow FLT3-L cultures and assessed whether their  
416 phenotype changed (Extended Data Fig. 6). All of the YFP<sup>+</sup> CCR7<sup>-</sup> pDCs increased  
417 their expression of CCR7, with 37% becoming strongly positive, whereas YFP<sup>+</sup>  
418 CCR7<sup>+</sup> pDCs maintained a stable phenotype. A very small proportion of the YFP<sup>-</sup>  
419 pDCs became YFP<sup>+</sup>. Thus, this experiment confirmed the transition from YFP<sup>+</sup>

420 CCR7<sup>-</sup> to YFP<sup>+</sup> CCR7<sup>+</sup> pDC activation states, and the lack of reverse transition. The  
421 YFP<sup>+</sup> CCR7<sup>+</sup> pDCs also expressed higher levels of MHC-II, CD80, CD86, CD40 and  
422 PD-L1 (Fig. 8d). Hence, after termination of their IFN-I secretion, pDCs likely acquire  
423 the ability to engage CD4 T cells in an antigen-specific manner, by up-regulating the  
424 three signals required for this function.

425 We assessed the ability of different pDC activation states to induce the  
426 proliferation of CD4 T cells in an antigen-specific manner, as compared to pDCs from  
427 uninfected mice that are quite limited in this function. As CCR7 was upregulated  
428 preferentially on IFN-I<sup>-</sup>YFP<sup>+</sup> pDCs (Fig. 8c), as a surrogate purification strategy for  
429 this activation state we sorted YFP<sup>+</sup>CCR7<sup>+</sup> pDCs from 38h MCMV-infected mice. We  
430 sorted in parallel YFP<sup>-</sup>CD86<sup>-</sup>, YFP<sup>-</sup>CD86<sup>+</sup> and YFP<sup>+</sup>CCR7<sup>-</sup> pDCs (Fig. 8e).  
431 YFP<sup>+</sup>CCR7<sup>+</sup> pDCs were the only population harboring a significant higher ability for  
432 CD4 T cell activation than pDCs from uninfected mice (Fig. 8f). We evaluated by  
433 confocal microscopy whether and when pDCs migrated in the T cell area of the  
434 spleen of MCMV-infected mice. At 36h post-infection, splenic YFP<sup>+</sup> cells were in a  
435 large part IFN-I<sup>+</sup> (Fig. 8g and Extended Data Fig. 7a-c), and mainly located in the  
436 marginal zone (Fig. 8g-j and Extended Data Fig. 7d) as previously published<sup>3, 22</sup>. In  
437 contrast, at 48h, splenic YFP<sup>+</sup> cells were mostly IFN<sup>-</sup> and located in the T cell zone  
438 (Fig. 8g-k and Extended Data Fig. 7d). Hence, our scRNASeq, flow cytometry,  
439 functional and microscopy data converge towards the conclusion: during MCMV  
440 infection, after having produced IFN-I, pDCs undergo further activation promoting  
441 their cognate interactions with CD4 T cells. Our study therefore supports a novel  
442 model of the molecular and spatiotemporal regulation of pDC functions during viral  
443 infections (Extended Data Fig. 8).

## 444 Discussion

445 It has been claimed that pDCs only produce IFN-I, because the pDC-like cells  
446 or tDCs that can contaminate them fully accounted for their antigen presentation<sup>8-15</sup>.  
447 However, under proper stimulation conditions, bona fide pDCs can exert both  
448 functions<sup>16, 17</sup>. Different models have attempted to explain how. First, pDC functional  
449 plasticity could be developmental, with prominent IFN-I production by bone marrow  
450 pDC precursors, before their terminal differentiation inducing their egress to the  
451 periphery and switch to antigen presentation<sup>18</sup>. Alternately, pDC functional plasticity  
452 could be instructed by stimulation. IFN-I production versus antigen presentation could  
453 be induced in pDCs by distinct, eventually antagonist, stimuli<sup>44</sup>, or reflect the  
454 induction of a division of labor by a single stimulus. Indeed, in vitro exposure to the  
455 influenza virus can instruct initially identical human pDCs to diversify into distinct and  
456 non-interconvertible activation states, with some pDCs producing IFN-I and others  
457 presenting antigens<sup>17</sup>. Finally, antigen presentation by pDCs was proposed to result  
458 from their cell fate conversion into cDC-like cells/tDCs<sup>19, 20, 45</sup>, due to an activation-  
459 induced inversion of their *Tcf4/Id2* expression ratio. However, the studies supporting  
460 these models were performed in vitro or upon genetic inactivation of *Tcf4*, and under  
461 conditions unable to assess dynamically the fate of individual pDCs. Rigorously  
462 characterizing pDC functional plasticity and cell fate during viral infections in vivo thus  
463 remains an important challenge<sup>19, 20</sup>, which we addressed by combining fate-mapping  
464 of individual pDCs for IFN-I production with flow cytometry, scRNAseq,  
465 immunohistological imaging and an ex vivo assay for CD4 T cell priming.

466 We identified different pDC activation states during MCMV infection and  
467 deciphered their relationships, showing that the same individual pDCs first produce

468 IFN-I and then activate T cells in the spleen of infected mice, contrary to what was  
469 proposed in the previous models of pDC plasticity discussed above.

470 Steady state pDCs were transcriptionally homogeneous, as previously  
471 reported<sup>9, 11, 14, 46</sup>. Thus, it is unlikely that the minor pDC fraction producing IFN-I upon  
472 activation corresponds to a distinct subset already primed for this function at steady  
473 state, at least not in a manner detectable transcriptionally.

474 A recent study reported the gene expression profiles and anatomical locations  
475 of YFP<sup>+</sup> versus YFP<sup>-</sup> pDCs from CpG-injected *Ifnb1<sup>Eyfp</sup>* mice<sup>47</sup>. YFP<sup>+</sup> pDCs  
476 upregulated CCR7, were located in the T cell zone and strongly expressed genes  
477 coding for T cell-activating cytokines. Hence, professional IFN-I-producing cells were  
478 proposed to be a distinct and polyfunctional pDC subset, specialized in  
479 simultaneously producing many cytokines, in lymphoid organ T cell zones, to  
480 coordinate innate and adaptive cellular immunity<sup>47</sup>. However, this study was  
481 performed at bulk level and only at one time point, preventing any definite conclusion  
482 on gene co-expression in single pDCs and on their ability to simultaneously perform  
483 different functions. In contrast, here we show that the YFP<sup>-</sup> and YFP<sup>+</sup> pDC  
484 populations are both heterogeneous, encompassing distinct activation states, and  
485 that, in vivo during MCMV infection, IFN-I production by pDCs is dissociated in time  
486 and space from their acquisition of the ability to interact with T cells in a cognate  
487 manner.

488 We identified LIFR downregulation as a novel phenotypic marker of pDC  
489 commitment to IFN-I production. LIFR is specifically expressed by pDCs and can  
490 inhibit their IFN-I production<sup>48</sup>. Hence, the failure of most pDCs to strongly  
491 downregulate LIFR during infection may contribute inhibiting their IFN-I production.

492 Pseudo-time analysis identified gene modules dynamically regulated along the  
493 pDC activation trajectory. Genes in the TNF-to-NFKB signaling pathway were  
494 induced early during pDC commitment to IFN-I production, consistent with the cell-  
495 intrinsic role of *Ikkkb* in promoting this function<sup>22</sup> and suggesting that it occurs in part  
496 downstream to TNF stimulation which we proved experimentally. Yet, IFN-I and TNF  
497 play antagonistic roles in health and disease<sup>43</sup>. Autocrine TNF production by human  
498 pDCs in vitro does not impede their simultaneous production of IFN-I, whereas  
499 exposure to exogenous TNF prior to TLR triggering does<sup>43</sup>. TNF promotes pDC  
500 maturation<sup>4, 17, 43</sup>, which is tightly associated with IFN-I production shutdown, as  
501 reported on bulk pDCs<sup>44</sup> and here at the single-cell level. We observed three  
502 consecutive waves of TNF responses in pDCs during infection. The last wave  
503 correlated with the induction in pDCs of genes associated to antigen processing and  
504 presentation. Hence, TNF may play different roles on pDCs depending on its origin,  
505 concentration and timing of delivery as compared to that of other signals. We  
506 identified transient *Egr1* induction, and shutdown of oxidative phosphorylation, as  
507 candidate mechanisms for the induction and termination of pDC IFN-I production,  
508 respectively. Further studies are warranted to test these hypotheses.

509 Unexpectedly<sup>19, 49</sup>, pDCs did not die early after termination of their IFN-I  
510 production. They survived for over 12 hours, producing IL-12, upregulating CCR7 and  
511 acquiring the ability to activate naïve CD4<sup>+</sup> T cells. We identified a novel end-mature  
512 state of activated pDCs, resulting from a progressive transcriptional convergence  
513 towards tDCs, which started at the state of peak IFN-I production and occurred in  
514 parallel to an inversion of the *Tcf4/Id2* expression ratio. This physiological  
515 observation is reminiscent of the acquisition of cDC features caused in pDCs by their  
516 genetic inactivation for *Tcf4*<sup>50</sup>. However, end-matured pDCs maintained a clear

517 imprint of their lineage identity, rather than undergoing the proposed cell fate  
518 conversion into tDCs<sup>45</sup>.

519 In conclusion, our study uncovered the dynamic activation trajectory of pDCs  
520 during MCMV infection in vivo, leading us to propose a novel model of the functional  
521 plasticity of pDCs, explaining how individual pDCs exert different functions in a  
522 manner tightly orchestrated in time and space. IFN-I production is exerted first,  
523 transiently, at the site of virus replication in the marginal zone of the spleen, whereas  
524 antigen presentation occurs later, after a further activation of pDCs leading to their  
525 transcriptional, phenotypic and functional convergence towards tDCs, including their  
526 migration to the splenic T cell zone. Future work will examine whether similar pDC  
527 activation trajectories occur during other systemic viral infections, including in  
528 primates. Once suitable mouse models have been generated<sup>21</sup>, it will be possible to  
529 assess the importance of antigen presentation by pDCs for shaping antiviral adaptive  
530 immunity.

531

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556

557 **Author contributions**

558 Studies were designed by E.T. , M.D. , A.A. and T.-P.V.M., with help from A-C.V and  
559 P.M. Experiments were performed by A.A., E.T., M.V., N.C. and N.A., with help from  
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564

565 **Competing interests**

566 The authors declare no competing interests.

567

568 **Online content**

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573

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- 696

697 **Figure legends**

698

699 **Fig. 1. Bulk transcriptional profiling suggests the induction of distinct pDC**  
700 **activation states *in vivo* during MCMV infection. a**, pDC gating strategy within live  
701 single-cells. **b**, Percentages of IFN-I<sup>+</sup> cells within pDCs at indicated time points  
702 after MCMV infection. \*p<0.05, \*\*p<0.01 (One-way ANOVA). **c**, YFP and CD86 co-  
703 expression in pDCs isolated from uninfected (UN) or 36h MCMV-infected (IN)  
704 *Ifnb1<sup>EYFP</sup>* mice. **d**, Percentages of subpopulations within splenic pDCs of 36h MCMV-  
705 infected *Ifnb1<sup>EYFP</sup>* mice. The data are shown for n=9 individual mice pooled from 3  
706 independent experiments. **e**, Heatmap showing mRNA expression levels of selected  
707 genes (rows) across pDC subpopulations (columns), with hierarchical clustering  
708 using City block distance for cells and Euclidian distance for genes. **f**, Co-expression  
709 of IFN- $\alpha/\beta$  and YFP in pDCs isolated from one representative 36h MCMV-infected  
710 *Ifnb1<sup>Eyfp</sup>* mouse. **g**, Co-expression of IFN- $\alpha/\beta$  and YFP in pDCs isolated from *Ifnb1<sup>Eyfp</sup>*  
711 mice, at 0h (UN), 33h, 36h, 40h, 44h and 48h after MCMV infection. For each time  
712 point, one representative mouse is shown. **h**, Proportions of IFN- $\alpha/\beta$ <sup>+</sup>YFP<sup>-</sup>, IFN-  
713  $\alpha/\beta$ <sup>+</sup>YFP<sup>+</sup> and IFN- $\alpha/\beta$ <sup>-</sup>YFP<sup>+</sup> cells amongst pDCs, at indicated time points. **i**, Pie  
714 charts recapitulating the mean proportions of cells expressing IFN-I and/or YFP (see  
715 color key) amongst pDC positive for either molecule at different time points during the  
716 course of MCMV infection in *Ifnb1<sup>Eyfp</sup>* mice. For panels b, d and h, data are presented  
717 as mean $\pm$ s.e.m, and for panel i as mean percentage. Panels b and f-i show data from  
718 individual mice, with n=5 at 0h, 7 at 33h, 10 at 36h, 5 at 40h, and 3 at 44h and 48h,  
719 pooled from 2 (resp. 3) independent experiments for 33h and 40h (resp. 0h and 36h).  
720 One experiment was performed for 44h and 48h.

721

722 **Fig. 2. scRNAseq analysis of pDCs from 36h MCMV-infected mice confirms**  
723 **their heterogeneity and pinpoints to LIFR downregulation as a selective marker**  
724 **of IFN-I-producing pDCs. a**, Flow cytometry gating strategy for index sorting of pDC  
725 from one uninfected mouse (top) and from one 36h MCMV-infected mouse (bottom).  
726 Numbers in parentheses correspond to the number of cells sorted for each  
727 population. **b-c**, Principal component analysis (PCA) on 1,016 highly variable genes  
728 of single pDCs isolated from uninfected, **b**, or from one MCMV-infected mice (SS2  
729 dataset#1), **c**, encompassing 29 YFP<sup>+</sup> cells (red) and 65 YFP<sup>-</sup> cells (blue). **d**,  
730 Heatmap showing mRNA expression of selected genes, and YFP protein  
731 fluorescence intensity obtained from index sorting data, with hierarchical clustering  
732 using one minus Pearson correlation as distance metric for both cells and genes. **e**,  
733 Scatter plots showing *Ifnb1* expression vs YFP protein fluorescence intensity (left), or  
734 *Ifnb1* vs *Eyfp* mRNA expression (right). **f**, Violin plot showing *Lifr* expression in YFP<sup>-</sup>  
735 vs YFP<sup>+</sup> pDCs. **g**, Scatter plot showing *Ifnb1* vs *Lifr* mRNA expression. **h**, Flow  
736 cytometry analysis showing the downregulation of LIFR protein expression on IFN-  
737  $\alpha/\beta^+$  (middle) or YFP<sup>+</sup> (right) pDC isolated from 36h MCMV-infected *Ifnb1*<sup>EYFP</sup> mice,  
738 as compared to pDC isolated from uninfected animals (left). The dot plots shown are  
739 from one mouse representative of 10 animals. **i**, Relative median fluorescence  
740 intensity (MFI) of LIFR on IFN- $\alpha/\beta^-$ YFP<sup>-</sup> (black), IFN- $\alpha/\beta^+$ YFP<sup>-</sup> (blue), IFN- $\alpha/\beta^+$ YFP<sup>+</sup>  
741 (green) and IFN- $\alpha/\beta^-$ YFP<sup>+</sup> (pink) populations. The data are shown for n=10 individual  
742 animals pooled from 2 independent experiments, with overlay of mean $\pm$ s.e.m. values.  
743 \*\*\*\*p<0.0001 (One-way ANOVA with Tukey's post hoc test).

744

745 **Fig. 3. scRNAseq analysis identifies 8 different pDC activation states in vivo**  
746 **during MCMV infection. a**, Dimensional reduction performed using the UMAP

747 algorithm, and graph-based cell clustering, for 264 bona fide pDCs isolated from one  
748 control and one 36h MCMV-infected mice (SS2 dataset#2). **b**, Inverse hyperbolic  
749 arcsine (asinh) fluorescence intensity of YFP projected on the UMAP space. **c-d**,  
750 Expression of *Yfp* and *Ifnb1* on the UMAP space. **e**, Violin plots showing mRNA  
751 expression profiles of selected genes across all individual cells and in comparison  
752 between the clusters identified in (**a**), cluster 0= UN pDCs, clusters 1-4= MCMV *Eyfp*<sup>-</sup>  
753 YFP<sup>-</sup> pDCs, cluster 5= MCMV *Eyfp*<sup>+</sup>YFP<sup>-</sup> pDCs, cluster 6= MCMV *Eyfp*<sup>+</sup>YFP<sup>+</sup> pDCs,  
754 cluster 7= MCMV *Eyfp*<sup>-</sup>YFP<sup>+</sup> pDCs. **f**, Heatmap showing mRNA expression levels of  
755 selected genes (rows) across individual pDCs (columns), with hierarchical clustering  
756 using one minus Spearman rank correlation for cells and Kendall's tau distance for  
757 genes. The top differentially expressed genes between Seurat clusters are shown, as  
758 well as representative ISG and pDC-specific genes. *B2m* was included as an  
759 invariant control housekeeping gene. YFP and LIFR protein fluorescence intensities,  
760 obtained from index sorting data, are also shown on the top of the heatmap, as well  
761 as the infection status of the mice from which the pDCs were isolated, and the  
762 belonging of individual pDCs to the Seurat cell clusters. **g**, LIFR expression intensity  
763 projected on the UMAP space. **h**, Violin plots showing asinh fluorescence intensity of  
764 EYFP and LIFR across Seurat clusters. **i**, Violin plots showing mRNA expression  
765 profiles of selected genes across Seurat clusters.

766

767 **Fig. 4. Inference of the pDC activation trajectory during MCMV infection. a**,  
768 Monocle pseudo-temporal inference of the pDC activation trajectory for the SS2  
769 dataset#2. **b**, Expression of the YFP or LIFR proteins vs the IFN-I meta-gene along  
770 pseudo-time. Dots correspond to individual cells and a 6-order polynomial curve was  
771 fit to the data. **c**, RNA Velocity reconstruction of the pDC activation trajectory.

772 Projections of the velocity vector of each pDC are represented as arrows in the  
773 UMAP space. The black dotted ellipse corresponds to the new subcluster 8. **d**, Violin  
774 plots showing pseudo-time distribution across Seurat clusters, including subcluster 8.  
775 **e**, Expression along pseudo-time of the indicated genes vs the IFN-I meta-gene,  
776 each normalized to their maximal value. **f**, Predicted induction (red) vs termination  
777 (blue) of the transcription of selected genes as obtained using Velocity. **g**,  
778 Normalized expression along pseudo-time of *Egr1* (top) and *Id2* (bottom) vs the IFN-I  
779 meta-gene. **h**, UMAP and clustering analysis of the FB5P kinetics dataset after  
780 removal of contaminants. **i**, Pseudo-temporal inference of the pDC activation  
781 trajectory obtained using Monocle. **j**, Distribution in the UMAP space of the cells  
782 isolated at different time points after infection. The cell numbers analyzed for each  
783 time point (black dots) are indicated in parentheses. **k**, Violin plots showing the  
784 values for pseudo-time (top), *Eyfp* (middle) and YFP (bottom) expression, across cell  
785 clusters. **l**, *Eyfp* and YFP expression across pseudo-time. Individual cells are shown  
786 as dots and a polynomial curve was fit to the data. **m**, Calculus of the frequency over  
787 real-time of pDCs being in the different activation states (cell clusters) associated to  
788 IFN-I production. **n**, Heatmap showing the equivalences between the pDC clusters  
789 from the SS2 dataset#2 and the FB5P kinetics dataset based on the calculation of  
790 Jaccard Indexes reflecting their marker gene content overlap. One minus Pearson  
791 correlation was used as distance metric for hierarchical clustering.

792

793 **Fig. 5. Transcriptional convergence of pDCs towards tDCs over pseudo-time. a**,  
794 Meta-gene expression levels (y-axis) along pseudo-time (x-axis), in the FB5P kinetic  
795 dataset, for genes higher in steady state pDCs over tDCs (pDC\_versus\_tDC\_UP  
796 gene set, dark red dots and upper black curve) and for the genes lower in steady

797 state pDCs as compared to tDCs (pDC\_versus\_tDC\_DN gene set, violet dots and  
798 lower black curve). Individual cells are shown as dots and a polynomial curve was fit  
799 to the data. The gene sets were generated by reanalyzing public data (GEO Series  
800 GSE76132, see Supplementary Data 2). **b**, Expression level along pseudo-time of  
801 representative individual genes of the pDC\_versus\_tDC\_UP gene set. **c**, Expression  
802 level along pseudo-time of representative individual genes of the  
803 pDC\_versus\_tDC\_DN gene set. **d**, Expression level along pseudo-time of the pDC  
804 master transcription factor *Tcf4* (blue dots and black curve) and of its counter-  
805 regulated cDC1 master transcription factor *Id2* (pink dots and black curve). The  
806 expression of the IFN-I meta-gene is shown on each graph for comparison (pale red  
807 dots and gray curve).

808

809 **Fig. 6. Identification of annotated gene modules regulated along pseudo-time**  
810 **during pDC activation.** **a**, BubbleMap showing the gene sets with the most relevant  
811 and significant enrichment patterns along the pseudo-time of pDC activation  
812 trajectory. Dark red bubbles represent significant increased expression of the gene  
813 set (columns) across two consecutive pDC clusters along pseudo-time (rows). Dark  
814 blue bubbles represent significant decreased expression. Empty bubbles correspond  
815 to lack of significant differences in gene set expression between the pDC clusters  
816 compared. Hence, the expression pattern of each gene set along pseudo-time is  
817 visualized as a vertical succession of red or blue bubbles. **b,c** Normalized expression  
818 level (y-axis) along pseudo-time (x-axis) of the meta-genes corresponding to the  
819 selected gene sets (left) and of one of their representative genes (right) (pale orange  
820 dots and black curves). The expression of the IFN-I meta-gene is shown on each  
821 graph for comparison (pale red dots and gray curve). **d**, Normalized expression level

822 (y-axis) along pseudo-time (x-axis) of the metagene corresponding to the antigen  
823 presentation gene set, vs the IFN-I meta-gene. **e**, Heatmap showing the expression  
824 patterns along pseudo-time of individual genes selected from the analysis illustrated  
825 on panel (a), with hierarchical clustering of the genes using one minus Pearson  
826 correlation as distance metric. Belonging of the genes to the gene sets illustrated in  
827 **a-c** is shown in the grid on the right of the heatmap, with gene set 1, ISG; 2, IFN- $\gamma$   
828 responsive genes; 3, TNF signaling pathway and responsive genes; 4, pDC Myd88-  
829 dependent genes UP; 5, KEGG TLR signaling pathway; 6, Reactome Traf6-mediated  
830 IRF7 activation; 7, NF $\kappa$ B signaling pathways and responsive genes; 8, Zwarg class  
831 2 transiently induced by EGF; 9, CHO NR4A1 targets; 10, KEGG Oxidative  
832 phosphorylation; 11, GO antigen processing and presentation; 12,  
833 pDC\_versus\_tDC\_DN; 13, pDC\_versus\_tDC\_UP.

834

835 **Fig. 7. Cell-intrinsic TNF signaling promotes pDC IFN-I production.** **a**,  
836 Experimental protocol followed to evaluate the role of TNF signaling in pDC activation  
837 during MCMV infection. **b**, Percentages of IFN<sup>+</sup> cells in splenic pDCs isolated from  
838 isotype control (IC)-treated animals vs anti-TNFR2-treated mice. The data are shown  
839 for 10 individual animals pooled from 2 independent experiments, with overlay of  
840 mean $\pm$ s.e.m. values. **c**, Generation of mixed bone marrow chimera (MBMC) mice;  
841 CTR, control. Recipient C57BL/6 CD45.1 (5.1) mice were lethally irradiated and then  
842 reconstituted with equal proportions of bone marrow (BM) cells isolated from WT 5.1  
843 mice and from indicated CD45.2 (5.2) mice, either WT for CTR MBMC mice or  
844 TNFR1/2-KO (TNFRDKO) for TEST MBMC animals. Reconstituted MBMC mice were  
845 infected by MCMV and their splenic pDCs examined ex vivo for IFN-I production. **d**,  
846 Measuring the cell-intrinsic role of TNF signaling in promoting pDC IFN-I production.

847 Results are expressed as 5.2/5.1 ratio of the percentages of IFN-I-producing pDCs  
848 obtained from MCMV-infected CTR WT versus TEST TNFRDKO MBMC mice. The  
849 data are shown for 5 individual animals for each type of MBMC mice, pooled from 2  
850 independent experiments, with overlay of mean $\pm$ s.e.m. values. For b and d, the  
851 statistical analysis was performed using one-sided Mann-Whitney U test. e, Dot plots  
852 show representative data of intracellular IFN-I staining in 5.2<sup>+</sup> versus 5.1<sup>+</sup> pDCs  
853 isolated from the indicated MCMV-infected MBMC mice.

854

855 **Fig. 8. Characterization of the phenotype, function and micro-anatomical**  
856 **location of pDC activation states. a**, YFP vs CCR7 expression in splenic pDCs  
857 during MCMV infection. Data from one representative *Ifnb1<sup>Eyfp</sup>* mouse is shown for  
858 each time points. **b**, Frequency of YFP<sup>+</sup>CCR7<sup>+</sup> cells within splenic pDCs during  
859 MCMV infection. The data represent individual mice with n = 5 at 0h, 7 at 33h, 10 at  
860 36h, 5 at 40h, and 3 at 44h and 48h, from one experiment for 44h and 48h, and  
861 pooled from 2 (resp. 3) independent experiments for 33h and 40h (resp. 0h and 36h).  
862 **c**, Mean fluorescence intensity of IFN- $\alpha/\beta$  on YFP<sup>+</sup>CCR7<sup>+</sup> vs YFP<sup>+</sup>CCR7<sup>-</sup> pDCs  
863 isolated from 36h MCMV-infected mice. **d**, Relative median fluorescence intensity  
864 (MFI) of indicated markers on pDC subpopulations isolated from 36h MCMV-infected  
865 *Ifnb1<sup>Eyfp</sup>* mice. The data in c and d are from n = 10 (resp. 5) mice from two  
866 independent experiments (c) or one experiment representative of two (d). **e**, Flow  
867 cytometry sorting strategy for splenic pDC subpopulations from uninfected (UN) or  
868 38h MCMV-infected *Ifnb1<sup>Eyfp</sup>* mice, starting from live single CD11b<sup>neg</sup> Lin<sup>neg</sup> cells. **f**,  
869 Expansion of naïve CD4 OT-II cells upon co-culture with the indicated OVA peptide-  
870 pulsed pDC subpopulations. The graph shows individual data points pooled from 3  
871 independent experiments, each with 2-4 replicate co-cultures for each pDC

872 subpopulation. **g**, Immunohistological analysis of spleen sections from uninfected  
873 (UN) or MCMV-infected *Irfb1<sup>Eyfp</sup>* mice harvested at the indicated time points. Top  
874 right image, 5x zoom of the region delimited in the previous image. **h**, Definition of  
875 spleen zones for cell quantification (see online methods). **i**, Distribution of YFP<sup>+</sup> cells  
876 across the different spleen zones during the course of MCMV infection. **j**, Detail of  
877 the individual data collected and used for generating the graph of panel I. **k**, A similar  
878 analysis was performed as in panel (j) for the percentages of WP YFP<sup>+</sup> pDCs  
879 residing in the TCZ. For g-k, data were analyzed from 3 different mice, with 5  
880 different whole splenic sections per mouse (i.e. 15 images per time point). In b-d,f,i-k,  
881 data are shown as mean±s.e.m. and P values are from One-way ANOVA with  
882 Tukey's post hoc test, with \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

883

884 **On-line methods**

885 **Mice**

886 C57BL/6 and BALB/c mice were purchased from Janvier, France. Congenic CD45.1  
887 C57BL/6 mice were purchased from Charles River, Italy. All other mouse strains  
888 were bred at the Centre d'ImmunoPhénomique (CIPHE) or the Centre d'Immunologie  
889 de Marseille-Luminy (CIML), Marseille, France, under specific pathogen free-  
890 conditions and in accordance with animal care and use regulations. *Ifnb1*<sup>Eyfp</sup> (B6.129-  
891 *Ifnb1*<sup>tm1Lky</sup>) mice<sup>25</sup> were purchased from Jackson Laboratories, USA. *Tnfr1*<sup>-/-</sup> *Tnfr2*<sup>-/-</sup>  
892 (B6.129S-*Tnfrsf1a*<sup>tm1Imx</sup>; *Tnfrsf1b*<sup>tm1Imx</sup>/J, TNFRDKO) were provided by J.-L.D. All  
893 animals used were age-matched females (8 to 12 weeks old unless specified  
894 otherwise). The animal care and use protocols were designed in accordance with  
895 national and international laws for laboratory animal welfare and experimentation  
896 (EEC Council Directive 2010/63/EU, September 2010). They were approved by the  
897 Marseille Ethical Committee for Animal Experimentation (registered by the Comité  
898 National de Réflexion Ethique sur l'Expérimentation Animale under no. 14;  
899 authorization #11-09/09/2011 and APAFIS#1212-2015072117438525 v5).

900 **Virus**

901 Virus stocks were prepared from salivary gland extracts of 3-weeks old MCMV-  
902 infected BALB/c mice. All mice used in the experiments were infected i.p. with 10<sup>5</sup> pfu  
903 Smith MCMV and sacrificed at indicated time points.

904 **In vivo treatment with blocking antibodies**

905 To block TNFR2, mice were injected i.p. 24 hrs before sacrifice with 0.5 mg of  
906 Armenian Hamster IgG (isotype control) or of anti-TNFR2 mAbs (both from BioXCell).

907 **Generation of Mixed Bone Marrow Chimera (MBMC) mice**

908 Congenic CD45.1 mice were 7 Gy irradiated, then reconstituted with equal  
909 proportions of BM cells derived from CD45.1 animals and from CD45.2 mice either  
910 wild type (WT) or deficient for the indicated genes. Mice were used at least 8 weeks  
911 after BM reconstitution.

912 **Cell preparation, flow cytometry analysis and cell sorting**

913 Spleens were harvested and submitted to enzymatic digestion for 25 minutes at 37°C  
914 with Collagenase IV (Worthington biochemicals) and DNase I (Roche Diagnostics), in  
915 the presence of Brefeldin A (10 µg/ml, Sigma-Aldrich) when intracellular cytokine  
916 staining was required. Red blood cells were then lysed by using RBC lysis buffer (Life  
917 Technologies), and pDCs were enriched with the mouse plasmacytoid dendritic cell  
918 isolation kit (Miltenyi Biotech, France). Extracellular staining with BV650 anti-BST2  
919 (or alternatively with PerCP-eF710 anti-BST2), Alexa700 anti-CD3e, APC-Cy7 anti-  
920 CD11b, Alexa700 anti-Ly-6G, Alexa700 anti-NK1.1, Alexa700 anti-CD19 and BV421  
921 anti-CD11c (or alternatively with BV785 anti-CD11c) was performed in PBS 1X  
922 supplemented with 2 mM EDTA (Sigma-Aldrich) and 0.5% bovine serum albumin  
923 (BSA, H2B, Limoges, France). A complete list of the antibodies with clones and  
924 dilutions used for flow cytometry is provided in the Nature Research Reporting  
925 Summary. All extracellular stainings were performed for 20 min, at 4°C with the  
926 exception of anti-mouse CCR7 mAb which was performed at 37°C. Dead cell staining  
927 (LIVE/DEAD™ Fixable Aqua Dead Cell Stain, Life Technologies) was performed in  
928 PBS 1X as per the manufacturer's recommendations. For preservation of the YFP  
929 signal in pDC from *Ifnb1<sup>Eyfp</sup>* mice treated for intracellular staining, cells were first fixed  
930 with PBS 1x containing 2% of formaldehyde solution (Sigma-Aldrich) for 40 min,  
931 followed by incubation with Cytofix/Cytoperm (BD Biosciences) for 20 min. Cells were

932 then stained with specific anti-cytokine mAbs diluted in 1x Perm/Wash (BD  
933 Biosciences) following the manufacturer's recommendations. Purified anti-mouse  
934 IFN- $\alpha$  (RMMA1) and IFN- $\beta$  (RMMB1) were purchased from PBL Interferon Source;  
935 purified rat IgG was used as isotype control (Jackson Immuno Research); these  
936 mAbs were coupled to Alexa647 by using a monoclonal labelling kit (Southern  
937 Biotech)<sup>22</sup>. Samples were acquired on a LSR Fortessa X-20 (BD Biosciences,  
938 France) and analyzed with FlowJo software (FlowJo, LLC). For microarray studies,  
939 pDCs were sorted to high purity by flow cytometry on a FACS Aria II cell sorter (BD  
940 Biosciences, France) and directly collected in RLT buffer (Qiagen) supplemented with  
941 10%  $\beta$ -mercaptoethanol. For SS2 scRNAseq, cells were index sorted on a BD FACS  
942 Aria III in 96-well plates, with each well filled with 10 $\mu$ L of lysis buffer (TCL buffer,  
943 Qiagen, supplemented with 1%  $\beta$ -mercaptoethanol). For FB5P single-cell RNA  
944 sequencing, cells were index sorted on a BD FACS Influx in 96-well plates, with each  
945 well filled with 2 $\mu$ l FB5P lysis buffer, as described<sup>41</sup>.

#### 946 **Generation of FLT3-L bone marrow cultures**

947 Bone marrow cells were isolated from CD45.1 C57BL/6 mice and cultured for 6-7  
948 days at 2  $10^6$  cells/ml in 24-well plates in RPMI supplemented with 10% FCS, 1%  
949 glutamine (Gibco), 100U/mL Penicillin streptomycin, 1% non-essential amino acids,  
950 1% sodium pyruvate and 0.05 mM  $\beta$ -mercaptoethanol, with a 1/10 dilution of B16-  
951 FLT3-L culture supernatant.

#### 952 **Immunohistological analysis**

953 Spleen fragments isolated from uninfected or MCMV-infected *Ifnb1*<sup>Eyfp</sup> reporter mice  
954 were fixed for 2 hours at 4°C with Antigenfix (DiaPath), then washed in phosphate  
955 buffer (PB1X: 0.025 M NaH<sub>2</sub>PO<sub>4</sub> and 0.1 M Na<sub>2</sub>HPO<sub>4</sub>) for 1 hour, dehydrated in

956 30% sucrose overnight at 4°C, and embedded in OCT freezing media (Sakura  
957 Fineteck). 20µm-thick tissue sections were blocked in PB1X containing 0.5 %  
958 saponin, 2% BSA, 2% 2.4G2 supernatant and streptavidin/biotin blocking kit (Vector  
959 Laboratories), and first stained in PB1X, 0.5% saponin, containing the following  
960 antibodies: rat anti-mouse B220 (BD Bioscience) followed by Alexa546-conjugated  
961 goat anti-rat mAb (Molecular probes) and hamster anti-mouse CD3 (BD Bioscience)  
962 followed by Alexa594-conjugated goat anti-hamster mAb (Jackson Immunoresearch).  
963 Tissue sections were then blocked with 5% rat serum (Jackson Immunoresearch) in  
964 PB1X and stained in PB1X, rat serum 5%, 0.5% saponin with the following  
965 antibodies: Alexa488-conjugated rabbit anti-GFP/YFP (Thermofisher), biotin rat anti-  
966 mouse CD169 (MOMA1, AbCam) followed by eFluor450-conjugated streptavidin  
967 (Thermofisher), and Alexa647-conjugated rat anti-mouse IFN $\alpha/\beta$  (Thermofisher)<sup>22</sup>.  
968 Stained sections were mounted in ProLong Gold antifade reagent (Thermofisher)  
969 mixed with SYTOX Blue nucleic acid stain (Thermofisher), acquired on a LSM 880  
970 confocal microscope (Zeiss) and analyzed with ImageJ software. The red pulp (RP)  
971 was identified as the zone outside of the external boundary of CD169 staining. The  
972 marginal zone (MZ) was delimited between the external boundary of CD169 staining  
973 and the external boundary of B220 staining. The white pulp (WP) was identified as  
974 the zone included within the external boundary of B220 staining. The T cell zone  
975 (TCZ) corresponds to the WP area encompassing CD3 staining, whereas the B cell  
976 zone (BCZ) was identified within WP as selectively including B220 staining.

977

### 978 **Microarray data generation and analysis**

979 Pooled spleens of uninfected or MCMV-infected mice were used in order to obtain  
980 10,000 cells for each pDC subpopulation, with two independent duplicate samples

981 generated per condition. Total RNA was prepared as described<sup>22</sup> and converted to  
982 biotinylated double-stranded cDNA targets which were hybridized on GeneChip  
983 Mouse Gene 1.0 ST arrays (Affymetrix) that were scanned to extract raw data (.CEL  
984 Intensity files). Bioinformatics analyses were performed as previously described<sup>51, 52</sup>  
985 and specified in Figure legends. Heatmaps were generated using Gene-E  
986 (<http://www.broadinstitute.org/cancer/software/GENE-E/>).

### 987 **Single-cell RNA sequencing**

988 For SS2 scRNASeq, single cells were sorted into 96-well full-skirted Eppendorf plates  
989 chilled to 4°C, prepared with lysis buffer. Single-cell lysates were sealed, vortexed,  
990 spun down at 300g at 4°C for 1 min, immediately placed on dry ice and transferred  
991 for storage at -80°C. The SS2 protocol<sup>31</sup> was performed on single-cells as previously  
992 described<sup>53</sup>. Briefly, plates containing cell lysates were thawed on ice, followed by  
993 RNA purification and first strand synthesis. cDNA was then amplified by PCR for 22  
994 cycles and libraries were generated using ¼ of Nextera XT library preparation kit  
995 (Illumina) with custom barcodes as described<sup>14</sup>. Libraries were then pooled  
996 equimolarly and sequenced on an Illumina HiSeq 4000 sequencer (paired-end, 50-bp  
997 reads). FACS-based 5-prime end single-cell RNAseq (FB5P-seq) was performed as  
998 described<sup>41</sup>.

### 999 **Single-cell RNA sequencing data analysis**

1000 For SS2 scRNAseq, raw FASTQ files were aligned with STAR<sup>54</sup> against the  
1001 GRCM38.94 genome to which the sequence of *Eyfp* was added as an extra  
1002 chromosome (see Supplementary text for the *Eyfp* sequence). Gene-specific read  
1003 counts were calculated using HTSeq-count<sup>55</sup>. FB5P sequencing data were processed  
1004 to generate single-cell UMI counts matrices as described<sup>41</sup>. The counts matrix was

1005 loaded to R, and Seurat<sup>56</sup> was used for downstream analyses such as normalization  
1006 (LogNormalize), shared nearest neighbor graph-based clustering, differential  
1007 expression analysis and visualization, eventually followed by Monocle<sup>57, 58</sup> or  
1008 Velocity<sup>38</sup> for the inference of the pDC activation trajectory, as summarized in  
1009 Supplementary Data 1 and described below. Unless specified otherwise, gene  
1010 expression is shown as log-normalized values and protein expression as inverse  
1011 hyperbolic arcsine (asinh) of fluorescence intensity raw or scaled values, namely  
1012  $\text{asinh}(\text{fluorescence})$  for SS2 dataset#1,  $\text{asinh}(\text{fluorescence}/100)$  for SS2 dataset#2,  
1013 and  $\text{asinh}(\text{fluorescence}/10)$  for FB5P datasets. For dimensionality reduction, we  
1014 performed a uniform manifold approximation and projection (UMAP), using the  
1015 RunUMAP function. The different clusters were projected on the UMAP plot for  
1016 visualization. The differentially expressed genes were determined using the  
1017 FindAllMarkers function. The heatmaps were plotted using the Gene-E program. For  
1018 some of the datasets, a first analysis was performed to identify and exclude  
1019 contaminating cells, as summarized in the corresponding Figure legends and in  
1020 Supplementary Data 1.

### 1021 **Alignment of clusters from the SS2 dataset#2 and the FB5P kinetic dataset**

1022 In order to compare the clusters obtained in the SS2 dataset#2 and the FB5P kinetic  
1023 dataset, we first extracted the marker genes of each cluster for each dataset  
1024 separately, using the FindMarker function of Seurat, using the "bimod" statistical test  
1025 and an adjusted p-value cut-off of 0.25. We then computed the Jaccard Indexes (JI)  
1026 between each cluster of one dataset with any other cluster of the other dataset. The  
1027 JI measures similarity between finite sample sets, and is defined as the size of the  
1028 intersection divided by the size of the union of the sample sets:  $J(A,B) = |A \cap B| / |A$

1029 U B]. The JI matrix was then used to generate a heatmap with hierarchical clustering,  
1030 using Gene-E.

### 1031 **Monocle analysis**

1032 We used Monocle3 to investigate cell trajectories and the dynamics of gene  
1033 expression within these trajectories. The cells from the uninfected mouse were set as  
1034 the root of the trajectory and the input genes were selected using Seurat. We first  
1035 clustered and projected the cells onto a low-dimensional space (UMAP) calculated  
1036 either by Monocle or by Seurat. Monocle then resolved the activation trajectories and  
1037 calculated along them the pseudo-time for each cell. In some cases where several  
1038 trajectories were found, we exported the branches identified, using the centroid-  
1039 based principal graph generated by Monocle  
1040 (<https://gist.github.com/mochar/70e67bfba87ceedbd1435b83e6464ba2>). Each  
1041 individual path from the starting point (cells from non-infected mice) to each of the  
1042 end points of the constructed graphs were retrieved. The expression profile of genes  
1043 of interest were then projected on the cells of chosen branches. For certain gene  
1044 modules or gene sets, a meta-gene was defined as the average of log-normalized  
1045 values of all of its member genes that were included in the Leading Edge of at least  
1046 one of the GSEA results illustrated in Fig. 6a. The expression of this meta-gene was  
1047 then plotted, with each cell shown as a dot and a polynomial curve computed to  
1048 illustrate the trend in dynamical expression change of the gene set along the pseudo-  
1049 time. When specified, the expression levels of each meta-gene or gene was  
1050 normalized, to its maximal value, to ease comparison of the temporal evolution of  
1051 their expression pattern.

### 1052 **RNA velocity analysis**

1053 RNA velocity was performed using the Velocyto software. Velocyto predicts the future  
1054 direction in which cells are moving in the transcriptional space by estimating, for each  
1055 cell, the time derivative of RNA abundance<sup>38</sup>. Specifically, it computes the predicted  
1056 fate of each single cell based on the integration of its precise transcriptional state for  
1057 hundreds of intron-bearing genes, as assessed by their ratio of unspliced to spliced  
1058 RNA, in comparison with the other cells. The directionality of the transitions is  
1059 represented as vectors, whose length is proportional to the velocity of these  
1060 transitions (longer vectors meaning faster transitions). Annotation of the spliced and  
1061 unspliced reads was performed using velocyto.py on the SS2 dataset#2 and using  
1062 DropEst on the FB5P datasets. Only the cells and genes that had been kept in the  
1063 previous analysis using the Seurat R package were considered in the Velocyto  
1064 analysis. PCA was performed with pagoda2 (<https://github.com/hms-dbmi/pagoda2>),  
1065 with spliced expression matrix as input, and the cell-to-cell distance matrix was  
1066 calculated using Euclidean distance. RNA velocity was estimated using gene-relative  
1067 model with k-nearest neighbor cell pooling. The overall pattern generated by  
1068 projecting the RNA velocities of each individual cell onto the UMAP space enables  
1069 visualizing the corresponding model of the trajectory.

#### 1070 **BubbleGUM analysis**

1071 BubbleGUM, a high throughput gene set enrichment analysis (GSEA) software<sup>42</sup>  
1072 was used to assess the enrichment of selected transcriptomic signatures across  
1073 populations of the microarray data or clusters of the scRNAseq data. BubbleGUM is  
1074 composed of two modules; i) GeneSign, which generates statistically significant gene  
1075 signatures and ii) BubbleMap, which automatically assesses the enrichment of input  
1076 gene signatures between all possible pairs of conditions from independent datasets,  
1077 based on gene set enrichment analysis (GSEA) methodology<sup>59</sup>, and which generates

1078 an integrated graphical display. To run the final BubbleMap analysis, we used a total  
1079 of 24 selected gene sets (Supplementary Data 2). These gene sets had been  
1080 selected from an initial pool of 27 homemade GeneSets and of all of the 17,810 gene  
1081 sets from the MSigDB (msigdb.v6.2.symbols.gmt), upon iterative runs of GSEA and  
1082 BubbleGUM analyses to focus the final analysis on the gene sets that yielded  
1083 significant and meaningful enrichments and were not too redundant with one another.  
1084 BubbleMap was used with 1,000 gene set permutations, and with “difference of  
1085 classes” as a metric for ranking the genes since the data were expressed in log  
1086 scale. The results are displayed as a BubbleMap, where each bubble is a GSEA  
1087 result and summarizes the information from the corresponding enrichment plot. The  
1088 color of the Bubble corresponds to the condition from the pairwise comparison in  
1089 which the signature is enriched. The bubble area is proportional to the GSEA  
1090 normalized enrichment score (NES). The intensity of the color corresponds to the  
1091 statistical significance of the enrichment, derived by computing the multiple testing-  
1092 adjusted permutation-based p-value using the Benjamini–Yekutieli correction.

### 1093 **In vitro T cell activation assay**

1094 For each experiment, spleens were isolated from 10 *Ifnb1*<sup>Eyfp</sup> mice infected with  
1095 MCMV 38h before sacrifice. Splenocytes were pooled and submitted to magnetic  
1096 depletion of Lineage<sup>+</sup> cells. pDCs in the negative fraction were identified as live  
1097 single-cells, CD11b<sup>-</sup> Lin(CD3,CD19, Ly6G, NK1.1)<sup>-</sup> CD11c<sup>int</sup> SiglecH<sup>+</sup> BST2<sup>hi</sup> cells,  
1098 and sorted as YFP<sup>-</sup>CD86<sup>+</sup>, YFP<sup>-</sup>CD86<sup>-</sup>, YFP<sup>+</sup>CCR7<sup>-</sup> or YFP<sup>+</sup>CCR7<sup>+</sup>. Cells were  
1099 collected into complete RPMI supplemented with 10% FCS, 1% glutamine (Gibco),  
1100 100U/mL Penicillin streptomycin, 1% non-essential amino acids, 1% sodium pyruvate  
1101 and 0.05 mM β-mercaptoethanol. pDCs sorted from uninfected mice were used as  
1102 controls. Cells were spun down, resuspended in complete RPMI with 5 µg/mL OVA

1103 peptide (323-339; H-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-  
1104 Arg-OH) (Sigma-Aldrich) and plated at 2,000 cells in 100  $\mu$ L per well in a V-bottom  
1105 plate for 20 min at 37°C. Naïve CD4<sup>+</sup> T cells from Rag2KO x Tg OT-II x B6.Ly5.1  
1106 mice were isolated using a naïve CD4<sup>+</sup> T cell enrichment kit (Miltenyi Biotec), labeled  
1107 with 2.5  $\mu$ M of carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 min,  
1108 and blocked for 5 min at RT with 5x volumes of complete RPMI. Cells were spun  
1109 down and washed once again. 10,000 T cells/50  $\mu$ L were added to each well  
1110 containing sorted pDCs. Wells without pDCs were used as negative controls. After 4  
1111 days of co-culture, cells were washed in PBS, stained with Live Dead Aqua, APC-  
1112 Cy7 anti-CD4 and Alexa700 anti-CD3e mAbs. Cells were then resuspended in 75  $\mu$ L  
1113 of PBS supplemented with 1 mM EDTA (Sigma-Aldrich) and 0.5 % Bovine Serum  
1114 Albumin (BSA, H2B). 25  $\mu$ L of absolute counting beads (Beckman Coulter) were  
1115 added to each well before acquisition by FACS. The proliferation in the negative  
1116 control wells (background) was subtracted from each condition. For each  
1117 experiment, the fold expansion was calculated as the number of proliferating cells in  
1118 each condition divided by the mean number of proliferating cells (3 replicates per  
1119 experiment) in the wells with pDCs isolated from uninfected mice.

## 1120 **Statistical analyses**

1121 All quantifications were performed unblinded. Statistical parameters including the  
1122 definitions and exact value of n (number of biological replicates and total number of  
1123 experiments), and the types of the statistical tests are reported in the figures and  
1124 corresponding figure legends. Statistical analyses were performed using Prism  
1125 (GraphPad Software) or R statistical programming language. Statistical analysis was  
1126 conducted on data with at least three biological replicates. Comparisons between  
1127 groups were planned before statistical testing and target effect sizes were not

1128 predetermined. Error bars displayed on graphs represent the mean  $\pm$  SEM. Statistical  
1129 significance was defined as \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001; \*\*\*\*, P<0.0001.

1130

### 1131 **Data availability**

1132 Microarray and scRNAseq data have been deposited in the GEO repository under  
1133 accession codes GSE150664 and GSE151248, respectively. All the other data that  
1134 support the findings of this study will be available from the corresponding authors  
1135 upon reasonable request.

1136

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1159 **Additional information**

1160 Extended data is available for this paper at XXX.

1161 **Supplementary information** is available for this paper at XXX.

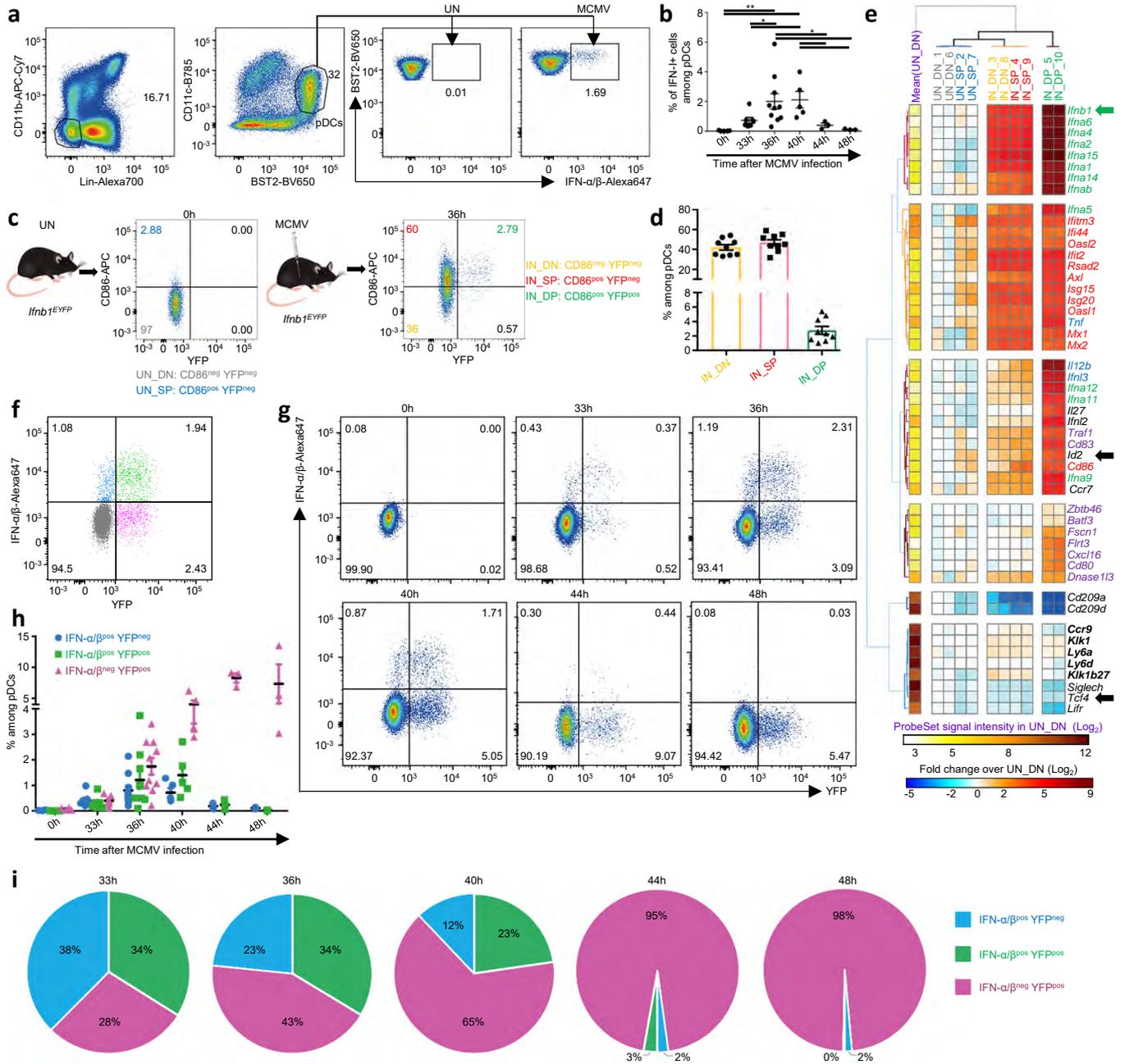
1162 **Correspondence and requests for materials** should be addressed to M.D. or E.T.

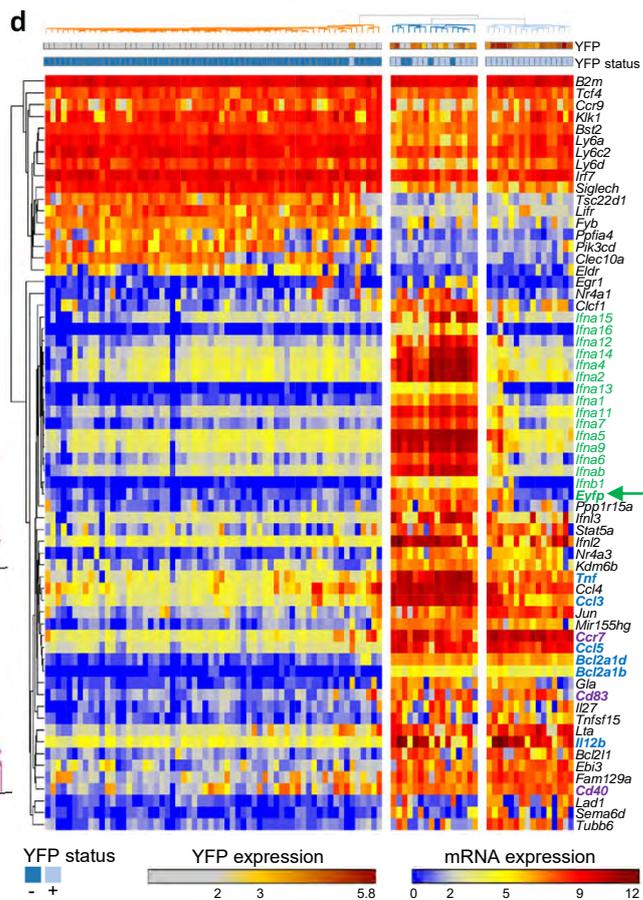
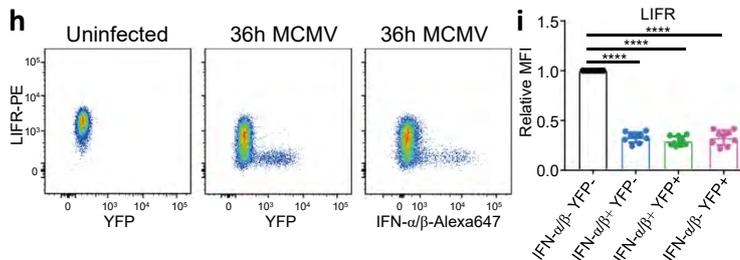
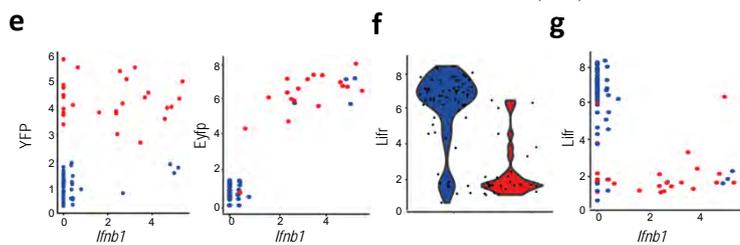
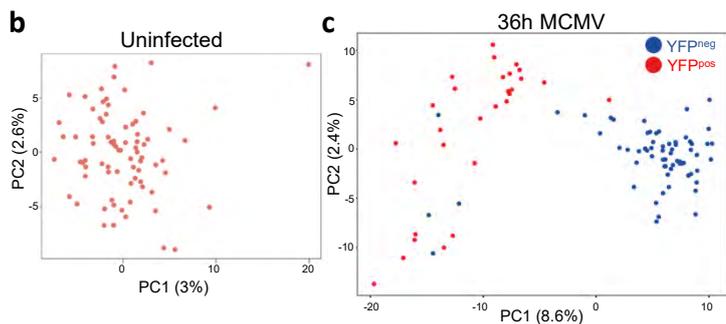
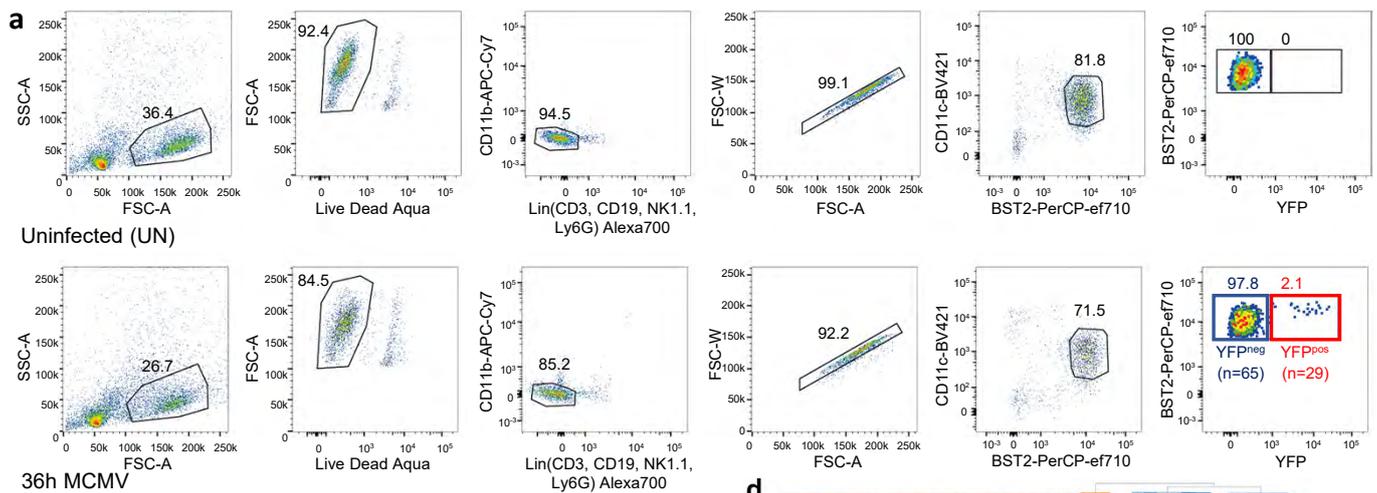
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1164 managed its editorial process and peer review in collaboration with the rest of the  
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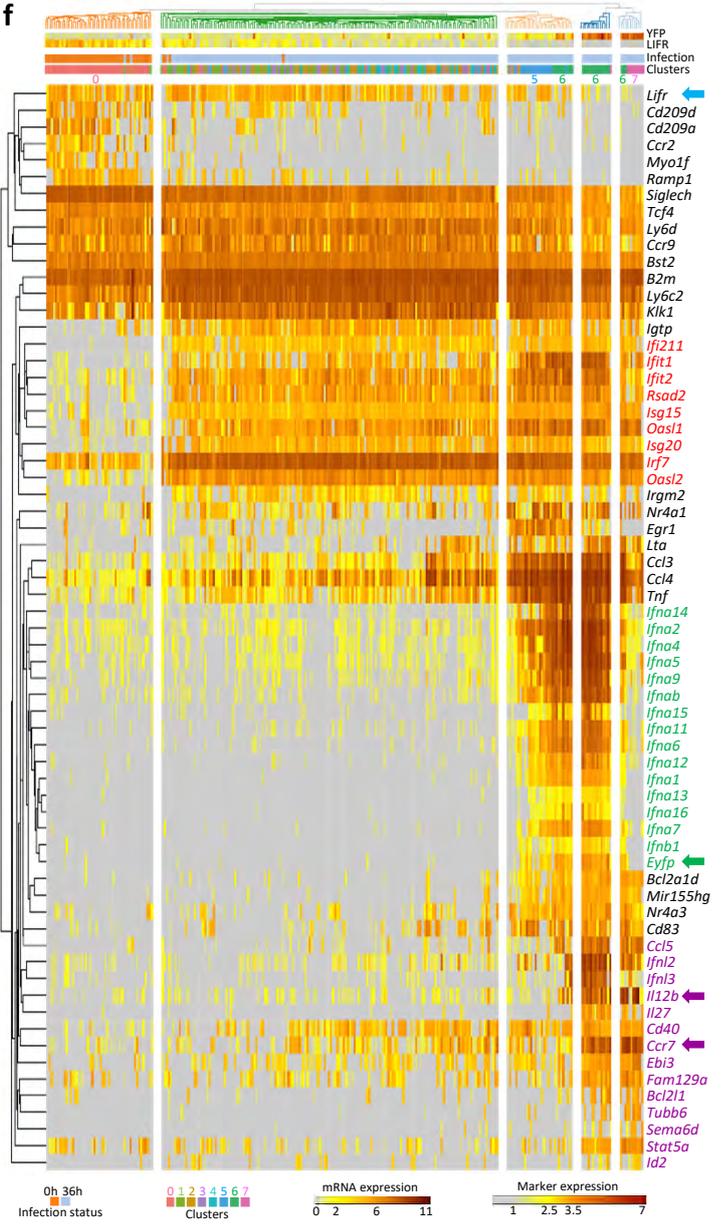
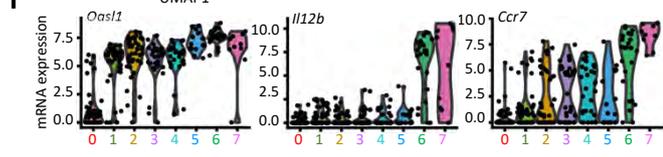
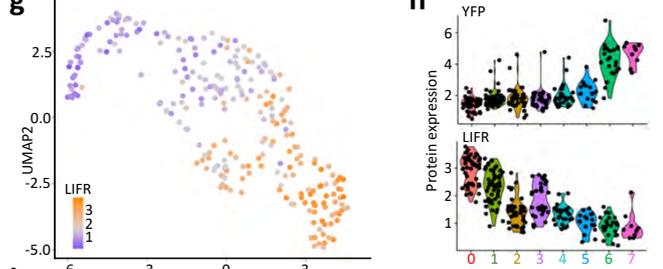
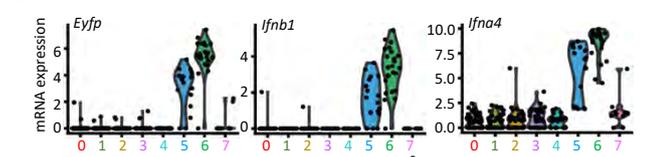
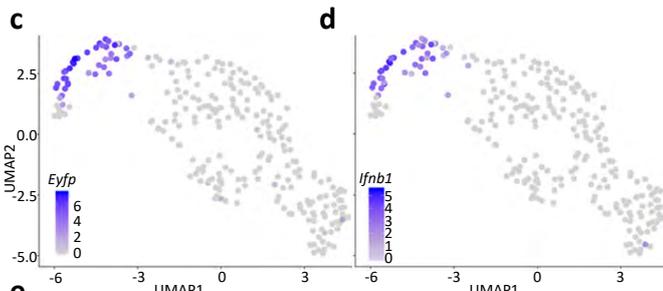
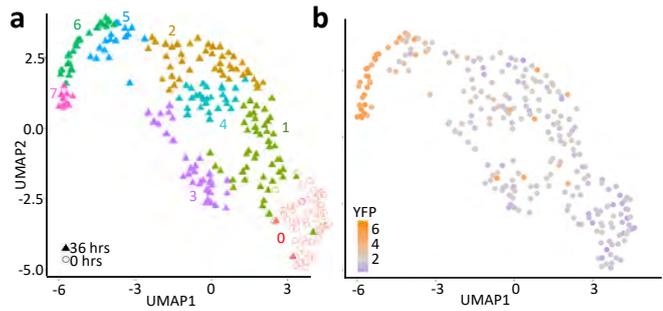
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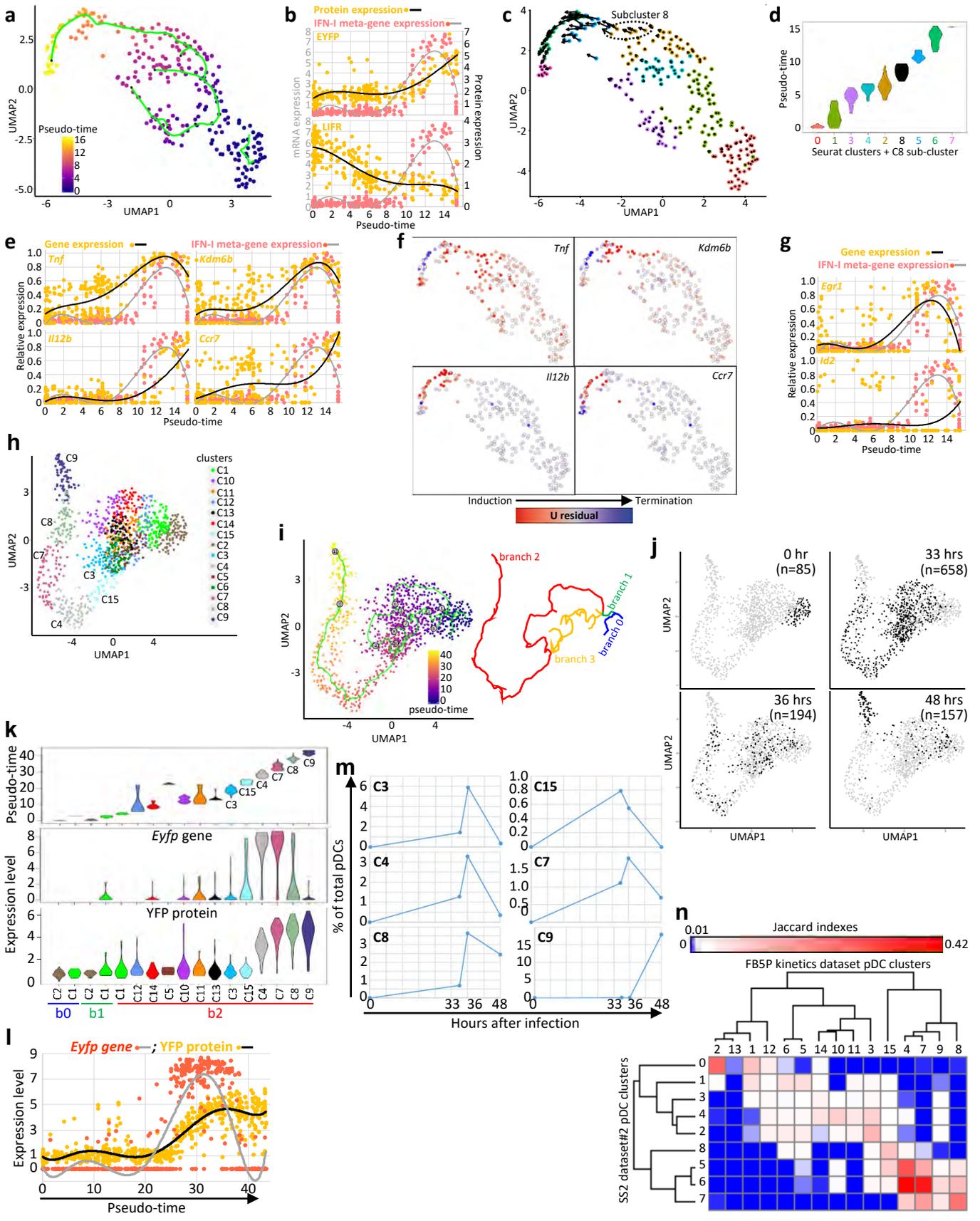
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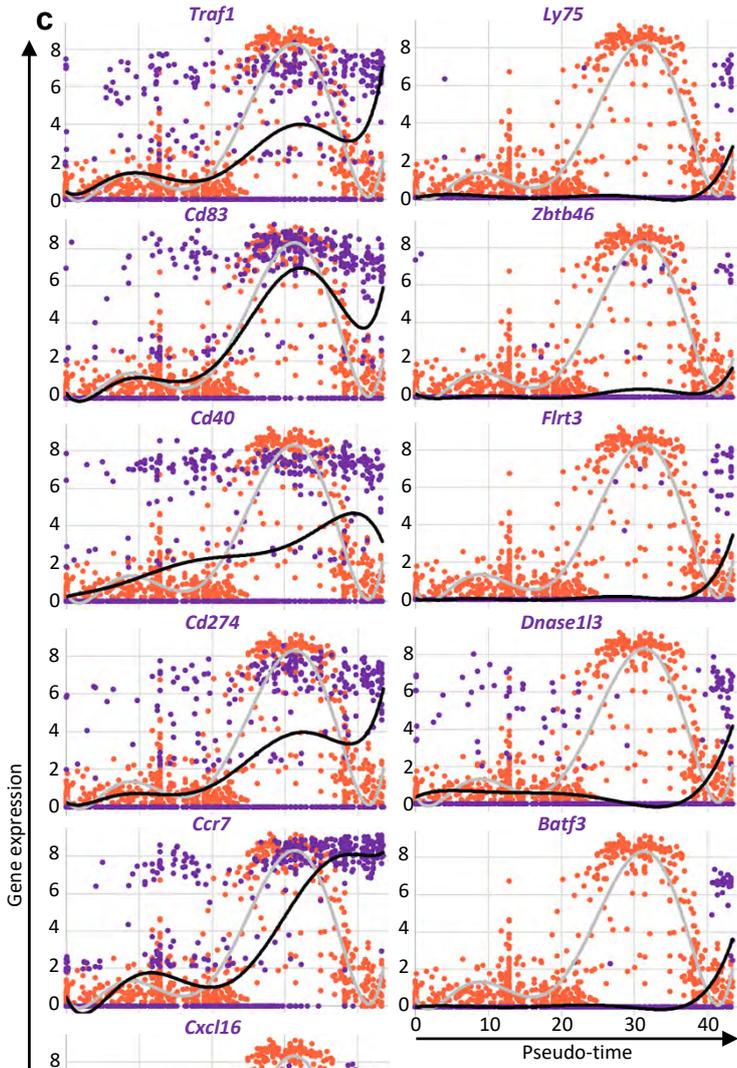
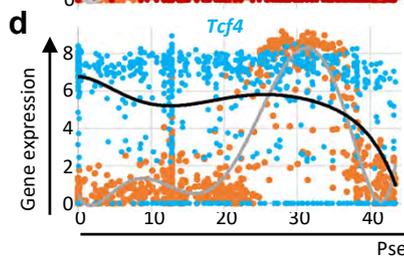
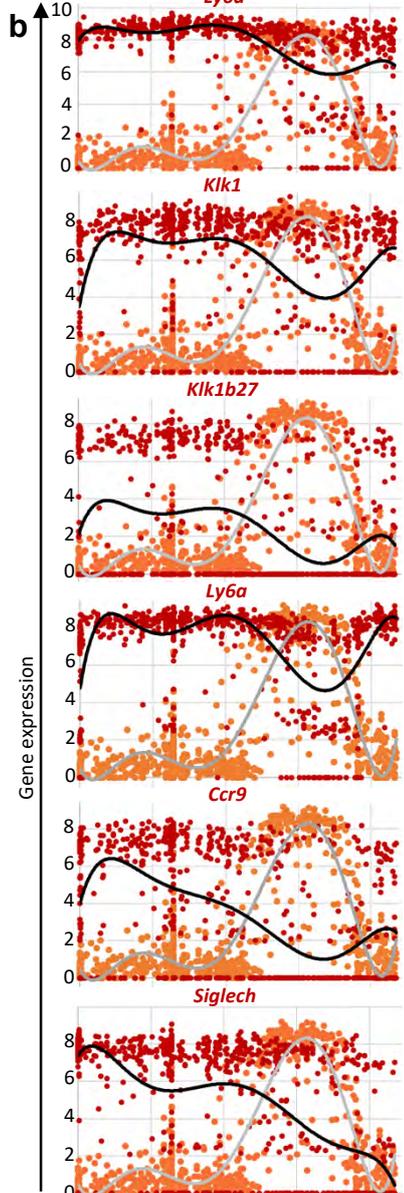
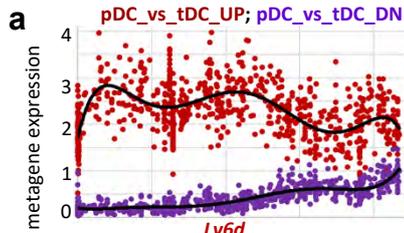
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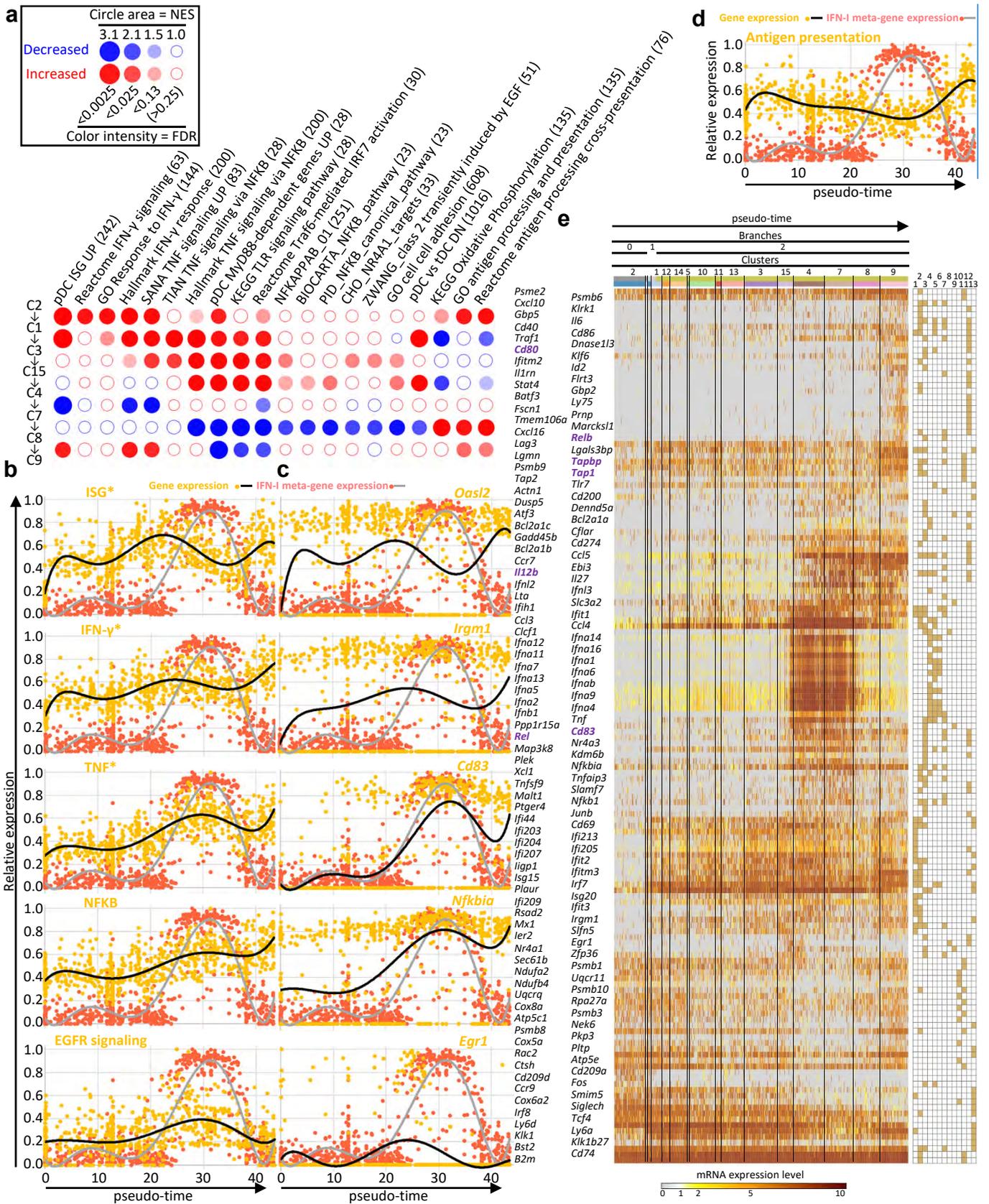


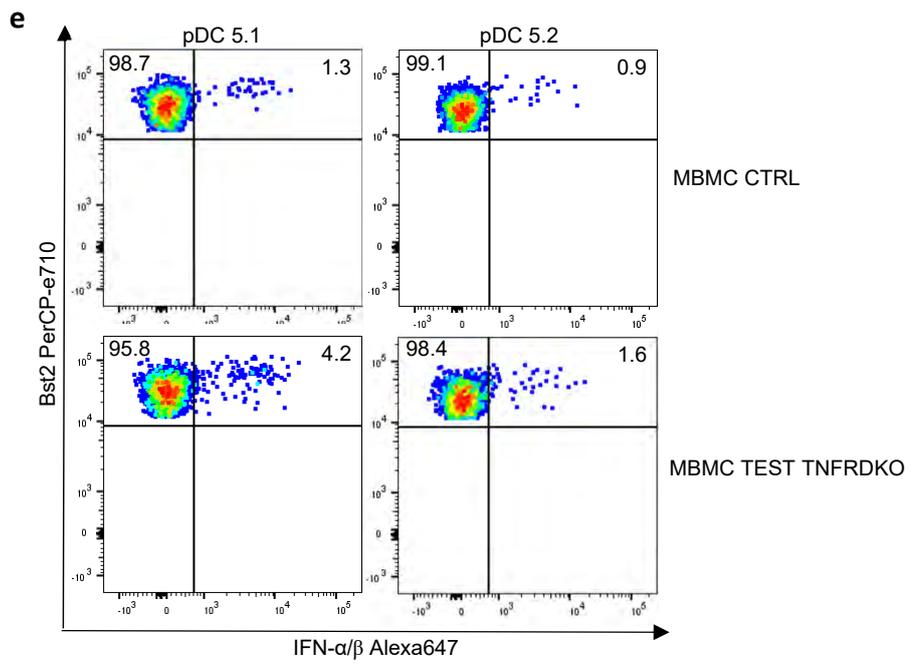
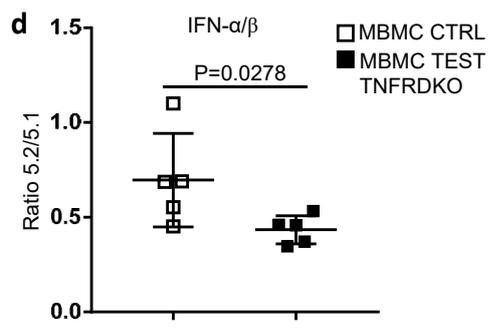
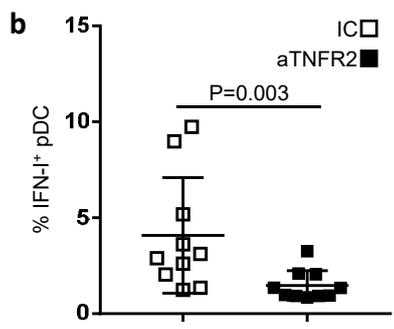
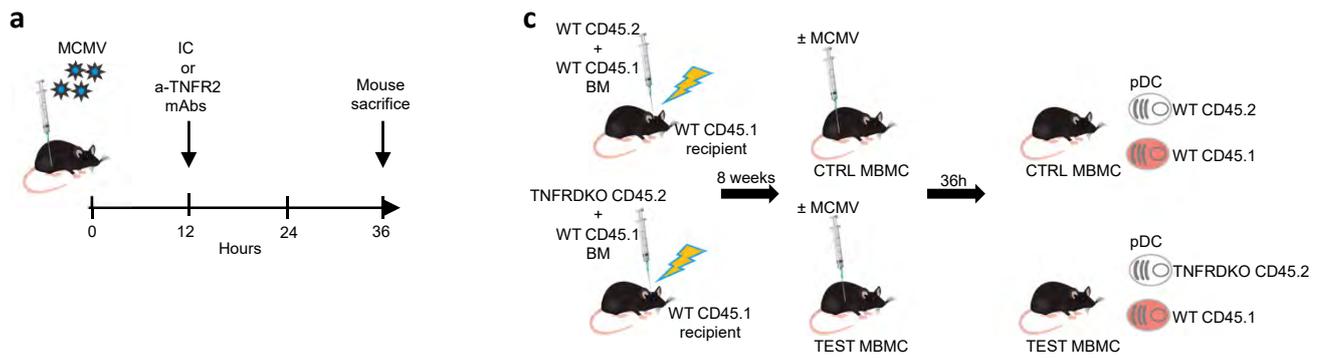
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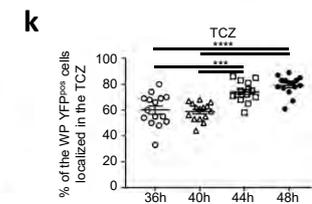
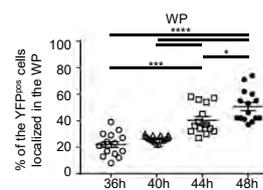
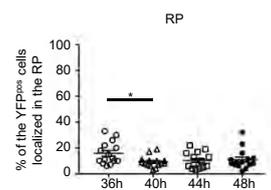
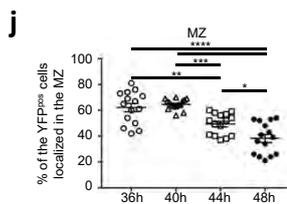
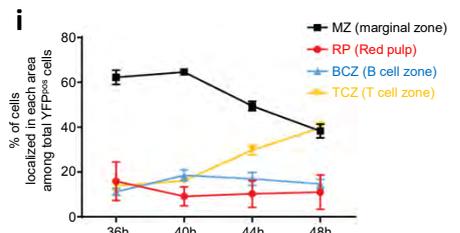
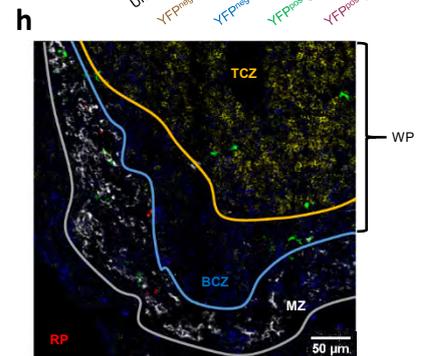
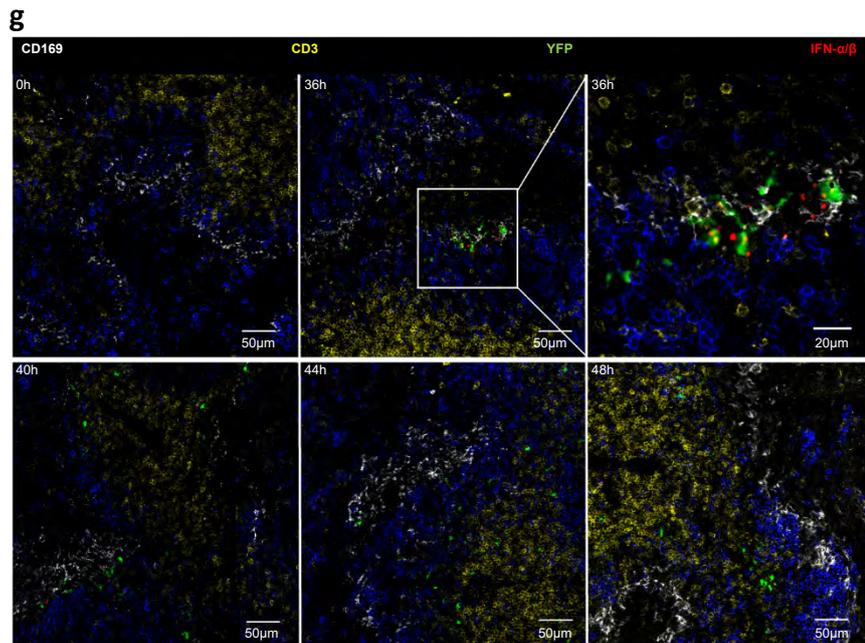
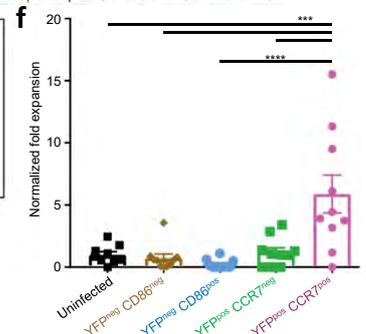
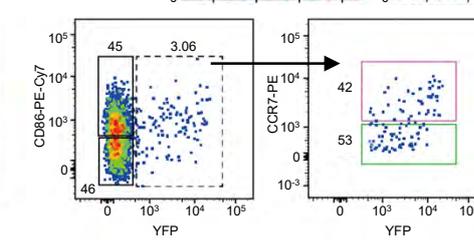
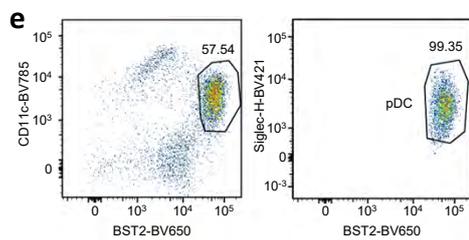
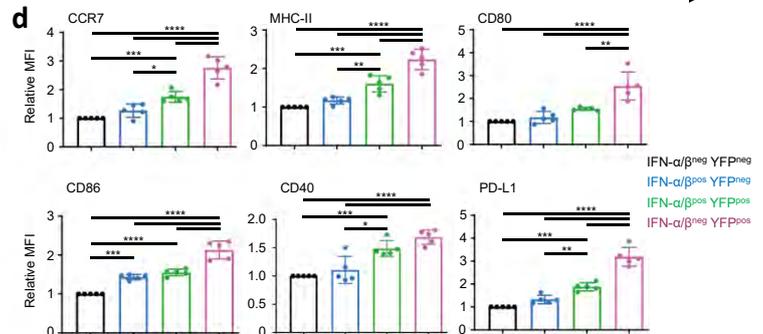
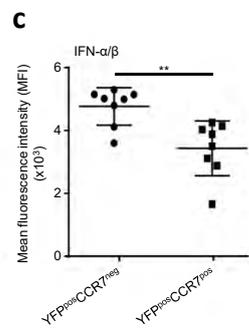
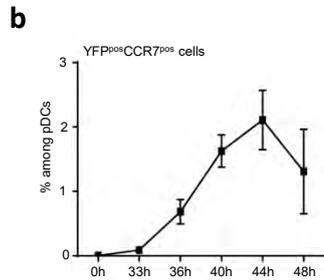
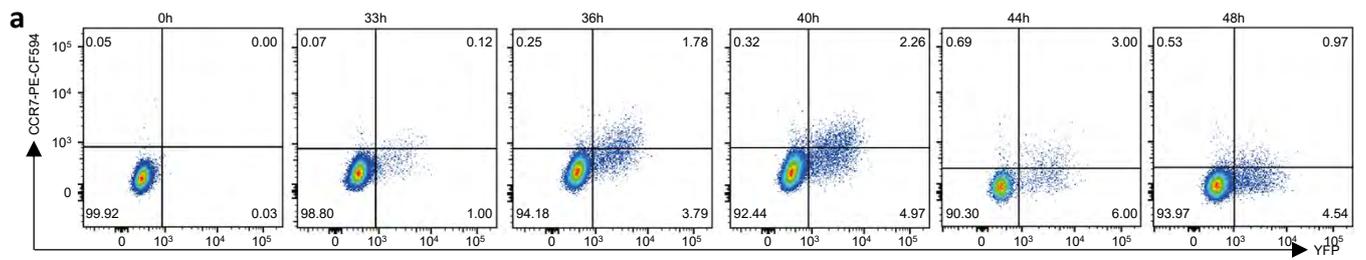
- pDCs over tDCs —
- tDCs over pDCs —

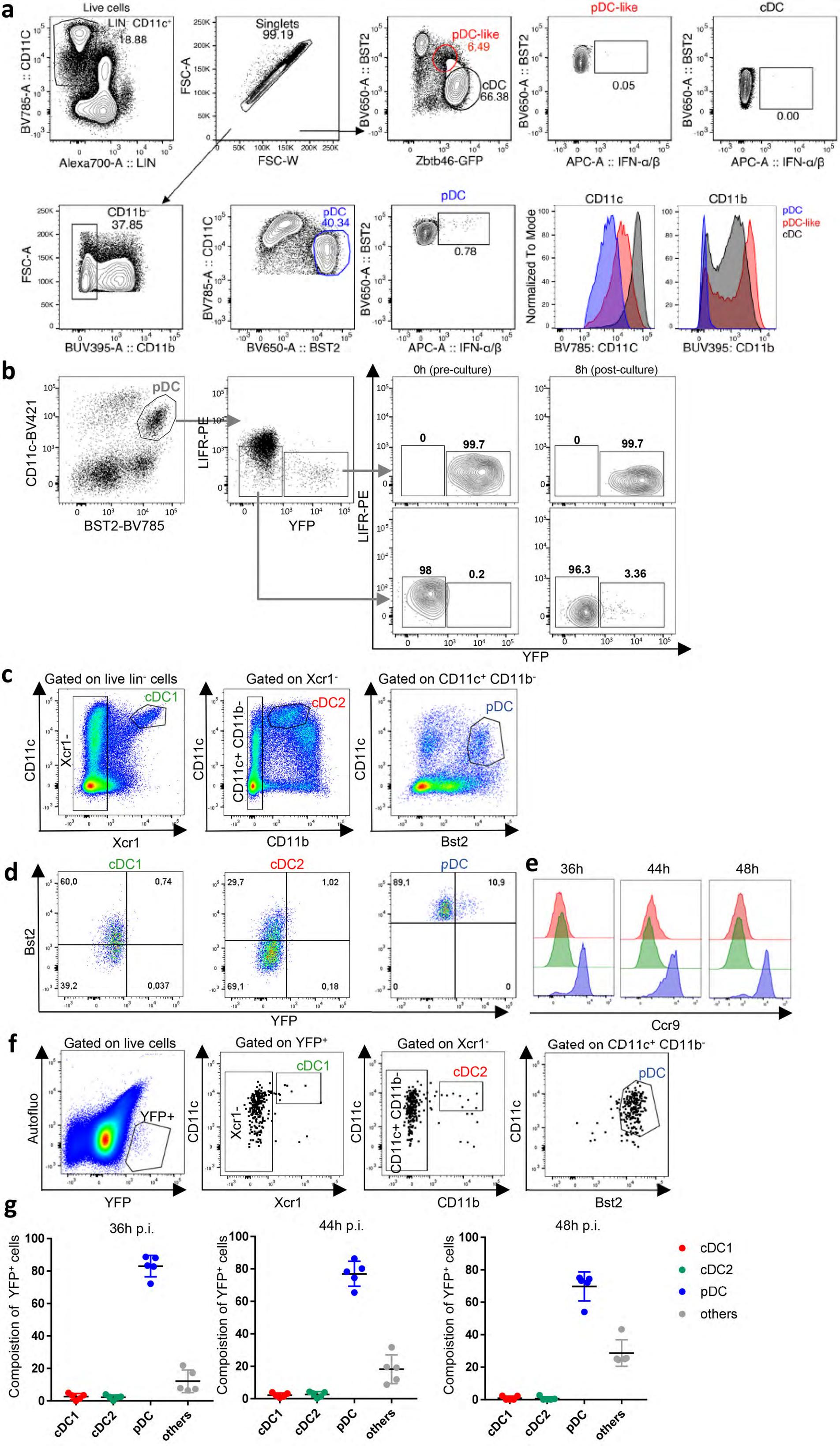
IFN-I meta-gene expression —

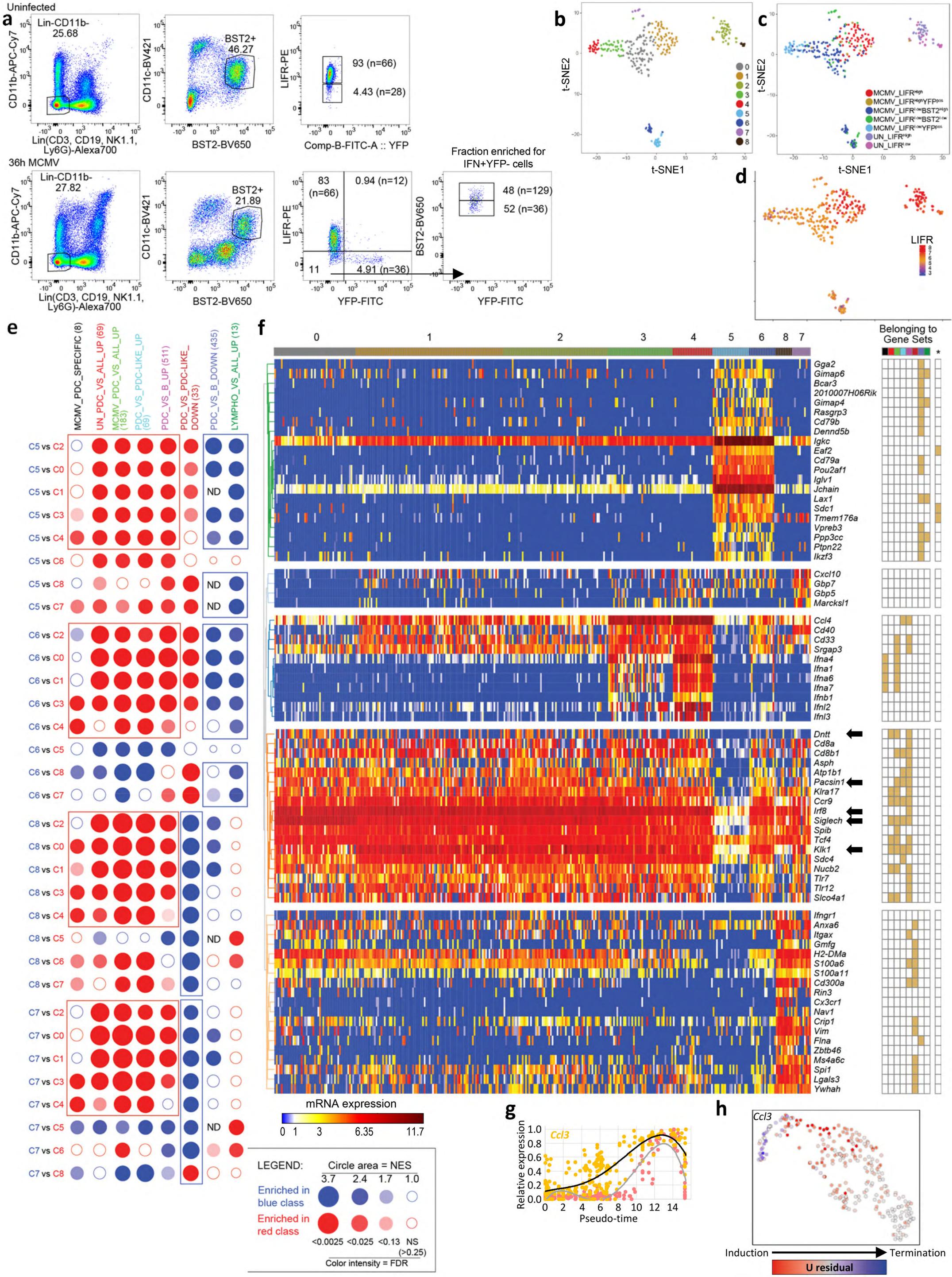
Tcf4 expression — Id2 expression —

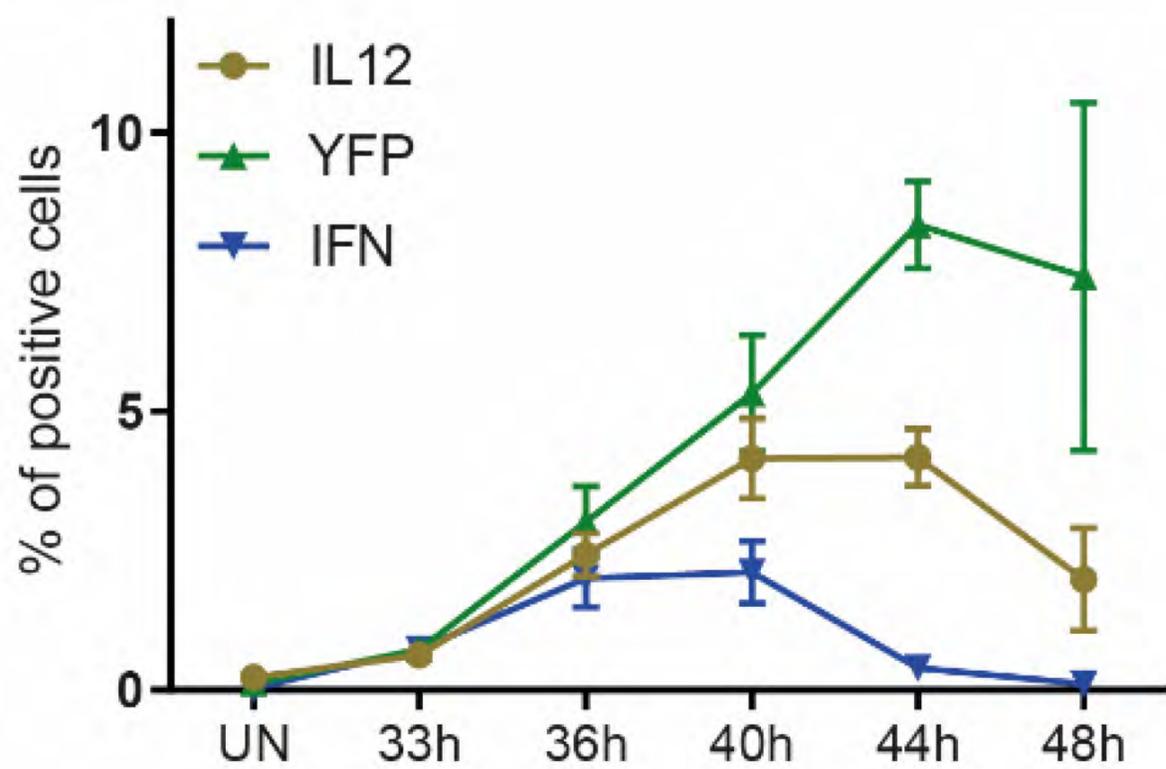
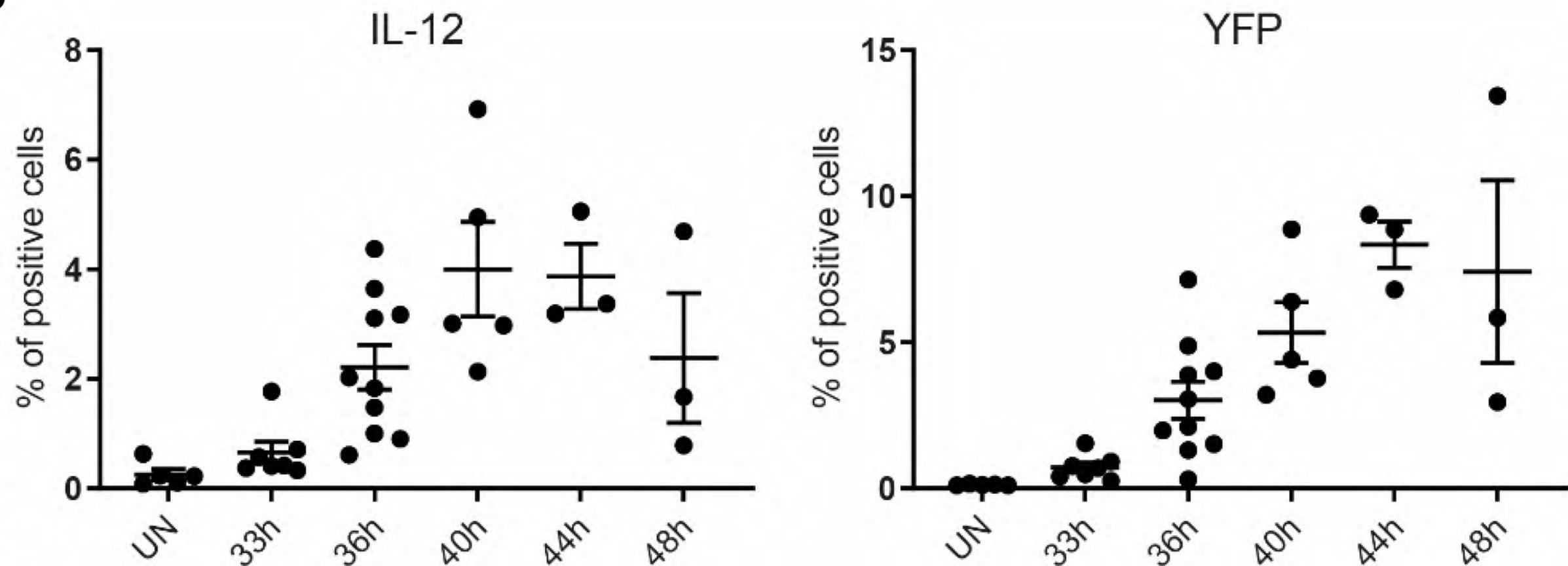
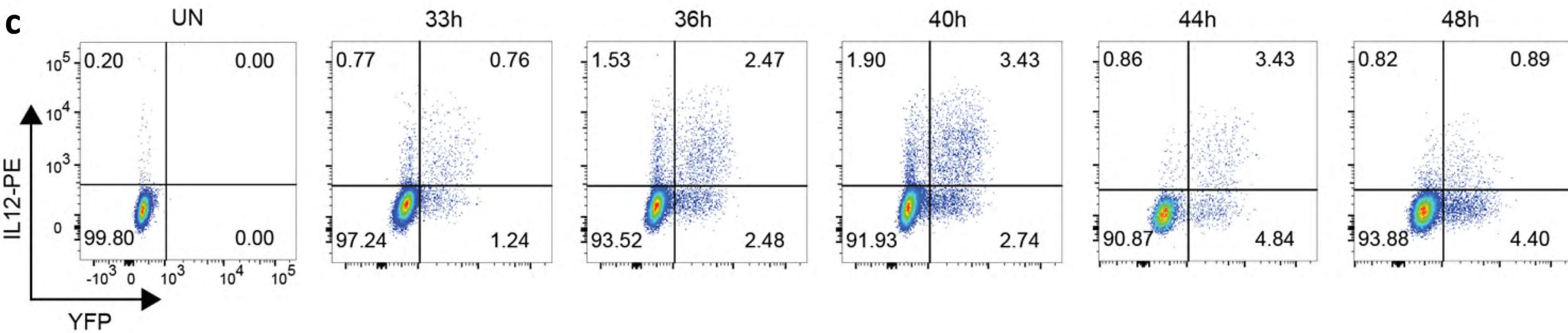


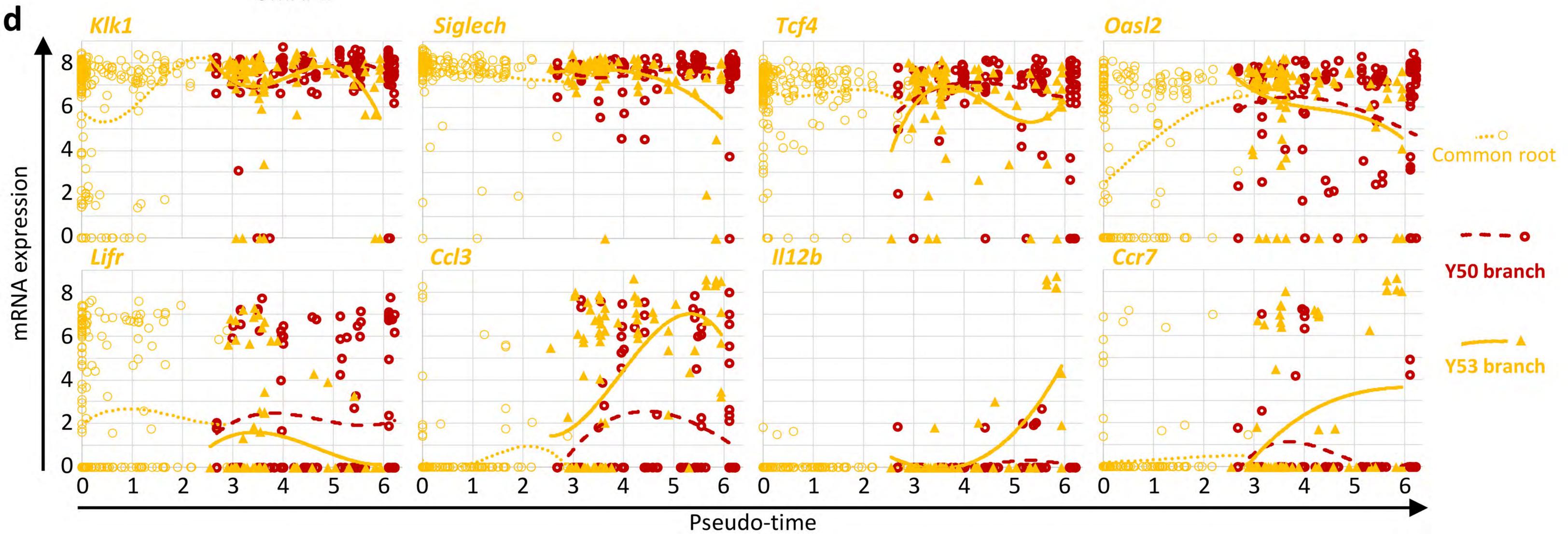
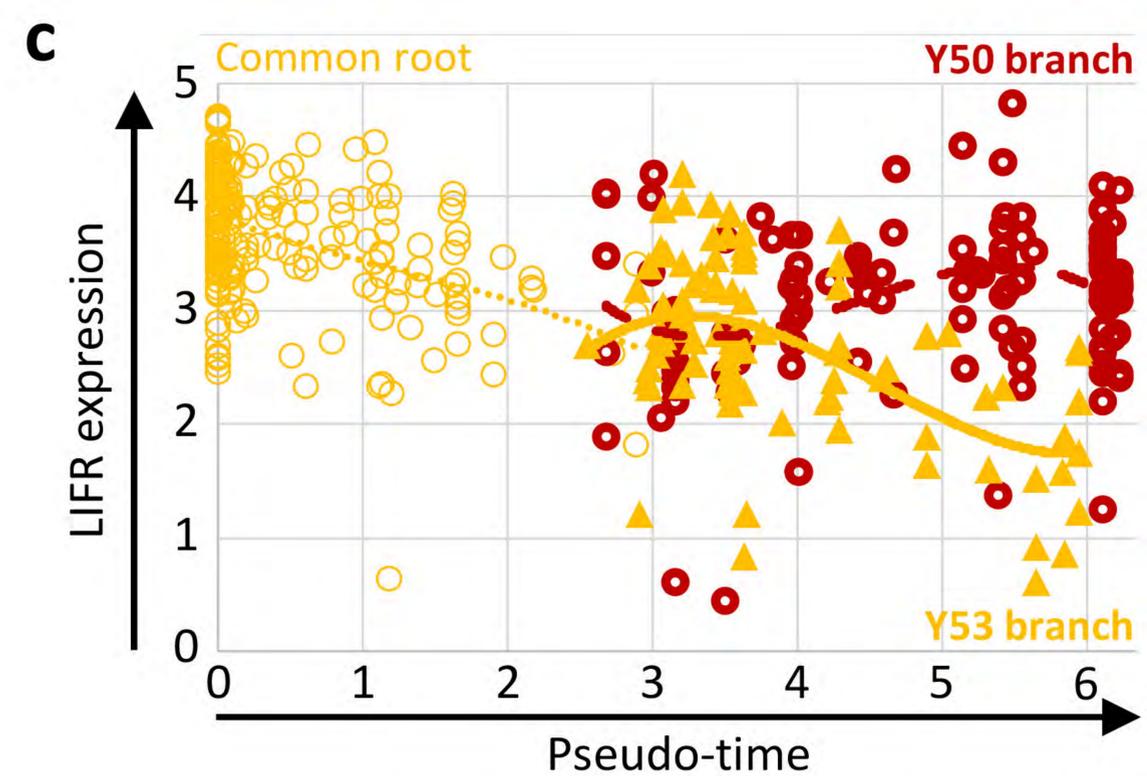
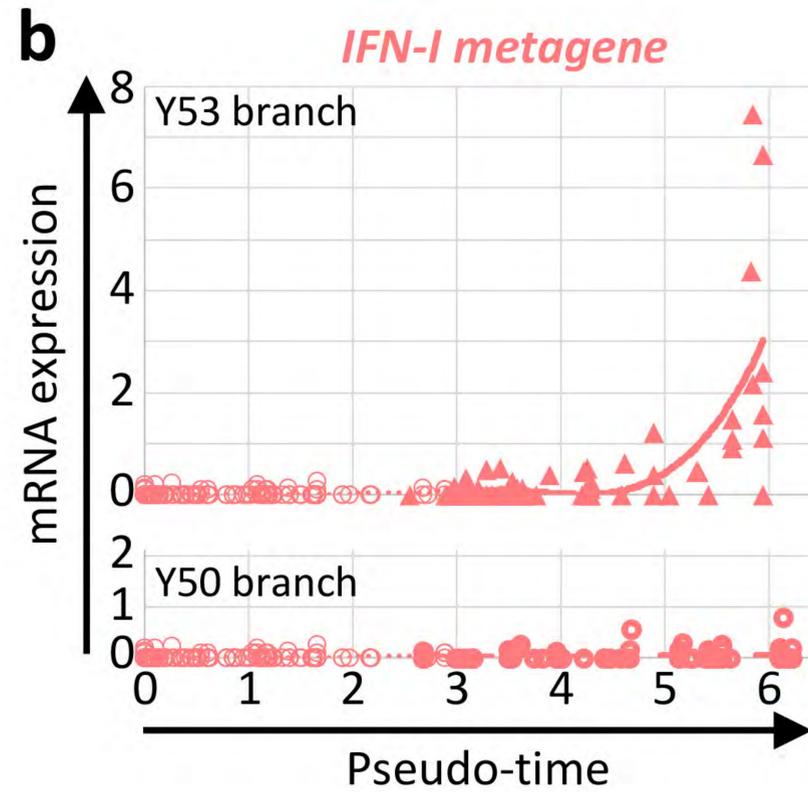
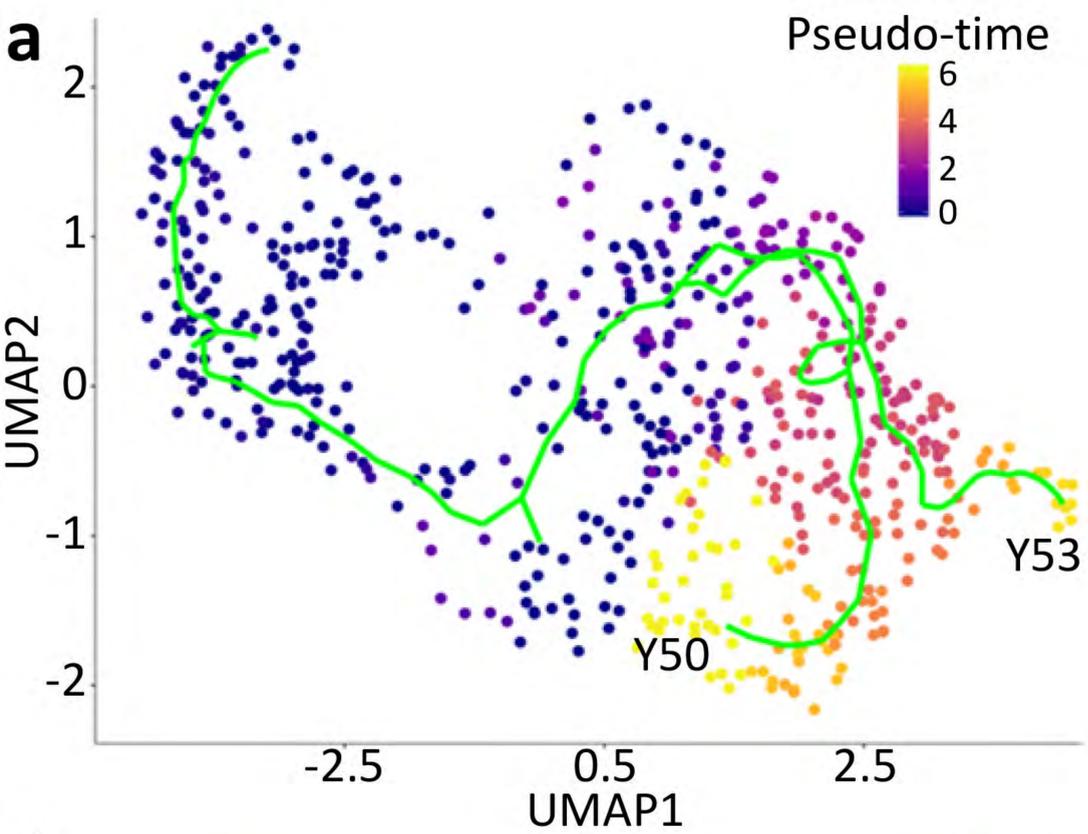


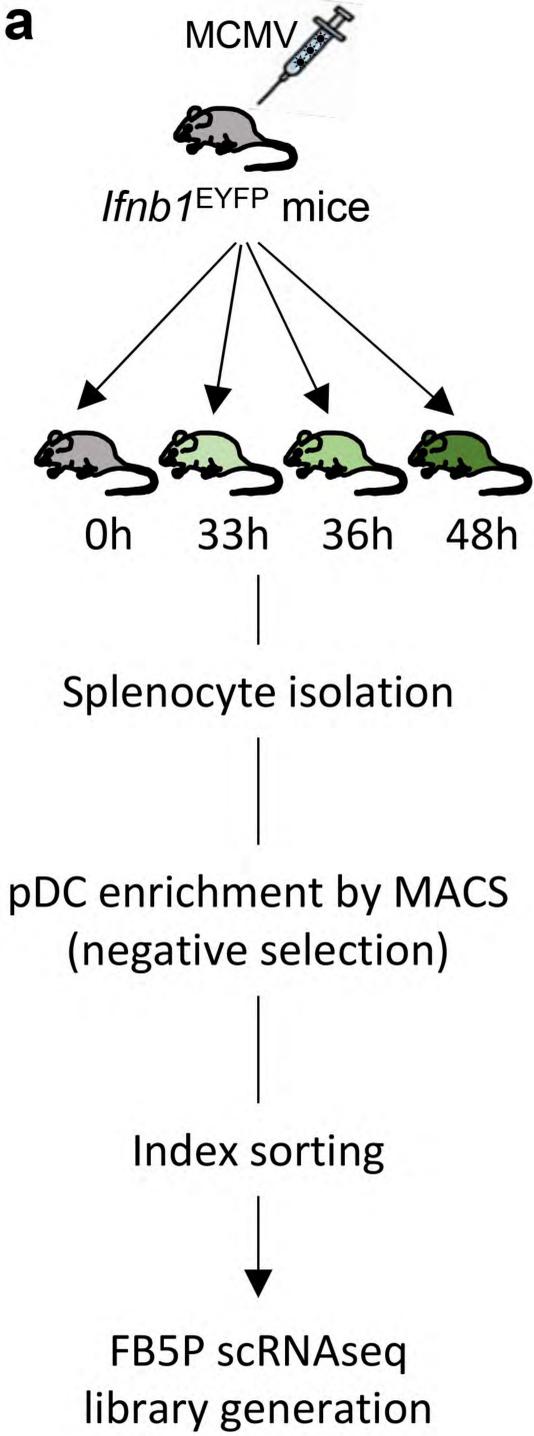






**a****b****c**

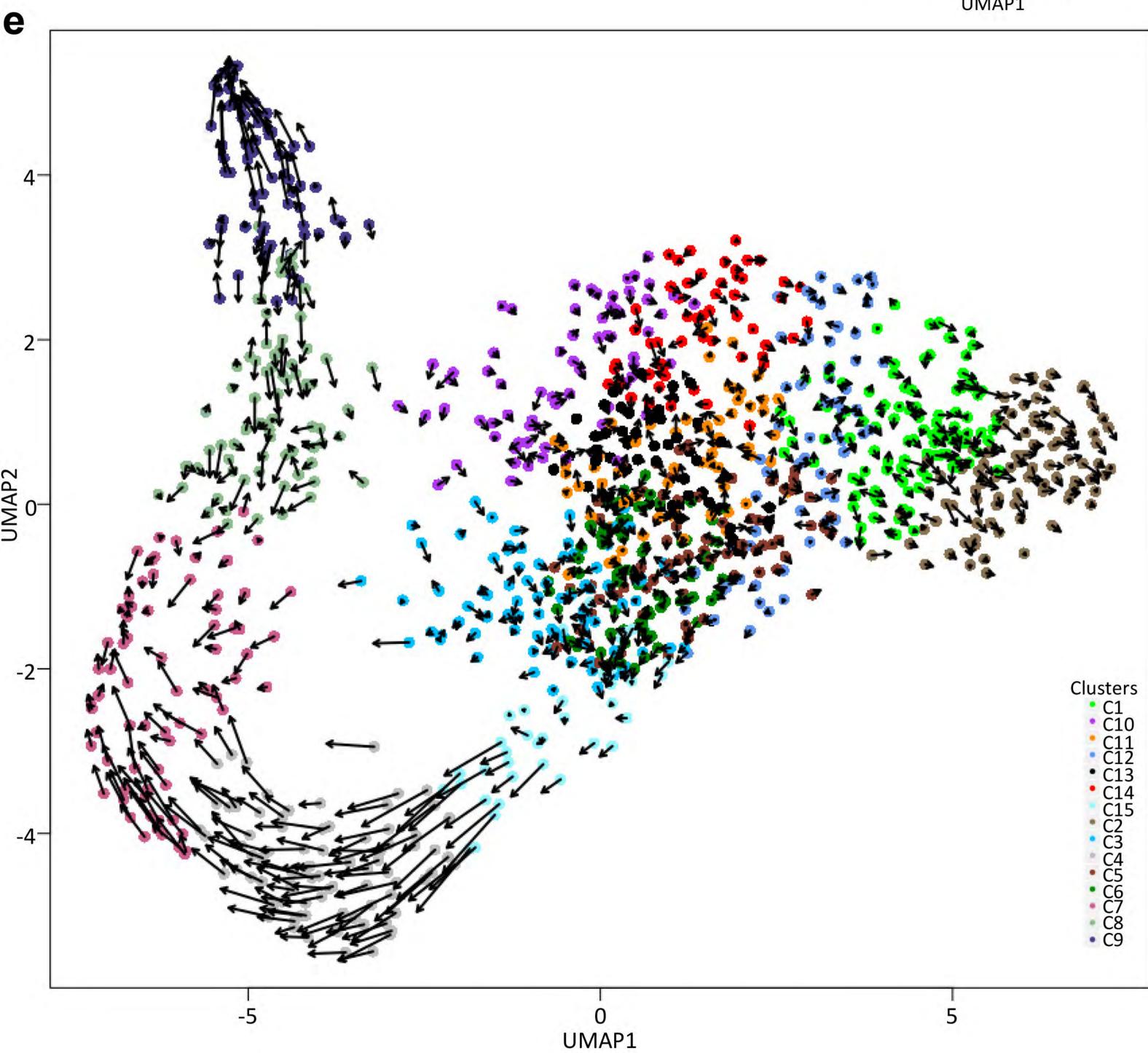
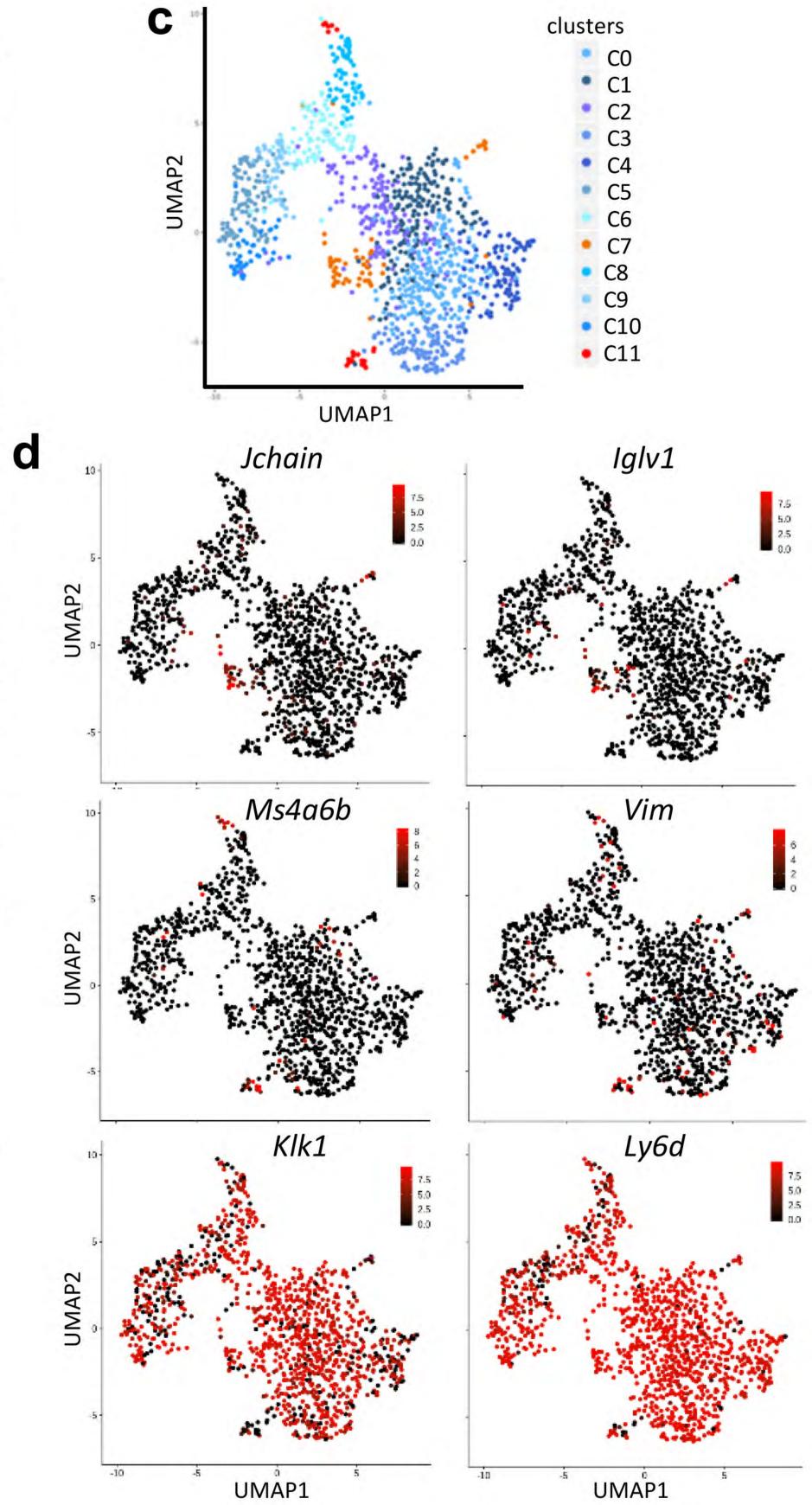


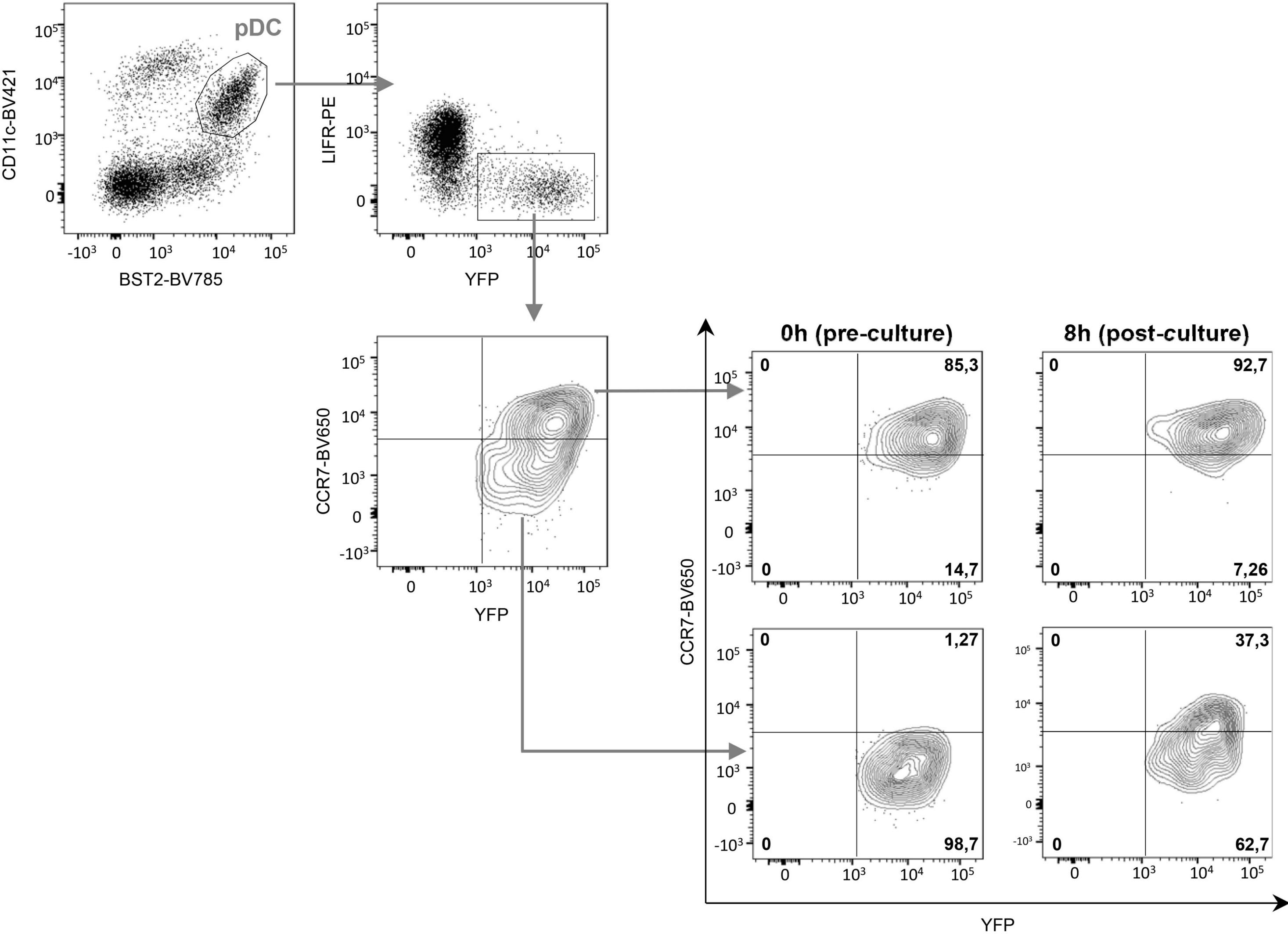


**b**

Time point	QC selection	Index sorting category			
		Bulk	EYFP+	LIFRlow	sum
0	total	94	0	0	94
	QC <sup>1</sup>	86	0	0	86
	pDC <sup>2</sup>	85	0	0	85
33	total	70	24	752	846
	QC <sup>1</sup>	63	20	611	694
	pDC <sup>2</sup>	62	18	578	658
36	total	70	24	188	282
	QC <sup>1</sup>	47	15	161	223
	pDC <sup>2</sup>	45	14	135	194
48	total	70	24	94	188
	QC <sup>1</sup>	64	22	84	170
	pDC <sup>2</sup>	59	21	77	157
total	total	304	72	1034	1410
	QC <sup>1</sup>	260	57	856	1173
	pDC <sup>2</sup>	251	53	790	1094

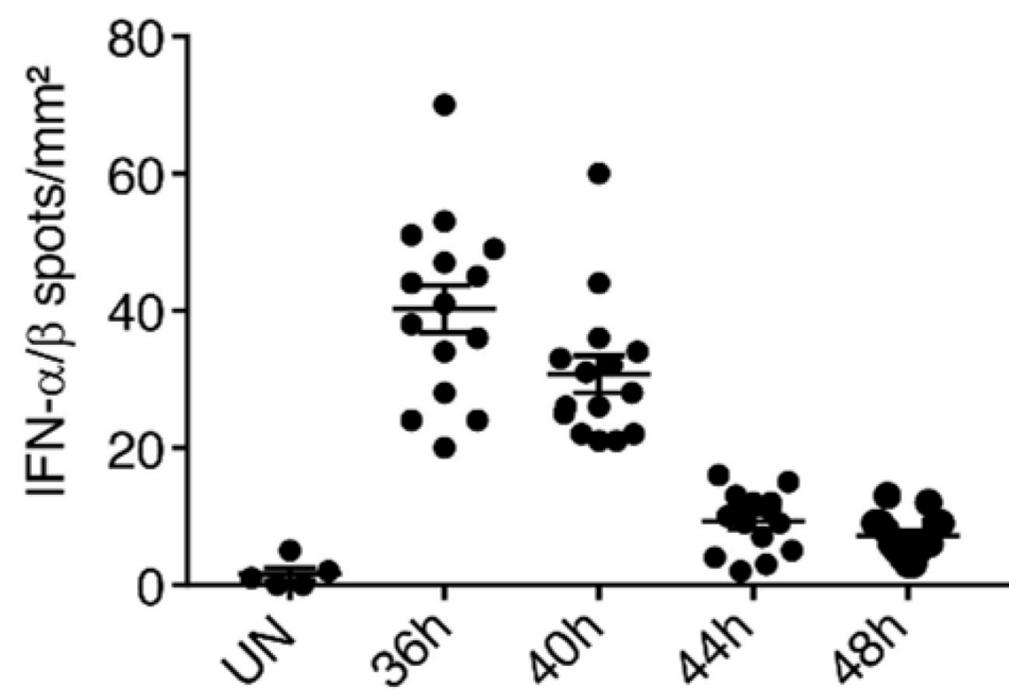
<sup>1</sup># cells that passed technical quality controls; <sup>2</sup>#cells that were identified as bona fide pDCs, as opposed to contaminating pDC-like pre-cDC/tDC or B cells.



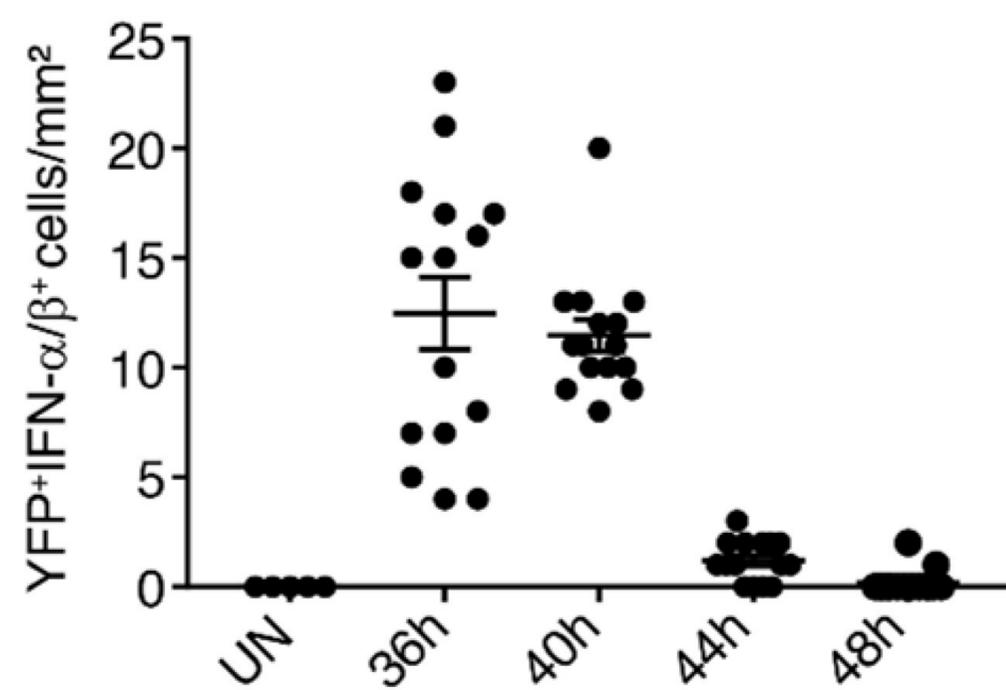


**a**

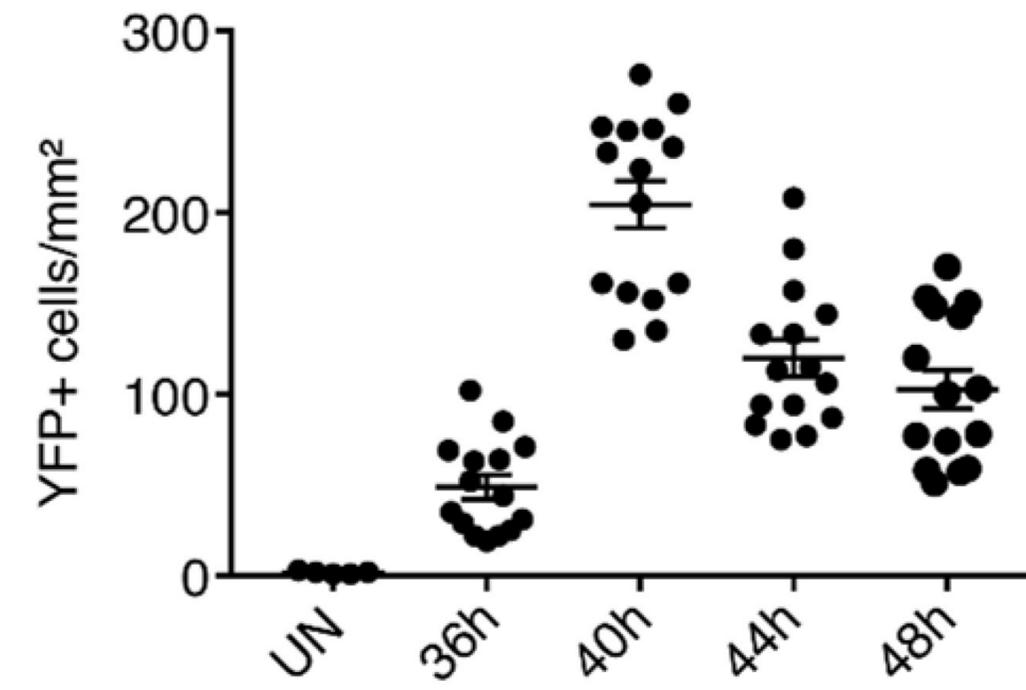
Whole spleen section

**b**

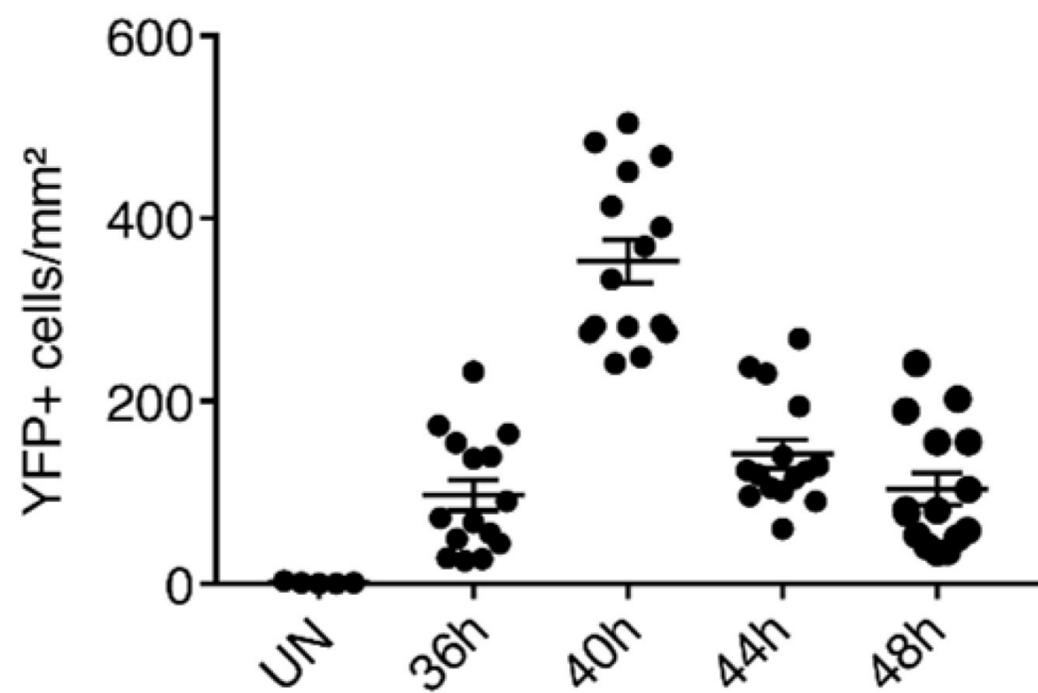
Whole spleen section

**c**

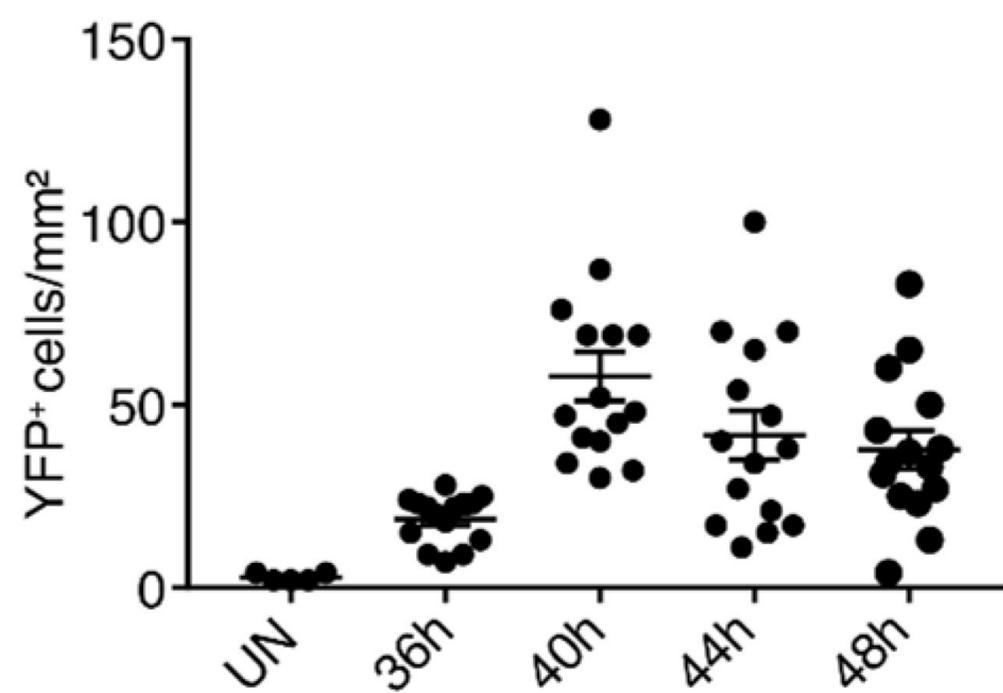
Whole spleen section

**d**

MZ



RP



WP

