Tissue clonality of dendritic cell subsets and emergency DCpoiesis revealed by multicolour fate mapping of DC progenitors

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Summary

Clonal relationships of cells in tissues can be determined by multi-colour fate mapping of progenitors coupled to probabilistic-based analysis. Here, we developed a combinatorial analysis approach to understand how the network of conventional dendritic cells (cDCs), key cells in immune surveillance, is established in tissues. We assessed the relatedness of cDCs occupying a given territory and show that precursors of cDCs (pre-cDCs) continuously enter tissues and proliferate to give rise to cDC clones. Most of these clones comprise a single cDC1 or cDC2 subtype, suggestive of pre-cDC commitment. Upon infection, a surge in the influx of pre-cDCs into the affected tissue dilutes clones and increases local cDC numbers. Our results indicate that tissue cDCs can be organised in a pathwork of closely positioned sister cells of the same subset whose co-existence is perturbed by local infection, when tissues actively communicate to the bone marrow the need for extra pre-cDCs to meet increased functional demand.

Introduction

Conventional dendritic cells (cDCs) are leucocytes that play a key role in innate immunity as well as in the initiation and regulation of T cell responses (Merad et al., 2013; Mildner and Jung, 2014). They comprise two basic sub-types, cDC1s and cDC2s, and form a network of immune sentinel cells in most tissues of mice and humans (Guilliams et al., 2014). cDC1s can be identified by expression of markers such as XCR1, DNGR-1 (aka CLEC9A), CD103 and low expression of CD11b (Guilliams et al., 2014; Merad et al., 2013; Mildner and Jung, 2014). They play a prominent role in cross-presentation of dead cell-associated antigens and in Th1 and cytotoxic T lymphocyte priming (Guilliams et al., 2014; Merad et al., 2013; Mildner and Jung, 2014). cDC1 differentiation depends on Irf8 and Batf family transcription factors, especially Batf3, as well as on Nfil3 and Id2 (Murphy et al., 2016). The other major DC subset, cDC2 (Guilliams et al., 2014) expresses high levels of CD11b and depends on Irf4, Irf2, Traf6, RelB and RBP-J for its differentiation (Murphy et al.,

2016). cDC2 are considerably heterogeneous and include, in the mouse, a subtype whose differentiation depends on Klf4 and induces Th2-dominated immunity (Tussiwand et al., 2015) , as well as gut CD103$^+$CD11b$^+$ DCs that prominently induce Th17 responses against pathobionts (Lewis et al., 2011; Persson et al., 2013; Schlitzer et al., 2013). Human cDC2 have similarly been shown to be heterogeneous (See et al., 2017; Villani et al., 2017) .

Irrespective of type, cDC1 and cDC2 all originate from cDC-committed haematopoietic progenitors in cone marrow (BM) known as the common (but, more accurately, the conventional) DC precursors (CDPs) (Bogunovic et al., 2009; Ginhoux et al., 2009; Naik et al., 2006; Liu:2009il Onai et al., 2007; Varol et al., 2009). CDPs give rise to pre-cDCs, which exit BM via the blood to seed lymphoid and non-lymphoid tissues(Bogunovic et al., 2009; Liu et al., 2009; Varol et al., 2009). Individual pre-cDCs were originally envisaged to be bi-potential and generate both cDC1 or cDC2 (Naik et al., 2007; Onai et al., 2007) . More recently, cDC1/cDC2 subset specification was shown to be able to occur, under at least certain circumstances, during the CDP to pre-cDC transition to give rise to committed pre-cDC1 or pre-cDC2 (Grajales-Reyes et al., 2015; Schlitzer et al., 2015; See et al., 2017). It remains unclear to what extent tissues are seeded by committed vs uncommitted pre-cDCs. Similarly, it is not known to which degree pre-cDCs arriving in non-lymphoid tissues have proliferative capacity and can undergo expansion to give rise to clones of differentiated cDC1 or cDC2 occupying a defined tissue territory.

The replacement of "old" tissue cDCs with "new" ones is thought to occur at a high rate as the half-life of cDCs in most tissues is 3-6 days (Liu et al., 2009). This steady-state cDC renewal is likely controlled at the level of the generation of pre-cDCs, their BM exit rate, their tissue seeding rate and the rate of pre-cDC differentiation into cDC1 and cDC2. It is also possible that pre-cDCs divide upon entering tissues(Naik et al., 2006), adding an additional factor to the regulation of cDC numbers. Importantly, there is indirect evidence to indicate that these parameters

are not immutable and that the global generation of cDC (cDCpoiesis) can increase upon loss of cDCs in the periphery (Hochweller et al., 2009) or in response to infection or tissue injury (Ballesteros-Tato et al., 2010; Farache et al., 2013; GeurtsvanKessel et al., 2008; Johansson et al., 2006; Nakano et al., 2017; Sevilla et al., 2004; Singh et al., 2008; Welner et al., 2008; Zuniga et al., 2004). While all these studies suggest that cDC numbers in tissues can be regulated by local demand, it is unclear whether this requires changes in putative local proliferation of pre-cDCs or, alternatively, communication with the BM and recruitment of additional precursors.

Confocal fluorescence microscopy is commonly used for analysis of tissue architecture and cell distribution (Paddock, 2013). When combined with multicolour fate mapping of cell precursors, it allows for analysis of single-colour cell clusters, which in turn informs on the clonal relationship of cells in tissues. This is particularly striking in organs such as the intestine, where intestinal stem cell precursors that display high proliferative capacity give rise to epithelial and other cells that remain adjacent to one another to form single-coloured villi (Snippert et al., 2010). However, when cell cycle progression is slower or progeny cells are motile, single-colour clustering is diluted. Analysis of less evident clustering can be carried out using algorithms that account for stochastic pattern formation (Ghigo et al., 2013; Jarjour et al., 2014; Mondor et al., 2016; Tay et al., 2017), although these are often restricted to two dimensional analysis. Moreover, the analysis needs to take into account different cell types, requiring cell phenotyping in addition to multi-colour fate mapping, which can rapidly reach the channel limitations of conventional confocal microscopy (Paddock, 2013).

Here, we use a workflow that employs spectral-resolved microscopy to allow for multiple fluorophore resolution followed by analysis of single-colour clustering in three dimensions to analyse the distribution and clonal architecture of cDCs in mice in which individual CDPs/pre-cDCs are fate-mapped with one of four possible fluorophores. We show that peripheral tissues such as lung and small intestine contain clones of sister cDCs that arise through local proliferation of immigrant pre-cDCs and

their progeny. Most of these clones consist of a single cDC subtype, consistent with early fate specification at the level of the CDP to pre-cDC transition. Notably, upon lung infection with influenza A virus, cDC numbers increase through an acute influx of pre-cDCs from BM, which dilutes pre-existing cDC clones. Thus, cDCpoiesis is a dynamic process responsive to emergency need, which is actively communicated from tissues to cDC progenitors in BM. This axis could be manipulated to increase immunity in vaccination or immunotherapy.

Results

Pre-cDCs and their progeny retain proliferative capacity in peripheral tissues

It is generally assumed that cDC progenitors actively cycle but that their cDC progeny are post-mitotic. To analyse the cell cycle status of pre-cDCs and cDCs in tissues, we stained cells in bone marrow (BM), spleen, mesenteric lymph node (mesLN), small intestine (SI) and lung for both DNA content and phosphorylated histone H3 (phospho-H3). This technique allows us to identify cells in four different phases of the cell cycle, namely G0/G1, S, G2 and M (Fig. 1A). Consistent with their high turnover rate, a large fraction of BM CDPs and pre-cDCs was found in S/G2/M phases at any given time (Fig. 1B). That contrasted with pre-cDCs and fully differentiated cDCs in spleen, LN, lung and SI, in which the fraction of S/G2/M cells was much smaller although, surprisingly, not negligible (Fig. 1D, F, H, J). As the DNA/phospho H3 staining does not distinguish cells that are still cycling (G1) from cells that have exited the cell cycle (G0), we carried out a separate analysis for Ki67, which is expressed in the G1, S, G2 and M phases but not in G0. As expected, 100% of BM CDPs and 80% of BM pre-cDCs were Ki67$^+$, consistent with cell cycle commitment (Fig. 1A, Fig. S1A). Notably, 40% of lung and SI pre-cDCs also stained for Ki67, indicating that this commitment is not fully lost upon BM exit (Fig. 1I, K, Fig. S1A). Similarly, we found that 30-40% of cDC1 and the two intestinal sub-types of cDC2 (CD103$^+$ CD11b$^+$ (double positive; DP) cDC2 and CD103$^-$ CD11b$^+$ cDC2)

were Ki67$^+$ (Fig. 1I, Fig. S1B and (Scott, 2014). In the lung, cDC1 and cDC2 stained for Ki67 at similar levels to cDCs in the SI (Fig. 1K, Fig. S1B). Overall, these data suggest that a substantial fraction of pre-cDCs that have entered peripheral tissues and of their cDC progeny remains in the G1 phase of the cell cycle and has therefore not become post-mitotic.

Multi-colour labelling of cDCs in Clec9a^{Confetti} mice

The above observations suggested the possibility that at least some tissue cDCs might be organised in patches of sister cells that arise through local cell division. To assess if this is the case, we resorted to multi-colour fate mapping of cDC precursors. We crossed Clec9a^{Cre} mice(Schraml et al., 2013) to Rosa26^{Confetti} mice(Snippert et al., 2010) (Fig. S2A; progeny henceforth called Clec9a^{Confetti}). Both CDPs and pre-cDCs express DNGR-1, encoded by the *Clec9a* gene, and mice expressing Cre recombinase under the control of the *Clec9a* locus can be used to trace the cDC lineage *in vivo*(Schraml et al., 2013). We expected that, in Clec9a^{Confetti} mice, cDC precursors would become stochastically labelled with one of four fluorescent proteins (CFP, GFP, YFP or RFP) and transfer the fluorophore to all daughter cells, allowing tracing of individual cDC clones. We validated Clec9a^{Confetti} mice by flow cytometric spectral analysis (which allows separation of closely related fluorophores, including GFP and YFP) of spleen, mesLN, SI and lung cell suspensions. In all organs, we found labelled cDC1 and cDC2 (Fig. S2B-D). The percentage of labelled cells in Clec9a^{Confetti} mice was lower than previously observed using Clec9a^{Cre} crossed to a Rosa26^{YFP} single fluorophore fate reporter strain(Schraml et al., 2013) (Fig. S2B), likely due to the complexity of the Rosa^{Confetti} locus, which reduces the efficiency of Cre-mediated recombination(Livet et al., 2007). This, together with the transience of Cre expression, leads to incomplete penetrance of the reporting event and labelling of only a fraction of cDCs, as previously reported. In contrast to cDCs, the frequency of labelled CD64$^+$ cells, generally considered to correspond to monocytes and macrophages

(MØs)(Guilliams et al., 2016), was very low (Fig. S2B), as expected(Schraml et al., 2013), although in lung, where CD64$^+$ cells vastly outnumber CD64$^-$ cDCs, they contributed to a larger fraction of all labelled cells (Fig. S2C). As noted(Snippert et al., 2010), the expression of the four fluorescent proteins in the Confetti reporter cassette was unequal, with clear underrepresentation of GFP$^+$ cDCs (Fig. S2C, D). Importantly, cDC1s, but not cDC2s, are DNGR-1$^+$ and hence express Cre recombinase after differentiation and become preferentially labelled in Clec9a-Cre-based reporter mice(Schraml et al., 2013), including Rosa$^{\text{Confetti}}$ (Fig. S2C, D). This also means that cDC1 can continue to recombine the inverted loxP sites of the Rosa$^{\text{Confetti}}$ locus, switching back and forth between expression of GFP and YFP or of RFP and CFP. This manifests itself as GFP$^+$YFP$^+$ or CFP$^+$RFP$^+$ double positive cells, which are disproportionately more abundant among cDC1 than cDC2 subsets (Fig. S2C, D). Despite these limitations, the flow cytometric spectral analysis clearly demonstrates that CDP/pre-cDCs in Clec9a$^{\text{Confetti}}$ mice can be stochastically labelled with different fluorophores that are faithfully transmitted to daughter cells resident in tissues.

Imaging of Clec9a$^{\text{Confetti}}$ mice reveals cDC distribution in three dimensions

To visualise the spatial arrangement of labelled cDCs and assess possible clustering by fluorophore indicative of local clonal expansion, we developed a protocol to fix and clarify tissues while preserving the Confetti fluorescent proteins (Fig. 2A). The method includes agarose embedding and vibratome sectioning, allowing one to cut 300µm sections, a thickness necessary to circumvent the scarcity of labelled cDCs in non-lymphoid tissues and to image enough cells for cluster analysis (Fig. 2A). It is also compatible with antibody staining of the sections (see below). Large tissue volumes (1020µm by 680µm in 100 z-steps of 3µm for SI, 1360µm by 1360µm in 40 z-steps of 5µm for lung) were then imaged by confocal microscopy using total spectrum acquisition (lambda mode scanning) followed by spectral unmixing to discriminate all fluorophores (Fig. 2A).

Validation of the system was initially carried out using lymphoid organs such as spleen and mesLN. It revealed an intricate network of Confetti$^+$ cells predominantly localised to T cell areas, as expected (Fig. 2B, C). However, the large number and high density of labelled cells in these organs precluded analysis of clustering based on fluorescent protein expression (Fig. 2B, C). We therefore focused on non-lymphoid tissues such as the SI and lung, where Confetti$^+$ cells were easily detected but sufficiently sparse to allow cluster analysis (Fig. 2D, E). Strikingly, visual inspection of images from SI and lung of Clec9a^{Confetti} mice revealed that Confetti$^+$ cells were often found in discrete single-colour clusters within individual villi or around airways, respectively (Fig. 2D, E and Movies S1, S2). Labelling was largely lost in Clec9a^{Confetti} mice crossed to Flt3l$^{-/-}$ mice (Fig. S3A, B and Movies S3, S4), which lack cDCs but not MØs(McKenna et al., 2000; Waskow et al., 2008)), confirming that Confetti$^+$ cells were bona fide cDCs. This was true even for the lungs, where a considerable number of Confetti$^+$ cells was found to be CD64$^+$ by flow cytometry (Fig S2D). To clarify the identity of CD64$^+$ cells, we carried out a separate flow cytometric analysis of Clec9a^{tdTomato} mice sufficient (WT) or deficient (Flt3l$^{-/-}$) in Flt3L. This revealed that Tomato-labelled CD64$^-$ cells were reduced in Flt3l$^{-/-}$ mice, as expected (Fig. S3C) and consistent with a reduction in total cDC numbers (Fig. S3D). However, Tomato$^+$ CD64$^+$ cells were also reduced in Flt3l$^{-/-}$ mice (Fig S3D) even though total CD64$^+$ cells, irrespective of CD11c and CD11b expression, were not (Fig S3D). These data are consistent with the possibility that CD64$^+$ cells expressing fluorescent protein in Clec9a lineage tracing mice represent atypical cDCs that express CD64, as previously suggested (Schraml et al., 2013). Nevertheless, to avoid confusion, CD64$^+$ cells were excluded from further analysis.

ClusterQuant 3D analysis indicates single-colour cDC clustering in Clec9a^{Confetti} mice

To quantify the clustering of cDCs in SI and lung, we developed a 3D version of the ClusterQuant software previously used for analysis of cell clusters in single tissue

planes(Ghigo et al., 2013). Briefly (Fig. 3A), the workflow involves different steps: (1) separation of 3D confocal images into individual z planes, corresponding to the optical slices used for image acquisition, (2) manual segmentation and annotation of cDCs in each plane, and (3) computation of 3D Voronoi polyhedrons using the x, y and z- cell coordinates. The polyhedron contains all voxels closer to the centroid of that Confetti$^+$ cell than to the centroids of all other cells and is used to compute neighbour and proximity relationships in the subsequent analysis steps (Fig. 3B, C and Movies S5, S6). To aid segmentation and generate shapes that approximate cell volumes, in step (2) it is necessary to draw borders along anatomic barriers (crypts and airways). We also randomly inserted dummy cells (displayed as grey cells) into the spaces between Confetti$^+$ cells, helping to remove artificially large Voronoi polyhedrons, avoiding false positive cluster formation and serving as a randomised control for the analysis (Fig. S4A, B, E – see below). Each Voronoi polyhedron was then assigned a colour based on the associated fluorescent protein except for double positive cells, which were assigned either purple (RFP$^+$ CFP$^+$) or orange (GFP$^+$ YFP$^+$). Adjacent Voronoi polyhedrons bearing the same colour were considered a cluster (i.e., a group of 2 or more cells of the same colour). The colours (but not the positions) of the Voronoi polyhedrons were then randomised in a step (4) through a Monte Carlo simulation with 10,000 possible scenarios. Comparison of the original (O) vs. simulated (S) images was carried out by extracting relevant parameters such as cluster number, cells per cluster and cluster compactness (Fig. 3A) to determine the probability that observed clusters could have arisen by chance.

In all cases, the ClusterQuant 3D analysis clearly revealed a pattern of single-colour cDC clustering in SI and lung that was not reproduced in randomised scenarios (Fig. 4). The difference between O and S images was statistically significant regardless of whether data were analysed as number of clusters relative to total cell number, fraction of total cells in clusters (Fig. 4A, B) or number of clusters per unit volume of tissue (Fig. S4C, D). This was true for both SI and lung and was observed

when images from all mice were pooled together (Fig. 4A, B) or when images were grouped per mouse (Fig. 4C, D). Confirming the validity of the 3D ClusterQuant algorithm, analysis of the randomly-placed dummy cells showed that they were not significantly clustered (Fig. S4E).

In additional analyses using statistical data binning with chi-squared tests, we examined the size distribution of clusters and normalised them to the value expected to be obtained by chance, which was calculated from the simulated scenarios. We found that the observed cDC clusters in both SI and lung were always larger than expected by chance (Fig. 4E, F, Fig. S4F, G). Indeed, in some cases, we observed very large clusters (> 15 cells) which are exceedingly improbable in a randomised scenario. However, it was also clear that cDCs were not always in clusters and in more than half of the cases appeared as isolated cells (Fig. 4G). Interestingly, we also found that clusters in the lung were significantly larger than in the small intestine, indicative of tissue differences (Fig. 4G, H). We conclude that at least some cDCs in SI and lung and, possibly, other tissues of Clec9a^{Confetti} mice form single-colour clusters, which must therefore represent clones of sister cells.

Single-colour cDC clusters are predominantly composed of a single cDC subset

We next asked whether cDC clones comprise multiple subsets (i.e., arise from uncommited pre-cDCs) or a single subset (i.e., originate from committed pre-cDC1s or pre-cDC2s or from dividing cDC1/2s) by analysing cluster composition in SI and lung from Clec9a^{Confetti} mice. We used staining with Abs against CD11b and CD103 to allow discrimination between cDC1 and cDC2 and Ab against CD64 to exclude CD64$^+$ cells (Fig. 5A-C and Movies S7, S8).

The distribution of fluorescent protein expression among cDC subsets in tissue sections of Clec9a^{Confetti} mice as assessed by microscopy (Fig. S5A) was concordant with that observed in cell suspensions analysed by spectral flow cytometry (Fig. S2C, D), cross-validating both experimental approaches. Cell composition analysis from

ClusterQuant 3D analysis revealed that single-colour clusters in both SI and lung often consisted of a single cDC subset (i.e., were "pure") although "mixed" clusters comprising cDC1 and cDC2 could also be observed (Fig. 5D, Fig. S5B). Pure clusters accounted for 80% of all clusters in the SI and 70% of clusters in the lung (Fig. 5D). We then carried out a separate analysis using a random distribution calculated probabilistically that considers the number of subsets and subset ratio in each tissue. This calculates the probability that any one cluster would contain a single cDC type merely by chance (Fig. S5E, F). This analysis revealed that the "purity" of cDC clusters was statistically significantly different from a chance event in all cases (Fig. 5E, F). Altogether, these data suggest that, during homeostasis, tissue cDC clones of sister cells are predominantly generated by local proliferation of incoming pre-committed cDCs and of their differentiated cDC progeny.

Single-colour cDC clusters are lost during infection

Next, we asked if cDC single-colour clustering (and, inferentially, cDC clonality) in tissues holds true under inflammatory conditions when an increase in cDC numbers is often observed(Ballesteros-Tato et al., 2010; GeurtsvanKessel et al., 2008; Johansson et al., 2006; Welner et al., 2008). Clec9a$^{\text{Confetti}}$ mice were intranasally infected with influenza A virus (strain X31), which, over the course of a week, caused a large increase in lung cDC1 and cDC2 numbers, as assessed by flow cytometry (Fig. 6A). This was accompanied by large scale infiltration of innate immune cells into the lungs but did not affect the specificity of the genetic labelling of cDCs in Clec9a$^{\text{Cre}}$ reporter mice (Fig. S6A-C). Images of infected lungs that were stained for viral proteins showed that the infection was patchy, with viral replication being confined to discrete foci (Fig. S6A and Movie S9), as reported (Fukuyama et al., 2015). At seven days post-infection, Confetti^{+} cells and CD64^{+} cells accumulated in much greater numbers around virus-containing foci than in uninfected regions (Fig. 6B, C). CD64^{+} cell infiltration was therefore used as a surrogate to discriminate high

(infected) and low (uninfected) infiltrated areas (Fig. 6C). Notably, 3D ClusterQuant analysis revealed that the cDC single-colour clustering that had been observed in steady state conditions was largely lost from high infiltrated areas after infection, which no longer showed a statistically-significant difference between O and S scenarios irrespective of whether the data were pooled or analysed by individual mouse (Fig. 6D, E, S7A and Movie S10). Low infiltrated areas retained some clusters of single-colour cDC in some of the mice but this did not reach statistical significance when all mice were considered together (Fig. 6F, G, Fig. S7B and Movie S11).

We extended the analysis by comparing cluster size distribution between uninfected lungs and areas of low or high infiltration in infected mice (Fig. 6H-K). This confirmed that the data fitted a random scenario (Fig. 6H, I) and established that there was only a slight increase in the frequency of cDC that were not in a cluster compared to the situation in uninfected mice (Fig. 6J). We found statistically significant differences at the level of larger clones, which could be preferentially found in lungs from uninfected mice and low infiltrated areas of lungs from infected mice compared to the high infiltrated areas of the latter (Fig. 4K). Altogether, these results show that the single-colour clustering of cDCs in lung is significantly reduced following influenza A virus challenge, predominantly in areas with active infection but also, to a lesser extent, in areas away from infectious foci.

Infection-driven loss of single-colour cDC clustering is due to an influx of pre-cDCs

The above data suggested that infection-driven increases in local cDC numbers were not likely to be a result of increased local clonal expansion. Indeed, flow cytometric analysis of cell cycle status showed that infection led, if anything, to a decrease in cDC1 and cDC2 in S/G2/M cell cycle phases and a reduction in cells positive for Ki67 (Fig. 7A, Fig. S7C). Pre-cDC in lung and pre-cDC and CDP in BM did not show any major changes in commitment to cell cycle (Ki67 staining, Fig. S7C, D) although more of them were found to be in S phase at d7 post infection

compared to uninfected mice (Fig. 7A, B). Therefore, the increase in cDC numbers in the lungs of mice post infection with influenza A virus (Fig. 6A) does not seem to be due to an increase in proliferation of cDCs or their immediate lung precursors.

To ask if it could be attributable to changes in the dynamics of lung seeding by pre-cDC, mice were examined at different time post-infection. At day 7 post-challenge, influenza virus infected mice had many more pre-cDCs in their lungs than uninfected controls (Fig. 7C). The increase in pre-cDCs in lungs was not obviously biased towards any one type of pre-committed pre-cDC (Grajales-Reyes et al., 2015; Schlitzer et al., 2015) (Fig. S7E), consistent with the fact that both cDC1 and cDC2 increased equally (Fig. 6A). In contrast to lung, there was loss of cDCs progenitors from BM, which reached their lowest numbers at day 3-4 post-infection and recovered by day 7 (Fig. 7D, E). As in lung, there was no major bias in terms of pre-cDC subset distribution in BM (Fig S7F).

These results suggested that high demand for cDCs induced by infection is met by rapid mobilisation of cDC progenitors from the BM. Consistent with that notion, there was a clear increase in total number of pre-cDCs circulating in peripheral blood of mice at 7 days post-infection, when the increase in lung pre-cDCs and cDCs became apparent (Fig. 7F), with no differences in commitment (Fig. S7G). To try to extend these data to humans, we carried out an analysis of transcriptome datasets from peripheral blood of patients prior to or post infection with influenza A virus(Zhai et al., 2015) using CIBERSORT (Newman et al., 2015). This revealed a marked increase in the blood frequency of DCs, annotated as "activated DCs" (Fig. 7G, H), consistent with a demand-driven increase in DCpoiesis. To assess whether pre-cDC numbers were also increased in human blood, as in mice, we assessed levels of transcripts for SEMA4D, a recently identified marker for cDC progenitors(Villani et al., 2017). SEMA4D transcripts were increased post infection (Fig. 7I). Importantly, although SEMA4D can also be expressed by T and B cells, T and B cell markers (*CD3E* or *CD79A*/B) were either unchanged o–r decreased in the same

datasets following influenza virus infection (Fig. 7I). Taken together, these data suggest that, in mice and humans, lung influenza virus infection leads to a local increase in cDCs that is met not by increased local clonal expansion of pre-cDC and their progeny but by communicating need to the BM, resulting in an efflux of pre-cDCs into blood and influx into lungs to meet demand.

Discussion

MØs and cDCs are immune cells that can be found in every tissue and play vital roles in maintaining organ homeostasis and in restoring tissue integrity upon infection or injury. Infection or inflammation are often accompanied by local increase in MØ and cDC numbers, which can be met through local proliferation or increased precursor recruitment. Tissue MØs are self-renewing and can proliferate more rapidly in response to injury-induced signals and cytokines (Minutti et al., 2017; Sieweke and Allen, 2013). Furthermore, blood monocytes can enter tissues upon demand and differentiate into cells that greatly resemble tissue MØs (Varol et al., 2015). Monocytes can also differentiate into cells that have cDC features but it is clear that such monocyte-derived DC-like cells are distinct from those that arise from regular cDCpoiesis and cannot substitute for them, for example in anti-viral or anti-tumour immunity (Briseño et al., 2016). Therefore, increased demand for cDCs in the periphery can only be met through increased generation of cDC precursors in BM or by greater mobilisation and/or proliferation of pre-cDCs in tissues. However, our understanding of cDCpoiesis, including tissue colonisation by pre-cDCs, is limited even in steady-state conditions. In addition, most studies of cDCpoiesis in mice rely on isolating defined progenitors and adoptively transferring them into other mice, where they may not find their way back to their niche. Here, we use a genetic model and develop a workflow of image analysis and three-dimensional clustering quantification to analyse how cDC precursors seed tissues at the single-cell level and in the absence of cell transfer. We compare the process at steady-state with that

induced by acute influenza virus infection. Our data reveal that pre-cDCs in the steady-state enter peripheral tissues and can divide locally before differentiating into cDCs, which themselves display residual proliferative capacity. This leads to formation of discrete clones of cDCs that are maintained in close proximity of each other, suggesting that much of the cDC network in tissues is in fact a patchwork of sister cells. Notably, we find that these sisters are predominantly composed of either cDC1 or cDC2, providing in vivo corroboration for the notion that cDC subset commitment can occur at the time of CDP to pre-cDC transition to give rise to blood pre-cDC1 and pre-cDC2(Grajales-Reyes et al., 2015; Schlitzer et al., 2015). Finally, we demonstrate that cDC generation is an elastic process that responds to external tissue demand by exporting pre-cDCs from BM at times of need. The rapid influx of such pre-cDCs into tissues and a reduction in local cell division leads to accelerated intermingling of clones and breaks up single-colour clustering in multi-colour fate-mapping mice.

Quantification of images from tissues of Clec9a[Confetti] mice revealed that 45% of cDCs in lung and 35% of cDCs in small intestine are in single-colour clusters of 2 or more cells. This pattern differs significantly from one obtained by chance and can only be interpreted by local clonal expansion and retention of sister cells and/or mother/daughter pairs in the same territory. It is remarkable that such single-colour clusters can be detected. Because cDCs are motile, especially when activated, it is likely that clustering of clones will dilute over time. Motility will additionally cause cDC clones to intermingle while cell death will lead to cluster dissolution. Finally, the fact that cDC1 can switch fluorescent protein expression can also break up single-colour clusters (although in some case this can be accounted for in the analysis as the half-life of the proteins is sufficiently long to result in double labelling). For all these reasons, the degree of cDC single-colour clustering that we observe in tissues is likely to be an underestimate of the true extent of tissue cDC clonality and many of the

isolated cells in tissues may therefore in fact have been previously been part of a single-colour cluster.

It is also remarkable that large clones are seen, including in one case a cluster of 23 cells positive for GFP, the most underrepresented fluorescent protein (Fig S5B). The issues discussed above (e.g., cDC migration) will disproportionately impact large clones, causing a bias towards detection of small single-colour clusters and isolated cells. The finding of some large clones suggests therefore that there may be defined tissue niches that allow for greater pre-cDC/cDC local proliferation or that prolong cDC half-life(Liu et al., 2007). Such niches may be more common in the lung than in SI as we noted a clear tendency for single-colour cDC clusters in that organ to be larger. Why clonality does not result in observable patches of cDC1 vs cDC2 in tissues can be understood from the fact that large clones are relatively rare and that, in our model, we visualise only a fraction of the tissue cDC network because of incomplete penetrance of the Cre-mediated recombination event. The superimposition of the mosaic pieces composed of cDC1 and cDC2 clones and single cells is expected to result in spatial mixing of the subsets. This may also explain why we do not see obvious single colour clusters in regions where cDCs are too abundant, such as T cell areas of lymph nodes and spleen. Mixing ensures that cDC with different preferences for antigen processing and presentation to distinct T cell subsets are present at any site of tissue damage or infection such that the ensuing adaptive response is properly diverse in peripheral tissues but not lymph nodes(Gerner et al., 2015).

Our analysis has centred on the cDC network but the methods we describe are generally applicable to any investigation of cell relationships in tissues. Confetti mice were used initially to describe the drift towards clonality in intestinal villi (Snippert et al., 2010) while ClusterQuant 2D has been successfully utilised in the analysis of clonality of Langerhans cells in epidermis and of stromal cells in lymph nodes (Ghigo et al., 2013; Jarjour et al., 2014). Notably, in all those cases, cells within a clone were in close proximity to one another and on a single plane, making the definition of a

cluster relatively straightforward. In the case of cDCs in three dimensions, clusters can be diffuse and their existence can only be ascertained because of the power of the statistical analysis, which compares the observed pattern to 10,000 randomised scenarios. We anticipate that the analysis pipeline described here will therefore be particularly useful in similar cases in which cells within a cluster are not necessarily juxtaposed in the three-dimensional space. Improvements to the analysis can be achieved through the use of a temporally-regulatable Cre driver (e.g., tamoxifen-inducible Cre) to permit pulse-chase experiments and evaluation of the kinetics of clonality establishment.

Experiments with cells *in vitro*, sorted *ex vivo* or transferred as bulk populations into mice have suggested that individual pre-cDCs can be either pre-committed to generate cDC1 or cDC2 or can be bi-potent and generate either cDC subset(Grajales-Reyes et al., 2015; Schlitzer et al., 2015) .Here, we revisited this question in Clec9a^{Confetti} mice where the composition of the Confetti$^+$ clones is analysed in an unperturbed state. We show that the majority of cDC clusters in lung and SI are pure cDC1 or cDC2 consistent with the possibility that they arise primarily from committed pre-cDC1s and pre-cDC2s. We also find that cDC1s and cDC2s retain residual capacity to proliferate after differentiation, which could contribute to the purity of the cDC clusters observed irrespective of pre-cDC commitment. The fact that cDC1s continue to express Cre could in theory introduce a bias towards detecting pure clusters of cDC1 expressing two fluorescent proteins surrounded by cDC2 expressing one of the two proteins. However, this was not observable in our images (data not shown) and the rarity of doubly labelled cDC1 events cannot explain the extremely high fraction of pure clusters detected. It should be noted that not all clusters are pure and that some mixed cDC ones are found in both SI and lung, possibly providing *in vivo* evidence for the existence of uncommitted pre-cDCs capable of seeding tissues and giving rise to sister cells of different fates. This did not

change upon infection (see below) but the regulation of pre-cDC fates in tissues in different conditions will be an interesting area for additional studies.

During inflammation, tissues have increased demand for cDCs (Ballesteros-Tato et al., 2010; GeurtsvanKessel et al., 2008; Johansson et al., 2006; Welner et al., 2008).. This could be met by increased proliferation and survival of pre-cDCs and/or cDCs already in tissues or by increased generation of pre-cDCs in BM and increased recruitment of those cells to tissues. Analysis of these issues has sometimes been clouded by the failure to distinguish cDCs from monocyte-derived cells. Here, we focus specifically on parameters of cDCpoiesis that cause alterations in tissue cDCs. We show that influenza A virus infection causes an increase in lung cDC numbers, which is not accompanied by increased local proliferation of pre-cDC or their differentiated progeny. Rather, it is accompanied by efflux of pre-cDCs from BM, which increase in number and frequency in peripheral blood and enter tissues, leading to increased seeding. In Clec9a^{Confetti} mice, this acute influx of pre-cDCs into lungs results in dissolution of pre-existing single-colour cDC clusters, a process that might be exacerbated by increased cDC mobility leading to increased intermingling of pre-existing clones and migration to mediastinal lymph nodes. Differently from a prior report (Ballesteros-Tato et al., 2010), we did not observe a selective increase in cDC2 in influenza virus-infected lungs and, consistent with that observation, pre-cDC in BM or lung did not display any infection-induced changes in phenotype suggestive of a bias towards cDC1 or cDC2 commitment (Grajales-Reyes et al., 2015; Schlitzer et al., 2015). Whether this relates to the strain of influenza virus used, the severity of the infection or the fact that the other study (Ballesteros-Tato et al., 2010) did not necessarily discriminate bona fide cDC2 from monocyte-derived cells remains to be established.

Our findings reveal that infected tissues can communicate to the BM their need for increased cDC, resulting in a release of pre-cDCs into blood and transient loss of cDC progenitors from their site of origin. By analogy with the increased release of

granulocytes into the circulation during infection or inflammation (Boettcher and Manz, 2017; Takizawa et al., 2012), the fact that cDC generation responds to demand might aptly be termed "emergency DCpoiesis". The signals that regulate emergency DCpoiesis are presently unknown but could include a combination of inflammatory cytokines, chemokines and direct sensing of microbial products by cDC progenitors. Future work to elucidate the molecular pathways that regulate emergency DCpoiesis is likely to reveal useful targets for immunotherapeutic approaches to cancer, infectious disease and autoimmunity.

Experimental Procedures

Mice

Clec9a-Cre , ROSA26-YFP(Srinivas et al., 2001), ROSA26-Confetti(Snippert et al., 2010), Flt3l$^{-/-}$ (Taconic Biosciences) and C57BL/6J mice were bred at The Francis Crick Institute in specific pathogen-free conditions. All transgenic mouse lines were backcrossed to C57BL/6J. Six to twenty-week-old mice were used in all experiments unless otherwise specified. All animal experiments were performed in accordance with national and institutional guidelines for animal care.

Infection with influenza virus

Mice were anaesthetised via inhalation of isoflurane. Mice were infected intranasally with 35000 TCID50 of influenza A X31 (H3N2) in 30μl of PBS. Mice were monitored daily for weight loss and signs of infection and sacrificed at day 1, 3 and 7 post-infection.

Cell isolation

Spleens, mesLNs and lungs were cut into small pieces and digested with Collagenase IV (200U/ml, Worthington) and DNase I (0.2mg/ml, Roche) in RPMI for 30 min (spleen and mesLN) or 60 min (lung) at 37°C. Digested tissues were strained through

a 70µm cell strainer (BD Bioscience) and washed with FACS buffer (3% foetal calf serum, 5mM EDTA, 0.02% sodium azide in PBS). For spleens, red blood cells were lysed with Red Blood Cell Lysis Buffer (2 min at RT; Sigma). For small intestines (SI) Peyer's patches and connective tissue were removed. SI were cut longitudinally and rinsed with PBS. The tissue was cut in 0.5cm pieces and incubated in 20ml of RMPI with 25mM HEPES, L-glutamine, penicillin/streptomycin, 50µM β-mercaptoethanol, 5mM EDTA (all Gibco), 3% foetal calf serum and 0.145mg/ml DL-dithiothreitol (Sigma) for 20min at 37°C with shaking. SI were subsequently vortexed for 30s and strained through a fine mesh tea strainer. Tissue fragments were washed three times in 10ml of RMPI with 25mM HEPES, L-glutamine, penicillin/streptomycin, 50µM β-mercaptoethanol and 2mM EDTA (all Gibco), while vortexing in between each washing step to remove intraepithelial lymphocytes. SI fragments were digested with collagenase IV (200U/ml, Worthington) and DNase I (0.2mg/ml, Roche) in 10 ml RPMI medium for one hour at 37°C. Digested tissues were strained through a 70µm cell strainer (BD). For the SI and lung, leucocytes were enriched by Percoll gradient centrifugation (GE Healthcare). Nine parts Percoll were combined with one part 10x PBS to obtain 100% Percoll. Cells were resuspended in 70% Percoll in PBS or HBSS/RPMI, overlaid with 37% and 30% Percoll and centrifuged at room temperature for 30min at 2000rpm without braking. Cells were collected at the 70/37% interface.

Flow cytometry

Up to 4 million cells were pre-incubated with blocking anti-CD16/32 in FACS buffer for 10 min at 4°C and then stained for 20 min at 4°C with staining cocktail in FACS buffer in the presence of anti-CD16/32. DAPI was used to exclude dead cells, except for when cells were fixed. In the latter case, dead cells were excluded by live/dead fixable blue or aqua dye (Invitrogen). For Ki67, DNA content (FxCycle, Invitrogen) and phospho-H3 staining cells were fixed and permeabilised using Foxp3 Fix/Perm buffer set (eBioscience, 00-5523-00). For DNA content examination, samples were

collected on a LSR Fortessa flow cytometer (BD Biosciences) or a SP6800 Spectral Analyser (Sony) and analysed using FlowJo 9 or 10 software (TreeStar Inc.). CDPs were defined as Lin$^-$ CD11c$^-$ MHCII$^-$ DNGR-1$^+$, pre-cDCs were defined as Lin$^-$ CD11c$^+$ MHCII$^-$ DNGR-1$^+$ CD135$^+$ in BM and as CD45$^+$ Lin$^-$ CD11c$^+$ MHCII$^-$ CD43$^+$ Sirpα^{int} in SI, lung and blood. Irrespective of tissue, pre-cDCs were further subdivided into pre-cDC1s (CD117$^{int/+}$ CD115$^-$), pre-cDC2s (CD117$^-$ CD115$^+$) and uncommitted pre-cDCs (CD117$^-$ CD115$^-$). cDC1s were defined as CD11c$^+$ MHCII$^+$ CD64$^-$ CD11b$^-$ and cDC2s were defined as CD11c$^+$ MHCII$^+$ CD64$^-$ CD11b$^+$ in lung while in SI cDC2 were additionally separated on the basis of CD103 staining and defined as CD103$^-$ cDC2s (CD11c$^+$ MHCII+ CD64- CD11b$^+$ CD103$^-$) and CD103$^+$ cDC2s (CD11c$^+$ MHCII+ CD64- CD11b$^+$ CD103$^+$). Gating strategies are shown in Fig. S11-13, including the discrimination of the four Confetti fluorophores by spectral analysis.

The following antibodies were used for flow cytometry:

Target	Fluorophore	Clone	Company	Concentration
CD8-alpha	APC	53-6.7	Biolegend	0.4µg/ml
CD11b	BV605	M1/70	Biolegend	1µg/ml
CD11b	APC eFluor780	M1/70	eBioscience	1µg/ml
CD11c	PerCP Cy5.5	N418	Biolegend	1µg/ml
CD11c	APC eFluor780	N418	eBiocience	1µg/ml
CD16/32	Unconjugated	2.4G2	BD Bioscience	1µg/ml
CD45.2	BV605	104	Biolegend	1µg/ml
CD45R (B220)	PE	RA3-6B2	BD Bioscience	0.5µg/ml

CD64	AF647	X5-4/7.1	Biolegend	2μg/ml
CD103	PECy7	2E7	Biolegend	4μg/ml
DNGR-1	PE	1F6	Biolegend	5μg/ml
I-A/I-E (MHCII)	AF700	M5/114.15.2	eBioscience	1μg/ml
Ly6c/Ly6g (Gr-1)	Pacific Blue	RB-8C5	Biolegend	1μg/ml
Ki67	BV786	B56	BD Bioscience	5μg/ml
Phospho-H3	AF647	D2C8	Cell Signaling	0.25μg/ml

Microscopy

Mice were perfused with 20ml of PBS and 10ml of Antigenfix (Diapath). 1 ml of melted 2% low melting point agarose (Invitrogen) in PBS was inserted through the trachea. Lungs, spleen and lymph nodes were then removed and fixed overnight in Antigenfix at 4°C. The proximal small intestine (mostly duodenum) was also removed and contents were flushed with ice cold PBS and Antigenfix, and fixed for 2h at 4°C. Small intestines were subsequently cut longitudinally, rolled into a "Swiss roll" and fixed overnight in Antigenfix at 4°C. After fixation, tissues were transferred to 30% sucrose in PBS for 24h at 4C. Lungs, spleen and small intestine were embedded in 4% agarose in PBS and 300μm sections were cut using a Leica VT1200S vibratome and collected in PBS. Lymph nodes were unsectioned and imaged as whole mounts. For antibody staining, tissue sections were blocked for 12h at RT in 1% bovine serum albumin, 0.3% Triton X-100 (Sigma) and 1% mouse serum (Sigma) in 0.1M Tris pH7.4. Sections were stained with rat anti-CD11b (clone 5C6, BIO-RAD, 1:100), rabbit anti-CD64 (027, Sino Biological, 1:1000) and goat anti-CD103 (AF1990, R&D, 1:100) or anti-M+NP-FITC (Oxoid, 1:100) in blocking buffer for 48h at 4°C. Sections were subsequently stained with donkey anti-goat IgG

(H+L) AF680 (Jackson ImmunoResearch) in blocking buffer overnight at RT and blocked with 1% goat serum (Sigma) in 0.3% Triton X-100 in 0.1M Tris pH7.4 overnight at RT. Sections were then stained with donkey anti-rat AF594 (Invitrogen) and goat anti-rabbit IgG (H+L) AF647 (Invitrogen) or donkey anti-rabbit IgG (H+L) AF594 and donkey anti-FITC AF647 (Jackson ImmunoResearch) in blocking buffer overnight at RT. Alternatively, small intestine sections were stained with anti-E-cadherin AF594 (DECMA-1, Biolegend) in blocking buffer overnight at RT. Spleen sections were stained with anti-CD169 (Biolegend) in blocking buffer for 48h at 4°C and subsequently stained with donkey anti-goat IgG (H+L) AF594.

Sections were mounted on slides in the clearing solution Rapiclear 1.47 (SunJin Lab) for image acquisition while lymph nodes were clarified instead using uDISCO(Pan et al., 2016) and mounted on a metallic glass-bottom dish. Images were acquired on a LSM880 inverted confocal microscope with a 25x oil immersion objective (Zeiss). Laser lines at 405nm, 458nm, 514nm, 561nm and 633nm were used to excite the fluorophores. Lambda mode scanning (detecting 410-687nm) was used to detect CFP, GFP, YFP, RFP, AF594 and AF647. AF680 was detected in a separate channel collecting 686-735nm. For all images, tile scans and z-stacks were acquired with a step size of 2.5 µm and a pinhole of 1 airy unit. Images were taken at 1024x1024 voxel density with a line averaging of 8. Fluorophores and autofluorescence were unmixed into separate channels using the unmixing algorithm provided in the Zen software (Zeiss). Single-stained slides were used to obtain the reference spectra of the different fluorophores. For quantification of CD64^{+} cells and infected cells, surfaces were generated with Imaris software by using the AF594 and AF647 channels respectively. Images and Videos were generated after adjusting channels using Imaris software.

The following antibodies were used for microscopy:

Target	Fluorophore	Clone	Company	Concentration
CD11b	Purified	5C6	BIO-RAD	10µg/ml

CD64	Purified	027	Sino biological	0.75ng/ml
CD103	Purified	Polyclonal goat	R&D	3.54µg/ml
CD169	Purified	Polyclonal rat	Biolegend	5 µg/ml
IgG Goat	AF680	Polyclonal donk	Jackson Immun	3.75µg/ml
IgG Rabbit	AF594	Poly4064	Biolegend	1.25µg/ml
IgG Rabbit	AF647	Polyclonal goat	Invitrogen	5µg/ml
IgG Rat	AF594	Polyclonal donk	Invitrogen	5µg/ml
M+NP	FITC	Not stated	Oxoid	1:100
FITC	AF647	1F8-1E4	Jackson Immun	4µg/ml

Clonality analysis

To quantitatively analyse the clustering of cells in 3 dimensions, we developed a 3D version of ClusterQuant 2D(Ghigo et al., 2013). The software is based on the CognitionMaster and GenericBusinessClient software implemented in Microsoft NET/C#(Wienert et al., 2013). ClusterQuant3D comprises three modules. The first module facilitates manual cell labelling in 3D image stacks. In addition to labelling observed cells, "dummy cells" (extended figure 5C) and borders (villi, airways, high and low infiltrated areas) are annotated to fill void spaces and guarantee realistic Voronoi region growing. In the second software module, a 3D Voronoi mesh is created around the annotated cells for visualisation, including rotation and the adjustment of different transparency settings to interactively explore the 3D representation. This module also offers an option to define equivalence rules which

can be used to group different cell classes into higher level classes, e.g. "YFP cDC1" and "YFP cDC2" into "YFP cDC". The third module performs a statistical evaluation of the spatial relationships among the cells represented in the Voronoi mesh. For each cluster of connected cells of the same type the features 1) mean cells per cluster and 2) compactness (defined as number of same class neighbours divided by number of all neighbours) are computed. The statistical significance/non-randomness of cluster size and compactness are evaluated with 10,000 Monte Carlo simulations for each test. To this end, cell positions are maintained but cell labels are randomly shuffled for each simulation and the resulting size/compactness measures are computed and compared to the original ones and used for p-value estimation.

Clone composition analysis.

Based on the estimated ratio of cells cDC1:cDC2 (2:1) in lung and under the assumption that clonal composition was random, the probability that a clone of a given size is composed of a single cell-type could be calculated. In the lung, the probability that a clone of size n is pure, is $p \, (pure) = p_{cDC1}{}^n + p_{cDC2}{}^n$ where p_{cDC1} and p_{cDC2} are the proportions of each cell type. From these probabilities and the known number of clones of each size, the total number of clones that were pure and mixed could be estimated for each mouse, assuming random mixing. Fisher's Exact test was then used to compare experimental versus random for proportion of pure and mixed clones. Similar analysis was conducted in the small intestine for the three groups of cells cDC1, cD103$^+$ cDC2 and cD103$^-$ cDC2 cells (ratio 2:4:1).

Statistics

Statistical analyses were performed using GraphPad Prism software (GraphPad), MATLAB (Mathworks) or RStudio. Statistical test used is specified in each figure legend.

Author contributions

Figure legends

Fig. 1. cDCs progenitors and cDCs divide in lymphoid and non-lymphoid organs.
(A) DNA content histogram (left) or dot plot with DNA content and phospho-H3 (right) of BM CDPs from one mouse as a representative example of cell cycle analysis. (B) Percentage of BM CDPs or pre-cDCs in G0/G1, S, G2 or M phases of the cell cycle determined as in (A). (C) Percentage of Ki67$^+$ CDPs and pre-cDCs in BM. (D) Percentage of splenic pre-cDCs and cDCs in G0/G1, S, G2 or M phases of the cell cycle determined as in (A). (E) Percentage of Ki67$^+$ splenic pre-cDCs and cDCs. (F) Percentage of mesenteric lymph node pre-cDCs, resident and migratory cDCs in G0/G1, S, G2 or M phases of the cell cycle determined as in (A). (G) Percentage of Ki67$^+$ mesenteric lymph node pre-cDCs and cDCs. (H) Percentage of intestinal pre-cDCs and cDCs in G0/G1, S, G2 or M phases of the cell cycle determined as in (A). (I) Percentage of Ki67+ pre-cDCs, cDC1s, cDC2s and DP cDC2s in SI. (J) Percentage of lung pre-cDCs and cDCs in G0/G1, S, G2 or M phases of the cell cycle determined as in (A). (K) Percentage of Ki67$^+$ pre-cDCs, cDC1s and cDC2s in lung, as indicated. Data in (B, D, F, H, J) are mean values from 6 C57BL/6 mice. Data in (C, E, G, I, K) are compiled from 12 C57BL/6 mice analysed in 2 independent experiments. Cells are gated as indicated in Materials and Methods.

Fig 2. Images of several Clec9a^{Confetti} using confocal microscopy with lambda mode acquisition. (A) Workflow of tissue processing, staining and imaging of Clec9a^{Confetti} organs. (B) 3D projection of a 300μm spleen vibratome section that was stained with anti-CD169. Confetti surfaces were generated with Imaris software to reduce autofluorescence. Zooming into T cell areas (1) was used to visualise Confetti+ cDCs (2). (C) 3D projection of a mesenteric lymph node clarified with uDISCO. Confetti surfaces were generated with Imaris software to reduce autofluorescence background. Square depicts selected zoom in area displayed at the bottom. (D) 3D projection of a 300μm vibratome section of the small intestine from a Clec9aCre/+R26Confetti/+

mouse stained with an antibody recognising E-cadherin to delineate the epithelium. (E) 3D projection of a 300μm vibratome section of the lung from a Clec9a$^{Cre/+}$R26$^{Confetti/+}$ mouse. Autofluorescence channel is displayed to visualise the lung structure. (D) 3D projection of a 300μm vibratome section of the small intestine from a Clec9a$^{Cre/+}$R26$^{Confetti/+}$ mouse stained with an antibody recognising E-cadherin to delineate the epithelium. (E) 3D projection of a 300μm vibratome section of the lung from a Clec9a$^{Cre/+}$R26$^{Confetti/+}$ mouse. Autofluorescence channel is displayed to visualise the lung structure.

Fig 3. Analysis of single-colour cDC clusters using ClusterQuant 3D. (A) Workflow of plane separation (1), cell annotation (2), 3D Voronoi generation (3) randomisation (4) and analysis using ClusterQuant 3D. (B) Original images from the small intestine (top) and the lung (bottom) were annotated using ClusterQuant 3D software and converted to Voronoi diagrams (middle), which were then randomised through a Monte Carlo simulation (right). Dashed lines indicate the structure of the SI (villi) using CD11b stain or lung (airways) using autofluorescence.

Fig 4. SI and lung cDCs are organised in spatially-restricted clusters of sister cells (A) From left to right: number of clusters of 2 or more cells normalised to number of cells, percentage of number of cells in clusters and cluster compactness from 24 small intestine images from 4 mice (Observed (O), orange) compared to simulations (S, grey). (B) Data as in (B) from 25 lung images from 5 mice (Observed (O), blue) compared to simulations (S, grey). (C) Data from (A) grouped per mouse. Lines link the observed and simulated scenarios associated with each mouse. Colours correspond to individual mice (Observed (O) and simulations (S)). (D) Data from B grouped per mouse. Lines link the observed and simulated scenarios associated with each mouse. Colours correspond to individual mice (Observed (O) and simulations (S)). (E) Proportion of

clusters of size 1-≥7 in observed (O, orange) versus simulated (S, dashed line) images from small intestine images of a representative mouse. (F) Proportion of clusters of size 1-≥11 in observed (O, blue) versus simulated (S, dashed line) images from lung images of a representative mouse. (G) Proportion of clusters size 1 (grey) or 2 or more in the small intestine (orange) or the lung (blue). Data are from the pool of all mice analysed. (H) Comparison of proportion of clusters with size 2-10 in the small intestine (orange) and in the lung (blue). Data are from the pool of all mice analysed. Statistical analysis in (B-E) used a paired t-test and in (F-G) used a chi-squared test.

Fig 5. Single-colour cDC clusters are mainly composed of a single cDC subset. (A) 3D projection of a Clec9^{Confetti} small intestine image (left) and lung image (right) stained with antibodies against CD103, CD11b and CD64. (B) Single z slices of individual cells from the small intestine of Clec9^{Confetti} mice; individual channels are shown on the right side of the merged image. (C) Single z slices of individual cells from the small intestine of Clec9^{Confetti} mice; individual channels are shown on the right side of the merged image. (D). Pie charts representing percentage of pure clusters across the 24 small intestine images (SI, orange, 80%) or 25 lung images (blue, 70%) analysed. Grey bar indicates the percentage of the remaining mixed clone (20 and 30% respectively). (E) Analysis of the proportion of pure clusters of cluster size 2-7 found in the small intestine of the 4 mice analysed (O, orange) or the small intestine pure cluster random distribution calculated probabilistically (S, grey). (F) Analysis of the proportion of pure clusters of cluster size 2-10 found in the lung of the 5 mice analysed (O, blue) or the lung pure cluster random distribution calculated probabilistically (r, grey). Error bars

correspond to variation across mice using 95% CI. Statistical analysis was carried out using a Fishers exact test.

Fig 6. Influenza A virus infection dilutes single-colour cDC clusters (A) Number of cDC1s (left) or cDC2s (right) from Clec9aCre/+ R26eYFP/+ mice infected with Influenza A virus (magenta) or non-infected (N.i., grey) as control. (B) 3D projection of a Clec9^{Confetti} lung section 7 dpi with Influenza A virus. (C) Quantification of confetti$^+$ cells, CD64$^+$ cells and X31 particles from images with high (magenta) and low (purple) infiltration of cells. (D) Number of clusters of 2 or more cells normalised to number of cells and percentage of cells in clusters from 20 high infiltrated lung images from 5 mice (Original (O), magenta) compared to simulations (S, grey). (E) Number of clusters normalised to number of cells (left) and percentage of clonal cells (right) in high infiltrated areas from infected lungs of 5 mice. Lines link the observed and simulated scenarios associated with each mouse. Colours correspond to individual mice (Observed (O) and simulations (S)). (F) Number of clusters of 2 or more cells normalised to number of cells and percentage of number of cells in clusters from 19 low infiltrated lung images from 4 mice (Original (O), purple) compared to simulations (S, grey). (G) Number of clusters normalised to number of cells (left) and percentage of cells in clusters (right) in low infiltrated areas from infected lungs of 4 mice. Lines link the observed and simulated scenarios associated with each mouse. Colours correspond to individual mice (Observed (O) and simulations (S)). (H) Proportion of clusters of size 1-11 in original (O, magenta) versus simulated (S, dashed line) images from the high infiltrated images of a representative mouse. (I) Proportion of clusters of size 1-7 in original (O, purple) versus simulated (S, dashed line) images from the low infiltrated images of a representative mouse. (J) Proportion of clusters size 1 (grey) or 2 or more in non-infected lungs (N.i., blue, Fig.4), and high (magenta) and low (purple)

infiltrated areas in the lungs of infected mice. Data are from the pool of all mice analysed. (K). Comparison of proportion of clusters of size 2-10 in non-infected lungs (N.i., blue, Fig.2), and high (magenta) and low (purple) infiltrated areas in the lungs of infected mice. Data corresponds to the pool of all mice analysed. Statistical analysis in (B-G) used a paired t-test and in (H-K) used a chi-squared test.

Fig 7. Influenza A virus infection increases lung cDC numbers by recruiting BM progenitors. (A) Percentage of lung pre-cDCs and cDCs in G0/G1, S, G2 or M phases of the cell cycle determined as in Fig 1. Data corresponds of mean values from 6 non-infected (top) or 6 infected mice with Influenza virus. (B) Percentage of BM CDPs and pre-cDCs in G0/G1, S, G2 or M phases of the cell cycle determined as in Fig 1. Data corresponds of mean values from 6 non-infected (top) or 6 7dpi with Influenza virus. (C) Numbers of lung pre-cDCs from non-infected (grey), 3dpi or 7dpi with Influenza A virus (magenta) mice. (D) Numbers of BM CDPs from non-infected (grey), 3dpi or 7dpi with Influenza A virus (magenta). (E) Numbers of BM pre-cDCs from non-infected (grey), 3dpi or 7dpi with Influenza A virus (magenta). (F) Numbers of blood pre-cDCs from non-infected (grey) 3dpi or 7dpi with Influenza A virus (magenta). (G) Relative mean percentage of cells from peripheral blood of patients pre and post infection with Influenza A virus. Percentages were obtained from microarray data using CIBERSORT. (H) Percentage of activated blood DCs in peripheral blood of 41 patients pre and post infection with Influenza A virus. Percentage was calculated as for J. (I) Expression of SEMA4D, CD3E, CD79A and CD79B in peripheral blood from patients before (pre, grey) or in the first 48h of symptoms after natural infection with influenza A virus virus (post, magenta). Statistical analysis in C-F was based on an unpaired t test in 6 mice/group. Statistics in H, I were based on a paired t-test. Gating was done as for

Figure 1 and specified in materials and methods, including blood pre-cDCs that were gated a lung pre-cDCs.

Legends to supplementary figures

Fig S1. Percentage of Ki67+ DC progenitors and cDCs in BM, SI and lung of C57BL/6 mice. (A) Representative dot plots from figure 1A-C of BM CDPs, BM pre-cDCs, SI pre-cDCs and lung pre-cDCs stained with anti-Ki67 antibody (right) or isotype control (left). (B) Representative dot plots from figure 1B-C displaying % of Ki67+ SI and lung cDC1s and cDC2s and CD103+ (DP) cDC2s from the SI compared to isotype control (left).

Fig S2. Flow cytometric analysis of Clec9aConfetti organs. (A) Schematic of genetic recombination occurring in the progeny of Clec9a$^{Cre/Cre}$ mice and Rosa26$^{Confetti/Confetti}$ mice. (B) Representative plots of splenic cDC1 and cDC2 cell populations depicting the gating strategy followed for Confetti$^+$ cells analysed by spectral cytometry. (C) Labelling frequencies in different cDC subsets and macrophages found in spleen, mesenteric lymph nodes, small intestine and lung. (D) Percentage of Confetti$^+$ cDC subsets and macrophages from total live cells in the same organs than in a. Cells were labelled as explained in Figure 1.

Fig S3. Analysis of Clec9a$^{Cre/+}$ Rosa26$^{Confetti/+}$ Flt3L$^{-/-}$ mice. (A) 3D projection of a 300μm vibratome section of the small intestine stained with anti-E-cadherin (white). (B) 3D projection of a 300μm vibratome lung section. Autofluorescence channel (white) is depicted to show lung structure. (C) Percentage of tdTomato$^+$ cells that are CD64$^+$ or CD64$^-$ in mice sufficient (WT) or deficient for Flt3L (Flt3l$^{-/-}$). (D) Number

of cDCs, $CD64^+$ $CD11c^+$ or $CD11b^+$ cells in the lungs of Clec9a$^{Cre/+}$ Rosa26$^{tdTomato/+}$ that are either sufficient (WT) or deficient for Flt3L (Flt3l$^{-/-}$).

Fig S4. ClusterQuant 3D analysis. (A) Original image from the small intestine (left) that was annotated inserting dummy cells (Fig. 2A) and converted to Voronoi diagrams, in this case displaying dummy cells as well (right). (B) Original image from the lung (left) that was annotated inserting dummy cells (Fig. 2A) and converted to Voronoi diagrams, in this case displaying dummy cells as well (right). (C) Clusters found per volume of the small intestine analysed in the images (left) or grouped per mouse (right). (D) Clusters found per volume of the lung analysed in the images (left) or grouped per mouse (right). (E) Number of dummy clusters per image (left) or grouped per mouse (right) from dummy cells that were randomly inserted in the lung images. (F) Proportion of clusters of size 1-7 in original (O, orange) versus simulated (S, dashed line) images from small intestine images of the other 3 mice analysed that were not shown in Fig. 4E. (G) Proportion of clusters of size 1-11 in original (O, blue) versus simulated (S, dashed line) images from lung images of the other 4 mice analysed that were not shown in Fig. 4F. Statistical analysis in C-E was done using a paired t test. In F and G was done using a chi-squared test.

Fig S5. Identification of Confetti$^+$ cDC subsets. (A) Number of annotated Confetti$^+$ cDC subsets and macrophages in all the images analysed grouped per mouse. (B) Representative 20 clones in the SI and the number and type of cDC subset found in it. (D) Representative 20 clones in the lung and the number and type of cDC subset found in each clone. (E) Diagram depicting calculation of random probability in the SI for

cluster size 2. (F) Diagram depicting calculation of random probability in the lung for cluster size 2.

Fig S6. Quantification of infected cells and cell infiltration during Influenza A virus infection. (A) Representative 3D projection of 300um lung sections from Clec9aConfetti mice 7d.p.i. with influenza A virus (X31 strain). Sections were stained with anti-CD64 (magenta) and anti-M+NP viral proteins (X31, orange). Merge with autofluorescence channel (white) is shown on the left, X31 alone (middle) or X31 Imaris surfaces (right) for quantification in high or low infiltrated areas from the same image. (B) Labelling frequency in cDC1s, cDC2s and macrophages in Clec9a$^{Cre/+}$ R26$^{eYFP/+}$ mice 7 d.p.i, with Influenza A virus (C) Number of CD64$^+$CD11c$^+$ cells, CD64$^+$CD11b$^+$Ly6C$^-$ or Ly6C$^+$ cells, neutrophils and eosinophils from Clec9a$^{Cre/+}$ R26$^{eYFP/+}$ mice infected with Influenza A virus (magenta) or non-infected (N.i., grey) as control. Neutrophils were gated as CD45$^+$CD64$^-$Gr-1$^+$ and eosinophils as CD45$^+$CD11b$^+$ and further gating using SSC-A and FSC-A parameters. The remaining cell populations were gated as described previously and specified in Materials and Methods. Statistical analysis was done using an unpaired t test.

Fig S7. Quantification of cDC cluster formation in lung sections from infected Clec9a^{Confetti} mice. (A) Representative image from a high infiltrated area (left) that is annotated using Clusterquant 3D software. Observed Voronoi diagrams are generated (middle) as well as simulations (right). (B) Representative image from low infiltrated area (left) that annotated using Clusterquant 3D software. Observed Voronoi diagrams are generated (middle) as well as simulations (right). (C) Percentage of Ki67$^+$ lung pre-cDCs, lung cDC1s and lung cDC2s of non-infected mice (grey) or 7dpi with influenza

A virus (magenta). (D) Percentage of Ki67$^+$ BM CDPs (left) or BM pre-cDCs (right) from non-infected mice (grey) or 3dpi with Influenza A virus (magenta). (E) Percentage of pre-cDCs that are pre-cDC1s, pre-cDC2s or uncommitted in the lungs of non-infected 3 or 7dpi with Influenza A virus. (F) Percentage of pre-cDCs that are pre-cDC1s, pre-cDC2s or uncommitted in the BM of non-infected, 3 or 7dpi with Influenza A virus. (G) Percentage of pre-cDCs that are pre-cDC1s, pre-cDC2s or uncommitted in the blood of non-infected, 3 or 7dpi with Influenza A virus. Pre-cDC1s, pre-cDC2s and uncommitted pre-cDCs were gated as reported (Grajales-Reyes et al., 2015; CD117$^{+/int}$, CD115$^+$ or CD117$^-$CD115$^-$ respectively). Statistical analysis was performed using unpaired t test. Stars in E-G indicate statistical significance compared to N.i. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

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