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Clonal multi-omics analysis reveals the aetiology of Flt3 ligand-induced emergency dendritic cell development

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Abstract

Regulation of haematopoietic stem and progenitor cell (HSPC) fate is crucial during homeostasis and under stress conditions. Here, we examine the aetiology of the Flt3 ligand (Flt3L)-mediated increase of type 1 conventional dendritic cells (cDC1s). Using cellular barcoding we demonstrate this occurs through selective clonal expansion of HSPCs that are primed to produce cDC1s and not through activation of cDC1 fate by other HSPCs. In particular, multi/oligopotent clones selectively amplify their cDC1 output, without compromising production of other lineages via a process we term *tuning*. We then develop *Divi-Seq* to simultaneously profile division history, surface phenotype, and transcriptome of single HSPCs. We discover that Flt3L-responsive HSPCs maintain a proliferative 'early progenitor'-like state, leading to selective expansion of multiple transitional cDC1-primed progenitor stages that are marked by Irf8 expression. These findings define the mechanistic action of Flt3L through clonal *tuning*, which has significant implications for other models of 'emergency' haematopoiesis.

Introduction

Various extrinsic stimuli such as infection and cytokine administration can induce emergency haematopoiesis, where the production of certain immune cell types drastically increases[1-3]. Early haematopoietic stem and progenitor cells (HSPCs) in the bone marrow (BM) can sense and respond to many environmental cues to actively contribute to this process[4-6]. Depending on the context, extrinsic factors, most notably cytokines, can influence HSPC survival, proliferation, lineage differentiation and/or other parameters to drive emergency haematopoiesis[7]. Recent single cell transcriptional profiling and lineage tracing studies have highlighted substantial lineage bias and lineage restriction within HSPCs that had previously been assumed to be multi- or oligo-potent[8-13]. While these findings have advanced our understanding of the generation of cellular diversity during steady-state haematopoiesis[11,14] there is a paucity of clonal-level information that accounts for skewing of lineage production during emergency haematopoiesis.

Theoretically, two non-mutually exclusive clone-level mechanisms could explain selective lineage expansion during emergency haematopoiesis (Fig. 1a). First, new HSPC clones can acquire fate output towards a particular lineage (see *clonal fate activation* in Fig. 1a), which could imply activation of otherwise quiescent clones, and/or clones acquiring an additional lineage priming program. Second, emergency stimuli could preferentially *expand* those HSPCs already primed to a particular lineage (see *clonal expansion* in Fig. 1a). Population-level analysis cannot adequately dissect the contribution of the aforementioned mechanisms. Instead, a systematic analysis of changes in lineage fate of HSPCs at the single cell level is required to resolve the clonal aetiology of emergency haematopoiesis.

Dendritic cells (DCs) are a critical immune cell type that can be categorized into three functionally specialized subsets: type 1 conventional DCs (cDC1), type 2 cDCs (cDC2s), and plasmacytoid DCs (pDCs)[15]. In particular, cDC1s play a unique role in activating cytotoxic T lymphocytes against intracellular pathogens and cancer cells[16-21]. However, cDC1s are a surprisingly rare immune cell type and are especially sparse within the tumour microenvironment[18]. Therefore, strategies to increase their abundance have important potential clinical utility. The cytokine *fms*-like tyrosine kinase 3 (Flt3) ligand (Flt3L) is essential for steady-state DC development[22-25], and when used at supra-physiologic concentrations it promotes 'emergency' DC generation with a particular accumulation of cDC1s[26,27] that can

enhance immunity against cancers[18,28,29] and infections[2,30-32]. Importantly, where in the haematopoietic pathway Flt3L exerts its action is still debated[33]: some studies inferring this can occur in early Flt3$^+$ HSPCs[34-38], whereas other models imply a predominantly late effect in cDC1 expansion[39,40]. This controversy allowed us to test the effect of a cytokine (Flt3L) that selectively expands a cell type (cDC1) but for which a receptor (Flt3) is present along multiple stages of haematopoiesis (Fig. 1b).

Here, we use cellular barcoding and *Divi-Seq* – a novel single cell multi-omics method – to provide insights into the control and regulation of DC fate during Flt3L-mediated emergency haematopoiesis. We identify that both early and late HSPCs that are already DC-primed are responsive to Flt3L, leading to the selective expansion of clonal cDC1 output via *Irf8*$^+$ transitional progenitor stages without compromising their fate to other lineages. These findings have significant implications for the understanding of extrinsic regulation of HSPC fate during emergency haematopoiesis, as well as defining the mechanisms underlying DC expansion during exogenous administration of Flt3L for anti-microbial treatment and immunotherapy[2,18,28-32].

Results

Flt3L drives emergency cDC1 generation from early HSPC populations

Before undertaking clonal level investigations, we systematically established the responsiveness of different HSPC populations[41] to Flt3L stimulation (Fig. 1b). First, we used Cell Trace Violet (CTV) to track cell proliferation of Flt3L-stimulated $Lin^{-}cKit^{hi}Sca1^{+}$ cells (LSKs) *in vitro* and found that LSKs divided more in the presence of high concentrations of Flt3L (Extended Data Fig. 1). We then sorted multiple HSPC subsets (Extended Data Fig. 2a) and examined their responsiveness to Flt3L *in vitro* and found modest to substantial levels of proliferation from several HSPC populations including multipotent progenitor (MPP) 3/4s and macrophage-DC progenitors (MDPs) proliferated in the presence of Flt3L (Fig. 1c, d), indicative of Flt3L responsiveness (Fig. 1e). No marked cell proliferation was observed in $Flt3^{-}$ common myeloid progenitors (CMPs), granulocyte/monocyte progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs), consistent with prior reports[42,43].

Next, we analysed which HSPC populations in the BM changed in number either 3 or 5 days after Flt3L administration (Fig. 1f and Extended Data Fig. 2b). As expected, MEP number remained unchanged at both timepoints (Fig. 1f). Conversely, we observed an increase in the production of the majority of early HSPCs such as long term-HSCs (LT-HSCs), MPP2s and MPP3/MPP4s on day 5, as well as more restricted progenitor populations including common lymphoid progenitors (CLPs), CMPs and GMPs/MDPs (Fig. 1f). This suggested that early multi-potent progenitors and/or their ancestors were responsive directly or indirectly to exogenous Flt3L stimulation *in vivo*.

To directly test whether early HSPCs respond to Flt3L and induce emergency DC generation *in vivo*, LSKs were transplanted into recipient mice that were sub-lethally irradiated 3 days prior to minimise irradiation-induced alteration of Flt3L levels. After transplantation, mice received daily injections of PBS or Flt3L for 10-12 days, followed by the analysis of donor-derived lineages in spleen on day 14 (Fig. 1g, h and Extended Data Fig. 3). Consistent with prior knowledge in non-transplantation settings[26,27], we observed a substantial increase in donor-derived DCs following LSK transplantation and Flt3L stimulation, most prominently for cDC1s (Fig. 1g, h). Only marginal changes were observed in the myeloid (eosinophils, monocytes and neutrophils), lymphoid (B cells) and erythroid (erythroblast) lineages (Fig. 1h). Furthermore, mice exposed to Flt3L at a later period (days 5-8 or days 9-12) had smaller

increases in DC generation than those receiving Flt3L early (days 1-4) (Fig. 1i). These results indicated that exogenous Flt3L can directly act on transplanted early HSPCs and/or their downstream progeny for preferential expansion of DCs, particularly cDC1s.

Together, our results demonstrate that Flt3L promotes proliferation of multiple early HSPC subsets in addition to the current paradigm of a 'late' effect of Flt3L in DC development[40]. These findings provided a rationale for investigating the control of LSK fate upon supra-physiologic levels of Flt3L stimulation at a clonal level.

Cellular barcoding reveals minimal contribution to emergency cDC1 generation via clonal fate activation

We next sought to understand the clonal aetiology of emergency DC development using cellular barcoding, which can inform i) the number and quantitative contribution of clones to a given lineage, and ii) whether lineages have common or separate clonal ancestors. We tagged individual LSKs with unique and heritable DNA barcodes and transplanted these into sub-lethally irradiated recipients followed by daily PBS or Flt3L treatment (Fig. 2a). Two weeks post transplantation, different splenic progeny populations were sorted and the barcode composition within each population was analysed after barcode PCR amplification and sequencing (Fig. 2a). A comparison of both cell number and barcode number in each population allowed an initial assessment of whether clonal fate activation and/or lineage-specific *clonal expansion* accounted for preferential DC generation (Fig. 1a).

Flt3L treatment led to a preferential increase in DC generation from barcoded LSKs, particularly cDC1s (Fig. 1h and Fig. 2b). There was no significant difference in the number of total barcodes between PBS and Flt3L treatment (Fig. 2c) and only a slight increase in cDC1s with Flt3L (Fig. 2d). However, while some cDC1 *fate activation* was possible, this 2-fold increase in clone numbers could not fully account for the average 12-fold increase in cDC1 numbers (Fig. 2b) and their output was very low (see later). Importantly, we did not observe any significant increase or decrease of barcodes in myeloid, lymphoid or erythroid lineages (Fig. 2d and Extended Data Fig. 4), indicating that Flt3L-mediated emergency cDC1 generation was not at the expense of other lineages. Importantly, these findings were reproducible in other experimental settings, including transplantation via intra-femoral injection to bypass any confounding role of migration after *i.v.* injection, or into a host

environment with a reduced dose of irradiation (2 Gy; day -3 irradiation) to mitigate irradiation-induced cytokine production that may alter lineage fate (Extended Data Fig. 4).

Although best known for analysis of single cell RNA-seq data, we instead analysed barcoding using t-Distributed Stochastic Neighbourhood Embedding (t-SNE) to systematically characterize changes in clonal fate distribution and size as described previously (Fig. 2e, f)[13]. Using DBSCAN we identified 17 clusters, hereby referred to as 'fate clusters' (Fig. 2g). We also generated a heatmap where barcodes were ordered by fate clusters and numerical output (columns) to the different cell types (rows), and annotated for the treatment group (Fig. 2h).

Barcodes from both conditions were present in all fate clusters, with little over- or under-representation apparent (Fig. 2f-i). We noted that the small increase in cDC1-generating barcodes (Fig. 2d) would largely be explained by Cluster 9 but where clones from Flt3L treated mice were extremely low in numerical output (Fig. 2h). It is possible that these clones were below the limit of detection in PBS conditions, but expanded sufficiently to pass that threshold after Flt3L treatment. These results suggest a negligible contribution of *clonal fate activation* (Fig. 1a) to explain Flt3L-induced emergency cDC1 development.

Enhanced clonal expansion drives emergency cDC1 generation

Next, we investigated whether changes in clone size accounted for emergency cDC1 generation (Fig. 2b). We compared the total numerical output of cDC1s from each cDC1-generating fate cluster between PBS- and Flt3L-treated mice noting substantial increases in most cDC1-generating clusters, particularly those with multi-lineage output (clusters 1-4) (Fig. 3a). These collectively accounted for the majority of cDC1 output in both PBS- (84%) and Flt3L (95%) treated conditions (Fig. 3b, c). This resulted in a large numerical increase of cDC1s by these fate clusters (14 to 23-fold increase; Fig. 3b).

We next investigated the extent of clonal expansion of multi-outcome clones towards cDC1s, compared to other cell types by first visualising the distribution of clone sizes to each cell type per fate cluster (Fig. 3d and Extended Data Fig. 5). We observed a global increase in the distribution of cDC1s produced per clone from the relevant HSPCs, suggesting the majority of clones were responding to Flt3L and not simply a few clonal outliers (Fig. 3d and Extended Data Fig. 5). Interestingly, the average fold increase in clonal cDC1 contribution was greater

in fate clusters that exhibited multi-lineage output (5 to 14-fold) compared to fate clusters with cDC-biased output (1 to 4-fold) (Fig. 3d and Extended Data Fig. 5). However, within those multi-/oligo-potent clusters, Flt3L treatment selectively impacted clonal cDC1 output with little impact on clonal contribution – lower or higher – to other cell types (Fig. 3d, e). In summary, our barcoding results revealed that Flt3L-mediated emergency cDC1 generation was mostly due to enhanced *clonal expansion*, and not *clonal fate activation* in a way that did not compromise HSPC output to other lineages (Fig. 1a).

***Divi-Seq* simultaneously profiles divisional history and molecular state of single HSPCs**

Having systematically examined the clonal properties of Flt3L-induced emergency DC development, we further investigated the molecular aetiology underlying this process using *Divi-Seq*; a novel single cell multi-omics profiling approach for simultaneous assessment of cell division history, surface marker phenotype and the transcriptome of single cells. Briefly, LSKs were CTV-labelled prior to transplantation into non-irradiated recipients, followed by daily injection of PBS or Flt3L for three days (Fig. 4a). This allowed a short enough time to track divisional history using CTV before extensive dilution, but with sufficient time to capture the early molecular hallmarks of emergency DC development. BM cells were then harvested and stained for various surface markers, and single donor-derived cells index sorted into wells of 384-well plates for single cell RNA-sequencing (scRNA-seq) (Fig. 4a). Approximately 2,000 CTV^+ cells were sorted across both treatment conditions from two independent experiments. After additional FACS pre-gating (Extended Data Fig. 6) and quality control filtering of data from scRNA-seq (Methods), 467 PBS-treated and 870 Flt3L-treated cells remained for downstream analysis. In the second experiment, we also enumerated the number of donor-derived cells from each recipient. We observed an overall increase (5-fold) in cell number from all three Flt3L-treated mice compared to PBS controls (Fig. 4b). In addition, we observed a more than 3-fold increase in the number of donor-derived cells in multiple phenotypically defined HSPC subsets including MPP2s, MPP3/4s, CLPs, CMPs and GMP/MDPs after Flt3L stimulation (Extended Data Fig. 6).

Next, we performed clustering with Seurat and defined each cluster based on their surface marker expression, cluster-defining marker genes (Supplementary Table 1) and similarity scores to known cell types using SingleR (Extended Data Fig. 7). This led to the identification of seven clusters, including three clusters with HSPC signatures (HSC/MPP-like, lymphoid

progenitor-like and myeloid progenitor-like) and four progenitor clusters closer to mature lineages (cDC-like, pDC-like, monocyte-like and neutrophil-like) (Fig. 4c, d). For example, HSC/MPP-like cells expressed the highest level of HSPC markers compared to other clusters, including cKit, Sca1 and CD150 (Fig. 4c, d). Conversely, most cDC progenitor-like cells expressed CD11c and had high expression of cDC1 genes such as *Id2* and *Cst3* (Cystatin C) (Fig. 4c). Thus, this cluster likely contained cells that were more transcriptionally similar to pre-cDC1s than pre-cDC2s[44,45].

Hyper-proliferation and maintenance of a 'progenitor-like' signature with supra-physiologic levels of Flt3L

The most profound increase in abundance after Flt3L treatment was within the transcriptionally cDC-like cluster (27-fold; Fig. 4e and Extended Data Fig. 6e). An approximate 10-fold increase in the number of cells from Flt3L-treated mice was also observed in both the lymphoid progenitor- and myeloid progenitor-like clusters, consistent with cDC1s being generated from myeloid and lymphoid pathways[42,43]. In contrast, the transcriptionally pDC-, monocyte- and neutrophil-like cells represented a small proportion of cells, which did not alter drastically in numbers after Flt3L exposure (Fig. 4e and Extended Data Fig. 6). Importantly, although a higher proportion of HSC/MPP-like cells was found in PBS conditions, the number of cells estimated in the BM was 2-fold higher in mice treated with Flt3L due to an overall increase in cellularity (Fig. 4b, e and Extended Data Fig. 6).

The increase in cell number could largely be explained by enhanced cell division, as demonstrated by a shift in the CTV profiles between treatments (Fig. 4f, g). Importantly, increased proliferation was observed in cells that were either transcriptionally similar to DCs (cDC- and pDC-like clusters), or progenitor-like cells (HSC/MPP-, lymphoid progenitor- and myeloid progenitor-like clusters) (Fig. 4f) or when defined using surface marker expression, particularly MPP3/4s, CLPs, CMPs and GMP/MDPs (Fig. 4g). Conversely, Flt3L exposure had minimal effect on the division of cells without DC potential, including neutrophil- and monocyte-like progenitors (Fig. 4f). These results demonstrated a selective pattern in how single HSPCs respond to exogenous Flt3L stimulation that reflects DC lineage specification.

We then interrogated the relationship between divisional histories of cells and surface marker expression (Fig. 5a) or their transcriptional similarity to different known haematopoietic populations (Fig. 5b). Interestingly, most PBS-treated cells down-regulated expression of

HSPC markers including cKit, CD48, CD16/32 and CD34 concurrent with cell division, whereas large numbers of Flt3L-treated cells divided more and maintained high levels of these markers (Fig. 5a). A strikingly similar pattern was also observed when comparing CTV profiles to transcriptional similarity scores against LT-HSCs, ST-HSCs and MPP3/4s (Fig. 5b). Consistently, the up-regulation of mature lineage markers including CD11b and CD11c and a CDP signature was associated with further cell division with Flt3L (Fig. 5a, b). Together, these results suggested that Flt3L-exposed HSPCs maintain their proliferative and self-renewal capacity in a 'progenitor-like' state during the early phase of emergency DC development.

Selective expansion of early cDC1-primed HSPCs

Interestingly, a unique $CD11c^{+}cKit^{+}$ (CK) population after Flt3L treatment was identified in our *Divi-Seq* data, which exclusively aligned with the transcriptionally cDC-like cluster (Fig. 6a-c). While a small number of CKs were also present in the endogenous compartment of PBS-treated BM, there was an average 6-fold increase in abundance in mice treated with Flt3L (Fig. 6d). As a prior study had reported a cKit-expressing pre-cDC1 population in steady-state BM[44], we hypothesized that this CK population enriched for pre-cDC1s that were preferentially expanded with Flt3L stimulation. To test their DC potential, we cultured CKs with Flt3L from either PBS- or Flt3L-treated BM, as well as MDPs as controls (Fig. 6e, f). After 6 days, while MDPs were able to generate all DC subsets, both PBS- and Flt3L-treated CKs were strongly cDC1-biased (Fig. 6e, f). Thus, *Divi-seq* identified a prospectively identifiable, selectively Flt3L-expanded population that preferentially gives rise to cDC1s.

To further understand what underlying molecular drivers could account for selective cDC1 expansion after Flt3L stimulation, we examined the expression of key known transcription factors that regulate cDC1 development including *Irf8, Id2, Batf3 and Zeb2* (Fig. 7a). An increase in *Id2* and *Batf3* expression and corresponding decrease in *Zeb2* expression was mainly found in cDC-like cells (Fig. 7b and Extended Data Fig. 8), consistent with the notion that these transcription factors regulate later stages of cDC1 development[46,47]. In contrast, *Irf8* expression is known to associate with cDC1 fate priming in both early multi/oligo-potent and committed DC progenitors[12,44,47-50]. We found that many myeloid and lymphoid progenitor-like cells, as well as some HSC/MPP-like cells, expressed intermediate to high level of *Irf8* from both conditions (Fig. 7b and Extended Data Fig. 8), which fit with prior knowledge of cDC1 derivation from multiple lineage pathways[42,43]. We also observed a slight shift in *Irf8* expression after a short exposure of Flt3L, particularly in the cDC-like cluster (Fig. 7a, b).

Upregulation of *Irf8*, *Id2* or *Batf3* expression was associated with a high number of cell divisions (Fig. 7c), consistent with our previous observation that Flt3L-treated cells maintain a hyperproliferative 'progenitor-like' signature (Fig. 5). Thus, our *Divi-Seq* analysis provided further insights into key changes in the cDC1 transcriptional network in dividing cells after Flt3L exposure.

Considering Irf8 is a key regulator and marker of cDC1 fate determination in multiple progenitor populations including MPP4s[12,48], we examined the abundance of *Irf8*-expressing cells within the different HSPC compartments using an orthogonal model of *Irf8*-GFP reporter mice. We performed flow cytometry on *Irf8*-GFP BM after 3 d exposure to either PBS or Flt3L. Consistent to our previous results (Fig. 1 and Fig. 6), we found a significant increase in the total number of $Lin^{-}cKit^{+}Sca1^{-}$ cells (LKs) and CKs after Flt3L stimulation (Fig. 8a, b). Interestingly, when separating these populations based on Irf8 expression, only $Irf8^{+}$ LKs (5-fold) and $Irf8^{hi}$ CKs (11-fold) increased in abundance, but not their $Irf8^{-/int}$ counterparts (Fig. 8c-e). Collectively, our results suggested that Flt3L stimulates selective hyperproliferation of cDC1-primed HSPCs with the numerical accumulation of Irf8-expressing progenitors *en route* to the generation of a large number of cDC1s.

Discussion

In this study, we interrogate the clonal and molecular aetiology of Flt3L-mediated emergency DC development using a comprehensive single cell multi-omics strategy. First, we demonstrate through multiple approaches that Flt3L can act during the early stages of haematopoiesis to promote DC development, in addition to its role late in the pathway[39,40]. Second, using cellular barcoding, we show that multi-lineage LSKs that were already capable of cDC1 generation selectively expand their DC-producing sub-branch to contribute to greater DC numbers. Third, we develop *Divi-Seq,* and demonstrate that Flt3L-responsive HSPCs can maintain a 'progenitor-like' transcriptional state, leading to the substantial and selective expansion of the DC lineage. Finally, we identify a Flt3L-mediated cDC1-primed trajectory that originates in LSKs and amplifies via Irf8-expressing intermediates *en route* to cDC1 generation (Fig. 8f).

Our findings provide important insights into the regulation of clonal HSPC fate via extrinsic cytokine signals. Whether cytokines influence lineage choice via mechanisms that are *instructive* (where a single multipotent cell is directly primed by the cytokine) vs *permissive* (where two daughters from a multipotent cell differ in their responsiveness to that cytokine) has been a central debate in haematopoiesis[51-58]. However, these models; 1) are often considered in settings where a cytokine is either present or absent, not where cytokine concentration varies *i.e.* upon emergency haematopoiesis; 2) assume the starting HSPC population is multipotent and homogenous, which many studies now demonstrate is not the case[8-11,14]; 3) may differ in their role depending on the stage of haematopoiesis. Thus, more nuanced models are required to explain clone-level changes during emergency haematopoiesis.

Here, we demonstrate that the major contribution to increased cDC1s during Flt3L-mediated emergency development was not reflected in higher barcode numbers, nor was there a decrease in the barcodes contributing to other lineages. Rather, a similar number of HSPC clones selectively expanded more to generate more cDC1s. Therefore, neither the *instructive* nor *permissive* models could adequately explain emergency cDC1 production, although we cannot exclude their role in the original establishment of DC fate during homeostasis through Flt3L signalling. Indeed, it has been demonstrated that the ectopic expression of Flt3 can instruct DC fate in ordinarily $Flt3^-$ MEPs[59]. The fundamental difference in our model is that, beyond homeostatic levels of Flt3L signalling, exposure to increased concentration of Flt3L does not further *activate* LSK clones that were otherwise dormant, nor does it *suppress* the production

of other lineages. Instead, we introduce a new parameter called *tuning* where differing cytokine levels might 'dial up' (clonal expansion) or 'dial down' (clonal contraction) production of a given cell type from HSPCs that have a pre-existing output for a certain lineage. Importantly, this clonal *tuning* mechanism can occur without necessarily changing the imprinted but heterogeneous fate potency of HSPCs established during homeostasis[8-11,14].

Considering that all haematopoietic lineages develop through Flt3-expressing stages[60], it was unclear why supra-physiologic levels of Flt3L stimulation preferentially expand cDC1s. Here, we identify upregulation of cDC1-specific genes including *Irf8, Id2* and *Batf3* and selective expansion of multiple Irf8-expressing progenitor stages, but not their Irf8^{-} counterparts, during the early Flt3L-mediated response. Although subtle changes were already evident amongst LSKs (Figs. 1, 7-8), the most prominent skewing was observed in downstream *Irf8*-GFP^{+} LKs and CKs in the BM (Fig. 8). Therefore, the exquisite selectivity of cDC1 expansion by supraphysiologic Flt3L is likely explained by a combination of factors including: 1) specific and continuous responsiveness of both early and late Irf8^{+} DC-primed progenitors; 2) induction of genes that promote cDC1 specification and suppress cDC2, pDC and lymphocyte development; and 3) downstream lymphoid and myeloid progenitors that eventually downregulate surface Flt3 and so are no longer responsive to Flt3L.

Thus, our findings provide insights into both cellular and molecular mechanisms of emergency cDC1 development. Future studies should address the underlying signalling and molecular characteristics that lead to the emergence of this cDC1 program, for example whether the lack of putative cooperating cytokines such as IL-7 for lymphoid development, or G-, GM- or M-CSF for myeloid development explains the DC selectivity, and/or whether the strength of signal promotes selectivity in fate. Whether similar principles apply for other emergency cytokines such as G-CSF for granulocyte expansion[3] remains to be determined. Worth noting is the caveat that barcoding experiments were performed in partially irradiated recipients. However, many of our key findings including the observed increase in stem and progenitor subsets after Flt3L injection (Figs. 1f, 7), and the results of *Divi*-Seq (Figs. 4-7) after transplantation of LSKs were performed in non-irradiated mice. Collectively, our findings provide a framework that significantly enhances our understanding of the clonal level control of HSPC fate, with implications for the maintenance or manipulation of DC numbers in health and disease.

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Author contributions

D.S.L. designed and performed most experiments, did the analysis and wrote the manuscript. S.T., D.A-Z., T.B., J.S., O.S., J.R., A.P.N assisted with experiments. L.T. and T.S.W. assisted with data analysis. N.D.H., A.P.N., S.L.N., S.Taoudi., M.E.R., P.D.H. provided critical input into experimental design and analysis. S.H.N conceptualized and supervised the study and wrote the manuscript.

Competing interests

J.R. and N.D.H. are founders and shareholders of oNKo-Innate Pty. Ltd. This project was partly funded through a research agreement between Gilead Sciences, Inc. and S.H.N. Other authors declare no competing interests.

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Figure Legends

Figure 1. Supra-physiologic levels of Flt3L drive expansion of BM HSPC populations and promote emergency cDC1 development.

a. Possible clonal explanations of emergency haematopoiesis. ***b.*** *Flt3 expression during different stages of DC development. CMPs and CLPs are heterogeneous in Flt3 expression.* ***c-e,*** *Purified BM HSPC populations were labelled with CTV and cultured with or without Flt3L for 3 days, followed by flow cytometry analysis. n = 5 wells per condition, pooled from two independent experiments.* ***c.*** *CTV profile of live cells (Concatenation of n=3 wells per condition from one representative experiment). CTV MFI and cell numbers under each histogram are labelled.* ***d.*** *Number of live cells per seeded cell.* ***e.*** *Flt3 levels (MFI) of each HSPC population vs Flt3L responsiveness scores. Left: division score = log2 (fold change in CTV MFI, PBS/Flt3L); Right: expansion score = log2 (fold change in cell numbers, Flt3L/PBS). Each dot depicts one HSPC population per experiment.* ***f.*** *Number of HSPC populations (one tibia and one femur) from mice treated with PBS (n = 9), or Flt3L (n = 6) for 3 or 5 days. Data pooled from three independent experiments.* ***g-h****, Analysis of splenic populations from mice transplanted with LSKs, followed by daily s.c. injection of PBS or Flt3L for 10-12 days.* ***g.*** *Flow-cytometric analysis of donor-derived DC subsets. Numbers depict percentage of cells from parent gate.* ***h.*** *Number of donor-derived DCs, myeloid and B cells (n = 20 mice per treatment, data pooled of seven independent experiments) or total erythroid blasts (n = 15 PBS- & n = 14 Flt3L-treated mice; data pooled of five independent experiments).* ***i,*** *Number of donor-derived splenic DC subtypes from mice receiving daily s.c. injection of PBS from day 1-12 or Flt3L from days 1-12, 1-4, 5-8 or 9-12 two weeks post transplantation of LSKs. n = 3 mice per treatment, data representative of four independent experiments. BM HSPC (**c-f**) and splenic (**g-i**) populations were gated as in **Extended Data Fig. 2**. and **3**, respectively. (**d, f, h & i**) show mean ± SEM, P-values from two-tailed unpaired t-tests with Holm-Sidak correction for multiple comparison.*

Figure 2. Cellular barcoding reveals minimal contribution of clonal cDC1 fate activation to emergency cDC1 generation

a. *Schematic of cellular barcoding experiment. Barcoded CD45.1$^+$ LSKs were transplanted 3 d after sublethal irradiation (5 Gy) of CD45.2 recipient mice, followed by daily s.c. injection of PBS or Flt3L days 4-13. Splenic populations including cDC1, cDC2, pDC, myeloid cells (Mye) and B cells (B) were isolated by FACS on day 14, lysed, and barcodes amplified by PCR,*

sequenced and analysed. n = 14 PBS- (3,436 barcodes) and 14 Flt3L-treated (4,199 barcodes) mice were analysed; data pooled from five independent experiments. ***b.*** *Fold change in number of donor-derived barcoded (GFP^+) splenic cells in Flt3L-treated mice compared to PBS controls on day 14.* ***c.*** *Total number of barcodes detected from each recipient mouse.* ***d.*** *Number of barcodes present in each progeny splenic population.* ***e.*** *t-SNE plots showing all barcoded clones (points), colour depicts clonal contribution to different progeny populations.* ***f.*** *t-SNE plots of PBS barcodes (left) or Flt3L barcodes (right).* ***g.*** *t-SNE coloured by fate cluster ID.* ***h.*** *Heatmap showing contribution to cell types (row) by individual barcodes (columns). Treatment and fate cluster ID from* ***g.*** *of each barcode is annotated.* ***i.*** *Number of barcodes present in each fate cluster. (**b-d, i**) show mean ± SEM, P-values from two-tailed unpaired t-tests with Holm-Sidak correction for multiple comparison.*

Figure 3. Selective clonal expansion is the major driver of emergency cDC1 generation.

a. *Number of cDC1s produced by each cDC1-generating fate cluster (1-9).* ***b-c.*** *Numerical* ***(b)*** *or percentage* ***(c)*** *contribution to cDC1s by each cDC1-generating fate cluster.* ***d.*** *Violin plots showing the number of cells produced per clone (clone size) from all PBS- or Flt3L-treat clones in each fate cluster (row). Each dot represents a barcode. Average fold change in clone size (Flt3L/PBS) and P-values from two-tailed unpaired t-tests are shown for each pair. The first 4 fate clusters are shown here, the remaining fate clusters are shown in* ***Extended Data Fig. 5****.* ***e.*** *Numerical (left) or proportional (right) output to cell type by PBS- or Flt3L-treated clones from each cluster (row). Data pooled of five independent experiments; n = 14 mice per treatment group. (**a-c, e**) show mean ± SEM, (**a**) show P-values from two-tailed unpaired t-tests with Holm-Sidak correction for multiple comparison.*

Figure 4. *Divi-Seq* reveals increased cell division in HSPCs during the early Flt3L response.

a. *Experimental set up of Divi-Seq. $CD45.1^+$ LSKs were CTV-labelled prior to transplantation into non-irradiated CD45.2 recipient mice, followed by daily s.c. administration of PBS or Flt3L for 3 days. BM was harvested on day 4 and stained for the indicated antibodies. Donor-derived cells were index-sorted for scRNAseq. Two independent experiments were performed and pooled for analysis. *: additional step and markers included in the second experiment.* ***b.*** *Estimated number of donor-derived $CD45.1^+CD45.2^-CTV^+$ cells from each mouse (tibia, femur and ilium) from the second experiment.* ***c.*** *Heatmap representation of 1,337 single cells*

(467 PBS and 870 Flt3L) after Seurat clustering. Each cell is annotated with the following information (in rows): 1) cluster ID; 2) surface marker expression; 3) CTV intensity; 4) treatment; and 5) highly expressed marker genes in each cluster. Full list of marker genes is shown in ***Supplementary Table 1****.* ***d.*** *UMAP visualization. Cells are coloured by treatment, cluster ID, CTV or marker expression.* ***e.*** *Cluster representation in PBS or Flt3L condition. Left: percentage; middle: number of cells analysed in Divi-Seq; right: estimated number of cells based on total donor-derived cells estimated in* ***b****.* ***f.*** *CTV profile of cells present in each cluster.* ***g.*** *CTV profile of cells from each HSPC population (see phenotypic definition in* ***Extended Data Fig. 6)****; CTV MFI and number of cells under each histogram (Concatenation of n=3 mice per treatment from the second experiment) is labelled. (****b & e****) show is mean ± SEM, n = 3 mice per treatment group from the second experiment. (****b****) P-values from two-tailed unpaired t-tests.*

Figure 5. Maintenance of a 'progenitor-like' state in Flt3L-treated cells.

a. *Comparison of CTV intensity against expression of each surface marker in PBS- or Flt3L-treated donor-derived cells. *: only cells from the second experiment are shown, as these markers were not included in the first experiment.* ***b.*** *Scatter plots comparing CTV intensity to similarity scores to LT-HSC, ST-HSC, MPP3/4 or CDP, calculating using SingleR based on single cell gene expression (see* ***Extended Data Fig. 7*** *for all cell types).*

Figure 6. Emergence of a cDC1 precursor after short term Flt3L exposure.

a-c. *Scatter plots comparing cKit and CD11c expression of donor-derived cells analysed in Divi-Seq. Right box highlights a $CD11c^{+}cKit^{+}$ (CK) population. Each dot represents a single cell, and colour depicts treatment (****a****) or cluster ID (****b****). Cells in (****c****) are coloured in grey, other than those from cDC-like cluster that are coloured by treatment.* ***d.*** *Flow-cytometric analysis comparing cKit and CD11c expression of PBS- or Flt3L-treated endogenous cells (left), numbers depict percentage of CKs of total endogenous cells. Quantification of cell number is shown on right.* ***e.*** *Flow-cytometric analysis of DC output from day 6 Flt3L culture of PBS-treated MDPs, PBS-treated CKs or Flt3L-treated CKs.* ***f.*** *Number of DCs generated after 6 days in culture with Flt3L. (****d****) Data show n = 3 PBS- and 3 Flt3L-treated mice from the second Divi-Seq experiment. (****e-f****) Data representative of two independent experiment; n = 3 per group. (****d, f****) show mean ± SEM, P-values from two-tailed unpaired t-tests with Holm-Sidak correction for multiple comparison.*

Figure 7. Flt3L treatment alters expression of cDC1 genes.

a. *UMAP visualization of Divi-Seq data. Cells are coloured by cluster ID (top) or expression of transcription factors implicated in cDC1 development including Irf8, Id2, Batf3 and Zeb2 (bottom).* ***b.*** *Violin plots showing gene expression of all cells, or cells from cDC-like or monocyte-like cluster. Violin plots of other clusters are shown in* ***Extended Data Fig. 8****. Each dot represents a cell. Colour depicts PBS or Flt3L treatment. Large black dot indicates mean expression.* ***c.*** *Scatter plots comparing gene expression to CTV intensity. (****a-c****) show Divi-Seq data.*

Figure 8. Flt3L stimulation selectively expand Irf8-expression cDC1-primed progenitors.

a*. Flow cytometric analysis of* $Lin^{-}cKit^{+}Sca1^{+}$ *(LSK),* $Lin^{-}cKit^{+}Sca1^{+}$ *(LK) and* $CD11c^{+}cKit^{+}$ *(CK) cells from Irf8-GFP mice treated with PBS or Flt3L for 3 days. Lin markers: CD11b, CD19 and CD127.* ***b-e****. Number of cells presented in each population.* ***b****. Fold change in cell numbers (Flt3L/PBS).* ***c****. Percentage of* $Irf8^{+}$ *and* $Irf8^{-}$ *LSKs and LKs, or* $Irf8^{hi}$*,* $Irf8^{int}$ *and* $Irf8^{-}$ *CKs. (****b-e****) show mean ± SEM, n = 4 mice per treatment, data pooled from two independent experiments. (****b-d****) P-values from two-tailed unpaired t-tests with Holm-Sidak correction for multiple comparison.* ***f****. Schematic summarizing the clonal aetiology of Flt3L-mediated emergency DC development, which stems from the 'tuning' of clonal expansion at different stages.*

Methods

Mice

All mice were bred and maintained under specific pathogen-free conditions (light/dark cycle: 6 am – 8 pm; temperature: 18 – 24 °C; humidity: 40 – 70%), and protocols were approved by the WEHI animal ethics committee (AEC2018.015, AEC2018.006, AEC2014.031). CD45.2 (C57BL/6) and CD45.1 (C57BL/6 Pep^{3b}) male mice aged between 8-16 weeks were used. *Irf8*-GFP (C57BL/6 background) male and female mice aged between 8 – 27 weeks were used. In most transplantation experiments, CD45.1 mice were used as donor and CD45.2 mice were used as recipients, and *vice versa* for some experiments.

Transplantation

Recipient mice were either not irradiated or sub-lethally irradiated (5 Gy, or 2 Gy where indicated) three days prior to transplantation to permit endogenous cytokine levels to reduce significantly prior to transplantation[61]. For Fig. 1g-i, LSKs ($CD11b^{-}cKit^{+}Sca1^{+}$) were resuspended in PBS and approximately 5×10^3 cells were transplanted intravenously (FBS free) into each recipient. For Figs. 2-3, LSKs were first barcode transduced (see below) and approximately 5×10^3 cells (~10% were actually barcode labelled) were transplanted into each recipient. In some barcoding experiments as indicated (Extended Data Fig. 4d-f), transplantation was performed via intra-BM injection of 5×10^3 cells per recipient. For this procedure, mice were first anaesthetised using isoflurane. The hind leg was wiped with 70% Ethanol and 20-30 μL of cells resuspended in PBS were injected directly into the marrow space of the proximal epiphysis of the tibia. Mice were then injected subcutaneously with 100 μl of buprenorphine solution (0.1 mg/kg) for pain suppression. For *Divi-Seq* experiments, BM cells were first CTV labelled and 3×10^4 (experiment 1) or 5×10^4 (experiment 2) CTV-labelled LSKs were transplanted into each non-irradiated recipient.

Cytokine Injection

PBS or Flt3L (BioXcell) was injected subcutaneous daily for 3-12 days as indicated. Flt3L was resuspended in PBS and injection was performed at 10 μg/mouse per day.

Tissue Preparation and Flow cytometry

Bone marrow cells from ilium, tibia and femur were collected by flushing with FACS buffer (PBS containing 0.5% FBS and 2 mM EDTA) through a 22-gauge needle. Spleens were mashed with FACS buffer through 70 μm cell strainers with 3 mL syringe plungers. Red blood cells were lysed by incubating with Red Cell Removal Buffer (RCRB; NH_4Cl; generated in-house) for 1– 2 minutes, followed by washing and resuspension with FACS buffer. In experiments that included analysis of erythroid cells, red cell removal was not performed. Cells were stained with antibodies of interest at 4 °C for at least 30 minutes. Secondary antibody staining and/or Magnetic-Activated Cell Sorting (MACS) enrichment was performed as indicated, according to manufacturer's protocol (Miltenyl Biotec). Propidium iodide (PI) was added to exclude dead cells prior to flow cytometry analysis or sorting. Flow cytometry analysis was performed on a BD Fortessa X20 (BD Biosciences; FACSDiva software; Version 8.0.1). Cell sorting was performed on a BD Influx, BD Fusion or BD FACSAria-II/III (BD Biosciences; FACSDiva software). Cell numbers were quantified by adding a known number of counting beads (BD Biosciences) and gated based on low forward scatter and high side scatter using flow cytometry. The percentage of beads recorded was then used to estimate the percentage of cells recorded over the total number of cells. Data analysis was performed using FlowJo 9.9.6 (Treestar) or R with data exported using FlowJo 9.9.6.

For transplantation experiments, BM cells were first stained with anti-cKit-APC then MACS enriched for $cKit^+$ cells using anti-APC magnetic beads. The cKit-enriched fraction was then stained with anti-Sca1 and -CD11b antibodies. LSKs were defined as $CD11b^-cKit^+Sca1^+$ cells. For the identification of different BM HSPC subsets, total BM cells were stained with a cocktail of antibodies, cells gated on Lin^- ($CD11b^-CD11c^-$) cells, then defined as LT-HSCs ($cKit^+Sca1^+Flt3^-CD150^+CD48^-$), ST-HSCs ($cKit^+Sca1^+Flt3^-CD150^-CD48^-$), MPP2s ($cKit^+Sca1^+Flt3^-CD150^+CD48^+$), MPP3s ($cKit^+Sca1^+Flt3^-CD150^-CD48^+$), MPP4s ($cKit^+Sca1^+Flt3^+CD150^-CD48^+$), CLPs ($cKit^{int}Sca1^{int}IL7R\alpha^+$), CMPs ($cKit^+Sca1^-Flt3^-IL7R\alpha^-CD16/32^{int}CD34^+$), GMPs ($cKit^+Sca1^-Flt3^-IL7R\alpha^-CD16/32^{hi}CD34^+$), MEPs ($cKit^+Sca1^-Flt3^-IL7R\alpha^-CD16/32^-CD34^-$), MDPs/CDPs ($cKit^+Sca1^-Flt3^+$). The gating strategy of BM HSPC populations is shown in Extended Data Fig. 2. For the identification of progenitor stages in the *Irf8*-GFP mapping experiment, total BM cells were stained with a cocktail of antibodies. LSKs were defined as Lin^- ($CD11b^-CD19^-IL7R\alpha^-$) $cKit^+Sca1^+$, LKs were defined as Lin^- ($CD11b^-CD19^-IL7R\alpha^-$) $cKit^+Sca1^{-+}$, and CKs were defined as $CD11c^+cKit^+$.

For the identification of mature splenic DC, myeloid, lymphoid and erythroid cell types, splenocytes were first stained with CD11c-APC, SiglecH-PE and CD11b-biotin, followed by MACS enrichment using anti-APC and anti-PE beads ($CD11c^+$ and $Siglec\text{-}H^+$ as the DC-enriched fraction). The flow through fraction was then incubated with anti-biotin beads and MACS enriched for $CD11b^+$ myeloid cells. The flow through cells from the second MACS enrichment contained lymphoid and erythroid cells. Populations were then defined as the following: cDC1s ($F4/80^{low/-}Siglec\text{-}H^-CD11c^+CD8\alpha^+Sirp\alpha^-$), cDC2s ($F4/80^{low/-}Siglec\text{-}H^-CD11c^+CD8\alpha^-Sirp\alpha^+$) and pDCs ($F4/80^{low/-}Siglec\text{-}H^+CCR9^+CD11c^{int}$) from the DC-enriched fraction; eosinophils (eos, $CD11c^-Siglec\text{-}H^-CD11b^+Siglec\text{-}F^+$), monocytes (mon, $CD11c^-Siglec\text{-}H^-CD11b^+Siglec\text{-}F^-Gr\text{-}1^{int}$) and neutrophils (neu, $CD11c^-Siglec\text{-}H^-CD11b^+Siglec\text{-}F^-Gr\text{-}1^{hi}$) from the myeloid-enriched fraction; B cells ($CD11c^-Siglec\text{-}H^-CD11b^-Ter119^-CD19^+$) and erythroid blasts (EryB; $CD11c^-Siglec\text{-}H^-CD11b^-Ter119^+CD44^{hi}FSC\text{-}A^{hi}$), CD45.1 and CD45.2 antibodies were used to distinguish donor-derived vs host-derived cells within each population. The gating strategy of splenic populations is shown in Extended Data Fig. 3.

For index sorting of donor-derived cells in the *Divi-Seq* experiment, BM cells were stained with antibodies against cKit, Sca1, Flt3, CD150, CD16/32, CD34, CD11c, CD45.1 and CD45.2 in the first experiment, with additional antibodies against CD48, CD11b and IL7Rα in the second experiment. Viable CTV^+ cells were index sorted into wells of 384-well plates containing pre-aliquoted Cel-Seq2 reagents. After sorting, potential dead or contaminating endogenous cells were excluded *in silico* using FlowJo 9.9.6 (Treestar) based on stringent FCS, SSC, PI and CD45.1 (donor marker) profile for downstream analysis. See Extended Data Fig. 6 for gating strategy.

CTV Labelling

CTV labelling was performed in the same batch of cells prior to splitting for transplantation into different recipients or culture in different wells. CTV labelling of cells was performed using the CellTrace Violet Cell Proliferation Kit (ThermoFisher) according to the manufacturer's instructions, with minor adaptation. First, 5 mM CTV stock solution was freshly prepared by dissolving the CTV powder in a single supplied tube with 20 μL of DMSO. To minimize toxicity and cell death, the stock CTV solution was diluted 1 in 10 using PBS.

Cells were washed in PBS to remove any residual FBS from previous preparations and resuspended in 500 µL of PBS. Next, 5 µL of diluted CTV solution (500µM) per 2×10^6 cells was added to the cell suspension and vortexed immediately. The cell suspension was wrapped in foil to avoid contact with light and incubated at 37 °C for 20 minutes. A large volume of cold FBS-containing buffer (10% FBS in PBS) was added to the cells and the cell suspension was incubated on ice for 5 minutes before centrifugation. Cells were washed and resuspended in PBS for transplantation, or in medium for DC culture.

Cell culture

All cell culture experiments were performed in 96-well round bottom plates (Bio-Strategy). For Extended Data Fig. 1, 1×10^3 CTV-labelled LSKs were seeded per well in 200 µL of RPMI 1640 media (Life Technologies) with either 2 µg/mL or 2 ng/mL freshly added Flt3L (BioXcell). For Fig 1c-e, between 2×10^2 to 5×10^3 cells from different CTV-labelled HSPC populations were seeded per well in 200 µL of RPMI 1640 media with 2 µg/mL freshly added Flt3L or without any cytokines. For Fig. 6e-f, between 4×10^3 to 4×10^4 cells from different populations were seeded per well in 200 µL of DC condition media (supernatant of day 3 BM culture in RPMI 1640 media) with 2 µg/mL freshly added Flt3L. Cells were harvested and analysed by flow cytometry at the time points as indicated. Numerical output per seeded cell was normalized for the different seeded cell number in Figs. 1d & 6f.

Barcode transduction

Barcode transduction was performed in the same batch of cells prior to splitting for transplantation into different recipients [8]. Freshly isolated LSKs were resuspended in StemSpan medium (Stem Cell Technologies) supplemented with 50 ng/mL stem cell factor (SCF; generated in-house by Dr Jian-Guo Zhang) and transferred to a 96-well round bottom plate at less than 1×10^5 cells/well. Small amount of lentivirus containing the barcode library (pre-determined to give 5-10% transduction efficiency) was added and the plate was centrifuged at 900 *g* for 90 minutes at 22 °C prior to incubation at 37 °C and 5% CO_2 for 4.5 or 14.5 hours. After incubation, cells were extensively washed using a large volume of FBS-containing buffer (10% FBS in PBS or RPMI) to remove residual virus. Cells were then washed once using PBS to remove FBS. Cells were resuspended in PBS and transplanted into recipient mice (5×10^3 cells per mouse) via intravenous injection or intra-BM injection. Thus, approximately 500 barcoded cells (average 10% transduction efficiency, 5×10^3 cells per mouse) was transplanted

into each recipient. Due to variation in transduction and engraftment between experiments, the actual average number of barcodes per mouse was around 250 – 300 (Fig. 2c), which represented approximately 10% of total available barcodes in the reference library (2608 barcodes).

Barcode amplification and sequencing

PCR and sequencing were performed as described previously[8]. Briefly, sorted populations were lysed in 40 µl lysis buffer (Viagen) containing 0.5 mg/ml Proteinase K (Invitrogen) and split into technical replicates. Barcodes in cell lysate were then amplified following two rounds of PCRs. The first PCR amplified barcode DNA using common primers including the TopLiB (5' – TGC TGC CGT CAA CTA GAA CA – 3') and BotLiB (5' – GAT CTC GAATCA GGC GCT TA – 3'). The second PCR introduced an 82-bp well-specific 5' end forward index primer (384 in total) and an 86-bp plate-specific 3' reverse index primer (8 in total) to each sample for later de-multiplexing *in silico*. The sequences of these index primers are available upon request. Products from second round PCR with index primers were run on a 2% agarose gel to confirm a PCR product was generated, prior to being cleaned with size selected beads (NucleoMag NGS) according to the manufacturer's protocol. The cleaned PCR products were pooled and sequencing was performed on the Illumina MiSeq or NextSeq platform.

Barcode data processing and quality control (QC)

Processing of barcode data was performed as previously described[8], which involved the following steps: 1) number of reads per barcode from individual samples was mapped to the reference barcode library, which contains 2608 actual unique DNA barcode sequences (available upon request) and counted using the *processAmplicons* function from edgeR package (Version 3.28.0)[62,63]; 2) samples with total barcode read counts of less than 10^4 were removed; 3) Pearson correlation between technical replicates from the same population was calculated and samples with coefficient of less than 0.6 were removed; 4) read counts were set to zero for barcodes with reads in one but not the other technical replicate; 5) read counts of each barcodes from technical replicates were averaged; 6) total read counts per sample was normalized to 10^6; 7) read counts per sample was transformed using hyperbolic arsine transformation. Note that steps 2 and 3 were not performed for barcoding data from either intra-BM or low dose experiment presented in Extended Data Fig. 4, due to low read counts and/or low correlation between technical replicates in a number of samples in these experimental settings.

Barcode data analysis

Barcodes from all biological replicates (regardless of PBS or Flt3L treatment) from five independent experiments were pool and analysed. t-SNE (Rtsne package; Version 0.15) was performed using the resulting normalized and transformed barcode read counts (*i.e.* proportional output to cell type per barcode) to visualize lineage bias of individual barcodes in two-dimensions. DBSCAN clustering (dbscan package; Version 1.1-5) was performed using the resulting t-SNE coordinates to classify barcodes. Heatmaps were generated to visualize lineage output of barcodes identified in each cluster. Barcode numbers present in each biological replicate within each cluster were counted and compared between PBS and Flt3L treatment. Clone size (number of cells generated per cell type per barcode) was calculated based on average estimated cell numbers (based on % recovery of counted beads) at the population level across all experiments and proportional output to cell type per barcode (normalized and transformed barcode read counts). Heatmap was generated using pheatmap package (Version 1.0.12). t-SNE and violin plots were generated using ggplot2 package (Version 3.2.1).

***Divi-Seq* library generation**

Divi-Seq libraries were generated using an adapted CEL-Seq2 protocol[64]. Briefly, cells were lysed in 0.2% Triton-X and first strand cDNA was generated. All samples were then pooled and treated with Exonuclease 1, followed by second strand DNA synthesis (NEB), *In vitro* transcription, RNA fragmentation, reverse transcription and library amplification. Library was then size selected using 0.8x followed by 0.9x ratio of sample to beads (NucleoMag NGS) according to the manufacturer's protocol. The amount and quality of the library was checked on a Tapestation (Agilent Technologies) using a high sensitivity D5000 tape (Agilent Technologies) before sequencing on the Illumina NextSeq high output (14bp read 1, 72 bp read 2 and 6bp index read).

***Divi-Seq* data processing and QC**

First, reads from individual sorted plates (two plates from experiment one and four plates from experiment two) were mapped to the GRCm38 mouse genome using the Subread aligner (Subread package; Version 1.30.5) [65] and assigned to genes using the scPipe package (Version 1.8.0) [66] with ENSEMBL v86 annotation. Next, QC was performed using the *detect_outlier* function in scPipe to remove a total of 622 low quality cells. Gene counts were normalized

using the *computeSumFactors* (Scran package; Version 1.14.3) and *normalize* functions (Scater package; Version 1.14.3), and gene names were annotated using the *convert_geneid* function in scPipe. Common genes (15,125 genes) detected across plates were merged into a gene count matrix that was combined with index sort information. Mutual nearest neighbors correction (*mnnCorrect* function) from the batchelor package (Version 1.2.4)[67] was applied to correct for batch effects of gene expression between plates. Surrogate Variable Analysis (*ComBat* function from sva package; Version 3.34.0) was used to correct for batch effects in surface marker expression between experiments. Together, these QC steps resulted in the generation of a final single cell dataset of 1,337 cells (467 from PBS and 870 from Flt3L condition).

Divi-Seq data analysis

Seurat (Version 3.1.1)[68] was used to identify major groups of cells. First, dimension reduction was performed using the *RunPCA* and *RunUMAP* functions. UMAP visulazation was generated using the resulting UMAP coordinates (Fig. 4). Next, cell cycle genes were regressed out using *CellCycleScoring* and *vars.to.regress* functions before clustering analysis using the *FindNeighbors* (based on the first 20 PCA components; k.param = 40) and *FindClusters* functions. Cluster-defined marker genes were extracted using the *FindAllMarkers* function (Supplementary Table 1) and top 8 marker genes (top 10 ranked by adjusted P-value, followed by top 8 ranked by average log fold change) were selected and expression values were scaled for heatmap generation (Fig. 4c; pheatmap package; Version 1.0.12). CTV intensity and surface marker expression values (Z-score using *scale* function in R) were overlayed on the corresponding single cells for comparison (Fig. 4c). To facilitate cell type annotation, SingleR (Version 1.0.0) [69] was applied to *Divi-Seq* data to compute a similarity score (Z-score using the *scale* function in R) of each single cell to population-based RNA-seq dataset (Immgen database).

Statistical and reproducibility

Statistical analysis was performed in Prism (GraphPad; Version 8) or R (Version 3.6.2). Two-sided unpaired Student's t test was performed with Holm-Sidak correction for multiple comparison when applicable, as indicated in text. Mean ± Standard Error of Mean (SEM) and P-values are reported. All experiments were repeated at least two times. The exact numbers of independent experimental repeats were stated in corresponding figure legends.

Data availability

Divi-Seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE147977. Barcoding data are available on https://github.com/DawnSLin/Emergency_DC_Development. All other data supporting the findings of this study are available from the corresponding author on reasonable request. Source data are provided with this paper.

Code availability

All R packages used are available online, as described in the Methods. Customized code used to analyse *Divi-Seq* and barcoding data are available on GitHub https://github.com/DawnSLin/Emergency_DC_Development.

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