Lymph node stromal cells support the maturation of pre-DCs into cDC-like cells via colony-stimulating factor 1

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Abstract
Conventional dendritic cells (cDCs) arise from committed precursor dendritic cells (pre-DCs) in the bone marrow that continuously seed the periphery. Pre-DCs and other upstream progenitors proliferate and mature in response to Fms-related receptor tyrosine kinase 3 ligand, which is considered the key cytokine for cDC development. However, other cytokines such as stem cell factor and colony-stimulating factor 1 (CSF1) were also shown to induce pre-DC maturation into DC-like cells. Yet, it is still only incompletely understood which cells contribute to cDC development once pre-DCs arrive in peripheral tissues. Here, we analysed the impact of lymph node (LN) fibroblastic stromal cells (FSCs) on the maturation of pre-DCs into cDC-like cells. We could demonstrate that ex vivo isolated LN FSCs cocultured with pre-DCs induce precursor maturation into DC-like cells, which were capable of efficiently promoting the proliferation of naïve CD4+ T cells. Interestingly, FSCs isolated from distinct LNs induced DC-like cells with highly comparable transcriptomes, characterized by the expression of signature genes of both ex vivo isolated DCs and macrophages. Finally, by performing supplementation and receptor blocking studies, we could demonstrate that CSF1 is a driving factor for LN FSC-mediated pre-DC maturation into DC-like cells. In summary, we could identify CSF1 as a stromal cell-derived factor that has the potential to support the maturation of pre-DCs into cDC-like cells within secondary lymphoid organs.

Keywords
cytokines, dendritic cell, lymphoid architecture
INTRODUCTION

Dendritic cells (DCs) are mononuclear phagocytes with potent antigen-presenting capacity that are capable of priming antigen-specific T cell responses. Various types of different DCs have been reported: Langerhans cells, which are currently considered as a partly self-maintaining tissue-resident macrophage subset [1], plasmacytoid DCs (pDCs), which are important in the antiviral response and capable of producing large amounts of interferons [2], and conventional DCs (cDCs), which are the most efficient antigen-presenting cells [3] and can further be subdivided into cDC1s and cDC2s [4].

Defining DC ontogeny has proven to be challenging, and DCs were previously accredited to the myeloid and lymphoid as well as their own separate lineage. However, recent discoveries have refined our understanding of DC ontogeny [5, 6]. During haematopoiesis, cDCs arise from the macrophage DC progenitor (MDP), which can eventually give rise to both macrophages as well as DCs via further downstream progenitors. The common DC progenitor (CDP) is the first progenitor with a confined potential to pDCs and cDCs. Progenies further downstream of CDPs are fate-restricted to either become precursor plasmacytoid dendritic cells (pre-pDCs) or precursor dendritic cells (pre-DCs), the latter solely giving rise to cDCs [7–9]. In the bone marrow, pre-DCs are defined as Lin−CD11c+MHCII+CD135−CD172α− cells, and based on transcriptomic data it has been suggested that they can already be committed to either the cDC1 (Siglec-H−Ly6C−) or cDC2 (Siglec-H−Ly6C+) lineage while still residing inside the bone marrow [10]. Once pre-DCs emigrate from the bone marrow, they populate peripheral tissues throughout the body where they undergo a limited amount of proliferation cycles before terminally differentiating into cDCs. These cDCs in turn undergo limited proliferation before being replenished by a new wave of incoming pre-DCs, which was recently elegantly demonstrated by a multicolor fate-mapping approach [11]. For lymph nodes (LNs), it was reported that pre-DCs enter the parenchyma through high endothelial venules (HEVs), initially are sessile and remain in close proximity to HEVs for several hours primarily in the medullary side of the LN at the interface between the T cell zone and B cell follicles. Subsequently, while still localized in vessel-rich areas, pre-DCs displayed a more migratory behaviour, and after a few days, the progeny of the pre-DCs was evenly distributed throughout the LN paracortex, fully integrated into the endogenous DC network and assumed a typical DC behaviour [8]. Those DCs entering the LN parenchyma via the blood circulation belong to the resident DCs (resDCs), which can be distinguished from migratory DCs (migDCs), which enter the LNs via the lymphatic system and carry antigen from the surrounding tissues [12]. In spleen and LNs, DCs undertake a limited number of divisions during a 10–14 day period, and daughter cells were shown capable of presenting antigens captured by their progenitors [13].

A multitude of protocols for the in vitro generation of DC-like cells were established in recent years. Common approaches include the culture of bone marrow cells, monocytes or blood mononuclear cells with granulocyte-macrophage colony-stimulating factor (GM-CSF, aka CSF2) often in combination with interleukin (IL)-4 [14–16], or the culture of bone marrow cells with FLT3L [17, 18]. During cDC ontogeny, FLT3L is considered as the most important factor promoting cDC development from pre-DCs and further upstream haematopoietic progenitors, and both FLT3L−/− as well as FLT3−/− mice show severely reduced cDC numbers [19, 20]. Yet, DC progenitors isolated from FLT3−/− mice were shown to mature in response to CSF1, and additional deletion of the CSF1 receptor (CSF1R) in these mice further reduced DC development [21]. Moreover, DC-like cells developed in cultures of bone marrow cells supplemented with CSF1 [22]. Thus, multiple factors are involved in cDC development, although CSF1−/− and CSF2−/− mice do not display a severely impaired DC development [23–26]. Importantly, both FLT3 and CSF1R are expressed not only on MDPs and CDPs, but also on pre-DCs [8, 27], and it has been suggested that a tradeoff between FLT3L versus CSF1 signalling strength in concert with other factors is driving monocyte/macrophage or DC fate choice [28].

Stromal cells (SCs) are well known producers of both FLT3L and CSF1 among other growth factors [29, 30]. Hence, SC feeder lines, such as OP9 and 5G3, are frequently utilized in cell cultures to promote the generation of DC-like cells from bone marrow or cord blood cells and progenitors [31–34]. SCs are key structural elements for organ tissue integrity, and recent evidence suggests that they are also capable of shaping organ-specific immune responses [35]. SCs of secondary lymphoid organs (SLOs) are especially well characterized and can be broadly categorized into blood endothelial cells (BECs), lymphoid endothelial cells (LEC) and fibroblastic stromal cells (FSCs). While BECs line blood vessels and HEVs, and LECs surround the lymphatic capillaries, FSCs, by far the most numerous of these subsets, subdivide SLOs into distinct zones. In LNs, different subsets of FSCs reside in B cell follicles and germinal centers, the T cell zone and the medulla, where they guide incoming cDCs and other immune cells with chemotactic gradients, foster immune cell survival and contribute to the initiation of immune responses [36]. However, how LN FSCs contribute to the maturation of pre-DCs into cDCs is only incompletely understood.
FIGURE 1  Legend on next page.
We here hypothesized that LN FSCs influence pre-DCs, which enter the LN parenchyma through the HEVs after leaving the blood circulation, and foster pre-DC maturation and subsequent integration into the resDC network in concert with other immune cells. To study the direct impact of LN FSCs on pre-DC maturation, we set up co-cultures with ex vivo isolated LN FSCs and pre-DCs. We could demonstrate that LN FSCs are capable of inducing pre-DC maturation into DC-like cells, which harbour features of both macrophages and cDCs. Furthermore, supplementation and receptor blocking studies revealed that CSF1 is a driving factor for the LN FSC-mediated pre-DC maturation into DC-like cells, suggesting that CSF1 as a stromal cell-derived factor is capable of supporting the maturation of pre-DCs into cDC-like cells within secondary lymphoid organs.

RESULTS

LN FSCs support pre-DC maturation and survival in vitro

In LNs, resDCs arise locally from pre-DCs that enter the LN parenchyma via the blood circulation [3]. We hypothesized that LN FSCs, which populate the cortical and paracortical LN areas where DCs interact with B and T cells, can promote pre-DC maturation and contribute to their transition and integration into the local resDC compartment. To experimentally address this hypothesis, we isolated CD45^-CD24^-CD31^-gp38^- FSCs from pLNs or mLNs (Figure S1a) and co-cultured them with Lin^-MHCII^-CD11c^-CD11b^-CD135^-CD172a^- pre-DCs [37] isolated from bone marrow (Figure S1b). These pre-DCs contained CD11c^-low and CD11c^-high cells (Figure S1a), likely representing distinct developmental stages during the differentiation of CDPs into pre-DCs [38]. Neither CD11c^-low nor CD11c^-high pre-DCs expressed c-Kit (Figure S1a), indicating that no c-Kit-expressing MDPs or common monocyte progenitors (cMoPs) were added to the co-cultures with LN FSCs [8, 10, 37, 39].

Next, we followed the maturation of pre-DCs within the co-culture over time by assessing the expression of MHCII and CD11b by flow cytometry (Figure S1a). Already at day 1, a substantial fraction of pre-DCs co-cultured with pLN FSCs or mLN FSCs had initiated MHCII expression, while pre-DCs cultured without LN FSCs (medium control group) only expressed moderate MHCII levels (Figure 1a,b). Two days later, on day 3, this difference became even more obvious as pre-DCs cultured in medium only were devoid of any MHCII expression, while pre-DCs co-cultured with either pLN FSCs or mLN FSCs both showed two clearly distinguishable CD11c^-MHCII^- subsets. Although pre-DCs cultured in medium only did not survive until day 5, the majority of pre-DCs co-cultured with pLN FSCs or mLN FSCs had developed into CD11c^-MHCII^- DC-like cells (Figure 1a,b). A similar expression pattern was observed for CD11b with a moderate induction on pre-DCs co-cultured with pLN FSCs or mLN FSCs on day 1, a clearly distinguishable CD11c^-CD11b^- subset on day 3, and a homogeneous CD11c^-CD11b^- DC-like population by day 5. Importantly, pre-DCs cultured in the absence of LN FSCs showed only neglectable CD11b expression on day 1 (Figure 1c,d). It is worth mentioning that both CD11c^-low and CD11c^-high pre-DCs gave rise to DC-like cells. Notably, CD11c^-low pre-DCs developed into MHCII^-highCD11b^- cells at lower frequency than CD11c^-high pre-DCs, indicating that CD11c^-low pre-DCs had a delayed developmental kinetic compared to their CD11c^-high counterparts (Figure S1b,c). Before exiting the bone marrow, pre-DCs are CD11c^- MHCII^-CD11b^- cells [37], and Siglec-H and Ly6C can be used for further subsetting to distinguish pre-DCs committed to the cDC1 (Siglec-H^-Ly6C^- pre-DCs) or cDC2 lineage (Siglec-H^+Ly6C^+ pre-DCs) [10]. On day 1 of the culture, pre-DCs still showed a similar expression pattern for Siglec-H and Ly6C as compared to ex vivo isolated pre-DCs from the bone marrow [10], and no differences were observed between pre-DCs cultured in the presence or absence of LN FSCs (Figure 1e,g). On day 3, pre-DCs co-cultured with pLN FSCs or mLN FSCs developed into clearly distinguishable Siglec-H^+Ly6C^+ or Siglec-H^-Ly6C^-.
subsets, while pre-DCs cultured in medium only showed a uniform Siglec-H^+ Ly6C^+ phenotype. By day 5, all pre-DCs had developed into the Siglec-H^-Ly6C^- subset (Figure 1e, g). In summary, our data demonstrate that both pLN FSCs and mLN FSCs are sufficient to support the maturation of pre-DCs into DC-like cells during in vitro co-culture with comparable kinetics and phenotypes, indicating conserved functional properties of LN FSCs for cDC maturation.

**LN FSC-raised DC-like cells promote naïve T cell proliferation**

In order to verify that the DC-like cells raised in the in vitro co-culture had acquired functional characteristics of cDCs, we tested their ability to promote naïve T cell proliferation. At day 3 or day 5 of the co-culture of LN FSCs with pre-DCs, ovalbumin (Ova)-specific CD4^+ T cells, isolated from Foxp3^{hCD2xRag2/-}xD011.10 mice and Ova_{323-339} peptide were added to the co-cultures, and the cells were incubated for an additional four days. Cells not receiving Ova peptide served as unstimulated controls. At the end of the cultures, cells were harvested and analysed by flow cytometry. T cells were pre-gated as alive, CD4^+ cells.

(a) Exemplary histograms depict the count of CPDviolet-labelled T cells. Numbers indicate the gMFI of CPDviolet. (b) Scatterplot summarizes the normalized gMFI of CPDviolet of indicated treatment conditions. For the normalization of the gMFI within each experiment, the lowest gMFI value was substracted by the highest and then each sample’s gMFI value was divided by this factor. Data pooled from four independent experiments with bars indicating mean ± SD of meaned technical unicates-triplicates. DC, dendritic cell; FSC, fibroblastic stromal cell; gMFI, geometric mean of fluorescence intensity; mLN, mesenteric lymph node; ns, not significant; Ova, ovalbumin; pLN, peripheral lymph node; pre-DC, precursor dendritic cell; SD, standard deviation.
FIGURE 3  CSF1 produced by LN FSCs is sufficient to support pre-DC maturation into DC-like cells. (a) Previously published RNAseq data for pLN FSCs and mLN FSCs (GEO accession number GSE116633) were mined for candidate genes. Heatmap displaying the expression levels (log2(RPKM) values) of Flt3l, Csf1 and Dll1. (b–h) Pre-DCs were sorted from pooled bone marrow and co-cultured for one, three or five days with either FLT3L or CSF1 added to the culture media. At the end of the cultures, cells were harvested and analysed by flow cytometry. DC-like cells were pre-gated as alive, CD11c+ cells (gate event number: 83-9876). (b) Exemplary dot plots depict the expression of CD11c and MHCII. (c) Scatterplot summarizes frequencies of MHCII expression among DC-like cells. (d) Exemplary dot plots depict the expression of CD11c and CD11b. (e) Scatterplot summarizes frequencies of CD11b expression among DC-like cells. (f) Exemplary dot plots depict the expression of Siglec-H and Ly6C. (g) Scatterplot summarizes frequencies of Ly6C expression among DC-like cells. (h) Scatterplot summarizes frequencies of Siglec-H expression among DC-like cells. Data pooled from 3 to 14 independent experiments with bars indicating mean ± SD of meaned technical unicates-triplicates. BECs, blood endothelial cells; CSF1, colony-stimulating factor 1; DC, dendritic cell; DLL1, delta-like 1; FLT3L, Fms related receptor tyrosine kinase 3 ligand; FSC, fibroblastic stromal cell; LECs, lymphatic endothelial cells; LN, lymph node; NA, not available; ns, not significant; pre-DC, precursor dendritic cell; RPKM, reads per kilobase of exon length per million reads; SD, standard deviation.
CSF1 produced by LN FSCs is sufficient to support pre-DC maturation and survival in vitro

We next thought to determine which factors produced by LN FSCs promote the maturation of pre-DCs into DC-like cells during in vitro co-culture. To this end, we first mined previously generated RNAseq data from pLN and mLN FSCs (GEO accession number GSE116633) [40]. Scf, Csf2 and Il-4, which were previously reported to promote the generation of DC-like cells from progenitors or monocytes in in vitro cultures [14–16], were not expressed by LN FSCs. The Notch ligand delta-like 1 (Dll1), which can support the generation of DCs from hematopoietic progenitors [41–43], was expressed in pLN and mLN FSCs, but at rather low levels (Figure 3a). Comparably low expression levels were also observed for Flt3l, a factor well-known for promoting cDC development [17–20]. In contrast, Csf1 which was previously shown to support the generation of DC-like cells from bone marrow cells [22], was expressed at high levels in both pLN and mLN FSCs (Figure 3a). Yet, FSCs are heterogeneous [40, 44], and a subset of FSCs expressing Grem1 has been demonstrated to foster resDC homeostasis [45]. We therefore mined previously published pLN and mLN non-endothelial SC scRNAseq data [40] and found Csf1 broadly expressed across a variety of SC subsets, including Grem1+ FSCs (Figure S1). Thus, Csf1 might be involved in the LN FSC-mediated maturation of pre-DCs into DC-like cells.

To address this assumption experimentally, pre-DCs were cultured for one, three and five days with Csf1 in the absence of LN FSCs, and pre-DCs cultured with FLT3L were taken as controls. Interestingly, pre-DCs cultured with Csf1 showed a comparable kinetic regarding the expression of CD11c, MHCII, CD11b, Siglec-H and Ly6C (Figure 3b–h) when compared to pre-DCs co-cultured with LN FSCs (Figure 1). However, pre-DCs cultured with FLT3L showed a significantly diminished expression of CD11b as well as a significantly increased expression of Siglec-H and Ly6C already at day 3 when compared to pre-DCs cultured with Csf1, and all pre-DCs cultured with FLT3L had died at day 5 in contrast to pre-DCs cultured with Csf1 (Figure 3b–h). Together, these data suggest that Csf1 as a LN FSC-derived factor is contributing to the maturation of pre-DCs into DC-like cells, while FLT3L alone cannot support the differentiation and/or survival of DC-like cells in vitro for an extended period of time.

DC-like cells raised in cultures with CSF1 or on LN FSCs display almost identical transcriptomes

Following these observations, we employed RNAseq to further investigate the identity of the DC-like cells generated under the different culture conditions. At day 3 of the respective cultures, the cells were harvested and sorted into CD45−CD11c−MHCIILow (in the following termed “MHCIILow”) and CD45−CD11c+MHCIIHigh (in the following termed “MHCIIHigh”) subsets (Figure S1b). In addition, Lin−MHCII−CD11c+CD11b−CD135−CD172a− pre-DCs were sorted from bone marrow as input control. Global inspection of the RNAseq data by hierarchical clustering of all 5175 differentially expressed genes (DEGs) revealed that, as expected, the transcriptomic signature of input pre-DCs was distinct from the transcriptomes of all cultured cells (Figure 4a). Interestingly, all MHCIILow samples as well as all MHCIIHigh samples clustered together, with pre-DCs more closely resembling MHCIIHigh when compared to MHCIIHigh samples. Among the MHCIILow samples, cells cultured with Csf1 were the most dissimilar whereas among the MHCIIHigh samples cells treated with FLT3L displayed the most distinct transcriptomic signature (Figure 4a). The similarity of MHCIIHigh cells with pre-DCs was further supported when genes previously reported to be strongly expressed in pre-DCs [10] were plotted (Figure 4b), indicating that MHCIILow cells were not fully differentiated, yet. Therefore, we focused the subsequent analyses on the MHCIIHigh samples.

When assessing markers for DC maturation and activation, all MHCIIHigh DC-like cells showed comparable expression of the costimulatory markers Cd40, Cd86 and Tnfsf9 when compared to ex vivo isolated LN resDCs and migDCs, while expression of Cd80 and Tnfsf4 was reduced in DC-like cells (Figure 4c). MHCIIHigh DC-like cells from all groups expressed genes involved in cross-presentation pathways [46–49]. Comparison to ex vivo DCs revealed a varying expression of individual pathway members, e.g., cultured DC-like cells expressed less Tap1 and Tap2 while they showed a higher expression of Clec4 family members (Figure 4d). We also investigated whether MHCIIHigh DC-like cells appeared more polarized toward cDC1 or cDC2 fate. However, since the cultivated cells expressed both cDC1 (Xcr1, Nfil13, Irf8, Id2, Clec9a, Cd8a, Bcl6) and cDC2 (Cd4, Clec4a4, Irf2, Irf4, Itgam, Klf4, Mgl2, Notch2, Sirpα, Zeb2) associated genes to some extent (data not shown), no subset identity could be clearly ascribed. Additionally, we observed that by day 5 of culture all cells developed into a homogeneous CD24+CD11b+ population (Figure S1), precluding the establishment of cDC1 versus cDC2 identity based on the expression of these surface markers.

While a large number of >3000 DEGs were identified when both pLN FSC- and mLN FSC-raised DC-like cells were compared with pre-DCs (Figure 4d, upper row left/middle), the direct comparison between pLN FSC- and mLN FSC-raised DC-like cells only revealed three DEGs (Ldlrad3, Rnf144a, Havcr1) (Figure 4d, upper row right),
FIGURE 4  Legend on next page.
suggesting that, at least in vitro, the LN FSC-mediated pre-DC to DC maturation process is a common feature conserved among LN FSCs from different locations. In stark contrast, DC-like cells generated upon culture of pre-DCs with CSF1 showed a large number of >800 DEGs when compared to FLT3L-treated cells (Figure 4d, lower row left). Most importantly, CSF1-treated cells more closely resembled LN FSC-raised DC-like cells (only 85 DEGs) when compared to FLT3L-treated cells (>1000 DEGs) (Figure 4d, lower row right/middle). Thus, based on the transcriptomic signatures, CSF1 remains the top candidate factor mediating pre-DC to DC maturation in in vitro co-cultures with LN FSC.

We next asked how closely the MHCIIHigh DC-like cells generated upon co-culture of pre-DCs with LN FSCs resemble ex vivo cDCs, since CSF1 has been also implicated in macrophage biology [28, 50]. To this end, we made use of previously generated RNAseq data from LN-derived resDCs and migDCs (GEO accession number GSE116633) [40] as well as splenic red pulp macrophages (RPMs; GEO accession number GSE156162). Global inspection of the RNAseq data by hierarchical clustering of all 1532 DEGs (Figure 5a) as well as the comparison of core signature genes for cDCs [51] (Figure 5b) and macrophages [52] (Figure 5c) revealed that all MHCIIHigh DC-like cells generated in vitro clustered together as expected, yet showed a comparable degree of similarity to ex vivo isolated resDCs and migDCs as well as RPMs. Thus, upon in vitro differentiation on LN FSCs, pre-DCs appear to acquire an ambiguous myeloid phenotype, a chimeric cell identity encompassing both cDC and macrophage features.

**Lymph Node Stromal Cell Supporting Pre-DCs**

**Figure 4** DC-like cells raised in cultures with CSF1 or on LN FSCs display almost identical transcriptomes. Pre-DCs were sorted from pooled bone marrow and co-cultured with either sorted mLNs or pLN FSCs or with either FLT3L or CSF1 added to the culture media. At day 3 of the co-culture, cells were harvested and sorted as CD45+CD11c+MHCIILow (henceforth termed “Low” groups) and CD45+CD11c+MHCIIHigh (henceforth termed “High” groups) DC-like cells. Lin- MHCII- CD11c+CD11b+CD135+CD172a- pre-DCs were sorted as input control. Subsequently, RNAseq analysis was carried out. DEGs were identified as log2(FC) ≥1, q-value ≤0.05. (a) Hierarchical clustering of all 5175 DEGs (mean-centered log2(RPKM) values). (b) Heatmap depicting the expression (mean-centered log2(RPKM) values) of characteristic pre-DC-specific genes based on previously published data [10]. (c) Heatmap displaying the expression (log2(RPKM) values) of selected genes. (d) Heatmap showing the expression (log2(RPKM) values) of genes involved in antigen cross-presentation pathways. (c-i-d) LN resDC and migDC RNAseq data (GEO accession number GSE116633) were previously published [40], and data for pLN and mLN were merged in the present analysis. (e) Volcano plots depict log2(FC) vs. −log10(padj) values in the comparison of pre-DC and pLN High (upper left), pre-DC and mLN High (upper middle), pLN High and mLN High (upper right), CSF1 High and FLT3L High (lower left), mLN High and FLT3L High (lower middle), mLN high and CSF1 High (lower right). Samples for RNAseq were generated in three independent experiments yielding three biological replicates per condition. CSF1, colony-stimulating factor 1; DEG, differentially expressed gene; FC, fold-change; FLT3L, Fms related receptor tyrosine kinase 3 ligand; FSC, fibroblastic stromal cell; migDC, migratory dendritic cell; mLN, mesenteric lymph node; padj, FDR-adjusted p-value; pLN, peripheral lymph node; pre-DC, precursor dendritic cell; resDC, resident dendritic cell; RPKM, reads per kilobase of exon length per million reads.
DISCUSSION

It has been elegantly demonstrated how pre-DCs seed peripheral tissues [11], and in particular how they enter LNs through HEVs to integrate into the cDC network [8]. However, relatively little is known about the cell types and the micromillieu that drive pre-DC maturation into DC-like cells and support their integration into the organ parenchyma of secondary lymphoid organs. Recently, it was shown that the ablation of Grem1+ FSCs leads to a
decreased homeostatic proliferation and survival of the pre-DC compartment, emphasizing the importance of the FSC compartment for the generation and maintenance of DCs in LNs [45]. In the present study, we demonstrate that LN FSCs by themselves are sufficient to drive pre-DC maturation into DC-like cells and support their survival via CSF1 signalling.

The cytokine CSF1 is more strongly implicated in macrophage than DC biology and therefore not commonly utilized for the in vitro generation of DC-like cells. Bone marrow cultures from wild type and FLT3L−/− mice treated with CSF1 were shown to produce large quantities of cDCs and pDCs [22]. DC deficiency is exacerbated in FLT3L−/− compared to FLT3−/− mice, and it was reported that in response to CSF1 bone marrow cells, CDP and pre-DC2s isolated from FLT3−/− mice can mature into cDCs [21]. Moreover, FLT3−/− cDC progenitors showed increased CSF1 signalling, indicating that the loss of FLT3 renders these cells more susceptible to alternative cytokines. Upon co-cultivation of pre-DC2s with FLT3L or CSF1 the cells developed into CD11c+MHCII+CD172α+CD24+ cells by day 5 of cultivation [21], closely resembling the phenotype of DC-like cells obtained in the co-cultures of the present study, which also expressed CD24 at day 5. It should be noted that it was previously reported that in some instances sorted pre-DCs can survive in cultures with FLT3L for five days or beyond [21, 53]. While these findings are in contrast to our

**FIGURE 6** Blocking of CSF1R prevents LN FSC-mediated pre-DC maturation into DC-like cells. Pre-DCs were sorted from pooled bone marrow and co-cultured for three or five days with either sorted mLNs or pLN FSCs. Co-cultures received either no additional treatment, isotype control antibody or anti-CSF1R blocking antibody as indicated. At the end of the cultures, the cells were harvested and analysed by flow cytometry. DC-like cells were pre-gated as alive, CD11c+ cells (gate event number: 222-1769). (a) Exemplary dot plots depict the expression of CD11c and MHCII on cells co-cultured with pLN FSCs (left) or mLN FSCs (right). (b) Scatterplots summarize frequencies of MHCII expression among CD11c+ cells for day 3 (left) and day 5 (right). Data pooled from 2 to 3 independent experiments with bars indicating mean ± SD of meaned technical duplicates. Samples where < 100 viable CD11c+ DC-like cells were recovered are indicated in orange. CSF1R, colony-stimulating factor 1 receptor; DC, dendritic cell; FSC, fibroblastic stromal cell; mLN, mesenteric lymph node; NA, not available; pLN, peripheral lymph node; pre-DC, precursor dendritic cell.
own data, it is possible that they are due to differences in the cell culture conditions. Long-term cultures of splenic SC lines with haematopoietic progenitors resulted in the generation of CD11b+ DC-like cells [34, 42, 54], which were dependent on SC line-derived CSF1 [31]. Here, by comparing previously generated RNAseq data from LN-derived DCs as well as splenic RPMs to LN FSC-raised DC-like cells we could demonstrate that LN FSC-raised DC-like cells displayed an ambiguous myeloid phenotype with both macrophage- and DC-like features. Thus, continuous CSF1 stimulation might cause a more ‘extreme’ phenotype in cDC fate-committed precursor cells and lead to the acquisition of selected macrophage features. However, this is a difficult aspect to infer from, since FLT3L-treated cells showed a very similar core signature gene expression pattern when compared to CSF1-treated cells, and all in vitro cultured cells were distinct in their transcriptomes from ex vivo LN-derived DCs and splenic RPMs. Thus, it is likely that these in vitro generated DC-like cells do not have a direct in vivo correlate. Yet, the findings from the present study and previously published reports together suggest that CSF1 signalling is sufficient to support the generation of CD11b+CD24+ DCs from haematopoietic precursors, and that stromal cells in general and LN FSCs in particular represent a cellular source of CSF1 to foster pre-DC maturation into DC-like cells.

In this regard, it is interesting to discuss the evidence for CSF1-mediated modulation of DCs and their precursors in further detail. LN LECs and splenic FSCs were recently discovered as the crucial source of CSF1 for the maintenance of the subcapsular sinus macrophage niche [55] and the RPM niche [56], respectively. But what about DCs? Several studies in CSF1−/−, CSF1R−/− or osteopetrotic (op/op) mutant mice that bear a recessive mutation causing complete CSF1 deficiency have assessed the DC frequency in SLOs and several tissues. The results from these studies are somewhat inconsistent, since some publications found no effects on DC frequency [23, 24, 57] and only noted an altered DC morphology with shorter projected cytoplasmatic processes [23], while others reported a 43% reduction in splenic CSF1−/− cDCs [58] or a reduction of dermis, lung and kidney CD11b+ CSF1R−/− DCs without displaying an apparent effect in the spleen [59], suggesting the existence of site-specific differences in the requirements for preDC differentiation. Additional deletion of CSF1R in FLT3−/− mice further diminished DC numbers [21], although this phenotype might in part be conferred through secondary effects on the DC pool [60]. Additionally, it was reported that in the pregnant mouse uterus CSF1 controls pre-DC extravasation rates, which in turn influenced local DC tissue density [61]. CSF1R fluorescent reporter mice label, next to macrophages and monocytes, also DCs [62–65], although DCs only dimly express the reporter and CSF1R could not be detected on the cell surface [62]. Yet, cDCs were found to express Csf1r mRNA [59, 66]. Thus, the available data indicated that CSF1 signalling may be important for the generation of cDCs, but is obsolete in fully matured cDCs, or that CSF1 provides an alternative pathway for cDC development in the absence of sufficient FLT3L signalling.

In conclusion, we here could demonstrate that LN FSCs are capable and sufficient to support pre-DC maturation into DC-like cells. We further identified CSF1 as the main factor nurturing this maturation process, providing first evidence that LN FSCs shape pre-DC maturation through this cytokine which is so far mostly associated with monocyte and macrophage biology. It will be interesting to further elucidate how SC-mediated CSF1 signalling affects cDC development, maintenance, phenotype and function. Moreover, considering the high heterogeneity of LN FSCs [40, 44], it will be important to define to what extent specific FSC subsets are involved in promoting the maturation of pre-DCs into DCs in vivo. We hope that our work can provide a basis for such endeavours.

MATERIALS AND METHODS

**Mice**

Foxp3hCD2xRag2−/− xDOI11.10 (BALB/c), Foxp3hCD2 xCD90.1 (BALB/c), Foxp3hCD2 (BALB/c) and CD90.1 (BALB/c) mice were bred and kept under SPF conditions in ventilated, isolated cages at the Helmholtz Centre for Infection Research (Braunschweig, Germany). Mice were housed and handled in accordance with good animal practice as defined by FELASA (Federation of European Laboratory Animal Science Associations) and the national animal welfare body GV-SOLAS (Society for Laboratory Animal Science).

**Antibodies, flow cytometry and cell sorting**

The following fluorochrome-conjugated antibodies were purchased from BD, Biolegend or eBioscience: (human)-CD2 (RPA-2.10), CD3 (17A2), CD4 (RM4-5), CD8α (53–6.7), CD11b (M1/70), CD11c (N418), CD19 (6D5), CD24 (M1/69), CD31 (390), CD45 (30-F11), CD45R (RA3-6B2), CD49b (DX5), CD117 (c-Kit) (2B8), CD135 (A2F10), CD172a (P84), gp38 (8.1.1), TER-119 (TER-119), MHCIId (M5/114.15.2), Siglec-H (eBio440c), Ly6-C (HK1.4), CSF1R blocking antibody (AFS98) and IgG2a κ (RTK2758). Flow cytometric analysis was performed as described recently [67]. In brief, single-cell suspensions
were washed with PBS before dead cells were labelled, using the LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Invitrogen) according to manufacturer’s instructions. Cell suspensions were incubated with 10 μg/ml Chrom Pure rat IgG (Jackson ImmunoResearch Laboratories) and 10 μg/ml anti-mouse CD16/CD32 antibody (BioXcell) to inhibit unspecific antibody binding and block designated Fc receptors. For staining of surface antigens, cells were washed and then incubated with the corresponding antibodies dissolved in PBS/0.2% BSA (Sigma-Aldrich) for 15 min on ice. Cells were washed and samples were resuspended in PBS/0.2% BSA before labelled cells were acquired on a LS Fortessa (BD Bioscience) flow cytometer with the Diva software (BD Bioscience). Acquired data were analysed utilizing the FlowJo software (BD Bioscience). Cell sorting for co-cultures as well as RNAseq analyses was performed at the cell sorting facility of the Helmholtz Centre for Infection Research on FACS Aria IIu (BD Bioscience), FACS Aria II SORP (BD Bioscience) and FACS Aria-Fusion (BD Bioscience).

**LN FSC isolation**

LN FSCs were isolated from skin-draining pLN (inguinal and axillary) and gut-draining mLN (small intestinal and colon/caecum-draining). The respective LNs were resected and digested in RPMI 1640 medium (Gibco) containing 0.2 mg/ml collagenase P (Roche), 0.15 U/ml dispase (Roche) and 0.2 mg/ml DNase I (Roche) as described previously [40]. In the majority of the experiments, LNs were taken from CD90.1 (BALB/c) mice, and in a few experiments also from Foxp3<sup>hCD2</sup>xCD90.1 (BALB/c) and Foxp3<sup>hCD2</sup> (BALB/c) mice. In brief, mLN or pLNs were pooled from multiple mice and cut into pieces (BALB/c) and Foxp3<sup>hCD2</sup> (BALB/c) mice. Bones were then placed into PBS/0.2% BSA containing 5 mM EDTA. CD45<sup>-</sup> was stained using fluorochrome-coupled antibodies and magnetic labeling of CD45<sup>-</sup> enriched by autoMACS (Miltenyi Biotec) separation after digestion. After digestion, cells were kept at 4°C in the water bath with frequent resuspension. Cell rich supernatants were filtered through a 100 μm sieve and cells were resuspended in PBS/0.2% BSA before dead cells were labelled, using the LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Invitrogen). T cell isolation

Single-cell suspensions from spleen and LNs of Foxp3<sup>hCD2</sup>xRag2<sup>−/−</sup>xDO11. 10 mice were generated by meshing resected organs through a 100 μm sieve and cells were washed in PBS/0.2% BSA. Erythrocytes were lysed as described above before samples were washed. CD4<sup>+</sup> T cells were enriched with CD4 (L3T4) MicroBeads via autoMACS separation (Miltenyi Biotec). Co-culture of LN FSC and pre-DCs

Pre-DC isolation

Whole bone marrow was isolated from femurs and tibias. Bones were resected and cleaned from muscle and connective tissue before the tips at both ends of each bone were cut off. In the majority of the experiments, bones were taken from CD90.1 (BALB/c) mice, and in a few experiments also from Foxp3<sup>hCD2</sup>xCD90.1 (BALB/c) and Foxp3<sup>hCD2</sup> (BALB/c) mice. Bones were then placed into PBS/0.2% BSA containing 0.2 mg/ml collagenase P (Roche), 0.15 U/ml dispase (Roche) and 0.2 mg/ml DNase I (Roche) as described previously [40]. In the majority of the experiments, LNs were taken from CD90.1 (BALB/c) mice, and in a few experiments also from Foxp3<sup>hCD2</sup>xCD90.1 (BALB/c) and Foxp3<sup>hCD2</sup> (BALB/c) mice. In brief, mLN or pLNs were pooled from multiple mice and cut into small pieces before being digested in the digestion buffer for 20 min at 37°C in the water bath with frequent resuspension. Cell rich supernatants were filtered through a 100 μm sieve into ice cold PBS/0.2% BSA containing 5 mM EDTA (Roth), and remaining tissue pieces underwent further digestion rounds until complete disintegration. After digestion, cells were kept at 4°C in PBS/0.2% BSA containing 5 mM EDTA. CD45<sup>+</sup> cells were enriched by autoMACS (Miltenyi Biotec) separation after magnetic labeling of CD45<sup>+</sup> cells using anti-CD45 MicroBeads (30-F11, Miltenyi Biotec) or anti-CD45 Nanobeads (30-F11, Biolegend). Subsequently, the CD45<sup>-</sup> fraction was stained using fluorochrome-coupled antibodies and CD45<sup>-</sup>/CD24<sup>-</sup>/CD31<sup>-</sup>/gp38<sup>-</sup> FSCs were sorted by flow cytometry (Aria II, 100 μm nozzle) into round bottom 96-well plates. Co-culture of LN FSC and pre-DCs

Round bottom 96-well plates were coated with 0.5 μl of 50 μg/ml Fibronectin (Roche) diluted 1:20 in PBS from stock solution. For the 1 mg/ml Fibronectin stock solution, Fibronectin was dissolved in 2 M Urea (Sigma). Plates were centrifuged for 1 min at 400 g at RT before incubation for 1 h at 37°C and 5% CO<sub>2</sub>. Subsequently, plates were washed once with 150 μl cRPMI (RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS) (Gibco), 25 mM HEPES (Biochrom AG), 1 mM sodium pyruvate (Gibco), 0.05 mg/ml penicillin (Gibco), 0.05 g/ml streptomycin (Gibco)).
mg/ml streptomycin (Gibco) and 50 μM β-mercaptoethanol (Gibco)) before 150 μl cRPMI were added to the plates. FSCs were isolated from pooled mLNs or pLN samples as described above and 2000 LN FSCs per well were directly sorted by flow cytometry into the prepared plates. At the same time, pre-DCs were isolated from whole bone marrow of the same mice as described above and sorted by flow cytometry into Eppendorf tubes. After cell sorting, the plates containing LN FSCs were centrifuged for 1 min at 200 g at RT in order to gently spin-down the cells. Then, 3000 pre-DCs were added to each culture well containing either mLN or pLN FSCs or they were added to empty wells. In addition, wells containing only pre-DCs received either 50 ng/ml CSF1 or 200 ng/ml FLT3L. Plates were once more centrifuged for 1 min at 200 g at RT before incubation for one, three or five days at 37°C and 5% CO₂. At the end of the cultures, the cells were harvested and analysed by flow cytometry.

**T cell proliferation assay**

LN FSC and pre-DC co-cultures were seeded as described above, but in 100 μl cRPMI volume. On day 3 and 5 of the co-culture, CD4⁺ T cells from Foxp3<sup>hCD2<sub>x</sub>Rag2<sup>−/−</sup>x DO11.10</sup> mice were freshly isolated as described above, labelled with CO₂. At the end of the cultures, the cells were harvested and analysed by flow cytometry.

**In vitro CSF1R blocking assay**

For CSF1R blocking experiments, LN FSC were seeded as described above. Dedicated wells received either 5 μg/ml IgG2a κ (RTK2758) as an isotype control or 5 μg/ml CSF1R blocking antibody (AFS98). After addition of 3000 pre-DCs per well, plates were incubated for 5 min at RT followed by centrifugation for 1 min at 200 g at RT. Plates were incubated for three or five days at 37°C and 5% CO₂. At the end of the cultures, the cells were harvested and analysed by flow cytometry.

**RNAseq and scRNAseq**

At day 3 of the respective cultures, cells were harvested and wells from each condition were pooled. Subsequently, both viable CD4⁵⁺CD11c⁻²MHCIİ<sup>low</sup> and CD4⁵⁺CD11c⁺MHCIİ<sup>high</sup> DC-like cells were sorted by flow cytometry as described above into RTL⁺ lysis buffer (Qiagen), containing 10 μl/ml β-mercaptoethanol (Rothe). An aliquot of sorted Lin⁻MHCIİ⁺CD11c⁻CD11b⁻CD135⁺CD172α⁻ pre-DCs isolated during the seeding of the respective cell culture was used as an input control. Using the RNeasy Plus Micro kit (Qiagen), total RNA was extracted according to manufacturer’s instructions. RNA was converted to cDNA with the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Clontech Laboratories) according to manufacturer’s instructions, which was followed by a PCR product purification with Agencourt AMPure XP magnetic beads (Beckman Coulter). All libraries were prepared with the Nextera XT DNA Library Prep Kit (Illumina). Primers from the IDT for Illumina Nextera XT DNA DU Indexes Set A (Illumina) were used for indexing. Combined PCR product clean-up and size selection was performed with the Agencourt AMPure XP magnetic beads. For all intermediate isolation products in this process, RNA and cDNA quality and content, as well as the correct size of cDNA library fragments was verified using the Fragment Analyser 5200 (Agilent) profiles and Qubit measurements. The generated libraries were sequenced at the genome analytics facility of the Helmholtz Centre for Infection Research on an Illumina NovaSeq6000 using 50 bp paired-end reads. For data analysis, read quality of sequenced libraries was evaluated with FastQC. Sequencing reads were aligned to the reference mouse genome assembly GRCm38 using STAR, and aligned reads to annotated genes were quantified with htseq-count. Calculated read counts were processed with DESeq2 for the quantification of differential gene expression. For visualization, raw read counts were converted to RPKM (reads per kilobase of exon length per million mappable reads) values, and only genes with an annotated Gene Symbol were depicted in plots. For the identification of DEGs, indicated log₂(FC) values and a q-value ≤ 0.05 were utilized, while for depicted signature genes an RPKM threshold was applied. All depicted plots were generated with R using heatmap and ggplot2. DC-like cell sequencing data from co-cultures can be found under the GEO accession number GSE184661. scRNAseq data of pLN and mLN non-endothelial SCs were previously generated and analysed by Pezoldt et al. [40], and deposited under the GEO accession number GSE116633. All depicted plots were generated in R using Seurat and ggplot2 and all marker genes are plotted across previously identified non-endothelial SC clusters.
Statistical analysis

Prism software (GraphPad) was utilized for statistical analysis of flow cytometry data. For all figures, if not stated otherwise, each data point represents a single independent co-culture condition with the mean value of 1–3 technical replicates or an independent sort of pooled wells for RNAseq. When two unmatched groups were compared, a two-tailed Mann–Whitney test was applied. In case more than three groups were compared, a Kruskal-Wallis-Test was used followed by Dunn’s multiple comparisons as a post hoc test. If not indicated otherwise, samples with < 100 viable CD11c+ DC-like cells or T cells were excluded from the analyses. Data are presented as mean ± SD, and p-values below a threshold of 0.05 were considered as significant; *p < 0.05; ** p < 0.01; ***p < 0.001; ****p < 0.0001; ns = not significant.

AUTHOR CONTRIBUTIONS
Carolin Wiechers performed the experiments and interpreted data. Michael Beckstette and Carolin Wiechers performed bioinformatics analysis. Joern Pezoldt, Jochen Huehn and Barbara U. Schraml interpreted data, designed research and provided expertise. Carolin Wiechers and Jochen Huehn designed research, interpreted data and wrote the manuscript.

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CONFLICT OF INTEREST
The authors declare no competing commercial or financial interests in relation to the work described.

DATA AVAILABILITY STATEMENT
The RNAseq data reported in this paper are available under the GEO accession number GSE184661.

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