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Research Article

Flt3L, LIF, and IL-10 combination promotes the selective in vitro development of ESAM^{low} cDC2B from murine bone marrow

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The development of two conventional dendritic cells (DC) subsets (cDC1 and cDC2) and the plasmacytoid DC (pDC) in vivo and in cultures of bone marrow (BM) cells is mediated by the growth factor Flt3L. However, little is known about the factors that direct the development of the individual DC subsets. Here, we describe the selective in vitro generation of murine ESAM^{low} CD103^{-} XCR1^{-} CD172a^{+} CD11b^{+} cDC2 from BM by treatment with a combination of Flt3L, LIF, and IL-10 (collectively named as FL10). FL10 promotes common dendritic cell progenitors (CDP) proliferation in the cultures, similar to Flt3L and CDP sorted and cultured in FL10 generate exclusively cDC2. These cDC2 express the transcription factors *Irf4*, *Klf4*, and *Notch2*, and their growth is reduced using BM from *Irf4*$^{-/-}$ mice, but the expression of *Batf3* and *Tcf4* is low. Functionally they respond to TLR3, TLR4, and TLR9 signals by upregulation of the surface maturation markers MHC II, CD80, CD86, and CD40, while they poorly secrete proinflammatory cytokines. Peptide presentation to TCR transgenic OT-II cells induced proliferation and IFN-γ production that was similar to GM-CSF-generated BM-DC and higher than Flt3L-generated DC. Together, our data support that FL10 culture of BM cells selectively promotes CDP-derived ESAM^{low} cDC2 (cDC2B) development and survival in vitro.

Keywords: dendritic cells · cDC2 subset · Flt3L · LIF · IL-10

Introduction

Under steady-state conditions, mainly cDC1, cDC2, and pDC subsets can be found in peripheral and lymphoid tissues [1–3]. Dendritic cells (DC) derived from monocytes (MoDC) are scarcely found in mice and humans during steady-state conditions and are typically a product of emergency myelopoiesis under inflammatory, allergic, or infectious conditions [4–7]. More recently, the cDC2 cells have been further subdivided into ESAM^{high} cDC2A and ESAM^{low} cDC2B, which so far appear with a restricted tissue-specific distribution in the spleen and intestine [8, 9]. Both cell types most likely form separate DC subsets leading to term the granulocyte-macrophage stimulating factor (GM-CSF)-dependent ESAM^{high} cDC2 rather DC3, as revealed from human studies

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[10–12]. Remarkably, the loss of Runx3 converts the transcriptional signature of ESAM^{high} cDC2 into one that resembles ESAM^{low} cDC2 [13].

Several growth factors, cytokines, and other soluble mediators have been described to promote DC development in vitro. The generation of murine MoDC can be mediated in vitro predominantly by GM-CSF [14, 15], while human MoDC require additional IL-4 or TNF [16–19]. The use of Flt3L in bone marrow (BM) cultures has been first described for CD8α$^+$ cDC1 and CD11b$^+$ cDC2 generation [20, 21] and later also for pDC generation [22]. Together, the Flt3L-based culture allowed the generation of pDC, cDC1, and cDC2 from BM precursors, leading to a bulk mixture of these cells [23]. Protocols combining Flt3L with GM-CSF [24] or Delta-like-1 [25] have been reported to promote selective cDC1 outgrowth from BM cells. Both cDC1 and cDC2 subsets develop from monocyte dendritic cell progenitors (MDP) and common dendritic cell progenitors (CDP) and then further into their subtypes [26, 27]. While Flt3L treatment of MDP promotes their differentiation into CDP [28], GM-CSF treatment of MDP generates monocytes that can form MoDCs [7].

Although the different subsets of cDCs and pDC can be determined regarding their transcription factor requirements, surface markers, and functions [3, 29], the critical growth factors beyond Flt3L that drive bifurcation into either pDC, cDC1, or cDC2 remain unclear. Only for cDC1, a culture method combining Flt3L and GM-CSF has been shown to polarize the cultures toward CD103$^+$ cDC1 development [24].

Here we describe a modified protocol of the Flt3L mediated in vitro generation of bulk pDC, cDC1, and cDC2 from mouse BM [20, 21]. The addition of LIF and IL-10 to such Flt3L cultures resulted in the specific development of the ESAM^{low} cDC2 subset. The cDC2 enrichment was accompanied by increased apoptosis of developing cDC1 but also enhanced progenitor proliferation at the CDP stage. Both Flt3L and FL10 cultured DC responded poorly on TLR4 and TLR9 stimulation with upregulation of MHC II, costimulatory molecules, and cytokine release but were similarly effective to stimulate CD4$^+$ T cell proliferation and IFN-γ$^+$ Th1 polarization after LPS-induced maturation. Together, the FL10 protocol implies a critical role for LIF and IL-10 on the in vitro generation of ESAM^{low} cDC2 and provides a method for an enriched generation of this DC subset.

Results

BM cultures with FL10 are highly enriched for the ESAM^{low} cDC2B subset

Since the effects of LIF and IL-10 on different DC subsets have been described, we tested them in combination with Flt3L in BM cultures. We compared two standard protocols for the in vitro generation of DC from BM with GM-CSF [15] and Flt3L [21] with the FL10 cultures. The Flt3L culture was modified according to the culture conditions that appeared optimal for FL10 by reducing the culture period from 9 to 8 days, the starting cell density from 1.5–3 × 10^6 cells/ml to 1 × 10^6 cells/ml, and 5–7% CO_2 instead of 10% CO_2 at 37°C. The concentration of Flt3L at 200 ng/ml was the same, but we generally used commercial human Flt3L instead of in-house-generated mouse Flt3L [21].

The phenotypical appearance of Flt3L and FL10 cultures after 8 days was similar and did not contain large DC clusters, unlike GM-CSF cultures (Fig. 1A). Instead, FACS analyses revealed major differences between Flt3L and FL10 cultures. First, the frequency of CD11c$^-$ cells was clearly higher in the FL10 cultures and also the percentage of Annexin V$^+$ dead cells as compared with Flt3L cultures, which may be indicative of a selection process in the FL10 cultures (Fig. 1A). The B220$^+$ CD11c$^-$ cell populations were not further investigated but may represent contaminating B cells (Fig. 1A). Second, the addition of Flt3L alone generated CD11c$^+$ B220$^+$ pDC, while FL10 cultures did not form a distinct population and remained at a lower frequency (Fig. 1B and E). Third, CD11c$^+$ B220$^-$ Ly6C$^+$ CD64$^-$ monocytes and CD11c$^+$ B220$^-$ Ly6C$^-$ CD64$^+$ MoDC were reduced or not detectable in FL10 cultures as compared with those with Flt3L (Fig. 1B and E). Forth, the CD11c$^+$ B220$^-$ Ly6C$^-$ CD64$^-$ XCR1$^+$ CD103$^+$ CD11b$^-$ cDC1 subset and XCR1$^-$ CD11b$^-$ double negative cDC appeared only in Flt3L but not FL10 cultures (Fig. 1B and E). Finally, the only DC population that was highly enriched in FL10 cultures were CD11c$^+$ B220$^-$ Ly6C$^-$ CD64$^-$ XCR1$^-$ CD103$^-$ CD11b$^+$ cells compatible with a cDC2 phenotype (Fig. 1B, D, and E). The expression of CD172a (SIRPα) further supported the cDC2 profile (Fig. 1C).

The cDC2 can be further subdivided into subtypes expressing ESAM at high or low levels [8]. Here we found that both Flt3L and FL10 cultures generated only cDC2 with low surface levels of ESAM, unlike the spleen where ESAM^{low} and ESAM^{high} cDC2 can be detected, while cDC1 were always negative (Fig. 1C). Since little is known about the up- or downregulation of the ESAM marker on cDC, we conclude that both the Flt3L and FL10 in vitro cultures induce the ESAM^{low} cDC2B subtype.

Since we noted increased Annexin V$^+$ dead or dying cells in the FL10 cultures, we further dissected them into the respective DC subtypes. We found that cDC1 were enriched within the Annexin V$^+$ cells, while cDC2, pDC, and MoDC remained largely unaltered or reduced (Fig. 1F). Thus, the addition of LIF, IL-10, or both appears to selectively promote apoptosis of cDC1 when analyzed on day 8 of culture.

The phenotypical characterization indicated that FL10 cultures are enriched for ESAM^{low} cDC2 with the complete absence of MoDC, cDC1, and DN DC and a reduction of pDC and monocytes. Of note, the overall purity of living cDC2 in these cultures was still only around 35% (Fig. 1E). This was mainly due to a high frequency of Annexin V$^+$ cells, mostly among cDC1 and pDC but also cDC2 (Fig. 1E) and the presence of B220$^+$ CD11c$^-$ cells, likely representing B cells, as well as other not further defined B220$^-$ CD11c$^-$ cells, indicating that the use of this protocol does not reveal pure cDC2. Together, these data point to an important role for LIF and IL-10 to specifically direct ESAM^{low} cDC2B subset survival or development in vitro.

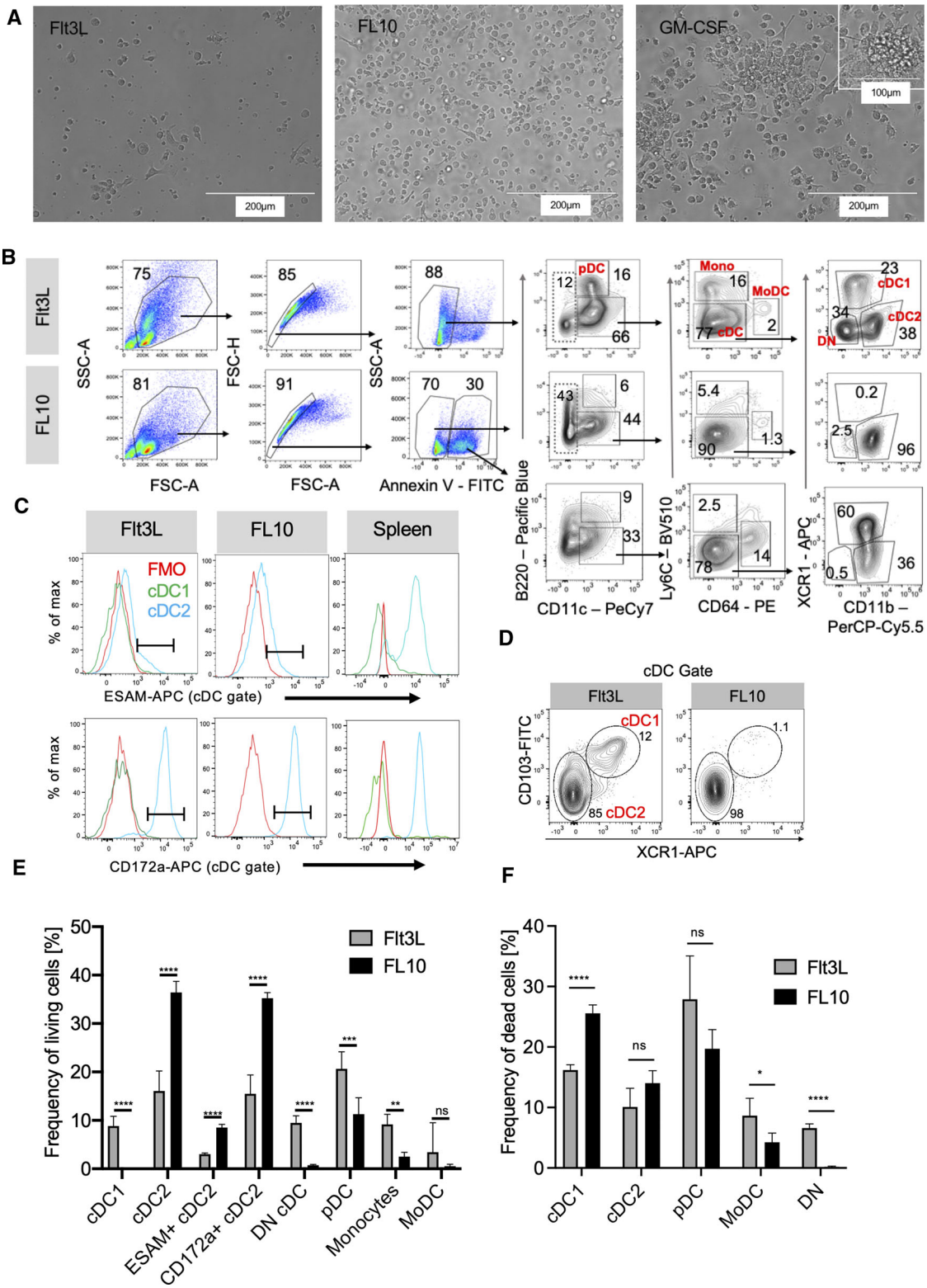

Figure 1. Phenotypic comparison of FL10 generated cDC2 with Flt3L cultures. Fresh BM cells were cultured either in Flt3L, FL10, or GM-CSF-containing media. After 8 days, cells were analyzed. (A) Microscopic phase contrast image of bone marrow cultures at day 8 of differentiation in Flt3L, FL10, or GM-CSF containing media. Only cells cultured in GM-CSF show the formation of cell clusters (insert). (B) Representative flow cytometry plots at day 8 of bone marrow cells cultured either in Flt3L or FL10 containing media. The gating strategy here and all further experiments included FSC-A/SSC-A, FSC-H/FSC-A doublet exclusion, and gating on life (Annexin V^-) or dead (Annexin V^+) cells. Then, conventional DCs were identified as $CD11c^+$, $B220^-$, $CD64^-$ $Ly6C^-$ cells and were further subdivided into cDC1 ($XCR1^+$) and cDC2 ($CD11b^+$). Designation of cell type in red color text. (C) cDC2 were further characterized by ESAM and CD172a (SIRPα) staining. As controls ex vivo isolated spleen cDC and Flt3L

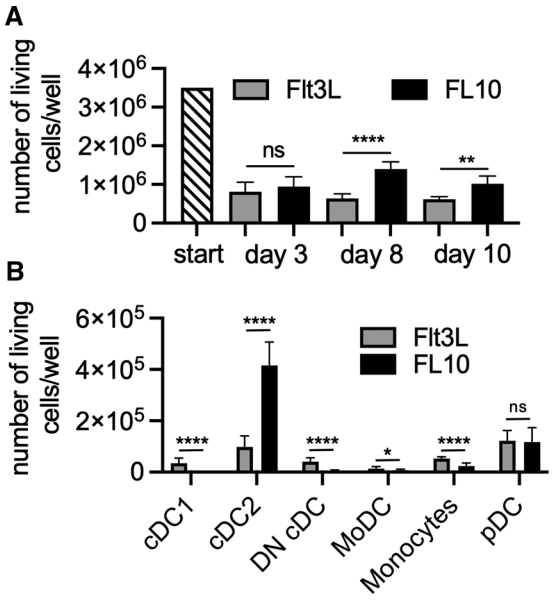

Figure 2. Optimal cDC2 generation in FL10 cultures at day 8. (A) Flt3L or FL10 cultures were analyzed on days 3, 8, or 10 to assess DC progenitor expansion. After 8 days of culture in either Flt3L or FL10 medium, cells were harvested, and living cells were counted per well in a Neubauer chamber using Trypan Blue. (B) Absolute numbers of cDC2 cells/well after 8 days in either Flt3L or FL10 media were calculated using cDC2 frequencies from flow cytometry analysis as in Figure 1B, and the numbers revealed in Figure 2A. Statistics with the unpaired two-sided *t*-test. (A) shows the results of 5 experiments and (B) of 12 experiments. All data is shown as mean + SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. cDC1 and cDC2, conventional dendritic cell subsets 1 and 2; DC, dendritic cells; FL10, combination of Flt3L LIF and IL-10; Flt3L, FMS-like tyrosine kinase-3 ligand; IL-10, interleukin-10.

FL10 cultures induce higher cell yields but similar CDP expansion than Flt3L cultures

Since we noted a higher frequency of apoptotic cells in FL10 cultures, we wondered whether this combination of cytokines might exert a general reduction of cellular yields when compared with Flt3L cultured BM cells, thus simply promoting cDC2 survival. In contrast, we found that the total cellular yield of FL10 cultures on day 8 was about double the cell number per well as obtained with Flt3L and relatively lower on days 3 and 10 (Fig. 2A). The absolute cell number of cDC2 appeared to increase even to about fourfold (Fig. 2B). These data indicate that FL10 cultures do allow not only selective survival of cDC2 but also that cDC2 generation is selectively enhanced with a maximum at day 8.

To further corroborate the FL10-promoted expansion of cDC2, we analyzed the frequencies and proliferation of selected myeloid progenitors earlier in the culture on day 3. While monocyte dendritic cell progenitors (MDP) expanded only moderately (Fig. 3A, C, and D), the successor population of common dendritic cell progenitors (CDP) [30] strongly increased in their frequency and cell number in Flt3L and FL10 cultures as compared with their counterparts from fresh BM (Fig. 3A, C, and D). This indicates that CDP are the major expanding population under these in vitro conditions. The proliferation rate measured at day 3 by Ki67 staining of the MDP and CDP remained comparable in Flt3L and FL10 cultures as detected in fresh BM (Fig. 3B and E). In contrast, the common monocyte progenitors (cMoP), which represent the latest proliferating cell type before development into nonproliferating monocytes [31], were reduced both in frequency and Ki67 expression when compared with fresh BM (Fig. 3B and E).

To demonstrate that Flt3L and FL10 generated cDC2 are indeed derived from CDP, we sorted congenic CD45.2^{+} CDP from either d3 Flt3L and FL10 cultures and added them to the respective day 3 Flt3L and FL10 culture conditions derived from CD45.1^{+} genotype. Analysis on day 8 indicated that FL10-derived CDP developed selectively into cDC2, while Flt3L-derived CDP generated all three cDC populations although cDC1 and DN cDC were less efficient (Fig. 3F).

Together, these data demonstrate that FL10-cultured cDC2 are derived from CDP which represent the major expanding progenitor at d3 in both Flt3L and FL10 cultures as compared with MDP and cMoP.

Culture parameters influencing FL10 culture conditions

To further explore the factors influencing the generation of cDC2 in vitro, we dissected the FL10 components LIF and IL-10 in combination with Flt3L. While the addition of LIF or IL-10 clearly increased the proportion and cell number as compared with Flt3L cultures, IL-10 showed only a moderate and not statistically significant effect for the specific generation of cDC2 and the triple combination appeared better than any dual combination (Fig. 4A–C).

Next, we compared whether the selection of recombinant human or recombinant murine Flt3L would have differential effects when used in FL10 for the generation of cDC2. The use of human Flt3L alone clearly promoted cDC2 frequencies as compared with murine Flt3L alone, but both types of Flt3L used in combination with LIF and IL-10 further increased cDC2 frequencies (Fig. 4D).

Since the batch of FCS and its ill-defined components may dramatically influence murine GM-CSF-dependent BM-DC

cultured cDC1 are shown. (D) cDC1 equivalently express XCR1 and CD103 under Flt3L but they are lacking under FL10 culture conditions. (E) Flt3L or FL10 day 8 cultures were analyzed for their frequencies of the individual cell populations within the living (Annexin V^{-}) cells, or (F) their frequencies of individual cell populations within dead (Annexin V^{+}) cell gates. (A–D) are representative for >10 independent experiments. (F–G) show the results of four experiments. Statistics were performed by unpaired two-sided *t*-test. All data are shown as mean + SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. BM, bone marrow; cDC1 and cDC2, conventional dendritic cell subsets 1 and 2; CDP, common dendritic cell progenitors; cMoP, common monocyte progenitor; DC, dendritic cells; FL10, combination of Flt3L LIF and IL-10; Flt3L, FMS-like tyrosine kinase-3 ligand; GM-CSF, granulocyte-macrophage stimulating factor; IL-10, interleukin-10; LIF, leukemia inhibitory factor.

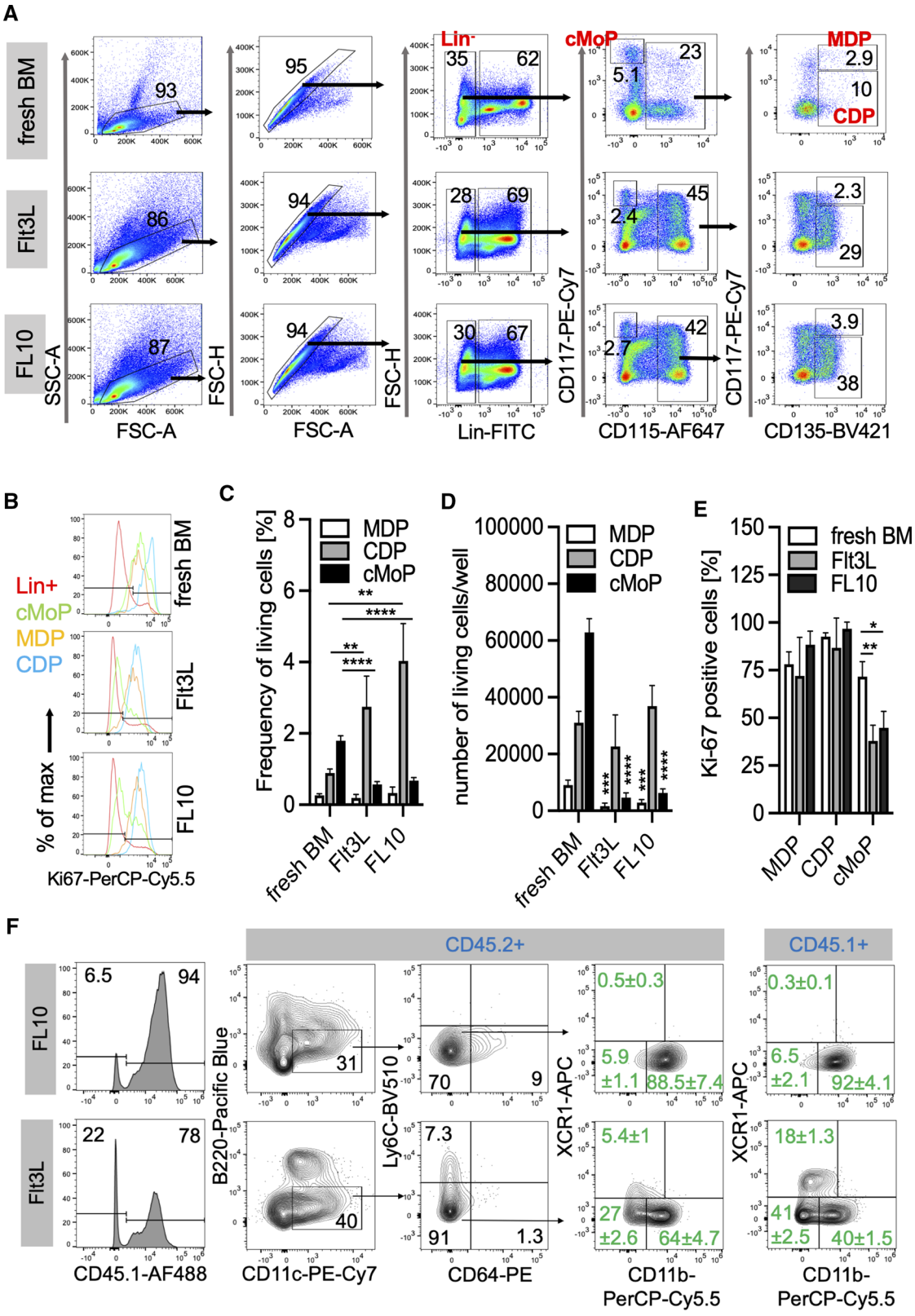

Figure 3. cDC2 in Flt3L and FL10 cultures are derived from expanding CDP. (A) Representative flow cytometry plots of fresh BM cells and d3 cultures in Flt3L and FL10. Gating strategy with FSC-A/SSC-A, doublet exclusion, and lineage marker exclusion (CD3, NK1.1, Ly6G, CD19, CD11c). cMoP were identified as $CD115^{-}$ $CD117^{+}$, CDP as $CD115^{+}$ $CD135^{+}$ $CD117^{-}$ and MDP as $CD115^{+}$ $CD135^{+}$ $CD117^{+}$ cells. (B) Representative histogram overlays showing Ki67 staining in Lin^{+} cells as negative control, cMoP, MDP, and CDP of fresh BM and d3 cultures with Flt3L or FL10. (C) Statistical assessment of d3 progenitor cell frequencies and (D) cell number. Significance was assessed by comparison to the respective cell type in fresh BM. The data

generation [32], we tested whether dialysis of FCS or the use of different FCS batches or a serum-free FCS substitute, would influence cDC2 generation. Our data indicate that dialysis of our standard FCS inhibits cDC2 generation, indicating that small molecules <20 kDa within the FCS play an important role in cDC2 generation (Fig. 4E). Some batches of FCS from different suppliers showed a similar outgrowth of cDC2 as compared with our standard FCS from SIGMA, and one batch, as well as the serum-free FCS substitute Pannexin, reduced the cDC2 frequency to about half of the other batches (Fig. 4F).

Together, the LIF plus IL-10 combination was superior to the other conditions or additives tested, and no difference was detected between human or mouse Flt3L within the FL10 combination.

ESAM^{low} cDC2 have a unique transcriptional signature and display a partial IRF4 requirement

The development of DC subsets is directed by the subsequent expression of transcription factors guiding them into the cDC lineage and further into the respective cDC1 and cDC2 subsets [3, 33, 34]. Therefore, the expression of selected transcription factors that are indicative of DC subtypes was quantified by qRT-PCR. FL10 cultures were compared with cDC1, cDC2, and pDC from sorted Flt3L cultures (Fig. 5A).

Our results obtained for sorted cDC1, cDC2, and pDC are similar to the relative expression profiles available through the public database (ImmGen ULI RNAseq) for the respective ex vivo isolated DC subsets from the spleen (Fig. 5B).

A high expression of *Irf4* is characteristic of cDC2 independent of their ESAM expression [3]. FL10-cultured cells expressed mRNA for *Irf4* at lower but not significantly different levels as compared with sorted cDC2 from Flt3L cultures (Fig. 5A). The transcription factors *Klf4* and *Notch2*, indicative for cDC2, are both up-regulated in FL10 cultures (Fig. 5A). Although the transcription factor *Notch2* promotes ESAM^{high} cDC2 development [8, 35], the ESAM^{low} cDC2 subset depends on *Klf4* [36]. Here, Klf4 shows only a nonsignificant trend for higher expression than in sorted Flt3L-generated cDC2, and the inverse situation is detectable for Notch2 transcription.

The expression of transcription factors that are not related to cDC2, such as *Batf3*, which is required for cDC1 development in vivo [37] and in vitro [24], and *Tcf4*, encoding the pDC marker E2-2, which is essential for pDC development in mice [38], were expressed only at low transcriptional levels in FL10 cultured cells (Fig. 5A). Since the expression of *Batf3* has now been accepted to be also expressed in cDC2 [3] and this is also reflected by the data from the ImmGen database, the expression of this marker may not contribute to distinguish cDC1 from cDC2.

The common cDC zink finger transcription factor *Zbtb46* [39, 40] was expressed but in significantly lower amounts as compared with sorted cDC2 from Flt3L cultures (Fig. 5A). Together, the mRNA profile for transcription factor expression of FL10 cultures is highly similar to the corresponding cDC2 cells sorted from Flt3L cultures (Fig. 5A) or the ImmGen database (Fig. 5B). However, the selective induction of the ESAM^{low} cDC2 subsets by FL10 conditions is not or only marginally supported by the qRT-PCR data.

In vivo, cDC2 generation largely depends on the transcriptional activity of *Irf4* in precursors of cDC (pre-cDC) since their presence in *Irf4*$^{-/-}$ mice is strongly reduced [41]. Similarly, here FL10 cultures with BM-derived from *Irf4*$^{-/-}$ mice showed mild but significantly reduced frequencies of cDC2 as compared with wild-type BM cells (Fig. 5C). The dependency on *Irf4* was stronger in Flt3L cultures (Fig. 5C), indicating that LIF and IL-10 may partially compensate for the need for *Irf4* transcriptional activity in vitro.

FL10-generated cDC2 show maturation but impaired cytokine production on TLR3, TLR4, and TLR9 agonists

Response to pathogens by upregulation of MHC II, costimulation, and proinflammatory cytokine production are important DC functions. Previous transcriptional analysis of the TLR expression by cDC1 and the two cDC2A and cDC2B subsets indicated that cDC2B express higher mRNA levels for TLR4 and TLR9 as compared with cDC2A and cDC1 highest levels of TLR9 [9].

Untreated FL10 cultures showed a higher spontaneous maturation as shown by their MHC II and CD86 surface expression, as compared with GM-CSF or Flt3L cultures (Fig. 6A–C). After stimulation of FL10 DC cultures with the TLR3 agonist Poly I:C, the TLR4 agonist LPS or the TLR9 agonist CpG oligonucleotides, we detected a clear response by surface upregulation of MHC II and CD86 molecules with all stimuli on all DC subsets but somewhat less by CpG (Fig. 6A–C). All stimuli also induced IL-6 but only low IL-12p40 cytokine production in cDC2 from Flt3L and FL10 cultures when compared with MoDC from GM-CSF cultures. LPS appeared to be the strongest cytokine inducer in cDC2 while CpG appeared the weakest, and GM-CSF-cultured DC failed to respond to Poly I:C (Fig. 6D–H). This was similar when IL-12p70 and TNF

shown in (C) and (D) were analyzed and gated as described in (A). (E) Statistical assessment of the frequencies of Ki67$^+$ proliferating progenitors at d3. The cells were gated as in (C) and analyzed as in (D) for Ki67. (F) CDP were sorted from d3 Flt3L or FL10 cultures of CD45.2$^+$ BM cells and 5000–10,000 cells were added to respective d3 congenic CD45.1$^+$ cultures. Flow cytometry was performed at d8. Black numbers indicate percentages for the actual gates. Green numbers are mean percentages of $n = 3$ cultures ± SD in their gates. (A–E) The results of four independent experiments are shown. (F) The results of $n = 3$ cultures from two independent experiments are shown. Statistics with the unpaired two-sided *t*-test. All data is shown as mean + SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. BM, bone marrow; cDC1 and cDC2, conventional dendritic cell subsets 1 and 2; CDP, common dendritic cell progenitors; FL10, combination of Flt3L LIF and IL-10; Flt3L, FMS-like tyrosine kinase-3 ligand; MDP, monocyte dendritic cell progenitors.

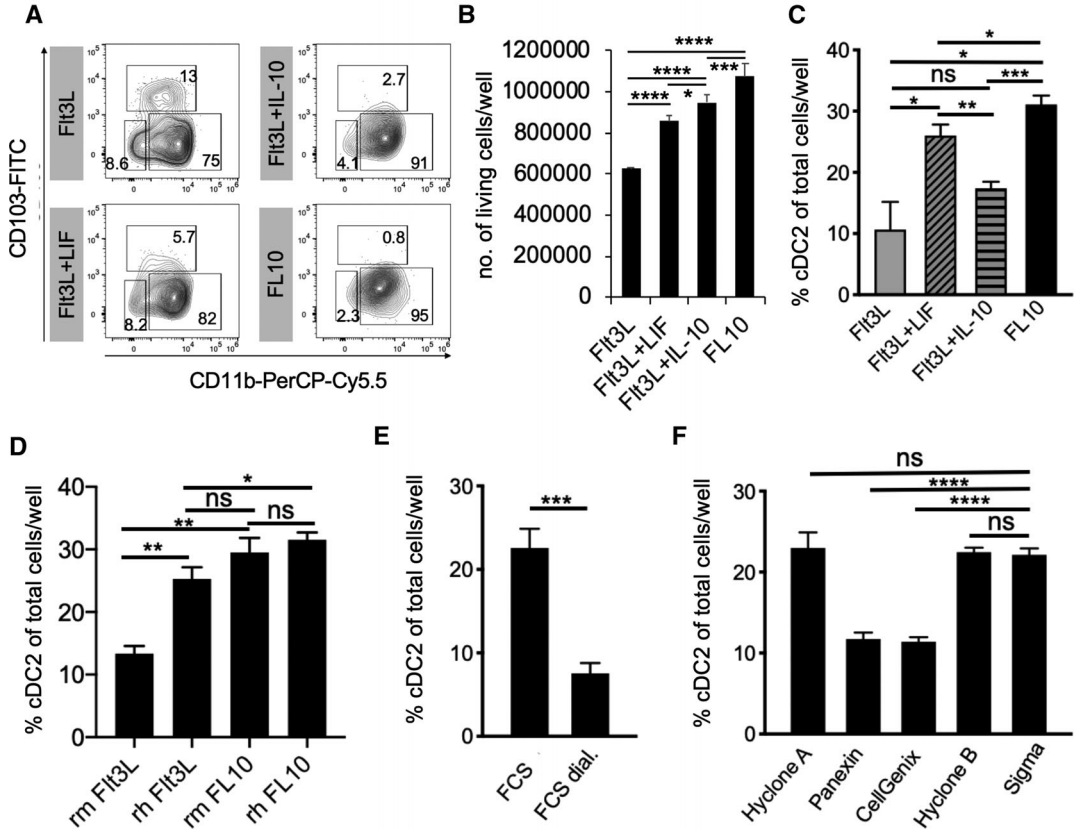

Figure 4. Additional factors influencing the generation of cDC2 in vitro. (A) BM cells were cultured until day 8 with the indicated cytokines and then analyzed for the indicated markers within the cDC gate by flow cytometry. (B) Statistical evaluation of yielded cell numbers in experimental settings as in (A). (D) Statistical evaluation of cell frequencies in experimental settings as in (A). (E) BM cells were cultured until day 8 with recombinant murine (rm) or recombinant human (rh) Flt3L alone or in combination with LIF and IL-10 and then analyzed for the frequency of cDC2 by flow cytometry. (F) BM cells were cultured with FL10 in media containing dialyzed or nondialyzed FCS and on day 8 analyzed for the frequency of cDC2 by flow cytometry. (E) BM cells were cultured with FL10 in media containing different batches of FCS from different providers or the serum-free additive Pannexin. Cultures were analyzed on day 8 for the frequency of cDC2 by flow cytometry. (A–E) The results of three independent experiments are shown. Statistics with the unpaired two-sided *t*-test. All data is shown as mean + SD. ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p < 0.05$, ns = not significant. BM, bone marrow; cDC1 and cDC2, conventional dendritic cell subsets 1 and 2; FL10, combination of Flt3L LIF and IL-10; Flt3L, FMS-like tyrosine kinase-3 ligand; LIF, leukemia inhibitory factor.

production was analyzed in supernatants from GM-CSF and FL10 cultures (Fig. 6I and J). Thus, FL10-generated cDC2 appear to mature readily with respect to surface markers and moderately for cytokine release after Poly I:C, CpG, or LPS stimulation but is similar to Flt3L cultured cDC2.

FL10 cDC2 induce robust CD4$^+$ T-cell proliferation and IFN-γ production in vitro

Since we observed that FL10 cDC2 responded better to the Th1 stimulus LPS than CpG or Poly I:C, we tested OVA-loaded and LPS-matured FL10 cultures for their capacity to prime CD4$^+$ OT-II T cells and to drive their polarization into Th1 cells. Although cDC2 produced only moderately IL-12p70 after LPS treatment, as indicated above, the induction of proliferation of antigen-specific CD4$^+$ Vβ5$^+$ TCR transgenic T cells was similar as compared with Flt3L or GM-CSF DC cultures (Fig. 7A and B). The induction of IFN-γ^+ cells was similar to GM-CSF cultures and higher than by Flt3L cells, while IL-2 release, reversely, low when compared with GM-CSF culture conditions but comparable with Flt3L cultures (Fig. 7C and D). IL-13$^+$ CD4$^+$ T cells were not induced by FL10 cultured cDC2, unlike by bulk DC generated in Flt3L and GM-CSF (Fig. 7E). These data indicate that FL10-generated and LPS-stimulated cDC2 bear a high potential to induce CD4$^+$ T-cell proliferation and IFN-γ^+ Th1 polarizing capacity.

Discussion

Here we report that the addition of LIF and IL-10 to Flt3L cultures of murine BM cells selectively promotes the generation of ESAM^{low} cDC2B as indicated by their surface marker profile, the preferential proliferation of CDP and MDP, and their transcription factor expression pattern (*Irf4, Batf3, Zbtb46, high Klf4, low Notch2*,). Small molecules within different FCS batches but also

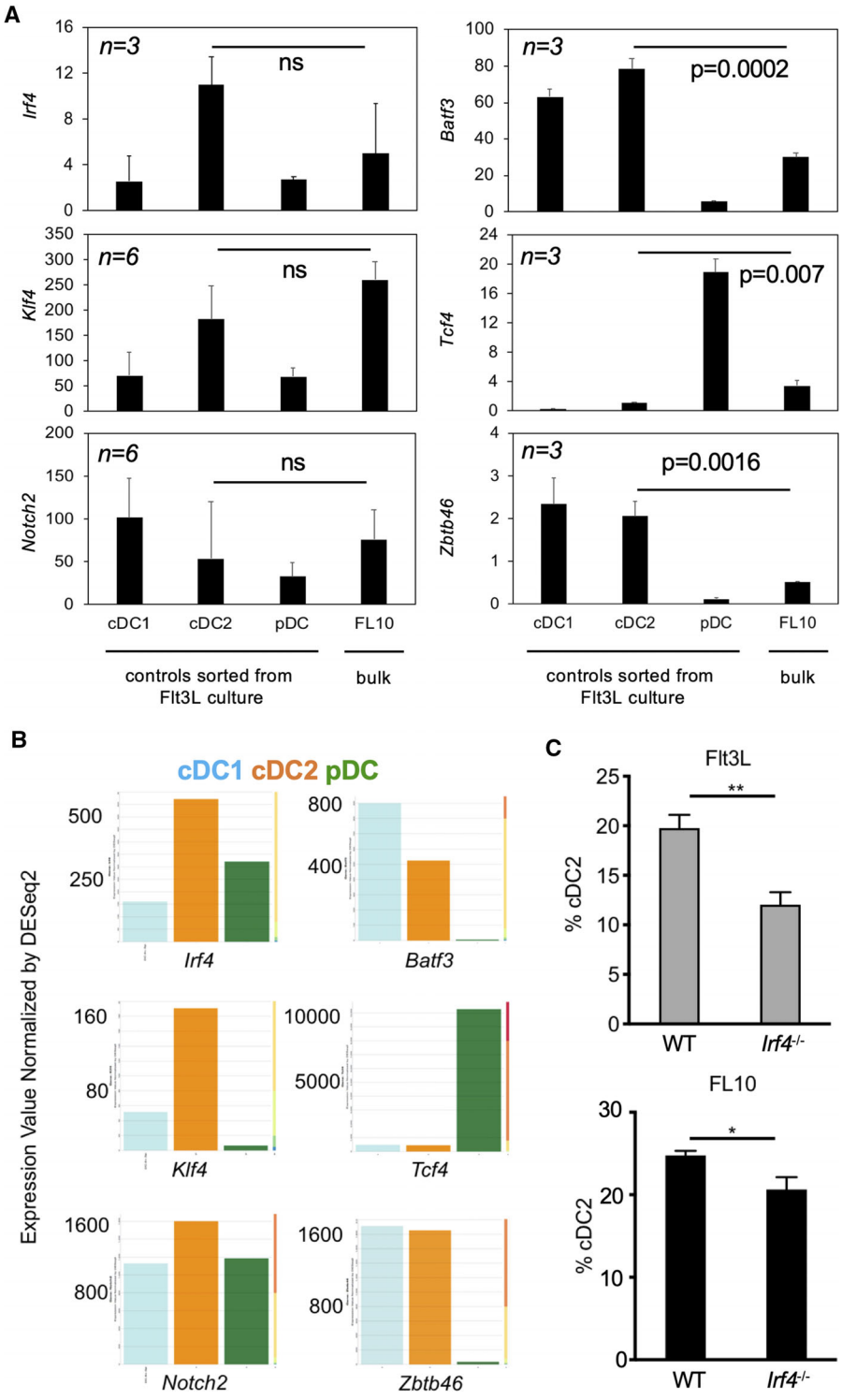

Figure 5. Transcription factor expression profiles of FL10 cultures and partial dependency on *Irf4* supports cDC2 generation. (A) FL10 cultures harvested at day 8. RNAs were isolated, and gene expression of the indicated transcription factors was quantified by RTqPCR. Control DC populations were generated accordingly by sorting cDC1, cDC2, and pDC from bulk Flt3L cultures. The transcript amounts of the transcription factors were normalized to *Gapdh* expression of the RNA sample. (B) RNA-seq data of the indicated DC subsets revealed from Gene Skyline (ImmGen.org). (C) FL10 cultures from wild-type (WT) and Irf4$^{-/-}$ mice were analyzed on day 8 by flow cytometry for their cDC2 frequencies. (A) and (C) show the results of three independent experiments. Statistics with the unpaired two-sided t-test. All data is shown as mean + SD. p-values comparing sorted cDC2 with cDC2 in FL10 cultures are indicated. $^{**}p < 0.01$, $^{*}p < 0.05$. cDC1 and cDC2, conventional dendritic cell subsets 1 and 2; FL10, combination of Flt3L LIF and IL-10; Flt3L, FMS-like tyrosine kinase-3 ligand; RTqPCR; real time quantitative polymerase chain reaction.

the serum-free additive Pannexin allow this cDC2 generation, and BM cells from *Irf4*$^{-/-}$ mice develop less efficiently into cDC2 than from their wild-type counterparts. They responded to LPS, Poly I:C, or CpG by maturation marker upregulation and proinflammatory cytokine production and they stimulated peptide-specific T cell proliferation to a similar extent as compared with DC cultures generated with Flt3L or GM-CSF while their generation of IFN-γ producing T cells was even superior. Together, our data indicate that LIF and IL-10 shift mixed cDC1/cDC2/pDC Flt3L cultures toward ESAM^{low} cDC2B generation in vitro.

The marker profile expressed by d8 FL10 cultures (B220^{-} CD11c^{+} Ly6C^{-} CD64^{-} XCR1^{-} CD103^{-} CD11b^{+} CD172a^{+}) clearly

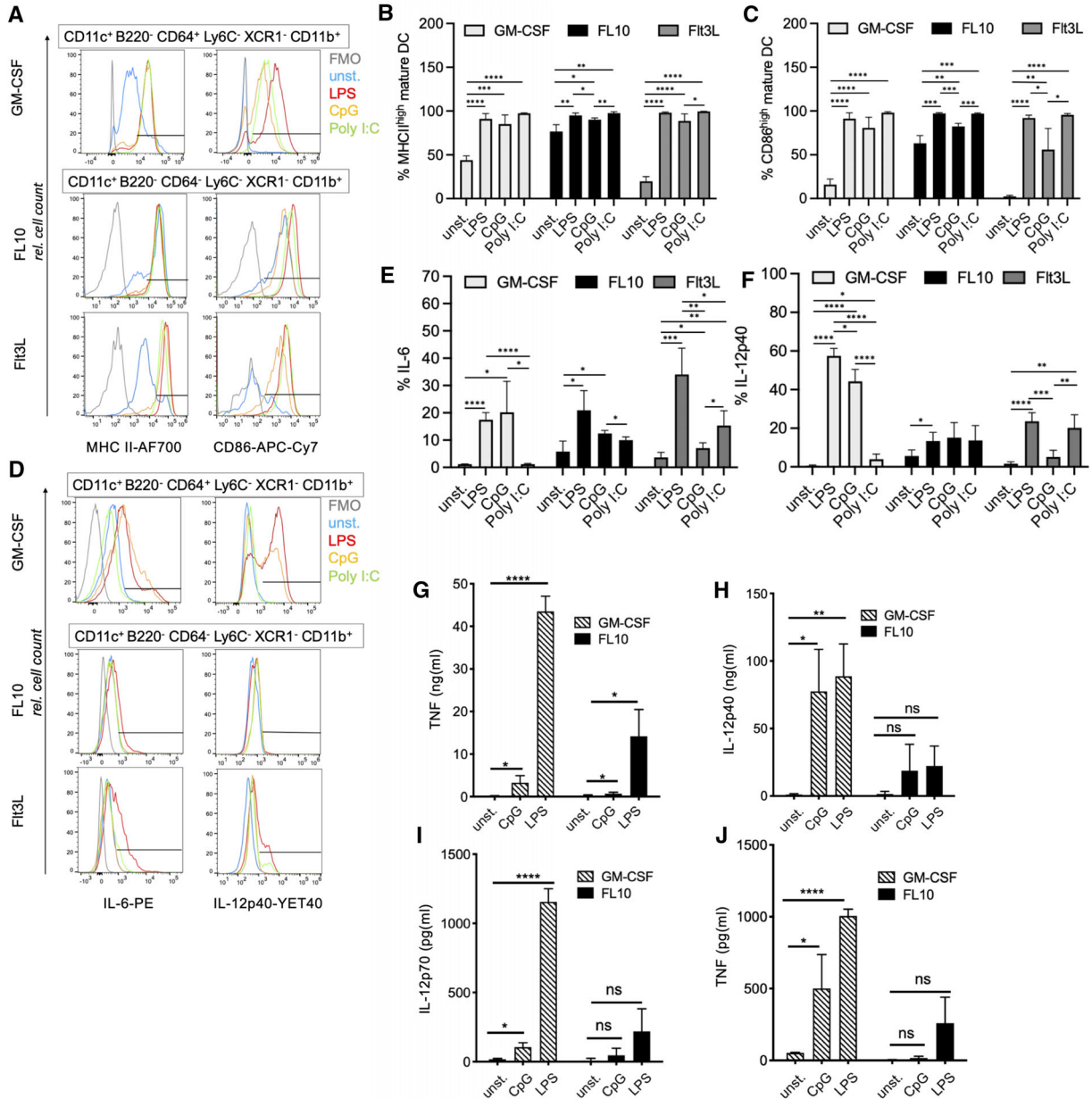

Figure 6. FL10 cultures can be matured as indicated by induction of surface markers and cytokine production. BM cells were cultured with GM-CSF, Flt3L, or FL10. On day 8 the cells were transferred to a 24-well plate at a density of 1×10^6 cells/ml and stimulated with Poly I:C, CpG, or LPS for 16 h in the absence of the initial growth factors. (A–F) Flow cytometry for surface or intracellular markers or cytokines or the Yet40 reporter for IL-12p40 was performed with subgating on the DC subsets as in Figure 1B, and positive staining was evaluated according to FMO controls. (A) and (D) represent staining examples and (B, C, E, F) the respective statistical evaluations as indicated. Data were derived from four cultures in two independent experiments. (G–H) The indicated cytokines in the culture supernatants were then measured by ELISA. The statistical evaluation of three independent experiments. All statistics were performed with the unpaired two-sided *t*-test shows the comparison of unstimulated with stimulated cells. $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, ns = not statistically significant. BM, bone marrow; cDC1 and cDC2, conventional dendritic cell subsets 1 and 2; ELISA, enzyme-linked immunosorbent assay; FL10, combination of Flt3L LIF and IL-10; Flt3L, FMS-like tyrosine kinase-3 ligand; GM-CSF, granulocyte-macrophage stimulating factor.

supports the cDC2 identity [2]. Especially, the CD172a/SIRPα expression supports the cDC2 generation since it is also a functional molecule required for the generation of cDC2 in mice [42]. cDC2 have been subdivided into ESAM^{high} and ESAM$^{low/negative}$ expressing subsets, and in the adult spleen, ESAM^{high} cDC2 represent the predominant subtype [8]. ESAM^{high} cDC2 have been further suggested to be derived from Runx3-dependent MDP or CDP, while only ESAM$^{low/negative}$ cDC2 is derived from CDP [13]. Our data revealed that the majority of cDC2 from FL10 cultures does express ESAM at low levels. However, in FL10 culture, MDP and CDP remained at similar proliferation rates at d3, as indicated by their Ki67 expression. Together with our finding that *Notch2*

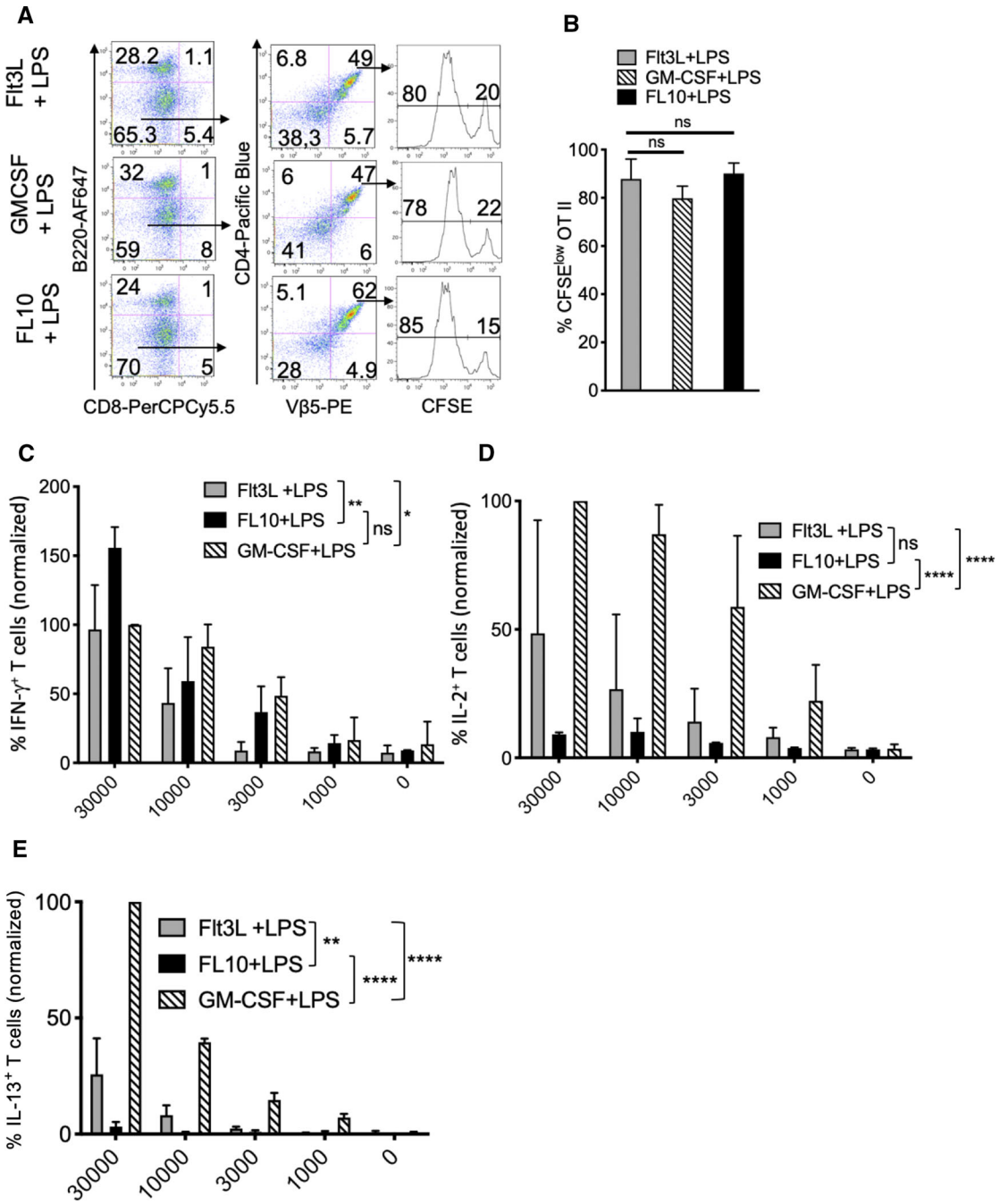

Figure 7. FL10 cultures show similar T-cell proliferation potential as compared with GM-CSF or Flt3L cultures but altered cytokine secretion profile. (A) BM cells were differentiated in the presence of Flt3L, FL10, or GM-CSF for day 8 and then activated with LPS for 16 h. Then 3×10^3 activated DC from each condition were incubated for another 5 days together with 2×10^7 CFSE labeled bulk OT-II cells and 1 mM $OVA_{323-339}$ peptide. Then flow cytometry analyses were performed as indicated in the representative data sets. (B) Statistical evaluation of three independent experiments as in (A), analyzed with unpaired two-sided *t*-test. ns = statistically insignificant. (C, D, E) After the activation of the OT-II $CD4^+$, T cells described under A, IFN-γ, IL-2, and IL-13 from the culture supernatants were measured via ELISA. Normalization was performed using the values with GM-CSF at 30,000 DC/well. For the statistical evaluation of three experiments, a two-way analysis of variance was used. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns = not statistically significant. BM, bone marrow; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; FL10, combination of Flt3L LIF and IL-10; Flt3L, FMS-like tyrosine kinase-3 ligand; GM-CSF, granulocyte-macrophage stimulating factor.

mRNA expression is rather low, but for *Klf4* relatively high, our culture method most likely generates the *Klf4*-dependent ESAM^{low} cDC2 subset.

The observed maintenance of MDP and CDP proliferation and loss of cMoP proliferation in FL10 cultures was similar to what could be observed in Flt3L cultures, indicating no general shift away from cDC generation, and the CDP sorting experiment indicated the specific cDC2 expansion from CDP in both FL10 and Flt3L cultures. However, we also observed an increased proportion of Annexin V$^+$ dead or dying cells in the cultures that were high among cDC1 and pDC and lower for cDC2, indicating a selective survival advantage of cDC2 under the FL10 culture conditions. Although the effect of LIF and IL-10 for MoDC and pDC generation and function have been directly addressed [43–48], there are fewer and less clear data available for their effect on Flt3L dependent cDC [49, 50]. The combination of Flt3L (also termed Flk2-ligand) with LIF has been described early to promote hematopoietic cell growth but without clearly specifying the developing cell types [49]. The cDC-restricted transcription factor *Zbtb46* has been found to downregulate LIF receptor expression on splenic CD4$^+$ cDC2 [39], and accordingly, LIF receptor expression of spleen DC was found to be restricted to pDC and absent on cDC [45]. Here, the FL10 cultures show relatively low Zbtb46 expression that may explain their responsiveness to LIF. LIF has been reported to suppress the maturation of murine GM-CSF generated BM-DC [43] and the development of pDC [45], which is in agreement with the reduced frequencies and increased apoptosis of pDC in FL10 cultures. IL-10 activity is generally considered to inhibit DC maturation and thereby promote tolerogenic DC functions, but mostly human, or mouse GM-CSF-dependent MoDC have been investigated [46, 51]. In a murine model of pulmonary tuberculosis, IL-10 inhibited mature CD11b$^+$ cDC2 function [52]. However here, IL-10 was added to Flt3L cultures during DC generation from progenitors and not during maturation. A higher rate of CDP proliferation and no increased apoptosis of cDC2 was observed in FL10 cultures as compared with Flt3L cultures and their maturation potential was similar in the absence of the initial growth factors Flt3L, LIF, and IL-10. Together, this indicates a cDC2 growth-promoting role of IL-10 in the FL10 cultures.

Experimental culture protocols to generate murine DC in vitro rely mostly on FCS-containing media. We have explored before that the quality of FCS batches dramatically can modify the MoDC generation in BM cultures with GM-CSF reaching form inhibition of DC generation to high yield but with complete spontaneous maturation [32]. Here we tested different FCS batches from different providers, and we found some concordance in cDC2 enrichment among them, while on batch and also the serum-free additive Pannexin still promoted cDC2 growth but revealed lower cDC2 frequencies. This indicates that similar to BM-derived GM-CSF MoDC cultures [53] and also successful FL10-dependent cDC2 generation depends on the appropriate selection of the FCS batch. The loss of cDC2 percentage by dialysis of a suitable batch of (always heat-inactivated) FCS indicates that undefined small and heat-resistant FCS components contribute to successful cDC2 generation. Recently, different levels of eicosanoids in FCS batches have been found to strongly influence the growth of the macrophage cell line U937 [54]. Thus, differences in the eicosanoid composition could contribute to DC growth in general and thus also investigated here.

DC maturation or activation by pathogens or their components is a hallmark of DC biology. Although all DC subsets generally share this function, there are differences in their expression of pattern recognition receptors which contributes to guide selective activation of individual DC subsets and thereby their potential to activate and polarize CD4$^+$ T cells into Th1, Th2, and Th17 [29]. Although cDC2 are generally considered to be prone to induction of Th2 responses as evidenced by in vivo infection studies, there is also evidence that rather pathogen-driven DC activation directs Th polarization in mice, and Th1, Th2, and Th17 responses can be induced by all DC subtypes [55, 56]. cDC2 have been demonstrated to raise strong Th1 responses against bacterial stimuli [56] and during *M. tuberculosis* infection in mice [52]. Spleen ESAM^{low} cDC2 (also termed cDC2B) have been shown to express TLR4 and TLR9 transcripts in contrast to ESAM^{high} cDC2 (also termed cDC2A) [9]. Consequently, FL10 stimulated ESAM^{low} cDC2B cultures were responsive to LPS, Poly I:C, and CpG. The upregulation of MHC II and CD86 was similar in all three DC culture conditions. While the frequency of IL-6 producing cDC2 was comparable to GM-CSF and Flt3L cultures after 16 h of stimulation, the frequency IL-12p40 was lower than from GM-CSF generated DC but still similar to Flt3L generated cDC2. When ELISA supernatants were measured the FL10 culture produced less of all cytokines tested than MoDC from GM-CSF cultures. These differences between the methods may indicate differences in the kinetics of cytokine release or additional release in the supernatants from contaminating cells and need further investigations.

Surprisingly, however, their potential to stimulate CD4$^+$ T cell proliferation was equal among all three DC culture conditions. For the DC-mediated induction of cytokine induction by T cells, the FL-10 generated and LPS-matured DC generated the highest frequency of IFN-γ producing CD4$^+$ T cells with low IL-2$^+$ and IL-13$^+$ T-cell frequencies. This indicates that FL-10-generated and LPS-matured ESAM^{high} cDC2 are highly efficient in Th1 polarization in vitro. This effect was clearly more pronounced than what could be observed with LPS-matured DC derived from Flt3L or GM-CSF cultures that showed a more unpolarized Th0 profile with high frequencies of CD4$^+$ T cells producing IL-2, IL-13, and IFN-γ. This may indicate that pathogen stimulation of ESAM^{low} cDC2 cultures alone is not sufficient to enable their cytokine release, but additional signals from T cells such as through CD40-CD154 interaction may be required [57, 58]. Alternatively, other signals than secretion of the Th1-polarizing cytokine IL-12, such as CD70 surface expression, may direct Th1 induction by the ESAM^{low} cDC2 [59].

In conclusion, our data provide strong evidence that the addition of LIF and IL-10 to Flt3L-dependent BM cultures promotes the selective growth and survival of ESAM^{low} cDC2B in vitro as indicated by their expression profiles of surface markers and transcription factors. This protocol does, however, not provide

a pure population of cDC2 since about half of the cells do not differentiate into $CD11c^+$ DC and many of them undergo apoptosis. The $ESAM^{low}$ cDC2B can be matured and, as a bulk population, show a strong potential to activate $CD4^+$ T cells in vitro. Further experiments will have to demonstrate whether LIF and IL-10 play similar roles for cDC2 generation in vivo and their human counterparts.

Materials and methods

Mice

If not stated otherwise, C57BL/6J wild-type mice, *Irf4*$^{-/-}$ mice, Yet40 reporter mice [60], kindly provided by Gottfried Alber and OT-II mice (F. Carbone, Melbourne) aged 6–12 weeks were used. Wild-type mice were initially obtained from Charles River, Sulzfeld, and bred in our facility under pathogen-free conditions.

Generation of DCs from bone marrow cells

For the generation of GM-CSF-derived BM-DCs, BM cells were seeded at a density of 3×10^6 per 10 cm Petri dish containing 10 ml R10 standard medium if not otherwise indicated consisting of RPMI 1640 medium (Sigma Lot RNBF8515) with 10% heat-inactivated FCS (Sigma LOT 045143270), 2 mM L-Glutamine, 100 U/ml Penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 10% culture supernatant from a murine GM-CSF transfected myeloma cell line at 37°C with 7% CO_2 as described in detail before [15]. For some experiments, standard FCS was used from CellGenix (Cat No: 20801-0500, Lot No: 1299E), Hyclone A (Cat No: SV30160.02, Lot No: RB35937), Hyclone B (Cat No: SV30143 Lot No: RVA35873), Sigma (Cat No: F7524 Lot No: 045143270), or the serum-free FCS substitute Pannexin 10% (Pan Biotech, Cat No: P04-96900, Lot No: 7000417). On day 3, a further 10 ml of R10 medium containing 10% GM-CSF culture supernatant was added to the plates. On day 6, the bone marrow cells were fed by carefully removing 10 ml of old R10 medium and adding 10 ml of fresh R10 medium with 10% GM-CSF culture supernatant. The loosely adherent and nonadherent cells were harvested and used on day 8.

For the generation of Flt3L- or FL10-derived BM-DCs, fresh BM cells were seeded at 3.5×10^6 cells/well in a six-well plate in 3.5 ml R10 media. Human Flt3L (200 ng/ml, Immunotools) was added alone or in addition with human LIF (10 ng/ml, Immunotools) or murine IL-10 (100 ng/ml, Immunotools), or in a triple combination of Flt3L, LIF, and IL-10 (termed FL10). After 5 days, 3.5 ml R10 medium with the same cytokine concentrations were added to the cells. In some experiments, murine Flt3L (200 ng/ml, Immunotools) was used. On day 8, the cells were harvested and used. For FCS dialysis, the tubes (exclusion size 20 kDa, Nadir) were filled with FCS and agitated in 1 l of PBS with 3 times changes every 4–6 h and then sterile filtered (0.45 μm).

Stimulation of DC

Bone marrow cultures were harvested on day 8 and transferred into a 24-well plate at a density of 1×10^6 cells/ml in the absence of the initial growth factors but the presence of LPS (0.1 μg/ml (for FACS) or 1 μg/ml (for ELISA), SIGMA) or virus-oriented phosphodiester cytidine-phosphate-guanosine oligodeoxynucleotides (CpG 1668, 1 μM 5′-TCCATGACGTTCCTGATG-3′, Sigma) or Poly I:C (50 μg/ml, Pharmacia) for 16 h. As a control, cells in their six-well plate remained untreated. The cells were then measured through flow cytometry and the supernatants in ELISA assays.

Preparation of single-cell suspensions from spleen and lymph nodes

Popliteal, inguinal, and cervical lymph nodes and spleens were isolated from mice under sterile conditions and transferred to 6 cm Petri dishes containing cold PBS. The organs were cut into pieces with scissors and digested for 20 min at room temperature with 1 mg/ml DNase I and 1 mg/ml collagenase IV. Single-cell suspensions were obtained by mixing spleen and lymph node cells with a syringe. The cell suspension was then filtered through a nylon cell sieve which was fixed on a 50 ml tube. The cells were then washed for 5 min at 1000 rpm and at room temperature. Erythrocyte lysis was carried out for the spleen cell suspension by resuspending the cell pellet in a 0.8% ammonium chloride buffer.

Flow cytometry and cell sorting

The following antibodies were used: Annexin V – FITC and AF647, B220 - Pacific Blue or AF647 clone RA3-6B2, CD11c – FITC or Pe-Cy7 clone N418, Ly6C – BV510 clone HK1.4, CD64 – PE clone X54-5/7.1, XCR1 – APC or APC-Cy7 clone ZET, CD11b – PerCP-Cy5.5 clone M1/70, CD172a – APC clone P84, ESAM – APC clone 1G8/ESAM, CD3 – FITC clone 145-2C11, NK1.1 – FITC clone PK136, Ly6G – FITC clone 1A8, CD19 – FITC clone 1D3/CD19, CD117 – Pe-Cy7 clone 2B8, CD115 – AF647 clone AFS98, CD135 – BV421 clone A2F10, Ki67 – PerCP-Cy5.5 clone 16A8, MHC II – AF700 clone M5/114.15.2, CD86 – APC-Cy7 clone GL-1, IL-6 – PE clone MP5-20F3, CD4 – Pacific Blue clone GK1.5, CD45.1 – AF488 clone A20, CD8 – PerCP-Cy5.5 clone 53–6.7 (all Biolegend). Vβ5 – PE clone MR9-4, CD103 – FITC clone M290 (both BD). The cells were stained in flow cytometry buffer consisting of 0.1% bovine serum albumin, 0.1% NaN_3 in PBS. 5×10^5 cells were stained in 50 μl with antibodies against surface markers for 30 min at 4°C in the dark. To remove unbound antibodies, the cells were washed once by centrifugation for 5 min at 1200 rpm and 4°C with 400 μl of flow cytometry buffer. The samples were stored in 100 μl 1% formaldehyde. Intranuclear staining of Ki67 or intracellular cytokine staining was performed following the manufacturer's protocol for the Anti-Mouse Foxp3 Staining Set (eBiosciences). Flow cytometry was performed with a FACS

LSR II (Becton Dickinson) or Attune NxT (ThermoFisher). The data obtained were analyzed with FlowJo software. Cell sorting was performed with an ARIA III (Becton Dickinson). The methods used adhered to published guidelines [61].

Real time quantitative polymerase chain reaction (RTqPCR)

The cellular RNAs were isolated using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek) according to the manufacturer's instructions. The RNA amounts were measured by spectroscopy. For RTqPCRs the following primers and hydrolyzation probes were used: GAPDHs: GCGAGATCCCTCCAAAATCA, GAPDHa: ATGGTTCACACCCATGACGA, GAPDHprobe: [6FAM]GGAGCCAAAAGGGTCATCAT[BHQ1], Batf3s: AGAAGGCTGACAAGCTCCAC, Batf3a: CCTTCAGCTTCGAAATCTCC, Batf3probe: [6FAM]AGCACGAGAGCCTGGAGCA[BHQ1], Notch2s: AACCTGTGGGACAGGATGC, Notch2a: TCACTTGTCCCAGAACCAATC Notch2probe: Universal ProbeLibrary #110 (Roche); IRF4s: AGCACCTTATGGCTCTCTGC, IRF4a: TGACTGGTCAGGGGCATAAT, IRF4probe: [6FAM]ATGCCACCCCATGACAGG[BHQ1], Klf4s:-CGGGAAGGGAGAAGACACT, Klf4a: GAGTTCCTCACGCCAACG, Klf4probe: Universal ProbeLibrary #62 (Roche), Tcf4s: CATATTTGTGGCCATTGAAGG, Tcf4a: CAGCTCTTTGTCCGTCCCTA, Tcf4probe: [6FAM]AAAATGCATCACCAACAGCGAAT-[BHQ1], Zbtb46s: AGAGAGCACATGAAGCGACA, Zbtb46a: TGCACACTTTGCACACGTACT, Zbtb46probe: [6FAM]CCACAGCAAGGACAAGAAGTACG[BHQ1]. The RNA process control kit (ROCHE, Germany) was used for cDNA synthesis and amplification. The PCR efficiency was determined with RNA dilutions rows and the absolute amounts with a synthesized control.

Enzyme-linked immunosorbent assay

DC culture supernatants were used in ELISA kits for IL-12p40 (Biolegend), IL-12p70 (BD), IL-6 (Biolegend), TNFα (Biolegend), IL-13 (R&D), IL-2 (R&D), IFN-γ (Biolegend). They were analyzed for cytokine content according to the manufacturer's protocols. The culture supernatants were centrifuged at 14,000*g* for 10 min and 4°C and stored at −20°C until they were used for ELISAs. The absorbance for all ELISAs was measured at 450 nm with a SpectraMax Plus microplate reader (Molecular Devices) and analyzed by SoftMax® Pro 6.1 software (Molecular Devices).

T-cell assays

FL10 cultures on day 8 were activated with LPS (1 μg/ml) and loaded with 1 μM $OVA_{323\text{-}339}$ peptide (China Peptides) for 16 h. As a source of $CD4^+$ OT-II cells, total bulk lymph node and spleen preparations were labeled with CFSE (5 μM, 10 min, RT, Invitrogen) and added at 2×10^5/well (96-well plate, flat bottom) to titrated amounts of DC. After 5 days, the proliferation was measured by flow cytometry.

Statistical analysis

Statistical analyzes were performed using Prism 6.0 software (GraphPad Prism). The unpaired, two-tailed Student's *t*-test was used when records from two independent groups were normally distributed. Statistical analysis was generated using two-way analysis of variance (ANOVA), in which the influence of two different categorical independent variables on one continuous dependent variable was examined. *p* values below 0.05 ($^*p < 0.05$) were considered significant.

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Author Contributions: LC, JS, and LK performed the exptoeriments and analyzed the data, HR and ML provided the *Irf4*$^{-/-}$ mice, HR, ML, HH, JB, AK, and MBL designed the experiments and supervised the data analyses, JB and MBL wrote the manuscript.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abbreviations: **BM**: bone marrow · **cDC1 and cDC2**: conventional dendritic cell subsets 1 and 2 · **CDP**: common dendritic cell progenitors · **cMoP**: common monocyte progenitor · **DC**: dendritic cells · **FL10**: combination of Flt3L LIF and IL-10 · **Flt3L**: FMS-like tyrosine kinase-3 ligand · **GM-CSF**: granulocyte-macrophage stimulating factor · **IL-10**: interleukin-10 · **LIF**: leukemia inhibitory factor · **MDP**: monocyte dendritic cell progenitors · **pDC**: plasmacytoid DC

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