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CUTTING EDGE

Cutting Edge: Generation of Splenic CD8$^+$ and CD8$^-$ Dendritic Cell Equivalents in Fms-Like Tyrosine Kinase 3 Ligand Bone Marrow Cultures[1]

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We demonstrate that functional and phenotypic equivalents of mouse splenic CD8$^+$ and CD8$^-$ conventional dendritic cell (cDC) subsets can be generated in vitro when bone marrow is cultured with fms-like tyrosine kinase 3 (flt3) ligand. In addition to CD45RA^{high} plasmacytoid DC, two distinct CD24^{high} and CD11b^{high} cDC subsets were present, and these subsets showed equivalent properties to splenic CD8$^+$ and CD8$^-$ cDC, respectively, in the following: 1) surface expression of CD11b, CD24, and signal regulatory protein-α; 2) developmental dependence on, and mRNA expression of, IFN regulatory factor-8; 3) mRNA expression of TLRs and chemokine receptors; 4) production of IL-12 p40/70, IFN-α, MIP-1α, and RANTES in response to TLR ligands; 5) expression of cystatin C; and 6) cross-presentation of exogenous Ag to CD8 T cells. Furthermore, despite lacking surface CD8 expression, the CD24^{high} subset contained CD8 mRNA and up-regulated surface expression when transferred into mice. This culture system allows access to bona fide counterparts of the splenic DC subsets. The Journal of Immunology,* 2005, 174: 6592–6597.

The delineation of dendritic cell (DC)[4] subsets has highlighted their unique functional specializations, which play important roles in the control of immune responses (1). Three functionally distinct subsets can be defined in steady-state mouse spleen and include the plasmacytoid pre-DC (pDC), CD8$^+$ and CD8$^-$ conventional DC (cDC) subsets, which appear to be distinct and not precursor-product related in the steady-state (2–4). The diminutive numbers of DC, and difficulty in their isolation, has often precluded study of their development and function. For example, at most 1×10^6 CD8$^+$ cDC can be recovered with high purity from one mouse spleen after an elaborate purification protocol (5). A culture method for generating higher yields of these subtypes would make them more accessible.

There are several well-established procedures for generating DC in culture from bone marrow (BM) precursors or from blood monocytes using GM-CSF and IL-4 (GM/IL-4 DC) (6). However, GM/IL-4 DC do not seem to show the heterogeneity in DC phenotype and function found with splenic DC. In fact, it is not clear whether GM/IL-4 DC have any counterparts among steady-state DC in vivo. In relation to this, a recent study found the development of steady-state DC was Stat3 dependent, whereas GM/IL-4 DC development from monocytes was Stat3 independent, suggesting that these two groups of DC have distinct developmental origins (7).

Culture of BM with fms-like tyrosine kinase 3 (flt3) ligand (FL) is a more recent method that allows the generation of both cDC and pDC in large numbers (now referred to as FL-DC) (8–10). The dependency on FL for DC generation in this culture system correlates with the observations that FL$^{-/-}$ mice have reduced DC numbers (11), that injection of mice with FL greatly increases DC numbers (12, 13), and that only early BM progenitors that express flt3 are effective precursors of DC (14). Some initial observations suggested that FL-cDC may further divide into subsets, but their relationship to splenic DC subsets was not further investigated (10, 15).

In this study, we show that despite the presence of some CD11b on all cDC from FL cultures, and the absence of surface CD4 and CD8 expression, FL-DC could be clearly segregated by surface markers into the equivalents of steady-state splenic pDC, CD8$^+$ cDC, and CD8$^-$ cDC. The shared properties between the FL-DC subsets and the splenic DC subset counterparts included surface marker expression, transcription factor expression and dependence for development, ability to cross-present cellular Ag to CD8 T cells, expression of TLR and

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[4] Abbreviations used in this paper: DC, dendritic cell; pDC, plasmacytoid pre-DC; cDC, conventional DC; BM, bone marrow; GM/IL-4 DC, GM-CSF and IL-4 DC; FL, fms-like tyrosine kinase 3 (flt3) ligand; MHC I, MHC class I; MHC II, MHC class II; IRF, IFN regulatory factor; CyC, cystatin C; SIRP, signal regulatory protein; WT, wild type.

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chemokine receptors, and production of cytokines and chemokines in response to TLR stimulation. This system should allow access to large numbers of the DC subsets for further study. In particular, up to 25×10^6 of the $CD8^+$ cDC equivalents can be generated from culturing the BM of one mouse with FL.

Materials and Methods

Mice

C57BL/6J (Ly5.2), C57BL/6 Pep^{3b} CD45.1 (Ly5.1) OT-I, OT-II, bm1, and IRF-$8^{-/-}$ mice (from I. Horak (Free University of Berlin, Berlin, Germany) and A. Kallies (Walter and Eliza Hall Institute)) were bred under specific pathogen-free conditions and used at 7–10 wk of age.

FL-DC preparation

FL-DC were generated as described (8, 10, 16), with some modifications. BM was extracted, and red cells were removed by a 30-s exposure to 0.168 M NH_4Cl and washed three times. Cells were cultured at $1.5–3 \times 10^6$ cells/ml in modified mouse osmolarity RPMI 1640 culture medium (DC medium) (17) containing 300 ng/ml murine FL (generated in-house), for 8–10 days at 37°C in 10% CO_2. Four-color staining with mAbs to CD11c, CD45RA, CD24, and CD11b was used to separate the DC subsets.

Splenic DC preparation

Recovery of splenic DC was performed as previously described (2, 5). Briefly, spleens were digested with DNase and collagenase, and light-density cells were collected after centrifugation in 1.077 g/cm^3 Nycodenz. Cells were incubated with rat mAbs against mouse CD19 (ID3), CD3 (KT31.1), Thy1.1 (T24/31.7), TER-119, and Ly6G (1A8), and non-DC depleted using anti-rat Ig magnetic beads (Qiagen).

Flow cytometry

DC were stained with varying combinations of mAbs to CD11c (N418-A594 or allophycocyanin), CD45RA (14.8-PE), CD11b (M1/70-Cy5), CD24 (M1/69-FITC or biotin), CD4 (GK1.5-allophycocyanin), CD8 (YTS169.4-FITC, PE, or allophycocyanin), signal regulatory protein (SIRP)-α (p84-biotin) and Ly5.2 (S450-15.2-FITC), with second-stage staining with streptavidin-PerCP-Cy5.5 or streptavidin-PE. Cell sorting and analysis was performed on a FACSVantage^{SE} DiVa, FACStar^{Plus}, LSR (BD Biosciences), or MoFlo (DakoCytomation) instruments.

In vivo FL-DC transfers

Between 0.4 and 2×10^6 sorted FL-DC subsets from Ly5.2 BM cultures were injected i.v. into nonirradiated Ly5.1 mice. Three days later, spleens were harvested and enriched for DC before flow cytometric analysis.

Ag presentation assays

OVA-specific CD8 (OT-I) or CD4 (OT-II) T cells were obtained from lymph nodes by depletion of non-CD8 or non-CD4 T cells, respectively, and CFSE labeled, as described elsewhere (18, 19). For T cell activation, 5×10^3 sorted DC were incubated with a titration of either SIINFEKL (MHC class I (MHC I)) peptide for 45 min at 37°C and then washed three times, or a titration of $OVA_{323–339}$ (MHC class II (MHC II)) peptide (Auspep) present during the duration of the experiment. For cross-presentation assays, 2.5×10^4 sorted DC were cultured with varying numbers of gamma-irradiated bm1 splenocytes that were previously coated with 10 mg/ml OVA protein and washed twice. For all cultures, 5×10^4 T cells were added and cultured in DC medium for 60 h with 10 ng/ml GM-CSF (PeproTech). At this time, cells were stained for Vα2-PE and CD8-allophycocyanin or CD4-allophycocyanin, and PE^+allophycocyanin$^+$ $CFSE^{low}$ cells were quantitated by flow cytometry.

Western blot analysis

Western blot of cell lysates was performed as described (20). Actin and cystatin C (CyC) were detected using anti-actin (Sigma-Aldrich) and anti-human CyC (DakoCytomation) rabbit sera.

RT-PCR

RT-PCR was performed on the purified DC subsets using primers for IFN regulatory factor (IRF)-4, IRF-8, CD4, and CD8. Real-time PCR for TLRs and chemokine receptors was performed using the QuantiTect SYBR Green PCR kit (Qiagen). Primer sequences and PCR conditions are available on demand.

DC activation

Sorted DC (1×10^5) were cultured in duplicate, either in 200 μl of DC medium alone or with 1 μg/ml R848 (Invitrogen Life Technologies), 1 μM CpG 2216 (Geneworks), or 1 μg/ml LPS (Sigma-Aldrich) for 24 h. For IL-12 p40/p70 and IFN-α production, DC were cultured in DC medium with 100 ng/ml GM-CSF, 20 ng/ml IL-4, and 20 ng/ml IFN-γ, with or without 0.5 μM CpG 2216, for 48 h.

ELISA

ELISAs were performed as previously described (21) with capture mAbs to IL-12 p40/p70 (C16.6 or R29A5), IL-6 (MP5-20F3), RANTES (53433), MIP1α (39624.11), or IFN-α (RMMA-1), and detection via biotinylated mAbs to IL12 p40/p70 (C17.8), IL-6 (M35-32C11), RANTES (R&D Systems), MIP-1α (R&D Systems), or polyclonal rabbit Ab to IFN-α (PBL).

Results and Discussion

Phenotypic characterization of FL-DC and splenic DC subsets

A total of $\sim 5 \times 10^6$ DC is usually isolated from one mouse spleen, whereas $\sim 50 \times 10^6$ DC could be generated from culturing the BM of one mouse with FL (Fig. 1*A*). To assess phenotype, DC derived from FL-stimulated BM cultures were compared in surface Ag expression to freshly isolated splenic DC. Both FL culture-derived and splenic $CD11c^+$ DC contained distinct $CD45RA^-$ cDC and $CD45RA^+$ pDC populations (Fig. 1*A*), as others have noted previously (8, 9, 16). However, because the cDC component had been shown to express both surface CD11b and CD205 (8), but not CD4 or CD8, the markers normally used to segregate the splenic $CD8^+$ and $CD8^-$ cDC subtypes were not applicable for FL-cDC subset discrimination (1, 17) (Fig. 1).

To assess whether FL-cDC nevertheless contained subsets, we analyzed for other surface markers differentially expressed by spleen cDC. These included CD24, a surface molecule selectively expressed by $CD8^+$ cDC (2, 5, 22), and SIRP-α, recently identified by microarrays as being selectively expressed by $CD8^-$ cDC (M. Lahoud, A. Proietto, and K. Shortman, manuscript in preparation) and differentially expressed among rat

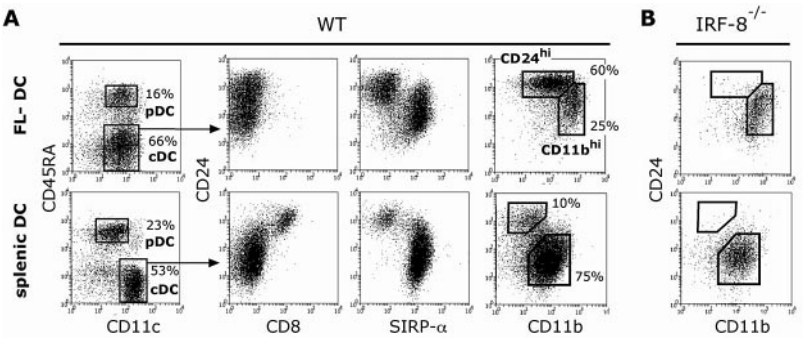

FIGURE 1. Surface markers on FL and spleen DC subsets in WT and IRF$8^{-/-}$ mice. *A*, FL cultures (which yielded 50×10^6 DC per mouse BM) and splenic DC (5×10^6 DC recovered per mouse spleen) from WT mice were stained with the indicated combinations of surface markers and analyzed by flow cytometry. *B*, IRF$8^{-/-}$ FL-DC and splenic DC were stained for similar surface markers. Analysis is representative of two to three individual experiments.

DC (23). FL-cDC expressed these markers, as well as CD11b, at different levels, which allowed their further division into two subtypes: $CD24^{high}SIRP\text{-}\alpha^{low}CD11b^{low}$ and $CD24^{low}SIRP\text{-}\alpha^{high}CD11b^{high}$ (Fig. 1*A*, *top panels*). We found that four-color staining for CD11c, CD45RA, CD11b, and CD24 was the ideal combination for discrimination of the three FL-DC subtypes.

An almost identical separation of subsets using the same combination of markers was found with splenic DC, but with differences in the relative proportions of each (Fig. 1*A*, *lower panels*). In particular, there was a larger proportion of $CD24^{high}$ cells among the FL-DC, the putative $CD8^+$ cDC equivalent. Also, whereas the splenic cDC subsets are selective in their surface expression of CD11b and CD205 in the steady state (5), all FL-cDC expressed some levels of each (Fig. 1*A*) (8, 9). This is not surprising considering that all splenic cDC up-regulate CD11b and CD205 upon in vitro culture (17). In view of these similarities in aspects of surface phenotype, we tested whether the FL-DC and splenic DC populations were developmentally and functionally equivalent. The terminology used hereafter for the three subsets is pDC, $CD24^{high}$ DC ($CD8^+$ cDC equivalents), and $CD11b^{high}$ DC ($CD8^-$ cDC equivalents).

DC subset differences in IRF-8$^{-/-}$ mice

IRF-8 transcription factor knockout mice retain splenic $CD8^-$ cDC but have reduced numbers of pDC and $CD8^+$ cDC (Fig. 1*B*, *lower panel*) (24). To assess whether a related defect occurred within FL-DC, we cultured BM from IRF-$8^{-/-}$ and wild-type (WT) mice with FL and compared the DC produced. Among the $IRF8^{-/-}$ FL-DC, there was comparable production of $CD11b^{high}$ DC but greatly reduced numbers of pDC and $CD24^{high}$ DC compared with WT FL-DC (Fig. 1*B*, *top panel*, and our unpublished data). Interestingly, a converse deficit in DC subset production has been reported using IRF-$4^{-/-}$ mice, which have an absence of $CD4^+$ DC in vivo and an absence of $CD11b^{high}$ DC in FL BM cultures (15).

IRF expression

Not only do the different spleen DC subsets require particular IRFs for their normal development, they also vary in their expression of these transcription factors in the steady state: the $CD8^+$ DC mainly express IRF-8, $CD8^-$ DC mainly express IRF-4, whereas pDC express both (15, 25). The FL-DC subsets were examined for IRF-4 and IRF-8 mRNA. Indeed, the expression pattern of IRFs in FL-DC subsets correlated with those in splenic DC (Fig. 2*A*), in line with previous studies (15).

Expression of CD4 and CD8

Even though unstimulated FL-DC do not express surface CD4 or CD8α, unlike their putative in vivo counterparts, mRNA expression of these molecules was tested. $CD24^{high}$ DC and pDC did express some CD8 transcript, whereas $CD11b^{high}$ DC did not. Only pDC expressed CD4 transcript (Fig. 2*A*). To see whether surface expression might be dependent on the normal in vivo DC environment, we transferred each purified FL-DC subset in vivo. After 3 days, we compared host and donor-derived splenic DC for surface CD4 and CD8 expression (Fig. 2*B*). Almost 75% of the recovered $CD24^{high}$ DC had up-regulated CD8, but very few expressed CD4. Transferred FL-pDC up-regulated both CD4 and CD8, so they then resembled their in vivo counterparts (2). However, transferred $CD11b^{high}$ DC up-regulated very little surface CD4 or CD8 in this time (15).

Ag presentation and cross-presentation

To assess the capacity of the cDC subsets to activate naive T cells, DC were coated with synthetic OVA peptides for MHC I or II, and then incubated with OT-I CD8 T cells or OT-II CD4 T cells, respectively. FL-DC showed comparable efficiency to their splenic DC counterparts in stimulating the proliferation of naive CD8 (Fig. 3*A*) and CD4 T cells (*B*). Cross-presentation, a process whereby exogenous Ags are taken up and presented via MHC I, is conducted efficiently by splenic $CD8^+$ cDC but not $CD8^-$ DC nor pDC (19, 26). To examine whether any of the FL-cDC populations were able to cross-present, we incubated each subset with OT-I cells and cellular Ag in the form of OVA protein-coated gamma-irradiated splenocytes from bm1 mice (unable to present OVA to OT-I cells). Of the FL-DC, only the $CD24^{high}$ subset was able to cross-present with efficiency similar to splenic $CD8^+$ cDC (Fig. 3*C*).

CyC expression

In the steady state, the splenic $CD8^+$ cDC are unique among the DC subsets in their expression of the cysteine protease inhibitor CyC (20), although the precise role of CyC in $CD8^+$ cDC biology is still controversial (20, 27). We found that only $CD24^{high}$ FL-DC expressed significant levels of CyC (Fig. 3*D*).

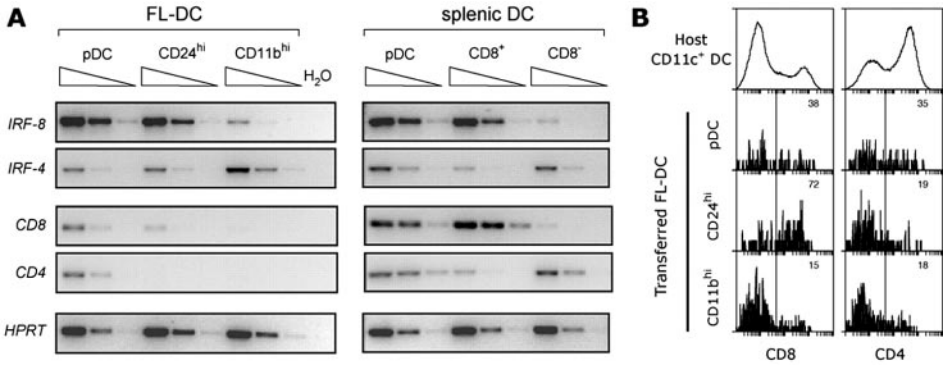

FIGURE 2. IRF, CD4, and CD8α expression. *A*, IRF-4 and IRF-8, CD4 and CD8 mRNA expression levels were detected by semiquantitative PCR among the FL and spleen DC subsets. *B*, Ly5.2 pDC, $CD24^{high}$ and $CD11b^{high}$ FL-DC were sorted to high purity and injected i.v. into nonirradiated Ly5.1 recipients. Three days later, recipient splenic DC were enriched and assessed for indicated markers on both host and donor-derived DC. Results are representative of two to three independent experiments.

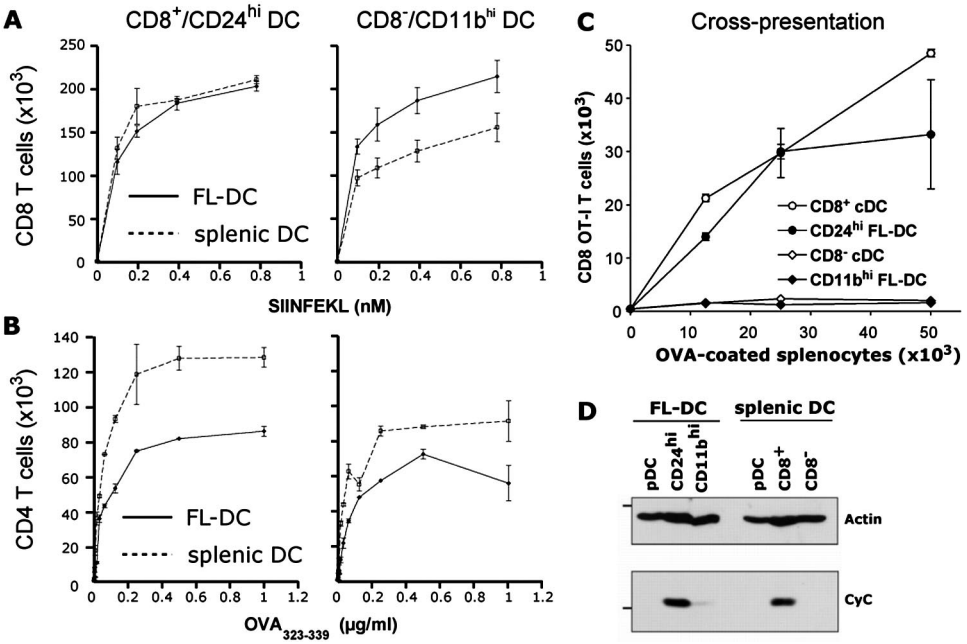

FIGURE 3. T cell activation, cross-presentation and CyC expression. A total of 5×10^3 sorted FL-DC or splenic DC subsets were incubated in duplicate with varying concentrations of peptide SIINFEKL for 45 min, and then washed (*A*), or $OVA_{323–339}$ peptide, before the addition of 5×10^4 CFSE-labeled OT-I or OT-II T cells (*B*). *C*, Varying numbers of OVA protein-coated bm1 splenocytes were incubated with 2.5×10^4 sorted DC subsets and 5×10^4 OT-I. Divided OT-I cells were quantitated after 60 h in each case. *D*, Equal numbers of each DC subset were sorted, and a Western blot was performed against CyC and actin. Results are representatives of two to three individual experiments.

TLR expression

The splenic DC subsets have unique TLR expression patterns, which enable them to directly respond to particular TLR ligands (28, 29). Splenic pDC express TLR7 and -9 but not TLR3 or -4; $CD8^+$ cDC express TLR3, -4, and -9 but not TLR7; $CD8^-$ cDC express TLR4, -7, and -9, but not TLR3. Using real-time PCR, an almost identical pattern of TLR mRNA expression was found between the FL-DC and splenic DC subset equivalents (Fig. 4*A*).

Chemokine receptor expression

The spleen DC subsets show quantitative differences in chemokine receptor expression, with pDC being the highest expressors of CXCR3 and CCR9, and $CD8^-$ cDC the highest expressors of CCR6 and CX3CR1, whereas $CD8^+$ cDC express some CXCR3 and CX3CR1 (29). We found comparable mRNA expression of most of these receptors between the FL-DC and splenic DC (Fig. 4*B*). One striking difference was the lack of CCR6 expression in $CD11b^{high}$ FL-DC compared with splenic $CD8^-$ cDC, which suggests an in vivo factor may be required for its normal expression.

Cytokine and chemokine production

A major functional difference between the splenic DC subsets is their capacity to produce particular cytokines. Cytokine production in response to TLR engagement depends on the TLR expression pattern of the DC subset, the TLR ligand, and on other factors such as the cytokine environment. Moreover, the splenic DC subsets differ in the cytokines they produce to the same TLR ligand (21, 29). For example, only pDC and $CD8^-$

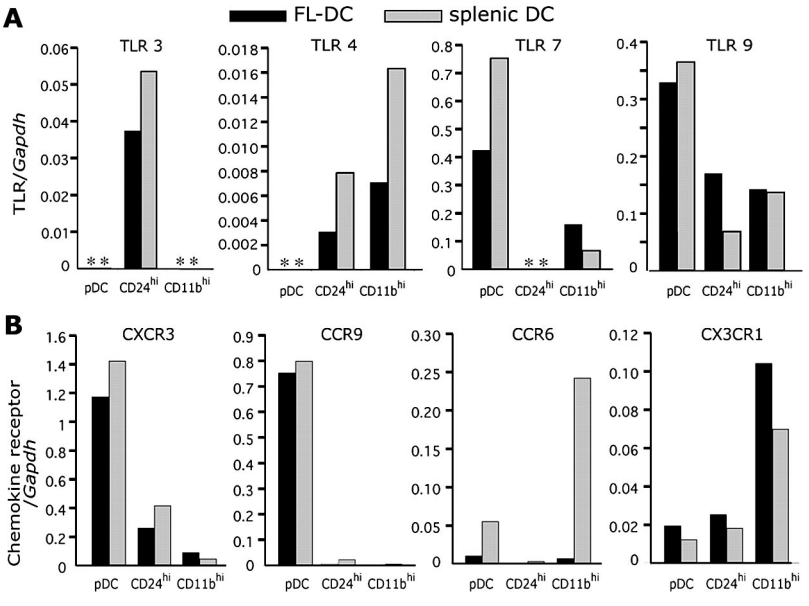

FIGURE 4. TLR and chemokine receptor expression. Real-time PCR was performed on sorted pDC, $CD24^{high}$ DC, or $CD11b^{high}$ DC for TLRs (*A*) and chemokine receptors (*B*). All values are expressed relative to GAPDH with asterisk (*) indicating levels below detection. Data are representative of two independent experiments.

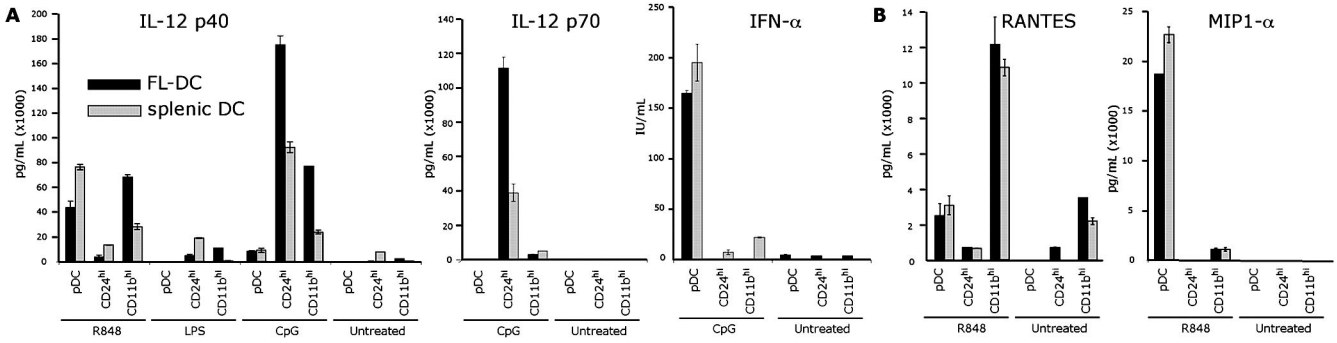

FIGURE 5. Cytokine and chemokine production. Sorted DC (1×10^5), as per Fig. 4, were cultured in duplicate with or without the indicated TLR agonists for 48 h in medium containing GM-CSF, IL-4, and IFN-γ (*A*), or 24 h in complete medium (*B*). Supernatants were analyzed by ELISA for the indicated cytokines and are representative of two to five independent experiments.

cDC express TLR7 and produce cytokines in response to the TLR7 ligand, R848. In contrast, all splenic DC express TLR9, but when stimulated with the TLR9 ligand, CpG DNA, pDC produce the highest levels of IFN-α, whereas $CD8^+$ cDC produce the highest levels of IL-12 p70. FL-DC subsets were compared with their splenic DC counterparts for cytokine production, in response to R848, CpG 2216, or LPS, in conditions known to induce cytokine production, and the supernatants were analyzed by ELISA. The patterns of cytokine production by the FL-DC subsets reflected their splenic DC equivalents (Fig. 5*A*).

Chemokine production in response to the TLR7 ligand R848 was comparable between FL-DC and splenic DC (Fig. 5*B*). In particular, $CD11b^{high}$ DC were the major producer of RANTES, whereas pDC were the major producers of MIP-1α. The $CD24^{high}$ DC subsets showed negligible chemokine or IL production in response to R848, correlating to their lack of TLR7 expression.

Conclusions

We have shown that FL BM cultures produce DC subsets that, despite differences in CD4 and CD8 expression, are close equivalents of the steady-state splenic pDC, $CD8^+$ cDC, and $CD8^-$ cDC subtypes. Importantly, key functional differences are conserved between the FL-DC and spleen DC equivalents. The major novel finding in this study is the generation of a functionally distinct spleen $CD8^+$ cDC equivalent, and a strategy for its isolation in high yield. We thus propose that, in contrast to GM/IL-4 DC, FL cultures better facilitate the study of steady-state DC subset ontogeny and function. Finally, FL-stimulated cultures may be useful for identifying and producing human DC counterparts to the murine DC subsets.

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Disclosures

The authors have no financial conflict of interest.

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