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Fibroblasts feeder niche and Flt3 Ligand as a novel inducer of plasmacytoid dendritic cells development in vitro

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ABSTRACT

Plasmacytoid dendritic cell (pDC), plays central role in antiviral immunity. The aim of this study was to assess the effect of Flt3 ligand (FL) alone or with L929 fibroblast feeder or L929 conditioned media on differentiation of mouse bone marrow (BM) cells into pDC in vitro. Murine BM cells were cultured with FL or with L929 or conditioned media for 9 days. The differentiated cells were analyzed using flow cytometry for PDCA-1, B220 and CXCR4. The relative expression of Stat3, CXCR4, CXCR7, IFN-β, TGF-β and Runx2 in differentiated cells determined by real time PCR. The development of pDC showed up to 19% increase after co-culture of BM cells with fibroblast feeder. Upregulation of Stat3, Runx2 and CXCR4 due to the presence of fibroblast feeder with FL in culture results in improved pDC development. Furthermore, 30% L929 supernatant along with Flt3 ligand was able to derive pDC up to 8.9% in comparison with FL alone, which was 6.6% in vitro. Thus, for the first time we introduced L929 fibroblast feeder as a niche producer of M-CSF and probably other growth factors and chemokines, which promotes the development of pDC in vitro along with FL, similar to in vivo niche.

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1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that modulate the outcome of immune response toward immunity or tolerance. There are a large variety of DC subsets according to surface phenotype, function, and tissue distribution [1]. DC can be classified into 2 classes: classical or conventional DC (cDC) and plasmacytoid DC (pDC), but the plasticity and origin of them remains unclear [2]. pDC or Type 1 interferon-(α, β, ω)-producing cells (IPCs) are a haematopoietic cell population with a characteristic plasma cell-like morphology found in many tissues in mouse, including blood, thymus, bone marrow, liver, and the T-cell areas of lymphoid organs [3,4]. Due to the critical role of pDC in immunity against viral infections, autoimmunity and tolerance, they have recently been the focus of attention. IPCs selectively express toll-like receptors (TLR-7 and TLR9), and quickly release high levels of type 1 interferon in contact with viral stimulation [5]. pDC can be directly identified in lymphoid and non-lymphoid organs by the specific expression of mPDCA-1 (murine plasmacytoid dendritic cell antigen-1) [6]. PDC have been recently considered as immunomodulating cells with a potential to induce tolerance [7]. PDC also function as tolerogenic cells by expressing indoleamine 2,3-dioxygenase (IDO), inducible costimulator ligand (ICOS-L), and/or programmed death 1 ligand (PD-L1), which can provoke the development of regulatory T-cells (Treg) and suppression of self- and alloreactive cells [1]. Hereby, we propose a method to study pDC development from bone marrow (BM) cultures. However, precise regulation mechanism of pDC development is not fully understood. Thus, the in vitro generation of murine pDC is a useful tool to further investigate pDC biology and function in the immune system.

1.1. Developmental biology of pDC

As mentioned in the literature, the use of FL in vitro can derive DCs plus pDC [8]. Investigation of progenitor cells generating M-CSF–dependent DC revealed that common lymphoid progenitors (CLPs) were efficient producers of pDC. Common myeloid progenitors (CMPs)

generated pDC 10-fold less efficiently but they were more efficient producers of cDC [9]. Onaei et al. in 2013 showed that Macrophage and DC progenitors (MDPs) and common DC progenitors (CDPs) are BM progenitors with DC differentiation potential [10,11]. However, both MDPs and CDPs generate large numbers of cDC but few pDC, indicating the likely presence of another committed pDC progenitor [10]. Researchers have explained DC progenitors with a major pDC differentiation potential. Although both MDPs and CDPs express the macrophage colony stimulating factor receptor (M-CSFR), the progenitors are limited to a M-CSFR$^-$ fraction detected as Linc-Kit$^{int/lo}$Flt3$^+$M-CSFR$^-$in development of pDC. PDC are produced from hematopoietic stem cells (HSCs) in BM through intermediate progenitors [12]. As M-CSFR$^-$ DC progenitors do not express M-CSFR, whereas MDPs and CDPs do express it, FL and M-CSF promote pDC development from CDPs [4,10]. The family of some tyrosine kinase receptors have the same function, including c-fms, the receptor for M-CSF and Flt3 for FL [9].

Thus, our findings confirmed approach to pDC differentiation pathways and ontogeny of these cells according to other researches [10]. Herein, an FL culture system of BM cells on fibroblast feeder (L929 cell line) has shown efficient development of pDC in vitro. In this FL plus fibroblast feeder system, FL is expected to be a crucial growth factor for survival, biology and development of pDC. Moreover, in the current study, probably M-CSF and other feeder derived factors play a role in pDC differentiation as well as FL. Fancke et al. confirmed that mice deficient in FL can alternatively provoke the generation of pDC in vitro and in vivo together with the M-CSF but independent of endogenous FL [9]. Kohara et al. demonstrated that the number of pDC and probably their earliest progenitors is strictly reduced in the absence of CXCR4, the primary physiologic receptor for CXC chemokine ligand 12 (CXCL12), also known as stromal cell–derived factor-1 (SDF-1) [13]. CXCL12 induces a significant increase in pDC produced from hematopoietic cells, and pDC and their progenitors migrate to CXCL12. In this study, we have suggested that L929 fibroblastic niche, containing CXCL12, induces significant differentiation of pDC-derived hematopoietic cells. So, these progenitors are more prone to migrate to CXCL12 in such accessible niche [13]. In addition, most pDC are in contact with CXCL12 presumably through abundant L929 fibroblast cells in the in vitro culture feeder system, like the interstitial space of BM [13–15]. These cells are likely to express essential growth factors like FL and M-CSF or other chemokine and receptors needed for pDC development in vitro in contact with HSCs and polarize to pDC in bone marrow. Thus, our findings identified CXCL12 as a key controller of pDC development associated with cellular niches, providing new methods for pDC generation. Some other transcription factors are involved in pDC development in synergism with FL, including Stat3, Runx2 and IFN I [4,10,16,17]. Current in vitro culture systems enable generation of mouse pDC in large scale, as their condition resemble the in vivo conditions for development and likely better function of pDC.

According to research conducted by Brasel et al., anti–interleukin-6 (IL-6) antibody significantly reduced the DC generated in vitro by FL, suggesting a role for IL-6 in the development of DC from BM precursors [8]. Stem cell factor (SCF) shows similar effects as FL in supporting DC development in vitro [13]. In this study, we report that large numbers of BM-derived murine PDCA-1 + B220 + pDC can be generated using FL and probably M-CSF or other growth factors from fibroblastic feeder as a natural niche for development of pDC.

The network, plasticity, potential pathway and origin of pDC development have not been elucidated. This study was conducted with the aim of assessing the important role of fibroblasts in developmental biology of pDC in vitro.

It has been shown in human and mouse that HSCs can be differentiated to pDC and conventional DC in the BM [18]. HSCs can differentiate to either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs) [19,20]. It was found that monocyte DC progenitors (MDPs) further differentiate to both pDC and cDC that originate from CMPs. Fms-like tyrosine kinase-3 ligand (Flt3-L) is an essential cytokine

in DC development, and Flt3 as its receptor is broadly expressed by DC progenitors in BM. FL derives the differentiation of common DC progenitors from MDPs to either pDC or pre-DC [19].

2. Materials and methods

2.1. BM cells Isolation

Murine BM cells were obtained from C57BL/6 mice. The mice were prepared from Pasteur Institute of Iran (Tehran, Iran). BM cells were isolated by flushing femurs with 2 mL phosphate-buffered saline (PBS) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Life Technologies). They were centrifuged once and then resuspended in tris-ammonium chloride at 37 °C for 2 min to lyse red blood cells. The cells were centrifuged again and then resuspended in RPMI1640 culture medium rich in DC, supplemented with essential and nonessential amino acids, 1 mmol/L sodium pyruvate, 2.5 mmol/L Hepes buffer pH 7.4, 50 μmol/L 2-mercaptoethanol (2-ME), 100 U/mL penicillin, 100 mg/mL streptomycin, 0.3 mg/mL L-glutamine, and 10% FBS (all media and supplement from Gibco).

2.2. Cell culture for pDC differentiation

BM cells were cultured in DC Media containing 100 ng/mL mouse FL (Peprotech) for 9 days at 1×10^6/mL in 6-well plates (SPL from life science). Briefly, the culture was incubated at 37 °C in a humidified atmosphere containing 5% CO2 in air. PDC were harvested from the culture by pipetting and removing nonadherent cells. Then, each well was twice washed by PBS in room temperature to remove loosely adherent cells, which were pooled with the nonadherent ones.

2.3. L929 Feeder and conditioned media

L929 feeder cell layer consists of adherent viable and bioactive cells cultured on 6-well plates and subjected to three hours of treatment with mitomycin-c (sigma) to arrest cell growth. This layer is able to provide extracellular matrix, and growth factors can be critical in developing and maintaining pDC differentiation. These cells are used as an underlay on which BM cells are grown in a co-culture system. To provide L929-conditioned media, the supernatant was harvested after 3–4 days on RPMI 1640 with 80–90% confluency, and was used in 30%v/v for culture of pDC.

2.4. Flow cytometric analysis

PDC from harvested cultures were centrifuged once and resuspended in cell-staining medium (SM) consisting of PBS supplemented with 2% heat-inactivated calf serum (Gibco), 2% heat-inactivated mouse serum, 10 mg/mL 2.4G2 anti-Fc receptor mAb (Ebioscience) and 0.02% sodium azide (Sigma). The cells were blocked with SM at 4 °C for 20 min before incubation with mAbs, and were incubated with mAbs for 35 min at 4 °C at 1×10^6 cells per sample in a 100-μL volume. All mAbs were provided from Ebioscience. The following mAbs were used: PDCA-1(CD317)-FITC, Rat IgG2b K Isotype Control FITC, B220

Table 1
Gene and primer sequences for Real time PCR.

Gene	Forward	Reverse
Beta 2 M	5′TTC AGT CGC GGT CGC TTC AGT C 3′	5′CAA TGT GAG GCG GGT GGA ACT G 3′
Runx2	5′ AAT GCC TCC GCT GTT ATG 3′	5′ TCT GTC TGT GCC TTC TTG G 3′
Stat-3	5′ GGA GGA GAG GAT CGT GGA G 3′	5′ ACC AGC AAC CTG ACT TTC G 3′
CXCR-4	5′ CTC CTG ACT ATA CCT GAC 3′	5′ GCT TAG AGA TGA TGA TGC 3′
CXCR-7	5′ GAG CAC AGC ATC AAG GAG 3′	5′ CCA ACA TAC CAG GAA GAC C 3′
TGF-β1	5′ATT CCT GGC GTT ACC TTG G 3′	5′ CCT GTA TTC CGT CTC CTT GG 3′
IFN-β	5′ ACA GCC CTC TCC ATC AAC 3′	5′CAT CTT CTC CGT CAT CTC C 3′

(CD45R)-APC, CXCR4-APC and Rat IgG2a K Isotype Control APC. We also evaluated cell surface expression of CXCR4 on the suspended differentiated cells. Cells were stained with control antibody, anti-mCXCR4 and were subjected to flow cytometric analysis. Flow cytometry was done using Attune® Acoustic Focusing Cytometer (life technologies), BD FACS Canto and analysis with Flowjo software.
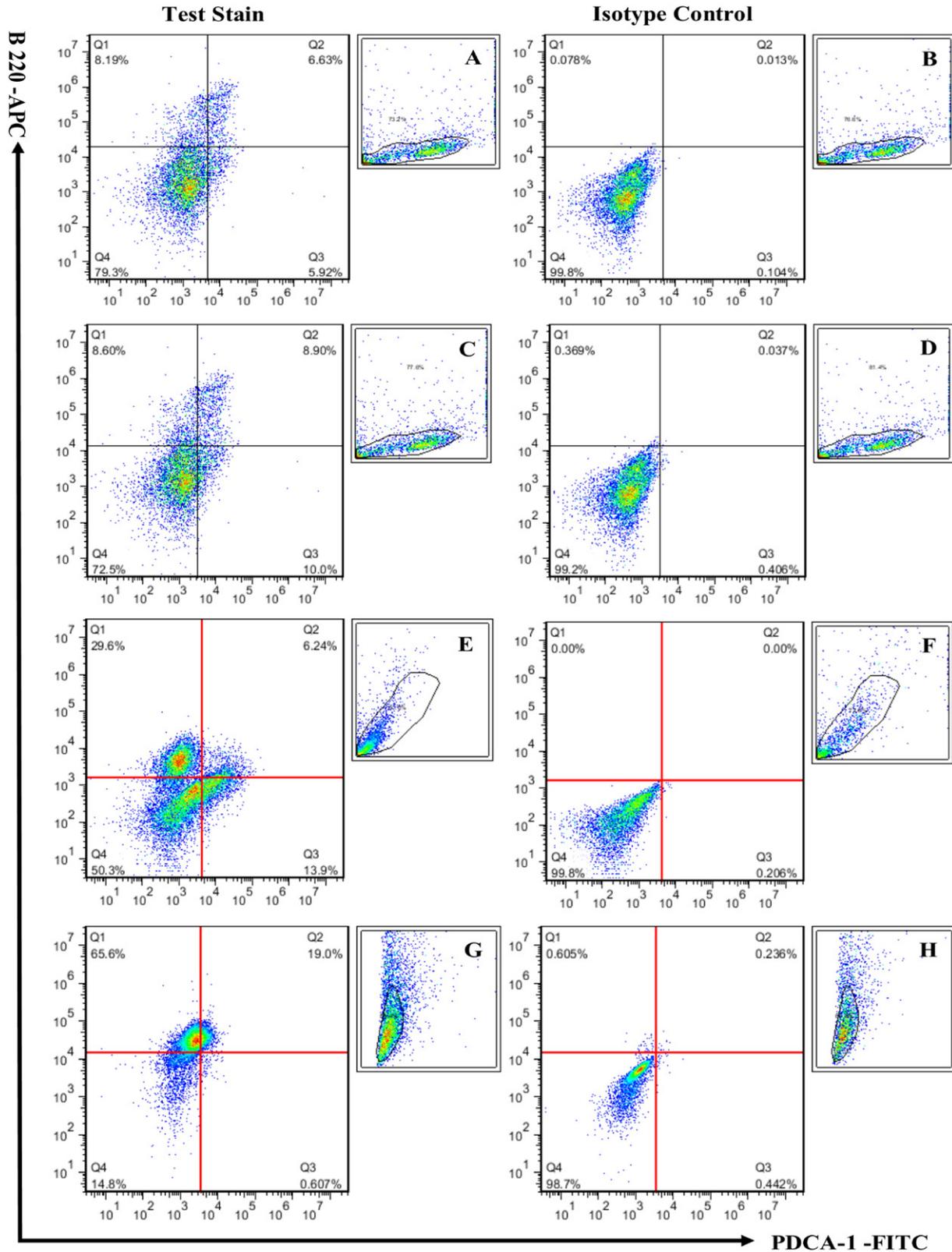

Fig. 1. Flow cytometry analysis of pDC differentiation. Double staining flow chart of analyzed sample for pDC differentiation in four groups. BL1-A channel is considered as a detector of anti PDCA-1-FITC and RL1-A for anti B220-APC. In the left column, the results are illustrated in quadrant Q2 zone as percent pDC differentiation with positive double staining. The left column corresponds to test stain and right column the isotype control. Each row shows one group in analysis. The flow charts include A and B FL alone, C and D FL + L929 CM, E and F fibroblast feeder (L929) alone and G and H for fibroblast feeder (L929) + FL, respectively. Back gating is indicated in right of each Quadrant. Data are representative of three experiments with similar results.
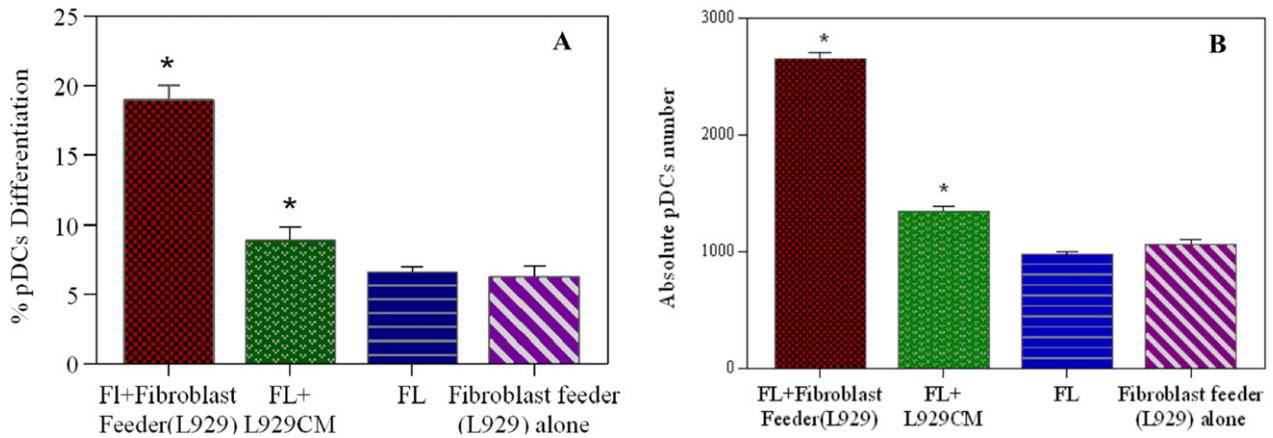
Fig. 2. A. Comparative pDC differentiation between the four Groups. The percent pDC differentiation was determined by flow cytometry and compared between the groups by ANOVA. *p < 0.05 indicated significant difference between groups. Error bars indicate s.d., and all experiments were done in triplicate. B. Comparative absolute number of pDC between four Groups. The absolute number of pDC was evaluated by flow cytometry and was compared between the groups by ANOVA. *p < 0.05 indicated significant difference between groups, respectively. Error bars indicate s.d., and all experiments were done in triplicate.

2.5. In vitro M-CSF production

M-CSF cytokine level in cell culture supernatants from L929 fibroblast feeder was tested using the ELISA kit in presence or absence of FL (R&D systems, USA). The tests were performed according to the manufacturer's instructions.

2.6. RNA extraction and cDNA synthesis

Total RNA was extracted using RNA extraction kit (Trizol, invitrogen) from the suspended differentiated cell population. Briefly, the cell pellets were lysed, 200 µl of chloroform was added, and the samples were incubated for 10 min at room temperature. After centrifugation (12,000 RPM for 15 min at 4 °C), total RNA was precipitated from the aqueous upper phase using 700 µl isopropanol and then re-suspended in 50 µl RNase free water and stored at -80 °C. The amount of extracted RNA was detected by absorbance at 260 nm.cDNA synthesis from 2 µg of total RNA was done in 50 mM Tris-HCL (pH = 8.3), 75 mM KCL, 3 mM MgCl, 10 mM dithiothreitol, 300 µM dNTP and 2.7 mM random primers (Vivantis) in a final volume of 46 µl. The samples were heated at 65 °C for 10 min and rapidly chilled on ice, and 100U of M-MuLV reverse transcriptase was added (Vivantis). The final mixture was incubated at 37 °C for 60 min and then heated at 95 °C for 5 min.

2.7. Real-Time PCR

Following total RNA extraction, the RNA was diluted to 5 µg/µl in water and used for cDNA synthesis. Real-time PCR was performed in 72-well, clear optical reaction plates with optical adhesive covers (Qiagen). To measure the gene expression in different samples of cDNA from differentiated suspended population, primers were designed for genes of interest using the oligo analyzer 6 online (http://eu.idtdna.com/analyzer/applications/oligoanalayzer/) program. The primers set up in this study are shown in Table 1.

The master reaction mixture included the following: 6.25 µl of SYBR Green PCR Master Mix (Takara Bio), 10 pmol of primer (1 µl), 1 µl of cDNA as template and 4.5 µl of nuclease free water. We investigated some of the genes involved in differentiation of pDC, like Stat3, CXCR4, CXCR7, IFN-β, TGF-β and Runx2 in differentiated cells. Cycling was performed on a Qiagen thermocycler (Rotor-GeneQ, Qiagen) using SYBR Green detection and a three-step PCR protocol. The samples were run in triplicate, and thresholds were set for all genes that were analyzed, with a reference standard to calculate $\Delta CT = CT\,(\text{gene}) - CT$
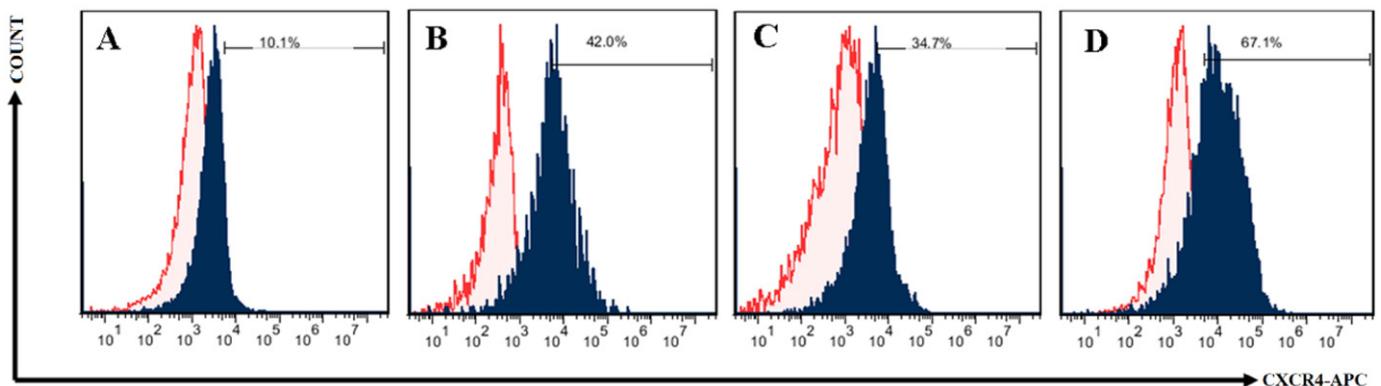

Fig. 3. Flow cytometry analysis of the cell surface CXCR4 expression in four different groups. The staining with isotype control antibody is shown as the red line and the staining with the anti-CXCR4-Apc antibody is shown as the blue solid profile. A) In the presence of FL growth factor alone. B) FL + L929 CM. C) fibroblast feeder (L929 cell) alone. D) fibroblast feeder (L929cell) + FL growth factor.
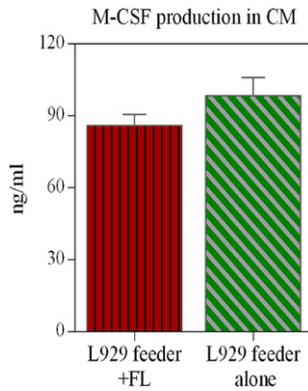
Fig. 4. The M-CSF concentration in L929 fibroblast feeder CM. The M-CSF level assessed by ELISA in culture media of L929 fibroblast feeder in presence and absence of FL. Data are expressed as the mean ± sd of three independent experiments. Data showed no significant difference between the two groups.

(standard). β2-microglubolin expression level was evaluated as an internal control. Finally, fold changes or expression levels relative to control were considered for analysis.

3. Statistical analysis

One-way ANOVA was used for statistical analysis of four groups of flow cytometry data. Analysis of ct values from real time RT-PCR was done by REST software [21] (http://rest.genequantification.info) and RT2 profiler software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

4. Results

These results clearly demonstrate L929 fibroblast feeder cells as a live, bioactive and rich source for a high level of expression of M-CSF and other growth signals. The culture supernatant along with FL (100 ng/ml) supports the significant threefold increase in proliferation of early hematopoietic progenitor cells (from 6.6% to 19%) towards pDC lineage differentiation (Fig. 2). The flow cytometry results of differentiated cells stained with Anti mPDCA-1-FITC and B220-APC as well as analysis with Flowjo software showed that differentiation potential of four groups of cultures, including: FL, FL plus L929 conditioned media (CM) 30%v/v, L929 fibroblast feeder alone and FL plus L929 fibroblast feeder was 6.6%, 8.9%, 6.2 and

19%, respectively (Fig. 1). To detect CXCR4 expression on cells, immunofluorescence staining was performed using APC-conjugated anti-mCXCR4 MoAb and APC-Isotype Ab. The percentage of CXCR4 positive cells show by histograms in four groups of cultures, including FL, FL plus L929 conditioned media (CM) 30%v/v, L929 fibroblast feeder alone and FL plus L929 fibroblast feeder was 10.1%, 42%, 34.7% and 67.1%, respectively (Fig. 3).

4.1. M-CSF production by fibroblast feeder

Along with in vitro differentiation of pDC in culture, the conditioned media from L929 fibroblast feeder cells were analyzed for M-CSF in presence or absence of FL (Fig. 4). Cytokine assays indicated that L929 fibroblast feeder produced a high level of M-CSF, as much as 86 and 98 ng/ml in presence and absence of FL, respectively. There was no significant difference for M-CSF level in presence or absence of FL (Fig. 4).

4.2. Real time RT PCR and gene expression analysis

We analyzed the association between crucial transcription factors, growth factors and chemokine receptors involved in signaling and development of differentiated cells toward pDC. The related genes, mentioned in the previous section, were evaluated by real time PCR. The relative expression and fold changes of target genes expression, such as CXCR4, Runx2 and Stat3 was significantly increased in fibroblast feeder plus FL group than the other two other groups (Fig. 5A), including FL plus L929CM and FL (p < 0.05). Fold change in expression of other genes such as CXCR7, TGFβ and IFN-β was examined in this study, and there was no statistically significant difference.

Comparison of gene fold changes of fibroblast feeder L929 plus FL group vs FL plus L929CM group was performed as shown in the scatter plot report (Fig. 5B). Significant increase in relative expression level of CXCR4, Runx2 and Stat3 genes was plotted (p < 0.05).

The heat map is illustrated in Fig. 6 to better visualize the expression pattern of considered genes, including upregulation and downregulation. These maps help to explore and interpret the results effectively, e.g. up regulation of Runx2, Stat3 and CXCR4 in this study.

5. Discussion

Feeder cell layers provide an intact and functional extracellular matrix as well as matrix-associated factors that secrete cytokines into the conditioned medium. Such feeder cells have the capacity to support
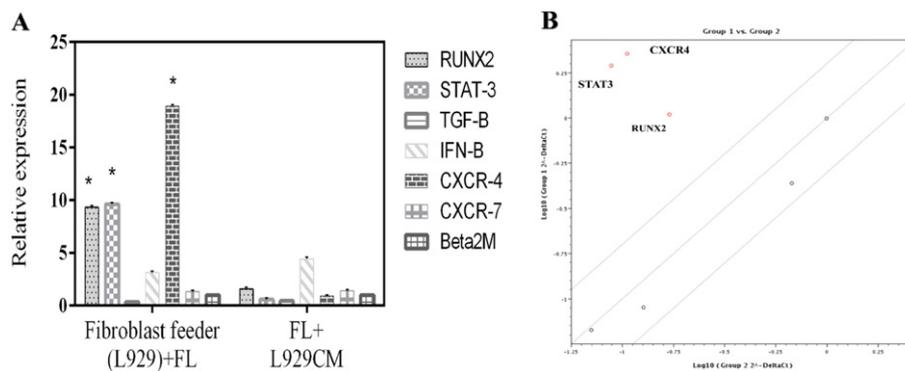

Fig. 5. A. The relative expression of genes engaged in pDC differentiation. The relative expression of the considered gene in pDC development in two main treated groups (fibroblast feeder/L929 + FL and FL + L929CM) compared with control group (FL). Each column is shown as the log of relative expression level of desired gene. Beta2M gene expression is set as an internal control and reference gene. Control group is a baseline for all gene expressions not indicated. The relative expression of Runx2, Stat3 and CXCR4 was significantly up-regulated in the fibroblast feeder/L929 + FL group when compared with the FL + L929CM and control groups. Error bars indicate s.d., and all experiments were performed in triplicate. Differences in expression were analyzed for the genes of interest using real-time PCR and P value < 0.05 was considered as significant up-regulation. B. The scatter plot visualizing logarithmic up regulation of gene expression (CXCR4, Runx2 and Stat3) in the two groups. A scatter plot of gene expression is represented as the log of the relative expression level of each gene (2 − ΔCt) between the FL + L929CM as a Group2 (x-axis) and fibroblast feeder (L929) + FL (y-axis) assumed as group 1. Statistically Significant Up-regulation was detected for three genes (Red): Runx2, Stat3, and CXCR4.
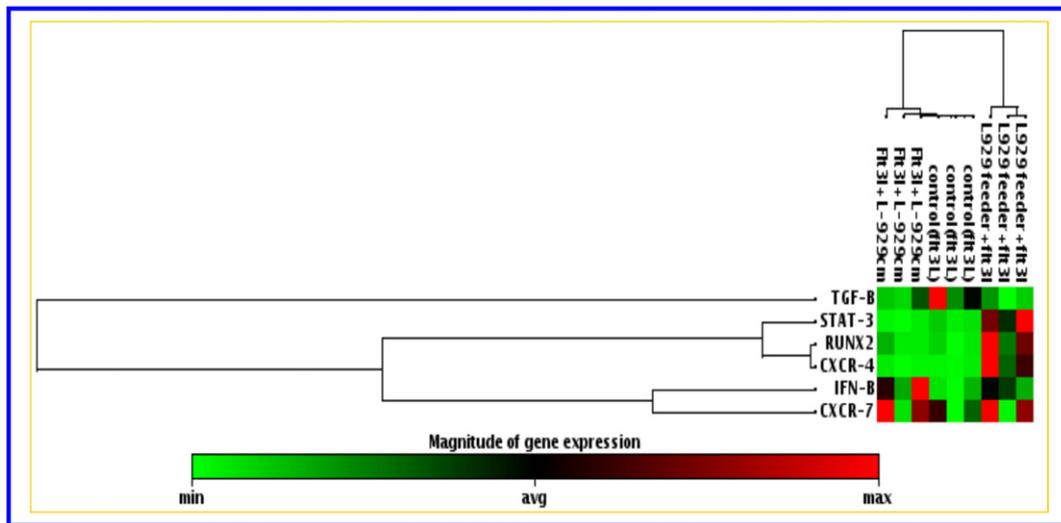
Fig. 6. Schematic illustrations showing heat map of gene expression. Comparison of the co-regulation of pDC development likely related with the expression transcription factors, cytokines and chemokine receptor across the treated samples and control group. RT-PCR assays were performed using three sets of cDNA samples that were prepared independently and resulted in the same pattern of relative expression.

in vitro survival and growth of cells that would require, for example, the presence of a variety of soluble or membrane-bound growth factors and receptors. Likewise, this system is economically affordable, and the cells are likely to remain live and fresh for future treatments. Purified growth factors and conditioned medium of cell lines can be used with less efficiency in some instances to make grow difficult cells independent of the presence of feeder cell layers. The CDP in murine BM express FL receptor (Flt3/CD135) and M-CSF receptor (M-CSFR/CD115), consistent with the ability of M-CSF to drive cDC and pDC development. Thus, in this study, we aimed to confirm the potential effect of M-CSF in pDC differentiation as a living system similar to feeder cells. We explain that the percent of pDC and perhaps their earliest progenitors is highly increased (3fold) in the presence of L929 and dependent signaling and growth factors such as M-CSF, CXCL12, FL, TGF-β, SCF, IL-6 and IFN-β [17,22,23]. CXCR4 or other chemokines and receptors on BM cell are the receptors for CXCL12 in L929 niche. Thus, we suggest the recruitment and migration of the progenitor cells close to fibroblastic niche. CXCL12 induces a significant increase in pDC generated from BM cells, and pDC and their progenitors most likely migrate to CXCL12 and can cause a burst of differentiation [13]. Our data indicate that level of CXCR4 expression, detected by flow cytometry increased with fibroblast feeder system plus FL consistent with RT-PCR analysis of these expression. In addition, most pDC are in contact with CXCL12-abundant fibroblast L929 cells in the in vitro culture feeder system similar to interstitial space of BM. Besides, in vivo, these cells express essential growth factors, like FL, M-CSF, which are required for pDC development in contact with HSCs and polarize to pDC differentiation in BM. Thus, we consider fibroblast feeder and likely M-CSF or CXCL12 as a key regulator of pDC development by cellular niches, providing new methods for pDC generation. Therefore, IL-6 and SCF released from fibroblasts are growth factors for DC and probably pDC development [8,23]. Indeed, it seems that fibroblast cells, like BM microenvironment, can crosstalk with HSCs to determine their fate in commitment and differentiation. These findings provide further support for the hypothesis that fibroblasts are appropriate niche producers of pDC in vitro or in vivo. Further studies on the current topic are therefore recommended. In this study, we examined the requirements for pDC development from several perspectives, including cytokine and chemokine requirements, development from BM precursors, and transcription factor dependence of pDC. This is the first study reporting a surprising advantage in use of an appropriate niche like fibroblasts for better development of pDC.

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