Brief Definitive Report

The Development of Murine Plasmacytoid Dendritic Cell Precursors Is Differentially Regulated by FLT3-ligand and Granulocyte/Macrophage Colony-Stimulating Factor

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Abstract

Plasmacytoid predendritic cells or type 1 interferon (IFN)-producing cells (IPCs) have recently been identified in mice. Although culture systems giving rise to different murine dendritic cell subsets have been established, the developmental regulation of murine plasmacytoid IPCs and the culture conditions leading to their generation remain unknown. Here we show that large numbers of over 40% pure $CD11c^+CD11b^-B220^+Gr\text{-}1^+$ IPCs can be generated from mouse bone marrow cultures with FLT3-ligand. By contrast GM-CSF or TNF-α, which promote the generation of $CD11c^+CD11b^+B220^-$ myeloid DCs, block completely the development of IPCs. IPCs generated display similar features to human IPCs, such as the plasmacytoid morphology, the ability to produce large amounts of IFN-α in responses to herpes simplex virus, and the capacity to respond to ligands for Toll-like receptor 9 (TLR-9; CpG ODN 1668), but not to ligands for TLR-4 (lipopolysaccharide [LPS]). Unlike human IPCs which produce little IL-12p70, mouse IPCs produce IL-12p70 in response to CpG ODN 1668 and herpes simplex virus. This study demonstrates that the development of murine $CD11c^+CD11b^-B220^+Gr\text{-}1^+$ IPCs and $CD11c^+CD11b^+B220^-$ myeloid DCs is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. Human IPCs and mouse IPCs display different ability to produce IL-12p70. Large numbers of mouse IPCs can now be obtained from total bone marrow culture.

Key words: pre-DC2 • IPC • T lymphocyte • antiviral immune response • innate immunity

Introduction

Plasmacytoid predendritic cells (pre-DC2) or type 1 IFN-producing cells (IPCs) represent a unique cell type of the hematopoietic system (1–5). IPCs in both humans and mice display a plasmacytoid morphology and have the capacity to produce large amounts of type 1 IFN in response to viral and bacterial stimulation (1–5). In addition, IPCs have the potential to differentiate into dendritic cells, which are capable of inducing naive T cell proliferation (6–9). Human IPCs display a unique phenotype: $CD4^+$ $CD45RA^+BDCA2^+CD123^{2+}CD11c^-Lin^-$ (6–9). Recently, mouse IPCs have been identified by their capacity to produce large amounts of type 1 IFN in response to stimulation by virus or bacteria (3–5). Unlike human IPCs, mouse IPCs express CD11c, and markers for B cells (B220) and granulocyte (GR-1), but do not express high CD123. In addition, mouse IPCs express high levels of CD45RB and Ly6c. The identification of mouse IPCs further supports the concept that IPCs may represent a pivotal effector cell type in antiviral immunity and opens the possibility to study their function in vivo.

During the past years, the developmental pathways of mouse DC subsets and the molecular regulation of their generation has been extensively studied. Mouse myeloid DCs were generated from mouse bone marrow (BM) or from peripheral blood monocytes with GM-CSF, or GM-CSF plus IL-4 (10). Mouse "lymphoid DCs" were generated from thymic lymphoid precursors in the presence of a combination of IL-1β, TNF-α, IL-7, stem cell factor (SCF), and IL-3 (11). However, the developmental regulation of mouse IPCs and the culture conditions leading to the generation of large numbers of mouse IPCs are currently unknown.

Previous studies demonstrated that injection of FLT3-ligand and G-CSF could significantly increase the number of

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IPCs in the blood of human volunteers (12, 13). In addition, FLT3-ligand but not G-CSF was shown to induce a proportion of human $CD34^+CD45RA^-$ early hematopoietic stem cells to differentiate into IPCs in culture (14). In this study, we demonstrate that the development of murine $CD11c^+$ $CD11b^-B220^+Gr\text{-}1^+$ IPCs is promoted by FLT3-ligand in total BM cultures. GM-CSF or TNF-α inhibited the development of murine IPCs, but promoted the development of $CD11c^+CD11b^+B220^-$ myeloid DCs. Unlike human IPCs that do not have the ability to produce IL-12p70, murine IPCs readily produce IL-12 in response to viruses or bacteria. It is now possible to generate large numbers of over 40% pure IPCs in mouse total BM culture with FLT3-ligand.

Materials and Methods

Mice. Female BALB/c mice (Taconic Farm) at 6–8 wk old were used as a source of BM, for the generation of mouse DCs in cultures.

Cell Isolation and Culture. BM cells were isolated by flushing femurs and tibiae with RPMI supplemented with 10% heat inactivated FCS. The BM cells were then passed through a 70-μm cell strainer, centrifuged, and resuspended in a tris-ammonium chloride buffer (Sigma-Aldrich) at 37°C for 5 min to lyze red blood cells. The cells were centrifuged and resuspended at 10^6 cells/ml in culture medium consisting of RPMI 1640, 10% FCS, 1 mmol/liter sodium pyruvate, hepes, penicillin, streptomycin, and 2-mercaptoethanol supplemented with different cytokines: murine FLT3-ligand (100 ng/ml; DNAX Research Institute), GM-CSF (100 ng/ml; Kenilworth), FLT3-ligand plus TNF-α (10 ng/ml; R&D Systems), FLT3-ligand plus GM-CSF, and GM-CSF plus TNF-α. Every 5 d of culture, half of the medium was removed and fresh cytokine-supplemented culture medium was added back into cultures.

Flow Cytometric Analysis and Cell Sorting. Cells were harvested at day 10 of culture and stained with anti-CD11c-PE mAb, anti-CD11b-APC mAb and FITC-labeled anti-B220 mAb, anti-CD45RB, anti-Ly6c, anti-GR1, anti-CD4, anti-CD8, anti-CD80, anti CD86, anti-CD40, and anti-MHC class II mAbs (all from BD PharMingen). For cell sorting the cells were stained with anti-B220 FITC mAb, anti-CD11c-PE mAb, anti-CD11b-APC mAb, and $CD11c^+CD11b^-B220^+$ and $CD11c^+CD11b^+B220^-$ were sorted.

In Vitro Stimulation and Quantitation of Cytokine Production. Sorted $CD11c^+CD11b^-B220^+$ and $CD11c^+CD11b^+B220^-$ cells were cultured for 24 h at 10^5 cells/200 μl in round-bottom 96-well culture plates in the presence of: irradiated HSV at 30 PFU/cell, Toll-like receptor (TLR)-4 ligand LPS from *Salmonella minnessota* serotype RE595 (Sigma-Aldrich) at 10 μg/ml, and TLR-9 ligand CPG-ODN 1668 (TCCATGACGTTCCGATGCT; MWG Biotech) at 1 μM. CPG was added twice at 0 and 12 h to the culture. For quantitation of cytokine production, cell-free supernatants were collected after 24 h and analyzed with the following ELISA kits: mouse IFN-α (PBL-Biomedical), mouse TNF-α (R&D Systems), and mouse IL-12p70 (R&D Systems).

Results

Murine $CD11c^+CD11b^-$ DC Subset Generated in FLT3-Ligand-supplemented BM Cultures Displays a Phenotype of Murine IPCs. FLT3-ligand was shown to induce the generation of large numbers of $CD11c^+$ DCs in mice (15). However, it was not determined whether IPCs were generated in this system. We investigated whether FLT3-ligand could induce the generation of mouse IPCs in cultures of total BM cells. Murine BM cells were cultured in the presence of 100 ng/ml FLT3-ligand for 20 d. During the first 5 d of culture, a rapid loss of B cells ($CD19^+$), T cells ($CD3^+$), NK cells ($DX5^+$), and granulocytes ($GR1^+CD11c^-$) was observed by flow cytometric analysis (Fig. 1 A). However, there was a dramatic increase in the percentage of $CD11c^+$ cells, from 1.2% before culture to 37% at day 5 of culture and to a maximal level of over 92% after day 10 of culture (Fig. 1 A). The total cellularity of the cultures reached its maximum at day 10, equaling the BM cell input number at day 0 (Fig. 1 B). Because mouse IPCs were recently shown to express CD11c, B220, GR-1, CD45RB, and Ly6c, but not CD11b (3, 4, 5), we investigated whether the $CD11c^+$ cells generated in culture contained cells with IPC phenotype by three color flow

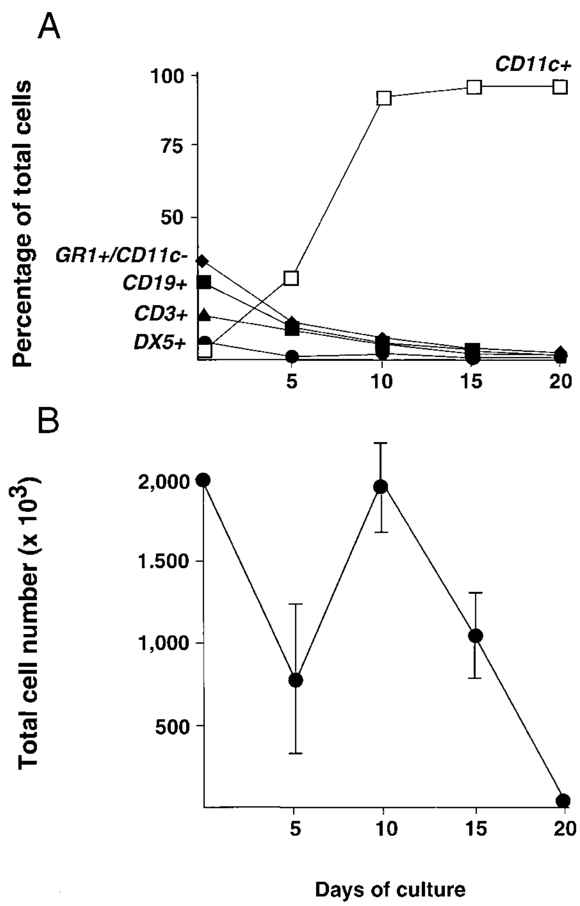

Figure 1. (A) Percentage of $CD3^+$, $CD19^+$, $DX5^+$, $GR1/CD11c^-$, and $CD11c^+$ cells analyzed by flow cytometry. During the first 5 d of culture, a rapid loss of B cells ($CD19^+$), T cells ($CD3^+$), NK cells ($DX5^+$), and granulocytes ($GR1^+CD11c^-$) was observed. However, there was a dramatic increase in the percentage of $CD11c^+$ cells. (B) Total cell number harvested from FLT3-ligand–supplemented BM cultures at day 0, 5, 10, 15, and 20. There was an initial cell loss at day 5 of cell culture. However, cell number was recovered by day 10 and then decreased after. Represented is the mean ± SD of three independent experiments.

cytometry. Fig. 2 A shows that two subsets of DCs, CD11c$^+$CD11b$^-$ and CD11c$^+$CD11b$^+$ are generated in FLT3-ligand supplemented BM cultures. The CD11c$^+$ CD11b$^-$ subset, which represented ~1% of total BM cells before culture, increased to 16% of total cultured cells at day 5, peaked at day 10 with 45%, and then decreased to 21% at day 15 of culture (Fig. 2 A). The CD11c$^+$ CD11b$^-$ subset expressed high levels of B220, CD45RB, Ly6c, and GR-1, low levels of MHC class II, and undetectable levels of CD80 and CD86 (Fig. 2 B), the typical phenotype of mouse plasmacytoid DC precursors (3–5). The CD11c$^+$CD11b$^+$ subset which represented ~0.2% of total BM cells before culture, increased to 13% of total cultured cells at day 5, to 32% at day 10, and to 49% at day 15 of culture (Fig. 2 A). The CD11c$^+$CD11b$^+$ subset did not express B220 and expressed lower levels of CD45RB, Ly6c, and GR-1, but expressed significant levels of CD80, CD86, and MHC class II, the typical phenotype of splenic myeloid DC subsets (16–18). Both CD11c$^+$ cell populations lacked lineage markers for B cells (CD19), T cells (CD3), NK cells (DX-5), and erythroid cells (TER-119) (data not shown). Therefore, FLT3-ligand induced the generation of over 90% pure CD11c$^+$ cells at day 10 of murine BM cultures. While ~50% of the CD11c$^+$ cells displayed the phenotype of the splenic CD11b$^+$ myeloid DC subset, the other 50% of the CD11c$^+$ cells displayed the phenotype of IPCs.

CD11c$^+$CD11b$^-$B220$^+$ DC subset displays a plasmacytoid morphology and produce a large amounts of IFN-α in response to Herpes simplex virus. We separated the CD11c$^+$ cells derived from day 10 BM culture with FLT3-ligand into two subsets, CD11c$^+$CD11b$^-$B220$^+$ and CD11c$^+$CD11b$^+$B220$^-$, by three-color immunofluorescence cell sorting. Whereas the CD11c$^+$CD11b$^-$B220$^+$ subset displayed plasmacytoid morphology, the CD11c$^+$ CD11b$^+$B220$^-$ cells displayed a morphology, similar to that of the splenic CD11c$^+$CD11b$^+$ myeloid DCs (data not shown). The CD11c$^+$CD11b$^-$B220$^+$ subset produced large amounts of IFN-α in response to HSV (2,884 ± 24; 2,664 ± 22; 2,160 ± 32 pg/ml/10^6 cells, from three separate donors; Fig. 3). By contrast, the CD11c$^+$CD11b$^+$B220$^-$ myeloid DC subset only produced low amounts of IFN-α in response to HSV (34 ± 0; 108 ± 6; 25 ± 10 pg/ml/10^6 cells; Fig. 3). In addition, the CD11c$^+$CD11b$^-$B220$^+$ subset, but not the CD11c$^+$ CD11b$^+$B220$^-$ myeloid DC subset produced a moderate amounts of TNF-α in response to HSV (Fig. 3). These data indicate that CD11c$^+$CD11b$^-$B220$^+$ cells generated in culture are IPCs, a key cell type in antiviral innate immunity.

Unlike Human IPCs, Murine CD11c$^+$CD11b$^-$B220$^+$ IPCs Produce IL-12 in Response to HSV and CpG. Previous studies demonstrated that human IPC had a poor ability to produce IL-12p70, compared with monocyte-derived DCs in response to CD40-ligand or microbial stimulation (19–21). Mouse CD11c$^+$CD11b$^-$B220$^+$ IPCs generated in cultures produced a large amounts of IL-12p70 in response to CpG ODN 1668 (2,092 ± 16; 1,282 ± 10; 1,586 ± 50 pg/ml/10^6 cells from three separate donors), which was 4–5 times more than that produced by the CD11c$^+$CD11b$^+$B220$^-$ myeloid DCs (168 ± 8; 150 ± 0.4; 46 ± 17 pg/ml/10^6 cells; Fig. 3). In addition, the CD11c$^+$CD11b$^-$B220$^+$ IPCs, but not the CD11c$^+$ CD11b$^+$B220$^-$ myeloid DCs produced a moderate amount of IL-12p70 in response to HSV (Fig. 3). These data confirm the recent reports that freshly isolated mouse IPCs produce both IFN-α and IL-12 in responses to viruses (4), or bacteria (5).

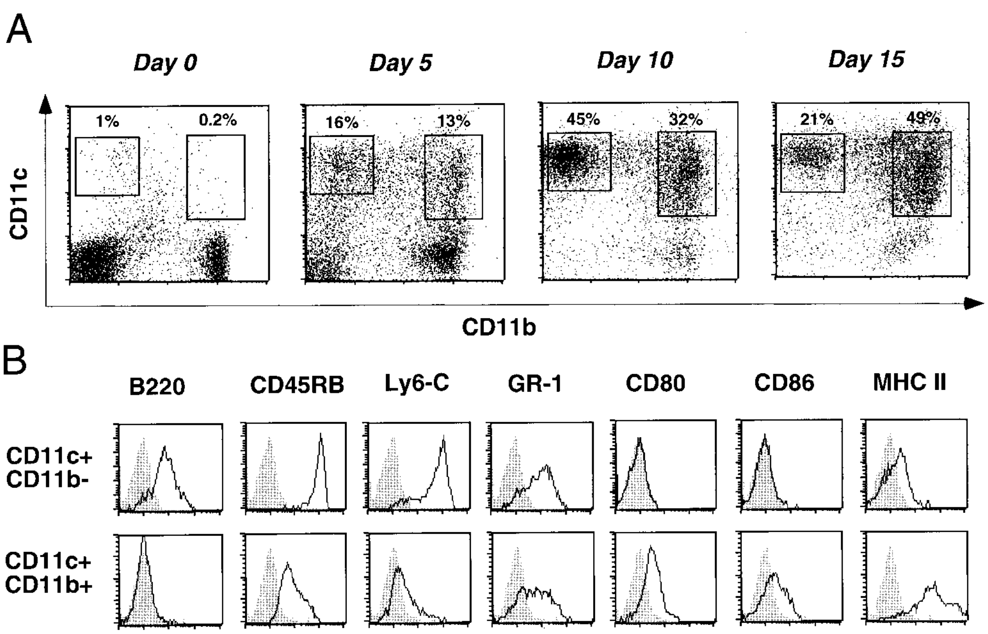

Figure 2. Generation of CD11c$^+$ CD11b$^-$B220$^+$ IPCs and the CD11c$^+$CD11b$^+$B220$^-$ myeloid DCs from FLT3-ligand-supplemented BM cell cultures. Three-color fluorescence flow cytometry analysis shows that CD11c$^+$ CD11b$^-$ cells represent 1% of total BM cells before culture, and 16% at day 5, 45% at day 10, and 21% at day 15 of culture with FTL3-ligand (A). The CD11c$^+$ CD11b$^-$ cells display a typical phenotype of mouse IPCs, being B220^{2+}CD45RB^{2+}Ly6c^{2+}Gr-1^{2+} CD80$^-$CD86$^-$MHC class II^{low} (B). The CD11c$^+$CD11b$^+$ cells represent 0.2% of total BM cells before culture, and 13% at day 5, 32% at day 10, and 49% at day 15 of culture with FTL3-ligand (A). The CD11c$^+$CD11b$^+$ cells display a phenotype similar to that of the splenic myeloid DCs, being B220$^-$ CD45RB$^+$Ly6c$^+$Gr-1^{+-} CD80$^+$ CD86$^+$MHC class II^{high} (B). The data shown are representative of six experiments.

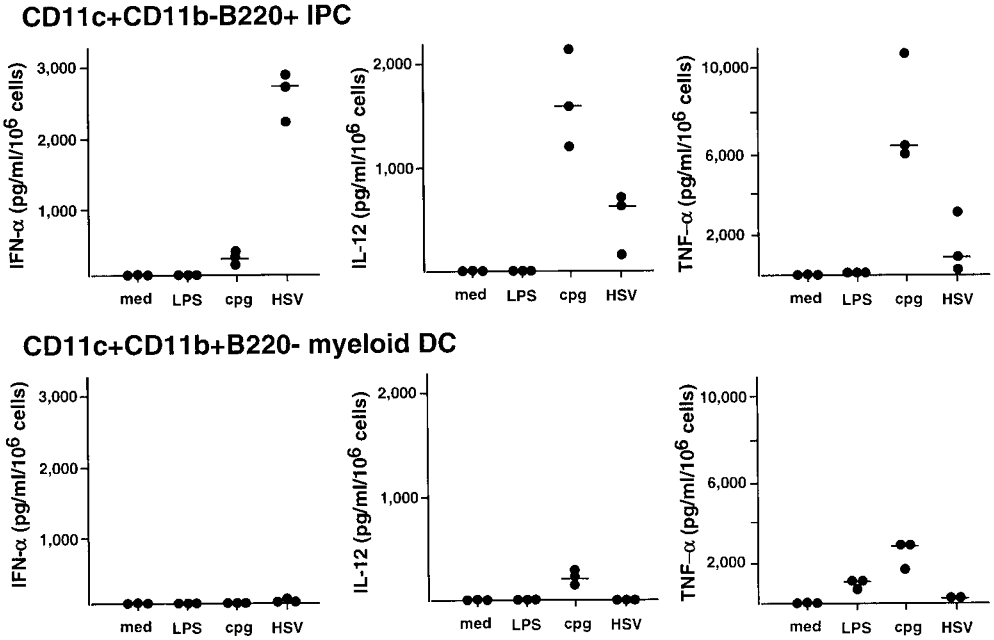

Figure 3. Cytokine production by the CD11c$^+$CD11b$^-$B220$^+$ IPCs and the CD11c$^+$CD11b$^+$ B220$^-$ myeloid DCs in cultures with medium, LPS, CpG ODN 1668, and HSV. Each solid circle represents a separate donor. The sensitivity of ELISA for detecting each cytokine: IFN-α (<12.5 pg/ml), TNF-α (<25 pg/ml), and IL-12 (15 pg/ml).

CD11c$^+$CD11b$^-$B220$^+$ IPCs Respond Poorly to LPS. Recent studies have shown that human IPCs preferentially respond to the TLR-9 ligands (bacterial CpG ODN), but do not respond to the TLR-4 ligands (LPS), by producing IFN-α and TNF-α, and differentiate into DCs (19–21). While CD11c$^+$CD11b$^-$B220$^+$ IPCs produced large amounts of IFN-α, IL-12p70, and TNF-α (6,014 ± 270; 10,570 ± 522; 5,754 ± 211 pg/ml/10^6 cells from three separate donors) in response to CpG ODN 1668 (Fig. 3), and differentiate into mature DCs (data not shown), they produced neither IFN-α, nor IL-12p70, and a very low amount of TNF-α (234 ± 10; 78 ± 0.4; 82 ± 14 pg/ml/10^6 cells) in response to LPS (Fig. 3). CD11c$^+$CD11b$^-$B220$^+$ IPCs did not differentiated into mature DCs and died after 24 h of culture with LPS. By contrast, the CD11c$^+$CD11b$^+$B220$^-$ myeloid DCs produced 6 to 13 times more TNF-α (1,366 ± 103.6; 1,008 ± 39.6; 1,298 ± 69 pg/ml/10^6 cells) than CD11c$^+$CD11b$^-$B220$^+$ IPCs (Fig. 3), and displayed an increased ability to induce antigen-specific naive T cell proliferation following activation by LPS (data not shown).

GM-CSF and TNF-α Inhibit the Generation of CD11c$^+$CD11b$^-$B220$^+$ IPCs in FLT3-ligand-supplemented BM Cultures. GM-CSF represents a key DC growth and differentiation factor both in vivo and in vitro (22). GM-CSF or GM-CSF plus TNF-α, cytokines used classically to gen-

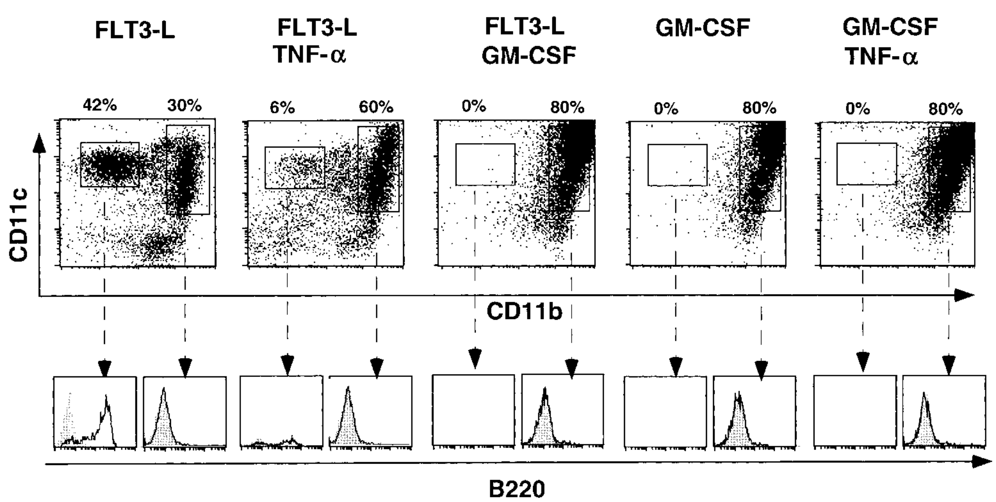

Figure 4. GM-CSF and TNF-α promotes the generation of CD11c$^+$CD11b$^+$B220$^-$ myeloid DCs and inhibits the FLT3-ligand-mediated generation of CD11c$^+$CD11b$^-$B220$^+$ IPCs. Freshly isolated BM cells were cultured for 10 d with FLT3-ligand, GM-CSF, or FLT3-ligand plus GM-CSF, FLT3-ligand plus TNF-α, or GM-CSF plus TNF-α. Three-color fluorescence flow cytometry analyses shows that both CD11c$^+$CD11b$^-$B220$^+$ IPCs (42%) and CD11c$^+$CD11b$^+$B220$^-$ myeloid DCs (30%) were generated at day 10 of BM culture with FLT3-ligand as shown in Fig. 2. Addition of TNF-α lead to a decrease of CD11c$^+$CD11b$^-$B220$^+$ IPCs from 42 to 6%, and an increase of CD11c$^+$CD11b$^+$B220$^-$ myeloid DCs from 30% to 60%. Addition of GM-CSF lead to a complete blockage of the generation of CD11c$^+$CD11b$^-$B220$^+$ IPCs, and an increase of CD11c$^+$CD11b$^+$B220$^-$ myeloid DCs from 30 to 80%. In total BM cultures with either GM-CSF, or GM-CSF plus TNF-α, only CD11c$^+$CD11b$^+$B220$^-$ myeloid DCs, but not CD11c$^+$CD11b$^-$B220$^+$ IPCs were generated. The data shown are representative of three experiments.

erate human and murine DCs in vitro, induced a single population of CD11c$^+$CD11b$^+$B220$^-$ myeloid DCs but no CD11c$^+$CD11b$^-$B220$^+$ IPCs in total BM culture (Fig. 4). The CD11c$^+$CD11b$^+$B220$^-$ DCs generated with GM-CSF or GM-CSF plus TNF-α displayed a similar phenotype to CD11c$^+$CD11b$^+$B220$^-$ DCs generated with FLT3-ligand (Fig. 2). Addition of GM-CSF or TNF-α to the FLT3-ligand cultures inhibited the generation of CD11c$^+$CD11b$^-$B220$^+$ IPCs (Fig. 4). These results demonstrate that the development of IPCs and CD11c$^+$ CD11b$^+$B220$^-$ DCs are regulated by different hematopoietic cytokines.

Discussion

In this study, we report that large numbers of over 40% pure murine IPCs can be generated in FLT3-ligand–supplemented BM cultures within 10 d. The study suggests that FLT3-ligand and GM-CSF/TNF-α have opposing effects on the development of CD11c$^+$CD11b$^-$B220$^+$ IPCs and CD11c$^+$CD11b$^+$B220$^-$ myeloid DCs, and that FLT3-ligand represents a key cytokine for IPC development in both humans and mice.

One striking difference between human and mouse IPCs is that mouse IPCs readily produce both IL-12 p70 and IFN-α, whereas human IPCs only make IFN-α in response to viral and bacterial stimulation. IFN-α has the ability to induce IFN-γ production from T cells in humans, but not in mice, because a mutation in STAT2 gene in mice, which results in a loss of type 1 IFN-induced STAT4 activation in mouse T cells (23). It is therefore highly likely that human but not mice IPCs have lost the ability to make IL-12p70 in response to virus and bacteria, due to the functional redundancy of human IFN-α and IL-12p70 in their ability to induce IFN-γ production in human T cells.

It has been proposed that human and mouse IPCs (pre-DC2) play a central role in antiviral innate immunity. Indeed, depletion of IPCs was found to be associated with progression of HIV-infected subjects to AIDS (24–26). Furthermore, it has been suggested that mature DCs derived from IPCs (pre-DC2) may induce adaptive immune responses with regulatory functions (27, 28). Recent studies suggest that inappropriate activation of IPCs may be associated with the pathophysiology of systemic lupus erythematosus (29, 30). The generation of large numbers of CD11c$^+$CD11b$^-$B220$^+$ IPCs by FLT3-ligand-supplemented BM cultures will permit further studies of their development and their in vivo function in the innate and adaptive immunity.

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