The Journal of Immunology

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J Immunol 2005; 174:2573-2581; ;
doi: 10.4049/jimmunol.174.5.2573
http://www.jimmunol.org/content/174/5/2573

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

IFN Regulatory Factor-4 and -8 Govern Dendritic Cell Subset Development and Their Functional Diversity

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Dendritic cells (DCs) are bone marrow (BM)-derived APCs central to both innate and adaptive immunity. DCs are a heterogeneous cell population composed of multiple subsets with diverse functions. The mechanism governing the generation of multiple DC subsets is, however, poorly understood. In this study we investigated the roles of closely related transcription factors, IFN regulatory factor (IRF)-4 and IRF-8, in DC development by analyzing *IRF-4*$^{-/-}$, *IRF-8*$^{-/-}$, and *IRF-4*$^{-/-}$*IRF-8*$^{-/-}$ (double-knockout) mice. We found that IRF-4 is required for the generation of CD4$^+$ DCs, whereas IRF-8 is, as reported previously, essential for CD8α^+ DCs. Both IRFs support the development of CD4$^-$CD8α^- DCs. IRF-8 and, to a lesser degree, IRF-4 contribute to plasmacytoid DC (PDC) development. Thus, the two IRFs together regulate the development of all conventional DCs as well as PDCs. Consistent with these findings, IRF-4, but not IRF-8, was expressed in CD4$^+$ DCs, whereas only IRF-8 was expressed in CD8α^+ DCs. CD4$^-$CD8α^- DCs and PDCs expressed both IRFs. We also demonstrate in vitro that GM-CSF-mediated DC differentiation depends on IRF-4, whereas Fms-like tyrosine kinase 3 ligand-mediated differentiation depends mainly on IRF-8. Gene transfer experiments with double-knockout BM cells showed that both IRFs have an overlapping activity and stimulate a common process of DC development. Nonetheless, each IRF also possesses a distinct activity to stimulate subset-specific gene expression, leading to the generation of functionally divergent DCs. Together, IRF-4 and IRF-8 serve as a backbone of the molecular program regulating DC subset development and their functional diversity. *The Journal of Immunology,* **2005, 174: 2573–2581.**

Dendritic cells (DCs)[3] are bone marrow (BM)-derived professional APCs. They play central roles in the induction of both innate and adaptive immunity (1). DCs recognize various pathogens and their components through pattern-recognition receptors such as TLRs and produce a variety of cytokines. They capture and process Ags to present antigenic peptides associated with MHC molecules to T cells, eliciting the Th1 and Th2 responses as well as inducing tolerance. In addition, DCs regulate other immune cells, including B and NK cells. The extensive diversity of DC function is partly attributed to the presence of multiple DC subsets (2, 3).

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Received for publication June 17, 2004. Accepted for publication December 13, 2004.

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[3] Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; BMDC, BM-derived DC; DKO, double knockout; DN, double negative; Flt3L, Fms-like tyrosine kinase 3 ligand; IAD, IRF association domain; ICSBP, IFN consensus sequence-binding protein; IRF, IFN regulatory factor; KO, knockout; Lin$^-$, lineage marker negative; Mmp12, matrix metalloproteinase 12; OVAp, OVA peptide; PDC, plasmacytoid DC; SCF, stem cell factor; WT, wild type.

At least six DC subsets have been identified in the mouse (4). Conventional CD11c^{high} DCs are divided into three subsets: CD4$^+$CD8α^-CD11b^{high}, CD4$^-$CD8α^+CD11b^{low}, and CD4$^-$CD8α^-CD11b^{high} (double negative (DN)) subsets. The spleen also contains CD11c^{int}B220$^+$CD11b$^-$ plasmacytoid DCs (PDCs), the major type I IFN-producing cells (5–7). The thymus also has these subsets, except for CD4$^+$CD8α^- DCs. Lymph nodes contain two additional subpopulations that are derived from epidermal DCs (Langerhans cells) and interstitial DCs.

Although the developmental course and the origin of DC subsets are not fully elucidated, it is thought that each DC subset arises from a separate developmental pathway (8, 9) that can be originated from both the common myeloid progenitors and common lymphoid progenitors (10–12). However, the molecular mechanism governing the generation of multiple DC subsets has remained poorly understood.

Transcription factors of the IFN regulatory factor (IRF) family play critical roles in the development and function of immune cells. IRF-8/IFN consensus sequence-binding protein (ICSBP) is a hemopoietic cell-specific, IFN-γ-inducible IRF that controls myeloid cell development (13, 14). *IRF-8*$^{-/-}$ mice are immunodeficient and develop chronic myelogenous leukemia-like disease (15). Recently, our laboratory and others found that IRF-8 is also essential for the development of several DC subsets, namely, CD8α^+ DCs, PDCs, epidermal DCs, and dermal DCs (16–19). *IRF-8*$^{-/-}$ mice, although deficient in these subsets, retain CD4$^+$ and DN DCs at normal levels, the major DC subsets in the spleen. IRF-4 is another immune system-specific member of the family, and among other IRF members it exhibits the greatest structural similarity with IRF-8 (20, 21). Both IRF-4 and IRF-8 interact with the same partner transcription factors, such as PU.1, and regulate transcription through similar DNA elements, although the two factors appear to have distinct partners as well (22). IRF-4 regulates

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B cell differentiation as well as T cell function (23), yet its role in DCs has remained unknown.

Based on the predominant expression in hemopoietic cells and the structural and functional similarity with IRF-8, we hypothesized that IRF-4 regulates DC development, perhaps through distinct pathways not affected by IRF-8. In this study, by analyzing *IRF-4*$^{-/-}$, *IRF-8*$^{-/-}$, and *IRF-4*$^{-/-}$*IRF-8*$^{-/-}$ mice, we demonstrate that IRF-4 and IRF-8 indeed regulate subset development and functional diversity in DCs through common and selective roles.

Materials and Methods

Mice

IRF-4$^{-/-}$*IRF-8*$^{-/-}$ mice on a C57BL/6 background were generated by intercrossing *IRF-4*$^{+/-}$*IRF-8*$^{+/-}$ mice that had been obtained by crossing *IRF-4*$^{-/-}$ and *IRF-8*$^{-/-}$ mice (15, 23). Age- and sex-matched mice of each genotype were used for experiments. C57BL/6 OTII TCR transgenic mice were obtained from Taconic Farms. Mice were housed under specific pathogen-free conditions and were used at 7–9 wk of age in accordance with institutional animal care guidelines.

Cell preparation and culture

DC-enriched, low density cell fractions from Liberase CI (Roche Biochemicals)-treated spleens and thymi were prepared using 30% BSA in PBS as previously described (17). CD11c$^+$ DCs were purified by immunomagnetic cell sorting using MACS CD11c microbeads (Miltenyi Biotec) according to the manufacturer's protocol. The purity of CD11c$^+$ cells was >90%. White blood cells from the peripheral blood were prepared using ACK lysing buffer (Quality Biological). OTII TCR transgenic T cells were negatively purified from lymph nodes by immunomagnetic cell depletion by AutoMACS (Miltenyi Biotec) using FITC-conjugated Abs against I-A$^b\beta$, CD24, CD16, CD8, B220, and NK1.1, followed by anti-FITC microbeads (Miltenyi Biotec). T cells (1×10^6) were labeled with CSFE (Molecular Probes), and were cocultured with 2×10^5 DCs in 0.5 ml with 10 nM $OVA_{329-339}$ peptide (OVAp; ISQAVHAAHAEINEAGR; Research Services Branch, National Institute of Arthritis and Infectious Diseases, National Institutes of Health). On day 3 T cells were restimulated with 10 ng/ml PMA and 1 μM ionomycin for 4 h, and 2 μM brefeldin was added during the final 2 h of stimulation. The cells were fixed with 2% paraformaldehyde and permeabilized with PBS supplemented with 0.1% BSA plus 0.1% saponin, and intracellular staining for IFN-γ and IL-4 was performed. BM mononuclear cells were prepared by a density gradient centrifugation on Histopaque-1083 (Sigma-Aldrich). Lineage marker-negative (Lin$^-$) cells were purified by immunomagnetic cell depletion using the lineage cell depletion kit (Miltenyi Biotec). GM-CSF-mediated, BM-derived DCs (BMDCs) were generated from BM Lin$^-$ cells in the presence of 5 ng/ml GM-CSF (BioSource International), 25 ng/ml stem cell factor (SCF; PeproTech) and 25 ng/ml TNF-α (PeproTech) as previously described (24). SCF was withdrawn from day 7, and BMDCs were analyzed on day 12. BMDCs generated with GM-CSF alone from BM mononuclear cells were also selectively dependent on IRF-4 for their development (data not shown). Fms-like tyrosine kinase 3 ligand (Flt3L)-mediated BMDCs were generated from BM mononuclear cells cultured for 9 days in the presence of 100 ng/ml Flt3L (PeproTech) as previously described (25). DCs were stimulated with 1 μg/ml *Escherichia coli*-derived LPS (Sigma-Aldrich), 1 μg/ml CpG oligomer DNA (1826 or D19) (18), or 100 μg/ml poly(I:C) (Amersham Biosciences) for 14 h (splenic DCs) or 24 h (BMDCs).

Flow cytometry

For detection of surface markers, cells were blocked with anti-mouse FcγR Ab (CD16/CD32, clone 2.4G2; BD Pharmingen), followed by incubation with specific Abs or isotype controls at 4°C for 30 min. Incubation with biotinylated Abs was followed by CyChrome-conjugated streptavidin (BD Pharmingen). The specific Abs used were FITC-conjugated anti-CD11c (clone HL-3) and B220 (RA3-6B2); PE-conjugated anti-I-A^b (AF6-120.1), CD80 (16-10A1), CD4 (RM4-5), and CD11b (M1/70); CyChrome-conjugated anti-CD8α (53-6.7) and B220; and biotin-conjugated anti-CD11c (all from BD Pharmingen). For detection of IRF-4 and IRF-8, surface marker-stained cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% saponin (Sigma-Aldrich), blocked with 5% normal donkey serum, and stained with goat anti-IRF-4 (M-17) or IRF-8 (C-19) Ab (Santa Cruz Biotechnology) or control normal goat IgG, followed by incubation with FITC-conjugated donkey anti-goat Ab (Jackson ImmunoResearch Laboratories). Stained cells were acquired in a FACSCalibur (BD Biosciences), and data were analyzed using FlowJo software (TreeStar).

Real-time RT-PCR and ELISA

RNA extraction, cDNA synthesis, real-time quantitative PCR using an ABI PRISM 7000 sequence detection system (Applied Biosystems), and primers for *GAPDH* were described previously (26). Other primer sequences used for PCR were: *MHC II* (sense, 5′-TAT GTG GAC TTG GAT AAG AAG-3′; antisense, 5′-ACA AAG CAG ATA AGG GTG TTG-3′), *CIITA* (sense, 5′-GGC AGC CAG ACC GTG TTC T-3′; antisense, 5′-GCC ATC TTG GGC CTC TAG CT-3′), *Itgae* (sense, 5′-TTG ACT CTT CAG GCG TAG CTT CT-3′; antisense, 5′-CCA CCG TAA AGT TGA GGA ACA TC-3′), and *Mmp12* (sense, 5′-GTG CCC GAT GTA CAG CAT CTT-3′; antisense, 5′-GGT ACC GCT TCA TCC ATC TTG-3′). Supernatants from stimulated DCs were analyzed for IFN-α and IL-12p40 by ELISA using commercially available kits (PBL and BD Pharmingen).

Retroviral transduction

Retroviral pMSCV-puro and pMSCV-ICSBP-puro vectors and preparation of retroviruses were described previously (13). pMSCV-IRF-4-puro was constructed by inserting mouse *IRF-4* cDNA into pMSCV-puro. BM Lin$^-$ cells (2×10^5 cells/ml) from double-knockout (DKO) mice were cocultured in the presence of Flt3L with WT BM mononuclear cells as feeders (1×10^6 cells/ml in the upper chamber) that were separated by microporous membranes using the Cell Culture Inserts system (0.45-μm pore size filter; BD Biosciences). Lin$^-$ cells were transduced with retroviruses by spinoculation (2500 rpm for 1 h at 33°C, twice) in the presence of 4 μg/ml polybrene (Sigma-Aldrich) on days 1 and 2. On day 4, feeder cells were removed, and puromycin (2 μg/ml) was added to select transduced cells. Transduced cells were analyzed on day 9.

Results

DC subset-specific expression of IRF-4 and IRF-8

We first examined the expression of IRF-4 and IRF-8 proteins in splenic DC subsets (Fig. 1). Immunostains of DC-enriched, low density cells showed that in the CD4$^+$ DC subset, IRF-4 was expressed, but IRF-8 was undetectable. Conversely, in CD8α^+ DCs, IRF-8 was abundantly expressed, but IRF-4 expression was almost absent. In the remaining subsets, both IRF-4 and IRF-8 were expressed. DN (CD4$^-$CD8α^-) cells expressed low levels of IRF-4, with a subpopulation of high IRF-8 expression. CD11c^{int}B220$^+$ PDCs expressed high IRF-8 and detectable IRF-4. Together, the expression of IRF-4 and IRF-8 is subset-selective, and DC subsets can be arranged according to the expression patterns of the two factors (Fig. 1).

Selective DC subset deficiency in IRF-4$^{-/-}$ *and* IRF-8$^{-/-}$ *mice*

To study the roles of the two IRFs in DC development, we analyzed *IRF-4*$^{-/-}$, *IRF-8*$^{-/-}$, and *IRF-4*$^{-/-}$*IRF-8*$^{-/-}$ (DKO) mice.

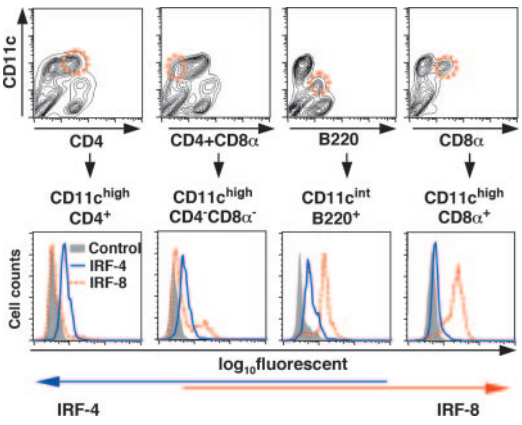

FIGURE 1. Expression of IRF-4 and IRF-8 in DC subsets. *Upper panel*, DC-enriched, low density cells from WT spleens were stained for the indicated surface markers and were analyzed by flow cytometry. *Lower panel*, Cells in the *upper panel* were stained for IRF-4 and IRF-4, and their expression in individual DC subsets (circled in red) is shown.

Fig. 2*A* shows subset analysis among the CD11c^{high} conventional DCs in the spleen. In *IRF-4*$^{-/-}$ mice, the number of CD4$^+$ DCs was severely diminished relative to that in the wild-type (WT) spleen (12.5-fold), while the number of CD8α^+ cells remained unchanged, and that of DN DCs was modestly reduced (Fig. 2*A*). Thus, the total number of CD11c^{high} conventional DCs in *IRF-4*$^{-/-}$ spleen was 2-fold less than that in WT spleen (Fig. 2*C*; CD11c^{high} cells). In contrast, a selective reduction in CD8α^+ DCs was observed in *IRF-8*$^{-/-}$ mice as previously reported (16, 17), whereas the number of CD4$^+$ DCs was only slightly increased, and that of DN DCs was unaffected (Fig. 2*A*). DKO mice retained only DN DCs, but the cell number in this subset was reduced >2-fold relative to that in the WT counterpart. This resulted in a 7.5-fold reduction in conventional DCs in DKO spleens (Fig. 2*C*). Furthermore, these DKO DCs did not express significant levels of MHC II even in the CD11c^{high} population, whereas single IRF-KO DCs showed only a modest reduction (Fig. 2*B*). As a result, the number of CD11c^{high}MHCII$^+$ DCs in DKO spleens fell 26-fold compared with that in WT spleens (Fig. 2*C*). Real-time RT-PCR analysis revealed that MHC class II transcript levels were barely detectable in DKO DCs. This was attributed to impaired expression of the *CIITA* gene that encodes the master transactivator of *MHC II* genes (Fig. 2*D*). These results suggest that the DKO DCs have extensive abnormality.

Previous work showed that IRF-8 plays an important role in PDC development (16, 18). The present analysis revealed that IRF-4 also has a role in PDC development. Namely, CD11c$^+$B220$^+$CD11b$^-$ PDCs were decreased in *IRF-4*$^{-/-}$ spleens by ~50% relative to those in WT spleens (Fig. 3). A greater reduction in PDCs was seen in *IRF-8*$^{-/-}$ spleens, as expected. Supporting the role for IRF-4, the number of PDCs in DKO mice was less than that in *IRF-8*$^{-/-}$ mice. As for the thymus, which does not contain CD4$^+$ DCs, DKO thymi contained few DN DCs and did not have CD8α^+ DCs and PDCs (data not shown). These data indicate that IRF-4 and IRF-8 are required for the development of distinct DC subsets; IRF-4 and IRF-8 are essential for CD4$^+$ DCs and CD8α^+ DCs, respectively. At the same time, both IRFs contribute to the generation of PDCs and DN DCs, suggesting overlapping activities of the two IRFs. These requirements correlate well with their expression patterns (see a model in Fig. 10).

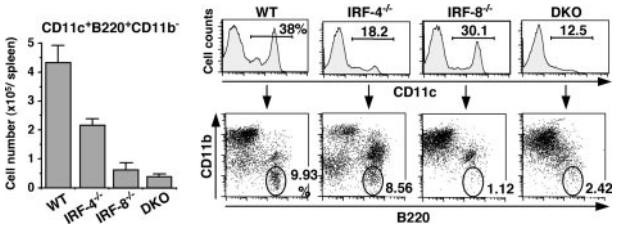

FIGURE 3. Analysis of PDCs in IRF-KO mice. The numbers of CD11c$^+$B220$^+$CD11b$^-$ PDCs per spleen in IRF-null mice (the mean of three experiments ± SD) are shown (*left panel*). A representative flow cytometric analysis is shown in the *right panel*.

Lack of IL-12p40 and IFN-α induction in IRF-8$^{-/-}$ *and DKO DCs*

To assess DC functions in IRF-KO mice, we tested the production of IL-12p40 and IFN-α, two cytokines important for innate immunity. IL-12p40 is a component of IL-12 and IL-23, cytokines that drive Th1 responses, and is reported to be produced in large quantity by CD8α^+ DCs (27). IFN-α is an antiviral cytokine produced primarily by PDCs (5–7). These cytokines are induced in response to TLR signaling. In Fig. 4, freshly isolated splenic CD11c$^+$ DCs were stimulated with LPS, CpG, or poly(I:C), ligands for TLR4, -9, and -3, respectively, and production of the cytokines was measured by ELISA. The cell population of the purified CD11c$^+$ cells was essentially the same as that in the *lower right panel* of Fig. 3. Note that the percentage (but not the absolute number per spleen) of PDCs in *IRF-4*$^{-/-}$ cells was similar to that in WT cells due to the decrease in total CD11c$^+$ cell number in *IRF-4*$^{-/-}$ spleens lacking the CD4$^+$ subset. IL-12p40 and IFN-α were strongly induced in response to TLR stimuli in both WT and *IRF-4*$^{-/-}$ DCs to a comparable level. In contrast, neither cytokine was induced significantly in *IRF-8*$^{-/-}$ and DKO DCs, indicating the importance of IRF-8 for the production of these cytokines.

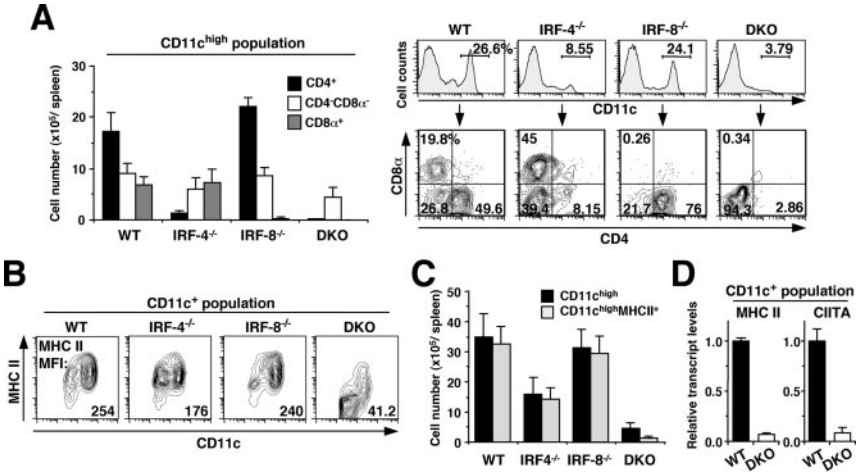

FIGURE 2. Analysis of conventional DC subsets in IRF-KO mice. *A*, *Left panel*, The gated CD11c^{high} cells of low density cell fraction from spleens were analyzed for CD4 and CD8α expression and were compiled for the total number of cells in each DC subset per spleen. The values are the mean of four experiments ± SD. *Right panel*, A representative flow cytometric analysis. *B*, MHC II (I-A^b) expression in gated splenic CD11c$^+$ DCs. The value in each panel indicates the mean fluorescent intensity of MHC II. Data are representative of four independent experiments. *C*, The numbers of total CD11c^{high} and CD11c^{high}MHC II$^+$ cells per spleen. Values represent the mean of four experiments ± SD. *D*, Expression of *MHC II* and *CIITA* transcripts. Splenic CD11c$^+$ DCs were purified by immunomagnetic cell sorting, and RNA expression was analyzed by real-time RT-PCR in duplicate. Values represent relative expression levels normalized by GAPDH.

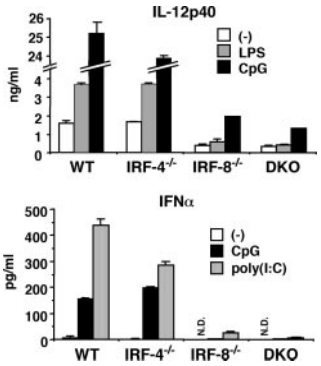

FIGURE 4. Cytokine production in IRF-KO splenic DCs. CD11c$^+$ DCs were purified from spleens by immunomagnetic cell sorting and were stimulated ex vivo at 1×10^6 cells/ml for 14 h with the indicated TLR ligands. Supernatants were tested for the production of IL-12p40 and IFN-α by ELISA in duplicate.

These results are consistent with the selective deficiency in CD8α^+ DCs and PDCs in *IRF-8*$^{-/-}$ mice.

Impaired Th cell priming by IRF-KO DCs

We also assessed the ability of IRF-KO DCs to induce proliferation of T cells. T cells from OTII transgenic mice expressing chicken OVAp-specific TCR were cultured with IRF-KO DCs in the presence of OVAp. When T cells were cultured with WT DCs, most T cells (83%) underwent multiple rounds of cell division in an OVAp-dependent manner (Fig. 5*A*). T cells cultured with *IRF-4*$^{-/-}$ or *IRF-8*$^{-/-}$ DCs led to a modest reduction in the percentage of cells that underwent cell division (74%). However, when cultured with DKO DCs, only 37% of cells underwent cell division, indicating the requirement of IRF-4 or IRF-8 for stimulating T cell proliferative responses.

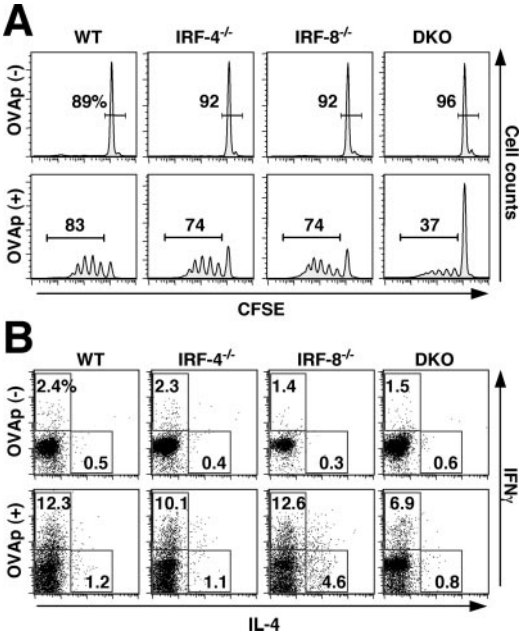

FIGURE 5. T cell responses to IRF-KO DCs. CD11c$^+$ splenic DCs from IRF-KO mice were purified by immunomagnetic cell sorting and were cultured with OVAp and CSFE-labeled CD4$^+$ T cells from OTII transgenic mice for 3 days. T cells were stained for IFN-γ and IL-4 and were analyzed by flow cytometry. *A*, Measurement of proliferation by CSFE dilution. *B*, Intracellular staining for IFN-γ and IL-4.

We also assessed the ability of IRF-KO DCs to regulate T cell differentiation to Th1 or Th2 cells (Fig. 5*B*). Cultures with WT DCs and OVAp generated T cells producing IFN-γ, but not IL-4. This is consistent with the fact that the Th1/Th2 balance is greatly biased to Th1 in the C57BL/6 mouse strain. However, a significant number of IL-4-producing T cells was observed when cultured with *IRF-8*$^{-/-}$ DCs in an OVAp-dependent manner. In contrast, IL-4$^+$ T cells were not generated when cultured with *IRF-4*$^{-/-}$ or DKO DCs. This indicates that 1) the DC subsets retained in *IRF-8*$^{-/-}$ spleen, namely, CD4$^+$ DCs and DN DCs expressing IRF-4, promote Th2 differentiation in the absence of other DC subsets; and/or 2) the DC subsets missing in *IRF-8*$^{-/-}$ spleen, namely, CD8α^+ DCs and/or PDCs, suppress Th2 differentiation. The fact that DKO DCs did not produce IL-4$^+$ T cells supports the requirement of IRF-4 for Th2 differentiation. T cell fractions producing IFN-γ were similar among WT and IRF-4/IRF-8 single-KO mice. The basis by which *IRF-8*$^{-/-}$ DCs produced comparable IFN-γ^+ T cells as WT DCs despite their defects in IL-12 p40 is at present unclear. The lower percentage of IFN-γ^+ cells in T cells cultured with DKO DC is probably due to the lower ratio of Ag-stimulated cells, because the proportions of IFN-γ^+ cells among proliferating T cells were similar between those cultured with WT and DKO DCs. Together, IRF-4 and IRF-8 differentially regulate DC's ability to prime T cell differentiation.

Role of IRF-4/IRF-8 recaptured in DC development in vitro

Several growth factors are capable of stimulating DC development in vivo in a subset-selective manner (28, 29). GM-CSF selectively promotes CD8α^-CD11b^{high} DCs that predominantly express IRF-4, whereas Flt3L expands broader subsets, especially the CD8α^+CD11b^{low} subset and PDCs, in which IRF-8 expression is predominant. Similarly, in vitro, GM-CSF promotes generation of CD11b$^+$ conventional DCs from BM cells, whereas Flt3L supports the development of both CD11b$^+$ conventional DCs and PDCs (24, 30–32). We determined whether DC generation supported by the growth factors depends on respective IRFs.

More than 50% of WT cells that arose in the GM-CSF-based culture (see *Materials and Methods*) were MHC II$^+$CD11c$^+$ (Fig. 6*A*). Although the percentage was lower, *IRF-8*$^{-/-}$ culture also contained MHC II$^+$ cells (~25%). In contrast, the generation of MHC II$^+$CD11c$^+$ cells was essentially absent in *IRF-4*$^{-/-}$ and DKO cultures. *IRF-4*$^{-/-}$ and DKO cells were also defective in CD80 expression and did not induce this molecule and MHC II after stimulation with LPS and CpG (Fig. 6*B*). These results indicate that DC development in the presence of GM-CSF depends on IRF-4, in agreement with the role for IRF-4 in CD4$^+$ DCs in vivo. That CD11c was expressed even in *IRF-4*$^{-/-}$ and DKO cells may indicate that IRF-4 acts presumably after commitment to the DC lineage. As might have been expected, WT DCs generated in this culture system expressed IRF-4, whereas IRF-8 expression was low (Fig. 7).

A nearly opposite situation was observed with Flt3L culture with respect to the roles of IRF-4 and IRF-8, in that the generation of MHC II$^+$CD11c$^+$ DCs from *IRF-8*$^{-/-}$ progenitors was strongly inhibited, whereas that from *IRF-4*$^{-/-}$ cells was not grossly affected (Fig. 6*A*). Induction of MHC II by LPS and CpG and that of CD80 by CpG were also severely affected in *IRF-8*$^{-/-}$ cells, but not in *IRF-4*$^{-/-}$ cells (Fig. 6*B*). In line with the requirement for IRF-8, the majority of WT DCs generated in this culture expressed IRF-8 (Fig. 7). Importantly, however, DKO culture contained many fewer MHC II$^+$ cells than *IRF-8*$^{-/-}$ culture (Fig. 6*A*), indicating a role for IRF-4. Furthermore, MHC II induction by LPS and CpG in DKO cells was almost completely abolished, whereas a small residual activity was noted with *IRF-8*$^{-/-}$ cells (Fig. 6*B*).

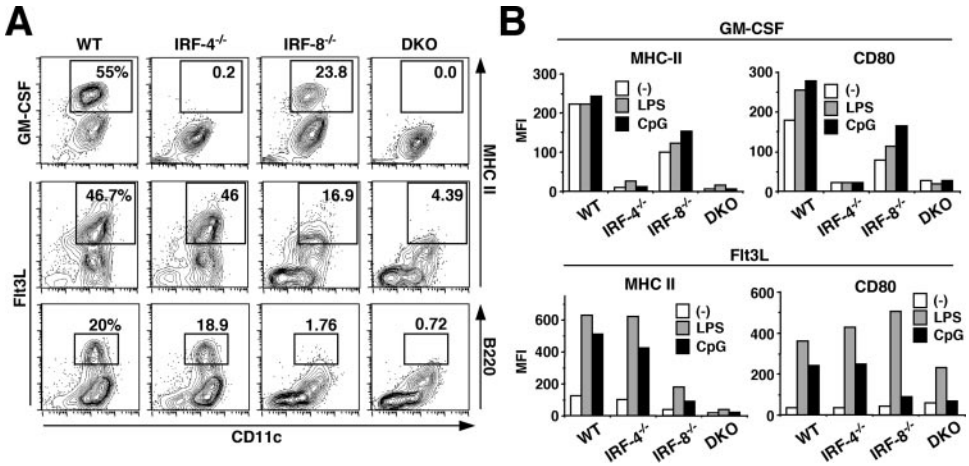

FIGURE 6. In vitro DC development from IRF-KO bone marrow progenitor cells. *A*, DCs were generated from WT or IRF-null BM progenitor cells in the presence of either GM-CSF, SCF, and TNF-α for 12 days or Flt3L for 9 days. Cells were analyzed by flow cytometry for the expression of CD11c, MHC II (I-A^b), and B220. *B*, The expression of MHC II and CD80 in BM-derived DCs in response to TLR ligands. Cells were stimulated with LPS or CpG during the final 24 h of culture and were analyzed for the expression of MHC II and CD80. The values indicate the mean fluorescent intensity. Data are representative of three independent experiments.

The role of IRF-4 in Flt3L-mediated DC development was also supported by the observation that *IRF-4*$^{-/-}$ DCs developed in Flt3L culture contained fewer CD11b^{high} cells than WT DCs (Fig. 8). Notably, cells in this population were *IRF-4*$^+$*IRF-8*$^-$ (Fig. 7), reminiscent of CD11b^{high}CD8α^- (CD4$^+$ and DN) DCs in vivo. It is of note that CD4 and CD8α marker expression was extinguished in culture, making it impractical to directly assess the generation of CD4$^+$ and CD8α^+ DCs under these in vitro conditions. In the Flt3L culture, a modest, but discernible, contribution of IRF-4 to PDC development, noted in vivo (Fig. 3), was recaptured; although a few PDCs were generated from *IRF-8*$^{-/-}$ cells (but fewer than WT and *IRF-4*$^{-/-}$ cells), essentially no PDCs were generated from DKO cells (Fig. 6*A*). We noted that a substantial fraction of *IRF-8*$^{-/-}$ and DKO cells derived in the presence of Flt3L were CD11c$^-$, suggesting that IRF-8 may have a role in a relatively early stage of DC differentiation.

These results show that DC development in the presence of GM-CSF depends on IRF-4, whereas that in the presence of Flt3L mainly depends on IRF-8 to generate distinct DC subtypes, paralleling their subset-selective role in vivo (see a model in Fig. 10).

Common activities of IRF-4 and IRF-8

From the above results one can envisage that IRF-4 and IRF-8, in addition to acting on common targets, might act on genes that specify phenotypic properties of distinct DC subsets. To begin studying common and specific activities of IRF-4 and IRF-8, we investigated the development of DKO DCs after reconstitution with IRF-4 or IRF-8. Lin$^-$ DKO BM progenitor cells transduced with IRF-4 or IRF-8 vector were allowed to develop in the presence of Flt3L. Previous data presented in Figs. 6–8 indicated that Flt3L supports the generation of conventional DCs and PDCs, in which both IRF-4 and IRF-8 play roles.

Introduction of either IRF-4 or IRF-8, but not control vector, rescued the generation of MHC II$^+$CD11c$^+$ DCs, concomitant with the rescue of *CIITA* expression (Fig. 9, *A* and *C*). IRF-transduced DCs also gained the ability to up-regulate MHC II and CD80 expression in response to LPS (Fig. 9*B*), indicating that the two IRFs have a common activity to support basic DC differentiation accompanying the expression of MHC II and CD80. Although the responsiveness to LPS was restored by either IRF, only IRF-8 efficiently restored CpG induction of these markers, indicating that the establishment of the TLR9 signaling pathway in DCs requires IRF-8, consistent with our recent findings (33).

Introduction of IRF-8 or IRF-4 resulted in the generation of B220$^+$ DCs as well, although the latter was less efficient than the former (Fig. 9*A*). This suggests that the two factors have the potential to direct the development PDCs, as defined by B220 expression. In support of this, introduction of either factor into *IRF-8*$^{-/-}$ progenitor cells generated B220$^+$ cells, although IRF-4 was again less efficient than IRF-8 (histograms not shown, percentages of B220$^+$ cells in MSCV-, IRF-8-, or IRF-4-transduced culture were 1.96, 9.15, and 6.3%, respectively). We noted in these experiments that the frequency of B220$^+$ DCs generated from DKO and *IRF-8*$^{-/-}$ cells upon IRF introduction was lower than that in WT culture. The limited restoration of PDC development by IRF introduction may be explained by the fact that ectopic IRF-8 protein expressed in PDCs was ~3-fold lower than that of endogenous IRF-8 (data not shown). The level of ectopic IRF-4 was, in contrast, ~2 times higher than that of endogenous IRF-4, again suggesting that endogenous IRF-4 has a relatively minor role in PDC development.

In agreement with what was observed for the Flt3L culture, MHC II and CD80 expression was rescued by either IRF-4 or IRF-8 in the presence of GM-CSF as well (data not shown).

Specific activities of IRF-4 and IRF-8

We next assessed whether IRF-4 and IRF-8 activate genes specific for distinct DC subsets. We found that only IRF-8, but not IRF-4, induced *integrin* α_E (*Itgae*) transcripts, the gene specifically expressed in CD8α^+ DCs (34), but not in CD4$^+$ or DN DCs (Fig. 9*C*). In contrast, only IRF-4, but not IRF-8, induced *matrix metalloproteinase 12* (*Mmp12*) gene expression, which was shown to be expressed more abundantly in CD4$^+$ DCs than in CD8α^+ DCs (35). Although the roles of these genes in DCs are not yet fully clarified, they may be important for DC tissue localization and migration. Integrin α_E is a subunit of integrin $\alpha_E\beta_7$ and was originally found to be expressed in some T cells. Integrin $\alpha_E\beta_7$ binds to E-cadherin expressed on epithelial cells and is indispensable for generating or maintaining mucosal T cells (36). Mmp-12 is an enzyme that degrades extracellular matrix and is essential for tissue migration of macrophages (37). Thus, the two IRFs direct the expression of subtype marker genes as well as those that characterize the functionality of DC subtypes.

We found that introduction of IRF-8, but not IRF-4, into DKO cells efficiently restored the induction of IL-12p40 after stimulation with LPS or CpG (Fig. 9*D*). Similarly, IRF-8, but not IRF-4, conferred the ability to produce IFN-α upon DKO cells in response to CpG and poly(I:C). This result was striking, given the potential of IRF-4 to generate B220$^+$ DCs, suggesting that PDCs generated by IRF-4 and IRF-8 are functionally distinct. These results are in

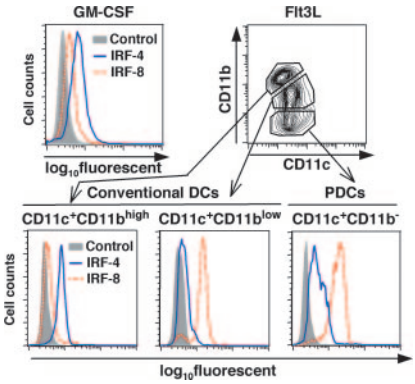

FIGURE 7. Analysis of IRF-4/IRF-8 expression in BMDCs. DCs generated from WT BM mononuclear cells in the presence of GM-CSF or Flt3L were stained with CD11c, CD11b, IRF-4, and IRF-8 as described in Fig. 1. For Flt3L-mediated BMDCs, the expression profiles of IRF-4 and IRF-8 in two subpopulations of conventional DCs ($CD11c^+CD11b^{high}$ and $CD11c^+CD11b^{low}$) and a PDC ($CD11c^+CD11b^-$) population are shown.

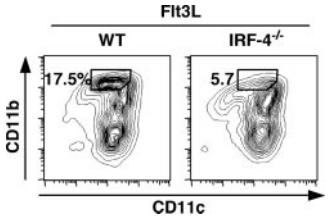

FIGURE 8. A reduction in the $CD11b^{high}$ subpopulation in *IRF-4*$^{-/-}$ BMDCs generated in the presence of Flt3L. BMDCs generated in the presence of Flt3L from WT and *IRF-4*$^{-/-}$ mice were analyzed for CD11c and CD11b expression. The percentages of $CD11c^+CD11b^{high}$ population are shown. Data are representative of three independent experiments.

agreement with data shown in Fig. 4, in that IRF-8, but not IRF-4, is required for the production of these cytokines in splenic DCs. The unique role of IRF-8 observed in DKO cells is corroborated by results with *IRF-8*$^{-/-}$ cells, in which induction of the cytokines was rescued by introduction of IRF-8, but not IRF-4 (data not shown). Together, the ability of IRF-8 to stimulate the production of these cytokines is a property not replaceable by IRF-4, verifying that DCs induced by IRF-4 and those induced by IRF-8 are functionally divergent.

Discussion

Our results show that IRF-4 and IRF-8 are differentially expressed in DC subsets and are required for their development. The two IRFs together affect the development of all conventional DCs as well as PDCs (Fig. 10). We also showed that IRF-4 and IRF-8 have both common and distinct activities to promote shared as well as subset-specific pathways of gene expression, thus illustrating a central role for the two IRFs in DC development.

Common activities of IRF-4/IRF-8

The following evidence indicates that the two IRFs have common activities. 1) Although single IRF-KO mice showed subset-selective defects, DKO mice were deficient in all splenic DC subsets, including the DN subset and PDCs, the subsets regulated by both IRF-4 and IRF-8. 2) Both IRFs, when introduced into DKO progenitor cells, promoted the differentiation of conventional DCs as well as PDCs. The two IRFs enhanced the ability to respond to LPS and up-regulated the expression of MHC II and costimulatory molecules. Thus, both IRFs are capable of inducing molecules that provide features common in all DCs, such as MHC II, CD11c and costimulatory molecules, as well as those that help specify features of PDCs, such as B220. This indicates that IRF-4/IRF-8, through their common activities, stimulate the basic process of the development of conventional DCs as well as PDCs (Fig. 10, vertical axis). The activities common in IRF-4/IRF-8 may represent a mechanism by which DCs, as a cell type, acquire common features despite their heterogeneity.

That the two IRFs have shared activities may not be surprising, given their extensive structural similarity in the N-terminal DNA binding domain (DBD) and the C-terminal IRF association domain (IAD). Indeed, these two IRFs share partner proteins with which they interact. An Ets transcription factor, PU.1, is a plausible candidate for a common partner that participates in DC development. This factor is essential for the development of myeloid cells, B cells, and DCs. PU.1 is reported to be essential for $CD8\alpha^-$ DCs, although another report indicates that PU.1 is important for other types of DCs as well (38, 39).

Selective activities of IRF-4/IRF-8

The two IRFs displayed clear subset selectivity, in that the development of $CD4^+$ DCs required IRF-4, whereas that of $CD8\alpha^+$ DCs required IRF-8. We also found that DC generation in vitro in the presence of GM-CSF depended on IRF-4, whereas that in Flt3L mainly relied on IRF-8. Interestingly, the subtype-selective roles of the IRFs correlated well with their subtype-specific expression profile, indicating that the differential expression of the IRFs constitutes a basis of their selective role. However, our observations also emphasize that IRF-4 and IRF-8 have intrinsically separate activities and act on separate target genes. For example, introduction of IRF-4 and IRF-8 induced subtype-selective genes such as *Itgae* and *Mmp12*, respectively, without substituting for each other. Another striking example of selective roles of IRF-4/IRF-8 was the expression of IL-12p40 and IFN-α, cytokines essential for innate immunity, in that only IRF-8, not IRF-4, imparted the capacity to induce these cytokines upon transduced DCs. Together, IRF-4 and IRF-8 most likely achieve their selective activities through their subtype-selective expression pattern and their ability to regulate distinct target genes (Fig. 10, horizontal axis). In view of previous findings that IRF-8 enhances *IL-12p40* promoter activity in macrophages (40–42), it is possible that this factor directly activates *IL-12p40* transcription in DCs as well. The basis by which IFN-α production in PDCs requires IRF-8 is currently unknown. Because IRF-3 and IRF-7 are required for *IFN-*$\alpha\beta$ promoter activities in other cell types, it will be of importance to determine how IRF-8 participates in the induction of *IFN-*α transcription in DCs.

Our current study also suggests that IRF-4 and IRF-8 inversely affect the Th1/Th2 balance by regulating DCs. In agreement with this, it has been suggested that $CD8\alpha^+$ DCs drive Th1 responses, whereas $CD8\alpha^-$ DCs stimulate Th2 responses (43–45). Furthermore, *IRF-4*$^{-/-}$ mice are biased to Th1 responses (defective in mounting Th2) (46, 47), whereas *IRF-8*$^{-/-}$ mice are biased to Th2 responses (impaired in developing Th1 responses) (reviewed in Ref. 14). In addition to the impairment in DCs, *IRF-4*$^{-/-}$ T cells have intrinsic defects in undergoing Th2 differentiation (46, 47), whereas *IRF-8*$^{-/-}$ T cells have the capacity for normal Th1/Th2 differentiation (14).

That IRF-4 and IRF-8 play separate roles may be explained by the fact that IRF-4 and IRF-8 have notable amino acid sequence differences in the region between the DBD and the IAD as well as within the IAD. Through these differences, the two factors may

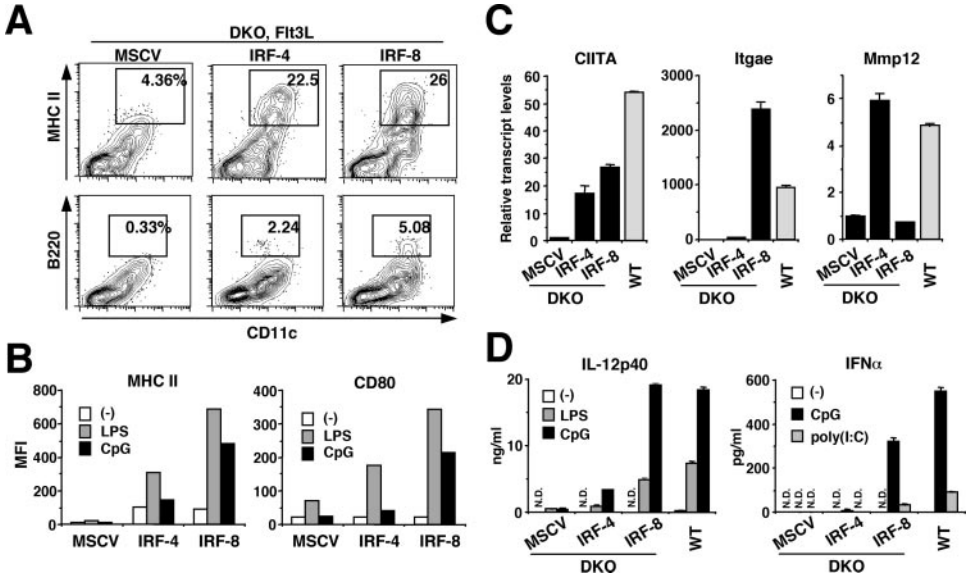

FIGURE 9. Restoration of DC phenotypes after IRF-4/IRF-8 gene transfer. *A*, DKO BM Lin$^-$ cells were transduced with control MSCV-puro, MSCV-IRF-8, or MSCV-IRF-4 retrovirus; cultured in the presence of Flt3L; and analyzed for the expression of CD11c, MHC II, and B220 on day 9. *B*, Transduced cells were stimulated with LPS or CpG for 24 h and were analyzed for the expression of MHC II and CD80. *C*, Transduced cells were analyzed for the expression of *CIITA*, *integrin* α_E (*Itgae*) and *Mmp12* by real-time RT-PCR in duplicate. *D*, Transduced cells were stimulated with LPS, CpG, or poly(I:C) for 24 h at 1×10^6 cells/ml. Supernatants were analyzed by ELISA for production of IL-12p40 and IFN-α in duplicate. N.D., not detected. Data are representative of at least three independent experiments.

interact with different partners, gaining different DNA binding specificity, thus activating their own sets of target genes. For example, it was shown that IRF-8, but not IRF-4, interacts with IRF-1 and IRF-2 (22). It is possible that IRF-8 cooperates with IRF-1 and/or IRF-2 to stimulate selective genes, as exemplified for *IL-12p40* (41).

Mode of IRF-4/IRF-8 actions

Our results indicate that the activities of IRF-4 and IRF-8 are cell-intrinsic, because 1) the requirement of IRF-4/IRF-8 for subset development correlated with their subset-specific expression both in vivo and in vitro; 2) defects in DKO cells were not amended by coculture with WT BM cells (see *Materials and Methods*); and 3) upon gene transfer, the IRFs rescued DC development, inducing genes specifying DC functions that were not expressed in IRF-KO mice.

The central roles that IRF-4 and IRF-8 play in directing DC development revealed in this work pose a question as to how the expression of the two IRFs is regulated at the progenitor cell level. Recently, it was shown that STAT3 is activated by Flt3L and is indispensable for the promotion of DC development mediated by Flt3L, but not by GM-CSF (48). This work is interesting in view of the dependence on IRF-8 in DC development in the presence of Flt3L. Because the *IRF-8* promoter has an active IFN-γ-activation site, STAT3 may link Flt3L signaling to IRF-8. In contrast, the *IRF-4* gene has functional κB elements (49). Interestingly, RelB, a member of the NF-κB/Rel family, is expressed in CD4$^+$ and DN DCs, but is low in CD8α^+ DCs (35), and is important for the development of CD4$^+$ DCs (50, 51). This raises the possibility that RelB might regulate IRF-4 expression. Other transcription factors shown to play roles in DC development include Ikaros C and Id2, which are required for CD8α^- DCs and CD8α^+ DCs, respectively (52, 53). Clarifying the relationship between these transcription factors may shed additional light on the DC developmental program.

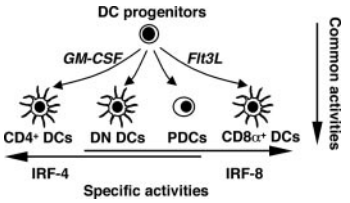

FIGURE 10. A model for the roles of IRF-4 and IRF-8 in DC development. DC subsets can be arranged according to the expression of and requirement for the IRFs. Through common and specific activities, the two IRFs together regulate development of the conventional DC subsets as well as PDCs. The roles of IRF-4/IRF-8 are recaptured in GM-CSF- and Flt3L-mediated DC development in vitro.

Recently, IRF-2 was reported to be critical for the development of CD4$^+$ DCs (54, 55). IRF-2 acts by inhibiting IFN-$\alpha\beta$ suppression of DC development. The mode of IRF-2 actions, however, seems quite different from that of IRF-4/IRF-8 actions. First, IRF-2 expression is ubiquitous among various cell types, whereas IRF-4/IRF-8 expression is hemopoietic cell-specific and DC subset-selective. CD4$^+$ DCs express IRF-4 that does not interact with IRF-2, but not IRF-8 that does interact with IRF-2. Second, the fact that IRF-4/IRF-8 stimulate different genes to direct development of divergent DC subtypes indicates that they manifest their specific activities independently of IFN-$\alpha\beta$ signaling. Furthermore, the C-terminal region of IRF-2 shares little similarity with that of IRF-4/IRF-8, and no common interacting proteins have been identified. Nonetheless, it is clear that the IRF family plays multiple roles in DC development through diverse processes. After we completed this work, another group reported that IRF-4 is required for the development of CD4$^+$ DCs, consistent with our results (56).

Defining DC subtypes by IRF-4 and IRF-8

Our results suggest that DC subtypes, regardless of whether they are generated in vivo or in vitro, can be distinguished by the expression and requirement of IRF-4/IRF-8. Defining DC subtypes by IRF expression may be more practical than by classifying them by surface markers alone, because expression of the IRFs indicates an underlying "cause" of the generation of DC subtype and their functions, rather than a "result" represented by surface markers. The role of IRF-4/IRF-8 in human DC subtypes is less clear than that in mouse DCs at present. However, it is noteworthy that *IRF-4* and *IRF-8* transcripts are expressed in human DCs as well (57, 58) (our unpublished observations). *IRF-4* was shown to be significantly up-regulated in human monocytes during DC differentiation (59). These data may support the possibility that IRF-4/IRF-8 play similar roles in human DC subsets as in the mouse subsets. Given

that DCs are a promising tool for immunotherapy of various diseases, examining IRF-4/IRF-8 expression in DCs prepared in various protocols may help reveal DC's functional parameters. Additionally, by introducing IRF-4/IRF-8 through gene transfer, it may become possible to modify the function of DCs.

In conclusion, IRF-4 and IRF-8, by acting in common and separate manners, serve as a backbone of DC development. Our work provides a new framework for understanding the molecular bases of DC subset development and their functional diversity.

Acknowledgments

We thank Tak W. Mak for *IRF-4*$^{-/-}$ mice, and Pratima Thotakura, Rebecca Kahane, and Ranmal Samarasinghe for technical assistance.

Disclosures

The authors have no financial conflict of interest.

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