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Plasmacytoid Dendritic Cells Activate Lymphoid-Specific Genetic Programs Irrespective of Their Cellular Origin

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Summary

The developmental origin of type I interferon (IFN)-producing plasmacytoid dendritic cells (PDCs) is controversial. In particular, the rearrangement of immunoglobulin heavy chain (IgH) genes in murine PDCs and the expression of pre-T cell receptor α (pTα) gene by human PDCs were proposed as evidence for their "lymphoid" origin. Here we demonstrate that PDCs capable of IFN production develop efficiently from both myeloid- and lymphoid-committed progenitors. Rearranged IgH genes as well as RAG transcripts were found in both myeloid- and lymphoid-derived PDCs. The human pTα transgenic reporter was activated in both myeloid- and lymphoid-derived PDCs at a level comparable to pre-T cells. PDCs were the only cell population that activated murine RAG1 knockin and human pTα transgenic reporters outside the lymphoid lineage. These results highlight a unique developmental program of PDCs that distinguishes them from other cell types including conventional dendritic cells.

Introduction

Dendritic cells (DCs) are one of the most important cell types in the immune system due to their unique ability to orchestrate an acquired immune response (Banchereau and Steinman, 1998). Murine DCs have been classified into two populations, myeloid and lymphoid, on the basis of anatomic localization, cellular origin in transplantation studies, and cell surface phenotypes (Shortman and Liu, 2002). In murine hematopoiesis, early studies showed that $CD11c^+MHCII^+CD11b^+CD8\alpha^-$ "myeloid" DCs can be efficiently derived from myeloid progenitors along with granulocytes and macrophages (Inaba et al., 1993), whereas $CD11c^+MHCII^+CD11b^-CD8\alpha^+$ "lymphoid" DCs can arise from thymic T cell progenitors (Ardavin et al., 1993). Further analyses revealed that DCs of these two phenotypes can originate both from the earliest myeloid progenitors (common myeloid progenitors, CMPs [Akashi et al., 2000]) and from the earliest lymphoid progenitors (common lymphoid progenitors, CLPs [Kondo et al., 1997]) (Traver et al., 2000). The potential to develop into the DC lineage is maintained in pro-T cells and granulocyte/monocyte progenitors (GMPs) but is absent in pro-B cells or megakaryocyte/erythrocyte progenitors (MEPs) (Manz et al., 2001; Wu et al., 2001), although some DC potential may be retained in more primitive B cell precursors (Izon et al., 2001). Thus, DC developmental potential mainly resides within early granulocyte/monocyte and T lymphoid developmental pathways.

Plasmacytoid dendritic cells (PDCs) were initially identified in humans (Cella et al., 1999; Grouard et al., 1997; Jarrossay et al., 2001; Kadowaki et al., 2000, 2001). These cells morphologically resemble antibody-secreting plasma cells and were therefore termed PDCs. Upon activation, they quickly acquire mature DC morphology and phenotype without significant cell proliferation (Liu, 2001; Shortman and Liu, 2002). PDCs secrete massive amounts of type I interferons (IFN-α and -β) when stimulated by unmethylated CpG DNA or by viruses (Cella et al., 1999; Grouard et al., 1997; Jarrossay et al., 2001; Kadowaki et al., 2000, 2001; Siegal et al., 1999) through the function of Toll-like receptors such as TLR7 and TLR9 (Kadowaki et al., 2001) and are thus believed to be critical mediators of antiviral immune response. PDCs also play a role in adaptive immunity by directing either T helper cell type 1 (Th1) or Th2 development (Boonstra et al., 2003), as well as by generating T regulatory cells that induce T cell tolerance (Gilliet and Liu, 2002). Recently, a murine counterpart of human PDCs has been identified and shown to exhibit similar functional properties (Asselin-Paturel et al., 2001; Bjorck, 2001; Martin et al., 2002; Nakano et al., 2001). Like human PDCs, mouse PDCs express high levels of CD45RA (B220) and low levels of MHC class II (MHCII). Mouse PDCs express low levels of the dendritic cell marker CD11c, and the majority of them are $CD8\alpha^+$. In addition, mouse PDCs are $Ly\text{-}6C^+$, a myeloid cell marker recognized by the Gr-1 antibody that also reacts with Ly-6G on mature granulocytes and monocytes.

The lineage origin of PDCs is controversial. On the one hand, murine PDCs have been reported to carry rearranged immunoglobulin heavy chain (IgH) D-J but not T cell receptor (TCR) Dβ-Jβ loci (Corcoran et al., 2003). Another apparently lymphoid characteristic of PDCs is the expression of the pre-T cell receptor α (pTα) in the human thymic PDCs (Bendriss-Vermare et al., 2001; Res et al., 1999). The pTα protein pairs with the TCRβ chain to form the pre-TCR (Groettrup et al., 1993)

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and plays a critical role in the efficient generation of mature T cells (Fehling et al., 1995). Thus, PDCs manifest classical lineage markers of both T and B cells, which was proposed to reflect their lymphoid past (Corcoran et al., 2003). On the other hand, human PDCs can be generated at least from myeloid progenitors expressing receptors for macrophage colony-stimulating factor (M-CSF) (Olweus et al., 1996). Mice deficient in ICSBP, a critical transcription factor for monocyte development (Tamura et al., 2000), display the loss of PDCs and CD8α^+ DCs (Aliberti et al., 2003; Schiavoni et al., 2002), which can be restored by enforced expression of ICSBP (Tsujimura et al., 2003). A recent report showed that lymphoid progenitors give rise to cells of B220$^+$CD11c^{lo} PDC phenotype more efficiently than myeloid progenitors (D'Amico and Wu, 2003). In this study, however, the function of the resulting cells was not evaluated. It is thus still unclear whether the lymphoid characteristics of PDCs reflect their lymphoid origin.

We have identified and purified progenitors of myeloid or lymphoid-restricted potentials including CMPs and CLPs (Akashi et al., 2000; Kondo et al., 1997). In the current study, we analyzed directly the PDC potentials of these progenitor populations. Our data show that lymphoid-restricted CLPs and pro-T cells as well as myeloid-restricted CMPs and GMPs can generate IFN-producing PDCs with IgH D-J rearrangement. Furthermore, recombination activation gene-1 (RAG1) transcripts were found in both myeloid and lymphoid PDCs. The majority of PDCs including CMP-derived PDCs activated RAG1 locus in mice carrying a knockin reporter for murine RAG1. Furthermore, in transgenic mice carrying a human pTα reporter construct, human pTα was strongly activated in PDCs irrespective of their lymphoid or myeloid origin. Taken together, our data demonstrate that IFN-producing PDCs can originate from both lymphoid and myeloid pathways and that the lymphoid characteristics of PDCs represent a unique "ectopic" activation of the lymphoid programs. PDC-specific activation of the human pTα reporter in murine hematopoiesis in turn suggests that previously defined murine PDCs are faithful counterparts of human PDC subsets.

Results

PDC Potentials of Murine Progenitors Correlate with Their Conventional DC Potentials

We estimated the relative contribution of myeloid and lymphoid pathways to PDC development by in vivo reconstitution assays. Myeloid- and lymphoid-committed progenitors including CMPs, GMPs, and CLPs (CD45.2) were purified and transplanted into C57B6-CD45.1 mice along with 300 host-type (CD45.1) Sca-1$^+$c-Kit$^+$ hematopoietic stem cells (HSCs) (Spangrude et al., 1988) to provide radioprotection. Myeloid or lymphoid differentiation potentials of these lineage-restricted progenitors have been described, previously (Akashi et al., 2000; Kondo et al., 1997; Na Nakorn et al., 2002). We injected 2×10^4 CLPs or CMPs, and analyzed their PDC progeny on day 10, 15, and 20 on FACS by using the phenotypic definition of Lineage (Lin: CD19, CD3, NK1.1, TCRβ, and sIgM) -negative, B220$^+$, and CD11c$^+$ (Asselin-Paturel et al., 2001; Bjorck, 2001; Nakano et al., 2001). The highest number of PDC progeny was obtained on day 15 in both cases. In both CMP- and CLP-injected mice, CD11c$^+$ donor-derived progeny (CD45.2$^+$) in the spleen contained a significant fraction of B220$^+$ PDCs as well as B220$^-$ DCs (Figure 1A). PDC and DC development was observed also in mice injected with 1×10^5 GMPs or 1×10^4 pro-T cells, whereas 1×10^5 MEPs or pro-B cells did not give rise to detectable numbers of PDC or DC subsets (Figure 1A and data not shown). Thus, PDC development appears similar to conventional DC development (Manz et al., 2001; Traver et al., 2000; Wu et al., 2001) in that they can be efficiently derived from both granulocyte/monocyte and early T lymphoid pathways.

A recent study showed that a circulating population expressing CD11c and B220 but not MHCII can give rise to PDCs, CD8α^+ DCs, and CD8α^- DCs (del Hoyo et al., 2002). CD11c$^+$I-A^{b-} DC precursors expressing B220 and CD11b were also found in the blood of mice injected with CMPs or CLPs (Figure 1A, right). These CMP- or CLP-derived CD11c$^+$I-A^{b-} DC precursors gave rise to B220$^+$CD11c$^+$ PDCs in the culture with Flt-3L (Brawand et al., 2002) for 2 days (data not shown), suggesting that at least a fraction of PDCs can originate from CMPs and CLPs via the common DC precursor stage.

Figure 1B shows phenotypic characteristics of CMP- or CLP-derived PDCs and DCs. Within the CD11c$^+$ DC fraction in mice reconstituted with CMPs, Gr-1 expression was only found in B220$^+$ PDCs. CMP-derived PDCs expressed only negative to low levels of CD11b and DC maturation/activation markers such as CD40, CD80, and CD86. A majority of PDCs expressed CD4 and CD8α. In both CMP- and CLP-derived PDCs, the expression level of I-A^b was relatively low as compared to B220$^-$ DCs. Thus, phenotypic characteristics of CMP- and CLP-derived PDCs were identical to those of B220$^+$CD11c$^+$ PDCs isolated from the normal spleen (Figure 1B, bottom). Purified B220$^+$CD11c$^+$ cells derived from CMPs or CLPs produced significant amount of IFN-α upon exposure to herpes simplex virus (HSV) in vitro, indicating that they are functional PDCs (Figure 2A).

On a per cell basis, transplanted CMPs and CLPs were more efficient than GMPs and pro-T cells for both PDC and DC production (Table 1). CMPs and CLPs gave rise to 2- to 3-fold higher numbers of conventional DCs as compared to PDCs. Total mouse bone marrow cells contains ~10-fold more CMPs than CLPs. Accordingly, the estimated contribution of myeloid progenitors (CMPs and GMPs combined) to the PDC pool was ~95% and ~30% in the spleen and the thymus, respectively (Table 1).

Comparison of Lineage-Specific Gene Expression in Myeloid and Lymphoid PDCs

We next tested the myeloid or lymphoid characteristics of CMP- and CLP-derived PDCs and DCs by evaluating lineage-related gene expression by semiquantitative RT-PCR (Akashi et al., 2000). As shown in Figure 2B, all PDC and DC populations expressed Flt-3 and myeloid cytokine receptors such as IL-3Rα and GM-CSFRα at similar levels. PDCs expressed higher levels of IL-7Rα, an essential cytokine receptor for T and B cell development (Akashi et al., 1997; Peschon et al., 1994), regardless of their myeloid or lymphoid origin. Both lymphoid-

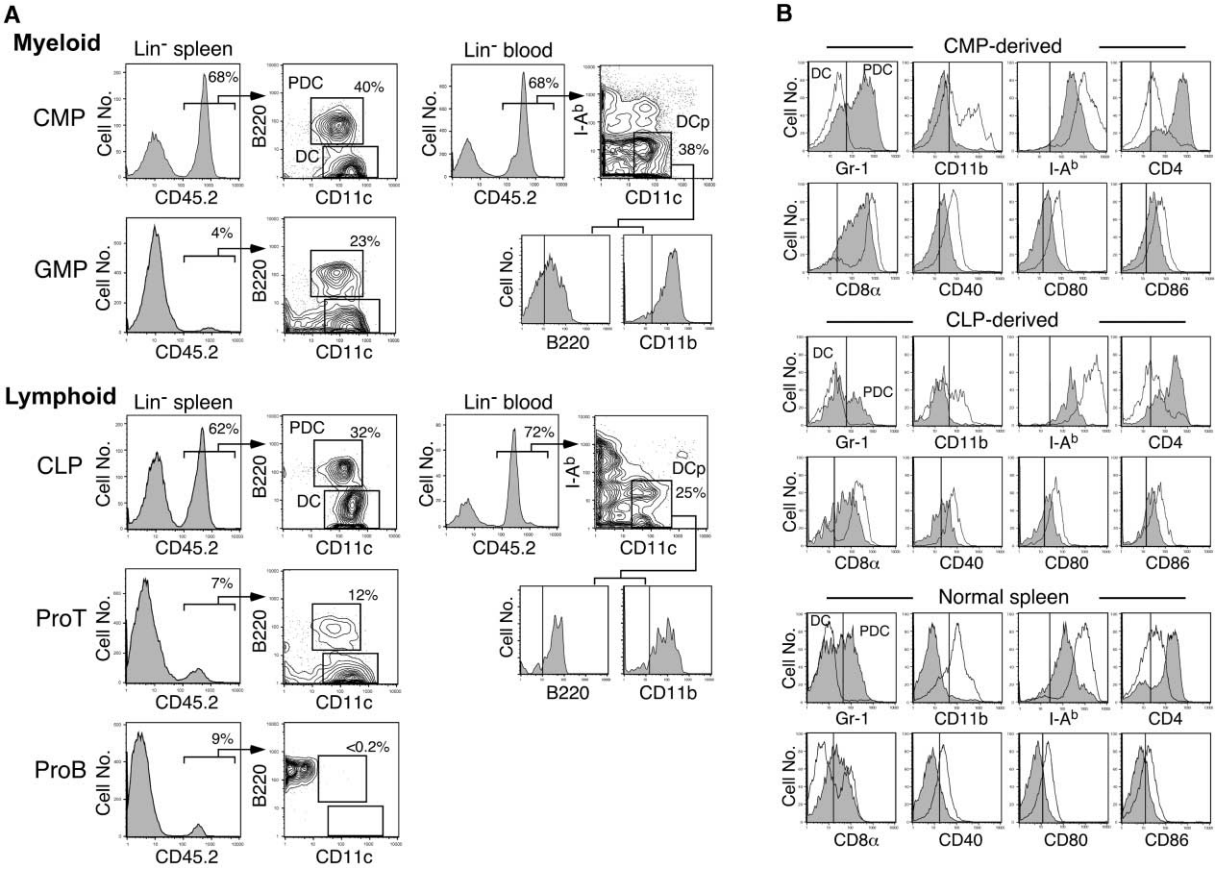

Figure 1. Development of PDCs from Myeloid and Lymphoid Progenitors In Vivo

(A) DC analyses 15 days after transplantation of each purified progenitor subset are shown. Spleen cells were enriched for positive expression of CD11c, and blood cells were enriched for negative expression of CD3 and CD19 prior to the phenotypic analyses (see Experimental Procedures). Lin (CD19, CD3, NK1.1, TCRβ, and sIgM)$^-$ CD45.2$^+$ cells in the spleen were analyzed for B220 and CD11c. Either transplanted CMP, GMP, CLP, or pro-T cells differentiated into both PDCs and DCs in the spleen (left panels). The CD11c$^+$MHCII$^-$ DC precursor population was detectable in the Lin$^-$ blood of mice reconstituted with CMPs or CLPs (right panels). CD11c$^+$MHCII$^-$ DC precursors expressed B220 and CD11b.

(B) DC/PDC-related antigen expression of CMP- or CLP-derived PDCs (closed histogram) and conventional DCs (open histogram) is shown. CMP, common myeloid progenitor; GMP, granulocyte/monocyte progenitor; CLP, common lymphoid progenitor; DCp, DC precursor.

and myeloid-derived PDCs expressed higher amounts of TLR9 (Kadowaki et al., 2001; Okada et al., 2003) which recognizes unmethylated CpG DNA leading to IFN-α production.

PU.1 and RelB are essential for CD8α$^-$ DC development (Guerriero et al., 2000; Wu et al., 1998), whereas ICSBP and Id2 are required for CD8α$^+$ DC generation (Aliberti et al., 2003; Hacker et al., 2003). ICSBP is essential also for PDC development (Schiavoni et al., 2002). All of these transcription factors were expressed in both PDCs and DCs irrespective of their origin (Figure 2B). Pax-5 and GATA-3, the master B and T lymphoid transcription factors, respectively (Nutt et al., 1997; Ting et al., 1996), were not expressed in any DC or PDC populations. Spi-B, a myeloid/B lymphoid transcription factor (Ray et al., 1992) was expressed only in PDCs as previously reported (Bendriss-Vermare et al., 2001). Notch-1, which plays a critical role in αβT cell commitment and differentiation (Deftos et al., 2000; Robey, 1999), was expressed in PDCs at higher levels than DCs. Spi-B and Notch-1 were expressed in both CMP- and CLP-derived PDCs at similar levels. Thus, the expression patterns of these dendritic cell-related genes were similar in myeloid- and lymphoid-derived PDCs.

Myeloid- and Lymphoid-Derived PDCs Rearrange IgH D-J Locus

We then tested the rearrangement status of IgH D-J genes in myeloid- and lymphoid-derived PDCs. Consistent with a previous report (Corcoran et al., 2003), freshly isolated PDCs but not DCs possessed IgH D-J rearrangements (Figure 2C, top). Purified Gr-1$^+$ or CD4$^+$ Lin$^-$B220$^+$CD11c$^+$ PDCs were also rearranged IgH genes (Figure 2C, bottom), indicating that the IgH rearrangements should not be due to contamination of B cells within the B220$^+$CD11c$^+$ PDC gate. TCR Dβ-Jβ gene rearrangement was not detected in PDCs or DCs (data not shown). CMPs and CLPs did not rearrange IgH genes (Figure 2C, top). Interestingly, a significant fraction of both CMP-derived and CLP-derived PDCs rearranged IgH D-J genes (Figure 2C, top), indicating

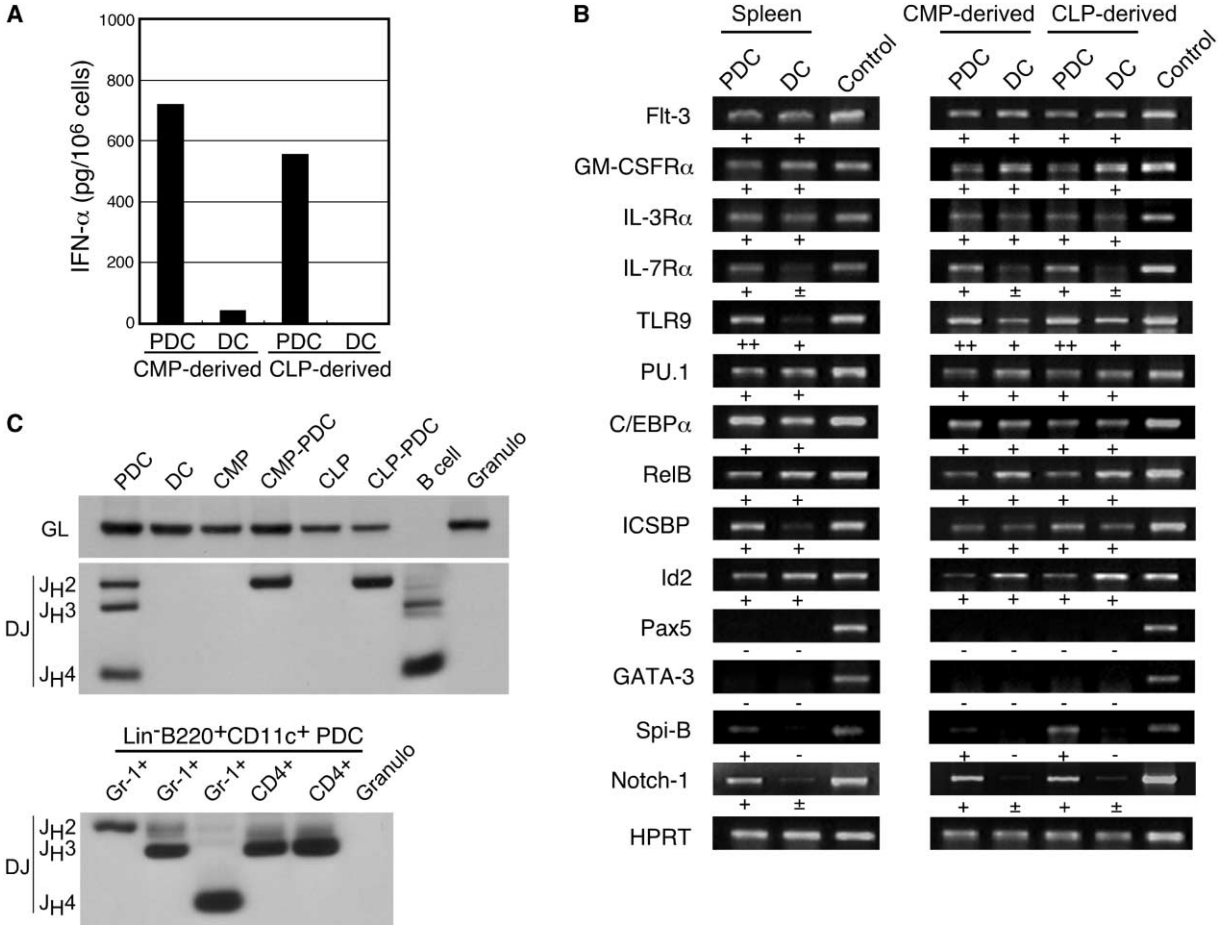

Figure 2. IFN Production and Gene Expression Profiles of PDCs and DCs of Myeloid or Lymphoid Origin

(A) IFN-α levels in the supernatant of PDCs cultured with HSV. Note that only PDCs produce significant amounts of IFN-α irrespective of their lineal origin.

(B) RT-PCR analyses of lineage or differentiation related genes including cytokine receptors and transcription factors. The symbols under each lane depict the relative amounts of transcripts in each population compared to control cDNA (2×10^5 cells) by the ratio of pixel density units of target cDNA/pixel density units of control cDNA: <0.1 (−), 0.1–0.5 (±), 0.5–1.5 (+), >1.5 (++).

(C) D-J rearrangement of IgH gene in PDCs. IgH D-J rearrangement was detected by genomic PCR using primer sets for D_HQ52 element. Freshly isolated PDCs but not DCs, CMPs, or CLPs rearranged IgH D-J locus. Both CMP-derived PDCs (CMP-PDC) and CLP-derived PDCs (CLP-PDC) rearranged at least at the JH2 locus of IgH (upper panels). Samples containing 300 freshly isolated Gr-1$^+$B220$^+$CD11c$^+$ or CD4$^+$B220$^+$CD11c$^+$ PDCs also rearranged IgH genes (bottom).

that the rearrangement of IgH genes occurs during the transition from the CMP or CLP stages to mature PDC stage. Thus, IgH D-J rearrangements in PDCs do not necessarily reflect their lymphoid origin but rather arise during late stages of PDC development.

PDCs Activate RAG1 Transcription Irrespective of Their Origin

Since the IgH rearrangements depend upon activation of RAG, we hypothesized that RAG might be expressed in PDCs. To test this model, we analyzed mice carrying a reporter for RAG1 transcription, in which enhanced green fluorescent protein (EGFP) is knocked into the RAG1 locus (Kuwata et al., 1999). In heterozygous RAG1-EGFP knockin mice, the majority of CLPs and proT cells expressed GFP, whereas there were no detectable GFP$^+$ cells in myeloid progenitors including CMPs and GMPs (Figure 3A). GFP was highly expressed in pre-T and pre-B cells. A fraction of NK (data not shown), T, and B cells maintained GFP expression. We found that the majority of spleen and thymic PDCs but not DCs expressed a low level of GFP (Figure 3B). Interestingly, in the analysis of the bone marrow and spleen, the activation of RAG1-GFP outside the lymphoid (T, B, and NK) system was only found in the PDC fraction. GFP was also expressed in PDCs developed from CMP in vivo (Figure 3C), but CD11c$^+$MHCII$^-$ DC precursors in the blood did not express GFP (Figure 3A). RT-PCR analyses showed that CMP- and CLP-derived PDCs, but not DCs, possessed RAG1 and RAG2 (data not shown) transcripts (Figure 3D). The expression level of RAG genes was low, since we could detect RAG1 and RAG2 transcripts in PDCs only by nested RT-PCR. Altogether, these data suggest that the IgH rearrangements in PDCs result from the ectopic low-level expression of RAG genes in the committed PDCs.

Table 1. Generation of Dendritic Cell Progeny 15 Days after Transplantation

Progenitors	Injected Cell No.	Progeny No. Spleen $B220^+CD11c^+$ PDC	Spleen $B220^-CD11c^+$ DC	Thymus $B220^+CD11c^+$ PDC	Thymus $B220^-CD11c^+$ DC	DC/PDC (Spleen)	Progenitor No. per Mouse*	Extrapolated Yield of PDCs Spleen	Extrapolated Yield of PDCs Thymus
Myeloid									
CMP	2×10^4	$0.8 \pm 0.2 \times 10^5$	$1.9 \pm 0.4 \times 10^5$	$0.1 \pm 0.02 \times 10^3$	$2.4 \pm 0.8 \times 10^3$	2.3	20×10^4	83.0×10^4	0.1×10^4
GMP	10×10^4	$0.2 \pm 0.1 \times 10^5$	$0.7 \pm 0.4 \times 10^5$	ND	ND	3.3	40×10^4	8.0×10^4	ND
Lymphoid									
CLP	2×10^4	$0.3 \pm 0.1 \times 10^5$	$1.1 \pm 0.2 \times 10^5$	$0.2 \pm 0.06 \times 10^4$	$3.5 \pm 1.3 \times 10^4$	3.4	2×10^4	3.1×10^4	0.2×10^4
Pro-T	1×10^4	$0.6 \pm 0.5 \times 10^3$	$9.1 \pm 2.3 \times 10^3$	ND	ND	14.5	3×10^4	0.19×10^4	ND

PDC, plasmacytoid dendritic cell; DC, conventional dendritic cell; CMP, common myeloid progenitor; GMP, granulocyte/monocyte progenitor; CLP, common lymphoid progenitor; ND, not detectable. Results are given as means ± SD in three experiments, each consisting of three to five animals.
*Progenitor number refers to total cell numbers in the femurs and tibias of one mouse (CLP, CMP, GMP) or in the thymus (Pro-T) of 6- to 8-week-old animals.

PDCs Activate Human pTα Transcription Irrespective of Their Origin

In humans, pTα is expressed in PDCs at a high level comparable to thymocytes (Bendriss-Vermare et al., 2001). In contrast, murine pTα expression was barely detectable in PDCs, and its level was significantly lower than in thymocytes (Corcoran et al., 2003). By RT-PCR, we could detect very low levels of murine pTα transcripts in both CLP- and CMP-derived PDCs (Figure 4A). Thus, it has been suggested that pTα expression may not reflect their origin. Furthermore, this observation raised the possibility that murine $B220^+CD11c^+$ PDCs may not represent the counterpart of human PDCs. Alternatively, the transcriptional regulation of the human and murine pTα genes may be inherently different. To distinguish between these possibilities, we again turned to the analysis of reporter mouse strains.

Normal transcriptional regulation of human genes has been reproduced in transgenic mice that carry large human genomic fragments such as yeast or bacterial artificial chromosomes (BAC) (Kaufman et al., 1999; Okuno et al., 2002; Peterson et al., 1993). Therefore, we analyzed BAC transgenic mice carrying either mouse or human pTα loci in which the first exons were replaced with an EGFP gene (Reizis and Leder, 1999, 2001). In murine pTα-EGFP transgenic mice, GFP expression was restricted to lymphoid cells in the thymus and was not found at detectable levels in spleen or bone marrow cells including PDCs (Figure 4B). In contrast, in human pTα-EGFP transgenic mice, GFP^+ cells were found in a significant fraction of thymic cells including $B220^+CD11c^+$ PDCs and immature thymocytes including pro-T and pre-T cells (Figure 5A). Strikingly, in the bone marrow and the spleen, GFP^+ cells almost exclusively displayed the $B220^+CD11c^+$ PDC phenotype (Figure 5A). These results indicate that the human pTα is activated in PDCs during mouse hematopoiesis, possibly due to some regulatory element specific to the human gene, and confirm that previously defined murine PDCs (Asselin-Paturel et al., 2001; Bjorck, 2001; Nakano et al., 2001) represent the human PDC counterpart.

The expression of the human pTα transgene in PDCs was heterogeneous, with the fraction of GFP^+ PDCs proportional to the transgene copy number (data not shown). In the transgenic line analyzed, the expression was found in ~30% of PDCs in the thymus, spleen, and bone marrow (Figure 5B). The expression patterns of Gr-1, CD11b, MHCII ($I\text{-}A^q$), CD4, and CD8α were similar between GFP^+ and GFP^- PDCs (Figure 5B), and both PDCs subsets produced IFN-α upon HSV treatment (Figure 5C). Moreover, expression levels of lymphoid genes such as IL-7Rα, Spi-B, and Notch-1 did not significantly differ between GFP^+ and GFP^- splenic PDCs (Figure 5D).

We next tested whether the activation of human pTα occurs specifically in lymphoid-derived PDCs. In human pTα-EGFP transgenic mice, GFP expression was initiated at the CLP stage (Figure 6A): GFP^+ cells were found in 2% and >60% of CLPs and pre-T cells, respectively, whereas myeloid progenitors such as CMPs and GMPs did not express GFP. GFP was not upregulated in circulating $CD11c^+MHCII^-$ DC precursors (Figure 6A). GFP^- CLPs and CMPs were purified from human pTα-EGFP transgenic mice and were transplanted into lethally irradiated hosts. As shown in Figure 6A, both CMPs and

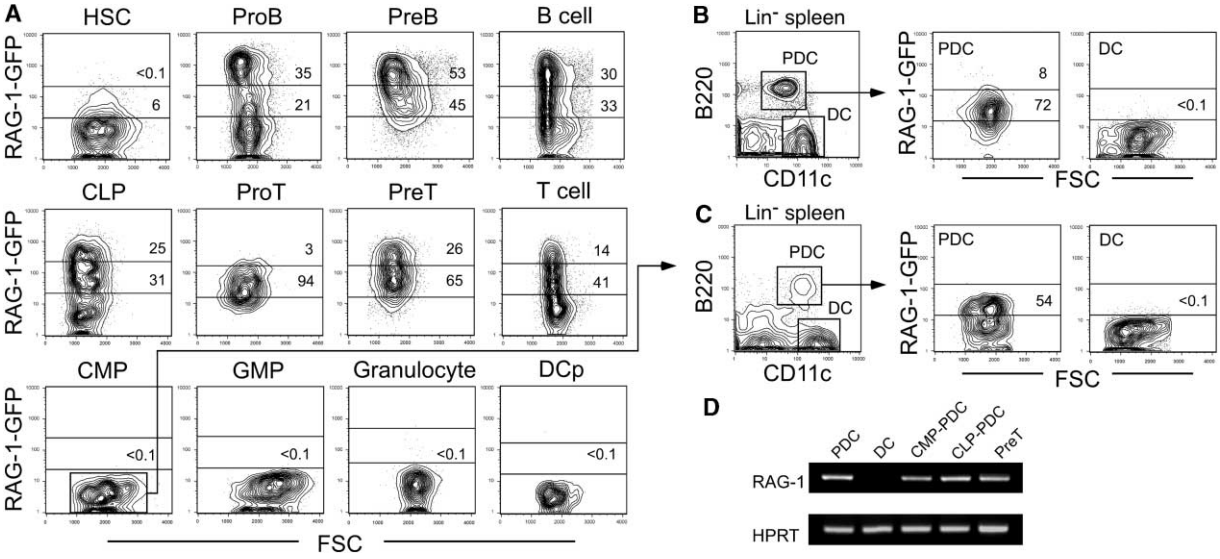

Figure 3. Analysis of RAG1-EGFP Knockin Mice

(A) Expression of RAG1-EGFP in each purified progenitor subsets. DCp, DC precursors.

(B) Steady-state PDCs but not DCs were EGFP$^+$.

(C) RAG1-EGFP was expressed in CMP-derived PDCs in the spleen. EGFP expression was evaluated 15 days after transplantation of purified EGFP$^-$ CMPs into congenic hosts.

(D) The nested RT-PCR analysis of RAG1 in purified populations. Both CMP-derived PDCs (CMP-PDC) and CLP-derived PDCs (CLP-PDC) possessed RAG1 transcripts.

CLPs gave rise to PDCs, which contained a similar proportion (~25%) of GFP$^+$ cells in each case. Thus, human pTα transcription is upregulated after cells differentiate into PDCs irrespective of their lineal origin.

Discussion

In this study, we provide a formal proof that functional PDCs originate from both myeloid and lymphoid devel-

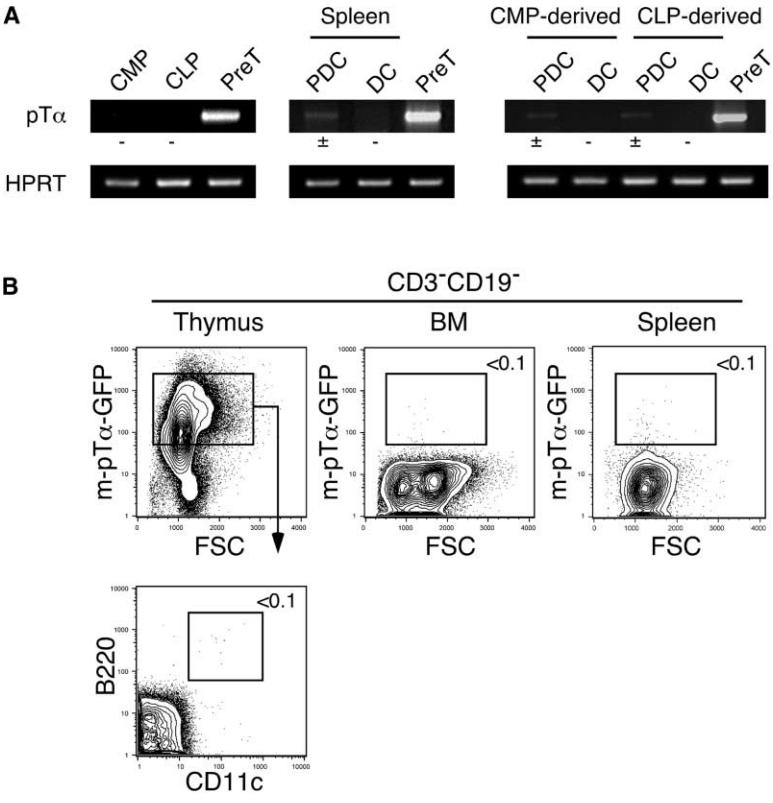

Figure 4. Analysis of Murine pTα Expression in PDCs

(A) RT-PCR analyses of pTα in purified PDC and DC subsets. Both myeloid and lymphoid PDCs express murine pTα transcript albeit at a very low level.

(B) Analysis of GFP$^+$ cells in murine (m) pTα-EGFP transgenic mice. GFP expression is only seen in B220$^-$CD11c$^-$ immature thymocytes, and none of the CD3$^-$CD19$^-$ spleen and bone marrow cells express GFP at a detectable level on FACS.

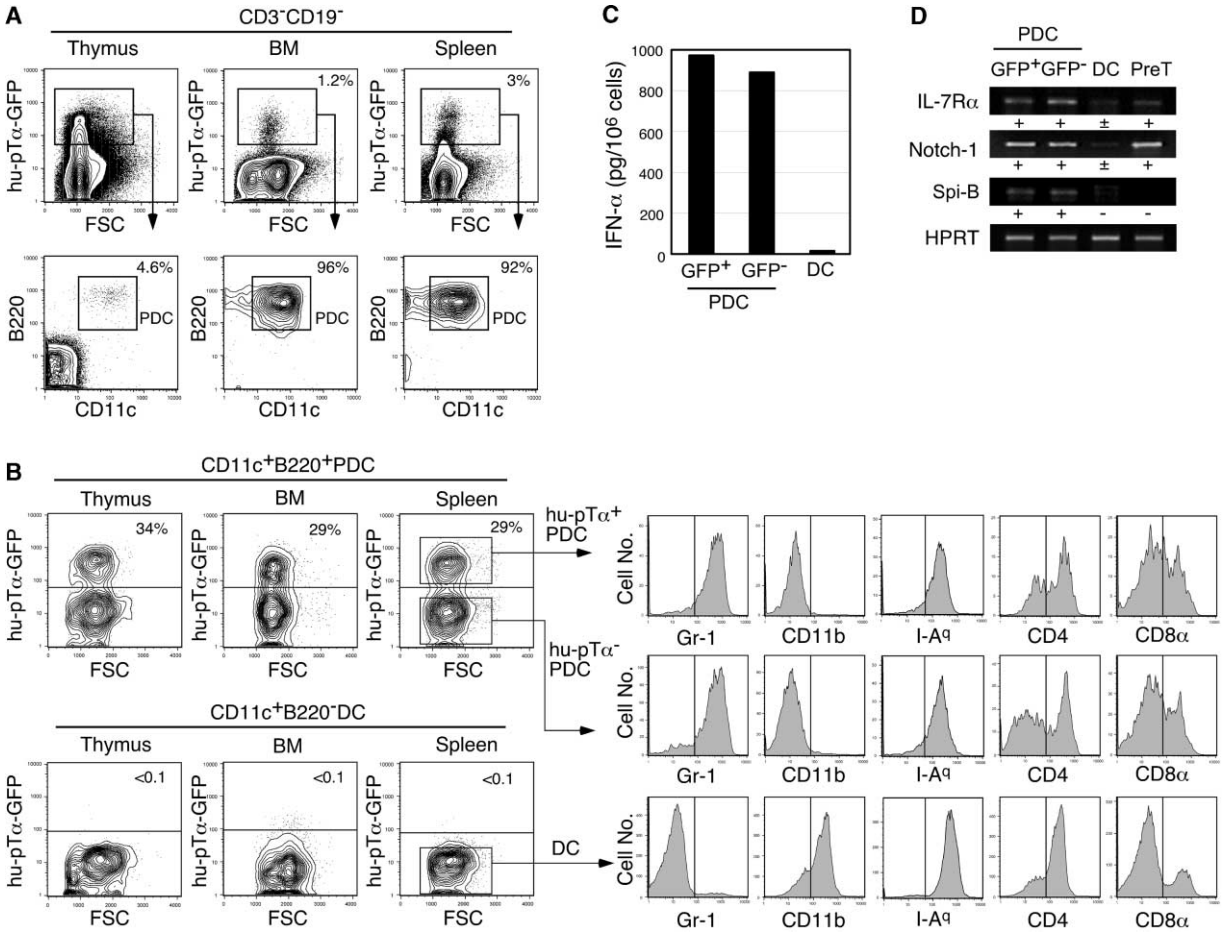

Figure 5. Analysis of Mice Harboring a Human pTα Transgenic Reporter

(A) Analysis of GFP$^+$ cells in human pTα-EGFP transgenic mice. In the thymus, the GFP$^+$ population consists of B220$^+$CD11c$^+$ PDCs as well as B220$^-$CD11c$^-$ immature thymocytes. In contrast, GFP$^+$ cells are exclusively B220$^+$CD11c$^+$ PDCs in the bone marrow and the spleen.

(B) GFP expression in PDCs and DCs in thymus, bone marrow, and spleen cells of human pTα-EGFP transgenic mice (left panels). The expression of other DC-related antigens in spleen PDCs and DCs is also shown (right panels). PDCs display the similar expression pattern of these antigens regardless of human pTα-EGFP transgene activation.

(C) IFN-α levels in the supernatant of human pTα-GFP-positive or -negative PDCs cultured with HSV.

(D) Expression profiles of lymphoid-related genes in human pTα-GFP-positive or -negative PDCs. BM, bone marrow; hu-pTα, human pTα.

opmental pathways. PDC potential is maintained along early T lymphoid stages (i.e, CLPs and pro-T cells) and early granulocyte/monocyte stages (i.e., CMPs and GMPs) (Figure 6B). CMP- and CLP-derived PDCs appear equally functional, producing IFN-α when challenged with a virus. Both CMPs and CLPs efficiently gave rise to PDCs and DCs, and the frequency of PDC generation within total DC outputs from each progenitor was similar (Table 1). Although a very small fraction (<0.1%) of CMPs maintain B cell potential (Akashi et al., 2000), PDCs and conventional DCs developed also from GMPs, which are completely devoid of lymphoid potential. Pro-B cells could not differentiate into either PDCs or DCs. Thus, PDC potential is closely associated with conventional DC potential (Manz et al., 2001; Traver et al., 2000) throughout the hematopoietic hierarchy. CMPs and CLPs could be still heterogeneous for PDC and DC potential since Flt-3-expressing CMPs and CLPs were reported to produce PDCs and DCs more efficiently than Flt-3-negative ones (D'Amico and Wu, 2003; Karsunky et al., 2003). In this study, however, we did not subfractionate each progenitor population by Flt-3 expression because Flt-3-negative subsets still could produce significant numbers of DCs and PDCs (D'Amico and Wu, 2003). It is possible that Flt-3 expression represents an immediate expansion potential of each progenitor subset. By using purified myeloid and lymphoid progenitors that include both Flt-3-positive and -negative subsets, we demonstrate here that the myeloid pathway should produce the majority of PDCs, as in the case of conventional DCs (Table 1) (Manz et al., 2001).

Myeloid and lymphoid cells generally differentiate along independent pathways, where CMPs and CLPs likely represent the earliest committed branchpoints (Akashi et al., 2000; Kondo et al., 1997). Therefore, it was conceivable that myeloid- or lymphoid-derived DC/PDC subsets might be phenotypically and functionally distinct. In this study, however, we could not find any apparent phenotypic or functional differences between myeloid- and lymphoid-derived subsets. Interestingly,

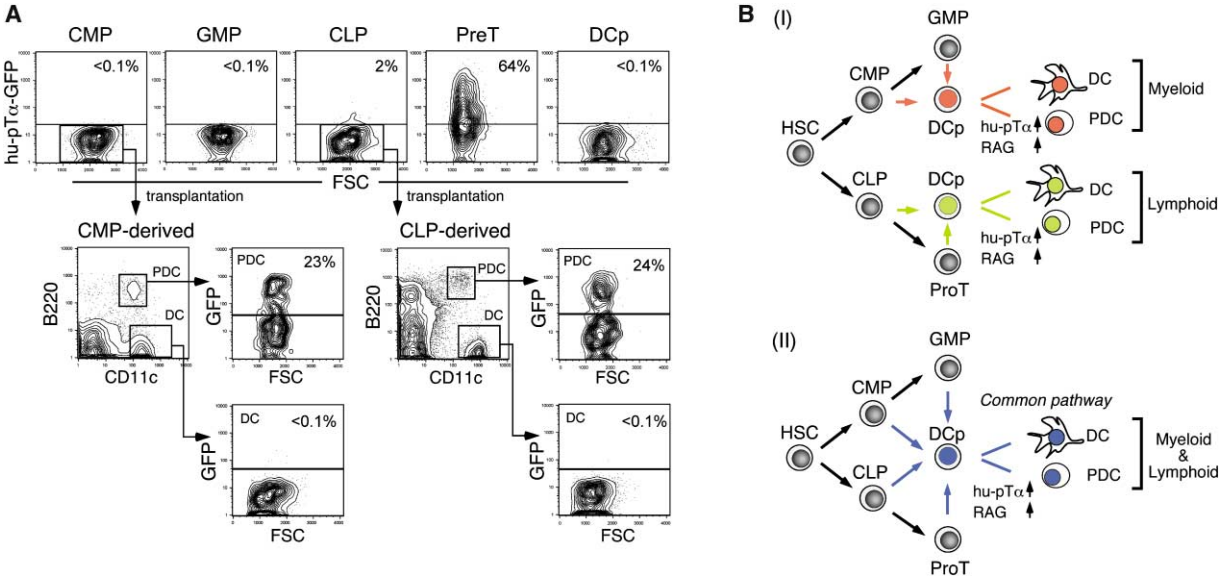

Figure 6. Function and Origin of PDC Populations Segregated by the Activation of the Human pTα Transgene

(A) The expression of human pTα-EGFP in PDCs derived from CMPs or CLPs. In human pTα-EGFP transgenic mice, pre-T cells but not CMPs or CLPs upregulate GFP (upper panels). GFP^- CMPs and CLPs give rise to GFP^+ PDCs in vivo after transplantation (bottom panels).

(B) Models of PDC and DC development from myeloid and lymphoid progenitors. According to the myeloid versus lymphoid developmental scheme (Akashi et al., 2000; Kondo et al., 1997), myeloid progenitors (CMPs and GMPs) and lymphoid progenitors (CLPs and pro-T cells) give rise to independent DC precursors (DCp), respectively (I), where DC and PDC populations should consist of myeloid and lymphoid subtypes. In contrast, if myeloid and lymphoid progenitors use an identical pathway for DC development via common DCp (II), their DC and PDC progeny should also be identical regardless of their original lineages. The induction of human pTα and RAG1 expression has been proposed to occur at late stages of PDC development from both lymphoid and myeloid progenitors in either model. HSC, hematopoietic stem cell.

myeloid-related cytokine receptors such as GM-CSFRα and IL-3Rα were expressed in CLP-derived PDCs and DCs at levels similar to CMP-derived ones, respectively, suggesting the reactivation of these myeloid cytokine receptors during the lymphoid to DC/PDC differentiation. It is important to note that CLPs and pro-T cells can be converted into mature granulocytes and monocytes simply by ectopic expression of GM-CSF signaling (Iwasaki-Arai et al., 2003; Kondo et al., 2000). In this context, DCs and PDCs may develop from CLPs and pro-T cells by using their residual myeloid differentiation programs (Iwasaki-Arai et al., 2003).

PDCs, however, also activate lymphoid programs during their development from myeloid and lymphoid progenitors. Indeed, B/myeloid-related Spi-B as well as T lymphoid-related Notch-1 was expressed at higher levels in CMP- and CLP-derived PDCs than conventional DCs (Figure 2B). Moreover, IgH rearrangement was detected in both myeloid- and lymphoid-derived PDCs (Figure 2C). Importantly, PDCs were the only cell types that expressed GFP outside the lymphoid lineage in RAG1-GFP mice (Figure 3), and both lymphoid and myeloid PDCs possessed RAG1 and RAG2 transcripts, although their expression levels were lower than lymphoid progenitors. Thus, the ectopic activation of RAG genes in both myeloid- and lymphoid-derived PDCs might result in the rearrangement of IgH genes. Another important lymphoid marker, pTα, is also expressed in both CLP- and CMP-derived PDCs, although their expression levels were low as compared to thymocytes (Figure 4). It remains to be established whether the activation of these lymphoid developmental programs is in any way relevant for the unique functions of PDCs.

By PCR analysis, the expression of pTα in the mouse PDCs appeared very low. Consistent with this, PDCs in murine pTα-EGFP mice did not express significant levels of GFP, while pre-T cells had GFP at a very high level. In human PDCs (Bendriss-Vermare et al., 2001), pTα levels are comparable to those observed in thymic T cell precursors (Res et al., 1999). Indeed, the human pTα transgene was strongly expressed in a fraction of PDCs, as represented by the expression of GFP at levels comparable to thymic precursors (Figure 5). These expression patterns were observed in multiple independent transgenic lines, including seven human pTα-EGFP and four mouse pTα-EGFP lines (B.R. et al., unpublished data). Therefore, the significant upregulation of pTα in PDCs appears to result from properties intrinsic to the human pTα gene. Our analysis showed that similar percentages of CMP- and CLP-derived PDCs activated the human pTα transgene, and both human $pT\alpha^+$ and $pT\alpha^-$ PDCs can produce equal amounts of IFN-α. Furthermore, when we crossed human pTα transgenic mice with athymic nu/nu mice, PDCs in athymic human-pTα-EGFP transgenic mice also became GFP^+ (B.R. et al., unpublished data). This indicates that the pTα activation does not require thymic T cell maturation or exposure to thymic microenvironments and is therefore independent of T cell development. Taken together, our results show that the expression of the human pTα by PDCs does not reflect their derivation from lymphoid precursors. The fact that the activation of human-pTα-EGFP reporter occurs exclusively in PDCs outside the lymphoid system suggests that the phenotypically defined murine PDCs (Asselin-Paturel et al., 2001; Bjorck, 2001; Martin et al., 2002; Nakano et al., 2001)

fully correspond to the human PDC counterpart (Cella et al., 1999; Grouard et al., 1997; Jarrossay et al., 2001; Kadowaki et al., 2000).

It is important to note that the GFP protein might degrade at a slower rate as compared to native proteins, and therefore, the low level of GFP expression in PDCs could represent the RAG1 activation at a developmental stage prior to the PDC stage. Both CMPs and CLPs generate CD11c$^+$MHCII$^-$ DC precursors (del Hoyo et al., 2002) (Figure 1A), which further differentiate into both PDCs and conventional DCs in vitro (H.S. et al., unpublished data). The RAG1 or the human pTα reporter, however, was not activated in the DC precursors, and the level of RAG1 transcripts was very low in mature PDCs. It is thus possible that these lymphoid genes are mainly activated during the transition from the DC precursor to mature PDC stages (Figure 6B). Furthermore, although it remains unknown whether all DCs and PDCs are generated from the DC precursor population, the capability of CMPs and CLPs to generate DC precursors suggests the existence of the common DC/PDC pathway, where myeloid and lymphoid DC pathways merge together at the common DC precursor stage to give rise to mature DCs and PDCs (Figure 6B, bottom). In this model, equivalent DC subsets may thus be independently generated by both lineal pathways, and this may explain our inability to detect unique DC attributes from each pathway.

If early lymphocyte-specific genes such as RAG and pTα are unlikely to play any role in PDC function, why are they expressed in mature PDCs? Since PDCs are part of the innate immune system, they are likely to predate antigen receptor-bearing lymphocytes in evolution. Therefore, the genetic program of their development would be more ancient than that of lymphocytes and may represent a "prototype" genetic pathway of immune cell development. Later in evolution, parts of such a pathway could be utilized in the development of specific lineages such as T and B lymphocytes. It is tempting to speculate that the expression of early lymphocyte genes in PDCs reflects the ancient mechanisms of transcriptional control that can activate both lymphoid- and myeloid-specific genes during PDC development. The characterization of these mechanisms of gene expression in PDCs should be a focus of future experimentation.

In conclusion, our data demonstrate that the gene expression program of PDCs is unique and includes the activation of both myeloid- and lymphoid-related genes. In particular, mature PDCs express at least some genetic programs primarily associated with early lymphocyte development, including RAG and pTα transcripts and rearranged IgH genes. This lymphoid program is independent of the cellular origin of PDCs, as it is observed in PDCs derived from both myeloid and lymphoid progenitors. Therefore, it is likely to reflect specific transcriptional mechanisms operating in committed PDCs rather than their affiliation with the lymphoid lineage. The expression of early lymphoid genes appears as a unique property of PDCs that clearly distinguishes them from other mature cell types in the immune system. Future studies should elucidate the molecular basis and possible functional significance of lymphoid gene expression in PDCs.

Experimental Procedures

Mice

C57B6 (CD45.1 or CD45.2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). RAG1-EGFP knockin mice have been described elsewhere (Igarashi et al., 2001; Kuwata et al., 1999). Human or murine pTα-EGFP transgenic mice (FVB-CD45.1) were described previously (Reizis and Leder, 1999, 2001). F1 (FVB-CD45.1xC57B6-CD45.2) mice were used as hosts for transplantation of pTα-EGFP transgenic cells. To generate pTα bacterial artificial chromosome transgenic mice, BAC clones containing either mouse or human pTα loci (90 and 113 kb, respectively) were modified to replace the first pTα exon with an enhanced green fluorescent protein gene (Reizis and Leder, 1999, 2001). The upstream pTα enhancer was left intact. The resulting BAC clones were used to generate multiple independent transgenic mouse lines, which manifested copy number-dependent EGFP expression with the same pattern for each construct (data not shown). One line derived from each BAC with a similarly high transgene copy number (16–17 copies in hemizygous state as determined by quantitative genomic Southern hybridization) was chosen for detailed analysis. Mice were bred and maintained in the Research Animal Facilities at the Dana Farber Cancer Institute or Harvard Medical School, in accordance with institutional guidelines.

Sorting of Progenitors and Dendritic Cells

Sorting of myeloid progenitors was accomplished by staining bone marrow cells with purified rat anti-IL-7Rα chain monoclonal antibodies (A7R34) (eBioscience, San Diego, CA) and purified or PE-Cy5-conjugated rat antibodies specific for the following lineage markers: CD3 (CT-CD3), CD4 (RM4-5), CD8 (5H10), B220 (6B2), Gr-1 (8C5), Ter119, and CD19 (6D5) (Caltag, Burlingame, CA). IL-7Rα^+Lin$^+$ cells were removed with sheep anti-rat IgG-conjugated magnetic beads (Dynabeads M-450; Dynal A.S., Oslo, Norway), and the remaining cells were stained with PE-Cy5-conjugated goat anti-rat IgG (Caltag). Cells were then stained with PE-conjugated anti-FcγRII/III (2.4G2), FITC-conjugated anti-CD34 (RAM34), APC-conjugated anti-c-Kit (2B8), and biotinylated anti-Sca-1 (E13-161-7) monoclonal antibodies (Pharmingen, San Diego, CA), followed by avidin-APC-Cy7 (Caltag). Myeloid progenitors were sorted as IL-7Rα^-Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRII/III^{lo} (CMPs), IL-7Rα^-Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRII/III^{hi} (GMPs), and IL-7Rα^-Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^-$FcγRII/III^{lo} (MEPs) as described previously (Akashi et al., 2000). HSCs and CLPs were sorted as IL-7Rα^-Lin$^-$Sca-1^{hi}c-Kit^{hi} and IL-7Rα^+Lin$^-$Sca-1^{lo}c-Kit^{lo} populations, respectively (Kondo et al., 1997). Pro-T and pro-B cells were sorted as CD3$^-$CD4$^-$CD8$^-$NK1.1$^-$IL-7Rα^+c-Kit$^+$CD25$^+$ and CD43$^+$B220$^+$IgM$^-$NK1.1$^-$ cells, respectively.

DCs were isolated from spleen, thymus, and bone marrow as described elsewhere (Manz et al., 2001; Wu et al., 2001). In brief, spleens and thymi were cut into small fragments and digested with collagenase and DNase under repeated agitation, followed by the addition of EDTA. Enzymatic digestion was avoided when isolating DCs from bone marrow samples. To enrich DC populations, CD11c$^+$ cells were positively enriched with CD11c (N418) MicroBeads and MACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). In some experiments, DCs were enriched after immunomagnetic depletion of B cells and T cells with anti-CD19 (6D5), anti-IgM (1B4B1), anti-CD3 (CT-CD3), and sheep anti-rat IgG-conjugated magnetic beads (Dynal). Cells were stained with anti-CD11c (HL3) and anti-B220 (RA3-6B2) as well as a lineage cocktail including anti-CD3 (CT-CD3), anti-TCRβ (H57-597), anti-CD19 (6D5) (Caltag), anti-IgM (1B4B1) (eBioscience), and anti-NK1.1 (PK136) (Pharmingen). PDCs and DCs were purified as Lin$^-$B220$^+$CD11c$^+$ cells and Lin$^-$B220$^-$CD11c$^+$ cells, respectively. These DC populations were further analyzed by anti-Gr-1 (RB6-8C5), anti-CD11b (M1/70), anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-I-A^b (AF6-120.1), anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL1), and anti-I-A^q (KH116). Streptavidin-conjugated PE, Cy5-PE, and APC-Cy7 (Caltag) were used to visualize biotinylated antibodies.

Cells were sorted using a highly modified double laser (488 nm/350 nm Enterprise II + 647 nm Spectrum) high-speed FACS (Moflo-MLS, Cytomation, Fort Collins, CO). Data were analyzed with FlowJo software (Treestar, Inc., San Carlos, CA).

Measurement of IFN-α from PDCs

FACS-purified B220$^+$CD11c$^+$ PDCs and B220$^-$CD11c$^+$ DCs were cultured with HSV (10 pfu/cell) in IMDM supplemented with 10% fetal calf serum (FCS) and 10^{-4} M 2-mercaptoethanol. Culture supernatants were collected 24 hr after the initiation of cultures and were analyzed by mouse IFN-α ELISA kit (Performance Biomedical Laboratories, New Brunswick, NJ).

In Vivo Reconstitution Assays

Purified progenitors (CD45.2) from C57B6 or RAG1-GFP knockin (C57B6) mice were intravenously transplanted into congenic mice (CD45.1) after a lethal dose (950 rad) of irradiation, together with 300 Sca-1$^+$c-Kit$^+$ host-type (CD45.1) stem cells to provide radioprotection. Mice were sacrificed on day 15, and donor-derived progeny were analyzed using the CD45.2 donor marker. For reconstitution assays of human pTα-EGFP transgenic progenitors (FVB-CD45.1), GFP$^-$ CMPs or CLPs were injected intravenously into F1 (FVB-CD45.1xC57B6-CD45.2) mice with 300 Sca-1$^+$c-Kit$^+$ stem cells of F1 mice. Donor-derived cells were detected as CD45.1$^+$CD45.2$^-$ cells.

Analysis of Gene Expression from Total RNA

Total RNA was extracted from 1000 purified cells for each population and was reverse transcribed to cDNA (Akashi et al., 2000). An aliquot of cDNA was analyzed for specific genes. PCR products were subjected to electrophoresis on a 1.8% agarose gel, followed by ethidium bromide staining. PCR amplification for each experiment was repeated for two or more independently prepared cDNA samples. Relative gene expression quantification was accomplished by comparing the level of any transcript in the target samples to that in control cDNA prepared from 2×10^5 whole bone marrow cells or thymocytes (Akashi et al., 2000). PCR cycles for each target gene were at a point when the reaction was in the exponential phase to obtain a linear correlation between pixel density units of the PCR product and the amount of control cDNA (Akashi et al., 2000). Primer sequences for each specific gene are shown in Supplemental Table S1 at http://www.immunity.com/cgi/content/full/21/1/43/DC1.

IgH Gene Rearrangement Analysis

Genomic DNA was extracted from 4000 double-sorted cells by incubation at 56°C for 45 min in 50 μl of 1× PCR buffer containing 0.5% Tween 20 and 100 μg/ml proteinase K, followed by 10 min at 94°C to inactivate proteinase K. PCR primers specific for D_HQ52 element were used to amplify rearranged IgH D-J as described previously (ten Boekel et al., 1995). PCR products were separated on an agarose gel, transferred onto nylon membranes, and subjected to Southern blotting using standard procedures. To detect TCRβ rearrangement, the extracted DNA was amplified with the primers for Dβ1-Jβ1 and Dβ2-Jβ2 as reported (Iwasaki-Arai et al., 2003).

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