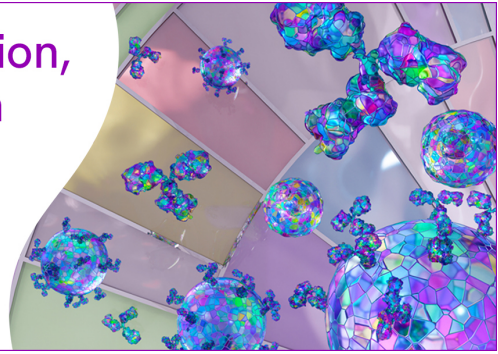


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# The Lymphoid Past of Mouse Plasmacytoid Cells and Thymic Dendritic Cells<sup>1</sup>

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There has been controversy over the possible lymphoid origin of certain dendritic cell (DC) subtypes. To resolve this issue, DC and plasmacytoid pre-DC isolated from normal mouse tissues were analyzed for transient (mRNA) and permanent (DNA rearrangement) markers of early stages of lymphoid development. About 27% of the DNA of CD8<sup>+</sup> DC from thymus, and 22–35% of the DNA of plasmacytoid pre-DC from spleen and thymus, was found to contain IgH gene D-J rearrangements, compared with 40% for T cells. However, the DC DNA did not contain IgH gene V-D-J rearrangements nor T cell Ag receptor  $\beta$  gene D-J rearrangements. The same DC lineage populations containing IgH D-J rearrangements expressed mRNA for CD3 chains, and for pre-T $\alpha$ . In contrast, little of the DNA of the conventional DC derived from spleen, lymph nodes, or skin, whether CD8<sup>+</sup> or CD8<sup>-</sup>, contained IgH D-J rearrangements and splenic conventional DC expressed very little CD3 $\epsilon$  or pre-T $\alpha$  mRNA. Therefore, many plasmacytoid pre-DC and thymic CD8<sup>+</sup> DC have shared early steps of development with the lymphoid lineages, and differ in origin from conventional peripheral DC. *The Journal of Immunology*, 2003, 170: 4926–4932.

The developmental origin of dendritic cells (DC)<sup>3</sup> has been a puzzling and controversial issue. The original concept that all DC were of myeloid origin was first questioned when a lymphoid-restricted precursor population, isolated as the earliest T lineage cell in the adult mouse thymus, was found to produce thymic CD8<sup>+</sup> DC on transfer to irradiated recipients (1). Around 70% of this T precursor population formed DC progeny in culture, but the lack of corresponding clonal data on T precursor potential meant no firm conclusions could be made (2). A lymphoid-restricted precursor population purified from human bone marrow was also shown to form DC in culture (3). These observations led to the unwarranted generalization that all murine CD8<sup>+</sup> DC were of lymphoid origin whereas all murine CD8<sup>-</sup> DC were of myeloid origin. Recently, the availability of lymphoid-restricted and myeloid-restricted precursor populations from mouse bone marrow (4, 5) has made it possible to test their potential to form splenic CD8<sup>+</sup> and CD8<sup>-</sup> DC. Results from the Stanford (6, 7) and Hall Institute (8) laboratories indicated that both myeloid and lymphoid precursor populations have the potential to produce both CD8<sup>+</sup> and CD8<sup>-</sup> DC, albeit with some bias in proportions. Thus, there appears to be substantial developmental flexibility at the stage of these precursor populations, with production of splenic CD8<sup>+</sup> or CD8<sup>-</sup> DC being determined not at this early level, but further downstream. The preponderance of myeloid precursors in

bone marrow then suggested that most splenic DC, whether CD8<sup>+</sup> or CD8<sup>-</sup>, should be of myeloid origin.

Even the proposed linkage between CD8<sup>+</sup> DC development and T cell development within the thymus, for which there was stronger evidence, has since been questioned. Precursor cells from growth factor receptor-deficient mice (*c-kit*<sup>-</sup>  $\gamma$ *c*<sup>-</sup>) and from Notch 1-deficient mice, both unable to produce T lineage cells, have nevertheless been shown to produce thymic CD8<sup>+</sup> DC (9, 10). This raised the possibility that the early precursor population isolated from the thymus of normal mice and considered to be a common T cell-DC precursor actually consisted of separate T cell and DC precursors, merely similar in surface phenotype.

In view of the apparent developmental flexibility, even the finding that many precursor populations retain a capacity to produce DC in culture, or on transfer to an irradiated recipient, leaves open the question of the actual origin of the DC found in a normal steady-state animal. Are any DC derived from lymphoid precursors under these conditions? One way to answer this is to examine DC freshly isolated from a normal mouse for relics of a lymphoid or a myeloid past. This requires markers more specific for these lineages than the surface proteins used so far. A pertinent example is the finding of mRNA for the early T lineage marker pre-T $\alpha$  in human thymus plasmacytoid DC precursors (p-preDC) (11), and a range of B cell gene transcripts in human tonsil p-preDC (12), all of which point to a developmental link with lymphoid cells. In the present study, we use CD3 chain mRNA and pre-T $\alpha$  mRNA expression in DC as transient indicators of a past T lineage relationship, and D-J rearrangements of IgH genes in DC as an indelible clonal marker of a past lymphoid orientation. We find the conventional DC of mouse thymus, but not those of spleen, show these signs of a lymphoid past. However many of the p-preDC of both spleen and thymus display these lymphoid-origin markers.

## Materials and Methods

### Mice

C57BL/6 mice, 5–7 wk of age, male and female, bred under specific pathogen-free conditions at the Walter and Eliza Hall Institute, were used in most experiments. For bone marrow reconstitution experiments the recipients were 8- to 10-wk-old mice, either C57BL/6 or C57BL/6 Ly5.1-Pep<sup>3b</sup>,

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; p-preDC, plasmacytoid DC precursor.

given two doses of gamma irradiation (each 5.5 Gy, 3 h apart). Donors of bone marrow were 5- to 7-wk-old C57BL/6, or CD3 $\epsilon$  null C57BL/6 mice (CD3 $\epsilon^{\Delta 5/\Delta 5}$ ) or Rag-1 null C57BL/6 mice (13). In some experiments, embryonic day 15 C57BL/6 mice were used as donors of fetal liver. The recipients were given  $2-5 \times 10^6$  bone marrow or fetal liver cells i.v. and the thymic DC populations examined 3 wk later.

#### DC and p-preDC purification

The isolation, immunofluorescent staining, and sorting of conventional quiescent DC and plasmacytoid pre-DC from spleen and thymus has been described elsewhere (14–16). Briefly, tissues were cut into small fragments then digested 25 min at room temperature with collagenase-DNase, then treated with EDTA for 5 min. All subsequent procedures were at 0–4°C in a Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free medium. Light density cells were isolated by centrifugation in a Nycodenz medium. Cells not of the DC lineages were depleted by an immunomagnetic bead procedure after coating the cells with mAb against CD3, Thy1, CD19, GR-1, and TER-119. Before immunofluorescent staining, autofluorescent and dead cells were removed by rapid presorting. After appropriate staining the conventional quiescent DC were defined and sorted as CD45RA<sup>-</sup>CD11c<sup>high</sup> cells or as CD11c<sup>high</sup>MHC-II<sup>high</sup> cells, and subdivided and sorted as either CD8 $\alpha^+$  or CD8 $\alpha^-$  cells in the thymus, or as CD4<sup>-</sup>8 $\alpha^-$ , CD4<sup>+</sup>8 $\alpha^-$ , CD4<sup>-</sup>8 $\alpha^+$  cells in the spleen. In the case of thymic DC, the 20% of DC showing low staining for CD8 $\alpha$  were considered as CD8 $\alpha^-$ , because this low staining has been shown to be due to pick-up of CD8 $\alpha\beta$  from thymocytes (14). The p-preDC were defined and sorted as CD45RA<sup>+</sup>CD11c<sup>int</sup> cells. All preparations were 97–99% pure on reanalysis.

#### Isolation of dermal and epidermal DC from mouse ear skin

Split skin from the ears of 20 mice was cultured for 48 h in medium containing 6-CKine and the cells exiting the ears were collected, as described in detail elsewhere (16). They were immunofluorescent-stained for MHC II and DEC-205 and sorted as MHC-II<sup>high</sup>DEC-205<sup>high</sup> epidermal DC and MHC-II<sup>high</sup>DEC-205<sup>int</sup> dermal DC.

#### Isolation of macrophages and granulocytes

After preparing spleen cell suspensions as above, or after washing out the peritoneum with saline, macrophages were immunofluorescent-labeled and sorted as CD11b<sup>high</sup>F4/80<sup>high</sup> cells. Granulocytes were isolated from bone marrow suspensions by immunofluorescent staining and sorting as GR-1<sup>high</sup>, high light side scatter cells.

#### Development of DC in culture from thymic “low CD4” precursors

The “low CD4” early T precursors, around 0.03% of thymocytes, were isolated as described previously (8, 17). They were cultured with a mix of seven cytokines in modified RPMI 1640 medium under the conditions described previously for the production of DC (2). Cells were harvested after 1–4 days of culture and any dead cells were removed by density centrifugation (18) before analysis.

#### CD3 mRNA expression by *in situ* hybridization on single cells

The detailed procedure and the <sup>35</sup>S-labeled probes used have been described in detail elsewhere (19, 20). Briefly, DC lineage cells or control cells were sorted onto poly-L-lysine-coated slides, depositing four samples each of 10<sup>4</sup> cells on each slide. The samples were dried, dehydrated, fixed, proteinase K-digested and acetylated, then hybridized with anti-sense <sup>35</sup>S-labeled probes for CD3 $\epsilon$ , CD3 $\gamma$ , and CD3 $\delta$  mRNA. After washing and dehydrating, the slides were dipped in photographic emulsion then exposed at –70°C for 10–20 days. The slides were then fixed, lightly counterstained, and then scanned under light microscopy at 200-fold magnification. Cells associated with >10 grains above background were considered positive.

#### Assay for CD3 $\epsilon$ , CD3 $\delta$ , and pre-T $\alpha$ mRNA by RT-PCR

Total cytoplasmic RNA was extracted from purified DC populations using a Qiagen RNeasy Mini kit (Qiagen, Clifton Hill, Australia) and contaminating genomic DNA was removed with RNase-free DNase I (Qiagen). First-strand cDNA synthesis from aliquots of various concentrations of the extracted RNA was performed in a final volume of 25  $\mu$ l using a Superscript II RNase H<sup>-</sup> Reverse Transcriptase kit (Invitrogen-Life Technologies, Mount Waverley, Australia). The PCR of optimal sensitivity for CD3 $\epsilon$  and CD3 $\delta$  expression was performed in a final volume of 50  $\mu$ l containing 7  $\mu$ l of cDNA, 2 mM MgCl<sub>2</sub>, 1 $\times$  thermoreaction buffer, dNTP mixture (0.2 mM of each), 120 pmol of each oligonucleotide primer, and

2.5 U AmpliTaq DNA polymerase (PerkinElmer, Rowville, Australia). Each set of 35 cycles consisted of 15 s at 94°C (for the first cycle this step was 1 min), 15 s at 58°C, and then 1 min at 72°C (for the final cycle this step was 5 min). The PCR for pre-T $\alpha$  expression was performed in a final volume of 50  $\mu$ l containing 7  $\mu$ l of cDNA, 1.5 mM MgCl<sub>2</sub>, 1 $\times$  thermoreaction buffer, dNTP mixture (0.26 mM of each), 200 pmol of each oligonucleotide primer, and 2.5 U AmpliTaq DNA polymerase (PerkinElmer). Each set of 35 cycles consisted of 15 s at 94°C (for the first cycle this step was 1 min), 15 s at 60°C and then 1 min at 72°C (for the final cycle this step was 5 min). The PCR for GAPDH, a control for the amount of cDNA, was performed in the same volumes as outlined above but with altered concentrations of MgCl<sub>2</sub> (1.5 mM) and oligonucleotide primers (30 pmol of each). Cycle conditions were also the same except that a lower annealing temperature of 50°C was used. The PCR for  $\beta$ -actin, also a control for the amount of cDNA, was performed under the same conditions as for CD3 $\epsilon$  and CD3 $\delta$  PCR. A GeneAmp 9700 DNA thermal cycler (PE Biosystems, Foster City, CA) was used for the PCR. Ten microliters of each PCR product was electrophoresed through a 2% agarose gel containing ethidium bromide. The sequence and position of the oligonucleotides used as primers for the PCR were as follows: CD3 $\epsilon$  forward primer 216–235, 5'-TCT CGG AAG TCG AGG ACA GT-3'; CD3 $\epsilon$  reverse primer 515–496, 5'-GCT CAT AGT CTG GGT TGG GA-3'; CD3 $\delta$  forward primer 10–29, 5'-AGC GGG ATT CTG GCT AGT CT-3'; CD3 $\delta$  reverse primer 389–370, 5'-GTC TCA TGT CCT GCA AAG CA-3'; pre-T $\alpha$  forward primer 219–240, 5'-ACA CTG CTG GTA GAT GGA AGG C-3'; pre-T $\alpha$  reverse primer 609–588, 5'-CGA GCA GAA GCA GTT TGA AGA G-3'; GAPDH forward primer 229–249, 5'-GAA CGG GAA GCT TGT CAT CAA-3'; GAPDH reverse primer 512–493, 5'-CTA AGC AGT TGG TGG TGC AG-3';  $\beta$ -actin forward primer 25–45, 5'-GTG GGC CGC TCT AGG CAC CAA-3';  $\beta$ -actin reverse primer 564–541, 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3'.

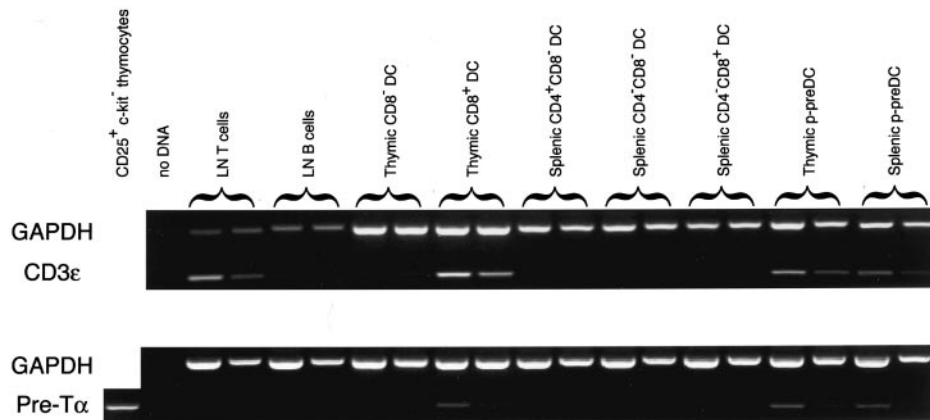
#### PCR assays for Ig and TCR gene rearrangements

Sorted cells were washed once with PBS, resuspended at a concentration of 10<sup>6</sup> cells/ml in PCR lysis buffer, and processed as described elsewhere (21). Primers for germline C $\mu$ , for D<sub>H</sub> to J<sub>H</sub> and for V<sub>H</sub> to D<sub>H</sub>J<sub>H</sub> rearrangements, and the PCR conditions used have been described (21). Amplification of the Rag-1 and Oct-2 genes served as controls to estimate template concentrations. DNA from all lysates was titrated to give an equivalent amount of product in the control reactions, and to ensure that the reactions were performed within the linear range of the assay. Oct-2 primers were: 5'-GCATCGAGACGAATGTCCGCTTCG; 3'-GTAGCTGGTCCGGCTTTCCCGGCT. Rag-1 primers were: 5'-GGGACCGTCGCAAGAGAAGACTCAGGC; 3'-GGACTTCACTGGGCTCTCCAGGTC. The Oct-2 and Rag-1 PCRs were performed for 30 cycles using the following program: 94°C, 1 min; 70°C, 2.5 min. Primers used for the TCR D $\beta$  to J $\beta$  rearrangement were: D $\beta$ 2, 5'-GTAGGCACCTGTGGGGAAGAAACT-3'; J $\beta$ 2, 5'-TGAGAGCTGTCTCTACTATCGATT-3'. The PCRs for TCR $\beta$  rearrangement were performed for 30 cycles at 94°C, 1 min; 62°C, 2 min; 72°C, 2 min. Ten microliters of each 25- $\mu$ l reaction was run on an agarose gel, transferred to nylon membranes, and hybridized with specific probes, using standard procedures. PCR products were quantified using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

## Results

### CD3 $\epsilon$ and pre-T $\alpha$ mRNA in DC lineage cells

mRNA encoding components of the CD3 complex, and encoding pre-T $\alpha$ , are expressed at early stages of T cell development. We argued that if certain DC derive from precursors with T lineage potential, some of these DC might still express CD3 chain mRNA or pre-T $\alpha$  mRNA, even though the proteins are not involved in DC function and DC do not express surface CD3 proteins. RT-PCR on RNA derived from purified, sorted CD11c<sup>+</sup>, or MHC-II<sup>+</sup> DC revealed clear bands corresponding to CD3 $\epsilon$  mRNA and to pre-T $\alpha$  mRNA if the RNA was from thymic CD8<sup>+</sup> DC, but not if the RNA was from thymic CD8<sup>-</sup> DC nor from any splenic DC, including the CD8<sup>+</sup> DC splenic DC (Fig. 1). Based on the intensity of the bands and titrations of the RNA, thymic CD8<sup>+</sup> DC gave a signal for CD3 $\epsilon$  that averaged 26% that of a similar number of T cells, and for pre-T $\alpha$  32% that of early CD4<sup>-</sup>CD8<sup>-</sup>c-kit<sup>+</sup>CD25<sup>+</sup> thymocytes. The CD3 $\epsilon$  mRNA signal could not be detected with any reliability in thymic DC by *in situ* hybridization on single cells,



**FIGURE 1.** CD3 $\epsilon$  and pre-T $\alpha$  mRNA transcripts in DC subpopulations. RT-PCR was performed on titrated RNA extracted from known amounts of cells. For CD3 $\epsilon$  expression, lymph node T cells (RNA from 100 and 50 cells) served as positive control, lymph node B cells (RNA from 100 and 50 cells) as a negative control. For the test DC subpopulations, the RNA levels were equivalent to 500 and 250 cells. For pre-T $\alpha$  expression, CD25<sup>+</sup>c-Kit<sup>-</sup>CD3<sup>-</sup>CD8<sup>-</sup> thymocytes served as a positive control and T cells and B cells as a negative control, with these controls and the DC subpopulations all assayed on the RNA equivalent of 1000 and 500 cells. In all cases, GAPDH was used as a housekeeping gene control for cDNA loading. Two such experiments gave equivalent results. The same distribution of CD3 $\epsilon$  RNA expression was also obtained in several earlier experiments with higher RNA loading but lower RT-PCR sensitivities.

presumably because of a high and variable background and the lower sensitivity of this system.

Both CD3 $\epsilon$  and pre-T $\alpha$  mRNA were also detected in plasmacytoid cells, from both thymus and spleen (Fig. 1). The signal for CD3 $\epsilon$  averaged 13 and 10% that obtained from a similar number of T cells for thymic and splenic p-preDC, respectively. The signal for pre-T $\alpha$  averaged 44 and 26% that of CD4<sup>-</sup>CD8<sup>-</sup>c-Kit<sup>+</sup>CD25<sup>+</sup> thymocytes for thymic and splenic p-preDC, respectively. These results for mouse thymic p-preDC were therefore in line with the expression of pre-T $\alpha$  mRNA by human thymic plasmacytoid cells (11).

#### Expression of CD3 $\epsilon$ mRNA during DC development in culture

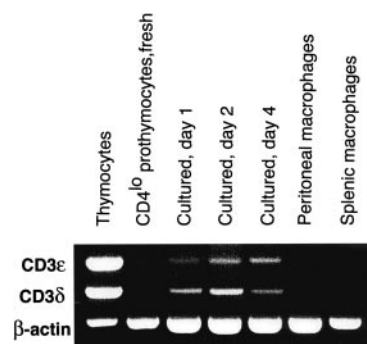
We argued that expression of CD3 mRNA might only be transient in DC, the signal detected in Fig. 1 being predominantly from recently formed cells. If so the signal should be highest in the first few days after differentiation from an early lymphoid precursor, because the average lifespan of thymic DC is around 10 days (22). To test this we induced the development of DC from the "low CD4" early precursor cells purified from mouse thymus (17, 23) by culturing these precursors with an effective mix of seven cytokines (2). This culture system produces the rapid death of around 30% of the precursors, but all of the surviving 70% of cells produce small clones of DC over the next 4 days, with DC morphology evident in most cultured cells within 1–2 days (2). No T cells or T lineage cells are detected at any stage of the culture. We cultured the cells from 1 to 4 days, removed all dead cells, then either extracted RNA and analyzed for CD3 $\epsilon$  or  $\delta$  mRNA by RT-PCR, or sorted the cells onto slides and conducted in situ hybridization for single cell expression of CD3 $\epsilon$ ,  $\gamma$ , or  $\delta$  mRNA.

The early T precursor population from thymus (CD4<sup>low</sup> precursor) showed only very low expression of CD3 $\epsilon$  or  $\delta$  mRNA. However, this rose to give readily detectable bands on RT-PCR analysis of the viable cells after 1 day in culture (Fig. 2), even when using conditions with a lower PCR sensitivity than those of Fig. 1. There was usually a decline in intensity of the bands obtained from days 2 to 4 of culture. Although it could be argued that some transiently induced T lineage cells which had not yet died might give the signal seen at day 1, all cells in these cultures at days 2 and 4 had a dendritic appearance and expressed DC markers, as we had previously shown (2).

In this situation it was possible by in situ hybridization to detect in the cultured cells CD3 $\epsilon$ , CD3 $\gamma$ , and CD3 $\delta$  mRNA, despite the relatively high background and the lower sensitivity of this method (Table I). The results agreed with the RT-PCR data, in that grain counts over the precursor cells were initially low, but rose to high levels within 1 day of culture. There was then a decline in grain counts, so positive cells could not be detected above the background grain count by day 4. This individual cell approach allowed the important conclusion that the vast majority of cells in the early stages of culture expressed the CD3 mRNA, rather than all the signal being derived from a minor component. Thus the signal was derived from the developing DC, and not from some minor T cell contaminant.

#### D-J rearrangements of IgH genes in DC lineage cells

Because mRNA for CD3 $\epsilon$  chains or for pre-T $\alpha$  were only transient markers of a lymphoid relationship, more likely to be expressed in



**FIGURE 2.** CD3 $\epsilon$  and CD3 $\delta$  mRNA expression during DC production in culture from early prothymocytes. The earliest prothymocytes (CD4<sup>low</sup> precursors) were isolated and sorted to high purity from adult mouse thymus, then cultured in the presence of cytokines known to induce DC production. Cells were harvested at 1, 2, and 4 days of culture, all dead cells removed, RNA extracted, and RT-PCR for CD3 $\epsilon$  and CD3 $\delta$  mRNA expression performed on an amount of cDNA equivalent to 1000 harvested viable cells. Thymocytes served as a positive control, macrophages as a negative control, and RT-PCR for  $\beta$ -actin served as a control for cDNA loading. Two separate assays gave similar results. The RT-PCR conditions used gave less sensitivity than those of Fig. 1.



Table I. Acquisition of CD3 mRNA by individual cells during DC development in culture from early T precursors<sup>a</sup>

mRNA Probed	Percent Positive Cells				
	Thymocytes (control)	CD4 <sup>low</sup> precursors (not cultured)	Cultured CD4 <sup>low</sup> precursors		
			Day 1	Day 2	Day 3
$\beta$ -Actin (control)	99 $\pm$ 1	99 $\pm$ 1	98 $\pm$ 2	100 $\pm$ 0	100 $\pm$ 0
CD3 $\epsilon$	97 $\pm$ 2	2 $\pm$ 3	90 $\pm$ 6	26 $\pm$ 23	2 $\pm$ 4
CD3 $\delta$	85 $\pm$ 7	4 $\pm$ 6	94 $\pm$ 5	56 $\pm$ 20	0
CD3 $\gamma$	95 $\pm$ 5	3 $\pm$ 9	97 $\pm$ 1	95	0

<sup>a</sup> Early prothymocytes (CD4<sup>low</sup> precursors) were purified from the adult mouse thymus and cultured with a mix of seven cytokines to induce the development of DC. Cells with DC morphology were evident by day 2 and no T lineage cells were detected. The cultured cells were harvested, the 30% dead cells removed, and the viable cells deposited on slides and processed for single cell in situ hybridization with the appropriate antisense probes. Where SDs are given, three to six individual samples were counted. Negative controls of cells with sense probes, or macrophages with antisense probes, always gave <5% positives.

the earlier cells of a lymphoid-derived DC lineage, we sought a permanent clonal marker which would be found in all lymphoid-derived cells. It is known that T cells, as well as B cells, carry D-J rearrangements of IgH genes (24), rearrangements that therefore occur at the level of the common B cell-T cell precursor. Any DC lineage originating from this same precursor should therefore bear such rearrangements as an indelible record in the DNA of all progeny cells.

We used a PCR-based approach to detect IgH gene rearrangements in the DNA of DC (Fig. 3). By comparing the intensities of rearranged vs rearranged plus germline bands we obtained an estimate of the extent of such rearrangements (Table II). The controls showed, as expected, almost complete D-J and V-D-J IgH gene rearrangements for purified B cells, and we set this value as 100%. We obtained around 40% D-J, but no V-D-J, rearrangements for T cells and thymocytes. Granulocytes showed no visible bands corresponding to D-J rearrangements and only a marginal Phosphor-Imager reading; we used the granulocyte DNA reading to set a value we would no longer consider as significant. Faint bands of D-J rearrangements were seen in isolated tissue macrophages. This was not investigated further because at most only a small proportion of macrophages might carry such rearrangements. However, this may fit with the view that some macrophage cell lines are related to B cells and clonally related populations of macrophages and B cells carrying Ig gene rearrangements can be generated in culture from retrovirus-infected bone marrow (25).

The total DC isolated from mouse thymus gave in three preliminary experiments clear bands representing IgH D-J rearrangements, but no V-D-J IgH rearrangements or Ig $\kappa$  rearrangements. Such rearrangements were then found to be concentrated in the major CD8 $\alpha^+$  thymic DC population (Fig. 3a). In this respect CD8<sup>+</sup> thymic DC resembled T cells and thymocytes, although on quantitation the extent of IgH allele rearrangement was variable, and averaged 27%, a little less than for T cells (Table II). The minor CD8 $\alpha^-$  thymic DC population showed fainter bands of rearrangement, which might have been due to overlap with the CD8 $\alpha^+$  DC, given the difficulties in clear separation of these groups due to CD8 $\alpha\beta$  pickup (14). Splenic DC and LN DC showed much less evidence of D-J IgH rearrangements. No clear bands and no significant level of rearrangements were measured in DNA from splenic CD8<sup>-</sup> DC. Importantly, only faint bands were obtained with DNA from splenic CD8<sup>+</sup> DC (Fig. 3a), in contrast to thymic CD8<sup>+</sup> DC. DC which migrated out of mouse skin in culture (both dermal- and epidermal-derived) showed no significant level of IgH rearrangements (Fig. 3a). Thus, thymic CD8<sup>+</sup> DC showed clear signs of a lymphoid origin, in contrast to splenic CD8<sup>+</sup> DC and to other peripheral DC.

There has been considerable evidence for a lymphoid origin of human p-preDC (11, 12, 26). We found the DNA of mouse thymic p-preDC showed clear bands corresponding to IgH D-J rearrangements, at a level similar to that of thymic CD8<sup>+</sup> DC (Fig. 3b). It was of particular interest that splenic p-preDC also showed clear evidence of IgH D-J rearrangements (Fig. 3b), almost to the same extent as thymic p-preDC, but in marked contrast to other splenic DC lineage cells.

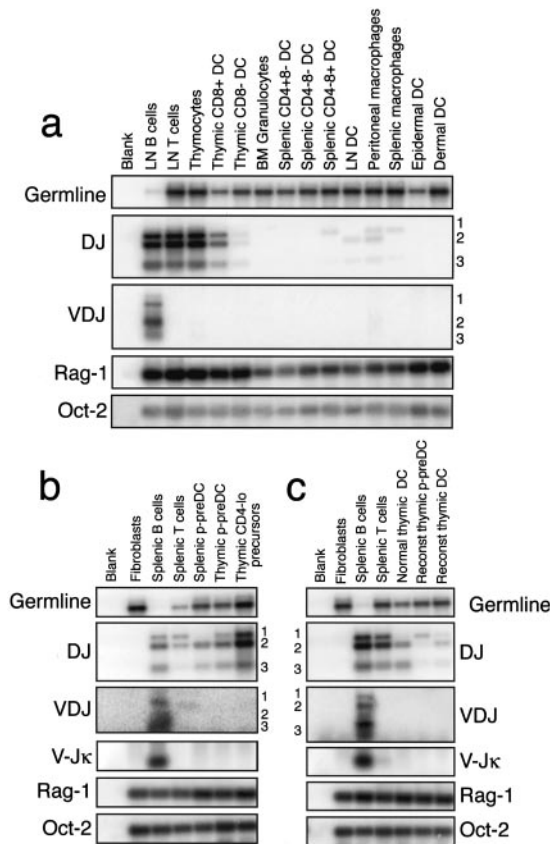
#### Thymic DC IgH D-J rearrangements in irradiated, reconstituted mice

It was notable that the extent of IgH D-J rearrangements in p-preDC and thymic DC was less than that of T cells and <50%, indicating that some thymic DC developed from precursors that were either earlier in the lymphoid pathway than the point of IgH D-J rearrangements, or were myeloid precursors. This pointed to some flexibility in the origin of these DC. To see whether the balance was altered under different conditions, we assessed the level of D-J rearrangements in CD8<sup>+</sup> DC and p-preDC from the thymus of mice that had been irradiated then reconstituted for 3 or 5 wk with bone marrow cells. Although bands corresponding to IgH D-J rearrangements were still visible, the extent of rearrangement of the isolated DNA was now much less in both the mature thymic DC and in thymic p-preDC at 3 wk (Fig. 3c; Table II) as well as at 5 wk (data not shown) after reconstitution. Similar results were obtained after reconstitution of mice with fetal liver precursors (data not shown). The thymic DC of such reconstituted mice also had little CD3 $\epsilon$  mRNA detectable by RT-PCR (data not shown).

#### Controls for pickup by DC of mRNA and DNA from T lineage cells

Thymic DC pick-up low levels of surface Ags from thymocytes (14) and CD8<sup>+</sup> DC from spleen have been shown to phagocytose apoptotic cells (27). Therefore, it was important to check that the previous results on thymic DC were not due to acquisition of mRNA for CD3 $\epsilon$  or pre-T $\alpha$ , or of rearranged IgH gene DNA, from T lineage cells via ingestion of apoptotic thymocytes. This possibility was checked in several ways.

The first approach was to construct bone marrow chimeras in irradiated Ly5.1 host mice, using normal Ly5.1 bone marrow together with either mutant CD3 $\epsilon$  null Ly5.2 bone marrow, or mutant Rag-1 null Ly5.2 marrow. The thymic DC from the chimeric mice were then purified and sorted into Ly5.1<sup>+</sup> and Ly5.2<sup>+</sup> groups 5 wk after bone marrow transfer. The Ly5.2<sup>+</sup> DC were then checked for the acquisition of CD3 $\epsilon$  mRNA by the CD3 $\epsilon$  null thymic DC, or of rearranged IgH genes by the RAG-1 null thymic



**FIGURE 3.** D-J rearrangements of IgH genes in DC lineage cells. PCR was performed on DNA from a range of DC subtypes and control cells, using primers for germline  $C_{\mu}$ , for  $D_H$  to  $J_H$  and for  $V_H$  to  $D_HJ_H$  rearrangements. Amplification of Rag-1 and Oct-2 genes served as controls for template concentrations. All cell samples were from C57BL/6 mice. Lymph node (LN) B cells served as the positive controls for both D-J and V-D-J rearrangements. T cells and thymocytes served as the example of a non-B cell lymphoid lineage cell showing partial D-J, but no V-D-J, rearrangements. Fibroblasts and bone marrow (BM) granulocytes served as negative controls for germline status. A comparison of the PhosphorImager readings of the D-J rearrangement bands compared to the D-J bands plus germline bands provided an estimate of the extent of D-J rearrangements, as summarized in Table II. *a*, Various conventional DC subsets either purified and sorted directly from tissues, or in the case of dermal and epidermal DC, purified and sorted from cells exiting culture mouse ear skin, are compared with other freshly isolated and purified cell types. *b*, The plasmacytoid cells (p-preDC) freshly isolated from spleen and thymus are examined. *c*, The DC lineage cells isolated from C57BL/6 mice irradiated then reconstituted with C57BL/6 bone marrow cells (Reconst) are compared with normal mouse DC populations (in *a-c*), to show the reduced level of IgH D-J rearrangements in reconstituted vs normal mice.

DC. This would indicate acquisition of these markers from the normal  $Ly5.1^+$  thymocytes in the chimeric thymus. However, in one such experiment testing for CD3 $\epsilon$  acquisition, and two experiments testing for rearranged DNA acquisition, no such acquisition of normal thymocyte RNA or DNA by the mutant DC was detected (data not shown). Because the chimeric thymuses were large and fully reconstituted, apoptotic cortical thymocytes would have been present and the sensitivity of the PCR readout should have been sufficient to detect even 10% of the signal given by normal thymic DC.

A second control was to check for rearranged TCR genes in the thymic DC of normal mice, because if the D-J rearranged IgH DNA signal was caused by the uptake of thymocytes, then extensive TCR gene rearrangements should also be evident. This had

already been tested by Southern analysis (28) and no TCR $\beta$  D-J gene rearrangements had been detected in the DNA of thymic DC. Two new experiments, now using a PCR-based procedure for detecting TCR $\beta$  D-J gene rearrangements in the same thymic DC DNA samples showing IgH D-J gene rearrangements, agreed with our earlier studies (28). No TCR $\beta$  gene rearrangements were detected in the thymic CD8 $^+$  or CD8 $^-$  DC, although they were readily detected in thymocytes from the same mice (Fig. 4).

## Discussion

The original debate on the origin of different DC subtypes concerned their possible derivation from myeloid-committed vs lymphoid-committed precursors. Although the nature of the debate has shifted with the increasing evidence for developmental flexibility at this early precursor level, none of the experiments to date had provided clear clonal evidence for a lymphoid origin of any particular DC subtype. Our DNA rearrangement studies now provide such evidence for thymic CD8 $^+$  DC and for thymic and splenic plasmacytoid cells. This "marker" represents a permanent imprint of a past event that is a crucial step in B lymphocyte development. Both B and T lymphocytes have this imprint, whereas granulocytes and most tissue macrophages do not. This evidence for a lymphoid origin of some DC is considerably strengthened by the close agreement between the three markers used, IgH gene rearrangements, CD3 chain mRNA expression, and pre-T $\alpha$  mRNA expression.

One caveat is the possibility that a myeloid-committed precursor might come into a sufficiently strong T cell-inducing environment in the thymus to have T lymphocyte genes activated and IgH rearrangement initiated, without actually gaining full capacity to form lymphoid cells. We consider this bystander acquisition of lymphoid characteristics unlikely. The finding that CD8 $^-$  thymic DC have a much reduced level of these markers and that splenic p-preDC do have them is one argument against the 'bystander' model. Conversely, the absence of lymphoid markers in splenic DC does not prove that all these DC were of myeloid origin. Such DC could represent an earlier branch off a lymphoid pathway, before IgH rearrangements and CD3 or pre-T $\alpha$  expression. We have not used any markers which could serve as relics of a DC myeloid past, to positively identify cells of myeloid origin.

Our major concern was whether the lymphoid origin markers we found in thymic DC had been acquired by phagocytosis of apoptotic thymocytes. The extent of the IgH gene rearrangements found made this less likely, but additional controls were needed. To some extent the DC themselves provided an internal control, because in each tissue some DC lineage subsets expressed the markers whereas others did not. It could be argued that certain DC subtypes are specialized for uptake of apoptotic cells, so these selectively acquired the markers. However, this argument cannot be made for the expression of CD3 mRNA during DC development in culture from purified precursors, because T lineage cells were absent and the vast majority of developing DC expressed some CD3 mRNA. The bone marrow chimera experiments where CD3 $\epsilon$  null thymic DC did not contain CD3 mRNA, or where the Rag-1 null thymic DC did not contain DNA with IgH D-J rearrangements, should exclude phagocytosis of apoptotic thymocytes as an explanation of marker acquisition in vivo. However the impact of these controls is weakened by the fact that in these reconstituted mice even the DC derived from normal bone marrow showed a marked reduction in these markers of a lymphoid past, so a clear positive internal control was missing. Probably the most stringent control was the analysis of TCR rearrangements, and the finding that thymic DC from normal mice, containing DNA with IgH D-J rearrangements, displayed no D-J rearrangements of the TCR $\beta$  locus, nor (in previous studies) of the TCR $\gamma$  locus. Pickup

Table II. The extent of IgH gene D-J rearrangements in DC subtypes and control cells

Cell Type	Relative DJ-Rearranged IgH Alleles <sup>a</sup>	SD	Number of PCR Assays	Number of Cell Preparations
B cells	1.00		13	4
Fibroblasts	0.00	0.00	4	1
Granulocytes	0.02		1	1
T cells	0.42	0.10	10	3
Thymocytes	0.39	0.09	5	3
CD4 <sup>low</sup> prothymocytes	0.50		1	1
Thymic CD8 <sup>+</sup> DC	0.27	0.11	4	3
Thymic CD8 <sup>-</sup> DC	0.16	0.08	4	3
Splenic CD4 <sup>-</sup> 8 <sup>+</sup> DC	0.03	0.00	2	2
Splenic CD4 <sup>-</sup> 8 <sup>-</sup> DC	0.01		1	1
Splenic CD4 <sup>+</sup> 8 <sup>-</sup> DC	0.02	0.01	4	4
LN DC (total)	0.02		1	1
Skin-derived DC (total)	0.02	0.01	3	3
Thymic p-preDC	0.35	0.06	3	1
Splenic p-preDC	0.22	0.01	3	1
BM-reconstituted thymic DC (total)	0.06	0.01	2	2
BM-reconstituted thymic p-preDC	0.09		1	1

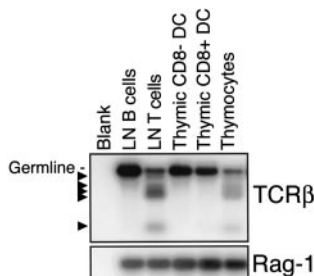
<sup>a</sup> The values were obtained from a series of experiments similar to Fig. 4, all performed in the linear range of the assay. The level of D-J rearrangements was estimated as the PhosphorImager readings on the D-J bands divided by the D-J plus germline bands. These values were then normalized to the value for B cell DNA in the same experiment, taken as 1.0.

of DNA from thymocytes would have introduced the TCR rearrangements readily detected in thymocytes. Pickup of DNA from B cells, a less likely phenomenon, would have introduced V-D-J as well as D-J rearrangements and no V-D-J rearrangements were seen in DC. Our conclusion is that the lymphoid-related markers are inherent to the thymic DC, and to p-preDC.

The finding that both myeloid-restricted and lymphoid-restricted precursor populations have a capacity to generate DC in culture, and to generate both CD8<sup>-</sup> and CD8<sup>+</sup> DC on reconstitution of irradiation-depleted mice (6-8), left open the question of how much of this potential is normally realized and which precursor cells contribute to DC production in normal mice. Our results on DC directly isolated from normal mice help to answer this question, although for DC development there is no direct link yet established between the activation of the lymphoid genes we use as markers and the surface markers used to define myeloid-restricted and lymphoid-restricted precursors. Nevertheless, one view is that because myeloid precursors predominate in bone marrow these would be the source of most DC in organs such as the spleen,

whereas because lymphoid precursors predominate in the thymus these would be the source of most DC in the thymus. Our present data support this view, because of the mature DC types, only thymic DC showed evidence of a lymphoid past, in the form of CD3 mRNA and pre-T $\alpha$  mRNA, and of IgH D-J gene rearrangements. These markers were present in a significant proportion of thymic DC. The exact proportion is not clear because we do not know whether a 50% DNA rearrangement would imply that all cells had one chromosome rearranged or half the cells had both chromosomes rearranged. However, because the proportion of DNA rearranged was <50%, some thymic CD8<sup>+</sup> DC clearly lacked these rearrangements. If the thymic CD8<sup>+</sup> DC with measured mean 27% IgH D-J rearrangements are compared with T cells with measured mean 40% IgH D-J rearrangements, 68% of normal adult mouse thymic CD8<sup>+</sup> DC could be considered to be of the same origin as T cells. The thymic CD8<sup>+</sup> DC lacking IgH D-J rearrangements may have branched off from a lymphoid pathway earlier than those with D-J rearrangements, or may have had a separate, perhaps myeloid, developmental origin. In this context, our data on the kinetics of thymic DC development (22) point to two different origins for thymic DC, one probably from an endogenous early prothymocyte, the other probably from the bloodstream.

The finding that these relics of a lymphoid past were much reduced in thymic DC of irradiated then reconstituted mice presumably reflects a flexibility in developmental origin. The thymic DC in these reconstituted animals could all branch off earlier from the lymphoid stream, before IgH rearrangement and expression of CD3 $\epsilon$  or pre-T $\alpha$  message. Alternatively, they could derive from an entirely separate developmental stream, possibly from myeloid precursors. This implies that the precise origins of thymic DC could vary with age or physiological state. The shift in apparent DC origin after irradiation and bone marrow reconstitution poses problems for experimental research, because results on DC origin obtained from bone marrow-reconstituted mice, such as the previous studies on the effects of conditional Notch 1 deletion on thymic DC and p-preDC development (9), might not reflect the steady state situation in normal adult animals.



**FIGURE 4.** Lack of TCR $\beta$  gene D-J rearrangements in the DNA extracted from thymic DC. A PCR assay was performed using probes specific for D-J rearrangements in TCR $\beta$  genes. T cells and thymocytes served as positive controls, B cells as a negative control. Amplification of the Rag-1 gene served as a control for template loading. Two such PCR assays on the same samples used in Fig. 3 gave similar negative results, confirming earlier data using a direct Southern hybridization assay.

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