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CD4 and CD8 Expression by Dendritic Cell Subtypes in Mouse Thymus and Spleen[1]

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The dendritic cells (DC) of mouse spleen and thymus were examined for expression of CD4 and CD8. Provided care was taken to avoid selective extraction or selective depletion of DC subpopulations, three main types of DC were detected in mouse spleen: a major new population of $CD4^+8^-$ DEC-205^{low} $CD11b^{high}$ DC, together with the previously described $CD4^-8^-$ DEC-205^{low} $CD11b^{high}$ DC and $CD4^-8\alpha\alpha^+$ DEC-205^{high} $CD11b^{low}$ DC. The CD4 on the surface of the $CD4^+$ splenic DC subpopulation was produced by the DC themselves, and CD4 RNA transcripts were present. Likewise, the CD8α on the surface of the splenic $CD8^+$ DC was shown to be a product of the DC themselves, in agreement with earlier evidence. All three spleen DC types would be considered as mature, based on expression of CD80, CD86, and CD40 as well as on T cell stimulating function. Mouse thymuses appeared to contain two DC types; both were DEC-$205^{high}$$CD11b^{low}$, but they differed in the level of CD8$\alpha\alpha$ expression. However, as well as this authenticated marker expression, immunofluorescent staining was also found to reflect a series of artifacts, due to the autofluorescence of contaminating cells and due to pickup of CD4 and CD8$\alpha\beta$. By constructing mice chimeric for the hemopoietic lineages using mixtures of wild-type bone marrow with $CD4^{null}$ or $CD8\alpha^{null}$ bone marrow, a marked pickup by thymic DC of Ags derived from thymocytes was demonstrated. *The Journal of Immunology,* **2000, 164: 2978–2986.**

Dendritic cells (DC)[3] collect, transport, and then present Ags to T lymphocytes (1–3). DC are essential for the initiation of primary T cell responses. They may also regulate the nature and the extent of the immune responses they initiate (3–9). Although all DC share features related to their common Ag processing and T cell activation machinery, there is evidence that they differ both in lineage origin and in aspects of their function (10). Accordingly it is important, as a first step, to recognize and segregate the different DC types found in tissues. The eventual aim is to determine the developmental origin of these DC types, to recognize different developmental or activation stages within any one DC lineage, and to understand the subtle differences in their interactions with T cells.

The expression of certain cell-surface markers has already pointed to substantial DC heterogeneity. We reported that a subpopulation of mouse spleen DC display on their surface CD8 as an $\alpha\alpha$ homodimer and, in accordance with this, express mRNA for CD8α but not for CD8β (11). This splenic $CD8\alpha^+$ DC population is DEC-205^+ $CD11b^-$, in contrast to a second splenic DC population that is $CD8\alpha^-$ DEC-205^- $CD11b^+$ (12). Lymph node DC appear still more heterogeneous and include an additional population that is $CD8\alpha^{low}$ DEC 205^+ (12–14). By contrast, our analysis of mouse thymus DC revealed what appeared to be a single group of $CD8\alpha^+$ DEC-205^+ DC, albeit with a wide range of CD8α surface staining (12, 15).

Murine $CD8\alpha^+$ DC appear to represent a lymphoid-related DC lineage separate from the conventional myeloid-related $CD8\alpha^-$ DC (10). $CD8\alpha^+$ thymic or splenic DC can be produced in recipient mice on transfer of an early thymic lymphoid precursor population with little, if any, capacity to form myeloid cells (16, 17). The murine lymphoid-related DC lineage differs from myeloid-related DC both in the cytokine requirements for development and in transcription factor control (18, 19). However, the expression of CD8α by the lymphoid-related DC does not appear to be essential for either their development or their function (18, 20), so it may be a relic of their particular developmental history. No CD8 expression has been detected on human DC (21).

Initially we detected only low levels of CD4 on murine DC (11), as did Crowley et al. (22). In what at that time appeared as a striking contrast, a proportion of human and rat DC stained strongly for surface CD4 (21, 23, 24). However, Salomon et al. (13) have since reported CD4 staining of a subgroup of mouse lymph node DC, and Pulendran et al. (25) have noted the occurrence of CD4 staining on a subgroup of Flt3 ligand-treated mouse spleen DC. We now find that a major population of mouse spleen DC expresses relatively high levels of CD4, a population previously eliminated from our preparations by the immunodepletion steps used to ensure high DC purity. However, we find that not all apparent CD4 or CD8 staining represents authentic expression by the DC because both autofluorescence and passive pickup of these molecules onto the DC surface confuse the staining results. Accordingly, we have conducted a critical analysis of the CD4, the CD8α, and the CD8β staining of mouse thymus and spleen DC. Using these markers and appropriate controls, we identify three distinct populations of DC in mouse spleen and two populations of DC in mouse thymus.

Materials and Methods

Mice

All mice were bred under specific pathogen-free conditions at the Walter and Eliza Hall Institute animal breeding facility. They were used at 5–7 wk

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[3] Abbreviations used in this paper: DC, dendritic cell; PI, propidium iodide; int, intermediate.

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of age. Most experiments used female mice of the C57BL/6J Wehi strain. For constructing bone marrow chimeras, the recipients were C57BL/6 Ly-5.1 Pep^{3b} mice. The bone marrow donors included the above strains, $CD8^{-/-}$ C57BL/6 mice (originally obtained from Dr. T. Mak), and $CD4^{-/-}$ C57BL/6 mice (provided by Dr. W. Heath; originally obtained from Dr. T. Mak). For the allogeneic T cell stimulation experiments, the CD4 T cells were derived from CBA CaH Wehi mice.

Bone marrow chimeric mice

The C57BL/6 Ly-5.1 mice were gamma-irradiated (two doses of 550 rad, 3 h apart) and then were injected i.v. with 3×10^6 bone marrow cells from wild-type Ly-5.1 mice together with 3×10^6 Ly-5.2 bone marrow cells from $CD4^{-/-}$ mice, $CD8\alpha^{-/-}$ mice, or wild-type C57BL/6 mice. For each experimental group reconstituted with a mix of Ly-5.1 wild-type and Ly-5.2 $CD4^{null}$ or $CD8^{null}$ bone marrow, a parallel control group reconstituted with a mix of Ly-5.1 wild-type and Ly-5.2 wild-type bone marrow was set up. Five to seven weeks after bone marrow transfer, the chimeric mice from each group were pooled, and the DC were isolated from the spleens and thymuses for analysis.

DC suspension and isolation media

These were all isoosmotic with mouse serum and identical with those described previously (11).

DC isolation procedure

The method was similar to our previous procedures (11, 12) with modifications to avoid loss of any CD4-bearing DC. Spleens (normally eight) were cut into small fragments and then digested with frequent mixing for 25 min at room temperature (22°C) in 10 ml modified RPMI 1640-FCS medium containing collagenase (1 mg/ml; type II; Worthington Biochemical, Freehold, NJ; verified as free of trypsin-like protease activity) and DNase I (Boehringer Mannheim, Mannheim, Germany). To disrupt DC-T cell complexes, EDTA (1 ml, 0.1 M (pH 7.2)) was added, and mixing continued for 5 min. Undigested fibrous material was removed by filtration through a stainless steel sieve. All subsequent steps were at 0–4°C using a divalent metal-free balanced salt solution containing EDTA (EDTA-BSS). Cells were recovered from the digest by centrifugation, the pellet was resuspended in a 1.077 g/cm^3 isoosmotic Nycodenz medium and centrifuged at $1700 \times g$ for 15 min, and then the low-density fraction was collected. The low-density cells (3–5% of the total) were diluted in EDTA-BSS, recovered by centrifugation, and then incubated for 30 min with the following mAb: anti-CD3 (KT3-1.1); anti-Thy 1 (T24/31.7, a pan-Thy 1); anti-Gr1 (RB68C5); and anti-erythrocyte (TER-119). All mAb were titrated and used at near-saturating concentrations, except anti-Thy 1, which was used at 25% of this level to avoid removing any DC that had absorbed low levels of Thy 1. Free mAb was removed by centrifuging the DC through a cushion of EDTA-FCS, and then the cells coated with mAb were removed using anti-rat Ig coupled magnetic beads (Dynabeads, Dynal, Oslo, Norway). The beads and cells in a 5:1 ratio, respectively, were mixed as a concentrated slurry by continuous slow rotation for 20 min. The slurry was then diluted with EDTA-BSS, and the beads and bound cells were removed with a Dynal magnet using several removal steps. The splenic DC (around 85% pure) were suspended in EDTA-BSS for presorting, staining, and analysis or sorting. For the isolation of thymic DC a similar procedure was used in this study, starting with thymuses and doubling the initial quantities to give a DC preparation only 20–30% pure. However, because thymic DC were finally shown to lack the $CD4^+$ $F4/80^+$ population, it was possible to produce an equivalent population of DC 80% pure by using the following mAb in the depletion mixture: anti-CD3 (KT3-1.1); anti-Thy 1 (T24/31.7, a pan-Thy 1); anti-CD4 (GK1.5); anti-CD8β (53-5.8); anti-CD25 (PC/61); anti-CD11b (Mac-1α; M1/70.15); anti-F4/80 (F4/80); anti-Gr1 (RB68C5); and anti-erythrocyte (TER-119). Anti-Thy 1, anti-CD4, anti-CD8β, anti-CD11b, and anti-F4/80 were all used at 25% of the usual near-saturation level to avoid depleting cells that either absorbed or expressed low levels of these markers on the surface.

Immunofluorescent labeling of DC preparations

The mAb, the fluorescent conjugates, and the labeling procedure have all been given in detail elsewhere (11, 12). The hybridomas were all grown and the mAb were purified and conjugated in this laboratory. The mAb used as pan-DC markers for segregating and sorting DC from non-DC contaminants were: anti-CD11c (N418), used as a FITC or Cy5 conjugate, and/or anti-class II MHC (N22), used as a Cy5 or Texas Red conjugate. Anti-class II MHC, which normally stains DC very strongly, was deliberately conjugated at less than maximal levels to keep fluorescence at saturation staining to only medium-high levels with which accurate compensation for fluorescence in other channels could be maintained. CD11c staining alone was used where DC were to be used for stimulating CD4 T cells to avoid blocking class II MHC. The mAb normally used to divide the DC into subpopulations were: anti-CD8α (YTS169.4), used as a FITC or an Alexa 594 conjugate; anti-CD4 (GK1.5), used as a PE or an Alexa 594 conjugate; anti-DEC-205 (NLDC145), used as a FITC or a biotin conjugate; and anti-CD11b (M1/70), used as a FITC, biotin, or Cy5 conjugate. Other mAb used were as follows: anti-CD8β (53-5.8), used as a FITC or biotin conjugate; anti-CD80 (16-10A1), used as a FITC or biotin conjugate; anti-CD86 (GL1), used as a FITC or biotin conjugate; anti-CD40 (FGK45.5), used as a FITC or biotin conjugate; anti-CD24 (M1/69), used as a FITC or biotin conjugate; anti-CD49d (PS/2), used as a FITC or biotin conjugate; anti-CD49f (EA-1; obtained from B. Imhof, Department of Pathology, Université de Génève, Switzerland), used as a FITC or biotin conjugate; and anti-F4/80 (F4/80), used as a FITC or biotin conjugate. The second-stage stain for the biotin-conjugated mAb was PE-Streptavidin (PharMingen, San Diego, CA).

Propidium iodide (PI) was included at 1 μg/ml in the final wash after immunofluorescent staining to label dead cells. DC were labeled in EDTA-BSS-FCS medium and before analysis were passed through a 26-gauge needle or repetitively passed through a pipette tip to minimize doublet formation.

Flow cytometric analysis, sorting, and presorting of DC

The fluorescent-labeled DC preparations were analyzed on a FACStar^{Plus} instrument (Becton Dickinson, San Jose, CA). Color compensation was initially set using Calibrite beads (Becton Dickinson) and then was checked using appropriate stained cell controls. Up to four fluorescent channels were used for the immunofluorescence staining (FL1 for fluorescein, FL2 for PE, FL3 for Cy5, and FL4 for Texas Red or Alexa 594), with the FL5 channel set to exclude PI-positive dead cells and autofluorescent cells. Care was taken in setting this gating to ensure that any cells staining very brightly in FL3 and spilling over into FL5 were not gated out as dead cells.

In many cases the DC preparations were presorted rather than just gated to remove autofluorescent cells with the presorting being performed before immunofluorescent staining and analysis. Presorting was conducted at high speed on a MoFlo instrument (Cytomation, Fort Collins, CO) or on the FACStar^{Plus} instrument using the FL1 and FL2 channels. In some cases the thymic DC preparations were labeled with anti-CD11b before presorting, and cells expressing high levels of CD11b were excluded along with the autofluorescent cells. This same channel of fluorescence could then be used for anti-class II MHC on subsequent staining because only cells showing high fluorescence were included as DC. Such presorting of thymic DC preparations was not required once it was established that immunomagnetic bead depletion employing anti-CD4, anti-F4/80, and anti-CD11b could be performed without eliminating any significant thymic DC population.

Short-term culture of DC

To wash passively bound proteins from the DC surface, the DC-enriched preparation, after presorting to remove autofluorescent cells but before labeling and sorting, was incubated for 16 h at 37°C in modified RPMI 1640-FCS medium, in a 10% CO_2-in-air atmosphere, and at a concentration of 5×10^5 cells/ml. The DC were then harvested, and dead cells were removed by selecting light-density viable DC using the Nycodenz centrifugation procedure described above. Recoveries of viable DC were around 50%.

DC-allogeneic T cell mixed leukocyte cultures

The mixed leukocyte culture system for determining the stimulatory capacity of DC was as described previously (5, 6). Briefly, DC were sorted from the C57BL/6 mice as $CD11c^+$ rather than MHC Class II^+ cells. Various numbers of DC were cultured with 20,000 CBA mouse lymph node CD4 T cells and purified as described previously (5) in 200 μl medium in V-well plates. At various times the cultures were pulsed for 6 h with [^{3}H]TdR, and then thymidine incorporation into cellular DNA was measured by liquid scintillation counting. The data shown are for day 3 of culture, the response peak, but similar relative stimulatory activity was observed at all time points.

Assay for DC adhesion properties

DC sorted from spleen DC preparations on the basis of class II MHC, CD4, and CD8α expression were suspended in a HEPES-buffered mouse osmolarity RPMI 1640 medium containing 10% FCS. The suspensions were placed in shallow chambers consisting of coverslips fastened to glass slides by double-sided tape along two opposite edges. After filling the chambers with the DC suspension, the other two edges of the coverslip were sealed with nail polish. After 1 h of incubation at 37°C, the DC in the chambers were viewed under phase contrast microscopy using a ×40 objective, and adherent cells were counted.

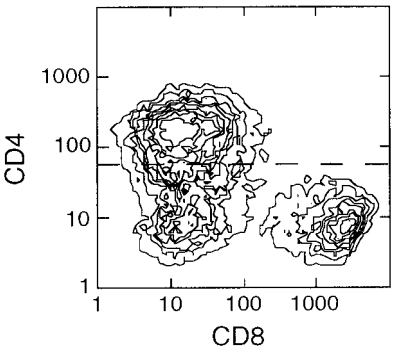

FIGURE 1. Immunofluorescent staining of spleen DC for CD4 and CD8α. DC were extracted and enriched from the spleens of C57BL/6 mice and were stained (in this particular experiment) with anti-class II MHC (Cy5 conjugate), anti-CD4 (PE conjugate), and anti-CD8α (Texas Red conjugate). The cells were gated for class II MHC^{high} cells with the high forward scatter of DC. Dead cells staining with PI and the autofluorescent cells (8% of the total in this particular preparation) were gated out using the FL5 channel. The CD4 and CD8α fluorescence distribution was then plotted for these gated DC. The result is typical of over 12 of these analyses. The broken line is an estimate of the cut-off point of the earlier isolation procedure (12), above which DC expressing CD4 and F4/80 were lost due to immunomagnetic bead depletion with anti-CD4 and anti-F4/80.

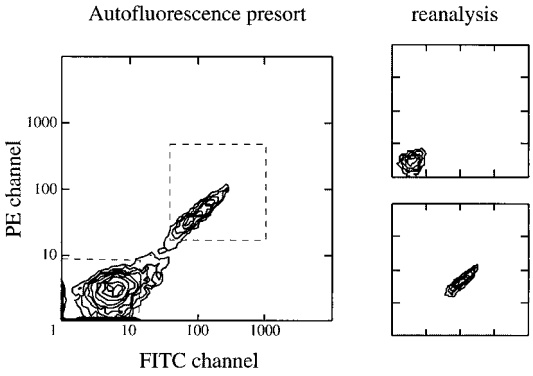

FIGURE 2. Autofluorescence and presorting of spleen DC preparations. An unstained spleen DC preparation was directly analyzed for fluorescence in the PE channel (FL2) and the FITC channel (FL1) of the FACStar^{Plus} instrument. The autofluorescent cells and the nonautofluorescent cells were then sorted using the gates indicated, reanalyzed before staining as indicated, and used for further studies. The level of autofluorescent cells in this preparation (30%) was high, but around one-third of all preparations were in this range.

RT-PCR for CD4 mRNA expression in DC

Total cytoplasmic RNA was prepared from purified DC populations using Qiagen RNeasy Mini Kit (Qiagen, Clifton Hill, Australia). The first-strand cDNA synthesis from extracted RNA was performed in a final volume of 50 μl using an AMV Reverse Transcription System (Promega, Madison, WI). The PCR was performed in a final volume of 30 μl containing 5 μl cDNA (equal to 1000 cells), $MgCl_2$ (1.5 mM), thermo-reaction buffer (1×), dNTP mixture (0.2 mM each), 1 μg of each oligonucleotide primer, and 1 U of *Taq* polymerase (Promega). Each set of 25–35 cycles consisted of 1 min at 94°C (for the first cycle this step was 4 min), 1 min at 60°C, and then 1 min at 72°C (for the final cycle this step was 11 min). A DNA thermal cycler was used (Perkin-Elmer/Cetus, Norwalk, CT). A sample (15 μl) of each reaction was electrophoresed through a 2% agarose gel. PCR for β-actin was performed (using the same PCR conditions) as controls for the amount of cDNA. The relative intensity of each CD4 PCR product band was then normalized on the basis of the ratio of the intensity of the CD4 band compared with the corresponding β-actin band. The sequence and the position of the oligonucleotides used as primers for the PCR were as follows: CD4 forward primer 828-846, 5′-GAG AGT CAG CGG AGT TCT C-3′; CD4 reverse primer 1156-1135, 5′-CTC ACA GGT CAA AGT ATT GTT G-3; β-actin forward primer 25-45, 5′-GTG GGC CGC TCT AGG CAC CAA-3′; β-actin reverse primer 541-564, 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′.

Results

CD4-bearing DC in mouse spleen

In earlier studies we had seen only marginal staining of mouse spleen DC for CD4 (11), even when anti-CD4 was omitted from the immunomagnetic bead depletion procedure used to remove nondendritic lymphoid and myeloid cells from the preparation. However, when both CD4 and F4/80 were omitted during this depletion step, the overall yield of DC in the enriched preparation doubled (to a mean of 2.5×10^6 DC per spleen), and over half of the DC obtained stained for CD4 (Fig. 1). In all respects other than the appearance of this additional CD4$^+$ population, the properties of the CD4$^-$CD8α^+ and the CD4$^-$CD8α^- DC were identical with those we have reported previously. The proportion of splenic DC that were CD4$^+$ was higher than with lymph node DC of which only around 15% were CD4$^+$ (data not shown). The CD4-bearing DC were among the CD8α^- DC rather than the putative lymphoid-related CD8α^+ DC, as shown in Fig. 1. This figure also provides an estimate of the cutoff point where the anti-CD4 and anti-F4/80 immunomagnetic bead procedure eliminated the high-staining CD4$^+$ DC in our earlier studies.

When immunomagnetic bead depletion was omitted entirely, no further major increase in DC yield was obtained, and the population distribution remained as in Fig. 1, with no new subpopulations apparent. However, longer sorting or analysis was then required to reject the high level of contaminants.

Autofluorescent cells in DC-enriched preparations

One cost of omitting immunomagnetic bead depletion, or reducing the number of mAb used for depletion, was a reduced purity before sorting and, as a consequence, a reduced resolution and an increasing difficulty in eliminating all contaminants. This was particularly the case for autofluorescent cells, which sometimes were at high levels in the DC preparations. Fig. 1 represents a favorable case in which most of the 8% autofluorescent cells could be gated out along with PI-positive dead cells during analysis. However, in many cases as illustrated in Fig. 2, which shows the autofluorescence of an unstained DC preparation, autofluorescent cells represented up to 30% of the DC-enriched sample. The autofluorescence overlapped both the class II MHC and the CD11c fluorescent staining used to define DC, as well as the CD4 and CD8 fluorescent staining used to separate DC subpopulations. Autofluorescence was brightest in the most useful PE and FITC channels. It could not be completely gated out using other channels without concurrent loss of DC because of limitations in the accuracy and linearity of the color-compensation systems. The autofluorescence was a property of intact, viable cells that could not be gated out on the basis of high PI staining. Accordingly, the most effective (albeit time-consuming) approach was to presort to eliminate autofluorescent cells before immunofluorescent staining.

The autofluorescent cells from mouse spleen DC preparations were presorted from the nonautofluorescent cells (Fig. 2), and the surface phenotype of both was examined (data not shown). All class II MHC^{high}, CD11c^{high}, DEC-205$^+$, and CD40$^+$ cells that could be classed as mature DC were within the nonautofluorescent fraction. The autofluorescent cells were positive but low for class II MHC and were almost negative for the DC markers CD11c, DEC-205, and CD40. They expressed both CD80 and CD86. They were F4/80 strong positive and most, but not all, were strongly

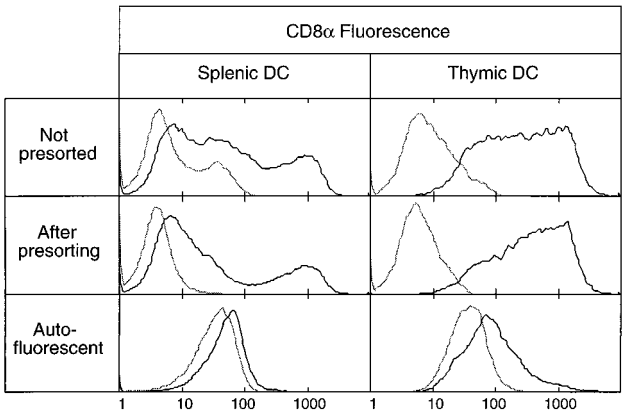

FIGURE 3. Intermediate CD8α fluorescence of spleen and thymus DC preparations. The DC preparations before presorting or after presorting to separate autofluorescent cells and other contaminants as in Fig. 2 were immunofluorescence stained for class II MHC and CD8α. They were gated for apparent high class II expression, a gate that would have included many of the high autofluorescence background cells. The level of CD8α fluorescent staining was then compared with the background fluorescence of samples lacking only the anti-CD8α stain.

positive for CD11b. Giemsa staining of cytospin preparations indicated that most autofluorescent cells had a morphology resembling macrophages. In contrast to DC, the autofluorescent cells showed a strong and permanent (>3-day) adherence to the plastic surface of culture vessels. We conclude that the majority of the autofluorescent cells were macrophages, although a proportion may have been immature DC.

In the case of thymic DC preparations, omission of CD4 and F4/80 immunomagnetic bead depletion caused still greater contamination of the DC preparations. As well as the autofluorescent cells, CD11b^{high} (Mac-1^{high}) nonfluorescent macrophages and thymic lymphoid precursor cells caused problems in subsequent analysis and sorting. This was overcome in some experiments by staining the cells with anti-CD11b and eliminating the CD11b^{high} cells, the smaller-sized lymphoid cells, and the autofluorescent cells in the presort. Possible CD11b^{low} DC were left in the preparation. The fluorochrome and the channel originally used for the CD11b stain could be used again for staining class II MHC on subsequent analysis or sorting because only class II MHC^{high} cells were then selected as DC. These maneuvers, although used in this study, finally proved unnecessary because, as shown later, thymic DC lacked the CD4$^+$, F4/80$^+$, and CD11b$^+$ DC subsets found in spleen. Therefore, a full immunomagnetic bead depletion was a safe procedure for thymic DC.

Apparent CD8^{int} cells in DC preparations

One recurring puzzle had been the appearance in DC preparations of cells selected as class II MHC$^+$ or CD11c$^+$ DC and apparent staining at intermediate levels for CD8α, rather than showing the clear-cut negative or strong positive CD8α staining shown in Fig. 1. This usually was seen when autofluorescent cells appeared in the background, as shown in Fig. 3, making it difficult to assess the significance of the apparent CD8α staining. As shown in Fig. 3, presorting the autofluorescent cells from the splenic DC preparations removed most of the cells that produced the peak of apparent CD8^{int} DC, although some low-level staining with the anti-CD8α mAb sometimes persisted, as in Fig. 3. In the case of thymic DC, the effect was less pronounced, but again some of the apparent lower staining with anti-CD8α mAb could be attributed to the autofluorescent cells (Fig. 3). Some DC, especially those in the thymus, express the low-affinity Fcγ receptor CD16/CD32. Blocking this receptor during staining caused some slight further reduction in the low-level staining with anti-CD8α, but most of this staining remained.

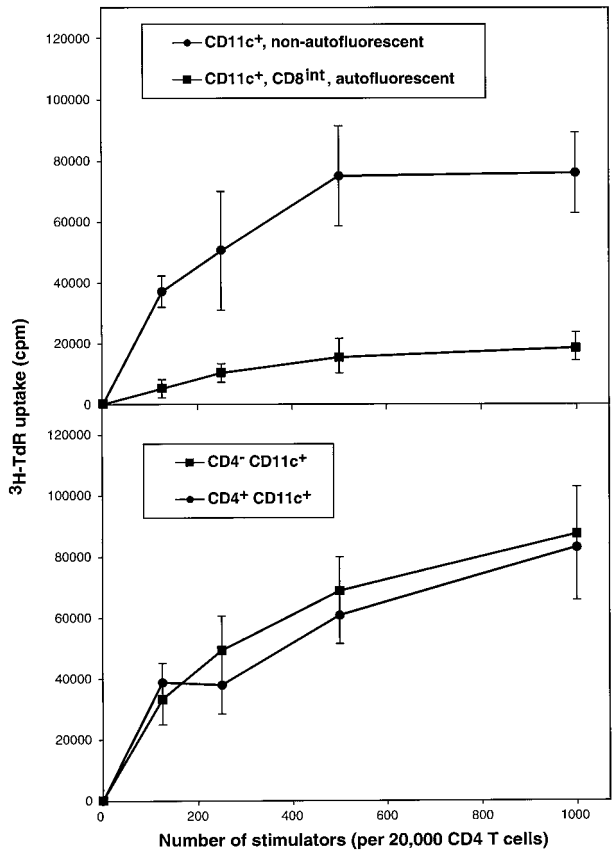

FIGURE 4. The ability of cells sorted from splenic DC preparations to activate allogeneic T cells. The mixed leukocyte cultures were between C57BL/6 spleen DC fractions and CBA lymph node CD4 T cells. Proliferation was assessed at day 3 of culture. The DC preparations (not presorted) were labeled with anti-CD11c and either anti-CD8α or anti-CD4. In the *upper panel*, the cells that were gated as CD11c$^+$ and CD8 intermediate (as in Fig. 3) and that were predominantly autofluorescent cells were sorted and compared with the total CD11c$^+$ population in which autofluorescent cells were gated out. In the *lower panel*, the CD4$^+$ and CD4$^-$ CD11c$^+$ cells with autofluorescent cells gated out but otherwise unsegregated were sorted and compared. Similar results were obtained in a second series of experiments.

To further check the nature of these cells (mostly autofluorescent) that could be mistaken for CD11c$^+$ CD8α^{int} splenic DC, they were sorted directly from an enriched splenic DC preparation and were tested for their capacity to stimulate allogeneic CD4 T cells in a mixed leukocyte reaction. They were compared with DC that were gated to remove autofluorescent cells and were sorted as CD11c$^+$ DC but were not separated further. The autofluorescent and apparent CD11c$^+$ CD8α^{int} cells showed a relatively poor ability to stimulate allogeneic T cell proliferation compared with the nonautofluorescent DC (Fig. 4). A low level of cross-contamination with authentic DC or the presence of some immature DC could have explained the residual stimulatory activity. Accordingly, most of the apparent CD8^{int} DC in splenic DC preparations were considered to be non-DC artifacts due mainly to autofluorescence. Presorting to eliminate autofluorescent cells was then used in most subsequent experiments.

Table I. *Adherence properties of spleen DC subpopulations*[a]

Spleen DC Population	Adherent (%)	Nonadherent (%)
$CD4^+8\alpha^-$	79 ± 5	21 ± 5
$CD4^-8\alpha^-$	32 ± 4	68 ± 4
$CD4^-8\alpha^+$	8 ± 2	92 ± 2

[a] DC were purified and sorted from spleen and then incubated for 1 h at 37°C in glass-slide chambers. The proportion of adherent cells was determined under phase-contrast microscopy, scoring at least 100 DC per slide. Values represent the mean ± range of pooled data from two experiments, with two slide chambers per population per experiment.

Properties of the $CD4^+$ DC of spleen

The major group of $CD4^+$ splenic DC we had previously lost from our preparations was assessed for its ability to stimulate proliferation in a mixed leukocyte reaction to verify that these cells could be classified as mature DC. As shown in Fig. 4, the DC-bearing CD4 were as effective at stimulating allogeneic CD4 T cells as the total populations lacking CD4 when $CD11c^+$ splenic DC were sorted simply on the basis of CD4 expression.

The splenic DC-bearing CD4 were all $CD8\alpha^-$, with the putative lymphoid-related $CD8\alpha^+$ DC lacking significant CD4 staining (Fig. 1). Therefore, splenic DC could be subdivided into the following three populations: $CD4^+8^-$, $CD4^-8^-$, and $CD4^-8^+$. One distinguishing feature of the splenic $CD4^+8^-$ DC was their adherence properties (Table I). After 1 h at 37°C, the majority of the $CD4^+8^-$ DC adhered and spread out on a glass or plastic surface, much like macrophages did but with the maintenance of a convoluted outer membrane with dendritic extensions. Despite these adherence properties, the $CD4^+8^-$ DC showed little capacity to phagocytose zymosan particles (data not shown). Although some $CD4^-8^-$ and $CD4^-8^+$ DC also adhered, the proportion was always much lower (Table I). Thus, isolation of splenic DC based on an initial adhesion to glass or plastic, as in an earlier DC isolation protocol (26), likely would have selected for the $CD4^+8^-$ DC (those above the broken line in Fig. 1).

The surface phenotype of the splenic DC populations

The surface antigenic phenotypes of the three spleen DC subpopulations were compared using a panel of mAb in three- and four-color immunofluorescent staining (Figs. 5 and 6). The splenic DC, including the $CD8\alpha^+$ DC, were all $CD8\beta^-$. The $CD8\alpha^+\beta^-$ DC were $CD4^-$ and $CD11b^-$ DEC-205^+, in accordance with our earlier results (12). The $CD4^-8^-$ and the $CD4^+8^-$ DC were indistinguishable by most markers, both differing from the $CD8\alpha^+$ DC in being $CD11b^+$ and DEC-205^- or DEC-205^{low}. They also both differed from the $CD8\alpha^+$ DC in expressing lower levels of the heat-stable Ag CD24 and the α6 integrin CD49f.

All three DC subtypes shared many common DC markers, including high levels of class II MHC, CD11c, and the α4 integrin CD49d (Fig. 6). They were all positive and expressed similar levels of the costimulator molecules CD40, B7.1 (CD80), and B7.2 (CD86) (Fig. 6). Thus, they could all be classed as mature DC, in agreement with their ability to activate T cells. The only feature found to distinguish the $CD4^+$ DC, apart from the expression of CD4 itself and the adhesion properties (Table I), was a higher level of expression of the F4/80 Ag (Fig. 6). Nevertheless, this level was considerably lower than it was on macrophages. Some F4/80 fluorescence was obtained with all DC populations, and even the $CD4^-8^-$ DC showed clear F4/80 staining. However, the differences in expression were sufficient to produce extensive depletion of the $CD4^+8^-$ DC with anti-F4/80 and immunomagnetic beads and allowed recovery of most $CD4^-8^-$ and $CD4^-8^+$ DC.

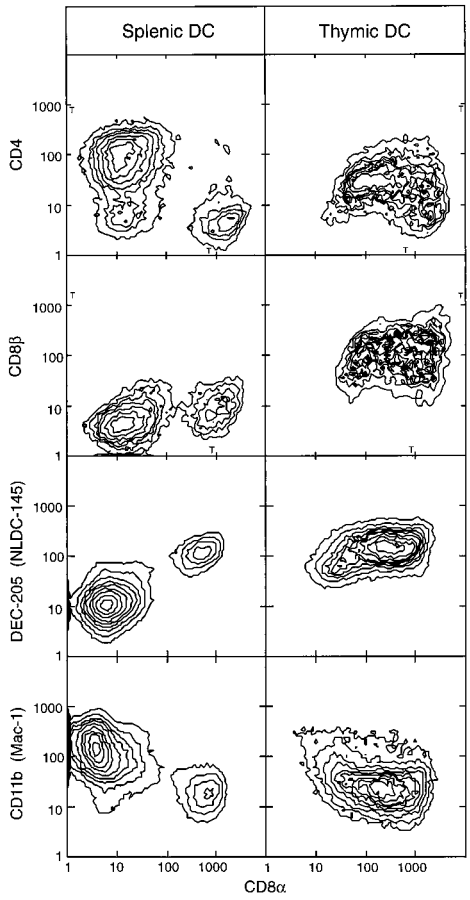

FIGURE 5. Comparison of the staining of spleen and thymus DC for CD8α correlated with other markers. The DC were purified and presorted to remove autofluorescent cells and other contaminants. They were then immunofluorescence labeled with anti-class II MHC and mAb against the other markers in four-color immunofluorescent staining. The DC were gated as class II MHC^{high} cells with the characteristic light scatter of DC and exclusion of PI. The level of fluorescence staining for other markers is then given for these gated cells. The "T" on the contour graphs indicates the modal level of fluorescence obtained on side-by-side staining of naive splenic T cells, for comparative purposes. The results are typical of three to seven of these analyses.

Immunofluorescent staining of thymic DC

In contrast to splenic DC, thymic DC were not segregated into clear subpopulations by immunofluorescent staining. As reported previously (12, 15), a wide spread of CD8α staining was obtained from low to the same high levels as T cells and splenic $CD8\alpha^+$ DC (Fig. 5). Staining for both CD8β and CD4 was obtained but at levels well below those characteristic of T cells and thymocytes (Fig. 5). However, the majority of thymic DC were $CD11b^{low}$ and DEC-205^{high}, resembling in this respect the $CD8\alpha^+\beta^-$ splenic DC (Fig. 5). Only a trail of $CD8\alpha^{low}$ thymic DC showed lower staining for DEC-205 and higher staining for CD11b, approaching but never equivalent to the splenic $CD8\alpha^-$ populations. Staining for a series of other markers and gating for CD8α expression (Fig. 7) showed that $CD8\alpha^{low}$, $CD8\alpha^{int}$, and $CD8\alpha^{high}$ thymic DC all expressed the markers expected of mature DC and all were generally similar to the $CD8\alpha^{high}$ DC of spleen; only the $CD8\alpha^{low}$ DC showed some differences for a proportion of the cells present, including some cells that were $F4/80^{low}$ $CD49f^{low}$, like $CD8\alpha^-$ splenic DC, and some cells that were class II MHC^{high}, $CD11c^{int}$, and $CD86^{high}$, like activated DC.

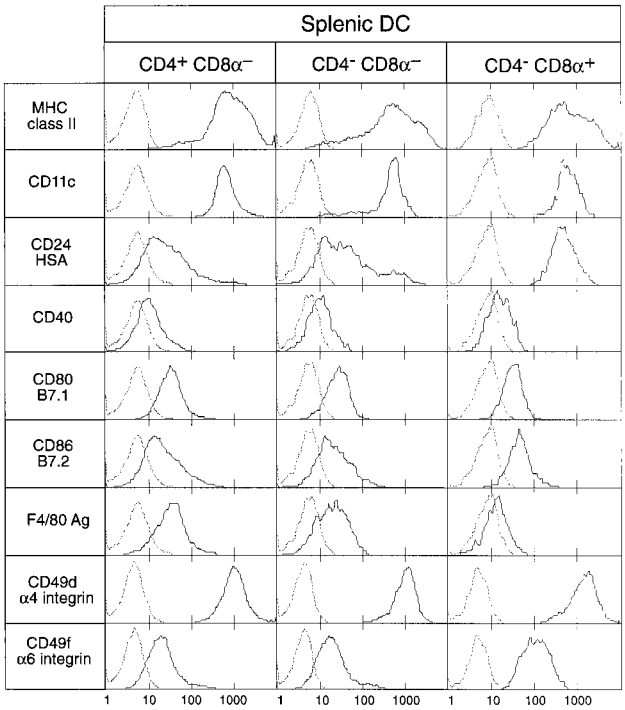

FIGURE 6. The staining of spleen DC subpopulations for other markers. The DC were prepared and stained in four fluorescent colors as in Fig. 5. The DC were gated as class II MHC^{high} cells as in Fig. 5 or as CD11c^{+} cells and additionally were gated for CD4 and CD8α expression to segregate the subpopulations. The level of staining of the gated subpopulations for the fourth marker is then presented, compared with a background with only this fourth mAb omitted. These backgrounds were identical with those obtained using isotype-matched control conjugated mAb. The DC were gated as class II MHC^{high} cells in all panels except the top, in which the class II MHC staining is given for DC gated as CD11c^{+} cells. Note that class II MHC staining was very high, so a less than optimally conjugated mAb was used to ensure the staining was on scale. The results have been confirmed in two to four separate experiments.

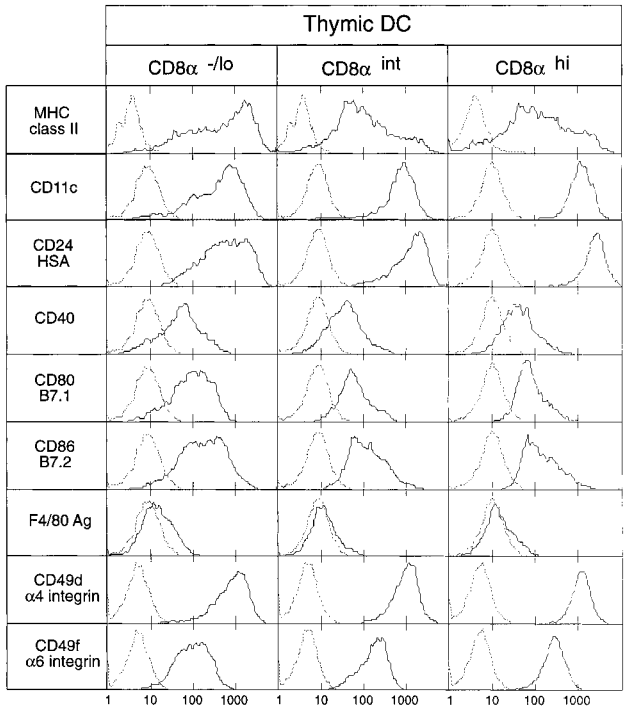

FIGURE 7. The staining of subgroups of thymus DC for a range of markers. The approach was similar to that of Fig. 6, except that the DC were subdivided only on the basis of the level of staining with CD8α. Two experiments gave similar results.

Tests for authentic marker expression: chimeras using marker-null mice

The moderate staining for CD4 and CD8β on thymic DC (Fig. 5) led to suspicion that these Ags may have been picked up from associated thymocytes, as we had demonstrated previously for the moderate level of Thy 1 on the surface of thymic DC (27). Accordingly, a critical test for Ag pickup was applied to the CD8α, CD8β, and CD4 staining of all the DC, using CD8α^{null} mice (in which neither CD8α nor CD8β should be expressed on the cell surface) and CD4^{null} mice. In both of these gene knockout mouse strains it could be demonstrated (using other markers) that all DC subtypes were present despite lacking their characteristic markers (20). Bone-marrow chimeras were constructed by injecting irradiated mice with equal numbers of Ly-5.2, CD8α^{null}, or CD4^{null} and Ly-5.1 wild-type bone marrow cells (or as control chimeras, with Ly-5.2 wild-type and Ly-5.1 wild-type bone marrow cells) and then analyzing the DC populations 5 wk later using the Ly-5 marker to separately analyze the DC progeny. The test was to see whether the marker expression disappeared when the gene was disrupted (in which case the expression had been authentic) or if the staining persisted (in which case it had been picked up from wild-type cells, presumably T lineage cells). The DC in the chimeras derived from the wild-type bone marrow gave staining results similar to those already demonstrated for normal, nonirradiated mice in both the test and control chimeras. The results for the DC derived from the CD8α^{null} or CD4^{null} bone marrow are compared with these side-by-side wild-type DC in Fig. 8.

On splenic DC, CD8α staining largely disappeared from the "CD8α^{high} DC" derived from CD8α^{null} bone marrow, indicating that the expression had been authentic. A variable but usually very slight level of CD8α staining above background persisted, indicating a very low level of Ag pickup. On thymic DC, most of the CD8α^{high} staining also disappeared, again indicating authentic CD8α expression; however, the staining did not drop to the background, with all DC now showing a low to intermediate staining, corresponding to the lower staining levels seen for about half of the DC from wild-type bone marrow (Fig. 8). Thus, the CD8α staining of thymic DC appeared to be a mix of high-level authentic expression by about half of the DC together with low-level pick-up of Ag from associated thymocytes by all DC.

CD8β staining was not seen above marginal levels on splenic DC but was seen at moderate levels on thymic DC. This staining of thymic DC persisted at the same level on the DC derived from CD8α^{null} bone marrow, in which the absence of the CD8α chain should prevent expression of CD8β on the cell surface (Fig. 8). Thus, all CD8β staining on DC could be attributed to Ag pickup.

On splenic DC, CD4 staining largely disappeared from the large population of "CD4^{+} DC" when the CD4 gene was disrupted, indicating that this had been authentic CD4 expression (Fig. 8). In contrast, the moderate staining of thymic DC with anti-CD4 persisted at normal levels even when the CD4 gene was disrupted, indicating that all the CD4 on thymic DC, just like all the CD8β on these cells, could be attributed to pickup from associated thymocytes.

Tests for authentic marker expression: overnight incubation

Another test for authentic DC surface-marker expression was to check whether the marker persisted, rather than being washed off,

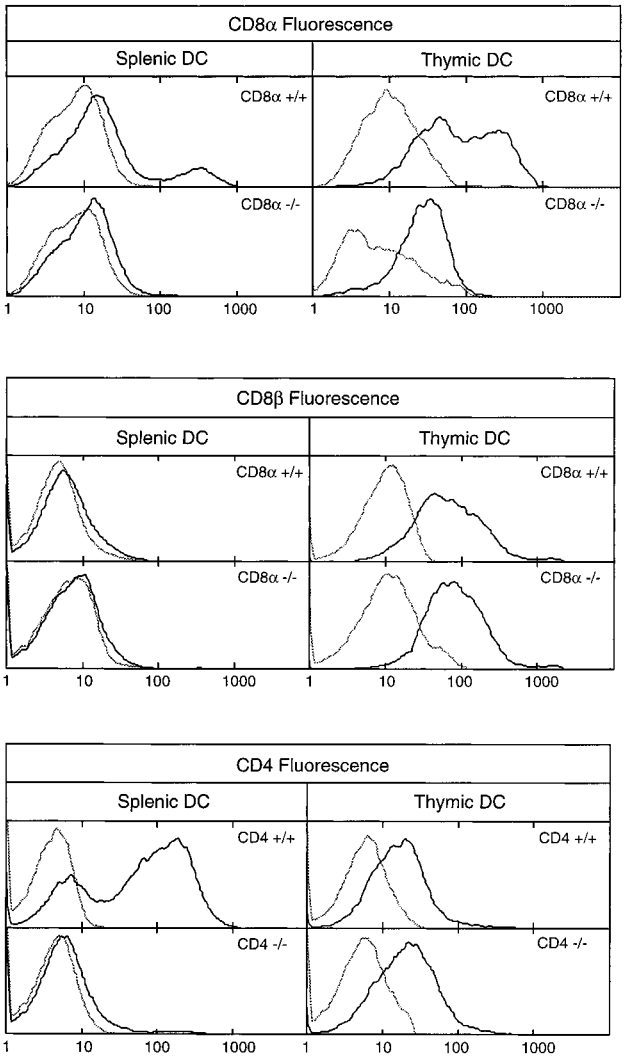

FIGURE 8. Staining for CD8α, CD8β, and CD4 on DC from bone-marrow chimeric mice. Chimeras were constructed by injecting irradiated mice with mixtures of bone marrow from wild-type mice and from CD8α^{null} (*upper two panels*) or CD4^{null} (*lower panel*) mice, with the wild-type and the null mice differing at the Ly-5 locus. The DC were purified and then labeled in three or four fluorescent colors without presorting. The stains used were anti-class II MHC (to distinguish DC), anti-Ly-5.2 (to segregate the DC derived from wild-type or null bone marrow), and anti-CD8α and either anti-CD8β or anti-CD4 (to determine the level of staining on the gated populations). Autofluorescent cells were gated out along with PI-positive cells as much as possible during analysis, but not all autofluorescence could be eliminated. In each case two experiments were conducted and produced equivalent results.

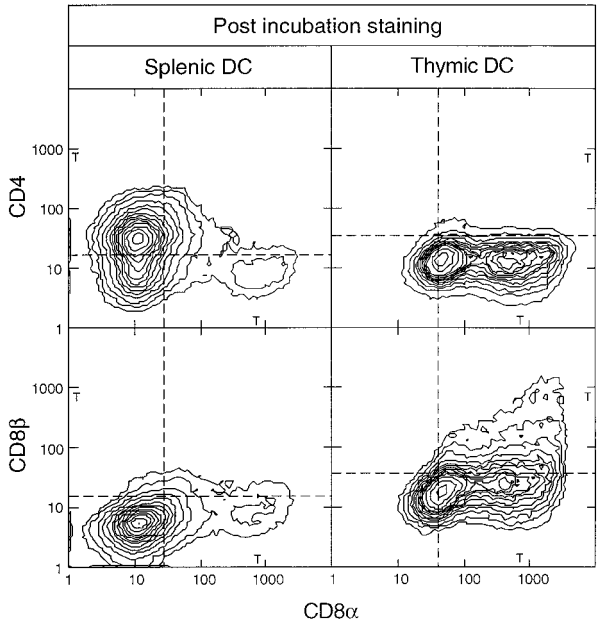

FIGURE 9. Staining for CD4, CD8α, and CD8β after overnight culture of DC. DC were purified, presorted to eliminate autofluorescent cells and contaminants as in Fig. 2, and then incubated in culture medium overnight. The next morning the DC were harvested, dead cells (~50% of the total) were removed, and the DC were stained and analyzed as in Fig. 5. A second experiment produced similar results.

after a short culture period. However, this approach had the disadvantages of incomplete elimination of absorbed Ags, changes in authentic marker expression due to DC maturation in culture, and the known differential loss of the CD8α^+ DC due to their slightly faster rate of death in culture (28). The basic CD4 and CD8α staining pattern of splenic DC persisted after culture, an argument for authentic marker expression (Fig. 9). The level of CD4 staining dropped noticeably but did not disappear, a result compatible with "maturation." The level of CD8α staining remained high, as we had noted previously (12, 15), but the relative number of CD8α^+ DC decreased, compatible with a higher proportion of CD8α^+ DC being among the 50% of DC that died on overnight culture. The CD4 and CD8β staining of thymic DC was markedly reduced by overnight culture (Fig. 9) and in some experiments was almost completely eliminated, a result more compatible with surface adsorption than with real synthesis and expression. Of particular note was the CD8α staining of thymic DC after incubation because the DC then clearly fell into two staining populations (Fig. 9). One group displayed persistent high CD8α staining compatible with authentic expression, as was seen with CD8α^+ splenic DC. Although the number of such CD8α^{high} thymic DC appeared to be reduced, this may have been due to differential death in culture. The other group after incubation showed low but still above background CD8α staining. It could not be ascertained whether these were CD8α^- DC that had not lost all the CD8 acquired from thymocytes or whether their low CD8α staining was a consequence of low but still endogenous CD8α synthesis.

Tests for authentic marker expression: PCR for mRNA

A final test for authentic marker expression was to determine whether the DC expressed mRNA coding for the surface molecules stained. Such tests had already been performed for CD8α and CD8β expression with the conclusion that thymic DC and splenic CD8α^+ contained mRNA for CD8α but not for CD8β (11). This approach was extended for CD4 expression, using RT-PCR (Fig. 10). Splenic CD4$^+$8$^-$ DC clearly contained RNA transcripts for CD4, a fact that was evident even with a low-sensitivity PCR, although the level of mRNA appeared less than that seen with thymocytes. This would accord with a lower CD4 surface expression on these DC compared with that on T cells. With a low-sensitivity PCR, no band corresponding to CD4 mRNA could be seen with thymic DC, CD4$^-$8$^-$ DC, or CD4$^-$8$^+$ DC. However, by increasing the sensitivity with more PCR cycles, all DC populations showed evidence of some CD4 mRNA, although at much lower levels than with the CD4$^+$8$^-$ splenic DC population.

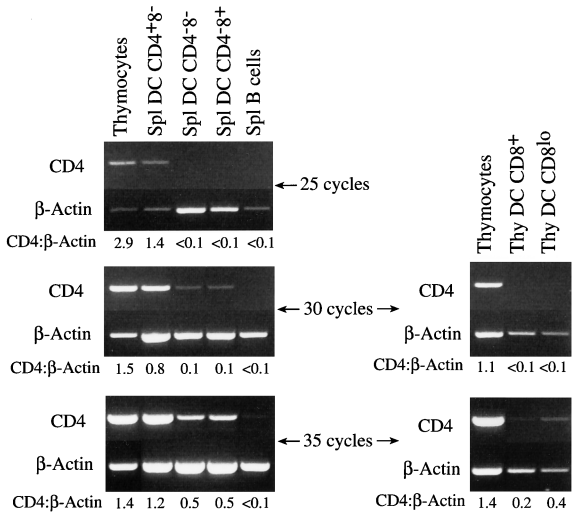

FIGURE 10. RT-PCR for CD4 RNA transcripts in DC. DC were purified and sorted into the subgroups shown, RNA was extracted, and RT-PCR was performed at various levels of sensitivity according to the number of PCR cycles. The figure shows the ethidium bromide-stained agarose gels with the PCR product from 500 cells. β-Actin was used as a housekeeping gene control for comparison with CD4 mRNA expression, and the relative intensity of the CD4 band compared with the β-actin band is given for comparison. Despite extraction of equivalent numbers of purified DC, thymic DC always gave a fainter band for β-actin mRNA than did splenic DC or the other cells tested. The results were consistent over three separate experiments.

Discussion

This study has revealed a further level of heterogeneity among mouse DC populations, at least as judged by surface-marker expression. There appear to be three rather than two types of DC in mouse spleen and two rather than one type of DC in mouse thymus. Although we have not considered the DC populations of lymph nodes in this study, it is already clear from earlier studies that they include still more subgroups than found in spleen (12–14). Further work will be needed to determine the biological basis of this DC population heterogeneity. This will be a sufficiently challenging task without the false leads provided by the inadequacies of DC isolation procedures or the various immunofluorescent staining artifacts, all of which we encountered in the present study and would undoubtedly be a limitation of similar studies in other laboratories. Although these are merely technical problems, they need to be recognized and eliminated before the real basis of DC heterogeneity in surface-marker expression can be explored.

One important variable already recognized by most workers in the field is the procedure used for DC isolation, which can select particular DC subsets or alter their phenotype. In our earlier procedures we paid the price for overzealous elimination of all macrophage-like and lymphoid cells by the loss of a major subgroup of CD4-bearing F4/80$^+$ adherent DC. However, insufficient DC enrichment before analysis makes flow cytometry and sorting difficult due to overlap of markers with other more numerous cell types to the presence of autofluorescent cells. It is of interest that this study has also pointed to a limitation of the earlier, adhesion-dependent isolation procedure of Steinman and coworkers (26), namely a strong selection for this same CD4-bearing adherent DC population. Thus, the earlier studies on mouse spleen DC from this laboratory and from the Rockefeller University laboratory would have been addressing different DC types with little overlap.

Another important problem when nonlymphoid cells are extracted from lymphoid tissues is autofluorescence. We have now demonstrated that the mature DC themselves are not markedly autofluorescent, at least when freshly extracted from tissues. Accordingly, the problem is finding the optimal means of eliminating the autofluorescent cells. Although reserving a fluorescent channel to gate out such cells is one solution, the fluorescence overlap between analysis channels and the limitation of color compensation procedures means this is a less-than-perfect option. Prior elimination of autofluorescent cells, for example by presorting, is a more effective but time-consuming option.

A problem that is directly associated with the DC themselves is the pickup of surface Ags from other cells. This is especially marked with thymic DC, which exist in the thymus tightly associated with thymocytes as "rosettes" (29, 30) and which are in an environment replete with dying thymocytes. It appears that DC are able to pick up onto their outer surface Thy-1 (27), CD4, and CD8$\alpha\beta$. The CD8$\alpha\beta$ picked up is in addition to the CD8$\alpha\alpha$ that they make themselves as an authentic surface protein. This "pick-up" may be no more subtle than fragments of thymocyte cell membranes attached to the DC surface. Alternatively, there could be more specific binding of CD8$\alpha\beta$ to class I MHC and CD4 to class II MHC. There is no evidence that this pickup is of any functional significance.

These artifacts have previously obscured the analysis of thymic DC populations and have led to the picture of a single DC group with a wide distribution of CD8 expression. It now appears that there are two more distinct groups, one CD8$\alpha\alpha^{high}$ and one CD8$\alpha\alpha^{low}$ or even CD8$\alpha\alpha^-$. However, both of these are CD8β^-, CD4$^-$, DEC-205$^+$, and CD11b^{low} and appear very similar by a series of other surface markers. They may all be one basic DC lineage, some of which have been exposed to factors inducing CD8α expression and others that have not. In favor of this view is the finding that all DC grown with high efficiency in culture from the thymic DC/T cell early precursor population lack CD8α expression, whereas DC produced from the same precursor population on intrathymic transfer into an irradiated recipient generate the normal spread of CD8 expression seen in intact mice (16–18). However, we cannot exclude the possibility that one of these DC subtypes found in the thymus is of different origin and might migrate into the thymus from the bloodstream.

The splenic DC seem less prone to pickup of T cell Ags, perhaps because of a less-tight association with T cells or because many are not located in the T cell zones of the spleen. In particular, the CD4 on the surface of about half of the splenic DC appears to be authentic expression of CD4 synthesized by the DC themselves. This resolves an apparent discrepancy between murine DC and those of human or rat origin: it is now clear that all three species have a proportion of DC expressing CD4. As we have indicated previously (11), the high CD8α on the surface of about one quarter of splenic DC reflects true synthesis by these DC, an apparent point of difference with other species. What does this expression of CD4 or CD8 imply?

We have argued previously that the lymphoid marker CD8 on splenic and thymic DC reflects their origin from a lymphoid precursor cell similar to the early lymphoid precursor we isolated from mouse thymus (16, 17, 31). Although formal clonal proof of a common lymphoid/DC precursor is still missing, it is clear that these DC represent a distinct lineage with distinct cytokine requirements and transcription factor control (18, 19). They also have a very much greater potential to produce IL-12 than either of the CD8$^-$ DC populations (Ref. 25 and H. Hochrein, manuscript in preparation). Although CD4 is also considered as a lymphoid marker, CD4 on DC does not necessarily reflect a lymphoid origin. All the other markers, including F4/80 and CD11b, suggest that these CD4$^+$ but CD8α^- DC resemble the CD4$^-$8α^- DC, which

are presumed to be of myeloid origin. It should also be noted that CD4 can also be expressed by monocytes.

The simplest explanation would be that these $CD4^+8^-$ splenic DC are an earlier and less mature form of the $CD4^-8^-$ myeloid DC type. By this argument, they should appear as immature by other markers and should on maturation lose CD4 to become the $CD4^-8^-$ DC. However, several facts argue against this. First, although CD4 expression does drop on incubation of these cells, the DC remain clearly $CD4^+$ with little evidence of an increase in the number of $CD4^-8^-$ DC. Second, the $CD4^+8^-$ DC show expression similar to that of the $CD4^-8^-$ DC of the maturation markers class II MHC, CD80, and CD86, and they appear fully mature in their capacity to stimulate allogeneic T cells. Finally, in preliminary labeling experiments to determine the turnover and lifespan of these DC types in vivo, there is no evidence for a precursor-product relationship between $CD4^+8^-$ DC and $CD4^-8^-$ DC (A. Kamath, D. Tough, and K. Shortman, manuscript in preparation). Therefore, they may represent a third independent developmental stream. One possibility is that they, rather than the $CD8^+$ DC, correspond to the human $CD4^+$ plasmacytoid DC2 lineage (32). The lineage relationships between these three splenic DC types and their individual biological functions are currently under active investigation.

References

1. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol. 9:271.*
2. Bell, D., J. W. Young, and J. Banchereau. 1999. Dendritic cells. *Adv. Immunol. 72:255.*
3. Hart, D. N. J. 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood 90:3245.*
4. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*
5. Süss, G., and K. Shortman. 1996. A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. *J. Exp. Med. 183:1789.*
6. Kronin, V., K. Winkel, G. Süss, A. Kelso, W. Heath, J. Kirberg, H. von Boehmer, and K. Shortman. 1996. A subclass of dendritic cells regulates the response of naive CD8 T cells by limiting their IL-2 production. *J. Immunol. 157:3819.*
7. Rissoan, M.-C., V. Soumelis, N. Kadowski, G. Grouard, F. Briere, R. de Waal Malefyt, and Y.-J. Liu. 1999. Reciprocal control of T helper and dendritic cell differentiation. *Science 283:1183.*
8. Maldonado-López, R., T. de Smedt, P. Michel, J. Godfroid, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain, and M. Moser. 1999. $CD8\alpha^+$ and $CD8\alpha^-$ subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J. Exp. Med. 189:587.*
9. Pulendran, B., J. L. Smith, G. Caspary, K. Brasel, D. Pettit, E. Maraskovsky, and C. R. Maliszewski. 1999. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc. Natl. Acad. Sci. USA 96:1036.*
10. Shortman, K., and C. Caux. 1997. Dendritic cell development: multiple pathways to Nature's adjuvants. *Stem Cells 15:409.*
11. Vremec, D., M. Zorbas, R. Scollay, D. J. Saunders, C. F. Ardavin, L. Wu, and K. Shortman. 1992. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J. Exp. Med. 176:47.*
12. Vremec, D., and K. Shortman. 1997. Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes on incubation and differences between thymus, spleen and lymph nodes. *J. Immunol. 159:565.*
13. Salomon, B., J. L. Cohen, C. Masurier, and D. Klatzmann. 1998. Three populations of mouse lymph node dendritic cells with different origins and dynamics. *J. Immunol. 160:708.*
14. Anjuère, F., P. Martin, I. Ferrero, M. López Fraga, G. Martinez del Hoyo, N. Wright, and C. Ardavin. 1999. Definition of dendritic cell subpopulations present in the spleen, Peyer's patches, lymph nodes, and skin of the mouse. *Blood 93:590.*
15. Wu, L., D. Vremec, C. Ardavin, K. Winkel, G. Suss, H. Georgiou, E. Maraskovsky, W. Cook, and K. Shortman. 1995. Mouse thymus dendritic cells: kinetics of development and changes in surface markers during maturation. *Eur. J. Immunol. 25:418.*
16. Ardavin, C., L. Wu, C.-L. Li, and K. Shortman. 1993. Thymic dendritic cells and T cells develop simultaneously within the thymus from a common precursor population. *Nature 362:761.*
17. Wu, L., C.-L. Li, and K. Shortman. 1996. Thymic dendritic cell precursors: relationship to the T-lymphocyte lineage and phenotype of the dendritic cell progeny. *J. Exp. Med. 184:903.*
18. Saunders, D., K. Lucas, J. Ismaili, L. Wu, E. Maraskovsky, A. Dunn, D. Metcalf, and K. Shortman. 1996. Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte-macrophage colony-stimulating factor. *J. Exp. Med. 184:2185.*
19. Wu, L., A. D'Amico, K. D. Winkel, M. Suter, D. Lo, and K. Shortman. 1998. RelB is essential for the development of myeloid-related $CD8\alpha^-$ dendritic cells but not of lymphoid-related $CD8\alpha^+$ dendritic cells. *Immunity 9:839.*
20. Kronin, V., D. Vremec, K. Winkel, B. J. Classon, R. G. Miller, T. W. Mak, K. Shortman, and G. Süss. 1997. Are $CD8^+$ dendritic cells veto cells? The role of CD8 on dendritic cells in the regulation of CD4 and CD8 T cell responses. *Int. Immunol. 9:1061.*
21. Winkel, K., F. Sotzik, D. Vremec, P. U. Cameron, and K. Shortman. 1994. CD4 and CD8 expression by human and mouse thymic dendritic cells. *Immunol. Lett. 40:93.*
22. Crowley, M. T., K. Inaba, M. D. Witmer-Pack, S. Gezelter, and R. M. Steinman. 1990. Use of the fluorescence activated cell sorted to enrich dendritic cells from mouse spleen. *J. Immunol. Methods 133:55.*
23. Sotzik, F., Y. Rosenberg, A. W. Boyd, M. Honeyman, D. Metcalf, R. Scollay, L. Wu, and K. Shortman. 1994. Assessment of CD4 expression by early T-precursor cells and by dendritic cells in the human thymus. *J. Immunol. 152: 3370.*
24. Liu, L., M. Zhang, C. Jenkins, and G. G. MacPherson. 1998. Dendritic cell heterogeneity in vivo: two functionally different dendritic cell populations in rat intestinal lymph can be distinguished by CD4 expression. *J. Immunol. 161:1146.*
25. Pulendran, B., J. Lingappa, M. K. Kennedy, J. Smith, M. Teepe, A. Rudensky, C. R. Maliszewski, and E. Maraskovsky. 1997. Developmental pathways of dendritic cells in vivo: distinct function, phenotype, and localization of dendritic cell subsets in FLT3 ligand-treated mice. *J. Immunol. 159:2222.*
26. Crowley, M., K. Inaba, M. Witmer-Pack, and R. M. Steinman. 1989. The cell surface of mouse dendritic cells: FACS analysis of dendritic cells from different tissues including thymus. *Cell. Immunol. 118:108.*
27. Shortman, K., L. Wu, C. Ardavin, D. Vremec, S. Sotzik, K. Winkel, and G. Süss. 1995. Thymic dendritic cells: surface phenotype, developmental origin and function. In *Dendritic Cells in Fundamental and Clinical Immunology*. J. Banchereau and D. Schmitt, eds. Plenum, New York, p. 21.
28. Winkel, K. D., V. Kronin, M. F. Krummel, and K. Shortman. 1997. The nature of the signals regulating CD8 T cell proliferative responses to $CD8\alpha^+$ or $CD8\alpha^-$ dendritic cells. *Eur. J. Immunol. 27:3350.*
29. Kyewski, B. A., R. V. Rouse, and H. S. Kaplan. 1982. Thymocyte rosettes: multicellular complexes of lymphocytes and bone marrow-derived stromal cells in the mouse thymus. *Proc. Natl. Acad. Sci. USA 79:5646.*
30. Shortman, K., D. Vremec, A. D'Amico, F. Battye, and R. Boyd. 1989. Nature of the thymocytes associated with dendritic cells and macrophages in thymic rosettes. *Cell. Immunol. 119:85.*
31. Wu, L., M. Antica, G. R. Johnson, R. Scollay, and K. Shortman. 1991. Developmental potential of the earliest precursor cells from the adult thymus. *J. Exp. Med. 174:1617.*
32. Grouard, G., M. Rissoan, L. Filgueira, I. Durand, J. Banchereau, and Y.-J. Liu. 1997. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J. Exp. Med. 185:1101.*