The Surface Phenotype of Dendritic Cells Purified from Mouse Thymus and Spleen: Investigation of the CD8 Expression by a Subpopulation of Dendritic Cells

By David Vremec, Michelle Zorbas, Roland Scollay, Dolores J. Saunders, Carlos F. Ardavin, Li Wu, and Ken Shortman

From The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia

Summary

A new procedure for rapid isolation of dendritic cells (DC) was devised, involving collagenase digestion of tissues, dissociation of lymphoid-DC complexes, selection of light-density cells, then depletion of lymphocytes and other non-DC by treatment with a mixture of lineage-specific monoclonal antibodies (mAbs) and removal with antiimmunoglobulin-coupled magnetic beads. This enriched population (~80% DC) was further purified when required by fluorescence-activated cell sorting for cells expressing high levels of class II major histocompatibility complex (MHC). The isolated DC were characterized by immunofluorescent staining using a panel of 30 mAbs. Thymic DC were surface positive for a number of markers characteristic of T cells, but they were distinct from T-lineage cells in expressing high levels of class II MHC, in lacking expression of the T cell receptor (TCR)-CD3 complex, and having TCR β and γ genes in germline state. Splenic DC shared many markers with thymic DC, but were negative for most T cell markers, with the exception of CD8. A substantial proportion of DC from both thymus and spleen expressed CD8 at high levels, comparable with that on T cells. This appeared to be authentic CD8, and was produced by the DC themselves, since they contained CD8α mRNA. Thymic DC presented both the CD8 α and β chains on the cell surface (Ly-2^+3^+), although the α chain was in excess; the splenic DC expressed only the CD8 α chain (Ly-2^+3^-). It is suggested that the expression of CD8 could endow certain antigen-presenting DC with a veto function.

Dendritic cells (DC),[1] first described by Steinman et al. (1, 2), are a minor population of irregularly shaped cells in lymphoid organs, distinguishable from both lymphocytes and macrophages. DC constitutively express high levels of both class I and class II MHC antigens (1, 2). They are highly efficient at presentation of antigen and stimulation of T lymphocytes (1–7). Those within the thymus are believed to effect deletion of developing T cells with self-reacting potential (8, 9). The surface antigenic pattern of thymic DC has been shown to differ somewhat from that of splenic DC (10–12), but it is not clear whether this implies the cells have a different origin, or a different function, or whether they simply represent different development states of the same functional lineage. There is evidence that it is the developmental state of the T cell that determines whether the T cell–DC interaction leads to T cell proliferation or to T cell death (13). As part of a study of the interaction between developing T cells in the thymus and thymic stromal elements (14), we have isolated thymic DC and compared them with those isolated from the spleen.

The usual procedure for isolating DC involves enrichment in a low buoyant density fraction by centrifugation, followed by selection as cells that show an initial adherence but then release from the vessel surface when cultured overnight (10). We have used such a procedure to isolate DC from mouse thymus (15). However, we were concerned that the association of many thymic DC with thymocytes to form dense and nonadherent rosettes (14, 16) would prevent their isolation in this way, and that overnight culture stage might change their phenotype (7). In addition, we noted a disturbing incidence of thymocytes intimately bound to the enriched DC,

[1] *Abbreviations used in this paper:* BSS, balanced salt solution; DC, dendritic cell; HSA, heat-stable antigen.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/07/0047/12 $2.00
Volume 176 July 1992 47–58

as well as a raised autofluorescent background. All these problems led us to question the apparent expression of certain T cell markers by thymic DC preparations. Accordingly, we developed a procedure that incorporated a step to dissociate DC-lymphocyte complexes, and which allowed a rapid enrichment of DC by immunomagnetic bead depletion, without the culture step. The resulting DC preparations were free of lymphocytes but still displayed several markers normally found on T cells. We have examined this critically and in particular verified the expression of surprisingly high levels of CD8 by a subpopulation of both thymic and splenic DC.

Materials and Methods

Animals. The mice were specific pathogen-free 5–7-wk-old females bred at The Walter and Eliza Hall Institute animal facility. Unless otherwise specified, the strain was C57BL/6J Wehi (Thy-1.2, Ly-2.2). In some experiments, the C57BL Ka (Thy-1.1, Ly-2.2) or the CBA CaH Wehi (Thy-1.2, Ly-2.1) strains were used.

mAbs. Most hybridomas were grown and the mAbs purified and conjugated in this laboratory. The mAbs used, the hybridoma clone numbers, and their antigenic specificities are listed in Table 1.

Immunofluorescent Staining and Flow Cytometry. The staining, flow cytometry analysis, and sorting procedures were similar to those described previously (42). All DC preparations were preincubated with a rat Ig–mouse Ig mix to minimize nonspecific binding. In the routine, procedure cells were stained with fluorescein-conjugated mAb against MHC class II (M5/114 for C57BL/6, 14.4.4S for CBA), with PE-conjugated mAb against CD8α (53–6.7), and with a third biotin-conjugated mAb from Table 1, using as the second-stage reagent a Texas red–streptavidin conjugate (Caltag Laboratories, South San Francisco, CA). The single-color stains used the biotin-conjugated mAb and Texas red–streptavidin second stage. Propidium iodide (1 μg/ml) was included in the final cell wash. Multiparameter analysis and sorting utilized a FACStar Plus®, while single parameter analysis utilized a FACScan® (Becton Dickinson & Co., Mountain View, CA). Forward light scatter and propidium iodide staining gates were set to exclude dead cells.

Cell Suspension Media. Cells were suspended in a mouse osmolarity, pH 7.2, Hepes-buffered balanced salt solution (BSS) containing 5% FCS (BSS-FCS). For digestion procedures, the medium was RPMI 1640, modified to mouse osmolarity, with additional pH 7.2 Hepes buffering, containing 2% FCS (RPMI 1640-FCS). To avoid reassociation of DC with T cells, the medium used was EDTA-BSS-FCS, a balanced salt solution isoosmotic with mouse serum but lacking Ca^{2+} and Mg^{2+} and including 5 μM EDTA; to this was added 5% FCS-EDTA, FCS that had Ca^{2+} and Mg^{2+} chelated by the addition of 1 ml 0.099 M EDTA per 10 ml serum.

Mechanically Dissociated Lymphoid Cell Suspensions. Suspensions containing predominantly lymphoid cells were prepared from spleen or thymus by teasing into cold BSS-FCS, settling to remove large debris, then washing by spinning through a layer of FCS. The cells were then suspended in cold BSS-FCS.

DC Isolation. Thymuses (10–30) or spleens (5–20) were teased apart or chopped into small fragments with scissors, then agitated in 10 ml BSS-FCS by rapidly pipetting up and down a wide-bore Pasteur pipette. After a brief settling to collect large stromal fragments, the supernatant was collected and lightly centrifuged (centrifuge accelerated up to 1,000 rpm, then immediately switched off) to collect small fragments and lympho-stromal complexes. The supernatant containing free cells was removed. This process was repeated three to four times, using the washings of the large stromal fragments to wash the small-fragment pellet, which was again collected by light centrifugation. The washed large and small stromal fragments were then pooled. The pooled stromal fragments were then digested with collagenase (1 mg/ml; Boehringer-Mannheim, Mannheim, West Germany) and DNAse (0.02 mg/ml; grade II bovine pancreatic DNAse I; Boehringer-Mannheim), in 7.5 ml RPMI 1640-FCS for 25 min at 22°C with continuous agitation. This procedure follows that previously used to isolate thymic rosettes (14, 43). The rosettes were then dissociated by adding 0.6 ml of 0.099 M EDTA, pH 7.2, and continuing the incubation for 5 min. To avoid rosette reformation, all separation steps from this point to the last depletion were carried out in EDTA-BSS-FCS, and any serum used was FCS-EDTA.

Cold FCS-EDTA was layered underneath the digest, and the cells were recovered by centrifugation at 500 *g* for 7 min. The cells were resuspended in cold isoosmotic Metrizamide solution (Nyegaard Diagnostics, Oslo, Norway), pH 7.2, density 1.075 g/cm^3, containing 5 mM EDTA, and a low-density cell fraction selected by centrifugation for 10 min at 1,700 *g*, as described elsewhere (44).

The recovered low-density cells were treated with a cocktail containing titrated levels of the following mAbs to coat non-DC: anti-CD3, KT3-1.1; anti-CD4, GK1.5; anti-Thy-1.2, 30-H12, or for Thy-1.1 mice, 19F12; anti-IL-2Rα, PC61; anti-Gr-1, RB68C5; anti-Mac-1α, M1/70.15; antimacrophage F4/80 antigen, F4/80; anti-B220, RA36B2; antierythroid, TER119; anti-FcR II, 2.4G2. Further details are in Table 1. Most mAbs were used at near-saturation levels, as for optimal cell staining. However, the mAbs specific for Thy-1, Mac-1, and FcRII, which are expressed at low levels on DC, were titrated in trial experiments and used at reduced levels so that only cells expressing high levels of these antigens were depleted. Incubation was for 25 min at 4°C, after which the cells were washed by layering over and spinning through FCS-EDTA. The cells were then depleted twice with anti-Ig-coated magnetic beads (using a 1:1 mixture of anti-rat Ig and anti–mouse Ig coupled Dynabeads, Dynal, Oslo) first at 7:1 then at 3:1 bead-to-cell ratios. The bead and cell mix was resuspended as a slurry in EDTA-BSS-FCS and incubated for 20 min at 4°C with continuous slow rotation. The mix was then diluted with 5 ml EDTA-BSS-FCS, and the beads were removed magnetically, using two removal cycles. In many experiments this depleted preparation was used directly, with gating during analysis for class II MHC bright (or CD8 bright) cells to selectively analyze DC. In other experiments the preparation was stained and sorted for class II MHC bright cells, to provide a pure DC preparation.

In some cases mechanically released free DC were also isolated from the free lymphoid cell suspension, by beginning isolation at the density separation step (see above).

Phase Contrast Microscopy. Purified DC were suspended at 2 × 10^4 cells/ml and incubated for 12–24 h at 37°C as 15-μl hanging-drop cultures in the wells of inverted Terasaki trays. The medium was pH 7.2 Hepes-buffered RPMI 1640 containing 10^{-4} M 2-ME, 10% FCS, and 10% of a supernatant of Con A–stimulated rat spleen cell cultures. The gas phase was 10% CO_2 in air, and water saturated. The trays were then turned over and incubated for a further 30 min to allow the DC to settle onto the flat bottom of the wells. The cells were examined at 400-fold magnification using a Nikon Diaphot inverted phase microscope.

Gel Hybridization Analysis for TCR Gene DNA Rearrangement. DNA was prepared from 5 × 10^5 cells as described previously (45). For TCR β gene rearrangement, DNA was digested with HindIII and hybridized with a Cβ probe. For TCR γ gene rearrangement, DNA was digested with EcoRI and hybridized with

a Cγ probe. Kidney DNA served as a germline control, showing two germline bands at 9.4 and 3.0 kb for Cβ genes, and three germline bands at 13.4, 10.5, and 7.5 kb for Cγ genes.

RNAse Protection Assay for CD8 mRNA. An EcoRI/PstI fragment of a Ly-2.2 clone (46) was cloned into the pGEM3 vector polylinker, and used as a template for the transcription of a 284-bp [^{32}P]UTP-labeled riboprobe (Promega Biotec, Madison, WI). The probe was eluted from a polyacrylamide gel and hybridized overnight with sample RNA at 42°C in 80% formamide, 0.4 M NaCl, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) buffer, pH 6.4, 10 mM EDTA. Single-stranded RNA was digested with RNAse A and RNAse T1, then samples were phenol extracted, precipitated with 10 μg tRNA, and subjected to electrophoresis on a 7% polyacrylamide gel. Bands were detected by autoradiography or on a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Results

Development of the Isolation Procedure. The procedure was developed for thymic DC, but was applicable to splenic DC. The tissue disruption conditions had been established for optimum yield of DC-thymocyte rosettes (14, 43). Teasing, agitation, and washing of the tissue removed 75% of lymphoid cells, thereby enriching the DC in the stromal residue. However, this procedure also released some DC, in the case of spleen ~30% of all recoverable DC; the implications of this are considered later. Collagenase digestion of the residual stroma at room temperature released both free DC and DC-lymphocyte rosettes (14, 16). The digest was treated with EDTA to completely disrupt rosettes (14), and the presence

Table 1. *The mAbs Used for DC Purification and Characterization*

Antigen	Alternative designation or specificity	Hybridoma clone	Reference or source
CD8 α chain	Pan Ly-2	53-6.7	17
	Pan Ly-2	YTS169.4	Caltag Laboratories
	Ly-2.1	49-11.1	18
	Ly-2.2	D9	19
CD8 β chain	Pan Ly 3	53-5.8	17; Pharmingen (San Diego, CA)
CD4		GK1.5	20
Class I MHC	H-2K^{k}	11-4.1	21
	H-2K^{d,b,q,r,s} D^{d}	34.1.2S	22
Class II MHC	I-A^{k,s,r}	ER-TR2	23
	I-A^{b,d,q} I-E^{d,k}	M5/114	24
	I-E/C^{k,d,p,r}	14-4.4S	25
CD2		RM2-2	26
CD45	Ly 5	AL14A2	27
CD45R	B220	RA36B2	28
CD44	Pgp-1	1M7.81	29
CD25	IL-2Rα	PC61	30
CD3		KT3-1.1	31
TCR γ/δ		GL3-1A	32
Thy 1	Thy-1.1	19-F12	33
	Thy-1.2	30-H12	17
HSA	J11d	M1/69	34
FcR II		2.4G2	35
LFA-1		I21/7.7	36
Mac-1	Mac-1α	M1/70.15	34
F4/80	Macrophage	F4/80	37
Gr-1	Granulocyte	RB68C5	38
TER-119	Erythroid	TER-119	Kina et al., manuscript in preparation
33D1	Dendritic	33D1	39
NLDC145	Dendritic	NLDC145	40; BMA (Augst, Switzerland)
MTS 35		MTS 35	41; R. Boyd (Monash University, Melbourne, Australia)

of EDTA during subsequent steps prevented the DC from reforming complexes with lymphocytes. The DC were then enriched on the basis of their low buoyant density by centrifugation in metrizamide medium (44), a modification of established procedures (1). A mean of 92% of lymphocytes sedimented at the density used, whereas 80% of DC floated.

The DC were still a minority of the light-density fraction. Lymphocytes, macrophages, and most other contaminating cell types were removed by coating the cells with a mixture of mAbs, each recognizing specific lineage markers, mixing the coated cells with anti-Ig-coupled magnetic beads, then removing the unwanted cells with a magnet. The expression of low levels of some T cell and macrophage markers by thymic DC (11) was an initial concern. However, it became apparent that the magnetic bead depletion technique removed only cells expressing high levels of surface antigens, and with adjustment of mAb concentration, an appropriate cut-off could be set. Accordingly, the mAbs recognizing Thy-1, Mac-1, and FcR were used at moderate, rather than saturating, levels. To check the depletion specificity of the mAbs used, they were omitted one at a time from the depletion cocktail, and the changes in both total cell recovery and cell surface phenotype were assessed. Omission of any one mAb caused an increase in contaminants, extensive in some cases (e.g., anti-Thy-1), minor but still detectable in others (e.g., anti-IL-2R). However, in no case was DC recovery significantly improved by omission of one mAb. In contrast to the mAbs finally used in the depletion cocktail, the addition of anti-CD8 in trial experiments resulted in a major drop in the recovery of class II MHC bright DC, and accordingly, it was excluded from the cocktail.

The twice-depleted preparations contained 75–95% DC, defined as the class II MHC bright cells. A low level of lymphocyte, macrophage, and epithelial cell contaminants (~10%) was detected on smeared and stained samples. Recoveries of DC from the collagenase-digested washed stromal residue ranged from 1 to 5 $\times$ 10^5 per thymus, and 5 to 9 $\times$ 10^5 per spleen. This preparation was used directly for flow cytometric analysis, gating for MHC class II bright cells to selectively analyze the DC component. Higher purity preparations for molecular or functional analysis were readily obtained by positive sorting for class II MHC bright cells, which reduced the level of class II MHC dull cells, and the level of evident lymphocyte and macrophage contamination, to <1%.

Morphological Appearance of DC Preparations. If examined immediately under phase-contrast microscopy, only ~20% of the cells in the final preparation showed typical DC morphology, the majority appearing as medium- to large-sized round cells with only a few fine protrusions. However, the typical DC morphology of convoluted shape, with cell extensions in the form of dendrites or veils, began to appear after short periods of culture. Survival was best and dendritic morphology most evident in low-density hanging-drop cultures, in a medium containing the mixture of cytokines released by mitogen-activated lymphocytes. After overnight culture of the depleted, but not sorted, DC preparations, 60–80% of the cells in the thymic preparations, and 30–60% of the cells in the splenic preparations, showed obvious DC morphology, as shown in Fig. 1. Since this was less than the level of class II MHC bright cells, these either included some non-DC or included cells of the dendritic lineage that were not able to express DC morphology in short-term culture.

Dendritic Markers on DC Preparations. To further check if

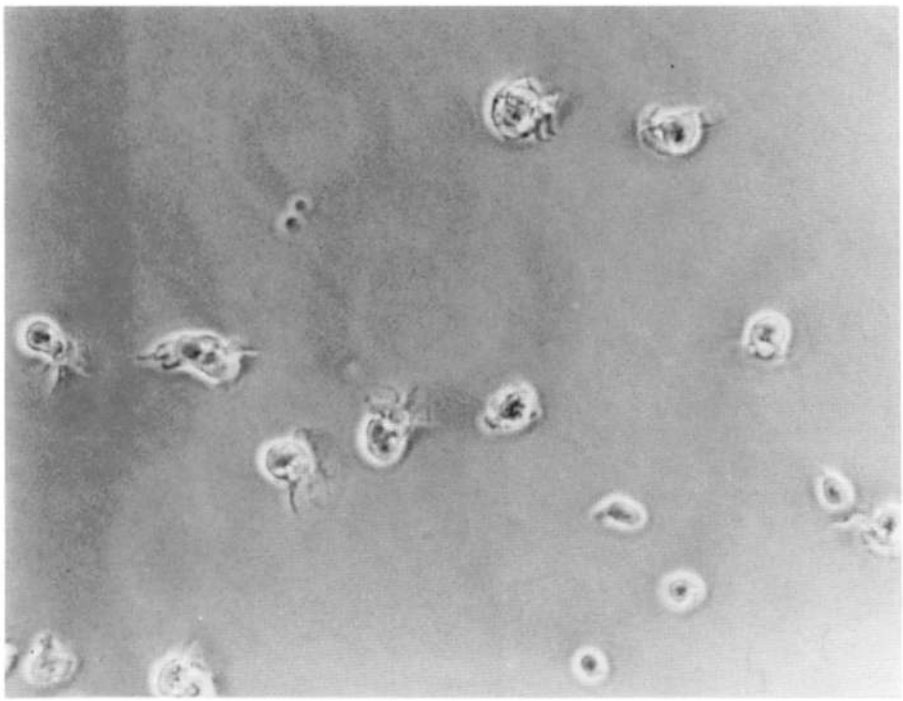

Figure 1. The appearance of cells in the thymic DC preparations after 15 h of culture. The preparations were not sorted to remove residual contaminants. The cells were incubated in hanging drop cultures, but then inverted and allowed to settle on the base of Terasaki tray wells. Some cells remained round but many adopted a convoluted shape with dendrites, veils, and other cell extensions. The cultures were photographed under phase contrast (×100).

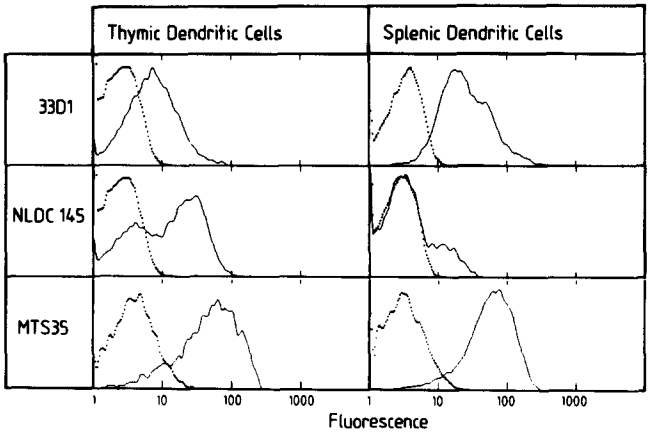

Figure 2. The staining of DC preparations with DC-specific mAbs. The preparations were not sorted to remove residual contaminants. Biotinylated mAbs were used with PE-avidin second stage in these single-parameter analyses. The dotted line represents the second stage alone background. For each fluorescence distribution profile similar results were obtained in two to four separate experiments.

the cells isolated were of the dendritic lineage, the preparations were stained with mAbs recognizing antigens reported to be specific for DC (Fig. 2). Although some of these gave relatively weak staining, the results were clear-cut. Most thymic and splenic DC stained positively with 33D1, a result that differs from other reports indicating that thymic DC do not stain with this mAb (10, 11). Both thymic and splenic DC preparations included cells positive with NLDC 145, a mAb known to stain the interdigitating type of DC (40). Generally, two peaks were seen, one negative to low and one of higher staining, with thymic DC always showing more of the clearly positive staining cells. Finally, both DC preparations were strongly positive with MTS 35. The antigen recognized by this mAb is also found on many lymphoid cells, but among the thymic stromal cell populations, it has been found to be selective for thymic DC (41).

Overall, the results with DC-specific mAbs indicated that the majority of cells in our preparations were a form of DC, but the spectrum of DC isolated differed from that usually obtained. Further evidence that the preparations were predominantly DC is the very high level of class II MHC on the majority of cells in both preparations (Figs. 3 and 4).

General Surface Markers on Thymic DC. The expression of a range of surface markers on the thymic DC preparation (before sorting for class II MHC bright cells) is shown in Fig. 3. The majority of cells stained very brightly for MHC class II (brighter than B cells); the MHC class II^{low} cells were considered to represent the non-DC contaminants. The thymic DC expressed high levels of MHC class I, CD45 (Ly-5), CD44 (Pgp-1), and LFA-1. They also expressed high levels of the heat-stable antigen (HSA; J11d), already reported as a characteristic of thymic DC (10, 11). All these markers correlated with MHC class II fluorescence, so gating for MHC class II bright cells removed most of the negative cells (not

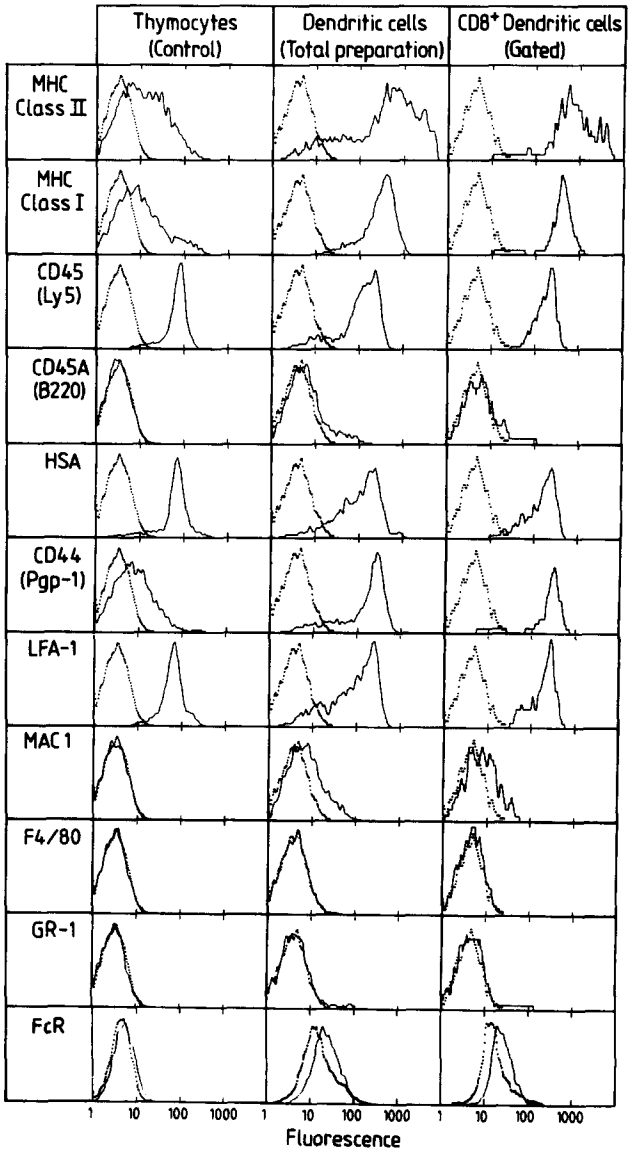

Figure 3. The level of various surface antigens on thymic DC. The cells were stained in three fluorescent colors for MHC class II, CD8, and the antigen to be studied. The dotted line gives the background, with only the biotinylated mAbs under analysis being omitted. (*Left*) The staining profile of a thymocyte suspension, as a control for staining intensities. (*Middle*) The staining profile of the total thymic DC preparation (not sorted to remove residual contaminants). (*Right*) The distribution after gating the DC preparation for cells expressing high levels of CD8. Gating for MHC class II bright cells gave almost identical profiles to CD8 gating. Each staining and analysis was performed two to four times with similar results.

shown). The DC were negative for the granulocyte marker Gr-1, the macrophage marker F4/80, and the B cell marker B220. However, they did express low levels of Mac-1 and the FcR II.

General Surface Markers on Splenic DC. The DC preparations isolated from spleen resembled closely those from thymus

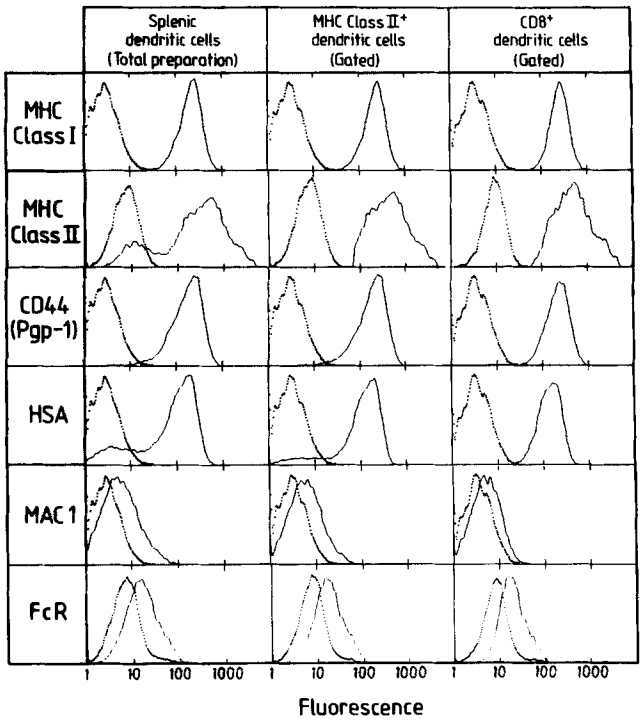

Figure 4. The level of various surface antigens on splenic DC. Conditions were similar to Fig. 3, and the thymocytes of Fig. 3 serve as a control for staining intensities. (*Left*) The results for the total splenic DC preparation (not sorted to remove residual contaminants). (*Middle*) The gated results for cells expressing high levels of MHC class II. (*Right*) The gated results for cells expressing high levels of CD8. Note the existence of a small population of HSA$^-$ class II MHC bright cells, but the absence of HSA$^-$ CD8 bright cells. Each staining and analysis was performed two to four times with similar results.

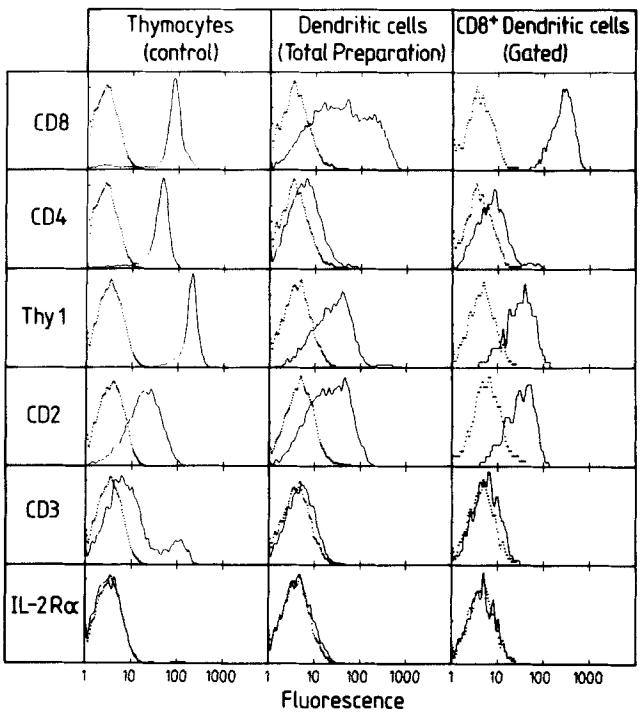

Figure 5. The distribution on thymic DC of surface antigens usually found on T cells. The presentation is similar to Fig. 3. The CD8 fluorescence profiles presented are based on staining with mAb YTS 169.4, with the different anti-CD8 53-6.7 mAbs used to gate for CD8 bright cells. As in Fig. 3, gating for CD8 bright cells gave very similar results to gating for MHC class II bright cells. Each staining and analysis was performed two to three times with similar results.

in expression of the series of markers discussed above (Fig. 4). There was generally a higher proportion of MHC class II–negative contaminants. It was notable that, as for thymic DC, the majority of cells were strongly positive for HSA, in contrast to the usual result with splenic DC preparations (10, 11). However, there was a small subgroup of HSA$^-$ cells, even after gating for MHC class II bright cells to eliminate contaminants. Thus, a minor subpopulation of our splenic DC resembled the majority population obtained by others.

T Cell Markers on Thymic DC. Published studies indicate that thymic DC express a number of surface antigens normally characteristic of T cells (10–12), and our own earlier studies confirmed the strong expression of CD8 (15). Our new isolation procedure was designed to reduce the possibility that this was simply due to bound thymocytes. We verified by phase contrast microscopy that there were no aggregates or rosettes in the DC preparations, and Giemsa-stained cytocentrifuge preparations showed no signs of the tight binding of thymocytes to some DC we had noted in earlier preparations. Nevertheless, our thymic DC still expressed some T cell markers, as shown in Fig. 5. There was no staining with anti-CD3, nor with antibody against the IL-2Rα chain, confirming the absence of T cells. However, the DC appeared to express very low levels of CD4, moderate levels of Thy-1 and CD2, and a range of levels of CD8, from zero to as bright as on T cells. The cells with the highest levels of CD8 showed the same pattern of markers seen for the majority DC population, and for the MHC class II bright cells (Figs. 3 and 5). These CD8$^+$ cells therefore expressed DC markers, and in separate experiments were found to show typical DC morphology after short periods in culture.

Specificity Tests for T Cell Markers on Thymic DC. To check whether the binding of mAb to DC was specific, especially where low level fluorescence was seen, thymic DC were isolated from different mouse strains, and allotype-specific mAbs were used for staining in a crisscross protocol. The staining of thymic DC for the Thy-1 antigen was specific, since no significant fluorescence above background levels was seen when anti-Thy-1.2 mAbs were used to stain Thy-1.1 DC, or when anti-Thy-1.1 mAbs were used to stain Thy-1.2 DC (data not shown). Likewise, the staining with mAbs recognizing the CD8α chain (Ly-2) was specific, since the anti-Ly-2.1 and the anti-Ly-2.2 mAbs showed the appropriate binding specificity, and no significant staining above background was seen with the inappropriate allotype (Fig. 6). Positive staining for CD8α was obtained with four different mAbs (Figs. 5 and 8), indicating the staining was not due to any accidental crossreactivity. In each case the CD8α staining of DC ex-

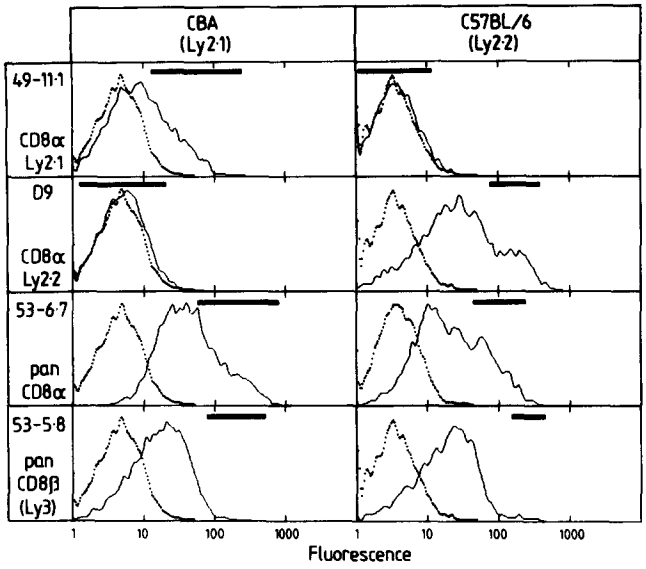

Figure 6. The specificity and nature of the staining for CD8 on thymic DC. The results are gated for the ~90% MHC class II bright cells in the thymic DC preparation. The dotted line gives the background fluorescence of samples with only the biotinylated anti-CD8 mAb omitted. The horizontal bars represent the range of fluorescence of the positive peak when thymocytes of the same strain are stained with the same antibodies. This result was confirmed in three other experiments.

tended to cells as bright as the staining of thymocytes with that Ab. Finally, the thymic DC were also stained with mAbs recognizing the CD8β chain (Ly-3), indicating the existence of some α/β CD8 heterodimer resembling that typically found on T cells; however, the level of staining with anti-Ly-3 was always well below that of the thymocyte controls, suggesting the α/α homodimer was the main CD8 form.

CD8 mRNA in Thymic DC. To determine whether the surface CD8 was synthesized by the DC themselves, rather than being picked up from previously associated thymocytes,

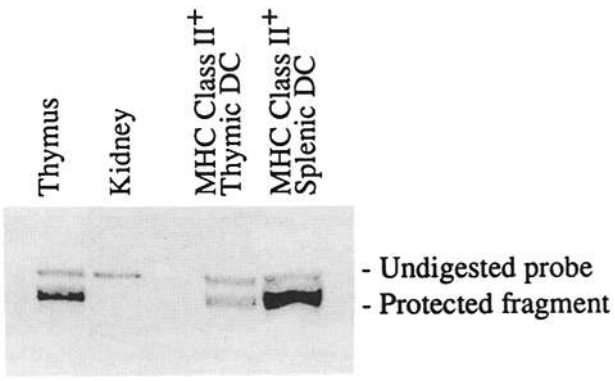

Figure 7. RNAse protection assay for CD8α mRNA in DC. The DC preparations from both thymus and spleen were sorted to provide >99% MHC class II bright cells. RNA was prepared from 5 × 10^5 cells. The ^{32}P-labeled probe was an antisense fragment of Ly-2.2, which protects a mRNA fragment to produce a band at 270 bp, the positive signal. Some undigested probe persisted as a band at 284 bp.

the presence of the CD8α chain message in purified and sorted thymic DC preparations was checked using an RNAse protection assay (Fig. 7). Using a CD8α probe, the sorted, MHC class II bright DC exhibited a clear protected band, although the level appeared to be somewhat less than that seen in thymocytes. Sorting of the CD8$^+$ from the CD8$^-$ fractions of the MHC class II bright cells showed most of this mRNA was in the CD8$^+$ fraction.

Are Thymic DC Deviant T Cells? The occurrence of many T cell markers on the thymic DC suggested they might have developed as a branch off one of the developing thymic T cell lineages. Accordingly, the rearrangement status of the TCR β and γ C region genes was assessed, since all but the very earliest T precursors in the thymus show rearrangement at these loci (47). However, restriction enzyme digestion, gel electrophoresis, and hybridization with Cβ or Cγ probes revealed that both these genes were in germline state in most thymic DC (Fig. 8). In the case of the Cγ probe, a very faint band, additional to the three strong germline bands, was sometimes seen around 14 kb. This did not correspond to any of the rearranged bands seen with thymocytes, and it was not clear whether it represented a rare DC rearrangement, a contaminant cell, or an artifact. Overall, these results indicate that most DC had not rearranged their TCR genes, and they confirm that the preparations were not contaminated with thymocytes or T precursor cells.

T Cell Markers on Splenic DC. In common with the thymic DC, those isolated from spleen were negative for CD3 and IL-2Rα (Fig. 9). In contrast with thymic DC, the splenic DC were also negative for CD4, and were either negative or very low for Thy-1 and CD2. Thus, most T cell markers

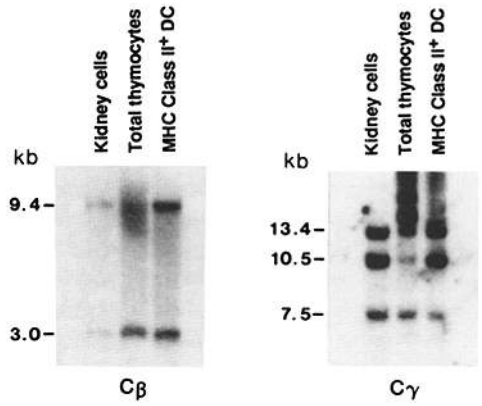

Figure 8. Status of TCR β and γ genes in thymic DC. The DC preparation was sorted to provide >99% MHC class II bright cells. DNA was prepared from the purified thymic DC as described in Materials and Methods. For TCR β gene rearrangement, DNA was digested with HindIII and hybridized with a Cβ probe. Kidney DNA served as a germline control, showing two germline bands of 9.4 kb (Cβ1) and 3.0 kb (Cβ2). As only the 9.4-kb band changed on rearrangement, the ratio of intensity of these two bands served as rough measure of the extent of rearrangement. For TCR γ gene rearrangement, DNA was digested with EcoRI and hybridized with a Cγ probe. Kidney DNA served as a germline control, showing three germline bands at 13.4, 10.5, and 7.5 kb. Cγ rearrangement was detected by the appearance of discrete additional bands, as shown in the thymus control sample.

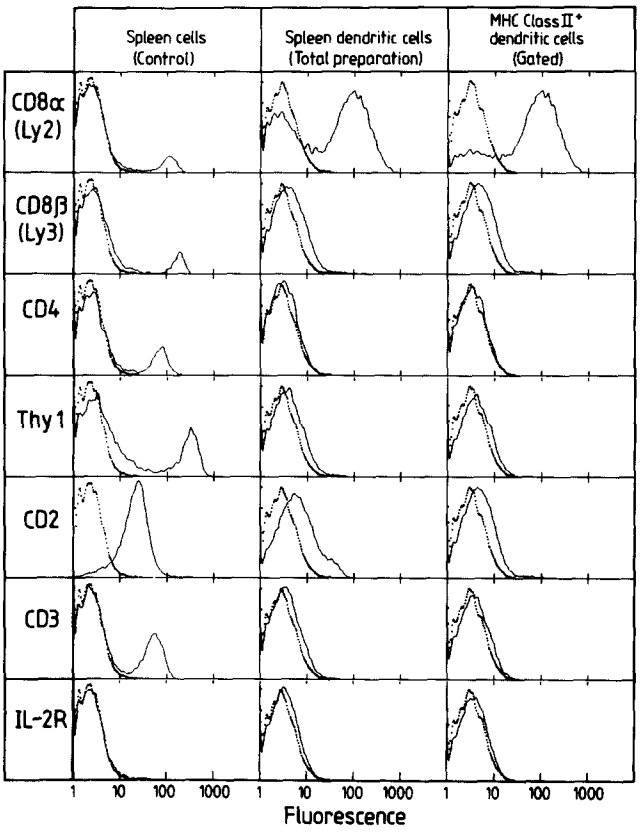

Figure 9. The distribution on splenic DC of surface antigens usually found on T cells. The presentation is similar to that of Figs. 4 and 5. (*Left*) The staining of a spleen lymphoid cell suspension, the 30% T cells in the suspension serving as a staining control. (*Middle*) The results for the total depleted splenic DC preparation (not sorted to remove residual contaminants). (*Right*) The same data gated for the 80% MHC class II bright cells. Similar results were obtained three times for CD8, and twice for the other markers.

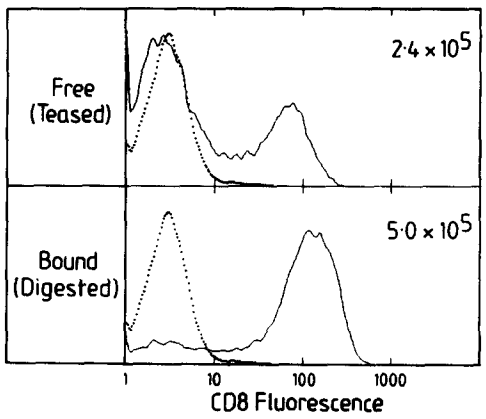

Figure 10. A comparison of the CD8 expression by loosely and tightly tissue-bound splenic DC. The free, or loosely bound, DC were released from tissue by teasing the spleens apart, followed by vigorous mechanical agitation. The bound DC were then released from the washed stromal residue by collagenase digestion. The preparations were stained for both CD8 and class II MHC, and the results presented are gated for the class II MHC bright DC. The dotted line gives the background for cells stained for MHC class II, but with anti-CD8 omitted. The numbers of DC recovered per spleen are given for this experiment. The results are typical of four such experiments.

were absent from these cells. However, an unexpectedly high proportion expressed high levels of CD8.

The expression of CD8 on the splenic DC was examined in more detail (Fig. 9). Rather than a continuum, as with thymic DC, the population fell into two groups, a major CD8 bright and a minor $CD8^-$ group. Gating for MHC class II bright cells usually eliminated some of the $CD8^-$ cells, but confirmed that the division into a majority of $CD8^+$ and a minority of $CD8^-$ cells applied to the dendritic component of the preparation. Despite the expression of CD8α (Ly-2), the splenic DC expressed no, or at most very low, levels of CD8β (Ly-3). The clear peak of both $CD8\alpha^+$ and $CD8\beta^+$ mature T cells seen among total spleen cells in side-by-side staining tubes confirmed this absence of CD8β from the splenic DC (Fig. 9).

CD8 mRNA in Splenic DC. The issue of whether the CD8α was synthesized by the splenic DC was examined by assaying for mRNA using an RNAse protection assay. A clear band of CD8α mRNA was also observed in the MHC class II bright sorted splenic DC (Fig. 7), the level being even higher than seen with thymocytes.

Differences between Free and Tissue Bound Splenic DC. The population of splenic DC we isolated was dominated by a $CD8^+$ HSA^+ population, with $CD8^-$ HSA^- cells as a minor subgroup. Since this was the converse of the distribution reported by others (11), we checked which aspect of the various separation procedures produced the bias. DC first isolated by our procedure from partially washed splenic stromal fragments were separated into cells showing transient adherence to plastic surfaces (59%) and those completely nonadherent (41%). The transiently adherent cells were 43% $CD8^+$, and the nonadherent cells were 57% $CD8^+$. Thus, there was some enrichment of $CD8^-$ DC by the adherence procedure, but not enough to explain the dominance of $CD8^-$ DC reported by others. Little difference was seen between the two DC types when a higher density medium was used in the centrifugation step in order to recover more DC. In contrast, marked differences were seen between the DC released from spleen by mechanical disruption and those requiring additional enzyme digestion and EDTA treatment. A comparison of the DC recovered from our normally discarded disrupted spleen washings (~30% of all recoverable DC) with those recovered by our usual digestion procedure is given in Fig. 10. The mechanically released DC were predominantly, but not exclusively, $CD8^-$, whereas the collagenase released DC were, as before, predominantly $CD8^+$. Our original procedure therefore gave a sample of DC strongly biased by selection for tissue-bound cells.

Discussion

Subpopulations of Splenic DC and Their Recovery after Different Isolation Procedures. One practical conclusion from this study is that the separation procedure used can strongly bias the subpopulation distribution of isolated DC. Although the DC yield was high, our original procedure, involving mechanical disruption of the tissue and washing out all free lymphoid cells before collagenase digestion and EDTA treatment, had caused the selective loss of ~30% of splenic DC. Conversely, procedures that do not use collagenase and then EDTA

to release DC would be expected to produce a population biased towards those with a loose tissue association. Especially with thymic tissue, the failure to disrupt DC-lymphocyte rosettes with the chelating agent would cause significant loss of DC, since the rosettes would be lost in the dense fraction during selection for low-density DC. In spleen, the tightly tissue-bound DC are predominantly $CD8^+$, whereas the loosely bound DC include more $CD8^-$ than $CD8^+$ cells. The strongly bound splenic DC are also HSA^{high} and $NLDC145^{high}$, which fits with the conclusions of others that this phenotype corresponds to the interdigitating DC (11, 40). The loosely bound splenic DC presumably include the HSA^{low} $NLDC145^{low}$ DC population that has been considered as a motile, migratory stage (10).

Another likely source of bias is the preferential attachment of certain DC populations to plastic surfaces during the adherence step of the usual isolation procedure. We observed some selection for $CD8^-$ DC by this procedure and have recently noted a strong selection for $33D1^+$ DC. In addition, if rosettes are not disrupted before this step, rosette-associated DC will be lost, since dendritic rosettes are nonadherent (14, 16). It is also possible that the incubation at 37°C itself may result in changes in the surface phenotype, since changes in DC viability and function due to a period of culture have been reported (7, 48). Our rapid, direct isolation procedure was designed to avoid this adherence step. Similar considerations led Crowley et al. (48) to develop a procedure for direct sorting of DC from the low-buoyant density spleen fraction using the mAb N418.

Our focus in this study has been on the class of DC known to present antigen to T cells. It should be noted that the separate lineage of follicular dendritic cells (FDC) (49), which present antigen to B cells, are not evident in our preparations. They have been deliberately selected out by the inclusion of mAb specific for the FcR during the depletion step.

Our final separation procedure is rapid and produces a high yield of DC. The bias in subpopulation distribution can be avoided if the readily released, as well as the tissue-bound, DC are collected. For many applications, the use of magnetic bead depletion avoids the need for cell sorting. However, since selection by magnetic bead depletion is by negative criteria, certain infrequent cell types may be concentrated in the preparation along with the DC, and some undepleted contaminants remain. Positive sorting for cells bearing high levels of MHC class II, or on the basis of other markers, is essential for high purity. Because DC are already the majority population, sorting time is greatly reduced compared with direct sorting from an undepleted suspension.

Thymic DC and T Cells Markers. The DC we isolated from the thymus shared many properties with the tissue-bound interdigitating population we isolated from the spleen, in being HSA^{high}, $NLDC145^+$ $MTS35^+$ $CD45^+$, $CD44^+$ FcR II^{low}, $Mac\text{-}1^{low}$, $F4/80^-$, $Gr\text{-}1^-$, and $B220^-$. These markers agree well with those reported by others using different isolation procedures (10–12). However, most of the thymic DC we isolated stained positively with mAb 33D1, in contrast with most reports, although some nonadherent $33D1^+$ thymic DC have been noted previously (11). This difference could result from the selective effects of different isolation procedures as discussed above, but it could also reflect an increased sensitivity in detection of a relatively weak-staining mAb.

The major differences we found between thymic and splenic DC, even when paired for the same "interdigitating DC" markers, were in the expression of a series of characteristic T cell surface markers. The central issue of CD8 expression is discussed in detail below. Thymic DC also had on their surface moderate levels of Thy-1 and CD2, and low levels of CD4; these were absent or at much lower levels on the corresponding splenic DC. This was not due to T cell or thymocyte contamination, as evident from the lack of CD3 staining, the lack of IL-2Rα staining, the lack of characteristic T cell or thymocyte TCR gene rearrangements in the DNA, the distribution of other markers, and the absence of associated thymocytes in stained preparations. For Thy-1 and CD8, the allotype controls indicated the staining was specific, and the absence of staining on splenic DC prepared by the same procedure reinforces this point. These T cell markers (except for CD8) have not been detected previously by others, nor detected by us using the standard isolation procedures (15).

We have two alternative explanations for the low expression of these T cell markers on our thymic DC, but not on our splenic DC. Due to their residence or development in the thymus, thymic DC may be subject to the same inducing factors as developing T cells, and so synthesis of a low level of these surface components may be induced either as an accidental bystander effect, or as a necessary requirement for function or persistence within the organ. The second, alternative explanation is that these surface components are not synthesized by the DC, but are picked up from the membranes of associated thymocytes. The difference between thymic and splenic DC would then be due to the tight association of thymic DC with thymocytes in rosettes. The failure to detect these markers following the standard DC cell isolation procedures would then be due to the exclusion of rosette-associated DC from the preparation, or due to the washing off of the bound material during the incubation procedure. This explanation can be excluded for CD8α chains, but it is a reasonable one for the other T cell markers present at lower levels on the DC surface.

CD8 Expression by DC. The most intriguing of the T cell markers expressed by DC is CD8. Nussenzweig et al. (50) and Crowley et al. (10, 48) had already reported the expression of CD8 by many thymic DC and by what was considered a minor population of splenic DC. We had already noted the presence of this marker on thymic DC using standard isolation procedures (15). With our new isolation procedure selective for the strongly tissue-associated splenic DC, the $CD8^+$ DC were the dominant population. We estimate that in the spleen as a whole about half the DC express CD8, a proportion at least as high as in thymus. In addition, the level of CD8 expressed is about as high as on $CD8^+$ T cells and thymocytes. This forced us to check the reality of this CD8 expression and consider its biological significance.

It appears the surface molecule stained is authentic CD8,

since it is recognized by many anti-CD8 mAbs, and its distribution in different mouse strains shows the appropriate allotype specificity. The high surface level, and the fact that the form of CD8 on DC differs from that on the surrounding T cells, makes it unlikely that it is merely picked up from T cells. The presence of CD8α mRNA in both splenic and thymic DC confirms that the α chain is synthesized by the DC themselves.

An intriguing and novel observation is that while thymic DC do express some CD8 β chain, the α chain appears to be in excess, and the α chain is the predominant form on splenic DC. Thus, rather than the α/β heterodimer form of CD8 characteristic of most TCR α/β T cells, the dominant form of CD8 characteristic of most TCR α/β T cells, the dominant form of CD8 on DC is the α/α homodimer. The CD8 α/α homodimer is the form commonly seen on TCR γ/δ T cells and on the few α/β T cells that develop extrathymically (51). To date there is no evidence that the homodimer and heterodimer forms of CD8 differ in function.

Significance of CD8 Expression by DC. The high level of CD8 expression by DC, unique outside the T lineage, suggests a functional role. Hambor et al. (52) have pointed out that as well as its accepted role in T lymphocyte stimulation as a binding receptor for MHC class I, CD8 can serve as an inhibitory ligand depressing immune responses. Such a role has been delineated by Sambhara and Miller (53), who have found that CD8 is required for a cell to exhibit veto function. T cells recognizing antigen presented by the MHC of a veto cell are signaled to die if, as well as receiving a signal through their TCR, they receive a signal through their MHC class I as a result of its interaction with the CD8 of the veto cell. Cells transfected with CD8α chain alone appear to acquire veto function (52, 53). In most systems the veto cell has been a CD8-bearing T cell. However, the efficiency of DC in antigenic peptide presentation via class I or class II MHC should, if the DC expresses high levels of CD8, lead to an equally efficient veto function.

This reasoning would provide a good explanation of the role of CD8 on thymic DC. As well as an ability to initiate the death of immature thymocytes by a simple TCR-mediated signal, deletion could be extended to more mature thymocytes in the medulla by adding a CD8 interaction signal transmitted through class I. However, in this light, the finding of a large population of $CD8^+$ DC in the spleen comes as a surprise. If the splenic $CD8^+$ DC do function as veto cells, the implication of having two distinct DC subgroups in the spleen, one very effective at presenting antigen and stimulating T cells, the other at presenting antigen and deleting T cells, must be considered. We are currently studying the separate immunological activities of these subpopulations of DC.

Dr. Frank Battye, Mr. Ralph Rossi, and Ms. Robyn Muir provided crucial expertise in flow cytometry. We thank Dr. R. Miller for discussing his results on veto cells, and Dr. R. Steinman for discussion and advice on dendritic cells.

This work was supported by the National Health and Medical Research Council, Australia, and by the C. H. Warman Research Fund.

Address correspondence to Ken Shortman, The Walter and Eliza Hall Institute of Medical Research, PO Royal Melbourne Hospital, Victoria 3050, Australia.

Received for publication 27 December 1991 and in revised form 26 March 1992.

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