FIG. 3 Surface phenotype of transferred transgenic TCR$^+$ CTL in persistently infected mice. 10^3 transgenic TCR$^+$ T cells were transferred on day 15 after infection with 10^7 PFU LCMV-D into C57BL/6 mice (Fig. 2B) and their kinetics were followed after direct staining by flow cytometry. Top, expression of the transgenic TCR (Vα2/Vβ8) on CD8$^+$ splenocytes at the indicated time points after transfer. Splenocytes from normal C57BL/6 mice and TCR-transgenic mice were used as controls. Bottom, CD3 and CD8 expression of the same samples.
METHODS. Transgenic TCR expression was determined by triple staining: cells were first incubated with unconjugated anti-Vα2 monoclonal antibody[42] followed by phycoerythrin (PE)-conjugated goat anti-rat immunoglobulin (Southern Biotechnology, Birmingham). After a blocking step with normal rat immunoglobulin, the staining was completed with anti-CD8-FITC (Becton Dickinson) and biotinylated anti-Vβ8 (Pharmingen) and revealed by avidin-RED 613 (Gibco, BRL). CD3/CD8 double staining was done with unconjugated anti-CD3 monoclonal antibody[43] and FITC-conjugated goat anti-rat immunoglobulin (CALTAG, San Fransisco). After a blocking step, it was completed by biotinylated anti-CD8 (Becton-Dickinson) and finally by avidin-PE (TAGO, Burlingham). Viable cells were gated by a combination of forward light scatter and 90° side scatter and were analysed on an EPICS profile analyser (Coulter, Hialeah, FL) with a scale extending over 4 $\log_{10}$.

deletion of CTL was dependent on virus dose and kinetics. Additional experiments showed that exhaustion depended on the LCMV isolate (LCMV-D or Cl-13 deleted CTL, but LCMV-Armstrong did not) and on major histocompatibility complex (MHC) and non-MHC genes of the host (not shown).

This drastic example of peripheral deletion of a well defined class I restricted antigen-specific effector of T-cell response adds to examples of anergy[20,21], partial disappearance of T cells[19,22] or downregulation of either TCR and/or of coreceptor[18,19] expression. Note that in contrast to these results, TCR-expressing T cells specific for superantigens[21-24] or for the male H-Y antigen[19] have been found in frequencies well above background after day 15.

Why do exhaustively induced CTLs disappear? Because transferred transgenic CTLs expanded vigorously, even at times when the endogenous response had already disappeared (on day 15, Fig. 2B) interleukins[25,26] or antigen presentation[27,28] apparently do not seem to be the limiting factor. Also, attempts to prevent exhaustion by repeated infusions of interleukin-1, interleukin-2, interferon-γ or concentrated interleukin-rich supernatants have been unsuccessful (data not shown). The possibility that specific CTLs lysed CTLs because they were infected by LCMV[29] seems unlikely because of the demonstrated specificity (Table 1) and because neither endogenous nor transgenic CTLs were found here (or earlier[30]) to be infected by LCMV (data not shown).

Together, these data show that, depending on the virus isolate and dose, most or all antiviral CTLs may become induced if noncytopathic viruses spread early, rapidly and widely in the host; then CTLs proliferate and disappear completely after a short period of 3-5 days of 'anergy'. The data suggest that T-cell memory represents continuous induction of T cells by antigen depots. Our findings explain mechanistically, but not yet molecularly, the puzzle of why high-dose infection with LCMV causes low CTL responses[8,9,31-33] and after intracerebral infection (or after transient immunosuppression[34,35]) fails to cause lethal choriomeningitis in adult mice[4,5]. A similar mechanism of exhaustion of T-cell responses against widely spreading noncytopathic viruses may lead to a hepatitis B virus carrier status in a few immunocompetent men[36], or the disappearance of anti-HIV CTLs during final AIDS[37,38], may induce the veto phenomenon[39] or cause high zone tolerance to model antigens[40]. □

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Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population

Carlos Ardavin*, Li Wu, Chung-Leung Li & Ken Shortman†

The Walter and Eliza Hall Institute of Medical Research, PO Royal Melbourne Hospital, Melbourne, Victoria 3050, Australia

DENDRITIC cells, a minor cell population in lymphoid tissues, are specialized for presentation of antigenic peptides to T lymphocytes[1]. Thymic dendritic cells are involved in the deletion of self-reactive T lymphocytes[2,3]. Although all dendritic cells are ultimately of bone-marrow origin[4-7], it has not been clear whether thymic dendritic cells are produced in the adult thymus from a precursor cell or whether they migrate there preformed from the periphery. Recently we isolated from adult mouse thymus a population of early T precursors that could still form B lymphocytes, but not erythroid or myeloid cells, when transferred intravenously[8,9]. Here we show that these thymic lymphoid precursor cells, as well as bone-marrow haematopoietic stem cells, are able to form both dendritic cells and T-cell progeny when transferred into an irradiated thymus. Such linked development may ensure that developing T cells are negatively selected predominantly by self antigens presented on newly formed thymic dendritic cells.

The minute population of early-T precursors we previously isolated from adult mouse thymus resembled bone marrow haematopoietic stem cells (BMSC) in surface phenotype, except

*Present address: Department of Cell Biology, Faculty of Biology, Complutense University, 28040 Madrid, Spain.
† To whom correspondence should be addressed.

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that they expressed Sca-2 and low levels of CD4 (ref. 8). This 'low-CD4 precursor' population had T-cell antigen receptor (TCR) genes in a germ-line state, produced $\alpha\beta$ and $\gamma\delta$ T cells following intrathymic (i.t.) transfer, but also produced B cells if it seeded bone marrow following intravenous (i.v.) transfer[8,9]. In a search for the precursors of thymic dendritic cells (DC), we tested these thymic lymphoid precursors, as well as early BMSC[10], because despite the accepted bone-marrow origin of DC[4-7], the derivation of DC from the same stem cell as other blood cell lineages had not been proven. These two precursor populations were isolated from adult mice of the *Ly 5.2* allotype and injected into irradiated congenic *Ly 5.1* mice. At various times after transfer, DC were released[11] from the recipient thymus tissue, then enriched[11] so that the very small numbers of DC progeny could be detected. Donor-derived DC were identified as Ly 5.2-bearing cells expressing very high levels of class II major histocompatibility complex (MHC) and the DC-specific marker NLDC 145 (refs 11, 12) (Fig. 1).

When 100 purified BMSC[10] were injected into one lobe of an irradiated recipient thymus, 11,000 DC progeny were found in the injected lobe 3 weeks later (Fig. 1), together with 23×10^6 T-lineage cells (as previously shown[13]; Fig. 2). The recipient spleens were also analysed to check for any leakage of BMSC from the injected thymus; no DC progeny (or B-cell or myeloid progeny) were ever found in the spleen after i.t. transfer. DC progeny were found in recipient spleens (Fig. 3), as well as in recipient thymuses, however, after i.v. transfer. These results demonstrate the BMSC origin of both thymic and splenic DC and that the adult thymus can support full DC development.

Intrathymic transfer of the thymic low-CD4 precursor population led to production in the thymus of DC (Fig. 1), as well as T-lineage cells[8,9] (Fig. 2). The recoverable yield from 10^4 injected low-CD4 precursors was 5×10^6 T-lineage cells and 4×10^3 DC, a 2,000:1 ratio similar to that obtained with BMSC, and similar to the ratio of thymocytes to DC in the normal thymus[11]. DC progeny were maintained at this level from 7 to 22 days post-transfer, but they had disappeared from the thymus by 28 days, together with the thymocyte progeny. There is also a rapid turnover of DC in rat thymus[14]. DC progeny were produced in the spleen in parallel with thymic DC production when the low-CD4 precursor cells were transferred i.v. (Fig. 3).

The DC nature of these class II MHC^{++} NLDC-145$^+$, Ly 5.2$^+$ cells in the recipient thymus was confirmed by other criteria (data not shown). These cells showed the high forward and side light-scatter characteristics of DC. They were negative for B220 and low-to-negative for Mac-1, excluding B cells and macrophages that also express class II MHC. They did not express significant levels of TCR-$\alpha\beta$, TCR-$\gamma\delta$, CD3, CD4 or Thy 1. About 60% of them expressed high levels of CD8-α, but only low levels of CD8-β, a characteristic of thymic DC[11]. Finally, when sorted and incubated at 37 °C in culture medium to allow recovery of normal surface configuration, 90% had typical DC morphology[11], with irregular shape and cytoplasmic processes.

Additional separation parameters were used to ensure the DC progeny were derived from the low-CD4 precursor population itself, rather than from contaminating multipotent stem cells or pre-existent DC (data not shown). As the low-CD4 T precursors are Sca-2$^+$ and BMSC are Sca-2$^-$ (refs 8, 9, 15), the preparation was labelled and sorted for Sca-2 expression; almost all DC precursor activity was in the major Sca-2$^+$ fraction. As cells of the low-CD4 precursor preparation are almost entirely class II MHC$^-$ (refs 8, 9), they were labelled and sorted to remove any last traces of class II MHC$^+$ cells; all DC precursor activity was recovered in the class II MHC$^-$ fraction. In addition, cells of the low-CD4 precursor population did not express the DC markers NLDC-145 or N418, and did not have DC morphology.

To determine whether later T precursors with rearranged TCR-γ and -β genes could also form DC, we isolated the CD3$^-$4$^-$8$^-$ IL-2Rα^+ precursor population from the adult mouse

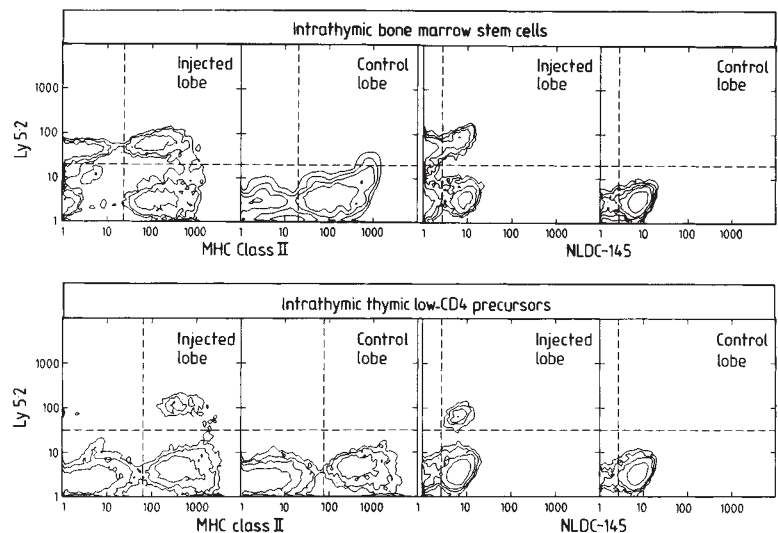

FIG. 1 The reconstitution of thymic dendritic cells by bone marrow haematopoietic stem cells or by thymic low-CD4 precursor cells. The precursor populations were purified from Ly 5.2 mice and injected into the left thymic lobe of irradiated Ly 5.1 mice. Fifteen days later thymic DC were prepared from the injected and uninjected lobes and analysed for donor-derived Ly 5.2$^+$ cells expressing class II MHC and the DC marker NLDC-145. The direct comparison of the injected lobe with the stained cells from uninjected right lobe, used as a negative control, served to correct for background fluorescence when analysing such a minor stromal component in irradiated mice. Note that both the control and the injected lobes of the irradiated recipients contained some host-derived DC, presumably from precursors which survived the irradiation dose. The class II MHC stain was extremely bright, requiring careful colour compensation, and a drop in instrument sensitivity compared to normal staining. This same setting was used for the weaker NLDC-145 stain, resulting in lower fluorescence but also very low background fluorescence. The contour lines in the diagrams are $\log_2$ probability density plots, with 0.2% as the lowest contour. These results at day 15 are typical of 18 separate experiments, with analyses done 7–22 days after transfer.

METHODS. Precursor cells were from 4–6 weeks C57BL/Ka Thy 1.1 (Ly 5.2) mice. Early BMSC were isolated[10] by immunomagnetic bead depletion of bone marrow cells bearing mature lineage markers, then sorting for Ly 6A/E$^+$ cells showing low-to-medium fluorescence after incubation with rhodamine 123. The low-CD4 precursor population was initially isolated from thymus[8,9] by first depleting thymocytes bearing CD3, CD8, CD2, IL-2Rα, Gr-1, TER-119, Mac-1 and B220 (cytotoxic then immunomagnetic bead procedures), then sorting for Thy 1$^+$, HSA^{++}, H-2K^{+++} cells. In later experiments, sorting for class II MHC$^-$ was substituted for the class I MHC^{+++} parameter, to ensure that there were no preformed DC. The precursor cells (10^2 BMSC, or 10^4 low-CD4 precursors) were injected into the left thymic lobe of γ-irradiated (7.5 Gy) recipient mice (C57BL/6 Ly 5.1-Pep^{3b}, 7–8 week)[8,9], using 8 recipients per experiment. The thymuses were then removed at various times, injected lobes being pooled for the test and uninjected lobes pooled as the control. DC were isolated[11] by digesting chopped thymic lobes with collagenase, disrupting DC–T cell complexes with ethylene diamine tetra-acetate, selecting light-density cells by centrifugation in metrizamide medium, and then depleting T-lineage cells, B cells and macrophages (using antibodies against CD3, CD4, Thy 1, IL-2Rα, F4.80, FcR, Gr-1 and TER-119, together with anti-mouse-Ig and anti-rat-Ig coated magnetic beads). About 0.1% of thymus cells was recovered. This DC suspension was then stained[8,9] with fluorescein-conjugated anti-Ly 5.2 (clone AL1-4A2), together with biotinylated anti-class II MHC (clone M5/114, specificity I-A^{b,d,q} I-E^{d,k}) or NLDC-145 (ref. 12) (BMA, Augst, Switzerland), using phycoerythrin–avidin as the second stage. Flow cytometric analysis was carried out on a FACScan (Becton Dickinson) using low-angle light scatter and propidium iodide staining to gate out dead cells and debris.

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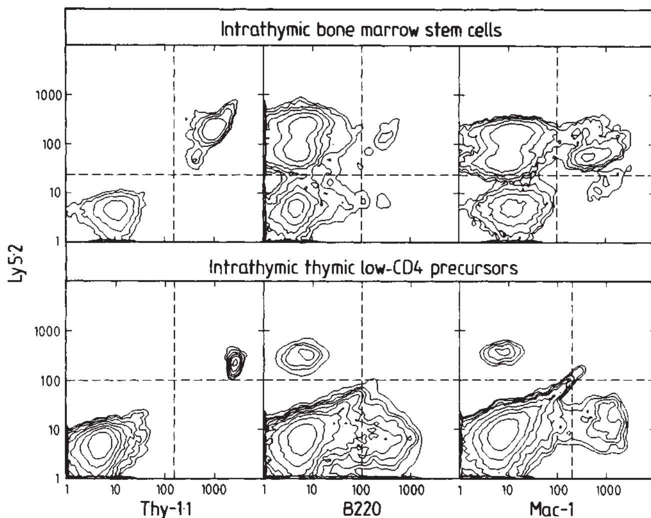

FIG. 2 Reconstitution of thymic T-cell, macrophage and B-cell populations by bone marrow haematopoietic stem cells or by thymic low-CD4 precursor cells. Recipient thymus lobes were analysed 17 days after i.t. transfer of precursor cells. Details are as given in Fig. 1 legend, except that thymus lobes were mechanically disrupted[8,9] rather than digested, and to analyse T-lineage reconstitution (Thy 1.1 stain), the suspension was counted and stained directly. Note that because the recipients were of the Thy 1.2 allotype, host-derived thymocytes are not visible. Before analysis of macrophage (Mac-1$^+$) or B-cell (B220$^+$) populations, the suspension was depleted of most thymocytes by treatment with cytotoxic antibodies against CD3, CD4 and CD8, and complement, followed by removal of damaged cells by a metrizamide-density cut[8,9]; about 0.5% of the thymus cells were recovered. Cells were stained with anti-Ly 5.2, together with lineage-specific biotinylated monoclonal antibodies (anti-Thy 1.1, clone 19-F12; anti-Mac-1, clone M1/70.15; anti-B220, clone RA3-6B2), as for Fig. 1[8,9]. Analysis was on a FACStar Plus. Contour lines are as in Fig. 1. Results are typical of 4 experiments.

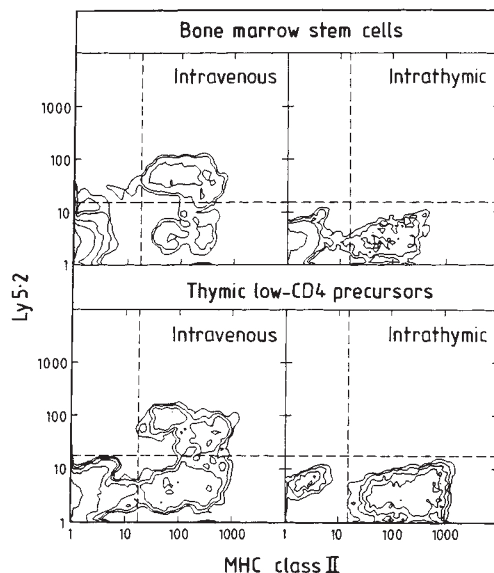

FIG. 3 Reconstitution of splenic dendritic cells by bone marrow haematopoietic stem cells or by thymic low-CD4 precursor cells. Precursor populations were transferred to irradiated recipients by i.t. or i.v. routes, then DC isolated from the recipient spleens 15 days later (as for thymic DC; see Fig. 1) and analysed. Conditions were as outlined for Fig. 1, except that for intravenous transfer 2×10^2 BMSC, or 3×10^4 low-CD4 precursor cells, were injected into recipients that had received two doses of γ-irradiation (each 5.5 Gy, 3 h apart), along with 4×10^4 recipient type bone marrow cells to ensure survival of recipients[9].

thymus[16]. When these were transferred i.t. at a 10-fold higher concentration than the low-CD4 precursors, the levels of T-lineage progeny were as expected[16] but no DC progeny were detected (data not shown).

We considered that thymic low-CD4 precursors could be the origin of all bone-marrow-derived thymus cells, including thymic B cells and macrophages. When 100 BMSC were injected i.t., Mac-1$^+$ macrophage progeny were readily detected 15–22 days later, together with a very small amount of B220$^+$ B-cell progeny (Fig. 2); the latter were also Ly 6$^+$, ThB$^+$ and s-Ig$^+$, so were true B cells but few in number, agreeing with earlier studies[13]. In contrast, when 10^4 thymic low-CD4 precursors were transferred i.t., no macrophage or B-cell progeny were detected 15–28 days later, although there was extensive T-lineage reconstitution (Fig. 2). This is further evidence for the absence of multipotent haematopoietic stem cells in this preparation and argues for a special developmental relationship between thymic DC and T cells. The inability to generate thymic macrophages is in accordance with the lack of myelopoiesis when thymic low-CD4 precursors are injected i.v.[9]. More surprising is the failure to generate thymic B cells, because normal B-cell progeny are generated in bone marrow and spleen on i.v. transfer[9]. But the thymus is evidently inefficient at supporting B-cell development, and thymic B cells may be a distinct lineage of mainly Ly 1 B cells[17].

Overall, the thymic DC lineage in the adult mouse appears to separate from the T lineage later than the multipotent stem cell but before TCR gene rearrangement. Thymic DC appear to differentiate from an intrathymic precursor, rather than arriving pre-formed as a class II MHC$^+$ or NLDC-145$^+$ DC, which simply expand in numbers. These thymic DC precursors were indistinguishable from the low-CD4 precursor population representing earliest adult intrathymic precursors of both $\alpha\beta$ and $\gamma\delta$ T cells[8,9]. But we still lack suitable clonal assays to determine whether this population contains separate but phenotypically similar T-cell and DC precursors, or whether it represents a single type of precursor having both lymphoid and DC developmental potential.

These results have implications for T-cell repertoire selection within the thymus. They suggest that each new cohort of T-lineage cells generated in the thymus is accompanied by (at a 10^3-fold lower frequency) a parallel cohort of newly generated thymic DC, endogenously derived from a similar if not identical precursor, and having a similar lifespan. This implies that the developing T cells interact mainly with newly formed thymus-restricted DC, rather than with any DC entering from the circulation which might present exogenous foreign antigens (see also ref. 14). This would ensure that the developing T cells are purged only of cells reactive with intrathymic and predominantly self antigens. Other mechanisms[18] would then be needed to produce peripheral tolerance to self antigens not expressed within the thymus itself. □

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