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Development of T Lymphocytes at Extrathymic Sites

Mariastefania Antica[1]* and Roland Scollay[2†]

T lymphocytes expressing both CD4 and CD8 are the predominant cell type in the thymic cortex but are extremely rare outside the thymus of normal mice. In this article, we show that if precursor thymocytes ($CD4^-CD8^-$) from fetal or adult donors are injected i.v. into irradiated recipients, some of these cells will lodge in lymph nodes and develop into both $CD4^+CD8^+$ (double-positive) and $CD4^+$ or $CD8^+$ (single-positive) cells. This phenomenon also occurred in thymectomized recipients, strongly suggesting it is genuine extrathymic development. Prethymic precursors (e.g., fetal liver), were unable to use the lymph node for T cell development, without thymic processing. The data suggest that given unusual circumstances (irradiation or thymectomy and availability of appropriate precursors), the lymph nodes can support T cell development. ***The Journal of Immunology*****, 1999, 163: 206–211.**

The majority of T cells develop in the thymus, although in recent years evidence has accumulated that thymus-independent T cell development can occur, particularly in the intestine (1, 2) and possibly in the liver (3, 4). Within the thymus, T lymphocyte precursors go through several clearly defined stages, starting as $CD4^-CD8^-$ cells, then acquiring both CD4 and CD8 to become double-positive (DP)[3] cells (~80% of all thymocytes), and finally losing either CD4 or CD8 to become mature single-positive (SP) cells, located predominantly in the thymic medulla. The cells that leave the thymus to populate secondary lymphoid tissues are essentially all SP cells. Indeed, the DP stage is confined to the thymic cortex (reviewed in (5), and in normal circumstances DP cells are absent or rare in the peripheral lymphoid tissues.

However, a number of published studies suggest that DP cells may occur outside the thymus. For example, it has been claimed that SP lymphocytes, when activated in vitro, acquire the lost accessory molecule to become DP (6, 7), although our own analyses, which included very careful controls, found DP cells to be very rare in both mouse and human cultures (8). DP cells have also been reported in lymph nodes of neonatal mice (9). All studies agree that DP cells are absent or very rare in normal adult peripheral lymphoid tissues, although they may be detected after drug treatment (10, 11). In both cases, they appear to be exported from the thymus. Finally, it has been reported that in mice in which the *Hlx* homeobox gene was constitutively expressed (as a transgene) in lymphocytes, disrupted development resulted in the appearance of DP cells, probably thymus derived, in the lymph nodes and spleen (12).

The work presented in this article is an extension of our previous studies in which various precursor populations were used to reconstitute irradiated recipients, and we have published a number of reports on the process of thymic reconstitution and colonization of peripheral tissues with mature SP progeny of injected donor cells. However, we have also noticed the appearance of "thymus-like" DP cells in the lymph nodes of animals reconstituting after intrathymic injection of thymic double-negative (DN) precursors. Here we report this phenomenon in a system that shows the reproducible presence of DP cells in lymph nodes of irradiated, reconstituted mice. Although the mechanisms involved remain unclear, it would seem that lymphocyte development, including DN, DP, and SP stages, can occur in lymph nodes (but not spleen) in small but significant numbers. The thymus is not necessary for this to take place, so it represents a true extrathymic pathway.

Materials and Methods

Animals

Male and female C57BL/Ka Thy-1.1 (Thy-1.1, Ly-5.2) and Ly-5 congenic recipient mice (C57BL/6-Ly-5.1-Pep3^b (Thy-1.2, Ly-5.1)), maintained under specific-pathogen-free conditions at the Walter and Eliza Hall Institute (Melbourne, Australia) and Centenary Institute Animal Facilities (Sydney, Australia) were used for all the experiments. Embryos were aged by vaginal plug assessment, day 0 being the day of plug detection. For i.v. lineage reconstitution, C57BL/Ka Thy-1.1 mice of different ages were used as donors and C57BL/6-Ly-5.1-Pep3^b mice at 8–12 wk of age were used as recipients.

Cell suspensions

Mice were killed with CO_2, and organs were removed immediately into cold mouse tonicity BSS containing 5% FCS. Cells were separated with a stainless steel sieve.

mAbs and reagents

The Abs used for magnetic bead depletion were: anti-CD8, clone 53-6.7 (13); anti-CD4, clone GK1.5 (14); anti-CD3, clone KT3–1.1 (15); anti-B220, clone RA3-6B2 (16); anti-Gr-1, clone RB6-8C5 (17); anti-Mac-1, clone M1/70.15 (18). All the above were used as either culture supernatants or ascitic fluid. The purified Abs used for immunofluorescent staining were: anti-Thy-1.1, clone 19F12 (19); anti-Ly-5.2, clone ALI-4A2 (20); and anti-TCR-$\alpha\beta$, clone H57-597 (21), used as a direct FITC conjugates or biotinylated; anti-heat stable Ag (HSA), clone M1/69 (18), used as a PE conjugate. The fluorescent reagents used for second stage staining were PE-avidin, Texas Red (TR)-avidin, and PE-, allophycocyanin-, or TR-anti-rat Ig (all from Caltag Laboratories, San Francisco, CA).

Preparation of donor $CD4^-CD8^-$ thymocytes

The procedure for purification of $CD4^-CD8^-$ thymocytes was similar to that described previously (22), and involved depletion of $CD4^+$ and $CD8^+$ cells with magnetic beads. For day 15 (E15) embryonic thymocytes, the depletion stage was omitted, because all cells were $CD4^-CD8^-$. Thymocyte suspensions from 24–80 E18 thymuses or 5–10 adult thymuses from Ly-5.2 mice were subjected to depletion. The thymocytes were incubated

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[3] Abbreviations used in this paper: DP, double positive; SP, single positive.

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Table I. *Effect of donor age on reconstitution*[a]

Donor	Recipient Tissue	Donor-Derived Cells						
				% Donor cells of phenotype[d]				
		% positive[b]	No. × 10^{-5}[c]	$CD4^-8^-$	$CD4^+8^+$	$CD4^+8^-$	$CD4^-8^+$	*n*
E15	Thymus	1.3 ± 1.5	5 (2–13)	8	16	61	16	4
	LN	30 ± 14	191 (46–246)	21	46	22	12	4
E18	Thymus	1.4 ± 0.5	20 (4–66)	3	60	31	6	6
	LN	35 ± 10	2 (1–3)	38	36	19	7	6
Adult	Thymus	43 ± 4	329 (279–399)	1	92	5	2	3
	LN	62 ± 4	12 (7–20)	20	61	9	9	3

[a] Appearance of donor-derived lymphoid cells in recipient thymus and lymph nodes 14 to 15 days after i.v. transfer of 1.5 × 10^6 $CD4^-CD8^-$ cells from E15, E18, and adult mouse thymuses. Results are means of determinations on 3–6 (*n*) individual recipients.
[b] Mean ± SD.
[c] Mean and range.
[d] Errors are within 20%.

with a mixture of anti-CD8 and anti-CD4 Abs, and the labeled cells were removed with anti-Ig-coated magnetic beads (Dynabeads, Dynal, Oslo, Norway). Analysis after depletion showed that the purity of the depleted population was >97%.

Lineage reconstitution assay

The procedure was similar to that described by Spangrude et al. (20). The Ly-5.1 recipient mice were lethally irradiated (two doses of 5.5 Gy γ-irradiation with a 3-h interval between) 1–4 h before i.v. transfer. Donor cells from Ly-5.2 mice were injected i.v., along with 4 10^4 Ly-5.1 (recipient type) unfractionated bone marrow cells, to ensure long term survival of the recipients. Antibiotics were added to the drinking water. At various times after transfer, the spleen, lymph nodes, and thymus were collected from the recipients. Cells were stained in two colors with donor-specific anti-Ly-5.2 (FITC-conjugated) together with biotinylated lineage-specific Abs, followed by PE-avidin for the second stage, as described above. In some experiments, four-color staining was used for analysis.

In some experiments, the donor-derived cells were sorted, on the basis of Ly-5, CD4, and CD8 staining, directly onto a microscope slide and analyzed under a fluorescent microscopy. For each of the experiments described, a control of i.v. transferred age-matched fetal liver cells was performed.

Thymectomy

Mice 4–5 wk old were thymectomized 3 wk before the i.v. transfer of DN thymocytes. Animals were assessed at sacrifice for residual thymus, and none was detected in any case.

Analysis and staining of recipient tissues

Thymuses, lymph nodes (pooled axillary, inguinal, brachial, submandibular, and mesenteric), and spleens of recipient animals were removed, and cell suspensions were prepared as previously described (20, 23). After a wash, the cells were incubated with FITC-, allophycocyanin-, TR-, and biotin-labeled Abs for 20 min. The cells were again washed and incubated with PE-conjugated streptavidin. After 20 min, the cells were again washed and resuspended in BSS with propidium iodide (1 μg/ml). All incubation and washes were performed on ice. Flow cytometric analysis was performed on FACStar[Plus] (Becton Dickinson, Mountain View, CA). The dual laser instrument allows four fluorescent parameters and two light scatter parameters to be recorded for each analyzed cell. For analysis, 0.5–2 10^5 cells were collected in each file. The cells of interest were selected by software gating, and Ag expression was determined. Propidium iodide-labeled dead cells were excluded from the analysis. Cells were considered negative if they fell under the curve of the negative control.

Results

Thymic and lymph node reconstitution following i.v. injection of various precursor populations

Irradiated Ly-5.1 mice were injected i.v. with $CD4^-CD8^-$ cells purified from thymuses of 15- and 18-day-old Ly-5.2 embryos and adult mice. Table I shows the percentage and absolute numbers of donor-derived cells in the recipient thymus and lymph nodes. Interestingly, the number of donor-derived cells in recipient thymus increases with donor age, whereas the number of donor derived cells in lymph nodes decreases. Donor cells were also present in spleens in similar proportions (data not shown). The data confirm our previous finding (22) that embryonic thymus is a relatively

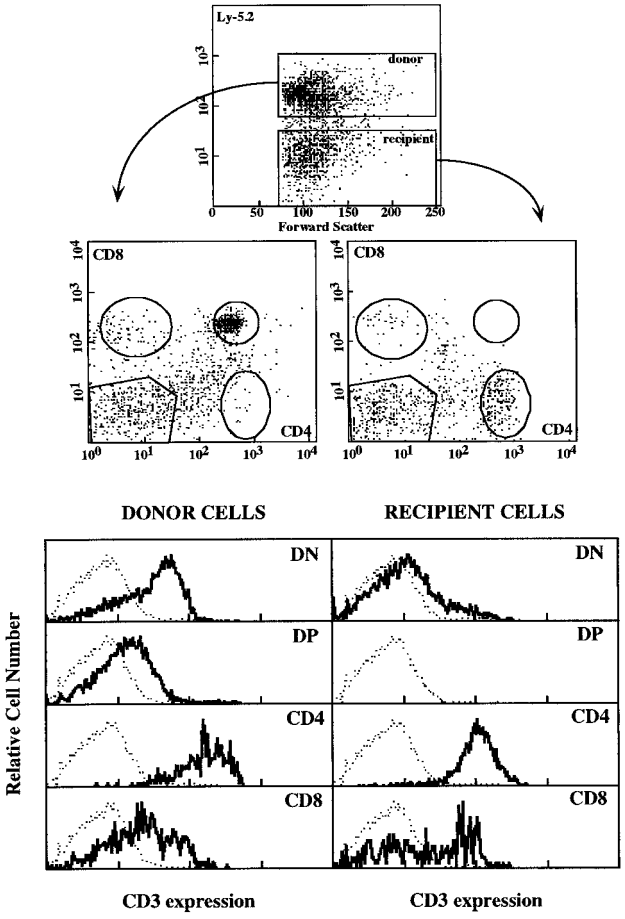

FIGURE 1. Flow cytometric analysis of lymph node cells from previously thymectomized mice 14 days after iv transfer of 1.5 10^6 E18 DN thymocytes. Upper dot plot, all lymph node cells; lower left plot, donor-derived cells (selected Ly-5.2-positive cells); and lower right plot, recipient-derived cells (selected Ly-5.2-negative cells). Histograms represent CD3 expression on selected donor derived vs recipient-derived DN, DP, and SP (CD4 or CD8 positive) cells. For details see *Materials and Methods*.

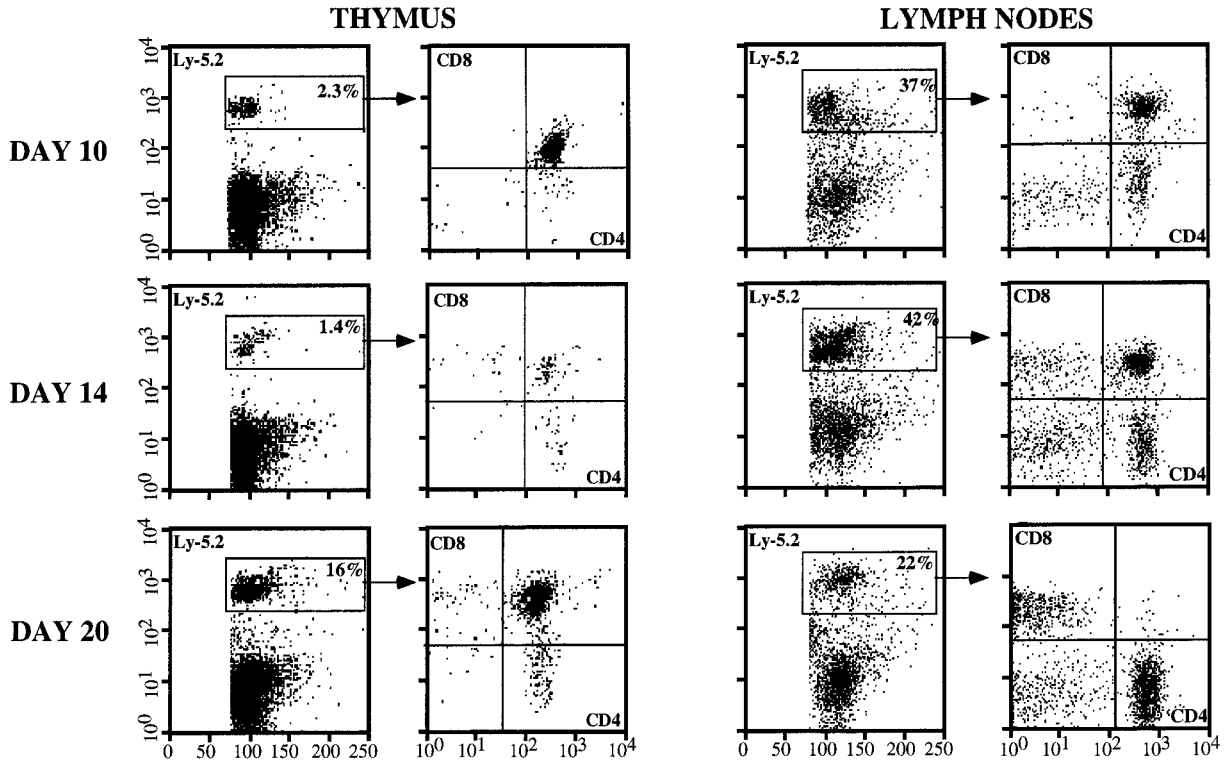

FIGURE 2. Kinetics of reconstitution. A representative result of thymus or lymph node cells from mice 10, 14, and 20 days after i.v. transfer of 1.5 10^6 E18 DN thymocytes.

poor source of precursors for thymus upon i.v. injection but that significant numbers of donor-derived cells accumulate in lymph nodes (and spleens (not shown)).

CD3, CD4, and CD8 expression on donor-derived cells

Fig. 1 shows a representative FACS analysis of the recipient lymph node. Analysis of the CD3, CD4, and CD8 expression on these donor-derived cells 2 wk after i.v. injection is shown in Table I and Fig. 1. Both thymus and lymph node show a substantial proportion of DP and SP cells derived from both adult and embryonic donors. Examples of CD4 and CD8 profiles are shown in Fig. 2. Donor-derived DP cells were never found in the spleens, and DP cells of recipient type were never found in the lymph nodes. All the lymph node DP cells were donor derived. Donor-derived lymph node $CD4^+$ and $CD8^+$ cells express also CD3 which is the same level as on the recipient SP cells (Fig. 1). Interestingly, DN donor-derived cells are mostly $CD3^{low}$, indicating that the donor-derived cells go through a developmental stage before becoming DP. This is not the case in the recipient DN cells because they represent mostly B cells.

To assess the kinetics of appearance of these normally intrathymic populations (i.e., the DP cells), we looked at various times after reconstitution. Table II shows an example of the progeny derived from E18 donor cells, but similar results were obtained with E15 and adult donors (data not shown). Donor-derived cells were first detected at 7 days with mainly DP cells present, but also significant numbers of DN cells (22%). After 2 wk, the number of DP cells declined whereas SP cell number increased. This continued, and by 3 wk, DP cells were down to <10% and SP increased when calculated in percentage as well as in absolute cell number.

Four-color immunofluorescence analysis of the DP donor cells showed them to be very similar to thymocytes, being mostly small

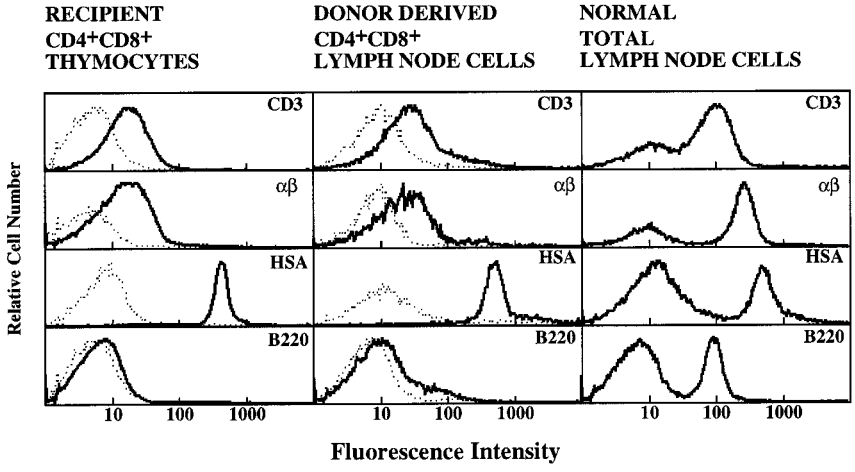

FIGURE 3. Histogram plots of selected donor-derived $CD4^+CD8^+$ cells stained with Abs to CD3, TCR-$\alpha\beta$, HSA, and B220 (middle row) compared with recipient DP thymocytes (left row), and normal lymph node cells (right row). Dotted lines are isotype-matching negative controls. HSA^+ cells in normal total lymph nodes are mainly B cells.

Table II. *Kinetics of reconstitution*[a]

Recipient Tissue	Day of Assay	% Positive	No. × 10^{-5}	% Donor Cells of Phenotype				n
				$CD4^-8^-$	$CD4^+8^+$	$CD4^+8^-$	$CD4^-8^+$	
Thymus	7/8	9	3.3	14	78	4	5	6
	10	4	16.1	7	81	10	1	5
	14	1.4	20.2	3	60	31	6	6
	20	8	29.1	11	59	24	8	2
LN	7/8	14	0.3	22	66	3	8	6
	10	30	0.7	30	52	13	5	5
	14	35	2.1	38	36	19	7	6
	20	21	11.4	27	9	45	20	2

[a] Appearance of donor-derived lymphoid cells in recipient thymus and lymph nodes at various times after i.v. transfer of 1.5×10^6 $CD4^-CD8^-$ thymocytes from E18 donors. Results are means of 2–6 (*n*) individual recipients. Errors are within 20%.

Table III. *Reconstitution in thymectomized mice*[a]

Day of Assay	% Positive[b]	No. × 10^{-3}	% Donor Cells of Phenotype[b]				n
			$CD4^-8^-$	$CD4^+8^+$	$CD4^+8^-$	$CD4^-8^+$	
10	66 ± 10	460	57 ± 5	27 ± 5	11 ± 2	5 ± 0.5	4
14	47 ± 15	250	54 ± 18	25 ± 20	26 ± 19	9 ± 0.6	3
23	35	530	60	5	15	21	1

[a] Appearance of donor-derived cells in lymph nodes of thymectomized recipient mice 10–23 days after i.v. transfer of 1.5×10^6 $CD4^-8^-$ cells isolated from E18 donor thymuses. Results are means of determinations on 1–4 (*n*) individual recipients.
[b] Mean ± SD.

(like thymocytes) and smaller than peripheral SP T cells. This can be seen in day 14 forward scatter vs the Ly-5 profile in Figs. 1 and 2. These cells were high for HSA, and low or negative for CD3 and TCR-$\alpha\beta$ expression. The SP cells were more like mature T cells, with CD4 or CD8 expression (Fig. 1), high TCR expression, and low Thy-1 and HSA expression (not shown).

Lymph node DP cells are derived only from thymic precursors

In all the experiments, fetal liver precursors were injected into parallel groups of mice as a control. These reconstituted all tissues well, although with slower kinetics (data not shown). Donor-derived T cells appeared in lymph nodes (and spleen) only after SP cells appeared in the thymus. In no case and at no time point were significant numbers of donor-derived DP cells observed in lymph nodes.

Lymph node DP cells do not come from recipient thymuses

Clearly the question arose whether these lymph node DP cells had leaked from the irradiation-damaged recipient thymuses. To test this, we thymectomized a group of recipients 3 wk before irradiation and reconstitution with E18 $CD4^-CD8^-$ thymocytes. The results are shown in Table III, with examples of FACS profiles in Fig. 4. Replicate animals were assayed individually, and DP cells were present at all times assayed; development into SP cells was also apparent in all animals. DP cells appeared less numerous than in the normal animals, but this conclusion could not be categorically stated based on the small sample. However, it seems clear that a significant number of these DP cells had arisen in the lymph nodes, directly from the E18 DN thymocytes which had migrated there without passage through the thymus. These DP cells can differentiate further into $CD3^+$ SP cells in this lymph node environment. The presence of DP cells in lymph nodes in all the situations assayed is summarized in Table IV. The data represent ~80 recipient mice, and results were consistent. DP donor cells were always present and were always phenotypically like DP thymocytes. In addition to Ag analysis, in the experiments with thymectomized animals, DP cells were purified and sorted from lymph nodes by flow cytometry. The identity of the sorted cells as viable DP lymphocytes was confirmed by fluorescence microscopy.

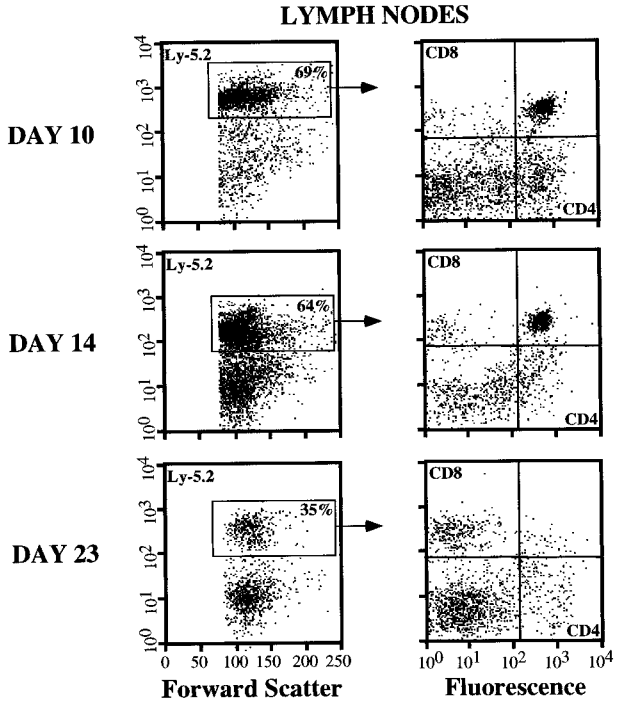

FIGURE 4. Dot plots of a representative result of lymph nodes from thymectomized mice 10, 14, and 23 days after i.v. transfer of DN thymocytes stained with Abs to Ly-5.2, CD4, and CD8. The right row represents CD4 and CD8 expression on selected donor-derived cells (Ly-5.2 positive). More details about the size of the groups and percentages of donor-derived CD4 and CD8 cells are given in Table III.

Table IV. *Percentage of donor-derived cells that are $CD4^+CD8^+$ in recipient lymph nodes*[a]

	% and No. ($\times 10^{-3}$) of Donor Cells That Were $CD4^+CD8^+$ in Recipient Lymph Nodes[b]				
Day of Assay	7/8[c]	10	12	14/15	20/21
Donor age					
E15	51 (1)	82 (54)	38 (21)	46 (88)	ND
E18	66 (20)	52 (32)	ND	36 (76)	9 (100)
E18 (TX[d])	ND	27 (124)	ND	25 (62)	5 (26)
Adult	14 (4)	ND	54 (257)	61 (741)	4 (62)

[a] Recipient mice were analyzed at several time points after i.v. injection of the DN thymocytes (1.5×10^6 donor cells per recipient mouse) from donors of various ages.
[b] The results represent mean values of 3–8 recipient mice from two experiments. Errors are within 15%.
[c] Percentage and number ($\times 10^{-3}$).
[d] TX, Thymectomized recipient.

Discussion

In this study, we demonstrate the appearance of $CD4^+CD8^+$ DP lymphocytes, which closely resemble typical cortical thymocytes, and mature SP lymphocytes in the lymph nodes of irradiated mice that had been i.v. injected with $CD4^-CD8^-$ thymocytes from fetal or adult donors. Their appearance even in thymectomized recipients strongly suggests that at least some of them arise by an aberrant extrathymic development pathway. The microenvironments that allow this to occur in lymph nodes are apparently not present in the spleen, because we have never detected these cells there. Furthermore, it seems that development of DP and SP cells in lymph nodes does not reproduce the whole spectrum of thymic processes, because early stem cells (as in fetal liver) were unable to develop in this way. Only thymocytes (already committed to intrathymic development) appear able to develop into DP and further in SP cells at extrathymic sites.

In our previous studies of reconstitution with fetal and adult thymocytes (22), in which both i.v. and intrathymic injection was used, DP cells were not detected in lymph nodes for two reasons. In some cases, we had analyzed mixed lymph node and spleen that was dominated by more numerous spleen cells, and spleens do not seem to support DP cell growth. In other cases, where we analyzed lymph nodes alone, although we detected donor-derived cells in small numbers, CD4 and CD8 expression was not assayed. However, this phenomenon of donor-derived DP and SP cell occurrence in lymph nodes is seen consistently after i.v. injections of thymocytes.

This model probably does not reflect the normal situation with an intact thymus, because immature thymocytes would not normally be in the blood or in an irradiated recipient. Because our data show that lymph nodes can support T cell development and on the other hand prethymic precursors were unable to use the lymph node for T cell development, without thymic processing, we believe that what we describe here is a different pathway from extrathymic intestinal or liver T cell development (1–4).

Nevertheless, the data demonstrate that the thymus is not the only tissue capable of supporting development of T cells from early precursors, and if the committed pre-T cells can reach lymph nodes, they can in fact develop there. In accordance with these results is a recent publication from Clegg et al. (24) showing that oncostatin M can support T cell development at extrathymic sites. An alternate interpretation of our data could be proposed in the light of a recent study of Cibotti et al. (25). The authors described development of DP to SP T lymphocytes in vitro in the absence of thymic epithelium due to inducer molecules. Our results confirm and further extend these findings in an in vivo system with precursor thymocytes that are even at an even earlier stage of development (DN cells). Coinducer molecules described by Cibotti et al. (25) may function by nonspecifically enhancing transduction of TCR signals at least at the DP stage. Therefore, it is possible that also the development from DN to DP and SP cells we noticed in our experiments was due to signals provided by lymph node cells.

The capability of lymph nodes to support T cell development provides new possibilities for treatment of immunodeficiencies in cases where the thymus cannot perform its primary function. It has been shown that the T cells in the spleen can undergo Ag-driven positive and negative selection (26). Whether the lymph node microenvironment is also capable of directing positive and negative selection is the subject of future experiments.

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