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Thymic and Extrathymic T Cell Development Pathways Follow Different Rules¹

Rafik Terra, Nathalie Labrecque, and Claude Perreault²

Separation between primary and secondary lymphoid organs is a universal feature in jawed vertebrates. Strikingly, oncostatin M (OM)-transgenic mice present massive extrathymic T cell development, localized exclusively in the lymph nodes (LN). According to the prevailing paradigm, the thymus is the main source of T lymphocytes in gnathostomes mainly because thymic epithelial cells have a unique ability to support early steps in T cell development. It is therefore remarkable that productive T cell development occurs in the OM⁺ LN, despite the absence of epithelial cells. The present study shows that in the OM⁺ LN: 1) MHC class I expression strictly on hemopoietic cells is sufficient to support the development of a diversified repertoire of CD8 T cells; 2) the efficiency of positive selection of specific TCR-transgenic T cells is not the same as in the thymus; 3) negative selection is very effective, despite the lack of an organized thymic-like medulla. Furthermore, our data suggest that extrathymic T lymphocytes developing in the OM⁺ LN undergo extensive postselection expansion because they live in the microenvironment in which they were positively selected. This work illustrates how the division of labor between primary and secondary lymphoid organs influences the repertoire and homeostasis of T lymphocytes. *The Journal of Immunology*, 2002, 169: 684–692.

The T cell repertoire is shaped by discrete central and peripheral events: positive and negative selection in the thymus and differential survival/expansion of postthymic T cells in the secondary lymphoid organs (1, 2). According to the strength of signal paradigm, thymocyte-positive selection results from a weak TCR signal, whereas no signal results in death by neglect and a strong signal results in negative selection (1, 3). Studies in transplantation chimeras and in vitro thymus culture suggest that thymocytes are positively selected mainly on MHC-peptide complexes expressed by thymic cortical epithelial cells (4–6). Nevertheless, a small subset of T cells stands as an exception to this rule: CD1d-dependent NKT cells expressing a semiinvariant TCR composed of a V α 14-J α 18 rearrangement are positively selected on double-positive (DP)³ thymocytes rather than thymus epithelial cells (7). Furthermore, studies using relatively constrained models have shown that fibroblasts and hemopoietic cells can support positive selection of mainstream thymocytes when they were located in the thymus environment (8–11). Importantly, positive selection of T lymphocytes by hemopoietic cells is largely thymus dependent because practically no T lymphocytes developed in athymic hosts (10, 11).

In contrast with positive selection, thymocyte negative selection is initiated by strong TCR signals triggered by MHC-restricted interactions mainly with dendritic cells and to a lesser extent with thymic medullary epithelial cells (12, 13). Although cortical epi-

thelial cells can induce deletion of some thymocytes (14, 15), this negative selection is probably limited to a subset of DP thymocytes that bind with high avidity to the most abundant self peptides expressed by cortical epithelial cells (13, 16–18). Teleologically, one highly controversial issue with regard to the shaping of the T cell repertoire is whether there is any reason for the division of labor between discrete subsets of cortical and medullary stromal cells (13, 19). In other terms, if the strength of the TCR signal dictates whether thymocytes will be positively or negatively selected, is there any advantage in positive and negative selection being induced mainly by interactions with cortical epithelial cells and medullary dendritic cells, respectively?

Following export from the thymus, naive T cells require continued engagement by MHC molecules to survive in the periphery and for homeostatic expansion (1, 20–22). Notably, T cells with different TCRs display major discrepancies in their ability to undergo homeostatic expansion (reviewed in Ref. 23). Thus, the T cell repertoire shaped in the thymus is subject to further molding in the peripheral lymphoid organs (2). The nature of the specific peptides involved in peripheral survival/expansion remains controversial. Some studies suggest that peptides involved in peripheral expansion may be identical with those that support intrathymic positive selection, while other studies suggest that they are structurally different, but share the same affinity or avidity (24–26). However, one indisputable point has unclear implications: the postselection T cell repertoire is anatomically secluded from the site in which it has been positively selected. Because of the blood-thymus barrier, mature T lymphocytes never reenter the thymus cortex (27, 28).

Two cytokines of the IL-6 family, leukemia inhibitory factor (LIF) and oncostatin M (OM), can cause thymic involution. In vivo administration of LIF and OM by i.p. injection to mice over 3 days is sufficient to induce profound thymic atrophy with loss of cortical thymocytes (29). Accordingly, LIF- and OM-transgenic mice present a profound thymic atrophy (30, 31). Unexpectedly however, LIF- and OM-transgenic mice were shown to develop massive extrathymic T cell development, localized exclusively in the lymph nodes (LNs) (30–32). Thus, abundant pT α transcripts are detectable in the LNs of OM-transgenic mice, but not in their

Guy-Bernier Research Center, Maisonneuve-Rosemont Hospital, Montreal, Quebec, Canada

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² Address correspondence and reprint requests to Dr. Claude Perreault, Guy-Bernier Research Center, Maisonneuve-Rosemont Hospital, 5415 de l'Assomption Boulevard, Montreal, Quebec, Canada HIT 2 M4. E-mail address: c.perreault@videotron.ca

³ Abbreviations used in this paper: DP, double positive; β_2 m, β_2 -microglobulin; DN, double negative; HSA, heat-stable Ag; LIF, leukemia inhibitory factor; LN, lymph node; OM, oncostatin M; SP, single positive; TREC, TCR excision circle.

wild-type counterparts (32). Furthermore, $\sim 215 \times 10^6$ Thy-1⁺CD4⁺CD8⁺ cells are present in the mesenteric LNs of 12-wk-old OM-transgenic mice (31). Studies of adult thymectomized recipients of fetal liver grafts have shown that the lymphopoietic pathway modulated by OM is truly thymus independent (31). The paracrine influence of OM is sufficient to induce T cell development in the LNs: nontransgenic fetal liver-derived progenitors generate CD4⁺CD8⁺ cells as well as mature T cells in the LNs of nontransgenic recipients, whereupon OM is supplied in a paracrine manner by coinjected OM-transgenic hemopoietic cells (31). The OM-dependent extrathymic pathway generates both CD4⁺ and CD8⁺ T cells, which are diverse in terms of V β usage and show a more rapid turnover rate (5-bromo-2'-deoxyuridine pulse-chase assays) than thymus-derived T lymphocytes in wild-type mice (31). Of note, the mature progeny (CD4⁺8⁻ and CD4⁻8⁺ T cells) of the OM-dependent extrathymic pathway shares properties of classical mainstream T lymphocytes: 1) CD4⁺ and CD8⁺ elements are NK1.1⁻ (31), unlike NK1.1⁺ T cells that are positively selected by interactions with hemopoietic cells in normal mice (33); 2) the CD8⁺ T cells express $\alpha\beta$ CD8 heterodimers (R. Terra, unpublished observation), as opposed to CD8 $\alpha\alpha$ T cells that have been reported to differentiate extrathymically in the intestines and liver (34).

OM can transform the LN into a primary lymphoid organ whose ability to support T cell development and to seed peripheral compartments is similar to that of a normal thymus (31, 32). This fact raises fundamental questions for two reasons. The OM⁺ LN is devoid of thymic epithelial cells, and DP T lymphocytes are admixed with single-positive (SP) T cells without any thymus-like corticomedullary segregation (I. Louis and C. Perreault, manuscript in preparation). Moreover, mature SP T cells such as those from the spleen readily recirculate from the blood to the OM⁺ LN (31). We address two pressing issues. In the OM-conditioned LN, which cell type can replace thymic epithelial cells in supporting the positive selection of T cell progenitors? Do positive and negative selection in the LN follow the same rules as in the thymus, and will the T cell repertoires generated in the thymus and the LN be similar? The need to investigate these queries is compelling for at least two reasons. The extrathymic T cell differentiation pathway that emerges following chronic exposure to OM has potential therapeutic interest for individuals with impaired thymus function. Moreover, because the OM⁺ LN functions as a primary and secondary lymphoid organ, these studies should allow us to evaluate to which extent the canonical separation between primary and secondary lymphoid organs influences T lymphocyte development and homeostasis.

Materials and Methods

Mice

C57BL/6J (B6), B10.D2-H2^dH2-T18^cHc¹/nSnJ (B10.D2), B6.SJL-Ptprc^ePep3^b/BoyJ (Ly-5^a) (B6.SJL; Ly-5.1⁺), and MHC class I-deficient (B6.129-B2 m^{mi1}) (35) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). H-Y TCR-transgenic mice (C57BL/10AiTac-Rag^{tm1}-TgN (TCRHY)N11) (36) were obtained from Taconic Farms (Germantown, NY). The 2C TCR-transgenic mice on a C57BL/6 background were from D. Y. Loh (Washington University School of Medicine, St. Louis, MO) (37). OM-transgenic mice on a C57BL/6 background have been previously described (31, 32). H-Y TCR/LckOM-, 2C TCR/LckOM-, and LckOM β_2 -microglobulin^{-/-} ($\beta_2m^{-/-}$)-transgenic mice were obtained by breeding heterozygous LckOM males with heterozygous H-Y TCR, 2C TCR, and homozygous $\beta_2m^{-/-}$ female mice, respectively. The genotype was confirmed by PCR assay using 200 ng genomic tail DNA and the following primers: LckOM 5' to 3', AGTCCCGTACTGCAGGAACA and GCTCACACCATTAAGTGC; H-Y TCR 5' to 3', GAGCCTCCAGTTCTCTCA and AAAGATGAGCTTCGCAGACC; 2C TCR 5' to 3', GTGACACAGCCCGATGCTCG and TCCAAATGTACAGCGCACTTG; and

$\beta_2m^{-/-}$, a mixture of three primers 5' to 3', 1) GCTATTCGGCTATGACTGGG, 2) TATCAGTCTCAGTGGGGGTG, and 3) CTGAGCTCTGTTTTCTGCTG. Mice were bred at the Guy-Bernier Research Center, maintained in autoclaved microisolator cages, and provided with autoclaved food and water, according to the standards of the Canadian Council on Animal Care.

Thymectomy and fetal liver cell transplantation

At 4–5 wk of age, mice were anesthetized by i.p. injection of 75 mg/kg sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada), and the thymus was removed with a suction cannula introduced over the suprasternal notch. Visual inspection at the time of sacrifice verified completeness of thymectomy in each animal. Cell transplantation was performed at least 2 wk after surgery. Hemopoietic chimeras were created by i.v. injection of 5×10^6 fetal liver cells, collected on day 13 postcoitum, into irradiated (12 Gy) recipients. MHC I⁺ recipients of $\beta_2m^{-/-}$ fetal liver cells were injected i.p. with 200 μ g anti-NK1.1 Ab (PK136; BD Pharmingen, Mississauga, Ontario, Canada) on days -2 and -1 to prevent NK cell-mediated rejection of MHC class I-deficient donor cells (38). Chimeras were analyzed 80–90 days after transplantation.

Abs and flow cytometry

For each sample, 10^6 cells were stained with Abs and analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, Mountain View, CA). The following Abs obtained from BD Pharmingen were used: PerCP anti-CD4 (RM4-5), APC anti-CD8 α (53-6.7), biotinylated anti-V β 3 TCR (KJ25) detected with FITC-streptavidin, FITC anti-V β 5.1.2 TCR (MR9-4), FITC anti-V β 6 TCR (RR4-7), FITC anti-V β 7 TCR (TR310), FITC anti-V β 8.1,2 TCR (MR5-2), FITC anti-V β 9 TCR (MR10-2), FITC anti-V β 10^b TCR (B21.5), FITC anti-V β 11 TCR (RR3-15), FITC anti-V β 13 TCR (MR12-3), FITC anti-V β 14 TCR (14-2), FITC anti-V β 17^a TCR (KJ23), FITC anti-Thy-1.2, FITC anti-Ly-5.1, FITC anti-Ly-5.2, FITC anti-CD44 (IM7), FITC anti-CD122 (5H4), FITC anti-heat-stable Ag (HSA), FITC anti-1B11 (CD43), FITC anti-CD25, FITC and PE anti-CD62L (Mel-14), FITC anti-CD5 (53-7.3), FITC anti-TCR α/β , FITC and PE anti-CD3 ϵ , biotinylated anti-Ly-5.2 detected with PE-streptavidin and PE anti-CD69. Rat IgG2a, κ was used as control Ab. Anti-H-Y TCR (T3.70) and anti-2C TCR (1B2) mAb (kind gifts from H. S. Teh and J. Wu, respectively) were detected with rat anti-mouse IgG1 PE.

Results

Positive selection of CD8 T lymphocytes in the OM-conditioned LN

We wished to determine whether extrathymic positive selection in the OM-conditioned LN is supported by hemopoietic or nonhemopoietic cells (that is, fibroblasts or endothelial cells). Our study of T cell differentiation used four types of hemopoietic chimeras in which adult-thymectomized lethally irradiated (12 Gy) Ly-5-congenic recipients were transplanted with OM-transgenic fetal liver cells. Chimeras differed in that MHC class I expression was either 1) normal (MHC I⁺ \rightarrow MHC I⁺); 2) deficient on both hemopoietic (donor-derived) and nonhemopoietic (recipient) cells ($\beta_2m^{-/-}$ \rightarrow $\beta_2m^{-/-}$), or selectively deficient on either 3) hemopoietic ($\beta_2m^{-/-}$ \rightarrow MHC I⁺) or 4) nonhemopoietic (MHC I⁺ \rightarrow $\beta_2m^{-/-}$) cells. Although the first two groups served as positive and negative controls, the last two groups allowed us to estimate the role of nonhemopoietic vs hemopoietic cells in positive selection of CD8⁺ T lymphocytes. OM-dependent extrathymic T cell development involves all LNs, but is particularly massive in the mesenteric LNs. Thus, all analyses of LN cells in this work were performed on mesenteric LNs. As expected, positive selection of CD8 T cells was defective in ($\beta_2m^{-/-}$ \rightarrow $\beta_2m^{-/-}$) chimeras (Fig. 1, A and B). A key finding was that positive selection of CD8 T cells proceeded normally in (MHC I⁺ \rightarrow $\beta_2m^{-/-}$) chimeras (Fig. 1, A and B) and generated a TCR repertoire with a diversified V β profile (Fig. 1C). Thus, expression of MHC I molecules on hemopoietic cells is sufficient for positive selection of OM⁺ T cells. At face value, the presence of significant proportions of SP CD8 T cells in ($\beta_2m^{-/-}$ \rightarrow MHC I⁺) chimeras suggested that nonhemopoietic

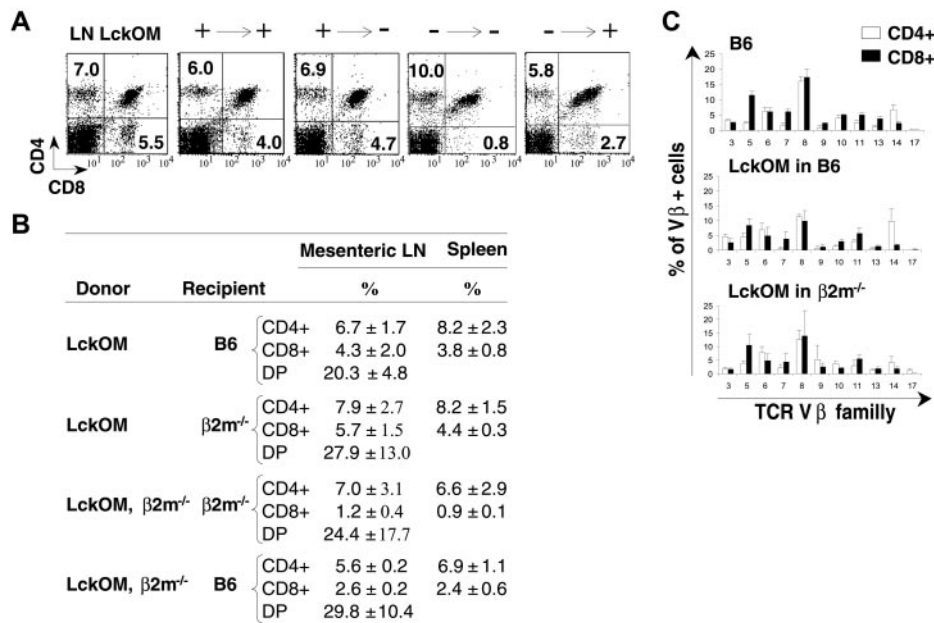


FIGURE 1. The role of hemopoietic and nonhemopoietic cells in the OM-dependent extrathymic positive selection of MHC class I-restricted T cells. **A**, CD4/CD8 profile of LN lymphoid cells in LckOM mice and adult-thymectomized lethally irradiated (12 Gy) Ly-5-congenic recipients transplanted with OM-transgenic fetal liver cells. Chimeras were analyzed on day 80 posttransplant. They differed in that MHC class I expression was either 1) normal (+→+), 2) deficient on both hemopoietic (donor-derived) and nonhemopoietic (recipient) cells (-→-), or selectively deficient on either 3) hemopoietic (-→+) or 4) nonhemopoietic (+→-) cells. MHC I⁺ recipients of β2m^{-/-} fetal liver cells were injected i.p. with 200 μg anti-NK1.1 Ab on days -2 and -1 to prevent NK cell-mediated rejection of MHC class I-deficient donor cells (38). Acquisitions were gated on lymphoid cells. The analysis is one representative experiment of three to six. **B**, Mean ± SD proportion of DP and SP T cells in the LN and spleen of the above-described chimeras (three to six mice per group). **C**, Vβ profile of CD4 and CD8 SP T cells in the spleen of B6 mice and hemopoietic chimeras (three to five mice per group).

cells can also support positive selection, albeit with reduced efficiency (Fig. 1, *A* and *B*). However, these results must be regarded as inconclusive because expression of Ly-5.1 and Ly-5.2 markers showed that 1–3% of LN hemopoietic cells were of recipient type (data not shown). Because residual host hemopoietic cells in the latter group were MHC I⁺, they could have supported positive selection of CD8 T cells.

Extrathymic differentiation of H-Y TCR-transgenic T lymphocytes

To study the process of positive and negative selection during extrathymic T cell differentiation, we analyzed T lymphocyte populations in single (H-Y TCR)- and double-transgenic (H-Y TCR and OM) male and female mice (Fig. 2; Tables I, II, and III). These mice possessed a C57BL/6 background and were not RAG deficient. Note that the fact that the OM⁺ LN is both a primary and secondary lymphoid organ complicates the interpretation. In contrast to SP T cells in the thymus, SP T cells in the OM⁺ LN are a combination of cells generated in situ admixed with recirculating T cells.

Positive selection in female mice. In single-transgenic (H-Y TCR) female mice, the proportion of CD8 SP T cells expressing the H-Y TCR (T3.70⁺) was ~97% in the thymus and ~36% in the spleen (Fig. 2*A*). In addition, the CD8-CD4 ratio dropped from ~3 in the thymus to ~0.6 in the spleen. This is consistent with previous reports that in H2^b female mice, T cells bearing the transgenic H-Y TCR are positively selected in the thymus, but expand poorly in the periphery (39, 40). In contrast, in double-transgenic (H-Y TCR and OM) mice, the proportion of T3.70⁺ cells among CD8 SP T cells was only ~25% in the LNs (the site in which T cells develop) and ~6% in the spleen (Fig. 2*B*). When estimating the proportion of T3.70⁺ cells among CD8 SP T cells in the OM⁺ LN, one caveat is that the repertoire generated in situ is admixed

with recirculating SP T cells. Nevertheless, the reduction in the proportion of T3.70⁺ cells was also conspicuous in LN DP T cells of OM-transgenic mice, although less marked than at the SP stage (Fig. 2, *A* vs *B*). Moreover, in contrast with that found in the thymus of single-transgenic mice, no T3.70^{high} cells were present among LN DP T cells from OM⁺ mice (see insets of Fig. 2, *A* and *B*). In addition, when compared with the thymus, the OM⁺ LN was unable to generate a normal pool size of CD8 T cells in secondary lymphoid organs. The number of T3.70⁺ SP CD8 T cells in the spleen of double-transgenic female mice was decreased 9-fold relative to single-transgenic mice (Tables I and II). These data indicate that in female mice, irrespective of their origin, H-Y TCR⁺ T cells do not thrive well in secondary lymphoid organs: positive selection is poor in the OM-transgenic LN, and T3.70⁺ SP T cells of thymic or extrathymic origin do not expand well in the periphery. The phenotype of T3.70⁺CD8⁺ T cells in the OM-transgenic LN also differed from that of their thymic-derived counterparts (Fig. 2, *A* vs *B*). In the OM⁺ LN, only a small proportion of these cells was HSA⁺, while the proportion of CD44^{high} and CD122⁺ cells was much increased.

Negative selection in male mice. As previously reported (41), we confirmed that T3.70⁺ T cells are negatively selected at the DP stage in the thymus of H2^b male mice (Fig. 2*C*). The number of DP T cells and the ratio of CD8-CD4 T cells in the thymus are severely decreased, and the expression of CD8 is decreased on SP T cells. Yet, consistent with observations by Rocha and von Boehmer (40), SP CD8^{low} H-Y TCR⁺ T cells that escaped intrathymic negative selection expanded well in the periphery in which ~97% of CD8 T cells were T3.70⁺. In fact, the absolute numbers of spleen T3.70⁺ CD8 T cells, greater in male than female mice (Tables I and II), illustrate how extensively homeostatic expansion can remold the postselection repertoire. Similarly, the number of

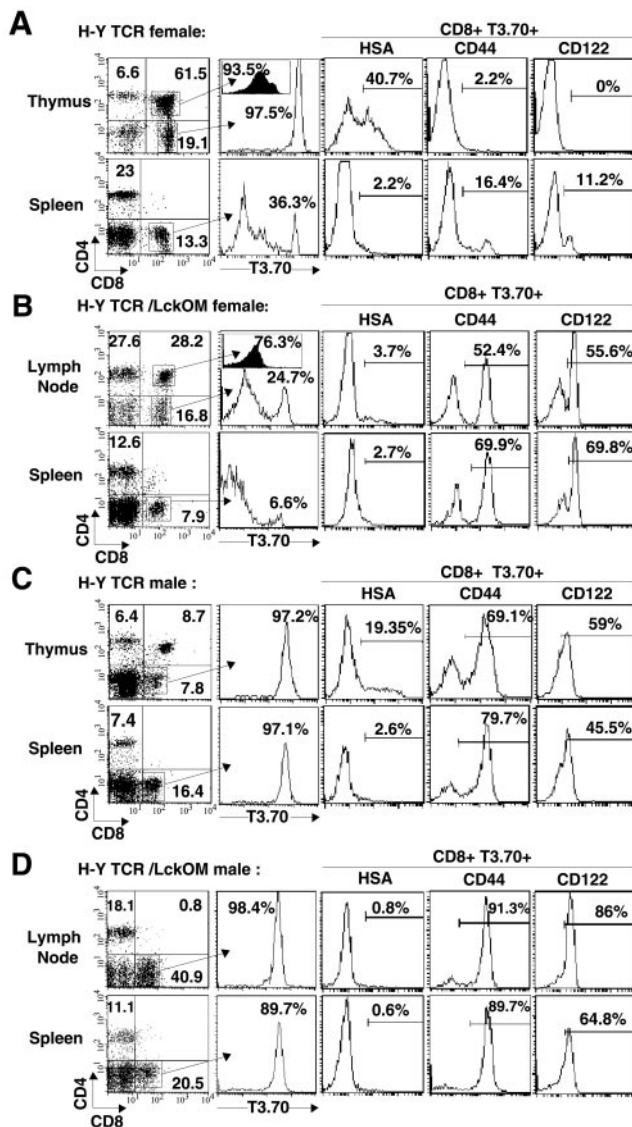


FIGURE 2. Positive and negative selection of H-Y TCR-bearing (T3.70⁺) T cells in the thymus of single-transgenic (H-Y TCR) mice and in the LN of double-transgenic mice (H-Y TCR and OM). CD4/CD8 profiling depicted in the *left panels* was performed on lymphoid cells in the thymus and spleen, and on gated Thy-1.2⁺ cells in the LN. Histograms in the second column show the proportion of T3.70⁺ T cells among SP or DP T cell populations. Expression of HSA, CD44, and CD122 was evaluated on CD4⁺CD8⁺T3.70⁺ T cells. The data are one representative experiment of three.

DP T cells was drastically decreased in the LN of OM⁺ male mice, yet CD8^{low} SP T cells that escaped negative selection expanded well in the periphery (spleen) (Fig. 2D and Tables I and II). Thus, negative selection of H-Y TCR⁺ T cells takes place very efficiently in the LN of OM⁺ mice. In comparison with T3.70⁺ CD8 T cells originating from the thymus of single-transgenic female mice, those found in single- and double-transgenic male mice showed an up-regulation of CD44 and CD122 and a paucity of HSA⁺ cells. The latter findings were more marked in T cells of LN than those of thymus origin (Fig. 2, C and D).

Extrathymic differentiation of 2C TCR-transgenic T lymphocytes

During intrathymic development, the 2C TCR is positively selected by H2K^b and negatively selected by H2L^d (42, 43). To evaluate whether the development of 2C⁺ T lymphocytes obeys

the same rules in the LN as in the thymus, we analyzed T lymphocyte populations in single (2C TCR)- and double-transgenic (2C TCR and OM) H2^b and H2^{b/d} RAG-competent mice (Fig. 3; Tables I, II, and III).

Positive selection in H2^b mice. In both the thymus of 2C⁺ mice and the LN of 2C⁺OM⁺ mice, the majority of SP T lymphocytes were CD8⁺, of which practically all expressed the 2C TCR (1B2⁺) (Fig. 3). Hence, positive selection of 2C⁺ T lymphocytes is very efficient in the OM⁺ LN, which is indeed at least as effective as the thymus in maintaining the size of the spleen CD8 T cell compartment (Tables I and II). Compared with the thymus, few 1B2⁺CD8⁺ T cells were HSA⁺ in the OM⁺ LN (Fig. 3), similar to our observations on T3.70⁺ T cells (Fig. 2). Yet, CD44 and CD122 activation markers were not up-regulated on OM⁺2C⁺ LN CD8 T cells (Fig. 3B) as they were on OM⁺ H-Y TCR⁺ CD8 T cells (Fig. 2B). We showed previously that development of non-TCR-transgenic T cells in the OM⁺ LN was thymus independent (Fig. 1) (31). That this is also the case for 2C TCR-transgenic T lymphocytes was revealed by analyses of T lymphocyte populations in irradiated adult-thymectomized Ly-5.1⁺ recipients reconstituted with fetal liver cells from Ly-5.2⁺ double-transgenic (2C TCR and OM) donors (Fig. 4). Our observation of efficient selection of 2C⁺ T cells in these chimeras confirmed their thymic independent generation.

Negative selection in H2^{b/d} mice. T cells bearing 2C TCR were negatively selected in H2^{b/d} mice, both in the thymus of OM-nontransgenic mice and in the LN of OM⁺ mice. Indeed, in both cases, the proportion of CD4⁺CD8⁺ and of SP CD8⁺ T lymphocytes was drastically decreased, and the percentage of CD8⁺ T cells that were 1B2⁺ was significantly lower than in H2^b mice (Fig. 3, C and D). Furthermore, the size of the spleen 1B2⁺ CD8 T cell pool was drastically reduced in both single- and double-transgenic H2^{b/d} mice (Fig. 3, C and D; Tables I and II).

Development of double-negative TCR-transgenic T cells

Double chain TCR $\alpha\beta$ transgenes expressed at the double-negative (DN) stage alter early ontogeny especially when affinity of the TCR for its ligand is strong, that is, conditions of negative selection. In this situation, increased numbers of DN cells are exported in the periphery. These TCR $\alpha\beta$ ⁺ DN T cells have properties of $\gamma\delta$ T cells, and their development is MHC independent (44–46). This prompted us to evaluate whether a similar expansion of TCR⁺ DN T cells was present in OM-transgenic mice. We indeed detected TCR⁺ DN T cells in OM⁺ mice. Some differences were observed between the numbers of TCR⁺ DN T cells in the thymus of OM⁻ mice vs the LN of OM⁺ mice (Tables I and II). However, it is difficult to speculate about the significance of these data because the LN of OM⁺ mice contains not only cells generated in situ, but also recirculating cells. Nonetheless, comparing the numbers of H-Y and 2C TCR⁺ DN T cells in the spleen of OM⁻ and OM⁺ mice was more informative and allowed us to make three points (Tables I and II). In both OM⁺ and OM⁻ mice, DN TCR⁺ T cells were more abundant when the mice expressed the nominal Ag recognized by the TCR (H-Y TCR male and 2C TCR H2^{b/d}). This is consistent with previous reports in OM⁻ mice (44, 45). In mice lacking the nominal Ag (H-Y TCR female and 2C TCR H2^b), the number of spleen TCR⁺ DN T cells was greater for the 2C than the H-Y TCR. Finally, the number of splenic TCR⁺ DN T cells was of similar magnitude in OM⁻ and OM⁺ mice. Indeed, among the four groups of mice (H-Y TCR female and male, 2C TCR H2^b, and

Table I. Numbers of H-Y TCR⁺ T cells in lymphoid organs following thymic vs extrathymic development^a

	H-Y TCR Female	H-Y TCR/LckOM Female	H-Y TCR Male	H-Y TCR/ LckOM Male
T3.70 ⁺ cells	Thymus	LN	Thymus	LN
DN	18.3 ± 4.7	15.7 ± 2.9	9.8 ± 2.3	24.5 ± 17.6
DP	63.0 ± 26.9	22.9 ± 12.7	0.2 ± 0.2	0.3 ± 0.2
	Spleen	Spleen	Spleen	Spleen
DN	1.9 ± 0.8	2.6 ± 2	30 ± 15.5	11.4 ± 5.4
SP CD8	5.1 ± 0.9	0.6 ± 0.5	19.3 ± 3.9	16.3 ± 2.9

^a Absolute numbers (mean × 10⁶ ± SD) of H-Y TCR-bearing (T3.70⁺) DN, DP, and SP CD8 T cells in the lymphoid organs of single-transgenic (H-Y TCR) and double-transgenic (H-Y TCR and OM) mice. Three to four mice per group.

H2^{b/d}), numbers of TCR⁺ DN T cells in OM⁺ mice corresponded to 38%–130% of those in OM⁻ mice. Thus, no dramatic differences were found in the TCR⁺ DN T cell development in OM⁺ relative to OM⁻ mice.

CD5 expression on T lymphocytes of thymic vs extrathymic origin

CD5 is a cell surface glycoprotein that functions as a negative regulator of TCR-mediated signaling (47). CD5 is up-regulated at crucial points during thymocyte development by pre-TCR and TCR engagement, and the level of CD5 surface expression is directly related to pre-TCR and TCR signaling intensity. Thus, CD5 surface levels were found to vary considerably among mature SP thymocytes and peripheral T cells that express distinct TCRs. The level of CD5 expression paralleled the avidity of the positively selecting TCR-MHC-ligand interaction (48, 49). Our data imply that positive selection in the OM⁺ LN is much more effective for lymphocytes bearing the 2C TCR than the H-Y TCR. Thus, by measuring CD5 expression, we sought to estimate the strength of the signals transmitted by these two types of TCR during thymic vs extrathymic development.

Expression of CD5 was decreased on DP and SP CD8 T3.70⁺ T lymphocytes developing in the OM⁺ LN relative to intrathymic T3.70⁺ T cells (Fig. 5A). This is consistent with the idea that H-Y-specific T cells receive TCR signals of lesser intensity in the periphery than in the thymus. Notably, low expression of CD5 was not a general characteristic of all T cells that matured in the OM⁺ LN: T3.70-negative T cells present in the LNs of H-Y TCR⁺OM⁺ female mice expressed high levels of CD5 (Fig. 5A). Following development in the thymus or in the OM⁺ LN, DP and CD8 SP T cells bearing the 2C TCR expressed higher levels of CD5 than H-Y TCR⁺ T cells. When compared with thymic T lymphocytes, 2C TCR⁺ T lymphocytes in the OM⁺ LN expressed barely or substantially decreased levels of CD5 at the DP and the CD8 SP stage, respectively. In sum, two major findings emerged from evaluation of CD5 expression. First, in the OM⁺ LN, expression of CD5 and presumably the strength of TCR signals were greater on 2C TCR⁺ than on H-Y TCR⁺ T lymphocytes (Fig. 5C). Data on both the

efficacy of positive selection (Fig. 2) and CD5 expression (Fig. 5) suggest that for T lymphocytes bearing the H-Y TCR, the efficacy of positive selection in the OM⁺ LN is low because they receive only weak TCR signals. Second, T lymphocytes bearing the 2C or the H-Y TCR express lower levels of CD5 when they develop in the OM⁺ LN than in the thymus.

Expression of differentiation/activation markers by extrathymic T lymphocytes

Naive T lymphocytes do not normally recirculate into the thymus. Only a few activated/memory T cells in the S phase of the cell cycle reenter the thymus medulla (but not the cortex) at time of infection (28, 50). In the LNs of OM-transgenic mice, however, SP T lymphocytes live in the environment in which their differentiation/selection took place. Thus, one fundamental difference between T lymphocytes developing in the thymus vs the OM⁺ LN is that in the latter case, the postselection T cell compartment is steadily confronted with the very cells that induced positive selection.

The phenotype of DP T cells found in the OM⁺ LN was similar to that of DP thymocytes of wild-type mice concerning expression of HSA, CD3, CD4, CD5, CD8, and CD44 (Fig. 6A). When compared with wild-type thymocytes, SP T cells in the OM⁺ LN showed several distinctive features: expression of CD3 was decreased, while that of CD44 was up-regulated; few CD4 or CD8 cells were HSA⁺; and few CD8 cells were CD69⁺ (Fig. 6A). In addition, the intensity of CD5 staining was not decreased on polyclonal populations of CD4 and CD8 T lymphocytes found in the OM⁺ LN (Fig. 6A) as it was on transgenic T cells bearing the H-Y and 2C TCR (Fig. 5). In fact, the mean CD5-labeling intensity was increased on CD8 SP T cells, being ~151 in the OM⁺ LN and ~118 in the wild-type thymus (Fig. 6A).

The CD44^{high} phenotype of SP T cells in the OM⁺ LN, confirmation of a previous report (31), suggests previous TCR interaction with heretofore unidentified ligands. This concept was supported by the fact that spleen SP T cells in OM⁺ mice showed other features typical of previous Ag encounter: 1) CD122 expres-

Table II. Numbers of 2C TCR⁺ T cells in lymphoid organs following thymic vs extrathymic development^a

	2C TCR H2 ^b	2C TCR/LckOM H2 ^b	2C TCR H2 ^{b/d}	2C TCR/LckOM H2 ^{b/d}
1B2 ⁺ cells	Thymus	LN	Thymus	LN
DN	8.6 ± 3.9	23.0 ± 2.6	6.6 ± 1.3	17.7 ± 6.1
DP	4.7 ± 3.2	31.4 ± 22.1	<0.1	<0.1
	Spleen	Spleen	Spleen	Spleen
DN	12.3 ± 5.7	6.6 ± 4.3	20.4 ± 9.2	15.7 ± 8.8
SP CD8	28.3 ± 10.3	35.3 ± 22.4	1.4 ± 1.1	8.0 ± 5.1

^a Absolute numbers of 2C TCR-bearing (1B2⁺) DN, DP, and SP CD8 T cells in the lymphoid organs of single-transgenic (2C TCR) and double-transgenic mice (2C TCR and OM). Three to four mice per group.

Table III. Numbers of Thy-1.2⁺ cells in single- and double-transgenic mice (three to four mice per group)

Mice	Organ	Cells (mean × 10 ⁶ ± SD)
LckOM	LN	194 ± 79.2
H-Y TCR female	Thymus	130 ± 39.4
H-Y TCR/LckOM female	LN	78.2 ± 15.7
H-Y TCR male	Thymus	13.2 ± 3.2
H-Y TCR/LckOM male	LN	94.9 ± 38.1
2C TCR H2 ^b	Thymus	28.3 ± 10.6
2C TCR/LckOM H2 ^b	LN	155.5 ± 80.9
2C TCR H2 ^{b/d}	Thymus	12.1 ± 2.6
2C TCR/LckOM H2 ^{b/d}	LN	46.3 ± 12.4

sion was strikingly increased on CD8 T cells, and 2) CD4 T cells showed decreased levels of CD62L and up-regulation of CD69 and of O-glycans recognized by 1B11 Ab (Fig. 6B).

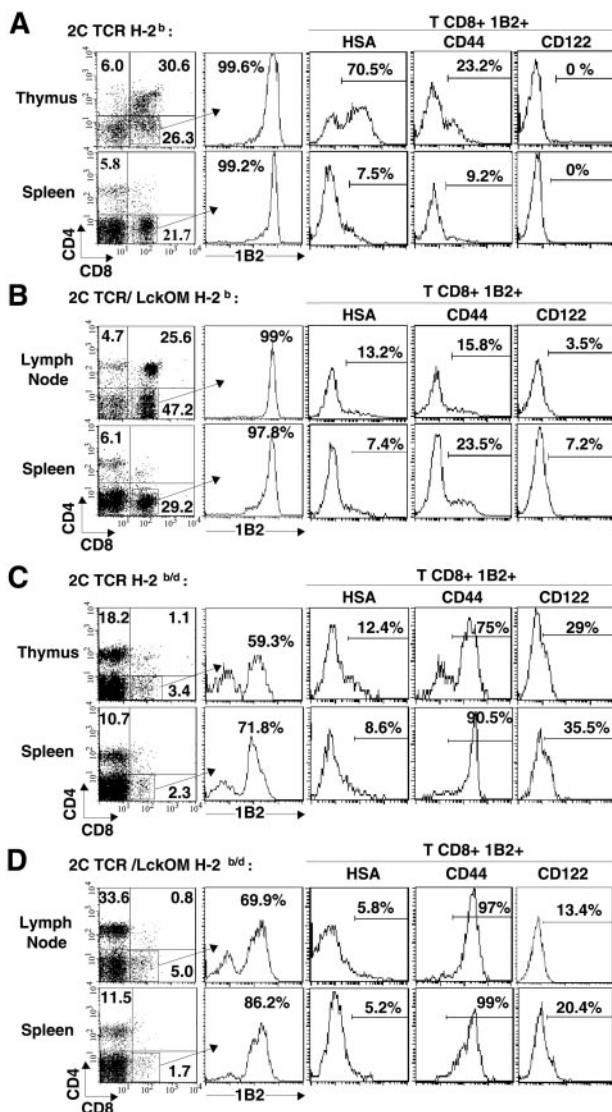


FIGURE 3. Positive and negative selection of 2C TCR-transgenic T lymphocytes (1B2⁺) in the thymus of 2C⁺ mice and in the LN of 2C⁺OM⁺ mice of H2^b or H2^{b/d} genotype. CD4/CD8 profiling depicted in the *left panels* was performed on lymphoid cells in the thymus and spleen, and on gated Thy-1.2⁺ cells in the LN. Histograms in the second column show the proportion of 1B2⁺ T cells among CD8 T cells. Expression of HSA, CD44, and CD122 was evaluated on CD4⁺CD8⁺T3.70⁺ T cells. The analysis is of one representative experiment of three.

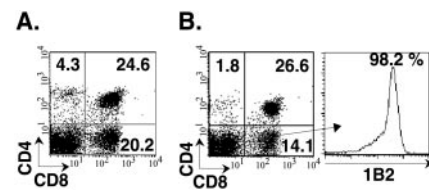


FIGURE 4. Development of OM⁺ 2C TCR⁺ T lymphocytes in the LN is thymus independent. *A*, LN of double-transgenic mice (2C TCR and OM). Acquisitions were gated on lymphoid cells. *B*, Irradiated adult-thymectomized B6.SJL (Ly-5.1) mice were reconstituted with fetal liver cells from double-transgenic (OM and 2C TCR) B6 (Ly-5.2) donors, and chimeras were analyzed on day 80 posttransplant. Acquisitions were gated on Ly-5.2⁺ lymphoid cells. Histogram on the *right* shows the proportion of 1B2⁺ T cells among CD8 SP T cells. These data are from one representative experiment of three.

All these results indicate that two inferences are possible regarding extrathymic T lymphocytes that develop in the OM⁺ LN. First, SP T cells steadily confronted with cells that display the MHC-peptide mixture that was instrumental in their positive selection have a phenotype similar to that of T lymphocytes undergoing homeostatic expansion (24, 51). Second, the intensity of TCR signals (CD5 levels) received during the DP→SP transition is normal (CD4) to high (CD8), even though CD3 levels (and those of TCRαβ, not shown) of SP T cells are relatively low. Decreased CD3 expression could represent a compensatory mechanism either to decrease the strength of the TCR signal at the time of selection or to dampen the ongoing stimulation of postselection SP T lymphocytes by self epitopes.

Discussion

The present study shows that notwithstanding the lack of both thymic epithelial cells and a thymus-like corticomedullary partition, the OM⁺ LN is able to support positive and negative selection of T lymphocytes. Interestingly, seminal experiments from Scollay's lab have demonstrated that: 1) when CD4[−]CD8[−] thymocytes harvested from the thymus of fetal or adult donors are injected i.v. into irradiated athymic recipients, some of these cells lodge in lymph nodes and develop into both DP and SP T lymphocytes; 2) this process does not occur in the spleen; 3) no T cell development is found in the LN when fetal liver cells rather than CD4[−]CD8[−] thymocytes are injected (52). Accordingly, what prevents the normal LN from sustaining T cell development must be its failure either to attract T cell progenitors or to support some early event at the CD4[−]CD8[−] stage. Otherwise, the LN would be able to support differentiation events downstream of this early step (productive seeding). How can OM transform the LN into

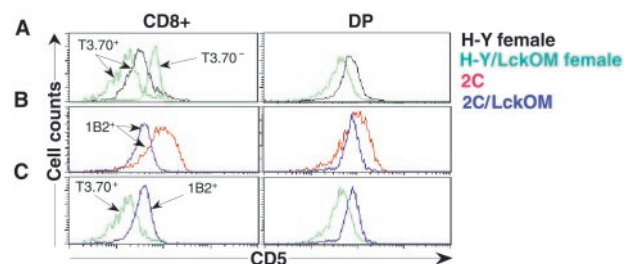
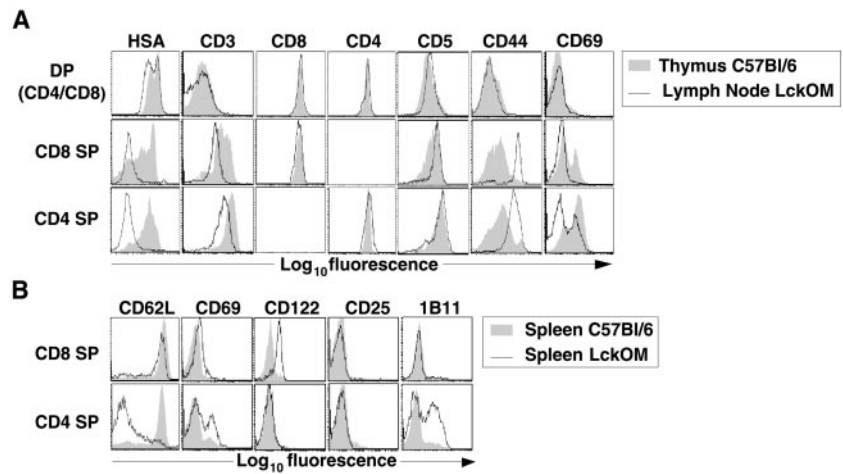


FIGURE 5. CD5 expression on thymic and extrathymic T lymphocytes. Overlay histograms depict the CD5-labeling intensity of DP and of CD8 SP T lymphocytes bearing the 2C TCR (1B2⁺) or the H-Y TCR (T3.70⁺). Cells were from the thymus of TCR single-transgenic mice or from the LN of double-transgenic (TCR and OM) H2^b mice. *A*, H-Y TCR⁺ vs H-Y TCR⁺OM⁺ female mice. *B*, 2C TCR⁺ vs 2C TCR⁺OM⁺ mice. *C*, 2C TCR⁺OM⁺ vs H-Y TCR⁺OM⁺ female mice.

FIGURE 6. Expression of differentiation/activation markers by T lymphocytes of thymic and extrathymic origins. *A*, DP, CD8 SP, and CD4 SP T cell populations from LckOM LN and C57BL/6 thymus were assayed for surface expression of HSA, CD3, CD8, CD4, CD5, CD44, and CD69. *B*, CD8 SP and CD4 SP T cell populations from the spleen of LckOM and C57BL/6 mice were assayed for surface expression of CD62L, CD69, CD122, CD25, and 1B11. Data, depicted as overlaid histograms, are representative of three experiments.



a primary T lymphoid organ that can positively select a diversified repertoire of T lymphocytes? This challenging question has yet to be answered. A plausible hypothesis at this point is that the crucial effect of OM is to induce productive seeding of the LN by immature T cell progenitors, the same early limiting event delineated by Antica and Scollay (52).

Positive selection

One salient finding emerging from our experiments in hemopoietic chimeras is that MHC I⁺ hemopoietic cells were sufficient to support positive selection of a polyclonal repertoire of extrathymic CD8 T cells (Fig. 1). It has been shown that hemopoietic cells can support intrathymic positive selection, although, with the exception of CD1d-dependent NKT cells, the importance of intrathymic positive selection by hemopoietic cells (as opposed to epithelial cells) is still a matter of debate (10, 11). Clearly, however, extrathymic hemopoietic cells do not normally support any significant level of T cell-positive selection (10, 11). That OM allows LN hemopoietic cells to induce positive selection of developing T lymphocytes is remarkable.

Positive selection did not proceed in the same fashion in the thymus and the OM⁺ LN, at least for CD8 SP T cells. Indeed, taking CD5 expression as a surrogate marker for the avidity of the positively selecting TCR-MHC-ligand interactions, we found a discrepancy between T cells bearing a transgenic TCR vs polyclonal T cells (Figs. 5 and 6). CD8 SP T lymphocytes bearing the 2C or the H-Y TCR expressed lower levels of CD5 when they developed in the OM⁺ LN than in the thymus (Fig. 5). In contrast, in the case of non-TCR-transgenic polyclonal CD8 SP T cells, the CD5 staining intensity was superior for those developing in the OM⁺ LN than in the thymus (Figs. 5 and 6). In general, there is no reason why the OM⁺ LN would select polyclonal T cells more efficiently than TCR-transgenic T cells. The most straightforward explanation is that expression of relevant MHC-peptide complexes by cells supporting positive selection dictates the efficacy of positive selection and that the MHC-peptide complexes that positively select the H-Y and 2C TCR are less abundant in the LN than in the thymus cortex. Yet, other MHC-peptide ligands expressed in the LN do generate TCR signals of optimal quality for proper positive selection of a diverse repertoire. Thus, the inference is that positive selection is supported by different sets of MHC-peptide complexes in the thymus vs the LN and that the two postselection repertoires are different, even though they may overlap. We also note that when T cells develop in a thymus in which MHC class I expression is limited to hemopoietic cells, 2C⁺, but not H-Y TCR⁺CD8 T

cells are positively selected (10, 11). This observation also supports the concept that epithelial cells and hemopoietic cells express different sets of MHC I-associated peptides and that the peptide mixture of hemopoietic cells better supports selection of 2C than H-Y TCR⁺ T cells.

Negative selection

Negative selection of extrathymic T lymphocytes bearing the H-Y or 2C TCR was very effective in the OM⁺ LN (Figs. 2 and 3). If anything, depletion of H-Y TCR⁺ DP T cells was more drastic in the OM⁺ LN than in the thymus (Fig. 2). This finding provides proof of principle for the concept that negative selection does not require a dedicated APC (53). Although thymus stromal cells have a unique ability to promote positive selection, their capacity to induce negative selection is shared by peripheral APCs. Put simply, negative selection occurs in the thymus because immature T lymphocytes are produced there. It could possibly occur in any organ, although it may require a certain abundance of hemopoietic APCs. We note that following negative selection, the fate of CD8 T cells bearing the 2C and the H-Y TCR was different. Irrespective of their thymic or extrathymic origin, the few H-Y TCR⁺ T cells that escaped negative selection (in male mice) expanded considerably in the periphery, whereas 2C TCR⁺ T cells did not (in H2^{b/d} mice) (Tables I and II). This demonstrates how extensively peripheral homeostatic mechanisms can remold the postselection repertoire by inducing a major expansion of T cells specific for some, but not all self epitopes.

The activated/memory phenotype of extrathymic T cells

One salient characteristic of SP extrathymic T lymphocytes that developed under the influence of OM is their phenotype, which is typical of Ag-experienced or memory T lymphocytes (Fig. 6). This idea fits well with our previous reports using *in vivo* 5-bromodeoxyuridine pulse-chase experiments in (non-TCR-transgenic) LckOM mice: their extrathymic T cells proliferate very rapidly and have a high turnover rate (31), as would be expected for T cells with a memory phenotype (54). However, 5-bromodeoxyuridine-labeling experiments have not been performed in TCR-transgenic OM⁺ mice. Therefore, we could not formally evaluate the peripheral survival and expansion of T cells bearing the H-Y and 2C TCR in OM⁺ mice. Interestingly, while the vast majority of our extrathymic T cells displayed a memory phenotype (Figs. 2 and 6), we observed one noticeable exception: most extrathymic CD8 T cells bearing the 2C TCR had a naive phenotype (Fig. 3). This means that acquisition of the memory phenotype was not a direct

consequence of exposure to OM, but was dependent on the TCR clonotype. Collectively, these data suggest that, with a few possible exceptions (e.g., the 2C TCR), extrathymic T cells in the OM⁺ LN have a memory phenotype and a high turnover rate because they undergo homeostatic expansion induced by chronic exposure to the LN MHC/peptide mixture that entailed their positive selection. That being the case, we recently observed that SP T cells derived from the OM⁺ LN behave functionally like memory T cells following in vitro and in vivo Ag priming (M. E. Blais, G. Gérard, and C. Perreault, manuscript in preparation). Nevertheless, that most 2C⁺ T cells in OM⁺ mice did not display an activated phenotype was surprising because 2C T cells can undergo homeostatic peripheral expansion (24). One explanation would be that the OM⁺ LN produces enough 2C⁺ T cells to fill peripheral niches without the need for peripheral expansion.

Aside from their memory phenotype, it is remarkable that the vast majority of extrathymic SP LN T lymphocytes were HSA^{low} (Fig. 6). To some extent, this is because the OM⁺ LN contains recirculating T cells in addition to T cells generated in situ (31). Interestingly, in the thymus of wild-type mice, the CD44^{high} HSA^{low} phenotype is shared by the single subset of thymocyte positively selected on hemopoietic cells: α -GalCer-CD1d-reactive T cells (55). These data lead us to propose that the CD44^{high} HSA^{low} phenotype is characteristic of T lymphocytes positively selected on hemopoietic cells. We envision at least two nonmutually exclusive explanations for the low proportion of HSA⁺ SP T cells in the OM⁺ LN. Expression of HSA, which was high on DP T lymphocytes, might be down-modulated more rapidly following positive selection on hemopoietic cells. Alternatively, HSA⁺ T cells may simply be diluted by rapidly proliferating HSA⁻ T lymphocytes because the latter cells undergo vigorous postselection proliferation. A parallel with studies of TCR excision circles (TREC) illustrates the latter mechanism. T cell division was indeed identified as the most important factor for decreasing TREC content (56). Thus, in the presence of normal thymic output, chronic Ag stimulation leads to low TREC levels (57, 58).

Another important feature of extrathymic SP LN T lymphocytes was their low levels of TCR/CD3. Strong TCR ligation by peptide/MHC complexes leads to TCR down-regulation whose probable role is to protect T cells from overstimulation (59, 60). TCR down-modulation, which is mediated by the intracellular retention and degradation of ligated complexes (61), is an effective means of tolerance to extrathymic Ags. Thus, in transgenic mice expressing low levels of the H2K^b Ag exclusively on hepatocytes, tolerance of Ag-reactive CD8 T cells was induced by TCR down-modulation (62). Interestingly, extrathymic T cells (whose site of development is unclear) found in nude mice or in irradiated adult-thymectomized hosts reconstituted with hemopoietic progenitors are indeed TCR^{low/int} (63). We therefore propose that TCR/CD3 down-modulation found in SP T cells developing in the OM⁺ LN is a general characteristic of extrathymic T cells. This TCR/CD3 down-modulation is required to prevent overstimulation of T lymphocytes that live in the microenvironment in which their positive selection took place.

Thymic and extrathymic T cell development pathways follow different rules. Extrathymic T cells that develop along the OM-dependent LN pathway are different from classic T cells in terms of repertoire selection, turnover kinetics, and expression of activation/maturation markers. These findings beg the question: what is the functionality of these extrathymic T cells? Athymic mice reconstituted with OM⁺ hemopoietic stem cells can reject allogeneic cancer cells (32) and do not show increased incidence of infections (at least when housed in a specific pathogen-free environment). In addition, we recently obtained evidence that they can

generate protective antiviral responses (M. E. Blais, G. Gérard, C. Perreault, manuscript in preparation). Hence, OM-induced extrathymic T cells appear to be quite functional. The thymus constitutes the primary T lymphoid organ in all jawed vertebrates, and in evolutionary terms is much more ancient than the LN. The thymus appeared in Chondrichthyes, the first vertebrates known to elicit adaptive immune responses, while LNs occur strictly in endothermic vertebrates and reach their full development only in eutherian mammals (64). The fact that extrathymic T cells generated in the LN under the influence of OM can replace the mainstream thymic T lymphocytes without any obvious disadvantage means that several features unique to the thymus are not essential to generate a functional T cell repertoire (e.g., thymic epithelial cells, corticomedullary segregation, blood-thymus barrier preventing the reentry of mature T lymphocytes). In other words, a primary T lymphoid organ does not have to share all the features of the thymus. This concept will lead us to further studies of the OM-dependent pathway and its potential medical relevance for the treatment of congenital and acquired immune deficiencies.

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