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*J Immunol* 2008; 180:2299-2312; doi: 10.4049/jimmunol.180.4.2299

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Why T Cells of Thymic Versus Extrathymic Origin Are Functionally Different1

Marie-Ève Blais,*† Sylvie Brochu,*† Martin Giroux,*† Marie-Pier Bélanger,*† Gaël Dulude,‡ Rafick-Pierre Sékaly,§ and Claude Perreault2*†§

Age-related thymic involution severely impairs immune responsiveness. Strategies to generate T cells extrathymically are therefore being explored with intense interest. We have demonstrated that T cells produced extrathymically were functionally deficient relative to thymus-derived T cells. The main limitation of extrathymic T cells is their undue susceptibility to apoptosis; they thus do not expand properly when confronted with pathogens. Using oncostatin M-transgenic mice, we found that in the absence of lymphopenia, T cells of extrathymic origin constitutively undergo excessive homeostatic proliferation that leads to overproduction of IL-2 and IFN-γ. IFN-γ up-regulates Fas and FasL on extrathymic CD8 T cells, thereby leading to their demise by Fas-mediated apoptosis. Moreover, IFN-γ and probably IL-2 curtail survival of extrathymic CD4 T cells by down-regulating IL-7R and Bcl-2, and they support a dramatic accumulation of FoxP3+ regulatory T cells. Additionally, we show that wild-type thymus-derived T cells undergoing homeostatic proliferation in a lymphopenic host shared key features of extrathymic T cells. Our work explains how excessive lymphopenia-independent homeostatic proliferation renders extrathymic T cells functionally defective. Based on previous work and data presented herein, we propose that extrathymic T cells undergo constitutive homeostatic proliferation because they are positively selected by lymph node hemopoietic cells rather than by thymic epithelial cells. The Journal of Immunology, 2008, 180: 2299–2312.

We have known for more than 40 years that the primary function of the thymus is to produce T lymphocytes (1). However, despite enormous progress in our understanding of molecular and cellular processes involved in T cell development, the ultimate role of the thymus stroma still eludes us (2). By ultimate role, we mean the ecologically relevant and evolutionarily selected function of the thymus in host defense (3). Indeed, although commonly taken for granted, the conservation of the thymus as the primary T lymphoid organ in all animals with an adaptive immune system is remarkable. In contrast, about 10 different organs have served as primary sites of hematopoiesis (including “B cell poiesis”) in jawed vertebrates (4, 5). At face value, conservation of the thymus as the primary lymphoid organ suggests that intrathymic development endows T cells with elusive evolutionarily selected functional attributes (6). The reason why the thymus unquestionably provides a unique environment for T cell precursor differentiation remains largely unknown. This is problematic because progressive thymus atrophy ultimately affects all ageing subjects and can even impinge on younger subjects affected by several serious illnesses (7–10). Therefore, strategies to create thymus substitutes are being pursued with intense interest (11–14).

Unexpectedly, studies from Zúñiga-Pflücker’s group have shown that a monolayer of stromal cells can support all steps of T cell development (13, 15). Thus, the three-dimensional thymic microenvironment and the presence of thymic epithelial cells are not essential for T cell development. In line with this, a cryptic extrathymic T cell development pathway has been described in lymph nodes (LN) of wild-type (WT) mice. Indeed, studies by Guy-Grand et al. using transgenic mice bearing a GFP gene placed under the control of the RAG2 promoter showed that LNs (and to a much lesser extent Peyer’s patches) were the sole sites of extrathymic T lymphopoiesis in mice (16). In euthymic mice, this pathway is inhibited by the progeny of the classic thymic pathway. However, the extrathymic LN pathway is amplified in athymic mice and in mice with severe T cell depletion (16, 17). Nevertheless, in nontransgenic athymic mice, extrathymic T cell development is extremely limited (16). Therefore, because athymic mice present severe T cell lymphopenia, it has been difficult to compare the protective value of thymically and extrathymically produced T cells. That caveat was resolved by using oncostatin M (OM)-transgenic mice. Transgenic expression or injection of OM induces total thymic atrophy that is compensated by a massive production of T cells in LNs that ectopically recapitulates all the development stages normally found in the thymus (18, 19). OM has two crucial effects on LNs. OM induces a COX-2-dependent angiogenesis of high endothelial venules that entails a massive accumulation of common lymphoid progenitors in LNs (20). Furthermore, OM increases the clonogenic potential of LN-resident common lymphoid progenitors (3, 16) necessary for the development of OM-expressing thymus substitutes.

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Received for publication October 22, 2007. Accepted for publication December 11, 2007.

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1 This work was supported by the Canadian Institutes of Health Research (Grant MOP 42384). M.E.B. and M.G. are supported by training grants from the National Cancer Institute of Canada and the Cole Foundation, respectively. C.P. and R.P.S. hold Canada Research Chairs in Immunobiology and in Human Immunology, respectively.

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3 Abbreviations used in this paper: LN, lymph node; FasL, Fas ligand; GMFI, geometric MFI; HP, homeostatic proliferation; LCMV, lymphocytic choriomeningitis virus; OM, oncostatin M; qPCR, quantitative real-time PCR; TREC, TCR excision circle; Treg cell, regulatory T cell; WT, wild type.

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progenitors (21). Therefore, induction of extrathymic T cell development by OM is not a transgenic idiosyncrasy. OM simply amplifies a cryptic T cell development pathway that is operative in LNs of nontransgenic mice. Because OM-transgenic mice have no quantitative CD4 or CD8 T cell deficit (19), they provide a unique model to evaluate the function of T cells generated extrathymically. We emphasize that for the sake of brevity, we hereafter refer to T cells generated in the thymus as thymus-derived T cells and to T cells generated extrathymically as extrathymic T cells.

T cell development in OM-transgenic mice is truly thymus independent, as shown by reconstitution of athymic nude mice by OM-transgenic hemopoietic stem cells (18). In contrast, T cell development in OM-transgenic mice is LN dependent. This was demonstrated by breeding OM-transgenic mice with aly/aly mice, which have no LNs but have a normal spleen (22). When tested at 12 wk of age, spleens of OM-transgenic aly/aly mice were essentially devoid of T cells (Fig. 1A). Extrathymic T cells generated in the LNs of OM-transgenic mice display a polyclonal Vβ repertoire (19). We have previously demonstrated that even though OM-transgenic mice have no CD4 or CD8 T cell lymphopenia, their extrathymic T cells are functionally deficient and cannot substitute for thymus-derived T cells (6, 19, 23). Extrathymic CD4 T cells fail to expand normally following Ag stimulation and do not provide adequate B cell help following infection with lymphocytic choriomeningitis virus (LCMV) or vesicular stomatitis virus (23).

The function of extrathymic CD8 T cells is somewhat better than that of their CD4 counterparts. Following Ag presentation, extrathymic CD8 T cells swiftly initiate proliferation and IFN-γ production, but they undergo premature contraction and hence accumulate to lower levels than thymus-derived T cells (23). Accordingly, extrathymic CD8 T cells provide only transient control of LCMV infection (23). Overall, the Achilles’ heel of extrathymic T cells resides in their rapid and massive apoptosis following antigenic stimulation (6). The primary objective of this work was therefore to discover why extrathymic T cells are overly susceptible to apoptosis and are consequently less fit than classic thymus-derived T cells.

Notably, OM-induced extrathymic CD4 and CD8 T cells behave like “innate” T cells (24): they have a memory-like phenotype (CD4+CD44hiCD62Llo and CD8+CD44hiCD122hi) (19, 25), rapidly secrete high amounts of IFN-γ upon Ag recognition, exhibit an accelerated turnover rate in BrdU pulse-chase assays (19), and display exceedingly low levels of TCR excision circles (Fig. 1, A and C). Thus, although OM-transgenic mice are not T cell lymphopenic (19), their extrathymic LN-derived T cells undergo vigorous “homeostatic” proliferation (HP) under steady-state conditions (in the absence of infection). Our second objective was therefore to determine whether the functional behavior of extrathymic T cells was due to their excessive HP. To this end, we compared features of extrathymic T cells from OM-transgenic
mice to those of thymus-derived T cells undergoing HP in lymphoprogenic mice. Our work explains why extrathymic T cells cannot substitute for thymus-derived T cells and provides a rationale for conservation of the thymus as the sole primary T-lymphoid organ.

Materials and Methods

Mice

C57BL/6d (referred to as CD45.2 WT mice), B6.129P2-Ifngtm1Mom/J (TCRβ-KO mice), B6. SJL–PtprcPep3β/Boy1 (B6.SJL; referred to as CD45.1 WT mice), C3H/HCl (H2b+), B6.129S7-Iftgαtm1aMi/J (referred to as IFN-γR KO), and B6.129S7-Iftgαtm1aMi/J (Iftgα−/−) mice were initially obtained from The Jackson Laboratory. We purchased Pdyl/ly from CRL Japan, splenic cells, and CD1-Foxp3+ mice were purchased from the Charles River Laboratories. OM-transgenic mice on a C57BL/6J background have been previously described (19). In OM-transgenic mice, expression of bovine IFN-γ was driven by the proximal promoter that is active primarily in immature thymocytes. Iftgα−/− OM-transgenic mice were generated by breeding Iftgα−/− females with OM-transgenic males. All mice were bred at Institute for Research in Immunology and Cancer and maintained under specific pathogen-free conditions according to the standards of the Canadian Council on Animal Care. Experimental protocols were approved by the Comité de Déontologie Animale of the University of Montréal. Mice between 8 and 15 wk of age were used.

Antibodies

All Abs were purchased from BD Biosciences except anti-IL-7Rα (eBioscience). Cells were stained as previously described (23). Bc1-2 and cytokine intracellular staining was achieved by using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions. Before intracellular cytokine staining, cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h in the presence of 2.5 μg/ml brefeldin A. This step is necessary to allow cytokine accumulation in the Golgi but insufficient to induce de novo cytokine production in naive cells. Intracellular FoxP3 staining was performed with a regulatory T cell staining kit from eBioscience.

Quantification of TCR excision circles (TRECs)

TRECs from C57BL/6d and OM-transgenic mice were quantified as previously described (26).

Homeostatic proliferation

Homeostatic proliferation in lymphoprogenic mice was induced according to classic methods (27). CD4 CD44low and CD8 CD44low spleen T cells from WT mice were enriched using an EasySep T cell enrichment kit (Invitro-Gen: Molecular Probes), followed by purification by flow cytometry. A total of 106 cells (5 × 104 CD4 T cells and 5 × 104 CD8 T cells) were transferred into nonirradiated TCRβ-KO mice and analyzed by flow cytometry 14 or 30 days later. For competitive assays, 5 × 105 CD8 T cells from the spleen of recipient mice 4.5 days later and were analyzed using the same Abs and flow cytometry settings. In control groups, B6.SJL mice were infected with OM-transgenic or C57BL/6d hosts.

Quantitative real-time PCR (qPCR): cDNA preparation

Thymus-derived and extrathymic CD4 and CD8 spleen T cells were purified by flow cytometry according to CD44 expression. Total RNA of purified cells was extracted and reverse-transcribed in a final volume of 100 μl using the High-Capacity cDNA Archive Kit with random primers (Applied Biosystems) as suggested by the manufacturer. Gene expression level was determined using primer and probe sets from Applied Biosystems (ABI Assays on Demand, http://www.appliedbiosystems.com). PCR for 384-well plate formats were performed using 2 μl of cDNA samples (20–50 ng), 5 μl of the TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 μl of the TaqMan Gene Expression Assays (20×, Applied Biosystems), and 2.5 μl of water in a total volume of 10 μl. The mouse GAPDH and/or HPRT (hypoxanthine phosphoribosyltransferase) predeveloped TaqMan assay were used as endogenous controls.

Detection and analysis

The ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) was used to detect the amplification level and was programmed to an initial step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All reactions were run in triplicate and the average values were used for quantification. The mouse GAPDH, ACTB (β-actin), or 18S ribosomal RNA were used as endogenous controls. The relative quantification of target genes was determined by using the △△Ct method. Briefly, the Ct (threshold cycle) values of target genes were normalized to an endogenous control gene (GAPDH) (△Ct = CtTarget − CtGAPDH) and compared with a calibrator (human reference RNA) (△DeltaCt = △CtSample − △CtCalibrator). Relative expression (RQ) was calculated using the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems) and the formula RQ = 2−△△Ct.

Expression of IL-7Rα and Bcl-2 in adoptively transferred cells

Extrathymic CD4 T cells (CD45.2+) from spleen of OM-transgenic mice were enriched using EasySep T cell enrichment kit, then isolated by FACS sorting. Cells were stained with the following Abs: anti-IL-7Rα, anti-Bcl-2, anti-CD4, and anti-CD45.2. Expression of IL-7Rα and Bcl-2 in donor cells was assessed before injection of 2 × 106 cells into nonirradiated B6.SJL mice (CD45.1+). Donor-derived cells were harvested from the spleen of recipient mice 4.5 days later and were analyzed using the same Abs and flow cytometry settings. In control groups, B6.SJL cells were injected into OM-transgenic or C57BL/6d hosts.

Detection of soluble cytokines

IFN-γ concentrations were assessed with the In Vivo Cytokine Capture Assay according to the manufacturer’s instructions (eBioscience). Briefly, mice were injected with a biotin-labeled Ab specific to the targeted cytokine. Mice were sacrificed 24 h later and the cytokine/antibody complex was captured from sera on ELISA plate microwells coated with Ab specific to a different epitope of the same cytokine. The complex was detected by streptavidin HRP followed by the addition of a chromogenic solution. IL-2 was detected in spleens of naive mice using BD Cytometric Bead Arrays (BD Biosciences).

In vitro suppression assay

To test for suppression of allogeneic MLR by individual T cell subsets, spleen cells from WT or OM-transgenic mice were sorted according to their CD4 and CD25 expression. Graded numbers of sorted test T cells were combined with 5 × 105 B6.SJL CFSE-labeled effector (E) splenocytes (2.5 μM) and 5 × 105 allogeneic irradiated (2800 rad) stimulator (S) C3H/HeJ splenocytes. Cells were incubated at 37°C with 5% CO2 for 4 days and analyzed by flow cytometry using FCS (De Novo Software) and ModFit softwares (Verity Software House).

Statistics

Differences between groups were tested using Student’s t test (*, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001). Error bars represent SDs.

Results

Apopototic profile of extrathymic T cells

All studies reported in this article were performed on spleen T cells. Unless stated otherwise, extrathymic T cells were harvested from the spleen of OM-transgenic mice, and thymus-derived T cells were isolated from the spleen of WT C57BL/6d mice. In initial experiments, we used annexin V labeling to quantify the apoptosis rate among thymus-derived and extrathymic T cells of adult uninfected mice. The proportion of apoptotic cells was significantly increased in both CD4 (7-fold) and CD8 (4-fold) extrathymic T cells compared with thymus-derived T cells. Approximately 17% of CD4 and 8% of CD8 extrathymic T cells were annexin V+ (Fig. 1D). Practically all extrathymic T cells in OM-transgenic mice are CD44high (Fig. 2A). In contrast, only a small proportion of thymus-derived CD4 (~15%) and CD8 (~20%) T cells express high CD44 levels. We observed that with extrathymic (CD44high) T cells, and in accordance with previous studies (28), thymic CD44high T cells displayed much higher apoptosis rates than did CD44low T cells (Fig. 2B). Because thymus-derived CD44low and
CD4\textsuperscript{high} T cell subsets have different apoptosis rates, they were analyzed separately in additional experiments.

Factors affecting T cell viability vary depending on cell type and status (29). Survival of naïve T cells is regulated by a Bcl-2/Bim-dependent route, and Bcl-2 levels are primarily regulated by IL-7 signals. Survival of activated T cells is regulated not only by members of the Bcl-2 family, but also by death receptors, in particular Fas. To uncover the molecular mechanisms involved in apoptosis of extrathymic T cells, we first evaluated expression of IL-7R\textsubscript{α} and Bcl-2 transcripts in thymus-derived T cells and extrathymic T cells (symbolized by the letter E). CD4 and CD8 T cells harvested from the spleen of WT and OM-transgenic mice were purified by flow cytometry and frozen in TRIzol (Invitrogen Life Technologies) before proceeding to mRNA extraction and cDNA amplification. Increased apoptosis of T cells undergoing HP. Fold change values for proportion of annexin V\textsuperscript{+} cells and expression of IL-7R\textsubscript{α}, Bcl-2, and Fas in extrathymic T cells and T cells undergoing HP. Results are depicted as ratios relative to CD44\textsuperscript{low} thymus-derived T cells (red line) on a log\textsubscript{10} scale. F, Increased expression of FasL mRNA in extrathymic T cells. Fold change values for FasL transcripts relative to CD44\textsuperscript{low} WT T cells. A uniform color code is used in A-F for thymus-derived CD44\textsuperscript{high} (orange) and CD44\textsuperscript{low} (red) T cells, extrathymic (blue) T cells, and thymus-derived T cells in HP on day 14 (black) or 30 (gray) posttransfer. Three to five mice per group. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
that expression of these three key molecules for T cell survival is similar in CD4\textsuperscript{high} T cells irrespective of their thymic or extrathymic origin, and such expression differs from that of thymus-derived CD4\textsuperscript{low} T cells. Additionally, our data indicate that expression of IL-7R\textalpha, Bcl-2, and Fas is differentially regulated in CD4 and CD8 T cells. They also suggest that down-regulation of IL-7R\textalpha/Bcl-2 and up-regulation of Fas are instrumental in apoptosis of extrathymic CD4 and CD8 T cells, respectively. Notably, key features of OM-transgenic T cells were also found in nontransgenic extrathymic T cells. Indeed, CD4 and CD8 T cells from nu/nu mice showed an increased frequency of apoptotic cells that correlated with low IL-7R\textalpha and Bcl-2 expression in CD4 T cells and increased Fas expression in CD8 T cells (Fig. 3).

Thymus-derived CD4\textsuperscript{high} T cells include two types of cells: genuine memory cells that have encountered foreign Ag, and memory-like cells engendered by HP (30). As mentioned in the Introduction, OM-transgenic extrathymic T cells undergo excessive HP in the absence of lymphopenia. We therefore sought to compare the apoptotic profile of extrathymic T cells with that of thymus-derived T cells undergoing HP. HP of thymus-derived T cells was induced in all experiments reported herein by injection of 10\textsuperscript{6} CD4\textsuperscript{low} thymus-derived spleen T cells from WT mice in nonirradiated TCR\beta-KO mice. Recipient mice were analyzed during early and late stages of HP, that is, 14 and 30 days posttransfer. At both time points, CD4 and CD8 thymus-derived T cells undergoing HP showed high apoptosis rates (annexin V\textsuperscript{+} cells), similar to those of extrathymic T cells (Fig. 2E). On day 14, and to a lesser extent on day 30 of HP, IL-7R\textalpha and Bcl-2 were down-regulated and Fas was up-regulated on CD4 T cells in HP, a pattern similar to that of extrathymic and thymus-derived CD4\textsuperscript{high}CD4\textsuperscript{low} T cells (Fig. 2E, left). Expression profiling of CD8 T cells yielded a more complicated picture, as it disclosed both similarities and discrepancies between extrathymic T cells and thymus-derived T cells in HP (Fig. 2E, right). Thus, similar to extrathymic CD8 T cells, thymus-derived CD8 T cells in HP up-regulated IL-7R\textalpha on day 30 and showed a modest up-regulation of Fas on day 14. However, while Bcl-2 expression was increased in extrathymic CD8 T cells, thymus-derived T cells in HP down-regulated Bcl-2 on days 14 and 30. Because binding of Fas to its ligand induces apoptosis of T cells in HP (31), we next quantified expression of Fas ligand (FasL) transcripts in CD4 and CD8 T cell subsets. Extrathymic CD8 T cells presented a distinctive and major up-regulation of FasL transcripts that was not found in thymus-derived CD4\textsuperscript{high} T cells or in thymus-derived T cells in HP (Fig. 2F). Thus, thymus-derived T cells in HP shared most but not all features of extrathymic T cells.

**Down-regulation of IL-7R\textalpha and Bcl-2 in extrathymic CD4 T cells is not cell-autonomous**

To determine whether down-regulation of IL-7R\textalpha expression impinged on apoptosis of extrathymic CD4 T cells, we determined the proportion of annexin V\textsuperscript{+} cells among IL-7R\textalpha\textsuperscript{low} and IL-7R\textalpha\textsuperscript{high} extrathymic CD4 T cells. The CD4 population with a high apoptosis rate resided in the IL-7R\textalpha\textsuperscript{low} fraction (19.1%) and not in the IL-7R\textalpha\textsuperscript{high} subset (3.5%), suggesting that down-regulation of IL-7R\textalpha is instrumental in apoptosis of extrathymic CD4 T cells (Fig. 4A). Several environmental factors such as γδ T cells, glucocorticoids, and TCR triggering can decrease IL-7R\textalpha expression (32, 33). To assess whether cell-extrinsic factors were responsible for down-regulation of IL-7R\textalpha on extrathymic CD4 T cells, we transferred extrathymic CD4 T cells from OM-transgenic mice (CD45.2\textsuperscript{+}) into nonirradiated WT (CD45.1\textsuperscript{+}) mice and evaluated IL-7R\textalpha expression on transferred cells. We first verified in a negative control group that IL-7R\textalpha expression was essentially unchanged on WT CD45.1\textsuperscript{+} cells transferred into WT CD45.2\textsuperscript{+} recipients (Fig. 4B, top). In contrast, expression of IL-7R\textalpha was increased by 105% on extrathymic CD4 T cells transferred into WT mice (Fig. 4B, middle). In keeping with this observation, IL-7R\textalpha expression on extrathymic T cells increased 3.8-fold following overnight incubation in serum-free medium (data not shown).
Moreover, when thymus-derived T cells (from WT mice) were transferred into OM-transgenic mice, expression of IL-7Rα decreased by 64% (Fig. 4B, bottom). Of significance, we found a close correlation between expression of IL-7Rα and Bcl-2 in adoptively transferred T cells (Fig. 4C). Furthermore, decreased expression of IL-7Rα and Bcl-2 in WT T cells transferred into OM-transgenic mice correlated with low cell recovery relative to OM+ T cells transferred into WT hosts (Fig. 4D). This finding shows that apoptosis of extrathythic CD4 T cell is induced by the OM-transgenic environment. Collectively, these results lead us to conclude that down-regulation of IL-7Rα on extrathythic CD4 T cells is a reversible non-cell-autonomous feature that reduces levels of Bcl-2 and entails a high apoptosis rate.

**IL-2 and IFN-γ production and accumulation in OM-transgenic mice**

The above results suggest that apoptosis of extrathythic CD4 T cells is due to repression of IL-7Rα by cell-extrinsic factors, and they point to the Fas pathway as being instrumental in apoptosis of extrathythic CD8 T cells. IL-2 and IFN-γ are known to decrease IL-7Rα expression and to up-regulate Fas and FasL (32, 34–37). We therefore evaluated expression of these cytokines in thymus-derived and extrathythic T cells by intracellular cytokine staining. After a brief in vitro culture in presence of PMA, ionomycin, and brefeldin A, we detected production of IFN-γ in >80% of extrathythic CD8 T cells but in only 14% of thymus-derived CD44low CD8 T cells (Fig. 5, A and B). A more modest, but highly significant overproduction of IFN-γ was also found in extrathythic CD4 T cells. Additionally, the proportion of IL-2-producing cells was 38.8% for extrathythic T CD4 cells vs 3.4% for CD44low thymus-derived T cells (Fig. 5, A and B). Moreover, we found that, as with extrathythic T cells, thymus-derived CD44high T cells and thymus-derived T cells in HP secreted increased amounts of IL-2 and IFN-γ (Fig. 5, A and B). Thus, among thymus-derived T cells in HP (on day 14 after injection in TCRβ-KO mice), 45% of CD8 T cells produced IFN-γ and 31% of CD4 T cells produced IL-2 (Fig. 5B).

We next asked whether overproduction of IL-2 and IFN-γ by extrathythic T cells would lead to extracellular accumulation of these cytokines in OM-transgenic mice. Concentrations of IL-2 in OM-transgenic spleens were twice those in WT spleens, whereas serum IFN-γ levels were increased 4-fold in OM-transgenic mice relative to WT mice (Fig. 5C). Binding of IFN-γ to its receptor induces repression of Ifngr2 transcription in activated T cells (38). We therefore quantified levels of Ifngr2 transcripts to determine whether IFN-γ-specific signaling was induced in extrathythic T cells in vivo. We found that, indeed, the quantity of Ifngr2 transcripts was considerably decreased in CD4 and CD8 extrathythic T cells relative to thymus-derived CD44low T cells (Fig. 5D). Again, thymus-derived CD44high and thymus-derived T cells in HP were similar to extrathythic T cells except for CD8 T cells in HP, in which amounts of Ifngr2 transcripts were not significantly decreased (Fig. 5D).

**Increased survival of extrathythic CD8 T cells in absence of IFN-γ**

IFN-γ can modulate T cell survival by up-regulating Fas and FasL and/or by down-regulating IL-7Rα. To further investigate the contribution of IFN-γ on apoptosis of extrathythic T cells, we generated IFN-γ-deficient (Ifng−/−) OM-transgenic mice. Ifng−/− OM-transgenic mice and standard OM-transgenic mice share key phenotypic features: they display a severe thymic atrophy, support
a massive extrathymic T cell development in their LNs, and show similar cellularities in lymphoid organs (data not shown). To assess the survival of $\text{Ifng}^{-/-}$ extrathymic T cells, we then compared expression of IL-7R, Bcl-2, and Fas in standard OM-transgenic mice vs $\text{Ifng}^{-/-}$ extrathymic CD4 T cells expressed more IL-7R and Bcl-2 and less Fas than did $\text{Ifng}^{+/-}$ extrathymic T cells (Fig. 6A, left). However, these differences did not translate into a reduced apoptosis rate for CD4 T cells (Fig. 6B). IFN-γ deficiency did not affect expression of IL-7Rα and Bcl-2 and down-regulated Fas expression. This is consistent with the increased cell recovery following transfer of OM− CD4 T cells in WT recipients (Fig. 4D).

**Lack of IFN-γ receptor improves survival of thymus-derived CD8 T cells in HP**

To test whether survival of T cells undergoing HP was also affected by IFN-γ, we assessed survival of IFN-γ-responsive and IFN-γ-unresponsive T cells in a competitive assay. We induced HP by cotransferring equal numbers ($5 \times 10^5$) of IFN-γ receptor-deficient T cells (IFN-γR-KO) and WT T cells (IFN-γR+) into TCRβ-KO mice (Fig. 7A). Lack of IFN-γR in CD4 cells had no significant impact on expression of IL-7Rα, Bcl-2, and Fas (Fig. 7B, left) or on the apoptosis rate (Fig. 7C). Hence, the relatively modest effects of IFN-γ on extrathymic CD4 T cells (Fig. 6A, left) proved survival. In CD4 extrathymic T cells, IFN-γ deficiency did not significantly improve survival, but it nevertheless up-regulated IL-7Rα and Bcl-2 and down-regulated Fas expression. This is consistent with the increased cell recovery following transfer of OM− CD4 T cells in WT recipients (Fig. 4D).

**FIGURE 5.** Overproduction of IL-2 and IFN-γ by extrathymic T cells. A and B, Intracellular staining for IL-2 and IFN-γ. In A, data are shown from one representative experiment (numbers represent percentage of positive cells), and in B, the means ± SD for three to six mice per group are shown. C, Accumulation of IFN-γ and IL-2 in serum and spleen of OM-transgenic mice, respectively. D, Decreased expression of $\text{Ifngr2}$ mRNA in extrathymic T cells. $\text{Ifngr2}$ mRNA expression in purified CD4 and CD8 T cell subsets. Three to six mice per group. A uniform color code is used in A, B, and D for thymus-derived CD44hi (orange) and CD44lo (red) T cells, extrathymic (E; blue) T cells, and thymus-derived T cells in HP on day 14 (black) or 30 (gray) posttransfer. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. 

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did not extend to thymus-derived CD4 T cells in HP (Fig. 7B, left). In contrast, IFN-γR-KO CD8 T cells undergoing HP expressed more IL-7Ra and Bcl-2 and less Fas than did their IFN-γR+ counterparts (Fig. 7B, right). Moreover, the proportion of apoptotic CD8 T cells was reduced by 50% in IFN-γR-KO compared with IFN-γR+ cells (Fig. 7C). Increased survival of IFN-γR-KO CD8 T cells led to preferential accumulation of this subset: 77% of cells recovered were IFN-γR-KO as opposed to only 22% for WT T cells (Fig. 7D). In contrast, proportions of IFN-γR-KO and WT CD4 T cells were almost identical, showing that this subset is less affected by IFN-γ than are CD8 T cells. Thus, for CD8 T cells, IFN-γ has a significant effect on survival of both extrathymic T cells (Fig. 6B) and thymus-derived T cells in HP (Fig. 7C).

Regulatory T cell enrichment in extrathymic T cells and thymus-derived T cells in HP

A cardinal feature of extrathymic T cells and thymus-derived T cells in HP is that they produce high amounts of IL-2 and IFN-γ (Fig. 5). Along with their capacity to regulate expression of prosurvival and proapoptotic molecules, IL-2 and IFN-γ can modulate the generation and function of regulatory T (Treg) cells. Because Treg cells are enriched in the CD25+ subset of CD4 T cells, we examined the frequency of CD4+CD25+ T cells in extrathymic T cells and thymus-derived T cells undergoing HP. Compared with WT mice, we discovered a 4-fold increase in the proportion of CD4+CD25+ T cells in extrathymic T cells and a 3-fold increase in thymus-derived T cells undergoing HP (Fig. 8, A and B). Remarkably, enrichment in CD4+CD25+ T cells during HP did not occur in IFN-γR-KO T cells (Fig. 8, A and B). Thus, the proportion of...
CD4<sup>+</sup>CD25<sup>+</sup> T cells was increased in extrathymic T cells and thymus-derived T cells in HP and, at least in the latter case, this phenomenon was IFN-γ dependent.

Treg cell function depends strictly on FoxP3 expression (39). We therefore compared FoxP3 expression by CD4 T cells in our experimental groups. While 7.5% of total CD4 T cells were FoxP3<sup>+</sup> in WT mice, the proportion rose to 20% for thymus-derived CD4<sup>+</sup> in HP (day 14) and reached the surprising level of 40% for extrathymic CD4<sup>+</sup> T cells (Fig. 8, A and B). Accumulation of FoxP3<sup>+</sup> cells during HP was abrogated in CD4 T cells derived from IFN-γR-KO donors (Fig. 8, C and D, upper panels). As expected, the vast majority of thymus-derived CD4<sup>+</sup>CD25<sup>+</sup> T cells were FoxP3<sup>+</sup>. Likewise, the proportion of CD4<sup>+</sup>CD25<sup>+</sup> T cells that were FoxP3<sup>+</sup> was ~90% for extrathymic T cells and thymus-derived T cells undergoing HP (Fig. 8, C and D, middle panels). That proportion decreased to 50% for IFN-γR-KO thymus-derived T cells in HP. Analysis of CD4<sup>+</sup>CD25<sup>-</sup> T cells revealed notable differences between thymus-derived and extrathymic T cells. While the proportion of CD4<sup>+</sup>CD25<sup>-</sup> T cells that were FoxP3<sup>+</sup> was only 2.7% among thymus-derived T cells, it increased to 20.9% in extrathymic T cells and 10.4% in thymus-derived T cells in HP (Fig. 8, C and D, lower panels). Again, expansion of FoxP3<sup>+</sup>CD4<sup>+</sup>CD25<sup>-</sup> T cells did not occur in IFN-γR-KO T cells.

Taken together, these results demonstrate a dramatic increase in the proportion of FoxP3<sup>+</sup> cells among extrathymic CD4 T cells. Of note, the absolute number of spleen CD4 T cells in OM-transgenic mice is similar to that of WT mice (19). Therefore, absolute numbers of FoxP3<sup>+</sup> cells per spleen were increased 4-fold in OM-transgenic mice relative to WT mice (data not shown). This increase resulted from two factors: an accumulation of CD4<sup>+</sup>CD25<sup>-</sup> cells that are practically all FoxP3<sup>+</sup>, and the presence of a significant proportion of FoxP3<sup>+</sup> cells in the CD4<sup>+</sup>CD25<sup>-</sup> compartment. A similar increase in the proportion of FoxP3<sup>+</sup> cells was observed in thymus-derived T cells in HP. In the latter case, expansion of FoxP3<sup>+</sup>CD4<sup>+</sup> T cells was clearly shown to be IFN-γ dependent.

Recent studies showing that human and mouse FoxP3<sup>+</sup> Treg cells express low levels of IL-7Rα provide suggestive evidence that FoxP3 may repress transcription of IL-7Rα (40, 41). This begs the question: Is the high proportion of FoxP3<sup>+</sup> cells among extrathymic CD4 T cells (Fig. 6B) responsible for their decreased expression of IL-7Rα (Fig. 2C)? We found that IL-7Rα expression was lower at the surface of FoxP3<sup>+</sup> than of FoxP3<sup>-</sup> thymus-derived CD4 T cells (data not shown). Conversely, FoxP3<sup>+</sup> and FoxP3<sup>-</sup> extrathymic CD4 T cells displayed similar levels of cell-surface IL-7Rα (data not shown). Thus, decreased expression

FIGURE 8. Increased frequency of Treg cells correlates with IFN-γ overproduction. A and B, Proportions of CD4<sup>+</sup>CD25<sup>+</sup> T cells in four experimental groups: thymus-derived and extrathymic T cells harvested from WT and OM-transgenic mice, respectively; WT and IFN-γR-KO T cells undergoing HP in nonirradiated TCRβ-KO mice (day 14). C and D, Proportion of FoxP3<sup>+</sup> cells among spleen CD4 T cells in the four experimental groups. A and C, One representative experiment of three. Numbers represent percentages of positive cells. B and D, Mean values of three to five mice per group. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

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of IL-7Rα in extrathymic CD4 T cells is not limited to FoxP3+ cells.

Suppressive activity of extrathymic Treg cells

We next sought to determine whether extrathymic CD4+CD25+ T cells had suppressor function. We performed MLR between B6.SJL (H2b, CD45.1+) responding effector cells labeled with CFSE and irradiated C3H/HeJ (H2k, CD45.2+) stimulator cells. Thymus-derived (WT) or extrathymic (OM-transgenic) CD45.2+CD4 spleen T cells were FACS-sorted according to CD25 expression and graded numbers were added to the MLR (Fig. 9A). We monitored CD8 T cell recovery and proliferation to evaluate the suppressive activity of added CD4 T cells (S). As expected, thymus-derived CD4+CD25− had no deleterious impact on the recovery of effector T cells (Fig. 9B, left). In fact, at the E:S ratio of 1:1, thymus-derived CD4+CD25− T cells enhanced the number of effector cells recovered. Extrathymic CD4+CD25+ T cells had no effect on the MLR. They did not significantly hamper recovery of effector cells, and consistent with previous observations (23), they had minimal, if any, helper activity at the E:S ratio of 1:1 (Fig. 9, B and D, left and Fig. 7). The key finding was that extrathymic CD4+CD25+ T cells showed a clear and dose-dependent inhibitory effect on the recovery of effector cells (Fig. 9, B and D). The suppressive effect of extrathymic CD4+CD25+ T cells was illustrated as follows: they inhibited the recovery of effector cells (Fig. 9B) by inhibiting their proliferation (Fig. 9, C and D), and they mitigated up-regulation of CD44 on effector cells (Fig. 9C). When added in low numbers (E:S ratio of 1:0.06) the

FIGURE 9. Extrathymic CD4+CD25+ T cells show high in vitro suppressive activity. A, Experimental design for the suppression assay. An MLR was generated by coculturing CFSE-labeled H2b effector cells (CD45.1+) and H2k APCs (CD45.2+) for 4 days. Sorted CD4+CD25− or CD4+CD25+ thymus-derived or extrathymic T cells were added to the MLR at graded effector-to-suppressor (E:S) cell ratios. B, Recovery of CD8 effector T cells following addition of graded numbers of CD4+CD25− (left) or CD4+CD25+ (right) thymus-derived (red) or extrathymic (blue) T cells to the MLR. C, CFSE profile (upper panels) and CD44 expression (lower panels) of effector cells in absence (control) or presence of CD4 T cells at an E:S ratio of 1:0.25. Numbers indicate percentages of undivided cells and of cells in the eighth division peak. One representative experiment of three. D, Extrathymic CD4+CD25− (left) and CD4+CD25+ (right) T cells inhibit the proliferation of CD8 effector cells. Percentage of inhibition was calculated as follows using the proliferation index (PI) provided by ModFit: [(PI MLR + added cells) − (PI MLR no added cells)]/PI MLR no added cells). E, Expression of CD103 on thymus-derived and extrathymic CD4+FoxP3+ T cells. Data are representative of three to five independent experiments.
suppressive effect of extrathymic CD4⁺/CD25⁺ T cells was even greater than that of thymus-derived CD4⁺/CD25⁺ T cells (Fig. 9D, right).

Treg cells can be segregated in two functional subsets based on CD103 (integrin α/β₇) expression (42–44). CD103⁺ Tregs have a naive-like phenotype and reside in LNs where they can suppress naive T cell proliferation. Because of differential expression of selectin ligands and chemokine receptors, CD103⁺ Tregs seek inflamed sites and exhibit high suppressive potential in various inflammation models. We found that the proportion of CD103⁺ cells was 70% in extrathymic CD4⁺FoxP3⁺ cells vs only 16% in CD4⁺FoxP3⁺ thymus-derived T cells (Fig. 9E). Thus, the dramatic accumulation of extrathymic CD4⁺FoxP3⁺ Treg cells in OM-transgenic mice essentially involves CD103⁺ elements endowed with powerful suppressive potential. The impressive in vitro suppressive activity of extrathymic CD4⁺CD25⁺ T cells was independent of OM. Indeed, although mature T cells from OM-transgenic mice may secrete low amounts of OM, CD4⁺CD25⁺ T cells did not produce more OM than did the nonsuppressive CD4⁺CD25⁻ T cells, excluding a direct suppressive action of OM in the MLR (data not shown).

Discussion

Extrathymic T cells cannot substitute for conventional thymus-derived T cells (23). A significant implication is that having the ability to generate T cells and to sustain normal numbers of T cells are not sufficient to ensure immunocompetence (6). The main drawback of extrathymic T cells is that they are unduly susceptible to apoptosis and therefore do not expand properly when confronted with a pathogen. The present work demonstrates that apoptosis of extrathymic CD4 T cells is due to repression of IL-7Rα and Bcl-2 by cell-extrinsic factors, while apoptosis of extrathymic CD8 T cells is death receptor factors (Fig. 10). Extrathymic T cells undergo excessive HP that leads to overproduction and accumulation of IL-2 and IFN-γ. IFN-γ plays a pivotal role in homeostasis of extrathymic T cells. High levels of IFN-γ up-regulate Fas and FasL on extrathymic CD8 T cells and thereby lead to their death by death receptor-mediated apoptosis. Moreover, IFN-γ curtails the survival of extrathymic CD4 T cells by down-regulating IL-7Rα and Bcl-2. Overproduction of IL-2 (and possibly of other cytokines of the IL-2 family (32)) probably contributes to down-regulation of IL-7Rα on extrathymic CD4 T cells, but further studies are needed to directly assess this point.

Taken together, three features provide incontrovertible evidence that extrathymic T cells undergo exalted HP in absence of lymphopenia: their memory-like phenotype (25), their exuberant proliferation rate (19), and the paucity of TCR excision circles (Fig. 1, B and C). Accordingly, we report herein that classic thymus-derived T cells undergoing HP in a lymphopenic host shared key features of extrathymic T cells: high apoptosis rate of CD4 and CD8 T cells; down-regulation of IL-7Rα and Bcl-2, overproduction of IL-2, and accumulation of FoxP3 Treg cells in the CD4 population; and overproduction of IFN-γ and up-regulation of Fas on CD8 T cells. This discrepancy is probably due to the fact that we studied thymus-derived T cells only in the acute phase of HP, and that up-regulation is a late event in thymus-derived CD8 T cells undergoing HP. In accordance with this assumption, Fas/FasL interactions have been shown to
induce apoptosis of thymus-derived T cells in another experimental model of HP (31).

Extrathymic CD8 T cells share all features of thymus-derived “innate” CD8 T cells: they undergo “spontaneous” HP in nonlymphopenic animals, they display a memory-like phenotype, and they exhibit immediate effector cytokine production (6, 24, 45). Two main types of innate thymus-derived TCRαβ CD8 T cells have been described: 1) in WT mice, T cells whose TCR is specific for peptides presented by MHC class Ib molecules (24, 46); and 2) CD8 T cells that develop in the thymus of mutant mice deficient for Tec-family tyrosine kinases Itk and Rlk (47–49). A key feature of innate thymus-derived T cells is that they are positively selected by hemopoietic cells whereas conventional TCRαβ CD8 T cells are positively selected by thymic epithelial cells (24, 46). It has been proposed that CD8 T cells selected on hemopoietic cells may play a unique role in immune responses (6, 24). This would be contingent upon rapid generation of effector function and, in particular, swift and massive secretion of IFN-γ. Evidence suggests that positive selection on hemopoietic cells entails high avidity interactions leading to the induction of comesodermin or T-bet transcription factors. Up-regulation of comesodermin or T-bet would then dictate the peculiar homeostatic and functional behavior of innate CD8 T cells (24). We previously reported that extrathymic CD8 T cells in OM-trangenic mice are positively selected by LN hemopoietic cells (25). This supports the concept that the nature of the cells supporting positive selection dictates whether TCRαβ CD8 T cells will be innate or conventional T cells. This begs a question: Why do thymic epithelial cells have the unique ability to induce generation of conventional T cells? In other words, what is the critical difference between positive selection on thymic epithelial cells vs other cells? This question might be directly addressed by studying polyclonal T cells positively selected on thymic epithelial vs hemopoietic cells. Also, answers may lie, at least in part, in the fact that thymic cortical epithelial cells express a unique form of proteasome (the thymoproteasome) that probably generates low-affinity MHC class I ligands compared with other proteasomes (50). It is tempting to speculate that MHC class I molecules associated with low-affinity peptides may induce weaker TCR signals in developing T cells positively selected on thymic epithelial vs hemopoietic cells. Also, answers may lie, at least in part, in the fact that thymic cortical epithelial cells express a unique form of proteasome (the thymoproteasome) that probably generates low-affinity MHC class I ligands compared with other proteasomes (50). It is tempting to speculate that MHC class I molecules associated with low-affinity peptides may induce weaker TCR signals in developing T cells positively selected on thymic epithelial vs hemopoietic cells.

When confronted with pathogens, extrathymic CD4 T cells do not expand properly and fail to provide help to B cells and CD8 T cells (23). We report herein that FoxP3 is expressed by ~40% of extrathymic CD4 T cells, most of which are CD25 + and CD103 +. Furthermore, extrathymic CD4 + CD25 + T cells display potent suppressive activity in vitro. To the best of our knowledge, the magnitude of (extrathymic) Treg cell expansion found in OM-trangenic mice is unprecedented. Nevertheless, it is perfectly consistent with the fact that expansion of Treg cells is induced by three key factors that coalesce in extrathymic T cells: HP, production of IL-2, and IFN-γ (51–56). Generation of Treg cells whenever T cells are activated by foreign or self-Ags (e.g., during HP) is important to prevent autoimmunity (57–60). Because ~40% are Treg cells, it makes sense that extrathymic CD4 T cells show limited expansion following Ag recognition and are unable to help CD8 T cells and B cells (23). One further possibility for future exploration is that Treg cells may induce apoptosis of extrathymic T cells. Indeed, extrathymic T cells show signs of activation, and Treg cells display perforin-dependent cytotoxicity against autologous-activated CD4 and CD8 T cells (61). Why are extrathymic CD4 T cells totally unfit to generate protective responses when confronted with pathogens? In tetraparental aggregation chimeras, thymus-derived CD4 T cells positively selected on hemopoietic cells are unable to generate functional antiviral responses (62). We therefore posit that positive selection by thymic epithelial cells is absolutely required to produce functional CD4 T cells. The unique ability of thymic epithelial cells to promote generation of functional CD4 T cells might be due to expression of a unique range of MHC class II-associated self-peptides (63). Indeed, in contrast with most peripheral cells, thymus cortical epithelial cells use lysosomal cathepsin L in place of cathepsin S to generate MHC class II-associated peptides (64). Furthermore, deletion of the cathepsin L-encoding gene in mice hindered positive selection of CD4 T cells (64). Similar to what has been predicted for the thymoproteasome and MHC class I-associated peptides (50), cathepsin L might generate peptides with a low affinity for MHC class II molecules that in turn would induce weak TCR signals required for generation of conventional TCRαβ CD4 T cells. Stronger TCR signals (below the negative selection threshold) lead to induction of FoxP3 and generation of Treg cells (65). Thus, the exceedingly high proportion of Treg cells among extrathymic CD4 T cells could result from positive selection on LN hemopoietic cells as opposed to thymic epithelial cells.

T lymphocytes are the sole hematolymphoid cell lineage that is not generated in main hemopoietic organs. In all vertebrates with an adaptive immune system, this task is outsourced to the thymus. This raises two fundamental questions: Why must T cells be produced in a different microenvironment, and why is it always in the thymus? By contrast, vertebrates have been using various organs for hematopoesis and B cell poiesis: bursa of Fabricius, cranium, gonads, gut, heart, kidney, liver, meninges (4, 66). Moreover, experimental mouse models have shown that Notch signals could transform the mammalian bone marrow in a primary T lymphoid organ (13, 67–69), whereas OM, leukemia inhibitory factor, and possibly Wnt4 could do the same for LNs (18, 21, 70). Clearly, it might not be so difficult to produce T cells extrathymically, but our work demonstrates that extrathymic T cells are functionally deficient and suggests that they could even have a deleterious effect on conventional T cells. Extrathymic CD4 T cells are completely ineffective by themselves. Moreover, the exceedingly high proportion of Treg cells among extrathymic CD4 T cells could hinder expansion of conventional T cells in infected animals. Besides, extrathymic CD8 T cells are essentially innate T cells. The key issue now is to discover how interactions between stromal cells and lymphoid cells generate functionally different progeny in the thymus vs extrathymic sites. The answer will most probably come from a better understanding of molecular interactions that take place between stromal cells and T-lineage cells in discrete thymic subcompartments (71–73). Finally, because T cells can literally be produced “in a dish” (74), there have recently been efforts to develop effective ex vivo strategies to generate T-lineage cells for patients with impaired thymic function (12, 14, 75). Our work demonstrates that the function of such extrathymic T cells needs to be carefully assessed and that for the time being, positive selection on thymic epithelial cells remains an absolute prerequisite for generation of conventional TCRαβ T cells. Additionally, we show that preservation of T cell function necessitates restoration of normal numbers of T cells. Otherwise, even genuine thymus-derived T cells will undergo lymphopenia-induced HP and thereby acquire the same defects as extrathymic T cells.

Acknowledgments
We are grateful to Daniele Gagné for advice on flow cytometry and cell sorting, Pierre Chagnon for help with qPCR, and to the staff of Institute for Research in Immunology and Cancer animal care facility (University of Montréal) for assistance. We thank M. Guimond and N. Labrecque for helpful comments, and J. A. Kashul for editorial assistance.
Disclosures
The authors have no financial conflicts of interest.

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