The Journal of Immunology

RESEARCH ARTICLE | FEBRUARY 15 1998

Early Intrathymic Precursor Cells Acquire a CD4^{low} Phenotype[1] FREE

Alison M. Michie; ... et. al

J Immunol (1998) 160 (4): 1735–1741.
https://doi.org/10.4049/jimmunol.160.4.1735

Early Intrathymic Precursor Cells Acquire a CD4^{low} Phenotype[1]

Alison M. Michie, James R. Carlyle, and Juan Carlos Zúñiga-Pflücker[2]

CD4^{low} cells are a population of lymphoid lineage-restricted progenitor cells representing the earliest precursors present in the adult thymus. Paradoxically, thymic progenitors with a similar phenotype in fetal mice and adult RAG-2-deficient (RAG-2$^{-/-}$) mice lack this characteristic low-level expression of CD4. We now show that radiation-induced differentiation of CD4$^+$CD8$^+$ double positive thymocytes in RAG-2$^{-/-}$ mice results in the appearance of low levels of CD4 on thymocytes that are phenotypically identical to CD4^{low} progenitor cells present in the normal adult thymus. This suggests that CD4 surface expression can be passively transferred from double positive cells to early progenitor thymocytes. Analysis of mixed bone marrow chimeras, reconstituted with hematopoietic stem cells from both CD4$^{-/-}$ (CD45.2) and CD4^{wt} (CD45.1) congenic mice, revealed a CD4^{low} phenotype on cells derived from CD4$^{-/-}$ bone marrow cells. Furthermore, these CD4$^{-/-}$-derived "CD4^{low}" progenitors were capable of reconstituting lymphocyte-depleted fetal thymi, with all thymocytes displaying a CD4$^{-/-}$ phenotype. This directly demonstrates that genetically CD4-deficient thymic progenitor cells can passively acquire a CD4^{low} phenotype. Moreover, CD4 expression on CD4^{low} progenitor thymocytes is sensitive to mild acid treatment, indicating that CD4 may not exist as an integral cell surface molecule on this thymocyte population. Our findings demonstrate that low-level CD4 surface expression can be passively acquired by intrathymic progenitor cells from the surrounding thymic microenvironment, suggesting that other cell surface molecules expressed at low levels may also result from an acquired phenotype. ***The Journal of Immunology*****, 1998, 160: 1735–1741.**

Mature T cells are generated in the thymus from bone marrow (BM)-[3] or fetal liver-derived hematopoietic stem cells (1). Several distinct differentiation stages have been defined during thymocyte development (1–4). The earliest population of progenitor thymocytes within the adult thymus, termed thymic lymphoid progenitors (TLP), phenotypically resembles BM-derived hematopoietic stem cells; however, these early immature thymocytes exhibit lymphoid lineage-restricted precursor potential (1, 2, 5, 6).

Although TLP cells were originally considered to belong to the CD4, CD8, and CD3/TCR negative (triple negative, TN) subset of thymocytes, they were later reported to express CD4 at low but detectable levels in the adult thymus; hence, these cells have been termed "CD4^{low}" progenitors (7). These TLPs can be identified by a CD117$^+$(c-*kit*)CD44$^+$CD90^{low}(Thy-1)CD25$^-$CD3$^-$CD4^{low} CD8$^-$ surface phenotype (2). Surprisingly, thymocytes displaying a similar overall phenotype as CD4^{low} progenitors, but that are derived from the fetal mouse thymus or from adult recombinase-activating gene-2-deficient (RAG-2$^{-/-}$) mouse thymus, do not express CD4 (2, 8, 9).

To understand the phenotypic differences between adult mouse thymocytes and those of early fetal or adult RAG-2$^{-/-}$ mice, we considered the possibility that the CD4^{low} phenotype may result from the passive acquisition of CD4 molecules by TLPs from CD4$^+$CD8$^+$ double positive (DP) or CD4 single positive (SP) cells, which are present in the adult thymic environment but absent in the fetal or RAG-2$^{-/-}$ thymic milieu. In support of this hypothesis, previous studies using radiation-BM-chimeras have demonstrated an acquired expression of allotypic MHC class I and class II determinants from other cells within the local thymic environment (10). Moreover, CD8 molecules have also been shown to passively adhere to cells within the thymus (11). Indeed, our findings demonstrate that the CD4^{low} surface expression observed on a subset of progenitor thymocytes in the adult thymus can result from a passive acquisition and not endogenous expression of CD4 molecules.

Materials and Methods

Animals

RAG-2$^{-/-}$ mice (12) were bred in our animal facility. Breeding pairs of CD4$^{-/-}$ mice backcrossed into the C57BL/6 background were obtained from Dr. T. Mak (Ontario Cancer Institute) (13) and were maintained in our animal facility. Timed-pregnant Swiss-NIH mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). C57BL/6-Ly 5.1 (CD45.1) congenic mice were provided by Drs. P. Ohashi (Ontario Cancer Institute) and P. Poussier (Wellesley Hospital Research Institute) and bred in our animal facility. Sublethal gamma-irradiation of RAG-2$^{-/-}$ mice or lethal gamma-irradiation of CD4$^{-/-}$ mice was conducted at 750 cGy or 950 cGy, respectively, as previously described (14, 15).

Flow cytometry and FACS

Abs were purchased from PharMingen (San Diego, CA). Staining of cells was performed as previously described (15). Briefly, single-cell thymocyte suspensions were prepared in HBSS (without phenol red) containing 1% BSA and 0.1% sodium azide (FACS buffer). Cells (10^6/100 μl) were incubated on ice for 30 min with 10 μl of the appropriate FITC-, phycoerythrin, cychrome-, or APC-conjugated mAbs and washed twice in ice-cold FACS buffer before analysis. Analysis was performed using a

Department of Immunology, University of Toronto, Toronto, Ontario, Canada

Received for publication October 17, 1997. Accepted for publication October 30, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Medical Research Council of Canada (MRC) and the National Cancer Institute of Canada (J.C.Z.-P.), and by an MRC studentship (J.R.C.) and an MRC scholarship (J.C.Z.-P.).

[2] Address correspondence and reprint requests to Dr. J. C. Zúñiga-Pflücker, Department of Immunology, University of Toronto, Medical Sciences Building, Toronto, Ontario, M5S 1A8, Canada. E-mail address: jc.zuniga.pflucker@utoronto.ca

[3] Abbreviations used in this paper: BM, bone marrow; TLP, thymic lymphoid progenitor; dGuo, deoxyguanosine; DP, double positive thymocyte (CD4$^+$CD8$^+$); FTOC, fetal thymic organ culture; MFU, mean fluorescence unit; RAG-2, recombinase-activating gene-2; SP, single positive; TN, triple negative thymocyte (CD3$^-$CD4$^-$CD8$^-$).

0022-1767/98/$02.00

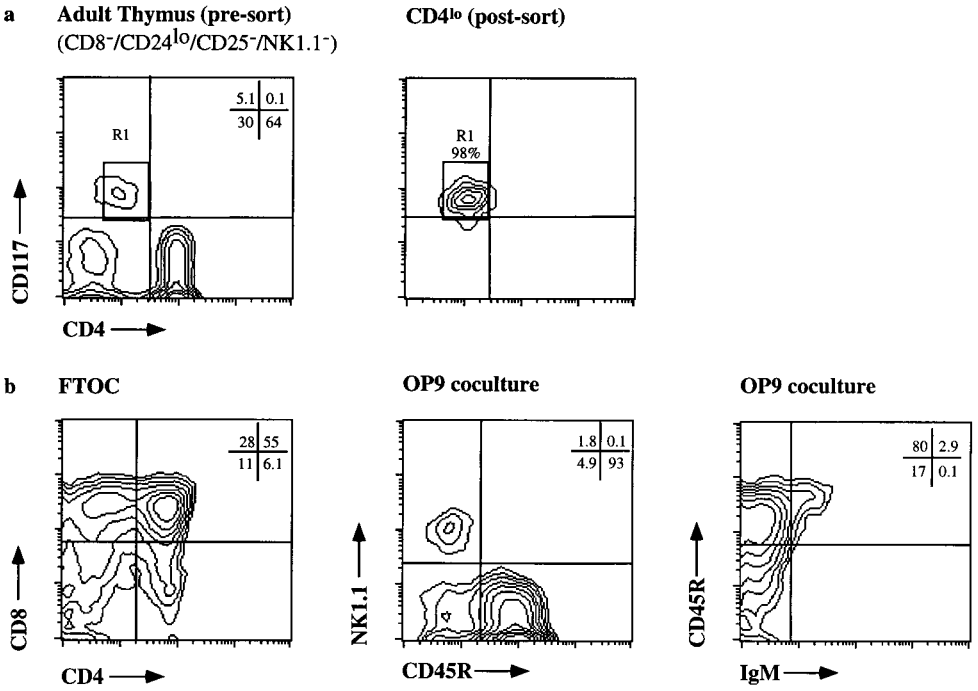

FIGURE 1. Generation of T, B, and NK cells from CD4^{low}CD117$^+$ cells. *a*, Thymocytes, depleted of CD8$^+$CD24^{high}CD25$^+$NK1.1$^+$ cells by Ab-complement treatment, were sorted for the CD117$^+$CD4^{low}CD90^{low} population (R1 gate) and used as precursors for the generation of mature T, B, or NK cells. *b*, Sorted CD117$^+$CD4^{low}CD90^{low} thymocytes (R1 gate) were reconstituted into alymphoid fetal thymic lobes (1000 cells/lobe) and assayed after 10 to 12 days in FTOC. Two-parameter flow cytometric analysis of the lobes (CD8 vs CD4) revealed the presence of T cell lineage-committed cells. Two-parameter flow cytometry revealed the generation of B and NK cells. NK1.1 vs CD45R (B220) staining revealed the presence of pre-B cells and NK-lineage cells in OP9 cocultures at day 7. CD45R vs mIgM staining revealed the presence of IgM-expressing B cells and pre-B cells in the cultures 4 days after the addition of LPS. Percentages of each population are indicated on the *upper right corner*.

FACSCalibur (Becton Dickinson and Co., Mountain View, CA) flow cytometer with CELLQUEST software; data were live gated by size and lack of propidium iodide uptake. For cell sorting, a Coulter Elite (Coulter Corp., Hialeah, FL) cytometer was used. Thymic single-cell suspensions were prepared and stained for FACS as described above, but no NaN_3 was added to HBSS. Sorted cells were >98% pure, as determined by postsort analysis.

Fetal thymic organ culture (FTOC)

Sorted populations were washed twice with DMEM supplemented with 12% FCS, 2 mM glutamine, 10 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 110 μg/ml sodium pyruvate, 50 μM 2-ME, and 10 mM HEPES, pH 7.4 (FTOC medium). Lymphocyte-depleted thymic lobes were prepared by culturing day 15 fetal thymic lobes from timed-pregnant RAG-2$^{-/-}$ mice in FTOC medium containing 1.35 mM deoxyguanosine (dGuo), as previously described (16, 17). Briefly, host FTOCs were cultured with dGuo for 4 to 6 days, then dGuo-containing medium was replaced with FTOC medium for 1 day, and then lobes were rinsed twice, resuspended in 8 μl of FTOC medium, and placed in Terasaki plates. Twenty microliters of FTOC medium containing 0.8 to 1 $\times$ 10^3 CD117$^+$ CD90^{low}CD4^{low} reconstituting cells were then added to dGuo-treated alymphoid fetal thymic lobes. The plates were inverted and cultures were incubated at 37°C in a humidified incubator containing 5% CO_2 in air for 24 to 48 h. Lobes were then transferred to FTOCs for 10 to 12 days. Cell suspensions from reconstituted thymic lobes were analyzed by flow cytometry as described above.

Preparation of CD4^{low} progenitor cells

Thymic single-cell suspensions were prepared from neonatal mice (4–14 days) in FTOC media. Thymocytes were depleted of CD8$^+$ (YTS-169), CD24^{high} (heat-stable Ag) (J11d.2), CD25$^+$ (IL-2Rα) (7D4), and NK1.1$^+$ (PK136) cells by Ab-complement-mediated lysis. Viable cells were recovered by buoyant density centrifugation using Lympholyte-M (Cedarlane, Hornsby, Ontario, Canada). CD4^{low} progenitor cells (CD117$^+$CD4^{low}CD90^{low}) were then isolated from the resulting CD8$^-$CD24^{low}CD25$^-$NK1.1$^-$ cell population by flow cytometric cell sorting (Fig. 1*a*; R1 gate), as described above.

In vitro generation of B and NK cells with OP9-BM stromal cells coculture

The generation of B and NK lymphocytes from TLPs by coculture with the BM-derived stromal cell line, OP9 (16, 17), has been previously described (18). Briefly, sorted CD117$^+$CD4^{low}CD90^{low} cells were washed twice in FTOC media and then placed onto a confluent layer of OP9 cells (1000 cells/well of a six-well plate) in FTOC media containing stem cell factor, IL-7, IL-3, and IL-6 (50 ng/ml of each cytokine). The cells were cultured for 11 days, and then IL-7 and LPS (15 μg/ml) were added for 4 days. The presence of NK1.1-expressing cells was assessed after 7 days of coculture with OP9 cells, while differentiation of mature IgM$^+$ B cells was evaluated at day 4 post-LPS by two-parameter flow cytometry.

Generation of CD4^{low}-expressing cells in the RAG-2$^{-/-}$ thymus

RAG-2$^{-/-}$ mice were sublethally gamma-irradiated (750 cGy) on day 0, with control mice remaining unirradiated. After 4 days, 1.25 $\times$ 10^7 BM cells from RAG-2$^{-/-}$ mice were injected i.v. into sublethally gamma-irradiated (750 cGy) and unmanipulated RAG-2$^{-/-}$ recipients (four mice/group). Sixteen days postirradiation, the thymi were removed and cells expressing CD8, CD24, CD25, and NK1.1 were lysed by Ab and complement depletion. The resulting population was analyzed by FACS for the presence of CD4^{low}CD117$^+$ cells.

BM reconstitution

BM was prepared from CD45.2 (CD4$^{-/-}$) and CD45.1 (CD4^{wt}) mice and injected i.v. into lethally γ-irradiated (950 cGy) CD45.2 (CD4$^{-/-}$) mice at 6 $\times$ 10^7 cells/mouse (three mice/group). After 28 days, donor-derived thymocytes were Ab and complement depleted of CD8-, CD24-, CD25-, and NK1.1-expressing cells, and CD117$^+$CD4^{low}CD90^{low} cells were isolated by flow cytometric cell sorting as described above.

Acid washing of thymocytes

Thymocytes, enriched for CD4$^-$CD8$^-$-double negative cells by Ab-complement-mediated depletion of CD8$^+$CD24^{high}CD25$^+$NK1.1$^+$-expressing cells, were washed in saline (0.9% NaCl solution), and then resuspended in

PBS or acid-washing solution (0.131 M citric acid, 0.066 M Na_2HPO_4, pH 3.3) as previously described (19). The cells were incubated at room temperature for 2 min in the acid solution, and then washed in PBS and prepared for FACS analysis as described above.

Results

$CD4^{low}$-progenitor cells exhibit lymphoid lineage-restricted precursor potential

$CD4^{low}$ progenitor thymocytes represent the earliest BM-derived immigrants to the thymus. The $CD4^{low}$ population of early precursor thymocytes, characterized by Wu and Shortman as a $CD44^+CD90^{low}CD25^-CD8^-CD3^-$ population, makes up 0.05% of adult thymocytes, and retains multipotency toward all lymphocyte lineages (T, B, and NK cells), as well as a novel subset of thymic dendritic cells (7, 20). However, these cells have lost myeloid and other hematopoietic lineage potentials (5–7).

We isolated a population of cells that phenotypically resemble the $CD4^{low}$ progenitor cells ($CD117^+CD44^+CD90^{low}CD24^{low}$ (heat-stable Ag)$CD25^-CD4^{low}CD8^-CD3^-NK1.1^-$) by a process of Ab-complement-mediated cell lysis of total thymocytes for $CD8^+CD24^{high}CD25^+NK1.1^+$ cells, followed by cell sorting for $CD4^{low}CD117^+CD90^{low}$ cells (Fig. 1*a*; R1 gate). These cells were assayed for their ability to give rise to T cells by placing them in FTOC. Freshly sorted $CD4^{low}$ cells were transferred to alymphoid dGuo-treated RAG-$2^{-/-}$ fetal lobes. After 10 days in FTOC, reconstitution of the lobes was evident, as populations of $CD4^+CD8^+$ DP cells (55%), and $CD4^+$ (6%) and $CD8^+$ (28%) SP thymocytes were found to be present, indicating that these $CD4^{low}$ cells possess T cell lineage potential (CD8 vs CD4; Fig. 1*b*). Control lobes that were not reconstituted with donor cells remained devoid of T lineage cells (data not shown).

To confirm the multipotent reconstituting ability of $CD4^{low}$ cells, we assayed for the generation of B and NK cells from sorted progenitor cells (Fig. 1*a*; R1 gate), by coculturing $CD4^{low}$ cells with the BM-derived stromal cell line, OP9 (Fig. 1*b*). This method of generating B and NK lineage cells has been successful in demonstrating that fetal TLP cells can give rise to B and NK cells after coculture with OP9 cells (18). Analysis of $CD4^{low}$ cells cocultured with OP9 cells revealed a large population of $CD45R^+$ (B220) cells and a smaller population of NK1.1-expressing cells, indicating the presence of NK lineage cells (2%) and pre-B cells (93%) (NK1.1 vs CD45R; Fig. 1*b*). Four days after the addition of LPS to the OP9 coculture system, surface IgM was detected by FACS analysis, indicating that mature B cells are generated in the cocultures (CD45R vs IgM; Fig. 1*b*). To demonstrate the clonal diversity of B cells generated from $CD4^{low}$ cells in the OP9 coculture system, DNA was extracted from these cells and subsequent PCR analysis revealed multiple products corresponding to D-J and V-DJ rearranged DNA from the IgH loci, indicating the generation of a diverse B cell repertoire (data not shown).

Coculture of fetal liver cells, which display a similar phenotype to $CD4^{low}$ progenitor cells ($CD117^+CD44^+CD90^{low}CD25^-CD3^-CD4^-CD8^-$), with OP9 cells gave rise to Mac-1 (CD11b)-expressing cells, indicative of the generation of myeloid cells. However, $CD4^{low}$ progenitor cells were unable to generate myeloid lineage cells under similar coculture conditions (data not shown and Ref. 18). Therefore, our means of isolating $CD4^{low}$ progenitor cells results in a population that displays functional characteristics similar to those identified by Wu and Shortman, in that they are lymphoid lineage restricted, giving rise to mature T, B, or NK lymphocytes (Fig. 1*b*), but are unable to give rise to myeloid and erythroid lineage cells (data not shown and Refs. 5–7).

Progenitor thymocytes from RAG-$2^{-/-}$ mice acquire a $CD4^{low}$ phenotype following radiation-induced differentiation of $CD4^+CD8^+$ cells

The thymus of adult RAG-2-deficient mice (RAG-$2^{-/-}$) resembles that of immature day 15 fetal mice, with most thymocytes arrested at the $CD117^-CD44^-CD25^+CD24^{high}CD3^-CD4^-CD8^-$ stage of T cell development (12). A maturation block occurs at the onset of TCR-β-chain rearrangement due to the lack of recombinase activity (12). We noted that RAG-$2^{-/-}$ thymocytes lack low levels of CD4 expression on early progenitors. Flow cytometric analysis of thymocytes from RAG-$2^{-/-}$ mice, depleted of $CD25^+CD24^{high}$ cells by Ab-complement-mediated lysis, revealed a population of $CD117^+$ precursor-type thymocytes, which should contain the $CD4^{low}$ thymic progenitor subset (Fig. 2*a*). However, when analyzed for the expression of CD4 with anti-CD4 or isotype-matched control Abs, we failed to detect significant CD4 surface expression (Fig. 2*a* and data not shown). We hypothesized that the lack of CD4 surface expression on early progenitor thymocytes of RAG-$2^{-/-}$ mice could result from the absence of CD4-bearing cells within the thymic environment, as these cells may be required to either actively induce an endogenous low-level expression of CD4, or provide an exogenous source of CD4 molecules to be passively acquired by progenitor thymocytes. This hypothesis is further supported by previous reports indicating that $CD4^{low}$ cells are not detected during thymic ontogeny until day 17 of gestation (9), which corresponds to the time when $CD4^+CD8^+$ (DP) thymocytes become detectable.

To test this hypothesis, we took advantage of our previous finding that thymocytes from RAG-$2^{-/-}$ mice progress to the DP stage following exposure to sublethal γ-radiation (Fig. 2*b* and Refs. 14 and 21). Flow cytometric analysis of progenitor thymocytes ($CD117^+CD90^{low}CD25^-CD24^{low}CD8^-$) from RAG-$2^{-/-}$ mice 16 days after sublethal γ-irradiation (750 cGy and reconstitution with syngeneic BM) showed the appearance of $CD4^{low}$ cells within this precursor population (Fig. 2*b*). The level of CD4 surface expression, detected as the mean fluorescence intensity of CD4, increased almost twofold (5.9 mean fluorescence units (MFU) to 11 MFU) in comparison to control adult RAG-$2^{-/-}$ mice. Moreover, the presence of CD4-bearing cells within the thymus appeared to elevate the overall CD4 fluorescence intensity (3.0 MFU to 4.7 MFU; Fig. 2). This observation is reminiscent of a previous report by Shores, Sharrow, and Singer, which showed that CD8 can be passively and nonspecifically acquired by all cells within the thymus (11).

We previously reported that DP cells are first detected in the RAG-$2^{-/-}$ mouse thymus, at 14 days postirradiation, and that DP thymocytes make up the majority of all thymocytes (60–80%) for up to 5 wk after treatment (14, 21). A time course analysis revealed that the detection of the $CD4^{low}$ phenotype among the progenitor-type thymocytes correlated with the appearance of DP thymocytes at day 14 following γ-irradiation (data not shown; see Refs. 14 and 21). Thus, as with fetal development, the appearance of the $CD4^{low}$ phenotype coincides with that of DP thymocytes in the thymic environment.

A $CD4^{low}$ phenotype can be passively acquired from the thymic microenvironment

To investigate the possibility that the $CD4^{low}$ expression on thymocyte progenitors results directly from a passive acquisition of CD4 molecules from CD4-bearing cells within the thymic microenvironment, we generated mixed BM chimeras. Unfractionated BM cells from C57BL/6-CD45.1 ($CD4^{wt}$) and CD4-deficient ($CD4^{-/-}$, CD45.2) mice were injected i.v., either separately or

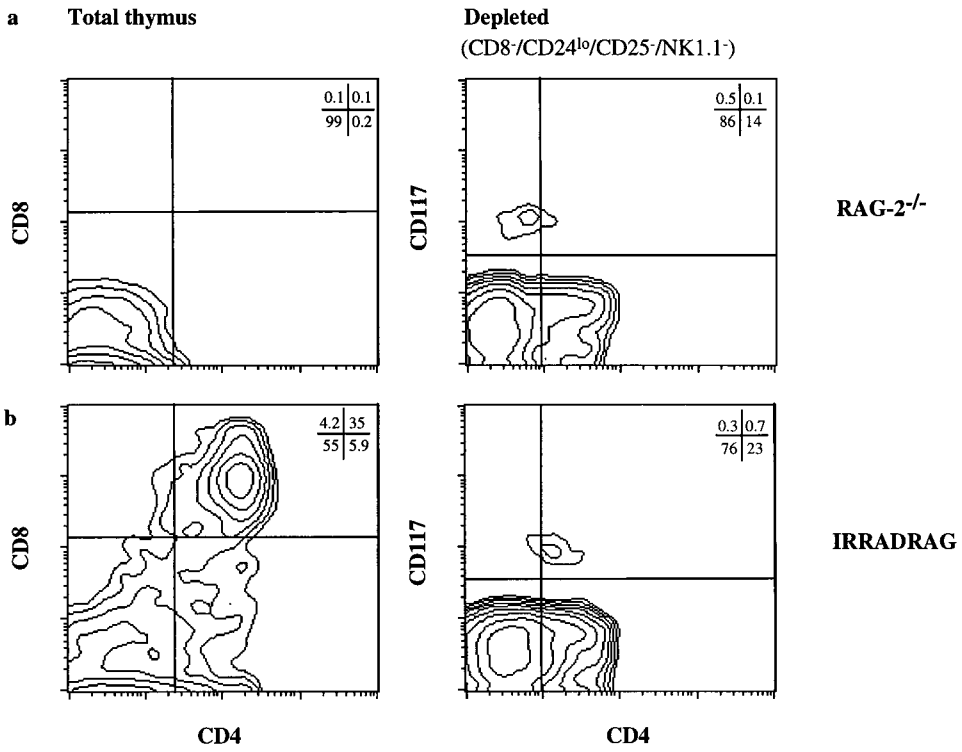

FIGURE 2. Progenitor thymocytes from RAG-$2^{-/-}$ mice acquire a $CD4^{low}$ phenotype following the gamma-irradiation-induced appearance of $CD4^+CD8^+$ cells. *a*, Two-parameter flow cytometric analysis of total thymi from control RAG-$2^{-/-}$ mice (RAG) of CD8 vs CD4 cell surface expression (*left panel*), and CD117 vs CD4 analysis in thymocytes depleted of $CD25^+CD24^{high}NK1.1^+$ cells by Ab-complement treatment (*right panel*). *b*, Two-parameter flow cytometric analysis of total thymocytes from sublethally gamma-irradiated (750 cGy) (see *Materials and Methods* for details) RAG-$2^{-/-}$ mice (IRRADRAG) of CD8 vs CD4 cell surface expression (*left panel*), and analysis of surface expression of CD117 vs CD4 in thymocytes depleted of $CD25^+CD24^{high}NK1.1^+$ cells by Ab-complement treatment (*right panel*). Percentages of each population are indicated in the *upper right corner*. Mean fluorescence intensity of CD4 expression for control vs irradiated $CD117^+$ thymocytes was 5.9 and 11 MFU.

together at a ratio of 1:3 ($CD4^{wt}$:$CD4^{-/-}$), into lethally irradiated $CD4^{-/-}$ CD45.2 host mice. After 4 wk, thymocytes were depleted of $CD8^+CD24^{high}CD25^+$ cells by Ab-complement-mediated cell lysis and the remaining thymocytes were analyzed for CD4, CD117, and CD45.1 or CD45.2 surface expression (Fig. 3). To better visualize the CD4 expression within the $CD117^+$ population of cells, $CD4^{high}$ SP cells were excluded from the histogram analysis. However, high-level CD4 staining is shown to illustrate that the R3 gated cells were indeed of a $CD4^{low}$ phenotype (Fig. 3*a*, *lower panel*).

As expected, analysis of thymocytes isolated from BM chimeras reconstituted with CD45.1 ($CD4^{wt}$) BM revealed the presence of progenitor cells expressing low levels of CD4 (Fig. 3*a*; $CD117^+$ vs $CD45.1^+$ on R1 gated cells). In contrast, all thymocytes from chimeras reconstituted with $CD4^{-/-}$ BM lacked CD4 expression (Fig. 3*c*; $CD117^+$ vs $CD45.2^+$ on R2 gated cells). An isotype-matched control is shown to indicate negative staining (Fig. 3*c*, *lower panel*). Analysis of mixed BM-chimeric mice showed that reconstitution of both CD45.1- and CD45.2-donor-derived progenitors occurred (Fig. 3*b*; CD117 vs CD45.2), as demonstrated by the presence of $CD45.2^-$ ($CD4^{wt}$, $CD45.1^+$) and $CD45.2^+$ ($CD4^{-/-}$) populations. $CD117^+CD45.2^-$ ($CD4^{wt}$) and $CD117^+CD45.2^+$ ($CD4^{-/-}$) gated populations (Fig. 3*b*; gates R1′ and R2′, respectively) were analyzed for CD4 expression. Figure 3*b* shows that low-level CD4 expression was evident on both donor-derived progenitor populations, i.e., those expressing CD45.1 ($CD4^{wt}$) and CD45.2 ($CD4^{-/-}$)-derived thymocytes. The presence of a $CD4^{low}$ phenotype on genetically CD4-deficient progenitor thymocytes demonstrates that CD4 molecules can be passively acquired from DP or $CD4^+$ SP cells within the thymus, thus appearing as "$CD4^{low}$" cells.

To test the origin and precursor potential of the $CD4^{low}$ cells present in the thymus of mixed BM-chimeric mice, $CD117^+CD4^{low}$ cells were sorted, according to CD45 allelic expression and $CD4^{low}$ phenotype, as indicated in Figure 3 (R1′ or R2′ and R3). The resulting populations were used to reconstitute dGuo-treated RAG-$2^{-/-}$ FTOCs. After 10 to 12 days in FTOC, the thymic lobes were analyzed by flow cytometry. dGuo-depleted FTOCs that did not receive precursor cells remained devoid of T lymphocytes (Fig. 4*a*), whereas FTOCs reconstituted with $CD4^{wt}(CD45.1^+)CD4^{low}$ or $CD4^{-/-}(CD45.2^+)CD4^{low}$ precursor cells gave rise to mature thymocytes as demonstrated by CD4 vs CD8 staining (Fig. 4, *b* and *c*). The $CD4^{wt}$ ($CD45.1^+$) population of cells resulted in the generation of mature DP thymocytes, as well as $CD4^+$ and $CD8^+$ SP T lymphocytes (Fig. 4*b*). However, the sorted $CD4^{low}CD45.2^+$ ($CD4^{-/-}$) cells gave rise to a large population of mature $CD8^+$ thymocytes, but due to their $CD4^{-/-}$ origin, lacked CD4 surface expression (Fig. 4*c*). Analysis of CD5 surface expression, a marker present on all $CD4^+$ cells (DP/SP), confirmed that there were "DP-like" cells within the $CD8^+$ population (data not shown). These results clearly show that CD4-deficient thymocytes from mixed chimeric mice, which have acquired the $CD4^{low}$ phenotype, serve as T cell precursors. Thus, the acquisition of the $CD4^{low}$ phenotype by $CD4^{-/-}$ thymocytes correlated with these cells also possessing progenitor function.

CD4 is noncovalently associated with the surface of $CD4^{low}$ progenitor cells

To further demonstrate that CD4 passively adheres to the surface of the $CD4^{low}$ progenitor population, we exposed these cells to a mild acid solution to disrupt hydrogen bonds and destabilize salt

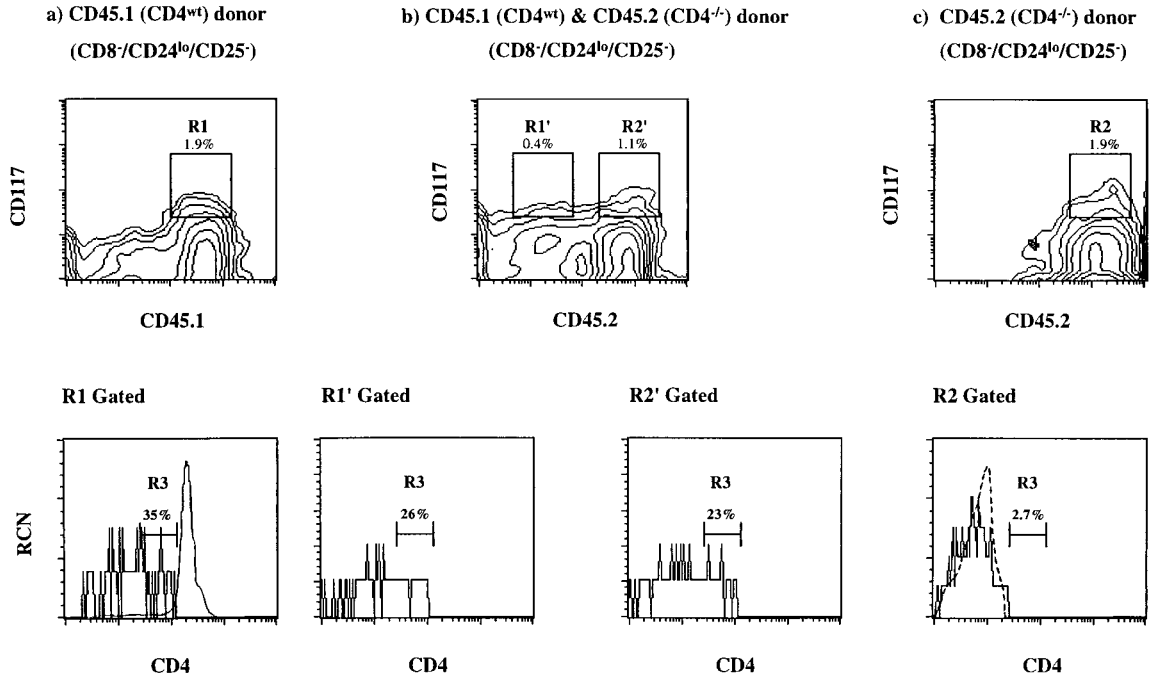

FIGURE 3. Progenitor thymocytes derived from CD4$^{-/-}$ mouse stem cells acquire a CD4^{low} phenotype in mixed BM-CD4$^{-/-}$ plus CD45.1 (CD4^{wt})-chimeric mice. Thymocytes, Ab-complement depleted of CD8$^+$CD24^{high}CD25$^+$-bearing cells from host CD4$^{-/-}$ (CD45.2) BM chimeric mice, were stained for cell surface expression of CD117 vs CD45.2 (CD45.1 was used when CD4^{wt} (CD45.1$^+$) donor cells were used to reconstitute host mice). CD4$^+$ lymphocytes were excluded from the histograms to aid in the visualization of CD4^{low} cells. A profile of CD4 staining and negative control staining with an isotype-matched Ab have been included in *a* and *c*, respectively. Lethally irradiated CD4$^{-/-}$ mice (950 cGy) were reconstituted with total BM stem cells from CD4^{wt} (CD45.1) congenic mice (*left panel*; *a*), CD4$^{-/-}$ (CD45.2) mice (*right panel*; *c*), or both CD4$^{-/-}$ (CD45.2) and CD4^{wt} (CD45.1) mice at a mixture of 3:1, respectively (*middle panel*; *b*). Analysis for CD4 cell surface expression (*lower panels*) from gated CD117$^+$ CD45.2$^-$ (CD45.1$^+$CD4^{wt}-derived thymocytes; R1′) or CD45.2$^+$ (CD4$^{-/-}$-derived thymocytes; R2′) precursor populations (sort gate indicated in *upper panel*) are shown. CD117$^+$CD4^{low} precursor thymocytes can be detected within the CD4$^{-/-}$-derived thymocytes (R2′ plus R3). Percentages of CD4^{low} progenitor cells are indicated in each histogram. CD117$^+$CD4^{low} thymocytes with CD45.2$^-$ (CD45.1$^+$; R1′ plus R3 gate) or CD45.2$^+$ (R2′ plus R3 gate) expression were gate sorted and used in FTOC reconstitution assays as indicated (see Fig. 4).

bridges, while leaving covalent bonds intact. This technique has been employed previously to release MHC class I-bound peptides from binding sites of human and mouse class I molecules, while maintaining cellular viability (22). Thus, molecules noncovalently bound to the cell surface are "washed off" upon acid treatment, while transmembrane-bound proteins will remain on the cell surface.

Mild acid washing of double negative-enriched cells (CD8$^-$CD24^{low}CD25$^-$ thymocytes) resulted in a reduction of the mean fluorescence intensity on CD4^{low} (CD117$^+$CD90^{low}) cells, compared with the unwashed control (13 MFU to 8.0 MFU; Fig. 5). Indeed, the overall fluorescence intensity of CD4 was also reduced in the CD117$^-$ population of cells (4.8 MFU to 3.3 MFU; Fig. 5). The fluorescence intensity of CD8 was also reduced upon acid washing (data not shown), supporting the previous report by Shores, Sharrow, and Singer that CD8 is passively acquired by double negative thymocytes after being shed from CD8-bearing cells within the adult thymus (11). Importantly, the mean fluorescence intensity of other cell surface markers, such as CD117 and CD2, remained unchanged on thymocytes upon acid washing (Fig. 5 and data not shown), indicating that this technique specifically affects passively adhered molecules. These experiments indicate that low-level expression of CD4 on the surface of "CD4^{low}" progenitor cells, and other cells within the thymus, is not necessarily associated to the cell via its transmembrane region. Therefore, we hypothesize that the CD4 associating with the cell surface is not endogenously derived, but can be passively acquired from the surrounding thymic milieu.

Discussion

In this study we demonstrate that low-level CD4 expression on the surface of lymphoid lineage-restricted CD4^{low} progenitor cells is not necessarily endogenously expressed as described previously (23), but can be passively acquired upon generation of CD4-bearing thymocytes within the thymic environment. Our major lines of evidence to support this notion are: 1) the acquisition of low-level CD4 expression on precursor thymocytes following the generation of γ-irradiation-induced CD4$^+$CD8$^+$ DP cells in RAG-2$^{-/-}$ mice; 2) introduction of CD4-bearing thymocytes on a CD4$^{-/-}$ background allows the generation of previously lacking CD4^{low} "expression" on progenitor cells unable to endogenously express this marker; and 3) the CD4^{low} phenotype can be "washed off" under mild acidic conditions.

Our conclusions are further supported by the observation that CD4^{low}CD117$^+$ cells are first detected after the development of DP cells during fetal ontogeny (2, 9). This was thought to be due to the inability of the CD4 locus to be transcribed early in fetal development, suggesting that low-level CD4 expression appears with maturity (24, 25). However, the absence of CD4^{low} expression on CD117$^+$ thymocytes in adult RAG-2$^{-/-}$ animals is not consistent with this hypothesis. Moreover, our results indicate that the temporal acquisition of low-level CD4 expression on this precursor population follows the development of CD4-bearing cells in RAG-2$^{-/-}$ and CD4$^{-/-}$ mouse thymocytes (Fig. 2*b*; Fig. 3), a situation that recapitulates early fetal ontogeny.

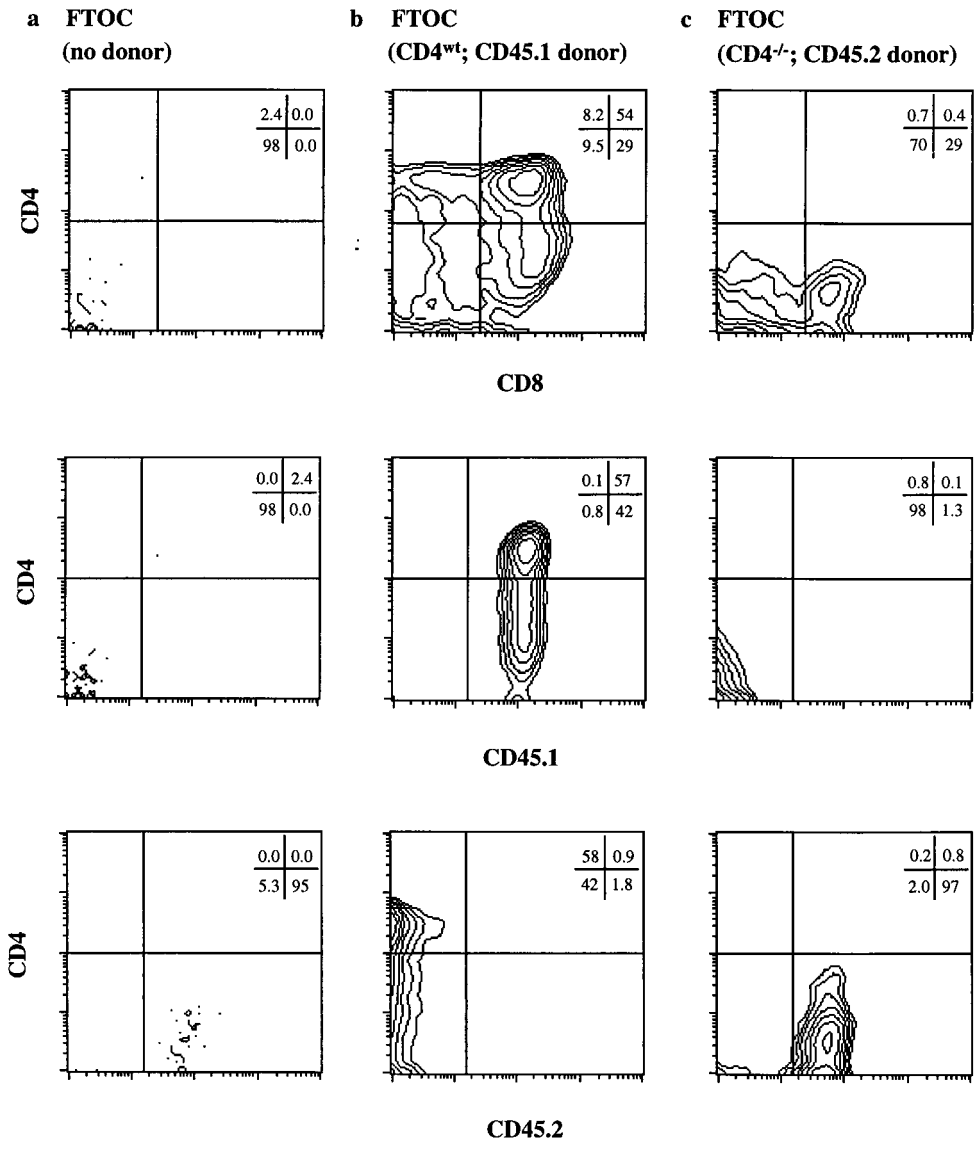

FIGURE 4. In vitro reconstitution of fetal RAG-2$^{-/-}$ lobes with CD117^{+}CD4^{low} cells from CD4$^{-/-}$ (CD45.2)- or CD4^{wt} (CD45.1)-derived precursor thymocytes from mixed chimeric mouse thymus. Sorting gates used for the isolation of CD45.2^{-} (R1′ plus R3 gate) and CD45.2^{+} (R2′ plus R3 gate) precursor cells are indicated in Figure 3. The RAG-2$^{-/-}$ fetal lobes were reconstituted with FTOC medium alone (*a*), CD45.1^{+} CD117^{+} CD4^{low} precursor cells (*b*), or CD45.2^{+} CD117^{+} CD4^{low} precursor cells (*c*). Thymocytes were stained for the expression of CD4 vs CD8 (*top panel*), CD4 vs CD45.1 (*middle panel*), and CD4 vs CD45.2 (*lower panel*). Donor-derived thymocytes accounted for >98% of cells in FTOC-RAG-2$^{-/-}$-reconstituted thymi. Percentages of each population are indicated in the *upper right corner*.

Our data are also supported by studies showing that MHC molecules and CD8 can passively adhere to all cells in the thymus (10, 11). More recently, thymic precursors have been reported to express low levels of CD8, as well as CD4 (23). However, reverse transcriptase-PCR analysis showed that mRNA for only CD4 could be detected, again suggesting that the CD8^{low} expression was the result of an acquired phenotype (11, 23). Our own reverse transcriptase-PCR analysis of purified, FACS-sorted CD4^{low} cells failed to show any evidence of expression of CD4 mRNA (data not shown). Indeed, CD4^{low} cells represent only 0.05% of the thymus, whereas CD4^{+} cells constitute ~90% of the total thymus. After removal of CD8^{+}CD24^{high}CD25^{+} cells, CD4^{high} cells represent ~65% of the remaining thymocytes, while CD4^{low} progenitor cells represent only ~2 to 3%. Therefore, the detection of CD4 mRNA in previous studies may be due to CD4^{low} progenitor cells being contaminated with more mature, endogenously expressing CD4-bearing thymocytes. Thus, by selecting a genetic background that is incapable of endogenous CD4 expression (CD4$^{-/-}$), we have shown that CD4 expression at the mRNA level is not required for progenitor cells to attain a CD4^{low} phenotype.

We have not addressed the manner in which CD4 is attached to the surface of this precursor population. However, as these cells are large, slow-cycling thymocytes, in comparison to the majority of rapidly cycling TN thymocytes (26), it is possible that the slower turnover of surface constituents enables them to acquire and retain CD4 at higher levels, thus giving the appearance of a noticeable CD4^{low} phenotype. Also, we have not yet established whether the CD4 that is shed from the DP and SP thymocytes has a role in T cell development. However, due to the absence of this marker on human progenitor cells in the thymus (27), and the lack of any evidence of a functional role for CD4 at this early stage in T lymphocyte development (13), it would appear that CD4 is randomly acquired on the surface of these lymphocyte-restricted mouse precursor thymocytes.

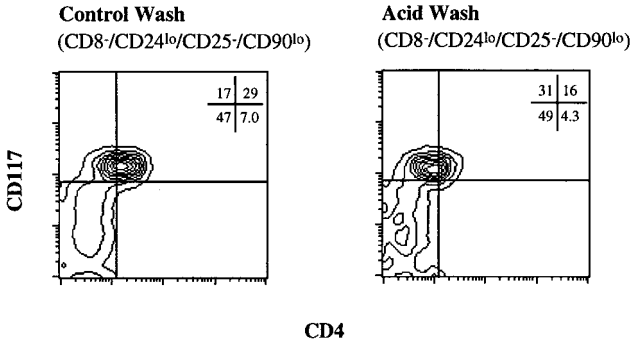

FIGURE 5. $CD4^{low}$ phenotype on progenitor cells is sensitive to mild acid treatment. Two-parameter flow cytometric analysis was conducted on thymocytes, enriched for the $CD4^{low}$ progenitor cells, as described in *Materials and Methods*. CD4 surface expression was analyzed on control or acid-washed thymocytes. CD4-SP thymocytes were gated out before analysis. There was almost a 40% loss detected in mean fluorescence intensity of CD4 surface expression (13 to 8 MFU), while CD117 fluorescence intensity remained unaltered. Percentages of each population are indicated in the *upper right corner*.

Although the phenotypic identification of $CD4^{low}$ progenitor thymocytes has provided a valuable tool in the cellular and molecular characterization of events controlling early thymopoiesis, our studies demonstrate that low-level fluorescence staining need not represent endogenous expression of a particular marker. Indeed, when studying low-level expression of cell surface molecules, it is important to consider the overall surface phenotype within the context of the cells' environments. In particular, we demonstrate that progenitor thymocytes acquire a $CD4^{low}$ phenotype only in the presence of other CD4-bearing cells, and that this can be a passive physical acquisition of surface phenotype rather than a genetically controlled cell autonomous event.

Acknowledgments

We thank Dr. Philippe Poussier for his critical reading of the manuscript and C. Smith for technical assistance with cell sorting.

References

1. Shortman, K., and L. Wu. 1996. Early T lymphocyte progenitors. *Annu. Rev. Immunol. 14:29.*
2. Zúñiga-Pflücker, J. C., and M. J. Lenardo. 1996. Regulation of thymocyte development from immature progenitors. *Curr. Opin. Immunol. 8:215.*
3. Godfrey, D. I., and A. Zlotnik. 1993. Control points in early T-cell development. *Immunol. Today 14:547.*
4. Zlotnik, A., and T. A. Moore. 1995. Cytokine production and requirements during T-cell development. *Curr. Opin. Immunol. 7:206.*
5. Ardavin, C., L. Wu, L. Chung-Leung, and K. Shortman. 1993. Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature 362:761.*
6. Wu, L., M. Antica, G. R. Johnson, R. Scollay, and K. Shortman. 1991. Developmental potential of the earliest precursor cells from the adult thymus. *J. Exp. Med. 174:1617.*
7. Wu, L., R. Scollay, M. Egerton, M. Pearse, G. J. Spangrude, and K. Shortman. 1991. CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus. *Nature 349:71.*
8. Matsuzaki, Y., J. Gyotoku, M. Ogawa, S. Nishikawa, and Y. Katsura. 1993. Characterization of c-kit positive intrathymic stem cells that are restricted to lymphoid differentiation. *J. Exp. Med. 178:1283.*
9. Antica, M., L. Wu, K. Shortman, and R. Scollay. 1993. Intrathymic lymphoid precursor cells during fetal thymus development. *J. Immunol. 151:5887.*
10. Sharrow, S. O., B. J. Mathieson, and A. Singer. 1981. Cell surface appearance of unexpected host MHC determinants on thymocytes from radiation bone marrow chimeras. *J. Immunol. 126:1327.*
11. Shores, E. W., S. O. Sharrow, and A. Singer. 1991. Presence of CD4 and CD8 determinants on CD4-CD8-murine thymocytes: passive acquisition of CD8 accessory molecules. *Eur. J. Immunol. 21:973.*
12. Shinkai, Y., G. Rathbun, K.-P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A. M. Stall, and F. W. Alt. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell 68:855.*
13. Rahemtulla, A., W. P. Fung-Leung, M. W. Schilham, T. M. Kundig, S. R. Sambhara, A. Narendran, A. Arabian, A. Wakeham, C. J. Paige, R. M. Zinkernagel, R. G. Miller, and T. W. Mak. 1991. Normal development and function of $CD8^+$ cells but markedly decreased helper cell activity in mice lacking CD4. *Nature 353:180.*
14. Zúñiga-Pflücker, J. C., D. Jiang, P. L. Schwartzberg, and M. J. Lenardo. 1994. Sublethal γ-radiation induces differentiation of $CD4^-/CD8^-$ into $CD4^+/CD8^+$ thymocytes without T cell receptor β rearrangement in recombinase activation gene $2^{-/-}$ mice. *J. Exp. Med. 180:1517.*
15. Zúñiga-Pflücker, J. C., and A. M. Kruisbeek. 1990. Intrathymic radioresistant stem cells follow an IL-2/IL-2R pathway during thymic regeneration after sublethal irradiation. *J. Immunol. 144:3736.*
16. Nakano, T. 1995. Lymphohematopoietic development from embryonic stem cells in vitro. *Semin. Immunol. 7:197.*
17. Nakano, T., H. Kodam, and T. Honjo. 1994. Generation of lympho-hematopoietic cells from embryonic stem cells in culture. *Science 265:1098.*
18. Carlyle, J. R., A. M. Michie, C. Furlonger, T. Nakano, M. J. Lenardo, C. J. Paige, and J. C. Zúñiga-Pflücker. 1997. Identification of a novel developmental stage marking lineage commitment of progenitor thymocytes. *J. Exp. Med. 186:173.*
19. Storkus, W. J., H. J. Zeh III, M. J. Maeurer, R. D. Salter, and M. T. Lotze. 1993. Identification of human melanoma peptides recognized by class I-restricted tumor-infiltrating T lymphocytes. *J. Immunol. 151:3719.*
20. Wu, L., C.-L. Li, and K. Shortman. 1996. Thymic dentritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. *J. Exp. Med. 184:903.*
21. Jiang, D., M. J. Lenardo, and J. C. Zúñiga-Pflücker. 1996. p53 prevents maturation to the $CD4^+CD8^+$ stage of thymocyte differentiation in the absence of T cell receptor rearrangement. *J. Exp. Med. 183:1923.*
22. Storkus, W. J., H. J. Zeh III, R. D. Salter, and M. T. Lotze. 1993. Identification of T cell epitopes: rapid isolation of class I-presented peptides from viable cells by mild acid elution. *J. Immunother. 14:94.*
23. Ismaili, J., M. Antica, and L. Wu. 1996. CD4 and CD8 expression and T cell antigen receptor gene rearrangement in early intrathymic precursor cells. *Eur. J. Immunol. 26:731.*
24. Owen, J. J. T., and E. J. Jenkinson. 1984. Early events in T lymphocyte genesis in the fetal thymus. *Am. J. Anat. 170:301.*
25. Havran, W. L., and J. P. Allison. 1988. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature 335:443.*
26. Pénit, C., B. Lucas, and F. Vasseur. 1995. Cell expansion and growth arrest phases during the transition from precursor ($CD4^-8^-$) to immature ($CD4^+8^+$) thymocytes in normal and genetically modified mice. *J. Immunol. 154:5103.*
27. Sotzik, F., Y. Rosenberg, A. W. Boyd, M. Honeyman, D. Metcalf, R. Scollay, L. Wu, and K. Shortman. 1994. Assessment of CD4 expression by early T precursor cells and by dendritic cells in the human thymus. *J. Immunol. 152:3370.*