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Two differential pathways from double-negative to double-positive thymocytes

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SUMMARY

Murine thymocytes are divided into four major populations on the basis of expression of CD4 and CD8 antigens. The bulk of evidence favours the view that $CD4^-CD8^-$ cells can develop into $CD4^-CD8^+$ and $CD4^+CD8^-$ cells via the $CD4^+CD8^+$ stage in the thymus. However, $CD4^-CD8^+$ and $CD4^+CD8^-$ thymocyte subsets contain not only $CD3^+$ mature cells but also $CD3^-$ immature cells, which seem to be intermediate cells between $CD4^-CD8^-$ and $CD4^+CD8^+$ cells. Here we demonstrate mouse strain differences in the proportion of immature single-positive thymocyte subsets in thymus at the steady or developing state. In C3H mice, immature $CD4^+CD8^-$ is dominant in proportion over $CD4^-CD8^+$ in foetal thymus and in donor-derived thymocytes at an early stage of bone marrow transplantation. On the other hand, immature $CD4^-CD8^+$ is dominant over $CD4^+CD8^-$ during T-cell development in the case of B10.BR mice. An intermediate pattern was shown in the case of F_1 mice. Both of these immature single-positive subsets gave rise to double-positive cells after 24 hr culture. These results suggest that there exist two distinct differential pathways; one is from $CD4^-CD8^-$ cells to $CD4^+CD8^+$ cells via $CD4^-CD8^+$ cells, and another is via $CD4^+CD8^-$ cells, and that an application of the 'CD8 pathway' or 'CD4 pathway' seems to be genetically destined by BM-derived cells but not by thymic stromal cells.

INTRODUCTION

Thymocytes are divided into four major populations on the basis of expression of CD4 and CD8 antigens. In adult mice, cells negative for CD4 and CD8 antigens contain the earliest T-cell precursors capable of giving rise to phenotypically and functionally all T cells.[1,2] Cells bearing both CD4 and CD8 (double-positive cells, $CD4^+CD8^+$) make up most thymocytes and correspond to cells found predominantly in the cortex. Although double-positive cells, about one-half of which express low levels of TcR/CD3 complex,[3,4] are assumed to be critically involved in positive and negative selection in the thymus, their role in T-cell ontogeny remains elusive.[5–7]. $CD4^+CD8^-$ or $CD4^-CD8^+$ (single-positive) medullary subsets contain immunocompetent cells[8,9] and express high levels of TcR/CD3 complex.[3,4] They are phenotypically and functionally similar to mature T lymphocytes existing at the peripheral lymphoid organs. However, recent experiments revealed that the CD8 single-positive subset in the thymus contains immature cells that are immediate precursors of double-positive thymocytes.[10–12] On the other hand, although it was believed that $CD4^+CD8^-$ thymocytes are comparatively homogeneous and appear at the late stage in T-cell development,[3,12–15] an immature $CD3^-$ subset of $CD4^+CD8^-$ thymocytes was also reported.[16,17] In the present study, we demonstrate that there exist two types of immature single-positive thymocytes, which are $CD4^+CD8^-CD3^-$ and $CD4^-CD8^+CD3^-$. They are detected in thymus at the steady state, and become prominent at the developing stage of differentiation, prior to an appearance of $CD4^+CD8^+$ cells. The frequency of an appearance of each immature single-positive subset is dependent on genetic backgrounds of precursor T cells themselves but not on the thymic environment in which they develop. The implications of these findings for pathways of intrathymic T-cell development are discussed.

Abbreviations: BM, bone marrow; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PE, phycoerythrin.

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MATERIALS AND METHODS

Mice

Female AKR/J(AKR,H-2^k, Thy-1.1) mice were obtained from Seiwa Experimental Animals (Nakatsu, Japan). Female C3H/HeN(C3H, H-2^k, Thy-1.2), C57BR/10BR(B10.BR, H-2^k, Thy-1.2) and (C3H/HeN × C57BL/10BR)F_1 were from Japan SLC, Inc. (Shizuoka, Japan). Timed pregnant mice were obtained from our own vivarium or purchased from Japan SLC Inc. The day of finding a vaginal plug was designated as Day 0 of

embryonic development. Foetal thymus lobes were dissected from the embryos using a stereoscopic microscope and fine forceps for microsurgery.

Chimeras

Eight-week-old AKR mice were irradiated with 950 rads, and 6 hr later these mice were reconstituted with 4×10^6 bone marrow (BM) cells from 8-week-old C3H, B10BR and (C3H × B10.BR)F_1 mice. The BM cells were treated with anti-CD4(GK1.5)[18] and anti-CD8 monoclonal antibody (mAb) (anti-mouse Lyt-2.1 or anti-mouse (Lyt-2.2 Meiji Institute of Health Science, Tokyo, Japan) and complement, then were washed three times with Hanks' balanced solution before reconstitution.

Cell preparation from chimera thymus

Thymocytes were purified according to the panning method by Wysocki & Sato[19] with minor modification. Cell suspensions were poured onto anti-Thy-1.1 mAb-coated dishes. After incubation for 70 min at 4°C, non-adherent cells were removed and the bound cells were recovered by flushing. After two cycles of selection, more than 97% of the recovered cells were Thy-1.1 or Thy-1.2 cells. In some experiments, Thy-1.2 cells were further separated into $CD8^-$ and $CD8^+$ cells by panning method using anti-CD8 mAb-coated dishes. To obtain $CD4^+$ cell-depleted cells, thymocytes were incubated with anti-CD4 antibody (GK1.5) at 4°C for 30 min and poured onto an anti-rat IgG-coated dish. Non-adherent cells were recovered as CD4-depleted thymocytes.

Flow microfluorometry

Phycoerythrin (PE)-conjugated anti-CD4 mAb and FITC-conjugatedanti-CD8 mAb were purchased from Becton-Dickinson (Mountain View, CA). The hybridoma secreting anti-CD3 mAb (clone 145-2C11; kindly provided by Dr J. A. Bluestone) was grown *in vitro* and the supernatant was precipitated by ammonium sulphate and was purified through a protein A column. Purified anti-CD3 mAb was conjugated with FITC in our laboratory.

All samples are treated with ammonium chloride to remove erythrocytes and analysed on a FACSCAN flow cytometer (Becton-Dickinson). Dead cells and debris were excluded from analysis by selective gating based on anterior and right angle scatter. In most experiments, 5×10^4 flow cytometer events were analysed, using Consort 30 software. All data were collected and displayed on a log scale of increasing green and red fluorescence intensity. Data were presented as two-dimensional contour maps. To obtain percentages of the thymocyte subpopulations, total counts were integrated in selected areas of the contour plots. To perform three-colour analysis, FITC-conjugated anti-CD3, PE-conjugated anti-CD4, biotin-conjugated anti-CD8 and streptavidin-conjugated duochrome were used. One-hundred and eighteen thousand cells were analysed per sample. CD3 expression on thymocyte subpopulations was determined by software gating using two staining parameters (CD8 and CD4).

Short term in vitro *culture*

CD8-depleted cells and CD4-depleted cells from donor-derived thymocytes were separated from host thymus on Day 12 after BM transplantation by the panning method. One million cells of each subset in 200 μl were cultured in a 5% CO_2 incubator for 24 hr in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% foetal calf serum (FCS). After 24 hr of culture, the cells were harvested and stained with PE-CD4 and FITC-CD8.

RESULTS

Strain variation in CD3 expression on single-positive cells in adult thymus

Previously, we have reported a preferential appearance of the $CD3^-$ subset of $CD4^+CD8^-$ thymocytes at an early stage of BM chimera, which is detected in exponentially proliferating C3H donor-derived cells.[16] To confirm CD3 expression by CD4 single-positive thymocytes in a steady state, cells from adult untreated mice were analysed by three-dimensional flow-fluorocytometry. Typical fluorescent results are shown in Fig. 1. Proportions of single-positive cells among total thymocytes and those of $CD3^-$ cells among $CD4^+CD8^-$ cells are summarized in Table 1. In normal adult C3H, approximately 30% of $CD4^+CD8^-$ were $CD3^-$, whereas in B10BR, most of the $CD4^+CD8^-$ thymocytes were $CD3^+$. Contrary to that, the proportion of $CD3^-$ cells among $CD4^-CD8^+$ thymocytes was

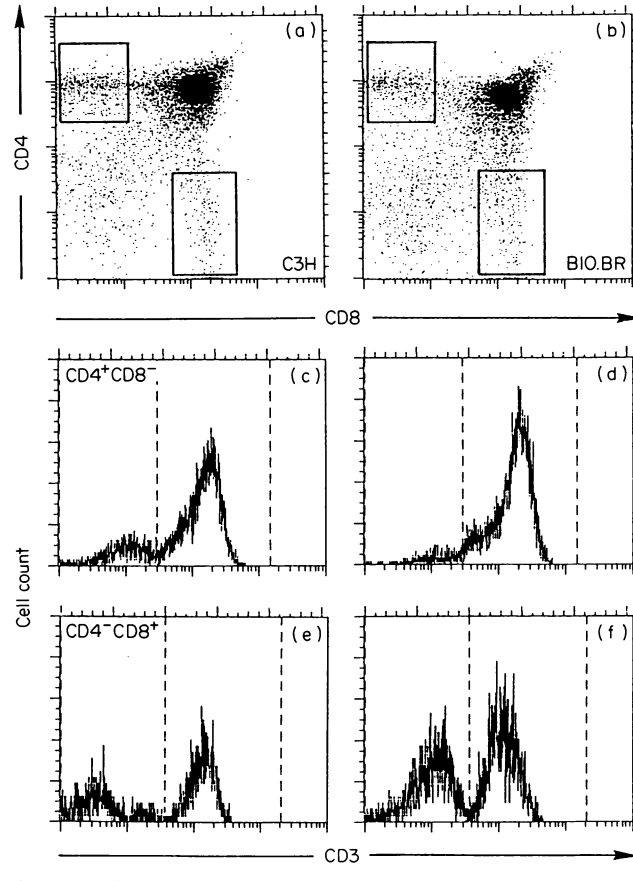

Figure 1. Three-colour analysis of untreated adult C3H(a,c,e) and B10.BR(b,d,f). CD4 versus CD8 expression on whole thymocytes is given (a,b). Single-positive thymocyte subpopulations were determined by software gating. CD3 expression on $CD4^+CD8^-$ cells (c,d) and $CD4^-CD8^+$ cells (e,f) is presented by a single-parameter histogram. Over 5000 cells were counted in each histogram.

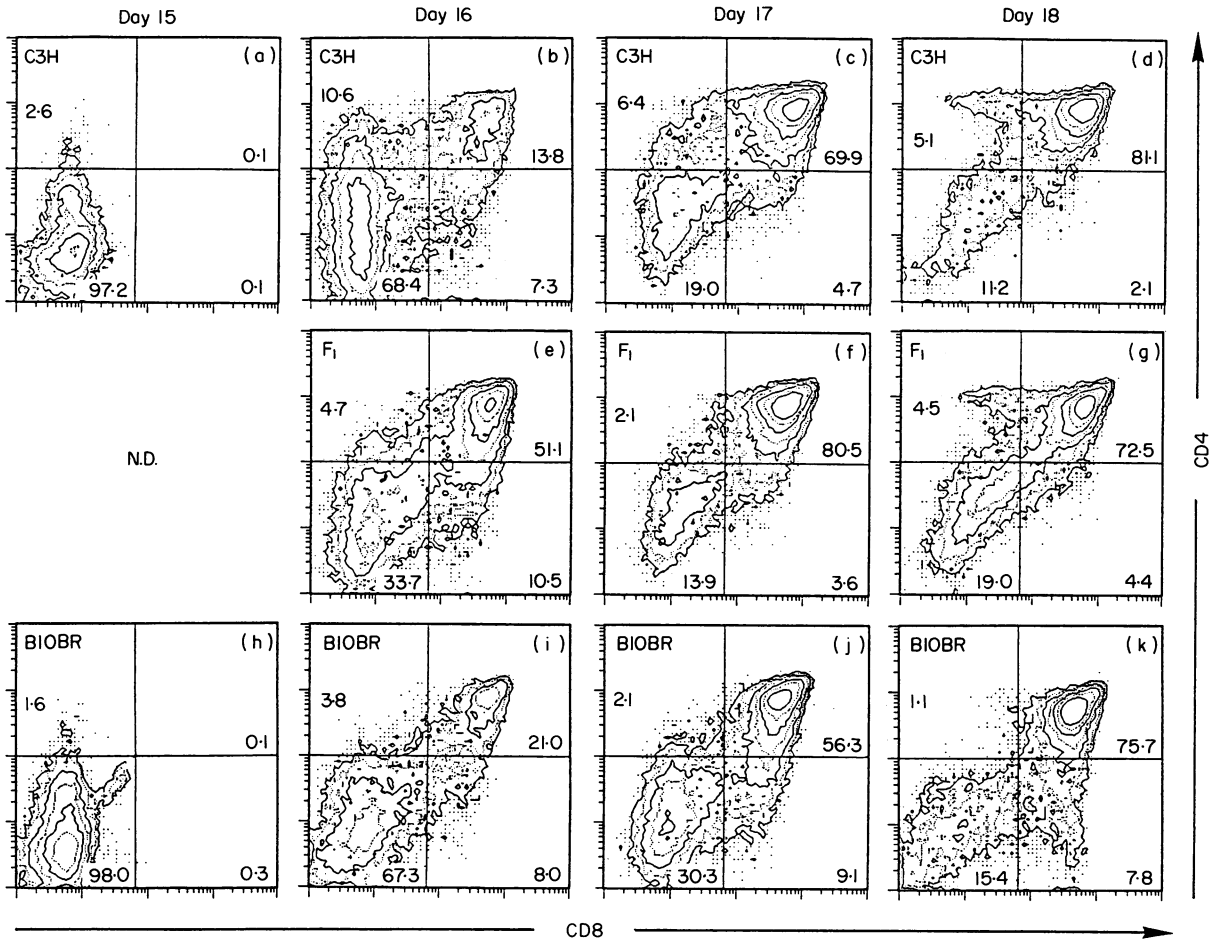

Figure 2. Ontogeny of expression of CD4 and CD8 on foetal thymocytes on Day 15(a,h), Day 16(b,e,i), Day 17(c.f.j) and Day 18(d,g,k). Foetal thymus lobes from C3H(a,b,c,d), (C3H × B10.BR)F_1(e,f,g) and B10.BR(h,i,j,k) were dissected from the embryos using a stereoscopic microscope and fine forceps for microsurgery. Recovered cells were prepared for two-colour flow microfluorometry.

Table 1. CD3 expression of single-positive thymocytes in adult mice

Mouse strain	C3H/He	B10.BR
Total cell counts ($\times 10^{-7}$)	9·7±2·0	21·3±5·0
% $CD4^+CD8^-$/total thymocytes*	11·6±1·1	8·6±0·8
% $CD4^-CD8^+$/total thymocytes†	3·2±0·4	3·6±0·6
% $CD3^-/CD4^+CD8^-$ thymocytes‡	29·1±6·7	11·4±3·5
% $CD3^-/CD4^-CD8^+$ thymocytes§	16·5¶±13·3	32·1±11·2

CD3 expression was determined by immunofluorescent staining with 145-2C11, as described in the Materials and Methods. Nine to 10 mice were examined and the mean value ± SD is given.

*,† Phenotypic subsets were determined by quadrant on the screen of two-dimensional analysis.

‡,§ Each subset was determined by software gating, as shown Fig. 1.

¶ $P < 0.025$.

relatively lower in C3H than that in B10.BR. Single-positive T cells in lymph nodes were shown to contain few $CD3^-$ cells (less than 2%, data not shown) in simultaneous assay.

Appearance of immature single-positive cells during foetal ontogeny

Considering that immature cells are more evident in a growing state rather than in a steady state, it is reasonable to analyse developing thymus. In order to confirm a strain variation in T-cell ontogeny, foetal thymocytes from C3H, B10.BR and (C3H × B10.BR)F_1 were investigated. Figure 2 shows the sequential expression of CD4 and CD8. From Day 15 to Day 18 of foetal development, a proportion of double-negative cells decreased while that of double-positive cells increased. On Day 16 and Day 17, an appreciable number of $CD4^+CD8^-$ cells appeared in C3H foetal thymus (Fig. 2b,c). On the other hand, $CD4^-CD8^+$ cells were dominant in proportion over $CD4^+CD8^-$ cells in B10.BR mice (Fig. 2i,j). The appearance of these single-positive cells preceded an accumulation of double-positive cells. The single-positive cells on Day 16 and on 17 did not express CD3 (data not shown). $CD3^+$ cells were mainly confined to the double-negative subset until Day 18 of gestation (data not shown).[3]

Development of donor-derived thymocytes in radiation BM chimera

To determine whether such strain variation in the appearance of

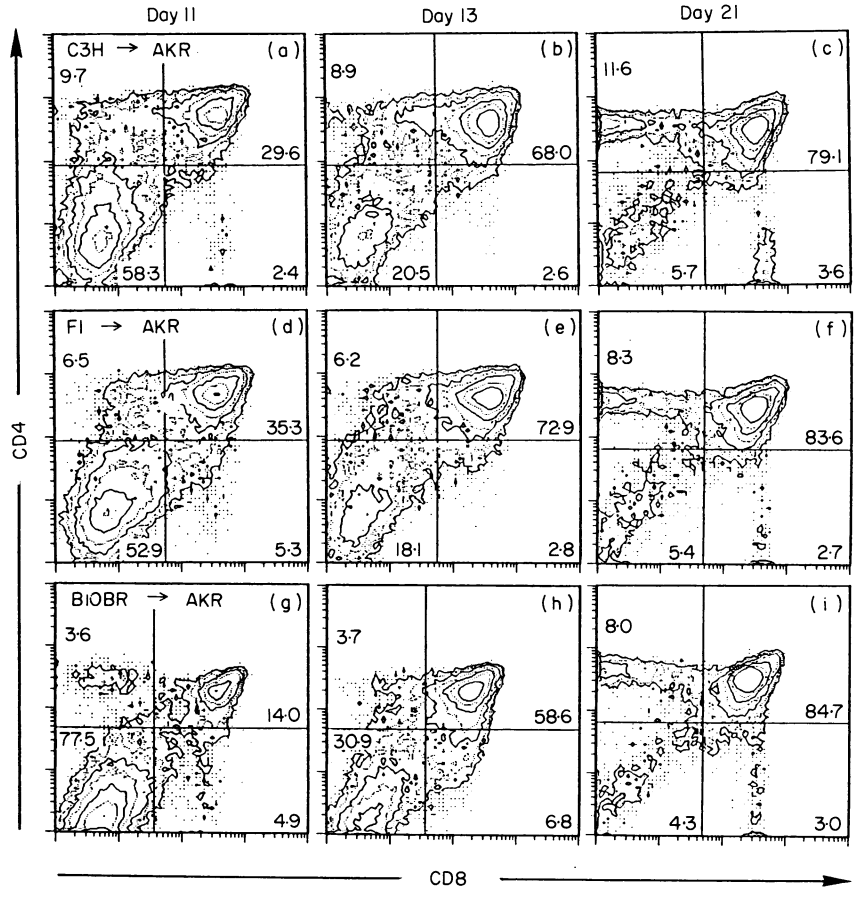

Figure 3. Ontogeny of expression of CD4 and CD8 on donor-derived thymocytes on Day 11(a,d,g), Day 13(b,e,h) and Day 21(c,f,i) after BM transplantation. C3H(a,b,c), (C3H × B10.BR)F_1(d,e,f) and B10.BR(g,h,i) mice were prepared as source of BM cells. Each sample was obtained from chimeric thymus by the panning method as described in the Materials and Methods. All applied samples contained < 1% Thy-1$^+$ (host-derived) cells.

immature single-positive cells is determined by thymic stromal cells or by BM-derived cells, we investigated the development of donor-derived thymocytes in radiation BM chimera. C3H, B10BR or (CRH × B10.BR)F_1 were used as a donor, and H-2-compatible Thy-1-disparate AKR strain was used as a host. As shown in Fig. 3, C3H donor-derived thymocytes preferentially gave rise to immature CD4$^+$CD8$^-$ cells (Fig. 3a,b). On the other hand, the CD4$^-$CD8$^+$ subset was dominant in proportion over the CD4$^+$CD8$^-$ subset in B10BR donor-derived cells on Day 13 (Fig. 3h). (C3H × B10.BR)F_1 donor-derived cells contained an intermediate proportion of both of two immature single-positive subsets. As far as expression of CD4 and CD8, the differentiation pattern of donor-derived thymocytes mimick that of foetal ontogeny. In other words, the appearance of immature single-positive thymocytes seems to be dependent upon BM-derived cells rather than the thymic environment, such as thymic stromal cells.

Strain variation in the ability to give rise to double-positive cells in short-term culture

Supposing that the early-appearing single-positive cells are descendants of double-negative cells, it is useful for understanding T-cell development to elucidate the fate of these cells. Donor-derived thymocytes on Day 12 after BM transplantation, which consist mainly of immature J11$^+$ [16] cortical-type thymocytes,[20] were treated with the panning plate. Both of the CD4-depleted cells and CD8-depleted cells were collected as samples, which were not coated by any antibodies because only non-adherent cells were recovered. The separated cells were incubated at 37°C in RPMI-1640 with 10% FCS in the absence of added mitogens or growth factors. Data from CD8-depleted (C3H × B10.BR)F_1-derived cells were not demonstrated because of the poor purity of recovered cells, which could be caused by co-expression of Lyt-2.1 and Lyt-2.2 on (CRH × B10.BR)F_1 cells. Figure 4 shows CD4 and CD8 expression of the separated cells before and after short-term culture. CD8-depleted cells derived from C3H gave rise to an appreciable number of double-positive cells, which reached 8·9% of recovered cells (Fig. 4a,b) after 24 hr incubation. On the other hand, CD4-depleted cells derived from C3H produced much less double-positive cells, 2·6% of the recovery (Fig. 4e,f). In the case of B10.BR-derived cells, over 11% of recovered cells were double-positive from the culture of CD4-depleted cells (Fig. 4i,j) with only 3·5% from CD8-depleted cells (Fig. 4c,d). CD4-depleted cells from (C3H × B10.BR)F_1-derived thymocytes gave rise to a considerable number of double-positive cells associated with an appearance of CD4-single positive cells (Fig. 4g,h).

The difference in ability to give rise to double-positive cells is

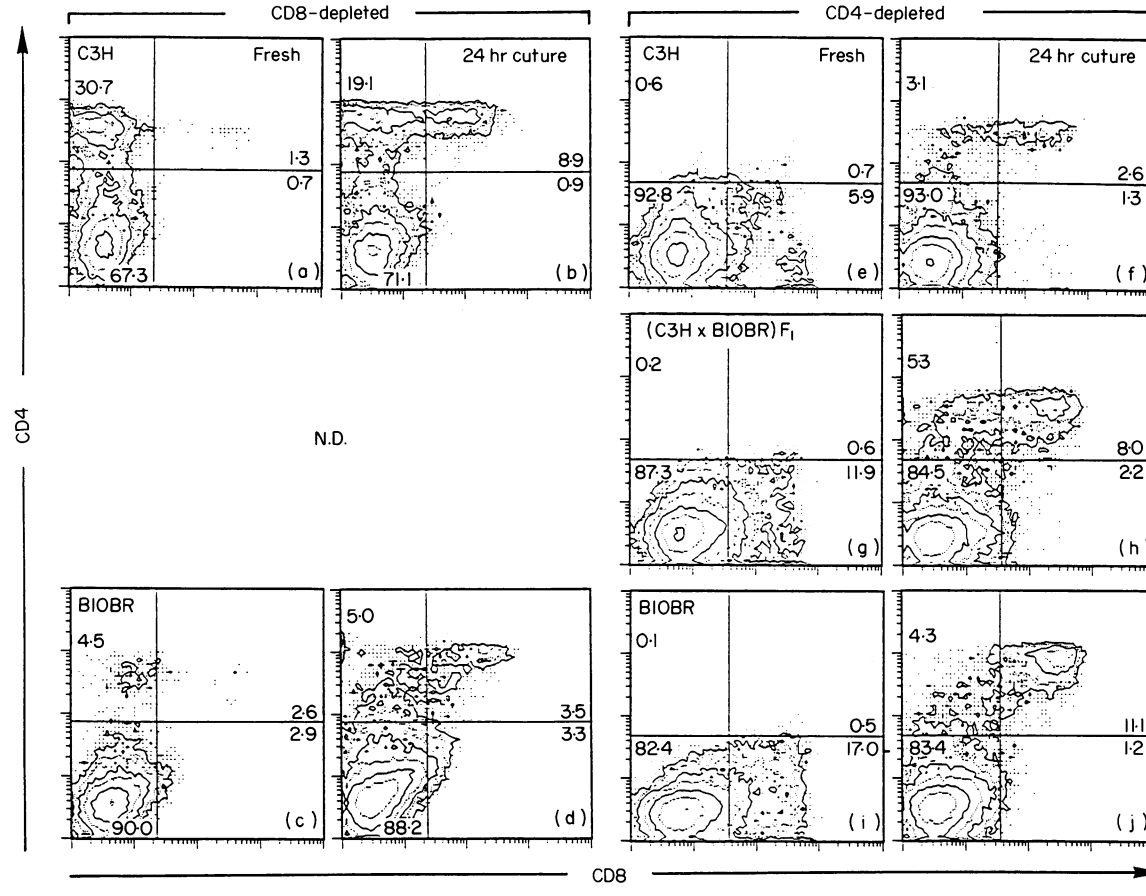

Figure 4. *In vitro* differentiation of donor-derived thymocytes on Day 12 after BM transplantation. C3H(a,b,e,f), (C3H × B10.BR)F_1(i,j) and B10.BR(c,d,g,h) donor-derived cells were collected and purified into CD4-depleted(a–d) and CD8-depleted (e–j) cells, which contained $<2\%$ $CD4^+$ cells or $<2\%$ $CD8^+$ cells, respectively. Each subset was analysed for CD4 and CD8 expression immediately (a,c,e,g,i) and after 24 hr culture (b,d,f,h,j).

mainly due to a difference in the proportion of immature single-positive cells in the applied samples, because the potential to yield double-positive cells by purified double-negative cells is much less than purified immature single-positive cells in short-term culture.[16,21]

DISCUSSION

We have detected a strain difference in the proportion of the $CD3^-$ subset in single-positive cells in the thymus of untreated adult mice, and in the appearance of immature single-positive cells during T-cell development. In C3H mice, immature $CD4^+CD8^-$ is dominant in proportion over $CD4^-CD8^+$ not only in foetal and adult thymus but also in donor-derived thymocytes at an early stage of BM transplantation. On the other hand, immature $CD4^-CD8^+$ is dominant in proportion over $CD4^+CD8^-$ during T-cell development in the case of B10.BR mice. An intermediate pattern was shown in the case of (CRH × B10.BR)F_1 mice.

The processes which lead to the development of mature T cells are not well understood. Haematopoietic stem cells begin to colonize the thymic rudiment at days 10–11 of gestation. The earliest foetal thymocytes express neither CD4, CD8 nor the TcR/CD3 complex.[3] The first CD3-associated TcR to appear in thymocyte development is TcR$\gamma\delta$.[22] Subsequently, TcR$\alpha\beta$-bearing cells represent the major $CD3^+$ thymocyte population. The accessory molecules are first expressed on Days 15–16 of development.[3] The double-positive cells increase in proportion to make up roughly 80% of the total thymocytes on foetal Day 19. Several reports suggested that the appearance of double-positive thymocytes was preceded by a population of cells that were $CD4^-CD8^+CD3^-$.[9,15,23] $CD4^-CD8^+CD3^-$ cells, which were also seen in the adult thymus, appear to represent thymocytes on the way to becoming $CD4^+CD8^+$ but have not yet expressed CD4.[10–12,24–26] All the isolated $CD4^-CD8^+CD3^-$ cells expressed CD4 molecules in short-term culture without any growth factors or mitogens.[10,11,21] Further, intrathymic injection of $CD4^-CD8^+CD3^-$ cells into Thy-1 congeneic mice gave rise to not only $CD4^+CD8^+$ cells but also $CD4^+CD8^-CD3^+$ cells *in vivo*.[14] These reports suggest a high developmental potential of the $CD3^-$ subset in $CD4^-CD8^+$ thymocytes.

Compared with $CD4^-CD8^+$ cells, there is little information for the heterogeneity of $CD4^+CD8^-$ thymocytes. Fowlkes referred to the phenotypic and functional heterogeneity in $CD4^+CD8^-CD3^+$ cells in her review.[24] Shortman *et al.* reported that $CD4^+CD8^-$ thymocytes are heterogeneous in HSA(J11d) expression.[12,27] However, $CD4^+CD8^-CD3^-$ has not been

described in detail. In the present study, we have found that CD4 single-positive cells contain an immature $CD3^-$ subset in normal adult and foetus as well as in the early stage of BM chimeras.[16] This subset also has the ability to give rise to double-positive cells in short-term *in vitro* cultures,[16] as well as the $CD4^-CD8^+CD3^-$ subset. They are also detected in the thymus of sublethally irradiated mice. In C3H mice, over 60% of $CD4^+CD8^-$ thymocytes are $CD3^-$ on Day 9 after sublethal (600 rads) irradiation (data not shown).

The $CD4^+CD8^-CD3^-$ thymocyte subset was detected preferentially in adult C3H mice but was hardly detected in B10.BR mice during T-cell development. Conversely, the immature CD8 single-positive cells were detected more abundantly in B10.BR mice than in C3H mice, as described in the Results. As noted in the previous report, the appearance of two immature single-positive subsets is clearly influenced by the mouse genetic background. As far as we have investigated, MRL and BALB/c mouse strains have shown similar patterns to (C3H × B10BR) mice. B57BL/6($H\text{-}2^b$) was similar to B10BR (data not shown). CBA/N showed a C3H-like appearance, which was supported by Hugo *et al.*[17] In addition, the result that T precursors from $(C3H \times B10.BR)F_1$ mice produced both of the two types of immature single-positive cells may suggest co-expression of two differentiation pathways.

Analysis after short-term culture revealed that both immature single-positive subsets represent immediate precursors of double-positive cells. Thus, it is suggested that there exist two kinds of pathways from $CD4^-CD8^-$ cells to $CD4^+CD8^+$ cells via $CD4^-CD8^+$ cells or via $CD4^+CD8^-$ cells in T-cell development in mice. The number of dividing cells in transition to $CD4^+CD8^+$ cells through this 'CD8 pathway' appears to be considerably greater than the number of dividing cells on a corresponding 'CD4 pathway',[13] like B10.BR or other strains. However, like C3H mice, there may be some mouse strains that use the 'CD4 pathway' preferentially for T-cell development. Strain difference in the appearance of the $CD3^-$ subset in single-positive thymocytes in untreated adult mice may be attributed to the strain difference in choice of differential pathways.

There are still several significant questions. (i) What determines the application of the 'CD8 pathway' or 'CD4 pathway'? In other words, does the 'pathway' simply provide the order for expression of CD4 and CD8? (ii) Is there any difference between double-positive cells derived from each pathway? (iii) Whether the immature single-positive cells develop to mature single-positive without passing through the double-positive stage or not. Our data on BM chimera revealed that it is T-cell precursors but not the thymic environment that determines the choice of pathway. However, extensive purification or cell cloning of each immature single-positive subset would be needed in order to answer the remaining questions.

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