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Identification of human T-lymphoid progenitor cells in $CD34^+CD38^{low}$ and $CD34^+CD38^+$ subsets of human cord blood and bone marrow cells using NOD-SCID fetal thymus organ cultures

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Summary. In contrast to myeloid and B-lymphoid differentiation, which take place in the marrow environment, development of T cells requires the presence of thymic stromal cells. We demonstrate in this study that human $CD34^+$, $CD34^+CD38^+$ and $CD34^+CD38^{low}$ cells from both cord blood and adult bone marrow reproducibly develop into $CD4^+CD8^+$ T cells when introduced into NOD-SCID embryonic thymuses and further cultured in organotypic cultures. Such human/mouse FTOC (fetal thymic organ culture) thus represents a reproducible and sensitive system to assess the T-cell potential of human primitive progenitor cells. The frequency of T-cell progenitors among cord-blood-derived $CD34^+$ cells was estimated to be 1/500. Furthermore, the differentiation steps classically observed in human thymus were reproduced in NOD-SCID FTOC initiated with cord blood and human marrow $CD34^+$ cells: immature human $CD4^{low}CD8^-$sCD3$^-$TCR$\alpha\beta^-CD5^+CD1a^+$ T cells were mixed with $CD4^+CD8^+$ cells and more mature $CD4^+CD8^-$TCR$\alpha\beta^+$ cells. However, in FTOC initiated with bone marrow T progenitors, <10% double-positive cells were observed, whereas this proportion increased to 50% when cord blood $CD34^+$ cells were used, and most $CD4^+$ cells were immature T cells. These differences may be explained by a lower frequency of T-cell progenitors in adult samples, but may also suggest differences in the thymic signals required by bone marrow versus cord blood T progenitors. Finally, since cytokine-stimulated $CD34^+CD38^{low}$ cells retained their ability to generate T cells, these FTOC assays will be of value to monitor, when combined with other biological assays, the influence of different expansion protocols on the potential of human stem cells.

Keywords: T lymphopoiesis, stem cells, NOD-SCID mice, thymus, cord blood.

Evaluation of the haemopoietic potential of human donor cells in transplantation protocols is usually based on the quantitation of colony-forming cells (CFC) and long-term culture initiating cells (LTC-IC) (Sutherland *et al*, 1989) *in vitro*, and more recently human cells reconstituting NOD-SCID recipients (Cashman *et al*, 1997; Conneally *et al*, 1997; Pflumio *et al*, 1996; Wang *et al*, 1997). Except for the *in vivo* NOD-SCID assay, which detects both B-lymphoid and myeloid progenitors, most of the *in vitro* assays identify an exclusive myeloid differentiation, and only during the past years have *in vitro* conditions been designed which successfully allow human $CD34^+CD38^{low}$ cells to differentiate into $CD19^+$ pro-B cells (Berardi *et al*, 1997; Hao *et al*, 1998; Galy *et al*, 1995a; Rawlings *et al*, 1997), and $CD56^+$ NK cells (Carayol *et al*, 1998). However, the identification of the T-cell potential of human pluripotent stem cells has remained a major obstacle both *in vitro* and *in vivo*. Feeders of thymic stromal cells do not reproducibly support T-cell differentiation *in vitro* (Rosenzweig *et al*, 1996) and the most successful strategy so far is based on the colonization by test cells of intact human or murine embryonic thymic lobes, which are then either kept in *in vitro* organotypic culture (fetal thymic organ culture; FTOC) (Jenkinson & Anderson, 1994; Merkenschlager *et al*, 1992) or grafted into a SCID recipient (Barcena *et al*, 1994; Galy *et al*, 1995b; Péault *et al*, 1991). Both human (Barcena *et al*, 1994; DiGustio *et al*, 1994; Galy *et al*, 1994, 1995b; Péault *et al*, 1991) and murine (Blom *et al*, 1997; Plum *et al*, 1994; Verhasselt *et al*, 1998;

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Yeoman *et al*, 1993) FTOC have enabled the identification of T-cell progenitors among adult as well as fetal human $CD34^+$ suspensions. In murine thymus, however, cell proliferation was lower than in human FTOC, and the production of single-positive $CD8^+$ cells was compromised because murine class I molecules are poor effectors of the CD8 selection. Nevertheless, these studies demonstrated that human T-lymphoid differentiation could proceed in a murine thymic environment *in vitro* and recent data suggest that this might occur also *in vivo* in NOD-SCID recipients (van der Loo *et al*, 1998; Yurasov *et al*, 1997). Since active human B lymphopoiesis and myelopoiesis take place *in vivo* in NOD-SCID mice (Cashman *et al*, 1997; Kollmann *et al*, 1994; Pflumio *et al*, 1996; Vormoor *et al*, 1994), and *in vitro* in the presence of murine stromal feeders (Berardi *et al*, 1997; Galy *et al*, 1995a; Rawlings *et al*, 1997), one can conclude that a murine environment can support the differentiation of primitive human haemopoietic cells in all lymphoid and myeloid pathways at least when cells from a fetal source are used.

We have previously described *in vitro* conditions suitable for B-lymphoid, NK and myeloid differentiation of $CD34^+CD38^{low}$ human cells (Berardi *et al*, 1997; Carayol *et al*, 1998; Croisille *et al*, 1994). These were based on the coculture of human cells with a murine stromal cell line in the presence of selected human cytokines. Since identification of stem cells requires assessment of their T-cell potential, we investigated in this study the capacity of embryonic thymic lobes from NOD-SCID mice to support T-cell differentiation from human $CD34^+$ cells. We report that NOD-SCID FTOC cultures reproducibly identify T-cell progenitors in both $CD34^+CD38^{low}$ and $CD34^+CD38^+$ cells from adult bone marrow and cord blood. These progenitors differentiated following the orderly sequence of events which occurs in human thymus. We further show that a short stimulation of $CD34^+CD38^{low}$ cells by a combination of cytokines including IL-3 did not alter their T-cell potential, an observation which may be clinically relevant. Mouse/human hybrid FTOC therefore emerge as a useful tool to add to the panel of biological assays developed to assess the full lymphoid and myeloid differentiative potential of primitive human stem cells.

MATERIALS AND METHODS

Mice. NOD-LtSz-scid/scid (hereafter called NOD-SCID) were originally obtained from Dr John Dick (Hospital for Sick Children, Toronto, Ontario, Canada). Mouse breeding pairs were housed in the animal facilities of the Institut Gustave Roussy under sterile conditions in air-filtered containers. 14–16 d pregnant NOD-SCID mice were used in most of the experiments. The age of development of the embryos was calculated taking as day 0 the morning on which the mating plug was observed. In experiments performed by microinjection, thymuses from newborn mice were used.

Collection and fractionation of human cord blood (CB), bone marrow (BM) and mobilized peripheral blood (PB) mononuclear cells (MNC). Cord blood samples from full-term newborns were collected (Hospital Saint-Vincent de Paul, Paris, France) with the informed consent of the mothers. Samples were diluted 1:2 in PBS without magnesium (−Mg) and calcium (−Ca) prior to separation over Ficoll/Hypaque (Lymphoprep, Nyegaard, Oslo; density 1·077 g/ml). All further manipulations of low-density CB-MNC were done in PBS–0·5% BSA. Low-density CB-MNC were subjected to a standard CD34 immunomagnetic bead separation using the miniMACS system following the manufacturer's guidelines (Miltenyi Biotec, Calif.). Bone marrow cells were extracted from bone fragments collected during surgery for hip replacement as previously described (Croisille *et al*, 1994) and $CD34^+$ cells were obtained from light-density cell suspensions following the procedure described above for cord blood specimens. In order to exclude contamination of the purified $CD34^+$ cells by mature T cells, $CD34^+$ cells obtained after bead separation were further purified by cell sorting. Sorting of $CD34^+$, $CD34^+CD38^{low}$ and $CD34^+CD38^+$ fractions was performed using a FACS-Vantage equiped with an argon ion laser (Innova 70–4-Coherent radiation, Palo Alto, Calif.) tuned to 488 nm and operating at 500 mW. A morphological gate including all the $CD34^+$ cells was determined on two-parameter histograms side scatter (SSC) versus forward scatter (FSC). Positivity or negativity for CD38 among the $CD34^+$ cells was determined using control cells labelled with the PE-CD34 (HPCA2, Becton Dickinson) Moab and an irrelevant IgG1 MoAb. Compensation was set up as described above. The $CD34^+CD38^{low}$ population represented 10–15% of the total $CD34^+$ population. It is well known that expression of CD38 is more heterogenous on $CD34^+$ cells from bone marrow than on cord blood or PB $CD34^+$ cells. $CD34^+$ cells with no expression of CD38 (as defined by the irrelevant IgG) represented 1–5% of the $CD34^+$ cells in bone marrrow but were almost undetectable among cord blood $CD34^+$ cells.

In all experiments, $CD34^+$, $CD34^+CD38^+$ and $CD34^+CD38^{low}$ cells were incubated with murine thymic lobes immediately after their purification. In some experiments $CD34^+CD38^{low}$ cells were incubated for 7 d with murine stromal MS-5 cells (Itoh *et al*, 1989) and cytokines; FTOC were initiated with $CD34^+$ cells sorted from the cultured cells. In these experiments, recombinant (r) human (hu) cytokines included rhuSCF (stem cell factor, 20 ng/ml, kindly provided by Amgen, Thousands Oaks, Calif., U.S.A.), rhu-IL-3 (2–100 ng/ml), rhu-IL-6 (100 U/ml), rhu-GM-CSF (granulocyte-macrophage colony-stimulating factor, 10 ng/ml), PEG-rhu MGDF (megakaryocyte growth and differentiation factor,10 ng/ml, kindly provided by Amgen, Thousands Oaks, Calif.), rhu-Flt3-L (10 ng/ml), rhu-IL-2 (5 ng/ml) and rhu-IL-7 (20 ng/ml) (all from Diaclone, Besançon, France).

Fetal thymic organ culture (FTOC). FTOC cultures were done as initially described by Jenkinson & Anderson (1994) and Merkenschlager & Fisher (1992), except that thymi were collected from NOD-SCID mice. Since the SCID mutation blocks T-cell development in NOD-SCID mice at an early stage, removal of the endogenous population of thymocytes was unnecessary.

Colonization of the thymic tissue by human cells was facilitated by the hanging-drop procedure. Each thymic lobe was incubated in Terasaki wells for 2 d in 25 μl of complete medium: RPMI 1640 supplemented with 10% heat-inactivated human serum, 5% fetal calf serum (FCS, Stem Cell Technologies, Vancouver, Canada), 100 IU/ml penicillin,

100 μg/ml streptomycin, 2 mM L-glutamine. 10–50 000 $CD34^+$ cells purified from cord blood or bone marrow were added to each well. The plates were immediately inverted to allow the formation of hanging drops and incubated undisturbed in a humidified incubator (5% CO_2 in air, 37°C). After 48 h, thymic lobes were transferred onto floating nucleopore filters (Isopore membrane, 25 mm in diameter, pore size 8 μm, Millipore SA, France) in six-well plates in 2·5 ml of complete medium and cultured for 28–35 d at 37°C in air supplemented with 5% CO_2 with a weekly medium change. Cytokines [rhu-IL-2 (5 ng/ml), 20 ng/ml rhu-IL-7 and 50 ng/ml rhu-SCF] were included only during the first 48 h, essentially to prevent loss of human cells by apoptosis (Kondo *et al*, 1997) and to promote the survival of early T-cell progenitors (Akashi *et al*, 1997).

At the end of the culture period, human cells were recovered from the thymic lobes by mechanical disruption of the tissue between two sieves. Initial experiments showed that this procedure extracted most viable cells from the thymus. Cells extracted from the different lobes were pooled except in limiting dilution experiments where lobes were individually processed.

Phenotype of human cells collected from cultured thymic lobes. Pooled cells extracted from thymic lobes were incubated with the following murine MoAbs recognizing human antigens and coupled to phycoerythrin (RPE), fluorescein isothyocyanate (FITC) or PE-Cy-5. The MoAbs used recognized the following human antigens: CD45 (CD45-FITC, Immunotech), CD4 (CD4-FITC, Pharmingen; CD4-PE, Becton Dickinson; CD4-PE-Cy-5, Pharmingen), CD8 (CD8-FITC, Becton Dickinson; CD8-PE, Pharmingen), CD3 (CD3-FITC, Immunotech and Coulter), TCR αβ (TCR αβ-PE, Immunotech), TCR γδ (TCR γδ-PE, Immunotech), CD7 (CD7-FITC, CD7-PE, both from Pharmingen), CD34 (CD34-FITC, Immunotech; CD34-PE, Becton Dickinson), CD38 (CD38-FITC, Immunotech), CD1a (CD1a-PE, Cymbus Bioscience), CD56 (CD56-PE, Immunotech; CD56-PE-Cy-5, Immunotech), CD19 (CD19-PE, Becton Dickinson), CD11b (CD11b-FITC, Immunotech), CD13 (CD13-FITC, Immunotech), CD14 (CD14-PE, Immunotech), CD36 (CD36-FITC, Immunotech) and CD33 (CD33-FITC and CD33-PE, Cymbus Bioscience). Lack of reactivity of the mouse anti-human MoAbs with NOD-SCID murine cells was ruled out in pilot experiments by labelling either marrow or thymic cells from untreated NOD-SCID mice with the above MoAbs. The morphological characteristics of mouse T-precursor cells were defined by staining with antibodies specific for murine antigens. Background levels were measured using isotypic controls. Analysis was done on a FACS-Sort (Becton Dickinson). When two-colour labelling was performed, compensation was set up with single-stained samples. Low forward scatter elements were excluded from the analysis by gating them out and 10 000 events were collected and analysed using the Cellquest software (Becton Dickinson). Labelling was performed in 50 μl of α-MEM/10% FCS for 20 min on ice. In some experiments the intracytoplasmic expression of CD3 was determined using a cell permeabilization kit (Harlan, Sera-Lab Ltd, Crawley Down). Negative controls included cells processed simultaneously and incubated with a non-relevant IgG.

RESULTS

NOD-SCID FTOC support the differentiation of human $CD34^+$, $CD34^+CD38^{low}$ and $CD34^+CD38^+$ T-cell progenitor cells

In a first series of experiments we showed the equal ability of FTOC established with deoxyguanosine-depleted thymic lobes from immune competent (C57BL × Balb-C) or with untreated lobes from immune deficient (SCID and NOD-SCID) murine

Table I. Results of FTOC experiments performed with $CD34^+$, $CD34^+CD38^{low}$ and $CD34^+CD38^+$ cells isolated from human cord blood and human bone marrow.

			Percentage of cells‡ (mean ±SEM)			
Cell fraction	Exp. (pos./total)*	Nucleated cells/lobe (mean ±SEM)†	$CD45^+$	$CD4^+$ total	$CD4^+CD8^+$ (% in $CD4^+$)§	$CD4^-CD8^+$
Cord blood						
$CD34^+$	9/9	20 400 ± 4800	60¶	49·4 ± 5·4	43·5 ± 10	2·4 ± 0·8
$CD34^+CD38^{low}$	6/7	7500 ± 2100	57·7 ± 11	41·3 ± 8	26·6 ± 9·2	1·1 ± 0·7
$CD34^+CD38^+$	6/6	14 700 ± 6200	81·6 ± 4·4	56 ± 10	44·3 ± 7	3 ± 0·8
Bone marrow						
$CD34^+$	4/5	11 500 ± 1400	35; 61	24·2 ± 1·8	10 ± 2	0·6 ± 0·3
$CD34^+CD38^{low}$	2/2	8700; 11 900	30·8; 50	22; 37	4·2; 3	0; 0
$CD34^+CD38^+$	2/2	5000; 18 100	23·3; 47	29; 13·9	7·7; 18	0

* An experiment was considered positive when DP $CD4^+CD8^+$ cells were observed.

† Numbers show the number of nucleated cells/lobe calculated from the total number of nucleated cells recovered from all the pooled thymic lobes divided by the total number of lobes.

‡ Cells were analysed in the lymphoid gate (defined in Fig 1). Numbers represent the mean (±SEM) % of human cells determined in the indicated number of positive experiments.

§ The proportion of $CD4^+CD8^+$ cells in the total $CD4^+$ fraction was calculated.

¶ When results were from fewer than three experiments mean and SEM were not calculated and raw data are shown.

embryos to support the development of double-positive $CD4^+CD8^+$ cells from cord blood $CD34^+$ cells (data not shown). For practical reasons, all further experiments were performed with NOD-SCID thymuses.

In nine separate experiments we assessed the capacity of embryonic thymic lobes from day 14 NOD-SCID embryos to support T-cell development from cord-blood-derived $CD34^+$ cells (Table I). Usually 10–15 thymic lobes were seeded per experiment, and each lobe was incubated with 20–50 000 sorted $CD34^+$ cells. After 30 d an average of 20 000 nucleated cells was recovered per lobe, 60% of which were labelled with an anti-human CD45 antibody. Even though these numbers were below the numbers of input cells, since only a few of these $CD34^+$ cells entered into the thymus (Plum *et al*, 1994), active cell proliferation probably occurred. $CD4^+$ cells were recovered in each experiment and represented 49·4 ± 5·4% (range 28·4–82%) of nucleated cells. Half (43·5 ± 10%, range 4·5–82%) of these were double-positive (DP) $CD4^+CD8^+$ cells (Table I and Fig 1A). Single-positive $CD4^+$ cells included a majority of immature T cells prior to the DP stage and a minority of mature T cells (see below detailed phenotypic analysis).

In order to determine whether T-cell progenitors were also present in the $CD34^+CD38^{low}$ fraction which is enriched in primitive B lymphoid and myeloid progenitors (Berardi *et al*, 1997; Croisille *et al*, 1994), we initiated FTOC experiments with $CD38^{low}$ cells (10–15 000 cells/lobe) ($n = 7$) and the corresponding $CD38^+$ cells (10–15 000 cells/lobe) ($n = 6$) separately. Cells were incubated with two different combinations of human recombinant cytokines during the 48 h of hanging drop: either IL-2, IL-7 and SCF ($n = 5$) or the same three plus IL-3, GM-CSF, IL-6, PEG-rhu-MGDF and Flt3-L ($n = 2$). Since both cocktails yielded similar results, the results were pooled.

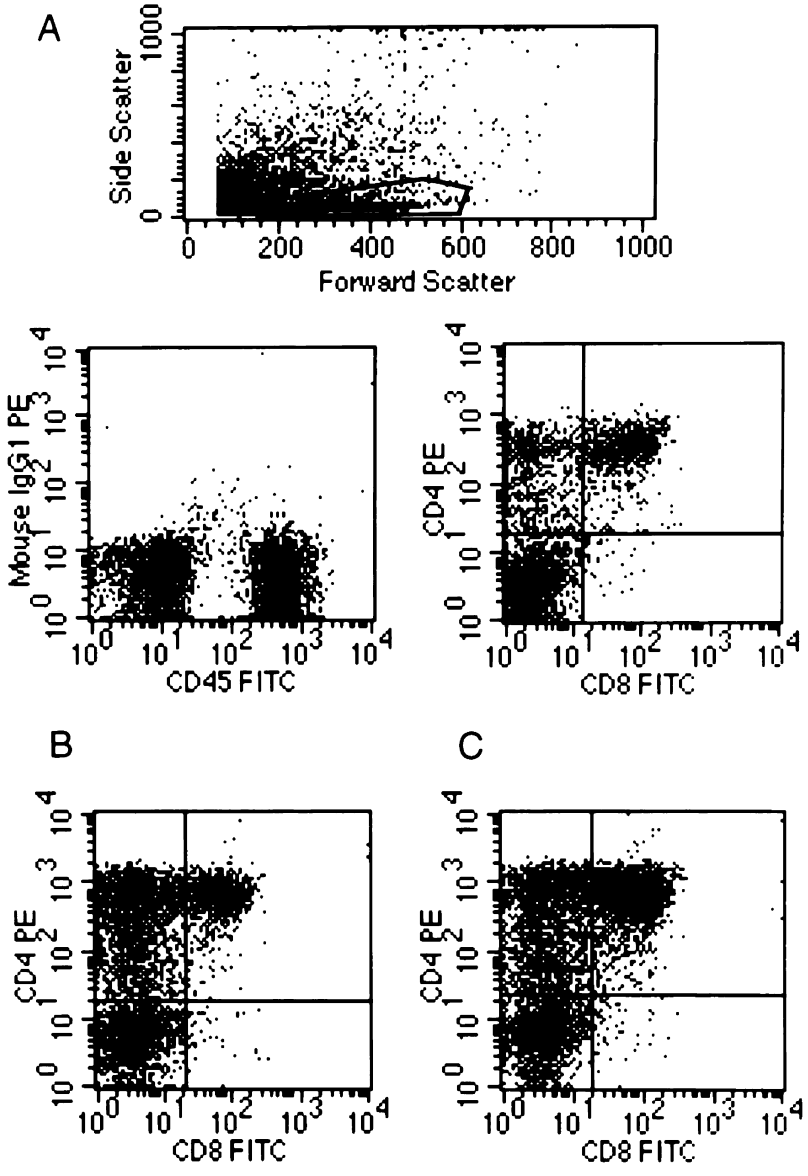

Fig 1. Phenotype of nucleated cells recovered from NOD-SCID thymic lobes reconstituted with cord blood $CD34^+$ cells. NOD-SCID embryonic thymuses were incubated with (A) 10 000 $CD34^+$ cells, (B) 10 000 $CD34^+CD38^{low}$ cells, and (C) $CD34^+CD38^+$ cells. After 30 d in FTOC culture, cells recovered from thymuses were labelled with a panel of T-cell markers specific for human cells and analysed in the lymphoid gate defined in panel (A). Shown here are the results of labelling with anti-human CD45, and two-colour labelling with anti-human CD4 and anti-human CD8.

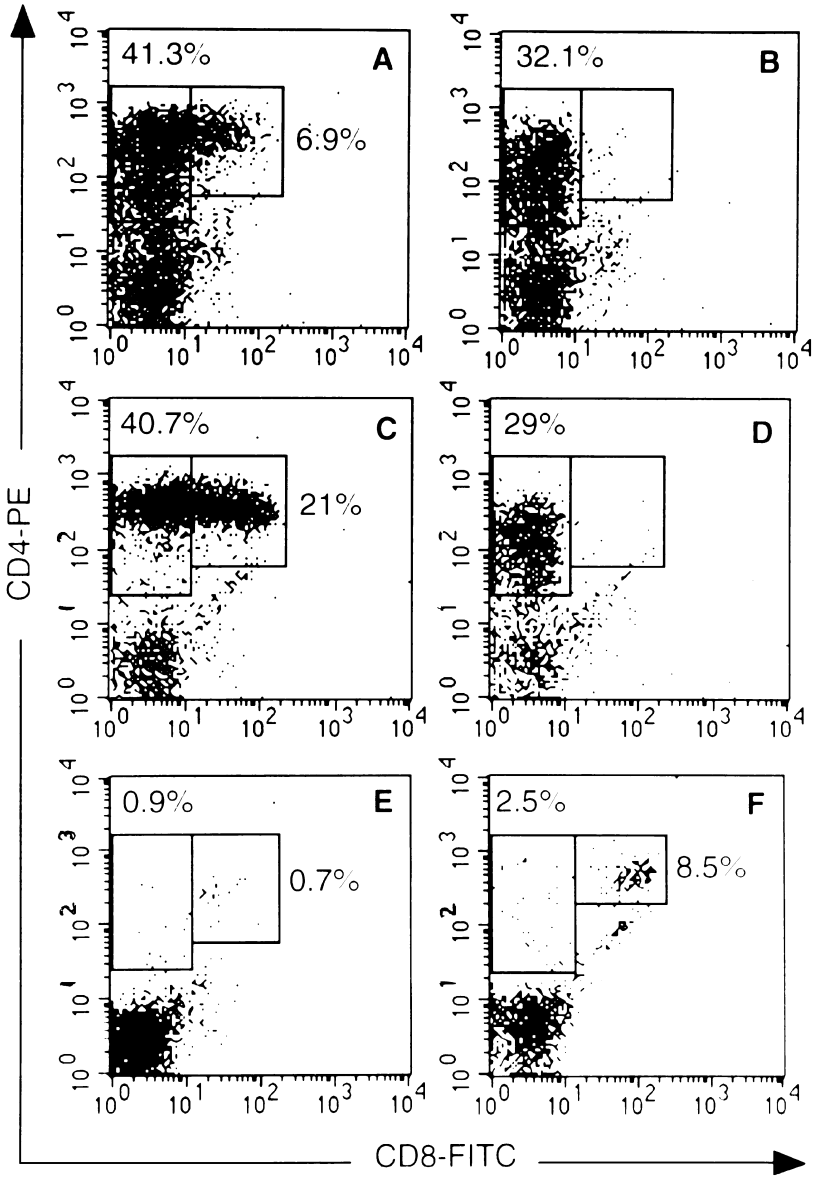

Fig 2. Phenotype of human cells recovered in individual NOD-SCID thymuses seeded with low numbers of cord blood $CD34^+$ cells. Thymuses were incubated with either 2000 $CD34^+$ cells/lobe (A, B), 500 $CD34^+$ cells/lobe (C, D), 100 $CD34^+$ cells/lobe (E), or 100 $CD34^+$ cells plus 5000 irradiated $CD38^+$ cells/lobe (F). After 30 d in culture each lobe was individually analysed by flow cytometry after two-colour labelling with anti-CD4-PE and anti-CD8-FITC. Positivity was based on the presence of significant numbers of $CD4^+$ cells or $CD4^+CD8^+$ cells. Analysis was performed in the morphological gate indicated in Fig 1A.

As shown in Table I, in 6/7 experiments where NOD-SCID FTOC were seeded with $CD38^{low}$ cells (Fig 1B) and in all six experiments seeded with $CD34^+CD38^+$ cells (Fig 1C) high proportions of $CD4^+$ cells (41·3 ± 8% for $CD38^{low}$ and 56 ± 10% for $CD38^+$ cells) were reproducibly generated after 30 d. DP $CD4^+CD8^+$ cells represented 26·6 ± 9·2% of all $CD4^+$ cells in FTOC seeded with $CD34^+CD38^{low}$ cells and 44·3 ± 7% in FTOC seeded with $CD34^+CD38^+$ cells (Table I and Fig 1C). Thus these results demonstrated that progenitors with the ability to differentiate along the T-cell pathway in NOD-SCID FTOC existed in both $CD34^+CD38^+$ and $CD34^+CD38^{low}$ cord blood suspensions. However, some differences were noted depending on the phenotype of input cells. First, the proportion of $CD4^{low}$ (mean fluoresence intensity MFI <100) within the $CD4^+$ population was higher in FTOC seeded with $CD38^{low}$ cells (31·3 ± 7·6%) than in experiments performed with $CD38^+$ (16·4 ± 3·2%) or $CD34^+$ cells (22·2 ± 4·6%). Second, as mentioned above, the proportion of $CD4^+CD8^+$ cells within the total $CD4^+$ fraction was higher when FTOC were initiated with $CD38^+$ (44·3 ± 7%) or $CD34^+$ (43·5 ± 10%) cells as compared to $CD38^{low}$ cells (26·6 ± 9·2%).

Estimation of the frequency of T-cell progenitors among $CD34^+$ cord blood cells by limiting dilution experiments

Limiting dilution analysis was then used in two experiments to determine the frequency of $CD34^+$ cells which generate T cells in FTOC. Individual thymic lobes were incubated with 2000 (nine lobes), 500 (18 lobes) or 100 (19 lobes) cord-blood-derived $CD34^+$ cells. Each lobe was examined individually after 30 d in culture. All control lobes seeded with 20 000 cells contained $CD4^+CD8^+$ cells. All nine lobes initiated with 2000 cells contained $CD4^{bright}$ cells (27 ± 4·4% of nucleated cells) (Figs 2A and 2B) and 6/9

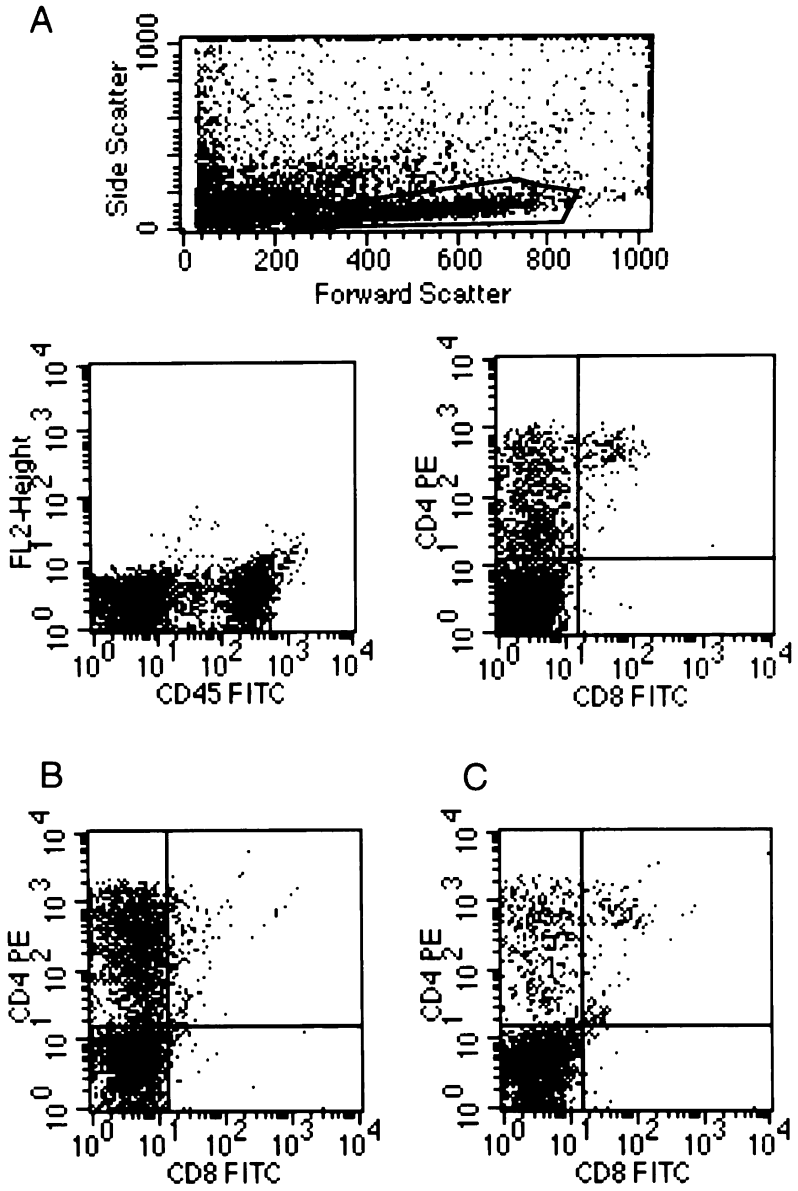

Fig 3. Phenotype of nucleated cells recovered from NOD-SCID thymic lobes reconstituted with adult bone marrow $CD34^+$ cells. NOD-SCID embryonic thymuses were incubated with (A) 10 000 $CD34^+$ cells, (B) 10 000 $CD34^+CD38^{low}$ cells, and (C) $CD34^+CD38^+$ cells. After 30 d in FTOC culture, cells recovered from thymuses were labelled with a panel of T-cell markers specific for human cells and analysed in the lymphoid gate defined in panel (A). Shown here are the results of labelling with anti-human CD45, and two-colour labelling with anti-human CD4 and anti-human CD8.

$CD4^+CD8^+$ cells (Fig 2A). Out of 18 lobes initiated with 500 cells, 11 contained $CD4^+$ cells and seven $CD4^+CD8^+$ cells (Figs 2C and 2D), and $CD4^+CD8^+$ cells were detected in 3/19 lobes seeded with 100 cells (Fig 2E). Based on these results, we calculated that 1/500 $CD34^+$ cord blood cells would give rise to T cells in FTOC. However, since many steps were uncontrolled in these FTOC, such as the number of $CD34^+$ cells effectively entering the thymus or the requirement for accessory cells, this frequency was likely to be underestimated. This was suggested by preliminary results which showed that the addition of 5000 irradiated $CD38^+$ cells to 100 $CD34^+$ test cells improved the proportion of positive lobes [5/10 lobes seeded were positive, three contained $CD4^+CD8^+$ cells (Figs 2F) and the other two contained $CD4^{bright}$ cells].

Identification of T-cell progenitors in the $CD34^+$, $CD34^+CD38^{low}$ and $CD34^+CD38^+$ fractions of adult bone marrow

In five separate experiments NOD-SCID thymic lobes were incubated with $CD34^+$ adult marrow cells in conditions similar to those designed for FTOC experiments performed with cord blood cells. As shown in Table I, $CD4^+CD8^+$ cells were detected in only four of these five experiments. Within the $CD4^+$ population the proportion of $CD4^+CD8^+$ cells was low ($10 \pm 2\%$) when compared to that observed for cord blood experiments ($43{\cdot}5 \pm 10\%$) (Table I, and Fig 3A). In two experiments both $CD34^+CD38^{low}$ (Fig 3B) and $CD34^+CD38^+$ (Fig 3C) cells incubated in hanging drop with NOD-SCID thymic lobes led to similarly low proportions (<20%) of $CD4^+CD8^+$ cells (Table I). Interestingly, the

majority of $CD4^+CD8^-$ cells ($65{\cdot}5 \pm 6\%$) were weakly labelled, independently of the phenotype of the input cells as if there were a block in the progression of differentiation towards the $CD4^+CD8^+$ stage. In contrast, $61{\cdot}4 \pm 4\%$ of $CD4^+CD8^-$ cells collected from FTOC established with cord blood cells were brightly labelled.

In contrast to positive results obtained with adult bone marrow samples, we failed to identify double positive $CD4^+CD8^+$ T cells in three experiments where NOD-SCID thymuses were incubated with $CD34^+$ cells from cytokine-mobilized peripheral blood and the proportion of $CD4^+$ in these thymuses was constantly below 10% (data not shown).

Human T-cell differentiation within NOD-SCID thymuses recapitulates the normal steps of human thymopoiesis

In order to evaluate whether human T-cell differentiation in NOD-SCID thymic lobes proceeded through the same ordered sequence as that described in the human thymus, we performed a detailed phenotype of cells collected from thymic lobes reconstituted with cord blood- or bone marrow-derived $CD34^+$ cells. Results are illustrated in Fig 4 and Table II. A detailed phenotypic analysis of T cells recovered from FTOC could not be performed in each experiment because numbers of cells collected were too low, but the expression of the T-cell-specific markers CD4, CD8, TCR$\alpha\beta$ and CD3 was systematically examined. Three stages of maturation were recognized: a $CD4^+CD8^-$ immature stage, preceeding the DP $CD4^+CD8^+$ stage, and a small compartment of positively selected mature $CD4^+CD8^-$ lymphocytes.

Most $CD4^+CD8^-$ cells coexpressed CD1a (92%, $n=1$), CD5 ($40 \pm 18\%$, range 16–94%, $n=4$), CD38 (99%, $n=1$) and intracytoplasmic CD3 (100%, $n=3$), thus proving their

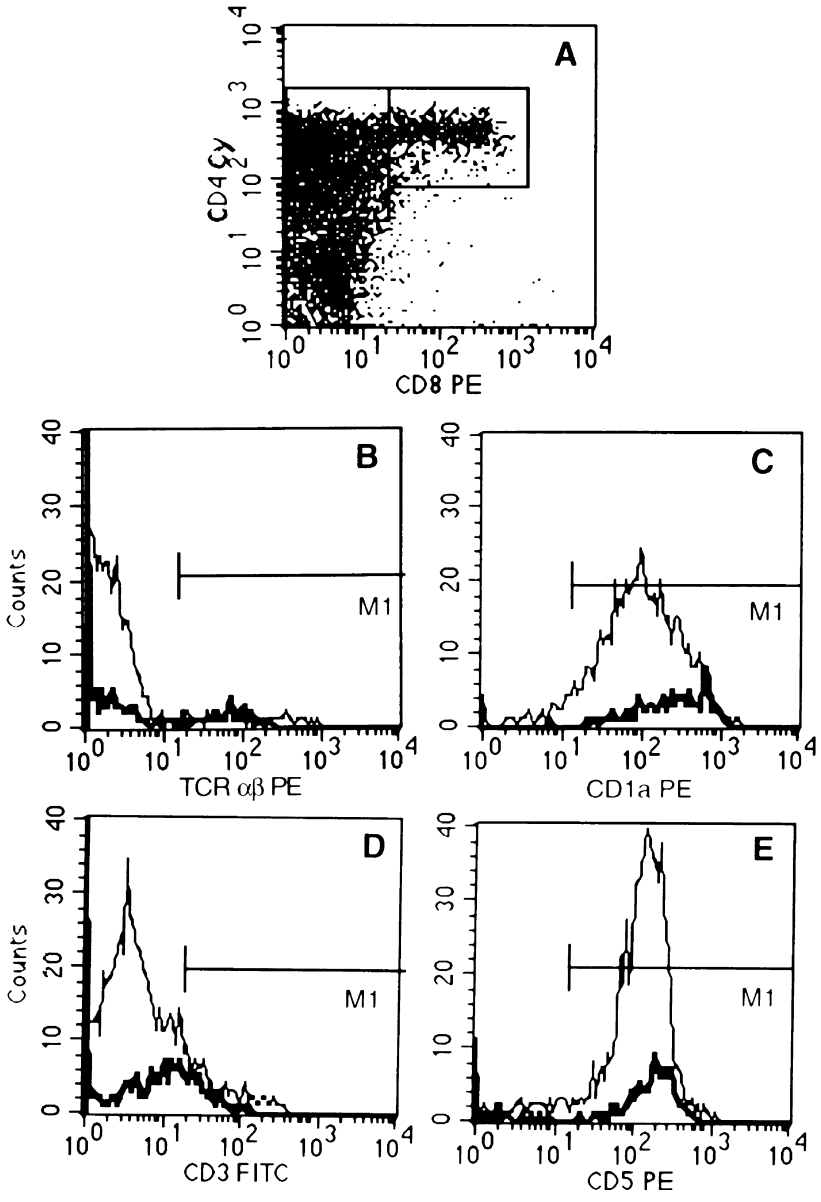

Fig 4. Expression of T-cell-specific markers on $CD4^+CD8^-$ and $CD4^+CD8^+$ populations observed in NOD-SCID FTOC initiated with $CD34^+$ cord blood cells. In this representative experiment three-colour labelling was performed and the expression of TCR$\alpha\beta$ (B), CD1a (C), sCD3 (D) and CD5 (E) was analysed separately on both $CD4^+CD8^-$ and $CD4^+CD8^+$ populations defined in panel A. Histograms show the distribution of the antigen on either $CD4^+CD8^+$ (thick line) or $CD4^+CD8^-$ cells (thin line). M1 delineates positivity based on results obtained with irrelevant antibodies.

Table II. Expression of T-cell markers on the $CD4^+CD8^+$ and $CD4^+CD8^-$ populations generated in FTOC culture of $CD34^+$ cord blood or bone marrow cells.

	Percentage of positive cells*			
	$CD4^+CD8^+$	(*n*)†	$CD4^+CD8^-$	(*n*)
TCR $\alpha\beta$	69 ± 12	(6)	2·8 ± 0·4	(6)
TCR $\gamma\delta$	1·8	(1)	2·3	(1)
CD5	86 ± 4	(4)	40 ± 18	(4)
CD1a	99·5	(1)	92	(1)
sCD3	64 ± 7·5	(5)	8 ± 3	(5)
iCD3	100	(3)	100	(3)
CD38	96·5	(1)	99	(1)
CD34	4	(1)	1·2	(1)
CD7	–	–	32 ± 8	(4)

* Numbers refer to the mean (± SEM) proportion of $CD4^+CD8^+$ or $CD4^+CD8^-$ cells co-expressing the indicated marker.

† *n* indicates the number of experiments.

lymphocytic nature. Rare $CD4^+CD8^-$ cells expressed CD34 (1·2%, $n=1$), and TCR$\gamma\delta$ (2·3%, $n=1$). CD7 was expressed on 32 ± 8% of $CD4^+$ cells ($n=4$), but CD25 and CD69 were not present. These cells most probably correspond to the $CD4^+CD8^-CD1a^+CD5^+sCD3^-TCR\alpha\beta^-$ immature SP stage prior to the DP $CD4^+CD8^+$ previously described in the human thymus (Hori *et al*, 1991). CD11b ($n=6$), CD13, CD14, CD33 and CD36 were not coexpressed by $CD4^+$ cells thus excluding that some $CD4^{low}$ cells belong to the granulomonocytic lineage.

Fewer than 10% $CD4^+$ T cells were mature selected T cells, which expressed TCR $\alpha\beta$ (2·8 ± 0·4%, range 1–4%, $n=6$) and sCD3 (8 ± 3%, range 2·4–20%, $n=5$). Single-positive $CD8^+$ cells were not detected, as previously reported in these human/mouse hybrid FTOC.

$CD4^+CD8^+$ cells included both TCR$\alpha\beta$-positive (69 ± 12%, $n=6$) and sCD3-positive (64 ± 7·5%, $n=5$) cells and TCR$\alpha\beta$-negative cells. Both subsets, probably representing successive maturation stages, were found in all experiments (Table II and Fig 4). Almost all $CD4^+CD8^+$ cells coexpressed CD5 (86 ± 4%, range 77–96%, $n=4$), CD1a (99·5%, $n=1$) and CD38 (96·5%, $n=1$). Only rare DP cells expressed CD34 (4%, $n=1$), or TCR$\gamma\delta$ (1·8%, $n=1$).

Although all three stages of T-cell maturation were detected in FTOC seeded by cord blood and bone marrow cells, FTOC initiated with adult marrow cells yielded a lower proportion of $CD4^+CD8^+$ than FTOC initiated with cord blood $CD34^+$ cells (10% *v* 45% respectively, see Table I and Figs 1 and 3). Second, most $CD4^+$ cells produced by bone marrow $CD34^+$ cells and their $CD38^{low}$ or $CD38^+$ subsets expressed intermediate or low levels of CD4 (MFI 5×10^1) in contrast to the bright $CD4^+$ staining observed in FTOC seeded with cord blood cells (Figs 1A and 3A).

$CD34^+CD4^-$ human cells were present in a significant proportion in all experiments seeded with cord blood $CD34^+$ or $CD34^+CD38^{low}$ cells (15 ± 7%, range 2–45%, $n=5$) and bone marrow cells (15·6%, $n=1$). $CD4^-CD11b^+$ cells (4 ± 0·6%, range 1·6–5%, $n=5$) and $CD56^+$ natural killer

Table III. Results of FTOC experiments performed with $CD34^+CD38^{low}$ cells isolated from human cord blood and adult bone marrow and used either fresh or after 7 d of prestimulation with human cytokines.

	Experiment			Percentage of cells (mean ±SEM)‡			
Source of cells	Positive/total*	Exp.	Nucleated cells/lobe (mean ±SEM)†	$CD45^+$	$CD4^+$ total	$CD4^+CD8^+$ (% in $CD4^+$)	$CD4^-CD8^+$
Cord blood							
Fresh	3/3	a	17 000	76·1	56·5	68·3	1·8
		b	4700	nd	22·1	13·3	0
		c	2800	nd	18·4	5·4	0
Cultured§	3/3	a	7300	39·8	26·8	24·3	3·8
		b	8500	60·8	43·3	27·7	0
		c	4900	46·2	31	27	1·5
Bone marrow							
Fresh	2/2		8700	30·8	22	4·2	0
			11 900	50	37	2·9	0
Cultured	1/4		20 800	45·7	30	45	4

* An experiment was considered positive when DP $CD4^+CD8^+$ cells were observed.

† Numbers show the number of nucleated cells/lobe calculated from the total number of nucleated cells recovered from all the pooled thymic lobes divided by the total number of lobes.

‡ Cells were analysed in the lymphoid gate (defined in Fig 1). Numbers represent for each experiment the percentage of positive cells for the indicated antigen. a, b, c refer to matched individual cord blood experiments.

§ $CD34^+CD38^{low}$ cells were first cultured 7 d with eight cytokines and 10–20 000 $CD34^+$ cells sorted after this period were incubated with thymic lobes.

cells (5%, $n=2$) were also present, but neither $CD14^+$ monocytes nor $CD19^+$ B-lymphoid cells.

Short-term treatment with IL-3 and human cytokines does not abrogate the T-cell potential of $CD34^+CD38^{low}$ cells

In the next series of experiments we investigated whether T-cell potential could be maintained in conditions which promote cell expansion, i.e. in the presence of cytokines. More specifically, we assessed the influence on the development of human T-lymphoid progenitor cells of interleukin-3, since in the mouse this cytokine abrogated expression of the lymphoid potential (Ball *et al*, 1996; Hirayama & Ogawa, 1995). In seven experiments (four performed with adult marrow and three with cord blood), $CD34^+CD38^{low}$ cells were incubated with a combination of eight cytokines (including 2 ng/ml IL-3) in the presence of stromal cells previously shown to support lymphoid differentiations (Berardi *et al*, 1997; Carayol *et al*, 1998). After 1 week in culture, we observed an average 40–50-fold expansion in total nucleated cells and a 10-fold expansion in the numbers of $CD34^+$ cells. $CD34^+$ cells were sorted and used to initiate FTOC experiments as described for fresh cells. As indicated in Table III, fresh and cultured cells generated identical proportions of total $CD4^+$ cells and of $CD4^+CD8^+$ cells. $CD34^+CD38^{low}$ cells from fresh cord blood produced on average 2600 DP cells, and cultured $CD34^+$ cells 1290 DP cells suggesting that there was no major loss in the potential of cultured cells. In contrast, cultured $CD34^+$ marrow cells yielded $CD4^+CD8^+$ cells in only one of the four experiments performed (Table III). It is therefore difficult to conclude on the maintenance of T-cell potential after cytokine stimulation of marrow $CD34^+CD38^-$ cells.

Finally, we ruled out a deleterious effect of IL-3 on the development of T cells in two FTOC experiments initiated with cells cultured with high concentrations of IL-3 (up to 100 ng/ml). This indicated that IL-3 had no inhibitory effect on T-cell differentiation, an observation which was in agreement with our additional data on the lack of inhibition of IL-3 on the production of both B cells and NK cells from $CD34^+CD38^-$ progenitors (data not shown).

DISCUSSION

A major obstacle to the identification of the full differentiative potential of candidate stem cells is the lack of an assay that will support simultaneous differentiation in all lymphoid and myeloid pathways. We (Berardi *et al*, 1997) and others (Baum *et al*, 1992; Galy *et al*, 1995a; Hao *et al*, 1998) have shown that myeloid and B-lymphoid potentials can be simultaneously expressed *in vitro* from human single cells, but expression of the T-cell potential of these cells remains difficult to assess, although not impossible, as recently shown in the mouse (Kawamoto *et al*, 1997; Kondo *et al.*, 1997).

In this study we have shown that NOD-SCID FTOC offers a very reproducible system to monitor *in vitro* the T-cell potential of various human stem cell fractions, even from as low as 100 cells. Thus $CD34^+$, $CD34^+CD38^{low}$ and $CD34^+CD38^+$ cells from both adult marrow and cord blood differentiated into T cells in NOD-SCID FTOC, and at least 0·02% of cord blood $CD34^+$ were T-cell progenitors. Phenotypic analysis of the produced T cells revealed both immature $CD4^+CD8^-sCD3^-CD1a^+CD5^+$ cells and DP $CD4^+CD8^+$ stage (Hori *et al*, 1991; Kraft *et al*, 1993) including $TCR\alpha\beta^+$ and $TCR\alpha\beta^-$ subsets. This indicated that the initial steps of T-cell differentiation which occur in murine FTOC reproduce those found during normal human thymocyte differentiation (Kisielow & Von Boehmer, 1995; Spits *et al*, 1995). However, in these hybrid FTOC further positive selection resulting in the production of mature single positive T cells $sCD3^+TCR\alpha\beta^+CD4^+$ or $CD8^+$ was compromised by the poor reactivity of murine class I and to a lesser degree class II molecules with human cells (Blom *et al*, 1997).

Our rationale for using the NOD-SCID strain was based on accumulating evidence that NOD-SCID mice are the most sensitive recipients for transplantable human cells with multiple potentials (Cashman *et al*, 1997; Pflumio *et al*, 1996) and are particularly suitable for human B-lymphoid differentiation (Hogan *et al*, 1997; Pflumio *et al*, 1996), and may also support T-cell differentiation *in vivo* (van der Loo *et al*, 1998; Yurasov *et al*, 1997). Furthermore, the successful development of T cells from fetal liver and cord blood progenitors in murine FTOC has been demonstrated. Thymic tissue from both immune competent and immune deficient embryonic mice (Blom *et al*, 1997; Plum *et al*, 1994; Res *et al*, 1996; Yeoman *et al*, 1993) has been used, and while this study was in progress unambiguous demonstration that $CD34^+CD38^-$ and $CD34^+CD38^+$ cord-blood-derived cells differentiate into T cells in hybrid mouse/human FTOC has been shown (Blom *et al*, 1997; Verhasselt *et al*, 1998). We confirm here that the FTOC procedure, using murine thymus, offers a useful *in vitro* alternative to the *in vivo* SCID-hu assay, which was the first to convincingly identify the T-cell potential of human primitive progenitor cells (Barcena *et al*, 1993; DiGustio *et al*, 1994; Galy *et al*, 1995b; Galy *et al*, 1994; Péault *et al*, 1991).

In contrast to the abundant litterature on the lymphoid potential of fetal cells, very little is known regarding the lymphoid potential of primitive progenitors from adult tissues. We have shown for the first time that both $CD34^+CD38^{low}$ and $CD38^+$ subsets from adult marrow could develop into T cells in NOD-SCID FTOC, whereas mobilized peripheral-blood-derived cells did not.

However, T-cell differentiation from adult marrow $CD34^+$ cells was less efficient than from cord blood $CD34^+$ cells. Thus, a low proportion (~10%) of $CD4^+CD8^+$ cells was recovered from thymic lobes after 35 d in culture and the expression of CD4 was low as compared to the bright CD4 labelling of T cells derived from cord blood $CD34^+$ cells. Preincubation of marrow $CD38^{low}$ cells with cytokines for 7 d did not improve these results (and even suggested a decrease in T-cell potential after cell stimulation, in contrast to the maintenance of T-cell potential in similar experiments performed with cord blood cells). Two parameters might contribute to these differences: first it is probable that the frequency of T-progenitor cells is lower in adult populations than in their fetal counterparts, a hypothesis which is

supported by the data obtained by Galy *et al* (1995) and Plum *et al* (1994). Lower frequencies of all categories of primitive progenitor cells in adult haemopoietic tissues when compared to fetal or neonatal tissues have been largely documented in a wide variety of biological assays, both in mouse (Pawliuk *et al*, 1996) and man (Lansdorp *et al*, 1993), including in the *in vivo* NOD-SCID-SRC/CRU assay (Conneally *et al*, 1997; Wang *et al*, 1997). Thus, in our experience 8–10% of $CD34^+CD38^{low}$ cord blood cells but <0·05% of $CD34^+CD38^{low}$ adult bone marrow cells are bipotent B-lymphoid-myeloid progenitors (Berardi *et al*, 1997).

Second, it is also possible that important signals required by adult human T-cell progenitors, but not fetal progenitors, are lacking in the NOD-SCID thymic environment. This might explain why we failed to detect T cells in NOD-SCID FTOC initiated with adult peripheral blood $CD34^+$ cells, whereas these cells generated high numbers of $CD4^+CD8^+$ cells in the SCID-hu assay (Galy *et al*, 1994), which suggests that additional signals are delivered by the human thymus environment. Alternatively, accessory cells present within $CD34^+$ cord blood cells, but not adult $CD34^+$ cells, may also play a role, such as dendritic cells which are the predominant population early during the culture (Plum *et al*, 1994; Verhasselt *et al*, 1998).

Finally, we also showed that in conditions where active cell divisions occur, cord-blood-derived $CD34^+CD38^-$ cells did not immediately lose their T-cell potential. Importantly, IL-3, which in the murine system has been reported to inhibit T-lymphoid and B-lymphoid differentiation of murine stem cells (Ball *et al*, 1996; Hirayama & Ogawa, 1995), had no such effect on human lymphoid differentiation, neither in the T cell (this study) nor B or NK differentiation pathways (unpublished observations). This suggests that early stem cells may be successfuly amplified without a major loss of potential, and NOD-SCID FTOC may therefore prove an invaluable additional tool to investigate the function of human stem cells subjected to treatment with regulatory molecules.

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