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Reconstitution of SCID mice with haemopoietic precursors: a detailed analysis of γδ T-cell reconstitution

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SUMMARY

A well-known characteristic of γδ T cells is that they are produced in waves during ontogeny, with cells expressing T-cell receptor Vγ5 appearing early in fetal thymic ontogeny, followed by Vγ6, then by other γδ T-cell types. In addition, evidence exists to suggest that the potential of haemopoietic precursors to generate different types of γδ T cells changes in ontogeny. We have used these observations as the basis for an extensive study of the potential for haemopoietic precursors isolated from fetal liver, neonatal spleen and adult bone marrow to reconstitute severe combined immunodeficient (SCID) mice. Mice that were reconstituted as newborns with fetal liver cells most closely resembled normal C.B-17 mice with respect to both lymphocyte numbers and subsets, while mice reconstituted with adult bone marrow had fewer cells than normal mice. This deficit spanned both T and B cells in all organs examined. Among the γδ T-cell subsets examined, the ability to reconstitute $V\gamma4^+$ cells was particularly dependent on the ontogenic age of the reconstituting presursors, with fetal liver cells having the greatest capacity to generate $V\gamma4^+$ cells, and adult bone marrow cells the least. The vast majority of the T cells produced in the reconstituted mice were of donor origin, and the level of reconstitution was found to be dependent upon some factor other than the presursor frequency.

INTRODUCTION

T cells expressing the γδ form of the T-cell receptor (TCR) are the first to appear in ontogeny.[1] These cells express a receptor comprised of Vγ5 and Vδ1 gene products,[2–5] and become, almost exclusively, the dendritic epidermal cells of the skin.[6–9] These TCR $V\gamma5V\delta1^+$ cells are produced exclusively during fetal life, requiring both a fetal thymic microenvironment and a fetal source of precursor cells to develop.[10] T cells expressing TCR Vγ6 appear in the thymus later in pre-natal life,[11] and like $V\gamma5^+$ cells, they are rare or undetectable in the adult thymus.[2] They have however, been isolated from the adult liver,[12] tongue and female reproductive tract.[13] Other γδ types can be detected around the time of birth. Their distribution is much broader than those cells produced early in ontogeny, and little is known about the microenvironmental and precursor requirements for the production of these cells. A hint that some $V\gamma1^+$ cells isolated from adult liver may be derived from a prenatal source came from the recent work of Ellis-Roark *et al.*[12] who found that the VDJ junctional sequences of a significant proportion of hybridomas derived from adult liver had short N regions – a characteristic of fetally derived cells.

Severe combined immunodeficient (SCID) mice provide a good model in which to study the production of T cells as they are devoid of endogenous T cells. Despite the absence of lymphocytes, adult SCID mice require irradiation before significant reconstitution can occur.[14] As irradiation may alter/damage thymic stromal elements, and neonatal mice can be reconstituted with fetal liver or adult bone marrow without the necessity of irradiation,[15,16] we chose newborn mice as recipients. Reconstitution was assessed by flow cytometric analysis at 8 weeks of age. At this age mice should have achieved maximal reconstitution while still being sufficiently young not to be complicated by a 'leaky' phenotype in which small numbers of SCID-derived T and B cells can be detected.[17] Using this system we were able to show that reconstitution of lymphocyte subsets, including γδ T-cell subsets, was dependent upon the source of progenitor cells.

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Abbreviations: BSS, balanced salt solution; FCS, fetal calf serum; i-IEL, intestinal intraepithelial lymphocytes; i.p., intraperitoneal; mAb, monoclonal antibodies; PEAV, phycoerythrin avidin; SCID, severe combined immunodeficient; TCR, T-cell receptor for antigen.

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MATERIALS AND METHODS

Mice

C.B-17/IcrTac-SCIDfDF mice were bred and maintained in our animal facility in a flexible film isolator (Harlan Isotec,

Bicester, Oxon, UK) maintained under positive pressure. C.B-17/IcrTacfDF mice were bred and maintained in a separate positive pressure flexible film isolator. The original breeding pairs for both strains were purchased from Taconic (Germantown, NY 12526). B6.PL.Thy-1(a)Cy mice were bred from pairs kindly provided by Dr U. Staerz (National Jewish Centre for Immunology and Respiratory Medicine, Denver, CO).

Reconstitution of SCID mice

Neonatal C.B-17 SCID/ SCID mice were reconstituted within the first 4 days of birth by intraperitoneal (i.p.) injection of 10^7 fetal liver cells, 10^6 neonatal spleen cells, or 10^6 adult bone marrow cells. Fetal liver cells were obtained from C.B-17 ICR mice at day 12–14 of gestation where day 0 was taken as the day of detection of the vaginal plug. A cell suspension was prepared by teasing the cells through a nylon mesh into balanced salt solution containing 5% fetal calf serum (BSS/FCS). Neonatal splenocytes were obtained from mice less than 24 hr old, and a cell suspension was prepared as for fetal liver. In addition, red blood cells were removed by NH_4Cl treatment[18] followed by removal of dead cells.[19] Adult bone marrow cells were obtained by flushing the femurs of 8–12-week-old mice with BSS/FCS, and preparing a suspension as per neonatal spleen.

Cell suspensions and cell counts

Thymus, lymph node, spleen and liver cell suspensions were prepared by teasing cells through a wire mesh into BSS/FCS. Cells were washed through FCS. In addition, splenocytes were depleted of red blood cells and dead cells as above. Red blood cells were also removed from liver cell suspensions and dead cells and debris was removed by centrifugation over 1·091 g/cm^3 metrizamide as previously described.[20] Intestinal intraepithelial lymphocytes (i-IEL) were prepared using a modified version of the method described by Schrader *et al.*[21] Briefly, small intestines were flushed of their contents, cut into 1-cm lengths (at which time the Peyer's patches were removed and discarded), then slit lengthwise. The pieces were further washed by gentle pipetting with medium. The epithelium was gently scraped off then vigorously pipetted to dissociate clumps. The resultant suspension was allowed to settle over 50% FCS for 30 min to allow separation of large epithelial clumps. Smaller epithelial clumps were removed by passage through a dead-cell removal column[19] or a nylon mesh. The majority of the remaining epithelial cells were removed by centrifugation over 1·091 g/cm^3 metrizamide. Preparation of i-IEL was performed in tissue culture medium containing 5% FCS and 0·1 M dithiothreitol.

Flow cytometric analysis of $\gamma\delta$ subsets in thymus, lymph nodes and spleen was performed on cells enriched for $\gamma\delta$ cells by complement-mediated depletion of $CD4^+$ and $CD8^+$ cells using the monoclonal antibodies (mAb) 172.4 (anti-CD4[22]) and D9 (anti-CD8[23]) as previously described.[20] In addition, CD4/CD8-depleted lymph node and spleen cells were depleted of B cells using sheep anti-mouse immunoglobulin-coated immunomagnetic beads (180 μl of sixfold concentrated beads per 10^7 cells) (Amerlex M®, Amersham, UK).

Viable nucleated cells were enumerated using a haemocytometer and the vital dye eosin (BDH, Poole, UK).

Flow cytometric analysis

Cells were stained for flow cytometric analysis in 96-well U-bottom tissue culture trays (Costar, Cambridge, MA) at 4° in BSS/FCS. The exact staining protocols used are indicated in the figure legends. The following mAb were used: anti-CD4 (GK1.5[24]), anti-CD8, (53.6.7[25]), anti-CD3 (KT3[26]), anti-TCR $\gamma\delta$ (GL3[27]), anti-TCR Vγ4 (UC3-10A6[28]), anti-TCR Vγ5 (F536[9]), anti-TCR Vγ7 (GL1[27]), anti-TCR Vδ4 (GL2[27]), anti-TCR Vδ6 (17C[29]), anti-Thy-1.2 (30H12[25]) and anti-Thy-1.1 (19E12[30]). The latter two reagents were kindly provided by Dr S. Candais. Anti-CD4 and anti-CD8 reagents were purchased from Becton Dickinson (San José, CA), and the remainder of the reagents were grown, purified and conjugated in this laboratory. Phycoerythrin–avidin (PEAV) (Caltag Laboratories, South San Francisco, CA) or phycoerythrin–Texas Red–avidin (Southern Biotechnology Associates, Inc., Birmingham, AL) were used as secondary reagents for biotinylated antibodies. Cells were analysed on a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA). Lysis II® software was used for both collection and analysis of the data. Viable nucleated cells were gated on the basis of low-angle light scatter and propidium iodide staining. These parameters and right-angle light scatter also allowed exclusion of remaining epithelial cells in i-IEL preparations.

RESULTS

Reconstitution of T cells in SCID mice by haemopoietic precursors isolated from fetal liver, neonatal spleen and adult bone marrow

We compared three different sources of haemopoietic precursors for their ability to reconstitute SCID mice when injected i.p. within the first 4 days of life. The results in Table 1 show that a gradation of reconstitution was observed with fetal liver cells giving closest to normal lymphoid reconstitution followed by neonatal spleen, and while bone marrow gave the poorest reconstitution, reconstitution still occurred to a significant degree. Interestingly in some situations, $\gamma\delta$ T cells appeared to be reconstituted less well than $\alpha\beta$ cells. This was particularly evident in mice reconstituted with adult bone marrow, and in the liver irrespective of the precursor source, where not only fewer than normal $\gamma\delta$ cells were recovered, but also the proportion of T cells expressing the $\gamma\delta$ receptor was decreased. The number of i-IEL recovered from reconstituted mice varied considerably between individuals (as it did between normal C.B-17 individuals) but was within the normal range. If many i-IEL are indeed extrathymically derived as proposed by a number of groups,[31–34] it is clear from our results that fetal liver, neonatal spleen and adult bone marrow all contain the requisite precursor cells for the production of a near normal i-IEL phenotype.

It is possible that the number of haemopoietic stem cells present in the three different inocula may be different, accounting for the difference in the reconstituting capacity of fetal liver and adult bone marrow cells. To address this possibility, we injected neonatal SCID mice with 10^7 adult bone marrow cells instead of the usual dose of 10^6 The increase in precursor number only resulted in a maximum twofold increase in the number of cells produced (Table 1). These data show that

Table 1. Numbers of cells recovered from thymus, lymph nodes, spleen, liver and i-IEL of normal C.B-17 mice and C.B-17 SCID mice reconstituted neonatally with 10^7 fetal liver cells, 10^6 neonatal spleen cells and $10^6/10^7$ adult bone marrow cells. The total number of cells was determined using a haemocytometer and eosin, while the proportion of $CD3^+$ and $\gamma\delta^+$ cells was determined by flow cytometric analysis of cells stained with anti-CD3–FITC + anti-γδ–biotin, followed by PEAV. Results are derived from five to seven individual mice per reconstitution regime

Reconstitution regime	Organ	Total cells ($\times 10^{-7}$)	$CD3^+$ cells ($\times 10^{-6}$)	$\gamma\delta^+$ cells ($\times 10^{-4}$)
Normal	thymus	11±4	17±6	19±5
Fetal liver		17±3	36±8	31±11
Neonatal spleen		17±5	29±7	24±12
Adult bone marrow (10^6)		6±2	18±4	8±4
Adult bone marrow (10^7)		13±3	24±4	23±13
Normal	lymph node	2·9±0·7	20±6	9·6±2·3
Fetal liver		2·8±0·7	22±6	10±2·3
Neonatal spleen		2·0±0·6	14±5	6·7±3·1
Adult bone marrow (10^6)		1·0±0·3	8±2	4·7±1·9
Adult bone marrow (10^7)		2·0±0·5	15±4	8·2±2·7
Normal	spleen	5·3±0·8	20±4	13±3
Fetal liver		3·7±0·8	16±4	15±4
Neonatal spleen		2·8±0·8	13±4	7·5±2·7
Adult bone marrow (10^6)		1·2±0·3	7±2	3·7±1·4
Adult bone marrow (10^7)		2·4±1·0	12±4	8·7±2·9
		($\times 10^{-5}$)		
Normal	liver	7·9±3·1	0·30±0·08	2·4±0·7
Fetal liver		8·6±2·9	0·33±0·10	1·5±0·7
Neonatal spleen		3·6±1·7	0·18±0·11	0·6±0·4
Adult bone marrow (10^6)		5·1±1·4	0·22±0·08	0·7±0·3
Adult bone marrow (10^7)		3·2±0·7	0·15±0·40	0·5±0·2
Normal	i-IEL	15±6	11±5	38±22
Fetal liver		14±11	10±8	39±35
Neonatal spleen		11±5	8±3	25±14
Adult bone marrow (10^6)		20±5	22±18	29±11
Adult bone marrow (10^7)		16±5	10±5	49±24

precursors derived from different sources are inherently different in their capacity to generate T cells.

B-cell reconstitution

While the main focus of this study is reconstitution of the T-cell compartment, SCID mice are also inherently deficient in B cells,[35] and based on published observations,[15,16] one would predict that B cells should also be reconstituted in our system. Although Cowing and Gilmore[16] reported full B-cell reconstitution in mice that had received adult bone marrow neonatally, we found significantly lower than normal numbers of B cells in both lymph nodes and spleen (Table 2). All reconstituted mice were demonstrated by enzyme-linked immunosorbent assay (ELISA) to have high levels of serum IgM and IgG (data not shown). As with T-cell reconstitution, fetal liver cells gave the best B-cell reconstitution and adult bone marrow the worst. The number of B cells recovered from fetal liver recipients was 50% of normal while mice receiving adult bone marrow had 20-fold lower numbers of B cells than normal C.B-17 mice (Table 2). Furthermore, in a similar trend as for T cells, a 10-fold increase in the number of bone marrow cells injected, only resulted in a fourfold increase in the number of B cells recovered (Table 2). B cells were reconstituted less well than T cells although the same heirarchy of precursor potential for both subsets was observed.

Table 2. Number of B cells recovered from normal C.B-17 mice and from SCID mice reconstituted with 10^7 fetal liver cells, 10^6 neonatal spleen cells and $10^6/10^7$ adult bone marrow cells. The total number of cells was determined using a haemocytometer and eosin, and the proportion of B cells indicated by flow cytometric analysis of cells stained with anti-immunoglobulin-FITC. Results are derived from five to seven individual mice per reconstitution regime

Reconstitution regime	Lymph node ($\times 10^{-6}$)	Spleen ($\times 10^{-6}$)
Normal	5·6±1·6	22±3
Fetal liver	3·1±0·8	11±2
Neonatal spleen	2·1±1·1	8±4
Adult bone marrow (10^6)	0·3±0·3	1±0·4
Adult bone marrow (10^7)	1·6±0·5	4±2

The vast majority of reconstituted T cells are of donor origin

A number of groups have reported that the presence of mature T cells in SCID mice promotes a degree of recipient T- and

B-cell differentiation.[36–39] It was therefore important to determine whether the mature lymphocytes recovered from reconstituted mice were of donor or host origin. We therefore reconstituted neonatal C.B-17 SCID mice (Thy-1.2) with neonatal spleen cells from B6.PL.Thy-1(a)Cy (Thy-1.1) mice. At 8 weeks of age mice reconstituted under these conditions very closely resembled mice reconstituted with C.B-17 neonatal spleen cells in terms of the number and type of cells recovered (data not shown). The data in Fig. 1 show Thy-1.1 and Thy-1.2 staining on thymus, lymph nodes, spleen, liver and i-IEL. Clearly, in all these organs, the majority of the cells were of the donor Thy-1.1 phenotype while very few Thy-1.2$^+$ cells were detected in any organ. A few Thy-1.2$^+$ cells were observed in the thymus. Further investigation showed the Thy-1.2$^+$ cells were CD3$^-$, and only 15% were CD4$^+$CD8$^+$ (data not shown). Significant numbers of Thy-1.2$^+$ cells were sometimes also detected in the liver. These cells were also found to be CD3$^-$ (data not shown). Clearly the majority of mature T cells generated upon reconstitution of our SCID mice are of donor origin.

CD4/CD8 subsets in reconstituted mice

T cells can be divided into two major subsets based on the expression of CD4 and CD8. Bosma *et al.*[15] and Cowing & Gilmore[16] demonstrated good T-cell reconstitution of SCID mice following neonatal transfer of fetal liver or adult bone marrow cells. In addition, Hilbert *et al.*[40] showed normal CD4/CD8 thymocyte subsets in SCID mice reconstituted with bone marrow. The data in Fig. 2 show CD4 and CD8 expression in the thymus, lymph nodes and spleen of mice reconstituted with the three different types of precursors compared to normal C.B-17 mice. It is clear that CD4$^+$CD8$^+$, CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ subsets are present in normal proportions in all reconstituted mice. The variation in the proportion of CD4$^-$CD8$^-$ cells in lymph nodes and spleen results from the differential reconstitution the B-cell compartment (see Table 2).

Differential reconstitution of γδ T-cell subsets

We showed in Table 1 that total γδ cells were not reconstituted to normal numbers in many instances. In light of the observation that different subsets of γδ cells are produced at different stages in ontogeny,[1–9] and precursor potential varies with the origin of the presursor,[10] it was possible that the deficit in γδ cell numbers may be due to a deficit in a particular subset(s). We investigated the subsets of γδ cells present in reconstituted mice with the available mAb. The data in Table 3 show the proportion of γδ cells in the thymus, lymph nodes, spleen, liver and i-IEL that express Vγ4, Vγ5, Vγ7, Vδ4 and Vδ6. Clearly Vγ4$^+$ cells are not reconstituted as well as other γδ

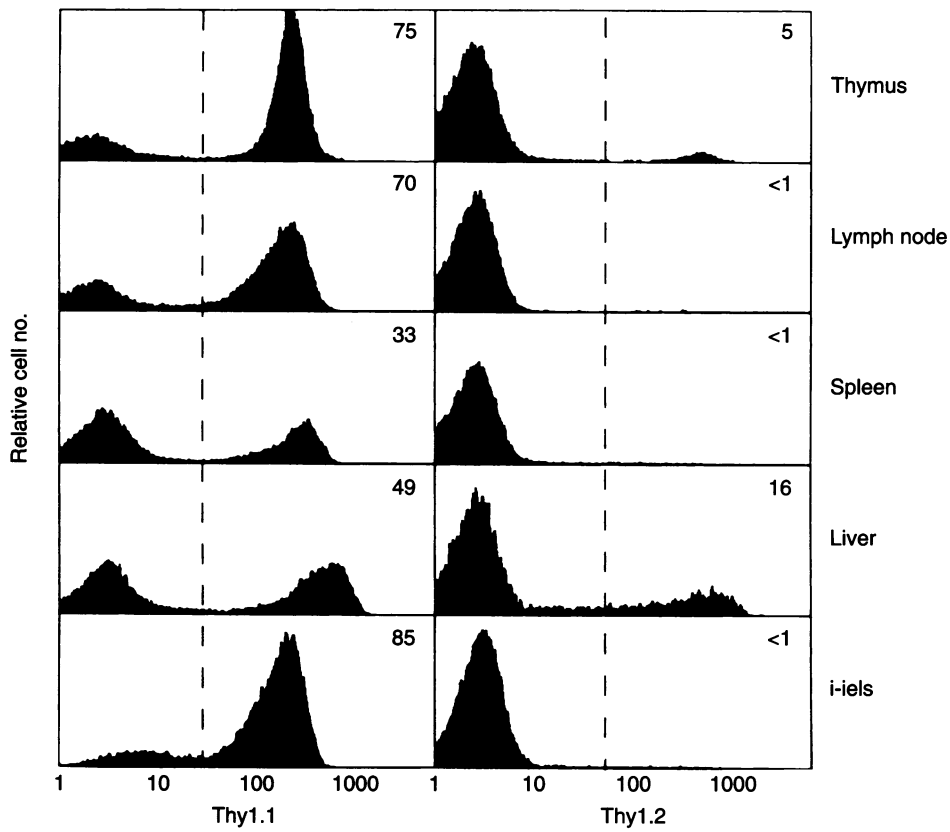

Figure 1. Log fluorescence staining of Thy-1.1 and Thy-1.2 on thymus, lymph nodes, spleen, liver and i-IEL of 8-week-old SCID mice reconstituted as neonates with 10^6 B6.PL.Thy-1(a)Cy neonatal spleen cells. Cells were stained with either anti-Thy-1.1–biotin followed by PEAV, or anti-Thy-1.2–fluorescein isothiocyanate. Results are typical of four individual mice. The numbers in the top right-hand corner of each profile represent the percentage of cells expressing Thy-1 as determined after subtraction of background staining.

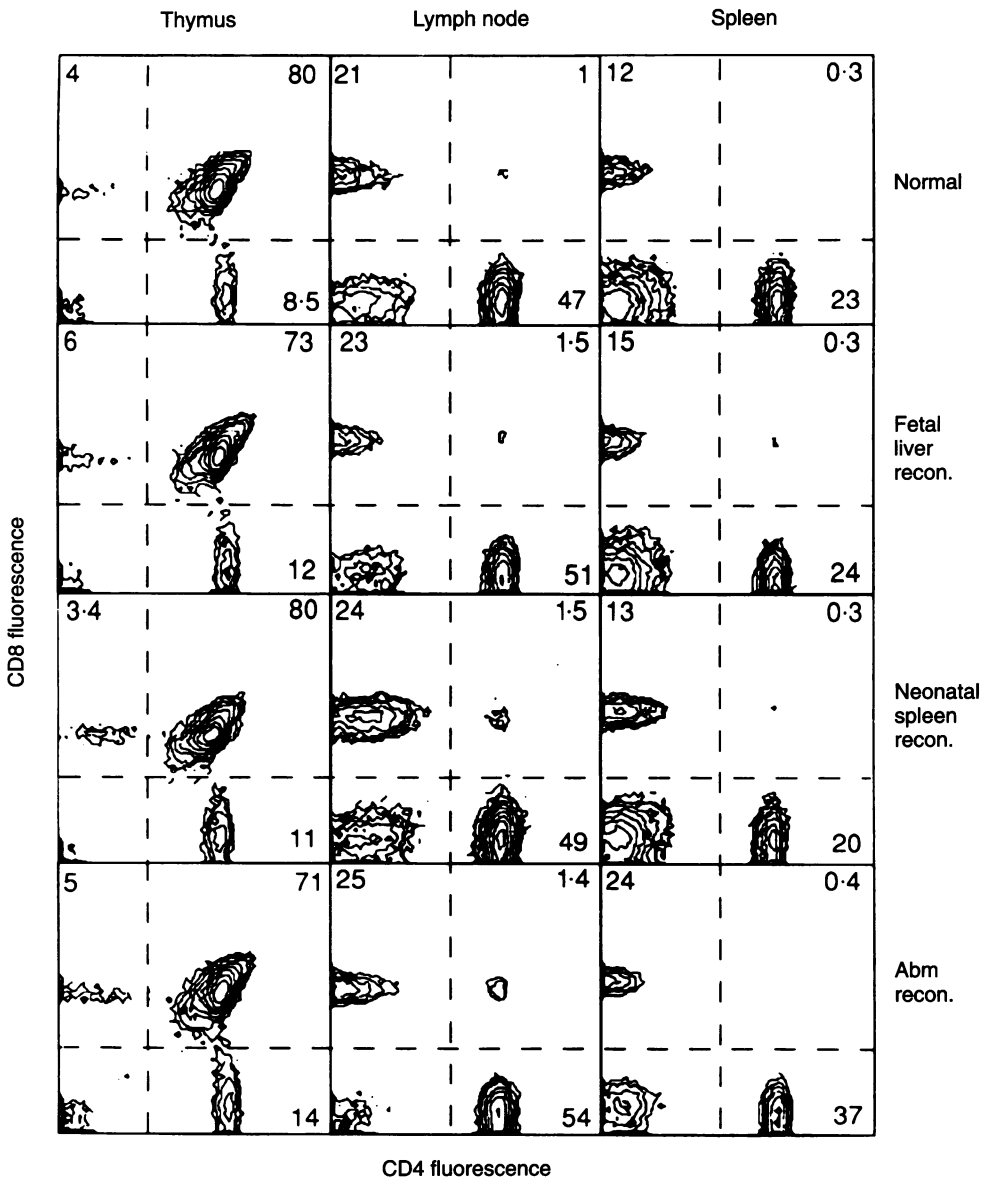

Figure 2. Fluorescence staining of CD4 and CD8 on thymus, lymph nodes and spleen of normal C.B-17 mice and 8-week-old SCID mice reconstituted as neonates with 10^7 fetal liver cells, 10^6 neonatal spleen cells, or 10^6 adult bone marrow cells. The percentage of each subset of the whole is shown. Cells were stained with anti-CD4–phycoerythrin + anti-CD8–fluorescein isothiocyanate. Results are typical of those obtained for five to seven individual mice per reconstitution regime. Data are plotted on a 4-log decade scale.

subsets, particularly in mice that received neonatal spleen or adult bone marrow. Some reduction in $V\gamma4^+$ cells was evident in all organs examined. In addition, adult bone marrow seems to have little capacity to produce liver $V\delta4^+$ cells although significant numbers of $V\delta4^+$ cells were present in other organs. It should be remembered that the total number of liver γδ cells in the mice reconstituted with newborn spleen or adult bone marrow was much less than in normal mice. Therefore even those subsets found to be present in normal or elevated proportions were frequently decreased in number.

The female reproductive tract is another anatomical site at which a significant proportion of T cells express the γδ TCR. Immunoperoxidase staining of uterine sections showed normal numbers of TCR αβ and TCR γδ cells in mice reconstituted with fetal liver or neonatal spleen. Mice reconstituted with adult bone marrow had significantly fewer γδ cells per section than normal mice, and therefore a lower αβ:γδ ratio (data not shown). Overall, reconstitution of neonatal SCID mice with γδ T cells was surprisingly normal with some qualitative and quantitative variation depending on the reconstitution regime.

DISCUSSION

A number of studies have shown that the lymphoid compartment of SCID mice can be reconstituted to significant levels after neonatal transfer of haemopoietic precursors.[15,16] These studies assessed functional reconstitution, but did not investigate reconstitution of different lymphocyte subsets in detail.

Table 3. Percentage of $V\gamma4^+$, $V\gamma5^+$, $V\gamma7^+$, $V\delta4^+$ and $V\delta6^+$ cells among total $\gamma\delta$ cells in thymus, lymph nodes, spleen, liver and i-IEL of normal mice and SCID mice reconstituted with 10^7 fetal liver cells, 10^6 neonatal spleen cells and 10^6 adult bone marrow cells. Seven separate samples (for thymus, lymph nodes and spleen these were depleted of $CD4^+$ and $CD8^+$, while immunoglobulin-positive cells also depleted from the latter two populations) were stained for each organ/reconstitution with the following biotinylated antibodies: anti-$\gamma\delta$, anti-V$\gamma4$, anti-V$\gamma5$, anti-V$\gamma7$, anti-V$\delta4$, anti-V$\delta6$, or no antibody. After washing, the cells were incubated with PEAV. Results are from three to five independent experiments per reconstitution regime each involving four to eight mice

Reconstitution	Organ	Vγ4	Vγ5	Vγ7	Vδ4	Vδ6
Normal	thymus	47±3	0	16±2	19±1	12±2
Fetal liver		42±2	0	17±3	24±3	14±4
Neonatal spleen		22±3	0	16±2	11±1	15±3
Adult bone marrow		20±3	0	11±2	8±1	18±2
Normal	lymph node	48±5	0	8±1	20±1	3±1
Fetal liver		29±3	0	8±5	19±5	4±1
Neonatal spleen		21±0	0	14±8	17±3	9±3
Adult bone marrow		15±6	0	9±4	23±9	10±4
Normal	spleen	31±4	0	12±2	13±2	4±2
Fetal liver		20±2	0	13±6	9±4	6±1
Neonatal spleen		16±2	0	14±1	16±9	7±2
Adult bone marrow		11±1	0	12±2	12±2	ND
Normal	liver	30±6	1±0	40±1	17±7	17±9
Fetal liver		28±7	2±1	38±11	10±3	29±16
Neonatal spleen		20±10	1±0	21±3	5±0	25±4
Adult bone marrow		15±1	2±1	25±7	2±1	37±19
Normal	i-IEL	11±2	0	64±6	20±1	18±3
Fetal liver		8±2	0	69±4	20±4	19±3
Neonatal spleen		3±3	0	67±3	17±4	28±5
Adult bone marrow		6±6	0	55±9	16±7	19±3

Our results show that donor-derived $CD4^+$ and $CD8^+$ cells exist in normal proportions in reconstituted mice although a gradation of the absolute number of cells generated was seen, with fetal liver cells generating closest to normal numbers of cells, and mice receiving adult bone marrow being the least well reconstituted. These differences could not be fully overcome by increasing the inoculum 10-fold, indicating inherent differences in the ability of haemopoietic precursors from different sources to reconstitute the lymphoid compartments of a SCID mouse. One possible explanation for the different reconstitution potential of fetal liver, neonatal spleen and adult bone marrow, stems from the work of Lemischka *et al.*[41], who found that upon reconstitution, haemopoietic stem cells can persist for long periods in a quiescent state that seems to be at least partially under the control of other stem cells. One could hypothesize that perhaps adult bone marrow-derived precursors are more susceptible to a quiescent state induced by host stem cells, while fetal liver precursors are least susceptible. A less involved but related explanation would be that ontogenically older precursors have an inherent diminished capacity to expand and differentiate.

The adult bone marrow and neonatal spleen cells used as a source of precursors contain a small number of mature T cells and B cells. It is therefore conceivable that these mature cells could repopulate the SCID mice. While other investigators have been able to establish conditions under which adoptively transferred mature T cells will survive in SCID mice, mature cells do not reconstitute SCID mice very well under the conditions we have used. When we transferred mature T cells into syngeneic SCID mice, only 5% of the initial innoculum could be recovered 8 weeks post-transfer (data not shown). While it is possible that some T cells in the reconstituted SCID may have been transferred as mature cells, the vast majority of T cells would have to have been produced post-transfer.

We found that, overall $\gamma\delta$ T cells were reconstituted as well as $\alpha\beta$ cells. This was not the case in the liver, and is consistent with the hypothesis that many liver $\gamma\delta$ cells are of fetal or early neonatal origin. Data from Ellis-Roark *et al.*[12] showing that many of the TCR Vγ junctional sequences of liver-derived hybridomas are simple and therefore fetal-like, are also consistent with a fetal/perinatal origin of a proportion of liver $\gamma\delta$ cells. Our observation that some hepatic $\gamma\delta$ cells are reconstituted after transfer of haemopoietic precursor cells into neonatal mice, combined with the finding that $\gamma\delta$ cells emigrate from the adult thymus to the liver (K.A.K., unpublished observations), indicate that the fetal/neonatal thymus is not the sole source of hepatic $\gamma\delta$ cells and that some subsets of hepatic $\gamma\delta$ cells are produced by the postnatal thymus.

Studies by Ikuta *et al.*[10] have shown that the development of $V\gamma5^+$ dendritic epidermal cells requires both a fetal source of precursor cells and a fetal thymic microenvironment. While it is clear that cells expressing the $\gamma\delta$ form of the TCR are the first to appear in the thymus in ontogeny, $\gamma\delta$ cells are also produced by the adult thymus.[42] The relative contribution of the adult, fetal and neonatal thymus to the peripheral $\gamma\delta$ T-cell pool has been speculative to date. Our results show some interesting and hitherto unknown patterns of reconstitution of $\gamma\delta$ cells by different sources of precursors. In particular,

Vγ4$^+$ cells were not reconstituted to normal levels. This observation was true in all organs examined for all three precursor sources, being most pronounced in mice reconstituted with adult bone marrow, and least severe in mice reconstituted with fetal liver cells. This suggests that in normal mice a subset of Vγ4$^+$ cells are produced early in life and persist. An explanation for the partial reconstitution of Vγ4$^+$ cells may lie in the order they are found on the chromosome. The two most 3′ V-regions, Vγ5 and Vγ6 cease to be utilized around the time of birth. The next most 3′ V-region is Vγ4 which seems to be somewhat decreased in use in the adult. There is no evidence to suggest that the most 5′ of V-regions, Vγ7, is suppressed in the adult. The Vγ1 and Vγ2 gene segments are not contiguous with these Vγ segments. They are to be found 3′ of JγlCγ1, and presumably are not a target for a Vγ5/6/4 'suppressor element'. Some correlation between Vδ4 cell production and precursor source was also observed. In particular, hepatic Vδ4$^+$ cells were poorly reconstituted in mice that received neonatal spleen or adult bone marrow, suggesting liver Vδ4$^+$ cells are a separate/non-overlapping population of cells to those in other peripheral lymphoid sites. The relationship between Vδ-gene usage and relative position on the chromosome has not been studied.

While T cells were reconstituted differentially and to varying degrees, it is clear that they were reconstituted to a greater degree than B cells. Reconstitution of different B-cell subsets, such as the CD5-type B cells versus so-called conventional B cells was not investigated. Data from Hayakawa *et al.*[43] would suggest that CD5-type B cells are of fetal and neonatal origin, and therefore donor-derived cells of this type would be predicted to be absent in our reconstituted animals.

Clearly the production of lymphoid cells is the result of a highly regulated process dependent upon a complex interplay between various factors including the ontogenic age of the precursor cell, thymic/bone marrow microenvironment and perhaps also the status of peripheral lymphoid tissues. Consequently, lymphocyte production through all phases of ontogeny is required to generate a lymphoid pool of normal composition. The mechanisms that control this process remain to be fully elucidated. In addition to expanding our knowledge of γδ T-cell production, these studies, in combination with previous studies, provide useful information on how one might generate mice that lack certain components of the lymphoid compartment, and may be utilized in the investigation of various immune responses.

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