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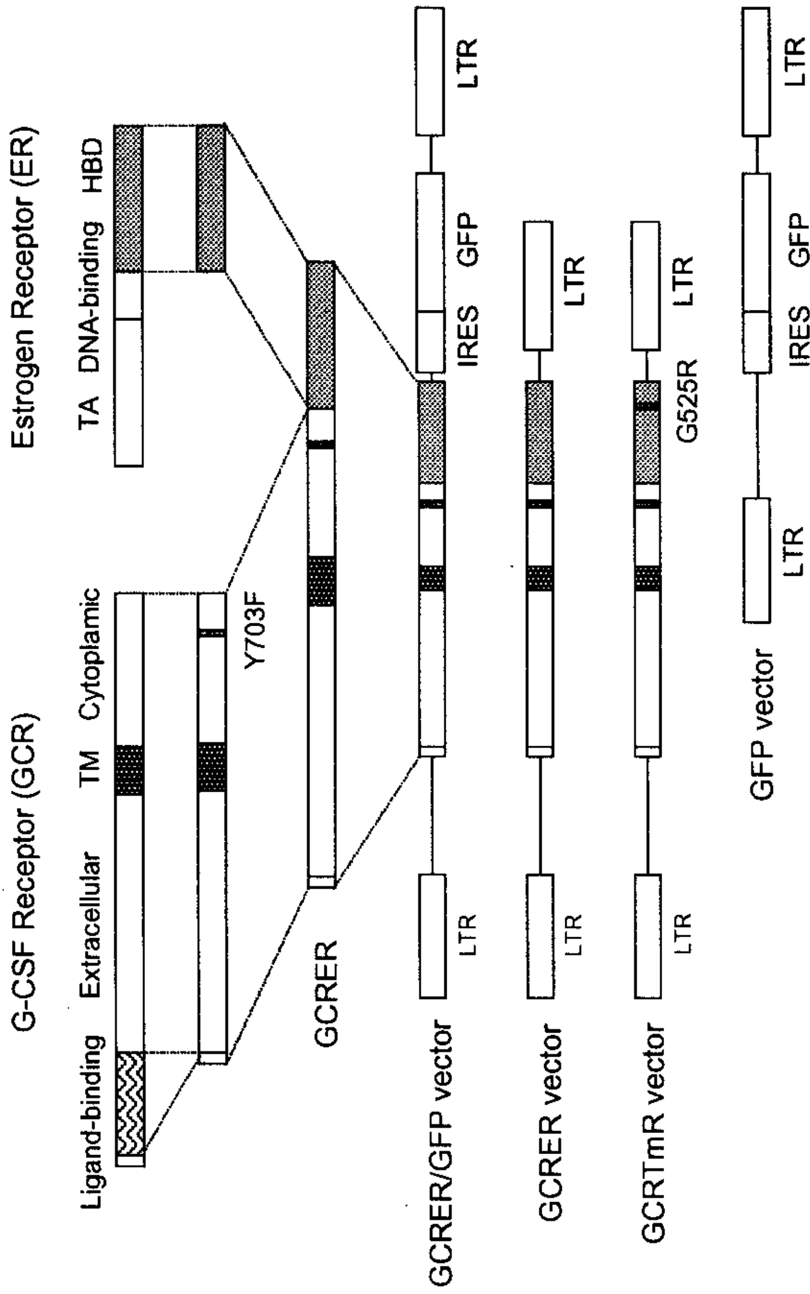
Table 1. Monkeys enrolled in the study

	Animal	Vector	Transduction efficiency (%)		Drug	Route	Peak concentrantion (nM)	Result
			$CD34^+$ cells*	CFU**				
Control study	C1 (female)	GFP	20	ND	(-)	NA***	NA	NA
	C2 (female)	GFP	13	70	(-)	NA	NA	NA
Single marking study	S1 (female)	GCRER/GFP	37	10	Estradiol	Pellet implantation	390	↗
	S2 (female)	GCRER/GFP	40	27	Estradiol	Intramuscular injection	900	→
Dual marking study	D1 (female)	GCRER	1	2.6	Toremifene	Oral intake	130	↗
		Nonexpressing PL II	3	8.6				→
	D2 (male)	GCRTmR	4.7	8.3	Toremifene	Oral intake	1600	↗
		Nonexpressing PL II	1.6	2.2				→
	D3 (male)	GCRTmR	19	38.5	Hydroxytamoxifen	Oral intake	70	→
		Nonexpressing PL I	42	34.0				→
	D4 (male)	GCRTmR	46	24.4	Hydroxytamoxifen	Oral intake	80	→
		Nonexpressing PL I	37	31.0				→

*The fractions of provirus-positive $CD34^+$ cells were examined by semiquantitative PCR.
** Individual colony DNA was examined for vector sequence by PCR.
***NA, not applicable.

Table 2. Genomic DNA sequences flanking the LTR in animal S1

Unique insertion	Provirus	Flanking genomic DNA
Clone 1	3'LTR cgggggtctttca	AACCGTCCGGTAGAA
Clone 2	3'LTR cgggggtctttca	CAGCAAAAAGCTATC
Clone 3	3'LTR cgggggtctttca	CATCACACTGGCGGT
Clone 4	3'LTR cgggggtctttca	TCCGAGCTCGGTACC
Clone 5	3'LTR cgggggtctttca	TTGCTGATCTGCGGT
Clone 6	3'LTR cgggggtctttca	TTGTGGGACAATGAG
Clone 7	3'LTR cgggggtctttca	AGCCGGGGCAGGGT
Clone 8	3'LTR cgggggtctttca	CTCGTCTGCTTGATC
Clone 9	3'LTR cgggggtctttca	TTGCTGGTGGAACTG
Clone 10	3'LTR cgggggtctttca	GCCACCCTGCAGCGC
Clone 11	3'LTR cgggggtctttca	TATAATCGGAGGGCT
Clone 12	3'LTR cgggggtctttca	TCATTTCCCTAGTAG
Clone 13	3'LTR cgggggtctttca	AGCCAATTTCTTTAA
Clone 14	3'LTR cgggggtctttca	AAATCGAGCTTAATA


G-CSF Receptor (GCR)
Ligand-binding
Extracellular
TM
Cytoplamic
Y703F
Estrogen Receptor (ER)
TA
DNA-binding
HBD
GCRER
GCRER/GFP vector
LTR
IRES
GFP
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G525R
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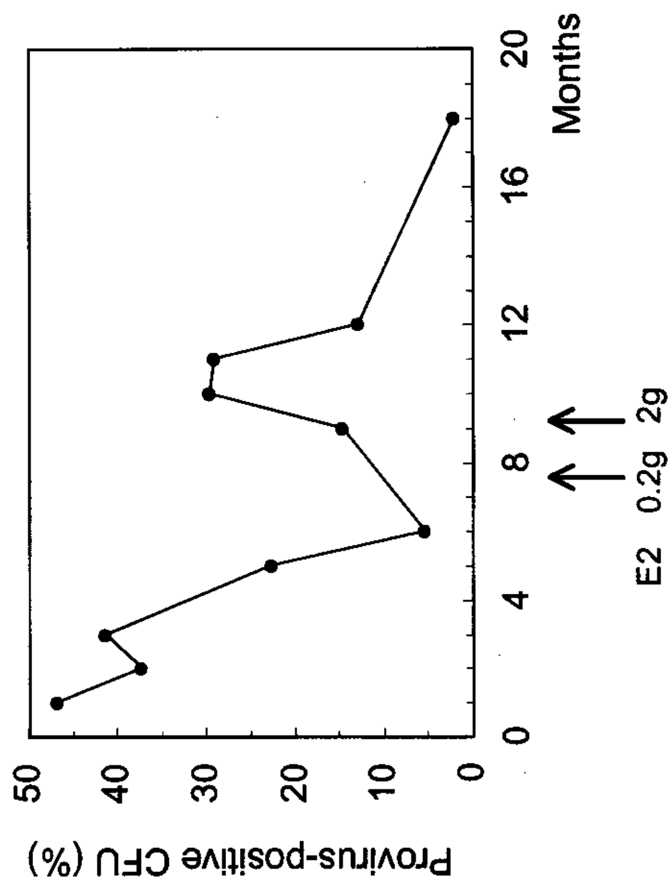
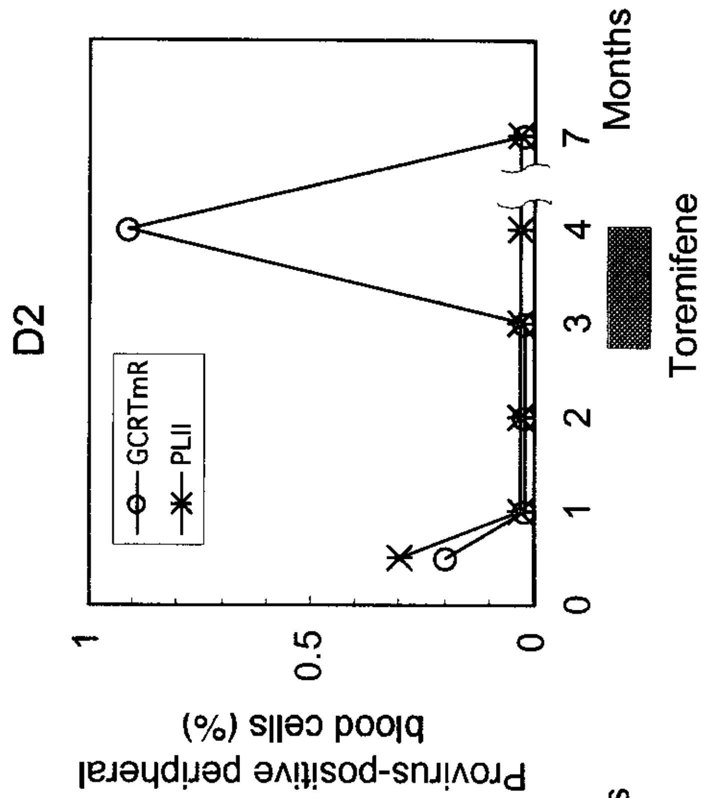
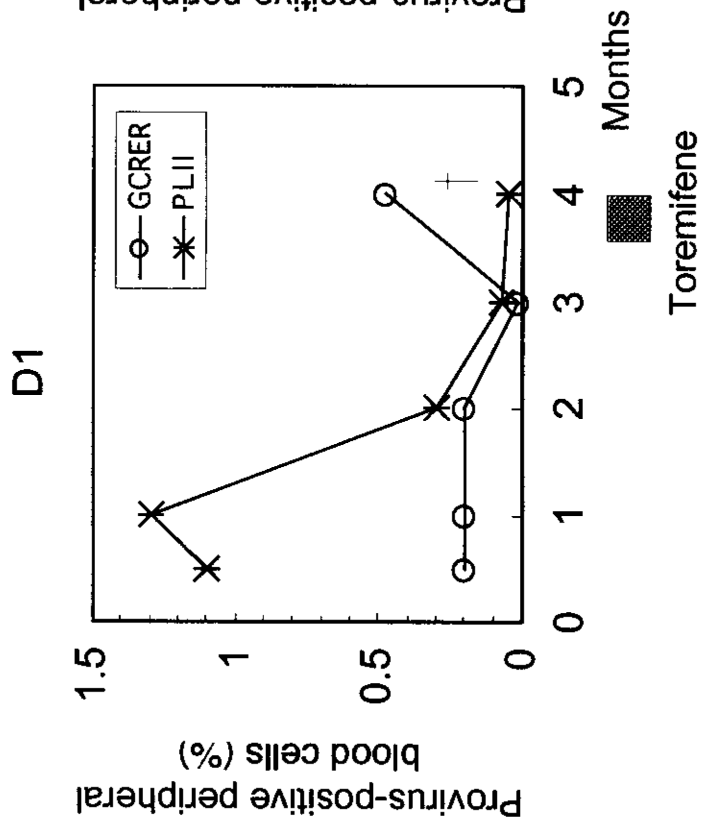
GFP vector
LTR
IRES
GFP
LTR


Provirus-positive CFU (%)
50
40
30
20
10
0
0
4
8
12
16
20
Months
E2
0.2g
2g

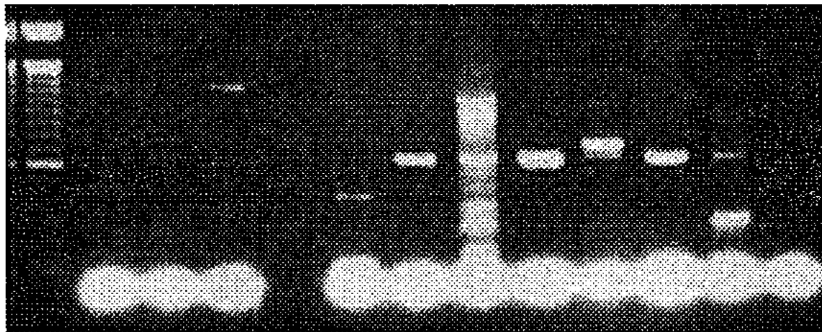


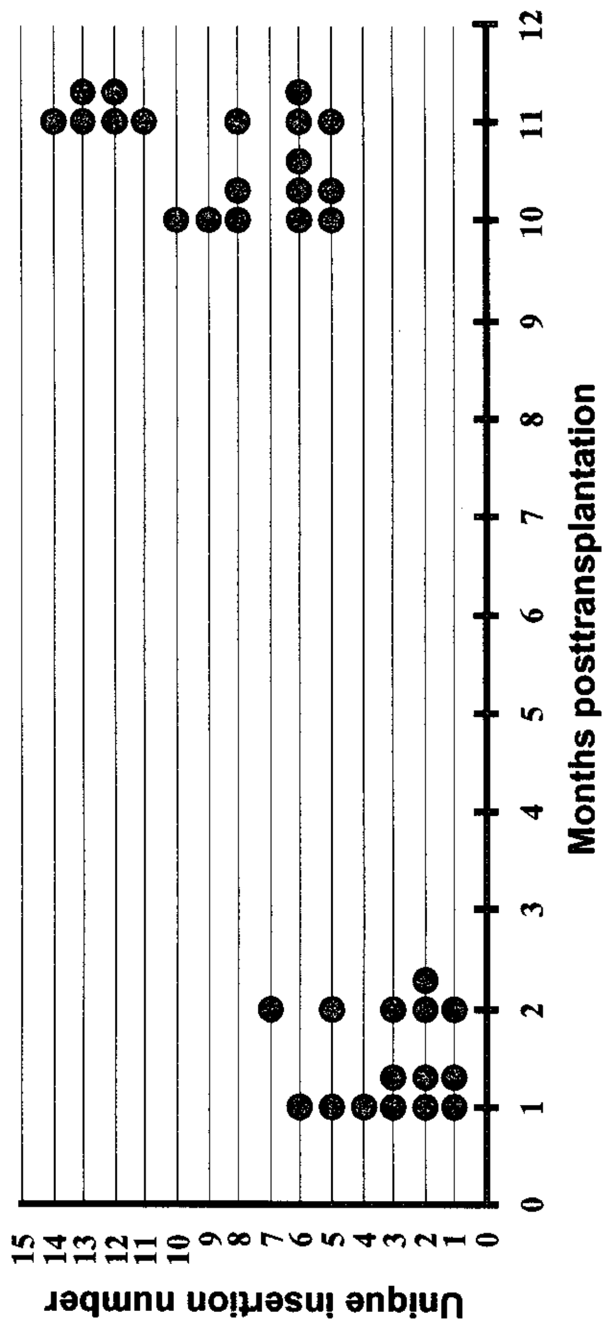
D2
GCRTmR
PLII
Provirus-positive peripheral blood cells (%)
1
0.5
0
0
1
2
3
4
7
Months
Toremifene



D1
GCRER
PLII
Provirus-positive peripheral blood cells (%)
1.5
1
0.5
0
0
1
2
3
4
5
Months
Toremifene

M 1 2 3 4 5 6 M 7 8 9 10 11




Unique insertion number
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Months posttransplantation
0
1
2
3
4
5
6
7
8
9
10
11
12

Selective growth advantage of wild-type lymphocytes in X-linked SCID recipients

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Running Title:

Selective advantage of γc^+ lymphocytes in X-SCID

Summary:

The cytokine receptor common gamma chain (γc) plays a pivotal role in multiple interleukin signaling, and γc gene mutations cause an X-linked form of SCID (X-SCID). Recently, γc gene transfer into the autologous X-SCID BM achieved appreciable lymphocyte reconstitution, contrasting with the limited success in previous gene therapy trials targeting hematopoietic stem cells. To understand the mechanisms underlying this success, we examined the repopulating potential of the wild-type (WT) BM cells using an X-SCID mouse model. Limited numbers of WT cells were infused into non-ablated WT and X-SCID hosts. Whereas no appreciable engraftment was observed in WT recipients, donor-derived lymphocytes well repopulated in X-SCID, reaching 37% (10^6 cells given) and 53% (10^7 cells given) of the normal control value 5 months post-BMT. A lineage analysis showed a predominance of the donor-derived lymphocytes ($CD4^+$ T, $CD8^+$ T, B and NK cells) in X-SCID while the donor-derived granulocytes and monocytes poorly engrafted. These results showed a selective advantage of WT cells in X-SCID, and that the advantage was restricted to lymphocytes. In human gene therapy for X-SCID, an analogous growth advantage would greatly enhance the repopulation of lymphocytes derived from a very small number of γc gene-supplemented precursors.

Keywords:

X-linked severe combined immunodeficiency

common gamma chain

bone marrow transplantation

lymphocyte reconstitution

growth advantage

Introduction

The cytokine receptor common gamma chain (γc) is shared among the receptors for interleukin (IL)-2, -4, -7, -9, -15, and plays a pivotal role in transmitting these cytokine signals.[1,2] Many of these signals are indispensable for lymphoid development and activation. Accordingly, mutations in the X-linked γc gene result in severe developmental failure of lymphoid tissues and immune function, a disorder known as X-linked SCID (X-SCID).[3,4] T cells and natural killer (NK) cells are profoundly diminished in those afflicted with this disorder, and B cells are nonfunctional although present in number. The affected patients suffer from recurrent and severe infections, usually leading to death in infancy or early childhood.

Until recently, the only curative means for X-SCID has been allogeneic BMT. This procedure usually reconstitutes T cells and NK cells, but frequently the humoral immunity is only partially reconstituted, and many patients depend upon regular immunoglobulin supplementation.[5] The reason for this B cell refractoriness is still unknown; preexisting B cells may prevent the donor cells from engrafting under the non-ablative BMT protocols currently used for X-SCID.

Recently, Fischer *et al* showed that hematopoietic stem cell gene transfer was effective in treating X-SCID.[6] Autologous BM CD34$^+$ cells were retrovirally transduced and reinfused into patients, and a successful T and NK cell reconstitution was observed in most cases. Whether B cell function can be fully reconstituted is to be clarified in a long-term study. The apparent success of X-SCID stem cell gene transfer makes a sharp contrast to the limited success in previous clinical trials targeting hematopoietic stem cells. Although recent advances in gene transfer such as newly introduced early-acting cytokines (TPO and Flt3 ligand) and the recombinant fibronectin fragment (CH296) may have augmented gene transfer efficacy,[7] it is unlikely that the Moloney-based retrovirus vector has achieved a dramatic increase in transduction efficiency into human hematopoietic stem cells.

One possibility that may account for their success is the pathogenesis of X-SCID and

the function of γc. Once hematopoietic stem cells or lymphoid progenitors are transduced with a functional γc gene, these cells can exclusively utilize the growth signals via IL-7 and other cytokines. That is, the therapeutic gene itself confers a tremendous growth advantage on the transduced cells, a situation rarely encountered in stem cell gene therapy for other diseases. To examine the strength of the selective advantage that lymphocytes with normal γc expression have over γc-deficient cells, we injected limited numbers of wild-type (WT) BM cells into non-ablated WT and X-SCID mice, and monitored the engraftment of the donor cells.

Materials and methods

Animals

Inbred C57BL/6-Ly5.2 mice were purchased from Clea Japan (Tokyo, Japan). The congenic C57BL/6-Ly5.1 mice are maintained in the animal facility of Jichi Medical School (Tochigi, Japan). A murine X-SCID model was created by targeted disruption of the X-linked γc gene.[8] The X-SCID mice were back-crossed to a C57BL/6-Ly5.2 background and an inbred colony was established. Meanwhile, congenic X-SCID mice were established by crossing with C57BL/6-Ly5.1 mice. The animals were maintained in a specific pathogen-free environment in the institutional animal facility and treated following institutional codes for animal rights.

BMT

BM cells were obtained from 6-week-old WT C57BL/6-Ly5.2 mice by flushing the femora with α-Minimum Essential Medium (Life Technologies, Grand Island, NY, USA), and the low-density mononuclear cells were obtained by density centrifugation on Lympholyte-M (Cedarlane, Hornby, Ontario, Canada). Either 10^5, 10^6 or 10^7 cells were injected into WT C57Bl/6-Ly5.1 and X-SCID C57BL/6-Ly5.1 recipients (8- to 10-week-old) via the tail vein without prior myeloablation.

Blood count and flow cytometry

Mouse peripheral blood was obtained by tail clipping and a complete blood count was made with a PC-608 particle counter (Erma, Tokyo, Japan). Prior to flow cytometry, RBC were lysed with ACK buffer (150 mM NH_4Cl, 12 mM $KHCO_3$, and 0.125 mM EDTA) for 15 minutes on ice. WBC were stained with MoAbs specific for Ly5.1, Ly5.2, Gr1, Mac1, CD4, CD8, B220, IgM, TCRβ and NK1.1 (Pharmingen, San Diego, CA, USA), and analyzed with a FACScan (Becton-Dickinson, San Jose, CA, USA).

Results

Lymphocyte reconstitution

The X-SCID mouse model was created by a partial replacement of the γc gene with a *neo* cassette, resulting in a deletion of the γc cytoplasmic domain required for signal transduction.[8] Similar mutations are found in a subset of X-SCID patients, whose phenotypes are indistinguishable from those with null mutations.[4] In mice, hemizygous males and homozygous females have a hypoplastic thymus and other lymphoid tissues, and are markedly lymphopenic. In addition to a profound reduction or absence of T cells and NK cells as observed in human X-SCID patients, the mice show significant B lymphopenia. B cell reduction is commonly observed in murine X-SCID models created with different targeting constructs,[8-10] suggesting that mouse B cells are more profoundly dependent on γc-mediated signals than their human counterparts. In the older X-SCID mice used in this study, a relative accumulation of $CD4^+$ T cells was observed, but the reason for this aberrant lymphopoiesis is yet to be determined.[8]

Before BMT, the X-SCID mice were severely lymphopenic, with total lymphocyte counts of 150 - 450/μl (1 - 3% of WT control; Table 1). A single infusion of 10^6 or 10^7 WT BM cells led to a marked increase of lymphocytes in these animals. One month following BMT, mice having received 10^6 or 10^7 cells showed a significant increase ($p < 0.02$ and p <

0.001) of total lymphocytes, and the lymphocyte counts steadily increased. Five months after BMT, lymphocyte numbers in the X-SCID recipients were 35 ± 11% and 73 ± 8% of the WT control, with infusion of 10^6 cells and 10^7 cells, respectively (Table 1 and Figure 1a). The X-SCID mice infused with 10^5 cells had increased numbers of lymphocytes following BMT, and the increase was close to being significant. During the observation, the lymphocyte counts in the WT recipients were unchanged.

Lymphocyte reconstitution was observed in all the subsets examined ($CD4^+$ T, $CD8^+$ T, B and NK cells). Figure 1 shows the absolute counts of lymphocyte subsets during the observation period, and Table 1 summarizes the end-point results at 5 months post-BMT. At this point, relative lymphoid reconstitution in the X-SCID mice infused with 10^6 cells was 28 ± 12%, 68 ± 23%, 25 ± 10% and 16 ± 6% for $CD4^+$ T, $CD8^+$ T, B and NK cells. At the same time, the X-SCID mice having received 10^7 cells had 52 ± 9%, 111 ± 27%, 87 ± 11% and 53 ± 16% of the $CD4^+$ T, $CD8^+$ T, B and NK cells of the WT controls. That is, $CD8^+$ T cells and B cells were within the normal range and $CD4^+$ T cells and NK cells were half-normal, after 10^7 cells were given to the X-SCID mice.

Overall WBC chimerism

Successful lymphocyte reconstitution in the X-SCID recipients was concordant with donor cell engraftment. Donor-derived Ly5.2 cells rapidly repopulated in the X-SCID mice, and as early as 1 month post-BMT, WBC chimerism was 9.5 ± 6.1%, 21.2 ± 9.5% and 51.0 ± 12.8% following infusion of 10^5 cells, 10^6 cells and 10^7 cells, respectively. The WBC chimerism increased and peaked at 3 months after BMT, plateauing thereafter. Five months after BMT, donor-derived WBC engrafted 11.2 ± 10.4%, 54.2 ± 9.5% and 75.0 ± 7.0% in the X-SCID recipients having received 10^5 cells, 10^6 cells and 10^7 cells, respectively (Figure 2). In contrast, the donor cells did not engraft in the WT recipients. The Ly5.2 WBC never reached 1% in the WT Ly5.1 mice, even after 10^7 cells were transplanted. Assuming that the total number of BM cells in a mouse is 2 x 10^8, 10^7 accounts for only 5%. Considering the

homing efficiency following tail vein i.v., the actual number of engrafting cells would be even fewer. Therefore, without preconditioning, graft rejection by immunocompetent mice is a likely consequence. On the other hand, the observed high donor chimerism in X-SCID was quite remarkable, indicating that the WT cells were extremely competitive in the X-SCID recipients.

Lineage chimerism

Although the WT cells engrafted in the unconditioned X-SCID recipients, not all the hematopoietic lineages repopulated equally well. In a series of flow cytometric analyses, donor cell chimerism was evaluated by counting the Ly5.2 cells in each lineage, namely granulocytes ($Gr1^{high}/Mac1^{+}$), monocytes ($Gr1^{low\text{-}mid}/Mac1^{+}$), $CD4^{+}$ T cells ($CD4^{+}/CD8^{-}$), $CD8^{+}$ T cells ($CD4^{-}/CD8^{+}$), B cells ($IgM^{+}/B220^{+}$) and NK cells ($NK1.1^{+}/TCR\beta^{-}$). Figure 3 shows such an analysis at 5 months post-BMT. The predominance of the donor-derived lymphocytes was remarkable, as was expected from the robust lymphocyte reconstitution. Following infusion of 10^6 and 10^7 cells, all the examined lymphocyte subsets were completely donor-derived. Even infusion of 10^5 WT cells resulted in a complete chimerism in $CD8^{+}$ T and NK cells, and more than 50% chimerism in $CD4^{+}$ T and B cells. On the other hand, donor-derived granulocytes and monocytes had a lower occurrence in the X-SCID recipients. For example, in the mice having received 10^7 WT cells, donor-derived granulocyte and monocyte numbers were $4.0 \pm 1.5\%$ and $15.8 \pm 4.7\%$. The results shown in Figure 3 suggested that a growth advantage among the γc-positive cells in X-SCID was greatly biased toward lymphocytes. Because lymphoid cells solely depend on γc-mediated signals, the advantage of γc-positive cells over X-SCID cells is overwhelming. Myeloid cells primarily depend on other cytokine signals, and preexisting granulocytes and monocytes can prevent the same lineage cells from engrafting after unconditioned BMT. It is of note, however, that the engraftment of myeloid cells was better in the X-SCID recipients than in the WT animals. This may suggest that the Ly5-congenic cells were immunogenic in the uncompromised hosts,

and that the reason for the Ly5.2 lymphocyte proliferation in the Ly5.1 X-SCID was twofold (see Discussion).

Discussion

In the present study, we showed that WT (γc^+) lymphocytes had a selective advantage in X-SCID recipients after non-myeloablative BMT. As long as we have tested, the extent of donor cell repopulation was dose-dependent, and infusion of limited numbers (10^6 or 10^7) of WT BM cells resulted in a significant repopulation of all the examined lymphoid subsets such as $CD4^+$ T, $CD8^+$ T, B and NK cells. We assumed that WT cells corresponding to only 5% of the total BM cells could reconstitute the hosts' lymphoid system to a half- to near-normal level. It is of interest to compare the engrafting potential of WT cells in other types of immunodeficiency disorders. Rohrer and Conley reported a similar competitive repopulation study using Btk tyrosine kinase-deficient X-linked immunodeficient (*xid*) mice.[11] They transplanted limited numbers of WT BM cells together with excess *xid* cells into lethally irradiated *xid* mice. An approximately 10-fold enrichment of WT B cells over *xid* cells was observed, and the humoral immunity was reconstituted. In our study, WT cells showed an apparently stronger selective advantage in X-SCID mice than in *xid* mice. Presumably, the dependency of the lymphoid lineage on the γc-mediated signal is much greater than that of B cells on the Btk-mediated signals. Such an enormous proliferative potential of the γc-positive lymphoid progenitor was suggested in an atypical case of X-SCID. Reportedly, a reversal event in the mutated γc gene resulted in a functional lymphoid precursor generating at least 1,000 T cell clones, and the disease phenotype being significantly ameliorated.[12,13]

Besides the engrafting potential of the donor cells, one should consider the immunogenicity of the donor cells and the host's immune status in BMT experiments. Although the chimerism was low, we observed that WT BM-derived granulocytes and monocytes engrafted better in X-SCID recipients than in WT animals (Figure 3). Since myeloid cells primarily depend on growth and differentiation signals other than γc-mediated