

B-cell function in canine X-linked severe combined immunodeficiency

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Abstract

Canine X-linked severe combined immunodeficiency (XSCID) is due to mutations in the common gamma (γ c) subunit of the IL-2, IL-4, IL-7, IL-9 and IL-15 receptors and has a similar clinical phenotype to human XSCID. We have previously shown that the block in T-cell development is more profound in XSCID dogs than in genetically engineered γ c-deficient mice. In this study we evaluated the B-cell function in XSCID dogs. In contrast to the marked decrease in peripheral B-cells in γ c-deficient mice, XSCID dogs have increased proportions and numbers of peripheral B-cells as observed in XSCID boys. Canine XSCID B-cells do not proliferate following stimulation with the T-cell-dependent B-cell mitogen, pokeweed mitogen (PWM); however, they proliferate normally in response to the T-cell-independent B-cell mitogen, formalin-fixed, heat-killed *Staphylococcus aureus*. Canine XSCID B-cells are capable of producing IgM but are incapable of normal class-switching to IgG antibody production as demonstrated by in vitro stimulation with PWM and immunization with the T-cell-dependent antigen, bacteriophage Φ X174. Similar results have been reported for XSCID boys. Thus, it appears that γ c-dependent cytokines

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have differing roles in human and canine B-cell development than in the mouse making the XSCID dog a valuable model for studying the role of these cytokines in B-cell development and function.
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1. Introduction

Severe combined immunodeficiency (SCID) represents a heterogeneous group of genetic diseases characterized by the absence of humoral (B-cell) and cell-mediated (T-cell) immune function which usually results in death by 1–2 years of age (Rosen et al., 1995). Over the past few years, the genes for many forms of SCID have been identified. The most common form of SCID is X-linked SCID (XSCID) which is due to mutations in the common gamma (γ c) subunit of the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15 (Takeshita et al., 1992; Noguchi et al., 1993a, b; Puck et al., 1993; Russell et al., 1993; Kondo et al., 1993a, b; Giri et al., 1994; Russell et al., 1995). Thus, the XSCID phenotype is the complex result of multiple cytokine defects. The shared usage of the γ c by receptors for growth factors that are essential for normal B- and T-cell development and function explains the profound immunologic abnormalities and clinical severity of the disease.

XSCID boys develop severe infections usually between 3 and 6 months of age, a time when maternal antibody is declining. The immunologic abnormalities have been recently reviewed (Conley et al., 1990; Gougeon et al., 1990; Conley, 1991; Buckley et al., 1993; Matthews et al., 1995; Rosen et al., 1995). At the time of diagnosis, affected boys have markedly reduced or absent peripheral T-cells that fail to proliferate in response to antigenic or mitogenic stimulation. Peripheral B-cells are present in normal or increased numbers but fail to mature and function normally. B-cells from XSCID boys are capable of producing IgM *in vitro* and *in vivo*, however they are unable to class switch from IgM to IgG (Small et al., 1989; Conley et al., 1990; Gougeon et al., 1990; Conley, 1991; Buckley et al., 1993). Although XSCID B-cells do not proliferate following stimulation with anti- μ or pokeweed mitogen, T-cell-dependent B-cell mitogens (Small et al., 1989; Conley et al., 1990; Gougeon et al., 1990), they are capable of proliferating following stimulation with heat-killed, formalin-fixed *Staphylococcus aureus* Cowan I strain (SAC) that has been documented to be a T-cell-independent B-cell mitogen in humans (Forsgren et al., 1976; Schuurman et al., 1980; Dosch et al., 1980; Falkoff et al., 1982; Small et al., 1989; Gougeon et al., 1990). Since bone marrow transplantation is currently the only treatment for XSCID, the natural history of human XSCID has been difficult to study.

Our laboratory has identified and characterized a naturally-occurring X-linked severe combined immunodeficiency in dogs (reviewed by Felsburg et al., 1998). The γ c mutation in our XSCID colony, which was derived from a single female carrier, is a 4 bp deletion in exon 1 that results in a predicted truncated protein of 21 aa instead of the normal 373 aa, in essence representing a naturally occurring γ c 'knock-out' (Henthorn et al., 1994; Felsburg et al., 1998). The clinical, immunologic and pathologic features of canine XSCID are virtually identical to human XSCID. During the neonatal period,

XSCID dogs have few, if any, peripheral T-cells and an increased number of peripheral B-cells. Some XSCID dogs do develop phenotypically mature, nonfunctional peripheral T-cells by 8–10 weeks of age, the biological equivalent of three human years (Glickman and Domanski, 1986), however the absolute number of peripheral T-cells remain significantly decreased compared to normal age-matched normal dogs. The purpose of this study was to document the utility of SAC as a T-cell-independent B-cell mitogen in the dog and to determine whether B-cell function in XSCID dogs is similar to that in XSCID boys.

2. Materials and methods

2.1. Dogs

The XSCID dogs used in this study were derived from a breeding colony established from a single carrier female (Jezyk et al., 1989; Felsburg et al., 1998). All of the affected males have the same γ c mutation, a 4 bp deletion in exon 1, and were diagnosed shortly after birth by a PCR based mutation detection assay using DNA isolated from whole blood (Henthorn et al., 1994).

2.2. Isolation of cells

Peripheral blood was collected by venipuncture into a heparinized syringe. The peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of diluted heparinized blood over a discontinuous gradient of Hypaque–Ficoll (Wunderli and Felsburg, 1989). Lymphoid tissue was obtained following humane euthanasia. Thymocytes, tonsil lymphocytes and lymph node cells were obtained by mincing the tissue into a single cell suspension with forceps. The resulting cell suspension was filtered through a fine mesh filter and washed twice with HBSS. Splenocytes were obtained in a similar manner except that after filtration, cells were centrifuged and resuspended in ammonium chloride lysis buffer (Sigma, St. Louis, MO) as previously described (HogenEsch and Felsburg, 1989).

B-cells were isolated by panning (Somberg et al., 1994). Bacterial grade Petri-dishes were coated with 10 μ g/ml affinity purified goat anti-dog IgG (Sigma, St. Louis, MO) in 0.05 M Tris–HCl buffer, pH 9.0, for 90 min at room temperature. The Petri-dishes were washed five times with PBS, incubated for 30 min with PBS/10% FCS, followed by two washes in PBS. Lymph node cells were added in 5 ml of RPMI 1640/5% FCS, supplemented with 25 mM HEPES, at a concentration of 5×10^6 cells/ml. After 1 h of incubation at room temperature with brief swirling at 30 min, the nonadherent cells were removed and the dishes gently washed four times with RPMI. Adherent cells were then gently removed with a cell scraper.

2.3. Flow cytometry

PBMC were stained for flow cytometric analysis as previously described (HogenEsch and Felsburg, 1989; Somberg et al., 1996). B-cells were quantitated using FITC

conjugated F(ab')₂ goat anti-dog IgG (heavy and light chain specific; Cappel, Durham, NC). The canine CD 3 monoclonal antibody, CA17.2A12, was used to quantitate T-cells (Moore et al., 1994). The secondary antibody was phycoerythrin-labeled anti-mouse IgG (Fisher Scientific, Pittsburgh, PA). Analysis gates were adjusted to 2% positive staining with negative controls. For each sample, 10,000 cells were analyzed using a Becton Dickinson FACSCalibur (Becton Dickinson, San Jose, CA).

2.4. Quantitation of serum IgG

Serum immunoglobulin concentrations were measured by radial immunodiffusion using affinity-purified heavy-chain specific antisera to canine IgA, IgG, and IgM (Bethyl Laboratories, Montgomery, TX).

2.5. Proliferation assays

The response of PBMC to in vitro mitogenic stimulation with PHA-P (2.5 µg/ml), PWM (5 µg/ml), and heat-killed, formalin-fixed *Staphylococcus aureus*, Cowan strain (varying concentrations) was performed as previously described (HogenEsch and Felsburg, 1989; Felsburg et al., 1997). All mitogens were purchased from Sigma, St. Louis, MO. Sixteen hours before the end of the culture period, 0.5 mCi ³H-thymidine (6.8 Ci/mmol; New England Nuclear, Boston, MA) was added to each well. At the end of the incubation period, the cells were harvested onto glass fiber filters and the incorporation of radioactivity measured by liquid scintillation spectrometry. The results are expressed as counts per minute (CPM).

2.6. Assessment of specific antibody production

Bacteriophage ΦX174 was administered intravenously in a dose of ~3 × 10⁹ PFU/kg. A secondary immunization was given 6 weeks after the primary immunization. Phage clearance and specific phage-neutralizing antibody activity, expressed as the rate of phage inactivation (*K*-value, *K*_v), was determined as previously described (Ochs et al., 1974; Felsburg et al., 1997). IgG antibody was measured as antibody activity that was resistant to treatment with 2-mercaptoethanol.

2.7. Assessment in vitro immunoglobulin production

PBMC were cultured in quadruplicate in 96-well flat bottom plates (Costar, Cambridge, MA) at 1 × 10⁵ cells/well in RPMI 1640 and 10% FCS and stimulated with 5 µg/ml PWM. Control wells consisted of media alone. The plates were incubated for 7 days at 37°C in a humid, 5% CO₂ atmosphere. Supernatants were collected and analysed by an indirect ELISA technique (HogenEsch and Felsburg, 1989). ELISA plates were coated overnight at room temperature with optimal concentrations of the IgG fraction of heavy chain specific rabbit anti-dog IgG and IgM (Rockland, Gilbertsville, PA) in bicarbonate buffer, pH 9.6. Standard curves were constructed for each plate using two-fold dilutions of purified dog IgG and IgM (kindly supplied by Dr. Robert Schwarzman).

Supernatants were diluted 1:5 and 1:25 in PBS-Tween and 75 μ l was added to the wells in triplicate. After 2 h of incubation at room temperature, the plates were washed three times with PBS-Tween and incubated for 2 h at room temperature with peroxidase-labeled, heavy chain specific rabbit anti-dog IgG or rabbit anti-dog IgM (Rockland, Gilbertsville, PA). The plates were read in a Molecular Dynamics V_{\max} plate reader at 490 nm.

3. Results

3.1. Phenotypic analysis of canine XSCID PBMC

Newborn canine XSCID dogs exhibit an identical peripheral blood lymphocyte phenotype as XSCID boys consisting of an increased proportion of B-cells and a severely reduced proportion or absence of peripheral T-cells (Fig. 1). As XSCID dogs age there is an increase of the proportion of peripheral T-cells with a concomitant decrease in the proportion of peripheral B-cells, however, the absolute number of peripheral T-cells remains \approx 20% of normal. This proportion of peripheral B- and T-cells, as well as the absolute numbers, remain relatively constant in XSCID dogs raised in a gnotobiotic environment for up to 4 years (data not shown).

3.2. Serum immunoglobulin concentrations in XSCID dogs

During the first 2–4 weeks of life, there is little difference in the serum immunoglobulin concentrations between normal and XSCID dogs (Table 1). The fact that IgG is relatively normal in the XSCID dogs in the neonatal period is due to maternal transfer of IgG through the colostrum. Between 4 and 8 weeks of age there is a rapid decline in maternal antibody. Table 1 also illustrates that at 8–12 weeks of age, when there little maternal antibody present, XSCID dogs produce comparable levels of IgM as normal dogs, but little IgG.

3.3. In vitro immunoglobulin production by XSCID B-cells

Analysis of in vitro immunoglobulin production following stimulation with PWM demonstrated that XSCID B-cells are capable of producing normal amounts of IgM, but are incapable of producing significant amounts of IgG (Fig. 2). These results are similar to the serum immunoglobulin profiles observed in XSCID dogs.

3.4. Specific antibody response

To assess the ability of XSCID dogs to produce specific antibody, four XSCID dogs raised in a gnotobiotic environment were immunized with the T-cell-dependent antigen, bacteriophage Φ X174, at 8 months of age. Two age-matched normal gnotobiotic littermates were used as controls. Fig. 3 demonstrates that the normal dogs produced a primary and secondary antibody response to Φ X174 and that the secondary antibody response was predominantly IgG antibody. Although the XSCID dogs were capable of

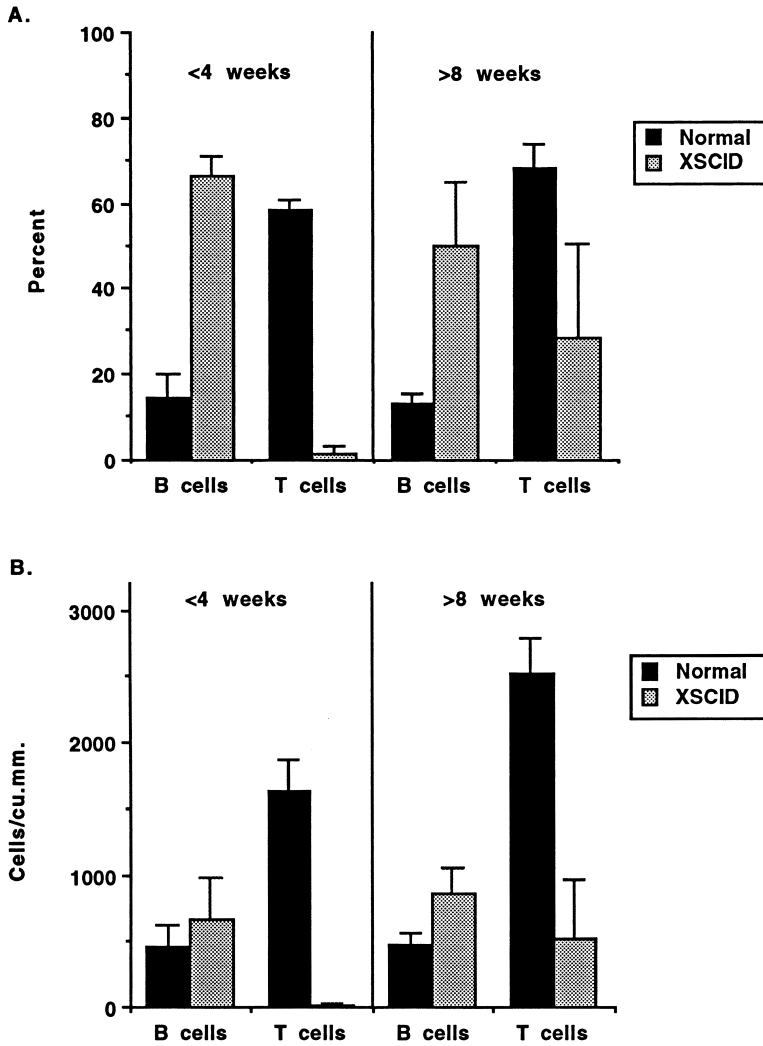


Fig. 1. Proportion (A) and absolute number (B) of peripheral B- and T-cells in XSCID and age-matched normal dogs.

Table 1
Serum immunoglobulin concentrations in XSCID dogs (mg/dl)

	Age <2 weeks		Age 8–12 weeks	
	Normal	XSCID	Normal	XSCID
IgM	27±16	30±14	140±49	115±51
IgG	1016±136	886±242	1196±256	120±36
IgA	<10	<10	24±9	<10

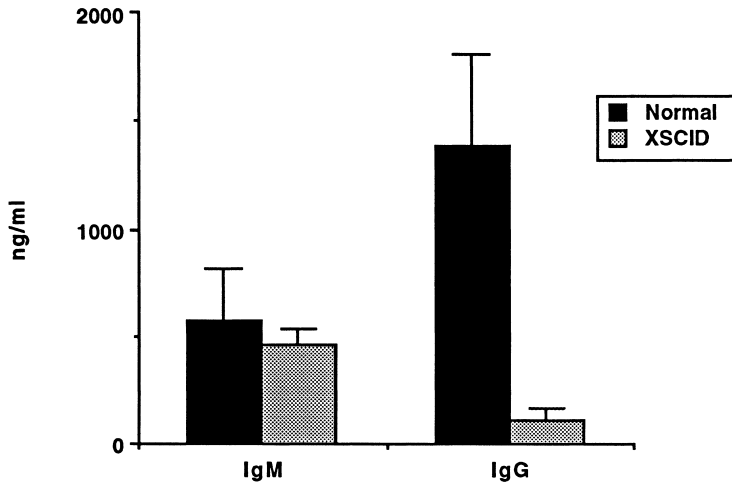


Fig. 2. In vitro immunoglobulin production by PWM-stimulated peripheral blood mononuclear cells from XSCID and age-matched normal dogs.

mounting a primary and secondary antibody response to Φ X174, the antibody titers were very low and the antibody produced was almost exclusively IgM.

3.5. Proliferative response of XSCID B-cells

Table 2 illustrates that, although phenotypically mature T-cells may be present, XSCID PBMC fail to proliferate in response to the T-cell mitogen, PHA, and to the T-dependent B-cell mitogen, PWM. This inability to proliferate in response to these two mitogens persists in XSCID dogs raised in a gnotobiotic environment for up to 4 years (data not shown).

Since SAC has been shown to be a T-cell independent B-cell mitogen for human B-cells, we were interested in determining whether this mitogen is also a T-cell independent B-cell mitogen for canine B-cells and therefore would be useful for evaluating the proliferative capability of canine XSCID B-cells in the absence of functional T-cells.

Table 2

Proliferative response of peripheral blood mononuclear cells and splenocytes from XSCID and age-matched normal dogs

Mitogen	Peripheral blood		Spleen	
	Normal	XSCID	Normal	XSCID
Medium	144±25 ^a	99±10	360±61	318±48
PHA	34,731±5662	622±267	43,715±5755	986±256
PWM	21,565±5136	814±248	ND ^b	ND
SAC	13,255±4925	10,590±7986	49,571±9400	42,124±8522

^a CPM±S.D.

^b Not done.

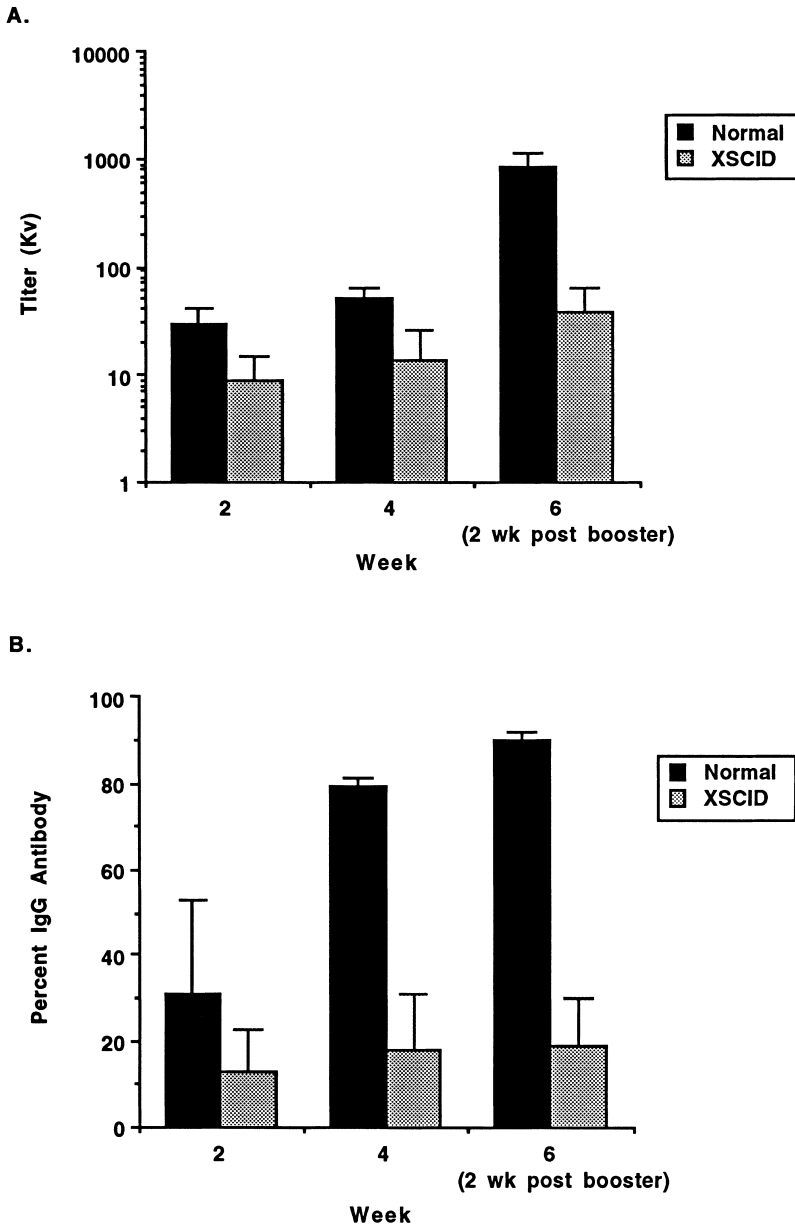


Fig. 3. Neutralizing antibody titers in XSCID and age-matched normal dogs following immunization with bacteriophage Φ X174. (A) Total antibody response. (B) Percent of IgG specific antibody.

Table 3

Proliferative response of normal canine lymphocytes from various lymphoid tissues^a

Cells	Medium	PHA	PWM	SAC
PBMC	220±80 ^b	37,256±4974	28,465±4131	8845±3914
Spleen	325±110	51,624±9226	40,274±6246	34,306±6022
Lymph node	422±134	55,056±8191	36,756±4792	29,518±6296
Tonsil	410±126	44,328±7104	30,092±5137	23,671±4911
Thymocytes	316±85	24,718±5322	11,562±3562	586±281

^a *n*=4.^b CPM±S.D.

Preliminary studies revealed that the optimal concentration of SAC for stimulating normal canine PBMC and spleen cells was a final concentration of 1:10,000 v/v (data not shown). This concentration is similar to that reported to be optimal for stimulating human B-cells.

The proliferative response to SAC was evaluated in lymphoid tissues containing varying proportions of B-cells and compared with their proliferative response to PHA and PWM (Table 3). The thymus was included since it consists almost exclusively of cells of the T-cell lineage (<1% B-cells). The proliferative response to SAC was highest in those lymphoid tissues containing the highest proportion of B-cells — the spleen, tonsil and lymph nodes, and lower in PBMC that contain fewer B-cells. As expected, thymocytes did not proliferate in response to stimulation with SAC.

To document that SAC was capable of stimulating canine B-cells in the absence of T-cells, B-cells were purified from lymph node preparations by a panning technique. The resultant B-cell population consisted of >95% B-cells and the non-B-cell population contained <2% B-cells. Table 4 demonstrates that the purified B-cell preparations proliferated in response to stimulation with SAC, but not to T-cell dependent mitogens, PHA and PWM. On the other hand, the non-B-cell population containing <2% B-cells did not proliferate in response to stimulation with SAC.

We next evaluated the ability of canine XSCID B-cells to proliferate in response to stimulation with SAC. Table 2 illustrates that peripheral blood B-cells and splenic B-cells from XSCID dogs have a normal proliferative response to SAC, but not to PHA. These data also confirm the T-cell independence of the SAC induced B-cell response.

Table 4

Proliferative response of purified normal canine lymph node B-cells^a

Cells	Medium	PHA	PWM	SAC
Unseparated	384±167 ^b	37,596±7845	31,671±5630	16,097±5797
B-cells ^c	292±145	2342±1153	1963±1266	27,914±8027
Non-B-cells ^d	406±201	45,616±9104	28,186±6172	971±329

^a *n*=5.^b CPM±S.D.^c 94.4±1.8%.^d 2.2±1.3%.

4. Discussion

This study confirms our previous findings that neonatal XSCID dogs have peripheral blood lymphocyte phenotypes virtually identical with those of the majority of human XSCID patients, characterized by elevated numbers of B-cells and few, if any, T-cells (Conley et al., 1990; Conley, 1991). Some XSCID dogs develop phenotypically mature, nonfunctional T-cells with age, although the absolute number of T-cells remain $\approx 20\%$ of normal, including three XSCID dogs raised in a gnotobiotic environment for 4 years (Felsburg et al., 1998). This appears to be in contrast to human XSCID patients, however, the natural course of human XSCID is difficult to study because of the need to bone marrow transplant human patients as soon as a diagnosis is made. In one XSCID boy who was raised in a gnotobiotic environment for 12 years, a similar age-related increase in nonfunctional T-cells was observed beginning around 2 1/2 years of age (South et al., 1977; Mukhopadhyay et al., 1978; Noguchi et al., 1993a). Based upon the comparison of biologic aging of humans and dogs, the age-related increase in T-cells occurs at approximately the same time in both human and canine XSCID (Glickman and Domanski, 1986; Somberg et al., 1996). Although an age-related increase in the proportion of splenic T-cells has been observed in $\gamma c^{-/-}$ mice, this increase is in contrast to that observed in canine and human XSCID in that the absolute number of splenic T-cells in $\gamma c^{-/-}$ mice is ≈ 3.5 times that of normal mice (Cao et al., 1995).

The results of this study also show that B-cell development in canine XSCID is similar to that reported for human XSCID, and differs from that of $\gamma c^{-/-}$ mice. In humans and dogs, a mutant γc appears to have little effect on B-cell development since XSCID dogs and boys have increased numbers of peripheral B-cells. The increased numbers of B-cells persist for up to 4 years in XSCID dogs raised in a gnotobiotic environment. Additionally, peripheral IgM⁺ B-cells from carrier females exhibit random X-chromosome inactivation, but the more mature IgG⁺ or IgA⁺ B-cells show nonrandom X-chromosome inactivation suggesting that a mutant γc does not interfere with the early stages of human B-cell development (Conley et al., 1988). In contrast, $\gamma c^{-/-}$ mice show a marked decrease in the number of B-cells in the peripheral circulation and bone marrow (Cao et al., 1995; DiSanto et al., 1995; Ohbo et al., 1996). This block in B-cell development appears to be at the transition of pro-B-cells to pre-B-cells (Cao et al., 1995; DiSanto et al., 1995). Since $\gamma c^{-/-}$, IL-7^{-/-}, IL-7R^{-/-}, and JAK3^{-/-} mice all exhibit a similar block in B-cell development, it has been proposed that IL-7 is a nonredundant cytokine that is critical for the early stages of B-cell development in the mouse (Peschon et al., 1994; Cao et al., 1995; DiSanto et al., 1995; von Freeden-Jeffrey et al., 1995; Nosaka et al., 1995; Thomis et al., 1995; Park et al., 1995; Ohbo et al., 1996; Leonard, 1996). This is in contrast to the findings in XSCID boys and dogs suggesting that either IL-7 is not required for B-cell development in humans and dogs or that IL-7 may function through a γc -independent pathway. Pribyl and LeBien (1996) have recently shown that human B-cell development can occur *in vitro* in a IL-7 independent manner suggesting that IL-7 may play little or no role in human B-cell development.

Our results also demonstrate that, similar to human XSCID patients, serum IgG and IgA concentrations are severely depressed in XSCID dogs, but serum IgM concentrations may be normal. Canine XSCID B-cells can be induced to produce near normal amounts

of IgM *in vitro* following stimulation with a T-cell-dependent B-cell mitogen, but the IgG response is <10% of normal. Similar findings have been reported for XSCID boys (Small et al., 1989; Gougeon et al., 1990). We have also shown that dogs immunized with the T-cell-dependent neoantigen, bacteriophage Φ X174, can mount a minimal specific IgM antibody response, but little specific IgG antibody even after a secondary immunization. Buckley et al. (1993) reported similar findings in XSCID boys who were bone marrow transplanted and did not engraft donor B-cells. These results show that human and canine XSCID B-cells are, at least, partially functional since they can produce IgM, but appear to be defective in their ability to class-switch to other immunoglobulin isotypes. The fact that XSCID B-cells are capable of limited class-switching suggests that there may be γ c-independent pathways for this to occur. For example, it has been recently shown that human B-cells lacking a functional γ c, including XSCID B-cells, are capable of class-switching to IgE in response to IL-4 (Matthews et al., 1995; Fujiwara et al., 1997). The proposed mechanism for this phenomenon is that in the absence of a functional γ c, IL-4 is signaling through a Type II IL-4R consisting of the IL-4R α and IL-13R α chains or through homodimerization of the IL-4R α chains (Izuhara et al., 1996; Keegan et al., 1996; Lai et al., 1996; Fujiwara et al., 1997; Taylor et al., 1997). The mechanism for the limited class-switching to IgG in XSCID B-cells needs to be resolved.

This study also shows that although canine XSCID B-cells fail to proliferate when stimulated with the T-cell-dependent B-cell mitogen, PWM, they are capable of normal proliferation when stimulated with SAC. The data presented in this paper strongly suggest that SAC is a T-cell-independent B-cell mitogen in the dog as has been reported for human B-cells (Forsgren et al., 1976; Schuurman et al., 1980; Dosch et al., 1980; Falkoff et al., 1982). Purified B-cells respond to SAC, whereas, non-B-cells were unable to proliferate in response to SAC although they maintained their ability to respond to documented T-cell mitogens. The ability of canine XSCID PBMC to proliferate normally in response to SAC and their inability to proliferate in response to T-cell mitogens also lends credence to the T-cell-independent nature of SAC. Similar results have been reported for XSCID boys (Small et al., 1989; Gougeon et al., 1990). Matthews et al. (Matthews et al., 1995, 1997) have shown that purified XSCID B-cells can proliferate normally *in vitro* when stimulated with CD40 ligand or anti- μ in the presence of IL-4, but not in the presence of IL-2 or IL-15, showing that the loss of the γ c does not diminish the sensitivity of human B-cells to IL-4. This response, in the absence of a functional γ c, may be due to IL-4 signaling through a Type II IL-4R or through homodimerization of IL-4R α since both mechanisms have been shown to induce the phosphorylation of the insulin-related substrate 1 (IRS-1) which plays a major role in IL-4 mediated proliferation (Keegan et al., 1994, 1996; Lai et al., 1996; Taylor et al., 1997). In contrast to human XSCID, B-cells from γ c^{-/-} mice fail to respond to stimulation with anti- μ and IL-4 (Cao et al., 1995). Collectively, these studies show that B-cell proliferation in humans and dogs can occur in the absence of a functional γ c.

In summary, the greater severity of the B-cell defect in γ c^{-/-} mice suggests that γ c-dependent cytokines have differing roles in human and canine B-cell development than in the mouse, and that the XSCID dog therefore represents a more appropriate model to study the role of the γ c in human B-cell development and function. Much of the proposed functions of the γ c in human B-cells are derived from studies using EBV-transformed

B-cell lines or other transfected immortalized cell lines. It remains to be determined in a biologically relevant system how the lack of a functional γ C affects normal B-cell development and function. With the exception of the two XSCID patients of Matthews et al. (Matthews et al., 1995, 1997), these studies are difficult to perform in human XSCID patients due to the urgent need for bone marrow transplantation as soon as the disease is diagnosed in affected boys. XSCID dogs provide a ready source of γ C-deficient B-cells in which to evaluate the true biologic significance of the γ C in B-cell development and function.

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