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Canine X-Linked Severe Combined Immunodeficiency

A Model for Investigating the Requirement for the Common Gamma Chain (γc) in Human Lymphocyte Development and Function

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Abstract

Our laboratory has identified and characterized an X-linked severe combined immunodeficiency (XSCID) in dogs that is due to mutations in the common gamma (γc) subunit of the interleukin-2 (IL2), IL4, IL7, IL9, and IL15 receptors. Canine XSCID, unlike genetically engineered γc-deficient mice, has a clinical and immunologic phenotype virtually identical to human XSCID. It appears that species-specific differences exist in the role of the γc and its associated cytokines in mice compared to their role in humans and dogs, suggesting γc-deficient dogs may be a more relevant model for studing the role of the γc in humans. We are utilizing this model for a variety of studies to address:

1. Fundamental questions concerning the role of the γc in cytokine regulation and lymphocyte development.
2. The pathogenesis of XSCID.
3. Strategies for improving bone marrow transplantation outcome.
4. Development and evaluation of strateies for gene therapy.
5. Human hematopoietic stem cell development.

Key Words

Dog (canine)
γc
Immunodeficiency
B cell
T cell

Introduction

Severe combined immunodeficiency (SCID) represents a heterogeneous group of genetic disorders characterized by the absence of T and B cell function, which usually results in death during infancy *(1)*. In the past 10 yr, the genes responsible for most forms of SCID

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have been identified, cloned, and their function and expression characterized. The most common form of SCID is X-linked SCID (XSCID), which is due to mutations in the common gamma (γc) subunit of the receptors for interleukin-2 (IL2), IL4, IL7, IL9, and IL15 *(2,3)*. Thus, the XSCID phenotype is the complex result of multiple cytokine defects. The shared usage of the γc 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 present in the first year of life because of severe, recurrent, or persistent infections that generally begin between 3 and 6 mo of age, at a time when maternally derived antibody has virtually disappeared. The most striking clinical feature is a failure to thrive. The immunologic abnormalities have been recently reviewed *(1,4–8)*. At the time of diagnosis, affected boys have markedly reduced or absent T cells that fail to proliferate in response to stimulation. Peripheral B cells are present in normal or increased numbers and have a virgin (IgM^+) phenotype, but they fail to mature and function normally. The primary B cell defect is assumed to be the inability to class-switch from IgM to IgG, since bone marrow-transplanted XSCID boys who do not engraft donor B cells fail to produce IgG antibody following immunization with bacteriophage ΦX174 *(7)*. Although a mutant γc has a profound effect on early human T cell differentiation, the observation that XSCID boys have normal or elevated numbers of IgM^+ B cells and that peripheral IgM^+ B cells from carrier females exhibit random X-chromosome inactivation *(9)* suggests that a mutant γc does not interfere with the early stages of human B cell development.

Since the discovery of the gene responsible for XSCID, γc-deficient mice have been created by homologous recombination *(10,11)*. These mice appear to develop as well as their littermates. The thymus is hypoplastic ,and the cellularity is approx 2–5% of normal. Interestingly, thymocyte differentiation does not appear to be arrested, since there is no significant alteration in the proportion of thymocyte subsets. Young γc-deficient mice lack peripheral T cells, but an age-related increase in the proportion and absolute numbers of splenic T cells has been reported *(11)*. The peripheral T cells do not respond to mitogenic stimulation. In contrast to human XSCID patients, γc-deficient mice also have greatly diminished peripheral B cells due to a block in the expansion of pre-B cells in the bone marrow. Thus, there are immunologic differences between human XSCID patients and γc-deficient mice—a mutated γc appears to have a greater effect on T cell development in humans *(10)* and a greater effect on B cell development in mice *(10,11)*. These findings suggest that species-specific differences may exist in regard to the role of γc-dependent cytokines and their receptors in the development and function of B cells and T cells in humans and mice.

The purpose of this article is to review another animal model of γc deficiency—canine X-linked severe combined immunodeficiency that was identified by our laboratory. Canine XSCID is a naturally occurring disease that results in a clinical and immunologic phenotype virtually identical to human XSCID and is due to mutations in the γc gene, making it a true homolog of the human disease *(12–16)*. The mutation in our colony is a 4-bp deletion in the signal peptide region resulting in a frame-shift with a premature termination codon in exon 1. The predicted product is a truncated protein of 21 amino acid (aa) instead of the normal 373 aa, in essence representing a naturally occurring γc "knockout." A breeding colony of XSCID dogs was developed from a single carrier female resulting in all affected dogs having the same genetic defect.

Clinical and Pathologic Features

As in XSCID boys, the most striking clinical feature is a failure to thrive or "stunted" growth. Problems with infections in the neonatal period are rare because of the presence of maternal antibody. Recurrent or chronic infections begin to appear between 8 and 12 wk of age with clinical signs that include pyoderma, diarrhea, and respiratory infections. These infections, usually of bacterial origin, are nonresponsive to antibiotic therapy and become generalized, resulting in severe pneumonia or overwhelming, systemic infections. XSCID dogs reared in a conventional environment rarely survive past 3–4 mo of age. The natural history of the disease has probably been modified by the infectious disease precautions taken in our closed colony, thereby limiting the viral and opportunistic infections to which these dogs are highly susceptible. Several XSCID dogs inadvertently vaccinated with a modified live canine distemper virus vaccine died 2–3 wk later of vaccine-induced distemper.

We have described the occurrence of acute monocytic leukemia, an extremely rare disease in the dog, in an XSCID dog that had been raised in a gnotobiotic environment for 20 mo, the biological equivalent to a 20- to 23-yr-old human *(17)*. This case represents the first reported case of malignancy in canine XSCID, the first case of acute monocytic leukemia in SCID, and the first documented malignancy in XSCID that is not associated with immunotherapy.

The major pathologic feature of canine XSCID is a small, dysplastic thymus *(18)*. Grossly identifiable lymph nodes are absent in the majority of XSCID dogs. When present, these small nodes show little organization and few, if any, typical small lymphocytes. All XSCID dogs lack grossly identifiable tonsils. Histologically, the tonsilar crypts reveal a profound lymphoid hypoplasia with a paucity of small to medium lymphocytes. Peyer's patches cannot be idenified grossly or histologically.

Immunologic Abnormalities

As in the documented cases of human XSCID, profound lymphopenia is not a prominent feature of canine XSCID. The majority of dogs have lymphocyte counts >1000/mL. FACS analysis reveals that newborn XSCID dogs have peripheral blood lymphocyte phenotypes that are identical with those of the majority of human XSCID patients, characterized by normal or elevated proportions of B cells and markedly reduced or absent T cells (Table 1). We have also shown that some XSCID dogs are capable of developing increased proportions of phenotpyically mature T cells around 8–10 wk of age, which is the biological equivalent of approximately three human years *(14,16)*. This in contrast to the reports that XSCID boys have few, if any, T cells. If human XSCID patients were capable of developing T cells as seen in canine XSCID, one would expect to observe this between 2 and 3 yr of age. The natural history of human XSCID has been difficult to study because of the urgent need for bone marrow transplantation if the patient is going to have a chance to survive. However, one untreated XSCID patient raised in a gnotobiotic environment developed normal proportions of T cells after $2\,^1/_2$ yr of age. Thus, T cells can develop in human XSCID comparable to those observed in canine XSCID. An age-related increase in the proportion of splenic T cells has also been observed in γc-deficient mice, but in contrast to XSCID dogs and the one human patient, the absolute number of T cells also increased to over 500% of normal *(11)*. Even in XSCID dogs that had relatively normal percentages of peripheral T cells, the absolute number averaged only 30% of normal, including three XSCID dogs raised in a gnotobiotic environment for 3 yr *(16)*.

An interesting finding is that as soon as T cells begin to appear in XSCID dogs, they rapidly switch from a $CD45RA^+$ (naive) phenotype to a $CD45RA^-$ (activated or memory phenotype),

Table 1. Proportion of peripheral B and T cells in XSCID dogs

Cells	<4 Wk		>8 Wk	
	Normal	XSCID	Normal	XSCID
B cells	14.3 ± 15.9[a]	66.3 ± 4.6	12.9 ± 2.5	49.7 ± 15.1
T cells	58.2 ± 4.6	1.4 ± 1.9	68.1 ± 5.8	23.7 ± 21.4
$CD45RA^+$ T cells[b]	92.5 ± 2.6	88.1 ± 4.6	90.6 ± 3.2	8.0 ± 0.6

[a]Percent ± SD.

[b]Percent of T cells that are $CD45RA^+$.

a phenomenon that is reported to be proliferation-dependent in normal individuals. Although it is clear that the early T cells observed in XSCID dogs are recent thymic emigrants, it is unclear whether the increased proportions seen in older XSCID dogs are due to increased release of thymocytes that rapidly switch their CD45RA phenotype or to extrathymic expansion of the few $CD45RA^+$ peripheral T cells present during the first few weeks of life. Since CD45RA expression on peripheral T cells of XSCID boys or γc-deficient mice has not been reported, it is unknown whether a similar switch in CD45RA expression occurs in these species.

One of the characteristic findings in XSCID dogs is an absent or markedly depressed lymphocyte blastogenic response to T cell mitogens and specific antigens. Table 2 illustrates that canine XSCID peripheral blood mononuclear cells (PBMC) exhibit a significant lack of proliferation in response to stimulation through the T cell receptor (phytohemagglutinin [PHA]) and when the necessary second messengers for cellular proliferation are directly provided by the combination of phorbol ester, PMA, and calcium ionophore (PMA/CI) that bypass signals delivered through ligand–receptor interaction. It is difficult to tell at this time whether the marginal response to PMA/CI observed in the XSCID dogs is due to B or T cells since PMA/CI is capable of activating both. Addition of human recombinant IL2 has little effect on the proliferative response to PHA in XSCID dogs, although it is interesting to note that the percent augmentation in the XSCID cultures (47%) is comparable to the 39% increase in the normal dog cultures (Table 2). The poor proliferative response of XSCID T cells can partially be attributed to the defective expression of functional IL2 receptors *(14)*.

XSCID B cells are capable of proliferation when stimulated with formalin-fixed, heat-killed *Staphylococcus aureus*, a T-independent, B cell mitogen (Table 2). Similar results have been reported for human XSCID patients *(5,8)*. To address further the functional capability of XSCID B cells, we immunized gnotobiotic normal and XSCID dogs with the T cell-dependent neoantigen, bacteriophage ϕX 174, at approx 1 yr of age. Table 3 illustrates the total antibody and IgG-specific antibody titers following primary and secondary immunization. Although XSCID dogs can produce minimal amounts of specific antibody, it is almost exclusively IgM. On the other hand, the specific antibody response in normal dogs is primarily IgG. These results emphasize the inability of XSCID B cells to undergo isotype class-switching, which is the result of the lack of a functional IL4 receptor. The minimal amount of IgG antibody produced is most likely due to the interaction of IL4 with its γc-independent receptor.

Table 2. Proliferative response of XSCID peripheral blood mononuclear cells

Mitogen	Normal	XSCID
Medium	124 ± 15[a]	99 ± 10
PHA	22,942 ± 4289	773 ± 235
PMA/CI	14,857 ± 3044	2376 ± 1252
SAC	10,590 ± 4925	13,255 ± 7986
PHA	24,147 ± 3919	814 ± 397
PHA + rIL2	33,626 ± 8621	1195 ± 642[b]

[a]CPM (mean ± SEM).
[b]$p = 0.001$ (XSCID PHA vs PHA + IL2)

Table 3. Neutralizing antibody in XSCID dogs following immunization with ϕX174

	Total antibody		IgG antibody	
Immunization	Normal	XSCID	Normal	XSCID
Primary	50 ± 14	5 ± 2	38 ± 12	0.6 ± 1.0
Secondary	456 ± 180	15 ± 8	392 ± 100	1.8 ± 1.2

[a]Results expressed as neutralizing antibody titer (kv).

Defective Thymocyte Development in XSCID Dogs

Because of the paucity of peripheral T cells, XSCID appears to be a disease primarily affecting the differentiation and maturation of the T cell lineage. A major issue that has not been previously addressed is what is occurring in the thymus of human XSCID patients. During thymopoiesis, human thymocytes undergo an ordered expression of cell-surface antigens on developing thymocytes as well as a controlled mitogenesis of thymocytes at discrete stages of development. Proliferation is one of the most important events occurring during early thymocyte development. The differentiation of $CD4^-CD8^-$ (DN) thymocytes to $CD4^+CD8^+$ (DP) thymocytes is proliferation-dependent, whereas the maturation of DP thymocytes to mature $CD4^+CD8^-$ or $CD4^-CD8^+$ (SP) thymocytes is proliferation-independent. A steady expansion in the number of thymocytes takes place from the DN stage to the $CD3^+$DP stage. Proliferation ceases in the DP population following the surface expression of CD3.

We have studied the thymi of 24 XSCID and 24 age-matched littermates between 4 and 10 wk of age *(14)*. In normal dogs, the thymocyte subsets were found in proportions similar to those described in humans, suggesting that the same ordered maturation process occurs in the dog thymus. However, in the postnatal XSCID thymus, a mutated γc severely affects thymocyte development. The thymus from XSCID dogs is approx 8% the weight of age-matched normal dogs, and the total number of thymocytes in the thymus of XSCID dogs is approx 0.3% of the thymocytes present in the thymus of age-matched normal dogs (Table 4). The reduction in cellularity is

Table 4. Characterization of XSCID thymocytes

Cells	Normal	XSCID
Thymocytes	203 ± 63[a]	0.63 ± 1.2
$CD4^-CD8^-$	13.1 ± 6.1	45.6 ± 22.2
$CD4^+CD8^+$	68.7 ± 12.4	30.7 ± 20.6
$CD4^+CD8^-$	11.6 ± 7.8	10.5 ± 8.6
$CD4^-CD8^+$	2.9 ± 1.8	2.2 ± 4.4

[a]Thymocytes × 10^8 ± SD.

approx 15-fold greater than that observed in the γc-deficient mice. In contrast to γc-deficient mice, there are profound alterations in the thymocyte subsets in XSCID dogs. The proportion of DN thymocytes in XSCID dogs is increased 3.5-fold and the DP population is decreased 2.3-fold. Interestingly, there is no significant difference in the proportion of SP thymocytes. When one takes into consideration the size and cellularity of the XSCID thymuses, one can appreciate there is a paucity of all thymocyte subsets in the XSCID dogs. Our data demonstrate a profound alteration in the expansion and differentiation of DN thymocytes in XSCID thymi.

In addition to the significantly reduced cellularity in the postnatal XSCID thymus, we have shown that there is an increased proportion of DN cells (2.5-fold) that appear to be in the thymoblast stage of development. These results suggest that postnatal XSCID thymocytes have a reduced ability to enter the mitotic phase of the cell cycle. The dramatic reduction in cellularity and the alteration in the proportion of DN and DP thymocytes in the XSCID thymus are highly suggestive of a proliferation defect limiting the expansion of the pool of thymocytes and impeding their differentiation. It appears that the transition of DP to SP cells, which is proliferation-independent, occurs normally in XSCID dogs in the absence of a functional γc.

The proliferative response of XSCID thymocytes following mitogenic stimulation through the T cell receptor with Con A was severely depressed when compared with the response of normal thymocytes, however, the XSCID thymocytes responded normally to PMA/CI (Table 5). These findings are similar to those that have been observed in γc-deficient mice *(10,11)*.

Bone Marrow Transplantation Studies

The single, largest immunologic problem in human XSCID patients following bone marrow transplantation (BMT) is the engraftment of few, if any, donor B cells with resultant poor reconstitution of humoral immune function *(19,20)*. Pretransplant chemotherapy (cytoablation) has been recently shown to enhance donor B cell engraftment and reconsititution of humoral immune function in XSCID boys following BMT (Weinberg, personal communication). We have recently shown that XSCID dogs can be BMT, resulting in full immunologic reconstitution and engraftment of donor B and T cells without the need for cytoablative therapy *(21)*.

XSCID dogs were BMT between 2 and 3 wk of age using unfractionated bone marrow cells from either DLA-identical or DLA-haploidentical littermate donors at a dose of 1.0–1.5 × 10^8 nucleated cells/kg. The kinetics of immune reconstitution and the engraftment of donor B and T cells were essentially the same in all transplanted dogs regardless of whether the

Table 5. Proliferative response of XSCID thymocytes

Mitogen	Normal	XSCID
Medium	267 ± 238[a]	255 ± 143
ConA	25,612 ± 5231	2088 ± 602
PMA/CI	8749 ± 4696	7324 ± 5346

[a]CPM (mean ± SEM).

donor was dog leukocyte antigen (DLA)-identical or DLA-haploidentical.

T cells became evident 1 mo post-BMT and reached normal levels, both percentage and absolute numbers, by two months post-BMT. Since T cells derived from transplanted stem cells would be expected to be naive, we examined the CD45 isoform usage following BMT. At 1 mo post-BMT, approx 70% of the T cells were $CD45RA^+$ (naive T cells) and by 2 mo post-BMT, >90% of the T cells were $CD45RA^+$, suggesting that the T cells were recent thymic emigrants. The T cell proliferative response to PHA significantly increased 1 mo post-BMT and was normalized by 2 mo post-BMT. The generation of T cells and T cell function is similar to that observed following BMT of human XSCID patients.

Serum IgG concentrations started to increase by 2–3 mo post-BMT and reached normal, age-matched levels by 5 mo post-BMT. When serum IgG concentrations reached normal levels, all transplanted dogs were immunized with bacteriophage ΦX174. The transplanted XSCID dogs were not only capable of producing antigen-specific antibody, but more importantly, were also capable of class-switching to IgG (Fig. 1).

The origin of flow-sorted B and T cells was determined at 6 mo post-BMT by assessing the genotype of the γc gene using a PCR-based mutation assay. The T cell population in all transplanted dogs was exclusively of donor origin, whereas the B cell population demonstrated mixed chimerism ranging from 30–50% donor B cells.

An interesting clinical finding is 70% of the transplanted dogs developed cutaneous papillomas approx 1 yr post-BMT. Prior to this time, we have never observed papillomas in our colony, and none of the carrier females or normal males in the colony have developed papillomas. Interestingly, it appears that papillomas are also a frequent problem in transplanted XSCID boys who are 5 yr, or more post-BMT *(20)* and in a kindred of XSCID boys with a missense mutation in exon 7 of the γc that diminishes JAK3 binding who can survive into adolescence and adulthood *(3,22)*.

Transplantation of XSCID Dogs with Human $CD34^+$ Fetal Liver Cells

Three neonatal XSCID dogs were transplanted with human $CD34^+$-enriched fetal liver cells obtained from 13 wk fetal livers by negative immunomagnetic selection using monoclonal antibodies (MAbs) to human glycophorin to eliminate erythroid cells, and to mature human B cells, T cells, and myeloid cells. The resultant cell preparation contained 22% $CD34^+$ cells. Each dog received 2×10^6 cells/kg (~0.44×10^6 $CD34^+$ cells/kg) by iv injection.

The dogs were monitored at monthly intervals for the presence of human cells by flow cytometry using MAbs against human CD3 (mature T cells), CD19 (mature B cells), and CD11b (myeloid cells). The MAbs used in this study did not crossreact with normal dog leukocytes. No human cells were detected at 30 d

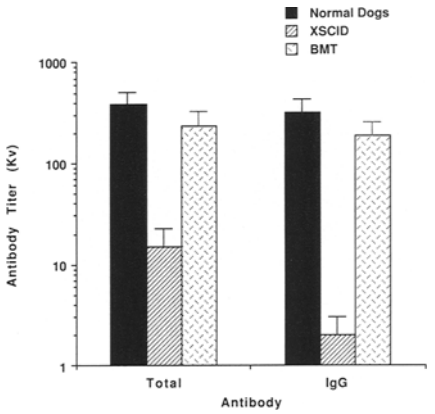

Fig. 1. Total and IgG-specific antibody titers in bone marrow transplanted XSCID dogs following secondary immunization with bacteriophage ΦX174.

posttransplantation in any of the dogs. At 60 d, one dog showed ~3% human cells, primarily $CD11b^+$ cells, in the peripheral circulation. However, at 90 d posttransplantation, 9–29% of the PBMC in the three dogs were of human origin (Fig. 2). The $CD3^+$ cells were $CD45RA^+$, indicating that the cells were recent thymic emigrants derived from immature progenitors.

The ability of the PBMC to respond to mitogenic stimulation was evaluated in the dog with the most human cells at 90 d posttransplantation. The PHA response in the dog was 6950, compared to the typical XSCID response of <1000, suggesting the the engrafted human T cells were functional.

Histologic examination of two of the dogs revealed that the spleen had a relatively normal architecture as compared to untreated XSCID dogs in which the spleen is devoid of any lymphoid follicles or germinal centers. The thymus demonstrated a corticomedullary demarcation with thymocytes present in the outer region of the cortex, which has not been seen in any untreated XSCID dogs.

Although these studies are very preliminary, the results suggest that the bone marrow and thymic microenvironments in the XSCID dog are capable of supporting the differentiation and maturation of human B and T cells.

Future Directions

A central role for the γc, and the cytokines with which it interacts, in T cell development is demonstrated by the profound T cell defect in human and canine XSCID. However, the fact that limited T cell development does occur in human and canine XSCID suggests that aspects of T cell development can occur independently of a functional γc. Much of the proposed functions of the γc, and its downstream signaling molecules in lymphoid cells are derived from studies using transfected fibroblasts or immortalized lymphoid cell lines. These studies propose that a functional γc is essential for signal transduction in lymphoid cells in response to those cytokines associated with the γc *(3)*. It remains to be determined in a biologically relevant system whether signaling can occur in lymphoid cells in the absence of a functional γc. These studies are difficult to perform in human XSCID patients because of the urgent need for bone marrow transplantation and the lack of access to human XSCID thymocytes. It is becoming clear that the cytokines associated with receptors containing the γc are capable of signaling in nonlymphoid cells through pathways different from those attributed to lymphoid cells. We are actively

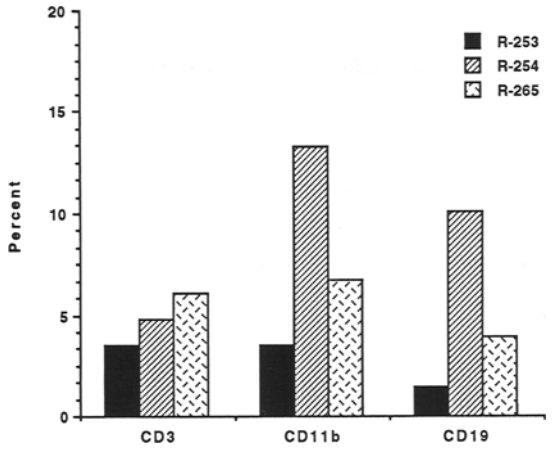

Fig. 2. Presence of human peripheral blood mononuclear cells in XSCID dogs transplanted with human $CD34^+$ fetal liver cells.

evaluating whether the limited T cell development observed in XSCID may be owing to one or more cytokines signaling through non-conventional signaling pathways that are independent of a functional γc. Since γc-dependent cytokines have differing roles in human and canine B cell development than in the mouse, the XSCID dog is also being used to study the role of these cytokines in B cell development and function.

The immune response to papillomavirus infections, an ever-increasingly important infection in humans due to its association with various cancers, is poorly understood. The fact that bone marrow transplanted XSCID dogs that possess a fully competent systemic humoral and cell-mediated immune system have a significant problem with papillomas suggests that (1) a γc-dependent cell lineage(s), most likely localized to the skin, is involved in the immune response to papillomavirus infection, and (2) this lineage is not being reconstituted following bone marrow transplantation. We are initiating studies using this model to identify and characterize the defective cell population(s) that will not only further our understanding of the immunobiology of papillomavirus infection, but will also provide insight into the differentiation and maturation pathway of this cell lineage(s).

Since bone marrow transplantation is presently the only treatment available to cure patients with XSCID, strategies to improve donor B cell engraftment in human patients without the need for pretransplant conditioning are necessary in order to achieve optimal long-term survival. Historically, the dog has been a valuable model for bone marrow transplantation, with many of the advances achieved in the dog being directly transferrable to human clinical protocols. Because all of our transplanted XSCID dogs have engrafted donor B cells and reconstituted normal humoral immune function without the need for cytoablative therapy, we are using this unique model to determine which variable(s) contributes to the successful engraftment of donor B cells. We are also using the XSCID model to develop and evaluate strategies for *in utero* bone marrow transplantation.

XSCID dogs represent an ideal large animal preclinical model for developing and evaluating strategies for human gene therapy. Current studies include the analyzis of transduction, engraftment, and functional correc-

tion of the immune system in XSCID dogs receiving autologous $CD34^+$ marrow cells transduced with a conventional retroviral vector. We will also be using this model to test novel vectors for the ability to give activation-independent expression in resting T cells and to prevent vector silencing—two problems in current human clinical gene therapy trials.

The ability to study the development and function of the human immune system, as well as the pathogenesis and treatment of diseases of the immune system, is difficult because of practical and ethical limitations. Animal models, such as the mouse, are only approximate models that may not be relevant to human. In addition, for many diseases affecting human, no animals models exist since these diseases only affect human. Various strategies have been used to attempt to study the human immune system in SCID mice. However, it is evident that the mouse does not provide the necessary microenvironment for providing a stable, functional human immune system. Preliminary studies in XSCID dogs suggest that the canine bone marrow and thymic microenvironments, unlike those in the mouse, are capable of supporting the development of mature, circulating human B- and T cells when transplanted with human fetal liver hematopoietic stem cells. We are pursuing studies to document the utility of the XSCID dog as a model for studying human lymphopoiesis and immune function (XSCID-hu dog). A major advantage of using the XSCID dog is that, due to its size, it represents an experimental small animal model in which repeated blood samples can be collected in order to study the kinetics of lymphopoiesis, response of the immune system to infectious agents, and to test therapeutic agents in the same animal over time. The XSCID-hu dog would also be a valuable experimental model in which to test strategies for human gene therapy.

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