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SCID pigs: An emerging large animal NK model

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ABSTRACT

Severe Combined ImmunoDeficiency (SCID) is defined as the lack or impairment of an adaptive immune system. Although SCID phenotypes are characteristically absent of T and B cells, many such SCID cellular profiles include the presence of NK cells. In human SCID patients, functional NK cells may impact the engraftment success of life saving procedures such as bone marrow transplantation. However, in animal models, a T cell-, B cell-, NK cell+ environment provides a valuable tool for asking specific questions about the extent of the innate immune system function as well as emerging NK targeted therapies against cancer. Physiologically and immunologically the pig is more similar to the human than common rodent research animals. This review discusses why the T- B- NK+ SCID pig may offer a more relevant model for development of human SCID patient therapies as well as provide an opportunity for systematic exploration of the role of NK cells in artiodactyl immunity.

Severe combined immunodeficiency: Mutations and cellular profiles

SCID is naturally found in humans, mice, horses, dogs, and recently pigs[1-5]. It is characterized chiefly by lymphopenia (absence of T cells and often B cells), but also by a lack of thymocytes, a missing or small thymus, and abnormalities to additional immune tissues[6].

Although the SCID condition is defined by the central phenotype of lacking T and B cells, SCID causative mutations can affect different stages along the lymphoid development or function pathway. These include alterations that affect developmental cytokine signaling, lymphocyte precursors, and/or inhibit the creation of the T cell receptor (TCR) and B cell receptor (BCR) complexes. NK cells are innate lymphocytes and develop from common lymphoid precursors shared by T and B cells. Since NK cells do not make receptors requiring somatic recombination, they can be unaffected by causative SCID mutations that inhibit such steps required in production of TCRs and BCRs. Thus, the stage of development that a causative SCID mutation affects will determine the presence or absence of NK cells. SCID defects and phenotypes are well described in an excellent review by Cossu (2010), but we will briefly describe some broad differences in cellular profiles, emphasizing mutations that lead to the presence or absence of NK cells.

The most common form of human SCID is an X-linked defect in the common gamma chain (CD132), which is present in the interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21 receptors and encoded by the *IL2RG* gene[2,6]. Without the common gamma

chain for the IL-7 and IL-15 receptors, neither T cells nor NK cells can develop. However, B cell development is independent of the common gamma chain, thus a mutation in this gene creates a T cell-, B cell+, NK cell- (T-B+NK-) phenotype[2,6]. A defect in Janus Kinase 3 (JAK3), which alters intracellular signaling from the common γ chain (γc), also causes a T-B+NK- SCID phenotype[2]. Another classic form of SCID with a different cellular deficiency comes from mutations in the Recombination Activation Genes (RAG) 1 or 2. RAG genes are lymphoid specific and their gene products form a complex that binds to recombination signal sequences flanking V (variable), D (diversity), and J (joining) segments[7]. The functional RAG1/2 complex induces double stranded breaks (DSB)s in the DNA and creates an inter-strand hairpin loop. In RAG1- or RAG2-defective individuals, this cleavage is inhibited, and T or B lymphocyte cannot produce a TCR or BCR, respectively[7]. However, as somatic recombination is not required for development of NK cells, most RAG deficient patients are T-B-NK+. Mutations in the Artemis gene have been found naturally in humans and pigs, and also causes a T-B-NK+ phenotype[2,8]. The Artemis gene encodes an endonuclease that cleaves the hairpin loop left by RAG complex, an essential next step in rearrangement to create the TCR and BCR[9,10]. Due to this role of Artemis in DNA repair of DSB involving non-homologous end joining (NHEJ), Artemis-deficient patients and animal models are sensitive to radiation (radiosensitive)[2,11,12]. Also radiosensitive and sharing a T- B- NK^+ cellular profile are SCID patients with defects of the DNA-dependent kinase catalytic subunit (DNA-PKcs) protein. After cleavage by RAG proteins and hairpin loop repair by Artemis, DNA-PKcs proteins are activated by DNA ends and are critical components of DNA repair during NHEJ[4,8,11]. DNA-PKcs mutations were actually identified as causative defects of SCID phenotypes in Arabian horses, Jack Russell terrier dogs, and mice before such mutations were recognized in humans[1,2,4,5]. Other types of naturally occurring SCID have been documented including a defect in adenosine deaminase (ADA), IL7Rα (IL-7 receptor) as well as defective CD3γ, CD3δ, CD3ζ, and/or CD3ε, all subunits of the mature TCR on the cell surface[2].

NK Cells: Cell-surface markers, immune function, as engraftment inhibitors, and as cancer therapy targets

NK cells have many cell surface markers in common with cytotoxic T cells such as CD8a+(glycoprotein associated with cell-cell interactions), and CD2+(T cell linage surface antigen), but are externally CD3-[13-16]. Common surface receptors associated with NK cells include CD56 (neural cell adhesion molecule) and CD16 (Fc-γ IIIA receptor). The latter receptor binds antibody–bound cells which are impaired and thus targeted for cytotoxic "killing" through Antibody Dependent Cellular Cytotoxicity (ADCC)[17]. Unlike cytotoxic T cells, NK cells do not require activation by specific antigens presented by MHC complexes, but rather can lyse targets based on cells lacking or down regulating self MHC class I molecules, which can provide protection against virally infected or cancer cells[16,18]. NK cell function is commonly measured against target cells (cytotoxicity) but can also be analyzed through cytokine production and response to activation signals[9,19,20]. NK cell lysis of target cells can be accomplished by cell to cell binding of FAS or TRAIL 'death' ligands, ADCC, or receptor activating granule exocytosis of proteins such as perforin and granzyme B, both of which can also be measured as an indication of NK cell activation[13,21,22]. In addition to cytotoxic roles, activated NK cells are a major producer of cytokines such as IFN-γ[13,20,23]. NK cell production of IFN-γ enables communication and activation among NK cells and other immune cells including macrophages, dendritic cells, T cells, and B cells[18,24]. Activation of NK cells has been demonstrated *in vitro* using individual or combinations of cytokines, including interleukin (IL)-2 and IFN- α[25] as well as IL-15, IL-12, and IL-18[9,19,20]. In specific combinations or collectively, these attributes have been used to measure the activity of NK cells in various SCID backgrounds.

Though NK cells in a T-B- environment may offer some immune protection to the host, for some SCID patients the presence of functional NK cells may negatively impact the success of critical procedures such as bone marrow transplantation (BMT). NK- type SCID individuals have enhanced survival with allogeneic stem cell transplantations compared with NK+ SCID patients[26], while the presence or absence of B cells did not impact success in such patients. In addition, 33% of NK+ SCID stem cell recipients required additional procedures, including pre-transplant myeloablation and/or radiation, while only 8% of NK- recipients required such procedures[26]. Moreover, SCID patients that are NK+ and radiosensitive (Artemis, DNA-PKcs), may be dangerously sensitive to commonly utilized pre-transplant radiation treatments, further decreasing BMT success rates[2,26].

However, in the context of NK immunology and SCID research, a circulating lymphocyte environment composed only of NK cells can be a valuable tool. NK cells are recognized for their anti-tumor activity and thus methods for activating such cells in the patient or in providing NK cells as therapeutics is a very active area of cancer research[13]. For example, T-, B-, NK+ environments are valuable for development of NK specific therapies including IL-2 and IFN-α supplementation. Supplementation of IL-2 therapy is intended to elicit an increased response (proliferation, increased cytotoxicity) from NK cells[27]. Intraperitoneal-injected human NK cells activated with IL-2 show significantly greater anti-tumor activity (decreased tumor burden) in ovarian cancer mouse models compared

with non-activated NK cells[28]. IFN-α is associated with viral defense and anti-tumor activity and thus IFN-α activation of NK cells has become a potential focus for cancer and viral therapy[29]. Stimulation of IFN-α production results in increased cytotoxicity of human NK cells and increased perforin gene expression[29].

As well, T-B-NK+ systems can offer opportunities to develop mechanistic insight into innate immune function. Interestingly, recent studies used contact hypersensitivity in SCID mice to show immune memory localized to liver-resident NK cells[22,23,30,31]. Adoptive transfer studies in SCID mice showed that educated NK cell populations are responsible for such memory, and that these cells were found in the liver[22,30]. Upon a second exposure, NK cells exhibiting memory-like behavior accumulated in the site of re-challenge and show a heightened activation state as measured by IFN-γ production and upregulation of activation receptors[22,30]. Importantly, such NK memory can also be elicited by vaccines for different viruses, and transfer of these memory NK cells into naïve mice can provide protection against re-infection without the presence of the adaptive immune system[23,31]. A better understanding of innate memory mechanisms can enhance our understanding of vaccine response mechanisms and aid in vaccine development.

In summary, understanding the cellular profile of any given SCID environment is important for focusing therapy efforts in human SCID patients as well as for utilizing the full potential of research animals lacking an adaptive immune system. In the last section, we provide an overview of established and developing SCID model systems with an emphasis on the advantages of a large animal pig model.

Large animal models: The opportunities of the SCID Pig

As a research model, SCID systems offer insight into the mechanism of SCID disease, offer valuable tools for development of biomedical therapies, and present an unique opportunity to explore the capabilities of the innate immune system. In an effort to harness this model potential, the SCID condition had been transgenically introduced into mice, rats, and recently swine[12,32-38]. The pig offers a large animal model with more similar genetics, anatomy, and physiology to humans. For example, the porcine immune system resembles that in the human for 80% of analyzed parameters, compared to a human to mouse parameter match of only 10%[39,40]. This suggests an advantage for pigs as a biomedical model for immunology and biomedical research and therapy development.

Though the SCID phenotype is artificially achievable by a variety of genetic manipulations, swine researchers have focused on targeting *IL2RG* and/or the RAG 1/2 knockouts[12,32-37]. Engineered IL2RG SCID pigs have been created using serial nuclear transfer[37], zinc finger nucleases (ZFNs)[12], or the clustered regularly interspaced short palindromic repeats (CRISPR)/cas9 system[34] technologies. Similar to human SCID patients, IL2RG SCID pigs have shown an X-linked heritability[12,37] and also display the typical T-B+NK- cellular phenotype[12,37]. More suitable for NK specific research questions are the RAG targeted SCID pig models which have been achieved using transcription activator-like effector nucleases (TALENs) and/or somatic cell nuclear transfer (SCNT)[32,33,35]. As described above, RAG knockouts typically result in a T-B-NK+ phenotype; however, the NK(+) RAG knockout in combination with the NK (-) common gamma chain IL2RG knockout produces T-B-NK- SCIDs[33,36]. Interestingly, NK cells from RAG-SCID mice display a different surface marker profile, increased cytotoxic activity, and proliferation deficiencies compared to NK cells from wild type mice[7,41]. To date, no such defect has been reported in the RAG-SCID pig models[32,33,35].

The only naturally occurring SCID pig has two different recessive mutations within the Artemis gene; these mutations cause a SCID phenotype in the homozygous or compound heterozygous state[8]. These SCID pigs have a phenotype very similar to human Artemis SCID patients, as they lack B and T cells but have a functional population of NK cells capable of cytotoxic lysis of numerous tumor target cells lines, perforin production, and response to activating cytokines[9]. As seen in human Artemis patients, fibroblasts from Artemis SCID pigs are also radiosensitive[8]. Also, consistent with classic characteristic of SCID models, the Artemis SCID pigs are able to host xenotransplants. The Artemis SCID pig failed to reject human melanoma (A375-Sm) and pancreatic carcinoma (PANC-1) cell lines injected subcutaneously into the ear[42]. Given the substantial differences in animal models, including SCID models, it is useful to have additional such models for regenerative and cancer medicine. We propose that this and other SCID pig models may be more physiologically similar to SCID humans, and such newly discovered or created models offers a valuable research model for clinical testing, procedure improvement, as well as therapeutics.

One of the landmark uses of SCID mice models has been the creation of "humanized" mouse models in which human hematopoietic stem cells (HSCs) are introduced to a given SCID host and allowed to differentiate into components of the immune system[43]. Coveted for the lack of xenograft rejection and human tissue differentiation, humanized mice can provide the environment to harbor and allow differentiation of human HSC[43], allowing a model of human immune response to host species-restricted pathogens such as HIV or hepatitis C virus[44]. Early research on various strains of humanization of SCID mouse variants identified that potential mouse cell to human cell interactions were interfering with engraftment success. Evidence showed

mouse phagocytes could be directly killing developing human NK cell precursors and/or human NK precursors were not recognizing murine cytokines necessary for NK cell lineage development[45]. Although specific B cell and T cell responses could be measured, these humanized mice presented weak or non-significant NK and myeloid cell compartment development. Phagocytosis by host macrophages is largely influenced by the interaction of host SIRPα and CD47 on donor cells, which initiates an anti-phagocytosis signal in the SIRPα+ phagocyte and increasing survival of CD47 expressing cells (most nucleated cells including human stem cell and human NK cell precursors)[45]. The phagocytosis problem was largely corrected with the original Non-Obese Diabetic (NOD) mouse, which is recognized as a gold standard of engraftment modeling, due mostly to its modified SIRPα which has an increased affinity for donor human CD47 compared even to human SIRPα, thus encouraging survival of donor stem cells and their descendants[45]. It is well documented that the "rescue" of NK and myeloid cell differentiation and replication can also be accomplished with supplementation of combinations of human IL-15, erythropoietin, G-CSF, IL-3, and/or IL-4 through direct injection or through transgenesis[46]. Although attempts at humanization of available pig has not yet been published, mature teratomas developed after injection of human pluripotent stem cell injection into RAG mutant SCID pigs[35]. The success of the SCID mouse for xenotransplantation, cancer therapy, human specific disease modeling, and stem cell therapies is well documented[43,44,46] and can be expected to extend to swine models. Swine immune parameters more similar to the human, as described above, and in addition, the swine immune gene component or "immunome" is very similar to humans[47].

Another advantage of the pig model is the anatomical and pharmacological similarity to the human, which is especially valuable for establishing drug and therapy dosages. Above we discuss the use of IL-2 supplementation as cancer therapy by activating anti-tumor cytotoxic cells such as NK cells (and if present, CD8+ T cells). IL-2 injections have been responsible for complete regression (all measurable tumor cleared) in 7% of renal cancer and melanoma patients, and an additional 10% saw partial regression (clearance of at least 50% of tumor burden)[27]. However, IL-2 supplementation is extremely dose sensitive; too much IL-2 will have toxic effects, including the development of Vascular Leak Syndrome (VLS)[48]. VLS can be fatal and in severe cases causing cardiac and pulmonary failure[48]. In work observing human cancer patients receiving IL-2 therapy, 65% had to adjust or stop treatment due to VSL complications[48]; therefore, it is imperative to establish a safe yet efficient dosage. IL-2 supplementation therapy is already being established in pigs and has also been shown to be beneficial for decreased Graft versus Host Disease (GvHD) following a mismatched bone marrow transplant in miniature swine[49].

In conclusion, there are useful SCID pig models available that may further advance the work accomplished in SCID mice in a system more similar to a human environment. The T-B-NK+ SCID pig model provides a new opportunity for advancement of NK cell biology. The presence of functional NK cells in a deficient immune system enables the exploration of challenges faced by NK+ SCID human patients and potential procedural improvements, the development of NK cell specific therapies, and the exploration of mechanisms independent of the adaptive immune system.

Conclusions

Recognizing the capacity of the SCID natural killer cell remains a crucial component in understanding the innate immunity present in any given SCID environment. The presence of NK cells influences engraftment and stem cell transplantation in SCID patients. In addition, characterizing SCID resident NK cells is important for animal model development and may define how current models can be best utilized for biomedical, cancer, and therapy uses. Given the physiological similarities of swine to humans, pigs as immune-deficient models have notable advantages and vast potential as a tool for immunologic, therapy advancement, and biomedical research.

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Conflict of Interest

The authors do not have any conflicts of interest with work described in this manuscript.

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