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NK cells are intrinsically functional in pigs with Severe Combined Immunodeficiency (SCID) caused by spontaneous mutations in the Artemis gene

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ABSTRACT

We have identified Severe Combined Immunodeficiency (SCID) in a line of Yorkshire pigs at Iowa State University. These SCID pigs lack B-cells and T-cells, but possess Natural Killer (NK) cells. This SCID phenotype is caused by recessive mutations in the *Artemis* gene. Interestingly, two human tumor cell lines, PANC-1 and A375-SM, survived after injection into these SCID pigs, but, as we demonstrate here, these cells, as well as K562 tumor cells, can be lysed *in vitro* by NK cells from SCID and non-SCID pigs. NK cells from both SCID and non-SCID pigs required activation *in vitro* with either recombinant human IL-2 or the combination of recombinant porcine IL-12 and IL-18 to kill tumor targets. We also showed that SCID NK cells could be activated to produce perforin, and perforin production was greatly enhanced in NK cells from both SCID and non-SCID pigs after IL-2 cytokine treatment. While CD16+, CD172- NK cells constituted an average of only 4% in non-SCID pigs, NK cells averaged 27% of the peripheral blood mononuclear cell population in SCID pigs. We found no significant differences in killing activity per NK cell between SCID and non-SCID pigs. We conclude that survival of human cancer cells in these SCID pigs is not due to an intrinsic defect in NK cell killing ability.

1. Introduction

Severe combined immunodeficiency (SCID) can be caused by genetic defects in over 30 genes (Cossu, 2010). Several mouse lines have been developed through genetic modification to model known SCID phenotypes in humans (Xiao et al., 2009). In addition, SCID-type defects have been identified to occur spontaneously in several animal species, including the dog (Meek et al., 2001), mouse (Barthels et al., 2013), and horse (Perryman, 2004; Leber et al., 1998).

Recently, a novel SCID phenotype was serendipitously discovered in a line of Yorkshire pigs that was selected for increased feed efficiency at Iowa State University (Cai et al., 2011) during a large-scale Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) challenge study (Cino-Ozuna et al., 2012). Upon necropsy of piglets that died unexpectedly early in the study, abnormal lymph node and thymus structures as well as very low antibody titers were detected, indicating a SCID-like phenotype. A genome-wide association analysis of 172 pigs within the selection line pedigree that was segregating the SCID phenotype identified a 5.6 Mb region on *Sus scrofa* chromosome 10, which contains the *Artemis* (*DCLRE1C*) gene (Waide et al., 2015). Defects in *Artemis* are known to specifically affect the mechanism of recombination of the T-Cell Receptor (TCR) and B-Cell Receptor (BCR) complexes (Cossu, 2010). The *Artemis* gene encodes an endonuclease that cleaves the hairpin loop created by the RAG1 and RAG2 proteins during somatic rearrangement of these two complexes (Schuetz et al., 2014). Due to the clear relevance of this gene for the SCID phenotype observed, molecular genetic analyses of this gene was performed, revealing two independent mutations in *Artemis*. Both alleles have a Mendelian recessive mode of inheritance and cause the SCID phenotype in either the homozygous or compound heterozygous state (Waide et al., 2015).

As seen in other species with a defect in *Artemis* (Schuetz et al., 2014), these pigs lack B-lymphocytes and T-lymphocytes but produce Natural Killer (NK) cells (Ewen et al., 2014; Waide et al., 2015). NK cells are innate lymphocytes cytolytic to cells not presenting 'self' Major Histocompatibility Complex I (MHC class I), including virally infected cells or tumor cells with down-regulated MHC class I (Vivier et al., 2011). NK cell lysis of target cells can be accom-

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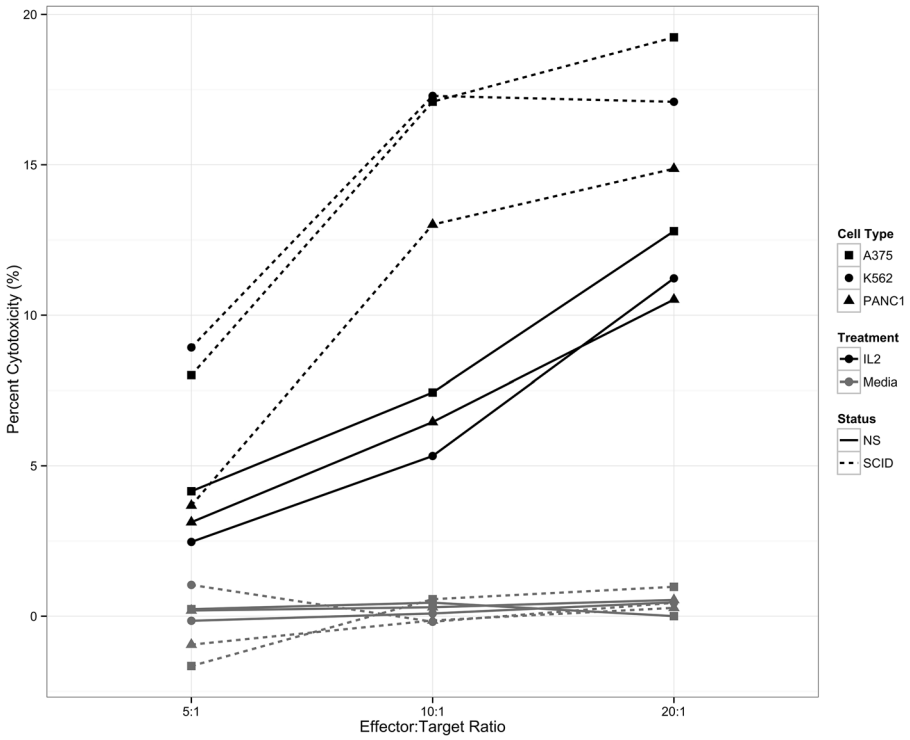

Fig. 1. Human cancer cell lines are killed by IL-2 activated porcine NK cells. PBMCs from SCID and non-SCID piglets incubated in the presence or absence of recombinant human IL-2 (rh IL-2) and tested for cytotoxicity against A375-SM, K562, or PANC-1 human cell lines. Percent Cytotoxicity was reported for 5:1, 10:1, and 20:1 effector:target ratios. SCID PBMCs had the highest percent cytotoxicity (dotted line) compared to non-SCID (NS) littermate cells (solid line). PBMCs not treated (activated) with IL-2, but rather treated with media alone, did not lyse target cells from any of the tumor lines (gray lines). Figure created with ggplot2 (Wickham, 2009).

plished by exocytosis of granular proteins perforin, granzyme B, and other pro-apoptotic proteins (Maher et al., 2002). The NK cell recognizes surface changes in tumor or virally infected cells, binds via activating receptors (for review see Pregram et al., 2010), and releases granular contents including perforin, which form pores in the target cell. Perforin alone can result in osmotic lysis of the target cell. However, perforin–induced membrane changes can allow granzyme B, co-localized in the granule, to enter the target cell and induce programmed cell death or apoptosis. (Maher et al., 2002; Bolitho et al., 2007). Resting murine NK cells have very low levels of perforin and granzyme B, but exposure to an activating signal increases the production of each protein (Fehniger et al., 2007). NK cells in mice deficient for granzyme B and perforin have minimal cytotoxic activity, even if activated by cytokines *in vitro* (Fehniger et al., 2007). Activation of porcine NK cells has been demonstrated using numerous cytokines, including recombinant human IL-2 and IFN-α (Mori et al., 1998). Activation of NK cells is also well documented with IL-15, IL-12, and IL-18 (Fehniger et al., 2007) (Pintarič et al., 2008).

As an animal model for human research, the SCID pig may be more advantageous than other species, as the domestic pig is more physiologically and immunologically similar to humans (Meurens et al., 2011) and has an "immunome" with greater homology to humans than mice (Dawson et al., 2013). Basel et al. (2012) showed that the SCID pigs did not reject two human tumor cell lines, PANC-1 (pancreatic carcinoma) and A375-SM (melanoma). As these immune-compromised pigs possessed phenotypically identifiable NK cells, yet failed to reject cancer target cells, the authors concluded that the NK cells are not functional due to lack of appropriate cytokine stimulation from absent T-cells. However, no studies have directly measured intrinsic killing activity of NK cell from the Artemis-mutated SCID pigs.

The presence or absence of functional NK cells in SCID patients can impact the success of clinical procedures, such as bone marrow transplantation, and likelihood of Graft versus Host Disease (Hassan et al., 2014). Since SCID models with detectable NK cells have been predicted (Lunn et al., 1995) or reported (Buckley et al., 1997) to be functionally impaired or operative, it is important to determine the functionality of the NK cells within the porcine *Artemis* SCID model. This information will be relevant to the utilization of the SCID pig model in preclinical studies. This paper addresses the role of NK cells in SCID pigs by defining their cytolytic activity, responses to cytokine activation *in vitro*, and production of perforin.

2. Results

Peripheral blood mononuclear cells (PBMCs) from SCID and non-SCID piglet littermates were first tested for their ability to recognize and kill several types of human tumor targets, including those previously shown to survive in SCID pigs (Basel et al., 2012): A375-SM (melanoma); PANC-1 (pancreatic carcinoma); as well as K562 (chronic myelogenous leukemia) cells, a standard target cell for human and porcine NK cell research. Cells were plated at three different effector to target (E:T) ratios (5:1, 10:1, 20:1) with effectors of PBMCs (used as a source of NK cells) and the three cancer cell type targets. Percent Cytotoxicity in PBMCs required cytokine activation (human recombinant IL-2) to kill tumor cells *in vitro*, as no lysis of any of the three cell types tested was observed for cells treated with medium alone at any E:T ratio. The difference in killing at every E:T ratio for cells treated with IL-2 versus medium alone was significant (p-value < 0.0001) (Fig. 1). Activated PBMCs had similar levels of cytotoxicity for all cancer target cells across all E:T ratios, and percent cytotoxicity was not significantly different between cancer target cell types (p-value = 0.065).

Flow cytometric analysis of PBMC's was used in combination with these assays to correlate NK cell activity directly to NK cell effector concentration. We defined NK cells as PBMCs that