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JAK3-deficient mini-pigs exhibit impaired lymphoid organogenesis, intestinal structure, and leukocyte/cytokine production

Pil-Soo Jeong^{a,c,1}, Hae-Jun Yang^{a,d,1}, Young-Ho Park^{a,e,1}, Yeung Bae Jin^{f,1}, Bong-Seok Song^a, Jung Joo Hong^{b,e}, Seung Hwan Lee^g, Jong-Hee Lee^{b,e}, Kyung Seob Lim^a, Kang-Jin Jeong^b, Philyong Kang^a, Hwal-Yong Lee^b, Hee-Chang Son^a, Han-Na Kim^f, Seung-Min Ha^{a,e}, Eun-Ha Hwang^b, Jae-Jin Cha^a, Yena Jung^{a,h}, Seon-A Choi^{a,i}, Sanghoon Lee^{a,j}, Sang-Rae Lee^k, Seung-Chan Lee^l, Kyung Soo Kang^{l,m}, Chang-Gi Hur^l, Yong Woo Jungⁿ, Deog-Bon Koo^c, Young-Kug Choo^d, Jin-Man Kim^o, Bo-Woong Sim^{a,e,*}, Sun-Uk Kim^{a,e,*}

^a Futuristic Animal Resource & Research Center, Korea Research Institute of Bioscience and Biotechnology, Cheongju, Republic of Korea

^b National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Cheongju, Republic of Korea

^c Department of Biotechnology, Daegu University, Gyeongsan, Republic of Korea

^d Department of Biological Science, College of National Sciences, Wonkwang University, Iksan, Republic of Korea

^e Department of Functional Genomics, KRIBB School of Bioscience, University of Science and Technology (UST), Daejeon, Republic of Korea

^f Department of Laboratory Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju, Republic of Korea

^g Department of Life Science, Chung-Ang University, Seoul, Republic of Korea

^h Research Institute, huMetaCELL Inc., Bucheon, Republic of Korea

ⁱ Department of Companion Animals, Chungcheong University, Cheongju, Republic of Korea

^j Laboratory of Theriogenology, College of Veterinary Medicine, Chungnam National University, Daejeon, Republic of Korea

^k Department of Pharmacology, Ajou University School of Medicine, Suwon, Republic of Korea

^l Bio Division, APURES Inc., Pyeongtaek, Republic of Korea

^m Department of Bio Life Sciences, Shingu College, Seongnam, Republic of Korea

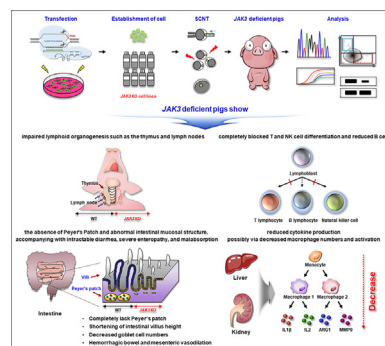
ⁿ College of Pharmacy, Korea University, Sejong, Republic of Korea

^o Department of Pathology, Cancer Research Institute and Infection Signaling Network Research Center, Chungnam National University School of Medicine, Daejeon, Republic of Korea

HIGHLIGHTS

- We first generated JAK3-deficient mini-pigs with severe combined immunodeficiency.
- JAK3-deficient mini-pigs exhibited disruption of the development of immune organs and lymphoid cells.
- JAK3 plays important role in monocyte/macrophage differentiation and macrophage activation.
- JAK3-deficient mini-pigs showed intestinal abnormalities including intractable diarrhea and severe enteropathy.
- JAK3-deficient mini-pigs exhibit phenotypic characteristics and clinical symptoms similar to those of patients.

GRAPHICAL ABSTRACT



* Corresponding authors at: Futuristic Animal Resource & Research Center, Korea Research Institute of Bioscience and Biotechnology, Chungcheongbukdo 28116, Republic of Korea. Futuristic Animal Resource & Research Center, Korea Research Institute of Bioscience and Biotechnology, Chungcheongbukdo 28116, Republic of Korea.

E-mail addresses: embryont@kribb.re.kr (B.-W. Sim), sunuk@kribb.re.kr (S.-U. Kim).

¹ These authors contributed equally to this work.

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ABSTRACT

Introduction: Severe combined immunodeficiency (SCID) mini-pigs are a highly versatile model for human disease research and regenerative medicine.

Objectives: This study aims to generate a novel JAK3-deficient mini-pig model with a human-like immune system and to elucidate how JAK3 plays an important role in immune system.

Methods: JAK3 and RAG2 knockout (KO) mini-pigs were generated using CRISPR/Cas9 and somatic cell nuclear transfer. These mini-pigs were transferred to a sterilized isolator within a specific pathogen-free facility. Phenotypic characteristics and clinical manifestations were analyzed through histological and hematological analysis of SCID mini-pigs to explore the unique role of JAK3 in immune functions.

Results: JAK3 KO was characterized by defects in T and NK cells, very low levels of B cells, and a complete absence of thymus and lymph nodes. Notably, JAK3 KO mini-pigs had significantly reduced numbers of monocytes in peripheral blood, macrophages in tissue, and inflammatory cytokines, suggesting that JAK3 KO can induce a broad immunodeficiency that extends to the myeloid system as well as the lymphoid. Moreover, JAK3 KO mini-pigs had intestinal abnormalities similar to those of patients.

Conclusion: These results suggest that JAK3 KO mini-pigs can be used as an effective model for the development of therapies for SCID patients, as well as for regenerative medicine applications such as the development of patient-specific artificial organs.

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Introduction

Severe combined immunodeficiency (SCID) describes a genetic disorder characterized by disruption of the development or functions of major immune cells such as T, B, and natural killer (NK) cells. SCID is classified by cytokine signaling disorders, V(D)J recombination disorders, pre-T cell receptor signaling disorders, and increased lymphocyte apoptosis [1]. It can be caused by mutations in at least 12 genes, including interleukin-2 receptor gamma (*IL2R γ*), recombination activating gene 1/2 (*RAG1/2*), and Janus kinase 3 (*JAK3*) [2]. Despite the different causes, most patients with SCID have a small thymus or lack thymocytes, lymph nodes, or tonsils. They also commonly exhibit high susceptibility to infection, ultimately resulting in death [3,4]. Although many strategies for treatment of SCID are being developed (e.g., stem cell and bone marrow transplants, and enzyme replacement therapy), patients with SCID remain at risk for severe or fatal infections [5].

JAK3, a tyrosine kinase affiliated with the Janus family of kinases, plays an important role in the immune system as a receptor subunit of various cytokine receptors including interleukins (ILs) 2, 4, 7, 9, 15, and 21 [6]. Because JAK3 is predominantly expressed in hematopoietic and epithelial cells, its role in cytokine signaling is thought to be more limited than that of other JAKs [7]. JAK3 functions in cytokine-dependent gene regulation and lymphocyte activation by regulating early cytokine signaling and downstream STAT activation [8]. Disruption of JAK3 causes autosomal SCID and is responsible for approximately 7 % of SCID in human patients [9]. Patients with JAK3-deficient SCID (JAK3-SCID) show a similar phenotype to that of patients with X-SCID, characterized by the absence of T and NK cells and normal or increased B cells with impaired function (T⁻B⁺NK⁻). This phenotype manifests because JAK3 and the common gamma chain (γ c) of *IL2R γ* are linked components in the same signaling pathway [10]. Mice with JAK3 and *IL2R γ* deficiency exhibit a lack of T and NK cells, as well as significantly reduced mature B cells in the bone marrow and blood [11]. Moreover, both JAK3 and *IL2R γ* knockouts (KOs) result in several changes that cause severe damage to thymus development and the absence of ileal Peyer's patches and lymph nodes [11–13]. Despite similar phenotypes, some researchers have reported that JAK3 KO mice differ from *IL2R γ* KO mice. In particular, JAK3 KO mice exhibit more severe thymic hypoplasia than *IL2R γ* KO mice. Moreover, thymic corpuscles were detected in the thymic medulla of *IL2R γ* KO mice but not in JAK3 KO mice [14].

Because they permit engraftment of human cells and tissue, many SCID animal models have been created to implement regenerative engineering, including xenotransplantation, and to aid in the understanding of incurable diseases of patients, as well as the development of therapeutics [1,15,16]. Such models could be beneficial for the authentication of stem cell therapies in cancer research [17]. Mice have been widely used as models of SCID (e.g., *RAG1* [18], *RAG2* [19], *IL2R γ* [20], and JAK3-deficient [12] mice) for biomedical study of the human immune system [21], cancer [22], stem cell therapy [23], and infectious diseases [24]. However, SCID mice exhibit a slightly different phenotype than that of human patients with SCID because of differences in body size, genetics, and immune system [25,26]. Pigs are a valuable human disease model with many advantages, such as anatomical, physiological, and hematological similarities to humans, although they are expensive compared to mice due to the need for advanced handling and large-scale rearing facilities [27]. In particular, pigs are a suitable SCID model because the immune system is more similar to that of humans (> 80 %) relative to mice (< 10 %) [28]. Therefore, the use of pig models of SCID will help overcome the limitations of mice, improving our understanding of the human immune system.

Herein, a novel JAK3 KO mini-pig model was generated using CRISPR/Cas9 and somatic cell nuclear transfer (SCNT). Phenotypic characteristics and clinical manifestations were analyzed through histological and hematological analysis of SCID mini-pigs to explore the features of JAK3 deficiency in mini-pigs. The cellular phenotypes of JAK3 KO mini-pigs include absence of T and NK cells, as well as low numbers of B cells (T⁻B^{low}NK⁻). Moreover, JAK3 KO mini-pigs show more severe immunological incompetence (e.g., absent or significantly reduced thymus or spleen) compared to RAG2 KO mini-pigs. Notably, in JAK3 KO mini-pigs, no lymph nodes or Peyer's patches were detected, although these were only slightly reduced in RAG2 KO mini-pigs. Overall, we found that lymphocytes and other leukocytes (e.g., monocytes and macrophages) were significantly reduced in JAK3 KO mini-pigs compared to wild-type (WT) mini-pigs, and that clinical manifestations observed in patients with JAK3-SCID were similar to those observed in JAK3 KO mini-pigs [29,30].

Materials and methods

Additional methods are provided in the [supplementary information](#).

Ethics statement

This study was carried out in strict accordance with the recommendations of the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Institutional Animal Care and Use Committee (Approval No. KRIBB-AEC-18130). The SCID mini-pigs used in the experiments except for the ectopic transplantation experiment to determine the presence or absence of immunodeficiency were performed either euthanized due to a sudden deterioration of condition, or as sudden death individuals.

Animal source and welfare

All mini-pigs were reared in sterile stainless pens under controlled environmental conditions including a temperature of $21 \pm 2^\circ\text{C}$, relative humidity of $50 \pm 5\%$, light intensity of 300 lx, and ventilation at 10–20 times per hour. Animal health (including virus and bacterial infections) was periodically monitored and managed by swine veterinary experts. Experiments using mini-pigs were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health. For euthanasia, mini-pigs were injected with ketamine (5 mg/kg intravenously, Yuhan, Seoul, Republic of Korea) followed by KCl injection (75–150 mg/kg, Dai Han Pharm. Co, Chungcheongbuk-do, Republic of Korea) and all efforts were made to minimize animal suffering by institutional veterinary experts, in accordance with NIH guidelines.

Results

Establishment of SCNT with targeted mutations in *JAK3* via CRISPR/Cas9

To disrupt the *JAK3* gene in mini-pig fibroblasts, we designed two single-guide RNAs (sgRNAs) based on exon 2 near the start codon of the coding sequence region to induce a frameshift mutation (Fig. 1a). To obtain enriched *JAK3* KO cells, we used a previously reported method with a surrogate reporter system (Supplementary Fig. 1) [31]. This approach resulted in the clear identification of cells expressing both monomeric red fluorescent protein and enhanced green fluorescent protein. The Fluorescence was visible or detectable within 24–48 h post-transfection by fluorescence microscopy (Fig. 1b) and flow cytometry (Fig. 1c). We constructed a *JAK3* KO cell line using sgRNA #2 because it had relatively few off-targets and higher efficiency (Fig. 1d). Then we plated double-positive CRISPR/Cas9-expressing mini-pig kidney derived fibroblast cells (mPKDFCs) at one cell per well into a 96-well plate, using a BD FACSAria III sorter, to obtain single-cell clones (Fig. 1e). After 4 weeks of culture, six positive colonies were obtained from a total of 20 cell colonies by sequencing. Among these colonies, three were biallelic deletions ($\Delta 17$ and $\Delta 1$) while three displayed biallelic insertions (+1 bp) (Fig. 1f). *JAK3* mutant cells with frameshift mutations (#7) (Fig. 1f) were predicted to create premature stop codons and exhibit normal karyotypes (Supplementary Fig. 2). To examine the non-specific mutations induced by CRISPR/Cas9 editing of the *JAK3* gene, off-targets of the sgRNAs were analyzed by deep sequencing and Sanger sequencing to identify off-target activity. We confirmed that off-target candidates had three mismatch sequences compared to the original sgRNA targets in WT mini-pigs. Genotyping by amplification and sequencing of the 10 predicted off-target sites showed that no mutant induction occurred in the targeted sites (Supplementary Fig. 3).

Generation of *JAK3*-deficient cloned mini-pigs

Donor cells with a *JAK3* biallelic mutation (*JAK3* KO) were used for SCNT (Fig. 2a). *JAK3* KO donor cells yielded four pregnancies from eight transfers (Supplementary Table 1). Thirty-four male cloned mini-pigs were obtained from four recipients by hysterectomy and transferred immediately into a sterilized isolator in a specific-pathogen-free (SPF) facility (Fig. 2b and Supplementary Fig. 4). Sequencing analysis showed a deletion of 17 bp at the target site, as in the donor cells (Fig. 2c). Expression of *JAK3* mRNA in the spleen and liver tissues of *JAK3* KO mini-pigs was confirmed to be nearly absent (Fig. 2d and Supplementary Fig. 2b). In addition, Western blotting analysis of spleen tissue lysates from these mini-pigs further confirmed the loss of *JAK3* expression in *JAK3* KO mini-pigs. The phosphorylated signal transducer and activator of transcription 5 (STAT5), a well-known downstream effector of *JAK3* signaling, was also absent (Fig. 2e). These findings indicated our *JAK3* KO mini-pigs showed both loss of function and loss of expression of the *JAK3* gene.

Phenotypic features of SCID in *JAK3* KO mini-pigs compared to *RAG2* KO mini-pigs

To analyze the unique roles of *JAK3* deficiency in the immune system, we generated *RAG2* biallelic mutation (*RAG2* KO) mini-pigs using CRISPR/Cas9 and SCNT, following the same methodology for *JAK3* KO mini-pigs (Supplementary Figs. 5, 6, and Supplementary Table 1). The survivability of previous *RAG2* KO mini-pigs varied from 12 to 48 days, depending on the strain and rearing environment [17]. We prolonged the lifespan of SCID mini-pigs by means of hysterectomy using a sterile bubble, as well as rearing in an SPF environment. When this method was used, the median lifespan of *RAG2* KO mini-pigs was 15 days, with a maximum of 250 days (Fig. 3a and Supplementary Table 2). However, *JAK3* KO mini-pigs had a poor survival outcome. The median lifespan of *JAK3* KO mini-pigs was 3 days, with a maximum of 90 days (Fig. 3a and Supplementary Table 2).

Gross analysis revealed that both *RAG2* and *JAK3* KO mini-pigs lacked a thymus and had a smaller spleen compared to WT mini-pigs (Fig. 3b). However, these organs were significantly smaller in *JAK3* KO mini-pigs than in *RAG2* KO mini-pigs (Supplementary Table 3). Notably, lymph nodes were observed in *RAG2* KO mini-pigs, but not in *JAK3* KO mini-pigs (Fig. 3b and Supplementary Figs. 7, 8). Next, differences in the internal and cellular structures of the spleen and thymus in each group were analyzed by hematoxylin and eosin (H&E) histological analysis. Compared to WT mini-pigs, *RAG2* and *JAK3* KO mini-pigs showed markedly disrupted development of the splenic white/red pulp surrounding the central arteries. In *RAG2* KO mini-pigs, the thymus was also hypoplastic and consisted of an epithelial rudiment with no lymphocytes. In *JAK3* KO mini-pigs, the thymus was completely absent (Fig. 3c).

To assess the presence of immune cells in spleen tissues of these SCID mini-pigs, we performed immunohistochemical staining. In *RAG2* KO mini-pigs, CD3-positive (CD3⁺) and CD20⁺ cells were either absent or did not reach the levels observed in WT splenic tissue, whereas CD335⁺ cells were observed. By contrast, *JAK3* KO mini-pigs exhibited some CD3⁺ and CD20⁺ cells, but no CD335⁺ cells (Fig. 3d). These results were consistent with fluorescence-activated cell sorting (FACS) analysis. CD3⁺ and CD45RA⁺ cells were dramatically reduced in peripheral blood mononuclear cells (PBMCs), whereas CD16⁺ cells were observed in *RAG2* KO mini-pigs, indicating a lack of T and B lymphocytes (but not NK cells) in PBMCs. Overall, CD16⁺ cells were nearly undetectable in *JAK3* KO mini-pigs compared to WT and *RAG2* KO mini-pigs (Fig. 3e

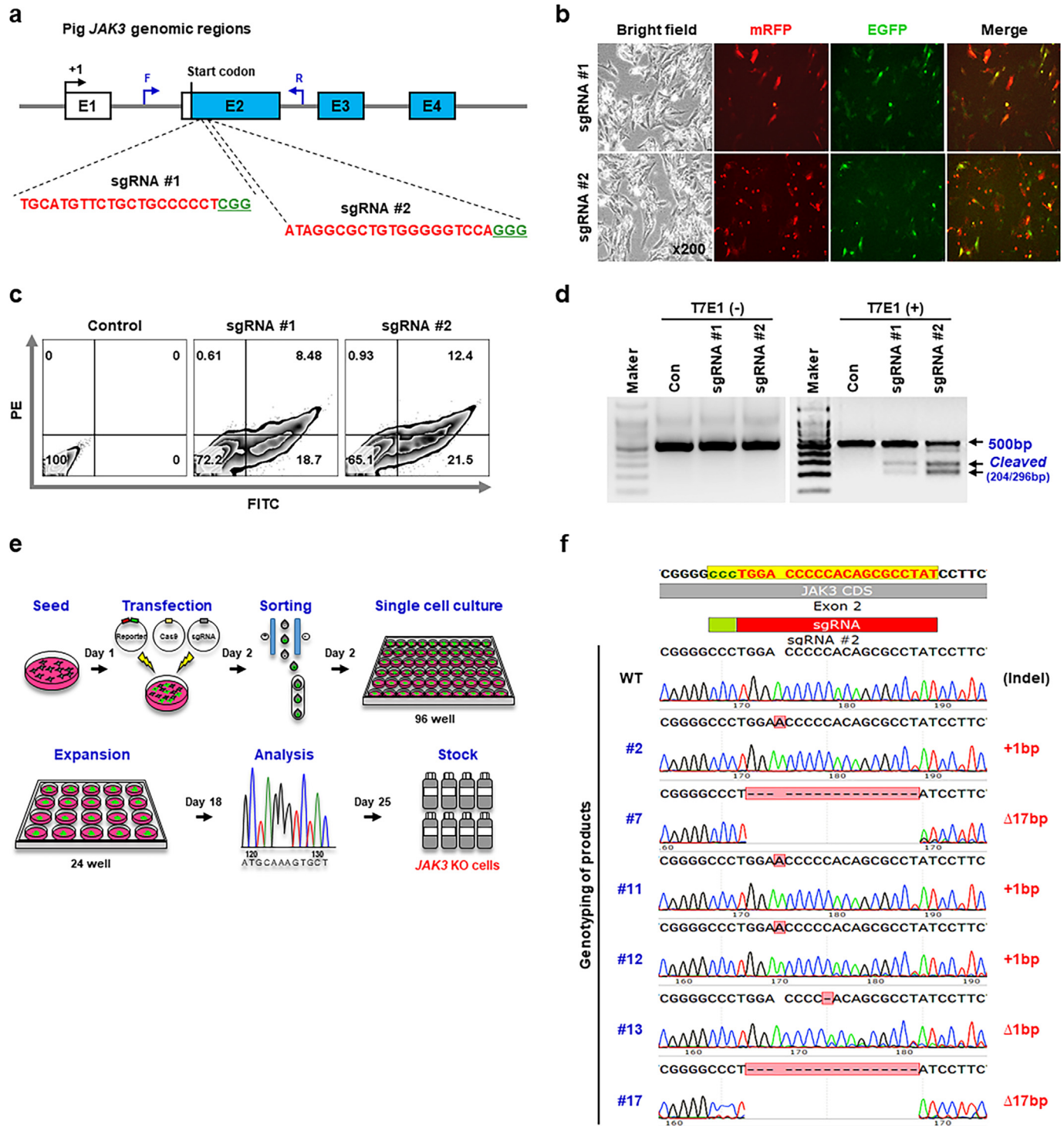


Fig. 1. CRISPR/Cas9-mediated establishment of *JAK3* KO cell lines of mPKDFCs. **a**, Schematic diagram of the sgRNA targeting exon 2 of the *JAK3* gene. sgRNA targeting sites are indicated in red. Protospacer adjacent motif sequences are indicated in green and underlined. +1 indicates transcription start site. Blue arrows (F, forward; R, reverse) represent PCR primers to identify the *JAK3* indel region. **b**, mPKDFCs were co-transfected with Cas9 plasmid (1 μ g), sgRNA plasmid (1 μ g), and RGS reporter plasmid (2 μ g). Images were obtained at 48 h post-transfection (red fluorescence indicates RGS reporter; green fluorescence indicates RGS2 reporter editing). Magnification: 200x. **c**, FACS analysis of enhanced green fluorescent protein and monomeric red fluorescent protein expression in plasmid-transfected mPKDFCs. PE indicates RGS2 reporter plasmid-transfected mPKDFCs. FITC indicates edited RGS2 reporter plasmid-transfected mPKDFCs. **d**, Analysis of CRISPR/Cas9 system efficiency in mPKDFCs by T7E1 assay. Targeted region was PCR amplified and digested by T7 endonuclease 1. **e**, Schematic diagram of establishment of mPKDFC-derived *JAK3* mutation cell lines. **f**, Sequences of established *JAK3* mutation cell lines. Blue numbers of established cell lines indicate order of cloning. Insertions and deletions (indels) are indicated by red numbers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and Supplementary Fig. 9), indicating that the lack of NK cells could be a factor in the reduced lifespan of the *JAK3* mini-pig model. NK cells are an important cause of morbidity and mortality in human patients and mice with NK cell-deficient immunodeficiency [32].

Finally, to validate these results, we used variable (V), diversity (D), and joining (J) [V(D)J] recombination to determine whether T cell receptor (TCR) and B cell receptor (BCR) rearrangements occurred in SCID mini-pigs [33]. In RAG2 KO mini-pigs,

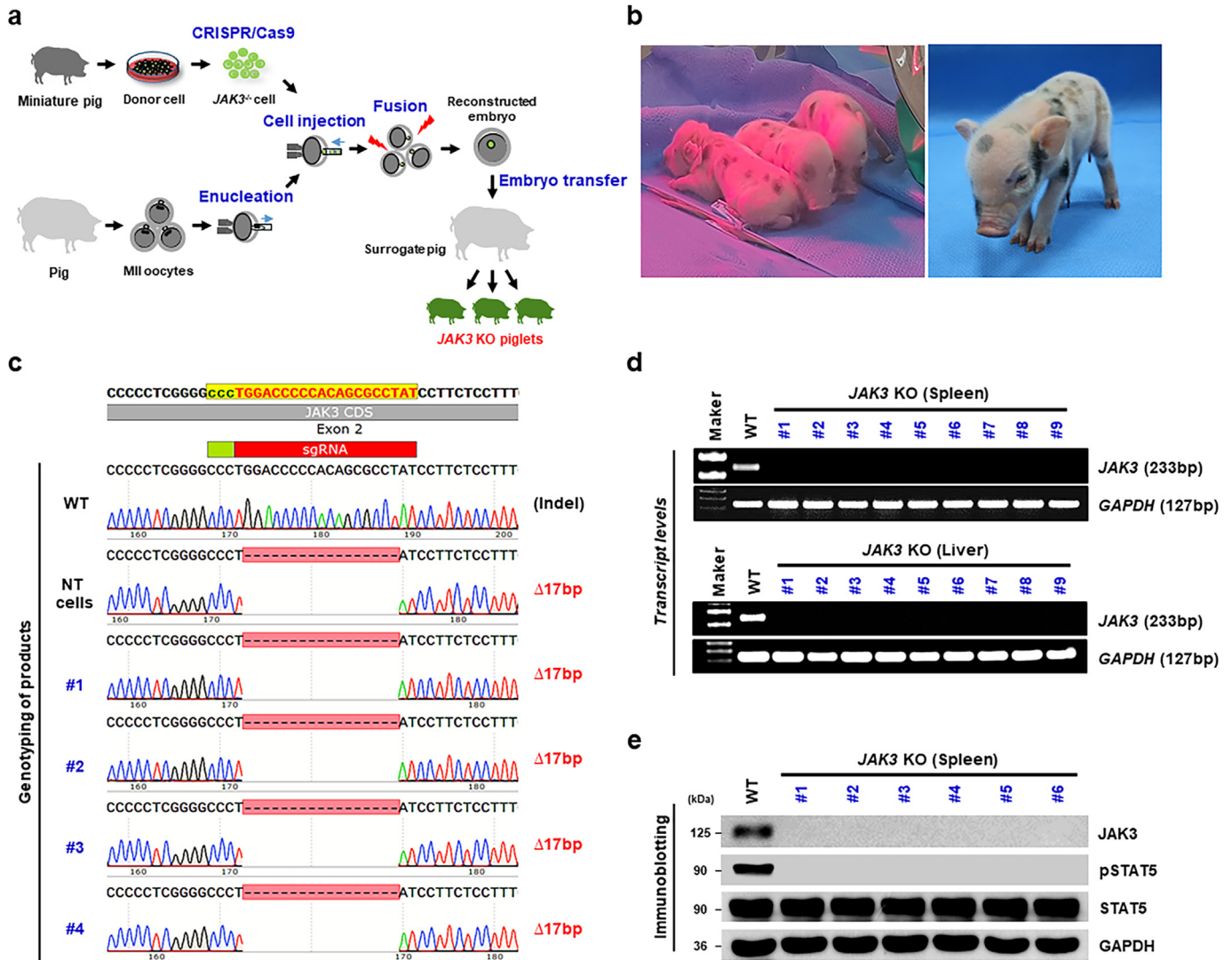


Fig. 2. Generation of *JAK3*-deficient mini-pigs. **a**, Schematic strategy used to generate *JAK3*-deficient mini-pigs. SCNT was conducted to generate *JAK3* KO male mini-pigs. **b**, Photograph of *JAK3*-deficient mini-pigs obtained by hysterectomy, 1 day before expected date of birth. **c**, Genotypes of *JAK3*-deficient mini-pigs by Sanger sequencing. Red dots indicate 17 base-pair (bp) deletion mutations in the sequence of *JAK3*-deficient mini-pigs. Blue numbers indicate cloned piglet ID. Insertions and deletions (indels) are indicated by red numbers. **d**, Semi-quantitative RT-PCR analysis of *JAK3* gene expression in spleen and liver tissues of WT ($n = 1$) and *JAK3*-deficient ($n = 9$) newborn mini-pigs. Blue numbers indicate cloned piglet ID. **e**, Western blot for *JAK3* protein and STAT signaling in the spleens of WT ($n = 1$) and *JAK3*-deficient ($n = 6$) mini-pigs. Blue numbers indicate cloned piglet ID. All data are representative of at least three independent experiments with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

both TCR and BCR were blocked. Conversely, only TCR was blocked in *JAK3* KO mini-pigs (Fig. 4). These results indicated that *RAG2* KO mini-pigs lacked or exhibited significantly reduced immune organs, cellular phenotypes of $T^+B^-NK^+$, and did not induce V(D)J recombination consistent with previous studies [34] and that *JAK3* KO mini-pigs are phenotypically important for thymus and lymph node formation, as well as for the $T^+B^{low}NK^-$ phenotype; some T cells formed, but TCR rearrangement was unsuccessful.

Cancer cell xenotransplantation in *JAK3* KO mini-pigs

After ectopic transplantation, a subcutaneously inoculated human melanoma cell line (LOX-IMVI) formed a tumor at the inoculation site by 16 days in *RAG2* and *JAK3* KO mini-pigs. In WT mini-pigs, it did not develop into tumors, even when more cancer cells were injected. (Supplementary Fig. 10a). These results are consistent with the findings of previous reports, in which *RAG2* KO mini-pigs could be xenotransplanted [34]. However, our study was the first to show that xenografts can be achieved in a *JAK3* KO mini-pig model. Notably,

immunohistochemistry staining of tumor sections revealed fewer infiltrations of NK cells into tumors formed in *JAK3* KO mini-pigs compared to *RAG2* KO mini-pigs (Supplementary Fig. 10b). Similarly, expression of NK cell-associated genes (e.g., *TBX21*, *EOMES*, *NKG2D*, *NKp44*, and *NKp46*) in tumors was observed in *RAG2*, but not *JAK3*, KO mini-pigs (Supplementary Fig. 10c). Taken together, our results showed that the reduced NK cell numbers may affect survival and tumor formation in *JAK3* KO mini-pigs.

JAK3 KO mini-pigs showed significantly reduced numbers of monocytes, macrophages, and platelets, as well as reduced macrophage activation and pre-inflammatory cytokine production

JAK3 is expressed in NK and activated T cells but is also important for myeloid lineage cells [35]. Therefore, we investigated the numbers and functions of monocytes and macrophages in *JAK3* KO mini-pigs. We first detected reduced numbers of lymphocytes in complete blood counts of both SCID mini-pigs. However, the reduction in monocytes in *RAG2* and *JAK3* KO mini-pigs did not dif-

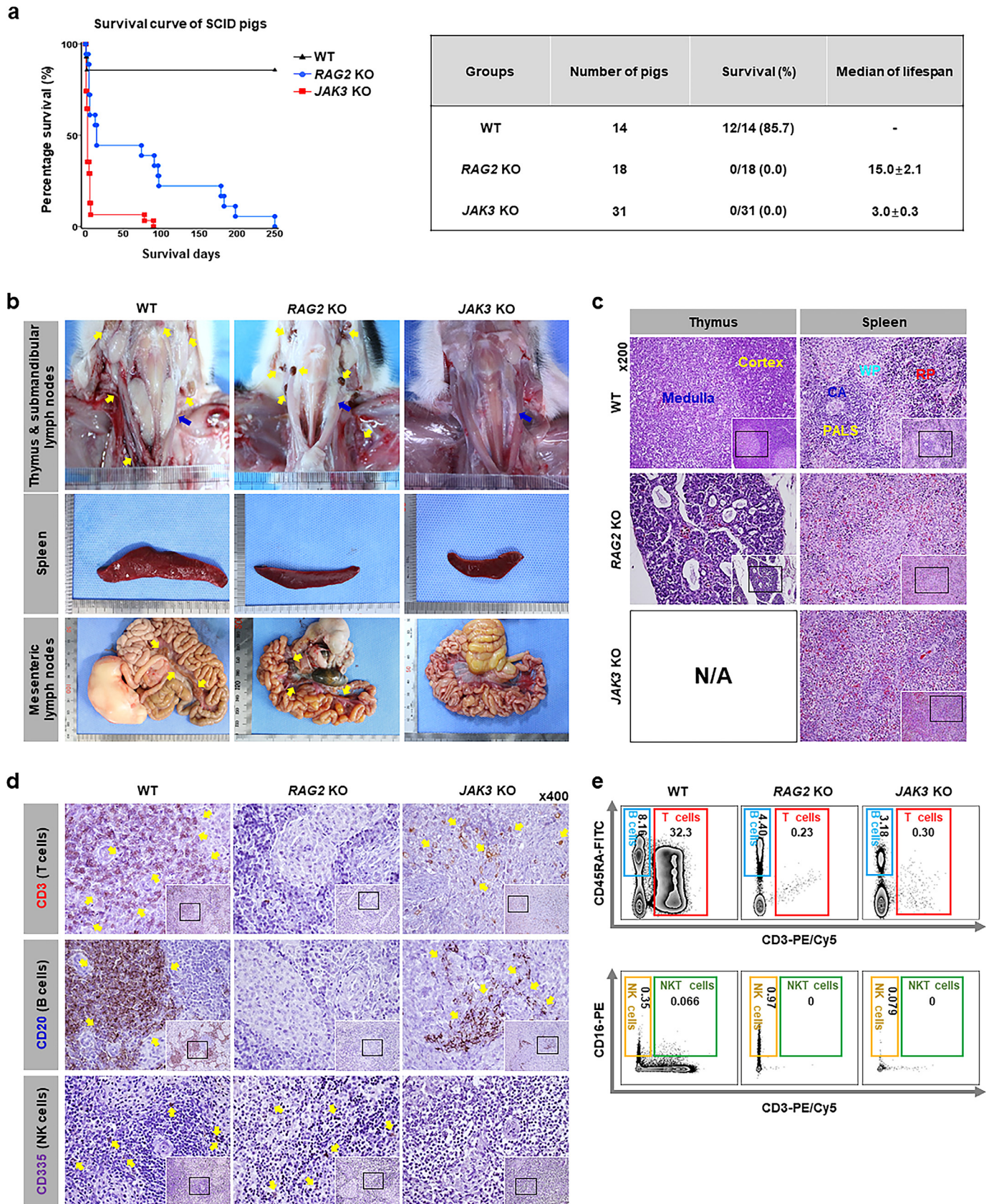


Fig. 3. Phenotypic characterization of SCID mini-pigs. **a**, Survival curves of WT ($n = 14$), RAG2 KO ($n = 18$), and JAK3 KO ($n = 31$) mini-pigs. **b**, (top) Gross appearance of thymus (blue arrow), submandibular lymph nodes (yellow arrows), (middle) spleen, and (bottom) mesenteric lymph nodes (yellow arrows) in WT ($n = 4$), RAG2 KO ($n = 5$), and JAK3 KO ($n = 8$) mini-pigs. **c**, Histological analysis of the thymus and spleen in WT, RAG2 KO, and JAK3 KO mini-pigs. CA, central artery; PALS, periarterial lymphoid sheath; RP, red pulp; WP, white pulp. Magnification: main panels, 200x; inserts, 100x; N/A, not applicable. **d**, Immunohistochemical analysis of CD3, CD20, and CD335 in the spleen. Magnification: main panels, 400x; inserts, 100x. Yellow arrows indicate positive cells. **e**, FACS analysis of CD45RA, CD16, and CD3 in PBMCs from WT, RAG2 KO, and JAK3 KO mini-pigs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

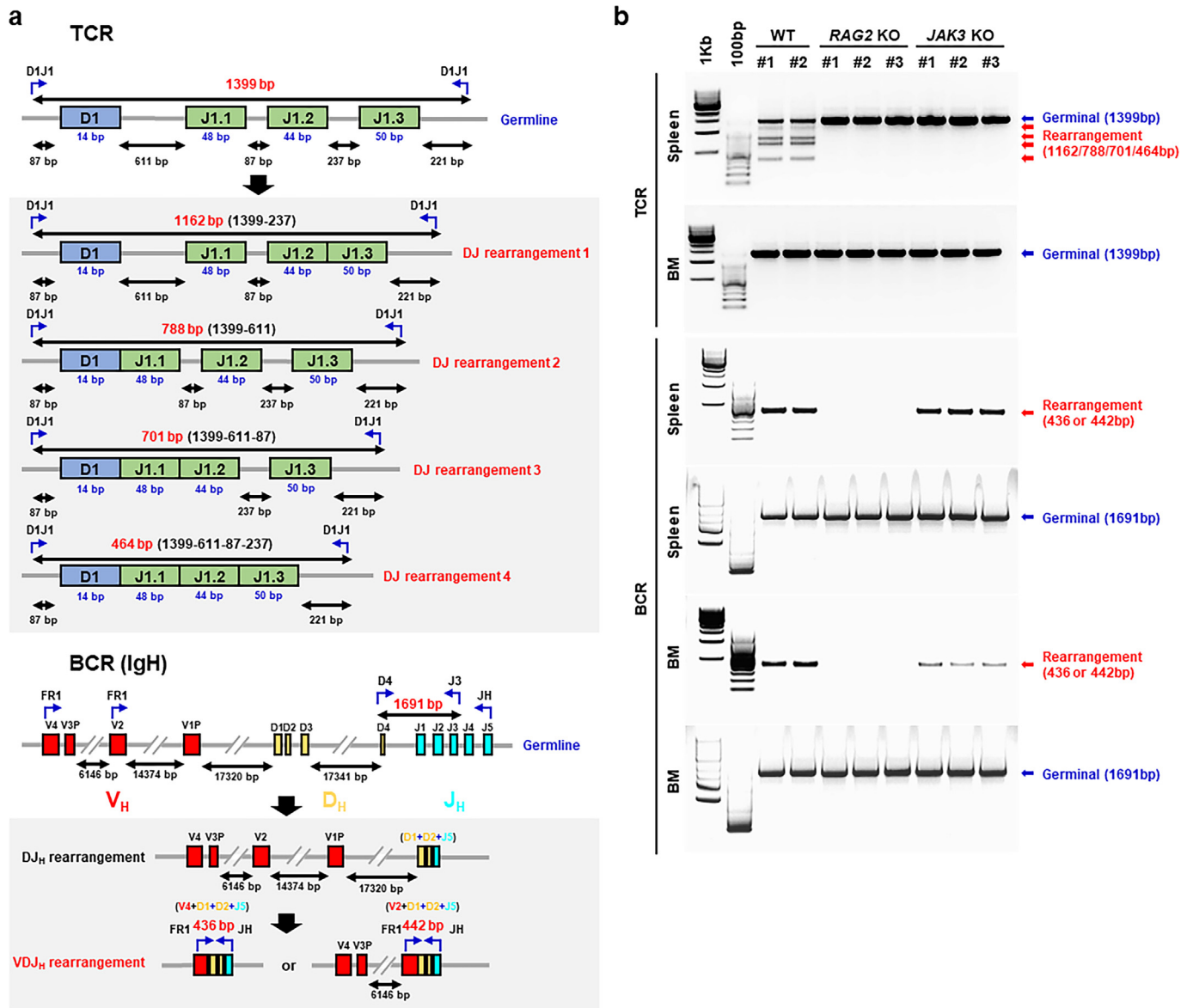


Fig. 4. V(D)J recombination analysis of SCID mini-pigs. **a**, (top) Schematic diagram of TCR rearrangement. One PCR primer was used to identify the germinal band and four types of TCR rearranged bands. (bottom) Schematic diagram of BCR rearrangement at the IgH locus. BCR is a two-step process in which a D gene segment first recombines with a J segment; a V gene then recombines with the DJ block. Blue arrows indicate PCR primers to identify V(D)J rearrangement. **b**, V(D)J recombination analyses of TCR and BCR rearrangement in the spleen and bone marrow (BM) from WT ($n = 2$), RAG2 KO ($n = 3$), and JAK3 KO ($n = 3$) mini-pigs. All data are representative of at least three independent experiments with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for significantly between the two SCID models. Further, platelets were not significantly different from WT mini-pigs (Fig. 5a and Supplementary Table 4). JAK3 KO mini-pigs also showed significantly reduced expression levels of genes known to be important for tissue-resident macrophages (CD68, CD169, and CD64) in the kidneys (Fig. 5b) and liver (Supplementary Fig. 11a). Accordingly, we found higher numbers of CD68⁺ cells and increased CD169 levels in kidney tissues from RAG2 KO mini-pigs compared to WT mini-pigs. However, these cells and marker levels were significantly reduced in JAK3 KO mini-pigs (Fig. 5c-e).

Furthermore, most macrophage 1 (M1)- and macrophage 2 (M2)-specific marker expression in kidney and liver tissues was increased in RAG2 KO mini-pigs compared to WT mini-pigs. However, only 7 of 11 were significantly reduced in the kidneys of JAK3 KO mini-pigs (Fig. 5f) and 10 of 11 were significantly reduced in the livers of JAK3 KO mini-pigs (Supplementary Fig. 11b). Similar results were

obtained when expression levels of macrophage genes in tumor tissues were compared between JAK3 KO and RAG2 KO mini-pigs (Supplementary Fig. 11c). Furthermore, there was greater production of nitrite/nitrate by inducible nitric oxide synthase, a known M1 marker, in RAG2 KO mini-pigs than in WT and JAK3 KO mini-pigs (Fig. 5g). Concentrations of several inflammatory cytokines (e.g., IL-1 β , IL-2, CXCL10, IL-10, and IL-12) were low in WT and JAK3 KO mini-pigs, but high in RAG2 KO mini-pigs. In particular, CXCL10 and IL-12 levels were significantly lower in JAK3 KO mini-pigs than in WT mini-pigs (Fig. 5h). Consistent with a previous study [36], these results showed that JAK3 is essential for the central cytokine signaling pathway, which is involved in monocyte to macrophage differentiation and macrophage activation. To the best of our knowledge, this is the first observation of macrophage changes in SCID mini-pigs due to loss of JAK3 modulation of macrophage activation and production of several cytokines.

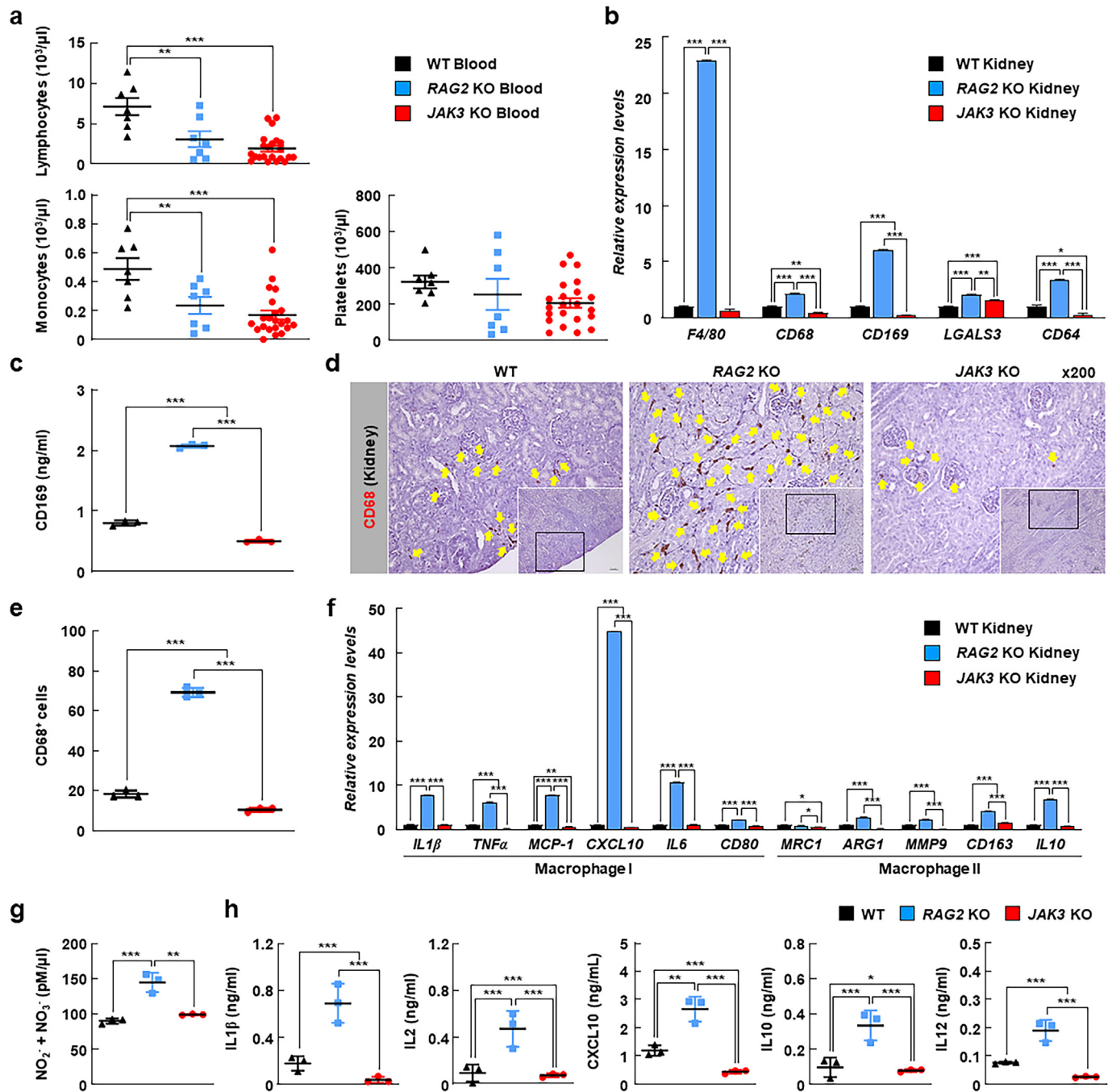


Fig. 5

Fig. 5. Characterization of monocyte and macrophage polarization in SCID mini-pigs. **a**, Comparison of lymphocyte, monocyte, and platelet counts. Blood was obtained from WT ($n = 7$), RAG2 KO ($n = 8$), and JAK3 KO ($n = 22$) mini-pigs. **b**, qRT-PCR analysis for expression of macrophage markers in kidney tissues of WT, RAG2 KO, and JAK3 KO mini-pigs ($n = 3$ per group). **c**, ELISA analysis of CD169 in kidney tissue lysates of WT, RAG2 KO, and JAK3 KO mini-pigs ($n = 3$ per group). **d**, Immunohistochemistry analysis of anti-CD68 macrophages in kidney tissues. **e**, CD68⁺ cells in kidney tissue of WT, RAG2, and JAK3 KO mini-pigs ($n = 3$ per group). **f**, qRT-PCR analysis of macrophage 1 (M1)- and macrophage 2 (M2)-specific marker expression in kidney tissues from WT, RAG2 KO, and JAK3 KO mini-pigs ($n = 3$ per group). **g**, Measurement of total nitrite and nitrate (NO₂/NO₃) levels and **h**, ELISA analysis of released cytokines in kidney tissue lysates from WT, RAG2 KO, and JAK3 KO mini-pigs ($n = 3$ per group). Data are from at least three independent experiments and values represent means \pm standard deviations; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Holm-Sidak multiple comparisons. All data are representative of at least three independent experiments with similar results.

Clinical symptoms of SCID mini-pigs differed according to genetic differences

In pediatric patients with SCID, oral candidiasis, persistent diarrhea with growth disorders, malnutrition, and/or interstitial pneumonia are the most frequent symptoms of infection leading to

diagnosis [37]. Thus, we investigated whether there were any comparable clinical symptoms in our models based on the significant difference in survivability between the two types of SCID mini-pig. SCID mini-pigs with suddenly worsened conditions were euthanized at 3–15 days after hysterectomy. Subsequently, there were many instances of pneumonia and inflammatory diseases in RAG2

KO mini-pigs (89 %, $n = 9$). However, fewer instances of inflammatory disease were observed in *JAK3* KO mini-pigs (21 %, $n = 24$) (Fig. 6a), indicating that *JAK3* deficiency interrupted inflammatory cytokine signaling. Representative H&E images of lung sections revealed numerous inflammatory cell infiltrations (including macrophages) in *RAG2* KO mini-pigs. These involved the alveolar septum, as well as spaces with hemorrhage and congestion. However, the lungs of *JAK3* KO mini-pigs were thickened with increased alveolar matrix in some areas, accompanied by a reduction in alveolar space.

Conversely, we observed intestinal manifestations (e.g., intractable diarrhea, severe enteropathy, and malabsorption) in the majority of *JAK3* KO mini-pigs. In particular, in *JAK3* KO mini-pigs that died suddenly, both the small and large intestines were full of gas. Furthermore, we observed vascular congestion in the mesenteric artery, as well as obvious hemorrhage and gangrenous discoloration in the intestine (Fig. 6b, c, and Supplementary Fig. 12a, b). We presume that the corresponding problems occurred during postnatal growth, because normal intestines were observed in animals that had been euthanized immediately after hysterectomy (Supplementary Fig. 12c). Notably, a clinical enteropathy phenotype has been reported in patients with *JAK3* mutations, consistent with our *JAK3* KO mini-pigs [38]. Furthermore, histopathological analysis revealed that villi were short in *JAK3* KO mini-pigs. Moreover, altered differentiation was present throughout the intestinal epithelium, with a marked reduction in goblet and irregular brush borders, as well as a dramatic lack of Peyer's patches (Fig. 6c, e-g). Overall, immunoglobulin (Ig) A-secreting plasma cells were rarely detected in intestinal lamina propria, in contrast to WT mini-pigs (Fig. 6d). However, IgD and IgM levels were almost normal in other tissues (e.g., spleen and kidney) of *RAG2* and *JAK3* KO mini-pigs, whereas IgA and IgE showed different patterns (Supplementary Fig. 13). Although markedly reduced numbers of IgA-secreting plasma cells and levels of IgA in spleen tissue were observed in *RAG2* KO mini-pigs, these mini-pigs did have some change in intestinal mucosal structure compared to WT, but much less significant compared to *JAK3* KO mini-pigs.

To assess whether normal differentiation occurred in the intestinal epithelium of *JAK3* KO mini-pigs, we performed immunostaining and quantification of proliferating cell nuclear antigen (PCNA). Some PCNA⁺ cells were observed in the entire crypt-villus axis, as well as in the base of crypt-like structures in *JAK3* KO mini-pigs, whereas they were observed only in crypts in WT and *RAG2* KO mini-pigs (Fig. 6c, h). These findings suggested dysregulation of intestinal epithelial proliferation [39]. Based on these results, we presume that clinical features differ depending on whether *RAG2* or *JAK3* is deleted. Inflammation-related diseases were mainly observed in *RAG2* KO mini-pigs, whereas *JAK3* KO mini-pigs showed gastrointestinal disorders, including undifferentiated crypt cells, which led to the development of protracted diarrhea associated with failure to thrive.

Discussion

Patients with SCID have common clinical symptoms, but several studies have revealed differences due to genetic differences [37,40,41]. Therefore, many mouse models with SCID-related genes have been generated to identify the characteristics of SCID and its underlying mechanisms. However, SCID mouse models with *JAK3* mutations are relatively poorly understood compared to models investigating other SCID-related genes (e.g., *IL2R γ* , *RAG1*, and *RAG2*). In addition, mice differ from humans in terms of immunological features such as ILs, cytokines, and inflammatory responses [25,42]. We report here a *JAK3* KO model using mini-pigs with immunological similarity to humans. Notably, we found that

both SCID mini-pigs had phenotypic characteristics and clinical symptoms similar to those of patients. We believe that *JAK3* KO mini-pigs will be useful for understanding the mechanisms of human SCID and may constitute an effective model for treatment of SCID in human patients.

First, we endeavored to more clearly identify the roles of SCID-related genes by improving the existing SCID model generation method and rearing conditions. Microinjection of the CRISPR/Cas9 system directly into fertilized embryos has been widely used to produce gene-edited pigs. However, microinjection methods have disadvantages including different genotypes, phenotypes, mosaicism, and low efficiency [43,44]. In addition, the CRISPR/Cas9 system exhibits considerable problems with off-target effects [45]. Conversely, SCNT has several advantages including the generation of genetically identical offspring, selection of animal sex, and understanding of mechanism-related gene functions [46]. The results of the present study showed that SCID mini-pigs with a consistent genotype and phenotype could be efficiently produced using SCNT methods and single cell clone cultures. Furthermore, donor cells were identified using deep sequencing and Sanger sequencing to confirm that no mutation induction occurred at off-target sites. Overall, SCID pigs lack an intact immune system, and early death cannot be avoided due to various infections in conventional housing conditions [17,47,48]. Several research groups have recently attempted to extend lifespan by improving various methods such as delivery of SCID pigs and rearing facilities [49,50]. Despite these efforts, SCID pigs do not thrive and their lifespans remain short. We delivered by hysterectomy, which maintains a more aseptic environment than caesarean section, and reared the SCID mini-pigs in an SPF facility to avoid infections and prolong lifespan (Supplementary Fig. 4). Our results showed that this procedure could extend the lifespan of SCID mini-pigs compared to existing methods. Additionally, we could more accurately analyze the effects of SCID-related genes without changes due to infection. To the best of our knowledge, this is the first report of the production of SCID mini-pigs via these methods.

Patients with *JAK3* deficiency generally exhibit immunodeficiency features such as defects in immune cells and organs. Similar phenotypic features have been reported in mouse models [12]. Moreover, *JAK3* KO mice reportedly have no peripheral lymph nodes or Peyer's patches [51]. Notably, these immunological phenotypes are similar to the phenotypes of *IL-7* or *IL-7* receptor KO mice [52]. *JAK3* is constitutively associated with the *IL-7* receptor. Thus, defects of immune organs and cells in *JAK3* KO mice may be attributable to the absence of the *IL-7* signaling pathway, which plays an important role in organogenesis involving the thymus, lymph nodes, and Peyer's patches [53,54]. Another possible explanation for the defective immunological phenotype in *JAK3* KO mice includes changes to chemokine receptor signaling. Chemokines are small molecules that act as secondary proinflammatory mediators induced by immune responses. Importantly, chemokines interact with G-protein-coupled transmembrane receptors [55]. A previous study revealed that the JAK/STAT signaling pathway is involved in chemokine receptor signaling [56–58]. Furthermore, immunological phenotypes of *JAK3* KO mice are similar to those of mice with C–C chemokine receptor type 7 deficiency, which affects lymph node organogenesis and T lymphocyte homing. Thus, *JAK3* KO mice might lack C–C chemokine receptor type 7-mediated signaling [59,60]. In addition, *JAK3* is strongly expressed not only in thymocytes but also in cultured thymic epithelial cells, suggesting that *JAK3* may play a role in supporting thymocyte development in the signaling pathway of thymic epithelial cells [12]. In the present study, *JAK3* KO mini-pigs had a defective immunological phenotype, including absence of T and NK cells, low numbers of B cells, absence of the thymus, and a small spleen, compared to WT and *RAG2* KO mini-pigs. Moreover, they did not develop submandibu-

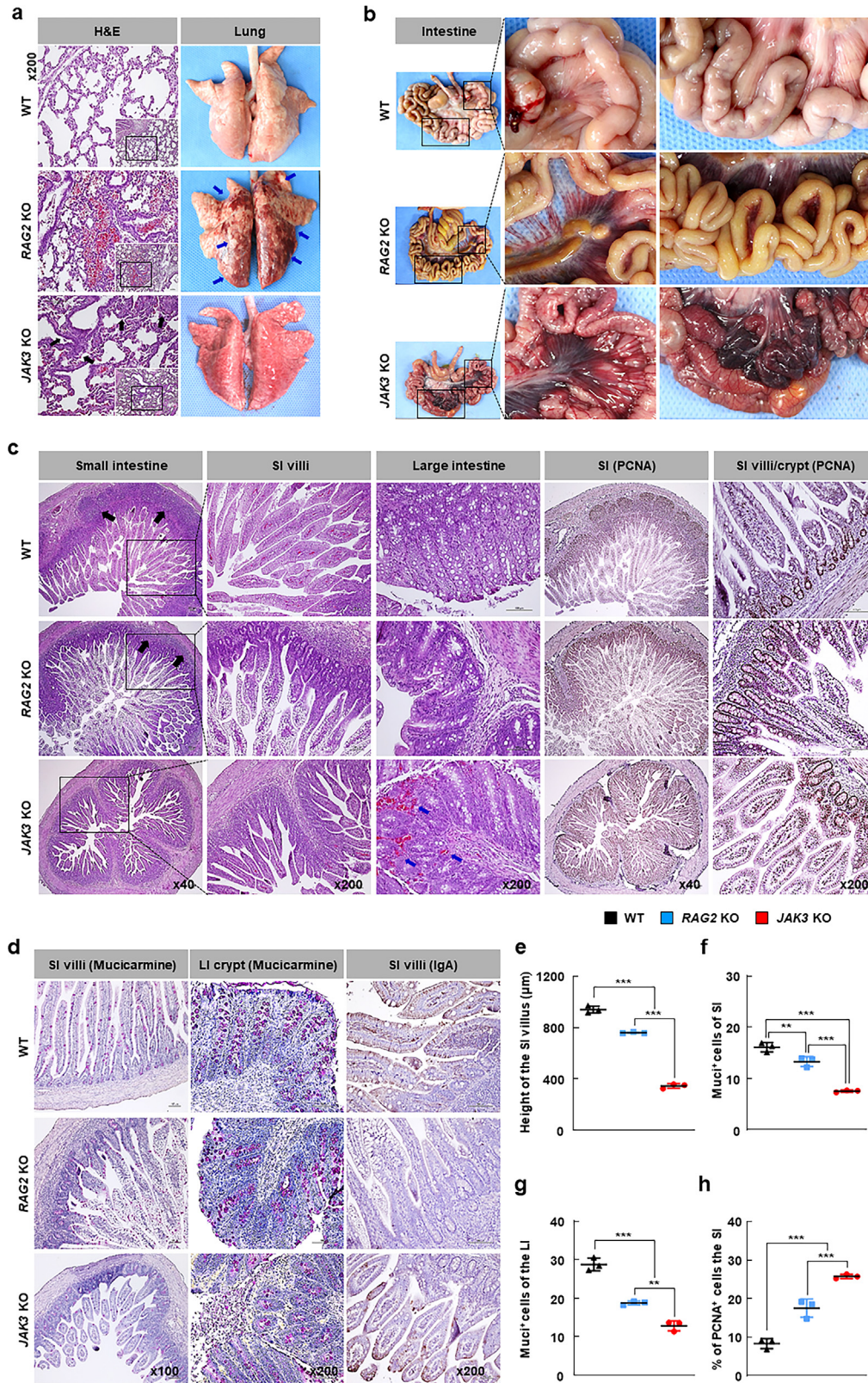


Fig. 6. Differences in major symptoms between types of SCID mini-pigs. Gross pathology and histopathology of **a**, interstitial pneumonia with multifocal reddish areas (blue arrows) in a RAG2 KO mini-pig. Black arrows indicate areas thickened with increased alveolar matrix. Magnification: main panels, 200x; inserts, 100x. **b** and **c**, Representative images of H&E-stained sections and PCNA immunohistochemistry staining of small intestine (SI) and large intestines from WT, RAG2 KO, and JAK3 KO mini-pigs. Intestines with hemorrhage and congestion are from JAK3 KO mini-pigs. Blue arrows indicate hemorrhage. Black arrows indicate Peyer's patches. Magnification: 40x or 200x. **d**, Representative images of mucicarmine-stained sections of SI villi and large intestine (LI) crypt, as well as IgA immunohistochemistry staining of SI villi, in WT, RAG2 KO, and JAK3 KO mini-pigs. Magnification: 100x or 200x. **e**, Height of each villus was measured from top of villus to villus-crypt junction in ileum. Average height values of villi were obtained from 12 villi in each intestinal segment from WT, RAG2 KO, and JAK3 KO mini-pigs ($n = 3$ per group). Goblet cells in SI villi **f**, and LI villi **g**, were counted after mucicarmine staining. Average numbers of goblet cells were obtained from 12 villi or crypts in each intestinal segment. **h**, Average numbers of PCNA⁺ cells were obtained from 12 SI villi in each intestinal segment. Data are from at least three independent experiments and values represent means \pm standard deviations; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Holm-Sidak multiple comparisons. All data are representative of at least three independent experiments with similar results from WT, RAG2 KO, and JAK3 KO mini-pigs ($n = 3$ per group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lar, mesenteric, and inguinal lymph nodes, or Peyer's patches, whereas the sizes of these organs are only slightly reduced in *RAG2* KO mini-pigs. Although further investigations are needed, the immunological defects in *JAK3* KO mini-pigs appear to indicate impairment of IL-7 and chemokine signaling.

Most patients with SCID easily contract pneumonia or infectious diseases, and may fail to thrive, resulting in early death without hematopoietic stem cell or bone marrow transplantation [3,41]. Patients with *JAK3*-SCID experience persistent diarrhea, gastrointestinal disorder, and failure to thrive [37,40]. However, patients with *IL2R γ* -SCID develop multiple recurrent opportunistic infections including pneumonia and sepsis, as well as hepatomegaly and splenomegaly [40,61]. Here, we raise important questions regarding the clinical features of patients with *JAK3*- and *IL2R γ* -SCID. *JAK3* has specificity for transmitting activation signals only through γ c receptors; thus, we expected to observe similar clinical manifestations in *JAK3*- and *IL2R γ* -SCID patients. However, these patients have been reported to have some differences in clinical manifestations. For example, protracted diarrhea and malabsorption cause severe survival threats in patients with *JAK3*-SCID, whereas these symptoms are rarely observed in patients with *IL2R γ* -SCID. In addition, macrophages are normally observed in *IL2R γ* KO pigs [62], but were markedly reduced in our *JAK3* KO mini-pigs. Previous studies have shown that *JAK3* plays an important role in myeloid differentiation. In particular, it accelerates monocyte differentiation in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) [63] and IL-6 [36]. Thus, *JAK3* is presumably regulated by other signaling pathways, suggesting complete loss of *JAK3* may be more severe than *IL2R γ* dysfunction. Notably, this is the first description of *JAK3* KO mini-pigs in terms of the presence of various gastrointestinal disorders (e.g., enteropathy, malabsorption, and histological abnormalities of the digestive system leading to the development of protracted diarrhea) associated with failure to thrive, as observed in patients with *JAK3*-SCID. Moreover, histopathological analysis revealed that *JAK3* KO mini-pigs had shortened villi, markedly reduced numbers of goblet cells, irregular brush borders, a dramatic lack of Peyer's patches, and an overall higher number of PCNA-positive cells in the entire crypt-villus axis, indicating that normal differentiation did not occur in the intestinal epithelium of these mini-pigs. This is consistent with a previous study, which reported that *JAK3* deficiency reduces the expression of villin and carbonic anhydrase, markers of intestinal epithelial cell differentiation, which leads to intestinal epithelial damage [64]. Therefore, we consider the clinical manifestations in our *JAK3* KO mini-pigs to be similar to those in patients with *JAK3*-SCID and suggest that these mini-pigs may be a suitable animal model for patients with *JAK3*-SCID.

Conclusion

In conclusion, we successfully developed *JAK3* KO mini-pigs with a distinct immunological phenotype, including abnormalities in lymphocytes (T, B, and NK cells) and leukocytes (monocytes and macrophages), and impaired inflammatory cytokine production. Unlike *RAG2* KO mini-pigs, *JAK3* KO mini-pigs exhibit severe developmental defects in lymphoid organs such as the thymus, lymph nodes, and Peyer's patches, as well as intestinal abnormalities characterized by intractable diarrhea and severe enteropathy. In contrast, *RAG2* KO mini-pigs predominantly present with inflammatory symptoms, such as pneumonia, highlighting distinct causes of mortality between the two models. This study revealed that *JAK3* KO mini-pigs exhibit immunodeficiency features that are distinct from conventional SCID models, including altered lymphocyte populations, reduced numbers of macrophages, and impaired cytokine signaling. Furthermore, a comparison of the phenotypes observed in *IL2R γ* SCID model animals and patients suggests that

JAK3 defects may have a more severe impact than *IL2R γ* dysfunction. The phenotype of *JAK3* KO mini-pigs closely resembles that of human patients with *JAK3* mutations, establishing them as a valuable model for studying human immune and lymphoid diseases. Current investigations are focused on the potential for immune reconstitution through human stem cell and immune cell transplantation experiments, as well as on studying the role of *JAK3* and its immunological interactions during T-cell and NK cell development. Future research directions include the generation of multiple combination models of *JAK3* KO mini-pigs with other immunodeficiency genes, to establish a diverse spectrum of immunodeficiency models. These models are expected to serve as a platform for precision medicine research and to advance understanding of the development and function of the immune system.

Compliance with Ethics Requirements

This study was carried out in strict accordance with the recommendations of the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Institutional Animal Care and Use Committee (Approval No. KRIBB-AEC-18130). The SCID pigs used in the experiments except for the ectopic transplantation experiment to determine the presence or absence of immunodeficiency were performed either euthanized due to a sudden deterioration of condition, or as sudden death individuals.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2025.04.036>.

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