

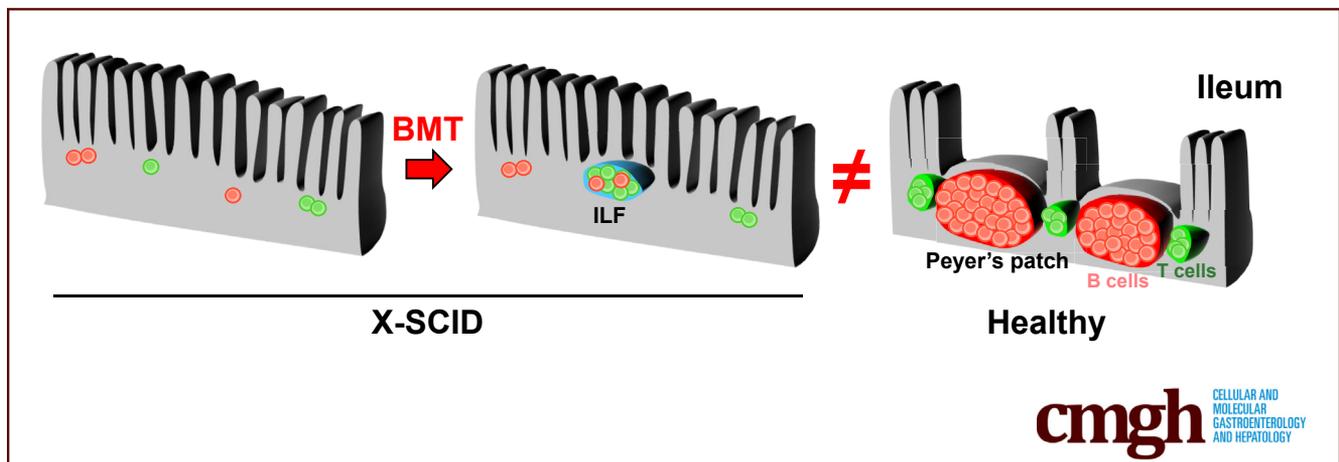
ORIGINAL RESEARCH

Elucidation of the Effects of a Current X-SCID Therapy on Intestinal Lymphoid Organogenesis Using an In Vivo Animal Model



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SUMMARY

The current BMT therapies for X-SCID patients is insufficient to induce the organogenesis of intestinal lymphoid tissues that are associated with numerous functions in vivo.

BACKGROUND & AIMS: Organ-level research using an animal model lacking *Il2rg*, the gene responsible for X-linked severe combined immunodeficiency (X-SCID), is clinically unavailable and would be a powerful tool to gain deeper insights into the symptoms of patients with X-SCID.

METHODS: We used an X-SCID animal model, which was first established in our group by the deletion of *Il2rg* gene in pigs, to understand the clinical signs from multiple perspectives based on pathology, immunology, microbiology, and nutrition. We also treated the X-SCID pigs with bone marrow transplantation (BMT) for mimicking a current therapeutic treatment for patients with X-SCID and investigated the effect at the organ-level. Moreover, the results were confirmed using serum and fecal samples collected from patients with X-SCID.

RESULTS: We demonstrated that X-SCID pigs completely lacked Peyer's patches (PPs) and IgA production in the small intestine, but possessed some dysfunctional intestinal T and B cells. Another novel discovery was that X-SCID pigs developed a heterogeneous intestinal microflora and possessed abnormal plasma metabolites, indicating that X-SCID could be an immune disorder that affects various in vivo functions. Importantly, the organogenesis of PPs in X-SCID pigs was not promoted by BMT. Although a few isolated lymphoid follicles developed in the small intestine of BMT-treated X-SCID pigs, there was no evidence that they contributed to IgA production and microflora formation. Consistently, most patients with X-SCID who received BMT possessed abnormal intestinal immune and microbial environments regardless of the presence of sufficient serum IgG.

CONCLUSIONS: These results indicate that the current BMT therapies for patients with X-SCID may be insufficient to induce the organogenesis of intestinal lymphoid tissues that are associated with numerous functions in vivo. (*Cell Mol Gastroenterol Hepatol* 2020;10:83–100; <https://doi.org/10.1016/j.jcmgh.2020.01.011>)

Keywords: Peyer's Patches; IgA; Microflora; X-SCID; BMT.

X-linked severe combined immunodeficiency (X-SCID) is a serious immune disorder caused by mutation of the interleukin (IL)-2 receptor gamma chain gene (*IL2RG*) located on the X chromosome.^{1–3} Because of dysfunctional B cells and the near-complete absence of T and natural killer cells, X-SCID is lethal within the first 2 years of life unless the immune system can be reconstituted.⁴ Patients with X-SCID often develop infectious diseases (eg, pneumonia, candidiasis) caused by *Pneumocystis jirovecii*, *Candida albicans*, etc., within 3 months of birth because of a failure in the development of an immune system that is capable of inducing acquired humoral and cellular immune responses.^{5,6} The treatments required for the survival of patients with X-SCID include bone marrow transplantation (BMT) with HLA-matched (or closely matched) bone marrow cells or gene therapy to replace the mutated *IL2RG* gene with a normal one.^{7,8} Given that the safety of gene therapy requires careful examination because of a few cases of leukemia as an adverse event after the therapy, BMT has become the most common treatment for patients with X-SCID to cure their symptoms.^{9,10} However, it should be emphasized that a large number of BMT-treated patients with X-SCID still require intravenous immunoglobulin (IVIg) prophylaxis, even after BMT, to sustain serum IgG levels >400 mg/dL.¹¹ Consequently, the current therapeutics with BMT for patients with X-SCID appear to be the most effective procedure to reconstitute measurable levels of hematopoietic cells in peripheral blood.¹² However, further research, particularly using an in vivo X-SCID animal model at the organ level, is required to understand the effect of BMT on the functional maturation of the immune system, including antibody production.¹³

Our group was the first to establish a porcine X-SCID model by generating genetically modified pigs lacking the gene for *Il2rg*, which encodes the common gamma chain (γ c) for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21.¹⁴ The functional region of *Il2rg* on the X chromosome (exon 6) was deleted in this model by homologous recombination with the targeting vector, resulting in the loss of γ c activity in *Il2rg*-deficient pigs.¹⁴ Using a genome editing strategy, another group subsequently succeeded in establishing an *Il2rg*-lacking X-SCID pig model whose phenotype was identical to that of our original model.¹⁵ There are 6 ILs recognized by γ c that play key roles in multiple important immune activities, including the development of hematopoietic cells.¹⁶ Thus, there are serious immune abnormalities in the immune system of *Il2rg*-deficient pigs, such as negligible levels of T and natural killer cells in the peripheral blood.¹⁴ Although B cells are abundant in *Il2rg*-deficient pigs, 3 major immunoglobulin (Ig) subclasses, namely, IgG, IgM, and IgA, are undetectable in the peripheral blood, indicating that the B cells are incapable of producing Igs.¹⁴ The phenotypic features of the immune system of *Il2rg*-deficient pigs are identical to the clinically identified symptoms of patients with X-SCID.¹⁴ Therefore, *Il2rg*-deficient pigs are a suitable X-SCID animal model for designing in vivo studies that cannot be conducted using samples

obtained from patients with X-SCID (eg, pathologically addressing the influence of X-SCID on the organogenesis of secondary lymphoid tissues in which mature immune cells function).

Peyer's patches (PPs), a secondary lymphoid tissue in the gastrointestinal immune system, play a key role in initiating intestinal immune responses, such as IgA production.^{17,18} The current understanding of PP organogenesis is that it is initiated during the embryonic stage by the interaction of fetal liver-derived lymphoid tissue inducer (LTi) cells, which are a subset of type 3 innate lymphoid cells, with nonhematopoietic lymphoid tissue organizer cells at the PP anlagen.^{19–21} Upon stimulation by LTi cells, lymphoid tissue organizer cells express several chemokines (eg, CXCL13, CCL19, CCL21) and adhesion molecules (eg, VCAM1, ICAM1) necessary for recruitment and maintenance of T, B, and other hematopoietic cells, all of which are involved in forming PPs.^{21,22} It should be noted that γ c is an essential receptor for the survival of LTi cells in the gastrointestinal tract because intestinal epithelial cell-derived IL-7, a ligand for γ c, is a key cytokine required to maintain intestinal LTi cells.²³ Therefore, in this and other clinical studies, it is important to note that the initial stage of PP organogenesis, which occurs during the embryonic stage, is completely absent in patients with X-SCID because of the absence of γ c-dependent LTi cells in the gastrointestinal tract.

In this study, we used X-SCID pigs that exhibited the equivalent immune features of patients with X-SCID to demonstrate (1) the abnormality of lymphoid tissue organogenesis, especially PP development, at the organ level; (2) the influence of X-SCID on the development of intestinal immune, microbial, and nutritional environments in vivo; and (3) the association of incomplete PP organogenesis with an abnormality of intestinal microflora, even after mimicking a current therapeutic treatment for patients with X-SCID. Moreover, using samples collected from patients with X-SCID, we demonstrated that the phenotype of X-SCID pigs was comparable to that of patients with X-SCID. Considering these results, we strongly recommend that the current therapeutic procedure for patients with X-SCID should be further improved to include the development of lymphoid tissues with complete morphological and functional features.

*These authors contributed equally to this work.

Abbreviations used in this paper: BMT, bone marrow transplantation; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; Ig, immunoglobulin; IL, interleukin; ILFs, isolated lymphoid follicles; IVIG, intravenous immunoglobulin; LTi, lymphoid tissue inducer; NARO, National Agriculture and Food Research Organization; PCA, principal component analysis; PCR, polymerase chain reaction; PP, Peyer's patch; RT, room temperature; WT, wild-type; X-SCID, X-linked severe combined immunodeficiency.



Most current article

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2352-345X

<https://doi.org/10.1016/j.jcmgh.2020.01.011>

Results

Organogenesis of Lymphoid Organs Was Impaired in X-SCID Pigs

To investigate the developmental abnormalities of immune tissues in X-SCID pigs at the organ level, 9 tissues, including both immune and nonimmune organs, were harvested from wild-type (WT) and X-SCID pigs and subjected to a general pathological study by staining with hematoxylin and eosin. An apparent difference between WT and X-SCID pigs was observed in the ileum and mesenterium. In the ileum, we observed the development of PPs consisting of a large number of lymphoid follicles in the WT pigs, whereas X-SCID pigs did not possess such follicular structures (Figure 1A). By contrast, a sufficient number of lymphoid follicles were not observed in the colon of both WT and X-SCID pigs (Figure 1B). Mesenteric lymph nodes were only found in WT (not X-SCID) pigs (Figure 1C). In the spleen, there were white pulps in both WT and X-SCID pigs. However, the development of germinal centers was only found in white pulps of WT (not X-SCID) pigs (Figure 1D). No apparent differences were noted when nonlymphoid tissues (ie, liver, lung, pancreas, kidney, and heart) were compared between WT and X-SCID pigs (Figure 1E–I). These results indicate that X-SCID pigs possess serious abnormalities in the organogenesis of lymphoid organs.

X-SCID Pigs Lacked PPs but Occasionally Possessed Obscure Accumulations of T and B Cells in the Ileum

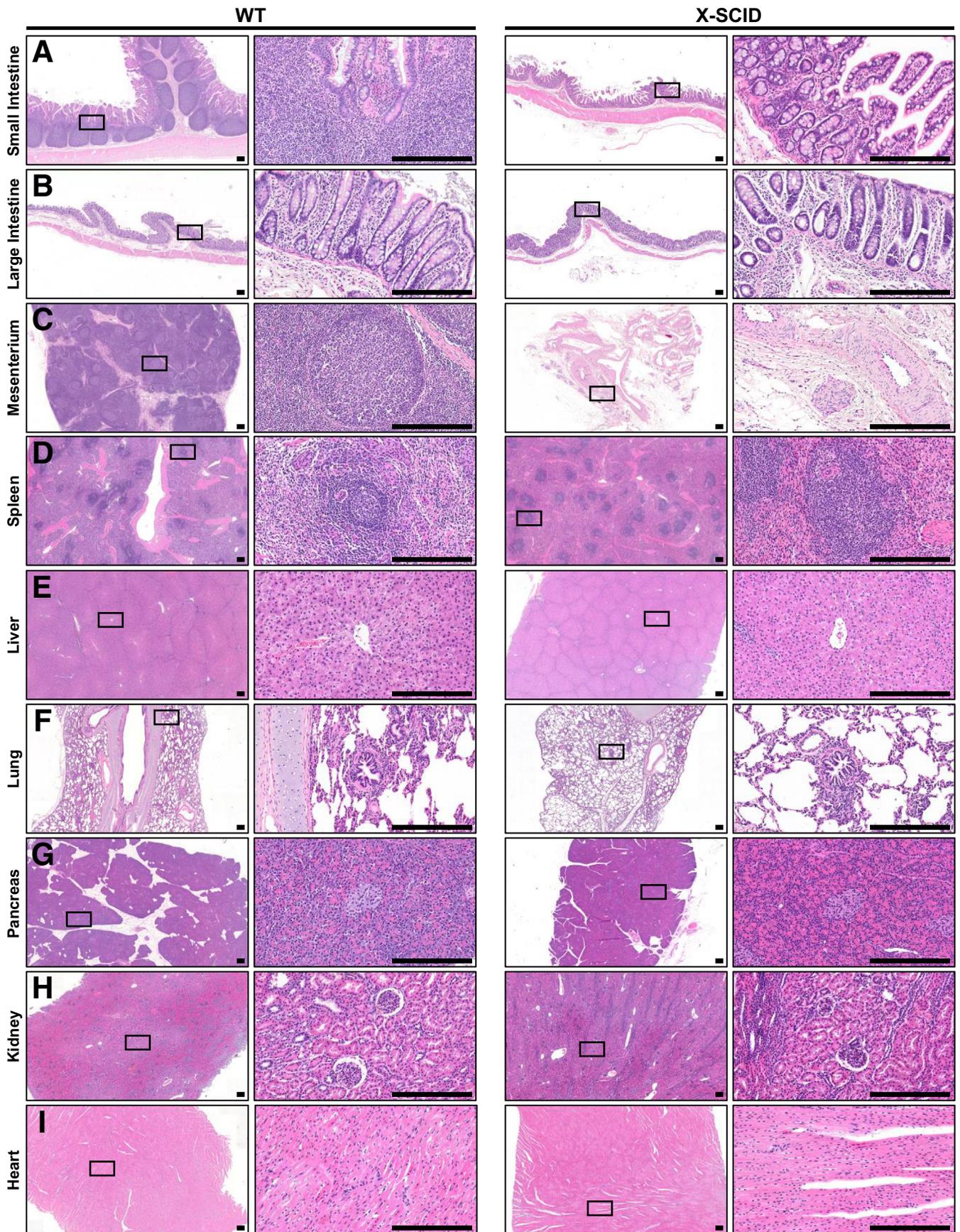
Given that the organogenesis of PPs was completely abolished in X-SCID pigs, the absence of lymphocytes in the ileum was further confirmed via flow cytometry using cells isolated from the ileum of WT and X-SCID pigs. In WT pigs, CD21⁺ B cells and CD3⁺ T cells were abundant, indicating that PP-derived cells, including B and T cells, were accurately detected in the analysis (Figure 2A). By contrast, regardless of the absence of PPs, small proportions of CD21⁺ B cells and CD3⁺ T cells were unexpectedly found in the ileum of X-SCID pigs (Figure 2A). Therefore, to confirm the presence of B and T cells in the ileum of X-SCID pigs, immunohistochemical analyses were performed using anti-CD20 and anti-CD3 antibodies. These antibodies were not the same as those used for the flow cytometry analysis; however, the 2 antibodies were appropriate for immunohistochemical detection of B and T cells in tissue sections. In the ileum of WT pigs, CD20⁺ B cells were abundant in the follicular regions of PPs and CD3⁺ T cells were detected in the subfollicular regions surrounding the follicular regions (Figure 2B). Expectedly, such continuous follicular (B cell) and subfollicular (T cell) regions were not detected in the ileum of X-SCID pigs. However, a few B and T cells were detected in some X-SCID pigs (Figure 2B). These results indicate that X-SCID pigs lack PPs but might recruit a small number of B and T cells into the ileum.

X-SCID Pigs Lacked the Ability to Generate Antibodies in the Gut Immune System

One of the important functions of PPs in WT pigs is the initiation of an immune response to produce IgA, which is the most abundant Ig subclass secreted in the gastrointestinal tract.^{17,18} To investigate the influence of the PP deficiency on IgA production, we performed immunohistochemistry to examine cells producing 3 major Ig subclasses (ie, IgA, IgG, and IgM) in the ileum. In the follicular region of WT PPs, especially in the central area, IgM⁺ (not IgA⁺ and IgG⁺) cells that appeared to be mature naïve B cells expressing surface IgM were abundant (Figure 3A). Above the central area, sufficient IgA⁺, some IgM⁺, and a few IgG⁺ cells, all of which were strongly recognized by the corresponding antibody and thus appeared to be fully differentiated plasma cells, were observed (Figure 3A). In the lamina propria of WT intestinal villi, numerous IgA⁺, some IgM⁺, and a few IgG⁺ cells were also observed (Figure 3A). However, such Ig⁺ plasma cells were rarely detected in X-SCID pigs regardless of the Ig subclass. Moreover, subsequent enzyme-linked immunosorbent assay (ELISA) analyses to detect the amounts of IgA, IgG, and IgM revealed that those Igs were almost undetectable in feces collected from X-SCID pigs (Figure 3B). By contrast, consistent with the abundant numbers of Ig⁺ plasma cells in WT pigs, IgA, IgM, and IgG were readily detectable in feces collected from WT pigs (Figure 3B). These results indicate that X-SCID pigs could not induce normal differentiation of B cells into plasma cells for producing Igs in the gastrointestinal tract.

X-SCID Pigs Possess an Unorganized Intestinal Microflora

Since intestinal IgA plays a crucial role in establishing a symbiotic relationship between the host and microorganisms in the intestine, we investigated the influence of an intestinal IgA deficiency on the development of microflora in the gastrointestinal tract. A FISH analysis revealed that a similar level of microbial cohabitation exists in the colonic lumen of both WT and X-SCID pigs (Figure 4A). A quantitative polymerase chain reaction (PCR) analysis to measure the copy number of the conserved bacterial gene *tuf* in feces indicated equivalent numbers of microorganisms in both WT and X-SCID pigs (Figure 4B). A subsequent metagenomics analysis following the sequencing of 16S rRNA genes demonstrated the presence of bacterial diversity in feces of both WT and X-SCID pigs (Figure 4C). Specifically, multiple microorganisms belonging to the orders of *Bacteroidales*, *Acidaminococcales*, *Clostridiales*, etc., were identified in feces of both WT and X-SCID pigs (Figure 4C). However, principal component analysis (PCA) suggested that the intestinal microflora of WT pigs was uniform, even among individuals, whereas that of X-SCID pigs was relatively heterogeneous with huge variations among individual pigs (Figure 4D). These findings were confirmed statistically using the Monte Carlo method with a significant difference (Figure 4E). Specifically, the *P* value of the *F* statistic of {X-SCID No. 1, ..., No. 10} and {WT No. 1, ..., No. 10} was



estimated experimentally under uniform distribution over all partitions of the union I of $\{X-SCID \text{ No. } 1, \dots, \text{No. } 10\}$ and $\{WT \text{ No. } 1, \dots, \text{No. } 10\}$ into I_1 and I_2 of equal size to verify if the intestinal microflora of X-SCID Nos. 1–10 varied with a higher degree of variance than those of WT Nos. 1–10. The F statistic of I_1 and I_2 was defined as $\frac{\sum_{i \in I_1} \sum_s (r_{i,s} - \bar{r}_{I_1,s})^2}{\sum_{i \in I_2} \sum_s (r_{i,s} - \bar{r}_{I_2,s})^2}$. In the calculation formula, $r_{i,s}$ represents the ratio of species s in the intestinal microflora of individual i , $\bar{r}_{I_j,s}$ denotes the mean of $r_{i,s}$ over all individuals i in I_j , and the number of species s considered was 916. The P value was calculated by drawing a pair of I_1 and I_2 halving I uniformly at random 10,000 times. The histogram of the 10,000 F statistics obtained from the present study indicated that the F statistic of such a random pair of I_1 and I_2 was according to a distribution quite similar to the F -distribution with parameters both set to approximately 40. The F statistic of $\{X-SCID \text{ No. } 1, \dots, \text{No. } 10\}$ and $\{WT \text{ No. } 1, \dots, \text{No. } 10\}$ was 2.269, and among the 10,000 random pairs of I_1 and I_2 , the number of pairs whose F statistics were at least 2.269 or at most $1/2.269$ was 63. Consequently, the P value estimated from the Monte Carlo experiment was .0063, indicating that the intestinal microflora of X-SCID pigs was more variable than that of WT pigs. These results suggest that intestinal IgA facilitates establishment of a uniform microbial environment in the gastrointestinal tract. Thus, the absence of intestinal IgA in X-SCID pigs may be a major factor responsible for the high degree of variability in the intestinal microflora.

Immune Deficiency of X-SCID Pigs Affected Both Resident Bacteria and Metabolic Functions

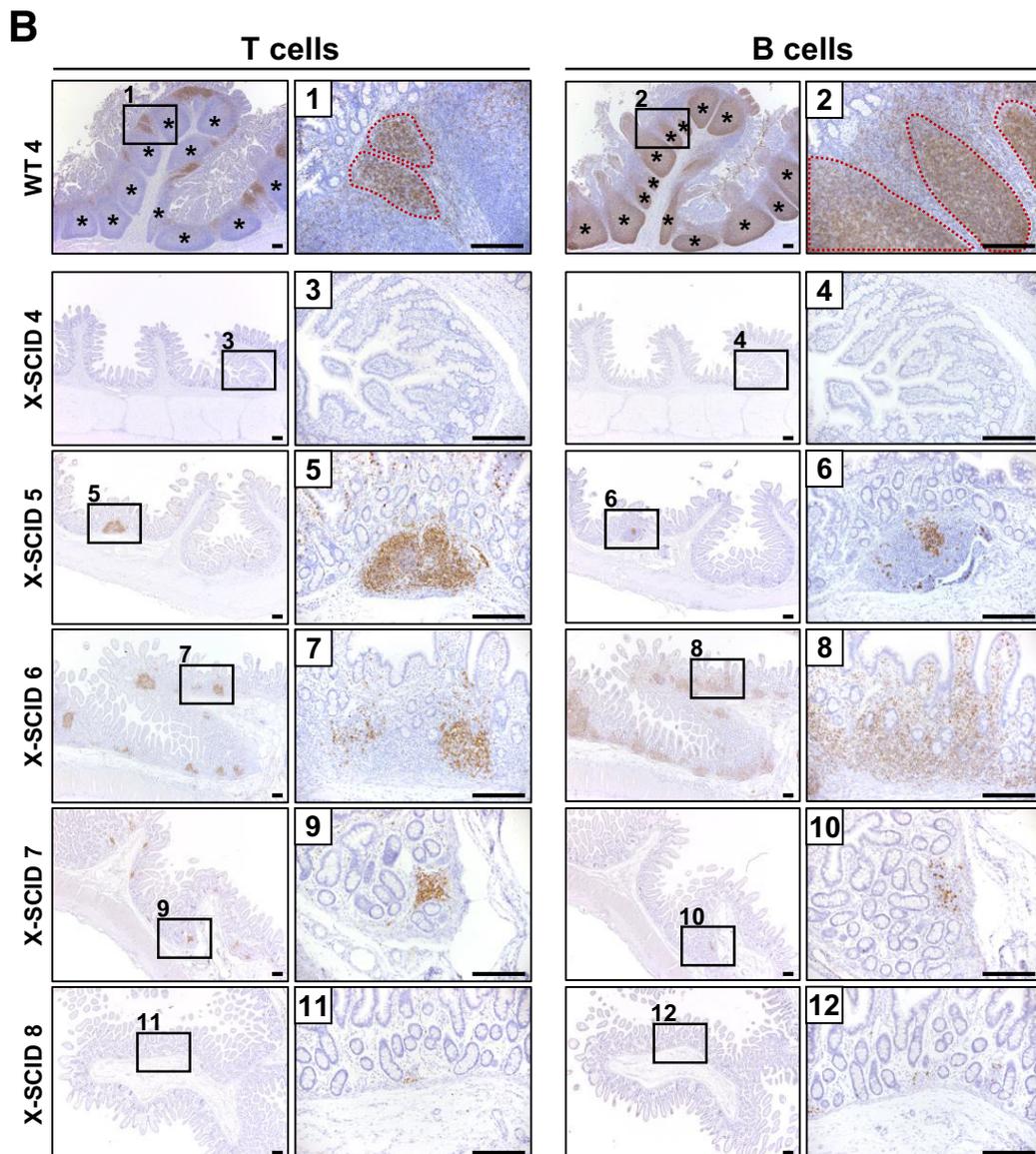
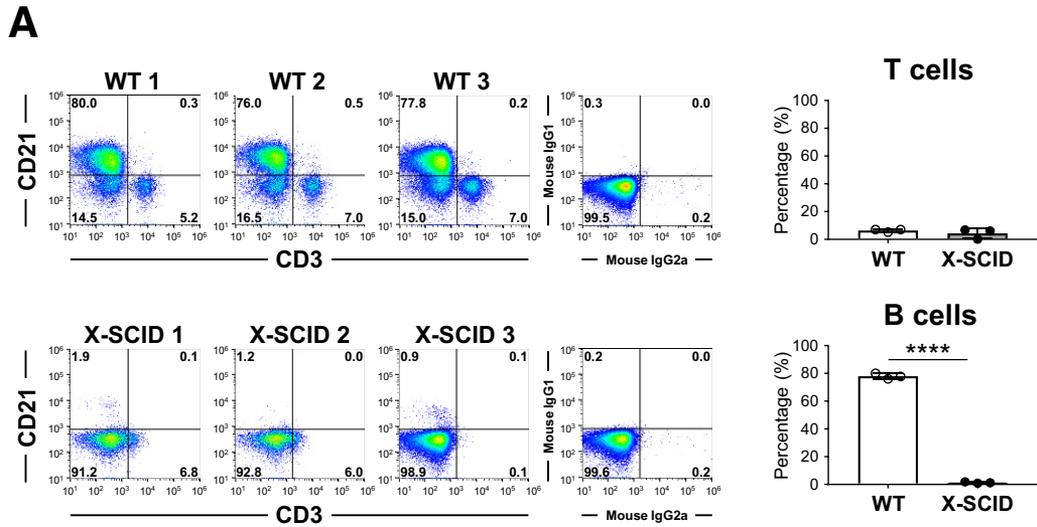
Because the intestinal microflora cohabiting in the gastrointestinal tract influences host metabolism, we performed a metabolomics analysis to comprehensively analyze metabolites in the plasma of both WT and X-SCID pigs. We detected 235 peaks, including 136 cations and 99 anions. The heat map created by a comparison of each peak and its PCA showed that the metabolic profiles of WT and X-SCID pigs were not identical (Figures 5A, B). Of the 235 metabolites, the amounts of 37 molecules were different between WT and X-SCID pigs (Supplementary Table 1). Specifically, in X-SCID pigs, the amounts of 34 metabolites were less, whereas the amounts of 3 metabolites were greater when compared with those in WT pigs (Supplementary Table 1). Using the Kyoto Encyclopedia of Genes and Genomes, 2 particular pathways for the generation of glycerophospholipids and glycine metabolism were identified to be mostly influenced by immune deficiency (Figure 5C). Specifically, the amounts of 7 molecules identified as metabolites related to glycerophospholipid synthesis (ie,

glycerol 3-phosphate, ethanolamine, phosphoethanolamine, choline, phosphocholine, serine, and glycine) were all lower in X-SCID than in WT pigs. These results indicate that X-SCID is an immune disease, as well as a systemic disorder with multiple abnormalities in numerous organs.

BMT to X-SCID Pigs Did Not Facilitate the Organogenesis of PPs and Thus Created Atypical Intestinal Immune and Microbial Environments

In clinical practice, BMT is the most common standard immune procedure for patients with X-SCID.¹² The effect of the BMT on development of immune functions is assessed by analyzing the reconstitution of hematopoietic cells in peripheral blood. However, the effect of BMT on organogenesis of lymphoid tissues in patients with X-SCID remains a mystery, although such lymphoid tissues play a central role in the immune system. Given that X-SCID pigs are the most suitable model for addressing lymphoid organogenesis, we examined the organogenesis of PPs in X-SCID pigs 3–5 years after transplantation with bone marrow-derived hematopoietic stem cells obtained by depletion of lineage marker positive cells (Figure 6A). Reconstitution of peripheral lymphocytes, especially T cells, was clearly observed in BMT-treated X-SCID pigs (Figure 6B). In addition, consistent with the results from our previous study that demonstrated an increased and sustained levels of IgM, IgG, and IgA in the serum of X-SCID pigs 3–34 weeks after the BMT,¹⁴ levels of Igs, especially IgG, were readily detectable in serum 3–5 years after the BMT (Figure 7A). Most importantly, a few isolated lymphoid follicles (ILFs) were present in the ileum of all 3 BMT-treated X-SCID pigs examined (Figure 7B). Moreover, their structure was completely different from the morphological features of WT PPs, which consisted of continuous lymphoid follicles (Figure 1A). Specifically, although both B and T cells were present in the ILFs of BMT-treated X-SCID pigs, follicular (B cell) and subfollicular (T cell) regions, both of which develop in WT PPs, were not detected (Figure 7B). Given that intact X-SCID pigs possessed a small number of isolated lymphoid aggregates with obscure T and B cell regions (Figure 2B), we examined the chimerism of donor and recipient to confirm whether the donor-derived cells formed the ILFs in the ileum of BMT-treated X-SCID pigs. As a green fluorescent protein (GFP)-expressing transgenic pig was used as a donor in 1 BMT trial (BMT3), the tissue sections of recipient X-SCID pigs were stained with an anti-GFP antibody to determine the origin of cells present in the ILFs. More than one-half of the cells were recognized by the anti-GFP antibody (Figure 7B), which means that the ILFs were

Figure 1. (See previous page). Influence of *Il2rg* deficiency on the development lymphoid and nonlymphoid tissues. Lymphoid and nonlymphoid tissues harvested from WT ($n = 5$) and X-SCID ($n = 5$) pigs were subjected to hematoxylin and eosin staining. A major difference in terms of the presence or absence of lymphoid follicles was observed when (A) the ileum, not (B) the colon, was compared between WT and X-SCID pigs. (C) Mesenteric lymph nodes were completely absent in X-SCID pigs. (D) White pulps developed in the spleen of both WT and X-SCID pigs, but germinal centers were only found in WT pigs. There were no obvious differences in the morphology of the (E) liver, (F) lung, (G) pancreas, (H) kidney and (I) heart between WT and X-SCID pigs. Scale bars = 250 μm .



composed of cells that originated from both the recipient and donor and were therefore distinct from the isolated lymphoid aggregates present in intact X-SCID pigs. These results indicate that the donor-derived bone marrow cells could migrate into the ileum of recipient X-SCID pigs, but were not involved in the normal organogenesis of PPs. Next, we investigated the immune function and microflora in the gastrointestinal tract of BMT-treated X-SCID pigs. As expected, a large amount of IgA, followed by IgM (not IgG), was detected in feces collected from all 3 WT pigs (Figure 7C). Interestingly, although similar levels of Igs were detected in the feces of 1 BMT-treated X-SCID pig (BMT1), the amounts were extremely low in the feces of 2 other BMT-treated X-SCID pigs (Figure 7C). As WT PPs play a key role in inducing Ig class-switching from IgM to IgA, as demonstrated in Figure 3A, the capability of ILFs in BMT-treated X-SCID pigs to induce Ig class-switching was further histologically examined. Although IgA⁺ cells were abundant in WT PPs (Figure 3A), such class-switched IgA⁺ cells were rarely detected in the ILFs of BMT-treated X-SCID pigs (Figure 7D). By contrast, in the lamina propria of intestinal villi, a decent number of IgA⁺ cells were present in all 3 BMT-treated X-SCID pigs examined (Figure 7D). Moreover, the intestinal microflora was not identical between BMT-treated X-SCID pigs and age-matched WT pigs (Figure 7E, F). These results indicated that the ILFs developed in BMT-treated X-SCID pigs could be immunologically immature in terms of Ig class switching from IgM to IgA. By contrast, ILF-independent IgA production may have been induced in the gastrointestinal tract of BMT-treated X-SCID pigs regardless of the quantity of IgA detected in feces. However, BMT to X-SCID pigs could not strongly facilitate the development of WT-like microflora in the gastrointestinal tract.

Current Therapeutics for Patients With X-SCID Remain to Be Improved to Achieve Functional Intestinal Immune and Microbial Environments

To demonstrate that the findings inferred from our studies using the X-SCID pig model provided a deeper insight into the improvement of current therapeutics for patients with X-SCID in clinical practice, we investigated the intestinal immune and microbial environments of patients with X-SCID after BMT treatment. Fecal and serum samples were collected from 5 patients with X-SCID who were treated for BMT 3–8 months after birth. There were 2 patients with X-SCID, 3 and 15 years of age, who still required IVIG therapy because of insufficient Igs in their serum. The

other 3 patients, 4, 11, and 29 years of age, had received no immune procedures. Because of the IVIG therapy received by the 2 patients with X-SCID, a sufficient amount of IgG was detected in the serum of all 5 patients with X-SCID, although the amounts of IgM and IgA were extremely low in the 2 patients with X-SCID who received IVIG continually (Figure 8A). A subsequent magnetic resonance imaging showed no obvious structure of the thymus in the 2 patients (Figure 8B). One patient, 29 years of age, developed the thymus although it was atrophied and replaced mostly with adipose tissue (Figure 8B). Most importantly, the 2 patients (Nos. 1 and 4) developing immature systemic immune system did not have detectable amounts of IgA in their feces, whereas the amounts of IgA were detectable in the feces of the other 3 patients (Nos. 2, 3, and 5) although the values differed in quantity (Figure 8C). Moreover, 1 patient who produced a sufficient level of IgA in feces had developed a diverse intestinal microflora, whereas the other 4 patients who had no or little IgA in feces had possessed limited diversity of microflora in their gastrointestinal tract (Figure 8D). These results indicated that, the current therapeutics using BMT for patients with X-SCID must be further improved regarding lymphoid organogenesis to facilitate development of intestinal immune and microbial environments.

Discussion

The aim of our research using an X-SCID animal model was to imitate *in vivo* studies that cannot be conducted using X-SCID patient samples, such as lymphoid tissues, because of the difficulty of clinical extraction before and after the treatment. Our particular interest was to utilize X-SCID pigs to investigate development of secondary lymphoid tissues and its associated biological functions induced by BMT, which is the most common therapeutic procedure for patients with X-SCID. In clinical practice, the success or failure of BMT treatment for patients with X-SCID is evaluated by testing peripheral blood. Thus, most studies regarding the improvement of BMT procedures for patients with X-SCID have been designed to increase the reconstitution of hematopoietic cells in peripheral blood and also to decrease the risk of graft-versus-host disease after the therapy.^{4,11,24} However, to our knowledge, no clinical studies have focused on understanding BMT-induced organogenesis of lymphoid tissues in patients with X-SCID. Therefore, alternative strategies for patients with X-SCID to induce further functional maturation of lymphoid tissues by BMT (or other procedures) have not yet been considered.

Figure 2. (See previous page). **Analyses of immune cells in the ileum of X-SCID pigs.** (A) Flow cytometry analyses indicated that a large number of CD21⁺ B cells and some CD3⁺ T cells were found in WT pigs developing PPs (n = 3, WT 1–3), whereas very few B and T cells were also observed in X-SCID pigs (n = 3, X-SCID 1–3). (B) In addition, immunohistochemical analyses demonstrated that in the ileum (especially in PPs) of WT pigs (n = 5, WT 4–8), CD20⁺ B cells and CD3⁺ T cells formed follicular and subfollicular regions, respectively. Images obtained from a representative (WT 4) of the 5 WT individuals analyzed are shown. In contrast to WT pigs, the distribution of B and T cells in X-SCID pigs (n = 5, X-SCID 4–8) was variable among individuals. No or few B and T cells were observed in 2 X-SCID pigs (X-SCID 4 and 8), whereas a few B and T cells were detected in 3 X-SCID pigs (X-SCID 5–7) regardless of the absence of distinct follicular and subfollicular regions. Scale bars = 200 μ m. Asterisks show follicular area. Dotted lines demonstrate regions stained by either anti-CD3 or anti-CD20. ****P < .0001.

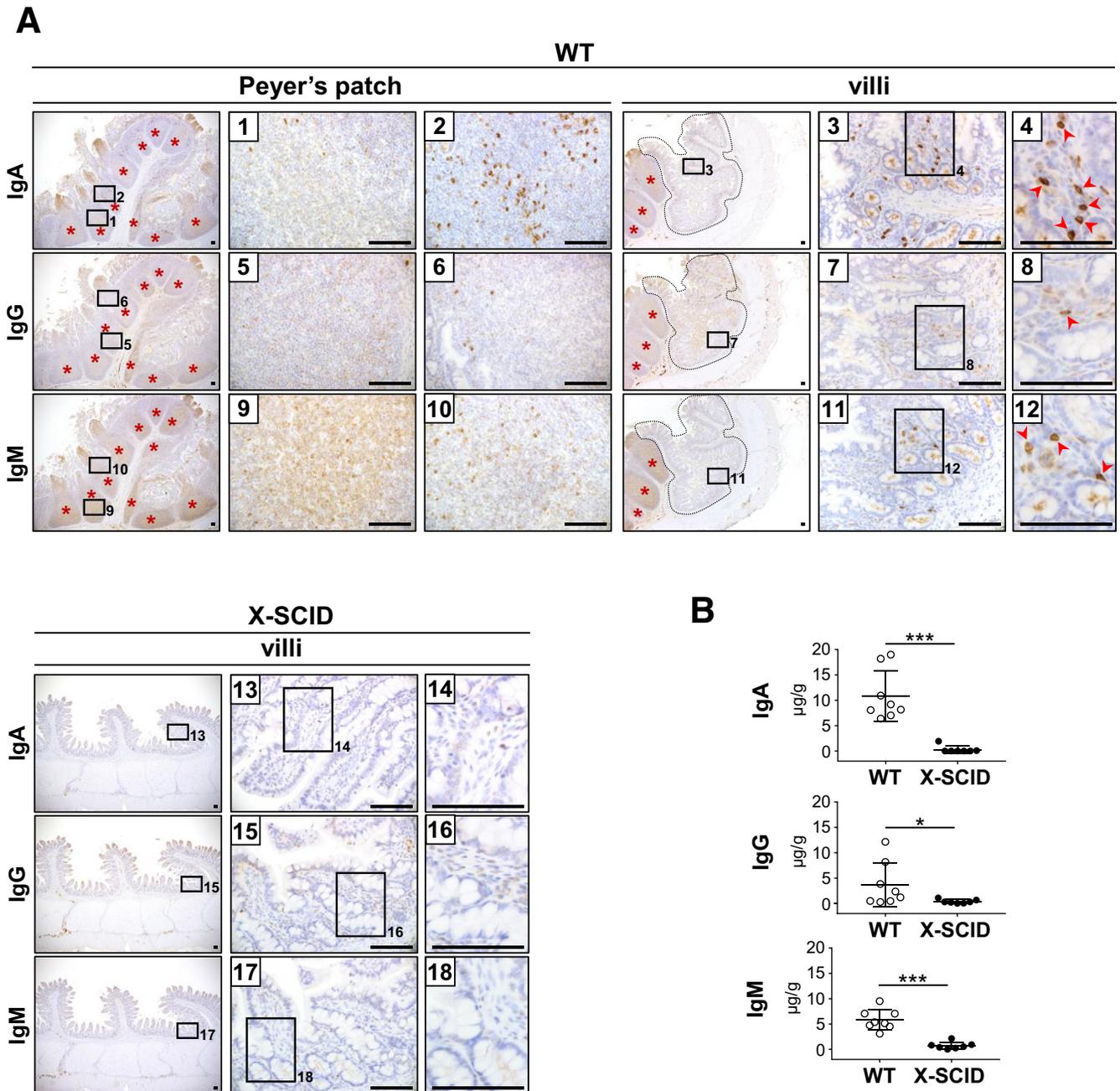


Figure 3. Lack of antibody production in the ileum of X-SCID pigs. (A) Tissue sections of ileum harvested from WT ($n = 5$) and X-SCID ($n = 5$) pigs were stained with either anti-IgA, anti-IgG, or anti-IgM. In the PPs of WT pigs, IgA⁺ cells were abundant, and there were some IgM⁺ and a few IgG⁺ cells above the follicular area, in which B cells expressing surface IgM were present. In the lamina propria of intestinal villi, a large number of IgA⁺ cells, some IgM⁺ cells, and a few IgG⁺ cells were found. However, such Ig⁺ cells were rarely detected in the ileum of X-SCID pigs. (B) The amounts of Igs (ie, IgA, IgG, IgM) in feces from WT ($n = 8$) and X-SCID ($n = 7$) pigs were measured by ELISA. WT pigs produced a large amount of IgA, followed by IgM and IgG in their feces, whereas X-SCID pigs had mostly undetectable levels of Igs in feces. Asterisks show the follicular area. Scale bars = 100 μm . * $P < .05$, *** $P < .001$.

One of the novel findings of this study is evidence for differences in the immune status of lymphoid tissues in the absence of the *I12rg* gene in X-SCID pigs at the organ level. Patients with X-SCID possess sufficient (but nonfunctional) B cells in peripheral blood. Therefore, the distribution of B cells in lymphoid tissues after infiltration from peripheral blood must be determined to assess the efficacy of BMT on

development of lymphoid tissue. As X-SCID pigs do not develop PPs (Figure 1), the number of B cells isolated from the ileum of intact X-SCID pigs was extremely low, with a statistically significant difference when compared with that of developing PPs in WT pigs (Figure 2). Nevertheless, a minimal number of B cells was detected in X-SCID pigs by flow cytometry (Figure 2). Additionally, an

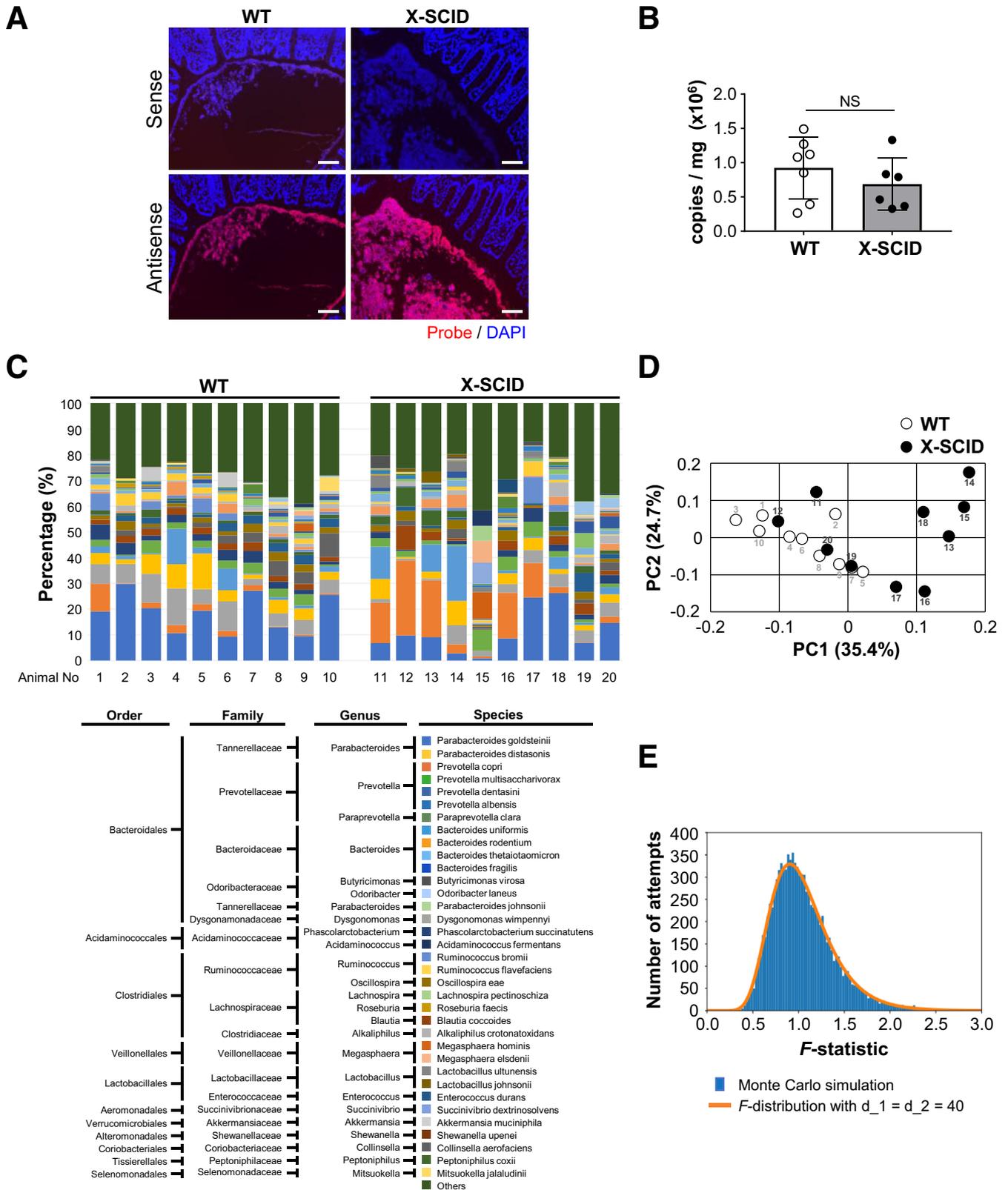
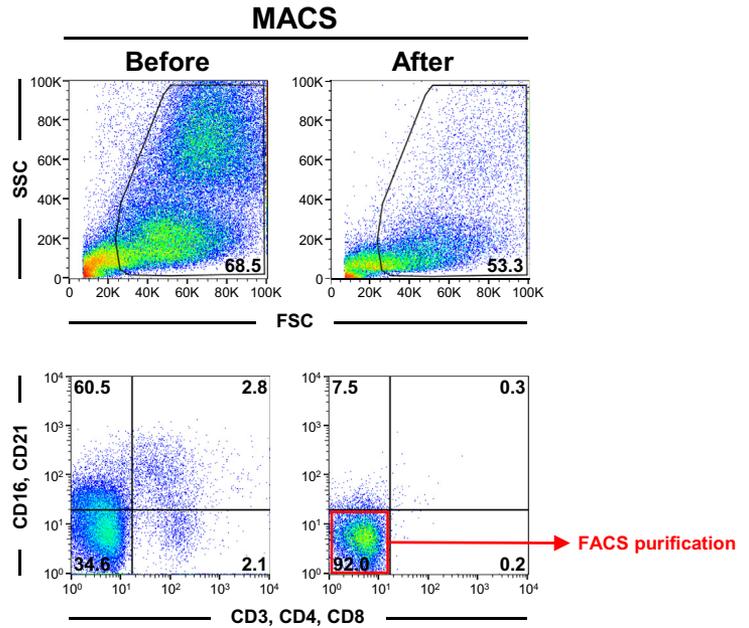


Figure 4. Development of nonidentical microbial environments in the gastrointestinal tract of X-SCID pigs. (A) A FISH analysis, using a common bacterial probe, EUB338, showed that both WT (n = 3) and X-SCID (n = 3) pigs possessed abundant microorganisms in the colon. (B) A quantitative analysis of the numbers of microorganisms in the feces of WT (n = 7) and X-SCID (n = 6) pigs demonstrated that the numbers were identical between WT and X-SCID pigs. (C) A metagenomics analysis to analyze 16S rRNA gene sequences demonstrated that numerous species of microorganisms were present in the feces of both WT (n = 10) and X-SCID (n = 10) pigs. (D) A further PCA showed that the microbial environment in the gastrointestinal tract of X-SCID pigs is much more heterogeneous compared with that of WT pigs. (E) F statistics and F distribution analyzed using the Monte Carlo method further supported the hypothesis, with $P = .0062$. Scale bars = 100 μm . PC; principal component.

A



B

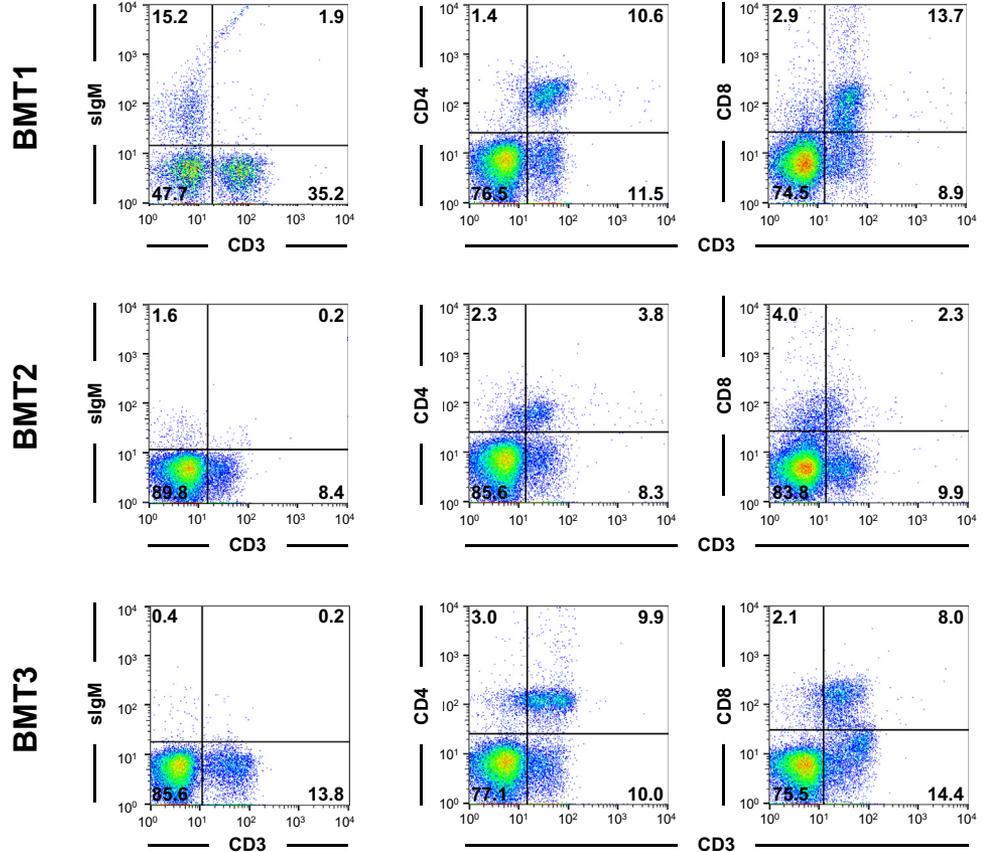


Figure 6. Reconstitution of hematopoietic cells in peripheral blood by BMT. (A) CD3⁻, CD4⁻, CD8⁻, CD16⁻, and CD21⁻ cells present in WT bone marrow were obtained as bone marrow-derived hematopoietic stem cells using a magnetic-activated cell sorting (MACS) followed by a fluorescence-associated cell sorting (FACS) for transplantation into X-SCID pigs. (B) The efficacy of BMT for X-SCID pigs was evaluated by flow cytometry based on the frequencies of CD3⁺ T cells and sigM⁺ B cells, and T cell subsets, which included CD4⁺ helper and CD8⁺ cytotoxic T cells, in peripheral blood. FSC, forward scatter; SSC, side scatter.

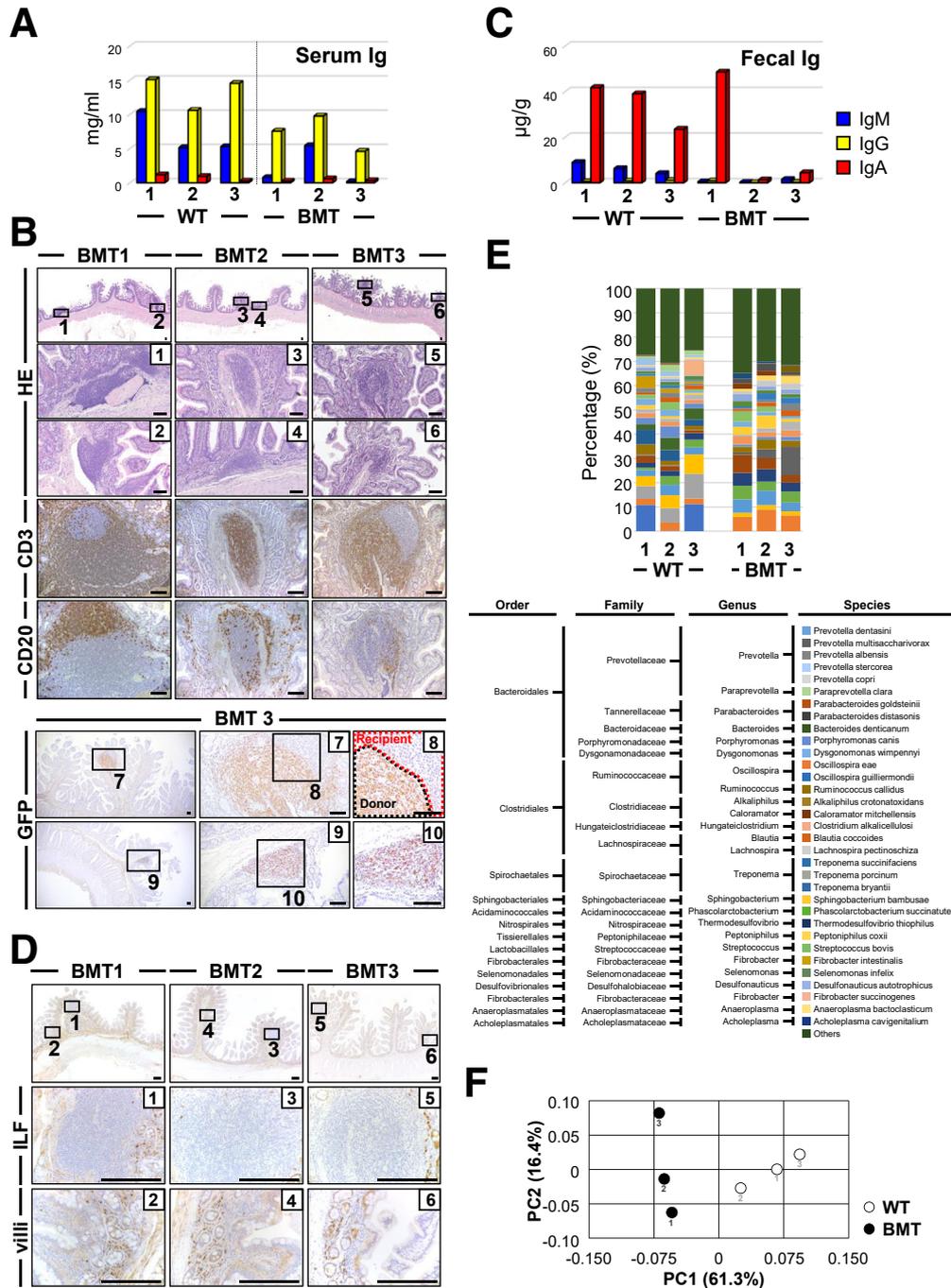


Figure 7. The effect of BMT for X-SCID pigs on the development of the lymphoid tissue structure and intestinal immune and microbial environments. (A) IgG was detected in the serum of both age-matched WT pigs (n = 3) and BMT-treated X-SCID pigs (n = 3). (B) Histological analyses showed that only a few ILFs, which are composed of abnormally accumulated CD3⁺ T cells and CD20⁺ B cells, developed in the BMT-treated X-SCID pigs. (B) One trial (BMT3), using a GFP-expressing transgenic pig as the donor of BMT, showed that the ILFs were composed of both donor and recipient cells because some (not all) cells in the ILFs were recognized by the anti-GFP. (C) In addition, abundant levels of Igs, especially IgA, were found in the feces of WT pigs, whereas only 1 of 3 BMT-treated X-SCID pigs had IgA in the feces. Immunohistochemical analyses showed that few class-switched IgA⁺ cells were present in the ILFs. (D) However, detectable levels of IgA⁺ cells were scattered in the villi of all 3 BMT-treated X-SCID pigs. (E) A metagenomics analysis demonstrated that the intestinal microflora was not identical between BMT-treated X-SCID pigs and age-matched WT pigs. Scale bars = 100 µm. HE, hematoxylin and eosin.

microflora exerts a considerable effect on lipid and amino acid metabolism, we identified significant differences in the amounts of multiple metabolites between WT and X-SCID

pigs (Figure 5). Two metabolic pathways that were profoundly affected by γ c deficiency were related to glycerophospholipid synthesis and glycine metabolism. The

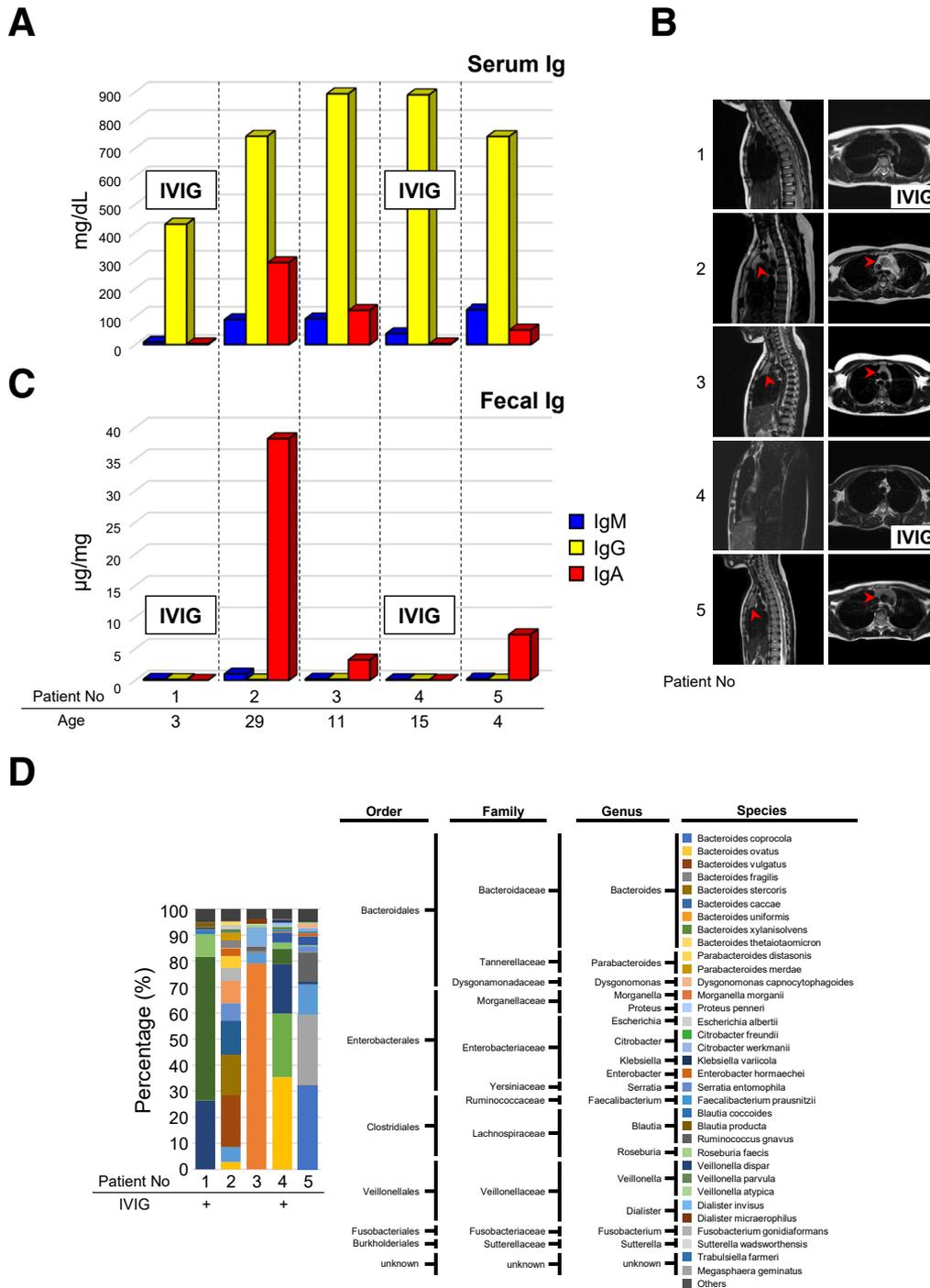


Figure 8. The effect of BMT to patients with X-SCID on the development of the lymphoid tissue structure and intestinal immune and microbial environments. Serum samples were collected from BMT-treated patients with X-SCID (n = 5) and used to assess the amounts of Igs (ie, IgM, IgG, and IgA). (A) Although 2 of 5 patients (Nos. 1 and 4) were receiving continuous IVIG, all patients had serum Igs, especially IgG. (B) Magnetic resonance imaging revealed that the 2 patients receiving continuous IVIG did not possess an obvious thymic structure. (B) One patient (No. 2), 29 years of age, developed the thymus, but the structure was atrophied and replaced mostly with adipose tissue. Arrowheads show the thymus. Fecal samples were collected from BMT-treated patients with X-SCID (n = 5) and used to assess the amounts of Igs (ie, IgM, IgG, and IgA) and the development of intestinal microflora. (C) No Igs were detected in fecal samples from the 2 patients (Nos. 1 and 4) receiving IVIG, and only small amounts of IgA were detected in feces from 2 other patients (Nos. 3 and 5). (C) By contrast, sufficient IgA was detected in feces from 1 patient (No. 2). (D) A metagenomics analysis demonstrated that only the patient who produced a high amount of IgA in feces developed a diverse intestinal microflora.

former pathway resulted in the phosphorylation of ethanolamine and choline, whereas the latter pathway involved the interconversion between glycine and serine that also participated in glycerophospholipid synthesis. Specifically, the amounts of ethanolamine and choline were equivalent between WT and X-SCID pigs, whereas the amounts of their metabolites (ie, phosphor-ethanolamine and phosphor-choline as well as glycine and serine) were lower in X-SCID pigs than in WT pigs. This might be due to the low expression of choline phosphotransferase and ethanolamine phosphotransferase, responsible for the phosphorylation of ethanolamine and choline, respectively,³⁰ as well as impaired metabolism of glycine and serine.³¹ Alternatively, we speculate that phosphor-ethanolamine and phosphor-choline are actively utilized in X-SCID pigs to synthesize glycerophospholipid for strengthening cell membranes in an abnormal situation. Although the results from metagenomic and metabolomic analyses must be further addressed, an X-SCID animal model represents a fundamental breakthrough in studies to advance our understanding of the multiple clinical symptoms observed in patients with X-SCID.

As an *in vivo* animal model for developing immunotherapy for patients with X-SCID, X-SCID pigs provided novel insights into the effect of BMT on lymphoid tissue organogenesis in the gastrointestinal tract. Specifically, BMT for X-SCID pigs is capable of not only reconstituting adequate hematopoietic cells but also producing Igs, especially IgG, in peripheral blood.¹⁴ However, this was insufficient to induce PP development with a complete structure formed by T and B cells because only a few ILFs composed of both donor and recipient cells were present in the ileum (Figure 7). Additionally, BMT was imperfect to induce IgA production in the gastrointestinal tract. Such an immune abnormality may continuously affect the intestinal microbiome (Figure 7). The failure of PP development by BMT might be due to PP organogenesis being initiated during the embryonic stages. Given that the current procedure of BMT for patients with X-SCID is carried out within 2 years of birth at the latest, we performed BMT using neonatal X-SCID pigs. The organogenesis of ILFs (not PPs) is initiated after birth and the role of ILFs is to induce T cell-independent IgA production against intestinal microorganisms.³² However, few class-switched IgA⁺ cells were detected in the ILFs of BMT-treated X-SCID pigs. Nevertheless, IgA⁺ plasma cells were observed in the intestinal villi, although their presence did not correspond to the amount of intestinal IgA detected in their feces (Figure 7). These results indicate that, in recipient X-SCID pigs, donor-derived cells could be involved in the development of ILFs. However, the structure does not function to induce B cell differentiation for IgA production. By contrast, PP- and ILF-independent IgA production may occur in B-1 cells of BMT-treated X-SCID pigs.³³ However, IgA quantity and quality, which probably influence antigen specificity, were unstable in BMT-treated X-SCID pigs because of the completely different properties of intestinal B cells compared with those in WT pigs that possess PP-dependent, ILF-dependent, and B1 cell-derived IgA production in the gastrointestinal tract.

Human studies using serum and fecal samples from BMT-treated patients with X-SCID demonstrated the universality of clinically valuable findings obtained *in vivo* from X-SCID pigs (Figure 8). IgA production in the gastrointestinal tract was completely absent in 2 of 5 BMT-treated patients with X-SCID who received continuous IVIG. Although the level of serum IgG was sufficient, only a small amount of intestinal IgA was detected in feces sampled from 2 other patients. Moreover, the 4 patients who had no or little intestinal IgA developed unbalanced intestinal microflora, suggesting that an abnormality of the intestinal immune system might affect the microbial environment in the gastrointestinal tract. Taken together, our hypothesis inferred from all results obtained from both the *in vivo* animal model and humans is that BMT does not contribute to PP development in patients with X-SCID because of a mismatch with PP organogenesis initiated by LT_i cells during the embryonic stage, but it facilitates the formation of a few ILFs with an abnormal structure and function because of the accumulation of unusual cells at the ILF anlagen. Therefore, to facilitate the complete lymphoid organogenesis required for development of immune and multiple other functions in the gastrointestinal tract, a novel therapeutic approach for patients with X-SCID must be developed to complement the missing elements, especially PP development. One challenging, but potentially viable approach might be prenatal BMT to induce PP organogenesis during embryonic development.^{34,35} Thus, our current ongoing research is focused on demonstrating the efficacy of prenatal BMT using X-SCID pig embryos.

In conclusion, using an X-SCID animal model, we succeeded in performing clinically valuable *in vivo* research and obtained novel findings that could be invaluable for improving the current therapeutics for patients with X-SCID to induce organogenesis of functional PPs that are directly or indirectly associated with multiple functions in both immunology and microbiology.

Materials and Methods

Animals

An X-SCID animal model was established in the National Agriculture and Food Research Organization (NARO).¹⁴ In this study, *Il2rg*^{-/-} male pigs born at the animal facility of NARO from an *Il2rg*^{+/-} female pigs mated to an *Il2rg*^{+/-} male pig were used as an animal model of X-SCID. Littermate WT pigs (*Il2rg*^{+/-} male and *Il2rg*^{+/-} female animals) were used as control animals. WT and X-SCID pigs were weaned at 4 weeks old and sacrificed at 6–13 weeks old, before the condition of the X-SCID pigs worsened because of their immunodeficiency. To investigate the influence of *Il2rg* deficiency on the immunological, microbiological, and nutritional statuses, multiple tissues, blood, and feces were sampled when the pigs were sacrificed.

Samples

Multiple tissues classified as digestive, respiratory, vascular, urinary, and immune organs, such as ileum, colon, spleen, lung, liver, pancreas, kidney, heart, and mesenterium,

were harvested from the WT and X-SCID pigs. The tissues were fixed in 4% (w/v) of paraformaldehyde (Nacalai Tesque, Kyoto, Japan) overnight at 4°C and embedded in paraffin intended for sectioning using histochemical analyses. Fecal samples were used in a metagenomics analysis to determine the intestinal microflora present in the pigs and for an ELISA analysis to examine the amount of fecal Igs. Plasma samples were used for a metabolomics analysis to comprehensively compare the amounts of multiple metabolites between the WT and X-SCID pigs.

Histology

Tissue sections (5 µm) were dewaxed using 70%, 80%, 90%, 95%, 100%, and 100% (v/v) ethanol and then stained with hematoxylin and eosin to study the influence of *Il2rg* deficiency on the histological features of each tissue examined. To investigate the presence of immune cells (ie, T and B cells) in each tissue, the sections were treated with 5% (w/v) of blocking reagent (PerkinElmer, Waltham, MA) for 30 minutes at room temperature (RT) and incubated with either rabbit anti-CD3 monoclonal antibodies (1:100, SP7; Abcam, Cambridge, United Kingdom), rabbit anti-CD20 polyclonal antibodies (1:100; Biocare Medical, Pacheco, CA), rabbit IgG isotype control antibodies (1:100, EPR25A; Abcam), or universal negative control serum (undiluted; Biocare Medical). The sections were then treated with Histofine Simple Stain MAX PO (R) (Nichirei Biosciences, Tokyo, Japan) at RT for 1 hour, and the signal was developed with DAB (Dojindo Molecular Technologies, Rockville, MD). Finally, counterstaining with hematoxylin was performed. To assess the presence of intestinal microorganisms histologically, the colonic tissue sections were subjected to FISH with either 2 pmol/µL of EUB338 probe (5'-Cy3-GCTGCCTCCCGTAGGAGT-3') or 2 pmol/µL of non-EUB338 probe (5'-Cy3-CGACGGAGGGCATCCTCA-3') for 2 hours at 46°C. After washing, the sections were counterstained with 1 µg/mL of DAPI (Dojindo). Tissue images were obtained using either a VS120 (Olympus, Tokyo, Japan), BX63 (Olympus), or BZ-9000 (Keyence, Osaka, Japan) microscope.

Enzyme-Linked Immunosorbent Assay

The concentrations of IgG, IgM, and IgA were determined by ELISA. Briefly, 96-well ELISA plates (Thermo Fisher Scientific, Waltham, MA) were coated overnight at 4°C with 500 ng/mL of goat anti-pig IgG, IgM, or IgA polyclonal antibodies (Bethyl Laboratories, Montgomery, TX) and blocked for 1 hour at RT with 1% (w/v) of bovine serum albumin and 0.05% (v/v) of Tween-20. Serially diluted samples and standard reference serum with known concentrations of IgG, IgM, and IgA (Bethyl Laboratories) were individually added to each well and incubated for 2 hours at RT. After washing, the plates were treated with 100 ng/mL of Ig subclass-specific goat polyclonal antibodies conjugated with horseradish peroxidase (Bethyl Laboratories) as secondary antibodies for 1 hour at RT. The signal was developed using a tetramethylbenzidine microwell peroxidase substrate system (KPL; SeraCare Life Sciences, Milford, MA), and the absorbance of each well at 450 nm was measured using a plate reader.

Flow Cytometry

Mononuclear cells were isolated from the ileum of WT and X-SCID pigs by enzymatic digestion using 0.5 mg/mL of collagenase and 5 U/mL of deoxyribonuclease I (both Sigma-Aldrich, St Louis, MO) for 1 hour at 37°C after removal of the epithelial layer using 1 mM of EDTA and 1 mM of DTT. The isolated cells were stained at 4°C for 30 minutes with fluorescein isothiocyanate-conjugated mouse anti-pig CD3ε antibody (5 µg/mL, BB23-8E6-8C8; BD Biosciences, San Jose, CA) and allophycocyanin-conjugated anti-human CD21 antibody (20 µL/test, B-ly4; BD Biosciences), which cross-reacts with pig CD21. Fluorescein isothiocyanate-conjugated mouse IgG2a (5 µg/mL, G155-178; BD Biosciences) and allophycocyanin-conjugated mouse IgG1 (20 µL/test, MOPC-21; BD Biosciences) were also used as isotype controls. Cell viability solution (10 µL/test; BD Biosciences) was added to each stain to exclude dead cells from the analyses. The stained cells were analyzed using an Accuri C6 flow cytometer (BD Biosciences), and the data were analyzed using FlowJo v10 software for multicolor analysis (FlowJo LLC, Ashland, OR).

Metagenomic Analysis

Genomic DNA was extracted from the feces of 10 WT (58.6 ± 12.9 days old) and 10 X-SCID (56.3 ± 15.9 days old) pigs using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). As reported previously,³⁶ the V3 and V4 regions of the bacterial 16S rRNA gene were amplified by PCR using PrimeSTAR HS DNA polymerase (Takara Bio, Shiga, Japan) and the following 5 primers: forward (5'-TGCTCTCCGATCTGACCCTACGGGNGGCWGCAG-3') and reverse (5'-CGCTCTCCGATCTCTGNNNGACTACHVGGGTATCTAATCC-3', 5'-CGCTCTCCGATCTCTGNNNGACTACHVGGGTATCTAATCC-3', 5'-CGCTCTCCGATCTCTGNNNGACTACHVGGGTATCTAATCC-3', and 5'-CGCTCTCCGATCTCTGNNNGACTACHVGGGTATCTAATCC-3'). The individual adaptor tag sequences, highlighted by a single underline, were inserted at the 5' region of the forward and reverse primers. The joint sequence, consisting of 3–6 random bases, highlighted by a double underline, was inserted between the adapter tag and the 16S rRNA sequences in the reverse primers. The PCR fragments amplified by the first round of PCR from each fecal DNA sample were subjected to a second round of PCR using the following forward and reverse primers (5'-AATGATACGGCGACCACCGAGATCTACACXXXXXACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG-3') and (5'-CAAGCAGAA GACGGCATAACGAGATxxxxxxxxxGTGACTGGAGTTCAGACGTG TGCTCTCCGATCTG-3'), respectively, including a 5- or 9-base index, shown as "XXXXX" or "xxxxxxxxx," to distinguish each sample based on the individual index sequence. All PCR products were sequenced using the MiSeq platform (Illumina, San Diego, CA) with a MiSeq Reagent Kit v2 (500 cycles) (Illumina). The data were analyzed utilizing BaseSpace software (Illumina) to identify bacterial species from each sequencing run. A PCA was also carried out using the statistical program Python for comparison of intestinal microflora between WT and X-SCID pigs. A quantitative PCR analysis using a Bacteria (*tuf* gene)

Quantitative PCR Kit (Takara) was performed to determine the copy numbers of the bacterial-specific gene *tuf* in genomic DNA obtained from the feces samples.

Metabolomics Analysis

Metabolite extraction and metabolome analysis were conducted at the Human Metabolome Technologies, Inc (Tsuruoka, Japan). Briefly, plasma samples (50 μL /animal) obtained from WT and X-SCID pigs were diluted in 450 μL of methanol containing 10 μM each of standard metabolites and centrifuged after mixing it with 500 μL of chloroform and 200 μL of distilled water. An aliquot (400 μL) of the resultant supernatant was subjected to ultrafiltration using an ultrafree-MC centrifugal filter unit. The filtrate was dried and then resuspended in 50 μL of distilled water. The rehydrated samples were analyzed by a capillary electrophoresis-time-of-flight/mass spectrometry system with fused silica capillaries (internal diameter 50 μm \times 80 cm; Agilent Technologies, Santa Clara, CA) as reported previously.³⁷

Bone Marrow Transplantation

Three X-SCID pigs (recipients), which were transplanted in our previous study¹⁴ with hematopoietic stem cells obtained from bone marrow of either WT or transgenic pigs expressing GFP (donors) by depletion of lineage positive cells, were also used in this study for analyzing lymphoid tissue development at the organ level. The ileum was harvested from the recipients 3–5 years after the BMT to histologically assess the development of PPs. Feces were also collected to measure the amounts of IgG, IgM, and IgA by ELISA and to determine the development of intestinal microflora using the metagenomics analysis described previously.

Human Study

Clinical investigations were performed in accordance with the principles of the Declaration of Helsinki. Serum and fecal samples were collected from 5 patients with X-SCID (3, 4, 11, 15, and 29 years old) who received BMT 3–8 months after birth. The concentrations of IgG, IgM, and IgA in serum and fecal samples were examined by turbidimetric immunoassays and ELISA, respectively. Fecal samples were collected using a stick-type stool collection kit and suspended in 500 μL of phosphate-buffered saline. The supernatant was collected following centrifugation to obtain soluble proteins including Igs. The concentration of total soluble proteins in the supernatant was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific), and the amounts of IgG, IgM, and IgA were determined using human IgG, IgM, and IgA ELISA kits (Abcam) according to the manufacturer's protocols. Bacterial DNA was extracted from fecal samples and subjected to a metagenomics analysis to investigate the bacterial species present in the feces of BMT-treated patients with X-SCID. Magnetic resonance imaging was performed to study the thymic structure of BMT-treated patients with X-SCID.

Statistics

Statistical analyses were performed by *t* test using Prism 7 software (GraphPad Software, San Diego, CA) in Figure 2A, 3B, and 4B; Welch's *t* test in Figure 5C and Supplementary Table 1; and the Monte Carlo method in Figure 4E.

Study Approval

All experiments using human samples collected from patients with X-SCID were conducted in accordance with the protocols approved by the Ethics Committee of Tohoku University Graduate School of Medicine. All experiments using animals were conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the Institute of Agrobiological Sciences, NARO.

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Received October 4, 2019. Revised January 26, 2020. Accepted January 27, 2020.

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Acknowledgments

The authors thank F. Ishikawa (RIKEN Center for Integrative Medical Sciences) and the technical staff (Prime Tech Ltd) for their contribution in generating X-SCID pigs. We also thank the staff (Olympus) and M. Suzuki (Tohoku University Institute for Disaster Reconstruction and Regeneration Research) for their technical support with a virtual slide system VS120 (Olympus).

Conflicts of interest

The authors disclose no conflicts.

CRedit Authorship Contributions

Tomonori Nochi, Ph.D. (Conceptualization: Lead; Data curation: Lead; Formal analysis: Lead; Funding acquisition: Lead; Investigation: Lead; Methodology: Lead; Project administration: Lead; Resources: Lead; Software: Lead; Supervision: Lead; Validation: Lead; Visualization: Lead; Writing – original draft: Lead) Shunichi Suzuki, Ph.D. (Data curation: Lead; Formal analysis: Lead; Investigation: Lead; Methodology: Lead) Shun Ito, MS (Formal

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Funding

This study was mostly supported by Rare/Intractable Disease Project of Japan from The Japan Agency for Medical Research and Development (AMED). This study was also supported in part by Grants-in-Aid for Scientific Research (A) (18H03969), Grants-in-Aid for Challenging Research (Exploratory) (17K19882), Grants-in-Aid for young scientists (A) (16H06207), and Core-to-Core Program (Advanced Research Networks) from Japan Society for the Promotion of Science, Program for Interdisciplinary Research from Frontier Research Institute for Interdisciplinary Sciences at Tohoku University, and the Grant for Joint Research Project of the Institute of Medical Science, The University of Tokyo.