RESEARCH REPORT
SWINE HEALTH

Title: The contribution of adaptive immunity to Porcine Reproductive and Respiratory Syndrome virus infection -**NPB # 13-187**

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Industry Summary:
Objectives: The purpose of our studies was to understand how adaptive immunity contributes to the susceptibility of nursery-age piglets to porcine reproductive and respiratory syndrome virus (PRRSV). Typically, adaptive immunity, comprised of T and B cells, is central to the host's ability to control and clear viral infections, through the generation of antibodies and cell-mediated immunity. However, a previous study conducted by our research group observed that pigs that were deficient in T and B cells, due to a genetic defect that led to severe combined immunodeficiency (SCID), developed less severe infections when exposed to PRRSV than normal littermates. This led us to speculate that some component of adaptive immunity was actually enhancing the ability of PRRSV to infect normal pigs. Therefore, we sought to utilize pigs with SCID in order to understand viral infection in the absence of adaptive immunity. Additionally, we proposed to selectively add back a population of T cells from normal donors in order to understand how these cells may be contributing to the susceptibility of young animals to PRRSV.
Method: We received pregnant sows from Iowa State University and, upon farrowing, screened the litters for the genetic defect and identified normal and SCID pigs. For our study that looked at acute PRRSV infection in SCID pigs, one litter was exposed to virus shortly after weaning. Serum was collected a numerous timepoints to assess levels of viral infection, and to monitor anti-viral responses. At the peak of acute infection, animals were sacrificed, and bronchoalveolar lavage fluid (BALF) and lung tissue were collected for analysis. Lung samples were subjected to immunopathological assessment to score tissue damage, while serum and BALF samples were monitored for anti-viral cytokine production.
In studies where we sought to repopulate SCID piglets with T cells, we adoptively transferred either mature T cells, or T cell precursors from normal pigs into SCID animals by intravenous injection. We collected blood samples at various timepoints to monitor whether T cells had become engrafted in the recipient pigs. At the end of the experiments, we collected blood, BALF, lymph nodes, spleen, gut, and thymus tissue to evaluate the level of T cell reconstitution. Tissue samples were subjected to immunopathological and immunohistochemical analysis for the detection of T cells. In addition, lymphocytes were isolated from these tissues, stained with antibodies, and analyzed by flow cytometry in order to determine what subsets of T cells were present in these animals.
Research Findings: As we had observed in a prior study, animals with SCID once again developed lower levels of circulating virus than their normal littermates. Pathology was also lower in many of the SCID pigs, which was surprising given their lack of adaptive immunity. Furthermore, these animals also produced lower levels of anti-viral cytokines, suggesting that the lower viremia loads were not a result of a more vigorous immune response. We also did not detect T cell-derived cytokines in serum of BALF, suggesting these mediators were not yet contributing to viral clearance, or suppressing the acute anti-viral immune response.
In reconstitution experiments, we detected the successful engraftment of T cells in SCID piglets that received T cell precursor cells, but not in those that received purified, mature T cells. These recipients developed a full

repertoire of the T cell population, including a subset known to dampen immune responses, and one that may play a role in regulating macrophage permissiveness to PRRSV. Further studies may now be performed using our T cell reconstitution procedure to evaluate what role of T cells may play during acute PRRSV infection.
These results demonstrate that the pig SCID model has tremendous potential and utility for understanding how the different facets of the immune system contribute to PRRSV infection. We have developed a critical component of the model, as T cell reconstitution studies may lead to the identification of new host targets, and the generation of more successful vaccines in the future.

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Scientific Abstract:
Porcine Reproductive and Respiratory System is an economically detesting disease, costing the pork industy millions of dollars in losses every year in the United States. Despite intensive research over the last twenty years, effective containment of the causative virus (PRRSV), and vaccination strategies against PRRSV have not yet been successfully developed. What has been established is that the virus has numerous viral evasion strategies, including the ability to subvert innate immunity, escape effective antibody responses, and may have the ability to recruit and manipulate immunosuppressive immune cells to dampen anti-viral responses. Additionally, vaccine development has been hampered by the extremely high mutation rate of the virus, resulting in ineffective long-term antibody-mediated protection.
The network of interactions between the PRRRSV and host's immune responses is complicated, and effective models to study and dissect innate immunity and/or adaptive anti-viral responses have been lacking in pigs. Many other viruses have been successfully studied in mouse models, where components of the immune system can be selectively manipulated, either through genetic manipulation (such as transgenic, and knockout strategies), adoptive transfer of immune cell subsets, or with antibody-mediated cell depletion studies.
Recently, we have described a line of pigs with a spontaneous, naturally-occurring genetic defect that give rise to animals with severe combined immunodeficiency (SCID). These animals have almost no circulating T and B cells in peripheral blood, and lack normal T and B cell zones in lymph nodes, spleen, thymus, and gut. However, natural killer cells, granulocytes, and monocyte/macrophage population are present.
We have proposed to use this novel, SCID porcine biological model to understand the interaction of PRRSV and innate and adaptive immunity. Previous work has established that during acute infection, viremia levels in SCID pigs is lower that those of normal littermates. This suggested that cells of the adaptive immune systems were either modulating macrophages to a more PRRSV-permissive phenotype, or were dampening anti-viral responses during acute infection. Therefore, we initiated a comprehensive analysis of PRRSV infection in SCID (vs control) animals by infecting animals with a GFP-expressing virus. We proposed that we would be able to quantify the number of infected porcine alveolar macrophages (PAMs) in bronchoalveolar lavages (BALF) in these animals, and determine if higher numbers of macrophages were present in samples derived from normal animals. We also endeavored to assess whether there were significant differences in the anti-viral responses between the control and SCID piglets by assessing the phenotype of macrophages, and the production of cytokines.
This study effectively reproduced the lower viremia loads observed in a previous study; however, we were unable to quantify the number of infected macrophages, as the GFP signal was not detected. Of interest, infection levels among SCID animals varied, and animals with higher viremia loads had higher levels of PRRSV-induced immunopathology and higher anti-viral cytokine responses. Nearly half of the SCID, those with lower viremia, did not have any evidence of lung pathology, and had lower levels of anti-viral cytokines. As T cell-derived cyokines were nearly undetectable in normal animals, this suggested that PRRSV-related lung pathology might be caused by cytokines produced by cells of the innate immune system. However, these observations do not rule out the possibility that T cells are still playing a role in modulating innate immunity. Additionally, PAMs derived from uninfected SCID piglets demonstrated higher levels of activation, as measured by expression level of SLA-II. This more "activated" phenotype may render PAMs more resistant to PRRSV infection. Whether this phenotype is dependent on the presence of T cells will be an area of future investigation.
In order to directly investigate the role of T cells in PAM permissiveness and acute viral infection, a pig model where T cells can be selectively reconstituted is needed. Therefore, we sought to develop a strategy where we could adoptively transfer T cells from genetically-matched donors into recipient SCID pigs in order to reconstitute the T cell population. Initially, we sought to transfer mature T cells, using fluorescent activated cell sorting and purification of CD3+ T cells. However, this strategy was unsuccessful. For our subsequent experiment, we adoptively transferred unlabelled thymocytes from SLA-matched normal donors into SCID recipients. After 28 days post-transfer, we began to measure a small level of T cell engraftment. By 49 days, at the end of the experiment, significant levels of T cells could be detected in peripheral blood, lymph nodes, spleen, and gut tissues. Furthermore, all T cell subsets, including regulatory T cells, were represented in the T cell population. Also of note, recipients did not show evidence of contaminating B cells, validating that the adoptive transfer of thymocytes was a viable methodology to specifically establish T cell reconstitution. While other groups have successfully generated B and T cell reconstitution using bone marrow from normal animals, this was the first study to ever successfully selectively engraft the T cell population. This model can now be implemented into future studies to evaluate the contribution of T cells to PRRSV infection, their role in dampening anti-viral responses, modulating macrophage permissiveness, and ultimately, determine whether this population may be a novel target for vaccine development.

Introduction:

Porcine Reproductive and Respiratory System remains a devastating disease for the pork industry, despite intense efforts over the last twenty years to combat the causative agent, PRRSV. The high evolutionary rate of the virus, along with its many immune subversion strategies has proven to be a challenge for the development of effective vaccines and anti-viral treatments. Indeed, numerous mechanisms by which the virus suppresses immune responses have been proposed, but adequate biological models have not been available to appropriately test these theories. A SCID porcine model (where animals that lack adaptive immunity) may provide a new and powerful tool to dissect viral interactions with innate or adaptive immunity, and reveal new host targets for more effective anti-PRRSV vaccine strategies.

The purpose of the studies described in this report aimed to identify the factors that contribute to the acute infection and replication of PRRSVs in the alveolar macrophages of young pigs. In a previous study, we had observed that viremia levels were actually lower in SCID piglets than their normal littermates. Therefore, we hypothesized that adaptive immunity was enhancing acute PRRSV infection and replication, either by modulating macrophage permissiveness, or by suppressing anti-viral immune responses.

Firstly, we proposed to further investigate acute PRRSV infection in SCID pigs by utilizing a GFP-expressing virus and to quantify the number of infected GFP+ macrophages. Additionally, we sought to evaluate the phenotype and the levels of cytokine production by porcine alveolar macrophages, as this may affect the degree of viral permissiveness.

Secondly, we sought to evaluate the role of T cells, particularly regulatory T cells (Tregs), during acute PRRSV infection, as these cells may skew macrophages to a more permissive phenotype. We proposed to develop a novel T cell reconstitution methodology in SCID piglets, where donor cells from normal littermates would be adoptively transferred into SCID recipients. If T cell engraftment in SCIDs could be established, we could ask the question of whether the presence of T cells, and perhaps Tregs, would return viremia levels similar to those observed in normal piglets. If T cell reconstitution resulted in higher viral replication in SCID piglets compared to untreated SCID animals, this would suggest that T cells, and perhaps Tregs, are potentially viable new targets for vaccine development.

Objectives:

Objective 1: Characterize acute PRRSV replication in the SCID pig. Preliminary results suggest that PRRSV replication during acute infection is actually reduced in nursery-aged SCID pigs compared to normal littermates. This suggests that adaptive cells, such as T cells, are involved in regulating the permissiveness of macrophages to infection. We plan to further characterize PRRSV replication in the SCID pig using GFP-labeled virus.

Objective 2: We will determine the role of regulatory T cells (Tregs) in regulating permissiveness to PRRSV infection. Tregs are a unique subset of cells that produce cytokines that, under normal circumstances, maintain immune homeostasis and prevent autoimmunity. Preliminary studies have indicated that Tregs may increase the number of permissive macrophages in young pigs, and may account for the increased viremia and enhanced clinical signs in young pigs. We will utilize the SCID pig model to determine if Tregs affect macrophage permissiveness by reconstituting SCID animals with Tregs, and comparing the resulting levels of viremia between T cell –bearing and non-reconstituted SCID controls.

Materials & Methods:

Objective 1

The aim of this experiment was to characterize acute PRRSV infection in SCID and normal piglets. We received one pregnant sow for the study; in this experiment 7 SCIDs and 2 normal piglets were utilized from this litter, as 1 other normal piglet was euthanized prior to weaning due to health issues. At day 22 post-farrow, piglets were weaned and infected with a GFP-expressing strain of PRRSV. Sera to assess viremia levels were collected on days 0, 4, 7, and 10 post infection. Piglets were necropsied at days 10 post-infection, and peripheral blood and BALF were collected; lungs, and lymph nodes were collected for pathological assessment. We attempted to enumerate the number of infected macrophages in BALF by flow cytometry, staining cells with anti-CD163 antibody and measuring levels of

GFP+CD163+ cells. Additionally, BALF was frozen and later assessed for cytokine production using a 13-plex pig cytokine kit from EMD Millipore and our Magpix Luminex multi-analyte instrumentation.

Objective 2

Experiment 1 – The aim of this experiment was to develop the T cell reconstitution model in SCID pigs. This model could then be used in future experiments to answer the question of the whether T cells enhanced the permissiveness of porcine alveolar macrophages to PRRSV infection.

Method: The study began with one pregnant sow and one litter of piglets; 3 of which we identified to be of the SCID phenotype, 5 were normal carriers. All animals were SLA-typed by Dr. Sam Ho at Life of Michigan. Two normal, SLA-matched donors were identified for adoptive transfer of T cells into SCID recipients. On day 9 and 10 post farrow, donor pigs were sacrificed and peripheral blood, lymph nodes, and spleens were harvested from these animals. The lymphocytes were isolated from tissues and cells were stained with fluorescently-conjugated anti-CD3e antibody, and a live/ dead discriminator dye. We purified viable, mature T cells using fluorescent activated cell sorting, and approximately 0.5 – 1 $x10^7$ were immediately and intravenously injected into each recipient pig. One SCID piglet did not receive an adoptive transfer, and acted as the non-reconstituted control. Three carrier pigs remained, and acted as "normal" controls. Peripheral blood was collected from piglets for the monitoring of T cells at day16 post transfer. Whole blood (100ul) was stained with anti-CD3 antibodies, red blood cells were lysed, and washed cells were analyzed by flow cytometry. We assessed the frequency of CD3+ T cells within the lymphocyte population, and also calculated the concentration of these cells per uL of whole blood. At 27 days post reconstitution, all animals were necropsied; peripheral blood and bronchoalveolar lavages (BALF) were collected, and spleen, lymph nodes, thymus, and gut ileum were submitted to pathology. Blood lymphocytes were characterized by flow cytometry, and levels of T cells and B cells were measured. Alveolar macrophages from BALF were also characterized by flow cytometry, after cells were stained with anti-SLA II, anti-CD163, and anti-CD172 antibodies. Some cells were stained with anti-CD163 and purified by cell sorting. These cells were further cultured in the presence of a stimulant, PMA, to assess cytokine production. Sections from the thymus, lymph nodes, spleen, and gut were evaluated by H&E and by immunohistochemical staining with an anti-CD3e antibody.

Experiment 2 – The aim of this experiment was to develop the T cell reconstitution model; however, thymocytes were used as source of T cells, rather than FACS purified, CD3-labeled T cells (as above). The study used one pregnant sow and her litter comprised of 13 piglets; 7 SCID and 6 normal. Once again, all piglets were SLA-typed at Life of Michigan. A total of 4 SLA-matched and gender matched SCID piglets were identified for thymocyte reconstitution. On day 11 post-farrow, thymi from matched donor piglets were collected and processed into single cell suspensions. Thymocytes, ranging from 0.5 to 8 $x10^8$ cells were injected intravenously into recipients. On days 16, 28, 35, and 49 post adoptive transfer, peripheral blood was collected from piglets and T cell reconstitution was monitored by flow cytometry, after staining whole blood samples with anti-CD3, anti-CD4, anti-CD8, and anti-γδ antibodies. At some time-points, we also stained cells with anti-FoxP3 antibody to measure the levels of Tregs. At day 49, the remaining piglets (1 normal, 1 SCID, and 3 reconstituted) were necropsied (as in Experiment 1). Peripheral blood and BALF was collected for flow cytometry analysis; thymus, spleen, lymph nodes, and ileum were collected for pathological analysis. Additionally, ileum and thymi were processed to generate single cell suspensions and assessed by flow cytometry. Alveolar macrophages were stained with antibodies, as above, to evaluate their phenotype, and cultured to assess cytokine production.

Results:

Objective 1: Characterization of acute PRRSV replication in the SCID pig.

Preliminary results suggested that PRRSV replication during acute infection was actually reduced in nursery-aged SCID pigs compared to normal littermates. This suggested that adaptive cells, such as T cells, could have been involved in regulating the permissiveness of macrophages to infection. We aimed to further characterize PRRSV replication in the SCID pig using GFP-labeled virus.

Viremia

At total of 9 piglets were infected at day 22 post-farrow with a GFP-expressing strain of PRRSV. Sera were collected at days 0, 4, 7, and 10 days post infection to monitor virema loads, using a PCR-based assay. As shown in Figure 1, viremia was detected in all animals at all time-points. Furthermore, higher average viremia loads were detected in normal animals compared to SCIDs, reproducing the results of an earlier study.

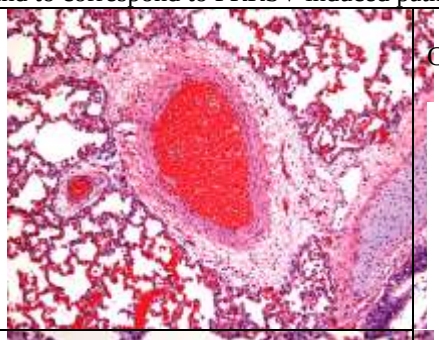
We attempted to evaluate the number of GFP+ virus-infected porcine alveolar macrophages, using flow cytometry analysis, in order to determine whether higher viral loads correlated with higher numbers of infected cells. However, we did not detect any GFP+ alveolar macrophages from bronchoalveolar lavages at day 10 of infection. Potentially, while viremia load may peak by day10 of infection, alveolar macrophage populations may already be in decline, and earlier time-points may be more appropriate to detect GFP+ infected cells, a modification that will be incorporated in future experiments.

Lung Pathology

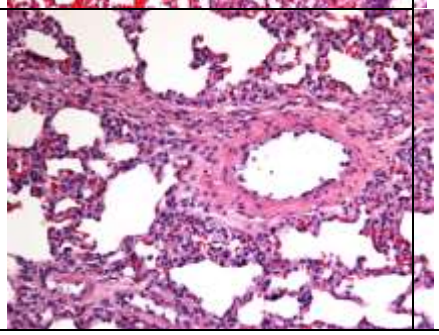
At days 10 post-infection, animals were necropsied and lung tissues were evaluated by histopathology (Fig. 1). Lung tissues derived from normal animals were characterized by infiltrates of lymphocytes and plasma cells in the alveolar spaces, moderate perivascular edema, and moderate interstitial pneumonia. However, rather surprisingly, 3/ 7 piglets with the SCID phenotype demonstrated no microscopic changes in their lung tissue. The remaining 4 SCID piglets showed some evidence of interstitial pneumonia, with mild to moderate perivascular edema. Of interest, lung pathology was more significant in animals where viremia levels were elevated, while animals with lower viremia did not generate PRRSV-related lung lesions. This suggested that either the virus was directly inducing lung damage, or that the higher viremia levels induced a more rigorous immune response that led to increased lung pathology.

Figure 1 – Acute PRRSV infection in normal and SCID piglets. A&B) Histopathology of lungs from a normal (A) and a SCID piglet (B) at day 10 post infection. Lung damage was observed in the control animals, while no abnormalities were detected in the tissues from a SCID pig. C) Levels of viremia were evaluated by PCR, where normal pigs displayed higher levels of virus compared to SCID animals. Within the SCID cohort, three animals remained free of immunopathology, while the remaining four displayed mild to moderate lung pathology – enhanced viremia levels were found to correspond to PRRSV induced pathology. Mean ± S.E.M.

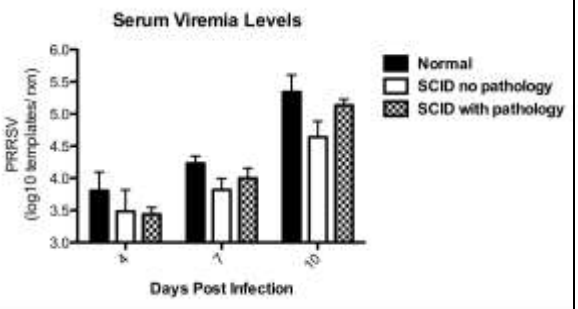
A) Normal
Evidence of PRRSV-related immunopathology, characterized by lymphocyte infiltration and perivascular edema.



B) SCID
The lung from this SCID animal showed no signs of immunopathology.



C)

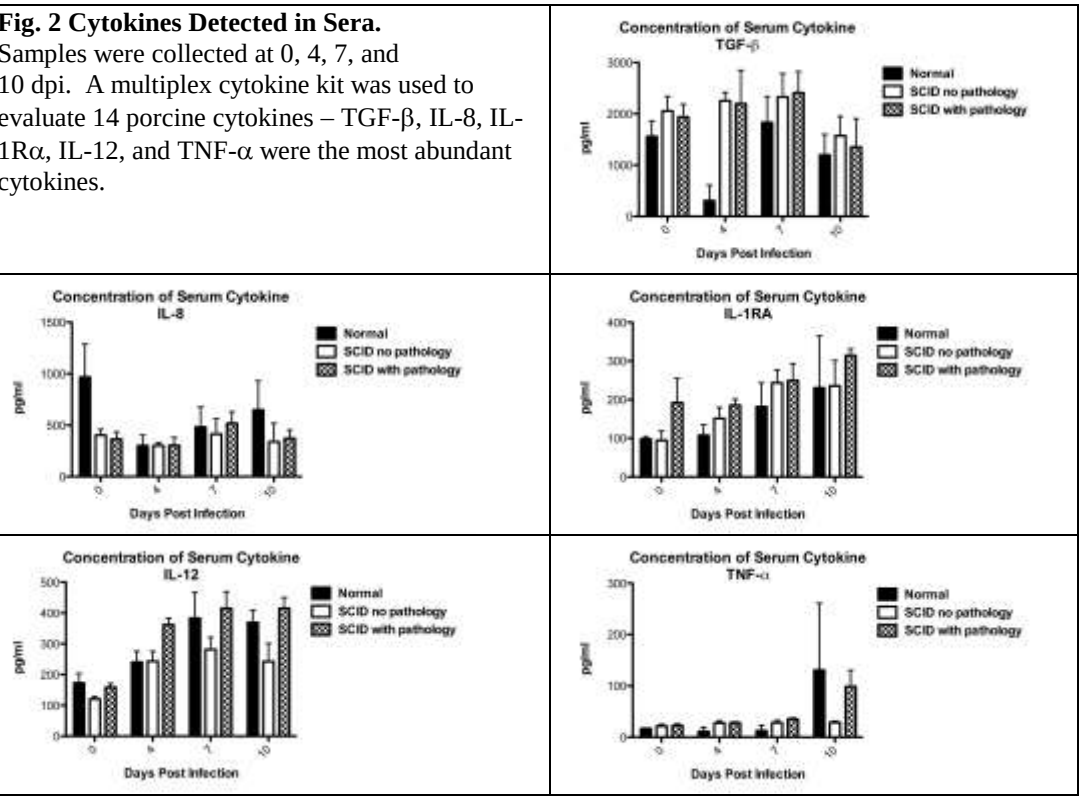


Cytokines

To ascertain the mechanism underlying the differing levels of viremia and PRRSV-related immunopathology, cytokine levels were evaluated in sera (days 0, 4, 7, and 10 post-infection) and bronchoalveolar lavages (day 10 post-infection) (Fig. 2 & 3). Prior to infection, high basal levels of TGF-β were detected in the sera of all animals, and remained high following PRRSV infection; however, this cytokine was nearly undetectable in BALF at ten days post infection. This finding suggested that anti-inflammatory TGF-β was likely not affecting viral replication, or mitigating lung damage. Other than IL-8, we did not detect any significant differences in the basal levels of serum cytokines between normal and SCID animals. However, following infection, levels of anti-viral cytokines such as IL-1RA, IL-12 and TNF-α increased, with IL-12 and TNF-α levels most closely mirroring higher viremia loads and immunopathology.

In BALF, the most prominent cytokines at day 10 post-infection were IL-1RA and IL-18. Higher levels of these cytokines were also detected in animals with higher viral loads and immunopathology. Other anti-viral and inflammatory cytokines detected at more moderate levels in BALF included IL-12, IL-1α, and IL-1β. Of interest, these cytokines were not detectable in animals with healthy lungs.

Fig. 2 Cytokines Detected in Sera.
Samples were collected at 0, 4, 7, and 10 dpi. A multiplex cytokine kit was used to evaluate 14 porcine cytokines – TGF-β, IL-8, IL-1Rα, IL-12, and TNF-α were the most abundant cytokines.



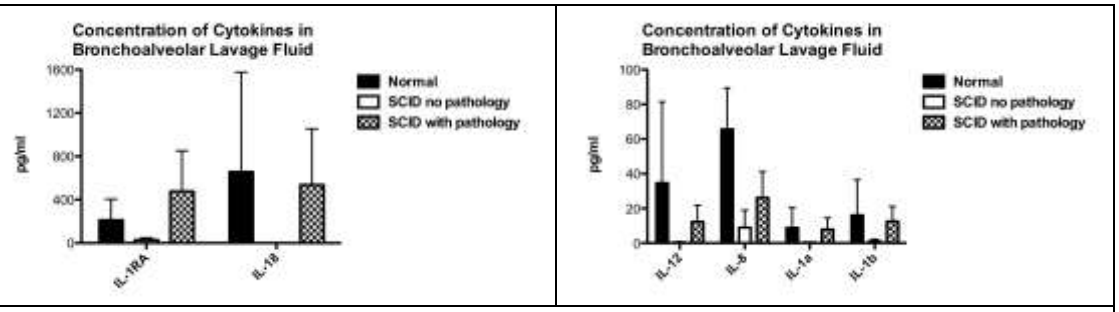
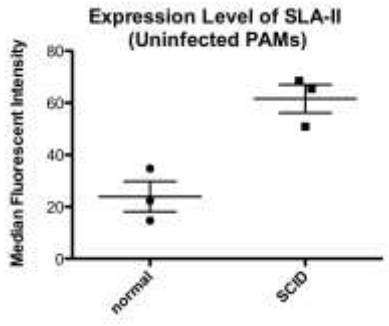


Fig. 3 Cytokines Detected in Bronchoalveolar Lavages
Lavages were collected at 10 dpi. A multiplex cytokine kit was used to evaluate 14 porcine cytokines; IL-1RA and IL-18 were the most abundant cytokines, while IL-12, IL-8, IL-1α, and IL-1β were present at moderate concentrations. Mean ± S.E.M.

The data generated in this experiment suggested that the immunopathology observed in the lungs of the normal animals and half of the SCID animals, could be related to inflammatory cytokines predominantly produced by cells of the innate immune system. Cytokines typically produced by activated T cells, such as IL-2, IL-10, IFN-γ, and IL-4, were extremely low or undetectable in both sera and BALF of normal animals. While these observations seem to rule out T cell cytokines affecting PRRSV-induced lung damage, cellular interactions between T cells and macrophages could still modulate macrophage permissiveness and produce the higher viremia loads detected in normal animals. In the future, studies using reconstituted SCIDs may provide more insight as to whether these interactions play a major role in viral replication.

Additional factors that could influence viremia, such as the number or phenotype of macrophages prior to infection, were also evaluated. As demonstrated in Figure 4, flow cytometric analysis of the phenotype of uninfected porcine alveolar macrophages detected higher levels of SLA-II, a molecule associated with macrophage activation, on the surface of cells derived from SCID animals. Thus, this may provide evidence that, in SCID

animals, activated macrophages may be more resistant to PRRSV infection. To further investigate this observation, we have recently stained additional PAMS from normal, SCID, and T cell reconstituted SCID animals, and these data will be evaluated in the near future. We have also FACS purified BALF-derived CD163+ macrophages from uninfected normal and SCID animals, and stimulated these cells in culture. We plan to assess the cytokine levels in these cryopreserved cell culture supernatants, and determine whether the profile of cytokines produced by the macrophages of SCID animals differ from those produced by cells from normal piglets. Furthermore, in studies of earlier litters, we have also observed lower concentrations of circulating monocytes in the peripheral blood of SCID animals, which could limit the source of PRRSV-permissive cells (Fig. 4B). Therefore, we plan to retrospectively analyze the monocyte populations of animals in this study, and determine if there are significant differences in the concentration of circulating monocytes, and if these levels correlate with levels of viremia.



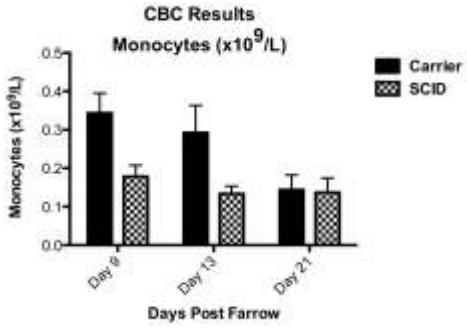


Figure 4

A) Levels of SLA-II expressed on unifected PAMs. Macrophages from normal and SCID animals were stained with anti-SLA II, anti-CD163, or isotype control antibodies. Cells with high forward and side properties were assessed for median fluorescent intensity levels of SLA-II (median fluorescent intensity of SLA II – median fluorescent intensity of isotype). Cells within this gate were >90% CD163+. Mean ± S.E.M

B) Concentration of monocytes in peripheral blood, detected by cell blood count analysis. Monocyte populations from 15 normal carriers and 13 SCIDs were evaluated at numerous time-points after birth, and expressed as cells x 10^9/ L blood.
Mean ± S.D.

Objective 2: We will determine the role of regulatory T cells (Tregs) in regulating permissiveness to PRRSV infection. Regulatory T cells are a unique subset of cells that produce cytokines that, under normal circumstances, maintain immune homeostasis and prevent autoimmunity. Preliminary studies have indicated that Tregs may increase the number of permissive macrophages in young pigs, and may account for the increased viremia and enhanced clinical signs in nursery age pigs. We proposed to utilize the SCID pig model to determine if T cells affect macrophage permissiveness by reconstituting SCID animals with T cells, and comparing the resulting levels of viremia between T cell –bearing and non-reconstituted SCID controls.

Experiment 1: T cell reconstitution using mature, FACS purified T cells

In our first reconstitution experiment, at day 10 post-farrow, we adoptively transferred up to $1x10^7$ CD3+ T cells into two recipient SCID piglets. The mature T cells were isolated from the peripheral blood, lymph nodes, and spleen of SLA-matched siblings, stained with anti-CD3 antibody, and purified using fluorescent activated cell sorting (FACS). One SCID remained untreated, and three normal piglets were kept as controls. The engraftment of T cells in peripheral blood was assessed using anti-CD3 antibody staining and flow cytometry techniques.

Results : Peripheral Blood

At day +12 post adoptive T cell transfer, an untreated SCID piglet contained 9 cells /uL of CD3+ T cells in whole blood (which constituted 11% of lymphocytes), the normal animals averaged 1632 cells/ uL (52 – 71% of lymphocytes), and animals injected with mature T cells ("reconstituted") SCIDs averaged 30 cells/ uL (8-11% of lymphocytes) (Figure 5A & B).

At day +27 post adoptive T cell transfer, the untreated SCID control contained 38 cells/ uL of CD3+ T in whole blood (which constituted 10% of lymphocytes), normal animals averaged 1830/ uL of whole blood (55 - 67 % of lymphocytes), and reconstituted SCIDs averaged 37 cells/ uL of blood (7-17% of lymphocytes).

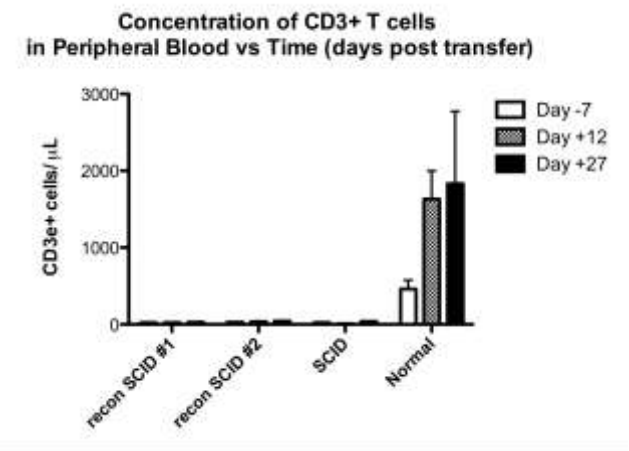


Fig 5A – Kinetics of the peripheral blood CD3+ T cell population derived from SCID, "reconstituted" SCID, and normal piglets. Cell concentrations were calculated by flow cytometric analysis. Mature T cell adoptive transfer ("reconstitution") took place at day 10 post-farrow.

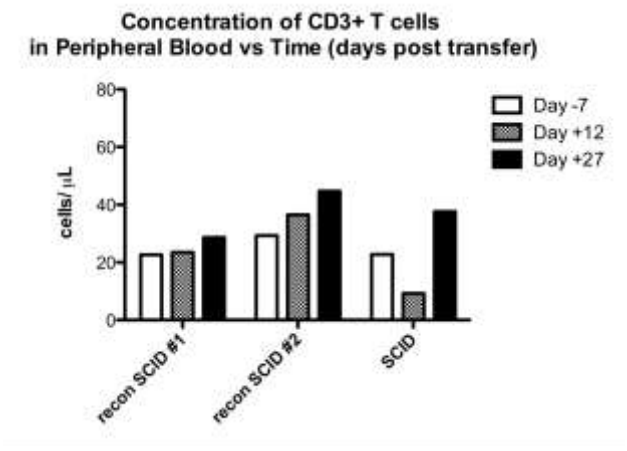
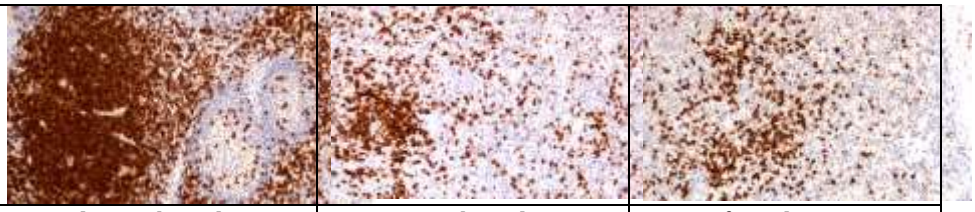


Fig 5B – Kinetics of the peripheral blood CD3+ T cell population derived from SCID, and "reconstituted" SCID piglets. Cell concentrations were calculated by flow cytometric analysis. Mature T cell adoptive transfer ("reconstitution") took place at day 10 post-farrow.

Results: Lymphoid Tissues

Immunopathological and immunohistochemical analysis further confirmed that there was very little evidence of T cell reconstitution in these treated SCID animals, when compared to control animals (Figure 6).

Figure 6
Immunohistochemistry of Lymph Nodes stained with anti-CD3e+ antibody (brown).



Normal Lymph Node. CD3+ T cells are predominantly localized to T cell follicles.	SCID Lymph Node. Only a few CD3+ cells, scattered throughout the tissue.	SCID after adoptive transfer of CD3+ T cells (day 27 post treatment). Like untreated SCIDs, only a few CD3+ cells are observed, and the cells are scattered throughout the tissue.

Summary:

We concluded from these data that a number of modifications would be implemented in a second reconstitution study. These included: 1) a larger dose of transferred cells; 2) longer engraftment time; and 3) a source of unlabelled cells, as the CD3 antibody may have triggered innate cells to attack the adoptively transferred CD3+ T cells.

Experiment 2: T cell reconstitution using Adoptive Transfer of Thymocytes

Therefore, in our second experiment, we elected to adoptively transfer up to $8x10^8$ thymocytes per recipient, as this would provide a relatively pure source of T cells and T cell precursors (no B cells were detected in these preparations). Additionally, we did not label the thymocytes with antibody, and we extended the post-adoptive transfer treatment period up to 49 days. In total, three SCIDs received adoptive transfer of thymocytes, three SCIDs did not receive treatment and served as negative controls, and one normal animal was kept as a positive control. Of note, 2/3 untreated SCIDs were euthanized prior to the end of the study due to health issues. However,

reconstituted SCIDs remained reasonably healthy throughout the course of the experiment, with only intermittent antibiotic treatments needed to treat minor bacterial infections.

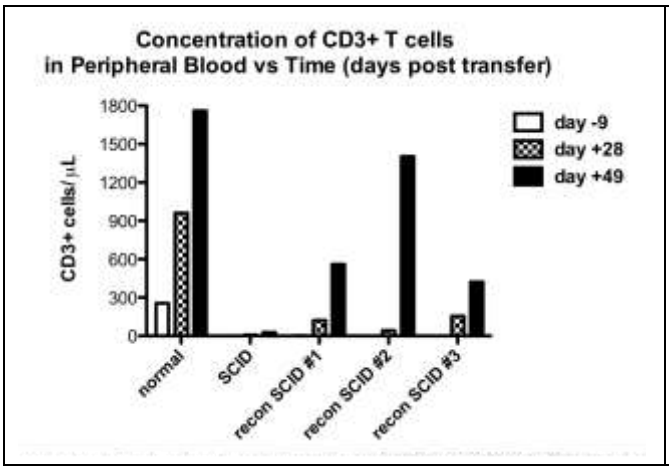
Result: Peripheral Blood

At 16 days post adoptive transfer, untreated SCIDs contained 3 cells/ uL of CD3+ T cells in whole blood (0.15 – 3.8% of lymphocytes), the normal control contained 1041 cells/ uL blood (58% of lymphocytes), and treated SCID animals averaged 29 cells/ uL (8.44 – 27.44% of lymphocytes) (Figure 7A).

By day 49 post adoptive transfer, the remaining untreated SCID animal contained 27 cells/ uL of CD3+ T cells in blood, the normal animal contained 1756 cells/ uL (72% of lymphocytes), and treated SCID animals averaged 796 cells/ uL (59.36- 68.95% of lymphocytes).

Using multi-parameter flow cytometry, we were able to further characterize the peripheral blood CD3+ T cell populations in our animals. As demonstrated in Figure 7B, a relatively similar frequency of CD8+ single positive (SP) cells were observed in normal, SCID, and treated SCID groups, ranging from 27 – 52% of CD3+ cells in treated SCID, 38% in untreated SCID (although very few CD3 cells were detected), and 33% in normal animals. The CD4+ single population (SP) was similar in normal (11% of CD3+ T cells) and SCID (11.4%) animals, but was depressed in reconstituted SCIDs (1.9 – 7.0%). Of interest, CD4+CD8+ double positive T cells were strikingly higher in SCID (46% of CD3+ cells) and reconstituted SCIDs (38.5 – 70.8%) when compared to the normal control (15.6%). These cells are generally regarded as activated, or memory T helper cells in normal animals; however, no signs of graft versus host were observed in reconstituted animals. Likely, this phenotype is a consequence of the lymphopenic environment, as adoptively transferred naive T cells in murine SCID animals will typically display an activated phenotype until T cell homeostasis is achieved. Also of note, the CD4-CD8- double T cell population was greatly reduced in both SCID (4.1% of CD3+ cells) and treated SCID (0.11 – 2.4%) animals, compared to the normal control (41.8%). These constitute a population of γδ T cells, a TCR1+CD8- subset (41.5% of CD3+ cells) that are generated from fetal liver *in utero* (Figure 7C). An alternative γδ T cell population, characterized by TCR1+CD8+ staining was only slightly altered in SCID (18.3% of CD3+ cells) and reconstituted SCIDs (0.9 – 11.5%) when compared to normal animals (10.0%).

We also assessed the level of regulatory T cells (CD3+CD4+CD25+FoxP3+) cells in our animals, as this may be a population that modulates macrophage permissiveness to PRRSV infection. As demonstrated in Figure 7D, Tregs (FoxP3+CD25hi) constituted 2.4% of CD4+CD3+ T cells in normal animals. In SCID animals, adoptive transfer of thymocytes gave rise to a lower, but detectable Treg population (0.8 – 1.9% of CD4+CD3+) in peripheral blood. The development of T cell subsets, especially Tregs, following adoptive T cell transfer into SCID recipients is critical to establishing this reconstitution model as a means to evaluate the role of T cells and Tregs during PRRSV infection.



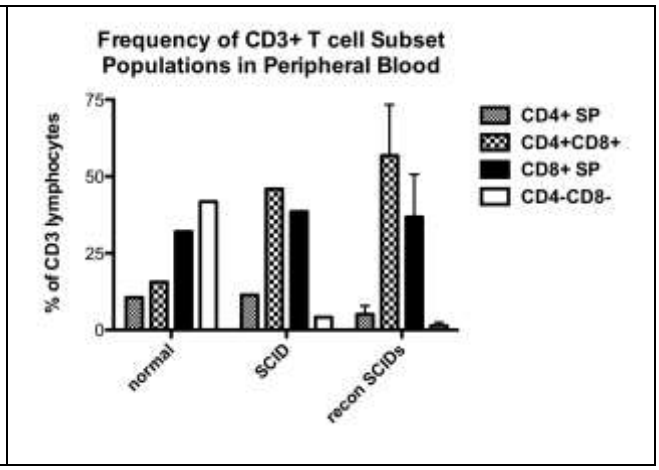
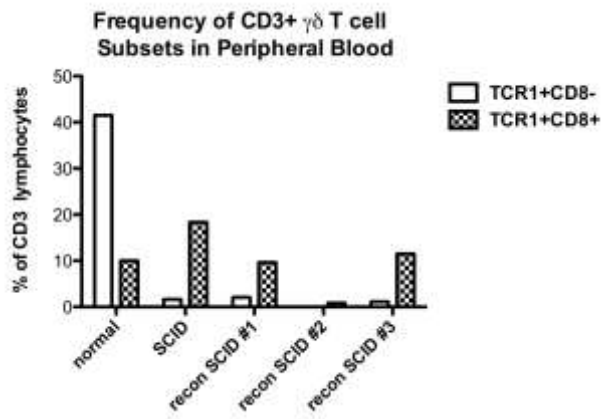
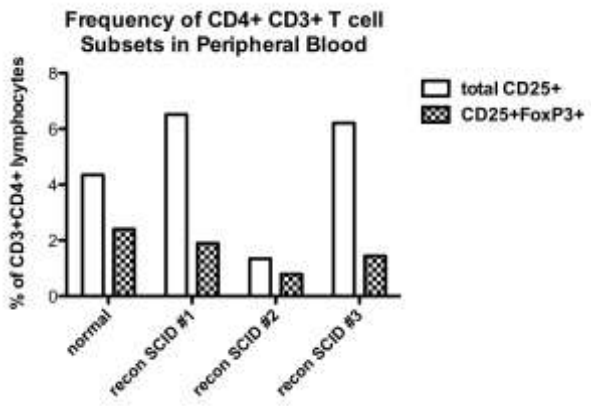


Figure 7

A) Concentration of CD3+ T cells detected in peripheral blood following adoptive thymocyte transfer. Detectable levels of T cell reconstitution began appearing at day 28 post-transfer, and steadily increased until necropsy, at day 49.

B) Frequency of T cell subsets detected within the CD3+ lymphocyte population in peripheral blood. Cells were stained with anti-CD3, anti-CD8a, and anti-CD4 antibodies, then analyzed by flow cytometry. Mean ± S.E.M





C) Frequency of γδT cell subsets detected within the CD3+ lymphocyte population in peripheral blood. Cells were stained with anti-CD3, anti-γδ (TCR1), and anti-CD8a antibodies, then analyzed by flow cytometry.

D) Frequency of Treg cell subsets detected within the CD3+CD4+ lymphocyte population in peripheral blood. Cells were stained with anti-CD3, anti-CD4, and anti-CD25 antibodies, then fixed and permeabilized to allow staining of intracellular FoxP3, then analyzed by flow cytometry.

We also assessed levels of CD21+ cells, and determined that our reconstituted animals did not show any significant evidence of B cell engraftment. This was important, as it demonstrated that the thymocyte adoptive transfer did not contain B cell contaminants that could compromise T cell reconstitution (data not shown).

Results: Assessment of Lymphoid Tissues by Immunohistochemistry

Tissues were collected from normal, SCID, and thymocyte-treated SCID animals at day 49 post adoptive transfer, and submitted for pathological assessment. As demonstrated in Figure 8, elevated levels of tissue-resident CD3+ cells were detected by immunohistochemistry in reconstituted animals, when compared to untreated SCID piglets. While the level of T cells observed in thymocyte-treated SCIDs were somewhat lower from the normal control animal, this data clearly established the engraftment of CD3+ cells in a number of lymphoid tissues, and corroborated the flow cytometric data generated from peripheral blood.

Analysis of Gut Tissue Flow by Cytometry Analysis

Furthermore, we isolated lymphocytes from the ileum gut tissue, and analyzed the cells by flow cytometry. As demonstrated in Tables 1 & 2, the number of CD3+ T cells recovered from reconstituted SCIDs, while lower than the control, yielded much higher numbers than untreated SCIDs. All T cell subsets were represented in reconstituted SCIDs, including CD4+, CD8+, and γδ T cell populations, as well as a portion of CD4+CD8+ double positive cells.

Thymic Tissue

The analysis of thymic tissues also revealed detectable changes in the thymocyte populations of reconstituted SCID animals, when compared to the untreated SCID control (Table 3). In SCID animals, most thymocytes become arrested at an early stage of T cell development, prior to successful TCR rearrangements. These immature cells are characterized by a CD44 low, CD3-, TCR1-, CD1+, and CD4-CD8- double negative phenotype. In contrast, thymocyte reconstituted SCID animals showed elevated frequencies of CD3+ and TCR1+ cells, and a reduction of CD44low, CD1+, and CD4-CD8- double negative cells. Additionally, H&E staining of thymic tissue demonstrated sporadic foci of thymocytes in treated SCID, similar to cortical structures found in normal tissues. This may be evidence of the engraftment of functional thymocyte precursors, which may lead to long-term T cell reconstitution in treated piglets.

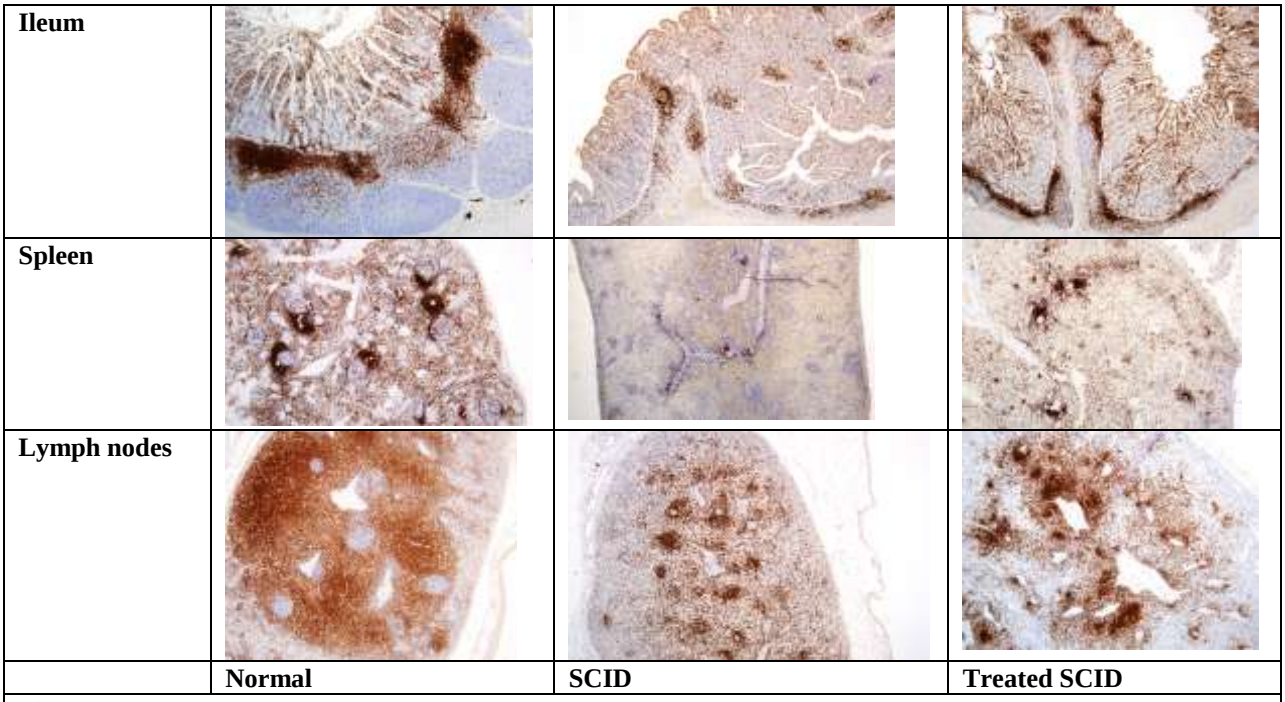
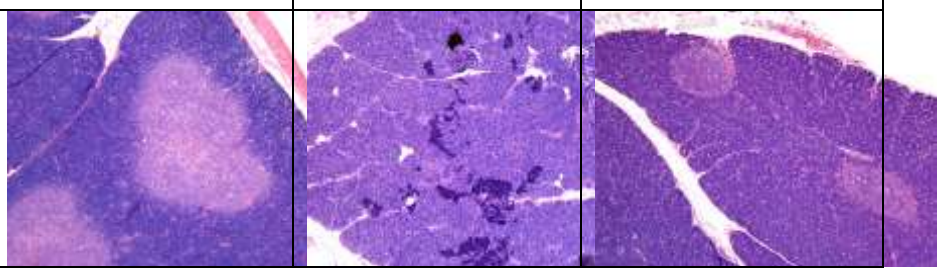


Figure 8
Immunohistochemistry of lymphoid tissues stained with anti-CD3 antibody from normal, SCID, and T cell reconstituted SCID animals.
(Top) Ileum, derived from normal animals, was characterized by T cells distributed throughout the layer of epithelial villi, and in foci at the luminal side of the lamina propria. Only small numbers of CD3+ cells were observed throughout the epithelial and lamina propria layers of SCIDs. Following adoptive thymocyte transfer, increased numbers of CD3+ cells were detected throughout the epithelial layer, and concentrated pockets were developing at the lamina propria junction.
(Middle) Spleen tissue, derived from normal animals, was characterized by a predominant staining of T cells within defined follicles. However, in SCIDs very few CD3+ T cells were observed. In contrast, anti-CD3 antibody staining of spleens derived from treated SCIDs was observed to have small foci forming, despite a lack of B cell follicle architecture.
(Bottom) Lymph nodes showed similar results to the spleen, as CD3+ staining of reconstituted SCID tissues demonstrated the formation of more organized, concentrated centers of T cells, despite the lack of organized B cell regions.

TABLE 1 – Mean Number of Intraepithelial Lymphocytes Recovered from Ileum Tissue			
Marker (% of CD45 cells)	Normal (N=1)	SCID (N=1)	Recon SCID (N=3)
CD3+	601, 729	1847	144, 416
CD4+	306, 904	767	89, 631
CD8+	274, 173	1049	46, 020
TCR1+ (γδ T cells)	29, 816	401	4, 143

TABLE 2 – Mean Number of Lymphocytes Recovered from Lamina Propria Ileum Tissue			
Marker (% of CD45 cells)	Normal (N=1)	SCID (N=1)	Recon SCID (N=3)
CD3+	3, 557, 227	n.d.	741, 471
CD4+	2, 072, 114	1640	521, 754
CD8+	1, 631, 627	1861	369, 084
TCR1+ (γδ T cells)	130, 322	1036	67, 613

Table 3 - Analysis of Thymic Tissues			
Phenotype (% of thymocytes)	**Normal**	**SCID**	**Recon SCID (frequency range)**
CD3+ (cells with TCR rearrangement)	10.33%	0.68%	1.82 – 2.41%
TCR1+ (γδ cells with TCR rearrangement)	3.95%	0.16%	0.59 – 1.88%
CD4-CD8- (immature thymocytes)	14.57%	81.44%	35.58 – 81.99%
CD44^{low} (immature thymocytes)	52.82%	96.26%	86.16 - 89.89%
CD1+ (% of TCR1+ cells)	22.13%	84.51%	9.45 – 25.75%
H & E stain			

Summary:

We have concluded from the data generated in our second reconstitution experiment:

1) adoptive transfer of SLA- and gender-matched thymocytes from normal littermate is a viable method for clean T cell reconstitution of SCID piglets, without evidence of B cell contamination.
2) donor thymocyte population matured into various peripheral blood T cell subsets, including CD4+, CD8+, γδ (TCR1+), and Treg populations
3) donor thymocytes matured and localized into lymphoid tissues, such as lymph nodes, spleen, and gut.

Another thymocyte reconstitution experiment, with a PRRSV challenge, was planned in parallel with the experiment described above. However, only one of the two sows became pregnant, and so we plan to do this experiment in the future.

Discussion:

This project sought to further investigate the role of adaptive immunity during the course of PRRSV infection, and determine whether T cells contribute to acute PRRSV infection and replication. For our first experiment, we aimed to further characterize PRRSV infection in piglets with severe combined immunodeficiency (SCID), animals that lack adaptive immunity (ie – no T and B cells). We were able to reproduce results from an earlier experiment, where lower viremia loads were detected in SCID piglets compared to SCID littermates. Immunopathology in lung tissue was also lower in SCID animals, a finding that was rather unexpected considering these animals are immune deficient. Higher levels of pro-inflammatory cytokines were present in the bronchoalveolar lavage fluid of infected, normal animals, which may contribute to the lung damage we observed. What still remains unknown is why viremia levels are higher in normal pigs, despite a more robust anti-viral immune response. Of note, alveolar macrophages derived from healthy, uninfected SCID pigs displayed a higher level of SLA-II, a molecule whose expression is up-regulated upon activation. We speculate that the higher level of "basal activation" in PAMs from SCID pigs may render them more resistant to PRRSV infection. We plan to further investigate this finding by measuring cytokine production in frozen BALF samples of uninfected, healthy normal and SCID animals. Additionally, we have cryopreserved PAMs from these animals, and additional markers may be analyzed in the future.

One explanation of the differing susceptibilities of SCIDs to PRRSV is the presence of T cells. A subset of cells, known as regulatory T cells (Tregs), are a small but potent population of cells that function to maintain immune homeostasis and prevent autoimmunity. We hypothesized that these cells may be down-regulating or skewing alveolar macrophages to a more permissive phenotype for PRRSV infection. In order to study the effects of T cells and PRRSV permissiveness, we sought to develop a novel biological model to investigate this relationship. In this

proposal, we have established a viable methodology to adoptively transfer thymocytes from normal donors into genetically-matched SCID recipients. Immunohistochemical and flow cytometry-based techniques established that we attained successful engraftment of all T cell subsets, including regulatory T cells. Therefore, we have developed a promising and exciting new model to directly determine whether macrophage permissiveness is due to their interactions with T cells. We have collected data on the phenotype of PAMs from these reconstituted SCID, as well as BALF samples that we plan to analyze in the near future. This data may provide evidence that T cells, and the activation or suppression of macrophages, are potential new targets for more effective vaccines in the future.