

METHODS Methods

~~Animal Procedures~~ ~~P. gunnii~~

All bandicoots ~~were~~ collected by Zoos Victoria staff on an opportunistic basis ~~and with~~ approval by the Zoos Victoria Animal Ethics Committee. Up to 2 mL of whole blood was taken from the femoral vein of each bandicoot during routine procedures and health checks ~~and~~. It was then stored in 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8 (EDTA-50 µL per mL of blood) tubes at 4°C until collected for processing.

~~Animal Procedures~~ ~~SCID Mice~~

All experiments involving severe combined immunodeficiency (SCID) mice were approved by ~~The~~ University of Melbourne's Animal Ethics Committee (Ethics ID: 1212578.1).

NOD-SCID.IL2ry mice were obtained from the Walter and Eliza Hall Institute of Medical Research (WEHI) and housed in the small animal facility of the Faculty of Veterinary Science at the University of Melbourne ~~small animal facility~~. ~~Mice~~ The mice were housed in filter boxes in compliance with government guidelines and were provided with standard rodent feed and water as required.

~~Isolation of P. gunnii~~ ~~WBCs~~ ~~white blood cells (wbc)~~

~~The~~ bandicoot blood was transferred from the collection tubes into 15 mL Falcon tubes (BD Biosciences). ~~The~~ ~~s~~ Samples were washed ~~three~~ times with 10 mL wash buffer (phosphate buffered saline [~~P~~B~~S~~] + 2% fetal calf serum [~~F~~C~~S~~]) via centrifugation (600 ~~relative centrifugal force~~ [RCF] ~~ref~~, 5 minutes at 4°C). ~~The~~ ~~s~~ Supernatant was discarded and ~~the~~ samples were resuspended in 2 mL PBS (Scientific Services, Murdoch Childrens Research Institute [~~M~~C~~R~~I]).

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Cell separation was performed using a 70/30% percoll pH 8.5–9.5 gradient (Sigma) by centrifugation (600 ~~ref~~RCF, 10 minutes, room temperature [~~RT~~]). ~~Wbc~~White blood cell (WBC) interphase was isolated, transferred to new clean ~~new~~ 15 mL Falcon tubes and washed ~~three~~3 times with 5 mL PBS and centrifuged (600 ~~RCF~~ref, 5 minutes, 4°C).

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~~The Wbc~~WBCs were suspended in 1 mL PBS ~~and~~, counted on a hemocytometer with Trypan blue stain (Sigma) and washed twice with sterile saline via centrifugation (600 ~~ref~~RCF, 5 minutes, 4°C).

~~Intraperitoneal~~IP (IP) Injection of *P. gunnii* ~~WwBbCse~~

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The

~~Wbc~~WBCs were suspended in a ~~500 µL~~ total volume of 500 µL sterile saline (in accordance with the University of Melbourne's ~~Animal Ethics Committee guidelines~~ ~~sterile saline~~. ~~Control~~The control mice were intraperitoneal (IP) injected with 500 µL of sterile saline only. IP injection was ~~made in at~~ the lower ~~right~~ section of the abdomen using a 25 ~~gauge~~ needle.

Euthanasia and ~~O~~rgan/~~C~~ell ~~C~~ollection

~~The m~~Mice were euthanised by CO₂ asphyxiation. Blood was collected into EDTA (50 µL per 1 mL sample) tubes by cardiac puncture. Peritoneal cells were collected by peritoneal wash with 10 mL cold wash buffer. Spleens, livers, kidneys and lungs were collected for DNA extraction and analysis.

~~The Wbc~~WBCs were separated from ~~the~~ blood and stored in Phenol (Amresco®) at ~~-~~20°C.

Peritoneal cells were collected via centrifugation (600 ~~ref~~RCF, 5 minutes, 4°C) and stored in Phenol at ~~-~~20°C.

Livers, kidneys and lungs were weighed, photographed and snap-frozen at ~~-~~80°C.

2.3 Splenocyte Isolation

The spleens were placed in a petri dish in wash buffer and ground between two frosted slides until homogenised. Disrupted spleens were then transferred into a 15 mL Falcon tube and the cells were collected via centrifugation (600 RCF, 5 minutes, 4°C). The supernatant was discarded. The cells were resuspended in the remaining liquid and red blood cell lysis was performed by adding 9 mL sterile water per tube for 10 seconds, then adding 1000 µL of 10x PBS (Scientific Services, MCRI). The tube was inverted several times and the cells were collected via centrifugation (600 RCF, 5 minutes, 4°C). The cells were counted using hemocytometer and Trypan blue stain (1:2 dilution) and stored in Phenol at 20°C.

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3 DISCUSSION

SCID mice have been used as animal models for many years, including as models for placental mammals such as humans and cetaceans [40–43]. To date, a successful marsupial-SCID mouse model has not been documented. The Eastern Barred Bandicoot (EBB) would benefit greatly from the production of such a model given its endangered status and susceptibility to *Toxoplasma gondii* (*T.gondii*).

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This study aimed to create a marsupial-SCID mouse, by first developing tools for detection of bandicoot cells within SCID mice.

3.1 Developing Tools for Detecting Bandicoot Cells within Marsupial-SCID Mice

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Available antibodies were tested using flow cytometry against bandicoot cells for cross reactivity. All antibodies tested were not cross reactive with bandicoot, and therefore not of use in this study. As marsupial-specific reagents and tools are difficult to source [1, 29, 30], tools were created to determine the presence of bandicoot cells in SCID mice.

As genomic PCR primers for bandicoot are not commercially available, these were designed. Three genes (TRIM3, BRCA1 and PRKCA) were examined for their suitability as genomic primers for bandicoot detection. At completion of temperature gradient analysis, TRIM3 was chosen as the genomic primer for use in this study, with an optimal PCR temperature of 62°C. In addition, it was ascertained that TRIM3 is not SCID mouse cross reactive. This primer set was used for the remainder of the study along with the mouse house-keeping primer set, GAPDH. The TRIM3 primer set created in this study contributes a valuable tool to the small amount-number of marsupial reagents available for the study and analysis of marsupial immune systems.

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The sensitivity of TRIM3 was not experimentally quantified due to sample and time constraints. For further studies, an experiment to measure the ability of TRIM3 to detect bandicoot at varying concentrations in SCID mice is suggested. By "spiking" SCID mouse cells with serially diluted bandicoot cells and testing DNA purified from these with the TRIM3 primer, the experimental sensitivity of the assay could be determined. For example, SCID mouse cells would be "spiked" with 10%, 1%, 0.1%, 0.01% and 0.001% bandicoot cells. After PCR amplification with TRIM3, results would be visualised with gel electrophoresis. If bands were visible for all percentages except at 0.001%, it could be said in future experiments with visible bands that bandicoot is present at 0.01% or higher. Likewise, if bandicoot was not detected, it could be said that bandicoot was not detected and if it is present in the SCID mouse, was present at a concentration below 0.01%.

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Generating Marsupial-SCID Mice

mice

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With the documentation of successful SCID mouse models using human, cetacean, rat and dog [35, 39–43], this study aimed to create marsupial-SCID mice. SCID mice were injected with EBB ~~wbe~~-WBCs and sampled at various time points.

Previous studies reviewed have sampled at a variety of time points after injection, ranging from 30 minutes to 13 weeks [40–42]. Time points for successful reconstitution were also varied, however but did show a trend towards greater reconstitution between 5 and 8 weeks. For this study, time points for collection after IP injection were set between 2 weeks and 8+

weeks. Collection and data analysis ~~were~~ limited by bandicoot sample availability and, as a result, final sample time points were at 14, 51 and 63 days after IP injection.

At ~~time point~~ 14 days after IP injection, bandicoot DNA was successfully detected in the ~~w~~~~b~~~~e~~~~W~~~~B~~~~C~~~~s~~ of ~~two~~2 of the ~~four~~4 experimental mice. No bandicoot DNA was detected in any of the experimental mice spleen or peritoneal wash samples. It is noted that the positive control of bandicoot DNA ~~positive control~~ for the spleen and peritoneal samples did not show a visible band. Upon examination, this was due to incorrect DNA extraction of the bandicoot sample used for that particular positive control. This positive control was discarded and another was used for the remainder of the study, where all bandicoot positive controls showed clear bands with TRIM3. However, as ~~the~~ positive control failed in this instance (and due to the lack of a sample, the PCR could not be repeated), no final conclusions can be made regarding the presence of bandicoot cells in the peritoneal wash and spleen 14 days after IP injection.

At collection time points of 51 and 63 days after IP injection, bandicoot DNA was not detected in any of the experimental mouse ~~w~~~~b~~~~e~~~~W~~~~B~~~~C~~, spleen or peritoneal wash samples.

At all time points, in particular 51 and 63 days, variation in ~~the organ sizes of organs were~~ was seen. The most prominent was the diminished size of experimental mouse spleens compared with the controls. ~~Due~~Owing ~~to~~ ~~the~~ ~~risk~~ ~~of~~ ~~graft versus host disease~~ (GvHD) ~~in~~ ~~in~~ ~~chimeric~~ ~~SCID~~ ~~mice~~ [28], ~~experimental~~ ~~mice~~ were checked daily and appeared in good health. At all time points, organs were removed and assessed. Although spleens in the later time points did show a reduction in size compared ~~with~~ the controls, they did not exhibit physical signs suggestive of GvHD. Previous studies [35, 43] showed an increase in spleen size (splenomegaly) and necrosis due to GvHD after cell engraftment. This is inconsistent with the findings of this study. It is concluded that GvHD was ~~not unlikely to~~ be the cause of the reduced size and cell count of experimental spleens, although it is unclear why this reduction occurred. It is suggested that the general health and organs of all experimental mice be closely monitored and assessed for signs ~~suggesting of~~ GvHD in any further study involving SCID chimeras.

The possibility of long-term reconstitution and host-cell integration into the donor ~~mouse~~ system ~~were~~ was not examined in this pilot study. Further marsupial-SCID studies should examine ~~t~~he role of growth factors and cytokines in immune system development and

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integration between graft and host ~~need to be examined in further marsupial SCID studies.~~ Cytokines are synthesised by immune cells (and sometimes other cells) and have a key role in interactions between the immune system and other organs ~~—~~ [44]. Limitations in immune integration in chimeric models have been documented, and are thought to occur due to cytokine and growth ~~—~~factor differences between host and graft [38, 45]. These differences can lead to the diminished ability of mouse cytokines to bind and interact with donor *P. gunnii* cytokine receptors. It can also lead to the opposite; ~~;~~ that is, the diminished ability of donor *P. gunnii* cytokines to bind and interact with host ~~—~~mouse cytokine ~~receptors~~receptors. Communication may be interrupted one way (host cytokines cannot bind to donor receptors) or both ways (host cytokines cannot bind to donor receptors and donor cytokines cannot bind to host receptors) [44]. The possible lack of cross reactivity and communication in cytokines between SCID host and marsupial graft could lead to diminished efficacy in long-term immune integration and reconstitution.-

As bandicoot samples were only received opportunistically from Zoos Victoria, the irregular nature and ~~a~~ lack of these samples led to limitations throughout this study. A planned mixed lymphocyte reaction (MLR) to observe and characterise interactions between SCID mouse and bandicoot cells was not completed; time ~~—~~points were limited; and experimental sample sizes s for each time ~~point—point~~ ~~was—were~~ quite limited. Limited sample sizes restricted any meaningful statistical analysis of data to the composite data for spleen size and splenocyte count only. A maximum of 2 mL blood was set for bandicoot samples s due to the animal's size, and ~~so~~ experiments were planned with an expectation of obtaining such values. However, ~~in reality, while~~ Melbourne Zoo ~~was while~~ extremely generous, it was unable to provide 2 mLs on all occasions and sample volumes were typically between 0.5 mL and 1 mL of whole blood. This limited the ~~amount—number~~ of *P. gunnii* ~~wbe~~ WBCs available to inject per mouse. *P. gunnii* samples could not be ~~“pooled”~~ due to the high risk of graft-versus-graft disease. ~~This would be likely; if mixing and injecting the immune cells of two~~ ~~or more animals are mixed together, as~~ immune responses between the cells of ~~different bandicoots would be~~ highly probable. Therefore, ~~the samples was—were~~ injected ~~on a into~~ ~~mice~~ one bandicoot per mouse basis.

In this study, *P. gunnii* ~~wbe~~ WBC amounts injected into SCID ~~—~~ mice

ranged from 0.8×10^6 ~~—~~ 9.0×10^6 ~~—~~ with an average of 3.2×10^6 . Previous studies ~~iesy~~ showed eds that ~~—~~ the

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higher the number of engrafted cells, the more successful the engraftment [40], with injected cell amounts ranging from $10^{-60} \times 10^6$ [40, 42], ~~and to~~ as high as $4^{-10} \times 10^7$ [41, 43]. ~~With As~~ all amounts injected in this study ~~being were~~ lower than those amounts ~~that~~ ~~resulted in~~ successful engraftment in the literature, it is not ~~entirely~~ surprising that a ~~greater frequency higher amount of successful~~ engraftment was not seen. The ~~two~~ mice that did show successful engraftment of *P. gunnii* cells 14 days ~~post~~ IP injection received 2.5×10^6 and 1.6×10^6 cells, respectively. However, the ~~two~~ mice in

the same 14-day post-IP injection cohort that did not successfully engraft ~~received~~ comparable amounts: 0.8×10^6 and 2.1×10^6 cells, respectively. Therefore, ~~in further studies~~ ~~should investigate further~~ the effects of both injected cell numbers and the amount of time post-injection ~~must be investigated further~~.

It is ~~further also~~ suggested that ~~for any further marsupial SCID chimeric study~~, a less endangered and perhaps larger animal be used for investigating the feasibility of the model ~~in future marsupial-SCID chimeric studies~~. This would remove the limitations of ~~the low sample availability of samples and cell counts for IP injection~~. With access to more readily available marsupial samples, further time ~~points~~ (mid-way between 14 and 51 ~~days~~ is suggested) and larger sample sizes per experiment ~~could~~ be included. ~~This would increase the likelihood of meaningful statistical analyses would be possible~~, and ~~the adequate assessment of~~ the success of the marsupial-SCID chimera ~~more adequately assessed~~. ~~However, despite this study's~~ several limitations, the presence of bandicoot DNA detected in the ~~wbc~~ WBCs of ~~two~~ out of ~~four~~ experimental mice at the 14-day time ~~point~~ is encouraging ~~.~~

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Methods

Animal Procedures—*P. gunnii*

All bandicoots were collected by Zoos Victoria staff on an opportunistic basis with approval by the Zoos Victoria Animal Ethics Committee. Up to 2 mL of whole blood was taken from the femoral vein of each bandicoot during routine procedures and health checks. It was then stored in 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8 (50 µL per mL of blood) tubes at 4°C until collected for processing.

Animal Procedures—SCID Mice

All experiments involving severe combined immunodeficiency (SCID) mice were approved by the University of Melbourne's Animal Ethics Committee (Ethics ID: 1212578.1). NOD-SCID.IL2ry mice were obtained from the Walter and Eliza Hall Institute of Medical Research (WEHI) and housed in the small animal facility of the Faculty of Veterinary Science at the University of Melbourne. The mice were housed in filter boxes in compliance with government guidelines and were provided with standard rodent feed and water as required.

Isolation of *P. gunnii* WBCs

The bandicoot blood was transferred from the collection tubes into 15 mL Falcon tubes (BD Biosciences). The samples were washed three times with 10 mL wash buffer (phosphate buffered saline [PBS] + 2% fetal calf serum [FCS]) via centrifugation (600 relative centrifugal force [RCF], 5 minutes at 4°C). The supernatant was discarded and the samples were resuspended in 2 mL PBS (Scientific Services, Murdoch Childrens Research Institute [MCRI]).

Cell separation was performed using a 70/30% percoll pH 8.5–9.5 gradient (Sigma) by centrifugation (600 RCF, 10 minutes, room temperature [RT]). White blood cell (WBC) interphase was isolated, transferred to new clean 15 mL Falcon tubes and washed three times with 5 mL PBS and centrifuged (600 RCF, 5 minutes, 4°C).

The WBCs were suspended in 1 mL PBS, counted on a hemocytometer with Trypan blue stain (Sigma) and washed twice with sterile saline via centrifugation (600 RCF, 5 minutes, 4°C).

IP Injection of *P. gunnii* WBCs

The WBCs were suspended in a total volume of 500 μ L sterile saline (in accordance with the University of Melbourne's Animal Ethics Committee guidelines). The control mice were intraperitoneal (IP) injected with 500 μ L of sterile saline only. IP injection was at the lower-right section of the abdomen using a 25-gauge needle.

Euthanasia and Organ/Cell Collection

The mice were euthanised by CO₂ asphyxiation. Blood was collected into EDTA (50 μ L per 1 mL sample) tubes by cardiac puncture. Peritoneal cells were collected by peritoneal wash with 10 mL cold wash buffer. Spleens, livers, kidneys and lungs were collected for DNA extraction and analysis.

The WBCs were separated from the blood and stored in Phenol (Amresco®) at -20°C . Peritoneal cells were collected via centrifugation (600 RCF, 5 minutes, 4°C) and stored in Phenol at -20°C . Livers, kidneys and lungs were weighed, photographed and snap-frozen at -80°C .

Splenocyte Isolation

The spleens were placed in a petri dish in wash buffer and ground between two frosted slides until homogenised. Disrupted spleens were then transferred into a 15 mL Falcon tube and the cells were collected via centrifugation (600 RCF, 5 minutes, 4°C). The supernatant was discarded. The cells were resuspended in the remaining liquid and red blood cell lysis was performed by adding 9 mL sterile water per tube for 10 seconds, then adding 1000 μ L of 10x PBS (Scientific Services, MCRI). The tube was inverted several times and the cells were collected via centrifugation (600 RCF, 5 minutes, 4°C). The cells were counted using hemocytometer and Trypan blue stain (1:2 dilution) and stored in Phenol at -20°C .

Discussion

SCID mice have been used as animal models for many years, including as models for placental mammals such as humans and cetaceans [40–43]. To date, a successful marsupial-SCID mouse model has not been documented. The Eastern Barred Bandicoot (EBB) would benefit greatly from the production of such a model given its endangered status and susceptibility to *Toxoplasma gondii* (*T.gondii*).

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The sensitivity of TRIM3 was not experimentally quantified due to sample and time constraints. For further studies, an experiment to measure the ability of TRIM3 to detect bandicoot at varying concentrations in SCID mice is suggested. By ‘spiking’ SCID mouse cells with serially diluted bandicoot cells and testing DNA purified from these with the TRIM3 primer, the experimental sensitivity of the assay could be determined. For example, SCID mouse cells would be spiked with 10%, 1%, 0.1%, 0.01% and 0.001% bandicoot cells. After PCR amplification with TRIM3, results would be visualised with gel electrophoresis. If bands were visible for all percentages except at 0.001%, it could be said in future experiments with visible bands that bandicoot is present at 0.01% or higher. Likewise, if bandicoot is not detected, it could be said that it is present in the SCID mouse at a concentration below 0.01%.

Generating Marsupial-SCID Mice

With the documentation of successful SCID mouse models using human, cetacean, rat and dog [35, 39–43], this study aimed to create marsupial-SCID mice. SCID mice were injected with EBB WBCs and sampled at various time points.

Previous studies have sampled at a variety of time points after injection, ranging from 30 minutes to 13 weeks [40–42]. Time points for successful reconstitution were also varied, but did show a trend towards greater reconstitution between 5 and 8 weeks. For this study, time points for collection after IP injection were set between 2 and 8+ weeks. Collection and data analysis were limited by bandicoot sample availability and, as a result, final sample time points were at 14, 51 and 63 days after IP injection.

At 14 days after IP injection, bandicoot DNA was successfully detected in the WBCs of two of the four experimental mice. No bandicoot DNA was detected in any of the experimental mice spleen or peritoneal wash samples. It is noted that the positive control of bandicoot DNA for the spleen and peritoneal samples did not show a visible band. Upon examination, this was due to incorrect DNA extraction of the bandicoot sample used for that particular positive control. This positive control was discarded and another was used for the remainder of the study, where all bandicoot positive controls showed clear bands with TRIM3. However, as the positive control failed in this instance (and due to the lack of a sample, the PCR could not be repeated), no final conclusions can be made regarding the presence of bandicoot cells in the peritoneal wash and spleen 14 days after IP injection.

At collection time points of 51 and 63 days after IP injection, bandicoot DNA was not detected in any of the experimental mouse WBC, spleen or peritoneal wash samples.

At all time points, in particular 51 and 63 days, variation in organ size was seen. The most prominent was the diminished size of experimental mouse spleens compared with the controls. Owing to the risk of graft versus host disease (GvHD) in chimeric SCID mice [28], experimental mice were checked daily and appeared in good health. At all time points, organs were removed and assessed. Although spleens in the later time points did show a reduction in size compared with the controls, they did not exhibit physical signs suggestive of GvHD. Previous studies [35, 43] showed an increase in spleen size (splenomegaly) and necrosis due to GvHD after cell engraftment. This is inconsistent with the findings of this study. It is concluded that GvHD was unlikely to be the cause of the reduced size and cell count of

experimental spleens, although it is unclear why this reduction occurred. It is suggested that the general health and organs of all experimental mice be closely monitored and assessed for signs of GvHD in any further study involving SCID chimeras.

The possibility of long-term reconstitution and host-cell integration into the donor mouse system was not examined in this pilot study. Further marsupial-SCID studies should examine the role of growth factors and cytokines in immune system development and integration between graft and host. Cytokines are synthesised by immune cells (and sometimes other cells) and have a key role in interactions between the immune system and other organs [44]. Limitations in immune integration in chimeric models have been documented, and are thought to occur due to cytokine and growth-factor differences between host and graft [38, 45]. These differences can lead to the diminished ability of mouse cytokines to bind and interact with donor *P. gunnii* cytokine receptors. It can also lead to the opposite; that is, the diminished ability of donor *P. gunnii* cytokines to bind and interact with host-mouse cytokine receptors. Communication may be interrupted one way (host cytokines cannot bind to donor receptors) or both ways (host cytokines cannot bind to donor receptors and donor cytokines cannot bind to host receptors) [44]. The possible lack of cross reactivity and communication in cytokines between SCID host and marsupial graft could lead to diminished efficacy in long-term immune integration and reconstitution.

As bandicoot samples were only received opportunistically from Zoos Victoria, the irregular nature and lack of these samples led to limitations throughout this study. A planned mixed lymphocyte reaction (MLR) to observe and characterise interactions between SCID mouse and bandicoot cells was not completed; time points were limited; and experimental sample sizes for each time point were limited. Limited sample sizes restricted any meaningful statistical analysis of data to the composite data for spleen size and splenocyte count only. A maximum of 2 mL blood was set for bandicoot samples due to the animal's size, and experiments were planned with an expectation of obtaining such values. However, while Melbourne Zoo was extremely generous, it was unable to provide 2 mL on all occasions and sample volumes were typically between 0.5 mL and 1 mL of whole blood. This limited the number of *P. gunnii* WBCs available to inject per mouse. *P. gunnii* samples could not be 'pooled' due to the high risk of graft-versus-graft disease: if the immune cells of two or more animals are mixed together, immune responses between the cells are highly probable. Therefore, the samples were injected on a one bandicoot per mouse basis.

In this study, *P. gunnii* WBC amounts injected into SCID mice ranged from 0.8×10^6 – 9.0×10^6 , with an average of 3.2×10^6 . Previous studies showed that the higher the number of engrafted cells, the more successful the engraftment [40], with injected cell amounts ranging from 10 – 60×10^6 [40, 42], to as high as 4 – 10×10^7 [41, 43]. As all amounts injected in this study were lower than those amounts that resulted in successful engraftment in the literature, it is not surprising that a greater frequency of successful engraftment was not seen. The two mice that did show successful engraftment of *P. gunnii* cells 14 days post-IP injection received 2.5×10^6 and 1.6×10^6 cells, respectively. However, the two mice in the same 14-day post-IP injection cohort that did not successfully engraft received comparable amounts: 0.8×10^6 and 2.1×10^6 cells, respectively. Therefore, further studies should investigate further the effects of both injected cell numbers and the amount of time post-injection.

It is also suggested that a less endangered and perhaps larger animal be used for investigating the feasibility of the model in future marsupial-SCID chimeric studies. This would remove the limitations of the low availability of samples and cell counts for IP injection. With access to more readily available marsupial samples, further time points (mid-way between 14 and 51 days is suggested) and larger sample sizes per experiment could be included. This would increase the likelihood of meaningful statistical analysis and the adequate assessment of the success of the marsupial-SCID chimera. However, despite this study's several limitations, the presence of bandicoot DNA detected in the WBCs of two out of four experimental mice at the 14-day time point is encouraging.