Animal Model

Immunological Characterization of Human Vaginal Xenografts in Immunocompromised Mice

Development of a Small Animal Model for the Study of Human Immunodeficiency Virus-1 Infection

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A small animal model for the *in vivo* study of human immunodeficiency virus-1 and other fastidious infectious agents in human host target tissues is critical for the advancement of therapeutic and preventative strategies. Our laboratory has developed a human vaginal xenograft model that histologically recapitulates features of the human vaginal epithelial barrier. Vaginal xenografts were surgically implanted into C.B.-*Igh-1*^b/*IcrTac-Prkdc*^{scid} (SCID) and NOD/LtSz-*scid/scid* (NOD/SCID) mice, with and without human peripheral blood mononuclear cell reconstitution. Immunohistochemical staining of vaginal xenografts demonstrated that in the SCID strain healed vaginal xenografts did not retain intrinsic human immune cells at baseline levels, whereas the NOD/SCID strain supported retention of intrinsic human immune cell populations within the xenografts for at least 2 months after engraftment. In peripheral blood mononuclear cell-reconstituted NOD/SCID mice with vaginal xenografts, flow cytometric analyses detected human immune cell populations in the peripheral blood and immunohistochemical methods detected infiltration of human CD45+ cells in the mouse spleens and vaginal xenografts for at least 2 months after reconstitution. This optimized NOD/SCID human vaginal xenograft model may provide a unique small animal *in vivo* system for the study of human immunodeficiency virus-1 transmission and infection. (*Am J Pathol* 2001, 159:2331–2345)

The mucosal immune system in the female reproductive tract seems to play a prominent, yet not completely de-

fined, role in the transmission of human immunodeficiency virus (HIV)-1.^{1–6} More than 2 decades into the HIV epidemic, there is still considerable controversy about the role of HIV-1 cellular targets within mucosal tissues and the predominant mechanism of HIV-1 trafficking across mucosal surfaces after sexual exposure to virus.⁷ Identifying more precisely the roles of these different cell types within the vagina and the conditions under which infectivity can be established are critical to understanding HIV-1 transmission. Therefore, there is an urgent need for a model system that contains these HIV-1 target cell populations within intact vaginal epithelium. In such a model system, the functional roles and interactions of these immune cell components in HIV-1 transmission could be studied.

Our laboratory has demonstrated that a variety of human epithelial tissues can be successfully grafted to orthotopic, subcutaneous, and renal capsule sites in immunocompromised mice.^{8,9} Uninfected vaginal xenografts recapitulate the histological and cytochemical features of normal human vagina. Grafts infected with human papillomavirus (HPV) or herpes simplex virus-2 exhibit pathological features and patterns of virus macromolecular synthesis identical to those observed in patient lesions. Such xenografts have also been successfully used to demonstrate microbicide-mediated abrogation of HPV infection as well as herpes simplex virus-2 infection. However, when SCID mice with healed subcutaneous human vaginal xenografts were exposed to HIV-1 IIIB, xenografts were unable to support viral infection (M. K. Howett, unpublished data). It was subse-

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quently hypothesized that HIV-1 immune cell targets were not retained within the vaginal xenograft throughout the graft-healing process.

To address this hypothesis and to optimize the vaginal xenograft model for appropriate use as an HIV-1 model, an evaluation of the human immune cell components within this *in vivo* system was completed. We demonstrate that the ability of intrinsic human immune cell populations to be retained within the human vaginal xenograft tissue after transplantation varies according to the host animal strain used. SCID animals were unable to retain human immune cell populations within the healed xenograft, whereas NOD/SCID animals were successful in retaining baseline levels (equivalent to freshly excised human vaginal tissue) of human CD45+, CD4+, CD8+, CD68+, CD1a+, and CD21+ cell populations. NOD/SCID mice with healed human vaginal xenografts were also reconstituted with human peripheral blood mononuclear cells (PBMCs) to maintain systemic HIV-1 target cell populations throughout these animals and to increase the number of HIV-1 target cells within the vaginal xenograft. Using an optimized reconstitution protocol, we achieved significant levels of reconstitution in the peripheral blood and in the spleens of NOD/SCID mice. We have also observed overall increased levels of human immune cell populations within the vaginal xenografts of PBMC-reconstituted NOD/SCID mice. The NOD/SCID vaginal xenograft model, with or without PBMC reconstitution, can be used to further study the natural history of pathogens that infect vaginal epithelium and allows for efficacy and toxicity testing of candidate vaginal microbicides to prevent the transmission of sexually transmitted diseases (STD).

Materials and Methods

Animals

Six-week-old female C.B.-*lgh-1^b/lcrTac-Prkdc^{scid}* (Taconic Farms, Germantown, NY) mice were used for initial experiments. Animals were maintained under specific pathogen-free conditions and were fed sterilized rodent Purina 5K52 diet. Six-week-old female NOD/LtSz-*scid/scid* mice (The Jackson Laboratory, Bar Harbor, ME) were maintained under identical specific pathogen-free environmental conditions. The diet for the NOD/SCID animals consisted of sterilized rodent diet NIH 31M and acidified water, pH 3.0. Research involving all animals conformed to the "Guiding Principles in the Care and Use of Animals" approved by the Council of the American Physiological Society. Experiments using animals were approved by The Penn State College of Medicine Institutional Animal Care and Use Committee.

Human Xenografts

Vaginal Tissue

Tissue was isolated from patients undergoing vaginal reconstructive surgery (resected vaginal wall) or hysterectomy (vaginal cuff). Preliminary visual examination was

performed on all vaginal samples, and only grossly normal samples were used. Specimens were held at 4°C for <3 hours in serum-free cell culture medium (Minimum Essential Medium; Life Technologies, Inc., Rockville, MD) containing 0.05% sodium bicarbonate (Life Technologies, Inc.) and 10 mmol/L of HEPES (Life Technologies, Inc.), as well as the following antibiotics: penicillin (100 U/ml; Sigma Chemical Company, St. Louis, MO), streptomycin (0.08 mg/ml, Sigma), gentamicin (0.4 mg/ml, Life Technologies, Inc.), and fungizone (2.5 µl/ml, Life Technologies, Inc.) before grafting. The vaginal wall was cut into split-thickness grafts, ~2 cm × 2 cm × 0.5 mm in size, consisting of the vaginal epithelial layer with minimal stroma. The tissue was then rolled into a tubular configuration with the epithelial layer directed inward and placed within the subcutaneous space on the backs of the immunocompromised mice. At this time, the grafted animals were also implanted with silastic tubes (2.5 mm in length, 0.078 inches in inner diameter, and 0.125 inches in outer diameter) (Dow Corning, Midland, MI) containing a 1:1 mixture of Silastic Brand Medical Adhesive Silicon Type A (Dow Corning) and 17β-estradiol [1,3,5(10)-estratriene-3,17β; Sigma] prepared as previously described.⁸ Estrogen pellets were formulated to release sufficient hormone to achieve circulating peripheral blood estrogen levels of 200 pg/ml, which correlates with levels found in premenopausal women. This hormone level sustains growth of vaginal xenografts into healthy, fully differentiating epithelium. In addition, the subcutaneous placement of the grafts in a tubular configuration allows for re-entry into the lumen for future HIV-1 inoculation experiments. Grafts are vascularized and re-epithelialized ~14 days after implantation. All protocols using human tissues (vaginal and PBMC) were conducted in accordance with Penn State College of Medicine Clinical Investigation Committee guidelines and were approved by the Institutional Review Board.

PBMCs

Reconstituted animals were grafted with vaginal tissue as previously described and subsequently received systemic grafting of human PBMCs from a separate donor. Donor PBMCs were separated by density gradient centrifugation of buffy coat on Histopaque-1077 as recommended by the product protocol (Sigma). Cryopreserved purified mononuclear cell populations were also obtained commercially for experiments with NOD/SCID mice (Poietics, San Diego, CA). Mice received an intraperitoneal injection with 1×10^7 , 5×10^7 , or 9×10^7 total PBMCs in a volume of phosphate-buffered saline (PBS) <0.5 ml. The entire procedure was completed within 3 hours of patient blood donation or thawing of cryopreserved stock cells. To determine the optimal timing for dual vaginal and PBMC xenografting, three experimental groups were initially established: 1) vaginal graft first, then PBMC graft; 2) PBMC graft first, then vaginal graft; and 3) simultaneous grafting (both grafts within 24 hours of each other). There was always a 2-week interval between implantation of the vaginal grafts and PBMC reconstitution, with the exception of the simultaneous grafted group.

Table 1. Monoclonal Antibodies Used for Immunohistochemical Analyses

Name	Clone number	Class	Binding specificity	Staining kit	Optimal dilution	Antigen retrieval solution
CD45	2B11, PD7/26	IgG1, kappa	Leukocytes	Animal Research Kit	1:50	Citric buffer, pH 6.0
CD4	P3-NS1-Ag 4-1	IgG1	T-helper cells	VectaStain Elite Kit	1:25	EDTA, pH 8.0
CD8	C8/144B	IgG1, kappa	Cytotoxic/suppressor T cells	Animal Research Kit	1:40	Citric buffer, pH 6.0
CD68	KP1	IgG1	Monocytes, macrophages	Animal Research Kit	1:50	Citric buffer, pH 6.0
CD1a	O10	IgG1	Langerhans' cells	Universal Staining Kit	Prediluted	Citric buffer, pH 6.0
CD21	IF8	IgG1	Mature B lymphocytes	VectaStain Elite Kit	1:40	Pronase E digestion

Reconstitution status was then assessed every 2 weeks after PBMC injection, for as long as 2 months. Final analyses of the vaginal xenografts were performed 4 weeks after implantation of the vaginal grafts. Therefore, animals that received PBMC grafts before vaginal grafts were analyzed 4 weeks after the vaginal graft was implanted (6 weeks after PBMC grafting). Animals that received vaginal grafts first were inoculated with PBMCs 2 weeks later. Final analyses on these animals were performed 4 weeks after implantation of vaginal grafts. In the optimized reconstitution protocol, PBMCs were injected 2 weeks after vaginal xenografting. In successfully reconstituted animals, human immune cells were observed in the peripheral circulation by fluorescence activated cell sorter (FACS) analyses and later, by immunohistochemical staining (anti-human CD45) of mouse spleens. Only animals that tested positive by FACS analyses were considered to be successfully reconstituted and were used for data collection.

Immunohistochemical Analyses

Vaginal mucosa was isolated from human donors as described previously and immediately processed for immunohistochemical analyses to determine baseline levels and distribution patterns of human immune cell populations within the vagina. At selected time points after xenotransplantation, human vaginal xenografts and select organs of experimental mice were harvested. Tissues were processed by standard formalin fixation and paraffin-embedding methods. Formalin-fixed sections were performed because of excellent tissue morphology and because of safety considerations for sectioning of fresh, xenografted, and eventual HIV-1-infected vaginal tissue. Paraffin-embedded samples were cut to 4 μ m thickness, and sections were stained with hematoxylin and eosin (H&E) for morphological analysis. Tissue sections were also stained for the presence of human cell surface receptors using the following mouse monoclonal antibodies: anti-CD45 (DAKO, Carpinteria, CA), anti-CD4 (Novocastra Laboratories, Newcastle, UK), anti-CD8 (DAKO), anti-CD68 (DAKO), anti-CD1a (Immunotech, Marseille, France), and anti-CD21 (DAKO), as described in Table 1. All anti-CD45, CD8, and CD68 staining was performed using the Animal Research Kit (ARK, DAKO) as per the manufacturer's instructions. Tissues that were stained with anti-CD4 and anti-CD21 antibodies used the VectaStain Elite kit (Vector Laboratories Inc., Burlingame, CA), and samples stained with anti-CD1a used the Uni-

versal Staining Kit (Immunotech) as per the manufacturer's instructions. Briefly, all tissue sections were baked in a vacuum oven at 55°C for 1 hour to evaporate paraffin. Samples were then dehydrated and rehydrated by incubation in xylene and a graded alcohol series. Endogenous peroxidases were destroyed by immersion in 3% hydrogen peroxide in methanol for 10 minutes. Tissues that were stained with anti-CD4 antibody were incubated in a 0.5% hydrogen peroxide/methanol solution to avoid irreversible damage to the CD4 receptor. After each appropriate antigen retrieval step (recommended by antibody manufacturer), samples were blocked with 10% horse serum (VectaStain Elite kit), peroxidase solution (ARK kit), or Universal Protein Blocking Solution (Universal Staining Kit) for 1 hour at room temperature. Respective primary antibodies were diluted in PBS as per the manufacturers' recommendations and a total volume of 150 μ l was added to each section for the appropriate incubation period. All subsequent steps were performed at room temperature. After two 5-minute washes with PBS, tissue sections were incubated with biotinylated anti-mouse secondary antibody for 30 minutes. Samples were again washed twice with PBS for 5 minutes each. Streptavidin-peroxidase conjugate incubations were performed for 30 minutes. Color visualization of the complex was achieved by incubating tissue sections with diaminobenzidine tetrahydrochloride containing Ni^{2+} for 5 minutes. All tissue sections were briefly rinsed in water and counterstained with eosin as routinely performed by others.^{10,11}

A technical obstacle when staining human tissues that were grafted into mouse hosts is that the secondary (anti-mouse IgG) antibody may stain mouse immunoglobulin within the human xenografts. As a precaution, this problem was circumvented by preincubating primary and secondary antibodies together (in equimolar amounts) before the reaction of the complex with the target of the primary antibody. This approach lowers the risk of the secondary antibody-induced background reactivity. In addition, staining with secondary antibody alone did not stain normal NOD/SCID mouse tissues or human xenografts from NOD/SCID animals presumably because this mouse strain is agammaglobulinemic. Staining tissue sections with the secondary antibody alone served as negative controls. Mouse spleens from female BALB/c mice and NOD/SCID mice and human tonsil samples also served as appropriate tissue controls.

Flow Cytometry Analyses

The presence of human immune cells in the peripheral blood of PBMC-reconstituted and -unreconstituted animals was determined by FACS analyses. Approximately 150 μ l of whole blood was collected in heparinized tubes at 2-week intervals after PBMC injection. Blood was obtained from the retro-orbital plexus of each mouse during the course of the experiment and by cardiac puncture at the time of organ and xenograft harvesting. Whole blood was stained to measure the number of cells expressing the following human cell-surface markers CD14/45 and CD3/4/8 (antibodies from Becton Dickinson, San Jose, CA). After staining of the blood for 45 minutes at 4°C, the cells were washed with 2 ml of PBS and pelleted. Samples were then treated with FACS Lysing Solution (Becton Dickinson) to remove red blood cells, washed twice with PBS, and fixed in 2% paraformaldehyde. Blood aliquots from each animal, in each experiment, were also stained with a human heavy chain isotype (γ 1/ γ 1) antibody as an internal control. This isotype control served to detect nonspecific binding of the heavy chain portion of the antibody to PBMC cell surfaces. In addition, blood samples from immunocompromised animals (not reconstituted with human PBMCs) also served as a negative control for all of the human-specific antibody staining. These IgG controls, therefore, defined background staining limits and quadrant markers. Cell phenotypes were assessed by bivariate plots of FL-1 and FL-2 in logarithmic mode using CELLQuest software (Becton Dickinson). Dead cells and residual red blood cells were excluded from final analyses by appropriate lysing steps and by electronic gating.

Results

Healing Time Course of Human Vaginal Xenografts in SCID Animals

Our laboratory developed a model that utilizes intact human vaginal epithelium and associated stroma to recapitulate the mucosal epithelial barrier normally found *in vivo*. Successful engraftment of human vaginal epithelial tissues in athymic and SCID mice has been previously reported.⁸ Once grafted into the SCID animal, the vaginal tissue underwent a brief period of degeneration of the epithelial superficial layers; however, the basal layer of cells remained intact. This process began at approximately day 3, and continued until 7 days after engraftment (Figure 1; A to C). Fourteen days after engraftment, the differentiated epithelial layers of the vaginal xenograft were restored, and the stratified squamous epithelium was histologically similar to normal vaginal tissue (Figure 1D). The cytochemical and morphological phenotypes of the xenografts were extensively characterized and were representative of normal vagina with respect to glycogen levels, estrogen and progesterone receptor levels, and lactoferrin expression. In addition, levels of glutamylcysteine synthetase, glutathione-S-transferases and Mn and Cu/Zn superoxide dismutases in vaginal xenografts were

also representative of freshly isolated normal vaginal tissue. Vaginal xenografts were vascularized by 14 days after engraftment into SCID animals and detectable levels of circulating mouse IgG were found within the xenografts for at least 90 days after implantation (M. K. Howett, manuscript in preparation).

Human Vaginal Xenografts in SCID Animals Are Unable to Retain Baseline Levels of Intrinsic Human Immune Cells

To determine whether SCID animals were capable of retaining intrinsic human immune cell populations within vaginal xenografts, immunohistochemical analyses were performed on harvested xenografts throughout the graft-healing process. The first objective, however, was to establish the average baseline level of human immune cell populations for normal vaginal donor tissue before grafting into SCID mice. Staining for CD45 (leukocyte common antigen) indicated that high levels of human immune cells were present throughout the vaginal donor tissue (Figure 2A). The donor vaginal tissue obtained for xenografting resembles the immune cell profiles previously observed in healthy, vaginal tissues.¹²

Vaginal xenografts were harvested from SCID mice at 0, 3, 7, and 14 days, as well as 1 and 2 months after engraftment. Each time course experiment was performed using tissue from a single vaginal donor, and a total of three experiments were completed with 12 animals per experiment. Immunohistochemical studies were performed on harvested xenografts using antibody directed against human CD45. These results demonstrated that the vaginal xenografts in the SCID animals contained intrinsic CD45+ cells at the time of implantation (time 0) and to day 7 after engraftment (Figure 2; A to C). However, CD45+ cell populations within the vaginal xenografts were considerably diminished relative to baseline levels by 14 days after engraftment (Figure 2D). Human CD45+ cell populations were not restored 2 months after engraftment.

To immunologically reconstitute vaginal xenografted SCID mice, human PBMCs were used as described in Materials and Methods. Reconstituted animals that were positive for human lymphocytes 2 weeks after PBMC inoculation retained stable levels of positive human immune cells in the peripheral blood up to 2 months after PBMC engraftment. Approximately 30% of the SCID animals with vaginal xenografts were successfully reconstituted with human lymphocytes when administered 5×10^7 PBMCs per animal in a volume of PBS <0.5 ml. This was not considered an acceptable success rate for reconstitution. The overall number of positively reconstituted animals, as measured by FACS analyses, dramatically increased to 71% when 9×10^7 PBMCs per animal were injected (Figure 3A). However, there was also an increase in the mortality rate of animals receiving the higher concentration of PBMCs. Approximately 50% of these positively reconstituted animals died because of what were believed to be B-cell lymphomas before the 2-month experimental period was completed. The devel-

Figure 1. The establishment of human vaginal xenografts in immunocompromised mice. Representative H&E-stained sections of vaginal xenografts harvested at 0 (A), 3 (B), 7 (C), and 14 (D) days after engraftment demonstrate the graft-healing process. The initial deterioration of the vaginal epithelium begins ~3 days after engraftment and continues down to the basal layer of cells ~7 days after engraftment. However, by 14 days after engraftment, the differentiated vaginal epithelium is restored within the xenografts. Histological and cytochemical analyses demonstrate that human vaginal xenografts are representative of healthy human vaginal tissues. Original magnifications, $\times 200$.

opment of B-cell lymphomas in reconstituted SCID animals has been reported by others.^{13–16} In general, if the blood of a reconstituted mouse consisted of $>60\%$ human-specific CD45+ cells, the animal died before the 2-month experimental period was completed and extensive tumor growth was observed in the animal's liver, spleen, thymus, and lymph nodes (Figure 3; B to D). We also observed that animals that developed such complications exhibited a skewed reconstitution ratio of 4-fold to 5-fold higher levels of CD8+ compared to CD4+ cells in the peripheral blood.

The timing of each xenograft (blood and vagina) into the SCID animals did effect the overall success rate of PBMC reconstitution. The results of two independent experiments demonstrated that simultaneous engraftment of both xenografts (within 24 hours of each other) did not result in any positively PBMC-reconstituted animals (data not shown). SCID animals that were allowed a 2-week time interval between engraftments were able to be support human immune cell populations in the peripheral blood up to 2 months after inoculation. The overall quantity of PBMCs in the peripheral blood of reconstituted

Figure 2. Immunohistochemical human CD45 staining of human vaginal xenografts in SCID animals. Vaginal xenografts were surgically implanted into SCID mice as described in Materials and Methods. Grafts were harvested immediately after implantation into SCID animals at day 0 (**A**), day 3 (**B**), day 7 (**C**), and day 14 (**D**) after engraftment. Immunohistochemical staining demonstrates that human CD45-positive cell populations were present at time 0 (**A**) and were maintained within the tissue to day 7 (**B** and **C**) after engraftment. However, by 14 days after engraftment, the intrinsic human immune cell populations were significantly reduced (**D**). **Arrows** indicate CD45+ human immune cells. Original magnifications, $\times 200$.

animals, however, was low. Further experiments will be required to fully understand why simultaneous grafting may inhibit PBMC reconstitution in SCID animals.

Immunohistochemical analyses on the vaginal xenografts of reconstituted SCID animals were performed to monitor the levels of human CD45+ immune cells within these tissues. Three major observations were recorded at the conclusion of these 2-month experiments. First, vaginal xenografts of SCID animals, which were not reconstituted with PBMCs, did not stain positively for human lymphocytes by immunohistochemical analyses. Second,

animals that were positive for human lymphocytes in the peripheral blood by FACS analyses did have a slight increase in the numbers of human CD45+ -stained cells within the vaginal xenografts. However, the numbers of CD45+ cells did not reach the baseline levels observed in freshly excised vaginal tissue. Finally, animals that showed >60% of human PBMCs in their peripheral blood and that developed lymphomas, contained higher levels of CD45+ cells in the vaginal xenografts; however, these animals are not ideal because of the high mortality rate because of lymphomas.

Figure 3. Representative PBMC-reconstituted SCID mouse with advanced lymphoma development. All data panels collected from one representative animal injected with 9×10^7 PBMCs. The presence of human immune cells in peripheral blood was measured by FACS analysis 2 months after inoculation (**A**). The first graph demonstrates very high levels of human CD45+ lymphocytes in the peripheral blood. In addition, the second graph demonstrates higher levels of reconstituted CD8+ T-cell populations over CD4+ T cells. On autopsy, the thymus (**B**), liver (**C**), and spleen (**D**) demonstrated diffuse lymphomas infiltrated with dysmorphic, enlarged cell populations (arrows). Original magnifications, $\times 400$.

Human Vaginal Xenografts in NOD/SCID Animals Retained Intrinsic Baseline Levels of Human Immune Cells

To further optimize the vaginal xenograft model, we investigated alternative immunodeficient mouse background strains that have been specifically engineered to tolerate xenograft transplantation. Manipulation of the SCID strain has led to the development of more severely immunodeficient mouse strains that also possess enhanced receptivity of xenografts. Previous data demonstrated that when NOD/SCID mice were used as recipients of human lymphoid cells, these animals resulted in a 5- to 10-fold higher number of positively reconstituted animals compared to SCID animals. In addition, the number of engrafted PBMCs recovered from NOD/SCID ani-

mals were significantly increased over the rates observed in SCID mice.¹⁷ The NOD/SCID strain was originally chosen because we hypothesized that they would permit a higher success rate and higher circulating levels of human PBMCs in the PBMC-reconstituted animals.

To determine that the NOD/SCID host strain was capable of supporting healthy vaginal xenografts, the graft-healing process was evaluated by analyzing the xenografts at 0, 3, 7, and 14 days and 3, 4, 6, and 8 weeks after engraftment. A total of four animals per time point were evaluated in two separate experiments, each of which used tissue from a single vaginal donor. H&E staining was performed on the harvested xenografts to visualize tissue morphology. The healing process of the vaginal grafts in NOD/SCID animals was similar to that observed in the SCID animals. Epithelial tissue degener-

Figure 4. The establishment of human vaginal xenografts in NOD/SCID mice. NOD/SCID-immunodeficient mice were able to tolerate human vaginal xenograft tissue transplantation without rejection. H&E staining of vaginal tissue samples harvested 2 months after engraftment (**E** and **F**) demonstrates that the histology of the grafts was well preserved in this animal strain. The graft-healing time-course samples demonstrate that, overall, the process is similar to that observed in the SCID strain: time 0 (**A**), day 3 (**B**), day 7 (**C**), and day 14 (**D**). The epithelial layer is generally restored throughout the graft by 3 weeks after engraftment in the NOD/SCID strain. Original magnifications: $\times 200$ (**A–D** and **F**), $\times 100$ (**E**).

Figure 5. Immunohistochemical analyses of human vaginal xenografts in NOD/SCID mice. Immunohistochemical staining for human CD45 on harvested xenografts demonstrated that the intrinsic levels of human immune cells within the vaginal xenografts were conserved throughout the course of the graft-healing process in NOD/SCID animals. Tissues were harvested and stained with H&E at days 0 (**A**), 3 (**B**), 7 (**C**), and 21 (**D**) after engraftment. **Arrows** indicate CD45+ human immune cells. Original magnifications, $\times 200$.

ation began at day 3 after engraftment and continued to ~ 7 days after engraftment (Figure 4). However, in NOD/SCID animals, complete restoration of the epithelial layer across the entire xenograft surface area was not optimal until 3 weeks after engraftment as compared to the 2-week time period observed in SCID hosts.

To determine whether the NOD/SCID host strain retained intrinsic human immune cell populations within the vaginal xenograft, immunohistochemical staining of the harvested tissues with anti-human CD45 antibody was performed. These results demonstrated that the intrinsic human immune cell populations were retained through-

out the healing process. More importantly, the human CD45+ cell populations were present at baseline levels (equivalent to time 0 freshly excised tissue), up to 2 months after engraftment (Figure 5). In addition to the numbers of immune cells being equivalent to baseline levels, the overall pattern of immune cell distribution within the tissue remains constant throughout the course of graft healing and is representative of normal vagina.

To determine specific human immune cell populations retained in the vaginal grafts, additional immunohistochemical analyses were performed. Initially, a comprehensive study was conducted to establish a baseline

Figure 6. Immunohistochemical analyses of specific human immune cell populations within the vaginal xenografts. **Column A** represents freshly excised human vaginal tissue. **Column B** represents vaginal xenografts from the same donor as in **column A**, 21 days after engraftment in NOD/SCID animals. Human CD4+ cells were identified on 0 (**A**) and 21 days (**B**) and retained to 2 months after engraftment. CD8+ cells (**C** and **D**) and CD68+ monocytes/macrophages (**E** and **F**) were also detected within the vaginal xenografts throughout the graft-healing process. Finally, CD1a Langerhans' cells (**G** and **H**) and CD21 mature B lymphocytes (**I** and **J**), were also detected within these human xenografts throughout the graft-healing process. The immune cell populations were retained at baseline levels (equivalent to freshly excised vaginal tissue) throughout the 2-month monitoring process. Original magnifications, $\times 200$.

distribution profile of specific immune cell populations in the human vaginal mucosa. A total of 10 tissues were immediately processed after surgical removal from the patient. These vaginal samples were stained with specific antibodies against the human surface markers CD4, CD8, CD68, CD1a, and CD21 (Figure 6, column A). CD4⁺ cells were distributed throughout the epithelial submucosa and suprabasal layers of all tissue samples. CD8⁺ cells were, in general, more numerous than CD4⁺ cells. CD8⁺ cells were identified throughout the epithelium; however, they were mainly concentrated as a distinct single band at the basal layer, which is consistent with previously reported findings.¹⁸ CD68⁺ monocytes/macrophages were found in low numbers and were mainly concentrated below the basal epithelial layer in the stroma. CD1a⁺ Langerhans' cells were found in relative abundance and dispersed throughout the vaginal mucosa, being both suprabasal and basal in location. In addition, the processes of Langerhans' cells were shown to extend into the epithelial layer. Finally, low numbers of CD21⁺ B cells were identified in the vaginal tissue. These cells were concentrated mainly below the basal layer with only a few cells located in the epithelial layers. Profiles of immune cell populations within the human vagina from healthy women have been previously studied.^{12,19}

Vaginal xenografts were harvested at 0, 3, 7, 14, and 21 days and 1 and 2 months after engraftment into NOD/SCID animals. These vaginal graft specimens were stained with specific antibodies directed against the human surface markers CD4, CD8, CD68, CD1a, and CD21. Human CD4⁺ cells, a primary HIV-1 target cell population, were retained at baseline levels, up to 2 months after engraftment (Figure 6, A and B). In addition, CD8⁺ cells (including cytotoxic T lymphocytes), CD68⁺ macrophages, CD1a⁺ Langerhans' cells, and CD21⁺ B cells were all detected within the vaginal xenografts throughout the 21-day graft-healing process (Figure 6; C to J). These cell populations were identified in their natural locations and were retained at baseline levels up to 2 months after engraftment, comparable to normal vaginal tissue.

PBMC Reconstitution of NOD/SCID Strain Was More Successful than SCID Strain

To systemically reconstitute NOD/SCID animals containing vaginal xenografts, animals were xenografted with vaginal tissues, allowed 2 weeks for graft healing, and subsequently reconstituted with 5×10^7 PBMCs per animal via intraperitoneal inoculation. Reconstituted and unreconstituted NOD/SCID animals were monitored by flow cytometric analysis for the presence of human lymphocytes within the peripheral blood. Although human immune cells were not identified in the periphery of unreconstituted NOD/SCID animals, the vaginal xenografts themselves continued to stain positively by immunohistochemistry for anti-human CD45, CD4, CD8, CD68, CD1a, and CD21, as described in Figure 6. FACS analyses on PBMC-reconstituted NOD/SCID animals demonstrated

Figure 7. Representative FACS analyses of peripheral blood from NOD/SCID mice: unreconstituted or reconstituted with 5×10^7 human PBMCs per mouse (same PBMC donor). **Column 1:** Donor PBMC profile before injection into NOD/SCID mice. **Column 2:** Representative unreconstituted NOD/SCID mouse at 2 months after inoculation of PBS alone. **Column 3:** Representative PBMC-reconstituted NOD/SCID mouse at 2 months after inoculation. Human CD14/45 and CD4/8 expression was measured in **rows A and B**, respectively.

that reconstitution of human CD45⁺, CD4⁺, and CD8⁺ populations were successfully established and maintained in the peripheral blood of these animals for at least 2 months after PBMC inoculation (Figure 7). As with PBMC-reconstituted SCID animals, human CD14⁺ monocytes were never observed in the peripheral blood of PBMC-reconstituted NOD/SCID animals. Approximately 80% of NOD/SCID mice were successfully reconstituted by this method, gating an average of 30% total human lymphocytes in each animal. Immunohistochemical staining was performed on harvested spleens of both unreconstituted and reconstituted NOD/SCID animals 2 months after PBS or PBMC injection, respectively. Human-specific CD45⁺ cell populations were detected in the mouse spleens of only the reconstituted animals (Figure 8, C and E).

Every NOD/SCID animal, with or without PBMC reconstitution, retained baseline levels of each human immune cell population within the vaginal xenografts up to 2 months after engraftment (Figure 8B). In a small percentage (<4%) of reconstituted animals, the overall numbers of human immune cells within the vaginal xenografts was increased relative to baseline levels (Figure 8, D and F). The tissue morphology, including the vaginal xenografts, of these more highly reconstituted animals was normal as determined by H&E staining. For the purposes of assembling this model system, increased numbers of infiltrating HIV-1 susceptible target cells may prove beneficial for initiating transmission of the virus.

Discussion

The advancement of anti-HIV therapeutic and preventative strategies is critically dependent on the identification of the roles of different cell types within the vagina during HIV-1 transmission and the conditions under which virus infection can be established. More than 2 decades into the HIV epidemic, it is still not well defined which cells within the vagina serve as targets for HIV infection and

Figure 8. Immunohistochemical analyses of unreconstituted and PBMC-reconstituted NOD/SCID animals with healed vaginal xenografts. CD45 immunohistochemical staining of harvested spleens from NOD/SCID animals 2 months after injection with PBS or human PBMCs. Animals injected with PBS alone (unreconstituted) did not stain positively for human immune cell populations in the spleen (**A**). Animals injected with 5×10^7 human PBMCs stained positively for human CD45 expression in the spleen 2 months after injection (**C** and **E**). The vaginal xenografts from both unreconstituted and of reconstituted animals (**B**, unreconstituted; **D** and **F**, reconstituted) stain positively for human CD45 expression 2 months after engraftment. Graft tissues from reconstituted animals possess increased numbers of human immune cells compared to the unreconstituted sample. Original magnifications: $\times 200$ (**A–D**), $\times 100$ (**E** and **F**).

which of these cell populations are critical for the sexual transmission of the virus. Therefore, there is an urgent need for a model system that contains these HIV-1 target cell populations so that the roles of individual cell types and the interactions between these immune cell components in the context of HIV-1 transmission can be more clearly examined.

Initially, we determined the average baseline levels of immune cell populations within human vaginal donor tissue. Various factors influence immune cell numbers and locations within the human vagina. Such factors include phase of menstrual cycle, hormone levels, and the presence of infectious agents.^{20–23} Fluctuation of these factors may lead to increased numbers and perturbations in the distribution of the normal ratio of HIV-susceptible target cells in the vagina. Defining a baseline level of intrinsic human immune cells for each donor tissue was also important to establish an internal control for each xenograft experiment. Immune cell levels did vary slightly among individual vaginal donors. All xenograft tissues within each experiment were derived from a single vaginal donor. Therefore, to monitor the levels of immune cells within a set of xenografts throughout the course of graft healing, normal tissue from the same donor (ungrafted) served as a baseline control for each experiment.

Although SCID mice xenografted with vaginal tissue represent a suitable model for HPV infection and for studies of inactivation of HPV by microbicides, human vaginal xenografts in SCID animals were not able to support HIV-1 infection.⁸ Initial attempts to directly infect the lumen of healed vaginal xenografts in SCID animals with HIV-1 IIIB were unsuccessful (M. K. Howett, unpublished observations). As a result, we hypothesized that susceptible HIV-1 target cell populations were no longer present in the vaginal xenografts after the graft-healing process; the absence of such immune cell populations would preclude HIV-1 infection. This hypothesis was strongly supported by immunohistochemical analyses of vaginal xenografts harvested from SCID animals throughout the course of the graft-healing process.

Severe combined immunodeficient (SCID) mice are devoid of cellular as well as humoral immune function because of defective V(D)J recombination and aberrant DNA double-strand break repair.²⁴ Although SCID mice do possess progenitor lymphoid cells, these precursor cells cannot generate functional immunoglobulin and T-cell receptor chains and, therefore, fail to develop into mature B and T cells. SCID mice, however, do exhibit normal to elevated natural killer cell activity, functional macrophages, and elevated hemolytic complement activity.^{25–28} In addition, >25% of SCID mice spontaneously develop partially restored immune function. Mice with this leaky SCID phenotype eventually develop minute levels of B cells and rudimentary antibody responses.^{29,30} Leaky functional SCID mouse T cells have also been shown to efficiently reject allogeneic SCID mouse skin grafts.³¹ Both the leaky lymphocyte phenotype and the presence of native immune cell function components, such as natural killer cells, may contribute to the limited

survival of human lymphoid cells grafted into these animals.²⁸

Previously, human peripheral blood leukocytes have been successfully engrafted into SCID mice to study HIV infection.^{32–34} However, PBMC reconstitution of the SCID mouse varies considerably among methods of reconstitution, laboratories, experiments, source of grafts, and even individual mice.³⁵ The rate of success for reconstitution of SCID animals using human PBMCs remain controversial with some authors noting overall low reconstitution rates, signs consistent with graft *versus* host disease, inverted CD4/8 T-cell ratios, B-cell lymphomas, and high mortality rates.^{13,35} Furthermore, because of these conflicting reports, it is difficult to delineate a consistent reconstitution protocol that would allow for reproducibility and accurate comparison of data.

Our attempts to establish a vaginal xenograft system in the PBMC-reconstituted SCID strain have also resulted in less than optimal rates and levels of reconstitution. Overall, only 30% of SCID animals containing vaginal xenografts were positive for human CD45 within their peripheral blood up to 2 months after PBMC reconstitution. Immunohistochemical staining of vaginal xenografts in SCID animals reconstituted with lower concentrations of PBMCs (1×10^7 to 5×10^7) demonstrated that immune cell components were not restored back to baseline levels within the vaginal xenografts. Higher concentrations of human PBMCs were injected in the SCID animals in an effort to increase the overall success rate of reconstitution systemically in the animals and to concomitantly increase the likelihood of restoring human immune cells within the xenograft. Approximately 50% of the animals that received higher concentrations (9×10^7) of PBMCs contained detectable peripheral blood levels of human CD45 but began to die before the 2-month experimental time period had elapsed. Autopsy of these animals showed that the body cavities contained numerous tumors believed to be Epstein-Barr virus-induced lymphomas, an observation reported by others when reconstituting SCID mice with human PBMCs at concentrations higher than 5×10^6 per mouse.^{13–16}

Another consideration in designing this model was the potential for graft *versus* host disease. Graft *versus* host disease is particularly interesting in our dual xenograft model system because there is potential for a graft (human vaginal xenograft/human PBMCs) *versus* host (mouse) reaction as well as a graft (vaginal xenograft/human PBMCs from a different donor) *versus* graft (human PBMCs from a different donor/vaginal xenograft) reaction. One mechanism to circumvent the graft *versus* graft obstacle would be to use PBMCs harvested from the same vaginal donor, in which case, the two xenografts would be HLA-matched. However, the utility of SCID mice in these grafting experiments may be limited by their normal natural killer cell activity and the leaky B- and T-lymphocyte response, both of which may precipitate rejection of grafted human tissues.

To optimize the vaginal xenograft model, we investigated alternative immunodeficient mouse strains that have been specifically engineered to tolerate xenograft transplantation. The NOD/SCID mouse was originally de-

veloped by crossing the C.B.-17-Sz-*scid* background onto the NOD/Lt substrain of NOD/Shi.²⁸ The NOD/SCID strain is characterized by absent B- and T-cell function, deficit in natural killer cell function, absence of circulating complement, and defects in the differentiation and function of antigen-presenting cells. We demonstrate that the NOD/SCID mouse strain, although severely immunocompromised, is fully capable of supporting human vaginal xenografts up to 2 months after engraftment. The vaginal xenografts were morphologically similar to normal vaginal tissue, and immunohistochemical studies using anti-human CD45, CD4, CD8, CD68, CD1a, and CD21 indicate that vaginal xenografts established in the NOD/SCID strain can maintain endogenous levels of human immune cell populations up to 2 months after graft healing.

PBMC reconstitution of NOD/SCID animals resulted in a significant increase in the positive reconstitution rate compared to identical treatment of SCID animals. More importantly, the mortality rate of successfully reconstituted NOD/SCID animals was not altered as a result of PBMC engraftment; this rate is dramatically different from the results obtained in SCID reconstitution experiments. In addition, the ability of human immune cells within NOD/SCID animals to migrate and home to the appropriate target organs, such as the mouse spleen, was demonstrated by immunohistochemical analyses. Reconstitution PBMC clearance kinetic analyses and trafficking studies are currently ongoing in our laboratory to further explore these events in this model system.

In <4% of reconstituted NOD/SCID animals, there was a dramatic increase in the number of human CD45+ immune cell populations within the vaginal xenograft. This may have been the result of trafficking of PBMC-injected lymphocytes to the vaginal graft through the vascular network, potentially in response to favorable cytokines within the graft milieu. Because donor vaginal tissue and donor PBMCs were not HLA-matched, we cannot exclude the possibility that increased numbers of immune cells in the vaginal xenograft may represent some type of low-level graft rejection. Alternatively, this reaction may represent a delayed hyperacute response, delayed because the host strain does not contribute to the immune response between the two HLA-unmatched xenografts. In addition, cross-reactive recognition may support the idea of alloreactive responses between the two xenografts. We observed, however, that there is no destruction of the vaginal grafted tissue and that epithelial differentiation proceeded normally for a period of up to 2 months after engraftment as determined by histological analyses. Regardless of the mechanism, the increased lymphocyte infiltration into the vaginal epithelium may be a beneficial effect of reconstitution because it may serve to increase potential HIV-1 target cells within the target tissue. We therefore anticipate higher and more sustained levels of HIV-1 replication in these animals.

The flow cytometric data showed a reconstituted lymphocyte population in positive SCID and NOD/SCID animals (as measured by CD45); however, a CD14+ monocyte population was never observed within the peripheral blood of these animals despite its presence in donor

PBMC samples. The absence of human monocyte populations in the peripheral blood of PBMC-reconstituted animals has been reported by others.³⁶ Evidence exists for CD34+ stem cell populations to support long term engraftment in NOD/SCID animals and to become terminally differentiated.³⁷ It is, therefore, unclear what happens to the monocytes initially present in the PBMC populations after injection into the animals. The absence of human monocytes within the mouse peripheral circulation may become problematic for infection of the model system for monitoring systemic HIV-1 infection. When considering the initial steps of HIV-1 transmission, however, it is critical to emphasize that immunohistochemical staining with CD68 antibody detected positive CD68+ cell populations, which include monocytes and macrophages, within the vaginal xenograft tissue at baseline levels up to 2 months after injection. Successful reconstitution of CD4+ and CD8+ cells was supported by positive staining of the peripheral blood of animals by flow cytometric analyses. The localization of these cells within the vaginal xenografts of reconstituted animals has also been demonstrated by immunohistochemical staining. These data support our model system may be an attractive small animal model for the study HIV within the context of the human vaginal microenvironment.

These immune cell populations each have been postulated to play a critical role in the transmission of HIV-1 and their presence within the vaginal xenograft provides encouraging evidence that this model will support HIV-1 infection. Our laboratory has subsequently confirmed that this model supports HIV-1 replication (T. M. Kish, manuscript in preparation). This xenografting system offers for the first time a small animal model for studying HIV-1 transmission within the context of natural host tissue; this model also allows for the dissection of the role(s) of multiple cell subpopulations in HIV-1-infected genital mucosal tissue.

The use of this model system may be further exploited to study vaginal HIV-1 transmission in the context of co-infections by STD pathogens. Our laboratory has confirmed that HPV and herpes simplex virus-2 infection of the vaginal xenograft model mimics viral pathogenesis normally observed *in vivo*. Evidence suggests that when analyzing the transmission rate of viruses that cause STDs, HIV-1 is not transmitted as efficiently as herpes simplex virus, HPV, or hepatitis viruses.²³ It is estimated that only 1 of every 500 incidences of vaginal intercourse with an HIV-infected partner results in transmission of HIV-1. However, if one or both partners are infected with another STD pathogen, susceptibility to HIV-1 transmission is substantially increased. This vaginal xenograft model provides a unique system to determine STD pathogen transmission rates and to better understand the impact that co-infections may have in transmission rates. This model also allows for the study of the life cycle of fastidious pathogens within the context of the host human vaginal tissue.

In conclusion, we present an innovative model system that provides the human immune cell components within vaginal tissue at baseline levels found *in vivo*. This system also allows for the complete characterization of the complex interactions between many of the cell populations that are hypothesized to have a functional role in the initial

steps of transmission of the HIV-1 virus. Furthermore, this system can be used to test potential vaginal microbicides for their ability to block the initial steps of viral transmission and may also provide a small animal model for testing anti-retroviral therapeutics. Full development and exploitation of this model may also relieve the financial, logistic, and ethical dilemmas associated with SIV/HIV experimentation in higher primates.

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