Immune Competency of a Hairless Mouse Strain for Improved Preclinical Studies in Genetically Engineered Mice

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Abstract

Genetically engineered mouse models (GEMM) of cancer are of increasing value to preclinical therapeutics. Optical imaging is a cost-effective method of assessing deep-seated tumor growth in GEMMs whose tumors can be encoded to express luminescent or fluorescent reporters, although reporter signal attenuation would be improved if animals were fur-free. In this study, we sought to determine whether hereditable furlessness resulting from a hypomorphic mutation in the Hairless gene would or would not also affect immune competence. By assessing humoral and cellular immunity of the SKH1 mouse line bearing the hypomorphic Hairless mutation, we determined that blood counts, immunoglobulin levels, and CD4+ and CD8+ T cells were comparable between SKH1 and the C57Bl/6 strain. On examination of T-cell subsets, statistically significant differences in naive T cells (1.7 versus 3.4 × 10^6 cells/spleen in SKH1 versus C57Bl/6, P = 0.008) and memory T cells (1.4 versus 0.13 × 10^6 cells/spleen in SKH1 versus C57Bl/6, P = 0.008) were detected. However, the numerical differences did not result in altered T-cell functional response to antigen rechallenge (keyhole limpet hemocyanin) in a lymph node cell in vitro proliferative assay. Furthermore, interbreeding the SKH1 mouse line to a rhabdomyosarcoma GEMM showed preserved antitumor responses of CD56+ natural killer cells and CD163+ macrophages, without any differences in tumor pathology. The fur-free GEMM was also especially amenable to multiplex optical imaging. Thus, SKH1 represents an immune competent, fur-free mouse strain that may be of use for interbreeding to other genetically engineered mouse models of cancer for improved preclinical studies. Mol Cancer Ther; 9(8); 2354–64. ©2010 AACR.

Introduction

The mouse strain SKH1 carries an autosomal recessive hypomorphic mutation called hr in the gene Hairless (Hr; ref. 1). This mutation is caused by a proviral insertion of the murine leukemia virus at the Hr locus (Fig. 1; ref. 1). These mice undergo hair loss before weaning, a feature that could dramatically improve preclinical therapeutic investigation for genetically engineered mouse models (GEMM) of cancer. Specifically, GEMMs can be bred to the SKH1 strain to achieve furlessness, a feature that improves serial optical imaging of reporter genes and contrast agents in live animals (2). In transgenic mouse models of human disease, tumors or tissues are often genetically engineered to express luciferase or fluorescent proteins as optically detectable reporters to determine the health, proliferation, or migration (metastasis) of the cell population or tissue of interest. When wild-type mice are imaged, fur reduces luminescent and fluorescent reporter gene signal by >10-fold (3). Because even skin alone can reduce optical signal by 90% (4), the detection of small tumors or metastases using optical imaging is often quite challenging. Mice without fur would allow better imaging; however, for preclinical models to be physiologically accurate to human disease, fur-free GEMMs must also be immune competent. The Hr gene encodes the protein Hr, which is highly expressed in the skin and brain and acts as a transcriptional corepressor for multiple nuclear receptors, including thyroid hormone receptor, retinoic acid receptor, and the vitamin D receptor (5). The absence of the repressor protein Hr in hr mice alters transcription of gene

Acknowledgments

Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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References

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doi: 10.1158/1535-7163.MCT-10-0207

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products that function in keratinocyte differentiation (5). In addition to changes in hair and skin development, mutations that affect keratinocyte gene expression may alter thymus development and cell-mediated immunity, as dramatically illustrated by the homozygous nude phenotype due to disruption of Foxn1 (6). Thus, mutations in the Hr gene have the potential to seriously impact immunologic function, which underlies the purpose of our study evaluating the immune function of the SKH1 mouse line.
First described by Brooke in 1926 (7), the homozygous SKH1 mouse line has been maintained in commercial breeding facilities without immunologic precautions for years if not decades (C.B. Clifford, personal communications). This apparent immune competence may be in part because homozygous SKH1 mice still produce Hr transcript at 5% of normal levels (8), despite an insertion of murine leukemia virus (MuLV) pmv-43 in exon 6 of Hr (1), pmv-43 itself is believed to result from a recombination of xenotropic and polytropic classes of endogenous murine leukemia virus based upon restriction mapping (9, 10). Humans are also known to carry mutations of the Hr gene and have been reported to have skin and hair phenotypes (OMIM 602302 and ref. 11), but no associations with immunodeficiency or non-skin cancer predisposition have been made. Therefore, the goal of this study was to ascertain the immunologic differences, if any, between mice homozygous for the autosomal recessive Hr mutation, SKH1, and a control C57Bl/6 mice line to ascertain whether the SKH1 mouse line can be used for preclinical therapeutic models in GEMMs. Our assessment of humoral and cellular immune competence revealed relatively little functional immunologic differences for the SKH1 strain in comparison with C57Bl/6.

Materials and Methods

Mice

All animal procedures were conducted in accordance with the Guidelines for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Health Science Center at San Antonio (UTHSCSA). The SKH1 strain mice carrying the homozygous Hr mutation hr (also called Hairless/hr or Hairless/hr/hr) and the comparison C57Bl/6 control mice were purchased from Charles River Laboratories. For all studies, 8- to 12-week-old female mice were used unless otherwise specified. Tissues were harvested at necropsy following euthanasia under approved methods. The Pax3: Fkhr alveolar rhabdomyosarcoma mouse model used for transgenic experiments was previously described (3, 12–14). PCR genotyping for the Hr mutation was done using primers am05 (viral LTR) 5′-GGTACTGGACGCAGCTTTTCG-3′, am06 (Hr exon 6) 5′-TGTAGCCGTGGGAGCAGGACGAGCATG-3′, and am07 (Hr intron 6) 5′-CTCCTGTTTGGTTGCTAT-3′ which produce a 350 bp product for the wild-type allele and a 250 bp product for the SKH1 mutant allele. For tumor-bearing animals, tumor dimensions were measured with digital calipers and the volume was calculated from the formula \( \pi/6 \times \text{length} \times \text{width} \times \text{height} \).

Complete blood counts

At the academic site, complete blood counts were done on a VetScan HM2 Hematology System (Abaxis). Six mice (3 males and 3 females) ages 160 to 200 days were evaluated per strain. At the commercial site, complete blood counts were done on a Hemavet 950 Veterinary Hematology Analyzer (Drew Scientific). Five SKH1 mice (sexes not specified) ages 180 to 240 days and 68 C57Bl/6 mice (34 males and 34 females) ages 56 to 70 days were evaluated. Statistical analysis was done with the Wilcoxon rank-sum test.

Immunizations

Animals were immunized with a 1:1 emulsion of keyhole limpet hemocyanin (KLH; Sigma-Aldrich) in complete Freund’s adjuvant. Animals received 25 μg of KLH s.c. at the base of the tail. For some groups of mice, draining lymph nodes were collected after 7 days and cell suspensions were made by mechanical disruption and passage through a mesh screen for measurement of cellular responses. Other groups of mice were bled 1 week after immunization and then immunized 30 days after primary immunization with 5 μg KLH in solution i.p. One week after secondary immunization, mice were bled again.

Immunoglobulin and antibody measurements

Serum IgM was measured using a commercial colorimetric enzyme-linked immunosorbent assay (ELISA) assay kit (Immuno-Tek, Zepto-Metrix Corp.). Quantification was by comparison with a standard curve supplied with the kit. Serum IgA was measured using a similar ELISA kit (Alpha Diagnostic International). Specific anti-KLH antibody responses were analyzed using commercial solid phase ELISA kits detecting anti-KLH IgM (5 days postimmunization) and IgG (2 weeks postimmunization; Life Diagnostics, Inc.). Quantification was by comparison with a standard curve using a reference serum supplied with the kits. For each assay, 11 to 15 mice per strain were evaluated. Statistical analysis was done using a two-sample t-test for each assay.

Immunophenotyping

Cell suspensions were made from spleen and thymus by mechanical disruption and passage through a mesh filter. Lymphocyte phenotyping was done using the following directly conjugated monoclonal antibodies, all supplied by the same manufacturer (BD Biosciences): PE-Cy7 hamster anti-mouse CD3ε, APC-Cy7 rat anti-mouse CD4, PerCP-Cy5.5 rat anti-mouse CD8α, APC rat anti-mouse CD25, PE rat anti-mouse CD44, PE rat anti-mouse CD49b, and R-PE rat anti-mouse CD62L. Labeled cells were analyzed using a BD FACSarai instrument (BD Biosciences) in the UTHSCSA core flow cytometry laboratory. Individual small lymphocytes were gated by forward and side light scatter by strategies as presented in Supplementary Fig. S1A to F (in these examples, Supplementary Fig. S1A to C are for spleen and Supplementary Fig. S1D to F are for thymus). One sample each of unstained and single-color stained cells was used to set thresholds for populations of interest. The remaining samples were then analyzed for the
indicated markers by multicolor staining. Supplementary Figure S1B is a representative dot plot of CD3 T cell, and CD19 B cell analysis of spleen, whereas Supplementary Fig. S1E shows an example of CD4 and CD8 single-positive, double-positive, and double-negative T-cell populations in thymus. Supplementary Figure S1C and S1F illustrate the relationship of the subpopulations used to generate the data shown in the tables. For each assay, 5 mice per strain were evaluated. Statistical analysis was done using the Wilcoxon rank-sum test.

Cellular immune responses

Lymph node cells from unimmunized mice were placed in 96-well plastic tissue culture plates and incubated with 0.02 μg/mL phytohemagglutinin for 5 days. Lymph node cells from KLH-immunized mice were incubated in 96-well plates containing titrated doses of KLH for 7 days. Sixteen hours prior to harvest, cells were pulsed with 1 μg tritiated thymidine (²H-TdR). Cells were collected on glass fiber filters and ²H-TdR incorporation was measured by liquid scintillation spectrometry. For each assay, 4 to 5 mice per strain and 14 to 20 overall replicates were evaluated. KLH diluent (vehicle) was used as a negative control and anti-CD3 was used as a positive control. Statistical analysis was done using a linear mixed effects model with random coefficients of intercept and treatment (capturing overall variations among mice and variations due to changes over different treatments). Correlation structure was assumed to be different for two groups. Log-transformation was used on ²H-TdR values to make the data normally distributed.

Delayed-type hypersensitivity assay. C57Bl/6 (n = 5) and SKH1 (n = 5) mice were initially sensitized by a s.c. injection at the base of the tail with 25 μg of KLH in 50 μL of a 1:1 emulsion of PBS:complete Freund’s adjuvant. Seven days later the mice were challenged with 5 μg of KLH in 10 μL PBS injected s.c. in the left ear or with 10 μL PBS injected s.c. in the right ear. Two days later the thickness of both ears was measured to determine the in vivo immune response of the B6 and SKH1.

Quantitative reverse transcriptase-PCR for hairless

Total RNA was isolated from mouse tail tissue using Trizol (Invitrogen) according to the manufacturer's specifications. RNA was then purified using the RNeasy mini prep kit (Qiagen) with the optional DNAse I treatment. Total RNA according to the manufacturer’s instruction. Real-Time PCR System (PE Applied Biosystems). Quantification of Hr expression was done using SYBR Green PCR Master Mix (PE Applied Biosystems) and the forward primer th009 (exon 6) 5′-ACCACCGACTCTTGAAACCACC-3′ and the reverse primer th010 (exon 7) 5′-GGAGACAAACTGGGTCAGGA-3′ to generate a 188 bp product predicted by National Center for Biotechnology Information sequence gi 531706. Expression of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was determined using ABI TaqMan Fast Universal PCR Master Mix, with forward primer 5′-TGACACCACTCCTTAG-3′, reverse primer 5′-GGATGCAGGGATGATGTTC-3′, and probe 5′-FAM-CAGAAGACTGGATGCCCCCTC-TAMRA-3′, and the level of mRNA expression of Hr was normalized to Gapdh. Statistical analysis was done using a two-sample t-test.

Histopathology

Histology using H&E and immunohistochemistry was done as previously described (3). Because tumor heterogeneity and necrosis can bias quantitation of natural killer (NK) cells and macrophages, those cell populations were only quantitated in nonmucosal areas.

Multiplex live animal imaging

Optical imaging for this experiment was carried out using the Xenogen IVIS Spectrum system (Caliper-Lumaspec). For the imaging experiment in Fig. 5, a mouse of the genotype Myf6Cre;R26Ros+/WT, Pax3+/-Fam/WT; Trp53R172Hr/Lusapm/+F2-10 Rosa26LacI-P3Fm/WT, F2-10 Lusapm/WT, HrSKH1/SKH1/+ bearing a lower limb tumor was injected i.v. via tail vein with 100 μL of 0.2 mmol/L IR-820 (Sigma-Aldrich) in PBS as previously described (2) and 100 μL of 2 nmol/L MMPSense 680 (VisEn Medical, Inc.). This mouse was imaged after 24 hours with camera settings at 1-second exposure time, 4 x 4 binning, 12.6 cm field of view, and f/stop of 1/2. The data were acquired and analyzed using the manufacturer's Living Image 3.2 software. The animal was imaged using the same anesthesia protocol of 2% isoflurane in 100% oxygen at 2.5 liters per minute. Body temperature was maintained at 37°C by a heated stage. The images were acquired using epi-illumination at an excitation wavelength of 680 nm and an emission wavelength of 720 nm for MMPSense 680, and at an excitation wavelength of 710 nm and an emission wavelength of 820 nm for IR-820. After recovery on a heatpad, the mouse was injected i.p. with 200 μL of 15 mg/ml D-luciferin (firefly potassium salt). Ten minutes after injection, the mouse was imaged with aforementioned camera settings with no excitation and open emission filter.

Results

SKH1 mice develop progressive, rostral-caudal alopecia

The proviral insertion site of pmv43 into exon 6 of Hr is well defined (Fig. 1A), and thus the recessive SKH1 allele can be readily genotyped by PCR (Fig. 1B; ref. 1). SKH1 mice express 8.5% of the normal full-length Hr transcript (Fig. 1C). This reduction of the correct transcript is the result of aberrant splicing of the Hr gene caused by the provirus (8). Mice develop progressive alopecia that begins at the eyes, forelimbs, and nose, then proceeds caudally by 4 weeks of life (Fig. 1D). This rostrocaudal wave pattern is consistent with the wave pattern of hair follicle activity in mice and has been previously reported.

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for this hr mutant of Hr (15,16). The commercially available SKH1 strain is considered “outbred,” because by single nucleotide polymorphism-based assays approximately 75% of loci are homozygous for one allele and because the colony is maintained by an outbred breeding scheme.8

Blood counts are similar between SKH1 mice and C57Bl/6 mice

Because the commercially available SKH1 mouse strain is considered outbred (having first been established from a colony at the Skin and Cancer Hospital of Temple University in 1986),8 we sought to carry out a full immunologic evaluation of this HairlessSKH1/SKH1 mouse strain in its current outbred state (hereafter referred to as the SKH1 mouse line). As our control mouse line, we chose the C57Bl/6 (hereafter referred to as the B6 mouse line) because C57Bl/6 is most commonly used in murine immunologic studies. We began our immunologic analyses with complete blood counts conducted independently for mice housed at academic and commercial sites (Table 1 and Supplementary Table S1, respectively). Only at the academic site were leukocyte counts different between SKH1 and B6 mice, in that the percentage of lymphocytes was lower in SKH1 than in B6 mice (70 versus 83%, P = 0.05), although absolute lymphocyte counts were not different between strains. No other statistically significant trends were seen in independently compared complete blood counts. All subsequent experimental data for the SKH1 and B6 were obtained at the academic site.

Humoral immunity of SKH1 mice is comparable with C57Bl/6 at baseline and after immunization

The next step of our evaluation was to examine humoral immunity. For immunoglobulins, baseline IgM levels were lower in SKH1 than in B6 mice (Fig. 2A; 2.66 versus 3.49 ng/mL, P < 0.0001). Baseline serum IgG and IgA levels were not significantly different (Fig. 2B and C). CD19-positive B cells from the spleen were comparable between SKH1 and B6 mice (Fig. 2D). Following immunization with KLH antigen, levels of anti-KLH IgM were lower in 5 days postimmunization for SKH1 mice when compared with B6 mice (Fig. 2E; 0.12 versus 0.11 ng/mL, P = 0.02), consistent with decreased baseline IgM levels for SKH1 mice. However, 2 weeks postimmunization anti-KLH IgG levels were appropriately elevated and similar in B6 and SKH1 mice (Fig. 2F). Thus, humoral immunity was similar between SKH1 and B6 mice except for lower IgM (but not IgG) levels in SKH1 mice.

Alterations in T-cell subset expression in SKH1 mice were not associated with functional defects

We evaluated T-cell subset distribution in thymus and spleen using multiplex flow cytometry. In comparison with B6, the numbers and percentages of the major thymocyte subsets CD4/CD8 double-negative, double-positive, and single-positive CD4 and CD8 subsets were not significantly different in SKH1 mice (Fig. 3A). These results suggested that the effect of the hr mutation on epithelial cells does not disturb thymocyte differentiation.

In the peripheral lymphoid compartment, represented by the spleen, the number of CD3+ cells was mildly increased (Fig. 3B; 2.3 versus 4.5 × 10^3 cells/spleen in SKH1

### Table 1. Hematologic assessment of HairlessSKH1/SKH1 mice

<table>
<thead>
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<th>C57Bl/6</th>
<th>HairlessSKH1/SKH1</th>
<th>P</th>
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<tr>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td></td>
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<tr>
<td>WBC count (×10^3 cells/μL)</td>
<td>5.04 (1.84)</td>
<td>7.04 (1.28)</td>
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<td>Lymphocyte count (×10^3 cells/μL)</td>
<td>4.25 (1.71)</td>
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<td>Monocyte count (×10^3 cells/μL)</td>
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<td>0.27 (0.17)</td>
<td>0.11</td>
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<td>% lymphocytes</td>
<td>83.20 (5.22)</td>
<td>70.12 (9.47)</td>
<td>0.05</td>
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<tr>
<td>% of monocytes</td>
<td>2.03 (1.20)</td>
<td>3.97 (2.76)</td>
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<tr>
<td>RBC count (×10^12 cells/μL)</td>
<td>10.89 (0.34)</td>
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<td>Hematocrit</td>
<td>40.63 (1.92)</td>
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<td>Mean corpuscular volume (fL)</td>
<td>37.33 (1.21)</td>
<td>41.83 (1.83)</td>
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<td>Mean corpuscular hemoglobin (pg)</td>
<td>14.20 (0.54)</td>
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<td>Mean corpuscular hemoglobin concentration (g/dL)</td>
<td>38.03 (1.00)</td>
<td>37.73 (1.16)</td>
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<tr>
<td>RBC distribution width (%)</td>
<td>20.32 (0.52)</td>
<td>17.83 (1.01)</td>
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<tr>
<td>Platelet (×10^3 cells/μL)</td>
<td>414.00 (117.13)</td>
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<td>Mean platelet volume (fL)</td>
<td>6.08 (0.12)</td>
<td>6.27 (0.26)</td>
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</table>

NOTE: Six mice (3 males and 3 females) ages 160 to 200 days were evaluated per strain. Statistical approaches are described in Materials and Methods. Values in bold indicate indices exhibiting significant differences between C57Bl/6 and Hairless mice.

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8 CB Clifford, personal communications.
versus B6 mice, \( P = 0.02 \)), whereas the major CD4 and CD8 subsets were decreased (Fig. 3B). This finding was correlated primarily with a significant decrease in naïve T cells (Fig. 3B; 1.7 versus 3.4 \( \times 10^5 \) cells/spleen in SKH1 versus B6 mice, \( P = 0.008 \)) and memory T cells (Fig. 3B; 1.4 versus 0.13 \( \times 10^6 \) cells/spleen in SKH1 versus B6 mice, \( P = 0.008 \)). CD25+ regulatory T-cell numbers were also mildly decreased (Fig. 3B; 6.0 versus 4.7 \( \times 10^5 \) cells/spleen in SKH1 versus B6 mice, \( P = 0.04 \)), consistent with the mild reduction in peripheral lymphocytes seen in complete blood counts. Despite these changes in peripheral T-cell numbers and subset distribution, lymph node in vitro proliferative response to antigen rechallenge (KLH) was preserved (Fig. 3C). Likewise, positive control responses to anti-CD3 stimulation were similar to B6, with SKH1 exhibiting a significant increase even compared with B6 (Fig. 3C).

To verify that the in vitro T cell–dependent immune response correlated to in vivo immune functions, a delayed-type hypersensitivity assay was done using KLH, the same antigen used for the in vitro tests. Inflammatory response to KLH following initial sensitization and subsequent challenge did not differ significantly between the SKH1 and the B6 mice (Fig. 3D).

**Tumor histology for tumor-bearing GEMM mice with or without hr mutations is comparable**

To evaluate dendritic cell function in tumor-bearing mice harboring homozygous SKH1 mutations (HR SKH1/SKH1), we carried out histology and immunohistochemistry on tumors from **Myf6CreNcbi/WT Pax3Pax5Fm/Pax5Fm Trp53F2-10/Trp53F2-10 Rosa26Rosa26** mice harboring wild-type HR alleles or homozygous SKH1 mutation (Fig. 4). The tumors were essentially indistinguishable at the histologic level between the two groups. The tumors in both groups were composed of sheets of epithelioid cells arranged in nests and sheets. The lesional cells had enlarged, rounded, or polygonal nuclei with clumpy chromatin and variably prominent nucleoli. There was significant nuclear pleomorphism and variable numbers of larger cells with more abundant cytoplasm consistent with rhabdomyoblasts. Mitotic activity including atypical mitotic figures was brisk. Histologic and immunohistochemical features of myogenin positivity were unchanged by the presence of homozygous SKH1 mutation (\( n = 4 \) per cohort; representative results are given in Fig. 4A and B). Both cohorts of mice had varying degrees of tumor-associated necrosis that influenced the degree of CD56+ NK cell or CD163+ macrophage infiltration (Fig. 4C and D). The range for the maximum number of CD56+ cells...
seen per tumor in a nonnecrotic field of view was 3 to 189 versus 13 to 276 for SKH1 versus control, respectively (the rhabdomyosarcoma control animals are on a mixed background of C57Bl/6 and SV/J129). Similarly, for CD163+ cells the range was 12 to 99 versus 64 to 101 for SKH1 versus control, respectively. Thus, the presence or absence of CD56+ NK cells and CD163+ macrophages in tumors was unaffected by SKH1 mutation. Tumor growth rates were also indistinguishable between mice bearing alveolar rhabdomyosarcoma tumors whether these mice carried 0 or 2 SKH1 mutations at the Hr locus \((P = 0.95; \text{Supplementary Fig. S2})\). Tumors in mice homozygous for SKH1 mutations were often also easier to monitor as a result of furlessness.

The SKH1 mouse line allows multiplex optical imaging

As proof of principle that interbreeding homozygous SKH1 mutation into a genetically engineered model can improve optical imaging of tumor-bearing mice, multiplex imaging was done to detect viable rhabdomyosarcoma tumor cells from a luciferase allele (Rosa26\(^{lucapm}\)) while concurrently examining capillary leak using IR-820 (2) and matrix metalloprotease activity in the same animal (Fig. 5A and B). Viable tumor cells were seen at the periphery of the tumor, as well as at an ectopic site in the left upper quadrant (Fig. 5A and B, panels second from left, arrows). Capillary leak was seen throughout the left lower limb (Fig. 5A and B, panels second from right), but matrix metalloprotease activity was seen only in the tumor’s proximally advancing edge at the ankle (Fig. 5A and B, rightmost panels, arrows). MMPsense signal in the abdomen was attributed to alfalfa-containing mouse chow (Supplementary Fig. S3). At necropsy, the metastatic lesion was determined to be a lymph node (Fig. 5C), and the primary tumor and lymph node

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**Figure 3.** Cellular immunity of SKH1 versus B6 mice. A, thymus cell counts for T-cell subpopulations. No statistical differences were found. B, peripheral (splenic) cell counts for CD3 T cells, CD8 T cells, CD49+ NK cells, CD4+CD25+ regulatory T cells, CD44\(^{lo}\) CD62L\(^{hi}\) naive T cells, and CD44\(^{hi}\) CD62L\(^{lo}\) memory T cells. C, ex vivo leukocyte proliferation following KLH immunization. Pos, positive; Neg, negative; CTL, control. D, in vivo immune response following KLH immunization (no significant difference was seen between B6 and SKH1 mice). Data presented in A or B are absolute numbers of cells per thymus or spleen, respectively. For all experiments, \(n = 5\) for B6, \(n = 5\) for SKH1.
metastasis were both histopathologically diagnosed as alveolar rhabdomyosarcoma (Fig. 5D-I).

Discussion

In our studies we have presented an immunologic evaluation of the Hairless<sup>SKH1</sup>/SKH1 mouse (SKH1 strain) in comparison with C57Bl/6 controls. In the current outbred state of the SKH1 strain, overall blood leukocyte counts and differential were essentially similar to C57Bl/6. In the evaluation of humoral immunity, a consistent but mild decrease in preimmunization and postimmunization IgM levels was found in SKH1 mice, but postimmunization IgG levels were the equivalent to C57Bl/6. The evaluation of cellular immunity revealed statistically significant modest differences in several lymphocyte subsets, but, importantly, a functional proliferation assay of immunized lymphatic cells restimulated with a foreign antigen ex vivo was as robust for SKH1 mice as for C57Bl/6 mice. When homozygosity for the Hairless<sup>SKH1</sup> mutation was interbred to a conditional, genetically engineered mouse model of alveolar rhabdomyosarcoma, the degree of macrophage and NK cell infiltrates were not altered. In addition, tumor phenotype (histology and tumor growth rate) was unchanged. Thus, the Hairless<sup>SKH1</sup> mutation represents a heritable, easily genotyped fur-free phenotypic trait that does not significantly alter immune function for preclinical disease models. Furthermore, the fur-free nature of genetically engineered mice harboring homozygous Hairless<sup>SKH1</sup> mutation is especially amenable to multiplex optical imaging that can analyze tumor extent, capillary integrity, and biochemical (enzymatic) activity in vivo concurrently and/or serially.

The SKH1 mouse represents one of a spectrum of hypomorphic (mild), autosomal recessive mutations in the <i>Hr</i> gene that nevertheless result in alopecia (recently reviewed in ref. 17). Mice with severe mutations in the Hairless gene are designated Rhino (also called, hrrh). A targeted null allele of Hairless is very similar to Rhino (5). Historically, humoral and cellular immunity evaluations of Hairless mice were done as early as 1974 (18–20); however, a contemporary evaluation of the SKH1 strain in its current outbred state has not been carried out. Here, we establish the SKH1 strain to be functionally immune competent in its current outbred state.

Reports dating back to 1969 suggest an increased incidence of T-cell leukemia in 8- to 10-month-old <i>hr/hr</i> mice (21, 22), which has been attributed to cooperativity with other endogenous viruses (21, 23), but not reactivation of <i>pmv43</i> because the integrated provirus would not be expected to itself reactivate. However, in a commercially operated isolator-based breeding colony of more than 500 breeders, observed daily and routinely kept to approximately 6 to 8 months of age, no mice with lymphoproliferative neoplasia have been found in the last 5 years (CB Clifford, personal communications). This dramatic difference in the rate of hematopoietic cancer may reflect either a change in the endogenous (retroviral) burden over time, or that environmental factors in the past led to more lymphoma than is now present. Mouse colony environments are now more controlled than in the past, and thus infectious and noninfectious causes of chronic inflammation are fewer, which we speculate could influence the rates of lymphoma in predisposed strains. The improved ability to discern lymphoma from reactive lymphoid proliferation due to infectious causes (e.g., mycoplasma) is another possible contributor to the differences in apparent lymphoma rates over time. However, a genetic predisposition to leukemia may nonetheless be present, given that homozygous <i>hr</i> mice historically develop leukemias at much higher incidence than heterozygous <i>hr</i> mice or wild-type mice (21–23). Knowing if or how the Hairless gene functions as a tumor suppressor in the hematopoietic lineage remains an interesting future topic of study. Whether a Hairless mutation would confound each specific genetically engineered mouse model of human cancer would need to be investigated on a
Figure 5. Multiplex optical imaging of a Hairless rhabdomyosarcoma model. A, left to right: photograph, luciferase luminescence (scale $2.8 \times 10^5$ to $8.0 \times 10^8$ p/s/cm$^2$/sr), IR-820 fluorescence (scale $3.2 \times 10^5$ to $1.6 \times 10^9$ p/s/cm$^2$/sr), and MMPsense fluorescence (scale 0 to $2.8 \times 10^9$ p/s/cm$^2$/sr) for a mouse bearing a rhabdomyosarcoma of the left foot. Viable tumor is shown with arrowheads. *, metastatic lesion. MMPsense activity is shown by an arrow at the advancing front of the tumor. Apparent MMPsense signal in the abdomen is artifact attributable to alfalfa in mouse chow. B, repeat imaging for a more restricted field of view. Scales: luminescence ($2.8 \times 10^4$ to $8.6 \times 10^8$ p/s/cm$^2$/sr), IR-820 fluorescence ($2.1 \times 10^7$ to $3.4 \times 10^9$ p/s/cm$^2$/sr) and MMPsense fluorescence (0 to $4.9 \times 10^9$ p/s/cm$^2$/sr). C, necropsy photomicrograph showing an enlarged s.c. lymph node that overlay the spleen. D, H&E of primary tumor consistent with rhabdomyosarcoma (×400). E to F, immunohistochemistry positive for nuclear myogenin and cytoplasmic desmin, respectively (×400). G, H&E of lymph node shown in A, B, and C (×20). H, close-up of G (×200). I, myogenin-positive rhabdomyosarcoma cells in the lymph node (×400).
case-by-case basis. In the case of rhabdomyosarcoma, the biology of tumors seems essentially unchanged in the cohorts that we examined. Moreover, although the role of Hairless (a nuclear receptor corepressor) is becoming increasingly well characterized at the molecular level (5, 24), the role of Hairless as a tumor-initiating mutation in the absence of a secondary carcinogen is less obvious in mouse or man (OMIM 602302 and ref. 11). Fortunately, however, from an experimental point of view, the proviral integration would be expected to be stable; although the pmv43 integration into the Hr locus was first discovered by a rare revertant resulting from recombination of the long terminal repeats (1), the rate of reversion of MuLV proviruses has been shown to be very low in examples such as the d^2 coat color insertional mutation in DBA mice (a somatic reversion rate of 9 × 10^{-7} events per animal; ref. 25). The driving purpose of this immunologic evaluation of the SKH1 mouse strain was to characterize a furlessness trait that does not interfere with immune competence but could be genotyped and interbred to genetically engineered mouse models of human cancer so that optical imaging could be improved. As stated earlier, fur reduces luminescent and fluorescent reporter gene signal by >10-fold (3). Heritable furlessness would have utility in both academic medicine and the pharmaceutical industry where preclinical therapeutic studies using genetically engineered mice are of increasing importance. The alternative use of depilatory agents is time intensive, can be associated with hypothermia, and often leads to hyperpigmented macules that themselves attenuate fluorescent or luminescent signal emanating from tumors. The C57Bl/6J-Tyrc-2J/J mouse strain, which carries an autosomal recessive Tyrosinase (Tyr) mutation, provides another alternative to SKH1 mice, in that C57Bl/6J-Tyr^{-2}/J mice are albino and their fur is unpigmented (26). Use of C57Bl/6J-Tyr^{-2}/J mice carries a different set of considerations, in that these mice undergo retinal degeneration (27) and their use does not necessarily overcome the entirety of reporter gene signal attenuation or scatter associated with fur. Thus, the SKH1 mouse line presents one very practical approach to improved optical imaging in genetically engineered mice without sacrificing immune competence. We believe the SKH1 mouse line will be of significant value to cancer research. We recognize that the use of Hairless mutation mice for small animal optical imaging is already extending to fields outside oncology (28, 29), and we hope that this contemporary immunologic evaluation will extend use of the SKH1 immunocompetent, fur-free mouse strain for a multitude of preclinical cancer studies using GEMMs.

**Disclosure of Potential Conflicts of Interest**

C. Keller: funding in part was provided by Charles River Laboratories for this investigator-initiated study. No other potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Dr. Thom Saunders, Dr. John Sundberg, Dr. John Coffin, and Dr. Jonathan Stoye for advice in these studies, and Lee Ann Zarzabal for statistical support.

**Grant Support**

Funding for this investigator-initiated study was provided in part from Charles River Laboratories and 5R01CA133229-02 to C. Keller. C. Keller is a member of the Cancer Therapy Research Center (2P30CA054174-17). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 03/01/2010; revised 05/20/2010; accepted 06/11/2010; published OnlineFirst 07/27/2010.

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