haematologica
Journal of the Ferrata Storti Foundation

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*Received: June 24, 2024.*
*Accepted: September 13, 2024.*

*Citation: Sheyar Abdullah, Anthony Sabulski, Zahra Hudda, Assem Ziady, Nathan Luebbering, Lucy Giordullo, Elizabeth Odegard, Jason T. Blackard, Steve Kleiboeker, Michelle Altrich, Sonata Jodele, Alix E. Seif, Stella M. Davies, and Benjamin L. Laskin. Susceptibility to BK polyomavirus-associated hemorrhagic cystitis in children undergoing allogeneic transplant. Haematologica. 2024 Sept 19. doi: 10.3324/haematol.2024.286163 [Epub ahead of print]*

**Susceptibility to BK polyomavirus-associated hemorrhagic cystitis in children undergoing allogeneic transplant**

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**Running head:** Susceptibility to BK cystitis after transplant

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**Data sharing statement:** Requests for data can be sent by email to the corresponding author

**Funding:** This work was supported by National Institutes of Health grant R01 DK125418 to BLL and JTB

**Disclosure of Conflicts of Interest (related to this work)**

Steve Kleiboeker and Michelle Altrich are employees of Eurofins Viracor who ran the BK polyomavirus PCR testing on blood and urine samples free of charge. No other authors have a relevant conflict of interest.

**Authorship contributions**

SA designed research studies, conducted experiments, analyzed data, and wrote the manuscript. AS designed research studies, analyzed data, acquired data, and critically reviewed the manuscript. ZH acquired data, analyzed data, and critically reviewed the manuscript. AZ designed research studies, analyzed data, and critically reviewed the manuscript. NL designed research studies, conducted experiments, and analyzed data. LL acquired data. EO and JB designed research studies, analyzed data, and critically reviewed the manuscript. SK and MA conducted experiments, acquired data, and critically reviewed the manuscript. SJ and AS designed research studies, acquired data, analyzed data, and critically reviewed the manuscript. SD designed research studies, analyzed data, and wrote the manuscript. BL designed research studies, acquired data, analyzed data, and wrote the manuscript.

BK polyomavirus (BKPyV) detected after hematopoietic stem cell transplant (HSCT) is associated with hemorrhagic cystitis.[1] Cystitis causes dysuria and may lead to urinary obstruction and an increased risk of death.[2] We prospectively studied 193 children and young adults at two large pediatric centers and observed that BKPyV was frequently detected in the urine (viruria=87%) and blood (DNAemia=50%) after allogeneic HSCT, but only 22% developed cystitis.[3] Reported risk factors for cystitis include cyclophosphamide, busulfan, mismatched human-leukocyte antigen (HLA) grafts, DNAemia, log-fold increases in viruria, and delayed immune reconstitution.[2] However, the exact mechanisms leading to BKPyV-associated cystitis and the reasons why only some patients with BKPyV develop cystitis are unknown. We hypothesized that host factors would be associated with cystitis and focused on polymorphisms in innate viral defense proteins including apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC3)[4] and toll-like receptor 3 (TLR3).[5] To test our hypothesis, we analyzed stored blood samples from our existing cohort. Our cohort of 193 subjects, ≥2 years old, were prospectively enrolled before allogeneic HSCT at the Children's Hospital of Philadelphia or Cincinnati Children's Hospital Medical Center from April 2013 to May 2018.[3] Cystitis was assessed by reviewing the medical record and was defined as grade ≥2: detectable BKPyV with urinary symptoms and visible hematuria.[2] The Institutional Review Boards at our two centers approved the study and participants provided informed consent and assent. Eurofins Viracor (Lenexa, KS) previously tested blood and urine samples from subjects for BKPyV using a quantitative polymerase chain reaction (PCR).[3] To test if genetic polymorphisms in viral defense proteins were associated with susceptibility to cystitis, genomic DNA was extracted from neutrophils collected pre-HSCT, to reflect recipient genome, using the QuickGene extraction system (Autogen, Holliston, MA). The presence of a 29.5 kb deletion in the *APOBEC3* gene was assessed with a PCR breakpoint assay[6] using a TaqMan PCR Master Mix kit (Qiagen, Hilden, Germany). Briefly, separate PCR assays were performed with primers specific to the deletion (700bp product) and insertion (490bp product) alleles. These products were analyzed using a 1.5% agarose gel. Analyses of single nucleotide polymorphisms (SNPs) in other viral defense genes (*TLR3*, melanoma differentiation-associated protein 5 [*MDA5*], retinoic acid-inducible gene I [*RIG1*], and interferon regulatory factor 3 [*IRF3*])[5,7] were performed using TaqMan SNP genotyping mix and assays (Applied Biosystems, Waltham, MA). To measure the expression of *APOBEC3A* and *APOBEC3B* genes, quantitative PCR was performed on RNA extracted from mononuclear cells pre-HSCT. Plasma APOBEC3B concentrations pre-HSCT were measured using enzyme-linked immunosorbent assays

(MyBioSource, San Diego, CA), with limits of detection ranging from 0.625 to 20 ng/mL. We performed a correlative murine study to test if differential expression of TLR3 would explain the lower risk of cystitis in younger children. Wild-type murine formalin-fixed paraffin embedded bladder biopsies were sectioned at 4.5 µm onto positive charged slides and baked at 60 degrees for 30 minutes. Slides were stained using the BenchMark DISCOVERY platform with a TLR3 antibody (Abcam, 62566) at 1:200 using a citrate HIER pretreatment and a 40 min incubation. Continuous variables are presented as medians (interquartile range, IQR) and were examined with the Wilcoxon rank-sum test or signed-rank test. Categorical variables were examined with the Chi-square, Fisher's exact, or Cochran-Armitage test. The independent variable was defense protein polymorphisms. Cystitis was the primary outcome and secondary outcomes were BKPyV at 1-month post-HSCT, separately defined as DNAemia (>0 copies/mL) or billionuria (>$10^9$ copies/mL). The study was designed to have 90% power to detect an odds ratio of 2.5 for cystitis in children with a deleted *APOBEC* allele compared to wild type. Analyses were performed using STATA (17.0), and a two-sided p-value <0.05 was considered significant. The 193 subjects had a median age of 10.2 years and 58.0% were male. The most common underlying diagnoses were malignancy (37%), bone marrow failure (27%), and immunodeficiency (21%). Conditioning included cyclophosphamide and busulfan in 56.5% and 43.5% of subjects, respectively (28.0% received both). Acute graft versus host disease developed in 38.3% of subjects. As we previously published, by 1-month post-HSCT, 59/162 (36.4%) participants had BKPyV viruria ≥$10^9$ copies/mL (billionuria), and 73/185 (39.5%) had DNAemia >0 copies/mL. Grade ≥2 cystitis developed in 43/193 (22.3%) subjects at a median of 34 days (IQR 25-54 days) post-HSCT.[3] The *APOBEC3* deletion polymorphism (Figure 1A) was detected in 28/174 (16.1%) recipients in their pre-HSCT DNA. The remaining 146/174 (83.9%) recipients did not have the deletion polymorphism and 19/193 (9.8%) could not be assessed. As shown in Figure 1B, median recipient pre-HSCT *APOBEC3A* expression – reported as the inverse of the delta cycle time – was significantly decreased in subjects with the deletion (0.054 [IQR 0.052-0.055]) as compared to those with the wild type (0.058 [IQR 0.056-0.060], p<0.0001). Median *APOBEC3B* expression was decreased in subjects with the deletion (0.039 [IQR 0.038-0.042]) as compared to those with the wild type (0.041 [IQR 0.040-0.044], p=0.07). Measuring protein expression (Figure 1C), we observed that pre-HSCT, median plasma APOBEC3B concentrations were significantly decreased in subjects with the deletion (0.27 ng/mL [IQR 0-1.13 ng/mL]) compared to those without the deletion (0.89 ng/mL [IQR 0.65-1.52 ng/mL], p=0.04). The presence of the

deletion was not associated with billionuria (p=0.27) or DNAemia (p=0.45) 1-month post-HSCT. However, the *APOBEC3* deletion was more frequent among subjects who later developed cystitis (odds ratio 2.7, 95% confidence interval 1.2-6.5, p=0.02). SNPs in the viral sensor genes *MDA5, RIG1, IRF3, and TLR3* were also genotyped using recipient pre-HSCT samples (Table 1). Subjects with an A/A genotype at *TLR3* SNP rs5743305 had a lower risk of cystitis compared to those with an A/T or T/T genotype (p=0.02). The other three tested *TLR3* SNPs were not associated with cystitis. The tested *IRF3*, *MDA5*, and *RIG1* SNPs were also not associated with cystitis. We previously observed that older age was associated with a higher risk of cystitis.[3] Of the 38 children <6 years of age in our cohort, only 5/38 (13.2%) developed cystitis and no child <4 years of age developed cystitis. Expression of TLR3 in specific organs varies by age but the bladder has not been studied.[7,8] We therefore hypothesized that age-dependent TLR3 expression may be associated with cystitis. Due to challenges in accessing bladder tissue from healthy children, we performed an immunohistochemistry analysis of TLR3 in bladders obtained from mice at day 3, day 21, and month 4 of age (Figure 2). TLR3 was highly expressed in all layers of the neonatal bladder. Expression remained constant in the urothelium but declined in other layers of the bladder with age, being largely absent in the submucosa of adult mice. The mechanisms leading to cystitis are understudied. It is known that cellular immune responses are important to control BKPyV after HSCT and solid organ transplant.[9] We hypothesized that polymorphisms in innate immune proteins would be associated with cystitis after HSCT. We observed that polymorphisms in defense (*APOBEC*) and sensor (*TLR3*) proteins were associated with susceptibility to cystitis. APOBEC3 are DNA cytosine deaminases that provide antiviral defenses by mutating viral genomes.[10] Importantly, others have shown that expression of BKPyV large T-antigen up-regulates *APOBEC3B* expression in cultured cells, supporting an important, although perhaps paradoxical, relationship between virus and host.[11] We established that the *APOBEC* deletion was associated with reduced RNA and protein expression of *APOBEC3A* and *3B*, in agreement with a single prior study.[12] Homozygosity for the *APOBEC* deletion is infrequent in population studies, and we observed no homozygotes in our study.[6] We then examined polymorphisms in other anti-viral defense proteins that occur with a clinically relevant minor allele frequency to identify additional relevant genetic variants. We identified an increased risk of cystitis associated with a functional SNP in *TLR3* (rs5743305), but no such variants in *MDA5*, *RIG1*, or *IRF3*. rs5743305 has been associated with infections in patients with leukemia[13] and clearance of hepatitis B virus.[14] This SNP is located in the promoter of *TLR3*.

Work by others suggests reduced transcription from the variant allele in the setting of breast cancer, providing support that our observation is biologically relevant and not a result of testing multiple comparisons.[15] Cystitis rarely occurs in young children and older age was associated with an increased risk of cystitis in our cohort.[3] Age-dependent expression of *TLR3* in the intestines of small children is associated with susceptibility to rotavirus.[7] These data led us to speculate that bladder expression of TLR3 might also be age-dependent, and as younger children infrequently develop cystitis, that expression of TLR3 will decrease with age. We examined the expression of TLR3 in healthy mice at the equivalent of infant, adolescent, and adult ages and observed decreases in submucosal bladder TLR3 expression in the older mice. A strength of our study is the availability of samples collected prospectively from a large, clinically annotated cohort of children receiving HSCT, which we believe is the first such study ever undertaken. An important challenge in studying BKPyV-associated cystitis is the lack of an animal model, increasing the value of our carefully constructed human cohort. In summary, our findings offer potential novel approaches to protection from BKPyV-associated cystitis, potentially by induction of relevant innate immune host defenses.

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**Table 1. Risk of hemorrhagic cystitis by immune defense protein genotype**

| | Risk of hemorrhagic cystitis by genotype | | | p-value |
|---|---|---|---|---|
| ***MDA5*** | | | | |
| rs35667974 | T/T (n = 170) | T/C (n = 5) | C/C (n = 1) | |
| HC | 40 (23.5%) | 1 (20.0%) | 0 (0%) | 0.61 |
| rs1990760 | T/T (n = 56) | T/C (n = 73) | C/C (n = 47) | |
| HC | 13 (23.2%) | 17 (23.3%) | 11 (23.4%) | 0.98 |
| rs3747517 | C/C (n = 95) | T/C (n = 63) | T/T (n = 18) | |
| HC | 20 (21.1%) | 15 (23.8%) | 6 (33.3%) | 0.30 |
| rs35744605 | C/C (n = 175) | A/C (n = 1) | - | |
| HC | 41 (23.4%) | 0 (0%) | - | 1.0 |
| ***RIG1*** | | | | |
| rs9695310 | G/G (n = 43) | G/C (n = 91) | C/C (n = 42) | |
| HC | 11 (25.6%) | 19 (20.9%) | 11 (26.2%) | 0.95 |
| rs3739674 | G/G (n = 30) | G/C (n = 77) | C/C (n = 69) | |
| HC | 8 (26.7%) | 16 (20.8%) | 17 (24.6%) | 0.98 |
| ***IRF3*** | | | | |
| rs7251 | C/C (n = 65) | G/C (n = 79) | G/G (n = 27) | |
| HC | 16 (24.6%) | 21 (26.6%) | 3 (11.1%) | 0.29 |
| rs2304205 | A/A (n = 87) | A/C (n = 60) | C/C (n = 21) | |
| HC | 23 (26.4%) | 14 (23.3%) | 3 (14.3%) | 0.27 |
| ***TLR3*** | | | | |
| rs5743305 | T/T (n = 71) | A/T (n = 89) | A/A (n = 16) | |
| HC | 22 (31.0%) | 18 (20.2%) | 1 (6.3%) | 0.02 |
| rs3775291 | C/C (n = 93) | T/C (n = 63) | T/T (n = 20) | |

| | | | | |
|---|---|---|---|---|
| HC | 20 (21.5%) | 16 (25.4%) | 5 (25.0%) | 0.60 |
| rs3775290 | C/C (n = 97) | T/C (n = 62) | T/T (n = 17) | |
| HC | 21 (21.7%) | 14 (22.6%) | 6 (35.3%) | 0.33 |
| rs3775296 | C/C (n = 116) | A/C (n = 53) | A/A (n = 7) | |
| HC | 23 (19.8%) | 16 (30.2%) | 2 (28.6%) | 0.16 |

Data shown as n (% with hemorrhagic cystitis [HC]) and p-values calculated with Cochran-Armitage test for trend except for *MDA5* rs35744605 (Fisher exact test). Genotypes are ordered in the columns as homozygote-heterozygote-homozygote at each locus.

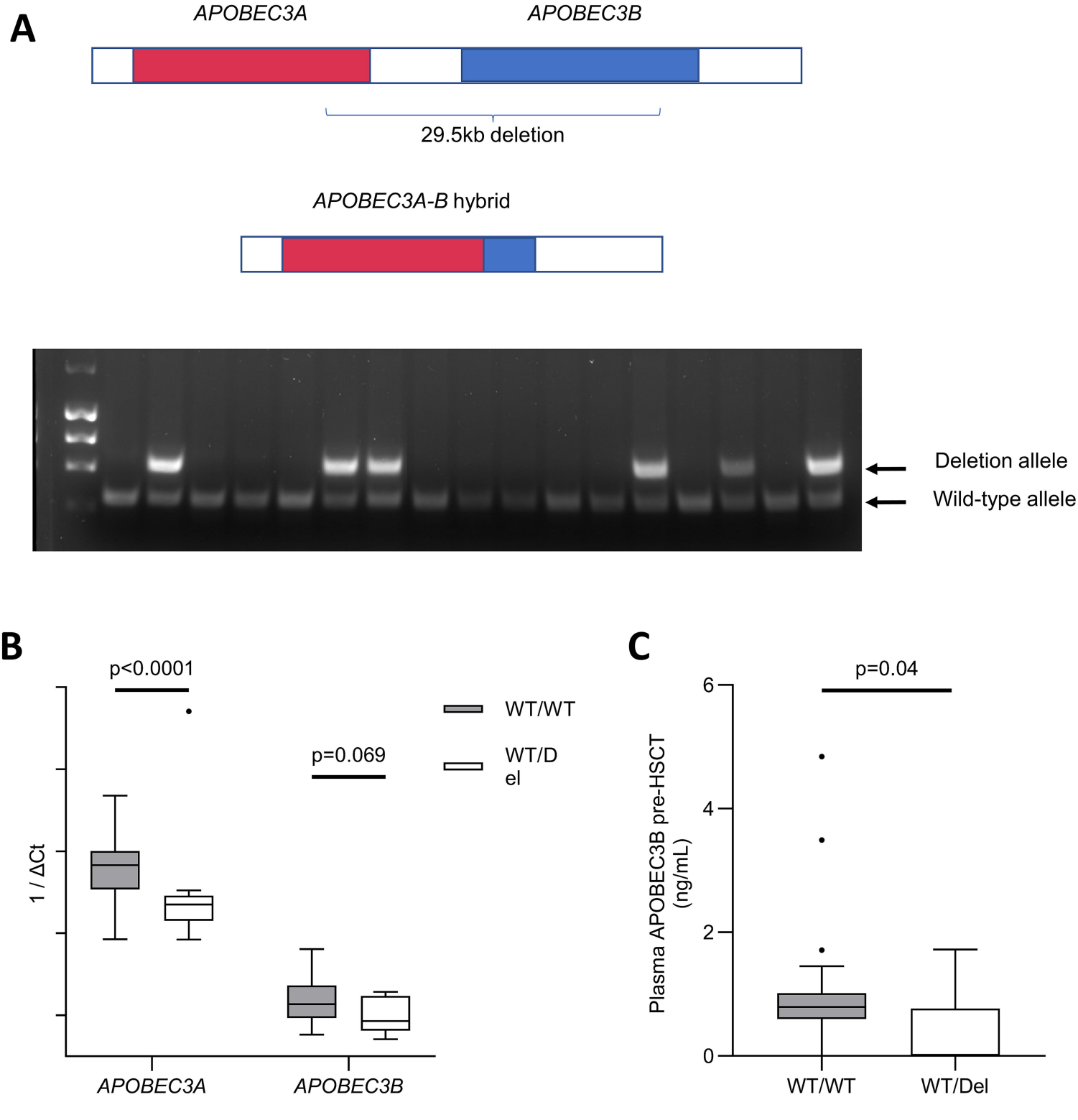
**Figure Legends**

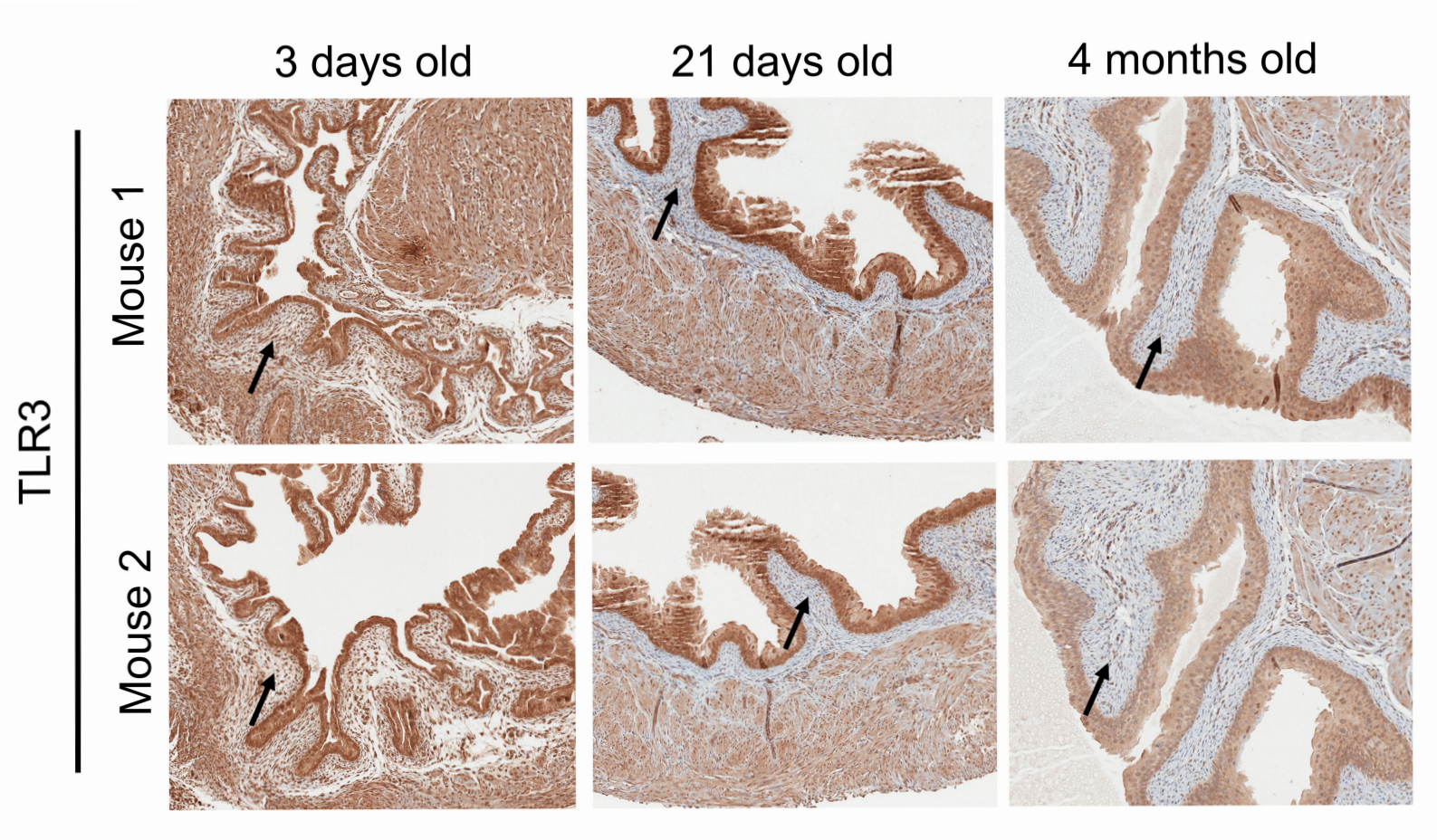
**Figure 1. *APOBEC3* deletion polymorphism**

(A) A 29.5kb region spanning *APOBEC3A* and *APOBEC3B* is removed, forming a hybrid transcript that is distinguishable using a PCR assay, the results of which are shown on a 1.5% agarose gel. The deletion polymorphism is associated with (B) a significant reduction in *APOBEC3A* expression ($p<0.0001$) and reduced *APOBEC3B* expression ($p=0.069$) and (C) significantly lower levels of plasma APOBEC3B concentration before allogeneic transplant ($p=0.04$) (p-values by Wilcoxon rank-sum).

**Figure 2. Expression of TLR3 in healthy mouse bladder tissue varies by age**

The figure shows representative images of immunohistochemistry staining of TLR3 (brown staining indicates TLR3 expression and arrows show submucosa) in two neonatal (3 days old), two adolescent (21 days old), and two adult (4 month) mice bladders. TLR3 is highly expressed in all layers of the neonatal bladder as demonstrated by the diffuse brown staining in the 3-day-old mouse bladders. Expression remains constant in the urothelium but declines with age, as shown by the decreased intensity of the staining in the 21-day-old and 4-month-old mouse bladders. TLR3 was absent in the lamina propria and muscularis of the 21-day-old and 4-month-old mice (blue staining with arrows).


A
APOBEC3A
APOBEC3B
29.5kb deletion
APOBEC3A-B hybrid
Deletion allele
Wild-type allele
B
p<0.0001
p=0.069
WT/WT
WT/Del
1 / ΔCt
APOBEC3A
APOBEC3B
C
p=0.04
Plasma APOBEC3B pre-HSCT (ng/mL)
6
4
2
0
WT/WT
WT/Del


3 days old
21 days old
4 months old
TLR3
Mouse 1
Mouse 2