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Possible Role of a Cell Surface Carbohydrate in Evolution of Resistance to Viral Infections in Old World Primates

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Due to inactivation of the α1,3-galactosyltransferase gene (*GGTA1*, or the α1,3GT gene) approximately 28 million years ago, the carbohydrate αGal (Galα1,3Galβ1,4GlcNAc) is not expressed on the cells of Old World monkeys and apes (including humans) but is expressed in all other mammals. The proposed selective advantage of this mutation for these primates is the ability to produce anti-Gal antibodies, which may be an effective immune component in neutralizing αGal-expressing pathogens. However, loss of α1,3GT expression may have been advantageous by providing natural resistance against viral pathogens that exploited the α1,3GT pathway or cell surface αGal for infection. Infections of paired cell lines with differential expression of α1,3GT showed that Sindbis viruses (SINV) preferentially replicate in α1,3GT-positive cells, whereas herpes simplex viruses type 1 and type 2 (HSV-1 and HSV-2) preferentially grow in cells lacking α1,3GT. Viral growth and spread correlated with the ability of the different viruses to successfully initiate infection in the presence or absence of α1,3GT expression. GT knockout (KO) suckling mice infected with SINV strains (AR339 and S.A.AR86) experienced significant delay in onset of disease symptoms and mortality compared to wild-type (WT) B6 suckling mice. In contrast, HSV-2-infected GT KO mice had higher viral titers in spleen and liver and exhibited significantly more focal hepatic necrosis than WT B6 mice. This study demonstrates that α1,3GT activity plays a role in the course of infections for certain viruses. Furthermore, this study has implications for the evolution of resistance to viral infections in primates.

The enzyme α1,3-galactosyltransferase (α1,3GT), coded for by the *GGTA1* gene, is responsible for the addition of the terminal galactose (Gal) unit to αGal-capped carbohydrate chains (Galα1,3Galβ1,4GlcNAc) found on cell surface glycoproteins and glycolipids in the tissues of most mammals (1, 2). Catarrhines (Old World monkeys and apes, including humans) are unique among mammals because they do not express αGal on their cell surfaces. Among these primates, several mutations are found on the *GGTA1* locus that result in its inactivation. One of these mutations is a loss-of-function mutation shared by all crown catarrhines, and it has been molecularly clocked to approximately 28 million years ago (3). This must have occurred prior to the time of divergence of the Old World monkeys and apes. All other crown groups of primates—platyrrhini (New World monkeys), strepsirrhini (lemurs and lorises), and tarsiers (4, 5)—retain an actively expressed α1,3GT gene and therefore have enzymatic activity that catalyzes production of the terminal galactose sugar.

Primates that lack αGal expression produce a natural antibody, anti-Gal, which recognizes the αGal epitope (2, 6, 7). The IgG isotype directs cells of the innate immune system, including macrophages, neutrophils, and NK cells, to exert their cytotoxic effects on αGal-expressing tissues, and the IgM isotype induces complement-mediated lysis of αGal-positive cells (6, 8). Certain pathogens express αGal on their surfaces; these include bacteria (including those found in primate guts) (4, 7, 9), protists (10–12), and viruses derived from hosts that express αGal. Parasite growth is inhibited by antibody-dependent complement-mediated damage (11, 13, 14). Fresh sera with functional complement activity from catarrhines can also inactivate αGal-expressing viruses, including various retroviruses (15–19) and other enveloped viruses, such as lymphocytic choriomeningitis virus (20, 21) and pseudorabies virus (22). Sera from other mammals, including New World monkeys, rats, mice, and guinea pigs, do not lyse αGal-expressing viruses, as these species do not naturally make anti-Gal (15–17). It is hypothesized that loss of *GGTA1* gene expression was advantageous to catarrhines because it permitted immune recognition of pathogens expressing αGal; as such, suppression of the α1,3GT gene would have been necessary for losing the immune tolerance to αGal and thus gaining the ability to produce anti-Gal (2). The ability of anti-Gal to neutralize pathogenic agents via the complement system has been heavily researched. However, glycan-based interactions of pathogens and αGal-positive host cells have received only limited investigation.

Pathogens commonly exploit host cell surface carbohydrates or glycosylation pathways in the course of infections, and αGal is no exception. Toxin A, produced by *Clostridium difficile*, binds to the αGal moiety on host cells and causes cytotoxic effects in these cells (23, 24). Host cell surface glycans are also preferred binding sites for viruses to utilize as receptors (25), including monosaccharides like sialic acid, which competes with αGal for the same residues on terminal glycan chains (7, 26, 27). The primate evolutionary record yields good evidence of alterations in sialic acid loci in response to pathogenic pressures, notably, changes to the *ST6GAL1* and *CMAH* genes in response to influenza A virus and *Plasmodium reichenowi* infections, respectively (28–31). In certain primate species, carbohydrate expression patterns of sialic acid have undergone major genetic changes, while the same structures have remained conserved in the majority of vertebrates (28, 30).

Received 30 April 2013 **Accepted** 26 May 2013
Published ahead of print 5 June 2013
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doi:10.1128/JVI.01118-13

This indicates that selective pressures on carbohydrate domains can be species specific. It is of interest to consider that because αGal residues can utilize the same position on glycoconjugates as sialic acid, they may perform corresponding functions during pathogen infections and therefore may also be subjected to similar selection pressure from pathogens. Host expression of α1,3GT (and αGal) could therefore be an important determinant of resistance or susceptibility to viruses or other pathogens that could utilize the α1,3GT pathway as part of their infection cycle. This aspect of the α1,3GT gene—the differential ability of pathogens to infect host cells through utilization of binding sites or receptors containing αGal—has yet to be explored. The goal of the present study was to determine whether expression of the α1,3GT gene, and consequently αGal, plays a role in cell susceptibility to viral infections.

MATERIALS AND METHODS

Mice. GT knockout (KO) mice (C57BL/6J × DBA/2J × 120sv) were generated by disruption of the α1,3GT gene by homologous recombination (32). Breeding pairs were kindly donated to our laboratory by Uri Galili at the University of Massachusetts Medical School (UMMS). GT KO mice were backcrossed onto the C57BL/6J background for well over 10 generations and bred at the UMMS animal facilities. Experimental mice that are double knockouts for the *GGTA1* gene do not naturally produce anti-Gal in sera (8). Low levels of IgG and IgM antibodies are produced after initial exposure to antigenic epitopes, and a robust antibody response is achieved only by multiple immunizations (8). Adult C57BL/6J mice, acquired from Jackson Laboratories, were used as control mice. C57BL/6J suckling mice were bred at the UMMS animal facilities.

Cell lines. The BL6 (bladder 6) cell line, a subclone of the highly invasive B16 melanoma cell line, was isolated from the bladder wall of C57BL/6 mice (33, 34). Cells were acquired from Uri Galili at UMMS. Continuous selection for metastatic capabilities resulted in BL6 cells that spontaneously lost biosynthetic α1,3GT activity (35). These melanoma cells were later transfected with the murine α1,3GT gene cDNA to create a cell line, designated BL6αGT, with characteristics similar to those of the parental cell line, except for αGal expression on the cell surface (35). We used the BL6αGT (α1,3GT$^+$) cells as controls for BL6 (α1,3GT$^-$) cells and regularly monitored both lines for surface expression of αGal by staining with monoclonal anti-Gal antibody, M86 (Enzo Life Sciences), and secondary allophycocyanin (APC)- or R-phycoerythrin (R-PE)-conjugated goat anti-mouse IgM (Invitrogen). Alternatively, fluorescein isothiocyanate (FITC)-conjugated BS lectin (BSl-B_4) from *Bandeiraea simplicifolia* was used as an αGal-binding lectin (Sigma-Aldrich). Fluorescence was measured using a BD Biosciences LSR II flow cytometer with fluorescence-activated cell sorting (FACS) Diva software and analyzed using FlowJo software (TreeStar Inc., Ashland, OR). The two cell lines have similar replication cycles, and growth was closely monitored by colony shape and cell morphology. Experiments with different viruses were often done simultaneously in parallel cultures to control for any potential problem concerning a fragile cell line unable to host a viral infection. Both cell lines were cultivated as monolayers in GIBCO minimum essential medium (MEM) and supplemented with 10% fetal calf serum (FCS), 2.5% penicillin and streptomycin, and 2.5% L-glutamine.

Viruses. The following enveloped viruses were used in these studies: Sindbis virus (SINV) strain AR339 (36); SINV strains TE12-GFP (green fluorescent protein), TR339, HR, and S.A.AR86, obtained from Dennis T. Brown at North Carolina State University, grown in Vero cells, and stored at −80°C; herpes simplex type 2 (HSV-2) strain MS (37); HSV-1 strain KOS1.1 (38); and the HSV-1 KOS 1.1 GFP ICP8 mutant (39). The HSV-1 GFP strain has a mutation of the early DNA-binding protein ICP8, which functions in viral nucleic acid and protein localization to replication compartments as well as regulation of late gene expression in host cells (39). The recombinant virus is able to effectively initiate infection, but DNA replication and late gene expression in host cells are affected, and subsequent virus production is significantly reduced compared to that of wild type HSV-1 (39–41). Viruses were propagated in Vero cells (African green monkey kidney cell line) or L929 cells (C3H mouse fibroblast cell line) and harvested at the peak of infection (SINV strains at 24 h postinfection [hpi] and HSV at 48 hpi). Cell debris was removed from culture fluid by centrifugation. Culture fluid from virus-infected cells was titrated by plaque assay on Vero cell monolayers. The concentration of virus (multiplicity of infection or MOI) used for infections in our assays was based on titrations in Vero cells. Vero cells, a fibroblast cell line isolated from an African green monkey (*Chlorocebus* sp.), are interferon deficient and suitable for growing viruses (42). These cells do not express αGal on their surfaces, and viruses propagated in Vero cells do not carry αGal carbohydrates on their envelopes. L929 cells express αGal on their surfaces, and viruses grown in L929 cells have αGal incorporated into their envelopes (21). Vero and L929 cells were maintained as monolayers in MEM supplemented with 10% FCS, 2.5% penicillin and streptomycin, and 2.5% L-glutamine.

Time courses and adsorption assays. BL6 and BL6αGT cells were seeded in 6-well plastic plates and left to adhere for 24 h. Before infection, media were removed and replaced with 1 ml of complete MEM. Virus propagated on Vero cells was diluted to a multiplicity of infection (MOI) of from 0.01 to 1.0 in MEM, added to the BL6 and BL6αGT monolayers, and left for adsorption periods of 60 min for time courses or from 15 to 180 min for adsorption assays. Culture fluid with unadsorbed virus was removed, and cells were washed twice and refed with 2 ml MEM. For time courses, culture fluid from infected cells was collected up to 72 hpi and titrated by plaque assay on Vero cell monolayers. Infected monolayers with GFP viruses were observed using a Nikon Eclipse fluorescence microscope, and the images were analyzed with NIS-Elements Advanced Research imaging software; alternatively, cells were harvested by dispersion with trypsin and analyzed by FACS analysis at each time point. For adsorption assays, cells were harvested from 7 to 48 hpi and prepared for FACS analysis.

HSV-2 infections in adult mice and gamma irradiation. Male GT KO and C57BL/6J (WT B6) mice from 8 to 19 weeks of age were used in various experiments where no gamma irradiation was administered. Mice were inoculated intraperitoneally (i.p.) with 3.75×10^5 or 5×10^5 PFU of HSV-2. Infected mice were examined daily for physiological or behavioral changes and sacrificed by cervical dislocation at 3, 4, and 5 days postinfection (dpi). Abdominal fat pad, spleen, and liver were harvested and examined for pathology, and virus from organ suspensions was titrated by plaque assay on Vero cell monolayers. For *in vivo* HSV-2 infections with gamma-irradiated animals, male GT KO and WT B6 mice 8 to 14 weeks of age received 9 to 11 Gy. Immediately after irradiation, mice were inoculated i.p. with 3.75×10^5 PFU of HSV-2. Infected mice were observed and sacrificed as described above at days 2, 3, 4, 5, and 6. Organs were harvested, examined, and titrated as described previously.

SINV survival studies. GT KO and C57BL/6J suckling mice were infected via different routes and doses of different SINV strains as follows: 8-day old mice were inoculated intracranially (i.c.) with 200 PFU SINV strain AR339, and 13-day old mice were inoculated subcutaneously (s.c.) with 1.68×10^3 PFU. Different inoculation routes and ages were used to determine susceptibility to infection. Pregnant females were monitored for day of birth, but because births from GT KO and WT B6 pups were not synchronized to the day, infections of mice were staggered. Entire litters (3 to 8 pups) were infected when pups came of age, excluding runts. Two or three litters per mouse strain were used for each experiment at each dose, SINV strain, and age of infection. Mice were weighed daily and monitored for symptoms of disease, including paralysis, lethargy, and other possible signs of neurological involvement, such as spasms and shaking.

Statistical methods. Survival data were analyzed by the log-rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon survival test using GraphPad InStat software. Analyses of individual HSV-2 *in vivo* experiments were done using GraphPad InStat Student's *t* tests and chi-squares,

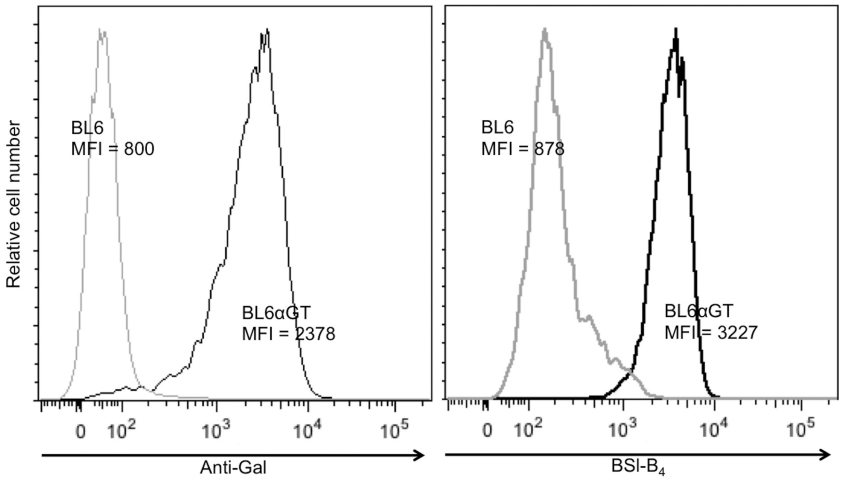

FIG 1 Expression of αGal on BL6 and BL6αGT cells. Cells were stained with anti-Gal (IgM) or BS lectin ($BSl\text{-}B_4$), both of which bind αGal. The gray peak represents BL6 cell expression of αGal, and the black peak represents that of BL6αGT cells. BSl can bind αGal monomers, while anti-Gal requires multiple sites for binding. MFI, mean fluorescence intensity of αGal-positive cells.

and analyses of pooled experiments were completed using Minitab statistical software analysis of variance (ANOVA) mixed models with random assumption, factoring dose, age, and experimental trial differences. Significance was set at a *P* value of ≤0.05.

RESULTS

Viral growth kinetics in the presence or absence of *GGTA1* gene in the host cell. If α1,3GT activity is important in the course of viral infections, then viral replication should be contingent on whether host cells express *GGTA1*. Time course infections were performed using paired cell lines—one that actively expressed the α1,3GT gene (BL6αGT) and one that did not (BL6). Figure 1 shows that the BL6 cell line did not react with anti-Gal antibody (left) or bind BS lectin (right), which also reacts with αGal. The BL6αGT line bound both anti-Gal and BS lectin. This confirms the differential cell surface expression of αGal at the time of these experiments; this is an important issue, as continued passage of the BL6αGT line may result in decreased expression over time. Table 1 shows representative data indicating that several viruses screened (vesicular stomatitis virus [VSV], lymphocytic choriomeningitis virus [LCMV], Pichinde virus [PV]) grew equally well (as measured by PFU titers from culture fluid) in both cell lines. Any variations in the growth of these viruses that did occur between the two cell lines were inconsistent. However, differences in viral replication between the cell lines were consistently observed for all tested SINV strains (AR339, S.A.AR86, TR339, HR, and TE12-GFP), as well as for HSV-1 and HSV-2. Notably, the SINV strains often grew to 10- to 20-fold-higher titers in the presence of α1,3GT, whereas the HSV strains grew often to 100-fold-higher titers in the absence of α1,3GT (Fig. 2). SINV growth differences between cell lines were observable early in the time course—after a single round of viral replication (Fig. 2B). SINV-infected BL6αGT monolayers also exhibited more cytopathic effects (CPE) than did BL6 cells. BL6 cell monolayers, in contrast, were much more susceptible to HSV-1 and HSV-2 infections (Fig. 2C) and exhibited greater CPE. Experiments with SINV and herpesviruses

TABLE 1 Replication of viruses in BL6 cells[a]

Virus (hpi)	Titer (log_{10} PFU [mean ± SD])[b]	
	BL6αGT (+)	BL6 (−)
VSV (24)	7.1 ± 0.07	6.8 ± 0.16
PV (48)	5.7 ± 0.14	5.6 ± 0.35
LCMV (48)	6.8 ± 0.04	7.0 ± 0.03

[a] Viruses grown in either αGal-positive or αGal-negative BL6 cells were titrated on Vero cell monolayers. Experiments were completed in duplicate and are representative of 2 to 4 tests/virus.
[b] Peak titer for that time course. The MOI was 1.0 for VSV and PV and 0.1 for LCMV.

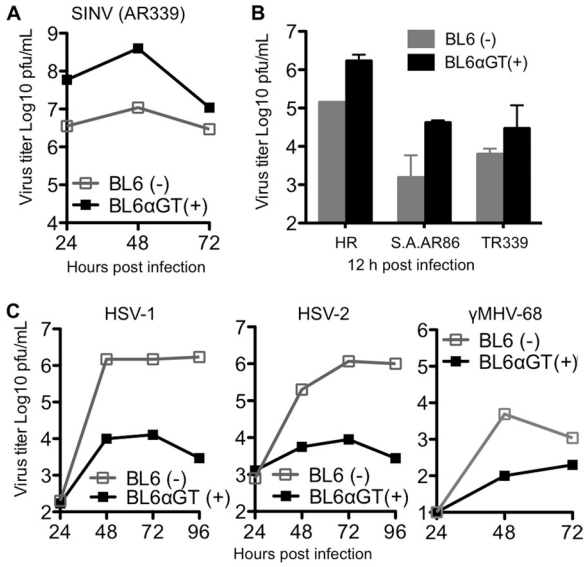

FIG 2 Growth kinetics of SINV and herpesviruses in BL6 cell lines. Results are representative of >5 experiments for HSV and SINV and of 2 tests for γMHV-68. Virus was diluted to an MOI of 1.0 in MEM, added in 100 μl (SINV strains) or 100 to 400 μl (herpesviruses) to the BL6 and BL6αGT monolayers, and left for an adsorption period of 60 min. (A and C) During time courses, culture fluids from cell monolayers were collected every 24 h for 72 h postinfection (hpi) (SINV AR339 and γMHV-68) or 96 hpi (HSV-1 and -2). (B) For 12-h replication experiments, cell monolayers were infected with SINV viruses at an MOI of 0.1, and culture fluid was collected, starting at 12 hpi.

TABLE 2 Replication of viruses in SK cells[a]

Virus (hpi)	Titer (log_{10} PFU[b])	
	SK-GT (+)	SK (−)
HSV-1 (48)	4.7	**6.9**
HSV-2 (48)	3.5	**4.5**
SINV AR339 (24)	**7.3**	6.0
SINV TE12-GFP (24)	**6.2**	5.5

[a] HSV and SINV viruses grown in either αGal-positive or αGal-negative SK cells were titrated on Vero cell monolayers. The MOI for HSV-1, HSV-2, SINV AR339, and SINV TE12-GFP was 1.0. These results were obtained with single samples but are representative of >2 experiments.
[b] The bold numbers are the higher titers.

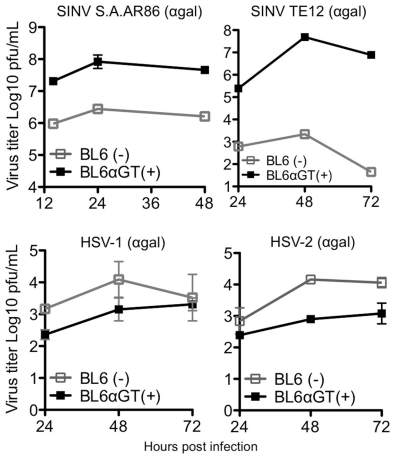

FIG 3 Growth kinetics of αGal-expressing viruses in the BL6 cell lines. Results are representative of 2 separate experiments completed in duplicate. SINV and herpesviruses were propagated in αGal-positive L929 cells to produce virions expressing αGal. Time courses were completed as previously described. SINV(αGal) was used at an MOI of 0.1 and HSV(αGal) at an MOI of 0.01. Culture fluid was harvested every 12 h for 48 hpi [SINV(αGal)] or every 24 h for 72 hpi [HSV(αGal)].

were frequently done simultaneously in parallel cultures, with the same skewed results. A third herpesvirus, murine gammaherpesvirus 68 (γMHV-68), also replicated better in α1,3GT-negative cells (Fig. 2C). Because related viruses behaved similarly during infections of each cell type, viral preference for expression or nonexpression of α1,3GT in host cells appears to be a general characteristic of certain viruses and, at least in the systems studied here, is virus dependent but not virus strain dependent.

Similar patterns of SINV and HSV-1 and -2 syntheses were seen in paired α1,3GT-modified human melanoma cell lines, SK-N-MC pLXN (SK) (inactive αGT gene) and SK-N-MC clone 10 (SK-GT) (active αGT gene) (21) (Table 2). Thus, whether or not the cell was of human or mouse origin, the presence of an active α1,3GT gene had the same effect. Taken together, these data strongly suggest that, whereas host cell α1,3GT gene activity does not universally influence the course of all viral infections, it is an important factor in the replication of certain viruses.

Viral expression of αGal does not affect host cell susceptibility to SINV and HSV strains. Enveloped viruses incorporate host cell carbohydrates onto their envelopes during egress from cells, and this in turn can affect interactions with subsequent host cells. We established previously (21) that various enveloped viruses, including SINV, acquire αGal after passages in mouse fibroblast L929 (α1,3GT-positive) cells. To determine whether viral infectivity for our two cell lines was affected by the presence of αGal on the virion, we completed time course assays of BL6 and BL6αGT cell lines with HSV-1 and -2 and several SINV strains that had been propagated in L929 cells (designated SINVαGal, HSV-1αGal, and HSV-2αGal). The SINVαGal virus strains still preferentially replicated in α1,3GT-positive cells, while HSV-1αGal and HSV-2αGal preferentially grew in α1,3GT-negative cells (Fig. 3). Further, when the same MOI was used, infections of BL6 cells with SINV S.A.AR86 virus propagated in αGal-expressing L929 cells and in non-αGal-expressing Vero cells resulted in similar viral titers (Table 3). L929-grown virus and Vero-grown virus also replicated to similar levels in BL6αGT cells (Table 3). This showed that the cell in which the virus was produced does not alter infection levels in αGal-positive and -negative hosts. These results also indicate that host cell susceptibility to various SINV strains and to HSV-1 and -2 is affected by host cell differences in α1,3GT gene expression but not by variable αGal expression on the virions.

The level of viral replication correlates with the proportion of infected cells in the population. Susceptibility to viral infections that is dependent on α1,3GT expression should be a general characteristic of the entire cell population. High viral titers, as measured from culture fluid, however, could indicate either of two phenomena: (i) many infected cells producing moderate levels of virus or (ii) a subpopulation of infected cells producing large amounts of virus. In order to investigate whether the amounts of virus in culture fluid correlated with the fraction of infected cells in the population, we used SINV and HSV-1 recombinants that express a green fluorescent protein (SINV TE12-GFP and HSV-1 GFP ICP8 mutant) during replication, and we performed time course assays on BL6 and BL6αGT cells. HSV-1 GFP has reduced replication potential due to an ICP8 gene mutation that interferes with late gene expression but not with its ability to initiate infection; therefore, differences in viral yields between cell types can still indicate differences in cell susceptibility to infection. Further, GFP is expressed as a delayed early gene product, and so fluorescence in host cells is a useful measure of a first round of infection. Infected cells were directly measured via FACS, and virus from culture fluid was quantified by plaque assay. As seen with wild-type strains, SINV TE12-GFP replicated better in BL6αGT cells (Fig. 4A), whereas HSV-1 GFP replicated better in BL6 cells (Fig. 4B). Under fluorescence microscopy, GFP SINV-infected BL6αGT cell monolayers had relatively large areas of fluorescing cells compared to SINV-infected α1,3GT-negative cells (Fig. 4C). Conversely, HSV-1 infected BL6αGT cell monolayers exhibited relatively small foci of fluorescing cells compared to HSV-1-infected BL6 cells (Fig. 4D). Time course studies using FACS analy-

TABLE 3 Replication of S.A.AR86 and S.A.AR86(αGal) in BL6 cells[a]

Virus	Titer (log_{10} PFU [mean ± SD])[b]	
	BL6αGT (+)	BL6 (−)
SINV S.A.AR86	**7.5 ± 0.03**	5.5 ± 0.10
SINV S.A.AR86(αGal)	**7.4 ± 0.05**	5.7 ± 0.10

[a] BL6 and BL6αGT were infected with SINV S.A.AR86 expressing or not expressing αGal on its envelope at an MOI of 0.1. Results are representative of one experiment completed in duplicate, and titers are consistent with results from other time courses with S.A.AR86 at similar MOIs.
[b] Peak titer obtained at 24 hpi during a 48-h time course. Higher titers are in bold.

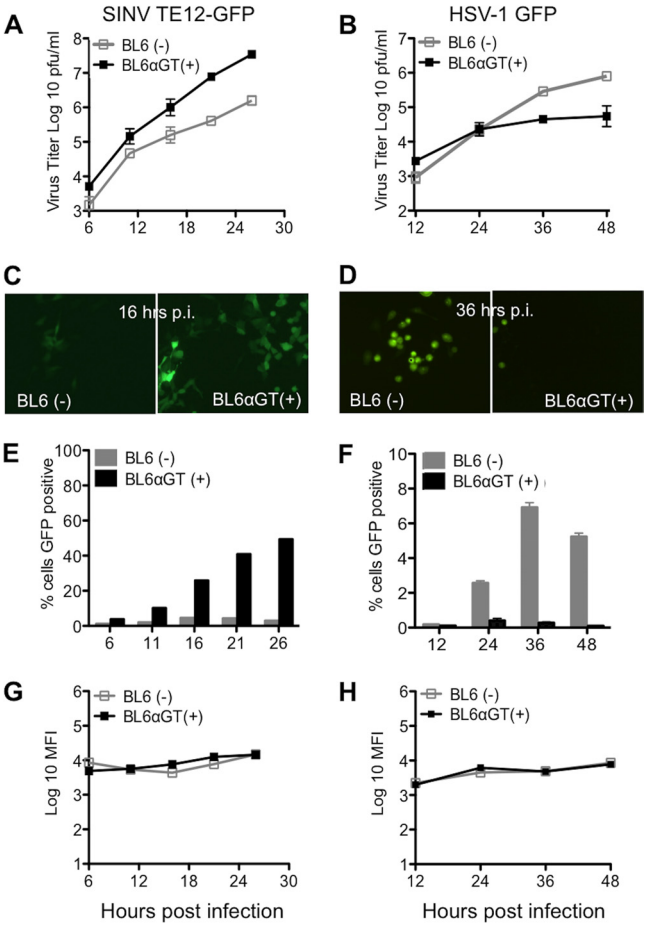

FIG 4 Replication of GFP viruses in BL6 cell lines. Time courses on BL6 and BL6αGT cells were performed as described in the text. Results are representative of >5 experiments for SINV GFP and 3 experiments for HSV GFP. SINV TE12-GFP was used at an MOI of 1 and HSV-1 GFP at an MOI of 0.1. (A and B) Culture fluid was collected from SINV-infected monolayers for titration every 5 h beginning at 6 hpi (A) and from HSV-infected monolayers every 12 h for 48 hpi (B). (C to F) Infected monolayers were observed under a fluorescence microscope to visually monitor infection (C and D), and cells were harvested for FACS analysis (E and F). (G and H) Mean fluorescence intensity of GFP expressing cells was measured via FACS Diva software.

sis showed that a substantially greater fraction of SINV-infected α1,3GT-positive cells expressed GFP (Fig. 4E), compared to a small subpopulation of BL6 cells, even after multiple rounds of replication. In contrast, a much greater proportion of HSV-1-infected BL6 cells expressed GFP, and only a small fraction of BL6αGT cells were GFP positive for the duration of the time course (Fig. 4F). Comparable mean fluorescent intensity (MFI) levels of GFP were observed across both cell lines during SINV and HSV infections (Fig. 4G and H), suggesting that once cells are productively infected, virus is synthesized at similar levels in both cell lines. These data suggest that differences in viral replication between host cell types are due to differential success in virus establishing a productive infection.

Differences in host cell susceptibility are evident early after viral infection. Cells were incubated with either GFP virus for various time periods, and infected cells were harvested prior to a first round of replication—thus measuring initial infection levels—and analyzed via FACS. In HSV-1 assays, longer adsorption time resulted in a small increase in infected BL6 cells, but the infection on BL6αGT monolayers was almost negligible (Fig. 5A). In GFP-SINV assays, longer adsorption time resulted in higher fractions of α1,3GT-positive infected cells but not of α1,3GT-negative cells; infection levels in these cell populations did not increase with adsorption times longer than 120 min (Fig. 5B). Additionally, it is of interest that following multiple rounds of replication (Fig. 5C), the proportion of GFP SINV-infected cells in the BL6 population declined. This was not a result of lysed cell monolayers, as at 36 hpi the BL6 monolayers appeared more intact than BL6αGT cell monolayers (Fig. 5D); the monolayer simply appeared to resist infection. These data support our findings that α1,3GT gene activity is a determining factor in the successful initiation of SINV and HSV infections and points to a mechanism early in virus-host cell interactions.

GT KO mice are more susceptible than WT mice to HSV-2 infections. The experiments described above showed that active expression or nonexpression of the α1,3GT gene plays an important role in the course of viral infections *in vitro*. However, the role of the α1,3GT gene or αGal expression must be relevant *in vivo* in order to have implications for primate evolution. To test whether our *in vitro* data could be corroborated *in vivo*, we used C57BL/6J (WT B6) mice, which naturally express the *GGTA1* gene, and *GGTA1* knockout mice (GT KO), which lack it. Mice aged 8 to 19 weeks were inoculated by the i.p. route with different doses of HSV-2. From i.p. inoculations, HSV-2, which is not highly pathogenic in B6 mice, spreads via blood to visceral organs. Spleen, liver, and visceral fat pads proximal to the injection site were harvested at various dpi to quantify virus. At day 3 postinfection (p.i.), viral loads from splenic suspensions were often higher in KO mice than in WT mice, but this difference was not always statistically significant. At day 3 p.i., virus from liver suspensions was detected in a greater proportion of KO mice (83.4%) compared to WT mice (45.5%) ($x^2 = 5.9$, df = 1, $P < 0.02$), and macroscopic hepatic lesions commonly associated with HSV-2 infections following i.p. inoculation (43, 44) were more frequently observed in KO mice (27.7%) than in WT mice (5.5%); however, differences in organ viral load between the two mouse types were not statistically significant. By day 4 p.i., virus from liver suspensions was detected in a significantly greater proportion of KO mice (88.2%) than WT mice (35.3%) ($x^2 = 10.09$, df = 1, $P < 0.002$). Viral load was also significantly higher in KO mice than WT mice, regardless of mouse age or viral dose administered (Fig. 6A), and a greater proportion of KO mice exhibited hepatic necrosis (29.4%) than WT mice (11.8%). By day 5 p.i., KO and WT mice had largely cleared the infection from all organs tested. These data show differences in resistance to HSV-2 between KO and WT mice, reflected in the amount of virus isolated from liver and from the level of hepatic necrosis observed in the infected mice.

To test whether differences in susceptibility to HSV-2 infection were seen in mice with compromised immune systems, mice were given a high dose of gamma irradiation directly prior to infection. Replication of HSV-2 was considerably enhanced in the spleens and livers of these immunocompromised KO mice but only slightly improved in WT mice. At day 2, the virus was barely detectable in spleens of WT mice, but KO mice had mean viral titers up to 80-fold higher (Fig. 6B). By 5 dpi, all KO mice exhibited numerous large focal necrotic lesions and elevated viral load in livers (Fig. 6B). In contrast, only a few WT mice presented small hepatic lesions and relatively low (or undetectable) viral titers.

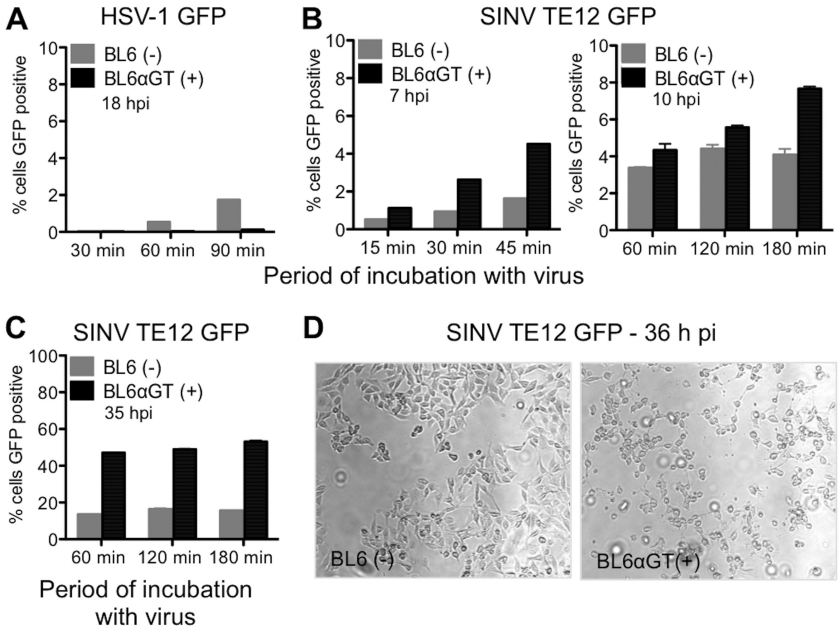

FIG 5 Virus adsorption to BL6 and BL6αGT cell lines. Results are representative of >5 experiments with SINV GFP and 3 for HSV GFP. Virus was diluted to an MOI of 1.0 (SINV TE12-GFP) or MOI of 0.1 (GFP HSV-1) in MEM and added in 100 μl to the BL6 and BL6αGT monolayers for various periods. Culture fluid with unabsorbed virus was removed after each incubation endpoint, and cells were washed twice with medium and refed with 2 ml MEM. At harvest, cells were prepared for FACS analysis. (A) GFP HSV-1 was incubated with cells for 30, 60, or 90 min, and monolayers were harvested at 18 hpi, prior to one round of replication. (B) GFP SINV was incubated with cells for 15 to 180 min, and cells were harvested from 7 to 10 hpi, prior to one round of replication. (C) GFP SINV was incubated with cells for 60, 120, or 180 min, and cells were harvested at 35 hpi, after multiple rounds of infection. (D) GFP SINV-infected cell monolayers approximately 36 hpi under light microscopy.

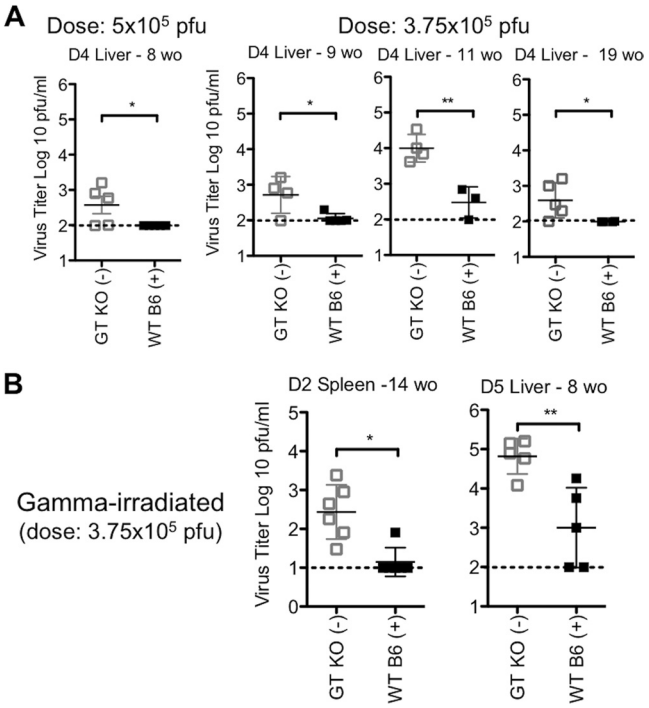

FIG 6 HSV-2 infections of *GGTA1* KO and WT B6 mice. Mice were inoculated intraperitoneally with HSV-2. Organs for titration (PFU/organ) from nonirradiated mice were harvested at days 3, 4, and 5 and at day 2, 3, 4, 5, or 6 from γ-irradiated mice. wo, weeks old. (A) Nonirradiated mice 8 weeks of age were inoculated with 5×10^5 PFU in 400 μl medium, and those 9, 11, or 19 weeks of age were inoculated with 3.75×10^5 PFU in 300 μl medium. (B) Gamma-irradiated mice 8 to 14 weeks old were inoculated with 3.75×10^5 PFU in 300 μl medium. *, $P \leq 0.05$; **, $P \leq 0.005$.

Thus, the results are consistent with the concept that the differences in viral load were due to inherent replication of the virus rather than to unrecognized differences in immune responses in these two strains of mice.

SINV-infected WT suckling mice have accelerated symptom onset and death compared to KO counterparts. Adult mice are resistant to most SINV strains and can control infections with an alpha/beta-interferon (IFN-α/β) response, but in neonates, which have a weak IFN response (up to 14 days of age), most SINV strains induce a severe, lethal viral encephalitis (45, 46). In order to determine host differences in response to various SINV infections, mice 6 to 20 days of age were inoculated i.c., because the virus is neurotropic, or s.c., to mimic the natural route of vector-borne infection. For these experiments, we used the less virulent AR339 strain and the more virulent S.A.AR86 strain. We tested a variety of ages for infection and found that mice infected at day 6 were not cared for by their mothers, and mice infected at day 20 were resistant to the lethal effect of either strain. Infections of mice from 8 to 14 days of age resulted in 100% mortality, regardless of the presence or absence of *GGTA1*. However, significant differences were observed in the mean survival period prior to death and onset of pathological symptoms. Intracranial infections of 8-day-old mice with the more virulent SINV S.A.AR86 resulted in a rapid onset of spasmodic behavior and lethargy. All WT mice died by day 3, and a few KO mice survived until day 4, but this difference was not statistically significant. Figure 7A shows the results of an experiment where 8-day-old WT mice infected i.c. with SINV AR339 had a shorter mean survival time than KO mice (3.0 versus 4.0 days). WT mice exhibited spasms, trembling, and unsteady locomotion as early as 24 h after inoculation and suc-

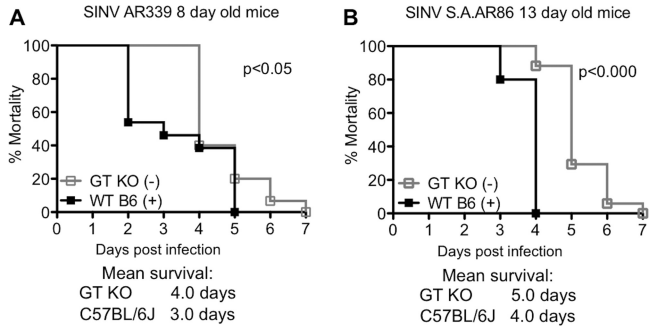

FIG 7 SINV infections of α1,3GT KO and WT B6 weanling mice. Eight-day-old mice were inoculated intracranially with approximately 200 PFU SINV AR339 strain in 10 μl of media (2 litters KO, $n = 15$; 2 litters WT, $n = 13$) (A) or 13-day old mice were inoculated subcutaneously with approximately 1.68×10^3 PFU of SINV S.A.AR86 in 30 μl (3 KO litters, $n = 17$; 2 WT litters, $n = 10$) (B).

cumbed to infection beginning at 2 dpi. In contrast, KO mice experienced morbidity around day 2 and 3 and began dying at day 4. Thirteen-day-old KO mice inoculated s.c. with S.A.AR86 had a longer mean survival time than WT mice (5.0 versus 4.0 days) and experienced weight loss later than WT mice (day 3 versus day 2) (Fig. 7B). Shaking and hind limb paralysis were observed by day 2 in WT mice and on day 4 in GT KO. At 14 days of age, both KO and WT mice began dying on day 4 p.i., survived until day 6 p.i., and showed no significant differences in survival times and symptomology. Altogether, these data point to differences in SINV infection kinetics between susceptible GT KO and WT B6 suckling mice, reflecting the pattern of susceptibility to SINV seen with cultured cells.

DISCUSSION

Here we show that a mutation that occurred in the common ancestor of Old World monkeys and apes approximately 28 million years ago had the capacity to significantly alter their susceptibility to viral infections, and we suggest that this change of susceptibility to infection may have provided an evolutionary advantage to individuals harboring this mutation. Clearly, the viruses examined in this study would not have been the putative selection agents of 28 million years ago, so this study should be taken as a proof of principle of what could have happened rather than as an account of what did happen. Our current work shows that loss of α1,3GT expression rendered cells more resistant to some viruses but more sensitive to others. Perhaps the loss of the αGal moiety rendered certain basal catarrhines more resistant to a virus like SINV, which we show grew to 10- to 20-fold-higher titers in cells expressing the sugar and which was more virulent in αGal-expressing suckling mice. It is more difficult to explain how a mutation that enhanced susceptibility to some viruses, like HSV-1 and HSV-2, could provide a selective advantage. HSV may have simply evolved specificity for hosts that did not express α1,3GT, and it is expected that some viruses would have evolved to accommodate the changes in primate carbohydrate structure. However, it is of interest that herpesvirus infections can sometimes benefit the chronically infected host. Recent work with mice has shown that herpesvirus latency can induce a state of heightened NK cell activity that results in swifter action against new infections, and certain human herpesvirus infections may exert similar effects (47). Furthermore, the periodic activation of latent or persistent viruses can induce chronic IFN-γ secretion and macrophage activation, which can provide cross protection against acute bacterial infections (48). Therefore, depending on the viral agent, a mutation in αGal expression that results in change of host susceptibility to that virus might provide a beneficial function to the host, but could do so by separate mechanisms.

Loss of a functional *GGTA1* gene in catarrhines permitted their expression of anti-Gal antibody (2, 17), an effective component of the innate immune system against a variety of αGal-expressing pathogens, via a complement-dependent lysis (6, 11, 13, 15, 16, 18, 19–21). As a result, catarrhines may have been indirectly protected from pathogens naturally expressing αGal or from zoonotic transmission of certain viruses acquired from α1,3GT-positive species because of the αGal antigens expressed on the pathogen's surface (2, 5, 6). However, despite the benefits conferred by anti-Gal, *GGTA1* gene activity is conserved in the majority of mammals, and in these species, its function appears to be under great physiological constraint (3). The function of αGal in animal metabolism is not yet known, and other consequences of the inactivation of this locus have not been identified. Perhaps inactivation of *GGTA1* gene was detrimental to the individual and may have been evolutionarily possible only after its enzymatic role had been filled (3). Homozygous knockout (KO) pigs (49) and KO mice (27) have been successfully generated and bred, producing normal-sized litters that do not experience greater mortality than their WT counterparts. A few abnormal health conditions can occur later in life, such as early-onset cataracts (3), but *GGTA1* loss is compatible with life. This indicates that basal catarrhines could have experienced loss of α1,3GT function without an elevated risk of mortality or morbidity. We speculate that the loss of αGal from tissue surfaces may have played a protective role during the evolution of the catarrhine lineage, and the benefits of losing gene function may have outweighed any negative effects.

We propose here a mechanism by which evolution may have acted on the *GGTA1* locus, directly altering host cell susceptibility to infection. We show that *in vitro* inactivation of the α1,3GT gene in the host can confer direct protection against viruses such as SINV by markedly inhibiting infection, regardless of αGal expression or nonexpression on the infecting viruses. For young mice, infections with most SINV strains are lethal, but we show here that at least in a distinct window of time, suckling mice that express α1,3GT experience disease symptoms sooner and have shorter mean survival times than GT KO mice. In contrast, inactivation of the α1,3GT gene in a host cell or in mice result in an enhanced infection potential by various herpesviruses. These experiments confirm that that differential expression of α1,3GT can directly influence host susceptibility to different viral infections.

The mechanism by which activity of the *GGTA1* gene alters the course of viral infections is likely by inhibiting the ability of a virus to initiate infection. Differences in efficiency of initial infection were seen with both SINV and herpesviruses, but once productively infected, the cells appeared to express similar amounts of viral protein, as indicated by GFP expression. We found that treatment of αGal-expressing cells with α-galactosidase cleaved αGal from the cell surface and rendered them more resistant to SINV infection. However, this enzyme also further reduced the low level of infectivity in αGal-negative cells (data not shown). One possible explanation for this phenomenon is the low-level production of αGal by an additional glycosyltransferase, the iGb3S enzyme, as

has been observed in rats (50) and in GT KO mice and pigs (51). Humans do not express this enzyme (51), and any expression of terminal galactose on human tissues is generally associated with a pathological state (2, 52). In KO mice, the αGal disaccharide expressed via iGb3S synthesis is restricted to glycolipids, and detection by BS lectin or anti-Gal is poor, but sites are recognized by anti-Gal monoclonal antibodies fused to NS-1 hybridoma cells (51). BL6 cells could potentially express barely detectable levels of iGb3S-derived αGal residues, the presence (and removal) of which may have some effect on infection in these cells. We are further investigating this observation.

The involvement of αGal in the course of viral infections is thus an avenue that warrants further investigation. Cellular glycans and host glycosylation pathways play an important role in viral infections (30, 53, 54), and in this way the distribution of host cell carbohydrates may even determine host range and tissue tropism (55, 56). SIN viruses have widespread host repertoires and utilize the ubiquitously expressed protein NRAMP2 for binding and entry into host cells (57, 58). It is unlikely that αGal would be a required receptor utilized in entry for SINV strains because loss of α1,3GT expression limits but does not preclude viral infection. Further, insect cells such as those of *Aedes* mosquitos, which SINV infects, lack terminal glycosylation from galactosyltransferases and sialyltransferases (59). The most common terminal glycan expressed on SINV virions propagated in *Aedes*-derived cells is mannose (60). On mammalian cells, αGal may instead participate in attachment or binding of SINV in a process analogous to how the virus may utilize heparan sulfate (61); these receptors may promote initial adherence to host cells until higher-affinity receptors are engaged (55). Loss of heparan sulfate on host cells reduces SINV infection but does not abrogate it. HSV-1 and -2 entry into cells is a multistep process initiated by viral glycoproteins binding to cellular heparan sulfate proteoglycans (62–65) followed by binding to entry receptors, such as the herpesvirus entry mediator (HVEM) protein or nectin-1 and nectin-2, and includes other virion–cellular-protein interactions (64–66). Active expression of the α1,3GT gene strongly impairs infection of both herpes serotypes. Experimental blocking of viral glycoprotein interactions with cognate cellular proteins disrupted the entry processes and inhibited infection (66, 67). It is conceivable that the presence of αGal on the cellular surface could alter the interactions of binding or entry receptors and result in reduced infection efficiency for these viruses.

Catarrhines evolved during the Oligocene epoch, a period of vast geological and climatic change that coincides with a great chronological gap in the primate paleontological record (68). The scarcity of fossil evidence makes it difficult to discern phylogenetic relationships between ancestral and descendant taxa and to even estimate conclusive divergence dates for Old World monkeys and apes (69–71). However, molecular studies indicate that this was a period of active genetic evolution for these primates (5, 70). The significance of the *GGTA1* inactivation 28 million years ago is therefore of great interest for studies of primate evolution because of the implications for the evolution of resistance to viral infections and for the phylogenetic divergence of primates.

Conservation work shows that it is often the case that synergistic causes are responsible for reducing the number of individuals in populations to the brink of extinction (72). If the hypotheses tested during this research are correct, the loss of α1,3GT expression plus the production of anti-Gal may have given αGal-negative catarrhines an advantage over αGal-positive catarrhines in controlling pathogens that could exploit the α1,3GT pathway. Perhaps the additive pressures of habitat changes, climatic change (68), and pathogen infections had a considerable effect on basal catarrhines still expressing the α1,3GT gene, placing them at an evolutionary disadvantage. Experimental evidence on the significance of α1,3GT activity and αGal expression in viral disease such as that presented here may shed light on the processes that helped shape catarrhine evolution during the Oligocene epoch.

ACKNOWLEDGMENTS

We thank Uri Galili for helpful suggestions and donation of GT KO mice and mouse bladder melanoma cell lines, Dennis Brown for Sindbis virus strains (S.A.AR86, TR339, HR, and TE12-GFP), David Knipe and Evelyn Kurt-Jones for HSV-1 GFP ICP8, and Michael Sutherland for help with statistical analysis.

This work was partly funded by the National Science Foundation SBE DDRIG grant no. 0925717 to I.A.R. and Laurie Godfrey and NIH research grant CA34461 to R.M.W.

The views expressed are those of the authors and not necessarily those of the granting agencies.

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