

Stable genetic integration of a red fluorescent protein in a virulent Group A *Streptococcus* strain

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Abstract

There are several advantages, both *in vitro* and *in vivo*, in utilizing bacteria that express a fluorescent protein. Such a protein can be transiently incorporated into the bacteria or integrated within the bacterial genome. The most widely utilized fluorescent protein is green fluorescent protein (GFP), but limitations exist on its use. Additional fluorescent proteins have been designed that have many advantages over GFP and technologies for their incorporation into bacteria have been optimized. In the current study, we report the successful integration and expression of a stable fluorescent reporter, mCherry (red fluorescent protein, RFP), into the genome of a human pathogen, Group A *Streptococcus pyogenes* (GAS) isolate AP53(S-). RFP was targeted at the atg codon of the *fcR* pseudogene that is present in the *mga* regulon of AP53(S-). Transcription of critical bacterial genes was not functionally altered by the genomic integration of mCherry. Host virulence both *in vitro* (keratinocyte infection and cytotoxicity) and *in vivo* (skin infection) was maintained in AP53(S-)-RFP. Additionally, survival of mice infected with either AP53(S-) or AP53(S-)-RFP was similar, demonstrating that overall pathogenicity of the AP53(S-) strain was not altered by the expression of mCherry. These studies demonstrate the feasibility of integrating a fluorescent reporter into the bacterial genome of a naturally virulent isolate of Group A *S. pyogenes* for comparative experimental studies.

INTRODUCTION

Bacterial cells that have been engineered to produce a fluorescent reporter signal have served as valuable tools to track individual bacterial strains during infection processes, both *in vitro* and *in vivo*. This has allowed visualization of bacterial dynamics, even in complex mixed microbial populations, in order to investigate their localization, colonization, and survival [1–7]. One concern with utilizing the most studied fluorescent protein, green fluorescent protein (GFP), for these purposes is that it requires oxygen for fluorescence, which limits its application to select microenvironments [8]. However, recent studies have identified a GFP from Unagi eel that does not require oxygen for fluorescence [9]. Since the discovery and utilization of GFP, other variants have been developed that are more suitable for *in vivo* studies. Monomeric Cherry (mCherry) is a red fluorescent protein (RFP) that is an engineered variant of the naturally existing tetrameric Discosoma red (DsRed) protein [10]. While still

requiring oxygen for its fluorescence properties, the advantages of mCherry are its speed of fluorescence development, pH tolerance, and photostability. Traditional use of this protein in plasmid expression systems in bacteria requires the presence of antibiotic selection for plasmid maintenance. Integration of the fluorescent protein into a specific region of the genome can eliminate the need for antibiotic selection and would allow greater stability and consistency in bacterial-based studies. This has been achieved by targeted insertion techniques [6, 11–14]. In the current study, we demonstrate the successful targeted integration of mCherry at the atg site of the *fcR* pseudogene within the *mga* regulon of the human skin infection isolate Group A *Streptococcus pyogenes* strain AP53/CovR+S- [AP53(S-)]. Our method will be highly adaptable for engineering reporter strains in other Group A *Streptococcus* (GAS) isolates, particularly those that have been newly isolated from clinical specimens.

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Abbreviations: DIC, differential interference contrast; FBS, fetal bovine serum; GAS, Group A *Streptococcus*; GFP, green fluorescent protein; hPg, human plasminogen; RFP, red fluorescent protein; SCO, single crossover.

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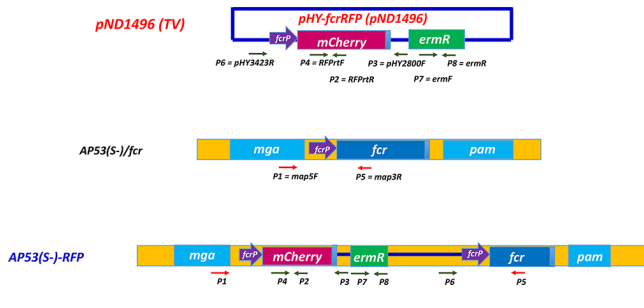


Fig. 1. Generation of targeting vector for expression of RFP in AP53(S-). The sequences 5' of the *fcr* pseudogene and mCherry (RFP) coding sequence were targeted at the *fcr* pseudogene locus in AP53(S-) GAS strain utilizing the pCR TOPO vector. This *fcr*-mCherry DNA cassette was then cloned into the temperature-sensitive plasmid pHY304, which also contained the erythromycin resistance gene (*erm*^r), at *NotI/BamHI* sites within the multiple cloning locus, to generate the GAS-RFP targeting vector (pND1496). The pND1496 plasmid was used to generate AP53(S-)-RFP stable cell lines through a single crossover event at the atg of the *fcr* gene within the *mga* regulon locus.

METHODS

Plasmid design

Targeted insertion of the mCherry coding sequence in GAS AP53(S-) was accomplished by homologous recombination at the atg site of the *fcr* pseudogene coding sequence. The AP53 *fcr* gene contains a single base mutation resulting in a stop codon in an otherwise complete gene. The *fcr* gene in

Table 1. Primers for the AP53(S-)-RFP stable line targeting vector and mapping

FcrRFP-NotF	5'-cgggccgcgtgacagatgttatggggta-3'
FcrRFP-NotR	5'-tctctcctcgcccttctcaccattgtttactccttatttctcaattc-3'
FcrRFP-RFPF	5'-gaaattgagaataaggagtaacaatggtgagcaaggcgaggagga-3'
FcrRFP-RFPR	5'-gctagaaaagatagtgtaattgggcttactgtacagctcgtcatgc-3'
FcrRFP-Map5F (=P1)	5'-GACTCCTAGTGATAGAGAGAAG-3'
FcrRFP-Map3R (=P5)	5'-CCCCTAAATCAGTCTTAAGCCC-3'
pHY2800F (=P6)	5'-CAAATTCCTACTAAGCGCTCG-3'
pHY3423R (=P3)	5'-CATTAATGCAGCTGGCAGCAGAG-3'
ermF (=P7)	5'-GGATATTCACCGAACACTAGGG-3'
ermR (=P8)	5'-GACGATATTCTCGATTGACCC-3'
RFPrtF (=P4)	5'-CAGTTCATGTACGGCTCCAAGG-3'
RFPrtR (=P2)	5'-CCATGGTCTTCTTCTGCATTACG-3'

Primers in bold were used to generate the insert for the targeting vector.

Table 2. Primers for RT-PCR of other bacterial genes

Gene	Primer sequence	Positions from ATG (size, bp)
<i>fbpA</i>	F: 5'-CAAACCTGAGCGTTGGGGTAA-3'	45–400 (356)
	R: 5'-GTGTCTCCTGCACGGATG-3'	
<i>pam</i>	F: 5'-AGTCGTAGGAGCAGGGTT-3'	81–439 (359)
	R: 5'-GCTTATCCGCTAATTTGTCT-3'	
<i>speB</i>	F: 5'-CAGCAGCTATCAAAGCAGGTGCAC-3'	146–429 (284)
	R: 5'-CTCAGCGGTACCAGCATAAGTAG-3'	
<i>sk2b</i>	F: 5'-GGAACAGTCAAGCCTGTCCAAGCT-3'	55–369 (315)
	R: 5'-GTAGCCGTCGTTACTGTGAACGTT-3'	
<i>covS</i>	F: 5'-GTTAGAGTTCGTCTTTCTGAGGTG-3'	873–1113 (241)
	R: 5'-GACCATGCAAGCCAGGAGATGATTC-3'	
<i>srtA</i>	F: 5'-GTACAGAGAGTGTCTTGAAGCAC-3'	233–492 (260)
	R: 5'-CCCATTTTGTGCTCTTTCAAGCGG-3'	
<i>hasA</i>	F: 5'-GAGCCATTTAAAGGAAAGCCACATG-3'	163–457 (295)
	R: 5'-CGTCAGCGTCAGATCTTTCAAATGC-3'	
<i>slo</i>	F: 5'-GCTAGTACAGAAACCACAACGAC-3'	124–532 (409)
	R: 5'-GATAGGTCCTATCAGTGACAGAGTC-3'	
<i>plr (gapdh)</i>	F: 5'-GACGGTACTGAAACAGTTATCTC-3'	420–654 (235)

other strains does not contain a stop codon within the coding sequence of the gene [15]. For insertion of mCherry, a 418 bp sequence upstream of *fcr*/ATG (*fcr* promoter) linked to the mCherry coding sequence with the *fcr* termination (26 bp downstream of *fcr*/TAA) was subcloned into the pCR-TOPO vector (Invitrogen). This *fcr*-mCherry DNA cassette, which contained *NotI* (5') and *BamHI* (3') restriction sites, was then cloned into the temperature-sensitive plasmid pHY304 (from M. J. Walker, Queensland, Australia), which also contained an erythromycin resistance gene (*erm*^r), at *NotI/BamHI* sites within the multiple cloning locus, to generate the GAS-RFP targeting vector (pND1496). The pND1496 plasmid was used to generate AP53(S-)-RFP stable cell lines.

Generation of a stable GAS-RFP cell line

GAS-competent cells were made by utilizing a sucrose protocol [16] from a natural isolate of GAS strain AP53 (AP53/CovS-, from G. Lindahl, Lund, Sweden) [17]. The AP53(S-)-RFP stable cell line was generated by electroporating pND1496 and the GAS cells in the presence of 4 μg erythromycin ml⁻¹, and targeted into the *fcr* gene allele within the *mga* regulon of the GAS genome (Fig. 1). Chromosomal integration was achieved by single crossover (SCO) at 30 °C (the optimal temperature for plasmid replication) and then switched to 37 °C for screening for Em^r. Confirmation of SCO was achieved by PCR (primers are listed in Table 1). The expression of mCherry by the targeted GAS strains was verified by utilizing fluorescence

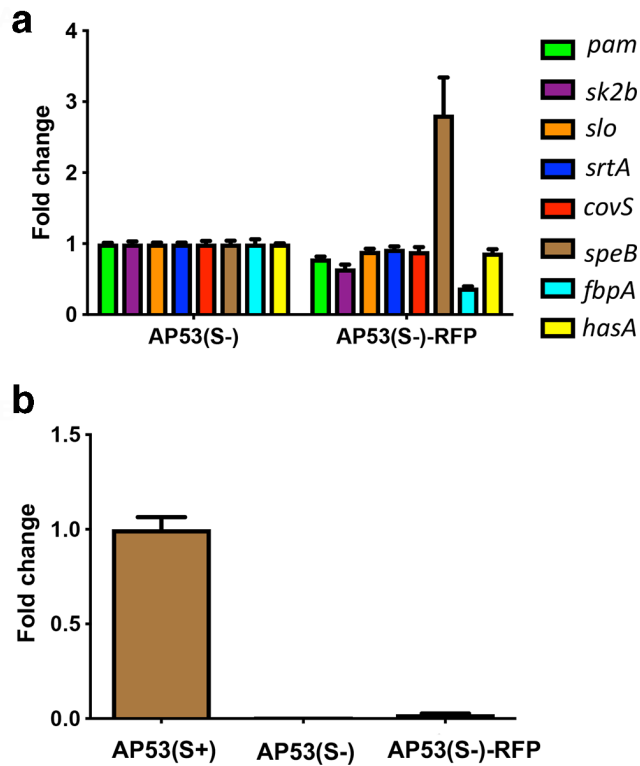


Fig. 2. RT-PCR of transcripts of RFP and other bacterial genes in AP53(S-) and AP53(S-)-RFP. $\Delta\Delta CT$ analyses were performed on a number of AP53(S-) and AP53(S-)-RFP genes. (a) Gene transcript values for AP53(S-) were assigned a value of 1 to normalize comparative changes in AP53(S-)-RFP. (b) SpeB gene transcript value for AP53(S+) was assigned a value of 1 to normalize comparative changes in AP53(S-) and AP53(S-)-RFP. *gapdh(plr)* served as a housekeeping gene. $n=4$ for each gene.

microscopy (Nikon ECLIPSE TE 2000-S) with the Texas Red channel to visualize RFP expression and maintenance of bacterial morphology in fluorescent cells. RT-PCR of mRNA purified from GAS was also performed to specifically verify mCherry transcription in the AP53(S-)-RFP stable cells.

Analysis of pleiotrophic effects in GAS AP53(S-)-RFP on other bacterial genes

Determination of any pleiotrophic effects of mCherry coding sequence insertion at the Fcr pseudogene of the GAS genome on other genes was determined by RT-PCR. Total RNA was extracted at log phase growth from AP53(S-) and AP53(S-)-RFP. Eight example virulence-associated genes and a housekeeping gene were selected for RT-PCR [*sortase A (srtA)*, *fibronectin binding protein A (fbpA)*, *hyaluronan synthase A (has A)*, *streptolysin O (slo)*, *streptococcal pyrogenic exotoxin B (speB)*, *plasminogen-binding group A streptococcal M-like protein (pam)*, *streptokinase 2b (sk2b)*, *cluster of virulence sensor (covS)*, *the house keeping gene plasminogen receptor (plr)*/ *glyceraldehyde 3-phosphate dehydrogenase (gapdh)*] to compare RNA transcript levels from the parent strain and the fluorescent strains. Results were normalized to the

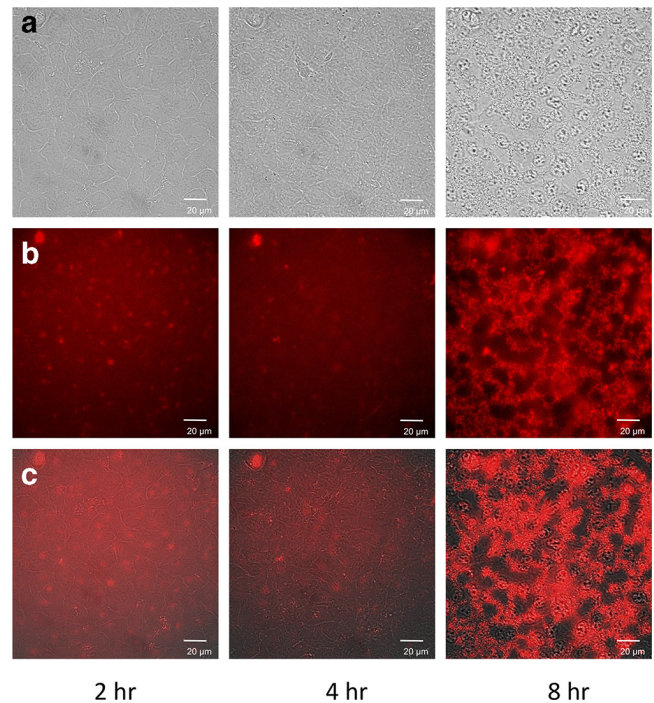


Fig. 3. AP53(S-)-RFP growth on human keratinocytes. Confluent human keratinocytes (HaCaT cells) were infected with AP53(S-)-RFP bacteria at a m.o.i. of 1. Images were taken at 2, 4, and 8 h post-infection. (a) DIC channel. (b) RFP channel. (c) Merged. Magnification, 600 \times .

housekeeping gene, *gapdh*. Primers for the nine genes are listed in Table 2.

GAS AP53(S-)-RFP infection of keratinocytes

Human keratinocytes (HaCaTs, a spontaneously transformed immortalized keratinocyte cell line from adult human skin) were grown to confluency in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS). Cells were grown in a 35 mm dish with a glass coverslip (MatTek) for imaging. Prior to infection, HaCaT cells were washed with PBS and new DMEM+FBS was added. HaCaT cells were infected with GAS strain (AP53(S-)-RFP) at a m.o.i. of 1 bacterium per host cell. Cells and bacteria were grown in the environmental chamber of the Eclipse Ti Inverted Microscope (Nikon) at 37°C and 5% CO₂. Images were taken of the cells every 10 min for 8 h with NIS-Elements software (Nikon) using the differential interference contrast (DIC) and RFP channels. Selected images from time points of 2, 4, and 8 h were processed using ImageJ, at a magnification of 600 \times .

Keratinocyte cytotoxicity after AP53(S-) and AP53(S-)-RFP infection

Human keratinocytes (HaCaT cells) were grown to 90% confluency in 24-well cell culture treated plates in DMEM (Gibco) supplemented with 10% FBS. AP53(S-) and AP53(S-)-RFP GAS strains were grown in Todd Hewitt broth supplemented with 2% yeast extract (THY) at 37°C overnight.

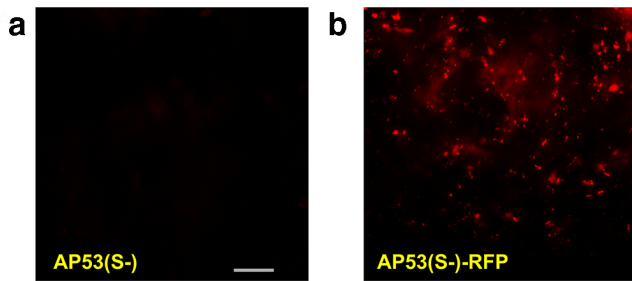


Fig. 4. Histology of skin from a wound infection site in mice 48 h post-infection. Mice were subcutaneously infected with $1-3 \times 10^7$ c.f.u. of (a) AP53(S-) and (b) AP53(S-)-RFP. Skin wound tissue was removed for fluorescence analysis. Red fluorescent bacteria are shown in (b). Bar, 25 μ m (a). Magnification, 400 \times .

Prior to infection, spent medium was removed from the HaCaT cells and then washed with PBS, and new medium was added. Overnight bacterial cultures were centrifuged at 3500 r.p.m. for 5 min and the pellets were resuspended in new THY media. Optical densities of the cultures were normalized, and bacteria were added to the HaCaT cells for a m.o.i. of 10 bacteria per host cell. Infected HaCaT cells were incubated at 37°C and 5% CO₂ for 6 h. At the conclusion of the infection, the supernatant was removed from the HaCaT monolayer, and the cells were washed with PBS. The wash was aspirated, and 250 μ l of a 4 μ M ethidium homodimer solution in PBS (Molecular Probes) was added to each well. Cells were incubated in the dark for 30 min. The level of fluorescence was determined using a plate reader set to 528 nm excitation and 617 nm emission with a cutoff value of 590 nm. After the first reading, 0.1% (w/v) saponin was added to each well of the plate. Plates were rocked in the dark for 20 min before a second fluorescence reading with the same settings. Percentage membrane permeabilization values were obtained for each well by dividing the initial fluorescence reading (post-treatment) by the second fluorescence reading (post-saponin). An ANOVA test was used to determine statistical significance using GraphPad Prism software.

GAS AP53(S-)-RFP infection in mice and *in vivo* assessments of pathogenesis

GAS is a human pathogen and is weakly virulent in mice. To develop a GAS infection model in mice, the human plasminogen transgene was incorporated into the murine genome [18]. Additionally, GAS expresses a plasminogen activator, namely streptokinase, which only activates human plasminogen (hPg). With the expression of hPg, mice are susceptible to GAS infection and have been used in a number of studies. Therefore, for these studies, WT C57BL6/J mice expressing the hPg transgene (from D. Ginsburg, Ann Arbor, MI, USA) [18] were anaesthetized with isoflurane and then injected, subcutaneously, with $1-3 \times 10^7$ c.f.u. per mouse of AP53(S-) or AP53(S-)-RFP. Infected murine skin samples were taken at 48h and were then fixed in 4% paraformaldehyde for 3 h and subsequently cryoprotected in 15% then 30% sucrose for 2 h

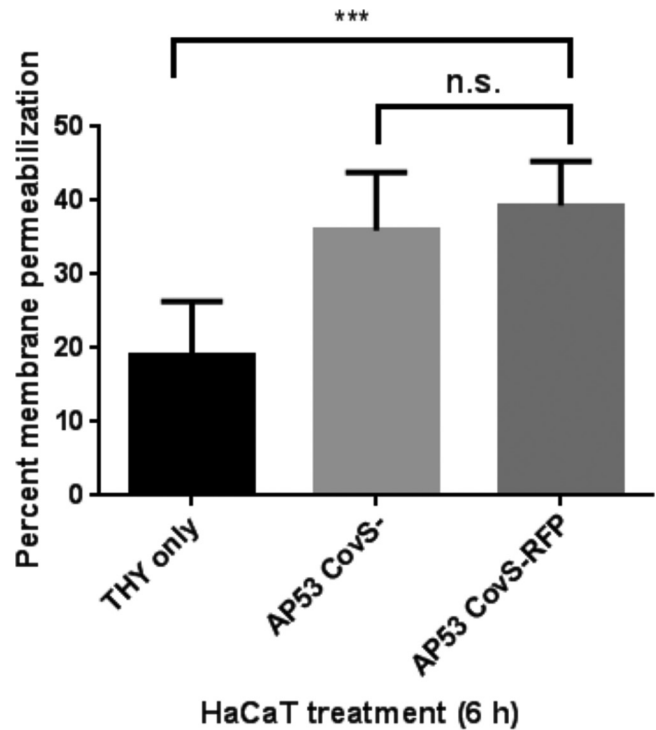


Fig. 5. Membrane permeabilization of human keratinocytes following GAS infection. Human keratinocytes (HaCaT cells) were infected with AP53(S-) and AP53(S-)-RFP GAS strains for 6 h. Membrane permeabilization was assessed using fluorescence readings after ethidium homodimer treatment. *** $P < .001$.

each, respectively. Samples were then embedded in Optimal Cutting Temperature (OCT) compound for 15 min, flash frozen in liquid nitrogen and stored at -80°C . Cryosections (10 μ m) were made (Shandon motorized cryostat), mounted on supercharged slides and coverslipped with prolong gold mounting medium. Fluorescence images were then immediately acquired with a Nikon TE 2000-S microscope and Nikon Elements AR 3.2 imaging software using the Texas Red channel. All images were acquired and processed uniformly. Representative images are shown. Magnification was 400 \times .

Mouse survival studies

Mice were infected, subcutaneously, with $1-3 \times 10^8$ c.f.u. AP53(S-) and AP53(S-)-RFP, as described above, and survival was monitored as a function of time. Survival differences between the two GAS strains were evaluated by a log rank test.

Ethical considerations

The animal studies described in this work were approved by the University of Notre Dame Institutional Animal Care and Use Committee.

RESULTS AND DISCUSSION

The AP53(S-) GAS strain is a highly virulent strain initially isolated from a patient who developed necrotizing fasciitis.

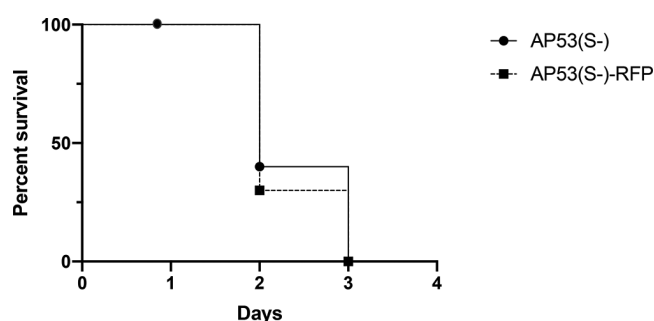


Fig. 6. Survival of human plasminogen transgenic mice after AP53(S-) or AP53(S-)-RFP infection. C57BL/6J mice expressing hPg were infected, subcutaneously, with $1-3 \times 10^8$ c.f.u. of AP53(S-) ($n=5$) or AP53(S-)-RFP ($n=10$) and survival was monitored over a 3-day period.

Its enhanced virulence is due to an inactivating mutation in the sensor component of the cluster of virulence (cov) responder (R)/sensor (S) two-component gene regulatory system (covRS) [15, 19]. In the present study, we generated a stable fluorescence-labelled AP53(S-) GAS strain by single site insertion at the *fcr* pseudogene with the coding sequence of mCherry in GAS strain AP53(S-). Utilizing a plasmid that contained sequencing 5' of the *fcr* gene, mCherry coding sequence and *fcr* termination sequences, the mCherry cassette inserted at the *fcr* locus in the AP53(S-) GAS strain (Fig. 1). Fluorescence microscopic analysis demonstrated that the bacteria express mCherry. Insertion of the mCherry cassette in the multiple gene regulator of GAS (*mga*) locus did not affect, to any significant extent, the transcription of other critical genes (Fig. 2a). In particular, we analysed transcription levels of prominent GAS virulence genes (*srtA*, *fbp*, *hasA*, *slo*, *speB*, *pam*, *sk*, *covS*), which for some (*pAM*, *sk2b*, *speB*, *fbpA*) were affected by insertion of the mCherry gene into the GAS AP53 strain. However, the increase in *speB* transcripts in AP53(S-)-RFP was insignificant compared to transcript levels in AP53(S+) (Fig. 2b). Infected cultured keratinocytes (Fig. 3) and skin from AP53(S-)-RFP-infected mice (Fig. 4) demonstrated that the AP53(S-)-RFP cells were detectable by their fluorescence properties. Keratinocyte infection by GAS AP53(S-)-RFP showed similar dynamics and host cytotoxicity to the parental GAS AP53(S-) strain (Fig. 5). AP53(S-)-RFP and control AP53(S-) were comparable in infected mice survival studies, with similar histology characteristics of immune cell infiltration and desquamation, demonstrating no effect on AP53(S-) endogenous virulence capacity (Fig. 6). These results demonstrate that a fluorescent protein can be targeted into the genomic component of a virulent strain of GAS, resulting in bacteria that endogenously and stably express a fluorescent protein without significantly altering the virulence capacity relative to the parental strain.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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