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Decreased reactivation of a herpes simplex virus type 1 (HSV-1) latency associated transcript (LAT) mutant using the in vivo mouse UV-B model of induced reactivation

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Abstract

Blinding ocular herpetic disease in humans is due to herpes simplex virus type 1 (HSV-1) reactivations from latency, rather than to primary acute infection. The cellular and molecular mechanisms that control the HSV-1 latency-reactivation cycle remain to be fully elucidated. The aim of this study was to determine if reactivation of the HSV-1 latency associated transcript (LAT) deletion mutant (dLAT2903) was impaired in this model, as it is in the rabbit model of induced and spontaneous reactivation and in the explant TG induced reactivation model in mice. The eyes of mice latently infected with wild type HSV-1 strain McKrae ($LAT^{(+)}$ virus) or dLAT2903 ($LAT^{(-)}$ virus) were irradiated with UV-B and reactivation was determined. We found that compared to $LAT^{(-)}$ virus, $LAT^{(+)}$ virus reactivated at a higher rate as determined by shedding of virus in tears on days 3 to 7 after UV-B treatment. Thus, the UV-B induced reactivation model of HSV-1 appears to be a useful small animal model for studying the mechanisms involved in how LAT enhances the HSV-1 reactivation phenotype. The utility of the

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Conflict of interest.

All of the authors (Lbachir BenMohamed, Nelson Osorio, Ruchi Srivastava, Arif A. Khan, Jennifer L. Simpson, Steven L. Wechsler) declare that they have no conflict of interest.

model for investigating the immune evasion mechanisms regulating the HSV-1 latency/reactivation cycle and for testing the protective efficacy of candidate therapeutic vaccines and drugs are discussed.

Keywords

HSV-1; LAT; UV-B; recurrent herpetic disease; eye; animal model; immunology; virology

INTRODUCTION

Following primary infection at the eye, herpes simplex virus (HSV-1) replicates, travels up innervating neuronal axons to the trigeminal ganglia (TG) where it establishes a life-long latent infection in sensory neurons. Herpes simplex virus (HSV-1) stromal keratitis (HSK) is a leading cause of corneal blindness in the United States (Smith *et al*, 1980). HSK is an immuno-pathological process that is triggered by sporadic reactivation of HSV-1 from a latent state in sensory neurons, transport down neuronal axons, and shedding in tears (Chentoufi and Benmohamed, 2012; Meyers, 1975; Stuart *et al*, 2004). This leads to recurrent bouts of inflammation and progressive corneal scarring (Nesburn, 1983). However, the viral/host factors involved, and the underlying innate and adaptive immune mechanisms and their kinetics, remain to be fully elucidated. Major symptoms of recurrent herpes disease in the eye include clouding of the cornea and neovascularization, both of which can impair vision and lead to loss of sight. Over 400,000 people in the United States have a history of recurrent herpetic ocular disease (Farooq and Shukla, 2012; Xu *et al*, 2002). Estimates are that up to 90% (Farooq and Shukla, 2012; Kaufman *et al*, 2005; Xu *et al*, 2006) of adults in the United States harbor latent HSV-1, making them susceptible to developing HSK (Kumaraguru *et al*, 1999; Rowe *et al*, 2013).

The HSV-1 latency associated transcript gene (LAT) is expressed during latency (Rock *et al*, 1987; Spivack and Fraser, 1987; Stevens *et al*, 1987; Stroop *et al*, 1984) and is the only viral gene that is consistently detected as being abundantly transcribed at this time (Rock *et al*, 1987). The remaining 80 plus viral genes are shut down during latency. In mice, small amounts of mRNA from some of these genes are sometimes detected during latency. This is likely a result of sporadic abortive reactivations in single neurons, sometimes called “molecular reactivation” (Feldman *et al*, 2002; Margolis *et al*, 2007). Experiments in mice and rabbits have shown that LAT plays a critical role in enhancing the reactivation phenotype (Hill *et al*, 1990; Leib *et al*, 1989; Perng *et al*, 1994; Trousdale *et al*, 1991). This appears to be a function of LAT’s anti-apoptosis activity (Inman *et al*, 2001; Perng *et al*, 2000), since wild type levels of reactivation can be restored to a $LAT^{(-)}$ virus by inserting one of several different alternative anti-apoptosis genes in place of LAT (Jin *et al*, 2008; Jin *et al*, 2007; Jin *et al*, 2005; Perng *et al*, 2002). LAT may also contribute to latency/reactivation *via* numerous immune evasion mechanisms, such as its ability to directly or indirectly delay or interfere with interferon production (Peng *et al*, 2005), protect against $CD8^+$ T-cell killing by blocking granzyme B induced apoptosis (Jiang *et al*, 2011), promote exhaustion of $CD8^+$ T-cells (Allen *et al*, 2011; Chentoufi *et al*, 2011), and increase HVEM

expression (Allen *et al*, 2014) (herpes virus entry mediator, a member of the tumor necrosis family) which can act as a switch to decrease T cell function.

Mouse studies of HSV-1 reactivation from latency have been limited by the fact that spontaneous reactivation of HSV-1 accompanied by return of the reactivated virus to the eye, either does not occur, or occurs at a rate too low for study (Feldman *et al*, 2002; Gebhardt and Halford, 2005). Thus, most HSV-1 reactivation studies in mice have been done ex vivo using the trigeminal ganglia (TG) explant induced reactivation model (Deshmane *et al*, 1993; Devi-Rao *et al*, 1994; Leib *et al*, 1989; Perng *et al*, 2001; Spivack *et al*, 1995). This is an ex vivo system in which reactivation is induced by sacrificing the mouse, removing the TGs, cutting them into small pieces, culturing the pieces in tissue culture media, and monitoring the media for the appearance of reactivated infectious virus. Whether the ex vivo TG explant induced model of HSV-1 reactivation reliably mirrors the in vivo situation in humans is unclear. HSV-1 reactivation in mice can also be induced by other methods including hyperthermia, sodium butyrate, cyclophosphamide plus dexamethasone, cadmium, cellophane, xylene, retinoic acid, iontophoresis of epinephrine, dimethyl sulfoxide, and physical restraint (Blyth *et al*, 1980; Cook *et al*, 1991; Halford *et al*, 1996; Harbour *et al*, 1981; Higaki *et al*, 2003; Higaki *et al*, 2002; Hill *et al*, 1982; Himmelein *et al*, 2011; Neumann *et al*, 2007; Sawtell and Thompson, 1992; Toma *et al*, 2008; Zlotnik *et al*, 1970). A potential drawback of these models is that there are no reports of recurrent eye disease being induced. Thus these models may not be optimal for the study of therapeutic interventions aimed at preventing recurrent HSK.

Fortunately, an in vivo HSV-1 reactivation model in mice in which significant recurrent corneal disease is induced has been developed (Laycock *et al*, 1991). In this model, mice are ocularly infected with HSV-1 to establish cohorts of latently infected mice. The eyes of the latently infected mice are subsequently irradiated with UV-B light. This induces reactivation of the virus and its return to the eye, as determined by the detection of infectious HSV-1 in the tears of approximately 50% of eyes between days 3 and 7 post UV-B exposure. Recurrent herpetic eye disease also occurs at high levels in this model. The use of this UV-B model appears to be mostly limited to the lab that developed this model and investigators from that lab (Keadle *et al*, 2008; Keadle *et al*, 1997; Keadle *et al*, 2002a; Keadle *et al*, 2002b; Keadle *et al*, 2005; Keadle *et al*, 2002c; Keadle *et al*, 2001; Keadle *et al*, 2000; Laycock *et al*, 1991; Morris *et al*, 2012a; Stuart and Keadle, 2012a; Stuart *et al*, 2008; Walker *et al*, 1998). A recent video (Morris *et al*, 2012a) demonstrating the UV-B model convinced us to attempt the model in our lab. We were particularly interested in determining if wild type ($LAT^{(+)}$) virus would reactivate at a higher rate than $LAT^{(-)}$ virus following UV-B induced reactivation, as is the case for spontaneous and in vivo induced reactivation in rabbits (Hill *et al*, 1990; Perng *et al*, 1994) and for TG explant induced reactivation (Leib *et al*, 1989) and heat stress induced reactivation (Sawtell and Thompson, 1992) in mice.

We report here that: (*i*) We were successful in performing the UV-B model of HSV-1 induced reactivation in latently infected mice and the results were consistent with those previously reported (Laycock *et al*, 1991; Morris *et al*, 2012a); and (*ii*) Compared to $LAT^{(-)}$ virus (dLAT2903), wild type ($LAT^{(+)}$) HSV-1 reactivated at a higher rate in the UV-B mouse model, as determined by shedding of virus in tears on days 3 to 7 after UV-B

treatment. The usefulness of the UV-B light mouse model in investigating the possible LAT-mediate immune evasion mechanisms that regulate HSV-1 latency/reactivation and in testing the efficacy of candidate therapeutic vaccines and drugs are discussed.

MATERIALS AND METHODS

Cell lines

Rabbit skin (RS) cells were maintained in Eagle minimal essential medium (MEM) with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1mM sodium pyruvate, 10% fetal bovine serum (Promega Scientific), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Sigma, St. Louis, MO).

Viruses

All viruses were triple plaque purified and passaged only two or three times in rabbit skin (RS) cells prior to use. $LAT^{(+)}$ wild-type (wt.) HSV-1 strain McKrae is the parental virus for the $LAT^{(-)}$ mutant dLAT2903. Both viruses have been previously described (Perng *et al*, 1994).

Mice

Eight- to 10-week-old female C57BL/6 mice (Jackson Labs) were used in all studies. All animal studies conform to the UC Irvine IACUC guidelines and the guidelines of the US National Institute of Health.

Infection of mice

Ocular infection of mice was performed as described by (Morris *et al*, 2012a) using 1×10^6 pfu of either $LAT^{(+)}$ McKrae or $LAT^{(-)}$ dLAT2903 per eye. Briefly, mice were anesthetized and corneas were scarified (i.e., the epithelium was lightly scratched) in a crosshatched pattern of 4 to 5 vertical and 4 to 5 horizontal scratches using a 25-gauge needle. Each mouse received an i.p. injection of 0.5 ml of pooled serum containing HSV-1 neutralizing antibodies with a 50% plaque reduction neutralization titer of approximately 1:128. The serum we used differed from that previously used (Morris *et al*, 2012a) as we used pooled serum from rabbits latently infected with HSV-1 instead of pooled human serum. In the pilot study (experiment 1 in Fig. 2 below) only the right eyes of mice were infected, consistent with the description of the UV-B mouse model (Morris *et al*, 2012a). In experiment 2 in Figs. 2 and 3 below and in the experiment shown in Fig. 4 below, both eyes were infected.

UV-B irradiation of mouse eyes, monitoring shedding of reactivated HSV-1 in tears, and monitoring recurrent eye disease

UV-B irradiation was done exactly as described (Morris *et al*, 2012a), including using the same UV-B light source, except that in some experiments both eyes were used. Briefly, anesthetized mice were placed on a TM20 Chromato-Vu transilluminator (UVP, San Gabriel, CA), which emits UV-B at a peak wavelength of 302 nm. Each mouse was positioned on a piece of cardboard containing a hole that is the same size as the mouse's eye. This allowed just the eye to be irradiated by the UV-B source. Each eye was irradiated

with 250 mJ of UV-B light cm^2 (approximately 1 minute exposure on the transilluminator). On days 3, 4, 5, 6, and 7 post UV-B irradiation, eyes were swabbed and tear films plated on RS cells for detection of reactivated virus as we previously described for monitoring lytic virus replication in eyes of rabbits and mice and spontaneously reactivated virus in eyes of rabbits (Jin *et al*, 2005; Perng *et al*, 1994). Recurrent eye disease was monitored at various times post UV-B irradiation as described (Morris *et al*, 2012a).

RESULTS

Inducing reactivation of HSV-1 latency by UV-B irradiation as judged by detection of infectious virus in tears of mice

We first did a small pilot study using 9 infected and 2 uninfected eyes following the published procedure (Morris *et al*, 2012a) as closely as possible. An outline of the time course of the UV-B induced reactivation experiments in this report is illustrated in **Fig. 1**. As described in *Materials & Methods*, nine C57BL/6 female mice were anesthetized. The right cornea of each mouse was lightly scarified and infected with 1×10^6 pfu/eye of the McKrae strain of HSV-1 as eye drops. Just prior to infection each mouse received an i.p. injection of 0.5 ml of immune serum containing HSV-1 neutralizing antibody to partially protect against acute eye disease and death. Thirty days post infection the nine infected eyes were irradiated with 250 mJoules of UV-B (302 nm). The right eyes of 2 naïve age- and sex-matched control mice were subjected to the same UV-B irradiation. The only known variance from the published procedure was that we used anti-HSV-1 pooled rabbit immune serum instead of pooled human immune serum to help protect the infected mice against the primary HSV-1 infection. Following UV-B irradiation, tear swabs were collected daily for 7 days and plated on indicator cells (RS cells) to detect the presence of infectious virus that had reactivated in the corresponding TG and returned to the eye. **Fig. 2A** (Expt. 1) shows the cumulative percent of eyes that had at least one virus positive tear culture in this study. Infectious virus indicative of latent virus that reactivated in the corresponding TG and returned to the eye was detected in approximately 55% of the eyes in this experiment. These results are similar to those previously reported (Laycock *et al*, 1991; Morris *et al*, 2012a) and show that UV-B irradiation induces reactivation of HSV-1 that can be detected by shedding of virus in tears.

We also determined the cumulative number of virus positive tear film cultures (**Fig. 2B**; Expt. 1). This takes into account multiple days on which an eye shed virus, and is one of the standard analyses we perform when analyzing spontaneous reactivation in rabbit eyes (Perng *et al*, 1994). Since in this, and subsequent experiments, we never detected virus in any mouse eye prior to day 3 post UV-B treatment or after day 7 post UV-B treatment, the total number of cultures used for these calculations excluded days 0, 1, and 2 and days 8 and later. The calculation was done as follows: [(number of virus positive tear film cultures on that day + the number of positive cultures on all preceding days) / (total number of tear film cultures evaluated from day 3 to day 7)] × 100%. Thus, the denominator was 9 eyes × 5 swabs (days 3, 4, 5, 6, 7) or 45.

To confirm the results in the pilot study, and to determine if using both eyes rather than just one eye per mouse would produce similar results, both eyes from 10 mice (20 eyes/group)

were either infected with wt McKrae or mock infected (naïve mice). All other manipulations were identical to those in the pilot study. The HSV-1 reactivation results are shown in **Fig. 2A** and **2B** (Expt. 2). Both the cumulative percent of eyes shedding reactivated virus and the cumulative percent of virus positive cultures were virtually identical to those in the smaller pilot study. These results confirmed that we were able to perform the mouse UV-B induced reactivation model, as judged by induction of shedding virus in mouse tears.

Inducing recurrent eye disease using UV-B

In addition to inducing shedding of virus in tears, the UV-B model is also reported to induce recurrent herpetic disease as judged by stromal clouding (Laycock *et al*, 1991; Morris *et al*, 2012). To confirm that we were also able to reproduce this result, eye disease was determined in the eyes of the mice in experiment 2 before and after UV-B irradiation. One day prior to UV-B, all eyes in both groups were scored on a scale of 0 to 4 by examination under a dissecting microscope, as previously described (Morris *et al*, 2012). Five eyes had significant disease prior to UV-B irradiation. This was a result of the primary ocular infection. These eyes were removed from the study because the existing disease would make it impossible to score disease induced by UV-B reactivation of HSV-1. All 20 eyes in the control group were normal prior to UV-B. Following UV-B irradiation, the amount of disease in individual eyes was evaluated and scored on the days indicated in **Fig. 3**, by an individual who was masked as to which group the eye was from. Between days 10 to 31 post UV-B irradiation the average eye disease score was significantly higher in the HSV-1 infected group compared to the uninfected control group. These results were also similar to those previously reported (Morris *et al*, 2012a), indicating that the UV-B model was working as expected.

UV-B irradiation induces more virus reactivation (shedding) in mice latently infected with wild type LAT$^{(+)}$ virus compared to the LAT$^{(-)}$ virus dLAT2903

It is well established that in rabbits the HSV-1 LAT plays an important role in both the in vivo spontaneous and induced reactivation phenotypes. Similarly LAT plays an important role in induced reactivation of HSV-1 in the mouse TG explant induced model and in the mouse heat stressed model. However, some LAT mutants do not behave similarly in the in vivo rabbit model and the ex vivo mouse TG explant model (Loutsch *et al*, 1999; Maggioncalda *et al*, 1994). It was therefore of interest to determine if LAT plays a significant role in the in vivo UV-B reactivation model in mice.

We therefore infected both eyes of 15 mice per group with either wild type McKrae (LAT$^{(+)}$ virus) or dLAT2903 (LAT$^{(-)}$ virus), which was derived from McKrae. All procedures were as described above. Following UV-B irradiation, virus reactivation (shedding in tears) was determined and analyzed as in Figure 1 (**Table 1**, **Fig. 4A** and **4B**). Reactivation of LAT$^{(+)}$ virus was significantly greater than LAT$^{(-)}$ virus by both analyses. The results for the percent of virus positive cultures (induced reactivation) seen from day 3 to 7 after UV-B irradiation are reminiscent of the percent of virus positive tear cultures seen in rabbits from day 31 to 56 post infection (spontaneous reactivation) (Perng *et al*, 1994). Thus, the UV-B induced reactivation mouse model readily distinguishes between LAT$^{(+)}$ and LAT$^{(-)}$ viruses

and provides an additional model for determining the reactivation phenotype of HSV-1 mutants.

DISCUSSION

Previous studies have used the UV-B mouse model of induced HSV-1 reactivation to investigate the protective efficacy of vaccines and drugs (e.g., acyclovir), and to determined immune mechanisms that protect from or promote recurrent HSK (Keadle *et al*, 2008; Keadle *et al*, 1997; Keadle *et al*, 2002a; Keadle *et al*, 2002b; Keadle *et al*, 2005; Keadle *et al*, 2002c; Keadle *et al*, 2001; Keadle *et al*, 2000; Laycock *et al*, 1991; Morris *et al*, 2012a; Stuart and Keadle, 2012a; Stuart *et al*, 2008; Walker *et al*, 1998). To our knowledge the effect of the HSV-1 LAT gene has not previously been investigated in the UV-B model.

LAT is both an indicator of HSV-1 latency and a critical gene in the latency/reactivation life cycle of HSV-1 (Hill *et al*, 1990; Leib *et al*, 1989; Perng *et al*, 1994; Rock *et al*, 1987; Spivack and Fraser, 1987; Stevens *et al*, 1987; Trousdale *et al*, 1991). Unlike other HSV-1 genes whose expression is tightly regulated in a cascade fashion of gene expression (i.e., α, β, and γ genes), LAT is expressed at all times. The LAT promoter functions in the absence of any other HSV-1 gene (Zwaagstra *et al*, 1989; Zwaagstra *et al*, 1990) and LAT can be detected in tissue culture almost immediately after infection (Spivack and Fraser, 1988a; Spivack and Fraser, 1988b). LAT expression remains on and LAT RNA accumulates such that at late times after tissue culture infection LAT is abundant. During latency in animal models and in humans, LAT is the only viral gene that is consistently and abundantly detected (Deatly *et al*, 1987; Rock *et al*, 1987; Stevens *et al*, 1987). A low level of lytic cycle gene expression that is sometimes detected in ganglia during latent infection of mice (Feldman *et al*, 2002) is likely due to a small number of neurons undergoing abortive reactivation. Detection of lytic cycle gene expression in rabbits and in humans is likely due to viral reactivation in a small subset of neurons since spontaneous reactivation occurs in rabbits and humans.

LAT has anti-apoptosis activity (Perng *et al*, 2000) and can also act as an immune evasion gene (Allen *et al*, 2011; Allen *et al*, 2014; Chentoufi *et al*, 2012; Chentoufi *et al*, 2011; Jiang *et al*, 2011; Peng *et al*, 2005). Both of these LAT functions likely play a role in how LAT enhances the reactivation phenotype. Mouse models that require either the induction of reactivation or the detection of reactivation to take place ex vivo cannot fully take into account effects of LAT's immune evasion activity on HSV-1 reactivation and recurrent disease. The rabbit model of HSV-1 latency/reactivation appears to more closely resemble the human situation, since both spontaneous reactivation and induced reactivation take place completely in vivo. Unfortunately, because of limited availability of rabbit immunological reagents, and knock-in and knock-out rabbits, the ability to study the underlying cellular and molecular immune mechanisms in rabbits does not approach the robustness of such studies in mice. Thus, a fully in vivo reactivation model in the mouse in which the influence of LAT on the reactivation phenotype can be investigated, would be a valuable asset for deciphering the cellular and molecular immune mechanisms that regulate HSV-1 latency/reactivation.

Most adults carry latent HSV-1, shed reactivated virus frequently, but remain asymptomatic having no recurrent disease (Kumar *et al*, 2009; Schacker *et al*, 1998; Wald *et al*, 1995). In contrast, a small proportion of individuals are symptomatic and have frequent recurrent disease (Kumar *et al*, 2009; Schacker *et al*, 1998; Wald *et al*, 1995). Cross talk between the immune system and the virus orchestrate HSV latency/reactivation (Allen *et al*, 2011; Allen *et al*, 2014; Chentoufi *et al*, 2012; Chentoufi *et al*, 2011; Ghiasi *et al*, 1992; Jiang *et al*, 2011; Peng *et al*, 2005). Although most herpetic disease is due to viral reactivation, rather than to primary acute infection (Nesburn, 1983; Nesburn *et al*, 1998a; Nesburn *et al*, 1998b), the vast majority of experimental models investigating the immune mechanisms that orchestrate recurrent herpes disease have used the mouse model of primary acute infection, instead of more relevant models of recurrent disease (Morris *et al*, 2012b; Stuart and Keadle, 2012a; Webre *et al*, 2012). The extrapolation to human recurrent disease is uncertain, because the protective and pathological mechanisms that operate during primary herpetic disease are, in most part, different from those that operate during recurrent herpetic disease (Morris *et al*, 2012a; Stuart and Keadle, 2012a; West *et al*, 2014). The UV-B model of HSV-1 induced reactivation is more likely to immunologically and pathologically mimic human recurrent ocular herpetic disease (Morris *et al*, 2012a; Stuart and Keadle, 2012a; Webre *et al*, 2012).

In addition to explant cultivation of TG and UV-B irradiation of eyes, other methods have also been used to induce reactivation of HSV-1 in mice. These include hyperthermia, sodium butyrate, cyclophosphamide plus dexamethasone, cadmium, cellophane, xylene, retinoic acid, iontophoresis of epinephrine, dimethyl sulfoxide, and physical restraint (Blyth *et al*, 1980; Cook *et al*, 1991; Halford *et al*, 1996; Harbour *et al*, 1981; Higaki *et al*, 2003; Higaki *et al*, 2002; Hill *et al*, 1982; Himmelein *et al*, 2011; Neumann *et al*, 2007; Sawtell and Thompson, 1992; Toma *et al*, 2008; Zlotnik *et al*, 1970). In these models, HSV-1 reactivation was assessed either by the presence of infectious virus in the ganglia in vitro or shedding of virus in tears. Shimeld (Shimeld *et al*, 1989; Shimeld *et al*, 1990) compared immunosuppressive drugs (cyclophosphamide plus dexamethasone) to UV-B irradiation. Both induced HSV-1 reactivation judged by shedding of virus in tears, but only UV-B induced recurrent eye disease. To our knowledge, only UV-B has been reported to induce significant recurrent HSK, making it the most useful mouse model for investigating the cellular and immunological mechanisms involved in recurrent herpetic eye disease. The UV-B method used in the present study was developed by Shimeld *et al*. (Shimeld *et al*, 1989; Shimeld *et al*, 1990; Shimeld *et al*, 1996a; Shimeld *et al*, 1995; Shimeld *et al*, 1996b; Shimeld *et al*, 1997) and adopted and modified by (Laycock *et al*, 1994; Laycock *et al*, 1991; Laycock *et al*, 1993; Pepose *et al*, 1992; Stuart and Keadle, 2012b) and (Morris *et al*, 2012a). The present study extends the UV-B model by showing that $LAT^{(+)}$ (wt HSV-1) has a higher frequency of reactivation in this model than does $LAT^{(-)}$ HSV-1.

It should be noted that the UV-B induced reactivation model is not limited to C57BL/6 mice. With the NIH strain of mice UV-B induced reactivation is 70–90% as determined by detection of infectious virus in tear films (Keadle *et al*, 1997; Laycock *et al*, 1991; Stuart *et al*, 2004). UV-B successfully induced HSV-1 reactivation in latently infected HLA transgenic mice made on either a C57BL/6 or a BALB/c genetic background (not shown).

In the studies reported here, the recently described detailed procedures for the UV-B mouse model of HSV-1 reactivation (Morris *et al*, 2012a) were followed as closely as possible. We found that UV-B irradiation of the eyes of latently infected mice induced reactivation of the $LAT^{(+)}$ virus more efficiently than it induced reactivation of the $LAT^{(-)}$ virus, as judged by detection of infectious virus in tears between days 3 to 7 post UV-B. The ability of LAT to enhance the reactivation phenotype in the in vivo UV-B induced reactivation model was similar to the ability of LAT to enhance reactivation in other mouse models and in rabbits. In addition, the sensitivity of the UV-B mouse model to distinguish between $LAT^{(+)}$ and $LAT^{(-)}$ viruses was highly reminiscent of the rabbit model. Specifically, the percent of reactivated virus positive cultures between days 3 and 7 post UV-B irradiation was 9% for $LAT^{(+)}$ virus and 3% for $LAT^{(-)}$ virus. This decreased reactivation with $LAT^{(-)}$ virus to approximately 33% that of $LAT^{(+)}$ virus is similar to what is seen for spontaneous reactivation in latently infected rabbits (Perng *et al*, 1994). In our lab, this decreased spontaneous reactivation rate of $LAT^{(-)}$ viruses in the rabbit model is very consistent, while in the mouse TG explant model the difference between $LAT^{(+)}$ and $LAT^{(-)}$ viruses is sometimes not as clear cut. Published results for at least one LAT mutant also suggest that the in vivo rabbit model may be more sensitive in detecting HSV-1 mutational effects on the reactivation phenotype than ex vivo mouse models (Loutsch *et al*, 1999; Maggioncalda *et al*, 1996). For these and other reasons discussed above, we feel that this fully in vivo mouse UV-B induced reactivation model of HSV-1 should be a highly useful addition to deciphering the mechanism(s) by which LAT enhances the reactivation phenotype and for investigating the cellular and molecular immune mechanisms involved in HSV-1 reactivation in general.

Every year approximately 20,000 individuals in the United States develop symptoms of recurrent, painful and potentially blinding ocular herpetic disease due to sporadic spontaneous reactivation of HSV-1 from latently infected sensory neurons of the TG (Kumar *et al*, 2009; Schacker *et al*, 1998; Wald *et al*, 1995). To date, there is no licensed therapeutic vaccine that can effectively stop or reduce HSV-1 reactivation from latency. Current long term anti-viral drug therapies (e.g. Acyclovir and derivatives) reduce recurrent ocular disease in symptomatic individuals by only ~40%, and do not eliminate virus reactivation (HEDS, 1998). Identifying the mechanisms that lead to HSV-1 reactivation from latently infected sensory neurons, the root of recurrent disease, would help in developing more effective immunotherapies to prevent or reduce viral shedding in tears and, hence, reduce recurrent herpetic disease and blindness (reviewed in (Kuo *et al*, 2014). The protective efficacy of candidate therapeutic vaccines and drugs against ocular herpes must first be pre-clinically tested in reliable animal models of recurrent ocular herpetic disease. We have found that the UV-B in vivo model of HSV-1 induced reactivation also works in our “humanized” HLA-A*0201 transgenic mouse model of ocular herpes (not shown). This will allow us in future studies to test the therapeutic efficacy of candidate vaccines based on human HLA-A*0201-restricted epitopes (Khan *et al*, 2015).

In conclusion, there are two principal findings in this report: (*i*) we successfully reproduced the UV-B model of HSV-1 induced reactivation; and (*ii*) we found that HSV-1 reactivation induced by UV-B in the mouse model was increased in the presence of LAT.

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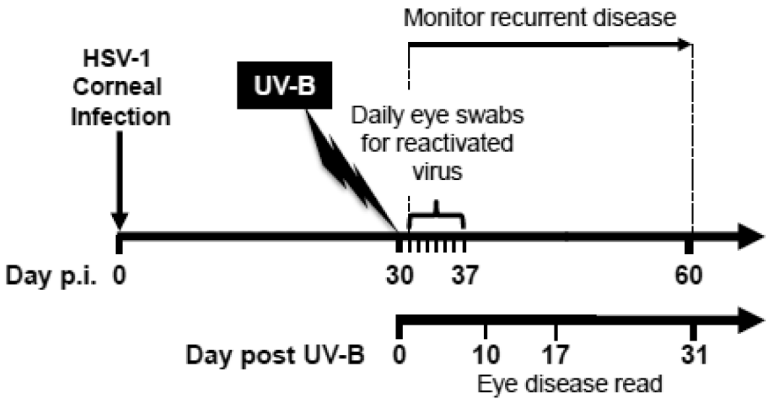

Figure 1. Schematic representation of the time line used in the UV-B model

Mice were infected with HSV-1 as described in Materials & Methods closely adhering to the published model as shown in a video (Morris *et al*, 2012a). Tears were collected on the indicated days (days 0-7). Eye disease was scored on the indicated days (days 10, 17, 31).

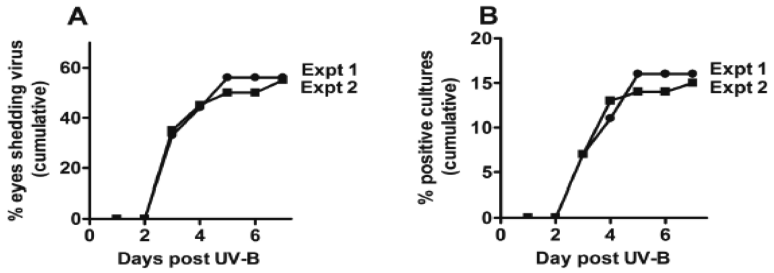

Figure 2. UV-B induced reactivation of virus from mice latently infected with HSV-1 strain McKrae

Mice were infected and ~30 days later when latency was well established, virus was induced by UV-B irradiation of eyes as described in Materials & Methods and (Morris *et al*, 2012a). Panel A: The cumulative percent of eyes that shed UV-B induced reactivated virus within one week of UV-B irradiation. Panel B: The cumulative percent of virus positive cultures (daily eye swabs plated on indicator cells). Expt. 1: Results of the right eyes of 9 mice. Expt. 2: Results of both eyes from 10 mice (20 eyes).

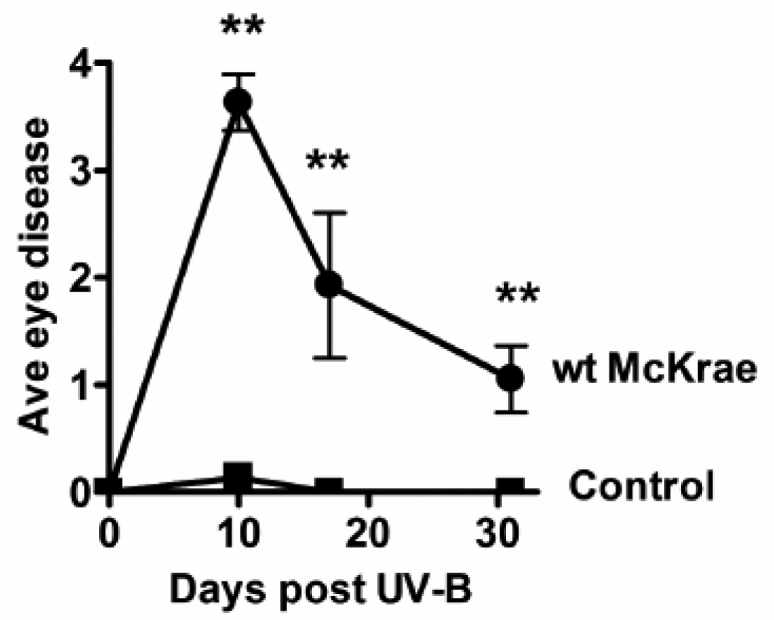

Figure 3. Recurrent eye disease following UV-B irradiation

The eyes from the experiment 1 shown in figure 2 were monitored for eye disease (clouding on a scale of 0 to 4 (Morris *et al*, 2012a)) on days 10, 17, and 31 post UV-B irradiation. Groups: wt McKrae, 15 eyes; Control, naïve age matched mice receiving the same UV-B irradiation treatment, 20 eyes. "**" Indicates highly significant differences on that day with a P value <0.001 as determined by a two sided Fisher exact test.

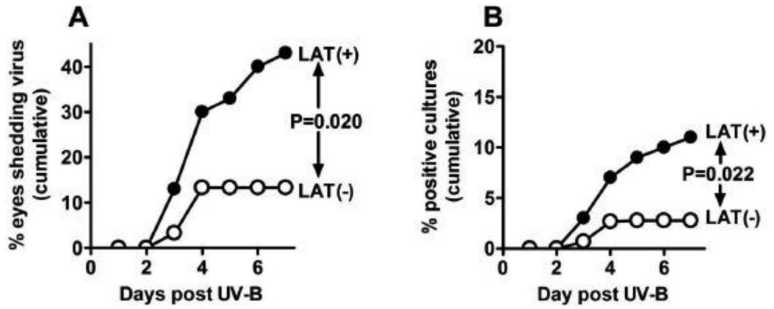

Figure 4. UV-B induced reactivation of LAT$^{(+)}$ versus LAT$^{(-)}$ virus

Fifteen mice/group (30 eyes) were infected, subjected to UV-B irradiation 30 days p.i., and induced reactivation analyzed as described in the legend to Figure 2. P values were determined by 2-sided Fisher exact test.

Table 1

UV-B induced reactivation in $LAT^{(+)}$ versus $LAT^{(-)}$ mice[a].

Virus	No. eyes shedding reactivated virus/no. eyes (%)	No. reactivated virus positive cultures/no. cultures (%)
$LAT^{(+)}$ (wt McKrae)	13/30 (43%)	16/180 (9%)
$LAT^{(-)}$ (dLAT2903)	4/30 (13%)	5/180 (3%)
P value (Fisher exact)	P=0.02	P=0.022

[a]Mice were ocularly infected as described in Materials & Methods and the legend to figure 4 with $LAT^{(+)}$ (wild type McKrae) virus (the parental virus for dLAT2903) or $LAT^{(-)}$ (dLAT2903) virus. Reactivation was induced by UV-B irradiation as described in Materials & Methods.