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Blockade of PD-1 and LAG-3 Immune Checkpoints Combined with Vaccination Restores the Function of Antiviral Tissue-Resident CD8$^+$ T_{RM} Cells and Reduces Ocular Herpes Simplex Infection and Disease in HLA Transgenic Rabbits

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ABSTRACT Chronic viruses such as herpes simplex virus 1 (HSV-1) evade the hosts' immune system by inducing the exhaustion of antiviral T cells. In the present study, we found that exhausted HSV-specific CD8$^+$ T cells, with elevated expression of programmed death ligand-1 (PD-1) and lymphocyte activation gene-3 (LAG-3) receptors were frequent in symptomatic patients, with a history of numerous episodes of recurrent corneal herpetic disease, compared to asymptomatic patients who never had corneal herpetic disease. Subsequently, using a rabbit model of recurrent ocular herpes, we found that the combined blockade of PD-1 and LAG-3 pathways with antagonist antibodies significantly restored the function of tissue-resident antiviral CD8$^+$ T_{RM} cells in both the cornea and the trigeminal ganglia (TG). An increased number of functional tissue-resident HSV-specific CD8$^+$ T_{RM} cells in latently infected rabbits was associated with protection against recurrent herpes infection and disease. Compared to the PD-1 or LAG-3 blockade alone, the combined blockade of PD-1 and LAG-3 appeared to have a synergistic effect in generating frequent polyfunctional Ki-67$^+$, IFN-γ^+, CD107$^+$, and CD8$^+$ T cells. Moreover, using the human leukocyte antigen (HLA) transgenic rabbit model, we found that dual blockade of PD-1 and LAG-3 reinforced the effect of a multiepitope vaccine in boosting the frequency of HSV-1-specific CD8$^+$ T_{RM} cells and reducing disease severity. Thus, both the PD-1 and the LAG-3 exhaustion pathways play a fundamental role in ocular herpes T cell immunopathology and provide important immune checkpoint targets to combat ocular herpes.

IMPORTANCE HSV-specific tissue-resident memory CD8$^+$ T_{RM} cells play a critical role in preventing virus reactivation from latently infected TG and subsequent virus shedding in tears that trigger the recurrent corneal herpetic disease. In this report, we determined how the dual blockade of PD-1 and LAG-3 immune checkpoints, combined with vaccination, improved the function of CD8$^+$ T_{RM} cells associated with a significant reduction in recurrent ocular herpes in HLA transgenic (Tg) rabbit model. The combined blockade of PD-1 and LAG-3 appeared to have a synergistic effect in generating frequent polyfunctional CD8$^+$ T_{RM} cells that infiltrated both the cornea and the TG. The preclinical findings using the established HLA Tg rabbit model of recurrent herpes highlight that blocking immune checkpoints combined with a T cell-based vaccine would provide an important strategy to combat recurrent ocular herpes in the clinic.

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Herpes simplex virus 1 (HSV-1), a neurotropic member of the *Alphaherpesvirus* family, is among the most prevalent and successful human pathogens (1–4). HSV-1 infects over 3.72 billion individuals worldwide and can cause potentially blinding recurrent keratitis (2, 5, 6). After a primary acute infection of the cornea, HSV-1 can cause a spectrum of ocular diseases such as herpetic keratitis, blepharitis, conjunctivitis, and neovascularization. At the end of the acute phase, HSV-1 travels up sensory neurons to the trigeminal ganglia (TG), where it establishes lifelong latency in its host (7–11). Reactivation of latent virus from neurons of the TG, anterograde transportation to nerve termini, and reinfection of the cornea can cause potentially blinding keratitis and is the major issue with HSV-1 infection globally (12–15).

A dynamic cross talk between the virus and CD8$^+$ T cells within the latently infected TG is involved in restraining reactivation of HSV-1 from latency (7, 8, 10, 11, 16). HSV-specific CD8$^+$ T cells are selectively activated and retained in the tissues of latently infected TG (8, 10, 11), although the exact mechanisms are yet to be fully elucidated. While HSV-specific CD8$^+$ T cells can significantly reduce reactivation (7, 11), apparently by interfering with virus replication and spread (7, 10, 11), yet HSV-1 can manage to reactivate even in the presence of an often-sizable pool of virus-specific CD8$^+$ T cells in the TG, apparently by interfering with the quality and quantity of CD8$^+$ T cells that reside in the TG (8, 11, 17). Thus, the antiviral CD8$^+$ T cells are kept functionally restricted by persistent presence of the virus, using among several mechanisms, functional exhaustion of T cells, which is usually the result of prolonged exposure of T cell to viral antigens, as occurs during productive or abortive replication attempts in chronic infections (18, 19).

While the majority of HSV-infected humans remain asymptomatic (ASYMP) after virus reactivation, a minor proportion are symptomatic (SYMP), manifesting severe recurrent herpetic disease (20, 21). A few recent investigations have shed light on the molecular mechanism of reactivation (12–15). Repetitive HSV-1 latent/reactivation cycles, sporadic events that occur in latently infected TG, cause the elimination or partial impairment of antiviral T cells (16, 22, 23). This is usually the result of prolonged exposure of T cells to high levels of viral antigens during the chronic phases of latency/reactivation cycles (16, 24, 25). Controlling reactivation by overcoming T cell exhaustion has thus become a hopeful therapeutic strategy (16, 25–27). The discovery of a plethora of exhaustion markers, including programmed death-1 (PD-1) and lymphocyte activation gene-3 (LAG-3; also known as CD223), has paved the way to therapeutically target them to restore T cell functions in chronic infections (26, 27).

Although not much is known about the exhaustion states of HSV-1-specific CD8$^+$ T cells in ASYMP and SYMP individuals, our recent report identified that HSV-1 epitope-specific LAG-3$^+$ CD8$^+$ T cells and PD-1$^+$ CD8$^+$ T cells appeared to be more frequent in SYMP individuals than in ASYMP individuals, which may be a potential cause of suboptimal immunity, often associated with symptomatic shedding (26–28). Moreover, using a mouse model of recurrent herpetic keratitis, we demonstrated that combination of therapeutic immunization and blockade of LAG-3 during the reactivation phase led to the restoration of function of tissue-resident antiviral CD8$^+$ T cells and significantly protected against recurrent ocular herpes infection and disease (26–28). However, the degree of applicability of these findings to humans needs to be tested using better models that mimic humans. To this end, in the present study we used a rabbit model of ocular herpes to elucidate the effectiveness of blocking PD-1 and LAG-3 pathways of exhaustion, either alone or in combination, in restoring antiviral immunity in the TG and the cornea and in protection against herpetic infection and disease. We have previously reported in our rabbit model that expression of the latency-associated transcript gene (LAT) of HSV is associated with a broad repertoire of functionally exhausted antiviral CD8$^+$ T cells retained in the TG (28). In addition, we used the

TABLE 1 Cohorts of HLA-A*02:01-positive, HSV-seropositive SYMP and ASYMP individuals enrolled in the study

Subject-level characteristic	All subjects ($n = 30$)	ASYMP subjects ($n = 15$)	SYMP subjects ($n = 15$)
Gender, no. (%)			
Female	16 (63)	8 (63)	8 (63)
Male	14 (37)	7 (37)	7 (37)
Race, no. (%)			
Caucasian	22 (69)	10 (75)	10 (63)
Non-Caucasian	8 (31)	5 (25)	5 (37)
Median age, yr (range)	40 (23–57)	42 (26–55)	39.5 (23–57)
HSV status, no. (%)			
HSV-1 seropositive	30 (100)	15	15
HSV-2 seropositive	0 (0)	0	0
HSV-1/HIV-2 seropositive	0 (0)	0	0
HSV seronegative	0 (0)	0	0
HLA, no. (%)			
HLA-A*02:01 positive	30 (100)	15	15
HLA-A*02:01-negative	0 (0)	0	0
Herpes disease status, no. (%)			
Asymptomatic (ASYMP)	15 (50)	15 (50)	0 (0)
Symptomatic (SYMP)	15 (50)	0 (0)	15 (50)

recently developed HLA transgenic (Tg) rabbit model, to test whether immune checkpoint blockades will reinforce the effect of vaccination with a neurotrophic human adeno-associated virus subtype 8 (AAV8) vector engineered to coexpress multiple $CD8^+$ and $CD4^+$ immunodominant asymptomatic herpes epitopes in neurons. Overall, our findings suggest that the dual blockade of PD-1 and LAG-3 demonstrates a synergistic effect and boosts the effect of vaccination toward combating ocular herpes infection and disease in the rabbit model, which can translate into a potential therapeutic strategy to combat symptomatic herpes in humans.

RESULTS

HSV-specific PD-1^+ $CD8^+$ and LAG-3^+ $CD8^+$ T cells are frequent in symptomatic patients with recurrent ocular herpetic disease. The characteristics of the SYMP and ASYMP study populations used in the present study with respect to gender, age, HLA-A*02:01 frequency distribution, HSV-1/HSV-2 seropositivity, and status of the ocular herpetic disease are presented in Table 1 and are detailed in Materials and Methods. Since HSV-1 is the main cause of ocular herpes, only individuals who are HSV-1 seropositive and HSV-2 seronegative were enrolled in the present study. HSV-1-seropositive individuals were divided into two groups: (i) ten HLA-A*02:01-positive, HSV-1-infected ASYMP individuals who have never had any clinically detectable herpes disease, and (ii) ten HLA-A*02:01-positive, HSV-1-infected SYMP individuals with a history of numerous episodes of well-documented recurrent clinical herpes diseases, such as herpetic lid lesions, herpetic conjunctivitis, dendritic or geographic keratitis, stromal keratitis, and iritis consistent with recurrent herpes stromal keratitis (rHSK), with one or more episodes per year for the past 5 years. Only SYMP patients who were not on acyclovir or other antiviral or anti-inflammatory drug treatments at the time of blood sample collections were enrolled. One patient had more than two severe recurrent episodes during the last ten years that necessitated multiple corneal transplantations.

The frequencies of HSV-1 $gB_{183–191}$ epitope-specific $CD8^+$ T cells from SYMP and ASYMP ($n = 5$, each) individuals were analyzed by flow cytometry for the expression of LAG-3 and PD-1. As shown in Fig. 1A, there were no observed differences in the frequencies of $gB_{183–191}$ epitope-specific $CD8^+$ T cells between ASYMP (0.37%) and SYMP (0.25%) individuals. A tetramer specific to the immunodominant $gB_{183–191}$ epitope was used to decipher the expression of LAG-3 and PD-1 uniquely on HSV-

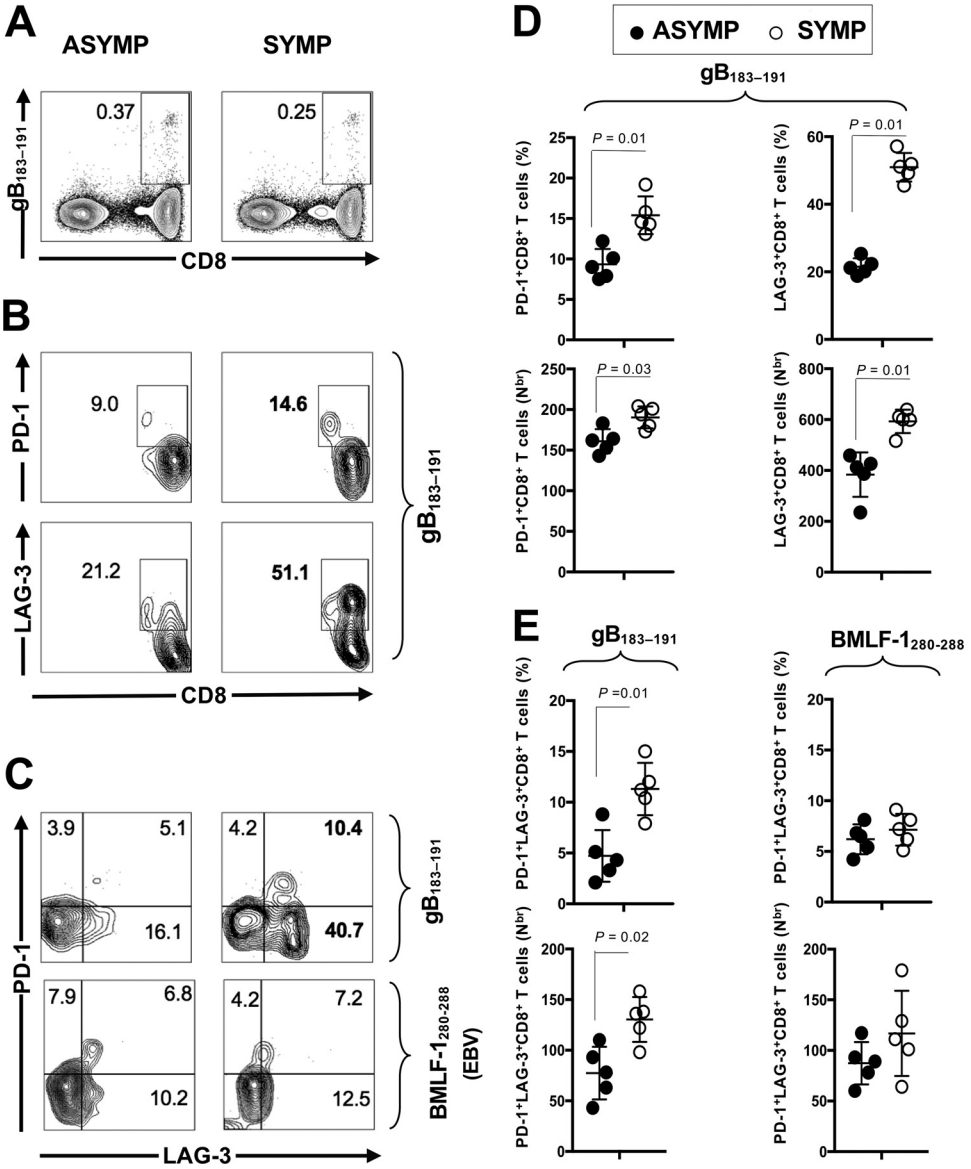

FIG 1 Frequency of HSV-1 $gB_{183–191}$ epitope-specific LAG-3$^+$ CD8$^+$ T cells and PD-1$^+$ CD8$^+$ T cells in ASYMP versus SYMP individuals. (A) Representative FACS plot of the frequencies of HSV-1 $gB_{183–191}$ tetramer-specific CD8$^+$ T cells in ASYMP versus SYMP individuals. (B) Representative FACS plot of the frequencies of HSV-1 $gB_{183–191}$ tetramer-specific PD-1$^+$ CD8$^+$ T cells and LAG-3$^+$ CD8$^+$ T cells in ASYMP and SYMP individuals. (C) Representative FACS plot of the frequencies of HSV-1 $gB_{183–191}$ tetramer-specific PD-1$^+$ LAG-3$^+$ CD8$^+$ T cells in ASYMP and SYMP individuals. (D) Average percentages (upper panels) and absolute numbers (lower panels) of HSV-1 $gB_{183–191}$ tetramer-specific PD-1$^+$ CD8$^+$ T cells and LAG-3$^+$ CD8$^+$ T cells. (E) Average percentages (upper right panels) and absolute numbers (lower right panels) of HSV-1 $gB_{183–191}$ tetramer-specific PD-1$^+$ LAG-3$^+$ CD8$^+$ T cells in ASYMP and SYMP individuals and average percentages (upper left panels) and absolute numbers (lower left panels) of EBV BMLF-1280-288 tetramer-specific PD-1$^+$ LAG-3$^+$ CD8$^+$ T cells in ASYMP and SYMP individuals. The results are representative of two independent experiments in each individual. The indicated *P* values, calculated using an unpaired *t* test, show statistical significances between SYMP and ASYMP individuals.

specific CD8$^+$ T cells (instead of bulk CD8$^+$ T cells). In contrast to the frequencies of total HSV-1 $gB_{183–191}$ epitope-specific CD8$^+$ T cells (regardless of exhaustion), more frequent phenotypically exhausted $gB_{183–191}$ epitope-specific LAG-3$^+$CD8$^+$ T cells and PD-1$^+$ CD8$^+$ T cells, which expressed LAG-3 and/or PD-1 markers of exhaustion, were detected in SYMP individuals as opposed to less frequent $gB_{183–191}$ epitope-specific LAG-3$^+$ CD8$^+$ T cells and PD-1$^+$ CD8$^+$ T cells detected in ASYMP individuals (Fig. 1B to E). Moreover, as shown in Fig. 1C and E, elevated coexpression of PD-1 and LAG-3 were detected in $gB_{183–191}$ epitope-specific CD8$^+$ T cells from SYMP patients compared to

the moderate to low expression of PD-1 and LAG-3 on $gB_{183–191}$ epitope-specific $CD8^+$ T cells from ASYMP healthy individuals.

Altogether, these results indicate that PD-1 and/or LAG-3 markers of exhaustion are abundantly expressed on HSV-1 $gB_{183–191}$ epitope-specific $CD8^+$ T cells from SYMP patients that are clinically diagnosed with the repetitive recurrent ocular herpetic disease. The present result, using the immunodominant $gB_{183–191}$ epitope from the glycoprotein B (gB) is in agreement with the functional impairment of $CD8^+$ T cells specific to another epitope from the tegument protein VP11/12 ($VP11/12_{66–74}$-specific $CD8^+$ T cells), previously reported in SYMP individuals (5). Since LAG-3 and PD-1 markers are strong determinants of functional exhaustion, this indicates that the exhaustion of HSV-specific $CD8^+$ T cells in SYMP individuals may cause the apparent loss of protective immunity seen in SYMP individuals, an outcome that is often associated with symptomatic shedding.

Because of ethical and practical complexities in obtaining tissue-resident $CD8^+$ T_{RM} cells from human cornea and trigeminal ganglia (TG), we were limited to using circulating $CD8^+$ T cells in humans. However, the phenotype and function of human blood-derived $CD8^+$ T cells may not reflect tissue-resident $CD8^+$ T_{RM} cells. For these reasons, in order to determine the phenotypic and functional exhaustion of cornea- and TG-resident HSV-specific $CD8^+$ T_{RM} cells, we utilized in the remainder of this study our rabbit model of recurrent ocular herpes. Moreover, using the rabbit model of recurrent herpes permitted us to determine the effect of blocking PD-1 and LAG-3 pathways (with or without a therapeutic vaccine) in reversing the exhaustion of HSV-specific $CD8^+$ T cells and whether such treatment will cure or at least reduce ocular herpes infection and disease.

Combined blockade of PD-1 and LAG-3 is associated with a reduction in viral replication and disease in HSV-1-infected rabbits. We studied the effect of blocking PD-1 and LAG-3 pathways of immune exhaustion, using antagonistic monoclonal antibodies (MAbs), on viral infection, disease, and the antiviral $CD8^+$ T cell response following ocular challenge with HSV-1. A group of 20 WT New Zealand (NZW) rabbits were ocularly infected with 2×10^5 PFU of HSV-1 strain McKrae (Fig. 2A). After infection, the rabbits were divided as follows: (i) αPD-1 ($n = 5$), intravenously (i.v.) injected with 200 μg of anti-PD-1 MAb at three different time points, i.e., days 3, 5, and 7 postinfection (p.i.); (ii) αLAG-3 ($n = 5$), i.v. injected with 200 μg of αLAG-3 MAb on days 3, 5, and 7 p.i.; (iii) αPD-1 plus αLAG-3 ($n = 5$), i.v. injected with 200 μg of αPD-1 MAb plus 200 μg of αLAG-3 MAb on days 3, 5, and 7 p.i.; and (iv) mock ($n = 5$) control, i.v. injected with saline. Eye swabs were collected daily, and disease was monitored. As shown in Fig. 2B, there was a significant decrease of viral titer detected on day 8 following terminal blockade in the combination of αPD-1/αLAG-3-treated group compared to the mock-treated group. Furthermore, on the same day, the disease severity was far less in the combination blockade group versus the mock-treated group (Fig. 2C).

Combined blockade of PD-1 and LAG-3 strengthens the antiviral $CD8^+$ T cell responses following ocular herpes infection in rabbits. We next determined whether blockade would improve the antiviral immune response and control of virus replication. On day 14 p.i., the rabbits were sacrificed; the spleens, corneas (the site of primary infection), and TG (the site of latency) were harvested, and functional profiles of the $CD8^+$ T cells were determined (Fig. 3A). A significantly higher frequency of IFN-γ^+ $CD8^+$ T cells, $CD107^+$ $CD8^+$ T cells, and Ki-67^+ $CD8^+$ T cells were detected in the cornea (Fig. 3B and C), TG (Fig. 4A and B), and spleen (Fig. 4C and D) in the combination αPD-1/αLAG-3-treated group compared to the mock-treated group ($P < 0.05$).

Altogether, these results indicate that (i) combined blockade of PD-1 and LAG-3 leads to significant control of viral replication, as well as the manifestation of ocular herpetic disease, and (ii) the observed protection is underpinned by the betterment of functional profile of $CD8^+$ T cells.

Combined blockade of PD-1 and LAG-3 leads to an increase in $CD8^+$ T cell infiltrates in HSV-1-infected TG and cornea following ocular herpes infection. To gain

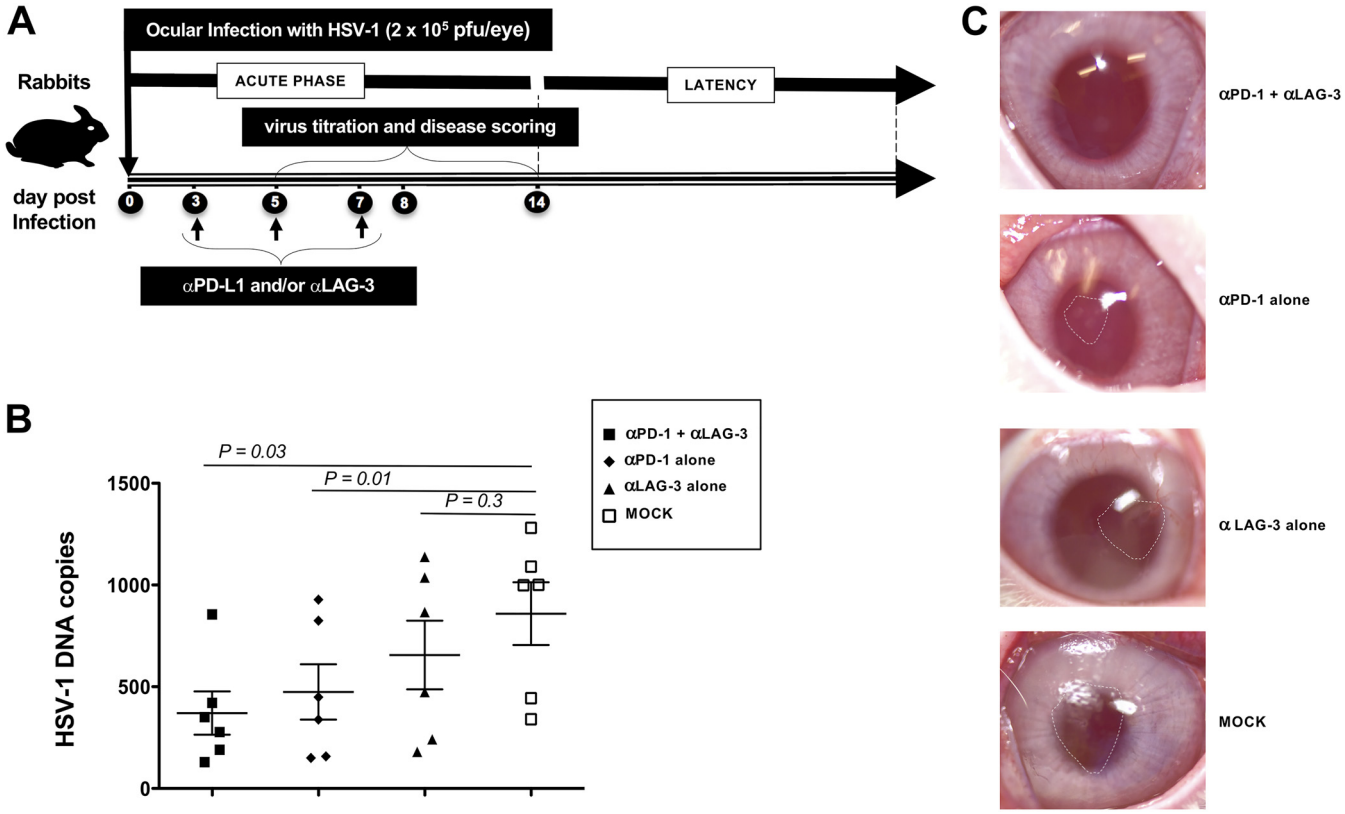

FIG 2 Effect of the PD-1 and LAG-3 blockade on recurrent ocular herpes infection and disease in HSV-1-infected rabbits. (A) Schematic representation of HSV-1 ocular infection, blockade with PD-1 and LAG-3 MAb treatment, and virological analyses in New Zealand rabbits ($n = 20$) following ocular infection on day 0 with 2×10^5 PFU of HSV-1 (strain McKrae). (B) Quantification of infectious virus particles on day 8 following terminal blockade in eye swabs by standard real-time PCR to detect viral DNA in various treatment groups. (C) Representative images showing ocular disease detected in rabbits in various treatment groups on day 8 following terminal blockade. The results are representative of two independent experiments. The indicated *P* values, calculated using the unpaired *t* test, show the statistical significance between various treated groups versus the mock-treated group.

direct insight into the effect of blockades on tissue cell inflitrations, we determined the percentage and number of $CD8^+$ T cells in the TG and cornea. $CD8^+$ T cells were quantitated by immunostaining. As shown, although PD-1 or LAG-3 blockade alone appeared to increase the amount of infiltrates of T cell types in the TG (Fig. 5, top row) and cornea (Fig. 5, bottom row), the αPD-1/αLAG-3 combination bolstered their numbers conspicuously. The increased accumulation of T cells in TG and cornea suggested enhanced migration and proliferation of protective T cells following immune checkpoint blockades. Altogether, this result indicates that while single immune checkpoint blockade improves the immune response and reduces the disease to some extent, dual checkpoint blockades significantly and synergistically amplified the effect.

PD-1 and LAG-3 dual checkpoint blockade, combined with peptide vaccination with HSV-1 HLA-A*0201-restricted $CD8^+$ T cell epitopes, reduced ocular herpes infection and disease in HSV-1-infected HLA transgenic rabbits. Next, we determined whether immune checkpoint blockades will reinforce the effect of vaccination using our HLA Tg rabbit model. HLA Tg rabbits ($n = 16$) were immunized with a topical ocular treatment of a human multiepitope vaccine, as shown in Fig. 6A and as detailed in Materials and Methods. Using the optimal CamKIIA promoter, we constructed a neurotrophic recombinant nonreplicating adeno-associated virus subtype 8 (AAV8) vector expressing 10 immunodominant human $CD8^+$ T cell epitopes and 3 $CD4^+$ T cell epitopes. HLA Tg rabbits were immunized twice, at 2-week intervals, with the multiepitope vaccine construct. At 10 days after the second immunization, rabbits were infected with 2×10^5 PFU of HSV-1/eye. Soon after the second immunization and before infection, the rabbits were segregated as follows: (i) vaccination + blockade

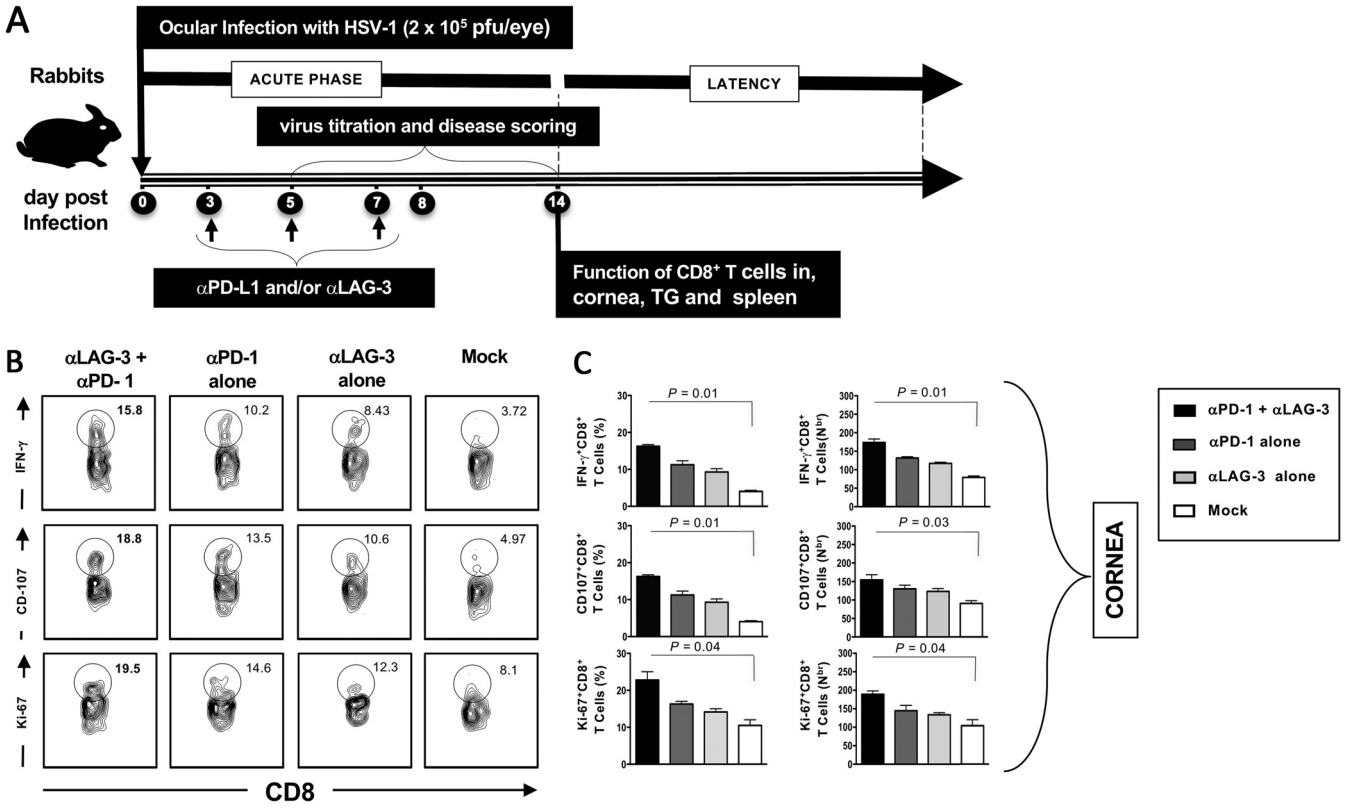

FIG 3 Increased tissue-resident antiviral CD8$^+$ T cells in the cornea of HSV-1-infected rabbits following blockade of PD-1 and LAG-3 immune checkpoints is associated with reduction in the severity of herpetic disease. (A) Schematic representation of HSV-1 ocular infection, blockade with PD-1 and LAG-3 MAb treatment, and immunological analyses in New Zealand rabbits ($n = 20$) following ocular infection on day 0 with 2×10^5 PFU of HSV-1 (strain McKrae). (B and C) Representative FACS plots, average percentages, and average absolute numbers of IFN-γ^+ CD8$^+$ T cells, CD107$^+$ CD8$^+$ T cells, and Ki-67$^+$ CD8$^+$ T cells in various treatment groups in the cornea. The indicated *P* values, calculated using the unpaired *t* test, show the statistical significance between the αPD-1/αLAG-3-treated group and the mock-treated group.

(αPD-1 + αLAG-3; $n = 10$), prophylactically vaccinated 2 weeks apart (days 0 and 15) before infection and i.v. injected with 200 μg of αPD-1 MAb plus 200 μg of αLAG-3 MAb on days -3, -5, and -7 before infection and on days 3, 5, and 7 p.i.; (ii) vaccination only ($n = 10$), prophylactically vaccinated 2 weeks apart (days 0 and 15) before infection; (iii) blockade only (αPD-1 + αLAG-3; $n = 10$), i.v. injected with 200 μg of αPD-1 MAb plus 200 μg of αLAG-3 MAb on days -3, -5, and -7 before infection and on days 3, 5, and 7 p.i.; and (iv) mock ($n = 10$) control, i.v. injected with saline. Eye swabs were collected daily, and disease monitored. At 12 days p.i., blood was drawn from rabbits in each group, and the frequencies of tetramer$^+$ CD8$^+$ T cells against the vaccine epitopes were determined. As shown in Fig. 6B to D, we noted that (i) the combination of vaccination and blockade markedly decreased the viral titer and disease severity compared to the mock-treated group and (ii) a significantly high frequency of HSV-specific CD8$^+$ T cells corresponding to three tetramers, ($gB_{183-191}$, $VP11/12_{702-710}$, and $VP13/14_{544-552}$) was observed in the vaccine-plus-blockade group compared to the mock-treated group. Vaccine alone or blockade alone also had only a marginal effect.

Taken together, these results indicate that synergistic blockade of the PD-1 and LAG-3 immune checkpoint can appreciably improve the effect of vaccination in combating HSV-1 disease severity and thus may be a potential therapeutic avenue against herpes infection.

DISCUSSION

Tissue-resident memory T cells (T_{RM}) have become remarkably important for preventing viral infections/replications through "barrier" tissues and "immune closed compartments," such as the cornea and the TG tissues, the sites of acute and latent

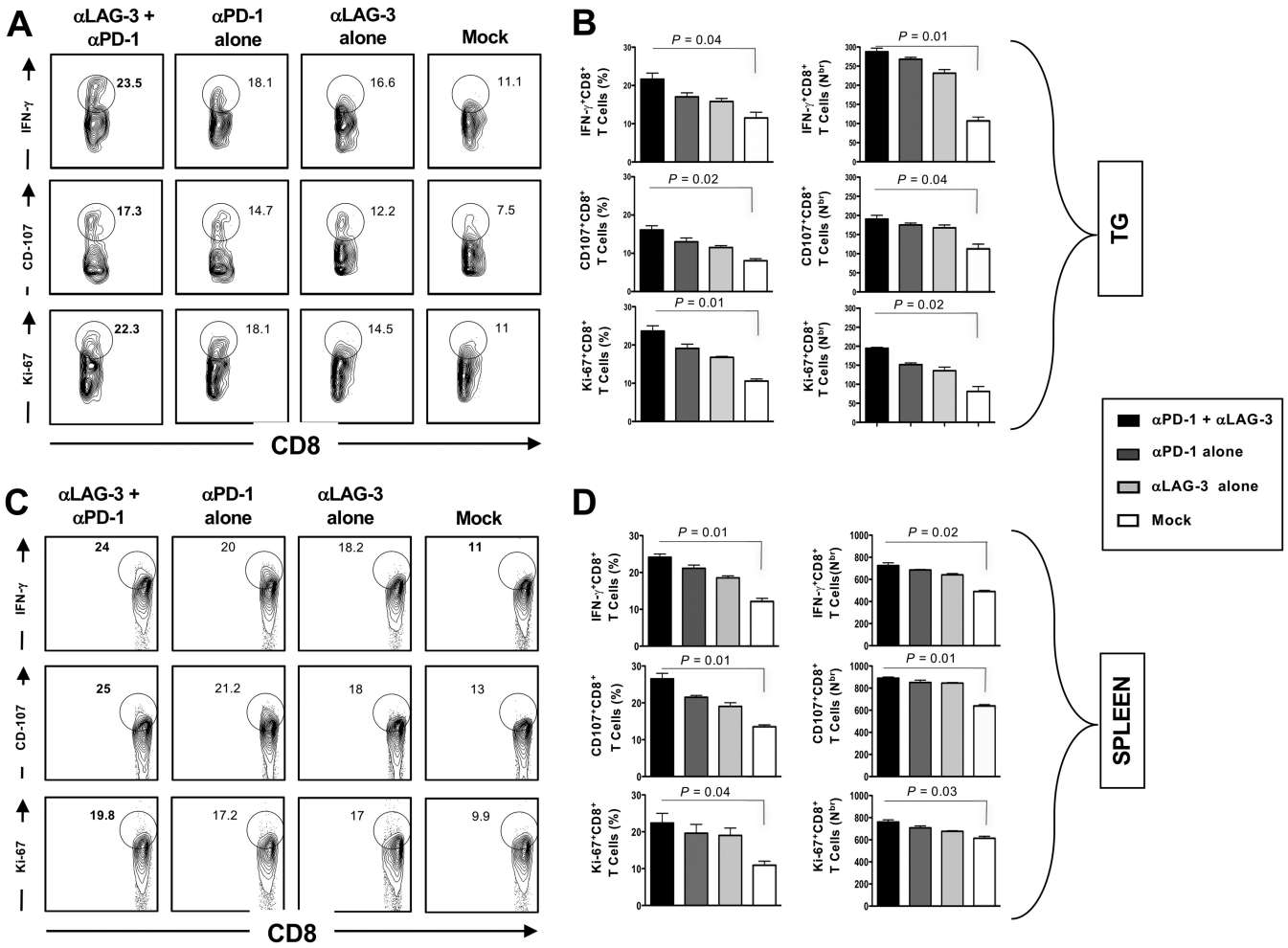

FIG 4 Blockade of PD-1 and LAG-3 improves the function of tissue-resident antiviral CD8$^+$ T cells associated with a reduction in the severity of herpetic disease in HSV-1-infected rabbits. Representative FACS plots, average percentages, and average absolute numbers of IFN-γ^+ CD8$^+$ T cells, CD107$^+$ CD8$^+$ T cells, and Ki-67$^+$ CD8$^+$ T cells in various treatment groups in the TG (A and B) and spleen (C and D) are shown. The indicated *P* values, calculated using the unpaired *t* test, show the statistical significance between αPD-1/αLAG-3-treated groups and the mock-treated group.

HSV-1 infections, respectively (6, 26, 27, 29). These T_{RM} cells directly kill infected cells or recruit other immune cells to assist in protecting these tissues (30–32). In contrast to the well-studied "migratory" circulating memory T_{EM} and T_{CM} cells (which transit through the blood and lymph in order to hunt for sites of viral infection), T_{RM} cells do not (or transiently) leave the barrier tissues. Since T_{RM} cells are so valuable for protective immunity against herpes infection, direct generation of T_{RM} cells by vaccines provides an exciting opportunity to enhance protective immunity. However, HSV-specific CD8$^+$ T cells, with abundant expression of PD-1 and LAG-3, are common in symptomatic patients, with frequent recurrent ocular herpetic disease, suggesting phenotypic and functional exhaustion of antiviral T_{RM} cells. In the present study, we took advantage of the PD-1 and LAG-3 exhaustion markers that are expressed in cornea and TG tissue-resident T_{RM} cell populations and demonstrated that blockade of these immune checkpoints at the timing of our choice (e.g., when cells first arrive in the TG and cornea or else during latency, 30 days p.i.), and in the location of our choice (e.g., cornea and TG) increased the number of functional CD8$^+$ T_{RM} cells associated with protection from recurrent ocular herpes infection and disease.

Most of the potentially blinding recurrent herpes stromal keratitis (rHSK) occurs in latently infected humans following spontaneous reactivation of HSV-1 from latently infected sensory neurons of TG that led to virus shedding in tears. Spontaneous

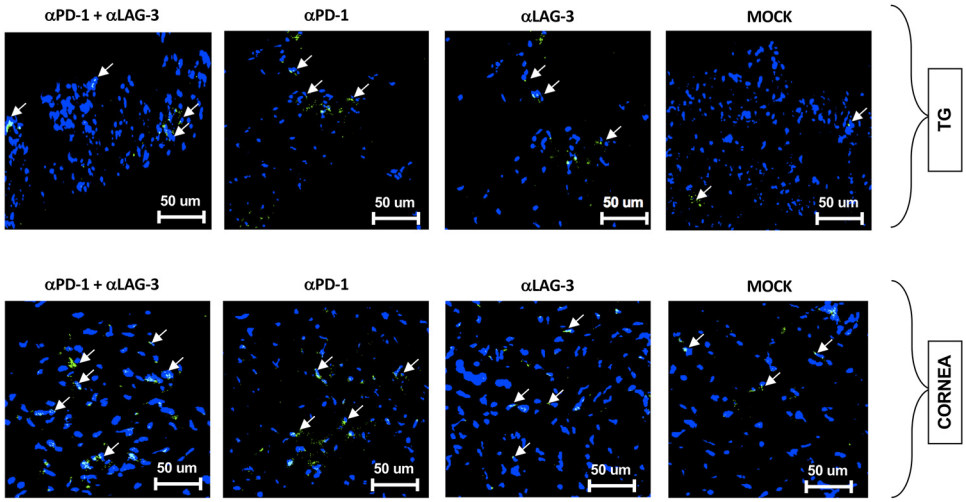

FIG 5 Combination blockade of PD-1 and LAG-3 increases the size of CD8$^+$ T cell infiltrates in the TG and corneas of HSV-infected rabbits. The TG and corneas of all treatment groups were harvested following sacrifice. Tissue sections were fixed, embedded in OCT, and immunostained. Sections from all groups of rabbits were costained using DAPI and with a MAb specific to rabbit CD8$^+$ T cells in the TG (top row) and cornea (bottom row). Fluorescence microscopy images are shown (magnification, ×20). Blue, DAPI (DNA stain); green, CD8$^+$/CD4$^+$ cells. The data are representative of two independent experiments.

reactivation and virus shedding in tears does not occur in HSV-1 latently infected mice TG. To avoid the inherent drawbacks in the mouse model of herpes infection, an alternative rabbit model in which HSV-1 reactivation and virus shedding in tear film occur spontaneously was developed (7). The present study, to our knowledge, is the first to report that blockade of the PD-1 and LAG-3 immune checkpoints produces robust protection against spontaneous HSV-1 reactivation and virus shedding in tears. This was associated with an increased number of functional tissue-resident CD8$^+$ T_{RM} cells in both latently infected TG and cornea. The result suggests immune checkpoint blockade as an innovative approach to treat blinding recurrent herpetic disease. Moreover, we demonstrated that the HLA transgenic rabbit model is capable of mounting a strong "human-like" CD8$^+$ T cell response specific to immunizing human HLA-restricted epitopes that correlate with protection against recurrent ocular herpes. Using this HLA transgenic rabbit, in which the human leukocyte antigen (HLA), a major component of T cell immunity, is replaced by the identical component taken from a human counterpart (i.e., HLA-A*0201 class I molecule), we demonstrated that a prime/pull therapeutic vaccination, together with the blockade of PD-1 and LAG-3, resulted in even more functional CD8$^+$ T_{RM} cells in the TG and cornea. This therapeutic vaccination/blockade combination produced a robust protection against HSV-1 reactivation and virus shedding in tears (Fig. 6). Thus, the prime/pull vaccine, combined with the blockade of the immune checkpoint, appears to be an innovative approach to treat blinding recurrent herpetic disease.

Efficient inhibition and clearance of HSV-1 infection rely on both innate and adaptive immune responses, with CD8$^+$ T cell immunity playing an important role in inhibiting HSV-1 reactivation from latently infected neurons of the TG (1, 33–35). The availability of functional antiviral CD8$^+$ T cells during various herpes latency/reactivation cycles is an important aspect of the adaptive herpes immunity. However, as an immune evasion strategy and to adapt to its host, the virus appeared to manage to induce phenotypic and functional exhaustion of antiviral CD8$^+$ T_{RM} cells that reside in the cornea and TG. HSV-specific CD8$^+$ T cells become less functional, as the infection persists, and may gradually lose their effector function, including proliferation, cytotoxicity, and cytokine production. In the absence of a sizable pool of virus-specific functional CD8$^+$ T_{RM} cells, the HSV-1 can reactivate from the TG and shed in tears causing recurrent ocular herpes. In this study, we demonstrated that reversion of CD8$^+$

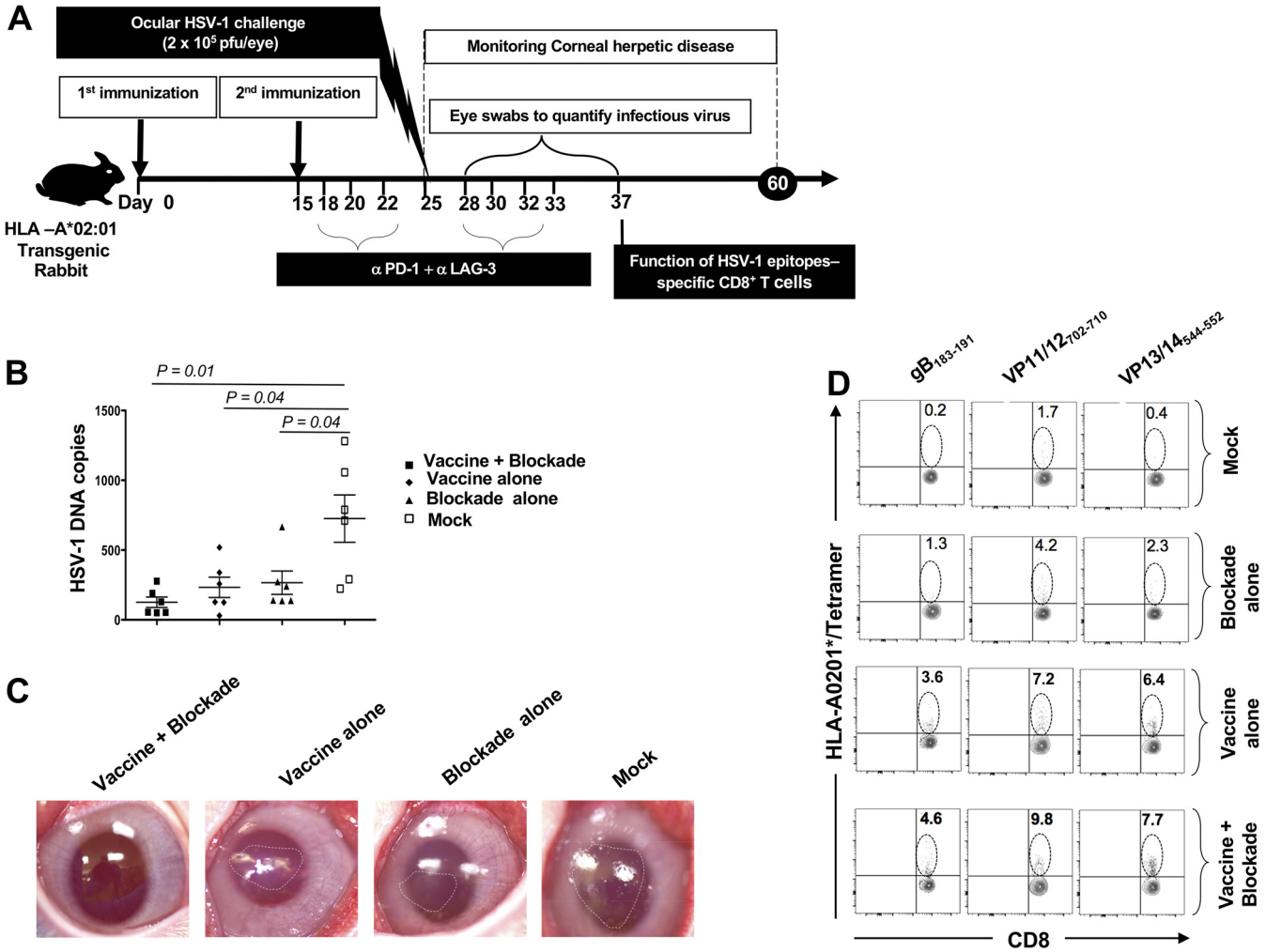

FIG 6 Combined blockade of PD-1 and LAG-3 improves the protective efficacy of herpes vaccine in HSV-1-infected HLA transgenic rabbits. (A) Schematic representation of HSV-1 ocular infection, multiepitope vaccination regimen, blockade with PD-1 and LAG-3 MAb treatment, and virological and immunological analyses in HLA Tg rabbits ($n = 12$), as detailed in Materials and Methods. (B) Quantification of infectious virus particles on day 8 p.i. following terminal blockade in eye swabs by standard real-time PCR to detect viral DNA in various treatment groups. (C) Representative images showing ocular disease detected in rabbits in various treatment groups following terminal blockade. (D) HLA Tg rabbits were bled on days 12 p.i., and the frequencies of epitope-specific $CD8^+$ T cells were determined using the corresponding HLA-A*02:01-peptides/tetramers. Shown are representative FACS plots of the frequencies of $tetramer^+$ $CD8^+$ T cells specific to three tetramers ($gB_{183-191}$, $VP11/12_{702-710}$, and $VP13/14_{544-552}$) in PBMCs from rabbits of each group. The number at the top of each box represents the percentage of epitope-specific $CD8^+$ T cells. The results are representative of two independent experiments. The indicated *P* values, calculated using the unpaired *t* test, show the statistical significance between various treated groups versus the mock-treated group.

T cell exhaustion during HSV-1 reactivation led to less ocular herpes disease in the rabbit model, a strategy that holds significant promise in the design of new immunotherapies for HSV-1. Similar to rabbits, we also demonstrated the exhaustion of $CD8^+$ T cells in HSV-1-seropositive SYMP humans, which expressed inhibitory receptors, such as PD-1 and LAG-3. IN addition to the expression of PD-1, a high expression of LAG-3 was also detected in HSV-1-specific $CD8^+$ T cells from the corneas and TG of rabbits, which helped us to distinguish between exhaustion and activation (28).

Even though the TG is generally an "immune closed compartment," $CD4^+$ and $CD8^+$ T cells can infiltrate virus-infected TG (28, 35). An immune surveillance role for cornea- and TG-resident HSV-specific $CD8^+$ T cells has been established (11, 16, 25, 36–38). When rabbits are exposed to stress, there is an enhancement of HSV-1 reactivation that can cause the development of recurrent corneal disease (28, 39, 40). Tissue-resident $CD4^+$ and $CD8^+$ T_{RM} cells appear to be involved in controlling virus reactivation from latently TG (28, 39). To our knowledge, this is the first report in the HLA Tg rabbits that shows (i) LAG-3-related functional exhaustion of HSV-specific $CD8^+$ T cells in both the

corneas and TG of latently infected wild-type New Zealand and HLA Tg rabbits and (ii) a significant increase in functionally exhausted LAG-3$^+$ CD8$^+$ T cells in SYMP patients, as well as in latently infected HLA Tg rabbits that developed increased virus shedding and severe recurrent corneal herpetic disease following reactivation from latency.

The present study in HSV-1-infected New Zealand and HLA Tg rabbits also strengthens our previous findings in mice showing that HSV-1 infection results in the accumulation of virus-specific exhaustion of CD8$^+$ T cells expressing PD-1 in latently infected TG (16, 25, 28). More importantly, we show in HLA Tg rabbits that a synergic therapeutic blockade of the PD-1 and LAG-3 immune checkpoints, in combination with a therapeutic immunization, restored the function of HSV-specific CD8$^+$ T_{RM} cells that reside in the corneas and TG of latently infected rabbits. Moreover, this was associated with a reduction in recurrent ocular herpes following reactivation in latently infected HLA Tg rabbits. Thus, in addition to the previously described immune evasion mechanisms in mice (15, 16, 25, 28, 41), our data in HLA Tg rabbits confirm the novel mechanism by which HSV-1 evades the control by host's antiviral tissue-resident CD8$^+$ T cells through interfering with the function of PD-1$^+$ LAG-3$^+$ CD8$^+$ T cells.

We previously reported high levels of PD-1 and LAG-3 expression in human CD8$^+$ T cells specific to the HSV-1 VP11/12$_{66-74}$ tegument epitope. Using different cohorts of SYMP and ASYMP individuals, the present study confirms and extend these finding by reporting high levels of PD-1 and LAG-3 expression in human CD8$^+$ T cells specific to the HSV-1 gB$_{183-191}$ epitope. The lack of PD-1 and LG-3 upregulation detected on the bulk CD8$^+$ T cells suggests that the observed CD8$^+$ T cell exhaustion is restricted to HSV-specific PD-1$^+$ LAG-3$^+$ CD8$^+$ T cells. These findings are consistent with the potential role of PD-1 and LAG-3 pathways in the exhaustion of HSV-specific CD8$^+$ T cells. This prompted us to test in the HLA Tg rabbit model of recurrent ocular herpes whether MAbs blocking the PD-1 and LAG-3 pathways would reverse dysfunction associated with protection from recurrent ocular herpes (27). The PD-1 receptors are PDL-1 and PDL-2, while the LAG-3 receptor's main ligand is major histocompatibility complex class II (MHC-II) (42). LAG-3 receptor appears to bind with higher affinity to its ligand MHC-II, while the binding of CD4 molecule to MHC-II appears to be moderate to low, depending on the epitope presented. Therefore, a competition between LAG-3/MHC-II and DC4/MHC-II is expected to destabilize the TCR/CD4/MHC-II interaction (43, 44). In doing so, the LAG-3 receptor negatively regulates CD4$^+$ T cell function and homeostasis (45, 46). Moreover, the LAG-3 receptor also appears to regulate CD8$^+$ T cell function and homeostasis (47). However, the underlying cellular and molecular mechanisms of the LAG-3 pathway in CD8$^+$ T cells remain to be fully elucidated. In this report, using the rabbit model of recurrent ocular herpes, we found that the exhaustion of HSV-specific CD8$^+$ T cells by the LAG-3 and PD-1 inhibitory pathways was nonredundant, since blockade of LAG-3 and PD-1 receptors appeared to synergistically improve the function of HSV-1 gB$_{183-191}$ epitope-specific CD8$^+$ T cells. Reversing the exhaustion of HSV-specific CD8$^+$ T cells following blockade of PD-1 and LAG-3 pathways in wild-type or HLA Tg rabbits was associated with a significant reduction of HSV-1 load and recurrent corneal disease. Thus, antiviral CD8$^+$ T cell responses during recurrent ocular herpes appear to be regulated by complex patterns of coexpressed PD-1 and LAG-3 inhibitory receptors.

While still in its early stages, basic and clinical data suggest that blockade of immune checkpoints can be beneficial in the treatment of chronic infections (48–50). Furthermore, besides classical PD-1 and LAG-3, other inhibitory receptors such as TIM-3 and TIGIT are potential checkpoints that can be manipulated for the treatment of chronic infections (49). However, caution should be taken when blocking immune checkpoint pathways that help keep the body's immune responses in check and prevent autoimmunity. Both PD-1 and LAG-3 play important roles during the normal immune response to prevent autoimmunity. Releasing the "brakes" on the immune system by blocking PD-1 and LAG-3 pathways can overactivate effector T cells, and this might cause tissue damage. Nevertheless, in the present preclinical study in rabbits, we found that MAb therapies blocking LAG-3 and PD-1 immune checkpoint safely and efficiently led to

significant reductions of recurrent corneal herpetic disease without apparent unwanted side effects. The improved clinical outcome of LAG-3 and PD-1 blockade in rabbits with established recurrent herpes was directly associated with a multifaceted enhancement of both the numbers and function of antiviral tissue-resident $CD8^+$ T cells. Moreover, we report that combination of a therapeutic blockade of LAG-3/PD-1 immune checkpoint and therapeutic vaccination in HLA Tg rabbits lead to the generation of functional HSV-specific $CD8^+$ T cells in latently infected TG and cornea associated with an even more reduction in virus reactivation and recurrent disease in latently infected rabbits, following reactivation.

The increased $CD8^+$ T_{RM} cell exhaustion in the corneas and TG of rabbits latently infected with HSV-1 could be the result of an increase in the load of viral antigens locally. However, during latency, less than 1 neuron/TG had detectable viral antigens (10, 51, 52). Therefore, even though $CD8^+$ T_{RM} cells in the TG of HLA Tg rabbits are much more sensitive to antigens, the low and unsustained levels of antigens are unlikely to result in exhaustion of $CD8^+$ T_{RM} cells in this model. Thus, it seems unlikely that the apparent exhaustion of HSV-specific $CD8^+$ T_{RM} cells detected in TG of HLA Tg rabbits was due to a load of viral antigens. Additional factors, such the high rate of virus reactivation in rabbit TG, may contribute to consistent $CD8^+$ T_{RM} cell stimulation and hence to their exhaustion. It is likely that TG-resident HSV-specific $CD8^+$ T_{RM} cells could have a higher functional avidity and hence an ability to respond to a low density of epitopes than their $CD8^+$ T_{CM} and $CD8^+$ T_{EM} cell counterparts circulating in the periphery (22). Alternatively, the observed higher exhaustion of $CD8^+$ T_{RM} cells may suggest that there are a lot more viral antigens present in the rabbit TG and cornea latently infected with HSV-1 than had been previously reported. Nevertheless, using our HLA transgenic mouse and HLA transgenic rabbit models (8, 53), we are currently in the process of assessing the exhaustion of $CD8^+$ T_{RM} cells specific to a set of immunodominant and subdominant human HSV-1 epitopes from tegument, regulatory, and surface proteins that are recognized by $CD8^+$ T_{RM} cells in these "humanized" animal models of induced and spontaneous recurrent ocular herpes, respectively.

In summary, using the HLA Tg rabbit model of recurrent ocular herpes, the present study demonstrates, for the first time, that the TG and cornea contain infiltrates of exhausted $PD\text{-}1^+$ $CD8^+$ and $LAG\text{-}3^+$ $CD8^+$ T_{RM} cells that are specific to immunodominant HSV-1 epitopes. Moreover, the study demonstrates that higher numbers of functional HSV-specific $CD8^+$ T_{RM} cells can be generated in both the TG and corneas of HSV-1 latently infected rabbits following a therapeutic administration of cross-reactive MAbs that blocked the LAG-3 and PD-1 immune checkpoints. Importantly, such therapy was associated with a significant reduction of virus reactivation and less virus shedding in tears, and this translated into less severe recurrent herpetic disease. Finally, we also report for the first time that in HSV-1 latently infected HLA Tg rabbits a combination of the LAG-3 and PD-1 immune checkpoint blockade, together with a therapeutic peptide vaccination, leads to the generation of even more numbers of functional HSV-specific $CD8^+$ T_{RM} cells in both the cornea and the TG. This was associated with improved protection from recurrent herpes infection and disease in treated HLA Tg rabbits. Altogether, the present preclinical study using the HLA Tg rabbit model of recurrent ocular herpes suggests that blockade of the LAG-3 and PD-1 pathways in combination with a therapeutic vaccination may have great therapeutic promise. The finding opens up the possibilities of designing novel combination therapies for herpes-infected symptomatic patients.

MATERIALS AND METHODS

Human study population. All clinical investigations in this study were conducted according to the Declaration of Helsinki. All subjects were enrolled at the University of California, Irvine, under approved Institutional Review Board-approved protocols (IRB#2003-3111 and IRB#2009-6963). Written informed consent was received from all participants prior to inclusion in the study.

During the last 15 years (i.e., January 2003 to June 2019), we screened 955 individuals for HSV-1 and HSV-2 seropositivity. Patients were divided into SYMP and ASYMP groups based on the inclusion criteria, as previously described (2–5, 20). Among the large cohort of SYMP and ASYMP individuals, 30 HLA-A*02:01-positive patients (15 ASYMP and 15 SYMP) were enrolled in this study (Table 1). SYMP and

ASYMP groups were matched for age, gender, serological status, and race. The HLA-A2 status was confirmed by peripheral blood mononuclear cell (PBMC) staining with 2 μl of anti-HLA-A2 MAb (clone BB7.2; BD Pharmingen, Inc., San Diego, CA) at 4°C for 30 min. The cells were washed and analyzed by flow cytometry using a LSRII (Becton Dickinson, Franklin Lakes, NJ). The acquired data were analyzed with FlowJo software (BD Biosciences, San Jose, CA).

Human peripheral blood mononuclear cell isolation. Individuals (negative for HIV and HBV and with or without any HSV infection history) were recruited at the UC Irvine Institute for Clinical and Translational Science (ICTS). Between 40 and 100 ml of blood was drawn into Vacutainer Tubes (Becton Dickinson). The serum was isolated and stored at −80°C for the detection of anti-HSV-1 and HSV-2 antibodies, as we have previously described (54). PBMCs were isolated by gradient centrifugation using leukocyte separation medium (Life Sciences, Tewksbury, MA). The cells were then washed in phosphate-buffered saline (PBS) and resuspended in complete culture medium consisting of RPMI 1640 and 10% fetal bovine serum (FBS; Bio-Products, Woodland, CA) supplemented with 1× penicillin/streptomycin/L-glutamine, 1× sodium pyruvate, 1× nonessential amino acids, and 50 μM 2-mercaptoethanol (Life Technologies, Rockville, MD). Freshly isolated PBMCs were also cryopreserved in 90% fetal calf serum and 10% dimethyl sulfoxide in liquid nitrogen for future testing.

Human T cell flow cytometry. The following anti-human antibodies were used for the flow cytometry assays: CD3 A700 (clone SK7; BioLegend, San Diego, CA), CD8 PE-Cy7 (clone SK1; BioLegend) PD-1 FITC (clone EH12.2H7; BioLegend), and LAG-3 PerCPCy5.5 (clone 11C3C65; BioLegend). For the surface stain, MAbs against cell markers were added to a total of 1×10^6 cells in 1× PBS containing 1% FBS and 0.1% sodium azide (fluorescence-activated cell sorting [FACS] buffer) for 45 min at 4°C. After washing twice with FACS buffer, the cells were fixed in PBS containing 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). For each sample, 100,000 total events were acquired on the BD LSRII. Ab capture beads (BD Biosciences) were used as individual compensation tubes for each fluorophore in the experiment. To define positive and negative populations, we used fluorescence minus controls for each fluorophore. Furthermore, we optimized gating by examining known negative cell populations for background expression levels similar to that used in our previous work (9). Briefly, we gated single cells, dump cells, viable cells (Aqua Blue), lymphocytes, $CD3^+$ cells, and $CD8^+$ cells before finally gating human epitope-specific $CD8^+$ T cells using HSV-specific tetramers. Data analysis was performed using FlowJo software (BD Biosciences). Statistical analyses were done using GraphPad Prism version 5 (La Jolla, CA).

Tetramer/gB peptide staining. Fresh PBMCs were analyzed for the frequency of $CD8^+$ T cells recognizing the $gB_{183–191}$ peptide/tetramer complexes, as we previously described (55–58). The cells were incubated with $gB_{183–191}$ peptide/tetramer complex for 30 to 45 min at 37°C. The cell preparations were then washed with FACS buffer and stained with FITC-conjugated anti-human CD8 MAb (BD Pharmingen). The cells were then washed and fixed with 1% paraformaldehyde in PBS and subsequently acquired on a BD LSRII. Data were analyzed using FlowJo version 9.5.6 (TreeStar, Ashland, OR).

HLA transgenic and New Zealand wild-type rabbits. An HLA transgenic rabbit colony was bred at the University of California—Irvine (UC Irvine) (8, 28, 39, 59–61), and the New Zealand White (NZW) rabbits, purchased from Western Oregon Rabbit Co., were used for all the experiments. All rabbits were housed and treated in accordance with ARVO (Association for Research in Vision and Ophthalmology), AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care), and NIH (National Institutes of Health) guidelines. A colony of human leukocyte antigens (HLA) transgenic (Tg) rabbits maintained at UC Irvine was used for all experiments. The HLA Tg rabbits retain their endogenous rabbit MHC locus and express human HLA-A*02:01 under the control of its normal promoter (8, 28, 39, 59–61). Prior to this study, the expression of HLA-A*02:01 molecules on the PBMCs of each HLA Tg rabbit was confirmed by FACS as described previously (8). Only rabbits with high HLA expression in >90% of PBMCs were used in these studies. Thus, all of the HLA rabbits used for these experiments had a similarly high-level expression of HLA-A*02:01. This avoided potential bias due to the variability of HLA-A*0201 molecule levels in different animals. High expression of HLA-A*02:01 molecules is expected (i) to force rabbit $CD8^+$ T cells to use human HLA-A*02:01 molecules at both thymic educational and peripheral effector levels (8) and (ii) to minimize the competition between rabbit MHC class I molecules and human HLA-A*02:01-restricted responses (8). New Zealand White rabbits (non-Tg control rabbits) were used as controls.

Design and construction of AAV8 vector. Human adeno-associated virus subtype 8 (AAV8) was used in this study, as we previously described (35, 39). Human HLA-restricted HSV-1-specific $CD8^+$ and $CD4^+$ T cell epitopes were cloned along with open reading frame for the chemokine CXCL10 and the cytokine interleukin-2 for targeted expression to neuronal cells in rabbit TG and corneas. AAVs were purified by CsCl gradient ultracentrifugation, followed by desalting and viral titers (GC per milliliters) were determined by real-time PCR, as we described previously (35, 39).

Herpes simplex virus production and ocular herpes challenge. The HSV-1 (strain McKrae) was used in this study. The virus was triple plaque purified and prepared as previously described (35, 39). Groups of New Zealand White rabbits and HLA Tg (8 to 10 weeks) received an ocular HSV-1 challenge (2×10^5 PFU, McKrae strain) without scarification (28, 39, 59, 62–64). Following ocular infection, rabbits were monitored for ocular herpes, virus infection, and disease (28, 39, 59, 62–64).

PD-1 and LAG-3 blockade. Cross-reactive anti-PD-1 MAb (RMPI-14) and anti-LAG-3 MAb (C9B7W) were purchased from BioXcell (West Lebanon, NH). NZW and HLA Tg rabbits were ocularly infected with 2×10^5 PFU of strain McKrae and received i.v. injections of 200 μg of anti-PD-1 MAb and/or anti-LAG-3 MAb on scheduled days, as illustrated in Fig. 2A, 3A, and 6A.

Rabbit corneal disease clinical scores. Rabbits were examined for ocular disease and survival for 30 days following blockade MAb treatment after the infection. The ocular disease was determined by a masked investigator, and pictures were taken before the infection and following the blockade postinfection. A standard 0 to 4 scale was used: 0, no disease; 1, 25%; 2, 50%; 3, 75%; and 4, 100% staining.

Detection of rabbit ocular infectious virus. Tears were collected from both eyes using a Dacron swab (type 1; Spectrum Laboratories, Los Angeles, CA) following the commencement of blockade postchallenge. Individual swabs were transferred to a 2-ml sterile cryogenic vial containing 1 ml of culture medium and stored at −80°C until use. The HSV-1 titers in tear samples were determined by standard real-time PCR as previously described (65–67).

Flow cytometry assays on rabbit T cells. PBMCs were analyzed by flow cytometry. The following antibodies were used: mouse anti-rabbit CD8 (clone MCA1576F; Bio-Rad), mouse anti-human CD107^{a} (clone H4A3; BioLegend), CD107^{b} (clone H4B4; BioLegend), Ki-67 (clone 20Raj1; BioLegend), and anti-mouse-IFN-γ (clone XMG1.2; BioLegend). For surface staining, MAbs against various cell markers were added to a total of 1×10^6 cells in PBS containing 1% FBS and 0.1% sodium azide (fluorescence-activated cell sorting [FACS] buffer) and left for 45 min at 4°C. For intracellular staining, MAbs were added to the cells and incubated for 45 min on ice and in the dark. Cells were washed again with Perm/Wash and FACS buffer and then fixed in PBS containing 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). For the measurement of CD107$^{a/b}$, IFN-γ, and Ki-67, cells were first stimulated *in vitro* with either phytohemagglutinin (PHA; 5 μg/ml) or peptides (10 μg/ml). Briefly, 1×10^6 cells were transferred into a 96-well flat-bottom plate and stimulated with PHA or peptides in the presence of BD GolgiStop (10 μg/ml) for 6 h at 37°C. At the end of the incubation period, the cells were transferred to a 96-well round-bottom plate and washed once with FACS buffer. Surface and intracellular staining were done as we described previously (28, 39, 59, 62–64). LSRII acquired a total of 50,000 events (Becton Dickinson, Mountain View, CA), followed by analysis using FlowJo software (TreeStar).

Rabbit CD8$^+$ T cell tetramer assays. For tetramer-specific CD8$^+$ T cell responses, PBMCs were analyzed for the frequency of CD8$^+$ T cells specific to each of the CD8$^+$ T cell epitopes incorporated in the multiepitope vaccine by using the corresponding HLA-A2-peptides/tetramers, provided by the NIH tetramer facility (8, 28, 39, 59–61). A human beta-2-microglobulin was incorporated into the tetramers, since no rabbit beta-2-microglobulins are currently available. Briefly, the cells were first incubated with 1 μg/ml of each of the three phycoerythrin-labeled HLA-A2-peptides/tetramers at 37°C for 30 to 45 min. The cells were washed twice and then stained with 1 μg/ml of FITC-conjugated mouse anti-rabbit CD8 MAb (clone MCA1576F; Bio-Rad). After two additional washes, the cells were fixed with 2% formaldehyde in FACS buffer. LSRII acquired a total of 50,000 events (Becton Dickinson), followed by analysis using FlowJo software (TreeStar).

Rabbit immunohistochemistry. Rabbit TG was cut into 8-μm-thick sections using a cryostat. Sections were washed with 1× PBS for 15 min and blocked using 10% FBS in 1× PBS for 1 h. Sections were stained using anti-rabbit CD8$^+$ and CD4$^+$ antibody (1:200) overnight at 4°C. After secondary fluorescent staining, sections were washed with 1× PBS and mounted after DAPI (4′,6′-diamidino-2-phenylindole) staining (1:10,000 dilution). Immunofluorescence infiltration of CD4$^+$ with CD8$^+$ T cells was examined using a Keyence BZ-X700 fluorescence microscope at ×40 magnification and imaged using z-stack.

Statistical analyses. Data for each assay were compared by analysis of variance (ANOVA) and Student *t* test using GraphPad Prism version 5 (La Jolla, CA). Differences between the groups were identified by ANOVA and multiple-comparison procedures, as we previously described (68). The data are expressed as means ± the standard deviations (SD). Results were considered statistically significant at $P < 0.05$.

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We declare that no conflicts of interest exist.

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