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Immune Regulation of Transgene Expression in the Brain: B Cells Regulate an Early Phase of Elimination of Transgene Expression from Adenoviral Vectors

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ABSTRACT

Cellular immune mechanisms that regulate viral gene expression within infected brain cells remain poorly understood. Previous work has shown that systemic immunization against adenovirus after vector delivery to the brain results in complete loss of brain cells infected by adenoviral vectors. Although T cells play an important role in this process, we demonstrate herein that B cells also significantly regulate transgene expression from the CNS. After the systemic immunization against adenovirus of animals injected via the brain with an adenoviral vector 30 days earlier, we uncovered substantial infiltration by $CD19^+$ B cells of the area of the brain transduced by the virus. This suggests the involvement of B cells in the adaptive immune response-mediated loss of transduced cells from the brain. Confocal analysis of these brains demonstrated physical contacts between transduced brain cells and $CD19^+$ cells. To test the hypothesis that B cells play a causal role in the loss of infected cells from the brain, we demonstrated that animals devoid of B cells were unable to eliminate transgene expression at early time points after immunization. This demonstrates that B cells play a necessary role in the loss of transgene expression at early, but not late, time points postimmunization. Thus, these data have important implications for our understanding of the role of B cells as immune effectors during the immune-mediated clearance of viral infections from the CNS, and also for understanding mechanisms operating in brain autoimmunity, as well as for the potential safety of clinical gene therapy for brain diseases.

INTRODUCTION

VIRAL VECTORS CAN BE USED to express specific proteins within predetermined brain cells for experimental or therapeutic (i.e., gene therapy) purposes (1–3). However, priming of the systemic host immune system against any antigens present within viral vectors, and including the marker transgene or therapeutic transgene, generates an immune response that causes brain inflammation and in some cases destruction of infected cells (4–15). Although when used as tumor immunotherapy the immune response to viral vectors may be acceptable or even desirable, deleterious immune responses have halted various clinical trials (16–18). The long-term viability of clinical gene therapy for the treatment of neurological diseases therefore depends on approaches that will not elicit significant immune-mediated attack of transduced cells. How immune mechanisms detect and

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respond to viral vectors in the brain needs to be understood to predict and control untoward and deleterious immune responses. This implies a comprehensive understanding of the cellular and molecular mechanisms by which the immune system detects and eventually eliminates exogenous DNA sequences and proteins introduced into the CNS is thus needed. This in turn will allow the design of safer, less toxic, and nonimmunogenic vectors.

B cells recognize antigens through their B cell receptors (BCRs). After B cells encounter antigen, these cells can undergo clonal selection and are able to further differentiate into plasma cells with the potential for secretion of specific antibodies into serum, or become memory B cells. The humoral antibody response to viral vectors in the brain has been examined previously but is not completely elucidated (19). Although the presence of immune cells in the central nervous system (CNS) or of antibodies in cerebrospinal fluid (CSF) is usually associated with disease states in which the blood–brain barrier (BBB) has been compromised, activated B cells, or activated T cells, are capable of entering the brain parenchyma (20).

Nevertheless, the immunoprivileged status of the brain, refers to the fact that the systemic immune response cannot be primed from infectious or particulate antigen delivered directly and selectively into the brain parenchyma, and is further evidenced by the prolonged survival in normal brain of allogeneic tumors and tissue transplants (21). However, it has been more recently shown by Cserr and Knopf that intrathecal antibody synthesis in response to soluble antigens, such as ovalbumin, within the brain can be elicited in healthy animals with normal, noncompromised BBB permeability (22). Thus, brain immune reactivity depends essentially on two factors. One, whether antigens are injected selectively into the brain parenchyma (does not prime a systemic immune response), or into the brain ventricles (does prime a systemic immune response), and whether the antigen injected is particulate and will thus remain restricted to the injection site in the brain parenchyma (and will not prime a systemic immune response), or can diffuse and eventually reach the brain ventricular system (and prime a systemic immune response) [reviewed in (23–26)].

In the present study, we examined the involvement of B cells in the adaptive immune response to brain cells transduced by adenoviral vectors. Illustrated by the presence of infiltrating $CD19^+$ cells, contacts between $CD19^+$ cells and transduced cells in the brain after systemic immunization against a first-generation adenoviral vector, and the significant delay in the capacity of the systemic immune response to clear transduced cells from the brains of Igh-$6^{-/-}$ animals, our work demonstrates that B cells play an important role in the regulation of the immune response initiated against these vectors in the brain.

MATERIALS AND METHODS

Adenoviral vectors

Production and characterization of RAdhCMV/HSV1-TK (RAd-TK, where TK is thymidine kinase) and RAd-HPRT (where HPRT is hypoxanthine–guanine phosphoribosyltransferase) have been described in detail previously (27,28). The presence of contaminating wild-type virus (29), and endotoxin/lipopolysaccharide (LPS) content (30), have also been described previously.

Animals, surgical procedures, and immunization schedules

Wild-type C57BL/6 and Igh-$6^{-/-}$ mice, all on a C57BL/6 background (Jackson Laboratory, Bar Harbor, ME), were anesthetized with ketamine (75 mg/kg) and medetomidine (0.5 mg/kg). Animals were injected via the right striatum (stereotactic coordinates: 0.05 mm anterior, 0.22 mm lateral from bregma, and 0.32 mm ventral from dura) with 1×10^7 infective units (IU) of RAd-TK in a 0.5-μL volume. These experiments were all postimmunization experiments, that is, 30 days after viral vector injection into the CNS, animals were immunized systemically (intraperitoneal injection) with 3.28×10^8 IU of RAd-HPRT in 100 μL. At experimental end points, mice were anesthetized with ketamine (50 mg/kg) and xylazine (50 mg/kg) and perfused with oxygenated Tyrode's solution alone for molecular studies, or perfused–fixed with oxygenated Tyrode's solution followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were postfixed for 48 h before immunohistochemistry. All animal experiments were performed after approval by the Cedars-Sinai Medical Center (Los Angeles, CA) Institutional Animal Care and Use Committee.

Immunohistochemistry and immunofluorescence

Striatal sections (50 μm) were cut with a vibratory microtome (Vibratome, St. Louis, MO) and used for immunohistochemistry. Immunoreacted sections were quantified with a Zeiss Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany), or examined with a confocal microscope (Leica DM IRE2; Leica Microsystems, Wetzlar, Germany). Antibodies used included rabbit polyclonal anti-TK (diluted 1:10,000) and rat anti-mouse CD19 (diluted 1:500) from Serotec (Oxford, UK). Secondary antibodies were biotin-conjugated goat anti-rabbit IgG (diluted 1:800; Dako, Carpinteria, CA), Texas red-conjugated goat anti-rat IgG (diluted 1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA), and Alexa 488-conjugated goat anti-rabbit (diluted 1:1000; Invitrogen Molecular Probes, Eugene, OR).

Quantification and stereological analysis

The optical fractionator probe for unbiased stereological cell estimation in the striatum of mice injected with RAd-TK was used as described (31), using Stereo Investigator software version 5.0 (MicroBrightField, Williston, VT) with a Zeiss Axioplan 2 microscope. Data were expressed as the absolute number of positive cells in each anatomical region analyzed, as described (31). Results were expressed as means ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) after a post-hoc Tukey's test analysis.

Quantification of cell contacts

Contacts between immune cells ($CD19^+$ immunoreactive cells) and transduced cells (TK-immunoreactive cells) were assessed and quantified in mice by confocal microscopy, by at least two investigators experienced in confocal analysis of brain tissue, from areas where immune cells and TK-expressing cells overlapped anatomically. The number of contacts in sections at each of the time points examined was defined with a Leica DM IRE2 microscope with the ×63 oil objective and Leica confocal software (Leica Microsystems). A series range for each section was determined by setting an upper and lower threshold, using the *Z/Y* Position for Spatial Image Series setting, and microscope settings were established and maintained by Leica technicians for optimal resolution. Contacts were defined as areas where colocalization of both markers occurs between two cells in single 0.5-μm-thick optical sections; contacts were present over at least two or three 0.5-μm optical sections in the *z* axis. In each single 0.5-μm layer, total numbers of $CD19^+$ immune cells, TK-expressing cells, and contacts were determined with Leica software. Results were expressed as (*1*) the percentage of immune cells contacting TK-immunoreactive cells, (*2*) the percentage of TK-immunoreactive cells that had contacts, and (*3*) the mean number of immune cells that contact each TK cell.

Statistical analysis

All statistical analyses were performed by one-way ANOVA followed by a Tukey's test analysis.

RESULTS

Systemic immunization against adenovirus after injection of first-generation adenoviral vectors into the brain results in $CD19^+$ cell infiltration into the CNS

We first performed experiments to examine the ability of $CD19^+$ cells to infiltrate the CNS on induction of an adaptive immune response by systemic immunization with adenovirus. Systemic immunization is necessary because priming does not occur after the careful delivery of adenovirus, or other viruses, or infectious particulate antigens, directly into the brain parenchyma (23,25, 32–34). C57BL/6 mice were initially injected via the striatum with 0.5 μL of PBS containing 1×10^7 IU of RAd-TK. Thirty days after the striatal injection, control animals were perfused–fixed and experimental animals were immunized systemically with RAd-HPRT. Systemic immunization was conducted with RAd-HPRT to study immune responses against adenovirus, without taking into account any existing immune responses against the transgenes, which were mismatched.

At 14 and 30 days postimmunization animals were perfused–fixed, and 50-μm-thick serial brain sections were cut on a Vibratome to examine the influx into the brain of B cells at early times postimmunization. Immunofluorescence analysis was performed on free-floating sections, and confocal microscopy was used to determine the presence of immune cells infiltrating the area of the brain containing infected cells. These studies demonstrated infiltration of $CD19^+$ cells exclusively into areas of the brain that contained infected cells expressing the TK marker gene (Fig. 1A–F). Stereological quantification of $CD19^+$ cells in the CNS of these animals demonstrated that virtually no $CD19^+$ cells were present in the brains of control animals (0 days) that were not immunized with RAd-HPRT; however, statistically significant numbers of $CD19^+$ cells had infiltrated the brains of experimental groups after initiation of an adaptive immune response by immunization with RAd-HPRT at 14 and 30 days after immunization (Fig. 1G).

$CD19^+$ cells establish specific contacts with transduced cells in the CNS of C57BL/6 mice

To determine the anatomical distribution of infiltrating immune cells in relation to transduced brain cells, we used confocal microscopy to assess the existence of physical contacts between $CD19^+$ cells and TK-transduced cells. Contacts were defined as close apposition, in at least two consecutive 0.5-μm optical confocal sections, of a transduced cell (TK-immunoreactive) and an immune cell (CD19-immunoreactive). No infiltrating $CD19^+$ cells, or contacts with transduced cells, were detected in nonimmunized animals (0 days). Quantification included the percentage of $CD19^+$ cells contacting transduced cells, the percentage of transduced cells with $CD19^+$ cell contacts, and the average number of $CD19^+$ cells per transduced cell (Fig. 2A–C). The quantitative analysis indicates that >25% of $CD19^+$ cells contacted infected cells, and that in turn a minority of infected cells was contacted by $CD19^+$ cells. Thus, although direct con-

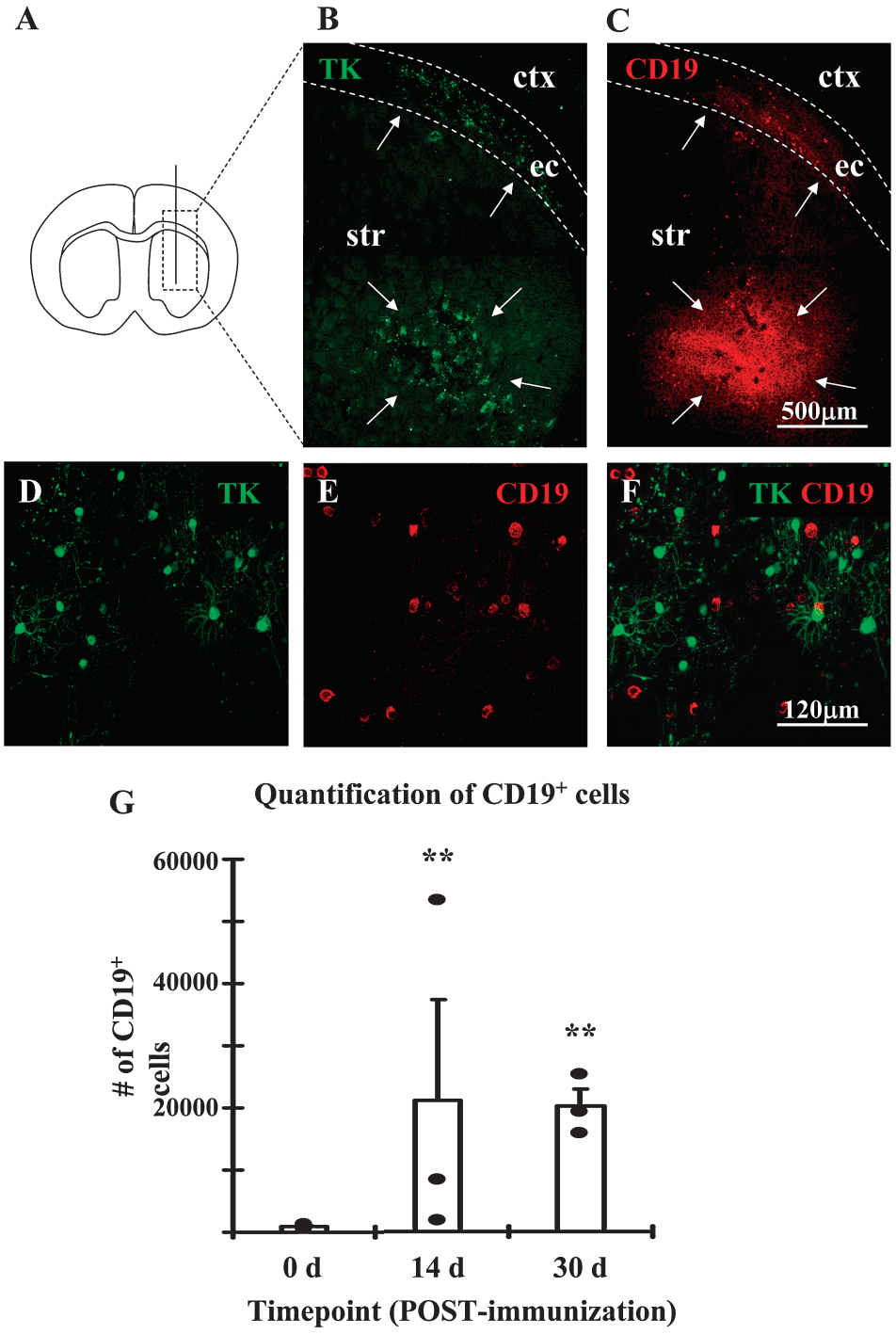

FIG. 1. Qualitative and quantitative microscopy analysis of immune cell infiltration at time points postimmunization, using stereological quantification. (**A**–**C**) $CD19^+$ B cells selectively infiltrate the area of the brain infected with adenoviral vectors. (**A**) Schematic view of the brain at the level of the striatum; the boxed area illustrates the injection site, which is shown in (**B**) (section immunoreacted for the marker transgene TK) and (**C**) (section immunoreacted for $CD19^+$ B cells). str, striatum; ctx, neocortex; ec, external capsule. (**D**–**F**) Infiltration of $CD19^+$ B cells in C57BL/6 mice injected with RAd-TK and immunized with RAd-HPRT 30 days later [(**D**) TK (green); (**E**) $CD19^+$ (red); (**F**) merged], shown 30 days after the immunization analyzed by confocal microscopy. (**G**) Stereological quantification of $CD19^+$ T cells in the brains of animals injected with RAd-TK and immunized with RAd-HPRT, shown in the absence of immunization (0 days) and 14 and 30 days postimmunization. $^{**}p < 0.01$ versus controls.

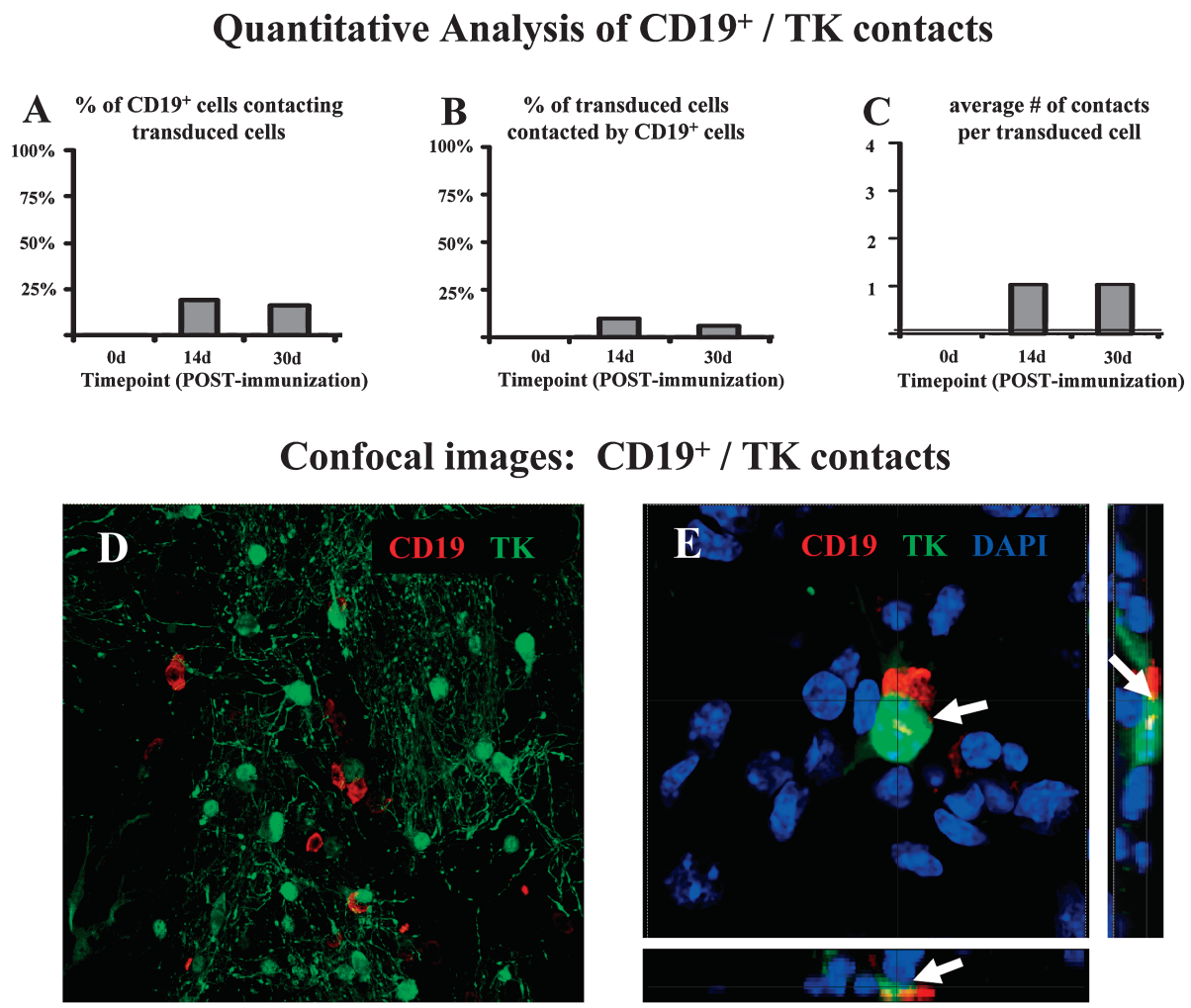

FIG. 2. Qualitative and quantitative laser confocal microscopy analysis of $CD19^+$ immune cell interactions with adenovirally transduced brain cells. (**A–C**) Quantitative measures of contacts between $CD19^+$ T cells and transduced cells: (**A**) percentage of $CD19^+$ cells contacting TK cells; (**B**) percentage of TK cells with $CD19^+$ contacts; (**C**) average number of $CD19^+$ T cell contacts per TK-expressing cell. This demonstrates that a minority (up to 20%) of $CD19^+$ cells contact transduced cells; only 10% of transduced cells are contacted by a $CD19^+$ cell; and on average only one $CD19^+$ cell will contact a target transduced cell. (**D**) Projection image of a group of transduced neurons expressing TK (green), with closely apposed $CD19^+$ cells (red). (**E**) Single contact in a 0.5-μm-thick optical confocal section ($CD19^+$ [red]; TK [green]; DAPI [blue nuclei]), at 30 days postimmunization; thinner (0.5 μm) optical sections are shown to demonstrate actual contacts, which are indicated by the white arrows.

tacts exist, the majority of B cells infiltrating the brain region containing infected cells do not establish direct anatomical contacts with infected cells. Confocal microscopy analysis illustrates the specific contacts between $CD19^+$ cells and TK-transduced cells (Fig. 2D, projection image; Fig. 2E, 0.5-μm confocal layer). These data suggest that effects of B cells on infected cells may be a result of direct contact with infected cells, or result from the release of particular mediators, or from the capacity of B cells to recruit other effectors into the brain through their function as antigen-presenting cells.

B cells mediate the elimination of adenovirus-encoded transgene expression in the brain

To determine whether B cells play a role in the elimination of infected cells from the brain, we examined the effects of systemic immunization on the loss of infected cells in Igh-$6^{-/-}$ animals (Jackson Laboratory), which lack mature B cell populations. All Igh-$6^{-/-}$ transgenic mice were on the C57BL/6 background. After intrastriatal injection of RAd-TK, evidence of TK-transduced cells could be seen in nonimmunized control and immu-

nized Igh-6$^{-/-}$ mice at all time points analyzed (Fig. 3A). However, unbiased stereological quantification of transduced cells in the brains of control and RAd-HPRT-immunized animals demonstrated no significant loss of transduced cells at 30 days postimmunization, but a significant loss of transduced cells at 60, 90, and 120 days postimmunization (Fig. 3B). However, in control C57BL/6 animals that were immunized against adeno-

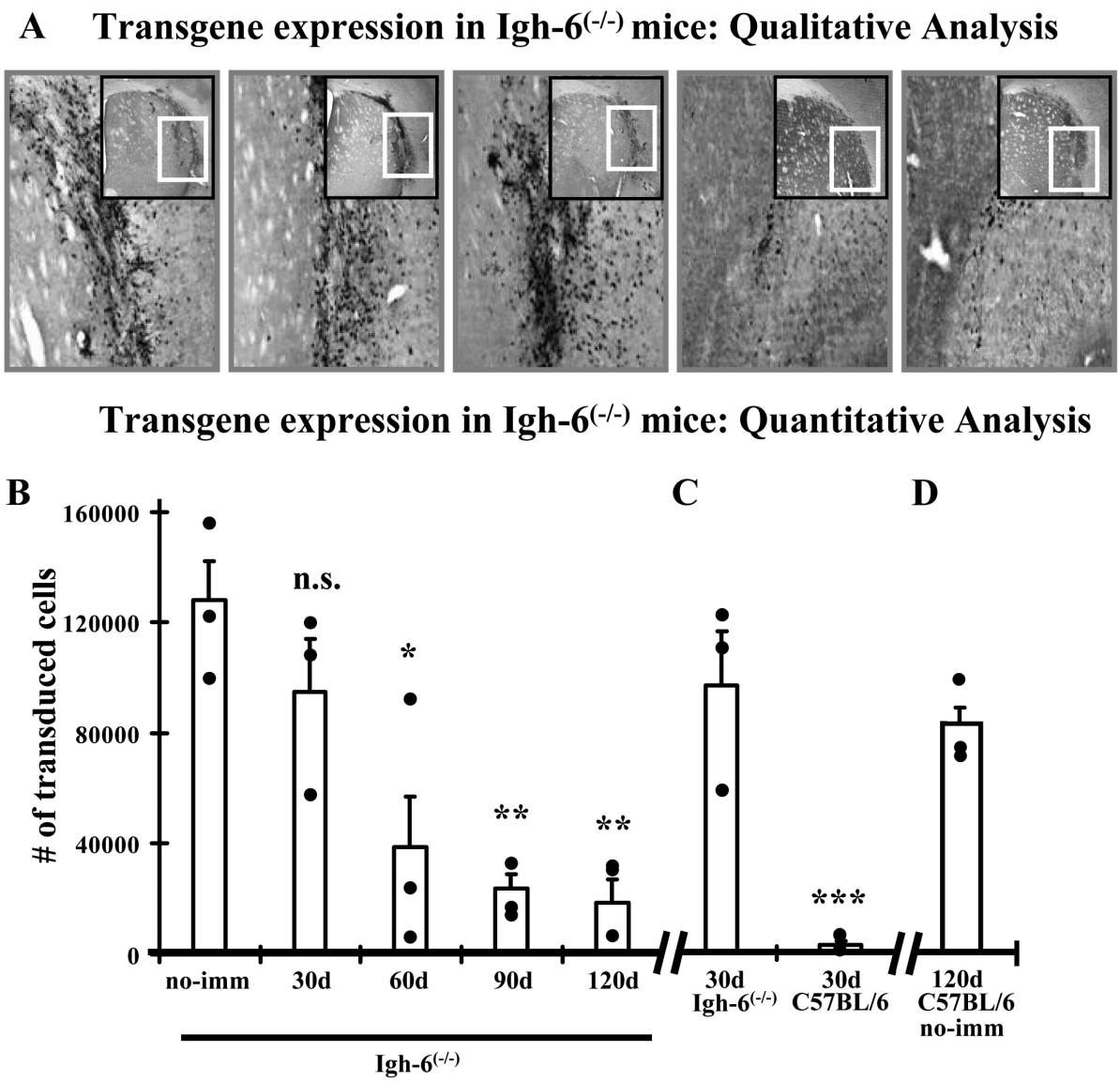

FIG. 3. Qualitative and quantitative analysis of TK expression in the CNS of animals devoid of B cells. (**A**) Immunohistochemistry images (*insets*, lower power) of TK transgene expression in the striatum of Igh-6$^{-/-}$ mice. *Left* to *right:* No immunization, and 30, 60, 90, and 120 days postimmunization. TK expression persists 30 days after systemic RAd-HPRT immunization and clearly decreases to significantly lower levels by 90–120 days postimmunization. (**B**) Stereological quantification of TK-immunoreactive cells in the striatum of nonimmunized Igh-6$^{-/-}$ mice (no-imm), or HPRT-immunized Igh-6$^{-/-}$ mice at 30–120 days postimmunization. (**C**) Stereological quantification of TK-immunoreactive cells in the striatum of HPRT-immunized Igh-6$^{-/-}$ mice compared with control HPRT-immunized C57BL/6 mice, both shown 30 d postimmunization. Notice that transgene expression has been eliminated in control immunized C57BL/6 mice, whereas there has been no decrease in the number of TK-immunoreactive cells in immunized B cell-deficient Igh-6$^{-/-}$ mice. (**D**) Stereological quantification of TK-immunoreactive cells in the striatum of C57BL/6 mice 120 days after sham immunization, showing continued transgene expression in the absence of immunization.

virus transgene, expression had already been eliminated by day 30 postimmunization (Fig. 3C) (and remained at low levels for up to 1 year; data not shown). In control C57BL/6 animals that were sham immunized, transgene expression levels remained stable (Fig. 3D). Thus, the elimination of transgene-expressing cells was significantly delayed in Igh-6$^{-/-}$ mice. This suggests that B cells play an important role in the early phase of elimination of transgene expression in the brain, but are not necessary for the sustained long-term loss of infected cells from the brain (Fig. 3B).

DISCUSSION

Our experiments demonstrating the influx of B cells into the brains of immunized animals, their specific contacts with target infected cells, and the delay in elimination of transgene expression in mice devoid of these cell types, indicate that B cells play an important role in the regulation of expression from viral genomes within infected brain cells. Understanding the cellular and molecular mechanisms by which the immune system regulates transgene expression from virally infected brain cells is crucial for understanding how the immune system regulates expression from viral genomes that infect the CNS. Here we examine expression of a marker gene encoded by a viral vector as a model to elucidate the mechanisms eventually leading to the inhibition of viral gene expression and viral clearance from the CNS. We also believe this work will contribute to enhancing the efficacy of clinical trials in neurological gene therapy that use viral vectors as therapeutic tools, and thus rely on continued expression from the viral vectors, eventually even in the presence of immune responses (8–10,13,35–37). Using a mouse model of brain immune responses to adenoviruses, we have previously demonstrated that CD4$^+$ and CD8$^+$ T cells are involved in the elimination of transgene expression from the brain through a combination of cytotoxic and noncytotoxic regulatory mechanisms, and that this response occurs whether animals are exposed systemically to virus before or after the delivery of viral vectors to the brain.

It has been shown that humoral immune responses can lead to the death of brain cells and neurons (38) and that intrathecal antibody synthesis within the brain can be elicited in healthy animals with normal BBB permeability (21,22). It has also been reported that the number of antigen-specific CD8$^+$ T cells is reduced in Lymphocytic Choriomeningitis Virus (LCMV)-infected mice that lack B cells, indicating that interactions may occur between B cells and other immune cells (39). Here, we examine the role of B cells in the adaptive immune response to adenoviral vectors. We demonstrate actual contacts between virally infected brain cells and CD19$^+$ B cells, and provide evidence that the eventual loss of adenovirus-transduced brain cells in the brain involves B cells.

The CD19 antigen has been shown to be restricted to the B cell lineage and was used as a pan-B cell marker to identify B cells (40). Examination of the influx of B cells into the CNS was performed by immunohistochemistry, using immunoreactivity for CD19 as a marker of B cells within the brain. Quantification of immunohistochemistry showed that few CD19$^+$ cells are present in the normal naive brain before systemic activation of the adaptive immune response by RAd-HPRT immunization, but B cells will infiltrate only those areas of the brain containing infected cells. Stereological quantification detected CD19-immunoreactive B cells within the CNS 14 days after immunization and these cells were still present 30 days after immunization. Analysis by flow cytometry, using a CD45R/B220 antibody, which is also expressed on some activated T and natural killer (NK) cells (41), as well as plasmacytoid dendritic cells (42), confirmed that cells expressing B cell antigens are able to infiltrate the CNS after systemic anti-adenoviral immunization. An increase in the number of CD45R/B220$^+$ cells was detected in the CNS of Rad-HPRT-immunized mice 14 days after immunization (data not shown).

Further investigation of the relationship between infiltrating immune cells and CNS cells expressing the adenovirus-encoded transgene was done by confocal analysis to examine the existence of cell–cell contacts in detail. Quantification of such contacts revealed that specific contacts exist between CD19$^+$ B cells and transduced cells in the brains of mice only after immunization with RAd-HPRT, indicating that the adaptive immune system must be activated in order for CD19$^+$ cells to enter the brain and engage infected cells. Only a minority (approximately 15–20%) of infiltrating CD19$^+$ cells had established contacts with transduced cells by 14 and 30 days after immunization, and even fewer transduced cells (approximately 10–15%) were contacted by these same CD19$^+$ cells. However, the small number of CD19$^+$ cells that infiltrate and the absence of contacts in control animals indicate that immunization against viral vectors and the induction of an adaptive immune response are able to induce B cell infiltration into the CNS, resulting in specific contacts between immune cells and transduced cells. Whether individual B cells can contact various infected cells over time remains to be determined, using *in vivo* imaging technologies.

Data from Igh-6$^{-/-}$ transgenic mice indicate that B cells play a role in the early regulation of transgene expression from viral vectors. Our unpublished data have shown that transgene expression in the brain from first-

generation vectors is nearly abolished in C57BL/6 mice by 30 days after immunization, and remains low for up to 1 year later. We also demonstrated that T cells are involved in the elimination of transgene expression from the brain, as $CD4^{-/-}$ and $CD8^{-/-}$ transgenic animals were unable to clear transgene expression after immunization. Use of Igh-$6^{-/-}$ animals, devoid of B cells, demonstrates that there are still significant numbers of transduced cells in the brain 30 days postimmunization, a time point at which transgene expression is nearly eliminated in immunized C57BL/6 animals. However, at the 60-day postimmunization time point, there is a significant decrease in the number of transduced cells, indicating that the role of B cells may be functionally necessary only at early stages of the adaptive immune response. This indicates that B cells play a role in the early elimination of transgene expression from the infected brain, but do play lesser or no roles in the elimination of transgene expression from viral vectors that occurs at the later time points.

The precise role of B cells in the adaptive immune response to viral vectors remains to be elucidated. One of the possibilities is that B cells, circulating in the blood and lymph under normal conditions, are also circulating through the CSF. On peripheral exposure to adenovirus and induction of an adaptive immune response B cells specific for adenoviral antigens are able to migrate to the site of previous adenoviral infection in the CNS and act as an antigen-presenting cell. The phenomenon of B cell trafficking to the brain in rats infused with antigen has been described previously (43) for resting B cells in brain studies of immune tolerance (44,45). Activation of B cells has also led to the induction of chemokine genes, leading to the recruitment of regulatory cells (46). As such, B cells could act by facilitating the entry of other immune effectors into the brain, and their role would be taken up by different antigen-presenting cells at later time points. This would explain the delay in the elimination of transgene expression in B cell-deficient animals.

Although the precise mechanism by which the $CD19^{+}$ B cells act on brain cells infected with adenovirus has yet to be elucidated, the experiments described conclusively illustrate that on injection of first-generation adenoviral vectors into the brain, and subsequent immunization, an increase in infiltrating $CD19^{+}$ B cells is detected. Together with a significant delay in the elimination of transgene expression from the brains of mice deficient in B cells, these data provide conclusive evidence that B cells access the brain as part of the systemic immune response, do recognize the presence of infected cells within the brain, and significantly contribute to their elimination. The molecular mechanisms by which B cells do so remain to be explored in further detail.

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