Decreased Vector Gene Expression from E2b Gene-Deleted Adenovirus Serotype 5 Vaccines Intensifies Proinflammatory Immune Responses

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ABSTRACT Recombinant adenovirus serotype 5 (Ad5) vectors are promising vaccine candidates due to their intrinsic immunogenicity and potent transgene expression; however, widespread preexisting Ad5 immunity has been considered a developmental impediment to the use of traditional, or conventional, E1 and E3 gene-deleted Ad5 (Ad5[E1−/]) vaccines. Even in the presence of anti-Ad5 immunity, recent murine and human studies have confirmed E2b gene-deleted Ad5 (Ad5[E1−/E2b−/]) vaccines to be highly efficacious inducers of transgene-specific memory responses and significantly less toxic options than Ad5[E1−/] vaccines. While these findings have been substantially confirmed, the molecular mechanisms underlying the different reactions to these vaccine platforms are unknown. Using cultures of human peripheral blood mononuclear cells (hPBMCs) derived from multiple human donors, we found that Ad5[E1−/E2b−/] vaccines trigger higher levels of hPBMC proinflammatory cytokine secretion than Ad5[E1−/] vaccines. Interestingly, these responses were generated regardless of the donors’ preexisting anti-Ad5 humoral and cell-mediated immune response status. In vitro hPBMC infection with the Ad5[E1−/E2b−/] vaccine also provoked greater Th1-dominant gene responses yet smaller amounts of Ad-derived gene expression than Ad5[E1−/] vaccines. These results suggest that Ad5[E1−/E2b−/] vaccines, in contrast to Ad5[E1−/] vaccines, do not promote activities that suppress innate immune signaling, thereby allowing for improved vaccine efficacy and a superior safety profile independently of previous Ad5 immunity.

KEYWORDS adenoviruses, gene transfer, humoral immunity, immunization, innate immunity, tumor vaccines, vaccines, viral immunity

The study and characterization of recombinant adenovirus (Ad) vaccine platforms, particularly Ad serotype 5 (Ad5) vaccine platforms, are of great interest for their development and clinical application for human vaccination. Ad5-based vaccines are attractive for a multitude of reasons, including their broad human cell tropism, relative ease of manufacturing for scaled production, and low potential for adverse side effects (1). Additionally, numerous studies have demonstrated the tremendous ability of Ad5-based vaccines to create robust, transgene-specific cell-mediated immunity against antigens derived from pathogens, such as HIV (2–8), the malaria parasite (9, 10), and Mycobacterium tuberculosis (11, 12), as well as many tumor-associated antigens (13–17). Although the advantages of these vaccines are abundant, there has been some hesitation toward their practical use due to the high prevalence of preexisting Ad5 immunity in the human population (18–20). Several strategies to evade preexisting immunity toward Ad5 have been attempted, including the use of alternative delivery
methods (21, 22), modification of viral surface proteins (23), and the use of varied Ad serotypes (10, 24, 25), and each of these has had various levels of success.

Another proposed strategy to combat this issue is to delete additional genes from the Ad5-based vaccine platform. Traditional, or conventional, recombinant Ads are engineered to lack the early E1 and E3 gene regions (Ad5[E1-]), and these deletions substantially inhibit but do not completely eliminate the ability of these viral vectors to replicate and translate viral proteins (26). Conversely, multiply deleted recombinant Ads have further removal of early gene regions, such as E2b (Ad5[E1-,E2b-]), a gene that typically encodes the DNA polymerase and preterminal region of Ad5 (1, 27). In studies comparing these two types of Ads, Ad5[E1-,E2b-] vaccines have been found to promote the decreased expression of viral proteins, persist longer in vivo, and induce less virus-associated toxicity following in vivo administration (16, 28, 29). Furthermore, multiple murine, nonhuman primate, and human studies have shown that the anti-Ad5 preexisting immunity that inhibits the efficacy of Ad5[E1-] vaccines can be circumvented through the use of Ad5[E1-,E2b-] platforms, allowing for induction of a robust immune response against the vaccine transgene target (5, 14–16, 30).

Currently, the mechanisms underlying the differences between these Ad5 vaccine platforms are unclear, although several hypotheses have been raised. For example, it has been reported that CD8+ T cells specific to Ad5 E2b gene-derived epitopes can be present in humans following Ad5 infection (31, 32). While these epitopes are not as immunodominant as some of the naturally formed antibodies against other Ad5 components (e.g., hexon), anti-Ad5 E2b gene antibodies may still compromise the therapeutic response of Ad5-based vaccines (23, 33). It has also been observed that Ad5[E1-] vaccines instigate the leaky expression of the same viral proteins, while E2b gene-deleted vaccines do not (26, 34). Consequently, it has been hypothesized that viral protein production from Ad5[E1-] vaccines accelerates anti-Ad5 memory responses toward the recombinant Ad-infected cells, thereby shortening the transgene expression time and decreasing therapeutic efficacy.

Another hypothesis to consider is that the two types of Ad5 vaccines may produce divergent inductions of the innate immune response early after initial administration. Previous microarray studies confirmed that Ad5[E1-] infection instigates a unique hepatocellular gene expression pattern compared to that instigated by fully deleted Ad5 vaccines, which lack any viral genome-derived gene expression, and that these differences in immune-related gene expression peaked at 6 h postinfection (hpi) (35). Acknowledging that further viral gene deletions within recombinant Ad5 (rAd5) vaccines can change immune reactions toward them, we measured the levels of viral and host gene expression, as well as infection-induced cytokine secretion, from multiple independent samples of human peripheral blood mononuclear cells (hPBMCs) early after Ad5[E1-] or Ad5[E1-,E2b-] infection. The results of these studies suggest that the improved therapeutic value of the Ad5[E1-,E2b-] vaccine over conventional recombinant Ad5 vaccines may be due to differential activation of the innate immune response.

RESULTS

Ad5[E1-,E2b-] vaccines induce an increased proinflammatory cytokine response upon hPBMC infection. To determine if exposure to the Ad5[E1-] or Ad5[E1-,E2b-] vaccine provokes different levels of innate immune cytokine secretion, we obtained and utilized several individual, deidentified human blood samples. Upon receiving the samples, hPBMCs were isolated via a Ficoll-Paque gradient, cultured, and mock infected or infected with the Ad5[E1-] or Ad5[E1-,E2b-] vaccine platform. At 72 hpi, medium was collected and used for enzyme-linked immunosorbent assay (ELISA) measurement of interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-6 levels. These particular proinflammatory cytokines were selected for quantification due to their significant and early production from hPBMCs following Ad infection (36). Interestingly, statistical comparison of the mean values for all samples revealed that Ad5[E1-,E2b-] treatment of hPBMCs induced larger amounts of IL-1β (P < 0.05) and
TNF-α (P < 0.001) secretion than Ad5[E1−] treatment of hPBMCs (Fig. 1). Furthermore, Ad5[E1−] treatment of hPBMCs did not appear to induce levels of IL-1β or TNF-α secretion significantly higher than those produced by mock-treated cells under these experimental conditions. No observed difference between Ad5[E1−] and Ad5[E1−,E2b−]-induced secretion of IL-6 from hPBMCs occurred; however, both Ad5[E1−] and Ad5[E1−,E2b−] triggered higher levels of IL-6 production than mock infection. Overall, these findings suggest that Ad5[E1−,E2b] hPBMC infection may elicit a stronger, early proinflammatory cytokine response than similar infections with an Ad5[E1−] vaccine platform, an observation that was consistent across samples from several human subjects.

**Ad5-induced proinflammatory cytokine responses from human blood samples are not correlated to preexisting Ad5 immunity.** In the past, concern over Ad5-based vaccinations has been raised because of the high levels of preexisting Ad5 immunity present in certain human populations (24, 37). Previous studies have shown that the presence of anti-Ad5 IgG antibodies or cell-mediated immunity can prevent the development of transgene immunity following vaccination with Ad5[E1−] transgene-expressing vaccines (7, 8, 38), yet this effect does not seem to be problematic during the use of Ad5[E1−,E2b−] vaccines (5, 6, 14–17).
To investigate if Ad5[E1−] or Ad5[E1−,E2b−] infection-induced cytokine expression correlated to preexisting anti-Ad5 antibody levels, serum was collected from the previously specified human blood samples prior to hPBMC isolation. An anti-Ad5 IgG ELISA was performed, and the levels of anti-Ad5 IgG were plotted against the same subject’s hPBMC IL-1β, TNF-α, and IL-6 secreted cytokine levels at 72 h following Ad5[E1−] or Ad5[E1−,E2b−] infection. Correlation analysis revealed no association between preexisting anti-Ad5 IgG levels and IL-1β, TNF-α, or IL-6 secretion amounts (Fig. 2A) with either Ad5[E1−] (Fig. 2B) or Ad5[E1−,E2b−] (Fig. 2C) treatment.

Additionally, we wished to examine if preexisting anti-Ad5 cell-mediated immunity could be correlated to Ad5[E1−] or Ad5[E1−,E2b−] infection-induced cytokine production after 72 h, as CD8+ T lymphocytes have also been found to be an integral part of anti-Ad5 immunity (38). To do this, hPBMCs were stimulated with UV-inactivated Ad5[E1−] and analyzed by flow cytometry for quantification of gamma interferon (IFN-γ)-expressing CD3+CD8+ T cells responsive to Ad5 capsid proteins, as previously described (4, 39). CD3+CD8+ cells which expressed intracellular IFN-γ were considered anti-Ad5-specific CD8+ T cells, and the percentage of these cells was plotted against the samples’ respective IL-1β, TNF-α, and IL-6 cytokine concentrations. Similar to the humoral response, correlation analysis revealed no association between the percentage
of anti-Ad5-specific CD8+ T cells and any of the measured cytokine secretion amounts induced by Ad5[E1−] (Fig. 3A) or Ad5[E1−,E2b−] (Fig. 3B) infection, suggesting that the stimulated memory T cell responses and cytokine secretion induced by Ad5 vector recognition cannot alone predict the amount of IL-1β, TNF-α, and IL-6 expression from hPBMCs.

We also considered the possibility that the larger amounts of proinflammatory cytokines induced by the Ad5[E1−,E2b−] vaccine than by the Ad5[E1−] vaccine may be associated with the level of preexisting anti-Ad5 antibodies and/or CD8+ T cells. To examine these relationships, correlation analyses were completed as mentioned above, with the exception that the values obtained for anti-Ad5 immunity were plotted against a ratio of the amount of cytokine secretion induced by Ad5[E1−,E2b−] to that induced by Ad5[E1−] for each subject at 72 hpi. These analyses revealed that the levels of neither preexisting anti-Ad5 IgG antibodies (see Fig. S1A in the supplemental material) nor anti-Ad5 CD8+ T cells (Fig. S1B) were correlated to cytokine expression ratios. Furthermore, there was no correlation between the levels of preexisting anti-Ad5 IgG antibodies and anti-Ad5 CD8+ T cells for each subject (Fig. S2). Overall, these results suggest that while proinflammatory cytokine induction differs between the Ad5[E1−] and Ad5[E1−,E2b−] vaccines, this response seems to occur independently of preexisting anti-Ad5 humoral or cellular immunity.

**Ad5[E1−,E2b−] infection results in diminished Ad gene expression.** To evaluate the effect that E2b gene function has on Ad5 vaccine-induced gene expression, we infected HEK293 cells for 24 h with equivalent doses of Ad5[E1−], Ad5[E1−,E2b−], or a control vaccine, the helper-dependent Ad5 (HD-Ad5) vaccine, which lacks any viral genes (40). HEK293 cells constitutively produce the Ad5 E1 protein (41), allowing the Ad5[E1−] vaccine platform but not the Ad5[E1−,E2b−] or HD-Ad5 vaccine platform to replicate. Subsequently, we found that HEK293 cells expressed significantly larger amounts of E4, virus-associated (VA) gene I (VAI), VAI, and hexon RNA when they were infected with the Ad5[E1−] vaccine vector than when they were infected with the Ad5[E1−,E2b−] vaccine vector (P < 0.001) (Fig. 4A). Statistically, Ad5[E1−,E2b−] gene transcript levels were no different from those measured from mock-infected or HD-Ad5-infected cells, with the exception of those of the early gene E4 (P < 0.001).
To determine if these viral gene expression differences would also be seen following infection of cells which do not express the Ad5 E1 gene, buffy coat-derived hPBMCs were plated and mock infected or infected with an equivalent dose of Ad5[E1−], Ad5[E1−,E2b−], or HD-Ad5. RNA was collected at 24 hpi for RT-PCR quantification of Ad gene expression. (B) hPBMCs were plated at 5 × 10^6 cells/well on 6-well plates with RPMI medium supplemented with 2% FBS, 1× PSF, 5 ng/ml IL-12, and 10 ng/ml IL-18 and either mock infected or infected with 10,000 vp/cell Ad5[E1−], Ad5[E1−,E2b−], or HD-Ad5. RNA was collected at 6 hpi for RT-PCR quantification of Ad gene expression. Bars denote means ± standard errors. *, **, and *** significant differences (P < 0.05, P < 0.01, and P < 0.001, respectively) between the indicated group and mock-treated samples; #, ##, and ### significant differences (P < 0.05, P < 0.01, and P < 0.001, respectively) between Ad treatments.

To further verify that these Ad gene expression variations were not due to unequal amounts of Ad5 particles being introduced and/or entering the hPBMCs, sample DNA and RNA were collected simultaneously at 6 hpi to allow for adequate rAd5 PBMC infection and gene expression (42). Relative viral genome quantification was conducted by quantitative PCR measurement of the amount of Ad5 hexon gene DNA, as described previously (43). The amounts of Ad5 DNA and RNA collected from the same cell-containing wells were directly compared to examine if the amount of virions could be the sole variable promoting increased Ad5 gene expression. Comparison of viral DNA from Ad5[E1−]- and Ad5[E1−,E2b−]-treated cells revealed no mean differences in the amount of viral genomes present in either group (Fig. S3A), nor was it found that rAd5 virion quantities alone could account for sample variability in Ad5 gene expression patterns (Fig. S3B).
The Ad5[E1−] and Ad5[E1−,E2b−] vaccine platforms induce distinct innate immune gene expression profiles. To examine the induction of human immune-related genes by different Ad5 vaccine platforms, hPBMCs were either mock infected or infected with the Ad5[E1−], Ad5[E1−,E2b−], or HD-Ad5 vaccine at equivalent doses. RNA was isolated from infected hPBMC samples at 6 hpi and used to measure the levels of expression of the early innate immune response genes IFIT1, IL-12β, RIG-I, and IFN-β; these genes were selected due to their robust expression from hPBMCs and diverse representation of multiple innate immune signaling pathways (44, 45). Interestingly, exposure to the Ad5[E1−,E2b−] vaccine platform induced significantly more IFIT1 and IL-12β gene expression in hPBMCs than exposure to the Ad5[E1−] vaccine platform (P < 0.001 and P < 0.01, respectively) (Fig. 5). The levels of IFIT1 and IL-12β expression triggered by Ad5[E1−,E2b−] infection were also significantly greater than those triggered by HD-Ad5 infection (P < 0.05 and P < 0.001, respectively) or mock infection (P < 0.001). Conversely, Ad5[E1−] did not induce significantly larger amounts of IFIT1 or IL-12β gene expression than the other treatments and actually induced statistically significantly smaller quantities of IFIT1 than the HD-Ad5 control vaccine platform (P < 0.01). Infection of hPBMCs with Ad5[E1−,E2b−] also promoted RIG-I gene expression at levels greater than those noted in mock-infected cells (P < 0.05). Alternatively, hPBMCs infected with Ad5[E1−] or HD-Ad5 did produce higher levels of IFN-β gene expression than mock-infected hPBMCs (P < 0.01 and P < 0.05, respectively), and while the difference was not statistically significant, this result suggests increased induction of
IFN-β expression from cells treated with the Ad5[E1−] or HD-Ad5 vaccine platform than from cells treated with the Ad5[E1−,E2b−] vaccine platform.

**DISCUSSION**

Recombinant adenovirus vectors are promising, highly characterized platforms from which novel vaccines can be produced. Conventional Ad5[E1−] vaccines possess the ability to promote strong immunologic responses against their expressed transgenes, but the same properties also trigger host antiviral responses, which can lead to increased toxicity, limited persistence, and decreased efficacy in the face of preexisting anti-Ad5 immunity (7, 28, 29, 34, 46). Alternatively, multiply deleted Ad5[E1−,E2b−] vaccines have been found to overcome several of these obstacles. Head-to-head comparisons of traditional Ad5[E1−] vaccines and E2b gene-deleted, Ad5[E1−,E2b−] vaccines have shown that the latter produce virtually no hepatotoxicity after systemic administration (29), persist and express transgenes for longer durations than Ad5[E1−] vaccines (28, 34), and remain therapeutically efficacious in hosts harboring substantial preexisting anti-Ad5 immunity (5, 14–16, 30).

It has been largely assumed that the rationale for these differences in recombinant Ad5 vaccines is that Ad5[E1−,E2b−] vaccines do not produce substantial amounts of viral proteins upon infection; thereby, they are able to more adeptly evade host antiviral responses (26, 34). While this may serve as a partial contributor toward these divergent responses, multiple recombinant Ad5 studies have shown that anti-Ad5 immunity alone is not predictive of a recombinant Ad5-based vaccine’s efficacy (11, 14, 47).

To investigate the underlying immunologic and mechanistic differences between Ad5[E1−] and Ad5[E1−,E2b−] vaccine responses, we chose to use a human PBMC experimental model. hPBMCs were harvested from multiple human blood samples, a choice that allowed for a diverse population to be sampled from while also providing a generous statistical power. Our analysis of early innate cytokine secretion from hPBMCs following recombinant Ad5 vaccine infection revealed the more robust production of IL-1β and TNF-α from cells treated with the Ad5[E1−,E2b−] vaccine platform than those treated with the Ad5[E1−] vaccine platform but no difference between these treatments in the induction of IL-6 secretion (Fig. 1). These divergent proinflammatory cytokine inductions may explain the functional differences between the Ad5[E1−] and Ad5[E1−,E2b−] vaccines. For example, IL-1β and TNF-α enhance downstream adaptive memory immune responses, such as antigen-dependent CD4+ T cell clonal expansion (48). Alternatively, IL-6 promotes skewing of the immune response toward the Th2 response, both by stimulating IL-4 production and by inhibiting CD4+ T cell responsiveness to IFN-γ stimulation (49). Further testing also revealed that an individual hPBMC sample’s Ad5 vaccine-induced cytokine concentrations could not be attributed to preexisting anti-Ad5 antibodies (Fig. 2) or CD8+ T cells (Fig. 3) alone; neither variable was found to have any correlation with the IL-1β, TNF-α, or IL-6 amounts produced by the individual samples following Ad5 vaccine platform infection. These findings are not to imply that preexisting anti-Ad5 immunity does not affect downstream adaptive cytokine responses to rAd5 vectors but instead suggests that these are not the only variables that affect cytokine expression differences between Ad5[E1−] and Ad5[E1−,E2b−] vectors. In future studies, it would be of interest to compare anti-Ad5 antibody levels through a specific Ad5 neutralization assay to quantify the exact titers against cytokine responses as well (50); however, in the spectrum of this study, we focused on obtaining measurements of the relative optical density at 450 nm (OD450), which allowed us to simply conclude that preexisting anti-Ad5 antibodies are not the only variable that determines the immunologic differences between the Ad5[E1−] and Ad5[E1−,E2b−] vectors.

Additionally, the *in vitro* innate immune gene expression patterns of Ad5[E1−] and Ad5[E1−,E2b−]-infected hPBMCs were measured (Fig. 5), revealing that Ad5[E1−,E2b−] infection produced significantly higher levels of IL-12 and IFIT1, both of which promote Th1 and NF-κB pathway activation. Conversely, Ad5[E1−] hPBMC infection...
induced a significant amount of IFN-β gene expression but triggered no substantial induction of IL-2 or IFIT1 expression. These innate immune differences may begin to explain the differential efficacy of the Ad5[E1−,E2b−] vaccine from that of the Ad5[E1−] vaccine and its longer viral persistence in vivo. For example, it is known that IL-12 expression enhances dendritic cell activation, promotes CD4+ T cell Th1 differentiation, and boosts IgG antibody production, all of which are beneficial immune actions in the development of a substantial vaccination response (51). Alternatively, it has been found that increased levels of IFN-β production following viral vaccine administration can inhibit mucosal phagocyte IL-12 responses, thereby decreasing the efficiency of vaccine-induced phagocyte killing and major histocompatibility complex class II expression (12).

In these experiments, HD-Ad5 was used as a control vector; it functions well in this role as it possesses the outer structure of Ad5 but contains no Ad5 genetic material. It should be noted that helper-dependent recombinant Ad vectors, such as HD-Ad5, have also been used in vaccine and gene therapy trials but have been found to trigger relatively low levels of immunogenicity in comparison to conventional Ad5 vectors (35). Interestingly, the innate immune gene expression levels measured in Ad5[E1−] versus HD-Ad5 vaccine vector-infected cells were usually the same; in the case of IFIT1, however, HD-Ad5 actually induced significantly larger amounts of expression than Ad5[E1−]. It was important to confirm that the amounts of our HD-Ad5 infectious input genomes were equivalent to those of our other rAd5 vectors to verify this robust difference (see Fig. S3 in the supplemental material), as these gene expression results may suggest that Ad5[E1−] vectors could actually be inhibiting inflammatory responses upon hPBMC infection or elaborating Ad-derived immune evasion strategies.

Furthermore, it was found that infection by the Ad5[E1−,E2b−] vaccine platform produced much lower levels of Ad gene expression than infection by the Ad5[E1−] vaccine platform, such as virus-associated (VA) RNA genes VAI and VAII of Ad5. Ad VA RNAs have been characterized to be potent double-stranded RNA activators of type I interferon expression (52), yet they also act as NF-κB signaling inhibitors (53, 54). Moreover, Ad VA RNAs interact with RNA interference (RNAi)-related enzymes to inhibit endogenous RNAi activities while simultaneously promoting the synthesis of VA RNA-derived small interfering RNA and microRNA (55–57), the production of which has been shown to impact many aspects of cell biology, including antiviral immune function (58, 59). The decreased levels of expression of Ad gene products by Ad5[E1−,E2b−] vaccines may minimize these and other types of inhibitory effects, some of which (e.g., NF-κB inhibition) are known to be directly detrimental to the ability of vaccines to induce the development of immunity to specific antigens (51, 60, 61). Products from other Ad5 genes have also been shown to directly affect human cellular signaling pathways; for example, hexon proteins can interact with dynein-dependent transport and the coagulation cascade (62, 63). These results suggest that Ad5[E1−] vaccines could actually inhibit beneficial inflammatory responses upon their administration to human hosts though the elaboration of Ad-derived immune evasion strategies.

In summary, the Ad5[E1−] and Ad5[E1−,E2b−]-based vaccine platforms were each found to induce unique, early innate responses along with differences in virus-derived gene expression during hPBMC infection. These findings help to shed light on the immune balancing act that recombinant virus-based vaccines face when they are used for vaccination; the triggering of innate immunity can promote transgene-specific memory responses and vaccine efficacy, yet it can also activate antiviral responses and predispose them to preexisting immune responses that diminish vaccine potency. While adaptive immune responses triggered by previous Ad5 exposure may still contribute to the lower efficacy of Ad5[E1−] vaccines than Ad5[E1−,E2b−] vaccines, these results suggest that early adenoviral biological activities may also play a role in these differences. It is vital that future studies also be conducted to compare Ad5[E1−] and Ad5[E1−,E2b−] vectors containing various transgenes, as these may also influence the immunologic differences that these two vaccine platforms produce. A better understanding of the immune responses toward innovative recombinant Ad vaccines,
such as the Ad5[E1−,E2b−] platform, may allow for further optimization for the safe and effective use of this and many other virus-based vaccines.

MATERIALS AND METHODS

Recombinant adenovirus vaccine vector construction. Transgene-lacking recombinant Ad5 vector platforms Ad5[E1−] (64) and Ad5[E1−,E2b−] (26) were built and propagated as previously described, using a pAdEasy-based system (26, 65). Viral particle (vp) titers were determined by spectrophotometry. Ad5 gene deletions were confirmed by PCR, as previously described (26). The helper-dependent adenovirus (HD-Ad5) FAdhGAA was kindly provided by Philip Ng (Baylor College of Medicine) (40).

Cell isolation, culture, and adenovirus infection. Deidentified blood samples (Stanford Blood Bank, Palo Alto, CA) were obtained, spun to isolate serum from the rest of the blood product, and then used to isolate hPBMCs using a Ficoll-Paque Plus gradient (GE Healthcare Life Sciences, Piscataway, NJ), as described in the manufacturer’s protocol. In experiments where serum was not required, deidentified buffy coats were used as a source of hPBMCs (Stanford Blood Bank) and were isolated in the same manner. hPBMCs were plated at 5 × 106 cells/well on 6-well plates and incubated in RPMI medium supplemented with 2% fetal bovine serum (FBS), 1× penicillin-streptomycin-fungizone (PSF), 5 ng/ml IL-12, and 10 ng/ml IL-18. HEK293 cells were seeded at 3.5 × 105 cells/well in 6-well plates in Dulbecco modified eagle medium (DMEM) supplemented with 10% FBS and 1× PSF. Ad5 vectors were administered in a volume of 50 μl/well phosphate-buffered saline (PBS) into fresh cell medium and incubated at 37°C until harvest. Mock treatment was treatment with PBS alone.

ELISA. Anti-Ad5 ELISAs were performed on serum as previously described (66). Briefly, 5 × 104 vp/well Ad5[E1−] was plated on a high-binding 96-well flat-bottom plate, and the plate was incubated overnight at 4°C. The plates were rinsed with washing buffer (PBS containing 0.05% Tween) and incubated with blocking buffer (PBS containing 3% bovine serum albumin) for an hour at room temperature. Serum was diluted in PBS (1:200), plated, and incubated at room temperature for an hour. Wells were rinsed with washing buffer, coated with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin antibody (Bio-Rad, Hercules, CA), and developed with tetramethylbenzidine (TMB; Sigma-Aldrich, St. Louis, MO). The development reaction was halted by adding 1 N phosphoric acid to the wells. The absorbance at 450 nm of the plates was analyzed using an automatic microplate reader.

ELISA analysis. Anti-Ad5 ELISAs were performed on serum as previously described (66). Briefly, 5 × 104 vp/well Ad5[E1−] was plated on a high-binding 96-well flat-bottom plate, and the plate was incubated overnight at 4°C. The plates were rinsed with washing buffer (PBS containing 0.05% Tween) and incubated with blocking buffer (PBS containing 3% bovine serum albumin) for an hour at room temperature. Serum was diluted in PBS (1:200), plated, and incubated at room temperature for an hour. Wells were rinsed with washing buffer, coated with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin antibody (Bio-Rad, Hercules, CA), and developed with tetramethylbenzidine (TMB; Sigma-Aldrich, St. Louis, MO). The development reaction was halted by adding 1 N phosphoric acid to the wells. The absorbance at 450 nm of the plates was analyzed using an automatic microplate reader.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/CVI.00061-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.
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