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*J Immunol* 2010; 184:5475-5484; Prepublished online 21 April 2010;
doi: 10.4049/jimmunol.0903808
http://www.jimmunol.org/content/184/10/5475
Augmentation of Primary Influenza A Virus-Specific CD8+ T Cell Responses in Aged Mice through Blockade of an Immunoinhibitory Pathway

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Immune responses diminish with age resulting in an increased susceptibility of the elderly to infectious agents and an inability to mount protective immune responses to vaccines. Immunosenescence affects multiple aspects of the immune system, including CD8+ T cells, which control viral infections and are assumed to prevent the development of cancers. In this study, we tested if CD8+ T cell responses in aged mice could be enhanced through a vaccine that concomitantly expresses Ag and a molecule that blocks an immunoinhibitory pathway. Specifically, we tested a vaccine based on a replication-defective chimpanzee-derived adenovirus vector expressing the nucleoprotein (NP) of influenza A virus as a fusion protein with the HSV type 1 glycoprotein D, which through binding to the herpes virus entry mediator, blocks the immunoinhibitory herpes virus entry mediator B and T lymphocyte attenuator/CD160 pathways. Our results show that the vaccine expressing a fusion protein of NP and glycoprotein D induces significantly higher NP-specific CD8+ T cell responses in young and aged mice compared with the vaccine expressing NP only.

of the virus envelope that is essential for virus entry into host cells (29, 30) was the first known HIV-1 ligand (29, 31). Contact residues for gD-HVEM (31–33), BTLA-HVEM (34), and CD160-HVEM (28) have been identified. The N terminus of gD binds to a site close to the BTLA and CD160 binding sites on HVEM and soluble gD can block BTLA–HVEM interactions (32) and is also likely to block HVEM–CD160 interactions. HVEM is in addition expressed on CD4+CD25+ FoxP3+ Tregs and is thought to contribute to their direct inhibition of effector T cells expressing BTLA/CD160 (35). Blockade of HVEM (AdC68). The vector expressed the nucleoprotein (NP) of influenza virus, and which carries an immunodominant CD8+ T cell epitope that is highly preserved between different strains of influenza A virus. Our results show that fusion of NP into gD augments a virus-driven CD8+ T cell responses to NP, not only in young but also in aged mice.

Materials and Methods

Mice

Female 6-wk-old C57BL/6 mice were purchased from Taconic Laboratories (Rockville, MD) and housed at the Animal Facility of the Wistar Institute (Philadelphia, PA). For studies with young mice, animals were tested or immunized at 8–10 wk of age. For experiments with old mice, animals were aged at the Wistar Institute and enrolled into experiments once they were 21 mo old. All experiments were performed according to institutionally approved protocols.

Viruses and vectors

E1-deleted recombinant AdC68 vectors were generated from a molecular clone. The cDNA encoding the NP gene of the A/PR8 strain under the control of the CMV promoter was inserted into a pShuttle vector (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). The correct in-frame cloning of NP encoding gene was confirmed by restriction analysis. To construct AdC68gDNP vector the gDNP chimeric gene was amplified by PCR using the pgDNP vector as a template and the following primers: Fw 5′-ATTGCCGGGCCGCTTACCGCGAACCGAAGGCG3′ and Rv 5′-ATGGCGCGGCGCCTTACCGCACCCG3′. DNA fragments were digested with ApaI and cloned into the ApaI site of the pRE4 vector (37). The correct in-frame cloning of NP encoding gene was confirmed by restriction analysis. To construct AdC68gDNP vector the gDNP chimeric gene was amplified by PCR using the pgDNP vector as a template and the following primers: Fw 5′-ACACCAAAAGTTATGCGGGGCCGTCCGCG3′ and Rv 5′-AGGGCCAAAAGTTATGCGGGGCCGTCCGCG3′. After digestion with HindIII, the gDNP chimeric gene was cloned into the pShuttle vector. The correct in-frame cloning of NP encoding gene was confirmed by restriction analysis and nucleotide sequencing, then subcloned into the molecular clone of the E1-deleted chimpanzee-derived Ad vector 68 using PsiI-SceI and I-CeuI sites as described (38). The full-length gD was cloned into pShuttle and from there into the viral molecular clone using similar approaches generating AdC68gD. E1-deleted viruses were rescued on HEK293 cells. Virus was further propagated on HEK293 cells, purified and titrated as described previously (38). Vector batches were quality controlled by determining virus particle to infectious units ratios, by testing for replication competent Ad and genetic integrity of the vectors was determined by Southern blotting of purified viral DNA. Expression of the NP protein was confirmed on infection of HEK293 cells by Western blot analysis. Both AdC68NP and AdC68gDNP expressed NP in HEK293 cells. Expression was quantified by real-time RT-PCR and levels of transcripts induced by the two vectors were similar (data not shown). For this assay HEK293 cells were infected with serial dilutions of the Ad vectors. Twenty-four hours later, infected cells were harvested and RNA was isolated and reverse-transcribed. The housekeeping gene Gapdh was quantified from each sample by real-time PCR. Samples adjusted to equal amounts of Gapdh and quantified the real-time PCR for NP, resulting in an amplification of 11.95 bp. The following primers were used for the PCR: Fw 5′-CCTAAATGAGACCCAAATGAGA-3′ and Rv 5′-CACCCTCGCCCTTTGTAGA-3′, respectively. Standards for the NP gene (10−5–101 copies) were amplified in parallel. The real-time PCR consisted of cycles at 95°C for 5 s, 53°C for 10 s, 72°C for 10 s, and 83°C for 4 s.

A/PR8 virus was grown in embryonated chicken eggs and titrated for mean lethal dose (LD50) in adult C57BL/6 mice

Immunization and infection of mice

Female C57BL/6 mice were immunized i.m. at 8–10 wk of age with 1010 virus particles (vps) of AdC68NP or AdC68gDNP vector diluted in sterile PBS. For booster immunization mice were inoculated intranasally with 0.5 LD50 of A/PR8 virus.

Isolation of lymphocytes

Lymphocytes were isolated from blood via a submandibular bleed into 1 ml 4% sodium citrate and 1 ml RPMI 1640. Lymphocytes were purified by gradient centrifugation with 1 ml Histopaque-1083 (Sigma-Aldrich, St. Louis, MO). RBCs were lysed with ACK lysis buffer (Life Technologies, Grand Island, NY) treatment for 1–5 min. Lymphocytes were isolated from spleen and liver as described previously (39). Lymphocytes were isolated from lungs as follows. Lungs were perfused with 5–10 ml PBS, isolated, and homogenized with a screen and plunger. Cells were resuspended in 5 ml 1.5 mM EDTA in PBS and incubated for 45 min at 37°C. Lymphocytes were then enzymatically dissociated in 5 ml 1.4 mg/ml collagenase Type I (Life Technologies, Carlsbad, CA) in RPMI 1640 with 1% FBS and incubated for 30 min at 37°C and vortexed intermittently. After centrifugation, RBCs were lysed for 1 min with ACK lysis buffer (Invitrogen, Carlsbad, CA) and washed in RPMI 1640 with 1% FBS.

Intracellular cytokine staining and tetramer staining

Intracellular cytokine staining (ICS) was performed as described previously (38). Abs used for ICS were PerCP-Cy5.5-labeled anti-CD8, Pacific Blue-labeled CD127 (eBioscience, San Diego, CA), Pacific Blue-labeled anti-CD19 (eBioscience), PE-labeled anti-CD69 (Biolegend, San Diego, CA), FITC-labeled anti–IFN-γ, APC-labeled anti-IL-2, and PE-labeled anti–TNF-α. For tetramer (tet) staining cells were stained with APC-labeled NP tetramer (National Institutes of Health Tetramer Core Facility at Emory University, Atlanta, GA) for the immunodominant H-2b class I binding epitope of NP (aa366–374: ASNENKAIVY). They were costained with the following additional Abs: AlexaFlour700-labeled anti-CD44 (BioLegend, San Diego, CA), PerCP-Cy5.5-labeled anti-CD8, Pacific Blue-labeled CD127 (eBioscience, San Diego, CA), Pacific Blue-labeled anti-CD19 (BioLegend), PE-labeled anti-CD160 (Accurate Chemical, Westbury, NY), FITC-labeled anti-CD26L, FITC-labeled anti-CD49 (BioLegend), FITC-labeled anti–Gr-1 (BioLegend), PE–Texas Red-labeled anti-CD4 (Caltag Laboratories, Burlingame, CA), PE-Cy7–labeled anti–PD-1 (BioLegend), PE–Cy7–labeled anti–CD11b (BioLegend). Abs to different markers carrying the same dye were used at least in triplicate. Flow cytometry, at least 300,000 events were acquired on a BD LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). To determine frequencies of NP-specific CD8+ T cells, lymphocytes were analyzed from individual mice and data show averages ± SDs. To determine phenotypes of lymphocyte subsets, data from individual mice of a given group were concatenated in FlowJo to ensure that numbers of events sufficed for the analyses of small subpopulations. In analyses of leukocyte subsets, lymphocytes were first gated on CD4+ and CD8+ cells, CD4+CD8− cells were gated on CD19+ and CD19− cells, CD19+ cells were gated on Gr-1+ and Gr-1− cells (or in separate experiments CD94+ and CD94− cells), Gr-1+ cells (or CD94+ cells) were gated on CD11b+ cells.

In vivo cytolytic T cell assay

Vaccinated and sham-vaccinated mice were injected with 2×10⁵ synthetic peptides. Half of them had been pulsed with the NP peptide and labeled with a high concentration of CFSE, the other half had been pulsed with the SIINFEKL peptide and labeled with a low concentration of CFSE. Twenty-four hours later, mice were bled and events of low and high CFSE expression were determined from pooled PBMCs. Twenty-four hours after injection, mice were euthanized and CFSE peak were analyzed on spleenocytes. Splenocytes were in addition stained with a live cell dye, Abs to CD4, CD8, and the NP peptide to determine percentages of NP-responsive CD8+ T cells. Percent-specific lysis was calculated as described (39).

Statistical analyses

Each experiment was conducted repeatedly with 3–10 mice. Statistical significance of differences between two groups was calculated by paired Student t tests and between more than two groups by ANOVA.
$p$ values obtained from multiple comparisons were corrected for (Bonferroni correction) to avoid type 1 errors.

**Results**

**PBMCs in young and aged mice at baseline**

Aging is, in part, influenced by the environment, and mice aged in different facilities can show differences in their immune profiles. We therefore initially characterized the composition of leukocyte subsets in blood to define differences at baseline between young ($n = 15$) and aged ($n = 14$) C57BL/6 mice. As shown in Fig. 1, numbers of CD4$^+$ and CD8$^+$ T cells were lower in blood of aged mice, whereas those of Gr-1$^+$ granulocytes and neutrophils and CD11b$^+$ monocytes, dendritic cells, and NK cells were markedly increased. Although numbers of B cells (CD19$^+$) were higher in young than aged mice, this difference failed to reach statistical significance.

CD8$^+$ T cells in blood were further characterized for expression levels of differentiation markers, including CD44, which is low on naive cells and increased on those that are Ag-experienced, and CD62L, an adhesion molecule that is highly expressed on naive and central memory T cells. In addition, we tested for the coinhibitory markers PD-1, BTLA, and CD160, which are linked to functional impairment of Ag-experienced T cells. As shown in Fig. 2A, CD44 was higher on CD8$^+$ T cells from the aged mice and a large proportion of their T cells expressed reduced levels of CD62L, indicating, as described previously (40), a relative paucity of naive T cells in this group. A significant difference was also seen for the expression of PD-1, which was lower on aged than on young CD8$^+$ T cells. Expression levels for BTLA and CD160 were indistinguishable for the two groups. To test if the coinhibitory markers were differentially expressed on naive cells, CD8$^+$ T cells were gated on those that were CD44$^{low}$ (Fig. 2B). Again, naive CD8$^+$ T cells from aged mice expressed lower levels of PD-1 than those from young mice, whereas expression levels of BTLA and CD160 remained comparable. These results suggest that during immunosenescence some immunoinhibitory pathways, such as the PD-1–PD-L1/L2 pathway, may become impaired whereas others, such as the HVEM-BTLA/CD160 pathway, appear to remain relatively unaffected.

**Magnitude of the NP-specific CD8$^+$ T cell response in young and old mice**

To assess if manipulation of the HVEM-BTLA/CD160 pathways could improve the CD8$^+$ T cell response to a specific Ag in aged mice, young and old mice were vaccinated once i.m. with the AdC68NP or AdC68gDNP vector given at $10^{10}$ vps. Blood was collected at different times thereafter and frequencies of NP-specific CD8$^+$ T cells were determined by staining of cells with a tet specific for the immunodominant epitope of NP. As shown in Fig. 3A, young mice immunized with the AdC68NP vector ($n = 10–44$, different numbers were analyzed for the different time points) developed a detectable NP-specific CD8$^+$ T cell response by day 10 after vaccination, which increased further by day 14 and then gradually declined. Young mice immunized with the AdC68gDNP vector ($n = 10–49$) developed an NP-specific CD8$^+$ T cell response with similar kinetics. Frequencies of NP-specific CD8$^+$ T cells induced in young mice by the AdC68gDNP vaccine were significantly higher at all time points tested than those induced by the AdC68NP vaccine. In aged mice ($n = 10–28$), the AdC68NP vaccine failed to induce a detectable NP-specific CD8$^+$ T cell response by day 10. Two weeks after immunization NP-specific CD8$^+$ T cells became detectable in aged mice and like in young mice, they then gradually declined. Frequencies of NP-specific CD8$^+$ T cells were, except for one time point (week 8), lower in aged than in young mice vaccinated with the AdC68NP vaccine. On vaccination with the AdC68gDNP vaccine ($n = 10–29$) aged mice developed an NP-specific CD8$^+$ T cell response by day 10. The response increased further by weeks 2 and 4 and then decreased. Frequencies induced by the AdC68gDNP vaccine were higher than those induced by the AdC68NP vaccine in aged mice; they were also higher than those induced by AdC68NP vaccine in young mice and this difference reached statistical significance as of week 4 after immunization. As shown in Fig. 1, aged mice have significantly lower counts of CD8$^+$ T cell in blood compared with young mice, and a comparison of frequencies of a specific CD8$^+$ T cell subset may thus be misleading. We therefore calculated numbers of NP-specific CD8$^+$ T cells per $10^7$ PBMCs (Fig. 3B). Absolute numbers of NP-specific CD8$^+$ T cells mirrored their frequencies and again in both young and aged mice the AdC68gDNP vaccine induced a more robust response at all time points tested compared with the AdC68NP vaccine.
To ensure that the NP-specific CD8+ T cell response induced by the vectors was driven by the immunodominant peptide within NP, we repeated the experiment in young mice and included a group that was immunized with the AdC68gD vector. Frequencies of NP-specific CD8+ T cells were monitored by tet staining or ICS from blood for a period of 3 mo. Naive mice were tested in parallel. At all of the time points tested, NP-specific CD8+ T cells could be detected in mice immunized with the AdC68NP or AdC68gDNP vector. Mice immunized with the AdC68gD vector showed background frequencies of NP-specific CD8+ T cells that were comparable to those in naive mice (data not shown).

The vaccine-induced response showed a high degree of variability in aged mice with frequencies of NP-specific CD8+ T cells ranging from <0.1%, which is comparable to preimmunization frequencies, to >20%. To determine whether inclusion of gD into the vaccine to NP affected the percentages of mice that developed a response on vaccination, we calculated how many mice in the four different vaccine groups were non- or low responders, that is, developed frequencies of NP-specific CD8+ T cells <1% (Fig. 3C). By 4 wk after vaccination, all of the young mice immunized with either of the two vaccines had frequencies of NP-specific CD8+ T cells >1%. In contrast, >30% of the aged mice vaccinated with the AdC68NP

FIGURE 3. CD8+ T cell responses to NP in young and aged mice vaccinated with the AdC68gDNP or AdC68NP vaccine. Groups of young and old mice were vaccinated with 10^10 vps of AdC68NP or AdC68gDNP vectors. They were bled at different times there after to measure CD8+ T cell responses to NP by tet staining. The data shown reflect results that were combined from several experiments. A, Percentages of NP-specific CD8+ T cells within 10^7 PBMCs. C, Percentages of mice in the four different groups that had frequencies of NP-specific CD8+ T cells >1% over all CD8+ T cells at 4 wk or 3 mo after immunization. D, Percentages of mice in the four groups that had percentages of NP-specific CD8+ T cells >10% over all CD8+ T cells at 4 wk and 3 mo after immunization. E, Average frequencies of NP-specific CD8+ T cells (± SD) in different tissues of vaccinated young and aged mice are shown. The numbers within the graphs show the p value (by two-tailed Student t test) of differences between mice of a given age group vaccinated with AdC68NP or AdC68gDNP.
vaccine scored as low/nonresponders. Inclusion of gD reduced this percentage to ∼17%. By 3 mo after vaccination, frequencies of NP-specific CD8+ T cells remained >1% in all of the young AdC68gDNP vaccinated mice but had declined to <1% in ∼15% of AdC68NP vaccinated young mice. In aged AdC68gDNP vaccinated mice, percentages of low/nonresponders slightly decreased by month 3 in comparison with week 4, whereas they increased to >50% in AdC68NP vaccinated aged mice. A comparison of mice that developed high frequencies (>10%) of NP-specific CD8+ T cells (Fig. 3D) showed that at 4 wk after immunization >50% of young and aged mice immunized with the AdC68gDNP vaccine showed such high responses. Only 25% of young mice and <5% of aged mice immunized with the AdC68NP developed frequencies of NP-specific CD8+ T cells >10%. By 3 mo after immunization, percentages of high responders declined in most groups and this decline was especially pronounced in aged mice vaccinated with the AdC68gDNP vaccine, which at this time point became comparable to the AdC68NP vaccinated old group. Percentages of high responders remained >25% in the AdC68gDNP vaccinated young group. Overall, these results show that inclusion of gD into the vaccine not only augments mean frequencies and numbers of NP-specific CD8+ T cells in aged mice but also reduces the percentages of low/nonresponders.

To ensure that the observed increase of NP-specific CD8+ T cells was not just restricted to blood, we primed young and aged mice with the AdC68NP or AdC68gDNP vectors and analyzed NP-specific CD8+ T cell frequencies from spleen, lungs, and liver (Fig. 3E). At 4 wk after vaccination, AdC68gDNP vaccinated young and aged mice showed significantly higher frequencies of NP-specific CD8+ T cells in lung and spleen. The difference did not reach significance in livers (only tested for aged mice) due to very high variability of frequencies in this compartment.

Cytokine secretion by young and aged NP-specific CD8+ T cells
To determine the functionality of NP-specific CD8+ T cells in young and aged mice and to assess if inclusion of gD into the immunogen affected T cell functionality, blood-derived CD8+ T cells from mice immunized once 3 mo previously with 1010 vps of the AdC68NP or AdC68gDNP vaccine were tested for cytokine production, namely, IFN-γ, TNF-α, and IL-2, on in vitro stimulation with the NP peptide. As shown in Fig. 4A, >50% of NP-reactive CD8+ T cells from young mice produced IFN-γ and ∼30% produced IFN-γ together with TNF-α. Cells that produced all three cytokines or IL-2 in combination with TNF-α or IFN-γ were rare. NP-specific CD8+ T cells from mice immunized with the AdC68gDNP vaccine showed an increase in cells producing IFN-γ and TNF-α and a decrease in those producing IFN-γ only. Frequencies of NP-specific CD8+ T cells producing other combinations of cytokines were similar to those in young mice immunized with the AdC68NP vaccine. NP-specific CD8+ T cells from aged mice produced, at equal ratios, IFN-γ alone or IFN-γ together with TNF-α. The proportions of NP-specific CD8+ T cells producing all three cytokines or TNF-α only were higher in aged than in young mice immunized with the AdC68NP vector although this difference failed to reach statistical significance. Absolute percentages of CD8+ T cells producing cytokines in response to NP were higher in young and aged mice immunized with the AdC68gDNP vector due to increases in CD8+ T cells producing IFN-γ and TNF-α and, to a lesser extent, in those producing IFN-γ only (Fig. 4B). It should be pointed out that frequencies of NP-specific CD8+ T cells measured by ICS were well below those detected by tet staining. This could reflect that a large portion of tet+CD8+ T cells lacked functions or equally likely that they had functions that were not tested for. Notwithstanding, proportions of tet/cytokine+ CD8+ T cells were

FIGURE 4. Cytokine production by NP-specific CD8+ T cells. Young and old mice were vaccinated with 1010 vps of AdC68NP or AdC68gDNP vector. Three months later, PBMCs were tested for production of IFN-γ (G), IL-2 (I), and/or TNF-α (T) by ICS. A, Boolean combinations of G, I, and T for the four groups are shown. The gates show the mean distribution of populations with distinct functionalities. B, Percentages are shown of CD8+ T cells over all CD8+ T cells producing the different functions in response to the NP peptide. Closed black squares show results for individual mice, red X shows averages. C, The GMFI ± SD for the intracellular stains for IFN-γ and TNF-α.
Cytokines were higher in NP-specific CD8+ T cells from young and aged mice. Levels of the predominant cytokines (i.e., IFN-γ and TNF-α) produced per cell were determined by measuring the geometric mean fluorescent intensity (GMFI) of the dyes labeled to the matching Abs (Fig. 4C). Average GMFI for both cytokines were higher in NP-specific CD8+ T cells from young and aged mice immunized with AdC68gDNP compared with the AdC68NP vaccine; nevertheless these differences did not reach statistical significance on multivariate adjustments of p values.

Overall, these data show that both vaccines induced CD8+ T cells with similar cytokine profiles in young and aged mice; differences between groups were overall subtle.

**In vivo lysis by young and aged NP-specific CD8+ T cells**

To further determine whether CD8+ T cells induced by the gD-expressing vaccine remain functional, we tested for their capacity to lyse target cells in vivo. To this end old and young mice were vaccinated with AdC68gD, AdC68NP, or AdC68gDNP given at $10^{10}$ vps per mouse. Fourteen days later, mice were injected with CFSE-labeled target cells and the degree of their lysis was assessed 20 and 24 h later (Fig. 5). At the earlier time point, we tested for target cell lysis by analyzing loss of CFSE+ NP-peptide pulsed cells in pooled PBMCs. At this time the highest degree of lysis was observed in young AdC68gDNP immunized mice, followed by old mice vaccinated with the same construct. In either age group, mice immunized with AdC68NP exhibited less lysis of the NP peptide-pulsed target cells. By 24 h after transfer, young mice immunized with either vaccine and old mice vaccinated with AdC68gDNP showed near complete lysis of the NP peptide-pulsed cells, whereas aged mice vaccinated with the AdC68NP vector showed significantly lower levels of lysis compared with AdC68gDNP vaccinated old mice. In parallel, we tested splenocytes for numbers of NP-tet+ CD8+ T cells. In either age group, mice that had been immunized with AdC68gDNP had higher numbers of NP-specific CD8+ T cells compared with those vaccinated with the AdC68NP vaccine. Aged mice had slightly higher numbers of specific CD8+ T cells in their spleens compared with young mice, but nevertheless showed at least for the AdC68NP-vaccinated group significantly less lysis indicating an age-associated defect in the lytic capacity of aged CD8+ T cells as has been reported previously (41, 42).

**FIGURE 5. In vivo lysis.** Groups of three young and old mice were vaccinated with $10^{10}$ vps of AdC68gD (controls), AdC68NP or AdC68gDNP vector. Two weeks later, mice were injected with CFSE-labeled splenocytes pulsed with the NP peptide or as a control with SIINFEKL. A, Mice were bled 20 h later to assess loss of NP-pulsed CFSE-labeled cells from pooled blood samples. Mice were then euthanized and the same measurements were taken from spleens of individual mice (black bars). The latter assay allowed to assess differences by two-tailed t test and p values are shown above the graphs. B, Frequencies of NP-specific CD8+ T cells over all CD8+ T cells were determined in parallel by staining spleen samples with the NP-tet and Abs to CD8 and CD44. The graph shows average numbers of NP-specific CD8+ T cells ± SD in spleens of the different groups.

**The effect of a booster immunization on NP-specific CD8+ T cells from young and old mice primed with the AdC68NP or AdC68gDNP vaccine**

To assess if mice primed with the AdC68gDNP vaccine continued to have higher frequencies of NP-specific CD8+ T cells after a booster immunization, young and old mice were vaccinated with $10^{10}$ vps of the AdC68NP or AdC68gDNP, 3 mo later vaccinated mice as well as age-matched naive mice were boosted with a sublethal dose of A/PR8 virus. Frequencies and numbers of NP-specific CD8+ T cells were monitored from blood before and at three time points after the boost (Fig. 6). At the time of euthanasia, namely, 20 d after the boost, frequencies as well as numbers of NP-specific CD8+ T cells were in addition measured from spleens, lungs, and livers. Shortly before booster immunization, young and old mice vaccinated with the AdC68gDNP vaccine had significantly higher frequencies and numbers of NP-specific CD8+ T cells in blood compared with age-matched AdC68NP-vaccinated mice. By day 20 after challenge, frequencies of NP-specific CD8+ T cells were again significantly higher in both age groups primed with the AdC68gDNP vector, whereas numbers of NP-specific CD8+ T cells in blood were only significantly higher in the aged mice. The same trend was seen in other tissues, namely, spleens, lungs, and livers where at the time of euthanasia the aged mice showed significantly higher frequencies of NP-specific CD8+ T cells on AdC68gDNP vaccination. This difference did not reach significant in young mice. Overall the results show that inclusion of gD into the vaccine did not impair the primed CD8+ T cells’ ability to expand up antigenic re-exposure.

**Phenotypes of NP-specific CD8+ T cells in young and aged mice**

We tested for differences in NP-specific CD8+ T cell expression levels of PD-1, BTLA, and CD160 in young and old mice immunized with the AdC68NP or AdC68gDNP vectors. CD44low CD8+ T cells from age-matched naive mice were analyzed in parallel and those from young mice are shown in Fig. 7A. By 2 wk after immunization, NP-specific CD8+ T cells from young mice immunized with either AdC68NP or AdC68gDNP showed a reduction in PD-1 expression relative to naive CD8+ T cells. PD-1 was slightly lower on NP-specific CD8+ T cells from the aged mice and this difference was statistically significant ($p = 0.034$ after multivariate adjustment) for mice immunized with the AdC68gDNP vector. NP-specific CD8+ T cells of all 4 vaccine groups, that is, young and old immunized with AdC68NP or AdC68gDNP showed a reduction in BTLA expression by 2 wk after immunization and there was no marked difference between the groups. CD160 remained unchanged on NP-specific CD8+ T cells from young mice but was reduced on those from aged mice and the difference in CD160 expression between young and aged mice was statistically significant for mice immunized with AdC68gDNP ($p = 0.043$ after multivariate adjustment). These data show that age as well as the inclusion of gD into the vaccine affects expression levels of immunoinhibitors on vaccine-induced CD8+ T cells. A shift in kinetics of Ag-driven activation of CD8+ T cells may have contributed to the observed differences and we therefore repeated the...
experiment at 8 wk after vaccination (Fig. 7B). In young mice, PD-1 was, as had been seen earlier, markedly lower on NP-specific than on naive young CD8+ T cells. Comparing the two young vaccine groups, PD-1 levels were higher on NP-specific CD8+ T cells from the young AdC68NP group. Nevertheless, this difference failed to reach statistical significance after multivariate correction of $p$ values ($p = 0.12$). Comparing age groups, PD-1 levels at this time point were significantly lower on young NP-specific CD8+ T cells from mice immunized with the AdC68NP vaccine compared with NP-specific CD8+ T cells from the corresponding old mouse group ($p = 0.0081$ after multivariate adjustment). This difference was not seen in mice immunized with the AdC68gDNP vaccine. BTLA was expressed in both young and aged mice at reduced levels on NP-specific CD8+ T cells and this reduction was more pronounced on those from aged mice vaccinated with the AdC68gDNP vector. Levels of CD160 were significantly lower on NP-specific CD8+ T cells from aged mice immunized with the AdC68NP vaccine than on those from aged mice immunized with AdC8gDNP ($p = 0.00072$ after multivariate adjustment). Comparing expression levels of CD160 between CD8+ T cells from young and aged mice showed that aged mice vaccinated with AdC68NP had significantly higher levels of CD160 compared with those from young mice.

**FIGURE 6.** Expansion of NP-specific CD8+ T cells after prime-boost immunization. Young and old mice were vaccinated with $10^{10}$ vps of AdC68NP or AdC68gDNP vector. Three months later, they were challenged intranasally with a subethal dose of A/PR8 virus. Frequencies of NP-specific CD8+ T cells were measured from blood 2 and 9 wk after priming, at 2 d before the challenge (D-2) and at 5, 8, and 20 d after challenge (Dx PC) by tet staining. The graph in A shows average frequencies in blood, the graph in B shows numbers of NP-specific CD8+ T cells per $10^7$ PBMCs. The $p$ values (by Student $t$ test) for significance of differences between the two vaccine groups are shown underneath the graphs. At the time of euthanasia on day 20 after the boost, lymphocytes isolated from spleens, lungs, and livers were tested for frequencies (C) and numbers (D) of NP-specific CD8+ T cells by tet staining. The $p$ values (by Student $t$ test) for significance of differences between the two vaccine groups are shown within the graphs.
(p = 0.00042 after multivariate adjustment). Overall differences of the expression levels of the immunoinhibitors were subtle (<10-fold) between the groups.

Discussion

Influenza is one of the top 10 causes of death in older adults with an average annual death toll of ~44,000 in the United States (43). Although a trivalent inactivated influenza vaccine is commercially available and recommended for use in the elderly, its efficacy in reducing influenza-related morbidity and mortality is still debated (44–47). Neutralizing Abs to the viral hemagglutinin and to a lesser extent the neuraminidase are assumed to protect against influenza in trivalent inactivated influenza-vaccinated individuals; protection is specific for a virus subtype and not effective against antigenic shift or drift strains.

Other more cross-reactive immune responses, such as CD8+ T cells, may provide resistance to influenza virus infections in humans, as shown by epidemiological studies, and trials are under way to develop so-called universal influenza A vaccines based on conserved viral proteins, such as the matrix 2 protein ectodomain for induction of non-neutralizing, protective Abs and the NP for induction of CD8+ T cells. To what degree cross-reactive immune responses, such as CD8+ T cells, may provide resistance to influenza A virus infection in the aged or rather exacerbate disease by causing immunopathology remains to be established.

A number of studies have shown that CD8+ T cell responses in the aged are impaired both in humans and mice. Although some studies suggest that the age-associated defects in CD8+ T cell responses relate to impaired Ag-presentation (48, 49), hypofunctionality of CD4+ T cells (50, 51) or loss of naive T cells after thymic involution (40, 52) and a decline in the proliferative capacity of hematopoietic stem cells (53), other studies suggest an intrinsic defect in aged CD8+ T cells (54) potentially by cell senescence and its associated changes in the cells’ genome and epigenome. Immunoinhibitory pathways may become more predominant on aging. In humans, frequencies of circulating CD4+ and CD8+ Tregs increase (55) and aged Ag-experienced CD4+ T cells express increased levels of immunoinhibitory molecules, such as PD-1 and CTLA-4 (56). Notwithstanding, autoimmunity is more common in the aged, arguing against an increase in immunoinhibition on immunosenescence.

To explore if manipulation of an immunoinhibitory pathway could augment a vaccine-induced CD8+ T cell response in aged mice, we tested AdC68 vectors expressing the NP of influenza virus either in its native form or fused to the C terminus of HSV-1 gD. HSV-1 gD binds close to the BTLA/CD160 binding sites on HVEM thus interrupting these immunoinhibitory pathways (33). BTLA, unlike other immunoinhibitors, such as PD-1, is expressed on naive T cells and thus presumably regulates adaptive immune responses at an early stage after activation. In addition, BTLA plays a role in the development of memory and regulates homeostatic CD8+ T cell proliferation (57). BTLA also contributes to the control of early innate immune responses and mice that lack BTLA develop an enhanced cytokine response by NKT cells (58, 59). HVEM on Tregs and BTLA on effector T cells allow for their interactions (35) and again gD would be expected to block Treg-mediated suppression through direct cell to cell contact. The role of CD8+ T cell regulation of CD8+ T cell differentiation is not well characterized but available studies suggest that it plays an immunoinhibitory role (28) and that is becomes upregulated on CD8+ T cells that progress toward exhaustion (60).

Our initial data show that on Ag-experienced and naive circulating CD8+ T cells of aging mice levels of BTLA and CD160 remain unchanged, whereas PD-1 expression is reduced. Although a simple analysis of expression levels does not permit assessment of potential differences in downstream signaling mechanisms, it nevertheless suggests that these pathways may remain relatively intact in CD8+ T cells from the aged. This was confirmed by functional studies, which showed that gD-mediated blockade of HVEM–BTLA/CD160 interactions augments a primary CD8+ T cell response in both young and aged mice. Inclusion of gD into the immunogen does not appear to affect the kinetics of the response in young and aged mice, as absolute numbers of NP-specific circulating CD8+ T cells (Fig. 3A, 3B) peaked around week 2–4 after vaccination with either of the two vaccines, nor does gD affect the longevity of the CD8+ T cell response. Ad vectors persist at low levels in activated T cells and remain transcriptionally active. The continued presence of Ag, albeit at low levels, maintains high frequencies of activated Ad transgene product-specific CD8+ T cells that commonly show no or only a marginal decline in numbers once the bulk of infected cells has been eliminated. This was again observed here in young mice that had stable numbers of circulating NP-specific CD8+ T cells in blood between 2 and 14 wk, although frequencies declined. In aged mice, in contrast, frequencies and absolute numbers of NP-specific CD8+ T cells in blood started to decrease after week 4 after vaccination, suggesting that CD8+ T cell responses cannot be maintained in the aged. Thus, although gD, presumably through blockade of HVEM-associated immunoinhibitory pathways increases the magnitude of Ag-driven CD8+ T cell responses in aged mice, it fails to reduce their attrition.

BTLA has been shown to decrease differentiation of CD8+ T cells into central memory cells. CD8+ T cells induced by the transgene product of an Ad vector only very gradually transition into central memory and by 3 mo after vaccination, the latest time point that we tested, >95% of NP-specific CD8+ T cells from AdC68NP or
AdC68gDNP vaccinated young and old mice remain CD62L<sup>low</sup> and CD127<sup>lo</sup> (data not shown) as is typical for effector-like CD8<sup>+</sup> T cells, indicating that neither age nor blockade of the HVEM-BTLA/CD160 pathways affects the continuous activation of CD8<sup>+</sup> T cells through Ag persisting at low levels. Cytokine secretion pattern are also similar in young and aged mice immunized with AdC68NP or AdC68gDNP vectors, indicating that aged CD8<sup>+</sup> T cells induced by the AdC68 vector are capable of producing a spectrum of cytokines similar to that produced by young CD8<sup>+</sup> T cells and that inclusion of gD into an immunogen has no major effect on CD8<sup>+</sup> T cell functionality. On a per cell basis, CD8<sup>+</sup> T cells from aged mice produce slightly higher levels of IFN-γ than those from young mice. CD8<sup>+</sup> T cells from aged or young mice vaccinated with the AdC68gDNP vaccine produce higher levels of IFN-γ and TNF-α compared with those from mice immunized with the AdC68NP vector. However, these differences are subtle and fail to reach statistical significance. CD8<sup>+</sup> T cell-mediated cytolyis was higher in both young and aged mice vaccinated with the AdC68gDNP vector compared with those that had received the AdC68NP vector indicating again that NP-specific CD8<sup>+</sup> T cells induced in presence of gD were functional. However, it should be pointed out that comparing numbers of NP-specific CD8<sup>+</sup> T cells in spleens to the overall levels of target cell lysis, NP-specific CD8<sup>+</sup> T cell from young mice seemed to be more lytic compared with those from the aged, a defect that has been reported previously (41), and this was not corrected by inclusion of gD into the vaccine. Vaccine-induced NP-specific CD8<sup>+</sup> T cells expanded on a booster immunization and by day 20 in aged mice their frequencies and numbers were higher in those that had been primed with the AdC68gDNP vector. Expansion of NP-specific CD8<sup>+</sup> T cells in aged mice appeared to be delayed however, compared with that of young mice, which showed a robust increase earlier, namely, by day 8 after the boost. This apparent defect in rapid expansion of CD8<sup>+</sup> T cells confirms previous results which showed reduced proliferation of naive and memory mouse CD8<sup>+</sup> T cells in response to Ag or mitogen (14, 60, 61) As remains to be investigated this albeit slight delay in expansion may nevertheless have profound effects of the aged CD8<sup>+</sup> T cells’ ability to provide resistance to a rapidly proliferating pathogen, such as influenza virus.

We analyzed expression levels of immunoinhibitory markers on vaccine induced CD8<sup>+</sup> T cells from young and aged mice to determine whether aged CD8<sup>+</sup> T cells are more prone to differentiate toward exhaustion, as would be indicated by an increase in expression of PD-1 and potentially CD160, and to furthermore test if expression of these markers is affected by inclusion of gD into the immunogen. At 2 mo after vaccination, levels of all 3 immunoinhibitors are higher on AdC68NP-induced NP-specific CD8<sup>+</sup> T cells from aged mice in comparison with those on the corresponding populations from young mice. Differences are subtle but reach statistical significance for PD-1 and CD160, two markers that have been linked to immunological exhaustion.

This relative age-associated increase in immunoinhibitors is not seen on NP-specific CD8<sup>+</sup> T cells from young and aged mice vaccinated with the AdC68gDNP vaccine. If indeed the marginally higher levels of immunoinhibitors on NP-specific CD8<sup>+</sup> T cells from AdC68NP vaccinated aged mice are biologically meaningful and signal their differentiation toward functional impairment, which in turn might be prevented by gD, remains to be investigated in more depth.

In summary, our studies show that inclusion of gD into a vaccine designed to induce a CD8<sup>+</sup> T cell response to a conserved Ag of a common pathogen can augment a primary CD8<sup>+</sup> T cell response in aged mice. As the HVEM-BTLA/CD160 pathway is highly conserved between mice and humans, we would expect an equal benefit in humans. Humans naturally infected with HSV-1 encounter gD, and the protein, which has no known toxicity in humans, can induce an Ab response. As we reported previously, the enhanced immune response elicited in mice by vaccines expressing an Ag fused to gD is not inhibited by pre-existing immunity to HSV-1 gD (62). One clear advantage of enhancing a vaccine driven immune response by blocking an immunoinhibitory pathway by an antagonist that is directly encoded by the vaccine is that the effect will be localized, and is thus not expected to have global effects on the immune system, although this has to be studied in more detail in appropriate models of autoimmune prone mice. This is in contrast to inhibition of regulatory pathways by Abs, as is being explored for blockade of Treg or the PD1-PDL1/L2 pathways in cancer or chronic infections, which especially in vulnerable populations, such as the aged, may carry the risk of promoting autoimmune reactions.

Acknowledgments
We thank C. Barth and C. Cole for assisting in preparation of the manuscript. We also thank Drs. G. Freeman, A. Sharp, W. Weninger, and E.J. Wherry for helpful discussion.

Disclosures
H.C.J.E. and M.O.L. have pending patents for the use of gD in vaccines. H.C.J.E. has a pending patent for the AdC68 vector.

References