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*J Immunol* 2013; 190:3854-3858; Prepublished online 6 March 2013; doi: 10.4049/jimmunol.1202790

http://www.jimmunol.org/content/190/8/3854

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/03/07/jimmunol.1202790.DC1

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Cutting Edge: Rapid Boosting of Cross-Reactive Memory CD8 T Cells Broadens the Protective Capacity of the Flumist Vaccine

Bram Slüetter,* Lecia L. Pewe,* Peter Lauer,† and John T. Harty*‡,§

Memory CD8 T cells recognizing conserved proteins from influenza A virus (IAV), such as nucleoprotein, have the potential to provide protection in individuals who lack the proper neutralizing Abs. In this study, we show that the most potent CD8 T cell–inducing influenza vaccine on the market (Flumist) does not induce sufficient numbers of cross-reactive CD8 T cells to provide substantial protection against lethal nonhomologous IAV challenge. However, Flumist-primed CD8 T cells rapidly acquire memory characteristics and can respond to short-interval boosting to greatly enlarge the IAV-specific memory pool, which is sufficient to protect mice from nonhomologous IAV challenge. Thus, a current vaccine strategy, Flumist, may serve as a priming platform for the rapid induction of large numbers of memory CD8 T cells with the capacity for broad protection against influenza. The Journal of Immunology, 2013, 190: 3854–3858.

Despite the availability of a seasonal vaccine, influenza A virus (IAV) continues to be a heavy burden on society and healthcare, infecting between 2 and 10% of the North American population and causing up to 500,000 annual deaths worldwide (1). A major reason for the limited effectiveness of the vaccine is the high rate of mutation in the IAV hemagglutinin (HA) and neuraminidase (NA) proteins. This results in rapidly decreasing protection by neutralizing Abs induced by prior seasonal vaccines (2). Therefore, a vaccine that protects against a wide variety of IAV subtypes (heterosubtypic immunity [HI]) would be highly desirable. In contrast to the sequence variations in IAV surface proteins HA and NA, which are selected by the immunological pressure of neutralizing Abs, internal viral components, like nucleoprotein (NP) and matrix protein 1/2, are remarkably conserved over a wide range of subtypes (3). Therefore, in the absence of neutralizing Abs, NP-specific memory CD8 T cells may control IAV, thereby mitigating disease symptoms and providing a first line of defense against a possible influenza pandemic. The relatively new (9 y on the market) cold-adapted live attenuated nasal influenza vaccine, Flumist, induces higher CD8 T cell responses than do the injectable IAV vaccines (4); therefore, it has been speculated to provide HI (5). It is unknown whether Flumist vaccination induces sufficient cross-reactive memory CD8 T cells to provide resistance to nonhomologous IAV infection or whether multiple Flumist vaccinations increase the number of these broadly protective memory CD8 T cells. In this study, we address the cross-protective potential of memory CD8 T cells induced by Flumist immunization and show that specifically enhancing cross-reactive CD8 T cells through heterologous boosting of Flumist-immune hosts provides a simple and potentially translational tool to broaden the protective capacity of this licensed vaccine.

Materials and Methods

Mice

Female BALB/c mice were acquired from the National Cancer Institute and housed under pathogen-free conditions. Postinfection (p.i.) mice were transferred to Biosafety level 2 housing. All animal studies and procedures were approved by the University of Iowa Animal Care and Use Committee, under Public Health Service assurance, Office of Laboratory Animal Welfare guidelines.

Immunization and challenges

Attenuated actA/inlB double-deficient Listeria monocytogenes expressing A/PuertoRico/8/34 (PR8) NP (LM-NP) was generated by Aduro BioTech, using the methodology described (6). Vaccinia virus (VV) expressing NP (VV-NP) was a gift from Dr. J. Bennink (National Institutes of Health, Bethesda MD). rNP was purchased from ImmuneTech (New York, NY). Flumist (MedImmune, Gaithersburg, MD) was purchased from the University of Iowa Hospital pharmacy. A total of 5 μl undiluted Flumist was introduced into each nostril while the mouse was conscious to ensure that the vaccine did not reach the lower respiratory tract (7). PR8 (H1N1) influenza virus was grown in chicken eggs, as described (8). Mice were challenged with a 10× LD50 in 50 μl PBS (2×105 TCID50), referred to as lethal dose throughout the article, while lightly anesthetized with isoflurane. Body weight was monitored daily, and mice were euthanized when they had lost 30% of their starting weight, in accordance with Institutional Animal Care and Use Committee guidelines.

The online version of this article contains supplemental material.

Abbreviations used in this article: HA, hemagglutinin; HI, heterosubtypic immunity; IAV, influenza A virus; LM-NP, Listeria monocytogenes expressing PR8 nucleoprotein; NA, neuraminidase; NP, nucleoprotein; p.i., postinfection; PR8, A/PuertoRico/8/34; VV, vaccinia virus; VV-NP, vaccinia virus expressing nucleoprotein.

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**Viral titers**

At the designated time points, infected mice were euthanized, and lungs were homogenized in 2 ml DMEM and stored at −80°C until further analysis. Serial dilutions of lung homogenates were co-cultured in 96-well plates with 1 × 10^5 MDCK cells/well and incubated at 37°C and 5% CO2. The next day, medium was replaced with supplemented DMEM containing 0.001% trypsin and incubated for an additional 72 h. To assess hemagglutination, supernatants were mixed with 0.5% v/v chicken RBCs suspended in PBS and incubated for an additional 72 h. To assess hemagglutination, supernatants were mixed with 0.5% v/v chicken RBCs suspended in PBS and incubated for an additional 72 h.

**Statistics**

Unless indicated otherwise, significance was calculated by one-way ANOVA with the Bonferroni posttest, using GraphPad Prism 4 for Macintosh. The p values <0.05 were considered significant.

**Results**

**Vaccination with Flumist does not induce sufficient numbers of memory CD8 T cells to protect mice from lethal heterosubtypic influenza challenge**

The live attenuated Flumist vaccine was reported to generate larger CD8 T cell responses compared with injectable inactivated or subunit vaccines (4, 5) and, therefore, could be considered the most potent and translatable current strategy to induce IAV-specific memory CD8 T cells. In line with this, nasal immunization of BALB/c mice with Flumist induced a detectable NP147-specific CD8 T cell response, reaching 1.5% of the circulating CD8 T cells at 8 d postimmunization. However, within 30 d, NP147-specific CD8 T cells in the circulation contracted to frequencies indistinguishable from naive mice by tetramer analysis (Fig. 1A). Interestingly, although the 2010/2011 Flumist contains an H1N1 (A/California/7/2009) IAV strain that was reported to induce neutralizing Abs against homologous challenge (9), serum from mice immunized with Flumist 40 d earlier did not contain any measurable neutralizing activity against PR8, also an H1N1 strain (Fig. 1B). In contrast, serum from mice that had recovered from a sublethal PR8 challenge exhibited substantial neutralization activity against PR8 (Fig. 1B, p < 0.05). Therefore, PR8 challenge of Flumist-immunized mice is similar to a scenario in which individuals are infected with an IAV strain that has undergone antigenic drift and is no longer subjected to neutralization by Abs provided by a prior infection or the seasonal IAV vaccination. To assess whether the CD8 T cells induced by vaccination with Flumist provide protection in this scenario, mice were challenged with a lethal dose of PR8 40 d after immunization with Flumist. No decrease in peak viral titers (p = 0.13, Fig. 1C) and no significant increase in survival was observed (p = 0.14, Fig. 1D) in Flumist-vaccinated mice. Some of the Flumist-immune mice started to recover by day 8 p.i., whereas unimmunized mice uniformly suffered from additional weight loss until day 10 p.i. (Fig. 1D), suggesting that Flumist vaccination provides some immunity to PR8. Recovery coincided with a substantial increase in the percentage of circulating NP147-specific CD8 T cells at day 7 p.i., which was indicative of a potent secondary expansion of these cells following PR8 challenge of Flumist-immune mice (Fig. 1E). Moreover, the limited protection that Flumist provides is further reduced by depletion of CD8 T cells prior to PR8 infection (Supplemental Fig. 1). Thus, immunization with Flumist results in a cross-reactive memory CD8 T cell population; however, this population seems numerically insufficient to control a nonhomologous IAV infection during the first days p.i. and takes ≥7 d to expand into numbers sufficient to limit disease in a fraction of immunized mice.

**Boosting can augment the Flumist-induced CD8 T cell response to provide protection against nonhomologous IAV challenge**

Our data suggest that the numbers of IAV-specific memory CD8 T cells induced by Flumist vaccination are not sufficient to provide substantial protection against a nonhomologous challenge. However, prime-boost strategies have been applied successfully in mice (10, 11) to induce large CD8 T cell responses and are currently being evaluated in primates (12). Because Flumist is administered annually, some individuals might have received prime-boost immunizations, but it is not known whether the number of NP-specific memory CD8 T cells increases upon Flumist boosting. To address this question, mice received a second Flumist immunization 7 mo after their first vaccination. An increase in the frequency of circulating NP147-specific CD8 T cells was observed shortly after boost (Fig. 2A), suggesting that pre-existing neutralizing Abs induced by the first immunization did not completely prevent nasal infection by Flumist. However, the magnitude of the secondary response did not exceed the primary response to Flumist, and no significant increase in the numbers of NP147-specific memory CD8 T cells was observed. Therefore, these data suggest that annual homologous boosting with Flumist is not an effective way to increase the IAV-specific memory CD8 T cell population.

To circumvent potential neutralizing Abs, we asked whether boosting with a potent heterologous vector would effectively expand the Flumist-primed NP147-specific CD8 T cell population. *actAlinB* double-deficient *L. monocytogenes* are currently under clinical development (13) as a potent inducer of CD8 T cell responses. When BALB/c mice were primed with Flumist and boosted 6 mo later with *actAlinB* double-
deficient LM-NP, a remarkable expansion in NP147-specific CD8 T cells was observed that established a memory population exceeding 20% of all circulating CD8 T cells (Fig. 2B). Upon challenge with a lethal dose of PR8 2 mo post-boost (day 232), prime-boosted mice showed little weight loss (Fig. 2C) and a 1000-fold reduction in viral titers 4 d p.i. (p, 0.0001, Fig. 2D) compared with mice that were primed only with Flumist. Interestingly, boosting Flumist-primed mice at this time point with an experimental subunit vaccine, rIAV NP combined with potent adjuvants [TLR3 agonist polyinosinic-polycytidylic acid (PolyIC) and anti-CD40 (14)], also resulted in robust expansion of NP147-specific CD8 T cells and the establishment of large populations of memory CD8 T cells (Fig. 2E). Thus, specifically enlarging the cross-reactive memory CD8 population by means of a live attenuated booster agent or a strongly adjuvanted subunit vaccine targeting conserved Ags appears to be an effective way of improving the protective potential of Flumist.

Flumist-induced CD8 T cell response can be boosted rapidly to provide protection against nonhomologous IAV challenge

LM-NP and Flumist prime a similar-sized NP147-specific CD8 T cell response in naive animals (1.5% NP147 specific, Fig. 2B). Nonetheless, only LM-NP is capable of boosting NP147-specific memory CD8 T cells in Flumist-primed mice. Inducing strong inflammation is an important requisite of an effective booster agent (15), because signal 3 cytokines, such as type I IFN and IL-12, cause additional accumulation of effector CD8 T cells (16). Although Flumist consists of a live replicating virus, the lack of boosting by reimmunization with Flumist may suggest that it does not induce strong inflammation. Consistent with this suggestion, although LM-NP elicited primarily KLRG1hi NP147-specific effector CD8 T cells, Flumist infection of naive mice induced primarily KLRG1low NP147-specific effector CD8 T cells (Fig. 3A), which suggests that the latter cells result from priming under low inflammatory conditions (17). Interestingly, a large representation of KLRG1low NP147-specific CD8 T cells 8 d post-Flumist vaccination also suggests that this population primarily consists of CD8 T cells with early memory characteristics (18). This could mean that CD8 T cells induced by immunization with Flumist may be boostable shortly after priming.

FIGURE 2. Heterologous boosting of Flumist-immunized mice 6–7 mo after prime induces massive expansion of NP147-specific CD8 T cells and robust protection against lethal PR8 challenge. BALB/c mice were primed with Flumist and were boosted after 225 d with Flumist (A) or after 179 d with LM-NP (107 CFU) (B). The NP147-specific CD8 T cell response was tracked over time in the blood. Arrow indicates time of boost with Flumist (A) or LM-NP (B). Flumist-primed mice were challenged with a lethal dose of PR8 45 d post-LM-NP boost, and morbidity (C) and lung viral titers on day 4 p.i. (D) were determined. (E) BALB/c mice were primed with Flumist and were boosted after 210 d with 50 µg rNP adjuvanted with 50 or 10 µg anti-CD40. The NP147-specific CD8 T cell response was tracked over time in the blood. *p < 0.05.

FIGURE 3. Flumist-primed NP147-specific CD8 effector T cells have an early memory phenotype and can be used in accelerated prime boost to generate protective immunity. (A) Representative flow plot of expression of KLRG1 on KdNP147+ CD8 T cells from blood 8 d after nasal immunization with LM-NP or Flumist. (B) Naive mice and mice primed with Flumist 7 d earlier were immunized with 107 CFU LM-NP (LM-NP and Flumist+LM-NP, respectively) or were left unboosted (Flumist). On day 50 post–LM-NP boost, mice were challenged with a lethal dose of PR8, and morbidity (C) and lung viral titers on day 4 p.i. (D) were determined. (E) Flumist-primed mice were boosted 7 d later with Flumist or 5 × 106 PFU VV-NP. *p < 0.05.
Indeed, similar to boosting after 7 mo, boosting Flumist-primed CD8 T cells with LM-NP 7 d postprime resulted in robust expansion of NP147-specific memory CD8 T cells and the establishment of a large NP147-specific memory population (Fig. 3B). In contrast, mice that received LM-NP without being primed with Flumist failed to establish a detectable NP147-specific memory CD8 T cell population (Fig. 3B). When challenged with a lethal dose of PR8 at a memory time point, Flumist-primed LM-NP–boosted mice rapidly controlled the IAV infection, exhibiting less morbidity (Fig. 3C) and significantly lower lung viral titers on day 4 p.i. compared with naïve mice or mice that only received Flumist (p < 0.0001, Fig. 3D). Importantly, when CD8 T cells in Flumist-primed LM-NP–boosted mice were depleted prior to PR8 challenge, no decrease in viral titers was observed (Supplemental Fig. 2), indicating that the protection against IAV was CD8 T cell mediated.

Finally, we assessed whether L. monocytogenes is the only vector capable of rapidly boosting Flumist-primed CD8 T cells. Although short-interval boosting with Flumist did not enhance the numbers of NP147-specific memory CD8 T cells (Fig. 3E), boosting with VV-NP resulted in expansion similar to LM-NP boosting. Thus, Flumist-primed NP-specific CD8 T cells could potentially be boosted rapidly by a relatively safe and established vaccine vector, such as VV-NP.

Discussion
Because of the constant need to reformulate seasonal vaccines and the threat of pandemics, the development of a universal IAV vaccine has been a major goal in the field of vaccine research. As the surface proteins HA and NA found in various subtypes are subject to rapid mutation, the conserved nature of internal viral components, like NP or matrix protein, has focused attention on the protective capacity of CD8 T cells with specificity for conserved IAV proteins. Although data support that influenza A infection elicits cross-reactive CD8 T cells capable of eliminating infected cells after a secondary heterologous infection (19), this process does not provide life-long immunity against future influenza encounters. This conundrum has raised doubts about whether CD8 T cells can provide robust cross-protection in humans (20), and clinical evidence of HI mediated by CD8 T cells is scarce. Intranasal immunization with cold-adapted attenuated IAV is currently the most efficacious licensed vaccine capable of inducing memory CD8 T cells (21, 22). Nonetheless, in BALB/c mice, vaccination with Flumist induced too few cross-reactive memory CD8 T cells to provide control of viral replication during the first days of nonhomologous IAV infection, and boosting with Flumist 7 mo later did not increase the number of NP147-specific memory CD8 T cells. Consistent with this, recent clinical studies actually suggest no increase in IAV-specific memory CD8 T cells in children that receive the seasonal influenza vaccination annually (23) and show that repeated vaccination actually interferes with the establishment of memory CD8 T cells after natural IAV infection due to the presence of neutralizing Abs (24). We show that when a strong heterologous booster agent, such as LM-NP or adjuvanted NP, is used, the numbers of cross-reactive memory CD8 T cells primed by Flumist can be dramatically increased to protect against a nonhomologous IAV challenge. Importantly, we also show that Flumist-induced CD8 T cells have the unexpected property of rapid acquisition of memory characteristics and the ability to respond to short-interval boosting. This is a surprising find, because generally, but not exclusively (25), immunization with live organisms is highly inflammatory and induces CD8 T cells with a short-lived effector phenotype (15, 26, 27) and delayed acquisition of memory characteristics. Therefore, it generally takes months to effectively boost CD8 T cells primed by viral infection (11, 26). However, Flumist-primed CD8 T cells could be boosted to reach protective levels as early as 7 d postpriming. This property of Flumist-induced CD8 T cells provides a potentially novel prime-boost strategy that could provide protective levels of CD8 T cells in a matter of days. This accelerated prime-boost strategy might be very useful in the case of a sudden pandemic flu outbreak; people who were vaccinated with Flumist last season or only very recently potentially could be protected by boosting with an NP-containing vector until a traditional homologous influenza vaccine is available. Although the booster methods used in this study are not approved for use in humans, both L. monocytogenes–based immunization (13) and VV-based immunization (28) have been administered safely to healthy volunteers, indicating that it may be possible to develop a safe, but potent, CD8 T cell–inducing vaccine. Because the amino acid sequence of IAV NP does not change drastically over time (3), a vector expressing NP has the potential to be mass produced and stockpiled until needed in case of a pandemic.

Acknowledgments
We thank Dr. J. Bennink for providing VV-NP.

Disclosures
P.L. is an employee of Adudo Biotech, which holds patents related to materials used in this study. The other authors have no financial conflicts of interest.

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