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*J Immunol* 2011; 186:4331-4339; Prepublished online 25 February 2011; doi: 10.4049/jimmunol.1003057

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http://www.jimmunol.org/content/suppl/2011/02/25/jimmunol.1003057.DC1

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Contributions of Antinucleoprotein IgG to Heterosubtypic Immunity against Influenza Virus

Mark W. LaMere,* Ho-Tak Lam,* Amy Moquin,† Laura Haynes,† Frances E. Lund,* Troy D. Randall,* and Denise A. Kaminski*

Influenza A virus causes recurring seasonal epidemics and occasional influenza pandemics. Because of changes in envelope glycoprotein Ags, neutralizing Abs induced by inactivated vaccines provide limited cross-protection against new viral serotypes. However, prior influenza infection induces heterosubtypic immunity that accelerates viral clearance of a second strain, even if the external proteins are distinct. In mice, cross-protection can also be elicited by systemic immunization with the highly conserved internal nucleoprotein (NP). Both T lymphocytes and Ab contribute to such cross-protection. In this paper, we demonstrate that anti-NP IgG specifically promoted influenza virus clearance in mice by using a mechanism involving both FcRs and CD8+ cells. Furthermore, anti-NP IgG rescued poor heterosubtypic immunity in B cell-deficient mice, correlating with enhanced NP-specific CD8 T cell responses. Thus, Ab against this conserved Ag has potent antiviral activity both in naive and in influenza-immune subjects. Such antiviral activity was not seen when mice were vaccinated with another internal influenza protein, nonstructural 1. The high conservation of NP Ag and the known longevity of Ab responses suggest that anti-NP IgG may provide a critically needed component of a universal influenza vaccine. The Journal of Immunology, 2011, 186: 4331–4339.

Seasonal influenza epidemics hospitalize 200,000 and kill 36,000 Americans annually (1–3). Vaccination with inactivated virus induces neutralizing Abs that are poorly effective against new strains with changes in external target Ags (2, 4–7). By contrast, prior influenza infection of mice induces immunity that can accelerate virus clearance of a secondary infection, even if the challenge virus is of a different subtype (heterosubtypic immunity [Het-I]) (8–15). Evidence for Het-I in humans exists as well (7, 16–19). Thus, a more universal influenza vaccine would best exploit the mechanisms of Het-I, which likely involve immune reactions to conserved viral Ags.

Recent advances have been made by inducing broadly neutralizing Ab against the conserved stem region of H1 and of H3, representative of typical seasonal circulating viruses and the 2009 H1N1 pandemic virus (20, 21). However, such Abs against the H1 stem are mainly effective against heterologous H1 viruses, and Abs against the H3 stem are mainly effective against heterologous H3 viruses (20, 21). Although incorporating such reactivity into current seasonal vaccines will likely enhance protection within subtypes, humans and livestock will remain susceptible to unforeseen zoonotic transfers from other subtypes (e.g., H5N1, H7N7, H9N2, and so on (22–24)), which pose a potential for future pandemics. Thus, cross-protection provided by highly conserved Ag, such as nucleoprotein (NP), would provide an additional level of insurance against these possibilities.

Unlike external viral Ags, the internal NP is >90% conserved among all influenza A strains (25, 26), including H5N1 avian viruses (e.g., GenBank accession number DQ493166) and the novel H1N1 virus (e.g., GenBank accession number ACP41106) that caused the 2009 influenza pandemic. Thus, the sequence stability of NP makes it an attractive candidate for vaccination. Indeed, systemic NP vaccination accelerates viral clearance and prevents mortality in mice challenged with various viral serotypes (27–33). The protective effects of NP vaccination correlate with the induction of NP-specific CD8 T cell responses (27–33). T cell depletion can abrogate NP-immune protection, and there is evidence that both CD8 and CD4 cells contribute (29, 31).

In addition to the contributions of T cells, accelerated viral clearance provided by Het-I or by NP immunization requires Ab (10, 34). Furthermore, NP-immune serum passively transfers protection to naive mice in an Ab-dependent manner (34). Therefore, NP-immune Ab can promote viral clearance. However, applying such cross-protective effects of NP immunity for human use requires a better understanding of how it works. In this paper, we show that anti-NP IgG specifically has antiviral activity that involves both FcRs and CD8+ cells. Importantly, this Ab can accelerate virus clearance in both B cell-deficient and B cell-sufficient mice. Vaccination with influenza nonstructural 1 (NS1) did not promote virus clearance, suggesting that NP has unique characteristics as a protective Ag. These new data help to clarify a mechanism for how these Abs may promote influenza virus clearance and its utility for cross-protective vaccination.

Materials and Methods

Animals and viruses
C57BL/6, μMT, μMT/FcR γc−/−, Aid−/−, and IFNα/βR−/− were bred and maintained in the University of Rochester vivarium. Th7−/− mice were purchased from The Jackson Laboratory and bred in our facility. The

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Received for publication September 13, 2010. Accepted for publication January 21, 2011.

This work was supported by National Institutes of Health Grants AI079537 (parent and American Recovery and Reinvestment Act supplement) (to D.A.K.), AI61511 (to F.E.L.) and by House Resolution 3222 (Department of Defense funding) (to L.H.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AID, activation-induced cytidine deaminase; ASC, Ab-secreting cell; DC, dendritic cell; HA, hemagglutinin; Het-I, heterosubtypic immunity; i.e., immune reaction; KLH, keyhole limpet hemocyanin; NP, nucleoprotein; Ns1, nonstructural 1; PA, acidic polymerase.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/$16.00 purchased from The Jackson Laboratory and bred in our facility. The
University of Rochester University Committee on Animal Resources approved all animal procedures. For some experiments, µMT and µMT/FcR γc−/− mice were treated with 950 rad [137]Cs, then reconstituted i.v. with 10^7 µMT bone marrow cells, and then allowed to reconstitute for 8 wk. Influenza H3N2 A/X31 and H1N1 A/PR8/34 were grown in embryonated hen’s eggs as described previously (12). Virus inoculum doses are given as LD50 as determined in C57BL/6 mice.

**Immune serum and Ab**

To generate donor immune serum, 8- to 10-wk-old C57BL/6 mice were immunized i.p. with 30 µg LPS adjuvant in the presence or absence of 100 µg purified recombinant influenza NP (generated as described in Ref. 34) on days 0, 10, 20, and when necessary, day 30. After 1:10^3 NP-reactive IgG titers were achieved, serum was collected every 14 d. Serum was stored at −70˚C until pooled for use. NP-specific IgG-secreting hybridomas (clones IC5-2A10-G1 [anti-NP IgG1], IC6-1H5-G2a [anti-NP IgG2a], and H19-L2-J2-G6 [anti-NP IgG2b]) were a gift from Dr. W. Gerhard (Wistar Institute, Philadelphia, PA). Low-endotoxin mAb was purified from hybridoma supernatants by BioXcell (Lebanon, NH). Control mAbs (clones MOPC21 [IgG1], Cl.18 [IgG2a], and MNP-11 [IgG2b]), rat anti-CD8, and control rat Ab (anti-keyhole limpet hemocyanin [KLH]) were purchased from BioXcell. Influenza PR8 NS1 amino acid sequence was used to synthesize codon-optimized cDNA for expression in bacteria (GeneArt). NS1 cDNA was cloned into pTrcHis (Invitrogen) as previously described for NP (34). Purification of bacterially expressed NS1 was performed by an inclusion body extraction protocol. Cell pellets were washed with Triton X-100 buffer, followed by resuspension in urea buffer. The released protein was then dialyzed into 1X PBS and sterile-filtered.

**Passive transfer and influenza challenge**

On day −1, relative to influenza challenge, µMT and µMT/FcR γc−/− recipient mice were given i.p. injections of either 400 µl donor serum (LPS-immune control or NP-immune) or a mixture of 300 µg each IgG mAb subclass. In separate experiments, µMT mice were treated with 100 µg rat anti-mouse CD8 (clone 2.43) or anti-KLH isotype control (clone LPS-immune or NP-immune) on days 0, 3, 7, and 10. Control mice were treated with 100 µl normal mouse serum on day −1. Controls for passive transfer included untreated mice or mice treated with 100 µl/or 1 g purified recombinant influenza NP (generated as described in Ref. 34) on days 0, 3, 7, and 10.

**Tissue analysis**

At the indicated times post-PR8 challenge, infected mice were sacrificed, and lung and spleen were collected for analysis. To measure virus, lungs were homogenized in 2.5 ml PBS with antibiotics and frozen until analysis by viral focus assay as described previously (34). Lung and spleen were stained for flow cytometry as described previously (34). Stained cells were analyzed with an Accuri C6 Flow Cytometer (Accuri). Data were analyzed by FlowJo Software (Tree Star). In other experiments, spleen cells were plated onto MultiScreen ELISPOT plates (Millipore) coated with 5 µg/ml either goat anti-mouse IgG (H+L) or rNP. After 6 h at 37˚C 5% CO2, plates were washed and probed with alkaline phosphatase-conjugated anti-mouse IgG at 4˚C overnight. Plates were then washed and Ab-secreting cells (ASC) were detected with 5-bromo-4-chloro-3-indolyl phosphate/NBT substrate (Moss).

**Statistical analysis**

Statistical analysis was performed with Prism 5.0a software using the tests indicated in the figure legends.

**Results**

NP-specific IgG cooperates with cellular immunity

The importance of B cells (12) and Ab repertoire (10) for influenza Het-I strongly suggests that Ab against conserved viral proteins promote virus clearance and is thus an attractive candidate for universal influenza vaccines. To determine whether such a strategy should involve diversified Ab, we asked whether Het-I requires expression of activation-induced cytidine deaminase (AID), which is required for somatic hypermutation and class-switch recombination (35). We infected C57BL/6 mice and AID−/− mice with H3N2 influenza X31, followed by a secondary challenge with a lethal dose of H1N1 PR8 (Fig. 1A). X31-immune C57BL/6 mice rapidly reduced lung viral load by day 5 and nearly cleared the virus by day 7 postchallenge (Fig. 1B), as expected (8–12, 14, 15). By contrast, AID−/− mice had consistently high viral titers without clearance throughout the time of observation (Fig. 1B). CD8 T cell responses in the lung (Fig. 1C, Table I), mediastinal lymph node, and spleen (data not shown) were equally or more robust in the AID−/− mice compared with C57BL/6. Thus, diversified (i.e., high-affinity, class-switched) Ab is required for rapid virus clearance in heterosubtypic-immune mice. Because the two viruses differ in their hemagglutinin (HA) and neuraminidase (NA) molecules, the reactivity responsible for this effect is likely against more conserved Ag(s) such as NP. As anti-NP IgG is present in influenza-immune C57BL/6 mice (12) but not in µMT nor AID−/− mice, this Ab can be considered a correlate of protection for mouse Het-I against influenza virus.

To evaluate what proportion of the Ab response to X31 priming in the C57BL/6 mice was accounted for by NP reactivity, we performed an ELISA on day-10 immune serum compared with a monoclonal NP-specific IgG Ab. Total serum IgG was increased in X31-immune infected mice compared with mice given PBS alone (Fig. 2A), as expected. In most of the infected mice, NP-reactive IgG was induced to a level near 60 µg/ml (Fig. 2B). This value would suggest that ∼3% of the serum IgG in day-10 immune mice is against NP (Fig. 2C). We also performed ELISpot analysis at this time point, which does not require a monoclonal standard (Fig. 2D–F). In this case, NP-reactive IgG ASC accounted for ∼0.5% of the total IgG response. We note that a large proportion of the increased total serum IgG response is due to a rise in the IgG2c subclass, which requires Myd88 expression in B cells per se (data
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Table I. Lung T cell numbers in heterosubtypically challenged mice

<table>
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<th></th>
<th>C57BL/6</th>
<th>AID&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>p Value&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>4</td>
<td></td>
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<tr>
<td>Total lung</td>
<td>4.90 × 10&lt;sup&gt;5&lt;/sup&gt; ± 3.94 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.00 × 10&lt;sup&gt;6&lt;/sup&gt; ± 3.68 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.0195</td>
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<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.33 × 10&lt;sup&gt;5&lt;/sup&gt; ± 1.82 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.21 × 10&lt;sup&gt;6&lt;/sup&gt; ± 1.45 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.0159</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; Dnp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.21 × 10&lt;sup&gt;5&lt;/sup&gt; ± 1.03 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.80 × 10&lt;sup&gt;5&lt;/sup&gt; ± 5.47 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.0159</td>
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Numbers are mean ± SD.

<sup>a</sup>Mann–Whitney U test.

not shown). Thus, a large proportion of the total IgG is likely polyclonal, suggesting that anti-NP IgG may be a larger fraction of the virus-specific response than is calculated in this paper.

To determine whether NP-specific Ab has antiviral activity, we injected NP-immune serum or purified anti-NP IgG mAb into B cell-deficient μMT mice 1 d before i.n. infection with influenza PR8. Unlike recipients of PBS alone or negative control (LPS adjuvant-immune) serum, recipients of NP/LPS-immune (NP-immune) serum had significantly reduced or cleared virus from the lung by day 10 (Fig. 3), consistent with previous results (34). Importantly, purified anti-NP IgG (a mixture of subclasses) also cleared virus from the lung by day-10 postchallenge infection (Fig. 3). Therefore, NP-specific IgG per se has significant antiviral activity, indicating that other components in the donor serum such as complement and cytokines make little, if any, contribution during serum transfer. Whether or not host-derived complement and cytokines contribute to the efficacy of the Abs remains to be determined.

Knowing that anti-NP IgG had antiviral activity, we next asked whether this Ab could indeed contribute to Het-I. We injected purified NP-specific IgG into naive and X31-immune μMT mice, which have poor Het-I (12). The mice were then rechallenged with high-dose PR8 (Fig. 4A). Compared with influenza-naive recipients, X31-immune recipients given control mAbs had modestly reduced viral titers (Fig. 4B), likely because of antiviral T cells in these B cell-deficient animals. That such activity was not apparent in AID<sup>−/−</sup> mice suggests additional, Ab-independent roles for B cell regulation of viral immunity that remain to be elaborated. If the X31-immune mice were injected with anti-NP IgG, the lung viral load was further reduced on day 5 and was cleared by day 6 (Fig. 4B). Therefore, anti-NP IgG cooperates with existing non-humoral immunity to rescue poor heterosubtypic viral clearance in B cell-deficient mice. Furthermore, X31-immune recipients of anti-NP IgG had higher numbers of NP-specific CD8 T cells in the spleen (Fig. 4C, 4D). Therefore, NP-specific IgG can enhance T cell responses, suggesting that CD8 T cells contribute to the antiviral mechanism of this Ab.

To determine whether CD8-expressing cells are functionally involved in the mechanism of NP-immune Ab, we depleted CD8<sup>+</sup> cells in μMT mice. Although some antiviral activity was detectable in CD8-depleted recipients of NP-immune serum, it was less substantial and did not clear the virus by day 10, as in the CD8-sufficient μMT recipients (Fig. 5). Therefore, CD8-expressing cells do indeed contribute to the antiviral mechanism of NP-immune Ab. As T cells are also required (34), this result suggests that NP-specific Ab cooperates with cytotoxic T lymphocytes to promote influenza virus clearance.

**Ag exposure and involvement of FcR suggest a role for NP-immune complexes**

The antiviral effects of anti-NP Ab were initially unexpected since the Ag is internal and anti-NP IgG does not neutralize virus by conventional definitions (36, 37). Nonetheless, our results strongly suggest that at some point during infection, NP is available to the Ag is internal and anti-NP IgG does not neutralize virus by conventional definitions (36, 37). Nonetheless, our results strongly suggest that at some point during infection, NP is available to

FIGURE 2. NP-reactive IgG response during X31 priming infection. C57BL/6 mice were treated i.n. with either sterile PBS or 0.25 LD<sub>50</sub> influenza X31 virus. At day 10, serum and spleen were collected for analysis. A–C. Serum IgG analysis by ELISA. A. Total serum IgG. B. NP-reactive serum IgG. Quantitative estimation in micrograms per milliliter was determined by comparing signal from diluted serum with that of a known monoclonal anti-NP IgG2a. C. Anti-NP IgG1 as a percentage of total serum IgG, based on values from A and B. D–F. Spleen ASC measured by ELISPOT analysis. D. Total IgG ASC in spleen. E. NP-reactive ASC in spleen. F. Anti-NP ASC as a percentage of total splenic IgG ASC.

FIGURE 3. Anti-NP IgG has antiviral activity. B cell-deficient μMT mice were injected i.p. with PBS alone, 400 μL LPS-immune serum or NP/LPS-immune serum (prepared as described in Materials and Methods), or with 300 μg each NP-specific IgG1, IgG2a, and IgG2b 1 d before i.n. infection with 0.13 LD<sub>50</sub> influenza PR8. Shown are lung viral titers on day 10 postinfection. The p values calculated by Student t test. Representative of at least three similar experiments.

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day 7, and had declined to undetectable levels by day 9, corresponding with the typical time of virus clearance (Fig. 6 and data not shown). Notably, NP was not detected at day 1 but increased as the infection proceeded, suggesting that the protein detected was derived from virus replication, as opposed to the allantoic fluid inoculum. NP was also detectable in nasal washes and in supernatants from MDCK cells infected in culture (data not shown). Therefore, NP protein appears to be released from infected cells both in vivo and in vitro. Such accessible NP would likely be available for recognition by Ab to form immune complexes capable of engaging further antiviral reactions.

The fate of Ab-bound Ag is determined by the Ig C region (38, 39). Immune complexes can activate the classical complement cascade (40) and can also engage FcR to trigger further immune reactions (38, 39). To determine whether FcR are important in our system, we crossed B cell-deficient mice with mice deficient in the common g-chain shared by the activating FcRs (FcγRI, RII, and RIV) (38, 39). μMT and μMT/FcγRI γ−/− mice were each injected with anti-NP IgG or with control IgG 1 d before influenza PR8 infection. Whereas μMT recipients of anti-NP IgG cleared virus by day 10, μMT/FcγRI γ−/− recipients had reduced virus titers by 10-fold, but had not cleared (Fig. 6B). Similar results were observed at day 10 if NP-immune serum was used instead of mAb (data not shown). NP-immune serum could also...

**FIGURE 4.** Anti-NP IgG cooperates with existing influenza immunity. A. Experimental design. μMT mice were treated i.n. with either PBS ("naive") or 0.25 LD₅₀ influenza X31 on day 0. Purified IgG was injected as in Fig. 2. Influenza PR8 was used at 2.5 LD₅₀ i.n. B, Lung viral titers of μMT recipient mice on the indicated day post-PR8 challenge. ***p < 0.0005 by Student t test. C, CD8⁺ T cells binding the indicated Db tetramer in the spleen of the indicated mice on day 5 (C) and day 6 (D) post-PR8 challenge. **p = 0.008 by Mann–Whitney U test. Representative of at least three similar experiments. BD, below the limit-of-detection.

**FIGURE 5.** CD8 depletion inhibits antiviral activity of NP-immune serum. μMT mice were treated with rat anti-KLH ("ctrl") or anti-CD8 every other day beginning on day 1 as indicated in Materials and Methods. CD8 depletion was confirmed in representative lung samples, as well as in spleen and mediastinal lymph node. Serum (400 μl) from LPS- or from NP/LPS-immune C57BL/6 mice was transferred into μMT recipients 1 d prior to infection with 0.13 LD₅₀ PR8. Lung viral titers in μMT recipients on day 10 postinfection. The p values were calculated by Student t test.

**FIGURE 6.** NP exposure and involvement of FcR. A. C57BL/6 mice were infected with 0.25 LD₅₀ influenza PR8 or treated with PBS i.n. Bronchoalveolar lavage was collected at the indicated time points. ELISA plates coated with anti-NP IgG mAb were incubated with the indicated samples. NP was detected with biotinylated serum IgG purified from NP-immune mice. n = 2 mice per PBS and 3 PR8 mice per time point. Representative of three similar experiments. B, Purified mAb (300 μg each IgG1, IgG2a, and IgG2b) was transferred into the indicated recipients 1 d prior to infection with 0.13 LD₅₀ PR8. Lung viral titers on day 10 post-infection. Representative of two similar experiments. The p values calculated by Student t test.

C, LPS- or NP-immune serum (400 μl) was injected i.p. into the indicated mice 1 d before i.n. infection with 0.13 LD₅₀ influenza PR8. D, Irradiated μMT and μMT/FcγRI γ−/− mice were each reconstituted with μMT bone marrow (BM). After 8 wk, the chimeras were injected i.p. with a mixture of 300 μg of the indicated IgG1, IgG2a, and IgG2b. One day later, the mice were challenged i.n. with 0.13 LD₅₀ influenza PR8.
reduce the virus in the lung by day 5 postchallenge, and did so in an FcR-dependent manner (Fig. 6C). Thus, one or more activating FcR is needed for the full antiviral activity of NP-specific Ab, suggesting that FcR engagement by anti-NP IgG triggers downstream antiviral reactions. The involvement of FcR early post-infection suggests that these molecules are functioning either in one or more innate-type immune cells or even in nonhematopoietic cells. Using radiation bone marrow chimeras, we found that FcR expression on nonhematopoietic cells is dispensable for the antiviral activity of anti-NP IgG (Fig. 6D), favoring the explanation of innate immune cell involvement. The nature of these cells and also the molecules contributing to the minimal activity seen in the FcR-deficient animals on day 10 (Fig. 6B) remain to be determined.

High-titer NP-immune Ab promotes virus clearance in B cell-sufficient mice

We next transferred NP-immune serum into intact (B cell-sufficient) C57BL/6 mice prior to influenza challenge. In preliminary studies, a single serum injection lacked the antiviral effect seen in μMT recipients (data not shown). Therefore, we performed multiple daily injections of NP-immune serum beginning 1 d before challenge infection (Fig. 7A). In this case, the NP-reactive IgG titer increased to donor levels after several injections, remaining high after the final dose on day 3 postinfection (Fig. 7B). Nonetheless, the lung viral load at day 8 differed little between recipients of NP-immune and control serum (Fig. 7C). We next injected the serum beginning 3 d prior to infection to ensure that recipient anti-NP IgG titer were comparable to donor levels by the time of challenge (Fig. 7D, 7E). On day 8 postchallenge, these C57BL/6 recipients of NP-immune serum had significantly reduced virus in the lung compared with recipients of control serum (Fig. 7F). A similar regimen of purified anti-NP mAb injections also enhanced virus clearance in C57BL/6 recipient mice (Fig. 8). Therefore, the antiviral effects of NP-immune Ab are not limited to B cell-deficient mice. Indeed, active immunization of B cell-competent mice with NP protein Ag accelerates virus clearance (27–33) using a mechanism that requires Ab production (34).

Although the reason μMT mice require less Ab for passive activity is unknown, the results in Figs. 7 and 8 likely explain a previous report that one dose of NP-immune serum did not protect B cell-sufficient BALB/c recipient mice (30). Furthermore, the results indicate that a high titer of Ab is needed to engage antiviral mechanisms early postinfection.

The ability of NP-immune Ab to work in C57BL/6 recipients allowed us to test for the requirements of downstream molecules, using gene-targeted mice on a B6 background. Influenza NP is an RNA-binding protein. Interestingly, Ab against self-RNA and RNA-binding proteins can induce IFN-α and -β production through FcR coengagement with TLR7 and exacerbate autoimmune disease (41, 42). Additionally, nonneutralizing Ab against other RNA viruses can induce IFN-α production from cultured monocytes by a similar mechanism (43–45). To test whether NP-specific Ab might function via these antiviral cytokines in an analogous manner, control and anti-NP IgG were each injected into C57BL/6 or into mice deficient in the common receptor for IFN-αβ. After sublethal PR8 infection, both C57BL/6 and IFNαR−/− mice given anti-NP IgG had reduced day-8 viral load compared with mice receiving control IgG (Fig. 8A). In fact, the antiviral effect appeared to be more consistent in the mutant recipients, suggesting that IFN-αβ may be inhibitory in this context. Interestingly, challenging the Ab recipients instead with a lethal dose of virus resulted in a slightly lesser effect of the Ab in the IFNαR−/− mice compared with C57BL/6 recipients (Fig. 8B). This modest difference suggests that in circumstances of lethal infection, as might be encountered with highly pathogenic avian H5N1 strains (22), an IFN-mediated mechanism might then be used by the Ab. However, for typical seasonal infections, the sublethal inoculation is more likely, and the mechanisms stimulated by anti-NP Ab probably use other pathways. Our ability to

![FIGURE 7](http://www.jimmunol.org/)

C57BL/6 mice were given 400-μl i.p. injections of LPS- or NP/LPS-immune serum according to the schedules indicated in A and D. B and E, Serum NP-reactive IgG in donors and recipients of immune and control serum. C and F, Day-8 lung viral titers in serum recipients. A–C, Mice injected beginning on day −1 relative to challenge with 0.25 LD₃₀ influenza PR8. D–F, Mice injected beginning on day −3 relative to challenge with 0.25 LD₃₀ influenza PR8.

![FIGURE 8](http://www.jimmunol.org/)

Three hundred micrograms of mixed IgG1, IgG2a, and IgG2b control or anti-NP IgG mAb were injected i.p. into the indicated mice on days −3 to +1 relative to influenza infection. A, On day 0, recipient mice were challenged i.n. with 0.25 LD₃₀ influenza PR8, and lungs were assayed for virus titers on day 8. B, On day 0, mice were challenged i.n. with 2.5 LD₃₀ PR8. On day 7 postinfection, lungs were assayed for viral titers. Each experiment was performed twice with similar results.

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**Figure 7**. C57BL/6 mice were given 400-μl i.p. injections of LPS- or NP/LPS-immune serum according to the schedules indicated in A and D. B and E, Serum NP-reactive IgG in donors and recipients of immune and control serum. C and F, Day-8 lung viral titers in serum recipients. A–C, Mice injected beginning on day −1 relative to challenge with 0.25 LD₃₀ influenza PR8. D–F, Mice injected beginning on day −3 relative to challenge with 0.25 LD₃₀ influenza PR8.

**Figure 8**. Three hundred micrograms of mixed IgG1, IgG2a, and IgG2b control or anti-NP IgG mAb were injected i.p. into the indicated mice on days −3 to +1 relative to influenza infection. A, On day 0, recipient mice were challenged i.n. with 0.25 LD₃₀ influenza PR8, and lungs were assayed for virus titers on day 8. B, On day 0, mice were challenged i.n. with 2.5 LD₃₀ PR8. On day 7 postinfection, lungs were assayed for viral titers. Each experiment was performed twice with similar results.
protect Tlr7−/− mice from sublethal challenge by active vaccination with NP further supports this conclusion (Supplemental Fig. 1).

Finally, we asked whether immunization with other influenza Ags besides NP can be beneficial against influenza virus. The NS1 gene product is produced by virally infected cells (46). Like NP, NS1 is an internal RNA-binding protein, although not incorporated into virions per se. NS1 immunization induced significant titers of Ag-reactive IgG in the serum of C57BL/6 mice; however, when NS1-immune mice were challenged with influenza PR8, no reductions in lung viral titers were observed (data not shown). NS1-reactive Ab was detectable in C57BL/6 mice that had previously cleared influenza X31 (Supplemental Fig. 2A, 2B). These Ab levels could be boosted by i.p. immunization with purified NS1 (Supplemental Fig. 2A, 2B). When challenged with influenza PR8, X31-immune mice had modestly decreased viral load, as expected. However, NS1-boostered X31-immune mice had significantly more virus than those receiving X31 alone (Supplemental Fig. 2C). Our results suggest that NS1 immunity does not provide protection against influenza virus but instead may be inhibitory for virus clearance. These results further suggest that some characteristic of NP makes it unique as a protective Ag.

Discussion

A universal influenza vaccine would best exploit the mechanisms of natural Het-I. In this paper, we show that Het-I requires Ig diversification, suggesting that high-affinity, class-switched Ab against conserved Ags makes a major contribution to its mechanism. Correspondingly, NP-specific IgG had antiviral activity in vivo that rescued poor Het-I in B cell-deficient mice. This activity correlated with an enhanced NP-specific CD8 T cell response and was dampened when CD8+ cells were depleted. Optimal antiviral activity was observed only when recipient mice expressed the common γ-chain of the activating FcR. Importantly, antiviral activity of these Abs was not limited to B cell-deficient mice and could reduce viral load upon lethal challenge by a mechanism partially involving responsiveness to IFN-α/β. Given the high conservation of the target Ag, these important new findings strongly suggest that immunization to induce NP-specific Abs of effector classes that can optimally engage such mechanisms would provide cross-protective immunity required for a genuinely universal influenza vaccine.

A previous study in AID−/− mice showed that despite delayed virus clearance, Ig class-switch recombination and somatic hypermutation are not required for surviving primary and homosubtypic secondary influenza infection (47). This result correlated with a robust and long-lived IgM response and equivalent virus-neutralizing titers in the serum compared with AID+/− mice (47). These observations suggest that nonmutated IgM responses are adequate for protection, consistent with previous findings that secreted natural IgM is required for protection (48).

Unlike neutralizing Ab, NP-specific IgG does not prevent virus entry into host cells (36, 37). Therefore, acceleration of viral clearance by the latter likely occurs by a more complex mechanism involving other effector activities. Such a mechanism would first require that the Ag, which typically functions inside of virions and inside of infected cells (26, 49), is accessible to the Ab. In fact, we detected NP in the supernatant of influenza-infected MDCK cells in culture, in the bronchoalveolar lavage, and in nasal washes of influenza-infected mice (Fig. 6A and data not shown). This exposure likely provides an opportunity for the Ag to interact with NP-specific Ab to form immune complexes that could then stimulate antiviral immune reactions. NP detected in our assay might be NP monomers or multimers, complexes with RNA and/or polymerase, or even associated with virions. Any of these structures could form beneficial immune complexes with specific Ab. Other reports show that cells cultured with influenza virus have NP present on the cell surface (50, 51). Cell-surface NP would provide an opportunity for Ab binding and complement-mediated lysis of infected cells, as has been shown in culture (51). Whether soluble or membrane associated, it is clear from the presence of NP-reactive Ab in influenza-experienced individuals (52–54) that NP is exposed to the humoral immune system sometime during an infection in humans as well.

Most anti-NP IgG antiviral activity in μMT mice required the FcR common γ-chain. Notably, NP-immune serum reductions in lung viral titers by 10-fold as early as day 5 postinfection in μMT mice but not in μMT/FcRγ−/− mice (Fig 6C). Thus, the major antiviral mechanism used by these Ab is engaged relatively early postinfection, using one or more of the activating FcRs. Several possible mechanisms might be engaged by NP-immune complexes binding to such receptors. Because FcRγ is expressed ubiquitously, its engagement on either hematopoietic cells or nonhematopoietic cells, such as infected epithelia, could stimulate antiviral reactions. However, anti-NP IgG still stimulated virus clearance in irradiated μMT/FcRγ−/− mice reconstituted with FcR-competent μMT bone marrow (Fig. 6D). Therefore, FcR engagement on nonhematopoietic cells is not necessary, and expression on immune cells is sufficient to trigger the antiviral mechanisms(s). Previously, serum Ab induced by a PR8 subunit vaccine protected mice from lethal homosubtypic challenge using a mechanism involving FcRγc, but not γ2a cells nor NK cells (55).

Such serum stimulated virus uptake by a macrophage cell line in culture (55). Cross-protective nonneutralizing Ab against influenza virus can also cooperate with macrophages in vitro to inhibit virus replication (13), although it is unclear whether this effect is due to anti-NP Ab per se and whether FcR are involved. Nonneutralizing Ab against other viruses can stimulate cytokine, chemokine, and cytolytic molecule production from cultured monocytes and NK cells (43–45, 56). Additionally, Ab-dependent cell-mediated cytotoxicity has significant antiviral potential in HIV (57, 58) and SIV infections (59, 60). It remains to be determined whether Ab against influenza NP or against the conserved internal proteins of other viruses uses Ab-dependent cell-mediated cytotoxicity to eliminate virus. Clearly, this and other nonneutralizing mechanisms could be advantageous for viruses that readily evade neutralizing Ab (6).

The FcR common γ-chain interacts with at least three α subunits that differ in their distribution among cell types and relative affinity for different IgG subclasses (38, 39, 61). In our experience, the most reliable antiviral regimen was to inject mice either with whole NP-immune serum or with a combination of anti-NP IgG1, IgG2a, and IgG2b mAb clones, representative of the subclasses detectable in the immune serum. In pilot experiments, each clone was capable of some antiviral activity on its own, with IgG2a being the most effective at stimulating virus clearance (data not shown). This observation is consistent with the fact that mouse IgG2a has the highest affinity for the activating FcRs, but the lowest affinity for the inhibitory FcyRIIB (38). However, in an unpredictable pattern among experiments, isotype-matched control IgG2a and anti-NP IgG2a each in combination with PR8 induced severe weight loss (data not shown). Thus, vaccine strategies to stimulate anti-NP Ab would likely be optimal using adjuvants that can induce combinations of both “inflammatory” (e.g., mouse IgG2a/c and human IgG1/2) and “regulatory” subclasses (e.g., mouse IgG1 and human IgG4) so that the antiviral mechanisms can be subsequently dampened before excessive pathology is experienced.
A unique characteristic of anti-NP IgG antiviral activity is the involvement of T lymphocytes (34), and in particular, CD8+ cells (Fig. 5) downstream of the Ab. The need for T cells likely explains previous reports that anti-NP mAb does not protect mice that lack both B and T cells (36, 37). These results collectively suggest that Ag from infected cells and specific Ab form immune complexes that engage FcR to trigger subsequent antiviral reactions that enhance, involve, and/or cooperate with T lymphocytes to eliminate virally infected cells in the lung. In fact, FcR engagement can enhance dendritic cell (DC) activation and Ag-presenting activity (62–68). Furthermore, immune complexes formed with anti-NP IgG2a can promote DC activation in vitro and enhance T cell responses when delivered in vivo (69). Despite the functional importance of T lymphocytes in our system, we did not find consistent evidence of an enhanced DC or antiviral T cell response in recipients of NP-immune serum (data not shown). Such paradoxical observations may suggest that another antiviral mechanism is engaged, without completely clearing virus in the absence of an antiviral T cell response. Alternatively, the Ab may be influencing a specific T cell response whose magnitude, kinetics, and/or mode of action (i.e., differentiation status) used for virus clearance is difficult to detect. In fact, in our previous study, the T cell response was suppressed for both NP and acidic polymerase (PA) in actively immunized mice, coincident with the clearance of replicating virus (34). This effect may occur because the precursor frequency of NP-specific T cells is extremely low in mice on a C57BL/6 background (70, 71). Correspondingly, anti-NP Ab-enhanced CD8 T cell responses are readily detected in influenza-immune recipients (Fig. 4C, 4D), which likely have an increased precursor frequency of NP-specific T cells. It is unknown whether other putatively antiviral Ab specificities could also work through T lymphocytes. If one speculates that, for example, Abs against PA or nonneutralizing Abs against HA could enhance PA- and HA-specific T cell responses (respectively), such Ab may not be protective, because HA- and PA-specific T cell responses poorly protect, and even delay viral clearance (72, 73). However, a T cell-independent role for anti-PA Ab cannot be excluded.

The experiments in this study used the PR8 influenza strain, and its reassortant derivative, X31, which has identical internal gene products. Thus, the anti-NP CD8 responses in our mice on a C57BL/6 background are likely against the same dominant NP366 peptide sequence presented in the Db MHC class I allele in both prime and challenge. However, in nature, humans with varying HLA molecules will encounter viruses whose sequences may vary at particular peptide epitopes. Nevertheless, immunization with whole NP protein also protects BALB/c mice whose dominant NP147 CTL epitope is presented by the Kd allele (27, 30). NP even protects outbred mice with undefined MHC I alleles (74). Thus, the protective effects are not limited to the DbNP366 specificity of PR8/X31 peptides. Because anti-NP IgG likely recognizes a distinct part(s) of the NP protein, the Ab is probably capable of cooperating with CD8 T cells in varying contexts. Further illustrating this point, the NP366 sequence of PR8 differs from that of the 2009 H1N1 pandemic strain A/CA/04/2009 at aa 371 (M→V). Although this is a small change, our Db tetramer with the PR8 sequence did not bind CD8 cells from pandemic virus-infected mice (data not shown). Nonetheless, we found that immunization of C57BL/6 mice with whole NP, based on the PR8 peptide sequence, still accelerated clearance of the 2009 strain in an Ab-dependent manner (M.W. LaMere, A. Moquin, F.E.H. Lee, R.S. Misra, P.J. Blair, L. Haynes, T.D. Randall, F.E. Lund, and D.A. Kaminski, submitted for publication). Therefore, the use of this Ab may not be restricted by the fine specificity of the participating CD8 T cells.

Compared with µMT, more anti-NP Ab was required to reduce viral titers in C57BL/6 mice, likely explaining a previous report that a single dose of NP-immune serum did not protect BALB/c mice (30). It is possible that B cell-competent recipients have competition between endogenous naive Ab monomers and NP–Ab immune complexes for occupying and engaging FcR. However, other factors cannot be excluded. Nonetheless, Figs. 7 and 8 show that the protective effects of anti-NP Ab are not limited to B cell-deficient mice. The requirement for high titers of anti-NP is disadvantageous for investigating Ab mechanisms and for passive therapy. However, active immunization to induce high-titer Ab against this conserved Ag would clearly be advantageous, because it could provide long-lived cross-protection that is not provided by vaccination with inactivated vaccines.

Unlike NP immunization, NS1 immunization did not accelerate influenza virus clearance. We observed delayed viral clearance in NS1-boosted influenza-immune mice. This effect may be due to an inhibitory effect of NS1-specific T lymphocytes, as has been described for influenza PA and HA CD8 epitopes (72, 73, 75). T cell-independent mechanisms might include Ab stabilization of the Ag either to enhance its proviral functions (46) or to clear NS1-associated dsRNA, in turn, preventing antiviral responses stimulated by this non-host RNA form. An additional possibility is that the early and transient expression of NS1 during an infection does not allow for engagement of protective Ab-mediated reactions. Regardless of the mechanism, our combined results suggest that some characteristic of NP makes it unique as a protective Ag. The need for CD8 cells and T lymphocytes in the mechanism of anti-NP Ab further suggests that a beneficial characteristic of a non-neutralizing Ab target is protective T cell epitopes. An examination of other influenza proteins such as M1, which like NP is more abundant, would help determine whether this is the case. This information is likely to be highly relevant in selecting candidate target Ag of other viruses.

Collectively, we have shown that NP-immune Ab has substantial antiviral activity that can contribute to mouse Het-I against influenza virus. Because Ab responses are known to be very long-lived (76, 77), investing in immunization with this highly conserved protective protein could be a major step in developing a truly universal influenza vaccine.


**SUPPLEMENTAL FIGURE 1.** NP-immunity against sublethal PR8 does not require TLR7. The indicated mice were immunized i.p. with 30 μg LPS ± 100 μg purified NP on days 0, 10, and 20. On day 40, the mice were infected with 0.25 LD₅₀ influenza PR8 i.n. Lungs were assayed for viral titers on day 8 post-infection.

**SUPPLEMENTAL FIGURE 2.** NS1 immunization does not promote virus clearance. *A*, Experimental design. C57BL/6 mice were infected i.n. with 0.25 LD₅₀ influenza X31 or PBS alone on day 0. On day 166, half of the X31-immune mice were boosted with 60 μg recombinant purified NS1 i.p. On day 228, all mice were challenged with 2.5 LD₅₀ influenza PR8 i.n. *B*, NS1-reactive IgG in mouse sera prior to PR8 challenge. *C*, Lung viral load at day 6 post-PR8 challenge.
LaMere, et al, Supplemental Figure 1

![Graph showing lung viral titer, log10, against immunization sub-lethal PR8. The x-axis represents different treatments (LPS, NP/LPS) and the y-axis represents viral titer. The graph compares C57BL/6 and TLR7−/− mice.](image-url)