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*J Immunol* 2008; 181:4168-4176; doi: 10.4049/jimmunol.181.6.4168
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A Novel Role for Non-Neutralizing Antibodies against Nucleoprotein in Facilitating Resistance to Influenza Virus

Damian M. Carragher, Denise A. Kaminski, Amy Moquin, Louise Hartson, and Troy D. Randall

Current influenza vaccines elicit Abs to the hemagglutinin and neuraminidase envelope proteins. Due to antigenic drift, these vaccines must be reformulated annually to include the envelope proteins predicted to dominate in the following season. By contrast, vaccination with the conserved nucleoprotein (NP) elicits immunity against multiple serotypes (heterosubtypic immunity). NP vaccination is generally thought to convey protection primarily via CD8 effector mechanisms. However, significant titers of anti-NP Abs are also induced, yet the involvement of Abs in protection has largely been disregarded. To investigate how Ab responses might contribute to heterosubtypic immunity, we vaccinated C57BL/6 mice with soluble rNP. This approach induced high titers of NP-specific serum Ab, but only poorly detectable NP-specific T cell responses. Nevertheless, rNP immunization significantly reduced morbidity and viral titers after influenza challenge. Importantly, Ab-deficient mice were not protected by this vaccination strategy. Furthermore, rNP-immune serum could transfer protection to naive hosts in an Ab-dependent manner. Therefore, Ab to conserved, internal viral proteins, such as NP, provides an unexpected, yet important mechanism of protection against influenza. These results suggest that vaccines designed to elicit optimal heterosubtypic immunity to influenza should promote both Ab and T cell responses to conserved internal proteins. The Journal of Immunology, 2008, 181: 4168–4176.
presence of NP on the cell surface (21). Furthermore, non-neutralizing Ab to HIV-1 can promote complement-mediated virolysis (22) and non-neutralizing Abs to Coxsackie B4 and poliovirus can induce secretion of the antiviral cytokine IFN-α/H9251 from human monocytes (23–25). Based on these data, it is clear that non-neutralizing Abs have the potential to elicit antiviral responses. However, the antiviral potential of non-neutralizing anti-NP Abs in vivo, particularly in T cell-competent mice, has remained largely unexplored.

Interestingly, our laboratory recently showed that immune serum from C57BL/6 mice infected with an H3N2 strain of influenza virus can passively promote viral clearance and reduce morbidity in immune B cell-deficient /H9262 MT mice after challenge with a heterosubtypic H1N1 strain (19). Abs in H3N2-immune serum do not cross-react with the H1 or N1 proteins and do not detect the external domain of M2 (M2e), suggesting that Abs against surface epitopes of influenza virus are not involved in the protective effect. By contrast, H3N2-immune serum has high titers of Ab reactive with NP (19), consistent with previous observations (26–30). These data suggest that anti-NP Abs play an important role in mediating cross-reactive protection against multiple serotypes and subtypes of influenza. Given the potentially beneficial effects of anti-NP suggested by all of these studies, it is unknown whether these Abs are responsible for and can confer protection from influenza virus. Given the dire need for improving cross-protective vaccination strategies against this disease, it is critical to fully understand how immunization with this conserved protein confers protection.

In this study, we show that Ab is necessary for NP immunization to confer protection in C57BL/6 mice and that NP-immune serum can transfer protection to naive recipient mice. Our results challenge the existing paradigm that T cell responses to conserved epitopes in internal proteins are the exclusive effectors of cross-reactive immunity to heterosubtypic strains of influenza virus and strongly suggest that Abs to these proteins are also an important component of the protective mechanism.

Materials and Methods

Protein production and purification

Recombinant NP protein was produced and purified as previously described (19). Briefly, the NP gene of influenza A/PR8/34 (PR8) was cloned into the pTricHis2c plasmid for expression in Escherichia coli. 6X His-tagged rNP protein was affinity-purified via the ProBond purification system (Invitrogen). Resulting protein was dialyzed into PBS and sterile-filtered.

Mice and procedures

All mice were on the C57BL/6 background and were bred and maintained at the Trudeau Institute. C57BL/6, B6.129P2-Cd40tm1Kik/J.
FIGURE 2. rNP immunization alters the kinetics of the CD8 T cell response after viral challenge. C57BL/6 mice were vaccinated i.p. with 30 μg of rNP and 20 μg of LPS or with LPS alone on days 0 and 10 and challenged with 500 EIU of PR8 on day 40. NP-specific (A) and PA-specific (B) CD8 T cells in the lung were measured by flow cytometry on the indicated day subsequent to challenge infection. NP-specific (C) and PA-specific (D) CD8 T cells were measured in the spleen. Mean ± SD of five mice per group. Representative of at least three similar experiments.

(CD40−/−), and B cell-deficient B6.129S2-Igh-6m1Cgn/J (μMT) mice were obtained from The Jackson Laboratory. Aid−/− mice were obtained from Dr. R. Gerstein (University of Massachusetts Medical School; Worcester, MA). Mice lacking the secretory exon of IgM (μS−/−) mice were obtained from Dr. R. Corley (Boston University; Boston, MA). Aid−/− and μS−/− mice were intercrossed to generate Ab-deficient Aid/μS mice. Mice were immunized i.p. with combinations of 20 μg of LPS with or without 30 μg of rNP at days 0 and 10. For influenza infections, mice were anesthetized with isofluorane USP (Webster Veterinary) and 500 egg infectious units (EIU) (0.2 LD50) of influenza PR8 were administered intranasally in 100 μl of sterile PBS. All procedures involving live animals were approved by the Trudeau Institute Institutional Animal Care and Use Committee and were performed in accordance with guidelines set by the National Research Council.

Virus foci assay

Madin-Darby canine kidney cells were grown in 96-well, flat-bottom plates until just confluent and then washed with HBSS. Homogenized lung samples were diluted in Zero Serum Media (Diagnostic Hybrids) supplemented with 4 μg/ml trypsin and applied to washed Madin-Darby canine kidney cells. Plates were centrifuged for 1.5 h at 800 g, washed, and cultured overnight in Zero Serum Media/trypsin at 33°C. The medium was removed and the cells were fixed with 80% acetone and allowed to dry. The wells were rehydrated with PBS, containing 2% FBS and 0.01% NaN3, and the cells were incubated in 3% FBS in PBS containing 10

Flow cytometry

Mice were sacrificed at the indicated times after infection and tissues were removed and mechanically disrupted by passage through wire mesh. Resultant cell suspensions were RBC-lysed and mesh-filtered. Cells were incubated in 3% FBS in PBS containing 10 μg/ml 2.4G2 to block Fc receptor binding, followed by staining with fluorochrome-conjugated CD8, CD62L (BD Biosciences), and MHC class 1 tetramers presenting NP366–374 or PA224–233 peptides (Trudeau Institute Molecular Biology Core Facility). Samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences).

Serum collection and ELISAs

Peripheral blood was obtained from either euthanized mice by severing the renal artery and pipetting into a 1.5-ml tube or from live mice via the lateral tail vein. After clotting for 30 min at 37°C, the precipitate was pelleted in a microcentrifuge and the serum was collected. NP-specific ELISAs were performed by coating plates with 2 μg/ml rNP. Serum samples were diluted in 3-fold serial dilutions in PBS with 10 μg/ml BSA and 0.1% Tween 20 before incubation on coated plates. Bound Ab was detected with HRP-conjugated goat anti-mouse IgM or goat anti-mouse IgG (Southern Biotechnology Associates).

Results

rNP immunization reduces influenza-induced morbidity and enhances viral clearance

To induce high titers of NP-specific Ab, we purified soluble rNP to use as an immunogen. This protein runs as a single band of ~53 kDa on a reducing SDS-PAGE gel (Fig. 1A). We i.p. immunized C57BL/6 mice with either 30 μg of purified rNP (plus 20 μg of LPS as an adjuvant that is known to stimulate strong B cell responses) or with LPS alone on days 0 (prime) and 10 (boost). This vaccination alone did not induce a NP-specific CD8 T cell response that was detectable by MHC class I tetramer staining and flow cytometry at various times after boosting (Fig. 1B and data not shown). However, the vaccination clearly induced high titers of NP-specific Ab in the serum as late as 39 days after priming (Fig. 1C). Thus, as expected, immunization with soluble rNP promotes a robust Ab response, but a limited CD8 T cell response.

To determine whether this apparently Ab-biased vaccine could still confer protection from an influenza virus challenge, the immunized mice were intranasally (i.n.) infected with a nonlethal dose of influenza PR8 virus (500 EIU, ~0.2 LD50) 1 mo after the boost (day 40 after priming). Mice immunized with LPS alone lost ~15% body weight by day 7 postinfection and had not yet recovered to their initial starting weight by day 11 (Fig. 1D). By contrast, mice vaccinated with rNP/LPS lost <5% of their initial weight and fully recovered by day 11 (Fig. 1D). The reduced morbidity in rNP-vaccinated mice was associated with significantly lower viral titers in the lungs on day 8 after infection (Fig. 1E). Therefore, as previously described (11, 31), immunization of C57BL/6 mice with rNP provides some measure of protection from sublethal challenge.
**FIGURE 3.** rNP-elicited protection from influenza requires CD40. C57BL/6 and CD40−/− mice were vaccinated i.p. with either 30 μg of rNP and 20 μg of LPS or with LPS alone on days 0 and 10. All groups were challenged i.n. with 500 EIU of PR8 on day 40. A. Relative body weights were determined. Mean ± SD of five mice per group. B. Viral titers were assayed in the lung at day 8 postinfection. Mean ± SD of five mice per group. C. NP-specific CD8 T cells in the lung were determined by flow cytometry on day 7 postinfection. D. Serum titers of NP-specific IgM and IgG were measured by ELISA in vaccinated mice on day 39 (1 day before infection). Mean ± SD of five mice per group. Representative of at least three similar experiments.

**FIGURE 4.** rNP vaccination-mediated reduction in viral titers requires Ab. C57BL/6 and Ab-deficient AID/−/− mice were immunized i.p. with either 30 μg of rNP and 20 μg of LPS or with LPS alone on days 0 and 10. All groups were subsequently challenged i.n. with 500 EIU of PR8 on day 40, and analyzed 8 days later. A. Titers of NP-specific IgM and IgG were measured by ELISA in serum. Mean ± SD of five mice per group. The low titers of anti-NP IgM in C57BL/6 mice are not consistently detected among experiments performed at this time point. B. Lung viral titers were measured. C. NP-specific CD8 T cells in the lung were determined by flow cytometry. Representative of two similar experiments.
Our laboratory previously showed that CD40 expression is required for optimal CD8 T cell responses to influenza virus (34). Thus, if CD8 T cells were contributing to rNP-immune protection, we would expect that rNP immunization would be ineffective in the absence of CD40 expression. Indeed, whereas C57BL/6 mice vaccinated with rNP/LPS lost very little weight after challenge
infection, rNP-immune $CD40^{-/-}$ mice lost as much weight as LPS-vaccinated C57BL/6 mice (Fig. 3A). Furthermore, rNP vaccination did not reduce lung viral titers in $CD40^{-/-}$ mice as in the controls (Fig. 3B). Thus, a protective immune response to vaccination with rNP/LPS requires CD40. However, the early NP-specific CD8 T cell response in the lung was observed in rNP-immune $CD40^{-/-}$ mice as well as in rNP-immune C57BL/6 mice, in comparison to LPS-immunized controls (Fig. 3C). Therefore, although an early CD8 T cell recall response occurs in the lung of rNP-immune mice, this response alone appears to be insufficient to protect mice that do not express CD40.

**Ab is required for rNP-immune protection**

Because CD40 expression is required for rNP-immune protection, but not for early CD8 T cell responses in our system (Fig. 3, A–C), we hypothesized that another CD40-regulated effector function was necessary. CD40 is essential for effective induction of germinal center reactions to protein Ags (35, 36) and for the accompanying class-switched, high-affinity, and long-lived Ab responses (37). In fact, rNP-immune $CD40^{-/-}$ mice had little to no NP-specific IgG in the serum (Fig. 3D). These results show that protection induced by rNP correlates with serum titers of NP-specific Ab.

To determine whether the loss of protection in the $CD40^{-/-}$ mice was due to the failure to generate Ab, we crossed mice with a mutation in activation-induced cytidine deaminase ($Aid^{-/-}$) (38) with mice lacking the secretory form of IgM ($\mu S^{-/-}$) (39). Because $Aid^{-/-}$ mice cannot isotype switch their Ab genes and $\mu S^{-/-}$ mice cannot secrete IgM, the resulting $Aid/\mu S$ mice have B cells, but cannot secrete Ab of any isotype. We vaccinated C57BL/6 and $Aid/\mu S$ mice with rNP/LPS or with LPS alone and challenged them with influenza virus on day 40. Fig. 4A shows that, even after vaccination and influenza infection, $Aid/\mu S$ mice do not generate any NP-specific Abs compared with vaccinated and infected C57BL/6 mice. As observed earlier, rNP-immune C57BL/6 mice had significantly lower viral titers than LPS-vaccinated controls on day 8 postinfection (Fig. 4B). However, rNP-immune, Ab-deficient $Aid/\mu S$ mice had viral titers that were as high as those in LPS-vaccinated control mice. Importantly, rNP-immune $Aid/\mu S$ mice still had an enhanced NP-specific CD8 T cell response that was still detectable at day 8 postinfection, when the modest recall response in the rNP-immune C57BL/6 mice had declined (Fig. 4C). It is likely that the higher Ag load (Fig. 4B) extends the expansion of existing memory T cells in the $Aid/\mu S$ mice, whereas the Ab in rNP-immune C57BL/6 mice prevents further expansion by promoting viral clearance. These results directly demonstrate that Abs are essential for rNP-elicited protection from influenza virus.

**rNP-immune serum protects $\mu MT$ mice from influenza-induced morbidity and enhances viral clearance in an Ab-dependent manner**

Although a previous study demonstrated that transfer of monoclonal anti-NP Abs to $scid$ mice did not protect from influenza virus (4), it was possible that these Abs would be protective if T cells were also present. To test this possibility, we transferred serum from rNP-vaccinated C57BL/6 donors to $\mu MT$ mice and challenged the B cell-deficient recipients with influenza virus the following day. Whereas recipients of LPS-immune serum continued to lose up to 25% of initial body weight through day 10 after infection, $\mu MT$ mice receiving rNP-immune serum lost only ~10% of their body weight and began to recover by day 8 (Fig. 5A). Moreover, lung viral titers on day 10 were reduced by ~100-fold in recipients of rNP-immune serum relative to those in mice that received control serum (Fig. 5B). Therefore, rNP-immune serum can convey protection against influenza challenge in T cell-competent MT hosts.

We next tested whether the transfer of rNP-immune serum affected CD8 T cell responses. The frequency of NP-specific CD8 T cells in the spleen was not significantly different in recipients of rNP-immune serum compared with control serum, and the frequency of PA-specific CD8 T cells was slightly lower in mice that received rNP-immune serum (Fig. 5C). Furthermore, the overall kinetics and magnitude of the CD8 T cell response in these mice remained largely unaffected compared with mice that received control serum (Fig. 5, D and E). Thus, the protection provided by rNP-immune serum does not correlate with a detectable modification of the CD8 T cell response.

To demonstrate that the protection conveyed by rNP-immune serum transfer is Ab-mediated, we immunized C57BL/6 and Ab-deficient $Aid/\mu S$ mice with rNP/LPS, transferred serum from these animals to naive $\mu MT$ recipients, and challenged them with influenza virus the following day. Recipients of rNP-immune serum from C57BL/6 mice lost only ~15% of their initial body weight and were recovering by day 11 postinfection; however, mice that received serum from rNP-immune $Aid/\mu S$ mice still lost >25% body weight and showed no recovery effects comparable to recipients of C57BL/6 control serum (LPS) (Fig. 6A). Additionally, rNP-immune serum from the $Aid/\mu S$ donors failed to reduce lung viral titers (Fig. 6B). These results clearly demonstrate that the protection against influenza infection conveyed by rNP-immune serum transfer is dependent upon Ab.

Finally, to address why previous studies found no protective effect of NP-specific Abs in $scid$ mice (4), we transferred rNP-immune serum into mice deficient in recombination-activating...
influenza resistance has been underappreciated. In part, this dis-
tinction (9, 14, 17, 31, 41), the potential for these Abs to facilitate
by vaccination with rNP.

An alternative strategy is to vaccinate with highly conserved
proteins in vaccine-mediated protection.

Collectively, these findings establish that Ab, in combination
with T lymphocytes, is a crucial effector underlying the protection
elicited by rNP vaccination. Furthermore, the results reveal a novel and
important role for non-neutralizing polyclonal Ab to internal,
conserved viral proteins in vaccine-mediated protection.

**Discussion**

Current influenza vaccines are designed to elicit neutralizing Ab
responses to external, mutation-prone molecules such as HA
and NA. However, these vaccines must be reformulated annu-
ally to account for antigenic drift, and they fail to provide sig-
nificant protection when the HA and NA proteins of the circu-
lating virus are substantially different than those in the vaccine.
An alternative strategy is to vaccinate with highly conserved internal influenza proteins, such as NP. Our data confirm that NP
vaccination alleviates morbidity and reduces viral load when the vaccinated C57BL/6 mice are challenged with live influenza virus. Although we immunized with a soluble protein Ag, we observed a modest acceleration of the NP-specific CD8
T cell response in the lung and spleen, suggesting that the Ag
was cross-presented and generated a memory precursor pool.
However, a rapid CD8 T cell expansion per se cannot be entirely
responsible for protection in these experiments, as CD40−/− and AID/μS mice, which make defective Ab responses, were not protected by rNP vaccination, despite the presence of responding NP-specific memory CD8 T cells in the lung. Furthermore, transferred rNP-immune serum reduces influenza-induced morbidity and viral load in an Ab-dependent fashion in otherwise naive μMT recipients, but not in naïve Rag1−/− recipients. Together, these data indicate that Abs play an unexpectedly important role in immune protection elicited by vaccination with rNP.

Despite high titers of specific Ab generated by NP vaccina-
tion (9, 14, 17, 31, 41), the potential for these Abs to facilitate influenza resistance has been underappreciated. In part, this dis-
regard is due to early studies that failed to show protective effects of NP-specific Abs in lymphopenic scid (4) and in BALB/c recipients (9). These results solidified the paradigm

that non-neutralizing Abs to NP do not contribute to protective
immunity from influenza virus. We have found that B cell-de-
cicient μMT mice can be protected by heterosubtypic immune serum (19) and, more specifically, by rNP-immune serum in an
Ab-dependent manner (Figs. 5–7). Compared with control mice such as C57BL/6, μMT mice are much more susceptible to the
pathogenic effects of influenza, likely due to poor Ab responses to
the virus (42), as well as a lack of influenza-reactive natural Ab (43). Thus, the protective effects that we transfer with a single dose of rNP-immune sera are readily observable in the absence of endogenous Ab responses. By contrast, intact recipient mice may require larger amounts of anti-NP Ab comparable to the titers observed in actively immunized C57BL/6 (Figs. 1 and 3) and BALB/c mice, which are each protected by NP vacc-
nation (Ref. 9 and data not shown). The failure to protect lymphopenic scid recipient mice with monoclonal anti-NP Ab in a previous report (4) is likely due to the absence of T cells. Importantly, we show that our polyclonal rNP-immune serum can protect T cell-sufficient μMT mice, but not T cell-deficient Rag1−/− mice (Fig. 7). This result not only explains the previous failure of anti-NP to protect lymphopenic scid recipients (4), but also demonstrates that T lymphocytes are an important component of the antiviral mechanism mediated by these Abs. The nature of the participating T cells, whether a characteristic of the CD8 response that was not detected or another T cell subset remains to be determined. Our data add to growing evidence that non-neutralizing Abs can contribute to protection from influenza challenge via multiple mechanisms (17, 19, 44, 45).

Another reason that the protective potential of anti-NP Abs has been dismissed is the assumption that effective Ab must neutralize virus and prevent its attachment to host cells. How-
ever, the Fc region of an Ab can activate a variety of antiviral effector functions (22, 24, 25, 46–49). In fact, non-neutralizing anti-influenza Abs, including anti-NP, have been shown to in-
duce complement-mediated cytolysis (21), increased T cell re-
ponses associated with enhanced dendritic cell function (20), and reduced viral replication in culture (45). Furthermore, NP-
LPS immunostimulating complexes given orally were shown to provide protection against virulent influenza challenge (50).

This protection correlated with high serum and bronchoalveolar lavage Ab titers, particularly IgA, and no CTL response was detected in the spleen or lung of challenged animals. Although no causal relationship was demonstrated, the results suggest that additional mechanisms may function depending upon the vaccine strategy and route of delivery. Therefore, NP-immune Ab in our model may be promoting early viral clearance through various mechanisms.

Influenza NP is internal to the virion and internal to influenza-
infected cells (51). Thus, it is not readily apparent how anti-NP
Abs in vaccinated mice would encounter this Ag. Nonetheless, anti-NP Abs are eventually generated during natural influenza vi-
rus infection (18, 19), indicating that this Ag is somehow exposed to the humoral immune system. This exposure may be via NP released from dying infected cells (52) or by its expression on the plasma membrane (53, 54). Therefore, interaction of vaccine-in-
duced anti-NP Abs with this NP early in the infection likely trig-
gers downstream effector mechanisms that blunt virus replication,
slow progression of the infection, and reduce morbidity.

In summary, our data clearly demonstrate that anti-NP Abs are essential for rNP-derived protection and that rNP-immune
serum can convey this protection to naive recipients. Impor-
tantly, these results show that a humoral immune response to a
single, conserved, internal protein of influenza virus makes a
significant contribution to protection. This information greatly enhances our understanding of how current influenza vaccines could be improved to provide cross-protective immunity in humans. If long-lived, this cross-protection would considerably reduce the effort and cost of providing annual immunizations, the cost of caring for infected individuals, and possibly provide a level of population immunity that would curtail the spread of pandemic influenza.

Acknowledgments

We thank Drs. Rachel Gersten and Ronald Corley for providing mutant mice and Drs. Frances Lund, Ravi Misra, and Javier Rangel-Moreno for helpful discussions and for reading this manuscript.

Disclosures

The authors have no financial conflict of interest.

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