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Universal Vaccine Based on Ectodomain of Matrix Protein 2 of Influenza A: Fc Receptors and Alveolar Macrophages Mediate Protection

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The ectodomain of matrix protein 2 (M2e) of influenza A virus is an attractive target for a universal influenza A vaccine: the M2e sequence is highly conserved across influenza virus subtypes, and induced humoral anti-M2e immunity protects against a lethal influenza virus challenge in animal models. Clinical phase I studies with M2e vaccine candidates have been completed. However, the in vivo mechanism of immune protection induced by M2e-carrier vaccination is unclear. Using passive immunization experiments in wild-type, FcRγ−/−, FcγRI−/−, FcγRIII−/−, and (FcγRI, FcγRIII)−/− mice, we report in this study that Fc receptors are essential for anti-M2e IgG-mediated immune protection. M2e-specific IgG1 isotype Abs are shown to require functional FcγRIII for in vivo immune protection but other anti-M2e IgG isotypes can rescue FcγRII−/− mice from a lethal challenge. Using a conditional cell depletion protocol, we also demonstrate that alveolar macrophages (AM) play a crucial role in humoral M2e-specific immune protection. Additionally, we show that adoptive transfer of wild-type AM into (FcγRI, FcγRIII)−/− mice restores protection by passively transferred anti-M2e IgG. We conclude that AM and Fc receptor-dependent elimination of influenza A virus-infected cells are essential for protection by anti-M2e IgG. The Journal of Immunology, 2011, 186: 000–000.

Vaccination against infectious diseases is one of the most effective medical interventions. Multiple mechanisms of immune protection exist, and which mechanism is predominant in a given vaccination/pathogen system is often unclear or controversial. Nevertheless, knowledge of the in vivo mechanism of action of vaccines could help to develop improved immune protection strategies against diseases for which effective vaccines are currently unavailable or inadequate. In addition, a better insight in the effector mechanisms is crucial for defining reliable correlates of protection for novel vaccines.

Influenza virus is responsible for seasonal influenza epidemics and infrequent, unpredictable pandemics. It has been estimated that each year 5–10% of the world population becomes infected with influenza, resulting in considerable public health and economic burdens (1). Annual influenza vaccination is recommended for specific population groups, such as the elderly and individuals suffering from diabetes or chronic respiratory or cardiovascular diseases (1, 2). Currently licensed influenza vaccines are tripartite and primarily aimed to provide protection by inducing Abs directed against influenza A and B virus hemagglutinin (HA) and possibly neuraminidase (NA) derived from two specific influenza A strains and one B strain. Hemagglutination-inhibiting Ab responses in serum are believed to correlate with protection, provided that the HA present in the vaccine corresponds fairly closely to that of the influenza strain circulating in the field (3–5). However, the antigenic properties of HA and NA from epidemic influenza viruses vary repeatedly over time, a process known as antigenic drift driven by escape selection from the adaptive immune response in the population (6, 7). Therefore, the composition of human influenza vaccines has to be revised each year (once for the northern and once for the southern hemisphere) and adapted according to the results of global surveillance performed by World Health Organization influenza reference laboratories. Surveillance and serological characterization of circulating influenza strains, recently supported by antigenic cartography, allows predicting with a reasonable success rate the identity of the epidemic influenza strains that are likely to circulate in a subsequent year, although vaccine mismatches still occur occasionally (8, 9).

Vaccines that target conserved influenza virus proteins can protect against multiple influenza A virus subtypes in animal models and, pending further successful clinical development, remain promising complements or alternatives for the HA-based, strain-specific vaccines currently used (10). We previously described a universal influenza A virus vaccine based on the conserved ectodomain of matrix protein 2 (M2e) (11). The M2e sequence is conserved across all known human influenza A viruses (12).
first 9 aa residues are almost completely conserved between human and avian influenza A viruses, and 7 out of the 15 remaining amino acid residues appear to be well tolerated at these positions in M2e. Mice vaccinated with M2e-carrier constructs and subsequently challenged with homosubtypic or heterosubtypic influenza virus infection have much reduced morbidity and decreased lung virus titers and are protected from virus-induced death (11). M2e-specific Abs are responsible for the protective immunity induced by M2e vaccination. Indeed, protective immunity can be transferred from vaccinated animals to naive recipients by serum (11, 13–16). Moreover, anti-M2e mAbs protect wild-type and immunodeficient scid recipient mice against influenza A virus infection (17–19). Although a contribution by M2e-specific T cell responses to protection cannot be ruled out, M2e-specific serum Abs are crucial for immune protection. But how anti-M2e Abs protect against an influenza A virus challenge is still unclear. M2 is sparsely present on virions but abundantly on virus-infected cells (20, 21), and influenza infectivity in tissue culture cannot be neutralized by anti-M2e Abs. The monoclonal anti-M2e Ab14C2 (IgG1) has been reported to inhibit plaque size growth of some influenza strains in vitro (20). However, other strains, such as PR8 and WSN, which were not susceptible to the 14C2 effect on plaque growth, were equally suppressed in vivo in M2e-VLP–immunized mice as in other strains (11). Because anti-M2e Abs do not neutralize influenza viruses, M2e-based vaccines are considered infection permissive.

Abs in immune serum can bind M2 on the surface of infected cells, but the ensuing mechanism of protection by anti-M2e Abs remains poorly understood. Contradictory findings have been reported regarding the involvement of Ab-dependent cell-mediated cytotoxicity (ADCC) by NK and/or NKT cells (15, 16). In addition, protection of mice by passive transfer of M2e–specific human IgG1 (homologous to murine IgG2a) mAb was dependent on a functional FcyRIII and complement pathway, whereas a human IgG4 (homologous to murine IgG1) anti-M2e Ab failed to protect mice (19). In contrast, Jegerlehner et al. (15) excluded a role for complement based on the observation that C3-deficient mice were as protected as wild-type mice by passively transferred mouse anti-M2e hyperimmune serum.

In general, mouse IgG2a and IgG2b Abs restrict viral infections better than IgG1 Abs (22, 23). This is in line with the requirement for Th1 cytokine responses, such as IFN-γ, for Ig class switching and associated enhancement of cellular antiviral responses (22–24). This is further confirmed by the observation that a subtype switch from IgG1 anti-M2e monoclonal 14C2 to the switch variant anti-M2e IgG2a improves its protective efficacy in vivo (17, 25).

We report on the crucial role of Fc receptors in anti-M2e IgG-mediated protection by using four different mutant BALB/c mouse strains with the same genetic background: FcRγ−/−, FcγRI−/−, FcγRII−/−, and FcγRI, FcγRIIId−/−. In addition, we demonstrate that both murine anti-M2e IgG1 and IgG2a/2b isoforms can protect mice from influenza A virus challenge. FcγRIIId−/− mice were also protected by IgG2a Abs directed against M2e, indicating that, at least for these isoforms, an Fc receptor-mediated mechanism did not involve NK cells, which, in mouse, exclusively express FcγRII. In contrast, anti-M2e IgG1 Abs failed to protect FcγRIIId−/− mice. Finally, by using an in vivo cell-depletion method and by adoptive transfer of wild-type alveolar macrophages into (FcyRI, FcγRIIId−/−) animals, we identified a critical role for alveolar macrophages in protection by anti-M2e Abs.

Materials and Methods

Mice

BALB/c mice were purchased from Harlan, BALB/c FcRγ−/− (encoded by FcεRIγ) mice were from Taconic Farms, and C57BL/6 mice were from Janvier. The mice were housed in individually ventilated cages in a specific-pathogen–free (SPF) animal house. The following mice were bred in an experimental animal facility of DMBR under non-SPF conditions and genotyped by a PCR protocol using genomic DNA from individual animals: FcγRI−/− (encoded by FcγRI), FcγRIIIb−/− (encoded by FcγRII), and FcγRI, FcγRIIIad−/− (all in a BALB/c genetic background). All mice were housed in a temperature-controlled environment with 12 h light/dark cycles and received water and food ad libitum. For experiments, adult SPF- (wild-type and FcγR−/−) BALB/c and non-SPF–kept strains were compared, the SPF-housed mice were transferred 3 wk in advance to the non-SPF environment. Eight-week-old female mice were used in all experiments. All animal experiments were done under conditions specified by law (European Directive and Belgian Royal Decree of November 14, 1993) and reviewed and approved by the Institutional Ethics Committee on Experimental Animals.

Passive immunization and virus challenge

To prepare high-titer M2e-immune serum, BALB/c mice were immunized with the vaccine construct 1818, an M2e–hepatitis B virus core (HBc) fusion protein containing three tandem copies of M2e fused to HBc, truncated at amino acid residue 150 (26, 27). Ten micrograms M2e–HBc vaccine was formulated with Ribi adjuvant (MPL + TDM; Sigma-Aldrich, St. Louis, MO) as previously described (11, 27), and mice were immunized three times i.p. at 3-wk intervals. Two weeks after the last immunization, the mice were killed by cervical dislocation, and blood was collected by cardiac puncture. Serum was prepared by clotting the blood at 37°C for 30 min followed by centrifugation. Anti-M2e immune ascites fluid was prepared by immunizing BALB/c mice i.p. with 1818 vaccine formulated with IFA. Between the second and third immunization, a 180/TG sarcoma cell suspension was injected i.p. to induce the formation of ascites fluid, which was harvested 12 d after injection of cells. The resulting ascites fluid contained anti-M2e IgG1, IgG2a, IgG2b, and IgG3 subtype Abs as determined with an M2e-peptide ELISA (27). Immune sera or purified IgG isotype fractions were injected i.p. (200 μl/mouse, unless stated otherwise) in naive mice. After 24 h, the mice were anesthetized with a mixture of xylazine (10 μg/g) and ketamine (100 μg/g) and challenged by intranasal administration of 50 μl PBS containing four LD90 of mouse-adapted X47 (PR8 × A/Victoria/5/75) or A/PR/8/34 influenza A virus, as indicated in the figure legends. Mortality and morbidity (weight loss) were monitored for 14 d after challenge.

IgG isotype separation

Total IgG from 30 ml anti-M2e immune ascites was bound on an MEP Hypercell column (Pall, Zaventem, Belgium) equilibrated with 50 mM NaH2PO4 (pH 8), using an Akta explorer chromatography system (GE Healthcare, Diegem, Belgium). Under these conditions, all IgGs present in the ascites fluid were captured on the column, whereas most contaminating proteins were in the flow-through fraction. After washing with water, IgGs were eluted from the protein A column using a pH step gradient isotypes were eluted from the MEP column using a pH step gradient isotypes was confirmed by using three mouse mAbs directed against DNP-specific Abs, and the eluted material was immediately neutralized with Tris-HCl buffer (pH 8.8). IgGs were then fractionated on an rProtein A-Sepharose FF column (GE Healthcare, Diegem, Belgium) equilibrated with 50 mM NaH2PO4 (pH 8), using an A˚kta explorer chromatography system (GE Healthcare, Diegem, Belgium). Under these conditions, all IgGs present in the ascites fluid were captured on the column, whereas most contaminating proteins were in the flow-through fraction. After washing with water, IgGs were eluted in 50 mM citric acid buffer (pH 3), and the eluted material was immediately neutralized with Tris-HCl buffer (pH 8.8). IgGs were then fractionated on an rProtein A-Sepharose FF column (GE Healthcare). Following equilibration with 50 mM NaH2PO4 buffer (pH 8), IgGs eluted from the MEP Hypercell column were loaded on the protein A column. IgG isoforms were eluted from the protein A column using a pH step gradient with buffers of pH 4.5, 4, and 3. At each pH step, elution was repeated three times with one column volume. The column was then flushed with one column volume of 50 mM NaH2PO4 buffer (pH 8) before elution at the next pH step. This purification procedure resulted in a pure IgG1 fraction eluting at pH 4.5, a mixture of IgG1 and IgG2a in the pH 4 eluate, and a mixture of predominantly IgG2b and residual IgG1 and IgG2a in the pH 3 eluate. This profile was determined using a M2e–peptide ELISA and a set of peroxidase-conjugated Abs specific for mouse isotypes IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Birmingham, AL). The specificity of these secondary Abs for the different mouse IgG isoforms was confirmed by using three mouse Abs directed against DNP (2,4-DNP) of different subclasses (IgG1, IgG2a, and IgG2b). The Abs were kindly provided by Dr. J.-P. Coutelier (Université Catholique de Louvain-la-Neuve, Louvain, Belgium). ELISA plates were coated with 1 μg/ml DNP-albumin to capture these IgG mAbs, and the captured Abs were subsequently used to evaluate the specificity of the subclass-specific secondary Abs used in this study (Supplemental Fig. 2).
Depletion of alveolar macrophages

Liposomes containing dichloromethylene diphosphonate (clodronate) or PBS were prepared as described previously (28). For liposome administration, BALB/c or C57BL/6 mice were anesthetized by i.p. injection with ketamine/xylazine and placed in a supine position at a head-up angle of ~30°. One million microliters PBS-liposomes or clodronate-liposomes were administered slowly intratracheally (i.t.) via the mouth using a gel loading tip attached to a Gilson pipette. Depletion of alveolar macrophages (AM) was determined in control experiments by bronchoalveolar lavage (BAL) 24 h after liposome administration under anesthesia with an i.p. injection of avertin (2.5% in low endotoxin PBS). A 23-gauge cannula was installed into the trachea, and cells were collected by washing the airway lumen with ~2 × 0.5 ml HBSS. After cyto centrifugation, cells were stained on cytocentrifugation preparations with May-Grünwald-Giemsa (Sigma-Aldrich). Twenty-four hours after PBS-liposome or clodronate-liposome administration, mice were injected i.p. with 400 μl anti-M2e–HbC hyperimmune or naive control mouse serum. After 24 h, a blood sample was taken from each mouse to determine the serum anti-M2e IgG titer by peptide ELISA, and mice were challenged with 4 LD50 mouse-adapted X47 virus as described above.

Adoptive transfer of alveolar macrophages

AM were isolated by BAL with HBSS–EDTA (HBSS with 0.5 mM EDTA) of BALB/c and (FcγRI, FcγRII)−/− mice. Twenty mice were sacrificed as donors for every group of 12 mice. A 23-gauge cannula was inserted into the trachea, and cells were collected by washing the airway lumen with ~3 × 0.5 ml HBSS–EDTA. The obtained BAL fluid was centrifuged, and cells were washed twice with PBS, counted, and resuspended in PBS at a density of 10^6 cells/ml. For adoptive transfer, mice (BALB/c wild-type and [FcγRI, FcγRII]−/−) were anesthetized by i.p. injection of ketamine/xylazine and placed in a supine position at a head-up angle of ~30°. One hundred microliters PBS containing 10^6 cells was administered slowly i.t. using a gel loading tip attached to a Gilson pipette. Wild-type mice received AM from (FcγRI, FcγRII)−/− mice and, reciprocally, (FcγRI, FcγRII)−/− mice received AM from wild-type mice. Twenty-four hours after injection, two times six mice were injected i.p. with 400 μl anti–M2e–HbC hyperimmune serum and two times six mice with 400 μl PBS. After 24 h, a blood sample was taken from each mouse to determine the serum anti-M2e IgG titer by peptide ELISA, and mice were challenged with 2 LD50 mouse-adapted X47 virus.

FACS analysis of single lung cell suspension

Single lung cell suspensions from mice treated with clodronate- or PBS-encapsulated liposomes were prepared and analyzed as follows. One day after treatment, mice were sacrificed by cervical dislocation, and lungs were removed aseptically, cut in small pieces, and incubated with collagenase D (100 μg/ml) in 500 μl PBS at 37°C. After 45 min, the cell suspensions were filtered through a 70-μm cell strainer and treated with a 190-μg/ml HBSS–EDTA. The obtained BAL fluid was centrifuged, and cells were resuspended in PBS (1 ml) with 10^6 cells was administered slowly i.t. using a gel loading tip attached to a Gilson pipette. Wild-type mice received AM from (FcγRI, FcγRII)−/− mice and, reciprocally, (FcγRI, FcγRII)−/− mice received AM from wild-type mice. Twenty-four hours after injection, two times six mice were injected i.p. with 400 μl anti–M2e–HbC hyperimmune serum and two times six mice with 400 μl PBS. After 24 h, a blood sample was taken from each mouse to determine the serum anti-M2e IgG titer by peptide ELISA, and mice were challenged with 2 LD50 mouse-adapted X47 virus.

Cell-mediated cytotoxicity assay

Splenocytes were prepared from mice sacrificed on days 1, 2, and 4 after i.t. treatment with clodronate- or PBS-encapsulated liposomes and assayed for NK and macrophage cytotoxicity using Yac-1 and L929 cells as targets, respectively. One million target cells were labeled with Calcein-AM (5 μM; Invitrogen) for 30 min and transferred to a 96-well round-bottom plate at 100,000 target cells per well. Spleen cells were then added as effectors at E:T ratios of 100, 50, 25, and 12.5. After 5 h of incubation at 37°C, the fluorescence at 485 nm in the cell supernatant was measured using a fluorometer (CytoFluor, PerSeptive Biosystems, Framingham, MA). The spontaneous release is defined as the fluorescence in the supernatant of Calcein-AM–labeled target cells without effectors; maximum release is the fluorescence in the supernatant of Calcein-AM–labeled target cells treated with 1% Triton X-100. The percentage of killing was calculated as follows: percentage of killing = [(fluorescence, experimental release) – (fluorescence, spontaneous release)]/[(fluorescence, maximal release) – (fluorescence, spontaneous release)]. All tests were carried out in triplicate.

Statistics

Statistical significance of differences between survival rates was analyzed by comparing Kaplan-Meier curves using the log-rank test and MedCalc Statistical Software (MedCalc, Mariakerke, Belgium). A value of p < 0.05 was considered statistically significant.

Results

Protection by anti-M2e IgG Abs requires FcγRI

Previous studies have demonstrated a strong correlation between the levels of anti-M2e IgG Abs in serum induced by M2e-based vaccines and protection against a potentially lethal influenza A virus challenge (11, 13, 16, 26). In addition, passive transfer of anti-M2e mAbs or M2e immune serum can protect mice from influenza A virus challenge (11, 18, 19). To better understand the in vivo protective mechanism of M2e-specific humoral immunity, we compared the protective efficacy provided by passive transfer of anti-M2e immune serum into wild-type and FcγRII−/− BALB/c mice (29). This FcγRII subunit is required for Ab-mediated phagocytosis by macrophages and for NK-dependent ADCC. Twenty-four hours after passive transfer and before challenge, anti-M2e IgG titers and Ab isotype distribution were similar in wild-type and FcγRII−/− animals (Fig. 1A). As expected, wild-type recipient mice were protected from challenge with 4 LD50 mouse-adapted X47 virus and displayed only transient weight loss (Fig. 1B, 1C). By contrast, all but one FcγRII−/− mice (n = 10) died of influenza infection despite the presence of mouse anti-M2e immune serum (Fig. 1B). This lack of protection was statistically highly significant (p < 0.0001 by Kaplan-Meier survival analysis) and was also reflected in the morbidity profile, with mutant mice displaying faster weight loss during the infection (Fig. 1C). In addition, on day six after challenge with 1 LD50 mouse-adapted X47 virus, the lung virus titer was 40 times higher in FcγRII−/− than in wild-type mice (data not shown). To exclude the possibility that FcγRII−/− mice are intrinsically more sensitive to influenza A virus challenge, the lethality of different viral challenge doses was compared in naive wild-type and mutant animals. As shown in Fig. 1D (and in agreement with Ref. 30), both mouse strains were equally susceptible to influenza A virus challenge. Because the FcγRII-γ-chain is essential for assembly and intracellular signaling of the activating receptors FcγRI, -III, and -IV, we conclude that one or more of these three known receptor(s) associated with the common γ-chain is/are essential for anti-M2e IgG-mediated immune protection.

FcγRI and FcγRIII play a partially redundant role in protection by M2e-specific Abs

To identify the FcγR(s) that mediate protection by M2e-specific Abs, we performed passive transfer experiments in FcγRI−/−, FcγRII−/−, and (FcγRI, FcγRII)−/− knockout mice, followed by lethal influenza A virus challenge. Wild-type and FcγRI−/− mice were included as controls. All animals had BALB/c genetic background and were of the same age (8 to 9 wk) and sex (female) to eliminate or reduce as much as possible any influence of these variables on the outcome (31). To allow optimal detection of protective efficacy, we used a relatively low-titer ascites preparation containing anti-M2e IgG1, IgG2a, IgG2b, and IgG3 isotype Abs. Twenty-four hours after passive transfer of this immune serum and before challenge, blood samples were taken from individual mice for analysis. An M2e-peptide ELISA revealed comparable IgG titers against M2e peptide in all five recipient mouse strains (n = 12 per group) on the day of challenge (Fig. 2A). In agreement with the results reported above, 10 out of 12 wild-type mice were protected following challenge with mouse-adapted X47 (H3N2) virus, whereas only 1 out of 12 FcγRI−/− mice survived the challenge, although the animals had similar anti-M2e Ab levels (Fig. 2B; p < 0.005). In this experiment, (FcγRI, FcγRIII)−/− mice were
significantly less protected than wild-type mice ($p = 0.005$) and FcγRIII$^{-/-}$ mice ($p = 0.01$). The kinetics of death after challenge in these double-knockout mice was also significantly slower compared with FcγRII$^{-/-}$ mice ($p = 0.05$). However, 2 wk after challenge, mortality was similar in the two strains. Protection from death in FcγRIII$^{-/-}$ mice (9 out of 12 mice survived) was similar to that in wild-type animals (10 out of 12 mice survived). Only 50% of the FcγRI$^{-/-}$ mice, however, survived the challenge, but this survival rate was not significantly different from that of the wild-type group ($p = 0.09$) or the (FcγRI, FcγRIII)$^{-/-}$ mice ($p = 0.21$). Similar results were obtained in a second passive transfer experiment followed by challenge with mouse-adapted PR8 virus (H1N1) (Supplemental Fig. 1). From these results, we conclude that loss of only FcγRI or only FcγRIII does not result in an appreciable drop in protection, whereas knockout of both FcγRI and FcγRIII strongly reduces protection by anti-M2e Abs against influenza virus.
challenge. Presumably, loss of FcγRI or FcγRIII alone is compensated sufficiently by other FcγRs. The difference in survival between the double-knockout and FcγRIγ-deficient mice was statistically significant in both experiments (p < 0.05, X47 challenge; and p < 0.005, PR8 challenge), suggesting a possibly auxiliary role for FcγRIV, which preferentially binds IgG2a and IgG2b Abs (32), in anti-M2e Fcγ-dependent immune protection.

Anti-M2e IgG1 protects wild-type but not FcγRIIγ−/− mice

A role for NK cells but not for complement (15) and a role for both complement and ADCC (19) in anti-M2e Ab-mediated protection have been reported. Following passive transfer of anti-M2e Abs, we observed only a small reduction in protection against influenza A virus challenge in mice lacking FcγRIII (Fig. 2B). The affinity of IgG for a particular Fc receptor differs according to the IgG isotype (33, 34). Therefore, the protective polyclonal M2e immune serum and both of the separated IgG isotype fractions were first characterized by i.p. injection in wild-type mice. Care was taken to administer equivalent anti-M2e titers of each given IgG isotype to all mice (Fig. 3A). One day after passive transfer, mice were infected with 4 LD50 PR8 virus. The total anti-M2e immune serum and the IgG1 fraction, as well as the IgG1 and IgG2a fraction, protected BALB/c mice against an otherwise lethal virus challenge dose (Fig. 3B).

Next, we compared the protection by total serum and by the IgG1 fraction in FcγRIIγ−/− BALB/c mice. The Fc-dependent in vivo activity of mouse IgG1 Abs is mediated almost exclusively by the low affinity FcγRIII receptor (35, 36). Consistent with the results mentioned above (Fig. 2B), FcγRIIγ−/− mice were well protected by total anti-M2e immune serum (Fig. 3C). By contrast, purified anti-M2e IgG1 failed to protect FcγRIIγ−/− mice (p < 0.005, Kaplan-Meyer survival analysis, compared with FcγRIIIγ−/− mice receiving total serum). Protection from a lethal influenza infection was restored in the FcγRIIγ−/− animals that received the pH 4 eluate fraction containing anti-M2e IgG1 and IgG2a, indicating that interactions between anti-M2e IgG2a Abs and possibly (an)other Fc receptor(s) can compensate for the loss of IgG1–FcγRIII cooperation in these knockout mice (Fig. 3B). On the basis of these results, we conclude that protection of mice against a lethal influenza A virus challenge by anti-M2e IgG1 Abs requires FcγRIII. However, IgG2a Abs, which bind with high affinity to FcγRI and FcγRIV, can confer full protection in the absence of FcγRIII.

Protection by anti-M2e Abs depends on alveolar macrophages

FcγRIII is expressed on macrophages, neutrophils, eosinophils, NK cells, and a subset of T cells (37). It has been shown that phagocytosis by macrophages of IgG1-opsonized but not IgG2a- and IgG2b-opsonized cells critically depends on FcγRIII (38). In addition, macrophages are known to contribute to phagocytosis of influenza virus-infected cells (39) and to clearance of influenza virus by Abs raised by immunization with a conventional subunit vaccine (30). Therefore, we assessed the contribution of AM to protection by anti-M2e IgG Abs. AM were eliminated by i.t. instillation in BALB/c mice of clodronate-loaded liposomes, whereas control mice received PBS-loaded liposomes. After 24 h, the mice were passively immunized with either control or anti-M2e immune serum and subsequently challenged with 4 LD50 mouse-adapted PR8 virus.

M2e immune serum and subsequently challenged with 4 LD50 mouse-adapted X47 virus. Serum anti-M2e IgG levels in mice treated with PBS-liposomes or clodronate-liposomes were
FIGURE 4. Alveolar macrophages are required for protection by anti-M2e IgGs. A, BALB/c mice were treated i.t. with PBS-loaded or clodronate-loaded liposomes (n = 12 per group). Twenty-four hours later, mice received murine anti-M2e immune serum by i.p injection. The left bar in the graph represents the anti-M2e IgG titer in the immune serum that was used for i.p injection. The two bars on the right show the mean M2e-specific IgG endpoint titers (± SD) in serum prepared from blood samples taken 2 d after anti-M2e serum injection of PBS- or clodronate-treated animals. B, Top panel: cytospin of cells in the BAL of mice treated with PBS-liposomes or clodronate-liposomes. Cells were stained with May-Grunwald-Giemsa. Insets: left panel, intact AM; right panel, apoptotic alveolar macrophage. Bottom panel: F4/80 expression determined by flow cytometry of single lung-cell suspensions prepared from mice treated i.t. with PBS-loaded or clodronate-loaded liposomes. The histograms are representative of six mice per group. C, Survival (top panel) and morbidity (bottom panel) of BALB/c mice receiving PBS or anti-M2e murine serum (anti-M2e IgG), depleted or not depleted of AM by i.t. administration of clodronate-loaded or PBS-loaded liposomes, and subsequently challenged with 4 LD₅₀ mouse-adapted X47 virus. Mice were treated with liposomes on
IgG in Fcγ Abs protect mice from influenza A virus challenge by a mechanism performed in C57BL/6 mice confirmed that AM are critical for the protection by anti-M2e IgG (Supplemental Fig. 3). Intratracheal treatment with clodronate-loaded liposomes did not affect the cytoidal activity of NK cells and macrophages in the periphery, as demonstrated using a YAC-1 and L929 cell killing assay, respectively (Fig. 4D). Therefore, we conclude that anti-M2e IgG Abs protect mice from influenza A virus challenge by a mechanism that requires AM.

We next reverted the impairment of protection by anti-M2e IgG in FcγR-deficient mice by adoptive transfer of wild-type AM in the lung compartment. (FcγRI, FcγRIII)−/− mice were only slightly better protected than FcγRIγ−/− mice against influenza A virus challenge after passive transfer of anti-M2e IgG (Fig. 2). Because (FcγRI, FcγRIII)−/− mice were breeding much better than FcγRIγ−/− mice, we used this double-knockout strain as recipients for wild-type AM. Reciprocally, wild-type mice received AM from (FcγRI, FcγRIII)−/− donors. After 24 h, the mice were passively immunized with either control or anti-M2e immune serum and challenged the following day with 2 LD50 mouse-adapted X47 virus (Fig. 5A). Anti-M2e IgG recipient mice had the same anti-M2e IgG serum titer on the day of challenge (Fig. 5B). Intratracheal instillation of wild-type AM from wild-type naive donor mice restored protection against X47 virus challenge in the presence of anti-M2e IgG (Fig. 5C). Anti-M2e IgG-treated wild-type mice that had received AM from (FcγRI, FcγRIII)−/− donors were also protected, whereas all PBS treated mice died. From this experiment, we conclude that adoptively transferred wild-type AM into the lungs of (FcγRI, FcγRIII)−/− mice restore protection by anti-M2e IgG in these knockout mice, indicating that Fc receptors on AM play a key role in immune protection.

Discussion

In this study, we demonstrate that Fc receptors are essential for anti-M2e IgG-mediated immune protection against influenza A virus challenge. To document the functional interaction between Fc receptors and anti-M2e IgGs, we used passive transfer of anti-M2e immune serum into wild-type mice and four knockout mouse strains, FcγRIγ−/−, FcγRI−/−, FcγRIII−/−, and (FcγRI, FcγRIII)−/−, matched for strain, sex, and age. To identify the role of the respective IgG isotype subclasses, we preferred to use a fractionated polyclonal M2e immune serum rather than isotype variants of one particular mAb because the humoral immune response against M2e vaccine is polyclonal (40). We identified an essential functional interaction between anti-M2e IgG1 Abs and FcγRIII in the protection in the mouse model, whereas IgG2a might operate via FcγRI and/or FcγRIII. Our results also provide evidence for a crucial role of AM in the mechanism of protection provided by anti-M2e Abs against influenza A virus challenge.

Our finding of a critical role for FcγRs in protection by anti-M2e IgGs against a potentially lethal influenza A virus challenge indicates that ADCC and Ab-dependent cell-mediated phagocytosis are the main mechanisms involved. Protection by M2e-based vaccines depends on Abs (11, 15, 16). Unlike anti-HA Abs, anti-M2e Abs do not prevent influenza virus infection in vitro, and reduction of plaque size growth in the presence of an anti-M2e mAb has been documented for certain strains but not for others, such as PR8 virus, a challenge virus that we also used in this study (11, 14, 20). M2 protein is expressed at least as abundantly as NA on the surface of infected cells, but much less M2 protein molecules are incorporated into the virion compared with HA or NA (12, 20). Therefore, anti-M2e IgGs probably provide protection by interacting with virus-infected cells rather than by interacting with viral particles. Through its Fc region, anti-M2e IgG can bind to and cross-link FcγRs expressed on myeloid cells. Depending on the balance between engagement of activating or inhibitory IgG-FcγRs, anti-M2e immune complexes could trigger immune effector cell activation, resulting in killing and/or phagocytosis of infected cells and thereby suppressing progeny virus formation and spread (34). Mice have the activating Fc receptors FcγRI, FcγRII, and FcγRIV and the inhibitory Fc receptor FcγRII. Individual Fc receptors bind IgG subclass Abs with different affinities, and the ratio of activating to inhibitory Fc receptor binding is thought to determine the biological efficacy of a given IgG subclass Ab (34). We found that elimination of the common γ-chain, which is essential for intracellular signaling of the three activating Fc receptors, abrogated protection by anti-M2e IgG. The loss of protection in the FcγRIγ−/− mice was nearly complete, and it is doubtful that an additional protection mechanism not involving FcγR contributes substantially. There was, however, a significant difference in protection between FcγRIγ−/− and (FcγRI, FcγRIII)−/− mice, suggesting an additional contribution to protection by an FcγRIV-mediated mechanism; FcγRIV is the most recently discovered FcγR family member (32). We also demonstrated that purified M2e-specific IgG1 failed to protect FcγRIII−/− animals from a lethal influenza challenge. This is in line with the reported phenotype of this knockout mouse and with the very low affinity of the Fc domain of IgG1 for FcγRI and FcγRIV (34, 35). It is of interest to note that protection by Abs induced by immunization with conventional HA- and NA-based vaccines also critically depends on Fc receptors, despite the well known fact that anti-HA Abs can have virus-neutralizing activity in vitro (30). Neutralization is measured in vitro and implies that the Abs can inhibit entry of virus into target cells. However, based on in vivo studies, it is likely that neutralizing antiviral Abs also act in concert with cellular effector functions—for example, by opsonizing virions, which are subsequently eliminated by phagocytes or by Ab-dependent killing of infected cells that display viral membrane proteins at the cell surface, as was proposed for anti-HIV surface Ag-specific Abs (41). Our results indicate that infected cells are
eliminated by anti-M2e IgG-mediated cellular cytotoxicity or phagocytosis because these cells express M2 abundantly at their surface early postinfection. This mechanism allows removal of infected cells before progeny virus budding and spread and explains the consistent drop in lung virus titers that we and others observed in independent studies using experimental vaccines eliciting anti-M2e Abs (11, 14–16, 18, 27, 42–44). Opsonization of virions by anti-M2e IgG followed by virion clearance might also contribute to in vivo immune protection but is probably less effective given the low abundance of M2 molecules compared with HA and NA on influenza viral particles (20).

Which cells are responsible for elimination of M2-expressing cells in the presence of anti-M2e Abs? Jegerlehner et al. (15) proposed an important role for NK-mediated Ab-dependent cytotoxicity in acquired anti-M2e immunity. However, two recent studies concluded that NK cells are not essential for protection by anti-M2e IgGs or following vaccination with M2 expression vectors (16, 43). All three studies made use of Ab administration to eliminate NK and NKT cells, either anti-NK1.1 (43) or anti-asialo-GM1 (15, 16), but used different mouse strains [C57BL/6 (15, 43) and BALB/c (16) mice] and different influenza virus challenge strains. Also, mice were actively immunized with an rM2e-HBc protein vaccine (15), with M2-DNA followed by a boost with an rM2-expressing adenoviral vector (16), or passively with anti-M2e mAbs (43). Considering these differences in experimental protocols, it remains unclear whether there is a role for NK cells in anti-M2e protective immunity induced by vaccination. Our results provide no evidence for a contribution by NK cells to protective immunity, because NK cells from FcγRIII−/− mice do not exhibit ADCC (38), and yet these mice were protected to the same extent as wild-type mice after passive transfer of anti-M2e IgG (Fig. 2B). However, an accessory role of NKs (e.g., acting in concert with AM and anti-M2e IgG) remains possible and will require additional studies.

We demonstrated that AM play a pivotal role in the protection by anti-M2e IgGs. AM are resident in the lung and are believed to be among the first immune cells that encounter microbes in the respiratory tract. These cells control the induction of primary cytotoxic T cell responses (45) and the level of antiviral lung cytokines, such as TNF, IFN-α, and IFN-γ during influenza virus infection of naive mice (46). It is likely that these innate antiviral functions were also affected by selectively depleting AM with i.t. instilled clodronate-loaded liposomes. However, in our experimental system, elimination of AM altered neither the morbidity nor the mortality profile of control mice following challenge (Fig. 4, Supplemental Fig. 3). It may be concluded that in combination with pre-existing anti-M2e Abs and, as demonstrated in this study, in a process requiring FcγRs, AM suppress influenza virus infection and spread by eliminating infected cells more effectively and rapidly than in naive animals. It has been shown that macrophages can eliminate apoptotic, influenza virus-infected cells in vivo by phagocytosis, a process that appears to be promoted by apoptosis of the target cells (39). Infiltration and activation of lymphocytes is rapidly initiated following influenza virus infection of naive lymphocytes (47). It is possible that scavenger cells that infiltrate the lungs soon postinfection also act in concert with anti-M2e IgGs to control and eliminate influenza virus. However, our results reveal that resident AM are the most critical effector cells responsible for anti-M2e immunoprotection. Anti-M2e IgG-dependent cytotoxicity directed against infected cells by ADCC and/or Ab-dependent cell-mediated phagocytosis could thus operate at a very early stage.

**FIGURE 5.** Wild-type AM can restore protection by anti-M2e IgG in (FcγRI, FcγRIII)−/− mice. A, Overview of the protocol for adoptive transfer of alveolar macrophage. Wild-type BALB/c mice (n = 12) and (FcγRI, FcγRIII)−/− mice (n = 12) received AM from (FcγRI, FcγRIII)−/− and wild-type BALB/c mice, respectively. Twenty-four hours later, six mice from each of these two groups received anti-M2e IgG and six mice PBS by i.p. injection. B, Anti-M2e serum IgG titers in wild-type and (FcγRI, FcγRIII)−/− mice prior to challenge. Serum was prepared from blood samples taken 24 h after i.p. injection of anti-M2e IgG in wild-type (n = 6) and (FcγRI, FcγRIII)−/− (n = 6) mice. The anti-M2e IgG1 and IgG2a titers of the pooled serum samples were determined by M2e peptide ELISA. The bars for the anti-M2e serum represent the anti-M2e IgG titer in the immune serum that was used for i.p. injection. C, Survival (top panel) and morbidity (bottom panel) of mice treated as in A after challenge with 2 LD50 mouse-adapted X47 virus. The morbidity curve shows the percentage mean body weight relative to the weight on the day of challenge (day 0) ± SD.
postinfection with influenza A virus, allowing maximal impact on virus propagation.

Ab-immune complexes trigger the classical pathway of complement activation, resulting in cell lysis. Jegerlehner et al. (15), examining survival after virus challenge of C3−/− mice, concluded that complement does not play an important role in protection by passively transferred murine anti-M2e immune serum, whereas Wang et al. (19), using passive transfer of a human anti-M2e mAb in C3−/− mice, concluded that complement was needed to control lung virus titers in challenged mice. Our results suggest only a negligible, if any, contribution by complement to anti-M2e Ab protection. Indeed, FcRγI−/− and (FcγRI, FcγRIII)−/− mice, which have normal complement, were not protected after X47 or PR8 challenge in the presence of murine anti-M2e IgGs.

What do these findings imply for further clinical development of M2e-based vaccines? People who have been exposed to influenza A virus infection will likely have HA- and NA-specific Abs in circulation that might sterically hinder anti-M2e Abs. However, this is highly unlikely because HA and NA are localized in GM1-rich microdomains on the surface of infected cells, whereas M2 is largely excluded from these raftlike structures, implying that anti-M2e Abs can bind M2 on the surface of infected cells where less HA and NA is expected (48, 49). For a prophylactic vaccine, it is important to define a measurable correlate of protection that allows prediction of the likelihood that an immunized subject will be protected from the deleterious consequences of infection by the corresponding pathogen. Supposing that our findings can be extrapolated to humans, this would imply that such a correlate should rely on a quantitative assay of phagocytosis or killing using M2-expressing cells as targets, (activated) FcγR-expressing cells such as macrophages as effectors, and human anti-M2e IgG from vaccinated individuals as the variable. The efficacy of an M2e vaccine in humans, defined as the reduction in incidence of illness following influenza A virus infection in an immunized group compared with a control group, remains to be demonstrated. The data reported in this study on the in vivo mechanism of action in an animal model for influenza highlight the essential interplay between FcγR-bearing cells and anti-M2e IgG in controlling the clinical outcome of the infection. It will be important to identify similar mechanisms of immune protection in humans to establish a true correlate of protection for this universal influenza A vaccine.

Acknowledgments

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Supplemental figure legends

Supplemental figure 1. Wild type, FcγRI\(^{-/+}\), FcγRIII\(^{-/+}\), (FcγRI, FcγRIII)\(^{-/-}\), and FcR\(\gamma^{\circ}\) mice received M2e immune serum by i.p. injection (n = 12 for each group) and were challenged 24 h later with 4LD\(_{50}\) of mouse-adapted PR8 virus. Strongest protection was observed in wild type, FcγRI\(^{-/-}\) and FcγRIII\(^{-/-}\) mice (92%, 92% and 83% survival, respectively). In (FcγRI, FcγRIII)\(^{-/-}\) mice survival was 60% and in the FcR\(\gamma^{\circ}\) group it was 16%. FcγRI\(^{-/-}\) and FcγRIII\(^{-/-}\) versus FcR\(\gamma^{\circ}\) \(p < 0.0005\) and \(p < 0.0005\), respectively; (FcγRI, FcγRIII)\(^{-/-}\) versus FcR\(\gamma^{\circ}\) \(p < 0.005\).

Supplemental figure 2. Characterization of anti-M2e immune serum fractions purified by stepwise pH gradient elution of IgGs from a protein A column. A, The amount of M2e-specific IgG subtypes (determined by ELISA) in the original serum was normalized to 1. The fraction eluting at pH 4.5 contained only IgG1; the fraction eluting at pH 4.0 contained IgG1 and IgG2a, and the fraction eluting at pH 3.0 contained mainly IgG2a and -2b, as well as residual IgG1 and IgG3. The anti-M2e IgG1 isotype titers in the pH 4.5 and pH 4.0 fractions were normalized to the IgG1 titer in the original serum, before passive transfer (figure 3). B, Specificity of the IgG isotype-specific secondary antibodies that were used for IgG subtype identification and quantification. Plates were coated with 10 μg/ml DNP-albumin conjugate to capture DNP-specific IgG1, IgG2a and IgG2b monoclonal antibodies, which were loaded in each plate in parallel serial dilutions. Each dilution series was subsequently probed with the subclass-specific secondary antibodies (Bioconnect) to be tested; the resulting OD\(_{450}\) values are depicted.

Supplemental figure 3. Survival (top) and morbidity (bottom) of C57BL/6 mice receiving PBS or anti-M2e murine serum (anti-M2e IgG), depleted or not depleted of alveolar macrophages by i.t.
administration of PBS-loaded or clodronate-loaded liposomes, and subsequently challenged with 4 LD$_{50}$ of mouse-adapted X47 virus. Mice were treated with liposomes on day –3, received naive or anti-M2e immune serum on day –1, and were challenged on day 0. The morbidity curve shows the percentage mean body weight relative to the weight on the day of challenge (day 0), ± SD.
Supplemental figure 1
Supplemental figure 2
Supplemental figure 3