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Enhancement of CD8$^+$ T Cell Immunity in the Lung by CpG Oligodeoxynucleotides Increases Protective Efficacy of a Modified Vaccinia Ankara Vaccine against Lethal Poxvirus Infection Even in a CD4-Deficient Host

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Immunostimulatory CpG oligodeoxynucleotides (ODN) have proven effective as adjuvants for protein-based vaccines, but their impact on immune responses induced by live viral vectors is not known. We found that addition of CpG ODN to modified vaccinia Ankara (MVA) markedly improved the induction of longer-lasting adaptive protective immunity in BALB/c mice against intranasal pathogenic vaccinia virus (Western Reserve; WR). Protection was mediated primarily by CD8$^+$ T cells in the lung, as determined by CD8-depletion studies, protection in B cell-deficient mice, and greater protection correlating with CD8$^+$ IFN-γ-producing cells in the lung but not with those in the spleen. Intranasal immunization was more effective at inducing CD8$^+$ T cell immunity in the lung, and protection, than i.m. immunization. Addition of CpG ODN increased the CD8$^+$ response but not the Ab response. Depletion of CD4 T cells before vaccination with MVA significantly diminished protection against pathogenic WR virus. However, CpG ODN delivered with MVA was able to substitute for CD4 help and protected CD4-depleted mice against WR vaccinia challenge. This study demonstrates for the first time a protective adjuvant effect of CpG ODN for a live viral vector vaccine that may overcome CD4 deficiency in the induction of protective CD8$^+$ T cell-mediated immunity. ***The Journal of Immunology,*** **2006, 177: 6336–6343.**

Immunostimulatory CpG oligodeoxynucleotides (ODN)[2] are similar to those in bacterial DNA and stimulate the immune response in favor of Th1 type and proinflammatory cytokine production (1–4). CpG ODN can be recognized by the TLR9 of the innate immune system, which triggers an immunomodulatory cascade of adaptive immunity. It was demonstrated that CpG ODN can enhance innate immune responses (5) and resistance against infectious disease and can serve as an adjuvant to improve adaptive immune responses against pathogens (6).

Although the efficacy of CpG ODN as vaccine adjuvants for DNA, protein, and peptide vaccines is well known (7–11), it was not known whether these would serve as effective adjuvants for live viral vector vaccines. Viruses carry their own TLR ligands, such as ssRNA or dsRNA, which binds to TLR7/8 or TLR3, respectively. Thus, it is not known whether CpG ODN, as a TLR9 ligand, would contribute further to immune induction. In this study, we addressed this question in the case of modified vaccinia Ankara (MVA) (12–14) as a vaccine against lethal vaccinia challenge, a model for smallpox. However, the results should be applicable to other viral vector vaccines expressing recombinant vaccine Ags (15–18).

Vaccination against smallpox is a major concern, because of the bioterrorism threat. "Dryvax" is the only licensed vaccine against smallpox. It is highly effective but has risk of adverse effects (19), and its use can be especially risky in immunocompromised patients with AIDS, other immunodeficiencies, and after organ transplant. A safer, effective vaccine is therefore needed for widespread use. Our early study (20) and studies from other groups (21, 22) demonstrated that MVA, at sufficient doses, provided protection against pathogenic vaccinia virus intranasal (IN) challenge of mice. MVA vaccination also can be effective against monkeypox (23). The MVA and Dryvax vaccines induced similar patterns of immune mechanisms of protection (20). Virus-neutralizing Ab was essential to protect against Western Reserve (WR) challenge, whereas effector CD4$^+$ or CD8$^+$ cells were not sufficient (20). Helper CD4 T cells, however, did contribute to protection against pathogenic vaccinia virus challenge (21). Recent studies identified several new class I HLA-A2-restricted CD8$^+$ T cell epitopes (24–26), and demonstrated that a single human HLA-A2-restricted CTL epitope can confer a survival advantage against lethal WR challenge in HHD-2 HLA-A2-transgenic mice (26). Several H-2K^b-restricted CD8 epitopes were described for C57BL/6 mice (27), and very recently for BALB/c mice as well (28). In this study, we show that use of CpG ODN with a live viral vector (MVA vaccine) not only increases the protective immune response, but also marshals an additional protective mechanism, the CD8$^+$ CTL. These CTL were not as effective for vaccine protection without the use of this TLR9 ligand as adjuvant. This approach might be useful to broaden the efficacy of viral vector vaccines whether for smallpox or for other diseases in which a recombinant viral Ag, such as HIV gp160, is inserted into the vector (16, 29–39).

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[2] Abbreviations used in this paper: ODN, oligodeoxynucleotide; MVA, modified vaccinia Ankara; IN, intranasal; DC, dendritic cell.

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Materials and Methods

Oligodeoxynucleotides

ODN were synthesized at the the Center for Biologics Evaluation and Research Core Facility. Immune stimulation was obtained by administering two CpG ODN (GCTAGA*CG*TTAGCGT and TCAA*CG*TTGA). The CpG motifs were switched to TpG or GpC in control ODN (GCTAGAT GTTAGGCT and TCAAGCTTGA). Neither endotoxin (measured by chromogenic *Limulus* amoebocyte lysate assay) nor protein (measured by bicinchoninic acid protein assay kit; Pierce Chemicals) were detectable in any ODN preparation. We used 25–100 μg of CpG ODN or control ODN per dose per animal.

Viruses

Vaccinia virus WR strain was originally obtained from the American Type Culture Collection. Vaccinia virus Wyeth, New York City Board of Health strain, was obtained from Wyeth Laboratories. Both were grown in HeLa cells and titered in BSC-1 cells. Vaccinia virus strain MVA, obtained from A. Mayr (University of Munich, Munich, Germany) (12, 13), was propagated and titered in chicken embryo fibroblast cells. This virus was a gift from Drs. B. Moss, P. Earl, and L. Wyatt (National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD) (23).

Mice, immunization, and challenge

Female BALB/c mice were purchased from the Frederick Cancer Research Center. The Jh knockout mouse carries a targeted deletion of the J_H locus, such that mice are homozygous for the absence of all four J_H gene segments, resulting in cells that cannot produce a complete, recombined version of the variable region of the H chain, and are therefore B cell deficient. This strain was made on the BALB/c background (Taconic Farms). For protection studies, BALB/c mice or B cell-deficient mice were immunized with different doses of MVA i.m. or IN. One month after immunization, mice were challenged with 10^6 PFU of WR, and individual body weight was measured daily. Mice with weight loss >25% were required to be euthanized, generally necessitating termination of the experiments around day 8, when the control group reached this level. Similar duration protection experiments for vaccinia have been used previously (20, 24). Depletion of $CD8^+$ or $CD4^+$ cells was done by i.p. treatment with mAb daily for 4 days (clone 2.43, 0.5 mg/mouse/day for CD8 and GK 1.5, 1 mg/mouse/day for CD4) (40, 41). Depletion was verified by FACScan analysis of peripheral blood cells to be >98% depleted.

Cell purification

Spleens were aseptically removed, and single-cell suspensions were prepared by gentle passage of the tissue through sterile screens. Erythrocytes were lysed with Tris-buffered ammonium chloride, and the remaining cells were washed extensively in RPMI 1640 (BioWhittaker) containing 2% FBS (Gemini Bio-Products) (42). Lungs were excised, avoiding the paratracheal lymph nodes, and washed twice in RPMI 1640. Intraparenchymal pulmonary mononuclear cell suspensions were isolated by collagenase/DNase digestion and Percoll gradient centrifugation as described previously (43).

IFN-γ ELISPOT

ELISPOT plates (Millipore) were precoated overnight with anti-IFN-γ Ab (Mabtech). Target cells (P815, a DBA/2 mastocytoma expressing class I but not class II H-2^d molecules) were infected overnight with vSC8 vaccinia virus (44), washed two times, and UV irradiated for 15 min. Splenic effector cells were mixed with infected target cells and centrifuged together in conical tubes for 3 min at 200 $\times$ *g*. Cells were cocultivated together for 1 h at 37°C and then transferred to the ELISPOT plate in a volume of 150 μl/well. After 24 h of cocultivation, IFN-γ spot-forming cells were developed by secondary anti-IFN-γ Ab (Mabtech), a Vectasin ABC kit (Vector Laboratories), and an AEC substrate kit (Vector Laboratories). Because our target cells (P815 cells) express only MHC class I, not class II, molecules (H-$2K^d$, D^d, and L^d), we expect that the majority of IFN-γ-producing cells are $CD8^+$ class I MHC-restricted T cells.

Vaccinia virus neutralization assay based on FACS

Vaccinia virus neutralization assay was done based on flow cytometric detection of GFP as described by Earl et al. (45).

Statistical methods

Statistical analysis was performed using a paired Student's *t* test comparing weight loss and survival in groups of immunized and unimmunized mice following vaccinia virus (WR) challenge (46).

Results

To study the role of CpG ODN as an adjuvant to enhance protection by MVA against WR, we immunized BALB/c mice IN with MVA at doses from 10^3 to 10^7 PFU in combination with CpG ODN or without CpG ODN. The protection was measured by prevention of weight loss of immunized animals. A minimum dose of 10^7 PFU of MVA alone given IN was required to induce complete protection against challenge with WR (Fig. 1*A*). However, in combination with CpG ODN, 10^5 PFU of MVA was sufficient to induce similar complete protection (Fig. 1*B*) ($p < 0.05$ on days 4, 5, 6, and 7 in mice immunized with 10^5 PFU of MVA alone vs 10^5 PFU of MVA plus CpG ODN). Thus, the adjuvant effect of CpG ODN increased the potency of the MVA vaccine 100-fold. The lack of long-term protection with CpG ODN alone (Fig. 2*A*) demonstrates that the protection is not an effect of the CpG ODN alone, and cannot be induced by treatment with control ODN (Fig. 2*A*) ($p > 0.05$ on days 4, 6, and 7 in mice immunized with 10^5 PFU of MVA alone vs 10^5 PFU of MVA plus control ODN). However, we found a limited role of CpG ODN alone in innate short-term protection (3 days after treatment with CpG ODN) of BALB/c mice against death due to pathogenic WR virus. CpG ODN alone (100 μg/dose) (without the MVA vaccine) provided short-term protection of BALB/c mice from death when challenged with WR, a mouse pathogenic strain of vaccinia virus (data not shown).

Another important consideration is how long this adjuvant effect of CpG ODN lasts relative to the time of immunization. To understand the role of this TLR9 ligand for protective immunity against pathogenic WR, we treated BALB/c mice with CpG ODN at different time points: 1 day before immunization with 10^5 MVA (group 1), CpG ODN together with 10^5 MVA (group 2), and CpG ODN 1 day after immunization with 10^5 MVA (group 3) (Fig. 2*B*). IN immunization with 10^7 PFU MVA in this case was used as the positive control. Three weeks after immunization, we challenged immunized BALB/c mice or unimmunized controls IN with 1 $\times$ 10^6 PFU WR, and measured the prevention of weight loss among

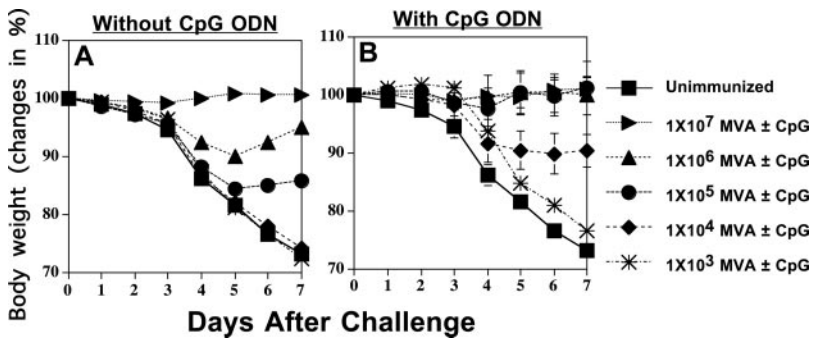

FIGURE 1. Protective efficacy of MVA immunization was improved by using CpG ODN as mucosal adjuvant for IN immunization in BALB/c mice. Groups of BALB/c mice (5/group) were immunized IN with 0, 10^3, 10^4, 10^5, 10^6, and 10^7 PFU without (*A*) or with (*B*) CpG ODN (25 μg/dose). One month later, mice were challenged IN with 10^6 PFU of WR vaccinia. Individual weight loss measured daily is presented as means for each group. These experiments were performed twice with comparable results.

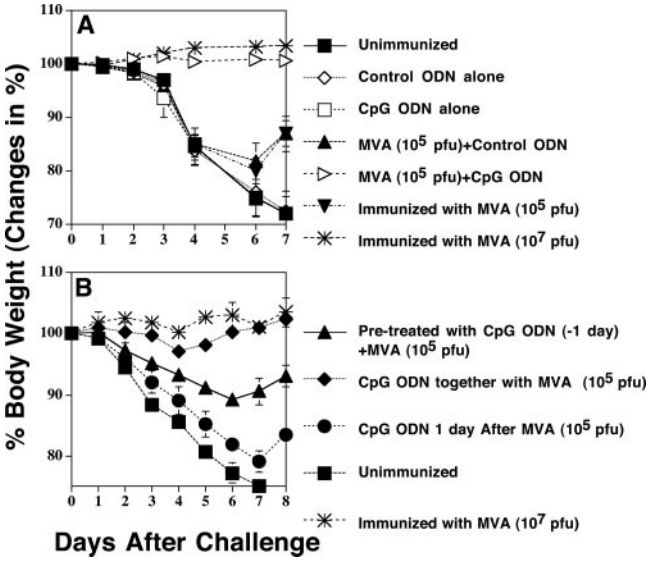

FIGURE 2. *A*, Protective efficacy of MVA immunization cannot be improved by using control ODN as mucosal adjuvant for IN immunization in BALB/c mice. Groups of BALB/c mice (5/group) were immunized IN with MVA 10^5 with or without control ODN (25 μg/dose). Three weeks later, mice were challenged IN with 10^6 PFU of WR vaccinia. Individual weight loss measured daily is presented as means for each group. *B*, CpG ODN is more effective when applied before MVA inoculation or together with MVA, but not after the MVA administration. Groups of BALB/c mice were immunized with MVA (10^5 PFU) and CpG ODN. In group 1, CpG ODN was injected IN 1 day before MVA administration; in group 2, CpG ODN was administered IN together with MVA; in group 3, CpG ODN was IN injected 1 day after immunization with MVA; in group 4, mice were immunized IN with MVA (10^7) alone; and in group 5, mice were unimmunized. Three weeks after the immunizations, animals were challenged IN with 1×10^6 PFU WR virus. Individual mouse weight measured daily is presented as means for each group. These experiments were performed twice with comparable results.

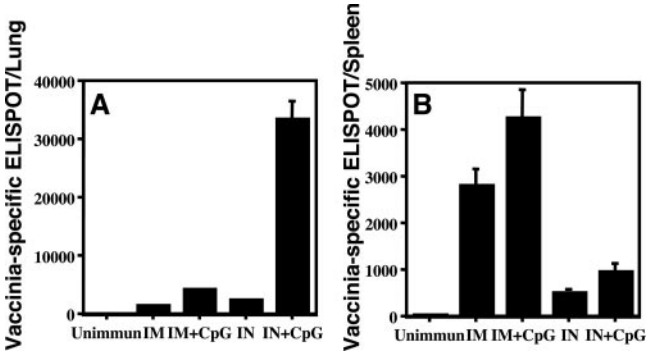

FIGURE 3. CpG ODN increased the number of vaccinia Ag-specific IFN-γ-producing cells after IN or i.m. immunizations. Ten BALB/c mice were IN immunized with MVA plus CpG ODN (5 mice) or without CpG ODN (5 mice), and another 10 BALB/c mice were given MVA by the i.m. route plus CpG ODN (5 mice) or without CpG ODN (5 mice). Nine days after the immunizations, we characterized the number of vaccinia-specific IFN-γ-producing T cells in the lung (*A*) and spleen (*B*) by IFN-γ ELISPOT assay.

immunized animals. Animals that started to regain weight before losing 25% of their body weight were found to recover and show long-term survival (data not shown). Immunization with 10^5 MVA plus CpG ODN together afforded the greatest protection against challenge with pathogenic WR, compared with CpG ODN administration 1 day before ($p < 0.05$ on days 6, 7, and 8 after challenge) or after MVA ($p < 0.01$ on days 6, 7, and 8 after challenge) (Fig. 2*B*). Hardly any protection was observed when MVA-immunized mice were treated with CpG ODN the day after immunization (Fig. 2*B*). This experiment indicated the importance of induction of innate immunity before or together with adaptive immunity, whereas elevation of innate immunity after immunization with MVA was ineffective.

To determine whether CpG ODN increased the number of IFN-γ-producing $CD8^+$ T cells concomitant with improvement of protective immunity, we used a vaccinia-specific IFN-γ ELISPOT assay. In these experiments, we used 10^6 PFU of MVA (both doses of MVA, 10^6, and 10^5, in combination with CpG ODN induced complete protection against IN challenge with 10^6 PFU of WR). Also, we studied the ability of CpG ODN to induce IFN-γ-producing cells in the lung and spleen after systemic (i.m.) immunization with MVA, and compared this with the effect of MVA+CpG ODN after IN immunization. Nine days after immunization, CpG ODN administered IN with MVA induced a significant increase in the total number of vaccinia-specific IFN-γ-producing $CD8^+$ T cells in the lung ($p < 0.001$) (Fig. 3*A*); however, the level of increase of vaccinia-specific IFN-γ-producing cells in the lung after CpG ODN with MVA given i.m. was significantly lower compared with CpG ODN with MVA given IN (Fig. 3*A*) ($p < 0.05$). A significantly greater number of vaccinia-specific IFN-γ-producing cells were found in the lungs of mice immunized IN with MVA (10^6 PFU) plus CpG ODN, raising the possibility that the IN CpG ODN altered the homing pattern of T cells (Fig. 3*A*). The pattern of vaccinia-specific IFN-γ ELISPOT-forming cells in the spleen was the reverse (Fig. 3*B*), with the highest numbers of Ag-specific IFN-γ-producing $CD8^+$ T cells observed after i.m. immunization. CpG ODN also significantly improved vaccinia-specific IFN-γ-producing cells in the spleen (Fig. 3*B*). Control ODN did not significantly increase the number of IFN-γ-secreting cells in the lung (data not shown).

The role of local $CD8^+$ CTL responses in the lung is not very well understood. Mice were immunized IN or i.m. with MVA (10^6 PFU) with or without CpG ODN. Fourteen days after immunization, mice were challenged IN with the high dose of WR (2×10^6). In this case, we used a high dose of WR virus for the challenge (2×10^6) just to determine the better route of immunization (i.m. vs IN) to protect against IN challenge with WR, because IN or i.m. immunization with 1×10^6 MVA plus CpG ODN induced complete protection against 1×10^6 PFU of WR. Clinical protection following IN vs i.m. immunizations was measured by prevention of weight loss of immunized animals. Complete protection against poxvirus-mediated disease was observed after IN immunization with CpG ODN (25 μg/dose) plus MVA (Fig. 4*A*). However, mice immunized IN with MVA alone survived after IN challenge with WR, but developed poxvirus-mediated disease marked by transient weight loss (Fig. 4*A*). Mice immunized i.m. (with or without CpG ODN) also survived after a high dose of WR challenge. However, weight loss in mice immunized i.m. was much more severe than in animals immunized IN (Fig. 4*A*). Thus, the route of MVA and adjuvant delivery could be very important for generation of protective immunity against poxvirus infection, especially with an optimal mucosal adjuvant.

A high number of IFN-γ vaccinia-specific ELISPOT-forming cells in the lung (not the spleen) after vaccination was associated with a high level of protection against WR challenge. We characterized the number of vaccinia-specific IFN-γ-producing ELISPOT-forming cells in the lung and spleen 6 days after challenge with WR. The rank order of Ag-specific $CD8^+$ cells in the lung

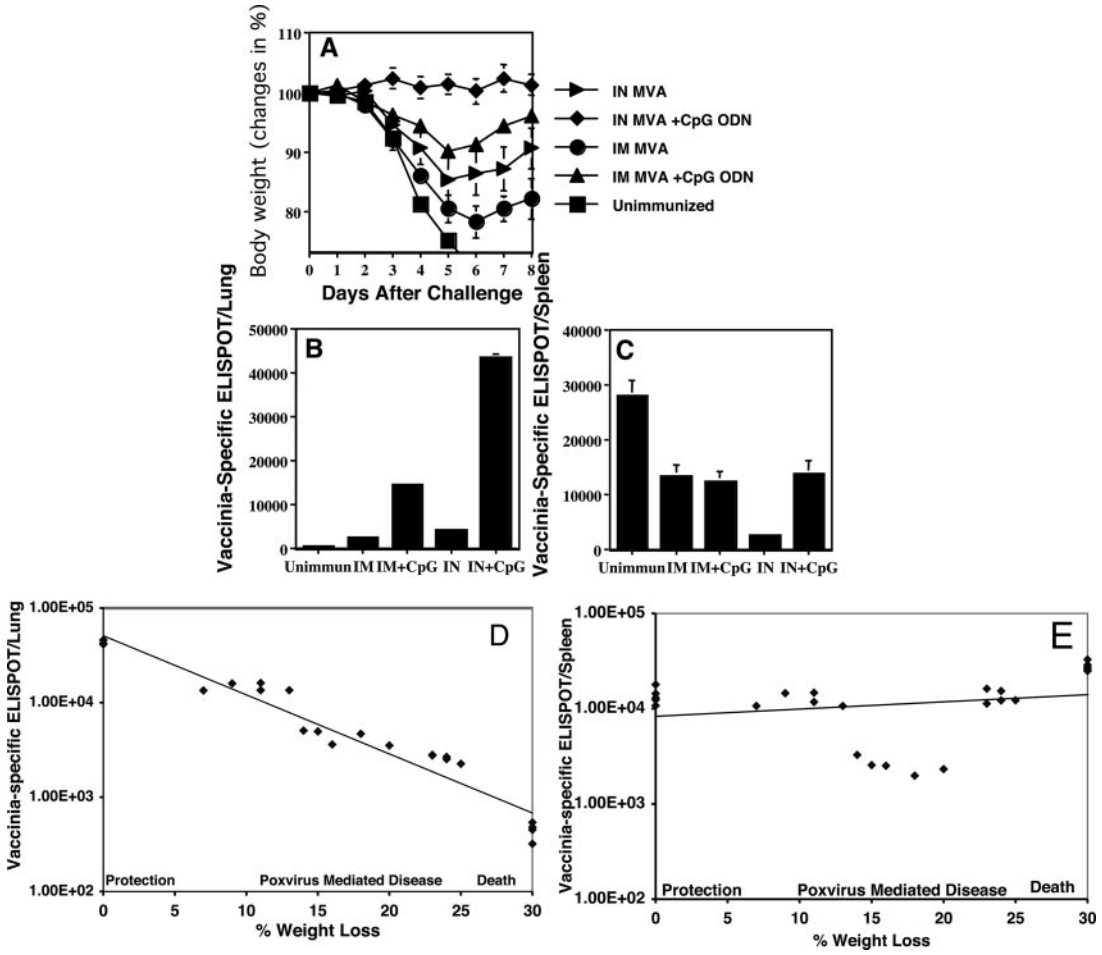

FIGURE 4. CpG ODN increases the protection (*A*) after challenge with pathogenic WR and the number of vaccinia-specific IFN-γ-producing T cells in the lung (*B*) and in the spleen (*C*). *D* and *E*, Protection against poxvirus-mediated disease correlated with IFN-γ-producing cells in the lung (*D*), not in the spleen (*E*). The BALB/c mice were IN or i.m. immunized with MVA vaccinia with CpG ODN (25 μg/dose) or without CpG ODN. Fourteen days after immunization, mice were IN challenged with WR (2×10^6 PFU). *A*, Individual weight loss measured daily is presented as means for each group. Six days after challenge, the vaccinia-specific IFN-γ-producing cells per organ were quantified in the lung (*B*) and spleen (*C*) by IFN-γ ELISPOT assay at 100,000/well. Correlations were determined between the poxvirus-mediated disease (from *A*) and IFN-γ-producing cells in the lung (*D*) and the spleen (*E*) before challenge (from Fig. 3, *A* and *B*), comparing animals from all five groups.

after challenge (Fig. 4*B*) correlated with the rank order of protection (Fig. 4*A*). However, the pattern of vaccinia-specific IFN-γ-producing cells in the spleen after IN challenge of immunized animals was different. Surprisingly, the highest number of vaccinia-specific IFN-γ-producing cells in spleen after WR challenge was observed in unimmunized mice (Fig. 4*C*). These data clearly demonstrate the importance of local CD8 CTL immunity in the lung, rather than CD8 CTL in a distant site such as the spleen. There is no functional $CD8^+$ CTL deficiency in unimmunized mice, but these CD8 CTLs are far away from the lung and cannot eradicate the pathogenic poxvirus and prevent poxvirus-mediated disease. The high number of IFN-γ-secreting cells in the spleen in unimmunized mice may potentially be explained by the presence of a high viral load in the mouse, which cannot be eradicated by the immune system. We analyzed the correlations between the numbers of vaccinia-specific IFN-γ-producing cells in the lung (Fig. 4*D*) and the spleen (Fig. 4*E*) after immunization (before challenge) (from Fig. 3) and the percentage of weight loss after IN challenge with WR (from Fig. 4*A*), including animals from all five groups within the correlation. A strong direct correlation ($r = -0.9$; $p < 0.001$) between the numbers of IFN-γ-producing cells in the lung and protection against weight loss was found (i.e., an inverse correlation with weight loss) (Fig. 4*D*). A high level of Ag-specific CD8 cells in the lung was associated with protection against IN challenge with WR. Using CpG ODN as the mucosal adjuvant significantly increased the protection and CD8 response in the lung. However, there was no correlation ($r = 0.44$; $p > 0.05$) between protection against death and vaccinia-specific IFN-γ-producing cells in the spleen (Fig. 4*E*). This correlation analysis suggests that the role of mucosal vs systemic $CD8^+$ T cells in protection are different, and that the major protective effect is associated with $CD8^+$ cells in the lung, but not in the spleen.

In contrast to our IFN-γ-producing cell findings, IN immunization with MVA plus CpG ODN did not significantly increase the titer of virus-neutralizing Abs in the serum (on days 14 and 21 after immunization) (Fig. 5, *A* and *B*) ($p > 0.05$). We challenged BALB/c mice with WR 3 wk after immunization and studied neutralizing Ab titers on day 3 after WR challenge (data not shown). WR challenge of mice immunized with MVA^+CpG ODN did not further increase neutralizing Ab titers ($p > 0.05$). Thus, we conclude that there is little or no improvement in vaccinia-neutralizing Ab production by use of CpG ODN as an adjuvant for MVA either in the primary response or in the recall response after challenge, and therefore this does not appear to be the mechanism of improved protection.

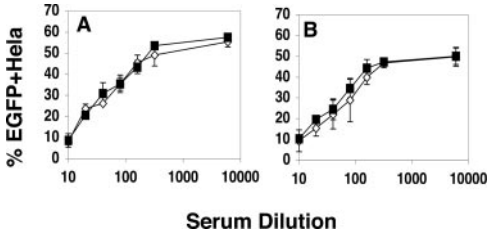

FIGURE 5. IN immunization with MVA plus CpG ODN did not significantly increase the titer of virus-neutralizing Abs in serum. Groups of BALB/c mice were immunized with MVA 10^6 PFU in presence of CpG ODN (■) or without CpG ODN (◇). Fourteen days (*A*) and 21 days (*B*) after immunization, individual sera were collected. Mouse serum was titrated (by 2-fold dilution). Ten serum dilutions were incubated with virus GFP (0.6×10^4 PFU) for 1 h in RPMI 1640 with BSA (no FCS) in 96-well plates. GFP virus was added in 180 μl of RPMI 1640 plus 20 μl of BSA and incubated for 1 h at 37°C. A total of 10^5 HeLa cells was added per well in 50 μl of medium and incubated for 16 h at 37°C. Infected HeLa cells were centrifuged, fixed by 0.5% formaldehyde, and analyzed by FACS. Plotted are percentages of infected cells vs serum dilution.

Although the improved T cell response correlated with improved protection, whereas Ab did not, these correlations do not prove cause and effect. To do so, we needed to deplete B cells or T cells to determine which was necessary for protection. As demonstrated by several independent studies, vaccinia-neutralizing Ab is sufficient to protect against WR and smallpox challenge (20). Ag-specific memory $CD8^+$ CTL alone were less effective for protection against pathogenic WR (20). However, after high-dose immunization with MVA (10^8 PFU), $CD8^+$ CTL can protect against WR challenge without Ab (21). We asked whether using CpG ODN as an adjuvant for mucosal immunization can improve protection mediated by $CD8^+$ CTL in the absence of Abs. We immunized B cell-deficient mice IN with MVA (10^7 and 10^8 PFU) with or without CpG ODN. Three weeks after immunization, we challenged mice IN with 10^6 of WR virus. We challenged the B cell-deficient mice 3 wk after immunization, because we would like to demonstrate the role of memory T cell response (improved by the adjuvant effect of CpG ODN) for protection against pathogenic poxvirus. Immunization of B cell-deficient mice with 10^8 PFU of MVA with or without CpG ODN provided complete protection against WR challenge ($p > 0.05$). In the case of B cell-deficient mice, immunization with 10^7 PFU of MVA was most informative, because when we challenged B cell-deficient animals immunized with only 10^7 PFU of MVA, we found that mice were not protected against disease, although they were protected against death (showing recovery of weight, indicating long-term survival), after pathogenic vaccinia challenge. However, B cell-deficient animals immunized with 10^7 PFU of MVA plus CpG ODN were almost completely resistant against WR challenge (Fig. 6) ($p < 0.05$ on days 4 and 6 after challenge). Thus, the adjuvant effect of CpG ODN in B cell-deficient mice can improve the efficacy of vaccination against smallpox through an Ab-independent mechanism, presumably $CD8^+$ CTL.

The mechanism of CpG ODN-augmented protection against WR challenge in BALB/c mice was similarly dependent on $CD8^+$ T cells. We IN immunized BALB/c mice with 10^5 PFU MVA (two logs lower dose of MVA compared with the dose of immunization for B cell-deficient animals) with or without CpG ODN. IN immunization with 10^7 PFU MVA in this case was used as the positive control for our protection experiment (Fig. 7*A*). One group of MVA-CpG-immunized mice was treated with anti-CD8 Ab (0.5-mg dose per mouse i.p. for 3 days before challenge). Anti-CD8 treatment abrogated the increased protection afforded by the CpG adjuvant (Fig. 7*A*) ($p < 0.01$ on days 5, 6, and 7 after challenge). Thus, the protection against pathogenic vaccinia virus challenge afforded by CpG ODN plus low-dose MVA in wild-type BALB/c mice is dependent on $CD8^+$ CTL, not Abs.

It is known that $CD4^+$ T cells have important regulatory functions for $CD8^+$ CTL and Ab responses and protective immunity against WR challenge (21). Help for $CD8^+$ CTL is mediated in part by activation of APC (47–51). We asked whether CpG ODN, which can also activate APC, can substitute for the $CD4^+$ T help and induce protection against WR challenge. BALB/c mice were treated daily with anti-CD4 Abs (GK 1.5, 1 mg/mouse/day) for 4 days before immunization, and then immunized with 10^6 PFU of MVA (group 1), or with 10^6 PFU of MVA plus CpG ODN (group 2). MVA-immunized, CD4-depleted mice were not protected against disease after IN challenge with WR, although they ultimately survived (Fig. 7*B*). However, CD4-depleted BALB/c mice immunized with MVA plus CpG ODN were completely protected against vaccinia-mediated disease ($p < 0.01$ on days 4, 5, and 6 after challenge). These studies suggested that CpG ODN can substitute for CD4 help, for the generation of protective immunity against smallpox.

Discussion

Although CpG ODN as ligands for TLR9 have been shown to improve responses induced by DNA, protein, and peptide vaccines, and inactivated virus Ag (3, 7, 52–55), it was not clear whether they could improve the response to a live viral vector vaccine that carries its own TLR ligands. We reasoned that CpG

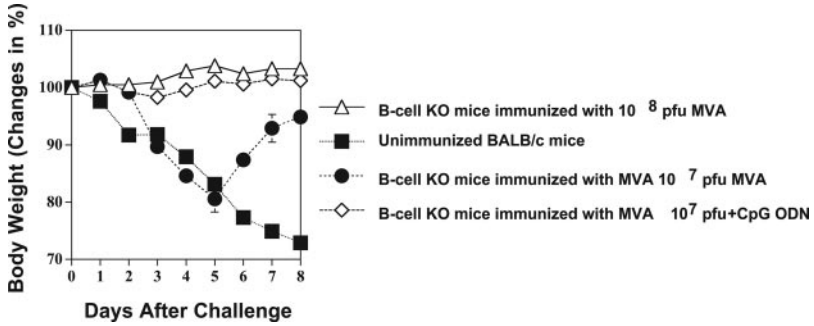

FIGURE 6. CpG ODN can improve protection against pathogenic WR challenge in MVA-immunized, B cell-deficient mice. Groups of B cell-deficient mice were immunized IN with 10^7 MVA together with CpG ODN or without CpG ODN; control BALB/c mice were unimmunized (five BALB/c mice). One group of B cell-deficient mice immunized with 10^7 PFU of MVA plus CpG ODN was treated with anti-CD8 Ab (0.5-mg dose per mouse i.p. for 3 days before challenge). Three weeks after immunization, mice were challenged IN with pathogenic WR virus. Individual mouse weight measured daily is presented as means for each group. These experiments were performed twice with comparable results.

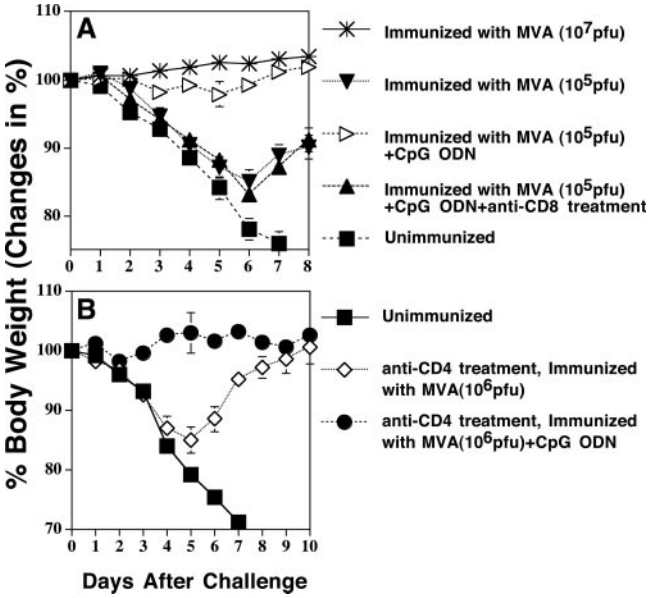

FIGURE 7. $CD8^+$ CTL are important for CpG ODN-augmented protection against WR challenge in BALB/c mice (*A*), and CpG ODN can substitute for CD4 help and improved protection in CD4-depleted mice (*B*). *A*, Groups of BALB/c mice were immunized IN with 10^5 in presence of CpG ODN or without CpG ODN. One group of BALB/c mice immunized with 10^5 PFU of MVA plus CpG ODN was treated with anti-CD8 Ab (0.5-mg dose per mouse i.p. for 3 days before challenge). Control BALB/c mice were immunized with MVA alone (10^7 PFU) or unimmunized (five BALB/c mice). *B*, BALB/c mice were treated daily for 4 days with anti-CD4 Abs (GK 1.5, 1 mg/mouse/day) before immunization. After CD4 depletion, mice were immunized IN with MVA (10^6 PFU) with CpG ODN or MVA alone (without CpG ODN). Three weeks after immunization, mice were challenged IN with pathogenic WR. Individual mouse weight measured daily is presented as means for each group. These experiments were performed twice with comparable results.

ODN represents a TLR ligand normally found in bacteria, not viruses. Therefore, we asked whether it might provide additional adjuvant effects that would complement those provided by natural viral TLR ligands.

In this study, we have shown that CpG ODN as an adjuvant for a live attenuated viral vector vaccine can indeed improve the immune responses and protection against mucosal viral challenge. It was also important to understand the major mechanisms of such protection. In this regard, we found that the major mechanism of CpG-improved protection is $CD8^+$ CTL mediated and that depletion of $CD8^+$ cells can abrogate protection against mucosal challenge with pathogenic WR. We cannot rule out some contribution of some other mechanisms of antiviral immunity, but depletion studies indicated that $CD8^+$ CTL (particularly $CD8^+$ CTL in the lung) were the major mediator necessary for protection against mucosal viral challenge after IN immunization with CpG-MVA. This finding contrasts with many studies using CpG ODN as an adjuvant for protein or peptide vaccines, in which protection was associated with an increase in Ab production (including neutralizing Ab and/or mucosal Ab) (8, 9, 56).

It was very important to apply CpG ODN together with the Ag for mucosal immunization, because the application of CpG ODN the next day after immunization was not effective. We interpret this as a requirement to induce TLR-ligand-dependent dendritic cell (DC) maturation in the presence of Ag, whereas DC activation and maturation later is much less effective (57–59). It is important to acknowledge that TLR9 not as widely distributed in primates' cells as in mice (60). Additional studies on improved resistance induced by immunization with MVA plus CpG ODN in a primate model are needed. Also, an effective vaccine should generate a long-term memory response against smallpox. We did not directly address this question in this study, but our challenge studies of wild-type mice (Fig. 2*B*) and B cell-deficient mice (Fig. 6) 3 wk after immunization with MVA plus CpG ODN suggest that this vaccine strategy may induce a memory response against pathogenic poxvirus.

Our previous studies (40, 61, 62) and studies of other groups (63, 64) indicated that mucosal immunization induced a better memory $CD8^+$ CTL response in the local mucosal sites and protection compared with systemic immunization. We believe that a higher number of IFN-γ-producing cells in the lung after IN immunization (compared with i.m.) does not reflect kinetic differences just in the initiation of immune responses, but will produce a high number of the long-term memory T cells in the local mucosa sites.

Surprisingly, immunization with CpG ODN plus MVA was able to induce protective $CD8^+$ CTL immunity by a Th cell-independent mechanism. Depletion of CD4 cells before CpG-MVA immunization did not abrogate the protection against WR challenge. This effect of CpG ODN may have applications for immunization against smallpox in patients with AIDS and other cases of immunodeficiency. A previous study by Cho et al. (65) demonstrated that a protein-immunostimulatory DNA sequence conjugate was more potent than other immunostimulatory DNA sequence-based vaccines because it induced Th-independent activation of CTL and facilitated exogenous Ag presentation on MHC class I. In our case, use of CpG ODN together with live attenuated vaccine for mucosal immunization demonstrated a similar CD4-independent mechanism for generation of protective immunity against a pathogenic vaccinia virus.

This present study indicated that CpG ODN could improve the link between the innate and adaptive antiviral immunity and protection against pathogenic challenge with WR. Treatment with TLR9 ligand alone (without specific Ag) protected mice for a brief period against lethal challenge with pathogenic WR, albeit not against disease. This phenomenon could be explained by a favorable cytokine profile produced by DC in the local tissue stimulated by CpG ODN, leading to a strong adaptive immune response (first of all $CD8^+$ CTL in the lung) after challenge with pathogenic WR. A similar effect of CpG ODN on innate protection against a lethal orthopoxvirus infection was described by Rees et al. (66). This study demonstrated that the CC chemokines RANTES and MIP-1β were elevated in the bronchoalveolar lavage from unimmunized mice treated IN with CpG ODN, and it is possible that these chemokines play a role in the innate protection against a lethal orthopoxvirus challenge (66).

In our previous study and in recent studies by other groups (20, 22), vaccinia-specific Ab was essential to protect against poxvirus-mediated disease, whereas effector $CD8^+$ cells were not necessary or sufficient. However, T cells could contribute to the protection against sublethal infection in unimmunized animals (20). We also demonstrated that a peptide vaccine inducing only a $CD8^+$ T cell response to an epitope presented by the MHC class I molecule HLA-A2.1 in HLA-A2.1 transgenic mice could protect animals from lethal challenge with WR (26). The current study indicated that the effect of CpG ODN on the response to a viral vector vaccine does not primarily involve the improvement of Ab responses, but rather an increase in the number of Ag-specific $CD8^+$ cells in the lung. A strong inverse correlation ($r = -0.9$) between the numbers of IFN-γ-producing $CD8^+$ cells in the lung and poxvirus-mediated disease was found, whereas no correlation ($r = 0.44$) between protection and vaccinia-specific $CD8^+$ T cells in the

spleen was observed. This correlation suggests that the major role in protection is attributable to $CD8^+$ cells in the lung. Future vaccine strategies against smallpox (and probably against some other pulmonary viruses) should focus on inducing a high level of Ag-specific $CD8^+$ cells in the lung. Thus, CpG ODN can be used as a mucosal adjuvant for a live attenuated vaccine, and the increased proportion of $CD8^+$ CTL in the lung after immunization IN with MVA plus CpG ODN can substantially contribute to protective immunity against pathogenic viral challenge and may improve the efficacy of a smallpox vaccine based on MVA or a vaccine for other diseases in which a recombinant viral Ag is inserted into this vector.

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Disclosures

The authors have no financial conflict of interest.

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