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CD4+ Th1 type immunity is implicated in resistance to global infectious diseases. To improve the efficacy of T cell immunity induced by human immunodeficiency virus (HIV) vaccines, we are developing a protein-based approach that directly harnesses the function of dendritic cells (DCs) in intact lymphoid tissues. Vaccine proteins are selectively delivered to DCs by antibodies to DEC-205/CD205, a receptor for antigen presentation. We find that polyriboinosinic:polyribocytidylic acid (poly IC) independently serves as an adjuvant to allow a DC-targeted protein to induce protective CD4+ T cell responses at a mucosal surface, the airway. After two doses of DEC-targeted, HIV gag p24 along with poly IC, responder CD4+ T cells have qualitative features that have been correlated with protective function. The T cells simultaneously make IFN-γ, tumor necrosis factor (TNF)-α, and IL-2, and in high amounts for prolonged periods. The T cells also proliferate and continue to secrete IFN-γ in response to HIV gag p24. The adjuvant role of poly IC requires Toll-like receptor (TLR) 3 and melanoma differentiation-associated gene-5 (MDA5) receptors, but its analog poly $IC_{12}U$ requires only TLR3. We suggest that poly IC be tested as an adjuvant with DC-targeted vaccines to induce numerous multifunctional CD4+ Th1 cells with proliferative capacity.

HIV | gag | adjuvant | MDA5 | TLR3

Cell mediated immunity is implicated in resistance to global infectious diseases like HIV, malaria, and tuberculosis (1–3). CD4+ Th1 helper cells are a key component of this immunity (4). They produce large amounts of IFN-γ and TNF-α (5), exert cytolytic activity on major histocompatibility complex (MHC) class II+ targets (6), and sustain functional CD8+ T memory cells (7, 8). HIV-specific CD4+ T cells that produce IL-2 can restore proliferation of antigen-specific CD8+ T cells *in vitro* (9). Further, HIV-specific CD4+ T cells that produce multiple cytokines such as IFN-γ and IL-2 (10–12), or proliferate and secrete IFN-γ upon encounter with HIV antigens (13), are reported to be associated with a better clinical outcome, whereas higher numbers of CD4+ memory T cells correlate with superior protection by SIV vaccines in monkeys (14). Therefore, effective vaccine development requires an understanding of how to generate CD4+ T cells in sufficient quantity and quality to provide protective immunity.

DCs are antigen presenting cells that induce strong T cell-based responses (15). For example, when a subset of DCs that express the endocytic receptor DEC-205 (16) is loaded with antigen ex vivo and reinfused into mice, the DCs expand antigen-specific helper T cells to primarily produce IFN-γ (17, 18). *In vivo*, DEC-205 expressing DCs (DEC+ DCs) mediate antigen presentation on both MHC class I and II products, leading to clonal expansion of CD8+ and CD4+ T cells respectively (19). We are developing an approach that targets antigens directly to DCs *in situ* using the DEC receptor (20–24), which is expressed on DCs in the T cell areas (25), particularly a subset (26) that often expresses CD8 (27). Delivery of vaccine proteins engineered into α-DEC mAbs greatly enhances the efficiency of antigen presentation (20, 21, 23, 24) and allows protein-based vaccines to induce large numbers of Th1 type CD4+ T cells (22–24), as well as CD4+ dependent protection to challenge with recombinant vaccinia virus (23).

Productive immune responses require both antigen uptake and DC maturation, because in the absence of maturation, DCs can induce T cell tolerance (20, 21). DC maturation can be achieved with adjuvants, including chemically defined ligands for pattern recognition receptors, such as the TLRs (28, 29). For example, HIV-specific, CD4+ and CD8+ immunity in mice (30) and monkeys can be induced by conjugating TLR7/8 ligand to HIV gag p41, whereas unconjugated HIV gag p41 is poorly immunogenic (31, 32). However, ligands for pattern recognition receptors have not been evaluated as adjuvants with DC-targeted HIV vaccines.

In our prior studies, we administered α-DEC fusion mAb together with a combination of an agonistic α-CD40-mAb and poly IC as a DC maturation stimulus (22–24). Poly IC is recognized by endosomal TLR3 expressed by DEC+ DCs (33) and by MDA5 cytosolic receptors (34). Poly IC has been tested as a single therapeutic agent at high doses in patients with malignant disease (35–37), and it acts as an adjuvant for antibody immunity (38, 39). Here, we show that poly IC alone is an effective adjuvant for a DC-targeted vaccine to yield CD4+ T cell immunity that is quantitatively and qualitatively robust and also protective in a lung infection model.

Results

Poly IC Is an Adjuvant for Strong CD4+ T Cell Responses to DEC-Targeted HIV gag p24. To examine the adjuvant properties of poly IC and monophosphoryl lipid A (MPLA) we measured serum cytokine responses. Poly IC (50 μg) induced more IL-6 relative to MPLA [supporting information (SI) Fig. 6*A*] and also elicited TNF-α and IFN-α. To evaluate the efficacy of these compounds as adjuvants for HIV gag-specific, CD4+ T cell responses, we administered each with α-DEC-p24 mAb twice i.p. over 4 wk. One week after the final injection, IFN-γ secretion was monitored in response to HIV gag p24 peptides by multicolor flow cytometry using the gating strategy shown in SI Fig. 7. Poly IC was a superior adjuvant to MPLA for inducing strong CD4+ T cell responses, achieving frequencies of IFN-γ secreting T cells that corresponded to 0.5–6% of total CD3+ CD4+ T cells (SI Fig. 6*B*). However, poly IC was

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Conflict of interest statement: R.M.S. is a consultant to Celldex, which is developing human DEC-205-based vaccines. All other authors declare no conflict of interest.

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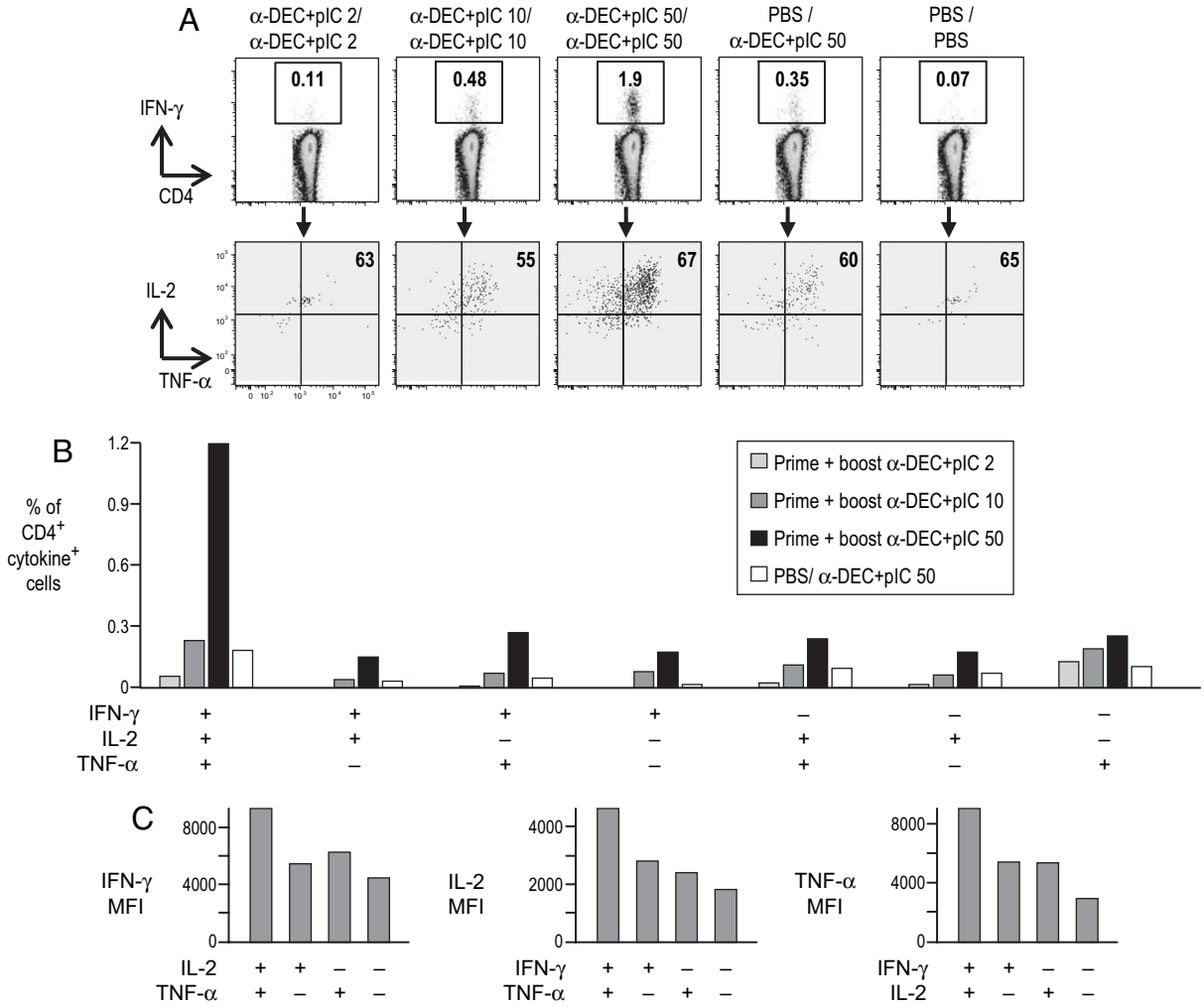

Fig. 1. Properties of HIV gag-specific CD4$^+$ T cells after prime-boost immunization with α-DEC-p24 and poly IC. C57BL/6 mice were injected s.c. with 5 μg of α-DEC-p24 and 2, 10, or 50 μg of poly IC or PBS, and boosted with the same conditions 6 wk later. One other group received 5 μg of α-DEC-p24 and 50 μg of poly IC once at the time of the boost. Two weeks later, HIV gag-specific, CD3$^+$ CD4$^+$, splenic T cells were analyzed for IFN-γ-, IL-2-, and TNF-α-secretion (*A*), percentage of CD4$^+$ T cells expressing each of seven possible combinations of IFN-γ, TNF-α, and IL-2 (*B*), and IFN-γ, TNF-α, and IL-2 MFIs of gag-specific cells expressing 3$^+$, 2$^+$, or 1$^+$ cytokines after prime-boost with α-DEC-p24 plus 50 μg of poly IC (*C*). Data are representative of two experiments with two mice pooled in each.

needed during both priming and booster doses of vaccine (data not shown).

α-DEC-gag Plus Poly IC Elicits Multifunctional and High Cytokine-Secreting CD4$^+$ T Cells. To assess the quality of CD4$^+$ T cells induced with poly IC, we injected mice with α-DEC-p24 and graded doses of poly IC over 6 wk. One group received α-DEC-p24 and poly IC only at the time of boost. Two weeks after the boost, the frequency of gag-specific, CD4$^+$ T cells producing IL-2, IFN-γ, or TNF-γ was greatest with two doses of α-DEC-p24 mAb and 50 μg of poly IC (Fig. 1*A* and SI Fig. 8*A*).

We then examined the capacity of the individual gag-specific T cells to secrete multiple cytokines. Such multifunctional T cells elicited by immunization prospectively correlate with protection in a mouse model of *Leishmania major* infection (5) and are found retrospectively to be increased in HIV infected individuals who have a better clinical outcome (10, 12). Fourteen days after prime-boost immunization with α-DEC-p24 and 50 μg of poly IC, ≈50% of the gag-specific CD4$^+$ T cells produced all three cytokines, IFN-γ, IL-2, and TNF-α (Fig. 1*B* and SI Fig. 8*B*). The total frequencies of cytokine producers was less if we used two doses of α-DEC-p24 and 10 μg of poly IC, or a single immunization with α-DEC-p24 and 50 μg of poly IC (Fig. 1*B*).

We also assessed the amount of each cytokine (median fluorescence intensity or MFI) made by gag-responsive cells, because this parameter correlates with protective CD4$^+$ immunity in the *L. major* model (5). We found that the MFI of cells producing 3 cytokines were higher for IFN-γ, IL-2, and TNF-α than the MFI of cells producing two cytokines or one cytokine (Fig. 1*C*). Therefore, the effector CD4$^+$ T cells induced with α-DEC-p24 and poly IC have features, like multifunctionality and high cytokine production, that are currently associated with superior Th1 immunity.

CD4$^+$ Effector T Cells Persist for Several Weeks After Prime-Boost Immunization. To assess the duration of HIV gag-specific cytokine-producing ("effector") CD4$^+$ T cells after prime-boost immunization with α-DEC-p24 plus poly IC, we analyzed mice 2 and 7 wk after the boost. As summarized for three experiments each in C57BL/6 and BALB/c mice, specific effector CD4$^+$ T cells persisted at least 7 wk, but decreased from week 2 to 7 (SI Fig. 9). The percentage of cells producing all three cytokines (IFN-γ, TNF-α, IL-2) remained stable from week 2 to 7 after the boost (data not shown). The stability of the multifunctional vaccine-induced T cells

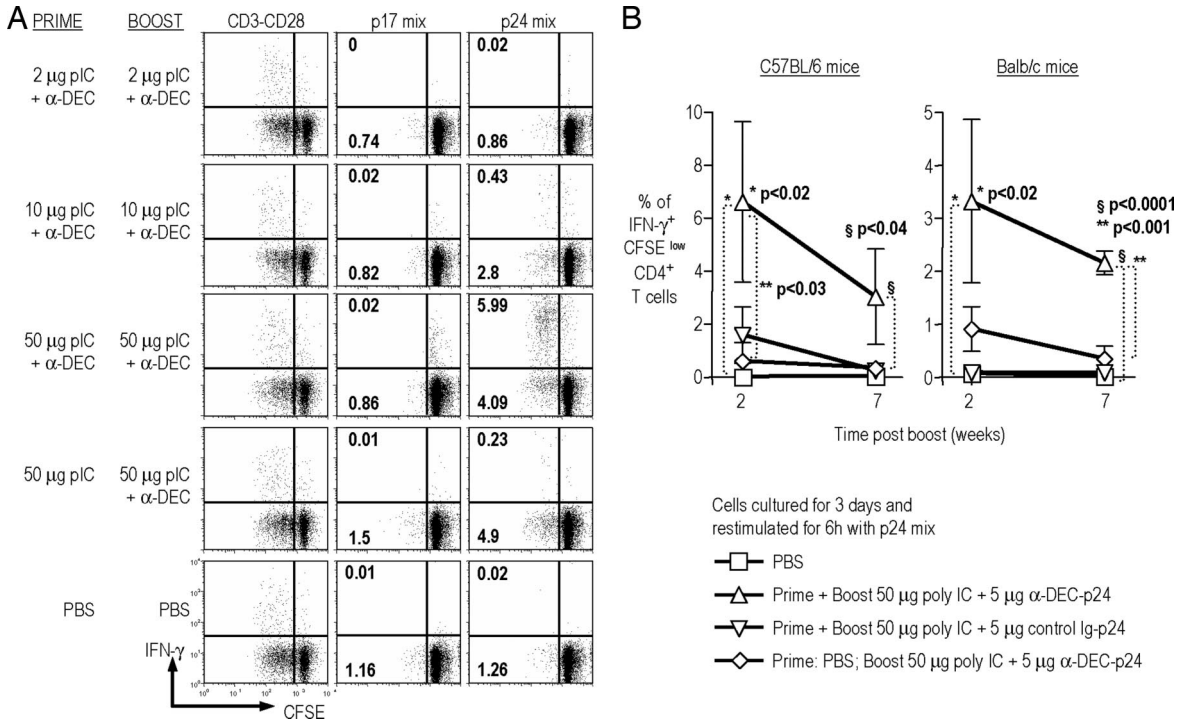

Fig. 2. Prime-boost immunization with α-DEC-p24 and poly IC induces long-lived IFN-γ-secreting and proliferating $CD4^+$ T cells. (*A*) As in Fig. 1, but 2 wk after boosting, bulk splenocytes were CFSE-labeled and stimulated with α-CD28 and gag p24 peptide mix, control gag p17 peptide mix, or α-CD3 for 3 days, whereupon the cells were restimulated for 6 h with HIV gag p24 peptides to detect IFN-γ in proliferated $CFSE^{low}$, $CD3^+CD4^+$ T cells. Data are the percentage of IFN-γ^+ or IFN-γ^- $CD3^+CD4^+$ proliferating T cells (*Top Left* and *Bottom Left*, respectively) from one of two similar experiments with two mice pooled in each. (*B*) As in *A*, but mean ± SD of the frequencies of IFN-γ-producing and proliferating $CD3^+CD4^+$ T cells in three experiments in BALB/c or C57BL/6 mice immunized twice with the indicated vaccines at a 6-wk interval, 2 and 7 wk after boost (except control Ig-p24; $n = 2$ in C57BL/6 and $n = 1$ in BALB/c mice).

is similar to that reported using *Leishmania* protein administered with CpG (5). Injection of two doses of control Ig-p24 or one dose of α-DEC-p24 plus 50 μg of poly IC resulted in lower frequencies of cytokine-producing $CD4^+$ T cells, which declined at week 2 to 7 (SI Fig. 9). Thus, prime-boost immunization induces high frequencies of durable multifunctional $CD4^+$ T cells.

Prime-Boost Immunization with α-DEC-gag and Poly IC Induces Proliferating $CD4^+$ T Cells. $CD4^+$ Th1 cell proliferation and IFN-γ production are reported to be associated with low HIV-1 RNA and proviral DNA loads (13). To determine whether these features could be induced by a DC-targeted vaccine, splenocytes were labeled with carboxyfluorescein succinimidyl ester (CFSE) to follow the successive halving of CFSE/cell with each division. Two wk after the boost, $CD4^+$ T cells were cultured for 3 days and challenged with p24 peptides. The T cells responded by proliferation and IFN-γ formation, specifically to gag p24 in the vaccine, and not the control gag p17 peptide mix. Fewer responding $CD4^+$ T cells were seen with two doses of α-DEC-p24 plus 10 or 2 μg of poly IC or one dose of α-DEC-p24 plus 50 μg of poly IC (Fig. 2*A*).

To assess the persistence of proliferative, HIV gag-specific $CD4^+$ T cells after prime-boost immunization, we did three experiments each in C57BL/6 and BALB/c mice and found that proliferative $CD4^+$ T cells persisted at least 7 wk but decreased from week 2 to 7 (Fig. 2*B*). Only low frequencies of proliferating $CD4^+$ T cells were identified after two doses of control Ig-p24 or one dose of α-DEC-p24 plus poly IC, and only at 2 but not 7 weeks after vaccination. Therefore, a prime boost with α-DEC-p24 and 50 μg of poly IC induces both long-lived IFN-γ secreting and proliferating $CD4^+$ T cells.

Poly IC also Serves as an Adjuvant for $CD4^+$ T Cell Responses to α-DEC-nef. To extend the concept of directed delivery of antigens to DCs *in situ* to other HIV antigens, we engineered the nef protein into the α-DEC-205 heavy chain. The fusion mAbs were expressed and contained heavy chains of ≈70 kDa as opposed to ≈50 kDa for unmodified mouse IgG1 (SI Fig. 10). When BALB/c mice were immunized with two doses of α-DEC-nef plus poly IC, $CD4^+$ T cells became responsive to one nef peptide pool; in contrast, two doses of "empty" α-DEC-205 mAb plus poly IC did not immunize (Fig. 3*A*). This strategy also elicited proliferating IFN-γ producing $CD4^+$ T cells (Fig. 3*B*), indicating good quantity and quality $CD4^+$ T cell responses to HIV nef as well as gag.

Prime-Boost Immunization with α-DEC-gag and Poly IC Elicits Protection at a Mucosal Surface. To determine if poly IC acts as an adjuvant for long lasting protective immunity at a mucosal surface, the lung, we challenged vaccinated BALB/c and C57BL/6 mice intranasally (i.n.) with recombinant vaccinia-gag virus 6–8 wk after prime-boost immunization with α-DEC-gag and poly IC. In initial protection experiments, we verified that a dose of 50 μg of poly IC gave optimal protection (SI Fig. 11*A*). The results from three experiments each in BALB/c and C57BL/6 mice show that mice injected with PBS lost weight and developed high titers of virus in the lung ($> 10^8$ PFU/ml) over 6–7 days (Fig. 4*A*). By two criteria, reduced weight loss and reduced growth of virus, two doses of α-DEC-p24 and 50 μg of poly IC provided better protection relative to two doses of control Ig-p24 or one dose of α-DEC-p24 plus poly IC.

As previously described, $CD4^+$ T cell depletion from vaccinated mice before the challenge with vaccinia-gag ablated protection (23) (SI Fig. 11*B*). In addition, DEC expression was essential because $DEC^{-/-}$ mice failed to be protected (Fig. 4*B*). However, $TLR3^{-/-}$ mice were fully protected to vaccinia-gag when immunized with two doses of α-DEC-p41 and poly IC (Fig. 4*B*), indicating that TLR3 was not essential for protection with poly IC adjuvant.

TLR3 Dependent Immunity to DC-Targeted HIV gag Vaccine and Poly $IC_{12}U$. We also examined the poly IC analog poly $IC_{12}U$ (trade name Ampligen) as an adjuvant, because poly $IC_{12}U$ exhibits minimal

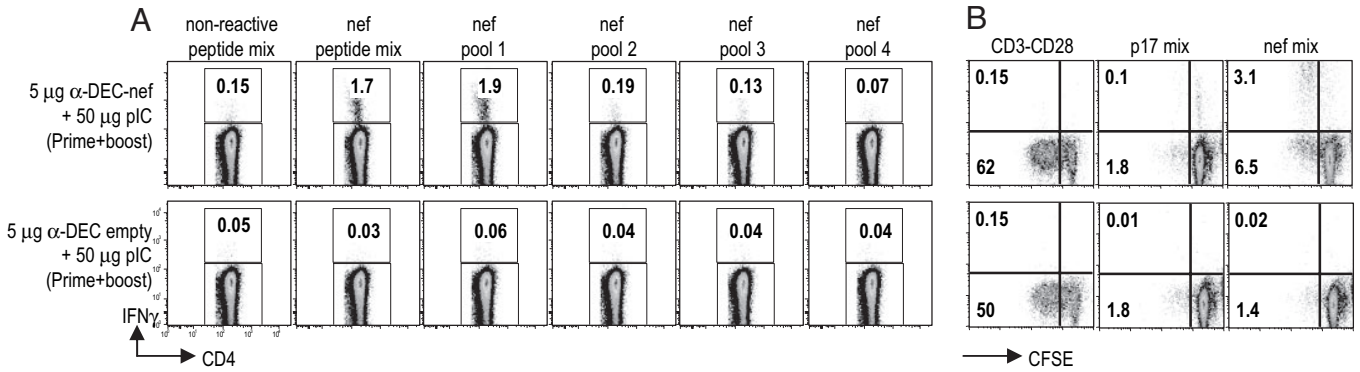

Fig. 3. α-DEC-nef and poly IC induces nef-specific $CD4^+$ T cell immunity. BALB/c mice were injected twice i.p. with 5 μg of α-DEC-nef or unconjugated α-DEC mAb and 50 μg of poly IC at a 6-wk interval. Nef-specific T cells were analyzed a week after boosting. (*A*) IFN-γ in spleen $CD3^+CD4^+$ T cells in response to HIV nef peptide mix, different nef peptide pools, or nonreactive peptide mix. (*B*) CFSE-labeled bulk splenocytes were stimulated with α-CD28 and nef peptide mix, nonreactive peptide mix or α-CD3 for 3 days, whereupon the cells were restimulated with HIV nef peptides to detect IFN-γ in proliferated $CFSE^{low}$, $CD3^+CD4^+$ T cells. Shown is the percentage of IFN-γ^+ or IFN-γ^- $CD3^+CD4^+$ proliferating T cells (*Upper Left* and *Lower Left*, respectively) from one of three experiments with two mice pooled in each.

toxicity even at high doses but shares immunomodulatory properties with poly IC (40, 41). CxB6 F1 mice were injected with two doses of α-DEC-p24 mAb plus increasing doses of poly IC or poly $IC_{12}U$, and we monitored the proliferative capacity of $CD3^+CD4^+$ T cells in response to HIV gag p24 peptides 1 wk after boosting. Both forms of poly IC induced $CD4^+$ T cell responses that were dose-dependent, although the immune response induced by poly IC was greater than with poly $IC_{12}U$ (Fig. 5*A*).

To determine the pattern recognition receptors required by poly IC and poly $IC_{12}U$, we tested wild-type, $TLR3^{-/-}$ and $MDA5^{-/-}$ mice. Poly IC had some adjuvant activity in $TLR3^{-/-}$ mice, whereas poly $IC_{12}U$ could not elicit $CD4^+$ IFN-γ secreting T cells in these animals (Fig. 5*B*). Both adjuvants elicited responses in $MDA5^{-/-}$ mice. Taken together the data indicate that TLR3 is essential for the adjuvant role of poly $IC_{12}U$, but poly IC may require both TLR3 and the cytosolic sensor MDA5.

Discussion

Dendritic cells are potent inducers of T cell-mediated immunity and are therefore attractive targets to improve vaccine efficacy. As an example, when vaccine proteins are selectively delivered by mAbs to DC endocytic receptors, antigen presentation and immune

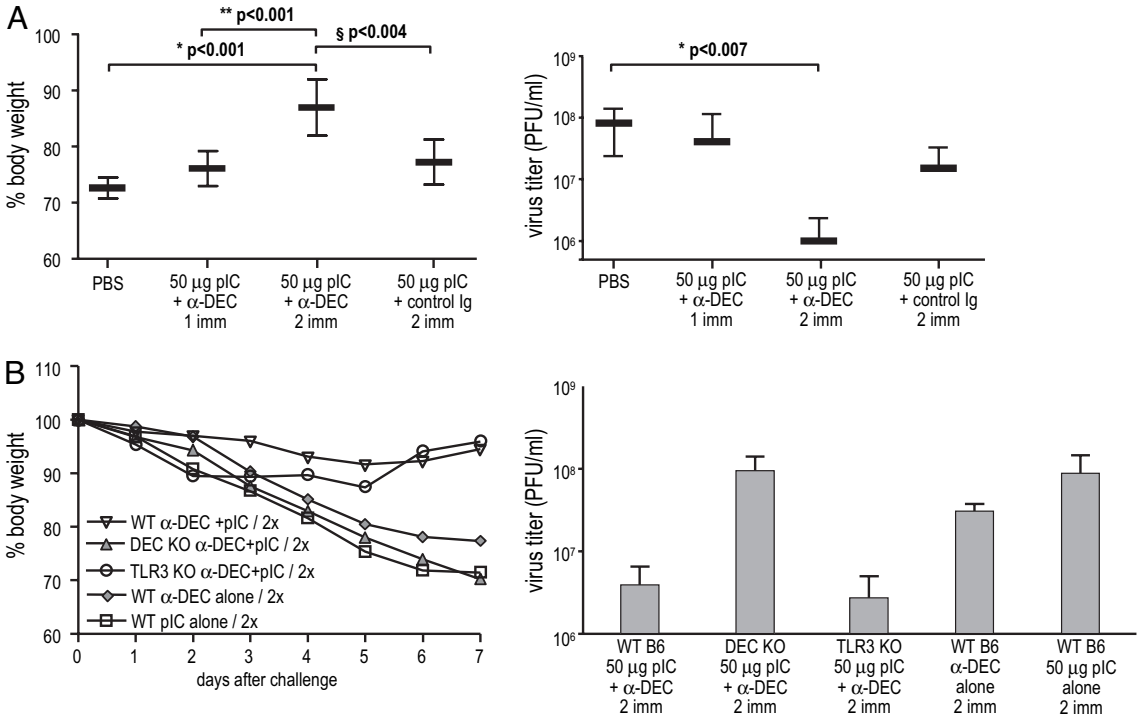

Fig. 4. Prime-boost immunization with 5 μg of α-DEC-gag and 50 μg of poly IC provides protective immunity to airway challenge with vaccinia-gag virus. (*A*) Average weight loss (*Left*) and lung virus titers (*Right*) as a mean ± SD after challenge from six experiments, three each in BALB/c and C57BL/6 mice (except control Ig-p24; $n = 2$ in BALB/c). Mice were primed and boosted at a 6-wk interval with the indicated vaccines. Another group was immunized with α-DEC-p24 and poly IC at the time of the boost only. Six to eight weeks after boost, mice were challenged i.n. with 5×10^4 PFU vaccinia-gag. (*B*) As in *A*, but C57BL/6, $DEC\text{-}205^{-/-}$, or $TLR3^{-/-}$ mice were immunized with α-DEC-p41 and challenged with vaccinia-gag. Data are one of two similar experiments. Data are average body weight (*Left*) or vaccinia plaque-forming titers in lung (*Right*) as mean ± SD ($n = 5$).

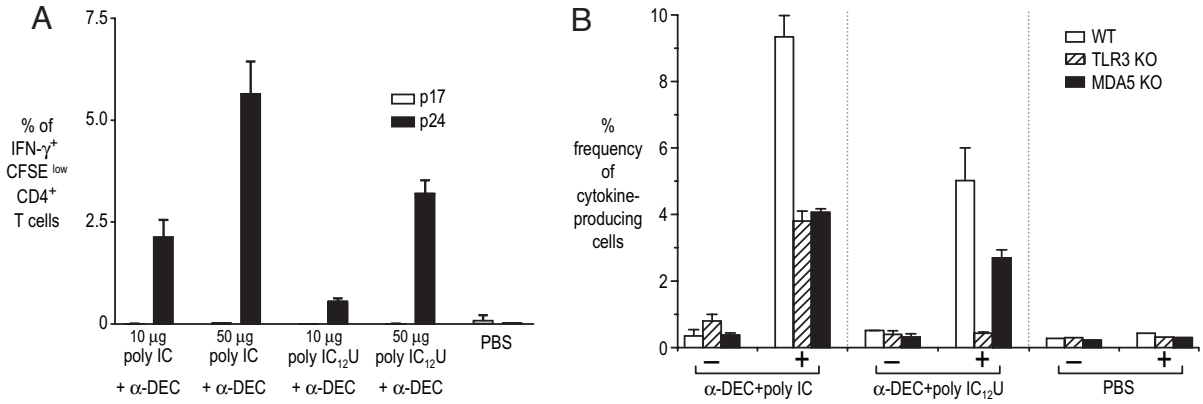

Fig. 5. Poly $IC_{12}U$ acts as an adjuvant for $CD4^+$ T cell immunity to α-DEC-p24 vaccine in a TLR3-dependent manner. (*A*) CxB6 F_1 mice were injected i.p. with 5 μg of α-DEC-p24 plus graded doses of poly IC (InVivoGen) or poly $IC_{12}U$ (Ampligen) or PBS, and boosted with the same conditions 6 wk later. The percentage of IFN-γ-producing and proliferating $CD3^+CD4^+$ T cells in response to HIV gag p17 or p24 mix 1 wk after boost are shown as mean $\pm$ SD (n = 4 mice). (*B*) IFN-γ-secretion in response to HIV gag p24 peptides by $CD4^+$ splenocytes in wild-type, $TLR3^{-/-}$, and $MDA5^{-/-}$ mice immunized with two doses of α-DEC-p24 plus 50 μg of poly IC (InVivoGen) or 250 μg of poly $IC_{12}U$ (Ampligen). Data are one of two similar experiments with two mice in each.

responses develop with much greater efficacy relative to nontargeted antigen. One receptor under study is DEC or CD205, which is expressed on DCs in the T cell areas (25, 42). Previously, we immunized mice with α-DEC-HIV gag p24 fusion mAb together with a combination of agonistic α-CD40-mAb and poly IC as the adjuvant (23), and we studied the primary $CD4^+$ T cell response to gag. Here, we used poly IC alone as a DC maturation stimulus, as a preclinical model for future proof of concept studies in monkeys and humans, and we studied several features of the quality of $CD4^+$ T cell immunity including memory. Our data reveal the potential of poly IC to serve as an adjuvant using a protein based vaccine that directly targets DCs and elicits long-lived and protective Th1 $CD4^+$ T cells of superior quality and quantity.

The $CD4^+$ T cell response with poly IC as an adjuvant was multifunctional, i.e., the T cells produced multiple cytokines such as IFN-γ, TNF-α, and IL-2, and in high amounts. We also found that the DEC-targeted vaccine induced high frequencies of IFN-γ-producing and proliferating $CD4^+$ T cells, a feature that to date is unique to this vaccine approach. Increased frequencies of multifunctional $CD4^+$ T cells elicited by immunization correlate with better protection in the *L. major* model (5). Further, they are found retrospectively to be increased in HIV infected individuals who have a better clinical outcome (10, 12).

Another interesting consequence of DC-targeted HIV gag p24 was the induction of long-lasting protective $CD4^+$ Th1 T cell immunity to vaccinia-gag. IFN-γ resists vaccinia infection (43), and $CD4^+$ Th1 cells lyse cytomegalovirus-infected MHC II^+ targets (6); both features may contribute to resistance induced by DEC targeted proteins together with poly IC.

Poly IC can be recognized by TLR3 endosomal (33) and MDA5 cytosolic receptors (34). We found that both TLR3 and MDA5 contributed to the adjuvant action and protective immunity with poly IC. In contrast, TLR3 was exclusively needed for the adjuvant role of poly $IC_{12}U$ (44).

Interestingly, we observed only weak $CD8^+$ T cell responses to 2 doses of poly IC adjuvanted, DC-targeted vaccine (data not shown). This result is surprising, given evidence that DEC^+ DCs efficiently cross-present ovalbumin to $CD8^+$ T cells, particularly ovalbumin delivered within α-DEC mAbs (21). In addition, poly IC induces type I interferons, which promote cross-presentation by DCs (45) and survival of $CD8^+$ T cells (46). More research is required to evaluate whether the $CD8^+$ T cell response induced by DEC-targeted vaccine can be improved with different DC stimuli or different vaccine formulations.

Although poly IC has been used as an adjuvant to enhance the immunogenicity to a vaccine protein in mice (38), its ability to induce $CD4^+$ T cell responses that are also protective has not been shown before. Targeting of poly IC-adjuvanted vaccine protein to DEC^+ DCs should favor the kind of Th1 $CD4^+$ T cell immunity now implicated in resistance to many global infectious diseases.

Materials and Methods

Mice. C57BL/6 (B6), BALB/c, and CxB6 F_1 were from Harlan. Mice deleted for TLR3, MDA5 and DEC-205 genes were kindly provided by S. Akira (Osaka University, Osaka, Japan), M. Colonna (Washington University, St. Louis, MO), and M. Nussenzweig (The Rockefeller University, New York, NY). Mice were maintained under specific pathogen-free conditions and used at 7–8 wk of age according to Institutional Animal Care and Use guidelines.

Fusion HIV Protein mAbs. HIV gag-p24, HIV gag-p41, and unconjugated α-DEC-205 mAbs were prepared as described in ref. 23. To clone HIV nef into the COOH terminus of α-DEC-205 mAb heavy chain (20), the nef sequence was amplified by overlapping PCR from a CCR5-tropic primary virus classified as a subtype C/B′ recombinant. Fusion HIV nef mAbs were expressed by transient transfection (calcium-phosphate) as for HIV gag fusion mAbs (23). All mAbs were characterized by SDS/PAGE and Western blotting using HRP sheep anti-mouse IgG (GE Healthcare, Buckinghamshire, U.K.) or HRP-anti-gag p24 (ImmunoDiagnostics, Woburn, MA). mAbs binding was verified on CHO cells stably transfected with mouse DEC-205 by FACS using PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). All mAbs were endotoxin-free in Limulus Amebocyte Lysate assay, QCL-1000 (Cambrex, Walkersville, MD).

HIV Peptides. A library of overlapping (staggered by 4 aa) 15-mer peptides spanning the entire HIV gag p17, p24 or nef proteins were synthesized by the Proteomics Resource Center (The Rockefeller University). The 30-member gag p17 and 60-member gag p24 libraries were resuspended at 1 mg/ml of each peptide in 100% DMSO, whereas the new 50-member nef library was divided into four pools of 12–13 peptides each, spanning amino acids 1–58 (pool 1), amino acids 59–106 (pool 2), amino acids 107–152 (pool 3), and amino acids 153–204 (pool 4) of HIV nef.

Immunizations. Mice were injected once or twice i.p. or s.c. in the hind footpads at 4- to 6-wk intervals with fusion mAb together with adjuvant, which was poly IC (2, 10, or 50 μg; InVivoGen, San Diego, CA), poly $IC_{12}U$ (10, 50, or 250 μg; Ampligen Hemispherx, Philadelphia, PA), or 30 μg of MPLA (InVivoGen).

***In Vivo* Cytokine Analysis.** Mice were injected i.p. with 50 μg of poly IC or 30 μg of MPLA and serum was collected at the indicated times to measure TNF-α and IL-6 by ELISA (eBioscience, San Diego, CA) and IFN-α by an ELISA kit from R&D Systems (Minneapolis, MN).

Assays for HIV-Specific Immune T Cells. Bulk splenocytes were either restimulated with the entire HIV gag p24 or HIV nef peptide mix, or a negative control

peptide mix (peptides at 1 μg/ml), in the presence of 2 μg/ml costimulatory α-CD28 (clone 37.51) for 6 h at 37°C, adding Brefeldin A (BFA) (10 μg/ml; Sigma–Aldrich, St. Louis, MO) for the last 5 h to allow accumulation of intracellular cytokines. Cells were washed, incubated 10 min at 4°C with 2.4G2 mAb to block Fcγ receptors, washed, and stained with Live/Dead Fixable Aqua viability dye (Invitrogen, Carlsbad, CA), Pacific blue-conjugated anti-CD3, PerCP-conjugated anti-CD4, and APC-Cy7-conjugated anti-CD8 (BD Biosciences, San Diego, CA) mAbs for 20 min at 4°C. Cells were permeabilized (Cytofix/Cytoperm Plus; BD Biosciences) and stained with Alexa Fluor 700-conjugated α-IFN-γ, APC-conjugated α-IL-2, and Alexa Fluor 488-conjugated α-TNF-α mAbs for 15 min at room temperature (BD Biosciences), resuspended in BD stabilizing fixative, and 100,000 live-CD3$^+$ cells were acquired on a BD LSR II flow cytometer. Data were analyzed with FlowJo Software (Tree Star, Inc., San Carlos, CA) and Spice 4.0 (M. Roederer, National Institutes of Health). To assess proliferation of primed T cells, bulk splenocytes were labeled with CFSE (10^7 cells per ml, 1 μM, 10 min, 37°C; Molecular Probes, Eugene, OR) and cultured with α-CD28 and either HIV gag p24, HIV nef peptides, nonreactive peptide mix or α-CD3 (0.1 μg/ml; positive control) in 96-well, round-bottom plates. After 3 days of culture, samples were restimulated for 6 h with p24 or nef peptides and α-CD28, adding BFA at 10 μg/ml for the last 5 h. The cells were stained with PerCP-conjugated α-CD3 and PE-conjugated α-CD4 as above, permeabilized, stained with APC-conjugated α-IFN-γ mAb for 15 min at room temperature (BD Biosciences), and acquired on a FACSCalibur with data analysis in FlowJo Software (Tree Star), collecting 40,000 high-CD3$^+$ events.

Protection to Airway Challenge with Recombinant Vaccinia-gag Virus. We applied 5×10^4 PFU i.n. to groups of four to five nembutal-anesthetized mice. In some cases, vaccinated C57BL/6 mice were depleted of CD4$^+$ T cells by injecting 100 μg of GK 1.5 mAb 1–3 days before challenge. To assess protection, we monitored body weights daily for 6–7 days and then harvested lungs to measure PFU on CV-1 cell monolayers.

Statistical Analysis. All comparisons between vaccine groups used a two-tailed Student's *t* test to compare the differences between means and standard deviation of the experimental groups shown in the figures. Analysis was performed with a Prism 3 program (Graphpad Software Inc., San Diego, CA).

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