Non-classical Natural Killer T Cells Modulate Plasmid DNA Vaccine Antigen Expression and Vaccine-elicited Immune Responses by MCP-1 Secretion after Interaction with a β2-Microglobulin-independent CD1d

Ralf Geiben-Lynn, John R. Greenland, Kwesi Frimpong-Boateng, and Norman L. Letvin

From the Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, and Harvard Medical School, Boston, Massachusetts 02215

The magnitude and durability of a plasmid DNA vaccine-induced immune response is shaped by immune effector molecules at the site of vaccination. In the present study, we show that antigen expression is modified by type II NKT cells, after interaction with a β2-microglobulin-independent CD1d receptor. After activation, during the first days following plasmid DNA vaccination, NKT cells release IL-5 and MCP-1, leading to a T helper 0 (T(0)) cytokine/chemokine profile and a stronger CD8+CD4+ T cell immune response. Our data indicate that this phenomenon was induced through the strong T(0) chemokine MCP-1 during the early phases of plasmid DNA vaccination because injecting the type II NKT cell-associated MCP-1 during the first 5 days led to 2–3-fold increases in vaccine-elicited T cell responses. This study demonstrates a critical role for NKT cells in plasmid DNA vaccine-induced immune responses. Manipulation of NKT cell function or co-administration of MCP-1 may represent novel methods for enhancing immune responses to plasmid DNA vaccines.

Plasmid DNA vaccine-induced T cell memory responses are potentiated by innate immune responses that are generated during the first days after vaccination (1). At that time Natural Killer (NK) and Natural Killer T (NKT) cells are attracted to the site of vaccine administration (2). Investigation into the function of both cell types following vaccination is important because in diverse biologic settings the release of NK or NKT cell-associated cytokines and chemokines has been shown to modulate adaptive T cell and humoral immune responses. Responding quickly, these cells have the capacity to produce cytokines and chemokines that help B cells produce antibodies, induce macrophages to develop enhanced microbialidal activity, and recruit T cells, neutrophils, eosinophils, and basophils to sites of infection and inflammation (3–9).

NK and NKT cells have distinct and complementary functions. NK cells can recognize and eliminate tumors and virus-infected cells (3–5). NKT cells participate in immune processes associated with self-tolerance, including tumor rejection (10, 11), suppression of anti-tumor responses (12), down-regulation of autoimmune inflammatory diseases (13), immune privilege (14), tolerance to allografts and xenografts (15–17), fetal-maternal tolerance (18), protection against graft-versus-host disease (19), and protection against pathogens such as Cryptococcus (20) and hepatitis B virus (21).

NK and NKT cells recognize target cells using distinct receptors. NK cell effector function is regulated by a balance between opposing signals delivered by inhibitory and activating receptors. A number of surface molecules expressed by NK cells, including CD2, CD16, CD69, and DNAM-1 have been implicated in the triggering of NK cell-mediated cytotoxicity. Additionally, the activating counterparts of the MHC-specific receptor p50 and the CD94/NKG2C molecules trigger NK-mediated cytotoxicity against MHC class I-expressing target cells. MHC class I negative targets are lysed after recognition by the cytotoxicity receptors Nkp46, Nkp44, and Nkp30 (3–5).

The majority of NKT cells express the NK lineage-specific receptor NK1.1 (CD161) and secrete cytokines upon recognition of CD1d. CD1d can present lipids and glycolipids as well as peptides to NKT cells. Invariant NKT (iNKT) cells or type I NKT cells make use of a restricted TCR repertoire, using an invariant TCR Vα14-Jα281 rearrangement and a limited set of TCR Vβ segments, suggesting that they recognize a limited set of CD1d-associated ligands (8, 9). A second group of CD1d-reactive NKT cells, referred to as type II NKT cells, use a diverse TCR repertoire that allows them to recognize a diversity of ligands presented on CD1d (8).

Because plasmid DNA antigen clearance has been shown to be β2m-independent (22), we sought to determine whether NK or NKT cells influence this process. The present studies demonstrate that β2m-independent CD1d-restricted type II NKT cells influence the speed of plasmid antigen clearance, and consequently the magnitude of the cellular and humoral immune response to DNA vaccination.

EXPERIMENTAL PROCEDURES

Mice—6–8-week female C57BL/6, CD1d KO (CD1.1/CD1.2 KO on BALB/c background), BALB/c, and beige mice (C57BL/
6-Lys(b) (23–25) were purchased from the Jackson Laboratory. Jα18 KO mice (iNKT KO), mice missing the Vα14-Jα18 NKT cells, were a gift from Dr. Mark E. Exley (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA). All animals were housed and maintained in accordance with the Guide for the Care and Use of Laboratory Animals (26), and all studies and procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Vectors and Immunization—The plasmid DNA-Luciferase (DNA-Luc) construct with the AL11-Gag tag was constructed as described previously (27). This vector contains the GL4.10 luciferase gene (Promega, Madison, WI) and the immunodominant H-2Dβ-restricted SIV-Gag AL11 epitope flanked by triple alanine spacers. Plasmid DNA was prepared using an endotoxin-free Qiagen Giga-prep kit. For immunizations, 50 μg of plasmid DNA in 100 μl of sterile saline were divided between quadriceps muscles by intramuscular (i.m.) inoculation. DNA endotoxin concentration was determined with the E-Toxate kit (Sigma), and was below 0.1 unit/μg in all vaccine preparations used in these studies.

Antibodies for Use in Flow Cytometric Analysis—Fluorescein isothiocyanate (FITC)-, allophycocyanin (APC)-, and phycoerythrin (PE)-labeled antibodies were used for the flow cytometric analysis. The dye-coupled antibodies anti-NK1.1-FITC (mC5-3), anti-CD8-APC (53-6.7), CD4-APC-Cy7 (GK1.5), and anti-CD3e-PE (JES6-5H4), anti-CD69-APC were purchased from BD Bioscience (San Jose, CA). CD1d-PBS57 and control PE-conjugated tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility (Emory University Vaccine Center, Atlanta, GA) and used as described earlier (28).

Ceramide Reagents—Various reagents, including D-glucosyl-β-1′-ceramide (C8), D-galactosyl-β-1′-ceramide (C8), D-glucosyl-β-1′-ceramide (C12), and D-galactosyl-β-1′-ceramide (C12), were purchased from AvantiLipids (Alabaster, AL). All ceramide reagents were first dissolved in DMSO, then injected via the intraperitoneal (intraperitoneal) route 3 days prior to plasmid DNA inoculations at a dose of 10 μg in 200 μl of sterile PBS, a dose at which the D-glucosyl-β-1′-ceramide (C12) has been shown to totally deplete NKT cell numbers (29). D-Glucosyl-β-1′-ceramide (C8), D-galactosyl-β-1′-ceramide (C8), and D-galactosyl-β-1′-ceramide (C12) were used as controls.

IL-13 and MCP-1 Administration—IL-13 and MCP-1 were purchased from PeproTech (Rocky Hill, NJ). Proteins were reconstituted in PBS and administered at 50, 100, 500, and 1000 ng together with 50 μg of the plasmid DNA-Luc construct in a total of 100 μl of PBS divided equally between the quadriceps muscles. A similar protocol was used for injecting the proteins via the i.m. route without the plasmid DNA at days 3 and 5.

Immunological Assays—H-2Dβ/AL11 tetramers were prepared and used to stain epitope-specific CD8+ T cells as previously described (30). Peripheral blood was collected and lysed with BD Pharmlyse buffer (BD Bioscience). The samples were then analyzed on a FACS Array (BD Bioscience), and CD8+ T lymphocytes were examined by staining with the Dβ/AL11 tetramer. CD8+ T lymphocytes from control mice immunized with untagged plasmid DNA-Luc exhibited ≤0.1% tetramer staining. Intracellular cytokine staining (ICS) was performed as previously described (31). For T cell stimulations, cells were incubated with a luciferase (Luc) peptide pool at 2 μg/ml for each peptide. The Luc peptide pool of 67 18-mers, overlapping by 10 amino acids, was synthesized by Quality Control Biochemicals (Hopkinton, MA). ELISpot assays were performed as previously described with a Luc peptide pool at a concentration of 2 μg/ml for each peptide (32).

In Vivo Bioluminescence Measurement—Mice were injected by intraperitoneal route with 100 μl of a 30 mg/ml solution of firefly luciferin (Xenogen, Alameda, CA) in PBS, as well as 100 μl of a 20 mg/ml ketamine and 1.72 μg/ml xylazine mixture. After 20 min, imaging was performed using the IVIS Series 100 (Xenogen) with an integration time of 1 min. Overlay images and luminescence measurements were made using Living Image softwareTM (Version2.50.1, Xenogen).

Measurement of Antigen Expression—To convert the in vivo bioluminescence Relative Light Units (RLU) of the different vectors into quantity of antigen expressed, we prepared a calibration curve with values of emitted light per min for different amounts of recombinant Luc protein (Promega). There was a linear correlation between amount of protein injected (10 ng to 50 μg) and light emitted, enabling us to calculate antigen expression from the different vectors according to the following formula: concentration of antigen in ng = antilog [(log RLU – 4.2)/0.76].

Cell Depletion and CD1d Signaling Blockade—Both NK and NKT cell depletion were performed with the anti-asialo GM1 antibody (ASGM1) (Wako Chemicals, Richmond, VA) using 50–μl infusions as described earlier (33). Nonimmune rabbit IgG (Sigma) was used as a control. The ASGM1 depletion achieved greater than 80% depletion of NK or NKT cells at days 14 and 21 as confirmed by monoclonal anti-CD3 and anti-NK1.1 antibody (BD Bioscience) staining and flow cytometric analysis. To block CD1d interaction the anti-CD1d antibody (3C11, eBioscience, San Diego, CA), which can recognize the β2m-dependent and β2m-independent forms of CD1d (34), was used and compared with an isotype control antibody (eBioscience). All antibodies were administered via the intraperitoneal route (250 μg antibody per mouse per inoculation) 3 days prior to plasmid DNA inoculation and every 3 days thereafter.

Cytokine/Chemokine Array—To test for 20 cytokines and chemokines in mouse serum the Quantibody™ Mouse Array 1 chip (cat. QAM-CYT-1) from RayBiotech (Norcross, GA) was used according the manufacturer’s protocol. This enabled testing for GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-17, KC, MCP-1, M-CSF, RANTES, TNF-α, and VEGF at the same time.

Data Analysis—Statistical differences were determined using the Mann-Whitney test. A p value of < 0.05 was considered to be significant. Statistical calculations were performed using the GraphPad PrismTM program (version 4.03). Error bars represent the S.E.
**RESULTS**

**DNA Vaccine Antigen Expression Damping Is Dependent on Innate Immune Cells**—We have previously shown that antigen expression during the first 3 days following plasmid DNA inoculation influenced the strength of the vaccine-induced memory T cell immune response (1). Because DNA vaccination is associated with the accumulation of both NK and NKT cells at the site of inoculation (2), we sought to determine whether these cell populations modulate plasmid DNA-elicted adaptive immune responses. We did this by depleting both NK and NKT cells following DNA vaccination to test the influence of these cells on vaccine antigen clearance and vaccine-elicted immune responses. This was accomplished by treatment of vaccinated mice with an anti-ASGM1 antibody. To measure vaccine antigen clearance in vivo and CD8+ T cell immune responses simultaneously without resorting to serial animal sacrifice, we immunized mice via the i.m. route with a plasmid DNA-Luc construct, which has the H-2D^b-restricted CTL epitope SIV-Gag AL11 added at the N terminus of the luciferase gene. Monoclonal antibody staining and flow cytometric analysis confirmed that greater than 80% of NK cells (p = 0.02, n = 4) and greater 95% of NKT cells (p = 0.01, n = 4) were depleted in peripheral blood after anti-ASGM1 treatment at day 14 (Fig. 1A) and day 21. The depleting efficacy of anti-ASGM1 antibody was lost at the 28 day time point, and NK and NKT cells recover to the levels seen in control animal levels. We found that NK/NKT cell depletion prevented the clearance of antigen expression normally seen by day 21 after plasmid DNA inoculation (Fig. 1B). To determine if NK/NKT cell depletion influenced the Gag-specific CD8+ T cell response, we stained the PBMC of these mice with the AL11-Gag tetramer. We observed a total absence of the SIV-Gag AL11-specific CD8+ T cell response (d14, p = 0.03, n = 4) in the anti-ASGM1-treated mice when effector cell staining reached the highest levels (Fig. 1C). T cell immune responses against the Luc-antigen were assessed by IFN-γ ELISPOT assay using the Luc peptide pool. We observed a total loss of Luc-specific T cell immune responses after NK/NKT cell depletion (Fig. 1D). These findings suggested that NK or NKT cells are important for eliciting strong DNA vaccine-induced T cell immune responses.

**DNA Vaccine Antigen Expression Damping Is Dependent on NKT Cells**—In light of the evidence that NK and/or NKT cells influence adaptive immune responses and antigen clearance in plasmid DNA-vaccinated mice, we designed experiments to define the contribution of each of these cell types to the kinetics of DNA vaccine antigen expression. We first evaluated plasmid DNA vaccination in CD1d KO mice, in which complete depletion of NKT cells is achieved through a homozygous CD1d-targeted mutation (35). NK cells and T,2 immune responses are unaffected in these CD1d KO mice (36). NKT-deficient CD1d KO mice were not able to clear DNA vaccine antigen expression at day 14 (p = 0.03, n = 4) and day 28 (p = 0.03, n = 4) following immunization, suggesting that CD1d may be important for the clearance of plasmid DNA antigen expression in myocytes (Fig. 2A). Similar results were observed in mice treated with an anti-CD1d receptor blocking antibody, demonstrating that interaction with CD1d is necessary for the seen effect. In these mice, antigen clearance was diminished in comparison to that seen in wild-type mice starting at day 21, and the difference in antigen clearance with the KO and wild-type mice achieved statistical significance at day 28 (p = 0.01, n = 4) (Fig. 2B). In contrast, using beige mice, in which NKT cells are functional but NK cells are defective (23–25), we observed no defect in antigen clearance following immunization during 28 days following immunization (Fig. 2C). These results suggest that NK cells are not necessary for DNA vaccine antigen clearance.

Having demonstrated with the CD1d KO mice and anti-CD1d receptor-blocking antibody-treated mice that NK cells influence both DNA vaccine antigen clearance and DNA vaccine-elicted adaptive T cell immune responses, we administered the CD1d ligand β-glucosyl-β1-1’ ceramide (C12) at a dose at which total depletion of NK cell function in vivo has been achieved without affecting NK cell function (29). This
treatment resulted in a decrease of plasmid DNA antigen clearance beginning at day 21 following vaccine administration (d21, \( p = 0.03, n = 4; \) d28, \( p = 0.03, n = 4 \)) (Fig. 2E) confirming plasmid DNA NKT cell dependence by another method. No effect on vaccine antigen clearance was seen following administration of the control ceramides \( \beta \)-glucosyl-\( \beta \)-1’-ceramide (C8) (d21, \( p = 0.69, n = 4; \) d28, \( p = 0.90, n = 4 \)), \( \alpha \)-galactosyl-\( \beta \)-1’-ceramide (C8) (d21, \( p = 0.40, n = 4; \) d28, \( p = 0.10, n = 4 \)), or \( \alpha \)-galactosyl-\( \beta \)-1’-ceramide (C12) (d21, \( p = 1.0, n = 4; \) d28, \( p = 0.06, n = 4 \)).

Using iNKT KO mice in which a mutation depletes \( V\alpha14-J\beta281 \) cells, we also evaluated which subset of NKT cells, the iNKT or type II NKT cells, influences plasmid DNA-Luc antigen expression. We observed a similar rate of damping of antigen expression in iNKT KO mice and wild-type mice, ruling out the possibility that iNKT cells play an important role in DNA vaccine antigen clearance (Fig. 2D). Together with earlier findings that DNA antigen clearance is \( \beta \)2m-independent (22), these findings suggest that the type II NKT mediate this function.

**Change of DNA Vaccine-elicited Immune Responses after Depletion of CD1d-expressing Cells—Monitoring CD8\(^+\) T cell immune by AL11-Gag tetramer staining, we found that the strength of immune response correlated with greater damping of antigen expression. Only mice with intact type II NKT cells showed wild-type CD8\(^+\) T cell immune responses. Both NK cell- and iNKT cell-depleted mice developed normal immune responses (Fig. 3, A and B), whereas a total loss of NKT cell function through additional depletion of type II NKT cells led to a decrease in CD8\(^+\) T cell

---

**FIGURE 2. Damping of luciferase expression from a plasmid DNA vaccine construct is NKT cell mediated.**

Luciferase antigen expression in A, wild-type and CD1d KO; in B, wild-type and anti-CD1d antibody-treated mice; in C, wild-type and beige mice; in D, luciferase antigen expression in wild-type and iNKT KO mice; in E, wild-type and \( \beta \)-glucosyl-\( \beta \)-1’-ceramide-treated mice. For luciferase antigen expression measurements, mice (4 per group) were inoculated with a plasmid DNA-Luc construct and luciferase expression in vivo was measured by IVIS imaging. For CD1d receptor blockade mice were treated by intraperitoneal administration with an anti-CD1d antibody 3 days prior to, and every third day after DNA-Luc inoculation for a 28-day period, and in vivo luciferase expression was measured. For ceramide treatment, mice were treated by intraperitoneal administration with 10 \( \mu \)g of ceramide or vehicle control. Mean \( \pm \) S.E. *, significant difference (\( p < 0.05 \)), Mann-Whitney test. Experiments were done twice. Representative data are shown.

**FIGURE 3. Plasmid DNA vaccine-induced CD8\(^+\) T cell response is NKT cell mediated.**

CD8\(^+\) T cell immune responses in A, wild-type and beige mice; in B, wild-type and inNKT mice; in C, wild-type and \( \beta \)-glucosyl-\( \beta \)-1’-ceramide treated mice. For CD8\(^+\) T cell immune response measurements, mice (4 per group) were inoculated with 50 \( \mu \)g of plasmid DNA-Luc. For ceramide treatment, mice were treated by intraperitoneal administration with 10 \( \mu \)g of ceramide or vehicle control. CD8\(^+\) T cell immune responses were measured with AL11-Gag tetramer staining. Mean \( \pm \) S.E. *, significant difference (\( p < 0.05 \)), Mann-Whitney test. Experiments were done twice. Representative data are shown.
mediated immune responses (Fig. 3C). Thus, the complete depletion of NKT cell function can also be achieved by β-glucosyl-β1-1’ ceramide (C12) treatment (29). This led to a total loss of SIV Gag AL11-specific Cytotoxic T Lymphocyte (CTL) response at day 14 (p = 0.03, n = 4) and day 21 (p = 0.03, n = 4) following vaccine administration (Fig. 3C). Treatment of control ceramides did not have an effect on the cellular immune response.

To further evaluate the contribution of CD1d-restricted T cell responses to a plasmid DNA vaccine-elicited immune response, we compared the Luc-specific T cell immune response generated by plasmid DNA luciferase immunization in wild-type and CD1d KO mice in more detail. We found that wild-type mice showed a 3–4-fold higher IFN-γ spot-forming cells (SFC) response than CD1d KO mice following exposure to Luc peptides one month after plasmid DNA inoculation (Fig. 4A). We also assessed the CD4$^+$ T cell immune response to Luc peptides in wild-type and CD1d KO mice by intracellular cytokine staining (ICS). Wild-type mice showed a 4-fold greater CD4$^+$ T cell immune response to the Luc peptides than CD1d KO mice (Fig. 4B). Together, these results suggest that type II NKT cells are necessary for the induction of vaccine antigen-specific CTL responses and clearance of antigen following DNA immunization.

To test the hypothesis that DNA vaccination activates NKT cells at the site of injection and these cells then migrate to the peripheral blood, we measured CD69 expression by CD1d$^+$ PBSS7 tetramer-positive cells in PBMC. We found an ~20-fold increase in activated NKT cells in PBMC 5 days after vaccination compared with unvaccinated animals (Fig. 4C). Plasmid DNA vaccination was associated with up-regulation CD69 surface expression from 0.4 to 10% (p = 0.03, n = 4).

**IL-5 and MCP-1 Secretion after NKT Cell Recognition of CD1d**—One possible explanation for this observed function of NKT cells is that these cells secrete a series of cytokines following recognition of CD1d that can polarize T helper cell responses (37). To test this hypothesis, we measured 20 cytokines and chemokines in sera of wild-type and CD1d KO mice following DNA immunization. We found that cytokines and chemokines were maximally increased at day 1 after inoculation and then returned to background levels at day 2 or 3. These kinetics are consistent with an innate immune response. Comparing the blood levels of these proteins in wild-type and CD1d KO mice, we found that both T$_{11}$-type and T$_{12}$-type cytokines decreased in the CD1d KO: GM-CSF (wt: 30.56 ± 15.3, CD1d: 5.28 ± 1.6, p = 0.15, n = 5), IL-2 (wt: 61.6 ± 41.5, CD1d: 10.84 ± 4.5, p = 0.10, n = 5), IL-5 (wt: 150.8 ± 61.8, CD1d: 28.5 ± 19.7, p = 0.03, n = 5), IL-13 (wt: 152.2 ± 114.9, CD1d: 14.6 ± 6.4, p = 0.06, n = 5) and the chemokine MCP-1 (wt: 99.9 ± 31.0, CD1d 17.0 ± 1.9, p = 0.008, n = 5) were expressed at higher levels in the wild type than in the CD1d KO mice (Fig. 5).

**NKT-associated Cytokines and Chemokines Augment CD8$^+$ T Cell Specific Immune Responses**—We finally tested whether the T$_{11}$-type chemokine MCP-1 more highly expressed following plasmid DNA vaccination in the wild type than in the CD1d KO mice, could be alone responsible for the enhancement of the DNA vaccine-induced SIV-Gag AL11-specific CD8$^+$ T cell immune response. We used T$_{12}$-type cytokine IL-13 as a control. We treated wild-type mice with each of the proteins by i.m. route on days 0, 3, and 5 following plasmid DNA-Luc inoculation and measured the SIV-Gag AL11-specific CD8$^+$ T cell responses. We found that MCP-1 increased the SIV-Gag AL11-specific CD8$^+$ T cell responses by 2–3-fold (Fig. 6A), whereas the IL-13 control had no effect on the DNA vaccine-elicited responses (Fig. 6B).
NKT Cells Modulate DNA Vaccine Potency

FIGURE 6. Improvement of CD8\(^+\) T cell immune response with recombinant MCP-1. SIV-Gag AL11 epitope-specific CD8\(^+\) T cell responses in vaccinated wild-type and NKT cell cytokine/chemokine-treated mice. C57BL/6 mice were injected with A, 500 ng MCP-1; B, 500 ng IL-13 on days 0, 3, and 5. Controls were treated with vehicle solution. Epitope-specific CD8\(^+\) T cell responses were measured by DP\(^+\)/AL11 tetramer staining of CD8\(^+\) T cells at the indicated times following plasmid DNA vaccine inoculation. Data are shown as mean ± S.E. Experiments were done twice. Representative data are shown.

DISCUSSION

Emerging data suggest that NKT cells are a heterogeneous population with a variety of immune functions. NKT cells have been classified into subtypes based on the degree of their TCR diversity and the antigen-presenting molecules that they recognize (8). Two of these subtypes, the classical or iNKT cells and the non-classical or type II NKT cells, interact with the CD1 receptor. The present study shows that type II NKT cells contribute to plasmid DNA antigen clearance and to changes in vaccine-induced cellular immune responses.

Our findings increase the range of biologic functions mediated by type II NKT cells found so far: (i) In ulcerative colitis, type II NKT cells are present in the lamina propria of the colon, and lyse intestinal epithelial cells (38), suggesting that they may play the role of effector cells in the pathogenesis of ulcerative colitis (39); (ii) in a transgenic mouse model of acute hepatitis B infection, type II NKT cells have been shown to cause liver cell injury (40); furthermore, (iii) type II NKT cells have been shown to regulate autoimmune diabetes in a TCR-transgenic mouse (41), (iv) they down-regulate tumor-immune surveillance in mouse models of cancer by enhancing TGF-\(\beta\) production (12, 42), and (v) may contribute to the pathogenesis of allergic asthma (43).

Our findings suggest that plasmid DNA vaccination activates NKT cells, which then migrate to the peripheral blood. This increased NKT activation is to levels half of that seen after Mycobacterium tuberculosis infections (28). Type II NKT cells express a semi-variant TCR, and, therefore, they can recognize a larger diversity of ligands presented on CD1d than iNKT cells. Most NKT cells recognize antigen presented by CD1d, an the MHC class I-like, \(\beta\)2m-associated molecule (44). A \(\beta\)2m-independent form of the CD1d (34, 45, 46) has been shown to play a major role in airway hyper-reactivity associated with allergic asthma (43) and in the pathogenesis of ulcerative colitis (38). The nature of antigens specifically recognized by type II NKT cells remains poorly understood, but include self-glycolipid 3-sulfated galactosylceramide, called sulfatide, and a number of nonlipidic small molecules (47, 48). Recent work has suggested that CD1d-restricted cells act as a cellular sensor system for the detection of inflammation in tissues (49). According to this paradigm, inflammation-associated lysophospholipids may be presented by CD1d independent of \(\beta\)2m during the early phases of plasmid DNA vaccination, and this may activate NKT cells and IL-5 and MCP-1 is released. This is consistent with an earlier observation that NKT cells migrate to the plasmid DNA inoculation site and cause inflammation, which results in a release of MCP-1 (2). This phenomenon was enhanced by the addition of the CpG motif, indicating an influence of this motif on CD1d receptor activation. IL-5 is a T\(_{11}\)-type cytokine and is cooperatively expressed with IL-4 and IL-13. Its expression is reduced in IL-4 KO mice or IL-13 KO mice, and is absent in mice that do not express IL-4 and IL-13 (50). IL-5 and IL-13 both are typically secreted after Stat6 pathway activation and the induction of the DNA-binding factor GATA-3 (12, 51–53). In contrast, MCP-1 is a strong T\(_{11}\)1 cytokine (54) and might suppress the actions of T\(_{11}\)2-type cytokines. The T\(_{11}\)0 pattern of cytokine/chemokine profile found directly after plasmid DNA vaccination is typical for CD4\(^+\) CD1d-restricted T cells, which are functionally distinct from double-negative NKT cells (55, 56). This underlines the unusual characteristic that NKT cells are able to make both T\(_{11}\)1- and T\(_{11}\)2-type cytokines simultaneously following stimulation in vivo (57–59) what seems at face value paradoxical, as T\(_{11}\)1 cytokines often antagonize the action of T\(_{11}\)2 cytokines and vice versa. Despite this T\(_{11}\)0-like cytokine pattern, NKT cells can “go both ways” as their activation in some cases can polarize the immune response in a T\(_{11}\)1 direction, while in other cases a T\(_{11}\)2 response is generated. Adding to the unpredictability surrounding the functional consequences of NKT cell activation, these lymphocytes have been implicated as immunosuppressive cells in some systems, usually via their production of the T\(_{11}\)2-type cytokines or IL-10, while in other systems, they appear to promote enhanced cell-mediated immunity via production of T\(_{11}\)1-type cytokines (60, 61).

The data generated in the present study suggest that MCP-1 released by NKT cells during the early phase of plasmid DNA vaccination polarize naïve CD4\(^+\) T cells and drive them toward a T\(_{11}\)1 type. Thus, the model seems to create an environment that favors the immune potentiating function, rather than the suppressive function of NKT cells. We have previously shown that these CD4\(^+\) T cells clear plasmid DNA antigen expression though a MHC class II-restricted Fas/FasL-dependent mechanism, and that depletion of CD4\(^+\) T cells leads to a reduction in the plasmid DNA antigen-specific CD8\(^+\) T cell immune response (22). Therefore, the NKT-cell enhanced CD4\(^+\) helper cells might also be responsible for the increase of the plasmid DNA antigen-specific CD8\(^+\) T cell immune response. A similar phenomenon was described earlier for HIV patients where CD4\(^+\) T helper cells increased the HIV-specific CD8\(^+\) T cell immune response (62). The current study demonstrates that NKT cells shape a plasmid DNA vaccine-induced cellular response and improve the effector and memory responses generated by these vaccines.

Acknowledgments—We thank Michelle Lifton, Beth Israel Deaconess Medical Center and Harvard Medical School, for advice, support, and reagents. We thank Dr. Mark E. Exley, Beth Israel Deaconess Medical Center and Harvard Medical School, for providing the iNKT KO mice. We thank Drs. Keith Reimann and James Whitney for critical reading of the manuscript.
