

Distinct Pathways of Humoral and Cellular Immunity Induced with the Mucosal Administration of a Nanoemulsion Adjuvant

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Nasal administration of an oil-in-water nanoemulsion (NE) adjuvant W₈₀5EC produces potent systemic and mucosal, Th-1- and Th-17-balanced cellular responses. However, its molecular mechanism of action has not been fully characterized and is of particular interest because NE does not contain specific ligands for innate immune receptors. In these studies, we demonstrate that W₈₀5EC NE adjuvant activates innate immunity, induces specific gene transcription, and modulates NF- κ B activity via TLR2 and TLR4 by a mechanism that appears to be distinct from typical TLR agonists. Nasal immunization with NE-based vaccine showed that the TLR2, TLR4, and MyD88 pathways and IL-12 and IL-12R β 1 expression are not required for an Ab response, but they are essential for the induction of balanced Th-1 polarization and Th-17 cellular immunity. NE adjuvant induces MHC class II, CD80, and CD86 costimulatory molecule expression and dendritic cell maturation. Further, upon immunization with NE, adjuvant mice deficient in the CD86 receptor had normal Ab responses but significantly reduced Th-1 cellular responses, whereas animals deficient in both CD80 and CD86 or lacking CD40 failed to produce either humoral or cellular immunity. Overall, our data show that intranasal administration of Ag with NE induces TLR2 and TLR4 activation along with a MyD88-independent Ab response and a MyD88-dependent Th-1 and Th-17 cell-mediated immune response. These findings suggest that the unique properties of NE adjuvant may offer novel opportunities for understanding previously unrecognized mechanisms of immune activation important for generating effective mucosal and systemic immune responses. *The Journal of Immunology*, 2014, 192: 2722–2733.

The lack of a well-characterized, effective, and safe mucosal adjuvant is a serious problem in the development of efficacious mucosal vaccines (1–3). Most vaccine adjuvants are known to target innate signaling pathways that control the development of adaptive humoral and cellular immune responses,

but the molecular mechanism of their action is still being characterized (4–10). Activation of TLRs has emerged as central to innate immune activation with various adjuvants, such as monophosphoryl lipid A and CpG oligonucleotides (7, 11–13). However, the importance of TLR signaling in the Ab responses was challenged recently (14). Additionally, it is unclear how these adjuvants function on mucosa, because mucosal tolerance may interfere with TLR-mediated innate receptor activation (15).

TLRs activate multiple signaling pathways, and their signaling is largely dependent on the MyD88 adaptor molecule, induction of stress-activated protein kinases, and the transcription factor NF- κ B (7, 16). This leads to the expression of various cytokines and costimulatory molecules and maturation of dendritic cells (DCs), which are critical for the initiation of adaptive immunity. DC maturation involves upregulation of the costimulatory CD40, CD80, CD86, and MHC class II surface molecules and IL-12 cytokine expression (17). TCR engagement and receptor–ligand interactions via CD40/CD40L, CD80/CD86, and CD28/CTLA-4 families of costimulatory molecules are critical for priming of naive CD4 T cells, regulation of T and B cell responses, and induction of adaptive immunity (13, 18). However, little is known about the effects of non-TLR ligand-based adjuvants on DC maturation and costimulatory molecule expression or about the IL-12/IL-4 cytokine milieu that influences T cell differentiation into distinct Th-1 and Th-2 subsets (19–22). Adjuvants, such as aluminum salts (alum) or MF59, were reported to be independent of TLR signaling, with alum, but not MF59, inducing Nalp3 inflammasome activation. However, the significance of these findings to adjuvant activity remains open to debate (23–26). Also, no TLR-based adjuvant appears to consistently produce a Th-17

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Abbreviations used in this article: AP, alkaline phosphatase; BD, BD Biosciences; DC, dendritic cell; i.n., intranasal(ly); MDDC, monocyte-derived DC; NE, nanoemulsion; PA, recombinant protective Ag of anthrax; PA-Alum, 20 μ g PA adsorbed onto 500 μ g aluminum hydroxide; PMB, polymyxin B; SEAP, secretory embryonic alkaline phosphatase; WT, wild-type.

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response, which is now thought to be important in protecting mucosal surfaces during infection (27–30). This suggests that activation of other mechanisms may be important, particularly on mucosal surfaces.

We recently developed a mucosal nanoemulsion (NE) adjuvant, W₈₀5EC, that does not contain known TLR or other innate receptor ligands but is able to enhance both humoral and cellular immunity (27, 31, 32). This NE adjuvant is simply an oil-in-water formulation of emulsified, highly refined soybean oil combined with nonionic and cationic surfactants and ethanol, and it is similar in size to many viruses that infect the nasal mucosa (32). Our studies (32–34) showed that nasal administration of the NE adjuvant mixed with Ag(s) induces robust mucosal and systemic Ab responses and unique Th-1–biased and Th-17–mediated cellular immunity without causing acute inflammation in the nasal mucosa. NE-based vaccines formulated with various viral and bacteria-derived Ags and administered intranasally (i.n.) produced protective immunity to a variety of pathogens, including anthrax, vaccinia, HIV, influenza, and hepatitis B surface Ag (32–35). Recently, we also demonstrated that W₈₀5EC NE increases Ag uptake and trafficking by epithelial cells and DCs, resulting in both apoptosis and DC maturation (36, 37). These findings suggest that W₈₀5EC NE produces unique activation of epithelial cells and DCs on the nasal mucosa.

The objective of this study was to investigate the role of innate immunological pathways in W₈₀5EC NE adjuvant–induced immune responses. We immunized mice with NE combined with recombinant protective Ag of anthrax (PA) (34) and assessed the activation of innate pathogen pattern recognition receptors; expression and function of DC costimulatory molecules CD40, CD80, and CD86; and IL-12/IL-12Rβ1 signaling. The results of these studies elucidate that MyD88-dependent and -independent mechanisms underlie adjuvant activity of W₈₀5EC NE and clarify the unique aspects of mucosally generated humoral and cellular immunity.

Materials and Methods

Adjuvant and Ag

The cationic W₈₀5EC NE was formulated with materials “generally recognized as safe,” was manufactured under good manufacturing practices, and was supplied by NanoBio (Ann Arbor, MI). W₈₀5EC NE contains Tween 80 (5%), ethanol (8%), cetyl pyridinium chloride (1%), soybean oil (64%), and water and is emulsified into droplets with a mean diameter of 400 nm using a high-speed emulsifier (32). The endotoxin content in the W₈₀5EC formulation was determined, using *Limulus* amoebocyte lysate tests, to be 0.13 EU/ml of 100% NE concentration ($\sim 1.3 \times 10^{-10}$ EU/ng), well below the current 10 EU/ng U.S. Food and Drug Administration reference standard for endotoxin (Certificate of Analysis, Croda). PA was purchased from List Biological Laboratories (Campbell, CA) as a lyophilized preparation of purified endotoxin-free protein. After reconstitution in sterile Milli-Q water, the protein aliquots were stored at -80°C until used.

Reagents

PBS was purchased from Cellgro (Mediatech, Plainfield, NJ). Deionized water was prepared using a Milli-Q Ultrapure Water Purification system (Millipore, Billerica, MA). Endotoxin-free water from InvivoGen (San Diego, CA) was used for all NE dilutions and vaccine preparations. BSA was purchased from Sigma (St. Louis, MO). LPS, polymyxin B (PMB), PMA, and ionomycin were purchased from InvivoGen. Alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG (H&L) and subclass-specific secondary Abs were purchased from Rockland Immunochemicals (Gilbertsville, PA).

Cells

Recombinant human HEK293-TLR-Blue clones and mouse RAW-Blue cells with a reporter plasmid expressing enzyme secretory embryonic AP (SEAP), under the control of an NF-κB/API-1-inducible promoter, were

purchased from InvivoGen. Cells were maintained in DMEM containing Zeocin and blasticidin, according to the manufacturer's recommendation. Mouse JAWS II DC and RAW264.7 macrophage cell lines were purchased from American Type Culture Collection (Manassas, VA) and cultured in conditions recommended by American Type Culture Collection.

TLR screening

TLR screening was performed using a panel of HEK293-TLR-Blue clones engineered to express only a single specific TLR and a SEAP–reporter plasmid activated with NF-κB, AP-1, and IRF7 transcription factors. Initial TLR ligand screening was performed by InvivoGen. Cells incubated with TLR-specific ligands were used as a positive control. Cell activation was evaluated as an increase in SEAP activity measured as absorbance at OD₆₅₀ nm, using QUANTI-Blue reagent (InvivoGen), according to the manufacturer's protocol.

Detection of NF-κB activation

To obtain nuclear and cytoplasmic extracts, 10⁷ JAWS II DCs or RAW264.7 cells were incubated in complete RPMI 1640 medium for 60 min at 37°C with subcytotoxic NE concentrations (0.001 or 0.01%), LPS (1 μg/ml), or PMA/ionomycin (200 ng/ml PMA and 1 μg/ml ionomycin). Cells were collected on ice, and cytosolic and nuclear extracts were prepared as described previously (38). For Western blots, 5 μg nuclear extracts were denatured and resolved on 4–12% PAGE-SDS and transferred onto Immobilon-P membrane (Millipore). After blocking with 5% dry milk in PBS-Tween, blots were incubated with rabbit anti-p105/p-p50 (Epicomics, Burlingame, CA), anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA), and either anti-β-actin (Novus Biologicals, Littleton, CO) or lamin a/c (Cell Signaling Technology) Abs, using the manufacturers' recommended conditions, and developed with AP substrate (NBT/BCIP; Thermo Scientific, Waltham, MA).

Microarray analysis

JAWS II DCs (10⁷ cells/culture) were incubated with 0.001% W₈₀5EC alone (NE) or mixed with 10 μg/ml PA (PA-NE). Control cultures either were left untreated (PBS) or incubated with 10 μg/ml PA (PA) or with 1 μg/ml TLR4 ligand LPS K12. Total cellular RNA was isolated from cells after 6 and 24 h of treatment using RNeasy RT (MRC, Cincinnati, OH). RNA was evaluated for integrity, and microarray analysis of >45,000 probe sets was performed using Affymetrix Mouse GeneChip 430 2.0 at the University of Michigan Comprehensive Cancer Center Microarray Core Facility. Gene transcription was analyzed using Bioconductor open access software and National Institutes of Health DAVID annotation program (39). Gene-expression data have been submitted to the Gene Expression Omnibus database under accession number GSE51804 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51804>).

RT-PCR assays

Gene-expression validation analysis was performed with real-time RT-PCR. The mRNA was isolated from JAWS II DCs, as described above. RT-PCR amplification was performed using the RT² First Strand Kit, RT² Profiler PCR Array (PAMM-225ZA) with 84 mouse genes involved in the NF-κB–signaling targets, and RT² SYBR Green qPCR Master Mix (all from SABiosciences a QIAGEN Company, Frederick, MD). The PCR array was run on the Eppendorf realplex2 Mastercycler ep gradient S. Data analysis was performed using the DDCt method (40). The fold-change for each gene from the control to the experimental group was calculated as $2^{-(\text{DDCt})}$.

Preparation of human monocyte-derived DCs

Human PBMCs were isolated from healthy donors, as described in Kryczek et al. (41). CD14⁺ monocytes were purified using paramagnetic beads (STEMCELL Technologies, Vancouver, BC, Canada) to >91% purity. Monocyte-derived DCs (MDDCs) were differentiated from CD14⁺ cells by incubation with 20 ng/ml GM-CSF and 5 ng/ml IL-4. On day six, the resulting DCs had uniform CD14⁺, CD1a⁺, CD11c⁺ phenotype and morphology confirmed by flow cytometry (41).

In vitro activation of DCs

MDDC cultures (2×10^6 cells/2 ml) were incubated for 48 h with 0.0001 and 0.001% W₈₀5EC NE. Control cells were left untreated (PBS) or were incubated in the presence of PMA/ionomycin (200 ng/ml PMA and 1 μg/ml ionomycin). Phenotypic analysis of surface markers was performed by staining with FITC- and/or PE fluorochrome–conjugated mAbs to HLA-DR, CD80, and CD86 and with appropriate isotype controls (BD Biosciences [BD], San Jose, CA), according to the manufacturer's protocol.

Cells were analyzed using an LSR II flow cytometer with DIVA software (both from BD).

Animals

Female MyD88^{-/-} knockout C57BL/6 mice were bred at the Unit for Laboratory Medicine facility at the University of Michigan. The MyD88^{-/-} mutant genotype was obtained by insertion of a neomycin cassette sequence in place of exons 4 and 5. Genotype of knockout mice was confirmed by genomic PCR using primers specific to intron 2 and neomycin cassette sequences (42). TLR2^{-/-}, TLR4^{-/-}, CD86^{-/-}, double CD80/CD86^{-/-} mutants, and wild-type (WT) C57BL/6 background mice (females, 6–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). CD40^{-/-}, IL-12 p35/p40^{-/-}, and IL-12Rβ1^{-/-} knockout mice generated in the C57BL/6 background were a generous gift from Dr. D.K. Bishop (University of Michigan Medical School). The germ-free Swiss-Webster mice were raised at the University of Michigan's germ-free colony, housed in soft-sided bubble isolators, and fed autoclaved water and laboratory chow ad libitum. All mice remained under sterile conditions for the duration of the study, as determined by aerobic and anaerobic culturing of bacteria on blood agar plates and by Gram staining of feces (from live mice) and/or cecal contents (at necropsy). Control Swiss-Webster mice with normal microbial flora were housed in standard microisolator conditions. All mice used in these studies were housed five to a cage under specific pathogen-free conditions with food and water available ad libitum, in accordance with the standards of the American Association for Accreditation of Laboratory Animal Care. All procedures involving animals were conducted in accordance with and with the approval of the University of Michigan University Committee on Use and Care of Animals.

Immunization procedures

Vaccine formulation containing 20 μg PA admixed with 20% W₈₀5EC (PA-NE) was prepared by mixing Ag solution with concentrated W₈₀5EC in PBS as diluent. Mice (*n* = 5) were immunized with two i.n. administrations of either PA-NE or control formulation PA-PBS 4 wk apart. The i.n. immunizations (5 μl/nare) were done using a micropipette tip applied to the nares while animals slowly inhaled the material. In some experiments, mice were immunized with i.m. injection of 20 μg PA adsorbed onto 500 μg aluminum hydroxide (PA-Alum). The i.m. immunizations consisted of 50 μl injected into mouse apaxial muscle using a 26G5/8 needle. Both i.n. and i.m. immunizations were performed in mice anesthetized with isoflurane using the IMPAC 6 anesthesia delivery system.

Blood and splenocyte collection

For immune response analysis, mouse sera and splenic lymphocytes were obtained as described previously (32, 34). Final blood samples and splenocytes were harvested at the end of the experiments, up to 12 wk after the primary immunization.

Determination of serum IgG Abs

Anti-PA IgG Ab detection was performed using a PA-specific ELISA, as described previously (34). Ab concentrations were calculated as end point titers, defined as the reciprocal of the highest serum dilution producing an absorbance at OD₄₅₀ nm above a cutoff value. The cutoff value is determined as OD of the corresponding dilution of the negative-control sera + 2 SD (43, 44).

Analysis of splenic cytokine expression

Freshly isolated mouse murine splenocytes were seeded at 4 × 10⁶ cells/ml (RPMI 1640, 2% FBS) and incubated with PA (10 μg/ml) or as controls with either PMA/ionomycin (200 ng/ml and 1 μg/ml, respectively) or LPS (1 μg/ml) for 72 h. Cell culture supernatants were harvested and analyzed for the presence of cytokines using LuminexMultiplex21 multianalyte profiling beads (Luminex, Austin, TX), according to the manufacturer's instructions.

Statistical analysis

Results are expressed as mean + SD. Statistical significance was assessed using the multiple-comparison Tukey test. In case of comparisons limited to two groups, the statistical significance was determined by the Student *t* test using 95% confidence limits. A *p* value < 0.05 was considered statistically significant.

Results

NE activates NF-κB via innate TLRs in vitro

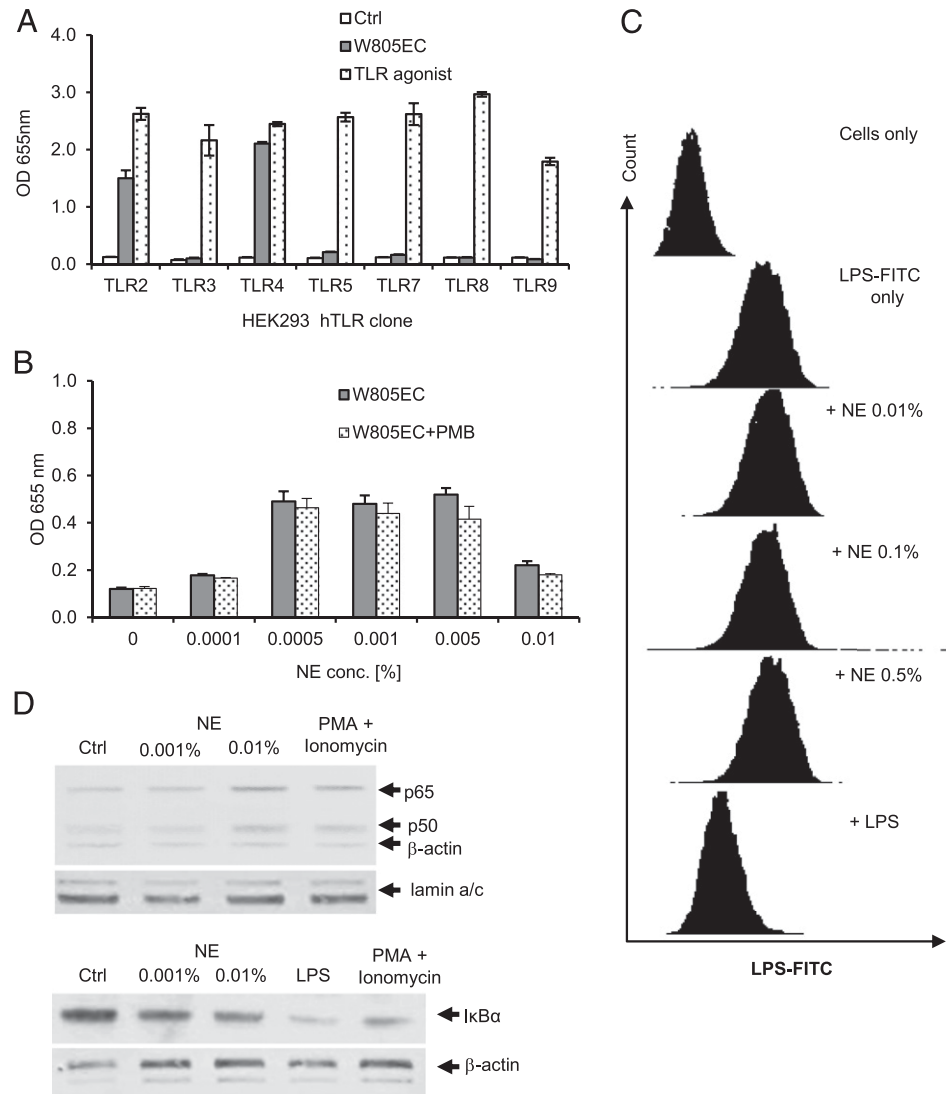
The TLR family of innate immune receptors activates both innate and adaptive immunity through NF-κB-mediated gene expression (7). To determine whether the W₈₀5EC NE adjuvant acts through

TLRs, the NE effect was tested in the cell-based assays using HEK293-TLR-Blue clones, each expressing a single human TLR2 through TLR9, and a reporter gene (SEAP) under the control of NF-κB. Incubation with W₈₀5EC significantly increased SEAP production only in cells expressing either TLR2 or TLR4 receptors (Fig. 1A). The NE-dependent NF-κB activation was confirmed in two other reporter cell lines that naturally express a broad range of TLRs: RAW-Blue mouse macrophages (Fig. 1B) and THP-1-Blue human monocyte cells (Supplemental Fig. 1A). To rule out that NF-κB activation by W₈₀5EC NE occurred from occult microbial contamination, we attempted to block activation using the LPS inhibitor PMB (45, 46). PMB treatment inhibited LPS-mediated activation but had no effect on NF-κB activation with NE (Fig. 1B, Supplemental Fig. 1B), suggesting that microbial contamination is not responsible for NE adjuvant activity. The lower SEAP levels at 0.01% NE are due to the cytotoxic effects of NE reducing cell viability in vitro. Furthermore, in vitro binding studies in RAW264.7 cells showed that NE does not interfere with LPS binding, suggesting that the LPS/MD-2/TLR4 binding site is not blocked by NE interactions (Fig. 1C, Supplemental Fig. 1C). These results suggest that the NE droplets may interact with cell membrane components, causing TLR activation followed by downstream TLR-signaling events (47). Western blots were used to determine activation of NF-κB subunits in either JAWS II DCs or RAW264.7 macrophage cells treated with W₈₀5EC NE (Fig. 1D, Supplemental Fig. 1D). W₈₀5EC treatment increased phosphorylation and nuclear translocation of p50 and p65 NF-κB subunits similar to PMA/ionomycin and LPS stimulation (Fig. 1D, *upper panel*). The mechanism of p50 and p65 activation involved degradation of the cytoplasmic IκBα (Fig. 1D, *lower panel*).

Differential gene expression with W₈₀5EC NE and TLR4 agonist

JAWS II DCs were used to further evaluate the immunostimulatory ability of W₈₀5EC NE by analysis of global gene expression changes in vitro. DCs were incubated with either W₈₀5EC (NE) alone or mixed with PA protein (PA-NE) as a prototype Ag. Control cultures were incubated with PA (PA) alone, stimulated with LPS (a TLR4 ligand), or with the addition of PBS (untreated). Cells were harvested after 6 or 24 h, and RNA was prepared for transcriptional analysis using mouse whole-genome microarray. Table I presents the number of gene transcripts changed with W₈₀5EC and LPS treatment in comparison with unstimulated controls (PBS-only treatment). At 6 h, >93% of the transcripts that were changed with NE or PA-NE treatments were common with the LPS treatment group (Fig. 2A). A divergent pattern of gene expression emerged at 24 h, showing only 20% transcripts that were common for W₈₀5EC and LPS treatments (Fig. 2B). The effect of W₈₀5EC on gene expression did not reflect a delayed pattern of LPS activation, because only 16.3% of genes modulated with NE at 24 h were common with those activated at the earlier time point with LPS (Fig. 2C). These results show the similarity of early W₈₀5EC NE effects and TLR4 activation. However, downstream molecular pathways are significantly different from those observed with LPS activation. Hierarchical clustering and functional analysis identified a significantly enriched category of defense immunity and cytokine activity GO:00051125 and difference in NF-κB pathway genes (Supplemental Fig. 2A, Supplemental Table I). Validation of the microarray findings was carried out using RT-PCR assays performed on a panel of NF-κB-signaling targets. This showed a pattern of gene upregulation consistent with LPS activation and the results from the microarray assay. W₈₀5EC induced early activation of Csf3, Cxcl3, Cxcl10,

FIGURE 1. Stimulatory activity of $W_{80}5EC$ NE in vitro. **(A)** NE activates an NF- κ B-dependent reporter in HEK293 clones expressing TLR2 or TLR4. Cultures of single TLR1–10 clones of HEK293-TLR-Blue cells were incubated for 24 h with 0.001% NE. Optimized concentrations of specific TLR agonists were used as positive controls. **(B)** NE activation is not caused by endotoxin contamination. RAW-Blue cells were preincubated for 20 min with PMB (10 μ g/ml), followed by the addition of the indicated NE concentrations for 24 h. SEAP was measured in triplicate samples of the cell culture supernatants, and results are presented as absorbance at OD₆₅₀ nm \pm SD. The cytotoxic effect of 0.01% NE in vitro is reflected in the lower level of SEAP activity seen in these experiments. **(C)** $W_{80}5EC$ NE does not compete with LPS binding. RAW267.4 cells were preincubated at 4°C for 1 h to prevent LPS internalization. Subsequently, 1 μ g/ml LPS-FITC was added for 10 min and competed with NE concentrations of 0.001, 0.01, and 0.05% or with 1 μ g/ml LPS of *Escherichia coli* K12. The cells were incubated for 1 h at 4°C, collected, and FACS analyzed for LPS-FITC binding using a BD LSR II flow cytometer. **(D)** Activation and nuclear translocation of p50 and p65 NF- κ B subunits in JAWS II DCs. Western blot detection of phosphorylated p50 and p65 subunits in nuclear extract from JAWS II (*upper blot*). NE induces I κ B α degradation (cytoplasmic extract, *lower panel*). NF- κ B and I κ B α detection were normalized with lamin a/c and β -actin, as indicated.



IL-1 α , IL-1 β , and IL-6 expression in a pattern that was similar, although at much lower levels, to that detected in LPS-treated JAWS II cells (Fig. 2D, 2E). Also, consistent with the microarray results, PA alone did not activate transcription (Supplemental Fig. 2B).

Role of TLR pathway in the mechanism of $W_{80}5EC$ NE adjuvant in vivo

Based on in vitro results, we directly addressed the role of TLR signaling in $W_{80}5EC$ NE adjuvanticity in vivo. MyD88^{-/-} mice

Table I. Changes in global gene expression in JAWS II DCs

JAWS II Microarray		No. of Changed Transcripts		
Time (h)	Treatment	Up	Down	Total
6	NE	127	114	241
	PA-NE	87	127	214
	PA-PBS	1	4	5
	LPS	887	838	1725
24	NE	702	452	1154
	PA-NE	725	425	1150
	PA-PBS	3	0	3
	LPS	631	293	924

Microarray analysis was performed using Affymetrix Mouse GeneChip 430 2.0 for detection of >45,000 transcripts. Significant change log₂ \geq 1; *p* value \leq 0.05, ANOVA.

(deficient in a common TLR adaptor protein), TLR2^{-/-} and TLR4^{-/-} mice (lacking expression of either one of these TLRs), and WT mice were immunized i.n. with either PA-NE or PA-PBS or i.m. with PA-Alum. Serum anti-PA IgG titers did not reveal any discernible difference between all three mutant mice and WT controls (Fig. 3A, 3B). Specifically, $W_{80}5EC$ adjuvant produced similar anti-PA IgG titers in MyD88^{-/-}, TLR2^{-/-}, TLR4^{-/-}, and WT mice, indicating that the innate TLR-MyD88 pathway is not required for the Ab response with mucosal administration of NE adjuvant. Furthermore, both MyD88^{-/-} and WT animals had an Ab response that was equal to control mice immunized i.m. with alum adjuvant (Fig. 3A). Nasal immunization of germ-free mice demonstrated that activity of $W_{80}5EC$ NE adjuvant also was not dependent on the presence of microbial components derived from normal commensal mucosal flora, because Ab titers in these animals were comparable to those obtained after parenteral immunization with alum adjuvant (Fig. 3C).

In contrast, analysis of cellular immunity demonstrated that MyD88 deficiency significantly affects the ability of $W_{80}5EC$ NE to mount the balanced Th-1 and Th-17 T cell responses demonstrated by WT animals (Fig. 4A). Ag-specific stimulation of WT splenocytes produced strong IFN- γ expression, which was severely reduced in MyD88^{-/-}, TLR2^{-/-}, and TLR4^{-/-} mutants (Fig. 4). Furthermore, analysis of the IgG subclass pattern in MyD88^{-/-} mice showed increased IgG1 and decreased IgG2b

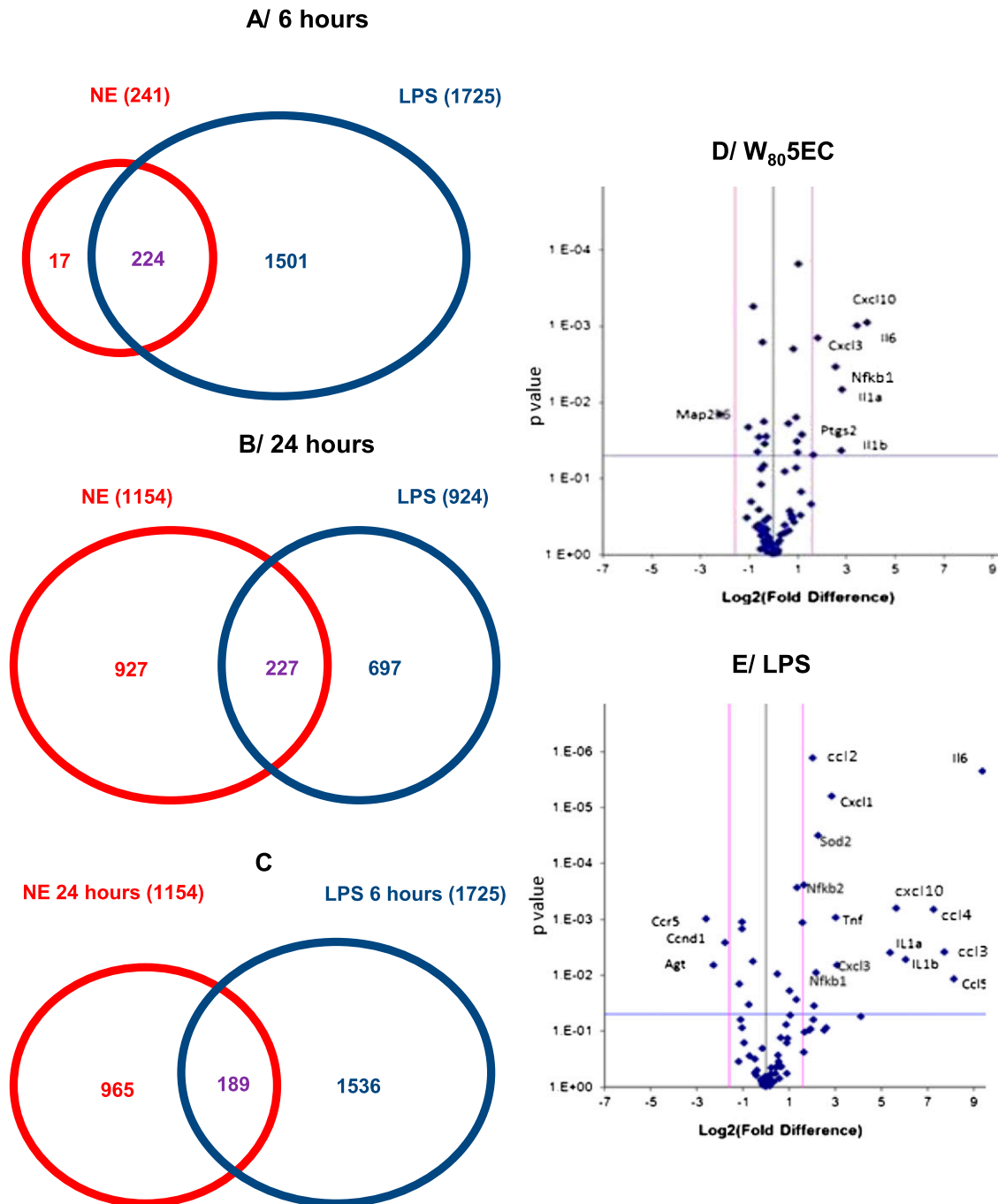


FIGURE 2. Analysis of global changes in gene transcription induced in JAWS II cells. Venn diagrams showing distribution of genes whose expression was changed >2 -fold ($\log_2 \geq 1$) after incubation with 0.001% W_{805EC} NE or 1 $\mu\text{g/ml}$ LPS for 6 h (A) or 24 h (B). (C) Common and unique genes modulated by W_{805EC} NE at 24 h and LPS at 6 h. The total number of genes modulated by either NE or LPS at each time point is shown in parentheses. RT-PCR verification of NF- κ B target gene expression in W_{805EC} NE-treated (D) and LPS-treated (E) JAWS II cells (RNA isolated after 6 h of treatment with 0.001% NE or 1 $\mu\text{g/ml}$ LPS). Microarray and PCR analysis were performed twice.

subclass titers (Supplemental Fig. 3A). A comparison of the Th-type pattern of cytokine expression in splenocytes from WT mice immunized with either PA-NE (i.n.) or PA-Alum (i.m.) clearly documents differences in the type of Th polarization of the cellular response. These data show high levels of the Th-1-type cytokines IFN- γ and IL-2 and low levels of the Th-2 type cytokines IL-5 and IL-13 in mice immunized with NE adjuvant. In contrast, mice immunized with alum, a classic Th-2-type adjuvant, produce characteristically high levels of IL-5 and IL-13 and low levels of IFN- γ and IL-2 (Supplemental Fig. 3B). Furthermore, TLR4 $^{-/-}$, but not TLR2 $^{-/-}$, animals had a significant de-

crease in IL-2 production compared with WT animals. MyD88 $^{-/-}$, TLR2 $^{-/-}$, and TLR4 $^{-/-}$ splenocytes were fully capable of IFN- γ secretion upon activation with PMA/ionomycin in vitro, indicating that reduction of IFN- γ is not due to an intrinsic defect in the expression of the IFN- γ gene (data not shown). Induction of the Th-17 response and IL-17 expression were completely abrogated in W_{805EC} NE-immunized MyD88 $^{-/-}$ mice (Fig. 4A), as we (27) showed previously in TLR2 $^{-/-}$ and TLR4 $^{-/-}$ mutants. MyD88 and TLR2 deficiency resulted in dominant Th-2 cellular immunity, with a significant increase in IL-5 and IL-13, as well as regulatory IL-10 cytokine, whereas TLR4 $^{-/-}$ splenocytes pro-

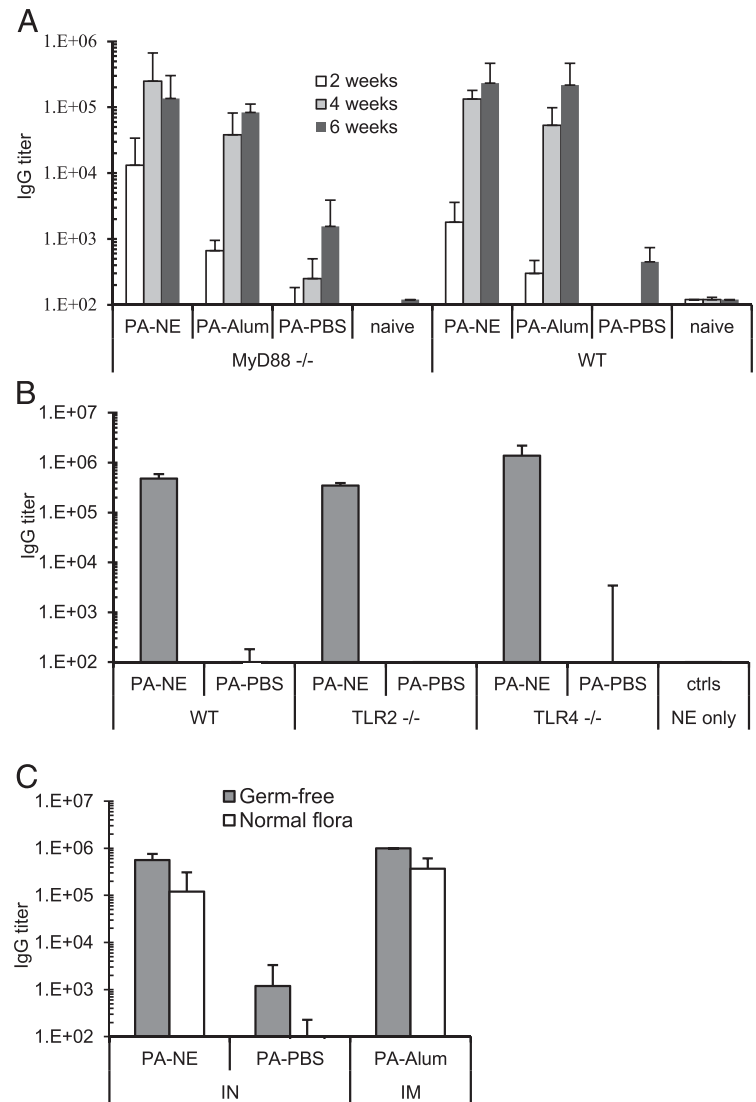


FIGURE 3. *W*₈₀5EC NE adjuvant does not require TLR-MyD88 signaling to induce Ab response. Comparison of IgG titers in MyD88^{-/-} (**A**) and TLR2^{-/-} and TLR4^{-/-} (**B**) mutants immunized i.n. with PA-NE, PA-Alum, or PA-PBS. (**C**) Comparison of IgG titers in normal and germ-free Swiss-Webster mice. Anti-PA IgG Ab titers were measured at 11 wk after primary immunization. Experiments were performed twice, with five animals/group.

duced lower induction of these cytokines than did TLR2^{-/-} mutants (Fig. 4B). Ag-specific expression of GM-CSF was not affected by the absence of MyD88 signaling. No Ag-specific cytokine secretion was observed in nonimmunized control splenocytes derived from any strain of mice.

*W*₈₀5EC NE induces upregulation of DC maturation markers *in vitro*

To assess the capability of *W*₈₀5EC NE to activate DC maturation, we evaluated the NE biological effect on human MDDCs. Analysis of the cell surface phenotype in these cells demonstrated that NE produced a consistent increase in DC maturation markers and costimulatory molecule expression (Fig. 5). MHC class II expression increased >2-fold when incubated with 0.001% NE, but it was unchanged at the lower concentration (Fig. 5, *left panels*). Furthermore, the population of either CD80 or CD86 (Bright) DCs increased ~2-fold at both NE concentrations (Fig. 5, *middle and right panels*). In general, the effect of *W*₈₀5EC NE on DC maturation phenotype was comparable to protein kinase C stimulation used as a positive control (Fig. 5, *bottom panels*).

*W*₈₀5EC NE adjuvant activity in mice deficient in CD80, CD86, and CD40 costimulatory molecules

Activation and upregulation of immunostimulatory surface molecules on DCs and other APCs are critical check points for the

initiation of Ag-specific adaptive immune responses (48). To investigate the requirement for costimulatory receptors in the mechanism of *W*₈₀5EC adjuvant, CD86^{-/-}, double CD80/CD86^{-/-}, and CD40^{-/-} mutant mice were immunized i.n. with PA-NE or a PA-PBS control. Serum IgG analysis showed that mice lacking CD86 expression had an Ab response on par with WT animals, whereas double CD80/CD86 and CD40 mutants failed to produce anti-PA Abs (Fig. 6A). Induction of cellular immunity was assessed by Ag-specific cytokine expression in splenocytes. Results demonstrate severely diminished production of Th-1-type cytokines IFN- γ and IL-2 in CD86^{-/-} and CD40^{-/-} mutants, whereas no Th-1- or Th-2-type cytokines were detected in CD80/CD86^{-/-} mutants (Fig. 6B). In contrast, CD86-deficient splenocytes produced elevated levels of Th-2-type cytokine IL-5, whereas IL-5, IL-10, and IL-13 were diminished in CD40^{-/-} mutants (Fig. 6B). These results suggest that the *W*₈₀5EC adjuvant may independently use CD80 and CD86 signaling and that CD80 compensates for the CD86 deficiency in the Ab response; however, it is not sufficient to maintain Th-1-biased cell immunity. Lack of both CD80 and CD86 molecules abrogated the immune response with the NE adjuvant. Similarly, the profound effect of the CD40^{-/-} mutation on both Ab and cellular responses demonstrates the critical and nonredundant role of this costimulatory molecule in the mechanism of *W*₈₀5EC NE adjuvant activity.

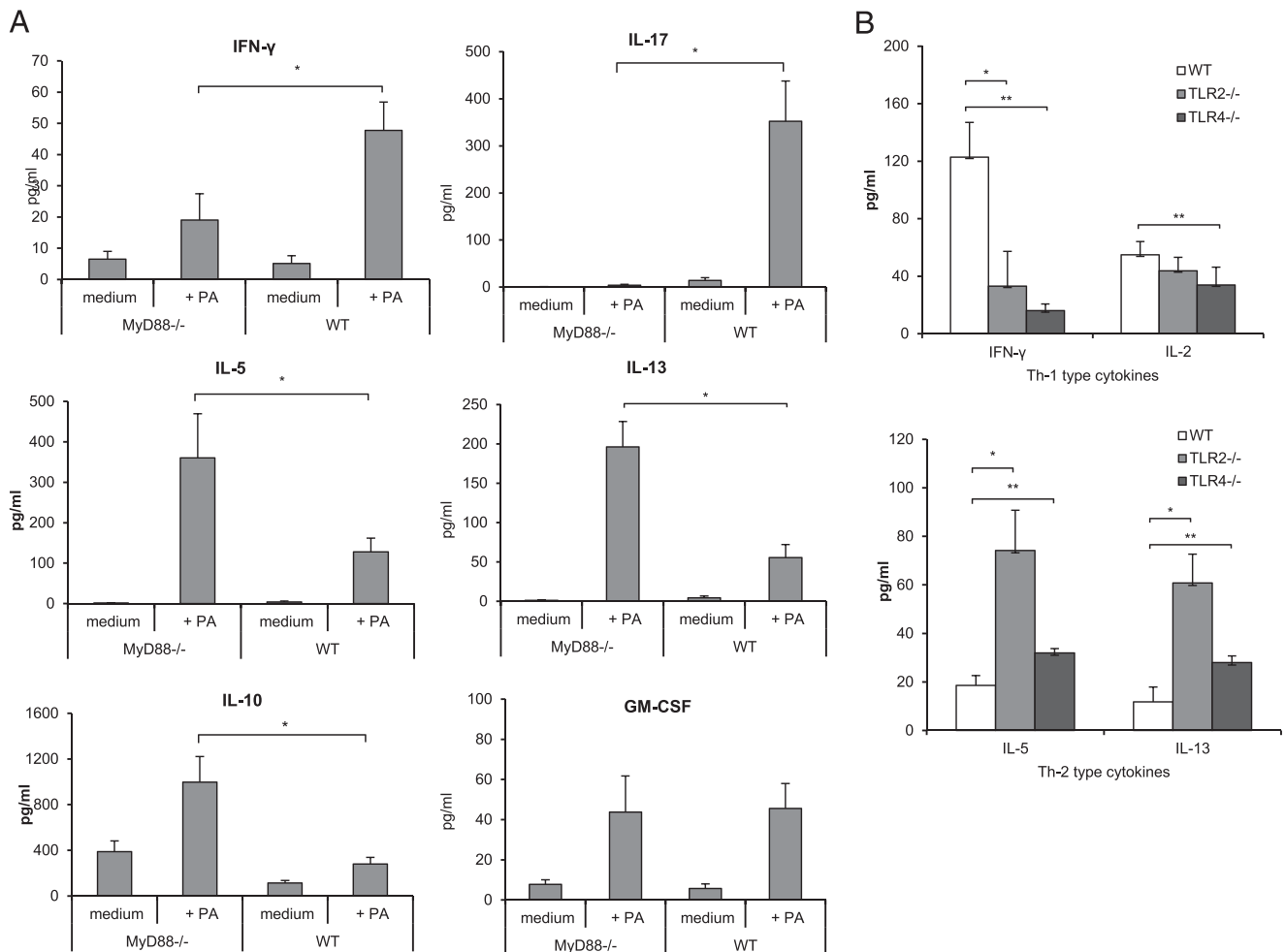


FIGURE 4. Effect of MyD88, TLR2, and TLR4 deficiency on cellular immunity in mice immunized i.n. with *W*₈₀5EC NE adjuvant. **(A)** Ag-specific Th-1-, Th-2-, and Th-17-type cytokine production in splenic lymphocytes in MyD88^{-/-} and WT mice. *Statistically significant difference between WT versus MyD88^{-/-} mice ($p < 0.05$). **(B)** Ag-specific Th-1- and Th-2-type cytokine expression in TLR2^{-/-} and TLR4^{-/-} mice. Cytokine expression was measured in the supernatants of splenocytes from mice immunized with PA-NE and stimulated with PA protein in vitro. Results are average cytokine concentration \pm SD. Experiments were performed twice, with five animals/group. Analysis by Student *t* test and multivariate Tukey test. *, **Statistically significant difference between cytokine production in WT versus TLR2^{-/-} ($p < 0.05$) and TLR4^{-/-} ($p < 0.05$) mutant mice, respectively.

*W*₈₀5EC NE adjuvant activity in IL-12- and IL-12R β 1-deficient mice

IL-12, a cytokine secreted mainly by activated APCs, is a potent stimulator of T cell function and polarization toward Th-1 immunity (49). We investigated the role of IL-12 signaling in NE adjuvant activity using mice deficient in both p35 and p40 IL-12 subunits (IL-12 p35/p40^{-/-}), as well as in mutants lacking cognate IL-12R β 1 (IL-12R β 1^{-/-}). Mice were immunized i.n. with PA-NE and with PA-PBS as control. Serum IgG analysis showed that IL-12 p35/p40^{-/-} mutants produced ~15-fold lower Ab titers than did WT mice, whereas the lack of IL-12R β 1 had no significant effect on the Ab response (Fig. 7A). T cell immunity assessed in vitro by analysis of Ag-specific cytokine expression in splenocytes from mutant mice showed that both mutants failed to produce Th-1-type cytokines and IL-17, whereas levels of IL-5 and IL-13 Th-2 cytokines were elevated in comparison with WT mice (Fig. 6B–D). These results demonstrate that *W*₈₀5EC NE adjuvant does not require an IL-12 environment to elicit robust IgG responses. However, IL-12 and downstream signaling via the IL-12R β 1 receptor are necessary for activation and for maintenance of Th-1-balanced and Th-17-type cellular immunity (27).

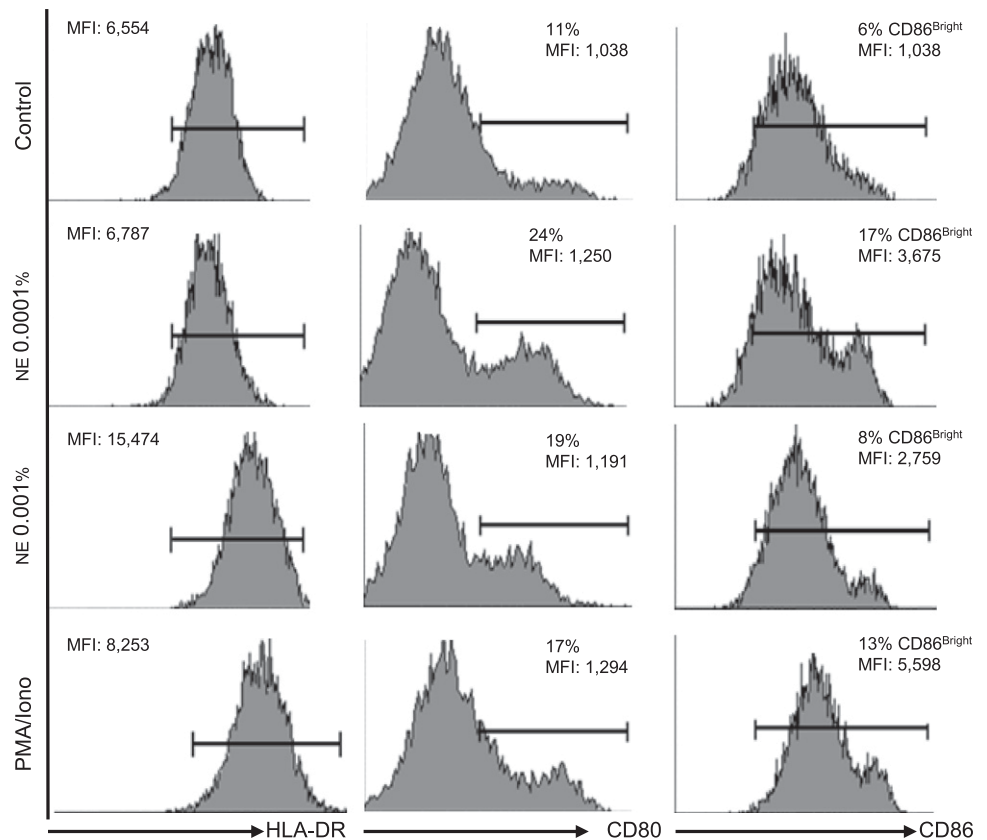
Discussion

Vaccine adjuvants are composed of diverse classes of materials that have poorly characterized mechanisms of action, although many of these compounds contain components that activate inflammation and innate immunity (10, 14). Some adjuvants are known to contain biological ligands of TLR that activate the innate response and induce humoral and cellular adaptive immunity (10, 14, 50–52). Other materials, like squalene, have been formulated in oil-in-water emulsions (such as M59 adjuvant) as candidates for human vaccine, but they have no known mechanism of action for their proinflammatory activity (53).

In this article, we present data on a novel NE adjuvant, *W*₈₀5EC NE, which is one of the first effective mucosal-acting adjuvants and does not contain specific proinflammatory or biological materials. Despite this, the mechanism of this NE adjuvant appears to involve both TLR-dependent and TLR-independent pathways for induction of adaptive humoral and cellular immunity.

In comparison with other emulsion-based adjuvants, *W*₈₀5EC NE is a simple formulation of four components: highly refined, heat-treated soybean oil, nonanionic Tween 80 and cationic cetyl pyridinium chloride surfactants, and ethyl alcohol, all emulsified in water to obtain a dispersion with a nanoscale lipid droplet size

FIGURE 5. $W_{80}5EC$ NE promotes DC maturation phenotype in vitro. Human MDDCs were incubated for 48 h with 0.0001% or 0.001% NE, as indicated. Cells cultured with no treatment (Control) or incubated in the presence of PMA/ionomycin were used as activation controls. Subsequently, analysis of HLA-DR (MHCII), CD80, and CD86 surface expression was performed on DC populations gated for $CD11c^+$ expression. Results shown are representative data from two experiments.



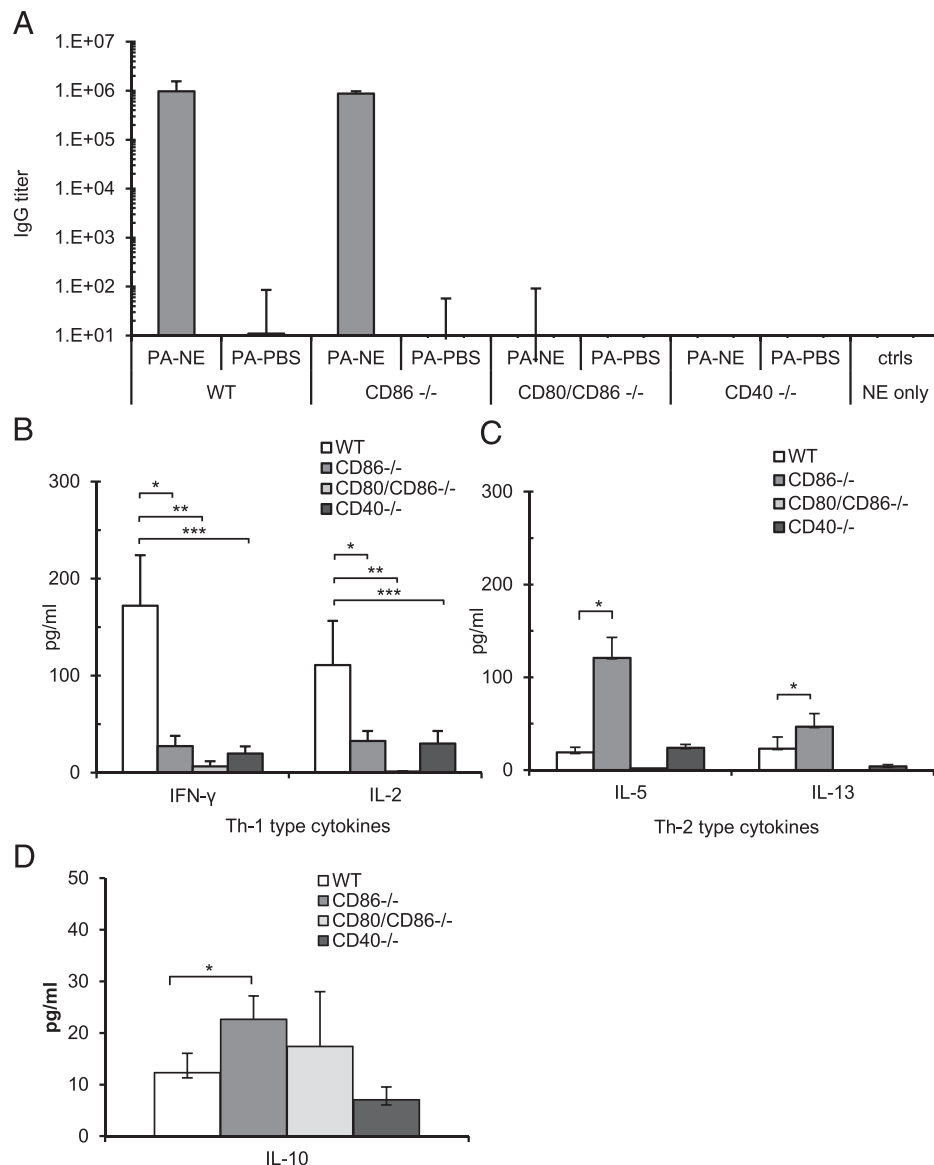
(53, 54). NE's unique mucosal adjuvant activity appears to be derived both from the high potential energy of this nanostructure that facilitates fusion with cells, as well as from its chemical composition (32, 54). Fusion of the emulsion with DCs in vitro induces $\text{I}\kappa\text{B}\alpha$ degradation and subsequent activation of p50 and p65 NF- κB subunits. The activation results also were confirmed in THP-1Blue and RAW-Blue NF- κB reporter lines and in DCs and macrophages transiently transfected with a different reporter plasmid expressing luciferase under control of a minimal NF- κB -inducible promoter (pNF- κB -Luc, Stratagene; data not shown). The ability to directly mediate NF- κB activation in various types of immune cells could be central to the NE mechanism of immunostimulation, because NF- κB functions as a pleiotropic regulator and transducer of the stimulatory signals for genes involved in a variety of immune responses (55–57).

Cell-based screening for TLR agonist activity and global analysis of gene expression suggest that $W_{80}5EC$ NE activity involves innate receptors TLR2 and TLR4 (Figs. 1A, 4). Although NE requires these receptors, it does not compete with the LPS for TLR binding, indicating that NE may activate TLR by nonligand-specific interactions; this could include aggregating receptors in lipid rafts, as is documented for TCR signaling (58, 59). In addition, the microarray analysis of global gene expression suggests that $W_{80}5EC$ NE appears to use TLR4 to initiate signaling pathways, but its downstream signaling appears to be distinct from LPS, at least from the resulting gene-activation patterns. Of interest, alum adjuvant also was reported to interact with DC membrane components to trigger DC signaling and promote $CD4^+$ T cell activation and humoral immune responses (60). In addition, surfactants like Tweens and lipids (including liposomes, as well as saturated and unsaturated fatty acids) can differentially modulate DC-signaling cascades to augment or modify immune activation (61–65). Thus, membrane perturbations of DCs and

other accessory cells involving lipids and surfactants may play an important role in $W_{80}5EC$ NE adjuvant activity.

Immune responses in either MyD88- or TLR2- and TLR4-deficient mice illustrate the importance of innate receptors in the complex and distinct mechanisms of $W_{80}5EC$ NE. Although TLR activation of innate immunity has been considered central to the Ab-enhancing effects of adjuvants (66), work by Namezee et al. (67) recently challenged this view. They demonstrated that mice deficient in MyD88 and TRIF, molecules critical for signaling by all TLRs, can still produce robust Ab in response to immunizations with either TLR-dependent or TLR-independent adjuvants (14). In these studies, we demonstrate for the first time, to our knowledge, that an absence of TLR2, TLR4, and the common (with exception of TLR3) TLR adaptor protein MyD88 has no effect on the ability of mucosal NE adjuvant to elicit a robust Ab response. In contrast, the absence of TLR-MyD88 signaling has a profound effect on NE-induced cellular immunity. Our data also suggest that, in $W_{80}5EC$ NE-immunized mice, TLR2 and TLR4 differentially affect the Th-1/Th-2 balance, because TLR4^{-/-} and MyD88^{-/-} mice had severely diminished Th-1- and Th-17-type cytokine production and reduced IgG2b Ab titers, whereas Th-1 cytokines in TLR2-deficient mice were affected to a lesser degree (27). Furthermore, the absence of TLR2 and MyD88 resulted in the elevated expression of IL-5, IL-10, and IL-13 cytokines, despite functional TLR4. In contrast to our results, when TLR2-deficient mice are used in a rheumatoid arthritis model or immunized with the YF-17D yellow fever vaccine, these animals have enhanced Th-1 immunity (68–70). Although previous reports indicated that MyD88^{-/-} mice develop Th-2 responses during *Chlamydia* infection and are protected from Th-1-type experimental autoimmunity (experimental autoimmune encephalomyelitis) (71, 72), distinct roles for TLR2- and TLR4-activating adjuvants have not been clearly defined (73). It is possible that this

FIGURE 6. $W_{80}5EC$ NE adjuvant activity in $CD40^{-/-}$, $CD86^{-/-}$, and $CD80/CD86^{-/-}$ mutant mice. **(A)** Ab response. Mice were immunized i.n. with either PA-NE or PA-PBS. Anti-PA IgG titers were measured at 12 wk after primary immunization. Ag-specific cellular response: Th-1- **(B)** and Th-2-type **(C, D)** cytokines were measured in the cell culture supernatants of splenocytes from mice immunized with PA-NE after 24 h of stimulation with PA. Results are average cytokine concentration \pm SD. Experiments were performed twice, with five animals/group; analysis by multivariate Tukey test. *, **, and ***Statistically significant differences in cytokine production in WT versus $CD86^{-/-}$ ($p < 0.05$), double $CD80/CD86^{-/-}$ ($p < 0.05$), and $CD40^{-/-}$ mutants ($p < 0.05$), respectively.



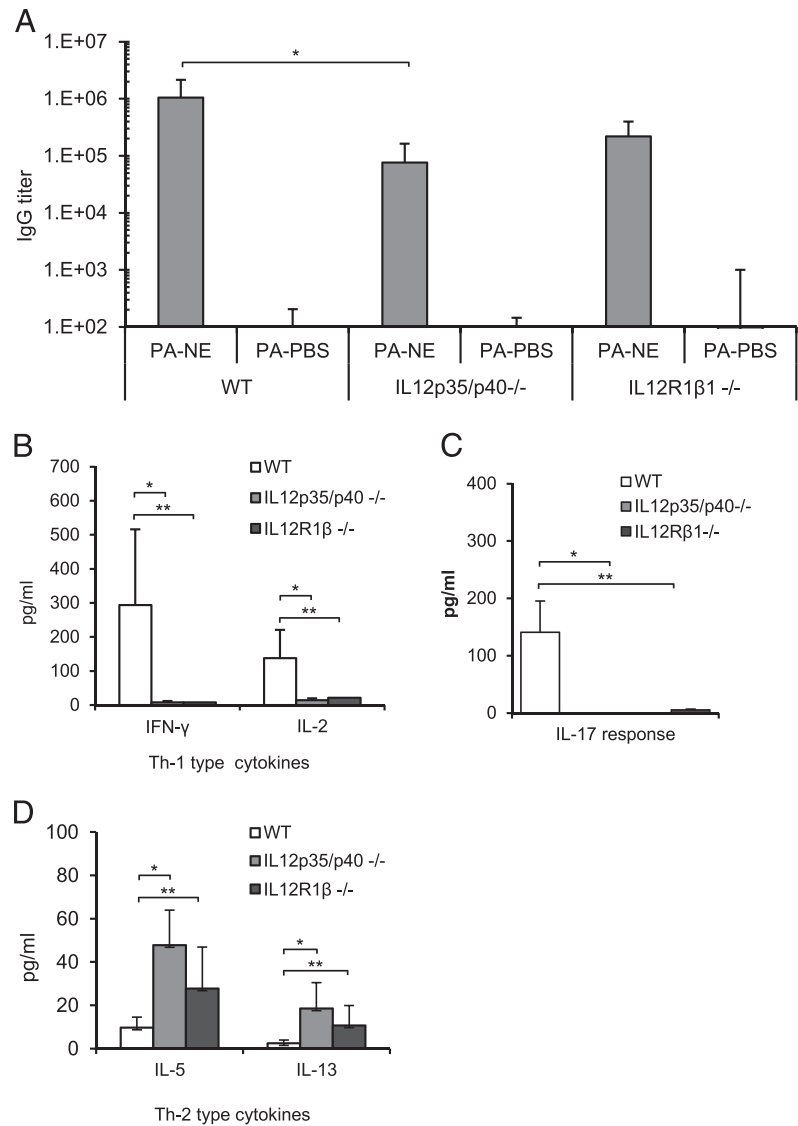
nuanced response reflects relative contributions of TLR2 (MyD88 dependent) and TLR4 (using both MyD88 and TRIF) activation with $W_{80}5EC$ adjuvant. Additional studies using animals with double TLR2/TLR4 or MyD88/TRIF defects will help to clarify the role of MyD88-dependent and -independent innate signaling in the mucosal mechanism of $W_{80}5EC$ NE.

In addition to TLR activation, DCs and IL-12 signaling play a critical role in the development of cell-mediated immunity (49, 74, 75). Consistent with other studies, our results show that IL-12 (p35/p40) and IL-12R β 1 receptor are required for NE-mediated Th-1-skewed T cell responses. However, because both IL-12-induced IFN- γ production and T cell proliferation are impaired in either IL-12 or IL-12R mutant mice, it is not entirely clear whether NE-mediated cellular responses depend solely on IL-12 cytokine expression or also require downstream signaling by IFN- γ (76). In addition, because IL-12 mutants do not produce IFN- γ in response to LPS-TLR4 stimulation, lack of Th-1 immunity suggests that NE's effects on DC activity, and subsequent naive T cell priming, may be mediated by TLR4 and/or IFN- γ . The absence of an IL-17 response in IL-12p35/40-deficient mutants is most likely due to a dual role for IL-12p40 as a common component with IL-23, which drives Th-17 differentiation. Our results

also suggest that NE activity may link IL-12 and MyD88 for the activation of mTOR to promote Th-17 via IL-1 and IL-23 signaling (77, 78). Similar to the TLR-MyD88 deficiency, lack of either IL-12 or IL-12R β 1 had no detrimental effect on Ab responses with NE adjuvant. Taken together, these results demonstrate that $W_{80}5EC$ NE adjuvant mediates a balanced cellular response involving both IL-12-dependent Th-1 and Th-17 cellular immunity and IL-12-independent humoral immunity, a process that is fundamentally different from the activity of alum adjuvant (79–81).

Multiple molecular signals are required for the development of an adaptive immune response. Ag presentation to the cognate TCRs or BCRs by APCs is the primary signal; however, the engagement of costimulatory molecules by APCs and T and B lymphocytes is also important (18). CD80, CD86, and MHC class II molecules are expressed by mature DCs, monocytes/macrophages, and B cells, and they modulate naive $CD4^+$ T cell functions by binding with CD28 or CTL-4 receptors (17). Recently, our group (36, 37) demonstrated that nasal administration of $W_{80}5EC$ NE adjuvant to mucosal surfaces increased Ag uptake and trafficking by epithelial cells and DCs, resulting in DC maturation. The current studies showed that $W_{80}5EC$ induced DC maturation in vitro, character-

FIGURE 7. W₈₀5EC NE adjuvant activity in IL12^{-/-} and IL-12Rβ1^{-/-} mice. **(A)** Ab response. Mice were immunized i.n. with PA-NE or PA-PBS vaccine. Anti-PA IgG titers were measured at 12 wk after primary immunization. * indicates statistically significant differences in IgG titers between WT and IL-12p35/p40^{-/-} mice. Ag-specific cellular response: Th-1-type **(B)**, Th-17-type **(C)**, and Th-2-type **(D)** cytokine production was measured in the cell culture supernatants of splenocytes from mice immunized with PA-NE and stimulated with PA. Results are average cytokine concentration ± SEM. Experiments were performed twice with five animals/group; analysis by multivariate Tukey test. *, **Statistically significant differences in cytokine production in WT versus IL-12p35/p40^{-/-} (*p* < 0.05) and IL-12Rβ1 (*p* < 0.05) mutant mice, respectively.



ized by the increased expression of B7 family molecules CD80 and CD86, as well as MHC class II (82, 83). CD80 and CD86 share ~27% external domain homology and activate the bidirectional signaling by engaging either the immunostimulatory CD28 or the inhibitory CTLA-4 receptors on T cells (18, 84). Consistent with other studies, mice lacking CD86 produced normal Ab titers, whereas double CD80/CD86 mutation completely abolished this response and severely reduced a Th-1 bias of immunity (85, 86). This suggests that NE activates either CD80 or CD86 signaling, and CD80 activation alone is sufficient for the B cell-dependent IgG response but not for Th-1-type cell immunity. In addition to diminished Th-1-type responses, all investigated mutations severely repressed development of Th-17 immunity after immunization with NE adjuvant, as demonstrated in our previous study (27).

In summary, we identified a unique set of signaling activities underlying the mechanism of the mucosal adjuvant W₈₀5EC NE (Table II). Activation of TLR through nonligand-mediated means and signaling through MyD88 and IL-12 pathways are indispensable for the induction-balanced Th-1 and Th-17 cellular immunity, but they are not necessary for an Ab response. Because of the unique activity of this material as a mucosal adjuvant, we believe that these data have important implications for under-

standing the signaling pathways involved in mucosal immunity. In addition, understanding the separate mechanisms by which W₈₀5EC NE adjuvant produces humoral and cellular types of immune responses will further facilitate the rational development of the most effective mucosal vaccines.

Table II. Summary: immunological checkpoints of Ab and cellular immune responses induced with W₈₀5EC NE mucosal adjuvant

Genotype	Adaptive Immune Responses			
	Humoral	Cellular		
		Ab	Th-1	Th-2
WT	+	+	+/-	+
MyD88 ^{-/-}	+	-	+	-
TLR2 ^{-/-}	+	-	+	-
TLR4 ^{-/-}	+	-	+/-	-
IL-12 ^{-/-}	+	-	+	-
IL-12Rβ1 ^{-/-}	+	-	+	-
CD40 ^{-/-}	-	-	-	-
CD86 ^{-/-}	+	-	+	-
CD80/CD86 ^{-/-}	-	-	-	-

+ and - signify induction of immune response.

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Disclosures

J.R.B., Jr. has an ownership stake in NanoBio Corp., the company that the University of Michigan has licensed to commercialize the NE technology examined in these studies. NanoBio Corp. was also paid under a contract to produce the formulations used in these studies. NanoBio Corp. had no role in the design of the studies, the data collection and analysis, the decision to publish, or the preparation of this manuscript. The other authors have no financial conflicts of interest.

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