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Dendritic Cell Differentiation Induced by a Self-Peptide Derived from Apolipoprotein E¹

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Dendritic cells (DCs) are professional APCs and potent stimulators of naive T cells. Since DCs have the ability to immunize or tolerize T cells they are unique candidates for use in immunotherapy. Our laboratory has discovered that a naturally processed self-peptide from apolipoprotein E, Ep1.B, induces DC-like morphology and surface marker expression in a murine monocytic cell line (PU5-1.8), human monocytic cell line (U937), murine splenocytes, and human peripheral blood monocytes. Microscopy and flow cytometric analysis revealed that Ep1.B-treated cells display decreased adherence to plastic and increased aggregation, dendritic processes, and expression of DC surface markers, including DEC-205, CD11c, B7.1, and B7.2. These effects were observed in both PU5-1.8 cells and splenocytes from various mouse strains including BALB/c, C57BL/6, NOD/Lt, and C3H/HeJ. Coadministration of Ep1.B with OVA antigenic peptide functions in dampening specific immune response to OVA. Ep1.B down-regulates proliferation of T cells and IFN- γ production and stimulates IL-10 secretion in immunized mice. Ep1.B-induced differentiation resulted in the activation of PI3K and MAPK signaling pathways, including ERK1/2, p38, and JNK. We also found that NF- κ B, a transcription factor essential for DC differentiation, is critical in mediating the effects of Ep1.B. Ep1.B-induced differentiation is independent of MyD88-dependent pathway of TLR signaling. Cumulatively, these findings suggest that Ep1.B acts by initiating a signal transduction cascade in monocytes leading to their differentiation into DCs. *The Journal of Immunology*, 2008, 181: 6859–6871.

Dendritic cells (DCs)⁴ play a key role in initiating and controlling the magnitude and the quality of adaptive immune responses (1). DCs induce T cells to differentiate to acquire function, especially to form CD8⁺ killer lymphocytes and an expanding array of CD4⁺ helper cells. This includes Th1, Th2, and Th17 T cells that resist various categories of infection, as well as Foxp3⁺ and IL-10⁺ T cells that silence immunity to harmless self and environmental Ags (2). The immunostimulatory capacity of DCs renders them attractive candidates to modulate immunity in malignancy, transplantation, and autoimmune diseases. DCs pulsed with tumor peptides elicit protective and antitumor immunity in mice (3). DCs are also potent inducers of T cell tolerance (4) and therefore offer a potential therapy for au-

toimmune diseases (5) and graft rejection (6). For example, in NOD mice, which spontaneously develop diabetes, DCs can induce the generation of regulatory T cells in vitro that have a therapeutic outcome in mice with overt diabetes (7).

In the murine system, it has been shown that monocytes can differentiate into DCs both in vivo (8–10) and in vitro (11, 12). Hematopoietic CD34⁺ progenitors cultured with GM-CSF and IL-4 can differentiate to DCs (13). DCs can also develop from peripheral blood monocytes when cultured with GM-CSF and IL-4. The resulting cells have the characteristics of immature DCs but they can mature in response to inflammatory stimuli such as LPS, TNF- α , IFN- γ , IL-1, or CD40L (14, 15) to up-regulate MHC II and costimulatory molecules. IL-4 exerts its DC differentiation effect by blocking monocyte differentiation toward macrophage through inhibition of M-CSF production and prevention of GM-CSF receptor α -chain down-regulation on differentiating CD34⁺ cells (16). At least two cytokines, GM-CSF and Flt3 ligand, induce the expansion of DCs in vivo (17–19).

Historically, the study of DCs has been limited due to the low number of these cells in vivo. For example, in both humans and mice only small numbers of DCs can be isolated from peripheral blood and secondary lymphoid organs (20). Efforts have been directed at increasing DC numbers using PBMCs and bone marrow-derived CD34⁺ cells as progenitors (13, 14, 21), although most clinical trials use ex vivo monocyte-derived DCs for vaccination purposes, as they are easily generated using a patient's peripheral blood. We now report a novel way to induce DC differentiation using a self-peptide derived from apolipoprotein E (ApoE).

ApoE is a 34-kDa glycoprotein with 299 aa that is expressed in a variety of tissues, including the liver, brain, kidney, and adrenal glands. ApoE is important for lipid metabolism and redistribution (22). Additionally, ApoE appears to be involved in cellular functions that are independent of its role in lipid homeostasis. For

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⁴ Abbreviations used in this paper: DC, dendritic cell; ApoE, apolipoprotein E; BMDC, bone marrow-derived DC; NAC, N-acetyl-L-cysteine; PVDF, polyvinylidene difluoride.

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example, it can inhibit proliferation of lymphocytes, smooth muscle cells, endothelial cells, and tumor cells (23–27). ApoE has two distinct but interacting structural and functional domains: the amino-terminal domain (residues 1–191) contains the low-density lipoprotein receptor (LDL-R) and LDL-R-related protein (LRP) binding domains, whereas the carboxyl-terminal domain (residues 216–299) contains the major lipid-binding determinants (28–30). The immunogenicity of two naturally processed self-peptides derived from the carboxyl terminus of ApoE, ApoEp1 (Ep1) (236–252) and ApoEp2 (Ep2) (268–284), eluted from murine MHC class II I-A^d has been reported by our laboratory (31, 32). We have discovered that a peptide fragment from Ep1, Ep1.B (239–252), can induce monocyte differentiation resulting in the generation of functional DCs *in vitro*. Since DCs are capable of activating naive T cells in the initiation of an immune response, they may be prime targets for modulation in immunotherapy.

Materials and Methods

Peptide synthesis and purification

Peptides were synthesized using the Merrifield solid-phase technique on an ABI 431A peptide synthesizer (Applied Biosystems) as described earlier (32). The peptides were purified using HPLC on a C18 reverse-phase semi-preparative column (SynChrom) with a linear water-to-acetonitrile gradient (33). Subsequently, the peptides were lyophilized and stored at –20°C until use. Peptides were reconstituted in double-distilled H₂O or 10% glucose and sterilized by passage through a 0.22- μ m filter before use. The composition and purity of the synthetic peptides were assessed by mass spectrometry at the University of Western Ontario Biological Mass Spectrometry Laboratory.

mAbs and reagents

The following anti-mouse fluorochrome (fluorescein or PE)-conjugated mAbs were purchased from BD Pharmingen for flow cytometry experiments: CD80, CD86, CD11c, and Mac-1. Unconjugated monoclonal rat anti-mouse DEC-205 and 33D1 (generously supplied by Dr. T. L. Delovitch, London, Ontario, Canada) were used with FITC-labeled goat anti-rat Ig (BD Pharmingen) as the secondary Ab. Monoclonal MK-D6 (anti-I-A^d) hybridoma was purchased from the American Type Culture Collection (ATCC) to detect MHC class II. PE-conjugated TLR-4 and FITC-conjugated TLR-9 were purchased from eBioscience. For human monocytic cell line, PE-conjugated mAbs CD80 (clone MEM233), CD83 (clone HB15e), and CD86 (clone BU63) used were from Caltag Laboratories. CDw123 (clone 9F5) and CD32/16 isotype-matched controls were purchased from BD Pharmingen or Caltag Laboratories.

The following were used as primary Abs for Western blot analysis: mouse monoclonal phospho-p44/42 MAPK E10 Ab and rabbit polyclonal phospho-p38 MAPK Ab, p38 MAPK Ab, SAPK/JNK Ab, and phospho Akt were all purchased from Cell Signaling Technology, while rabbit anti-ERK1/2 (MAPK) polyclonal Ab and polyclonal rabbit anti-active JNK Ab were purchased from Stressgen and Promega, respectively. The following were used as secondary Abs: HRP-conjugated goat anti-rabbit IgG (H+L) (Bio-Rad) and HRP-linked whole anti-mouse Ig (Amersham Life Science).

Mitomycin C and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich. LY294002 PI3K-Akt inhibitor, SP600125 JNK1/2 inhibitor, SB203580 p38 MAPK inhibitor, and PD98059 ERK kinase (MEK1/2) inhibitor were obtained from Calbiochem.

Cell culture

The murine monocytic cell line, PU5.1.8, was obtained from the ATCC. PU5.1.8 cells were cultured in DMEM (Invitrogen) containing 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin (Invitrogen), 10 mM HEPES, 5 \times 10^{–5} M 2-ME, and 10% heat-inactivated FBS (HyClone). The human monocytic cell line U937 (ATCC CRL 1593) was maintained in complete RPMI 1640 (Invitrogen) containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 5 \times 10^{–5} M 2-ME, and 10% heat-inactivated FBS. For purification of human monocytes, mononuclear cells were separated from peripheral blood with Histopaque 1077, and monocytes were enriched by depletion of nonmonocyte cells by a EasySep negative selection human enrichment kit (StemCell Technologies). For the preparation of bone marrow DCs, bone marrow progenitors harvested from tibia and femur were grown in RPMI-10 supplemented with GM-CSF (10 ng/ml), and Ep1.B (50 μ g/ml) was added to the culture on days 0 and 3.

Cells were matured overnight with LPS (1 μ g/ml) before purification with CD11c⁺ MACS magnet beads (Miltenyi Biotec).

Mice

BALB/cJ (H-2^d), C57BL/6J (B6) (H-2^b), C3H/HeJ (H-2^k), and NOD/Lt (H-2^g) mice between 4 and 12 wk of age were purchased from The Jackson Laboratory. B6.MyD88^{–/–} mice were bred at the Yale University School of Medicine (New Haven, CT). Mice were housed in the nonspecific pathogen-free (SPF) animal facility at the University of Western Ontario (London, Ontario, Canada).

Spleen cell preparation

Mice were sacrificed and their spleens were removed aseptically. Single-cell suspension was prepared by passing the spleen through a fine-mesh sieve. The erythrocytes were lysed in 5 ml ACK lysis buffer (0.15 M NH₄Cl, 1.0 mM Na₂EDTA (pH 7.3)) for 2 min at room temperature. After washing, cells were counted and resuspended in complete media for the assay.

Flow cytometry

Cells were incubated with CD32/16 Ab to prevent nonspecific Ab binding. Fluorochrome-conjugated primary mAb in FACS buffer (1% BSA in PBS) was added and cells were incubated for 45 min on ice. Primary incubation with unconjugated Ab was followed by a wash with PBS before adding fluorochrome-conjugated secondary Ab to the cells for an additional 30 min on ice. The samples were washed and resuspended in PBS and the fluorescence of the stained cells was determined. The data was acquired on a BD FACScan (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Mixed lymphocyte reactions

Primary allogeneic MLRs were performed using PU5-1.8 cells (BALB/c origin) as stimulators and naive C57BL/6 T cells as responders. Stimulator cells were preincubated for 72 h in either media alone or with 100 μ g/ml Ep1.B. Stimulator cells were then treated with mitomycin C (50 μ g/ml, 2 h at 37°C; Sigma-Aldrich) to inhibit proliferation. To isolate naive C57BL/6 T cells, single-cell suspensions of splenocytes were passed over a nylon wool column. Pretreated stimulator cells (2 \times 10⁵) were cocultured with responder cells (4 \times 10⁵) for 3 days at 37°C. After 72 h, cultures were pulsed with [³H]TdR (0.5 μ Ci/well) and incubated for an additional 18 h. Cells were harvested (Tomtec) and [³H]TdR incorporation was measured using a MicroBeta liquid scintillation counter (Wallac). Results are expressed as the mean cpm \pm SD from triplicate wells.

Proliferation assays and cytokine analysis

For proliferation assays, six groups of three 6-wk-old female NOD mice received footpad injections of either saline, 100 μ g Ep1.B or Ep1.N, 50 μ g OVA peptide, or a combination of Ep1.B + OVA (100 and 50 μ g, respectively) or Ep1.N + OVA (100 and 50 μ g, respectively) emulsified in IFA. To administer the footpad immunization, mice were anesthetized by methoxyflurane (Metofane) inhalation and then a volume of 50 μ l was injected into each footpad. Ten days postinjection mice were euthanized and their popliteal lymph nodes were removed and the cells were pooled. Peptide-primed cells (2 \times 10⁵) were incubated with one of the following: media alone, 5 μ g/ml Con A (Sigma-Aldrich), 100 μ g/ml of Ep1.B or Ep1.N, 50 μ g/ml OVA, or a combination of ApoE peptide and OVA (100 and 50 μ g/ml, respectively) in 96-well flat-bottom microtiter plates (BD Biosciences). The cultures were kept at 37°C in a 5% CO₂-in-air incubator for 72 h, and 50 μ l [³H]TdR (0.5 μ Ci/well) was added for an additional 18 h. The cells were then harvested (Tomtec) and [³H]TdR incorporation was measured using a MicroBeta liquid scintillation counter. Culture supernatants were collected at 24, 48, and 72 h and stored at –20°C until they were tested for IL-4, IL-10, and IFN- γ content using sandwich ELISA assays. ELISA assays were performed according to the manufacturer's (BD Pharmingen) protocol.

Western blotting

PU5-1.8 cells were cultured in OptiMEM reduced serum media (Invitrogen) at 37°C with either 100 μ g/ml Ep1.B or Ep1.N or 20 μ g/ml *Escherichia coli* LPS (Sigma-Aldrich) for 0, 10, or 60 min. Following the required incubation, cold PBS containing 0.4 mM Na₃VO₄ and 0.4 mM EDTA was added to the cells to cease stimulation. The cells were pelleted and lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.6), 5 mM EDTA, 1 mM Na₃VO₄, 25 μ M *p*-nitrophenyl-*p*'-guanidino-benzoate, and 10 μ g/ml of both leupeptin and aprotinin was added

Table I. Sequences of ApoE-derived peptides

Name	Sequence	Residues
Ep1	EEQ TQQ IRL QAE IFQ AR	236–252
Ep1.B	TQQ IRL QAE IFQ AR	239–252
Ep1.N	TQQ IRL QAE IAQ AR	239–252ΔA ₂₄₉ ^a
Ep1.R	RA QFI EAQ LRI QQT	252–239
Ep2	WANLVEKIQASVATNPI	268–284

^a Amino acid at position 249 was substituted with alanine.

to the cells for 30 min on ice. After lysis, the cells were pelleted at 4°C and sample buffer (4×) was added to the lysates. The samples were heated for 7 min at 97°C and were resolved on 10% SDS polyacrylamide gels. The gels were transferred to polyvinylidene difluoride (PVDF) (Roche Diagnostics) membranes and incubated for 2 h in blocking reagent (Roche Diagnostics) to prevent nonspecific binding of the Abs. The membranes were then probed with the indicated primary Ab overnight at 4°C. After thorough washing with TBST (0.01 M Tris, 0.15 M NaCl, 0.002% Tween 20

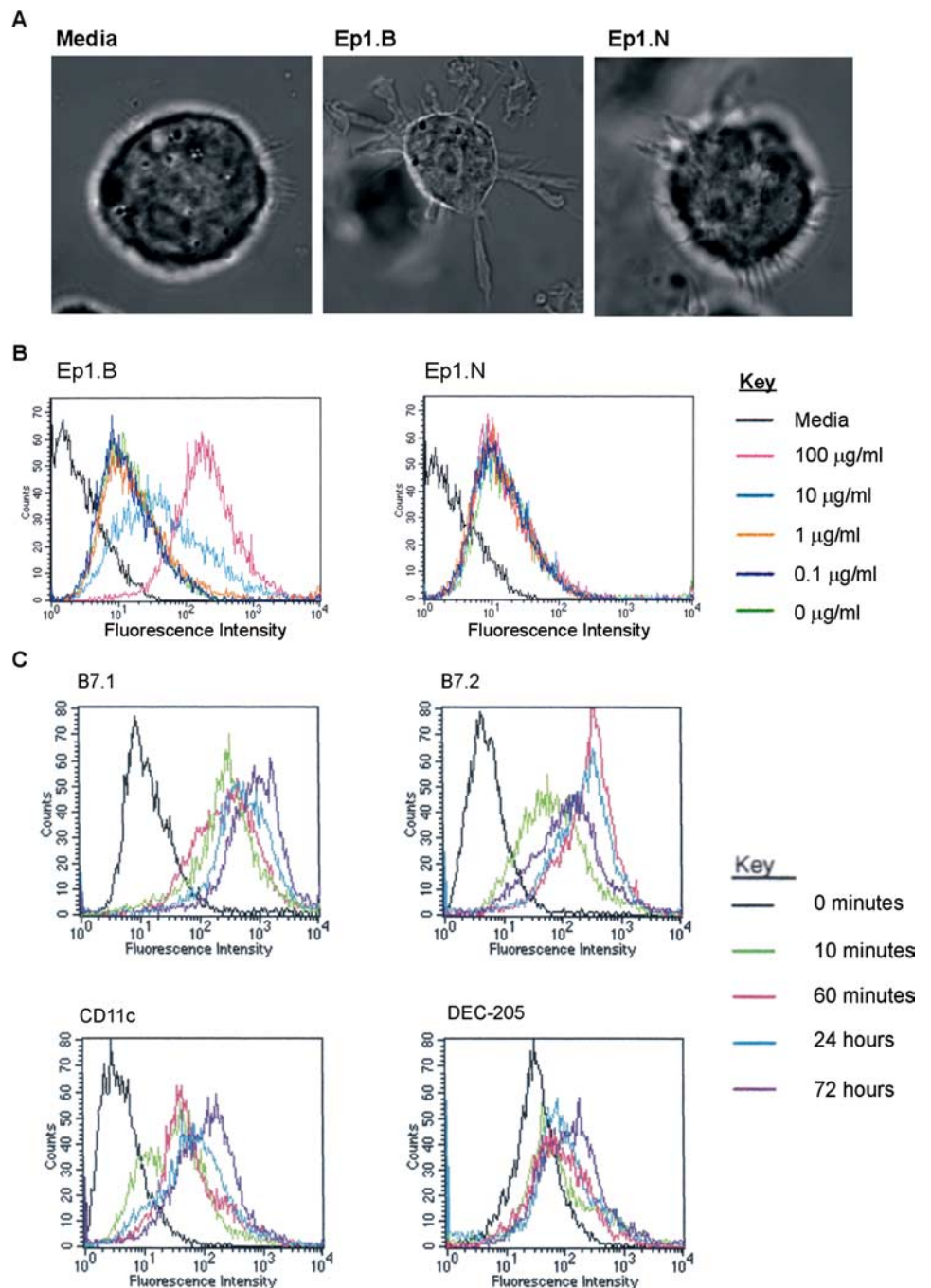
in double-distilled H₂O) the membranes were incubated with the appropriate secondary Ab for 30 min. Following a final wash step, protein signal was detected by chemiluminescence (Roche Diagnostics).

Results

Ep1.B peptide induces DC-like morphology

Monocytic PU5-1.8 cells derived from BALB/c mice were incubated with Ep1.B or the negative control Ep1.N peptide (which differs from Ep1.B by one amino acid in the C terminus, see Table I) to assess activity. After only 2 h, Ep1.B-treated cells began to detach from plastic plates and aggregate in suspension optimally at 100 μg/ml, while cells cultured in media alone or Ep1.N were unaffected. Within 48 h, morphologically Ep1.B-treated cells appeared less rounded, more granular, and displayed dendritic-like processes when compared with cells cultured in media alone or the negative control peptide (Fig. 1A). FACS analysis confirmed an increase in cell size and granularity (data not shown). To test

FIGURE 1. Ep1.B peptide induces DC-like morphology and phenotypic markers. *A*, PU5-1.8 cells (1×10^4) were incubated with media alone or 100 μg/ml of Ep1.B or control peptide Ep1.N in 24-well plates for 48 h. Following incubation the cells were mounted on microscope slides and the samples were viewed by phase-contrast microscopy using a scanning laser confocal microscope under ×60 oil immersion. *B*, To determine the optimal peptide dose, PU5-1.8 cells (1×10^6) were incubated with varying concentrations of Ep1.B in 24-well plates for 72 h and then stained with B7.1 for FACS analysis; 10,000 events were collected. *C*, To elucidate the optimal culture period, PU5-1.8 cells (1×10^6) were incubated for different periods of time with 100 μg/ml of Ep1.B in 24-well plates. Subsequently the cells were stained and analyzed by flow cytometry where 10,000 events were collected.



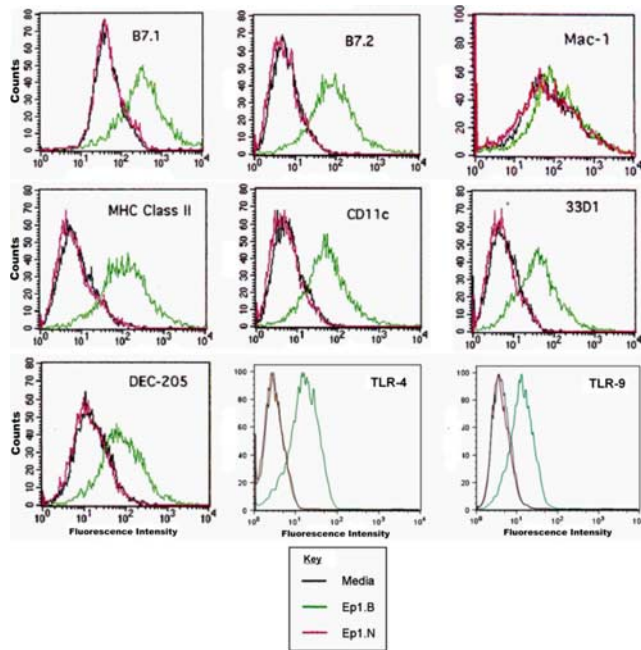


FIGURE 2. Ep1.B induces expression of DC surface molecules on mice monocytic cell line. PU5-1.8 cells (1×10^6) were incubated for 72 h in media alone or with 100 $\mu\text{g}/\text{ml}$ of either Ep1.B or Ep1.N in 24-well plates and then stained with various markers of DCs for FACS analysis. Specifically for TLR-9 staining, cells were permeabilized and intracellular staining was done and 10,000 events were collected.

whether the Ep1.B-induced phenotypic changes are dose-dependent, PU5-1.8 cells were cultured with various concentrations of Ep1.B peptide, and B7.1 expression was determined by FACS

analysis. It was found that 100 $\mu\text{g}/\text{ml}$ Ep1.B is the optimal concentration to induce B7.1 (Fig. 1B). This concentration of Ep1.B was found to be nontoxic by trypan blue exclusion. Based on the kinetics of changes in B7.1 expression it was determined that Ep1.B has an effect on treated cells that is apparent after only 10 min. However, following a time course, expression was determined to be optimal following 72 h culture with the peptide, as determined by induction of expression of B7.1, B7.2, CD11c, and DEC-205 (Fig. 1C).

Expression of DC surface molecules on Ep1.B-treated cells

Morphologically, Ep1.B-treated cells resembled DCs, as evidenced by their thin dendrites. The ability of Ep1.B to induce DCs was confirmed by FACS analysis. PU5-1.8 cells were incubated for 72 h with 100 $\mu\text{g}/\text{ml}$ Ep1.B or control peptide and then stained for FACS analysis. Surface expression of MHC class II, costimulatory molecules B7.1 and B7.2, and TLR-4 and TLR-9 was significantly increased above the background while expression of the macrophage marker Mac-1 was unaffected (Fig. 2). Additionally, a significant increase in the DC-specific molecules CD11c, 33D1, and DEC-205 expression was detected, suggesting that the monocytic PU5-1.8 cells have differentiated into DC-like cells. Surface marker expression on cells that were cultured with the negative control Ep1.N closely matched the background levels established by cells cultured in media alone (Fig. 2).

Ep1.B-induced changes were not due to LPS contamination

To rule out possible endotoxin contamination of the peptide preparation, the peptides were passed over a polymyxin B column before their addition to cell culture. Following 72 h of culture, the increases in surface marker expression on Ep1.B-treated cells following polymyxin B treatment were comparable to those observed

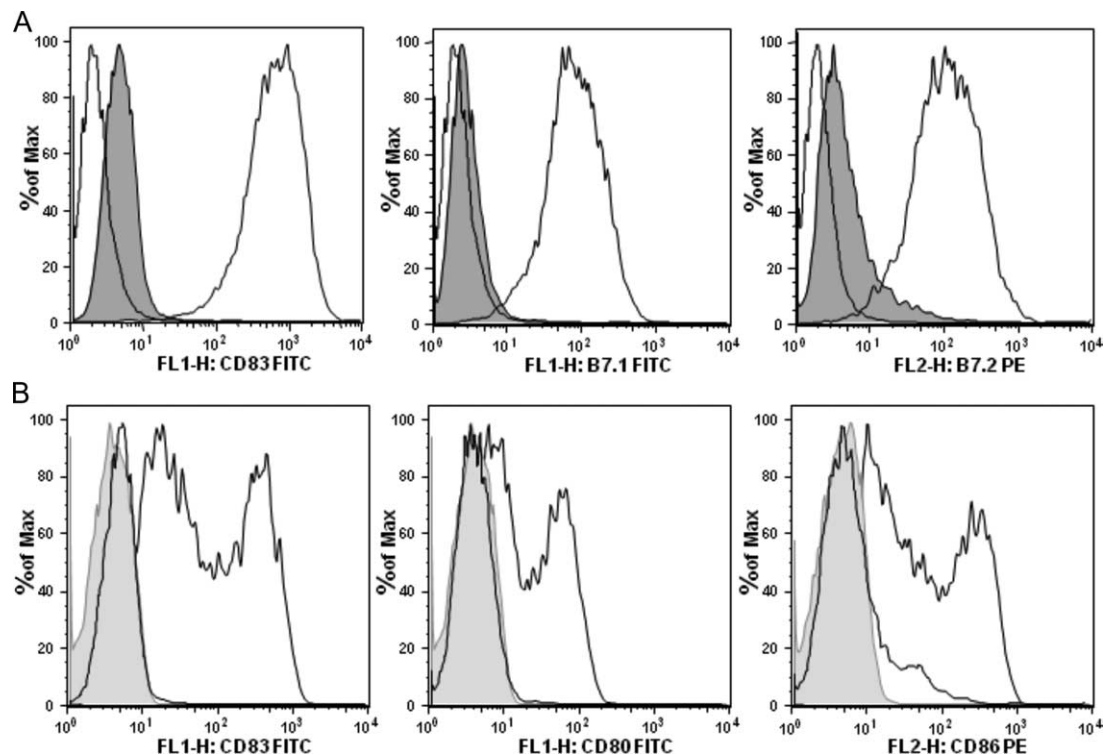


FIGURE 3. Ep1.B induces expression of DC surface molecules on human monocytes. *A*, Human monocytic cell line U937 cells (1×10^6) were incubated for 24 h with 100 $\mu\text{g}/\text{ml}$ of Ep1.B or media alone as negative control in 24-well plates and then stained for FACS analysis; 10,000 events were collected. *B*, Human monocytes were separated from peripheral blood and incubated for 72 h with 100 $\mu\text{g}/\text{ml}$ of Ep1.B or media alone as negative control in 24-well plates and then stained for FACS analysis. Open histogram, cells treated with Ep1.B; filled histogram, cells not treated with Ep1.B; dashed line, isotype control.

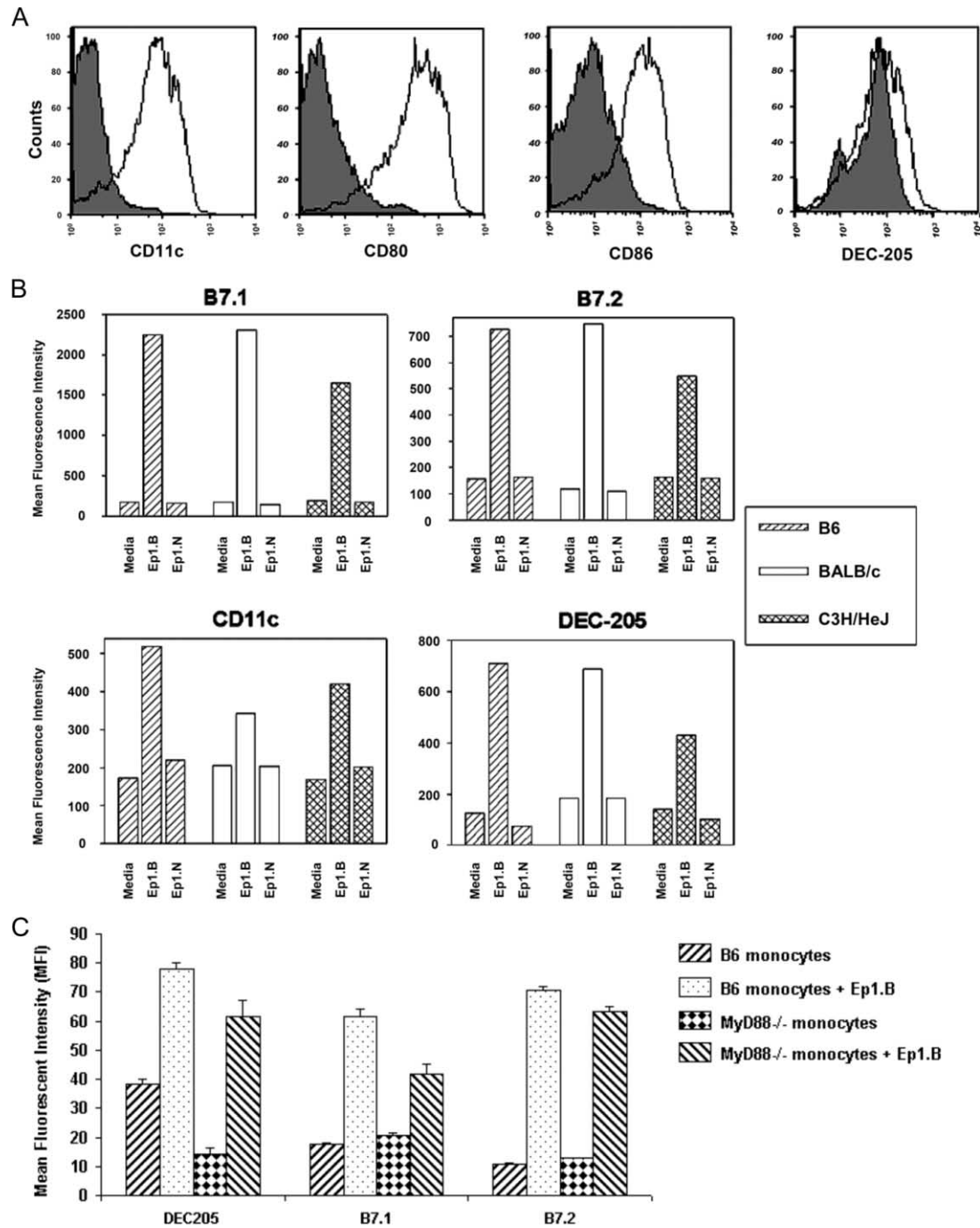


FIGURE 4. Induction of DC surface markers by Ep1.B is not strain specific or MyD88 dependent. *A*, Splenocytes (1×10^6) from NOD mice were incubated with $100 \mu\text{g/ml}$ Ep1.B for 72 h and analyzed for the expression of CD11c, CD80, and CD86 and DEC-205 by flow cytometry on splenocytes gated for CD11b⁺ cells. Open histogram, cells treated with Ep1.B; filled histogram, cells not treated with Ep1.B. *B*, Splenocytes (4×10^6) from C57BL/6, BALB/c, and C3H/HeJ were incubated for 72 h with $100 \mu\text{g/ml}$ Ep1.B or Ep1.N in 6-well plates and then stained for FACS analysis; 10,000 events were collected. *C*, Monocytes from spleen of B6.MyD88^{-/-} mice and control B6 mice were incubated for 72 h with $50 \mu\text{g/ml}$ Ep1.B and then stained with DEC-205, B7.1, and B7.2 for FACS analysis. Mean fluorescent staining for the expression of these molecules is shown.

in Fig. 2 (data not shown). This indicates that the changes in morphology and phenotype were not due to LPS contamination. As shown later (see Fig. 4*B*), this was further confirmed using C3H/HeJ mice that are unable to respond to LPS due to their spontaneous mutation in TLR-4 (34, 35).

Ep1.B induces DC differentiation from human monocytes

We treated the human monocytic cell line U937 with Ep1.B to see if this DC generation potential is also applicable to human

monocytes. Ep1.B induced up-regulation of DC-specific markers such as CD83, CD80, and CD86 (Fig. 3*A*). Induction of phenotypic changes corresponding to DC differentiation was accompanied by expression of the activation marker CD83 and costimulatory molecules CD80 and CD86. Furthermore, human monocytes were purified from peripheral blood by magnet beads and incubated with Ep1.B for 72 h. Ep1.B induced expression of CD80, CD86, and CD83 on the surface of these cells (Fig. 3*B*).

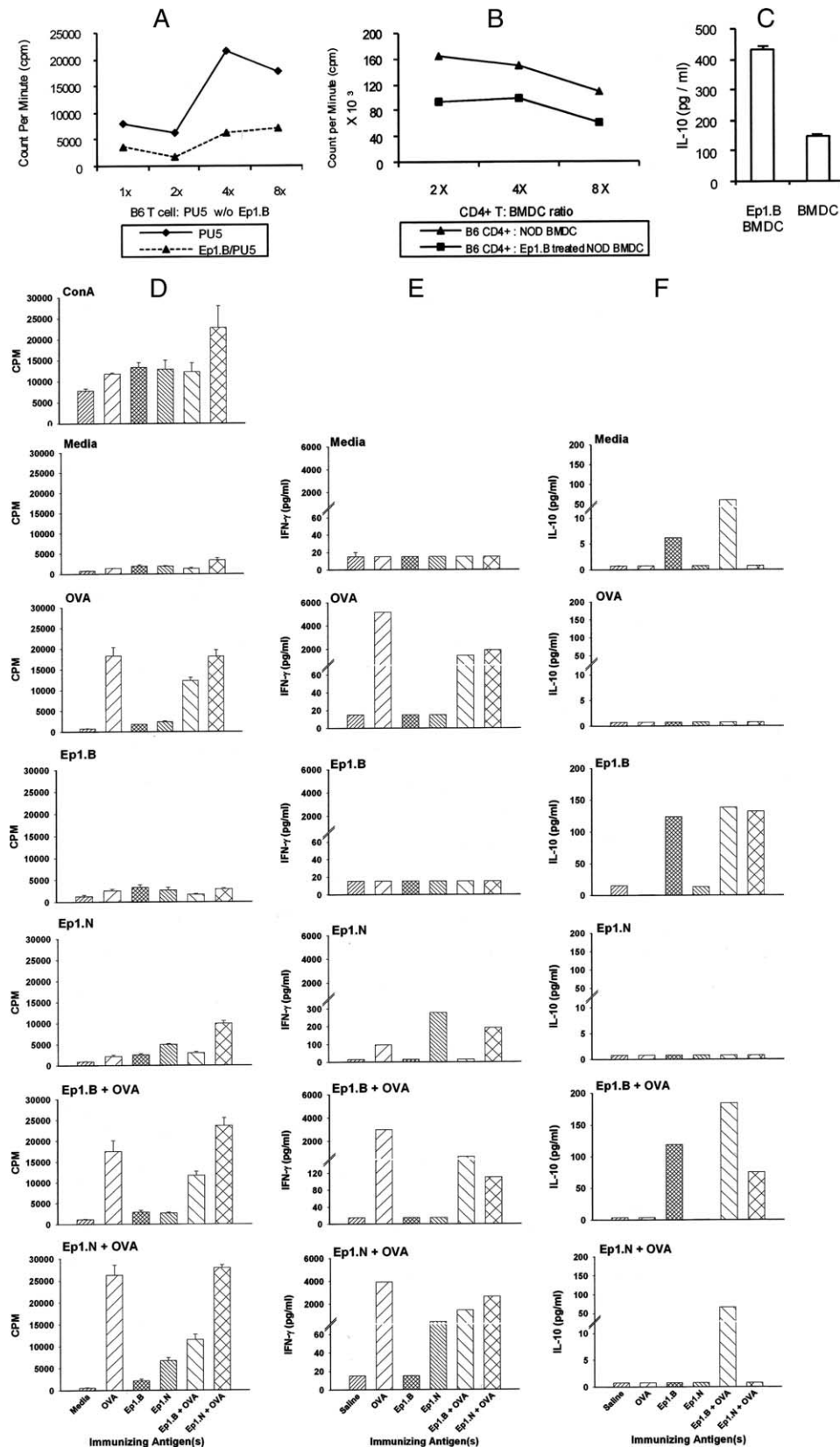


FIGURE 5. Ep1.B down-regulates proliferation and IFN- γ production and stimulates IL-10 secretion in immunized mice. *A*, Ep1.B-induced DCs down-regulate proliferation in MLR. PU5-1.8 cells (stimulators) were preincubated with 100 $\mu\text{g/ml}$ Ep1.B or Ep1.N peptide for 72 h, washed, and treated with mitomycin C. These cells were then cultured with T cells (responders) from C57BL/6 mice for 72 h at 37°C. Cultures were pulsed with [^3H]TdR (1.0 $\mu\text{Ci/well}$) and incubated for an additional 18 h. Cells were harvested and proliferation was assayed by measuring [^3H]TdR uptake using a MicroBeta counter. Results are expressed as the mean cpm \pm SD from triplicate cultures. *B*, In another set of experiments, Ep1.B (50 $\mu\text{g/ml}$) was added to days 0 and 3 of BMDC cultures from NOD mice, and following maturation with LPS (1 $\mu\text{g/ml}$), CD11c $^+$ DCs were purified with magnetic bead column and then plated in 96-well plates. Magnetic bead column-purified CD4 $^+$ T cells (10×10^5) from C57BL/6 mice were added to the culture for 3 days. T cell

Ep1.B-induced differentiation is not mouse strain specific

We next tested the effect of Ep1.B peptide on mice primary cells. Splenocytes from NOD mice were incubated with 100 $\mu\text{g/ml}$ Ep1.B for 72 h and analyzed using flow cytometry. Ep1.B induced the expression of DC markers on a CD11b⁺ gated monocyte population of spleen (Fig. 4A). Furthermore, unprimed splenocytes from BALB/c, C57BL/6, and C3H/HeJ mice were incubated with 100 $\mu\text{g/ml}$ Ep1.B or Ep1.N to determine whether the effects of Ep1.B are strain specific. Following 72 h of incubation, cells were stained and analyzed by flow cytometry (Fig. 4B). Splenocytes from the strains of mice tested responded to Ep1.B with similar affinity. Specifically, there was a significant increase in surface marker expression of the costimulatory molecules B7.1 and B7.2 in addition to the DC-specific markers CD11c and DEC-205 in the strains examined. There were no differences in surface molecule expression above the background (established by splenocytes cultured in media alone) for cells cultured in Ep1.N. This result shows that Ep1.B has an effect on primary cells that is not strain specific, suggesting that the putative Ep1.B receptor is not strain restricted. Importantly, C3H/HeJ mice have a mutation in the TLR-4 (*Tlr-4*) gene, responsible for responding to the lipid A moiety of LPS, making these mice LPS resistant. This further confirms that Ep1.B activity does not result from LPS contamination of the peptide preparation as discussed above.

Ep1.B-induced differentiation is not TLR dependent

The proximal events of TLR-mediated intracellular signaling are initiated by interactions with cytosolic adapters such as MyD88. MyD88 is a central adapter shared by almost all TLRs (36). As Ep1.B binds to the surface of cells, we performed experiments on MyD88^{-/-} mice to see if Ep1.B binds and activates TLRs through a MyD88-dependent pathway to induce differentiation of DCs from monocytes. We measured mean fluorescent intensity of DEC205, B7.1, and B7.2 surface molecules on spleen monocytes from C57BL/6 and MyD88^{-/-} mice cultured with Ep1.B for 72 h (Fig. 4C). Although there is a tendency for reduction of the Ep1.B effect in MyD88^{-/-} mice, absence of MyD88 did not abrogate the Ep1.B effect.

Alloreactivity of Ep1.B-induced DCs

A functional characteristic of DCs is their ability to induce primary MLRs. Since Ep1.B-treated cells have the costimulatory surface marker expression of DCs, we determined the alloreactivity capacity of Ep1.B-treated cells in MLR assays. To measure the T cell alloreactivity ability of Ep1.B-generated DCs, 2×10^5 untreated and Ep1.B-treated PU5-1.8 cells (BALB/c origin) were cocultured with 1×10^5 naive C57BL/6 T cells. Interestingly, allogeneic T cells displayed a significantly lower proliferative response to Ep1.B-treated PU5-1.8 cells compared with untreated PU5-1.8, suggesting that Ep1.B-treated monocytes differentiate into DCs with inhibitory properties (Fig. 5A). This is likely due to the in-

duction of tolerogenic DCs by Ep1.B. Alternatively, since untreated PU5-1.8 cells do not express MHC class II, it is assumed that the responding cells in the experiments in Fig. 5A are mostly CD8⁺ T cells recognizing allogeneic MHC class I. One possibility is that the Ep1.B peptide caused the down-regulation of MHC class I on PU5-1.8 cells. We measured the level of MHC class I K^d expression on PU5-1.8 cells after incubation with Ep1.B for 24 h. There was no difference in MHC class I expression before and after Ep1.B treatment (data not shown). Also, the same pattern of allostimulation occurred with primary cells from bone marrow-derived DCs (BMDCs) cultured in the presence of Ep1.B. There is a significant reduction in proliferation of allogeneic responder CD4⁺ T cells from B6 mice in coculture with Ep1.B-treated BMDCs of NOD mice (Fig. 5B). This could result from a change in the cytokine profile of Ep1.B-treated BMDCs, as supernatants of these cells have shown more IL-10 secretion (Fig. 5C). Furthermore, Ep1.B is also able to induce IL-10 production from T cells in recall response.

Ep1.B down-regulates proliferation and IFN- γ production and stimulates IL-10 secretion in immunized mice

As Ep1.B-induced DCs decreased proliferative response in MLRs, we wanted to know if immunization with Ep1.B would modify the immune response to antigenic peptides in vivo. For proliferation assays and cytokine ELISAs, 6-wk-old female NOD mice received footpad injections of either saline, 100 μg Ep1.B or Ep1.N, 50 μg OVA peptide, or a combination of Ep1.B + OVA (100 and 50 μg , respectively) or Ep1.N + OVA (100 and 50 μg , respectively) emulsified in IFA. After 10 days, the lymph nodes were harvested and primed cells were plated in triplicate with one of the following: media alone, 100 $\mu\text{g/ml}$ of Ep1.B or Ep1.N, 50 $\mu\text{g/ml}$ OVA, or a combination of Ep1.B or Ep1.N and OVA (100 and 50 $\mu\text{g/ml}$, respectively). Cells were assayed for T cell proliferation, while culture supernatants were collected and tested for IL-4, IL-10, and IFN- γ content using sandwich ELISA assays.

Cells from mice immunized with different Ags and cultured in vitro with media alone established background levels of cell proliferation, while incubation with 5 $\mu\text{g/ml}$ Con A, a T cell mitogen, served as a positive control to ensure that the cells were able to proliferate (Fig. 5D, Con A and media). Splenocytes from NOD mice did not exhibit a recall response when cultured with 100 $\mu\text{g/ml}$ Ep1.B following immunization with any of the Ags, suggesting that Ep1.B is nonimmunogenic (Fig. 5D, Ep1.B). Following in vitro culture with 100 $\mu\text{g/ml}$ Ep1.N there was modest proliferation in cells from mice immunized with either Ep1.N or a combination of Ep1.N + OVA (Fig. 5D, Ep1.N). Interestingly, for the recall response to OVA in vitro there was a significant decrease in proliferation for mice immunized with Ep1.B + OVA (Fig. 5D, OVA). This trend was also seen for the recall response to Ep1.B +

proliferation was measured by adding [³H]TdR in the last 18 h of culture. C, IL-10 levels in supernatants of primary culture of bone marrow progenitors of NOD mice with Ep1.B added on days 0 and 3 of culture, and supernatants were collected on day 6. D, Six-week-old NOD mice were divided into six groups of three and were given footpad injections of saline, 50 μg OVA, 100 μg Ep1.B, 100 μg Ep1.N, or a mixture of Ep1.B + OVA (100 and 50 μg , respectively) or Ep1.N in OVA (100 and 50 μg , respectively) emulsified in IFA. After 10 days the mice were euthanized and their draining lymph nodes were removed. Cells from the lymph nodes of each group of three mice were pooled for experiments. To assess proliferation, 2×10^5 cells from each group were cultured in 96-well plates and challenged with the immunizing Ag in addition to others, such that the cells were incubated for 72 h in either media alone, 5 $\mu\text{g/ml}$ Con A, 50 $\mu\text{g/ml}$ OVA, 100 $\mu\text{g/ml}$ Ep1.B or Ep1.N, or in a combination of Ep1.B + OVA (100 and 50 $\mu\text{g/ml}$, respectively) or Ep1.N in OVA (100 and 50 $\mu\text{g/ml}$, respectively). [³H]TdR was added to the culture for an additional 18 h of incubation. Proliferation was measured by [³H]TdR uptake using a MicroBeta counter. Results are represented as the mean \pm SEM from triplicate cultures. E and F, To detect cytokine production, 2×10^6 cells from each group were plated in the presence of the above-mentioned Ags, and supernatants from the cultures were harvested at 24, 48, and 72 h and tested for cytokine content. ELISA assays were used to detect IFN- γ (E) and IL-10 (F) production in the samples. Results are shown for the 24-h time point and are expressed as the mean of triplicate wells \pm SEM. x-axis of bar diagrams refers to peptides used for in vivo immunizations (Immunizing Ags), and headings of each diagram indicate peptides used for in vitro restimulation of the primed cells.

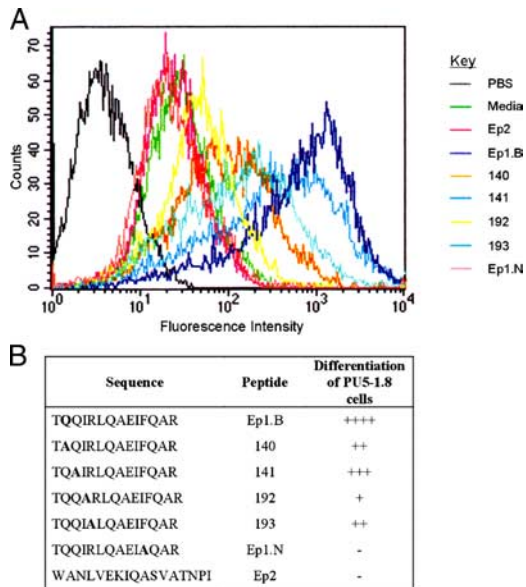


FIGURE 6. Effect of Ep1.B analogs on monocyte differentiation. *A*, PU5-1.8 (1×10^6) cells were incubated with 100 $\mu\text{g}/\text{ml}$ of Ep1.B or various Ep1.B analogs in 24-well plates for 72 h. The cells were then stained for CD11c expression and analyzed using flow cytometry. In each case, 10,000 events were collected. *B*, Differentiation was measured as an increase in CD11c expression on PU5-1.8 cells by flow cytometry. Fluorescent intensity for Ep1.B considered as a maximum (4+) and the analogs were assigned a grade of fluorescence intensity relative to Ep1.B fluorescence intensity.

OVA and Ep1.N + OVA. However, cells from mice immunized with OVA and Ep1.N + OVA exhibited significantly increased proliferation in culture with Ep1.N + OVA (Fig. 5D, Ep1.B +

OVA and Ep1.N + OVA). These results suggest that adding Ep1.B during priming down-regulates the proliferative response of NOD mice to OVA. This finding is not expected to be the result of competition for peptide binding to MHC, as a higher concentration of Ep1.B was used, or this phenomenon would be expected to be seen in mice immunized with Ep1.N + OVA. It is possible that Ep1.B induces the differentiation of DCs in vivo, which preferentially activate regulatory T cells, thereby accounting for the down-regulation of the immune response.

When the culture supernatants were tested for cytokine content, we found that IL-4 was undetectable in all of the samples. IFN- γ was detected in supernatants from cells cultured with OVA, Ep1.B + OVA, or Ep1.N + OVA from mice immunized with OVA, Ep1.B + OVA, or Ep1.N + OVA. A small amount of IFN- γ was also detected in mice immunized with OVA, Ep1.N, or Ep1.N + OVA and cultured with 100 $\mu\text{g}/\text{ml}$ Ep1.N. Notably, mice immunized with a combination of Ep1.B + OVA produced significantly lower amounts of IFN- γ than did mice immunized with OVA alone. This decrease in IFN- γ production was also seen for mice immunized with Ep1.N + OVA; however, the reduction was not as drastic (Fig. 5E). Conversely, cells from NOD mice immunized with Ep1.B alone or in combination with OVA produced IL-10 when cultured in the presence of these Ags. Little or no IL-10 was secreted in mice immunized with the other Ags with the exception of a moderate amount of IL-10 being produced in mice immunized with Ep1.N + OVA and cultured in vitro with Ep1.B or Ep1.B + OVA (Fig. 5F). Interestingly, where we observed a decrease in IFN- γ production in mice immunized with Ep1.B + OVA we saw a corresponding increase in IL-10 secretion in these animals. These results suggest that immunization with Ep1.B modulates the recall immune response of T cells by down-regulating proliferation and increasing anti-inflammatory cytokine secretion while OVA peptide promotes the existing Th1 profile.

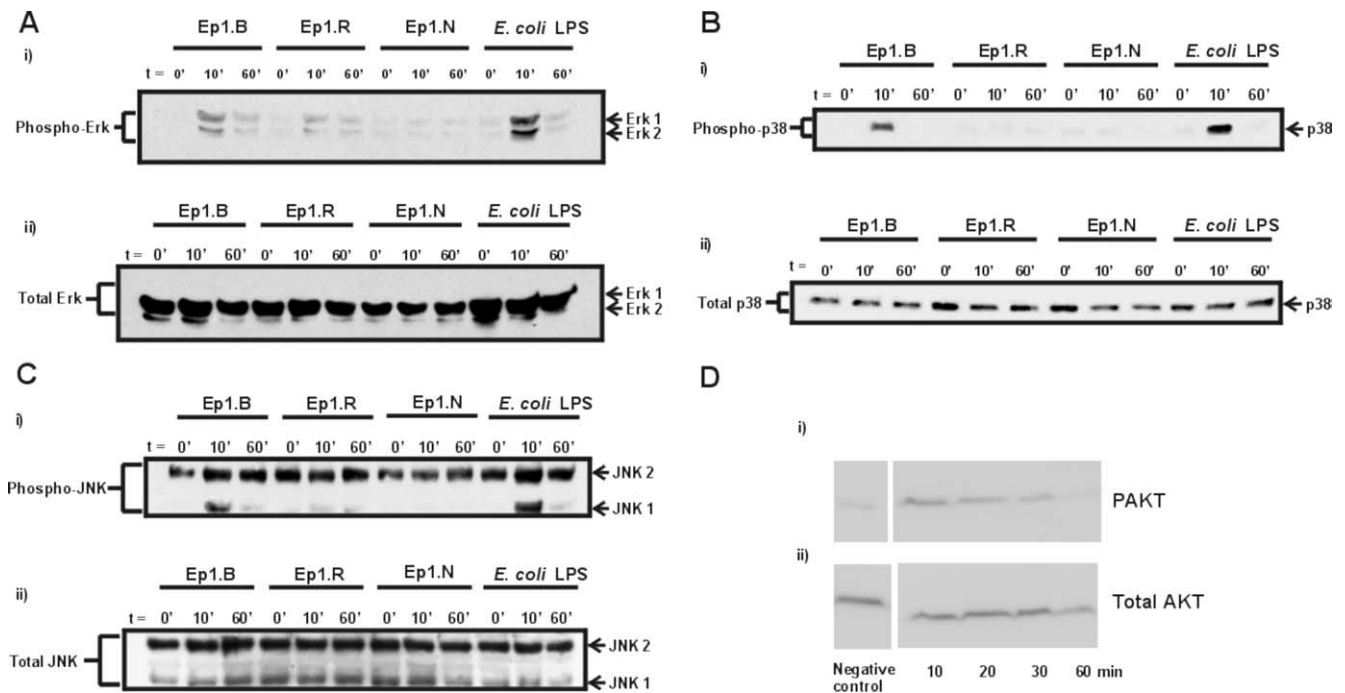


FIGURE 7. Ep1.B activates PI3K and MAPK signaling pathways. PU5-1.8 cells (3×10^6) were cultured in serum-free media at 37°C with 100 $\mu\text{g}/\text{ml}$ Ep1.B, Ep1.N, Ep1.R (a control peptide with reverse sequence of Ep1.B), or 20 $\mu\text{g}/\text{ml}$ *E. coli* LPS for 0, 10, or 60 min. Subsequently, the cells were lysed and the samples were resolved on 10% SDS-PAGE gels. The gels were transferred to PVDF membranes and immunoblotted for phospho-ERK (*Ai*), phospho-p38 (*Bi*), phospho-JNK (*Ci*), and phospho-Akt (*Di*). The membranes described above were then stripped and reprobed for total ERK (*Aii*), total p38 (*Bii*), total JNK (*Cii*), and total Akt (*Dii*).

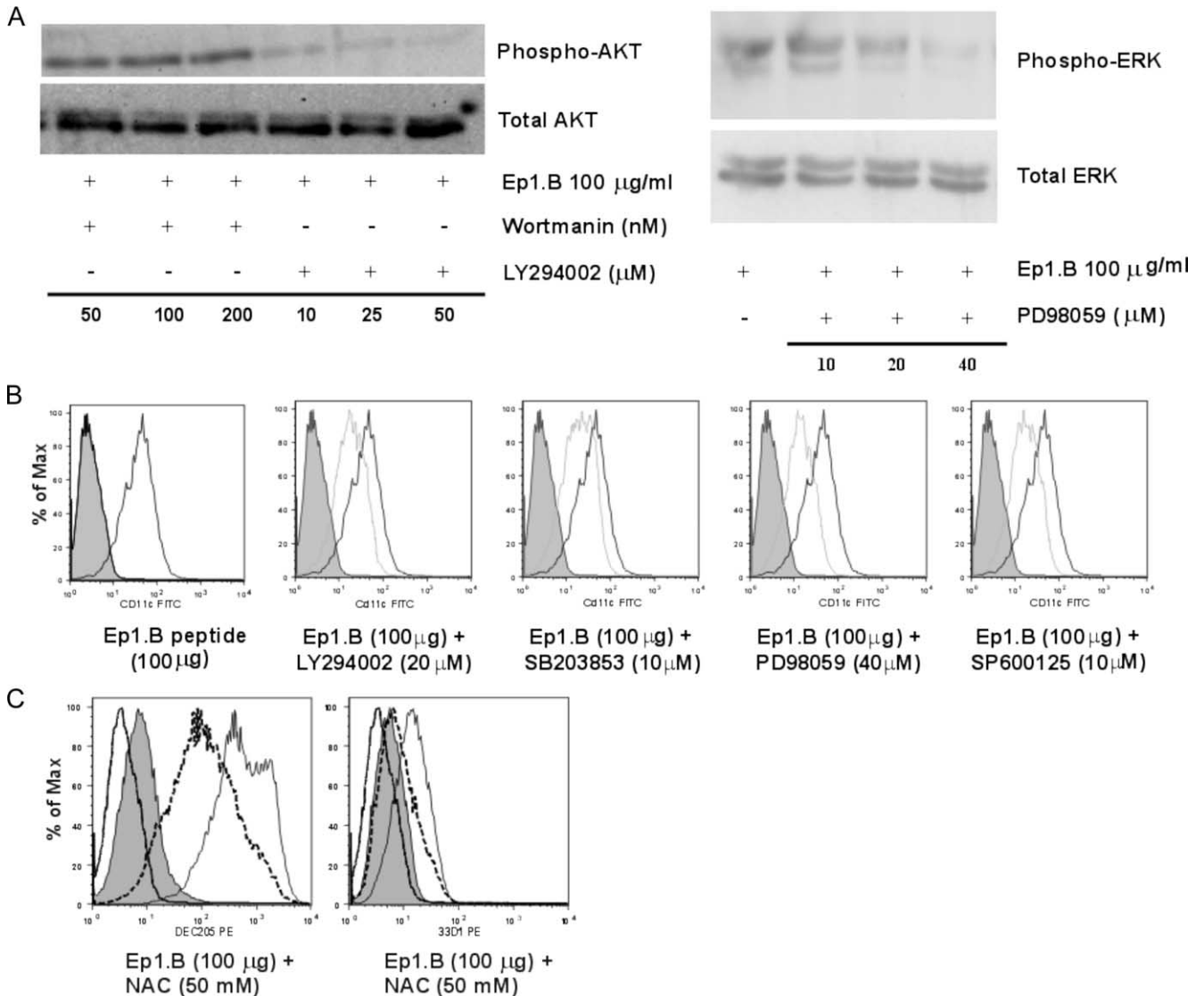


FIGURE 8. Specific inhibitors of PI3K and MAPK family and NF- κ B abrogate the effect of Ep1.B. *A*, PU5-1.8 cells (4×10^6) were cultured at 37°C for 1 h with various concentrations of PI3K inhibitor LY294002, p38 MAPK inhibitor SB203580, MEK inhibitor PD98059, and JNK inhibitor SP600245. Then, cells were stimulated with Ep1.B peptide (100 μ g/ml) for 10 min to determine an optimal concentration of the specific inhibitors. Subsequently, Western blots were done for phospho-Akt, phospho-JNK, phospho-p38 and phospho-ERK, and the results for phospho-Akt and phospho-ERK are shown. Wortmannin (400 nM), LY294002 (20 μ M), and PD98059 (20 μ M) were able to inhibit stimulation of PU5-1.8 cells with Ep1.B. *B*, The effect of inhibitors with optimal concentration on reduction of CD11c expression on PU5-1.8 cell line stimulated with Ep1.B peptide. Open histogram, cells treated with Ep1.B; histogram with dashed line, cells pretreated with inhibitor before stimulation with Ep1.B; filled histogram, cells not treated with Ep1.B. *C*, PU5-1.8 cells (1×10^6) were cultured for 2 h in the presence or absence of NAC (50 mM) and then stimulated with Ep1.B (100 μ g/ml). Following incubation, the cells were harvested and stained with DEC205 and 33D1 Abs. The samples were analyzed by flow cytometry where 10,000 events were collected. Open histogram, cells treated with Ep1.B; histogram with dashed line, cells pretreated with inhibitor before stimulation with Ep1.B; filled histogram, cells not treated with Ep1.B; and far left histogram, isotype control Ab.

Effect of Ep1.B analogs on monocyte differentiation

To determine whether any amino acid residues in the Ep1.B sequence are critical for its differentiating activity, alanine substitutions were performed along the length of the peptide. The resulting peptide analogs (Fig. 6) were tested on PU5-1.8 cells for DC differentiation. PU5-1.8 cells were cultured with 100 μ g/ml Ep1.B analogs for 72 h and then stained for FACS analysis. Increases in CD11c expression were used to measure the extent of PU5-1.8 differentiation into DCs since CD11c is a mouse DC-specific marker (Fig. 6A). As alanine residues were substituted for amino acids in the sequence of Ep1.B, the potency of the peptide was decreased as the alanine approached the C terminus (Fig. 6B). Importantly, Ep1.B activity was completely abolished when alanine was substituted for phenylalanine at position

11 in the peptide sequence. This peptide, termed Ep1.N, was used as the negative control peptide. Another control peptide, Ep2, derived from ApoE encompassing aa 268–284 also did not induce the differentiation of PU5-1.8 cells. The plus and minus scores represented in Fig. 6B are based on the extent of PU5-1.8 cell differentiation as measured by changes in CD11c surface marker expression using flow cytometry.

Ep1.B activates PI3K and MAPK signaling pathways

To determine the mechanism through which Ep1.B induces the differentiation of monocytes into DCs, we determined the activation of downstream signaling molecules such as members of the

MAPK superfamily. Western blots were performed to detect activation of MAPKs, including ERK, p38, and JNK. For this purpose, PU5-1.8 cells were cultured at 37°C with either 100 µg/ml Ep1.B or Ep1.N or Ep1.R (a peptide with reverse sequence of Ep1.B, Table I) or 20 µg/ml *E. coli* LPS for 0, 10, or 60 min. Following the required incubation, the cells were lysed and the lysates were resolved on 10% SDS-PAGE gels. The gels were transferred to PVDF membranes and then immunoblotted for the indicated proteins. It was observed that stimulation with Ep1.B resulted in the phosphorylation of ERK1 and ERK2 after 10 min and decreased after 1 h. This trend was also observed for the positive control: PU5-1.8 cells cultured with 20 µg/ml *E. coli* LPS, while ERK was not activated in cells cultured in Ep1.N (Fig. 7Ai). Similarly, p38 was activated in Ep1.B-treated cells within 10 min and was down-regulated after 1 h, as was observed for cells cultured with LPS, while there was no p38 activation in PU5-1.8 cells cultured with Ep1.N (Fig. 7Bi). Ep1.B also affects another member of the MAPK family, JNK. While levels of phospho-JNK2 were similar in all of the samples tested, phospho-JNK1 was up-regulated in cells treated with Ep1.B or *E. coli* LPS after a 10-min incubation. However, JNK1 levels were unaffected by Ep1.N (Fig. 7Ci). When the blots were stripped and probed for the levels of total ERK (Fig. 7Aii), p38 (Fig. 7Bii), and JNK (Fig. 7Cii), they were found to be similar, indicating that differences in the levels of the active forms of the MAPKs cannot be attributed to differences in protein loading. Therefore, Ep1.B activates ERK, p38, and JNK, while Ep1.N does not activate MAPK signaling pathways. Also, Ep1.B stimulates phosphorylation of Akt 10–30 min after stimulation of Ep1.B (Fig. 7, Di and Dii).

Furthermore, inhibition of those pathways by specific inhibitors and reversal of the induced phenotypic markers were used as additional evidence for the activation of signaling pathways by Ep1.B. For determination of signaling pathways used by Ep1.B, PU5-1.8 cells were treated with PI3K inhibitor LY294002, p38 MAPK inhibitor SB203580, MEK inhibitor PD98059, and JNK inhibitor SP600245. To determine an optimal concentration of the specific inhibitors in our experiments, we titrated the inhibitors and found that wortmannin (400 nM), LY294002 (20 µM), SB203580 (10 µM), PD98059 (20 µM), and SP600245 (10 µM) were sufficient to inhibit the phosphorylation activity of the kinases (Fig. 8A). While the PI3K inhibitor LY294002 (20 µM) could inhibit Akt phosphorylation, another PI3K inhibitor, wortmannin (200 nM), was not enough to inhibit reaction and a higher concentration (400 nM) was used (Fig. 8A). We found that these concentrations of inhibitors are not toxic to the cells by trypan blue staining and, on the other hand, they are being used in other studies (37–40). PU5-1.8 cells were cultured for 2 h in media containing these inhibitors, and then cells were stimulated with Ep1.B peptide. Cells cultured in media alone without the addition of Ep1.B established the background levels of surface marker expression. Fig. 8B shows the effect of various inhibitors on reduction of CD11c expression on PU5-1.8 cell line stimulated with Ep1.B peptide. We found that Ep1.B acts by initiating a signal transduction cascade in monocytes that results in the activation of PI3K and MAPK signaling pathways, including ERK1/2, p38, and JNK, thereby possibly instructing the differentiation of PU5-1.8 cells.

NAC, an inhibitor of NF-κB, prevents the activity of Ep1.B

Transcription factors of the Rel/NF-κB family, including p50, p52, and RelB, are expressed by, and have an important function in, the biology of DCs (41). Moreover, it has recently been shown that multiple signaling pathways that induce DC differentiation activate NF-κB (42). To determine whether incubation with Ep1.B results in NF-κB activation, PU5-1.8 cells were cultured for 24 h in media

alone or media containing 50 mM NAC, a potent inhibitor of NF-κB, in the absence or presence of 100 µg/ml Ep1.B. Consistent with previous results, incubating PU5-1.8 cells with 100 µg/ml Ep1.B resulted in significant increases in DEC205 and 33D1 surface expression (Fig. 8C). In addition to this observation, NAC was also found to be nontoxic to the cells by trypan blue staining. Importantly, when PU5-1.8 cells were cultured with 100 µg/ml Ep1.B and 50 mM NAC, a significant decrease in the levels of surface expression of DEC205 and 33D1 was observed (Fig. 8C). These results suggest that NF-κB is important in mediating the downstream effects of Ep1.B since inhibition of this molecule abrogates the ability of the peptide to up-regulate surface marker expression in PU5-1.8 cells.

Discussion

APCs are key regulators of the immune response, promoting or suppressing T cell responses depending on their lineage and functional state. Progress in the treatment of several clinical conditions, including transplantation, autoimmunity, infectious diseases, and cancer, can be greatly influenced by the development of agents that empower APCs to down-regulate or up-regulate the immune response (43). The recent development of methods to isolate DC precursors from blood and expand these cells in vitro to yield potent APCs has made this possible (44). Efforts directed at increasing DC numbers have shown that PBMCs and bone marrow-derived CD34⁺ cells, when cultured in the appropriate cytokine environment, are DC progenitors (13, 14, 21). However, for practical purposes most clinical trials use monocyte-derived DCs, as they are easily generated using a patient's peripheral blood. Although these results are encouraging, there are certain limitations to the use of DCs as therapeutics. For example, costly cytokine cocktails are required to generate DCs both in vitro and in vivo, and the composition of monocyte-conditioned medium, which can be used to induce final DC maturation, has unpredictable variations (45). Furthermore, there are potential side effects when administering large amounts of cytokines and growth factors in vivo to induce DCs.

In the present study we report that a novel self-peptide from ApoE, Ep1.B, can induce monocyte differentiation, resulting in the generation of DCs. This approach to monocyte differentiation offers several advantages over existing protocols to generate monocyte-derived DCs; 1) the peptide is derived from a naturally processed self-peptide (32), 2) Ep1.B is nonimmunogenic, 3) DC differentiation occurs rapidly within 72 h, 4) Ep1.B is relatively inexpensive when compared with the cytokine cocktails currently used to generate DCs, and 5) Ep1.B induces DC differentiation from monocytes in human cells. In this regard, after safety of Ep1.B peptide is proven, its application as an easy and effective way for ex vivo differentiation and maturation of peripheral blood monocytes for DC immunotherapy protocols could be considered.

Ep1.B-induced DCs possess morphological, phenotypical, and functional features of DC populations described in the literature (46). Ep1.B-treated cells display features characteristic of DC morphology, including decreased adherence to plastic, aggregation, and the presence of thin dendrites (Fig. 1A). Following 72 h of culture with Ep1.B, PU5-1.8 cells differentiate into DCs with a mature phenotype (Fig. 2). Notably, the increase in the DC-specific markers CD11c, 33D1, and DEC-205 provides evidence that monocytic PU5-1.8 cells have differentiated into DCs. The fact that there is no observable increase in Mac-1 expression suggests that PU5-1.8 cells are unable to differentiate into macrophage in the presence of Ep1.B, further implying that the peptide results in the generation of DCs.

The effect of Ep1.B has been seen in a various cell lines and primary cells (Figs. 3 and 4). The increase in DC-specific molecules is observed in culture of primary cells such as splenocytes from several mouse strains (Fig. 4) and bone marrow-derived monocytes (data not shown). Ep1.B also induces DC differentiation in the human monocytic cell line U937 (Fig. 3). The DC differentiation effect of Ep1.B on splenocytes could be on immediate precursors of splenic DCs in steady-state, which are not monocytes (9, 10). Contrary to this, monocytes contribute to the production of spleen DCs in inflammatory conditions. As the origin and type of DCs present in spleen and other lymphoid tissues in the steady-state and inflammatory state are different, it would be very interesting to know if Ep1.B administration can contribute to differentiation of DCs in steady-state and not inflammatory conditions. DCs generated *in vitro* with GM-CSF are representatives of inflammatory-derived DCs *in vivo*, while DCs generated in Flt3 ligand-stimulated cultures represent steady-state DCs (47). *In vitro* DC differentiation from human or mouse monocytes is well established. However, there is controversy over whether monocytes are precursors for DCs under physiological conditions (8, 12). Functional significance of *in vivo* DC derivation from monocytes, the extent of differentiation process in steady-state and pathological conditions, and DC subpopulations induced from monocytes are issues to be investigated for the effects of Ep1.B.

The results of functional experiments demonstrate that Ep1.B-induced DCs are rather tolerogenic. Injection of Ep1.B with other antigenic peptides such as OVA makes the responding T cells anergic with the deviation from Th1 cytokine profile (Fig. 5). Furthermore, this finding suggests that Ep1.B could be a potential candidate for generation of monocyte-derived DCs in physiological noninflammatory conditions, and the induction of IL-10 may be the key factor in its functional activity. This is not the first time that self-peptides have been found to play a role in DC differentiation. Two known immunosuppressive neuropeptides, the vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating polypeptide (PACAP), contribute to the development of bone marrow-derived tolerogenic DCs *in vitro* and *in vivo* (48, 49). The induction of tolerogenic DCs is mediated through the VPAC1 receptor and protein kinase A, and it correlates with the inhibition of I κ B phosphorylation and of NF- κ Bp65 nuclear translocation. Similar to the data on VIP, we think Ep1.B binds to an as yet to be determined receptor on the surface of DC progenitors to initiate signaling pathways for DC differentiation.

The receptor for Ep1.B is still unknown to us, but its effect could not be attributed to binding to TLR-4 or its natural ligand, MHC class II. Ep1.B could effectively stimulate differentiation of LPS-resistant splenocytes from C3H/HeJ mice. These mice have a mutation in their TLR-4 receptor gene such that the translated protein is unable to bind the lipid A moiety of LPS. This suggests that the Ep1.B effect on monocyte differentiation toward DC is not mediated by a LPS receptor. As the Ep1.B effect is not abrogated in monocytes from MyD88^{-/-} mice, which are deficient for the MyD88 adapter molecule required for TLR signaling, it is unlikely that the Ep1.B effect is through binding to TLRs and activation of the MyD88 signaling pathway in monocytes. We observed that spleen cells from MHC class II-null mice showed DC differentiation *in vitro* (data not shown), excluding the possibility of Ep1.B binding to MHC class II as a cause for the changes seen.

MAPK signaling cascades are one of the primary means of converting extracellular signals into intracellular responses (50). Our results suggest that Ep1.B can activate the MAPK signaling pathways in the PU5-1.8 cell line (Fig. 7). Of particular interest is the fact that Ep1.B activates ERK and p38. Studies have shown that p38 controls several aspects of DC maturation (51, 52), and ERK

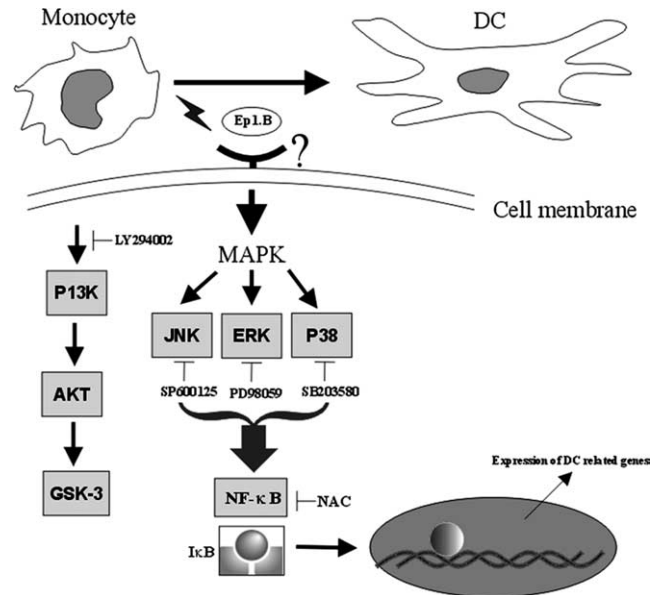


FIGURE 9. A model for monocyte to DC differentiation by Ep1.B. Ep1.B initiates a signal transduction cascade in monocytes, resulting in the activation of MAPK signaling pathways, including ERK1/2, p38, and JNK followed by NF- κ B activation, thereby instructing the differentiation toward DCs. Additionally, Ep1.B activates PI3K/AKT pathway that affects NF- κ B activation. The effect of various inhibitors on blocking individual signaling pathways is also shown.

lies upstream of the transcription factor NF- κ B. These MAPK signaling pathways differentially regulate all aspects of phenotypic maturation, cytokine production, and functional maturation of monocyte-derived DCs (53). Differential activity of p38 and ERK in DCs can induce different Th responses (54). Hence, determination of the ratio of activity of p38 and ERK in early progenitors of DCs treated with Ep1.B can shed light on the possible role of Ep1.B in generation of specific subsets of DCs. The importance of NF- κ B activation in DC differentiation is underscored by the fact that three different agents, which induce CD14⁺ monocyte to DC differentiation, all activate NF- κ B despite differences in the upstream signaling pathways (42). Similarly, in a separate experiment using a potent inhibitor of NF- κ B, NAC, we prevented Ep1.B-induced monocyte differentiation, implying that NF- κ B may be involved in this phenomenon (Fig. 8C). These findings are in accord with other studies demonstrating that inhibiting NF- κ B activation using NAC blocks the maturation of DCs (55, 56).

Also, we found that Akt is phosphorylated during treatment of PU5-1.8 cells with Ep1.B. Akt-1 is activated during proinflammatory stimulation of DC. Constitutively active glycogen synthase kinase (GSK-3) in immature monocyte-derived DCs inhibits expression of maturation markers on immature DCs. Phospho-Akt inhibits activity of GSK-3 and promotes expression of costimulatory molecules on DCs (57, 58). Thus, it seems that phospho-Akt plays a role in the maturation inducing function of Ep1.B rather than its differentiation inducing capability.

Cumulatively, these findings suggest that Ep1.B initiates a signal transduction cascade resulting in the differentiation of monocytes into DCs. As shown in Fig. 9, we propose that Ep1.B induces monocyte differentiation toward DCs through PI3K/AKT and MAPK pathways that leads to activation of NF- κ B and up-regulation of NF- κ B-controlled genes. The model is validated through various inhibitors of signaling cascade. It has been shown that different NF- κ B family members are involved in differentiation of

monocytes into either DCs or macrophages (59). The DC differentiating ability of Ep1.B could be a result of changes in the expression pattern of NF- κ B family members.

In conclusion, Ep1.B is capable of inducing the differentiation of monocytes into DCs. Despite the large body of evidence supporting the use of monocyte-derived DCs for immunotherapy, many issues need to be resolved to allow successful manipulation of the immune system. Recently, we reported the anti-atherogenic activity of Ep1.B in animal models. Ep1.B injection reduces neointimal hyperplasia after vascular surgery in rats and mice. Also, it prevents plaque growth during early plaque progression in ApoE-deficient mice (60, 61). It would be interesting to discover a correlation between the anti-atherogenic effect of Ep1.B and its effect on monocytes. One of the earliest events in atherosclerosis is the entry of monocytes into the arterial intima. These monocytes differentiate into macrophages and start to accumulate large amounts of lipid through the uptake of modified lipoproteins, which results in foam cell formation. Macrophage-derived foam cells in turn stimulate smooth muscle cell migration from the media into the intima, and in this way they contribute to the progression of atherosclerosis (62).

In the recent years, a number of DC subsets have been identified. DCs originating from different precursors in different tissues have different functions: some activate the immune system, while others induce tolerance. Ep1.B offers an alternative agent for immunotherapy through selective induction of DC subsets.

Disclosures

A patent on Ep1.B has been assigned to, and was licensed by, the University of Western Ontario with B. Singh and B. J. Rider as co-inventors. Other authors have no financial interests.

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