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A Toll-Like Receptor 2 Ligand Stimulates Th2 Responses In Vivo, via Induction of Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase and c-Fos in Dendritic Cells

Stephanie Dillon,* Anshu Agrawal,* Thomas Van Dyke,‡ Gary Landreth,§ Laurie McCauley,¶ Amy Koh,¶¶ Charles Maliszewski,¶ Shizuo Akira,# and Bali Pulendran2*†

The adaptive immune system can generate distinct classes of responses, but the mechanisms that determine this are poorly understood. In this study, we demonstrate that different Toll-like receptor (TLR) ligands induce distinct dendritic cell (DC) activation and immune responses in vivo. Thus, Escherichia coli LPS (TLR-4 stimulus), activates DCs to produce abundant IL-12(p70), but little IL-10, and stimulates Th1 and Tc1 responses. In contrast, Pam-3-cys (TLR-2 stimulus) elicits less IL-12(p70), but abundant IL-10, and favors Th2 and T cytotoxic 2 (Tc2) responses. These distinct responses likely occur via differences in extracellular signal-regulated kinase signaling in DCs. Thus, Pam-3-cys induces enhanced extracellular signal-regulated kinase signaling, compared with LPS, resulting in suppressed IL-12(p70) and enhanced IL-10 production, as well as enhanced induction of the transcription factor, c-Fos. Interestingly, DCs from c-fos−/− mice produce more IL-12(p70), but less IL-10, compared with control DCs. Therefore, different TLR ligands induce distinct cytokines and signaling in DCs, and differentially bias Th responses in vivo. The Journal of Immunology, 2004, 172: 4733–4743.

The adaptive immune system has evolved diverse responses to defend the host against a myriad of different pathogens. For example, immune responses against T cell-dependent Ags display staggering heterogeneity with respect to the cytokines made by Th cells and the class of Ab secreted by B cells (1–4). Thus, in response to intracellular microbes, CD4+ Th cells differentiate into Th1 cells, which produce IFN-γ; in contrast, helminths induce the differentiation of Th2 cells, whose cytokines (principally IL-4, IL-5, and IL-10) induce IgE and eosinophil-mediated destruction of the pathogens (1–4). At the individual T cell level, considerable heterogeneity of cytokine profiles can be seen with T cell clones, raising the possibility that the canonical Th1 and Th2 global phenotypes only represent two polar extremes of all possible single cell phenotypes (5).

Although there is much knowledge about the cytokines produced early in the response, and the transcription factors that determine Th polarization in T cells (3, 4), the early “decision-making mechanisms” which result in a given type of immune response are poorly understood. Several recent observations bear on this problem. First, distinct types of dendritic cell (DC)3 subsets can differentially induce Th1 and Th2 responses (6–8). For example, in mice, CD8α+ DCs can elicit Th1 cells, while CD8α− DCs can induce Th2/Th0 cells (6, 7). Second, the dose of Ag can play an important role in influencing the Th1/Th2 balance (9). Third, different microbial stimuli signal through distinct pattern recognition receptors on APCs (10–12). For example, LPS from Escherichia coli signals through Toll-like receptor (TLR)-4 (13), while TLR-2 appears to have a broad spectrum of ligands, including peptidoglycan of Staphylococcus aureus (14), lipoproteins from Mycobacterium tuberculosis (15, 16), and Sacharomyces cerevisiae zymosan (17). Fourth, different microbial stimuli differentially activate DCs to elicit distinct classes of immune responses (18–23). For example, Toxoplasma extracts (24) and E. coli LPS stimulate IL-12(p70) production in CD8α+ DCs and prime a Th1 response (25), and certain viruses induce IFN-α from plasmacytoid DCs and stimulate Th1 responses (26, 27). In contrast, schistosome egg Ags (28), filarial Ags (29), cholera toxin (30), lipoxins stimulated by Toxoplasma (31), certain forms of Candida albicans (32), or highly purified preparations of Porphyromonas gingivalis LPS (25), do not stimulate IL-12(p70), and favor Th2-like responses. Interestingly, a recent report suggests that P. gingivalis LPS signals via TLR-2 in murine macrophages (33). In this context, it is unclear whether signaling through distinct TLRs can shift the balance toward the Th2 phenotype. Although, our recent work with P. gingivalis LPS suggests that signaling through TLR-2 can stimulate Th2-like responses (25), it is not clear whether this is characteristic of all TLR-2 ligands, or whether this is a unique feature of P. gingivalis LPS. If indeed, signaling via different TLRs instructs DCs to elicit distinct Th responses, then the intracellular signaling...
pathways, which mediate such different outcomes, are not known. In this study, we address these questions, using a synthetic TLR-2 ligand, Pam3,cys-Ser-Lys12 (Pam-3-cys) (12), and highly purified preparations of *E. coli* LPS. Our data suggest that signaling via distinct TLR-2 conditions DCs to modulate the Th balance toward the Th2 pathway, via a mechanism involving enhanced induction of extracellular signal-regulated kinase (ERK) and the early growth transcription factor, c-Fos.

### Materials and Methods

#### Mice

* C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Male B6.PL-Thy 1 (B6.PL) mice were purchased from The Jackson Laboratory or were bred at the Rodent Vivarium of the Yerkes National Primate Center (Emory University, Atlanta, GA). B6129F1/Tac (B6129) mice were purchased from Taconic Farms (Germantown, NY). TLR-2 knockout mice (TLR-2 fl/fl) were purchased from Taconic Farms (Germantown, NY) in complete DMEM/H9262. 

* MHC class II followed by labeling with streptavidin allophycocyanin. For staining was performed with phospho-p38 MAP kinase Ab, phospho-p44/42 kinase Ab (Cell Signaling Technology, Beverly, MA). Band visualization was performed with secondary HRP-conjugated Ab (Cell Signaling Technology) and the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

#### In vitro culture

Total spleen cells from TLR-2 knockout or B6129 mice were depleted of RBC by RBC lysis and washed three times. Spleen cells were plated at 2 × 10^6 cells/ml in 48-well culture dishes with various concentrations of *E. coli* LPS, Pam-3-cys or *P. gingivalis* LPS and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 48 h. Culture supernatants were collected and assayed for the presence of IL-6, IL-10, IL-12, IL-17, IL-22, IL-23, and IL-25. Cytokine assays were performed with rat anti-*E. coli* LPS mAb, rat anti-Pam-3-cys mAb or rat anti-*P. gingivalis* LPS mAb from BD PharMingen, and IL-13 was measured by an ELISA kit from BioSource International, Camarillo, CA. Total protein (80 μg) was measured by β-scintillation spectroscopy. For cytokine assays, aliquots of culture supernatants were removed after 90 h, pooled, and assayed for the presence of IL-4, IL-5, IL-13, and IFN-γ by ELISA.

#### Microbial stimuli

* Highly purified *E. coli* LPS (strain 25922) and *P. gingivalis* LPS (A7436) were generous gifts from T.V.D. Pam-3-cys was obtained from G. Jung (Eberhard Karls Universitat Tübingen, Tübingen, Germany) and reconstituted in endotoxin-free water. All Ags were sonicated before use.

* For adoptive transfer studies, age-matched B6.PL recipient mice were given 2.5 × 10^6 of either OT-2 or OT-1 TCR transgenic T cells iv.

#### B6.PL mice reconstituted with OT-2 TCR transgenic T cells were injected i.p. with 50 μg of MHC class II-restricted OVA peptide (ISQVHAA-HAEINEAG; OVA132-143) in PBS alone, or PBS containing either 25 μg of *E. coli* LPS or 50 μg of Pam-3-cys. OT-1 TCR transgenic T cells reconstituted B6.PL mice were injected in a similar fashion except with 50 μg of MHC class I-restricted OVA peptide (SIINFEKL; OVA323-331). The OVA peptides were obtained from BioSynthesis (Lewisville, TX) and from Dr. B. Evavold (Emory University, Atlanta, GA).

* To investigate the effect of TLR ligands on DC in vivo, B6129 or TLR-2 knockout mice were injected with PBS containing either 25 μg of *E. coli* LPS or 50 μg of Pam-3-cys. Six hours later, the spleens were removed and a small portion was digested with Collagenase, Type 4 (1 mg/ml; Worthington Biochemical, Lakewood, NJ) in complete DMEM + 2% FBS for 30 min at 37°C. The RBC were lysed and the cell suspension was washed twice before analysis of cell surface expression of activation markers by flow cytometry.

#### Flow cytometry

* All Abs used were from BD Pharmingen (San Diego, CA). For analysis of activation of DC after injection of TLR ligands in vivo, RBC-lysed, collagenase-digested spleen cells were incubated at 4°C with FITC-conjugated CD11b mAb, PE-conjugated CD11c mAb, and either biotin-labeled CD86 of MHC class II followed by labeling with streptavidin allophycocyanin. For analysis of OT-2 cells, cell suspensions prepared from spleen cells were incubated at 4°C with allophycocyanin-conjugated CD4 and PE-conjugated Thy 1.2. OT-1 cells were analyzed in a similar fashion, but with allophycocyanin-conjugated CD8 and PE-conjugated Thy 1.2 Abs.

#### Purification of DC

* CD11c^+CD11b^- and CD11c^+CD11b+ DC subsets were purified from spleen cell suspensions from mice treated for 9 days with Flt-3 ligand (Immunex, Seattle, WA). Briefly, spleens from Flt-3 ligand-treated mice were dissected, cut into small fragments, and then digested with Collagenase, Type 4 (1 mg/ml) in complete DMEM + 2% FBS for 30 min at 37°C. Cells were washed twice and frozen in FBS + 10% DMSO (Sigma-Aldrich, St. Louis, MO). Thawed cells were washed twice and the CD11c^+DCs were enriched using magnetic beads from Miltenyi Biotec (San Diego, CA). The resulting purity of CD11c^+DCs was ~95%. The enriched DCs were stained with FITC-conjugated CD11c (BD Pharmingen) and PE-conjugated CD11b (BD Pharmingen) and sorted into the CD11c^+CD11b^- and CD11c^+CD11b+ DC subsets using a high speed modular flow cytometer (MoFlo, Cytometry, Fort Collins, CO).

#### CD40 ligand (CD40L) fibroblasts

* 3T3-C57BL6 fibroblasts (Ref. 35; a kind gift of Dr. P. Hwu, National Cancer Institute, Bethesda, MD) were grown in complete DMEM + 10% FBS + 2 mg/ml G418 sulfate. Cells were exposed to trypsin-EDTA and irradiated at 3000 rad (2535 cGy; Gammacell 3000 Elan; MDS Nordion, Ottawa, Ontario, Canada) to prevent further replication. Cells were plated at 4 × 10^4 cells per well in a 48-well plate and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 in air.

#### Western blotting

* CD11c^+DC (1 × 10^6) were stimulated for 20 min or 2 h with either *E. coli* LPS (10 μg/ml) or Pam-3-cys (100 μg/ml), in the presence of an anti-CD40 Ab (BD Pharmingen), and lysed with cell extraction buffer (Immunon Tassay kit; BioSource International, Camarillo, CA). Total protein (80–100 μg) was resolved on 10% SDS-PAGE gels and transferred to Immuno Blot polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Blotting was performed with phospho-p38 MAP kinase Ab, phospho-p44/42 MAP kinase Ab, p38 kinase Ab, or p44/42 kinase Ab (Cell Signaling Technology, Beverly, MA). Bands were visualized with secondary HRP-conjugated Ab (Cell Signaling Technology) and the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).
Recent work from Vogel and coworkers (33) suggests that our recent data suggest that E. coli LPS, but not P. gingivalis LPS, induces IL-12(p70) from the CD11c⁺CD11b⁺ DC subset. Given, the striking differences in immune responses stimulated by E. coli LPS vs P. gingivalis LPS (25), it was important to confirm that our preparation of P. gingivalis LPS did indeed signal through TLR-2. Therefore, we cultured splenic DCs from wild-type and TLR-2⁻/⁻ mice with E. coli LPS, P. gingivalis LPS, and Pam-3-cys for 48 h. Then the supernatants were assayed for IL-6. As shown in Fig. 1, induction of cytokines by P. gingivalis LPS, but not E. coli LPS, is severely impaired in TLR-2⁻/⁻ mice. Consistent with previous reports by Akira et al. (12), cytokine induction by the synthetic molecule Pam-3-cys is impaired in TLR-2⁻/⁻ mice.

We wondered whether the inability of P. gingivalis LPS, to induce IL-12(p70), was also a characteristic of other TLR-2 ligands, or whether it was a unique feature of P. gingivalis LPS. Therefore, splenic DC subsets were isolated, and cultured in vitro, in the presence of a CD40L-expressing fibroblast cell line (39), together with E. coli LPS or Pam-3-cys. Previous work suggests that CD40 cross-linking on DCs is required to elicit optimal IL-12(p70) and IL-10 induction by microbial stimuli (40). The concentrations of E. coli LPS and Pam-3-cys chosen were those which triggered equivalent levels of a proinflammatory cytokine such as IL-6 in both subsets (Fig. 2a). The supernatants from these cultures were harvested after 48 h, and the levels of IL-12(p70), IL-10, and IL-6 in culture determined by ELISA. As shown in Fig. 2a, E. coli LPS induced abundant levels of the Th1-inducing cytokine IL-12(p70), preferentially from the CD11c⁺CD11b⁻ subset, but relatively little IL-10, a cytokine known to favor Th2 responses in mice. In contrast to E. coli LPS, Pam-3-cys induced lower levels of IL-12(p70), but much greater levels of IL-10 from the CD11c⁺CD11b⁺ subset. However, both stimuli induced similar levels of a proinflammatory cytokine such as IL-6 in both DC subsets, suggesting that the doses of stimuli chosen activated the DC subsets to similar extents. The major difference between the stimuli appeared to lie in the differential induction of IL-12(p70) and IL-10. Neither stimulus induced IL-4 in DCs (23). It should also be noted that, the different DC subsets appeared to exhibit some intrinsic bias in the nature of the cytokines they secreted. Thus, consistent with our previous study (25), IL-12(p70) was induced, preferentially, in the CD11c⁺CD11b⁺ DC subset, and IL-10 was induced at higher levels in the CD11c⁺CD11b⁻ subset. In summary, the present data suggest that Pam-3-cys induces lower levels of the Th1-inducing cytokine IL-12(p70), and much higher levels of the Th2-inducing IL-10, than E. coli LPS.

Induction of IL-10 by Pam-3-cys occurs via a mechanism dependent on TLR-2 and MyD88

The induction of high levels of IL-10, a cytokine which induces Th2 or T regulatory cells, by Pam-3-cys was intriguing. It has been suggested that signaling via any of the TLRs results in Th1 cytokines (15, 16, 41), and mice deficient in the TLR adaptor protein MyD88 have been reported to have a selective impairment in Th1 responses (42, 43). Thus, it was important to determine whether IL-10 induction by Pam-3-cys was truly dependent on TLR-2 and MyD88, or whether this was mediated via some other accessory receptor. Splenic DCs were isolated from wild-type, TLR-2, and MyD88-deficient mice, and cultured with the different stimuli in the presence of the CD40L-expressing fibroblasts. As shown in Fig. 2b and c, IL-10 induction by Pam-3-cys is impaired in TLR-2-deficient DCs, as well as in MyD88-deficient DCs. Thus, Pam-3-cys induces IL-10, largely through a mechanism dependent on both TLR-2 and MyD88. Contrary to previous reports, this suggests that TLR-2 and MyD88 can also induce certain Th2 cytokines such as IL-10.

E. coli LPS and Pam-3-cys activate splenic DC11c⁺CD11b⁻ and CD11c⁺CD11b⁺ DC subsets in vivo

We next determined whether TLR-4 and TLR-2 ligands could activate splenic DC subsets in vivo, by injecting the ligands i.p. into wild-type or TLR-2-deficient mice, and examining the microenvironmental localization of DCs, and their expression of costimulatory molecules, 4 or 6 h after injection. As shown in Fig. 3, E. coli LPS and Pam-3-cys induce equivalent up-regulation of CD86 and MHC class II(A-β) on both CD11c⁺CD11b⁻ and CD11c⁺CD11b⁺ DCs in wild-type mice. In TLR-2-deficient mice, the induction of CD86 and A-β by Pam-3-cys was severely impaired, but the effects of E. coli LPS were unaffected. Therefore, the synthetic molecule Pam-3-cys appears to activate DCs in vivo, via TLR-2.

E. coli LPS and Pam-3-cys induce different classes of Ag-specific CD4⁺ T cell responses in vivo

Given the significant differences in the relative levels of IL-12(p70) and IL-10 induced by TLR-4 and TLR-2 ligands, we wondered whether they stimulated different types of CD4⁺ T cell immune responses in vivo. We addressed this question, using OVA-specific, MHC class II-restricted (I-β), αβ TCR transgenic mice (OT-2 mice) (36). In these mice, the CD4⁺ OVA-specific T cells express Vα2 and Vβ5, and recognize the amino acid 323–339 peptide fragment (hereafter denoted as OVA323–339) from OVA. TCR transgenic T cells were adoptively transferred into Thy-1 congenic B6.PL.Thy-1a (B6.PL) mice, such that they constituted a small, but detectable proportion of all T cells (44). In this study, the fate of OVA-specific, transgenic T cells was followed using the Thy-1.2 Ab, which stains the transferred cells, but not the host cells. Cells with the phenotype Thy-1.2⁺CD4⁺Vα2⁺Vβ5⁺ are considered OVA-specific CD4⁺ T cells. In some of the experiments, we simply used Thy-1.2 in combination with CD4, to detect the OVA-specific T cells.

The reconstituted mice were injected with 50 µg of OVA323–339 peptide alone, or OVA323–339 + E. coli LPS, or OVA323–339 + Pam-3-cys i.p. Injection of OVA323–339 alone did not induce any

**FIGURE 1.** Induction of IL-6 by P. gingivalis LPS is impaired in TLR-2⁻/⁻ mice. Total splenic cells from wild-type or TLR-2⁻/⁻ mice were cultured in vitro with various concentrations of E. coli LPS, P. gingivalis LPS, and Pam-3-cys. The total production of IL-6 was measured 48 h later. In the presence of P. gingivalis LPS or Pam-3-cys, spleen cells from TLR-2⁻/⁻ mice had severely reduced levels of IL-6. Representative of three independent experiments.
significant clonal expansion of the CD4⁺Thy-1.2⁺ cells in the spleens of mice (Fig. 4a). However, both *E. coli* LPS and Pam-3-cys significantly enhanced the clonal expansion of CD4⁺Thy-1.2⁺ cells. Previous work has shown that productive T cell immunity is elicited only when the Ag is injected with an adjuvant, and that injections of soluble Ags only result in a transient and abortive clonal expansion, in which Ag-specific T cells cannot be efficiently restimulated in vitro, with protein or peptide (44–46).
Thus, we examined the in vitro proliferative capacity of OVA-specific T cells from the various cohorts of mice, by culturing single cell suspensions of the spleen with varying concentrations of OVA. As shown in Fig. 4b, mice that received OVA323–339 + E. coli LPS or OVA323–339 + Pam-3-cys had greatly enhanced responses, compared with those that received OVA323–339 peptide alone.

Cytokine production by Ag-specific T cells was measured by assaying the culture supernatants from the cultures described above for IFN-γ, IL-4, IL-5, and IL-13. There were significant differences between mice injected with OVA323–339 peptide alone, or OVA323–339 + E. coli LPS, or OVA323–339 + Pam-3-cys (Fig. 4c). In cultures from mice injected with OVA323–339 + E. coli LPS, there were high levels of IFN-γ, and low levels of IL-4 (~8 pg/ml) and IL-5 (~30 pg/ml). In addition, there was a significant level of IL-13. Considering that the sensitivity of the cytokine ELISA is 8 pg/ml (dotted red line, Fig. 4c), the levels of IL-4 and IL-5 induced by E. coli LPS are either below or barely above the threshold of detection, and thus E. coli LPS biases the response toward the Th1 pathway. This Th1 induction by E. coli LPS was dependent on IL-12(p70), because its neutralization, in vivo, with an Ab, largely impaired IFN-γ production (data not shown). However, the induction of significant levels of IL-13, as observed previously (25), suggests that the response induced does not fit the “canonical Th1 profile.” In striking contrast to this response, in cultures from mice injected with OVA323–339 + Pam-3-cys, there were much lower levels of IFN-γ, significantly higher levels of IL-5 (~70 pg/ml) and IL-4 (~30 pg/ml), and similar levels of IL-13 as that induced by E. coli LPS. Although the absolute levels of cytokines induced varied from experiment to experiment, in every experiment Pam-3-cys induced a much greater Th2 bias than LPS. Thus, Pam-3-cys induces a response in which there is a preferential bias toward the Th2 pathway (Fig. 2), consistent with its effective induction in DCs of IL-10, a Th2-inducing cytokine (40). This response is unlikely to represent a T regulatory response, because Pam-3-cys was able to induce significant clonal expansion and in vitro proliferation (Figs. 4 and 5). Thus, although neither stimulus induces canonical Th1 or Th2 responses, they exert strikingly different influences on modulating the Th1/Th2 balance. This is further illustrated by the ratios of Th1:Th2 cytokines induced by the E. coli LPS vs Pam-3-cys (IFN-γ:IL-4, 975 vs 40; IFN-γ:IL-5, 162 vs 18; IFN-γ:IL-13, 4.5 vs 1.2) (data not shown).

### Figure 4

*E. coli* LPS and Pam-3-cys induce different classes of CD4+ T cell responses. B6.PL mice reconstituted with OT-2 TCR transgenic T cells were injected i.p. with class II-restricted OVA peptide, OVA323–339 (50 μg) + *E. coli* LPS (25 μg), OVA323–339 (50 μg) + Pam-3-cys (50 μg), or OVA323–339 alone (50 μg). Four days later, spleens were removed and clonal expansion of OVA323–339-specific T cells was determined (a). Unfractionated spleen cells were rechallenged in vitro with OVA323–339 and proliferation (b) and cytokine production (c) were determined. a. Both *E. coli* LPS and Pam-3-cys induced clonal expansion of OVA323–339-specific CD4+ T cells. Further, the in vitro proliferation capacity of the OVA323–339 T cells was greatly enhanced in spleen cells from mice that had received the TLR ligands compared with mice that received OVA323–339 alone (b). Higher levels of IFN-γ were detected in culture supernatants from mice injected with OVA323–339 + *E. coli* LPS than those from mice which had received OVA323–339 + Pam-3-cys (p < 0.01). In contrast, injections of OVA323–339 + Pam-3-cys induced relatively higher levels of IL-4 and IL-5 (p < 0.01) and similar levels of IL-13 (c). This is further illustrated by the ratios of Th1:Th2 cytokines induced by the *E. coli* LPS vs Pam-3-cys (IFN-γ:IL-4, 975 vs 40; IFN-γ:IL-5, 162 vs 18; IFN-γ:IL-13, 4.5 vs 1.2) (data not shown). Data are representative of four independent experiments.
**FIGURE 5.** *E. coli* LPS and Pam-3-cys induce different classes of CD8⁺ T cell responses. B6.PL (Thy1.1) mice were reconstituted with OT-1 TCR transgenic T cells, and then injected i.p with class I-restricted OVA peptide, OVA257-264 (50 μg) + *E. coli* LPS (25 μg), OVA257-264 (50 μg) + Pam-3-cys (50 μg), or OVA257-264 alone (50 μg). Four days later, spleens were removed and clonal expansion of OVA257-264-specific T cells was determined (a). Unfractionated spleen cells were restimulated in vitro with OVA257-264 and proliferation (b) and cytokine production (c) was determined. a. Both *E. coli* LPS and Pam-3-cys induced clonal expansion of OVA257-264-specific CD8⁺ T cells. Further, the in vitro proliferation capacity of the OVA257-264 T cells was greatly enhanced in spleen cells from mice that had received the TLR ligands compared with mice that received OVA257-264 alone (b). Higher levels of IFN-γ were detected in culture supernatants from mice injected with OVA257-264 + *E. coli* LPS than those from mice which had received OVA257-264 + Pam-3-cys (p < 0.01). In contrast, injections of OVA257-264 + Pam-3-cys induced relatively higher levels of IL-4, IL-5, and IL-13 (p < 0.05; c). This is further illustrated by the ratios of Tc1:Tc2 cytokines induced by the *E. coli* LPS vs Pam-3-cys (IFN-γ:IL-4, 260 vs 28; IFN-γ:IL-5, 685 vs 21 IFN-γ:IL-13, 22 vs 4) (data not shown). Data are representative of three independent experiments.

**E. coli LPS and Pam-3-cys induce distinct types of Ag-specific CD8⁺ T cell responses in vivo**

The propensities of *E. coli* LPS and Pam-3-cys to stimulate different classes of Th responses in vivo prompted us to consider whether these stimuli could induce distinct types of CD8⁺ T cell responses in vivo. We investigated this, using OT-1 mice (H-2Kᵇ-determined). A total of 2.5 × 10⁶ spleen cells from OT-1 mice were transferred into B6.PL (Thy1.2) mice. Cohorts of host mice were injected with either OVA257-264 + *E. coli* LPS or OVA257-264 + Pam-3-cys. Clonal expansion of OVA-specific CD8⁺ T cells (CD8⁺Thy-1.2⁺) was assessed by flow cytometry (Fig. 5a). Both *E. coli* LPS + OVA257-264 and Pam-3-cys + OVA257-264 enhanced the clonal expansion of OVA-specific CD8⁺ T cells.

We then examined the in vitro proliferative capacity of the OVA-specific CD8⁺ T cells from the various cohorts of mice, by culturing single cell suspensions of the spleen with varying concentrations of OVA257-264. As shown in Fig. 5b, mice that received an injection of either *E. coli* LPS + OVA257-264, or Pam-3-cys + OVA257-264 had greatly enhanced responses, compared with those that received OVA257-264 alone.

Cytokine production by Ag-specific T cells was measured by assaying the culture supernatants from the cultures described above for IFN-γ, IL-4, IL-5, and IL-13 (Fig. 5c). There were significant differences between mice injected with OVA257-264 peptide alone, or OVA257-264 + *E. coli* LPS, or OVA257-264 + Pam-3-cys. In cultures from mice injected with OVA233-339 peptide alone, there was little, if any, IFN-γ, IL-4, IL-5, or IL-13. In contrast, and consistent with previous reports (44, 45), in cultures from mice injected with OVA257-264 + *E. coli* LPS, there were high levels of IFN-γ, and much lower levels of IL-4, IL-5, and IL-13. Thus, *E. coli* LPS appears to skew the T cytotoxic (Tc) balance toward the Tc1 pathway. However, compared with the cultures from the mice injected with *E. coli* LPS, in cultures from mice injected with OVA257-264 + Pam-3-cys, there were lower levels of IFN-γ, but higher levels of IL-4, IL-5, and IL-13. Therefore, Pam-3-cys appears to shift the balance toward the Tc2 pathway. This is further illustrated by the ratios of Tc1:Tc2 cytokines induced by...
the *E. coli* LPS vs Pam-3-cys (IFN-γ:IL-4, 260 vs 28; IFN-γ:IL-5, 685 vs 21; IFN-γ:IL-13, 22 vs 4) (data not shown).

**Pam-3-cys induces enhanced phosphorylation of ERK1/2, which suppress IL-12(p70) and promote IL-10 in DCs**

To gain insights into the potential intracellular signaling mechanisms which may mediate the different DC responses, we focused on the MAP-kinase signaling pathway, one of the most ancient signal transduction pathways in mammalian cells (47–49). MAP kinases consist of three major groups: p38 MAP kinases, the ERKs (ERK1 and 2), and the c-Jun NH₂-terminal kinases 1 and 2. Previous reports indicate a critical role for MAP kinases in regulating Th1/Th2 balance in T cells (47), and emerging evidence suggests a role for these proteins in regulating cytokine production from APCs (50, 51). Therefore, we sought to determine the phosphorylation of p38 and ERK1/2 in DCs stimulated with various stimuli. As shown in Fig. 6, and consistent with previous studies (52, 53), both *E. coli* LPS and Pam-3-cys induced the phosphorylation of

**FIGURE 6.** *E. coli* LPS and Pam-3-cys induce different levels of phosphorylation of ERK1/2 MAP kinase, which regulates the induction of IL-12(p70) and IL-10. a, CD11c⁺-enriched DCs were incubated with *E. coli* LPS or Pam-3-cys for 20 or 120 min in the presence of an anti-CD40 agonistic Ab. The cells were lysed and levels of phosphorylated and total p38 and p44/42 (ERK1/2) were determined by Western blot analyses. Even with CD40 ligation alone, there was significant p38 phosphorylation, consistent with previous reports (54, 55). Both *E. coli* LPS and Pam-3-cys induced equivalent phosphorylation of p38. However, the magnitude of phosphorylation of p44/42 induced by Pam-3-cys was substantially higher compared with *E. coli* LPS induced phosphorylation. b, CD11c⁺ DCs from ERK1⁻/⁻ mice were cultured with either *E. coli* LPS, or Pam-3-cys, in the presence of the CD40L-expressing fibroblasts. ERK1⁻/⁻ DCs produced significantly lower levels of IL-10, compared with wild-type controls (p < 0.05). In contrast, the induction of IL-12(p70) by *E. coli* LPS was significantly enhanced in ERK1⁻/⁻ DCs stimulated with LPS (p < 0.01). Data are representative of three independent experiments. c, Inhibition of ERK1/2 signaling results in a decrease in IL-10 production from both *E. coli* LPS-stimulated DC and Pam-3-cys-stimulated DC, and an increase in IL-12(p70) production by DC stimulated with *E. coli* LPS. The MEK (MAPK/ERK kinase) 1/2 inhibitor, UO126 (10 μM), was added to CD11c⁺-enriched DC before the addition of the TLR ligands and culturing with CD40L-expressing fibroblasts. The production of cytokines was measured 24 h later. Data are representative of three independent experiments.
p38, but DCs cultured with anti-CD40 Ab alone also induced significant p38 phosphorylation, which is consistent with previous reports (54, 55). Blocking the activity of p38 with a synthetic inhibitor resulted in a profound inhibition of IL-12(p70) and IL-6, and to a lesser extent IL-10 (data not shown).

In the case of ERK1/2, the magnitude of phosphorylation by Pam-3-cys was significantly greater with LPS. At 20 min, the intensity values of the phospho-ERK1/2 band for DC alone, E. coli LPS, and Pam-3-cys are 23, 58, and 164, respectively, confirming that while both stimuli induced phospho-ERK1/2, Pam-3-cys induced it to a much greater degree. This was not a kinetic effect, because E. coli LPS did not induce stronger ERK activity at earlier time points. What effect does ERK1/2 phosphorylation have on the induction of IL-12(p70) and IL-10 in DCs? We investigated this, using highly selective, synthetic inhibitors of ERK1/2 (51) as well as DCs from ERK1-deficient mice (35). Unfortunately, it was not possible for us to study ERK2−/− mice, as such mice are not viable. DCs from ERK1−/− mice appeared to develop normally, and appeared to display normal viability (data not shown). As indicated in Fig. 6b, abrogation of ERK signaling results in significantly enhanced induction of IL-12(p70) by E. coli LPS, but not by Pam-3-cys. In contrast, the induction of IL-10 by both E. coli LPS and Pam-3-cys was significantly reduced in DCs from ERK1−/− mice (Fig. 6b). Notably, the induction of IL-6, a surrogate marker of DC viability and function, was unaffected by the lack of ERK1. Similar results were obtained using synthetic inhibitors of ERK1 and 2 signaling, although in this case, the diminution of IL-10 production in inhibitor treated DCs was markedly more pronounced, than in the ERK1−/− DCs (Fig. 6c); furthermore, the LPS induced enhancement of IL-12(p70) was also more marked (Fig. 6c). The failure to observe significant enhancement of the induction of IL-12(p70) by Pam-3-cys either suggests that the inhibitors of ERK1 and 2 only block incompletely, or that there is an additional ERK-independent pathway that suppresses IL-12(p70), which is induced by Pam-3-cys signaling via TLR-2. Nevertheless, the data do suggest that signaling via ERK1/2 in DCs is critical in regulating the production of IL-12(p70) and IL-10, and thus influencing the Th1/Th2 balance.

**Pam-3-cys enhances DC expression of the immediate early gene product c-Fos, which regulates the production of IL-12(p70) and IL-10**

How does the enhanced ERK1/2 signaling induced by Pam-3-cys result in suppression of IL-12(p70), and enhanced IL-10? Recent work (56), suggests that enhanced ERK signaling results in the phosphorylation and stabilization of the immediate early gene product c-Fos, in a fibroblast cell line. In fact, ERK phosphorylation appears to be essential for enhanced c-Fos expression (56). Thus, we determined expression of c-Fos, in DCs stimulated with the different stimuli, using an Ab directed against c-Fos. As shown in Fig. 7a, the level of expression of c-Fos (as assessed by the mean fluorescence intensity of staining) in DCs stimulated by Pam-3-cys is greater than in DCs stimulated with E. coli LPS, or in unstimulated DCs. Furthermore, c-Fos expression was maintained, even at 45 min poststimulation, in DCs stimulated with Pam-3-cys, but not with E. coli LPS. The mean fluorescence intensities of the levels of c-Fos expression, at 20 min for DCs cultured alone, or with LPS, or Pam-3-cys are 14.8, 8.5, and 17.1, respectively. At 45 min, the corresponding values are 10.1, 10.8, and 19.1, respectively (data not shown). Similar results were obtained with the Western blotting technique to detect c-Fos (data not shown). Therefore, stimulation of DCs by Pam-3-cys, which induce enhanced ERK1/2 signaling, results in increased c-Fos expression. It should be noted that even unstimulated DCs, or DCs stimulated with E. coli LPS, do express levels of c-Fos that are greater relative to the isotype control. However, the levels induced

**FIGURE 7.** Pam-3-cys induces enhanced DC expression of c-Fos, a critical regulator of IL-12(p70) and IL-10. a, CD11c+−enriched DCs were incubated with E. coli LPS or Pam-3-cys for 20 or 45 min. The cells were lysed and levels of c-Fos expression were determined by intracellular staining, followed by flow cytometry. The level of c-Fos expression induced by Pam-3-cys was substantially higher compared with that induced by E. coli LPS. The mean fluorescence intensities of the levels of c-Fos expression, at 20 min for DCs cultured alone, or with LPS, or Pam-3-cys are 14.8, 8.5, and 17.1, respectively. At 45 min, the corresponding values are 10.1, 10.8, and 19.1, respectively (data not shown). Similar results were obtained with the Western blotting technique to detect c-Fos (data not shown). b, DCs from c-<sup>fos</sup>−/− mice secrete much higher levels of IL-12(p70), and much lower levels of IL-10, compared with DCs from wild-type mice. Levels of IL-6 are unaffected. Representative of data from four different experiments.
FIGURE 8. A model for signaling networks involved in Th1/Th2 decision making by DCs. TLR-4 ligands induce potent p38 MAP kinase activation, and less ERK activation. p38 is critical for the induction of IL-12(p70), and to a lesser extent IL-10. In contrast, Pam-3-cys, a TLR-2 ligand induces a higher threshold of ERK1/2 signaling, which results in the stabilization of the transcription factor c-Fos, which potently suppresses IL-12(p70), and enhances IL-10, thus favoring a Th2 bias. Note that both stimuli induce ERK and c-Fos, though Pam-3-cys does so at higher thresholds, and furthermore c-Fos is also likely to be stabilized by other ERK-independent networks. Also, note that ultimately, the responses represent a bias toward the opposite ends of the Th1/Th2 spectrum, rather than canonical Th1 or Th2 responses.

What role, if any, does c-Fos play in the regulation of IL-12(p70) and IL-10? We addressed this using DCs isolated from c-fos<sup>b<sup>−/−</sup></sup> mice (38, 57). c-fos<sup>b<sup>−/−</sup></sup> mice die within 3–4 wk of birth, but DC development appeared to be normal in these mice (data not shown). Age and sex-matched littermate controls were used as negative controls. As indicated in Fig. 7b, DCs from c-fos<sup>b<sup>−/−</sup></sup> mice secrete much higher levels of IL-12(p70), and much lower levels of IL-10, compared with DCs from wild-type mice, in response to any of the stimuli tested. However, the levels of IL-6 do not appear to be affected by c-Fos. Therefore, c-Fos expression in DCs appears to play a fundamental and very specific role in regulating the production of IL-12(p70) and IL-10, cytokines which stimulate Th1 and Th2 responses, respectively.

Discussion

The present data suggest that distinct TLR ligands can activate DCs differently to elicit distinct signaling pathways and cytokine profiles, which regulate the Th1/Th2 balance. There is now emerging evidence that signaling via different TLRs can yield distinct functional responses. For example, a recent study suggests that activating murine macrophages in vitro with various TLR-2 ligands induced TNF-α secretion, whereas only LPS was capable of inducing IL-1β and NO secretion (52), although the functional significance of this difference in IL-1 and TNF production is not clear. Second, a study by Re and Strominger (53) suggests that activating human monocyte-derived DCs with different TLR agonists induces distinct cytokines, but the consequences that signaling of these different cytokines on adaptive immunity, or the signaling mechanisms which elicit the production of the different cytokines are not known. Third, our data suggests that highly purified P. gingivalis LPS, a TLR-2 ligand (33), fails to induce IL-12(p70) in murine DCs, but rather induces Th2 responses (25). This has recently also been shown to be the case with human monocyte-derived DCs (58). Fourth, it has recently been shown that triggering TLR-4, but not TLR-2, signaling mediates IFN-β-induced STAT1αβ-dependent gene expression in macrophages (59). Finally, a recent report suggests that triggering human monocyte-derived DCs or plasmacytoid DCs through TLR-7 results in differential induction of IL-12 and IFN-α (60).

Despite this emerging picture, knowledge about the signaling mechanisms which mediate such diverse functional responses is sparse. Furthermore, the consequences that signaling through different TLRs may have on the Th1/Th2 balance is not known. In this regard, our present observations offer some novel perspectives (Fig. 8). First, the data suggest that activation of innate immune cells via TLRs do not always result in polarized Th1 responses (15, 16, 41, 61), but can also bias the response toward the Th2 pathway. Although the Th1/Th2 paradigm has yielded insights of profound importance, emerging evidence suggests this framework may be an oversimplification of the complexity that underlies an immune response. For example, individual T cells display a rather complex spectrum of cytokine profiles, in which the canonical Th1 and Th2 cells represent only the very extreme ends of an axis (5). In this context, it must be emphasized that our data suggests that neither stimulus induces a typical Th1 or Th2 response. Rather, each stimulus appears to modulate the response toward opposite ends of the Th1/Th2 spectrum (Fig. 8). The notion that activating DCs via TLR-2 may bias toward Th2 responses is further supported by our recent data that other TLR-2 ligands such as peptidoglycan and zymosan, also induce abundant levels of IL-10, and little IL-12(p70), in DCs and favor a Th2 bias (data not shown). However, because these ligands also signal through certain other receptors (NOD1 in the case of peptidoglycan (62, 63), and dectin in the case of zymosan (64)), the interpretation of these results must be approached with caution.

The second perspective offered by the present data is a mechanism involving differential triggering of ERK MAP kinase, which may in part mediate the distinct DC responses triggered by the different TLR ligands. Here too, it cannot be overstated that the functional responses to DCs against different stimuli are likely to be far more plastic and complex (65, 66) than what has been proposed in the strictly linear “DC1-DC2” model. Indeed, it is very possible that this plasticity may well occur at the TLR level itself—thus, although different TLRs may have intrinsic signaling differences in certain cell types, such differences might be greatly influenced by the lineage origin of the cells, and the local microenvironment. Finally, the data highlight fundamental differences in the induction of...
the early growth transcription factor c-Fos, which is phosphorylated and stabilized by prolonged ERK1/2 signaling (56). Given the existence of “AP-1” transcription factor binding sites in the promoter region of the IL-12(p40) gene (67), it is likely that members of the AP-1 family, including fos-jun or jun-jun complexes, play critical roles in regulating IL-12. Indeed, our data suggest that inhibition of c-Fos results in strikingly enhanced IL-12(p70), and reduced IL-10 in DCs (Fig. 7).

It is interesting to consider the proximal signaling molecules that might mediate the distinct DC responses induced by TLR-4 and TLR-2 ligands. To date, TLR-2 is known to associate with two adaptor proteins, MyD88 and TIRAP (12, 68, 69), while TLR-4 is largely restricted to CD14+ DCs (Fig. 7).

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Furthermore, the data underscore novel therapeutic opportunities that will be gained by modulating critical parameters (e.g., TLRs, MAP kinases, transcription factors) in the immune therapy of cancer, allergy, autoimmunity, and transplantation.

References


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