

CUTTING EDGE

Cutting Edge: Dendritic Cells Copulsed with Microbial and Helminth Antigens Undergo Modified Maturation, Segregate the Antigens to Distinct Intracellular Compartments, and Concurrently Induce Microbe-Specific Th1 and Helminth-Specific Th2 Responses¹

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*To examine the ability of dendritic cells (DC) to discriminate between helminth and microbial Ag and induce appropriately polarized Th responses, mouse DC were copulsed with the helminth Ag, schistosome egg Ag (SEA), along with the bacterium *Propionibacterium acnes*, Pa, and transferred into wild-type mice. Strikingly, SEA/Pa-copulsed DC induced concurrent Pa-specific Th1 (but not Th2) responses and SEA-specific Th2 (but not Th1) responses. Although DC exposed to both Ag undergo many of the maturation-associated changes that accompany exposure to Pa alone, Pa-induced IL-12 production was inhibited by SEA. Examination of Ag uptake revealed that SEA and Pa are acquired via discrete pathways and enter non-overlapping intracellular compartments. Data suggest that segregation of SEA and Pa into distinct compartments, coupled with SEA-induced modifications of the DC maturation pathway, are significant components of the ability of DC to interpret signals inherent to SEA and Pa and induce appropriately polarized Th responses. *The Journal of Immunology*, 2004, 172: 2016–2020.*

In previous reports, we have shown that dendritic cells (DC)⁴ that have been exposed to *Propionibacterium acnes* (Pa) induce Th1 responses when injected into naive mice, whereas DC pulsed with *Schistosoma mansoni* egg Ag (SEA) induce Th2 responses (1). These polar opposite Th responses mimic the dominant responses seen during infection with these bacterial and helminth pathogens. The accepted model for Th1 response induction is that DC are activated by pathogens via

Toll-like receptor (TLR) ligation to produce IL-12 (2, 3), a cytokine of known importance for Th1 response development (4). However, there is evidence that DC-associated factors other than IL-12 also play a significant role in Th1 response initiation (5, 6). Less is known of how DC polarize Th2 responses (3, 7). The fact that many Th1 response-inducing Ag strongly activate DC via TLR engagement, whereas Ag that inherently induce Th2 responses appear to fail to do so (1, 3, 7), provides reason to believe that, independently of their ability to produce IL-12, the relative maturation status of DC may be related to the type of T cell response they induce. Indeed, there is a view that Th2 responses reflect a default that occurs when Ag fail to induce DC maturation (3, 7). In addition to differences in IL-12 production associated with DC activation, the maturation status of these cells could be influential in Th1/Th2 induction because of the known sensitivity of this Th differentiation pathway to Ag dose (8) and the recognized association of DC maturation with increased Ag processing and peptide presentation (9).

In this study, we investigate the ability of DC exposed to a combination of SEA plus Pa to mature and generate SEA- and Pa-specific Th responses in vivo. Interestingly we found that: 1) these copulsed DC were capable of inducing non-overlapping Th1 and Th2 responses to Pa and SEA, respectively; 2) SEA inhibits the ability of Pa to induce IL-12 production by DC; and 3) SEA and Pa are segregated into distinct intracellular compartments. These results suggest that differential processing of SEA and Pa, along with SEA-mediated modifications of the Pa-induced DC maturation pathway, explain the ability of DC to interpret signals inherent to SEA and Pa and to simultaneously induce Th responses appropriate to each Ag.

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⁴ Abbreviations used in this paper: DC, dendritic cell; Pa, *Propionibacterium acnes*; SEA, schistosome egg Ag; TLR, Toll-like receptor; Tf, transferrin; TFR, Tf receptor; LAMP2, lysosome-associated membrane protein 2; MHCII, MHC class II.

Materials and Methods

Animals and Ags

Female wild-type C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Pa was obtained from The Van Kampen Group (Hoover, AL) and endotoxin-free SEA was prepared as described previously (1, 10).

DC preparation

DC were cultured from bone marrow in the presence of GM-CSF (PeproTech, Rocky Hill, NJ) as described elsewhere (1, 11). For activation, DC were pulsed with 50 $\mu\text{g/ml}$ SEA, 10 or 50 $\mu\text{g/ml}$ Pa, or a mixture of these Ag at these concentrations for the final 18 h of culture.

Confocal microscopy and flow cytometry.

SEA was labeled with Alexa Fluor 647 (Molecular Probes, Eugene, OR) and filter-sterilized before use. Pa was stained with Syto-9, using the Live/Dead BacLight Bacterial Viability kit (Molecular Probes). Alexa Fluor 488 and 594 transferrin (Tf) conjugates (Molecular Probes) were used at 25 $\mu\text{g/ml}$. FITC-labeled rat anti-lysosome-associated membrane protein 2 (LAMP2) and unlabeled rat anti-LAMP2 Ab (BD PharMingen, San Diego, CA) were used at 2.5 $\mu\text{g/ml}$. Alexa Fluor 546-labeled goat anti-rat IgG (Molecular Probes) was used at 1 $\mu\text{g/ml}$. For microscopy, DC were plated in eight-well chamber slides (Lab-Tek, Naperville, IL) and cultured for 2 or 18 h in the presence of SEA, Pa, or a mix of both Ag. For Tf staining, DC were incubated at 37°C for 1.5 h with SEA, Pa, or both Ag, washed twice with, and resuspended in, phenol red and FCS-free RPMI (RPMI*), and pulsed with labeled Tf for 0.5 h at 37°C. After two RPMI* washes, cells were fixed in 3% paraformaldehyde in PBS and mounted in Fluoromount G (Electron Microscopy Sciences, Fort Washington, PA). For Ab staining, cells were fixed in paraformaldehyde in PBS for 20 min at room temperature and permeabilized by incubation in PBS, 0.075% saponin, 1.5% FCS plus the relevant Ab, for 45 min at room temperature. Using the same buffer, cells were subsequently washed, labeled with secondary Ab in PBS, washed again, mounted in Fluoromount G (Electron Microscopy Sciences), and analyzed using a Nikon Eclipse E-600 microscope/Bio-Rad (Hercules, CA) confocal system. Each image reported represents the computer-generated coalescence of consecutive sections collected at 0.5- μm interval Z-stacks throughout individual cells. Flow cytometric analysis of DC was performed using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) with CellQuest (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) software.

Determination of DC maturation state and Th response-priming ability

Expression of surface molecules on DC was quantified by flow cytometry using FITC-, allophycocyanin-, or PE-conjugated mAb specific for CD11c, MHC class II (MHCII), and CD80 (BD PharMingen). Cytokine ELISAs were performed on 18-h culture supernatants using paired mAb in combination with recombinant cytokine standards (BD PharMingen) as described previously (1).

Mice were injected i.p. with 5×10^5 DC that had been pulsed with SEA, Pa, both Ag (SEA/Pa), or neither Ag, as described elsewhere (1). Splenic T cell responses were measured by restimulating splenocytes with Ag and quantitating IL-5 or IFN- γ in 72-h culture supernatants using ELISAs (1).

Results

Since maturation has been associated with the ability of DC to induce Th1 responses, we assessed surface expression levels of MHCII and CD80 in the Ag-pulsed cells to determine whether the SEA/Pa-copulsed DC attained a phenotype more similar to that of DC pulsed with SEA or with Pa. As expected (1), Pa alone was found to induce up-regulation of surface expression of these markers, whereas SEA had little effect (Fig. 1A). In the DC pulsed with both Ag, SEA had no discernable effect, with the majority DC expressing high levels of the two markers (Fig. 1A). In light of the recent report that SEA can inhibit the ability of LPS to stimulate IL-12 production by DC (12), we questioned whether SEA suppresses the IL-12 production induced by Pa. As anticipated from previous findings (1), Pa, but not SEA, stimulation resulted in the production of IL-12p40/IL-12p70 (Fig. 1, B and C). However, the addition of SEA to Pa led to significant inhibition of IL-12 production (Fig. 1, B and C). These data were confirmed by real-time RT-PCR for IL-12p40 and p35 mRNA (data not shown). Thus, SEA was able to significantly inhibit certain aspects of Pa-induced maturation.

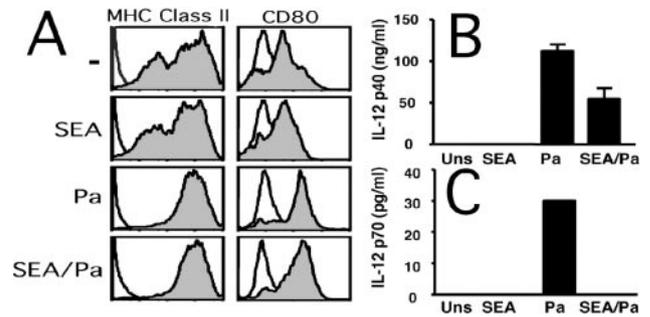


FIGURE 1. SEA does not inhibit Pa-induced increases in expression of maturation markers, but does suppress Pa-induced IL-12 production. *A*, Expression of MHC II and CD80 on DC maintained in medium alone (–) or pulsed with SEA, Pa, or SEA/Pa. DC exposed to SEA remained immature, whereas those pulsed with Pa or with SEA/Pa up-regulated expression of MHCII and CD80. Specific staining, filled histograms; isotype controls, open histograms. *B* and *C*, DC were unstimulated (Uns) or pulsed with SEA, Pa, or SEA/Pa, after which IL-12p40 (*B*) and IL-12p70 (*C*) levels in culture supernatants were measured. SEA did not induce IL-12 production and significantly inhibited ($p < 0.01$ in both cases; Student's *t* test) the ability of Pa to induce the production of this cytokine. Data points represent mean values \pm SD of multiple samples. Data from individual experiment are shown; the experiments were repeated five times with similar results.

To examine the Th responses induced by SEA/Pa-pulsed DC, we injected mice with DC that had been pulsed with SEA alone, Pa alone, or with both Ag. Seven days later, we restimulated splenocytes from these mice with SEA or Pa and measured the production of IFN- γ and IL-5. As expected (1), Pa-pulsed DC induced a Th1 response (Fig. 2A) and SEA-pulsed DC induced a Th2 response (Fig. 2B). Unexpectedly, DC pulsed with both Ag induced concurrent Pa-specific Th1 and SEA-specific Th2 responses (Fig. 2). There was no evidence of a Pa-specific Th2 response or SEA-specific Th1 response in animals that had received SEA/Pa-pulsed DC (Fig. 2). However, the intensity of the Th2 response induced by the copulsed DC was consistently diminished compared with that induced by DC pulsed with SEA alone (Fig. 2B); this reduction was not observed when mice were injected with a 1:1 mixture of DC pulsed with Pa alone

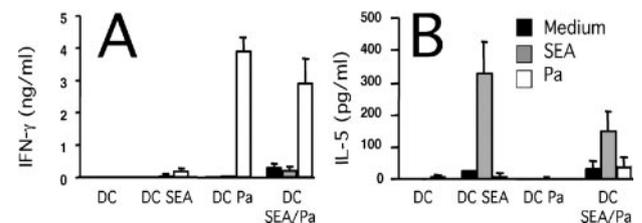


FIGURE 2. DC simultaneously induce Th1 and Th2 responses to distinct Ag. DC in medium alone or pulsed with SEA, Pa, or SEA/Pa were washed and injected into naive mice. One wk later, splenocytes were recovered and cultured in vitro with medium alone or with SEA or Pa. IFN- γ (*A*) and IL-5 (*B*), as indicators of Th1 and Th2 responses, respectively, were measured in 72-h culture supernatants. DC pulsed with Pa alone induced a Pa-specific Th1 response (*A*), but did not induce a Pa-specific Th2 response (*B*). In contrast, SEA-pulsed DC induced a SEA-specific Th2 (*B*), but not Th1 response (*A*). DC pulsed with both Ag (SEA/Pa) induced a SEA-specific Th2 response, albeit at a significantly lower ($p = 0.013$ by Student's *t* test) magnitude than that induced by DC pulsed with SEA alone (*B*), and also induced a Pa-specific Th1 response (*A*). However, the SEA/Pa-pulsed DC did not induce a SEA-specific Th1 or Pa-specific Th2 response (*A* and *B*). Data shown are from one experiment and are representative of five separate experiments.

and DC pulsed with SEA alone (data not shown), indicating that it was related to coacquisition of both Ag by DC.

Although we envisage that DC derived from bone marrow represent cells of a single lineage, it remained a possibility that the reason that populations of SEA/Pa-copulsed DC induce a Pa-specific Th1- and SEA-specific Th2 response is that the Ag are preferentially acquired by different subpopulations of DC. To examine this issue, we pulsed DC with fluorochrome-labeled Ag to determine whether individual DC simultaneously acquire both SEA and Pa. Flow cytometric analysis revealed that all DC within a population are capable of acquiring Pa or SEA when exposed to the individual Ag (Fig. 3*A*). Moreover, when incubated with SEA plus Pa, the majority of DC acquire both Ag (Fig. 3*A*).

We used confocal microscopy to carefully track SEA and Pa uptake by individual cells. Visualization of DC exposed to SEA plus Pa 18 h previously confirmed that individual cells are positive for both Ag (Fig. 3*B*, *a–c*). This analysis additionally showed that the two Ag segregate to distinct intracellular compartments (Fig. 3*Bc*), raising the possibility that SEA and Pa are handled very differently by DC. To begin to examine this issue, we asked whether SEA or Pa are taken up via the Tf receptor (TfR) pathway through which Tf is endocytosed in clathrin-coated pits and traffics through acidic endosomes (13). Using fluorochrome-labeled Tf to follow TfR internalization, we found SEA to be located within a Tf-negative compartment

(Fig. 3*B*, *d–f*), whereas Pa colocalized with Tf within DC (Fig. 3*B*, *g–i*). Similar results were obtained when DC were copulsed with SEA plus Pa (data not show). Next, we used colocalization with LAMP2 to ask whether SEA and Pa enter Ag-processing compartments (14). We found that in DC pulsed with SEA alone, Ag was sequestered to LAMP2-negative or -dull compartments (Fig. 3*B*, *j–l*). The addition of Pa to SEA resulted in a shift of all SEA to a LAMP2-dull compartment (Fig. 3*B*, *m–o*). In contrast, much of the Pa internalized in the absence of SEA markedly colocalized to a LAMP2-positive compartment (Fig. 3*B*, *p–r*), and the addition of SEA had little effect on this localization pattern (Fig. 3*B*, *s–u*). Thus, SEA and Pa enter DC via different pathways and localize to nonoverlapping intracellular sites.

Since DC maturation signals induce activation of lysosomal function and accumulation of peptide-MHCII complexes (15) and increased Ag dose favors Th1 response development, we examined whether increasing the strength of the Pa maturation signal given to copulsed DC from 10 to 50 $\mu\text{g/ml}$, while retaining the SEA concentration at 50 $\mu\text{g/ml}$, would change the pattern of Ag localization and/or the ability of the DC to induce a SEA-specific Th2 response. Compared with DC copulsed with SEA/Pa (10 $\mu\text{g/ml}$), DC copulsed with SEA/Pa (50 $\mu\text{g/ml}$) exhibited greater increases in surface expression of MHCII and CD80 (data not shown), indicating that they were more mature. In contrast to the situation in copulsed DC exposed to the

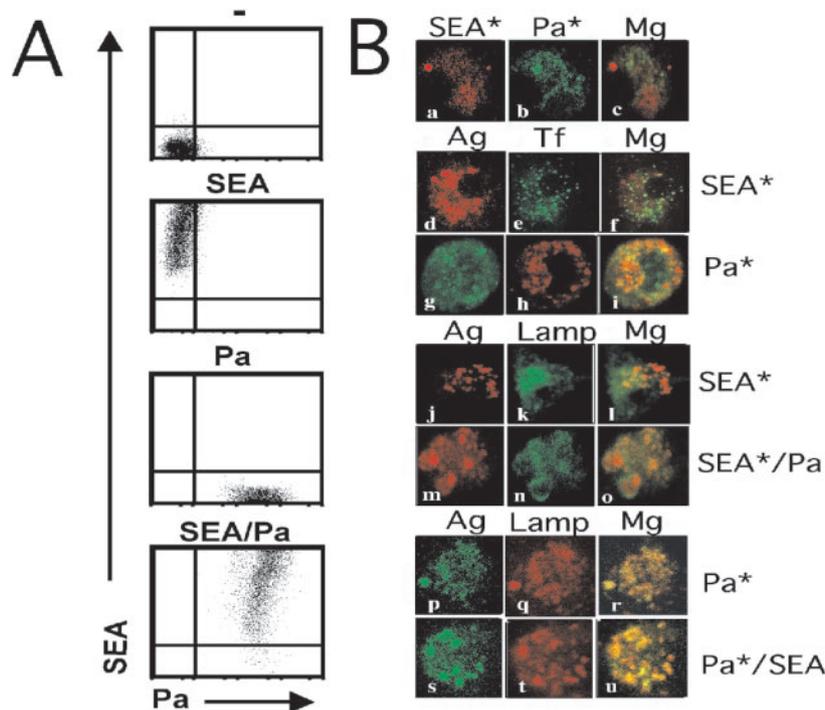
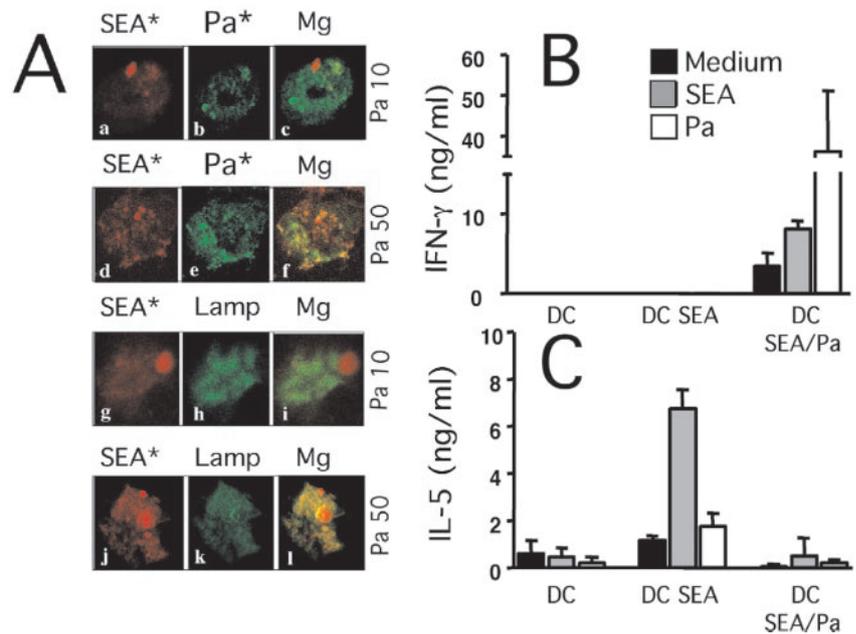


FIGURE 3. Individual DC simultaneously exposed to SEA and Pa acquire both Ag, but via distinct pathways and into nonoverlapping intracellular compartments. *A*, DC were grown in medium alone (–) or pulsed with Alexa Fluor-SEA, Syto9-Pa, or both Ag and were analyzed by flow cytometry. Dot plots show data from gated CD11c⁺ populations. *B*, Localization of SEA and Pa within DC. DC were pulsed with Alexa Fluor-SEA (*a*) and Syto9-Pa (*b*) and visualized by confocal microscopy. As indicated in *A*, individual cells acquired both Ag. Merged (Mg) images show sequestration of Ag into different compartments (*c*). Alexa Fluor-SEA (*d*)- and Syto9-Pa (*g*)-pulsed DC were allowed to endocytose Tf labeled with Alexa Fluor 488 (*e*) or 546 (*h*) for 0.5 h. Although SEA (red) and Tf (green) entered separate compartments (*f*), Pa (green) and Tf (red) entered the same compartment (*i*), evidenced by yellow/orange stain. DC pulsed with Alexa Fluor-SEA in the absence (*j*) or presence (*m*) of unlabeled Pa for 18 h were stained with Ab specific for LAMP2 (*k* and *n*). In the complementary experiment, DC pulsed with Syto9-Pa in the absence (*p*) or presence (*s*) of unlabeled SEA, for 18 h, were also stained with Ab specific for LAMP2 (*q* and *t*). Merged images of DC pulsed with SEA alone show that Ag and LAMP2 were mostly in separate compartments (*l*), but that the addition of Pa led to the localization of all SEA into a LAMP2-dull compartment (*o*). In DC pulsed with Pa alone, intense yellow indicates colocalization of Pa and LAMP2 (*r*) and the addition of SEA had no effect on this pattern (*u*). Asterisk indicates labeled Ag. Each image is representative of observations on >10 individual cells per experiment. The study was performed three times with similar results.

FIGURE 4. In DC exposed to SEA plus a high concentration of Pa (50 $\mu\text{g/ml}$), SEA and Pa colocalize into LAMP2-positive compartments. *A*, DC were pulsed with Alexa Fluor-SEA (*a* and *d*) plus Syto-9-Pa at 10 or 50 $\mu\text{g/ml}$ (*b* and *e*). Merged (*Mg*) images show that at a low Pa concentration (10 $\mu\text{g/ml}$), SEA and Pa are sequestered in different compartments (*c*), whereas at a high Pa concentration (50 $\mu\text{g/ml}$), the Ag are colocalized (*f*). DC pulsed for 18 h with Alexa Fluor-SEA (*g* and *j*) in the presence of unlabeled Pa (10 or 50 $\mu\text{g/ml}$) were stained with Ab for LAMP2 (*h* and *k*, respectively). Merged images show that in DC exposed to 10 $\mu\text{g/ml}$ Pa, SEA and LAMP2 are in different compartments (*i*), whereas in the presence of 50 $\mu\text{g/ml}$ Pa, SEA and LAMP2 colocalize (*l*). *B* and *C*, DC copulsed with SEA plus a high concentration of Pa (50 $\mu\text{g/ml}$) induce strong Pa-specific and weak SEA-specific Th1 responses and induce only weak SEA-specific Th2 responses. DC pulsed with medium alone, SEA, or SEA/Pa were injected into naive mice. After 7 days, splenocytes from injected mice were restimulated in the absence or presence of SEA or Pa. IFN- γ (*B*) and IL-5 (*C*) were measured in culture supernatants. The data shown are from one of two experiments that gave similar results.



low concentration of Pa, in DC copulsed with SEA/Pa (50 $\mu\text{g/ml}$) SEA localized extensively to a LAMP2-positive compartment (Fig. 4*A*, *j-l* cf with 4*A*, *g-i*) and in part colocalized with Pa (Fig. 4*A*, *d-f* cf with 4*A*, *a-c*). This change in the final localization of SEA that accompanied more extensive Pa-induced DC maturation was not reflected in a change in the way that the two Ag were acquired by the cells since SEA and Pa entered Tfr-negative and -positive compartments regardless (data not shown). We next assessed the consequences of the altered localization of SEA within the SEA/Pa (50 $\mu\text{g/ml}$) copulsed DC on the ability of these DC to induce SEA-specific Th responses. In marked contrast to the situation in mice injected with DC copulsed with SEA/Pa (10 $\mu\text{g/ml}$) animals injected with DC copulsed with SEA/Pa (50 $\mu\text{g/ml}$) failed to develop a SEA-specific Th2 response (Fig. 4*C*), but rather mounted a weak Th1 response to this Ag (Fig. 4*B*). Strong Pa-specific Th1 responses were induced in these animals (Fig. 4*A*).

Discussion

Recent findings indicate that Th response polarization is markedly influenced by DC, which interpret Ag-inherent characteristics and provide signals to T cells that favor appropriate polarization (1, 7, 16–18). Thus, it is clear that DC can discriminate between different Ag. Whether they can do so when presented with two distinct Ag simultaneously has remained unclear. It is clear that DC activated via TLR ligation often produce cytokines such as IL-12 that favor Th1 response polarization. Moreover, as DC mature in response to TLR ligation, they increase the concentration of MHCII/peptide on their surface (9, 19), creating a high dose peptide display that has also been shown to promote Th1 response development (6, 8). In contrast, previous studies have revealed that DC exposed to the Th2 response-inducing helminth Ag retain what appears to be an immature phenotype (1, 7, 20). Observations such as these led to the view that Th2 responses represent a default that occurs in the absence of TLR-mediated DC activation (3, 7).

We have compared how DC take up and are activated by SEA and Pa, Ag that inherently induce oppositely polarized Th re-

sponses. We expected that if SEA has little effect on DC, acting only as a passive Ag, Pa-induced activation should dominate when cells are simultaneously exposed to both Ag. Our data show clearly that SEA/Pa-copulsed DC undergo changes associated with TLR ligand-induced maturation, including up-regulation of MHCII, and CD80. However, the ability of SEA/Pa-pulsed DC to make IL-12 was impaired compared with that of DC pulsed with Pa alone. This result supports the recent report that SEA can have significant suppressive effects on the ability of DC to make IL-12 in response to the TLR4 ligand LPS (12). It is tempting to believe that the ability of SEA to suppress Pa-stimulated IL-12 production is linked to its ability to induce Th2 responses. This situation would be consistent with the observed absence of any effect of SEA on Pa-induced Th1 responses, since we know that DC need not make IL-12 to prime a Pa-specific Th1 response (5). Preliminary microarray analyses of transcript profiles of DC pulsed with SEA, Pa, or SEA/Pa have revealed a >3-fold repression by SEA of expression of a group of 17 genes induced by Pa (J. Sun, L. Cervi, A. Straw, and E. J. Pearce, unpublished data), indicating that the regulatory effects of SEA on Pa are broad ranging and suggesting that the suppression of the production of factors in addition to IL-12 could also play a role in permitting Th2 response development.

At this time it is unclear how SEA suppresses the ability of DC to make IL-12. However, recent reports have shown that SEA binds to DC-SIGN via core fucosylated glycans (21). This is of interest since DC-SIGN ligation by *Mycobacterium tuberculosis*-secreted mannose-capped lipoarabinomannan was recently shown to block maturation of mycobacteria-infected DC (22). Thus, a role for DC-SIGN in the SEA-mediated suppression of Pa-induced IL-12 production is plausible.

Our analysis of SEA and Pa uptake into DC revealed that these Ag are acquired via different pathways. Pa enters early endosomes that stain positively for Tfr, whereas SEA does not. Thereafter, the Ag remain in nonoverlapping compartments. The compartment that Pa enters is LAMP2 bright, which is

consistent with the bacteria being in an Ag-processing compartment. In contrast, some SEA within individual cells enters a LAMP2-dull environment, while the majority of the Ag appear to be in a LAMP2-negative compartment. Thus, the ways that SEA and Pa are trafficked intracellularly are quite different, and we speculate that this difference is linked to the contrasting antigenicity of the two Ag. One possibility, based on observed patterns of Ag/LAMP2 colocalization, is that processing of SEA is less extensive than that of Pa. Such a difference could lead to a higher density display of Pa-derived vs SEA-derived peptides, a situation that could be consistent with the observed abilities of Pa-pulsed DC to induce Th1 responses, SEA-pulsed DC to induce Th2 responses, and of SEA/Pa-copulsed DC to induce Ag-appropriate Th1 and Th2 responses. In accordance with these observations, our data show that, when copulsed DC are stimulated to an increased level of maturation by exposure to a higher concentration of Pa, SEA localizes to LAMP2-bright compartments, suggesting more extensive processing of this Ag. This could result in greater surface expression of MHCII complexed with SEA-derived peptides.

The ability of DC to mature in response to TLR ligands is clearly important for their ability to induce Th1 responses, but may not be important for Th2 response induction (1, 3, 23). It seems likely that the ability of SEA to suppress aspects of DC maturation plays a role in its inherent Th2 response-inducing characteristics. However, the fact that SEA/Pa-copulsed DC do not induce Pa-specific Th2 responses emphasizes that the segregated intracellular localization of SEA plays an equally, if not more important role in this regard. The observations that in highly matured DC (pulsed with SEA plus high concentrations of Pa) SEA is forced to localize to LAMP2-positive, Pa-positive compartments and that these DC fail to induce a strong SEA-specific Th2 response but rather induce a weak SEA-specific Th1 response lend support to this view.

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