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*J Immunol* 2003; 171:5837-5841; doi: 10.4049/jimmunol.171.11.5837

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Maturation of Dendritic Cell 2 Phenotype by a Helminth Glycan Uses a Toll-Like Receptor 4-Dependent Mechanism

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The biology of pathogen-associated molecular patterns (PAMPs) stimulating APCs to differentiate into a Th1-promoting phenotype has been well characterized. Conversely, not a single pathogen product that promotes a Th2 phenotype has been rigorously identified. Strong Th2 responses and dendritic cell 2 maturation are driven by helminth extracts, and carbohydrates have been shown to be responsible for much of this activity. In this study, we show that a helminth carbohydrate, lacto-N-fucopentaose III (LNFPIII) functions as an innate Th2 promoter via its action on murine dendritic cells, with the α1–3-linked fucose required for this activity. In contrast to Th1-type PAMPs, which activate extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases, the Th2 PAMP LNFPIII preferentially activates extracellular signal-regulated kinase. Furthermore, the ability of LNFPIII to drive DC2 maturation is dependent on signaling via Toll-like receptor 4. These data support a new understanding of how APCs integrate signaling pathways to produce a Th1- or Th2-promoting phenotype. The Journal of Immunology, 2003, 171: 5837–5841.

The ability of the immune system to mount the appropriate class of adaptive response to a pathogen or vaccine largely dictates resistance or susceptibility. Adaptive responses are driven by APCs, especially dendritic cells (DCs), which become activated upon encounter with pathogens or Ags. In 1989, Charles Janeway suggested that the innate immune system had evolved to recognize conserved molecular patterns across multiple pathogens. This recognition would both initiate an immediate response from innate responding cells and set the stage for the ensuing adaptive response. Janeway (1) also proposed that pathogen-expressed molecules that stimulate innate responses such as LPS be called pathogen-associated molecular patterns (PAMPs).

PAMPs such as LPS activate macrophages and DCs via interaction with Toll-like receptors (TLRs), resulting in translocation of NF-κB to the nucleus, activating a proinflammatory transcriptional program (2–4). Thus, PAMP ligation to TLRs on DCs provides a signal that drives the maturation of a DC1 phenotype, characterized by production of IL-12 and the ability to bias naive CD4+ T cells toward the Th1 compartment.

Although the mechanisms of Th1 PAMP activation on APCs are well studied, almost nothing is known about the recognition and activation mechanisms by which APCs are driven to promote Th2 responses. Furthermore, the receptors on DCs for Th2-inducing ligands have not been discovered, nor has the ensuing signaling associated with APC ligation of Th2 PAMPs been examined. In this regard, helminth parasites drive potent Th2 responses in vivo and represent ideal pathogens to study DC2 maturation. A soluble extract of Schistosoma mansoni eggs (SEA) has been shown by our laboratory and others to have Th2-promoting activities, consistent with the overall Th2-inducing nature of helminth infections (5). Recently, MacDonald et al. (6) demonstrated that SEA will drive the development of DC2s. However, specific molecules in SEA that drive DC2 maturation have yet to be identified, impeding studies on the biology of DC activation by Th2 PAMPs.

Using mAbs to S. mansoni SEA, our laboratory identified the human milk sugar lacto-N-fucopentaose III (LNFPIII) that contains the Lewisα glycan (7). Subsequently, we demonstrated that LNFPIII strongly promotes Th2 responses in vivo, functions as a Th2-driving adjuvant, as well as induces the in vivo recruitment of a population of suppressor macrophages (8, 9). These responses are dependent upon LNFPIII being presented as a glycoconjugate. Furthermore, our earlier work demonstrating the ability of LNFPIII to promote Th2 responses to a coadministered, unrelated Ag such as human serum albumin was the first demonstration of a molecularly defined Th2 adjuvant (8).

Because LNFPIII conjugates stimulate Th2 responses in vivo, we hypothesized that LNFPIII could stimulate DCs to mature to DC2 cells, in particular, DC2s capable of presenting an unrelated Ag (OVA peptide), to naive CD4+ T cells, driving their differentiation to Th2 cells. In this study, we demonstrate for the first time the maturation of DC2 cells by LNFPIII. We also present data that the DC2-maturing activity of LNFPIII-dextran (Dex) is dependent upon the fucose group, the absence of which abrogates all activity in vitro. We show that unlike the Th1 PAMP LPS, LNFPIII stimulation leads to preferential activation of primarily extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) and that LNFPIII maturation of DC2s is dependent on signaling through TLR4. Taken together, we have defined a molecule that can now be used to explore the mechanisms involved in DC2 maturation, and we provide evidence for a theory in which...
APCs can use the same innate receptors to promote either Th1 or Th2 responses by differential induction of signaling responses to discrete pathogen patterns.

Materials and Methods

Animals

Six- to twelve-week-old female BALB/c (bone marrow (BM) donors), BALB/c.C3-Flt3 receptor double-negative, and DO.11.10 TCR transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions. TCR-transgenic mice and OVA peptide (323-ISQAVHAAHAEINEAGR-339) were obtained from Sigma-Aldrich (St. Louis, MO).

Carbohydrates

LNFPIII (βGal[1-4]-(αFuc[1-3])-βGlcNAc[1-3]-βGal[1-4]-Glc) and lacto-N-neotetraose (LNnT) (βGal[1-4]-(αFuc[1-3])-βGlcNAc[1-3]-βGal[1-4]-Glc) (Fig. 1) were conjugated to a 40-kDa molecule of Dex (LNFPIII-Dex, LNnT-Dex) and were obtained along with Dex from Neose Technologies (Horsham, PA). The glycoconjugate consisted of 12–25 LNFPIII or LNnT molecules/molecule of Dex. Multiple batches of each glycoconjugate were tested and shown to have similar activity across batches, as quantified by several outputs, including soluble mediator production and kinase activation. Glycoconjugate activity declined with lower levels of substitution. Endotoxin assays by the Limulus Amebocyte Lysate test (Charles River, Charlesto, SC) on all batches of carbohydrate used showed an effective concentration of endotoxin of less than 0.5 ng/ml in each of our assays, but had no effect on LNFPIII-Dex in each of our assays. The 50 μg of LNFPIII-Dex used in most experiments refers to the weight of the conjugate, which for the majority of studies was 12 molecules of LNFPIII (molecular mass = 853.8)/40 kDa molecule of Dex. The total weight of conjugate is then ∼50 kDa; thus, at 50 μg/ml of conjugate, LNFPIII = 10 μg/ml.

Cell culture

Cells were cultured in MEM supplemented with 10% FCS (HyClone, Logan, UT), 100 U/mg penicillin, 100 μg/ml streptomycin, 0.005 mM 2-ME, and 2 mM glutamine (Sigma-Aldrich). Escherichia coli LPS was obtained from Sigma-Aldrich.

DC culture

DCs were prepared from BM essentially as described by Lutz et al. (10). Briefly, PBS was used to flush BM from femurs and tibias, using a 0.45-mm-wide-diameter needle. BM cells were washed and inoculated into bacteriological petri dishes at 2 × 10^6 total cells in 10 ml complete medium (see above). A total of 20 ng/ml of murine rGM-CSF (5 × 10^5 U/mg; Peprotech, Rocky Hill, NJ) was included in BM leukocyte culture on days 0, 3, and 6. A total of 10 ng/ml murine rGM-CSF was added on day 8. On day 6, the BM leukocyte culture was scraped from the petri dishes, and CD11c+ cells were isolated by MACS. Purified DCs were plated at 5 × 10^4 cells/well in 96-well plates and treated with LPS, LNFPIII, LNnT, or nothing on days 6 and 8. On day 10, medium was replaced with medium containing purified CD4+ T cells from the spleens of naive DO.11.10 TCR transgenic mice and OVA peptide (323-ISQAVHAAHAEINEAGR-339). Supernatants were harvested at 72 h post addition of OVA peptide.

ELISAs

IL-4 (pg/ml) and IFN-γ (ng/ml) sandwich ELISAs were performed using paired mAbs (BD PharMingen, San Diego, CA).

Western blot analysis

Phospho-specific ERK, total ERK, phospho-specific p38, and phospho-specific c-Jun N-terminal kinase (JNK) Abs were purchased from Cell Signaling (Beverly, MA). Western blots were performed using standard methodology. Due to the fact that GM-CSF alone induces a persistent ERK activation, DCs were not treated with GM-CSF on day 6, and rested for 2 days until day 8, at which point they were stimulated for the first time with LNFPIII-Dex, LNFPIII-Dex, LPS, or a bacterial extract containing multiple TLR ligands (BEx) (11). Extracts were separated by 12% Tris-HCl SDS-PAGE (Bio-Rad, Hercules, CA). Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blocked in 5% milk-TBS for 1 h at room temperature. Incubations were performed according to the manufacturer. Blots were developed with chemiluminescent substrate (Cell Signalling) and captured on a Kodak IS440CF Imaging Station with Kodak 1D software. Net intensities were calculated using this software. The intensity of bands from blots probed with anti-phospho MAPK Abs was normalized to a total ERK control blotted on the same blot using the Kodak 1D software.

Statistical analysis

Data were analyzed for significance using the ANOVA statistical software found in Microsoft Excel. Data are expressed as the mean ± SE. Significant differences (p < 0.05) are indicated with an asterisk.

Results

Generation and characterization of DC subsets

FACS analysis of LPS-treated cells revealed increased surface expression of CD40, B7.1, B7.2, as well as MHC class II when compared with nonstimulated DCs or those treated with LNFPIII-Dex (Table I). These results are similar to those of MacDonald et al. (6), who examined BM-derived DCs cultured with LPS or S. mansoni SEA. We next measured levels of IFN-γ and IL-4 in supernatants collected from cocultures of CD4+ T cells and OVA peptide-fed DCs stimulated with LPS or LNFPIII-Dex, to measure Th1 or Th2 responses, respectively. Coculture of CD4+ T cells with LPS-activated OVA-fed DCs resulted in a typical Th1-type cytokine response with low levels of IL-4 (Fig. 2A) and increased levels of IFN-γ (Fig. 2B). In contrast, CD4+ T cells cocultured with LNFPIII-Dex-activated OVA peptide-fed DCs produced increased levels of IL-4 (Fig. 2A) and simultaneously suppressed levels of IFN-γ (Fig. 2B) when compared with similar cocultures stimulated with LPS or with LNnT-Dex. These data show that, as expected, LPS stimulation biases immature DCs toward a DC1 phenotype and that LNFPIII-Dex stimulation of immature DCs drives an equally strong DC2 phenotype.

LNFPIII-Dex induces altered signaling in DCs as compared with LPS

The next question was to determine whether we could observe differences in signaling in DCs activated with LPS or LNFPIII-Dex. In addition to the glycoconjugates, we also tested for activity of monomers of LNFPIII and LNnT and found that neither induced significant activity in any experimental system tested (data not shown). Given the importance of the MAPK family in DC1 generation, we decided to examine activation of the three major families of MAPKs: ERK, JNK, and p38. As expected, LPS stimulation of DCs led to the phosphorylation of all three species of MAPKs.
MAPK. In contrast, LNFPIII-Dex induced strong phosphorylation of ERK, with weak phosphorylation of JNK and p38 (Fig. 3A). Thus, phosphorylation of the MAPKs following LNFPIII-Dex activation of DCs was quite different from that of LPS.

**TLR4 is required for LNFPIII-Dex activation of DCs**

Based on the signaling patterns we had observed, we decided to test a number of candidate receptors for LNFPIII-Dex. Although TLR4 has previously been exclusively associated with Th1-driving molecules, we found that the signaling we had observed in wild-type BM-DCs was abrogated in DCs derived from the BALB/c1ps-d mouse strain, which lacks a functional TLR4 protein. Fig. 3B shows that the response to LNFPIII-Dex is completely abrogated in these mice, providing strong evidence that LNFPIII-Dex signals through TLR4. We confirmed this finding using several other measures of TLR4 activity in a variety of cell types (data not shown).

The observation that LNFPIII-Dex was unable to activate cells from BALB/c1ps-d mice led us to test the hypothesis that the functional activity of LNFPIII-Dex in maturing DCs to DC2s is mediated by TLR4. BM-DCs from wild-type BALB/c mice or BALB/c1ps-d mice were matured in culture with LPS or LNFPIII-Dex using the protocol in Fig. 2. Examination of cytokines in supernatants from CD4+ T cells cocultured for 72 h with DCs matured with LNN-T-Dex (50 μg/ml), LNFPIII-Dex (50 μg/ml), or LPS (0.1 μg/ml) revealed that there were representative of at least three independent experiments, with n = 10 per experiment. Significance for LNFPIII-Dex and LPS samples was calculated with respect to LNN-T-Dex by ANOVA, and significant differences are indicated with an asterisk (p < 0.05).

**Discussion**

The major finding of our study was the demonstration that the defined pathogen-associated molecule, LNFPIII-Dex, drives in vitro differentiation of naive DCs to a DC2 phenotype via a mechanism dependent on TLR4. We further demonstrated that a molecule that is identical with LNFPIII-Dex, but lacks a fucose side chain (LNN-T-Dex), does not have any activity. Several groups have reported the prevalence of polyvalent Lewisx on various schistosome life cycle stages (reviewed in Ref. 12). In combination with previous data from ours and other labs, a consensus is building that TLR4 seems in conflict with numerous studies of LPS interactions with TLR4-inducing type 1 immunity. However, similar to our findings, another report has shown that signaling through TLR4 can mediate DC2 differentiation. In this study, MdD88 knockout-derived DCs were stimulated with LPS (14) and induced DC2 maturation, indicating that diverse APC phenotypes can arise depending on the adapter molecules activated by a TLR4 ligand. The molecular mechanism by which TLR4 interacts with structurally distinct PAMPs and responds to them differently probably has to do with how a given PAMP is presented to TLR4. Several accessory molecules have been associated with the extracellular domain of LPS.
TLR4, and variations in usage among the members that make up this complex may lead to different strengths of signal being transmitted through TLR4. To this end, recent work has shown that the Lewisx moiety present in LNFPIII is a strong ligand for DC-specific ICAM-3-grabbing nonintegrin, and we are currently investigating the potential role of this molecule in LNFPII-Dex signaling (15). An interpretation of this concept based upon our data is that LPS and accessory molecules drive stronger stimulation of TLR4 than LNFPII-Dex and whichever molecules it associates with; this strong stimulation leads to the generation of a proinflammatory burst from the APC. In contrast, LNFPII-Dex most likely drives a lower intensity stimulation leading to a less intense signaling cascade. This fits with the concept that helminth parasites present less of an immediate threat to the host than do other pathogens, and the cell surface accessory molecules that recognize helminth-expressed PAMPs will induce a low level signaling cascade. The intensity of TLR4 stimulation in this hypothesis would be an immunological decision point as to the strength and quality of the ensuing adaptive response. Interestingly, related work on a phosphatidyserine fraction from S. mansoni reported the ability to stimulate APCs to produce IL-10 via TLR2 stimulation, promoting T-regulatory cell maturation (16).

Another question is how the intracellular activity of TLR4 can mediate such diverse responses. Three adapter molecules have been identified that mediate TLR4 signaling (MyD88, TIR domain-containing adapter protein, and TIR domain-containing adapter inducing IFN-β/TIR-containing adapter molecule 1 (17–19). Furthermore, the report by Akira and colleagues (14) demonstrating that LPS drives DC2 differentiation of MyD88 knockout DCs supports the hypothesis that the downstream response is dependent upon which adapter molecules are recruited and activated.

The mechanism by which these adapter molecules are activated, perhaps even preferentially activated, has not been clearly elucidated, but it is clear that MyD88 and TIRAP are in the same pathway downstream of LPS signaling, and appear to be associated with a strong Th1 bias (20, 21). Less is known about TRIF, although it does mediate some of the observed NF-κB translocation in response to LPS (18). We are currently investigating the role of all three molecules in response to LNFPII-Dex.

The unique signaling pattern we observed in DCs in response to LNFPII-Dex provides further evidence that differential activation of adapter molecules may be occurring. We observed only strong ERK phosphorylation following stimulation with LNFPII-Dex, with little activation of p38 or JNK MAPK. Previously, strong activation of all three MAPKs was considered to be an essential feature of TLR signaling (19). It is rare that this primarily ERK pattern is ever observed and p38 MAPK activation has been implicated in increasing the transcriptional activity of NF-κB directly via the p65 unit (22). Thus, differential regulation of the MAPK family has the potential to produce a tremendous range of response phenotypes for APCs.

Current studies in our laboratory focus on these and other mediators of the innate immune response to understand how the precise regulation, activation, and kinetics of activation of various molecules all play a role in the ultimate decision of cell fate. These factors, as well as roles for potential novel pathways of activation downstream of TLR4 currently under investigation in our laboratory, will lead to a picture of how the initial responders of innate immunity process cell fate decisions based on environmental information, with great implications for both treatment of pathogens and immune dysregulation.

References

FIGURE 4. LNFPII-Dex-induced DC2 maturation is TLR4 dependent. IL-4 and IFN-γ production of OVA-specific CD4⁺ T cells cocultured for 72 h with DCs derived from either a wild-type BALB/c mouse or an lps-d TLR4 mutant mouse. Data are representative of at least three independent experiments, with n = 8 per experiment. Significance was calculated with respect to LNnT-Dex by ANOVA, and significant differences are indicated with an asterisk (p < 0.05).


