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Hyporesponsiveness of Intestinal Dendritic Cells to TLR Stimulation Is Limited to TLR4

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Dendritic cells (DCs) are crucial to intestinal immune regulation because of their roles in inducing protective immunity against pathogens while maintaining tolerance to commensal bacteria. Nonetheless, relatively little is known about intestinal DC responsiveness to innate immune stimuli via TLRs. We have previously shown that DCs migrating from the rat intestine in lymph (iLDCs) are hyporesponsive to LPS stimulation, thus possibly preventing harmful immune responses being induced to commensal flora. In this study, to understand how iLDC function is regulated by innate immune stimuli, we have characterized the expression and function of TLRs in iLDCs isolated from the thoracic duct lymph of mesenteric lymphadenectomized rats and compared these with DCs grown from bone marrow in the presence of Flt3 ligand. We show that iLDCs express mRNAs for all TLRs, but express significantly less TLR4 mRNA than bone marrow-derived DCs. Functionally, iLDCs could be activated by TLR agonists representing intestinal pathogen-associated molecular patterns, with the important exception of the TLR4 agonist LPS. Furthermore, we show that DCs in the intestinal wall interact directly with noninvasive bacteria (Bacillus subtilis spores), leading to an increase in the output of activated iLDCs into lymph, and that DCs containing spores are activated selectively. These data highlight a functional difference between TLR4 and other TLRs. As iLDCs can respond to TLR stimulation in vitro, there must be other mechanisms that prevent their activation by commensal bacteria under steady-state conditions. The Journal of Immunology, 2009, 182: 2405–2415.

The lumen of the gut is continuously exposed to a plethora of foreign Ags, most of which are ‘harmless’ and include food Ags as well as microbial products from gut commensal bacteria. Immune mechanisms have evolved to maintain immunological tolerance against such ‘harmless’ Ags to avoid chronic inflammation that could potentially lead to the development of inflammatory bowel disease (IBD). Nonetheless, the immune system has equally evolved the capacity to elicit protective mucosal and systemic immune responses against enteric pathogens. How this delicate immunoregulatory balance is maintained in the gut remains one of the major unanswered questions of mucosal immunology.

It is currently thought that dendritic cells (DCs) are crucial for immune regulation (1). DCs integrate qualitatively distinct signals that de facto inhibit or promote proinflammatory immune responses. In support of such a role is the fact that intestinal DCs can directly interact with Ags from the intestinal lumen, either by M cell-mediated uptake or by directly extending their dendrites between cells of the intestinal epithelium (2, 3). Considerable evidence shows that DC activation is crucially dependent on the detection of conserved microbial motifs, so-called pathogen associated molecular patterns (PAMPs), by pattern recognition receptors (PRRs), of which the family of TLRs are the most understood (4, 5). TLRs recognize a wide variety of PAMPs, and signaling through TLRs on DCs is critical for the initiation of adaptive immune responses (4, 6). However, relatively little is known about the capacity of intestinal DCs to detect and respond to TLR agonists. In the mouse, CD11c

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murine CD11c<sup>hi</sup>CD11b<sup>hi</sup> LP DCs express TLR5 and respond to stimulation with flagellin by the generation of IgA+B cells and Ag-specific IL-17 and IFN-γ producing CD4<sup>+</sup>T cells (11). This evidence showing that intestinal DCs may not be wholly unresponsive to TLR engagement is in agreement with our previous observation that a subset of rat intestinal lymph DCs respond to TLR7/8 stimulation (12).

The uncertainty regarding this key question of whether intestinal DCs respond to other innate immune stimuli in the steady state is likely related to the technical difficulties in isolating DCs directly from intestinal tissue. The isolation procedure itself may induce phenotypic and functional changes in the DC. To overcome these limitations, we collected lymph-borne DCs from the thoracic duct lymph of mesenteric lymphadenectomized (MLNX) rats, which allows the isolation of DCs that have recently left the intestine with minimal in vitro manipulation (13, 14). All rat intestinal lymph-borne DCs (iLDCs) express CD103 and high levels of surface MHC II and represent a heterogeneous population, phenotypically and functionally distinguished by the expression of CD172a and CD11b (12, 15). Typically, a 24-h collection of lymph from one rat contains 3–3.5 × 10<sup>8</sup> total cells; however, in the lymph of MLNX rats, CD103<sup>+</sup>MHC class II<sup>hi</sup> DCs comprise 0.1–0.25% of total cells or 3–9 × 10<sup>5</sup> DCs per rat per day (16). The CD172a<sup>+</sup>DCs have a weaker capacity for mixed lymphocyte reaction stimulation, survive poorly in culture, and were found to be responsible for the uptake and transfer of apoptotic enterocytes to the MLNs (15, 17). Using this methodology,
it has been shown that migrating rat intestinal DCs are not activated by LPS stimulation in vitro (18, 19). Furthermore, i.v. injection of LPS results in the emigration of intestinal DCs but does not induce these DCs to up-regulate the costimulatory molecules CD80 and CD86 (18). Consistent with these results, a recent study found very few rat CD103+/H11001 LP DCs stained positively for TLR4 in sections of the ileum (20).

Clarification of the capacity of intestinal DCs to express PRRs and respond to PAMPs represents a key question that could aid in the design of oral vaccines and adjuvants, as well as provide an indication of the pathways that may lead to intestinal inflammation. Using the system of thoracic duct cannulation of MLNX rats, we were able to analyze both the pattern of TLR expression and the functionality of intestinal DCs. We hypothesized that the hyporesponsiveness of intestinal DCs to LPS would be reflected by hyporesponsiveness to stimulation with other bacterial PAMPs. Contrary to this initial hypothesis, we demonstrate that although iLDCs show a lack of responsiveness to the TLR4 agonist LPS, they are able to up-regulate activation markers upon stimulation through other TLRs. Significantly, our results show that intestinal DCs can be activated both through TLR2 and TLR9, both major receptors of bacterial PAMPs.

Materials and Methods

Animals and surgical procedures

PVG RT1⁺ rats were bred and maintained under specific pathogen-free conditions at the Sir William Dunn School of Pathology, Oxford U.K. Experimental procedures were performed on animals between 8 and 20 wk old (except the rats used for mesenteric lymphadenectomy, which were 4–6 wk old). Mesenteric lymphadenectomy and thoracic duct cannulation were performed using established protocols as described previously (14). All procedures were conducted in accordance with U.K. Home Office guidelines.

Reagents

Cells were cultured in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Invitrogen), 50 μM 2-ME (Sigma-Aldrich), and 5% FCS (PAA Laboratories).

The following TLR agonists were used for stimulation: Pam2Cys, Pam3Cys (both from EMC Microcollections), poly(I:C) (GE Healthcare), CpG (ODN 2336; Coley Pharmaceuticals), flagellin (InvivoGen), resiquimod (R-848; InvivoGen), and purified Escherichia coli LPS (List Biochemicals) were dissolved or suspended in PBS. Spores of the Gram-positive organism Bacillus subtilis (strain PY79) were prepared as described previously (21) and heat inactivated.

Antibodies

mAbs to the rat Ags CD11b (clone OX42), CD103 (clone OX62), and CD172a (clone OX41) were purified from cell culture supernatants and conjugated to biotin, Alexa Fluor 488, or Alexa Fluor 647 using conjugation kits (Molecular Probes). PE-labeled anti-CD25 (clone OX39), PerCP-labeled anti-MHC class II (clone OX6), streptavidin reagents conjugated to FITC, PE, allophycocyanin, or allophycocyanin-Cy7 and mouse isotype control Abs conjugated to appropriate fluorochromes, were purchased from BD PharMingen. PE-labeled anti-CD80 (clone 3H5) and anti-CD86 (clone 24F) were purchased from Serotec.

Cell isolation

Thoracic duct leukocytes were collected from eight rats on ice in PBS with 10 mM EDTA and 20 U/ml heparin, passed through a 100-μm cell strainer (BD Biosciences), and RBCs were lysed with ACK (ammonium chloride potassium) lysis buffer. Cells were either stained and analyzed by flow cytometry or enriched for iLDCs using anti-CD103 MACS beads and autoMACS (Miltenyi Biotech). In certain experiments, cells were further purified by FACS using the MoFlo cell sorter (Dako).
Bone marrow-derived DCs (BMDCs)

Hind legs of male rats were removed and cells were flushed from the femur and tibia. The RBCs were lysed with ACK lysis buffer and the remaining cells were cultured in complete RPMI 1640 with 100 ng/ml Flt3L (Amgen) for 9 days.

Flow cytometry

Cell surface staining was performed in PBS with 2% FCS and 10 mM EDTA for 30 min on ice after blocking in 10% rat serum. Where a biotin-conjugated Ab was used, cells were further stained with a streptavidin-fluorochrome conjugate for 15 min. Samples were acquired on a FACSCalibur (BD Biosciences) or sorted and analyzed by the MoFlo cell sorter (Dako). Acquired data was analyzed using FlowJo software (version 7.2.2; Tree Star).

RNA extraction

Cells were suspended in TRizol (Invitrogen) and the RNA was extracted according to the manufacturer’s instructions. Contaminating genomic DNA was removed using the DNA-free kit (Ambion) in the presence of the RNase inhibitor RNasin (Promega). RNA was reverse transcribed using the Reverse-it RTase blend (ABgene).

Real-time quantitative PCR

cDNA was examined for the frequency of different transcripts using quantitative real-time PCR based on the TaqMan probe system or SYBR Green dye. All quantitative PCR (qPCR) reactions were performed in 10-μl volumes using 0.5 U of Taq polymerase (HotStar Taq; Qiagen) per reaction. All qPCR primers and dual-labeled probes are presented in Table I. Cycling parameters were 92°C for 30 s followed by annealing at 59°C for 30s and extension at 72°C for 15 s for 40 cycles. Fluorescence levels were detected by the ABI Prism 7900HT sequence detection system (Applied Biosystems) and analyzed using the SDS software (version 2.2; Applied Biosystems). The standard curve used for absolute quantification was generated by qPCR using serial dilutions of genomic DNA as template. Cyclophilin B was included as an internal control (housekeeping gene).

Microscopy

Cells were allowed to adhere to polytetrafluoroethylene-coated multislot microscope slides (C.A. Hendley Essex) and were fixed in acetone and blocked with 3% H2O2 followed by 10% rat serum before the addition of the primary Ab, anti-CD103. After washing, the primary Ab was detected using HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories). Tyramide Cy3 (PerkinElmer) was used to detect enzyme activity. Slides were counterstained with 4’,6’-diamidino-2-phenylindole (Molecular Probes) and mounted using Vectashield (Vector Laboratories). Labeled cells were viewed on a Leica TCS SP2 AOBS (acousto-optical beam splitter) confocal microscope (Leica) and analyzed using the Leica confocal software (LCS; Leica). Images from each detection channel were acquired separately as a gray scale image and then assigned colors and overlaid. Twenty images along the z-plane were acquired and used for three-dimensional reconstruction.

Cytokine detection

Rat IL-12p40 was detected by ELISA (BioSource International). Concentrations of IL-6, IL-10, TNF-α, and IL-12p70 were assessed by the Lincoplex multiplex cytokine bead assay (Linco) according to manufacturer’s instructions. Minimum detectable concentrations for all cytokines were <10 pg/ml. In some experiments, changes in the levels of IL-12p40, IL-12p35, IL-23p19, and IL-6 mRNA were assessed by qPCR. All primer sequences are listed in Table I.

Statistical analysis

For comparison of means between two groups, the data were analyzed using Student’s t test. For comparisons involving more than two data sets, an ANOVA was used. Values of p < 0.05 were considered significant, and Dunnett (for comparison to control group) or Bonferroni (for comparison...
of all pairs of groups) posttests were performed on the data sets. All statistical analysis was performed using GraphPad Prism and Microsoft Excel.

Results

Differential expression of TLR mRNAs by rat intestinal DC subsets

Given the fundamental role of TLRs, both in microbial recognition and control of tissue homeostasis (22–24), it was important to first establish the steady-state repertoire of TLRs expressed by intestinal DCs and to determine whether expression is subset-associated, which could reflect segregation of DC function in the intestine. iLDCs were collected from the pseudoafferent lymph of MLNX rats, enriched using anti-CD103 magnetic beads, and sorted by FACS as large CD103<sup>+</sup>/H<sub>11001</sub>MHC class II<sub>high</sub> cells (Fig. 1A). Three subsets of iLDCs were identified based on their expression of CD172a and CD11b, namely CD172a<sup>−</sup>/H<sub>11001</sub>CD11b<sup>−</sup>, CD172a<sup>+</sup>/H<sub>11001</sub>CD11b<sup>−</sup>, and CD172a<sup>−</sup>/H<sub>11001</sub>CD11b<sup>+</sup>.
CD172a\(^{+}\)CD11b\(^{+}\) (Fig. 1A). The expression of TLRs 1–11 and MD2 mRNA was analyzed by qPCR from each subset (sorted to \(\geq 95\%\) purity) and compared with expression from Flt3L-generated rat BMDCs.

Rat iLDCs expressed all of the TLR mRNAs tested (Fig. 1B) with the exception of TLR7. These data extend our previous observations (12) where we reported that TLR7 mRNA could not be detected in the CD172a\(^{+}\) subset, which also showed very low expression of TLR8 mRNA. Of note, although TLR7 transcripts were expressed by the two CD172a\(^{+}\) subsets, the level of expression was significantly lower than that in BMDCs. In contrast, the CD172a\(^{+}\) subsets exhibited levels of TLR8 mRNA comparable to those of BMDCs. Conversely, the CD172a\(^{-}\) subset showed a significantly higher level of TLR3 mRNA expression than either CD172a\(^{+}\) DC population. However, all three subsets of iLDCs expressed similar levels of TLR9 mRNA. No significant differences in the level of expression of TLRs 5, 6, 10, and 11 and MD2 were observed between any of the iLDC subsets or BMDCs.

Surprisingly, despite their previously demonstrated hyporesponsiveness to LPS stimulation (18), iLDCs were found to express mRNA for both TLR4 and MD2, crucial components of the LPS receptor complex. However, the level of TLR4 mRNA was significantly lower in all three iLDC subsets (\(p < 0.05\) for the CD172a\(^{+}\) subsets and \(p < 0.01\) for the CD172a\(^{-}\) subset) than in the LPS-sensitive BMDCs. In marked contrast, all iLDC subsets expressed high levels of TLR2 mRNA that were comparable to the levels expressed by BMDCs. Collectively, these data unequivocally demonstrate that in the steady state, rat iLDC express transcripts for all TLRs (with the exception of TLR7), although the levels of expression vary in different subpopulations.

**Intestinal DCs can be activated by TLR agonists in vitro**

Because commensal microbes may continually engage intestinal DCs under basal conditions, one might predict that they would be hypo-responsive to stimulation by microbial products to avoid spontaneous inflammation, as suggested by their nonresponsiveness to LPS stimulation (18, 25). However, the capacity of intestinal DCs to respond to the TLR5 agonist flagellin (7, 11) and the TLR7/8 agonist R-848 (12) suggests that the refractoriness of intestinal DC to phenotypic maturation and proinflammatory cytokine production driven by TLR engagement might not be universal. To resolve this issue, we examined DC activation by well-defined TLR agonists representative of motifs expressed by a broad range of microbes. Enriched iLDCs were cultured for 18 h in the presence or absence of Pam3Cys (TLR2 agonist), Poly (I:C) (agonist signaling through TLR3, although it can activate other cellular receptors), LPS (TLR4 agonist), flagellin (TLR5 agonist), R-848 (TLR7/8 agonist), and CpG (TLR9 agonist) and the level of activation markers CD25 and CD86 assessed by flow cytometry (Fig. 2). Remarkably, with the exception of LPS, a significant increase in the percentage of cells expressing CD25 or high levels of CD86 was observed after culture in the presence of these well-defined agonists for TLR 2, 3, 5, 7/8, and 9. These data strongly suggest that, despite putative continuous exposure to TLR agonists present in intestinal commensal bacteria, intestinal DCs respond to stimulation through TLR4 in a fundamentally distinct way from that of other TLRs.

Highly purified iLDCs are activated by TLR2 and TLR9 but not TLR4 agonists in vitro

Even though enriched iLDC preparations were used in the previous experiments, a possibility remained that the observed TLR2- and TLR9-mediated activation could be an indirect effect due to signals made by a population of contaminating cells. To address this issue, we sorted CD103\(^{+}\) MHC class II\(^{\text{high}}\) iLDCs by FACS (\(\geq 95\%\) purity) to eliminate any possible indirect effects on DC activation. FACS-purified iLDCs were stimulated with a range of representative TLR2 agonists including the triacylated lipopeptide...
Pam3Cys, which signals through the TLR1/2 heterodimer (26), diacylated lipopeptide Pam2Cys, which signals via the TLR 6/2 heterodimer (27), and heat-inactivated spores of B. subtilis, a Gram-positive bacterium that can activate DCs through TLR2 (A. G. C. Barnes, V. Cerovic, and L. S, Klavins, unpublished observations). Culture with each of the TLR2 agonists tested, but not with the TLR4 agonist LPS, led to an increased percentage of activated intestinal DCs across a range of doses (Fig. 3, A and C).

Given that intestinal DCs may also be exposed to DNA from commensal bacteria, we therefore investigated CD25, CD86, and CD80 expression following stimulation with CpG. As observed with TLR2 stimulation, CpG activated DCs in a dose-dependent manner (Fig. 3, B and C).

Because DCs are able to sample microflora and direct the nature of the developing adaptive immune response in part through TLR-dependant cytokine secretion, it remained important to assess cytokine production by iLDCs following stimulation with TLR2, TLR4, and TLR9 agonists over a range of concentrations. We found that cells incubated with Pam3Cys, Pam2Cys, B. subtilis spores, or CpG responded with enhanced secretion of IL-6 and IL-12p40 when compared with DCs cultured in medium alone, whereas those incubated with LPS showed no enhanced secretion (Fig. 3D). These data reflect the pattern of activation observed by analysis of the DC surface phenotype. Of note, no detectable secretion of IL-12p70 was observed in culture in the presence or absence of any microbial agonist (data not shown). Moreover, secretion of IL-10 was not reproducibly induced by TLR stimulation.

To assess whether the three identified populations of iLDCs varied in their ability to respond to TLR agonists, CD172a+, CD172a+CD11b−, and CD172a+CD11b+ iLDCs were purified separately by FACS, cultured in the presence and absence of representative TLR2, TLR4, and TLR9 agonists, and their culture supernatants were examined for cytokine production. For comparison, day 9 Flt3L-BMDCs were similarly stimulated with the same concentration of TLR agonist. As with our observation from total CD103+ iLDCs, all three subsets secreted IL-6 and IL-12p40 in response to the TLR2 agonist Pam3Cys and the TLR9 agonist CpG, but not the TLR4 agonist LPS (Fig. 4). Interestingly, the CD172a+, but not the CD172a+CD11b− or the CD172a+CD11b+ iLDC subset, secreted TNF-α when cultured with Pam3Cys or CpG, but not with LPS (Fig. 4). In some experiments, IL-10 production from all three subsets was increased following stimulation with TLR2 and TLR9 agonists, but never by LPS (not shown), although the titers never exceeded 70 pg/ml. Expression of IL-12p35 and IL-23p19 mRNAs, assessed by qPCR, demonstrated neither enhancement nor inhibition by TLR stimulation (data not shown).
Intraintestinal injection of B. subtilis spores induces a significant increase in the proportion of activated DCs migrating out of the intestine

Although TLR agonists (with the exception of LPS) were able to activate intestinal DCs in culture, a formal possibility remained that this result was an artifact of the removal of DCs from their normal microenvironment that could condition unresponsiveness to bacterial stimuli. To address this point, we used inactivated B. subtilis spores as a model of noninvasive bacteria in the intestine and examined whether the stimulatory effects on iLDCs observed in vitro could also be observed in vivo. MLNX rats were injected intraintestinally with $5 \times 10^{10}$ B. subtilis spores or PBS as control, and the thoracic duct lymph cells were collected over a 24-h period. DCs were identified as large CD103$^+$ MHC class II$^+$ cells and the three subsets (CD172a$^+$, CD172a$^+$CD11b$^+$, and CD172a$^+$CD11b$^+$) were analyzed for expression of the activation markers CD25, CD86, and CD80. Administration of B. subtilis spores did not alter the proportion or total number of intestinal DCs migrating in lymph compared with controls (data not shown). Nonetheless, a small but significant increase in the number of DCs bearing cell surface CD25 and CD86 ($p = 0.0153$ and $0.0195$, respectively) was detected in lymph 24 h after administering B. subtilis spores intraintestinally (Fig. 5, A and B). These data are consistent with the data generated using purified iLDCs in vitro described above. Furthermore, the same response was observed in all three DC subsets (Fig. 5C). Strikingly, these differences were not seen in the output of DCs isolated from the lymph between 24 and 48 h postinjection (data not shown), indicating that the activation observed was likely attributable to a direct effect on DCs resident in the intestine rather than DCs subsequently recruited to the intestine by the presence of the spores. Collectively, the data indicate that the intraintestinal administration of a model noninvasive Gram-positive organism leads to a significant increase in the activation phenotype of DC trafficking in intestine-draining lymph.

Migrating intestinal DCs directly interact with intraintestinally injected B. subtilis spores

Dendritic cells can adopt an activated phenotype due to signals (cytokines and cell-to-cell interactions) supplied by other cell types. However, DCs activated in this manner are unable to instruct the differentiation of effector T cells, unlike DCs activated by direct TLR stimulation (28). We therefore determined whether the observed phenotypic changes in migrating iLDCs were primarily due to direct contact of DCs with B. subtilis spores. To this end, MLNX rats were injected intraintestinally with $5 \times 10^{10}$ B. subtilis spores or PBS as control, and the thoracic duct lymph cells were collected during a 24-h period. DCs were identified as large CD103$^+$ MHC class II$^+$ cells and the three subsets (CD172a$^+$, CD172a$^+$CD11b$^+$, and CD172a$^+$CD11b$^+$) were analyzed for expression of the activation markers CD25, CD86, and CD80. Administration of B. subtilis spores did not alter the proportion or total number of intestinal DCs migrating in lymph compared with controls (data not shown). Nonetheless, a small but significant increase in the number of DCs bearing cell surface CD25 and CD86 ($p = 0.0153$ and $0.0195$, respectively) was detected in lymph 24 h after administering B. subtilis spores intraintestinally (Fig. 5, A and B). These data are consistent with the data generated using purified iLDCs in vitro described above. Furthermore, the same response was observed in all three DC subsets (Fig. 5C). Strikingly, these differences were not seen in the output of DCs isolated from the lymph between 24 and 48 h postinjection (data not shown), indicating that the activation observed was likely attributable to a direct effect on DCs resident in the intestine rather than DCs subsequently recruited to the intestine by the presence of the spores. Collectively, the data indicate that the intraintestinal administration of a model noninvasive Gram-positive organism leads to a significant increase in the activation phenotype of DC trafficking in intestine-draining lymph.
10^10 CFSE-labeled *B. subtilis* spores and thoracic duct lymph was collected during a 24-h period. DCs were identified as large CD103^+^ MHC class II^high^ cells by flow cytometry and evaluated for green fluorescence and CD25 expression.

Rats administered CFSE-labeled *B. subtilis* spores revealed a small but clearly distinct population of migrating DCs with a marked increase in green fluorescence when compared with the PBS-treated controls (Fig. 6, A and B). Moreover, the fluorescence was exclusively limited to the CD25^+^ iLDCs (Fig. 6, A and C), indicating preferential activation of the spore-bearing DCs. CFSE^+^ and CFSE^−^ DCs were sorted by FACS and the mRNA was analyzed for cytokine expression by qPCR. The CFSE^+^ DCs demonstrated a 3- to 5-fold higher level of IL-6 mRNA (Fig. 6D), although no significant difference in the expression of IL-10, TNF-α, IL-12p40, IL-12p35, or IL-23p19 was observed (data not shown). These data strongly suggest that the modified, activated phenotype of intestinal DCs seen after *B. subtilis* administration is, at least in part, due to direct activation.

Data from our laboratory established that DC activation by *B. subtilis* spores is crucially dependent upon their phagocytosis (A. G. C. Barnes and L. S. Klavinskis, unpublished data). To establish whether the administered *B. subtilis* spores were surface bound or internalized by migrating DCs, FACS-sorted CD103^+^ MHC class II^high^ cells from the lymph of spore-treated rats were examined by fluorescent confocal microscopy. Analysis of successive z-plane images and three-dimensional reconstruction (Fig. 6E; supplemental movie 1) revealed bright green-labeled spores clearly visible within the CD103^+^ DCs, demonstrating that *B. subtilis* spores are internalized by intestinal DC.

**Discussion**

In this study we have shed new light on the responsiveness of steady-state intestinal DCs to PAMPs commonly expressed by commensal bacteria. Recent studies using LPS have indicated that intestinal DCs (7, 18, 25) and macrophages (29, 30) are refractory to stimulation and suggest a possible mechanism for suppression of inflammatory signals from microflora continually present in the intestine. Contrary to this hypothesis, we demonstrate that intestinal DCs express the full repertoire of TLR mRNAs and correspondingly can be activated through these receptors by synthetic agonists representative of PAMPs present in the intestine, with the exception of LPS. Importantly, we show that intestinal DCs can directly interact with noninnvasive bacteria in the intestine and alter their phenotype in response. Our data suggests that the response of intestinal DCs to the engagement of TLR4 is fundamentally different from that of other TLRs.

Although rat iLDCs constitutively express the full repertoire of TLR mRNAs, differences in expression level among the three iLDC populations observed are likely related to their distinct functions. The CD172a^+^ subset of rat iLDCs, which is specialized for the uptake of apoptotic enterocytes (15), expressed low or non-detectable mRNA for both TLR7 and TLR8 (Fig. 1B). In addition, the CD172a^-^ iLDC subset showed higher levels of TLR3 mRNA expression than either CD172a^+^ population. A similar pattern of TLR3 and TLR7 expression is found in rat splenic CD172a^-^ CD4^-^ DCs (31) as well as in mouse CD8α^-^ DCs (32), which have also been shown to be specialized for apoptotic cell uptake (33, 34). Down-regulation of TLR7 could represent a possible mechanism of avoiding activation by host cellular ssRNA present in apoptotic cells, whereas TLR3, which is activated by dsRNA, may have evolved as a mechanism for surveying the viral status of phagocyted apoptotic cells (35). Thus differential expression of TLR7, TLR8, and TLR3 might equip CD172a^-^ DCs to switch from tolerance induction in the steady state to proinflammatory priming of Th1 cells and cross-priming of CTLs in virus infection.

The fact that all TLR agonists, with the exception of LPS, induced a significant increase in the proportion of activated intestinal DCs (Fig. 2) is consistent with the reported responsiveness of mouse LP CD11c^-^ DCs to the TLR5 agonist bacterial flagellin (7, 11) and with our previously published data on the activation of rat iLDCs by the TLR7/TLR8 agonist R848 (12). These data suggest that intestinal DCs are not completely hyporesponsive to TLR stimulation. Nonetheless, in agreement with the proposed tolerogenic role of steady-state intestinal DCs (36), iLDCs produced IL-10 in culture. However, the level of IL-10 was not consistently increased by stimulation with any TLR agonist over a range of concentrations (not shown). At the same time, stimulation through TLR2 and TLR9 increased the secretion of IL-6 and IL-12 p40 by all three subsets of iLDCs (Figs. 3D and 4). Interestingly, TNF-α was exclusively produced by the CD172a^-^ subset of iLDCs, further emphasizing the possibility that these cells may acquire a proinflammatory function upon activation. Likewise, Uematsu et al. found that bacterial flagellin stimulated mouse CD11c^-^ CD11b^-^ LP DCs to produce IL-6, IL-12p40, and also IL-12p70 (11). In sharp contrast, Monteleone et al., reported that mouse LP DCs were unable to produce IL-12p40 or IL-12p70 in vitro via an IL-10-mediated mechanism (8). The reason for this difference is unclear but could be caused by the distinct experimental systems used. Collectively, our data suggest that the cytokine output of iLDC is not hardwired but is instead continually modulated by signals from the environment.

The physiological significance and mechanism for the selective hyporesponsiveness of intestinal DCs to LPS remains largely unclear. One possibility is that TLR4 might require more rigorous regulation than other TLRs. Uniquely, TLR4 is the only TLR that activates both the MyD88/TIRAP- and the TRIF/TRAM-dependent signaling pathways (reviewed in Ref. 37) and could hence act as a far stronger signal for DC activation.

Although intestinal DCs can clearly be activated by TLR agonists in vitro, it is not inconceivable that in vivo they may be largely excluded from engaging commensal bacteria by the physical barrier of the intestinal epithelium. For this reason, heat-inactivated spores of *B. subtilis* (which express PAMPs dependent on both TLR2 recognition (our unpublished data) and likely other PRRs) were used to model as closely as possible the interaction of intestinal DCs and luminal bacteria in vivo. Strikingly, a significantly higher proportion of migrating iLDCs expressed activation markers from the rats administered *B. subtilis* spores intraintestinally. Moreover, the activated DC population contained internalized fluorescent spores, strongly suggesting that their activation is due, at least in part, to direct interaction with *B. subtilis*. These data offer a clear and direct demonstration that intestinal DCs can capture bacteria in the lumen of the intestine and transport them via the lymphatics to MLNs. This then raises the question as to why continuous TLR stimulation of intestinal DCs does not lead to the development of proinflammatory pathology. The conditioning of intestinal DCs by their local microenvironment might represent one potential mechanism. DCs conditioned by factors such as thymic stromal lymphopoietin (TSLP) (44) may allow intestinal DCs to maintain responsiveness to TLR stimulation but program them to respond in a noninflammatory manner.

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^3 The online version of this article contains supplemental material.
The complete abolition of TLR signaling in intestinal DCs may in fact be detrimental to the host. TLR2, along with its coreceptors, recognizes PAMPs from a broad array of organisms including Gram-positive (42) and Gram-negative bacteria (43), fungi (44), protists (45), helminths (46), and viruses (47). Down-regulation of this TLR-mediated surveillance mechanism as well as that of other TLRs in intestinal DCs could severely compromise the induction of protective immunity to pathogens and the initiation of localized mucosal immune responses against commensal bacteria (48). Additionally, however, expression and tonic engagement of TLRs by PAMPs in the intestine may contribute to the maintenance of intestinal homeostasis by having a protective role in the induction of IBDs (23, 49–51) or by acting as ‘natural adjuvants’ limiting the induction of tolerogenic mechanisms (52).

The precise mechanism that maintains the selective unresponsiveness of intestinal DCs to TLR4 stimulation is currently unknown. The data presented here unequivocally demonstrate that intestinal DCs are able to respond to TLR signals while maintaining unresponsiveness to LPS. Hence, the mechanism underpinning LPS unresponsiveness is undoubtedly upstream of the shared components of the TLR signaling pathways. An attractive hypothesis to accommodate the refractiveness of iLDC to LPS is that TLR4 is specifically underexpressed in intestinal DCs. Indeed, mRNA expression data (Fig. 1B) as well as independent data from mouse (7–9), human (10), and rat (20) all suggest that TLR4 is expressed at low levels in intestinal DCs and could thus represent a potential mechanism for maintaining LPS unresponsiveness. In addition, it will be important to analyze whether alternative mechanisms, such as down-regulation of other components of the LPS signaling complex or the presence of TLR4-specific inhibitors (53, 54), may also contribute to the insensitivity of intestinal DCs to LPS stimulation.

Although the lack of responsiveness to LPS stimulation may represent a mechanism for avoiding unwanted inflammatory responses in the intestine, we found that intestinal DCs can be activated by other TLR agonists, including TLR2 and TLR9. Important questions regarding the activation of intestinal DCs by TLR agonists remain to be addressed, particularly the functional outcome of their interaction with naïve T cells in the MLNs. Although steady-state intestinal DCs appear to mainly support the development of Th2 or regulatory T cells (36, 55), TLR5-stimulated intestinal DCs appear to mainly support the development of Th1 or Th2 cells (56). TLR5 stimulation may also contribute to the insensitivity of intestinal DCs to LPS stimulation (57).

In summary, our study significantly advances the current understanding of how intestinal DCs react to innate immune stimuli. Fundamental knowledge of which stimuli lead to the induction of protective immune responses in the intestine is central to enabling the rational design of novel mucosal adjuvants and vaccines. In addition, understanding the initial events that underpin immune responses could prove valuable in elucidating pathways that can lead to inflammation, which may inform the identification of novel therapeutic targets for the treatment of IBDs.

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