The Journal of Immunology

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J Immunol 2006; 176:5205-5212; ;
doi: 10.4049/jimmunol.176.9.5205
http://www.jimmunol.org/content/176/9/5205

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Regulation of Intestinal Dendritic Cell Migration and Activation by Plasmacytoid Dendritic Cells, TNF-α and Type 1 IFNs after Feeding a TLR7/8 Ligand

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Dendritic cells (DCs) migrating via lymph are the primary influence regulating naive T cell differentiation, be it active immunity or tolerance. How DCs achieve this regulation in vivo is poorly understood. Intestinal DCs are in direct contact with harmless or pathogenic luminal contents, but may also be influenced by signals from epithelial cells, macrophages, or other resident or immigrant cells. To understand the role of TLR7 and TLR8 in regulating intestinal DC function, we fed a TLR7/8 ligand (resiquimod (R-848)) to rats and mice and examined DC in pseudoafferent lymph (rat) and mesenteric lymph nodes (MLNs). Oral R-848 induced a 20- to 30-fold increase in DC output from the intestine within 10 h due to a virtually total release of lamina propria DCs. This resulted in an accumulation of DCs in the MLNs that in mice was completely TNF-α dependent. Surprisingly, intestinal lymph DCs (iL-DCs) released by R-848 did not up-regulate CD86, but did up-regulate CD25. In contrast, MLN-DCs from R-848-stimulated rats and mice expressed high levels of CD86. This DC activation in MLNs was dependent on type 1 IFNs. The major source of these rapidly released cytokines is plasmacytoid DCs (pDCs) and not classical DCs, because depletion of pDCs significantly reduces the R-848-stimulated increase in serum cytokine levels as well as the accumulation and activation of DCs in MLNs. These experiments show that TLR-mediated regulation of iL-DC functions in vivo is complex and does not depend only on direct iL-DC stimulation, but can be regulated by pDCs. *The Journal of Immunology,* **2006, 176: 5205–5212.**

The nature of the immune response to exogenous Ags depends on the context in which Ag is recognized by lymphocytes. Dendritic cells (DCs)[2] are rare cells present in all peripheral tissues that continually migrate via afferent lymph to the draining lymph nodes (LNs) where they present peripherally acquired Ags to naive T and B lymphocytes. Under steady-state conditions, this interaction induces T cell tolerance. However, microbial stimulation leads to DC activation and the subsequent generation of effector lymphocytes (1). Understanding how different peripheral conditions modulate DC migration and activation is crucial to understanding immune regulation. This is particularly true in the intestine, where DC can be in direct contact with luminal contents (2, 3). Studying the peripheral conditioning of intestinal DCs after in vivo stimulation is problematic, however, because techniques used to purify DCs can induce phenotypic and functional changes. Additionally, DCs represent heterogeneous populations of cells, and in LNs they can arrive via blood as well as afferent lymph. This makes the identification of migratory DC in LNs difficult unless there are specific markers such as Birbeck granules and langerin for Langerhans cells (LCs). There is thus limited information available about the factors that regulate activation and migration of intestinal DCs in vivo.

Cells of the innate immune system and other cells (e.g., enterocytes) express germline-encoded receptors that recognize surface molecules expressed by pathogens. These receptors include TLRs that induce active immune responses by direct or indirect activation of APCs, primarily DCs, which then express higher levels of costimulatory molecules and secrete proinflammatory cytokines (reviewed in Ref. 4). Single-stranded RNA is a natural ligand for TLR7 or TLR8 (depending on the species) (5, 6). Synthetic agonists for TLR7 and TLR8, such as imiquimod and resiquimod (R-848), are potent activators of innate cells (7). R-848 and related compounds stimulate murine splenic CD11c^{high} DCs both in vitro and in vivo after i.v. injection (8, 9). These DC express activation markers and secrete IL-12 and TNF-α, and in vivo migrate to T cell areas (TCAs) (8, 10). Preferentially, the CD11b^{+} subset of murine splenic CD11c^{high} DCs secretes cytokines after R-848 stimulation, and in contrast to CD11b^{-} DCs, these CD11b^{+} DCs express TLR7 (8, 9). Topical administration of imiquimod to rodents causes increased exit of LCs from the skin, but other migratory DC populations have not been studied (11).

Plasmacytoid DCs (pDCs) are specialized cells that secrete cytokines, in particular very large amounts of type I IFNs, in response to viral stimuli (12). Plasmacytoid DCs were initially isolated from human blood, but were later identified in rodent bone marrow (13), blood (14), spleen (15, 16), LN (15, 16), Peyer's patches (PPs) (17), and lung (18). Plasmacytoid DCs express high levels of transcripts for TLR7 and TLR9, explaining their strong responses to ssRNA (5, 6, 19) and DNA viruses (20, 21). Human (22, 23) and murine (9) pDCs also secrete cytokines in response to resiquimod stimulation in vitro. Recently, it has been shown that after i.v. administration of R-848, murine splenic pDC secrete IFN-α, up-regulate costimulatory molecules, and form clusters in

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Received for publication December 7, 2005. Accepted for publication February 9, 2006.

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[2] Abbreviations used in this paper: DC, dendritic cell; CBA, cytometric bead assay; iL-DC, intestinal lymph DC; LC, Langerhans cell; LN, lymph node; LP, lamina propria; MLN, mesenteric lymph node; MLNX, mesenteric lymphadenectomy; pDC, plasmacytoid DC; PP, Peyer's patch; R-848, resiquimod; TCA, T cell area; TDL, thoracic duct leukocyte; WT, wild type.

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TCAs (10). How pDC respond in the GALT to oral TLR7/8 ligands, however, has not yet been studied.

There is a great need for effective oral vaccines, and oral R-848 has been shown to have adjuvant properties in mice (24). DCs migrating in lymph are the cells that both transport Ag and transduce information that informs T cell differentiation. Thus, oral adjuvants probably work by inducing changes in the properties of migrating intestinal lymph DCs (iL-DCs). To investigate iL-DCs, we collected them by thoracic duct cannulation in rats that had previously been mesenteric lymphadenectomized (MLNX). This enabled us to study, with minimal in vitro manipulation, DCs that had very recently left the intestine (25).

In this study we have analyzed the effects of orally administered R-848 on intestinally derived DCs in lymph and mesenteric lymph node (MLN). To understand the cytokine-mediated interplay between pDCs and intestinal DCs, we have also used mice unable to respond to certain cytokines. We show that feeding R-848 leads to a 100-fold increase in the frequency of iL-DCs, representing a complete release of DC from the intestinal lamina propria (LP). The accumulation and subsequent activation of iL-DCs in the MLN are largely dependent on TNF-α and IFN-α, respectively. Finally, we show that activated pDCs are responsible for the rapid secretion of the bulk of these cytokines and that this secretion has effects on the accumulation and activation of DCs in the GALT.

Materials and Methods

Animals and surgical procedures

PVG (RT1^c) rats, IFN-$\alpha\beta$R$^{-/-}$ mice, and 129SvEv mice were bred and maintained under specific pathogen-free conditions at Sir William Dunn School of Pathology. Rats were males, 12–24 wk of age. Mice were age-matched females. All procedures were conducted in accordance with Home Office guidelines. MLNX of rats and thoracic duct cannulation were performed as described previously (25).

Reagents and mAbs

Cell cultures were grown in IMDM with 5% FCS, 50 mM 2-ME, 100 mg/ml penicillin, and 50 U/ml streptomycin (5% IMDM; all from Invitrogen Life Technologies). R-848 (InvivoGen) was dissolved in water and given orally (50 μg/rat and 10 μg/mouse). Human rCD40L trimer (hrCD40L) was provided by Amgen.

mAb 120.G8 (17) was obtained from Schering-Plough. Blocking hamster anti-murine TNF-α (TN3-19.12g1) (26, 27) was provided by Celltech.

mAbs to rat Ags, Igκ (OX12), TCR-α (R73), CD6 (OX52), CD8 (OX8), CD45RA (OX33), CD103 (OX62), and CD172a (OX41), were purified from cell culture supernatants and used unconjugated or conjugated to FITC or biotin. PerCP-labeled anti-MHC-II (OX6), PE-labeled anti-CD25 (OX39), and streptavidin-PE and -allophycocyanin were all purchased from BD Pharmingen. PE-labeled anti-CD86 (24F) was purchased from Serotec.

mAbs to mouse Ags, anti-CD16/32 (2.4G2), was purified from cell culture supernatants and used unconjugated. FITC-labeled anti-CD19 (1D3; Caltag Laboratories), PE-labeled anti-CD11c (HL3), PerCP-labeled anti-CD45R (B220), allophycocyanin-labeled anti-CD11b (M1/70) and Ly6C/G (GR-1), and biotinylated anti-CD86 (GL1) were all purchased from BD Pharmingen.

Isolation of cells

Thoracic duct leukocytes (TDLs) were collected on ice in PBS with 10 mM EDTA and 20 U/ml heparin. They were passed through a 70-mm pore size cell strainer (BD Biosciences), and RBCs were lysed with ammonium chloride-potassium carbonate lysis buffer.

LN and spleens were diced and treated for 30 min at 37°C in 5% IMDM with 1 mg/ml collagenase type IV (Roche) and 0.25 mg/ml DNase I (Roche). Cells were then passed through a cell strainer, and RBCs were lysed. For analysis of rat MLN-DC activation, MLNs were depleted of T and B cells using anti-CD6, -CD8, and -CD45RA, followed by goat anti-mouse Dynabeads (Dynal Biotech). Mouse DCs and pDCs were enriched using anti-CD11c MACS beads and AutoMACS (Miltenyi Biotec) according to the manufacturer's protocols. CD11c$^+$ cells were additionally purified by cell sorting using a MoFlo (DakoCytomation). The purity of the MoFlo-sorted cell populations was >95%.

FACS

Surface staining for FACS was performed in PBS with 2% FCS and 10 mM EDTA for 15 min on ice after blocking in 10% rat serum or anti-murine CD16/32. Cells were then fixed in 2% paraformaldehyde and analyzed using a FACSCalibur (BD Biosciences).

Tissue immunofluorescence

MLN and ileum containing PPs were removed under terminal anesthesia and snap-frozen. Sections (5 μm) were fixed in acetone, and fluorescently labeled sections were prepared and analyzed for the expression of CD103 and MHC class II as described previously (28). Where primary Abs against both CD103 and CD172a were used, both signals required amplification. Sections were blocked again after the tyramide reaction, and an alkaline phosphatase-based ELF-97 kit (Molecular Probes) was used to detect binding of anti-CD172a according to the manufacturer's instructions. The figures show representative images of many sections studied using tissues from at least two animals.

Quantitation of cytokine production

Production of mouse IL-6, IL-12p70, and TNF-α was measured by cytometric bead assay (CBA; BD Pharmingen), and rat TNF-α was measured by ELISA (BD Pharmingen) according to the manufacturer's protocols. IFN-α was measured by ELISA using a rat mAb against mouse IFN-α (RMMA-1; PBL Biomedical Laboratories) as capture Ab and a polyclonal rabbit anti-mouse IFN-α (PBL Biomedical Laboratories) as detection Ab. The biological activity of IFN-$\alpha\beta$ was confirmed by bioassay using a cytopathic effect reduction assay with L929 cells and cocal virus as a challenge virus, similar to that previously described (28).

Statistical analysis

Statistically significant differences between groups were determined by the nonparametric Mann-Whitney *U* test (for two groups) or one-factor ANOVA, followed by Tukey's test (for three or more groups).

Results

Oral R-848 induces rapid changes in the output of DCs and lymphocytes into intestinal and liver lymph

To assess the effect of a TLR7/8 agonist on iL-DCs, R-848 was fed to thoracic duct-cannulated MLNX rats. Lymph was collected on ice for 2-h periods. The iL-DCs were identified by microscopy as large veiled cells (Fig. 1*A*) and in FACS by high expression of MHC-II and CD103 (Fig. 1*B*). The frequency of DCs among all leukocytes started to increase by 2 h after feeding (data not shown) and was maximal by 4–8 h, an ~100-fold rise (Fig. 1*B*), when they represented >30% of lymph leukocytes. This dramatic increase in DC frequency reflected two factors. The total numbers of DCs exiting the intestine increased markedly, ~30-fold by 10–12 h after feeding (Fig. 1*C*). In addition, the increase in DC output was accompanied by an almost complete loss of lymphocyte output (data not shown). The numbers of DCs and lymphocytes in lymph returned to normal by 24 h. These experiments show that oral R-848 induces a dramatic increase in the numbers of DCs entering intestinal afferent lymphatics.

Anatomical localization of DCs in the intestine, PP, and MLN after oral R-848

To determine the origin and fate of DCs released by R-848, cryostat sections of intestine and MLNs were examined by immunofluorescence. MLN from control rats contained DCs (CD103$^+$, MHC class II$^+$) in the TCAs and interfollicular regions (Fig. 2*A*). By 12 h after oral R-848 administration, the numbers of DCs in both regions increased dramatically (Fig. 2*E*). This suggests that DCs were entering TCAs via interfollicular traffic areas, implying their origin from afferent lymph. The two main subsets of rat DCs, CD103$^+$/CD172a^{high} and CD103$^+$/CD172a^{low} DCs, can be differentiated in tissues microscopically. Consistent with previous data (29), CD172^{high} DCs are largely excluded from the central regions of TCAs of MLNs in control animals (Fig. 2*B*). By 12 h after

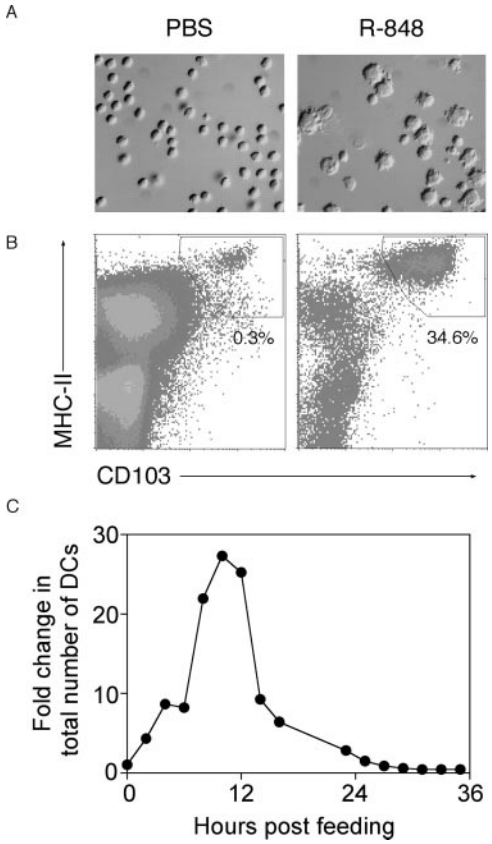

FIGURE 1. Effects of feeding R-848 on TDLs from MLNX rats. Thoracic ducts of MLNX rats were cannulated, and animals were fed PBS or R-848 (50 μg/rat) by gavage. TDLs were collected over 2-h periods. *A*, Cells collected between 4 and 6 h after feeding were examined by Nomarski interference microscopy (×40 objective). *B*, Cells were counted and stained for FACS. Numbers in the dot plots represent the percentage of iL-DCs ($CD103^+MHC\text{-}II^{high}$) among total TDLs collected between 4 and 6 h after feeding. *C*, The total number of iL-DCs was determined from the percentage of iL-DCs among total leukocytes per collection per rat as shown in *B*. The fold increase in total cell output were then calculated from the mean values of two R-848-fed animals compared with the mean values of two control animals. These results are representative of four independent experiments.

feeding R-848, however, both $CD172^{high}$ and $CD172^{low}$ DCs are plentiful in these regions (Fig. 2*F*). In the lamina propria in control rats, many large, irregularly shaped DCs ($CD103^+$, MHC class II^+) were present, particularly toward the distal ends of villi. By 12 h after feeding R-848, such DCs were no longer detectable (Fig. 2, *C* and *G*), consistent with the finding that large numbers of DC had entered afferent lymph. Examination of PPs from R848-treated animals showed that DCs ($CD103^+$ cells) were no longer present in subepithelial domes and were now abundant in interfollicular TCAs (Fig. 2, *D* and *H*). These experiments show that oral R-848 rapidly induces a complete loss of LP DCs and an altered distribution of PP DCs, associated with the appearance of CD172a-expressing DCs in the TCA of both MLN and PP.

Oral R-848 induces increased expression of CD86 by MLN-DCs, but not by iL-DCs

To determine whether oral R-848 caused intestinal DC activation, we collected iL-DC over 0–6, 6–12, and 12–24 h after feeding and labeled them for activation markers. At no time did these migrating DC show an increase in the expression of CD86, not even at the peak of output 6–12 h after feeding (Fig. 3*A*). The same results were obtained after staining of CD40 and CD80 (data not shown). In contrast, CD25 expression was already up-regulated on iL-DCs collected over 0–6 h (data not shown), and expression was maximal at 6–12 h compared with controls (Fig. 3*A*). The expression of CD25 by iL-DC then gradually waned to reach control levels by 36 h (data not shown).

In marked contrast to DCs released into intestinal lymph, DCs purified from MLN 18 h after feeding R-848 expressed increased levels of CD86 compared with MLN-DCs isolated from rats fed PBS (Fig. 3*B*). DCs purified from MLN also expressed increased levels of CD25. Thus, oral R-848 stimulates increased expression of CD86 by DCs in MLNs, but not in lymph, suggesting that factors acting locally in MLNs are responsible for DC activation.

In attempting to characterize the molecular bases of these changes in the rat, we were hampered by the lack of genetically modified strains and suitable reagents. Investigations were thus pursued in mice. To confirm that R-848 stimulated intestinal DC release in mice, both rats and mice were fed titrated doses of R-848 corrected for body weight. After 18 h, the percentages of DCs in spleen and MLN were determined by FACS (Fig. 3*C*). Rats and mice showed similar changes in the percentage of DCs in MLN after oral doses of R-848. Thus, R-848 has similar actions on intestinal DCs in both species.

Oral R-848 stimulates differential cytokine secretion by pDCs and DC subsets

It has been shown that i.v. administration of TLR7/8 ligands results in very rapid release of inflammatory cytokines into the blood (8). To determine whether oral R-848 had similar effects, serum cytokine levels were measured in mice 90 min after feeding R-848. High levels of IL-6, IL-12p70, TNF-α, and IFN-α were detected in the sera of all mice fed R-848 compared with controls (Fig. 4*A*).

To identify DCs as a source of these cytokines, we purified DC subsets ($CD11b^+$ DCs, $CD11b^-$ DCs, and pDCs) from murine MLNs 90 min after feeding R-848 (Fig. 4*B*). These DCs were cultured without any additional stimuli, and the levels of cytokines secreted were measured in supernatants 24 h later. $CD11b^+$ and $CD11b^-$ DCs secreted barely detectable amounts of IL-6, IL-12p70, and TNF-α (Fig. 4*C*), whereas pDCs produced at least 100-fold higher levels of all three cytokines. In addition, pDCs were the only cells able to produce detectable amounts of IFN-α, the activity of which was confirmed by bioassay (data not shown). In contrast to MLN $CD11b^+$ DCs, splenic $CD11b^+$ DC DCs rapidly secreted TNF-α after oral R-848 (Fig. 4*D*), confirming previous reports using i.v. injection of a TLR7/8 agonist (8).

These experiments show that oral R-848 stimulates rapid secretion of IL-6, IL-12p70, TNF-α, and IFN-α, and that in the MLN, these cytokines are produced by pDCs and not by resident $CD11b^+$ or $CD11b^-$ DCs.

TNF-α mediates release of intestinal DCs, but IFN-$\alpha\beta$ is required for increased expression of CD86 by MLN-DCs after oral R-848

To define the molecular basis of intestinal DC release and activation, experiments were performed using a blocking anti-TNF-α mAb and IFN-$\alpha\beta R^{-/-}$ mice. Wild-type (WT) mice were pretreated with an anti-TNF-α mAb and then fed R-848. Eighteen hours later, DCs were extracted from MLNs, and their absolute numbers were determined. Anti-TNF-α treatment completely abrogated the R-848-stimulated accumulation of DC in the MLN (Fig. 5*A*). In contrast, IFN-$\alpha\beta R^{-/-}$ mice that had been fed R-848 showed no significant abrogation of DC accumulation in MLN compared with WT mice. In the same experiments, the numbers of pDCs in the MLN were assessed. Oral R-848 caused no significant

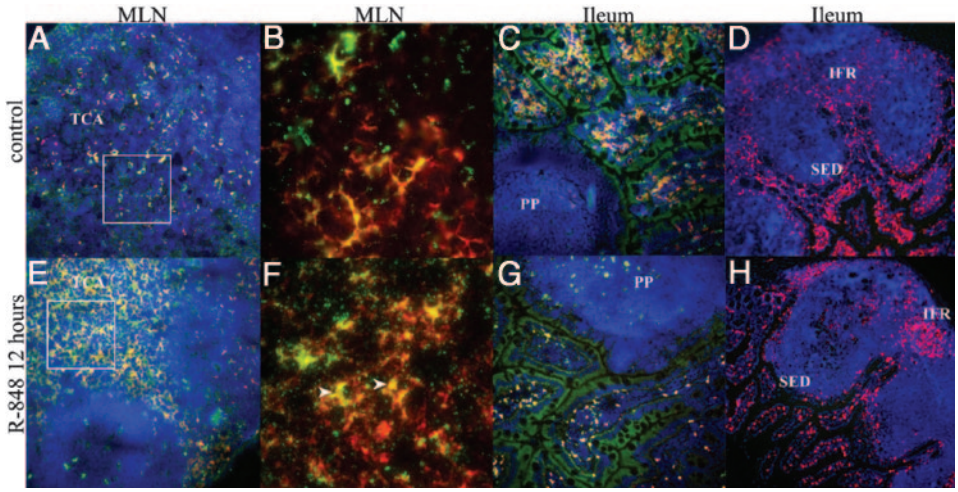

FIGURE 2. Effects of feeding R-848 on MLN-, PP-, and intestinal-DCs. Cryostat sections of MLN or ileum were stained with fluorescent Abs. Tissues were taken from untreated rats (*A–D*) and from rats 12 h after the administration of oral R-848 (*E–H*). Sections were stained with DAPI (blue) and CD103 (red) and photographed with a ×40 objective (*A*, *C*, *D*, *E*, *G*, and *H*). Sections in *A*, *C*, *E*, and *G* were double stained for CD103 and MHC class II (green). Squares within *A* and *E* correspond to the regions shown in *B* and *F*, which were stained for CD172a (green) and CD103 (red) and examined using a ×100 objective. Arrows indicate the locations of $CD103^+/CD172a^{high}$ cells in ×100 sections from R-848-treated animals (*E*). IFR, interfollicular region; SED, subepithelial dome.

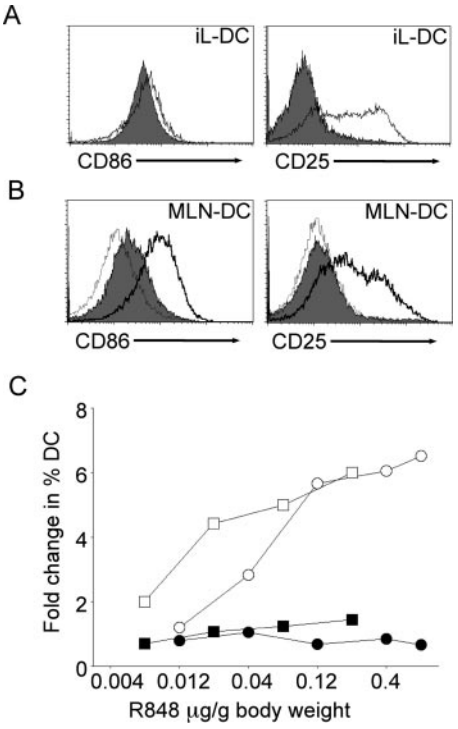

FIGURE 3. Effects of feeding R-848 on iL-DC activation and MLN-DC numbers and activation in rats and mice. *A*, TDLs were collected between 6 and 12 h after feeding PBS (■) or R-848 (□). The iL-DCs (identified as large $MHC\text{-}II^{high}TCR\alpha\beta^-IgK^-$ cells) were examined for CD86 and CD25 expression. Similar results were obtained at 24 h and when cells were gated as $CD103^+$ (data not shown). *B*, MLN-DCs (identified as $MHC\text{-}II^{high}CD103^+$ cells) were extracted by collagenase digestion 18 h after feeding PBS (filled histograms) or R-848 (open histograms) and examined for CD86 and CD25 expression. The dotted line shows isotype control staining. *C*, Eighteen hours after feeding titrated doses of R-848, MLN-DCs (○ and □) or spl-DCs (● and ■) were extracted by collagenase digestion from rats (□ and ■; identified as large $MHC\text{-}II^{high}CD103^+$ cells) or mice (○ and ●; identified as large $CD11c^{high}B220^-CD19^-$ cells). The percentage of DCs after feeding R-848 was compared with that in PBS-fed animals. Data are representative of at least two independent experiments.

change in pDC numbers in the MLN of WT and only a slight increase in IFN-$\alpha\beta R^{-/-}$ mice regardless of whether they were pretreated with anti-TNF-α mAb (Fig. 5*A*).

To determine the roles of TNF-α and IFN-$\alpha\beta$ in R-848-induced MLN-DC and pDC activation, CD86 expression was assessed. In WT mice pretreated with anti-TNF-α and fed R-848, the increase in CD86 expression on DCs was significant and was only slightly less than that in controls (Fig. 5*B*). In pDCs, the increase was significant and was also unaffected by anti-TNF-α pretreatment (Fig. 5*B*). In contrast, when IFN-$\alpha\beta R^{-/-}$ mice were given oral R-848, there was only a minor increase in CD86 expression on MLN-DCs and pDCs, and this increase was significantly less than that on WT MLN-DCs and -pDCs.

These experiments show that there are distinct molecular requirements for the effects of oral R-848 on intestinal DC migration and activation. The release of DCs from the intestine is dependent on TNF-α, whereas the activation of DCs in the MLN is dependent on type 1 IFN.

Cytokines secreted by pDCs mediate accumulation and activation of MLN-DCs

Because oral R-848 stimulates rapid and potent secretion of both TNF-α and IFN-α by MLN pDCs, and these cytokines have dramatic effects on classical MLN-DCs, it was an attractive hypothesis that pDCs influenced the migration and activation of intestinal DC. To test this, mice were treated daily for 3 days with the pDC-depleting Ab 120.G8 (17). This resulted in significant, but incomplete, depletion of pDCs in MLN (Fig. 6*A*). Four hours after the last injection, 120.G8-treated mice or controls were fed R-848, and serum levels of cytokines were measured 90 min later. Serum levels of IL-12p70, TNF-α, and IFN-α were significantly reduced in 120.G8-treated mice (Fig. 6*B*).

To assess the effects of pDC depletion on the release of intestinal DC by R-848, 120.G8-treated mice and control animals were fed R-848; 18 h later, MLN-DC numbers were determined. The increase in MLN-DC number normally stimulated by oral R-848 was significantly reduced in pDC-depleted mice compared with controls (Fig. 6*C*). MLN-DC activation after oral R-848, as assessed by CD86 expression, was also reduced significantly in mice treated with 120.G8 compared with controls (Fig. 6*D*).

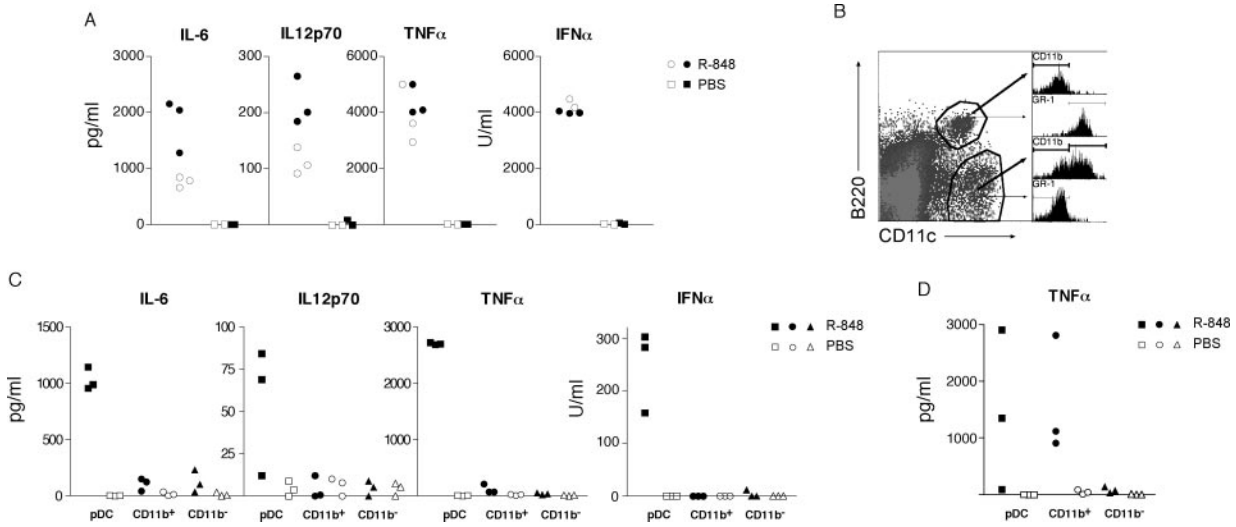

FIGURE 4. Cytokine secretion by murine MLN-DCs and pDCs after feeding R-848. *A*, Serum was collected from mice fed 90 min previously PBS (□ and ■) or 10 μg of R-848 (○ and ●). Sera were assayed by CBA or ELISA for cytokines. The graphs show two independent experiments, indicated by open or filled symbols. Each symbol represents one mouse. *B–D*, Mice were fed PBS (○, □, and △) or R-848 (●, ■, and ▲). After 90 min, MLN (*C*) and spleen (*D*) were removed, and after collagenase digestion, DC subsets were sorted by FACS. *B*, The gates used to isolate MLN pDC ($CD11c^{dim}B220^{+}GR\text{-}1^{+}CD11b^{-}$) and DCs ($CD11c^{high}B220^{-}GR\text{-}1^{-}CD11b^{+}$ or $^{-}$) are shown. MLN-pDCs (*C*) or spleen-pDCs (*D*; □ and ■), $CD11b^{+}$ DCs (○ and ●) or $CD11b^{-}$ DCs (▲ and △) were pooled from 7 to 10 mice that had been fed PBS (○, □, and △) or R-848 (●, ■, and ▲) 90 min previously. Sorted pDCs (9×10^4) or sorted DCs (1.5×10^5) were cultured for 24 h, and supernatants were assayed by CBA or ELISA. Data from two or three experiments are shown, where each symbol represents one independent experiment.

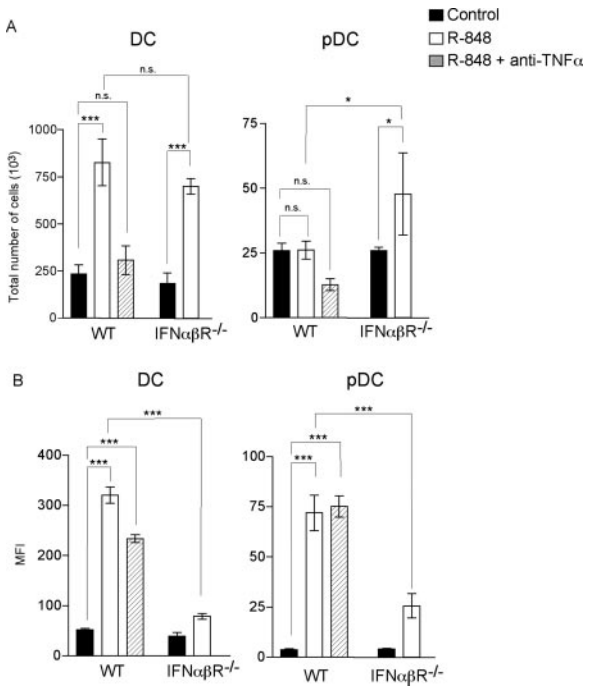

FIGURE 5. Roles of TNF-α and IFN-$\alpha\beta$R in activation of MLN-DCs after feeding R-848. WT or IFN$\alpha\beta R^{-/-}$ mice were fed R-848 (10 μg/mouse). Eighteen hours later, DCs were extracted by collagenase digestion from MLNs of control (■) or R-848-treated (□) mice, counted, and analyzed by FACS for CD86 expression. Some mice were given anti-TNF-α mAb (0.5 mg/mouse) i.v. 90 min before feeding R-848 (▨). *A*, Total numbers of DCs ($CD11c^{high}CD19^{-}B220^{-}$) and pDCs ($CD11c^{dim}CD19^{-}B220^{+}$). *B*, Expression levels of CD86 by DCs and pDCs. Results are presented as the mean ± SD of triplicate determinations and are representative of two independent experiments. Statistical analysis was performed using one-factor ANOVA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Together these data show that in response to oral R848, pDCs produce TNF-α and IFN-α, which contribute substantially to the release and activation of intestinal DC.

Discussion

The intestinal immune system is exposed to a multiplicity of harmless and pathogen-associated Ags and responds differentially to these Ags. DCs sample luminal contents, transport Ags, and transduce information crucial for regulation of the subsequent adaptive response. Numerous in vitro studies demonstrate that microbial stimuli acting, in particular, via TLRs and/or inflammatory cytokines, influence the migration and maturation of DCs. How these environmental cues are interpreted by DCs present in the intestine and associated lymphoid tissues in vivo is still not understood. To study the regulation of intestinal DC function with minimal manipulation, we used a rat model that permitted collection of DCs directly from intestinal lymph both in the steady state and after feeding a TLR7/8 ligand.

Steady-state iL-DCs comprise ~0.3–0.4% of TDLs in pseudoafferent intestinal lymph. Feeding R-848 leads to rapid and dramatic changes. By 4–8 h after feeding, iL-DCs constitute ~30–40% of TDLs. This 100-fold increase in frequency is due to a dramatic increase in the output of DCs from the intestine and a transient, but virtually complete, drop in the number of migrating lymphocytes. A second feeding of R-848, 24 h after the first, induces the same reduction in lymphocytes in thoracic duct lymph, but virtually no increase in iL-DC output (data not shown). This shows that the rats can still respond to R-848 and implies that all DCs in the gut were released after the first feeding. Topical administration of the related molecule, imiquimod, induces a decrease in LC number in the skin (11) as well as migration of intradermally injected immature DC to the draining LN (30). The kinetics of release in the skin, however, are very slow compared with those in the gut: it takes several days for the number of LCs to be halved, whereas we show that essentially all intestinal DC are

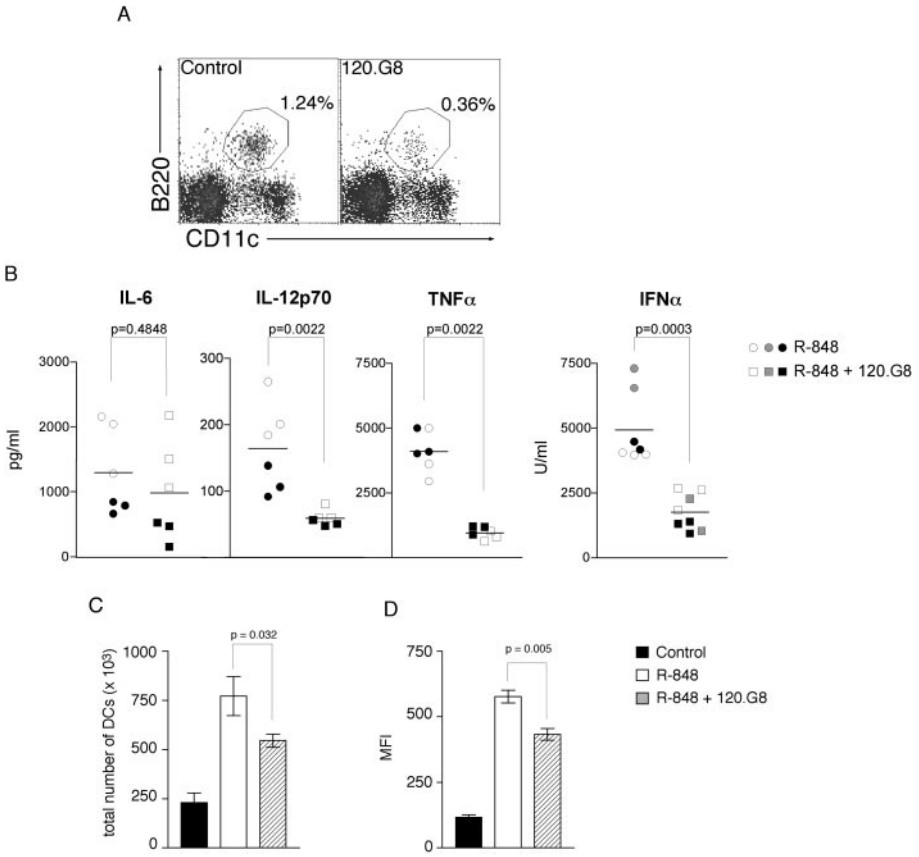

FIGURE 6. The role of pDCs in release and activation of MLN-DCs after feeding R-848. *A*, FACS analysis of CD19$^-$ cells from MLNs of mice that had received 120.G8 (0.5 mg/mouse) for 3 consecutive days (*right panel*) or control mice (*left panel*). Numbers in plots show the percentage of pDCs among CD19$^-$ cells. *B*, Cytokine levels in mouse sera 90 min after feeding R-848 (10 μg/mouse) to mice that had previously received 120.G8 (as described in *A*; (squares) or not (circles). The sera were assayed by CBA or ELISA. Two or three independent experiments are shown, indicated by open, gray, or black symbols. Each symbol represents one mouse, and the horizontal line is the mean value; *p* values were calculated using a nonparametric Mann-Whitney *U* test. Total numbers of (*C*) and expression levels of CD86 (*D*) by MLN-DCs (CD11c^{high}CD19$^-$B220$^-$) 16 h after feeding PBS (■), R-848 (□), or R-848 to mice that had previously been given 120.G8 (▨) as described in *A*. Results are presented as the mean ± SEM, where the control group consisted of three mice and the R-848-fed groups consisted of nine animals per group. The results are representative of three independent experiments. Statistical analysis was performed using a nonparametric Mann-Whitney *U* test.

released by 12 h after feeding R-848. The rapid release of intestinal DC compared with LCs could be due to a variety of causes, including the pharmacokinetics of the compound, differential expression of TLR7/8 by DCs themselves, and tissue-specific expression of TLRs by other immune or nonimmune cells.

The source of the increased numbers of iL-DC is most likely the LP of the small intestine. In support of this, in sections of this tissue taken from rats fed R-848 12 h previously, we cannot detect LP-DCs, which correlates with the lack of increase in iL-DCs after a second feeding of R-848, 24 h after the first. However, some iL-DCs could originate from PPs. If this occurs, it is likely to be a minor contribution, because a loss of DCs from the subepithelial dome of PPs appears to be balanced by the appearance of increased numbers of DCs in the TCA of PPs. Additionally, by flow cytometry we cannot detect changes in the total number of DCs in PPs in mice fed R-848 (our unpublished observations).

Increased numbers of both iL-DC subsets (CD172a^{high} and CD172a^{low}) released from the intestine correlated with the appearance of both these subsets in the TCA of MLN. This strongly suggests that the released iL-DCs migrate to the TCA of MLN. Moreover, these findings made after oral R-848 treatment are consistent with previous observations obtained after systemic administration of the TLR4 ligand LPS (29). Under steady-state conditions, CD172a^{high} DCs are excluded from the TCAs of lymphoid tissues. Importantly, and in contrast to the effects of i.v. LPS, oral R-848 induces migration of CD172^{high} DC into TCAs of PPs. In addition, murine PP-DCs express increased levels of costimulatory molecules after oral R-848 (our unpublished observations). The different responses of PP-DCs after i.v. LPS and oral R-848 treatments could reflect different local concentrations of the ligand with the two routes of administration. It could also reflect differences in TLR4, TLR7, and TLR8 expression between iL-DCs and PP-DCs, a hypothesis currently under investigation.

Steady-state iL-DCs are semimature, in that they express high levels of MHC-II and CCR7, but low levels of costimulatory molecules (our unpublished observation) (29). Oral R-848 stimulates little, if any, up-regulation of CD86 on iL-DCs at any time point after feeding. However, CD25 expression is up-regulated. We and others (31, 32) have previously shown that CD25 can be up-regulated by DCs from both rat and mouse after in vitro culture, and the receptor has been suggested to have a functional role in DC (33). In contrast, when MLN-DCs were analyzed after feeding R-848, they expressed uniformly high levels of CD86 as well as CD25 compared with MLN-DCs isolated from control animals. The iL-DCs may take more time to up-regulate CD86 than CD25. However, we did not observe increased levels of CD86 on iL-DCs even 24 h after R848 administration. An alternative possibility is that the signal(s) stimulating up-regulation of CD86 is not acting on DC peripherally, but only after they reach the MLN.

We have shown that in mice, oral R-848 stimulates a rapid increase in serum TNF-α, IL-6, IL-12p70, and IFN-α levels, which confirms previous experiments using i.v. TLR7/8 ligands (8, 10). In preliminary experiments using intestinal rat lymph, we have also been able to measure TNF-α (35–50 pg/ml) within 2 h after feeding R-848. We show that of these cytokines, TNF-α and IFN-α are central to the regulation of intestinal DC migration and activation, respectively. Although R848-mediated DC accumulation in the MLN was abrogated by blocking TNF-α in WT mice, DC accumulation was not impaired in IFN-$\alpha\beta$R$^{-/-}$ mice fed R-848. In contrast, IFN-α, but not TNF-α, stimulated CD86 expression on DCs after feeding R-848. The lack of activation was apparent in both CD11b$^+$ and CD11b$^-$ MLN-DCs (data not

shown). Moreover, after feeding R-848, IFN-$\alpha\beta R^{-/-}$ mice showed elevated levels of proinflammatory cytokines comparable to those in WT mice (data not shown). These results define distinct roles for TNF-α and type I IFNs on intestinal DC functions after feeding a TLR7/8 ligand.

Several cell types can produce IFN-α in response to viruses or virally derived products. The most potent producers, however, are pDCs (natural IFN-producing cells) that express TLR7 in mice, humans, and rats (9, 23, 34). Oral R-848 rapidly induces murine MLN-pDC to secrete high levels of IFN-α without additional stimuli and thus could contribute to DC activation. Indeed, partial depletion of pDCs significantly reduces the early systemic secretion of IFN-α and the subsequent activation of DCs in MLNs. However, we could not detect any rapid secretion of IFN-α by $CD11c^{high}$ MLN-DCs, indicating that an autocrine IFN-α loop, as previously described for activation of $CD11c^{high}$ DCs (35), is less likely after feeding R-848. Therefore, although we cannot exclude contributions from other cell types, TLR7 stimulation of murine pDCs after feeding R-848 causes activation of classical intestinal $CD11c^{high}$ DCs by a type 1 IFN-dependent pathway in vivo. A similar interplay between pDCs and DCs dependent on type 1 IFN has been suggested to occur during murine cytomegalovirus infections (36), where IFN-α secretion by pDCs is TLR9 dependent (20).

The pDCs are also a major early source of TNF-α, because purified pDCs from R-848-fed mice secrete high levels of this cytokine. Moreover, partial depletion of pDCs significantly reduces the serum levels of TNF-α stimulated by feeding R-848. Although other TLR7-expressing cells, such as monocytes/macrophages and DC subsets, may contribute to the rapid release of TNF-α, the significant reduction in TNF-α upon pDC depletion suggests a major contribution by pDCs. Our data for oral R-848 differ from those observed after i.v. administration of ssRNA, another TLR7 ligand, where pDC depletion had no effect on TNF-α levels, but reduced serum IFN-α (37). This suggests that the type of TLR7 ligand and/or the route of administration can influence TNF-α secretion. In addition, the expression of TLR7 could differ in systemic lymphoid tissues compared with that in GALT. In support of this, we show that MLN $CD11c^{high}$ DCs do not secrete TNF-α after feeding R-848, whereas splenic $CD11b^{+}$ DC rapidly do so. This selective secretion by splenic $CD11b^{+}$, but not $CD11b^{-}$, DCs correlates with the differential expression of TLR7 mRNA reported in the two subsets (8, 9). The lack of TNF-α secretion by $CD11b^{+}$ MLN-DC suggests that murine intestinal DCs, like rat iL-DCs, may not express TLR7 (U. Yrlid, V. Cerovic, S. W. F. Milling, C. D. Jenkins, L. S. Klavinskis, and G. G. MacPherson, manuscript in preparation).

It has recently been shown that pDCs recruited to the draining LN after a cutaneous herpes simplex virus infection assist $CD11c^{high}$ DC in the generation of CTLs via chemokines and cell-cell interactions (38). In this study we have shown, using our unique rat model that allows direct analysis of iL-DCs, that feeding the TLR agonist R-848 results in complete emptying of DC from the intestinal LP and a concurrent 30-fold increase in migrating intestinal DC. These migrating DC released from the intestinal wall enter T cell areas in MLNs. In mice we show that this DC release is mediated by TNF-α. The migrating DCs do not upregulate CD86 until they reach the MLN, and activation of DCs in MLNs requires type 1 IFN. Finally, we show that pDCs are important and necessary contributors of TNF-α and IFN-α that influence DC migration and activation. These experiments demonstrate a crucial role for TLR7/8 signaling and pDCs in regulating intestinal DC properties and functions in vivo.

Acknowledgments

We thank Prof. M. J. Wick and Drs. H. Uhlig and K. Maloy for their critical reviews of the manuscript, and Nigel Rust for his excellent assistance with cell sorting.

Disclosures

The authors have no financial conflict of interest.

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