Variable Requirement of Dendritic Cells for Recruitment of NK and T Cells to Different TLR Agonists

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TLRs initiate the host immune response to microbial pathogens by activating cells of the innate immune system. Dendritic cells (DCs) can be categorized into two major groups, conventional DCs (including CD8+ and CD8– DCs) and plasmacytoid DCs. In mice, these subsets of DCs express a variety of TLRs, with conventional DCs responding in vitro to predominantly TLR3, TLR4, TLR5, and TLR9 ligands, and plasmacytoid DCs responding mainly to TLR7 and TLR9 ligands. However, the in vivo requirement of DCs to initiate immune responses to specific TLR agonists is not fully known. Using mice depleted of >90% of CD11c+ MHC class II+ DCs, we demonstrate that cellular recruitment, including CD4+ T cell and CX5+DX5+ NK cell recruitment to draining lymph nodes following the footpad administration of TLR4 and TLR9 agonists, is dramatically decreased upon reduction of DC numbers, but type I IFN production can partially substitute for DCs in response to TLR3 and TLR7 agonists. Interestingly, TLR ligands can activate T cells and NK cells in the draining lymph nodes, even with reduced DC numbers. The findings reveal considerable plasticity in the response to TLR agonists, with TLR4 and TLR5 agonists sharing the requirement for DCs for subsequent lymph node recruitment of NK and T cells. The Journal of Immunology, 2007, 178: 3886–3892.

Dendritic cells (DCs) are APCs present in virtually every tissue, and are potent inducers of innate and adaptive immunity. Importantly, various subsets of DCs exist and drive specific immunological responses. Conventional DC subsets, as well as Ly6C+B220+PDCA-1+CD11chigh plasmacytoid DCs, use a variety of pattern recognition molecules, such as TLRs, to recognize pathogen-associated molecular patterns and distinguish self from nonself. These microbial recognition molecules, which include peptidoglycan, dsRNA, LPS, flagellin, ssRNA/nucleoside analogs, and unmethylated CpG DNA are recognized predominantly by TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9, respectively. Recognition of TLR ligands by DCs induces maturation and migration to secondary lymphoid tissue where they recruit, interact with, and activate various cell populations including T cells and NK cells. Recently, it has been shown that individual TLR agonists may induce differential responses through activation of distinct signaling cascades, driving Th1 or Th2 responses in CD4+ T cells. Even more recently, TLR-activated DCs have been shown to stimulate NK activity, which in turn can produce IFN-γ and provide an additional Th1 priming signal. Although much is known about TLR-dependent activation of T cells and NK cells, whether DCs play an obligatory role in this complex process in response to different TLR agonists is still unknown. For example, TLR7 (TLR7 is active in mice, whereas TLR8 is active in humans) and TLR9 ligands can activate plasmacytoid DCs and B cells (7, 8), whereas dsRNA can be recognized by various cells including DCs, fibroblasts, macrophages, and endothelial cells (9–11). All of these TLR agonists induce type I IFN production (12), which can induce chemokine-mediated cellular recruitment and substitute for costimulatory signals for CD4+ T cell activation.

In this study, we investigated the in vivo requirement for DCs in TLR-induced cellular recruitment to draining lymph nodes. Using a transgenic mouse model that can be selectively depleted of conventional CD11c+ MHC class II+ DCs (up to 90%), we show that cellular recruitment, including CX5+DX5+ NK and CD4+ T cell subsets, to draining lymph nodes of locally administered TLR4 and TLR5 agonists has an absolute requirement for conventional DCs. Interestingly, TLR3, TLR7, and TLR9 agonist-induced cellular recruitment, and activation of T cells and NK cells by any TLR agonist do not have the same absolute requirements for conventional DCs. Blocking type I IFN in TLR3 ligand-treated, DC-depleted mice inhibited the increase in total cellularity and recruitment of NK cells, but not CD4+ T cells, whereas blocking type I IFN in TLR7 ligand treated mice inhibited the increase of all cell types examined. In contrast, blocking type I IFN in TLR9 ligand-treated, DC-depleted mice had no effect on total cellularity and recruitment, demonstrating that redundant and differing pathways exist for activation and recruitment of innate and adaptive immune system components by different TLR agonists.

Materials and Methods

Mice

Transgenic B6.FVB-Tg [DTR/EGFP.57]Lan/I mice backcrossed to a BALB/c background that can be depleted of DCs have been referred to as CD11c-DTR mice (14). Mice were maintained in a breeding colony at the University of Florida College of Medicine Animal Facility under specific,
pathogen-free conditions. Mice were genotyped from tail DNA using published primer sequences. Age- and sex-matched littersmates between 8 and 12 wk of age not expressing the transgene were used as controls.

Reagents
LPS from Escherichia coli strain O111:B4 was purchased from Sigma-Aldrich. Synthetic dsRNA (polynosinic-polycytidylic acid (poly(I:C)) and CpG DNA oligodeoxynucleotide (ODN 1826) were obtained from InvivoGen. Flagellin derived from E. coli was prepared as previously described (15). The TLR7 agonist resiquimod (R848) was provided by 3M Pharmaceuticals. All Abs were purchased from BD Pharmingen except the plasmacytoid DC Ag-1 (PDCA-1; Miltenyi Biotec) and the NKG2D Abs (CX5; eBioscience). Recombinant murine IFN-α was prepared from PBL Biomedical Laboratories. Preparation of type I IFN blocking and control antiserum were as previously described (16).

Generation of bone marrow-derived DCs
DCs derived from murine bone marrow DCs were generated with murine recombinant GM-CSF (PeproTech), as previously described (17).

Depletion of DCs
To deplete CD11c+ DCs, transgenic CD11c-DTR and wild-type littersmates were treated with an i.p. injection of 4 ng per gram of body weight of diphtheria toxin (DT; Sigma-Aldrich), as previously described (14, 18). At the time of TLR administration (24 h), depletion of DCs and DC subsets in popliteal and inguinal lymph nodes was similar to depletion of splenic DCs observed in previous reports (18) (Fig. 1), with a 96% depletion of total DCs, over 90% depletion of classical DCs (CD8− DCs, and ~75% depletion of plasmacytoid DCs. For plasmacytoid DCs, only PDCA-1highB220high cells were considered, as a PDCA-1lowB220high cell population appeared following TLR treatment in ipsilateral lymph nodes, indicating increased expression of PDCA-1 on B cells. When examined, all of the PDCA-1highB220high cells were also CD11c+ in both ipsilateral and contralateral lymph nodes (data not shown).

Footpad injection and lymph node single-cell suspensions
Twenty-four hours after DT administration, mice were anesthetized and injected in the right hind footpad with 1 μg of flagellin or LPS, 0.1 μg of poly(I:C), 0.1 μg of resiquimod (R848), or 10 μg of CpG in 50 μl of PBS. Doses of TLR agonists were carefully titrated in preliminary studies to induce local immune response in the ipsilateral popliteal lymph node without inducing a response in the contralateral lymph node or spleen (15) and data not shown. When noted, 105 wild-type bone marrow-derived DCs were injected with or without LPS into the footpad, or 1 h before TLR ligand injection, 200 μl of type I IFN blocking or control antiserum was injected i.p. or i.v. (16). Twenty-four hours later, mice were sacrificed and both popliteal lymph nodes were harvested. The lymph nodes were dissected and processed to a single-cell suspension as previously described (15).

Flow cytometry
Lymph node cell suspensions were resuspended in 4% BSA flow buffer and blocked with CD16/CD32 Fc Abs. For T cell examination, FITC-conjugated CD69 Ab and allophycocyanin-conjugated CD4 Ab were used. For NK cells, PE-NK2D2 (C53) Ab, allophycocyanin-DX5 Ab, and FITC-CD69 were used. For plasmacytoid DCs, FITC-anti-CD68, MHC class II, or CD11c was combined with PE-PDCA-1 Ab and allophycocyanin-B220 Ab. To analyze DC loss 24 h after DT administration, spleen and lymph node cells were stained with FITC-MHC class II, PE-CD11c, and allophycocyanin-CD8 (conventional DCs) or with FITC-CD11c, PE-PDCA-1, and allophycocyanin-B220 (plasmacytoid DCs).

All samples were analyzed on a FACSCalibur (BD Biosciences) and a specialized software package (CellQuest; BD Biosciences). Isotype controls (BD Pharmingen) were used for all analysis. Dead cells were removed from analysis through 7-aminoactinomycin D (BD Pharmingen) staining.

Statistical analysis
Data were analyzed using the statistical software program StatView 5.0 statistical software package (Abacus Concepts) and are reported as the mean ± SEM. Differences were considered significant at p < 0.05, by either the paired Student’s t test or ANOVA.

FIGURE 1. Effect of DT on CD11c+ cell depletion in popliteal and inguinal lymph nodes of CD11c-DTR and wild-type mice. Wild-type (□) and CD11c-DTR (●) mice received the i.p. injection of 4 ng per gram of body weight of DT. Lymph nodes were removed at 24 h and total DC (totDCs; CD11c+, MHC class II+), CD8+ DC, CD8a− DC, and plasmacytoid DC (CD11c+, PDCA-1−, B220+) numbers were determined. A, Representative flow cytometric analysis demonstrating depletion of CD11c+ MHC class II+ cells in the popliteal lymph node of CD11c-DTR mice administered DT. B and C, Summary data showing depletion of all DC populations in both popliteal (B) and inguinal (C) lymph nodes of CD11c-DTR mice. Each value is obtained from two pooled lymph nodes in n = 4 mice. *, p < 0.05 in CD11c-DTR vs wild-type mice, as determined by Student’s t test.

Results
Injection of TLR agonists activates conventional and plasmacytoid DCs in draining lymph nodes
To explore whether TLR agonists induce DC recruitment to the draining lymph nodes and DC maturation is seen in vivo, we examined the draining and contralateral popliteal lymph nodes, following footpad injection of the TLR agonists. Adjuvants, such as TLR agonists, injected locally are known to induce DC maturation and migration to local lymph nodes where they create an environment conducive to creating immune responsiveness (15, 19). We first investigated whether footpad injection of TLR agonists increased the number and maturation state of conventional (CD11c− MHC class II+) DCs in the ipsilateral popliteal lymph node, compared with the contralateral popliteal lymph node.

We have previously shown that conventional CD8a− DCs and plasmacytoid DCs are recruited to draining lymph nodes following the footpad injection of recombinant flagellin, a potent TLR5 agonist (15). In this study, all of the TLR agents induced a recruitment of both total CD11c+ DCs and plasmacytoid (PDCA-1+)
B220) DCs into the draining (ipsilateral) popliteal lymph nodes, and increased their activation state (Fig. 2). Because these TLR agonists do not all directly activate plasmacytoid DCs, these data suggest that some activation occurs secondary to cytokine production by other cells. As shown in Fig. 1, we then used a novel in vivo model to transiently deplete mice of CD11c and MHC class II coexpressing DCs to determine their obligate requirement in the recruitment and activation of other cell type to draining lymph nodes of TLR agonist-treated mice.

DCs are required for maximal increases in lymph node cellularity in response to LPS or flagellin but not poly(I:C), resiquimod (R848), or CpG treatment

Because local administration of adjuvants increases total cellularity in draining lymph nodes, we tested whether DCs regulate the attraction of cells to the draining lymph nodes 24 h after the injection of different TLR agonists. In wild-type mice, injection of any TLR agonist into the footpad caused at least a 2-fold increase in total lymph node cellularity, and specific recruitment of CD4+ T cells and CX5+DX5+ NK cells in the ipsilateral vs contralateral lymph node (p < 0.05) (Fig. 3, left panels). However, in CD11c-DTR mice pretreated with DT, the injection of LPS and flagellin did not increase total cellularity (n = 4 mice for each group) (p = 0.4495 for LPS; p = 0.9917 for flagellin), or specific CD4+ T cell (p = 0.9347 for LPS; p = 0.8943 for flagellin) or NK cell recruitment (p = 0.089 for LPS; p = 0.9623 for flagellin), whereas poly(I:C), resiquimod (R848), and CpG still were capable of increasing cellularity, even with the extensive reduction of CD11c+ DCs (Fig. 3, right panels).

We next tested whether the cellular recruitment into the draining popliteal lymph node in response to LPS could be rescued in the CD11c-DTR mice by administering wild-type bone marrow-derived DCs at the time of DT administration. We used GM-CSF-cultured DCs because DCs generated using this protocol most resembled the immature conventional DC subtype being depleted in our model. This population of DCs contained 0.1% contaminating NK cells (data not shown). At 24 h after DC administration, these mice were either treated with LPS or untreated, and cellularity was examined by flow cytometry. Interestingly, LPS-treated
CD11c-DTR mice pretreated with wild-type DCs demonstrated a greater than 2-fold increase in total cellularity, whereas the CD11c-DTR mice that were treated with DCs, but not treated with LPS, demonstrated an insignificant, albeit modest, increase in total cellular and CD4 cellular recruitment ($p = 0.19$ total cellularity; $p = 0.10$ for CD4 cells) (Fig. 4A). Similarly, depletion with wild-type DCs restored the increase in CD4+ T cell and NK cell recruitment to draining lymph nodes of CD11c-DTR mice treated with LPS (Fig. 4B and C). Surprisingly, injection of wild-type DCs into CD11c-DTR mice without LPS injection was sufficient to induce some NK cell recruitment to the lymph node. These data demonstrate that the presence of the full complement of conventional DCs is absolutely required for maximal in vivo cellular recruitment to draining lymph nodes following TLR4 ligand treatment (and likely TLR5 ligand), but redundant mechanisms exist that do not require DCs in response to the other TLR agonists. Alternatively, the residual DC compartment following DT-mediated depletion, composed mainly of plasmacytoid DCs and more immature CD11clow DCs may be sufficient for the recruitment of cells to locally administer TLR3, TLR7, and TLR9 agonists.

**DCs are not required for in vivo T cell or NK cell activation by TLR agonists**

To test whether DCs are necessary for TLR-induced T cell and NK cell activation, we examined the expression of CD25 and CD69 on CD4+ and CD5+DX5+ cells. Preliminary data showed that CD69 expression was a more sensitive marker for T cell and NK cell activation 24 h following TLR ligand administration, and was used in subsequent experiments. In wild-type mice, we found that flagellin, LPS, resiquimod (R848), and CpG all induced significant activation of CD4+ T cells in the draining popliteal lymph node, whereas poly(I:C) did not (Fig. 5A). Unexpectedly, TLR agonist administration to CD11c-DTR mice also yielded significant activation of CD4+ T cells in the draining popliteal lymph node, whereas poly(I:C) did not (Fig. 5A). Unexpectedly, TLR agonist administration to CD11c-DTR mice also yielded significant activation of CD4+ T cells in the draining popliteal lymph node, as measured by CD69 expression (Fig. 5A). Furthermore, CD69 expression was increased on NK cells in both wild-type and CD11c-DTR mice in response to TLR agonists (Fig. 5B), demonstrating that DC depletion does not significantly alter TLR agonist-induced T cell or NK cell activation in vivo.

We also examined very briefly whether these TLR agonists polarized the CD4+ T cells to a Th1 or a Th2 phenotype. CD4+ T cells obtained from the ipsilateral and contralateral lymph nodes of wild-type and CD11c-DTR mice pretreated with the footpad injection of TLR agonists were incubated on anti-CD3-coated plates with GolgiStop, and Th1 and Th2 cytokines determined by flow cytometry. Footpad injections of resiquimod (TLR7 agonist) or flagellin (TLR5 agonist) alone did not result in significant Th1 or Th2 polarization, whereas TLR2 agonists (CpG and flagellin) significantly induced IFN-γ and IL-17 production, respectively. However, co-administration of TLR2 and TLR5 agonists resulted in a strong Th1 response, with increased IFN-γ, IL-17, and IL-23 production (Fig. 5C). These data suggest that DC depletion does not significantly alter the polarization of TLR agonist-induced T cell responses.
agonist) increased the number of both IL-4- and IFN-γ-secreting CD4+ T cells in both the ipsilateral and contralateral lymph nodes (data not shown).

Taken together, these data reveal that DCs regulate TLR4- and TLR5-mediated NK and T cell recruitment, but are dispensable for TLR3-, TLR7-, and TLR9-mediated NK and T cell recruitment. In addition, in vivo T cell and NK cell activation in response to TLR agonists do not require DCs, although we could not determine whether Th polarization of the CD4+ T cells varied with the different TLR agonists or the depletion of conventional DCs.

Type I IFN partially substitutes for DCs in TLR3- and TLR7-induced cellular recruitment

Because TLR3, TLR7, and TLR9 ligands all caused cellular recruitment in DC-depleted mice, we wished to examine alternative mechanisms. A potential mechanism for several TLR-induced immune responses, including DC maturation, depends on the production of type I IFN (20). In fact, IFN-induced chemokines, such as IP-10 and RANTES, are important recruiters of CD4+ T cells and NK cells (13). In preliminary studies, local footpad injection of IFN-α increased lymph node cellularity (data not shown) and specific recruitment of T cells and NK cells to the draining popliteal lymph nodes (Fig. 6). Furthermore, pretreatment with an antisera that neutralizes type I IFNs, but not a control antisera, completely inhibited IFN-induced T cell and NK cell recruitment in wild-type mice (Fig. 6). Interestingly, pretreatment of DC-depleted mice with anti-IFN antisera partially inhibited the recruitment of total cells, and completely inhibited the recruitment of NK cells to the draining lymph nodes of locally administered TLR3 ligand, but had no effect on CD4+ T cell recruitment in DC-depleted mice (Fig. 7, A–C). Somewhat similar results were seen with the TLR7 agonist, where blockade of IFN-α prevented the recruitment of both NK and T cells to the draining lymph nodes (Fig. 7, D–F). In contrast, pretreatment of DC-depleted mice with anti-IFN antisera had no effect on the recruitment of NK or CD4+ T cells in response to the TLR9 agonist, CpG (Fig. 7, G–I). These results demonstrate that type I IFN production may partially substitute for DCs in TLR3- and TLR7-induced (but not TLR9-induced) in vivo cellular responses, in this case cellular recruitment to draining lymph nodes.

To examine whether type I IFNs also contribute to the recruitment of cells to the lymph node in response to bacterial LPS in mice that have a full complement of DCs, wild-type mice were pretreated with anti-IFN antisera before the footpad injection of LPS. As shown in Fig. 8, passive immunization with anti-IFN antiserum partially attenuated the recruitment of total cells and NK cells, suggesting that although there is an absolute dependency on DCs for this response to LPS, endogenous type I IFN production also contributes to the cellular recruitment by these DCs.
Discussion

In this study, we have shown considerable plasticity in the DC response to different TLR agonists, in vivo, with conventional DCs being absolutely required for only TLR4- and TLR5-mediated recruitment to draining lymph nodes following local administration. In a previous study, we observed that flagellin activates DCs in TLR4 mutant mice, and flagellin signaling was completely inhibited by mutating an amino acid in the TLR5 binding domain (15). We used the same flagellin preparation in the present studies, which was essentially endotoxin-free, as determined by Limulus assay. Those findings suggest that the LPS and flagellin responses were specific to binding to their respective TLR, and not due to cross-reactivity.

Expectedly, local treatment with TLR agonists induced DC maturation in vivo, leading to increased cellular recruitment to the draining lymph nodes. All TLR agonists were similarly effective in this regard. These findings are therefore consistent with the observations of Kamath et al. (21) who showed that T cell activation is likely a common response to infection and can be mediated by the activation of IFN-α/β, which was essentially endotoxin-free, as determined by Limulus assay. Those findings suggest that the LPS and flagellin responses were specific to binding to their respective TLR, and not due to cross-reactivity.

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FIGURE 8. Effect of type I IFN blockade in wild-type mice treated with LPS. Wild-type mice were either pretreated with the i.p. injection of rabbit polyclonal antiserum against mouse type I IFN vs a control antiserum before the footpad injection of LPS. Total cell (A), CD4+ cell (B), and NK cell (C) responses were determined. Values are from n = 3 mice. *, p < 0.05 in anti-type I IFN vs control antiserum-treated mice, as determined by either Student’s t test or Wilcoxon ranked t test.

Discussion

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Disclosures
The authors have no financial conflict of interest.

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