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Peyer’s Patches Are Required for the Induction of Rapid Th1 Responses in the Gut and Mesenteric Lymph Nodes during an Enteric Infection1

Sue-fen Kwa,*† Peter Beverley,‡ and Adrian L. Smith*2*

The Peyer’s patches (PP) and mesenteric lymph nodes (MLN) are structural components of the gut-associated lymphoid tissues and contribute to the induction of immune responses toward infection in the gastrointestinal tract. These secondary lymphoid organs provide structural organization for efficient cellular interactions and the initiation of primary adaptive immune responses against infection. Immunity against primary infection with the enteric apicomplexan parasite, Eimeria vermiformis, depends on the rapid induction of local Th1 responses. Lymphotoxin (LT)-deficient mice which have various defects in secondary lymphoid organs were infected with E. vermiformis. The relative susceptibility of LTα−/−, LTβ−/−, LTα+/−β+/− mice and bone marrow chimeras, indicated that rapid protective Th1 responses required both PP and MLN. Moreover, the timing of Th1 induction in both MLN and gut was dependent on the presence of PP suggesting a level of cooperation between immune responses induced in these distinct lymphoid structures. The delay in Th1 induction was attributable to the delayed arrival of a broad range of dendritic cell subsets in the MLN and a substantial reduction of CD8α−CD11bhigh B220− dendritic cells in PP-deficient mice. The Journal of Immunology, 2006, 176: 7533–7541.

The gastrointestinal (GI)3 tract is a complex, compartmentalized organ equipped to protect against pathogens invading the host via the oral route. Within the GI tract exists an organization consisting of intraepithelial (IE) and lamina propria (LP) compartments, and the gut-associated lymphoid tissues (GALT) which include Peyer’s patches (PP) and mesenteric lymph nodes (MLN). These organized compartments contain immune cell populations which contribute to the active immune response against infection and the maintenance of oral tolerance toward gut commensal microbes and food Ags. The MLN form a chain-like structure of lymph nodes draining the GI tract via lymphatics and are the largest lymph nodes in the body. The PP are macroscopic nodules of immune cell aggregates distributed along the antimesenteric border of the intestine and vary in number, size, and shape across different species. In the human GI tract, PP numbers can reach ~300 (1) while in mice the average is 4–8.

The organogenesis of PP and MLN is dependent on lymphotoxin (LT) signaling but the requirements for LT are different. As a cytokine, LT exists functionally as a soluble homotrimer (LTα3) and a membrane-bound heterotrimer (LTαβ2) which are expressed by most hemopoietic cells (2, 3). MLN organogenesis is independent of TNF, TNFR2, and LTαβ but is dependent on LTα and LIGHT, another ligand of the LTβ receptor (4–6). PP are formed at embryonic day 15 and depend on the production of LTαβ2 by IL-7Ra+ CD4+ CD3− progenitor cells (7–9).

Deficiency in LT signaling is associated with structural defects in various knockout mice: LTα−/− mice have disorganized splenic architecture, no lymph nodes, and PP (10). LTβ−/− mice have a similar phenotype but possess residual cervical LN and MLN (11). Interestingly, the F1 progeny (LTα−/−β−/− double heterozygote) of LTα−/− and LTβ−/− parents are intact in all secondary lymphoid organs except PP which suggests a gene-dose influence on the formation of PP (Ref. 12 and see Fig. 1A).

Both the PP and MLN are considered important sites for the induction of immune responses against invading pathogens in the gut. These lymphoid structures share characteristics such as B cell follicles and T cell zones (13), similar to peripheral lymph nodes although PP lack the afferent lymphatics associated with MLN. Importantly, the structural organization in PP and MLN enables efficient cellular interaction and coordinates APC interaction with naive Ag-specific T cells. There are different requirements for PP and MLN during immune responses to infection and oral tolerance. MLN are essential for the induction of oral tolerance toward soluble Ag (14) and recent evidence suggests that LP-derived immunomodulatory dendritic cells (DC) migrating to the MLN may be involved (15, 16). The role of PP in the induction of oral tolerance and IgA Ab responses are less defined and depend on the type of study involved (17–19). There are many studies which describe immune responses in the PP during infection (20–22) but most suggest an accessory (nonessential) role for PP.

To date, few studies have addressed the absolute requirements for PP and MLN in the development of primary immune responses and their effect on protective immunity against infection. Although

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both MLN and PP function as inductive sites for the immune response during a gut infection, the contribution of each structure is not well-defined. Here, the role for PP-dependent and -independent immune responses against *Eimeria vermiformis*, a gut-residing apicoplast parasite, is examined using LT-deficient and bone marrow chimeric mice. *Eimeria* species are host-specific parasites and cause enteric coccidiosis in a wide spectrum of vertebrates (23). *E. vermiformis* invades the small intestinal enterocytes of the mouse and is effectively controlled by a Th1-type protective immune response brought about by IFN-γ-producing, MHC class II-restricted CD4+ T cells (24). Our results demonstrate that PP influence the efficiency of Th1 induction in the MLN and that coordinated PP-MLN-mediated immune responses are required to provide rapid and effective immunity against gut infection.

Materials and Methods

**Mice**

C57BL/6 (B6), Ly5.1 B6 congenic, LTAαβ−/−, LTBαβ−/−, LTAαβδ−/−, TCRβδ−/−, OT-II TCR transgenic (RAG1−/− B6 background) mice were bred in the specific pathogen-free facility at the Institute for Animal Health or purchased from Harlan. All mice used were on a B6 background and 7–14 wk of age unless indicated otherwise. Bone marrow chimeras were generated by irradiating recipients at 900 rad before injection i.v. 3.5 × 10^6 bone marrow cells in 200 μl of RPMI 1640 with 100 U/ml penicillin and streptomycin. Bone marrow chimeras were kept for 4 mo to allow reconstitution before infection. Reconstitution was checked by FACS analysis using the Ly5 marker and >90% reconstitution with donor bone marrow cells was attained with all chimeric mice made. All animal experiments were performed according to the United Kingdom Animals (Scientific Procedures) Act of 1986.

**Infection of mice and enumeration of oocyst numbers**

*E. vermiformis* parasites were propagated and enumerated as described previously (25). Mice were infected orally with 100 sporulated oocysts and 24 h fecal samples from individual mice were collected from day 7 postinfection for enumeration of oocysts. Homogenized faecal samples were diluted in saturated salt and oocysts enumerated microscopically using a McMaster parasite egg counting chamber. Counts were taken on a daily basis until no more oocysts were produced.

**FACS analysis and Abs**

Cells were stained for 10 min at room temperature (RT) and washed twice with PBS containing 1% FCS (PAA Laboratories) and 0.1% sodiumazide (Sigma-Aldrich) before analysis using the FACS Calibur instrument (BD Biosciences). Conjugated mAbs against CD45.1 (A20), CD4 (RM 4-5), CD8 (53–6.7), CD3ε (145–2C11), CD11c (HL3), CD45/B220 (RA3-6B2), CD80 (16-10A1), CD40 (3/23), CD11b (M1/70), IFN-γ, and αββ2 (DATK 32) were purchased from BD Biosciences.

**T cell adoptive transfer**

T cells from spleen and MLN were sorted using anti-CD90 microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. A total of 1 × 10^6 CD90+ cells were injected i.p. into recipient mice which were left for 7 days before infection. Purity of sorted cells was >90%. For tissue cell transfer, 2 × 10^6 MLN cells or 2 × 10^6 PP cells from day 8 postinfected B6 mice were injected i.p. into recipient mice which were kept for 7 days before challenge.

**Isolation of lymphocytes**

Cells were isolated from lymph nodes either by mechanical disruption or enzymatic digestion. IE lymphocytes (IEL) were isolated using a modified protocol as described previously (26). Briefly, the small intestine was excised of all PP, cut into 1-cm pieces and washed before incubation in HBSS (Sigma-Aldrich) supplemented with 10% FCS, 1 mM HEPES (Sigma-Aldrich), and 1 mM EDTA (Sigma-Aldrich) at 37°C in a gently shaking water bath for 20 min. After 10 s of vigorous vortexing, the supernatant was collected and cell pellet washed. IEL were purified using a gradient consisting of 44% and 67.5% Percoll (Amersham Biosciences). Gradients were centrifuged at 600 × g, for 25 min at RT and IEL were collected at the 44%/67.5% interphase and analyzed.

**Isolation of DCs**

DC were isolated as previously described (27). MLN were cut into several small pieces and digested with 1 mg/ml type III collagenase (Worthington Biochemical) and 0.5 mg/ml Dnase I (Sigma-Aldrich) at 37°C for 10 min followed by incubation at RT for 15 min. Following this, cell suspensions were washed with 0.079% EDTA to disrupt DC-T cell complexes and stop enzyme activity. A proportion of cells were stained for CD11c to evaluate total DC numbers before enrichment through a Nycoprep 1.0770 (Axis-Shield) gradient which was centrifuged at 600 × g for 25 min at RT. Further identification of DC subsets was conducted using anti-CD11b, CD8α, B220, CD80, and CD40. Total CD11c+ cells were calculated by multiplying the total cell count by the proportion of CD11c+ cells obtained by FACS analysis. These total CD11c+ numbers were used to calculate numbers within DC subsets with reference to the proportions (minimum of 1500 CD11c+ events collected) obtained after Nycoprep enrichment.

**RNA isolation and mRNA analysis**

RNA was isolated using the Qiagen RNeasy kit according to the manufacturer’s instructions. Tissue samples were stored in RNA later (Qiagen) before homogenization in lysis buffer using 1.0-mm glass beads and a bead beater. Quantitative RT-PCR was performed on mRNA using a RT-PCR mastermix (Eurogentec) and mRNA quantities were analyzed using the ABI Prism 7700 Sequence BioDetect instrument (Applied Biosystems). Relative levels of IFN-γ and IL18 mRNA were normalized against levels of CD3γ and hypoxanthine phosphoribosyltransferase (HPRT), respectively. Sequences of primers and fluorescent probes for IFN-γ, IL-18, CD3γ, and HPRT were described previously (28–30), although in this study, CD3γ probes were labeled with FAM-TAMRA. Cycling conditions consisted of an initial cycle of 2 min at 50°C, 30 min at 60°C, and 5 min at 95°C followed by 40 cycles of 20 s at 94°C and 60 s at 59°C. Results represent the relative fold difference between uninfected and infected samples and were calculated using the ΔΔcT method (CT method unless stated otherwise where ΔΔcT = (CT of cytokine of interest) − (CT of gene for normalization)). Fold difference = 2−(ΔΔcT of infected sample) − (mean ΔΔcT of uninfected samples).

**Ex vivo stimulation and cytokine assays**

A total of 1 × 10^6 cells were incubated at 37°C, 5% CO2 in 200 ml of RPMI 1640 supplemented with 50 mM 2-ME, 1 U/ml penicillin and streptomycin, and/or 10% FCS with oocyst lysate for 24 h. For DC/OT-II T-cell cultures, DC at a concentration 1 × 10^6 cells/ml were pulsed with 10 mg/ml OVA 323–339 peptide (AnaSpec) for 2 h at 37°C, 5% CO2 and washed before incubation for 48 h with 2 × 10^6 OT-II T cells in quadruplicate wells.

DC were obtained from the MLN of infected B6 mice and enriched by depleting T and B cells with anti-CD90 and anti-CD45R/B220 microbeads (Miltenyi Biotec). The CD11b-positive and -negative fractions were used without further fractionation. T cells from OT-II spleen and MLN were enriched by depletion using anti-MHC class II microbeads (Miltenyi Biotec). To measure cytokine production, supernatants from the cultures were analyzed using the BD Cytometric Bead Array kits (BD Biosciences).

**Intracellular cytokine staining**

Cells were cultured with oocyst lysate for 24 h as described above. A total of 10 μg/ml brefeldin A (Sigma-Aldrich) was added during the last 6 h of incubation. After washing, cells were surface stained for CD4, CD8α, and/or CD3ε before washing and vigorously resuspending them in 100 μl of brefeldin A (0.1 μg/ml) and 100 μl of 4% paraformaldehyde (Sigma-Aldrich). After 20 min of incubation, mAbs against CD8α, CD45, and CD11c were added and cells were washed twice before incubation at RT, cells were washed once more to stop permeabilization in 0.5% saponin (Sigma-Aldrich) for 10 min at RT. Cells were washed, resuspended, and stained for intracellular IFN-γ in 0.5% saponin for 30 min at RT. Two washes in 0.5% saponin and two washes in FACS wash was conducted to remove excess Ab and avoid nonspecific staining. Cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry.

**Statistical analysis**

Level of statistical significance was determined using ANOVA and Student’s t test.
Results

Specific LT deficiencies exacerbate infection with E. vermiformis

To examine the requirement for lymphotoxin and/or secondary lymphoid structures in the immune control of the enteric parasite, E. vermiformis, we measured infection in unmanipulated LT-deficient and B6 mice. The status of lymphoid structures in different LT-deficient mice has been described previously (10–12) and was confirmed at postmortem. Briefly, LT/−/− mice have no lymph nodes and PP while LT+/+ mice have similar defects but retain a residual MLN and cervical LN. LT+/−/− double heterozygote mice are intact in all lymphoid structures except PP unlike LT+/−/− or LT+/+ single heterozygote mice which are intact and retain PP (Fig. 1A). Intact B6 mice were relatively resistant to infection when compared with LT−/− and LT+/− mice, both of which were highly susceptible, producing ~35 million oocysts (Fig. 1B). LT+/−/− mice displayed an intermediate level of susceptibility and produced ~20 million oocysts/mouse. It is important to note that LT+/− and LT+/− single heterozygote mice which have PP were more resistant to infection than PP-deficient LT+/−/− mice (p < 0.02) (data not shown). The duration of parasite production (patent period) was longer with LT−/− (9.3 ± 0.3 days) and LT+/− (10.4 ± 0.5 days) mice than with LT+/−/− (8.3 ± 0.3 days) and B6 (8.6 ± 0.2 days) and the level of susceptibility in LT−/− and LT+/− mice was comparable to that seen in T cell-deficient TCRβ × δ−/− mice (24 and also Fig. 2). Complete immunity (evident by the absence of oocysts) developed in all LT-deficient mice which were rechallenged 1 mo after primary infection and this indicated that primary immune responses had occurred in all mice (Fig. 1C).

Both MLN and PP are required for the control of infection

The phenotype of LT-deficient mice may have been due to the defects in T cell production of LT or the developmental effects on secondary lymphoid structures. To examine these possibilities, T cells from LT+/− or B6 donors were adoptively transferred into TCRβ × δ−/− recipients which were subsequently infected. There

FIGURE 1. Specific LT deficiencies exacerbate infection with E. vermiformis. A, Small intestine from various LT-deficient mice. Arrows point to some of the PP found along the small intestine. Pictures without arrows indicate absence of all PP. B, Total oocyst output in mice during primary infection. Results represent a minimum of seven mice per group in one of three experiments. C, Total oocyst output per mouse during secondary infection. a–c, Groups labeled with the same italic letter are not significantly different while those labeled with different letters are significantly different (p < 0.02).

FIGURE 2. LTα expression by T cells is not required during infection. A total of 1 × 10^6 CD90+ sorted T cells from naive LT−/− or B6 donors were adoptively transferred into TCRβ × δ−/− recipients which were subsequently infected. Results show total oocyst output of individual mice from each group. **, Significantly different (p < 0.002) from all other groups.
was no difference in oocyst production between TCRβ × δ−/− recipients given T cells from either LTα−/− or B6 donors (Fig. 2). TCRβ × δ−/− mice given no cells remained highly susceptible to infection regardless of the origin of donor bone marrow cells. Reconstitution of LT−/− and LTβ−/− recipients with B6 bone marrow could not rescue their phenotype and these mice remained highly susceptible, producing large numbers of oocysts (Fig. 3, A and B). Both LTα−/−/β−/− recipients given either B6 or LTα−/−/β−/− bone marrow cells remained equally susceptible to infection (Fig. 3C). Although MLN are present in LTβ−/− mice, we observed that these were physically smaller and shorter in chain length compared with age-matched intact B6 (data not shown). In previous reports (31) reconstitution of LTβ−/− mice with B6 cells resulted in reconstitution of the MLN. However, although our chimeras were kept for 4 mo, MLN reconstitution only occurred partially as the MLN structure in LTβ−/− recipients given B6 cells contained fewer cells than B6 recipient chimeras (S.-f. Kwa and A. L. Smith, unpublished data). Hence, LTβ−/− recipients remained highly susceptible as full functional capacity of the MLN was not achieved. Despite reorganization in the spleen of LTα−/− and LTβ−/− mice given B6 cells (confirmed by microscopy), these recipients remained susceptible, indicating that splenic reorganization was insufficient to induce effective immunity against infection. LTα−/−/β−/− mice, which retained splenic organization and MLN, responded better than LTα−/− and LTβ−/− mice but were more susceptible than B6 mice. Collectively, these data indicate no requirement for bone marrow-derived cell-dependent expression of LT during infection and are consistent with a phenotype influenced by the presence or absence of functional MLN and PP.

Absence of PP results in delayed kinetics of Th1 immune responses

Immunity to primary infection with E. vermiformis is dependent on the timing of Th1 immune responses (25) and protective immunity developed by LT-deficient mice against secondary infections indicated that a primary immune response had been induced. Hence, it is possible that the susceptibility of LT-deficient mice seen during primary infection may be a result of delayed Th1 immune responses. To test this hypothesis, we assessed the timing of IFN-γ mRNA up-regulation in the ileum (where peak parasite infection occurs) and where possible, in the MLN of infected mice. In B6 mice, increased levels of IFN-γ mRNA in the ileum were detected at 6 days postinfection (DPI) onward whereas with LTα−/− mice, no response was detected until 10 DPI (Fig. 4A). LTα−/−/β−/− mice, which were intermittently susceptible, up-regulated IFN-γ mRNA in the ileum at 8 DPI, CD4+ IEL (sorted with magnetic beads) showed up-regulation of IFN-γ mRNA in B6 mice at 8 DPI but not in LTα−/− mice (data not shown) which also indicated that CD4+ IEL contributed to the IFN-γ mRNA expressed in the small intestine. In the MLN of LTα−/−/β−/− mice, IFN-γ mRNA was significantly up-regulated at 6 DPI, while in B6 mice significant production in the MLN occurred at 4 DPI (Fig. 4A). IFN-γ in the MLN was produced mainly by CD4+ T cells (p < 0.05) (Fig. 4B). At 6 DPI, there was a higher frequency of IFN-γ+ CD4+ T cells in the MLN of B6 than LTα−/−/β−/− mice, supporting the hypothesis that LTα−/−/β−/− mice have a delayed Th1 response to infection (Fig. 4C). Proportions of IFN-γ+ CD4+ T cells were similar in both LTα−/−/β−/− and B6 mice at 8 DPI (data not shown).

Stimulation of T cells with gut-derived DC leads to up-regulation of αβ (32, 33), a gut-homing integrin, which interacts with the mucosal vascular addressin cell adhesion molecule-1 (MadCAM-1) expressed by high endothelial venules and flat-walled venules in the gut (34, 35). In B6 mice, increased proportions of αCD4+ T cells were detected in the IE compartment from 8 DPI onwards while proportions in LTα−/− mice increased only at 14 DPI onward (Fig. 5A); similar proportions of the

Figure 3. Requirement for LT in structural organization. Bone marrow chimeric mice were made using LT-deficient and B6 mice as donors or recipients. Results show total oocyst output of individual mice from infected (A) LTα−/− (B) LTβ−/− (C) LTα−/−/β−/− bone marrow chimeric mice. Five to eight mice per group were used and data represents one of two experiments. ** Significant difference (p < 0.002) when compared with B6 recipient controls.
CD4⁺CD8⁻⁺CD8β⁻⁺ CD4⁺CD8α⁺⁺ IEL subsets expressed α₄β₇. An increase in α₄β₇⁺⁺CD4⁺ T cells was also found in the MLN of B6 mice at 6 DPI while the increase occurred later in LTα⁻⁺⁻β⁻⁺⁺ mice at 7 DPI (Fig. 5B). Gut-tropic T cells also use the chemokine receptor (CCR9) which recognizes TECK (CCL25) expressed by small intestinal epithelia (36). At 10 DPI, the portion of CCR9⁺⁺CD4⁺ IEL also increased in B6 mice but remained unchanged in LTα⁻⁻mouse (data not shown).

PP- and MLN-stimulated cells independently confer immunity

Although PP influence the rate of induction of IFN-γ responses in the MLN and are important for effective immune responses against E. vermiformis infection, susceptibility in LTα⁻⁻⁻β⁻⁻⁻ mice could also be due to a lack of in situ stimulation of appropriate parasite-specific T cell responses in the PP. There was a locally induced immune response in the PP of B6 mice where IFN-γ mRNA was up-regulated at 4 DPI (data not shown). Ex vivo Ag-stimulated PP cells from infected B6 mice also demonstrated up-regulation of IFN-γ and TNF-α at both 6 DPI and 8 DPI (Fig. 6, A and B) where levels were lower than MLN cells of the same mice stimulated under similar culture conditions (IFN-γ: 7835 ± 1343 pg/ml; TNF-α: 422 ± 51 pg/ml). Levels of IL4 did not increase significantly in ex vivo-stimulated PP cells (Fig. 6C) consistent with the induction of an Ag-specific Th1-type response.

To determine the extent of protection generated independently in the PP and MLN, we adoptively transferred 2 × 10⁶ PP or 2 × 10⁷ MLN cells from infected B6 donors into highly susceptible TCRβ⁻⁺⁻Δ⁻⁻ recipients which were subsequently infected. The numbers of MLN and PP donor cells transferred were selected according to the numbers found in B6 mice. In TCRβ⁻⁺⁻Δ⁻⁻ recipients given either B6 MLN or PP cells, oocyst numbers were significantly reduced (~6 million) compared with naive B6 mice (~10 million) or TCRβ⁻⁺⁻Δ⁻⁻ mice (~32 million) which both received no cells (Fig. 7). The length of the patent period was similar in TCRβ⁻⁺⁻Δ⁻⁻ mice given either PP (9.5 ± 0.4 days) or MLN (9.6 ± 0.9 days) cells and shorter than TCRβ⁻⁺⁻Δ⁻⁻ mice given no cells (12.9 ± 0.1 days) (p < 0.002). TCRβ⁻⁺⁻Δ⁻⁻ mice given LTα⁻⁻⁻β⁻⁻⁻ MLN cells also showed a significant reduction in oocyst output compared with recipients given no cells indicating that LTα⁻⁻⁻β⁻⁻⁻ MLN cells are capable of immune protection without the in vivo influence of PP. Therefore, both PP and MLN can function independently as sites for immune induction and generation of protective immune responses.

Delayed Th1 kinetics correlates with delayed presence of mature DC in the MLN

The influence of PP on the induction of MLN Th1 responses could be mediated by the migration of cell populations (e.g., DC) from the PP or other regions of the gut to the MLN. Hence, the kinetics and phenotype of DC populations in the MLN of PP-deficient and intact mice were examined during infection to determine any changes in the absence of PP. DC subsets accumulated in the MLN were initially defined using CD11c, CD8α, and molecules classically associated with activation (CD40, CD80). The majority of DC in the MLN of both LTα⁻⁻⁻β⁻⁻⁻ and B6 mice during infection were CD11c⁻⁺CD8α⁺⁺ and these were further analyzed. There were significant increases in mature DC expressing CD80 and/or CD40 at 4 DPI in the MLN of B6 mice while similar subsets increased later at 7 DPI in LTα⁻⁻⁻β⁻⁻⁻ mice (Fig. 8A). Abs to B220 and CD11b were used to further differentiate the CD11c⁺⁺CD8α⁺⁺ population and no delay was observed in the accumulation of CD11b⁺⁺B220⁺⁺ DC in the MLN (Fig. 8B). In contrast, there was a delayed arrival of CD11b⁺⁺B220⁺⁺ and CD11b⁺⁺B220⁺⁺ DC subsets in LTα⁻⁻⁻β⁻⁻⁻ mice. Moreover, a CD11b⁺⁺B220⁺⁺ DC population was noticeably reduced in the MLN of LTα⁻⁻⁻β⁻⁻⁻ mice at 6 DPI (Fig. 9A). Interestingly, in the PP of B6 mice, a small reduction in the number of CD11b⁺⁺B220⁺⁺ DC at 6 DPI was observed which inversely corresponds to the increase seen in the MLN (Fig. 9B). Despite the initial lack of CD11b⁺⁺B220⁺⁺ DC, there were increases in the
overall population of CD11b<sup>+</sup>B220<sup>−</sup> (includes CD11b<sup>high</sup> and CD11b<sup>lo</sup>) DC in the MLN of LT<sup>α−/− β−/−</sup> mice (although occurring later at 7 DPI) which suggests accumulation of similar populations that are derived from non-PP regions of the gut (e.g., LP).

Analysis of the CD11b<sup>+</sup>B220<sup>−</sup> DC fraction from the MLN of B6 mice at 6 DPI showed that this population expressed relatively higher mRNA levels of IL-18, a Th1-inducing cytokine (37, 38), than the CD11b<sup>+</sup>B220<sup>−</sup> DC fraction (Fig. 10A). IL-12p40 mRNA was undetectable in both fractions. The CD11b<sup>+</sup>B220<sup>−</sup> DC fraction isolated from the MLN of B6 mice at 6 DPI also stimulated higher levels of IFN-γ than the CD11b<sup>+</sup>B220<sup>−</sup> DC fraction in an OVA 323–339 peptide presentation assay using OT-II T cells (Fig. 10B). No differences were found in IL-2, IL-4, and IL-5 levels which remained low in both CD11b<sup>+</sup>B220<sup>−</sup> and CD11b<sup>+</sup>B220<sup>−</sup> cultures (data not shown). Clearly, PP influence the rate of DC accumulation in the MLN and may be one of the initial sources of CD11b<sup>+</sup>B220<sup>−</sup> DC which are mobilized to the MLN during the early immune response against E. vermiformis.

Discussion

The MLN and PP form the GALT, functioning as inductive sites and accommodating Ag presentation for the induction of T cell-mediated responses. We were able to explore the requirements for GALT structures during infection by using E. vermiformis which replicates in the small intestine. LTα<sup>−/−</sup>, LTβ<sup>−/−</sup>, and LTα<sup>−/− β−/−</sup> mice exhibited increased susceptibility to infection compared with PP-intact LTα<sup>−/−</sup>, LTβ<sup>−/−</sup>, and B6 mice and the level of susceptibility seen in LTα<sup>−/−</sup> and LTβ<sup>−/−</sup> mice resembled that

FIGURE 5. Delayed presence of αβ<sup>+</sup>CD4<sup>+</sup> T cells in LT-deficient mice during infection. A, αβ<sup>+</sup>CD4<sup>+</sup> T cell in the small intestinal IEL compartment of LTα<sup>−/−</sup> and B6 mice. B, αβ<sup>+</sup>CD4<sup>+</sup> T cells in the MLN of LTα<sup>−/− β−/−</sup> and B6 mice. FACS plots show the proportion of CD4<sup>+</sup> T cells expressing αβ<sup>+</sup> and represent one of four mice per time point in two experiments. All plots represent cells gated on CD4 and CD3. The bar chart depicts the changing numbers of αβ<sup>+</sup>CD4<sup>+</sup> T cells in the MLN which are calculated from differences in αβ<sup>+</sup>CD4<sup>+</sup> T cell numbers between infected and uninfected mice. Bars correspond to the mean and SEM of four mice per time point. *, Significant difference (p < 0.05) when compared with uninfected controls.

FIGURE 6. Th1 responses in PP during infection. A total of 1 × 10<sup>6</sup> PP cells were stimulated ex vivo with oocyst lysate for 24 h. Supernatants were measured for (A) IFN-γ, (B) TNF-α, and (C) IL-4 produced during stimulation. Each bar corresponds to the mean and SEM of four mice per time point. *, Significant difference (p < 0.05) when compared with uninfected controls.

FIGURE 7. Adoptive transfer of PP- and MLN-derived immunity. A total of 2 × 10<sup>5</sup> MLN or 2 × 10<sup>6</sup> PP cells from day 8 postinfected LTα<sup>−/− β−/−</sup> or B6 donors were injected i.p. into TCRβ<sup>−/−</sup> recipient mice which were subsequently infected. Results show total oocyst output of individual mice. a–c, Groups labeled with the same italic letter are not significantly different while those labeled with different letters are significantly different (p < 0.02).
of TCRβ × δ−/− mice. The use of T cell adoptive transfer and bone marrow chimeric mice substantiated the necessity for both organized MLN and PP lymphoid structures rather than immune cell-derived LT expression during infection. All LT-deficient mice (Fig. 1C) and bone marrow chimeras (data not shown) developed a high level of protection against reinfection indicating that primary immune responses had been initiated in all LT-deficient strains and demonstrating that protective immunity against a gut infection can be generated in the absence of organized GALT structures.

Immunity to primary infection with *E. vermiformis* is entirely dependent on the production of IFN-γ by MHC class II-restricted CD4+ T cells (24) and the increased susceptibility of LT-deficient mice can be explained by a delayed induction of Th1-type immune responses. Peak numbers of *E. vermiformis* in the gut are produced around 7–8 DPI and IFN-γ, a key component in the control of infection (24), was up-regulated too late to affect parasite replication in the small intestine of LTα−/− mice. Resistance to infection increased with the presence of organized MLN as seen in LTα+/−/− β+/− mice but the lack of PP rendered them more susceptible than intact B6 mice. Therefore in this infection, GALT structures are critical in influencing the organization of DC-T cell interactions to deliver rapid immune responses that efficiently control the parasite. It is unknown where the primary immune response was initiated in LTα−/− mice as these mice have no GALT but were still able to up-regulate IFN-γ at later time points. The increased proportion of αβ+ CD4 IEL in LTα−/− mice suggests stimulation by gut-derived DC which may migrate to unusual sites to present in the absence of MLN (e.g., spleen or bone marrow) although this is clearly less efficient. We cannot exclude the possibility that MAdCAM-1 expression in LTα−/− mice may be reduced in the absence of LTα3 signaling (4) and account for the delayed increase in αβ+ CD4 IEL. However, a delay in αβ+ CD4 T cells was also seen in the MLN of LTα+/−/− γ− mice (Fig. 5B) and the relative resistance of bone marrow chimeric mice (Fig. 3) and LTα+/− and LTβ+/− single heterozygote mice (data not shown) indicates that the effects of defective GALT structures override any possible role for reduced MAdCAM-1 levels.

In PP-deficient mice infected with *E. vermiformis*, an intact MLN did not confer complete protection against primary infection. Although there are alternative means for Ag-sampling (e.g., villus microfold cells, CX3CR1+ DC) and less conventional inductive sites such as isolated lymphoid follicles (39–41), these alternatives may not function as efficiently as PP during a gut infection. Isolated lymphoid follicles redevelop in LT-deficient mice reconstituted with LT-intact bone marrow (42) yet these mice remain relatively susceptible to infection (Fig. 3). Delays in Th1 responses within the MLN and small intestine in the absence of PP suggest that PP-mediated responses influence the rate of MLN-mediated responses where the dynamics of DC relocation within the PP (43, 44) and in the lymphatics draining the gut (45) are likely to have a downstream influence on T cell responses in the MLN. DC subsets arrived later in the MLN of LTα+/−/− β+/− mice which were also deficient in a CD11bhiB220− DC subset hence the delay in MLN Ag-specific responses may be attributable to the late arrival of Ag-presenting DC and lower numbers of the CD11bhiB220− subset in the MLN. As CD11bhi DC subsets have been described in the PP (46) and other evidence suggests that PP-DC migrate to MLN (44, 47, 48), it is possible that the lower numbers of the CD11bhiB220− DC subset in the MLN were related to the absence of PP. In intact B6 mice, the reduction in PP CD11bhi B220− DC at 6 DPI corresponded with increases in MLN which suggests mobilization of PP-DC to the MLN (Fig. 9B).

Functional examination of CD11b+ B220+ and CD11b− B220− DC fractions sorted from the MLN of infected B6 mice at 6 DPI showed significant differences where the CD11b− B220− DC fraction expressed higher levels of IL18 mRNA (although both fractions expressed IL18 mRNA) (Fig. 10A). The CD11b+ B220−
DC fraction also induced IFN-γ production during an OVA peptide presentation assay with OT-II T cells (Fig. 10B) and therefore the delayed arrival of Th1-inducing CD11b<sup>+</sup>B<sup>220</sup> DC could explain the delayed Th1 response in PP-deficient mice. Nevertheless, other DC subsets from non-PP regions of the gut that were recruited to the MLN are also likely to contribute to the induction of Th1 responses in the MLN. It is suggested that PP-DC may be critical during the early inductive phase of the Th1 response because PP-DC are more efficient at endocytosis than LP-DC (15) and are in close proximity to Ag-sampling microfold cells. In LTα<sup>−/−</sup>β<sup>−/−</sup> mice, Ag sampling is limited to non-PP sites which are not as efficient in propagating the immune response during the early stages of infection, especially when Ag is presented in limited quantities. In such a situation, the PP acts as a focal point where limited amounts of Ag can be sampled by microfold cells and transferred quickly to local DC. The instructional events that stimulate PP-DC to migrate to the MLN or remain in the PP (to present to local T cells) remain to be determined.

The data presented indicate that PP are essential for the rapid induction of gut Th1 responses, and we propose a cooperative role between PP and MLN in the development of effective immunity in the gut. The direct influence of PP on the rate of immune induction in the MLN highlights the integration and interplay of responses generated from distinct areas of the GALT and may be a beneficial outcome of PP-targeted oral vaccination strategies.

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Disclosures
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References


