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Three Lines of Defense: Macrophage Subset Compartmentalization in Salmonella-Infected Peyer's Patches

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Abstract:

Background: *Salmonella enterica* serovar Typhimurium is a major enteric pathogen that invades the host via M cells overlying Peyer's patches. Macrophages are critical for host defense, yet the anatomical distribution of distinct macrophage subsets within Peyer's patches during active infection remains incompletely characterized.

Methods: Female C57BL/6 mice were orally infected with *Salmonella* Typhimurium strain SL1344 following streptomycin pretreatment. At 7 days post-infection, cecal Peyer's patches were examined macroscopically and by immunohistochemistry using antibodies against F4/80, Moma1, and Moma2.

Results: Infected mice exhibited marked cecal hyperemia, wall thickening, and enlarged Peyer's patches. F4/80$^+$ macrophages densely accumulated in the subepithelial dome (SED) and were also observed in follicular periphery and perivascular areas. Moma1$^+$ cells were sharply delineated around B-cell follicles and vessels, with no staining in SED or interfollicular regions (IFR). Moma2$^+$ macrophages were widely distributed outside follicles, with highest density in IFR and around high endothelial venules.

Conclusion: Oral Salmonella infection induces compartmentalized redistribution of macrophage subsets in Peyer's patches. F4/80$^+$ cells serve as first-line phagocytes at the bacterial entry site, Moma1$^+$ cells function as barrier sentinels, and Moma2$^+$ cells localize to T-cell zones for antigen presentation. This functional topography informs mucosal vaccine design.

Keywords: Salmonella Typhimurium, Peyer's patches, macrophages, F4/80, Moma1, Moma2, immunohistochemistry, mucosal immunity

Introduction

Infections of the digestive system represent one of the most common and clinically significant categories of human disease worldwide (1). Enteric pathogens, including bacteria, viruses, and parasites, invade via the oral route and must overcome multiple host barriers, including gastric acidity, peristalsis, the intestinal mucus layer, the antimicrobial peptide barrier, and the epithelial monolayer (2). Among these pathogens, Salmonella enterica serovar Typhimurium is a Gram-negative, facultative intracellular bacterium that causes self-limiting gastroenteritis in immunocompetent individuals but can lead to severe systemic dissemination in infants, the elderly, or immunocompromised individuals (3,4). Following oral ingestion, Salmonella colonizes the distal small intestine and cecum, triggering acute neutrophilic inflammation, epithelial erosion, and robust mucosal immune responses (5). Understanding the cellular and molecular events that occur in the intestinal lymphoid tissues during such digestive system infections is critical for developing rationally designed oral vaccines and targeted immunotherapies (6).

Peyer's Patches: Anatomy, Histology, and the Central Role of M Cells

Peyer's Patches (PPs) are organized lymphoid nodules located along the antimesenteric wall of the ileum and, to a lesser extent, the jejunum and cecum (7). They are the primary inductive sites of the Gut-Associated Lymphoid Tissue (GALT) and are considered the immune sensors of the intestinal lumen (8). Histologically, each Peyer's Patch is compartmentalized into several highly specialized microdomains (9) (fig 1).

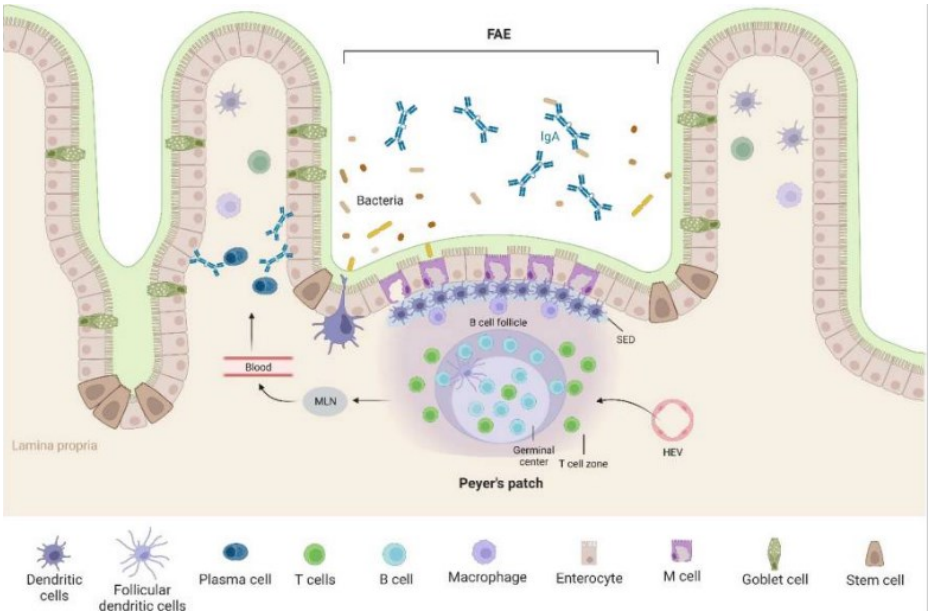

Figure 1: Schematic illustration of cellular composition of Peyer's patches (PPs). PP follicles are enclosed by follicle-associated endothelium (FAE) containing M cells that shuttle luminal antigen into the PP. Subepithelial dome (SED) below the FAE contains high density of antigen-presenting cells. Lymphocytes enter PP via high endothelial venules (HEV) and form large B cell follicles and small T cell zones. The interactions between B cells and T cells at the follicle-T cell zone lead to expansion and differentiation of B cells. The activated B cells form germinal center, generating IgA-secreting plasma cells. The generated effector cells leave the PPs through efferent lymphatics and enter the circulation via mesenteric lymph nodes (MLNs) (10). They home to the intestinal lamina propria from the blood circulation and the lamina propria plasma cells produce dimeric IgA that are transported across the epithelium. The secretory-IgA (S-IgA) can interact with bacteria in the gut lumen (11). (Photo from DOI: 10.1007/s13770-023-00543-y)

The Follicle-Associated Epithelium (FAE) and M Cells

The follicle-associated epithelium (FAE) is the specialized epithelial layer that overlies each Peyer's Patch dome, separating the luminal environment from the underlying lymphoid tissue (12). Unlike adjacent villus epithelium, the FAE contains fewer goblet cells, a thinner glycocalyx, and reduced expression of digestive enzymes (13). Its most distinctive feature is the presence of Microfold cells, or M cells (14).

M cells are specialized epithelial cells that constitute approximately 5–10% of the FAE (15). They possess a unique morphology characterized by:

- Short, irregular microvilli (rather than the tall, uniform brush border of enterocytes).

- A deep invagination or "pocket" on their basolateral surface, which harbors lymphocytes, dendritic cells, and macrophages (16).
- High transcytotic activity, allowing efficient transport of luminal material from the apical surface to the underlying pocket (17).

Functionally, M cells are the gatekeepers of mucosal immunity (18). They continuously sample the intestinal lumen, taking up soluble antigens, particulate matter, and intact microorganisms via phagocytosis, macropinocytosis, or receptor-mediated endocytosis (19). Transported cargo is delivered intact across the epithelial barrier and released into the subepithelial dome (SED), where it is rapidly acquired by resident phagocytes for antigen presentation (20).

However, M cells are also exploited by enteric pathogens (21). Salmonella enterica serovar Typhimurium, Shigella flexneri, Yersinia enterocolitica, and Listeria monocytogenes all specifically adhere to M cells via interactions between bacterial adhesins and M-cell surface receptors (e.g., GP2, glycoprotein 2) (22). Salmonella uses its type three secretion system (T3SS) to invade M cells, inducing membrane ruffling and cytoskeletal rearrangement (23). Bacteria are then transcytosed and released into the SED, alive and fully virulent (24). Thus, M cells serve as the primary portal of entry for Salmonella into the host mucosal immune system (25).

The Subepithelial Dome (SED)

Directly beneath the FAE lies the subepithelial dome (SED), a loose reticular network of stromal cells, extracellular matrix, and densely packed immune cells (26). The SED is the first immunological checkpoint encountered by Salmonella after M-cell translocation (27). It is rich in: $CD11c^+$ dendritic cells (both conventional and monocyte-derived). $CD68^+$ and $F4/80^+$ macrophages. Naïve and memory B cells. $CD4^+$ T cells (28).

The anatomical proximity of the SED to the M-cell pocket ensures rapid capture of incoming antigens and immediate initiation of the immune response (29).

B-Cell Follicles, Marginal Zone, and Interfollicular Regions

Below the SED reside one or more B-cell follicles, which may contain germinal centers in the presence of ongoing T-dependent immune responses (30). Surrounding each follicle is a marginal zone-like area and the subcapsular sinus, lined by $CD169^+$ ($Moma1^+$) metallophilic macrophages (31). Between adjacent follicles lie the interfollicular regions (IFR), which are predominantly populated by $CD3^+$ T cells and contain high endothelial venules (HEVs), the primary portals for lymphocyte recruitment from the bloodstream (32). This highly organized compartmentalization, FAE/M cells → SED → Follicles/IFR, creates an efficient assembly line for antigen capture, processing, presentation, and lymphocyte activation (33).

The Role of Macrophages in Peyer's Patches During Salmonella Infection

Macrophages are among the first immune cells to encounter Salmonella in the intestinal tissue and play a dual and critical role in host defense (34).

Macrophages as Innate Effector Cells

Macrophages recognize conserved bacterial components via pattern recognition receptors (PRRs), including Toll-like receptors (TLR2, TLR4, TLR5) and NOD-like receptors (NOD1, NOD2) (35). Ligation of these receptors triggers: Phagocytosis and formation of the phagolysosome, - Respiratory burst with production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (36). Secretion of pro-inflammatory cytokines: IL-1β, IL-6, IL-12, IL-23, and TNF-α (37). Classical (M1) polarization, characterized by enhanced microbicidal activity and antigen-presenting capacity (38). These responses are essential for containing bacterial replication and preventing systemic dissemination (39).

Macrophages as Antigen-Presenting Cells (APCs)

After phagocytosing and proteolytically processing Salmonella antigens, macrophages load peptide fragments onto MHC class II molecules and display them on the cell surface (40). They then migrate—or extend processes—into the interfollicular T-cell zones, where they present antigen to naïve $CD4^+$ T cells (41). This interaction, in the context of appropriate co-stimulation (CD80/CD86) and cytokine signals, drives Th1 and Th17 polarization, which is critical for controlling intracellular Salmonella infection (42).

Macrophage Heterogeneity and Subset-Specific Markers

Macrophages are not a homogeneous population. In murine lymphoid tissues, distinct subsets with specialized anatomical locations and functional roles have been identified using specific surface markers (43-44) (Table 1).

During Salmonella infection, these macrophage subsets are dynamically recruited and redistributed within Peyer's Patch compartments (48):

- F4/80$^+$ SED macrophages are strategically positioned to capture bacteria immediately after M-cell transport.

- Moma1$^+$ marginal sinus macrophages act as a second anatomical barrier, trapping bacteria that escape the SED and preventing lymphatic dissemination.

- Moma2$^+$ IFR macrophages are optimally located to interact with recruited T cells and drive adaptive immunity (49).

Gap in Knowledge and Study Aim

Despite the established importance of M cells as the portal of entry and macrophages as key mediators of immunity, the precise anatomical distribution and subset-specific localization of macrophages within Peyer's Patch compartments during active Salmonella infection remain incompletely characterized (50). Most studies have focused on splenic macrophages or in vitro models, and the in situ topography of F4/80$^+$, Moma1$^+$, and Moma2$^+$ cells in the infected gut-associated lymphoid tissue is not fully mapped (51).

This study aims to visualize and compare the accumulation patterns of F4/80$^+$, Moma1$^+$, and Moma2$^+$ macrophages in murine Peyer's Patches following oral Salmonella challenge, using immunohistochemical techniques. By integrating detailed anatomical mapping with subset-specific marker analysis, we seek to provide a functional topogram of the intestinal macrophage response during acute digestive system infection.

Materials and Methods

Animals and Infection Model

Female C57BL/6 mice (8 weeks old, 18–22 g) were obtained from a certified breeder and housed under specific pathogen-free conditions with a 12 h light/dark cycle and ad libitum access to autoclaved food and water. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Streptomycin Pretreatment: To enhance Salmonella colonization, mice were pretreated with streptomycin to disrupt the commensal microbiota (52). Twenty-four hours before infection (Day −1), each mouse received 20 mg streptomycin sulfate (Sigma-Aldrich) dissolved in 100 µL sterile water via oral gavage.

Bacterial Strain and Inoculum Preparation: Salmonella enterica serovar Typhimurium strain SL1344 (streptomycin-resistant) was grown overnight in LB

Table 1: Macrophages biomarker

Marker	Subset	Anatomical Location	Proposed Function	Ref
F4/80	Pan-macrophage	Subepithelial dome, lamina propria	Phagocytosis, bacterial killing, antigen capture	45
Moma1 (CD169)	Metallophilic macrophages	Marginal sinus, subcapsular sinus	Lymph filtration, antigen trapping, barrier function	46
Moma2	Inflammatory/tissue macrophages	Interfollicular regions, T-cell zones	Antigen presentation, T-cell priming, cytokine production	47

broth at 37°C with shaking, subcultured, and grown to late-log phase (OD600 ≈ 0.8–1.0) (53). Bacteria were pelleted, washed, and resuspended in sterile PBS to a final concentration of 1×10^8 CFU/mL. Inoculum dose was confirmed by serial dilution and plating on LB agar containing 100 µg/mL streptomycin.

Infection: On Day 0, mice were fasted for 3–4 hours, then orally gavaged with 100 µL of bacterial suspension (1×10^7 CFU) using a sterile 20 G stainless steel gavage needle. Control mice received 100 µL sterile PBS. Mice were monitored daily for clinical signs.

Tissue Collection: At 7 days post-infection, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. The cecum was excised, rinsed in ice-cold PBS, and processed for histological and immunohistochemical analysis.

Macroscopic Evaluation

Following excision, the cecum was carefully opened along the anti-mesenteric border, gently rinsed with ice-cold phosphate-buffered saline (PBS) to remove luminal contents, and pinned flat on a black wax-coated dissection pan. The mucosal surface was gently expanded and stabilized using fine insect pins to expose Peyer's patches without causing mechanical distortion, and the surrounding mucosal surface was evaluated under a stereomicroscope.

Immunohistochemistry Analysis

Cryosections (4 µm) were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10 minutes. Endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% hydrogen peroxide for 15 minutes at room temperature (54). After washing with PBS, non-specific antibody binding was minimized by incubating sections in 1% bovine serum albumin (BSA) in 0.1 M PBS for 1 hour. Sections were then incubated overnight at 4°C with primary antibodies directed against F4/80, Moma1, and Moma2, as detailed in Table 2. The following day, sections were washed and incubated with biotinylated secondary antibodies, followed by the Vectastain Standard HRP Kit (Vector Laboratories). Immunoreactivity was visualized using 0.05% 3,3′-diaminobenzidine (DAB) as the chromogen (55). Sections were then dehydrated through a graded series of ethanol, cleared in xylene, and mounted with coverslips using permanent mounting medium.

Results

Macroscopic Evaluation

Table 2: Primary Antibody Incubation:

Target / Marker	Antibody Clone	Host Species	Dilution	Source (Catalog No.)
Pan-macrophage	**F4/80** (clone BM8 or CI:A3-1)	Rat anti-mouse	1:100	BioLegend / Abcam
Metallophilic macrophages	**Moma1** (CD169, clone 3D6.112)	Rat anti-mouse	1:50	Abcam / Bio-Rad
Inflammatory/tissue macrophages	**Moma2** (clone MOMA-2)	Rat anti-mouse	1:50	Bio-Rad / Santa Cruz

Peyer's patches were identified as oval or round, opaque lymphoid aggregates protruding slightly from the surrounding mucosal surface. Peyer's patches in infected animals were enlarged and more opaque (Figure 1).

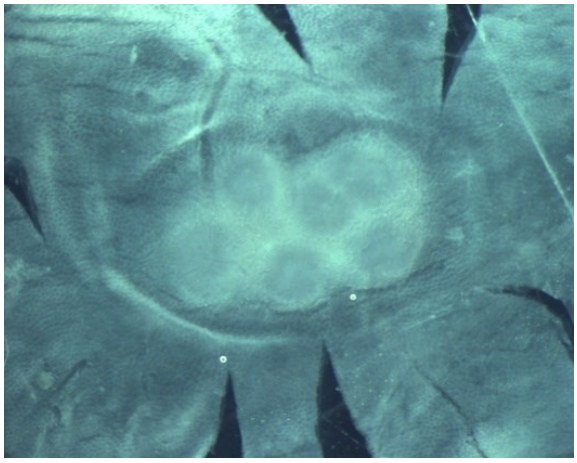

Figure 1. Macroscopic appearance of the murine cecum 7 days post-infection. The cecum was opened along the anti-mesenteric border, pinned flat, and imaged under a stereomicroscope at 5× magnification.

Immunohistochemical Findings

F4/80 Staining (Pan-Macrophage Marker)

In infected mice, dense clusters of F4/80$^+$ macrophages were observed predominantly in the subepithelial dome (SED) region. Moderate staining was also detected within the follicular periphery, interfollicular areas, and around blood vessels (Figure 2).

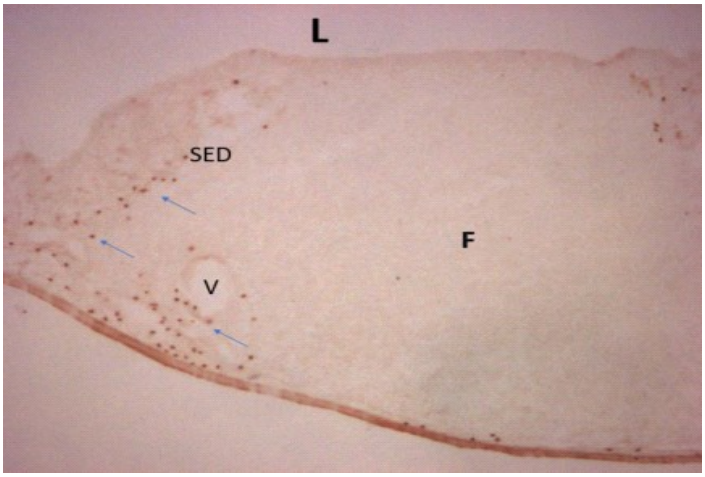

Figure 2. Immunohistochemical localization of F4/80$^+$ macrophages in Peyer's patch following Salmonella infection. Cecal sections from Salmonella-infected mice at 7 days post-infection were stained with anti-F4/80 antibody (clone BM8) using DAB as the chromogen (brown) and counterstained with Mayer's hematoxylin (blue). Dense clusters of F4/80$^+$ macrophages (arrows) are predominantly localized within the subepithelial dome (SED). Moderate staining is also observed in the follicular periphery and surrounding blood vessels (V). F, B-cell follicle; L, intestinal lumen; V, blood vessel. Original magnification 100×.

Moma1 Staining (Metallophilic Macrophages)

Moma1$^+$ cells were sharply delineated around blood vessels and surrounding B-cell follicles. No significant Moma1 staining was observed in the SED or interfollicular regions (IFR) (Figure 3).

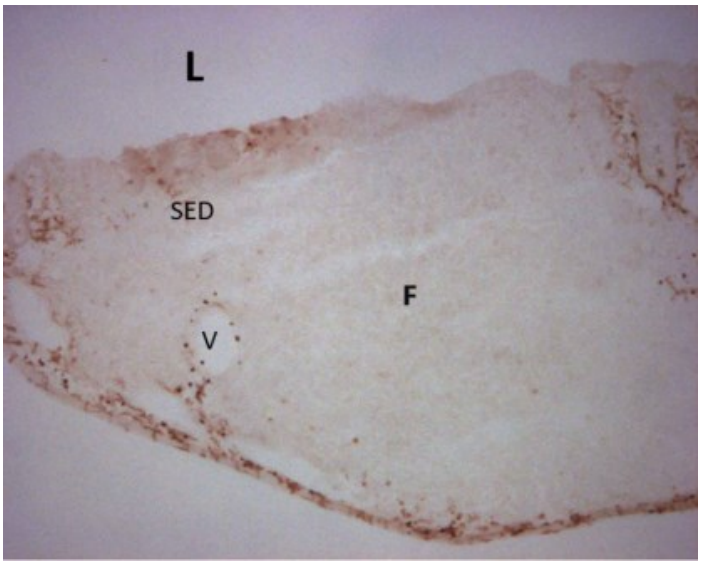

Figure 3. Immunohistochemical localization of Moma1$^+$ metallophilic macrophages in Peyer's patch following Salmonella infection. Cecal sections were stained with anti-Moma1 antibody (clone 3D6.112). Moma1$^+$ immunopositive cells (arrows) are sharply delineated around B-cell follicles (F) and adjacent to blood vessels (V). No significant staining is observed within the subepithelial dome (SED) or interfollicular regions (IFR). F, B-cell follicle; L, intestinal lumen; V, blood vessel. Original magnification 100×.

Moma2 Staining (Inflammatory/Tissue Macrophages)

Moma2$^+$ cells were widely distributed outside of the Peyer's Patch. The highest density was observed in the interfollicular regions (IFR) and around high endothelial venules (Figure 4).

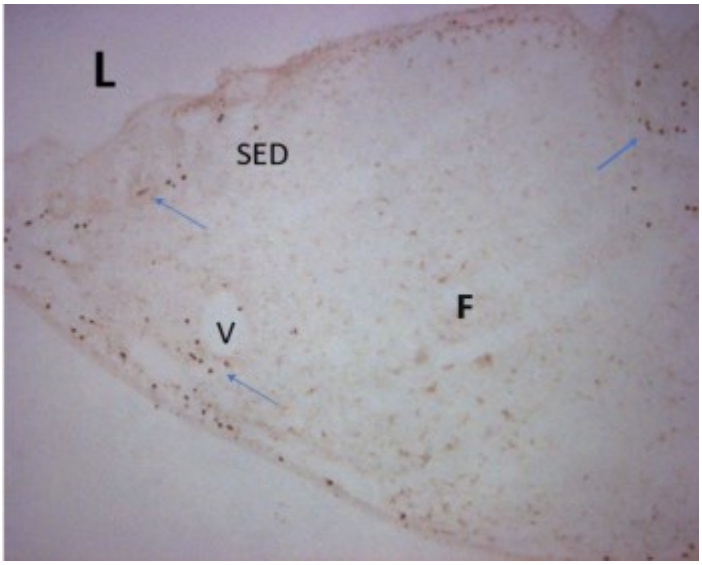

Figure 4. Immunohistochemical localization of Moma2$^+$ inflammatory macrophages in Peyer's patch following Salmonella infection. Cecal sections were stained with anti-Moma2 antibody (clone MOMA-2). Moma2$^+$ immunopositive cells (arrows) are widely distributed outside B-cell follicles (F), with highest density observed in the interfollicular regions (IFR) and surrounding high endothelial venules (V). These cells exhibit abundant cytoplasm and strong DAB reactivity. F, B-cell follicle; L, intestinal lumen; V, blood vessel. Original magnification 100×.

Discussion

This study provides a detailed immunohistochemical map of macrophage subset localization within murine Peyer's patches following oral Salmonella infection. Using F4/80, Moma1, and Moma2 as subset-specific markers, we demonstrate that Salmonella infection induces a compartmentalized redistribution of macrophages into distinct microanatomical niches that correspond to their specialized functions.

The dense accumulation of F4/80$^+$ macrophages in the subepithelial dome confirms that this compartment is the primary immunological checkpoint immediately downstream of M-cell-mediated bacterial translocation (27,29). As described in the Introduction, M cells transport Salmonella across the follicle-associated epithelium and release them intact into the subepithelial dome (24). Here, F4/80$^+$ macrophages are strategically positioned to capture and eliminate invading bacteria (45). The marked increase in F4/80$^+$ cell density in infected animals indicates significant recruitment of circulating monocytes, likely driven by local chemokines such as CCL2 and CX3CL1 (56). These recruited cells likely undergo classical M1 activation, characterized by enhanced bactericidal activity and pro-inflammatory cytokine secretion (38). Moderate F4/80 staining was also observed in the follicular periphery and around blood vessels, suggesting that perivascular macrophages may monitor hematogenous spread while follicular-associated macrophages participate in antigen transfer to B cells (57).

The restricted, sharp delineation of Moma1$^+$ cells around B-cell follicles and adjacent to vessels, with no staining in the subepithelial dome or interfollicular regions, closely resembles the localization of CD169$^+$ metallophilic macrophages in the splenic marginal zone and lymph node subcapsular sinus (31,46). Our findings suggest an analogous barrier function in Peyer's patches (58). During Salmonella infection, Moma1$^+$ macrophages preserve their anatomical integrity and do not migrate into inflamed compartments, indicating a role in lymph filtration and antigen trapping rather than direct microbicidal activity (59). By capturing bacteria and antigens that escape the subepithelial dome, these stationary sentinels likely prevent lymphatic dissemination and systemic infection (60). Perivascular Moma1$^+$ staining may represent an additional checkpoint monitoring vascular entry.

The widespread distribution of Moma2$^+$ macrophages outside B-cell follicles, with highest density in the interfollicular regions, directly supports their role in antigen presentation and T-cell activation (47). The interfollicular regions are T-cell zones containing naïve lymphocytes and high endothelial venules (32). Moma2$^+$ macrophages here are optimally positioned to acquire Salmonella antigens, process and present them via MHC class II, and provide co-stimulatory signals for naïve CD4$^+$ T cells (41). Their strong Moma2 reactivity and abundant cytoplasm are consistent with an

activated, antigen-presenting phenotype (61). These cells likely originate from inflammatory monocytes recruited via high endothelial venules, differentiating into TNF and iNOS-producing macrophages that amplify the local Th1 response (62). Moma2$^+$ cells were also observed outside Peyer's patches in the lamina propria and submucosa, possibly reflecting macrophage dissemination or distinct mucosal populations involved in epithelial defense (63).

Taken together, our findings reveal a spatially segregated, three-tiered macrophage defense system within Peyer's patches (Table 2). F4/80$^+$ macrophages in the subepithelial dome serve as first-line phagocytes for bacterial capture and killing. Moma1$^+$ metallophilic macrophages stationed at the marginal sinus and perivascular areas function as barrier sentinels preventing systemic spread. Moma2$^+$ inflammatory macrophages concentrated in the interfollicular regions act as antigen-presenting cells for T-cell priming and Th1/Th17 polarization (49). This division of labor allows Peyer's patches to simultaneously combat infection at the portal of entry, block lymphatic and vascular dissemination, and initiate adaptive immunity.

Table 2. Three-Line Defense System in Peyer's Patches

Line	Compartment	Macrophage Subset	Primary Function
1st	Subepithelial Dome (SED)	**F4/80$^+$**	Bacterial capture, phagocytosis, intracellular killing
2nd	Marginal sinus / Perifollicular	**Moma1**	Lymph filtration, antigen trapping, barrier function
3rd	Interfollicular Regions (IFR)	**Moma2**	Antigen presentation, T-cell priming, Th1/Th17 polarization

Our findings are consistent with previous reports describing CD169$^+$ macrophages as stationary sentinels in spleen and lymph nodes, and extend this concept to the gut-associated lymphoid tissue (46,58). The subepithelial dome accumulation of F4/80$^+$ macrophages corroborates studies of monocyte-derived intestinal macrophages during inflammation (56), while dense interfollicular Moma2$^+$ localization aligns with the established role of inflammatory macrophages in T-cell priming (62).

This study is primarily descriptive and topographical. We did not directly assess functional status such as bacterial killing capacity, cytokine production, or antigen presentation efficiency. Future studies should combine multiplex immunofluorescence with intracellular cytokine staining, phagocytosis assays using fluorescently labeled Salmonella, and subset-specific depletion models to determine the precise contribution of each macrophage population to bacterial control and host survival (64). Time-course analysis is also needed to map the kinetics of macrophage recruitment and redistribution (65).

Conclusion

In conclusion, this study provides the first comprehensive immunohistochemical map of F4/80$^+$, Moma1$^+$, and Moma2$^+$ macrophages in murine Peyer's patches during acute Salmonella infection. We demonstrate that F4/80$^+$ macrophages accumulate in the subepithelial dome as first-line phagocytes, Moma1$^+$ metallophilic macrophages remain stationed at the marginal sinus and perivascular areas as barrier sentinels, and Moma2$^+$ inflammatory macrophages populate the interfollicular regions as antigen-presenting cells. These findings have translational implications for oral vaccine design (6). Strategies that enhance F4/80$^+$ macrophage recruitment and activation may improve early bacterial clearance, agents that preserve Moma1$^+$ barrier integrity could prevent systemic dissemination, and adjuvants that boost Moma2$^+$ antigen presentation may potentiate T-cell responses and immunological memory (66).

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