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INVITED REVIEW

Development and organization of omental milky spots

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Summary

The milky spots in omentum are atypical lymphoid tissues that play a pivotal role in regulating immune responses in the peritoneal cavity. The milky spots act as central hubs for collecting antigens and particles from the peritoneal cavity, regulating lymphocyte trafficking, promoting the differentiation and self-renewal of immune cells, and supporting the local germinal centre response. In addition, the milky spots exhibit unique developmental characteristics that combine the features of secondary and tertiary lymphoid tissues. These structures are innately programmed to form during foetal development; however, they can also be formed postnatally in response to peritoneal irritation such as inflammation, infection, obesity, or tumour metastasis. In this review, I discuss emerging perspectives on homeostatic development and organization of the milky spots.

KEYWORDS
macrophage, milky spot, omentum, retinoic acid

1 | INTRODUCTION

Serous cavities, including the peritoneal, pleural and pericardial cavities, are fluid-filled spaces that hold internal organs. The peritoneal cavity is the largest serous cavity in mammals and accommodates most organs of the abdomen, including the stomach, spleen, intestines, pancreas and reproductive organs, whereas the pleural and pericardial cavities accommodate the lungs and heart respectively. The human peritoneal cavity contains 50–100 mL of transparent straw-coloured fluid that is continually secreted from the mesothelium lining the serous membranes.[1] This fluid moves through the diaphragm's motion and bowel peristalsis, playing a crucial role in lubricating the surface of abdominal organs.[2,3] The peritoneal cavity is normally sterile, whereas peritonitis, an inflammation of the peritoneum, can occur due to an infection caused by pathological or traumatic loss of intestinal wall integrity, cirrhosis, pancreatitis, abdominal surgery or peritoneal dialysis.[4,5] Infections within the peritoneal cavity pose a potent risk of disseminating these pathogens into the bloodstream and organs, leading to life-threatening sepsis. Indeed, intra-abdominal infection is the second most common cause of sepsis and requires prompt medical treatment.[6]

The exact development and progression of intra-abdominal sepsis remains largely unclear,[7,8] but it has become evident that visceral adipose tissue, omentum, plays a pivotal role in the defence mechanism and immune regulation within the peritoneal cavity. The omentum is an apron-like elongated adipose tissue that hangs from the stomach and is connected to the spleen, pancreas and colon.[9] In humans, it is a significant site for visceral fat storage. The size of the omentum varies depending on the storage of fat; it can range from 300 to 2000 g with a surface area of 300–1500 cm^2. The omentum can expand in size, particularly in obesity, and excess omental fat deposition is considered a risk factor for metabolic diseases.[10] In contrast, the omentum in mice is usually found as a thin, slightly elongated and vascularized fat attached to the stomach while it is

structurally similar to human omentum.[11] In addition to their ability to store fat, the omenta of both humans and mice are highly mobile organs that move across the cavity and have a unique ability to occlude the sites of peritoneal inflammation upon surgical wounds, ulcerated intestines and inflamed appendices.[12] Indeed, ileoanal anastomosis patients who undergo surgical removal of the omentum (omentectomy) tend to have worse outcomes,[13] and omentectomy in rats has been shown to reduce survival in experimental peritonitis.[14] Because of its critical function in the prevention of infection and injury, the omentum is often referred to as 'the policeman of the abdomen' which was first documented by British surgeon Morison in 1908.[15,16] In this review, I discuss the recent advances in our understanding of the unique ability of the omentum to regulate peritoneal immunity and homeostasis.

2 | FORMATION OF ATYPICAL LYMPHOID TISSUES IN THE OMENTUM

The omentum is markedly different from conventional adipose tissues, as it encompasses loosely arranged lymphoid follicles, known as 'milky spots', which are positioned directly beneath the mesothelial layer and encircled by adipocytes[17] (Figure 1). In human, Omentum harbours more than 100 milky spots with diameters ranging from 300 to 700 μm,[18] whereas the mouse omentum can harbour up to 80 aggregates under homeostatic conditions.[19] The milky spots compose unique niches to collect antigens, particles and pathogens in the peritoneal cavity and function as peripheral lymphoid tissues for the regulation of humoral immune responses.[20,21] Importantly,

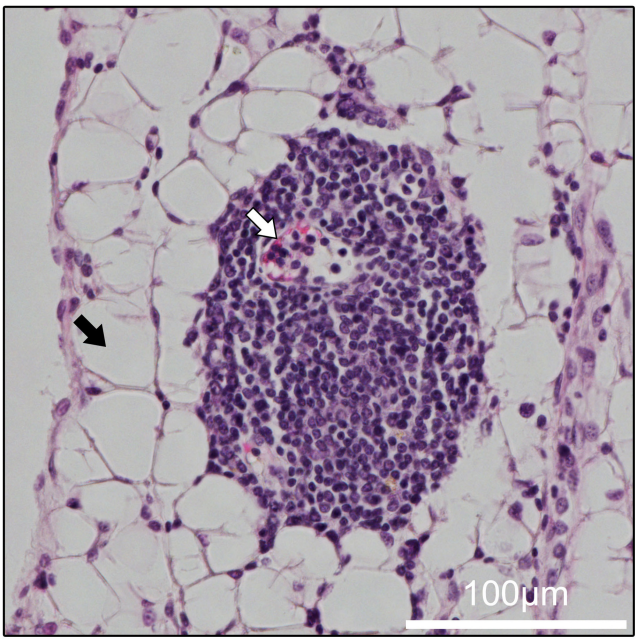

FIGURE 1 The milky spot in omentum. Haematoxylin and eosin (H&E) staining of the mouse milky spot. The milky spot is highly vascularized with blood vessels (white arrow) and is surrounded by adipocytes (black arrow). Scale bar, 100 μm.

Rangel-Moreno et al. revealed that the milky spots support local germinal centre B-cell responses including isotype switching, somatic hypermutation and limited affinity maturation, which in many ways resemble the follicles of secondary lymphoid tissues.[22]

The milky spots follow a developmental trajectory distinct from that of conventional lymphoid tissues. In human, the development of milky spots is first observed with the accumulation of monocytes and macrophages at 20 weeks of gestation, and true milky spots are detected at 35 weeks.[23] The structures of milky spots in mice and other mammals also appear during foetal development.[24,25] This suggests that the emergence of milky spots, like secondary lymphoid tissues, is an innately programmed process that has been predetermined, even though its development seems to occur much later than that of human lymph nodes, which start from 13 weeks of gestation.[26] During foetal life, haematopoietic lymphoid tissue inducer (LTi) cells accumulate at designated locations to initiate the development of lymph node and Peyer's patch.[27,28] LTi cells are essential for initiating the formation of secondary lymphoid tissues via their interaction with lymphoid tissue organizer (LTo) cells in a lymphotoxin-αβ–dependent manner. In contrast, omental milky spots develop in the absence of LTi cells, indicating that the development of milky spots uses regulatory mechanisms distinct from those used by lymph nodes and Peyer's patches.[22] Notably, the size and number of milky spots greatly increases in response to postnatal exposure to microbial products.[19] This phenomenon resembles the formation of tertiary lymphoid tissues that are formed postnatally in peripheral and non-lymphoid organs. The formation of tertiary lymphoid tissues is dependent on the presence of local chronic inflammation such as autoimmune diseases, chronic infection, rejection of organ transplantation and tumours.[29] Indeed, the formation of milky spots and other fat-associated lymphoid clusters (FALCs) such as mesenteric, gonadal, mesenteric, mediastinal and pericardial lymphoid clusters is enhanced by inflammation, which triggers the recruitment of myeloid cells that express tumour-necrosis factor (TNF) necessary for signalling via the TNF receptors in stromal cells.[19] While FALCs are generated after birth, with their formation being visible in mice at 2–3 weeks of age,[19] the structural similarity between milky spots and FALCs suggest that they perform similar functions in different fat storage locations.[17] The postnatal enhancement of these lymphoid clusters is at least partly dependent on the presence of commensal microbiota, as evidenced by that germ-free mice had fewer milky spots and FALCs than that in specific pathogen-free mice.[19,30] Thus, the milky spots seem to be atypical lymphoid tissues with a hybrid nature of secondary and tertiary lymphoid tissues; their formation can be initiated by both hard-wired developmental machinery and postnatal inflammation.[12,31]

3 | ROLES OF VITAMIN A IN THE MILKY SPOTS

The milky spots are made up of various types of immune cells, such as lymphocytes, monocytes, macrophages, neutrophils, eosinophils

and innate lymphoid cells (ILCs), while their cellular composition and positioning are distinct from that of conventional secondary lymphoid tissues. For example, the milky spots have been proposed to be an important source of macrophages in the peritoneal cavity. Peritoneal macrophages account for half of the immune cells in the peritoneal cavity, and play critical roles in the protection of the cavity through the elimination of invaded pathogens, programmed for 'silent' clearance of apoptotic cells[32] and promoting tissue repair responses upon infection and injury.[5,33] The milky spots provide a microenvironment in which peritoneal macrophage progenitor cells can home and proliferate.[34,35] Indeed, omentectomy reduces the number of peritoneal macrophages in rats,[36] and liposome-mediated peritoneal macrophage depletion triggers de novo macrophage proliferation in the milky spots.[37] We recently found that omentum-derived retinoic acid, a lipophilic molecule derived from vitamin A, acts on macrophages to induce the expression of the transcription factor GATA6, which is a master regulator for the functional specialization of peritoneal macrophages.[38] In mice lacking the GATA6 gene in macrophages (*Lysm*-cre/*Gata6*-flox), significant reduction in the number of peritoneal macrophages was observed.[39,40] On the other hand, there was massive accumulation of the macrophages in the milky spots due to impaired migration of macrophages in the absence of GATA6. Therefore, GATA6-dependent gene expression programme is crucial for macrophage migration from the omentum to the peritoneal cavity, while the precise mechanism governing macrophage migration remains unclear. Buechler et al. reported that omental mesothelial cells and fibroblasts that express the transcription factor Wilms' Tumor 1 (WT1) are the source of retinoic acid for the induction of GATA6 in macrophages.[41] Thus, retinoic acid is a key molecule that induces tissue-specific functional specialization of macrophages in the peritoneal cavity and the omentum.

The B-cell population in the milky spots also exhibits a unique cellular composition. The milky spots and peritoneal cavity enrich a subclass of B cells, termed B-1 cells, that display developmental, phenotypic and functional characteristics distinct from that of the conventional B cells (referred to as 'B-2' cells).[42] B-1 cells are characterized as $IgM^{hi}IgD^{lo}CD23^{lo}CD11b^{+}$ cells, and are further subdivided into $CD5^{+}$ B-1a and $CD5^{-}$ B-1b cells.[43] B-1 cells exhibit a BCR repertoire that is enriched with highly poly-specific receptors, enabling them to respond to both self-antigens and microbial antigens. They can be activated without T-cell help, and play a significant role in producing natural antibodies in serum. B-1 cells develop from haematopoietic progenitor cells in the omentum and liver during foetal development, and they primarily reside in the body cavities where they are maintained by self-renewal.[25,44,45] Thus, B-1 cells in the milky spots and peritoneal cavity account for a major B-cell population (35%–70%), whereas they are present at low frequencies (<2%) in other lymphoid tissues, such as lymph nodes and spleen. Notably, retinoic acid plays a critical role in the proliferation of B-1 cells in the peritoneal cavity through the induction of transcription factor nuclear factor of activated T cells c1 (NFATc1).[46] Therefore, the significance of omentum-derived retinoic acid extends beyond its role in macrophage polarization, as it also appears to play a critical part in B-1 cell self-renewal. Nevertheless, the exact function of $WT1^{+}$ stromal cells in this process remains to be explored.

In addition to their role in the production of natural serum antibodies, peritoneal B-1 cells also contribute to the production of IgA-secreting plasma cells in the gut.[47,48] Peritoneal and omental B-1 cells migrate to the gut in response to stimulation by gut microbiota products, followed by their differentiation into plasma cells.[49] Conversely, germ-free mice accumulate significantly more B-1 cells in the peritoneal cavity than mice kept under specific pathogen-free conditions.[30] These studies suggest that the interaction between peritoneal B-1 cells and the gut microbiota stimulates constant B-1 cell migration to the gut. In agreement with this idea, B-1 cells preferentially class-switch to IgA compared to conventional B-2 cells and marginal zone B cells, while the relative contribution of B-1 and B-2-derived IgA in vivo is controversial.[50,51] A direct comparison of peritoneal B-1a and B-1b cells demonstrated that IgA is mainly produced by B-1b cells.[52] TGF-β and retinoic acid, among the cytokines and other factors, are the most prominent factors to induce IgA class-switch as well as gut homing receptor expression in peritoneal B-1b cells.[53,54] We found that GATA6 in peritoneal macrophages regulates the expression of TGF-β-associated genes, including TGF-β2, LTBP1 which regulates extracellular matrix deposition of TGF-β and THBS1 which promotes activation of latent form of TGF-β.[38] $GATA6^{+}$ peritoneal macrophages induced class switching to IgA in peritoneal B-1 cells in the presence of retinoic acid, BAFF and LPS. Conversely, GATA6-deficient peritoneal macrophages failed to generate IgA^{+} B-1 cells in vitro, and the addition of recombinant TGF-β could restore the defect, suggesting GATA6 – TGF-β axis regulates IgA class switching of peritoneal B-1 cells. In addition, macrophage-specific GATA6-deficient mice showed substantial decrease in the number of IgA expressing B cells and the amount of faecal IgA, in the absence of secondary lymphoid.[55] In these mice, the number of B-1 cells in the peritoneal cavity was not affected, and total IgM, total IgA and phosphorylcholine-specific IgM in the serum were comparably detected in the serum, indicating that the reduction in IgA production was not due to B-1 cell–intrinsic alterations. Taken together, these studies demonstrate the significance of peritoneal lymphocytes in the generation of gut-associated IgA.

4 | STROMAL CELL NETWORK IN OMENTAL MILKY SPOTS

The development, organization and functioning of lymphoid tissues rely heavily on the formation of a structural framework constructed by stromal cells. The milky spots are supported by several stromal components, which comprise of a reticular network of fibroblastic stromal cells (FRCs), a glomerulus-like knot structure of blood vessels and a mesothelial layer that lines the milky spots.[56] FRCs are a type of specialized lymphoid tissue fibroblasts that are responsible for organizing the infrastructure of lymphoid tissue. FRCs are distinguished from other lymphoid tissue stromal cells by the expression of podoplanin (PDPN) and platelet-derived growth factor receptor-α

(PDGFRα, CD140a), and their lack of expression of PECAM-1 and CD45.[57] They also express molecules common to many myofibroblasts including desmin, CD90, CD105, α-smooth muscle actin (αSMA) and the antigen recognized by the ER-TR7 antibody.[58,59] The expression of chemokines CCL19 and CXCL13 is another characteristic of FRCs, and therefore, the genetic modification of FRCs in mice can be achieved by the induction of expression of Cre recombinase under the control of *Ccl19* or *Cxcl13* gene promoters.[60] In classical secondary lymphoid tissues, FRCs are heterogeneous cells with respect to morphology, localization and function. The specialized functions of each FRC subset serve to support and reinforce the intricate structure of lymphoid tissue.[12,61] These FRC subsets are exemplified by the T-cell zone reticular cells (TRCs) that are situated in the T-cell zone and produce interleukin-7 (IL-7) to support naive T cells; marginal zone reticular cells (MRCs) that are situated in the marginal zone in close contact with lymphatic endothelial cells and macrophages; interfollicular FRCs (IFRCs) that line the subcapsular sinus and the lymphatic vessels coursing between follicles; perivascular reticular cells (PRCs) that support high endothelial venule (HEV) barrier function and immune cell traffic from blood vessels into the lymphoid tissue parenchyma; and follicular dendritic cells (FDCs) that provide both antigen-driven and co-signals to B cells to support germinal centre function.

Compared to the secondary lymphoid tissues that are organized in strategic compartmentalization of B and T-cell zones, the milky spots display reduced structural complexity without a clear structural segregation or distinct arrangement of lymphocytes, and a dense cluster of B cells is intermingled with T cells and myeloid cells. Therefore, the precise function and the diversity of FRC subsets in milky spots had not been clarified until recently. Perez-Shibayama et al. achieved targeting of FRCs within the milky spots with the *Ccl19* promoter.[60] They showed that the MyD88-dependent innate immune sensing in FRCs triggers the recruitment of inflammatory monocytes, which is followed by the rapid reorganization of the milky spot structure. This process subsequently leads to the activation of B cells through $CD4^+$ T-cell-dependent immunoglobulin class switching. This study illustrated that FRCs in the milky spots are the fundamental component to regulate pathogen recognition, immune cell recruitment and lymphoid tissue remodelling, which facilitates the generation of protective peritoneal immunity. On the other hand, we recently found a small fraction of FRCs in the milky spots displayed high retinoic acid-producing capacity and the expression of the *Aldh1a2* gene, which encodes a rate-limiting enzyme regulating retinoic acid synthesis.[31] In addition to their expression of FRC-associated genes, they express the endothelial cell marker, *Tie2*, which is typically absent in FRCs. It is worth mentioning that FRCs with both retinoic acid-producing activity and TIE2 expression are not found in lymph nodes. In addition, *Aldh1a2*$^+$ FRCs are situated within the $Desmin^+$ filamentous network of FRCs,[62,63] implicating that *Aldh1a2*$^+$ FRCs may contribute to a unique lymphoid microarchitecture that is different from secondary lymphoid tissues. Diphtheria toxin receptor-mediated conditional ablation of *Aldh1a2*$^+$ FRCs resulted in the alteration in the milky spot architecture with a significant reduction in size and cellularity, indicating that *Aldh1a2*$^+$ FRCs play a critical role in supporting lymphoid tissue infrastructure. In agreement with this, the ablation of *Aldh1a2*$^+$ FRCs leads to a severe reduction in the number of circulating lymphocytes including $CD4^+$ T cells, $CD8^+$ T cells, and B-2 B cells.[31] In contrast, the impact of *Aldh1a2*$^+$ FRCs ablation on the numbers of stational or slower circulating cells such as B-1 B cells and macrophages is less pronounced. The adaptive transfer experiment indicated that the migration of blood-borne lymphocytes to the milky spots was significantly hindered by the ablation of *Aldh1a2*$^+$ FRC, whereas their migration to the lymph nodes was unaffected. Together, *Aldh1a2*$^+$ FRCs are required for the homeostatic recruitment of lymphocytes to the milky spots.

5 | LYMPHOCYTE TRAFFIC THROUGH THE MILKY SPOTS

Lymphocyte traffic through the lymphoid tissues is an essential homeostatic mechanism that regulates humoral and cellular immunity. In secondary lymphoid tissues, specialized blood vessels, called high endothelial venules (HEVs), serve to recruit blood-borne lymphocytes to the lymphoid tissue parenchyma, where they encounter cognate antigens. HEVs are comprised of high endothelial cells that are readily distinguished from other blood endothelial cells by their characteristic plump and cuboidal morphology.[64] By supporting high levels of lymphocyte extravasation from the blood circulation, HEVs are crucial for lymphocyte recirculation and immune surveillance for foreign invaders.[65] HEVs preferentially express genes that are associated with lymphocyte recruitment in order to perform their specialized functions as lymphocyte portals. These genes include peripheral node addressin (PNAd), which binds to the classic homing receptor for T and B lymphocytes, L-Selectin/CD62L.[66] The interaction between PNAd and L-selection is crucial for lymphocyte transmigration, particularly during the initial stages of the multistep adhesion cascade.[64] Thus, lymphocyte homeostasis in resting lymphoid tissues is maintained through the regulation of circulating lymphocyte entry by HEVs.

Similar to the conventional lymphoid tissues, some of the blood vessels in the milky spots express HEV markers including PNAd[22,31] (Figure 2). The formation and organization of the milky spots depend on HEVs, as evidenced by a significant decrease in the milky spot formation in lymphotoxin α (Lta)-deficient mice due to the impaired differentiation of HEVs.[22] Additionally, Buscher et al. demonstrated that peritonitis induced neutrophil transmigration across omental HEVs, and this process was dependent on PNAd, L-and E-selectin, and Mac-1.[67] Other studies have shown that mucosal addressin cell adhesion molecules (MAdCAM-1) is also expressed on HEVs in the milky spots.[31,68] MAdCAM-1 is a transmembrane protein typically expressed by HEVs in mucosal lymphoid tissues, such as Peyer's patches and mesenteric lymph nodes.[69] Importantly, α4β7 integrin, which is a ligand for MAdCAM-1, is required for homing of B2 lymphocytes to the milky spots, suggesting that molecular constituents

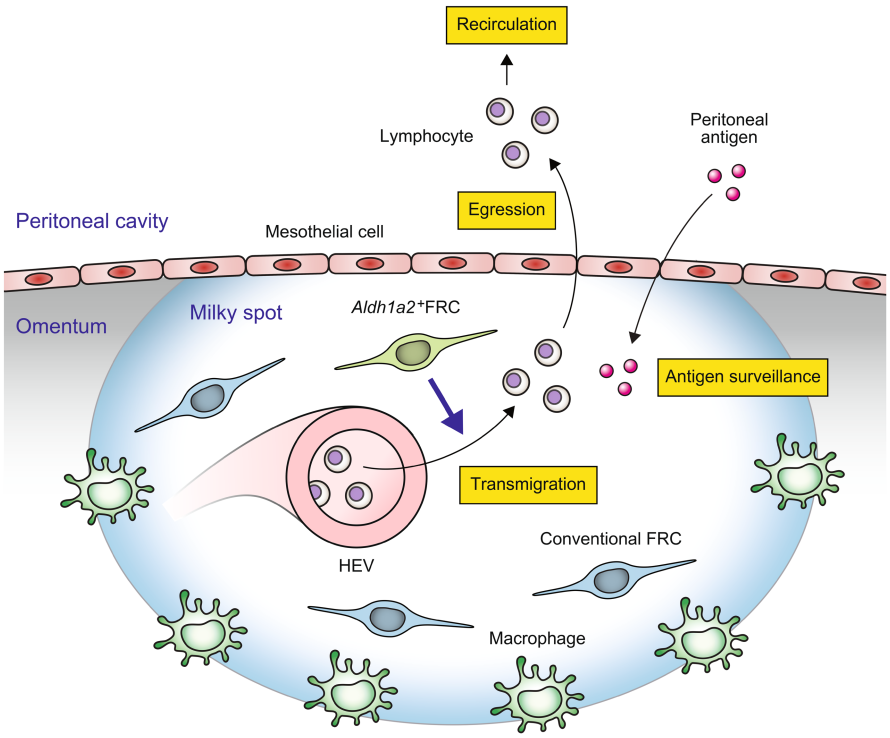

FIGURE 2 Lymphocyte circulation through the milky spots. High endothelial venules (HEVs) present in the milky spots facilitate the migration of blood-borne lymphocytes into the tissue parenchyma, where they encounter peritoneal antigen. The lymphocytes eventually leave the milky spots and enter the peritoneal cavity, where they are carried away through the lymphatic drainage system of the diaphragm, and then recirculate. *Aldh1a2*$^+$ FRCs are involved in HEV-mediated lymphocyte recruitment.

enabling B-cell entry into the milky spots share similarities with those of B-cell migration into mucosal lymphoid tissues.[68]

Lymphocyte trafficking to lymphoid tissues is influenced by a complex interplay between various molecular signals and interactions, including the expression of adhesion molecules, chemokine receptors and other signaling molecules.[70] The specific mechanisms and kinetics can vary depending on the context of the immune response and the type of lymphocytes involved. It is well established that several homeostatic chemokines including CCL19, CCL21, CXCL12 and CXCL13 have important functions in the trafficking and homing of lymphocytes through the secondary lymphoid tissues.[71,72] CCL19 and CCL21 are key chemokines that attract T cells and dendritic cells to T-cell zones and facilitate the interaction of these immune cells necessary for immune activation.[73] However, mice with a spontaneous mutation in plt (paucity of lymph node T cells) display well-developed milky spots in the omentum, despite not expressing CCL19 and CCL21.[22] In contrast, CXCL13 is absolutely required for the development and lymphocyte homing to the milky spots, which was demonstrated by the impaired formation of the milky spots in mice deficient in the CXCL13 or its cognate receptor CXCR5.[45,74] In secondary lymphoid tissues, CXCL13 is the primary chemokine that influences mature B cells and is mainly produced by FDCs located within B-cell follicles. However, FDCs do not appear to be present in the milky spots, and CXCL13 expression is largely limited to macrophages and mesothelial cells that cover the milky spots.[45,75] This demonstrates lymphoid tissue-specific variations in the expression of CXCL13 and the diversity of cellular sources contributing to its production in different immunological microenvironments.[26]

The chemokine CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is a key mediator of a variety of physiological processes, including chemotaxis, cell proliferation, migration and gene expression.[76,77] In conventional lymphoid tissues, CXCL12 is found on the luminal surface of HEVs where it promotes extravasation of blood-borne lymphocytes into the tissue parenchyma.[78,79] The presence of CXCL12 in the milky spots can be also found on HEVs,[31] and CXCL12 has emerged to be involved in the chemotaxis within the milky spots.[80,81] In line with this, the expression of CXCR4, the receptor for CXCL12, is widely expressed in lymphocyte populations in the milky spots.[31] Intriguingly, the ablation of *Aldh1a2*$^+$ FRCs severely reduced the expression of *Cxcl12* gene in the milky spots, whereas that of other homeostatic chemokines including *Cxcl13*, *Cxcl16*, *Ccl19* and *Ccl21* genes was unaffected, indicating that *Aldh1a2*$^+$ FRCs play a crucial role in the production of CXCL12. Mechanistically, *Aldh1a2*$^+$ FRC-derived retinoic acid induces the expression of the *Cxcl12* gene in endothelial cells. In addition, *Aldh1a2*$^+$ FRCs themselves produce CXCL12, which may be transported to the luminal surface of HEVs via a process called 'transcytosis'.[82,83] Together, the importance of *Aldh1a2*$^+$ FRCs in regulating the production of CXCL12 emphasizes their vital function in maintaining homeostatic milky spot formation (Figure 2).

It is worth mentioning that the milky spots appear to be devoid of efferent lymphatic vessel connections.[84] Alternatively, the peritoneal cavity has been proposed as a pathway for lymphocyte exit[45] (Figure 2). The surgical removal of the omentum prevents the migration of blood-borne lymphocytes into the peritoneal cavity, and the α4β1 integrin on B lymphocytes is responsible for their migration.[68] In addition, sphingosine 1-phosphate (S1P) has been shown to be involved in the egression of lymphocytes into the peritoneal cavity.[85] These peritoneal lymphocytes are subsequently directed towards either the mediastinal or perithymic lymph nodes, after

leaving the peritoneal cavity via the lymphatic vessels in the diaphragm.[86] Deficiency of CCR7, a receptor for CCL19 and CCL21, results in a massive accumulation of T and B cells in the peritoneal cavity, indicating that the CCL19/CCL21/CCR7 axis regulates lymphocyte egress from the peritoneal cavity.[74] Thus, the migration of lymphocytes through the peritoneal cavity is a complex mechanism involving chemokines, lipid mediators and various cell adhesion molecules.[87]

6 | COLLECTION OF PERITONEAL ANTIGENS IN THE MILKY SPOTS

The proper functioning of the immune system is highly contingent upon the efficient uptake of antigens to the lymphoid tissues. The milky spots serve as the primary location for gathering antigens from the peritoneal cavity,[22] capturing bacteria resulting from intestinal perforations,[30] and accumulating fluid from peritoneal dialysis.[88] However, the mode of antigen entry into the milky spots is quite distinct from that of conventional secondary lymphoid tissues. This difference highlights the omentum's unique nature as a specialized immunological site within the peritoneal cavity.[56]

In lymph nodes, the process of collecting antigens and leukocytes is facilitated by the drainage of afferent lymphatics into the subcapsular sinus (SCS), which is the space that separates the capsule from the cortical parenchyma of the lymph node[89] (Figure 3A). The structure of SCS is characterized by the presence of reticular fibres, which are surrounded by reticular cell processes and macrophages. SCS macrophages are strategically positioned at the lymph-tissue interface, where they capture the antigen-immune complex that is drained from afferent lymphatics.[90] Importantly, SCS macrophages exhibit low phagocytic and lysosomal activities, which appear to be important to retain opsonized antigens on their surface and transport them to B-cell follicles.[91,92] Together, SCS macrophages play a vital role in initiating immune responses within lymph nodes, as they function as specialized antigen-presenting cells and activate the adaptive immune system.[93]

In contrast, the collection of mucosal antigens is mediated by microfold cells (M cells), which are specialized epithelial cells that serve as a crucial gateway for the delivery of mucosal antigens to the underlying immune cells in Peyer's patches[94] (Figure 3B). M cells are equipped with receptors that specifically recognize soluble IgG, allowing for the uptake of antigens from the gut lumen.[95] Additionally, they have a unique ability to transport antigens to underlying lamina propria through transcytosis. After crossing the epithelium through transcytosis, antigens are transported to a special, pocket-like area situated beneath the M cell's basolateral membrane. This space is home to multiple lymphocyte and phagocyte populations and plays a crucial role in initiating mucosal immune reactions. Thus, M cells are essential for the induction of mucosal immune responses, providing the first line of defence against pathogens encountered in the gastrointestinal tract.

The milky spots neither have afferent lymphatics nor M cells. Instead, the process of collecting antigens from the peritoneal cavity is mediated by small pores or fenestrations found in a layer of mesothelial cells that cover these aggregates[56] (Figure 3C). These

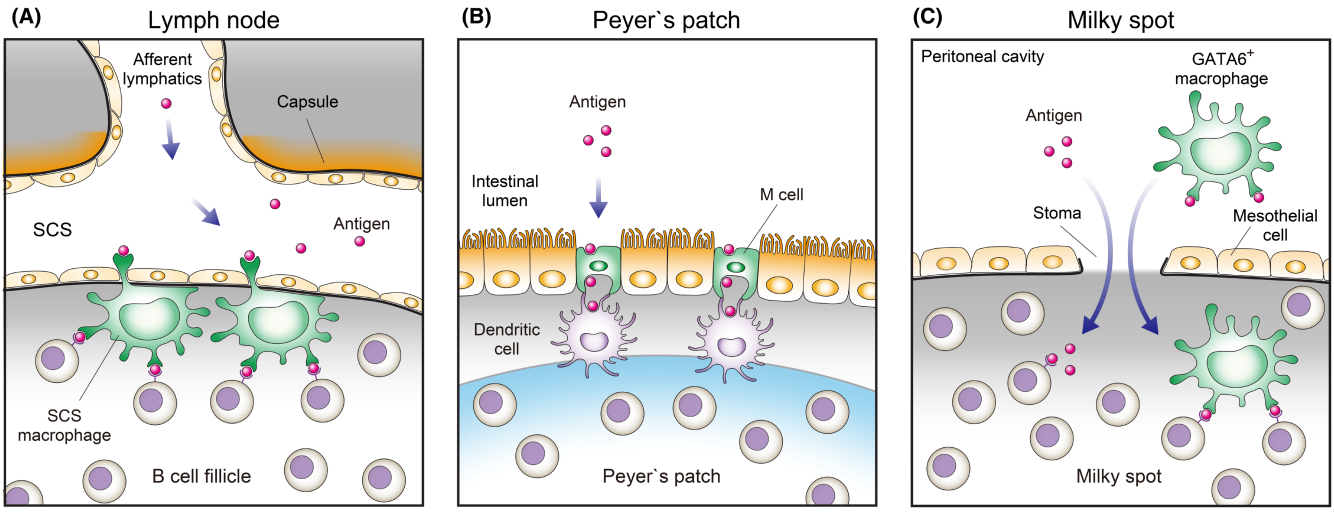

FIGURE 3 Entry of antigens to the lymphoid tissues. (A) Capture of lymph-borne antigens in the lymph nodes. The process of antigens entering the lymph nodes occurs via afferent lymphatic vessels, which transport lymph from the peripheral tissues towards the lymph nodes. These afferent lymphatic vessels empty into a region known as the subcapsular sinus (SCS). SCS macrophages serve as the first line of defence against lymph-borne antigens, delivering them to B-cell follicles to stimulate humoral immune responses. (B) Antigen uptake in the intestine. M cells, which are unique in their ability to take up antigens from the intestinal lumen, mediate transcytosis of gut luminal antigens. These antigens are then delivered to antigen-presenting cells such as dendritic cells to initiate mucosal immune response. (C) Antigen entry into the milky spots. The entry of antigens into the milky spots is facilitated by stomata, which are small pores located between mesothelial cells. Stomata allow direct flow of peritoneal antigens into the milky spots. Moreover, GATA6$^+$ macrophages located in the peritoneal cavity capture peritoneal antigens, which subsequently triggers their migration to the milky spots.

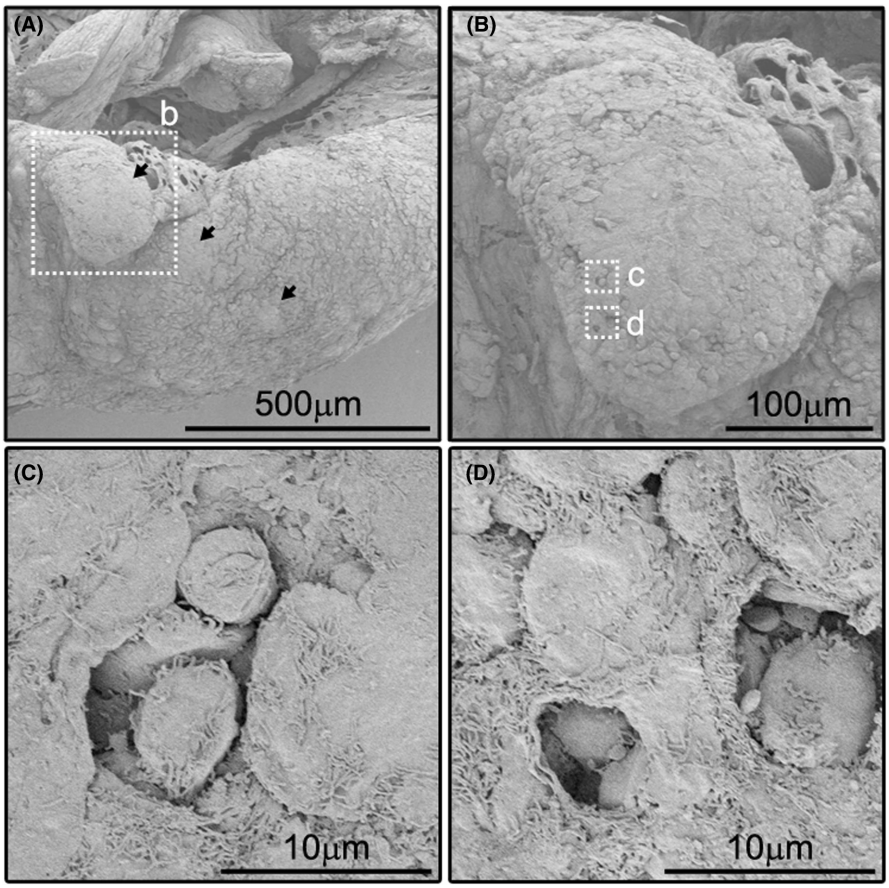

FIGURE 4 Scanning electron microscopy (SEM) of stomata of the milky spots. (A) The mouse milky spots are indicated by the black arrows. Scale bar, 500 μm. (B) Enlarged image of boxed milky spot. Scale bar, 100 μm. (C, D) Enlarged images of the boxed stomata. Scale bar, 10 μm.

intercellular pores (5–20 μm in diameter), known as 'stomata', enable the entry of free antigens and particles from the peritoneal cavity to the milky spots (Figure 4). Stomata are formed between the cubic mesothelial cells with 'paving stone-like' appearance which is clearly distinguishable from the flat mesothelial cells covering the other area of the omentum.[96,97] Besides the entry of free antigens through the stomata, GATA6$^+$ macrophages in the peritoneal cavity appear to play a role in the delivery of antigens to the milky spots.[22] Under steady conditions, GATA6$^+$-resident peritoneal macrophages either float in the peritoneal fluid or mildly attach to the serous membrane. However, in response to peritoneal infection or injury, they rapidly capture the peritoneal irritants and migrate to the milky spots, a phenomenon known as macrophage disappearance reaction (MDR).[38,98] The MDR implicates peritoneal macrophages as antigen-presenting cells that facilitate humoral immune responses in the milky spots.[99] Notably, GATA6$^+$ peritoneal macrophages express low or absent major histocompatibility complex (MHC) class II molecules and impair the ability to present processed antigens for CD4 T cell priming.[100,101] Instead, they enrich the expression of tethering receptors such as C-type lectin receptors and scavenger receptors,[102] which is reminiscent of the phenotype of SCS macrophages in lymph nodes. This implicates that GATA6$^+$ peritoneal macrophages may play a role in triggering T-cell-independent activation of B-1 cell immune responses.

7 | CONCLUSION REMARKS

The milky spots are unique lymphoid tissues that show characteristics of both secondary and tertiary lymphoid tissues. It appears that the mechanisms involved in the formation of the milky spot differ depending on whether it occurs during prenatal or postnatal. While this review highlights the homeostatic formation of the milky spots under steady-state conditions, many recent studies have documented and clarified the development of the milky spots upon the inflammation.[103] Future studies will reveal the specific and distinctive mechanisms underlying the formation of the milky spots. Meanwhile, the physiological relevance of the close relationship between milky spots and adipose tissues needs to be further investigated. In this regard, it will be also important to understand pathological relevance of the milky spots in obesity, which is associated with inflammation.

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CONFLICT OF INTEREST STATEMENT

The author declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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