Rom J Morphol Embryol 2022, 63(2):275–292 ISSN (print) 1220–0522, ISSN (online) 2066–8279 doi: 10.47162/RJME.63.2.01

REVIEW

RJME Romanian Journal of Morphology & Embryology http://www.rjme.ro/

Short histological kaleidoscope – recent findings in histology. Part II

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Abstract

This article focuses on the latest histological knowledge in the field regarding the peripheral lymphoid system [mucosa-associated lymphoid tissue (MALT), bronchus-associated lymphoid tissue (BALT), gut-associated lymphoid tissue (GALT)], the thymus stroma, some of the various corpuscles of the human body (Hassall's corpuscles in thymus, arenaceous corpuscles in pineal gland, *corpora amylacea* in prostate and other locations) and Fañanas glial cells in the cerebellum.

Keywords: corpuscles, glial cells, Hassall, arenaceous, *corpora amylacea*, Fañanas.

Introduction

Histology is a dynamic science that allows the permanent gathering of new scientific data from different medical fields, with the continuous modification of the known concepts about the structure of tissues and organs.

As already seen in Part I, the histological kaleidoscope papers present new insights on different histological issues; the present article focuses on the peripheral lymphoid system [mucosa-associated lymphoid tissue (MALT), bronchus-associated lymphoid tissue (BALT), gut-associated lymphoid tissue (GALT)], the thymus stroma [thymic epithelial cells (TECs)], the different types of corpuscles found in our body [*e.g.*, Hassall's corpuscles (HCs) in thymus, arenaceous corpuscles from the pineal gland (PG), *corpora amylacea* (CA) in prostate and other locations] and the quite mysterious cerebellum second characteristic population of glial cells, the Fañanas cells, all topics with structural data, lately enriched, due to modern advances in histology.

Mucosa-associated lymphoid tissue (MALT)

The lymphoid tissue is a composing part of the primary (central), secondary (peripheral), and tertiary (ectopic) lymphoid organs.

The central compartment contains the *primary lymphoid organs*, such as the red bone marrow, responsible for the production of B-lymphocytes, and the thymus, responsible for the production and selection of T-lymphocytes, to transform them both into immunocompetent lymphocytes [1].

The peripheral compartment consists of the *secondary (peripheral) lymphoid organs*, which appear during embryogenesis, regardless of the environmental antigen or an inflammatory process. These organs represent, in fact, the structural, anatomical support of the cellular reactions of the immune response. To this class belong the following structures: lymph nodes, spleen, tonsils, and MALT components, in which, because of antigenic exposure, effector B-lymphocytes, memory B-cell, plasma cells, memory, and T-effector lymphocytes are formed [1, 2].

The *tertiary lymphoid tissues* (ectopic lymphoid tissues) appear after birth without a genetic program of embryological development. They lack capsule and afferent lymphatics [1]. It is formed by the accumulation of lymphocytes under certain pathological conditions (autoimmune, inflammatory, tumor, bacterial, viral, or fungal infectious diseases) by activating various molecular pathways [3].

MALT was first described by Isaacson *et al.* as representing the organism's next path of defense after its passive anatomical and physiological barriers [4, 5]. MALT was defined as part of the immune system responsible for protecting the external mucosal membranes and the freely penetrable exterior of the gastrointestinal (GI) system from external pathogens [4, 6].

Overall, MALT is a complex of lymphoid cell nodules and diffuse lymphoid tissue found in the submucosal layers of the digestive, respiratory, and urogenital systems, and the thyroid, breasts, tonsils, salivary glands, skin [4, 7]. The cellular components are T- and B-lymphocytes and macrophages, responsible for capturing and reacting to antigens located at the mucosal access paths [8].

Regarding their distribution in the mucosal membranes of the body, MALT components are classified in: (*i*) BALT; (*ii*) tonsils (pharyngeal and palatine); (*iii*) auditory tube-,

nasal-, and larynx-associated lymphoid tissues (ATALT, NALT, and LALT); (*iv*) GALT; (*v*) conjunctiva-associated lymphoid tissue (CALT); (*vi*) other lymphoid nodules [4, 5, 8] (Table 1).

Table 1 – *Classification of MALT components*

	Components	Role
MALT	BALT	Protects the mucosal membranes of the bronchi and bronchioles
	ATALT	Defend the nasopharyngeal region
	NALT	Defend the nasopharyngeal region
	CALT	Defend the ocular region
	LALT	Defend the nasopharyngeal region
	GALT	Comprises diffuse lymphoid tissue in the gastrointestinal tract wall and PPs
	Tonsils (pharyngeal and palatine)	Form the Waldeyer's ring of the oropharynx
	Other lymphoid nodules	*E.g.*, genitourinary system

ATALT: Auditory tube-associated lymphoid tissue; BALT: Bronchus-associated lymphoid tissue; CALT: Conjunctiva-associated lymphoid tissue; GALT: Gut-associated lymphoid tissue; LALT: Larynx-associated lymphoid tissue; MALT: Mucosa-associated lymphoid tissue; NALT: Nasal-associated lymphoid tissue; PPs: Peyer's patches.

MALT comprises four main lymphoid parts in every region: *lamina propria*, *organized mucosal lymphoid tissue*, *intraepithelial lymphocytes* (IELs), and *regional lymph nodes* (mesenteric lymph nodes) [4].

In the *lamina propria* of the mucosa of the GI, genitourinary, and respiratory systems, there is diffuse lymphoid tissue made of lymphocytes and dendritic cells (DCs) [4, 8]. Within the *lamina propria*, macrophages, mature plasma cells, and sporadic B- and T-lymphocytes (IELs) can be found [4]. The role of these cells is to capture and process antigens, which secondly reach regional lymph nodes and start the immune response, eventually followed by the production of immunoglobulin (Ig)A, IgG, and IgM [4]. IELs are mainly situated between the epithelial cells of the villi and are represented by a mixed population of T-lymphocytes [4]. IELs of the *lamina propria* are heterogeneous, mainly formed of cluster of differentiation (CD)4(+) cells and some CD8(+) ones. The majority are CD3(+), CD5(+), and CD8(+), while 10% to 15% are CD3(+), but negative for CD4 and CD8. Few cells are CD3(+), CD4(+), and sporadic cells express CD56 [4].

Organized lymphoid tissue consists of single lymphoid nodules and aggregated lymphoid nodules [*e.g.*, tonsils and Peyer's patches (PPs)] [4, 7, 8].

Individual lymphoid nodules represent an accumulation of lymphocytes (predominantly B) situated in the mucosa and contain well-defined collections of small lymphocytes without any capsule [8]. In inactive conditions, without antigenic stimulation, they are not macroscopic observable and form the primary lymphoid nodules. After the antigen contact, as a response, they multiply and develop germinal centers (GCs) and encircle mantle cell zones, becoming secondary lymphoid nodules [4, 8]. The lymphoid follicles have almost the same composition and immunophenotype as those located in the lymph nodes. The single difference, in this case, is the increased marginal zone, which extends to the surface epithelium [4]. It was discovered that MALT marginal-zone cells had related histological aspects with those located in the spleen. The interfollicular area comprises T-cells and interdigitating DCs [4].

Aggregated lymphoid nodules are made of clusters of lymph nodules, the principal examples being the tonsils and PPs [4–8]. The aggregated lymphoid nodules of the PPs are primarily developed in the ileum [4, 8]. They are enveloped by a particular epithelium, the follicle-associated epithelium (FAE) [8]. FAE comprises enterocytes and interdigitated, microfold, epithelial cells (M-cells), and it is found between the PPs and the luminal microenvironment [4, 8]. M-cells capture and deliver by transcytosis: particles, antigens, bacteria, and viruses, from the intestinal lumen to the underlying immune cells (*e.g.*, DCs), responsible for transporting the material to the lymphoid tissue of the PPs [4, 8]. Within the FAE, the luminal bacterial level controls the ratio between the enterocytes, the primary cell population, and the M-cells [4, 8]. Therefore, the numerical level of M-cells was elevated in animals moved from pathogen-free housing to the normal environment. Furthermore, M-cells express IgA receptors, responsible for capturing and passing bacteria trapped by IgA [8].

Mesenteric lymph nodes share the same constitution as other lymph nodes, except the marginal zone around the follicles, which is perceptible and more significant [4].

Multiple adhesion molecules, their receptors, and chemokines control by their synchronized functions the configuration of the immune system in mucosal locations [4]. It looks like lymphocytes, with the ability to react to antigens in the MALT, gain homing capacities that allow them to return to the originate tissues [4]. Circulating lymphocytes' "homing" is controlled by a complex process regulated *via* several adhesive interactions between these cells and high endothelial venules (HEVs) [9]. Thus, in peripheral lymph nodes, the first phase of the immune encounter is regulated by a complex adhesion structure, named *peripheral node addressin* (PNAd), expressed on the endothelia of HEVs; it is formed by L-selectin and its carbohydrate ligand (6-sulfo sialyl Lewis X-capped glycoproteins), expressed on lymphocytes [9]. However, in GALT, this homing mechanism is controlled by the increased expression of $\alpha 4\beta 7$-integrin, which fixes on HEVs to a *mucosal addressin cell adhesion molecule-1* (MAdCAM-1) [4]. Moreover, MALT lymphoid cells present CD103, an $\alpha E\beta 7$-integrin, which has its ligand expression (an E-cadherin) on the basolateral surface of the epithelial cells [4].

Employing MALT, bacterial or viral antigens will initiate a complex immune response. Thus, the first response to an antigenic contact does not come from the systemic immune system but will be provided by the mucosal immune system, MALT, which is the first control area. Only when this one is deficient will the systemic immune system be activated; when the systemic immune system exceeds its physiological limits, it will enable the appearance of autoimmune diseases [10]. Since MALT works separately from the systemic immune system, blood tests without ancillary studies may only give partial functional details [11]. As an alternative, more practical examinations are the testing of secretory liquids, like the saliva (to detect IgA), or even direct analysis of the tissue [4, 5, 11].

On the other side, it was revealed that MALT could produce immune tolerance [4, 12]. As a result, the second antigenic contact leads to no reaction at secretory areas [11]. However, T-lymphocytes have been detected in the

spleen and suppressor factors in the bloodstream. This initiation of immune tolerance is principally directed to the inactivated proteins or dead microorganisms that interact with MALT [4, 11]. Therefore, vaccination *via* MALT must be made using vaccines with living microorganisms. The process of immune tolerance stimulation and varying reactions to living and lifeless microorganisms are only partially known, although they are significant for food allergies and the tolerance to food antigens [11].

In other regions, MALT has the function to preserve the equilibrium among immune sensitivity and immune tolerance [4, 12]. BALT maintains the tolerance to non-pathogen elements and the sensitivity to air pathogens [12]. GALT has a crucial role in sustaining the tolerance to commensal bacteria and food antigens [12].

Although numerous MALT regions present disorganized structures, they still possess most lymphocytes from the entire body, and subsequently, MALT represents an essential part of the immune system.

Nasal-associated lymphoid tissue (NALT)

NALT represents the organized lymphoid tissue within the nasal mucosa. It is composed of lymphoid follicles and infiltrative lymphocytes into the surface epithelium of all the various nasal cavity structures, principally in the middle concha [13, 14]. Different authors also include the nasopharyngeal tonsils in the structure of NALT [15].

Studies using the intranasal application of antigens to detect local immune reactions in NALT were performed on mice and rats [13]. The key role of NALT in host defense was demonstrated by nasal immunization with antigens and toxins that conduct the activation of antigen-specific IgA-secreting cells in nasal passageways and at other mucosal terminal locations [16]. The order of tissue development is chronologically distinctive for PPs, NALT, and peripheral lymph nodes [17]. Due to its strategic location, NALT is discovered before BALT [18]. NALT growth might depend on triggering signals supported by inhaled environmental antigens after birth [17]. It has been proposed that NALT and PPs play various roles in defense of respiratory and gut systems, possibly due to dissimilarities in location aspects and subcategory structure [13].

Larynx-associated lymphoid tissue (LALT)

LALT represents a physiological component of young children's larynx. LALT is an organized lymphoid tissue, mainly distributed in the supraglottic parts of the larynx [19]. This lymphoid structure shares all the histological features of MALT, like infiltrative lymphocytes of the surface epithelium, lymphoid follicles with GCs, and HEVs [19]. The lymphoid follicles of LALT present mainly B-lymphocytes, with few CD4(+) lymphocytes in their GCs. Surprisingly, in the parafollicular region, B-lymphocytes and both subset types of T-lymphocytes were highlighted in equal proportions [19].

Thus, LALT is an induction location for respiratory mucosal immunity [19].

Auditory tube-associated lymphoid tissue (ATALT)

ATALT represents the lymphoid tissue found within the auditory tube.

Antigen response in MALT is not usually detected in the normal animal or human middle ear [20]. One research of necropsy tissue indicates ATALT localization only in children's Eustachian tubes and middle ear [21]. In rats, the incidence of ATALT decreases as the middle ear is closer to the Eustachian tube, thus indicating an effector instead of an inductive immune function for the middle ear epithelium [20].

In normal conditions, the mucosa of the middle ear of animals and humans contains only rare immunocompetent cells, mainly mast cells and macrophages [22]. It was revealed that the number of immunocompetent cells gradually reduces as the normal middle ear gets closer to the nasopharynx through the Eustachian tube [20]. Electron microscopy (EM) examination of the human tympanic membrane found DCs within the basal epidermis. The existence of these antigen-presenting cells (APCs) within the healthy tympanic membrane indicates an inductive initial immune response [20], and much research is still needed to specify the role of ATALT as an immunological trigger in the middle ear.

Conjunctiva-associated lymphoid tissue (CALT)

CALT is the organized lymphoid tissue within normal conjunctiva [23, 24], with an age-dependent expression. Numerous reports show its main implication in the immunological defense of the eye surface [25]. In mice, CALT entirely arises from the nictitating membrane, but extensive morphological research on human tissue revealed a similar morphology of CALT (GCs B- and T-cells, M-cells lymphoepithelium, and HEVs) [23, 24]. In addition, non-invasive *in vivo* two-photon microscopy showed the cellular mechanisms in dynamics and their capacity to transfer antigens to the follicles [26].

CALT aspect is disease-specific and suggestive of its functional implication [23]. It seems that ocular allergies induce an enhanced and dry-eye syndrome, a global reduction of CALT [23]. Professional APCs modulate the expansion of CALT by their presence within the follicle, where they deliver the soluble antigen [23]. The precise role played by CALT in ocular surface pathologies requires additional investigations; nevertheless, the presence of active APCs that facilitates the antigen capture encourages the theory that CALT represents an immune defense trigger at the eye surface.

Bronchus-associated lymphoid tissue (BALT)

BALT, included in MALT, contains all specialized diffuse/clustered lymphoid structures or solitary lymphatic nodules located along the mucosa of the respiratory tract [3].

All segments of the lower respiratory tract, from the trachea to the alveoli, are permanently exposed by inhalation to continuous contact of antigens and pathogenic microorganisms. These invasions require the presence of immune solid defense mechanisms [2].

The histological structure of BALT is arranged in the form of lymphoid follicles. Thus, in their center are found the GCs, which contain B-lymphocytes and CD4(+) T-cells. Most BALT cells are B-cells expressing surface IgA and IgM. In the parafollicular areas are found cytotoxic CD8(+) T-lymphocytes, DCs, and specialized HEVs, through which

recirculate T-lymphocytes. Lymphocytes leave the blood and migrate to BALT in the walls of HEVs. HEV cells express adhesion molecules on the surface that can interact with specific receptors on the surface of lymphocytes. Plasma cells are found only around the periphery of BALT [3].

There is a dome-like protrusion in the subepithelial area, similar to PPs, covered by a specialized epithelium, without cilia, without goblet cells, but with M-cells, evidenced by immunohistochemical (IHC) methods; M-cells are like those of the dome epithelium of PPs. The presence of M-cells is essential for the transport of endocytosed particles and antigen molecules to the subepithelial lymphocytes and macrophages or towards professional APCs (*e.g.*, macrophages and immature DCs), provided that the tertiary BALT lymphoid structures do not have afferent lymphatics [1].

At the level of the dome area, there are the major histocompatibility complex (MHC) Class II(+) macrophages, DCs (capable of presenting the antigen), B-lymphocytes, and CD4(+) T-lymphocytes. Here, the DCs will present the antigen to B-lymphocytes, and the MHC Class II(+) macrophages will present it to the T-lymphocytes [1] (Figure 1).

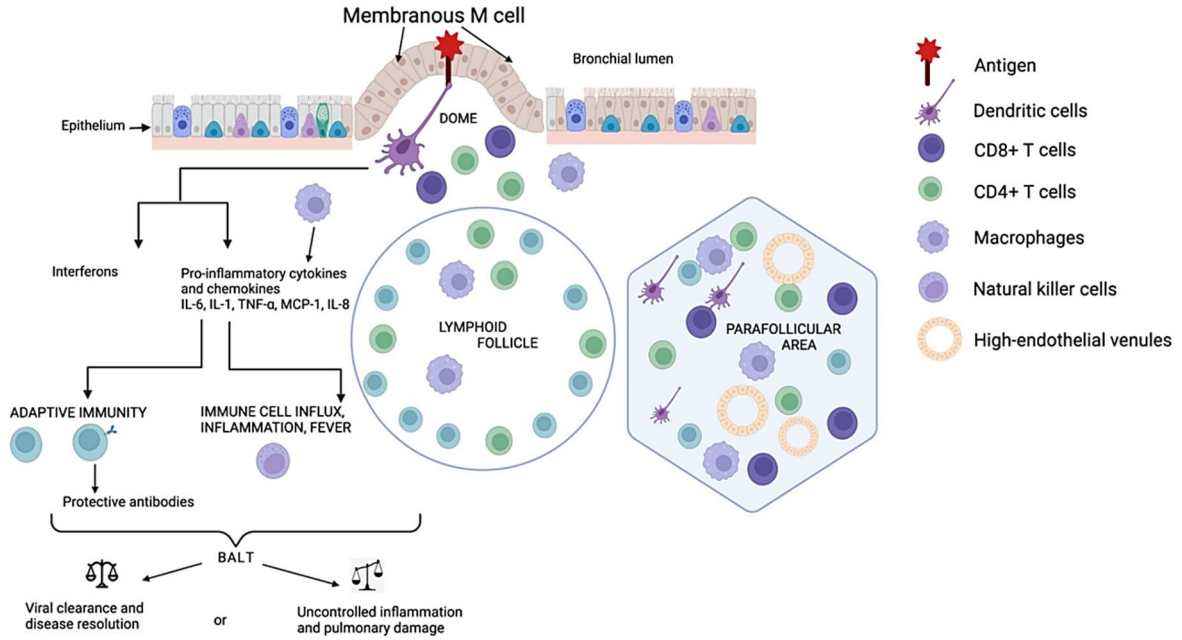

Figure 1 – ***Schematic drawing of BALT. Distribution of lymphocyte subsets: T4 is CD4(+) T-cells, T8 is CD8(+) T-cells, macrophages, dendritic cells, M epithelial cells. BALT: Bronchus-associated lymphoid tissue; CD: Cluster of differentiation; IL: Interleukin; M: Membranous; MCP-1: Monocyte chemoattractant protein-1; TNF-α: Tumor necrosis factor-alpha.***

At the same time, it has been revealed that relatively few BALT areas are present in healthy human lungs [2].

In contrast, in chronic lung disease, severe bronchitis or bronchiectasis, bacterial or viral pneumonia, mycoplasma infections, pulmonary complications of rheumatoid arthritis, obstructive bronchial tumors, BALT structures are very well developed. These have been named iBALT or "inducible BALT" because they always occur after the inflammatory process, caused by autoimmunity or infection [2].

The term iBALT was introduced by Randall's research team; they studied the immune response by demonstrating the spatial organization of B- and T-cells in iBALT, following viral *Influenza* infection in mice without secondary lymphoid organs (lymph nodes, spleen, or PPs) [2].

iBALT is present around the bronchi and bronchioles, near the pulmonary artery branches, or in some sections, exclusively perivascular. iBALT is not always associated with the airway, evident in the lung parenchyma [2].

In addition to iBALT, two forms of tertiary or ectopic lymphoid structures have been found in the lung. These are nodular inflammatory foci, consisting of myeloid cells, CD8(+) T-cells, and infectious granulomas; the granulomas are due to *Mycobacterium tuberculosis*, being characterized by a condensed area of macrophages, surrounded by B- and T-cells [27].

Strengthening the idea of the ectopic tissue, some studies have not revealed in the iBALT the presence of the epithelial dome covered by M-cells; a hypothesis was formulated regarding the existence of afferent lymphatics, which bring antigens in the vicinity of B-lymphocytes rich lymphoid follicles. The inflammatory process itself is the triggering factor for the formation of new lymphatic vessels. Interleukin (IL)-17, present mainly in bacterial infections, intervenes in the formation of iBALT, and promotes the expression of chemokine (C–X–C motif) ligand 13 (CXCL13) and chemokine (C–C motif) ligand 19 (CCL19), with a role in the recruitment of B- and T-lymphocytes. Likewise, in some viral infections, iBALT independently forms IL-17 [2].

After the inflammatory period, iBALT continues to be maintained due to fibroblastic stromal cells and lymphatic endothelial cells (ECs), which express cytokines such as IL-7 and B-cell activating factors (BAFFs). Their role is to promote the survival of memory B- and T-cells through mechanisms that involve the presence of lymphotoxin and signals from the tumor necrosis factor (TNF) superfamily [2]. The sustained and prolonged production of IL-17 causes macrophage and neutrophil buildup, resulting in chronic inflammation, which transforms a protective iBALT into a pathological iBALT [28].

T-lymphocytes [T-helper (Th)1, Th2, Th17, regulatory T-cells (Tregs)] control exacerbated inflammation and are crucial in the formation of iBALT, proving this by administering anti-CD4 treatments in several infection models, with consequent reduction of the lymphoid follicles in iBALT [28].

C–X–C motif chemokine receptor 3 (CXCR3), expressed by T-cells, numerically increases in chronic obstructive pulmonary disease (COPD) [1].

Fibroblastic reticular cells (FRCs) and follicular dendritic cells (FDCs) have been identified among specialized stromal cells. FRCs express CCL19 chemokine, which will target T-cells and further activate DCs to the parafollicular area. FDCs express the chemokine (C–X–C motif) ligand 12 (CXCL12) and CXCL13 chemokine, which attract B-cells and T-follicular helper (Tfh) cells and spatially organize them into the GCs of the lymphoid follicle [2]. FDCs depend on the lymphotoxin signaling pathway to differentiate and have the role of presenting antigen to B-cells, determining their activation and proliferation in GCs [3].

Of particular importance for the development and organization of the lymphoid tissue is activating the TNF superfamily members: TNF-α, lymphotoxin-α/β, and their receptors. These cytokines are involved in the differentiation of the antigen-transporting M-cells and the development of the postcapillary HEVs. Very important, they are responsible for the differentiation and maintenance of FDCs and FRCs stromal cells, which, in turn, will express chemokines that play a role in attracting lymphocytes [29].

In most viral infections, including the severe acute respiratory syndrome (SARS)–coronavirus (CoV), the immune response is more effective when iBALT is present. Hence, it can become a goal to obtain vaccines that can induce iBALT to initiate a specific antigen of humoral or cellular immunity against airborne pathogens. The protective effect can be explained by preventing the spread of infected cells from lymphoid follicles to the rest of the lung or other organs [28].

In the case of patients with non-small-cell lung cancer (NSCLC), the formation of iBALT around tumors increases the number of the DCs in the proximity of the neoplastic cells, indicating an active anti-tumor response, and is correlated with a better prognosis [29].

Other opinions state that iBALT could have a damaging effect in the case of chronic inflammation (COPD, rheumatoid lung disease), the production of autoantibodies leading in the end to autoimmune reactions, with the exacerbation of inflammation [10].

Among patients with pulmonary arterial hypertension, there has been an increase in the number and size of iBALT structures, correlated with the production of autoantibodies against fibroblasts and epithelial cells in these areas, and the consequence of increased local inflammation [28].

In the case of patients who underwent lung transplants, the presence of iBALT with active GCs can lead to graft rejection [30] due to the development of antibody-mediated rejection [31].

In cases of rheumatoid arthritis, an autoimmune disease, it has been observed that the elevated serum levels of rheumatoid factor and the presence of areas of vasculitis are associated with the development of tertiary lymphoid iBALT structures and with the worsening status of the disease [2, 29].

The development of iBALT can undertake several ways of formation and can be determined by a large variety of infectious stimuli (allergy, asthma, COPD, and even autoimmune diseases). New therapeutic approaches aimed at increasing immunity in the case of lung infections (while diminishing chronic pulmonary symptoms) can be developed by identifying immunological targets capable of limiting pathological iBALT, as they enhance protective iBALT structures. Thus, a better understanding of the protective role of iBALT, and the determination of its protective mechanisms will represent actual progress in the further development of therapies and vaccines for lung diseases.

Gut-associated lymphoid tissue (GALT)

The complex structure of the gut is represented by different lymphoid niches with organized and diffuse tissues [32].

GALT, a MALT variety, consists of secondary lymphoid structures [4] associated with the intestinal tract and approximately 300 m^2 [33]. The most significant structures in GALT, especially the multi follicular ones, can be seen with the naked eye, without specific staining methods, or magnification [34].

GALT helps maintain the balance and dynamics in the gut immune system [35]. The prominent role of GALT is to ensure the body's defense against potential pathogenic external factors that penetrate the mechanical barrier of the digestive mucosa. GALT regulates and adapts the immune response to the antigens to which the digestive system is exposed [36], possessing the highest number of immune cells in the human body [37].

Secondary lymphoid organs (spleen, lymph nodes, and MALT) allow cell-to-cell interactions and cytokine–cell interactions and provide the trapping and filtering of the antigens and the proliferation and maturation of the cells involved in the adaptive immune response. These cells represent the second line of defense in the body, ensuring protection in case of re-exposure to the same pathogen [38]. Thus, GALT serves as the site for adaptive immune cell priming. Cells' priming represents the first contact that antigen-specific Th-cell precursors have with an antigen, and it is essential to the Th-cells' subsequent interaction with B-cells in producing antibodies [39, 40].

Programmed in ontogeny, GALT develops during the prenatal period, and innate lymphoid cells (ILCs) are crucial in its development during embryogenesis [41]. Various external stimuli control the postnatal development of GALT (*e.g.*, microbial stimulation and nutrients).

In the human body, GALT represents the largest mass of cells with immune competence, generally consisting of *organized lymphoid tissue* (lymph nodes), *diffuse lymphoid tissue* (spread in *lamina propria* of the digestive tract), and *IELs* [35, 42]. Dysfunctions of the immune system of the GI tract and an abnormal GALT result in infectious and autoimmune diseases [43].

Specifically, GALT includes *IELs* (throughout the entire digestive tract), *crypto patches*, *diffuse lymphoid tissue* (in the *lamina propria* of the digestive tract) [2, 34, 38], several *isolated lymphoid follicles* (ILFs) (within the length of the intestine), *PPs* (in the ileum), *lymphoid follicles* (in the appendix), and *mesenteric lymph nodes* [34].

According to its structure and function, GALT can be divided into two distinct groups: GALT with diffuse structure/*effector sites* (intestinal *lamina propria* and IELs) [41] and GALT with dense structure/*inductive sites* (PPs, mesenteric lymph nodes, cecal patches, colonic patches, ILFs, and crypto patches) [2].

Lymphocytes accumulate intraepithelial in the villi and *lamina propria* of the intestine and can persist for a long time as effectors or regulatory cells [32].

IELs maintain the integrity of the intestinal epithelial barrier, respond to the invasion of infectious agents, and participate in tissue renewal [41].

GALT effector areas contain CD4(+) and CD8(+) T-lymphocytes [44]. A subpopulation of CD4(+) cells with regulatory function, which co-expresses CD25 [α-chain of interleukin-2 receptor (IL-2R)], has been identified in the intestinal mucosa. This subpopulation is generated by the contact of intestinal immune cells with luminal antigens. By releasing transforming growth factor-beta (TGF-β) and IL-10 and binding to cytotoxic T-lymphocyte antigen 4, these cells appear to play an important role in inducing and maintaining the immune tolerance in the gut [45]. A recent study showed that Tregs are directly activated by microbial lipopolysaccharides (proinflammatory products) [46]. It has also been shown that a subset of CD4 lymphocytes [CD45RB10CD25(+)] selectively expresses 4, 5, 7, 8 Toll-like receptors (TLRs) and thus provide a link between the innate and the adaptive immune response [47].

PPs respond to gut-derived antigens [2, 29, 48] due to specialized microscopical compartments involved in the efficacious response of the immune system and representing the site of the most intense activation of B-cells from the whole body [34, 47]. They are permanently exposed to the diversity of the intestinal microbiome and many food-derived antigens, acting as a connection between host and commensal bacteria and between host and gut pathogens [49, 50].

The following components characterize the structure of PPs: *individual follicles* (tens to hundreds, mainly constituted by many B-cells, maturated in the GCs) [34, 50], *macrophages* and *DCs* [41], *FAE* (with many IELs), and *associated subepithelial dome* (SED) [located between follicles and FAE and containing memory CD4(+) T-cells, and IgA, IgM, and IgG secretory B-cells] [34, 51]. The small T-cells are situated adjacent to the B-cell follicles in the parafollicular zone [36].

FAE is a specialized epithelium covering the luminal surface of PPs, containing many MHC Class II(+) enterocytes and the intestinal epithelial M-cells [36, 50, 52]. PPs M-cells represent between 10–20% of the FAE in humans, scattered throughout this overlying epithelium. M-cells form pockets on the basolateral sides [34] that harbor B- and T-cells, allowing direct interaction with the immune cells from the underlying SED [40]. Two-thirds of these B-lymphocytes express IgM, and one-third express IgD. Pockets T-lymphocytes are mainly represented by memory CD4(+) T-cells [46].

M-cells are specialized for luminal antigen sampling, recognizing, and taking luminal antigens [bacteria, viruses, and secretory (S)-IgA-bound antigen] [53] by expressing cell-surface receptors; after actively engulfing these antigens at their apical surface, they transport and exocytose them through their basolateral plasma membrane. The process is named transcytosis [34], and it facilitates the delivery of luminal antigens to resident DCs [50] and SED located macrophages [46]. At the follicle–T-zone interface, DCs present the antigen to SED located B-cells [39, 50], to trigger antigen-specific immune responses (*e.g.*, antigen-specific S-IgA production) [34, 41, 53]. Thus, M-cells contribute to maintaining gut immune homeostasis [54] (Figure 2).

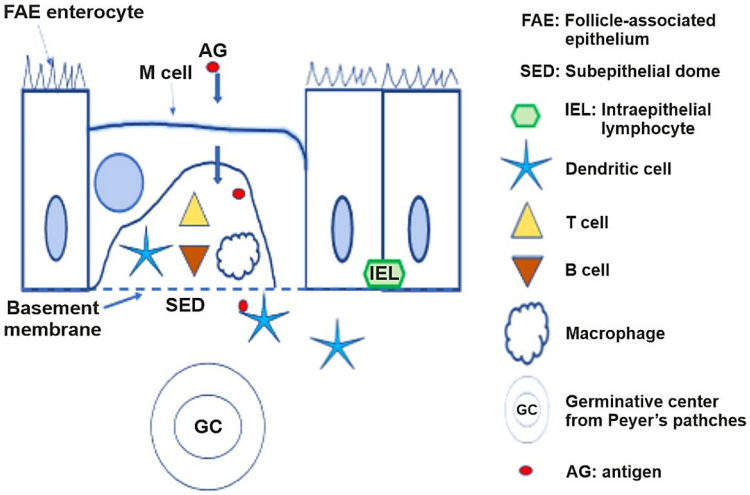

Figure 2 – ***FAE of PPs. M-cells mechanisms of transcytosis (after [57]). M: Membranous; PPs: Peyer's patches.***

S-IgA plays a leading role in the protection against pathogens and maintains a close bond with the gut microbial population.

Histological studies of human PPs have shown that active GCs are situated close to the SED [40]. According to their chronic microbial antigen exposure state, PPs display continual GCs activity [34]. In the GCs, B-cells are generated and maturated and give birth to plasma cells, these producing somatically mutated gut antigen-specific IgA antibodies [40, 50]. Encircling the GCs is an area of naïve IgD(+) B-cells, neighboring a larger memory B-cells interface zone (IgA and IgM isotypes) [34]. The extended perifollicular areas are T-cell zones with naïve and memory T-cells [50]. PPs GCs are organized into light and dark zones. The light zone contains a well-developed FDCs network orientated toward the SED [50] and retains and displays antigens to B-cells, using complement and Fc receptors [55]. The B-cells internalize, process, and present antigen to Tfh cells. This process is regulated by the follicular regulatory T-cells (Tfr) present in this light

zone [55]. A network of CXCL12-producing reticular cells (CRCs) is in the dark zone. The dark zone is the site of GCs B-cells proliferation and somatic hypermutation (SHM) [56].

Lymphocytes enter PPs from the circulation across HEVs [57] (as it was described before, in human circulating lymphocytes, $\alpha 4\beta 7$ binds to MAdCAM-1, expressed by HEVs) [34]. There are no afferent lymphatics in the human PPs [34]. However, within the submucosa of the intestine, the PPs present many lymphatic efferent vessels, *via* which lymphocytes and plasma cells leave the site [34, 57].

ILF consists of a single lymphoid follicle [34], much smaller in size (0.1–0.3 mm) than a PP [37].

Between the different segments of the intestine, the location, density, and cellular composition of ILF are very different [32].

ILF can be located entirely within the chorion, the mucosal ILF (M-ILF), or it can extend through the *muscularis mucosa* to enclose both the chorion and the submucosa, the submucosal ILF (SM-ILF); both M-ILF and SM-ILF are situated into the gut wall, throughout the length of the intestine [37, 49] and are adaptive immune organized inductive sites (intestinal adaptive immune responses being initiated in ILF) [32]. Also, the T-cells function as adaptive immune inductive sites [58], and most are CD4(+) T-cells [37]. The M-cells from the mucosa FAE contain in their "pockets" occasionally CD45(+) cells, and some cells express the glycoprotein-2 (GP-2) (M-cell marker of maturation) [32, 59]. In the areas of FAE covering the M-ILF and SM-ILF, the structure of SED is made of some B-cells containing GCs, [encircling by many antigen-presenting CD11c(+) cells], an associated MAdCAM-1(+) HEVs T-zone, and a neighboring lymphatic network [32, 60], all indicating the role of ILF as immune inductive sites [34, 49]. Therefore, the cell population in M-ILF and SM-ILF is represented over 90% by lymphocytes, the T-cells (compared with B-cells) being slightly numerous [34]. A "mixed cell zone" with no clear boundary is found between ILF and the environing chorion. At this interface away from the follicle, the cellular density is decreasing. Compared with PPs, the ILFs contain less memory CD4(+) T-cells and polyfunctional cytokine-producing cells [34]; yet, contrary to the chorion, in ILF, both CD4(+) and CD8(+) T-cell populations present more memory and naïve T-cells [34].

Recent studies have shown that, compared with GALT-free chorion, there is a different composition of T- and B-cells in the mucosa and submucosa of human ILF, highlighting its role in the intestine region-specific immune responses [58].

The dissection and characterization of these structures are difficult to achieve due to the inability to isolate them from the adjacent intestinal mucosa and, therefore, the immune structure and function of human GALT remain partially unknown [37, 58].

Novel cellular heterogeneities of the thymic epithelial cells and Hassall's corpuscles

Thymic epithelial cells (TECs)

TECs are derived from the endoderm of the third pharyngeal pouch and represent (based on their number and phenotype) the most important part of the thymus stromal cells [61]. Besides TECs, the thymic stromal cells include all non-T-lineage cells: fibroblasts/mesenchymal cells, macrophages, DCs, ECs, adipose cells, and B-cells [62, 63, 64].

During embryological development, the two separate lobes of the thymus are composed only of mesenchymal cells and TECs progenitors, enveloped by a neural crest cell-derived capsule [61]. In the human thymus, from week 8, vascular and mesenchymal cells start to infiltrate the thymus, and the medulla develops; from week 14–16, there is a clear distinction between the cortex and medulla, and naïve T-cells start to leave the thymus and populate the peripheral immune system. HCs are formed later, after the sixth month [63].

In TECs proliferation and immunophenotype differentiation, several transcription factors and signaling pathways are involved, many produced by the fetal mesenchymal cells, during the thymus development process: the forkhead family transcription factor [forkhead box N1 (FOXN1)], the earliest involved and the master regulator of TECs, the sonic hedgehog (Shh), the *fibroblast growth factor* (FGF) [61, 63], the bone morphogenetic protein (BMP), the Wnt (combined name from wingless and Int-1), that mediates the expression of FOXN1 and intervenes in T-cell development; the Wnt/β-catenin, which fine tuning is critical for embryonic organogenesis and which establish different TECs subpopulations [64, 65]. Some other transcriptional regulatory pathways of thymus and TECs development involve factors, such as paired box (PAX) 1 and PAX9, HOXA3 homeobox protein, and T-box transcription factor 1 (TBX1), but many more studies are needed to completely elucidate how these factors can be employed to restore or boost the thymic function [66].

The dynamic of epithelial progenitors in the thymus cortex is controlled by permanent interactions with developing thymocytes [67]. Different expressions for markers of the TECs progenitor cells were identified, such as CD24 and stem cells antigen-1 (Sca-1) (stem cell markers), CD205, β5T proteasome subunit, and IL7YFP [63, 67, 68]. There are rare identical subsets of progenitors for two types of TECs, such as placenta-expressed transcript 1 (PLET1)(+) Ly-51(+) [progenitor able to generate cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs)] and CD45(–) epithelial cell adhesion molecule (EpCAM)(+) (proved to be capable of self-renewal) [63]. Also, unipotent progenitors for cTECs [expressing EpCAM(+) CD205(+) and CD40(–)] and mTECs (expressing claudin-3 and -4) were described [63]. The lymphoepithelial interaction directly regulates mTECs progenitors [podoplanin(+)] residing at the cortico-medullary junction and contributes to the maintenance of mTEC compartment [67]. During early development, an intermediate population of mcTECs, expressing delta like non-canonical Notch ligand 2 (DLK2) can be evident in the late fetal and pediatric human thymus [69, 70]. It is possible that a specialized subset of the TECs progenitor cells resides within the cortical compartment in the postnatal thymus, but its bioavailability declines throughout life [68].

Three criteria (the structural characteristics, the versions of the proteasome, and the IHC profile) are used to subdivide TECs into four populations: subcapsular/subtrabecular epithelial cells, cTECs, mTECs, and HCs [63, 71].

The *subcapsular/subtrabecular TECs* link K1, K7, and K19 monoclonal anti-keratin antibodies, bound in a subcapsular/subtrabecular flat layer [72]. This subset of TECs has positive immunoreactivity for tyrosine hydroxylase (TH), α_1-adrenoceptor (α_1-AR) (both for TECs and the macrophages subcapsular/subtrabecular located), calcitonin gene-related peptide (CGRP), and bombesin [73, 74].

cTECs and mTECs are the main two types of TECs involved in immunological education of thymocytes and maintaining homeostasis of the thymic stromal environment. By EM, in the thymus cortex were identified four types of cTECs (types 1 to 4) and in the thymus medulla, three types of mTECs (types 5 to 7) [75].

Type 1 cTECs (*subcapsular*) are in contact with the connective tissue and presents a basal lamina; it has an irregular shape and small cellular prolongations, euchromatic nucleus, and delicate cytoplasmatic bundles of keratin tonofilaments.

Type 2 cTECs (*pale*) have a stellate shape, small cellular prolongations, substantial, oval, euchromatic nucleus, well-defined nucleolus, and delicate and sparse cytoplasmatic cytokeratin (CK) tonofilaments.

Type 3 cTECs (*intermediate*) have a polygonal, higher electron-dense nucleus, prominent nucleolus, abundant cytoplasm with bundles of tonofilaments and numerous secretory vacuoles, and massive extensions.

Type 4 cTECs (*dark*) have a very high electron-dense nucleus, with large heterochromatin blocks scattered all over, a sparse cytoplasm, with numerous organelles (Golgi complexes, large secretory vacuoles, multilamellar bodies, and lipid droplets), and the cellular prolongations are extensive and also packed with organelles.

Type 5 mTECs (*undifferentiated*) have a round shape, very fine cellular prolongations, marked euchromatic nucleus with the prominent nucleolus, a cytoplasm with mostly polyribosomes, and sparse and small other organelles.

Type 6 mTECs (*large*) have barely visible cellular prolongations, very abundant cytoplasm with intense metabolic and secretory activity (numerous transport vesicles, dilated rough endoplasmic reticulum, large Golgi fields, and clusters of small secretory vacuoles in a grape-like form or, larger and compressed vacuoles, and sometimes solitary cyst-like structures).

Type 7 mTECs (*spindle-shaped*) are small, with sparse cytoplasm, containing massive bundles of CK [75].

Immunophenotypically, in mice and humans, both types of TECs evidentiate and differentiate themselves by the expression of some keratins, other characteristic cell-surface markers, and the selective binding of *Ulex europaeus* agglutinin-1 (UEA-1) [76].

cTECs have long cytoplasmic processes, interacting between them and forming complexes called thymic "nurse cells" only postnatally detected. cTECs are characterized by the expression of specific genes [proteasome subunit beta 11 (*PSMB11*), serine protease 16 (*PRSS16*), C–C motif chemokine ligand 25 (*CCL25*)], delta-like 1 (DLL1) and delta-like 4 (DLL4) Notch receptor ligands (important for precursor cells to commit to the T-cell lineage), high levels of C–C chemokine receptor type 11 (CCRL1) and IL-7, cytokines [as the stem cell factor (SCF)], chemokines (CXCL12 and CCL25); they express also ERTR4, Ly-51, CD205, high level of CD49f, the CK8 and CK18 markers, express the proteasome subunit exclusively β5T (which mediates the presentation of ligands for MHC Class I molecules, an essential process for competent T-cells selection) and thymus-specific serine *protease* (TSSP/PRSS16), with a role in thymocyte-positive selection [64, 76–80]. Other factors expressed are CD83 (role to stabilize the MHC Class II expression), TSSP, cathepsin L protease (for the positive selection of CD4 T-cells), and low levels of the B7.1 and B7.2 costimulatory molecules [63, 68, 81, 82].

mTECs are oval, have short cytoplasmic processes and microvilli. mTECs are characterized by the expression of specific genes [*e.g.*, the forebrain embryonic zinc finger 2 (FEZF2) transcription factor – critical for the regulation of antigen expression; the autoimmune regulatory (AIRE) transcription factor – essential for the T-negative selection; the Spi-B transcription factor, a protein-coding gene], cathepsin S, CD40, low levels of CCRL1, higher levels of human leukocyte antigen (HLA) Class II, UEA-1, CD80, the CK4 and CK5 markers, CC19 and CCL21 chemokines (which shelter positively selected T-cells and mediate negative selection of T-cells), high levels of the B7.1 and B7.2 costimulatory molecules, as well as IL-15 (promotes Tregs development with IL-2) [63, 68, 80–82].

In the human thymus, mTECs exhibit highly heterogeneous profiles (displaying several self-antigens), thus contributing to the establishing of self-tolerance [64] in the context of a (not fully defined) high level of cellular heterogeneity [80].

Distinct mTEC subsets are represented by: AIRE(+) cells, corneocyte-like or keratinocyte-like cells forming HCs, CCL21(+) cells, thymic tuft cells, rare populations of ciliated thymic cells, neuroendocrine (NE)-like cells [80, 83], the muscle-like myoid cells, myelin(+) epithelial cells.

The *thymic tuft cells* represent a very rare and unique population of mTECs in humans, with characteristics of both mTECs and peripheral tuft cells, similar to tuft-like mTECs described in the mouse thymus. They are associated with cornified aggregates, able to present antigen, and contain a large variety of taste receptors [84]. These cells express *IL-25*, *MHCII* genes, CD74, doublecortin-like kinase 1 (DCLK1), and POU class 2 homeobox 3 (POU2F3) transcriptional factor [84, 85], but unfortunately, in humans, the markers used to define the tuft subtype in mouse, DCLK1 and POU2F3 although enriched, are not specific [69]. The identification of this minor and highly specialized subset of tuft mTECs suggests a compartmentalized medullary environment, which differentiates the immune niche and shapes the thymocyte's development by promoting IL-4-enriched surroundings [80, 84].

The *NE-like cells* express specific genes for brain-expressed X-linked 1 (*BEX1*), the neuronal transcription factor for neurogenic differentiation 1 (*NEUROD1*), and sex determining region Y (SRY)-box 2 (SOX2) transcription factor [80].

The *muscle-like myoid cells* express the *skeletal muscle-specific genes* and the master myogenic regulatory factor, myogenic differentiation 1 (*MYOD1*) and desmin (*DES*) [80, 86].

NE-like cells and myoid cells reported in several previous studies present an intermingled activity. In the human thymus, *HES6* Notch inhibitor, an inhibitor of

HES1 Notch target gene, is highly expressed in NE-like cells and myoid cells [80]. AIRE and FEZF2 control the level of transcription of NE self-peptides and tissue-restricted self-antigens in the TECs; their mutations are involved in autoimmune processes in peripheral organs [87]. NE-like cells and a subset of myoid cells were detected close to HCs [80]. The co-staining in the fetal thymus of the DES myoid marker with CK(+) represents an argument for the myoid cells' origin from epithelial cells during embryogenesis. The study of thymomas and thymic carcinomas, the transcriptome data, as well as the fetal co-expression of epithelial and myoid markers are also directing toward a branching point between NE-like cells and myoid cells [80].

The *myelin(+) epithelial cells* express the SRY-box 10 (SOX10) and SOX2 transcription factors, and myelin protein zero (MPZ) [80]. SOX10 is essential for peripheral nervous system (PNS) myelination [88].

Hassall's corpuscles (HCs)

HCs or *corpuscula thymica*, particular acidophilic structures of the thymic medulla, are vestiges of receded endodermal epithelium, with variable heterogeneity of the cellular component.

In human newborns, the main component of these spherical (onion-like) or oval bodies is a particular type of mTEC, displaying a concentric arrangement around a centrally placed mass that consists of macrophages, thymic DCs, myoid cells, and occasionally mast cells and lymphocytes. The lamellar-shaped appearance of these corpuscles represents the first diagnostic element for thymic tissue, and the CK AE1/AE3 IHC positivity (indicating the epithelial origin of mTEC's type and revealing their peripheral disposition in the HCs) is the second indicator of a certitude diagnostic. Elongated, spindle-shaped myoid cells, frequently observed during thymus development, were identified in the vicinity of HCs and, in some cases, as part of the HCs peripheral layer of cells, together with the S100-positive interdigitating DCs. The CD68(+) macrophages were also identified as taking part in the HCs, and rarely, CD117(+) mast cells were present in the surrounding area of HCs [89].

HCs have a variable size, between 20 to 100 μm, and their dimensions enhance with age. An interesting finding, previously reported, indicates that even in children within the same age range, the morphology and the shape of HCs may differ. In sick children with co-associated cardiac malformations (*e.g.*, atrioventricular and ventricular septal defects, or Fallot tetralogy), a particular observation was made; namely, bigger HCs with cystic distension and cellular remains. On the other hand, in children with transposition of great arteries, defects in the atrial septum, or atresia of the pulmonary valve, the morphology of HCs was consistent with those of normally developed ones [90]. The association of cardiac malformations with thymus morphological changes may be explained by the common origin in the vagal neural crest cells of both thymic mesenchymal cells and cardiac cells. Alterations in the migration routes and derivative lineage of these neural crest cells lead to aortopulmonary circulatory system defects, cardiac tissue malformation changes, and thymic medullary microenvironment imbalances [91].

In one of the first attempts to detail the morphology of HCs, Raica *et al*. described the presence of different evolutive stages, depending on the age of the patient. Under two years of age, a great number (more than five corpuscles/thymic lobule) but small in size, with deep eosinophilia and rarely degenerated or necrotic modifications, were seen. On the other hand, for patients between three to 21 years old, the number of HCs decreased (1–2/thymic lobule), but their size became larger, often displaying joint central parts filled with necrotic material, calcified debris, and cystic degeneration. Moreover, based on the disposition and phenotype of the epithelial cells, four particular embodiments of these corpuscles were described: *juvenile variant* – irregular small-sized corpuscle, conventional eosinophilic, with ellipsoidal or oval-shaped epithelial cells, and no degenerative changes; *immature variant* – with the spheroidal shape of the corpuscle, intense cytoplasmic eosinophilia of reticular cells, and lacking necrosis or cellular debris; *mature variant* – squamous epithelial cells bordering the corpuscles, with central necrosis and cystic degeneration; *senescent variant* – with increased corpuscle size, no epithelial cells, but with cystic degeneration, calcium deposits, apoptotic cells or necrosis. In the same patient, the four variants may coexist, but mature and senescent variants were only noticed in patients over six years old [92].

A peculiar finding is that CD20(+) B-lymphocytes were only detected in the thymic medulla or around HCs, where, together with the APCs (the thymic DCs), are thought to regulate thymic lymphopoiesis by enabling the self-tolerance process and negative selection of T-lymphocytes [93].

Morphological variations of the HCs were also reported on thymic tissue samples obtained from four patients with anti-acetylcholine receptor (AChR) antibodies in different types of *myasthenia gravis* (MG) with accompanying autoimmune Hashimoto's thyroiditis; they underwent thymectomy to diagnose a doubtful thymic lesion. In two patients with MG, the histopathological report showed thymic atrophy, with a substantial decrease in the thymic cortex section, degenerated cystic architecture with calcified HCs in late-onset MG patients, and distended cystic HCs in ocular-associated MG patients. The cysts origin, as modified HCs, is supported by the intense CK AE1/AE3 immunopositivity of cells lining the internal side of the cysts [94].

A recent investigation regarding the histological modifications that may appear in intravenous heroin drug addicts revealed the fact that in this subgroup, the thymus gland was overall atrophied, with fewer HCs, and significant dystrophic calcification of these structures. The regressive changes in HCs induce a lack in the maturation of naïve CD4(+) T-lymphocytes, sequentially decreasing their number and impairing proper thymus functioning [95].

Through the new immunophenotyping methods, we can conclude that the HCs are *corneocyte-like mTECs* and express characteristic genes [keratin 1 (*KRT1*), involucrin (*IVL*)], SOX2 transcription factor [66], exhibit cellular senescence, produce inflammatory cytokines and chemokines [including chemokine (C–X–C motif) ligand 5 (CXCL5)], and are developed under the control of *Aire* [83]. At the late stages of differentiation, post-*Aire* stages, mTECs maintain translation and exocytosis processes, upregulate

specific proteins for cornification, lose their nuclei, and display a distinct proinflammatory signature [96]. The HCs–mTECs also express thymic stromal lymphopoietin (TSLP) and have a critical role in the positive selection of self-reactive T-cells, mediated by the DCs, leading to the generation of CD4(+) CD25(+) Tregs [97].

TECs have a fundamental contribution to the distinct compartmentalization of the thymus [98] and to the development and maturation of the thymocytes. Mainly, the developing thymocytes can be distinguished by their CD4 and CD8 cell surface expression.

cTECs play vital roles in the initial phases of T-cell development and mediate the positive selection of thymocytes, which transform from CD4(–)/CD8(–) (double-negative) into CD4(+)/CD8(+) (double-positive).

mTECs mediate the negative selection of double-positive thymocytes into single-positive CD4(+) or CD8(+) thymocytes [62, 81]. Also, mTECs can serve as APCs for *Aire*-dependent antigens and, together with DCs and B-cells, mediate the self-tolerance by expressing and presenting tissue-restricted self-antigens [61, 64].

All the thymic stromal cells, including TECs, are critical for the development of cellular or T-cell-mediated immunity [99]. Contrary to secondary lymphoid organs devoided of epithelial cells (as lymph nodes), the thymus function (thymocytes education) depends on TECs. Moreover, TECs function is also modulated by the stimulatory crosstalk with thymocytes [98]. A reduced number of TECs or thymocytes–TEC crosstalk [by the lack of ephrin (Eph) and Eph signals] impair the development and homeostasis of thymic epithelium and can affect both thymic growth and thymocyte differentiation [100]. Another important factor is TECs integrity, which once affected (*e.g.*, by irradiation), changes thymocytes pool, proliferation, repair and renewal capacity, and their activity within the thymic microenvironment [82, 101]. Therefore, repairing an injured thymus is crucial for optimal immune responses against pathogens and tumor antigens and also for the immuno-therapeutic process [82].

The metabolic regulation of all thymic stromal cells and, in particular, of TECs fundamentally influences the thymic stromal microenvironment, the T-cell selection, and the thymus function. Regarding the thymic stromal micro-environment, TECs can produce a few components of the extracellular matrix (ECM) in a highly compartment-specific manner: collagen IV α-chains (cTECs) and fibronectin (mTECs), even though most of ECM is produced by fibroblasts and ECs. The complete characterization of ECM may have important implications for the regenerative techniques (matrices for organoids) [98]. Between other regulators of TECs functions, there are *signaling factors* [*e.g.*, mammalian target of rapamycin (mTOR), reactive oxygen species (ROS), autophagy] [99] and *growth factors* (*e.g.*, the keratinocyte growth factor, produced by thymic mesenchymal cells, which stimulates the TECs migration, proliferation, and differentiation and reduces TECs injury) [62]. Regarding the TECs metabolic activity, there are still questions about their involvement in T-cell trafficking.

TECs highly express the intracellular enzymes sphingosine-1-phosphate (S1P) and S1P-lyase (SPL) [102]. In mTECs and ECs, S1P is dephosphorylated by lipid phosphate phosphatase 3 (LPP3). Both LPP3 and SPL activities are required for mature thymocytes' egress into the bloodstream, where the mature thymocytes are lured by the high S1P concentrations derived from erythrocytes [102].

The TECs molecular characterization and their self-renew capacity are still not completely defined. Techniques such as flow cytometry, based on surface marker expression, and single-cell ribonucleic acid (RNA)-sequencing, can contribute to more comprehensive identification of human TECs subpopulations [69, 98].

TECs are sensitive to aging. The age-associated involution profoundly changes cTECs' cell size and morphology: cell cytoplasmic processes become difficult to detect, and cell volume reduces) [61]. The thymic involution starts faster than in other tissues, and at one year old, already TECs number begins to decrease; gradually, larger and larger thymus' parts are replaced with adipose tissue. The aging process determines the loss of the thymus cellularity and cannot be reversed. Puberty accelerates the thymocytes reduction at a rate of 3–5%. The environmental stressors, all the inflammatory processes, the immuno-suppressive agents, and cancer therapy (radio- and chemotherapy) accelerate the thymic involution [63]. TECs therapy can restore the function of TECs' thymic epithelial niches, can regenerate thymus function, and improve thymopoiesis in T-cell immune disorders or post-cancer therapy [67].

The regenerative approaches of the thymus (cell-based or organoid-based technologies or modulating endogenous repair pathways or new bio fabrication technologies – as embryonic stem cells-derived TECs) can all restore and enhance the thymus activity and recover T-cell immunity [63]. Mastering a better knowledge of cortical and medullary thymic epithelial compartments can lead to the thymopoiesis improvement, especially for the elderly and patients with autoimmunity or immunodeficiency disorders.

Pineal calcification, aging, and pathological changes

The pineal gland (PG) is a NE transducer that converts the information about light and dark status into a hormonal response, consisting of melatonin secretion. Melatonin, the main hormone synthesized by PG at nighttime, is released in the bloodstream and in the cerebrospinal fluid (CSF) of the third ventricle [103–105]. It modulates the circadian rhythm but also has an important neuroprotective function because of its antioxidant and anti-inflammatory role [103, 106, 107].

Pineal gland calcification (PGC), also called brain sand, pineal *acervuli*, or *corpora arenacea*, was first described in the 18th century by the Italian anatomist G.B. Morgagni and identified on skull radiography by Schiller in 1918 [108–110]. The process of calcification in the PG is a natural, physiological process, which is correlated to the decrease in melatonin secretion, aging, and pathological conditions, such as neurological and psychiatric disorders [103, 111–113].

The incidence of PGC is age-related and also seems to be different among the various population in different regions of the world [103, 111]. For example, the lowest incidence was reported in Africa (Gambia 1.3%; Nigeria 5%) [111], but in Turkey, a study on 12 000 subjects

revealed an incidence of 71.6% [103, 114]. It is generally accepted that the prevalence of PGC increases with age [103, 108, 109, 111]. However, some authors state that PGC can also be found in neonates, children, and young individuals, with an incidence that varies from 2% in the first decade of life to 53% in the third decade [103]. Yet, there are studies that deny the presence of PGC under the age of two years [115–117]. Some research papers investigate the relationship between PGC and gender, but the results are controversial; only a few authors reported an association with the male gender [106, 116], others did not find any differences between sexes [116].

Calcifications in the PG are located within the pineal parenchyma or connective tissue septa (*intrapineal corpora arenacea*) or in the pial capsule or arachnoid cover (*meningeal corpora arenacea*) [108, 111]. They mainly contain hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and may exhibit different growth patterns [103, 108, 111, 116, 118]. Some of the *acervuli* are small (up to 2–3 μm), regular in shape, round or globular (*globular pattern*), and others are larger (up to 1 mm), irregular in shape, with concentric, *lamellar pattern* (mulberry-like structures), which can form large aggregates [108, 111, 116]. Several studies suggest that the globular pattern is more frequent in young people and is mostly intrapineal located [108], while the concentric, lamellar pattern occurs in elderly people and can be mostly found in meningeal coverings [108].

The microscopic study of *corpora arenacea* in the Hematoxylin–Eosin staining identifies these calcifications as basophilic or purple structures [111]; an important method to identify the calcified concretions is von Kossa staining technique, which uses silver nitrate solution.

In addition, other methods such as histochemical staining, EM, transmission electron microscopy (TEM), scanning electron microscopy (SEM) are used to study the calcifications [111]. Some of these techniques reveal the presence of Ca^{2+} ions in the pinealocyte cytoplasm, as well as in the cell membrane [108]. Ca^{2+}-dependent adenosine triphosphatase (ATPase) plays an important role in maintaining calcium homeostasis within the pinealocytes, eliminating calcium in the extracellular space; any dysfunction of this enzyme may lead to pinealocytes lesions and low melatonin levels [111, 118].

The development of PGC is not clearly understood; various mechanisms and microenvironmental factors are suggested to be involved [111, 112].

Several theories have proposed the idea that tryptase, a protease stored in mast cells, has a role in the development initiation of PGC in the pineal connective tissue stroma, including in children [103, 112]. This statement is supported by studies that show a correlation between tryptase and calcification processes in other sites, including arterial calcifications [119].

Another mechanism may be the *dysfunction of the Ca^{2+}-dependent ATP-ase*, which may lead to intracellular accumulation of calcium in the pinealocyte; cell degradation will follow, with apoptosis and release of large amounts of calcium in the extracellular space, where they eventually form the intrapineal *corpora arenacea* [103].

Recent studies indicate that the formation of PGC resembles bone formation. A hypothetical mechanism to explain the development of PGC is based on the observation that melatonin stimulates the ossification process by *differentiation of mesenchymal stem cells* (MSCs) into osteoprogenitor cells and osteoblasts. The process is mediated through the MT_2 melatonin receptor, which promotes melatonin binding to MSCs and their differentiation into bone cells. In addition, it is suggested that melatonin stimulates the synthesis of type I collagen fibers and activates the gene expression of some bone proteins (*e.g.*, BMP2, alkaline phosphatase, osteocalcin, osteopontin, etc.), which eventually form the bone matrix [103, 118, 120, 121]. Several physiological or pathological factors may contribute to MSCs migration in the PG, such as PG rich blood supply, inflammation, hypoxia [103, 118, 121], sunlight exposure [105, 117], etc. (Figure 3).

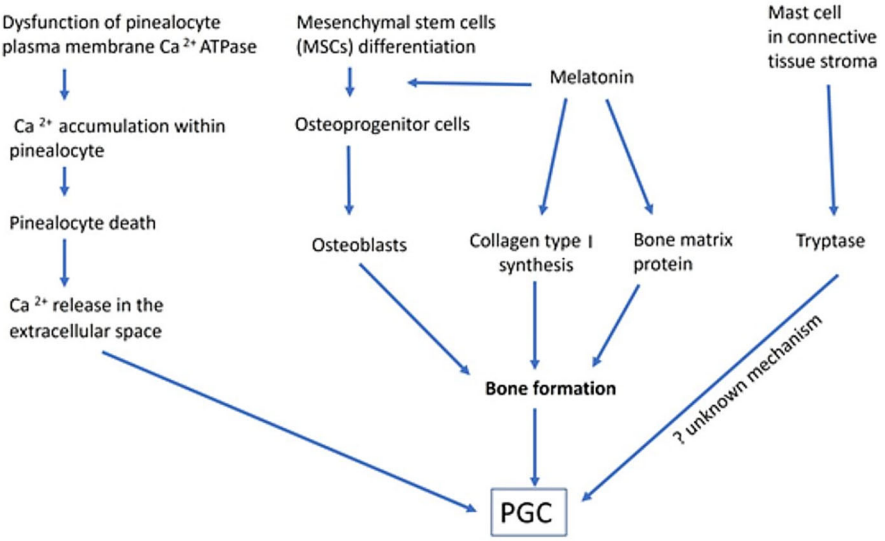

Figure 3 – ***Potential mechanisms formation for PGC. ATPase: Adenosine triphosphatase; PGC: Pineal gland calcification.***

The calcium concretions that accumulate in the gland will decrease the pinealocyte function, thus reducing the melatonin secretion [108, 111]. This fact is demonstrated by the observation that PGC is correlated with low levels of urinary melatonin metabolite 6-sulfatoxymelatonin [109, 110]. Melatonin, with its antioxidative role, might protect the neurons against oxidative stress, a theory vehiculated in neurodegenerative and neuropsychiatric diseases [103, 106, 111]. A low level of melatonin will expose the brain to oxidative stress and lesions, all the more so as the brain

is characterized by high oxygen consumption and a reduced ability of antioxidant enzymes to remove free radicals. This may explain the association between the decreased level of melatonin production and the enhancement of different pathological cerebral conditions.

The implication of pineal calcifications in the pathogenesis of different neurological diseases is not yet understood, but it was reported an association between PGC, low levels of melatonin and insomnia, sleep disorders, cognitive decline, schizophrenia, Alzheimer's disease (AD), multiple sclerosis, migraine, cerebral infarction, etc. [103, 106, 110, 111, 117]. Numerous recent studies suggest an important relationship between pineal calcifications and AD [103, 110, 111, 117] and state that melatonin administration may decrease the accumulation of β-amyloid protein senile plaques and neurofibrillary tangles, which represent the hallmarks of this disease [105, 110]. Moreover, experimental studies on AD mice reveal an association between pineal calcifications, PG dysfunction and decreased neurogenesis, and loss of neurons in the hippocampus, with cognitive decline and memory dysfunction [105].

Additional studies are needed to understand the development of pineal calcifications and their relationship with age and different pathologies, especially neurological and psychiatric disorders.

New aspects regarding the *corpora amylacea*

Corpora amylacea (CA) are spherical structures identified both in physiologically aged tissues and in various neurodegenerative disorders and prostatic inflammatory conditions. According to Purkinje, who first reported their presence in elderly human brain specimens in 1837, and subsequently to Virchow, in 1854, these granular entities were described as round concretions, existing in great number and size in aged brain tissue, without having pathological implications; as a result, being considered nonsignificant, they have left aside for many decades [122, 123].

Over the past 50 years, important information was brought into light regarding their specific origin, morphological appearance, and potential function. Initially, they were thought to be posthumous artifacts, products of the failed glycogenic process, or protein deposits of the hematogenous or lymphatic source. Further studies identified in their structure elements of nervous origin, such as clustered fragments of neurons, degenerated oligodendroglial cells, degraded astrocytes, or leaked blood components of impaired brain–blood barrier [124]. Later reports implied a correlation of CA with fungal infections [125] or acting as a shield against an autoimmune response to the inorganic waste deposited in their frame during formation [126].

Histological two-dimensional previous evaluations of their morphological aspect described CA, as amorphous, spherical, basophilic structures, with onion-like sheeting, concentrated in periventricular and subpial areas [127]. More recent work on normally aged human brain tissue samples, taken from deceased patients of non-neurological causes, put in evidence the complex three-dimensional structure of these CA. The authors characterized this particular assembly as single flattened disks-like entities entangled into an elaborate compact network into the neuropil and around blood vessels. Few individuals CA or less interconnected ones were observed within the neuropil, while the perivascular located CA displayed an aggregated pattern, sometimes elongated and fused, with no contact to the glial fibrillary acidic protein (GFAP)-positive astrocytic vascular end-feet [128].

Besides their normal increased number and size with age, in AD, in the periventricular white matter of these patients, the presence of multiple CA associated with a bacterial lipopolysaccharide was reported [129].

Besides AD, CA were also identified in Parkinson's disease or amyotrophic lateral sclerosis, but fewer in number and less immunoreactive to fungal proteins [130]. Nevertheless, microbial infections were incriminated in triggering an abnormal immune response, with increased synthesis and deposition of amyloid in brain disorders, such as multiple sclerosis [131] or Huntington's disease [132].

Aside from the central nervous system (CNS), the CA may be frequently detected as small concretions in the lumen of normal prostate gland [133] (Figure 4).

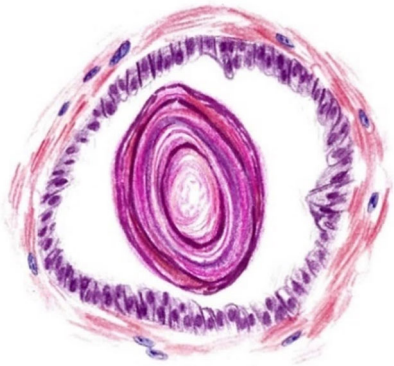

Figure 4 – ***Lamellar concretion of corpora amylacea in prostate gland.***

Found in great number in healthy adult males' prostate, they were described at first as benign multilayered bodies; however, over the years, their significance inflicted much debate. The most generally accepted origin is supposed to be linked to degenerated and impaired glandular epithelial cells, surrounded by lamellar, concentrical polyglucosan proteinaceous deposition of glandular secretions, mainly sulfated glycosaminoglycans [134]. However, CA, and their seemingly calcified variant, the prostatic calculi, possess in their organic matrix some acute inflammatory proteins, namely: lactoferrin, calprotectin, and myeloperoxidase; all these proteins are also present in neutrophils granules, indicating that CA may represent residues of previous prostatic infections [135, 136].

A recent exhaustive review regarding the composition-related origin and structural-depending functions of the CA, present in different human tissues, has proposed the use of new terminology for these CA: the "wasteosomes". The denomination "wasteosomes" or "waste products" would bring together previously reported heterogeneous content: a common Periodic Acid–Schiff (PAS)-positive glycan skeleton (carbohydrate structure) and variably Congo red-positive proteinaceous amyloid deposition (as cellular waste). This new theory makes sense, at least regarding the continuous growth of these irregular concentric depositions of the metabolic end-products with age [137].

Previously seldom described in prostate adenocarcinoma, a recent study reported, though, a higher CA occurrence within the tumoral and peritumoral prostatic tissue, yet correlated with less aggressive malignancy markers, but positively associated to chronic inflammatory conditions and body mass index [138].

Finally, contrary to all these many theories around them, CA remains mysterious enough, and much research is still needed to completely decipher their origin and implications in pathology.

Fañanas cells – the enigmatic cerebellar glia

In the cerebellum, at least two types of astrocytes have been recognized: Bergmann glia and the astrocytes in the granular layer. Though, in the early 20th century, an additional type of glia, the Fañanas cells, were first identified by Fañanas, in the deep molecular layer, close to Purkinje cells' bodies [139, 140]. Revealed by the Cajal's astrocyte staining (the gold-sublimate method), Fañanas cells were described as an astrocytic cell type, similar to Bergmann cells. Recently, Reichenbach & Wolburg reconsidered Fañanas glia as a subtype of Bergmann cells [141].

Even though Fañanas & Bergmann glia are not identical, their differentiation in light microscopy or EM is impossible based on the simple morphological criteria [142].

To identify the Fañanas glia in the cerebellar cortex, Goertzen & Veh used various techniques, including a modified Cajal's gold-sublimate method and immunocytochemistry for glial specific markers: GFAP, glutamine synthetase (GlnS), voltage-gated potassium channel 2.2 (Kv2.2), potassium channel-interacting protein 3 (KChIP3) and SOX2. Based on the localization in the proximity of Purkinje cells layer, the distribution of gold precipitates and the immunoexpression of Kv2.2, KChIP3 and SOX2 but not of GFAP, Fañanas cells were differentiated from the Bergmann glia [143].

The special modified Cajal's gold-sublimate technique enabled the detection of Fañanas cells, due to the black gold precipitates on the cell bodies and "feathered" processes in the inner third of the molecular layer. In the Purkinje cells layer, immunoreactivity for GlnS revealed adjacent Bergmann cells principal population, the presence of another glia type. Compared with Bergmann cells, with epithelioid cell bodies organized on a continuous row – hence the name "Golgi epithelial cells", the second type of glia had smaller cell bodies and more irregularly arranged, in a broader and discontinuous layer above Purkinje cells bodies. Moreover, the main cytoplasmic processes of Bergmann cells were mostly unbranched and radially extended towards the pial surface of the molecular layer. By contrast, the processes of Fañanas cells were more branched and "feathered", visible in the deep part of the molecular layer, closer to the Purkinje cells, and the granular layer, but difficult to identify in the middle and outer thirds of the molecular layer (Figure 5). The immunoreactivity to other markers also allowed the differentiation between the two types of glia in the Purkinje layer: the processes of Fañanas cells selectively stained with Kv2.2 and KChIP3 nuclei were immunopositive for SOX2, whereas Bergmann cells remained unstained [143].

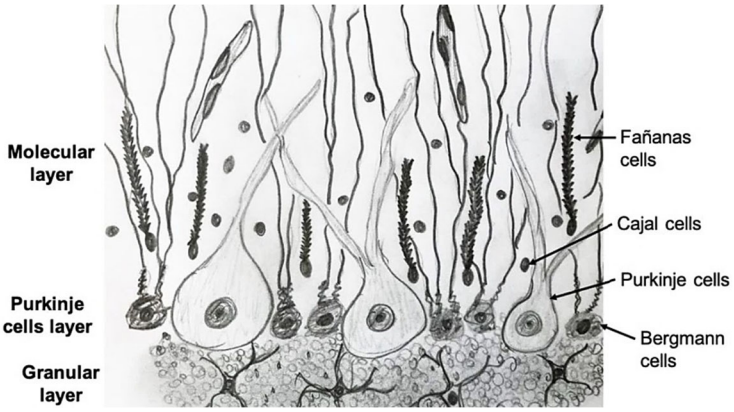

Figure 5 – ***Schematic drawing of Fañanas cells.***

The markers expression profile of Fañanas cells suggests that this astrocytic glia plays a role in maintaining cerebellar homeostasis. Kv2.2 is a protein ion channel implicated in the regulation of neurotransmitter release. KChIP3 binds calcium, deoxyribonucleic acid (DNA), and other proteins and regulates the function of potassium channels [143, 144]. SOX2 is a pluripotency transcription factor implicated in maintaining the undifferentiated state in stem cells; in the CNS, SOX2 is expressed by neural progenitor cells. Mandalos *et al*. demonstrated that alterations of SOX2 function in cerebellar glial cells could lead to neurological defects due to improper maturation of neuronal progenitors during cerebellum development and overproduction of immature granular neurons in the adult cerebellum [145].

Conclusions

Considering the continuous acquisition in Histology of new structural details of different tissues and organs, with new functional implications (highlighted by the recent molecular and IHC techniques), such accurate research on a given subject is essential in preclinical–clinical transition; the obtained information may be applied in different fields of modern medicine (acute and chronic inflammation, viral infections/including the SARS–CoV, pulmonary arterial hypertension, graft rejection, autoimmune diseases, cancer or rejuvenation).

Conflict of interests

The authors declare that they have no conflict of interests.

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Received: March 25, 2022

Accepted: November 3, 2022