

FALC stromal cells define a unique immunological niche for the surveillance of serous cavities

Authors: Lucy H. Jackson-Jones¹ and Cécile Bénézech²

¹ Division of Biomedical and Life Sciences, Lancaster University, Lancaster, LA1 4YQ, UK.

² Centre for Cardiovascular Sciences, University of Edinburgh, Edinburgh, EH16 4TJ, UK.

Corresponding author email: cbenezec@ed.ac.uk

Highlights:

- FALC stromal cells contribute to the barrier function of the mesothelium
- CCL19⁺ FRCs & CXCL13⁺ cover cells support the functional architecture of FALCs
- FALC stromal cells create an IL-33 rich environment to support ILC2 function
- Cover cells secrete RA required for cavity M ϕ phenotype & B cell function
- Innate-immune activation of FALC FRC mediates T-B cell interactions

Declarations of interest: none

Abstract:

The serous cavities contain specialised adipose tissues which house small clusters of immune cells known as fat-associated lymphoid clusters (FALCs). The continuous flow of fluid from the serous cavities through FALCs makes them unique niches for the clearance of fluid phase contaminants and initiation of locally protective immune responses during infection and inflammation. Development, and activation of FALCs both at homeostasis and following inflammation are co-ordinated by the close interaction of mesothelial and fibroblastic stromal cell populations with immune cells. In this review we discuss recent developments in FALC stromal cell biology and highlight key interactions that occur between FALC stroma and immune cells.

Introduction:

Internal organs are contained in three fluid-filled serous cavities: the peritoneum which envelops the abdominal viscera [1] the pleural cavity enclosing the lungs [2] and the pericardium which protects and anchors the heart [3]. These cavities are delimited by two membranes, the parietal serosa covering the wall of the cavity and the visceral serosa covering the organs. Fluid is secreted by the mesothelium lining these membranes ensuring lubrication of the serous cavities, free movement of organs and continuous flow of fluid. Fluid is drained out of the cavities through stomata found in the mesothelium of certain visceral adipose tissues, such as the membranous folds formed by the omentum and mesenteries in the peritoneal cavity, the adipose tissue of the mediastinum in the pleural cavity and the adipose tissue of the pericardium. Dedicated immunological niches called fat-associated lymphoid clusters (FALCs) develop under the intercellular pores formed by stomata [4,5] in the mesothelium of these visceral adipose tissues and contribute to the barrier function of the mesothelium and immune surveillance of serous cavities. FALCs were first identified in the omentum where,

historically, they were called milky spots [6], knowledge of their existence was later expanded to include other visceral adipose tissues [7-10]. FALCs are important immune activation and co-ordination hubs; possessing some functions akin to lymph nodes but distinct in that they are not encapsulated organs, enabling direct and more immediate contact with contaminants (pathogens, particulate material, PAMPs, DAMPs) within the cavities in which they reside. Recent studies are beginning to unpick the complex interactions between immune and stromal cells within adipose organs. The FALCs, as co-ordination hubs for body cavity immune responses represent an important site for the analysis of adipose stromal-immune interactions that take place just beyond the mucosal frontline.

1 Structure of FALCs and function of FALC stromal cells at homeostasis

1.1 Structure of FALCs

FALCs are a critical component of serous innate-B cell function [11]. The recruitment of B cells to FALCs is dependent on the homeostatic chemokine CXCL13 [12], which is expressed by stromal cells that form a 3-dimensional basket that envelopes FALC B cells [13]. Recent work from our lab confirmed that the expression of CXCL13 was limited to the loose lining of mesothelial cells covering the serous facing side of FALCs [14]. We called these cells CXCL13⁺ FALC cover cells. In addition to CXCL13, FALC cover cells express GP38/Podoplanin and a number of factors involved in the recruitment, adhesion or activation of immune cells [14] suggesting that FALC cover cells facilitate the extravasation of immune cells between the serous cavities and FALCs.

A distinct subset of stromal cells expressing the homeostatic chemokine CCL19 is found at the heart of the clusters [15]. These stromal cells form a reticular network, expressing GP38 and the fibroblastic marker PDGFR α , resembling fibroblastic reticular cells (FRCs) and referred to as FALC FRCs. Lymph nodes contain different subsets of FRCs contributing to

various structural and functional niches [16,17] including CCL19 expressing reticular cells found in the T-cell zone of lymph nodes and called T-reticular cells. While T cells are present in FALCs, there is no clear T-cell zone, indicating that FALC FRCs constitute a unique subset of lymphoid stromal cells with differing functional capacity to FRCs found within lymph nodes.

FALCs are highly vascularised [18-20] with the endothelial blood vasculature forming distinctive convolutions resembling the renal glomeruli. FALCs can support the differentiation of high endothelial venules (HEV). HEVs are essential for lymphocyte trafficking in secondary organs and in FALCs support the entrance of lymphocytes to the serous cavities from the circulation. In addition, HEVs are used by neutrophils to swiftly transit into the peritoneal cavity during peritonitis [21]. In contrast to lymph nodes which directly receive lymph through afferent collecting lymphatics, FALCs are not exposed to lymph but to the serous fluid entering through the mesothelial stomata. Collecting lymphatic vessels are found in the vicinity of FALCs to drain this fluid toward downstream lymph nodes [18]. See composition of FALCs in Figure 1A.

1.2 The expression of IL-33 by FALC stromal cells support ILC2 function in FALCs

Group 2 innate lymphoid cells (ILC2) were first identified in FALCs of the mesenteric adipose tissue [7], where in response to IL-33, they secrete IL-5 which in turns enables innate-B cell proliferation and IgM secretion [7,10]. We found that GP38⁺CD31⁻ FALC stromal cells produce IL-33, which is sequestered in their nucleus [10]. Recent reports have now shown that IL-33 is also present within mesenchymal cells interspersed within, and mesothelial cells covering, adipose tissues. IL-33 is emerging as a key regulator of adipose tissue homeostasis by controlling type 2 immune cell recruitment to adipose tissue [22-26]. In particular stromal adipose IL-33 promotes ILC2 secretion of IL-4 and IL-13 [22,25,26] triggering the expression

of *Ccl11* by mesenchymal adipose stromal cells which in turn increases recruitment of eosinophils to adipose tissue [23]. While $\text{PDGFR}\alpha^+\text{GP38}^+\text{CD31}^-$ adipose stromal cells have been shown to support the differentiation of ILC2 progenitors [22], it is still not clear what promotes the recruitment/survival of ILC2 in FALCs and the adipose tissue stromal cell niche nor what proportion of ILC2 are found in FALCs compared to the rest of the adipose tissue. Interestingly, adipose tissues rich in FALCs, such as the omentum, secrete high amounts of IL-33 compared to adipose depots poor in FALCs [10], suggesting that FALCs may concentrate IL-33 production and ILC2 action in adipose tissue. We recently confirmed by single-cell RNA sequencing the expression of IL-33 by mesothelial cells and mesenchymal cells [14].

1.3 The role of stromal cell derived retinoids at homeostasis

All serous cavities are home to a population of self-renewing cavity-resident macrophages [27-30], important for clearance of pathogens in serous cavities [27,31-33], tissue repair [34,35] and Ig production by innate-B cells [36,37]. The maintenance and functional identity of these cavity-resident macrophages rely on the transcription factor GATA6 [36,38,39]. Gata6 expression is induced by retinoic acid (RA), a derivative of retinol (vitamin A) [30,36]. Mesothelial cells and fibroblastic cells of the omentum and mesenteries express high levels of two rate limiting enzymes required for the conversion of retinol into RA, the retinaldehyde dehydrogenase 1 and 2 (*Raldh1* and *Raldh2*) [30,36]. The transcription factor Wilms tumour 1 (*Wt1*), which is required for the development of all visceral adipose tissues including the omental, pericardial and mesenteric depots [40] and is expressed by both $\text{PDGFR}\alpha^-$ mesothelial cells and $\text{PDGFR}\alpha^+$ fibroblasts in the omentum and mesenteries drive the expression of *Raldh1* and *Raldh2* [30]. Diphtheria-toxin mediated depletion of *Wt1*⁺ cells diminishes the frequency of serous-resident macrophages in the pericardial, pleural and peritoneal cavities confirming that *Wt1* expressing cells are critical for the maintenance of cavity-resident macrophages [30].

Single-cell RNA sequencing analysis of omentum stroma confirmed that mesothelial cells, FALC cover cells and adipose stromal cells are competent to secrete RA while FALC FRCs are not [14]. Secretion of RA by FALC cover cells may establish an RA rich environment important for FALC B cell function. RA directly induces the expression of the gut homing molecules $\alpha 4\beta 7$ Integrins and CCR9 by innate-B cells [41] while GATA6-dependent secretion of TGF- $\beta 2$ by peritoneal macrophages promotes IgA class switching in innate-B cells [36,42]. RA is also important for the maintenance and function of innate-B cells via control of NFATc1 expression [43,44]. See Figure 1B for RA production by FALC and adipose stromal cells.

2 Signals controlling the formation of FALCs and their maturation

2.1 During development and under homeostatic condition:

FALCs are first found in human omentum during foetal development [45] and appear in the mouse mesenteries during the first week after birth [9]. Recruitment of a subset of ROR γ t-dependent innate lymphoid cells (ILC) called lymphoid tissue inducer (LTi) cells expressing Lymphotoxin α (LT α) that engage the Lymphotoxin β receptor (LT β R) on mesenchymal cells is a critical step in the development of secondary lymphoid organs. In particular LT β R signalling induces CXCL13 expression by stromal cells of the lymph node anlagen, precipitating the recruitment of CXCR5⁺ LTi cells [46-50]. In contrast, the initiation of FALC formation is independent of ROR γ t and LT β R signalling [9,13]. While the formation of FALCs can be initiated in B and T cell deficient *Rag2*^{-/-} mice, FALCs are absent in *Rag2*^{-/-}*Il2r γ* ^{-/-} mice suggesting that a subset of ILCs different from LTi is required to trigger the initial formation of FALCs during normal development. FALCs do form in *Cxcl13*^{-/-} mice [13], but fail to recruit B cells. The expression of Cxcl13 is not affected in LT α ^{-/-} mice [13], indicating that Cxcl13 expression by FALC cover cells is not dependent on LT α /LT β R signalling. Additional work is required to determine what controls the expression of CXCL13 and the differentiation of FALC

cover cells. Interestingly, both RA [51] and interstitial fluid flow [52] have been shown to induce/reinforce CXCL13 expression during lymph node formation, two pathways which could be acting during FALC formation and which may have implications in disease states where there is increased serous fluid for example during peritoneal dialysis or malignant effusion [20].

FALCs are notably smaller and their number is reduced in *Tnfr1^{-/-}Tnfr2^{-/-}* mice [9] and in mice with a conditional deletion of Myeloid differentiation primary response 88 (MyD88) in *Ccl19⁺* FRCs [15] indicating an important role for TNF signalling and innate-immune sensing for the development of FALCs. MyD88 signalling controls transcriptional reprogramming of FALC FRCs leading to increased expression of homeostatic and inflammatory chemokines and extra-cellular matrix proteins [15]. Germ free mice also show reduced numbers of FALCs [9], indicating that the presence of commensal microbes or their by-products sets an innate inflammatory tone sensed by FALC FRCs to form and mature a full complement of FALCs in the serous cavities. Sensing of the environment by FALC FRCs is likely to be facilitated by the constant flow of serous fluid through FALCs bringing in pathogens and antigens.

2.2 Under acute inflammatory conditions

During peritonitis, neutrophil and inflammatory monocyte rapidly enter FALCs to access the peritoneal cavity, while peritoneal contaminants are captured by FALCs [6,14,19,20]. In addition to the activation of existing FALCs, peritonitis leads to swift *de novo* FALC formation. This is dependent on the production of TNF by inflammatory monocyte/macrophages and TNFR-signalling in stromal cells [9]. The initial recruitment of inflammatory monocytes into FALCs requires MyD88 dependent signalling within *Ccl19* expressing FALC FRC and the release of the monocyte chemoattractant CCL2 [15]. The cross-talk between monocyte/

macrophage and FALC FRCs thus plays a critical role in *de novo* FALC formation. Natural Killer T cells (iNKT), a subset of T cells enriched in visceral adipose tissue [53], are required to induce neo-formation of FALCs during acute inflammation, an effect mediated through IL-4 and IL-13 [9]. This suggests that a cross-talk between iNKT cells secreting IL-4/13 and FALC stromal cells expressing IL-4R is required to induce FALC formation.

3. FALC stromal cells support B cell immune responses

3.1 Innate-B cells

Upon sensing of inflammatory or infectious signals, serous B cells migrate into FALCs where they undergo rapid proliferation and start to release large amounts of poly-reactive IgM to protect the serous space [10,11,54]. The absence of IL-33 signalling via genetic deletion of *Il1rl1* (IL-33R) results in a failure to activate pericardial & mediastinal FALCs and a decrease in the levels of IgM locally within the pleural cavity in response to infection or allergic airway inflammation [10]. IL-33 does not act directly on FALC B cells but most likely on ILC2 [7], which respond by increased secretion of IL-5 enabling rapid B cell proliferation and IgM secretion (Figure 2A) [10]. Thus stromal-derived IL-33 is important not only during homeostasis but early in response to infection or inflammation within the serous cavities, the mechanisms leading to release of IL-33 by FALC stromal cells are still to be elucidated.

3.2 FALC FRC support T-cell dependent B cell immune responses

FALCs support T-cell dependent immune responses, including Ig isotype switching [9,13,17], somatic hypermutation, and limited affinity maturation [13], despite the lack of identifiable follicular dendritic cells. Although FALCs do not develop proper germinal centres [13], B cells rapidly acquire a germinal centre-like phenotype that can be assessed by the expression of first apoptosis signal (FAS) and the T and B cell activation marker GL7. Recent work by Perez-

Shibayama using FRC-restricted MyD88 ablation demonstrates that FALC FRC play a key role for CD4⁺ T cell-dependent B-cell activation, the initiation of a germinal centre reaction and IgG class switching during Salmonella infection. Recruitment of inflammatory monocytes into FALCs and bidirectional TNFR1/2 signalling between inflammatory monocytes and FRC mediates FRC activation and germinal centre formation (Figure 2B) [15]. Interestingly, the secretion of IgM by FALC B cells is not affected by FRC-restricted MyD88 ablation [15] indicating that innate-B cell function and T cell-dependent B cell immune responses are supported by distinct FALC stromal cell population. In support of this, IgM producing B cells are found in the periphery of FALCs while IgM^{low} B cells are found in the centre of the cluster [9,10].

Conclusions:

Here we discussed how the structure and function of FALCs is supported by unique subsets of stromal cells. FALCs are emerging as critical regulators of serous cavity immune function at homeostasis and during infection and inflammation. Recent studies have shed considerable light on the importance of the adipose stromal cell niche for the establishment and function of the immune compartment of the serous cavities and adipose tissues. Future studies will benefit from genetic tools that will enable targeted deletion and modification of specific subsets of adipose stromal cells including those within FALCs. As immune-adipose tissues are increasingly recognised for the key roles they play in the regulation of whole body homeostasis, metabolism & disease there is an urgent need to expedite the development of such tools.

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- of special interest, •• of outstanding interest

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	FALC FRC	FALC cover cells	Adipose stromal cells	Mesothelial cells	References
CD31	-	-	-	-	
GP38	+	+	+	+	
PDGFRα	+	-	+	-	[14,22],
CCL19	+	-	-	-	[14,15]
CXCL13	-	+	+	+	[13,14]
IL-33	+	+	+	+	[10,14,23-25]
CCL2	+	+	-	-	[14,15]
WT1	+	+	+	+	[14,30]
RA	-	+	+	+	[14,30]
CCL11	-	-	+	-	[14,23]

Table 1: Markers expressed by adipose and FALC stromal cells

Figure legends:

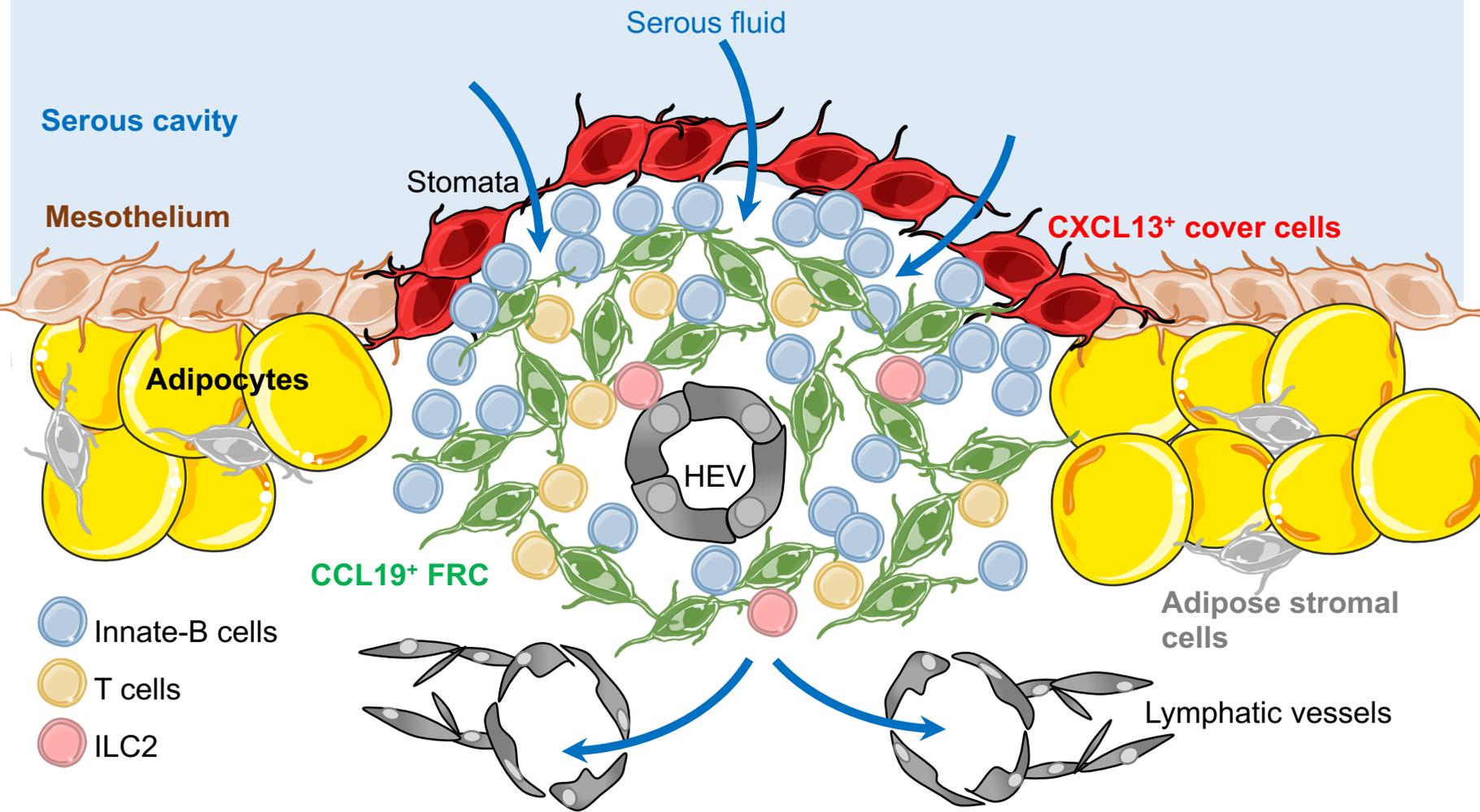
Figure 1: Structure of FALCs.

A, FALC stroma is made of two main stromal cell population: FALC FRCs expressing CCL19 and FALC cover cells expressing CXCL13. Serous fluid enters FALCs through stomata made by the loose lining of FALC cover cells and drain through collective lymphatic vessels in the vicinity of FALCs. Immune cells enter FALC from the circulation via HEVs and transit in and out of the serous cavity via stomata. **B**, Mesothelial cells, adipose stromal cells and FALC cover cells are competent to produce RA (1). RA induces the expression of GATA6 which is required to retain cavity-resident macrophages (2) and the secretion of TGF- β (3) which induces IgA class-switching (4). RA also acts directly on innate-B cells, inducing the expression of gut homing molecules (5) and is required for the expression of the transcription factor NFATc1 needed for the development and function of innate-B cells (6).

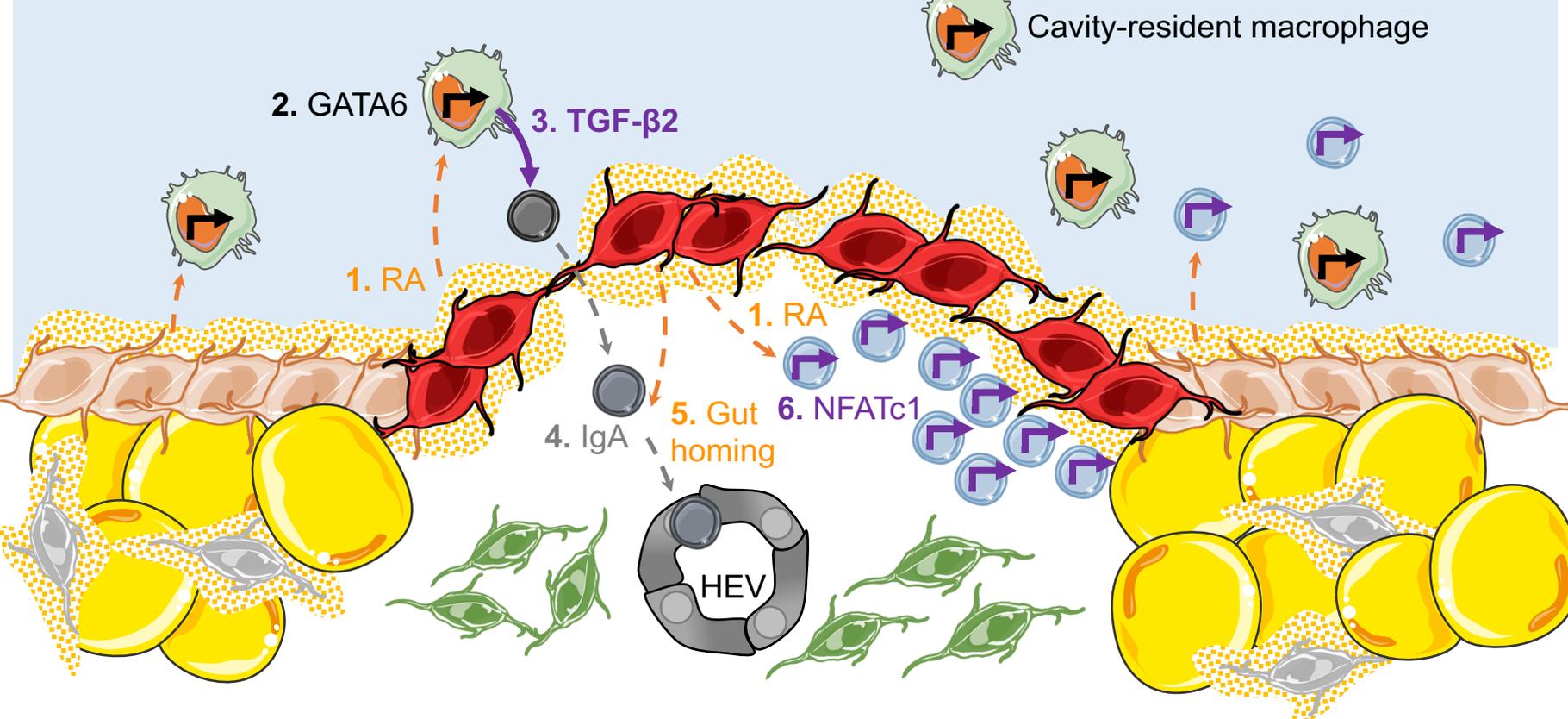
Figure 2: FALC stromal cells support B cell immune responses

A, FALC stromal cell support innate-B cell immune responses. CXCL13⁺ cover cells and CCL19⁺ FRCs express IL-33. Upon sensing of infectious/inflammatory signals (1), release of IL-33 (2) induces increased secretion of IL-5 by ILC2 (3) leading to innate-B cell activation and IgM secretion (4). **B**, FALC FRCs support T-cell dependent B cell immune response to peritoneal antigens. During serous infections, pathogens and antigens are filtered into FALCs (1) where MyD88 mediated-innate sensing of pathogens by CCL19⁺ FRCs (2) trigger CCL2 secretion (3) and inflammatory monocyte recruitment (4). FALC FRC-monocyte cross-talk through TNF-TNFR signaling (5) is required for T-B cell interactions (6), germinal center formation, class-switching, somatic hyper-mutation and affinity maturation (7).

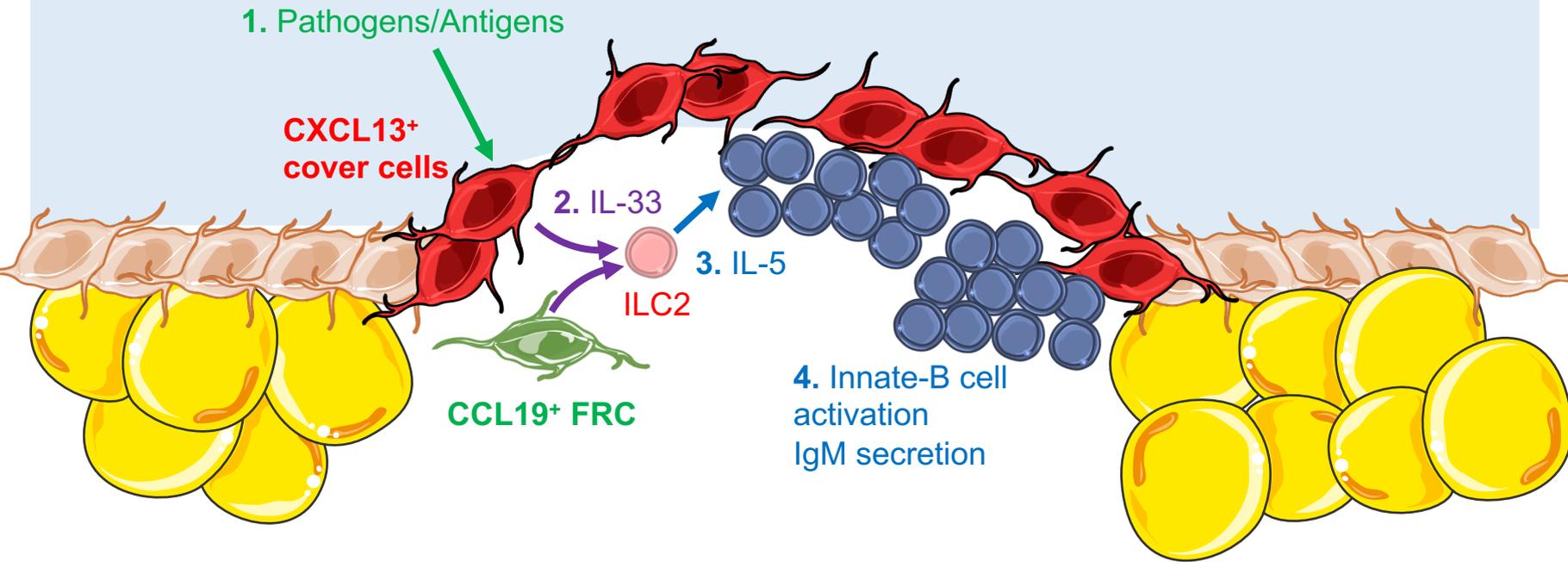
A. Stromal composition of FALCs



B. RA secretion by omental stromal cells



A. FALC stromal cells support innate-B cell activation



B. FALC FRCs support T-cell dependent B cell immune response to peritoneal antigens

