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Authors
Cautivo, Kelly M
Steer, Catherine A
Molofsky, Ari B

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Immune outposts in the adventitia: *One foot in sea and one on shore*

Kelly M. Cautivo[1], **Catherine A. Steer**[1], **Ari B. Molofsky**[1,2,#]

[1]University of California San Francisco, Departments of Laboratory Medicine

[2]Diabetes Center, San Francisco, CA, 94143, USA

Abstract

Advances in microscopy, genetically modified mice, and single-cell RNA sequencing have begun to deconvolute the composition and function of tissue immune niches. Here we discuss the evidence that the adventitia, the outermost layer of larger blood vessels, is a conserved niche and tissue immune outpost for multiple immune cells, including group 2 innate lymphoid cells (ILC2) and subsets of tissue-resident memory T cells, macrophages, and dendritic cells. We also describe the unique non-immune composition at adventitial regions, including fibroblast-like stromal cell subsets, lymphatic and blood endothelial cells, and neurons, and review how immune-stromal crosstalk impacts regional tissue immunity, organ adaptation, and disease.

Tissue immune niches: *when I was at home I was in a better place*

In secondary lymphoid organs (SLOs) such as lymph nodes and spleen, non-hematopoietic subsets are critical regulators of adaptive lymphocyte organization and function [1,2]. However, a myriad of innate and adaptive immune cells reside in non-lymphoid tissues and rarely recirculate in the blood or lymph. Many of these immune cells seed tissues early in life, establishing long-term residence, adopting unique phenotypes, and regulating optimal organ development, remodeling, and function [3,4]. These tasks occur within specialized microenvironments called niches, where local interactions with other immune cells and specialized non-hematopoietic cells optimize the detection of tissue perturbations, promoting adaptive coping mechanisms that either return the tissue to basal homeostasis or to an altered physiologic state.

Tissue immune niches are clearly heterogenous, which we conceptualize here as four broad classes illustrated within the respiratory tract (Fig. 1). We present these niche classes to

[#]**Corresponding Author Contact Information**: Ari B. Molofsky MD PhD, University of California San Francisco, 513 Parnassus Ave, HSW1201U Box 0451, San Francisco, CA, 94143, USA. Tel 1 (415) 476-1702., ari.molofsky@ucsf.edu.

Declaration of interests

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highlight similarities and differences with the adventitial niche, the focus of this review. **(A) Epithelial boundary niches**: microbial-rich epithelial barriers which house the greatest number and diversity of immune cells, lying both within and beneath the epithelial cells [5]. **(B) Parenchymal niches**: interstitial spaces surrounding organ specific parenchymal cells; these sites predominantly contain tissue resident-macrophages, microvasculature, and interstitial fibroblasts [6,7]. **(C) Mesothelial boundary niches**: found at the surface of internal organs, including the heart, lung, and the linings of the abdominal cavity, where mesothelial cells form an epithelial-like monolayer and support body-cavity macrophages [8,9]. **(D) Adventitial boundary niches**: present at the external layer of larger blood vessels and similar structures [10]. Although these four subsets are not entirely discrete, and contain organ specific properties and sub-niches, they are a useful conceptual framework for understanding immune – tissue interactions.

Vascular adventitia structure and composition: *what is the city but the people?*

The blood vasculature is organized hierarchically into networks of arteries, arterioles, capillaries, venules, and veins (Fig. 2A). The walls of larger arteries and veins have three concentric structural layers. The tunica intima is comprised of endothelial cells that directly contact blood. The tunica media consists of concentric rings of vascular smooth muscle cells and elastic fibers, building a muscular layer that is innervated by neurons and regulates blood volume and pressure. The tunica externa or **adventitia** is layered with dense extracellular matrix (ECM) that acts as a mechanical scaffold, housing a community of interacting cell types (Fig. 2B). The adventitia includes the vasa vasorum, a microvasculature of its own that provides oxygen and nutrients to the vessel wall. It is also traversed by lymphatic vessels that provide routes of immune cell trafficking and interstitial fluid drainage, and systemic communication is facilitated by neurons [11–13]. Adventitial regions are enriched with a variety of immune cells, including subsets of innate lymphoid cells (ILCs), resident-memory T cells (TRMs), macrophages, and dendritic cells (DCs), and are major sites for immune surveillance at rest and during inflammation [14]. To understand the immunologic properties of the adventitia, we next review adventitial stromal cells, critical immunomodulators of these tissue immune outposts.

Stromal cells: *a rose by any other name*

Tissues contain differentiated parenchymal cells that perform the primary functions of an organ (*e.g.* epithelial cells in skin, adipocytes in fat, neurons in brain). Although long overlooked, the stromal compartment regulates and supports parenchymal cells, building the shape and compartmentalization of an organ. The stromal compartment consists of stromal cells (SCs) that modulate ECM composition and function, regulate local parenchymal and immune cells, and potentially serve as progenitor cells that can give rise to a variety of differentiated mesenchymal populations [15,16]. SCs are heterogeneous populations of non-epithelial, non-endothelial cells, and include overlapping subsets with names including mesenchymal stromal/stem cells (MSCs), fibroblastic reticular cells (FRCs), fibroblasts, myofibroblasts, cancer associated fibroblasts (CAFs), and pericytes. Single

cell transcriptomics and functional analyses have begun to characterize SC subpopulations that differ in their progenitor, immunomodulatory, and fibrogenic phenotypes [1,10,17–19]. However, the relationship between SC subsets location and function are only beginning to emerge. Here we group SCs by microanatomic location: capillary-associated (pericytes), body cavity-associated (capsular SCs), parenchymal-associated (parenchymal SCs) and large vessel-associated (adventitial stromal cells, ASCs). ASCs are predominantly fibroblast-like cells that are responsive to various local stimuli, including hypoxia, mechanical stretch, cytokines, and growth factors, organizing, remodeling, and repairing the adventitial environment [11]. ASCs from multiple tissues express platelet-derived growth factor receptor α (PDGFRα) and podoplanin (Gp38), with variable combinations of Sca-1, Gli-1, CD34, and IL-33 [10]. Besides fibroblast-like ASCs, some α-smooth muscle actin (α-SMA)-expressing myofibroblasts and pericytes ($PDGFR\beta^{+}$ $NG2^{+}$) lie around the adventitial vasa vasorum of the human saphenous vein [20] and around larger blood vessels of multiple organs [16], although their potential immunomodulatory properties are not well understood.

Adventitial – Immune cell interactions: *we happy few, we band of brothers*

Adventitial spaces include diverse immune cells at steady state and expand in the context of immune challenges that include allergy, infection, atherosclerosis and organ transplantation [4,14,21,22]. Here we review the adventitial cells and signals that regulate immune cell survival and activation at these sites.

ASCs and Interleukin-33

Stromal cells from multiple organs express IL-33 during homeostasis and inflammation, with a particular enrichment in ASCs [10,23–27]. IL-33 is a nuclear-localized cytokine in the IL-1 family and functions as an alarmin, released with cell damage or stress, and signals via the T1/ST2 receptor (IL1RL1). IL-33 contributes to tissue development, remodeling, and resolution of inflammation, but in excess promotes allergic diseases, fibrosis, and certain cancers [28,29]. The major targets of IL 33 are tissue resident lymphocytes associated with type 2 *allergic* immunity, including group 2 innate lymphoid cells (ILC2s), Th2 TRMs, and a subset of Tregs (Fig. 3A). However,additional immune targets express lower levels of the ST2 receptor and respond to IL-33, including subsets of macrophages, DCs, and other lymphocytes. ASCs in the lungs produce IL-33 and TSLP, another cytokine implicated in type 2 immunity, and regulate the accumulation and activation of ILC2s during helminth infection [10]. Subsets of Tregs are also enriched in adventitial regions [10,30], directly expanding in response to IL-33 and IL-33-driven, ILC2-derived signals such as ICOSL and OX40L [30,31]. The signals that promote ASC IL-33 production and release remain largely unknown, and future work will be required to define if ASCs are the relevant source of IL-33 in various settings of tissue health and disease.

Adipose tissue ASC – immune crosstalk

A well-studied tissue for SC-lymphocyte crosstalk is the visceral adipose tissue. Several recent studies used single cell sequencing and IL-33 reporter mice to identify adipose tissue SCs as major producers of IL-33, regulating ILC2s and Tregs to impact adipose metabolic function [25–27,32–34]. This data builds on prior work that defined IL-33 and

type 2 associated immune cells as protective elements in mouse models of obesity, insulin resistance, and type 2 diabetes [35]. Adipose IL-33^{+} SCs were predominantly located around blood vessels, neurons, and fat-associated lymphoid clusters, similar to ASCs found in the lung and elsewhere [10]. Besides IL-33, adipose ASCs were also enriched in the expression of multiple cytokines and collagens (e.g. *Il6, Tnfa, Col1a1, Col3a1*), similar to previously defined vascular associated-SCs [17,19,36]. Adipose IL-33^{+} SCs were sufficient to promote ILC2 IL-5 and IL-13 production and subsequent recruitment of eosinophils [10,25–27,32,33], acting in part via contact-dependent LFA-1/ICAM-1 interactions [26]. Subsets of the IL-33-expressing SC expressed cadherin-11 [32,33], and cadherin-11–deficient mice had increased SC production of IL-33, with concomitant ILC2 activation and M2 macrophage expansion that helped control adipose tissue inflammation [32]. Similar to ILC2s, adipose Tregs are positively regulated by IL-33 [25–27,32–34]. Both Tregs and IL-33^{+} SC subsets increase with age, and SC-derived IL-33 (PDGFRα^{cre}; IL-33^{flox}) promoted adipose Treg accumulation [27]. Although the mechanisms of IL 33 release by SC remain largely unknown, tissue injury, non-apoptotic cell death, and mechanical stress could be critical [29]. Intriguingly, both age, sex, and diet influenced SC subset composition, with IL-33^{-neg} SCs showing increased adipogenic potential, suggesting a functional compartmentalization between immunoregulatory and adipogenic SCs [27]. ASC – lymphocyte interactions are not a one-way street. IL-13 produced from ILC2s drives ASC Eotaxin-1 production, contributing to eosinophil accumulation [26]. Similarly, Tregs appear to feed-back to control SC composition and IL-33 expression, a negative regulatory loop lost with obesity [27]. Despite the evidence for adipose SC – lymphocyte crosstalk, the exact mechanisms by which these conversations regulate adipose tissue and systemic metabolic function are still being defined [35].

Adventitial macrophages

Although a majority of tissue macrophages reside in parenchymal niche sites (Fig. 1), recent work described adventitial macrophages (Lyve1^{+}) localized to murine aortas in mice and humans, expressing MMP-9 and regulating the steady-state arterial matrix content and aortic diameter [37]. Using single cell analysis and imaging, two populations of interstitial macrophages were recently defined in several tissues, with Lyve1^{low} MHCII^{high} cells lying near nerve bundles, and Lyve1^{+} MHC-II^{low} cells residing in perivascular spaces where they helped maintain blood vessel integrity and antifibrotic activity [38]. Similar perivascular interstitial macrophages were previously described in the brain, skin, heart, and adipose tissue, where they regulate both homeostasis and inflammation [39–43]. However, many of these studies did not clearly differentiate between macrophages associated with microvasculature versus the macrovascular adventitia areas. Further work is required to more precisely define interstitial macrophage subsets and their interactions in the adventitia.

Dendritic cells

DC subset(s) have recently been found to be enriched within the adventitial space [10,44]. The proximity to lymphatic vessels within this space may be preferential for allowing trafficking of DCs after antigen encounter to draining lymph nodes. ILC2s residing in the adventitial space can regulate DC function in type 2 inflammation, promoting DC migration to lymph nodes and expression of chemokines that promote the accumulation of Th2 TRM

in mouse models of allergic asthma [10,44,45]. Future studies may clarify how DCs are maintained and regulated within these spaces and how they may interact with ASCs.

Lymphatics

Lymphatic vessels are enriched within adventitial spaces, draining interstitial fluid, antigens, and providing a path for immune cell trafficking to lymph nodes and systemic circulation. Lymphatic endothelial cells produce IL-7 in both humans and mice [10,46] and support the survival of lung Th2 TRM cells in response to allergic challenge and Th17 TRM cells after bacterial infection [46,47]. Sustained IL-7 and IL-15 produced by SCs is essential for the maintenance of ILCs and NK cells, respectively, even after development [48,49]. As such, lymphatic production of IL-7, and possibly other signals such as IL-33, may be mechanisms by which adventitial areas preferentially support subsets of tissue-resident lymphocytes [10,46] (Fig. 3B).

Neurons

Another layer of adventitial control may be exerted via neurons. For example, ILC2-activity is both negatively and positively regulated by neuron-derived signals (*e.g.* VIP, NMU, CGRP, catecholamines) in mouse models of helminth-driven and allergic inflammation [50–54] (Fig. 3C). Multiple neuronal subsets, including sensory, cholinergic, and catecholaminergic neurons, are enriched within the adventitial space, suggesting neuronal-lymphocyte crosstalk is prominent at these regions [21].

Adventitial remodeling with inflammation: *presume not that I am the thing I was*

During inflammation, adventitial cells respond to local aberrations and recruit immune cells, resulting in a temporary remodeling of the adventitial space. In chronic inflammatory settings, the adventitia expands and becomes a coordinating center for specialized stromal - immune cell interactions which can support TRM T cells and organize into lymphoid tissue-like aggregates called tertiary lymphoid organs (TLO). Next we review how ASCs are major regulators of these inflammatory changes.

Immune trafficking to the adventitial niche

ASCs express a range of chemokines such as Cxcl1, Cxcl12, Ccl2, Ccl7, Ccl8, Ccl11, and Ccl19, and could influence immune trafficking or positioning in the adventitia [10]. Several of these chemokines can potentially recruit neutrophils, monocytes, and eosinophils, or control DC trafficking from tissues to lymph nodes [55]. In additional to myeloid cell recruitment, CCL8 promotes IL-5^+ Th2 cell homing to mouse skin during allergic inflammation [56] as well as promoting IL-33 induced ILC2 motility within lung adventitial spaces [57]. As such, CCL8 may constitute one ASC-derived signal that promotes accumulation of ILC2s, Th2 TRM cells, and Treg subsets to adventitial spaces [10,58]. Although ASCs actively regulate adventitial immune composition, many of the precise signals and contributions are not well defined, nor it is clear how ASCs cooperate with macrophages that also produce similar chemokines.

Resident memory T-cells

Tissue-resident memory T cells (TRM cells) are generated after antigen exposure, residing in tissues with limited recirculation, and subsets of TRMs are enriched in adventitial spaces. In human brain, both CD4 and CD8 TRMs were found in close proximity to blood vessels, and immunofluorescence staining with laminin revealed they predominantly reside in the perivascular space [59]. Th2 TRMs reside in adventitial spaces in close proximity to SCs and are dependent on SC-derived IL-33 [10]. Th17 TRMs generated after lung bacterial infection may also be enriched at adventitial sites [47]. A more complete catalog of discrete 'flavors' of adventitial-resident lymphocytes is required, including the signals that regulate lymphocytic homing, survival and function.

Tertiary Lymphoid Organs (TLOs)

TLOs are induced lymph node-like structures in tissues, where T-cell TRMs and B-cells aggregate upon infection or damage [60]. TLOs, also called tertiary lymphoid structures, are shaped by specialized SCs that are induced by inflammatory cues. TLOs preferentially form in adventitial sites, remaining long after the inflammation has been resolved. During viral infection of the salivary gland, IL-13 acted on podoplanin$^+$ SCs to upregulate VCAM-1 and promote TLO formation [61]. In *Pneumocystis* infection, IL-13 induced CXCL13 production by pulmonary SCs to drive TLO formation [62]. As IL-13$^+$ ILC2s are enriched in adventitial spaces, these data support previous studies suggesting a role for ILC2s, and likely other adventitial immune cells and signals, in promoting TLOs [60]. TLOs can have a protective function, promoting more efficient responses to secondary exposures, but have also been associated with a variety of inflammatory diseases in humans and mice [60]. TLOs include a spectrum of structures that contain SCs, lymphatics, and tissue-resident lymphocytes, and future studies may better elucidate TLO formation, function, and dissipation.

Conclusions and future directions: *how far that little candle throws his beams*

Tissue-resident immune cells are organized into distinct niches, with potential advantages and limitations that we are beginning to appreciate. In the adventitial niche, locating immune cells near blood and lymphatic vessels allows for efficient surveillance of antigens or environmental perturbations, promoting regional immune responses while limiting the potential for more expansive tissue destruction. ASCs likely recruit and support diverse adventitial immune cells to coordinate these regional responses, but unknowns abound. What additional adventitial cells, cytokines, or signals engage in immune crosstalk? What are the upstream cues that regulate ASC function? How do adventitial spaces develop in early life, potentially shaping life-long immunity? How does immune cell mis-localization outside of adventitial niches impact tissue health and disease? Characterizing the complex network of adventitial-immune crosstalk in healthy mice and humans will help define the impact of adventitial remodeling during inflammation and in chronic diseases such as allergic asthma and fibrosis.

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Highlights

- Adventitial perivascular niches contain specialized stromal and immune cells
- Tissue-resident type 2 and regulatory lymphocytes are enriched in the adventitia
- Adventitia contain lymphatics, neurons, and fibroblast-like stromal cells
- Adventitial stromal cells are critical tissue immunomodulators
- Adventitial - immune crosstalk impacts tissue physiology and inflammatory response

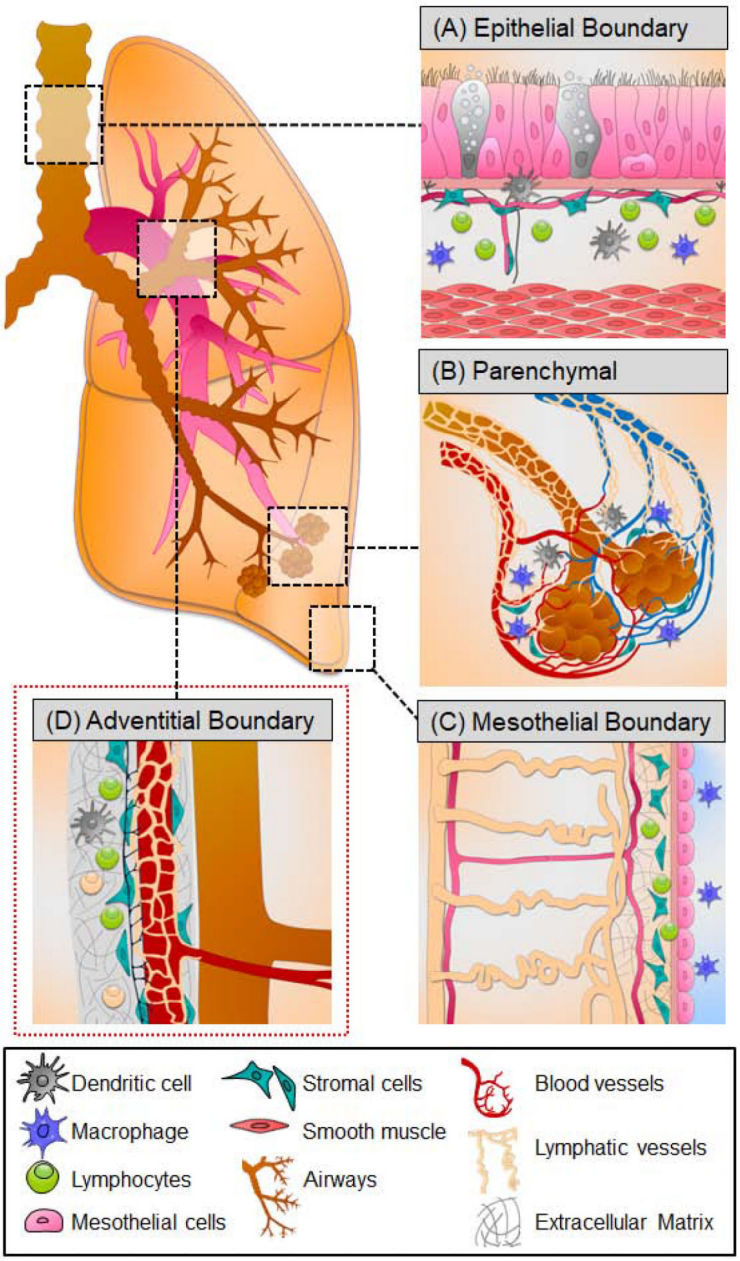

Figure 1: Immune cell niches of the respiratory tract.

Tissue immune cell niches categorized into four major classes. **(A) Epithelial:** Epithelial cells line the airways of the upper airways and lungs and immune surveillance occurs by immune cells that lie within or below epithelial layers. **(B) Parenchymal:** The interstitial space around the parenchymal cells, the lung alveoli harbor alveolar macrophages which are responsible for clearing the air spaces. **(C) Mesothelial:** Mesothelial cells form a layer, separating the lung from the pleural cavity. Lymphocytes lie beneath the mesothelial cells, and large cavity macrophages which lie within the pleural cavity are supported by mesothelial cells. **(D) Adventitial:** The adventitia supports large vessels and lung airways, providing a dynamic hub for multiple immune cells. Stromal cells reside in all the tissue

niches and aid in structural support and maintenance of the immune cells that reside in these niches.

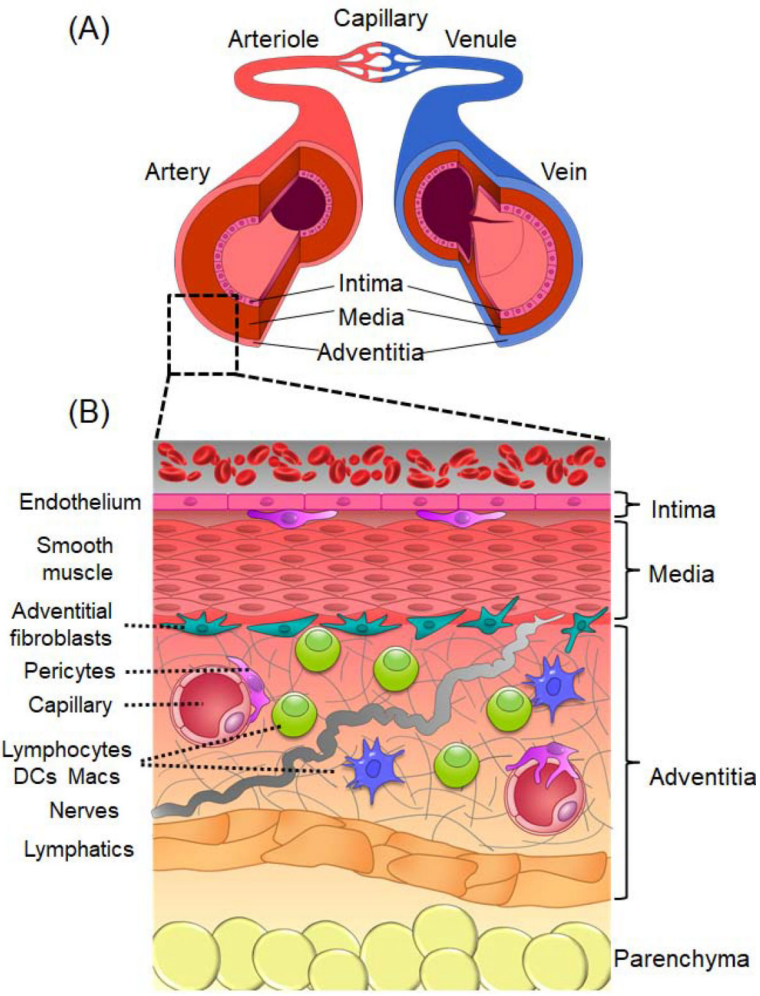

Figure 2: The adventitia provides scaffolding for blood vessels and a home to a variety of immune cells.

(A) The walls of large arteries and veins consist of three layers: the innermost tunica intima, the tunica media and the outermost tunica adventitia. **(B)** The tunica adventitia provides structural support for the vessel and is the bridge between the vasculature and surrounding tissue (parenchyma). The adventitia consists of dense connective tissue made up of collagen-rich extracellular matrix. It has its own microvasculature, is innervated, and enriched with lymphatic vessels, and provides a home for immune cells to receive supporting, activating and regulatory signals. These immune cells are supported by stromal cells including adventitial fibroblasts and pericytes.

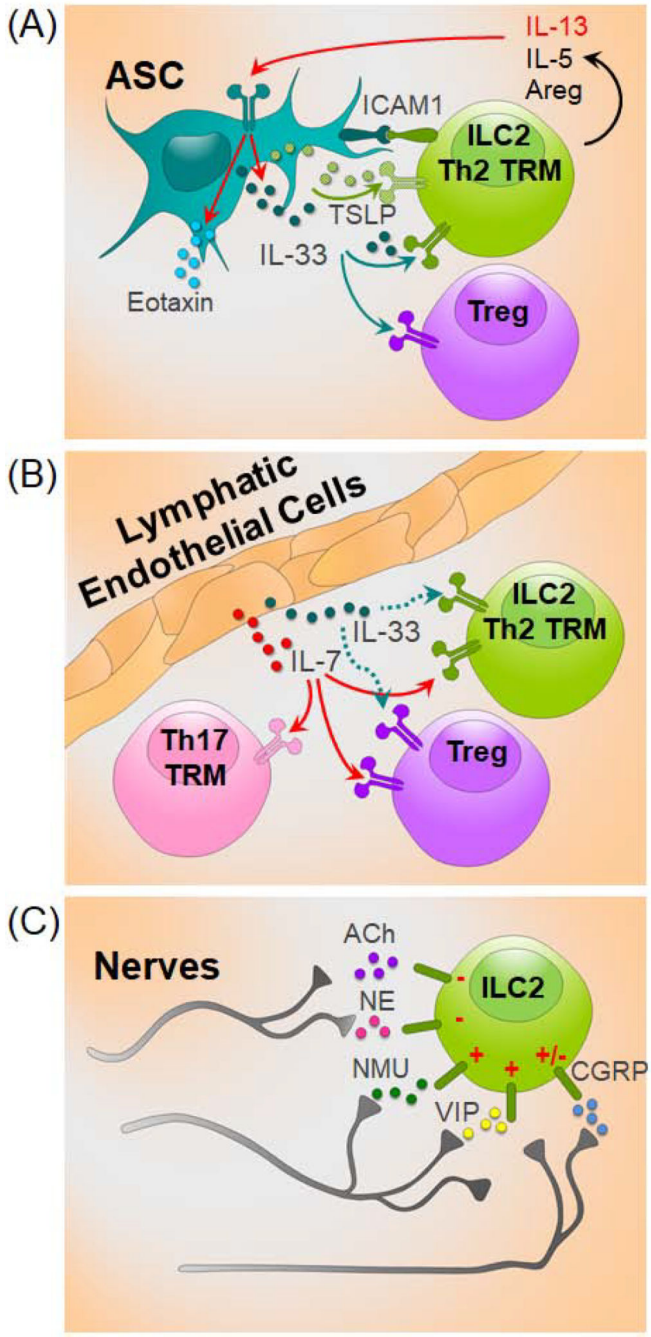

Figure 3: Stromal cell - immune cell crosstalk in adventitial spaces.

Immune cells receive signals from the stromal compartment within adventitial spaces for survival, activation and positioning. **(A)** Adventitial stromal cell (ASC)-derived IL-33 support ILC2s, Th2 cells and subsets of Tregs. ASCs can also support ILC2 function through ICAM-1/LFA-1 interactions and TSLP secretion. In a reciprocal manner, activated ILC2s release IL-13, and potentially additional signals (*i.e.* IL-5, Areg, Csf2), that feedback on ASCs to produce more IL-33 and Eotaxin-1. **(B)** Lymphatic endothelial cells are another potential source of IL-33 and provide the survival signal IL-7 for ILC2s, Th2 TRM

cells, Tregs, and other TRM cells. **(C)** Adventitial neurons can release neuropeptides and neurotransmitters which inhibit (NE, AcH), activate (NMU, VIP) or regulate (CGRP) ILC2 function. AcH: acetylcholine; NE: norepinephrine; β2AR: β2-adrenergic receptor; NMU: neuromedin U; VIP: vasoactive intestinal peptide; CGRP: calcitonin gene-related peptide.