J Physiol 602.19 (2024) pp 4789–4802

The Journal of Physiology

REVIEW-SYMPOSIUM

Neuroimmune modulation for targeting organ damage in hypertension and atherosclerosis

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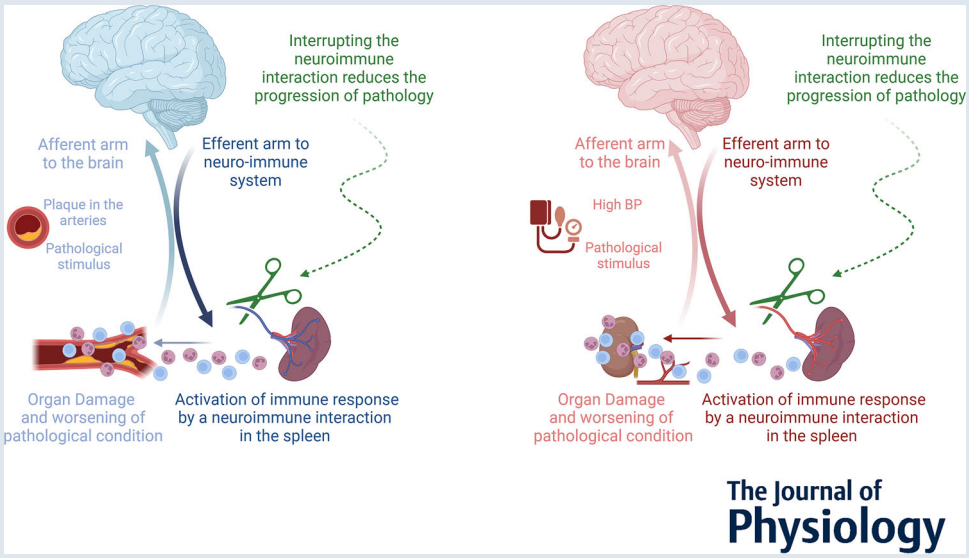
Handling Editors: Laura Bennet & Daniel Zoccal

The peer review history is available in the Supporting Information section of this article (https://doi.org/10.1113/JP284078#support-information-section).



Abstract The brain is essential for processing and integrating sensory signals coming from peripheral tissues. Conversely, the autonomic nervous system regulated by brain centres modulates the immune responses involved in the genesis and progression of cardiovascular diseases. Understanding the pathophysiological bases of this relationship established between the brain

Marialuisa Perrotta recently completed post doctoral training in the neurobiological bases of neuro-immune modulation of hypertension. In 2022, Dr Perrotta gained a position of assistant professor to start her own research activity, focused on the identification of brain areas involved in the immune response recruited by cardiovascular disease. **Daniela Carnevale** received her PhD in Neuroscience at the Italian Institute of Health and Catholic Sacred Heart University in Rome. In 2012, she started her academic career as an assistant professor at the faculty of Pharmacy and Medicine of Sapienza University at IRCCS Neuromed, where she established her lab after having won the ERC Starting Grant by the European Committee in 2017. As a full professor with tenure from 2020, she has received prestigious career awards. Her research focuses on how the nervous system modulates the immune response involved in cardiovascular diseases.

This review was presented as a plenary lecture at the international society for autonomic neuroscience (ISAN) meeting, which took place in Cairns, Australia, on 4–7 September 2022.

DOI: 10.1113/JP284078

and immune system is relevant for advancing therapies. An additional mechanism involved in the regulation of cardiovascular function is provided by the brain-mediated control of the renin–angiotensin system. In both cases, the communication is typically bidirectional and established by afferent and sensory signals collected at the level of peripheral tissues, efferent circuits, as well as of hormones. Understanding how the brain mediates the bidirectional communication and how the immune system participates in this process is object of intense investigation. This review examines key findings that support a role for these interactions in the pathogenesis of major vascular diseases that are characterized by a consistent alteration of the immune response, such as hypertension and atherosclerosis. In addition, we provide a critical appraisal of the translational implications that these discoveries have in the clinical setting where an effective management of neuroimmune and/or neuroinflammatory state might be beneficial.

(Received 10 May 2023; accepted after revision 27 August 2024; first published online 19 September 2024)
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Abstract figure legend Targeting the brain-to-spleen connection to reduce vascular damage in atherosclerosis and hypertension. The afferent and efferent arms of the circuit activated by pathological changes in atherosclerotic arteries are indicated in blue. The afferent and efferent arms of the circuit activated by hypertensive challenges are indicated in red. These circuits have two important hubs: one in the brain, where the pathological stimulus is perceived in specific areas that in turn activate peripheral autonomic efferent, and the second in the spleen, which activates egress of lymphocytes for subsequent infiltration in target organs. Created with BioRender.com.

The brain is an integrative station of afferent and sensory signals in hypertension and atherosclerosis

Recent work highlighted that hypertension and atherosclerosis share neural circuits involved in the regulation of peripheral immune responses, as well as vascular damage and dysfunction. Here, we discuss the commonalities and differences of these two frequent vascular diseases.

Brain signals in atherosclerosis. Atherosclerosis is characterized by plaque formation in the inner intimal layer of arteries. Mohanta et al. (2022) recently found axons and neuronal terminations in the adventitia of atherosclerotic arteries. Their study has shown that nerve endings emerged in the artery tertiary lymphoid organs (ATLOs) that aggregated around the adventitia of aged atherosclerotic apolipoprotein E knockout ($Apoe^{-/-}$) mice (Hu et al., 2015). These neuroimmune formations contain sensory and sympathetic fibres interacting with components of immune and vascular systems. Neurotropic retrogradely-migrating pseudorabies viruses (PRVs), belonging to the Bartha strain, were used to map the neural connections established between the adventitia and the central nervous system during the onset and progression of atherosclerosis (Mohanta et al., 2022). PRVs were injected into ATLOs and their expression was tracked back to the brain, starting from the dorsal root ganglion and then in distinct spinal cord and brain nuclei, including the intermediolateral neurons in the grey column of the spinal cord, the medullary neurons in the raphe pallidus nucleus, the gigantocellular reticular nucleus-alpha, the lateral paragigantocellular nucleus and the paraventricular hypothalamic nucleus (PVN) (Mohanta et al., 2022). Interestingly, the retrograde tracing also identified a brain network including the central nucleus of the amygdala, the rostral ventrolateral medulla (RVLM), the locus coeruleus and the dorsal motor nucleus of the vagus (DMV-10N) (Mohanta et al., 2022). Specifically, ensembles of neurons in the DMV-10N and in the medullary neurons of the raphe pallidus nucleus were stained by choline acetyltransferase, and hence were defined as cholinergic. Other neurons in the PVN and locus coeruleus were positively stained by tyrosine hydroxylase, and hence were defined as catecholaminergic. Collectively, the two neuronal ensembles regulate the activity of the parasympathetic and sympathetic nervous systems, respectively (Guyenet, 2006; Saper, 2002). When we analysed the efferent circuit activated in these brain regions, we identified an enhanced celiac vagus nerve activity that correlated with a similarly enhanced splenic nerve activity, comprising the parasympathetic and sympathetic nerves, respectively. The interaction between these parasympathetic and sympathetic nerves depended on an intact celiac ganglion (CG) innervation. Furthermore, this neural circuit was associated with the remodelling of innervations within the ATLOs of aged $Apoe^{-/-}$ mice, suggesting a role in sustaining

disease progression (Mohanta et al., 2022). To verify this last hypothesis, mice were denervated at the level of the CG and pathology's progression was monitored. In the absence of an intact CG, Apoe$^{-/-}$ mice showed collapsed ATLOs, reduced atherosclerosis progression and improved plaque stability (Mohanta et al., 2022). It is conceivable that an intervention to manipulate this pathway might have therapeutic implication in the attenuation of atherosclerosis.

Brain signals in hypertension. On a different note, earlier studies conducted in models of hypertension indicated that specific brain regions surrounding the third ventricle were involved in blood pressure regulation because their destruction prevented infiltration of T cells in target organs and blood pressure increase in response to angiotensin II (AngII) (Marvar et al., 2010). The subfornical organ (SFO), the organum vasculosum of the lamina terminalis (OVLT) and the area postrema are brain nuclei lining around the cerebral ventricles that are globally defined as circumventricular organs (CVOs). These neuronal ensembles have a leaky blood–brain barrier (BBB) and are the principal sensors of circulating substances such as AngII (Akhavanpoor et al., 2018; Ferguson & Bains, 1997; McKinley et al., 1998). These unique areas are enriched of AngII type 1a receptors (AT1aR) expressed by neurons, microglial cells and perivascular macrophages (Elsaafien et al., 2020; Faraco et al., 2016). Interestingly, Faraco et al. (2016) demonstrated that low doses of AngII inducing a moderate blood pressure increase, disrupted the BBB, allowing the blood-borne AngII to reach the perivascular space. To demonstrate that blood-borne AngII can entry the brain, they administered biotinylated AngII, 14 days after AngII infusion by osmotic minipumps. By confocal microscopy, the it was found that the labelled AngII peptide was in the proximity of the perivascular macrophages in the neurovascular unit. Clodronate liposomes mediated the depletion of perivascular macrophages and restored the neurovascular unit integrity. A similar effect was obtained by deleting the AT1aR in perivascular macrophages, suggesting that AT1aR-dependent effect of blood-borne AngII in the brain might depend on perivascular macrophages (Faraco et al., 2016). Another study showed that blood-borne AngII crossed the BBB, extravasating into the hypothalamus and the brainstem (Biancardi et al., 2014). Using fluorescent AngII, Biancardi et al. (2014) observed that the BBB permeability to AngII was increased in the PVN, in the nucleus of the solitary tract (NTS) and in the RVLM of spontaneously hypertensive rats. In particular, extravasated AngII was found in the proximity of neurons and microglial cells expressing AT1aR, highlighting a role for AT1aR in mediating the hypertensive effect of blood-borne AngII in the brain (Biancardi et al., 2014). To confirm this finding, Biancardi et al. (2014) showed that the AT1aR antagonists losartan prevented BBB disruption.

AT1aR localization and function in the cardiovascular system. Previously discussed evidence supports the crucial role for brain AT1R in maintaining cardiovascular physiology under normal physiological conditions. Higher levels of AT1R mRNA in the RVLM and in the NTS of hypertensive rats were found compared to normotensive controls (Song et al., 1994). Interestingly, using AT1aR-tdTomato reporter mice localized AT1aR in brain regions involved in the control of cardiovascular function, de Kloet et al. (2017). They showed that, under either baseline or hypertensive conditions, AT1aR overlapped with the neuronal marker HuC/D. In addition, in normotensive mice, the fluorescence of AT1aR in the PVN did not overlap microglia and astrocytes. In addition, in deoxycorticosterone (DOCA)-salt-treated hypertensive mice, there was no overlap between AT1aR-tdTomato fluorescence and microglial or astrocyte markers in the PVN, supporting the evidence that AT1aR are primarily expressed in neurons. Similar observations were found in brain nuclei around the cerebral ventricles such as the median preoptic nucleus (MnPO), the nucleus arcuate (ARC), SFO, area postrema and NTS, as well as the OVLT (Kinsman et al., 2020; Sumners et al., 2020). Brain regions expressing AT1aR, mainly localized around the cerebral ventricles, as those mentioned above, are functionally and structurally interconnected to form neural circuits activated by hypertensive challenges (McKinley et al., 2003). As an example, de Kloet et al. (2017) demonstrated that AT1aR in the PVN are specifically expressed by corticotropin releasing hormone (CRH) neurons that project to the median eminence. This neuronal population is involved in the co-ordination of neuroendocrine, cardiovascular and behavioural responses to stress (de Kloet et al., 2017). In theor work, it was shown that AT1aR in the PVN are not localized on pre-autonomic neurons but on neurosecretory neurons, suggesting that the activation of sympathetic outflow could be mediated by the interplay of other brain stations or by an internal neuronal circuitry of the PVN (de Kloet et al., 2017). Another study from the same group, utilizing fluorescence *in situ* hybridization to detect the vesicular glutamate transporter 2, which marks glutamatergic neurons, revealed that AT1aRs are predominantly expressed in glutamatergic neurons of the MnPO and the OVLT sending projections to brain regions involved in body fluid homeostasis and blood pressure regulation (Frazier et al., 2021). Overall, AT1R are predominantly expressed in neurons, particularly in glutamatergic neurons, that are able to influence other neurons in the centres of

cardiovascular control to mediate sympathetic responses and blood pressure increase (de Kloet et al., 2017).

PVN–SFO connections. Previous studies suggested the existence of connections between the SFO and the PVN established by angiotensinergic (or at least neurons responsive to AngII) projections (Li & Ferguson, 1993a, b). Although several observations support the notion that the PVN is influenced by AngII through SFO neurons, definitive evidence of a direct connection is still missing. Nonetheless, there are data in the literature that support this concept. For example, Tanaka et al. (2001) demonstrated that microinjections of AngII in the SFO provoked the release of noradrenalin from the PVN mediating drinking responses. In addition, a direct injection of the AngII antagonist saralasin or the alpha-adrenergic blocker phentolamine bilaterally in the PVN blocked the drinking behaviour, suggesting that both angiotensin and noradrenalin in the PVN participated to organize drinking responses (Tanaka et al., 2001). Considering that the PVN is located near the ventricular system, it is possible that micro-injections of the two drugs leaked into the CVO, hence reaching the SFO neurons where AngII mediates its effects. The PVN also contains neuronal populations that are involved in the control of stress circuits releasing adrenocorticotrophin hormone (ACTH) and cortico-sterone hormone (CORT). These neurons are regulated through the hypothalamic–pituitary–adrenal (HPA) axis (Baghai et al., 2002). Krause et al. (2011) demonstrated that, after injecting an anterograde neurotracer into the core of the SFO, there was a high expression of the tracer in the proximity of the parvocellular neurons of the PVN releasing ACTH and CORT. These neurons were positive for cFOS antibody, a marker of neuronal activation, during restrained stress in rats (Krause et al., 2011). These data highlight that SFO neurons project directly into neurons of the PVN activating in response to acute stress. In their work, with the aim of demonstrating whether AngII activates HPA axis by AT1aR during stress, Krause et al. (2011) injected a lentiviral vector directly into the SFO to delete AT1aR in this nucleus, observing decreased anxiety behaviour, concomitantly with a significant reduction of ACTH and CORT hormones in restrained stressed rats compared to control animals. These data demonstrate that the expression of AT1aR in neurons of the SFO was necessary to mediate the responses of PVN neurons to release stress hormones and regulate the HPA axis (Krause et al., 2011; Li & Ferguson, 1993a) Consistent with these findings, the stimulation of the SFO by AngII led to the activation of neural signals in the medial parvocellular neurons of the PVN (Donevan & Ferguson, 1988; Plotsky et al., 1988), further supporting the evidence of a SFO–PVN connection mediating the brain effects of AngII (Krause et al., 2011).

PVN-MnPO/OVLT connections. More recently, a connection from the lamina terminalis to the PVN, mediated by AngII and evoking arginine vasopressin (AVP) secretion, was identified as being responsible for a transient blood pressure increase in mice (Frazier et al., 2021). An elegant experiment was performed to identify the projections of MnPO/OVLT neurons expressing AT1aR. The delivery of an anterograde Cre-dependent neuronal adeno-associated viruses (AAV) expressing enhanced yellow fluorescent protein into the MnPO/OVLT of Agtr1a-Cre mice, led to visualization of enhanced yellow fluorescent protein in fibres projecting into the SFO in the bed nucleus of the stria terminalis, in the PVN, in the supraoptic nucleus, in the dorsomedial hypothalamus and in the commissural NTS (Frazier et al., 2021). Neurons projecting to the PVN were in the proximity of neurons that synthesize AVP, suggesting that this connection modulates the release of AVP in the circulation. Notably, *in vivo* optogenetic stimulation of PVN fibres originating from AT1aR-expressing neurons in CVO, increased blood pressure in Agtr1a-Cre mice with AAV-channel rhodopsin 2 administered into the MnPO/OVLT, confirming the importance of this glutamatergic connection for blood pressure regulation (Frazier et al., 2021). Other studies demonstrated that interrupting the glutamatergic projection from the MnPO to the PVN or inhibiting transmission using a selective knockdown of AT1aR in MnPO prevented the development of hypertension (Marciante et al., 2020; Shell et al., 2019). Interestingly, the parvocellular neurons of the PVN are those that exhibit pre-autonomic functions, projecting to the spinal cord by passing through the RVLM (Luther et al., 2002). A study demonstrated that pre-autonomic neurons in the PVN are of different morphology and are distinguished into three different classes that share the same characteristic regarding the expression of a low-threshold spike, as commonly observed in neurons projecting to the RVLM and controlling sympathetic drive (Luther et al., 2002). This property differentiated the parvocellular neurons from the magnocellular neurons also involved in releasing AVP (Stern et al., 2003). Nonetheless, the direct stimulation of glutamatergic AT1aR neurons in the MnPO/OVLT by an optogenetic approach failed to elicit EPSCs in parvocellular neurons of the PVN (Frazier et al., 2021). By delivering AAV-channel rhodopsin 2 into the MnPO/OVLT of Agtr1a-Cre mice and concomitantly administrating a retrograde neurotracer into the RVLM, Frazier et al. (2021) identified pre-autonomic neurons in the PVN, for which the majority exhibited the expected low threshold spikes, definitively indicating

that AT1aR neurons in the MnPO/OVLT send excitatory glutamatergic projections to the vasopressinergic neurons of the PVN that, in turn, release AVP into the systemic circulation, resulting in an increase in blood pressure levels. However, the optogenetic stimulation of AT1aR neurons in the MnPO/OVLT did not elicit EPSCs in parvocellular neurons, suggesting that glutamatergic projections did not directly synapse with pre-autonomic PVN neurons (Frazier et al., 2021). This result was partly unexpected but still in line with other evidence demonstrating the involvement of other brain nuclei in the regulation of sympathetic nerve activity in the cardiovascular system control. Indeed, an alternative pathway underling the sympatho-excitatory action of central AT1aR was promoted by the SFO, the main nucleus with excitatory projections into pre-autonomic PVN neurons (Llewellyn et al., 2012). AngII might activate pre-synaptic AT1aR to reduce GABA release, thereby disinhibiting pre-autonomic PVN neurons (Li & Pan, 2007, 2010). Overall, the PVN modulates peripheral sympathetic responses through direct projections to the intermediolateral neurons or indirect connections with the RVLM (Guyenet et al., 2013). Hormones such as AngII or AVP or oxytocin can participate in the neural modulation controlling the peripheral responses of the autonomic nervous system (Brown et al., 2020).

PVN–NTS connections. The pre-sympathetic neurons of PVN are also influenced by the afferent projections from the NTS (Affleck et al., 2012). Importantly, the cardiovascular afferents terminated within the dorsomedial part of the NTS, whereas the pre-sympathetic neurons of the PVN received this cardiovascular information via afferent projections from the NTS (Affleck et al., 2012). NTS projections were glutamatergic and terminated in the proximity of GABA neurons around the pre-sympathetic and magnocellular neurons of the PVN (Affleck et al., 2012). This work demonstrated that PVN neurons were influenced not only by upstream nuclei such as the SFO, the MnPO or the OVLT, but also by downstream nuclei of the brainstem in the regulation of cardiovascular system responses.

The routes of communications between the brain and peripheral organs in atherosclerosis and hypertension

Vagus–splenic axis. The progressive understanding of the mechanisms underlying neuroimmune interactions led to define disease's specific contexts where the peripheral nervous system establishes interfaces with immune cells. Notably, the sympathetic nervous system exerts key functions with respect to homeostasis of the cardiovascular system. Alterations of this system contribute to the onset of cardiovascular pathology and development of end-organ damage (Esler, 2015; Guyenet, 2006). We found that the vagus-celiac ganglion-splenic axis anatomically and functionally connects the brain to the splenic immune system (Carnevale et al., 2016; Carnevale et al., 2020; Kressel et al., 2020). In the context of atherosclerosis, celiac vagus nerve and splenic nerve activities, as measured by microneurography, increased with disease progression, indicating a correlation between neuronal activity, plaque formation and consequent inflammation (Mohanta et al., 2022). Interestingly, earlier data from our group showed that the same coupling of vagus–splenic electrophysiological activity is recruited by hypertensive stimuli (Carnevale et al., 2016), providing a support for the notion that atherosclerosis and hypertension might share common efferent neural circuits modulating immunity. We also observed that interrupting the splenic sympathetic drive by removal of the celiac ganglion or by denervation of the splenic artery prevented the development of hypertension in mice and protected them from T cell mobilization and infiltration in target tissues. The splenic sympathetic fibres comprise the link between neural circuits controlling cardiovascular and autonomic responses and immune cells in the spleen (Felten & Olschowka, 1987; Lori et al., 2017). One of the pioneer studies demonstrating the effect of AngII into the brain showed that I.C.V. injection of AngII increased the electrophysiological activity of the splenic sympathetic nerve and altered splenic cytokine gene expression in rats (Ganta et al., 2005). Interestingly, denervation of the splenic artery reduced the upregulation of splenic cytokines, demonstrating a direct link between brain AngII, the splenic nerve and splenic immunity (Ganta et al., 2005). Previous studies demonstrated that the vagus nerve was the primary mediator of neural circuits modulating the inflammatory response through the splenic nerve (Borovikova et al., 2000; Martelli et al., 2014; McAllen et al., 2022; Rosas-Ballina et al., 2011). Efferent vagus nerve fibres terminating in the celiac-superior mesenteric ganglion formed varicosity with cell bodies of sympathetic neurons innervating the spleen (Kressel et al., 2020). A selective optogenetic approach activating the DMV-10N or electrical activation of the cervical vagus nerve evoked action potentials in the splenic nerve (Kressel et al., 2020). Hence, the vagal efferents suppressed cytokine release from macrophages in the spleen, reducing peripheral inflammation (Rosas-Ballina et al., 2011). More importantly, bioelectronic stimulation of the celiac vagus nerve similarly activated the splenic nerve firing and promoted the egress of T CD8 effector cells (Carnevale et al., 2020). Indeed, bioelectronic stimulation of the celiac vagus nerve or the thermo-ablation of splenic fibres passing around the splenic artery might be investigated further as two innovative translational approaches modulating T lymphocyte activation and deployment toward target organs (Carnevale et al., 2016). This

approach has a high translational potential, suggesting new clinical strategies for resistant hypertension.

Central effects of AngII modulate the peripheral sympathetic outflow. The central nervous system and the immune system interact mutually in physiology and disease. Factors such as AngII, known to impact on both systems, might play multiple roles in hypertension (Perrotta et al., 2018a). An increase in circulating AngII can also cause the activation of proinflammatory cytokines in the circulation, leading to neuroinflammation. These proinflammatory cytokines penetrate the BBB at the level of the SFO (Wei et al., 2013). Interestingly, the intracarotid artery injection of tumour necrosis factor alpha (TNF-α) or interleukin 1 beta (IL-1β) elicited sympathetic responses and increased blood pressure in rats with an intact SFO, but not in rats with lesioned SFO (Wei et al., 2013). The evidence of a direct cellular connection between TNF-α and SFO neurons was demonstrated by an elegant experiment in which acute and chronic exposure to TNF-α modulated the electrophysiological activity of the sodium current in neurons of the SFO (Simpson & Ferguson, 2017). Once activated, neurons of the SFO enhanced the firing of autonomic neurons of the PVN to modulate blood pressure and efferent sympathetic outflow (Anderson et al., 2001). Another study showed that the administration of TNF-α directly into the SFO upregulated the expression of AT1R in both the SFO and the PVN and also increased renal sympathetic nerve activity (Wei et al., 2015), suggesting that TNF-α is important for mediating the effect of AngII in the activation of the efferent sympathetic outflow (Sriramula et al., 2008; Ufnal et al., 2006; Wei et al., 2015; Zera et al., 2008). The PVN consists of several types of neurons, with some of them projecting to the spinal cord and target tissues of the cardiovascular system with post-ganglionic neurons. Thus, the effect of proinflammatory cytokines in the SFO, which is directly connected with the PVN, is associated with an increase in the excitability of PVN neurons that in turn enhance the sympathetic nerve activity (Wei et al., 2015). AngII was also investigated as a neurotransmitter in the brain regions that control central autonomic pathways (Ferguson & Washburn, 1998). Moreover, it is also important to consider that circulating AngII plays an important role in sensitizing the SFO to circulating pro-inflammatory cytokines and vice versa. Other experimental studies also suggested that circulating AngII affects sympatho-excitation through combined effects of the SFO and the carotid body. In a model of intermittent hypoxia, increased sympathetic activity was hampered by systemic infusion of the AT1aR blocker losartan, demonstrating an underlying AngII-AT1aR signalling mechanism (Kim et al., 2018). Moreover, to demonstrate that these effects were brain-mediated, pharmacological inhibition of the SFO signalling was carried out using a GABAa receptor agonist directly injected in the SFO of rats with intermittent hypoxia. Although the sympathetic activity was reduced, the chemoreflex was unaltered as a result of an intact carotid body (Kim et al., 2018). Interestingly, both the SFO inhibition and carotid body denervation reduced the AngII effects on sympathetic drive under a condition of intermittent hypoxia. These data suggested that the carotid body also contributed to the central effects of AngII in mediating the sympathetic outflow in the periphery (Kim et al., 2018). Indeed, by autoradiography, it was possible to observe that the carotid body densely expressed AngII receptors, and thus is considered as a sensor of circulating AngII (Allen, 1998). In addition to AngII receptors, the carotid body expressed TNF-α receptor type I, which has the ability to sense TNF-α and other cytokines (Katayama et al., 2022). Systemic TNF-α administration had direct effects on carotid body afferents that in turn activated glutamatergic neurons in the NTS projecting to the RVLM to mediate the activation of splanchnic nerves (Katayama et al., 2022).

AngII effects on peripheral sympathetic nervous system. Other effects of circulating AngII on the sympathetic outflow are mediated by AT1aR expressed on sympathetic preganglionic neurons. Direct stimulation of AT1aR on sympathetic ganglia enhanced the electrophysiological activity of sympathetic neurons, increasing catecholamine release (Dendorfer et al., 2002). Further supporting this concept, an earlier work demonstrated that intrathecal injections of AngII caused a significant increase in blood pressure and sympathetic activity with an excitatory action on sympathetic neurons directly in the spinal cord of rats (Suter & Coote, 1987). The exact position of the catheter tip to inject AngII, aiming to obtain a change in blood pressure and sympathetic activity, was within the subarachnoid space T9–T11. To confirm this finding, the intrathecal injection of a competitive AngII receptor antagonist with partial agonistic activity saralasin was performed with positioning of the catheter tip as for AngII. It was found that the antagonist injection blocked these effects (Suter & Coote, 1987). Overall, these data demonstrated that AngII modulates peripheral sympathetic outflow through direct and indirect actions.

The neuro-immune interactions in hypertension and atherosclerosis

The peripheral nervous system mediates the bidirectional interaction established between the brain and immune cells in lymphoid and non-lymphoid organs, under physiological conditions and during disease (Abe et al., 2017). Immune cells express different subtypes of neural

receptors to respond to various neurotransmitters released in immune organs by peripheral sensory, sympathetic and parasympathetic neurons (Carnevale, 2022). As a primary lymphoid organ, the thymus is mainly innervated by noradrenergic nerves, whereas the bone marrow receives fibres from the sympathetic, parasympathetic and sensory systems. The spleen and the lymph nodes are secondary lymphoid organs mainly innervated by noradrenergic nerves. Tertiary lymphoid organs have a complex composition of cells, and they appear exclusively during pathology in the proximity of the affected tissue. They show neural networks, comprising sympathetic, parasympathetic and peptidergic neural fibres (Carnevale, 2022). Immune cells are regulated by the sympathetic nervous system (Bellinger et al., 1992; Bellinger et al., 2008; Udit et al., 2022), which predominantly releases noradrenaline in lymphoid tissues (Nance & Sanders, 2007). As an example, splenic immune cells bind both α and β-adrenergic ligands (Bidart et al., 1983; Rosas-Ballina et al., 2011).

The spleen in hypertension. Our previous observations demonstrated that AngII and DOCA-salt challenges induce the release of an angiogenic growth factor called placental growth factor (PlGF) in the spleen (Carnevale et al., 2014; Carnevale et al., 2016; Perrotta et al., 2018b). Mice lacking PlGF are spared from the effects of AngII and DOCA-salt, particularly from T cells infiltration in target tissues of elevated blood pressure and end-organ failure (Carnevale et al., 2016; Perrotta et al., 2018b). By exploiting selective surgeries of the splenic innervation, we showed that PlGF activation in the spleen was consequent to sympathetic activation (Carnevale et al., 2014). Subsequent studies were performed to analyse how the release of PlGF could be modulated by noradrenergic signalling in the spleen. In particular, bioelectronic medicine tools were utilized to stimulate the celiac vagus nerve, hence reproducing the effects induced by hypertensive stimuli. Electrically mediated release of noradrenaline in the spleen promoted an increase in PlGF expression and a concomitant reduction of CD3 T cell area, a condition that typically reflects an egress of these cells from the spleen towards target organs of hypertension (Carnevale et al., 2020). Phentolamine, an imidazoline, is a competitive alpha-adrenoceptor antagonist with similar affinities for the alpha-1 and alpha-2 sites. Propranolol is a non-selective, competitive antagonist at beta adrenoceptors, binding with high affinity to both beta-1 and beta-2 receptor subtypes. A spleen-restricted pre-treatment with phentolamine but not with propranolol hampered the responses activated by celiac vagus nerve stimulation, resulting in unaltered PlGF expression and T cell content compared to stimulated animals that received a vehicle control pre-treatment. Hence, despite having been simply considered as a reservoir of immune cells, the spleen is gaining more attention for the potential to utilize neural circuits and manipulate immune responses involved in disease progression.

The spleen and ATLOs in atherosclerosis. Notably, a key role of the spleen as a reservoir of immune cells was also demonstrated in the context of atherosclerosis where it contributes to organ damage and lesion growth (Potteaux et al., 2015). In this respect, Mellak et al. (2015) found that AngII treatment promoted the mobilization of splenic monocytes to develop and progress abdominal aortic aneurysm in Apoe$^{-/-}$ mice. Interestingly, we found that a splenic denervation obtained by celiac gangliectomy reduced splenic CD11b+ myeloid cells, decreased numbers and sizes of ATLOs, and reduced plaque sizes at the same time as increasing plaque stability (Mohanta et al., 2022). Taken together, these data indicated that an efferent neural pathway converging on the spleen through the vagus-splenic connection was a crucial modulator of immune cells recruitment in the context of atherosclerosis. As described above, ATLOs are structures that form in the lamina adventitia of affected arteries with an increased accumulation of immune cells and progression of atherosclerosis, similar to that observed in cancer and autoimmune diseases (Brea & Veiga-Fernandes, 2022). Such formations are absent in healthy conditions and specifically ensue in response to local inflammation, acquiring the ability to orchestrate different immune responses, dependent on the type of damage (Bery et al., 2022). On histological characterization, ATLOs were defined as structures containing several B cell subtypes, defined T cell areas, germinal centres, scattered innate immune cells, lymphatic vessels and high endothelial venules (Srikakulapu et al., 2016). More recently, it was found that ATLOs also contain axons growing during the progression of the disease. Interestingly, increased plaque formation and ATLO size were positively correlated with increased inflammation in the peripheral nervous system (Mohanta et al., 2022), suggesting that axon growth and the autonomic nervous system could be interconnected phenotypes. The manipulation of the splenic immune reservoir by the vagus–splenic axis can also be considered as an important tool by which to modulate pathology progression in atherosclerosis.

Vascular–immune interface and end-organ damage in atherosclerosis and hypertension

Given the increasing awareness of a key role played by inflammatory mechanisms in cardiovascular disease (CVD), there is an appealing possibility of ameliorating

the progression of target organ damage by developing immunomodulatory therapies. Experimental models targeting a variety of immune pathways have been useful in identifying the role and function of specific immune cells and mechanisms underlying hypertension and/or atherosclerosis.

Vascular-immune interfaces in hypertension. Mice deficient for Rag1 were the first model used to investigate the role of adaptive immune cells in hypertension. Rag1 is expressed in lymphocytes and its deficiency blocks the development and maturation of T and B cells. In a pivotal study conducted by Harrison's laboratory, Rag1 knockout mice were protected from Ang II or DOCA-salt induced hypertension (Guzik et al., 2007). The adoptive transfer of T cells, but not B cells, reestablished the hypertensive response. This concept paved the way for a series of subsequent studies that investigated the bases of the indispensable role of lymphocytes in hypertension. Immune and inflammatory responses play a decisive role in vascular remodelling, and the persistent exposure of the arterial vasculature to increased blood pressure contributes to vascular dysfunction, resulting in target organ damage (Drummond et al., 2019). The recruitment of monocytes and lymphocytes from peripheral blood to the vessel walls establishes vascular–immune interfaces and represents one of the primary events in the process of vascular inflammation. Our group developed a 3-D organ co-culture system that allows the co-incubation of resistance mesenteric arteries and immune cells. In this way, it becomes possible reproducing the vascular–immune interface that is formed during hypertension when activated lymphocytes infiltrate target peripheral resistance. In particular, we found that CD8 but not CD4 T cells isolated from AngII hypertensive mice increased the contractile properties of resistance arteries of naive mice (Carnevale et al., 2021), possibly explaining why CD8 but not CD4 knockout mice are protected from hypertension (Trott et al., 2014). Moreover, the analysis of CD8 and CD4 lymphocytes transcriptome by RNA sequencing highlighted specific pathways related to cell recruitment, tissue inflammation and calcium mobilization, which were altered in CD8 but not in CD4 T cells (Carnevale et al., 2021).

Vascular–immune interfaces in atherosclerosis. Notably, vascular–immune interfaces are key regulators of the atherosclerotic process, where the proliferation of activated immune cells recruited to the intimal layer of large and medium-sized arteries is a crucial event in the pathology (Wolf et al., 2015). Leukocyte subsets can differentially contribute to pro- and anti-atherogenic activities both within the atherosclerotic aorta and systemically (Weber et al., 2008). A combined approach of single-cell RNA-sequencing and mass cytometry (cytometry by time of flight) showed a heterogeneous phenotype of these immune cells: leukocytes in the aorta had a complexity similar to leukocytes in lymphoid organs but the various subpopulations can be also differentially regulated in health and disease and thus can be utilized as predictor of cardiovascular events in humans (Winkels et al., 2018). Indeed, tissue resident immune cells are responsible for disease development and progression equal to that of immune cells recruited from immune organs toward affected tissues (Winkels et al., 2018). Moreover, proteomic and transcriptomic single-cell analyses in atherosclerotic carotid artery plaques and blood from the same patient defined an atlas of immune cells and identified dysregulated pathways of innate and adaptive immune cells (Fernandez et al., 2019). Symptomatic patients with recent cardiovascular events showed a significant expansion of activated effector memory CD4+ T cell subsets in atherosclerotic plaques and distinctive alterations of CD4+ and CD8+ T cells associated with recent cardiovascular events. Conversely, T cells in asymptomatic patients were mostly activated in plaques. Similarly, macrophages of asymptomatic patients were activated and showed pro-inflammatory functions, whereas macrophages from plaques of patients with recent CV events displayed transcriptional signatures with distinct pro-inflammatory and reparative functions. Overall, each immune cell type has a specialized function in atherosclerotic plaques, being different between symptomatic and asymptomatic patients, indicating a unique interplay at the lesioned artery (Fernandez et al., 2019).

The aggregates of immune cells expanding adjacently to arterial segments with plaques and forming the ATLOs, in mice and humans (Mohanta et al., 2014; Mohanta et al., 2022), are characterized by distinct immune compartments executing specific functions. Among these, the recruitment of naïve T cells and the presence of lymphocyte subsets with opposite activities, including CD4+ and CD8+ effector and memory T cells, suggest that they participate in primary immune responses and in the organization of T and B cell responses in advanced atherosclerosis. Interestingly, vascular smooth muscle cells expressed lymphotoxin β receptors, which have an important role in mechanisms protecting against atherosclerosis progression and counteracting ATLO formation (Gräbner et al., 2009; Hu et al., 2015). Indeed, aged $Apoe^{-/-}LTbr^{-/-}$, hyperlipidaemic mice, with a deletion of lymphotoxin β receptor in smooth muscle cell, had an exacerbated atherosclerotic phenotype (Gräbner et al., 2009; Hu et al., 2015). *In vitro* studies revealed that the stimulation of the lymphotoxin β receptor on arterial smooth muscle cells promoted a change in their organization to resemble a lymphoid phenotype (Lötzer et al., 2010). Furthermore, ATLO cells expressed

the typical chemokines CXCL13 and CCL19 chemokines, similarly to that observed in lymphoid organs (Hu et al., 2015). Collectively, these data suggested that the signalling mediated by lymphotoxin β receptor in the smooth muscle was able to transduce a stimulus favouring ATLO formation (Mohanta et al., 2014; Mohanta et al., 2022). The comprehension of immune cell interactions and functions in atherogenic conditions may open the possibility to a better understanding of disease pathophysiology and progression and, consequently, the development of strategies for immunomodulatory therapies (Engelen et al., 2022).

Translational considerations

The concept of the residual inflammatory risk in CVD refers to the inflammatory status observed in peripheral organs of some patients affected by CVD, which persists despite good control of the primary risk factors with the currently available therapies (Ridker, 2016). This condition has been associated with an enhanced risk of further cardiovascular events. This basic concept was the underlying hypothesis that allowed conception of the CANTOS trial. In this trial, the reduction of cardiovascular events with canakinumab was higher in patients with a significant reduction of IL-6 levels and high-sensitivity C-reactive protein, suggesting that targeting of the IL-1β–IL-6–C-reactive protein pathway of innate immunity is beneficial (Ridker et al., 2017). Otherwise, the alternative but similar approach using low-dose methotrexate did not reduce levels of the IL pathway or C-reactive protein and cardiovascular events in the same type of patients enrolled in the CANTOS trial (Ridker et al., 2019). Although these clinical trials were a breakthrough in demonstrating the proof of concept that treatment of the residual inflammatory risk could achieve a better prognosis in CVD, we are still far from translating these approaches in current clinical practice.

IL-1β is also involved in inflammation associated with atherosclerosis. In particular, it was shown that statin therapy can reduce the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 inflammasome, as one of the principal regulators of chronic inflammation, as well as related-IL-1β expression (Wang et al., 2018). Also in this case, the use of drugs to reduce CVD progression is controversial and depends on the type of statins used. Some studies suggested that statins such as fluvastatin activate the inflammasome and caspase-1 (Henriksbo et al., 2014). By contrast, other studies showed that statins such as atorvastatin, typically used for coronary artery diseases, can also inhibit the inflammasome and caspase-1 (Satoh et al., 2014). These contradictory observations reveal the need to further investigate these aspects with respect to managing patient therapy and considering additional effects on the immune and inflammatory responses.

In this context, drugs inhibiting adrenergic signalling, such as β-blockers (Wiysonge et al., 2017), are worthy of mention. These are widely used in patients afflicted by CVD, with reported multiple beneficial effects, representing a first-line therapy for improving prognosis after a cardiovascular event (Wiysonge et al., 2017). In other contexts, the blockade of β-receptors by propranolol reduced cancer growth and angiogenesis and enhanced the response to the anti-inflammatory treatment ameliorating anti-cytotoxic T-lymphocyte associated protein 4 treatment (Fjæstad et al., 2022). However, although this effect could be interesting in terms of complementing the treatment of some forms of tumour and reducing the inflammatory state, β-blockers still have many systemic effects with respect to cell-specific immunomodulatory functions.

Dissecting the molecular mechanisms underlying the cross-talk between immune cells and the vasculature or developing tools to modulate the trafficking of specific inflammatory cells will be necessary to enable proposed new therapies for a personalized medicine approach targeting inflammation in affected vascular districts. As non-pharmacological approaches, bioelectronic tools or surgical interventions on specific neural pathway allow activation or inhibition of specific cells of the immune system to deepen their role in cardiovascular pathologies characterized by an imbalance of immune responses. Activation of the cholinergic anti-inflammatory pathway by vagus nerve stimulation is one of the most promising strategies for investigating neuroimmune interactions in CVD and attenuating inflammation. Tanaka et al. (2021) demonstrated that the selective stimulation of afferent vagal fibres generated the activation of splenic immune responses and promoted the protection of kidney injury in a model of ischemia reperfusion, conserving the beneficial effect of vagus nerve stimulation with respect to reducing inflammation. This finding provides a relevant strategy with respect to considering the neuroimmune interactions as potential therapeutic targets (Abe et al., 2017; Tanaka et al., 2021). As shown by this work, the activation of specific sympathetic neurons by vagus nerve stimulation is important for kidney protection and for suppressing inflammation (Abe et al., 2017; Bellinger et al., 2008; Katayama et al., 2022; Komegae et al., 2018; Lankadeva et al., 2020; Martelli et al., 2014; Martelli et al., 2019; McKinley et al., 2022; Tanaka et al., 2021). Interestingly, it is also conceivable that, under some pathological conditions, their activation could be deleterious. One example is represented by our results showing that the overactivation of splenic sympathetic nerve activity in AngII-induced hypertension induces an aberrant infiltration of immune cells in target

organs causing disease progression. Thus, multi-organ neuro-modulatory approaches that allow enhancement of some pathways and contemporary switching off of others could be a solution for selectively targeting the multiple aspects of CVD (Kiuchi et al., 2023). The sympathetic denervation could be useful to selectively interfere with different alterations of the cardiovascular system or to modulate underlying immune responses (Kiuchi et al., 2023).

Conclusions

The brain represents an integrative organ receiving afferent and sensory signals from the periphery during pathological changes. Disturbances in the periphery activate complex neural connections established between different nuclei in the brain, possibly eliciting reflex circuits that modulate immune responses in lymphoid organs (Perrotta & Carnevale, 2024). Indeed, peripheral nerves of the autonomic nervous system interact with immune cells to establish neuroimmune interfaces into lymphoid organs. Once activated by nervous stimuli, immune cells egress from lymphoid organs to infiltrate non-lymphoid tissues contributing to end-organ damage. The establishment of vascular–immune interfaces in peripheral target tissues is crucial to regulation of organ function, possibly contributing to disease progression. As an example, in hypertension, immune cells infiltrate the vasculature where they participate in vascular remodelling and dysfunction, finally resulting in target organ damage. In atherosclerosis, the proliferation of activated immune cells recruited to the intimal layer of large and medium-sized arteries is a crucial event in pathology progression and organ damage. Thus, an approach aiming to abrogate lymphoid organ innervation could be useful for modulating neuroimmune interactions during the progression of hypertension and atherosclerosis, this reducing end-organ damage. The increasing interest in the investigation of mechanisms linking nervous, immune and cardiovascular systems has fostered the development of new approaches and strategies for innovative neuroimmuno-modulatory therapies.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

M.P. researched data for the article and wrote the manuscript. D.C. conceptualized and discussed the content, reviewed the article and supervised the work. Both authors have read approved the final version of the manuscript submitted for publication.

Funding

This work was supported by the ERC Starting Grant SymPAtHY GA 759 921 of the European Commission granted to DC.

Acknowledgements

Open access publishing facilitated by Universita degli Studi di Roma La Sapienza, as part of the Wiley - CRUI-CARE agreement.

Keywords

autonomic nervous system, central nervous system, immunity, target organ damage, vascular inflammation

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