Cytokine Aerobics: Oxidation Controls Cytokine Dynamics and Function

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Summary

In this issue of *Structure*, Skeens et al. provide insights into the structure and dynamics of an oxidized form of the atypical cytokine macrophage migration-inhibitory factor (MIF). The study unveils a surprising conformational susceptibility of MIF to ambient redox alterations and identifies redox-sensitive residues and latent allostery sites with functional relevance.

Main text

Macrophage migration-inhibitory factor (MIF) is a multifunctional inflammatory cytokine and atypical chemokine (ACK). MIF is an important upstream modulator of the innate (and adaptive) host immune response, but, when deregulated, it promotes a variety of pathologies including acute and chronic inflammatory conditions, cardiovascular diseases, cancer, and autoimmunity (Calandra and Roger, 2003; Kang and Bucala, 2019; Schindler et al., 2018). The MIF protein is evolutionarily highly conserved with orthologs found in various kingdoms and species, including plants and unicellular parasites, suggesting that the MIF protein family has evolved over 800 million years ago and that MIF proteins may have originally exerted non-

cytokine intracellular functions. This may explain, at least in part, a number of the intriguing atypical molecular properties that have been identified for MIF and that differentiate it from conventional cytokines and chemokines (Schindler *et al.*, 2018). To this end, the MIF sequence does not contain an N-terminal signal sequence, and translated MIF resides in the cytosolic and nuclear cellular compartment, from where it is released into the extracellular space by an unconventional secretion pathway. Extracellular MIF signals through its cognate receptor CD74 (a type II membrane protein that also functions as an MHC class II chaperone) to regulate a variety of immune and inflammatory activities as well as tumor cell proliferation (***Figure 1***). MIF also engages in chemokine mimicry to bind to the CXC-type chemokine receptors CXCR2, CXCR4, and CXCR7/ACKR3, a signaling network important in atherogenic leukocyte recruitment responses, tumor cell migration, and pulmonary fibrosis (***Figure 1***) (Calandra and Roger, 2003; Kang and Bucala, 2019; Schindler *et al.*, 2018). While MIF's α/β-containing structural fold is somewhat reminiscent of the overall folds of interleukin-(IL)-1β and chemokines such as IL-8/CXCL8 (Sun et al., 1996), and while its surface-exposed regions partially mimic binding motifs for the aforementioned chemokine receptors, the sequence homology to the corresponding classical chemokine ligands is limited and MIF proteins cannot be structurally classified into any of the known cytokine or chemokine sub-classes (Calandra and Roger, 2003; Schindler *et al.*, 2018). In contrast, the three-dimensional architecture of the MIF trimer strikingly resembles that of bacterial tautomerases such as 4-oxalocrotonate tautomerase (4-OT) (Calandra and Roger, 2003; Sun *et al.*, 1996). Moreover, MIF shares with these enzymes an N-terminal proline residue that forms the core of a conserved catalytic tautomerase pocket. Herein and in the Structure paper by Skeens et al., this proline residue is termed Pro-1, but across the literature, it is also referred to as Pro-2, because the N-terminal methionine residue preceding Pro-2 in the gene encoding DNA sequence is post-translationally removed in most cells. While modification of the pocket by mutations, inhibitors, or oxidation/dehydrogenation of Pro-1 modulates some of MIF's receptor and signaling activities and pathogenic effects (Calandra and Roger, 2003; Kang and Bucala, 2019; Schindler *et al.*, 2018), MIF's tautomerase activity has so far only been demonstrated *in vitro*

and a substrate and physiological role in mammalian cells have yet to be identified, together insinuating that the tautomerase site mainly indirectly affects MIF's receptor-mediated cytokine activities. MIF's evolutionary connection also is a likely explanation for the conservation of a redox-active Cys-Xaa-Xaa-Cys (CXXC; Cys-Ala-Leu-Cys (CALC) in MIF) motif that it shares with thiol-protein oxidoreductases (TPORs) (Kleemann et al., 1998) and that has been linked to some of MIF's disease-related effects (Schindler *et al.*, 2018). Of note, recent work by Schinagl et al. identified Cys-80 as a redox-sensitive conformational switch in MIF, triggering the conversion of reduced MIF (redMIF) to oxidized MIF (oxMIF) (Schinagl et al., 2018), similar to the findings of the current *Structure* manuscript by Skeens et al. (Skeens et al., 2022). Furthermore, capitalizing on an oxMIF-specific antibody that specifically binds to an oxidized (disulfide) form of a CALC-spanning MIF peptide, they proposed a link between the switch residue cysteine 80, an unleashing of the redox-active CALC motif and pathogenic activities of MIF related to oxidative microenvironments (Schinagl *et al.*, 2018). However, the structural and atomic details of the redox-dependent conformational switch remained elusive.

In the current issue of *Structure*, Skeens et al. (2022) use solution nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry to elucidate the structural details of such a redox-dependent conformational switch in MIF and to test the hypothesis that MIF may act as an allosteric 'redox sensor' to respond to pro-oxidative inflammatory microenvironments as a means to fine-tune its downstream effector activities. With NMR spin relaxation experiments under oxidative *versus* redox-neutral and reducing solution conditions, and by employing site-specific mutagenesis of identified 'latent' redox-sensitive residues, they: i) found that MIF is extensively modified in an oxidative environment; ii) that it becomes remarkably dynamic under such conditions with motions observed in the µs-ms but also ps-ns time-scale; iii) that oxMIF exhibits the most pronounced conformational and dynamic changes; iv) determined MIF's redox potential to be in the near-to-physiological range (midpoint: E'_0 = -215 mV $\pm$ 15 mV); v) discovered that residues Cys-80 and Lys-66 serve as redox-sensitive – latent - allosteric sites to toggle MIF conformation and function; and vi) that mutation of these switches ablates MIF's tautomerase activity and attenuates its inflammatory neutrophil

recruitment activity in murine lungs *in vivo*. In conjunction, these results unveil previously unrecognized substantial oxidation-triggered changes in the conformation and dynamics of the MIF molecule and implicate these alterations as a molecular switch mechanism to fine-tune MIF effector functions.

There are commonalities between the 2018 study by Schinagl et al. and the current paper published in *Structure* (Schinagl *et al.*, 2018; Skeens *et al.*, 2022). Schinagl et al. also identified Cys-80 as a switch cysteine for the conversion from redMIF to oxMIF and implicated redMIF as a latent, inactive, form of MIF, and suggested that formation of oxMIF leads to a isoform of MIF with certain pathophysiological characteristics (Schinagl *et al.*, 2018). However, regarding MIF's effector activity, they solely focused on MIF's catalytic redox activity to suggest that an oxidation-dependent activation of MIF via Cys-80 leads to a conformational change and activation of the CALC redox motif. The mechanism, however, how CALC-mediated redox effects might affect MIF's downstream signaling activities has remained obscure. Skeens et al. did not study effects on CALC, also because the corresponding CALC-spanning peptide was not amenable to the mass spectrometry methods used in their study. Instead, they took a wholistic, impressively comprehensive, look at the redox signature of MIF and its conformational, dynamic and functional consequences. Most importantly, this provides us with detailed and unprecedented insight into the redox-dependent structure and dynamics of MIF at the molecular level. The dynamic measurements not only identified µs-ms dynamics predominant in oxMIF, but also changes in the ps-ns time-scale that can also be observed in redMIF. In addition to Cys-80, they identified Lys-66 as a critical redox-sensitive residue. While Lys-66 is not covalently redox-modified itself, it undergoes large changes in flexibility between the oxidized and reduced states of MIF. Although it remains to be clarified how precisely this residue engages in and mediates redox-triggered conformational changes, as it is positioned at the bottom of the first α-helix with a supposed prime role in stabilizing the internal structure of the MIF fold, we suggest to term it a 'secondary' redox switch residue. Importantly, in conjunction with their mutational experiments, Skeens et al. provide convincing evidence that Cys-80 and Lys-66 function as redox-sensitive latent allosteric sites (i.e. 'switch' residues) for

the generation of oxMIF that is accompanied by increased dynamics, refolding, and altered receptor binding properties. Thus, in contrast to the CALC redox concept suggested by Schinagl et al., the current study in *Structure* suggests that the conversion to oxMIF unleashes receptor binding and/or activation sites in MIF (Skeens *et al.*, 2022). The performed pulmonary neutrophil recruitment assay implies that this involves an activation of MIF/CD74 engagement (***Figure 1***), a notion that is in line with previous evidence on conformational dynamics and plasticity of MIF and a previously established connection between nanosecond dynamics of MIF and the MIF/CD74 binding interface (Pantouris et al., 2018), but this will have to be ultimately confirmed by direct evidence from receptor binding and signaling experiments. Still, structural alterations in the MIF tautomerase pocket (i.e. by inhibitor binding or mutations) have previously been shown to lead to reductions in receptor activation by MIF and MIF's tautomerase activity is influenced by an allosteric site at the central solvent channel also implicated in the current study by Skeens et al. (Pantouris *et al.*, 2018; Pantouris et al., 2020; Skeens *et al.*, 2022), lending further support to the redox-dependent latent allostery concept suggested here. Mechanistically, the switch residues might form covalent interactions with cysteine or lysine residues on CD74 that are proximal to the predicted interaction site between MIF and CD74, in line with previous suggestions (Meza-Romero et al., 2016). However, mechanisms of CD74 activation by redMIF have not been directly tested in the current study and at this point clearly cannot be excluded. Moreover, it is unlikely that CD74 is the only MIF receptor affected by redox modifications of MIF and altered dynamics in oxMIF. For example, CXCR4 is prominently expressed on the immune, endothelial, and parenchymal cells anticipated to populate disease-related pro-oxidative inflammatory microenvironments (***Figure 1***). Binding of MIF to this receptor also is affected by structural changes in MIF's tautomerase pocket and CXCR4 contains potentially redox-susceptible cysteine residues as well as lysines in its ectodomain that may be amenable to redox-dependent interactions with the identified MIF switch residues.

Together, the paper by Skeens et al. published in this issue of *Structure* provides compelling evidence for a novel mechanistic paradigm of how microenvironmental redox

conditions may influence MIF structure, dynamics, and function. Although several studies over the past two decades have established links between MIF and redox control in pathogenic conditions such as cancer, inflammatory diseases and ischemia/reperfusion injury, partially addressing underlying structure-activity relationships, Skeens et al. for the first time provide a comprehensive picture of the redox signature of MIF at the molecular level. This adds an important facet to our understanding of how the MIF protein network may be regulated. Currently incompletely understood mechanistic questions, as to which MIF receptor is preferentially activated by extracellular MIF in a given disease, cell, or tissue context may be partially governed by the specific redox signature of MIF. Oxidized MIF (or redMIF) may display a higher binding affinity or an enhanced EC_{50} value for a certain MIF receptor in a given redox microenvironment, thus paving the way for a certain signaling bias. Although the nuclear/cytosolic compartment generally exhibits a reducing redox potential, intracellular MIF protein-protein interactions might also be controlled by fluctuations in the cellular redox state.

The data obtained by Skeens et al. call for future studies to elucidate the structure-function relationships between specific redox signatures of MIF and its receptors (as well as its other binding partners) in more detail. Moreover, it will be interesting to explore if the MIF homolog MIF-2/D-DT or MIF orthologs such as plant-derived MIF/D-DT-like (MDL) proteins may have similar redox-sensitive conformational and dynamic properties as MIF. Lastly, the current study should aid in developing tailored therapeutic approaches with improved specificity and efficacy.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft (DFG) grant SFB1123-A3 and by the German Center for Cardiovascular Diseases (DZHK), partner site Munich, grant DZHK B 20-004 to J.B.

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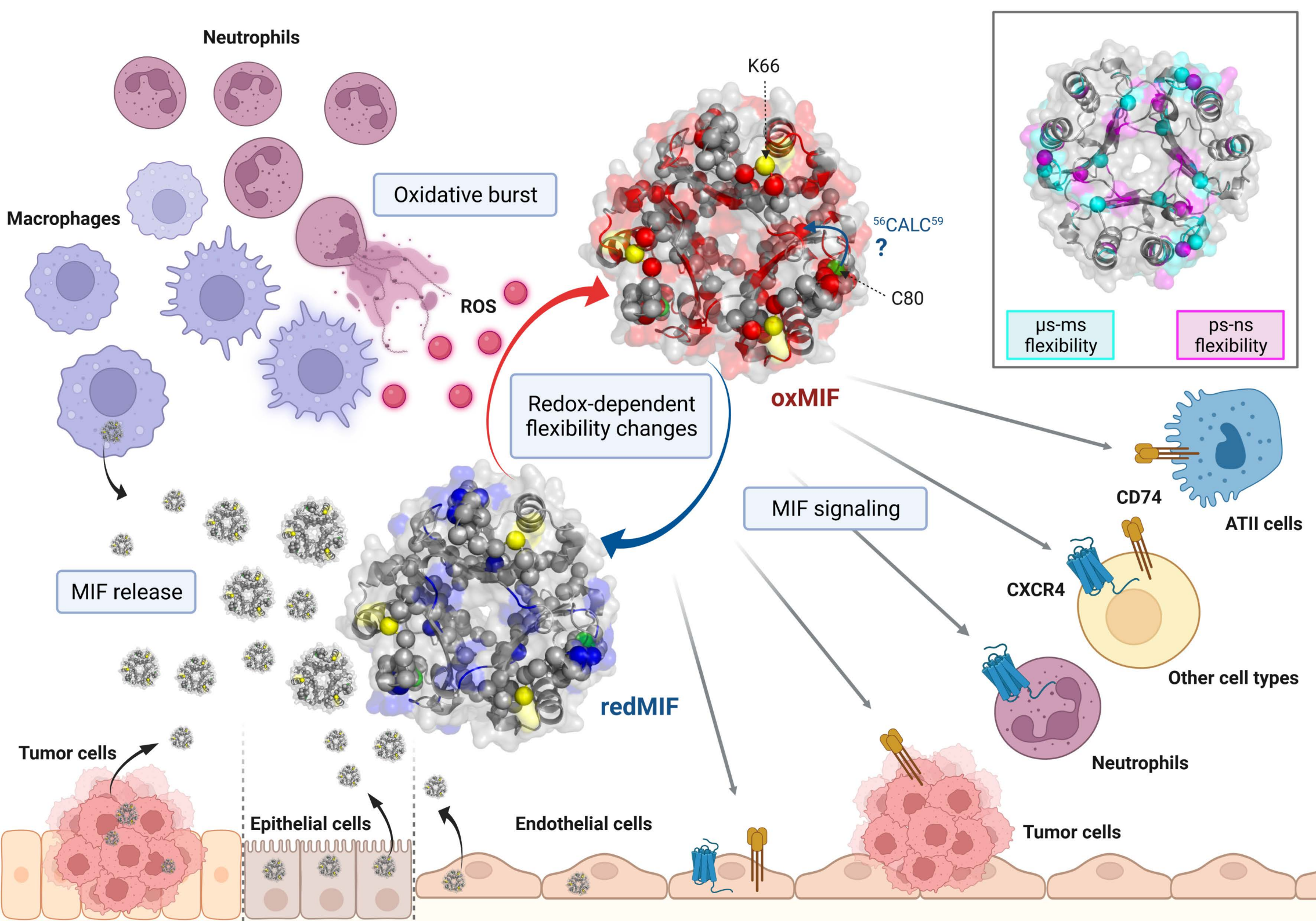
Figure legend

Figure 1: Structure and dynamics of MIF are altered by microenvironmental redox conditions with implications for MIF receptor interactions. MIF is known to be secreted by a variety of cell types, including macrophages, endothelial and certain epithelial cells as well as tumor cells. Once secreted, MIF is exposed to the local microenvironmental redox potential and may, for example, be oxidized in an inflammatory context where both macrophages and neutrophils promote oxidizing conditions via an oxidative burst, creating reactive oxygen species (ROS) and other pro-oxidative mediators. Skeens et al. show that wide areas of the MIF molecule selectively become more flexible under oxidizing (or reducing) conditions, marked in either red (in the "oxMIF" conformation) or blue ("redMIF" conformation). Their data further suggest that residues Cys-80 (C80, yellow spheres) and Lys-66 (K66, green spheres) act as redox sensors or switches between both conformations. A role for C80 as redox switch for a conformational rearrangement of the 56CALC59 motif of MIF, as previously suggested by Schinagl et al. (2018) is indicated as well. Residues of MIF that are involved in engaging its cognate receptor CD74 as well as its non-cognate receptors CXCR2 and CXCR4 are visualized as spheres. As these areas of MIF overlap significantly with residues changing their dynamics especially under oxidative conditions, becoming more flexible, this redox-dependent change in protein dynamics could have consequences for MIF's ability to signal through these receptors, which could be expressed on endothelial and tumor cells, but also on alveolar type II epithelial (ATII) cells, neutrophils, monocytes/macrophages, or other cell types. The upper-right inset highlights redox-sensitive residues of MIF that show either µs-ms (cyan) or ps-ns flexibility (magenta). Among these are residues I64, Y95 N97, N109 and S111 (depicted as spheres) that were shown previously to control CD74 activation and the enzymatic activity of MIF. The shown structures of MIF were adapted from the PDB entry *1MIF* structure using PyMOL version 1.8.2.2 (Schrödinger, LLC) and the figure was created with BioRender.com.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Endothelial cells
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