Epigenetic alterations in endothelial cells during sepsis and inflammation

New insights in sepsis-associated endothelial dysfunction?

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Abstract

Due to an excessive inflammatory response induced by the bacterial gram-negative compound LPS, the endothelial barrier integrity is disrupted in sepsis patients. Enhanced vascular permeability and thereby tissue leakage, can lead to multiple organ failure. Both genetic and epigenetic alterations underlie this endothelial dysfunction. Sepsis studies related to epigenetic alterations mainly focus on macrophages or monocytes, but the role of endothelial cells (ECs) is often nog included. This thesis aimed to generate a broader perspective about the epigenetic alterations in ECs underlying endothelial dysfunction. Therefore, we will combine the current knowledge of both epigenetic alterations in ECs after inflammation, disturbed blood flow, and sepsis.

Genes that are epigenetically altered can be divided into three groups: adhesion molecules, inflammatory cytokines, and genes associated with endothelial function. Of the last group, KLF2, Ang1, and VEGFR are promising therapeutic targets, as they regulate multiple downstream genes and endothelial integrity. Since several genes are regulated by both histone modifications and DNA methylation, combined treatment is necessary to increase the effect of the epigenetic inhibitors. A new system, CRISPR/dCas9, has shown to alter the epigenetic state in a highly specific way, thereby improving diseases like acute kidney injury (AKI) *in vivo*. Applications of this system could be used to target the aforementioned genes to restore endothelial function, although the secondary challenge to clear the bacteria remains.

In conclusion, a broad view of altered epigenetic mechanisms after inflammation is generated that can be used as therapeutic targets to overcome sepsis-induced endothelial dysfunction. Additionally, epigenetic editing by CRISPR/dCas9 seems to be more promising than the use of epigenetic inhibitors.

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Introduction

Sepsis is a life-threatening condition with organ dysfunction due to a dysregulated immune response after a severe infection, which is estimated to affect more than 30 million people worldwide each year despite advancements in medical therapy[1,2]. The incidence of sepsis has increased over the past 40 years, partly explained by the increase in age of populations worldwide and a greater recognition among experts[3,4]. Despite the mortality of sepsis has decreased in recent years, it is still around 20% and increases when people suffer from severer sepsis (26%), and the long-term mortality is even 50-70% due to additional morbidities (Fig 1)[2,5,6]. Furthermore, sepsis is the leading cause of death in intensive care units and is responsible for more than 260.000 deaths annually in the United States alone, and more than 5 million deaths worldwide[2,7–9].

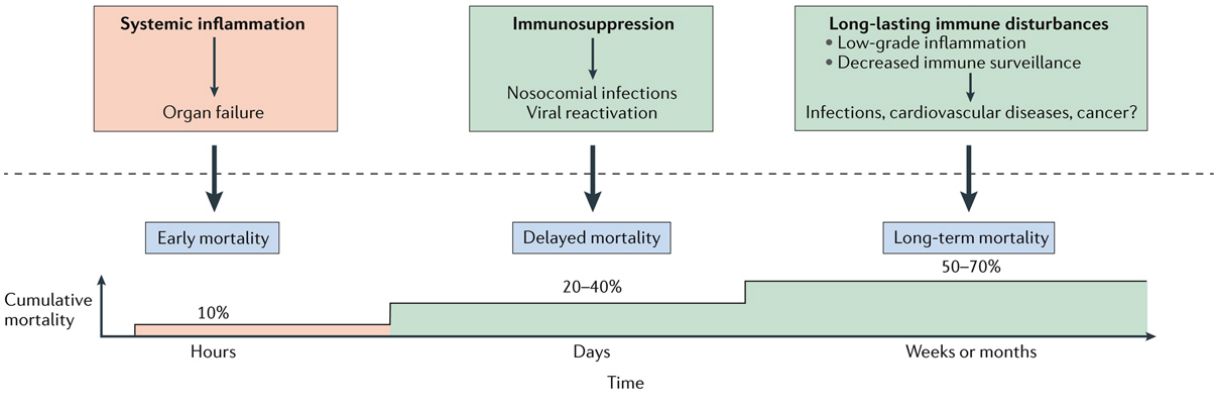

Figure 1. Sepsis-associated mortality[5].

The dysregulated inflammatory response in sepsis can arise from a variety of infections, including infections of the lung (pneumonia), skin (cellulitis), urinary tract, and abdomen, caused by bacteria, viruses, parasites, or fungi[10]. By recognizing pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), immune cells can respond to the primary infection[11]. In sepsis, the initial inflammatory response of the host is excessive, resulting in a cytokine-mediated hyper-inflammatory state[12]. Interestingly, in sepsis, there is simultaneously a compensatory anti-inflammatory immune response (Fig 2)[13,14]. Most patients with sepsis cannot return to normal immunological homeostasis, resulting in sustained immune suppression[5,15]. The induced immunosuppression results in a predisposition to secondary infections. Furthermore, the impaired immune response can result in among others, septic shock, endothelial dysfunction, and multiple organ dysfunction syndrome (MODS). The main immunological changes are summarized in figure 2[15].

Next to immune cells, endothelial cells (ECs) play a major role in regulating the immune response[16]. During inflammation, ECs gain expression of adhesion molecules such as intracellular adhesion molecule 1 (ICAM1), E-selectin, and vascular cell adhesion molecule 1 (VCAM1)[17]. These adhesion molecules are necessary for leukocytes to bind to ECs and to migrate towards the underlying tissue[18]. ECs are also involved in the release of pro-inflammatory cytokines, and in maintaining the barrier function of the endothelium[19]. Due to the excessive inflammatory response in sepsis, this barrier function is disrupted, contributing to the development of tissue and organ edema, hypotension, and MODS[20].

Recently, researchers showed that sepsis-induced epigenetic changes in monocytes, myeloid-derived suppressor cells (MDSC), and macrophages, both *in vitro* and *in vivo*[21–23]. However, the majority of epigenetic studies related to sepsis are focused on the alterations in immune cells, since some studies suggest these are the main drivers of sepsis-associated modifications[24]. The role of epigenetic alterations in ECs underlying the mechanisms behind endothelial dysfunction in sepsis is so far underexposed, as to our knowledge only one paper so far has reported on this subject[25]. Several papers are using histone deacetylases (HDAC) inhibitors that show beneficial responses both *in vivo* and *in*

vitro concerning ECs in sepsis, but the exact mechanisms remain unknown[26,27]. Despite the lack of studies aimed at epigenetic mechanisms in sepsis-induced inflammation, more papers are highlighting epigenetic changes in ECs in other (inflammatory) diseases. These studies aimed at the endothelium in inflammation have the potential to be translated to sepsis-associated inflammation and related syndromes. Therefore, this thesis aims to elucidate the epigenetic alterations and mechanisms in ECs underlying endothelial dysfunction and organ failure in sepsis. Additionally, we will discuss shortly the most promising epigenetic treatments.

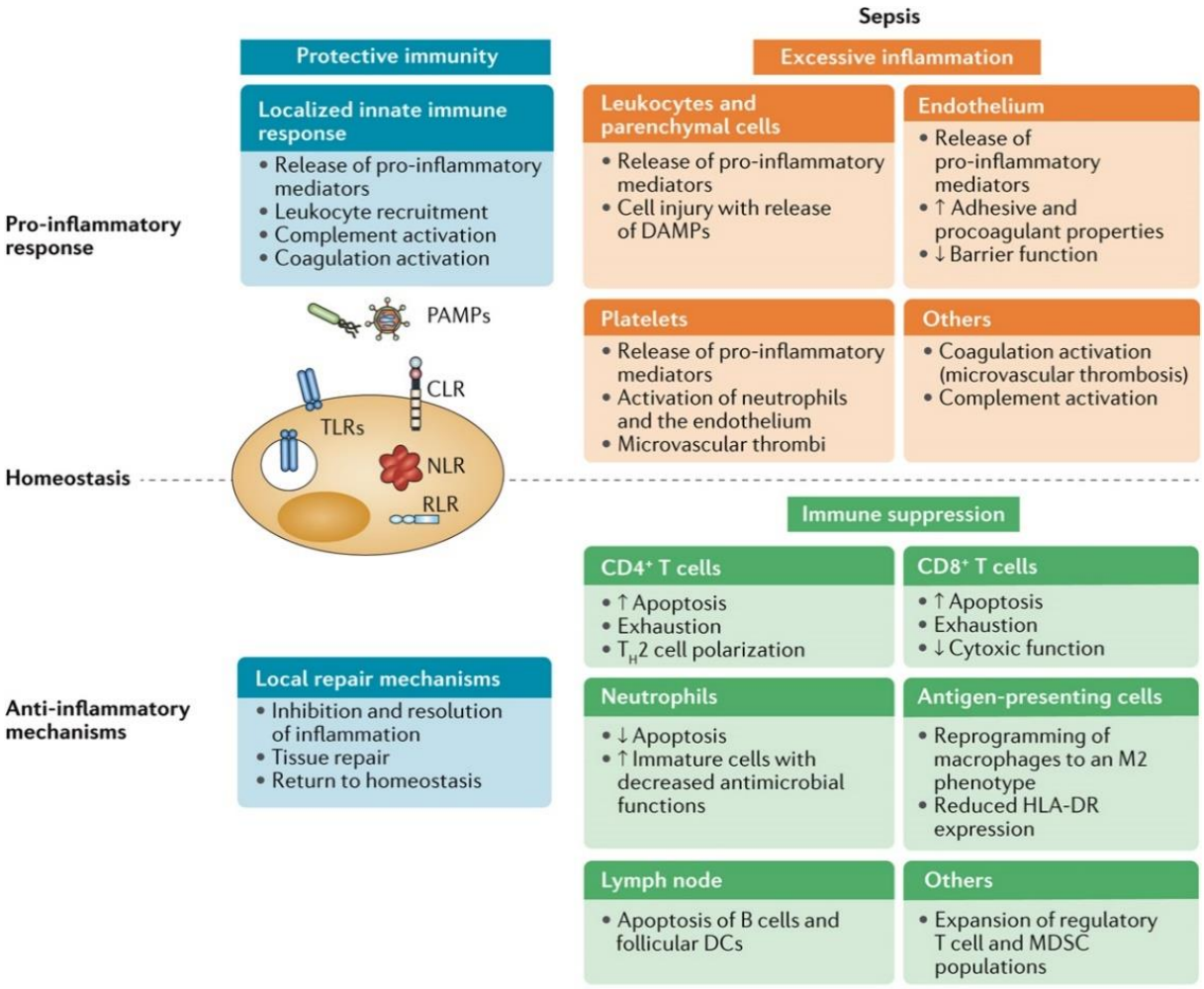

Figure 2. Sepsis-associated immunological responses[15].

Epigenetic mechanisms and regulation

In eukaryotic cells, the DNA is tightly wrapped around histones that regulate and protect the DNA, thereby regulating gene expression. This structure of DNA and histones is called chromatin. Chromatin in the nucleus can be in two different states: an open structure (euchromatin) that allows the recruitment of RNA polymerases and transcription factors (TFs) that initiates transcription, and a condensed chromatin (heterochromatin) that is not accessible for RNA polymerases and TFs, thereby repressing gene expression[28]. This structure is cell-type dependent and dynamic. Epigenetics encompasses the regulation of gene expression without alterations in the DNA sequence, although the definition of epigenetics differs between groups and is a point of discussion[29–31]. Epigenetic regulation is essential for cellular function, and epigenetic dysfunction is associated with many different malignancies, including cancer, pre-eclampsia, Crohn's disease, and asthma[32–35]. The main mechanisms involving epigenetic regulation by influencing chromatin accessibility are DNA methylation and histone modification.

DNA methylation

DNA methylation refers to the process in which a methyl group is transferred to the C-5 position of cytosine-guanine (CpG) motifs[36]. Enriched regions in the mammalian DNA are termed CpG islands and are present at loci associated with transcriptional regulation such as enhancers and promoters. CpG islands are present in 60-70% of the promoter regions in all human genes[37]. DNA methyltransferases (DNMTs) are the main regulators of DNA methylation and consist out of five members: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L[38]. DNMT1 methylation is responsible for transferring the DNA methylation status of the parent strand to the daughter strands during replication[39]. DNMT3A and DNMT3B are mainly responsible for *de novo* methylation of unmethylated CpG islands[39]. DNA methylation can be reversed by ten-eleven translocation (TET) proteins, that can oxidize 5-methylcytosine (5mC) in multiple steps to 5-carboxylcytosine (5caC)[40]. The oxidized cytosine is recognized and excised, followed by the replacement of an unmodified cytosine via base excision repair (BER)[41].

Histone modifications

Histones can be post-translationally modified, thereby modifying the availability of the DNA sequence for transcriptional machinery by altering the chromatin conformation and nucleosomal positioning[42]. Histones proteins can modify histones at their protruded histone tails, and these modifications are dynamic and change rapidly[43]. There are several types of modifications, and the most important modifications include acetylation, methylation, phosphorylation, and ubiquitylation. Some of these modifications disrupt the interactions between histones and DNA, causing the nucleosomes to unwind and thereby gene expression activation. Other mechanisms strengthen the histone-DNA interactions, thereby creating a condensed chromatin that causes gene silencing.

Histone methylation

Histone methylation occurs on the lysine (K) or arginine (R) residues of histones H3 and H4. These residues are present on the histone tails of H3 and H4, and five K residues are associated with methylation (H3K4, H3K9, H3K27, H3K36, and H4K20)[44]. Methylation of lysine is associated with both transcriptional activation (H3K4, H3K36, and H3K79) and repression (H3K9 and H3K27) depending on the methylation site, whereas arginine methylation promotes only transcriptional activation (Table 1)[45,46]. The functional flexibility of lysine methylation could be a result of the fact that it does not affect histone-DNA interaction directly, nor altering the histone charge[47].

Histone methylation is actively regulated and is a reversible process. The methylation and demethylation is mediated by histone methyltransferases (HMTs) and histone demethylases. There are a lot of enzymes and proteins involved in these processes [extensively reviewed in [48]], but the main mediators of lysine methylation are SET-domain containing proteins, and demethylation is mainly regulated by KDM proteins, such as KDM6A-C (H3K27) and KDM4A-D (H3K4).

Histone acetylation

Histone lysine acetylation and deacetylation is mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs add a negatively charged acetyl group to positively charged lysine residues of histones H3 and H4 (such as H3K9, H3K27, and H4K5), thereby changing the overall charge (Table 1)[49,50]. This weakens the interaction between the DNA and the histones, thereby opening the chromatin structure, allowing recruitment and binding of transcription factors and RNA polymerase, initiating gene transcription[51]. However, the exact effect of acetylation on chromatin structure and gene transcription is probably site-specific[52]. Still, histone acetylation mainly targets the promoter and enhancer regions (Fig 3)[53].

Table 1. Major epigenetic marks

Active		Repressive	
Mark	**Location**	**Mark**	**Location**
H3K4me1	Enhancers	H3K27me3	Promoters
H3K4me2	Promoters	H3K9me3	Pericentromeres, satellite repeats
H3K4me3	Promoters		
H3K9Ac	Enhancers, promoters		
H3K27Ac	Enhancers, promoters		

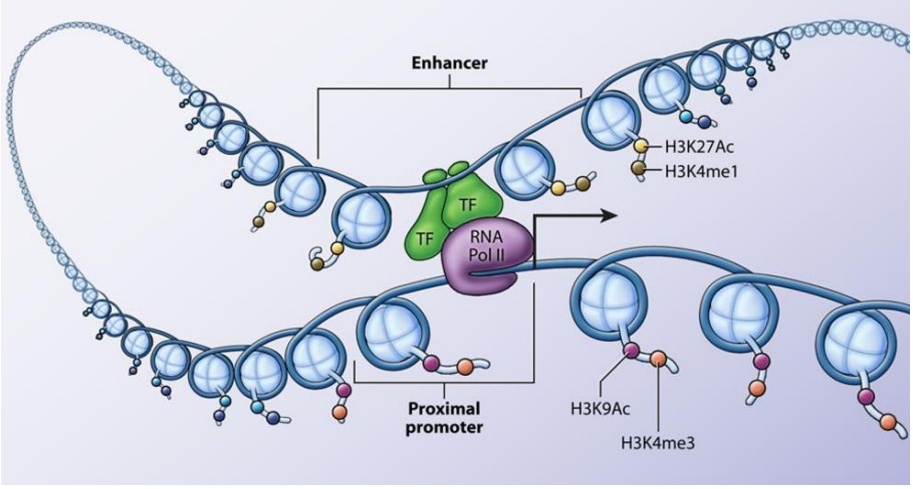

Figure 3. Enhancer and promoter regions and their corresponding histone marks[54].

The endothelium

ECs form a one-cell thick layer at the interior surface of the entire vascular system, which includes blood and lymphatic vessels. The endothelium regulates and affects many cardiovascular functions, including permeability, proliferation, innate and adaptive immunity, and the formation of new blood vessels[16]. ECs are structural and functionally heterogeneous since the structure and function differ per anatomical region of the cardiovascular system[16]. ECs are held together by tight- and adherens junctions and are anchored to a continuous basal membrane that provides the cells a structural and organizational stability[55]. Pericytes are cells that wrap around blood vessels and interact with ECs by direct or paracrine signaling[56].

Mechanisms controlling endothelial permeability

The endothelium forms a dynamic barrier between the blood and the tissue in most organs, and it allows and regulates fluid and solute molecules exchange. This barrier is maintained by cell-cell adhesions like adherens junctions (AJs) and tight junctions (TJs), the cell cytoskeleton, and the glycocalyx (Fig 4)[57]. TJ transmembrane proteins in ECs include occludin, claudin, and JAM (junctional adhesion molecules), which are anchored to the actin cytoskeleton of ECs via several zonula occludens (ZO) proteins[58]. The TJs make an impermeable barrier for the majority of large molecules. The main component of AJs is vascular endothelial (VE)-cadherin, and is only expressed in ECs[59]. VE-cadherin is responsible for the integrity and connection between neighboring ECs in a Ca2+-dependent structure[60]. Furthermore, VE-cadherin can influence the TJ organization, thereby being the main regulator of endothelial permeability[61].

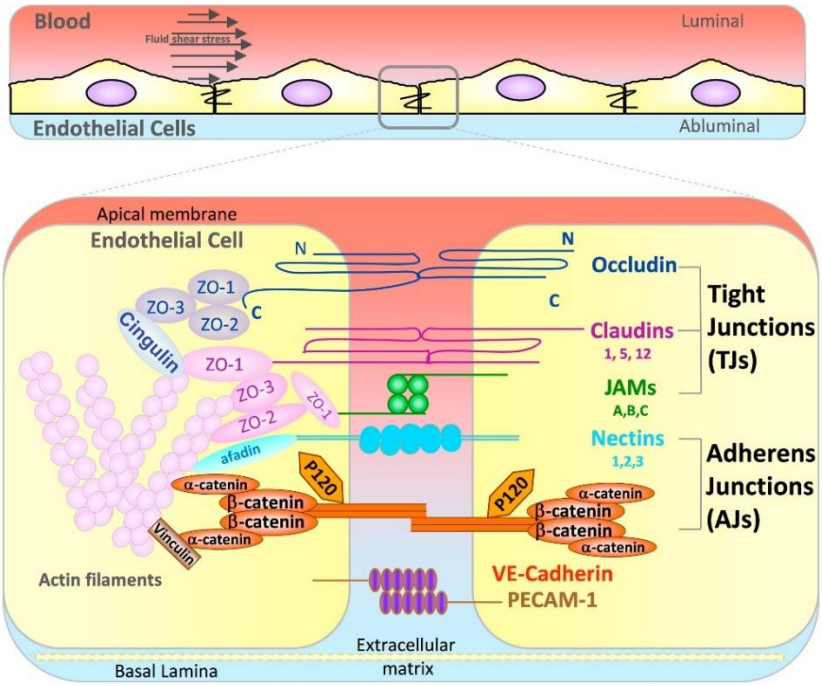

Figure 4. Endothelial cell-cell junctions. The main transmembrane proteins involved in cell-cell junctions are depicted in this figure. Occludins, claudins, and JAMs are associated with tight junctions, whereas nectins and VE-cadherin are adherens junctions. PECAM-1 is associated with neither type of junctions. The glycocalyx is not depicted in this figure but is lined at the luminal side of ECs[62].

Next to cell-cell adhesions, signaling pathways induced by vascular endothelial growth factor (VEGF) and angiopoietins (Ang1 and Ang2) and their receptors (VEGFR and Tie2 respectively) are responsible for endothelial function[63,64]. Ang1 is produced by pericytes, and during homeostasis, it clusters together to activate the Tie2 receptor which supports the barrier function and survival of ECs[65]. During inflammation, the competitive Tie2 antagonist Ang2 is upregulated[66]. This disrupts the normal Tie2 signaling, causing disassembly of cell-cell junctions and thereby promoting vascular leakage[67]. In addition to the Ang/Tie pathway, VEGF/VEGFR plays a role in endothelial permeability. VEGF is highly expressed during embryogenesis where it controls the formation of a primitive blood network during embryogenesis (vasculogenesis), and it plays a role in angiogenesis[68,69]. This is initiated by VEGF that stimulates endocytosis of VE-cadherin, resulting in destabilized AJs and increased endothelial leakage[70]. In sepsis patients, VEGF levels are shown to be increased that correlates with vascular permeability[71].

The inflammatory response in endothelial cells to LPS

Lipopolysaccharides (LPS), also known as endotoxin, is a major component of the outer membrane of gram-negative bacteria. LPS is the key component leading to septic shock, and more generally in inducing a strong immune response[72]. LPS binds to the Toll-like receptor 4 (TLR4), expressed on the outer membrane of ECs and monocytes[73]. The activation and role of TLR4 pathways in inflammation and sepsis are reviewed elsewhere[74,75]. In short, binding of LPS to TLR4 on ECs leads to activation of NF-κB, promoting intracellular inflammatory pathways. This results in an increased secretion of cytokines and chemokines such as IL-6, IL-10, MCP1, and G-CSF (granulocyte-colony stimulating factor). Furthermore, the expression of the adhesion molecules VCAM1, ICAM1, and E-selection on the surface of ECs is increased. NF-κB activation influences several factors mediating endothelial barrier function as well, since expression levels of claudins and occludins, key components of TJs, are decreased resulting in vascular leakage (Fig 5).

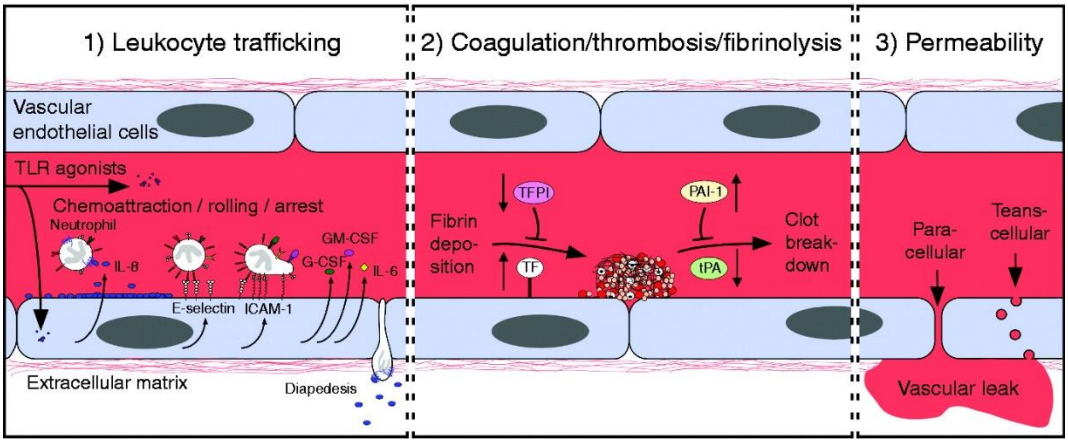

Figure 5. The results of endothelial activation by LPS via TLR4. 1) Pro-inflammatory cytokines are released by ECs and the expression of adhesion molecules is enhanced. 2) Not described in this thesis, but EC activation results in coagulation. 3) Claudin and occludin expression levels are decreased, resulting in increased vascular permeability[75].

Endothelial epigenetic alterations in sepsis

In sepsis, both immune and ECs undergo genetic and epigenetic changes[22,25]. Although endothelial dysfunction is one of the hallmarks of sepsis, epigenetic studies in sepsis mainly involved immune cells such as macrophages and monocytes, or even whole blood samples[21,23,76]. The role of epigenetics in ECs in sepsis is so far underexposed. Nevertheless, it has been shown that epigenetic regulation via histone modifications and DNA methylation are underlying (some of) the immunological alterations in sepsis[77]. Expression levels of pro-inflammatory cytokines were associated with histone acetylation levels after inflammation[78].

Endothelial dysfunction

After a localized infection at sites where bacteria reside, leukocytes and platelets adhere via adhesion molecules to the surface of ECs and migrate to the underlying tissue. Due to an excessive inflammatory state in sepsis, this response is exaggerated and the endothelial barrier is disrupted via destabilization of cell-cell interactions, which is one of the key phenomena in endothelial dysfunction in sepsis[79]. The loss of barrier integrity results in increased permeability and thereby leakage of fluid and intravascular proteins into the underlying tissues[80]. Vascular leakage can lead to tissue and organ edema, hypotension, shock, and multiple organ failure[20]. In addition to the loss of barrier integrity, ECs are excessively activated and adhesion molecules expression for leukocytes including E-selectin, VCAM1, and ICAM1 are upregulated[81,82]. The upregulation of these adhesion molecules results in initiating leukocyte-binding, rolling, and transmigration of leukocytes into the subendothelial tissue[18,83]. Genetic deletion of Icam1 in mice resulted in resistance to sepsis-induced organ failure, even with high doses of LPS, and reduced neutrophil infiltration in the liver and kidney[84,85]. Enhanced neutrophil infiltration in organs such as the lungs is associated with multiple organ failure[86,87].

Epigenetic alterations in angiogenic genes

Bomsztyk and colleagues were the first who looked into the epigenetic alterations underlying the downregulation of several angiogenic genes in a murine model of acute lung injury (ALI)-induced sepsis[25]. They analyzed epigenetic marks at the genes encoding for Ang1 (Angpt1), Tie2 (Tek), and VEGFR2 (Kdr) in the lung, kidney, and liver. The expression of these genes is known to be decreased in sepsis, and mRNA levels were decreased in the kidney, lung, and liver (Fig 6)[88–90]. To check if the ALI-sepsis mice developed increased endothelial permeability, they analyzed albumin (a plasma component) in fragments of the lung, kidney, and liver. Albumin levels were increased and comparable with other mouse models of sepsis, confirming endothelial dysfunction in the kidney, lung, and liver[91]. Another control was measuring the expression of Ngal (Neutrophil gelatinase-associated lipocalin),

produced by neutrophils and a well-known marker of acute kidney injury (AKI)[92]. Loss of the active mark histone 3 lysine acetylation (H3KAc) at the promoters of Angpt1, Tek, and Kdr was suggested to induce the downregulation of these genes since H3KAc is associated with gene transcription (Fig 7, right panel). Decreased H3KAc marks corresponded with a decrease of RNA Polymerase II (initiates transcription) at these genomic sites, especially in the lung (Fig 7, left panel). Other epigenetic marks that are associated with active gene transcription, H3K4me2 and H3K4me3, were less affected after inducing ALI, except for some loci in the lung. Interestingly, repressive H3K9me3 levels were not altered at these loci, and repressive H3K27me3 levels were slightly increased in especially the kidney. This suggests that the differences in gene expression are mainly dependent on the deacetylation of active marks (H3K9Ac/H3K14Ac) rather than an increase in repressive marks.

Although this was the first paper reporting epigenetic marks underlying altered gene expression in a sepsis-induced model, it has several limitations [as discussed by [93]] that makes the data difficult to interpret. The major limitation of the study is that the epigenetic marks were not measured in a cell-type-specific way. Several types of immune cells infiltrate the organs in sepsis and were analyzed together with pericytes and ECs, thereby making the data not specific and hard to interpret. Ngal is for example mainly expressed by neutrophils, angpt1 by pericytes, and Tek by ECs. Single-cell analysis or sorted cells will enable pronounced data that can be used as a potential drug target. Furthermore, as mentioned by the authors, they only used one time point (6 hours). Alterations in gene expression and thereby probably epigenetic regulation as well were shown in other studies to be time-dependent[90,94,95].

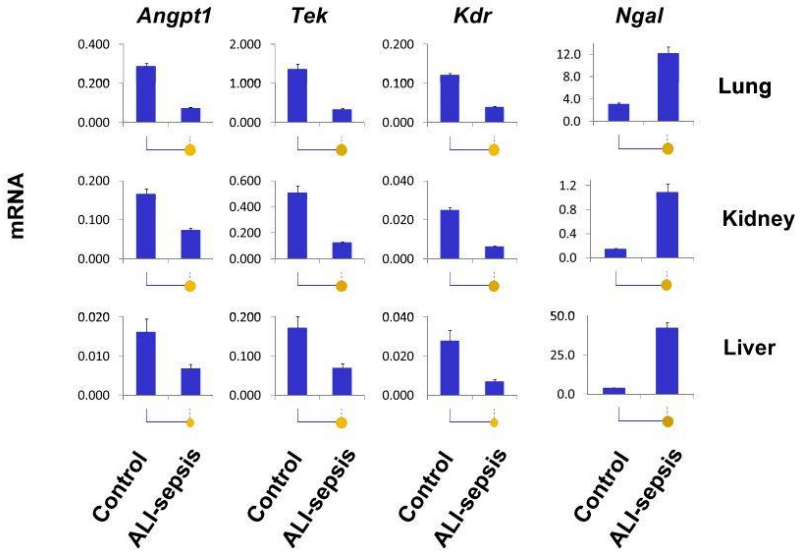

Figure 6. mRNA levels of Angpt1 (Ang1), Tek (Tie2), Kdr (VEGFR2), and Ngal (NGAL) in mice after acute lung injury induced sepsis (ALI-sepsis). Angpt1, Tek, and Kdr were downregulated as is also seen in sepsis. Statistical significance is shown by the circles: a small circle means $P < 0.05$ and a large circle $P < 0.01$. No line means no statistical difference[25].

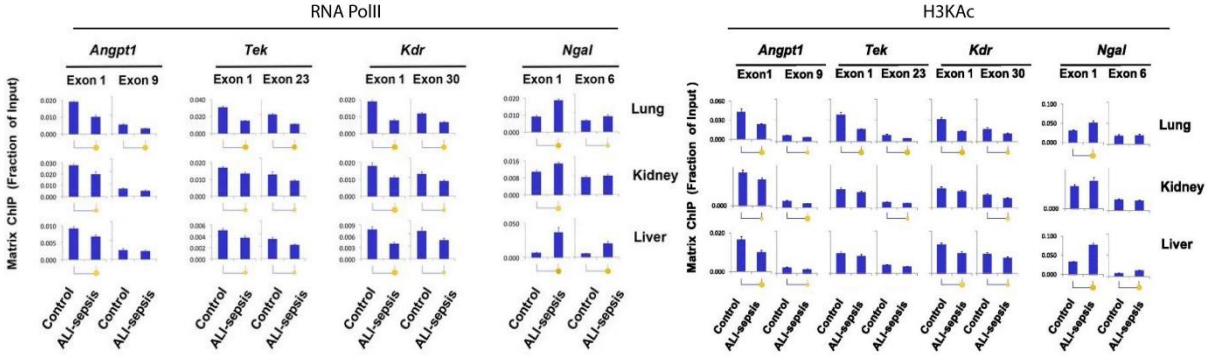

Figure 7. RNA PolII and H3KAc alterations at exons of Angpt1, Tek, Kdr, and Ngal in mice after ALI-sepsis. For each gene, the PolII and H3KAc marks at the first and last exon were analyzed via ChIP with α-PolII (left panel) and α-H3KAc (right panel)[25].

Another paper of the same group in 2018 was aimed to elucidate the epigenetic regulation of the transition of acute kidney injury (AKI) to chronic kidney disease (CKD)[96]. Since this paper was not aimed to analyze epigenetic changes in sepsis, it does not include epigenetic alterations in angiogenic genes like Ang1, Ang2, or Tek, but it does include Vcam1, Tlr4, and Kdr. mRNA levels in AKI induced mice of Ang1, Kdr, and Tek were downregulated as seen in sepsis. Furthermore, Ang2 was upregulated. This confirmed endothelial dysfunction in the AKI model, making it implacable for sepsis-related changes although these experiments were not cell-type specific. In accordance with previous work, downregulation of Kdr (Tie2) mRNA levels corresponded with decreased H3K27Ac marks rather than an increase in repressive marks as H3K27me3 or H3K4me2 (Fig 8)[25]. Remarkably, H3K27Ac marks were significantly reduced at the Vcam1 loci, despite a major upregulation of Vcam1 expression and increased RNA PolII levels (although not significant) (Fig 8). Additionally, other active marks as H3K4me2 and H3K4me1 were significantly reduced. Since VCAM1 is mainly expressed on ECs and not in immune cells or surrounding tissue, epigenetic marks present in other cells than ECs could interfere with the results, highlighting the importance of cell-specific analysis[97]. Next to Vcam1, the receptor for LPS, Tlr4 (Toll-like receptor-4) expression was upregulated after 2 hours of LPS treatment (Fig 8). None of the measured epigenetic marks showed a significant alteration, implicating that previously found Tlr4 and Vcam1 mRNA alterations were not induced by epigenetic mechanisms.

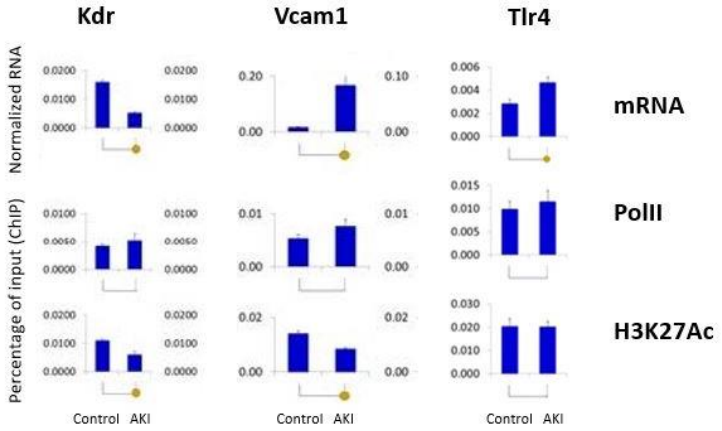

Figure 8. mRNA, PolII and H3K27Ac levels in AKI-induced mice. Kdr expression was downregulated after two hours of LPS induction and Vcam1 and Tlr4 are upregulated. Interestingly, H3K27Ac marks were decreased on exon 1 of Vcam1 and not altered on exon 1 of Tlr4[96].

Endothelial epigenetic alterations during inflammation and disturbed blood flow

Despite the lack of epigenetic studies regarding the endothelial in sepsis, epigenetic endothelial alterations in other diseases are gaining interest. These studies aimed at the endothelium in inflammation and cardiovascular diseases have the potential to be translated to sepsis-associated inflammation and related syndromes.

In vitro *and* in vivo *stimulation of inflammation*

LPS is together with TNFα and IL-1β commonly used to induce a pro-inflammatory activation of HUVECs[98]. Furthermore, LPS is linked to microvascular leakage, septic shock, and sepsis-induced organ failure and is also used to induce sepsis in murine models[75,99]. LPS-induced ALI was shown to alter DNA methylation genome-wide, suggesting a role for LPS in epigenetic alterations in the endothelium[100].

One of the genes that is epigenetically altered by LPS treatment in HUVECs, is the Kruppel-like factor 2 (KLF2)[101]. KLF2 is highly expressed in ECs and is a regulator of the endothelial barrier[102]. KLF2 induces eNOS expression and downregulates VCAM1 and E-selectin. LPS treatment of HUVECs resulted in the downregulation of protein and mRNA levels of KLF2 in a dose- and time-dependent manner (Fig

9). This was linked to enhanced CpG methylation in the promoter region of KL2, in which 12 out of 16 CpG sites were significantly affected (Fig 10). Usage of siRNAs for DNMT1 in combination with LPS resulted in the restoration of KLF2 expression, suggesting a role for DNMT1 in modulating methylation levels at the KLF2 promoter. The combination of LPS with a chemical inhibitor of DNA methyltransferases (AZA) restored most of the altered CpG methylation levels (Fig 10). AZA treatment also restored mRNA and protein levels of E-selectin, VCAM1, and eNOS. Although this study only restricted to HUVECs, these findings support the idea that LPS could induce hypermethylation of KLF2.

ICAM1 mediates next to VCAM1 and E-selectin the adhesion and migration of leukocytes towards the site of inflammation across the endothelium. TNF-α treatment in human brain microvascular endothelial cells (HBMVECs) enhanced expression and protein levels of ICAM1[103]. ChIP analysis of the ICAM1 promoter region in these cells showed a significant reduction of the suppressing marks H3K9me2, H3K9me3, and H3K27me3. H3K9Ac and H3K14Ac were not altered after TNF-α treatment (Fig 11). G9a and EZH2, two histone methyltransferases that mediates histone methylation (H3K9 and H3K27 respectively) and thereby transcriptional repression, were involved in ICAM1 expression[103–105]. Inhibition of G9a and EZH2 led to higher ICAM1 expression, whereas G9a overexpression in HBMVECs blocked ICAM1 expression after TNF-α treatment. The histone demethylase KDM4B is involved in the demethylation of H3K9me3 marks to induce gene transcription, which corresponds with increased binding of KDM4B to the ICAM1 promoter and decreased levels of H3K9 levels[103,106]. Furthermore, G9a and KDM4B were shown to regulate VCAM1 expression as well, suggesting that demethylation plays an important role in the expression of adhesion proteins after inflammation. Although more KDM subtypes (KDM1B and KDM7A) are upregulated when treated with TNF-α, only KDM4B is implicated to play a role in the epigenetic regulation of ICAM1[103,107].

The NF-κB signaling pathway is one of the main mediators of producing pro-inflammatory cytokines and upregulation of adhesion molecules, such as VCAM1, TNF-α, and IL-6[108]. One of the epigenetic regulators that was induced by NF-κB in murine macrophages after inflammatory stimuli was KDM6B (JMJD3)[109]. Also, KDM6B was recruited to the promoter regions of over 70% LPS-induced genes such as Tnf-α, Vegfa, and Icam1. KDM6B is another histone demethylase, catalyzing the demethylation of H3K27me2/3[110]. To study whether KDM6B was also involved in ECs, Yu et al. (2017) stimulated HUVECs with LPS, resulting in increased KDM6B expression within two hours. Both NF-κB and KDM6B were accumulated at the promoter region of target genes TNF-α, MMP9, IL-6, IL-1β, and COX2, except for ICAM-1 (Fig 12A, B)[111]. H3K27me3 levels were decreased at the same loci, implicating that KDM6B synergizes with NF-κB after LPS stimulation (Fig 12C). H3K4me3 levels of IL-6, IL-1β, and COX2 were decreased although expression levels were increased, indicating that LPS treatment has multiple effects on H3K4me3 (Fig 12D).

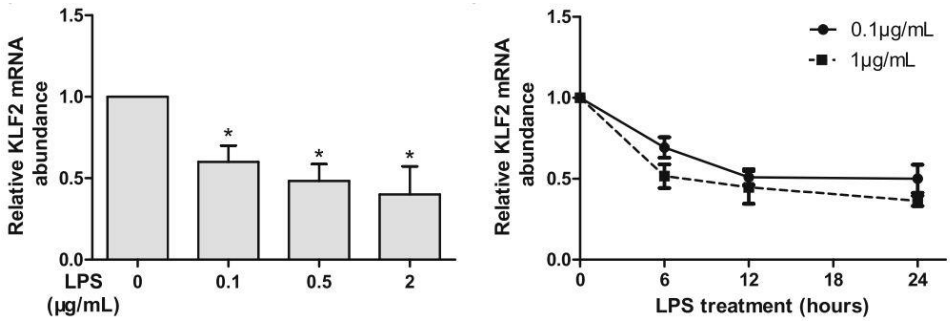

Figure 9. KLF2 expression is dose- and time-dependent of LPS treatment[101].

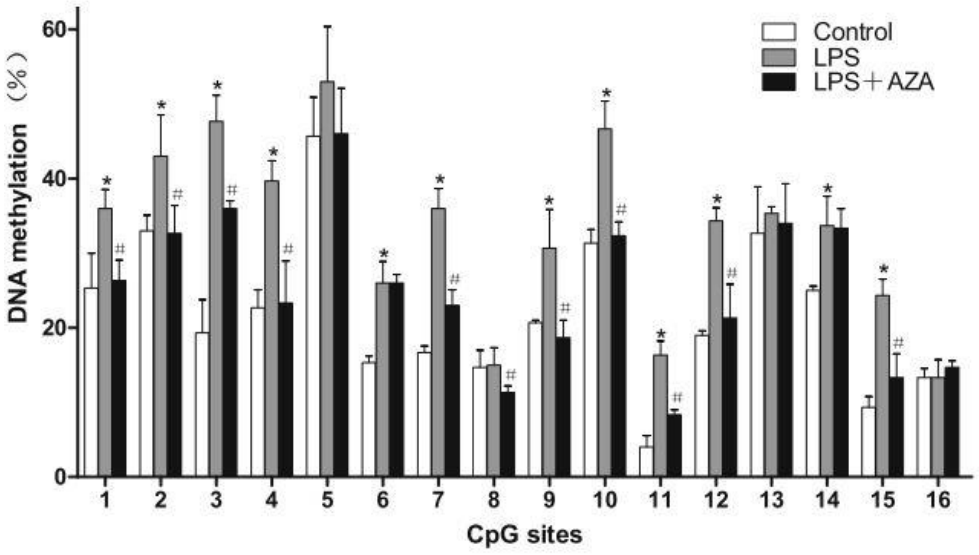

Figure 10. DNA methylation at CpG sites found at the promoter region of KLF2[101]**.**

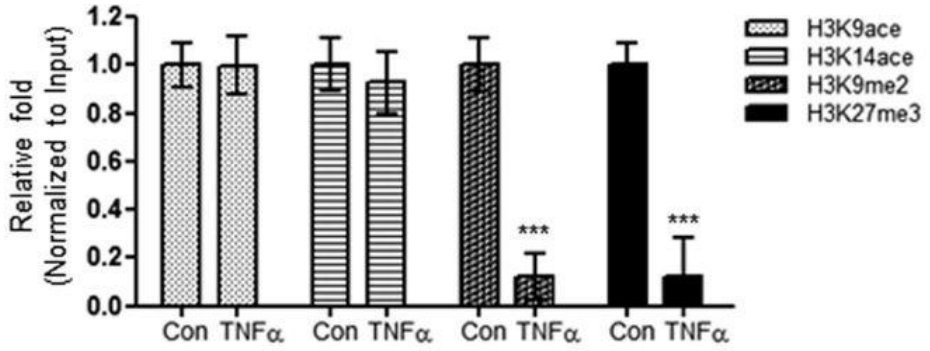

Figure 11. Epigenetic marks at the ICAM1 loci in HBMVECs, with and without TNF-α treatment. H3K9Ac and H3K14Ac marks were not altered after TNF-α treatment. H3K9me2 and H3K9me3 marks were significantly decreased[103].

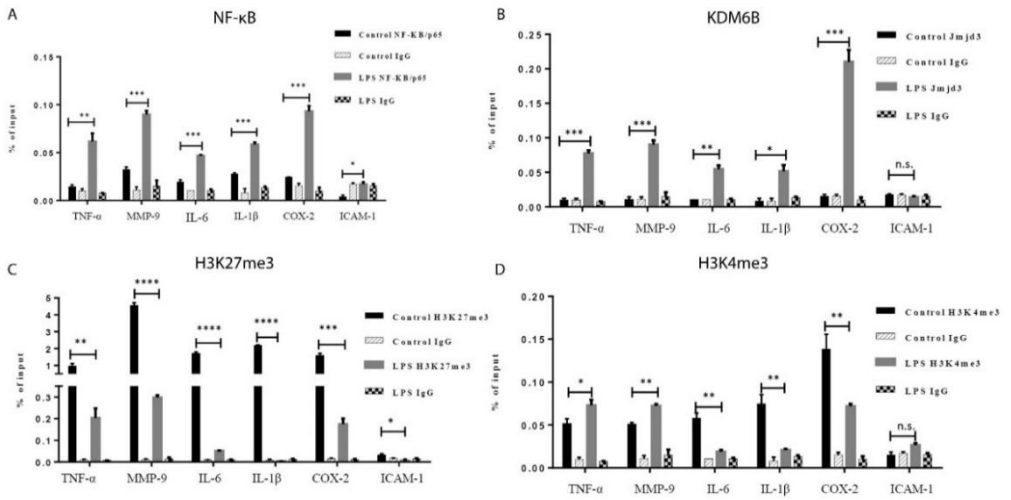

Figure 12. ChIP analysis of promoter regions of TNFa, MMP9, IL-6, IL-1β, COX2, and ICAM1. ChIP assay was performed in HUVECs with and without LPS treatment. (A) NF-κB. (B) KDM6B (C) H3K27me3 (D) H3K4me3[111].

Flow-dependent epigenetic regulation

Hypotension is one of the criteria to identify patients with sepsis as the blood flow in patients with sepsis is decreased because of vasodilation[1,112]. The frictional force in the endothelium is generated by blood flow and is called shear stress[113]. Stable or steady blood flow (s-flow) is necessary for regulating endothelial functions since it increased the expression of KLF2, eNOS, and Nrf2, important regulators of maintaining barrier integrity and the endothelial inflammatory response (Fig 13)[114,115]. On the other hand, disturbed blood flow (d-flow) is associated with endothelial dysfunction, as low shear stress resulted in increased endothelial permeability[113,116,117].

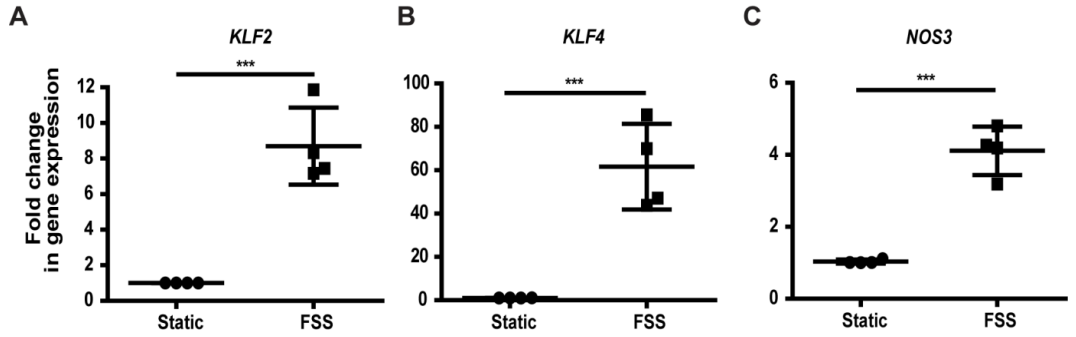

Figure 13. Shear stress upregulates gene expression of protective genes KLF2, KLF4, and NOS3 (eNOS). Cells were exposed for 72h to s-flow and shear stress[115].

Blood flow and shear stress regulate endothelial gene expression, partly via DNA methylation[118,119]. In stable blood-flow condition, DNMTs are downregulated, which allows transcription of genes that are key mediators of endothelial function, like Klf4 and Hoxa5[118,119]. Disturbed blood flow was associated with an upregulation of DNMTs (DNMT1 and DNMT3A) and increased DNA methylation, thereby suppressing gene transcription[118,120]. Cells pre-treated with the DNMT1 inhibitor AZA before being subjected to shear stress experiments showed no increased DNA methylation, indicating that DNMT1 played an important role in regulating gene expression induced by d-flow [120]. This was supported in another study, were AZA and DNMT1 siRNA reduced methylation and a decrease in endothelial inflammation *in vitro*[118]. *In vivo* experiments from the same study showed that the hypermethylated promoter regions induced by disturbed blood flow were rescued by AZA treatment, as the regions regained their normal methylation patterns. Furthermore, AZA treatment inhibited the formation of atherosclerosis in mice.

Histone modifications can regulate endothelial gene expression in a flow-mediated way, and dysregulation of (de)acetylation and (de)methylation can underlie vascular diseases like atherosclerosis[121,122]. The expression of the histone methyltransferases EZH2 is regulated by shear stress in HUVECs, as s-flow decreased EZH2 expression[115]. Furthermore, EZH2 was shown to regulate genes involved in cell adhesion like ICAM1. KD of EZH2 resulted in the activation of MAPK7 signaling, which has protective effects on the endothelium, even in the absence of shear stress[115,123]. Another study showed that both EZH2 and H3K27me3 were reduced by s-flow, both *in vitro* and *in vivo*, without affecting KDM6B expression[124]. Transcriptome data and RNA-sequencing showed an important function for the IGFBP5 gene, a mechanosensitive gene, induced by VEGF[125]. H3K27me3 silences this gene, but after treatment with EZH2 siRNA, the expression was upregulated. Interestingly, overexpression of IGFBP5 decreased ICAM1 and VCAM1 expression after induced inflammation of HUVECs via TNF-α, suggesting an anti-inflammatory role for IGFBP5 (Fig 14). This corresponded with a reduced adhesion of monocytes to ECs. These results, combined with the downregulation of IGFBP5 in murine skeletal muscle cells after inducing sepsis, make IGFBP5 a potential target for treatment[126].

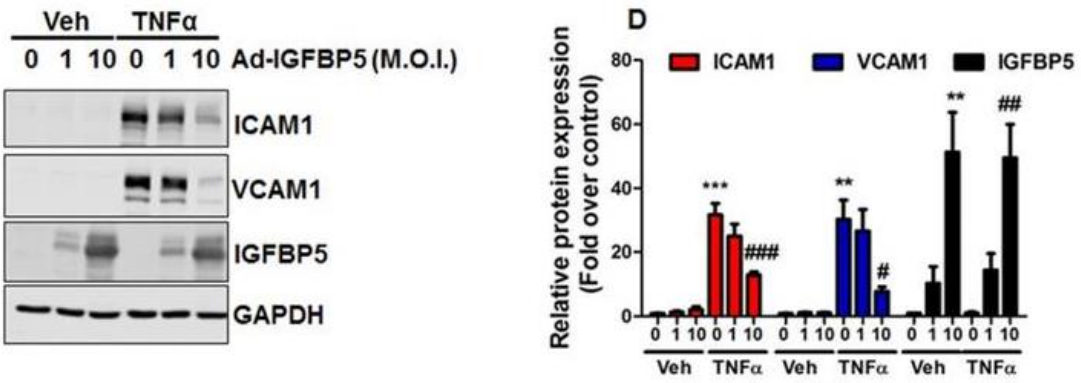

Figure 14. IGFBP5 overexpression inhibits inflammation in ECs. HUVECs were treated with TNF-α to induce inflammation. IGFB5 decreases both protein and mRNA expression of ICAM1 and VCAM1[124].

Shear stress mediates histone acetylation and deacetylation in endothelial dysfunction [reviewed in [127]]. Low and disturbed blood flow induced nuclear accumulation and sustained expression of HDAC-1/2/3 (class I) and HDAC-5/7 (class II) in ECs, while s-flow induced a nuclear export of HDAC-5/7 in a phosphorylation-dependent manner[128]. Increased HDAC1/2/3 levels downregulated Nrf2, whereas increased HDAC-3/5/7 was associated with downregulation of KLF2 via MEF2 acetylation (Fig 15).

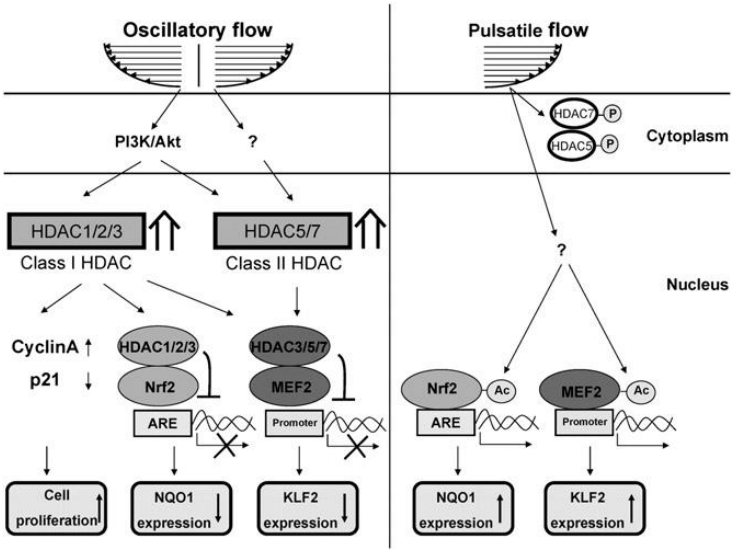

Figure 15. Summarizing picture of the influence of oscillatory flow (d-flow) and pulsatile flow (s-flow) on HDAC signaling and gene expression in ECs[128].

Table 2. Altered gene expression in sepsis, inflammation, and disturbed blood flow induced by epigenetic mechanisms.

Gene	mRNA	Mechanism	Model	Ref
		Sepsis and inflammation		
VCAM1	↑	Decrease of H3K27Ac and H3K4me2	AKI-mice	96
		Decrease of repressive histone marks (KDM4B)	HBMVECs	103
ICAM1	↑	Decrease of H3K9me2 and H3K27me3 (KDM4B)	HBMVECs	103
Tlr4	↑	Unknown	AKI-mice	96
TNF-α	↑	Decrease of H3K27me3 (KDM6B)	HUVECs	111
		Increase of H3K27Ac and H3K4me3	AKI mice	96
IL-6	↑	Decrease of H3K27me3 (KDM6B)	HUVECs	111
IL-1β	↑	Decrease of H3K27me3 (KDM6B)	HUVECs	111
KLF2	↓	DNA methylation (DNMT1)	HUVECs	101
Ang1	↓	Loss of H3KAc	ALI-mice	25
Tie2 (Tek)	↓	Loss of H3KAc	ALI-mice	25

VEGFR (Kdr)	↓	Loss of H3KAc	ALI-mice	[25]
		Loss of H3K27Ac	ALI-mice	[96]
Disturbed flow				
KLF4	↓	DNA methylation (DNMT3A)	HUVECs	[119]
Klf3	↓	DNA methylation (DNMT1)	Mice	[118]
Hoxa5	↓	DNA methylation (DNMT1)	Mice	[118]
IGFBP5	↓	Histone methylation (EZH2)	HUVECs & mice	[124]
Nrf2	↓	Histone acetylation (HDAC1/2/3)	HUVECs	[128]
KLF2	↓	Histone acetylation (HDAC5/7)	HUVECs	[128]
Steady flow				
KLF2	↑	Downregulation of EZH2	HUVECs	[115]
KLF4	↑	Downregulation of EZH2	HUVECs	[115]
NOS3	↑	Downregulation of EZH2	HUVECs	[115]

Therapeutic implications and future directions

The majority of studies related to epigenetic alterations in sepsis are focused on alterations in immune cells rather than ECs. Therefore, this thesis aimed to elucidate the epigenetic alterations and mechanisms in ECs that underlie endothelial dysfunction and organ failure. By including studies aimed at epigenetic alterations in the endothelium after inflammation and disturbed blood flow, a broader perspective on endothelial dysfunction sepsis is generated.

Both immune cells and ECs undergo several genetic and epigenetic alterations in sepsis. Roughly, you can divide the altered ECs genes into three groups: adhesion molecules, inflammatory cytokines, and genes associated with endothelial integrity [summarized in Table 2]. Adhesion molecules as VCAM1, ICAM1, and E-selectin are upregulated by ECs after inflammation, although only VCAM1 and ICAM1 are epigenetically regulated[96,103]. This is mainly mediated by KDM4B, which is upregulated by inflammation and leads to a reduction of H3K9 methylation at the promoter region of ICAM1. Of note, this was performed in brain microvascular endothelial cells (HMBVECs) instead of HUVECs, which is more common to study EC inflammation. Since the structure and function of ECs differ per region of the body, it is interesting to study whether these alterations are found in HUVECs as well[16]. Furthermore, alterations in genes associated with endothelial integrity were found, resulting in an increased endothelial permeability. Ang1, Tie2, and KLF2 were downregulated via epigenetic mechanisms. Interestingly, KLF2 was mediated by both histone acetylation and DNA methylation, whereas Ang1 and Tie2 were influenced by the loss of H3K acetylation[25,101,128]. The expression of inflammatory cytokines such as IL-6, TNF-α, and IL-1β were regulated via epigenetic mechanisms after inflammation as well. This was mainly mediated by upregulated levels of KDM6B, resulting in decreased levels of H3K27me3[111].

Epigenetic inhibitors

Several studies have used DNA methyltransferases and histone deacetylase inhibitors (HDACi) in their studies to confirm whether genes were epigenetically regulated or not[101]. Additionally, HDACi were used to study whether it improved the outcome of sepsis-induced models, such as mice and rats [summarized in [24,27]]. HDACi improved the endothelial function in kidneys, lungs, and liver by inhibiting the pro-inflammatory response in mice induced with hemorrhagic shock[26]. More specifically, inhibition of HDAC6 (Tub-A) in mice resulted in improved survival, and a reduction of inflammatory cytokines TNF-α and IL-6[129,130]. HDAC1/2 inhibition by TSA reduced pro-apoptotic gene expression and reduced inflammation in the lung[131,132]. In these studies, TSA was administered together with AZA, thereby inhibiting both HDAC and DNA demethylases. When sodium butyrate was used to inhibit HDAC1/2, this had a protective effect on the liver, kidney, and lung, and the rats showed an improved survival[133].

Although a variety of HDAC inhibitors used in sepsis-induced mouse models showed an improved outcome, the exact epigenetic regulatory mechanisms remain unknown. Since HDAC does not only target histone tails but also plays a role in essential cellular processes, administration of HDACi can result in off-target effects[134]. Additionally, a knockdown of a specific HDAC can be compensated

by other HDACs, suggesting the same when inhibitors are used[135]. Therefore, the use of specific HDACi and combined treatment is mandatory for fine-tuning dysregulated genes in sepsis and to reduce off-target effects.

KDM4B and KDM6B are two main histone demethylases involved in the inflammatory response in ECs[103,109,111]. They are responsible for the demethylation of H3K9 and H3K27 respectively, and increased KDM4B expression is associated with enhanced expression of adhesion molecules VCAM1 and ICAM1. Inflammatory cytokines like TNF-α and IL-6 are altered as well, proposed through the upregulation of KDM6B. Combined treatment with both KDM4B and KDM6B inhibitors could be a treatment to reduce the inflammatory state of ECs. KDM4B inhibition allows active demethylation of H3K4me2/3 at pro-inflammatory promoters via KDM1A, thereby reducing inflammation[136]. Furthermore, pretreatment with a KDM4B inhibitor (ML324) in HBMVECs stimulated with TNF-α decreased ICAM1 protein levels and reduced leukocyte adhesion[103]. However, KDM4B regulates multiple metabolic genes as well, and $Kdm4b^{-/-}$ mice are more prone to obesity and metabolic dysfunction[137]. Temporary treatment with KDM4B inhibitors could be useful to suppress adhesion molecule expression, but long-term treatment would not be beneficial as the risk of obesity increases in mice.

Inhibition of KDM6B in NK cells resulted in increased levels of H3K27me3 at the transcription start sites of pro-inflammatory cytokines[138]. Although KDM6B inhibition downregulated genes involved in NK cell killing, 48h of treatment did not have a significant impact on their ability to kill cancer cells *ex vivo*. Despite this is not tested in mice, inhibition of KDM6B would reduce the inflammatory state without affecting their functionality. Furthermore, inhibition led to IFN-γ suppression, which is proposed to mediate neutrophil function[138,139]. This is interesting as increased neutrophil infiltration into the surrounding tissues is linked to sepsis and can lead to organ failure[86,87]. In mice with induced sepsis, inhibition of KDM6B by GSKJ4 protected against early septic death[140]. Additionally, pro-inflammatory cytokines TNF-α, IL-6, and IL-1β were downregulated. The most promising result of this study was that the inhibitor promoted the clearance of bacteria in the blood, which is crucial for reducing inflammation and organ injury.

Another reason for combined treatment is the fact that multiple genes, like KLF2, are regulated by both DNA methylation as histone acetylation[101,128]. Treatment of LPS-stimulated macrophages with KDM6 inhibition (AZA) and HDACi (TSA) alters the induced inflammatory response by decreasing H3K9Ac levels and enhanced H3K27me3 levels at pro-inflammatory genes[131].

Epigenetic editing via CRISPR/dCas9

The broad spectrum of epigenetic alterations and high specificity in ECs in sepsis makes it difficult to treat. Histone inhibitors have their disadvantages, and therefore there is a need for new treatment possibilities. A novel technique to alter the dysregulated gene expression in sepsis is epigenetic editing via CRISPR technology. CRISPR is an adaptive microbial immune system that is edited for the use of, among others, (epi)genome editing in cells[141]. The engineered CRISPR/Cas9 system consists of a single guide RNA (sgRNA), which is a target-specific sequence, and a Cas9 protein. Cas9 has two nuclease domains, which generate double-strand breaks by cutting both DNA strands (extensively reviewed by [141,142]).

There is also a CRISPR system with inactivated endonuclease domains, also known as dead or deactivated Cas9, dCas9[141]. By inactivating its endonuclease domains via point mutations, it cannot cut the DNA, while maintaining their ability to target the desired DNA sequence. Therefore, dCas9 in combination with an attached eraser or writer can alter the epigenetic state of cells, without making cuts or mutations in the DNA. This makes epigenome editing less invasive than genome engineering. By fusion of dCas9 with a histone acetyltransferase, highly specific and robust gene activation was achieved *in vitro*[143]. *In vivo*, multiple phenotypes of human diseases has been improved in mouse models, including acute kidney injury (AKI) and Alzheimer[144,145].

A potential target of epigenetic editing via CRISPR/dCas9 would be KLF2, as viral overexpression leads in monocytes inhibited the induction of pro-inflammatory cytokines after LPS treatment in monocytes[146]. Since KLF2 is a main regulator of the endothelial barrier function and is

affected in inflammatory diseases like sepsis, KLF2 could be a novel promising target for treatment [102,147].

Conclusion

It can be discussed if the downregulation of the inflammatory response by immune cells is beneficial, as temporary treatment often does not clear the infection itself. Altering genes that mediate the endothelial barrier could be more promising as a quick action to prevent vascular leakage and thereby organ failure. Some examples of potential target genes would be KLF2, ANG1, and VEGFR. Furthermore, both immune cells and ECs mediate the inflammatory reaction in patients. An extra challenge is that in addition to the restoration of the epigenetic state, the bacteria have to be cleared as well.

To conclude, epigenetic editing via CRISPR/dCas9 seems to be the most promising therapeutic option, as it is already shown to improve AKI phenotypes *in vivo*. Although the effect is not yet studied in ECs and dCas9 has not been performed in HUVECs, it is an encouraging new option to study whether it can be used to treat sepsis. This is mainly since HDACi and other epigenetic inhibitors are not site-specific and target a wide range of cellular processes, although combined treatment can enhance sepsis-outcome as well.

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