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Relevance of the mRNA-binding protein KSRP for innate immune processes

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Declaration of authorship

I, Vanessa Bolduan, hereby declare that this dissertation entitled “Relevance of the mRNA-binding protein KSRP for innate immune processes” was written by myself without unauthorized external assistance and that only sources (materials, apparatus, literature) acknowledged in this work were used. I have not submitted the work presented for any scientific examination and I have not submitted the work or any part of it to any other faculty or department as a dissertation.

Mainz, September 2024

Vanessa Bolduan

Preface

The experiments presented in this dissertation were performed from November 2021 until August 2024 at the University Medical Center Mainz in the Department of Dermatology in the lab of Prof. Dr. Stephan Grabbe under the supervision of PD Dr. Matthias Bros.
The obtained data have been presented in two publications and one submitted manuscript attached to this thesis (see Appendix 1-3).

[1] K. A. Palzer*, **V. Bolduan***, R. Käfer, H. Kleinert, M. Bros, A. Pautz.
The Role of KH-Type Splicing Regulatory Protein (KSRP) for Immune Functions and Tumorigenesis.
Cells, **2022**, 11(9):1482
* Shared first authorship

[2] **V. Bolduan**, K.A. Palzer. C. Hieber, J. Schunke, M. Fichter, P. Schneider, S. Grabbe, A. Pautz, M. Bros.
The mRNA-Binding Protein KSRP Limits the Inflammatory Response of Macrophages.
International Journal of Molecular Sciences, **2024**, 25(7), 3884

[3] **V. Bolduan**, K.A. Palzer, F. Ries, N. Busch, A. Pautz, M. Bros
KSRP deficiency attenuates the course of pulmonary aspergillosis, associated with elevated pathogen-killing activity of innate myeloid immune cells.
submitted to Cells, **2024**

Key findings and their scientific relevance are summarized in the following sections.

Mainz, September 2024
Vanessa Bolduan

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Graphical Abstract

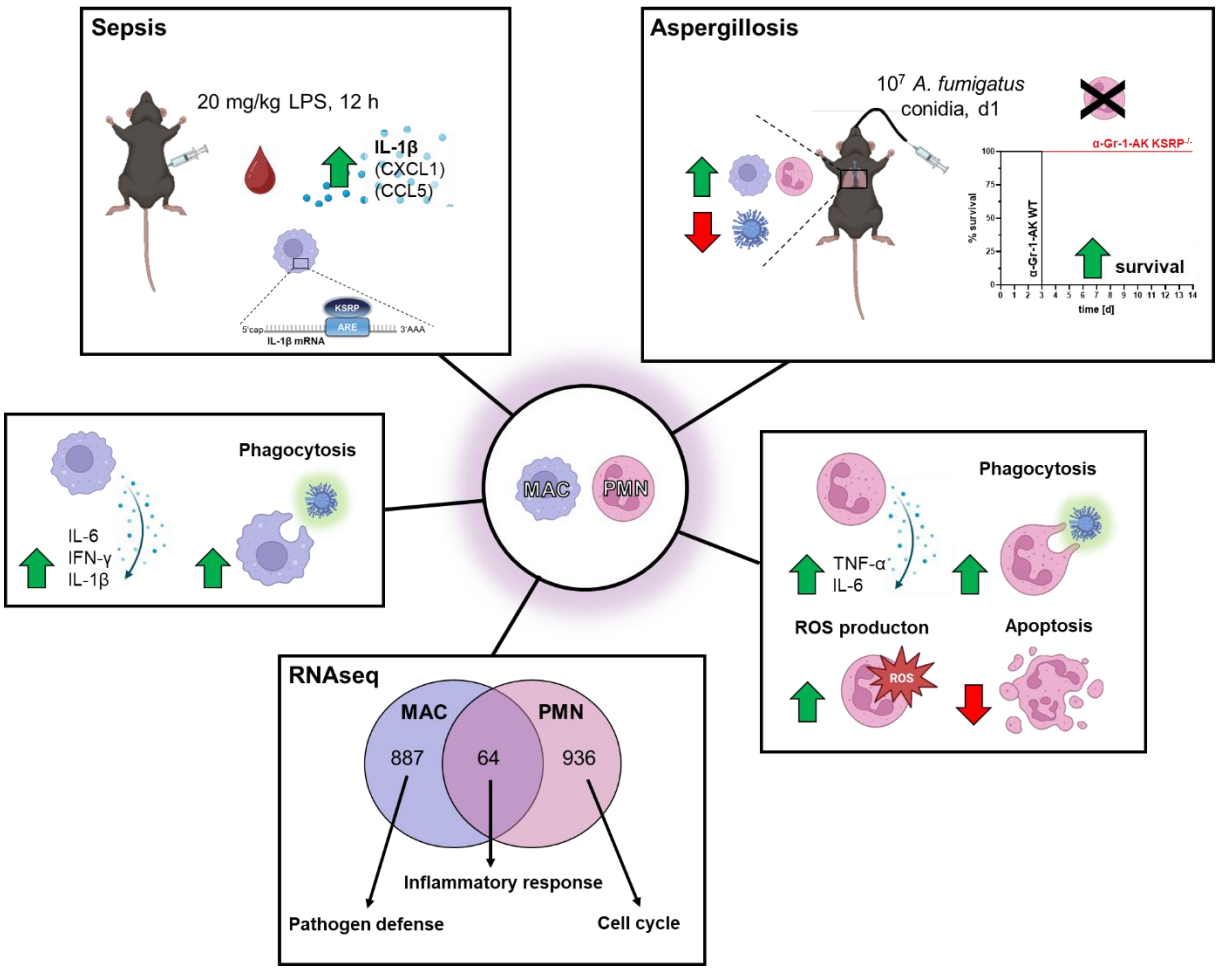

Figure 1. KSRP negatively regulates immune cell functions of PMN and MAC.
Shown are the key findings of this dissertation; created with BioRender.com. Sera from KSRP$^{-/-}$ mice present with elevated levels of IL-1β in an LPS-induced sepsis model. Consistently, KSRP was shown to regulate the stability of LPS-induced IL-1β mRNA in macrophages (MAC). In order to ascertain whether the absence of KSRP exerts beneficial effects in an infection model, invasive pulmonary aspergillosis (IPA) was induced in KSRP$^{-/-}$ and WT mice. KSRP$^{-/-}$ mice treated with *Aspergillus fumigatus* conidia (AFC) exhibited higher frequencies of polymorphonuclear neutrophils (PMN) and MAC along with lower fungal burden in the lung one day after inoculation. As PMN-deficient KSRP$^{-/-}$ mice, in contrast to WT mice, survived IPA, it was hypothesized that the elevated production of pro-inflammatory mediators and the increased pathogen-defense activity by MAC compensated for the lack of PMN function. In fact, KSRP-deficient PMN and MAC displayed increased production of pro-inflammatory cytokines following stimulation, accompanied by increased phagocytic uptake of AFC. In addition, PMN showed increased production of reactive oxygen species (ROS) in response to stimulation as well as reduced levels of apoptosis. A comparative analysis of RNA sequencing (RNAseq) data revealed that the majority of genes that were upregulated in PMN were found to be associated with the cell cycle control, while in MAC the upregulated genes were linked to anti-pathogen mechanisms. Moreover, both PMN and MAC overexpressed genes associated with the inflammatory response.

Abstract

The innate immune system is the body's first, rapid and nonspecific defense against pathogens. It involves phagocytic cells such as macrophages (MAC) and polymorphonuclear neutrophils (PMN) that recognize and eliminate invading pathogens. MAC/PMN are the first responders in acute infections and play critical roles in pathogen clearance and inflammation using various mechanisms such as phagocytosis, degranulation and the release of reactive oxygen species (ROS) to fight infection.

Inflammatory disorders are frequently characterized by a dysregulation of pro-inflammatory gene expression, which is controlled at the post-transcriptional level by RNA-binding proteins (RBP) via regulation of mRNA stability and translational efficacy. KH-type splicing regulatory protein (KSRP) is a multifunctional RBP that influences gene expression at various levels due to its ability to bind AU-rich motifs (ARE) within the 3´-untranslated region (3'-UTR) of mRNA species, which in many cases encode dynamically regulated proteins like cytokines. Consequently, it has been postulated that KSRP plays a role in the pathogenesis of inflammatory disorders. Due to the important role of MAC and PMN in innate immune responses, the overall aim of this dissertation was to delineate the role of KSRP for the immunophenotype and function of these innate cell types.

The results of this dissertation demonstrate that KSRP-deficient mice showed elevated levels of pro-inflammatory cytokine IL-1β in an LPS-induced sepsis model. In line, it was demonstrated that in MAC KSRP regulates LPS-induced IL-1β mRNA stability. To evaluate if KSRP deficiency has beneficial effects in an infection model, we induced invasive pulmonary aspergillosis (IPA) in KSRP-deficient ($KSRP^{-/-}$) and wild-type (WT) mice. KSRP-deficient mice treated with *Aspergillus fumigatus* conidia (AFC) demonstrated an increased frequency of PMN and MAC in lungs, accompanied by lower levels of fungal burden one day following inoculation. In addition, PMN and MAC from $KSRP^{-/-}$ mice exhibited elevated phagocytic uptake of AFC, along with increased ROS production by PMN following stimulation. A comparison of the RNA sequencing data revealed that 64 genes related to inflammatory and immune responses were commonly upregulated by PMN and MAC. The majority of the upregulated genes in PMN were associated with the cell cycle, while in MAC upregulated genes contribute to pathogen defense. In agreement with elevated expression of cell cycle-related genes, PMN from KSRP-deficient mice presented with reduced apoptosis levels. Taken together, the results of this dissertation indicate that KSRP contributes to negative regulation of cytokine expression and effector functions of PMN and MAC, thereby limiting their pathogen-killing activity. As a perspective, targeted inhibition of KSRP in innate myeloid cell types may help to improve the course of infections, especially in immunocompromised patients.

Zusammenfassung

Das angeborene Immunsystem stellt die erste, schnelle und unspezifische Abwehr des Körpers gegen Krankheitserreger dar. Es umfasst phagozytische Zellen wie Makrophagen (MAC) und polymorphkernige Neutrophile (PMN), welche in der Lage sind, eindringende Krankheitserreger zu erkennen und zu eliminieren. MAC und PMN stellen die erste zelluläre Reaktion auf Gewebeverletzungen und Infektionen dar. Zu diesem Zweck nutzen sie verschiedene Mechanismen wie Phagozytose, Degranulation und die Freisetzung reaktiver Sauerstoffspezies (*reactive oxygen species*, ROS), um Infektionen zu bekämpfen. Entzündliche Erkrankungen sind häufig durch eine Deregulation der proinflammatorischen Genexpression gekennzeichnet, welche auf posttranskriptioneller Ebene durch RNA-bindende Proteine (RBP) über die Regulierung der mRNA-Stabilität gesteuert wird.

Das KH-type splicing regulatory protein (KSRP) ist ein multifunktionales RBP, das die Genexpression auf verschiedenen Ebenen beeinflusst. Es ist in der Lage, AU-reiche Motive (ARE) innerhalb der 3′-untranslatierten Region (3′-UTR) von mRNA-Spezies zu binden, die in vielen Fällen für dynamisch regulierte Proteine wie Zytokine kodieren. In der Konsequenz wurde postuliert, dass KSRP eine Rolle bei der Pathogenese von Entzündungserkrankungen spielt. Aufgrund der essenziellen Rolle von PMN und MAC bei angeborenen Immunantworten war das Ziel dieser Dissertation, die Funktion von KSRP für den Immunphänotyp und die Funktion beider angeborener Zelltypen zu charakterisieren.

Die Ergebnisse der vorliegenden Dissertation demonstrieren, dass KSRP-defiziente Mäuse im Rahmen eines Lipopolysaccharid (LPS)-induzierten Sepsis-Modells eine signifikante Erhöhung des proinflammatorischen Zytokins IL-1β aufwiesen. Des Weiteren konnte nachgewiesen werden, dass KSRP die Stabilität der durch LPS induzierten IL-1β-mRNA reguliert. Im Folgenden wurde untersucht, ob eine KSRP-Defizienz in einem Infektionsmodell Vorteile hinsichtlich der Pathogenabwehr mit sich bringt. Zu diesem Zweck wurde in Wildtyp- (WT) und $KSRP^{-/-}$-Mäusen eine invasive pulmonale Aspergillose (IPA) induziert. Einen Tag nach der Behandlung mit *Aspergillus fumigatus*-Konidien (AFC) konnte bei KSRP-defizienten Mäusen eine erhöhte Frequenz von PMN und MAC, sowie eine geringe Pilzbelastung in der Lunge gefunden werden. Zudem zeigten PMN und MAC von $KSRP^{-/-}$-Mäusen eine gesteigerte phagozytische Aufnahme von AFC, zudem produzierten PMN mehr reaktive Sauerstoffspezies (ROS) nach Stimulation. Ein Vergleich der RNA-Sequenzierungsdaten ergab, dass 64 Gene, die mit Entzündungs- und Immunreaktionen assoziiert sind, von PMN und MAC gemeinsam nach Stimulation aufreguliert werden. Die Analyse der hochregulierten Gene in PMN ergab, dass ein Großteil von ihnen mit dem Zellzyklus assoziiert ist. Demgegenüber stehen die hochregulierten Gene in MAC, welche hauptsächlich am Abwehrmechanismus gegen Pathogene beteiligt sind. Daran anschließend, konnte eine verringerte Apoptose bei PMN von KSRP-defizienten Mäusen beobachtet werden. Die

Ergebnisse dieser Dissertation lassen den Schluss zu, dass KSRP eine negative Regulierung der Zytokinexpression und der Effektorfunktionen von PMN und MAC bewirkt, wodurch deren pathogenabtötende Aktivität eingeschränkt wird. Eine gezielte Hemmung von KSRP in diesen angeborenen myeloischen Zellen könnte zukünftig dazu beitragen, den Verlauf von Infektionen zu verbessern, insbesondere bei immungeschwächten Patienten.

1. Introduction

1.1 KH-type splicing regulatory protein (KSRP)

KSRP (K homology [KH]-type splicing protein, KHSRP) is a 747-amino acid long, single-stranded nucleic acid-binding protein that interacts with target ribonucleic acid (RNA) species in the nucleus and cytoplasm [5]. Figure 2 illustrates the structural organization of the KSRP protein, which can be divided into a central region and additional regions on either side [6]. The central region contains four KH domains that are responsible for the interaction with single-stranded nucleic acids [7]. While KH domain 3 preferentially binds to G-containing sequences, KH domains 1, 2, and 4 bind to various sequence motifs with moderate selectivity. In contrast to the central region, the N- and C-termini exhibit low complexity, are posttranslationally modified and contain elements that facilitate protein-protein interactions [6]. The N-terminal part comprises a proline-glycine-rich region and includes a nuclear localization signal, whereas the C-terminal part contains a glutamine-rich region with four Y-rich repeats [6, 8]. Consequently, the structure of KSRP is crucial for its ability to bind to various target genes.

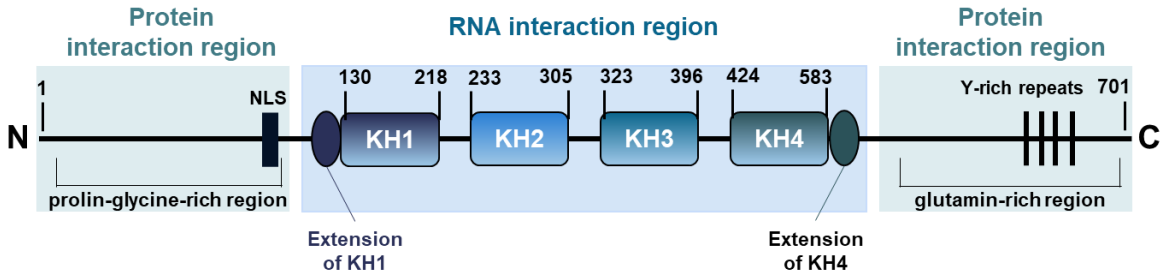

Figure 2. Schematic illustration of KSRP protein structure.
Highlighting the domain organization in the central region, responsible for RNA interaction, and the two flanking regions at the N- and C-termini, which are essential for protein interaction. Figure modified from **[1]**.

1.2 KSRP regulates gene expression on various levels

Regulation of gene expression involves multiple steps, including transcription, messenger RNA (mRNA) processing, nuclear export, translation, and protein degradation. Thereby the regulatory mechanisms can intervene at various levels and at different timepoints [9].

The first step in gene expression starts in the nucleus with the transcription of DNA into RNA by a DNA-dependent polymerase [10]. Transcription can be regulated in different ways [11]. An important mechanism of transcriptional regulation is the accessibility of DNA to protein complexes for initiation of gene expression. In this regard, transcription factors are able to bind DNA in the promoter region and thereby initiate, enhance or inhibit the process of RNA formation [12]. Post-transcriptional regulation occurs through the processing of precursor

mRNA (heterogeneous nuclear (hn)RNA) into mature mRNA or the transport of mRNA from the nucleus to the cytoplasm. The stability of transcripts can be influenced by RNA-binding proteins (RBP) or microRNAs (miRNAs) [13, 14]. The final step in gene expression is the translation of the mRNA into protein, which takes place in the cytoplasm. Translation can be enhanced or inhibited via various mechanisms [15]. Post-translational regulation includes diverse modifications of amino acids determining the activity and lifespan of the protein [16].

In 1996, KSRP was initially identified as a transcription factor that regulated expression of the c-myc oncogene [8]. For this, KSRP binds to the FUSE motif of the c-myc promoter and the four Y-rich regions within its C-terminus subsequently activate c-myc transcription [8]. One year later, KSRP was shown to function as a pre-mRNA splicing regulatory protein [17]. As part of a multi-protein complex, KSRP binds to an intronic splicing enhancer element of the proto-oncogene c-src, thereby regulating the alternative splicing of c-src pre-mRNA [17].

KSRP regulates gene expression on multiple levels. In the nucleus, it acts as a transcription [8] and splicing factor [17], while in the cytoplasm, it controls mRNA stability by promoting its decay [18-21] and inhibiting its translation [22] (Figure 3). In addition, KSRP exerts an indirect influence on gene expression by facilitating the maturation of a subset of miRNAs, which in turn modulate the expression of numerous genes [23-28]. Some of these regulatory mechanisms are described in more detail below.

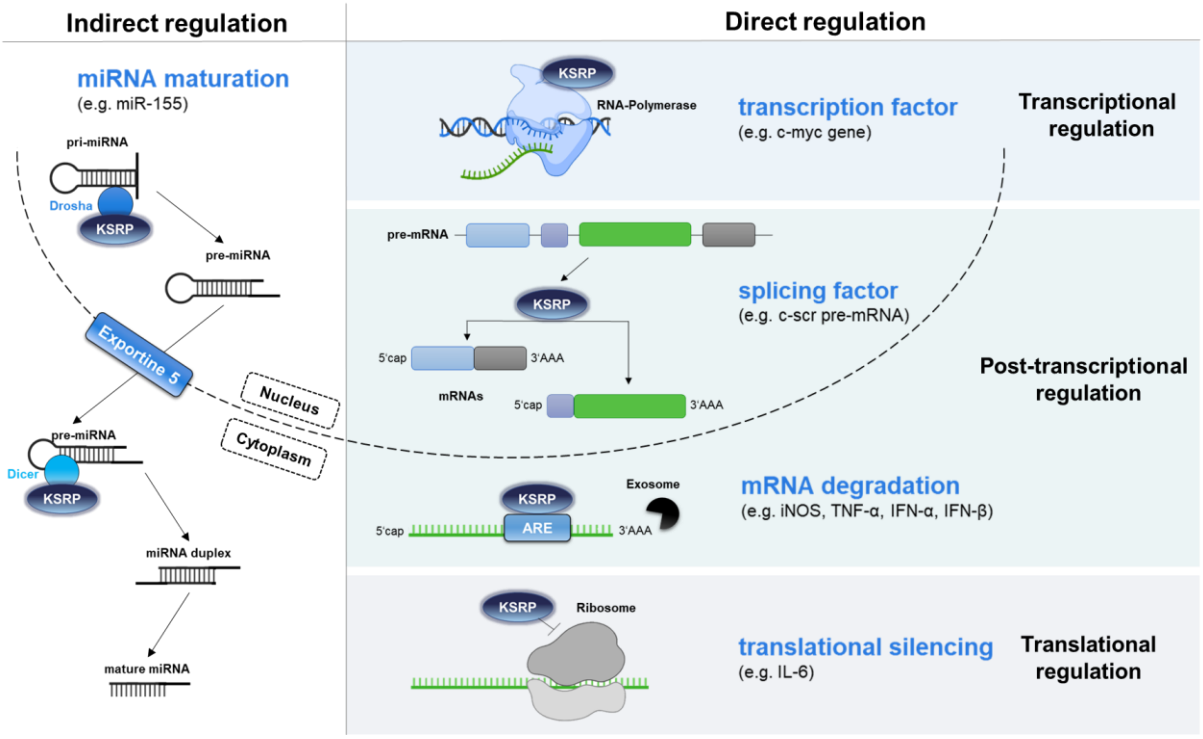

Figure 3. KSRP regulates gene expression at multiple levels.

In the nucleus, it acts as a transcription and splicing factor, while in the cytoplasm, it facilitates the rapid degradation of ARE-containing mRNA by recruiting enzymes and inhibits the translation of mRNA.

Additionally, KSRP supports miRNA maturation by interacting the ribonucleases Drosha and Dicer. Figure modified from **[1]**.

1.2.1 Post-transcriptional regulation

Post-transcriptional regulation occurs subsequent to gene transcription, preceding the translation of the resulting transcript into a protein. Consequently, the pre-RNA is subjected to various modifications, which regulate its stability, intracellular localization, and translational rate [29].

As 5’- and 3’-exonucleases are capable of degrading unprotected single-stranded RNA, two protective caps are added at the ends of the strand. The first of these is a 7-methyl-guanosine cap, also referred to as 5'-cap, which is added during the elongation of transcription at the 5’-end [30]. This cap protects the RNA from degradation by exonucleases and phosphatases and serves as a recognition signal for subsequent translation. Following the termination of transcription, RNA-polyadenylation occurs. The removal of the polyadenylation signal (5'-...AAUAAA...-3') results in the attachment of a poly(A) tail of up to 250 adenine nucleotides to the 3'-end by poly(A) RNA polymerases [31]. The poly(A) tail serves also to stabilize and transport the RNA out of the cell nucleus. Intron sequences present in the pre-mRNA are removed by splicing [32]. Finally, the mature mRNA is comprised of the 5'-cap structure, the 5'-untranslated region (5'-UTR), the protein-coding region and the 3'-UTR including the poly(A) tail. The UTRs play a significant role in post-transcriptional regulation. They regulate active export of mRNA from the cell nucleus, mRNA stability and subcellular localization, and the efficiency of translation. In the following sections, some steps of post-transcriptional control of gene expression are discussed.

1.2.1.1 Regulation of mRNA stability

The regulation of mRNA stability represents the most significant regulatory mechanism at the post-transcriptional level. The rate of mRNA degradation has a profound influence on the amount of protein produced and is typically initiated by the shortening of the poly(A) tail or by complete deadenylation. This step is carried out by the deadenylases poly(A)-specific ribonuclease (PARN) [33] and/or the deadenylase complex consisting of poly(A)-specific ribonuclease subunit 2 and 3 (PAN2/PAN3) [34]. The unprotected RNA can then be degraded in the 5'-3' and 3'-5' direction. For 5’-3’ degradation a cleavage of the 5’-cap structure is necessary, which is catalyzed by the enzymes of the mRNA decapping complex, comprising mRNA decapping enzyme 1 and 2 (DCP1/DCP2) [35]. The mRNA is then finally degraded by exoribonuclease 1 (XRN1) [36]. The 3’-5’ degradation is initiated by recruitment of the exosome, a protein complex with 3’-5’-endonucleolytic activity.

The degradation of mRNAs (or their stabilization) is regulated by *cis*-acting adenine-uracil-rich elements (AREs) in the 3'-UTR [37]. AREs are mainly found in short-lived RNAs, such as cytokines, proto-oncogenes, transcription factors and growth factors [38]. This suggests that regulation of gene expression at the level of mRNA stability is important for inflammatory and immunological processes, transcription as well as cell differentiation and proliferation [39].

AREs are classified into three categories based on their sequence and distinct functional properties [40]. Class I and II AREs contain multiple copies of the pentanucleotide AUUUA. Class I AREs are located near U-rich sequence stretches, promoting the even shortening of poly-(A)tails and thereby contributing to the degradation of mRNAs. They are primarily found in mRNAs that encode for transcription factors, such as c-fos and c-myc, as well as in mRNAs of certain cytokines, including interleukin (IL-) 4 and IL-6 [40]. Class II AREs contain contiguous and overlapping pentanucleotide sequences and cause an uneven deadenylation, resulting in transcripts with poly-(A)strands of different lengths. Examples of mRNAs with such inherent AREs are granulocyte macrophage colony-stimulating factor (GM-CSF), IL-2, tumor necrosis factor (TNF)-α and Interferon (IFN)-α [40]. Class III AREs differ from the other AREs by the absence of AUUUA elements. They are characterized by U-rich sequence segments and are found for example in the mRNA of the Jun protooncogene (c-jun) [40].

In general, *trans*-acting RBPs exert their effects by binding to *cis*-acting sequence motifs (e.g. ARE), influencing mRNA stability in either a positive or negative manner. For example, the human antigen R (HuR) has been shown to have a predominantly stabilizing function on the mRNA transcript half-life [41]. Furthermore, RBPs may also influence translational efficacy (e.g., T-Cell-Restricted Intracellular Antigen-1 [42]).

In contrast, tristetraprolin (TTP), the ARE/poly(U)-binding degradation factor (AUF1), and KSRP have been described as destabilizing factors [43-45]. It has been demonstrated that these RBPs are responsible for the recruitment of the exosome and thus promote mRNA degradation [44, 46].

As outlined before, many mRNAs encoding pro-inflammatory mediators that contain AREs in their 3'UTR are targets of KSRP-mediated mRNA decay, and are therefore often inherently unstable [47]. For instance, KSRP has been shown to decrease the stability of mRNAs encoding TNF-α, IL-8, type I and III IFN [20, 21] and inducible nitric oxide synthase (iNOS) [18] by binding to AREs in their 3'-UTR (Figure 3). Overall, KSRP appears to be a central component of ARE-mediated mRNA decay (AMD) [20]. Furthermore, in 2000, Lellek and colleagues identified KSRP as a constituent of the apolipoprotein B mRNA-editing enzyme-complex [48]. Another study identified approximately 100 target mRNAs of KSRP, including IL-6, IL-8, and Cyclooxygenase-2, whose expression levels were upregulated in KSRP-deficient cells [49]. However, KSRP-dependent mRNA degradation was observed in only 10% of all target mRNAs, suggesting additional mechanisms of KSRP-mediated gene regulation.

Overall, the significance of RBPs as regulators of mRNA stability for the control of gene expression is evident in the pathogenesis of inflammatory diseases. For instance, TTP-deficient mice exhibit spontaneous development of a chronic inflammatory phenotype, which has been attributed to the increased mRNA stability of pro-inflammatory cytokines, including TNF-α, GM-CSF, and IL-6 [50, 51]. Additionally, increased pro-inflammatory gene expression has been observed in AUF1-deficient mice [52]. Moreover, the elevated expression of numerous genes in inflammatory disorders has also been attributed to a disruption in mRNA stability [53].

1.2.1.2 Translation efficacy

KSRP exerts influence not only on mRNA stability but may also attenuate mRNA translation, thereby impairing the expression of genes that encode pro-inflammatory cytokines and chemokines. Dhamija and colleagues investigated the polysome profiles of cells with siRNA-mediated KSRP deficiency and observed that KSRP-deficient HeLA cells (human epithelial cells of a cervical carcinoma) exhibited elevated IL-6 protein levels. KSRP was demonstrated to interact with the AREs of IL-6 mRNA, thereby mediating its translational silencing [22].

1.2.1.3 Micro (mi)RNAs

miRNAs are small (21-23 bp) and highly conserved non-protein coding RNA molecules that play a critical role in post-transcriptional gene regulation. Consequently, they are constituents of RNA-induced silencing complexes (RISC) [54]. It is postulated that they regulate the expression of up to 30% of protein-coding genes [55]. Depending on the degree of sequence homology to the target-binding site translational repression and mRNA cleavage, are initiated [56]. In case of low base pairing translation is inhibited, whereas in case of high homology the mRNA is degraded [57]. Due to the possibility of RISC/mRNA interaction even at low complementarity, a single miRNA may target multiple distinct transcripts and a single gene might be under the control of several miRNAs, illustrating the complexity of this regulatory mechanism. Furthermore, miRNAs can compete or cooperate with RBPs in post-transcriptional gene regulation. Therefore, miRNAs play a significant role in diverse biological processes. With regard to the immune system, miRNAs influence the differentiation of immune cells and are involved in the activation of both innate and adaptive immune responses [58]. To ensure the proper functioning of biological processes, the expression profiles of numerous miRNAs are specific to different tissues and developmental stages.

KSRP plays a pivotal role in the processing of a subsets of miRNAs, particularly those with a GC-rich stem-loop structure in their immature precursor transcripts [59]. The KH domain 3 of KSRP exhibits selective binding to G-rich sequences, thereby enabling KSRP to interact with the ribonucleases Drosha and Dicer in the nucleus and cytoplasm (Figure 3). In the nucleus,

KSRP facilitates the cleavage of pri-miRNA into pre-miRNA. By interacting with exportin-5, KSRP promotes the transport of pre-miRNA to the cytoplasm. By binding to the terminal loop of pre-miRNA and interacting with Dicer, KSRP promotes the maturation of pre-miRNA into mature miRNA in the cytoplasm [59]. This process is essential for the maturation of specific miRNAs, including miR-155 [23], let-7a [24, 25] and miR-129 [26], which are critical in regulating immune processes. These findings suggests that KSRP plays a significant role in immune cell biology.

In conclusion, KSRP is a versatile RBP that regulates gene expression at various levels. Currently, the most significant biological functions of KSRP appear to be its involvement in mRNA decay and the maturation of miRNAs.

1.3 Regulation of KSRP activity

1.3.1 Transcript and mRNA level

KSRP activity is regulated at the transcriptional and post-transcriptional level by miRNAs and other RBP. Whereas transcriptional regulation of the KSRP gene expression remains largely unexplored, more detailed information about post-transcriptional regulation mechanisms are available (Figure 4). For example, binding of miRNA-206 to the 3'-UTR of KSRP results in decreased KSRP levels [60]. In mammary gland cells miRNA-27b-3p promoted KSRP mRNA decay [61]. Since KSRP inhibited transforming growth factor β (TGF-β)-induced epithelial-to-mesenchymal (EMT) transition, KSRP silencing was a prerequisite for EMT transition in mammary gland cells. In addition, Zhou et al. demonstrated in *Cryptosporidium parvum*-infected gut epithelial cells that KSRP is translationally silenced by miRNA-27b-3p, resulting in enhanced iNOS mRNA stability [62]. Interaction of HuR with the ARE of KSRP mRNA results in stabilization of KSRP mRNA. Hence, a knockdown of HuR resulted in reduced mRNA levels of KSRP [63].

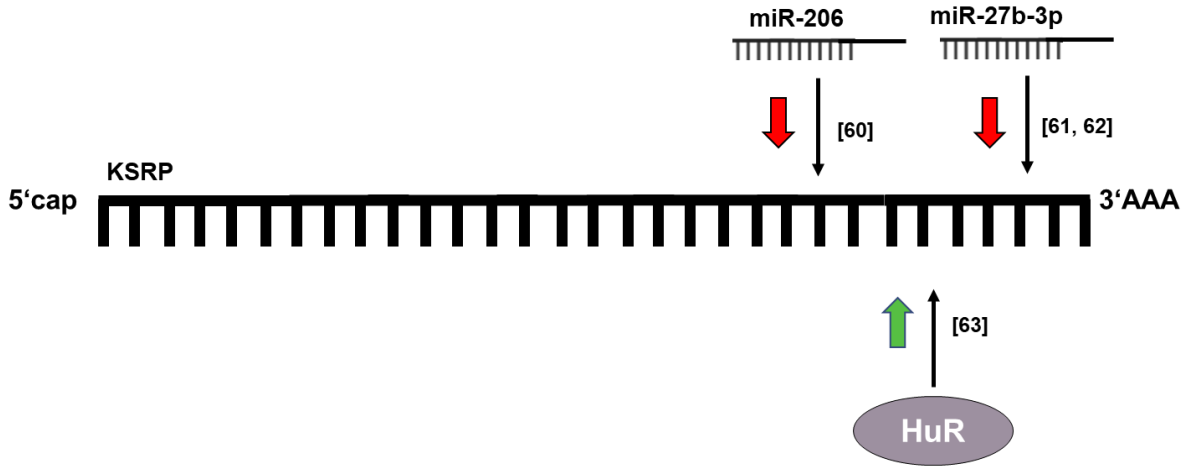

Figure 4. Summary of factors regulating KSRP expression on the post-transcriptional level.

1.3.2 Protein Level

KSRP activity is also regulated by several post-translational modifications, including phosphorylation, ubiquitination and interaction with long non-coding RNAs (lncRNAs).

1.3.2.1 Phosphorylation

Protein phosphorylation is one of the most common and important post-translational modifications. It is a reversible cellular regulatory mechanisms as many enzymes and receptors are activated or deactivated by phosphorylation or dephosphorylation events [64]. Phosphorylation is mediated by protein kinases that add a phosphate group (PO_4) to the polar group R of various amino acids [64]. Consequently, this addition results in a change of protein conformation, thereby facilitating the assembly and disassembly of protein complexes [64].
KSRP has multiple phosphorylation sites (see Figure 5) that are targeted by various kinases, including p38 mitogen-activated protein kinase (MAPK), protein kinase B (PKB), and ataxia telangiectasia mutated (ATM) kinase, which in turn regulate KSRP activity [47].

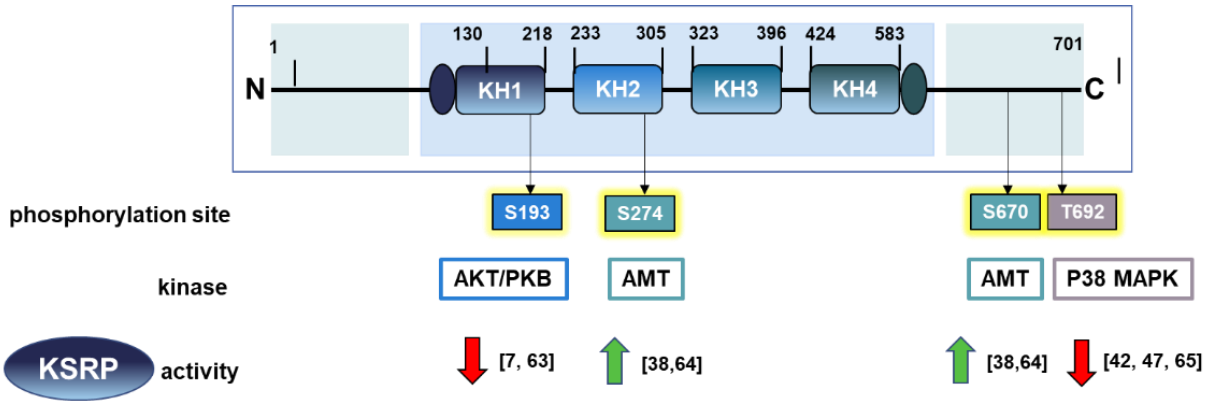

Figure 5. Modification of KSRP by phosphorylation.

PKB = protein kinase B, AMT = ataxia telangiectasia mutated kinase, MAPK = mitogen-activated protein kinase.

Phosphorylation of KSRP by PKB on serine 193 stimulated the unfolding of unstable KH1 which created a site for 14-3-3-ζ binding [65]. This allowed interaction of KSRP with 14-3-3-ζ, which resulted in nuclear accumulation of KSRP and accordingly to reduced KSRP-mediated mRNA destabilization [65]. Further, PKB-mediated phosphorylation of KSRP prevented its ability to associate with the exosome [66].
Additionally, DNA-activated ATM kinase was shown to phosphorylate KSRP at serine 274 and serine 670, respectively, thereby increasing the interaction of KSRP with pre-miRNAs, leading to increased biogenesis of KSRP-dependently processed miRNAs [20, 67].
p38 MAPK phosphorylated KSRP at threonine 692 and inhibited its binding to target ARE-containing mRNA, thus leading to a 2-3-fold increase in the stability of e.g. myogenic transcripts encoding myogenin and protein 21 [68] as well as to an IL-1β-dependent increase

in IL-8 expression [49].

KSRP negatively regulates prothrombin expression by binding to the upstream sequence element (USE) in the 3'-UTR of prothrombin mRNA [69]. Upon phosphorylation by activated p38 MAPK, KSRP dissociates from the USE, resulted in increased mRNA stabilization.

Similarly, the KSRP-dependent silencing of IL-6 can be reversed by the pro-inflammatory cytokine IL-1 [22]. Activation of p38 MAPK by IL-1 signaling results in the phosphorylation of KSRP, which reduces its binding to IL-6 mRNA.

Moreover, the natural compound resveratrol has been demonstrated to increase KSRP activity by inhibiting phosphorylation at threonine residue 692 by p38 MAPK [70]. This results in the increased degradation of various pro-inflammatory mRNAs, which may contribute to the anti-inflammatory effects of resveratrol. Additionally, resveratrol has been demonstrated to impede TGF-β-induced epithelial-to-mesenchymal transition (EMT) in mammary gland cells, a process that is dependent on KSRP [71].

In conclusion, the phosphorylation of KSRP at specific sites results in changes to KSRP activity, which in turn leads to an increase in the expression of pro-inflammatory cytokines.

1.3.2.2 Ubiquitination

Ubiquitination denotes the attachment of ubiquitin to a target protein, which plays an important role in protein functions, such as protein degradation or kinase activation. Ubiquitin is a 76-amino acid protein that can be conjugated to a protein as a single ubiquitin (e.g., monoubiquitination) or as a multiple ubiquitin (e.g., polyubiquitination) [72].

Ubiquitination of KSRP [73, 74] plays a critical role in the course of viral infection. KSRP is involved in enterovirus internal ribosome entry site (IRES)-driven translation through modification of IRES trans-acting factors (ITAFs) [73, 75]. *In vitro* experiments have demonstrated that KSRP activity is impaired through interaction with Kelch-like protein (KLHL)12. KLHL12 promotes polyubiquitination of KSRP at the lysine (lys)109, lys121 and lys122 residues in the C-terminal part of KSRP via linking to cullin 3-based ubiquitin-protein E3 ligase complex. This leads to suppressed EV71 translation and favors modulation of positively acting ITAFs [73].

Moreover, KSRP activity was found modified by ubiquitination through small ubiquitin-like modifier (SUMO) in human embryonic kidney and prostate cancer cells [76]. SUMOs are involved in regulation of target proteins by influencing their activity, stability, and localization [77]. SUMOylation of KSRP increased its accumulation in the cytoplasm, and at the same time resulted in attenuated maturation of miRNAs with G-rich stretches in the terminal loop (e.g., let-7 miRNA family) due to disassociation of KSRP from the pri-miRNA-Drosha complex.

Furthermore, KSRP seems to be a substrate of a multi-protein E3 ubiquitin ligase complex, containing F-box and WD repeat domain-containing 2 (FBXW2), S-phase kinase-associated

protein 1 and cullin-1 F-box protein (SCF). Wang and coworkers demonstrated in murine macrophages that FBXW2 directly engaged KSRP and triggered its ubiquitination by SCF, leading to degradation of KSRP [78].

To sum up, ubiquitination of KSRP appears to cause attenuation in KSRP activity (Figure 6).

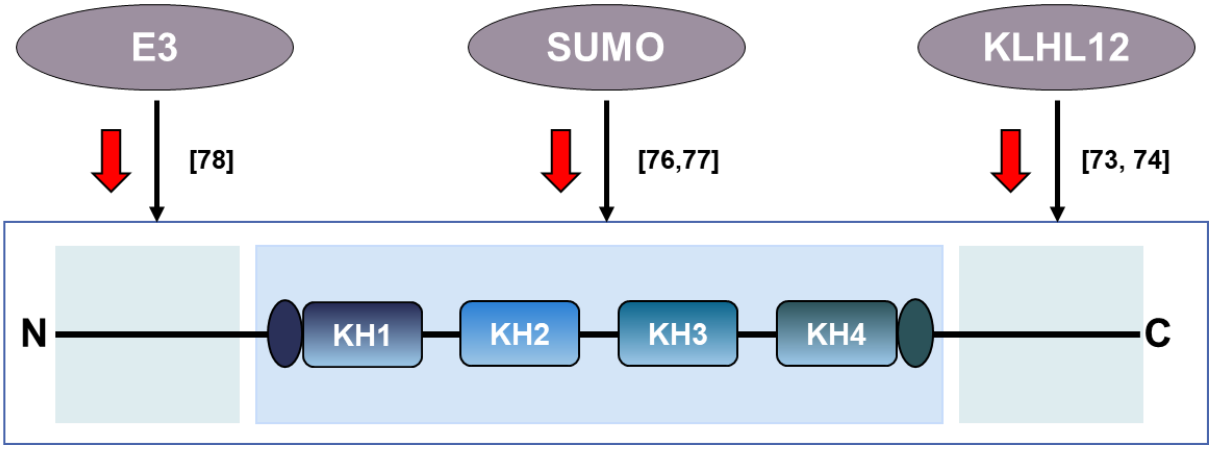

Figure 6. Modification of KSRP by ubiquitination.

KLHL12 = Kelch-like protein 12 , SUMO = small ubiquitin-like modifier.

1.3.2.3 Long Non-Coding RNAs

Long non-coding RNAs (lncRNA) are defined as RNAs longer than 200 nucleotides that are not translated into functional proteins and are expressed in multiple cell types and tissues [79, 80]. Some reports describe interaction of lncRNAs with KSRP that modulate KSRP-mediated mRNA decay (Figure 7).

KSRP activity in the cytoplasm was shown to be modified by interaction with the lncRNA H19 [81]. H19 engaged KSRP and this interaction favored KSRP-mediated destabilization of labile transcripts, such as myogenin mRNA, in mesenchymal C2C12 cells. Upon PKB activation, KSRP was released from H19. This resulted in enhanced stabilization of myogenin mRNA, but also promoted maturation of myogenic miRNAs, thus favoring myogenic differentiation. Furthermore, KSRP activity was modified by interaction with the lncRNA Epr in epithelial tissues [82]. Epr engaged KSRP and inhibited KSRP-mediated decay of Cdkn1a mRNA, thus favoring cell proliferation.

Furthermore, it has been demonstrated that axon-enriched long intergenic non-coding RNA (ALAE), regulating axon elongation, interacts with KSRP and suppresses its activity [83]. The promotion of axon elongation by ALAE is achieved through the enhancement of translation of growth-associated protein 43 (Gap43) mRNA. ALAE inhibits the ARE of Gap43 mRNA, preventing the binding of KSRP and thereby preventing the degradation of Gap43 mRNA. This results in the enhanced stabilization of Gap43 mRNA and the promotion of axon elongation.

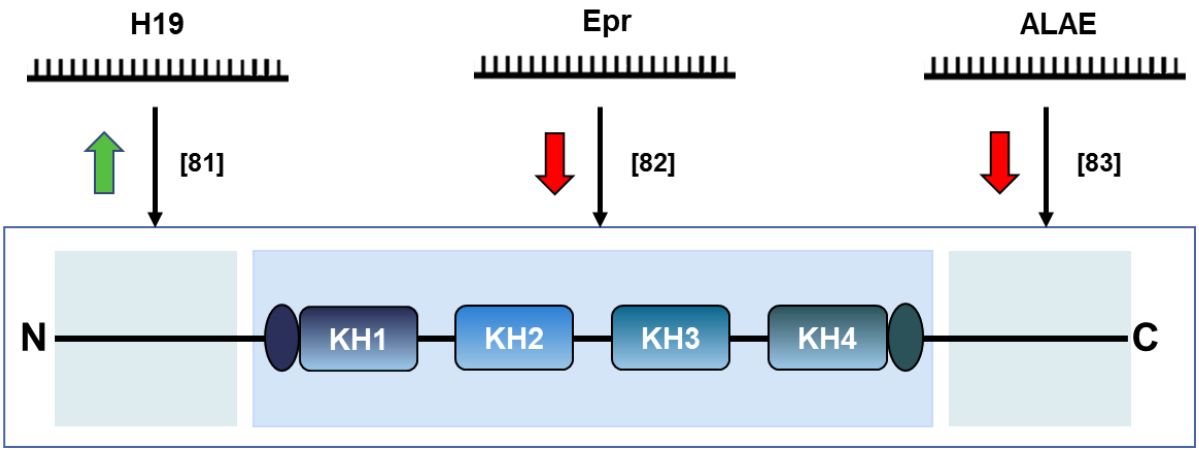

Figure 7. lncRNAs interacting with KSRP.

In summary, KSRP activity is regulated through multiple levels, including transcriptional and post-transcriptional mechanisms, such as phosphorylation, ubiquitination, and interactions with lncRNAs, occurring both in the nucleus and the cytoplasm.

1.4 KSRP as a regulator of innate immune responses

The immune system is responsible to defend an organism against dangers posed by invading pathogens, including bacteria, viruses and fungi, as well as malignant cells and protecting against foreign substances [84]. The immune system can be divided into two distinct arms [85]. The innate, non-specific arm, which reacts immediately after the recognition of an invading pathogen trough the complement system and/ or phagocytic cells (e.g. macrophages) [86]. In contrast, the adaptive, specific arm confers a delayed response that may require several days to fully develop but is able to form immunological memory [86].

1.4.1 Innate Immune System

The innate immune system is the evolutionarily older arm of the immune system and is a complex network of initial response to pathogenic organisms and environmental insults [84]. As the first barrier against pathogens, e.g. the skin, when getting disrupted by infection or lesion, the innate immune mechanisms in the skin begin to immediately kill the pathogen [85, 86]. The complement reaction is one of the fastest-acting mechanisms of the immune system [86]. The early events of complement activation, which are based on an enzymatic amplification cascade, can be triggered by one of three pathways [87]. The classical pathway is activated by antigen–antibody complexes, the alternative pathway by microbial cell walls, and the lectin pathway by the interaction of microbial carbohydrates with mannose-binding protein in the plasma [87]. Either through direct or indirect interaction, blood-resident inactive complement proteins encounter a pathogen and will rapidly bind to it. Upon binding to the

pathogen, a series of proteolytic cleavages will activate complement proteins, resulting in the formation of large multimeric complexes that in turn disrupt bacterial membranes, thereby killing the invading pathogen directly [88].

At the cellular level, the innate immune response is mediated by cells recruited from the blood, including polymorphonuclear granulocytes (PMN), monocytes and macrophages (MAC) [89]. These innate immune cells are able to sense and home to danger signals associated with damage and/or infection. In addition, they will continuously sample their local microenvironment through phagocytosis. Germline-encoded pattern recognition receptors (PRRs), like Toll-like receptors (TLRs) on innate immune cells, allow the host to recognize pathogen-associated molecular patterns (PAMPs), such as LPS, flagellin, single-stranded RNA, and unmethylated CpG initiating a cascade of cellular responses that results in the activation of cells.

PMN, MAC and dendritic cells (DC) also serve as antigen-presenting cells (APC) that present pathogen-derived antigens in combination with strong receptor-mediated co-stimulation (e.g. CD80/CD86) and the secretion of T cell-polarizing cytokines [90]. By this, T cells with an antigen-specific T cell receptor are activated and subsequently confer adaptive immune responses [91].

1.4.1.1 Dendritic Cells (DC)

DC are derived from bone marrow precursors and can be found in the blood, lymphoid organs, and various tissues, particularly those in close proximity to the external environment, such as the skin, lung, and intestine [92]. As phagocytic cells, they are capable of internalizing and destroying invading pathogens, processing and presenting derived antigens on the surface of both MHC class-I and –II molecules to T cells [93]. They are professional APC with their ability to prime naïve T cells, thereby initiating a targeted adaptive immune response [94].

1.4.1.2 Macrophages

Macrophages are innate immune cells that are present throughout the body [86]. Upon activation by pathogens or inflammation, they exert a dual role: They exhibit powerful pathogen-killing activities through e.g. phagocytosis or recruitment of immune cells to the inflammation site by releasing cytokines and chemokines [95], and serve as APC, thereby triggering antigen-specific T cell responses [96].

1.4.1.3 Granulocytes (Polymorphonuclear cells, PMN)

Granulocytes are composed of a group of three cell types that can be distinguished by the contents of their granules. These include neutrophils, basophils, and eosinophils. In general, these are short-lived cells (~5 days) that play an important role in early responses to parasites,

extracellular bacteria and tumors [97]. PMN are terminally differentiated, originate from the bone marrow and are equipped with a variety of PRR that enable them to detect pathogens [97]. Thereby PMN protect the host through a number of pathogen-killing mechanisms [98], including phagocytosis, degranulation, the production of reactive oxygen species (ROS), and the formation of neutrophil extracellular traps (NETs) [99]. Furthermore, PMN secrete chemokines and cytokines, which serve as indirect defense mechanisms by attracting and polarizing other immune cells [100-102].

1.4.1.4 Cytokines

To function properly, the immune system must tightly regulate the synthesis and release of cytokines [103]. As these are important regulators of immune cell function, a fast and robust expression is necessary to establish a swift immune response to an invading pathogen. However, cytokine expression must be controlled to avoid a dysregulated excessive immune response resulting in tissue destruction or autoimmune reactions. Therefore, cytokine expression is controlled by transcriptional, post-transcriptional and translational mechanisms **[1].** Cytokines are small secreted proteins that include a wide variety of signaling peptides, proteins, and glycoproteins. They are released by cells and have a specific effect on the interactions and communication between cells [104]. They may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some cases on distant cells (endocrine action). It is common for different cell types to secrete the same cytokine and for a given cytokine to act on several different cell types (pleiotropy) [104]. Cytokines are often produced in a cascade, as one cytokine stimulates its target cells to produce additional cytokines, acting synergistically or antagonistically. They are produced by many cell populations [104]. Cytokines can be classified into different groups based on the cell types that produce them. Due to their ability to attract other cells, chemokines are cytokines with chemotactic activities. Cytokines made by a leukocyte that act on other leukocytes are also called interleukins (ILs) and are the broadest group [86]. The tumor necrosis factor (TNF) family includes TNF-α and TNF-β; they mediate a variety of functions ranging from the regulation of cell differentiation to cell survival [86]. Interferons (IFN) are a subset of cytokines that are glycoproteins and are divided into three groups, type I (IFN-α and IFN-β) and type II (IFN-γ) and type III (IFN-λ).

1.5 Aims of the PhD thesis

Regulation of the functional activity of immune cells requires dynamic adaptation at the gene expression level. In this regard, the mRNA-binding protein KSRP has been shown to modulate immune cell functions via a reduction in target mRNA stability and the maturation of microRNAs. Given its central role in ARE-mediated mRNA decay (AMD) of e.g. pro-inflammatory mediators, KSRP has been recognized as a key negative regulator of inflammatory immune responses [21, 44, 105-107]. Although KSRP's regulatory importance is becoming clearer, its role in the immune system remains relatively unexplored.
As KSRP is ubiquitously expressed, including immune cells as PMN, MAC, DC, T cells and B cells, it is important to elucidate its functional role in those cells.

This dissertation aims to provide a comprehensive summary of the current knowledge on the multiple roles of KSRP as a regulator of gene expression, with a focus on its emerging importance in the induction and course of innate immune responses, and with a specific attention on PMN and MAC. Consequently, we examined the impact of KSRP deficiency on the immune phenotype and functions of myeloid innate immune cells *in vitro, in vivo*, and in corresponding *ex vivo* analyses to ascertain whether KSRP represents a potential target for immunotherapeutic interventions in infection.

2. Results

The data presented in this thesis have either been published or submitted to scientific journals. Key findings are summarized in the following sections, with the relevant publications and submitted manuscript provided in the Appendix.

2.1 KSRP deficiency led to increased production of pro-inflammatory cytokines in sepsis

During sepsis, systemic activation of the innate immune system by PAMPs results in a severe and persistent inflammatory response characterized by an excessive release of inflammatory cytokines, collectively referred as “cytokine storm” [108, 109]. Especially, IL-1β, along with IL-6 and TNF-α regulate early responses in sepsis progression [110]. Many pro-inflammatory and immune cell modulating genes are regulated post-transcriptionally by RBPs [13]. Given the pivotal role of innate myeloid immune cells in sepsis, such as PMN and MAC, the *in vivo* relevance of KSRP deficiency for cytokine/chemokine production was evaluated in an acute inflammation model. To this end, $KSRP^{-/-}$ and WT mice received an intraperitoneal injection (i.p.) of 5 or 20 mg LPS per kg bodyweight (Figure 8A). Accordingly treated $KSRP^{-/-}$ mice exhibited elevated IL-1β levels in sera 12 h post-injection (Figure 8B). This finding underscores that KSRP plays a role in limiting the extent of the sepsis-associated cytokine storm. In line with these results, time kinetic studies with KSRP-deficient bone marrow-derived MAC (BMDM) exhibited higher mRNA and protein expression levels of IL-1β following LPS stimulation (refer to **[2]**, Figure 2). Immunoprecipitation (IP) studies further indicate that KSRP directly binds to LPS-induced IL-1β mRNA in BMDM (Figure 8C).

In contrast, the regulation of IL-6, TNF-α, IFN-γ, IFN-α, and IFN-β mRNAs by KSRP in BMDM appears to be indirect (refer to **[2]**, Figure 3), occurring through mechanisms such as translational inhibition (refer to 1.2.1.2) or interaction with miRNAs (refer to 1.2.1.3).

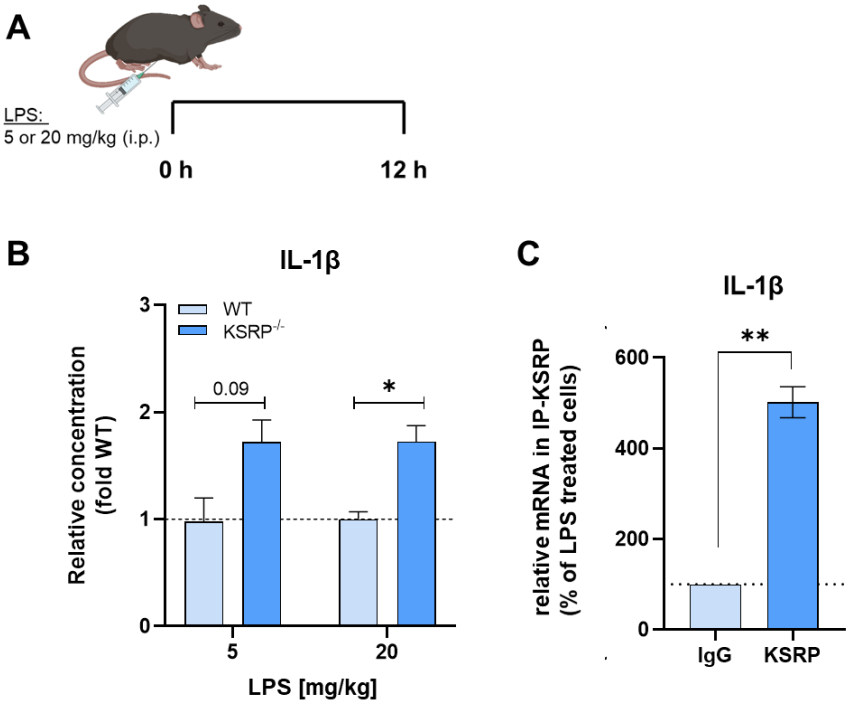

Figure 8. KSRP deficiency led to an increase in IL-1β production following treatment with 5 or 20 mg/kg LPS, may through direct binding to IL-1β transcript.
(A) WT or KSRP$^{-/-}$ mice received an intraperitoneal injection (i.p.) with 5 or 20 mg/kg bodyweight LPS and were sacrificed 12 h later. Created with BioRender.com. (B) Analysis of IL-1β levels 12 h after 5 or 20 mg/kg LPS injection. Shown are the means ± SEM of n = 4–6 analyses (* $p < 0.05$ versus WT cytokine expression; two-tailed Mann-Whitney test). (C) WT BMDMs were treated with LPS (1 µg/mL) for 6 h to stimulate the production of IL-1β. After cell lysis, RNAs bound to KSRP were immunoprecipitated using a specific antibody. To standardize the samples for subsequent analysis, 1 ng of *in vitro* transcribed luciferase RNA was added to each sample. The RNA was then purified, reverse transcribed into cDNA, and real-time PCR was used to quantify the mRNA levels of IL-1β and luciferase (for normalization). Data are presented as means ± SEM with individual data points for each animal (n = 3 analyses), showing the relative mRNA amounts bound to KSRP compared to IgG controls (100%) (** $p < 0.01$; one-sample t-test). Figure modified from **[2].**

2.2 KSRP deficiency attenuates the course of invasive pulmonary aspergillosis

Since we reported that KSRP deficiency led to a more pronounced inflammatory response by MAC *in vitro* and *in vivo* in a sepsis model **[2]**, we aimed to explore whether this could provide advantages in pathogen eradication in an infection model. To evaluate this hypothesis, we assessed in KSRP-deficient mice the course of invasive pulmonary aspergillosis (IPA), a major threat to immunocompromised individuals caused by the infection with the saprophytic fungus *Aspergillus fumigatus (A. fumigatus)* [111, 112]. In immunosuppressed patients, *A. fumigatus* can cause IPA, a potentially lethal disease that arises when inhaled conidia germinate in the lungs and develop into hyphae [113]. The innate immune system, mainly PMN and MAC are

considered the primary defense mechanism for the clearance of conidia and the prevention of the growth of *A. fumigatus* conidia (AFC) [114-116].

In the mouse IPA model, some animals were injected i.p. with an anti (α)-Gr-1 antibody to deplete PMN prior to intratracheally inoculation with *A. fumigatus* conidia (d0), to prove successful infection. Surprisingly, in contrast to the WT control animals, the neutropenic KSRP$^{-/-}$ mice showed only mild symptoms of disease (Figure 9A). All immunocompetent WT and KSRP$^{-/-}$ mice survived infection as monitored over 2 weeks (Figure 9A).

We observed a notable reduction in *A. fumigatus* burden in the lungs of KSRP$^{-/-}$ compared to WT mice (Figure 9B), which was associated with higher frequencies of PMN in BAL (Figure 9C) and of PMN and MAC in lung tissue (Figure 9D). In contrast, for eosinophilic granulocytes (EOS) and DC (refer to **[3]**, Figure 2D+E), as well as for T cells, B cells and natural killer (NK) cells (refer to **[3]**, Figure S1A) no genotype-dependent differences could be observed.

14 days following infection, the second group of mice was analyzed to assess late-onset of *A. fumigatus* infection. As previously stated, neutropenic KSRP$^{-/-}$ mice demonstrated unexpected mild disease symptoms.

These results suggest, that infection can be combated more effectively in KSRP-deficient mice than in WT mice by PMN and MAC. Of note, enhanced anti-pathogenic activity of the latter may even compensate for neutropenia (refer to 2.3 and 2.4).

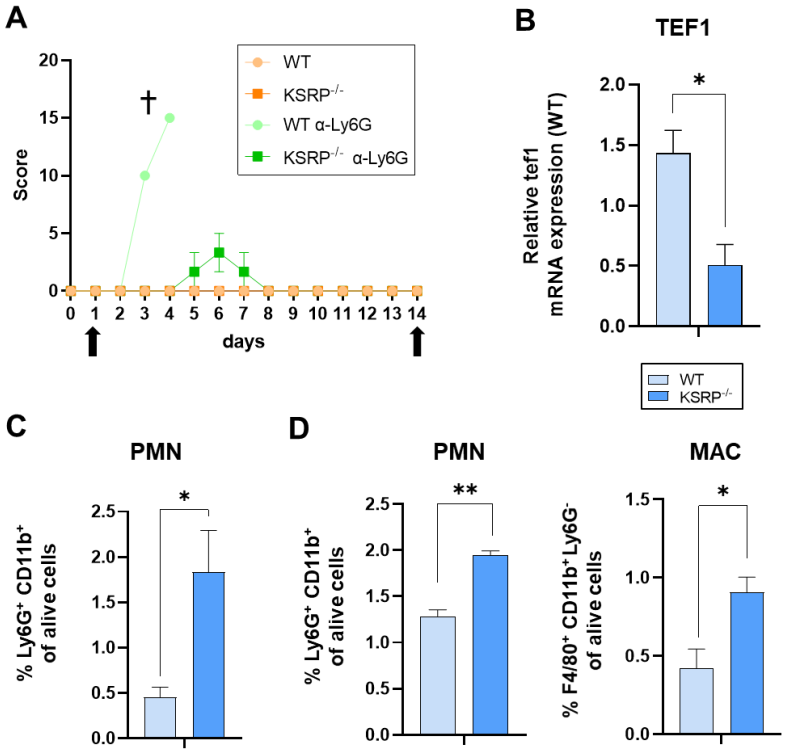

Figure 9. KSRP deficiency attenuates IPA progression.

(A) The clinical course of IPA monitoring was assessed of the different groups for 14 days. Parameters comprised breathing, reaction to pain overall appearance, hypothermia, strong weight loss, motoric disabilities and apathy. On day 1 and day 14 after inoculation mice were sacrificed and organs subjected to *ex vivo* analyses. (B) We prepared total RNA of lung tissue and measured specific TEF1 mRNA expression, as marker for AFC [117], using the qRT-PCR method and normalized to GAPDH mRNA

expression. One day post-inoculation flow cytometric analysis showed higher frequencies of PMN in BALF (C) and higher frequencies of PMN and MAC in the lung tissue (D). Data denote the mean ± SEM of 3 samples analyzed per group. Statistically significant differences between groups are indicated (** p < 0.01, * p < 0.05, two-tailed students t-test). Figure modified from **[3]**.

2.3 KSRP deficiency enhances effector functions of innate myeloid immune cells

PMN and MAC utilize a variety of mechanisms, including phagocytosis, degranulation, and the release of reactive oxygen species (ROS), to combat infections [99, 118]. We observed that KSRP-deficient PMN (Figure 10A) and MAC (Figure 10B) exhibited higher uptake of AFC than the corresponding wild type controls.

Further, our findings show that PMN from KSRP$^{-/-}$ mice produced significantly higher levels of ROS (Figure 10C), whereas stimulation with AFC yielded higher ROS production only in WT cells, still below then levels observed for KSRP-deficient PMN (Figure 10D).

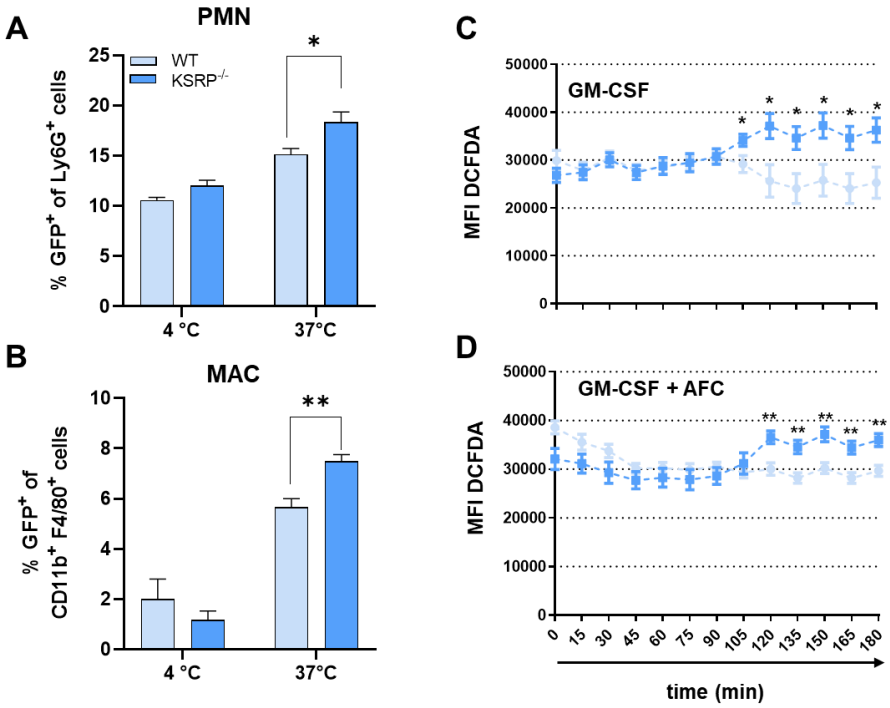

Figure 10. KSRP deficiency amplifies the effector functions of PMN and MAC.
(A) 1x10^5 PMN or MAC were cultured with 3x10^5 serum-preincubated GFP-fluorescent AFC at 4°C or 37°C. Following a 3 h incubation period, the frequency of GFP-positive PMN and MAC was determined by flow cytometry. Data represent the mean ± SEM of 6 samples analyzed/group (** p < 0.01, * p < 0.05; two-tailed students t-test. 1x10^5 PMN were stained with DCFDA, treated with indicated stimuli (100 ng/ml GM-CSF (C) and/or 1x10^5 AFC (D)) and measured in 15 min intervals for 3 h. Data represent the mean ± SEM of 9 samples analyzed/group (** p < 0.01, * p < 0.05; two-tailed students t-test). Figure modified from **[3]**.

2.4 KSRP deficiency in PMN and MAC results in upregulation of genes involved in pathogen defense

In light of the pivotal role of KSRP-dependent control of PMN and MAC functions (refer to 2.3) we performed comparative gene expression analysis to assess, which genes were congruently upregulated in case of KSRP deficiency. This analysis identified 64 shared upregulated genes (Figure 11A). Additionally, it revealed that PMN uniquely upregulated 936 genes, while MAC upregulated 887 genes (Figure 11A). Further bioinformatic analysis revealed that a fraction of genes congruently upregulated by either KSRP-deficient innate immune cell type is interconnected and involved in the positive regulation of innate immune responses (5.4 %), anti-viral responses (2.3 %), and anti-bacterial responses (1.7 %) (Figure 11B).

Moreover, GESA demonstrated elevated gene expression in KSRP-deficient PMN and MAC that are implicated in the IL-6 signaling pathway (Figure 11C+F). In line, we demonstrated, that inactivation of the KSRP gene enhances IL-6 protein expression in murine peritoneal cells, which are enriched in MAC, after LPS stimulation (refer to **[2]**, Figure 4).
In time kinetic studies, MAC exhibited upregulation of IL-6 mRNA expression (Figure 11G) and displayed higher levels of IL-6 protein (Figure 11E), whereas PMN showed no genotype-dependent differences in IL-6 mRNA levels (Figure 11D). Interestingly, KSRP-deficient PMN exhibited increased IL-6 secretion following LPS treatment (Figure 11E), indicating that KSRP not only affects mRNA stability but may also attenuate mRNA translation in a target mRNA-specific manner, thereby impairing gene expression (refer to section 1.2.1.2).
Furthermore, KSRP-deficient PMN demonstrated elevated levels of TNF-α mRNA (Figure 11D) and increased TNF-α secretion (Figure 11E), while KSRP-deficient MAC exhibited higher mRNA levels of IFN-γ (Figure 11G) and in agreement, higher IFN-γ protein levels following LPS stimulation (Figure 11H).

In addition, the differentially upregulated genes in KSRP-deficient PMN and MAC were examined for gene clusters using the STRING database (refer to **[3]**, Figure S3). The predominant categories of genes that were upregulated in PMN were related to DNA replication (29.4%), DNA repair (18.8%), the cell cycle (14.5%), and cellular metabolic processes (6.7%) (refer to **[3]**, Figure S3A). In contrast, MAC predominantly upregulated genes associated with anti-viral (22.4%), anti-bacterial (17.8%), and anti-fungal (13%) defense mechanisms (refer to **[3]**, Figure S3B).

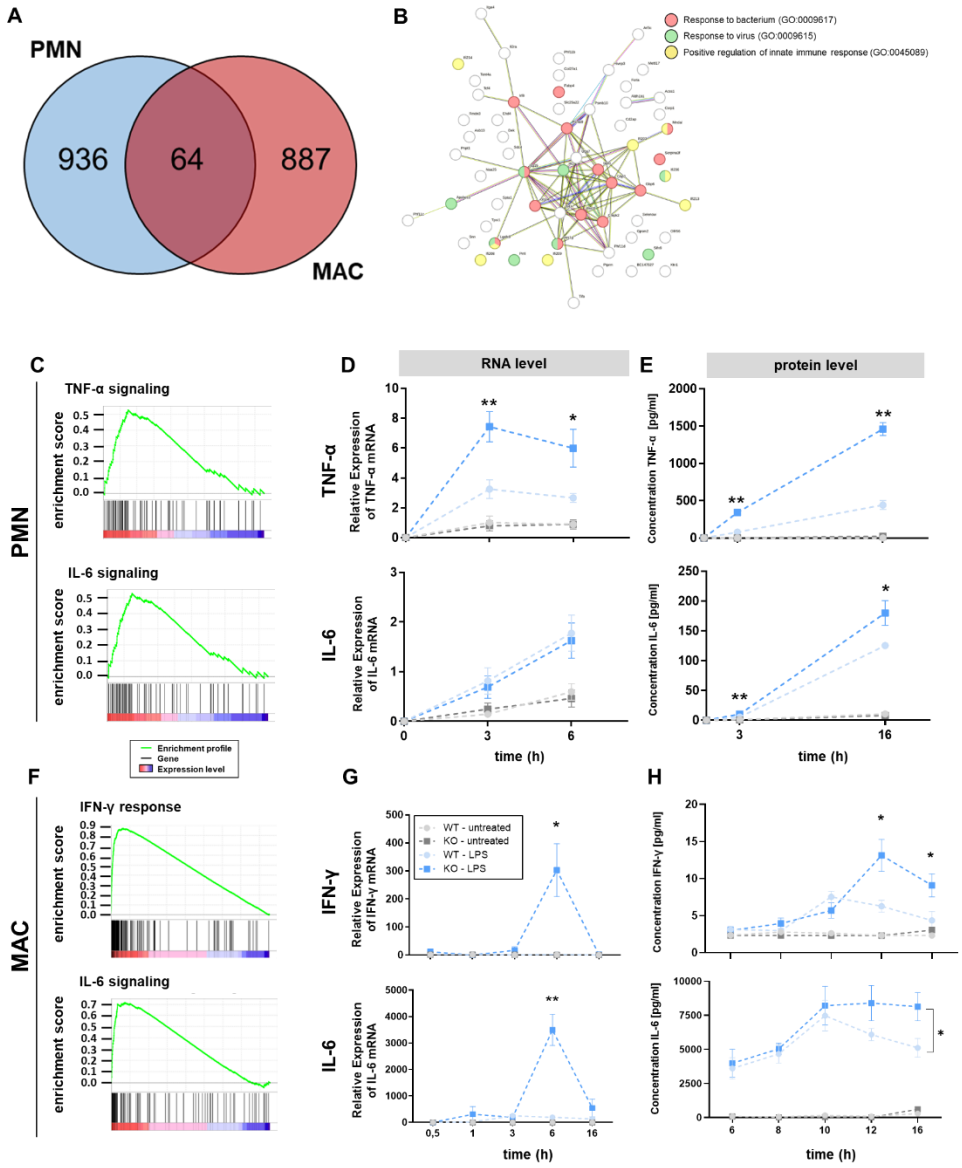

Figure 11. Upregulation of genes involved in pathogen defense in KSRP-deficient PMN and MAC following LPS-stimulation.

(A) Using Venn-Diagramm calculator we calculated same and differential upregulated genes after LPS stimulation within PMN and MAC.(B) Analyzing the 64 congruently upregulated genes between PMN and BMDM with STRING database, revealed genes are interlinked and contribute to positive defense regulation against pathogen and activation of innate immunity. To analyze the mRNA and protein expression of different immune relevant genes in cells of KSRP$^{-/-}$ or WT animals, PMN and MAC were stimulated with 1 µg/µl LPS for indicated time periods. (A) and (F) Gene set enrichment analysis (GSEA) revealed significantly regulated pathways involved in cytokine expression, such as TNF-α, IL-6 and IFN-γ (BH-adjusted $p < 0.05$). Total RNA was isolated from PMN (D) and MAC (G) and specific mRNA expression was measured using the qRT-PCR method normalized to GAPDH expression. Shown are the mean ± SEM of n = 9 analyses (PMN) and n= 3-4 analyses (MAC) (** $p < 0.01$, * $p < 0.05$; versus untreated WT cells; two-tailed students t-test). Supernatants of PMN (E) and MAC (H) were collected, and cytokine contents were measured using the Anti-Virus-Response LegendPlex-Kit from BioLegend. Shown are the mean ± SEM of n = 6-9 analyses (PMN) and n= 7-8 analyses (MAC) (** $p < 0.01$, * $p< 0.05$; two-tailed students t-test). Figure modified from **[2]** and **[3]**.

2.5 KSRP elevates PMN apoptosis

Since PMN exhibited an upregulation of genes linked to viability (refer to section 2.4), we conducted a flow cytometric analysis of PMN under basal conditions and following LPS stimulation. Notably, KSRP$^{-/-}$ PMN exhibit attenuated apoptosis compared to WT mice, suggesting that KSRP may play a role in promoting PMN apoptosis (Figure 12).

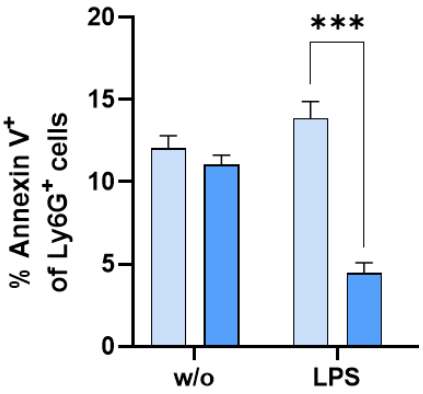

Figure 12. KSRP deficiency attenuated PMN apoptosis
1x10^6 PMN were cultured 6 h without (w/o) or with 1 µg/ml LPS. Subsequently, the samples were stained for subsequent flow cytometry analysis. The assessment of PMN apoptosis revealed a diminished expression of the apoptosis marker Annexin-V in stimulated KSRP-deficient PMN. Shown are the mean ± SEM of n = 6 analyses (*** p < 0.001; two-tailed students t-test). Figure modified from **[3].**.

3. Discussion

The innate immune response is rapid, non-specific, and serves as the initial line of defense to eliminate pathogens or injured cells, to initiate inflammation, recruit additional immune cells, and trigger the adaptive immune response [85]. Upon crossing an epithelial barrier within host tissues, a microorganism is typically recognized by phagocytic cells that are resident in the tissues [118]. Phagocytosis has thereby two important effects: firstly, to destroy the pathogen, and secondly, to release microbial proteins, which can be processed and presented as pathogen-specific antigens to cells of the adaptive immune system, specifically T cells [118].
MAC represent the predominant phagocyte population present in most body tissues under conditions of homeostasis [86]. The second group of phagocytes is comprised of PMN [119]. The recognition of an invading pathogen by danger receptors such as TLRs initiates a cascade of signaling pathways [120], resulting in rapid and robust expression of pro-inflammatory cytokines [121] and on functional level in the recruitment of PMN and MAC to sites of infection and inflammation [122]. Both cell types are essential for the clearance of pathogens and the resolution of inflammation [98]. A variety of mechanisms are employed by these cells to combat infections, including phagocytosis, degranulation, and the production of ROS [99, 118].
To avoid excessive immune reactions, such as such as a cytokine storm [123], extensive tissue damage [124], or an autoimmune response [125], cytokine expression has to be controlled strictly. The multifunctional RBP KSRP has been identified as a general negative regulator of inflammatory immune responses, dampening immune activation by inhibiting the production of pro-inflammatory cytokines. Consistent with its key role in ARE-mediated decay of pro-inflammatory mediators, it has been shown to limit cytokine production in activated immune cells by promoting the decay of relevant mRNAs, as demonstrated in both cell culture studies [18] and in primary cells from KSRP$^{-/-}$ mice [21, 70, 105]. Additionally, we observed increased production of Th2-associated cytokines in polyclonally stimulated KSRP-deficient T cells [106]. In addition, mice with complete KSRP deficiency in all tissues have shown enhanced anti-viral responses upon infection, along with increased production of type I interferons [19]. In line, we found in LPS-stimulated MAC from KSRP-deficient mice using the STRING Database, that the top 15 upregulated genes are interconnected and involved in regulating the innate immune response, particularly the cellular response to type I interferons **[2]**.
This regulation is achieved through various direct regulation mechanisms: the promotion of mRNA decay [18, 20, 21] and the inhibition of translation [22] of target mRNAs. Both functions are conferred by binding of KSRP to an ARE sequence within its target mRNA. Nevertheless, KSRP may also operate indirectly by facilitating the maturation of miRNA species, which subsequently inhibit gene expression [27, 28]. Despite growing evidence of KSRP's regulatory importance, its cell-type specific immunological function has only been investigated to limited extent so far.

Sepsis is a complex disturbance of the body's immune system, affecting the equilibrium between inflammatory and anti-inflammatory processes [126]. In the course of sepsis, there is a systemic release of pro-inflammatory cytokines by innate myeloid cells, including IL-1β, IL-6, and TNF-α [132]. The increased expression of these factors is, in part, due to elevated activity of various transcription factors (e.g., NF-κB, STATs, AP-1), which confer enhanced gene expression. However, genes that are involved in the regulation of inflammatory and immune responses are also subject to post-transcriptional regulation by RBP [127].
Our findings indicate *in vitro* elevated expression of IL-1β on mRNA and protein level in MAC, along with increased levels of IL-1β in the serum of KSRP-deficient mice in a LPS-induced *in vivo* sepsis model, suggesting that KSRP limits the cytokine storm during sepsis **[2]**. Similarly, Liu *et al*. showed that the RBP AUF1 provided protection against endotoxic shock by reducing the expression of pro-inflammatory cytokines, including IL-1β and TNF-α, through mRNA degradation [52]. Consistently, immunoprecipitation assays confirmed direct binding of KSRP to LPS-induced IL-1β mRNA in MAC **[2].** It would be of interest to observe the survival of $KSRP^{-/-}$ mice over a longer period of time in this context. At present, it can be postulated that KSRP-deficient mice in the sepsis model die at an earlier stage than WT mice as a result of an elevated cytokine storm. Moreover, it would be interesting to identify the innate myeloid cells responsible for cytokine production, for instance through combination of extracellular pan-surface marker (MAC: CD11b, F4/80, PMN: CD11b, Ly6G) with intracellular cytokine staining (IL-1β, IL-6 and TNF-α).

To investigate the regulatory differences between KSRP-deficient PMN and MAC, a comparative analysis of the upregulated genes after LPS stimulation was conducted. The analysis revealed that 64 genes were upregulated in both cell types, involved in positive regulation of the innate immune and pathogen defense response, suggesting elevated pathogen defense activity in the absence of KSRP **[3].**
In addition, the absence of KSRP in LPS-stimulated PMN and MAC has been observed to result in elevated levels of pro-inflammatory mediators, including IFN-γ, IL-6, and TNF-α **[2, 3]**. Notably, in KSRP-deficient PMN an increase in IL-6 was only detectable at the protein level **[3]**, suggesting that KSRP not only affects mRNA stability, but may also attenuate mRNA translation in a target mRNA-specific manner, thereby diminishing gene expression, as shown by Dhamija *et al.* The polysome profiles of HeLA cells with siRNA-mediated KSRP deficiency were examined, and it was found that KSRP interacts with the ARE of IL-6 mRNA and mediates its translational silencing. [22]. To ascertain whether KSRP is also responsible for translation silencing in PMN and MAC, an examination of the polysome profile of these cells could be conducted in a manner analogous to that employed by Dhamija *et al*.

Our findings align with those of previous studies, which have demonstrated that KSRP plays a role in regulating the expression of multiple pro-inflammatory mediators, including IL-1β [28, 128], IL-6 [22, 49], and TNF-α [22, 49, 128].
As we found elevated levels of IL-6 and TNF-α in supernatant of primary peritoneal cells from KSRP$^{-/-}$ mice, in line with upregulation of genes involved in type I interferon response in MAC, we proceeded with an IP, analyzing binding of KSRP to IL-6, TNF-α, IFN-γ, IFN-α, and IFN-β. However, no binding of KSRP could be detected, suggesting that KSRP regulates the expression of those cytokines in an indirect manner **[2]**. In addition to direct interactions leading to mRNA decay or translational inhibition, KSRP also regulates gene expression indirectly. It plays a key role in processing specific miRNAs, especially those with GC-rich stem-loop structures [59], such as miR-155 [23], let-7a [25] and miR-129 [26], which play crucial roles for immune regulation. To date, miRNA let-7a was reported to inhibit IL-6 expression in macrophages [129]. Further research is required to elucidate whether KSRP exerts an influence on the maturation of specific miRNAs in this context.

Given the results of stimulation-induced hyperactivation of innate immune cells in KSRP-deficient mice, such PMN and MAC, we aimed to explore whether the lack of KSRP could provide advantages in an infection model. To evaluate this hypothesis, we initiated IPA, a significant threat to immunocompromised individuals caused by the infection with *A. fumigatus* [111]. At the early stage control of fungal spreading is critically dependent on innate immune cell activity, such as PMN [114, 115] and MAC [116]. As phagocytosis is one key effector mechanism by which PMN and MAC clear pathogen infections [119], we first analyzed the effect of KSRP deficiency in this context. KSRP-deficient PMN and MAC exhibited increased phagocytic activity against GFP-labeled AFC **[3]**, indicating that PMN and MAC may prevent the growth of AFC *in vivo*. One day following infection, we observed that KSRP-deficient mice exhibited a reduced fungal burden in the lungs **[3]**. This was accompanied by elevated frequencies of PMN in BALF and of PMN and MAC in lung tissue, which suggests that these immune cells were able to clear AFC at a faster rate due to their increased phagocytic activity **[3]**. In addition, we recently demonstrated that KSRP attenuates the migration of PMN towards CXCL1 **[1],** the most crucial chemoattractant for PMN in the context of infection [130], serving as a potential explanation for the increased number of PMN in the BALF and lungs.
Somewhat surprisingly, PMN-deficient KSRP$^{-/-}$ mice survived the inoculation with AFC, whereas the WT control group died within the first three days, as expected [131]. These findings indicate that, additional factors may be responsible for the suppression of AFC to hyphae. Given that KSRP-deficient MAC showed stronger expression of pathogen defense-associated genes, which is in line with an enhanced pro-inflammatory response and exhibited increased phagocytic activity, it seems feasible to hypothesize that this immune cell population

may compensate at least in part for the loss of antibody-mediated PMN depletion **[3]**. To validate this hypothesis, detailed analysis are necessary to gain insight in cellular and molecular functions of KSRP that led to the survival. To this end, it would also be necessary to deplete MAC alone or in combination with PMN in KSRP-deficient mice. In addition, the pathogen-killing activity of both cell types could be quantified at an early time point (e.g. after one day), which would provide information on the survival of neutropenic KSRP$^{-/-}$ mice.

Furthermore, our findings revealed that KSRP deficiency resulted in increased ROS production by PMN **[3]**. It has been demonstrated that multiple targets of KSRP encode mRNAs that are regulated by oxidative stress [18, 46, 132]. The data presented here suggest that KSRP may negatively influence the regulation of gene expression in response to oxidative stress. This effect may be mediated by protein kinases, such as p38 or PKB/Akt, which phosphorylate and thereby inhibit KSRP in its ARE-mediated decay of target mRNAs [133].
Given the essential role of ROS in neutrophil extracellular trap (NET) formation, termed NETosis [134], it would be beneficial to examine the role of KSRP in PMN in this context as well. In addition, the impact of KSRP on ROS production by MAC could be investigated examined in a manner analogous to that employed for PMN.

A considerable number of genes are found to be enhanced in expression by stimulated KSRP-deficient PMN, which are linked to cell viability **[3]**. In accordance, KSRP$^{-/-}$ PMN were observed to exhibit diminished apoptosis, indicating that KSRP deficiency prolongs cell survival **[3]**. These findings additionally could contribute to an increased frequency of PMN in the BAL/lungs of IPA-treated KSRP$^{-/-}$ mice. This, in conjunction with elevated effector mechanisms, results in a notable enhancement of pathogen defense. As previously described by Ebner and colleagues, TTP-deficient PMN express higher levels of the TTP target Mcl1 mRNA, which codes for an anti-apoptotic factor particularly relevant for PMN [135]. Thereby, similar to your observed results in KSRP-deficient mice, TTP deficiency reduces PMN apoptosis upon stimulation, whereas it is not impaired under steady-state conditions [135].

Taken together, the data presented herein demonstrate that KSRP plays a pivotal role in negatively regulating anti-pathogen activity in PMN and MAC. In both cell types, KSRP deficiency led to the upregulation of genes associated with pro-inflammatory responses and pathogen defense. Besides direct regulation of gene expression via posttranscriptional mechanisms, KSRP also acts in an indirect manner by promoting the maturation of a subset of miRNA species [23-28], which in turn affect expression of multiple genes. Further studies are necessary to elucidate the mode of KSRP-mediated regulation of its target genes in MAC and PMN.

4. Outlook

The objective of this dissertation was to examine the potential significance of the RBP KSRP in the context of innate immune processes, particularly in PMN and MAC. The regulation of immune cell activity demands dynamic adaptation at the gene expression level. It has been postulated that KSRP exerts its influence on immune cell functions by post-transcriptionally reducing the mRNA stability of target genes, such as cytokines and facilitating the maturation of microRNAs. Despite increasing evidence of KSRP's regulatory significance, its role in the immune system has been explored to limited extent only. Given its ubiquitous expression, including immune cells such as PMN, MAC, DC, T cells, and B cells, it may constitute as an interesting target for the immunotherapeutic treatment.

Regarding therapeutic approaches, it may be possible to enhance KSRP activity in immune cells, such as PMN and MAC, but also T and B cells to temporarily attenuate unwanted inflammatory immune responses, including sepsis, autoimmune diseases, or allergies.

In this regard, a previous study indicated that KSRP deficiency leads to increased production of Th2-associated cytokines [106]. Further, we observed KSRP deficiency to exacerbate OVA-induced allergic asthma symptoms in comparison to WT mice **[4]**. The mechanisms by which KSRP exerts its influence on cytokine expression appear to involve transcriptional modulation. It appears that KSRP primarily suppresses IL-4 expression, which in turn attenuates IL-13 production, implicating that KSRP plays a role in regulating Th2-mediated immune responses. Interestingly, it was also demonstrated that resveratrol modulates KSRP mRNA binding activity, thereby enhancing mRNA degradation and resulting in anti-inflammatory effects [70].

Conversely, the inhibition of KSRP has the potential to enhance the body's defense against an infection in patients with limited innate immunity. This is as evidenced by the elevated pathogen-killing activities of KSRP-deficient innate myeloid cells, which facilitate the rapid clearance of pathogens. It is well established that PMN and MAC are related cell types that together orchestrate an immune response by performing effector functions and regulating other immune cells as well as each other [136].

In conclusion, the findings of this dissertation demonstrate that KSRP on the one hand, serve to prevent excessive tissue damage in the host, however on the other hand attenuates pathogen-killing activities. Further research is required to gain a comprehensive understanding of KSRP targets, particularly to ascertain whether this RBP exerts its influence on immune responses exclusively through its action only on effector cytokines or if it also affects the expression of key transcription factors. Similarly, further investigation is required to elucidate the functional role of KSRP in other immune cells, including NK cells and B cells. This should encompass both homeostatic and stimulatory conditions *in vitro*, as well as relevant disease models. To this end, it will be essential to use mice with conditional KSRP deficiency to investigate the cell type-specific roles of this protein.

5. References

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6. Appendix

6.1 Publication 1

The Role of KH-Type Splicing Regulatory Protein (KSRP) for Immune Functions and Tumorigenesis

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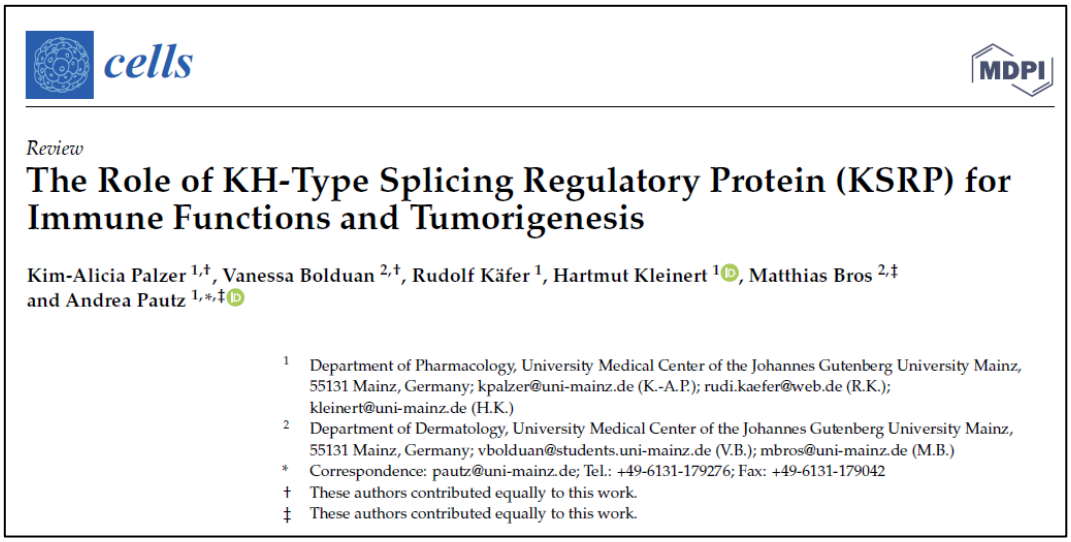

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Review

The Role of KH-Type Splicing Regulatory Protein (KSRP) for Immune Functions and Tumorigenesis

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Summary:

The review summarizes current knowledge about post-transcriptional control of gene expression, particularly through RNA-binding proteins like KH-type splicing regulatory protein (KSRP), playing a key role in modulating cytokine, chemokine, and growth factor expression, crucial for immune and tumor cell function. KSRP regulates mRNA stability by promoting mRNA decay, inhibiting translation, and enhancing microRNA maturation. It is essential for immune cell function, myeloid hematopoiesis, and maintaining immune homeostasis by controlling pro-inflammatory factors. KSRP also dampens T helper cell 2 responses during infection and is linked to tumor growth and metastasis, which is likely to be an important topic for further research.

Author contribution:

- Literature research about KSRP-related sections for effects in the immune system
- Preparation and writing of the manuscript together with Kim Palzer, Dr. Andrea Pautz and Dr. Matthias Bros
- Proofreading of the submitted manuscript

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Review

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Abstract: Post-transcriptional control of gene expression is one important mechanism that enables stringent and rapid modulation of cytokine, chemokines or growth factors expression, all relevant for immune or tumor cell function and communication. The RNA-binding protein KH-type splicing regulatory protein (KSRP) controls the mRNA stability of according genes by initiation of mRNA decay and inhibition of translation, and by enhancing the maturation of microRNAs. Therefore, KSRP plays a pivotal role in immune cell function and tumor progression. In this review, we summarize the current knowledge about KSRP with regard to the regulation of immunologically relevant targets, and the functional role of KSRP on immune responses and tumorigenesis. KSRP is involved in the control of myeloid hematopoiesis. Further, KSRP-mediated mRNA decay of pro-inflammatory factors is necessary to keep immune homeostasis. In case of infection, functional impairment of KSRP is important for the induction of robust immune responses. In this regard, KSRP seems to primarily dampen T helper cell 2 immune responses. In cancer, KSRP has often been associated with tumor growth and metastasis. In summary, aside of initiation of mRNA decay, the KSRP-mediated regulation of microRNA maturation seems to be especially important for its diverse biological functions, which warrants further in-depth examination.

Keywords: KH-type splicing regulatory protein; post-transcriptional gene regulation; mRNA decay; micro RNA; cytokine; antiviral response; T helper cell; tumorigenesis

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1. Introduction

To prevent an exaggerated immune response, tight control of the expression of pro-inflammatory mediators, such as cytokines or chemokines, is necessary. Since these are important regulators of immune cell function, rapid and strong expression is required to elicit a rapid response to an invading pathogen. Nevertheless, it is also important to resolve immune responses to counteract tissue destruction and to prevent autoimmune responses. Gene expression can be controlled by transcriptional and posttranscriptional mechanisms, and the latter comprise regulation of mRNA decay and translation efficiency. The 3-untranslated region (3′-UTR) of mRNA represents an important element in the post-transcriptional regulation of inflammatory cytokines/chemokines by RNA-binding proteins (RPBs) and micro (mi)RNA. In general, RPBs exert rather a stabilizing (human antigen R, HuR) or destabilizing (Tristetraprolin, TTP/KH-type splicing protein, K(H)SRP) effect on mRNA transcript half-life, and may affect its translational efficacy (e.g., T-Cell-Restricted Intracellular Antigen-1) [1,2].

KSRP (K homology [KH]-type splicing protein, KHSRP) is a single-stranded nucleic acid-binding protein which interacts with target RNA species in nuclear and cytoplasmic cell compartments [3]. In humans, the *KSRP* gene is located on chromosome 19p13.3, contains 21 exons, one transcript is listed in the RefSeq database (ENST00000600480.2), and encodes the 747 amino acid (aa) KSRP protein, as investigated in the literature. Some hints from the database indicate that additional transcripts may exist, but nothing is known about their biological significance.

The murine gene (ENSMUSG00000007670) is located on chromosome 17 [4] and contains 19 exons. However, the protein sequence of KSRP (in both species 747 aa) is highly conserved between both species. KSRP was first described in 1996 by Levens laboratory and categorized as a member of the far upstream element (FUSE)-binding protein (FBP) family, and was originally named FBP2 [5]. In addition to KSRP, two other members, named FBP1 and FBP3, belong to that family. FBP1 is involved in different cellular processes by regulating transcription, splicing and translation of target genes [6], whereas the biological function of FBP3 remains largely unknown [7].

The structure of the KSRP/FBP2 protein can be divided into a central region that is flanked on either side by one additional region (Figure 1) [8]. The central region contains four KH domains mediating the interaction with single-stranded nucleic acids [9]. Whereas KH domain 1, 2, and 4 bind to a large number of sequence motifs with moderate selectivity, KH domain 3 preferably binds to G-containing sequences. In comparison with the central region, the N- and C-termini are regions with low complexity and are post-translationally modified [8]. The N-terminal part comprises a proline-glycine-rich region, whereas the C-terminal part contains a glutamine-rich region, and both contain elements for protein interaction [5,8]. Moreover, the N-terminal part contains the nuclear localization signal, and the C-terminus harbors four Y-rich repeats. Thus, the structure of KSRP is important for its flexibility in terms of target gene binding. KSRP activity is regulated by phosphorylation and other modifications as outlined below.

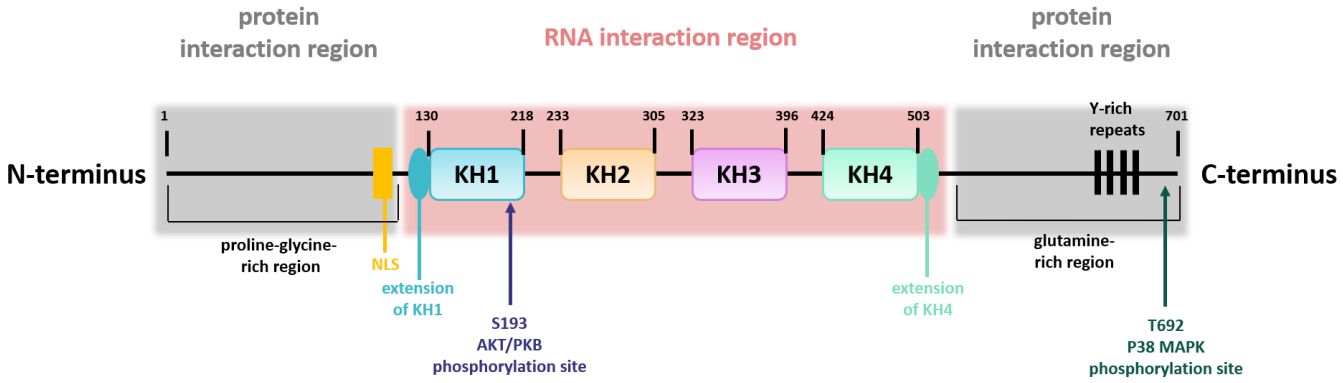

Figure 1. KSRP structure. Schematic overview of KSRP protein structure, including the domain organization in the central region, involved in RNA interaction, and the two N- and C-terminally flanking regions, which are necessary for protein interaction. Phosphorylation sites are indicated (own illustration inspired by [10]).

In the nucleus, KSRP acts as a transcription and splicing factor, and in the cytoplasm regulates mRNA stability by promoting its decay and translational silencing (Figure 2) [11]. In addition to regulation of gene expression via post-transcriptional mechanisms, KSRP promotes the maturation of a subset of micro (mi)RNA species, which, in turn, affect expression of multiple genes.

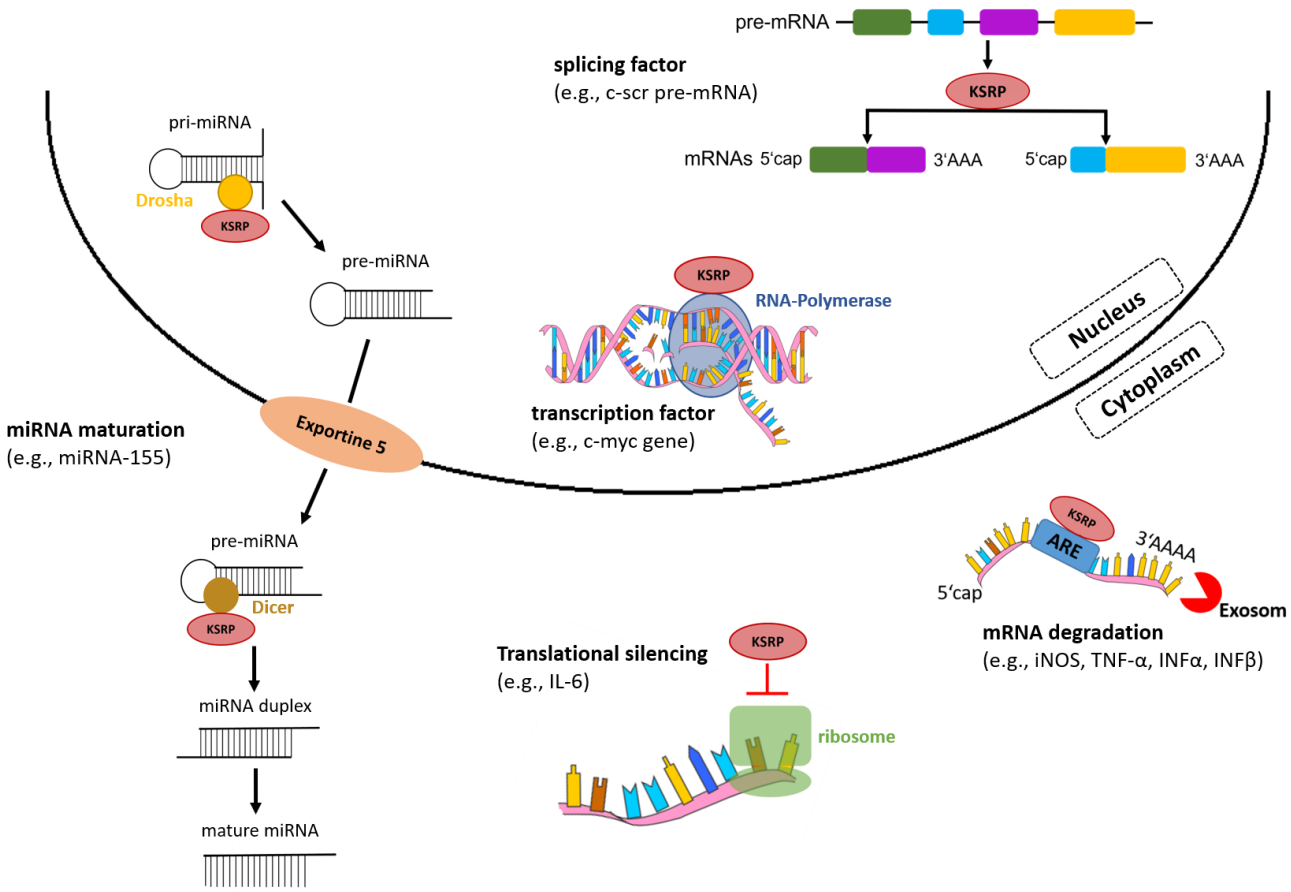

Figure 2. KSRP regulates gene expression on various levels. In the nucleus, KSRP functions as a transcription and splicing factor, and in the cytoplasm mediates rapid decay of ARE-containing mRNAs by recruiting enzymes and silences translation of mRNAs. Moreover, KSRP promotes miRNA maturation by interacting with the ribonucleases Drosha and Dicer (own illustration inspired by [12]).

This review aims to summarize current knowledge on the multiple roles of KSRP as a regulator of gene expression, with a focus on its emerging importance on the induction and course of innate and adaptive immune responses, in addition to tumorigenesis.

2. KSRP Regulates Gene Expression on Various Levels

2.1. Gene Transcription

In 1996 KSRP was originally identified as a transcription factor of the c-myc oncogene [5]. KSRP binds to FUSE motif, and the four Y-rich regions within the C-terminus of KSRP activate c-myc transcription [8]. One year later the ability of KSRP to act as a pre-mRNA splicing regulatory protein was demonstrated. As a component of a multi-protein complex, KSRP was found to bind to an intronic splicing enhancer element of the proto-oncogene c-src and to regulate the alternative splicing process of c-scr pre-mRNA [13].

2.2. mRNA Level

KSRP plays an important role in different steps of post-transcriptional control of gene expression, including the regulation of mRNA stability and translatability. In addition to modulation of post-transcriptional target gene expression, KSRP also promotes the biogenesis of distinct micro (mi)RNA species, which, in turn, may affect expression of numerous genes as outlined in the following.

2.2.1. mRNA Stability

Many mRNAs of pro-inflammatory mediators that possess AU-rich elements (AREs) in the 3′UTR are targets of KSRP-mediated mRNA decay and are, therefore, often inherently unstable [14]. For example, it has been demonstrated that KSRP decreases stability of the mRNAs encoding tumor necrosis factor-α (TNF-α), interleukin (IL)-8, type I and III interferons (IFN) [15,16] and inducible nitric oxide synthase (iNOS) [17] by binding to ARE in their 3′-UTR. For this, KSRP recruits the exosome multiprotein complex with 3′-5′-endonucleolytic activity and other enzymes involved in mRNA decay, such as the poly (A)-specific ribonuclease (PARN) [18], the deadenylase complex consisting of poly(A) specific ribonuclease subunit 2 and 3 (PAN2/PAN3) [19], and the 5′-3′-exonuclease 1 (XRN1) [20]. Moreover, KSRP recruits mRNA decapping enzymes such as decapping mRNA 2, which activates deadenylation of poly-A-tail of mRNA, and the decapping complex consisting of decapping mRNA 1 and 2 (DCP1/DCP2) [21,22]. KH domains 3 and 4 are necessary for KSRP-mediated mRNA decay, by binding to AREs with high-affinity and interacting with mRNA decay enzymes [9]. Both KH domains act independently of each other, resulting in a broad spectrum of target mRNAs [10]. However, KH domain 3 stabilizes the interaction of KH domain 4 to target mRNAs. Thus, altogether, KSRP seems to be a central component of the ARE-mediated mRNA decay (AMD) [15].

Moreover, in 2000, Lellek and coworkers identified KSRP as a component of the apolipoprotein B mRNA editing enzyme-complex [23]. Another study identified ~100 target mRNAs of KSRP, comprising, e.g., IL-6, IL-8 and Cyclooxygenase-2, whose expression levels were upregulated in KSRP-deficient cells [24]. However, KSRP-dependent mRNA degradation could only be detected in 10% of the ~100 target mRNAs, indicative of additional modes of KSRP-mediated gene regulation.

2.2.2. Translation Efficacy

Furthermore, KSRP not only enhances mRNA degradation, but also silences mRNA translation and consequently impairs expression of, e.g., proinflammatory cytokine or chemokine genes [25]. Dhamija and coworkers compared the polysome profiles of cells with siRNA-mediated KSRP deficiency and control cells. In KSRP-deficient cells there was increased IL-6 protein. KSRP was reported to interact with ARE of IL-6 mRNA and mediate its translational silencing. However, further investigations are necessary, as to date, only Dhamjia and coworkers have identified the ability of KSRP to regulate mRNA translatability.

2.3. miRNA Biogenesis

miRNAs are small non-protein coding RNAs, which play a critical role in post-transcriptional gene regulation as constituents of RNA-induced silencing complexes (RISC) [26]. miRNA inhibit gene expression by binding to target sequences that are located most often in the 3′-UTR of mRNAs [26]. Thereby, they initiate translational repression and/or mRNA cleavage, depending on the degree of sequence homology to the target-binding site [27]. A single miRNA can target multiple transcripts and a single gene can be under the control of multiple miRNAs.

KSRP is important for proper processing of a subset of miRNAs, especially of those that contain a GC-rich stem-loop structure in the immature precursor transcript [28]. KH domain 3 binds selectively towards G-rich sequences, and KSRP interacts with ribonucleases Drosha and Dicer in nucleus and cytoplasm, respectively (Figure 2). In the nucleus, KSRP cleaves pri-miRNA into pre-miRNA. Moreover, it promotes the transport of pre-miRNA into the cytoplasm by interacting with exportine-5. In the cytoplasm, KSRP promotes maturation of pre-miRNA into mature miRNA by binding to the terminal loop of pre-miRNA and interacting with the ribonuclease Dicer [28]. Among those miRNAs whose maturation requires KSRP are miR-155 [29], let-7a [30,31] and miR-129 [32], which exert important functions in the regulation of immune processes as outlined in Table 1. This suggests that KSRP has an important function in immune cell biology that should be evaluated in detail.

Table 1. Overview of immune cell functions mediated by miRNAs whose maturation is promoted by KSRP.

miRNA	miRNA Function in Immune Cells	References
miR-155	Expressed in stimulated antigen presenting cells and antigen receptor stimulated lymphocytes	[33,34]
	Hematopoietic lineage differentiation	[35]
	B cell differentiation, maturation, and antibody class switching	[36]
	Treg development	[37]
	Th cell polarization	[34,38]
miR-129	Inhibits runt-related transcription factor (RUNX)1, which determines myeloid differentiation of polymorphonuclear neutrophilic granulocytes (PMN) and monocytes	[39]
	M1-polarization of macrophages	[40]
	$CD4^+$ T cell proliferation	[41]
let-7a	Promotes IL-6 expression by macrophages	[42]
	Under hypoxic conditions, imprints a M2-like immunophenotype in macrophages	[43]

To summarize, KSRP is a versatile RNA-binding protein (RBP) which modulates gene expression at multiple levels promoted by its structural diversity. At the moment, KSRP-mediated mRNA decay and KSRP-mediated maturation of miRNAs seem to be the most important biological functions of the protein.

3. Regulation of KSRP Activity

3.1. Transcript and mRNA Level

KSRP activity is regulated at the transcriptional and post-transcriptional level. Whereas transcriptional regulation of KSRP gene expression remains largely unexplored, more information about post-transcriptional mechanisms exists. Table 2 presents a short overview about factors that regulate KSRP expression on mRNA level.

Table 2. Summary of factors that regulate KSRP expression on the post-transcriptional level.

Regulator	KSRP Expression	References
miRNA-206	downregulated	[44]
miRNA-27b-3p	downregulated	[45,46]
HuR	upregulated	[47]
survival motor neuron proteins	upregulated	[48]

3.2. Protein Level

KSRP activity is also regulated by several post-translational modifications, including phosphorylation and ubiquitination, in addition to interaction with long non coding RNAs (lncRNAs).

3.2.1. Phosphorylation

KSRP contains multiple phosphorylation sites (see Figure 1) that are engaged by various kinases such as p38 mitogen-activated protein kinase (MAPK), protein kinase B (PKB) and ataxia telangiectasia mutated (ATM) kinase, thereby regulating KSRP activity [14]. Here, we only present a short overview of post-translational KSRP modifications and the functional consequences (Table 3). For detailed information see [14,49].

Table 3. Modification of KSRP by phosphorylation.

Phosphorylation	KSRP Activity	Cellular Consequence	References
p38MAPK Threonine 692	suppressed	Inhibition of KSRR-mediated mRNA decay activity	[24,25,50]
PKB Serine 193	suppressed	Interaction of KSRP with 14-3-3-ζ leads to inhibition of KSRR-mediated mRNA decay activity	[51]
		No exosome interaction possible	[10]
ATM	activated	Increased biogenesis of KSPR-dependent processed miRNAs	[15,49]

In addition, KSRP negatively regulates the expression of prothrombin by binding to the upstream sequence element (USE) in the 3′-UTR of prothrombin mRNA [52]. Phosphorylation of KSRP via activated p38 MAPK results in dissociation of KSRP from the USE, yielding increased stabilization of prothrombin mRNA. Furthermore, the natural compound resveratrol has been demonstrated to increase KSRP activity by inhibiting threonine phosphorylation at residue 692 [53]. This, in turn, enhances the degradation of different pro-inflammatory mRNAs, which may explain some of the anti-inflammatory effects of resveratrol. Moreover, it has been described that resveratrol interferes with the transforming growth factor β (TGF-ß)-induced epithelial-to-mesenchymal transition in mammary gland cells, which depends on KSRP [54].

Phosphorylation of KSRP at its phosphorylation sites leads to reduced activity of KSRP and thus to an increased pro-inflammatory cytokine expression.

3.2.2. Ubiquitination

Ubiquitination of KSRP [55,56] attenuates its activity (as outlined in Table 4).

Table 4. Modification of KSRP by ubiquitination.

Ubiquitination of KSRP Mediated by:	KSRP	Cellular Consequence	References
Kelch-like protein 12	activity suppressed	Inhibition of KSRP-mediated internal ribosome entry site driven viral translation	[55,57]
Small ubiquitin-like modifier	activity suppressed	Attenuated KSRP-mediated maturation of miRNAs	[58,59]
Multi-protein E3 ubiquitin ligase complex	KSRP degradation		[60]

3.2.3. Long Non-Coding RNAs

Long non-coding RNAs (lncRNA) are defined as ncRNAs longer than 200 nucleotides and are expressed in multiple cell types and tissues [61]. Some reports describe interaction of lncRNAs with KSRP that modulates KSRP-mediated mRNA decay (Table 5).

Table 5. lncRNAs interacting with KSRP.

lncRNA	KSRP Activity	Consequence to Target mRNA	References
H19	upregulated	Increase in mRNA decay	[62]
Epr	suppressed	Inhibition of mRNA decay	[63]
ALAE	suppressed	Inhibition of mRNA decay	[64]

Altogether, KSRP activity is regulated at various levels by transcriptional and post-transcriptional mechanisms, e.g., by interaction with miRNA and other RNA-BP, in addition to post-translational mechanisms, including phosphorylation, ubiquitination and interaction with lncRNAs, in both nucleus and cytoplasm.

4. KSRP as a Regulator of Innate and Adaptive Immune Responses

PMN, monocytes/macrophages and dendritic cells (DC) recognize pathogens and are activated by pathogen-specific moieties [65] and soluble danger signals such as cytokines [66]. These innate immune cell types kill pathogens by various means, and in addition, serve as antigen presenting cells (APC) that present pathogen-derived antigens in the context of increased expression of costimulatory receptors and T cell-polarizing cytokines [67]. By this, T cells with an antigen-specific T cell receptor are activated and confer adaptive immune responses [68]. Due to its central role in AMD of pro-inflammatory mediators, KSRP was considered as an important negative regulator of inflammatory immune responses by limiting cytokine production of activated immune cells, since it promoted decay of the according mRNA, as observed in cell culture experiments [22] and when assaying primary cells isolated from KSRP$^{-/-}$ mice [16,69–71].

4.1. Innate Immune Cells

KSRP confers regulation of immune responses on several levels. As outlined above, KSRP was reported to promote granulocytic and at the same time to inhibit monocytic differentiation via processing of miR-129, and indirectly via attenuation of RUNX1 [39]. The modulation of innate immune responses by KSRP is mediated in part via regulation of type I and III interferon expression in immune and non-immune cells and modulation of retinoic acid-inducible gene (RIG-)I receptor signaling [16,72,73], in addition to other cytokines generated by innate immune cells as shown for monocytes/macrophages and PMN.

We observed that in the collagen antibody-induced arthritis (CAIA) disease, mouse model KSRP knock out (KSRP$^{-/-}$) mice developed markedly lower joint inflammation compared with wild type (WT) mice, accompanied by lower expression of pro-inflammatory cytokines. In general, KSRP$^{-/-}$ mice were less susceptible to CAIA induction and had a much less pronounced disease severity. Myeloid cells, such as macrophages or PMN, were reduced in peripheral blood mononuclear cells isolated from KSRP$^{-/-}$ mice, and in LPS-stimulated spleen cells isolated from KSRP$^{-/-}$ mice. Since these cells are critically involved in CAIA induction [74], the lower number of myeloid cells in KSRP$^{-/-}$ mice may account at least in part for this phenomenon [69]. In this regard, we also showed that the frequency of apoptotic CD11b^{+} cells was significantly enhanced in KSRP$^{-/-}$ mice.

Whereas DC are the most potent type of APC [75], PMN are rather specialized in direct eradication of pathogens [76], e.g., by phagocytosis, the release of reactive oxygen species (ROS) and pathogen-binding chromatin-based extracellular traps, termed NETosis [77]. Furthermore, activated PMN secrete numerous cytokines/chemokines to attract and polarize leukocytes [78]. PMN are the first immune cell population that immigrates infected/inflamed tissue [79]. We observed that KSRP deficiency improved the migration of PMN, which suggested that KSRP may attenuate PMN migration in vivo (Figure 3).

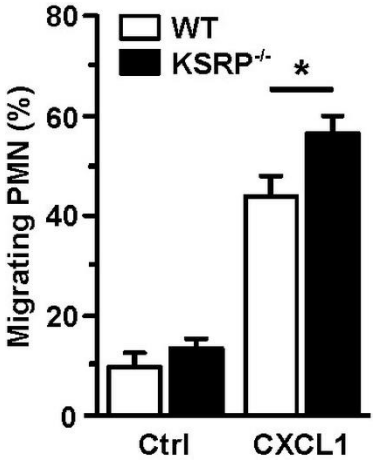

Figure 3. KSRP coregulates PMN migration. Bone marrow cells derived from WT and KSRP$^{-/-}$ mice towards C-X-C motif chemokine ligand 1 (CXCL1) were assessed in a transwell (Ø 5 µm) migration assay. Bone marrow derived cells were cultivated in 24-well plates with Iscove's Modified Dulbecco's Medium supplemented with 5% FCS, 1% penicillin/streptavidin, 2 mM L-Glutamin and 50 µM β-Mercaptoethanol, in a final concentration of 5 × 10^5 cells/mL. Seeded cells were stimulated with 250 ng/mL CXCL1. Control (Ctrl): w/o chemokine. After incubation for 4 h at 37 °C and 10% CO_2, the frequency of migrating Ly6G$^+$ PMN was assessed by flow cytometric analysis (mean + SEM, $n = 6$; * $p < 0.05$).

Altogether, several lines of experimental evidence indicate that KSRP regulates/influences the differentiation and function of PMN and monocytes/macrophages.

4.2. Adaptive Immune Cells

Only limited knowledge exists about the importance of KSRP for cells of the adaptive immune system. B cells and T cells are termed adaptive immune cells since these are activated in an antigen-specific manner, and thereby are able to evoke pathogen-specific immune responses. B cells provide a variety of important functions to the adaptive immune system including antibody production, antigen presentation, and cytokine secretion [80].

In adaptive immune responses, the cytokine environment is important for the activation and differentiation of CD4$^+$ T cells into distinct Th cell subsets (e.g., Th1, Th2, Th9 and Th17) [81]. Activated CD4$^+$ T cells play an important regulatory role as they are not only required for full activation of CD8$^+$ T cells [82], but also of B cells [83]. These helper functions are predominantly determined by Th-released cytokines. CD8$^+$ T cells give rise to cytotoxic T lymphocytes (CTL) which directly recognize and kill infected (or malignant) cells that present the CTL-specific antigen via major histocompatibility complex I [84].

We demonstrated that knockdown of the KSRP protein enhanced the proliferation of polyclonally stimulated CD4$^+$ T cells, but not of KSRP$^{-/-}$ CD8$^+$ T cells. Modulation of IL-2 expression, previously reported as a KSRP target in cultures of immortalized cell lines [22], seemed not to contribute to enhanced T cell proliferation, since we were not able to detect any difference in IL-2 production on mRNA or protein level between primary KSRP$^{-/-}$ and WT CD4$^+$ T cells. Another obvious finding was that upon polyclonal stimulation KSRP$^{-/-}$ CD4$^+$ T cells produced higher amounts of IL-4, IL-5, IL-9, IL-10 and IL-13 as compared with WT cells. This overall change in cytokine pattern indicates that KSRP serves to inhibit Th2 polarization [70].

In order to identify the molecular mechanisms responsible for the altered cytokine expression and proliferation of KSRP$^{-/-}$ CD4$^+$ T cells, we analyzed transcription factor expression in polyclonally stimulated CD4$^+$ T cells. We observed no genotype-specific differences in Th2-associated GATA3 expression (Figure 4). This observation suggested that KSRP regulated Th polarization by targeting other mRNA species either directly or via miRNA regulation [85].

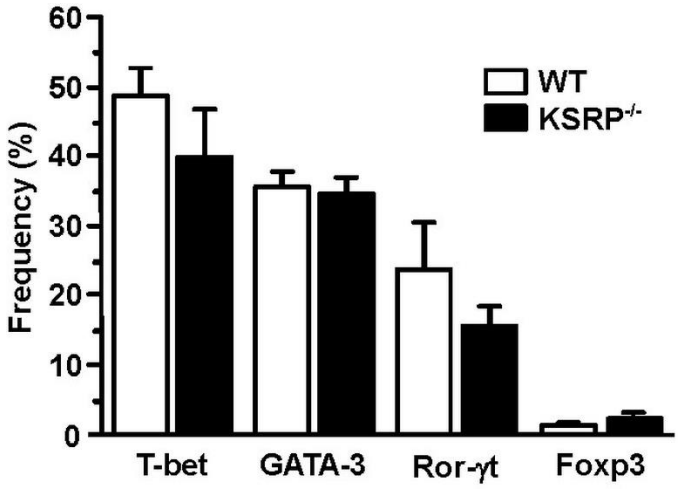

Figure 4. Expression of GATA-3 in stimulated CD4$^+$ T cells is not affected by KSRP deficiency. Splenic CD4$^+$ T cells (WT, KSRP$^{-/-}$) were isolated by magnetic bead separation, and were polyclonally stimulated with agonistic CD3- (1 μg/mL) and CD28- (2 μg/mL) specific antibodies for 72 h. Transcription factor expression was delineated by intracellular flow cytometric analysis. Data denote the frequencies of transcription factor-positive CD4$^+$ T cell (mean + SEM, n = 4).

All cytokine mRNA species whose expression is differentially regulated by KSRP in CD4$^+$ T cells contain ARE in their 3′-UTR, but whereas responsiveness of IL-10 and IL-13 mRNA for rapid degradation by other ARE-binding RBP such as TTP [86], AUF1 [87] and HuR [88] has been documented, much less is known about mechanisms of post-transcriptional regulation of IL-5 [89] and IL-9 [90] mRNA expression. We detected direct binding of KSRP to the IL-10 and IL-13 mRNA 3′-UTR, respectively, in pull down experiments, but not to the IL-5 and IL-9 mRNA 3′-UTR [70]. Further, we observed no direct effect of KSRP on the decay of either mRNA monitored. However, polyclonally stimulated KSRP$^{-/-}$ CD4$^+$ T cells displayed increased expression of IL-4 on mRNA and protein level as compared with the corresponding WT control, which may be explained by a longer IL-4 mRNA half-life [70]. Therefore, our data suggested that KSRP is a negative regulator of IL-4 expression. IL-4 on one hand is a master regulator of Th2 polarization [91], and on the other hand constitutes the prototypic Th2-associated cytokine [92]. Further studies are necessary to elucidate by which mechanisms KSRP modulates Th2 polarization on a molecular level.

In summary, KSRP is an important negative regulator of pro-inflammatory mediators by using its several functions, and is involved in immune response.

4.3. KSRP as a Negative Modulator of Immune Responses in Infection

In the case of immune responses in consequence to infections, the host innate immune system plays a significant role in the elimination of pathogen infection [93]. Danger receptors such as Toll-like receptors (TLR) play an essential role in the activation of innate immunity by recognizing specific patterns of microbial components and activating downstream intracellular signaling pathways such as nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) [94]. On one hand, the resulting expression of, e.g., pro-inflammatory cytokines needs to be fast and robust to establish a swift immune response to an invading pathogen [95]. On the other hand, cytokine expression has to be controlled strictly to avoid an excessive immune response resulting in a cytokine storm [96], extensive tissue destruction [97] or autoimmune reactions [98]. Due to its central role in AMD of mRNA species encoding pro-inflammatory mediators, KSRP is considered as an important negative regulator of inflammatory immune responses [15,22].

In this regard, KSRP was demonstrated to inhibit the activation of the retinoic acid-inducible gene (RIG-)I receptor, which is involved in antiviral defense mechanisms [73].

In the absence of KSRP RIG-I receptor induced antiviral signaling was enhanced and accordingly viral replication was reduced.

Type I interferons (IFN-α and IFN-β) play crucial roles in the innate immune response against viral infection [99,100]. Lin and colleagues detected in cells derived from KSRP$^{-/-}$ mice that type I interferons were upregulated, which implied that KSRP plays a crucial role in maintaining low basal IFN-α/β expression levels in the absence of stimuli [72]. Additionally, type I interferon levels were increased in KSRP$^{-/-}$ mice in response to viral infection as a result of decreased mRNA decay. Resulting from this increased expression of IFN-α and IFN-β, the KSRP$^{-/-}$ mice were more resistant to vesicular stomatitis virus and herpes simplex virus I than WT mice.

In another study, it was demonstrated that *Helicobacter pylori* infection in mice downregulated KSRP expression and upregulated expression of inflammatory-related genes such as C-X-C motif ligand 2 and TLR2. Increasing mRNA decay of pro-inflammatory factors through KSRP overexpression in *H. pylori* mice facilitated *H. pylori* proliferation and colonization and induced aggravated gastric inflammation and mucosal damage, implying that downregulation of KSRP is necessary for an effective innate immune response against *H. pylori* [101].

Interestingly, modulation of gene expression by KSRP plays an important role not only in Gram-negative pathogens. Lipoteichoic acid (LTA) from the Gram-positive *Staphylococcus aureus* (aLTA) also constitutes a potent immunostimulation agent. KSRP was downregulated by aLTA in a monocytic cell line (THP-1) at protein level [102]. However, there were no differences of KSRP expression at gene level. Zeng and colleagues hypothesized that aLTA, similar to LPS, may regulate expression of inflammatory genes at the transcriptional level via TLR2-mediated activation of NF-κB. At the post-transcriptional level, aLTA might downregulated the destabilizing factor KSRP. Through these two regulatory mechanisms, aLTA treatment could increase and stabilize the mRNAs, and consequently elevate cytokine production.

Investigations regarding *Salmonella enteritidis* infection in Caco-2 cells revealed also decreased KSRP expression, similar to *H. pylori* infection [103]. In addition, overexpression of KSRP in Caco-2 cells resulted in reduced levels of inflammatory factors. Interestingly, Nie and coworkers further demonstrated that the decreased expression of KSRP protein following *S. enteritidis* infection was diminished when blocking the NF-κB signaling pathway, revealing that changes in the expression of KSRP were regulated by this pathway.

However, infection does not result, in general, in subsequent downregulation of KSRP mRNA as shown for H69 cells infected with *Cryptosporidium parvum* [46]. Instead, infection by *C. parvum* activated TLR4/NF-κB signaling and increased miR-27b-3p expression, causing a translational suppression of KSRP in infected host epithelial cells. In turn, downregulation of KSRP stabilized iNOS mRNA and promoted production of nitric oxide, exerting antimicrobial activity, by epithelial cells.

Interestingly, negative regulation of pro-inflammatory factors also plays an important role in the prevention of hepatic fibrosis. Pro-inflammatory factors such as cytokines activate hepatic stellate cells and thereby contribute to the development of hepatic fibrosis [104]. Wang and coworkers showed that soluble egg antigen stimulation and *Schistosoma japonicum* infection increased KSRP mRNA and protein levels and downregulated miR-27b-3p expression in vitro and in vivo [105]. In accordance, both knockdown of miR-27b-3p and overexpression of KSRP attenuated *S. japonicum*-induced hepatic fibrosis in vivo, due to increased mRNA decay of proinflammatory factors mediated by KSRP.

Taken together, KSRP seems to be an important negative regulator of inflammatory immune responses in infection. While downregulation of KSRP is important for the generation of a robust immune response, against a pathogen, it is equally important to limit immune responses/production of proinflammatory factors through KSRP-mediated mRNA decay to keep the immune system homeostasis in balance.

4.4. KSRP as a Regulator of Auto-Inflammatory Diseases

As outlined above (see Section 4.1), we observed that, somewhat surprisingly, induction of CAIA, a well-established RA model in C57BL/6 mice, was attenuated in terms of disease onset and severity in KSRP$^{-/-}$ mice, as compared with WT mice [69]. In addition to the reduced number of PMN in KSRP$^{-/-}$ mice, the attenuated course of CAIA in KSRP$^{-/-}$ mice may be explained also by the intrinsic property of KSRP$^{-/-}$ CD4^{+} T cells to express preferentially Th2-related cytokines such as IL-4, IL-5, IL-10, and IL-13, which have been described to be important for the resolution of RA-associated inflammation in diseased joints [69,70,106].

We analyzed the role of KSRP also in a murine systemic lupus erythematosus (SLE) model. To this end, we made use of MRL-Fas^{lpr} mice, which spontaneously develop a SLE-like syndrome [107], and bred those with KSRP$^{-/-}$ mice. The derived MRL-Fas^{lpr}KSRP$^{-/-}$ mice presented with more severe symptoms of glomerulonephritis, indicative of important protective effects of KSRP in the regulation of immune homeostasis in the kidney [71]. Moreover, we detected that the knockout of KSRP might have different effects on disease progression depending on the organ manifestation. In contrast to glomerulonephritis, lymphadenopathy, a prominent disease symptom in MRL-Fas^{lpr} mice, was attenuated in Fas^{lpr}KSRP$^{/}$ mice, indicating a disease driving force of KSRP. The initial screen of the new mouse strain identified cell types (CD4^{+} IFN-γ^{+} T cells, FoxP3^{+} T cells) and targets (IL-1R, CD11a) of interest for further studies.

Concerning chronic inflammatory disease, Xia and coworkers provided evidence for a new mechanism by which liver epithelial cells maintain homeostasis during inflammation [108]. Previous studies indicated that increased levels of C-X3-C motif chemokine ligand 1 (CX3CL1) in the liver are associated with severe inflammatory liver disease [109,110]. In this study, CX3CL1 mRNA stability was demonstrated as directly regulated by KSRP through its interaction with ARE within the CX3CL1 mRNA 3′-UTR [108]. Thus, upregulation of KSRP destabilized CX3CL1 mRNA in liver epithelial cells. In addition, miR-27b-3p was identified as a negative regulator of immune reaction in response to IFN-γ stimulation: IFN-γ stimulation decreased miR-27b-3p expression and increased KSRP protein contents without changing its mRNA level in vitro and in vivo. Consequently, miR-27b-3p regulated the stabilization of CX3CL1 mRNA by attenuating KSRP mRNA translation efficiency. In agreement, downregulation of miR-27b-3p following IFN-γ stimulation resulted in KSRP induction, providing negative feedback regulation of chemokine expression in liver epithelial cells in response to inflammation.

With regard to autosomal recessive diseases such as cystic fibrosis (CF), caused by massive pro-inflammatory phenotype in the lung [111], Bhattacharyya and colleagues reported that deregulation of miR-155 might be involved in CF pathophysiology [112]. They detected high levels of miR-155 in cultured CF IB3-1 and primary lung epithelial cells and demonstrated the antagonistic role of the RNA-BP KSRP und TTP in the regulation of miR-155 biogenesis in CF cells. Suppression of KSRP led to inhibition of miR-155 maturation, whereas overexpression of TTP suppressed the processing of miR-155 through the induction of miR-1 in CF lung epithelial cells [112].

To sum up, the studies implicate that not only is KSRP an important regulator of immune reactions, KSRP itself is also regulated by miRNAs. Interaction with other RBPs also becomes obvious, raising the question of whether another destabilizing RBP can take over the function of KSRP in the case of KSRP deficiency. To answer this upcoming question, further studies are necessary.

4.5. KSRP Affects Tumorigenesis

Besides its role in shaping immune responses in case of autoimmunity and infection, KSRP also plays a role in tumor pathogenesis. Several reports have demonstrated an association of KSRP with different types of lung cancers. In small cell lung cancer (SCLC) increased KSRP protein levels were detected in tumor tissue, and this correlated with advanced tumor stage [113]. Knockdown of KSRP inhibited SCLC cell proliferation but had

no effect on cell migration or invasion. KSRP contributed indirectly to tumor progression by promoting miR-26a maturation that led to inhibition of the tumor suppressor phosphatase and tensin homolog. Bikkavilli et al. described enhanced expression of KSRP in non (N)SCLC tissue, which correlated with poor overall survival [114]. The oncogenic properties of KSRP were attributed to KSRP-mediated downregulation of the tumor suppressor Sprouty RTK signaling antagonist 4. In addition, Yan et al. described KSRP as a metastasis-associated molecule in NSCLC [115]. In that study, interaction of KSRP with heterogeneous nuclear ribonucleoprotein C was observed, which may promote tumor metastasis by activating the IFN-α-Janus kinases—signal transducer and activator of the transcription protein 1 signaling pathway. In contrast to the results of the aforementioned studies, interestingly, KSRP was also shown to act in an anti-tumorigenic manner in NSCLC. In this regard, Chien et al. detected reduced KSRP protein expression in tumor tissue and a strong correlation between KSRP expression and overall survival [116]. The authors proposed that KSRP was necessary to promote miR-23a maturation, thus leading to destabilization of early growth response 3 mRNA, resulting in inhibition of NSCLC cell mobility.

In colorectal cancer (CRC), enhanced expression of KSRP was found in tumor tissue and this was associated with a worse overall survival [117]. KSRP seemed to drive epithelial cell proliferation in primary and metastatic cells through control of cell cycle progression and promoted, e.g., angiogenesis by enhancing vascular endothelial growth factor secretion. In addition, enhanced KSRP expression in CRC cells was associated with resistance to 5-fluoruracil treatment. Mechanistically, KSRP was demonstrated to down-regulate mRNA levels of the tumor suppressor ERBB receptor feedback inhibitor 1, by enhancing the maturation of miR-501-5p [118].

KAI1 COOH-terminal interacting tetraspanin (KITENIN) contributed to tumor progression and poor clinical outcomes in various cancers including colorectal cancer, most probably by enhancing neoangiogenesis [119]. KSRP contributed to metastasis in CRC by stabilizing the functional KITENIN complex [120]. DKC1125 (disintegrator of KITENIN complex #1125) was reported to suppress KITENIN activity by direct binding to KSRP. This interaction led to destabilization of the KITENIN complex by recruiting the receptor for activated C kinase 1 and miRNA-124, thereby suppressing metastasis in CRC. Of note, KSRP could also have a protective function in CRC by destabilizing Homeobox protein C10 (HOXC10) mRNA [121]. In CRC and in CRC-initiating cells, high expression of circular HOX (cis-HOX) RNA has been detected. cis-HOX blocked KSRP-mediated HOXC10 mRNA destabilization, thus leading to activation of the tumor-promoting Wnt/b-catenin signaling pathway.

In glioblastoma multiforme (GBM) cells, KSRP was demonstrated to inhibit migration of GBM cells, and, therefore, re-sensitized them to chemotherapy [122]. High KSRP expression was detected in GBM patients who survived long after surgery, indicating a link between KSRP and a better overall survival. Moreover, one study linked KSRP to inducing apoptosis in glioma cells in a caspase-dependent manner [123].

The Follistatin-related protein 1 (FSTL1) primary mRNA transcript also encoded for miR-198, and the switch between expression of the FSTL1 protein and miR-198 is an important regulator of tumor metastasis and wound healing [124]. KSRP processed FSTL1 mRNA to generate miR-198 by binding to the FSTL1 3′-UTR [125]. In keratinocytes, TGF-β induced the expression of miR-181a, which bound to the 3′-UTR of KSRP mRNA and thereby promoted its decay. This resulted in impaired miR-198 but enhanced FSTL1 expression. In the case of temozolomide resistance in glioma, TGF-β increased FSTL1 protein expression and decreased miR198 expression without affecting miR-181a or KSRP expression [126]. In these cases, TGF-β enhanced the expression levels of lncRNAs H19 and HOXD cluster antisense RNA 2, which competitively bind to KSRP and prevent KSRP from participating in FSTL1/miR-198 switching. This also resulted in increased expression of O-6-methylguanine DNA methyltransferase, which correlated with bad prognosis and resistance to chemotherapy.

Additionally, in squamous cell carcinoma, KSRP mediated FSTL1/miR-198 processing constitutes an important factor for metastasis [125]. Downregulation of KSRP in malignant epithelial cells inhibited miR-198 processing and thus contributed to FSTL1 expression.

In cervical cancer, interaction of KSRP with lncRNA LINC01305 promoted tumor growth [127]. Inactivation of the breast cancer susceptibility gene 1 (BRCA1) plays a significant role in breast and ovarian cancers and qRT-PCR analyses indicated that KSRP was over-expressed in BRCA1 mutated tumors [128]. In different breast cancer cell lines, mutations of the tumor suppressor gene TP53 led to changes in proteasome gene expression and enhanced proteasomal activity. In this context, anti-oncogenic properties were attributed to KSRP [129]. The p53-mediated proteasomal dysfunction resulted in increased KSRP degradation, and this impaired expression of the tumor-suppressive miRNAs let-7a and miR-30c, whose maturation is dependent on KSRP as outlined above. In breast cancer cells, IL-1 β-mediated replacement of KSRP by the mRNA stabilizing factor HuR at the IL-8 3′-UTR has been described, which in light of the tumor-promoting role of IL-8 may contribute to cancer progression [130]. We have previously demonstrated concurrent binding of KSRP and HuR to the same target 3′-UTR [17].

In hepatocellular carcinoma (HCC) over-expression of FBPs was identified. As outlined above, KSRP, also known as FBP2, has been shown to promote c-myc transcription [5], and c-myc was demonstrated to contribute to HCC progression [131]. The peptidyl-prolyl isomerase Pin1 is over-expressed in several cancer tissues, and may promote tumorigenesis by regulating mRNA decay in cooperation with the ARE-binding proteins AUF1 and KSRP [132].

In esophageal squamous cell carcinoma (ESCC), increased KSRP expression levels were associated with worse overall survival [133]. KSRP promoted growth, migration, and invasion of ESCC cells by enhancing the maturation of cancer-associated miRNAs, such as miR-21, miR-130b, and miR-301. Accordingly, this reduced the expression of the according miRNA target mRNAs, such as bone morphogenetic protein 6, programmed cell death protein 4, and Metalloproteinase inhibitor 3, and promoted epithelial to mesenchymal transition. Additionally, in osteosarcoma cells, KSRP was significantly upregulated, and contributed to enhanced cell proliferation and migration [134]. Likewise, KSRP was associated with enhanced proliferation of melanoma cells [135]. Here, KSRP-mediated destabilization of killin mRNA, a p53-regulated DNA replication inhibitor. Further, KSRP promoted invasiveness and metastasis of pancreatic cancer cells by interaction with the small nucleolar RNAs SNORA18 and SNORA22, and thereby enhanced the number of cell protrusions [136].

Another investigation demonstrated, in mice, that lncRNA Neat1 interacted with KSRP to promote metastasis in soft tissue sarcomas [137]. It was assumed that the Neat1/KSRP complex functioned as an RNA splicing regulator to mediate tumor cell colonization of the lung. Additionally, KSRP may also be involved in papillary thyroid carcinoma (PTC) by interacting with lncRNA AB074169 (lncAB) [138]. In normal cells, lncAB blocked mRNA decay activity of KSRP, whereas in PTC tumor cells lncAB DNA was hypermethylated, resulting in enhanced mRNA degradation of p21 by KSRP, which, in turn, promoted cell proliferation.

Overall, KSRP seems to promote cell proliferation and metastasis, but results strongly depend on tumors investigated, and opposite effects have also been described. In the context of tumorigenesis, a number of different KSRP-mediated mechanisms and KSRP targets has been described. In addition to direct regulation of mRNA stability, KSRP-mediated miRNA maturation and interaction of KSRP with lncRNA on protein level have been observed in different tumor models.

5. Conclusions

KSRP constitutes an important regulator of both innate and adaptive immune cells in addition to tumorigenesis. To date, KSRP may be considered as an important break of immune activation by inhibiting the production of proinflammatory cytokines by the

initiation of mRNA decay and the promotion of miRNA maturation. This break is released in response to infection and also due to post-transcriptional modulation of KSRP activity. Aside from the role of KSRP to regulate the overall extent of immune activation, it also serves to finetune the character of an (adaptive) immune response, as reflected, e.g., by the intrinsic Th2 bias of $CD4^+$ T cells in case of KSRP deficiency. However, further in-depth analyses are required to gain full insight in KSRP targets, especially with regard to the issue of whether this RBP acts only on the level of effector cytokines, or orchestrates the shape of immune responses also, by affecting the expression of key transcription factors. Despite the emerging evidence of the regulatory importance of KSRP, its function has been addressed in few immune cell types to date. Therefore, further studies need to address the functional role of KSRP in other innate (e.g., NK cells, innate lymphoid cells) and adaptive (B cells) immune cell types under basal conditions and in response to activation in suitable disease models. With regard to the latter, it will be important to employ mice with conditional KSRP deficiency in order to address the cell type-specific role of this RBP. Finally, it will also be important to shed light on the exact role of KSRP in tumorigenesis; depending on the tumor type, KSRP may either block or promote tumor induction and progression.

Altogether, deeper understanding into the role of KSRP in the immune system and for tumor induction and progression is a necessary prerequisite for the development of drugs which, when applied by suitable nano-carriers, may allow control of KSRP in a cell type-specific manner for therapeutic purposes. According to our data, in particular the ability of KSRP to shift T helper cell polarization towards a distinct direction (enhancing KSRP activity favors Th1 response, inhibition of KSRP activity promotes Th2 response), may be an interesting tool to restore immune homeostasis in different chronic inflammatory diseases.

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Abbreviations

aa	Amino acid
ALAE	Axon-enriched long intergenic noncoding RNA regulating axon elongation
AMD	ARE-mediated mRNA decay
APC	Antigen presenting cell
ARE	AU-rich element
ATM	Ataxia telangiectasia mutated
AUF1	AU-binding factor 1
BRCA1	Breast cancer susceptibility gene 1
CAIA	Collagen antibody induced-arthritis
CD	Cluster of differentiation
CF	Cystic fibrosis
Cis-HOX	circular HOX
CRC	Colorectal cancer
CTL	Cytotoxic T lymphocyte
CX3CL1	C-X3-C motif chemokine ligand 1
CXCL1	C-X-C motif chemokine Ligand 1
DC	Dendritic cell
ESCC	Esophageal squamous cell carcinoma
FBP	Far upstream element binding protein
FSTL1	Follistatin-related protein 1

FUSE	Far upstream element
GBM	Glioblastoma multiforme
HCC	Hepatocellular carcinoma
HuR	Human antigen R
HOXC10	Homeobox protein C10
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KH	K homology
KITENIN	Kai 1 COOH-terminal interacting tetraspanin
KSRP	KH-type splicing regulatory protein
lncRNA	Long non-coding RNA
LPS	lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen actiaved protein kinase
miRNA	MicroRNA
NF-κB	Nuclear factor k-light-chain-enhancer of activated B cells
NSCLC	Non-small cell lung cancer
PKB	Protein kinase B
PMN	Polymorphonuclear neutrophilic leukocytes
PTC	Papillary thyroid carcinoma
RA	Rheumatoid arthritis
RBP	RNA-binding protein
RIG-I	Retinoic acid-inducible gene I
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RUNX1	Runt-related transcription factor 1
SCLC	Small cell lung cancer
SLE	Systemic lupus erythematosus
TGF-β	Transforming growth factor β
TTP	Tristetraprolin
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
Th	T helper cell
USE	Upstream sequence element
UTR	Untranslated region
WT	Wild type

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6.2 Publication 2

The mRNA-Binding Protein KSRP Limits the Inflammatory Response of Macrophages

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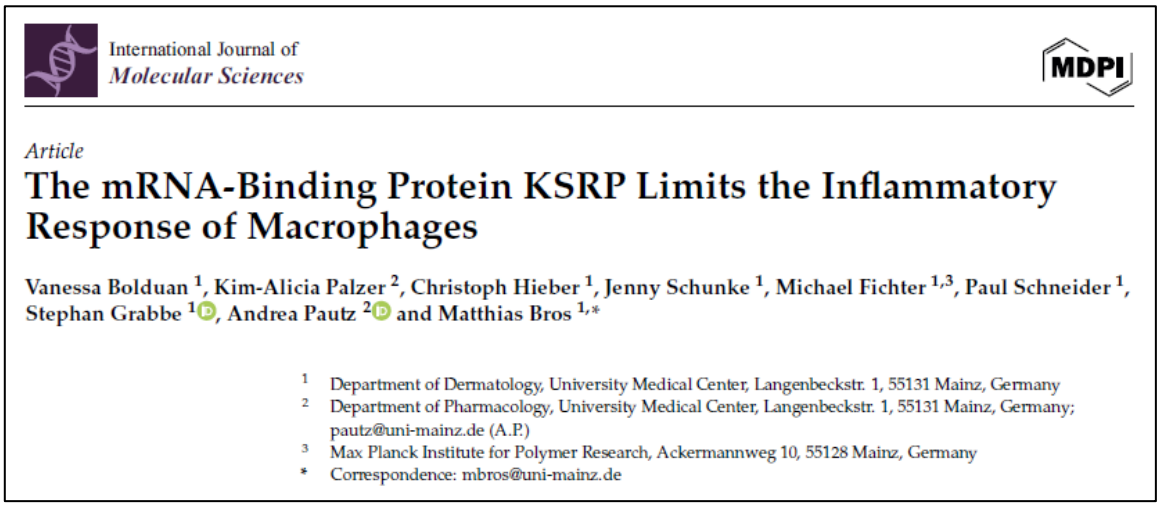

International Journal of *Molecular Sciences*

MDPI

Article

The mRNA-Binding Protein KSRP Limits the Inflammatory Response of Macrophages

Vanessa Bolduan [1], Kim-Alicia Palzer [2], Christoph Hieber [1], Jenny Schunke [1], Michael Fichter [1,3], Paul Schneider [1], Stephan Grabbe [1], Andrea Pautz [2] and Matthias Bros [1,*]

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Summary:

This study explored the role of KSRP in macrophage immune-phenotypes using bone marrow-derived macrophages (BMDMs) from wild-type (WT) and KSRP-deficient ($KSRP^{-/-}$) mice. RNA sequencing demonstrated that KSRP−/− BMDMs exhibited markedly elevated mRNA expression of genes associated with inflammatory and immune responses, particularly type I interferon responses, following LPS stimulation. Similarly, time-course studies demonstrated elevated mRNA and protein levels of IFN-γ, IL-1β, and IL-6 in KSRP-deficient macrophages following 6 h of LPS stimulation. Similar outcomes were observed in primary peritoneal macrophages from KSRP-deficient mice, which demonstrated elevated IL-6, TNF-α, CXCL1, and CCL5 protein levels in response to LPS stimulation. In a sepsis model, KSRP-deficient mice produced higher IL-1β levels than WT controls. In line, Immunoprecipitation revealed binding of KSRP to IL-1β mRNA. Overall, these findings suggest that KSRP serves as a pivotal negative regulator of cytokine expression in macrophages.

Author contribution:

- Design, conduction and evaluation of all biological experiments
- Preparation of graphs and figures
- Preparation and writing of the manuscript together with Dr. Matthias Bros
- Proofreading of the submitted manuscript

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Abstract: KH-type splicing regulatory protein (KSRP) is a single-stranded nucleic acid-binding protein with multiple functions. It is known to bind AU-rich motifs within the 3′-untranslated region of mRNA species, which in many cases encode dynamically regulated proteins like cytokines. In the present study, we investigated the role of KSRP for the immunophenotype of macrophages using bone marrow-derived macrophages (BMDM) from wild-type (WT) and KSRP$^{-/-}$ mice. RNA sequencing revealed that KSRP$^{-/-}$ BMDM displayed significantly higher mRNA expression levels of genes involved in inflammatory and immune responses, particularly type I interferon responses, following LPS stimulation. In line, time kinetics studies revealed increased levels of interferon-γ (IFN-γ), interleukin (IL)-1β and IL-6 mRNA in KSRP$^{-/-}$ macrophages after 6 h subsequent to LPS stimulation as compared to WT cultures. At the protein level, KSRP$^{-/-}$ BMDM displayed higher levels of these cytokines after overnight stimulation. Matching results were observed for primary peritoneal macrophages of KSRP$^{-/-}$ mice. These showed higher IL-6, tumor necrosis factor-α (TNF-α), C-X-C motif chemokine 1 (CXCL1) and CC-chemokine ligand 5 (CCL5) protein levels in response to LPS stimulation than the WT controls. As macrophages play a key role in sepsis, the in vivo relevance of KSRP deficiency for cytokine/chemokine production was analyzed in an acute inflammation model. In agreement with our in vitro findings, KSRP-deficient animals showed higher cytokine production upon LPS administration in comparison to WT mice. Taken together, these findings demonstrate that KSRP constitutes an important negative regulator of cytokine expression in macrophages.

Keywords: RNA-binding proteins; cytokines; chemokines; KSRP; macrophages; LPS-induced sepsis

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1. Introduction

Macrophages are present in all tissues of the body. When activated by pathogens or inflammation, macrophages exert various cytotoxic functions such as phagocytosis and attract immune cells to the site of inflammation by releasing cytokines and chemokines [1]. These properties make them a crucial component of the immune system. Activated macrophages secrete pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, as well as other pro-inflammatory mediators like nitric oxide (NO) and prostaglandins [2]. Moreover, macrophages serve as antigen-presenting cells and activate antigen-specific T cells [3].

Gene expression is orchestrated at the transcriptional level by transcription factors and long non-coding RNAs (lncRNA) that bind to cognate recognition sites within the gene promoter and enhancer/silencer regions, thereby regulating gene transcription [4]. In addition, genes which require dynamic regulation in response to stimuli are regulated at the post-transcriptional level [5]. In this regard, micro(mi)RNAs bind sequence-complementary

sequence stretches within their target mRNA to limit mRNA stability and translational efficacy, respectively [6]. In addition to miRNA, RNA-binding proteins (RBP) also exert post-transcriptional gene regulation [7]. One class of RBP preferably engages AU-rich elements (ARE), which are mainly located in the 3′ untranslated region (UTR) of mRNA species [8]. AREs are the most prevalent recruiting motifs for RBP [9] and contain one or more core pentamers (e.g., AUUUA), often arranged in tandem repeats, and preferably located in a U-rich region [10]. Generally, AREs are characteristic of mRNAs encoding short-lived proteins, such as pro-inflammatory cytokines, transcription or growth factors [11]. Upon binding to their target sequences, ARE-RBPs either enhance (e.g., human antigen R, HuR) or limit (e.g., Tristetraprolin, TTP) mRNA stability and translation efficacy (e.g., T-Cell-Restricted Intracellular Antigen-1, TIA-1), respectively [9,12]. By now, an increasing number of studies have revealed the important role of RBP regarding the regulation of immune cell phenotypes [13,14].

KSRP (K homology [KH]-type splicing protein) is a ubiquitously expressed single-stranded nucleic acid-binding protein that has been reported to act as an mRNA stability-limiting RBP [15]. Additionally, it functions as a transcription factor and a maturation factor for various miRNA species [12]. So far, little is known about the cell type-specific function of KSRP in the immune system. In accordance with its central role in ARE-mediated decay of pro-inflammatory mediators, KSRP-deficient mice displaying a complete knockout in all tissues have been shown to present stronger anti-viral responses upon infection accompanied by elevated production of type I interferons [16]. Accordingly, KSRP has been considered an important negative regulator of inflammatory immune responses. In this regard, KSRP has been reported to limit cytokine production of activated immune cells, since it promoted decay of the according mRNA, as observed in cell culture experiments [17] and when assaying primary cells isolated from KSRP$^{-/-}$ mice [13,18,19]. Further, we reported an increased production of Th2-associated cytokines by polyclonal stimulated KSRP-deficient T cells [20].

This study aimed to delineate the role of KSRP as a regulator of the immune phenotype of macrophages. We show that KSRP-deficient macrophages are characterized by a stronger inflammatory response to LPS stimulation in vitro and in vivo, suggesting that KSRP is an important negative regulator for pro-inflammatory cytokine expression under disease conditions.

2. Results

To date, little is known about the role of the RNA-binding protein KSRP on the gene regulation of innate immune cells, such as macrophages. Since KSRP seems to play a crucial role by regulating the mRNA expression of pro-inflammatory cytokines, we hypothesized that KSRP-deficient macrophages may show a stronger inflammatory response in response to LPS stimulation.

2.1. KSRP$^{-/-}$ Mice Displayed Higher mRNA Expression Levels of Stimulation-Induced Cytokines

First, we analyzed bone marrow-derived macrophages (BMDM) under basal conditions and in response to LPS stimulation by flow cytometry. No differences were observed between WT and KSRP$^{-/-}$ BMDM under basal conditions or in response to LPS stimulation with regard to BMDM cell numbers and activation marker expression (CD80, CD86, MHCII) (Figure S1). Furthermore, no genotype-dependent differences in metabolism were found in either condition (Figure S2).

To obtain a broader view on KSRP-mediated transcriptional changes we subjected BMDM to RNA sequencing. Under basal conditions, KSRP-deficient BMDM were characterized by differential regulation of a total of 142 genes (up: 86; down: 56) compared to the WT group, the top 10 regulated genes are shown in Figure S3. For instance, the transcription factor *Zfp384* (zinc finger protein 384), limiting cytokine/chemokine gene transcription in macrophages in response to viral infection [21], or *Zbtb2* (zinc finger and BTB domain containing 2) inhibiting NF-κB activation [22] were upregulated in KSRP-deficient

BMDM (Table S1). To assess the impact of KSRP on gene expression of pre-stimulated macrophages, BMDMs were treated with LPS as the most potent TLR4 ligand [23]. After 6 h of stimulation, BMDMs lacking KSRP expressed 951 genes to higher and 652 genes to lower extent compared to the corresponding control group (WT). Figure 1A shows the top 50 up- or downregulated genes. Interestingly, protein interaction analysis using STRING Database revealed that the top 15 upregulated genes in KSRP$^{-/-}$ mice (Figure 1B), are interlinked and contribute to the regulation of innate immune response, particularly with regard to the cellular response to type I interferons (Figure 1C). Gene set enrichment analysis (GESA) indicated higher expression of many genes involved in the inflammatory response in KSRP-deficient BMDM (Figure 1D).

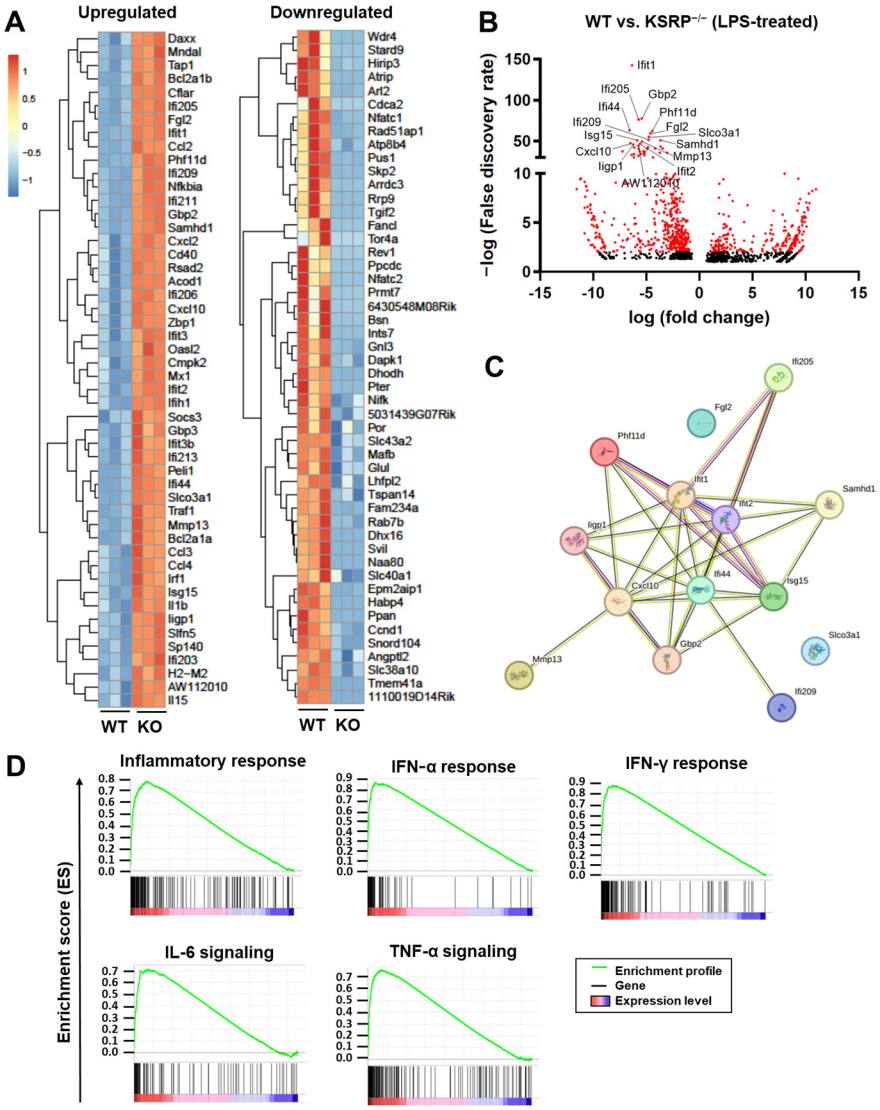

Figure 1. KSRP deficiency in BMDM results in upregulation of genes involved in inflammatory response, like IFN-α, IFN-γ, IL-6 and TNF-α signaling. LPS-stimulated BMDMs (WT and KSRP$^{-/-}$, each n = 3) were subjected to RNA-seq analysis. (**A**) Heatmap representation of the top 50 significantly upregulated (left panel) and significantly downregulated (right panel) genes in WT versus KSRP$^{-/-}$ BMDM (hierarchical clustering). The color legend denotes the level of gene expression (low: blue; high: red). (**B**) Volcano plot of significant ($p < 0.1$) quantified mRNA species. Significantly regulated genes (BH-adjusted $p < 0.05$ and log2 (fold-change) > 2) are given in red. The top 15 genes are named. (**C**) Results from analysis using STRING Database with the Top 15 upregulated gene in KSRP$^{-/-}$ mice. (**D**) Gene set enrichment plots of significantly regulated pathways (BH-adjusted $p < 0.05$).

2.2. KSRP$^{-/-}$ Mice Show Higher Protein Expression of IFN-γ, IL-1β and IL-6 in Response to Stimulation

Time kinetics studies confirmed higher levels of IFN-γ and IL-6 mRNA in KSRP$^{-/-}$ versus WT macrophages after 6 h and of IL-1β mRNA after 6 h and 16 h (Figure 2A). In agreement, KSRP$^{-/-}$ BMDM displayed higher IFN-γ levels after 12 h and 16 h, while IL-1β and IL-6 showed higher protein levels after 16 h (Figure 2B).

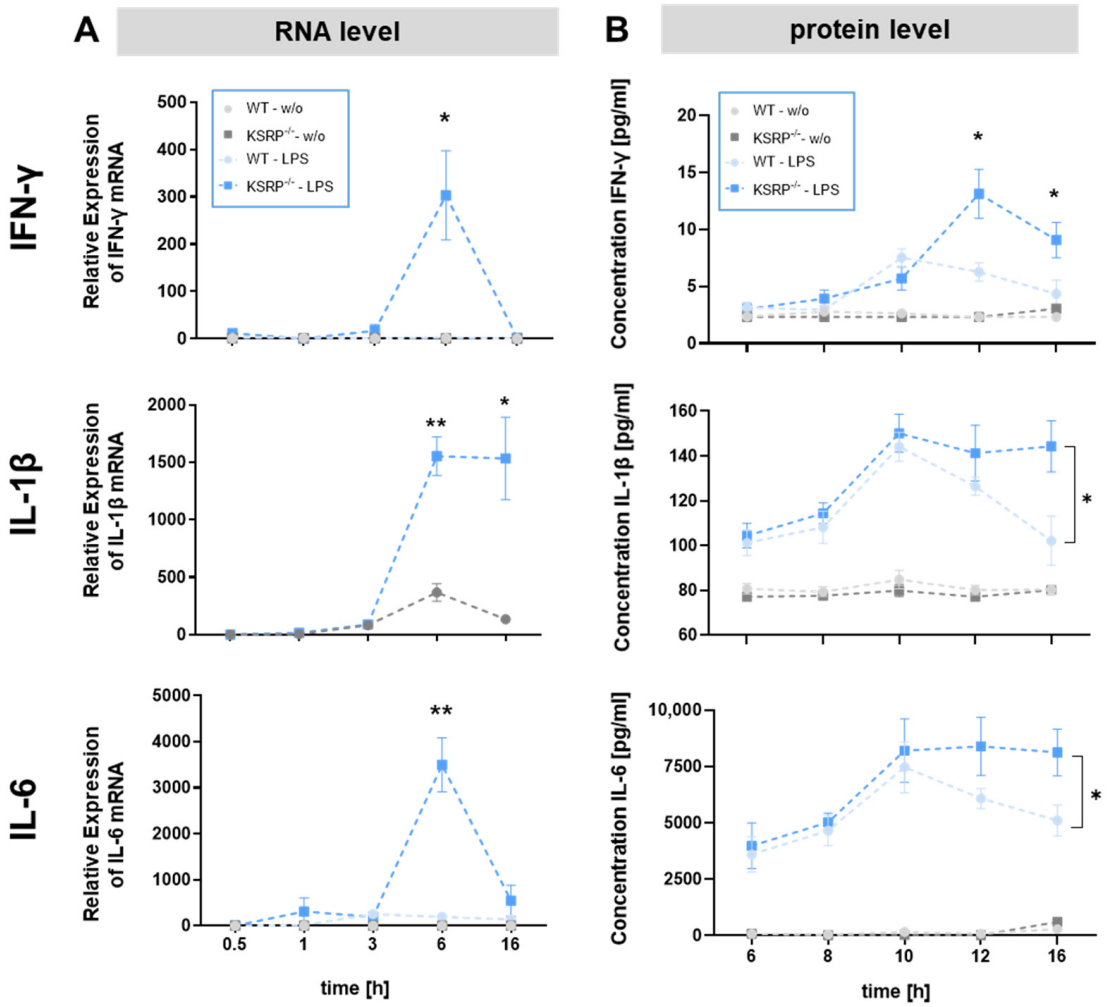

Figure 2. BMDM of KSRP$^{-/-}$ animals display higher mRNA expression and protein expression of IFN-γ, IL-1β and IL-6 in response to stimulation. (**A**) To analyze the mRNA expression of different immune relevant genes in cells of KSRP$^{-/-}$ or WT animals, BMDM were left untreated or were stimulated with 1 µg/µL LPS for different time periods (0.5 h, 1 h, 3 h, 6 h and 16 h). Subsequently, we prepared total RNA by homogenizing the sample in RLT plus lysis buffer and isolated the RNA using the RNeasy Plus Mini Kit. cDNA was synthesized by applying the iScript kit. Specific mRNA expression was measured using the qRT-PCR method and normalized to β2M mRNA expression. Shown are the mean ± SEM of n = 3–4 analyses (** $p < 0.01$, * $p < 0.05$; versus untreated WT cells; two-tailed Mann–Whitney test). (**B**) To analyze the protein expression of different immune-relevant genes in cells of KSRP$^{-/-}$ or WT animals, BMDM were left untreated or were stimulated with 1 µg/µL LPS for different time periods (6 h, 8 h, 10 h, 12 h and 16 h). Supernatants of BMDM were collected and cytokine levels were determined using the Anti-Virus-Response LegendPlex Kit from BioLegend (San Diego, CA, USA). Shown are the means ± SEM of n = 7–8 analyses (* $p < 0.05$; versus untreated cells; two-tailed Mann–Whitney test).

2.3. KSRP Binds Directly to IL-1β mRNA

To consider whether KSRP directly binds to the mRNAs of genes identified as stronger upregulated in stimulated KSRP-deficient versus WT BMDM, we used RNA immunoprecipitation. As KSRP is a multifunctional protein involved in various levels of gene regulation, this approach allowed us to investigate its role in mRNA regulation. KSRP directly binds to the mRNA of IL-1β, while the other examined mRNAs (IL-6, IFN-α, IFN-β, TNF-α, Ifit1, Gpb2) may not exhibit such binding (Figure 3). This indicates that IL-1b mRNA may directly regulated by KSRP.

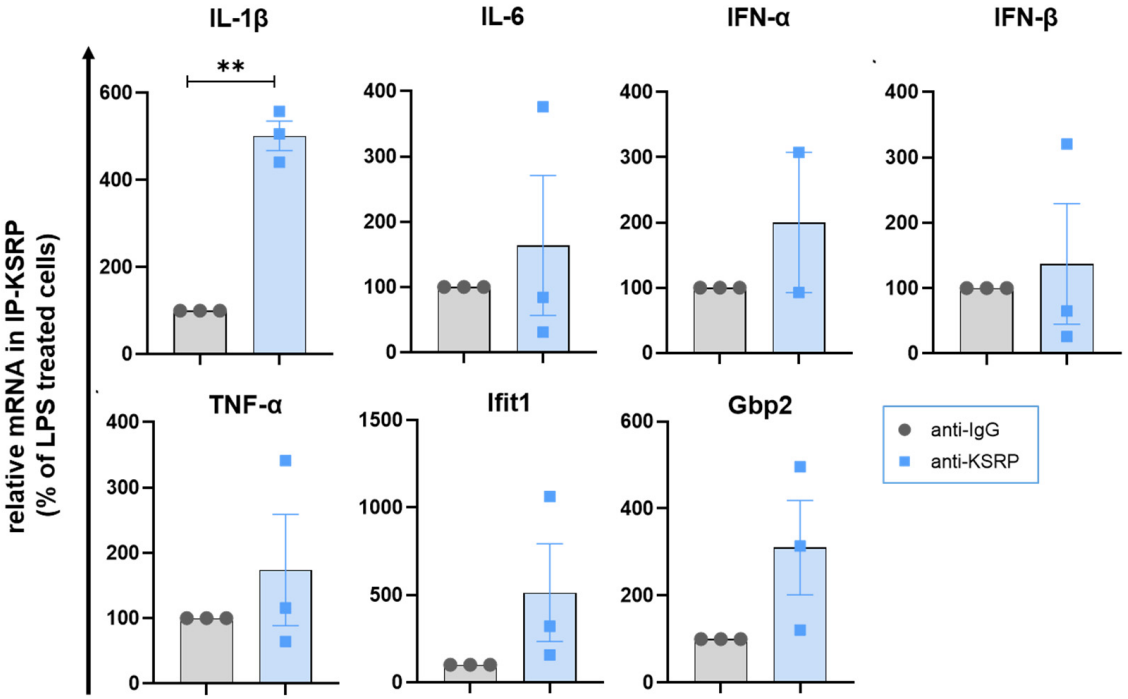

Figure 3. KSRP may regulates IL-1β mRNA stability by direct binding to its mRNA. WT BMDM were incubated with LPS (1 μg/mL) to induce pro-inflammatory chemo/cytokines for 6 h. After lysis of the cells, RNAs that bound to KSRP were immunoprecipitated with a specific antibody. To standardize the RNAs for the subsequent analyses, 1 ng of in vitro transcribed luciferase RNA was added to each sample. The RNA was purified, transcribed into cDNA and mRNA real time-PCR was used to determine the mRNA quantity of IL-1β, IL-6, IFN-α, IFN-β, TNF-α, Ifit1, Gbp2 and luciferase serving as a control for normalization. Shown are the means ± SEM and individual data points for each animal (n = 2–3 analyses) of the relative mRNA amounts bound to KSRP in relation to IgG controls (100%) (** $p < 0.01$; one-sample *t*-test).

2.4. KSRP$^{-/-}$ Primary Macrophages from the Peritoneum Produce Higher Levels of Pro-Inflammatory Cytokines in Response to Stimulation

Bollmann et al. observed higher mRNA expression of chemokine (C-X-C motif) ligand (CXCL)1, inducible nitric oxide synthase and TNF-α in response to stimulation with LPS in adherent primary KSRP$^{-/-}$ peritoneal cells compared to WT cells [18]. Therefore, we asked if expression of other (pro-inflammatory) cytokines was affected by KSRP deficiency as well. Hence, we also assessed primary macrophage-enriched cell suspensions obtained by peritoneal lavage and analyzed protein production of these cells. Regarding KSRP$^{-/-}$ peritoneal cells we detected an enhanced production of pro-inflammatory cytokines, such as CXCL1, CCL5, TNF-α and IL-6 (Figure 4), whereas expression levels of other mediator remained unaltered.

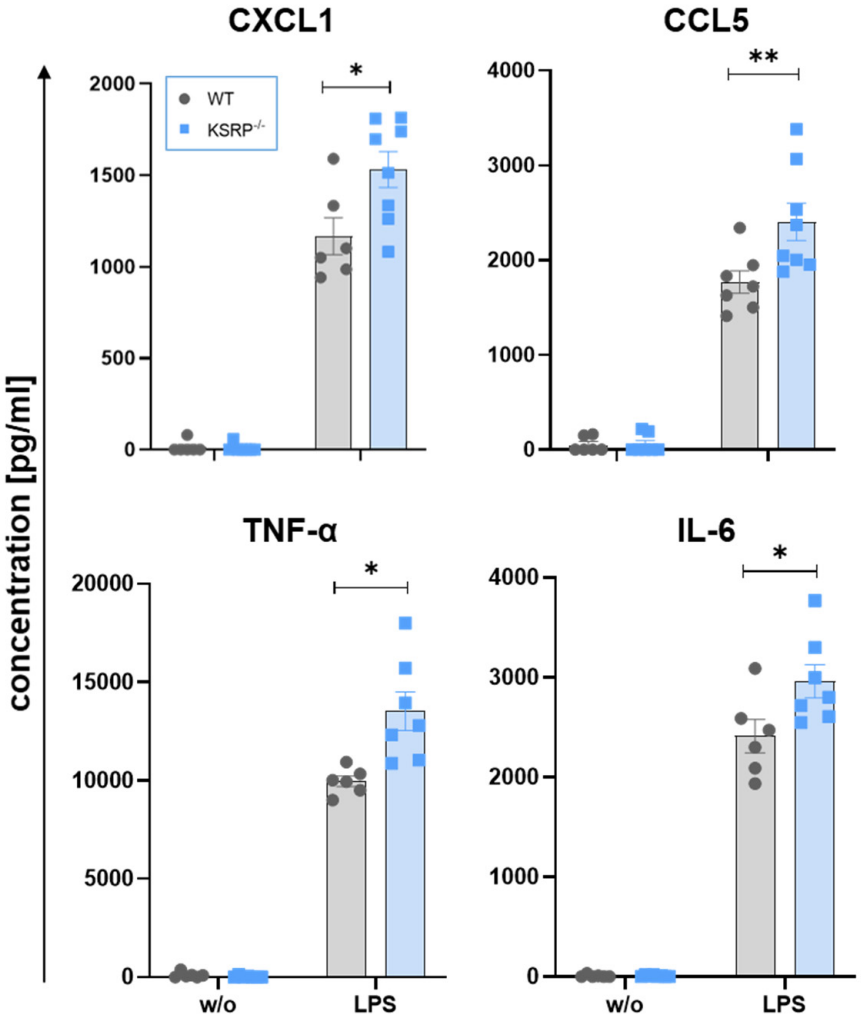

Figure 4. Inactivation of the KSRP gene enhances CXCL1, CCL5 (RANTES), TNF-α and IL-6 protein expression in murine peritoneal cells. Peritoneal cells were isolated from WT and KSRP$^{-/-}$ mice. Adherent cells (mostly monocytes/macrophages) were used for the experiments. Adherent peritoneal cells were incubated with LPS (1 µg/mL) to induce pro-inflammatory chemo/cytokines. After 6 h supernatant was used for analysis using the Anti-Virus-Response LegendPlex- Kit from BioLegend. Shown are the means ± SEM of n = 6–8 analyses (* $p < 0.05$; ** $p < 0.01$ versus untreated cells; two-tailed Mann–Whitney test).

2.5. KSRP$^{-/-}$ Display Tendencies to Higher Proinflammatory Cytokine Production in LPS-Induced Sepsis

Our results indicate, that KSRP may act as an important negative regulator of inflammatory responses of macrophages in vitro. Due to the pronounced role of macrophages in sepsis, we further analyzed the effect of KSRP deficiency in an in vivo LPS-induced acute inflammation model focusing on early KSRP-dependent effects. To this end, mice were injected intraperitoneally (i.p.) with LPS at sub-lethal doses to stimulate pro-inflammatory cytokine expression [24] (Figure 5A). 12 h after injecting 5 mg/kg LPS i.p., we obtained some effects for IL-1β in KSRP-deficient mice, however below significance. After injection of high dose LPS (20 mg/kg), sera analyses revealed a significant increase in IL-1β production in KSRP-deficient mice compared to WT mice (Figure 5B). A similar trend was found for the chemokines CXCL1 and CCL5 in response to the higher LPS dose (Figure 5B).

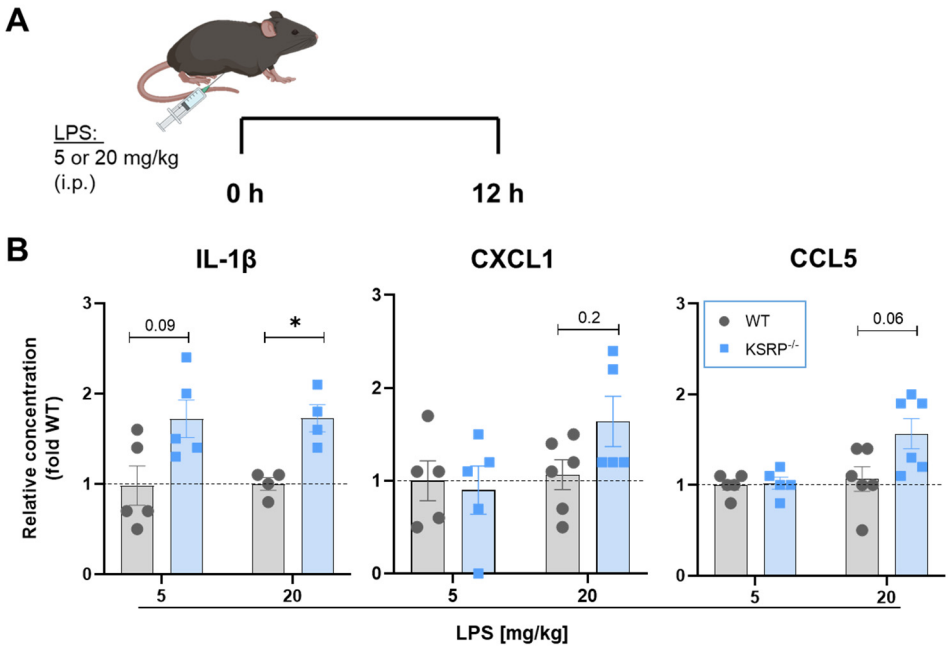

Figure 5. Inactivation of the KSRP gene increased cytokine production after treatment with 5 or 20 mg/kg LPS. (**A**) WT or KSRP$^{-/-}$ mice were treated with 5 or 20 mg/kg LPS i.p. for 12h. Created with BioRender.com. (**B**) Analysis of different pro-inflammatory cytokines involved in sepsis progression. Shown are the means ± SEM of n = 4–6 analyses (* $p < 0.05$ versus WT cytokine expression; two-tailed Mann–Whitney test).

In summary, these findings demonstrate that a deficiency of the RNA-binding protein KSRP in macrophages leads to a more rapid and increased production of pro-inflammatory cytokines in response to LPS stimulation. Therefore, KSRP constitutes an important negative regulator of cytokine production.

3. Discussion

Macrophages are innate immune cells that reside throughout the body. On the one hand they exert potent pathogen-killing activity by various mechanisms and on the other hand act as antigen-presenting cells, thereby inducing antigen-specific T cell responses [25]. To date, several RBPs like TTP, HuR, TIAR have been shown to influence the immunophenotype of macrophages [26–29]. By now, the multifunctional RBP KSRP has been identified as a general negative regulator of inflammatory immune responses by limiting cytokine production of activated immune cells via promoting decay [17,19,30] or inhibiting translation [31] of target mRNAs. Both functions are conferred by direct binding of KSRP to ARE within its target mRNA. However, KSRP may also act in an indirect manner by mediating the maturation of miRNA species, which in turn inhibit gene expression [32,33]. Due to the pronounced role of macrophages in innate and adaptive immune responses, we asked for the role of KSRP in shaping the immunophenotype of primary macrophages. We show that KSRP limits the inflammatory response of macrophages as evidenced by increased expression of inflammatory mediators in KSRP-deficient macrophages in response to stimulation in vitro. In line, KSRP$^{-/-}$ mice displayed elevated cytokine production in an acute inflammation model known to be mediated in large part by activated macrophages.

No major differences in the frequency of macrophages in the spleen and liver of KSRP$^{-/-}$ versus WT mice under homeostatic conditions were observed. Further, we noted no genotype-dependent differences in the expression of surface activation markers of macrophages under basal conditions or in response to LPS stimulation (Figure S1). Similarly, KSRP deficiency did not affect the glycolytic activity of unstimulated and stimulated macrophages, respectively (Figure S2). However, RNA sequencing analysis of KSRP-deficient BMDM under basal conditions showed, e.g., upregulation of *Zfp384*,

which limits cytokine responses at the transcriptional level [21] or *Zbtb2* (zinc finger and BTB domain containing 2) inhibiting NF-κB activation [22]. These findings suggest that KSRP-deficiency under basal conditions may be balanced in part by the upregulation of other immune response limiting genes. However, this assumption needs to be verified in further experiments.

RNA sequencing after 6 h of LPS stimulation revealed higher mRNA expression levels of genes involved in inflammatory responses related to IL-6 or TNF-α signaling in KSRP-deficient BMDM. Interestingly, the top 15 upregulated genes in KSRP$^{-/-}$ BMDM are interlinked and contribute to the cellular response of type I interferons. In line, Lin et al. demonstrated that KSRP is a crucial negative regulator of type I IFN gene expression at the post-transcriptional level by interacting with the 3′-UTRs of these mRNAs, as shown for mouse embryonic fibroblasts of KSRP$^{-/-}$ mice [16]. In our study, time kinetics studies revealed higher mRNA and protein levels of IFN-γ, IL-1β, and IL-6 after LPS stimulation of KSRP-deficient BMDM. To evaluate our observations, we analyzed also primary peritoneal cells that are enriched in macrophages. In accordance with our findings on BMDM, LPS stimulation resulted in a significant upregulation of IL6, TNF-α, CXCL1 (KC) and CCL5 (RANTES) expression in KSRP$^{-/-}$ peritoneal macrophages.

In agreement, previous studies have shown that KSRP regulates the expression of several pro-inflammatory mediators, including IL-1β [33,34], IL-6 [31,35], CCL5 [33], and TNF-α [31,34,35]. The results of our IP studies suggest direct binding of KSRP to LPS-induced IL-1β mRNA in BMDM, while IL-6, TNF-α, IFN-γ, IFN-α and IFN-β mRNAs were most probably regulated by KSRP in an indirect manner.

As outlined above, KSRP may regulate gene expression both in a direct and in an indirect manner. For instance, Li et al. observed stabilization of both IL-1β and TNF-α mRNA transcripts in LPS-stimulated astrocytes from KSRP$^{-/-}$ mice [34]. Showing binding of KSRP to according mRNAs, suggesting a direct manner of KSRP regulation. As mentioned, Lin and coworkers already revealed regulation of *IFNA4* and *IFNB* mRNA stability by KSRP through interaction with the 3′-UTR [16]. Interestingly, Winzen et al., identified ~100 target mRNAs of KSRP, including IL-6, IL-8 and cyclooxygenase-2, whose expression levels were higher in KSRP-deficient cells [35]. However, mRNA degradation was only detected in 10% of all KSRP targets, which may be explained in part by the additional function of KSRP to inhibit mRNA translation. Dhamija and colleagues assessed the polysome profiles of cells with siRNA-mediated KSRP deficiency. Thereby KSRP was found to interact with the ARE of IL-6 mRNA and mediate its translational silencing [31].

In addition to direct interaction with mRNA resulting in decay or translational inhibition, KSRP may also regulate gene expression in an indirect manner. KSRP plays a crucial role in processing of a subset of miRNAs, particularly those containing a GC-rich stem-loop structure in their immature precursor transcript [36]. Among those are miR-155 [37], let-7a [38] and miR-129 [39], which exert important functions in the regulation of immune processes. Ruggiero and colleagues demonstrated miRNA-mediated degradation of IL-1β and CCL5 mRNA in a mouse macrophage cell line [33]. Also miRNA let-7a was reported to inhibit IL-6 expression in macrophages [40]. Taken together, KSRP regulates gene expression on various levels. In the nucleus, it acts as a transcription and splicing factor, while in the cytoplasm it mediates rapid decay of ARE-containing mRNAs or silences translation of mRNA. In addition to direct regulation of gene expression via post-transcriptional mechanisms, KSRP also acts in an indirect manner by promoting the maturation of a subset of miRNA species, which in turn affect expression of multiple genes. With regard to our data, these results suggest that KSRP may regulate expression of a given genes in a direct or indirect manner, depending on the cell type and experimental conditions. Further studies are necessary to elucidate the mode of KSRP-mediated regulation of its target genes in macrophages.

Sepsis is a multilayered disturbance of systemic immunologic homeostasis of inflammation and anti-inflammation [41]. In the course of sepsis, there is a systemic release of pro-inflammatory cytokines [42]. The increased expression of these factors is in part

due to elevated activity of various transcription factors (e.g., NF-κB, STATs, AP-1) that confer enhanced gene expression. However, especially pro-inflammatory and immune cell function-modulating genes are also regulated at the post-transcriptional level by RBP [7].

In agreement with our in vitro findings, we demonstrate that KSRP$^{-/-}$ mice displayed higher IL-1β levels in sera derived from LPS-treated mice and observed similar effects for other proinflammatory mediators albeit below statistical significance. This finding suggests that KSRP contributes to limit the extent of the cytokine storm in macrophages in the course of sepsis. Likewise, Liu et al. showed that the RBP AUF1 protected animals from endotoxic shock by reducing the expression of the pro-inflammatory cytokines TNF-α and IL-1β through mRNA degradation [24]. Similar to KSRP also deficiency of AUF1 under steady state conditions did not influence mouse development [24].

Our observations suggest that KSRP may be part of an immunological negative feedback loop limiting immune responses to prevent excessive damages of the host's tissues both by direct mRNA binding but also via yet unknown indirect mechanisms. With regard to therapeutic approaches, KSRP activity could be temporarily enhanced to attenuate unwanted inflammatory immune responses, including sepsis. In this regard, we have already shown that resveratrol modulates KSRP mRNA-binding activity and thereby enhanced mRNA degradation, leading to anti-inflammatory effects [18]. Further studies are dedicated to elucidate the role of KSRP in this regard.

4. Materials and Methods

4.1. Mice

KSRP$^{+/-}$ mice on C57BL/6 background [16] were bred and maintained in the Central Animal Facility of the Johannes Gutenberg University Mainz under specific pathogen-free conditions. KSRP$^{-/-}$ (KO) and KSRP$^{+/+}$ (WT) animals were obtained by mating KSRP$^{+/-}$ animals. Genotyping of the animals was performed by polymerase chain reaction using the following primers: KSRP-wt-for GCGGGGAGAATGTGAAGG, KSRP$^{-/-}$-for CTCCGCCTCCTCAGCTTG, and KSRP-wt/$^{-/-}$ -rev GAGGCCCCTGGTTGAAGG. All animal procedures were performed in accordance with the institutional guidelines and have been approved by the National Investigation Office of Rhineland-Palatinate (approval ID: G17-1-061). Mice (8–14 weeks) of both sexes were used throughout all experiments.

4.2. Bone Marrow-Derived Macrophages (BMDM)

Bone marrow cells (4×10^5/mL) were seeded in 12-well cell cluster plates (1 mL) (Greiner Bio-One, Kremsmünster, Austria) in IMDM-based culture medium containing 5% FCS (PAN-Biotech, Aidenbach, Germany), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM ß-mercaptoethanol (all from Sigma-Aldrich, Deisenhofen, Germany), and supplemented with 10 ng/mL recombinant murine M-CSF (Miltenyi Biotec, Bergisch Gladbach, Germany). Culture media was replenished every 3 days of culture. BMDM were subjected to experiments on days 7–9 of culture unless indicated otherwise.

4.3. Isolation of Peritoenal Cells

Peritoneal cells were isolated from WT and KSRP$^{-/-}$ mice using a 27G needle and ice-cold PBS supplemented with 3% FCS. Afterwards, cells were seeded in 24-well cell cluster plates (500 µL, 10×10^5/mL) (Greiner Bio-One, Kremsmünster, Austria) in IMDM-base culture medium (see above) for 3 h at 5% CO_2, 37 °C. Non-adherent cells were removed by gentle washing with pre-warmed PBS to enrich adherent monocytes/macrophages. Replicate wells were treated with LPS (1 µg/mL). After 6 h cell supernatant was collected and analyzed for cytokine contents (see Section 4.4).

4.4. Cytometric Bead Array

The cytokine secretion of cultivated and pretreated cells as well as cytokine levels in murine sera were quantified using a multiplex bead-based immunoassay (LEGENDplex Mouse Anti-Virus Response Panel; 13-plex, BioLegend, San Diego, CA, USA) in accordance

with manufacturer's instructions. Samples were measured in an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA). Data analysis was performed using LEGENDplex Qognit software (v8.0, BioLegend, San Diego, CA, USA).

4.5. Immunoprecipitation-qRT-PCR Assay

Immunoprecipitation-qRT-PCR assay was performed as described [19] with slight modifications. WT BMDM (d7) were treated with LPS (1 µg/mL) for 6 h to induce pro-inflammatory chemokines and cytokines. KSRP/RNA complexes were isolated using KSRP-specific antibody (pAB anti-KSRP, Novus Biologicals, Centennial, CO, USA) and a corresponding isotype control antibody (anti-mouse IgG, Sigma-Aldrich, Deisenhofen, Germany) in parallel reactions. Total RNA was purified using the GeneJET RNA Cleanup and Concentration Micro Kits (Qiagen, Hilden, Germany), transcribed into cDNA and qRT-PCR was used to determine quantities of IL-1β, IL-6, IFN-α, IFN-β, TNF-α, Ifit1 and Gbp2 mRNA.

4.6. Analysis of mRNA Expression in Cells or Tissues of KSRP$^{-/-}$ or WT Animals

To analyze the mRNA expression of different immunorelevant genes in cells of KSRP$^{-/-}$ or WT animals total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Total RNA was subjected to RNA sequencing (see Section 4.7) and real-time PCR (see Section 4.8) For real-time PCR cDNA was synthesized by applying the iScript kit (Bio-Rad, Munich, Germany).

4.7. RNA-Sequencing and Bioinformatical Analysis

A total of 4 × 10^5 BMDM of WT and KSRP$^{-/-}$ mice were cultured with LPS (1 µg/mL) for 6 h. RNA was purified with the RNeasy Plus Mini Kit according to the manufacturer's protocol (Qiagen Hilden, Germany). NGS library prep was performed with Lexogen's QuantSeq 3′mRNA-Seq Library Prep Kit FWD (Lexogen, Vienna, Austria) following Lexogen's standard protocol with modifications for low Input RNA (≤10 ng) (015UG009V0260). Libraries were prepared with a starting amount of 6.9 ng and amplified in 22 PCR cycles. Libraries were profiled in a High Sensitivity DNA chip on a 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA, USA) and quantified using the Qubit dsDNA HS Assay Kit, in a Qubit Flex Fluorometer (Life technologies, Carlsbad, CA, USA). All 12 samples were pooled together with 12 samples from another project in equimolar ratio and sequenced on 1 NextSeq 500 Highoutput Flowcell, SR (Illumina, Inc, San Diego, CA, USA)for 1 × 85 cycles plus 6 cycles for the index read. RNA-Seq reads were aligned with STAR aligner (v2.7.3a; [43]) to the GRCm39 genome with the parameters—outStd SAM—outMultimapperOrder Random—outSAMattributes NH HI AS nM MD—outFilterMismatchNmax 999—outFilterMismatchNoverReadLmax 0.04. Using the featureCounts program of subread software (v2.0.0; [44]) the primary alignments were assigned to exons with default parameters. The GENCODE mouse annotation release M26 was used in all the steps. Further, with only the uniquely mapped reads differential expression analysis was performed using the bioconductor release 3.14 ([45]) and DESeq2 (v1.34.0; [46]) where genes showing a Benjamini–Hochberg-adjusted FDR < 0.1 were considered differentially expressed. Results were illustrated using the R heatmap package. GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA) was used to create volcano plots of differentially expressed genes. The gene set enrichment analysis of normalized gene counts was performed using the GSEA 4.2.3 software (standard settings, gene set database: h.all.v7.5.1 [47,48]). A false discovery rate (FDR) q-value < 0.05 was considered statistically significant. STRING database (v12) was used to analyze protein-protein interaction networks. Transcriptome data have been deposited in the GEO database, accession number GSE261444.

4.8. Real-Time PCR

cDNA of differentially pretreated macrophages was used for real-time PCR using the following primers: IL-1β (5′-GCCCATCCTCTGTGACTCAT-3′, 5′-AGGCCACAGGTATTTTGTCG-3′), IL-6 (5′-CCGGAGAGGAGACTTCACAG-3′, 5′-CAGAATTGCCATTGCACAAC-3′), IFN-γ (5′-GCTTGCAGCTCTTCCTCAT-3′, 5′-GTCACCATCCTTTTGCCAGT-3′) and β2-Mikroglobulin (β2M) (5′-CGGCCTGTATGCTATCCAGA-3′, 5′-GGGTGAATTCAGTGTGAGCC-3′). All primers were obtained from Eurofins Scientific (Luxembourg City, Luxembourg). Reaction mixtures had a final volume of 20 µL and included 200 ng cDNA, 70 nM of each primer, and 12.5 µL of 2× primaQUANT Master Mix high ROX (Steinbrenner Laborsysteme, Wiesenbach, Germany). Each sample was tested in duplicate. Thermal cycling conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min, followed by a melting curve stage of 95 °C for 15 s and 60 °C for 1 min using an ABI 7300 real-time PCR cycler (Applied Biosystems, Waltham, MA, USA). mRNA expression was normalized to β2M mRNA expression.

4.9. The LPS-Induced Sepsis Model

WT and KSRP$^{-/-}$ mice were injected with LPS (5 or 20 mg/kg bodyweight). After 12 h, blood samples were collected, centrifuged at 10,000× *g* for 8 min and sera were used for cytokine detection (see Section 4.4).

4.10. Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software v9.0 (GraphPad Software Inc., San Diego, CA, USA). Results were expressed as the mean ± standard error of the mean (SEM).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms25073884/s1.

Author Contributions: Conceptualization and methodology, V.B., K.-A.P., C.H. and M.B.; software, V.B., K.-A.P., C.H. and M.B.; validation, V.B., K.-A.P., C.H., A.P. and M.B.; formal analysis, V.B., K.-A.P., C.H., A.P. and M.B.; investigation, V.B., K.-A.P., J.S., M.F. and P.S.; resources, A.P., M.B. and S.G.; data curation, V.B., C.H. and M.B.; writing—original draft preparation, V.B. and M.B.; writing—review and editing, V.B., M.B., K.-A.P., J.S., M.F., P.S. and A.P., visualization, V.B., C.H. and M.B.; supervision, M.B., A.P. and S.G.; project administration, A.P. and M.B., funding acquisition, A.P., M.B. and S.G. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Material

The mRNA-binding protein KSRP limits the inflammatory response of macrophages

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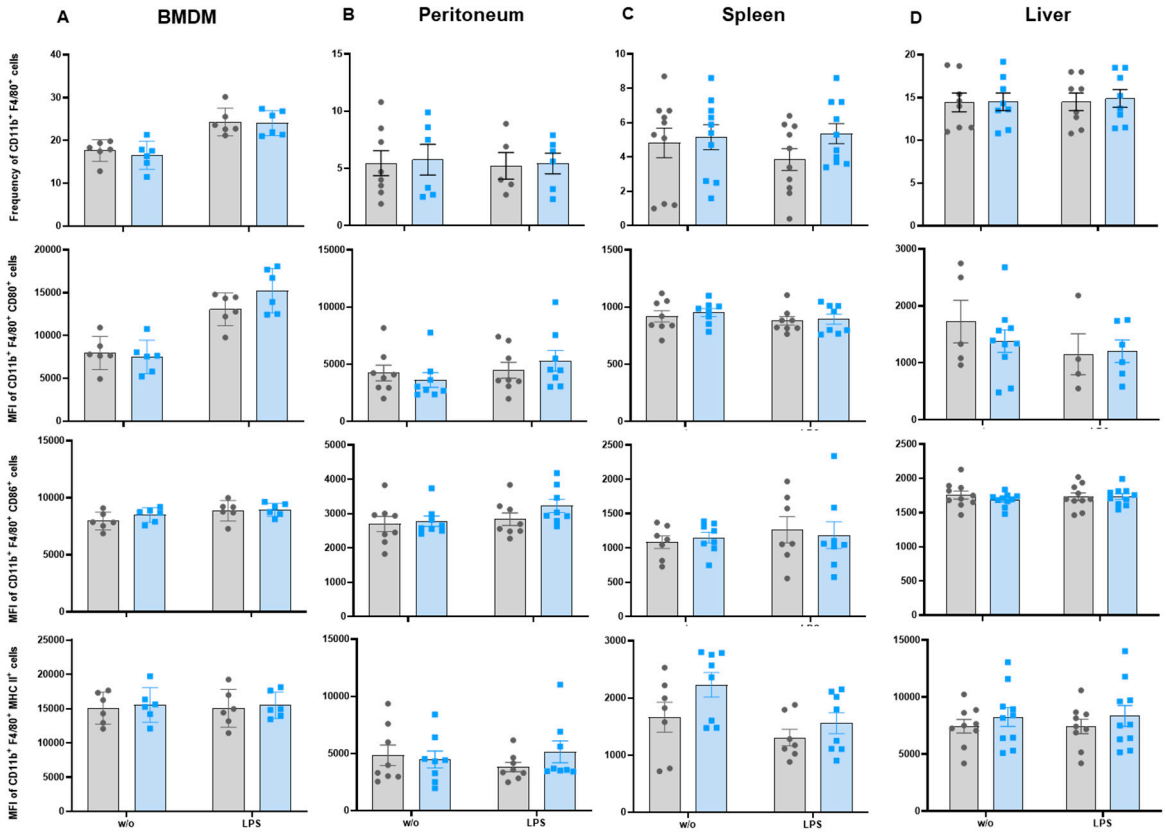

Fig. S1: KSRP mice show no differences concerning cell frequency and surface activation markers. Cells were isolated from the bone marrow and differentiated with 10 ng/ml M-CSF to BMDM or directly isolated from the different organs and unstimulaed (w/o) or stimulated (1 µg/µl LPS, 6h) used for flow cytometic staining. Analysis of Bone marrow-derived macrophages (BMDM) (A), Peritoneal macrophages (B), splenic macrophages (C) and liver macrophages (D) concerning macrophage freuqnecy and activation marker expression. and liver macrophages (D) concerning macrophage frequency and activation marker expression. Cells were stained for flow cytometric analysis as described earlier (Bednarczyk M et al., 2022.). To this regard, cells were incubated with FITC-conjugated anti-CD86 (GL-1) and anti-MHCII (M5/114.15.2), PerCP-eFluor710-conjugated anti-CD80 (16-10A1), APC-conjugated anti-F4/80 (BM8), eFluor450-conjugated anti-CD86 (GL-1), Super Bright 700-conjugated anti-CD11b (M1/70), PE-conjugated anti-CD80 (16-10A1) and PE-eFluor 610-

conjugated anti-MHCII (M5/114.15.2). Antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA), Thermo Fisher (Scientific, Waltham, MA, USA) or BioLegend (San Diego, CA, USA).

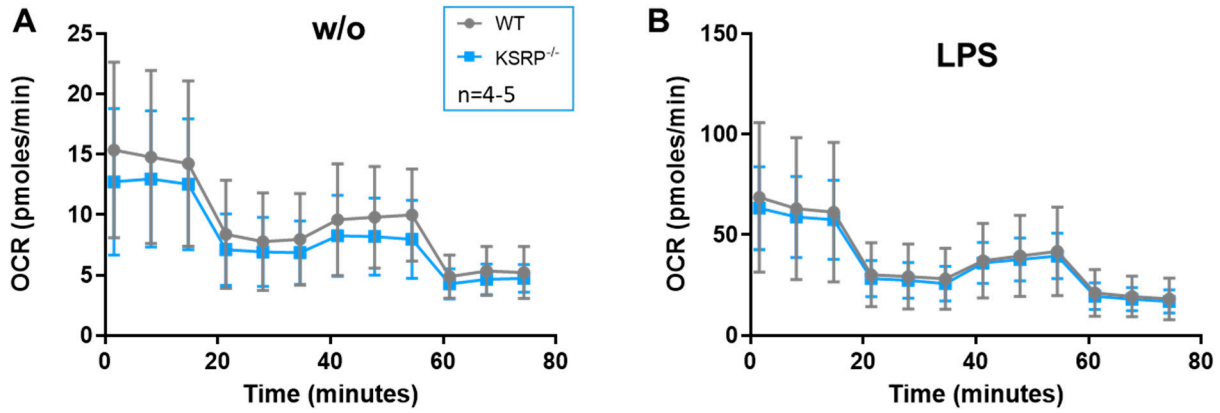

Fig. S2: KSRP BMDM show no differences concerning metabolic activity under basal conditons (A) and in response to LPS stimulation (B). Bone marrow progenitor cells were isolated from KSRP WT and KO mice and differentiated with M-CSF for 7 days to generate bone marrow-derived macrophages (BMDM). Seahorse Cell Mito Stress assays were performed according to the manufacturer's protocol and specifications for a Seahorse XFp Analyzer (Agilent, Santa Clara, CA, USA). In brief, the day before the assay, BMDM were plated at 30,000 cells per well in standard cell culture medium in Seahorse XFp Cell Culture Miniplates (Agilent, #103025-100). The following day, medium was exchanged for Seahorse assay medium consisting of XF RPMI (Seahorse XF RPMI medium, Agilent, #103576-100) supplemented with 1 mM pyruvate (Agilent, #103578-100), 2 mM glutamine (Agilent, #103579-100), and 10 mM glucose (Agilent, #103577-100). Cells were treated with 1 µg/µl LPS incubated for 6 h in a 37 °C 10% CO_2 incubator. Meanwhile, Seahorse sensor cartridges (Seahorse XFp FluxPak, Agilent, #103022-100) were loaded with 1.5 µM oligomycin, 1.0 µM FCCP, and 0.5 µM rotenone/antimycin A (Seahorse XFp Cell Mito Stress Test Kit, Agilent, #103010-100). Plates were placed into a Seahorse XFp Analyzer and the program "Seahorse XF Mito Stress Test" was ran.

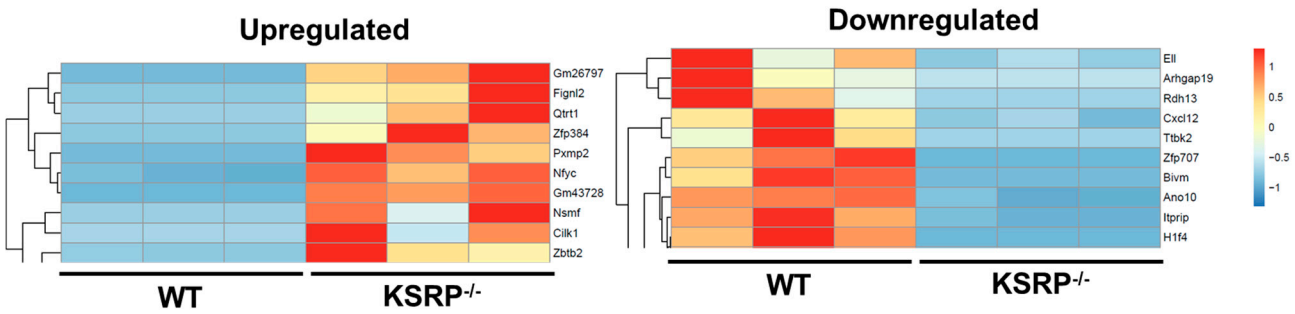

Fig. S3: Unstimulated BMDMs (WT and KSRP$^{-/-}$, each n=3) were subjected to RNA-seq analysis. Shown is a heatmap representation of the top 10 significantly downpregulated (left panel) and significantly upregulated (right panel) genes in WT versus KSRP$^{-/-}$ BMDM (hierarchical clustering). The color legend denotes the level of gene expression (low: blue; high: red).

Table S1: Top 10 up/down regulated genes under basal conditions of WT and KSRP$^{-/-}$ mice.

	Gene		Functions
	Symbol	Name	
Upregulated	Gm26797	predicted gene, 26797	-
	Fignl2	fidgetin like 2	enables microtubule-severing ATPase activity
	Qtrt1	queuine tRNA-ribosyltransferase catalytic subunit 1	catalytic subunit of tRNA-guanine transglycosylase. that confers tRNA modification by synthesizing 7-deazaguanosine queuosine (found in tRNAs that code for asparagine, aspartic acid, histidine and tyrosine)
	Zfp384	zinc finger protein 384	zinc-finger-containing transcription factor; shown to limit cytokine/chemokine expression in response to viral infection (**PMID: 32152414**)
	Pxmp2	peroxisomal membrane protein 2	peroxisomal channel; regulates lipid metabolism
	Nfyc	nuclear transcription factor Y subunit gamma	subunit of transcription factor binding CCAAT motifs
	Gm43728	predicted gene, 43728	-
	Nsmf	NMDA receptor synaptonuclear signaling and neuronal migration factor	
	Cilk1	ciliogenesis associated kinase 1	serine/threonine protein kinase
	Zbtb2	zinc finger and BTB domain containing 2	transcription factor (silencer); inhibits NF-kB activation (**PMID: 25609694**)
Downregulated	Ell	elongation factor for RNA Polymerase II	part of transcription elongation factor complex
	Arhgap19	Rho GTPase activating protein 19	regulates small GTPase-mediated signal transduction
	Rdh13	retinol dehydrogenase 13	catalyzes reduction/oxidation of retinoids; protects mitochondria against oxidative stress
	Cxcl12	C-X-C motif chemokine ligand 12	ligand of G-protein-coupled chemokine (C-X-C motif) receptor 4; broad immunological role
	Ttbk2	tau tubulin kinase 2	serine-threonine kinase; phosphorylates tau and tubulin proteins
	Zfp707	zinc finger protein 707	transcription factor
	Bivm	basic, immunoglobulin-like variable motif containing	-
	Ano10	anoctamin 10	calcium-activated chloride channel
	Itprip	inositol 1,4,5-trisphosphate receptor interacting protein	enhances sensitivity of inositol 1,4,5-trisphosphate receptor to intracellular calcium signaling
	H1f4	H1.4 linker histone, cluster member	engages linker DNA between nucleosomes to compact chromatin

6.3 Publication 3

KSRP deficiency attenuates the course of pulmonary aspergillosis, associated with elevated pathogen-killing activity of innate myeloid immune cells

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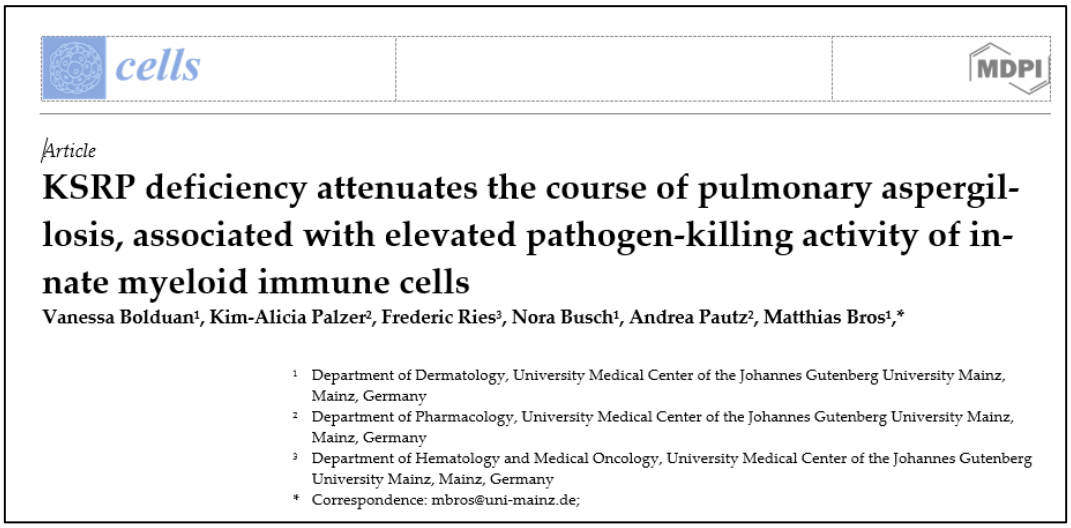

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Article

KSRP deficiency attenuates the course of pulmonary aspergillosis, associated with elevated pathogen-killing activity of innate myeloid immune cells

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Summary:

The study investigates the role of the mRNA-binding protein KSRP in modulating the functions of innate immune cells, specifically polymorphonuclear neutrophils (PMN) and macrophages (MAC), during invasive pulmonary aspergillosis (IPA). In KSRP-deficient ($KSRP^{-/-}$) mice, we detected lower fungal burden, along with increased PMN and MAC frequencies in the BALF and lungs. RNA sequencing revealed shared upregulation of 64 immune-related genes between PMN and MAC. Further *in vitro* studies showed that $KSRP^{-/-}$ PMN and MAC had enhanced phagocytosis and PMN displayed increased ROS production. As the majority of genes upregulated in PMN were involved viability, we analyzed apoptosis and found elevated levels of stimulated apoptotic PMN. Overall, the results suggest KSRP is a negative regulator of immune activity, with its deficiency enhances PMN and MAC effector functions and improving pathogen defense.

Author contribution:

- Design, conduction and evaluation of all biological experiments
- Preparation of graphs and figures
- Preparation and writing of the manuscript together with Dr. Matthias Bros
- Proofreading of the submitted manuscript

cells

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Submitted Article

KSRP deficiency attenuates the course of pulmonary aspergillosis, associated with elevated pathogen-killing activity of innate myeloid immune cells

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Abstract: The mRNA-binding protein KSRP (KH-type splicing regulatory protein) is known to modulate immune cell functions post-transcriptionally e.g. by reducing the mRNA stability of cytokines. It is known that KSRP binds AU-rich motifs (ARE) within the 3´-untranslated region of mRNA species, which in many cases encode dynamically regulated proteins like cytokines. Innate myeloid immune cells, such as polymorphonuclear neutrophils (PMN) or macrophages (MAC) eliminate pathogens by multiple mechanisms, including phagocytosis, as well as secretion of chemo- and cytokines. Here, we investigated the role of KSRP on the phenotype and functions of both innate immune cell types in mouse model of invasive pulmonary aspergillosis (IPA). Here, KSRP$^{-/-}$ mice showed lower levels of Aspergillus fumigatus conidia (AFC) and an increase in the frequencies of PMN and MAC in the lungs. Our results showed that PMN and MAC from KSRP$^{-/-}$ mice exhibited enhanced phagocytic uptake of AFC, accompanied by increased ROS production in PMN upon stimulation. As a comparison of RNA sequencing data revealed that 64 genes related to inflammatory and immune responses were shared between PMN and MAC. The majority of genes upregulated in PMN were involved in metabolic processes, cell cycle and DNA repair. In agreement, KSRP-deficient PMN displayed reduced levels of apoptosis. In conclusion, our results indicate that KSRP serves as a critical negative regulator of PMN and MAC anti-pathogen activity.

Keywords: Innate immunity, RNA-binding proteins; invasive pulmonary aspergillosis; phagocytosis; neutrophils; macrophages

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1. Introduction

KSRP (K homology [KH]-type splicing protein) is a multifunctional mRNA-binding protein (RBP) that has been demonstrated to limit mRNA stability through direct binding to AU-rich elements (ARE) located within the 3'-untranslated region (3'-UTR) of target mRNAs [1-4]. Moreover, it inhibits the translation of target mRNAs [5], serves as a transcription [6] and splicing factor [7], and functions as a maturation factor for various microRNA species [8-13]. To date, KSRP has been identified as a crucial negative regulator of inflammatory immune responses to avoid excessive immune reactions, such as cytokine storms [14], extensive tissue damage [15], or autoimmune responses [16]. However, the precise role of KSRP within the immune system remains to be determined [17].

The innate immune response acts as a rapid, non-specific first line of defense, eliminating pathogens, initiating inflammation, recruiting immune cells, and activating the adaptive immune response. When pathogens breach epithelial barriers, they are recognized by phagocytic cells, primarily macrophages (MAC) and polymorphonuclear neutrophils

(PMN) [18,19]. Both MAC and PMNs initiate immune responses by recognizing pathogens via receptors like Toll-like receptors (TLRs), triggering pro-inflammatory cytokine production [20], recruit immune cells to sites of infection and inflammation [21] and exhibit pathogen-killing activities through phagocytosis and the production of ROS [22,23]. Thereby, also infiltrating monocytes, which differentiate into MAC, appear to be of significant importance in this pathogen-killing process [24].

Aspergillus fumigatus (A. fumigatus), a ubiquitous saprophytic fungus, has emerged as the predominant airborne fungal pathogen affecting immunocompromised patients [25]. In these individuals A. fumigatus can cause lethal invasive pulmonary aspergillosis (IPA), commonly after inhalation of airborne conidia, which germinate in the lungs of immunocompromised hosts, sprouting there as hyphae [26]. The innate immune system is regarded as primary responsible for the clearance of conidia and the defense against the outgrowth of A. fumigatus conidia (AFC) [27]. The recruitment of PMN to the lung tissue is essential for an efficient clearance of A. fumigatus [28,29]. Nevertheless, resident leukocytes present in the alveolar lung tissue, including MAC, initiate an early response against invasive aspergillosis as well [30].

PMN and MAC mediate the killing of *A. fumigatus* by various effector mechanisms, which depend on the size of conidia and hyphae. The phagocytosis of small-sized AFC is enabled by the size of the conidia [31], while the size of the hyphae prevents phagocytosis.

In accordance with its pivotal function in ARE-mediated decay of pro-inflammatory mediators, our recent findings indicate that MAC lacking KSRP exhibit a more pronounced inflammatory response in an LPS-induced sepsis model [32]. In this study, we asked whether stimulation-induced hyperactivation of innate immune cells would also affect pathogen eradicating functions of PMN and MAC, in a model of aspergillosis.

We observed reduced AFC levels in KSRP-deficient mice in an *in vivo* IPA model, along with higher frequencies of PMN and MAC in the lung. Somewhat surprisingly, PMN-depleted KSRP-deficient mice survived IPA. Interestingly, comparison of MAC and PMN RNA sequencing revealed a moderate number of genes equally upregulated in KSRP-deficient PMN and MAC in response to stimulation, mainly involved in inflammatory and immune responses. In line, PMN and MAC of $KSRP^{-/-}$ mice displayed higher phagocytic uptake of AFC, and PMN generated more ROS following stimulation. The majority of PMN-specifically upregulated genes were related to the control of cellular metabolism, the cell cycle, and DNA repair. In line, we detected reduced level of apoptotic KSRP-deficient PMN in response to stimulation. In conclusion, our findings demonstrate that KSRP plays a vital role as a negative regulator of PMN and MAC anti-pathogen activity.

2. Materials and Methods

2.1 Mice

$KSRP^{+/-}$ mice on C57BL/6 background [33] were bred and maintained in the Central Animal Facility of the Johannes Gutenberg University Mainz under specific pathogen-free conditions. $KSRP^{-/-}$ (KO) and WT animals were obtained by mating $KSRP^{+/-}$ animals. Genotyping of the animals was performed by polymerase chain reaction using the following primers: KSRP-WT-for GCGGGGAGAATGTGAAGG, KSRP-KO-for CTCCGCCTCCTCAGCTTG, and KSRP-WT/KO-rev GAGGCCCCTGGTT-GAAGG. All animal procedures were performed in accordance with the institutional guidelines and have been approved by the National Investigation Office of Rhine-land-Palatinate (approval IDs: G23-1-037). Mice (8–14 weeks) of both sexes were used throughout all experiments.

2.2 Fungal Strains and Cultivation Condition

The WT (ATCC 46645) and green fluorescent protein (GFP)-expressing (AfS148) *Aspergillus fumigatus* strains [34] were cultivated in *Aspergillus* minimal medium (AMM) comprising 1% (w/v) glucose, 1% Hutner's trace element solution, and 1M Mg_2SO_4 (Carl

Roth, Karlsruhe, Germany), as previously described [35]. In brief, the conidia were incubated on AMM agar plates for four days at 37°C with 5% CO_2 in order to facilitate fungal growth. To prepare spore suspensions, the plates were rinsed with sterile water containing small glass beads (ø 4 mm; Carl Roth, Karlsruhe, Germany) to detach the conidia from the agar plate surface. The resulting spore suspension was filtered twice through a sterile 40-µm nylon mesh and subsequently stored in sterile water at 4°C.

2.3 Mouse Model of Invasive Aspergillosis

WT and KSRP$^{-/-}$ mice were anesthetized with a mixture of 14.5% Ketamine (50 mg/ml) and 5.7% Xylazine (0.2%) before being challenged with 10^7 *A. fumigatus* conidia (AFC, strain ATCC 46645) administered intratracheally, as previously described [36,37]. Briefly, a 22G indwelling venous catheter (Vasofix, B. Braun AG, Melsungen, Germany) was inserted into the trachea, and 100 µl of sterile fungal suspension was delivered through the catheter. To improve lung dispersion, the mice were mechanically ventilated at 250 breaths/min with a tidal volume of 300 µl for 2 minutes using an animal respirator (MiniVent, Hugo Sachs, March-Hugstetten, Germany), as previously described [35]. To assess the early immune response to fungal infection, 3 mice per group were sacrificed 24 hours post-infection. In another group (n=3 mice/group), the progression of systemic infection was monitored daily by clinical scoring evaluating weight, activity, breathing, general appearance (posture, skin, and fur condition), and survival over 14 days. Mice displaying severe symptoms, as determined by clinical scoring, were immediately euthanized in accordance with institutional animal ethics guidelines. Where indicated, PMN depletion was induced by intraperitoneal injection (i.p.) of an anti-Gr-1 antibody (150 µg, clone RB6-8C5; BioXCell, Lebanon, NH) one day before inoculation with the fungal suspension.

2.4 Immune Cell Isolation

Spleen cell suspensions from WT and KSRP$^{-/-}$ mice were obtained via mechanical homogenization with a sterile syringe plunger (Braun, Melsungen, Germany) on a 40 µm cell strainer (EASYstrainer™; Greiner Bio-One, Kremsmünster, Austria), washed twice with cold (phosphate-buffered saline) PBS, and red blood cells (RBC) were lysed with hypotonic Gey´s lysis buffer (155mM NH4Cl, 10mM KHCO3, 10µM EDTA at pH 7.4). 2 × 10^6/ml spleen cells were seeded in 1 ml IMDM-based culture medium containing 5% FCS (PAN-Biotech, Aidenbach, Germany), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM ß-mercaptoethanol (all from Sigma-Aldrich, Deisenhofen, Germany) and left untreated or stimulated with 1 µg/ml LPS and 1x10^6 AFC over-night (16h), respectively. Afterwards, cell culture supernatants were collected and analyzed for cytokine contents (see section 2.6).

Following IPA-treatment, lung tissue, the lung-associated lymph nodes and BALF were used for subsequent flow cytometric analysis (see Section 2.5). To this end, lung tissue was cut into small pieces and digested using 6 mg/ml collagenase IV and 0.5 mg/mL DNase I in RPMI for 1 h at 37 °C in a thermo shaker. Subsequently, EDTA was added (10 mM) and samples were incubated for an additional 2 min at 4 °C. The cell suspension was run through a 70 µm cell strainer and washed with PBS (300xg, 4 °C, 8 min). Erythrocytes were lysed using Gey's lysis buffer. The lymph node was mashed through a 40 µM cell strainer and washed with PBS, following the cells were seeded in 96-well plates. BALF was collected by rinsing the trachea with 600 µl cold PBS. Next, BALF was centrifuged to sediment cells. The fluid was used for cytokine content analysis (see section 2.6), and the cells of the BAL were seeded into 96-well plates for flow cytometric analysis (see section 2.5).

PMN were purified from bone marrow of WT and KSRP$^{-/-}$ mice using magnetic cell sorting using biotin-labeled Ly6G-specific antibodies and streptavidin-conjugated beads (both from Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's protocol. Freshly isolated PMN were used for different assays (see Section 2.10-2.12).

2.5 Flow Cytometric Analysis

For flow cytometric analysis freshly isolated or pretreated cells were seeded into 96-well culture plates, washed with staining buffer (PBS/2% FCS), and Fc receptors were blocked by incubation with rat anti-mouse CD16/CD32 antibody (clone 2.4G2) for 15 min at 4°C. Then, cells were incubated for 20 min at 4°C with antibodies (Table 1). All antibodies were obtained from Biolegend (San Diego, CA) or Thermo Fisher (Waltham, MA). Viability was assessed using Fixable-viability-dye (FVD), conjugated with APC eFluor780 or eFlour450 (ThermoFisher), according to the manufacturer´s instructions. Samples were analyzed using a flow cytometer (Attune™ NxT AcousticFocusing Cytometer), and data were processed using Attune™ NxT Software (all from Thermo Fisher).

Table 1. Antibodies used for investigations.

Surface marker	Dye	clone
CD86	FITC	GL-1
CD80	PerCP eFl780	16-10A1
CD11c	APC	N418
MHC II	Brilliant Violet 421 eFluor 450	M5/114.15.2
CD11b	FITC Super Bright 600- PE	M1/70
Ly6G	PE-eFluor 610 APC	1A8 RB6-8C5
CD62L	PE-Cyanine 7	MEL-14
F4/80	eFluor 506	BM8
CD3	eFluor 506	145-2C11
CD19	Super Bright 702 PerCP-Cyanine 5.5	1D3
NK1.1	PE	PK136
SiglecF	Brilliant Violet421	E50-2440
CD11c	Brilliant Violet421 APC	N418

2.6 Cytometric Bead Array

Cytokines were quantified using a multiplex bead-based immunoassay (LEGENDplex Mouse Anti-Virus Response Panel; 13-plex, BioLegend, San Diego, CA, USA) in accordance with manufacturer's instructions. Samples were measured in an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA). Data analysis was performed using LEGENDplex™ Qognit software (v8.0, BioLegend, San Diego, CA, USA).

2.7 Analysis of mRNA Expression in tissue or cells of KSRP$^{-/-}$ or WT animals

RNA of IPA-treated lung tissue was prepared by guanidinium isothiocyanate (GIT) chloroform extraction as previously described in [38]. To analyze the mRNA expression of different immunorelevant genes in PMN total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Total RNA of PMN was subjected to RNA sequencing (see Section 2.13) and real-time PCR (see Section 2.8), respectively.

2.8 Real-Time PCR

For real-time PCR cDNA was synthesized by applying the iScript kit (Bio-Rad, Munich, Germany). cDNA of differentially pretreated PMN was used for real-time PCR using the following murine primers: IL-6 (5′-CCGGAGAGGAGACTTCACAG-3′, 5′-

CAGAATTGCCATTGCACAAC-3′), TNF-a (5′CCACCACGCTCTTCTGTCTA-3′,5′-AGGGTCTGGGCCATAGAACT-3′), GAPDH (5′-CCATCACCATCTTCCAGGAG-3′, 5′-TTTCTCGTGGTTCACACCC-3′). cDNA of lung tissue of IPA-treated mice was analyzed using TEF1 (5′-CCATGTGTGTCGAGTCCTTC-3′, 5′-GAACGTACAGCAACAGTCTGG-3′). All primers were obtained from Eurofins Scientific (Luxembourg City, Luxembourg). Reaction mixtures had a final volume of 20 µL and included 200 ng cDNA, 70 nM of each primer, and 12.5 µL of 2× primaQUANT Master Mix high ROX (Steinbrenner Laborsysteme, Wiesenbach, Germany). Each sample was tested in duplicate. Thermal cycling conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min, followed by a melting curve stage of 95 °C for 15 s and 60 °C for 1 min using an ABI 7300 real-time PCR cycler (Applied Biosystems, Waltham, MA, USA). mRNA expression was normalized to GAPDH mRNA expression.

2.9 Bone Marrow-Derived Macrophages (BMDM, MAC)

Bone marrow cells (4 × 10^5/mL) were seeded in 12-well cell cluster plates (1 mL) in IMDM-based culture medium as previously described (section 2.3), and supplemented with 10 ng/mL recombinant murine M-CSF (Miltenyi Biotec). Culture media was replenished every 3 days of culture. BMDM were subjected to experiments on day 7 of culture unless indicated otherwise.

2.10 Fungal uptake by PMN and BMDM

Freshly isolated PMN or BMDM (day 7 of culture) were resuspended at a concentration of 1 x 10^6 cells/ml in cell culture medium, seeded into 96-well plates (100 µl/well), and incubated with GFP-fluorescent AFC [28] at the indicated ratios. This was done in parallel at 4°C and 37°C to distinguish between mere adhesion and energy-dependent uptake, respectively. Following a 3 h incubation period, samples were washed twice with each 500 µl cold PBS and incubated with anti-CD11b and anti-Ly6G (PMN) or anti-F4/80 (MAC)specific antibodies, as well as FVD eFluor 450, in order to assess the uptake of GFP-labeled conidia via flow cytometry. The gating strategy is shown in Figure S4.

2.11 Analysis of ROS Production

To quantify ROS production, freshly isolated PMN (10^6 cells/ml) were seeded into 96-well plates (100 µl/well), followed by a wash with 200 µl PBS. The cells were then resuspended in 100 µl of ROS detection solution, comprising 2 mM 2′-7′dichlorodihydrofluorescein (DCFDA, Alexis Biochemicals, Lausen, Switzerland) in PBS. Following a 20-minute incubation period at 37°C, the cells were washed with 200 µl PBS, centrifuged, and pelleted cells were resuspended in 200 µl PBS. Subsequently, the PMN were stimulated with 100 ng/ml Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF), 1 µg/ml LPS, or 10^5 AFC at 37°C, 5% CO_2, in triplicate. Median fluorescence intensities (MFI) were quantified using a SPARK multimode microplate reader (TECAN Trading AG, CHE) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm for a period of 180 minutes, with readings taken at 15-minute intervals.

2.12 Assessment of Neutrophil Apoptosis

Freshly isolated PMN (10^6 cells/ml) were incubated in cell culture medium in 96-well plates (100 µl/well) and treated for 6 h in parallel w/o or with LPS (1µg/ml) in order to assess spontaneous apoptosis (w/o) and apoptosis upon LPS-treatment. Then, samples were washed twice with 200 µl PBS and incubated with α-Annexin V (FITC), α-Ly6G (PE) and FVD (eFluor 450) according to the manufacturer´s protocol (ThermoFisher, Waltham, MA) to delineate early and late apoptotic cells. The complete gating strategy is illustrated in Figure S7.

2.13 RNA-Sequencing and Bioinformatical Analysis

A total of 10^6 PMN were cultured w/o or with LPS (1 µg/mL) for 6 h. RNA was purified with the RNeasy Plus Mini Kit according to the manufacturer's protocol (Qiagen Hilden, Germany). NGS library preparation was performed using Lexogen's QuantSeq 3'mRNA-Seq Library Prep Kit FWD (Lexogen, Vienna, Austria) following Lexogen's standard protocol with modifications for low input RNA (≤10 ng) (015UG009V0260). Libraries were prepared with a starting amount of 6.9 ng total RNA and amplified in 22 PCR cycles. Libraries were profiled in a High Sensitivity DNA chip on a 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA, USA) and quantified using the Qubit dsDNA HS Assay Kit, in a Qubit Flex Fluorometer (Life technologies, Carlsbad, CA). Samples were sequenced on 1 NextSeq 500 Highoutput Flowcell, SR (Illumina, Inc, San Diego, CA, USA) for 85 cycles plus 6 cycles for the index read. RNA-Seq reads were aligned with STAR aligner (v2.7.3a; [39]) to the GRCm39 genome with the parameters --outStd SAM --outMultimapperOrder Random --outSAMattributes NH HI AS nM MD --outFilterMismatchNmax 999 --outFilterMismatchNoverReadLmax 0.04. Using the featureCounts program of subread software (v2.0.0; [40]) the primary alignments were assigned to exons with default parameters. The GENCODE mouse annotation release M26 was used in all the steps. Further, with only the uniquely mapped reads differential expression analysis was performed using the bioconductor release 3.14 ([41]) and DESeq2 (v1.34.0; [42]) where genes showing a Benjamini–Hochberg-adjusted FDR < 0.1 were considered differentially expressed. Results were illustrated using the R heatmap package. The gene set enrichment analysis of normalized gene counts was performed using the GSEA 4.2.3 software (standard settings, gene set database: h.all.v7.5.1 [43,44]). A false discovery rate (FDR) q-value < 0.05 was considered statistically significant. STRING database (v12) was used to analyze protein-protein interaction networks [45]. The Venn diagram calculator (https://bioinformatics.psb.ugent.be/webtools/Venn/) was employed to calculate the number of equally and differentially expressed genes within the specified gene set. Transcriptome data have been deposited in the GEO database *(in process).*

2.14 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software v9.5.1 (GraphPad Software Inc., San Diego, CA, USA). Results were expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1 KSRP deficiency resulted in increased production of pro-inflammatory mediators after stimulation

Sepsis is a condition that arises from an excessive response of the body to an infection. In certain circumstances, components of the innate immune system, such as MAC and PMN, which typically protect the body from infection, can cause cell and tissue damage, leading to multiple organ failure, which is a principal clinical manifestation of sepsis [46]. We recently demonstrated that KSRP$^{-/-}$ mice secrete elevated levels of proinflammatory mediators in a LPS-induced sepsis model, indicating that KSRP typically limits inflammatory responses [32]. We sought to determine whether this could yield advantages in an infection model. To evaluate the hyperactivity of KSRP-deficient immune cells towards a pathogen, spleen cells derived from WT and KSRP$^{-/-}$ mice were incubated with *Aspergillus fumigatus*, which is known to cause fungal infection in the lungs of immunocompromised patients [25,47]. Here, the innate immune system, especially alveolar MAC and PMN, is the primary mechanism responsible for the clearance of conidia and the defense against the outgrowth of AFC [27,30].

Significant elevations in IFN-γ and IL-1β cytokine concentration were observed following AFC stimulation, while TNF-α, CCL2, CCL5 and CXCL1 displayed a trend towards significance (Figure 1). It is noteworthy that IL-1β, CCL2, CCL5 and CXCL1 cytokine levels were also significantly elevated following LPS stimulation (Figure 1). In conclusion, these results show that KSRP limits the cytokine response towards different TLR agonists (LPS - TLR-4 [48], AFC - TLR-2 and TLR-4 [25]), indicating that KSRP-deficient mice may show a stronger anti-pathological immune response.

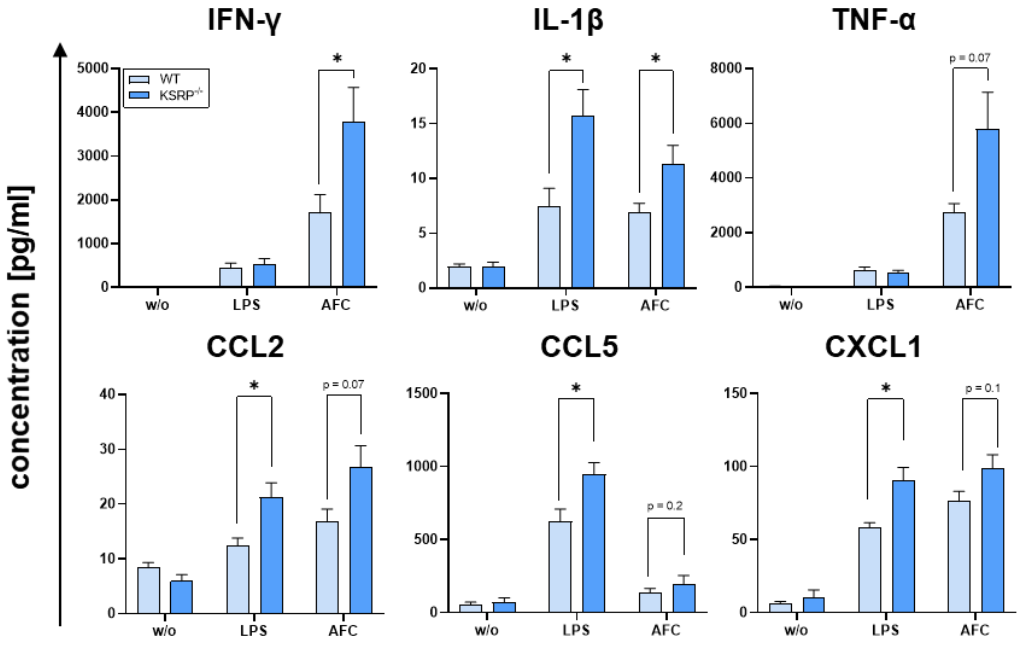

Figure 1. KSRP-deficiency led to higher production of pro-inflammatoy mediators following LPS or AFC stimulation. To analyze the protein expression of different immune relevant genes in spleen cells of KSRP$^{-/-}$ or WT animals, 1x10^6 spleen cells were stimulated with 1 µg/µl LPS or 1x10^6 AFC for 16 h. Supernatants of spleen cells were collected, and cytokine contents were determined using the Anti-Virus-Response LegendPlex- Kit from BioLegend. Shown are the mean ± SEM of n = 4-7 analyses (* $p < 0.05$; two-tailed students t-test).

3.2 PMN depletion in KSRP$^{-/-}$ mice led to resistance to A. fumigatus infection

To evaluate this hypothesis, we initiated IPA, a major threat to immunocompromised individuals caused by an infection with *A. fumigatus* [35]. Eradication of AFC critically depends on PMN and MAC activity at the early stage [28-30]. Some animals were injected intraperitoneal (i.p.) with an anti-Gr-1 antibody to deplete PMN prior to intratracheally inoculation with *A. fumigatus* conidia (d0), to prove successful infection (Figure 2A). Surprisingly, in contrast to the WT control animals, the neutropenic KSRP$^{-/-}$ mice showed only mild symptoms of disease (Figure 2B). All immunocompetent WT and KSRP$^{-/-}$ mice survived infection as monitored over 2 weeks (Figure 2B). Next, we investigated the initial innate immune response to *A. fumigatus* infection. To this end, we performed a comprehensive analysis of lung tissue, bronchoalveolar lavage fluid (BALF), and serum derived from infected mice one day post-infection (Group 1). First, we quantified fungal burden in lung tissue of WT and KSRP$^{-/-}$ mice by measuring AFC-specific elongation factor 1-alpha (TEF1) mRNA expression as previously described [49]. Quantitative polymerase chain reaction (qPCR) analysis demonstrated a notably lower AFC levels in KSRP$^{-/-}$ mice relative to WT mice (Figure 2C). Flow cytometric analysis revealed higher frequencies of PMN in BALF and PMN and MAC in lung tissue of KSRP$^{-/-}$ mice compared to WT mice (Figure 2D+E), whereas for eosinophilic granulocytes (EOS) and dendritic cells (DC) (Figure 2D+E), as well as for T cells, B cells and NK cells (Figure S1A) no genotype-dependent

differences could be observed. 14 days following infection, Group 2 of mice was analyzed to assess late-onset of *A. fumigatus* infection. As previously stated, neutropenic KSRP$^{-/-}$ mice demonstrated unexpected mild disease symptoms and were also analyzed 14 days post inoculation. The analysis of the lung lymph node revealed an increase in the number of T and B cells in the α-Gr-1 treated KSRP$^{-/-}$ mice in comparison to the KSRP$^{-/-}$ mice (Figure S1C). These data suggest, that infection can be combated more effectively by KSRP-deficient than WT animals.

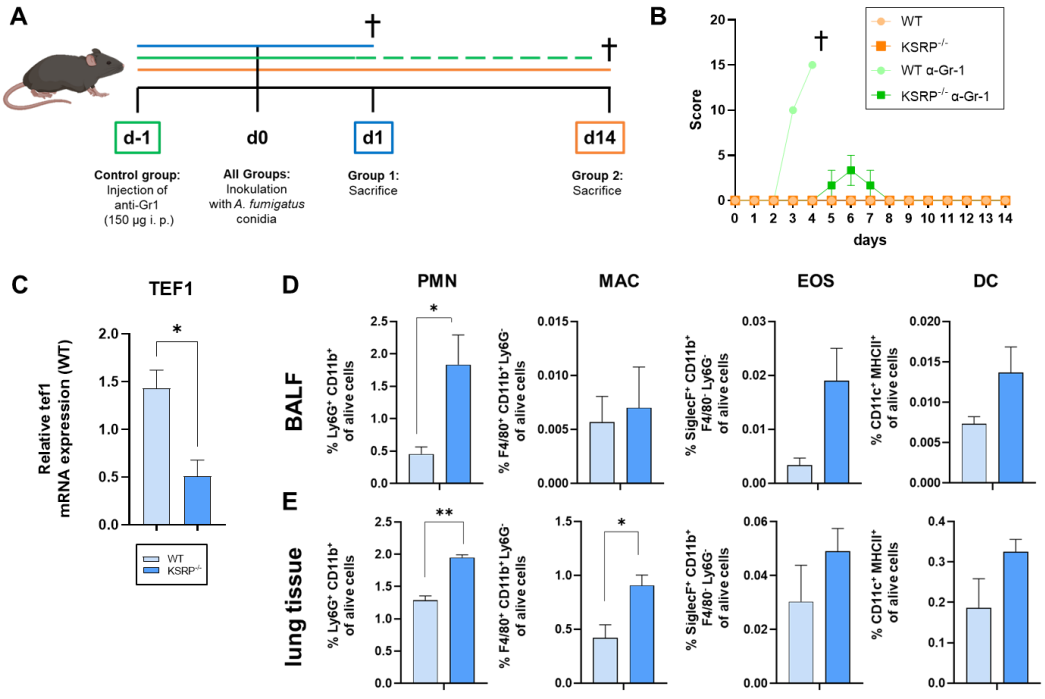

Figure 2. Depletion of PMN and inactivation of the KSRP gene led to resistance against A. fumigatus infection. (A) One day prior inoculation with fungal suspension PMN depletion was induced by i.p. injection of anti-Gr-1 antibody in control animals (green). 24h after inoculation the first group (blue) was sacrificed, while the second group (orange) was analyzed 14 days following inoculation of AFC. Created with BioRender.com. (B) The clinical course of IPA monitoring was assessed of the different groups for 14 days. Parameters comprised breathing, reaction to pain overall appearance, hypothermia, strong weight loss, motoric disabilities and apathy. (C) We prepared total RNA of lung tissue and measured specific TEF1 mRNA expression, as marker for AFC [49], using the qRT-PCR method and normalized to GAPDH mRNA expression. Lungs of KSRP$^{-/-}$ mice contains lower AFC-specific mRNA compared to WT mice one day post-inoculation. (D) Flow cytometric analysis showed higher frequencies of PMN in KSRP-deficient mice, whereas MAC, EOS, DC showed no genotype-depended differences in BALF. (E) Flow cytometric analyses of lung tissue displayed higher frequencies of PMN and MAC, whereas EOS and DC showed no genotype-depended difference one day after inoculation. Data denote the mean ± SEM of 3 samples analyzed per group. Statistically significant differences between groups are indicated (**P < 0.01, *P< 0.05, two-tailed students t-test).

3.3 *KSRP deficiency in PMN results in upregulation of genes involved in inflammatory response*

KSRP-deficient PMN exhibited a reduced expression of 485 genes and an elevation in the abundance of 1,070 genes in comparison to the corresponding control group (WT). Notably, GESA demonstrated that KSRP-deficient PMN exhibited downregulation of genes involved in metabolic processes, including oxidative phosphorylation and glycolysis (Figure S2).

In response to LPS stimulation, KSRP-deficient PMN demonstrated differential regulation of a total of 1,716 genes, comprising 716 upregulated and 1,000 downregulated

genes. GESA revealed elevated expression of multiple genes in KSRP-deficient PMN that are implicated in TNF-α and IL-6 signaling pathways (Figure 3A). In accordance with these findings, time-kinetics studies demonstrated that KSRP-deficient PMN exhibited higher levels of TNF-α mRNA after 3 hours following the initiation of LPS stimulation (Figure 3B). Additionally, KSRP-deficient PMN exhibited elevated TNF-α levels at both the 3- and 16-hour time points following LPS stimulation (Figure 3C). In contrast, no genotype-dependent differences were observed in IL-6 mRNA levels (Figure 3C). However, KSRP-deficient PMN exhibited increased IL-6 secretion following LPS treatment (Figure 3C), indicating that KSRP not only affects mRNA stability but may also attenuate mRNA translation in a target mRNA-specific manner, thereby impairing gene expression.

Since we recently reported that MAC following LPS stimulation produced higher levels of pro-inflammatory cytokines [32], we next investigated genotype-dependent transcriptional alterations between KSRP-deficient PMN and MAC.

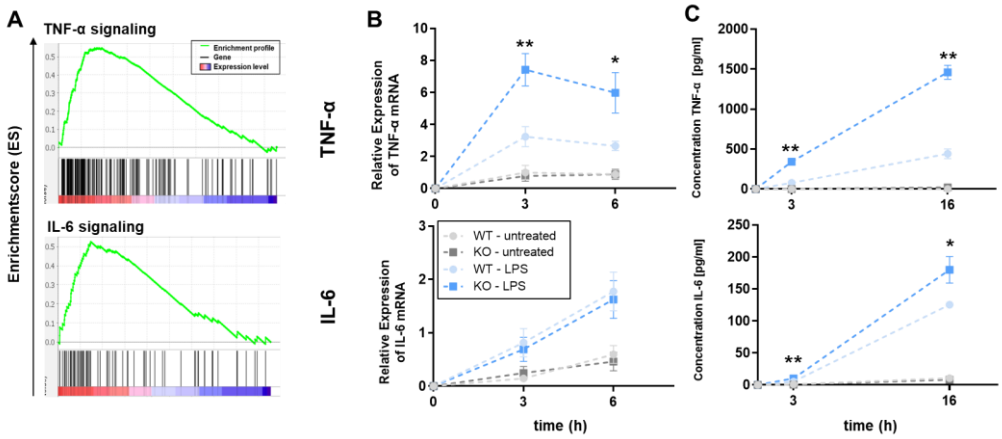

Figure 3. KSRP deficiency in PMN and BMDM results in upregulation of genes involved in inflammatory response in response to LPS-stimulation. (A) Gene set enrichment plots of TNF-α and IL-6 signaling pathways are significantly upregulated in PMN of KSRP$^{-/-}$ mice (BH-adjusted $p < 0.05$). (B) To analyze the mRNA expression of TNF-α and IL-6 in PMN of WT and KSRP$^{-/-}$ mice, PMN were stimulated with 1 µg/µl LPS for different time periods (3 h and 6 h). Then we prepared total RNA by homogenizing the sample in RLT plus lysis buffer and isolated the RNA using the RNeasy Plus Mini Kit. cDNA was synthesized by applying the iScript kit. Specific mRNA expression was measured using the qRT-PCR method and normalized to GAPDH mRNA expression. Shown are the mean ± SEM of n = 9 analyses (**P < 0.01, *P < 0.05; versus untreated WT cells; two-tailed students t-test). (C) To analyze the protein expression of TNF-α and IL-6 in PMN WT and KSRP$^{-/-}$ mice, PMN were stimulated with 1 µg/µl LPS for different time periods (3 h and 16 h). Supernatants of PMN were collected, and cytokine contents were determined using the Anti-Virus-Response LegendPlex- Kit from BioLegend. Shown are the mean ± SEM of n = 6-9 analyses (**P < 0.01, *P < 0.05; two-tailed students t-test).

3.4 KSRP-deficient PMN and MAC share upregualtion of genes involved in pathogen defense

To gain further insight into the similarities and differences in gene regulation in KSRP-deficient PMN and MAC regarding their eminent role in pathogen defense, we conducted a comparative analysis of all upregulated genes following LPS stimulation in both cell types. Our findings revealed that 64 genes were upregulated in both cell types. Additionally, PMN exhibited an upregulation of 936 genes, while MAC demonstrated an upregulation of 887 genes (Figure 4A).

STRING database analysis revealed that the 64 genes congruently upregulated by KSRP-deficient PMN and MAC are interlinked and contribute to positive regulation of innate immune response (5.4 %), anti-viral (2.3 %) and anti-bacterial immune response (1.7 %) (Figure 4B).

The genes that exhibited differential upregulation between PMN and MAC were also examined for gene clusters using the STRING database (Figure S3). This revealed that PMN mainly upregulated genes associated with DNA replication (29.4 %), DNA repair (18.8 %), the cell cycle (14.5 %) and cellular metabolic processes (6.7 %) (Figure S3A). In contrast, MAC exhibited an upregulation of genes associated with anti-viral (22.4%), anti-bacterium (17.8 %), and anti-fungal immune response (13 %) (Figure S3B).

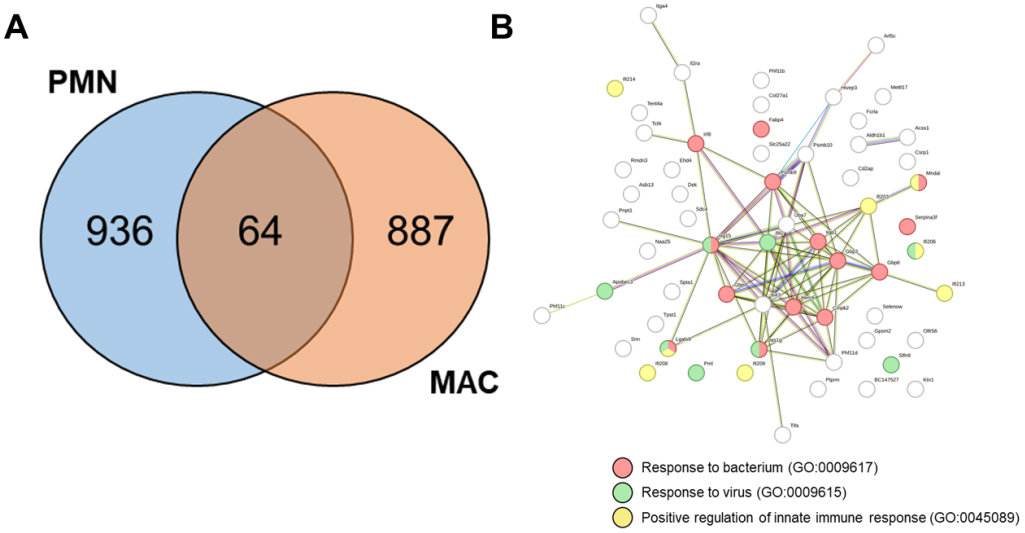

Figure 4. Upregulation of genes involved in pathogen defense in KSRP-deficient PMN and MAC following LPS-stimulation. (A) Using Venn-Diagramm calculator (https://bioinformatics.psb.ugent.be/webtools/Venn/) we calculated same and differential upregulated genes after LPS stimulation within PMN and MAC. (B) STRING database revealed that 64 equally upregulated genes between PMN and BMDM are interlinked and contribute to positive defense regulation against pathogen and activation of innate immunity.

3.5 KSRP knockout enhances PMN and MAC effector functions

3.5.1 Phagocytosis

To analyze potential effects of KSRP on phagocytic activity of PMN and MAC, as a major mechanism for pathogen killing, including *A. fumigatus* [50], we incubated GFP-labeled AFC with bone marrow-derived PMN and day-7-differentiated MAC from WT and KSRP$^{-/-}$ mice. Thereby, we observed that PMN (Figure 5A) and MAC (Figure 5B) from KSRP$^{-/-}$ mice exhibited significantly higher uptake of AFC at 37°C three hours after incubation. Exemplary flow cytometry data depicting the elevated uptake of GFP-fluorescent AFC by KSRP-deficient PMN (Figure 5C) and MAC (Figure 5D) are presented.

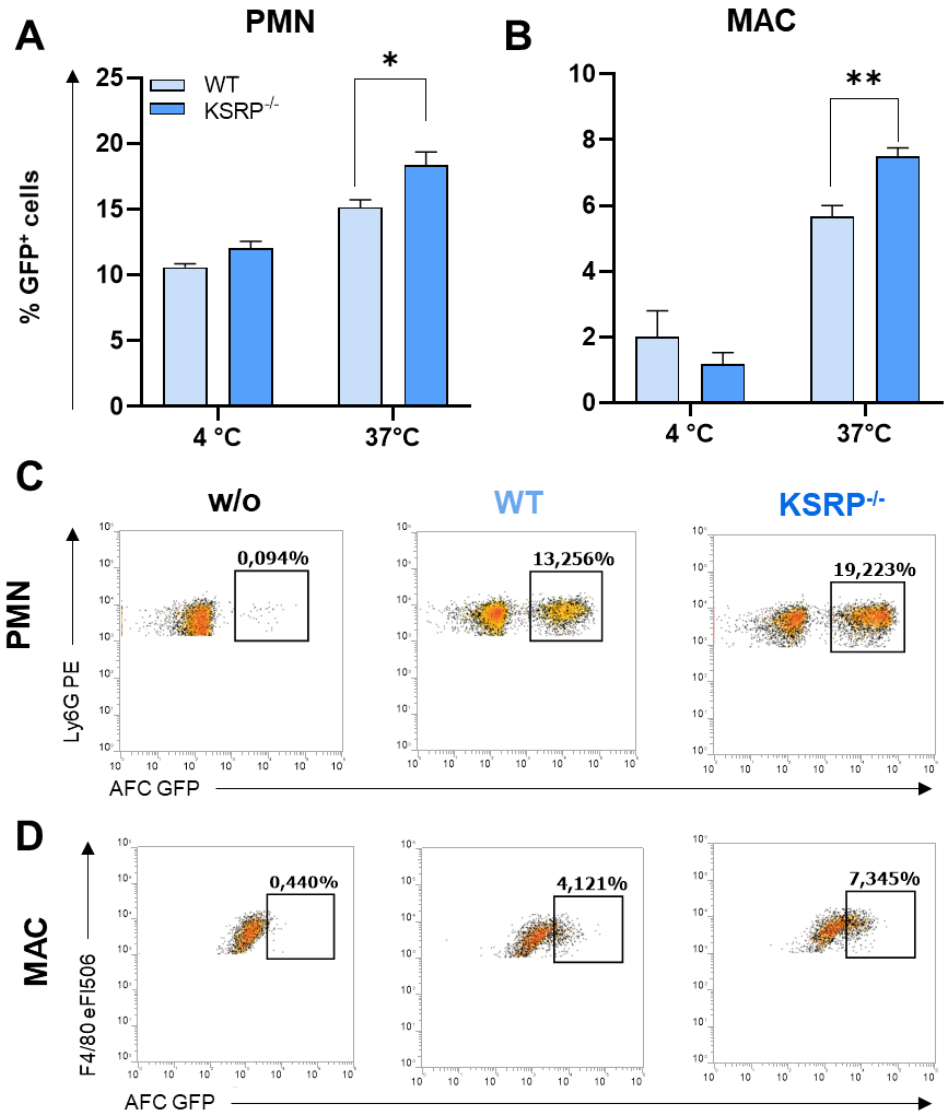

Figure 5. KSRP-deficiency enhances PMN and MAC phagocytosis. PMN were immunomagnetically isolated via Ly6G from bone-marrow of WT and KSRP$^{-/-}$ mice, whereas BMDM were differentiated from bone marrow with M-CSF for 7 days. (A) and (B) 1x10^5 PMN or BMDM were cultured with serum-pre incubated GFP-fluorescent AFC at 4°C or 37°C with indicated ratios. This was done both at 4°C and 37°C to distinguish between mere adhesion and energy-dependent uptake After 3 h the frequency GFP-positive PMN and BMDM was determined by flow cytometry. Data represent the mean ± SEM of 6 samples analyzed/group (**P < 0.01, *P < 0.05; two-tailed students t-test). Exemplary flow cytometry data depicting the pronounced uptake of GFP-fluorescent conidia by PMN (C) and BMDM (D) from KSRP$^{-/-}$ mice. The complete gating strategy is illustrated in Figure S4.

3.5.2 ROS production

We then examined ROS generation as another key effector mechanism in innate pathogen defense. Our findings show that PMN from KSRP$^{-/-}$ mice produced significantly higher levels of ROS (Figure 6A), whereas stimulation with AFC yielded somewhat higher ROS production only in WT cells, but still below the levels observed for KSRP-deficient PMN (Figure 6B). The same outcomes were observed when LPS was employed as the stimulus (Figure S5).

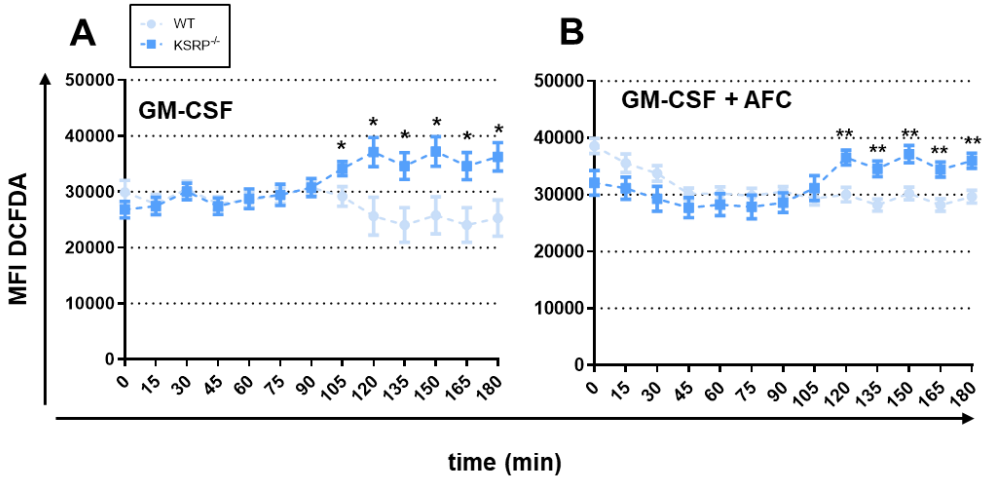

Figure 6. KSRP-deficiency enhances ROS production by PMN. 1x10^5 PMN were stained with DCFDA, treated with indicated stimuli (100 ng/ml GM-CSF (A) and/or 1x10^5 AFC (B)) and measured in 15 min intervals for 3 h. Data represent the mean ± SEM of 9 samples analyzed/group (**P < 0.01, *P < 0.05; two-tailed students t-test).

3.6 Stimulation of KSRP-deficient PMN attenuates apoptosis

As PMN displayed an upregulation of genes associated with cell viability, we accordingly analyzed PMN under basal conditions and in response to LPS and AFC stimulation by flow cytometry. Concerning the expression of early (CD62L or CD18) and late (CD80, CD86, MHCII) activation markers no genotype-dependent differences were observed under basal conditions and in response stimulation (Figure S6). Interestingly, the frequencies of PMN in bone marrow under steady-state conditions were not altered by KSRP deficiency (w/o stimulation), whereas stimulation with LPS led to higher frequencies of PMN in KSRP$^{-/-}$ mice (Figure 5A). In agreement, lower levels of KSRP$^{-/-}$ PMN were positive for the apoptosis marker Annexin V as compared to WT mice, indicating that KSRP may promote apoptosis of PMN (Figure 5B, C).

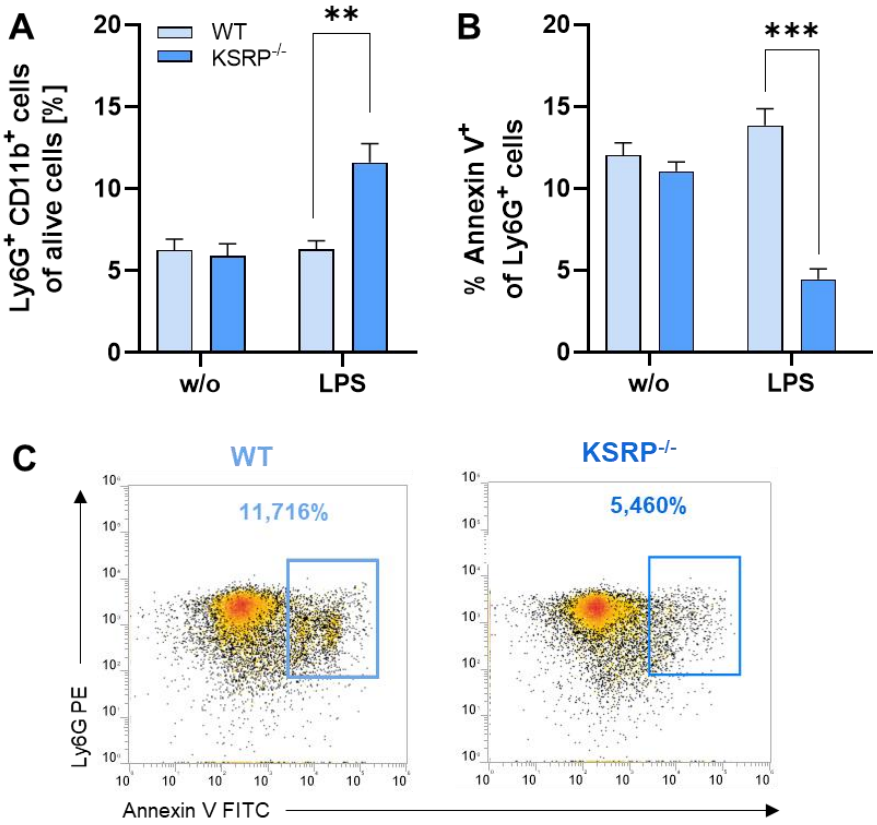

Figure 7. Stimulation with LPS leads to a reduced apoptosis in PMN of KSRP$^{-/-}$ mice. PMN were immunomagnetically isolated via Ly6G from bone-marrow of WT and KSRP$^{-/-}$ mice. 1x10^{6} PMN were cultured without or with 1 µg/ml LPS for 6h. Afterwards surface receptors were stained for flow cytometry analysis. (A) Stimulation leads to frequencies of PMN in KSRP$^{-/-}$ mice. Shown are the mean ± SEM of n = 9 analyses (**P < 0.01; two-tailed students t-test). (B) Assessment of PMN apoptosis revealed a lower expression of apoptosis marker Annexin-V in stimulated KSRP-deficient PMN. Shown are the mean ± SEM of n = 6 analyses (**P < 0.01; two-tailed students t-test). (C) Exemplary flow cytometry data showing attenuated apoptosis by PMN from KSRP$^{-/-}$ mice. The complete gating strategy is illustrated in Figure S7.

4. Discussion

Our previous research has demonstrated that innate immune cells of KSRP$^{-/-}$ mice generate elevated levels of proinflammatory mediators in an LPS-induced sepsis model, which suggests that KSRP plays a role in limiting inflammatory responses [32]. In this study we investigated whether an exacerbated innate immune response could offer benefits in an infection model. Therefore we used *Aspergillus fumigatus*, a pathogen responsible for the most common mold infection in the lungs of immunocompromised patients [47]. In this context, the innate immune system, particularly PMN [35] and MAC [25,30], serves as the primary defense mechanism responsible for clearing conidia and preventing the growth of AFC [27].

Previous studies have indicated that IFN-γ [51,52], IL-1β [53], TNF-α [52], CCL2 [54], CCL5 [55] and CXCL1 [56] play a pivotal role in the host defense mechanisms associated with IPA. In the presence of AFC, splenic immune cells lacking KSRP exhibit augmented production of select anti-fungal cytokines, such as IFN-γ, IL-1β, TNF-α and CCL2, suggesting that KSRP-deficient mice may demonstrate a more robust anti-fungal immune response.

In line, a comparative analysis of the upregulated genes in KSRP-deficient PMN and MAC after LPS stimulation demonstrated 64 shared upregulated genes. Using STRING data-

base we revealed that they are involved in positive regulation of the innate immune response and pathogen defense response, suggesting elevated pathogen defense activity in the absence of KSRP.

As phagocytosis is one key effector mechanism by which PMN and MAC clear pathogen infections [19], we analyzed the effect of KSRP-deficiency in this context. PMN and MAC derived from KSRP$^{-/-}$ mice exhibited increased phagocytic activity against GFP-labeled AFC, indicating that PMN and MAC may prevent the growth of AFC *in vivo*.

To assess this hypothesis, we initiated IPA in WT and KSRP$^{-/-}$ mice through AFC inoculation. One day after infection we observed, the KSRP-deficient mice exhibited a lower fungal load in lungs of KSRP-deficient animals which was accompanied by elevated frequencies of PMN in BALF and of PMN and MAC in lung tissue, suggesting a faster clearance of AFC by these immune cells. In addition, we recently demonstrated that KSRP attenuates the migration of PMN towards CXCL1 [57], the most crucial chemoattractant for PMN in the context of infection [58].

It was unexpected that the PMN-deficient KSRP$^{-/-}$ mice survived the inoculation with AFC, whereas the WT control group died within the first three days. These findings indicate that, additional factors may be responsible for the suppression of AFC to hyphae. In this regard our finding of elevated phagocytic activity of KSRP-deficient MAC *in vitro*, the enhanced MAC numbers in lungs of KSRP-deficient AFC-infected mice and the MAC-specific upregulation of gene sets involved in pathogen defense, suggests that this immune cell populations may compensate for the loss of antibody-mediated depleted of PMN.

While the innate immune response serves as the primary line of defense, the adaptive immune system is indispensable for controlling and eliminating the infection, particularly in immunocompromised individuals [67] such as the PMN-deficient mice. It is noteworthy that, 14 days post-inoculation, an increase in T and B cell frequencies were observed in lung-associated lymph nodes of KSRP-deficient mice. Further studies are necessary to delineate whether this difference is due to a KSRP-dependent intrinsically stronger immune activity of the adaptive immune system.

Furthermore, our findings revealed that KSRP deficiency resulted in elevated ROS production in PMN. The data presented here suggest that KSRP may influence the regulation of oxidative stress-induced gene expression. It has been demonstrated that multiple targets of KSRP encode mRNAs that are regulated by oxidative stress [59,60]. The data presented here suggest that KSRP may influence the regulation of oxidative stress-induced gene expression. This effect may be mediated by protein kinases, e.g. p38 or PKB/Akt that phosphorylate and thereby inhibit KSRP in its ARE-mediated decay of target mRNAs [61]. It is noteworthy that TTP, which also exhibits binding affinity for numerous ROS-regulated mRNAs (e.g., GM-CSF, c-fos, COX-2), was observed to be present at elevated levels in liver and skeletal muscle in mice fed a high-glucose diet, triggering inflammation and oxidative stress [61].

Moreover, RNA sequencing after 6 hours of LPS stimulation revealed higher mRNA expression levels of genes involved in inflammatory responses related to IL-6 or TNF-α signaling in KSRP-deficient PMN, which are also involved in IPA clearance. These findings could be confirmed by time kinetics studies, reveling higher mRNA and protein levels for TNF-α, while there were no genotype-dependent differences of IL-6 on RNA level. However, KSRP-deficient PMN displayed higher IL-6 concentrations after 3h and 16h. In line, Dhamija and colleagues already demonstrated KSRP to interact with the AREs of IL-6 mRNA, thereby mediating its translational silencing [5], suggesting KSRP acts not at transcriptional, but on translational level.

Quite a number of genes enhanced in expression by stimulated KSRP-deficient PMN is involved in signaling pathways that regulate cell cycle and DNA repair. In agreement, KSRP$^{-/-}$ PMN were characterized by attenuated apoptosis, suggesting KSRP-deficiency prolongs cell survival, leading in association with elevated effector mechanisms to pronounced pathogen defense. Ebner and co-workers already described that TTP-deficient

PMN express higher levels of the TTP target Mcl1 mRNA which codes for an anti-apoptotic factor particularly relevant for neutrophils [62]. Although TTP-deficiency reduces PMN apoptosis, the PMN homeostasis under steady-state conditions is not impaired, implying that TTP regulates PMN survival upon infection, but not during homeostatic differentiation or circulation [62].

Taken together, our results suggest that KSRP is a critical negative regulator of anti-pathogen activity in PMN and MAC. The inhibition of KSRP in PMN and MAC has the potential to enhance the body's defense against infection in patients with limited innate immunity. This may be achieved, for instance, through the use of RNA interference.

Supplementary Materials: The following supporting information can be downloaded at: XXX

Author Contributions: Conceptualization, V.B. and M.B.; methodology, V.B., K-A.P., F.R. and N.B.; software, V.B., N.B., and M.B.; validation, V.B., K-A.P., F.R., N.B., A.P. and M.B..; formal analysis, V.B. and M.B.; investigation, V.B., K-A.P., F.R., N.B., A.P., M.B.; resources, A.P. and M.B.; data curation, V.B. and M.B:.; writing—original draft preparation, V.B. and M.B.; writing—review and editing, V.B., K.-A.P., F.R., A.P. and M.B.; visualization, V.B. and M.B.; supervision, A.P. and M.B.; project administration, A.P. and M.B..; funding acquisition, A.P. and M.B. All authors have read and agreed to the published version of the manuscript

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Conflicts of Interest: The authors declare no conflict of interest.

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cells

MDPI

Supplementary Material

KSRP deficiency attenuates the course of pulmonary aspergillosis, associated with elevated pathogen killing activity of innate myeloid immune cells

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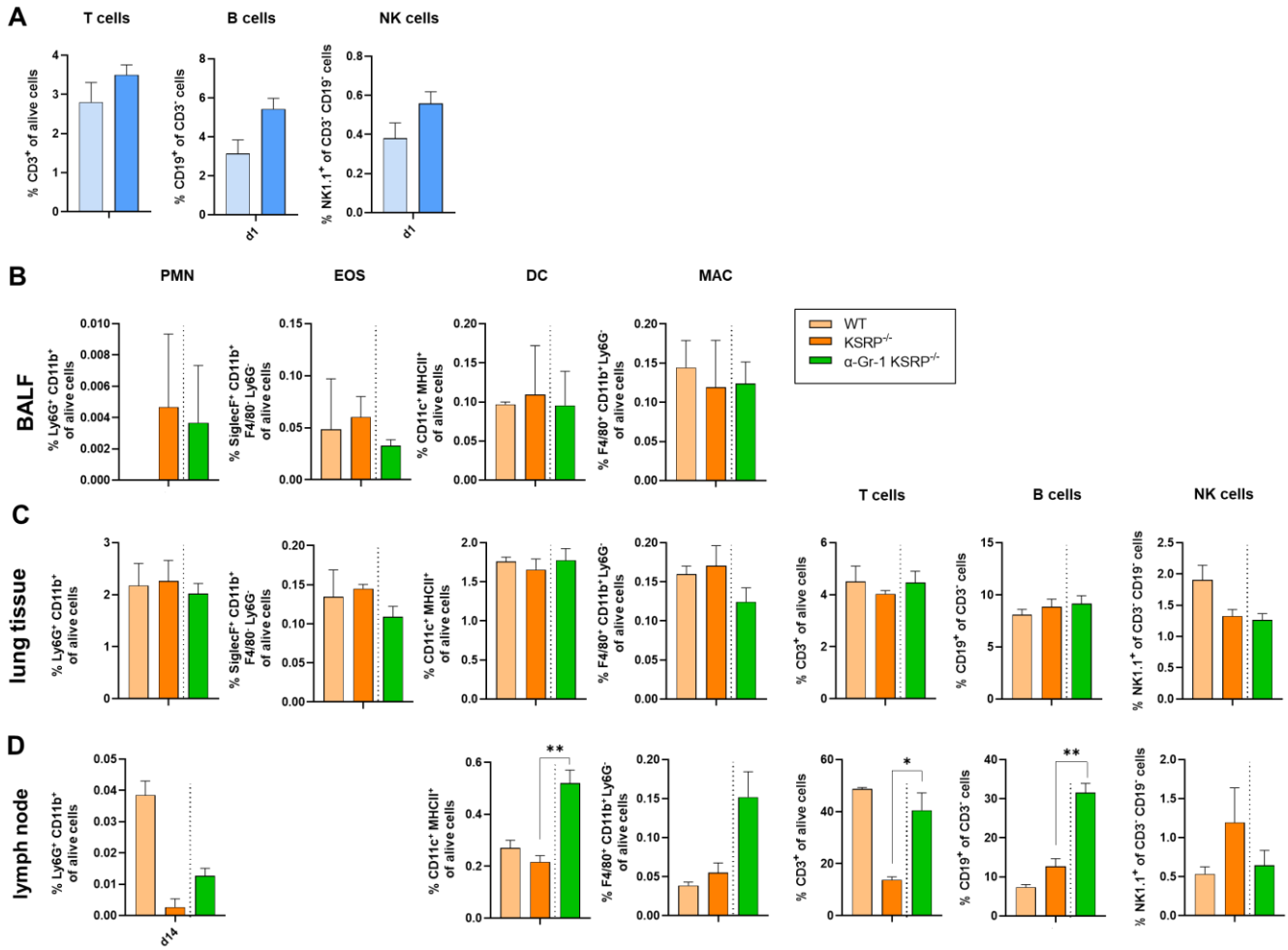

Figure S1. (A) No genotype-dependent differences were detachable regarding T, B and NK cells one day post inoculation. (B) Flow cytometric analyses of BALF, lung tissue (C) and the lung-associated lymph node (D
) revealed only significant levels of T and B cells in the lymph node. PMN, EOS, DC and MAC displayed no genotype-depended differences 14 days after inoculation.

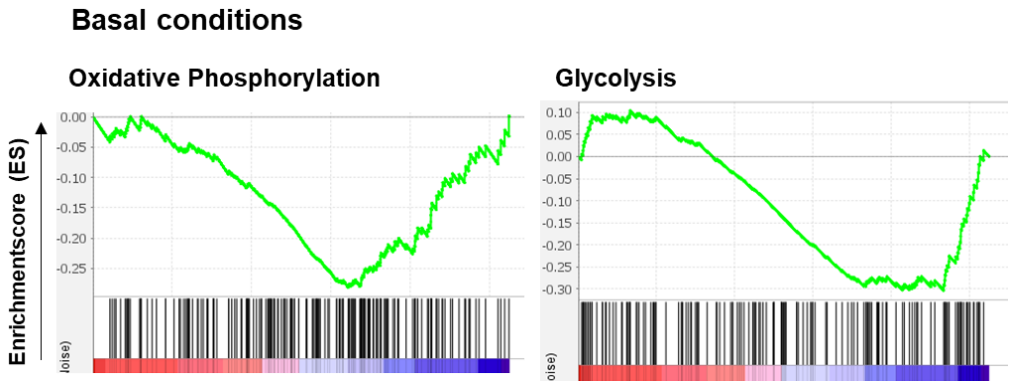

Figure S2. GESA revealed that KSRP-deficient PMNs showed without stimulation a downregulation of genes associated with metabolic pathways, such as oxidative phosphorylation and glycolysis.

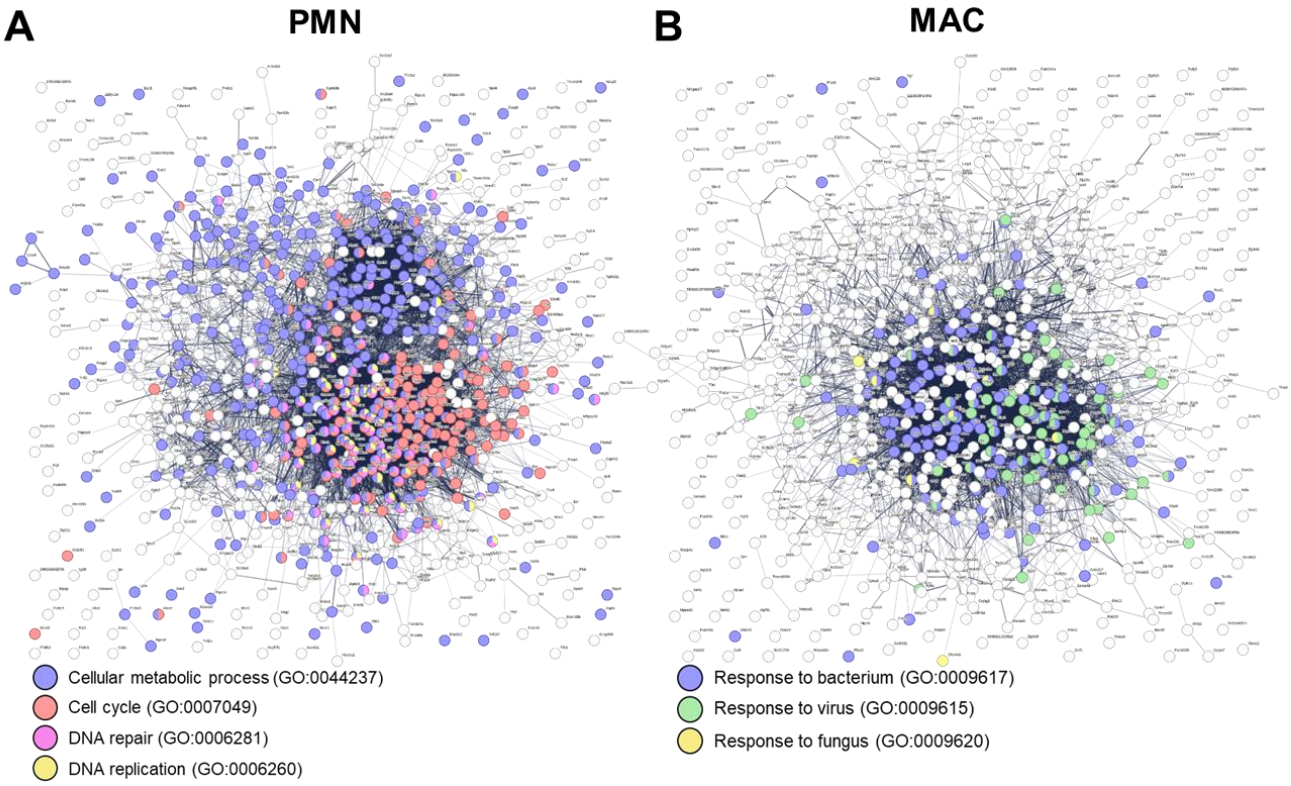

Figure S3 Genes showing differential upregulation between PMN and MAC were further analyzed for gene clusters using the STRING database. This analysis revealed that PMN (A) predominantly upregulated genes related to DNA replication (29.4%), DNA repair (18.8%), the cell cycle (14.5%), and cellular metabolic processes (6.7%). In contrast, MAC (B) displayed an upregulation of genes involved in defense against viruses (22.4%), bacteria (17.8%), and fungi (13%).

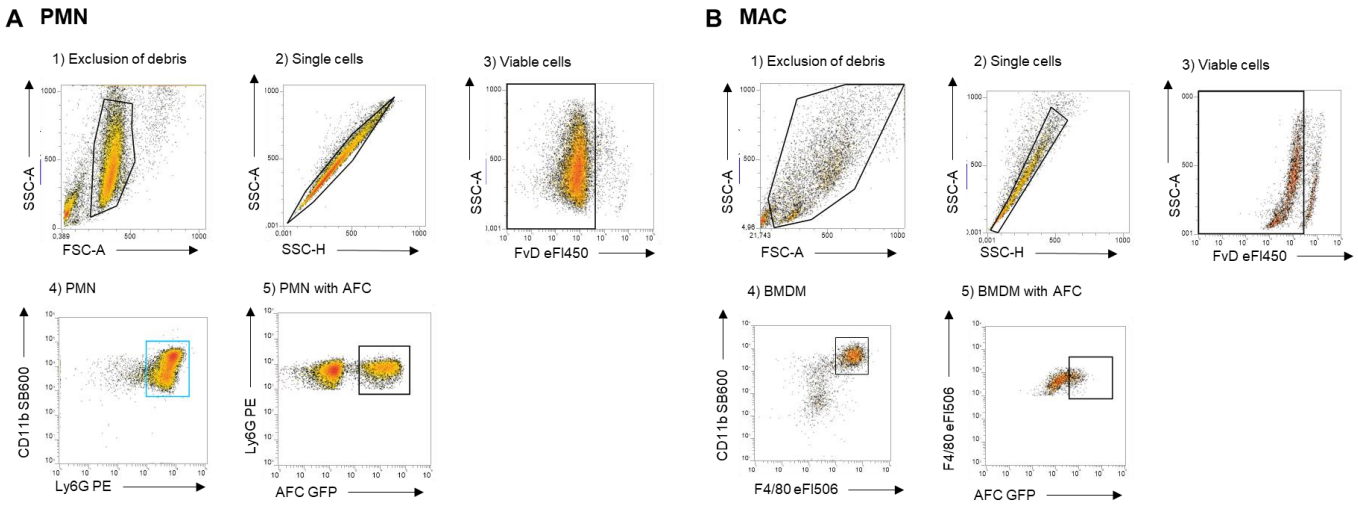

Figure S4. Gating strategy to evaluated phagocytic uptake of GFP-labeled AFC for PMN (A) and MAC (B).

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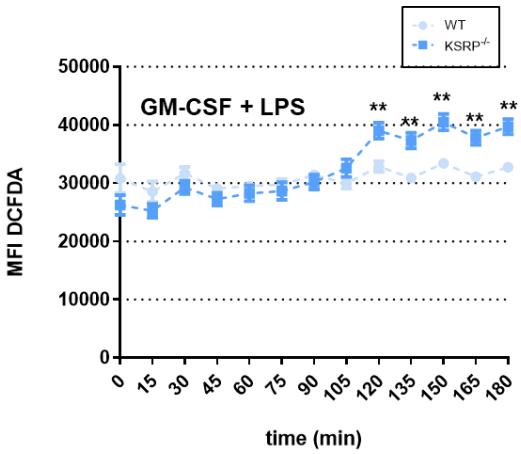

Figure S5. PMN from KSRP$^{-/-}$ mice produced significantly higher levels of ROS following LPS stimulation.

19

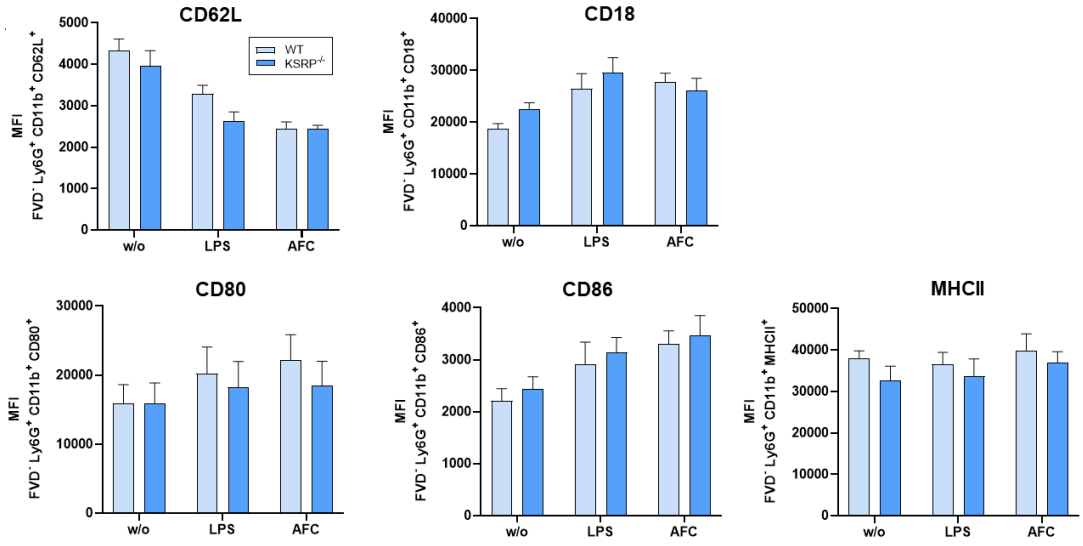

Figure S6. Stimulation of PMN from WT and KSRP$^{-/-}$ mice has no genotype-dependent impact on (early) surface activation marker expression.

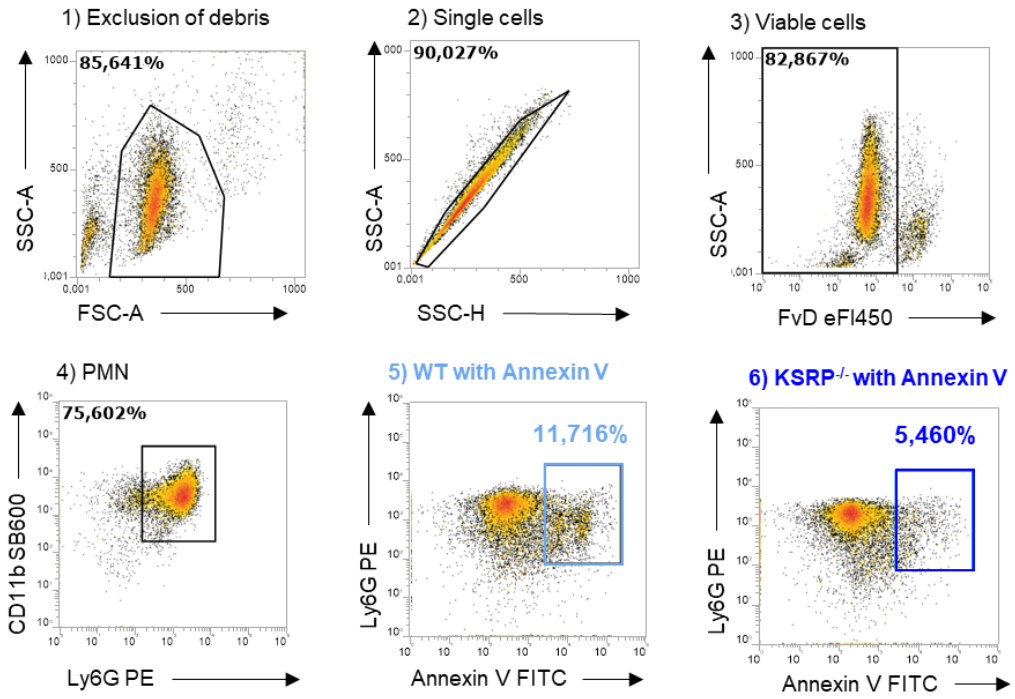

Figure S7. Gating strategy to evaluated apoptosis levels of PMN in WT and KSRP$^{-/-}$ mice.

6.4 Abbreviation list

5‘/3‘-UTR	5'/3’-untranslated region
A. fumigatus	*Aspergillus fumigatus*
aa	amino acid
AFC	*Aspergillus fumigatus* conidia
ALAE	regulating axon elongation
AMD	ARE-mediated mRNA decay
AMT	ataxia telangiectasia mutated kinase
AP-1	Activator protein 1
APC	antigen-presenting cells
ARE	adenine-uracil-rich element
AUF1	ARE/poly(U)-binding degradation factor
BALF	bronchoalveolar lavage fluid
BCR	B cell receptor
BMDM	Bone marrow-derived macrophages
CAIA	Collagen antibody-induced arthritis
CCL5 (RANTES)	CC-Chemokin-Ligand-5
CD	Cluster of Differentiation
CTL	cytotoxic T lymphocytes
CXCL1	C-X-C-motif ligand 1
DCFDA	2´-7´Dichlorodihydrofluorescein
DCs	Dendritic cells
DNA	deoxyribonucleic acid
EMT	epithelial-to-mesenchymal transition
FBP	FUSE-binding protein
FBXW2	F-box and WD repeat domain-containing 2
FUSE	far upstream element
GESA	Gene set enrichment analysis
GFP	Green fluorescent protein
GM-CSF	Granulocyte/Macrophage Colony-Stimulating Factor
hnRNA	Heterogeneous nuclear RNA
HuR	human antigen R
i.p.	intraperitoneally
IFN	Interferon
IL	interleukin
iNOS	Inducible nitric oxide synthase
IPA	Invasive pulmonary aspergillosis
IRES	enterovirus internal ribosome entry site
ITAFs	IRES trans-acting factors
KH	K homology
KLHL12	Kelch-like protein 12

KS(H)RP	KH-type splicing regulatory protein
lncRNA	Long non-coding RNA
LPS	lipopolysaccharide
lys	lysin
MAPK	mitogen-activated protein kinase
miRNA	microRNA
mRNA	messenger RNA
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
PAMPs	Pathogen-associated molecular patterns
PKB	protein kinase B
PMA	Phorbol-12-myristate-13-acetate
PMN	polymorphonuclear neutrophils
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
RISC	RNA-induced silencing complexes
RNA	ribonucleic acid
RBP	RNA-binding protein
ROS	Reactive oxygen species
SCF	S-phase kinase-associated protein 1 and cullin-1 F-box protein
STAT	Signal Transducers and Activators of Transcription
SUMO	small ubiquitin-like modifier
TCR	T cell receptor
TEF1	elongation factor 1-alpha
TGF-β	Transforming growth factor-β
Th	helper T cells
TLRs	Toll-like receptors
TNF	tumor necrosis factor
TTP	Tristetraprolin
USE	Upstream sequence element
WT	Wild type

Curriculum Vitae

Publication list

KSRP-related publications

- K. A. Palzer*, **V. Bolduan***, R. Käfer, H. Kleinert, M. Bros, A. Pautz. The Role of KH-Type Splicing Regulatory Protein (KSRP) for Immune Functions and Tumorigenesis. Cells, 2022, 11(9):1482.
  * Shared first authorship

- **V. Bolduan**, K.A. Palzer. C. Hieber, J. Schunke, M. Fichter, P. Schneider, S. Grabbe, A. Pautz, M. Bros..The mRNA-Binding Protein KSRP Limits the Inflammatory Response of Macrophages. International Journal of Molecular Sciences, 2024, 25(7), 3884

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