MicroRNAs and the resolution phase of inflammation in macrophages

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Toll-like receptors (TLRs) signal the presence of pathogens and tissue injury, triggering the inflammatory process in macrophages. The goal of inflammation is to resolve the injury and return the body to homeostasis. MicroRNAs are an important group of regulators of TLR signaling and several are induced by TLRs in macrophages. These TLR-induced microRNAs target signaling components in the TLR pathway, thereby producing a negative feedback loop, and they are therefore prime candidates for the initiation of repair. Importantly, their dysregualtion may be important for chronic inflammation, which in turn can lead to autoimmunity and cancer, as discussed in this *Viewpoint*.

The first line of defense against pathogens is composed primarily of innate immune cells – specifically phagocytes (macrophages and polymorphonuclear neutrophils). Once the inflammatory response is initiated, the system is brought back to homeostasis by negative regulators. Since there is now ample evidence to indicate that dysregulation of innate immunity can give rise to a range of inflammatory diseases, elaborate control mechanisms must exist to prevent its overactivation. These control mechanisms are likely to be triggered after the initial activation of innate immune receptors (such as the TLRs), their job being to restore the system to homeostasis. In the case of TLR activation, a large number of such controls have been identified, ranging from decoy receptors to phosphatases to deubiquinating enzymes [1]. Recently, microRNAs (miRNAs) have emerged as key regulators of TLRs, particularly in macrophages, and it is highly likely that they fine-tune signaling in order to allow for resolution of the inflammatory process.

miRNAs are typically small (21–22 nucleotides) noncoding RNAs, the majority of which are intergenic or intronic, although a minority of miRNAs are derived from protein-coding mRNAs [2]. miRNAs form a complex with the RNA-induced silencing complex (RISC) producing miRISCs that bind to complementary 3′ UTRs of target genes and thereby repress translation of mRNA, promote degradation, or stabilize the target mRNA [2]. Depending on the pathogen encountered, TLRs induce a number of miRNAs with expression of some miRNAs being decreased in response to TLRs [2].

Here, we discuss how miRNAs regulate TLRs, particularly in macrophages, a process likely to occur in the resolution phase of inflammation and speculate on the importance of miRNAs in diseases, which feature dysregulated innate immunity. We discuss three particular miRNAs – miR-155, miR-146a, and miR-21 – since these miRNAs have been strongly implicated in the regulation of TLRs in a number of cells including macrophages [3]. Interestingly, miR-155 and miR-146 are specifically present in LPS-induced macrophages, as compared with similarly activated polymorphonuclear neutrophils (PMNs), suggesting a particular role for these miRNAs in macrophages [4]. We also speculate on the potential novel therapies that target miRNAs in infection and inflammation that could be developed.

miR-155

The gene-encoding miR-155 is located on chromosome 21 in the B-cell integration cluster (BIC) [5]. BIC is highly conserved between humans and mice and is highly expressed in lymphoid organs. miR-155 expression is strongly induced in response to LPS or type I interferons, in both monocytes and macrophages of human or mouse origin, demonstrating that this miRNA participates in the innate immune response to both bacterial and viral infection [6, 7]. Furthermore, miR-155 is highly expressed in activated B and T cells and has been shown to play a role in regulating cytokine expression in the germinal center [8]. miR-155 is induced by either the MyD88 or the TRIF pathways through LPS or poly I:C stimulation [7].

Unlike the miRNAs discussed later in this *Viewpoint*, the evidence so far presented on miR-155 function indicates that it is likely to be pro- rather than anti-inflammatory. This is because one of the roles of miR-155 in macrophages is to allow the translation of tumor necrosis factor (TNF), a key pro-inflammatory cytokine [6, 9]. In resting macrophages, the 3′ UTR of TNF induces a self-repression, which is released upon LPS stimulation via the binding of miR-155. This has been shown in macrophages, where miR-155 overexpression results in increased TNF production and miR-155 deficiency results in lower levels of TNF [9]. Targeting miR-155 in macrophages would therefore limit TNF production and would be useful therapeutically in TNF-mediated disorders. An in vivo study has shown that B cells that overexpress miR-155 transgenically produce more TNF and the corresponding transgenic mice have an elevated susceptibility to LPS-induced septic shock [8]. miR-155-deficient B cells, on the other hand, fail to produce TNF [8].

As shown in Fig. 1, in macrophages, miR-155 is negatively regulated by IL-10, an anti-inflammatory cytokine [10]. Inhibition of miR-155 by IL-10 increases expression of Src homology2 (SH2) domain-containing inositol 5′-phosphatase 1 (SHIP1), a known target of miR-155 [11, 12]. Previously, SHIP1 has

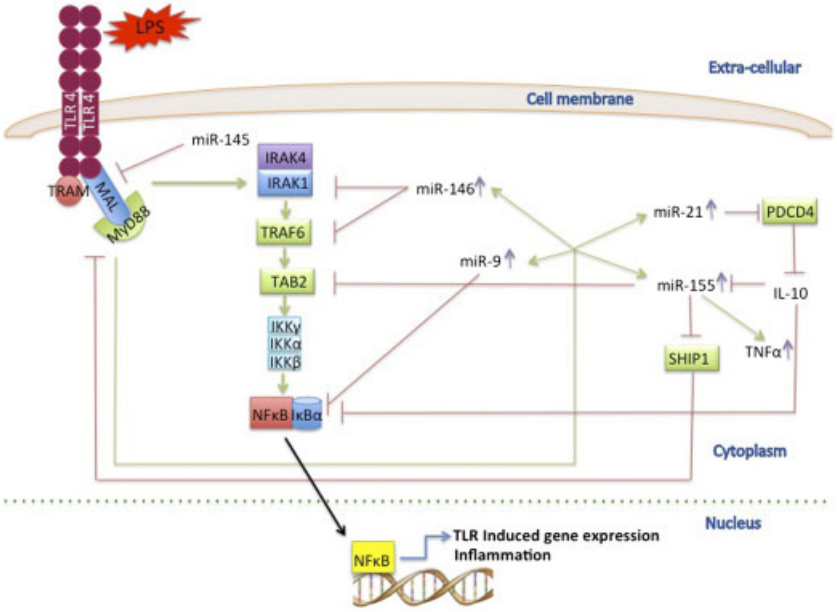

Figure 1. miRNA-mediated regulation of the NF-κB pathway in macrophages. LPS activates TLR4 and that initiates a cascade of events resulting in the induction of NF-κB. LPS stimulation also induces various miRNA that can regulate the NF-κB activation pathway: miR-146 blocks IRAK1 and TRAF6, miR-9 blocks NF-κB, miR-21 blocks PDCD4 and these three miRNA are anti-inflammatory; miR-155, on the other hand, is a pro-inflammatory miRNA, inducing TNF-α which further activates macrophages and blocks SHIP1. SHIP1 then negatively regulates the TLR-induced responses. IL-10 is an anti-inflammatory cytokine and is blocked by PDCD4. Upon miR-21 induction, PDCD4 is blocked and IL-10 blocks miR-155 and NF-κB. Hence, a negative regulatory loop forms. Overall, this figure illustrates how miRNAs work together to bring the system back to homeostasis postinflammation triggered by LPS.

been shown to function as a negative regulator of TLR-induced responses [13–15]. The action of SHIP1 is likely to be through a negative regulatory loop as the serine/threonine protein kinase AKT, which is downregulated by SHIP1, negatively regulates miR-155 [16]. Thus, after LPS stimulation, miR-155 expression increases, SHIP1 levels fall, and AKT activity increases; as AKT downregulates miR-155, the initial high miR-155 levels are brought back under control.

miR-155 KO mice have been shown to have an impaired immune response to *Salmonella typhimurium*, and these mice cannot be successfully immunized against this pathogen [17]. Further analysis revealed a defect in B- and T-cell activation, explaining the lack of immunization capacity in these mice. Furthermore, the failed T-cell response was, in part, due to the failure of DCs to present antigen and due to an altered Th1 response in which the $CD4^+$ T cells had impaired cytokine production [17]. This was most likely due to the failure of DCs to functionally activate costimulatory signals and defective antigen presentation; miR-155 may be responsible for the impaired cytokine production. A second study showed that miR-155 KO mice exhibit reduced numbers of germinal centre (GC) B cells, whereas miR-155-overexpressing mice showed elevated levels [8]. This study concluded that miR-155 achieves its response partly by regulating the expression of cytokines, e.g. TNF [8]. A third study with miR-155-deficient mice revealed elevated levels of activation-induced cytidine diamine (AID) [18]. AID is a strong mutation-causing component in the class switching process and therefore its activity needs to be tightly regulated [19]. AID initiates somatic hypermutation and is essential for class-switch recombination [19]. The gene-encoding AID contains a miR-155 binding site in its 3′ UTR [8, 18]. B cells undergoing class switching express high, but controlled, levels of miR-155; genetically modified mice with a mutation in the 3′ UTR binding site for miR-155 in the AID gene that blocks miR-155 binding show increased AID levels, compared with WT cells, and increased numbers of *Myx-Igh* translocations and, as a result, have disrupted affinity maturation. miR-155 thus closely regulates AID expression in cells to prevent hypermutational activity. These in vivo experiments confirm that miR-155 is especially important for B-cell development and identify AID as a key target.

miR-146

miR-146 is one of the most prominent miRNAs induced by LPS in macrophages [3, 20]. Resolvin D1, an anti-inflammatory lipid mediator, also induces miR-146 [21]. miR-146 expression is NF-κB dependent and, to date, IL-1R-associated kinase 1 (IRAK1), IRAK2, and TNFR-associated factor 6 (TRAF6) have been shown to be miR-146 targets [20]. As shown in Fig. 1, these targets are components of the NF-κB pathway and control NF-κB expression. *Irak1* has been validated as a target for miR-146 in in vivo studies [22]. Since IRAK1 and TRAF6 are required for NF-κB activation, there is therefore a negative regulatory loop in operation whereby NF-κB activation upregulates miR-146 that, upon maturation, downregulates IRAK1 and TRAF6 and thereby represses NF-κB. IL-1β, which is produced in response to LPS, triggers miR-146 production, which blocks NF-κB, and thereby participates in a negative regulatory loop modulating LPS-induced signals [23]. Furthermore, overexpression of miR-146 results in a decrease in various chemokines and cytokines, including CXCL8, CCL5 [23], IL-6, CXCL8 [24, 25], and IL-1β itself [26], and thereby prevents overactivation of inflammation and brings the system back to homeostasis.

Within 6 months of birth, miR-146a KO mice develop a spontaneous autoimmune-like disorder that leads to death [27]. These KO mice exhibit loss of immunological tolerance and their macrophages are hyper-responsive to

LPS. The mice also develop tumors in secondary lymphoid organs [27], which is likely to be due to chronic inflammation. miR-146a is therefore the best understood miRNA in terms of prevention of the damaging effects of inflammation, and its role could be potentially exploited to prevent certain inflammatory disorders and tumors.

miR-21

miR-21 is induced upon LPS stimulation via the MyD88 pathway in an NF-κB-dependent manner in macrophages [28]. As shown in Fig. 1, miR-21 controls inflammation by downregulating the translation of the pro-inflammatory tumor suppressor programmed cell death 4 (PDCD4) [28], an inhibitor of IL-10 production. Hence, miR-21 promotes IL-10 production upon LPS stimulation by regulating PDCD4. IL-10 is an anti-inflammatory cytokine that blocks NF-κB and allows the system to go back to a homeostatic state. miR-21 could therefore be another key miRNA in the resolution of inflammation.

miR-21 regulates NF-κB in a cell-specific manner. As shown in Fig. 1, miR-21 forms a negative regulatory loop in innate immune cells that keeps inflammation in check by limiting NF-κB expression through the upregulation of IL-10; IL-10 represses NF-κB. In contrast, in tumor cells, miR-21 downregulates phosphatase and tensin homologue (PTEN) and activates AKT, thereby maintaining/increasing NF-κB activity [29], and hence maintaining/promoting tumorogenesis. A number of miR-21 targets in tumor-associated genes have been identified and validated, including tropomyosin 1 (TPM1) [30], reversion-inducing-cysteine-rich protein with kazal motifs (RECK) [31], Fas ligand (FasL) [32], tumor-associated protein 63 (TAp63) [33], and heterogeneous nuclear ribonucleoprotein K (HNRPK) [33]. miR-21 is therefore seen as an important "Oncomir" and its activation by TLRs may provide yet another link between inflammation and cancer.

Prospects for immunomodulatory therapeutics

Given the level of research activity in the field of miRNAs, there is hope that new diagnostics or therapeutics might emerge for infectious and inflammatory diseases. The current best prospect is for hepatitis C virus (HCV) [34, 35]. The 5′ UTR of the HCV genome contains sequences essential for its replication including two binding sites for miR-122. The HCV has conveniently made use of liver-abundant miR-122 to facilitate its replication and translation [36–38]. A miR-122 antagomir, that specifically silences miR-122, has been found to inhibit replication of HCV genotypes 1a and 2a [37–39]. Furthermore, experimental data generated using HVC-infected chimpanzees demonstrate that the miR-122 antisense locked nucleic acid (LNA) SPC3649 is able to clear both the HCV 1a and the 1b genotypes [40]. These data hold much promise for novel anti-HCV therapies. In the case of HCV-induced inflammation, if the target site for miR-155 in the TNF 3′ UTR was to be blocked, this could provide a new strategy to limit TNF expression and TNF-associated activities. Another approach could be to specifically boost the effect that miR-21 has on PDCD4 and thus also generate an anti-inflammatory effect. These types of studies are worth pursuing, since targeting both miR-155 and miR-122 would effectively boost the resolution of inflammation.

A second example where the targeting of miRNAs regulated by TLRs might hold promise is in myelodysplastic syndrome (MDS). MDS results from the ineffective production of myeloid cells from stem cells in the BM and arises at the stage of primitive $CD34^+$ hematopoietic stem/progenitor cells due to ineffective hematopoiesis. One of the most common forms is the 5q-syndrome, which results in the deletion of a segment on chromosome 5, long-arm position 32 (5q32) [41–43]. The commonly deleted region at 5q32 contains 40 genes and a number of miRNAs, including miR-145 and miR-146a. Starczynowski et al. [41] found that 5q-MDS individuals had low levels of miR-145 and miR-146a, thereby confirming their deletion [41]. A key target for miR-145 is known to be the adapter Mal, which is required for signaling by TLR2 and, especially, TLR4 [42]. As mentioned in the *miR-146* section, miR-146 targets IRAK1 and TRAF6. The knockdown of miR-145 and miR-146a or, in particular, the enforced

Table 1. In vivo-verified mouse models investigating microRNA effects

Targets	Technique	Expression	Phenotype	Reference
miR-155	*bic*/miR-155 KO	Thymus, spleen	Increased lung airway remodeling, defective adaptive immunity, impaired DC antigen presentation	[18]
miR-155	*bic*/miR-155 KO	Thymus, spleen	Reduced germinal centre B cells, defective B and T cells	[8]
miR-155	miR-155 site KO in AID gene	Thymus, spleen	Impaired affinity maturation, deregulated AID and elevated CSR expression in splenic B cells	[20]
miR-146	miR-146 KO	Lymphoid organs	Healthy birth, develop autoimmune-like disorder within 6 months	[28]
miR-21	miR-21 KO/LNA-antimiR-21	Fibroblasts	No obvious phenotype	[44]
miR-21	Synthetic antagomir	Fibroblasts	Inhibits interstitial fibrosis and attenuates cardiac dysfunction	[45]

expression of TRAF6 in hematopoietic stem/progenitor cells transplanted into mice results in thrombocytosis, neutropenia, and megakaryocytic dysplacia [41]. These changes lead to the induction/overexpression of pro-inflammatory cytokines, such as IL-6, leading to chronic inflammation, which again appears to promote tumorogenesis in this disease. Other studies, e.g. [43], have failed to find a correlation between 5q-MDS and downregulation of miR-145–miR-146a, however; hence further analysis is needed. Nonetheless, blockade of the Mal/TRAF6 pathway could prove to be therapeutically useful in MDS.

Conclusions

Clearly, the targeting of miRNAs for therapeutic purposes is at an early stage; however, given the roles of miR-146a, miR-155, and miR-21 in the control of inflammation, and, in particular, in macrophage function, they remain of interest for future drug development. An important consideration is in vivo validation, and Table 1 summarizes this aspect for these miRNAs. As summarized in Table 1, deletion of miR-155, miR-146, and miR-21 has serious consequences in mice, e.g. autoimmune disease. These miRNAs therefore seem to regulate important functions and their deletion leads to numerous immune dysfunctions, e.g. miR-155 KO mice have defective DCs. Ultimately, the hope is that the extensive knowledge that is emerging on these important fine-tuners of inflammation might be brought to bear on the complex processes in the resolution of inflammation, and from there possibly to cancer, where dysregulation of inflammation plays an important role.

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Abbreviations: **AID**: activation-induced cytidine diamine · **BIC**: B-cell integration cluster · **IRAK1**: IL-1R-associated kinase · **MDS**: myelodysplastic syndrome · **miRNA**: microRNA · **PDCD4**: programmed cell death 4 · **SHIP1**: Src homology2 (SH2) domain-containing inositol 5′-phosphatase 1 · **TRAF6**: TNFR-associated factor 6

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