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Review

MicroRNAs: Fine-tuners of Type I Interferon-dependent inflammatory responses

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ABSTRACT

Inflammation, a physiological response to noxious stimuli of variable origin, is characterized by the expression and release of numerous cytokines, whose uncontrolled synthesis can rapidly lead to severe pathologies such as autoimmunity. Therefore, a tight regulation must be achieved in order to ensure, both temporally and spatially, accurate production of these molecules. Working in concert with regulatory proteins which have been described extensively, microRNAs appeared recently as novel actors in the network leading to an inflammatory context. To analyze globally the impact of miRNA on inflammatory responses of infectious origin, we performed viral inoculation in DICER-deficient mutant mice. In addition, using a genetic approach, in which a Dicer hypomorphic mutation was combined with the CD95 $^{lpr/lpr}$ allele, we investigated the consequences of low miRNA production on the initiation and development of lupus as a model of inflammatory settings triggered under sterile conditions. In this review, we discuss the data obtained with these two models within the frame of miRNA-dependent regulation of Type I Interferon-Stimulated genes.

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INTRODUCTION

Inflammation is an evolutionarily conserved mechanism [1] whereby specialized immune cells becomes activated in response to an external stimulus. Depending on the organism in which this phenomenon occurs, cell activation can take different forms: some cells may acquire phagocytic activity, while others start secreting signaling molecules. The trigger can also be multifaceted. It can be of infectious (viral, bacterial…) or non-infectious (sterile) origin and derive from the host itself (ATP, β-amyloid) or the environment (alum, UV radiation) [2, 3]. All these different triggers might also be considered under a unique term as "danger" signals [4].

Upon ligation to their cognate receptors - termed Pattern Recognition Receptors - these stimuli will activate intracellular signaling cascades which will ultimately induce changes in gene expression [5]. Among the numerous induced molecules that participate in the inflammatory process, TNF-α and Type I Interferons (IFNs) occupy a key position because they appear at both ends of a balance. If one or the other prevails, the resulting

disequilibrium leads to immunopathology [6]. In this regard, the example of Type I IFN secretion is particularly illustrative. Whereas their diminished expression is correlated with susceptibility to most viral infections [7], an excess of α/β IFN is associated to several autoimmune diseases, among which systemic lupus erythematosus (SLE) represents a paradigm for these pathologies [8].

Therefore, tight regulation of the expression of these molecules appears crucial to maintain homeostasis and to respond appropriately to external aggressions. Whereas the molecular events leading to the transcriptional induction of Type I IFNs have been extensively studied over the past decades [9], precise information regarding their negative regulation are scarce [10, 11].

It is now widely recognized that microRNAs (miRNAs) are important modulators of gene expression and affect many biological processes [12]. miRNAs are small, single-stranded non-coding RNAs whose biogenesis, for the majority of them, follows a so-called "canonical pathway" [13]. It involves nuclear processing of a primary (pri-miRNAs) RNA pol II-dependent transcript by the nuclease DROSHA and the double-stranded RNA-binding protein DGCR8. The resulting precursor (pre-miRNA) is then exported to the cytoplasm where further maturation into a 22-nt long duplex miRNA/miRNA* requires the RNAse III DICER. Finally, with the help of accessory proteins TRBP and PCT [14], one strand is loaded on the RNA-Induced Silencing Complex (RISC) which contains a protein of the Argonaute (AGO1-4) family with slicing activity. Within the RISC, the miRNA is guided towards its target mRNA sequence, usually located in the 3' untranslated (3'UTR) region. Variable mechanisms, including diminished mRNA stability or inhibition of translation, account for repressed target gene expression and depend on miRNA/mRNA base-pairing [15]. More than 2000 mature miRNA sequences are now described in humans (http://www.mirbase.org/cgi-bin/browse.pl?org=hsa, [16]) and it is estimated that each miRNA potentially regulates hundreds of genes [17]. However, the amplitude by which miRNAs impact gene expression is modest and is set between 1.2 to 4-fold [18], which favors their role as fine-tuners of gene expression rather than on/off switches. It also indicates that miRNAs likely reduce the transcriptional background of their target genes. Altogether, combined with the observation that they are expressed in immune cells, these features make miRNAs suitable candidates for the adjustment of a mechanism which requires accurate regulation: the inflammatory response.

Several miRNAs with opposing functions have been identified as important mediators in inflammation, among which miR-155 (pro-inflammatory) and miR-146 (anti-inflammatory) have been extensively studied [19]. While a large number of targets for both miRNAs had been predicted, hence suggesting wide regulatory functions, *in vivo* data actually reporting convincing evidence of their implication in inflammation were limited. Precise answers to this question were provided by the generation of knock-out mutant mice. Thus, consistent with its role as a promoter of inflammation, miR-155 $^{-/-}$ animals were shown to be resistant to experimental autoimmune encephalomyelitis [20], whereas the phenotype of miR-146 $^{-/-}$ mice, which exhibited hypersensitivity to LPS, is in line with the anti-inflammatory role attributed to this miRNA [21].

Recently, our laboratory evaluated the global contribution of miRNAs to the regulation of inflammatory responses. For this, we analyzed the innate immune response of naïve and virus-infected Dicer-deficient mice and compared it to that of wild-type animals [22]. The deregulation of several Type I Interferon-dependent genes in these animals prompted us to extend our analysis and to investigate the impact of lower miRNA maturation in Dicer mutant animals to the initiation and development of a prototypic Type I IFN-dependent autoimmune condition, systemic lupus erythematosus (SLE). In this review, we will discuss the results that we obtained on the role of miRNAs in the regulation of inflammatory responses under infectious (viral) or non-infectious (autoimmune) conditions, with special emphasis on Type I IFN-stimulated genes as miRNAs targets.

miRNAs in type I IFN-dependent innate antiviral defense

The prominent role of RNA interference (RNAi) - based antiviral immunity is now well established in plants, where it was first recognized [23] and in

invertebrates [24, 25]. However, in mammals, a protective function for non-coding RNAs has been debated. Several recent data indicated that miRNA actively participates in the defense against viral infection by directly targeting the genome of the virus [26, 27]. Accordingly, it was later demonstrated that the Hepatitis C virus (HCV) was able to highjack a host-derived miRNA, miR-122, for its own benefit [28, 29]. In addition, the demonstration that some viruses, such as herpesviruses and polyomaviruses, have acquired the capacity to express their own genome-encoded miRNAs [30, 31], strongly supports the idea that microRNAs are likely important modulators of antiviral defense. Indeed, miRNAs are perfect tools for the virus to control the host environment: they are non-immunogenic and their small size requires less coding capacity and enables rapid evolution to adapt to different host-derived mRNA targets [32]. However, and for the past 55 years, Type I (α/β) interferons have played a unique and central role in antiviral defense mechanisms [9]. Indeed, among the numerous interferon-stimulated genes (ISGs) are those encoding factors such as protein kinase R or the Mx proteins which are essential contributors to the antiviral affects mediated by these cytokines [33]. Moreover, a novel class of interferon, type III, with high antiviral potency has recently been identified [34]. Analyses of the cross talk between Type I IFN signaling and miRNAs reconcile these apparently divergent observations. Indeed, the resistance to LPS-mediated endotoxic shock of *β-ifn* knock-out mice demonstrated the central role of Type I IFN in this phenomenon [35], which illustrates the absolute requirement for a precise regulation of its expression. Many reports now indicate that microRNAs are important modulators of type I IFN production and as such, participate to a harmonious and well-coordinated antiviral response. A direct interaction between miR-26a and miR-34a with the ifn-β mRNA has been reported [36] which suggest that an initial modest 2- to 4-fold reduction of IFN-β production could result in profound alteration of type I IFN which is released in large amounts after amplification loops. Apart from this mechanism, many miRNAs were shown to interact with mRNAs encoding signaling proteins downstream of diverse Pattern Recognition Receptors, thereby regulating type I IFN expression indirectly. Interestingly, miR-155 can target IRAK-M, a negative regulator downstream of TLR7 [37] and SOCS1, a repressor of type I IFN signaling [38]. This combined action of miR-155 at different levels of type I IFN expression and in different cells likely contributes to its important pro-inflammatory effects. miR-146 exhibits opposite functions by targeting TRAF6, IRAK1 and IRAK2 [39]. This observation indicates that miR-146 negatively regulates type I IFN production triggered downstream of the viral infection sensor RIG-I. Furthermore, miR-146 induction upon Vesicular Stomatitis Virus (VSV) infection indicates that this miRNA participates in a feedback loop, thus avoiding excessive inflammation. Alternatively, miR-146 up-regulation could also reflect an evasion mechanism developed by the virus, since less type IFN favors viral replication. These examples illustrate that microRNAs control the expression of molecules involved in Toll-like Receptors (or other PRRs) signaling, thereby providing a mechanistic explanation for their potent regulatory functions of inflammatory responses [40]. The development and activation of antiviral effector cells is also subjected to miRNA-dependent regulation. Again, miRNAs exerting positive or negative effects on NK cells have been identified. Mice in which miR-150 is depleted exhibited impaired NK cells maturation and conversely, a gain of function approach with a miR-150 transgene promotes their development [41]. This contrasts with the role of miR-30e and miR-378 which target Granzyme B and perforin mRNAs, thus suppressing NK cytotoxic activity [42].

To globally apprehend the role of miRNA in type I IFN-dependent responses, we performed expression analysis of both miRNAs associated with inflammatory responses and Type I IFN-dependent genes in homozygous mutant mice (named Dicer $^{d/d}$) carrying a viable hypomorphic Dicer allele [22]. This unique mutant mouse results from the fortuitous integration of a β-gal-neomycin transgene designed for insertional mutagenesis purposes [27]. During embryogenesis, a low expression level of *Dicer* above a certain threshold in most animals permits the survival of adult mice. However, they are not obtained in the classical Mendelian ratios, thus indicating that the

mutation blocks the embryonic development of those which are characterized by insufficient *Dicer* expression. Using qRT-PCR macroarrays, we first observed, in RNA extracted from whole spleen or macrophages from peritoneal exudates, that almost 50% of the miRNAs involved in inflammation exhibited significant reduction. In parallel, we used a similar approach to simultaneously quantify the expression of 88 Interferon-stimulated genes (ISGs). Interestingly, we identified a subset of ISGs whose expression was significantly augmented and noted the absence of down regulated genes in cells isolated from *Dicer* mutants animals. Performing cross analysis of these data led us to demonstrate that CXCL10-encoding mRNA is likely targeted by miR-21 *in vivo* and that miRNA-dependent STAT1 regulation likely account for several ISGs higher expression in Dicer $^{d/d}$ macrophages and splenocytes. Recently, low but constitutive levels of IFN- β in healthy animals were reported to be mandatory for the maintenance of the hematopoietic stem cell niche or bone remodeling [43]. Our observations suggest that miRNAs are important factors involved in the regulation of this tonic signaling mediated by type I IFN whose importance in the maintenance of homeostasis remains poorly documented.

We next addressed the question relative to the role of miRNAs in Type I IFN signaling induced upon viral infection. For this, we analyzed the expression of inflammatory miRNAs and ISGs in naïve mice and upon mouse cytomegalovirus (MCMV) acute infection. We chose this model because it represents a unique case of a physiologically relevant example of host-pathogen interaction in the mouse. Indeed, mice are naturally infected by this virus and the animal pathology closely mimics that which is observed in patients infected by the human ortholog (HCMV). In wild-type animals, we identified three groups of ISGs: (1) those which exhibit induced expression upon viral infection, (2) ISGs expressed at the same level in naïve and infected animals and (3) those with reduced expression in MCMV-infected mice. The same clustering was performed for Dicer $^{d/d}$ mutants. Interestingly, we observed an overall reduced expression of ISGs in MCMV-infected mutants compared to wild-type mice. More specifically, the MCMV-dependent transcriptional induction of *Irf7*, a transcription factor central to MCMV innate defense, is strongly reduced in splenocytes and macrophages deficient in DICER protein. This defect is associated to lower IFN-β production, diminished NK cell activation and, ultimately, increased susceptibility to the viral infection, as evidenced by lower survival of mutant mice following *in vivo* MCMV inoculation. Altogether, our data enabled us to suggest a working model whereby miRNAs maintain low type I IFN production by targeting activators in healthy conditions. On the contrary, miRNA-dependent targeting of repressors permits rapid IFN synthesis when needed, i.e upon acute viral infection [22].

To decipher in more detail the molecular mechanisms underlying this lower resistance to viral infection of Dicer mutants, we tested the integrity of TLR signaling. As noted above, the regulation of the inflammatory properties of immune cells occurs in many instances upon miRNA interaction with mRNAs encoding components of TLR pathways [40]. Furthermore, efficient innate defense to MCMV requires intact TLR signaling [44, 45]. As illustrated in Figure 1, significant differences in the induction level of irf7 mRNA between wild-type and Dicer-deficient macrophages were not observed when the cells were stimulated with pure TLR agonists (LPS, a TLR4 ligand; poly I:C, a TLR3 ligand and unmethylated CpG DNA, a TLR9 ligand). However, MCMV activates multiple sensors *in vivo* [46] which likely act in a coordinated fashion for maximal antiviral efficacy. It is therefore conceivable that the phenotype characteristic of the Dicer mutant mice upon acute MCMV infection results from an accumulation of subtle alterations in isolated pathways as a result of low miRNA maturation, which, ultimately, leads to increased death rate.

miRNAs, type I IFN and SLE

With a prevalence of 40 cases/100,000 persons (depending on ethnicity) in the U.S, Systemic Lupus erythematosus (SLE) is considered as a prototype for immune complexes-driven systemic autoimmune diseases. The etiology of the disease is complex and implicates environmental as well as genetic factors [47]. In addition, it is speculated that microbial triggers also participate in the

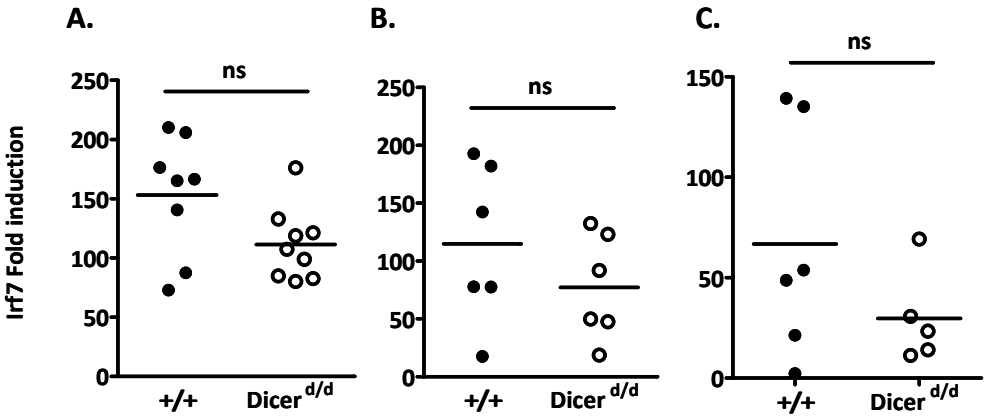

Figure 1. Dicer deficiency minimally impacts on ligand-specific TLR-dependent signaling. Peritoneal macrophages isolated from controls (black dots; +/+) or Dicer$^{d/d}$ (white dots) mice were left untreated or stimulated with various TLR ligands: **A.** LPS (800 pg/mL). **B.** Poly I:C (150 μg/mL). **C.** CpG DNA (15 μg/ml). Cell stimulation was monitored by the quantification of *irf7* expression by qRT-PCR normalized to that of *actin*. Data are expressed as fold induction representing the differential expression between untreated and stimulated cells ($2^{-\Delta\Delta Ct}$). Non significant differences (ns) were observed upon unpaired T-test analysis.

initiation/development of SLE in genetically predisposed individuals. The central role of type I IFN was ascertained by several arguments [48]: first, the observation of the level of these cytokines is elevated in the serum of patients suffering from lupus [49]. It was also shown that type I IFN receptor deficiency protects from the pathology observed in NZB/NZW mice [50]. Finally, a gene expression profiling study has described an IFN-α signature in peripheral blood of SLE patients [51]. Later on, polymorphisms in genes controlling Type I IFN production or genes acting in downstream pathways have been associated with the disease [52]. Recent genome wide association studies strengthened the role of IRF5 in disease susceptibility [53]. In line with these observations and given the role of miRNAs as modulators of interferon-dependent genes expression described earlier, perturbation in miRNA expression was linked to SLE [54, 55]. With regard to the specific miRNAs which were mentioned above and are the focus of intense research, expression studies uncovered that both miR-155 and miR-146a are up regulated in patients with SLE [56]. Urinary expression level of miR-155 correlated with proteinuria and disease severity index and miR-146a exhibited reverse correlation with TNF-α expression. This challenging observation (what is the significance of the over expression of two miRNAs with apparent antagonist activity?) nevertheless suggests that miRNAs could serve as potential non-invasive biomarkers whose quantification in the blood or urine may predict SLE evolution and/or treatment response [57]. Investigations performed in mice revealed a similar complexity. Profiling miRNAs expressed in splenocytes isolated from lupus-prone animals (MRL-lpr, B6-lpr or NZB/W) identified a common set of over expressed miRNAs which, again, includes miR-155. Individual qRT-PCR experiments demonstrated that miR-146a was specifically up regulated in splenic T cells of MRL-lpr mice [58]. Paradoxically, reduced Dicer expression was noted concomitantly to miR-155 over expression in regulatory T cells from MRL-lpr mice, suggesting that alternative pathways may enable the production of mature miRNAs under inflammatory conditions [59]. This hypothesis is in agreement with our own observations [22] that the maturation of a large proportion of miRNAs remains unaffected in Dicer $^{d/d}$ splenocytes and macrophages which are characterized by the weak, but constitutive, expression of inflammatory markers. Altogether, these data strongly suggest that miRNAs actively participate in the pathogenesis of SLE, but the precise mechanism of their action remains obscure.

This is in sharp contrast with Rheumatoid Arthritis (RA), another autoimmune disease of inflammatory origin for which a clear role for miR-155 has been attributed based on *in vivo* data in mice and humans. Over expression of miR-155 was detected in synovial macrophages from RA patients and concomitant diminished expression of Src homology 2-containing inositol phosphatase-1 (SHIP-1), a likely miR-155 target was noted [60]. Accordingly, genetically modified mice in which miR-155 had been deleted were totally resistant to Collagen-Induced arthritis [61].

To provide a model aiming at an *in vivo* analysis of the role of miRNAs in the pathogenesis of SLE, we favored a genetic approach. Dicer $^{d/d}$ mice do not spontaneously develop the classical markers of the disease. For instance, even in aged (1 year-old) mutant animals, auto antibodies were absent from the serum, the proteinuria remained similar to that of wild type mice and activated B cells were undetectable in spleens. However, we considered that the constitutive expression level of some ISGs observed in the mutant could result in the establishment of a pro inflammatory milieu which, in conjunction with additional risk factors, might favor SLE development. To test this hypothesis, we crossed Dicer $^{d/d}$ mice with animals carrying the lpr mutation in the *cd95* (*fas*) gene in the same background (C57BL/6). Double homozygous mutants were generated and compared with wild-types and homozygous mutants for single mutations upon assessment of several parameters. As expected, we observed splenomegaly and lymph nodes enlargement in mice carrying the lpr mutation (compared with wild type or single Dicer $^{d/d}$ mice), regardless of the *Dicer* gene status, wild-type or mutant. Similarly, FACS analysis of the cell population present in spleens harvested from cd95 $^{lpr/lpr}$; Dicer $^{+/+}$ and cd95 $^{lpr/lpr}$; Dicer $^{d/d}$ animals showed no significant difference at the level of double negative ($CD8^-CD4^-$) $B220^+$ cells or for memory B cells ($CD19^+$ IgM^{low}). Finally, the quantification of auto antibodies in the serum of the mice did not reveal any marked difference between these two genotypes. Next, we analyzed by qRT-PCR the expression of several ISGs. We first checked *irf7* expression in splenocytes (Figure 2A) and confirmed that this gene is over

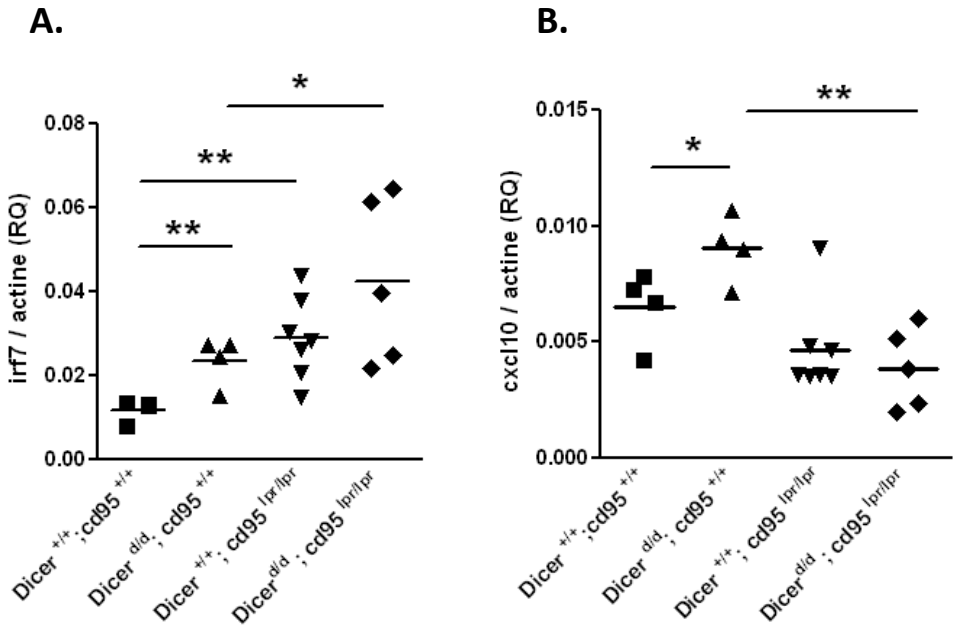

Figure 2. Genetic interactions between *Dicer* and *fas* genes on the development of lupus. **A.** *Irf7* and **B**. *cxcl10* expression were quantified by qRT-PCR in spleen extracts from controls and single and double mutants for the *Dicer* and *cd95* genes. Expression was normalized to that of *actin* and is expressed in Relative Quantity (RQ). Significant differences (*P<0.05; **P<0.001) were observed upon unpaired T-test analysis.

expressed in cells carrying the Dicer mutation. Interestingly, we also noted *irf7* up regulation in cd95 $^{lpr/lpr}$ cells (in the presence or not of the Dicer $^{d/d}$ mutation), which suggests that the lpr mutation also results in a type I IFN-dependent pro inflammatory milieu. The combination of both mutations does not appear to exhibit significant additive effects. A similar effect of the Dicer $^{d/d}$ mutation on *cxcl10* expression was observed (Figure 2B), but, surprisingly, *cxcl10* up regulation was not observed in Dicer $^{+/+}$; cd95 $^{lpr/lpr}$ splenocytes. More importantly, the presence of the lpr allele abolishes the effect of the Dicer hypomorphic mutation on *cxcl10* expression. We hypothesized that this different effect of the lpr mutation on *irf7* and *cxcl10* likely reflect cell-specific gene expression. Indeed, *irf7* is strongly up regulated in plasmacytoid dendritic cells (pDCs) triggered with type I IFN, whereas the chemokine CXCL10 is essentially secreted by cells of the monocytic lineage. Our observations therefore indicate that, while the Dicer mutation likely affects the properties of both cell types, the lpr allele of the *fas* gene could modify the behavior and/or proportion of pDCs in the spleen. Additional experiments using FACS analysis of the different dendritic cell population in the spleen of C57BL/6 $^{lpr/lpr}$ animals are required to explore this interesting phenotype in more details. ISG expression studies in isolated and sorted dendritic cell population would also be informative.

CONCLUSIONS

MicroRNAs have been the focus of intense attention in the past years and a large amount of data report their involvement in many physiopathological processes. Because the knock-out of *Dicer* is lethal, most animal models designed to investigate the role of miRNAs are mice in which a targeted conditional ablation of the floxed *Dicer* gene has been performed with the help of a promoter-specific CRE transgene. Our Dicer $^{d/d}$ mutant mice, which are characterized by a lower DICER expression and reduced miRNA maturation, represent an attractive alternative to the classical CRE/Lox approach. A major drawback commonly opposed to the use of hypomorphs is that such mutants exhibit weak phenotypes which are therefore difficult to assess and require large number of animals for quantification. This is the exact opposite to KO animals which usually enable a clear black/white discrimination when compared to controls. We rather consider, particularly in the case of *Dicer*, for which no human with a complete deficiency of this gene exists, that the Dicer $^{d/d}$ mice closely mimic physiological situations. Despite a residual DICER activity which is difficult to estimate, we observe that low miRNAs production results in pro-inflammatory, IFN-enriched conditions. Furthermore, under acute viral infection, Dicer mutant animals exhibit a significant alteration of multiple Interferon-stimulated genes expression which ultimately leads to increased susceptibility and death rate.

The impact of the Dicer hypomorphic mutation on the development on lupus triggered by the cd95 ^{lpr} allele is less clear. The double mutants showed no obvious modification of the disease severity (as judged by proteinuria which reflects kidney functions) as compared to cd95 $^{lpr/lpr}$ mice. Nor did we detect any changes at the macroscopic (organ size) or cellular (cell proportions) levels between mice carrying the cd95 ^{lpr} allele, with or without the Dicer $^{d/d}$ mutation. However, subtle differences in the pattern of expression of ISGs suggest that cell-specific miRNA- driven alterations might participate in the disease. An additional point making this genetic model difficult to assess is the weak lupus-like phenotype conferred by the cd95 ^{lpr} allele in the C57BL/6 background. In these conditions, visualizing improvement or worsening of the disease as a consequence of the Dicer $^{d/d}$ mutation was quite challenging.

Finally, as mentioned above, SLE is a multifactorial disease, the initiation of which requires extrinsic stimuli such as viral infections. More specifically, Epstein-Barr virus (EBV) infections have long been suspected to be an important initiator of the disease [62]. Importantly, EBV, like other herpesviruses, encodes its own set of miRNAs [63], whose functions, for the most part, remain obscure. Therefore, a very tantalizing avenue of research would be to consider that herpesvirus-derived miRNAs expressed during latency might alter the long-term inflammatory response of the

host and, in conjunction with additional host and environmental factors, create the appropriate conditions for the emergence of lupus or other auto inflammatory diseases.

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