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Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses

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Summary

Foxp3$^+$ regulatory T (Treg) cells maintain immune homeostasis by limiting different types of inflammatory responses. Here, we report that miR-146a, one of the miRNAs prevalently expressed in Treg cells, is critical for their suppressor function. The deficiency of miR-146a in Treg cells resulted in a breakdown of immunological tolerance manifested in a fatal IFNγ-dependent immune-mediated lesions in a variety of organs. This was likely due to augmented expression and activation of signal transducer and activator transcription 1 (Stat1), a direct target of miR146a. Likewise, heightened Stat1 activation in Treg cells subjected to a selective ablation of SOCS1, a key negative regulator of Stat1 phosphorylation downstream of IFNγ receptor, was associated with analogous Th1-mediated pathology. Our results suggest that specific aspects of Treg suppressor function are controlled by a single miRNA and that an optimal range of Stat1 activation is important for Treg-mediated control of Th1 responses and associated autoimmunity.

Introduction

A variety of pathologies are caused by the immune responses to “self” and environmental non-microbial antigens, to microbial antigens derived from commensal microorganisms, and to infectious agents. Regulatory T cells (Treg) limit “collateral damage” resulting from protective immunity to infection, suppress sterile inflammation, and immune responses to “self” and allergens (Belkaid and Tarbell, 2009; Lu and Rudensky, 2009; Sakaguchi, 2005). Suppressive function of Treg cells is indispensable for the immune homeostasis and survival

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of higher organisms - Treg cell ablation in healthy adult mice leads to a fatal, aggressive lympho- and myeloproliferative autoimmune syndrome (Kim et al., 2007). X chromosome-encoded transcription factor Foxp3 plays a pivotal role in differentiation, homeostasis, and function of Treg cells. Foxp3 loss-of-function mutations cause systemic immune-mediated lesions similar to those observed upon chronic Treg cell ablation (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Analyses of T cells expressing GFP-tagged null and functional *Foxp3* alleles and genome-wide analysis of Foxp3 binding genes showed that Foxp3 directly or indirectly controls several thousand genes in Treg cells (Fontenot et al., 2005; Gavin et al., 2007; Lin et al., 2007; Zheng et al., 2007; Lu et al., 2009; Marson et al., 2007). These studies also revealed that a number of genes constitutively up- or down-regulated in Treg cells in a Foxp3-dependent manner undergo corresponding changes, albeit transient and less prominent, in activated effector T cells. This sizable cluster of "Foxp3-amplified" genes includes those serving as hallmarks of Treg cells (CTLA4, CD25, and GITR) and playing important functional roles in their homeostasis and function (Gavin et al., 2007; Lin et al., 2007; Zheng et al., 2007; Marson et al., 2007). These results suggested that Foxp3 acts in an "opportunistic" manner by amplifying and stabilizing expression of genes beneficial to Treg cell function.

In addition to coding genes, the "Foxp3-amplified" cluster also includes several small non-coding RNA known as microRNAs (miRNAs) (Cobb et al., 2006). Notably, miRNAs serve as important regulators of Treg cell homeostasis and function in both basal and inflammatory settings (Chong et al., 2008; Liston et al., 2008; Zhou et al., 2008). Ablation of miRNA precursor-processing enzyme Dicer in Treg cells results in a reduction in their numbers and suppressive capacity in healthy mice harboring both Dicer-deficient and -sufficient Treg cells and a complete loss of suppressor function in diseased mice lacking wild-type Treg cells (Liston et al., 2008). Consequently, the generalized depletion of miRNAs in Treg cells leads to a fatal early-onset autoimmune pathology indistinguishable from that in Foxp3 mutant mice devoid of Treg cells (Chong et al., 2008; Liston et al., 2008; Zhou et al., 2008). These results and of the description of a subset of miRNAs differentially expressed in regulatory vs. effector T cells (Cobb et al., 2006) raised a question as to how individual miRNAs present in increased amounts in Treg cells contribute to distinct aspects of their homeostasis and function. In this regard, miR-155, a well known onco-miR (cancer-associated micro-RNA), is constitutively expressed in high amounts in Treg cells in a Foxp3-dependent manner, whereas T cells lacking Foxp3, B cells, and myeloid cells transiently up-regulate miR-155 upon activation (Androulidaki et al., 2009; O'Connell et al., 2007; Thai et al., 2007). Our previous studies showed that miR-155 confers heightened responsiveness of Treg cells to their key survival and growth factor, IL-2, thereby maintaining their numbers in a competitive environment. However, miR-155 is largely dispensable for Treg suppressor function (Lu et al., 2009).

Like miR-155, miR-146a is highly expressed in Treg cells and is induced upon activation of effector T cells and myeloid cells. In the latter, miR-146a acts as a negative feedback regulator to limit TRAF6 and IRAK1/2 mediated signaling in inflammatory settings (Hou et al., 2009; Taganov et al., 2006), whereas in activated human T cells, miR-146a has been suggested to oppose apoptosis and IL-2 production (Curtale et al., 2010). Thus, we explored a role for miR-146a in Treg cells and found that this microRNA is essential for the ability of Treg cells to restrain IFNγ-mediated pathogenic Th1 responses and associated inflammation. In Treg cells, miR-146a-mediated down-regulation of Stat1, a key transcription factor required for Th1 effector cell differentiation, was necessary for Treg ability to suppress Th1 responses. Furthermore, relieving negative regulation of Stat1 activation in Treg cells using an alternative genetic approach resulted in a breakdown of immune homeostasis similar to that observed in mice harboring miR-146a-deficient Treg cells. Thus, our results suggest that a specific miRNA over-represented in Treg cells ensures their suppressor function by

maintaining an optimal threshold of cytokine receptor-dependent activation of transcription factors crucial for a particular type of immune response.

Results

Elevated miR-146a expression in Treg cells

To identify miRNAs essential for controlling Treg cell function, we examined miRNAs differentially expressed between Foxp3$^+$ Treg cells and Foxp3$^-$ non-Treg CD4$^+$ T cells (Cobb et al., 2006)(data not shown). Among miRNA overrepresented in Treg cells, miR-146 was particularly prominent. This miRNA has been implicated in negative regulation of immune cell function, specifically in negative feedback regulation of myeloid cell activation (Taganov et al., 2006). To study the role of miR-146 in Treg cells, we first sought to confirm the high amounts of miR-146 in Treg cells. Since mature miR-146a and miR-146b sequences differ by two nucleotides, it was difficult to distinguish whether miR-146a or miR-146b or both are present in increased amounts in Treg cells. To address this issue, we performed stem-loop based Taqman real-time RT-PCR assay, which allowed us to distinguish miRNAs within the same family (Chen et al., 2005). As shown in Fig. S1A, the miR-146a expression in CD4$^+$CD25$^+$Treg cells was sharply augmented in comparison to that in CD4$^+$CD25$^-$CD62L^{hi} naïve T cells, while miR-146b expression was much lower. Analysis of Treg and non-Treg cell subsets isolated from miR-146a-deficient mice suggested that the small increase in miR-146b signal in Treg cells was likely due to miR-146a expression since miR-146b expression could no longer be observed in miR-146a-deficient Treg cells (Fig. S1A). In support of this notion, examination of the easily discriminated primary miRNA transcripts demonstrated that pri-miR-146a but not pri-miR-146b was elevated in Treg cells compared to naïve T cells(Fig. S1B). Thus, miR-146a, but not miR-146b, was highly up-regulated in Treg cells.

miR-146a deficiency resulted in increased numbers, but impaired function of Treg cells

To assess a role for miR-146a in Treg cells, we examined Foxp3$^+$ Treg cell subsets in the thymus and in the peripheral lymphoid organs of miR-146a-deficient mice. These mice develop severe lympho- and myeloproliferative syndrome at 6 months of age (M.B., K.T. and D.B., submitted). Therefore, we analyzed miR-146a-deficient and littermate control mice at 6-8 weeks of age prior to development of any clinical signs of autoimmune or inflammatory disease and found comparable sizes of different thymocyte and peripheral lymphoid and myeloid cell subsets (Fig. S1C-F and data not shown). Moreover, T cell activation status was also similar to that observed in the wild-type (WT) littermates (Fig. S1I and data not shown). In contrast to a reduction in Treg cell numbers observed in mice lacking miR-155 (Lu et al., 2009), miR-146a-deficient mice contained significantly increased numbers of Foxp3$^+$ Treg cells in the periphery, but not in the thymus (Fig. S1G and H). Consistent with its increased size, the miR-146a-deficient Treg cell subset exhibited heightened proliferative activity reflected in augmented expression of Ki67 and a modest increase in several activation markers (Fig. S1J and data not shown).

Since miR-146a is an important negative regulator of myeloid cell responses to cytokines and TLR ligands, it was possible that increased miR146a-deficient Treg cell numbers were due to a heightened activation status of dendritic cell (DC), but not a cell-intrinsic effect of miR-146a deficiency. It was also possible that altered immune effector function in *Mirn146a*$^{-/-}$ mice masked potential defects in miR-146a-deficient Treg cell-mediated suppression. To explore these possibilities we performed a series of bone marrow (BM) transfer studies (Fig. 1A). First, we transferred BM cells from miR-146a-deficient or -sufficient littermates mixed with BM cells from Ly5.1$^+$B6 mice at a 1:1 ratio into Rag2- or TCRβδ-deficient recipients. Analysis of the resulting chimeric animals (*Mirn146a*$^{-/-}$/B6)

allowed us to discriminate between cell-intrinsic and cell-extrinsic effects of miR-146a deficiency on increased Treg cell numbers. Second, by performing co-transfers of Ly5.1$^+$Foxp3KO BM cells in place of Ly5.1$^+$B6 BM, we generated mice (*Mirn146a*$^{-/-}$/Foxp3KO) in which all Treg cells lack miR-146a because Foxp3KO precursors fail to generate Treg cells, but all other BM derived cells originate from both miR-146a-deficient and -sufficient BM. These mice allowed us to examine the function of miR-146a-deficient Treg cells in a miR-146a-sufficient environment. Like chimeric mice reconstituted with Foxp3KO BM alone, *Mirn-146a*$^{-/-}$/Foxp3KO chimeras developed severe immune mediated pathology including conjunctivitis, blepharitis, and dermatitis as early as 5 weeks after BM transfer and became moribund soon thereafter (Fig. 1B). Histological examination of *Mirn146a*$^{-/-}$/Foxp3KO chimeras 6-7 weeks after transfer showed massive lymphocyte activation and tissue infiltration in the lung, liver, and skin similar to that observed in mice harboring Dicer-deficient Treg cells (Chong et al., 2008;Liston et al., 2008;Zhou et al., 2008). In contrast, there were no clinical or histological signs of immune mediated lesions in control *Mirn146a*$^{-/-}$/B6 chimeras where both miR146a-deficient and -sufficient Treg cells were present (Fig. S2).

The disease in *Mirn146a*$^{-/-}$/Foxp3KO was not due to reduced numbers of Treg cells. *Mirn146a*$^{-/-}$/Foxp3KO chimeras which developed autoimmune pathology, had increased numbers of Treg cells compared to control *Mirn146a*$^{+/+}$/Foxp3KO animals (Fig. 1C-E). Importantly, the difference in miR-146-deficient vs. -sufficient Treg cell numbers was also observed in *Mirn146a*$^{-/-}$/B6 and *Mirn146a*$^{+/+}$/B6 mice chimeric mice where miR-146a-deficient Treg cells compete with their wild-type counterparts (Fig. 1F and G). The observed increase in the proportion of the miR-146a-deficient Treg cell subset in both *Mirn146a*$^{-/-}$/Foxp3KO and *Mirn146a*$^{-/-}$/Ly5.1$^+$B6 mixed chimeras suggested that miR-146a deficiency increases Treg cell numbers in a cell-autonomous manner.

Despite their increased numbers, miR146a-deficient Treg cells were unable to restrain activation of miR-146a-sufficient effector T cells, which was exhibited by high proliferative activity, an increased CD62L^{low} cell subset, and high levels of the activation markers ICOS and CTLA4 (Fig. 1H). In agreement with the histolopathology data and lack of clinical signs of disease, flow cytometric analysis of control *Mirn-146a*$^{-/-}$/Ly5.1$^+$B6 chimeras showed that both miR-146a-sufficient and -deficient effector T cells were kept in check by the miR-146a-sufficient Treg cells present in these mice (Fig. 1I). These results suggested an indispensable role of miR-146a in Treg cell-mediated immunological tolerance.

miR-146a deficiency in Treg cells resulted in dysregulated IFNγ responses

To identify the underlying mechanisms responsible for early fatal immune-mediated disease observed in *Mirn146a*$^{-/-}$/Foxp3KO chimeras, we first sought to examine whether miR-146a deficiency results in a general impairment of Treg suppression function in inflammatory settings similar to Dicer deficiency in Treg cells (Liston et al., 2008). To examine this possibility, miR-146a-deficient Treg cells isolated from healthy *Mirn146a*$^{-/-}$/B6 or diseased *Mirn146a*$^{-/-}$/Foxp3KO mice were co-cultured with miR-146a-sufficient responder CD4$^+$ T cells. We found that miR-146a-deficient Treg cells isolated from healthy *Mirn146a*$^{-/-}$/B6 chimeras were as suppressive as their miR146-sufficient counterparts from control animals, whereas miR146-deficient Treg cells from diseased *Mirn146a*$^{-/-}$/Foxp3KO mice were more suppressive *in vitro*, likely due to their activation in an inflammatory environment (Fig. 2A and B). These results suggested that, unlike Dicer deficiency, lack of miR-146a did not diminish the overall suppressor capacity of Treg cells reflected by an *in vitro* suppression assay. Instead, a particular aspect of Treg suppressor function was likely impaired in the absence of miR-146a.

As mentioned earlier, miR-146a functions as a negative regulator of TLR/IL-1 signaling pathway by targeting TRAF6 and IRAK1/2 (Taganov et al., 2006; Hou et al., 2009). A recent study demonstrated that IL-1 stimulation results in IL-17 production in $CD4^+$ T cells including $Foxp3^+$ Treg cells (Chung et al., 2009). Moreover, a critical role of IRAK1 in promoting Th17 differentiation at the expense of TGFβ-dependent generation of peripheral $Foxp3^+$ Treg cells suggested a possibility that the lack of miR-146a might lead to heightened Th17 responses, which cause the fatal autoimmunity in *Mirn146a*$^{-/-}$/Foxp3KO chimeras (Maitra et al., 2009). Indeed, in agreement with previous studies we found increased levels of TRAF6 and IRAK1 in Treg and non-Treg T cells (Fig., S1K). However, we did not observe any change in either Foxp3 induction or Th17 differentiation *in vitro* in the presence or absence of miR-146a (Fig. S1L). More importantly, Th17 cell numbers were similar in *Mirn146a*$^{-/-}$/Foxp3KO and control *Mirn146a*$^{+/+}$/Foxp3KO and *Mirn146a*$^{-/-}$/B6 mice (data not shown). Further analysis of cytokine production showed that the loss of miR-146a in Treg cells resulted in increased production of the pro-inflammatory Th1 cytokine IFNγ by both miR146a-deficient and -sufficient CD4 and CD8 T cells whereas production of IL-4, IL-5, and IL-17 was unaffected (Fig. 2C; data not shown). This increase was not observed in the presence of miR146a-sufficient Treg cells in *Mirn146a*$^{-/-}$/B6 mice (Fig. 2F and data not shown). The selective dysregulation of Th1 responses in the presence of miR-146a-deficient Treg cells was in contrast to sharply increased production of multiple cytokines (IL-2, IL-4, IL-5, IL-17, and IFNγ) in the presence of Dicer-deficient Treg cells (Liston et al., 2008). Consistent with these findings, purified miR-146a-deficient Treg cells adoptively transferred together with Foxp3KO $CD4^+$ effector T cells into lymphopenic recipients failed to restrain Th1 responses (Fig. S3).

In addition to a marked increase in IFNγ production in miR-146a-sufficient and -deficient effector T cells, a sizable proportion of miR-146a-deficient $Foxp3^+$ Treg cells produced IFNγ in *Mirn146a*$^{-/-}$/Foxp3KO mice (Fig. 2C-E). Production of IFNγ and other effector cytokines by Treg cells is kept in check by Foxp3 likely as a “safety” feature, since Treg cells express TCR with an increased reactivity against “self” antigens (Hsieh et al., 2006). Loss of Foxp3 in Treg cells resulted in the acquisition of the ability to produce pro-inflammatory cytokines, and cause tissue lesions in the absence of functional Treg cells (Williams and Rudensky, 2007; Zhou et al., 2009). Thus, our current observations suggested that in addition to Foxp3, miR-146a prevents acquisition of Th1-like properties by Treg cells, i.e. restrains production of the pro-inflammatory cytokine IFNγ by Treg cells.

IFNγ blockade prevents the autoimmune disease in mice harboring miR-146a-deficient Treg cells

Next, we examined whether the augmented IFNγ response was responsible for the autoimmune disease observed in *Mirn146a*$^{-/-}$/Foxp3KO chimeric mice. To address this question, we neutralized the IFNγ activity by treating mice with IFNγ blocking antibody starting 3 wks after BM transfer (Fig. 3A). IFNγ blockade did not affect the increases in miR-146a-deficient Treg cell numbers (Fig. 3B), however, it rescued the disease observed in their presence. At the time of analysis, treated *Mirn146a*$^{-/-}$/Foxp3KO mice were disease-free and phenotypically indistinguishable from control *Mirn146a*$^{+/+}$/Foxp3KO mice (Fig. 3C; data not shown). In contrast, control IgG treated *Mirn146a*$^{-/-}$/Foxp3KO mice developed severe clinical signs of immune-mediated disease and tissue inflammation (Fig. 3C and data not shown). The effectiveness of IFNγ blockade was also manifested in normalization of the number of T cells expressing CXCR3, a Th1-specific chemokine receptor (Nakajima et al., 2002;Barbi et al., 2007), in IFNγ antibody-treated mice, whereas in IgG-treated animals, CXCR3 expression on effector and Treg cells remained high (Fig. 3C and data not shown). Importantly, antibody-mediated neutralization of IFNγ and rescue of Th1-mediated disease in *Mirn146a*$^{-/-}$/Foxp3KO mice were not associated with

measurable increases in IL-4 or IL-17 (Fig. 3D). These results suggested that miR-146a-deficient Treg cells were able to restrain Th2 and Th17, but not Th1 responses and that immune-mediated lesions observed in the presence of miR-146a-deficient Treg cells were IFNγ dependent. Since an increase in numbers of miR-146a-deficient Treg cells was unaffected by IFNγ neutralization, these experiments also imply that Treg cell homeostasis and suppressor function are likely controlled by miR-146a via distinct molecular mechanisms.

miR-146a regulates Stat1 in Treg cells

Next, we sought to explore molecular mechanisms underlying miR-146a-dependent Treg cell-mediated Th1 regulation. In addition to the aforementioned miR-146a targets, TRAF6 and IRAK1, a recent study has suggested that Stat1 is a miR-146a target in human PBMCs (Tang et al., 2009). Since Stat1 is a key transcription factor downstream of IFNγ receptor signaling, it was possible that Stat1 expression in miR-146a-deficient Treg cells was increased and could account for the IFNγ-/Th1-dependent immune mediated lesions observed in *Mirn146a*$^{-/-}$/Foxp3KO chimeras. However, unlike the 3′ UTR of the human *Stat1* gene, which contains a perfect match with the miR-146a seed sequence, no canonical target sequence was found in the mouse *Stat1* gene. Nevertheless, a highly conserved sequence with partial complementary to miR-146a was present in the mouse *Stat1* 3′UTR (Fig. 4A). Although computational algorithms relying on miRNA seed sequence analysis failed to identify mouse Stat1 as a miR-146a putative target, it was recently shown that some microRNAs, like miR-24, control multiple genes in the absence of canonical target seed sequences (Lal et al., 2009). Thus, it was possible that miR-146a might recognize mouse Stat1 through “seedless” yet conserved complementary sequences. Indeed, transfection of mouse Stat1 3′ UTR-containing luciferase reporter into miR-146a-sufficient, but not miR-146a-deficient T cells showed repression of reporter activity, which was abolished upon mutagenesis of the putative miR-146a binding site (Fig. 4B). Moreover, miR-146a overexpression in HEK293 cells reduced luciferase reporter activity (data not shown). Finally, we found ~3-4-fold increase in total Stat1 protein in both Treg and non-Treg cells lacking miR-146a. Together, these results suggested mouse Stat1 serves as a target of miR-146a in mouse T cells (Fig. 4C). Levels of phosphorylated Stat1 were also markedly increased in miR-146a-deficient cells. Consistent with the rescue from disease, Stat1 phosphorylation returned to basal level in chimeric mice treated with IFNγ antibody, whereas the total Stat1 amounts were only partially diminished (Fig. 4C). Elevated levels of TRAF6 and IRAK1 in miR-146a-deficient T cells were largely unaffected by IFNγ antibody treatment and, thus, demonstrate the specificity of the observed effects of IFNγ neutralization.

miR-146a controls Treg cell-mediated regulation of IFNγ response through targeting Stat1

To test the hypothesis that increased Stat1 expression in the absence of miR-146a is responsible for the observed failure of Treg cells to control Th1 response, we conducted a “loss-of-function” experiment by generating miR-146a-deficient mice harboring a single functional *Stat1* allele (*Mirn146a*$^{-/-}$ *Stat1*$^{+/-=}$). We co-transferred *Mirn146a*$^{-/-}$ *Stat1*$^{+/-}$ and Ly5.1^{+}Foxp3KO bone marrow cells mixed at 1:1 ratio into Rag2-d or TCRβδ-deficient recipients (*Mirn146a*$^{-/-}$*Stat1*$^{+/-}$/Foxp3KO). As shown in figure 4D, both Treg cells and non-Treg T cells isolated from *Mirn146a*$^{-/-}$*Stat1*$^{+/-}$/Foxp3KO mice exhibited ~50% reduction in total and phosphorylated Stat1 amounts compared to corresponding *Mirn146a*$^{-/-}$*Stat1*$^{+/+}$/Foxp3KO controls. As a result, *Mirn146a*$^{-/-}$*Stat1*$^{+/-}$/Foxp3KO mice developed a much milder and delayed immune-mediated pathology (Fig. 4E and data not shown). In agreement with a milder disease phenotype, we observed significantly reduced IFNγ secretion by both miR-146a-sufficient and -deficient effector CD4^{+} T cells. Furthermore, reduced Stat1 expression markedly diminished IFNγ production by miR-146a-

deficient Treg cells (Fig. 4F). Interestingly, reduced IFNγ production was accompanied by a substantial increase in IL-4 producing miR-146a-sufficient and –deficient effector $CD4^+$ T cells in *Mirn146a*$^{-/-}$*Stat1*$^{+/-}$/Foxp3KO chimeric mice (Fig. 4G). Together, these results provided genetic evidence that miR-146a ensures Treg cell-mediated control of Th1 responses at least in part through targeting Stat1 and that limiting Stat1 expression in miR-146a-deficient Treg cells moderates these responses.

IFNγ production by miR-146a-deficient T cells contributes to, but does not fully account for pathogenic Th1 responses in *Mirn146a*$^{-/-}$/Foxp3KO mice

The mixed bone marrow transfers using miR-146a-deficient hematopoietic stem cells revealed an important role for miR-146a in Treg cells and suggested that miR-146a deficiency in these cells leads to Th1 mediated disease. However, we cannot formally exclude potential contribution to the disease by other miR-146a-deficient cell subsets in addition to Treg cells. In addition, the generation of miR-146a-deficient "former" Treg cells due to the loss of Foxp3 by some Treg cells upon adoptive transfer into lymphopenic hosts raises a question as to whether miR-146a deficiency in Treg cells could solely account for dysregulation of Th1 responses (Fig. S3A). Since miR-146a deficiency resulted in a similar level of Stat1 up-regulation and activation in both Treg and effector T cells (Fig. 4C and D), it is plausible that IFNγ production resulting from unrestrained Stat1 activation in miR-146a-deficient T effector cells contributed to the disease in chimeric mice harboring miR-146a-deficient Treg cells. Consistent with this possibility, we found that miR-146a-deficient T cells exhibited elevated IFNγ, but not IL-4 production upon activation under non-polarizing Th0 condition (Fig.5A).

To dissociate a role of miR-146a in Treg cell-mediated suppression from its role in restraining IFN-γ production by T effector cells, we generated miR-146a-deficient mice lacking IFNγ (*Mirn146a*$^{-/-}$ *Ifng*$^{-/-}$). We transferred *Mirn146a*$^{-/-}$ *Ifng*$^{-/-}$ and Ly5.1$^+$Foxp3KO BM cells mixed at 1:1 ratio into Rag2- or TCRβδ-deficient recipients (Fig. 5B). These *Mirn146a*$^{-/-}$*Ifng*$^{-/-}$/Foxp3KO mice allowed us to assess a role of miR-146a in Treg cell-mediated suppression of pathogenic Th1 responses without a confounding effect of the IFNγ production by miR-146a-deficient cells. We found that even in the absence of IFNγ supplied by miR-146a-deficient cells, *Mirn146a*$^{-/-}$*Ifng*$^{-/-}$/Foxp3KO developed autoimmune disease accompanied by markedly augmented IFNγ production by miR-146a-sufficient $CD4^+$ and $CD8^+$ T cells (Fig. 5C and D). Consistent with the increased IFNγ production, miR-146a-sufficient effector $CD4^+$ T cells displayed an activated phenotype along with elevated CXCR3 expression (Fig. 5E). These results provide further support for the notion that miR-146a plays an indispensable role in Treg cell-mediated Th1 regulation. Nevertheless, it must be noted that miR-146a-sufficient T cells from *Mirn146a*$^{-/-}$*Ifng*$^{-/-}$/Foxp3KO mice produced less IFNγ and were less activated in comparison to those from *Mirn146a*$^{-/-}$*Ifng*$^{+/+}$/Foxp3KO mice. This observation suggested that IFNγ production by miR-146a-deficient cells contributed to, but does not fully account for the development of severe Th1 pathology in the presence of miR-146a-deficient Treg cells (Fig. 5C and D). Thus, miR-146a limits Th1 responses in two ways: by endowing Treg cells with the ability to suppress Th1 responses and by negatively regulating T effector cells in a cell-autonomous manner. Corresponding to this role in the Th1 response, we found higher levels of miR-146a in both T effector and Treg cells with Th1-like features ($CXCR3^+$T-bet^{hi}) in comparison to their $CXCR3^-$T-bet^{low} counterparts (Fig.5F-I).

Unrestrained Stat1 activation in Treg cells results in IFNγ-mediated Th1 pathology

Considering that each miRNA regulates multiple targets, it was important to examine the consequences of altering Stat1 activation in Treg cells by means other than the manipulation of miR146a expression. Therefore, we performed a "gain-of-function" experiment by

analyzing mice with a Treg-specific ablation of SOCS1, a negative regulator of Stat1. Although SOCS1 also controls other signaling pathways, loss of SOCS1-dependent negative regulation of Stat1 is the major contributor to the IFNγ-mediated immunopathology in mice with SOCS1 deficiency (Kubo et al., 2003; Alexander et al., 1999; Horino et al., 2008; Marine et al., 1999). As shown in Fig. 6A-F, increased Treg cell numbers were observed in *Foxp3*^{Cre}*Socs1*$^{fl/fl}$ mice. This is likely due to augmented IL-2R/Stat5 signaling (Fig. S4)(Lu et al., 2009; Zhan et al., 2009). However, despite this increase in Treg number, *Foxp3*^{Cre}*Socs1*$^{fl/fl}$ mice showed clinical signs of immune-mediated pathology including conjunctivitis, blepharitis, and dermatitis by 6 weeks of age (Fig. 6G). Furthermore, both SOCS1-sufficient effector T cells and SOCS1-deficient Treg cells in *Foxp3*^{cre}*SOCS1*$^{fl/fl}$ mice exhibited an activated phenotype (Fig. 6H, I and data not shown).

Next, we sought to examine effector mechanisms that account for the disease observed in *Foxp3*^{cre}*SOCS1*$^{fl/fl}$ mice. Like miR-146a-deficient Treg cells, SOCS1-deficient Treg cells had unimpaired *in vitro* suppression activity (Fig. 7A). As expected SOCS1 deficiency in Treg cells resulted in unrestrained Stat1 activation in Treg cells (Fig. 7B). And similar to our observations in *Mirn146a*$^{-/-}$/FoxP3KO chimeras, these mice exhibited selective dysregulated IFNγ production by both Foxp3^{+} and Foxp3^{-} CD4^{+} T cell as well as CD8^{+} T cells. (Fig. 7C and D). Although both *Mirn146a*$^{-/-}$/Foxp3KO chimeras and *Foxp3*^{cre}*SOCS1*$^{fl/fl}$ mice exhibited Th1-related pathology, clinical disease manifestations were significantly less severe in the latter mice. A likely explanation for the observed difference was that generation of BM chimeras required irradiation and was accompanied by transient lymphopenia. Indeed, we found that *Foxp3*^{cre}*SOCS1*$^{fl/fl}$ /Foxp3KO chimeras generated upon transfer of Foxp3KO and *Foxp3*^{cre}*SOCS1*$^{fl/fl}$ BM at 1:1 ratio into Rag2- or TCRβδ-deficient recipients (Fig. S5A) exhibited an accelerated kinetics and increased severity of autoimmune disease in a close resemblance to *Mirn146a*$^{-/-}$/Foxp3KO mice (Fig. S5B-D).

Together, our studies suggest that elevated miR-146a expression in Treg cells is required for control of IFNγ-mediated Th1 immune responses at least in part through targeting Stat1, a key molecule in the IFNγ signaling pathway. Our results further imply that excessive effector cytokine signaling in Treg cells may lead to their failure to suppress the corresponding response and consequently brings about severe autoimmunity.

Discussion

Diverse molecular mechanisms control the function of Foxp3^{+} Treg cells, which serve as critical guardians of immune homeostasis. Very recently, miRNA-mediated post-transcriptional regulation of gene expression in Treg cells has attracted considerable attention because of the devastating failure of immunological tolerance observed in mice with Treg cell-specific ablation of Dicer and Drosha-dependent miRNAs (Chong et al., 2008; Liston et al., 2008; Zhou et al., 2008). These mice, like *Foxp3*^{null} mice, which are genetically devoid of Treg cells, develop unprovoked Th1, Th2, and Th17 responses and die before 4 weeks of age from severe immune-mediated lesions in a variety of organs (Chong et al., 2008; Liston et al., 2008; Zhou et al., 2008). In an effort to identify specific miRNAs contributing to Treg function, we discovered that miR-155, whose elevated expression in Treg cells is driven by Foxp3, confers competitive fitness to Treg cells by increasing sensitivity of IL-2R signaling through targeting SOCS1. However, miR-155 was dispensable for suppressor function of Treg cells despite its non-redundant role in Treg homeostasis (Kohlhaas et al., 2009; Lu et al., 2009). Another recent study suggested that high level of cAMP expression in Treg cells is conditional upon low amounts of miR-142-3p and that forced expression of this miRNA in Treg cells attenuates their ability to suppress T cell proliferation *in vitro* (Huang et al., 2009). However, the striking loss of suppressor function

observed in Dicer-deficient Treg cells is most likely due to loss of miRNAs that are overrepresented in these cells (Liston et al., 2008). Here, we demonstrate an essential role for one miRNA, miR-146a, in Treg cell function *in vivo* by analyzing chimeric mice generated upon co-transfer of miR-146a-deficient and Foxp3-deficient or wild-type B6 bone marrow cells into lymphopenic recipients. The presence or absence of miR-146a-sufficient Treg cells represented the sole difference between the resulting *Mirn146a*$^{-/-}$/Ly5.1^{+}B6 and *Mirn146a*$^{-/-}$/Foxp3KO mixed chimeras. Chimeric mice harboring only miR-146a-deficient Treg cells, but not mice harboring both miR-146a-deficient and -sufficient Treg cells, succumbed to severe autoimmune disease early after bone marrow reconstitution demonstrating an indispensable role of miR-146a in Treg cell-mediated suppression.

Foxp3-dependent suppressor program implemented by Treg cells keeps in check different types of effector immune responses to “self” antigens and pathogens. Several recent studies revealed that Treg cells do not seem to execute a universal hard-wired functional program limiting all kind of inflammation and immunity but instead these cells exhibit distinct mechanistic requirements for suppression of particular classes of the immune response. For example, Irf4, a transcription factor required for Th2 differentiation, is expressed in Treg cells in a Foxp3 dependent manner and endows them with the ability to suppress Th2 responses (Zheng et al., 2009). Similarly, the expression of T-bet, another key transcription factor in Th1 effector cell differentiation, is induced in Treg cells in a Stat1-dependent manner in response to IFNγ signalling and enables them to proliferate, migrate to, and accumulate at the sites of Th1 responses (Koch et al., 2009). Likewise, we have found that Treg cell-restricted ablation of Stat3, a transcription factor required for Th17 induction, leads to uncontrolled Th17 dependent pathology (Chaudhry et al., 2009). These studies suggest that in Treg cells distinct suppressor mechanisms are tailored to fit particular tissue and inflammatory settings by the same transcription factors which guide effector T cell differentiation in response to similar cues. In this study, we extended this concept to miRNAs by demonstrating that fatal immune mediated lesions observed in the presence of miR-146a-deficient Treg cells were accompanied by sharply augmented Th1 responses and were dependent upon increased amounts of IFNγ.

Activation of the transcription factor Stat1 downstream of the IFNγ receptor facilitates differentiation of Th1 cells and forced expression of a Stat1-encoding transgene in T cells results in an IFNγ-mediated immunopathology (Siebler et al., 2003). A connection between miR-146a and Stat1, a key transcription factor in IFNγ response, was suggested by two recent studies implying that Stat1 serves as a miR-146a target in human lymphocytes and monocytes (Cameron et al., 2008; Tang et al., 2009). Despite the lack of a canonical miR-146a site in the 3′UTR of the mouse *Stat1* gene, it contains a highly conserved site with partial complementarity to miR-146a seed sequence and additional complementarity in the adjacent sequence. Luciferase reporter assays demonstrated that this putative miR-146a binding site is crucial for miR-146a-mediated repression of Stat1. In addition, we have currently employed the HITS-CLIP (high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation) approach to identify miR targets in T cells. Our preliminary data confirmed the proposed region for miR-146a binding in the mouse *Stat1* gene (data not shown). More importantly, the disease observed in mice harboring miR-146a-deficient Treg cells was largely alleviated when miR-146a deficiency was coupled with *Stat1* hemizygosity. Finally, important independent confirmation of this biological significance of restraining Stat1 activation in Treg cells for their ability to suppress Th1 responses was provided by our finding in *Foxp3*^{cre}*SOCS1*$^{fl/fl}$ mice and *Foxp3*^{cre}*SOCS1*$^{fl/fl}$ / Foxp3KO chimeras. These mice, which harbor SOCS1-deficient Treg cells, develop IFNγ-mediated Th1 immunopathology similar to that observed in the presence of miR-146a-deficient Treg cells. Together, these findings offer genetic support for the notion that

miR-146a ensures Treg cell-mediated regulation of Th1 responses, in a significant part, through targeting Stat1.

Importantly, analysis of *Mirn146a*$^{-/-}$*Ifng*$^{-/-}$/Foxp3KO and *Mirn146a*$^{-/-}$/Foxp3KO chimeric mice revealed that miR-146a-deficient Treg cells were unable to prevent pathogenic Th1 responses and associated disease regardless in the absence of IFNγ supplied by the miR-146a-deficient compartment. Nevertheless, these experiments also showed that IFNγ production by miR-146a-deficient T effector cells contributed to full-scale Th1 immunopathology in *Mirn146a*$^{-/-}$/Foxp3KO mice. It is noteworthy that miR-146a was the first identified miRNA with an increased expression in terminally differentiated Th1 cells (Monticelli et al., 2005). Thus, miR-146a-mediated Stat1 repression in activated T effector cells likely serves as a mechanism of negative feedback regulation of Th1 responses. Moreover, our observation of higher levels of miR-146a in both Treg and effector T cells with "Th1 like" features (CXCR3^{+}T-bet^{hi}) in comparison to their CXCR3^{-}T-bet^{low} counterparts suggested that Treg cells further increase miR-146a expression in Th1 environment to ensure effective suppression of Th1 responses. Consistent with this model, the presence of miR-146a-sufficient Treg cells in *Mirn146a*$^{-/-}$/Ly5.1^{+} B6 chimeric mice was able to keep in check of Th1-prone effector T cells derived from miR-146a-deficient compartment. Together, our data demonstrated an important role of miR-146a in both Treg cell-mediated Th1 regulation and in limiting Th1 responses in a T effector cell-intrinsic manner.

In addition to Stat1, miR-146a was previously shown to negatively regulate TLR/IL-1R signaling pathway in macrophages by targeting TRAF6 and IRAK1/2 (Hou et al., 2009; Taganov et al., 2006). Indeed, levels TRAF6 and IRAK1 proteins were increased in both miR-146a-deficient Treg and non-Treg T cells. It has been shown that IL-1R signaling facilitates Th17 differentiation and IL-17 production (Chung et al., 2009; Maitra et al., 2009). Furthermore, Th2 responses are also facilitated by signaling through IL-1R and IL-33R, another TRAF6/IRAK-dependent IL-1R family member (Ben-Sasson et al., 2009; Schmitz et al., 2005). Thus, according to the idea of "symmetry" between cues eliciting effector T cell differentiation and corresponding suppressor capability in Treg cells, miR-146a deficiency in Treg cells could also result in augmented Th17 or Th2 responses. However, the lack of Th2 or Th17 cytokine production in mice harboring miR-146a-deficient Treg cells and the unimpeded ability of miR-146a-deficient Treg cells to suppress *in vitro* proliferative responses of T cells demonstrated that miR-146a deficiency in Treg cells results in a selective impairment in their ability to regulate Th1 responses. Lack of a detectable increase in Th2 and Th17 responses in the presence of miR-146a-deficient Treg cells can be explained by the fact that IFNγ/Stat1 signaling, while promoting Th1 immune response, inhibits the differentiation and function of Th2 and Th17 cells (Hu and Ivashkiv, 2009). Thus, it is possible that the potential impact of dysregulated TRAF6/IRAK1 signaling in miR-146a-deficient Treg cells was masked by the dominant effect of elevated Stat1 expression and activation. In support of this idea, combined miR-146a deficiency with *Stat1* hemizygosity in Treg cells resulted in an increase in IL-4 producing Th2 cells in addition to the aforementioned decrease in IFNγ-producing Th1 cells.

Although the heightened Stat1 activation associated with miR-146a and SOCS1 deficiency in Treg cells impaired their ability to suppress Th1 responses, the numbers of miR-146a- and SOCS1-deficient Treg cells were markedly increased compared to their wild-type counterparts. Whereas an increase in size of SOCS1-deficient Treg subset is most likely a consequence of augmented IL-2R signaling and Stat5 phosphorylation (Lu et al., 2009), miR-146a deficiency did not alter Stat5 expression or activation (data not shown). In addition, miR-146a-deficient Treg cell number was not decreased by the attenuated Stat1 phosphorylation and inflammation that accompanied antibody-mediated blockade of IFNγ

signaling in chimeric mice, or Stat1 haploinsufficiency. These findings suggested that de-repression of miR-146a targets other than Stat1 likely contributed to the increased Treg cell numbers in miR-146a-deficient mice.

Our findings that miR-146a- or SOCS1-deficient Treg cells fail to control Th1 responses likely due to unrestrained expression and activation of Stat1 may appear to fit the aforementioned concept of "symmetry" in the integration of environmental cues by Treg and effector T cells. However, whereas in previous studies it was the lack of Th lineage specific transcription factors Stat3, Irf4, and T-bet in Treg cells that resulted in impaired suppression of Th17, Th2, and Th1 responses, respectively; in this study unrestrained activation of Stat1 led to immunopathology. Importantly, Stat1 as well as IFNγ receptor (IFNγR) expression in Treg cells are necessary for T-bet induction in Treg cells, a prerequisite for efficient suppression of Th1 responses (Koch et al., 2009). The observations that both the lack of Stat1 and unrestrained Stat1 activation resulted in a breakdown of immunologic tolerance suggested that Treg-mediated suppression of Th1 mediated immunopathology is dependent upon a certain optimal range of Stat1 activation by cytokines. Moreover, by limiting Stat1 amounts in Treg cells, miR-146a prevents their deviation into IFNγ-producing "Th1-like" cells and thereby, safeguards their function and identity in inflammatory settings.

Our findings also raise a question as to why miR-146a- or SOCS1-deficient Treg cells fail to control Th1 responses. Chemokine receptor CXCR3, a well characterized target of T-bet (Beima et al., 2006; Lord et al., 2005), is induced in Treg cells in a T-bet-dependent manner and endows them with the ability to migrate to the sites of Th1 responses, a prerequisite for their efficient regulation (Koch et al., 2009). However, CXCR3 expression on the surface of Treg cells was elevated in the absence of miR-146a or SOCS1 consistent with increased Stat1 activation. This finding leads to the next fundamental question as to how do miR-146a- or SOCS1-deficient Treg cells lose control of Th1 responses. It is possible that IFNγ secreted by Treg cells could facilitate pathogenic Th1 responses since a sizable fraction of both miR-146a- and SOCS1-deficient Treg cells produce IFNγ. In addition, our previous microarray and phenotypic analyses have shown that IFNγR is upregulated in $Foxp3^{+}$Treg cells vs. naïve T cells in a Foxp3-dependent manner. Furthermore, a recent study by the Belkaid group has demonstrated that during lethal *T. gondii* infection exposure of wild-type Treg cells to high amounts of Th1 inflammatory mediators superimposes Th1 effector program on Treg cells. Consequently, Treg cells produce IFNγ and potentially contribute to the tissue damage leading to death of the infected host (Oldenhove et al., 2009). Together, these findings raise a possibility that an autocrine loop amplifies Stat1 activation and IFNγ production in miR-146a- and SOCS1-deficient Treg cells, and that overt IFNγ signaling in miR-146a-deficient and SOCS1-deficient Treg cells results in the breakdown of a yet unidentified mechanism of Treg cell-mediated suppression of Th1 responses. In supporting this notion, reduced pathogenic Th1 responses were observed in chimeric mice harboring miR-146a-deficient Treg cells incapable of secreting IFNγ. Nevertheless, lack of IFNγ production by miR-146a-deficient non-Treg cells precludes clear conclusions drawn from these studies. Further genetic studies employing Treg cell-specific ablation of IFNγ and IFNγR are required to directly test this idea.

In summary, our studies demonstrated that a single miRNA, miR-146a, is indispensable for suppression mediated by Treg cells *in vivo*. Excessive activation of Stat1 in Treg cells is kept in check by miR-146a to ensure efficient control of spontaneous IFNγ-dependent Th1-mediated immunopathology and prevent deviation of activated Treg cells into IFNγ-producing Th1-like cells. Similar lesions observed in mice with Treg cell-specific ablation of SOCS1 lend further support to this notion. Our observations combined with the previously reported requirement for Stat1-dependent T-bet induction in Treg cells (Koch et al., 2009) suggest that a certain optimal range of Stat1 activation maintained by miR-146a is

important for Treg-mediated control of Th1 responses and that both lack of Stat1 and unrestrained Stat1 activation in Treg cells lead to a severe failure of immunologic tolerance.

Experimental procedures

Mice

Mirn146a$^{-/-}$ (Boldin et al., submitted), *Foxp3*^{KO} (Fontenot et al., 2003), *Foxp3*$^{YFP\text{-}cre}$ (Rubtsov et al., 2008), *SOCS1*^{fl} (Tanaka et al., 2008), and *Stat1*$^{-/-}$ (Meraz et al., 1996) mice were housed under SPF conditions. Disease development was monitored by frequent visual examination and histopathological analyses. All mice were used in accordance with guidelines from the Institutional Animal Care Committee of the Memorial Sloan-Kettering Cancer Center.

Quantitative PCR analysis

Total RNA (including microRNA) was prepared from FACS purified cells using miRNeasy kit (Qiagen). For detecting the miR-146a and miR-146b expression levels, Taqman "stem-loop" real-time RT-PCR was performed as demonstrated previously (Jazdzewski et al., 2008). For measuring primary miR-146a and miR-146b, first strand complementary DNA was synthesized by using iScript cDNA synthesis kit (Bio-Rad) followed by real-time PCR analysis (SYBR green; Applied Biosystems).

Generation of bone-marrow chimeras mice and *in vivo* IFNγ neutralization

Mixed bone marrow chimeras were generated as described elsewhere (Lu et al., 2009). Briefly, T cell-depleted bone marrow cells from mice of indicated genotypes were mixed at 1:1 ratio and injected into T cell-deficient *Rag2*$^{-/-}$ or in some cases *Tcrβ/Tcrδ*$^{-/-}$ irradiated (950 rads) hosts (3-5×10^6 total cells/recipient). Clinical signs, tissue histology as well as the immune cell subset composition in the thymus and secondary lymphoid tissues were examined 6 weeks after BM transfer or when mice became moribund. In some experiments, mixed BM chimeras were administrated i.p. with 0.5 mg neutralizing IFNγ antibody (clone XMG1.2) or isotype-matched control IgG weekly starting at 3 wks after BM transfer.

In vitro suppression assay

The ability of miR146-deficient and –sufficient Treg cells to suppress proliferative responses of T cells was assessed in a standard *in vitro* suppression assay (Liston et al., 2008). Briefly, FACS purified 4×10^4 naive Ly5.1$^-$ CD4$^+$CD25$^-$CD62L^{hi} T cells and *Mirn146a*$^{+/+}$ or *Mirn146*$^{-/-}$ Ly5.1$^-$CD4$^+$CD25^{hi} Treg cells isolated from the mixed BM chimeras were mixed at the indicated ratios and stimulated with CD3 antibody in the presence of irradiated (2000 rads) splenocytes . T cell proliferation was assessed by with ^{3}H-TdR incorporation (cpm) in triplicate cultures during the last 8 hr of culture.

Flow cytometry and cytokine secretion assays

Cell surface staining and flow cytometric analysis of CD4, CD8, CD25, CD62L, CD103, GITR, ICOS and CXCR3 (all eBioscience) expression were performed as described elsewhere (Fontenot et al., 2005). Intracellular staining of Foxp3, IL-4, IFNγ, CTLA-4 (all eBioscience) and Ki67 (BD bioscience) were performed following fixation and permeabilisation according to manufacturer′s instructions.

To measure T cell cytokine production, 2×10^6 splenocytes were stimulated in 24-well plates with PMA (50 ng/ml) and ionomycin (250 ng/ml) in the presence of "Golgi plug" (BD Biosciences) for 5 hrs at 37 °C before staining.

Luciferase reporter assay

$CD4^+$ T cells isolated from *Mirn146a*$^{-/-}$ or *Mirn146a*$^{+/+}$ mice were cultured in 12-well plates (1×10^6 cells/well) and transfected with psiCheck2 luciferase reporter plasmids (Promega) containing either wild-type or mutated Stat1 3′ UTR using Amaxa mouse T cell nucleofector kit (Lonza, Basel, Switerland). Cells were harvested 24 hr later and luciferase activity was assessed using Dual-Luciferase Reporter Assay System (Promega) according to manufacturer′s protocol.

Western blot analysis

Cell lysates from miR146a-sufficient or -deficient $Ly5.1^-CD4^+CD25^-CD62L^{hi}$ T cells and $Ly5.1^-CD4^+CD25^{hi}$ Treg cells FACS purified from the mixed BM chimeras were separated by SDS-PAGE (5×10^5/lane) subjected to Western blot analysis as described previously (Lu et al., 2009). In some experiments, $CD4^+Foxp3^-$ T cells or $CD4^+Foxp3^+$ T cells isolated from *Foxp3*^{cre}*SOCS1*$^{fl/fl}$ mice or control littermates were used. Antibodies against TRAF6 (04-451; Miliipore), IRAK1 (NBP1-03169; Novus), Stat1 (9172), phospho-Stat1 (9167), Stat5 (9363), phospho-Stat5 (#9359; all from Cell Signaling Technology) and β-actin (AC-74, Sigma) were used to visualize the corresponding proteins. Protein quantitation was performed using NIH Image J software (http://rsb.info.nih.gov/ij/).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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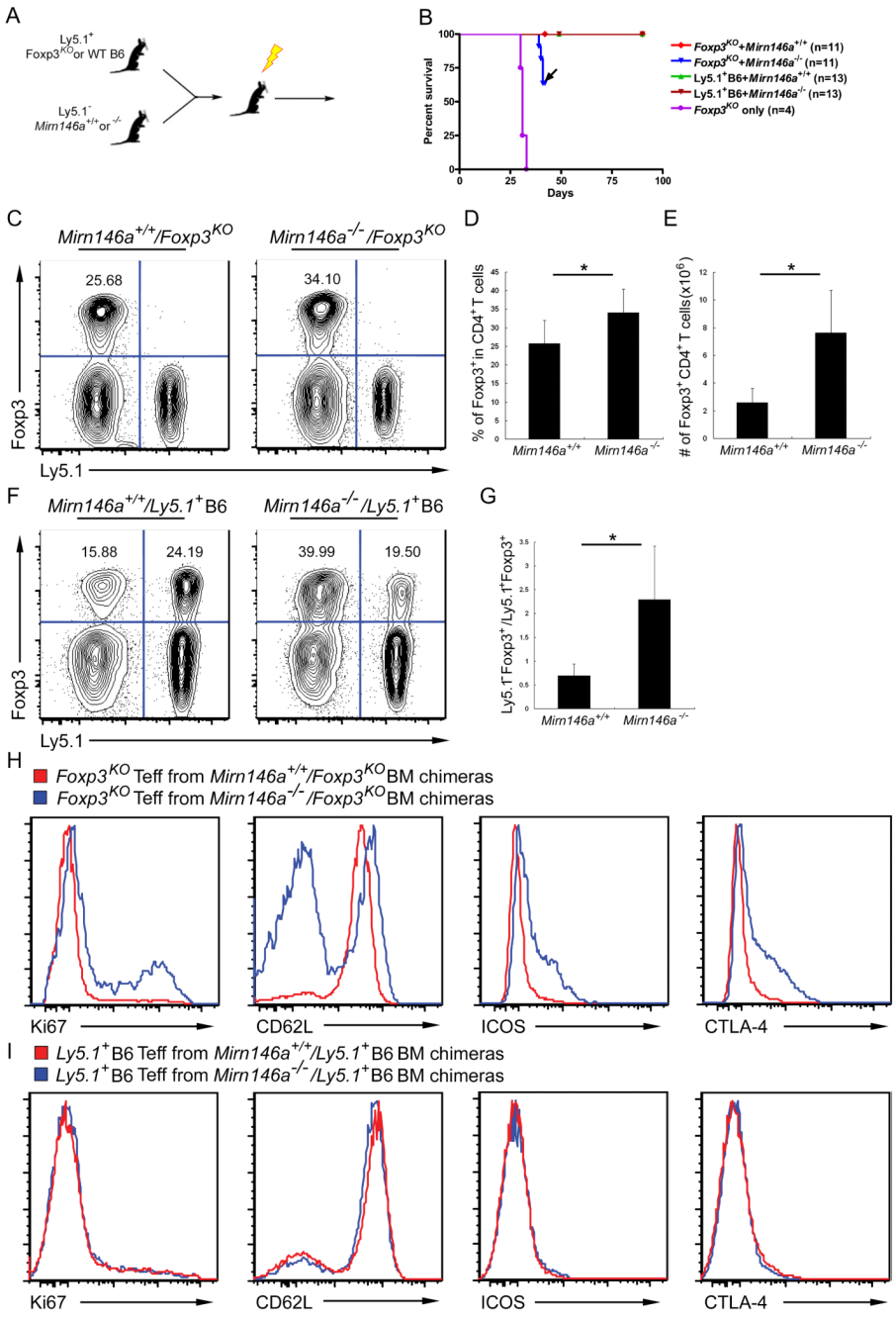

Fig 1. miR-146a-deficient Treg cells failed to effectively control immune homeostasis and restrain miR-146a-sufficient effector T cells

(A) Schematic of generation of mixed BM chimeras. **(B)** Kaplan-Meyer survival plot of mixed BM chimeras generated upon reconstitution of irradiated *Rag2*$^{-/-}$ (or *TCRβδ*$^{-/-}$) recipients with 1:1 mixtures of indicated BM cells. 40% of mice in *Mirn146a*$^{-/-}$/Foxp3KO group died before the day of analysis (arrow). The rest of the mice in this group along with other control groups were euthanized and analyzed. Splenic Treg cell frequencies in *Mirn146a*$^{-/-}$/Foxp3KO and *Mirn146a*$^{-/-}$/B6 chimeric mice generated upon transfer of *Mirn146a*$^{-/-}$ BM mixed with Ly5.1^{+} Foxp3KO BM **(C)** or Ly5.1^{+} B6 BM **(D)** 6-7 weeks after BM transfer. The proportion **(E)** and absolute numbers **(F)** of Ly5.1-Foxp3^{+} Treg cells

in indicated chimeric mice. **(G)** The ratios of Ly5.1^{-}Foxp3^{+} and Ly5.1^{+}Foxp3^{+} cells in indicated chimeric mice are shown. Expression of activation markers and Ki67 in miR-146a-sufficient Ly5.1^{+}Foxp3^{-} Teff cell subsets from *mirn146a*$^{-/-}$/Foxp3KO and *mirn146a*$^{+/}$/Foxp3KO **(H)** or *mirn146a*$^{-/-}$/B6 and *mirn146a*$^{+/+}$/B6 BM **(I)** 6-7 weeks after BM transfer. Data are representative of three independent experiments (n=11-13); values represent the mean +/− s.d., **P*<0.05. See also Figure S1 and S2.

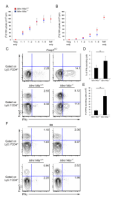

Fig 2. Selective dysregulation of IFNγ responses in mice harboring miR-146a-deficient Treg cells

miR-146a-deficient or -sufficient Treg cells isolated from **(A)** healthy *Mirn146a*$^{-/-}$/B6 or **(B)** diseased *Mirn146a*$^{-/-}$/Foxp3KO chimeric mice were co-cultured with wild-type responder CD4^{+} T cells at the indicated ratios for 72h in the presence of CD3 antibody and irradiated T cell-depleted splenocytes. **(C)** Frequencies of IFNγ secreting cells in Foxp3^{-}CD4^{+} and Foxp3^{+}CD4^{+} T cells isolated from diseased *Mirn146a*$^{-/-}$/Foxp3KO are shown. miR-146a-deficient T effector subset appears to make somewhat less IFNγ as compared to *Foxp3*^{ko} T cell subset because the latter contains an increased frequency of self-reactive T cells due to the presence of Treg "wannabe's" (Hsieh et al., 2006). The proportions **(D)** and absolute numbers **(E)** of IFNγ secreting cells in Foxp3^{+}CD4^{+} Treg cells isolated from diseased *Mirn146a*$^{-/-}$/Foxp3KO chimeric mice are numerated. Data are representative of three independent experiments (n=11-13); values represent the mean +/− s.d., **P*<0.05. **(F)** Frequency of IFNγ secreting cells in Foxp3^{-}CD4^{+} and Foxp3^{+}CD4^{+} T cells isolated from healthy *Mirn146a*$^{-/-}$/B6 mice are shown. Data are representative of three independent experiments (n=11-13). See also Figure S3.

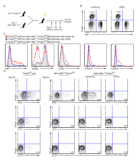

Fig 3. IFNγ blockade rescued immune mediated pathology in mice harboring miR-146a-deficient Treg cells

(A) Schematic of IFNγ neutralization experiments using BM chimeras. **(B)** Splenic Treg cell frequencies in chimeric mice treated with IFNγ neutralizing or control antibodies 6 weeks after BM transfer. **(C)** Expression of Ki67 and other activation markers in miR-146a-sufficient Ly5.1^{+}Foxp3^{-}CD4^{+} Teff cells isolated from *Mirn146a*$^{-/-}$/Foxp3KO mice with IFNγ neutralizing or control antibodies. **(D)** Frequency of IFNγ, IL-4, and IL-17 secreting cells in miR-146a-sufficient Ly5.1^{+}Foxp3^{-} CD4^{+} Teff cell subsers from indicated chimeric mice with or without αIFNγ treatment are shown. Ly5.1^{+}Foxp3^{-} CD4^{+} Teff cells isolated from chimeric mice reconstituted with Foxp3KO BM only served as positive control. Data are representative of two independent experiments (n=9-12).

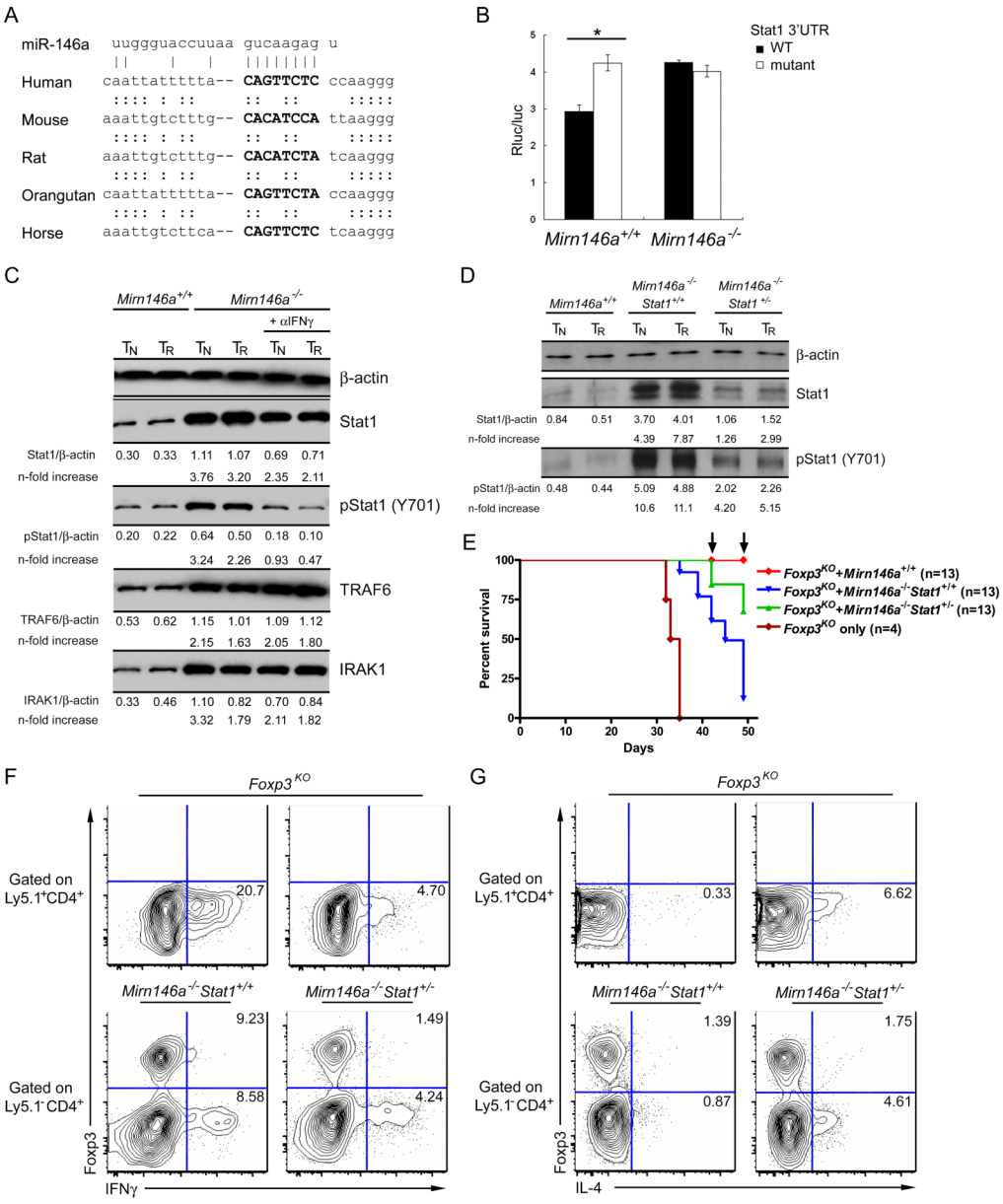

Fig 4. Loss of miR-146a-mediated Stat1 repression contributes to the IFNγ-mediated Th1 pathology in mice harboring miR-146a-deficient Treg cells

(A) Multiple species sequence alignment of the *Stat1* 3′ UTR including the putative miR-146a target site sequence (bold). **(B)** $CD4^+$ T cells isolated from miR-146a-deficient or -sufficient mice were co-transfected with a luciferase reporter construct containing wild-type or mutated *Stat1* 3′UTR and assessed for luciferase activity 24hrs after transfection. **(C)** Immunoblot analysis of the Stat1, TRAF6 and IRAK1 protein expression as well as the phosphrylation of Stat1. Densitometric values normalized on the basis of β-actin expression are indicated below the corresponding lanes; fold increase in normalized target protein expression in the absence of miR-146a with or without IFNγ neutralization in the indicated T cell subsets. Data are representative of two independent experiments. **(D)** Immunoblot analysis of total Stat1 as well as the phosphorylated Stat1 protein in mice with Stat1 hemizygosity. Densitometric values normalized on the basis of β-actin expression as well as fold increase in normalized target protein expression in the indicated T cell subsets are

shown below the corresponding lanes. **(E)** Kaplan-Meyer survival plot of *Rag2*$^{-/-}$ mice reconstituted with 1:1 mixtures of indicated BM cells. Arrows indicate the time of analysis. Frequency of **(F)** IFNγ or **(G)** IL-4 secreting cells in Foxp3^{-}CD4^{+} and Foxp3^{+}CD4^{+} T cell subsets in diseased *mirn146*$^{-/-}$*Stat1*$^{+/+}$/Foxp3KO and *mirn146*$^{-/-}$*Stat1*$^{-/+}$/Foxp3KO chimeric mice. Data are representative of two independent experiments (n=13).

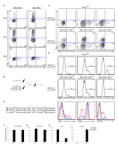

Fig 5. IFNγ production by miR-146a-deficient T cells partially contributed to, but cannot account for Th1 immunopathology in *Mirn146a*$^{-/-}$/Foxp3KO mice

(A) $CD4^+CD25^-CD62L^{hi}$ cells isolated from *Mirn146a*$^{-/-}$ mice or wild-type littermates were cultured *in vitro* under Th1/Th2 polarizing conditions. Intracellular IFNγ and IL-4 staining was assessed by FACS analysis. Data are representative of three independent experiments (n=4-6). **(B)** Schematic of generation of mixed BM chimeras. Frequencies of IFNγ secreting cells in **(C)** $CD4^+$ and **(D)** $CD8^+$ T cell population isolated from indicated chimeric mice are shown. **(E)** Flow cytometric analysis of expression of activation markers and Ki67 in miR-146a-sufficient $Ly5.1^+Foxp3^-CD4^+$ Teff cells from indicated chimeric mice are shown. Data are representative of three independent experiments (n=6-7). Real-time PCR analysis of **(F)** primary miR-146a, **(G)** mature miR-146a as well as **(H)** T-bet and **(I)** Foxp3 mRNA expression in FACS-purified $CXCR3^+$ and $CXCR3^-$ populations from $CD4^+CD25^+$ Treg cells (T_R) and $CD4^+CD25^-CD62L^{hi}$ R naïve T cells (T_N). Data are representative of two independent experiments.

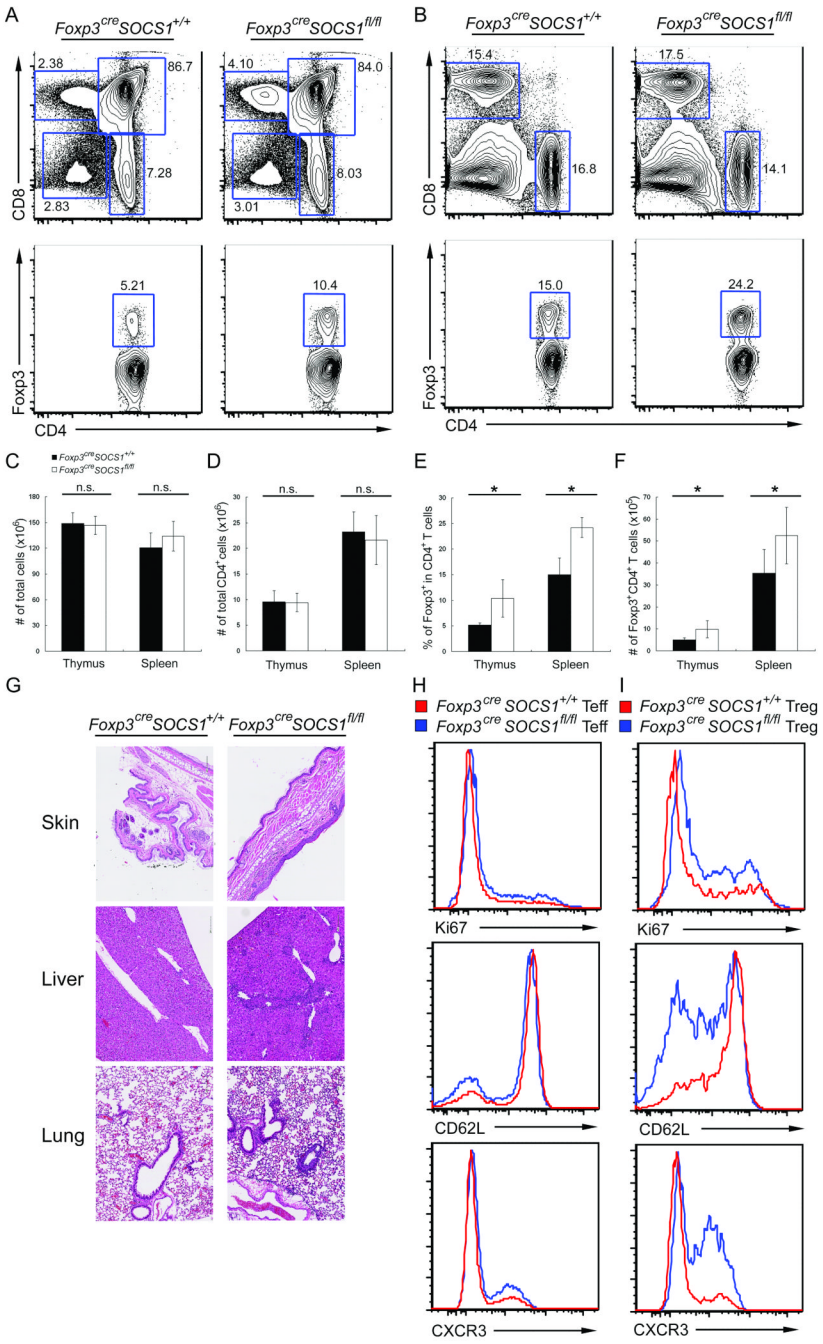

Fig 6. Phenotypic analysis of mice subjected Treg cell-specific SOCS1 ablation

Flow-cytometric analysis of **(A)** thymus and **(B)** spleen cells of 6-8 week-old *Foxp3^{cre}SOCS1$^{fl/fl}$* mice and wild-type littermates. Percentages of different thymocyte and splenocyte subsets are shown. **(C-F)** Cellularity of the thymus and spleen and the protportion and absolute numbers of thymus and splenic Foxp3^{+}CD4^{+} Treg cells in *Foxp3^{cre}SOCS1$^{fl/fl}$* mice or wild-type littermates are shown. Data are representative of three independent experiments (n=6-8); values represent the mean +/− s.d.; **P*<0.05. **(G)** H&E-stained sections of the skin, liver, and lung from the indicated chimeric mice. Flow-cytometric analysis of expression of Ki67 and other activation markers in **(H)** Foxp3^{-}CD4^{+} Teff cells and **(I)** Foxp3^{+}CD4^{+} Treg cells in 6-8 week old *Foxp3^{cre}SOCS1$^{fl/fl}$* mice (blue) or

wild-type littermates (red). Data are representative of two independent experiments (n=6-8). See also Figure S4.

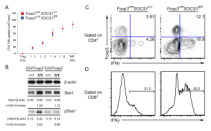

Fig 7. Unrestrained Stat1 activation in SOCS1-deficient Treg cells is associated with dysregulated IFNγ responses

(A) SOCS1-deficient or -sufficient Treg cells were co-cultured with wild-type responder $CD4^+$ T cells at the indicated ratios for 72 h in the presence of CD3 antibody and irradiated (2000 rads) T cell-depleted splenocytes. **(B)** Immunoblot analysis of total and phospho-Stat1. Densitometric values normalized on the basis of β-actin expression and fold increase in normalized target protein expression in the indicated T cell subsets are indicated below the corresponding lanes. **(C)** Frequencies of IFNγ secreting cells within $Foxp3^-CD4^+$ and $Foxp3^+CD4^+$ as well as $CD8^+$ subsets in *Foxp3*^{cre}*SOCS1*$^{fl/fl}$ mice or wild-type littermates. Data are representative of two independent experiments. See also Figure S5.