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1 **MicroRNA-182 is induced by IL-2 and promotes clonal expansion of**
2 **activated helper T lymphocytes**

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44 **ABSTRACT**

45 Upon activation by antigen, helper T lymphocytes switch from a resting state to
46 clonal expansion. This switch requires inactivation of the transcription factor
47 forkhead-box O1 (Foxo1), a suppressor of proliferation expressed in resting helper T
48 lymphocytes. In the early antigen-dependent phase of expansion, Foxo1 is
49 inactivated by antigen receptor-mediated post-translational modifications. Here we
50 show that in the late phase of expansion, Foxo1 is no longer post-translationally
51 regulated, but is inhibited post-transcriptionally by interleukin-2 (IL-2)-induced
52 microRNA-182 (miR-182). Specific inhibition of miR-182 in helper T lymphocytes
53 limits their expansion *in vitro* and *in vivo*. Our results demonstrate a central role for
54 miR-182 in the physiological regulation of IL-2-driven helper T cell-mediated immune
55 responses and open up new therapeutic possibilities.

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65 Activation of the T cell receptor (TCR) by antigen and costimulation via CD28
66 induces proliferation and IL-2 production of CD4⁺ helper T lymphocytes¹. Once
67 instructed to proliferate, the clonal expansion of helper T lymphocytes becomes
68 independent of further antigenic stimulation¹⁻² and is promoted by para- or autocrine
69 IL-2³. To initiate and perpetuate helper T cell proliferation, T cell activation signals
70 such as TCR-CD28 and IL-2 receptor (IL-2R) signaling have to inhibit Foxo1, which
71 blocks cell cycle progression in resting cells via induction of p27^{kip}⁴. Foxo1 also
72 induces the pro-apoptotic protein Bim⁴ which is blocked by Bcl-2 in resting but not in
73 activated helper T lymphocytes⁵. In the initial phase of activation Foxo1 protein is
74 rapidly inactivated by post-translational modification, i.e. phosphorylation, dependent
75 on TCR-CD28 signaling. This post-translational modification leads to exclusion of
76 Foxo1 from the nucleus and promotes its functional inactivation^{4, 6}. However, TCR
77 and IL-2R signaling are short-lived and it has been unclear as to how Foxo1 is
78 repressed in the late phase of clonal expansion.

79 Here we show that in the late phase of clonal expansion expression of Foxo1 is
80 regulated post-transcriptionally by miR-182, which is a member of the miR-183-96-
81 182 cluster⁷ and is induced through IL-2R signaling. Activated signal transducer and
82 activator of transcription 5 (STAT5) binds directly to the regulatory region of miR-182
83 and induces its expression. MiR-182 binds to a specific site of the 3'UTR of *Foxo1*
84 mRNA and reduces its expression. In activated helper T lymphocytes we found that
85 specific inhibition of miR-182 results in increased amounts of Foxo1 and decreased
86 clonal expansion. Constitutive overexpression of miR-182 in activated helper T
87 lymphocytes results in drastically increased clonal expansion. In a transfer model of
88 ovalbumin (OVA)-induced arthritis, inhibition of miR-182 in helper T lymphocytes

89 reduced disease severity. Thus, our results indicate a critical contribution of miR-182
90 in the regulation of helper T cell expansion.

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RESULTS

MiR-182 is induced in activated helper T cells

Murine naive ($CD4^+CD62L^{hi}CD25^-$) helper T cells were activated *in vitro* under T helper (T_H) 1, T_H2 and T_H17 polarizing conditions. Expression of miR-96, miR-182 and miR-183 was determined by quantitative PCR. MiR-96 was not detectable, neither in naive nor in activated cells (data not shown). MiR-182 and miR-183 were both expressed in naive and in activated cells (**Fig. 1a**). In naive cells, expression level of miR-182 was threefold higher than those of miR-183. Six days after activation, expression of miR-182 and miR-183 was drastically upregulated in helper T lymphocytes of all culture conditions relative to naive helper T cells (**Fig. 1a**). Expression of miR-182 was induced 50- to 200-fold and miR-183 expression increased 20- to 50-fold.

Irrespective of the polarizing culture conditions, expression of miR-182 increased between day one and two, reached a peak expression around day three (**Fig. 1b**) and decreased thereafter (**Supplementary Fig. 1a**). Non-activated naive $CD4^+$ T cells did not upregulate miR-182 expression (**Fig. 1b**). There was no significant difference between miR-182 expression of T_H0 , T_H1 , T_H2 and T_H17 cells. Similar miR-182 kinetics were observed for human naive ($CD45RA^+$) and memory ($CD45RO^+$) helper T cells (**Supplementary Fig. 1b, c**). The strong induction of miR-182 and miR-183 suggests a critical role of these miRNAs in activated helper T cells.

Foxo1 is a target of miR-182 in activated helper T cells

Computational target prediction using PicTar⁸ and TargetScan5.1⁹ algorithms revealed *Foxo1* as a putative target for the miRNAs of the miR-183-96-182 cluster. To validate *Foxo1* mRNA as a target of miR-96, miR-182 and/or miR-183, we cloned regions of the 3'UTR (>3000 nucleotides) of *Foxo1* mRNA, containing (i) the predicted conserved overlapping binding sites for miR-96 and miR-182 and the binding site for miR-183 (nucleotides 1 to 174), (ii) only the miR-183 binding site (nucleotides 55 to 132) or (iii) only the overlapping miR-96 and 182 binding sites (nucleotides 105 to 191) into a retroviral vector downstream of a reporter gene encoding truncated human CD4 (**Supplementary Fig. 2**).

Murine naive CD4⁺ helper T cells were activated for 36 h and transduced with the retroviral vectors. Reporter gene expression was suppressed by 20-40% over a culture period of four weeks in cells carrying the vectors containing the predicted overlapping miR-96-miR-182 binding site, but not in cells carrying the vector containing only the predicted miR-183 binding site (**Fig. 2a**).

The functional relevance of the predicted overlapping miR-96-miR-182 binding site was validated by mutating its sequence from GUGCCAAA to GACGGUAA. The observed suppression of reporter gene expression could be reverted in this mutant (miR-96-miR-182^{mut} bs) (**Fig. 2b**). These experiments identify *Foxo1* as a target of miR-182 in activated helper T lymphocytes. In these cells, *Foxo1* is targeted only by miR-182 but not by miR-96 which is not expressed.

Dynamics of regulation of *Foxo1* expression

We quantified *Foxo1* mRNA and miR-182 in activated murine and human helper T cells. As expected, the levels of miR-182 and *Foxo1* mRNA showed a negative correlation in murine (**Fig. 3a**) and in human helper T cells (**Supplementary Fig. 3**), with increasing miR-182 and decreasing *Foxo1* mRNA over time.

To further confirm the interaction of miR-182 and *Foxo1* we inhibited miR-182, by using either Locked Nucleic Acid (LNA) oligomers¹⁰ or antagomir oligonucleotides, both binding and inhibiting the mature miRNA. LNA-182 treatment of activated helper T cells blocked miR-182 expression by 60%, while *Foxo1* mRNA increased by 20% and *Foxo1* protein increased by 50% (**Supplementary Fig. 4a-c**). With antagomirs miR-182 could be blocked by more than 90% (**Fig. 3b**), while *Foxo1* mRNA increased by 60% and *Foxo1* protein increased by 50% (**Fig. 3c, d**).

Phosphorylated *Foxo1* is exported from the nucleus and is therefore inactive⁴. To evaluate the kinetics of *Foxo1* phosphorylation (P-*Foxo1*), we immunoblotted activated helper T cells. P-*Foxo1* was not detectable before T cell activation (data not shown), but was rapidly induced by activation, already after 1 h. The ratio of total *Foxo1* to P-*Foxo1* remained low for 66 h (**Fig. 3e**). Thereafter, the ratio of total *Foxo1* to P-*Foxo1* increased about fourfold for the rest of the observation period, until 112 h after onset of activation (**Fig. 3e**). The amounts of total *Foxo1* protein decreased gradually and remained low until 112 h after activation (**Fig. 3f**). These results indicate that suppression of *Foxo1* in activated helper T lymphocytes is mediated initially by post-translational modification, until about 66 h after onset of activation, and thereafter by transcriptional and/or post-transcriptional regulation.

IL-2 is required for the induction of miR-182

Naive helper T cells were activated *in vitro*, in the presence or absence of recombinant IL-2. IL-2 induced expression of miR-182 in a dose-dependent fashion (**Fig. 4a**). Conversely, expression of miR-182 was inhibited by 80% by blocking IL-2 and CD25 (IL-2 receptor α chain) with antibodies (**Fig. 4b**), suggesting that IL-2R signaling promotes miR-182 expression.

IL-2R signaling activates STAT5¹¹. Interestingly, the intergenic miR-183-96-182 cluster contains several phylogenetically conserved binding sites for STAT5. Upon activation of naive helper T cells, STAT5 binding to a predicted STAT5 binding site could be determined by chromatin immunoprecipitation (ChIP) (**Fig. 4c** and **Supplementary Fig. 5a-c**). Specific inhibition of STAT5 activity with the chromone-based inhibitor nicotinyl hydrazone (compound 6)¹², prevented induction of miR-182 in activated helper T cells (**Fig. 4d**) without altering IL-2 production (**Supplementary Fig. 5d**).

To analyze accessibility of the miR-182 locus, histone acetylation was determined by ChIP with antibodies specific for hyper-acetylated histone 4 (Ac-H4) under T_H1, T_H2 and T_H17 polarizing conditions. The miR-182 locus was hyper-acetylated after 24 h in activated naive helper T lymphocytes, pointing to a transcriptionally accessible conformation of the locus at this time. Subsequently, the hyper-acetylation declined gradually until it reached levels observed in naive cells after six days (**Fig. 4e**).

We wondered whether IL-2 signaling was sufficient to induce miR-182. Naturally-occurring regulatory helper T cells (nT_{Reg}) do not produce IL-2 upon activation, but express the IL-2R constitutively¹³. In these cells, expression of miR-182 could be induced by stimulation with recombinant IL-2, anti-CD3 and anti-CD28, but not by

stimulation with recombinant IL-2 alone (**Supplementary Fig. 5e**). Thus, induction of miR-182 expression in helper T cells requires both TCR-CD28 and IL-2R signaling.

MiR-182 promotes clonal expansion of helper T cells

The role of miR-182 in helper T cell expansion was analyzed in loss and gain of function experiments. Treatment of activated helper T lymphocytes *in vitro* with inhibitory antagomir-182 reduced the number of viable cells by 50% on day three and by 60% on day four, compared to a scrambled control (antagomir-scr) (**Fig. 5a**). The observed difference in cell numbers was due to increased cell death (**Fig. 5b**) and reduced proliferative capacity (**Fig. 5c**).

To test whether the treatment with antagomir-182 reduced helper T cell expansion *in vivo*, we treated ovalbumin (OVA)–specific helper T lymphocytes from DO11.10 mice with antagomirs and labeled them with carboxyfluorescein diacetate succinimidyl ester (CFSE) *ex vivo*. Subsequently, the helper T cells were transferred into Balb/c mice. One day after transfer, mice were immunized with OVA. On day four after immunization, lymph nodes and spleens of the mice were harvested and helper T cells analyzed. The number of viable helper T lymphocytes was reduced by 70% after antagomir-182 treatment (**Fig. 5d**). The CFSE labeling revealed a clear reduction in proliferation after antagomir-182 treatment (**Fig. 5e**).

Inhibition of miR-182 with LNAs led to a reduction of viable cells by 30% (**Supplementary Fig. 4d**). A similar effect could be achieved by blocking the miR-182 binding site in the Foxo1 3'UTR with binding site-masking LNAs (LNA-bs). This

treatment reduced the number of viable helper T cells by 20% (**Supplementary Fig. 4d**).

The effect of constitutive overexpression of miR-182 was tested *in vivo* with TCR-transgenic helper T lymphocytes from SMARTA mice that are specific for a lymphocytic choriomeningitis virus (LCMV)-derived peptide¹⁴. Activated SMARTA Thy1.1⁺ T cells were transduced with a retroviral vector encoding miR-182 (miR-182RV), a scrambled control vector (scrRV), or an empty vector (RV) (**Supplementary Fig. 6a**). Expression of miR-182 was 40-fold higher in miR-182RV- than in scrRV-transduced helper T cells (**Supplementary Fig. 6b**). Transduced SMARTA Thy1.1⁺ helper T cells were then adoptively transferred into naive C57BL/6 mice. On day six after transfer mice were infected with LCMV. On day eleven after transfer the expansion of Thy1.1⁺ helper T cells was analyzed. MiR-182RV-expressing helper T cells exhibited a six- to fourteen-fold increased expansion relative to scrRV or empty RV transduced helper T cells, respectively (**Supplementary Fig. 6c**).

MiR-182 inhibition reduces OVA-induced arthritis in mice

As inhibition of miR-182 reduced clonal expansion of helper T cells *in vitro* and *in vivo*, we next analyzed whether miR-182 inhibition impacts on the ability of helper T cells to induce inflammation in a murine model of arthritis. OVA-specific helper T lymphocytes from OT-II mice were treated with antagomir-182 *ex vivo* and adoptively transferred into Rag^{-/-} mice. One day after transfer, mice were immunized with OVA. On day seven after transfer, OVA was injected into the left knee joint and knee swelling determined. Knee swelling was reduced by about 35% in the mice that

received antagomir-182-treated helper T cells compared to control mice that received untreated cells (**Fig. 6a**). On day 15 after OVA challenge, the absolute numbers of CD4⁺ lymphocytes were determined in popliteal and inguinal lymph nodes. Those mice which received antagomir-182-treated cells had 60% fewer viable CD4⁺ lymphocytes (**Fig. 6b**).

The same experimental set-up was used to induce arthritis in Balb/c mice which were transferred with antagomir-182-treated helper T cells from DO11.10 mice. Knee swelling was reduced by about 40% with antagomir-182 treated helper T cells (**Fig. 6c**).

MiR-182 knockdown with antagomirs resulted in significantly lower histological scores of inflammation and tissue destruction (**Fig. 6d, e** and **Supplementary Table 1**). Infiltration of granulocytes and mononuclear cells into the inflamed tissue of the knee joint, bone and cartilage destruction and the formation of pannus were diminished in the mice with antagomir-182-treated cells compared to the control group (**Fig. 6e**).

DISCUSSION

In helper T lymphocytes the switch from the resting state in the absence of antigen, to proliferative expansion upon antigenic activation is tightly controlled by the transcription factor Foxo1¹⁵. Resting helper T cells express Foxo1, which induces interleukin-7 receptor (IL-7R), Bim and p27^{kip} 15-20 and, in a positive feedback loop, the expression of itself²¹⁻²². IL-7R signaling induces Bcl-2 to neutralize the pro-apoptotic effects of Bim⁵, whereas p27^{kip} blocks proliferation²³⁻²⁴. Thus, to enable

269 proliferation following antigenic stimulation, Foxo1 is blocked in activated helper T
270 cells¹⁵. IL-2 plays an important, yet not fully understood role in regulating clonal
271 expansion of activated helper T cells¹¹.

272 Immediately upon T cell activation, Foxo1 is blocked by TCR-dependent post-
273 translational phosphorylation⁴. However, TCR signaling ceases quickly and is
274 decreased by 90% after 8 h of activation, while helper T cells continue to
275 proliferate²⁵⁻²⁷. In this phase of clonal expansion, proliferation and survival of the
276 cells are dependent on IL-2³. An understanding of Foxo1 regulation in the IL-2-
277 dependent phase of expansion has been elusive. Here we show that IL-2-induced
278 miR-182 regulates Foxo1 at the post-transcriptional level, uncovering a further
279 mechanism by which IL-2 can function as a T cell growth factor. Ectopic
280 overexpression of miR-182 in helper T lymphocytes resulted in an about tenfold
281 enhanced clonal expansion, demonstrating the potential of proliferative regulation at
282 the post-transcriptional level|

283 Ectopic expression, however, might overestimate the relative contribution of miR-182
284 to the regulation of clonal expansion. Without physiological downregulation of miR-
285 182, inhibition of Foxo1 leads to a situation where all the expanded cells die (data
286 not shown), probably due to a lack of common γ -chain signals provided by IL-2 or IL-
287 7. Blocking physiological expression of miR-182 with antagomirs reduced clonal
288 expansion by 60% *in vitro* and by 70% *in vivo*. This is in accordance with data from
289 Dicer-deficient helper T cells which showed a 75% reduction in clonal expansion²⁸.
290 Thus, the IL-2-miR-182 axis constitutes an essential contribution to clonal expansion
291 of activated helper T cells. Predictably, this contribution depends on the dynamics of
292 IL-2 signaling during expansion. It remains to be determined to what extent this axis

contributes to clonal expansion of memory cells, some of which express little if any IL-2 on their own, e.g. T_H2 cells and T_H1 cells²⁹.

The 60- 70% relative contribution of the IL-2-miR-182 axis to clonal expansion of activated helper T cells reflects the final phase of expansion when neither TCR nor IL-2R signaling are available. In the preceding phase, IL-2R signaling induces miR-182 but it also directly regulates Foxo1 expression by inducing phosphorylation of Foxo1 via phosphatidylinositol 3-kinase (PI3K) and Akt⁴. In the initial phase, TCR-CD28 signaling induces IL-2R and also regulates Foxo1 expression directly, via the PI3K-Akt pathway⁴. During *ex vivo* activation of naive helper T cells, the TCR-CD28-dependent phase lasts for about one day, the directly IL-2-dependent phase lasts from day one to day three, while, as we show here, the IL-2-miR-182-dependent phase lasts from day two to day four. Both, the post-translational regulation by TCR-CD28 and IL-2, and the post-transcriptional regulation by miR-182 result in a disruption of the positive feedback loop of Foxo1 expression²¹⁻²² during clonal expansion. The regulation of miR-182 by IL-2 is obviously critical since helper T cells, after IL-2-driven clonal expansion, become IL-7-dependent for survival³⁰ and expression of the IL-7R is dependent on Foxo1¹⁹⁻²⁰. It is suggestive that prolonged downregulation of Foxo1 by constitutive expression of miR-182 leads to extinction of clonally expanded helper T cells.

In view of the critical role of STAT5 signaling for miR-182 induction, it remains enigmatic why only IL-2R signaling, but not IL-7R signaling induces miR-182. Both cytokines belong to the common cytokine receptor γ chain family and can activate STAT5¹¹. As we show here, STAT5 is the transcription factor that binds to the miR-182 locus. However, our results also suggest that IL-2R signaling on its own cannot induce miR-182. This was determined for regulatory T cells but is apparently also

true for the other T_H subsets since TCR signaling is always required to change the accessibility of the miR-182 locus for STAT5. No putative binding sites for NFAT or NF-κB are present in the miR-183-96-182 cluster. Thus, the molecular correlate of this “accessibility” remains yet elusive.

MiR-182 is encoded in a cluster with miR-183 and miR-96⁷. Interestingly, the *Foxo1* 3'UTR has overlapping binding sites for miR-96 and miR-182 and a binding site for miR-183⁷. For regulation of Foxo1 protein expression in activated helper T cells, however, miR-96 does not play a role, since it is not expressed at detectable levels, neither in naive nor in activated cells. MiR-183 is expressed in naive and in activated helper T cells. However, the predicted miR-183 binding site within the *Foxo1* 3'UTR did not confer suppression to a reporter gene in activated helper T cells. This observation could be explained by the fact that putative target sites are announced according to their sequence but might be inaccessible *in vivo* due to the secondary and tertiary structure of the mRNA³¹. A role for miR-182, but not miR-183 in post-transcriptional regulation of Foxo1 expression has also been shown for breast cancer cells. In these cells constitutive expression of miR-182 induces proliferation³².

It is known that miRNAs generally have multiple targets and miR-182 is also likely to target other mRNAs in addition to *Foxo1* mRNA. The proliferative effects of miR-182 found in this study, however, are due to *Foxo1*-targeting since masking of the specific miR-182 binding site within the *Foxo1* 3'UTR reproduced the effects observed with the inhibition of miR-182.

MiR-182, as we show here, provides distinct and decisive regulation of IL-2-induced clonal expansion of activated helper T cells and their survival as expanded cells by means of post-transcriptional regulation of Foxo1 expression. Our results showing

reduced severity of disease in a transfer model of OVA-induced arthritis open up a new therapeutic avenue for the control of unwanted helper T cell expansion in immune-mediated diseases by antagomirs³³. Conversely, mimics of miR-182 could be useful to inhibit Foxo1 in expanded helper T cells and thus eliminate these cells³⁴.

AUTHOR CONTRIBUTIONS

A.B.S. designed and performed experiments, analyzed data and wrote the manuscript; C.H. did experiments and analyzed data; E.S. designed and performed the OVA-induced arthritis experiments; A.K. and C.L. performed histology; A.N.H., I.P. and M.L. designed and analyzed LCMV experiments; R.R. performed the OVA-induced arthritis experiments; M.F. and T.H. analyzed CFSE measurements; J.D. and A.T. did human T cell experiments; F.F. provided cells; G.A.H. did inhibitory experiments; N.L., Z.F., U.B., A.B., J.G., W.C. and N.R. did miRNA screening experiments and/or analyzed them; N.R., M.L. and Z.F. discussed the results and commented on the manuscript; F.H. revised the manuscript; A.J. constructed scrambled overexpression vector; B.H., M.M., F.M.S. and R.B. provided technical support and conceptual advice; H.D.C. designed experiments and supervised research; A.R. designed study, supervised research and wrote the manuscript; M.F.M. designed the study, analyzed data and wrote the manuscript.

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FIGURE LEGENDS

Figure 1 MiR-182 is induced upon activation of primary naive helper T lymphocytes. (a) Expression of miR-182 and miR-183 measured by quantitative PCR in T_H1 , T_H2 and T_H17 lymphocytes that were differentiated *in vitro* with anti-CD3, anti-CD28 and irradiated splenocytes under their respective polarizing conditions for six days. *Ex vivo* naive helper T lymphocytes were analyzed on day 0. One representative out of two independent experiments is shown. (b) Kinetics of miR-182 expression in helper T lymphocytes were measured by quantitative PCR. Cells were activated *in vitro* with anti-CD3 and anti-CD28 and differentiated into indicated subsets. Naive helper T lymphocytes were cultivated without anti-CD3 or anti-CD28 stimulation. SDs are based on pools of three independent experiments.

Figure 2 Foxo1 is a target of miR-182. (a) Reporter assays using a reporter vector for the miR-183 bs (filled circles), a reporter vector for the overlapping miR-96-miR-

182 bs (open squares), a reporter vector for all three predicted bs (filled squares) and an empty reporter vector as control (open circles). The mean fluorescence intensity (MFI) of human CD4 was measured by flow cytometry and normalized to the MFI of the empty control vector. Lymphocytes were restimulated every six days with anti-CD3, anti-CD28 and irradiated splenocytes. All reporter assays were repeated and showed the same results. **(b)** Reporter assays using the empty reporter vector (control), the reporter vector for the overlapping miR-96 and miR-182 bs (miR-96-miR-182 bs) and a reporter vector with a mutated miR-96 and miR-182 bs (miR-96-miR-182^{mut} bs). The MFI of human CD4 was measured on day three post activation. SDs are based on pools of three independent experiments, **p<0.005.

Figure 3 Inactivation of Foxo1 by phosphorylation occurs rapidly upon activation and ceases after 66 h. **(a)** Expression kinetics of *Foxo1* mRNA (left axis) and miR-182 (right axis) in activated helper T lymphocytes, measured by quantitative PCR. **(b)** Expression of miR-182 day four after treatment with miR-182-specific antagomirs (antagomir-182) or scrambled control antagomirs (antagomir-scr), measured by quantitative PCR. SDs are based on pools of six independent experiments, ***p<0.001 **(c)** Expression of *Foxo1* mRNA on day four after treatment with miR-182-specific antagomirs (antagomir-182) or scrambled control antagomirs (antagomir-scr), measured by quantitative PCR. Two independent experiments are depicted. **(d)** Expression of Foxo1 protein on day four after treatment with miR-182-specific antagomirs (antagomir-182) or scrambled control antagomirs (antagomir-scr), measured by immunoblotting and densitometry. Two independent experiments are depicted. **(e)** Kinetics of the ratio of total Foxo1 to P-Foxo1 upon activation by anti-CD3 and anti-CD28 measured by immunoblotting and densitometry. SDs are

based on pools of four independent experiments. (f) Kinetics of total Foxo1 protein upon activation by anti-CD3 and anti-CD28 measured by immunoblotting. SDs are based on pools of four independent experiments.

Figure 4 IL-2 induces miR-182 expression. (a) Expression of miR-182 in T_H0 lymphocytes that were activated with plate-bound anti-CD3 and anti-CD28 for 48 h and cultured with indicated concentrations of recombinant IL-2, measured by quantitative PCR. One representative out of two independent experiments is shown. (b) MiR-182 expression in T_H0 lymphocytes that were activated for 48 h under standard conditions (control) or with anti-IL-2 and anti-IL-2R α antibodies (aIL-2+aIL-2R α), and in naive helper T cells (naive). SDs are based on pools of three independent experiments, * $p < 0.05$. (c) STAT5 immunoprecipitation of a predicted STAT binding site at the miR-182 locus in T_H0 cells that were activated with plate-bound anti-CD3 and anti-CD28 for 24 h. An intronic sequence of the *Foxo1* locus with no putative STAT binding site 0.7 kb upstream or downstream served as a negative control. A known STAT5 binding site of the *Cd25* promoter region served as positive control. (d) MiR-182 expression in T_H0 lymphocytes treated either with a chromone-based STAT5 inhibitor (compound 6) or vehicle (DMSO) as control, measured by quantitative PCR. SDs are based on pools of five independent experiments. (e) Kinetics of histone 4 acetylation at the miR-182 locus in T_H1 , T_H2 , T_H17 and naive helper T lymphocytes determined by chromatin immunoprecipitation.

Figure 5 Inhibition of miR-182 reduces T cell expansion *in vitro* and *in vivo*. (a) Absolute numbers of viable cells after treatment of anti-CD3-anti-CD28-activated helper T cells with antagomir-182 or antagomir-scr were measured by flow

cytometry. SDs are based on pools of three independent experiments (* $p < 0.05$). **(b)** Percentage of dead cells as determined by propidium-iodide staining after treatment of anti-CD3 and anti-CD28-activated helper T cells with antagomir-182 or antagomir-scr, measured by flow cytometry. SDs are based on three independent experiments (day three, ** $p < 0.005$; day four, * $p < 0.05$). **(c)** CFSE dilution analysis of CD4⁺ helper T lymphocytes after three days of treatment with antagomir-182 or antagomir-scr (*in vitro*). **(d)** Absolute numbers of viable helper T cells recovered from lymph nodes and spleen of Balb/c mice that were adoptively transferred with antagomir-treated and CFSE-labeled CD4⁺ helper T lymphocytes from DO11.10 mice, analyzed by flow cytometry on day four after immunization with OVA peptide. Each group contained three (antagomir-scr) to four mice (antagomir-182), standard errors of mean (SEM)s are depicted, ** $p < 0.005$. **(e)** Representative CFSE dilution analysis of helper T cells recovered from lymph nodes and spleen of Balb/c mice that were adoptively transferred with antagomir-treated and CFSE-labeled CD4⁺ helper T lymphocytes from DO11.10 mice, analyzed by flow cytometry on day two after immunization with OVA peptide.

Figure 6 Inhibition of miR-182 reduces severity of OVA-induced arthritis in

mice. (a,c) Knee swelling of OVA-immunized **(a)** Rag^{-/-} mice that were adoptively transferred with antagomir-182-treated or control OT-II cells and **(c)** Balb/c mice that were adoptively transferred with antagomir-182-treated or control DO11.10 cells after challenge with OVA peptide. **(a)** Each group consisted of three mice, SEMs are depicted ($p < 0.001$, two way ANOVA). **(c)** Each group consisted of five to seven mice, SEMs are depicted ($p < 0.001$, two way ANOVA). **(b)** Absolute numbers of viable CD4⁺ lymphocytes in pooled popliteal and inguinal lymph nodes. Each group

consisted of three mice, SEMs are depicted, $*p<0.05$. (d) Histological scores of arthritis-induced knee joints after transfer of antagomir-182-treated helper T cells from DO11.10 mice into Balb/c mice. Each group consisted of five mice, medians are depicted, $**p<0.005$. (e) Representative hematoxylin-eosin (HE) staining of knee joint sections showing increased fibroblast proliferation, mononuclear cell infiltration, bone and cartilage destruction and the formation of pannus in the control vs. antagomir-182-treated group (control, histological score 18; antagomir-182, histological score 13; maximum score 21).

METHODS

Mice

C57BL/6, DO11.10, Balb/c, SMARTAxThy1.1, OT-II and Rag^{-/-} mice were bred under pathogen-free conditions, handled in accordance with good animal practice as defined by the German animal welfare bodies and sacrificed by cervical dislocation. All animal experiments were performed in accordance with institutional, state, and federal guidelines (Landesamt Für Gesundheit und Soziales, Berlin, Germany).

Cell culture

Murine naive (CD4⁺CD62L^{hi}CD25⁻) T cells from spleens of 6-10 weeks old mice were purified and cultured as described previously³⁵. Unless stated otherwise, C57BL/6

mice were used. Naive helper T cells (6×10^5 cells/ml) were stimulated with plate-bound anti-CD3e (5 μ g/ml, clone 145-2C11, Miltenyi Biotec), soluble anti-CD28 (4 μ g/ml, clone 37.51, Miltenyi Biotec) and, if cells were cultivated > six days, CD90-depleted and irradiated (30 Gy) APCs (CD4⁺ fraction). Th1, Th2 and Th17 lineages were induced as previously described³⁶⁻³⁷. Th0 cells were induced by adding anti-IFN γ (10 μ g/ml, clone AN18.17.24), anti-IL-4 (10 μ g/ml, clone 11B11) and anti-IL-12 (10 μ g/ml, clone C17.8). Recombinant IL-2 (eBioscience), anti-IL-2R α (anti-CD25, 50 μ g/ml, clone pC61.5), anti-IL-2 (50 μ g/ml, clone JES6-1A12) or STAT5 inhibitor (compound 6, 200 μ M)¹² (were added as indicated.

Ex vivo human helper T lymphocytes were obtained from buffy coats from anonymous healthy adult donors, in accordance with local ethical committee approval, and stimulated as stated elsewhere³⁸.

Quantitative PCR

Total RNA was isolated using Trizol reagent (Invitrogen). For miRNA qRT-PCR cDNA was prepared from 200 ng of total RNA with the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems). Real time PCR was carried out using TaqMan MicroRNA Assays (Applied Biosystems) according to the manufacturer's recommendations. TaqMan U6 snRNA Assay (Applied Biosystems) was used to normalize expression values.

For mRNA qRT-PCR, 400 ng of total RNA were reverse transcribed using the Reverse Transcription Kit (Applied Biosystems). The following custom primers were used for SYBR Green based real-time PCR (Roche): murine Foxo1 fw, 5'-CGGGCTGGAAGAATTCAATTC-3' and murine Foxo1 rev, 5'-

509 AGTTCCTTCATTCTGCACTCGAA-3'; human Foxo1 fw, 5'-
 510 GCACACGAATGAACTTGCTG-3' and human Foxo1 rev, 5'-
 511 AAGAGCGTGCCCTACTTCAA-3'; murine HPRT fw, 5'-
 512 TCCTCCTCAGACCGCTTTT-3' and murine HPRT rev, 5'-
 513 CATAACCTGGTTCATCATCGC-3'; human HPRT fw, 5'-
 514 ACCCTTTCCAAATCCTCAGC-3' and human HPRT rev, 5'-
 515 GTTATGGCGACCCGCAG-3'. Expression values were normalized to HPRT
 516 expression ($2^{-\Delta Ct}$ method).

517 *Reporter assay*

518 To generate reporter vectors, sequences from *Foxo1*-3'UTR were cloned
 519 downstream of the human *cd4* gene into the pMSCV vector (BD Biosciences).
 520 Retroviral supernatants were generated and T cells were transduced as described
 521 before³⁹. Human CD4 expression was quantified by flow cytometry using
 522 fluorochrome-coupled anti-human CD4 antibody (clone TT1).

523 *Chromatin immunoprecipitation assay*

524 Chromatin immunoprecipitation was performed as described previously³⁶, using
 525 either polyclonal anti-acetyl-HistoneH4 (5 µg/ml, Millipore) or polyclonal anti-
 526 STAT5A/STAT5B antibody mixture (5 µg/ml, R&D Systems). The amount of
 527 immunoprecipitated DNA was determined by quantitative PCR. Putative binding sites
 528 in conserved regions of the miR-183/96/182 cluster (chr6:30115650-30119860 [-])
 529 were identified using the rvista webpage (<http://rvista.dcode.org/>), based on
 530 TRANSFAC professional V10.2 library. The following primers were used: pre-miR-
 531 182 fw, 5'-ACCGGGTCCCATTACCTTAC-3' and pre-miR-182 rev, 5'-
 532 GCCTCCTAAAACCACCCTA-3'; STAT5 fw, 5'-AGGGTGGCTAGAAGCCCTAA-3'

533 and STAT5 rev, 5'-TCTAGCCTGCATCACCTCT-3'; positive fw, 5'-
534 AGGAGGCAATCTGGGTCAG-3' and positive rev, 5'-
535 AAGCTGAGTCTCACATATCAGAA-3'; negative fw, 5'-
536 GTGCATTCCCTGGTGTATCC-3' and negative rev, 5'-
537 GATGTTGGGGACGAGAGAAG-3'.

538 *Overexpression of miR-182*

539 To generate the miR-182 overexpression vector miR-182RV a 347 bp fragment
540 harboring pre-miR-182 was amplified using the following primers: pri-182 fw, 5'-
541 GTTAACGTTAACTGTGGGAAGAGCGC-3' (including 2 HpaI restriction sites for
542 cloning) and pri-182 rev, 5'- CTCGAGAAAAACACCGAGAAGAGGTCGA-3'. The
543 pri-miR-182 fragment was cloned into the pQCXIX-vector (Clontech) under control of
544 the U6 promoter. GFP was inserted under the control of the EF1 α promoter, both
545 derived from pLVTHM from D. Trono (École Polytechnique Fédérale de Lausanne).
546 The control vector scrRV contains a scrambled version of miR-182 that was inserted
547 as synthetic oligo:

548 GAAGGCTCGAGAAGGTATATTTGCTGTTGACAGTGAGCGACGTTATGAATCTGC
549 GTGATGCTGTTGACAGTGAGCGTCACGCAGATTCATAACGTTGCCTACTGCCTC

550 G. Production of viral supernatants and T_H cell transduction were performed as
551 described for the reporter assays.

552 *Inhibition of miR-182 activity*

553 To inhibit miRNA-182 either naive helper T cells or helper T cells that were activated
554 for 24 h, were treated with specific antagomir-like oligonucleotides (Dharmacon). T
555 cells were resuspended in serum-free ACCELL medium (Dharmacon) with 1 μ M
556 antagomir-182 or antagomir-scr. After 1 h of incubation at 37°C in 5% CO₂ RPMI

557 medium supplemented with 1 μ M antagomir was added in threefold excess and cells
558 were cultivated as described above. As control, T cells were incubated for 1h in
559 ACCELL medium without addition of antagomirs.

560 Alternatively, miR-182 activity was inhibited with specific LNAs (Exiqon) that either
561 targeted miR-182 or masked the miR-182 binding site. Activated helper T cells were
562 transfected by nucleofection (Lonza) on day two post activation according to
563 manufacturer's recommendations. The final concentration of inhibitors was 1 μ M.

564 To monitor proliferation, cells were labeled with carboxyfluorescein succinimidyl
565 ester (CFSE) directly after sorting (according to ⁴⁰).

566 *Immunoblot*

567 Immunoblotting was performed as described previously⁴¹. Membranes were probed
568 with primary antibodies against total Foxo1 (clone C29-H4) and tyrosin-
569 phosphorylated Foxo1 (polyclonal, both from Cell Signaling) and β -actin (Santa
570 Cruz) for normalization. Immunoreactive bands were detected by
571 chemiluminescence using ECL reagent (GE Healthcare) and analyzed by
572 densitometry with Fuji LAS 3000 software.

573 *LCMV model*

574 Naive helper T cells from SMARTAxThy1.1 mice were activated for 36 h and
575 transduced with miR-182RV. GFP⁺CD4⁺ and GFP⁻CD4⁺ cells were sorted by FACS
576 on day four post activation. A total amount of 5×10^4 cells was transferred i.v. into
577 C57BL/6 mice. Six days after transfer mice were infected with 200 plaque forming
578 units (pfu) LCMV-WE. On day five after infection mice were bled and analyzed for
579 Thy1.1⁺ GFP⁺ helper T cells.

OVA-induced arthritis model

2x10⁶ OVA-specific naive helper T cells from OT-II mice and DO11.10 mice were injected i.v. into RAG^{-/-} recipients and Balb/c recipients, respectively. One day later recipients were immunized with cationized OVA (100 µg in 100 µl PBS/ mouse) at the tail base. The RAG^{-/-} recipients were challenged on day seven after transfer, while the Balb/c recipients were challenged on day fourteen after transfer. For re-challenge cationized OVA (60 µg) was injected i.a. in the left knee joint. Knee swelling was determined as delta swelling between the left knee joint and the contralateral control knee joint. Knee joints were processed as described before³⁹.

Statistics

All statistical analyses used the Student's *t*-test for paired or unpaired data, unless stated otherwise.

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Figure 1

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Figure 2

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Figure 3

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Figure 4

Figure 5

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Figure 6

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d

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Control

Antagomir-182