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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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108 RESULTS

109 **MiR-182 is induced in activated helper T cells**

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

120 Irrespective of the polarizing culture conditions, expression of miR-182 increased
121 between day one and two, reached a peak expression around day three (**Fig. 1b**)
122 and decreased thereafter (**Supplementary Fig. 1a**). Non-activated naive $CD4^+$ T
123 cells did not upregulate miR-182 expression (**Fig. 1b**). There was no significant
124 difference between miR-182 expression of T_H0 , T_H1 , T_H2 and T_H17 cells. Similar
125 miR-182 kinetics were observed for human naive ($CD45RA^+$) and memory
126 ($CD45RO^+$) helper T cells (**Supplementary Fig. 1b, c**). The strong induction of miR-
127 182 and miR-183 suggests a critical role of these miRNAs in activated helper T cells.

128

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

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

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

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

150

151 **Dynamics of regulation of *Foxo1* expression**

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

156

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

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

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

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

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

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

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

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

201

202 *MiR-182 promotes clonal expansion of helper T cells*

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

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

218

219 Inhibition of miR-182 with LNAs led to a reduction of viable cells by 30%
220 (**Supplementary Fig. 4d**). A similar effect could be achieved by blocking the miR-
221 182 binding site in the Foxo1 3'UTR with binding site-masking LNAs (LNA-bs). This

222 treatment reduced the number of viable helper T cells by 20% (**Supplementary Fig.**
223 **4d**).

224 The effect of constitutive overexpression of miR-182 was tested *in vivo* with TCR-
225 transgenic helper T lymphocytes from SMARTA mice that are specific for a
226 lymphocytic choriomeningitis virus (LCMV)-derived peptide¹⁴. Activated SMARTA
227 Thy1.1⁺ T cells were transduced with a retroviral vector encoding miR-182 (miR-
228 182RV), a scrambled control vector (scrRV), or an empty vector (RV)
229 (**Supplementary Fig. 6a**). Expression of miR-182 was 40-fold higher in miR-182RV-
230 than in scrRV-transduced helper T cells (**Supplementary Fig. 6b**). Transduced
231 SMARTA Thy1.1⁺ helper T cells were then adoptively transferred into naive C57BL/6
232 mice. On day six after transfer mice were infected with LCMV. On day eleven after
233 transfer the expansion of Thy1.1⁺ helper T cells was analyzed. MiR-182RV-
234 expressing helper T cells exhibited a six- to fourteen-fold increased expansion
235 relative to scrRV or empty RV transduced helper T cells, respectively
236 (**Supplementary Fig. 6c**).

237

238 *MiR-182 inhibition reduces OVA-induced arthritis in mice*

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

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

251 The same experimental set-up was used to induce arthritis in Balb/c mice which
252 were transferred with antagomir-182-treated helper T cells from DO11.10 mice. Knee
253 swelling was reduced by about 40% with antagomir-182 treated helper T cells (**Fig.**
254 **6c**).

255 MiR-182 knockdown with antagomirs resulted in significantly lower histological
256 scores of inflammation and tissue destruction (**Fig. 6d, e** and **Supplementary Table**
257 **1**). Infiltration of granulocytes and mononuclear cells into the inflamed tissue of the
258 knee joint, bone and cartilage destruction and the formation of pannus were
259 diminished in the mice with antagomir-182-treated cells compared to the control
260 group (**Fig. 6e**).

261

262 **DISCUSSION**

263 In helper T lymphocytes the switch from the resting state in the absence of antigen,
264 to proliferative expansion upon antigenic activation is tightly controlled by the
265 transcription factor Foxo1¹⁵. Resting helper T cells express Foxo1, which induces
266 interleukin-7 receptor (IL-7R), Bim and p27^{kip} 15-20 and, in a positive feedback loop,
267 the expression of itself²¹⁻²². IL-7R signaling induces Bcl-2 to neutralize the pro-
268 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

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

295 The 60- 70% relative contribution of the IL-2-miR-182 axis to clonal expansion of
296 activated helper T cells reflects the final phase of expansion when neither TCR nor
297 IL-2R signaling are available. In the preceding phase, IL-2R signaling induces miR-
298 182 but it also directly regulates Foxo1 expression by inducing phosphorylation of
299 Foxo1 via phosphatidylinositol 3-kinase (PI3K) and Akt⁴. In the initial phase, TCR-
300 CD28 signaling induces IL-2R and also regulates Foxo1 expression directly, via the
301 PI3K-Akt pathway⁴. During *ex vivo* activation of naive helper T cells, the TCR-CD28-
302 dependent phase lasts for about one day, the directly IL-2-dependent phase lasts
303 from day one to day three, while, as we show here, the IL-2-miR-182-dependent
304 phase lasts from day two to day four. Both, the post-translational regulation by TCR-
305 CD28 and IL-2, and the post-transcriptional regulation by miR-182 result in a
306 disruption of the positive feedback loop of Foxo1 expression²¹⁻²² during clonal
307 expansion. The regulation of miR-182 by IL-2 is obviously critical since helper T
308 cells, after IL-2-driven clonal expansion, become IL-7-dependent for survival³⁰ and
309 expression of the IL-7R is dependent on Foxo1¹⁹⁻²⁰. It is suggestive that prolonged
310 downregulation of Foxo1 by constitutive expression of miR-182 leads to extinction of
311 clonally expanded helper T cells.

312 In view of the critical role of STAT5 signaling for miR-182 induction, it remains
313 enigmatic why only IL-2R signaling, but not IL-7R signaling induces miR-182. Both
314 cytokines belong to the common cytokine receptor γ chain family and can activate
315 STAT5¹¹. As we show here, STAT5 is the transcription factor that binds to the miR-
316 182 locus. However, our results also suggest that IL-2R signaling on its own cannot
317 induce miR-182. This was determined for regulatory T cells but is apparently also

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

322 MiR-182 is encoded in a cluster with miR-183 and miR-96⁷. Interestingly, the *Foxo1*
323 3'UTR has overlapping binding sites for miR-96 and miR-182 and a binding site for
324 miR-183⁷. For regulation of *Foxo1* protein expression in activated helper T cells,
325 however, miR-96 does not play a role, since it is not expressed at detectable levels,
326 neither in naive nor in activated cells. MiR-183 is expressed in naive and in activated
327 helper T cells. However, the predicted miR-183 binding site within the *Foxo1* 3'UTR
328 did not confer suppression to a reporter gene in activated helper T cells. This
329 observation could be explained by the fact that putative target sites are announced
330 according to their sequence but might be inaccessible *in vivo* due to the secondary
331 and tertiary structure of the mRNA³¹. A role for miR-182, but not miR-183 in post-
332 transcriptional regulation of *Foxo1* expression has also been shown for breast
333 cancer cells. In these cells constitutive expression of miR-182 induces proliferation³².

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

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

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

346

347 **AUTHOR CONTRIBUTIONS**

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

361

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373

374 **FIGURE LEGENDS**

375

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

386

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

389 182 bs (open squares), a reporter vector for all three predicted bs (filled squares)
390 and an empty reporter vector as control (open circles). The mean fluorescence
391 intensity (MFI) of human CD4 was measured by flow cytometry and normalized to
392 the MFI of the empty control vector. Lymphocytes were restimulated every six days
393 with anti-CD3, anti-CD28 and irradiated splenocytes. All reporter assays were
394 repeated and showed the same results. **(b)** Reporter assays using the empty
395 reporter vector (control), the reporter vector for the overlapping miR-96 and miR-182
396 bs (miR-96-miR-182 bs) and a reporter vector with a mutated miR-96 and miR-182
397 bs (miR-96-miR-182^{mut} bs). The MFI of human CD4 was measured on day three post
398 activation. SDs are based on pools of three independent experiments, **p<0.005.

399

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

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

417

418 **Figure 4 IL-2 induces miR-182 expression.** (a) Expression of miR-182 in T_{H0}
419 lymphocytes that were activated with plate-bound anti-CD3 and anti-CD28 for 48 h
420 and cultured with indicated concentrations of recombinant IL-2, measured by
421 quantitative PCR. One representative out of two independent experiments is shown.
422 (b) MiR-182 expression in T_{H0} lymphocytes that were activated for 48 h under
423 standard conditions (control) or with anti-IL-2 and anti-IL-2R α antibodies (aIL-2+aIL-
424 2R α), and in naive helper T cells (naive). SDs are based on pools of three
425 independent experiments, *p<0.05. (c) STAT5 immunoprecipitation of a predicted
426 STAT binding site at the miR-182 locus in T_{H0} cells that were activated with plate-
427 bound anti-CD3 and anti-CD28 for 24 h. An intronic sequence of the *Foxo1* locus
428 with no putative STAT binding site 0.7 kb upstream or downstream served as a
429 negative control. A known STAT5 binding site of the *Cd25* promoter region served as
430 positive control. (d) MiR-182 expression in T_{H0} lymphocytes treated either with a
431 chromone-based STAT5 inhibitor (compound 6) or vehicle (DMSO) as control,
432 measured by quantitative PCR. SDs are based on pools of five independent
433 experiments. (e) Kinetics of histone 4 acetylation at the miR-182 locus in T_{H1}, T_{H2},
434 T_{H17} and naive helper T lymphocytes determined by chromatin immunoprecipitation.

435

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

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

455

456 **Figure 6 Inhibition of miR-182 reduces severity of OVA-induced arthritis in**
457 **mice. (a,c) Knee swelling of OVA-immunized (a) Rag^{-/-} mice that were adoptively**
458 **transferred with antagomir-182-treated or control OT-II cells and (c) Balb/c mice that**
459 **were adoptively transferred with antagomir-182-treated or control DO11.10 cells after**
460 **challenge with OVA peptide. (a) Each group consisted of three mice, SEMs are**
461 **depicted ($p < 0.001$, two way ANOVA). (c) Each group consisted of five to seven**
462 **mice, SEMs are depicted ($p < 0.001$, two way ANOVA). (b) Absolute numbers of**
463 **viable CD4⁺ lymphocytes in pooled popliteal and inguinal lymph nodes. Each group**

464 consisted of three mice, SEMs are depicted, * $p < 0.05$. (d) Histological scores of
465 arthritis-induced knee joints after transfer of antagomir-182-treated helper T cells
466 from DO11.10 mice into Balb/c mice. Each group consisted of five mice, medians are
467 depicted, ** $p < 0.005$. (e) Representative hematoxylin-eosin (HE) staining of knee joint
468 sections showing increased fibroblast proliferation, mononuclear cell infiltration, bone
469 and cartilage destruction and the formation of pannus in the control vs. antagomir-
470 182-treated group (control, histological score 18; antagomir-182, histological score
471 13; maximum score 21).

472

473

474

475 **METHODS**

476 *Mice*

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

482 *Cell culture*

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

485 mice were used. Naive helper T cells (6×10^5 cells/ml) were stimulated with plate-
486 bound anti-CD3e (5 μ g/ml, clone 145-2C11, Miltenyi Biotec), soluble anti-CD28 (4
487 μ g/ml, clone 37.51, Miltenyi Biotec) and, if cells were cultivated > six days, CD90-
488 depleted and irradiated (30 Gy) APCs (CD4⁻ fraction). Th1, Th2 and Th17 lineages
489 were induced as previously described³⁶⁻³⁷. Th0 cells were induced by adding anti-
490 IFN γ (10 μ g/ml, clone AN18.17.24), anti-IL-4 (10 μ g/ml, clone 11B11) and anti-IL-12
491 (10 μ g/ml, clone C17.8). Recombinant IL-2 (eBioscience), anti-IL-2R α (anti-CD25, 50
492 μ g/ml, clone pC61.5), anti-IL-2 (50 μ g/ml, clone JES6-1A12) or STAT5 inhibitor
493 (compound 6, 200 μ M)¹² (were added as indicated.

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

497

498 *Quantitative PCR*

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

505 For mRNA qRT-PCR, 400 ng of total RNA were reverse transcribed using the
506 Reverse Transcription Kit (Applied Biosystems). The following custom primers were
507 used for SYBR Green based real-time PCR (Roche): murine Foxo1 fw, 5`-
508 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.

580 *OVA-induced arthritis model*

581 2×10^6 OVA-specific naive helper T cells from OT-II mice and DO11.10 mice were
582 injected i.v. into RAG^{-/-} recipients and Balb/c recipients, respectively. One day later
583 recipients were immunized with cationized OVA (100 µg in 100 µl PBS/ mouse) at
584 the tail base. The RAG^{-/-} recipients were challenged on day seven after transfer,
585 while the Balb/c recipients were challenged on day fourteen after transfer. For re-
586 challenge cationized OVA (60 µg) was injected i.a. in the left knee joint. Knee
587 swelling was determined as delta swelling between the left knee joint and the
588 contralateral control knee joint. Knee joints were processed as described before³⁹.

589 *Statistics*

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

592

593

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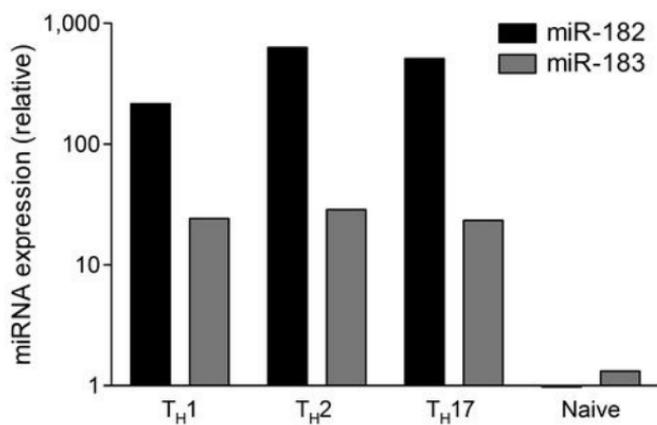
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Figure 1

a



b

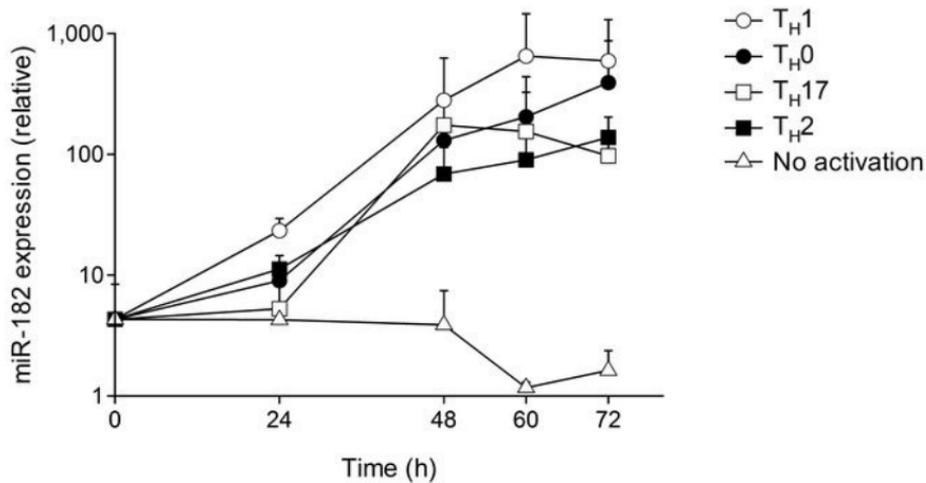
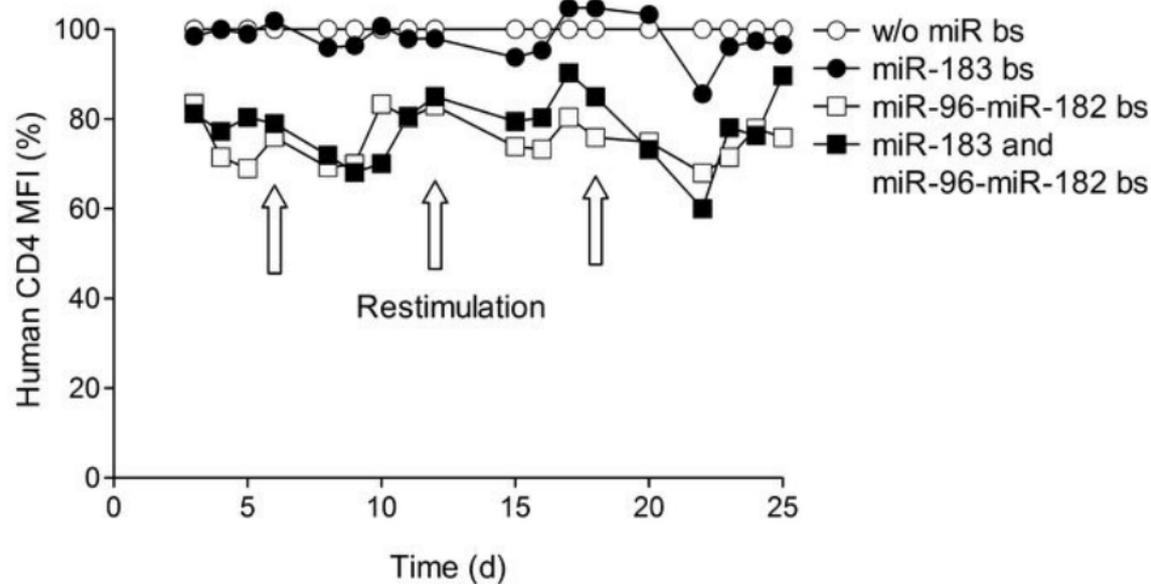


Figure 2

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b

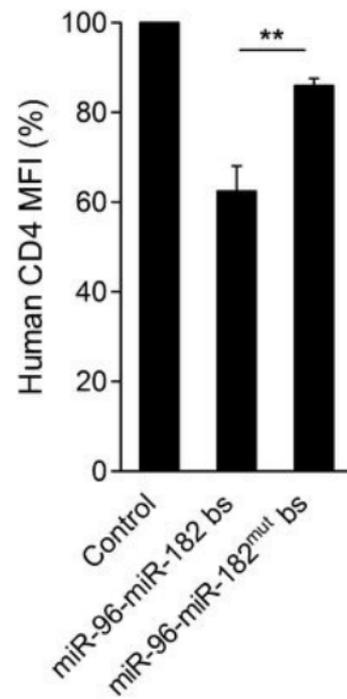
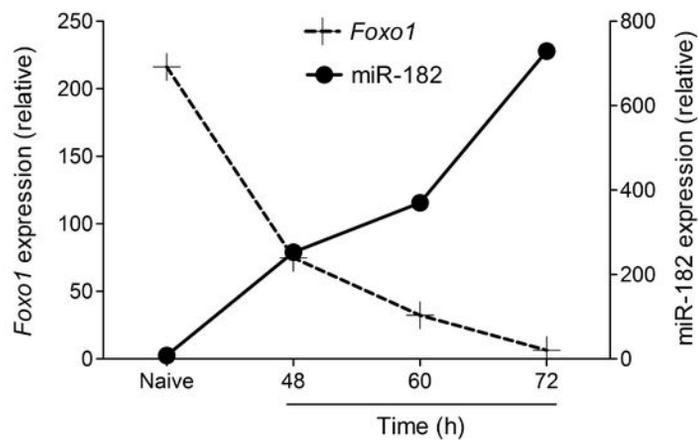
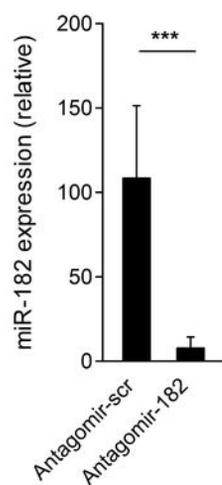


Figure 3

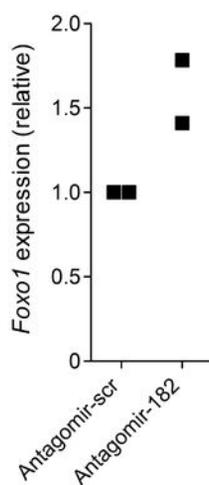
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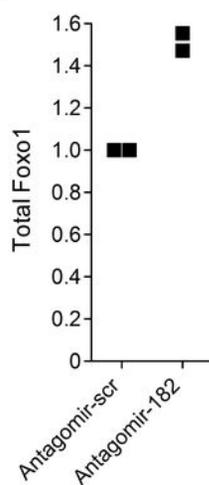
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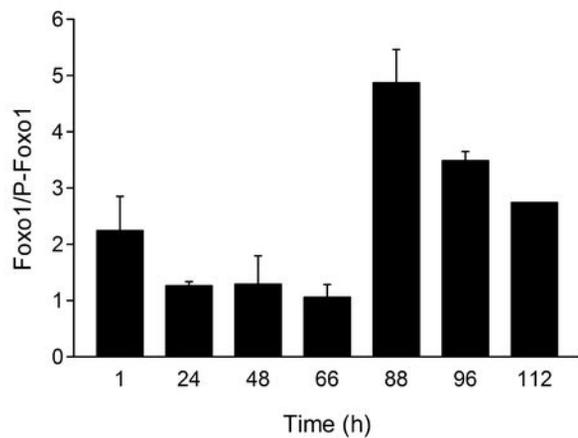
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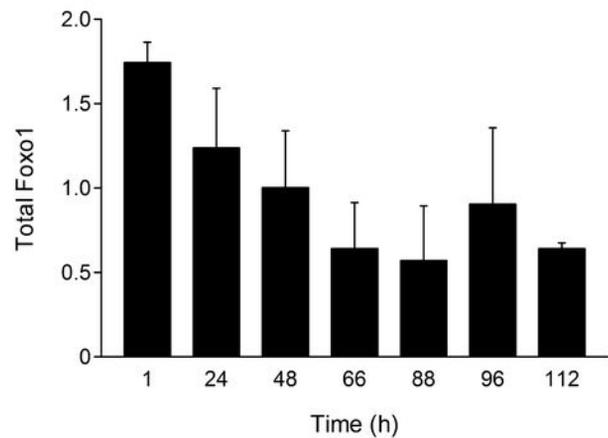


Figure 4

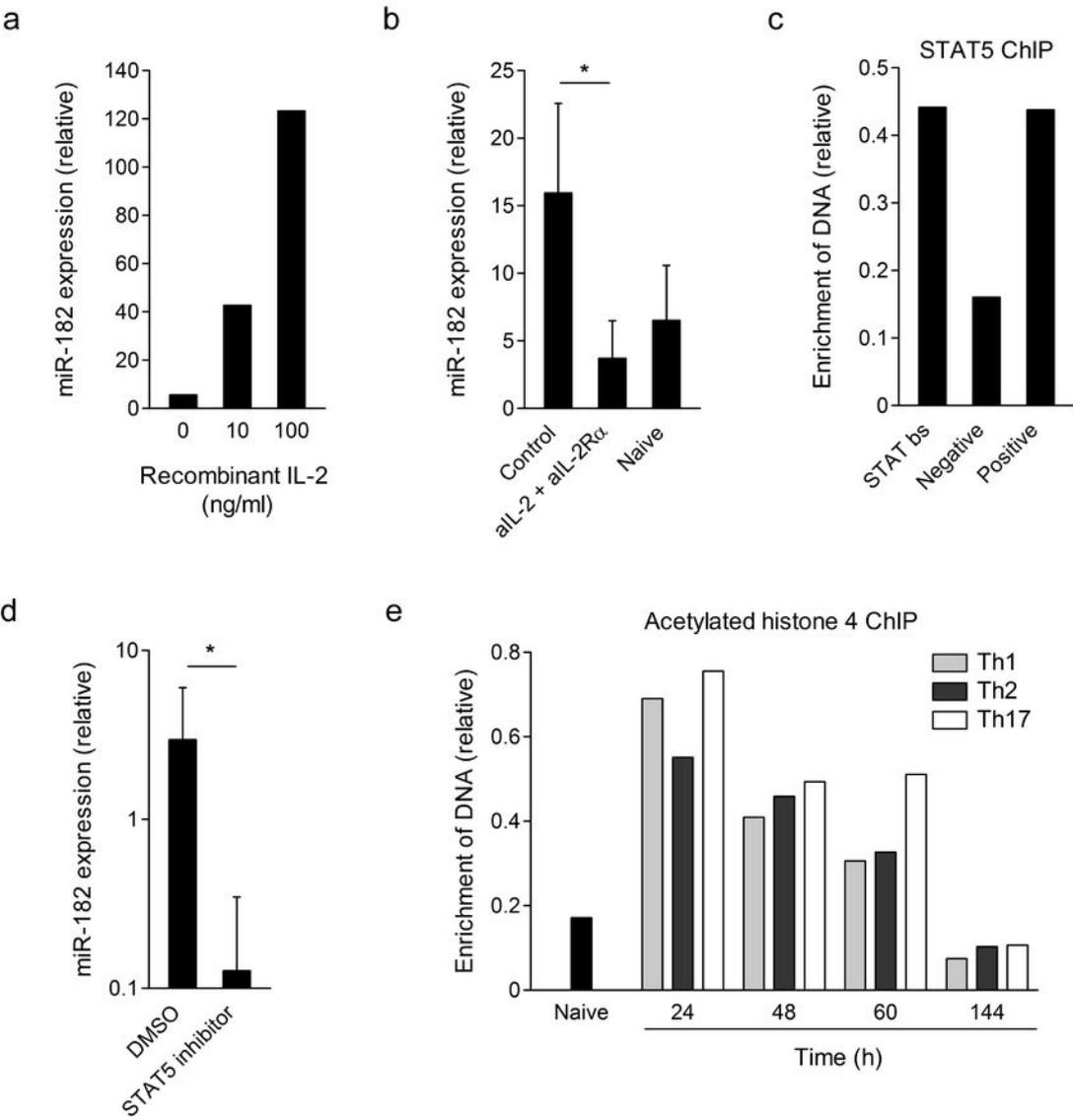
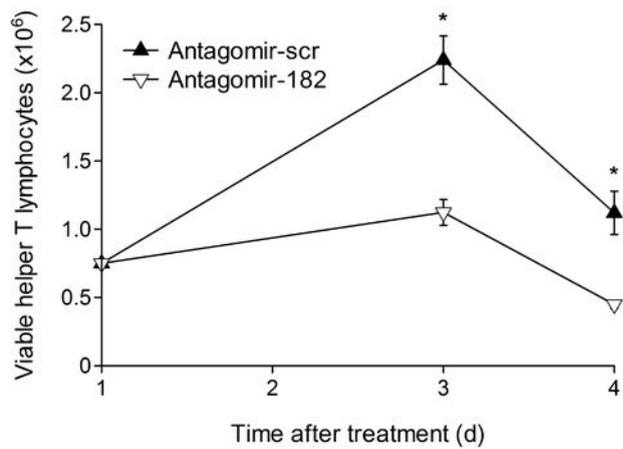
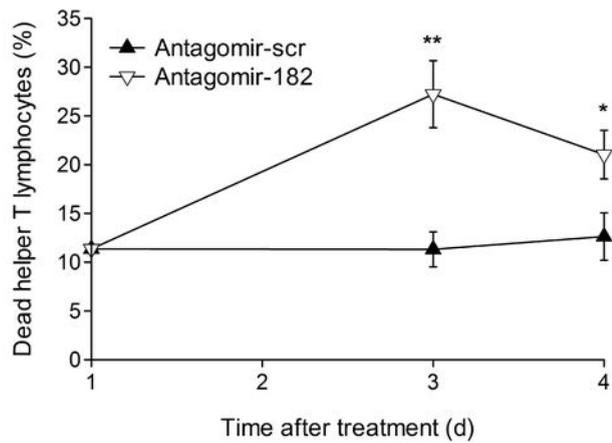


Figure 5

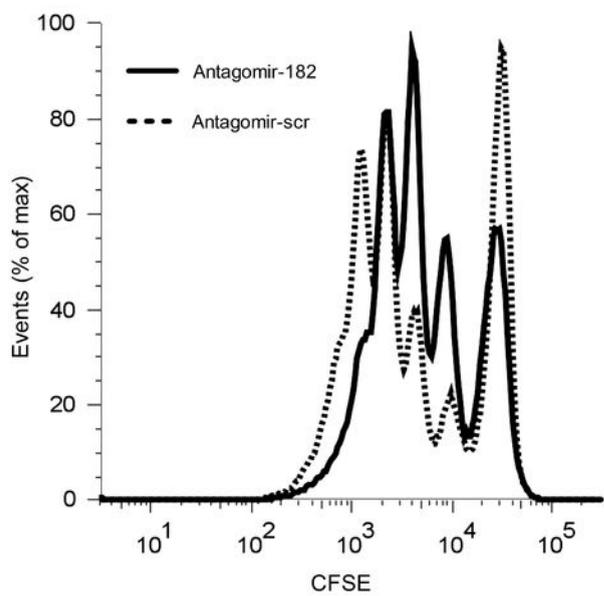
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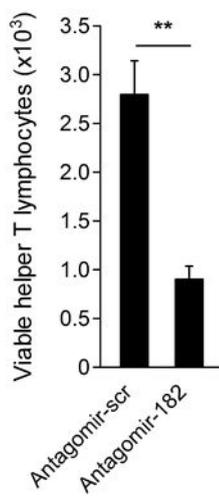
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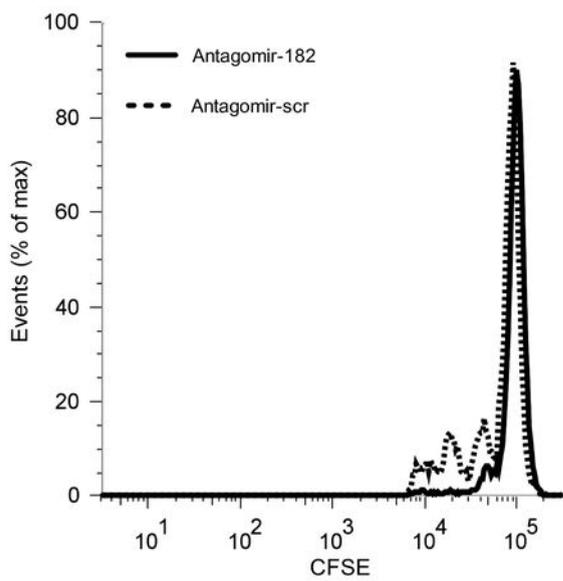
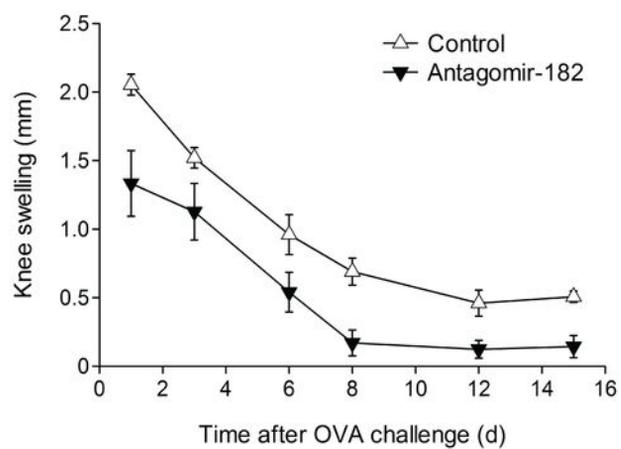
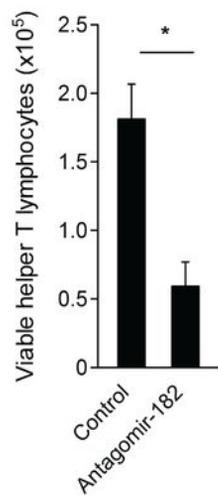


Figure 6

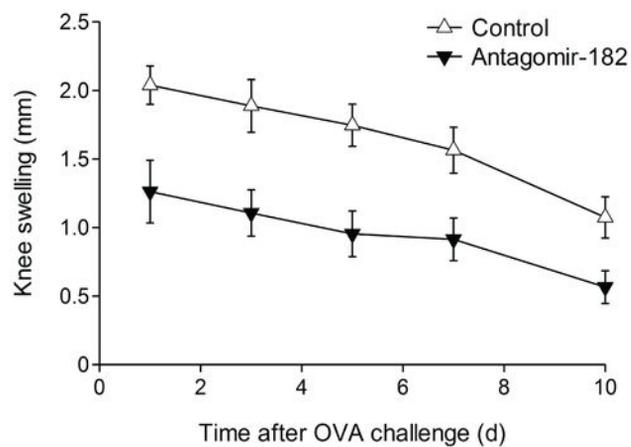
a



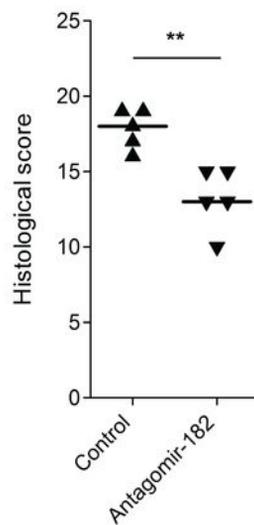
b



c



d



e

