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**Distinct microRNA signatures in human lymphocyte subsets  
and enforcement of CD4<sup>+</sup> T cell naïve state by miR-125b**

Riccardo L. Rossi<sup>1,5</sup>, Grazisa Rossetti<sup>1,5</sup>, Lynn Wenandy<sup>1,5</sup>, Serena Curti<sup>1</sup>, Anna Ripamonti<sup>1</sup>, Raoul J. P. Bonnal<sup>1</sup>, Roberto Sciarretta Birolo<sup>1</sup>, Monica Moro<sup>1</sup>, Maria C. Crosti<sup>1</sup>, Paola Gruarin<sup>1</sup>, Stefano Maglie<sup>1</sup>, Francesco Marabita<sup>1</sup>, Debora Mascheroni<sup>1</sup>, Valeria Parente<sup>1</sup>, Mario Comelli<sup>2</sup>, Emilio Trabucchi<sup>3</sup>, Raffaele De Francesco<sup>1</sup>, Jens Geginat<sup>1</sup>, Sergio Abrignani<sup>1,4</sup> & Massimiliano Pagani<sup>1</sup>

1. Istituto Nazionale Genetica Molecolare (INGM), Via F. Sforza 28, Milan 20122, Italy

2. Dipartimento di Scienze Sanitarie Applicate, Università di Pavia, Via A. Bassi, 21, Pavia 27100, Italy

3. Pio Albergo Trivulzio, Via Trivulzio 15, Milan 20146, Italy

4. IRCCS Ca'Granda Ospedale Maggiore Policlinico, Via F. Sforza 28, Milano 20122, Italy

5. These authors contributed equally to this work

Correspondence should be addressed to: [abrignani@ingm.org](mailto:abrignani@ingm.org); [pagani@ingm.org](mailto:pagani@ingm.org)

20 **ABSTRACT**

22 MicroRNAs are small non-coding RNAs that regulate gene expression post-  
transcriptionally. Here we applied microRNA profiling in seventeen human lymphocyte  
24 subsets to identify microRNA signatures that were distinct among various subsets and  
different from those of mouse lymphocytes. miR-125b, one of the signature microRNAs  
26 of naïve CD4<sup>+</sup> T cells, regulated expression of genes involved in T-cell differentiation  
*i.e.*, *IFNG*, *IL2RB*, *IL10RA* and *PRDMI*. Expression of synthetic miR-125b RNA and  
28 lentiviral vectors encoding pre-miR-125b in naïve lymphocytes inhibited differentiation  
to effector cells. Our data provide an atlas of microRNA expression in human  
30 lymphocytes, define subset-specific signatures and their target genes, and indicate T-cell  
naïve state is enforced by microRNAs.

32

## 34 INTRODUCTION

MicroRNAs (miRNA) are highly conserved non-coding single-stranded small RNA  
36 molecules that control gene expression post-transcriptionally by binding the 3'UTR of  
target mRNAs. This results in degradation or translation inhibition of target mRNAs<sup>1</sup>.  
38 miRNAs are expressed in a tissue-specific manner, and changes in their expression within  
a tissue have been associated with several human diseases, including cancer,  
40 inflammation, and chronic viral infections<sup>2-4</sup>.

Although differentiation and functions of T lymphocytes have been thoroughly  
42 investigated through comprehensive gene expression analysis<sup>5</sup> and several transcription  
factors have been identified as essential for the differentiation of naïve CD4<sup>+</sup> T cells into  
44 different effector subsets<sup>6</sup>, the molecular basis of CD4<sup>+</sup> T helper (T<sub>H</sub>) cell differentiation  
and of the process through which T cells acquire their effector function is incompletely  
46 understood.

miRNAs are differentially expressed during immune cells development and  
48 function<sup>7,8</sup>, and their importance has been highlighted by experiments showing that  
disruption of Ago2 (the core of RNA-Induced Silencing Complex) in hematopoietic cells  
50 results in impaired B cell differentiation<sup>9</sup> and that conditional deletion of Dicer in  
thymocytes induces a strong reduction of T lymphocytes in the thymus and periphery<sup>10,11</sup>.  
52 Moreover, several studies suggest a role for specific miRNAs in lymphocyte biology.  
miR-181 modulates T cell antigen receptor sensitivity<sup>12</sup>, miR-150 regulates the  
54 transcription factor c-Myb<sup>13</sup>, and miR-155 affects differentiation of CD4<sup>+</sup> T lymphocytes  
into T<sub>H</sub>1 cells, the development of regulatory T cells<sup>14,15</sup> and the promotion of T cell-  
56 dependent tissue inflammation<sup>16</sup>. miR-125b is up-regulated in human tonsil germinal



center B lymphocytes where it regulates Blimp-1 expression<sup>17</sup>. miR-125b overexpression  
58 in hematopoietic stem cells causes myeloid leukemia in mice<sup>18-20</sup>, and in humans miR-  
125b is over-expressed in hematological tumors such as acute lymphoblastic leukemia<sup>21</sup>  
60 and acute myeloid leukemia<sup>22</sup>.

Genome wide miRNA analyses have been performed in mouse lymphocyte subsets<sup>23</sup>,  
62 in human tonsil derived B lymphocyte subsets<sup>24</sup> and in total human CD4<sup>+</sup> and CD8<sup>+</sup> T  
cells<sup>25</sup>, however little is known on the miRNAs that regulate differentiation of human T  
64 cell subsets. We performed a broad analysis of miRNA expression in seventeen different  
highly purified human lymphocyte subsets and identified specific miRNA signatures. In  
66 particular, we focused on naïve CD4<sup>+</sup> T cells and found that miR-125b regulates a  
network of genes involved in CD4<sup>+</sup> T cell differentiation including cytokine receptors,  
68 cytokines and transcription factors.

## 70 RESULTS

### microRNA expression in primary human lymphocyte subsets

72 To assess miRNAs expression in human primary lymphocytes, seventeen T, B  
and natural killer (NK) cell subsets were purified by FACS according to established  
74 surface markers<sup>26-30</sup> from peripheral blood mononuclear cells (PBMCs) of three to six  
healthy donors (**Table 1a**). The purified CD4<sup>+</sup> T helper subsets were highly enriched for  
76 cells producing various marker cytokines (**Table 1b**), demonstrating the concordance  
between phenotype and effector functions. TaqMan miRNA Arrays were used to assess  
78 664 miRNAs by RT-qPCR in these purified lymphocyte subsets. An unsupervised  
hierarchical clustering analysis of all the 242 miRNAs that were found expressed in the  
80 subsets analyzed provides an unbiased categorization of samples belonging to NK, T-  
CD4<sup>+</sup>, T-CD8<sup>+</sup> and B lymphocytes, which matches the phenotypic classification of  
82 subsets (**Fig. 1a,b**).

Previous reports have analyzed miRNA expression in five human lymphocyte  
84 subsets (naïve and memory CD4<sup>+</sup> T cells, naïve CD8<sup>+</sup> T cells, naïve B cells and NK cells)  
<sup>24,25</sup>. Our analysis has identified most of the miRNAs (average of 78%) already described  
86 in a particular cell subset by these studies. However, most miRNAs detected by our  
analysis have not been previously identified (**Supplementary Fig. 1**). Thus, our data  
88 provide a thorough quantitative and qualitative “Atlas” (**Supplementary File 1**) of the  
miRNome expression of most human lymphocyte subsets.

### 90 microRNA expression signatures of human lymphocytes

Next we searched for miRNAs that are preferentially expressed in selected human  
92 lymphocytes subsets, i.e., signatures (**Supplementary Table 1**). We therefore compared

the miRNomes of all seventeen cell subsets, selected those miRNAs that had more than 3  
94 fold expression difference in a given subset (one way ANOVA  $p < 0.01$ ) and found that  
twenty-nine miRNAs displayed subset specific expression (Fig. 1c). Within signatures,  
96 we identified miRNAs that were not previously described as selectively expressed in any  
human T lymphocyte subsets, such as miR-125b, miR-193b and miR-188-5p in naïve  
98  $CD4^+$  T cells, and miR-381 in  $CD4^+$   $T_H1$  cells. In the case of a few miRNAs, such as  
miR-146a and miR-21 in  $T_{reg}$  cells, we confirmed their specific expression<sup>31,32</sup>.

100 We also looked for miRNAs that were differentially expressed among different  
differentiation stages of a single lineage, such as  $T_H1$ ,  $T_H2$ , and  $T_H17$  memory cells all  
102 deriving from naïve  $CD4^+$  helper T cells and compared each of these four subset with the  
other three. Twenty miRNAs were up-regulated and 5 down-regulated in naïve  $CD4^+$  T  
104 cells compared to  $CD4^+$  memory subsets. Four miRNAs were found differentially  
expressed in  $T_H1$ , two in  $T_H17$  and four were up-regulated in  $T_H2$  memory cells (**Fig. 1d**).

106 To validate the miRNA signatures identified in primary T lymphocytes *ex vivo*,  
we followed their change in expression after activation *in vitro*. Naïve  $CD4^+$  T cells were  
108 activated and miRNAs expression was monitored at different time points (up to 8 days).  
Nineteen of the twenty miRNAs that were specifically highly expressed in resting naïve  
110  $CD4^+$  T cells were down-regulated upon activation, while there was an increase in the  
expression of four out of five miRNAs that were highly expressed in resting  $CD4^+$   
112 memory cells (**Fig. 1e**). We conclude that human  $CD4^+$  T lymphocyte subsets change  
their specific miRNA signatures during differentiation from naïve to memory cells.

114  
**microRNA signatures in *Homo sapiens* and *Mus musculus***

116 To assess the degree of conservation between the human and mouse immune  
system-specific miRNAs, we compared the human miRNomes and signatures we  
118 identified with those recently published in mouse lymphocyte subsets<sup>23</sup>. Considering the  
data currently available for mouse lymphocytes, we were able to compare six subsets  
120 (CD4<sup>+</sup> naïve, CD4<sup>+</sup> T<sub>H</sub>1, CD4<sup>+</sup> T<sub>H</sub>17, CD4<sup>+</sup> T<sub>H</sub>2, CD4<sup>+</sup> T<sub>reg</sub> and NK). Using the miRBase  
as reference, we compared the human and mouse lymphocyte-miRNomes by a BLAST  
122 search for similarities between mature miRNA sequences. An average 61% of miRNAs  
expressed in the mouse lymphocyte subsets (**Supplementary Fig. 2**) were also expressed  
124 in the corresponding human lymphocytes. We focused our comparison on the forty-six  
miRNAs (**Supplementary Table 2**) that had been identified as signatures of the six  
126 human lymphocyte subsets (**Fig. 1c**) or as miRNA differentially expressed between naïve  
and effector CD4<sup>+</sup> T cell subsets (**Fig. 1d**). Twelve of these forty-six miRNAs did not  
128 have a mouse homologue<sup>33</sup> and twenty-two were not expressed in any of the six mouse  
lymphocyte subsets. Twelve of the forty-six miRNAs were expressed in mouse  
130 lymphocytes<sup>23</sup> (**Fig. 1f**) but only six were expressed with the same profile in man and  
mouse lymphocyte subsets (**Supplementary Fig. 3**).

132 To rule out differences that might be due to different methods used in the different  
studies, we used RT-qPCR Taqman Assays to measure the expression of four miRNAs  
134 specific for human naïve CD4<sup>+</sup> T cells (miR-125b, miR-188-5p, miR-193b and let-7c) in  
mouse naïve and memory CD4<sup>+</sup> T cells. All four miRNAs were highly expressed in  
136 human naïve CD4<sup>+</sup> T cells compared to human memory CD4<sup>+</sup> T cells, while only let-7c  
was expressed in high amounts in naïve CD4<sup>+</sup> T cells in mouse (**Fig. 1g**). We conclude  
138 there is a relatively good (61%) conservation of the overall lymphocyte miRNomes, but a

poor concordance (6 out of 46) of the lymphocyte miRNA signatures between man and  
140 mouse.

#### 142 **miR-125b targets genes in naïve CD4<sup>+</sup> T cells**

Next we sought to identify the mRNA targets regulated by miRNAs in naïve  
144 CD4<sup>+</sup> T cells that might be involved in the differentiation process toward effector-  
memory cells. We focused on miR-125b because it displayed the strongest differential  
146 expression between naïve CD4<sup>+</sup> T cells and effector-memory lymphocyte subsets (**Fig.**  
**1c, Fig. 2a**). We combined target prediction with gene expression analysis to identify  
148 those transcripts that exhibited an inverse relationship with the expression level of miR-  
125b (Anti-Correlated-Predicted-Target-mRNAs, ACEPTs) and found 72 ACEPTs for  
150 miR-125b (**Fig. 2b** and **Supplementary Table 3**). To assess the potential role of these 72  
transcripts in T cell differentiation, we predicted their biological functions with Ingenuity  
152 Pathway Analysis, which maps bio-molecular networks based on known pathways, gene  
ontology and interactions with reliable data curation. The 72 miR-125b ACEPTs are  
154 significantly enriched for the biological function “Cell-mediated immune response” and  
the canonical pathway “T Helper Cell Differentiation” (**Fig. 2c**). Furthermore, miR-125b  
156 ACEPTs generate a network of 133 gene-products (**Fig. 2d**) that include several  
molecules involved in T cell differentiation such as cytokines, cytokine receptors and  
158 transcription regulators. In conclusion, the integrated analysis of gene expression and  
target prediction suggests that miR-125b regulates key genes involved in naïve CD4<sup>+</sup> T  
160 cell differentiation.

## 162 **miR-125b suppresses CD4<sup>+</sup> T cell differentiation**

We assessed the inhibitory effect of miR-125b on 14 genes belonging to the miR-125b ACCEPT group and known to play a role in CD4<sup>+</sup> T cell differentiation. The 3' UTRs of these genes were cloned after a luciferase reporter gene and co-transfected into HEK-293T cells with a synthetic mature miR-125b double strand RNA or a scrambled control. miR-125b directly regulated four of the 14 UTRs, *i.e.*, interferon- $\gamma$  (*IFNG*), interleukin 2 receptor  $\beta$  (*IL2RB*), interleukin 10 receptor  $\alpha$  (*IL10RA*) and Blimp-1 (*PRDMI*) (**Fig. 3a**). Deletion of the miR-125b responsive elements in these UTRs resulted in the abrogation of the inhibitory effect of miR-125b (**Fig. 3b**). Because miR-125b and its precursors are down-modulated in naïve CD4<sup>+</sup> T cells after polyclonal activation *in vitro* (**Fig. 1e** and **Supplementary Figure 5**), we assessed the direct regulation of the four targets following miR-125b overexpression in primary cells. We assessed the expression levels of *IFNG*, *IL2RB*, *IL10RA* and *PRDMI* transcripts by RT-qPCR three days after the transduction of naïve CD4<sup>+</sup> T cells with lentiviral vectors expressing miR-125b (LV-125b) or a control lentivirus (LV-ctrl). Prolonged expression of miR-125b resulted in reduced levels (29 to 64%) of all four transcripts (**Fig. 3c**).

178 Compared with the human genes, the 3'UTR of the mouse *IFNG* and *IL10RA* genes do not have the predicted miR-125b responsive-elements, while the mouse *IL2RB* gene has one miR-125b responsive-elements compared to the three of the human gene. Such comparative analysis indicates that only the *PRDMI* mouse gene has the predicted miR-125b responsive-elements (**Fig. 3d** and **Supplementary Table 4**). These data demonstrate that miR-125b directly regulates the naïve T cells expression of genes required for the differentiation of effector T lymphocytes. Most of miR-125b target genes

186 identified in man lack the miR-125b responsive-elements in the 3'UTR of the mouse  
homologues, correlating with the poor expression of miR-125b in naïve CD4<sup>+</sup> T cells in  
mouse.

188

### **miR-125b enforces naïveness of CD4<sup>+</sup> T cells**

190 Because miR-125b directly regulates genes involved in the differentiation of naïve CD4<sup>+</sup>  
T cells, we assessed the impact of miR-125b on the acquisition of the effector-memory  
192 phenotype. Naïve CD4<sup>+</sup> T cells were activated by T cell receptor (TCR) engagement,  
transfected with synthetic miR-125b RNA (mimic-125b) and then stained with effector-  
194 memory marker mAbs. Three to five days after activation cells transfected with mimic-  
125b showed decreased surface expression of markers of T<sub>H</sub>1 and T<sub>H</sub>2 memory CD4<sup>+</sup> T  
196 cells, such as CXCR3 and CCR4. In parallel, we observed an increased expression of the  
naïve CD45RA<sup>+</sup> CD45RO<sup>-</sup> phenotype, while control markers (CD4 and MHC class I)  
198 were not affected (**Fig. 4a**). In addition, mimic-125b decreased the expression of the two  
cytokine receptors (IL-2R $\beta$  and IL-10R $\alpha$ ) that represent direct miR-125b targets (**Fig.**  
200 **4b**). Analysis of CD4<sup>+</sup> T cell proliferation with a Ki-67 mAb, which is expressed  
exclusively in proliferating cells, and using a CFSE dilution assay indicated that *in vitro*  
202 proliferation of naïve CD4<sup>+</sup> T cells was not affected by transfection with mimic-125b  
(**Fig. 4c**). These results indicate that miR-125b expression contributes to the maintenance  
204 of naïveness in primary human T cells and suggest that its down-modulation is associated  
with the acquisition of effector-memory phenotypes by CD4<sup>+</sup> T cells.

206

### **miR-125b regulates acquisition of effector function**

208 To assess whether prolonged expression of miR-125b affected the acquisition of  
effector function, naïve CD4<sup>+</sup> T cells were activated and transduced with lentiviral  
210 vectors encoding pre-miR-125b (LV-125b) or a control lentivirus (LV-ctrl). After 8 days  
of culture, cells were tested for effector function after stimulation for 6 hours with  
212 phorbol 12-myristate 13-acetate (PMA) plus ionomycin. Naïve CD4<sup>+</sup> cells activated in  
the presence of ectopic miR-125b displayed a reduced effector function as demonstrated  
214 by the decreased production of IFN- $\gamma$  and IL-13 detected both intracellularly (**Fig. 5a,b**)  
and in the culture supernatants (data not shown), whereas cells transduced with the  
216 control lentivirus showed effector function similar to untransduced controls. Comparable  
results were obtained using a lentivirus vector expressing a mutated miR-125b, as well as  
218 mimic-125b (**Supplementary Fig. 4**), miR-125b expression in cells transduced with LV-  
125b was similar to that observed in untransduced primary naïve cells (**Fig. 5c**),  
220 indicating that ectopic expression of miR-125b was within the physiological range. These  
results strongly suggest that miR-125b plays a key role in preventing the acquisition of  
222 effector functions by primary CD4<sup>+</sup> T cells.



## 224 **DISCUSSION**

226 Here we perform an analysis of the miRNomes in seventeen major human lymphocyte subsets and show that the specific miRNAs signatures that mark these subsets have a poor concordance between human and mouse lymphocytes. Identification of miRNA target genes shows that miR-125b, one of the miRNA that is highly specific for naïve CD4<sup>+</sup> T cells, directly regulates a set of genes important to the differentiation into effector-memory lymphocytes and its prolonged expression during differentiation reduces the acquisition of effector function.

232 miRNomes provide a molecular categorization of different lymphocyte subsets that is coherent with their phenotypic characterization and suggests that selected miRNAs play a role in regulating the differentiation of these cell subsets. The comparison of the subset-specific miRNomes identified in this study with the published human miRNomes<sup>34</sup> revealed that our approach identified up to 80% of the published miRNAs and identified additional miRNAs, possibly due to the sensitivity of the approach. Because TaqMan miRNA Arrays require only a few nanograms of RNA, this approach is suitable for very rare lymphocyte subsets, allowing us to obtain the widest available microRNA profile of human lymphocyte subsets.

242 The comparison of miRNomes obtained in various studies and from different species can be challenging, because the nomenclatures used in different technological platforms are not always aligned. To address this issue we first created a database that incorporates and integrates the different nomenclatures of the various miRBase versions. Secondly, because deep sequencing technology often identifies microRNAs with codes that are different from standard miRBase nomenclature, we used the mouse miRNA

genomic coordinates and mature sequences to translate the deep sequencing data into  
248 miRBase-compliant nomenclature. Thus, comparison of the miRNA signatures between  
mouse and man lymphoid subsets was possible and revealed a surprisingly low 13%  
250 concordance. In particular, three (miR-193b, miR-188-5p and miR-125b) of the four  
miRNAs that constitute a signature of naïveness in human CD4<sup>+</sup> T cells are almost absent  
252 in mouse cells.

The identification of miRNA target genes is challenging due to the fact that  
254 miRNAs bind to target mRNAs by incomplete sequence pairing over a short sequence.  
Several *in silico*, molecular and biochemical approaches have been used in the last few  
256 years to identify miRNA targets, each one with strengths and weaknesses<sup>35</sup>. Our approach  
is extremely helpful in restricting the number of putative target genes that can be  
258 predicted *in silico*. Indeed, using the miR-125b ACEPT gene list, we restricted our target  
gene search from the 2058 predicted target genes to 72 miR-125b ACEPT genes. Cloning  
260 of the whole 3'UTRs and dual-luciferase assay revealed that 4 out of 14 of these 72  
potential targets were directly regulated by miR-125b.

262 Our findings suggest that the naïve state is, at least in part, actively preserved by  
miR-125b that reduces the expression of important genes below the levels required to  
264 confer effector-memory identity to a naïve cells. The T<sub>H</sub>1 cytokine IFN- $\gamma$  represents a  
highly relevant target, because it is the most abundant effector cytokine in human  
266 memory T cells and it is not secreted by naïve T cells. The IL-2R $\beta$  chain is also critical  
for memory cell identity, because it mediates IL-15-dependent homeostatic self-renewal  
268 of memory T cells, while naïve T cells lack IL-2R $\beta$ , do not respond to IL-15 and their  
homeostasis depends on IL-7 and TCR tickling induced by self-MHC<sup>36-38</sup>. Blimp-1 is

270 known to promote terminal T cell differentiation<sup>39</sup>. miR-125b alone cannot account for all  
the complex series of changes in gene expression and functions that mark differentiation  
272 of naïve to memory T cells, and it is likely the other miRNA signatures of naïve CD4<sup>+</sup> T  
cells (miR-188-5p, miR-193b and let-7c) play a role. However, the importance of the  
274 miR-125b targets outline its relevance in the enforcement of the naïve state of CD4<sup>+</sup> T  
cells.

276 Although mouse and man mostly utilize these same genes for the differentiation  
of naïve to memory T cells, we found that three (IFN- $\gamma$ , IL10R $\alpha$  and IL2R $\beta$ ) of the four  
278 genes that are the targets of miR-125b in man, do not have the miR-125b responsive  
elements in their 3'UTR in mouse. This findings, together with the observation that  
280 mouse naïve T cells express very little miR-125b, suggest a dichotomy between the genes  
key to naïve T cell differentiation and their regulatory miRNAs between man and mouse,  
282 and underscore the necessity of studying the role of miRNAs in human lymphocyte  
differentiation primarily with human cells.

284 From a translational medicine point of view, the importance of deciphering the  
miRNome in human T lymphocyte is twofold. First, identification of miRNA signatures  
286 and definition of their target genes that specifically regulate differentiation of the various  
T cell subsets may provide novel therapeutic targets for immune-mediated diseases and  
288 give a better definition of subsets. Second, because miRNAs are released in a stable form  
outside cells<sup>40</sup>, it is possible that lymphocyte subset miRNA signatures circulate in the  
290 blood in a cell-free state, and could therefore be used as sensitive biomarkers of different  
T cell subsets that are active in distant tissues.

292

## **DATABASE ACCESSION NUMBERS**

294 Raw and normalized gene expression data are available for download in the GEO format  
with accession number GSE22880.

296

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302 CFI

All authors declare that they have no competing financial interests with this work.

304

## **AUTHOR CONTRIBUTIONS**

306 R.L.R, G.R. and L.W. designed and did experiments, analyzed data and wrote the  
manuscript; M.M. and M.C.C. did FACS experiments; R.J.P.B. performed bioinformatic

308 analyses; M.C. did statistical analyses; R.S.B., S.C., S.M., P.G., F.M., D.M. V. P. and  
A.R. did experiments and analyzed data; E.T. provided conceptual advice; R.D.F.

310 discussed the results, provided conceptual advice and commented on the manuscript; J.G.  
designed and supervised research and wrote the manuscript; S.A. and M.P. designed the

312 study, supervised research and wrote the manuscript.

314 **FIGURES LEGENDS**

**Table 1** Purification of human primary lymphocyte subsets. **(a)** Seventeen human lymphocyte subsets were isolated from Peripheral Blood Lymphocytes. Purity is that achieved after sorting; Sorting phenotype indicates surface marker combinations used for cell sorting. **(b)** Cytokines produced by the purified T helper cell subsets following polyclonal stimulation with PMA and ionomycin. Cells co-producing IFN- $\gamma$  and IL-4 or IFN- $\gamma$  and IL-17 were very low (< 1%) or undetectable (data not shown)

**Figure 1** microRNA expression describes and discriminates among different lymphocyte subsets. **(a)** Unsupervised hierarchical clusters of the miRNAs detected for each cell subset analyzed. 242 subset-specific miRNAs were selected using one-way Anova analysis (p-value 0.001). Yellow to blue gradient indicates expression values ( $\Delta$ CT log transformed and mean centered). The bracketed letters in the sample names indicates the individual donor. **(b)** Cell-lineages segregation and colour codes of various lymphocyte-subsets as in panel **a** **(c)** Heat map of average expression values (z-scores of  $\Delta$ CT) for mature miRNAs across all 17 lymphocyte subsets profiled. **(d)** z-scores heat map of signature miRNAs for naïve CD4<sup>+</sup>, T<sub>H</sub>1, T<sub>H</sub>17 and T<sub>H</sub>2 cell subsets. Differentially expressed miRNAs were selected with the comparative CT method ( $\Delta\Delta$ CT) and filtered for statistical significance Anova test (p < 0.05). Each one of the four subsets was in turn considered as reference and compared with the remaining three subsets. **(e)** Heat map of relative quantities (RQ) for 25 miRNAs assessed by RT-qPCR in naïve CD4<sup>+</sup> T cells activated with anti-CD3 and anti-CD28 mAbs at the indicated time points compared to time zero. **(f)** Expression of 46 miRNAs representing the human lymphocytes signatures

of the six lymphocyte subsets (naïve CD4<sup>+</sup> T cells, T<sub>H</sub>1, T<sub>H</sub>17, T<sub>H</sub>2 and NK cells) for  
338 which mouse data are published<sup>23</sup>. **(g)** Expression of miR-125b, miR-188-5p, mir-193b  
and let-7c assessed by TaqMan assays in naïve and memory CD4<sup>+</sup> T cells purified from  
340 human PBMC and mouse spleen.

342 **Figure 2** Identification of putative miR-125b target genes in naïve CD4<sup>+</sup> T cells and  
description of their functional relationships. **(a)** RT-qPCR data of miR-125b expression  
344 in lymphocyte subsets is shown as mean expression values. **(b)** Venn diagram of CD4<sup>+</sup> T  
naïve and memory cells transcripts and miR-125b predicted targets (TargetScan 5.1). 857  
346 are EPTs (Expressed Predicted Targets -in red-), Pie chart shows the distribution of EPT  
genes according to their memory/naïve (M/N) expression ratio. **(c)** Enrichment of the “T  
348 Helper Cell Differentiation” canonical pathway genes, according to IPA, in the EPT  
(M/N>1.5) group. Control groups were the expressed genes (M/N>1.5), EPT (M/N<1.5)  
350 and the predicted targets. Significance is plotted as  $-\log_2(\text{p-values})$ . **(d)** Molecular  
network of functional relationships extracted from IPA knowledge base for the EPT with  
352 a M/N>1.5. The graph shows direct interactions generated by genes that are “network  
eligible” according to IPA (dark blue); Molecules in the network that are predicted to be  
354 miR-125b targets are in light blue; In white other network molecules.

356 **Figure 3** miR-125b directly regulates genes key to T cell differentiation. **(a)** Dual  
luciferase assay of 14 genes predicted to be regulated by miR-125b. The luciferase  
358 constructs was transfected into HEK-293T cells together with synthetic mature miR-125b  
(black bars) or a synthetic scrambled control (white bars). An empty vector and a non

360 miR-125b target transcript (GAPDH) were used as negative controls while a vector with a  
tandem stretch of six miR-125b target sites (Sponges) was the positive control. The  
362 dashed line represents the threshold (75%) of luciferase activity. The values represent  
average  $\pm$  SEM (n=18). T-test results are indicated by (\*\*\*)  $P < 0.001$  relative to the  
364 scrambled transfected controls. **(b)** miR-125b miRNA responsive element was deleted in  
the 3'UTR of *IFNG*, *IL10RA*, *IL2RB* and *PRDMI*, and luciferase activity was assessed as  
366 described above. The values represent average  $\pm$  SEM (n=18) Anova test significance is  
indicated by (\*\*\*)  $P < 0.001$ . **(c)** Expression levels of *IFNG*, *IL10RA*, *IL2RB* and *PRDMI*  
368 transcripts were measured by RT-qPCR in activated naïve CD4<sup>+</sup> T cells transduced with  
lentiviral vectors encoding miR-125b (LV-125b) or mock control (LV-ctrl). The values  
370 represent average  $\pm$  SEM of 3 independent experiments. **(d)** Scheme of miR-125b  
responsive elements (colour coded) predicted by Targetscan5.1 in human and mouse  
372 *IFNG*, *IL10RA*, *IL2RB* and *PRDMI* transcripts.

374 **Figure 4** miR-125b preserves naïve state of CD4<sup>+</sup> T cells. Purified peripheral blood naïve  
CD4<sup>+</sup> T cells were stimulated with anti-CD3 and allo-PBMCs and nucleofected with  
376 mimic-125b or scrambled control. **(a)** Cells were stained at day 3 for CXCR3 and CCR4  
or at day 5 for CD45RA, CD45RO, CD4 and MHC I surface expression by flow  
378 cytometry. **(b)** Cells were analyzed after three days for IL10R $\alpha$ , and IL2R $\beta$  surface  
expression by flow cytometry. **(c)** Purified, unlabeled or CFSE-labeled naïve CD4<sup>+</sup> T  
380 cells were stimulated with anti-CD3 and allo-PBMCs and nucleofected with mimic-125b  
or scrambled control, proliferation was assessed respectively by Ki67 staining or CFSE  
382 dilution at day 5. Numbers indicate percentages. Similar results were obtained in three

independent experiments

384

**Figure 5** miR-125b regulates naïve CD4<sup>+</sup> T cell differentiation. **(a)** and **(b)** Purified naïve  
386 CD4<sup>+</sup> T cells were stimulated with anti-CD3 and anti-CD28 antibodies and transduced  
with lentiviral vectors encoding miR-125b (LV-125b), or mock control (LV-ctrl). After  
388 seven days, cells were re-activated with PMA and Ionomycin and the acquisition of  
effector function was assessed as production of IFN- $\gamma$  and IL13 by intracellular staining  
390 after 6 hours. Mean and statistical significance of six independent experiments are shown  
in panel b. **(c)** miR-125b expression levels measured by RT-qPCR in naïve CD4<sup>+</sup> T cells  
392 stimulated for seven days with anti-CD3 and anti-CD-28 antibodies, which have been  
transduced with control (LV-ctrl) or lentiviral vectors encoding miR-125b (LV-125b). As  
394 control we used untransduced primary naïve CD4<sup>+</sup> T cells and CD4<sup>+</sup> T<sub>H</sub>1 memory cells.  
The mean of 3 independent experiments is shown.

396



## METHODS

398

### **Purification of human lymphocyte subsets and naïve T cell differentiation experiments.**

400

Buffy-coat blood of healthy donors was obtained from the IRCCS Policlinico Ospedale Maggiore in Milano, Italy and PBMCs were isolated by Ficoll centrifugation. Lymphocyte subsets were purified by cell sorting on a FACS Aria (BD) using different combinations of surface markers (Table 1). The ethical committee of IRCCS Policlinico Ospedale Maggiore in Milano (Italy) approved the use of PBMCs of healthy donors for research purposes and informed consent was obtained from all the subjects involved in this study. For *in vitro* differentiation to effector cells, resting naïve CD4<sup>+</sup> T lymphocytes were purified (> 98%) by negative selection with magnetic beads and activated at 1x10<sup>5</sup> cells/well on 96 well MaxiSorp plates (Nunc) coated with anti-CD3 (0.1 µg/ml) and anti-CD28 (6 µg/ml) antibodies (BD) and IL-2 at 20 IU/ml (Novartis). After seven days, cells were stimulated for 6 hrs with PMA-Ionomycin and effector function was assessed as IFN-γ production by intracellular staining with Pb-conjugated anti-IFN-γ antibody (BD). IL-13 production was assessed with PE-conjugated anti-IL13 antibody (BD)

414

### **RNA isolation and miRNA-mRNA expression profiling.**

416 Total RNA was isolated using the mirVana Kit (Ambion). The Megaplex protocol with pre-amplification was performed. Pre-amplified RT product was amplified using TaqMan Low Density Arrays (Applied Biosystems). Gene expression was performed on naïve CD4<sup>+</sup> and memory T cells with Illumina direct hybridization assays: cRNA was generated according to the Illumina protocol (Ambion); Hybridization and scanning were

420

performed on an Illumina iScan System and data were processed with BeadStudio v.3.

422 Both raw and normalized gene expression data are available for download in the GEO  
format with accession number GSE22880.

424

### **Data treatment, statistics and bioinformatics**

426 The median of three endogenous controls was used as computed control and  $\Delta$ CT were  
calculated. Raw CT values are available in the supplementary materials. MiRNA  
428 expressed in at least two thirds of samples analyzed were selected with a 1-way ANOVA  
test. miRNAs signatures for specific cell subsets were determined as detailed in the  
430 supplemental experimental procedures.

TargetScan 5.1 was used for miR-125b target predictions; expressed predicted targets  
432 were selected comparing the predicted targets with the genes expressed in naïve and-or  
memory CD4<sup>+</sup>T cells from gene expression experiments. Pathway-function analysis was  
434 performed with IPA and statistical relevance was determined with the built-in Fisher  
exact test.

436

### **Molecular biology**

438 miRNA expression levels on specific human and mouse populations was made using  
Taqman MiRNA assays (Applied Biosystems) according to the manufacturer protocol.  
440 miR-125b direct target expression levels were assessed by TaqMan Gene Expression  
assays (Applied Biosystems). 3'UTRs of 14 human miR-125b target genes were  
442 amplified from Human Testes or Human Fetal Brain cDNA, transferred to psiCHECK-2  
vector (Promega). The sponge control vector was designed as a repeat of six miR-125b

444 responsive elements (MRE). Mutated 3'UTRs were obtained deleting the miR-125b MRE  
in the 3' UTR by PCR mutagenesis, except for IL2R $\beta$ 's construct that was synthesized  
446 (Genart AG). Precursor miR-125b encoding lentiviral vector (pLV-125b) was purchased  
by SBI. Primers sequences are described in the supplementary experimental procedures.

448

#### **Dual luciferase assay**

450 HEK293T cells were cotransfected with 10 ng of each psiCHECK-2 construct along with  
20nM miR-125b duplexes or scrambled control (Qiagen) using Lipofectamine2000  
452 (Invitrogen). After 24 hours, the cells were lysed and Firefly and Renilla Luciferase  
activities were measured with the Dual Luc Reporter System (Promega). The whole  
454 3'UTRs were cloned in psiCHECK-2 immediately downstream the Renilla luciferase  
gene. Results were expressed as the ratio of Luciferases activities.

456

#### **Naïve CD4<sup>+</sup> T cells nucleofection**

458 Purified CD4<sup>+</sup> naïve T Cells, unloaded or pre-loaded with CFSE, were nucleofected with  
miR-125b duplexes or scrambled control (Dharmacon) using T Cell Nucleofector kit  
460 (VAPA-1002 Lonza). Cells were plated in complete medium and stimulated with soluble  
 $\alpha$ CD3 0.1  $\mu$ g/ml (clone TR66) and allo-PBMCs in a 96 well U-bottom plate in the  
462 presence of IL2 100 U/ml,  $\alpha$ IL4,  $\alpha$ IFN- $\gamma$  and  $\alpha$ IL12 (R&D) antibodies at 2  $\mu$ g/ml.  
Differentiation and proliferation markers and miR-125b direct targets were analyzed after  
464 surface staining using the following fluorescence-labeled antibodies from BD  
Biosciences: APC-CD45RO (clone UCHL1), PECy7-CD45RA (clone-H1100), PE-  
466 CD122 (clone MIK $\beta$ 3), PB-CD4 (clone RPA-T4), PECy5-CD183 (clone 1C6/CXCR3),

PE-CCR4 (clone 1G1), PE-Cy5-HLA-ABC (clone G46-2.6), PE-CDw210 (clone 3F9). An  
468 intracellular staining was done according to the manufacturer's protocol for Ki67 using  
PE-Ki67 (clone B56). A FACSCantoII and FlowJo887 software were used for data  
470 acquisition and analysis.

#### 472 **Lentivirus production and transduction**

Lentiviral particles were produced according to the standard protocol indicated in the SBI  
474 User Manual instructions. Naïve CD4<sup>+</sup> T cells were activated as described above and  
simultaneously transduced with either pLV-ctrl vector or pLV-125b at MOI:  $1 \times 10^7$   
476 TU/ml in the presence of polybrene (8 $\mu$ g/ml). Cells were detached on day 3 and  
transduction efficiency measured by the percentage of GFP positive cells by flow  
478 cytometry. Transduced cells were then replated on uncoated wells and cultured as  
described above.

480

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**Table 1****a**

Lymphocyte subsets	Number of donors	Purity (%)	Sorting phenotype
CD4 <sup>+</sup> naïve	4	99,8 ±0,1	CD4+, CCR7+, CD45RA+, CD45RO-
CD4 <sup>+</sup> T <sub>H</sub> 1	6	99,9 ±0,05	CD4+, CXCR3+, CCR6-, CD161-
CD4 <sup>+</sup> T <sub>H</sub> 17	4	99,1 ±1	CD4+, CCR6+, CD161+, CXCR3-
CD4 <sup>+</sup> T <sub>H</sub> 2	4	99,7 ±0,3	CD4+, CRTH2+, CXCR3-
CD4 <sup>+</sup> Treg	3	99,0 ±0,8	CD4+, CD127dim, CD25+
CD4 <sup>+</sup> memory	3	99,8 ±0,2	CD4+, CD45RA-, CD45RO+
CD4 <sup>+</sup> T <sub>CM</sub>	3	98,4 ±2,8	CD4+, CCR7+, CD45RA-, CD45RO+
CD4 <sup>+</sup> T <sub>EM</sub>	3	95,4 ±5,5	CD4+, CCR7-, CD45RA-, CD45RO+
CD4 <sup>+</sup> T <sub>EMRA</sub>	3	96,5 ±4	CD4+, CCR7-, CD45RA+, CD45RO-
CD8 <sup>+</sup> naïve	3	99,3 ±0,2	CD8+, CCR7+, CD45RA+, CD45RO-
CD8 <sup>+</sup> T <sub>CM</sub>	3	98,3 ±0,8	CD8+, CCR7+, CD45RA-, CD45RO+
CD8 <sup>+</sup> T <sub>EM</sub>	3	96,8 ±0,9	CD8+, CCR7-, CD45RA-, CD45RO+
CD8 <sup>+</sup> T <sub>EMRA</sub>	3	98,1 ±0,3	CD8+, CD45RA+, CCR7-, CD45RO-
NK cells	3	99,2 ±0,3	CD56+, CD3-
CD5 <sup>+</sup> B cells	3	99,1 ±0,8	CD19+, CD5+
naïve B cells	3	99,9 ±0,1	CD19+, CD5-, CD27-
memory B cells	3	99,1 ±0,8	CD19+, CD5-, CD27+

**b**

	CYTOKINES (%)		
	IFN- $\gamma$	IL-17	IL-4
T <sub>H</sub> 1	<b>50.5</b>	0.9	0.6
T <sub>H</sub> 17	4.5	<b>13.1</b>	0.6
T <sub>H</sub> 2	0.6	0.6	<b>57</b>

**Figure 1**

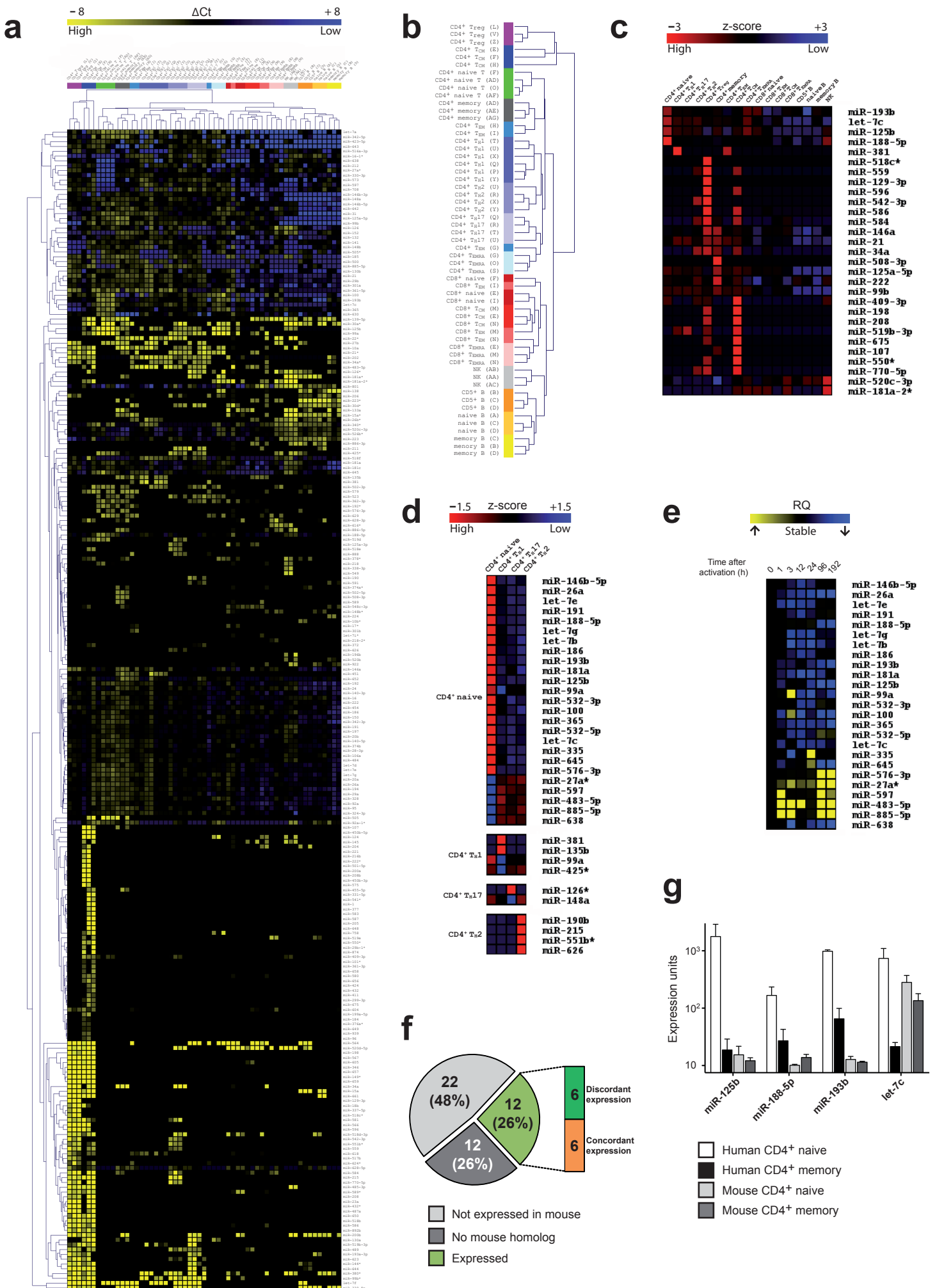


Figure 2

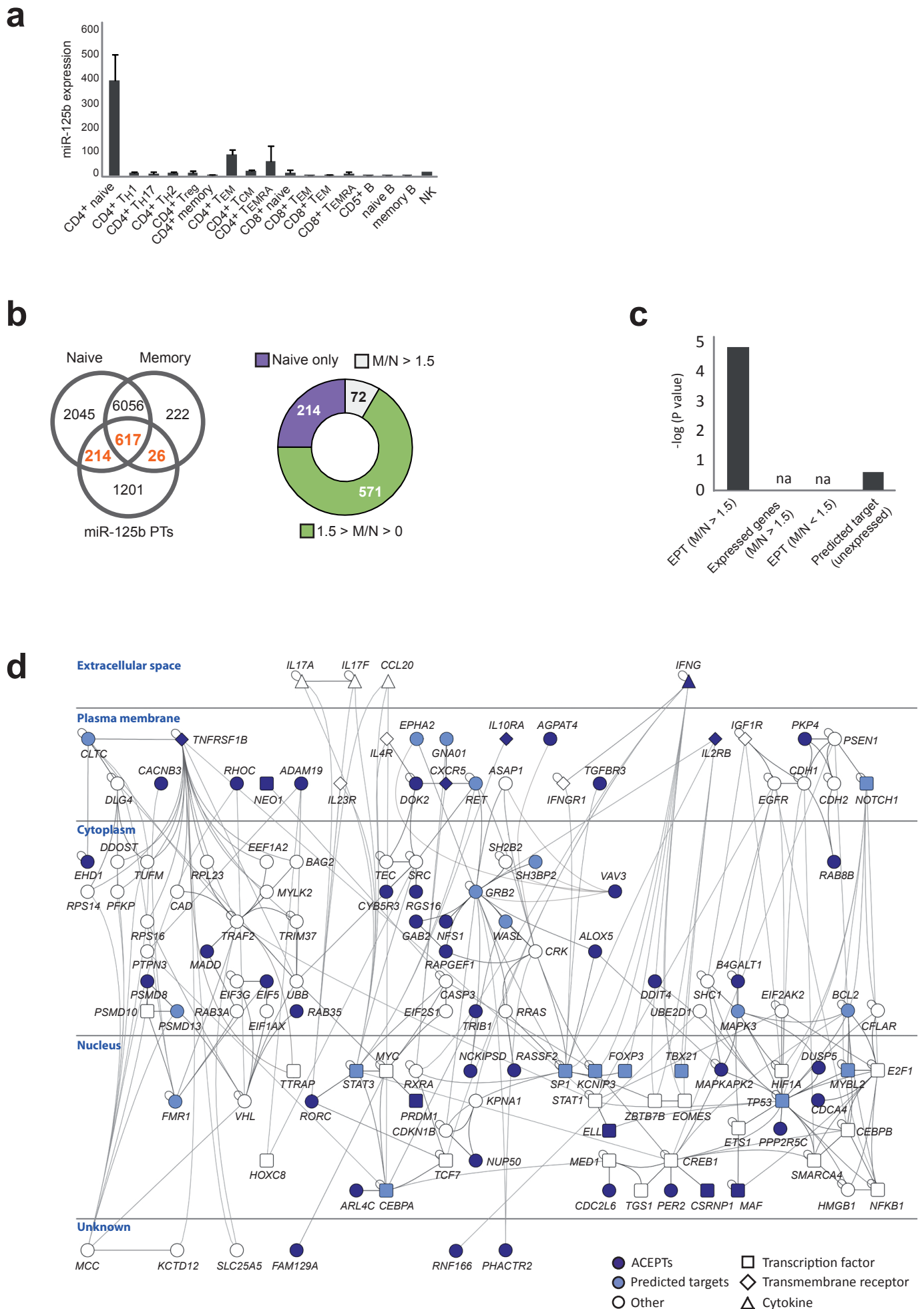
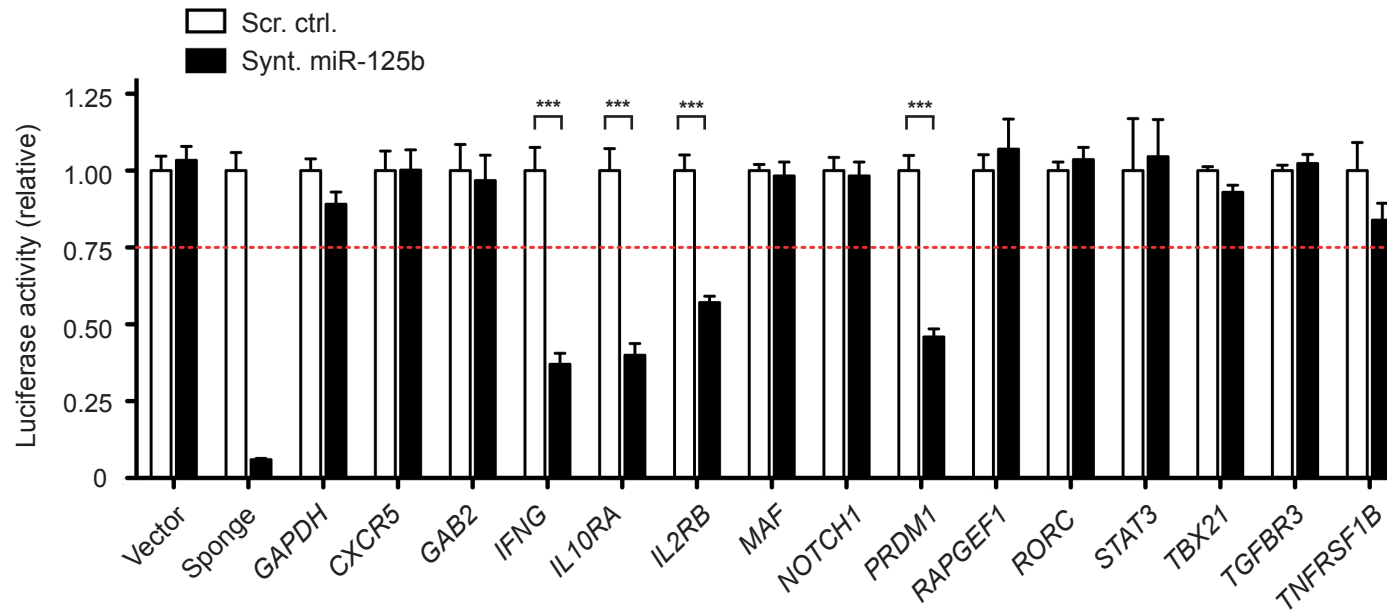
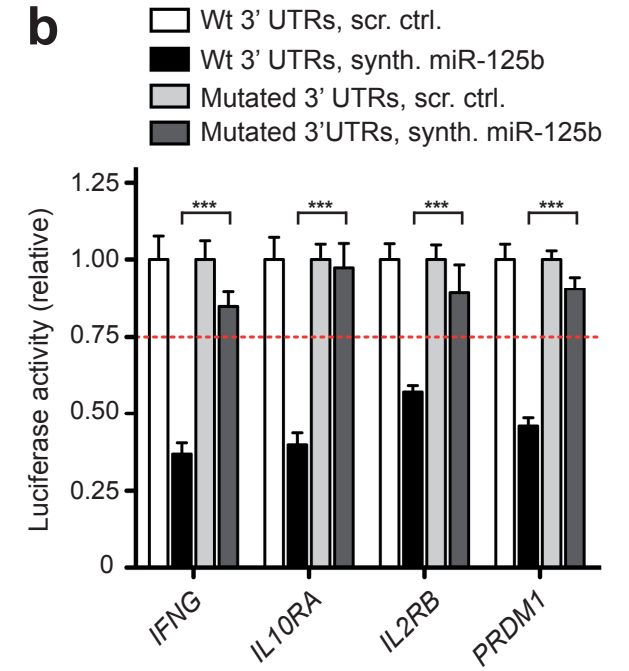


Figure 3

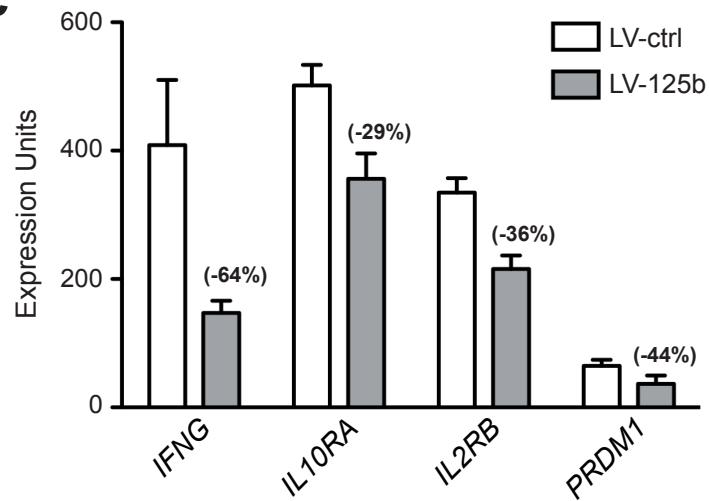
**a**



**b**



**c**



**d**

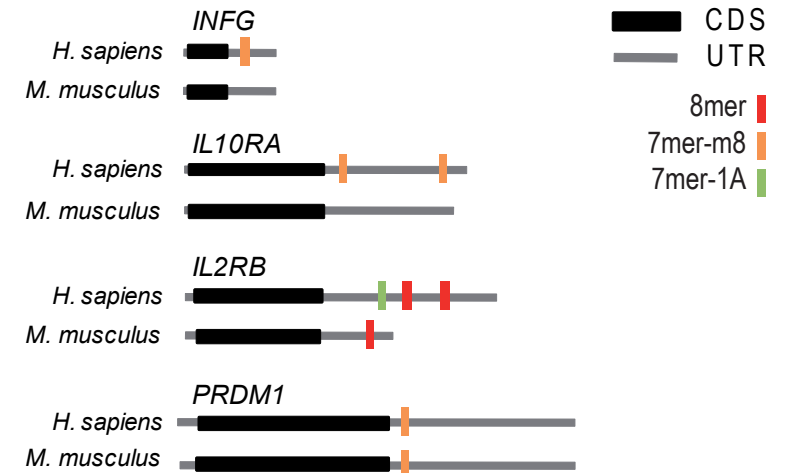


Figure 4

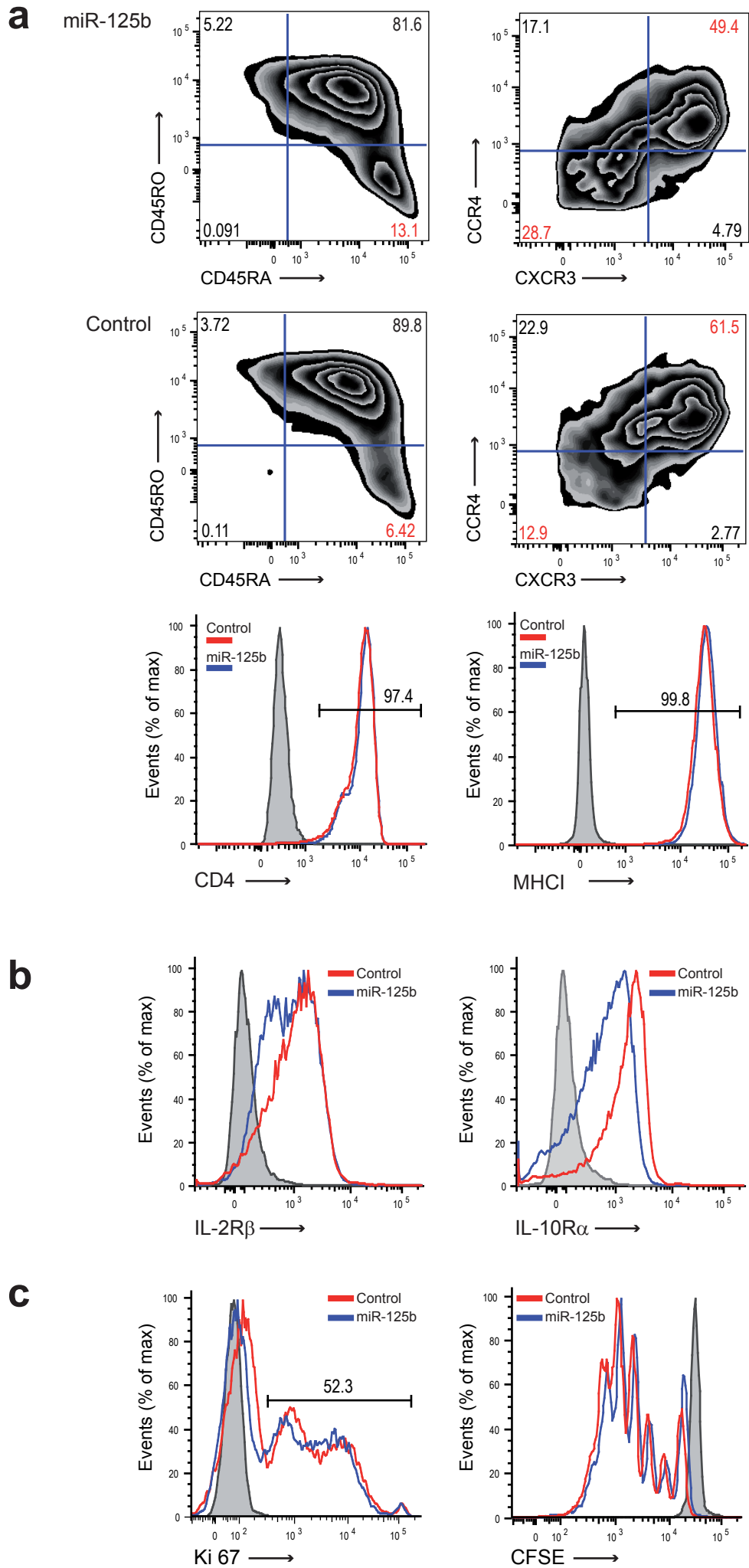


Figure 5

