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Distinct microRNA signatures in human lymphocyte subsets and enforcement of CD4^{+} T cell naïve state by miR-125b

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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 $CD4^+$ T cells, regulated expression of genes involved in T-cell differentiation *i.e., IFNG*, *IL2RB*, *IL10RA* and *PRDM1*. 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[1].
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[2-4].

Although differentiation and functions of T lymphocytes have been thoroughly
42 investigated through comprehensive gene expression analysis[5] and several transcription factors have been identified as essential for the differentiation of naïve $CD4^+$ T cells into
44 different effector subsets[6], the molecular basis of $CD4^+$ T helper (T_H) 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[7,8], 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[9] and that conditional deletion of Dicer in thymocytes induces a strong reduction of T lymphocytes in the thymus and periphery[10,11].
52 Moreover, several studies suggest a role for specific miRNAs in lymphocyte biology. miR-181 modulates T cell antigen receptor sensitivity[12], miR-150 regulates the
54 transcription factor c-Myb[13], and miR-155 affects differentiation of $CD4^+$ T lymphocytes into T_H1 cells, the development of regulatory T cells[14,15] and the promotion of T cell-
56 dependent tissue inflammation[16]. miR-125b is up-regulated in human tonsil germinal

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

Genome wide miRNA analyses have been performed in mouse lymphocyte subsets[23],
62 in human tonsil derived B lymphocyte subsets[24] and in total human $CD4^+$ and $CD8^+$ T cells[25], 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 $CD4^+$ T cells and found that miR-125b regulates a network of genes involved in $CD4^+$ 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[26-30] from peripheral blood mononuclear cells (PBMCs) of three to six healthy donors (**Table 1a**). The purified $CD4^+$ 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-$CD4^+$, T-$CD8^+$ 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 $CD4^+$ T cells, naïve $CD8^+$ T cells, naïve B cells and NK cells) [24,25]. 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[31,32].

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[23]. Considering the data currently available for mouse lymphocytes, we were able to compare six subsets
120 ($CD4^+$ naïve, $CD4^+$ T_H1, $CD4^+$ T_H17, $CD4^+$ T_H2, $CD4^+$ T_{reg} 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 $CD4^+$ T cell subsets (**Fig. 1d**). Twelve of these forty-six miRNAs did not
128 have a mouse homologue[33] 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[23] (**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 $CD4^+$ T cells (miR-125b, miR-188-5p, miR-193b and let-7c) in mouse naïve and memory $CD4^+$ T cells. All four miRNAs were highly expressed in
136 human naïve $CD4^+$ T cells compared to human memory $CD4^+$ T cells, while only let-7c was expressed in high amounts in naïve $CD4^+$ 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$^+$ T cells**

Next we sought to identify the mRNA targets regulated by miRNAs in naïve
144 CD4$^+$ 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$^+$ 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 asses 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$^+$ T
160 cell differentiation.

162 **miR-125b suppresses CD4$^+$ T cell differentiation**

We assessed the inhibitory effect of miR-125b on 14 genes belonging to the miR-
164 125b ACEPT group and known to play a role in CD4$^+$ T cell differentiation. The 3' UTRs of these genes were cloned after a luciferase reporter gene and co-transfected into HEK-
166 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-γ (*IFNG*) , interleukin 2
168 receptor β (*IL2RB*), interleukin 10 receptor α (*IL10RA*) and Blimp-1 (*PRDM1*) (**Fig. 3a**). Deletion of the miR-125b responsive elements in these UTRs resulted in the
170 abrogation of the inhibitory effect of miR-125b (**Fig. 3b**). Because miR-125b and its precursors are down-modulated in naïve CD4$^+$ T cells after polyclonal activation *in vitro*
172 (**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
174 levels of *IFNG*, *IL2RB*, *IL10RA* and *PRDM1* transcripts by RT-qPCR three days after the transduction of naïve CD4$^+$ T cells with lentiviral vectors expressing miR-125b (LV-
176 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*
180 gene has one miR-125b responsive-elements compared to the three of the human gene. Such comparative analysis indicates that only the *PRDM1* mouse gene has the predicted
182 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
184 required for the differentiation of effector T lymphocytes. Most of miR-125b target genes

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

188

miR-125b enforces naïveness of $CD4^+$ T cells

190 Because miR-125b directly regulates genes involved in the differentiation of naïve $CD4^+$ T cells, we assessed the impact of miR-125b on the acquisition of the effector-memory
192 phenotype. Naïve $CD4^+$ 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_H1 and T_H2 memory $CD4^+$ T
196 cells, such as CXCR3 and CCR4. In parallel, we observed an increased expression of the naïve $CD45RA^+$ $CD45RO^-$ 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-2Rβ and IL-10Rα) that represent direct miR-125b targets (**Fig.**
200 **4b**). Analysis of $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ cells activated in the presence of ectopic miR-125b displayed a reduced effector function as demonstrated
214 by the decreased production of IFN-γ and IL-13 detected both intracellularly (**Fig. 5a,b**) and in the culture supernantants (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 $CD4^+$ T cells.

224 **DISCUSSION**

Here we perform an analysis of the miRNomes in seventeen major human
226 lymphocyte subsets and show that the specific miRNAs signatures that mark these subsets have a poor concordance between human and mouse lymphocytes. Identification
228 of miRNA target genes shows that miR-125b, one of the miRNA that is highly specific for naïve $CD4^+$ T cells, directly regulates a set of genes important to the differentiation
230 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
234 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
236 miRNomes[34] revealed that our approach identified up to 80% of the published miRNAs and identified additional miRNAs, possibly due to the sensitivity of the approach.
238 Because TaqMan miRNA Arrays require only a few nanograms of RNA, this approach is suitable for very rare lymphocyte subsets, allowing us to obtained the widest available
240 microRNA profile of human lymphocyte subsets.

The comparison of miRNomes obtained in various studies and from different
242 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
244 incorporates and integrates the different nomenclatures of the various miRBase versions. Secondly, because deep sequencing technology often identifies microRNAs with codes
246 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 $CD4^+$ 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[35]. 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_H1 cytokine IFN-γ 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-2Rβ 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-2Rβ, do not respond to IL-15 and their homeostasis depends on IL-7 and TCR tickling induced by self-MHC[36-38]. Blimp-1 is

270 known to promote terminal T cell differentiation[39]. 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 $CD4^+$ 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 $CD4^+$ 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-γ, IL10Rα and IL2Rβ) 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[40], 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
316 lymphocyte subsets were isolated from Peripheral Blood Lymphocytes. Purity is that achieved after sorting; Sorting phenotype indicates surface marker combinations used for
318 cell sorting. **(b)** Cytokines produced by the purified T helper cell subsets following polyclonal stimulation with PMA and ionomycin. Cells co-producing IFN-γ and IL-4 or
320 IFN-γ and IL-17 were very low (< 1%) or undetectable (data not shown)

322 **Figure 1** microRNA expression describes and discriminates among different lymphocyte subsets. **(a)** Unsupervised hierarchical clusters of the miRNAs detected for each cell
324 subset analyzed. 242 subset-specific miRNAs were selected using one-way Anova analysis (p-value 0.001).Yellow to blue gradient indicates expression values (ΔCT log
326 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-
328 subsets as in panel **a (c)** Heat map of average expression values (z-scores of ΔCT) for mature miRNAs across all 17 lymphocyte subsets profiled. **(d)** z-scores heat map of
330 signature miRNAs for naïve $CD4^+$, T_H1, T_H17 and T_H2 cell subsets. Differentially expressed miRNAs were selected with the comparative CT method (ΔΔCT) and filtered
332 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
334 relative quantities (RQ) for 25 miRNAs assessed by RT-qPCR in naïve $CD4^+$ T cells activated with anti-CD3 and anti-CD28 mAbs at the indicated time points compared to
336 time zero. **(f)** Expression of 46 miRNAs representing the human lymphocytes signatures

of the six lymphocyte subsets (naïve $CD4^+$ T cells, T_H1, T_H17, T_H2 and NK cells) for
338 which mouse data are published[23]. **(g)** Expression of miR-125b, miR-188-5p, mir-193b
and let-7c assessed by TaqMan assays in naïve and memory $CD4^+$ T cells purified from
340 human PBMC and mouse spleen.

342 **Figure 2** Identification of putative miR-125b target genes in naïve $CD4^+$ 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 $CD4^+$ 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 $-\log_2$(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 transcipt (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 ± 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 *PRDM1*, and luciferase activity was assessed as
366 described above. The values represent average ± SEM (n=18) Anova test significance is indicated by (***) P<0.001. (**c)** Expression levels of *INFG*, *IL10RA*, *IL2RB* and *PRDM1*
368 transcripts were measured by RT-qPCR in activated naïve $CD4^+$ T cells transduced with lentiviral vectors encoding miR-125b (LV-125b) or mock control (LV-ctrl). The values
370 represent average ± SEM of 3 independent experiments. (**d)** Scheme of miR-125b responsive elements (colour coded) predicted by Targetscan5.1 in human and mouse
372 *INFG*, *IL10RA*, *IL2RB* and *PRDM1* transcripts.

374 **Figure 4** miR-125b preserves naïve state of $CD4^+$ T cells. Purified peripheral blood naïve $CD4^+$ 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 MHCI surface expression by flow
378 cytometry. (**b**) Cells were analyzed after three days for IL10Rα, and IL2Rβ surface expression by flow cytometry. (**c**) Purified, unlabeled or CFSE-labeled naïve $CD4^+$ 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 $CD4^+$ T cell differentiation. **(a)** and **(b)** Purified naïve

386 $CD4^+$ 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-γ 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 $CD4^+$ 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 $CD4^+$ T cells and $CD4^+$ T_H1 memory cells. The mean of 3 independent experiments is shown.

396

METHODS

398

Purification of human lymphocyte subsets and naïve T cell differentiation

400 **experiments.**

Buffy-coat blood of healthy donors was obtained from the IRCCS Policlinico Ospedale

402 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

404 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

406 research purposes and informed consent was obtained from all the subjects involved in this study. For *in vitro* differentiation to effector cells, resting naïve $CD4^+$ T lymphocytes

408 were purified (> 98%) by negative selection with magnetic beads and activated at $1x10^5$ cells/well on 96 well MaxiSorp plates (Nunc) coated with anti-CD3 (0.1 μg/ml) and anti-

410 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

412 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

418 Low Density Arrays (Applied Biosystems). Gene expression was performed on naïve $CD4^+$ and memory T cells with Illumina direct hybridization assays: cRNA was

420 generated according to the Illumina protocol (Ambion); Hybridization and scanning were

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 ΔCT 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 $CD4^{+}$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 IL2Rβ's construct that was synthesized
446 (Geneart 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 $CD4^+$ T cells nucleofection

458 Purified $CD4^+$ 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 αCD3 0.1 μg/ml (clone TR66) and allo-PBMCs in a 96 well U-bottom plate in the
462 presence of IL2 100 U/ml, αIL4, αIFN-γ and αIL12 (R&D) antibodies at 2 μg/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 MIKβ3), PB-CD4 (clone RPA-T4), PECy5-CD183 (clone 1C6/CXCR3),

PE-CCR4 (clone 1G1), PECy5-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 $CD4^+$ T cells were activated as described above and simultaneously transduced with either pLV-ctrl vector or pLV-125b at MOI: $1x10E^7$
476 TU/ml in the presence of polybrene (8µg/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
$CD4^+$ naïve	4	99,8 ±0,1	CD4+, CCR7+, CD45RA+, CD45RO-
$CD4^+$ T_H1	6	99,9 ±0,05	CD4+, CXCR3+, CCR6-, CD161-
$CD4^+$ T_H17	4	99,1 ±1	CD4+, CCR6+, CD161+, CXCR3-
$CD4^+$ T_H2	4	99,7 ±0,3	CD4+, CRTH2+, CXCR3-
$CD4^+$ Treg	3	99,0 ±0,8	CD4+, CD127dim, CD25+
$CD4^+$ memory	3	99,8 ±0,2	CD4+, CD45RA-, CD45RO+
$CD4^+$ T_{CM}	3	98,4 ±2,8	CD4+, CCR7+, CD45RA-, CD45RO+
$CD4^+$ T_{EM}	3	95,4 ±5,5	CD4+, CCR7-, CD45RA-, CD45RO+
$CD4^+$ T_{EMRA}	3	96,5 ±4	CD4+, CCR7-, CD45RA+, CD45RO-
$CD8^+$ naïve	3	99,3 ±0,2	CD8+, CCR7+, CD45RA+, CD45RO-
$CD8^+$ T_{CM}	3	98,3 ±0,8	CD8+, CCR7+, CD45RA-, CD45RO+
$CD8^+$ T_{EM}	3	96,8 ±0,9	CD8+, CCR7-, CD45RA-, CD45RO+
$CD8^+$ T_{EMRA}	3	98,1 ±0,3	CD8+, CD45RA+, CCR7-, CD45RO-
NK cells	3	99,2 ±0,3	CD56+, CD3-
$CD5^+$ 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-γ	**IL-17**	**IL-4**
T_H1	**50.5**	0.9	0.6
T_H17	4.5	**13.1**	0.6
T_H2	0.6	0.6	**57**

Figure1

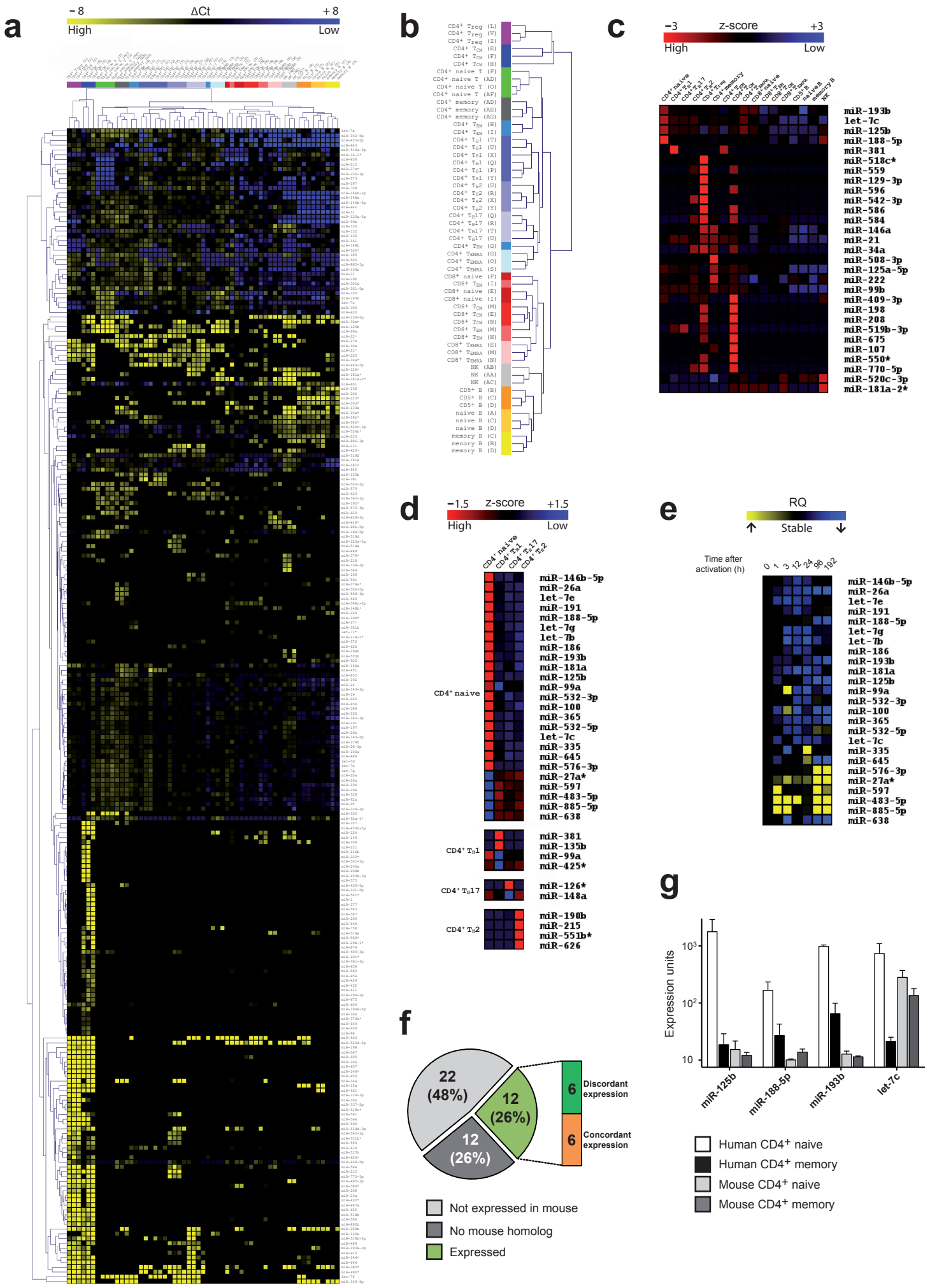

a
- 8
ΔCt
+ 8
High
Low
b
c
-3
z-score
+3
High
Low
miR-193b
let-7c
miR-125b
miR-188-5p
miR-381
miR-518c*
miR-559
miR-129-3p
miR-596
miR-542-3p
miR-586
miR-584
miR-146a
miR-21
miR-34a
miR-508-3p
miR-125a-5p
miR-222
miR-99b
miR-409-3p
miR-198
miR-208
miR-519b-3p
miR-675
miR-107
miR-550*
miR-770-5p
miR-520c-3p
miR-181a-2*
d
-1.5
z-score
+1.5
High
Low
CD4+ naive
CD4+ Th1
CD4+ Th17
CD4+ Th2
miR-146b-5p
miR-26a
let-7e
miR-191
miR-188-5p
let-7g
let-7b
miR-186
miR-193b
miR-181a
miR-125b
miR-99a
miR-532-3p
miR-100
miR-365
miR-532-5p
let-7c
miR-335
miR-645
miR-576-3p
miR-27a*
miR-597
miR-483-5p
miR-885-5p
miR-638
miR-381
miR-135b
miR-99a
miR-425*
miR-126*
miR-148a
miR-190b
miR-215
miR-551b*
miR-626
e
RQ
Stable
Time after activation (h)
0
1
3
12
24
96
192
f
22 (48%)
12 (26%)
12 (26%)
6
Discordant expression
6
Concordant expression
Not expressed in mouse
No mouse homolog
Expressed
g
Expression units
miR-125b
miR-188-5p
miR-193b
let-7c
Human CD4+ naive
Human CD4+ memory
Mouse CD4+ naive
Mouse CD4+ memory

Figure 2

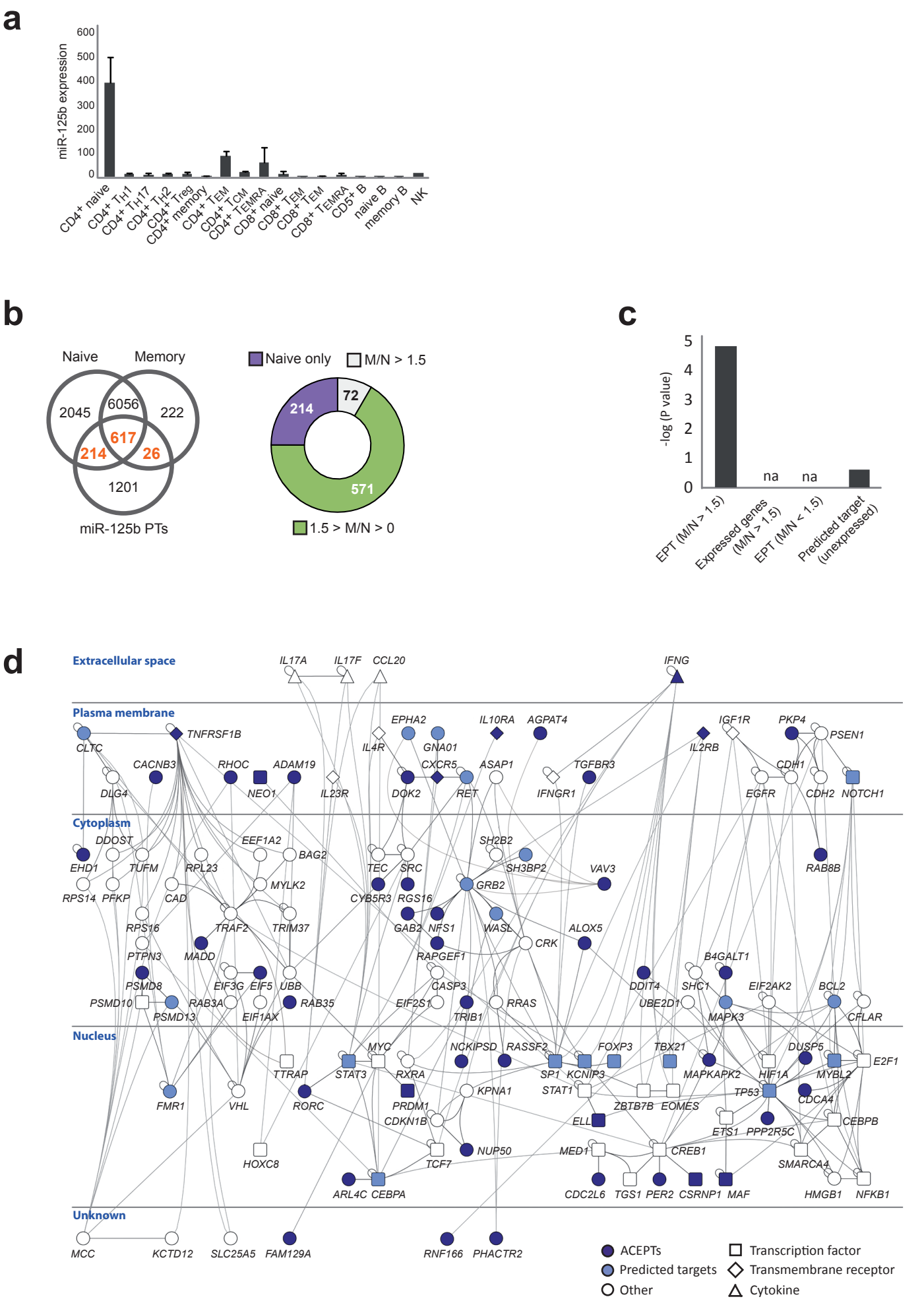

a
miR-125b expression
600
500
400
300
200
100
0
CD4+ naive
CD4+ TH1
CD4+ TH17
CD4+ TH2
CD4+ Treg
CD4+ memory
CD4+ TEM
CD4+ TCM
CD4+ TEMRA
CD8+ naive
CD8+ TEM
CD8+ TEM
CD8+ TEMRA
CD5+ B
naive B
memory B
NK
b
Naive
Memory
2045
6056
222
617
214
26
1201
miR-125b PTs
Naive only
M/N > 1.5
214
72
571
1.5 > M/N > 0
c
-log (P value)
5
4
3
2
1
0
na
na
EPT (M/N > 1.5)
Expressed genes (M/N > 1.5)
EPT (M/N < 1.5)
Predicted target (unexpressed)
d
Extracellular space
IL17A
IL17F
CCL20
IFNG
Plasma membrane
CLTC
TNFRSF1B
EPHA2
IL10RA
AGPAT4
IGF1R
PKP4
PSEN1
IL4R
GNA01
IL2RB
CACNB3
RHOC
ADAM19
CXCR5
ASAP1
TGFBR3
CDH1
DLG4
NEO1
IL23R
DOK2
RET
IFNGR1
EGFR
CDH2
NOTCH1
Cytoplasm
DDOST
EEF1A2
SH2B2
EHD1
TUFM
RPL23
BAG2
TEC
SRC
SH3BP2
VAV3
RAB8B
RPS14
PFKP
CAD
MYLK2
CYB5R3
RGS16
GRB2
RPS16
TRAF2
TRIM37
GAB2
NFS1
WASL
ALOX5
PTPN3
MADD
CRK
RAPGEF1
B4GALT1
PSMD8
EIF3G
EIF5
UBB
CASP3
DDIT4
SHC1
EIF2AK2
BCL2
PSMD10
RAB3A
RAB35
EIF2S1
RRAS
UBE2D1
PSMD13
EIF1AX
TRIB1
MAPK3
CFLAR
Nucleus
MYC
NCKIPSD
RASSF2
FOXP3
TBX21
DUSP5
E2F1
TTRAP
STAT3
RXRA
SP1
KCNIP3
MAPKAPK2
HIF1A
MYBL2
KPNA1
STAT1
TP53
FMR1
VHL
RORC
PRDM1
ZBTB7B
EOMES
CDCA4
CDKN1B
ELL
CEBPB
ETS1
PPP2R5C
NUP50
MED1
CREB1
HOXC8
TCF7
SMARCA4
ARL4C
CEBPA
CDC2L6
TGS1
PER2
CSRNP1
MAF
HMGB1
NFKB1
Unknown
MCC
KCTD12
SLC25A5
FAM129A
RNF166
PHACTR2
ACEPTs
Predicted targets
Other
Transcription factor
Transmembrane receptor
Cytokine

Figure 3

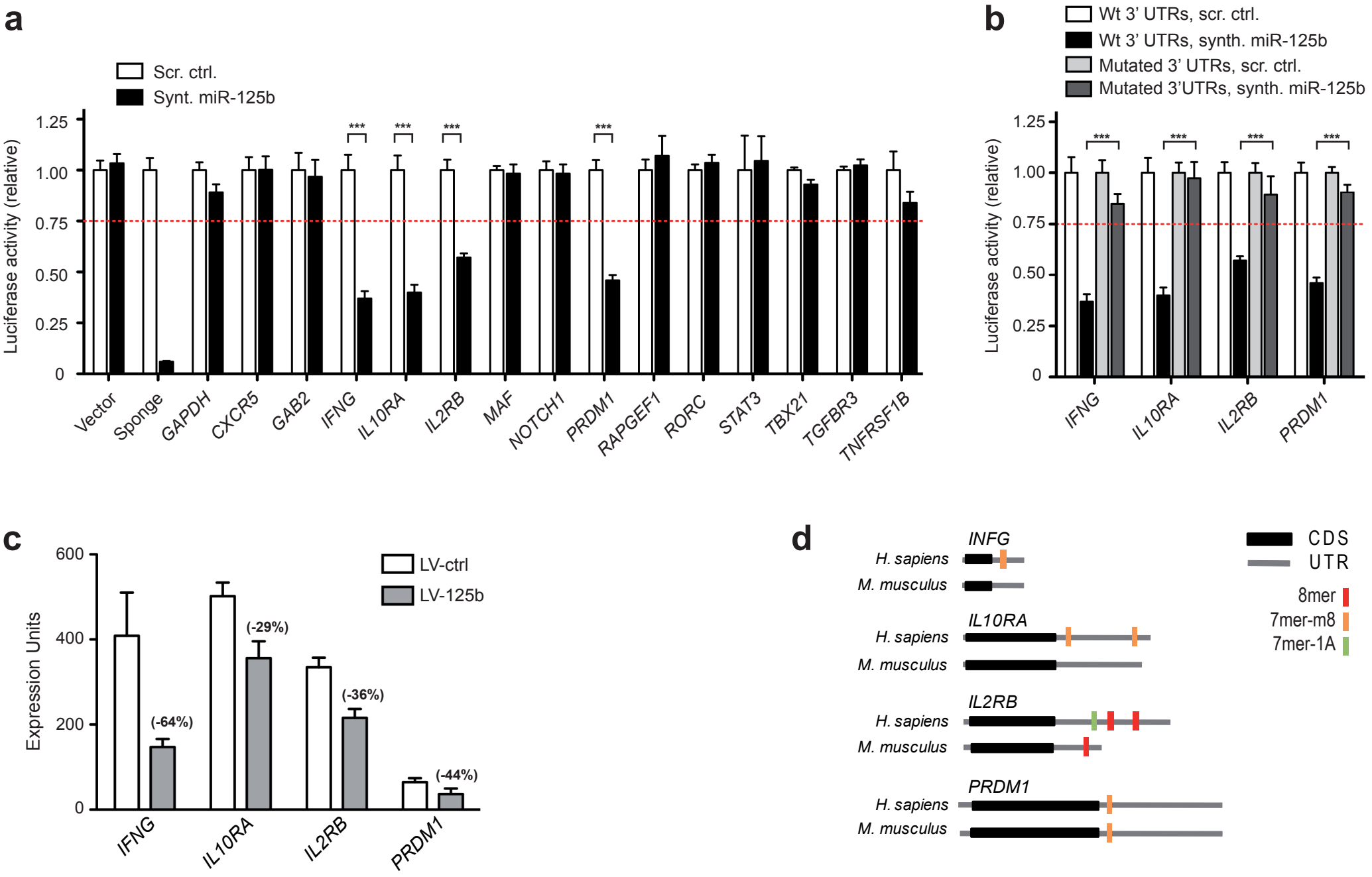

a
Scr. ctrl.
Synt. miR-125b
Luciferase activity (relative)
1.25
1.00
0.75
0.50
0.25
0
***
Vector
Sponge
GAPDH
CXCR5
GAB2
IFNG
IL10RA
IL2RB
MAF
NOTCH1
PRDM1
RAPGEF1
RORC
STAT3
TBX21
TGFBR3
TNFRSF1B
b
Wt 3' UTRs, scr. ctrl.
Wt 3' UTRs, synth. miR-125b
Mutated 3' UTRs, scr. ctrl.
Mutated 3'UTRs, synth. miR-125b
Luciferase activity (relative)
IFNG
IL10RA
IL2RB
PRDM1
c
LV-ctrl
LV-125b
Expression Units
600
400
200
0
(-64%)
(-29%)
(-36%)
(-44%)
IFNG
IL10RA
IL2RB
PRDM1
d
INFG
IL10RA
IL2RB
PRDM1
H. sapiens
M. musculus
CDS
UTR
8mer
7mer-m8
7mer-1A

Figure 4

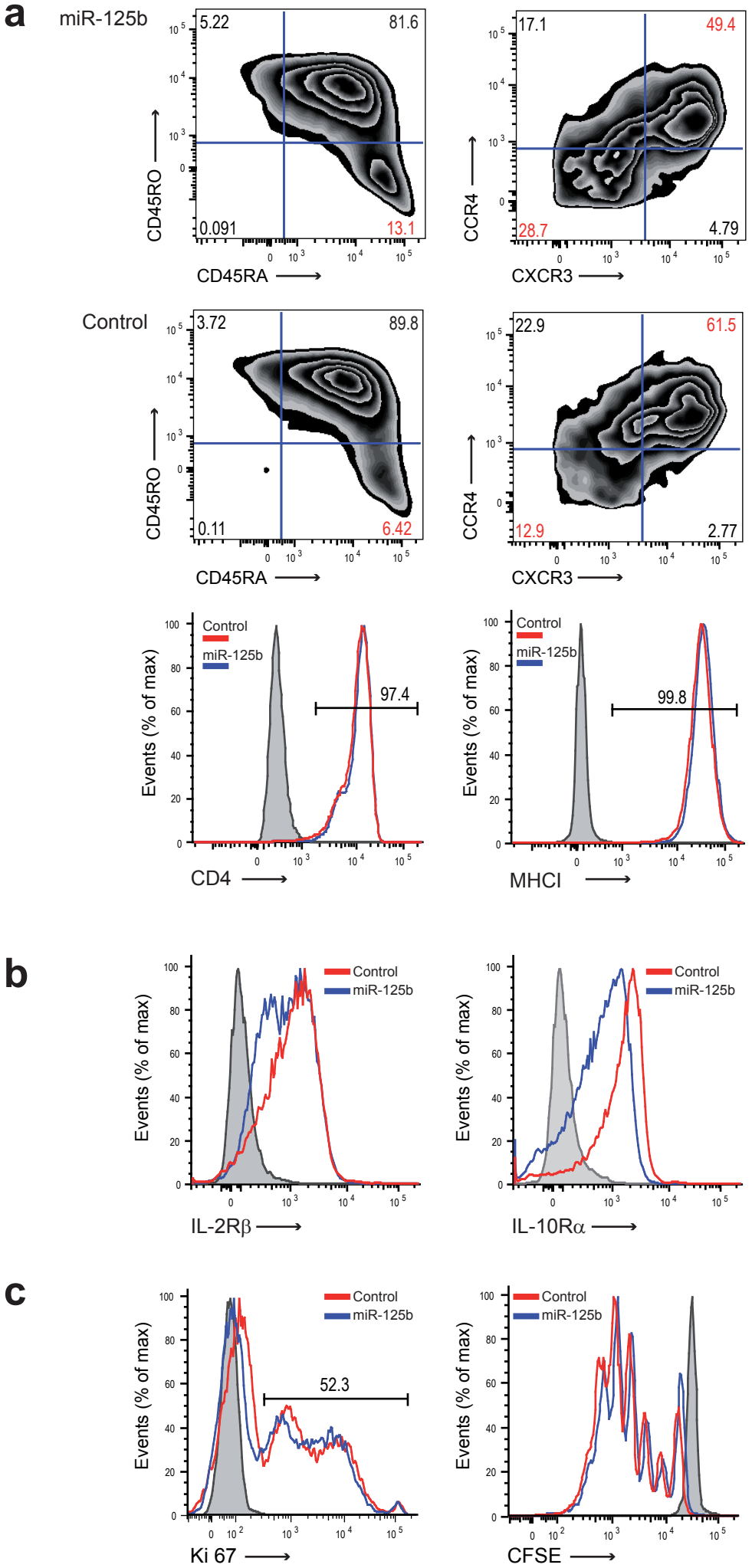
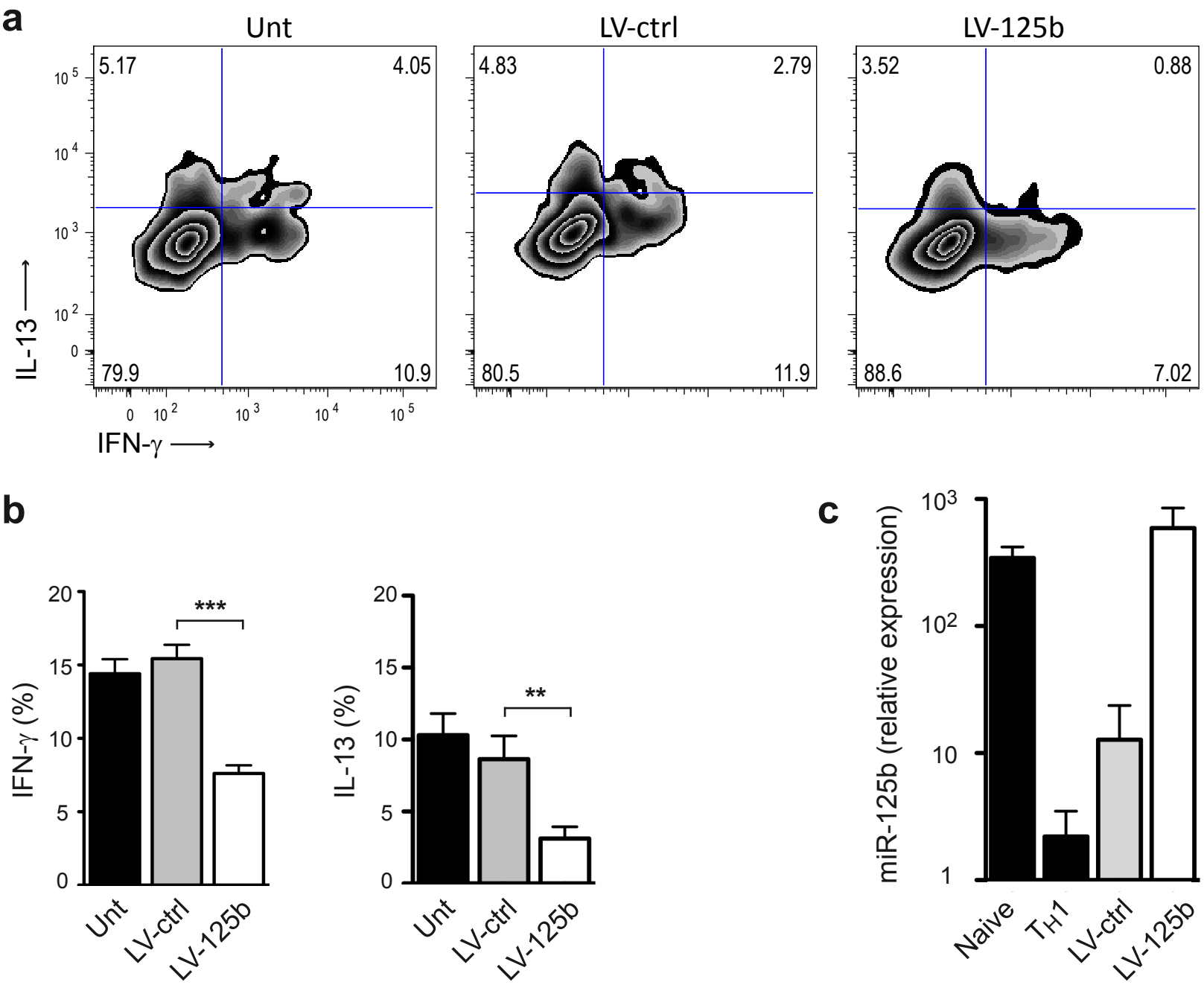

a
miR-125b
5.22
81.6
0.091
13.1
CD45RO
CD45RA
17.1
49.4
28.7
4.79
CCR4
CXCR3
Control
3.72
89.8
0.11
6.42
CD45RO
CD45RA
22.9
61.5
12.9
2.77
CCR4
CXCR3
Control
miR-125b
97.4
Events (% of max)
CD4
Control
miR-125b
99.8
Events (% of max)
MHCI
b
Control
miR-125b
Events (% of max)
IL-2Rβ
Control
miR-125b
Events (% of max)
IL-10Rα
c
Control
miR-125b
52.3
Events (% of max)
Ki 67
Control
miR-125b
Events (% of max)
CFSE

Figure 5


a
Unt
LV-ctrl
LV-125b
5.17
4.05
79.9
10.9
4.83
2.79
80.5
11.9
3.52
0.88
88.6
7.02
IL-13
IFN-γ
b
IFN-γ (%)
IL-13 (%)
***
**
Unt
LV-ctrl
LV-125b
c
miR-125b (relative expression)
Naive
TH1
LV-ctrl
LV-125b