# IRF5 promotes inflammatory macrophage polarization and Th1/Th17 response

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1 **IRF5 promotes inflammatory macrophage polarization and $T_H$1-$T_H$17 response**

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19 **Summary**

20 Genetic polymorphisms in the IRF5 gene, leading to increased mRNA expression, are

21 associated with a number of autoimmune diseases. We show that expression of IRF5 in

22 macrophages is reversibly induced by inflammatory stimuli and contributes to plasticity

23 of macrophage polarization. High levels of IRF5 are characteristic of M1 macrophages, in

24 which it directly activates transcription of IL-12p40/p35, IL-23p19 genes and represses

25 IL-10 gene. Consequently, these macrophages set up the environment for a potent $T_H1$-

26 $T_H17$ response. Global gene expression analysis demonstrates that exogenous IRF5 up-

27 or down-regulates expression of established phenotypic markers of M1 or M2

28 macrophages respectively. Our data suggest a critical role for IRF5 in M1 macrophage

29 polarization and defines a novel function for IRF5 as a transcriptional repressor.

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Macrophages are a heterogeneous population of immune cells that are essential for the initiation and resolution of pathogen- or tissue damage-induced inflammation [1]. They demonstrate remarkable plasticity that allows them to efficiently respond to environmental signals and change their phenotype and physiology in response to cytokines and microbial signals [2]. These changes can give rise to populations of cells with distinct functions, which are phenotypically characterised by production of pro-inflammatory and anti-inflammatory cytokines [3]. Based on the $T_H1$-$T_H2$ polarization concept [4] these cells are now referred to as M1 (classic) macrophages, that produce pro-inflammatory cytokines and mediate resistance to pathogens and tissue destruction, and M2 (alternative) macrophages, that produce anti-inflammatory cytokines and promote tissue repair and remodelling as well as tumour progression [3, 5].

The activation of a subset defining transcription factor (TF) is characteristic of a particular T cell lineage commitment: T-bet is associated with $T_H1$, GATA3 with $T_H2$, FOXP3 with T regulatory (Treg) cells and RORγT with $T_H17$ cells [6]. Dendritic cells (DCs) also employ subset-selective expression of IRF4 and IRF8 for their commitment. IRF4 is expressed at high levels in CD4$^+$ dendritic cells (DCs) but low in plasmacytoid DC (pDCs). As a consequence, the CD4$^+$ DC population is absent in $Irf4^{-/-}$ mice. Conversely, IRF8 is expressed at high levels in pDCs and CD8$^+$ DCs, thus $Irf8^{-/-}$ mice are largely devoid of these DC subsets [7]. However, transcription factors underlying macrophage polarization remain largely undefined. Activation of NF-κB p50 has been previously associated with inhibition of M1 polarizing genes [8], whereas CREB mediated induction of C/EBPβ has been shown to up-regulate M2-specific genes [9]. More recent evidence suggests that, in mice, IRF4 controls M2 macrophage polarization by stimulating the expression of selected M2 macrophage markers [10].

IRF5, another member of the IRF family, has diverse activities, such as activation of type I interferon (IFN) genes, inflammatory cytokines, including TNF, IL-6, IL-12 and IL-23, and tumour suppressors [11]. Consequently, IRF5 deficient mice are resistant to lethal endotoxic shock [12]. Human IRF5 is expressed in multiple splice variants with distinct cell type-specific expression, cellular localization, differential regulation and functions [13]. Moreover, genetic polymorphisms in the human IRF5 gene, leading to expression of several unique isoforms or increased expression of IRF5 mRNA, is implicated in autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjogren's syndrome, multiple sclerosis and inflammatory bowel disease [14-18]. Here we show a role for IRF5 in determining M1 macrophage lineage commitment. M1 macrophages are characterised by high level of IRF5, expression of which is induced during their differentiation. Forced expression of IRF5 in M2 macrophages drives global

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71     expression of M1-specific cytokines, chemokines and co-stimulatory molecules and leads

72     to a potent $T_H1$-$T_H17$ response. Conversely, the induction of M1-specific cytokines is

73     impaired in human M1 macrophages with levels of IRF5 expression reduced by siRNA

74     knock-down or in the peritoneal macrophages of the *Irf5*$^{-/-}$ mice. Our data suggest that

75     activation of IRF5 expression defines macrophage lineage commitment by driving M1

76     macrophage polarization and, together with the results of Satoh et al demonstrating a

77     role for IRF4 in controlling M2 macrophage markers [10], establish a new paradigm for

78     macrophage polarization and highlight the potential for therapeutic interventions via

79     modulation of the IRF5-IRF4 balance.

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## RESULTS

### IRF5 is highly expressed in human M1 macrophages

The M1 macrophage phenotype is induced by Interferon gamma (IFN-γ) followed by stimulation with bacterial products like lipopolysaccharide (LPS) or by treatment of monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) (**Supplementary Fig 1a** and Ref [19-21]). IRF5 mRNA expression was elevated in primary human monocytes or in monocyte-derived macrophages differentiated with GM-CSF (**Fig 1a**, **Supplementary Fig 1b**). Treatment of monocytes with GM-CSF but not macrophage colony stimulating factor (M-CSF) – an M2 differentiating factor – resulted in up-regulation of IRF5 mRNA expression within 4 h post stimulation (**Fig 1b**). To account for possible differences in macrophage *in vitro* differentiation protocols, we analysed the level of IRF5 in macrophages treated with either IFN-γ alone or in combination with LPS for 24h and found that these were similar to the ones in GM-CSF treated cells (**Supplementary Fig 1c**). The expression of IRF4, which controls M2 polarisation in mice [10], was equally induced during monocyte differentiation into M1 or M2 macrophages (**Fig 1a**). The expression of IRF3, another member of the IRF family central to the innate immune response, was not affected by differentiation into macrophage subtypes (**Fig 1a**). Thus, IRF5 is induced in pro-inflammatory M1 macrophages independently of the *in vitro* differentiation protocol, whereas the mRNA levels of IRF4 and IRF3 are comparable between the macrophage populations. There was no significant difference between M1 and M2 macrophages in their basal or LPS-induced levels of NF-κB p50 protein, previously implicated in macrophage polarisation towards M2 phenotype [8] (**Supplementary Fig 1d,e**).

### Expression of IRF5 in human macrophages is plastic

To examine whether IRF5 contributes to plasticity of macrophage polarisation, we attempted to convert one population into another by culturing M2 macrophages with GM-CSF and M1 macrophages with M-CSF. As expected, treatment of M2 macrophages with GM-CSF or IFN-γ led to production of M1 phenotypic markers upon LPS stimulation (M2->M1) (**Supplementary Fig 2a**). Conversely, treatment of M1 macrophages with M-CSF led to production of M2 phenotypic markers upon LPS stimulation (M1->M2) (**Supplementary Fig 2b**). M2->M1 conversion of macrophages increased IRF5 protein levels (**Fig 1c**, **Supplementary Fig 2c**), whereas M1->M2 macrophages had reduced levels of IRF5 (**Fig 1d**, **Supplementary Fig 2d**). Once again, the levels of IRF4 or IRF3 were unchanged (**Figs 1c, d**). These results demonstrate that expression of IRF5 is quickly adapted to environmental stimuli, and suggests that it participates in establishing macrophage plasticity.

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**IRF5 influences human M1 macrophage polarization**

We next investigated whether IRF5 would directly induce expression of M1 macrophage phenotypic markers. Bioactive IL-12p70 and IL-23 were detected in M2 macrophages infected with the adenoviral expression construct encoding for human IRF5 (variant 3/4) [13], but minimal with IRF3 or an empty vector [22] (**Fig 2a**). The up-regulation of IL-12p70 and IL-23 was reflected by a vast increase in secretion of the p40 subunit shared by the two cytokines (**Fig 2a**). The secretion of both IL-12p70 and IL-23 peaked at 24h post LPS stimulation and remained sustained up to at least 48h (**Supplementary Fig 3a**). We also observed a significant increase in production of other key pro-inflammatory cytokines such as IL-1β and TNF by IRF5-expressing macrophages (**Supplementary Fig 3b**). Remarkably, IL-10 production in the IRF5-expressing cells was noticeably reduced (**Fig 2a**). We also observed IL-10 protein inhibition in cells over-expressing IRF3 (**Fig 2a**), which might represent a negative feedback regulation of IL-10 expression [23], since the main direct target of IRF3, IFN-β, induces IL-10 [24]. The complementary experiment targeting endogenous IRF5 in M1 macrophages by RNA interference (RNAi) (**Supplementary Fig 3c**) resulted in significant inhibition of IL-12p70 and IL-23 and increase of IL-10 (**Fig 2b**). Secretion of IL-12p40 was also reduced in these cells (**Fig 2b**), consistent with the data obtained in mouse myeloid cells deficient in *Irf5* [12]. Taken together, IRF5 influences M1 macrophage polarisation by equipping the cells with an IL-12$^{hi}$, IL-23$^{hi}$, IL-10$^{lo}$ cytokine profile.


**IRF5 promotes human T$_H$1-T$_H$17 response**

One of the hallmarks of M1 macrophage polarisation is acquired antigen presenting features leading to efficient T$_H$1 response [20, 21]. To examine whether IRF5-aided the polarisation of T lymphocyte proliferation, fate or activation state, human M2 macrophages were infected with IRF5, IRF3 viral expression constructs or an empty vector and exposed to human T lymphocytes extracted and purified from peripheral blood of major histocompatibility complex (MHC) unmatched donors in a mixed lymphocyte reaction (MLR). Total T lymphocyte proliferation was determined 3 days after infection, while activation of specific T cell subsets was analysed by flow cytometry (**Supplementary Fig 4a**). Proliferation of T lymphocytes (**Supplementary Fig 4b**) was considerably higher when co-cultured with IRF5-expressing macrophages. Furthermore, only IRF5-expressing macrophages provided the cytokine environment necessary for T$_H$1 expansion and activation, assessed by increased number of IFN-γ producing CD4$^+$ cells (**Fig 3a** and **Supplementary Fig 4c**) and mRNA (**Supplementary 4d**) and protein (**Fig 3b**) expression of IFN-γ. In these cultures we also observed expansion and activation of T$_H$17 cells, assessed by increased number of IL-17 producing CD4$^+$ cells (**Fig 3d** and

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156 **Supplementary Fig 4e**), secretion of IL-17A (**Fig 3e**) and mRNA expression of IL-17A,
157 IL-17F, IL-21, IL-22, IL-26 and IL-23R (**Supplementary 4f**). In line with recent studies
158 demonstrating that IL-23 enhanced the emergence of a IL-17$^+$IFN-γ$^+$ population of T
159 cells [25], about 25% of IL-17$^+$ cells were also positive for IFN-γ (data not shown),
160 supporting a close developmental relationship of human $T_H$17 and $T_H$1 cells [26]. mRNA
161 expression of the $T_H$1 and $T_H$17 specifying transcription factors T-bet and RORγt was
162 significantly induced in T cells co-cultured with IRF5 expressing macrophages (**Figs 3c**
163 and **3f**). Of interest, expression of GATA3 and FOXP3 mRNA was reduced in the presence
164 of IRF5 expressing macrophages (data not shown). Hence, IRF5 promotes T lymphocyte
165 proliferation and activation of the $T_H$1 and $T_H$17 lineages, but does not induce $T_H$2 or $T_{reg}$
166 cell lineages.
167

168 **IRF5 directly induces transcription of human M1 genes**
169 IRF5 is a transcription factor which can bind to the regulatory regions of target genes
170 and modulate their expression. We next determined whether the role of IRF5 in
171 differential regulation of IL-12p70, IL-23 and IL-10 cytokine secretion was a direct
172 consequence of its function as a transcription factor. mRNA expression of IL-12p40, IL-
173 12p35 and IL-23p19 was strongly induced in M2 macrophages infected with adenoviral
174 vector constructs encoding for IRF5, but not IRF3 or an empty vector (**Fig 4a**).
175 Moreover, the IRF5-driven IL-12p40 mRNA expression was sustained until at least 16h
176 post LPS stimulation (**Supplementary Fig 5a**). Consistent with the protein secretion
177 data, expression of IL-10 mRNA was inhibited by IRF5 (**Fig 4a**). However, expression of
178 IL-10 mRNA was not altered by IRF3, suggesting the lack of a direct role for IRF3 in IL-
179 10 transcription. RNAi-mediated inhibition of endogenous IRF5 in M1 macrophages
180 reduced IL-12p40, p35 and IL23p19 mRNA expression 8h post LPS stimulation in cells
181 from multiple blood donors (**Fig 4b**). IL-12p40 was strongly inhibited throughout the
182 analysed time course, even 16h post LPS stimulation (**Supplementary Fig 5b**). The
183 expression of IL-10 mRNA was increased in the cells with knocked-down levels of IRF5
184 (**Fig 4b**).
185

186 To formally define the global expression profile induced by IRF5, we carried out genome-
187 wide expression analysis, in which M2 macrophages transduced with ectopic IRF5 were
188 compared to previously-defined human M1 and M2 macrophage subsets [21, 27]. We found
189 that expression of about 90% of known human polarization-specific markers was driven
190 by IRF5 (**Fig 4c**). IRF5 induced 20 M1-specific and inhibited 19 M2-specific genes
191 encoding cytokines, chemokines, co-stimulatory molecules and surface receptors (**Fig**
192 **4c**) resulting in higher or lower production of corresponding proteins (**Supplementary**

193 **Figs 5c,d**). Moreover, we identified a number of novel IRF5-regulated genes that are
194 likely to contribute to the main functional features of macrophage subsets, such as
195 phagocytosis and antigen presentation (**Supplementary Table 1**).

196

197 Next, we investigated the LPS-induced recruitment of IRF5 to the respective promoter
198 loci. All IRF family members share a well-conserved N-terminal DNA binding domain
199 (DBD) that recognises IFN-stimulated response elements (ISREs). A computational
200 analysis of the regions -2000 nt 5' upstream and +1000 nt downstream of the
201 transcription start site (TSS) of IL-12p40, IL-12p35, IL-23p19, IL-10 and other IRF5-
202 regulated genes (**Fig 5** and **Supplementary Table 1**) led to the identification of several
203 ISREs (**Supplementary Table 2**). Primers, encompassing these ISREs, were designed
204 and used in quantitative ChIP experiments in M1 macrophages stimulated with LPS for 0,
205 1, 2, 4, 8, and 24 h. We observed LPS-induced enrichment of IRF5 to the IL-12p40, IL-
206 12p35 and IL-23p19 promoter regions up to 8h post stimulation, matching the kinetics
207 of Pol II recruitment to the genes (**Fig 5a-c**). On the contrary, at the IL-10 promoter
208 region LPS-induced IRF5 recruitment took place between 1 and 4h post stimulation,
209 whereas Pol II could bind to the region only 8h post stimulation (**Fig 5d**), suggesting a
210 new inhibitory role for IRF5 in transcriptional regulation of selected genes. Taken
211 together, IRF5 regulates transcription of IL-12p40, IL-12p35, IL-23p19 and IL-10 genes
212 via recruitment to their promoter regions. It also influences the expression of the
213 majority of human lineage defining cytokines.

214

215 **IRF5 inhibits transcription of the human IL-10 gene**
216 To investigate whether IRF5 can directly repress transcription of the IL-10 gene, we used
217 an adenovirus construct with a gene-reporter in which the luciferase-reporter construct
218 was flanked with 195 nt 5' upstream of the IL-10 gene (IL-10-luc wt) [28]. The IL-10-luc
219 wt construct was co-infected with HA-tagged IRF5 or empty vector pENTR into M2
220 macrophages and luciferase activities were quantified. IRF5 expressing cells showed a
221 significant decrease in luciferase activity in both un-stimulated and 4h post LPS (**Fig 6a**).
222 To confirm the importance of IRF5 binding to the *IL10* promoter, we generated a mutant
223 of IRF5 lacking the DNA binding domain (IRF5 ΔDBD). The IRF5 ΔDBD was no longer
224 able to inhibit the IL-10-luc wt reporter (**Fig 6a**). To further explore the molecular
225 mechanism of IRF5-mediated suppression of *IL10* transcription, we introduced point
226 mutations into the identified ISRE (-182/-172 nt relative to the TSS) and co-infected the
227 IL-10-luc ISRE mut construct together with HA-tagged IRF5 and empty vector pENTR
228 into M2 macrophages. The IL-10-luc ISRE mut showed a different response to the wild
229 type in that ectopic IRF5 was no longer able to suppress luciferase activity (**Fig 6b**),
230 suggesting that IRF5 inhibits IL-10 by direct binding to the IL-10 promoter ISRE. This is

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231  opposite to the positive regulatory activity of IRF5 at the *TNF* [22] and *IL12p35* promoters

232  (**Supplementary Fig 6**). Therefore IRF5 can act not only as a transcriptional activator,

233  but also as a suppressor of selected target genes, in this case the anti-inflammatory

234  mediator IL-10. The mode of inhibition is mediated by direct binding of IRF5 to the

235  promoter region of *IL10* and likely engagement of yet to be identified novel co-factors.

236

237  **IRF5 plays a key role in mouse model of M1 inflammation**

238  Similar to their human counterparts, GM-CSF-differentiated mouse bone marrow derived

239  macrophages (GM-BMDMs) had higher levels of IRF5 protein expression compared to the

240  M-CSF-derived cells (M-BMDMs) (**Fig 7a**) and were the only cells secreting IL-12p70 and

241  IL-23 (data not shown). Consequently, GM-BMDMs from *Irf5*^{−/−} animals secreted

242  significantly less IL-12p70, IL-23 or more IL-10 in response to LPS stimulation (**Fig 7b**).

243  No difference in IL-10 secretion was observed in M-BMDMs from wild type or *Irf5*^{−/−}

244  animals (data not shown). To investigate the functional role of IRF5 in an *in vivo* model

245  of M1-polarized inflammation, *Irf5*^{−/−} mice were challenged with sub-lethal dose of intra-

246  peritoneal LPS. Within 3h there was a significant difference in the serum level of selected

247  cytokines between wild type and *Irf5*^{−/−} mice. Responses were consistent with the human

248  data i.e. in the *Irf5*^{−/−} mice there was reduced serum concentrations of IL-12p40, IL-23

249  (**Fig 7c**), TNF, as well as IL-6 (**Supplementary Fig 7**), but elevated IL-10 (**Fig 7c**).

250  Mice injected with PBS secreted no cytokines. The number of macrophages recruited in

251  the peritoneal cavity of LPS-challenged mice was similar in wild type and *Irf5*^{−/−} animals

252  (data not shown), but the expression of genes encoding M1 macrophage markers, i.e.

253  *Il12p35, Il12p40, Il23p19, Il1b, Tnf* and *Il6*, was significantly impaired in these cells (**Fig**

254  **7d**). The expression of genes encoding M2 markers in *Irf5*^{−/−} animals, i.e. *Il10,* arginase

255  1 (*Arg1*), *Fizz1 and Ym1*, was either significantly increased or showed a positive trend

256  (**Fig 7d** and data not shown). In addition, in splenocytes from the LPS-challenged *Irf5*^{−/−}

257  animals cultured *ex vivo* for an additional 48h we observed significantly reduced

258  production of IFN-γ and IL-17 (**Fig 7e**). In summary, our data together with the

259  previously reported role of IRF5 in LPS-induced lethal endotoxic shock [12], support a

260  major role of IRF5 in establishing pro-inflammatory macrophage phenotype in animal

261  models of M1-polarizing inflammation.

262

**Discussion**

Macrophages are key mediators of the immune response during inflammation. Plasticity and functional polarization are hallmarks of the macrophages resulting in phenotypic diversity of macrophage populations [29]. Taking into account that the deficiency of IRF5 in mice leads to diminished production of IL-12p40 and IL-23p19 [11, 12], universal markers of M1 macrophage subsets, we investigated whether IRF5 is involved in macrophage polarisation. We demonstrate that IRF5 is indeed a major factor defining macrophage polarization: it is highly expressed in M1 macrophages and induces a characteristic gene expression and cytokine secretion profile, and promotes robust $T_H1$-$T_H17$ responses. We also unravel a new regulatory role for IRF5 as an inhibitor of M2 macrophage marker expression. Finally, IRF5 contributes to macrophage plasticity, i.e. modulation of its levels leads to the conversion of one macrophage subset phenotype into the other.

The rapid and potent transcriptional response developed by macrophages encountering microbial stimuli, such as LPS, or subsequently cytokines, is orchestrated by many TFs. Among them are class III TFs, such as PU.1, C/EBPb, RUNX1, IRF8, which are lineage-specific transcriptional regulators turned on during macrophage differentiation [30]. The combinatorial expression of these proteins specifies the macrophage phenotype via constitutive activation or repression of genes and chromatin remodelling at inducible loci. For instance, PU.1 is required for maintaining H3K4me1 enhancer marks at macrophage-specific enhancers [31]. But only a small proportion of the macrophage transcriptome is altered by cell polarization [27] and among the genes differentially expressed between the M1 and M2 subsets are those regulated by IRF5, such as *IL12p40*, *IL12p35*, *IL23p19*, *IL1β*, *TNF*, *MIP1a*, *RANTES*, *CD1a*, *CD40*, *CD86*, *CCR7*. Another member of the IRF family, IRF4, known to inhibit IRF5 activation by competing for interaction with Myd88 [32], has been recently reported to control the expression of prototypical mouse M2 macrophage markers [10]. In human cells we found that the expression of IRF4 is equally induced by M-CSF or GM-CSF differentiation and is further enhanced by exposure to IL-4 [33]. IRF5 expression, on the other hand, is specifically induced by GM-CSF or IFN-γ, but is unresponsive to IL-4. Thus, IRF5 and IRF4 may be classified as class III TFs but with a caveat that they define specific macrophage subsets rather than the global macrophage lineage. NF-κB proteins, in particularly c-Rel and RelA, are important for expression of M1-specific cytokines [34, 35]. IRF5 and RelA cooperate in induction of the TNF gene [22]. It is interesting to speculate whetehr the genes encoding IL-12, IL-23 subunits and other M1-specific markers might be under similar joint transcriptional control. Thereby, IRF5 may participate in the combinatorial assembly with macrophage-specific TFs, e.g. PU.1, and environmentally induced NF-κB [31], to define the activity of specifically M1 enhancers.

301

302   The role of IRF5 in the inhibition of IL-10 gene transcription is novel and important in

303   view of its well documented immunosuppressive activity. *Il10*^{−/−} mice develop

304   spontaneous autoimmune diseases and show increased resistance to infection [36]. IL-10

305   represses immune responses by down-regulating inflammatory cytokines like TNF [37] and

306   is important for generation of $T_{reg}$ cells, that act to suppress activation of the immune

307   system and thereby maintain immune system homeostasis and tolerance to self-antigens

308   [38]. Major producers of IL-10 include M2 macrophages, B cells and T cells [39], whereas M1

309   macrophages and DCs are only weak producers [21]. Ectopic expression of IRF5 in M2

310   macrophages reduces IL-10 secretion upon LPS stimulation and also affects mRNA

311   expression of IL-10 and a number of other markers of human M2 macrophage

312   phenotype, such as mannose receptor C type I, insulin-like growth factor 1, CCL2,

313   CCL13, CD163, M-CSF receptor and macrophage scavenger receptor 1. Consistent with

314   other studies [40] we find no expression of the most widely used prototypical mouse M2

315   markers (Arg1, Ym1, Fizz 1) in human macrophages (data not shown), while their

316   expression in mouse LPS-elicited peritoneal macrophages showed a positive trend in the

317   absence of IRF5. Expression of some chemokines, defined as M1 (CXCL10) or M2

318   (CCL17, CCL18, CCL22) markers in mouse macrophages did not follow the expected

319   pattern of IRF5 dependence, i.e. induction for M1 and inhibition for M2, possibly

320   reflecting on the species-specific gene repertoire [41]. While human M1 but not M2

321   macrophages have been shown to secrete high levels of CCL22 [21], there is some

322   controversy in the literature as to whether CXCL10 is a marker of M1 or M2 macrophage

323   phenotypes [21, 24, 27], our data agree more with the latter model.

324

325   The swift modulation of IRF5 expression and cytokine production by CSFs can help to

326   explain the remarkable plasticity of macrophages in adjusting their phenotype in

327   response to environmental signals [2]. M-CSF is constitutively produced by several cell

328   types, including fibroblasts, endothelial cells, stromal cells and osteoblasts. It is likely

329   that this steady state production of M-CSF polarizes macrophages towards the M2

330   phenotype by keeping IRF5 expression low. By contrast, GM-CSF production by the

331   same cell types requires stimulation and occurs usually at a site of inflammation or

332   infection, which is also characterised by high levels of IFN-γ. Resolution of inflammation

333   may once again coincide with predominance of M-CSF and switch in IRF5-driven

334   cytokine production, as treatment of M2->M1 macrophages with M-CSF restores the

335   original M2 phenotype (M2->M1->M2). Activation of both the GM-CSF and IFN-γ

336   receptors stimulates the Janus kinase-signal transducer and activator of transcription

337   (JAK-STAT) pathway [29], and an ISRE element within the IRF5 promoter can bind STAT1

338   and STAT2 [13], suggesting a possible mechanism for GM-CSF- and IFN-γ-induced IRF5

339    expression. Consequently, high levels of IRF5 results in macrophage phenotype
340    polarization towards M1. IRF5 induces expression of IFN-γ mRNA pointing to an
341    autocrine loop in macrophage polarization.

342

343    IRF5-expressing macrophages promote T lymphocyte proliferation and activation and
344    drive them towards $T_H1$ and $T_H17$ phenotypes via secretion of IL-12 [42] and IL-23/IL-1β [43]
345    respectively. $T_H1$ cells constitutively express IFN-γ and T-bet, whereas $T_H17$ express
346    RORγT, IL-23R, IL-17A and IL-17F, IL-21, IL-22 and IL-26. All these $T_H1$ or $T_H17$ markers
347    are up-regulated in the presence of IRF5-expressing macrophages. Human $T_H17$ cells
348    seem to exhibit different features from murine $T_H17$ cells: while murine $T_H17$ originate
349    from a precursor common to $T_{reg}$ cells when IL-6 is produced in combination with TGF-β,
350    human $T_H17$ cells originate from $CD161^+CD4^+$ precursors in the presence of IL-23 and
351    IL-1β, with little involvement of IL-6 and indirect role for TGF-β [43]. Perhaps not
352    surprisingly, dependence of IL-6 expression on IRF5 is much greater in mouse
353    macrophages.

354

355    Both T cell subsets promote cellular immune function and have the capacity to cause
356    inflammation and autoimmune diseases, such as inflammatory bowel disease and
357    collagen-induce arthritis [44, 45]. Significantly, higher levels of *Irf5* mRNA have been found
358    in splenic cells from certain autoimmune-prone mouse strains than in non-autoimmune
359    mice [46], while IRF5 deficient mice show impaired production of $T_H1$ or $T_H17$ cytokines.
360    This points towards a possible broad effect of therapies targeting the induction of IRF5
361    expression by macrophages, for example by targeting IRF5-inducing stimuli. Related to
362    this, GM-CSF deficient mice fail to develop arthritis despite making a normal humoral
363    immune response to the arthritogenic stimulus [47] and the blockade of GM-CSF in wild-
364    type mice controls disease activity and levels of pro-inflammatory mediators in the joints
365    [48].

366

367    In summary, a distinct systemic role of IRF5 in macrophages is the orchestration of
368    transcriptional activation of pro-inflammatory cytokines, chemokines and co-stimulatory
369    molecules leading to efficient effector T cell response, rather than induction of a type I
370    IFN-induced transcriptional network [49]. Our data establish a new paradigm for
371    macrophage polarization and designate the IRF5-IRF4 regulatory axis as a new target for
372    therapeutic intervention: inhibition of IRF5 activity would specifically affect pro-
373    inflammatory cytokine expression and decrease the number of effector T cells.

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**AUTHORSHIP**

Contributions: T.K., T.S., K.B., and S.A. performed research; T.K., H.L., N.S., and I.A.U designed research and analysed data; T.K., M.F., T.H., and I.A.U. wrote the paper. Conflict of interest disclosure: The authors declare no competing financial interests.

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397

**Figure Legends**

399

400 **Figure 1:**

401 **IRF5 is highly expressed in M1-like macrophages and up-regulated by GM-CSF**

402 Total protein extracts were subjected to immunoblot analysis with antibodies against

403 IRF5, IRF4, or IRF3. Actin was used as a loading control. Representative blots of at least

404 4 independent experiments, each using cells derived from a different donor are shown.

405 (**a**) Monocytes (Mono) were collected at day 0 or differentiated into M1-like macrophages

406 with GM-CSF (50ng/ml) (GM-CSF) or M2-like macrophages with M-CSF (100ng/ml) (M-

407 CSF) for 5 days.  Cells were either left untreated or simulated with LPS for 24h.

408 (**b**) Monocytes were stimulated with GM-CSF (50ng/ml) or M-CSF (100ng/ml) for 2, 4, 8,

409 24 and 48h or left untreated. The level of IRF5 mRNA was measured by RT-PCR with a

410 corresponding TaqMan probe. Data shown are the mean $\pm$ SEM of 5 independent

411 experiments each using monocytes derived from a different donor:  *$p < 0.001$ (Two-way

412 ANOVA).

413 (**c**) For M2->M1 polarization, M2 macrophages were treated with or without GM-CSF

414 (50ng/ml) for 24h.

415 (**d**) For M1->M2 polarization, M1 macrophages were treated with or without M-CSF

416 (100ng/ml) for 24h.

417

418 **Figure 2:**

419 **IRF5 influences the production of macrophage lineage specific cytokines**

420 (**a**) M2 macrophages were infected with adenoviral vectors encoding IRF5, IRF3 or

421 empty vector (pENTR) and stimulated with LPS for 24h. The amount of secreted IL-

422 12p70, IL-23, IL-12p40 or IL-10 protein was determined by ELISA. Data show the trend

423 of cytokine secretion in 7-9   independent experiments each using M2 macrophages

424 derived from a different donor: *$p < 0.01$, **$p < 0.001$  (One-way ANOVA with Dunnett's

425 Multiple Comparison Post Test).

426 (**b**) M1 macrophages were transfected with siRNA targeting IRF5 (siIRF5) and stimulated

427 with LPS (10ng/ml) plus IFN-$\gamma$ (50ng/ml) for 24h. IL-12p70, IL-23, IL-12p40 or IL-10

428 secretion was compared to control cells transfected with non-targeting siRNA (siC). Data

429 shown are the mean $\pm$ SEM of 6-8 independent experiments each using M1

430 macrophages derived from a different donor:  *$p < 0.01$, **$p < 0.001$, (Student's t-test).

431

432 **Figure 3:**

433 **IRF5 promotes lymphocyte proliferation and $T_H1$-$T_H17$ response**

434 (**a**, **d**) M2-like macrophages were infected with adenoviral vectors encoding IRF5, IRF3

435 or empty vector (pENTR) and cultured with T lymphocytes from unmatched donors. After

436  4 days, cells were stimulated for 3h with PMA, ionomycin and Brefeldin A and IFN-γ and

437  IL-17 expression determined by immunocytochemistry staining. Data are shown as the

438  mean fluorescence intensity (MFI) ± SEM of 7 independent experiments.

439  (**b**, **e**) Supernatants after 4 days of co-culture were analysed for IFN-γ (**b**) and IL-17A

440  (**d**) production. Data are shown as the mean ± SEM of 6 (**b**) or 4 (**c**) independent

441  experiments. ~200pg/ml of IFN-γ and no detectable IL-17 was produced by M2

442  macrophages infected with adenoviral vectors encoding IRF5, IRF3 or empty vector

443  (pENTR).

444  (**c**, **f**) M2-like macrophages were infected with adenoviral vectors encoding IRF5, IRF3 or

445  empty vector (pENTR) and cultured with T lymphocytes from unmatched donors. T-bet

446  (TBX21) (**c**) and RORγT (RORC2) (**f**) mRNA expression was analysed after 2 days of co-

447  culture. Data are shown as the mean ± SEM of 6 independent experiments each using

448  cells derived from a different donor.

449  (**a-f**) *p< 0.05, **p<0.01, ***p<0.001 (One-way ANOVA with Dunnett's Multiple

450  Comparison Post Test).

451

452  **Figure 4:**

453  **IRF5 regulates mRNA expression of macrophage lineage specific cytokines**

454  (**a**) M2 macrophages were infected with adenoviral vectors encoding IRF5 or IRF3 and

455  basal cytokine mRNA expression was compared to empty vector (pENTR) control infected

456  cells. IL-12p40, IL-12p35, IL-23p19 or IL-10 mRNA levels in unstimulated cells were

457  analysed by q-PCR. Data shown are the mean ± SEM of 3-6 independent experiments

458  each using M2 macrophages derived from a different donor:  *p< 0.05, **p<0.01,

459  ***p<0.001 (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

460  (**b**) M1 macrophages were transfected with siRNA targeting IRF5 (siIRF5) and stimulated

461  with LPS (10ng/ml) for 8h or left untreated (IL-10). IL-12p40, IL-12p35, IL-23p19 or IL-

462  10 mRNA expression was compared to control cells transfected with non-targeting siRNA

463  (siC). Data shown are the mean ± SEM of 5-6 independent experiments presented as a

464  % of reduction in cytokine mRNA levels by siIRF5: **p<0.01, ***p< 0.001 (Student's t-

465  test).

466  (**c**) M2- macrophages from 4 different donors were infected with adenoviral vectors

467  encoding IRF5 or empty vector (pENTR) and global mRNA expression was analysed using

468  Illumina HumanHT-12 Expression BeadChips. Heatmaps showing the fold change in

469  M2+IRF5 cells relative to M2 cells at 0hr for sets of M1 and M2-specific genes described

470  in [21,27]. Red indicates higher expression in M2+IRF5 and green indicates higher

471  expression in M2 (scale shows the log2 fold change). M1-specific genes tend to be more

472  highly expressed in M2+IRF5 cells whereas M2-specific genes are downregulated by

473  IRF5.

15

474

**Figure 5:**

**IRF5 is directly involved in transcriptional regulation of lineage specific cytokines**

(**a-d**) M1 macrophages were left unstimulated or stimulated with LPS (10ng/ml) for 1, 2, 4, 8 or 24h followed by ChIP with antibodies specific to IRF5 (black bars), PolII (grey bars), or IgG control (white bars). Protein recruitment to the promoters of IL-12p40 (**a**), IL-12p35 (**b**), IL-23p19 (**c**) or IL-10 (**d**) was measured and presented as mean % input relative to genomic DNA (gDNA) ± SD of a representative experiment.

**Figure 6:**

**IRF5 inhibits transcriptional activation of the human IL-10 gene**

M2 macrophages were co-infected with (**a**) IL-10 wild type (IL-10-Luc wt) reporter plasmid or (**b**) the IL-10 plasmid in which site-specific mutations were introduced into the ISRE site at -180 to -173 (IL-10-Luc ISRE mut) and constructs coding for IRF5 (black bars), IRF5 DNA-binding mutant (IRF5ΔDBD) (grey bars) or empty vector (pENTR) (white bars). 24h post-infection, cells were left unstimulated (Un) or stimulated with LPS (10ng/ml) for 4h and luciferase activity was measured. Data are presented as the mean ± SEM from 3 independent experiments each using M2 macrophages derived from a different donor: *p<0.01 (One-way ANOVA with Dunnett's Multiple Comparison Post Test).

**Figure 7:**

**Impaired production of M1 and $T_H1$-$T_H17$ cytokines in *Irf5$^{-/-}$* mice**

(**a**) Bone-marrow cells from C57BL/6 mice were differentiated into M1 macrophages with GM-CSF (50ng/ml). On day 8, total protein extracts from adherent cells were subjected to immunoblot analysis with antibodies against IRF5.

(**b**) M1 macrophages from C57BL/6 mice (n=8) were stimulated with LPS (100ng/ml) for 24h and the amount of secreted IL-12p70, IL-23 and IL-10 was determined by ELISA. Data shown are the mean ± SEM of 8 samples from 3 independent experiments:  *p< 0.05, **p<0.01 (Student's t-test).

(**c**) Littermate wild type (n = 10) and *Irf5$^{-/-}$* (n = 10) mice were intraperitoneally injected with LPS (20ug/ml). Mice were sacrificed after 3h and serum concentrations of IL-12p40, IL-23 and IL-10 were measured by either ELISA (IL-12p40, IL-23) or BD$^{TM}$ cytrometric bead assay (IL-10). Data are shown as the mean ± SEM of 8-10 serum samples from 3 independent experiments: *p<0.05, ** p<0.01 (Student's t-test).

16

510     (**d**) mRNA levels of selected M1 and M2 markers were analysed in peritoneal cells from

511     LPS-injected mice in (**c**). Data are shown as the mean ± SEM of 10 samples from 3

512     independent experiments: *** $p < 0.001$, ** $p < 0.01$, *$p < 0.05$ (Student's t-test).

513     (**e**) Spleen cells from LPS-injected mice in (**c**) were cultured in the presence of anti-CD3

514     antibodies for 48h. The amount of secreted IFN-γ and IL-17a was determined by ELISA.

515     Data are shown as the mean ± SEM of 4-5 spleen cultures from two independent

516     experiments: *$p < 0.05$, ** $p < 0.01$ (Student's t-test).

517

518

519  **MATERIALS AND METHODS**

520  **Plasmids**

521  Expression constructs encoding full length human IRF3, IRF5v3/v4, and IRF5ΔDBD were

522  described in [22]. The vectors encoding IRF5 and IRF3 expressed similar levels of proteins,

523  but only IRF5 resulted in a significant increase in TNF secretion, while only IRF3 induced

524  type III IFNs [22]. IL-10 promoter driven luciferase-reporter constructs were previously

525  described [28]. The IL-12p35 wild-type and IL-12p35 ISRE mutant promoter constructs [50]

526  were a kind gift from Prof Xiaojing Ma (Cornell University, USA). The sequences and

527  restriction maps are available upon request.

528

529  **Mice**

530  The generation of *Irf5*$^{-/-}$ mice has been described [12]. For generation of BMDMs/GM-CSF,

531  bone marrow of wild-type or *Irf5*$^{-/-}$ was cultured in RPMI (PAA, USA) supplemented with

532  50ng/ml recombinant mouse GM-CSF (Preprotech, UK). After 8 days, adherent cells

533  were washed with PBS, re-plated and stimulated with 100ng/ml LPS (Alexis

534  Biochemicals, USA). For *in vivo* experiment, littermate wild-type and IRF5$^{-/-}$ mice were

535  intraperitoneally injected with 20ug LPS in 200ul sterile PBS. Mice were sacrificed after

536  3h and serum was collected. Spleens were removed and cultured in DMEM supplemented

537  with 10ng/ml anti-CD3 antibodies (BD Bioscience, USA) for 48h.

538

539  **Cell culture**

540  Enriched populations of human monocytes were obtained from the blood of healthy

541  donors by elutriation as described previously [22]. M1 and M2 macrophages were obtained

542  after 5 days of culturing human monocytes in RPMI 1640 (PAA, USA) supplemented with

543  50ng/ml GM-CSF or 100ng/ml M-CSF (Peprotech, UK). Cells were stimulated with

544  10ng/ml LPS (Alexis Biochemicals, USA) or 10ng/ml LPS plus 50ug/ml IFN-γ (Peprotech,

545  UK). For "priming" experiments M1 macrophages at day 5 were simulated for 24h with

546  M-CSF (100ng/ml). Similarly, M2 macrophages at day 5 were stimulated for 24h with

547  GM-CSF (50ng/ml); IFN-γ (50ng/ml) or LPS (10ng/ml) plus IFN-γ (50ng/ml).

548

549  **Measurement of cytokine production**

550  Cytokine secretion was quantified with specific ELISAs for human IL-12p40, IL-12p70,

551  IL-10, IFN-γ, TNF, CXCL10, IL-1β (BD Bioscience, USA); IL-23, CCL2 (eBioscience);

552  CCL5, CCL13, CCL22 (R&D Systems) and IL-17A, IL-4 (Insight Biotechnology). Mouse

553  cytokine secretion was quantified with specific ELISAs for Il-12p70, Il-23 and Il-10

554  (eBioscience); IFN-γ, IL-17a (BD Bioscience, USA) and serum levels of mouse IL-1β,

555  TNF, IL-6 and IL-10 were determined by BD$^{TM}$ cytrometric bead assay (BD Bioscience, USA)

556  on a FACS Canto II (BD Bioscience).

18

557

**Mixed lymphocyte reaction**

559  Human macrophages were plated in 96-well plate at 2 x 10$^4$ cells/well.  T lymphocytes

560  were isolated from the blood of healthy donors by elutriation, analysed by FACS and

561  used if purity was >90%.  T lymphocytes were added to macrophages at 5 x 10$^5$

562  cells/well. Control cultures contained medium, T lymphocytes or M2 macrophages alone.

563  After 72-96h of co-culture supernatants were collected for detection of cytokines.  For

564  proliferation experiments, cells were pulsed with 37,000 Bq of [3H]thymidine

565  (Amersham Biosciences, USA) 16h before harvest and DNA synthesis was measured by

566  [3H]thymidine incorporation using a Beckman beta scintillation counter (Beckman

567  Instruments, USA).

568

**RNA interference**

570  siRNA-mediated knockdown was performed using On-target plus SMART pool reagent

571  (Dharmacon, USA) designed to target human IRF5.  DharmaFECT I® (Dharmacon, USA)

572  was employed as the siRNAs transfection reagent according to manufacturers'

573  instructions.

574

**Adenoviral infection**

576  Infections of M2 macrophages were performed as described previously [22].

577

**RNA extraction and quantitative real-time RT-PCR**

579  Total RNA was extracted from cells using a QiaAmp RNeasy mini kit (Qiagen, Germany)

580  according to manufacturer's instructions. cDNA was synthesised from total RNA using

581  SuperScript® III Reverse Transcriptase (Invitrogen, USA) and 18-mer oligo dTs

582  (Eurofins MWG Operon, UK).  The gene expression was analysed by ΔΔCt method based

583  on the quantitative real-time PCR with TaqMan primer sets for human IL-12p35, IL-

584  12p40, IL-23p19, IL-10, IFN-γ, IL-17A/F, IL-21, IL-22, IL-26, IL-23R, TBX21 (for T-bet),

585  Mrc1, Arg1, Rentla (for Fizz1) and PO (Applied Biosystems) in an ABI 7900HT machine

586  (Applied Biosystems, USA). RORγt was detected by SybrGreen with the primer set for the

587  human RORC2 gene (RORC2_F1: TGAGAAGGACAGGGAGCCAA; RORC2_R1:

588  CCACAGATTTTGCAAGGGATCA).

589

**Luciferase gene reporter assay**

591  Infections of M2 macrophages were performed in 96-well plates in triplicate at a

592  multiplicity of infection of 50:1. Cells were seeded in serum-free, antibiotics-free RPMI

593  containing the desired number of viral particles in a final volume of 50µl.  Cells were

594  infected with expression constructs coding for IRF5, IRF5ΔDBD or empty vector and after

595    6 h followed by infection with IL-10 luciferase constructs. Cells were allowed to recover

596    for 24 hours before experimental assay. Co-transfections of HEK-293-TLR4/MD2 cells

597    with the IL-12p35 wild-type and IL-12p35 ISRE mutant constructs were performed as

598    described previously [22].

599

600    **Total protein extracts and immunoblots**

601    Total protein extracts were prepared as previously described [22]. Equal amounts of

602    proteins were resolved by SDS-PAGE and analysed with antibodies against IRF5 (ab2932

603    or ab21689, Abcam, UK), IRF3 (sc-9082x), IRF4 (sc-28696), p50 (sc-114x), RelA (sc-

604    372x), all form Santa Cruz, USA, and actin (A5541, Sigma, USA).

605

606    **Flow cytometry**

607    For surface staining of T cells, cells were stained for 30 min at 4 °C with anti-CD4-FITC

608    and anti-CD8-Per-CP-Cy5 (BD Bioscience). For intracellular cytokine staining (ICC), cells

609    were stimulated for 3-4h with phorbol myristate acetate (PMA), ionomycin and Brefeldin

610    A (Sigma-Aldrich). Cells were stained for cell surface markers, fixed in Cytofix (BD

611    Bioscience) and permeabilized using PBS containing 1% FCS, 0.01% sodium azide, and

612    0.05% saponin and stained with anti-IFN-γ-PB and anti-IL-17-PE (eBioscience). For

613    surface staining, macrophages were incubated for 30 at 4 °C with anti-CD40-APC

614    (eBioscience) and anti-CD163-PE (R&D Systems). Samples were run on a FACS Canto II

615    (BD Bioscience) and analysed using FlowJo software (TreeStar).

616

617    **Chromatin Immunoprecipitation**

618    ChIP assays were carried out essentially as previously described [22] using antibodies

619    against IRF5 (ab2932, Abcam, UK), Pol II (sc-899, Santa Cruz, USA) or IgG control

620    (PP64, Milipore, USA). The immuno-precipitated DNA fragments were then interrogated

621    by real-time PCR using SYBR®Premix Ex Taq II™ master mix (Takara Bio, USA) and the

622    following primers for IL12-p35 locus: (TCATTTTGGGCCGAGCTGGAG and

623    TACATCAGCTTCTCGGTGACACG); IL-12p40 locus: (TCCAGTACCAGCAACAGCAGCAGA and

624    GTAGGGGCTTGGGAAGTGCTTACCTT); IL-23p19 locus:

625    (ACTGTGAGGCCTGAAATGGGGAGC and ACTGGATGGTCCTGGTTTCATGGGAGA) and IL-10

626    locus: (CCTGTGCCGGGAAACCTTGATTGTGGC and

627    GTCAGGAGGACCAGGCAACAGAGCAGT). Data were analysed using an ABI 7900HT

628    software (Applied Biosystems, USA).

629

630    **Microarray, statistics and bioinformatics analyses.** Microarray, statistics and

631    bioinformatic analyses are described in **Supplementary Methods.**

632

633

**References**


634     **References**

635

636

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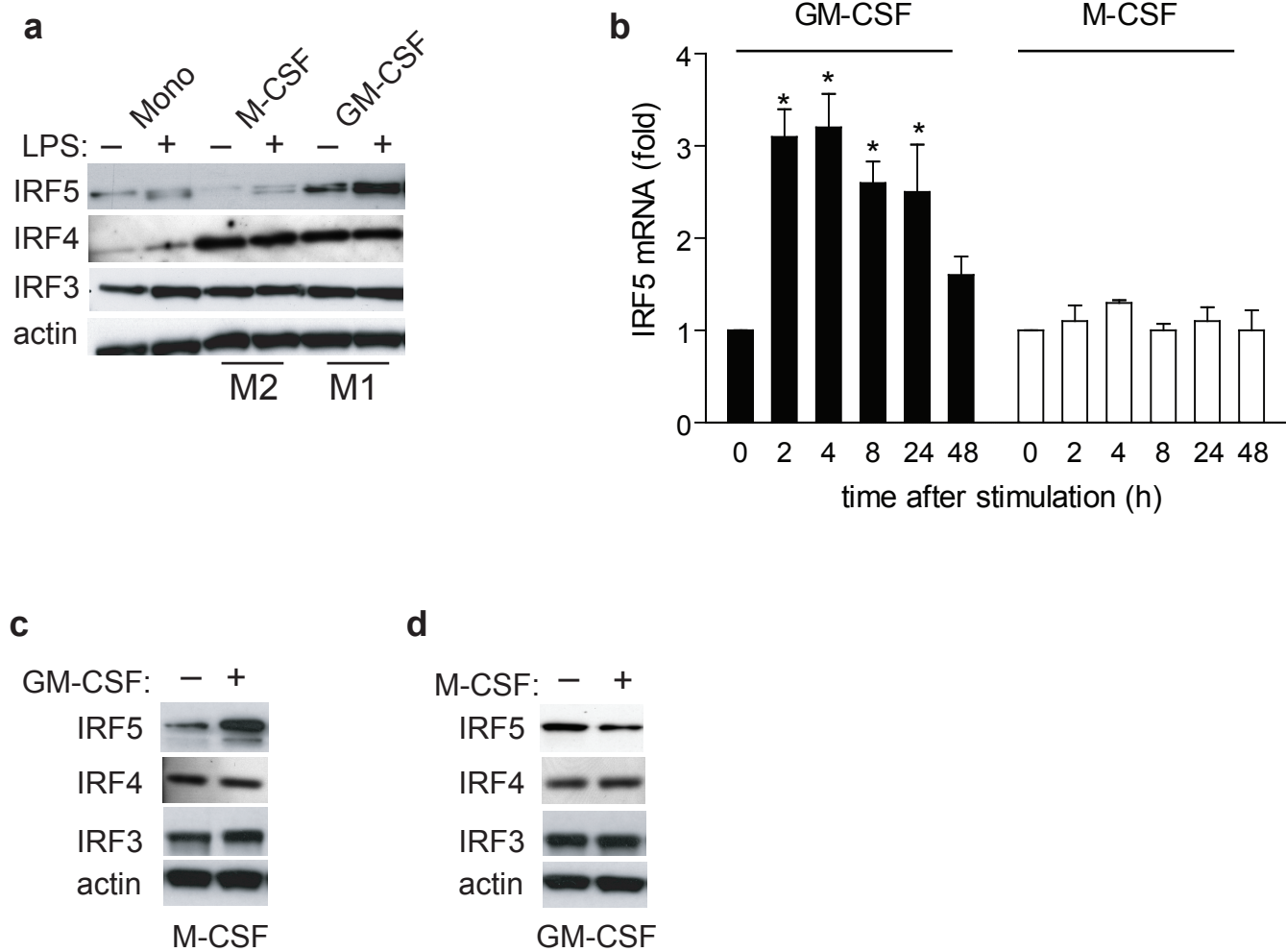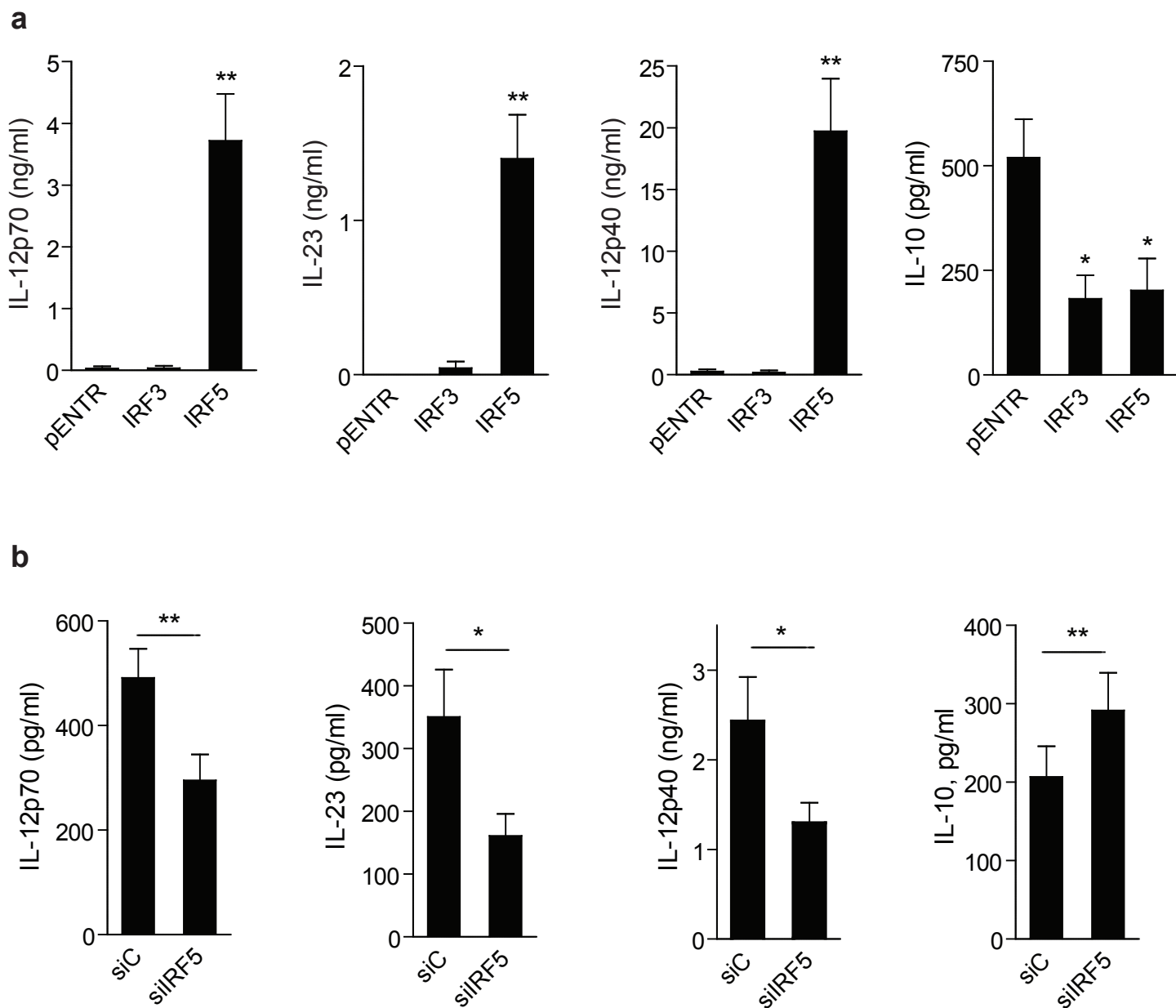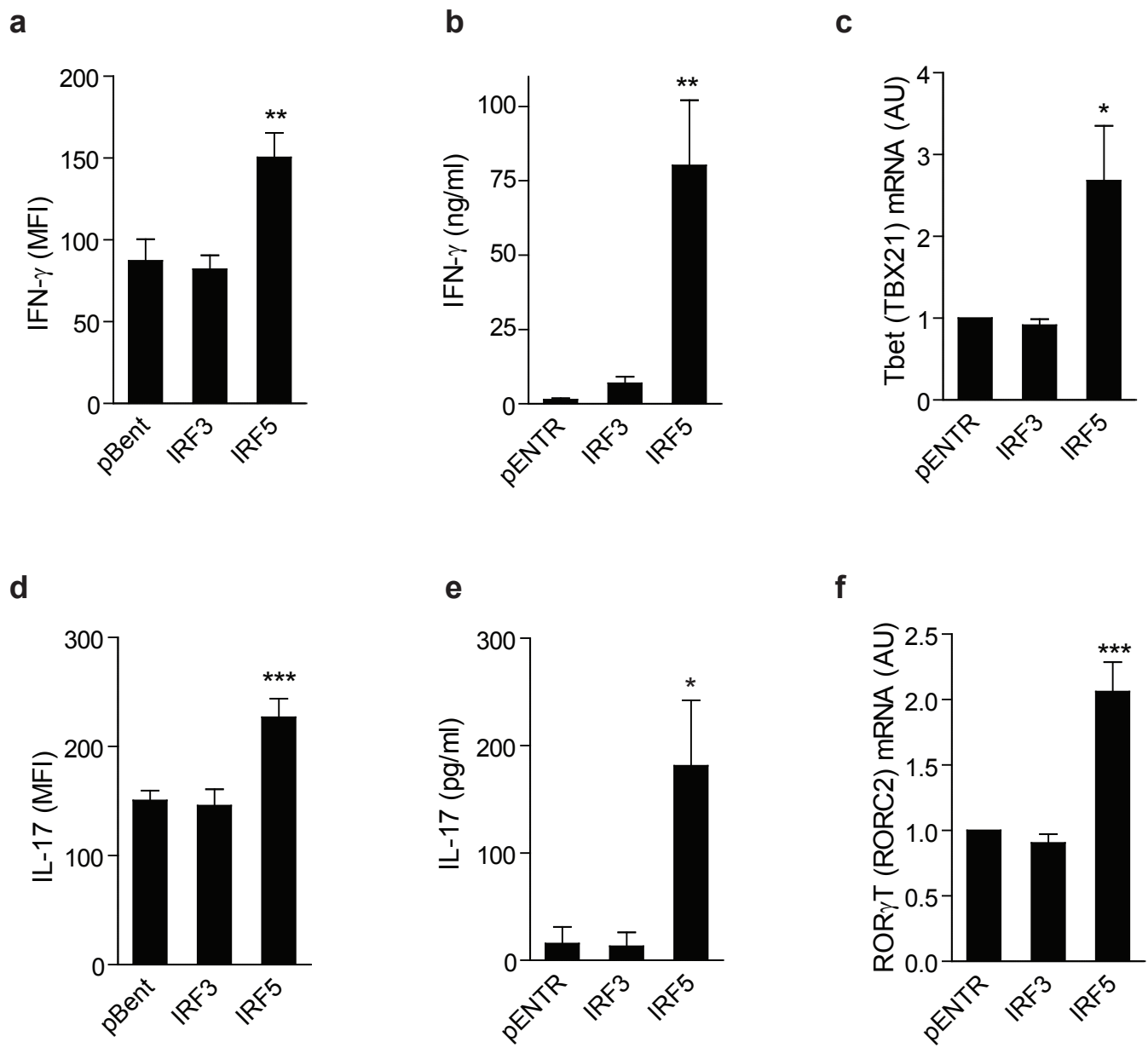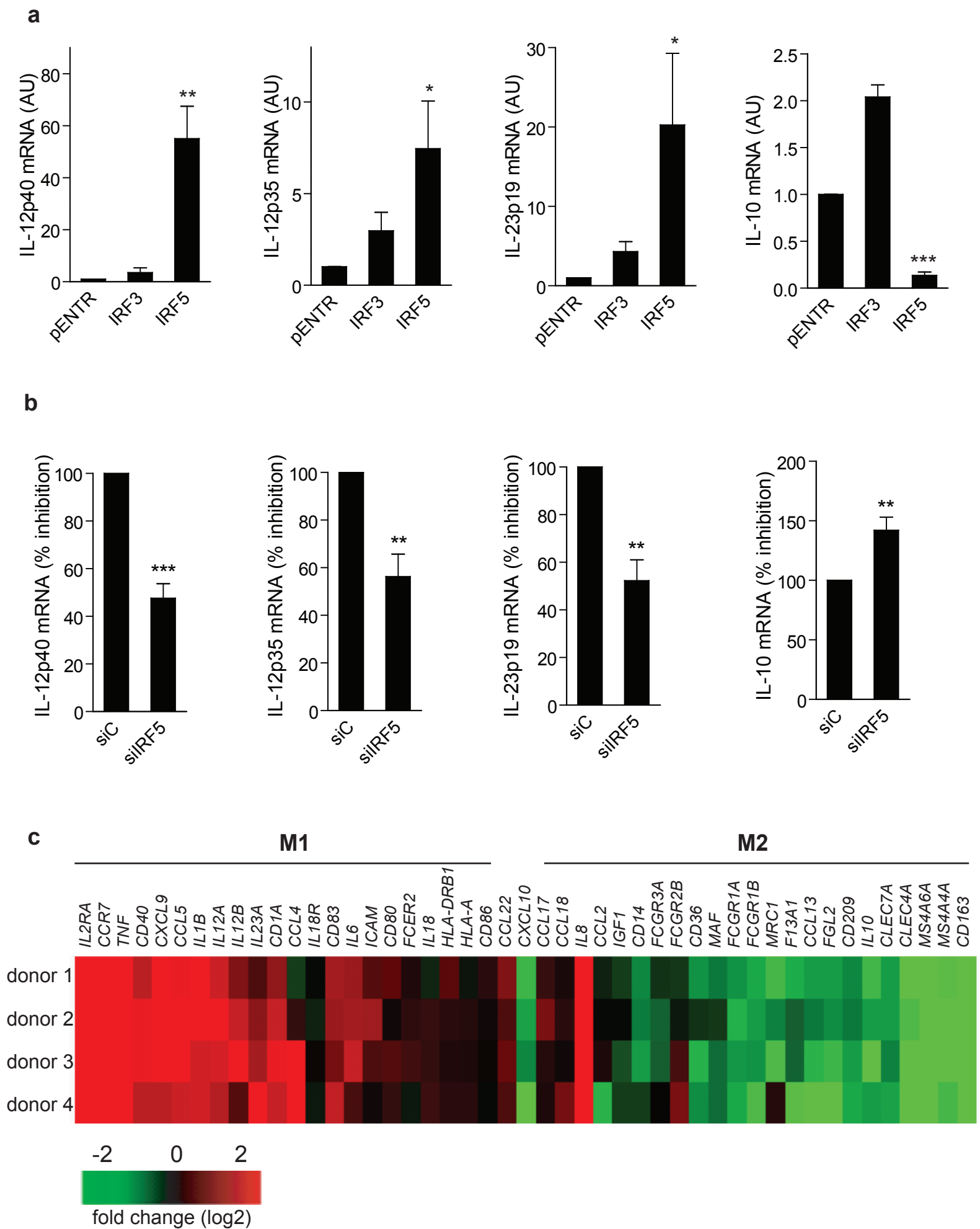767
768
769

Figure 1

**a**



**b**



Figure 2

**a**

IFN-γ (MFI)

pBent    IRF3    IRF5

**

**b**

IFN-γ (ng/ml)

pENTR    IRF3    IRF5

**

**c**

Tbet (TBX21) mRNA (AU)

pENTR    IRF3    IRF5

*

**d**

IL-17 (MFI)

pBent    IRF3    IRF5

***

**e**

IL-17 (pg/ml)

pENTR    IRF3    IRF5

*

**f**

RORγT (RORC2) mRNA (AU)

pENTR    IRF3    IRF5

***

Figure 3

**Figure 4**

**Figure 5**

**a**



**b**



□ pENTR  ■ IRF5  ▨ IRF5ΔDBD

**Figure 6**

Figure 7