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HAL Authorization

1 **IRF5 promotes inflammatory macrophage polarization and T_H1-T_H17 response**

2

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18

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.

30

31

32

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

44

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

58

59 IRF5, another member of the IRF family, has diverse activities, such as activation of type
60 I interferon (IFN) genes, inflammatory cytokines, including TNF, IL-6, IL-12 and IL-23,
61 and tumour suppressors ¹¹. Consequently, IRF5 deficient mice are resistant to lethal
62 endotoxic shock ¹². Human IRF5 is expressed in multiple splice variants with distinct cell
63 type-specific expression, cellular localization, differential regulation and functions ¹³.
64 Moreover, genetic polymorphisms in the human IRF5 gene, leading to expression of
65 several unique isoforms or increased expression of IRF5 mRNA, is implicated in
66 autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid
67 arthritis (RA), Sjogren's syndrome, multiple sclerosis and inflammatory bowel disease <sup>14-
68 18</sup>. Here we show a role for IRF5 in determining M1 macrophage lineage commitment. M1
69 macrophages are characterised by high level of IRF5, expression of which is induced
70 during their differentiation. Forced expression of IRF5 in M2 macrophages drives global

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.

80

81 RESULTS

82 IRF5 is highly expressed in human M1 macrophages

83 The M1 macrophage phenotype is induced by Interferon gamma (IFN- γ) followed by
84 stimulation with bacterial products like lipopolysaccharide (LPS) or by treatment of
85 monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF)
86 (**Supplementary Fig 1a** and Ref ¹⁹⁻²¹). IRF5 mRNA expression was elevated in primary
87 human monocytes or in monocyte-derived macrophages differentiated with GM-CSF (**Fig**
88 **1a, Supplementary Fig 1b**). Treatment of monocytes with GM-CSF but not
89 macrophage colony stimulating factor (M-CSF) – an M2 differentiating factor – resulted
90 in up-regulation of IRF5 mRNA expression within 4 h post stimulation (**Fig 1b**). To
91 account for possible differences in macrophage *in vitro* differentiation protocols, we
92 analysed the level of IRF5 in macrophages treated with either IFN- γ alone or in
93 combination with LPS for 24h and found that these were similar to the ones in GM-CSF
94 treated cells (**Supplementary Fig 1c**). The expression of IRF4, which controls M2
95 polarisation in mice ¹⁰, was equally induced during monocyte differentiation into M1 or
96 M2 macrophages (**Fig 1a**). The expression of IRF3, another member of the IRF family
97 central to the innate immune response, was not affected by differentiation into
98 macrophage subtypes (**Fig 1a**). Thus, IRF5 is induced in pro-inflammatory M1
99 macrophages independently of the *in vitro* differentiation protocol, whereas the mRNA
100 levels of IRF4 and IRF3 are comparable between the macrophage populations. There was
101 no significant difference between M1 and M2 macrophages in their basal or LPS-induced
102 levels of NF- κ B p50 protein, previously implicated in macrophage polarisation towards
103 M2 phenotype ⁸ (**Supplementary Fig 1d,e**).

104

105 Expression of IRF5 in human macrophages is plastic

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

118

119 **IRF5 influences human M1 macrophage polarization**

120 We next investigated whether IRF5 would directly induce expression of M1 macrophage
121 phenotypic markers. Bioactive IL-12p70 and IL-23 were detected in M2 macrophages
122 infected with the adenoviral expression construct encoding for human IRF5 (variant 3/4)
123 ¹³, but minimal with IRF3 or an empty vector ²² (**Fig 2a**). The up-regulation of IL-12p70
124 and IL-23 was reflected by a vast increase in secretion of the p40 subunit shared by the
125 two cytokines (**Fig 2a**). The secretion of both IL-12p70 and IL-23 peaked at 24h post
126 LPS stimulation and remained sustained up to at least 48h (**Supplementary Fig 3a**).
127 We also observed a significant increase in production of other key pro-inflammatory
128 cytokines such as IL-1 β and TNF by IRF5-expressing macrophages (**Supplementary Fig**
129 **3b**). Remarkably, IL-10 production in the IRF5-expressing cells was noticeably reduced
130 (**Fig 2a**). We also observed IL-10 protein inhibition in cells over-expressing IRF3 (**Fig**
131 **2a**), which might represent a negative feedback regulation of IL-10 expression ²³, since
132 the main direct target of IRF3, IFN- β , induces IL-10 ²⁴. The complementary experiment
133 targeting endogenous IRF5 in M1 macrophages by RNA interference (RNAi)
134 (**Supplementary Fig 3c**) resulted in significant inhibition of IL-12p70 and IL-23 and
135 increase of IL-10 (**Fig 2b**). Secretion of IL-12p40 was also reduced in these cells (**Fig**
136 **2b**), consistent with the data obtained in mouse myeloid cells deficient in *Irf5* ¹². Taken
137 together, IRF5 influences M1 macrophage polarisation by equipping the cells with an IL-
138 12^{hi}, IL-23^{hi}, IL-10^{lo} cytokine profile.

139

140 **IRF5 promotes human T_H1-T_H17 response**

141 One of the hallmarks of M1 macrophage polarisation is acquired antigen presenting
142 features leading to efficient T_H1 response ^{20, 21}. To examine whether IRF5-aided the
143 polarisation of T lymphocyte proliferation, fate or activation state, human M2
144 macrophages were infected with IRF5, IRF3 viral expression constructs or an empty
145 vector and exposed to human T lymphocytes extracted and purified from peripheral
146 blood of major histocompatibility complex (MHC) unmatched donors in a mixed
147 lymphocyte reaction (MLR). Total T lymphocyte proliferation was determined 3 days after
148 infection, while activation of specific T cell subsets was analysed by flow cytometry
149 (**Supplementary Fig 4a**). Proliferation of T lymphocytes (**Supplementary Fig 4b**) was
150 considerably higher when co-cultured with IRF5-expressing macrophages. Furthermore,
151 only IRF5-expressing macrophages provided the cytokine environment necessary for T_H1
152 expansion and activation, assessed by increased number of IFN- γ producing CD4⁺ cells
153 (**Fig 3a** and **Supplementary Fig 4c**) and mRNA (**Supplementary 4d**) and protein (**Fig**
154 **3b**) expression of IFN- γ . In these cultures we also observed expansion and activation of
155 T_H17 cells, assessed by increased number of IL-17 producing CD4⁺ cells (**Fig 3d** and

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_H17 and T_H1 cells ²⁶. mRNA
161 expression of the T_H1 and T_H17 specifying transcription factors T-bet and ROR γ t 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_H1 and T_H17 lineages, but does not induce T_H2 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 Δ DBD). The IRF5 Δ DBD 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

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 shock¹², support a
260 major role of IRF5 in establishing pro-inflammatory macrophage phenotype in animal
261 models of M1-polarizing inflammation.

262

263 **Discussion**

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

275
276 The rapid and potent transcriptional response developed by macrophages encountering
277 microbial stimuli, such as LPS, or subsequently cytokines, is orchestrated by many TFs.
278 Among them are class III TFs, such as PU.1, C/EBP β , RUNX1, IRF8, which are lineage-
279 specific transcriptional regulators turned on during macrophage differentiation ³⁰. The
280 combinatorial expression of these proteins specifies the macrophage phenotype via
281 constitutive activation or repression of genes and chromatin remodelling at inducible loci.
282 For instance, PU.1 is required for maintaining H3K4me1 enhancer marks at macrophage-
283 specific enhancers ³¹. But only a small proportion of the macrophage transcriptome is
284 altered by cell polarization ²⁷ and among the genes differentially expressed between the
285 M1 and M2 subsets are those regulated by IRF5, such as *IL12p40*, *IL12p35*, *IL23p19*,
286 *IL1 β* , *TNF*, *MIP1a*, *RANTES*, *CD1a*, *CD40*, *CD86*, *CCR7*. Another member of the IRF
287 family, IRF4, known to inhibit IRF5 activation by competing for interaction with Myd88
288 ³², has been recently reported to control the expression of prototypical mouse M2
289 macrophage markers ¹⁰. In human cells we found that the expression of IRF4 is equally
290 induced by M-CSF or GM-CSF differentiation and is further enhanced by exposure to IL-4
291 ³³. IRF5 expression, on the other hand, is specifically induced by GM-CSF or IFN- γ , but is
292 unresponsive to IL-4. Thus, IRF5 and IRF4 may be classified as class III TFs but with a
293 caveat that they define specific macrophage subsets rather than the global macrophage
294 lineage. NF- κ B proteins, in particularly c-Rel and RelA, are important for expression of
295 M1-specific cytokines ^{34, 35}. IRF5 and RelA cooperate in induction of the TNF gene ²². It is
296 interesting to speculate whether the genes encoding IL-12, IL-23 subunits and other M1-
297 specific markers might be under similar joint transcriptional control. Thereby, IRF5 may
298 participate in the combinatorial assembly with macrophage-specific TFs, e.g. PU.1, and
299 environmentally induced NF- κ B ³¹, to define the activity of specifically M1 enhancers.

300

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 infection³⁶. IL-10
305 represses immune responses by down-regulating inflammatory cytokines like TNF³⁷ 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
308³⁸. Major producers of IL-10 include M2 macrophages, B cells and T cells³⁹, whereas M1
309 macrophages and DCs are only weak producers²¹. 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 studies⁴⁰ 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 repertoire⁴¹. While human M1 but not M2
321 macrophages have been shown to secrete high levels of CCL22²¹, 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 signals². 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) pathway²⁹, and an ISRE element within the IRF5 promoter can bind STAT1
338 and STAT2¹³, 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-12⁴² and IL-23/IL-1 β ⁴³
345 respectively. T_H1 cells constitutively express IFN- γ and T-bet, whereas T_H17 express
346 ROR γ T, 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-1 β , 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 mice⁴⁶, 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 stimulus⁴⁷ and the blockade of GM-CSF in wild-
364 type mice controls disease activity and levels of pro-inflammatory mediators in the joints
365⁴⁸.

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 network⁴⁹. 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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378

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390

391

392 **AUTHORSHIP**

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

396

397

398 **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- γ (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) \pm 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 \pm SEM of 6 (b) or 4 (c) independent
441 experiments. \sim 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 ROR γ T (RORC2) (f) mRNA expression was analysed after 2 days of co-
447 culture. Data are shown as the mean \pm 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 \pm 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 \pm 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.

474

475 **Figure 5:**

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

478 (a-d) M1 macrophages were left unstimulated or stimulated with LPS (10ng/ml) for 1, 2,
479 4, 8 or 24h followed by CHIP with antibodies specific to IRF5 (black bars), PolII (grey
480 bars), or IgG control (white bars). Protein recruitment to the promoters of IL-12p40 (a),
481 IL-12p35 (b), IL-23p19 (c) or IL-10 (d) was measured and presented as mean % input
482 relative to genomic DNA (gDNA) \pm SD of a representative experiment.

483

484 **Figure 6:**

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

486 M2 macrophages were co-infected with (a) IL-10 wild type (IL-10-Luc wt) reporter
487 plasmid or (b) the IL-10 plasmid in which site-specific mutations were introduced into
488 the ISRE site at -180 to -173 (IL-10-Luc ISRE mut) and constructs coding for IRF5 (black
489 bars), IRF5 DNA-binding mutant (IRF5 Δ DBD) (grey bars) or empty vector (pENTR)
490 (white bars). 24h post-infection, cells were left unstimulated (Un) or stimulated with LPS
491 (10ng/ml) for 4h and luciferase activity was measured. Data are presented as the mean
492 \pm SEM from 3 independent experiments each using M2 macrophages derived from a
493 different donor: *p<0.01 (One-way ANOVA with Dunnett's Multiple Comparison Post
494 Test).

495

496 **Figure 7:**

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

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

501 (b) M1 macrophages from C57BL/6 mice (n=8) were stimulated with LPS (100ng/ml) for
502 24h and the amount of secreted IL-12p70, IL-23 and IL-10 was determined by ELISA. Data
503 shown are the mean \pm SEM of 8 samples from 3 independent experiments: *p< 0.05,
504 **p<0.01 (Student's t-test).

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

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 \pm 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 \pm 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 IRF5 Δ DBD 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-1 β (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-1 β ,
555 TNF, IL-6 and IL-10 were determined by BDTM cytometric bead assay (BD Bioscience, USA)
556 on a FACS Canto II (BD Bioscience).

557

558 **Mixed lymphocyte reaction**

559 Human macrophages were plated in 96-well plate at 2×10^4 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 5×10^5
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

569 **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

575 **Adenoviral infection**

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

577

578 **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 $\Delta\Delta C_t$ 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, Rent1a (for Fizz1) and PO (Applied Biosystems) in an ABI 7900HT machine
586 (Applied Biosystems, USA). ROR γ t was detected by SybrGreen with the primer set for the
587 human RORC2 gene (RORC2_F1: TGAGAAGGACAGGGAGCCAA; RORC2_R1:
588 CCACAGATTTTGCAAGGGATCA).

589

590 **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 50 μ l. Cells were
594 infected with expression constructs coding for IRF5, IRF5 Δ DBD 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 from 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 ACTGGATGGTCTGTTTCATGGGAGA) 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

634 **References**

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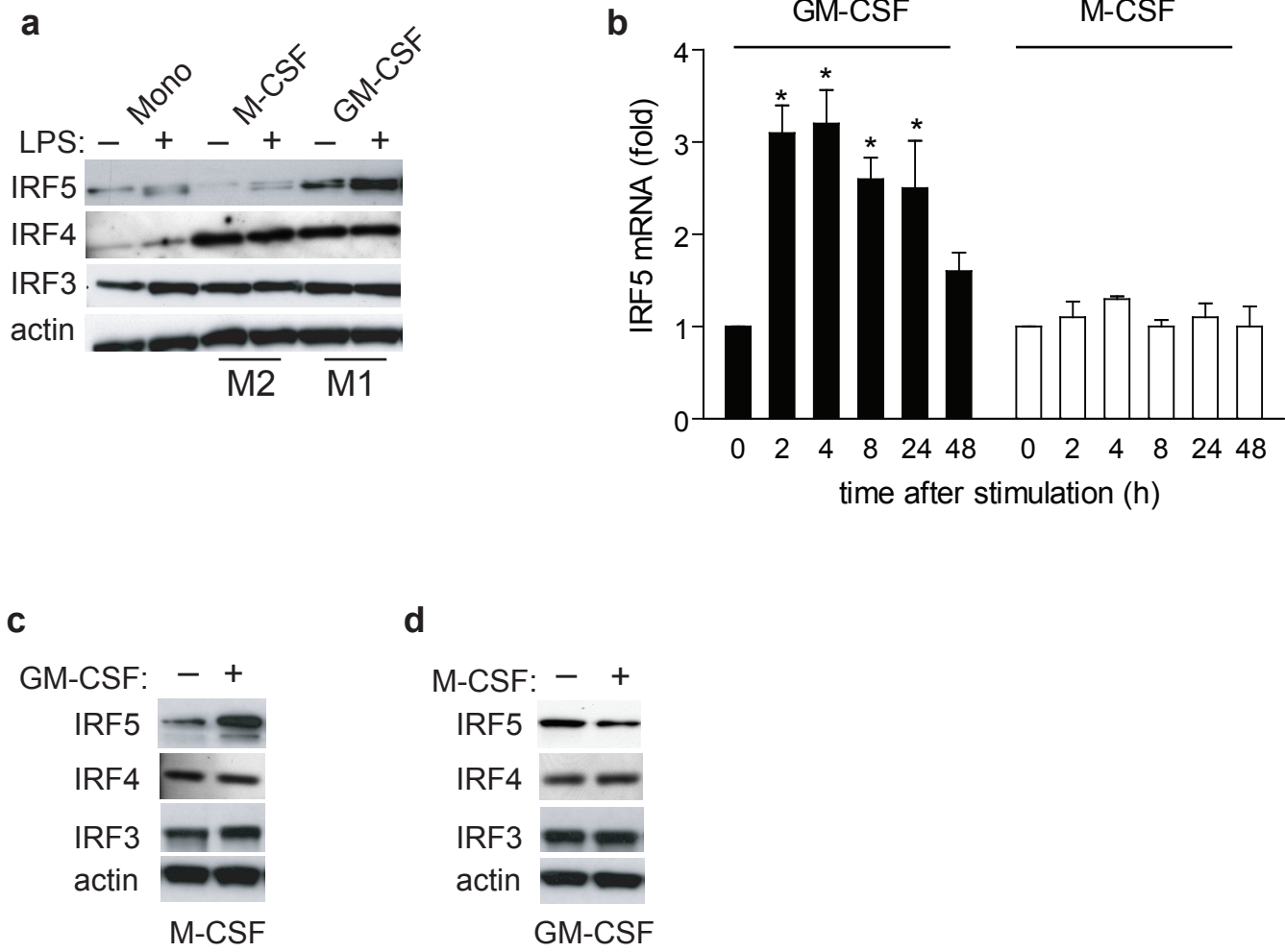


Figure 1

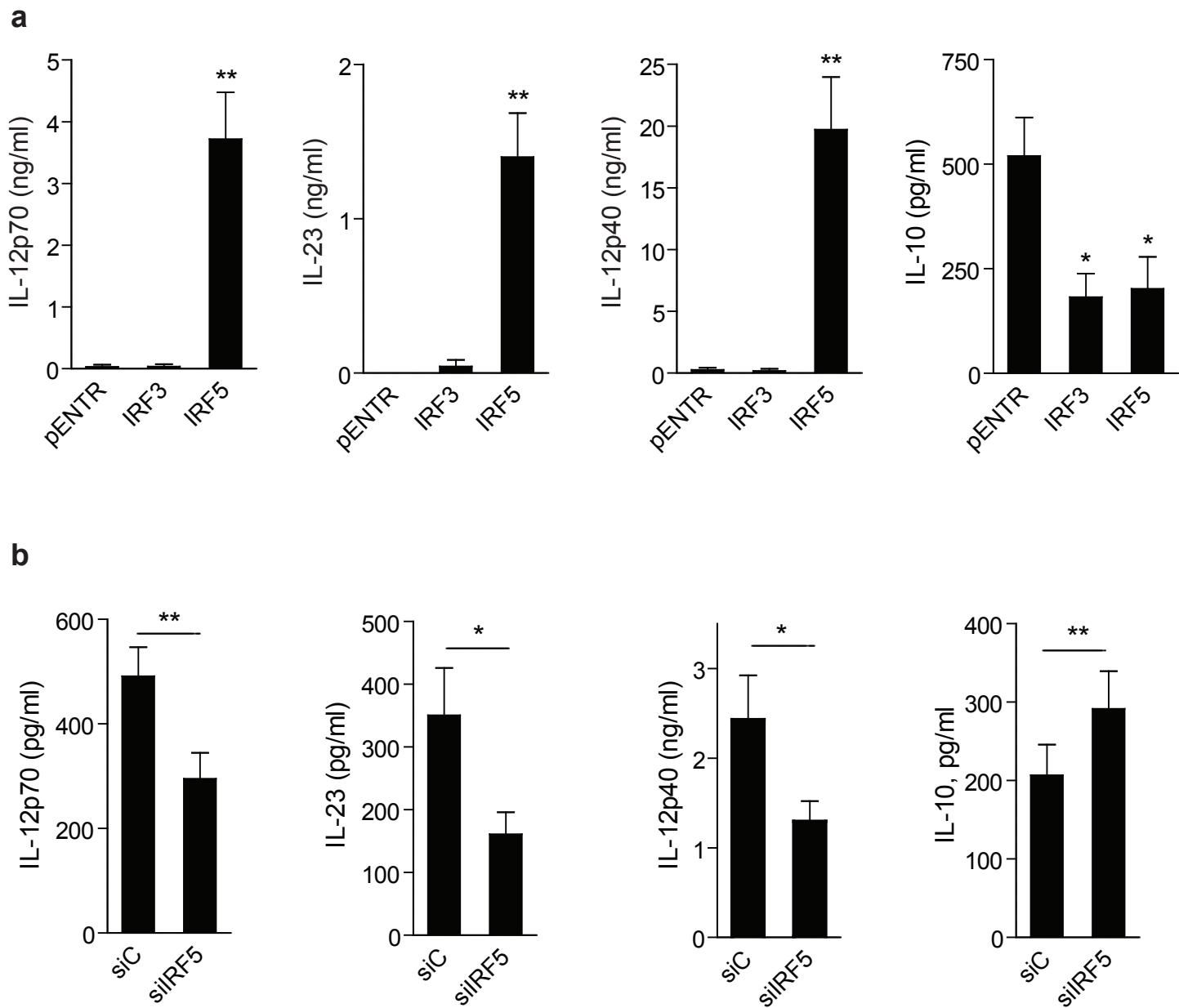


Figure 2

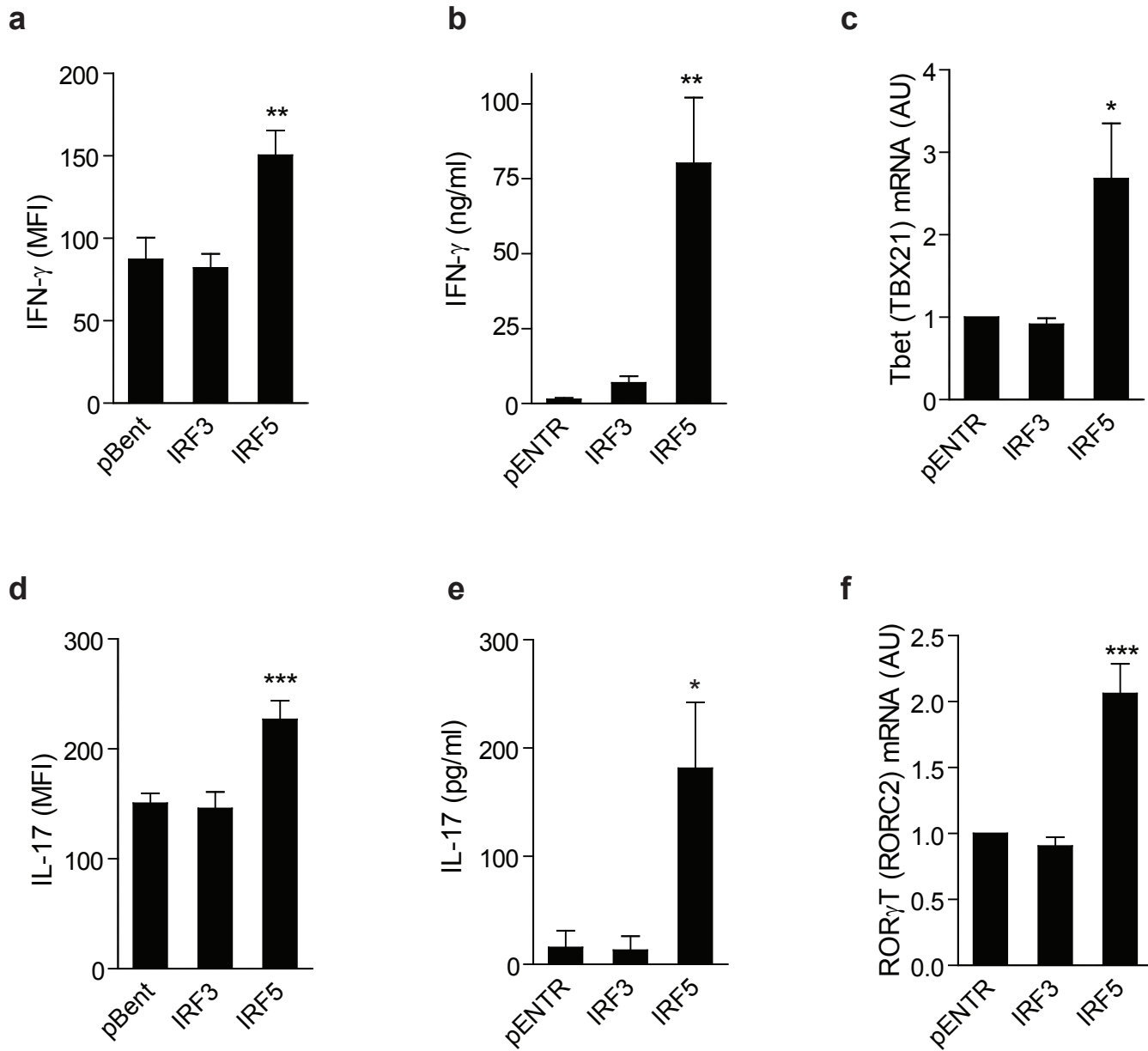


Figure 3

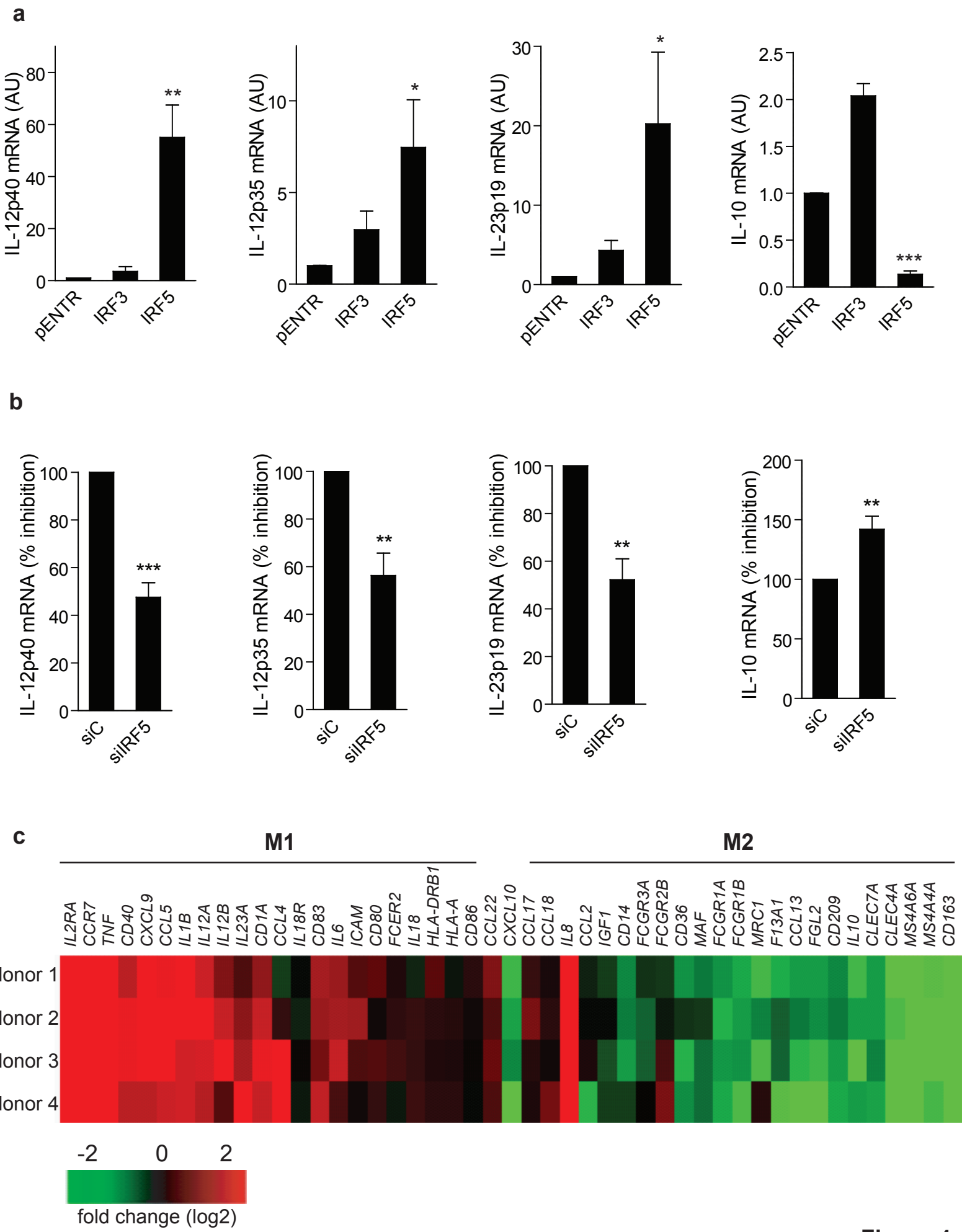


Figure 4

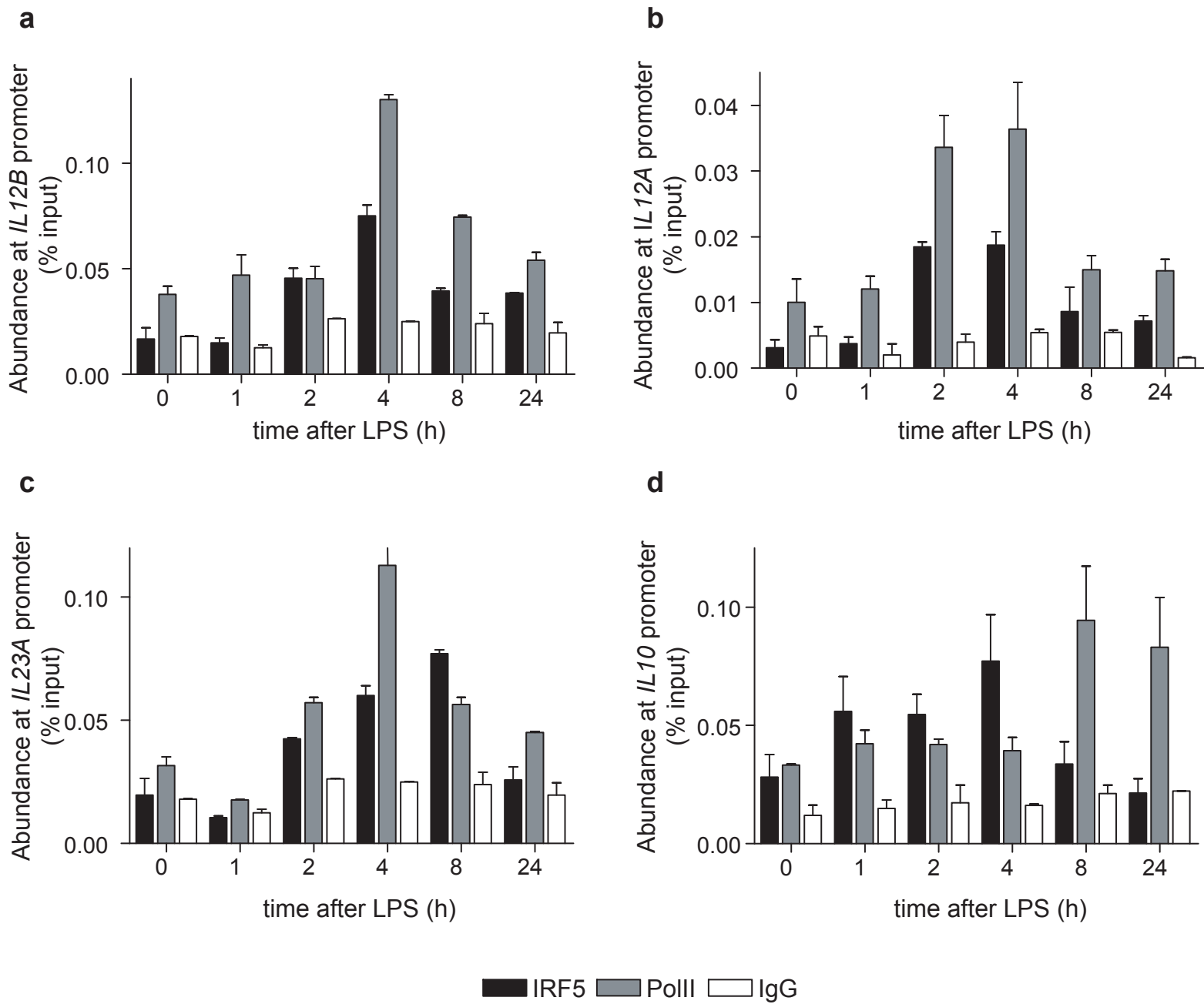


Figure 5

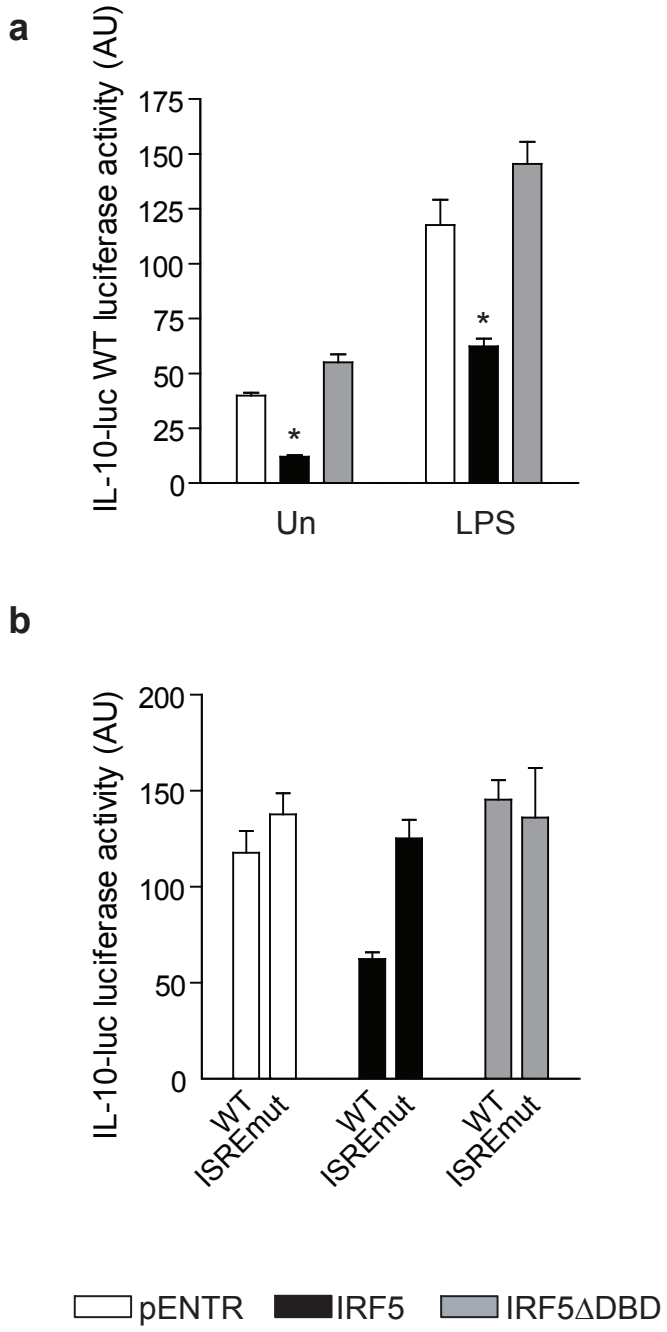


Figure 6

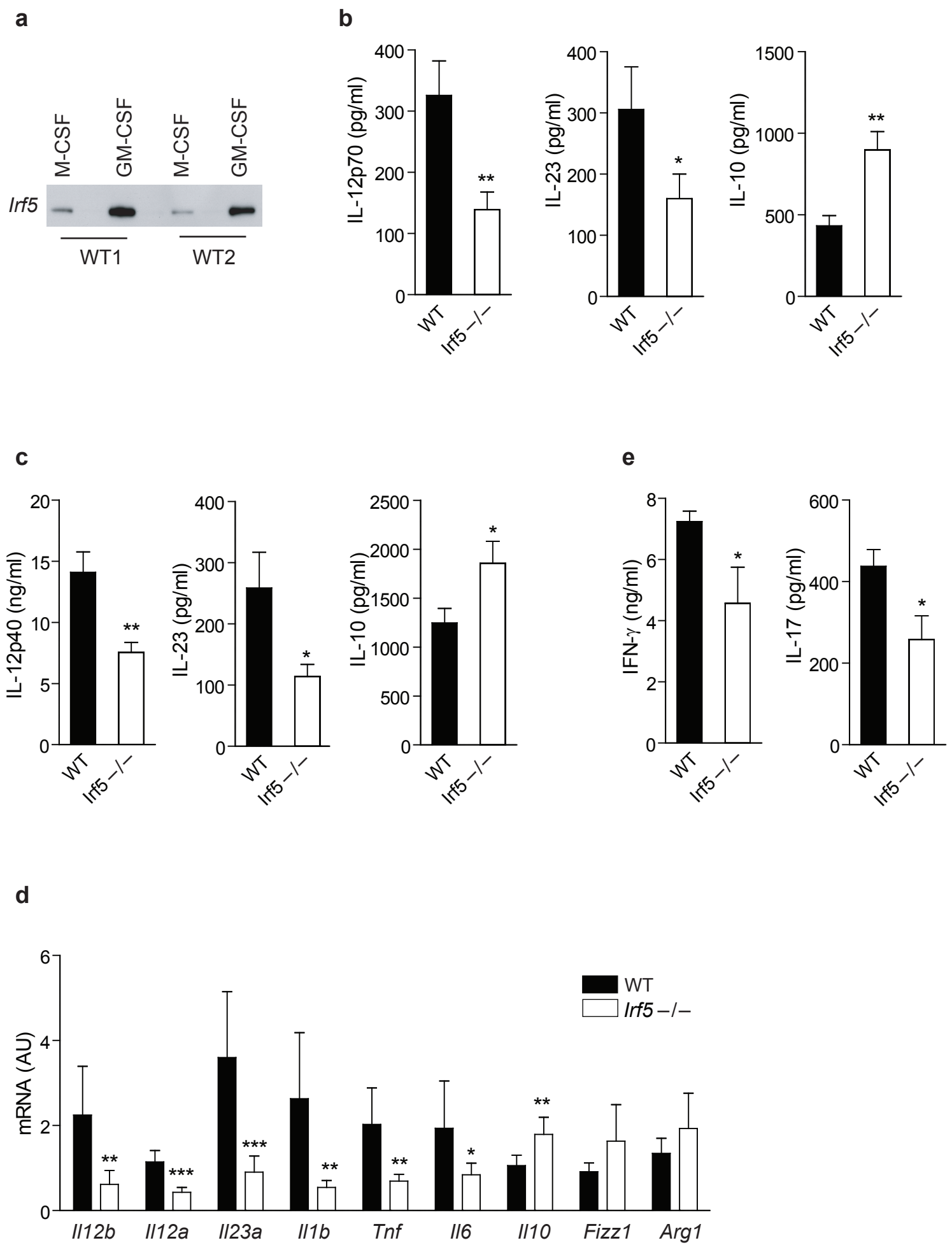


Figure 7