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1 **Complement regulator CD46 temporally regulates cytokine production**
2 **by conventional and unconventional T cells**
3
4

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42 **ABSTRACT**

43 **This study reveals a novel form of immunoregulation: engagement on CD4 T cells**
44 **of the complement regulator CD46 promotes T_H1 effector potential, but as**
45 **interleukin-2 (IL-2) accumulates, “switches” cells toward a regulatory phenotype,**
46 **attenuating IL-2 production *via* the transcriptional regulator ICER/CREM, and**
47 **upregulating IL-10 following interaction of the CD46-tail with SPAKinase.**
48 **Activated CD4 T cells produce CD46 ligands, and blocking CD46 inhibits IL-10**
49 **production. Furthermore, CD4⁺ T cells in rheumatoid arthritis fail to switch,**
50 **consequently producing excessive interferon- γ . Finally, $\gamma\delta$ T cells, which rarely**
51 **produce IL-10, express an alternative CD46-isoform and cannot switch.**
52 **Nonetheless, T cell receptor $\gamma\delta$ -CD46 co-engagement suppresses effector**
53 **cytokine production, establishing that CD46 employs multiple mechanisms to**
54 **regulate different T cell subsets during an immune response.**

55

56 The suppression of immune responses against self-antigen is vital to the limitation of
57 autoimmunity. Similarly, the timely contraction of T cell responses to infections is critical
58 for protection against immunopathologies arising from exuberant inflammation. The
59 cytokine interleukin-10 (IL-10) is critical in immunosuppression¹, inhibiting production of
60 the proinflammatory cytokines tumor necrosis factor (TNF) and IL-12 in macrophages
61 and dendritic cells (DCs)^{1,2}, and suppressing IL-2 and interferon- γ (IFN- γ) production by
62 effector T cells³. Thus, *Il10*^{-/-} mice succumb to colitis because of their inability to
63 regulate immune responses against gut flora⁴, and susceptibility to colitis was likewise
64 reported for human families carrying mutations in genes encoding IL-10 receptor
65 chains⁵. Moreover, while murine IL-10 deficiency accelerates the clearance of
66 *Toxoplasma gondii* or *Trypanosoma cruzi* infections, such mice succumb to tissue
67 damage caused by over-production of proinflammatory cytokines^{2,6}.

68

69 IL-10 can be produced by many cell types including DCs, macrophages, B cells, and T
70 cells, among which T_H2 cells and adaptive regulatory T (T_{REG}) cells, such as Tr1 cells,
71 have been implicated as prime sources^{1,7,8}. However, uncertainty as to the nature of
72 critical IL-10-producing cells *in vivo*, particularly in humans, has been fuelled by the

73 realization that under certain conditions, high amounts of IL-10 can be secreted by
74 some natural T_{REG} cells⁹, T_H17 cells¹⁰, and T_H1 cells¹¹⁻¹⁴. Indeed, IL10-producing T_H1
75 cells have sparked much interest because they appear key to regulating immune
76 responses to certain infections¹⁴⁻¹⁷, and because their induction might be a mechanism
77 by which tolerance is induced in the presence of persistent (self)-antigen¹⁸. Thus, there
78 is particular interest in understanding what regulates IL-10 production by T_H1 cells.

79
80 CD46 is a ubiquitously-expressed human type I transmembrane glycoprotein, originally
81 identified as a complement regulatory protein. We previously showed that co-
82 engagement of the T cell receptor (TCR) and CD46 on human CD4⁺ T cells induces
83 high IL-10 secretion, moderate IFN- γ production and granzyme B and perforin
84 expression^{19,20}. Indeed, this phenotype, plus the IL-10-dependent, FoxP3-independent
85 capacity to suppress bystander effector T cells establishes a similarity of CD3-CD46-
86 activated T cells to Tr1 cells²¹. Moreover, both CD46-dependent IL-10 induction and Tr1
87 generation are highly dependent on exogenous IL-2^{7,19,21}, suggesting that CD46 might
88 naturally be a key factor in the switch of T_H1 cells to a Tr1-like phenotype, however, this
89 has not been directly investigated.

90
91 CD46 binds the opsonins C3b and C4b and functions as a cofactor in their proteolytic
92 degradation by serine protease factor I²². CD46 also functions as a receptor for several
93 important human pathogens, such as *Streptococcus pyogenes*^{23,24}. Four isoforms of
94 CD46 arise by alternative splicing²⁵. All isoforms contain four conserved complement
95 control repeats (CCPs), followed by variant forms of a highly O-glycosylated region
96 (designated 'B' and 'C'), a transmembrane anchor, and one of two possible cytoplasmic
97 domains, designated CYT-1 and CYT-2 (**Supplementary Fig 1**). Thus, the four
98 commonly expressed CD46 isoforms are BC1 (denoting glycosylated regions B and C
99 connected to CYT-1), BC2, C1 and C2. Both CYT-1 and CYT-2 contain kinase
100 substrates, and are tyrosine-phosphorylated upon CD46 cross-linking of human CD4⁺ T
101 cells²⁶, that also activates the TCR adaptor proteins p120-CBL and LAT²³, Vav, Rac and
102 Erk²⁴, and additionally regulates FMS-like tyrosine kinase 3 (FLT3) and FLT3 ligand

103 expression²⁷. Thus CD46 activation has a high intrinsic potential to regulate T cell
104 function, and has been reported as a T cell co-stimulator.

105
106 Interestingly, interaction of T cells with antigen presenting cells (APCs) induces the
107 secretion of complement proteins (such as C3, factor B and factor D) and the
108 subsequent generation of complement activation fragments²⁹⁻³¹ by both cell populations.
109 Such a scenario might provide local ligands for CD46, thereby affecting cell fate.
110 Indeed, CD46 cross-linking by C3b and C4b during TCR activation can induce Tr1-like
111 cells¹⁹. Likewise, the presence of *S. pyogenes* during TCR activation *in vitro* promotes
112 IL-10-secreting Tr1-like cells²⁸. Nonetheless, cultures of Tr1-like cells induced by CD46
113 engagement paradoxically display high IFN- γ and other features of T_H1 cells³², raising
114 again the question of whether CD46 engagement is critically involved in T_H1-Tr1
115 “switching”¹⁹.

116
117 Whereas conventional CD4⁺ T cell activation defines the adaptive response, it is now
118 appreciated that the early phases of immune responses are substantively contributed to
119 by unconventional T cells of which $\gamma\delta$ cells are a prototype. Although such cells
120 proliferate, and display rapid and effusive effector function, little is known about how
121 their potential to promote immunopathology is controlled. Indeed, there is no clear case
122 for the existence of TCR $\gamma\delta$ ⁺ Foxp3⁺ T_{REG} cells under normal circumstances³³, and scant
123 evidence for induction of IL-10⁺ Tr1-like $\gamma\delta$ cells. Given its potential to induce IL-10 in
124 adaptive T cells, it is important to examine whether $\gamma\delta$ cells are responsive or not to
125 CD46-TCR co-stimulation.

126
127 Finally, it was also important to consider the significance *in vivo* of CD46-mediated
128 regulation of T cells by examining patients with inflammatory disease. By undertaking
129 these various analyses, the current study establishes that the IL-2-dependent TCR-
130 CD46 co-activation of human T cells is a powerful means to promote T_H1 cells and then
131 to switch them to IL-10 production. This pathway is absent in unconventional T cells and
132 is impaired in rheumatoid arthritis. At the same time, CD46 displays additional modes

133 of immunosuppression, permitting it to regulate an immune response across its
134 temporal progression.

135

136

137 **RESULTS**

138 **IL-10 secretion is regulated by CD46 and IL-2**

139 Although activation of purified human CD4⁺ T cells with stimulating monoclonal
140 antibodies (mAbs) to CD3 and CD46 in the presence of IL-2 induces high IL-10
141 secretion and confers a suppressive phenotype¹⁹, CD3-CD46-activated T cells can also
142 produce high amounts of IFN- γ (**Fig. 1a**,¹⁹). To better understand these seemingly
143 paradoxical effects of CD46, we varied the strength of the activating signals. While
144 changing the concentrations of the CD3 and CD46-specific mAbs did not significantly
145 affect IFN- γ or IL-10 secretion measured 72h post activation (data not shown), IL-10
146 production was strongly regulated by exogenous IL-2 (**Fig. 1b**). Specifically, in ≤ 0.5
147 U/ml IL-2, CD3-CD46 activation induced high IFN- γ secretion, compared to T cells
148 activated with either anti-CD3 or anti-CD3 plus anti-CD28. Anti-CD3 and anti-CD46 also
149 induced transient expression of IL-2, detectable at 24h following activation in the
150 absence of exogenous IL-2 (**Fig. 1c**). Increasing IL-2 beyond 5 U/ml did not further
151 increase IFN- γ production, but in IL-2 concentrations of 5-10 U/ml, CD3-CD46-activated
152 T cells from different donors all displayed strong IL-10 secretion in addition to IFN- γ ,
153 with the level of IL-10 produced increasing with the amount of IL-2 (**Fig. 1b**). This was
154 highly specific to IL-10, since no conditions promoted the production of IL-4, IL-5, or IL-
155 17 (data not shown). CD46-induced IFN- γ peaked 24h post activation and then steadily
156 declined, whereas IL-10 was barely detectable before 24h and peaked at 72h (**Fig. 1c**).
157 Thus, CD46-mediated IFN- γ production preceded IL-10 secretion even in high IL-2
158 cultures that are most conducive to IL-10 production.

159

160 **IFN- γ ⁺ and IL-10⁺ cells are successively induced**

161 We next investigated the single cell dynamics of CD3-CD46-induced IFN- γ and IL-10
162 secretion. Purified CD4⁺ T cells were activated with mAbs to CD3 alone or in
163 combinations with mAbs to CD28 plus CD46 in the presence of a neutralizing mAb to

164 IL-2, or either low (5 U/ml) or high (50 U/ml) doses of IL-2. Active cytokine secretion was
165 measured 36h post activation (**Fig. 1d**). Blockage of IL-2 inhibited cytokine production
166 under every condition analyzed. However, in the presence of IL-2, anti-CD3-CD46
167 activation or anti-CD3-CD28-CD46 activation induced three discrete T cell populations
168 with distinct secretion profiles: IFN- γ^+ IL-10 $^-$, IFN- γ^+ IL-10 $^+$ and IFN- γ^- IL-10 $^+$ cells (**Fig.**
169 **1d**). Cultures with 5 U/ml IL-2 contained higher frequencies of IFN- γ -secreting cells
170 (17.4% \pm 5.2% IFN- γ^+ IL-10 $^-$ and IFN- γ^+ IL-10 $^+$ [in CD3-CD46-activated cultures])
171 compared to IL-10-producing T cells (12.0% \pm 1.8%, IFN- γ^+ IL-10 $^+$ and IFN- γ^- IL-10 $^+$),
172 whereas 50 U/ml IL-2 induced a significant increase in IL-10 $^+$ cells (24.7% \pm 5.8%
173 IFN- γ^+ IL-10 $^+$ and IFN- γ^- IL-10 $^+$). By contrast, although CD3-CD28-stimulated cultures also
174 contained all three IFN- γ -IL-10-secreting T cell populations, there was no IL-2-
175 dependent change in the ratio of IFN- γ -producing vs. IL-10-positive T cells, and the total
176 number of IL-10-producing cells remained low even in high IL-2 (**Fig. 1d**). Although the
177 data in **Fig. 1d** reflect assays at 36h, cellular cytokine staining at 12h, 72h, and 90h post
178 stimulation also mirrored the results from our previous kinetic study (**Fig. 1c**): at 24h,
179 CD46 costimulation promoted mostly IFN- γ^+ IL-10 $^-$ cells, even in high IL-2, with the peak
180 of IL-10-producing cells arising subsequent to this. By 90h, the overall number of
181 cytokine-secreting cells declined substantially (data not shown).

182
183 The described experiments were performed with purified CD4 $^+$ T cell populations that at
184 minimum include naïve and memory CD4 $^+$ T cells, and natural T_{REG} cells. To exclude
185 the possibility that the observed CD46-elicited populations reflect selective reactivation
186 and/or expansion of one or more of these subsets, anti-CD3-CD46 activation protocols
187 were applied to highly purified naïve CD4 $^+$ T cells (CD4 $^+$ CD45RA $^+$ CD45RO $^-$ CD127 $^-$
188 CCR7 $^-$ CD25 $^-$). As before, IFN- γ^+ IL-10 $^-$, IFN- γ^+ IL-10 $^+$ and IFN- γ^- IL-10 $^+$ cells were
189 detected in the presence of high IL-2 for 36h (**Supplementary Fig. 2**). However,
190 activation of naïve T cell cultures induced a lower frequency of IL-10 $^+$ cells compared to
191 IFN- γ^+ cells (1.6% \pm 0.7% vs 5.6% \pm 1.9%; **Supplementary Fig. 2**). Conversely, use of
192 anti-CD3-CD46 to re-stimulate naïve cells previously activated and expanded with
193 mAbs to CD3 and CD46 substantially increased the proportion of both IFN- γ^+ IL-10 $^+$ and

194 IFN- γ ⁻IL-10⁺ T cells (**Supplementary Fig. 2**; right panels). Moreover, the frequencies of
195 IFN- γ ⁺IL-10⁺ and IFN- γ ⁻IL-10⁺ cells were significantly increased by each additional
196 CD3/CD46 re-stimulation. Again, no IL-4 or IL-5 was detected in any of these cultures
197 (data not shown). Secondary CD3-CD46 stimulation also increased IL-10 production,
198 albeit to a lesser extent, from T cells initially activated with anti-CD3 alone
199 (**Supplementary Fig. 2**; left panels). In short, the IL-2-dependent emergence over time
200 of IL-10-producing cells does not obviously reflect the selective response of a pre-
201 existing subset.

202

203 The capacity of the different cell populations induced by anti-CD3-CD46 to regulate T
204 cell activation was then assessed. CD4⁺ T cells were activated through CD3-CD46
205 ligation and subsequently sorted into IFN- γ ⁺IL-10⁻, IFN- γ ⁺IL-10⁺ and IFN- γ ⁻IL-10⁺ cell
206 subsets, which were then cultured for 18h in 1 U/ml IL-2. The supernatants of the three
207 cell types continued to contain high IFN- γ ; approximately equal amounts of IFN- γ and
208 IL-10; and high amounts of IL-10 with negligible IFN- γ , respectively (data not shown).
209 When freshly purified CD4⁺ T cells were cultured in the presence of these supernatants
210 together with immobilized cross-linking antibodies to CD3 and CD28, their proliferation
211 (measured at day 6) was substantially inhibited by the supernatants of the CD3-CD46-
212 induced IFN- γ ⁺IL-10⁺ cells, and of the IFN- γ ⁻IL-10⁺ T cells (**Fig. 1e**). The inhibition was
213 not evoked by supernatants from IFN- γ ⁺IL-10⁻ T cells, nor by supernatants from T cells
214 activated for 72h via CD3-CD28 (**Fig. 1d**). IL-10 upregulated by anti-CD3-CD46 is the
215 primary soluble mediator of this effect because regulation was almost completely
216 blocked by a neutralizing mAb to IL-10.

217

218 **CD46-induced IL10 originates in a T_H1 subset**

219 To assess whether CD46-induced IFN- γ ⁺IL-10⁺ and IFN- γ ⁻IL-10⁺ T cells originate from
220 the IFN- γ ⁺IL-10⁻ T_H1 cells that first arise, or whether each cell population arises with
221 different kinetics from separate cell populations (diagrammed schematically in **Fig. 2a**),
222 two sets of experiments were performed. First, purified CD4⁺ T cells were activated via
223 CD3 plus CD46 in high IL-2 in the presence of neutralizing mAbs to IFN- γ ; a condition

224 known to inhibit T_H1 development⁸. As expected, a significant decrease was observed
225 after 48h in the number of IFN- γ ⁺IL-10⁻ T cells (from 14% \pm 4% to 4% \pm 2.5%, **Fig. 2b**).
226 However, this treatment also substantially reduced the percentages of CD46-induced
227 IFN- γ ⁺IL-10⁺ cells from 16% \pm 2% to 2.5% \pm 1.5%, and of the IFN- γ ⁻IL-10⁺ cells from
228 18% \pm 3% to 1.5% \pm 0.5%. Thus, limiting T_H1 differentiation limits the “IL-10-switch”. IL-
229 10 neutralization did not decrease the cell numbers of any of the T cell populations
230 induced but rather slightly increased their representation (IFN- γ ⁺IL-10⁻ from 14% \pm 4%
231 to 17.5 \pm 4%; IFN- γ ⁺IL-10⁺ from 16% \pm 2% to 19.5% \pm 6.5%; IFN- γ ⁻IL-10⁺ from 18% \pm
232 3% to 18.5% \pm 2.5%, **Fig. 2b**). These data suggest at minimum that the continuous
233 production of IL-10 initially induced via CD3, CD46 and IL-2-mediated signals does not
234 depend on positive feedback by IL-10 itself.

235
236 Second, purified CD4⁺ T cells were activated via CD3 or CD3-CD46 in low levels of IL-
237 2, and the resulting IFN- γ ⁺IL-10⁻ cells isolated. When, after 4 days of expansion, the
238 cells were re-stimulated via CD3-CD46, they produced a high percentage of IFN- γ ⁺IL-
239 10⁺ and IFN- γ ⁻IL-10⁺ cells (**Fig. 2c**). Taken together, these two data sets strongly
240 suggest that CD46-induced IL-10-secreting T cells obligatorily derive from an initial ‘T_H1
241 phase’. In agreement with this, CD3-CD46-mediated re-activation of T_H1 cells primed
242 through CD3-CD28 activation of naïve T cells in the presence of IL-12 and neutralising
243 mAb to IL-4 (strong T_H1-skewing), also promotes IFN- γ ⁺IL-10⁺ and IFN- γ ⁻IL-10⁺ cells
244 (data not shown).

245
246 **CD3-CD46-induced IL-10 cells retain T_H1 markers**

247 T cell lineages are characterized by specific transcription factor expression and
248 activation: T_H1 differentiation is accompanied by STAT1 and STAT4 phosphorylation
249 and T-bet expression^{34,35}; T_H2 cells require STAT6 activation and expression of
250 GATA3^{34,35}; and natural T_{REG} cells are characterized by FoxP3 expression⁹. When
251 purified CD4⁺ T cells were activated via CD3-CD46 for 36h in high IL-2, all resultant
252 cells expressed a predominantly T_H1 profile (**Table I**). In mice, IL-10-secretion by T_H1
253 cells requires extracellular-signal regulated kinase (ERK) activation^{18,36}. Likewise,
254 CD46-dependent, TCR-independent induction of ERK1 and 2 in human CD4⁺ T cells

255 was previously reported²⁴. All three CD46-induced T cell populations contained high
256 amounts of pERK1 and 2 (**Table I**). Signaling through the IL-2 receptor complex induces
257 Janus kinase (JAK)-mediated STAT5 phosphorylation. Consistent with the fact that
258 CD46-induced IL-10 production is IL-2-dependent, pSTAT5 was detected in IFN- γ ⁺IL-
259 10⁻ and IFN- γ ⁺IL-10⁺ cells, although it was lower in IFN- γ ⁻IL-10⁺ T cells, perhaps
260 consistent with their limited proliferative capacity (¹⁹; data not shown).

261

262 **CD3-CD46 signals regulate IL-2 secretion**

263 Drawing further parallels with T_H1 cells, IL-2 expression was examined since it is a T_H1
264 hallmark reportedly lost by T_H1 cells that have the ability to co-secrete IL-10^{18, 36}. IL-2
265 expression had previously been detected in CD3-CD46-activated CD4⁺ T cell cultures
266 (**Fig. 1c**), and when examined in more detail, >85% of IFN- γ ⁺ cells actively secreted IL-2
267 at 36h post CD3-CD46 activation (**Fig. 3a**), whereas this was true for <25% of IL-10-
268 secreting cells (**Fig. 3a**;¹⁹). Of these, 50-60% co-expressed IFN- γ (data not shown).
269 Thus, CD3-CD46-activated cells shared with T_H1 cells the production of IL-2 that
270 ceased with the induction of the IL-10-secreting state.

271

272 Gene array comparisons of CD3-CD46-activated versus CD3-CD28-activated primary
273 CD4⁺ T cells revealed significantly higher levels of mRNA encoding the ICER/CREM
274 protein in CD46-activated cells (data not shown). Translocation to the nucleus of
275 ICER/CREM attenuates IL-2 gene transcription³⁷. Flow cytometry confirmed that CD3-
276 CD46 ligation induced ICER/CREM protein in all three subsets (**Fig. 3b** and **Table I**).
277 Unexpectedly, expression was highest in IFN- γ ⁺IL-10⁻ cells, which continue to express
278 IL-2 (**Table I**). However, immunoblotting of cytoplasmic and nuclear fractions revealed
279 consistent ICER/CREM nuclear translocation only after 48h of CD3-CD46 (**Fig. 3c**) or
280 CD3-CD28-CD46 activation (data not shown), coincident with the appearance of IL-10
281 in the culture medium (**Fig. 1c**). CD3 activation alone or CD3-CD28 activation did not
282 lead to measurable ICER/CREM nuclear translocation at any time point (data not
283 shown). To determine if ICER/CREM bound preferentially to the IL-2 promoter in IL-10⁺
284 cells, chromatin immunoprecipitation (ChIP) using an ICER/CREM-specific mAb was
285 applied to IFN- γ ⁺IL-10⁻ and IFN- γ ⁻IL-10⁺ cells purified 36h post CD3-CD46 activation. IL-

286 2 promoter-specific DNA sequences were precipitated only from IL-10⁺ cells, and not
287 from IFN- γ ⁺ T cells (**Fig. 3d**). Thus, CD3-CD46 activation induces strong ICER/CREM
288 expression in T_H1 cells but this is inactive in IFN- γ ⁺ cells that continue to produce IL-2.
289 Instead, active nuclear translocation occurs preferentially in IL-10⁺ cells, consistent with
290 their decreased IL-2 production. Note that IL-2 production is not attenuated secondarily
291 via IL-10 production because neither IL-10 neutralization during CD3-CD46 activation,
292 nor the addition of recombinant human IL-10 during CD3-IL-2 stimulation affected
293 ICER/CREM translocation or IL-2 production (data not shown).

294

295 **The CYT-1 domain of CD46 promotes IL-10 via SPAK**

296 As outlined above, CD46 is commonly expressed in four isoforms with two possible
297 cytoplasmic domains, CYT-1 and CYT-2 (**Supplementary Fig. 1**)²⁵. To determine
298 whether CYT-1 or CYT-2 differentially affect IL-10-switching, Jurkat T cells stably
299 expressing either the BC-CYT-1 (BC1) or BC-CYT-2 (BC2) isoforms were generated.
300 Untransfected Jurkat cells express only the C2 isoform and do not produce IFN- γ or IL-
301 10 upon CD3-CD46 activation in the presence of IL-2 (**Fig. 4a** and data not shown). By
302 contrast, this property was acquired by BC1-transfected cells but, importantly, not BC2-
303 transfected cells (**Fig. 4a**).

304

305 A yeast two-hybrid screen identified the Ste20 (SPS1)-related proline alanine-rich
306 kinase (SPAK) as an interaction partner with CYT-1 and CYT-2 (data not shown). SPAK
307 is a broadly expressed serine-threonine kinase implicated in mitogen activated protein
308 kinase (MAPK) regulation and T cell activation^{38,39}. Immunoprecipitation confirmed that
309 SPAK binds to CD46 constitutively in unstimulated primary CD4⁺ T cells (**Fig. 4b**).
310 Whereas CD3 activation completely disrupts the CD46-SPAK interaction within 15
311 mins., this is partially preserved when CD46 is also engaged (**Fig. 4b**, lanes 8-11).
312 SPAK also co-immunoprecipitated with CD46 (but neither CD3 nor CD28) in
313 unstimulated Jurkat T cells (data not shown). When SPAK expression was knocked
314 down in purified CD4⁺ T cells by siRNA, there was decreased capacity to produce IL-10
315 upon CD3-CD46 activation (**Fig. 4c**). Consistent with this, Jurkat cells stably transfected
316 with SPAK responded to CD3-CD46 activation by IL-10 production, whereas cells

317 transfected with a kinase-dead SPAK mutant (Δ SPAK) did not produce IL-10 under any
318 of the activation conditions tested (**Fig. 4d**). In short, the induction of IL-10 by CD46-
319 CD3 activation of human T cells is at least in part attributable to a signaling pathway
320 from the CYT-1-BC1 tail of CD46 *via* SPAK. This notion is supported by the finding that
321 protein knockdown of SPAK (**Supplementary Fig. 3a**) significantly reduced the
322 previously observed CD46-mediated hyperphosphorylation of ERK^{23,24,32}, a key factor in
323 IL-10 upregulation in T cells³⁶ (**Supplementary Fig. 3b**). The observation that SPAK
324 silencing abrogated both CD3- and CD46-induced JNK phosphorylation suggests that
325 SPAK might also contribute to JNK activation in a CD46-independent pathway. By
326 contrast, and as a specificity control, p38 phosphorylation in CD3 and CD3-CD46-
327 activated T cells remained largely unaffected by SPAK protein knockdown
328 (**Supplementary Fig. 3b**).

329

330 **CD46 also regulates the effector phase of $\gamma\delta$ T cell**

331 $V\gamma9V\delta2$ T cells, the major $\gamma\delta$ T cell subset in human peripheral blood, are primarily
332 biased towards a T_H1 -like phenotype, but the generation from these of Tr1-like cells has
333 not been convincingly demonstrated. If CD46-regulation is a major means of Tr1-like
334 cell induction, we reasoned that the scarcity of IL-10-producing $V\gamma9V\delta2$ T cells might be
335 associated with differences in the CD46 pathway. Indeed, although $V\gamma9V\delta2$ T cells
336 express CD46, albeit at lower levels than $CD4^+$ T cells (**Supplementary Fig. 4a**), these
337 different subsets strikingly differ by their expression pattern of CD46 variants. The BC2
338 isoform, which did not confer IL-10 production in CD3-CD46-stimulated Jurkat-
339 transfectants (**Fig. 4a**), is unequivocally the major isoform in $V\gamma9V\delta2$ T cells (**Fig. 5a**).
340 As expected, stimulation of these donors' peripheral blood mononuclear cells (PBMC)
341 with HMBPP (a $V\gamma9V\delta2$ T cell-specific agonist) provoked negligible IL-10 production,
342 and this was not increased by CD46 co-stimulation, even in high IL-2 (**Fig. 5b**).
343 However we noted some decrease in IFN- γ production (data not shown, but see below)
344 that contrasted with the promotion of IFN- γ by anti-CD3-CD46 in $CD4^+$ T cells (**Fig. 1a**
345 and **b**).

346

347 To further investigate this more direct immunosuppressive role of CD46, PBMC from
348 three additional donors were stimulated with HMBPP, with or without IL-2 and CD46
349 cross-linking. Again, CD46 did not induce IL-10 (data not shown), but consistently
350 decreased IFN- γ production and TNF secretion (**Fig. 5c**). This effect was not due to
351 CD46-induced cell death, because the proportions of apoptotic (AnnexinV⁺) and
352 necrotic (AnnexinV⁺ PI⁺) V γ 9V δ 2 T cells and the overall proportion of V γ 9V δ 2 T cells
353 were each comparable in PBMC stimulated with HMBPP and IL-2 in the absence or
354 presence of the anti-CD46 antibody (**Supplementary Fig. 4b**). A direct
355 immunosuppressive effect of CD46 on V γ 9V δ 2 cells was also evident in CD25 down-
356 regulation (**Fig. 5d**, upper panel), especially in the absence of IL-2, which was in stark
357 contrast to the response of CD4⁺ T cells (**Fig. 5d**, lower panel). To preclude potential
358 interfering effects of other subpopulations, flow-sorted V γ 9V δ 2 T cells (>95% pure) from
359 two donors were stimulated with HMBPP while CD4⁺ T cells from the same donors were
360 separately activated *via* CD3-CD28. While CD46 co-stimulation during these activation
361 conditions clearly allowed the detection of IFN- γ ⁺IL-10⁺ and IFN- γ ⁻IL-10⁺ CD4⁺ T cells,
362 such subpopulations were not detected among V γ 9V δ 2 T cells (**Fig. 5e**), and IFN- γ
363 production was again generally decreased in high IL-2 (**Fig. 5f**). Finally, the possibility
364 that IL-10 could be induced by CD46 re-stimulation of V γ 9V δ 2 T cells (as observed for
365 naïve CD4⁺ T cells, **Supplementary Fig. 2**) was investigated. A polyclonal T cell line
366 (>75% V γ 9V δ 2⁺), obtained after incubation of PBMC with HMBPP and IL-2, was thus
367 stimulated with HMBPP and/or IL-2 and CD46. Again, CD46 decreased both TNF and
368 IFN- γ secretion (**Supplementary Fig. 4c**), but could not induce significant IL-10
369 production (data not shown). While these results validate the finding that the BC1
370 isoform is necessary to switch cells to IL-10 producers, they also show that activation of
371 the BC2 isoform of CD46 directly inhibits T_H1-like responses by a major subset of
372 unconventional T cells that are strongly implicated in the early phases of immune
373 responses. Thus, CD46 appears to employ two distinct mechanisms to regulate the
374 production of pro-inflammatory cytokines.

375

376 **Defective CD46 signals might contribute to autoimmunity**

377 Further evidence that CD46 actively regulates IL-10 in human pathophysiology was
378 obtained from comparing the responses to CD3-CD46 activation in the presence of 25
379 U/ml IL-2 of CD4⁺ T cells from three healthy donors and three adult patients with
380 rheumatoid arthritis (RA). The baseline expression of CD3, CD46 and CD25 was similar
381 among all T cell samples (data not shown), yet at 36h post activation the cultures from
382 RA patients contained higher percentages of IFN- γ ⁺IL-10⁻ and IFN- γ ⁺IL-10⁺ but very few
383 IFN- γ ⁻IL-10⁺ T cells (**Fig. 6a**). In addition, while amounts of IFN- γ and IL-10 in the
384 supernatants of T cells from healthy donors were roughly similar, T cells from RA
385 patients produced ≥ 10 fold more IFN- γ than IL-10 (**Fig. 6b**) and supernatants derived
386 from these cells had no suppressive activity (data not shown). Because chronic
387 inflammatory conditions such as RA are often attributed to inappropriate responses to
388 persistent antigen, cytokine expression was examined after T cell re-stimulation with
389 CD3-CD46. Cultures of T cells from healthy donors showed increased switching to the
390 IFN- γ ⁻IL-10⁺ state, now containing approximately equal distributions of IFN- γ -producing
391 (IFN- γ ⁺IL-10⁻ and IFN- γ ⁺IL-10⁺) and IL-10-secreting (IFN- γ ⁺IL-10⁺ and IFN- γ ⁻IL-10⁺) T
392 cells (**Fig. 6c**), with a 1:1 ratio of IFN- γ and IL-10 secreted into the culture media (data
393 not shown). In contrast, re-stimulated cultures from RA patients lacked a significant IFN-
394 γ ⁻IL-10⁺ population, with a diminution even in IFN- γ ⁺IL-10⁺ cells relative to the cells'
395 initial stimulation (**Fig. 6c**). Thus, the ratio of IFN- γ :IL-10 in the culture media was now
396 $\sim 20:1$ (data not shown). Importantly, T cells from normal donors and RA patients
397 responded similarly to CD3-CD28 activation (**Supplementary Fig. 5a-c**) and T_H1 and
398 T_H2-driving cytokines (**Supplementary Fig. 5d**), showing that the observed
399 deregulation of IFN- γ vs. IL-10 expression in RA relates to a defect in CD46
400 responsiveness.

401

402 To further investigate IL-10-switching in arthritis, CD4⁺ T cells were isolated from the
403 synovial fluid of both inflamed knee joints of a juvenile arthritis patient. Of note, primary
404 T cells from synovial fluid mostly exhibit an activated CD25^{hi}, CD45RO⁺ phenotype,
405 although CD3 and CD46 expression levels were equivalent (data not shown). Similar to
406 the results obtained with blood-derived CD4⁺ T cells from RA patients, these synovial T
407 cells did not switch to the IFN- γ ⁻IL-10⁺ state after an initial CD3-CD46 stimulation (**Fig.**

408 **6d**); produced significantly more IFN- γ than IL-10 during expansion (**Fig. 6e**); were
409 mostly 'locked' into the IFN- γ^+ IL-10 $^-$ state upon restimulation (**Fig. 6f**); and secreted ~30
410 times more IFN- γ than IL-10 into the culture medium after restimulation (data not
411 shown). These data strongly suggest that the CD3-CD46-IL-2-mediated regulation of
412 the proinflammatory IFN- γ^+ (T_H1) phenotype to an immunoregulatory IL-10-only-
413 secreting phenotype is dysfunctional in RA and juvenile arthritis patients. Moreover, the
414 defect seems primarily to lie in the 'shut down' of IFN- γ production, since IL-10 secretion
415 itself by T cells from RA patients is induced almost normally upon CD46 engagement.

416

417 **Local complement production drives IL-10 secretion**

418 Evidence underpinning CD46 as a key regulatory axis was provided by the finding that
419 highly purified (APC-free) CD3-activated CD4 $^+$ T cell cultures contained the CD46 ligand
420 C3b (data not shown), and show high C3b deposition onto their cell surfaces (**Fig. 7a**).
421 C3b generation was increased by co-stimulation with either CD28 or CD46. The
422 prospect that this functionally engages CD46 was supported by the clear finding that
423 low level IL-10 production induced by CD3 or CD3-CD28-activation of CD4 $^+$ T cells was
424 reduced essentially to background levels by addition of soluble CD46 (**Fig. 7b**).

425

426

427 **DISCUSSION**

428 The irrefutable evidence for the importance of IL-10 in suppressing immunopathology
429 now extends to humans, where it has both biological and clinical implications⁴⁰. As a
430 result, there has been much interest in IL-10-producing cells. This area of study has
431 progressed from the concept of an IL-10 lineage, to one that embraces cells that switch
432 to IL-10 production from an earlier incarnation as IFN- γ -producing T_H1 cells. Cultures
433 that co-express IL-10 and IFN- γ have been clearly documented¹¹⁻¹⁴, and such a switch
434 would have the seeming advantage of inducing a regulatory cytokine and
435 simultaneously suppressing an effector cytokine based on the recognition of the same
436 antigen. Nonetheless, how such a switch might naturally occur, particularly in humans,
437 has not hitherto been examined in detail⁴⁰. This study makes the case that human T_H1
438 cells are promoted by the co-engagement of the TCR and CD46, but that as

439 environmental IL-2 increases, as would be the case in a flourishing effector response, a
440 switch to regulatory IL-10 production occurs, concomitant with diminished endogenous
441 IL-2 production.

442
443 Attesting to the importance of this mechanism, we find it to be defective in RA patients.
444 Moreover, it is striking that this switching also does not function in the main subset of
445 human $\gamma\delta$ cells, since such cells are known to express IL-10 only very rarely³³. CD46-
446 induced IL-10 production requires expression of CYT-1 of CD46; CYT-1 bearing
447 isoforms are expressed in CD4⁺ T cells but are undetectable in $\gamma\delta$ T cells. Together,
448 these findings would be consistent with the CD46-TCR activation regime being a
449 primary means of peripheral IL-10 induction tightly regulated by the respective CD46
450 isoform expression patterns. Interestingly, the analysis of $\gamma\delta$ cells does show an effect of
451 CD46-TCR co-engagement: instead of switching to IL-10 production, the cells display a
452 substantial suppression of effector function, and decrease in IL-2R expression. These
453 data permit us to propose that CD46 employs distinct mechanisms to regulate the
454 immune response from its beginning to its close: in the early stages it may promote IFN-
455 γ in conventional T cells, while controlling the rapidly activated effector functions of
456 unconventional T cells; in the later stages, it switches conventional T cells to regulatory
457 T cells.

458
459 It is now widely acknowledged that the complement system functions well beyond a
460 simple danger recognition and microbial clearance system and participates actively in
461 adaptive immune responses⁴¹. Two relatively new paradigms regarding complement
462 functions have renewed interest in this evolutionary old innate system: first, complement
463 not only plays a role in the induction of T cell responses but also in their contraction –
464 thus, consequently in immune homeostasis^{20,42,43}; second, the local production of
465 complement components by immunocompetent cells participates decisively in shaping
466 adaptive immune responses^{31,41,44}. The latter observation – mostly derived in mouse
467 models - is in good agreement with our novel observation that C3b is produced early
468 after CD3-activation of human CD4⁺ T cells and is increased upon co-stimulation. Thus,
469 our data allow us to suggest a model in which presentation of antigen and TCR

470 engagement induces the subsequent generation of CD46 ligands, providing a means for
471 CD46 activation early during T cell activation. Such activated cells are now poised to
472 integrate the third signal – high environmental IL-2 reflecting a successfully expanded
473 T_H1 response – and switch appropriately into the T_{REG} cell or contraction phase.

474

475 This scenario then raises the question about the hierarchy of CD46 activation
476 (specifically in relation to CD28 co-stimulation) in IL-10 induction by T_H1 cells. The
477 complete inhibition of CD3-CD28 co-stimulation-induced IL-10 production by
478 interference with ‘intrinsic’ CD46 activation suggests a dominant role for CD46 in IL-10
479 expression by T_H1 cells. Moreover, such ‘physiological’ CD46 engagement through C3b
480 or C4b produced by activated T cells might provide the molecular mechanisms by which
481 IL-10-secreting Tr1 cells are induced *in vitro* by other groups. The generation of ‘classic’
482 adaptive IL-10 (and IFN- γ)-producing Tr1 cells in culture requires minimally repetitive
483 CD3-CD28 activation (or exposure to DCs) of CD4⁺ T cells in the presence of high IL-
484 2^{7,45} - which are conditions conducive for CD46 engagement and CD46-induced IL-10
485 production.

486

487 Testing the notion that CD46 is important in regulating CD4⁺ and $\gamma\delta$ T cell responses in
488 a small animal model is currently hampered by the fact that rodents lack CD46
489 expression on somatic cells and that a murine molecule recapitulating CD46’s role in
490 TCR-IL-2-dependent IL-10 induction has not yet been identified²⁰. A previous study in
491 humans, however, connects significantly decreased IL-10 expression upon CD46
492 activation of CD4⁺ T cells with multiple sclerosis (MS)⁴⁶. Although the switch from the
493 T_H1 to Tr1 state was not examined in this study, low IL-10 production by T cells from MS
494 patients correlated with an abnormal increase in CYT-2 expression, consistent with our
495 finding that CYT-1 but not CYT-2 is required for IL-10 expression. These data combine
496 with our novel observation that T cells from RA patients are unable to promote the
497 CD46-mediated T_H1-Tr1 switch to implicate defects in this pathway in clinically
498 significant autoimmune and/or inflammatory conditions. It is now tempting to speculate
499 that a high intrinsic ‘signal threshold’ of T cells to switch from the T_H1 to Tr1 phenotype
500 upon IL-2-dependent CD46-activation might be a risk factor for autoimmunity.

501 Consistent with this is a recent correlation of certain CD25 polymorphisms with MS, RA
502 and type 1 diabetes⁴⁷. Conversely, a low intrinsic threshold for CD46-IL-2-mediated IL-
503 10 production might protect from autoimmunity but possibly at the price of an increased
504 risk for chronic infections.

505

506 Interestingly, the induction of IL-10 from naïve CD4⁺ T cells through TCR-CD46-IL-2
507 activation required repetitive stimulation of cells. This is reminiscent of the observation
508 that the tolerance-inducing switch of T_H1 cells into an IL-10⁺IFN- γ ⁺ state in mice requires
509 persistence of (self-)antigen¹⁸. Similarly, only continuous exposure of nonallergic
510 beekeepers to high doses of bee venom induces the switch from IFN- γ -secreting T_H1
511 cells into IL-10-producing Tr1-like cells indicated by the decrease of T cell-mediated
512 cutaneous swelling⁴⁸. After antigen withdrawal, bee venom-specific T cells again
513 produce only high IFN- γ and no IL-10 upon first reactivation and switch to IL-10
514 production only upon repetitive stimulation. CD46-activated T_H1 or Tr1 cells follow a
515 similar scheme: sorted IFN- γ ⁻IL-10⁺ T cells switch back to initial IFN- γ production after
516 expansion and restimulation (data not shown). These data are consistent with the
517 recent concept that IL-10-secreting cells might not necessarily represent a lineage but
518 rather the 'endpoint' of a successful effector T cell response. Indeed, they comply with
519 the facts that T_H1, T_H2 and T_H17 cells can produce IL-10, and that to date, no Tr1-
520 specific lineage marker has been identified. Consequently organs, such as the gut, skin
521 and lung, where there is chronic interaction between the host and the environment
522 might provide a local milieu – through continuous stimulation - that 'locks' T_H1 cells into
523 a regulatory state, potentially explaining the abundance of IL-10-secreting Tr1 cells in
524 these sites⁴⁹.

525

526 Although such T cell plasticity ensures the important flexibility to respond to
527 microenvironmental signals appropriately, it poses a major obstacle in the therapeutic
528 usage of IL-10-secreting T_H1 suppressor cells. Tr1 cells generated in a controlled *in*
529 *vitro* environment might reacquire a proinflammatory T_H1 phenotype after injection into
530 autoimmune or transplant patients. Identifying the 'molecular signature' that
531 characterizes the effector and the regulatory phase of CD46-induced T_H1 and Tr1 cells

532 might provide a means to actively ‘induce and lock’ these cells into the desired
533 functional state. Our capacity to identify specific signaling events required for IL-10
534 induction in T_H1 T cells (for example, the CD46-CYT-1-mediated activation of SPAK as
535 well as expression and nuclear translocation of ICER/CREM) could be a useful step in
536 creating a platform to monitor and potentially manipulate these pathways in the future.

537

538

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549

550

551 **AUTHOR CONTRIBUTIONS**

552 J.C., G.L.F., P.V., A.R., A.F., I.J., T.S. and C.K performed the experiments, discussed
553 that data and corrected the manuscript; G.L. provided financial support for I.J. and T.S.
554 and aided in data discussion; J.P.A. provided numerous vital reagents, helped
555 designing the experiments involving CD46-mediated signaling events, assisted in
556 interpreting the data and revised the manuscript; A.C. designed the experiments
557 involving the rheumatoid arthritis patients and provided the patient samples; A.H.
558 designed the experiments using $\gamma\delta$ T cells and revised the manuscript; C.K. designed
559 the study and wrote the manuscript.

560

561

562 **COMPETING INTEREST STATEMENT**

563 The authors declare no competing financial interest.

564

565

566 **FIGURE LEGENDS**

567 **Figure 1.** IL-2 regulates T_H1 vs. Tr1 effector function in CD3-CD46-activated CD4⁺ T
568 cells. (a) IFN- γ and IL-10 were detected in the supernatants of purified T cells activated
569 for 72 hours with the mAbs shown in combination with IL-2 (25 U/ml) (data represent
570 means \pm SD, $n=8$). CD46-induced IL-10 secretion requires high levels of exogenous IL-
571 2 (b; three independent donors shown), and is preceded by IFN- γ secretion (c).
572 Exogenous IL-2 was not provided when assessing IL-2 secretion, while 50 U/ml IL-2
573 was used when measuring IFN- γ or IL-10 secretion. Data represent means \pm SD ($n=3$).
574 (d) Cytokine secretion at 36h demarcated three distinct populations: IFN- γ^+ IL-10⁻, IFN-
575 γ^+ IL-10⁺, and IFN- γ^- IL-10⁺ (data representative of six experiments). (e): T cells activated
576 for 36h via CD3-CD46 in the presence of IL-2 (50 U/ml) were separated by flow
577 cytometry based upon their secretion profile (IFN- γ^+ IL-10⁻, IFN- γ^+ IL-10⁺ and IFN- γ^- IL-
578 10⁺) and cultured independently for further 18h (low IL-2). The resulting supernatants
579 from these expanded cultures were then each mixed with freshly purified CD4⁺ T cells
580 and the T cell plus supernatant mixtures activated via CD3-CD28 (+/- a neutralizing
581 anti-IL-10 mAb), and proliferation measured at day 6. As controls, freshly purified T cells
582 were also CD3-CD28-activated in the presence of fresh media or supernatants derived
583 from 72h CD3-CD28-activated T cells (left three bars). NA, non-activated; * $P < 0.05$, ** P
584 < 0.01 (Student's t -test).

585

586 **Figure 2.** CD46-IL-2 signals induce a switch from a T_H1 to a suppressive Tr1 phenotype
587 in CD4⁺ T cells. (a) CD46-induced suppressive IL-10-secreting cells might develop from
588 an initial T_H1 effector (1), or from a distinct cell subset (2). (b) Inhibition of T_H1 lineage
589 induction using a neutralizing mAb to IFN- γ blocked the production of IL-10 by cells
590 stimulated via CD3 and CD46 in the presence of 50 U/ml IL-2, whereas IL-10 blockade
591 was ineffective. Data are means \pm SD ($n=3$). (c) CD46-induced IFN- γ^+ IL-10⁻ cells give
592 rise to IFN- γ^+ IL-10⁺ and IFN- γ^- IL-10⁺ cells: purified T cells were activated via CD3 or

593 CD3-CD46 for 36h in the presence of IL-2 (5 U/ml), and IFN- γ ⁺IL-10⁻ cells isolated by
594 flow cytometry. Following expansion for 4 days with 5 U/ml IL-2, these cells were then
595 re-stimulated as shown, and secretion of IFN- γ and IL-10 assayed 18h post-activation
596 (data representative of four experiments). NA, non-activated; **P* < 0.05, ***P* < 0.01
597 (Student's *t*-test).

598

599 **Figure 3.** CD46-mediated signals contribute to the regulation of IL-2 expression by
600 CD4⁺ T cells. (a) CD46-induced IL-10 production is coupled with loss of IL-2 secretion.
601 T cells were stimulated for 36h via CD3-CD46, and active secretion of IFN- γ , IL-10 and
602 IL-2 measured. Note that the population of IL-10⁺IL-2⁺ cells in the lower right panel are
603 also IFN- γ ⁺ (not shown). Data representative of three experiments. (b) Intracellular flow
604 cytometry demonstrates increased ICER-CREM expression following 36h activation
605 with mAbs to CD3-CD46. Grey-filled histogram, isotype staining control. (c)
606 Immunoblotting of cytoplasmic (C) and nuclear (N) protein fractions reveals that CD3-
607 CD46-mediated activation of purified T cells induces nuclear translocation of ICER-
608 CREM after 48h. Data representative of two experiments. (d) ChIP analysis of CD3-
609 CD46-induced IFN- γ ⁺IL-10⁻ and IFN- γ ⁻IL-10⁺ cells demonstrates that ICER-CREM binds
610 to the IL-2 promoter uniquely in IL-10⁺ cells. The left panel shows PCR amplification of
611 an IL-2 promoter-specific sequence from the genomic DNA of each cell population
612 (control), with the right panel showing amplification of the same sequence from ICER-
613 CREM-precipitated DNA samples (data representative of three experiments). NA, non-
614 activated; PP DNA, ICER-CREM Ab-precipitated DNA.

615

616 **Figure 4.** The intracellular CYT-1 domain of CD46 and SPAK kinase are required for IL-
617 10 production in CD4⁺ T cells. (a) Jurkat T cells stably transfected with the CD46 BC1
618 isoform (Jurkat-BC1) are able to secrete IL-10 following 48h CD3-CD46 activation,
619 whereas Jurkat-BC2 are unresponsive. Data are means \pm SD (*n*=3). (b) Primary human
620 CD4⁺ T cells were activated for 15 minutes using the antibodies indicated, and cell
621 lysates used for immunoprecipitation with a CD46-specific or isotype control mAb.
622 Immunoblotting with an anti-SPAK antibody revealed an association between CD46 and

623 SPAK, but not between SPAK and CD3 (data not shown). Untreated lysates from non-
624 activated cells were loaded as control (“lysate”). Data representative of three
625 experiments. (c) siRNA-mediated knockdown of SPAK expression leads to reduced IL-
626 10 secretion by purified CD4⁺ T cells activated for 36 hours with mAbs against CD3-
627 CD46. As a control, T cells were either transfected with a non-specific siRNA or with
628 buffer alone (“mock”). Data represent means ± SD (*n*=3). (d) IL-10 production by CD3-
629 CD46-stimulated Jurkat T cells could be restored by over-expression of wild-type SPAK
630 (Jurkat-SPAK), but not a kinase-dead version (Jurkat-ΔSPAK). Data represent mean ±
631 SD (*n*=3). NA, non-activated; NT, non-transfected; ***P* < 0.01 (Student’s *t*-test).

632
633 **Figure 5.** CD46 directly regulates $\gamma\delta$ T cell function. (a) PCR analysis of CD46 isoform
634 usage by human $\gamma\delta$ T cells from three donors reveals a predominant expression of BC2,
635 compared with the variable expression pattern commonly observed in CD4⁺ T cells²⁶.
636 CHO cell lines transfected with single isoforms of CD46 are shown as control. (b, c, d)
637 PBMC were stimulated with HMBPP (10nM) +/- anti-CD46 mAb in the absence (open
638 bars) or presence (closed bars) of 100 U/ml IL-2. Cross-linking of CD46 failed to induce
639 IL-10 production by human $\gamma\delta$ T cells (b). However, it reduced the production of IFN- γ
640 and TNF (c), as well as CD25 expression (d), by V γ 9V δ 2 T cells, in marked contrast to
641 increased CD25 expression by CD4⁺ T cells. (e) Purified cultures of CD4⁺ or V γ 9V δ 2 T
642 cells (both >97% pure) were activated with anti-CD3 mAb or HMBPP respectively, in the
643 presence of anti-CD46 mAb and 100 U/ml IL-2. IFN- γ ⁺IL-10⁺ and IFN- γ ⁻IL-10⁺
644 populations were detected in CD4⁺ but not V γ 9V δ 2 T cells. (f) Production of IFN- γ by
645 purified V γ 9V δ 2 T cells was decreased by the anti-CD46 antibody in the absence (open
646 bars) and presence (closed bars) of IL-2. D1-D8 represent independent donors.

647
648 **Figure 6.** CD4⁺ T cells from rheumatoid arthritis (RA) patients are defective in the CD3-
649 CD46-induced IFN- γ -IL-10 switch. (a) Blood-derived T cells from three healthy donors
650 (HD A-C) and three RA patients (RA A-C) were CD3-CD46-activated (50 U/ml IL-2) for
651 36h and cytokine secretion assessed. Shown is the mid-value in percentages of each

652 population ($n=2$). **(b)** T cells were activated as described for panel **(a)**, and then
653 maintained in culture for 5 days with 50 U/ml IL-2. The levels of cytokines in the
654 supernatant were assessed at day 4. **(c)** Cells were then CD3-CD46 re-stimulated (50
655 U/ml IL-2) for 8h, and the percentage of cytokine-secreting cells (Cyt⁺) determined (mid-
656 value, $n=2$). **(d)** T cells from the synovial fluid of a juvenile arthritis (JA) patient are also
657 defective in CD3-CD46-induced IL-10 production. T cells from the synovial fluid of both
658 the left (JA-lk) and right (JA-rk) knees were analysed alongside blood-derived T cells
659 from two healthy donors (HD D and E) for: **(d)** the percentage of cytokine secreting cells
660 (mid-value, $n=2$) upon primary CD3-CD46 stimulation; **(e)** cytokine secretion during
661 expansion; and **(f)** the proportion of IL-10, IFN- γ , or IL-10-IFN- γ -secreting cells (mid-
662 value, $n=2$) upon secondary stimulation (as described for **(a)**, **(b)** and **(c)** respectively).

663
664 **Figure 7.** Engagement of CD46 by locally produced C3b drives IL-10 expression in
665 CD3-CD28-costimulated CD4⁺ T cells. **(a)** Purified CD4⁺ T cells were left non-activated
666 (grey filled histogram) or activated with the indicated immobilized mAbs for 12h as
667 indicated, at which point C3b surface deposition was assessed by flow cytometry. Using
668 ELISA, soluble C3b could be detected in the supernatants of all activated cell cultures
669 (data not shown). Data are representative of four experiments. **(b)** The addition of
670 soluble CD4 (sCD46) during activation of purified T cells with mAbs against CD3 +/-
671 CD28 inhibited the production of IL-10 in a dose-dependent manner, assessed at 36h
672 post-activation. Accordingly, the presence of immobilized C3b increased IL-10
673 production by these cells (data not shown). Data are representative of three
674 experiments. NS = data did not reach statistical significance.

675

676

677 **METHODS**

678 **Donor and blood samples**

679 Purified T cells were obtained from buffy coats (National Blood Service) or blood
680 samples from healthy volunteers. Informed consent was obtained from all subjects
681 included in the study and blood or synovial fluid were collected and processed with the

682 approval and in accordance of the King's College Ethics Committee guidelines
683 (Reference No: 06/Q0705/20). Adult patients with inflammatory arthritis (including
684 rheumatoid arthritis and juvenile idiopathic arthritis) were recruited to the study. All
685 patients had active disease with disease activity scores for 28 joints (DAS28) > 5.1,
686 representing moderately severe activity, in spite of therapy with combination disease
687 modifying anti-rheumatic drugs methotrexate, hydroxychloroquine and sulphasalazine.
688 Synovial fluid was obtained during therapeutic knee joint arthrocentesis.

689

690 **Cells, antibodies and recombinant proteins**

691 T cells were maintained as previously described¹⁹. Chinese hamster ovary (CHO) cells
692 and Jurkat T cells were purchased from the American Tissue Culture Centre and
693 cultured according to the manufacturer's protocol. Cell-stimulating Abs were bought
694 from BD Biosciences, San Diego, CA (anti-CD28; CD28.2), purified from a specific
695 hybridoma line (anti-CD3; OKT-3) or generated in house (anti-CD46; TRA-2-10²⁶). The
696 polyclonal rabbit SPAK antiserum was bought from Cell Signaling Technology, Inc.
697 (Danvers, MA) and the antibodies against phosphorylated and non-phosphorylated
698 ERK1/2, JNK and p38 from BD Biosciences. Neutralizing mAbs to human IL-2 (MQ1-
699 17H12), IL-4 (MP4-25D2), IL-10 (JES3-19F1) and IFN- γ (4S.B3), recombinant human
700 IL-4, IL-10, IL-12 and IFN- γ for *in vitro* T_H1 or T_H2 induction as well as fluorochrome-
701 conjugated Abs to phospho-Stat4, phospho-Stat5 and phospho-Stat6 were purchased
702 from BD Biosciences (and used with fixing and staining buffers suggested by the
703 manufacturer). Recombinant C3b was bought from Complement Technologies Inc.
704 (Tyler, TX). Soluble CD46 was generated by cloning the cDNA coding for SCRs 1-4 of
705 human CD46 into the pET15-b vector. BL21(DE3) bacteria were transfected with the
706 construct and recombinant soluble CD46 then purified from the inclusion bodies and
707 refolded according to a method described by White et al.⁵⁰. Functional activity of sCD46
708 was monitored in C3b binding and cofactor assays. Fluorochrome-conjugated mAbs to
709 human CD4, CD25, CD45RA, CD45RO, CCR7, and Foxp3 and AnnexinV-APC were
710 from BD Pharmingen (San Diego, CA). PE-labeled anti-mouse/human T-bet was
711 purchased from eBiosciences (Hatfield, UK). FITC-labeled anti-human pan $\gamma\delta$ TCR and
712 anti-human V δ 2 TCR were purchased from Beckman Coulter (Brea, CA). The anti-

713 CD46 mAb TRA-2-10 and anti-human ICER-CREM mAb (Abcam, Cambridge, UK) were
714 labeled with PE using the Invitrogen Zenon® Mouse R-phycoerythrin Mouse IgG₁
715 Labeling Kit (Invitrogen). Hydroxymethyl-butenyl-pyrophosphate (HMBPP), a
716 phosphoantigen specifically activating human V γ 9V δ 2 T cells, was kindly provided by
717 Dr. Hassan Jomaa (Justus-Liebig-Universität, Giessen, Germany).

718

719 **T cell isolation, $\gamma\delta$ T cell line, T_H1 and T_H2 cell generation**

720 CD4⁺ T lymphocytes were isolated from PBMC or synovial fluid using CD4 MicroBeads
721 (Miltenyi Biotec, Auburn, CA). Where indicated, T cells were isolated via cell sorting
722 after appropriate surface staining (CD4⁺, CD45RA⁺, CD45RO⁻, CD25⁻, CD127⁻ and
723 CCR7⁺ for naïve CD4⁺ T cells and pan anti- $\gamma\delta$ TCR for $\gamma\delta$ T cells). Purity of isolated
724 lymphocyte fractions was typically >97%. The polyclonal $\gamma\delta$ T cell line (>75% TCR V δ 2⁺)
725 was obtained after stimulation of PBMC with 10nM HMBPP and 100 U/ml IL-2. Fresh
726 medium +IL-2 was added every 2-3 days and cells were used for functional assays at
727 day 17. T_H1 and T_H2 cells were generated by activating and expanding sorted naïve
728 CD4⁺ T cells with mAbs to CD3 and CD28 (2 μ g/ml) with a function-neutralizing mAb to
729 IL-4 (10 μ g/ml) and rhIL-12 (10 ng/ml) for induction of a T_H1 phenotype and with anti-
730 IFN- γ (10 μ g/ml) and rhIL-4 (20 ng/ml) for T_H2 induction.

731

732 **T cell activation**

733 Purified CD4⁺ T cells were activated in 48-well culture plates (2.5 – 3.5 x 10⁵ cells/well)
734 coated with mAbs to CD3, CD28 and/or CD46 (2.0 μ g/ml each). Functional assays with
735 $\gamma\delta$ T cells were performed using the same protocol, except that HMBPP (10nM) was
736 used for stimulation.

737

738 **Cytokine measurements**

739 Cytokines were measured by using the T_H1/T_H2 Cytometric Bead Array (BD
740 Biosciences) or the human IFN- γ , IL-10 or IL-2 Cytokine Secretion Assay Kits (Miltenyi
741 Biotec) in combination as per manufacturer's protocol.

742

743 **Suppression assay**

744 Purified T cells were CD3-CD46-activated in the presence of rhIL-2 (5 or 50 U/ml) for
745 36h. Cells were then FACS-sorted based on their IFN- γ ⁺IL-10⁻, IFN- γ ⁺IL-10⁺ or IFN- γ ⁻IL-
746 10⁺ secretion profiles and cultured separately for 18 hrs with low IL-2. Supernatants
747 from these cultures were harvested and then transferred to freshly purified T cells. T
748 cell/supernatant mixtures were activated for 6 days with mAbs to CD3 or CD3 and
749 CD28 and cell proliferation measured employing the CellTiter 96[®] AQueous One Solution
750 Cell Proliferation Assay from Promega (Madison, WI).

751

752 **Chromatin Immunoprecipitation (ChIP) analysis**

753 Binding of ICER-CREM to the IL-2 gene promoter was assessed utilizing the MAGnify[™]
754 Chromatin Immunoprecipitation System (Invitrogen) according to the manufacturer's
755 protocol. A mAb to ICER-CREM conjugated to Protein G was used to
756 immunoprecipitate ICER-CREM-DNA complexes.

757

758 **RNA silencing**

759 siRNA targeting human SPAK (siRNA ID 898) and negative control siRNA were
760 purchased from Ambion (Austin, TX). siRNA was delivered into primary human CD4⁺ T
761 cells by electroporation (2 x 10⁶ cells ml/transfection buffer (Ambion); 3 μ g/ml siRNA;
762 200V and 325 mF using the Bio-Rad Gene Pulser [Bio-Rad Laboratories, Hercules,
763 CA]). Transfection efficiency and cell viability was consistently above 80% and 75%,
764 respectively, and protein knock down peaked at 36-48h post-transfection.

765

766 **PCR, RT-PCR and quantitative real-time RT-PCR analyses**

767 PCR. IL-2 promoter-specific primer pair: +301 to +510 promoter site: forward ⁵TTA CAA
768 GAA TCC CAA ACT³ - reverse ⁵TAGAGGCTTCATTATCAAA³.

769 RT-PCR. The CD46 isoform expression pattern was assessed in CD4⁺ and $\gamma\delta$ T cells by
770 using CD46-specific primers: forward ⁵GTGGTCAAATGTCGATTTCCAGTAGTCG³ -
771 reverse ⁵CAAGCCACATTGCAATATTAGCTAAGCCACA³)²⁶.

772 Quantitative real-time RT-PCR. For the analysis of GATA3 expression, RNA isolated
773 from T cells was subjected to reverse transcription and the amounts of cDNAs specific

774 for GATA3 determined using the ABI Prism 7700 Sequence Detection System and a
775 GATA3-specific primer pair (hs00231122_M1, Applied Biosystems Inc., Foster City,
776 CA). The individual samples were normalized using human 18S rRNA housekeeping
777 gene expression (Applied Biosystems Inc).

778

779 **Statistical analysis**

780 Statistical analyses were performed using the Student's two-tailed *t*-test (Excel software
781 [Microsoft, Redmond, WA]).

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Transcription factor	IFN- γ ⁺ IL-10 ⁻ (T _H 1)	IFN- γ ⁺ IL-10 ⁺ (T _{REG})	IFN- γ ⁻ IL-10 ⁺ (T _{REG})	T _H 1 ^a	T _H 2 ^a
T-bet	+++	+++	+++	++++	-
Gata3 ^b	-/+	+	+	-/+	++++
Foxp3	-	-	-	-	-
p-STAT4	++	+++	++	++++	++
p-STAT5	+++	+++	+	++++	++
p-STAT6	+	+	-/+	+	++++
p-ERK	++	+++	+++	++++	++
ICER/CREM	+++	++	+	++	++

955

956 **Table I. Expression profile of key T cell lineage transcription factors in CD46-**
957 **induced effector (T_H1) and suppressor (T_{REG}) cell populations.**

958

959

960 Purified CD4⁺ T cells were CD3-CD46-activated for 36 hrs in high IL-2 and the subsequent IL-10 and IFN- γ
961 γ Secretion Assays combined with intracellular FACS staining for the respective T cell lineage markers.

962 a, transcription factor expression levels were determined relative to the expression levels in either T_H1 or
963 T_H2 cells with +++++, very strong (highest expression observed); +++, strong; ++, moderate; +, low; -/+,
964 weak but detectable; -, not detectable. Purity of generated T_H1 and T_H2 cells (see Material and Methods)
965 was assessed by measuring the percentage of IFN- γ ⁺IL-4⁻ (T_H1) or IFN- γ ⁻/IL-4⁺ (T_H2) cells upon CD3-
966 CD28 restimulation and was > 85% in all assays.

967 b, due to the lack of a suitable Ab, mRNA GATA3 levels were determined using quantitative real-time RT-
968 PCR analysis.

969

970

Figure 1

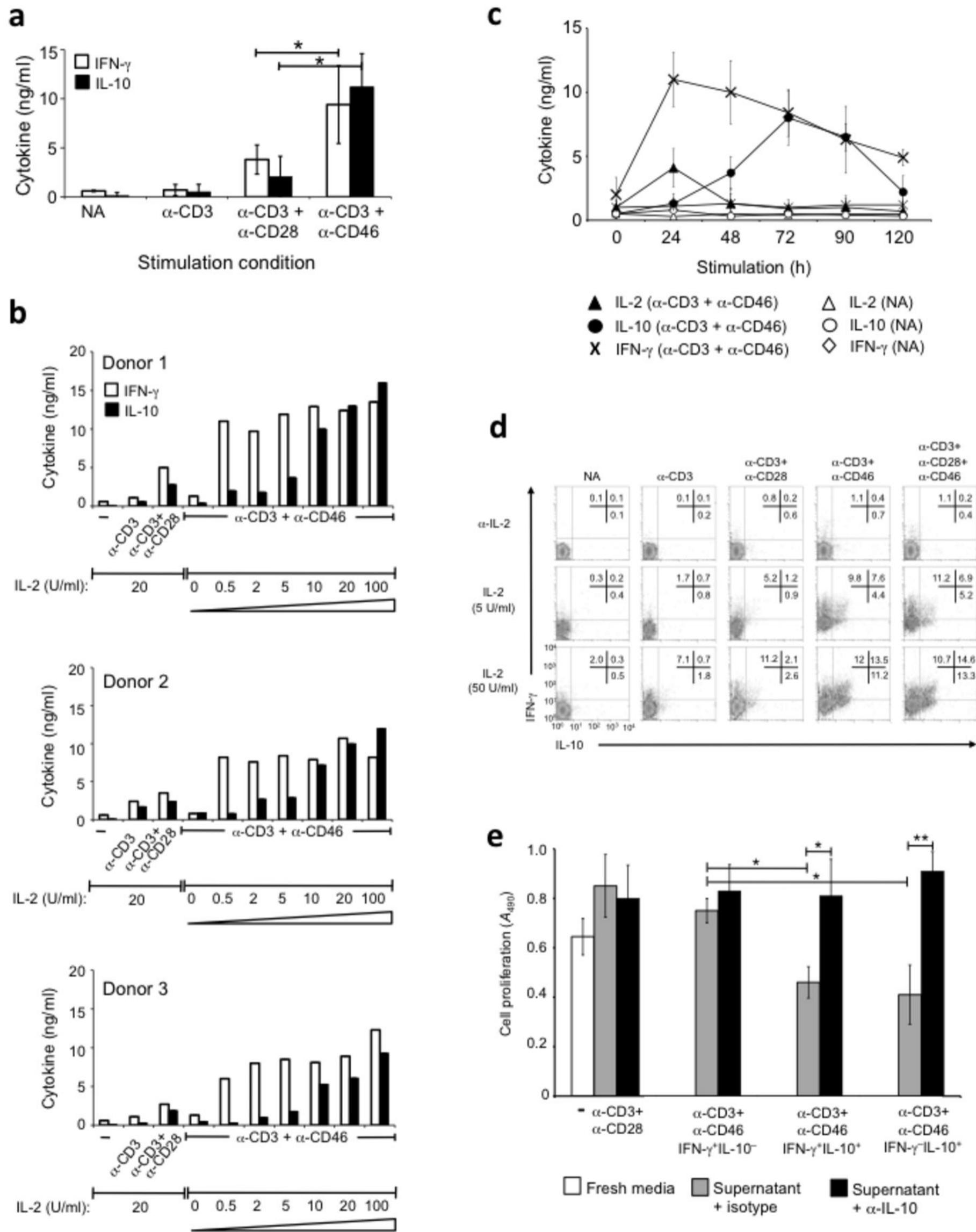


Figure 2

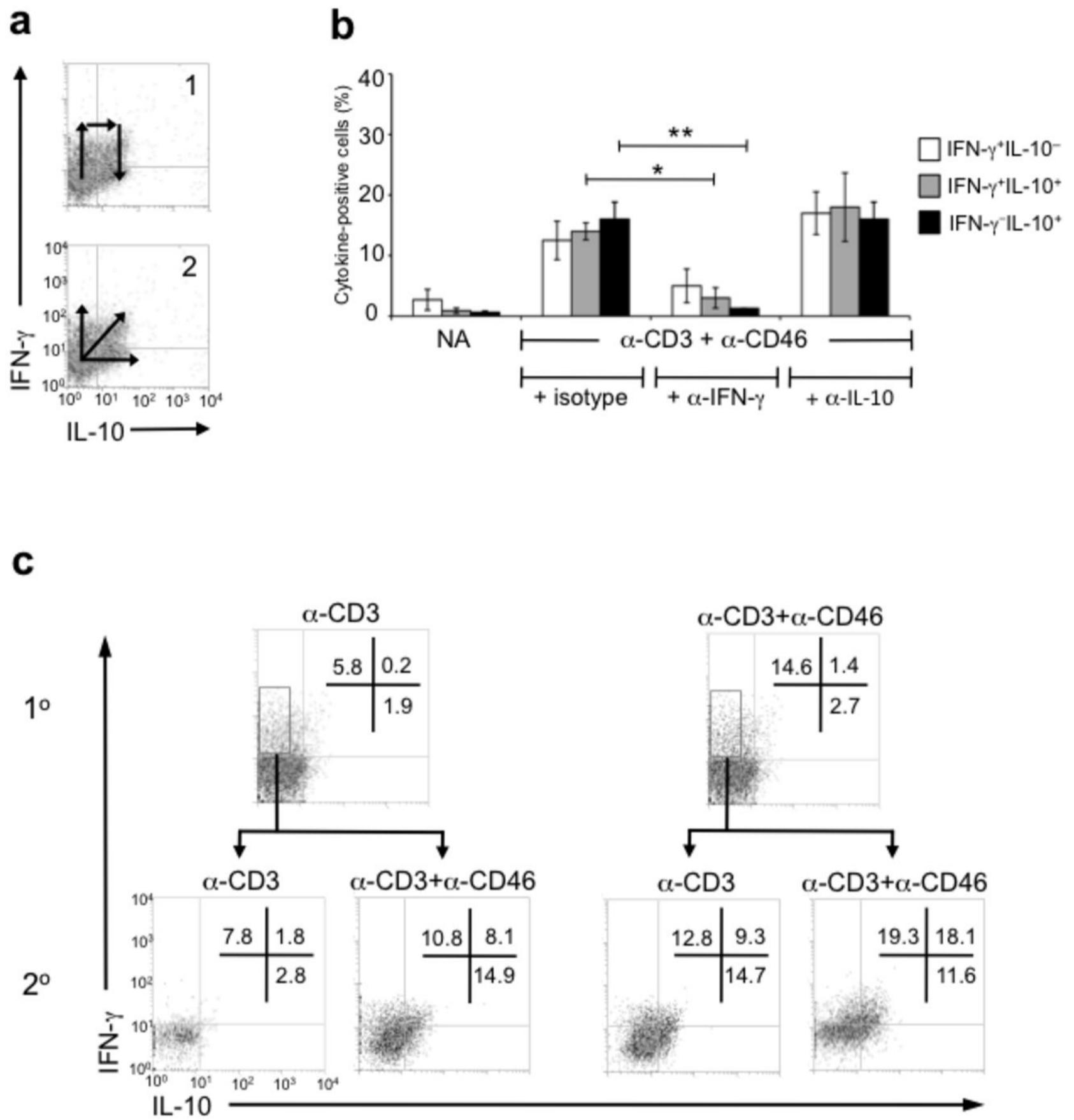


Figure 4

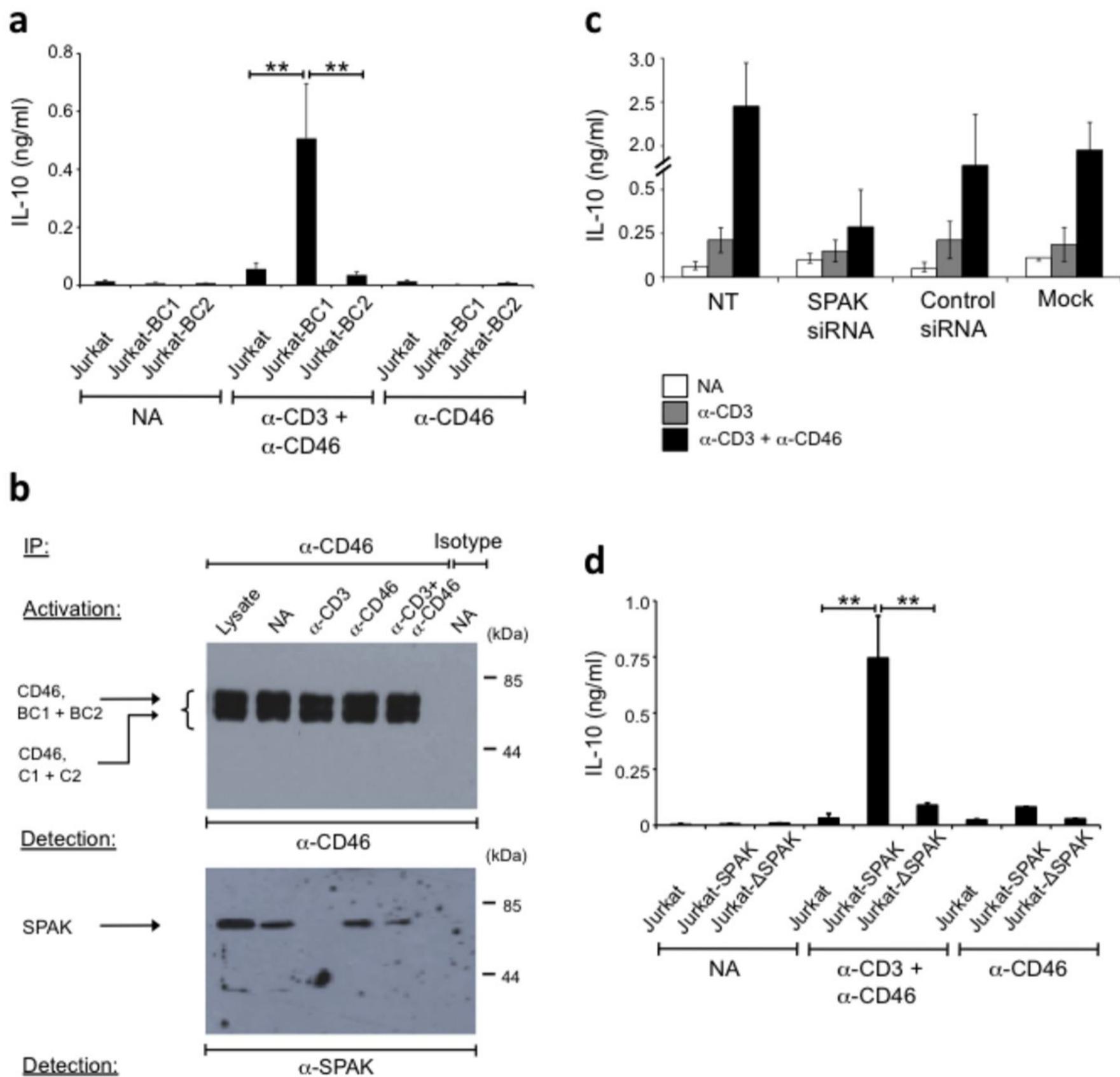


Figure 5

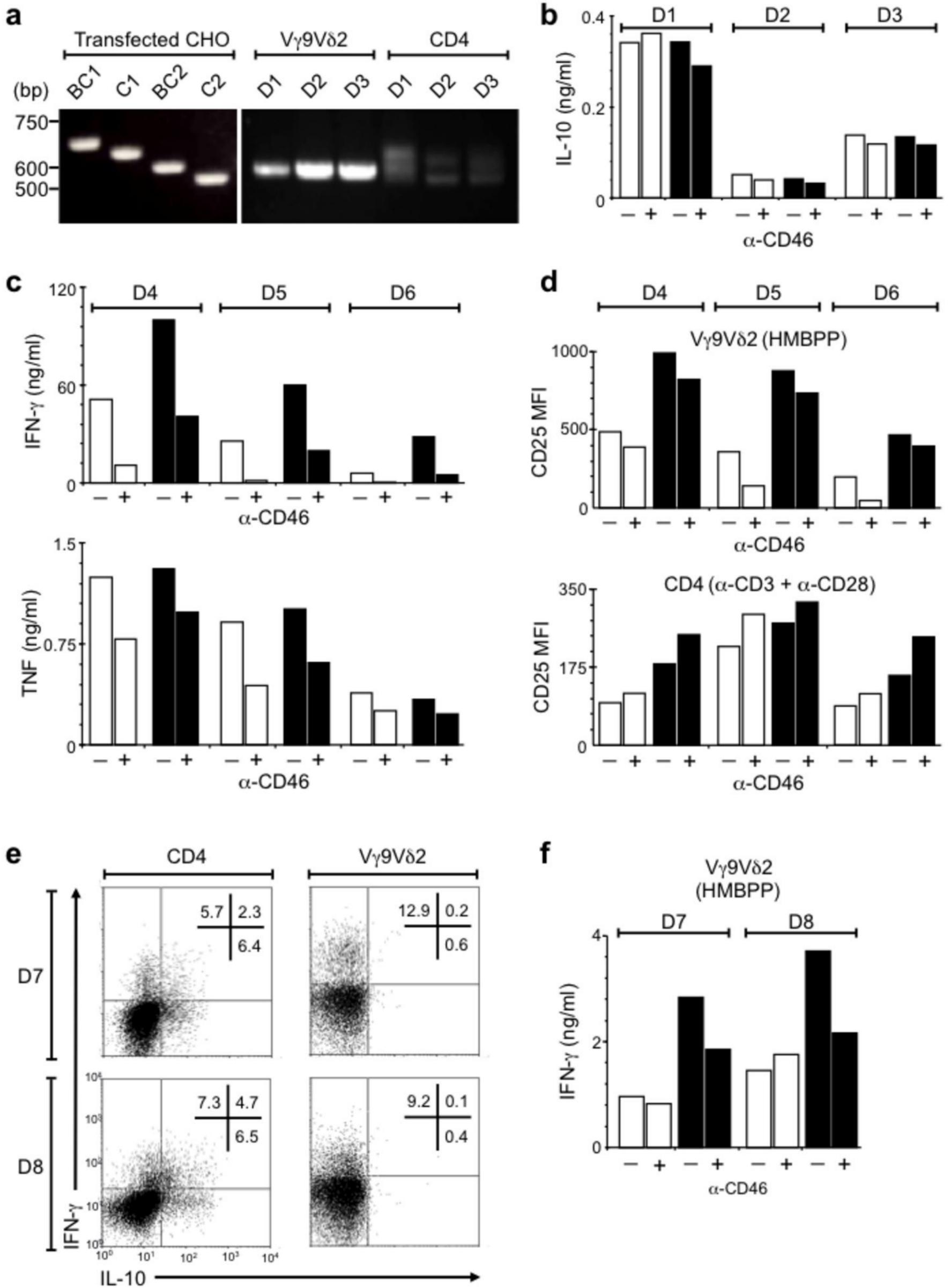


Figure 6

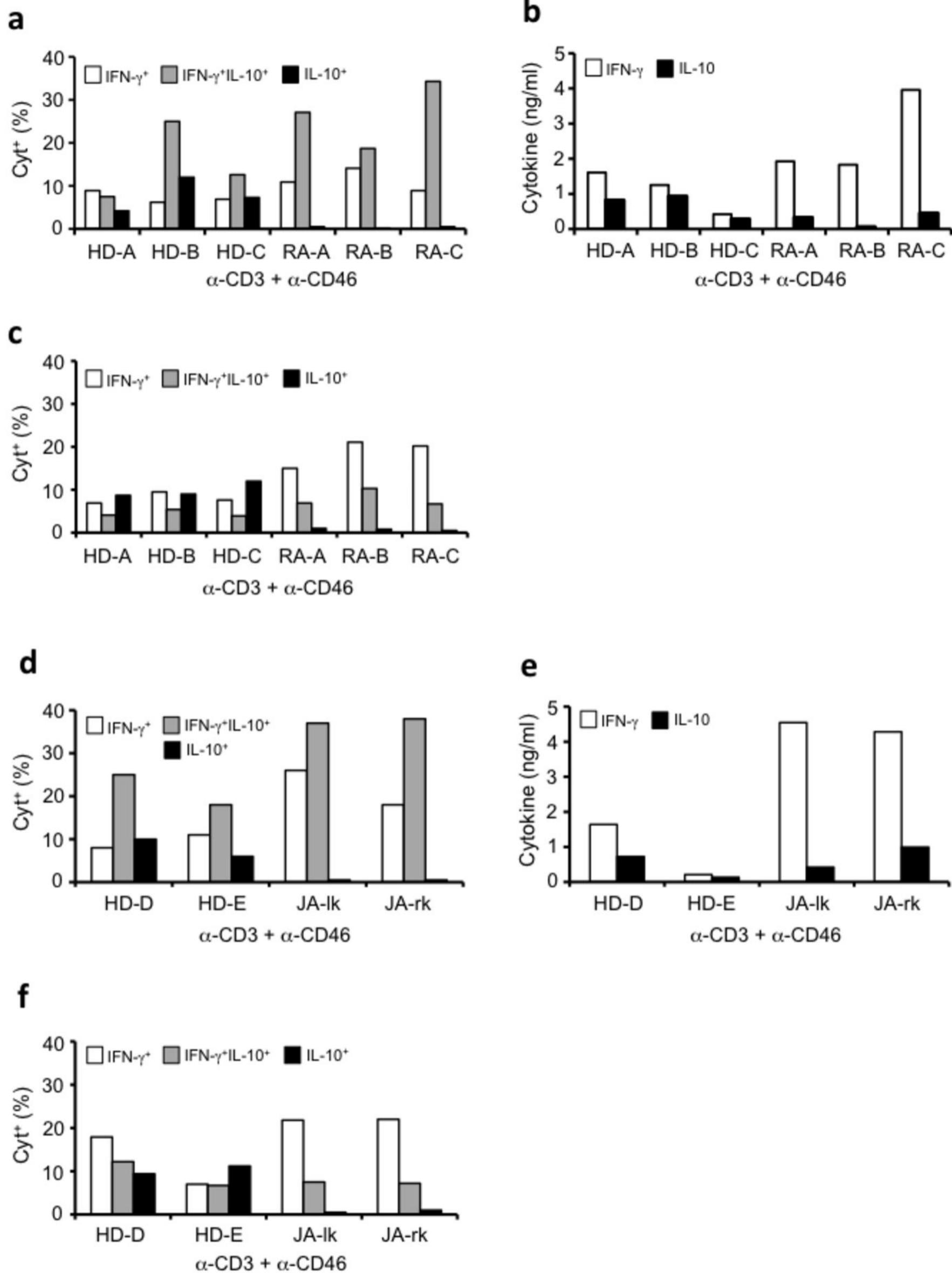
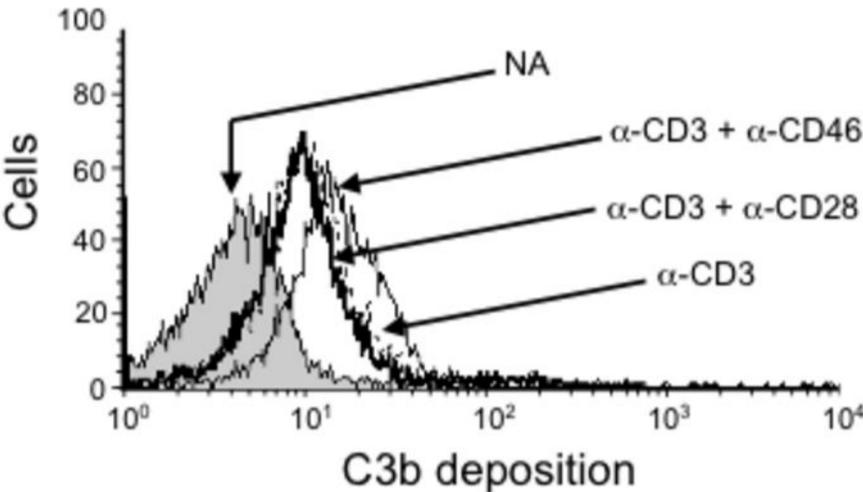


Figure 7

a



b

