1 Mitochondrial oxidative stress drives IL-12/IL-18-induced 2 IFN- γ production by CD4⁺ T cells and is controlled by Fas

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12 **Running title:** mROS and cytokine-induced IFN- γ in memory-like CD4⁺ T cells

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24 Abstract

25 Mitochondrial activation and mROS production are crucial for CD4⁺ T cell responses
 26 and have a role in naïve cell signaling after TCR activation. However, little is known
 27 about their role in recall responses driven by cytokine signaling. Here, we found that
 28 mROS are required for IL-12 plus IL-18-driven production of IFN- γ , an essential
 29 cytokine in inflammatory and autoimmune disease development. In particular, memory-
 30 like cells obtained after activation-induced differentiation showed faster and augmented
 31 mROS accumulation and increased IFN- γ production in response to IL-12 plus IL-18
 32 compared to naïve T cells. In contrast, mROS induction was similar in naïve and
 33 memory-like cells after TCR-dependent signaling. Taken together these results
 34 suggested that memory-like CD4⁺ T cells treated by IL-12 plus IL-18 attained
 35 conditions for an extraordinary mROS-producing potential. mROS inhibition
 36 significantly downregulated the production of IFN- γ and the expression of CD44
 37 activation marker, suggesting a direct mROS effect on the activation of memory-like T
 38 cells. Mechanistically, mROS was required for optimal activation of key signaling
 39 pathways that drive IFN- γ production after IL-12 plus IL-18 T cell stimulation, such as
 40 PKC- θ , AKT and STAT4 phosphorylation, and NF- κ B activation. Notably, we
 41 identified increased mROS as key promoters of hyperactivation and IFN- γ
 42 overproduction in Fas-deficient *lpr* memory-like CD4⁺ T cells compared to WT cells,
 43 following IL-12 plus IL-18 stimulation. mROS inhibition significantly reduced the
 44 population of disease-associated CD44^{hi}CD62L^{lo} *lpr* CD4⁺ T cells and their IFN- γ
 45 production. These findings uncover a previously unidentified role for Fas in regulating
 46 mitochondrial ROS production by memory-like T cells. This apoptosis-independent Fas
 47 activity might contribute to the accumulation of CD44^{hi}CD62L^{lo} CD4⁺ T cells that
 48 produce increased IFN- γ levels in *lpr* mice. Overall, our findings pinpoint mROS as

49 central regulators of TCR-independent signaling, and support mROS pharmacological
50 targeting to control aberrant immune responses in autoimmune-like disease.
51

52 Introduction

53 Upon TCR activation, mitochondria rapidly translocate to the immunological synapse,
54 leading to electron transport chain (ETC) activation (1) and mitochondrial reactive
55 oxygen species (mROS) production. Superoxide ($O_2^{\bullet-}$) is generated at mitochondrial
56 complexes I and III (2, 3), from where it can reach cytosol either directly through
57 mitochondrial membrane or after conversion to hydrogen peroxide (H_2O_2) (4). It has
58 been established more recently that mROS play crucial role as redox signaling
59 molecules in T cells, regulating IL-2 and IL-4 production upon TCR triggering (5, 6).

60 IL-12 and IL-18 are required to boost T_H1 responses and IFN- γ production after TCR
61 ligation (7, 8). In a mechanism evolved to provide an early source of IFN- γ and
62 contribute to the innate immune response, IFN- γ can be induced in a TCR-independent
63 manner, only by IL-12 and IL-18 (9, 10). It has been proposed that this “cytokine-
64 induced cytokine production” promotes chronic inflammation and autoimmunity (11,
65 12). Indeed, we previously demonstrated that IFN- γ production by *lpr* T cells creates a
66 loop by increasing the macrophage inflammatory potential (13). The signaling
67 mechanisms that drive IFN- γ production in response to IL-12 plus IL-18 are distinct
68 compared to those downstream of TCR, and lead to differential NF- κ B recruitment to
69 *Ifng* locus (14–16).

70 Although the role of mROS has been greatly appreciated downstream of TCR, their role
71 in recall responses of previously activated $CD4^+$ T cells and in IL-12 plus IL-18-
72 dependent signaling regulation remains unexplored. Excessive production of ROS is
73 thought to underlie some of the pathological immune reactions implicated in
74 autoimmunity. T cells from lupus patients exhibit mitochondrial hyperpolarization,
75 resulting in increased mROS production (17, 18). In a previous study, we demonstrated

76 that autoimmune traits in *lpr* mice were decreased by controlling autoreactive T cell
77 hyperactivation and their IFN- γ overproduction (13). However, the direct link between
78 mROS and IFN- γ hyperproduction in autoreactive T cells has not been made thus far.

79 Here, we hypothesized that mROS act as driving force to modulate signal transduction
80 leading to hyperproduction of IFN- γ . We analyzed the role of mROS in IL-12 plus IL-
81 18-induced IFN- γ production in naïve and in *in vitro*-differentiated memory-like CD4⁺
82 T cells, and found significantly higher amounts of mROS and IFN- γ in the later. We
83 demonstrated that mROS signal controls STAT4 and NF- κ B activation pathways.
84 Finally, we showed that Fas-deficient CD4⁺ T cells produce more mROS compared to
85 WT in response to IL-12 plus IL-18. This was translated to greatly increased IFN- γ
86 production and to higher proportions of CD44^{hi}CD62L^{lo} *lpr* CD4⁺ T cells compared to
87 WT. Our findings establish mROS as key component of TCR-independent IFN- γ
88 production and uncover an unknown role of Fas in regulating mROS and IFN- γ
89 production, providing rationale for targeting mROS in autoimmunity-related
90 hyperinflammation.

91 **Materials and Methods**

92 **Animals**

93 8-12 weeks old mice were used for CD4⁺ T cell isolation. C57BL/6 mice (WT) were
94 from Harlan Interfauna Ibérica and Fas-deficient C57BL/6-*lpr* mice (*lpr*) were from
95 Jackson laboratories. Mice were kept in SPF conditions. All animal experiments were
96 designed in compliance with European Union and national regulations, and were
97 approved by the CNB Bioethics Committee.

98 ***In vitro* CD4⁺ T cell activation and memory-like differentiation**

99 Naïve CD4⁺ T cells were purified from mouse spleens using Negative Isolation Kit
100 (Dyna Bead). Cell purity was >95% as measured by flow cytometry. For cell culture,
101 we used complete media containing 20 ng/ml human recombinant interleukin-2 (rIL-2,
102 PeproTech). Naïve CD4⁺ T cells (10⁶/ml) were stimulated with concavalin A (ConA;
103 1.5 µg/ml, Sigma) or with IL-12 (10 ng/ml) plus IL-18 (10 ng/ml) for indicated time
104 points. For memory-like differentiation, naïve CD4⁺ T cells were first stimulated with
105 ConA (1.5 µg/ml) for 24h, washed and cultured (0.5 x 10⁶/ml) in presence of 20 ng/ml
106 rIL-2 for 6 days. Differentiated memory-like cells were stimulated with ConA or with
107 IL-12 (10 ng/ml) plus IL-18 (10 ng/ml) for indicated time points. DPI (5 µM; Sigma),
108 rotenone (2.5 µM; Sigma) or antimycin A (4 µM) were added to cell culture 30 min
109 before the stimulus.

110 **Flow cytometry**

111 For detection of mitochondrial superoxide, cells were incubated with MitoSOX Red
112 (Invitrogen) at 5 µM final concentration for 30 min at 37 °C and stimulated for

113 indicated time points. Cells were then washed with PBS, centrifuged at $300 \times g$ for 5
114 min, and analyzed immediately using flow cytometry. MitoSOX Red fluorescence was
115 detected at the excitation and emission wavelengths of 488 and 585 nm, respectively.
116 To control for baseline fluorescence, samples from each experiment were stained
117 according to the above procedure and left unstimulated. MFI fold induction was
118 calculated by dividing stimulated by unstimulated values.

119 For measurement of mitochondrial membrane potential TMRM
120 (Tetramethylrhodamine, Methyl Ester, Perchlorate; ThermoFisher) was added to the
121 cells at 30 nM final concentration. After 30 min, cells were analyzed by flow cytometry.
122 Control cells were pre-incubated with 5 μ M FCCP for 10 min at 37 °C to depolarize the
123 membrane and show background staining.

124 Intracellular cytokine staining and cell cycle analysis were performed according to
125 standard procedure described in Supplementary Material. Stained cells were analyzed
126 on Gallios flow cytometer from Beckman Coulter. The data were analyzed using
127 FlowJo software (Tree Star).

128 **Immunoblotting**

129 Cells were treated as stated and the immunoblotting was performed as described in
130 Supplementary Material.

131 **EMSA**

132 EMSA was performed as described previously (19) and described in Supplementary
133 Material.

134 **Statistical Analysis**

135 Statistical significance was determined by unpaired 2-tailed Student's *t* test for
136 comparisons between two groups, or by 1- or 2-way ANOVA for multiple comparisons,
137 followed by Sidak's or Tukey post-hoc test. Differences were considered significant
138 when $p < 0.05$. All statistical analyses were conducted using Prism 8 software
139 (GraphPad).

140 Results

141 Memory-like CD4⁺ T cells produce increased mROS and IFN-γ in response to IL- 142 12/IL-18 compared with naïve cells

143 Naïve CD4⁺ T cells purified from mouse spleens were stimulated *ex vivo* with IL-12/IL-
144 18 to induce IFN-γ production independently of TCR, as previously described (20).
145 Alternatively, T cells were exposed to concavalin A (ConA), which is known for
146 mimicking the physiological TCR cross-linking leading to T cell activation and ROS
147 production (21). After primary TCR activation and IL-2 dependent culture, memory-
148 like CD4⁺ T cells were re-stimulated with either ConA or with IL-12/IL-18. As
149 previously described (22), naïve CD4⁺ T cells, characterized by CD62L^{hi}CD44^{lo}
150 phenotype, efficiently differentiated into central and effector memory cell subsets after
151 IL-2 expansion (Supplementary Figure 1). Upon 24 h of TCR stimulation, intracellular
152 staining showed significant but relatively low IFN-γ production both after primary and
153 secondary activation of naïve and memory-like cells, respectively (Figure 1A).
154 IL-12/IL-18 stimulation induced similar IFN-γ levels as ConA in naïve CD4⁺ cells
155 (Figure 1A). By contrast, the proportions of IFN-γ-producing cells after IL-12/IL-18
156 stimulation were strikingly higher in memory-like compared with naïve cells (Figure
157 1A). The data showed that IL-12/IL-18 induce IFN-γ more efficiently in memory-like
158 CD4⁺ T cells compared to TCR-dependent stimulation.

159 We measured mitochondrial superoxide production with MitoSOX Red in naïve and
160 memory-like T cells after TCR-dependent and -independent stimulation. After ConA
161 treatment, the percentage of MitoSOX^{hi} cells was significantly increased compared to
162 unstimulated cells, but we found no significant differences in mROS production
163 between naïve and memory-like cells (Figure 1B). In naïve cells, the proportions of

164 MitoSox^{hi} cells after IL-12/IL-18 stimulation were similar to those induced by ConA
165 (Figure 1B). However, IL-12/IL-18 induced significantly higher proportions of
166 MitoSOX^{hi} cells in the memory-like cell population compared to that of naïve cells
167 (Figure 1B). We also measured MitoSOX MFI and obtained similar results
168 (Supplementary Figure 2).

169 These data showed that memory-like cells displayed enhanced responses after IL-12/IL-
170 18 compared to ConA stimulation in terms of both IFN- γ and mROS production, while
171 the two types of stimulation had similar effects on naïve T cells.

172 **IL-12/IL-18-dependent IFN- γ production by naïve CD4⁺ T cells requires mROS** 173 **activation**

174 To investigate whether mROS are functionally related to IL-12/IL-18-induced
175 IFN- γ production by naïve CD4⁺ T cells, we inhibited mROS production using DPI
176 (diphenyleneiodonium) (23, 24). Alternatively, we used specific mitochondrial complex
177 I inhibitor rotenone.

178 Upon T cell stimulation we detected two cell populations: MitoSOX^{lo} and MitoSOX^{hi}
179 cells. While in MitoSOX^{hi} cell population MitoSOX MFI significantly increased at 30-
180 and 60-min post IL-12/IL-18 or ConA stimulation, MitoSOX^{lo} cells remained
181 unresponsive (Supplementary Figure 3 and data not shown). We therefore focused our
182 analyses on the MitoSOX^{hi} population, which represented activated T cells.

183 We analyzed early MitoSOX levels with or without mROS inhibitors after T cell
184 activation over a period of two hours. MitoSOX was significantly increased at 30 min
185 post stimulation and remained elevated for at least two hours, as indicated by MitoSOX
186 median fluorescence intensity (MFI) (Figure 2A). At all time points up to 2 h post-

187 stimulation, the activation-induced mROS increase was dramatically suppressed upon
188 DPI treatment, to mROS levels of unstimulated cells (Figure 2A). These data show that
189 DPI inhibits mROS production in cells that are responsive to IL-12/IL-18. Similar
190 results were obtained using rotenone (not shown).

191 IL-12/IL-18 stimulation of naïve T cells induced IFN- γ production in a low cell
192 proportion (~1.5%) that were also CD44^{hi}, at 24h post-stimulation (Figure 2B, left).
193 Importantly, DPI treatment reduced IFN- γ production by these cells (Figure 2B, left) in
194 a statistically significant manner (Figure 2B, right). However, DPI had no effect on the
195 expression of CD44 (Figure 2C). Overall, our results suggest that mROS control IFN- γ
196 production after activation of naïve CD4⁺ T cells.

197 **mROS inhibition lowers IFN- γ production by memory-like CD4⁺ T cells and** 198 **compromises their CD44^{hi} phenotype**

199 Memory-like CD4⁺ T cells generated after primary TCR activation and IL-2-dependent
200 culture significantly increased mROS production as early as 15 min post IL-12/IL-18
201 stimulation (Figure 3A). The impact of mROS on cytokine-induced signaling is likely
202 more relevant in memory-like than in naïve CD4⁺ T cells, as in the later mROS increase
203 was not detected until 30 min post-stimulation (Figure 2A).

204 DPI led to a prominent reduction of mROS, as detected by MitoSOX at all time points
205 tested after IL-12/IL-18 treatment (Figure 3A). Memory-like cells were more responsive
206 to IL-12/IL-18 stimulation compared to naïve cells, as ~10% of the total CD4⁺
207 population were CD44^{hi} cells and positive for IFN- γ at 24 h post-stimulation (Figure
208 3B). The mROS inhibitor DPI nearly abrogated IFN- γ production in memory-like T
209 cells (Figure 3B, left) in statistically significant manner (Figure 3B, right). In addition,

210 DPI led to significant downregulation of CD44 expression, suggesting that mROS
211 regulates the activation potential of memory-like T cells (Figure 3C). Of note, we didn't
212 observe any marked effect of DPI treatment on cell viability nor on cell cycle
213 distribution (Supplementary Figure 4A). Our results support the notion that mROS are
214 integral to the maintenance of activated CD44^{hi} phenotype and control the TCR-
215 independent IFN- γ production.

216 **Validation of DPI as an attenuator of IFN- γ production through inhibition of** 217 **mROS production**

218 Our results showed that DPI strongly inhibits mROS-dependent IFN- γ production by
219 memory-like CD4⁺ T cells. DPI is also known for inhibiting Nox enzymes (23, 25–27),
220 raising questions of whether the observed effects of DPI on IFN- γ production might be
221 due to mitochondria-unrelated processes. To address this point and validate the effect of
222 mROS generation on IFN- γ production we tested other respiratory chain blockers.
223 Specific complex I inhibitor rotenone significantly decreased IL-12/IL-18-induced
224 mROS production (Figure 4A), as well as the percentages of IFN- γ -producing and
225 CD44^{hi} cells (Figure 4B), thus providing similar results as DPI. We also used antimycin
226 A, an electron transport chain inhibitor acting at complex III. Although antimycin A has
227 been shown to increase mROS production (28), we found that in the present setting it
228 does not affect mROS at early time points post-IL-12/IL-18 stimulation (Figure 4A). In
229 addition, antimycin A was not effective in blocking IFN- γ production nor in reducing
230 CD44 expression, suggesting that an intact electron transport chain is not required for
231 IL-12/IL-18-induced IFN- γ production.

232 Other studies have shown that DPI treatment might affect the overall electron transport
233 activity and mitochondrial membrane potential (29). We therefore used potentiometric

234 TMRM dye to test whether DPI treatment affects the mitochondrial membrane potential
235 during memory-like CD4⁺ T cell activation. TMRM fluorescence showed that neither
236 IL-12/IL-18 nor DPI significantly affected mitochondrial membrane potential at early
237 time points after stimulation (Figure 4C). Thus, it seems that mROS inhibition did not
238 affect the overall electron transport rate, in accordance with previous studies (29).

239 **mROS-mediated signals activate PKC- θ , AKT, STAT4 and NF- κ B**

240 IL-12 and IL-18 act in synergy through different signaling pathways to activate IFN- γ
241 production (30). IL-12 receptor stimulation leads to STAT4 phosphorylation through
242 Jak2 and Tyk2, while IL-18 (also known as IFN- γ -inducing factor) activates the NF- κ B
243 pathway via IRAK (interleukin 1 receptor-associated kinase) and TRAF6 (TNF
244 receptor-associated factor 6) (20, 31, 32). We examined the components of these two
245 pathways in memory-like CD4⁺ T cells before and after treatment with mROS inhibitors
246 DPI or rotenone. Before IL-12/IL-18 treatment the NF- κ B pathway was kept in its
247 latent state, as seen by high protein levels of I κ B α (Figure 5 A, Ctrl). Stimulation
248 induced NF- κ B activation, as I κ B α levels dropped as early as 15 min and 30 min
249 (highest reduction) post-stimulation (Figure 5A and B). After DPI or rotenone treatment
250 this drop in I κ B α was halted, suggesting that NF- κ B activation is delayed in conditions
251 of decreased mROS. We also examined the activation of PKC- θ , as it has been
252 previously implicated in TCR-independent NF- κ B regulation (33). We found increased
253 PKC- θ phosphorylation as early as 15 min post-IL-12/IL-18 treatment (Figure 5A and
254 B). Both inhibitors reduced the phospho-PKC- θ levels, which was evident at 30 min
255 and more so at 1 h post-treatment. These data suggest that early mitochondrial oxidative
256 signal promotes PKC- θ and NF- κ B activation after IL-12/IL-18 signaling. In
257 accordance with this, electromobility shift showed elevated NF- κ B nuclear activity at

258 1h post-IL-12/IL-18 treatment, which was markedly decreased by DPI or rotenone
259 (Figure 5C).

260 STAT4 is key transcription factor recruited by IL-12-derived signal to regulate IFN- γ
261 (31). Phosphorylation of STAT4 was evident after IL-12 plus IL-18 stimulation (Figure
262 5D). Both after rotenone and DPI treatment, STAT4 phosphorylation was markedly
263 decreased early after IL-12/IL-18 stimulation (Figure 5D). This suggested a direct
264 mROS role in IL-12-dependent STAT4 activation. IL-18 in certain cell types activates
265 AKT (34), and mROS affects AKT phosphorylation (35). We detected increased AKT
266 phosphorylation after IL-12/IL-18 treatment (Figure 5D). mROS inhibition using
267 rotenone or DPI markedly decreased phospho-AKT levels (Figure 5D).

268 Altogether, these data further strengthen the notion that mROS trigger the early
269 signaling events downstream of IL-12 and IL-18 receptors leading to IFN- γ production
270 independently of TCR activation.

271 **IFN- γ hyperproduction in *lpr* CD44^{hi}CD62L^{lo} effector/memory CD4⁺ T cells is** 272 **related to increased mROS**

273 *lpr* (lymphoproliferation spontaneous mutation) mice are Fas (CD95) deficient and
274 show defective AICD (activation-induced cell death) of *in vitro* re-stimulated T cells
275 (36, 37). *In vivo*, one of the symptoms caused by Fas deficiency in *lpr* mice is the
276 accumulation of effector/memory CD44^{hi}CD62L^{lo} CD4⁺ T cells and their intrinsic
277 hyperactivation accompanied by hyperproduction of IFN- γ in response to IL-12/IL-
278 18 (13). To investigate the role of mROS in the responses of Fas-deficient memory-like
279 cells, we purified CD4⁺ T cells from WT and *lpr* mice and, after initial ConA
280 stimulation and IL-2 expansion, we stimulated the cells with IL-12/IL-18. Significantly

281 higher mROS levels were induced in *lpr* compared with WT cells, as seen by MitoSOX
 282 at 60 min after stimulation (Figure 6A). The mROS production was greatly diminished
 283 by DPI in both WT and *lpr* cells (Figure 6B). Of note, the proportions of CD44^{hi} cells
 284 were similar between WT and *lpr*, suggesting that Fas deficiency did not cause
 285 increased accumulation of memory-like cells under these experimental conditions and
 286 TCR-independent stimulation (Figure 6C). Accordingly, cell cycle analysis showed no
 287 apparent difference in apoptosis or proliferation in WT and *lpr* CD4⁺ T cells after IL-12
 288 plus IL-18 stimulation (Supplementary Figure 4B). Instead, *lpr* CD44^{hi} T cells were
 289 hyperactivated, as seen by significantly increased IFN- γ production compared to WT
 290 cells (~20 vs. 30% of the total CD4⁺ population) at 24 h post-IL-12/IL-18 (Figure 6D).
 291 DPI treatment significantly reduced the IFN- γ production in both WT and *lpr* CD44^{hi} T
 292 cells (Figure 6D). These results suggest that the increased mROS production drives the
 293 increased IFN- γ production by *lpr* CD44^{hi} T cells.

294 *lpr* CD4⁺ T cells showed increased proportions of CD44^{hi}/CD62L^{lo} effector/memory
 295 cells compared with the WT (~30 vs. 50%), which were further increased after IL-
 296 12/IL-18 treatment (~58 vs. 73%, Figure 6E). Although DPI did not affect CD62L^{hi} and
 297 CD62L^{lo} proportions in WT cells, it significantly reduced the population of *lpr*
 298 CD44^{hi}/CD62L^{lo} effector/memory cells, to the levels comparable to those in the WT cell
 299 population (Figure 6E). Altogether, our results corroborate that the increased mROS
 300 production drives not only the hyperactivation and increased potential for IFN- γ
 301 production in Fas-deficient CD4⁺ T cells, but also their intensified differentiation
 302 towards the effector/memory CD44^{hi}/CD62L^{lo} phenotype. This points to Fas as a
 303 regulator of mitochondrial ROS that ultimately controls the status of memory-like T
 304 cells independently of the proapoptotic role of Fas in such cells.

305 Discussion

306 It is established that TCR engagement induces mitochondrial activation and mROS
 307 production that modulates the redox state of signaling molecules governing the early
 308 events downstream of TCR induction. Here, we analyzed the role of mROS in recall
 309 responses of memory-like T cells. mROS induction was similar in naïve and memory-
 310 like cells after TCR-dependent signaling, while in response to IL-12 plus IL-18
 311 memory-like CD4⁺ T cells showed a great and highly significant increase in mROS and
 312 IFN-γ production compared to naïve T cells. This suggested that memory-like CD4⁺ T
 313 cells stimulated by IL-12 plus IL-18 acquired a singular capacity in producing mROS,
 314 and our study was geared at examining the effect of such TCR-independent triggering.
 315 We report the following major findings: 1) Inhibition of the increased mROS
 316 production by memory-like T cells after exposure to IL-12 plus IL-18 was directly
 317 associated to IFN-γ production and to maintaining the memory-like phenotype of
 318 stimulated cells; 2) The mechanism that drives IFN-γ production is regulated by the
 319 effect of mROS on the activation of the pathways that depend on IL-12 plus IL-18
 320 signaling; 3) Our results uncovered a previously unknown Fas role as a regulator of
 321 mROS induction, since *lpr* memory-like CD4⁺ T cells produced higher mROS levels
 322 compared to WT cells, leading to elevated IFN-γ and increased differentiation to
 323 effector/ memory T cells.

324 TCR stimulation of CD4⁺ cells triggers the early production of mROS, which act as
 325 secondary messengers in regulating IL-2 production and cell proliferation (6, 38).
 326 Additionally, at later stages of CD4⁺ T cells stimulation, mitochondrial activity and
 327 mROS production are increased (39). Mitochondria activation is associated with
 328 enhanced recall responses of memory CD8⁺ T cells after TCR-dependent restimulation,

329 but a possible mROS association with memory T cell responses was not assessed (40).
330 Nevertheless, cytosolic ROS was found increased in *in vitro* memory-like CD4⁺ T cells
331 after TCR dependent stimulation (5). In our study we found similar mROS levels in
332 naïve and memory-like CD4⁺ T cells early after TCR activation. We used IL-12 plus IL-
333 18 stimulation to investigate the role of mROS in memory-like CD4⁺ T cells, a protocol
334 that did not induce T cell apoptosis or proliferation, but greatly increased mROS in
335 memory-like compared to naïve T cells after activation. Overall, our findings suggest
336 that mROS production in T cells depends on their activation state and the stimulus type.

337 mROS levels were directly linked to the hyperactivation status of T cells in response to
338 IL-12 plus IL-18 induction, as inhibition of mROS production by DPI or Rotenone
339 reflected a greatly lowered IFN- γ expression and the diminished expression of the CD44
340 activation/memory marker. Apart from mROS, other aspects of mitochondrial activity
341 are needed for correct T cell function and TCR-induced activation. As part of the
342 immunological synapse, mitochondria participate in Ca²⁺ uptake and thus determine
343 amplitude and duration of Ca²⁺ signal. Mitochondrial uncoupling results in inhibition of
344 the TCR-induced Ca²⁺ signal due to diminished mitochondrial membrane potential and
345 reduced ability to prolong Ca influx (41). In our approach the mitochondrial membrane
346 potential remained unaffected by mROS inhibition, indicating that mROS is the
347 promotor of memory-T cell hyperactivation.

348 IFN- γ production is induced through synergy of signaling driven by IL-12R and IL-18R
349 stimulation pathways (30). mROS inhibition reduced IL-12R-dependent STAT4
350 phosphorylation and IL-18-dependent NF- κ B activation, indicating that mROS drive
351 signaling of both pathways. Kinetic analysis showed that mROS affects these pathways
352 early on after exposure of cells to IL-12 plus IL-18. Other molecules such as PKC- θ or

AKT linked to these pathways showed increased phosphorylation due to increased mROS. The redox conditions that are associated to increased mROS production, could be responsible for alteration in phosphorylation patterns, as kinase and phosphatase activities are redox-dependent. Whether the increased mROS activate specific pathway components or have a multiple effect in the phosphorylation and activation remains to be explored. Another important issue is to understand the reasons behind the specificity of mROS production after secondary IL-12 plus IL-18 stimulation. We hypothesize that components of the IL-12R or the IL-18R signaling pathways may drive ETC respiration. Indeed, PKC- θ (28) is shown to interact with and activate mitochondria, and Tyk2 that forms part of the Jak family and a component of the IL-12R signaling regulates mitochondrial activation (42).

Compared to normal T cells, *lpr* memory-like CD4⁺ overproduced mROS and IFN- γ after IL-12 plus IL-18 activation. Inhibition of mROS induction reduced not only the elevated IFN- γ but also the CD44^{hi}/CD62L^{lo} effector/memory cells that are responsible for the *in vivo* IFN- γ and lupus-like symptoms of *lpr* mice (13). Thus, our data suggest that Fas through non-apoptotic signaling (IL-12/IL-18) limits the production of mROS, compromising differentiation into effector/memory T cells and IFN- γ overproduction. This might provide a feedback mechanism to control T cell homeostasis under chronic inflammation and prevent development of autoimmunity. Therefore, uncontrolled mROS production by T cells in *lpr* mice or humans with ALPS (autoimmune lymphoproliferative syndrome) (43) might favor lymphadenopathy development, as mROS inhibition lowers Fas-dependent AICD (28). Other non-apoptotic Fas roles have been described, especially after primary T cell stimulation (44). Further studies are clearly needed for the better understanding of the mechanism that would explain the effect of Fas on mROS regulation. Overall, the results unveil a previously unknown apoptosis-

378 independent role of Fas in regulating mROS production and associated
379 hyperinflammatory outcomes. Our findings identify mROS as a driver of inflammation
380 in *lpr* disease and potentially other autoimmune disorders. These observations strongly
381 suggest that blockade of mROS provides a therapeutic strategy to decrease
382 inflammation in T cell-mediated inflammatory disorders.

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387 analysis and interpretation of the data and writing of the manuscript.

388 **Conflict of interest**

389 The authors declare no competing financial interest in relation to the work described.

390 **Availability of data and materials**

391 All data generated and analyzed during this study are included in this article.

392 **Authors' contributions**

393 DB and GR conceived and designed the study, analyzed the data and wrote the
394 manuscript. CMA and MAM provided input in designing the project. GR, PTZ, SCP
395 and RS performed the experiments. All authors read, discussed and approved the
396 manuscript.

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546

547 **Abbreviations used in this article:**

- 548 AICD: activation-induced cell death
- 549 ALPS: autoimmune lympho-proliferative syndrome
- 550 ConA: concavalin A
- 551 DPI: diphenyleneiodonium
- 552 ETC: electron transport chain
- 553 FCCP: carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
- 554 IRAK: interleukin 1 receptor-associated kinase
- 555 MFI: median fluorescence intensity
- 556 mROS: mitochondrial reactive oxygen species
- 557 Nox: NADPH oxidase
- 558 TMRM: tetramethylrhodamine, methyl ester, perchlorate
- 559 TRAF6: TNF receptor-associated factor 6
- 560 WT: wild-type

561 **Figure legends**

562 **Figure 1. IFN- γ production and mROS levels in memory vs. naïve CD4⁺ T cells.**

563 Naïve CD4⁺ T cells were isolated from WT mouse spleens and stimulated with ConA or
564 IL-12 and IL-18. To generate memory T cells, ConA-stimulated CD4⁺ cells were
565 expanded in IL-2 for 6 days and re-stimulated with either ConA or IL-12 and IL-18. **(A)**
566 Flow cytometry analysis of the intracellular staining showing the percentage of IFN- γ -
567 producing CD4⁺ naïve and memory T cells after 24 h stimulation with ConA or IL-12
568 and IL-18. **(B)** Flow cytometry analysis of mitoSOX red fluorescence showing the
569 percentage of mitoSOX^{hi} cells in naïve and memory cells at 1 hour after ConA or IL-12
570 and IL-18 stimulation. The graphs show mean $\pm$ SD ($n = 3$), * $p < 0.05$, ** $p < 0.01$,
571 *** $p < 0.001$, **** $p < 0.0001$, 2-way ANOVA (with Sidak's correction for multiple
572 comparison).

573 **Figure 2. DPI treatment lowers mROS levels and inhibits IFN- γ production**

574 **induced by IL-12 plus IL-18 in naïve CD4⁺ T cells.** Naïve CD4⁺ T cells from WT
575 mouse spleens were stimulated with IL-12 and IL-18 and analyzed by flow cytometry.
576 **(A)** MitoSOX red fluorescence staining showing mROS induction at early time points
577 after IL-12 plus IL-18 stimulation, and its reduction by DPI treatment. Gated on
578 mitoSOX^{hi} population. **(B)** Intracellular staining showing the frequency of IFN- γ -
579 producing CD44^{hi} cells after 24 h of IL-12 plus IL-18 stimulation, and its reduction by
580 DPI treatment. Gated on CD4⁺ cells. **(C)** Similar surface expression of CD44 activation
581 marker in control and DPI-treated cells after 24 h of IL-12 plus IL-18 stimulation.
582 Histograms and dot-plots are representative of at least 3 experiments performed. Graphs
583 show mean $\pm$ SD ($n=3$); * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$; two-way ANOVA with
584 Sidak's correction; # compared to 0 time point, * DPI treatment compared to control

585 (A); one-way ANOVA with post-hoc Tukey test (B) and (C). *n* represents values
586 obtained from different mice.

587 **Figure 3. DPI treatment lowers mROS levels and inhibits IFN- γ production in**
588 **differentiated memory-like CD4⁺ T cells.** CD4⁺ T cells from WT mouse spleens were
589 stimulated with ConA for 24 h, expanded in the presence of IL2 for 6 days, and
590 stimulated with IL-12 and IL-18. (A) mitoSOX red fluorescence showing increased
591 mROS production in the early time points of IL-12/IL-18 stimulation, and its reduction
592 after DPI treatment. (B) Intracellular staining showing reduced frequency of IFN- γ -
593 producing CD44^{hi} effector/memory T cells after DPI treatment; gated on CD4⁺ cells.
594 (C) Flow cytometry analysis showing decreased surface expression of CD44 after DPI
595 treatment. Dot plots and histograms are representative of at least 3 experiments
596 performed. Graphs show mean $\pm$ SD (*n*=3); **p*<0.05; ***p*<0.01; ****p*<0.001;
597 *****p*<0.0001, two-way ANOVA with Sidak's correction; # compared to 0 time point,
598 * DPI treatment compared to control (A); one-way ANOVA with post-hoc Tukey test
599 (B) and (C). *n* represents values obtained from different mice.

600 **Figure 4. The effect of different respiratory chain blockers on mROS generation,**
601 **IFN- γ production and mitochondrial membrane potential.** CD4⁺ T cells from WT
602 mouse spleens were stimulated with ConA for 24 h, expanded in the presence of IL2 for
603 6 days, and stimulated with IL-12 and IL-18. (A) mROS production in mitoSOX^{hi}
604 population was unaffected by antimycin A and reduced by rotenone treatment. (B) Flow
605 cytometry analysis showing the percentages of IFN- γ -producing and CD44^{hi} cells after
606 antimycin A and rotenone treatment; gated on CD4⁺ cells. (C) DPI treatment did not
607 affect mitochondrial membrane potential at early time points post IL-12 plus IL-18
608 treatment, as shown by TMRM fluorescence measured by flow cytometry. FCCP was

used to depolarize the membrane and show background staining (not shown). Graphs show mean $\pm$ SD ($n=3$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$, two-way ANOVA with Sidak's correction (A) and (C); one-way ANOVA with post-hoc Tukey test (B). n represents values obtained from different mice.

Figure 5. The effect of different inhibitors on signaling pathways triggered by IL-12 and IL-18. CD4⁺ T cells from WT mouse spleens were stimulated with ConA for 24 h, expanded in the presence of IL2 for 6 days, and stimulated with IL-12 and IL-18. Immunoblot analysis showing I κ B α and p-PKC- θ levels after DPI (A) and rotenone (B) treatment. (C) EMSA analysis showing reduced NF- κ B nuclear activity after DPI and rotenone treatment. (D) Immunoblot analysis of STAT4 and AKT phosphorylation after NAC, rotenone and DPI treatment. β -actin was used as loading control. Shown are representative gels of at least 2 experiments performed.

Figure 6. mROS drives increased IFN- γ production in *lpr* CD44^{hi}CD62L^{lo}CD4⁺ T cells. CD4⁺ T cells from WT and *lpr* mouse spleens were stimulated with ConA for 24 h, expanded in presence of IL-2 for 6 days and stimulated with IL-12 and IL-18. (A) Flow cytometry analysis showing increased mROS production in mitoSOX^{hi} *lpr* cells compared with WT at 60 min after IL-12 plus IL-18 stimulation. (B) The effect of DPI treatment on mROS production by WT and *lpr* cells at 60 min after IL-12 plus IL-18 stimulation. (C) Flow cytometry analysis showing unaffected CD44 surface expression in WT and *lpr* cells after IL-12 plus IL-18 treatment. (D) Intracellular staining showing increased frequency of IFN- γ -producing CD44^{hi} *lpr* cells compared with WT and their reduction after DPI treatment. (E) Flow cytometry analysis of CD62L and CD44 surface expression showing the increased proportions of *lpr* CD44^{hi}CD62L^{lo} cells compared with WT and their reduction after DPI treatment. Shown are representative

633 dot plots of 3 experiments performed. Graphs show mean $\pm$ SD ($n=3$); $*p<0.05$;
634 $**p<0.01$; $***p<0.001$; $****p<0.0001$, two-way ANOVA with Sidak's correction. n
635 represents values obtained from different mice.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6