1 # TNF may negatively regulate phagocytosis of Devil Facial Tumor disease
2 cells by activated macrophages

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23 **Abstract**

24 Macrophage phagocytosis of pathogens and tumour cells is an important early event in
25 protection against infectious disease and cancer. As tumour necrosis factor α (TNF) is an
26 important cytokine in macrophage activation, we investigated the involvement of TNF in
27 macrophage phagocytosis of tumour cells. We used Devil Facial Tumour Disease (DFTD)
28 cancer cells as the target tumour cells. The Tasmanian devil *(Sarcophilus harrisii*) population
29 is threatened by the transmissible DFTD. Using DFTD cells provided the opportunity to
30 determine if these cells can be phagocytosed and investigate requirement for TNF. As effector
31 cells, bone marrow derived macrophages (BMDMs), generated from C57BL/6 wild type
32 (B6.WT) and C57BL/6 TNF$^{-/-}$ (B6.TNF$^{-/-}$) mice were used. Phagocytosis of DFTD cells was
33 investigated by confocal microscopy and flow cytometry. DFTD cells were consistently
34 phagocytosed by B6.WT and B6.TNF$^{-/-}$ BMDMs with similar efficiency *in vitro*. Consequently
35 the DFTD cells are not resistant to phagocytosis. Following activation by exposure to IFNγ and
36 LPS or LPS alone, B6.TNF$^{-/-}$ BMDMs had higher phagocytic efficiency and lower nitric oxide
37 (NO) production compared to wild-type controls. In addition, NO seems to be unlikely to be
38 the involved in phagocytosis efficiency in IFNγ and LPS activated B6.TNF$^{-/-}$ macrophages and
39 consequences thereof. Our results indicate that TNF is not required for IFNγ and LPS or LPS
40 alone activation of macrophage phagocytosis. TNF may negatively regulate macrophage
41 phagocytosis of tumour cells.

42

43 **Introduction**

44 Tumour necrosis factor α (TNF) is a member of the tumour necrosis factor family with two
45 cognate receptors, TNF receptor 1 (TNFR1) and TNFR2 (Sedgwick et al., 2000). Under steady-
46 state conditions TNF is synthesised at low levels predominantely by T cells and macrophages,
47 but following challenge macrophages increase the production of TNF dramatically (Mannel et
48 al., 1980).
49 Macrophages are a heterogeneous population of innate immune cells with important roles in
50 tissue regeneration and immune defence (Hu et al., 2017). One of their evolutionary oldest
51 effector functions is phagocytosis, the engulfment of pathogens or cells debris, or more general,
52 particles followed by an uptake of the engulfed material in specialised vesicles termed phago-
53 lysosomes (Aderem and Underhill, 1999). This mechanism was originally described over 100
54 years ago (Tauber, 2003) and involves receptor recognition, cytoskeleton rearrangement and
55 phagosome maturation (Taylor et al., 2005). During the process of phagocytosis pathogens are
56 endocytosed and become engulfed as membrane coated phagosomes. The phagosomes then
57 fuse with lysosome to form an acidic environment to degrade the pathogens (Underhill and
58 Ozinsky, 2002). Actin polymerization is required for pathogen uptake and reactive oxygen and
59 nitrogen species contribute to the degradation of the pathogen (Aderem and Underhill, 1999).
60 In response to environmental stimuli, macrophages differentiate into classically activated,
61 proinflammatory (M1) macrophages or alternatively activated, anti-inflammatory (M2)
62 macrophages. M1 macrophages require exposure to the pro-inflammatory cytokines interferon
63 gamma (IFNγ) either alone or in combination with TNF or a Toll like receptor ligand such as
64 LPS. M2 macrophages require exposure to IL-4 and/or IL-13 (Murray et al., 2014). The
65 differentiation to either M1 or M2 macrophages is not only driven by cytokines but also
66 negatively regulated by TNF. The presence of TNF is necessary for macrophages to
67 differentiate to the M1 type. In absence of TNF a significant proportion of macrophages co-

68 expresses the M2-marker Arginase-1 (Arg1) interfering with the production of NO (Kratochvill
69 et al., 2015; Schleicher et al., 2016).
70 Interestingly, and in agreement with their different functions, M1 and M2 macrophages display
71 different levels of phagocytosis (Gordon and Martinez, 2010; Varin et al., 2010). IFNγ
72 activation of M1 macrophages enhances phagocytosis of apoptotic cells (Fernandez-
73 Boyanapalli et al., 2010). In contrast, IL-4 induced M2 macrophages exhibit impaired bacterial
74 phagocytosis due to reduced phagosome formation (Varin et al., 2010). Specifically, TNF
75 inhibits macrophage phagocytosis of apoptotic cells in a time and dose dependent manner
76 (Borges et al., 2009). TNF deficient macrophages have a reduced ability to endocytose
77 nontypeable *Haemophilus influenza* (Leichtle et al., 2010). However, TNF appears to enhance
78 IFNγ primed macrophage phagocytosis of apoptotic cells in a NO dependent manner
79 (Fernandez-Boyanapalli et al., 2010). Therefore, the effects of TNF in macrophage
80 phagocytosis under different activation states of macrophages are unclear. Consequently, we
81 investigated role of TNF in macrophage phagocytosis of tumour cells under different activation
82 conditions.
83 The tumour cells used in this study were Devil Facial Tumour Disease (DFTD) cells. The first
84 case of DFTD was recorded in 1996 and is primarily responsible for the catastrophic decline
85 of the Tasmanian devil (*Sarcophilus harrisii*) population (Jones et al., 2008). In 2016 a second
86 and independent DFTD was discovered and has been called DFT2 to distinguish it from the
87 first DFTD, now identified as DFT1 (Pye et al., 2016b). DFTD is a transmissible cancer
88 transmitted as an allograft by biting and is characterized by tumours located on face and neck
89 of diseased devils (Jones et al., 2008). Functional and histological analyses performed to date
90 indicate that the devil has a competent adaptive immune system (Kreiss et al., 2009). Since it
91 is pracytically and ethically problematic to harvest tissues from *S. harrisii* many aspects of the
92 innate immune system have not yet been explored and mouse models need to be established.

93 For example, it is unknown if DFTD cells can be phagocytosed by mouse macrophages and if
94 these cells after considering limitations can be used as model.
95 Therefore, we firstly determined if DFTD cells could be phagocytosed then examined the
96 activation status of macrophages and the effect of TNF on their ability to phagocytose DFTD
97 cells. We used bone marrow derived macrophages (BMDMs) isolated from wild type and
98 B6.TNF$^{-/-}$ mice and found that TNF inhibits macrophage phagocytosis efficiency following
99 activation by IFNγ/LPS and LPS.
100

101 **Materials and Methods**

102 **Animals**

103 The gene-targeted C57BL/6 (B6.TNF$^{-/-}$) mouse strains deficient for TNF were generated on a
104 genetically pure C57BL/6 (B6.WT) background as described (Körner et al., 1997). The
105 screening procedure followed the protocols described previously (Körner et al., 1997). All
106 animals were housed in pathogen-free conditions. Mice aged 8-16 weeks were used in all
107 experiments. The experiments were approved by the Animal Ethics Committee of University
108 of Tasmania (UTAS) under the ethics number A13934.

109 **Cell culture**

110 BMDM from B6.WT and B6.TNF$^{-/-}$ mice were generated from pelvic and femoral bones as
111 described (Schleicher and Bogdan, 2009). BMDM were cultured in RPMI 1640 medium
112 (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA),
113 10% L929-conditioned medium (containing M-CSF), 5% horse serum (Gibco, USA), 1%
114 penicillin and streptomycin (Gibco, USA), 1% nonessential amino acids (Gibco, USA) and 2-
115 mercaptotoethanol (Gibco, USA). After culture for 7 days, BMDM were untreated or treated
116 with the following substances: 20 ng/ml IFNγ (Peprotech, USA), 100 ng/ml LPS (Sigma, USA)
117 or a combination of IFNγ and LPS for 24 hours or 5 ng/ml IL-4 (Peprotech, USA) for 48 hours.
118 The purity of macrophages obtained was always at least 90% by using CD11b (PE-cy7,
119 eBioscience) and F4/80 (PerCP Cy5.5, Biolegend) in flow cytometry.
120 The Devil Facial Tumour Disease cell line, C5065, was established from primary tumour
121 samples (Pearse et al., 2012). Cells were grown in RPMI-1640 medium (Gibco, USA)
122 supplemented with 10% FBS (Bovogen Biological, Australia) and 5mM L-glutamine (Sigma,
123 USA). DFTD cells were maintained in a humidified 5% CO_2 incubator at 35 °C. Phagocytosis
124 assays required co-culturing of mouse macrophages with DFTD cells were maintained at 37 °C.

125

126 **Flow cytometry phagocytosis assay and calculations**

127 BMDM (effector cells) were labelled with CellTrace™ Violet (CTV, Invitrogen, USA), DFTD
128 cells (target cells) were labelled with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
129 (CFSE, Invitrogen, USA) according to manufacturer's instruction. BMDMs were resuspended
130 in cell culture medium at 10^6/ml. Serial dilution of 100 µl BMDMs were performed to obtain
131 effector to target ratios of 10:1, 5:1 with 10^4 target cells. The phagocytosis assays were
132 performed in duplicate or triplicate and incubated for 4 hours and 24 hours at 37 °C and for 4
133 hours at 4 °C for the negative control. The plates were analysed on a BD Canto II flow
134 cytometer and data calculations were performed with Flowjo V10.1 software (Tree star, USA),
135 and Graphpad Prism 5 for graphic display. To calculate percentage of DFTD cells
136 phagocytosed, three gates were identified. R1, total $CFSE^+$ tumour cells = total tumour cells.
137 R2, $CFSE^+/CTV^+$ dual labelled cells = tumour cells phagocytized by macrophages. R3,
138 $CFSE^+/CTV^-$ cells = free tumour cells. The formula used for calculating phagocytosis was as
139 follows: percentage of phagocytosis= R2/R1 x 100. For the 24 hours incubation (to avoid the
140 complication of cell division of DFTD cells), the absolute numbers of DFTD cells were
141 calculated by including all events (i.e. cells) or enumerating with a known number of cell count
142 beads (eBioscience, USA) added to enable absolute numbers to be determined by ratio. For the
143 bead count method, 5,000 beads were added to wells before the analysis by flow cytometry.
144 The number of cells and beads were recorded and the number of DFTD cells not phagocytosed
145 were calculated according to the ratio of analysed beads to 5,000. The number of DFTD cells
146 remaining = (5000/number of beads collected) x R3 cells. Spontaneous DFTD cell death during
147 short term assays was negligible.

148

149 **Cell staining and confocal microscopy for evidence of phagocytosis**

150 CTV labelled BMDMs were grown in 24 well plates on 0.2% gelatine (BD Bioscience, USA)
151 coated 12 mm coverslips. CFSE labelled DFTD cells were added when the coverslip was
152 almost confluent with BMDMs. After 4 and 24 hours incubation, cells were gently washed
153 with PBS. Cells were permeabilized with 0.25% Triton X-100 (Sigma, USA) for 20 min and
154 blocked with a 1% BSA (Sigma, USA) in PBS solution for 20 min. Antibody staining was
155 conducted for 30 min with a rabbit anti-mouse-LAMP1 antibody (BD Bioscience, USA). The
156 goat anti rabbit Alexa Fluor 594 (BD Pharmingen, USA) secondary antibody was applied for
157 30 min. The cells were washed three times in PBS and mounted using Dako mounting medium
158 (DakoCytomation, Australia). Confocal microscopy was performed using spinning disk and z-
159 stacks were taken in slices of 1 μm.

160 **Griess assay**

161 The content of NO in the cell culture media was determined by Griess assay as described
162 previously (Wilhelm et al., 2001). 50 μl cell culture medium was mixed with equal volumes of
163 Griess reagents (1% sulphanilamide in 2.5% H_3PO_4, Sigma, USA), then 50 μl Griess reagent
164 (0.1% napthylenediamide dihydrochloride, Sigma, USA) was added and incubated at room
165 temperature for 10 min. The absorbance at 540 nm was measured by a microplate reader (Bio-
166 Rad, USA) and compared to a standard nitrite curve using sodium nitrite (Sigma, USA).

167 **Statistical analysis**

168 Flow cytometry was conducted at least three times in duplicate or triplicate wells. Statistical
169 significance was determined by Student's unpaired t-test. P values of less than 0.05 were
170 considered statistically significant.

171

172

173 **Results**

174 **DFTD cells can be phagocytosed by macrophages from B6.WT and B6.TNF$^{-/-}$mice.**

175 To determine if DFTD cells could be phagocytosed, BMDMs from B6.WT and B6.TNF$^{-/-}$ mice
176 (CTV labelled; blue) were co-incubated with DFTD C5065 cells (CFSE-labelled; green). After
177 4 hours and 24 hours culture, phagocytosis was analysed by confocal microscopy (Figure 1).
178 Green DFTD cells could be identified within the blue macrophages from both B6.WT and
179 B6.TNF$^{-/-}$ mice. When *z*-stacks were analysed the green fragments were identified inside the
180 macrophages, suggesting the tumour cells had been engulfed (Fig 1). The degradation of
181 phagocytosed cellular material involves the process of phagosome maturation through
182 homotypic and heterotypic fusion of early endosomes, late endosomes and lysosomes (Zhou
183 and Yu, 2008). The presence of lysosomal-associated membrane protein 1 (LAMP1) was used
184 to discriminate a late phase of phagocytosis, after phagosomes/lysosome fusion (Tjelle et al.,
185 2000). As BMDMs from B6.WT and B6.TNF$^{-/-}$ mice can phagocytose DFTD cells, it suggests
186 that TNF is not essential for phagocytosis and that DFTD cells are not resistant to phagocytosis.

187

188 **TNF deficiency results in enhanced phagocytic capacity in IFNγ/LPS treated**
189 **macrophages.**

190 Confocal microscopy showed that DFTD cells are phagocytosed by BMDMs from B6.WT and
191 B6.TNF$^{-/-}$ mice, but flow cytometry was used to determine phagocytic efficiency. BMDMs
192 were labelled with CTV and DFTD cells were labelled with CFSE, then incubated at ratios of
193 E:T (10:1 and 5:1) at 37 ºC or 4 ºC for 4 hours. The phagocytosed DFTD cells were recognized
194 as CFSE and CTV double positive cells. In the absence of stimulation (control) the phagocytic
195 efficiencies of BMDMs from B6.WT and B6.TNF$^{-/-}$ mice were similar at both 10:1 (Fig 2A,

196 Suppl. Figure 1) and 5:1 (Fig 2B, Suppl. Figure 1) ratios. It suggests that TNF is not required
197 for macrophage phagocytosis of DFTD cells under steady-state conditions.

198 In order to determine TNF involvement in phagocytosis following macrophage activation,
199 BMDMs were stimulated with IFNγ and LPS, IFNγ alone, LPS alone or IL-4 alone. Following
200 IFNγ/LPS activation, macrophages from B6.TNF$^{-/-}$ mice displayed enhanced phagocytosis
201 efficiency at both 10:1 (Fig 2A, Suppl. Figure 1) and 5:1 (Fig 2B, Suppl. Figure 1) ratios,
202 compared with macrophages from B6.WT mice. However, BMDM macrophages from
203 B6.TNF$^{-/-}$ mice stimulated with IFNγ or IL-4 did not show a significant difference in phagocytic
204 efficiency, compared with BMDM from B6.WT mice (Fig 2A, 2B, Suppl. Figure 1). Compared
205 to untreated BMDMs, LPS treatment enhanced the phagocytic efficiency of BMDMs from
206 wild-type and B6.TNF$^{-/-}$ mice (Fig 2A, 2B, Suppl. Figure 1). But no significant difference was
207 shown between BMDMs from wild-type and B6.TNF$^{-/-}$ mice groups following LPS activation.

208 To confirm that the CFSE and CTV double positive cells represented phagocytosis rather than
209 cell binding, DFTD cells and macrophages were co-incubated at 4 ℃ for 4 hours (Fig 2C, 2D,
210 Suppl. Figure 1). The proportion of double positive cells was substantially reduced providing
211 support for phagocytosis at 37 ℃ rather than just binding to the cell surface.

212

213 **IFNγ/LPS, LPS activated BMDM from B6.TNF$^{-/-}$ mice phagocytose DFTD cells more**
214 **effectively than BMDM from B6.WT mice following 24 hours incubation.**

215 The phagocytosis and subsequent disappearance of DFTD cells was analysed with flow
216 cytometry after 24 hours incubation by enumerating the viable DFTD cells remaining. The
217 absolute number of free DFTD cells was calculated using cell count beads. IFNγ/LPS and LPS
218 alone activated BMDMs from B6.TNF$^{-/-}$ mice cultured with DFTD cells for 24 hours resulted
219 in fewer DFTD cells remaining compared to BMDMs from B6.WT mice at 10:1 and 5:1 ratios

220 (Fig 3A, 3B, Suppl. Figure 2). However, IFNγ or IL-4 activated BMDMs from B6.TNF$^{-/-}$ mice
221 had similar number of DFTD cells with wild-type controls at 10:1 and 5:1 ratios (Fig 3A, 3B,
222 Suppl. Figure 2). These results indicated that LPS and potentially IFNγ/LPS activated
223 macrophages from B6.TNF$^{-/-}$ mice phagocytosed more DFTD cells than macrophages from the
224 B6.WT mice. Thus, although phagocytosis by activated BMDMs was observed in the presence
225 of TNF, the absence of TNF appeared to increase the phagocytic activity.

226

227 **The production of NO is diminished from IFNγ/LPS or LPS activated B6.TNF$^{-/-}$**
228 **macrophages after incubation with DFTD cells for 24 hours.**

229 The effector molecule NO is an essential macrophage product and is cytotoxic to tumour cells
230 (MacMicking et al., 1997). As TNF regulates the production of NO in macrophages (Ding et
231 al., 1988), we determined whether NO is related to TNF regulation of macrophage
232 phagocytosis of DFTD cells. IFNγ/LPS, IFNγ or LPS treated macrophages from B6.WT,
233 B6.TNF$^{-/-}$ mice were co-incubated with DFTD cells for 24 hours and the NO contents in the
234 cell culture supernatant were measured. As shown in Fig 4, untreated macrophages from both
235 mouse genotypes did not produce any detectable NO. In response to IFNγ/LPS activation,
236 BMDMs from B6.TNF$^{-/-}$ mice produced less NO than wild type controls. This was evident at
237 10:1 (Fig 4A) and 5:1 (Fig 4B) ratios. Similarly, less NO was released from LPS activated
238 BMDMs from B6.TNF$^{-/-}$ mice than wild type controls (Fig 4A, 4B). The IFNγ treatment did
239 not alter NO production of B6.WT, B6.TNF$^{-/-}$ macrophages after incubation with DFTD cells
240 for 24 hours (Fig 4A, 4B). The production of NO might associate with the alteration of
241 phagocytosis in IFNγ/LPS and LPS alone treated B6.TNF$^{-/-}$ macrophages.

242

243 **TNF-associated alteration of macrophage phagocytosis during treatment with IFNγ/LPS**
244 **seems independent of NO production.**

245 To determine the association of NO with increased phagocytic activity in IFNγ/LPS treated
246 B6.TNF$^{-/-}$ macrophages. NO production was blocked by the inhibitor of NO synthesis, L-N6-
247 (1-Iminoethyl) lysine dihydrochlorideinducible (L-NIL). After the incubation with IFNγ/LPS
248 or IFNγ/LPS and L-NIL for 24 hours, BMDMs from B6.WT and B6.TNF$^{-/-}$ mice were co-
249 incubated with DFTD cells for 24 hours. The number of DFTD cells was measured by flow
250 cytometry and cell counting beads. As shown in Fig 5, L-NIL did not alter phagocytosis
251 following stimulation with IFNγ/LPS of macrophages from B6.WT or B6. TNF$^{-/-}$ mice. In
252 addition, compared to IFNγ/LPS treatment, L-NIL treatment did not alter the phagocytic ability
253 of macrophages from B6.WT or B6.TNF$^{-/-}$ mice (Fig 5). Thus, it suggests that NO was
254 unrelated to the altered phagocytic ability of IFNγ/LPS treated macrophages from B6.TNF$^{-/-}$
255 mice.

256

257 **Discussion**

258 Our study shows that DFTD cells are phagocytosed effectively by murine BMDMs and that
259 TNF modulates this ability. While we acknowledge the limitations imposed on far reaching
260 conclusions by the experimental combination of mouse macrophage and devil tumor cells the
261 main consequences of our findings are that an avoidance of phagocytosis by DFTD cells is
262 unlikely to contribute to immune escape and that TNF is not necessary for phagocytosis, but
263 negatively regulates phagocytosis by activated macrophages. Antibody-dependent
264 phagocytosis was not explored in this project because cross-species effects are unknown.

265 The absence of MHC-I expression on DFT1 cells prevents the activation of $CD8^+$ T cells and
266 thus interferes with the adaptive cellular immune response (Siddle et al., 2013). Nevertheless,
267 devils immunised with DFTD antigens (Kreiss et al., 2015; Tovar et al., 2017) and some
268 DFTD-inoculated devils (Pye et al., 2016a) can produce DFTD-specific antibodies which is in
269 line with antibody mediated opsonisation of DFT1 cells and subsequent phagocytosis of tumor
270 cells by macrophages. However, tumour cells can avoid phagocytosis by expressing inhibitory
271 molecules such as CD47 which is part of the immune checkpoint inhibitory pathway (Alvey
272 and Discher, 2017), and CD47 has been identified in transcriptome analysis from devil
273 mononuclear cells (Flies et al., 2017). Hence, there is the potential for DFT1 cells to express
274 this molecule. In the absence of monoclonal antibodies to identify inhibitory molecules and in
275 particular CD47, we conducted phagocytosis experiments of DFT1 cells by flow cytometry
276 and confocal microscopy using bone marrow-derived mouse macrophages in a proof of
277 principle approach. We could show that DFT1 cells can be phagocytosed supporting the notion
278 that antibody-opsonised DFT1 cells could also be sensitive to phagocytosis *in vivo* in the
279 Tasmanian devil after immunization (Kreiss et al., 2015; Pye et al., 2016a; Tovar et al., 2017).
280 After determining that mouse macrophages effectively phagocytose DFT1 tumour cells we
281 then investigated whether TNF was required. In the absence of stimulation, the presence of

282 TNF made no difference to the phagocytic efficiency. But following activation with IFNγ/LPS
283 or LPS (but not IFNγ), the presence of TNF reduced phagocytic activity. Although TNF is
284 regarded as a master regulator of inflammatory cytokine production (Parameswaran and Patial,
285 2010) there are reports that TNF can inhibit macrophage phagocytic activity. Exogenous TNF
286 has been shown to reduce the macrophage capacity to ingest apoptotic cells (McPhillips et al.,
287 2007). This inhibition appears specific for apoptotic cells as it is not seen with beads or
288 antibody-opsonized cells (McPhillips et al., 2007). The authors propose that within the
289 inflammatory environment, the reduced phagocytic ability may 'contribute to the local
290 intensity of the inflammatory response'. Macrophages stimulated with LPS produce TNF and
291 this would inhibit macrophage phagocytosis of apoptotic cells in an autocrine manner (Feng et
292 al., 2011). TNF is also produced by IFNγ/LPS activated macrophages (Cohen et al., 2015; Patel
293 et al., 2012). IFNγ on its own is unable to activate macrophages to produce TNF, but can do so
294 in the presence of LPS (Gifford and Lohmann-Matthes, 1987). This observation is
295 controversial as some studies have shown that IFNγ primed macrophages (Fernandez-
296 Boyanapalli et al., 2010; Vila-del Sol et al., 2008) can produce TNF. Furthermore, IFNγ
297 appears to restore an impaired ability of macrophages to phagocytose apoptotic cells in a nitric
298 oxide-dependent manner that requires TNF production (Fernandez-Boyanapalli et al., 2010;
299 Vila-del Sol et al., 2008). This might be due to the macrophages coming from different sources
300 and at different stages of maturation. A further caveat is the potential presence of traces of
301 contaminating TLR ligands. Already minute amounts can interfere with the activation of
302 macrophages. Finally, while TNF has no effect on phagocytosis by immature macrophages, it
303 can reduce phagocytosis by mature macrophages (McPhillips et al., 2007).

304 Phagocytosis of pathogens by macrophages requires NO and TNF dependent NO production
305 is required for the enhanced phagocytosis of apoptotic cells by IFNγ activated macrophages
306 (Fernandez-Boyanapalli et al., 2010). However, NO can impair phagocytosis of fluorescent

307 particles by affecting cytoskeletal assembly and pseudopod formation (Jun et al., 1996). TNF
308 is definitely involved in NO production as it induces iNOS expression via the NF- κB signaling
309 pathway (Ding et al., 1988; Patel et al., 2012). Furthermore, the expression of iNOS
310 downstream of IFNγ/LPS activation of macrophages requires TNF (Chan and Riches, 2001).
311 TNF dependent NO production is required for the enhanced phagocytosis of apoptotic cells by
312 IFNγ activated macrophages (Fernandez-Boyanapalli et al., 2010). We blocked production of
313 NO by using L-NIL, but inhibition of NO production did not alter phagocytosis efficiency in
314 IFNγ/LPS treated macrophages. It suggests that NO is independent of TNF presence in
315 IFNγ/LPS treated macrophages. Moreover, actin polymerization is an essential event in the
316 process of macrophage phagocytosis. It has been suggested that exogenous TNF decreases
317 actin reorganization in J774 macrophages (Peppelenbosch et al., 1999). The involvement of
318 actin and TNF inhibition of phagocytosis activity in IFNγ/LPS activated macrophages needs
319 to be further investigated. Finally, recent observations point to a new role for TNF in the
320 maintenance of a M1 phenotype in macrophages (Kratochvill et al., 2015; Schleicher et al.,
321 2016). Since we used TNF-negative macrophages in our experiments we cannot exclude the
322 possibility that a part of the cell population has differentiated to M2 macrophages with
323 consequences for their ability to produce pro-inflammatory mediators and phagocytose tumor
324 cells. This aspect has to be explored in further detailed studies.
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341

342

343 **Conflict of interest**

344 The authors state that they had no conflict of interest.

345

346 **Figures legends**

347 **Figure 1. Confocal microscope analysis of macrophage phagocytosing DFTD cells.** Co-
348 incubate CTV labelled BMDMs with CFSE labelled DFTD cells for 4 hours and 24 hours, cells
349 were fixed and permeabilized, then labelled with anti-LAMP1 antibody. Phagocytosis was
350 analysed in confocal microscopy. The z-stack (white arrow) demonstrated that tumour cells are
351 located inside B6.WT and B6.TNF$^{-/-}$ macrophages after 4 hours and 24 hours incubation (Green,
352 DFTD cells; Blue, macrophages; Red, LAMP1).

353

354 **Figure 2. IFNγ/LPS treated B6.TNF$^{-/-}$ BMDMs exhibited enhanced phagocytic efficiency**
355 **of DFTD cells compared to B6.WT BMDMs.** B6.WT and B6.TNF$^{-/-}$ BMDMs were treated
356 with IFNγ/LPS, IFNγ alone, LPS alone, or IL-4 alone. BMDMs were labelled with CTV and
357 DFTD cells were labelled with CFSE. After labelling, BMDMs were co-incubated with DFTD
358 cells for 4 hours. The percentage of phagocytosed DFTD cells in the incubation of 37 °C (A,
359 B) and 4 °C (C, D) was investigated by flow cytometry. The ratio of BMDMs: DFTD cells
360 indicates effector: target (E:T). Data were from a total of 3-7 mice within two or three
361 independent experiments (biological replicates), Δ $p < 0.05$ versus WT control, # $p < 0.05$
362 versus B6.TNF$^{-/-}$ control, * $p < 0.05$ versus WT versus B6.TNF$^{-/-}$, NS not significant.

363

364 **Figure 3: IFNγ/LPS, LPS activated B6.TNF$^{-/-}$ BMDMs are more effective at removing**
365 **DFTD cells than B6.WT BMDMs after 24 hours incubation.** BMDMs from B6.WT and
366 B6.TNF$^{-/-}$ mice were treated with IFNγ/LPS, IFNγ alone, LPS alone, or IL-4 alone. BMDMs
367 were labelled with CTV and DFTD cells were labelled with CFSE. After labelling, BMDMs
368 were incubated with DFTD cells for 24 hours, and the absolute number of viable DFTD cells
369 was calculated using flow cytometry with cell count beads. The ratio of BMDMs: DFTD cells
370 indicates effector: target (E:T). Data were from a total of 3-7 mice within two or three
371 independent experiments (biological replicates), * $p < 0.05$.

372

373 **Figure 4. Reduced NO production from IFNγ/LPS, LPS activated B6.TNF$^{-/-}$ macrophages**
374 **phagocytosis of DFTD cells.** B6.WT and B6.TNF$^{-/-}$ macrophages were stimulated with
375 IFNγ/LPS, IFNγ or LPS overnight. Macrophages were co-incubated with DFTD cells at 10:1(A)
376 and 5:1 (B) ratios, the concentration of NO in the cell culture supernatant was analysed in

377 Griess assay. The ratio of BMDMs: DFTD cells indicates effector: target (E:T). Data were from
378 3 mice (biological replicates), ΔΔ $p < 0.01$ versus WT control, ## $p < 0.01$ versus B6.TNF$^{-/-}$
379 control, * $p < 0.05$ versus WT versus B6.TNF$^{-/-}$.

380

381 **Figure 5. NO is unrelated to the TNF involved macrophage phagocytosis of DFTD cells.**
382 B6.WT and B6.TNF$^{-/-}$ BMDMs were treated with IFNγ/LPS or IFNγ/LPS with L-NIL
383 overnight. After incubation with BMDMs and DFTD cells for 24 hours, the numbers of DFTD
384 cells at E:T 10:1 (A), 5:1 (B) ratios were analysed by flow cytometry and cell counting beads.
385 The ratio of BMDMs: DFTD cells indicates effector: target (E:T). The numbers of DFTD cells
386 were calculated as methods described. Data were from a total of 3-5 mice within two or three
387 independent experiments (biological replicates), * $p < 0.05$, NS not significant.

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