Ongoing exposure to peritoneal dialysis fluid alters resident peritoneal macrophage phenotype and activation propensity

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Running title: Tissue resident macrophages in PD

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Abstract:

Peritoneal dialysis (PD) is a more continuous alternative to haemodialysis, for patients with chronic kidney disease, with considerable initial benefits for survival, patient independence and healthcare cost. However, longterm PD is associated with significant pathology, negating the positive effects over haemodialysis. Importantly, peritonitis and activation of macrophages is closely associated with disease progression and treatment failure. However, recent advances in macrophage biology suggest opposite functions for macrophages of different cellular origins. While monocyte-derived macrophages promote disease progression in some models of fibrosis, tissue resident macrophages have rather been associated with protective roles. Thus, we aimed to identify the relative contribution of tissue resident macrophages to PD induced inflammation in mice. Unexpectedly, we found an incremental loss of homeostatic characteristics, anti-inflammatory and efferocytic functionality in peritoneal resident macrophages, accompanied by enhanced inflammatory responses to external stimuli. Thus, alterations in tissue resident macrophages may render longterm PD patients sensitive to developing peritonitis and consequently fibrosis/sclerosis.

Introduction:

An estimated 5-10 million people worldwide die every year due to chronic kidney disease (Luyckx, Tonelli, & Stanifer, 2018). In Europe, an average of 850 people per million

population (pmp) require renal replacement therapy (RRT) and 120 new patients pmp commence treatment annually (Kramer et al., 2019). The average 5-year survival rate of patients receiving RRT is only 50.5%. This can be improved to over 90% if patients receive a kidney transplant, but rates of transplantation remain low (32 pmp), primarily due to organ availability and the majority of patients rely on dialysis as a therapy to substitute excretory kidney function (Kramer et al., 2019).

Peritoneal dialysis (PD), utilizing the body's own peritoneal membrane as a filter during dialysis, is a cost-effective alternative to haemodialysis (HD). Although PD has been associated with better initial survival rates (van de Luijtgaarden et al., 2016), lower costs for the health system (Chui et al., 2013) and increased patient autonomy (François & Bargman, 2014) as compared to HD, the incidence rate of PD over several decades has dropped in Europe (van de Luijtgaarden et al., 2016). There are a variety of reasons for this reduction in PD uptake, but the significant risk of adverse effects and in some cases fatal outcomes, has limited the general adoption of PD in adult patients across Europe (Bartosova & Schmitt, 2019). Treatment failure is commonly associated with repeated episodes of peritonitis (i.e. inflammation) and a progressive thickening and vascularisation of the peritoneum, leading to impaired filtration and thus reduced efficacy of PD (Bartosova & Schmitt, 2019; Morelle et al., 2015). In rare cases, the fibrotic changes to the peritoneum become so extreme that they form a fibrous cocoon encapsulating the internal organs leading to persistent or recurring adhesive bowel obstruction (Johnson et al., 2010; Moinuddin, Summers, Van Dellen, Augustine, & Herrick, 2014; Morelle et al., 2015). The recognition of this Encapsulating Peritoneal Sclerosis (EPS) is an indication to discontinue PD with the mortality approaching 50% one year after diagnosis (Danford, Lin, Smith, & Wolf, 2018). Management of EPS includes surgery but there is a relatively high frequency of symptom recurrence (Danford et al., 2018). In contrast, immunosuppressive treatments and the use of anti-fibrotic agents, like tamoxifen, have shown noticeable benefits to patient survival (Danford et al., 2018). Therefore, aberrant activation of the immune system appears to be linked to both alteration of peritoneal structure and PD treatment failure, as well as the progression of the fibrotic sequelae. Indeed, experimental rodent models of the disease, have suggested inappropriate or excessive activation of macrophages as a major cause of the pathology (Q. Li et al., 2018; Liao et al., 2017; Minutti et al., 2017; Jie Wang et al., 2013).

Recent advances in macrophage biology have highlighted the intrinsic heterogeneity of macrophage populations (Gordon & Plüddemann, 2017). Grossly simplified, macrophages can be split into tissue resident macrophages (MΦres) which are present in tissues during homeostasis, and monocyte-derived macrophages (MΦmono), which are recruited to the tissue during inflammatory conditions (Yang Zhao, Zou, Du, & Zhao, 2018). Both macrophage populations can and do respond to external stimuli, like infection, but they possess distinct response profiles and adopt distinct functional properties (Gundra et al., 2014; Ruckerl et al., 2017).

In health, peritoneal MΦres are essential for maintaining tissue homeostasis by silently removing apoptotic cells through efferocytosis (Allen & Ruckerl, 2017; Uderhardt et al., 2012) and by providing a source of tissue-reparative cells that infiltrate surrounding tissues (e.g. the liver) during injury (Jing Wang & Kubes, 2016). Importantly, upon encountering inflammatory signals, MΦres undergo the Macrophage Disappearance Reaction (MDR) (Barth, Hendrzak, Melnicoff, & Morahan, 1995). Following injection of inflammatory agents (e.g. bacterial antigens) or infection the number of peritoneal MΦres detectable in peritoneal lavage will drop significantly within a few hours (Accarias et al., 2016). The exact mechanism underlying MDR is still poorly understood, but it has been proposed that MΦres undergo activation-induced cell death (Ruckerl et al., 2017) or adhere to the mesothelial lining of the peritoneal cavity reducing their recovery (Jonjic et al., 1992; Jing Wang & Kubes, 2016; N. Zhang et al., 2019). Of note, the degree of detectable MΦres -loss directly correlates to the amount of inflammatory stimulus (e.g. cfu of bacteria) and the recruitment of inflammatory MΦmono (Ruckerl et al., 2017). Following resolution of the inflammation, MΦres can re-populate the peritoneal cavity and return to homeostatic numbers through proliferative expansion of remaining MΦres (Davies et al., 2013).

In models of fibrotic disorders of the lung or kidney, influx of monocytes and monocyte-derived macrophages has been linked to disease progression and induction of pathology (Braga et al., 2018; Misharin et al., 2017; Puranik et al., 2018). Similarly, in rodent models of peritoneal fibrosis, preventing the influx of monocytes or depleting all macrophages limits the degree of peritoneal thickening and improves glomerular filtration (Y.-T. Chen, Hsu, Lin, Pan, Liu, Wu, Tsai, Liao, Cheng, Chiang, Chen, Chu, & Lin, 2019a; Lee et al., 2012; Jie Wang et al., 2013). Moreover, injection of MΦmono, activated ex vivo using

110 bacterial antigens (i.e. Lipopolysaccharide), often referred to as M1 macrophages,
111 exacerbates disease progression (Q. Li et al., 2018). Together these data highlight the role
112 of inflammation and infiltration of MΦmono in the progression of peritoneal fibrosis and
113 seem to provide a cohesive picture explaining the enhanced risk of PD failure associated
114 with repeated episodes of peritonitis (Lambie et al., 2016). In this context, it is interesting
115 to note that continuing peritoneal irrigation and thus continual removal of peritoneal cells,
116 including any inflammatory infiltrate, in patients discontinuing PD, has been suggested to
117 prevent subsequent EPS formation (Moriishi et al., 2002).
118 Other studies have found a role for anti-inflammatory, IL-4 activated macrophages (also
119 called M2), characterised by the expression of CD206, Arg1 and Ym1, in promoting
120 peritoneal dialysis fluid (PD fluid) induced fibrosis (Bellón et al., 2011; Chandrasekaran et al.,
121 2019; Jie Wang et al., 2013). Additionally, chronic fibrotic kidney disease is linked to a
122 switch from inflammatory M1 to predominantly M2 activated macrophages (Han, Ma,
123 Tesch, Manthey, & Nikolic-Paterson, 2013). Indeed, both types of macrophage activation
124 seem to have the capacity to promote kidney fibrosis, possibly acting during different
125 phases of the pathology (Nikolic-Paterson, Wang, & Lan, 2015). Importantly, some
126 markers used to define M2 are differentially expressed on MΦres and MΦmono. For
127 example CD206 is expressed constitutively by MΦmono but not MΦres (Gundra et al.,
128 2014). Thus, the described role of M2 macrophages may merely reflect an enhanced
129 influx of MΦmono rather than IL-4 mediated activation. In fact, transfer of exogenously
130 activated M2 macrophages showed no impact on disease progression in a model of
131 peritoneal fibrosis (Q. Li et al., 2018) and even reduced pathology in a model of renal
132 fibrosis (Y Wang et al., 2007). Moreover, using different strategies to deplete
133 macrophages in kidney fibrosis yielded opposing results with regard to disease
134 progression indicating that depletion of different subsets of macrophage may lead to
135 different outcomes (Ferenbach et al., 2012). Indeed, renal MΦres as compared to
136 MΦmono have been shown to be protective in a model of kidney pathology (Puranik et
137 al., 2018). This suggests that the cellular origin of macrophages may play a prominent
138 part in determining their role during fibrotic disorders and influence the overall disease
139 outcome.
140
141 Here we analysed the effects of repeated PD fluid injection on macrophage population
142 dynamics and responses to activating signals. Our data indicate a significant change in
143 MΦres phenotype over time during PD fluid administration. MΦres lost expression of anti-

144 inflammatory and efferocytic markers correlating with the duration of PD fluid treatment.
145 Moreover, MΦres from PD fluid treated animals showed enhanced inflammatory
146 responses to external stimulation. Importantly, the enhanced inflammatory phenotype of
147 MΦres persisted even when PD fluid administration was stopped. Thus, repeated
148 exposure to PD fluid may render patients more susceptible to peritonitis, and by
149 extension to peritoneal fibrosis, due to exaggerated inflammatory responses of MΦres.
150
151 **Results:**
152
153 **Dialysis fluid induces the disappearance of tissue resident macrophages**
154
155 To determine the impact of peritoneal dialysis fluid (PD fluid) injection on peritoneal
156 macrophage populations (see suppl. fig 1 for gating strategy) we first characterised the
157 cellular dynamics induced after a single application of PD fluid. Six hours after PD fluid
158 injection, tissue resident macrophages (MΦres) underwent a pronounced disappearance
159 reaction with the number of F4/80 high MΦres reduced to approximately 30% of the
160 levels found in naive control animals (Fig 1A). Simultaneously, a significant influx of
161 neutrophils and Ly6C high monocytes were detected, indicative of an inflammatory
162 response (Fig 1B&C). Of note, similar to previous reports (Accarias et al., 2016) tissue
163 dwelling, monocyte-derived MΦmono (F4/80 low MHC-II high) also underwent a
164 disappearance reaction and were reduced in numbers by approximately 40 % (Fig 1D).
165 F4/80 high MΦres displayed limited signs of activation at this early time point post PD
166 fluid injection. No increase in expression of MHC-II could be detected (Fig 1E). In contrast
167 intracellular Ym1 and CD206 expression were significantly enhanced following PD fluid
168 injection, but expression was restricted to less than 10 % of cells (Fig 1F&G). The
169 disappearance of MΦres in this context seemed in part due to enhanced cell death as
170 indicated by increased levels of Annexin V staining (Fig 1H). By 24 hours post PD fluid
171 injection, the numbers of MΦres had returned to baseline levels and expression of CD206
172 was no longer significantly different, whereas Ym1 expression remained elevated
173 compared to naïve controls (Fig S2).
174
175 Overall, these data are consistent with the induction of low grade inflammation caused by
176 PD fluid injection accompanied by a significant change in the prevalence of various
177 myeloid cell populations within the peritoneal cavity.

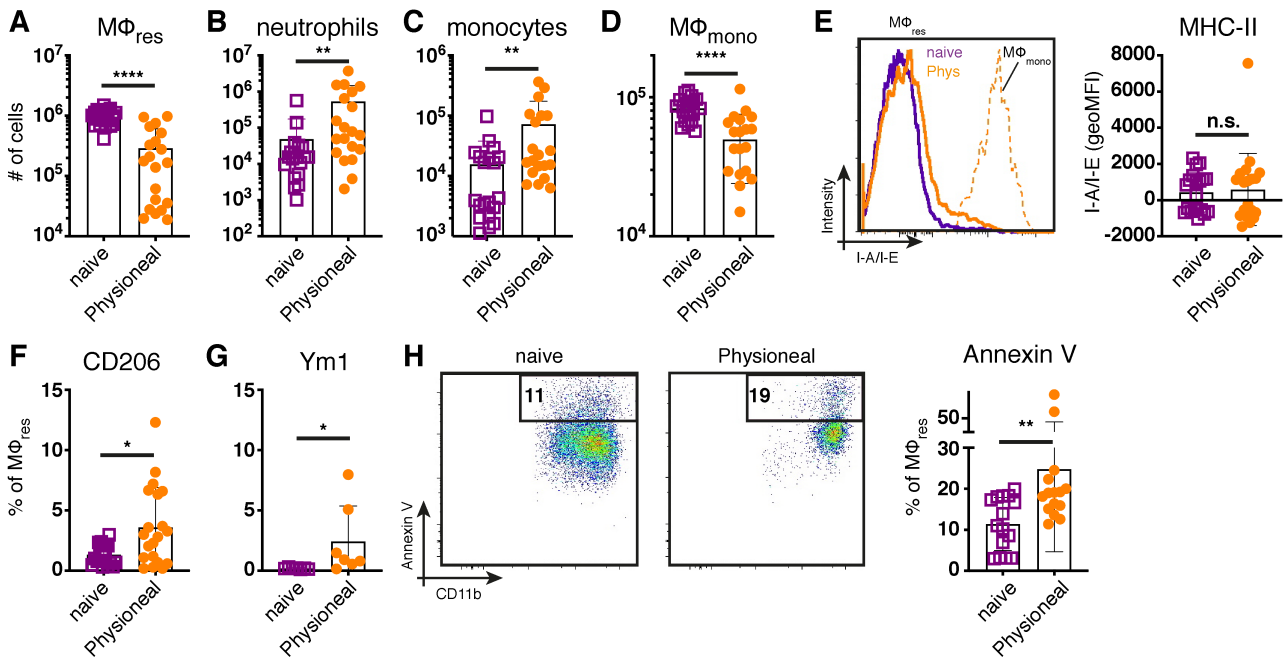

Figure 1: **Injection of dialysis fluid alters peritoneal myeloid cell composition**

C57BL/6 mice were injected with Physioneal (orange circles) or left untreated (purple squares) and whole PEC isolated 6 h later. Cells were counted and analysed by flow cytometry to determine the number of A) MΦres (lineage-CD11b+, F4/80high,MHC-II low), B) neutrophils (lineage +, SSC mid, MHC-II-,F4/80-,CD11b+), C) monocytes (lineage-CD11b+, F4/80-,MHC-II low, Ly6C high) and D) monocyte-derived macrophages (lineage-CD11b+, F4/80low,MHC-II high). E) Histogram and quantitative data of fluorescence mean intensity of MHC-II expression by MΦmono (orange, dashed line) or MΦres from Physioneal injected (orange, solid line) or naive animals (purple, solid line). F-H) Expression of CD206, Ym1 and binding of Annexin V by MΦres identified in A) assessed by flow cytometry. Datapoints depict individual animals and bars indicate mean and SD. Data pooled from 4 independent experiments using 3-5 animals per group. Data analysed using a Mann-Whitney-U test after transformation. n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ****: $p<0.0001$

lineage: TCRβ,CD19,Siglec-F, Ly6G

Repeated PD fluid treatment induces a gradual change in MΦres phenotype

We next sought to determine the long term effect of PD fluid administration on peritoneal macrophage populations. For this animals were injected once per day, 5 times a week with PD fluid i.p. and the peritoneal exudate cells (PEC) analysed 24 hours after 1, 4, 9 and 14 injections, respectively (Fig 2A). Over the course of the experiment the number of MΦres remained consistently lower in Physioneal treated animals as compared to naive mice (Fig 2B). Simultaneously, enhanced influx of Ly6C monocytes and successive accumulation of F4/80 low MΦmono could be detected in the peritoneal cavity following multiple rounds of PD fluid injection (Fig 2C& D).

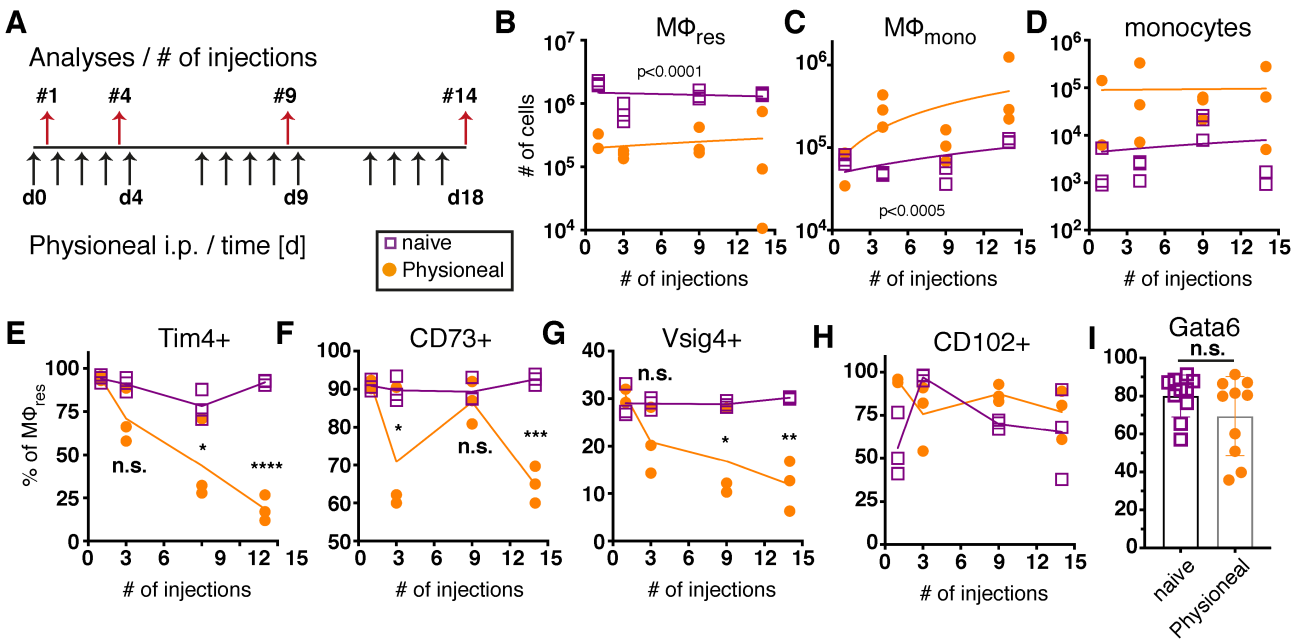

Figure 2: Repeated injection of PD fluid leads to a progressive change in MPres phenotype

C57BL/6 mice were injected with PD fluid (orange circle) or left untreated (purple square) 5 times a week for the indicated number of injections. At each timepoint whole PEC were isolated 24 h after the last injection and analysed by flow cytometry. A) Schematic depiction of experimental timeline. B-D) Number of MΦres, MΦmono and Ly6C high monocytes. E-H) Expression of MΦres associated cell-surface markers on F4/80 high macrophages. I) Intracellular expression of Gata6 in MΦres identified in A).

Datapoints depict individual animals and lines indicate (B-H) median or (I) mean with SD. Data from a single experiment using 3 animals per group per timepoint. Data analysed using 2-way ANOVA followed by Tukey's post-hoc test after transformation. n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ****: $p<0.0001$

Despite the overall reduced numbers of MΦres, repeated injection of PD fluid led to marked induction of Ki67 expression in MΦres, indicative of proliferative expansion (Fig. S3). This is in line with a previous report demonstrating repopulation of the peritoneal cavity by MΦres following an inflammatory insult through proliferation (Davies et al., 2011). Thus, it is likely that PD fluid injection leads to a repeated cycle of MΦres disappearance followed by re-population. Similarly, it is likely that the gradual influx of monocytes and MΦmono underlies cyclic fluctuations after each round of PD fluid injection. Thus, this data indicated that macrophage populations within the peritoneal cavity undergo dynamic but limited changes following PD fluid instillation, with a slight accumulation of MΦmono over time.

Closer investigation of the MΦres phenotype, however, revealed a progressive loss of characteristics that define the tissue resident phenotype. In particular markers associated with the efferocytic and anti-inflammatory function of MΦres were expressed at increasingly lower levels following repeated injection of PD fluid (Fig 2E - G). T cell

228 immunoglobulin and mucin domain containing 4 (Tim4), a molecule associated with the
229 efficient removal of apoptotic cells (Miyanishi, Segawa, & Nagata, 2012; Miyanishi et al.,
230 2007) as well as V-set and immunoglobulin domain–containing 4 (Vsig4), associated with
231 limiting inflammatory responses (J. Li et al., 2017; Yuan, Yang, Dong, Yamamura, & Fu,
232 2017)], are specifically expressed in MΦres during steady state conditions (Heng, Painter,
233 Immunological Genome Project Consortium, 2008). MΦres gradually lost expression of
234 these markers with increasing number of PD fluid injections (Fig 2 E & G). Similarly, CD73,
235 an anti-inflammatory effector molecule specifically expressed by MΦres (Haskó et al.,
236 2011; Rosas et al., 2014) was found to be significantly reduced after 14 injections of PD
237 fluid (Fig 2 F).
238 In contrast, expression of CD102 (Icam2) was not altered by PD fluid injection (Fig 2 H). A
239 similar phenotype (loss of MΦres marker expression with sustained CD102 expression)
240 has previously been described in mice lacking the transcription factor Gata6, indicating
241 that repeated PD fluid injection may exert its effects via affecting Gata6 expression
242 (Okabe & Medzhitov, 2014). However, no significant difference in Gata6 expression by
243 MΦres was found after 9 injections of PD fluid measured in subsequent experiments (Fig
244 2 I). Notably, Gata6 expression levels varied following PD fluid injection with diminished
245 expression detected in approximately 50 % of animals (Fig 2 I). Thus, any effect of PD
246 fluid injection on Gata6 expression may be transient or reflect the impact of other factors,
247 like the degree of inflammation.
248 Taken together, following repeated PD fluid injection, MΦres, while retaining their tissue
249 identity (F4/80 high CD102+ Gata6+), lose some of their functional characteristics and in
250 particular anti-inflammatory and efferocytosis associated functions.
251
252 **Repeated PBS injection but not chronic bacterial infection leads to a loss of MΦres**
253 **characteristics**
254
255 As shown in Figure 1 and previously reported (Q. Li et al., 2018), repeated injection of PD
256 fluid is associated with the induction of low grade inflammation as evidenced by influx of
257 neutrophils and Ly6C+ monocytes. Thus, to assess whether the loss of MΦres functional
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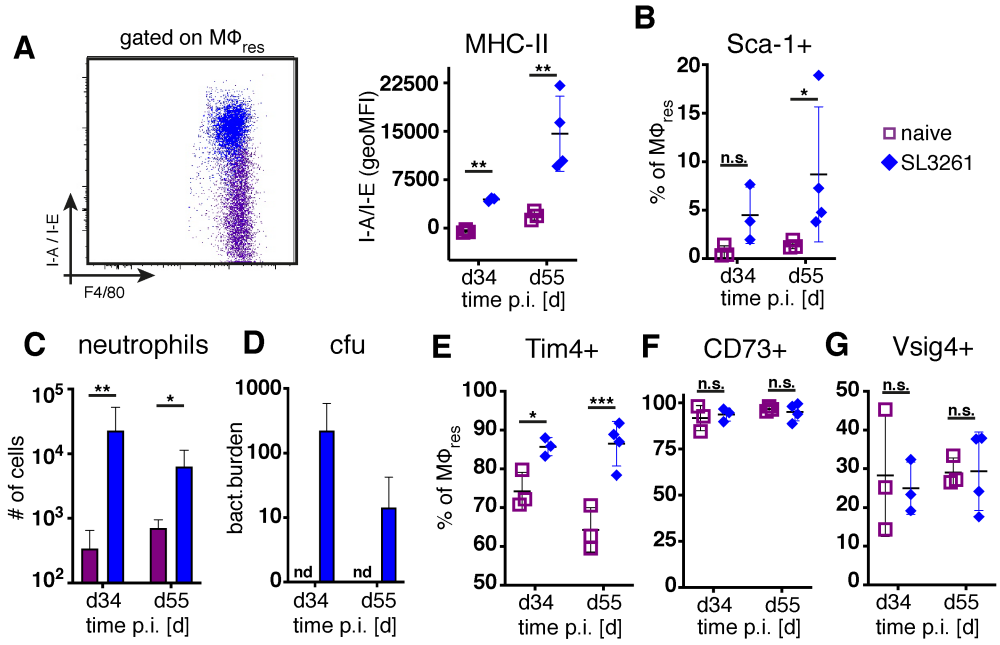


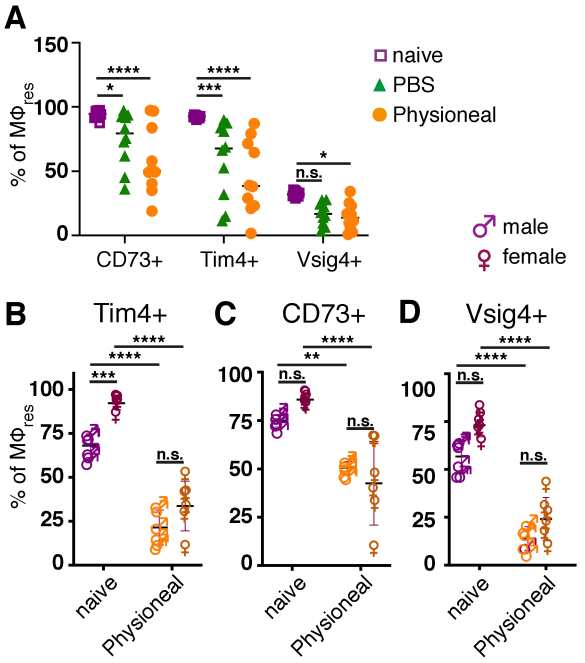
Figure 3: Chronic inflammation induced by S.Typhimurium infection does not phenocopy repeated PD fluid injection

C57BL/6 mice were infected orally with 1x10^8 cfu S.Typhimurium (orange squares) or left untreated (blue circles) and peritoneal cells analysed 34 (d34) and 55 days (d55) post infection. Dotplot (d55) and quantification of MHC-II (A) and Sca-1 (B) expression by MΦres assessed by flow cytometry C) Number of peritoneal neutrophils. D) Enumeration of bacterial colony forming units present in the peritoneal cavity. E-G) Expression of MΦres associated cell surface markers assessed by flow cytometry.
Datapoints depict individual animals and bars / lines indicate mean with SD. Data from a single experiment using 3-4 animals per group per timepoint. Data analysed using 2-way ANOVA followed by Tukey's post-hoc test after transformation. n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$

markers (i.e. Tim4, CD73, Vsig4) was due to the elicited inflammatory response, we analysed peritoneal exudate cells from animals subjected to prolonged bacterial infection. Animals were infected orally with attenuated *Salmonella enterica* ser. Typhimurium (SL3261, ΔaroA) and peritoneal cells analysed during the chronic phase of infection, 34 and 55 days post infection. In line with previous data (Ruckerl et al., 2017) and confirming the inflammatory environment, MΦres from SL3261 infected animals showed clear upregulation of MHC-II as well as enhanced expression of Sca-1 on day 55 (Fig 3A & B). Moreover, persistent influx of neutrophils was detected in the peritoneal cavity of SL3261 infected animals (Fig 3C) as well as live, cfu-forming bacteria (Fig 3D) confirming an ongoing pro-inflammatory activation.

However, unlike following repeated PD fluid injection, MΦres isolated from animals harbouring *S. Typhimurium* did not show any noticeable loss of Tim4, CD73 or Vsig4 expression in the chronic phase of the infection (Fig 3 E-G). Rather to the contrary MΦres

284 from SL3261 infected animals expressed elevated levels of Tim4 (Fig 3 E). Thus, chronic
285 inflammatory conditions alone did not cause the progressive loss of MΦres phenotype as
286 observed following repeated Physioneal injection.
287 Importantly, injection of sterile PBS instead of PD fluid induced similar, albeit less
288 pronounced changes in peritoneal macrophage phenotype (Fig 4A). This would indicate
289 that repeated disturbance of the peritoneal immune system alone, rather than specific
290 constituents of the dialysis fluid, was sufficient to drive the observed alterations in
291 peritoneal MΦres. However, PD fluid enhanced these effects (Fig 4A).



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293 **Figure 4: Repeated injection of sterile saline induces similar alterations to dialysis fluid**
294 A) Female C57BL/6 mice were injected 5 times a week for a total of nine injections with Physioneal (orange
295 circles), sterile PBS (green triangles) or left untreated (purple squares). 24 h after the last injection whole
296 PEC were isolated and analysed by flow cytometry for expression of MΦres associated cell-surface
297 markers. Data pooled from two independent experiments.
298 B) Male (♂) and female (♀) C57BL/6 mice were injected 5 times a week for a total of nine injections with PD
299 fluid (orange symbols) or left untreated (purple symbols). 24 h after the last injection whole PEC were
300 isolated and analysed as described for A).
301 Datapoints depict individual animals and lines indicate mean and SD. Data from a single experiment.
302 Data analysed using 2-way ANOVA followed by Tukey's multiple comparison test after transformation. n.s.:
303 not significant; *: $p<0.05$; **: $p<0.01$; ****: $p<0.0001$
304

305 Furthermore, it has been suggested that male peritoneal dialysis patients have a
306 significantly reduced survival rate on PD than female patients (Kitterer, Segerer, Braun,
307 Alscher, & Latus, 2017). Although the reason for this discrepancy is unknown and likely

308 due to multiple factors, differences in the inflammatory response may contribute to the
309 observed effects. Moreover, male and female mice differ considerably in the maintenance
310 and cellular dynamics of peritoneal macrophages (Bain et al., 2016). Thus, we assessed
311 the effect of repeated PD fluid injection on myeloid cell populations in male and female
312 mice. MΦres from naive male or female mice showed comparable expression levels of
313 CD73 and Vsig4. In contrast naive male mice showed considerably lower expression of
314 Tim4 (Figure 4B-D). However, independent of these differences in the steady state,
315 Physioneal induced the loss of Tim4, CD73 and Vsig4 in both sexes to a similar degree.
316 Taken together this data shows that repeated disturbance of the peritoneal environment
317 triggers low grade inflammation which alters the MΦres phenotype.
318
319 **Prolonged PD fluid injection alters the response of MΦres to external stimuli**
320
321 Our previous data had highlighted a significant loss of Tim4 as well as Vsig4 and CD73,
322 markers which have been associated with MΦres core functions (i.e. efferocytosis and
323 anti-inflammatory activity) (Lopez-Castejón, Baroja-Mazo, & Pelegrin, 2011; Miyanishi et
324 al., 2007; Yuan et al., 2017). Thus, we next aimed to assess whether repeated injection of
325 dialysis fluid altered macrophage functional responses.
326 To test the capacity of MΦres to take up and ingest apoptotic cells, whole PEC from
327 animals injected for various times with Physioneal or from naive controls were incubated
328 in the presence of apoptotic thymocytes labelled with pHrodo. pHrodo labelled cells emit
329 a very low, nearly undetectable fluorescent signal after staining, but will become brightly
330 fluorescent and clearly detectable by flow cytometry after encountering an acidic
331 environment, as found inside a phagolysosome (Miksa, Komura, Wu, Shah, & Wang,
332 2009). Thus, use of pHrodo allows the reliable detection of ingested apoptotic cells as
333 compared to labeled cells bound to the surface of a phagocyte.
334 Repeated injection of Physioneal gradually reduced the proportion of myeloid cells (total
335 CD11b+lin-) capable of ingesting apoptotic cells (Fig 5A). This was in part due to the
336 increased proportion of Ly6C high monocytes and F4/80 low, monocyte-derived
337 macrophages within the myeloid cell pool (Fig 2C&D), cells which possess reduced
338 efferocytic activity (Gautier, Ivanov, Lesnik, & Randolph, 2013). However, when the
339 analysis was restricted to MΦres a similar progressively reduced capacity to ingest
340 apoptotic cells was detected (Fig 5B). Thus, MΦres from Physioneal injected animals

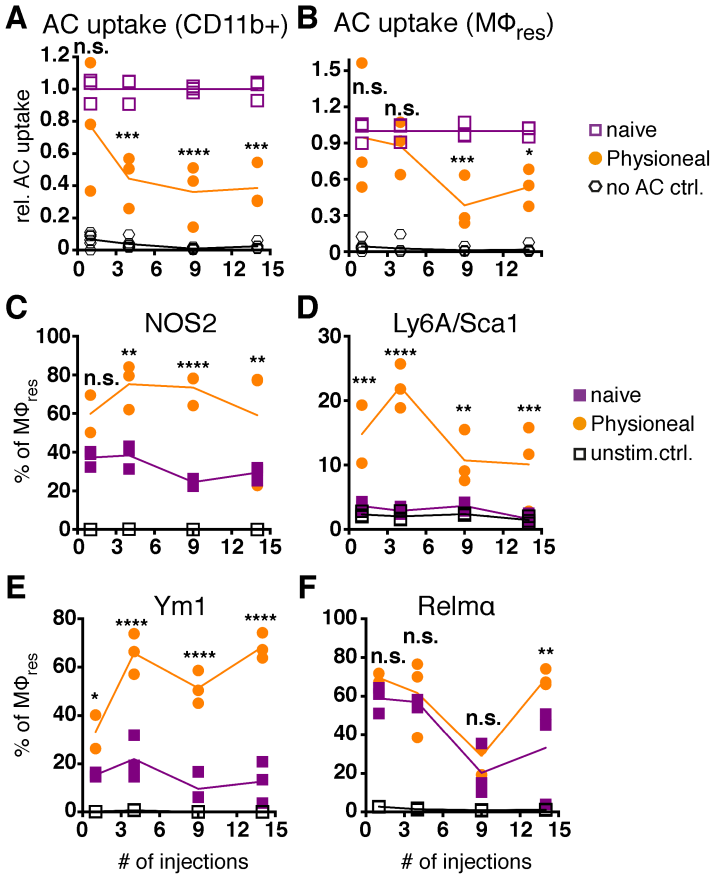


Figure 5: MΦres from PD fluid-injected animals show progressively enhanced responses to stimulation

C57BL/6 mice were injected with PD fluid (orange circle) or left untreated (purple square) 5 times a week for the indicated number of injections. At each timepoint whole PEC were isolated 24 h after the last injection and incubated in vitro in the presence of pHrodo labelled apoptotic thymocytes for 90 minutes (A & B) or stimulated with LPS/IFNγ (C & D; 6 h) or recombinant IL-4 (E & F; 24 h).

Uptake of apoptotic cells by total CD11b+ myeloid cells (A) or MΦres (CD102+I-A/I-E low) (B) as well as expression of MΦres associated cell-surface markers (C-F) assessed by flow cytometry.

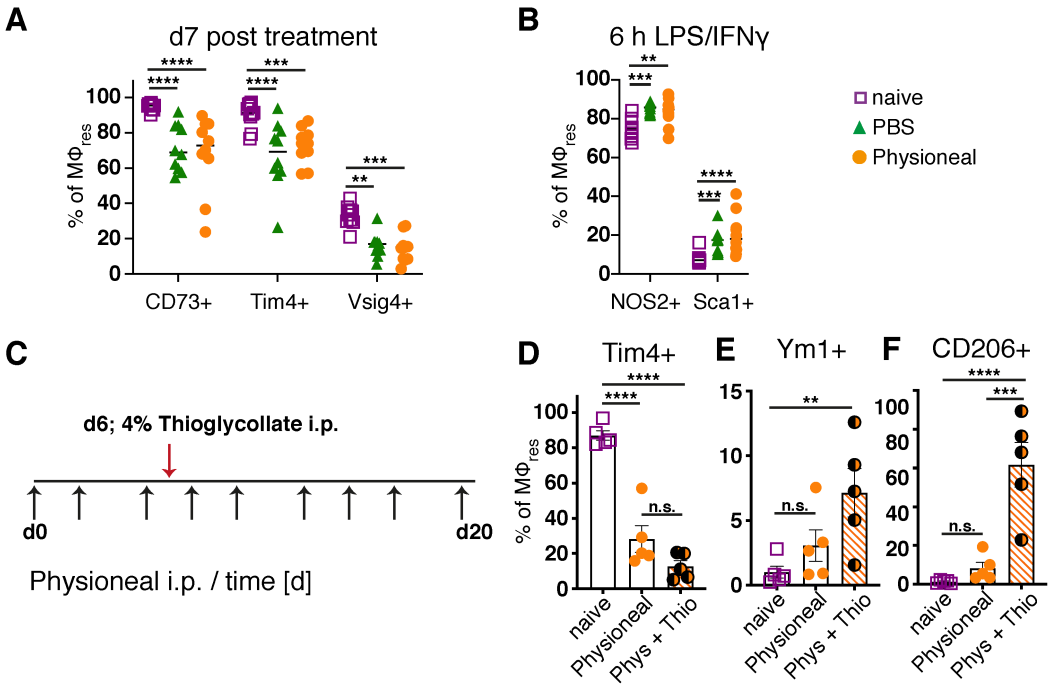
Datapoints depict individual animals and lines indicate mean and SD. Data from a single experiment. Data analysed using 2-way ANOVA followed by Tukey's post-hoc test after transformation. n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ****: $p<0.0001$

seemed to lose the functionality to carry out efferocytosis efficiently. Of note, this loss of efferocytic capacity was not restricted to the use of dialysis fluid, as repeated injection of sterile PBS induced a similar reduction in the capacity of MΦres to ingest apoptotic cells (Fig S4A).

To further analyse whether MΦres from Physioneal injected animals were in general less responsive to external stimuli, we subjected whole PEC to in vitro stimulation with LPS and rIFNγ. MΦres from PD fluid treated animals showed a gradually increasing inflammatory response towards bacterial stimulation (Fig 5C&D). The proportion of cells

362 expressing NOS2 or Sca-1, markers of pro-inflammatory, M1 activation (Menzies,
363 Henriquez, Alexander, & Roberts, 2010; S. Zhang, Kim, Batra, McKerrow, & Loke, 2010),
364 was consistently higher in cells from PD fluid treated animals as compared to cells from
365 naive animals (Fig 5C&D). Indeed, Sca-1 was not found to be upregulated on naive MΦres
366 after 6h stimulation with IFNγ/LPS in vitro, indicating repeated Physioneal injections
367 resulted in a stronger and more rapid response of MΦres to pro-inflammatory stimuli.
368
369 Importantly, an enhanced activation profile was not only observed in response to pro-
370 inflammatory stimuli, but also in response to rIL-4, a driver of M2, anti-inflammatory
371 macrophage activation (Ruckerl & Cook, 2019). MΦres stimulated with rIL-4 for 24h
372 showed increased expression of Ym1 and at a later timepoint also Relmα (Fig 5E&F).
373
374 Thus, MΦres altered their functional repertoire following repeated exposure to dialysis
375 fluid with a reduced efferocytic capacity and enhanced responses to both M1 and M2
376 polarising stimuli.
377
378 **Altered PD fluid-induced responsiveness of MΦres is maintained after treatment is**
379 **discontinued**
380
381 Next we examined whether the phenotypical and functional changes observed in MΦres
382 following repeated PD fluid injection were temporary or persisted even after treatment
383 ceased. For this mice were injected 5 times a week with Physioneal or PBS for a total of 9
384 injections, a timepoint when the altered MΦres phenotype is evident (Fig 2), and then
385 rested for seven days. Subsequently, the PEC were collected and analysed for the
386 expression of activation markers as well as re-stimulated in vitro to analyse their response
387 to external stimuli as described above. MΦres from discontinued Physioneal or PBS
388 injected animals maintained a reduced expression of CD73, Tim4 and Vsig4 compared to
389 naive controls (Fig 6A). Similarly, MΦres from Physioneal and PBS injected animals
390 showed an enhanced response to in vitro stimulation with LPS/IFNγ (NOS2, Sca1;
391 stimulated for 6 h) (Fig 6B). These data highlight that repeated PD fluid injection sensitise
392 peritoneal macrophages and, thus, potentially exacerbates the severity and the
393 detrimental sequelae of peritonitis events.
394

395 In order to verify that repeated exposure to PD fluid alters the response of peritoneal
396 macrophages to inflammatory stimuli in vivo we utilised a model of sterile inflammation.



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398 **Figure 6: PD fluid conditioned MPres maintain altered activation phenotype after treatment is**
399 **discontinued and show enhanced inflammatory responses in vivo**
400 (A) C57BL/6 mice were injected 5 times a week for a total of nine injections with Physioneal (orange circles),
401 sterile PBS (green triangles) or left untreated (purple squares). 7 days after the last injection whole PEC were
402 isolated and analysed by flow cytometry for expression of MΦres associated cell-surface markers.
403 B&C) The cells isolated in A) were subjected to in vitro stimulation with LPS/IFNγ (6 h, NOS2 & Sca1) and
404 analysed by flow cytometry.
405 D) Schematic depiction of the experimental timeline for experiments with intermittent induction of
406 inflammation.
407 E-G) Animals were injected 3 times a week for a total of nine injections with Physioneal (orange circles) or
408 left untreated (purple squares). Some Physioneal injected animals received after the third dose an additional
409 single i.p. injection of sterile 4 % thioglycollate (black & orange circles, shaded bars) to induce
410 inflammation. 24 h after the last injection whole PEC were isolated and analysed by flow cytometry.
411 Datapoints depict individual animals and lines indicate mean & SD. (A - C) Data pooled from 2 independent
412 experiments (except C from a single experiment). Data analysed using 2-way ANOVA followed by Tukey's
413 multiple comparison test after transformation. (E-G) Data from a single experiment analysed by 1-way
414 ANOVA.
415 n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$
416

417 Animals were subjected to repeated PD fluid injection (3 times per week for a total of 9
418 injections) and an inflammatory response was induced with a single dose of sterile 4 %
419 Brewer's modified thioglycollate medium at day 6, mimicking an intermittent event of

420 peritonitis (see Fig 6C). Twenty-four hours after the last PD fluid injection MΦres were
421 collected and analysed for the expression of Tim4, CD206 and Ym1. As expected,
422 repeated injection of dialysis fluid led to a significant loss of Tim4 expression on MΦres
423 with limited induction of CD206 or Ym1 expression (Fig 6D-F). Additionally, in line with our
424 in vitro findings (Fig 5), intermittent induction of inflammation did not further alter MΦres
425 phenotype as determined by Tim4 expression, but led to enhanced activation of MΦres
426 adopting a pro-fibrosis associated phenotype (Fig 6D-F). Importantly, expression of
427 CD206, which has been closely linked to induction of fibrosis (Bellón et al., 2011;
428 Chandrasekaran et al., 2019; Jie Wang et al., 2013), was thought to not be expressed by
429 MΦres (Gundra et al., 2014).
430 Overall these findings indicate that prolonged and repeated exposure to PD fluid renders
431 peritoneal macrophages more responsive to stimulation potentially exacerbating
432 detrimental inflammatory responses.
433
434 **Discussion:**
435
436 Macrophages are versatile cells implicated in many pathologies (Sica, Erreni, Allavena, &
437 Porta, 2015). They play both essential protective as well as detrimental / pathological
438 roles, often within the same disease setting (Guillot & Tacke, 2019; Ribatti, 2013; Wynn &
439 Vannella, 2016). Thus, the regulatory mechanisms governing macrophage responses have
440 become the focus of current research aiming to dissect these contradictory behaviours.
441 Moreover, scientists have proposed macrophages as an excellent target for therapeutic
442 interventions, as altering their phenotype may not only improve disease outcome, but
443 actively revert associated pathologies.
444
445 Recent discoveries have highlighted the inherent diversity of the macrophage pool,
446 indicating the presence of different types of macrophages with different ontogeny (Bain &
447 Jenkins, 2018), response profiles (Gundra et al., 2014) and functional roles (Ruckerl et al.,
448 2017). In particular, MΦres and acutely recruited MΦmono have been identified as
449 distinct mediators of pathology. During lung fibrosis recruited MΦmono have been shown
450 to be essential drivers of the fibrotic pathology and disease progression (Misharin et al.,
451 2017). Similarly, in murine models of peritoneal dialysis associated fibrosis, infiltration of
452 MΦmono has been shown to be detrimental to disease outcome (Y.-T. Chen, Hsu, Lin,
453 Pan, Liu, Wu, Tsai, Liao, Cheng, Chiang, Chen, Chu, & Lin, 2019b; Lee et al., 2012; Q. Li

454 et al., 2018; Jie Wang et al., 2013). Furthermore, significant changes in the prevalence of
455 specific macrophage/monocyte populations have been described in dialysis patients,
456 dependent on the history of peritonitis episodes (Liao et al., 2017). Thus, the pathology
457 inducing effect of peritonitis associated inflammation in peritoneal dialysis seem to be due
458 to the recruitment and accumulation of fibrosis promoting cells like MΦmono. Due to
459 legal restrictions on the maximum number of i.p. injections allowed, we did not observe
460 any clinical signs of fibrosis in our experimental setup. However, similar to the findings
461 above, we observed low-grade inflammation and infiltration of MΦmono in our system. In
462 addition, we demonstrated a drastic change in phenotype of MΦres. Importantly this
463 change in phenotype translated into an enhanced response profile boosting both pro-
464 inflammatory as well as M2 associated effector molecule production. Thus, the simple
465 dichotomy of *good* and *bad* macrophages cannot be upheld in these settings. The
466 increased risk of developing pathological sequelae following repeated episodes of
467 peritonitis in PD patients is likely due to the damaging effect of the inflammatory response
468 (Williams et al., 2002). Our data implies, that patients on longterm PD treatment are at an
469 increased risk to develop clinical symptoms of peritonitis, not only because of an
470 increased risk of exposure to bacterial antigens, but also due to an enhanced
471 inflammatory response of peritoneal resident cells. Whereas in healthy people small
472 numbers of infiltrating pathogens are silently removed by MΦres without triggering an
473 inflammatory response, PD fluid conditioned MΦres may have lost some of this regulatory
474 capacity leading to exacerbated pathology-promoting inflammation. Thus, targeting
475 MΦres to restore some of their homeostatic, anti-inflammatory capabilities may provide a
476 feasible approach to extend the therapeutic window of PD. Indeed, adding soluble factors
477 (e.g. soluble TLR2) to the dialysis fluid targeting the inflammatory response has previously
478 been suggested (Raby & Labéta, 2018) and could be adapted to specifically target
479 MΦres.
480
481 Intriguingly in PD patients receiving oral supplementation with vitamin D, a factor involved
482 in the expansion of peritoneal MΦres in mice (Song et al., 2016), antibacterial responses
483 were enhanced (Bacchetta et al., 2014), potentially through improved phagocytosis of
484 invading bacteria (Lishko, Moreno, Podolnikova, & Ugarova, 2016). MΦres in virtually all
485 tissues of the body are seeded during embryonic development and maintained locally
486 through proliferative expansion. However, in many tissues, including the peritoneal cavity,
487 these embryonic derived macrophages are, over time, replaced by monocyte-derived

488 cells adopting features of tissue resident cells (Bain et al., 2016). Thus, it may be feasible
489 to develop therapeutic approaches fostering the non-inflammatory removal of bacteria by
490 promoting MΦres differentiation. However, the conversion of MΦmono in the peritoneal
491 cavity under steady state seems inefficient and leads to a gradual accumulation of
492 Gata6+, F4/80 high MΦres with low expression of Tim4 (Bain et al., 2016). Our data from
493 *Salmonella enterica* ser. Typhimurium infected mice indicated that the late stages of
494 infection were linked to an increase in Tim4 expressing MΦres as compared to naive
495 animals. Similarly, previously published results from helminth infected animals indicate
496 enhanced expression of Tim4 on MΦres during the chronic phase of these infection
497 (Campbell et al., 2018). Hence, factors associated with the resolution of inflammation or
498 chronic Th2 immune responses may allow to 'rejuvenate' monocyte-derived MΦres or
499 promote the conversion of MΦmono. Further research is required to determine the
500 specific factors driving the MΦres phenotype as well as their potential use as therapeutic
501 adjunct during PD.
502
503 In this context it is important to consider that peritonitis or PD induced inflammation will
504 drive the disappearance of MΦres and, thus, may undo any previous accumulation /
505 conversion attempts. It has previously been suggested that the loss of MΦres is at least
506 in part due to enhanced cell death of resident cells during inflammation (Ruckerl et al.,
507 2017). Importantly, the degree of loss of MΦres is directly correlated to the degree of
508 inflammation as well as the number of MΦres present within the peritoneal cavity (Ruckerl
509 et al., 2017). Thus, targeted expansion of MΦres prior to or during PD fluid treatment may
510 provide an option to retain enhanced numbers of MΦres during inflammatory events. In
511 contrast, recent work suggests that the apparent reduction in the number of MΦres
512 detectable in the peritoneal wash is due to the adherence of these cells to the peritoneum
513 rather than an actual loss in numbers (N. Zhang et al., 2019). This is especially noteworthy
514 in the context of peritoneal dialysis as it would bring these cells in close contact to the
515 main site of pathology. Moreover, targeted disruption of the coagulation cascade reduced
516 the apparent loss of MΦres and prevented their adherence (N. Zhang et al., 2019)
517 potentially opening a window for therapeutic intervention. Currently neither approach
518 (increasing MΦres numbers or preventing the MDR) is likely suitable for use in human
519 patients due to systemic and off-target effects of the applied methodology. However,
520 both approaches indicate intriguing opportunities to develop novel techniques specifically
521 targeting peritoneal MΦres.

522

523 Independent of the therapeutic potential discussed above, analysing the immune cells
524 contained in the effluent of PD patients may yield a useful biomarker strategy. Changes in
525 the composition of myeloid cells as described by Liao et al. (Liao et al., 2017) as well as
526 the assessment of cellular activation markers as described in mice here, may allow risk-
527 based patient stratification. Patients likely to develop pathological sequelae of PD
528 treatment could then be prioritised for kidney transplant or transferred to haemodialysis
529 prior to PD failure or before overt pathological changes occur.
530

531 Lastly, our data comparing PBS injected animals to Physioneal 40 injection indicated a
532 limited impact of the composition of the PD fluid on the observed physiological changes.
533 This seems in contrast to the commonly held believe that the constituents of PD fluid, in
534 particular lactate, glucose-degradation-products (GDP) and low pH, are instrumental in
535 driving PD related pathology (Krishnan, Tam, Wu, Breborowicz, & Oreopoulos, 2005;
536 Mortier, Faict, Lameire, & De Vriese, 2005). However, our data is in line with a recent
537 report investigating the use of so-called biocompatible PD fluid in patients. Despite
538 considerably lower levels of GDP and lactate in these solutions, the morphological
539 changes observed were very similar to those seen in patients utilising standard PD fluid
540 and even an early increase in microvascular density was detected (Schaefer et al., 2018).
541 Of note, it has been suggested that this adverse effect of biocompatible PD fluid may be
542 due to an altered inflammatory response (Blake, 2018). Thus, together with our data this
543 suggests it may be more effective to prevent pathological changes by targeting the
544 elicited immune response to PD fluid instillation, even in the absence of peritonitis.
545

546 Taken together we have shown here that repeated exposure of the peritoneal
547 microenvironment to PD fluid instillation in mice led to a gradual change in phenotype as
548 well as activation response of peritoneal resident cells. These changes were relatively
549 long lasting and resulted in a more vigorous response to inflammatory triggers. Thus,
550 targeting MΦres and preventing excessive inflammatory responses in PD patients may
551 pose an exciting novel approach to limit PD-related pathology.
552

553 **Materials and Methods:**
554
555 **Ethics Statement**

556 All animal experiments were performed in accordance with the UK Animals (Scientific
557 Procedures) Act of 1986 under a Project License (70/8548) granted by the UK Home
558 Office and approved by the University of Manchester Ethical Review Committee.

559 **Mice and in vivo treatments**

560 Eight to 13 week old male and female C57BL/6 mice were obtained from a commercial
561 vendor (Envigo, Hillcrest, UK) and maintained in groups of 4-6 animals in specific
562 pathogen–free facilities at the University of Manchester. Experimental mice were
563 randomly allocated to treatment groups using a computer-based randomization
564 technique and age and sex matched. Euthanasia was performed by asphyxiation in
565 carbon dioxide in a rising concentration. Animal numbers were based on initial power
566 calculations of key determinants derived from preliminary experiments.
567 Animals were injected i.p. with 500 µL peritoneal dialysis fluid (Physioneal 40, 3.86%
568 glucose, Baxter HealthCare Ltd., Compton, UK) for the indicated number of injections.
569 Injections were carried out daily or every other day for three or five days per week for up
570 to 4 weeks (maximum 14 injections per animal). Control animals were left untreated or
571 received an equal volume of PBS i.p. as indicated.
572 For the induction of inflammatory responses PD fluid treated mice received intra-
573 peritoneal injections of 400 µL 4% Brewer modified thioglycollate medium (BD
574 Biosciences, San Jose, CA) or sterile saline as control. The attenuated *Salmonella*
575 *enterica* serovar Typhimurium strain SL3261 (ΔaroA) (Hoiseth & Stocker, 1981) was
576 cultured overnight at 37^0C in a shaking incubator from frozen stock in Luria-Bertani broth
577 with 50 µg/mL streptomycin. The following morning, culture was diluted in fresh Luria-
578 Bertani broth with 50 µg/mL streptomycin and incubated at 37^0C in a shaking incubator to
579 ensure the bacteria were in the growth phase. CFU/mL was estimated by the OD600
580 reading. Animals were pre-treated with 20 mg streptomycin 1 day prior to oral infection
581 with ~1x10^8 CFU *Salmonella* Typhimurium diluted in PBS. Infectious doses and
582 peritoneal bacterial burdens were enumerated by plating inocula or peritoneal exudate
583 cells in 10-fold serial dilutions in PBS on LB-Agar plates.

584 **Cell-isolation**

585 Peritoneal cavity exudate cells (PEC) were obtained by washing the cavity with 10 mL
586 lavage media comprised of RPMI 1640 (Sigma-Aldrich, Dorset, UK) containing 2 mM
587 EDTA and 1% L-Glutamine (Thermo Fisher Scientific, Waltham, MA). Erythrocytes were
588 removed by incubating with red blood cell lysis buffer (Sigma-Aldrich). Cellular content

589 was assessed by cell counting using Viastain AO/PI solution on a Cellometer ® Auto 2000
590 Cell Counter (Nexcelom Bioscience, Manchester, UK) in combination with multicolor flow
591 cytometry.

592 **Flow cytometry**

593 Equal numbers of cells were stained with Zombie UV viability assay (Biolegend, London,
594 UK). All samples were then blocked with 5 µg/mL anti CD16/32 (93; Biolegend) and heat-
595 inactivated normal mouse serum (1:10, Sigma-Aldrich) in flow cytometry buffer (0.5%
596 BSA and 2 mM EDTA in Dulbecco's PBS) before surface staining on ice with antibodies
597 to F4/80 (BM8), SiglecF (E502440, BD Biosciences), Ly6C (HK1.4), Ly-6G (1A8), TCRβ
598 (H57-597), CD11b (M1/70), CD11c (N418), I-A/I-E (M5/114.15.2), CD19 (6D5), CD115
599 (AFS98), CD73 (TY/11.8), CD102 (3C4 (MIC2/4)), Tim4 (RMT4-54), CD206 (MR6F3), Vsig4
600 (NLA14; Thermo Fisher Scientific), CD226 (10E5), Sca1/Ly6A (D7). All antibodies were
601 purchased from Biolegend unless stated otherwise.
602 Detection of intracellular activation markers was performed directly ex vivo. Cells were
603 stained for surface markers then fixed and permeabilized for at least 16 h using the
604 eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific).
605 Cells were then stained with directly labeled Abs to NOS2 (CXNFT; Thermo Fisher
606 Scientific), Arg1 (polyclonal, R&D Systems, Abingdon, UK), Ki67 (B56, BD Biosciences),
607 Annexin V (Biolegend), Gata-6 (D61E4; Cell Signaling Technology Europe, Leiden,
608 Netherlands) or purified polyclonal rabbit antiRelmα (PeproTech EC Ltd., London, UK) and
609 biotinylated anti-Ym1/2 (polyclonal, R&D Systems) followed by Zenon anti–rabbit reagent
610 (Thermo Fisher Scientific) or streptavidin BUV 737 (BD Biosciences), respectively.
611 Samples were acquired on a BD LSR II or BD FACSymphony using BD FACSDiva
612 software (BD Biosciences) and post-acquisition analysis performed using FlowJo v10
613 software (BD Biosciences).

614 **Cell-culture experiments**

615 For in vitro stimulation of PD fluid-conditioned cells, whole PEC were counted as
616 described above and seeded to 96-well U bottom plates at 3x10^5 cells per well in RPMI
617 1640 containing 5 % foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100
618 µg/mL streptomycin and stimulated with lipopolysaccharide (LPS, 100 ng/mL; *Salmonella*
619 *enterica* ser. Typhimurium; Sigma-Aldrich) and recombinant murine Interferon γ (IFNγ, 20
620 ng/mL PeproTech EC Ltd.) or medium alone for 6 h or with murine recombinant
621 Interleukin–4 (rIL-4, 20 ng/mL, PeproTech EC Ltd.) for 24 h and analysed for MΦ
622 activation markers by flow cytometry.

623 **Apoptotic cell uptake assay**

624 Uptake of apoptotic cells by peritoneal MΦres was assessed as previously described
625 (Miksa et al., 2009). Briefly, thymocytes were collected from naive animals by mincing
626 thymi through 2 µm gauze until completely homogenized. Erythrocytes were removed by
627 incubating with red blood cell lysis buffer (Sigma-Aldrich). Thymocytes were resuspended
628 at 1x10^7 cells/mL in complete DMEM and incubated in the presence of 0.1 µM
629 dexamethasone (Sigma-Aldrich) at 37 ºC for 18 h. This produced >90 % apoptosis, as
630 assessed by Viastain AO/PI staining measured on a Cellometer ® Auto 2000 Cell Counter
631 (Nexcelom Bioscience). Subsequently, apoptotic thymocytes were washed twice with
632 PBS and resuspended in PBS at 10^6 cells/mL containing 40 ng/mL pHrodo-SE (Thermo
633 Fisher Scientific) and incubated at RT for 30 min. Thereafter the cells were washed twice
634 with PBS and resuspended in RPMI containing 5 % foetal bovine serum, 2 mM L-
635 glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Unstained apoptotic cells
636 served as staining control.

637 **Statistical analysis**

638 Statistical analysis was performed using Prism 8 for Mac OS X (v8.2.1, GraphPad
639 Software, San Diego, CA). Differences between groups were determined by t-test or
640 ANOVA followed by Tukey's or Dunn's multiple comparison-test. In some cases data was
641 log-transformed to achieve normal distribution as determined by optical examination of
642 residuals. Where this was not possible a Mann-Whitney or Kruskal-Wallis test was used.
643 Percentages were subjected to arcsine transformation prior to analysis. Differences were
644 assumed statistically significant for *P* values of less than 0.05.

645

646 **Author contributions**

647 - Tara E Sutherland (Resources, Writing – Review & Editing, Funding Acquisition)
648 - Tovah N Shaw (Investigation, Writing – Review & Editing)
649 - Rachel Lennon (Resources, Writing – Review and Editing, Clinical advice)
650 - Sarah E Herrick (Conceptualization, Writing – Review and Editing)
651 - Dominik Ruckerl (Conceptualization, Formal Analysis, Validation, Investigation, Writing -
652 Original Draft Preparation, Writing – Review and Editing, Visualization, Project
653 Administration, Funding Acquisition)

654

655 **Competing interests**

656 The authors declare that they have no competing interests.

657

658 **Acknowledgements**

659 This work was supported by the Medical Research Council UK (MR/P02615X/1, DR) and
660 the Medical Research Foundation / Asthma UK (MRFAUK-2015-302, TES). TNS was
661 supported by a BBSRC Discovery Research Fellowship (BB/S01103X/1, TNS), RL was
662 supported by a Wellcome Trust Senior Fellowship award (202860/Z/16/Z, RL) and SEH
663 and DR were supported by an MRC Research Grant (MRC MR/S02560X/1, SEH).
664 We thank Dr. Gareth Howell from the Flow Cytometry Core Facility at the University of
665 Manchester for assistance with the flow cytometry analyses. We'd also like to thank
666 Sister Trish Smith from the Royal Manchester Children's Hospital for the provision of
667 Physioneal 40.
668

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670

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