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

Tara E. Sutherland[1,2], Tovah N. Shaw[1,2], Rachel Lennon[3,4], Sarah E. Herrick[1,4], Dominik Rückerl[1]

1 Lydia Becker Institute for Immunology and Infection, Faculty of Biology, Medicine and Health, Division of Infection, Immunity and Respiratory Medicine, University of Manchester, Manchester, UK
2 Manchester Collaborative Centre for Inflammation Research (MCCIR), University of Manchester, Manchester, UK
3 Wellcome Centre for Cell-Matrix Research, University of Manchester, Manchester, UK
4 Division of Cell Matrix Biology and Regenerative Medicine, Faculty of Biology Medicine and Health, School of Biological Sciences, University of Manchester, Manchester, UK

Running title: Tissue resident macrophages in PD

Corresponding author: Dr. Dominik Rückerl, Dominik.Ruckerl@manchester.ac.uk

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 (1). In Europe, an average of 850 people per million population (pmp) require renal

replacement therapy (RRT) and 120 new patients pmp commence treatment annually (2). 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 (2).

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 (3), lower costs for the health system (4) and increased patient autonomy (5) as compared to HD, the incidence rate of PD over several decades has dropped in Europe (3). 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 (6). 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 (6, 7). 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 (7-9). The recognition of this Encapsulating Peritoneal Sclerosis (EPS) is an indication to discontinue PD with the mortality approaching 50% one year after diagnosis (10). Management of EPS includes surgery but there is a relatively high frequency of symptom recurrence (10). In contrast, immunosuppressive treatments and the use of anti-fibrotic agents, like tamoxifen, have shown noticeable benefits to patient survival (10). 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 (11-14).

Recent advances in macrophage biology have highlighted the intrinsic heterogeneity of macrophage populations (15). 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 (16). Both macrophage populations can and do respond to

external stimuli, like infection, but they possess distinct response profiles and adopt distinct functional properties (17, 18).

In health, peritoneal MΦres are essential for maintaining tissue homeostasis by silently removing apoptotic cells through efferocytosis (19, 20) and by providing a source of tissue-reparative cells that infiltrate surrounding tissues (e.g. the liver) during injury (21). Importantly, upon encountering inflammatory signals, MΦres undergo the Macrophage Disappearance Reaction (MDR) (22). 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 (23). The exact mechanism underlying MDR is still poorly understood, but it has been proposed that MΦres undergo activation-induced cell death (18) or adhere to the mesothelial lining of the peritoneal cavity reducing their recovery (21, 24, 25). 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 (18). Following resolution of the inflammation, MΦres can re-populate the peritoneal cavity and return to homeostatic numbers through proliferative expansion of remaining MΦres (26).

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 (27-29). 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 (12, 30, 31). Moreover, injection of MΦmono, activated ex vivo using bacterial antigens (i.e. Lipopolysaccharide), often referred to as M1 macrophages, exacerbates disease progression (13). Together these data highlight the role of inflammation and infiltration of MΦmono in the progression of peritoneal fibrosis and seem to provide a cohesive picture explaining the enhanced risk of PD failure associated with repeated episodes of peritonitis (32). In this context, it is interesting to note that continuing peritoneal irrigation and thus continual removal of peritoneal cells, including any inflammatory infiltrate, in patients discontinuing PD, has been suggested to prevent subsequent EPS formation (33).
Other studies have found a role for anti-inflammatory, IL-4 activated macrophages (also called M2), characterised by the expression of CD206, Arg1 and Ym1, in promoting peritoneal dialysis fluid (PD fluid) induced fibrosis (12, 34, 35). Additionally, chronic fibrotic

109 kidney disease is linked to a switch from inflammatory M1 to predominantly M2 activated
110 macrophages (36). Indeed, both types of macrophage activation seem to have the
111 capacity to promote kidney fibrosis, possibly acting during different phases of the
112 pathology (37). Importantly, some markers used to define M2 are differentially expressed
113 on MΦres and MΦmono. For example CD206 is expressed constitutively by MΦmono but
114 not MΦres (17). Thus, the described role of M2 macrophages may merely reflect an
115 enhanced influx of MΦmono rather than IL-4 mediated activation. In fact, transfer of
116 exogenously activated M2 macrophages showed no impact on disease progression in a
117 model of peritoneal fibrosis (13) and even reduced pathology in a model of renal fibrosis
118 (38). Moreover, using different strategies to deplete macrophages in kidney fibrosis
119 yielded opposing results with regard to disease progression indicating that depletion of
120 different subsets of macrophage may lead to different outcomes (39). Indeed, renal MΦres
121 as compared to MΦmono have been shown to be protective in a model of kidney
122 pathology (29). This suggests that the cellular origin of macrophages may play a
123 prominent part in determining their role during fibrotic disorders and influence the overall
124 disease outcome.
125
126 Here we analysed the effects of repeated PD fluid injection on macrophage population
127 dynamics and responses to activating signals. Our data indicate a significant change in
128 MΦres phenotype over time during PD fluid administration. MΦres lost expression of anti-
129 inflammatory and efferocytic markers correlating with the duration of PD fluid treatment.
130 Moreover, MΦres from PD fluid treated animals showed enhanced inflammatory
131 responses to external stimulation. Importantly, the enhanced inflammatory phenotype of
132 MΦres persisted even when PD fluid administration was stopped. Thus, repeated
133 exposure to PD fluid may render patients more susceptible to peritonitis, and by
134 extension to peritoneal fibrosis, due to exaggerated inflammatory responses of MΦres.
135
136 **Results:**
137
138 **Dialysis fluid induces the disappearance of tissue resident macrophages**
139
140 To determine the impact of peritoneal dialysis fluid (PD fluid) injection on peritoneal
141 macrophage populations (see suppl. fig 1 for gating strategy) we first characterised the
142 cellular dynamics induced after a single application of PD fluid. Six hours after PD fluid

injection, tissue resident macrophages (MΦres) underwent a pronounced disappearance reaction with the number of F4/80 high MΦres reduced to approximately 30% of the levels found in naive control animals (Fig 1A). Simultaneously, a significant influx of neutrophils and Ly6C high monocytes were detected, indicative of an inflammatory response (Fig 1B&C). Of note, similar to previous reports (23) tissue dwelling, monocyte-derived MΦmono (F4/80 low MHC-II high) also underwent a disappearance reaction and were reduced in numbers by approximately 40 % (Fig 1D).

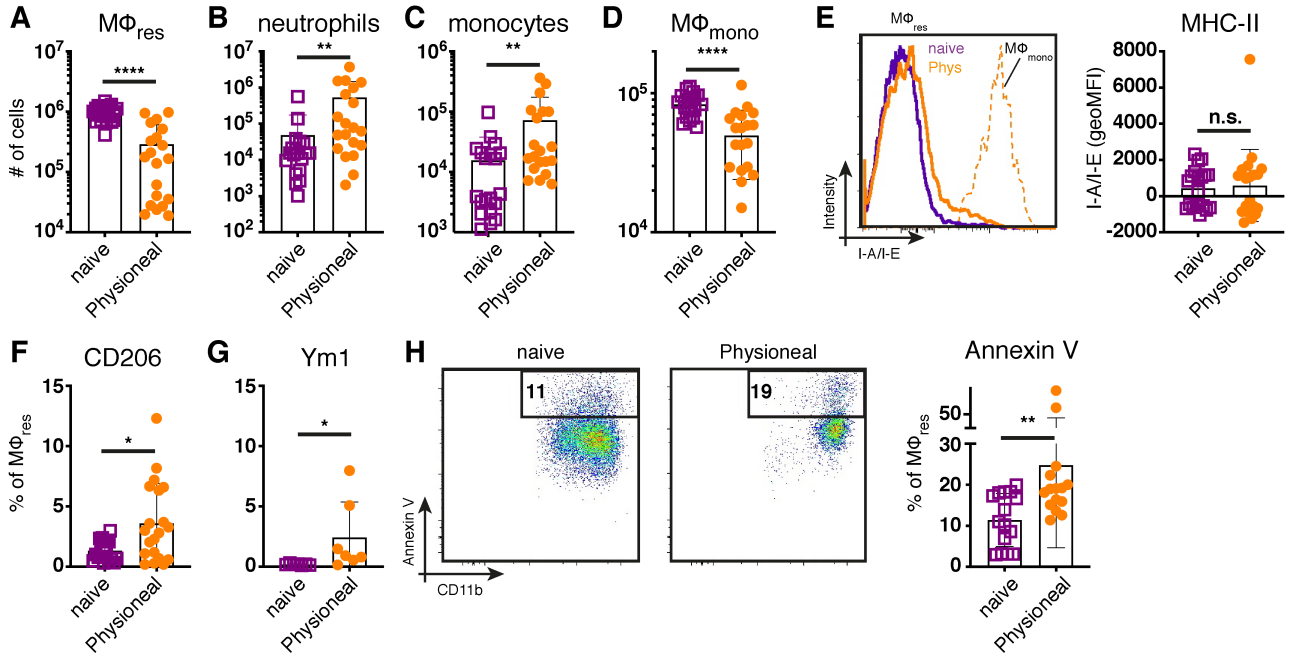


Figure 1: **Injection of dialysis fluid alters peritoneal myeloid cell composition**

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

F4/80 high MΦres displayed limited signs of activation at this early time point post PD fluid injection. No increase in expression of MHC-II could be detected (Fig 1E). In contrast

168 intracellular Ym1 and CD206 expression were significantly enhanced following PD fluid
169 injection, but expression was restricted to less than 10 % of cells (Fig 1F&G). The
170 disappearance of MΦres in this context seemed in part due to enhanced cell death as
171 indicated by increased levels of Annexin V staining (Fig 1H). By 24 hours post PD fluid
172 injection, the numbers of MΦres had returned to baseline levels and expression of CD206
173 was no longer significantly different, whereas Ym1 expression remained elevated
174 compared to naïve controls (Fig S2).
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.
178
179 **Repeated PD fluid treatment induces a gradual change in MΦres phenotype**
180
181 We next sought to determine the long term effect of PD fluid administration on peritoneal
182 macrophage populations. For this animals were injected once per day, 5 times a week
183 with PD fluid i.p. and the peritoneal exudate cells (PEC) analysed 24 hours after 1, 4, 9
184 and 14 injections, respectively (Fig 2A). Over the course of the experiment the number of
185 MΦres remained consistently lower in Physioneal treated animals as compared to naive
186 mice (Fig 2B). Simultaneously, enhanced influx of Ly6C monocytes and successive
187 accumulation of F4/80 low MΦmono could be detected in the peritoneal cavity following
188 multiple rounds of PD fluid injection (Fig 2C& D).
189 Despite the overall reduced numbers of MΦres, repeated injection of PD fluid led to
190 marked induction of Ki67 expression in MΦres, indicative of proliferative expansion (Fig.
191 S3). This is in line with a previous report demonstrating repopulation of the peritoneal
192 cavity by MΦres following an inflammatory insult through proliferation (40). Thus, it is likely
193 that PD fluid injection leads to a repeated cycle of MΦres disappearance followed by re-
194 population. Similarly, it is likely that the gradual influx of monocytes and MΦmono
195 underlies cyclic fluctuations after each round of PD fluid injection. Thus, this data
196 indicated that macrophage populations within the peritoneal cavity undergo dynamic but
197 limited changes following PD fluid instillation, with a slight accumulation of MΦmono over
198 time.
199

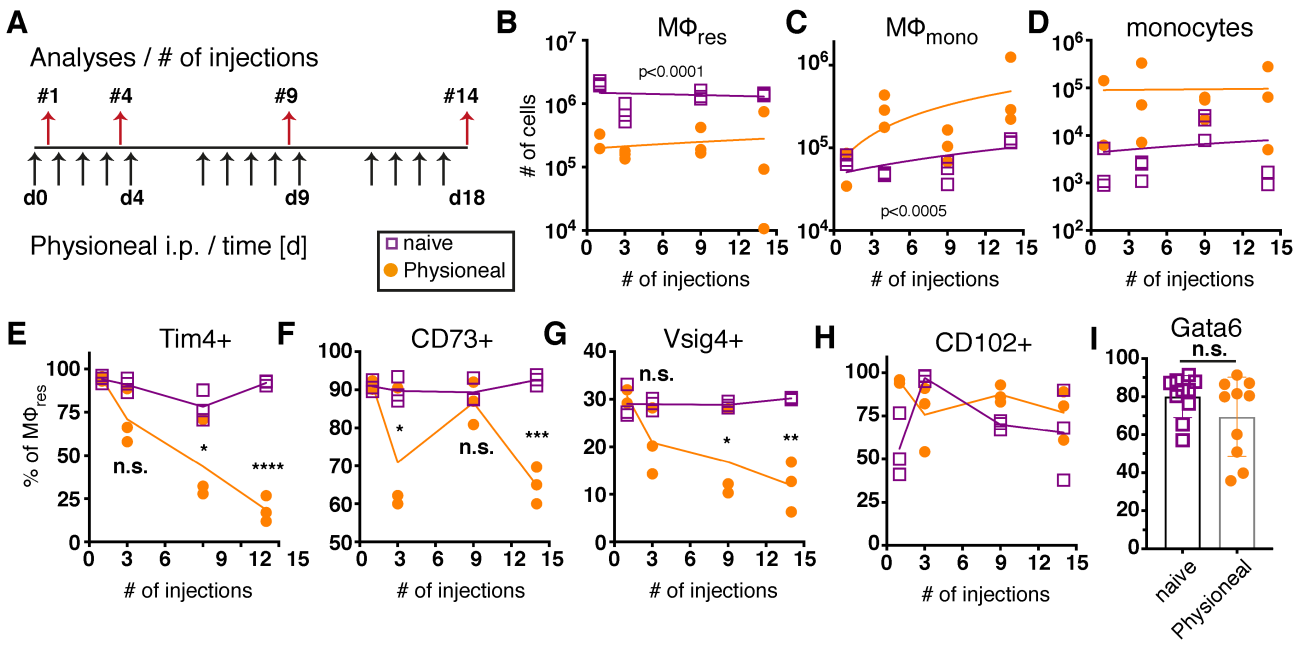


Figure 2: Repeated injection of PD fluid leads to a progressive change in MPres phenotype

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; *: $p<0.05$; **: $p<0.01$; ****: $p<0.0001$

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 immunoglobulin and mucin domain containing 4 (Tim4), a molecule associated with the efficient removal of apoptotic cells (41, 42) as well as V-set and immunoglobulin domain–containing 4 (Vsig4), associated with limiting inflammatory responses (43, 44)], are specifically expressed in MΦres during steady state conditions (45). MΦres gradually lost expression of these markers with increasing number of PD fluid injections (Fig 2 E & G). Similarly, CD73, an anti-inflammatory effector molecule specifically expressed by MΦres (46, 47) was found to be significantly reduced after 14 injections of PD fluid (Fig 2 F). In contrast, expression of CD102 (Icam2) was not altered by PD fluid injection (Fig 2 H). A similar phenotype (loss of MΦres marker expression with sustained CD102 expression) has previously been described in mice lacking the transcription factor Gata6, indicating

225 that repeated PD fluid injection may exert its effects via affecting Gata6 expression (48).
226 However, no significant difference in Gata6 expression by MΦres was found after 9
227 injections of PD fluid measured in subsequent experiments (Fig 2 I). Notably, Gata6
228 expression levels varied following PD fluid injection with diminished expression detected
229 in approximately 50 % of animals (Fig 2 I). Thus, any effect of PD fluid injection on Gata6
230 expression may be transient or reflect the impact of other factors, like the degree of
231 inflammation.
232 Taken together, following repeated PD fluid injection, MΦres, while retaining their tissue
233 identity (F4/80 high CD102+ Gata6+), lose some of their functional characteristics and in
234 particular anti-inflammatory and efferocytosis associated functions.
235
236 **Repeated PBS injection but not chronic bacterial infection leads to a loss of MΦres**
237 **characteristics**
238
239 As shown in Figure 1 and previously reported (13), repeated injection of PD fluid is
240 associated with the induction of low grade inflammation as evidenced by influx of
241 neutrophils and Ly6C+ monocytes. Thus, to assess whether the loss of MΦres functional
242 markers (i.e. Tim4, CD73, Vsig4) was due to the elicited inflammatory response, we
243 analysed peritoneal exudate cells from animals subjected to prolonged bacterial infection.
244 Animals were infected orally with attenuated *Salmonella enterica* ser. Typhimurium
245 (SL3261, ΔaroA) and peritoneal cells analysed during the chronic phase of infection, 34
246 and 55 days post infection. In line with previous data (18) and confirming the inflammatory
247 environment, MΦres from SL3261 infected animals showed clear upregulation of MHC-II
248 as well as enhanced expression of Sca-1 on day 55 (Fig 3A & B). Moreover, persistent
249 influx of neutrophils was detected in the peritoneal cavity of SL3261 infected animals (Fig
250 3C) as well as live, cfu-forming bacteria (Fig 3D) confirming an ongoing pro-inflammatory
251 activation.
252 However, unlike following repeated PD fluid injection, MΦres isolated from animals
253 harbouring *S. Typhimurium* did not show any noticeable loss of Tim4, CD73 or Vsig4
254 expression in the chronic phase of the infection (Fig 3 E-G). Rather to the contrary MΦres
255 from SL3261 infected animals expressed elevated levels of Tim4 (Fig 3 E). Thus, chronic
256 inflammatory conditions alone did not cause the progressive loss of MΦres phenotype as
257 observed following repeated Physioneal injection.

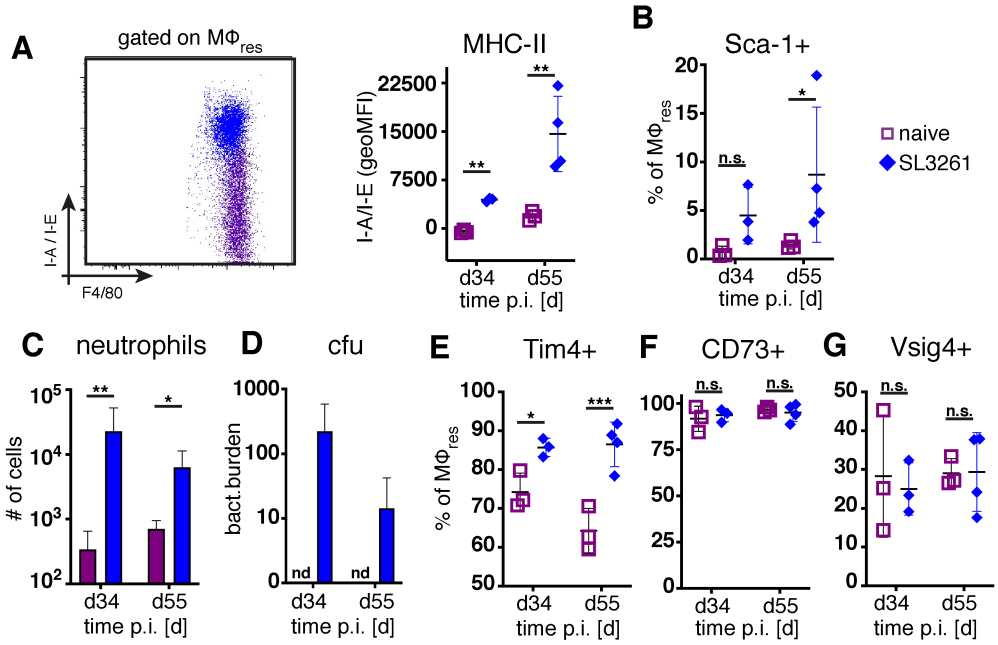


Figure 3: Chronic inflammation induced by S.Typhimurium infection does not phenocopy repeated PD fluid injection

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; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$

Importantly, injection of sterile PBS instead of PD fluid induced similar, albeit less pronounced changes in peritoneal macrophage phenotype (Fig 4A). This would indicate that repeated disturbance of the peritoneal immune system alone, rather than specific constituents of the dialysis fluid, was sufficient to drive the observed alterations in peritoneal MΦres. However, PD fluid enhanced these effects (Fig 4A).
Furthermore, it has been suggested that male peritoneal dialysis patients have a significantly reduced survival rate on PD than female patients (49). Although the reason for this discrepancy is unknown and likely due to multiple factors, differences in the inflammatory response may contribute to the observed effects. Moreover, male and female mice differ considerably in the maintenance and cellular dynamics of peritoneal macrophages (50). Thus, we assessed the effect of repeated PD fluid injection on myeloid cell populations in male and female mice. MΦres from naive male or female mice showed comparable expression levels of CD73 and Vsig4. In contrast naive male mice showed

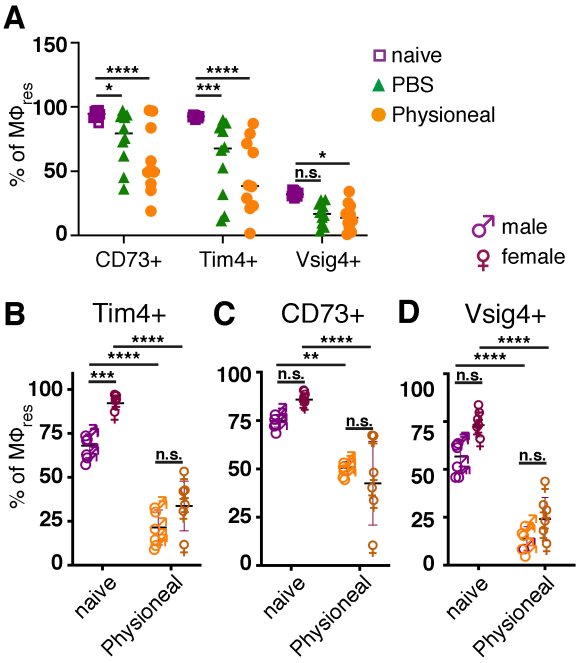


Figure 4: Repeated injection of sterile saline induces similar alterations to dialysis fluid

A) Female C57BL/6 mice were injected 5 times a week for a total of nine injections with Physioneal (orange circles), sterile PBS (green triangles) or left untreated (purple squares). 24 h after the last injection whole PEC were isolated and analysed by flow cytometry for expression of MΦres associated cell-surface markers. Data pooled from two independent experiments.

B) Male (♂) and female (♀) C57BL/6 mice were injected 5 times a week for a total of nine injections with PD fluid (orange symbols) or left untreated (purple symbols). 24 h after the last injection whole PEC were isolated and analysed as described for A).

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 multiple comparison test after transformation. n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ****: $p<0.0001$

considerably lower expression of Tim4 (Figure 4B-D). However, independent of these differences in the steady state, Physioneal induced the loss of Tim4, CD73 and Vsig4 in both sexes to a similar degree.

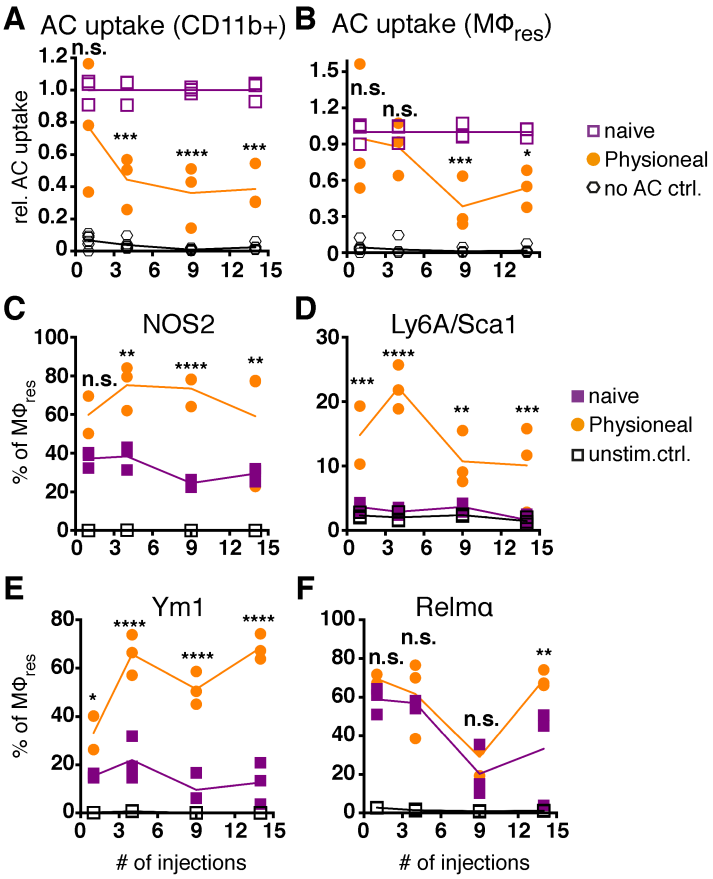
Taken together this data shows that repeated disturbance of the peritoneal environment triggers low grade inflammation which alters the MΦres phenotype.

Prolonged PD fluid injection alters the response of MΦres to external stimuli

Our previous data had highlighted a significant loss of Tim4 as well as Vsig4 and CD73, markers which have been associated with MΦres core functions (i.e. efferocytosis and

306 anti-inflammatory activity) (41, 44, 51). Thus, we next aimed to assess whether repeated
307 injection of dialysis fluid altered macrophage functional responses.
308 To test the capacity of MΦres to take up and ingest apoptotic cells, whole PEC from
309 animals injected for various times with Physioneal or from naive controls were incubated
310 in the presence of apoptotic thymocytes labelled with pHrodo. pHrodo labelled cells emit
311 a very low, nearly undetectable fluorescent signal after staining, but will become brightly
312 fluorescent and clearly detectable by flow cytometry after encountering an acidic
313 environment, as found inside a phagolysosome (52). Thus, use of pHrodo allows the
314 reliable detection of ingested apoptotic cells as compared to labeled cells bound to the
315 surface of a phagocyte.
316 Repeated injection of Physioneal gradually reduced the proportion of myeloid cells (total
317 CD11b+lin-) capable of ingesting apoptotic cells (Fig 5A). This was in part due to the
318 increased proportion of Ly6C high monocytes and F4/80 low, monocyte-derived
319 macrophages within the myeloid cell pool (Fig 2C&D), cells which possess reduced
320 efferocytic activity (53). However, when the analysis was restricted to MΦres a similar
321 progressively reduced capacity to ingest apoptotic cells was detected (Fig 5B). Thus,
322 MΦres from Physioneal injected animals seemed to lose the functionality to carry out
323 efferocytosis efficiently. Of note, this loss of efferocytic capacity was not restricted to the
324 use of dialysis fluid, as repeated injection of sterile PBS induced a similar reduction in the
325 capacity of MΦres to ingest apoptotic cells (Fig S4A).
326 To further analyse whether MΦres from Physioneal injected animals were in general less
327 responsive to external stimuli, we subjected whole PEC to in vitro stimulation with LPS
328 and rIFNγ. MΦres from PD fluid treated animals showed a gradually increasing
329 inflammatory response towards bacterial stimulation (Fig 5C&D). The proportion of cells
330 expressing NOS2 or Sca-1, markers of pro-inflammatory, M1 activation (54, 55), was
331 consistently higher in cells from PD fluid treated animals as compared to cells from naive
332 animals (Fig 5C&D). Indeed, Sca-1 was not found to be upregulated on naive MΦres after
333 6h stimulation with IFNγ/LPS in vitro, indicating repeated Physioneal injections resulted in
334 a stronger and more rapid response of MΦres to pro-inflammatory stimuli.
335 Importantly, an enhanced activation profile was not only observed in response to pro-
336 inflammatory stimuli, but also in response to rIL-4, a driver of M2, anti-inflammatory
337 macrophage activation (56). MΦres stimulated with rIL-4 for 24h showed increased
338 expression of Ym1 and at a later timepoint also Relmα (Fig 5E&F).

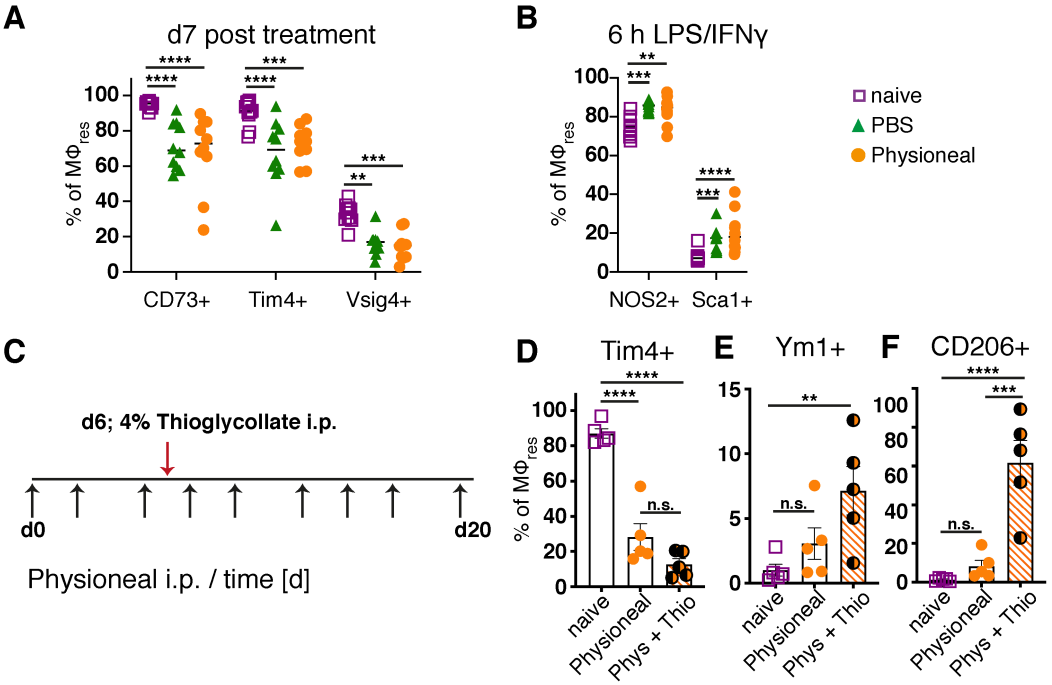
339 Thus, MΦres altered their functional repertoire following repeated exposure to dialysis
340 fluid with a reduced efferocytic capacity and enhanced responses to both M1 and M2
341 polarising stimuli.
342



343
344 **Figure 5: MΦres from PD fluid-injected animals show progressively enhanced responses to**
345 **stimulation**
346 C57BL/6 mice were injected with PD fluid (orange circle) or left untreated (purple square) 5 times a week for
347 the indicated number of injections. At each timepoint whole PEC were isolated 24 h after the last injection
348 and incubated in vitro in the presence of pHrodo labelled apoptotic thymocytes for 90 minutes (A & B) or
349 stimulated with LPS/IFNγ (C & D; 6 h) or recombinant IL-4 (E & F; 24 h).
350 Uptake of apoptotic cells by total CD11b+ myeloid cells (A) or MΦres (CD102+I-A/I-E low) (B) as well as
351 expression of MΦres associated cell-surface markers (C-F) assessed by flow cytometry.
352 Datapoints depict individual animals and lines indicate mean and SD. Data from a single experiment. Data
353 analysed using 2-way ANOVA followed by Tukey's post-hoc test after transformation. n.s.: not significant; *:
354 $p<0.05$; **: $p<0.01$; ****: $p<0.0001$
355

356 **Altered PD fluid-induced responsiveness of MΦres is maintained after treatment is**
357 **discontinued**
358

359 Next we examined whether the phenotypical and functional changes observed in MΦres
360 following repeated PD fluid injection were temporary or persisted even after treatment
361 ceased. For this mice were injected 5 times a week with Physioneal or PBS for a total of 9
362 injections, a timepoint when the altered MΦres phenotype is evident (Fig 2), and then
363 rested for seven days. Subsequently, the PEC were collected and analysed for the
364



365

366 **Figure 6: PD fluid conditioned MPres maintain altered activation phenotype after treatment is**
367 **discontinued and show enhanced inflammatory responses in vivo**
368 (A) C57BL/6 mice were injected 5 times a week for a total of nine injections with Physioneal (orange circles),
369 sterile PBS (green triangles) or left untreated (purple squares). 7 days after the last injection whole PEC were
370 isolated and analysed by flow cytometry for expression of MΦres associated cell-surface markers.
371 B&C) The cells isolated in A) were subjected to in vitro stimulation with LPS/IFNγ (6 h, NOS2 & Sca1) and
372 analysed by flow cytometry.
373 D) Schematic depiction of the experimental timeline for experiments with intermittent induction of
374 inflammation.
375 E-G) Animals were injected 3 times a week for a total of nine injections with Physioneal (orange circles) or
376 left untreated (purple squares). Some Physioneal injected animals received after the third dose an additional
377 single i.p. injection of sterile 4 % thioglycollate (black & orange circles, shaded bars) to induce
378 inflammation. 24 h after the last injection whole PEC were isolated and analysed by flow cytometry.
379 Datapoints depict individual animals and lines indicate mean & SD. (A - C) Data pooled from 2 independent
380 experiments (except C from a single experiment). Data analysed using 2-way ANOVA followed by Tukey's
381 multiple comparison test after transformation. (E-G) Data from a single experiment analysed by 1-way
382 ANOVA.
383 n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$
384

385 expression of activation markers as well as re-stimulated in vitro to analyse their response
386 to external stimuli as described above. MΦres from discontinued Physioneal or PBS
387 injected animals maintained a reduced expression of CD73, Tim4 and Vsig4 compared to
388 naive controls (Fig 6A). Similarly, MΦres from Physioneal and PBS injected animals
389 showed an enhanced response to in vitro stimulation with LPS/IFNγ (NOS2, Sca1;
390 stimulated for 6 h) (Fig 6B). These data highlight that repeated PD fluid injection sensitise
391 peritoneal macrophages and, thus, potentially exacerbates the severity and the
392 detrimental sequelae of peritonitis events.
393
394 In order to verify that repeated exposure to PD fluid alters the response of peritoneal
395 macrophages to inflammatory stimuli in vivo we utilised a model of sterile
396 inflammation.Animals were subjected to repeated PD fluid injection (3 times per week for
397 a total of 9 injections) and an inflammatory response was induced with a single dose of
398 sterile 4 % Brewer's modified thioglycollate medium at day 6, mimicking an intermittent
399 event of peritonitis (see Fig 6C). Twenty-four hours after the last PD fluid injection MΦres
400 were collected and analysed for the expression of Tim4, CD206 and Ym1. As expected,
401 repeated injection of dialysis fluid led to a significant loss of Tim4 expression on MΦres
402 with limited induction of CD206 or Ym1 expression (Fig 6D-F). Additionally, in line with our
403 in vitro findings (Fig 5), intermittent induction of inflammation did not further alter MΦres
404 phenotype as determined by Tim4 expression, but led to enhanced activation of MΦres
405 adopting a pro-fibrosis associated phenotype (Fig 6D-F). Importantly, expression of
406 CD206, which has been closely linked to induction of fibrosis (12, 34, 35), was thought to
407 not be expressed by MΦres (17).
408 Overall these findings indicate that prolonged and repeated exposure to PD fluid renders
409 peritoneal macrophages more responsive to stimulation potentially exacerbating
410 detrimental inflammatory responses.
411
412 **Discussion:**
413
414 Macrophages are versatile cells implicated in many pathologies (57). They play both
415 essential protective as well as detrimental / pathological roles, often within the same
416 disease setting (58-60). Thus, the regulatory mechanisms governing macrophage
417 responses have become the focus of current research aiming to dissect these
418 contradictory behaviours. Moreover, scientists have proposed macrophages as an

419 excellent target for therapeutic interventions, as altering their phenotype may not only
420 improve disease outcome, but actively revert associated pathologies.
421
422 Recent discoveries have highlighted the inherent diversity of the macrophage pool,
423 indicating the presence of different types of macrophages with different ontogeny (61),
424 response profiles (17) and functional roles (18). In particular, MΦres and acutely recruited
425 MΦmono have been identified as distinct mediators of pathology. During lung fibrosis
426 recruited MΦmono have been shown to be essential drivers of the fibrotic pathology and
427 disease progression (27). Similarly, in murine models of peritoneal dialysis associated
428 fibrosis, infiltration of MΦmono has been shown to be detrimental to disease outcome (12,
429 13, 30, 62). Furthermore, significant changes in the prevalence of specific
430 macrophage/monocyte populations have been described in dialysis patients, dependent
431 on the history of peritonitis episodes (11). Thus, the pathology inducing effect of peritonitis
432 associated inflammation in peritoneal dialysis seem to be due to the recruitment and
433 accumulation of fibrosis promoting cells like MΦmono. Due to legal restrictions on the
434 maximum number of i.p. injections allowed, we did not observe any clinical signs of
435 fibrosis in our experimental setup. However, similar to the findings above, we observed
436 low-grade inflammation and infiltration of MΦmono in our system. In addition, we
437 demonstrated a drastic change in phenotype of MΦres. Importantly this change in
438 phenotype translated into an enhanced response profile boosting both pro-inflammatory
439 as well as M2 associated effector molecule production. Thus, the simple dichotomy of
440 *good* and *bad* macrophages cannot be upheld in these settings. The increased risk of
441 developing pathological sequelae following repeated episodes of peritonitis in PD
442 patients is likely due to the damaging effect of the inflammatory response (63). Our data
443 implies, that patients on longterm PD treatment are at an increased risk to develop clinical
444 symptoms of peritonitis, not only because of an increased risk of exposure to bacterial
445 antigens, but also due to an enhanced inflammatory response of peritoneal resident cells.
446 Whereas in healthy people small numbers of infiltrating pathogens are silently removed by
447 MΦres without triggering an inflammatory response, PD fluid conditioned MΦres may
448 have lost some of this regulatory capacity leading to exacerbated pathology-promoting
449 inflammation. Thus, targeting MΦres to restore some of their homeostatic, anti-
450 inflammatory capabilities may provide a feasible approach to extend the therapeutic
451 window of PD. Indeed, adding soluble factors (e.g. soluble TLR2) to the dialysis fluid

452 targeting the inflammatory response has previously been suggested (64) and could be
453 adapted to specifically target MΦres.
454
455 Intriguingly in PD patients receiving oral supplementation with vitamin D, a factor involved
456 in the expansion of peritoneal MΦres in mice (65), antibacterial responses were enhanced
457 (66), potentially through improved phagocytosis of invading bacteria (67). MΦres in
458 virtually all tissues of the body are seeded during embryonic development and maintained
459 locally through proliferative expansion. However, in many tissues, including the peritoneal
460 cavity, these embryonic derived macrophages are, over time, replaced by monocyte-
461 derived cells adopting features of tissue resident cells (50). Thus, it may be feasible to
462 develop therapeutic approaches fostering the non-inflammatory removal of bacteria by
463 promoting MΦres differentiation. However, the conversion of MΦmono in the peritoneal
464 cavity under steady state seems inefficient and leads to a gradual accumulation of
465 Gata6+, F4/80 high MΦres with low expression of Tim4 (50). Our data from *Salmonella*
466 *enterica* ser. Typhimurium infected mice indicated that the late stages of infection were
467 linked to an increase in Tim4 expressing MΦres as compared to naive animals. Similarly,
468 previously published results from helminth infected animals indicate enhanced expression
469 of Tim4 on MΦres during the chronic phase of these infection (68). Hence, factors
470 associated with the resolution of inflammation or chronic Th2 immune responses may
471 allow to 'rejuvenate' monocyte-derived MΦres or promote the conversion of MΦmono.
472 Further research is required to determine the specific factors driving the MΦres
473 phenotype as well as their potential use as therapeutic adjunct during PD.
474
475 In this context it is important to consider that peritonitis or PD induced inflammation will
476 drive the disappearance of MΦres and, thus, may undo any previous accumulation /
477 conversion attempts. It has previously been suggested that the loss of MΦres is at least
478 in part due to enhanced cell death of resident cells during inflammation (18). Importantly,
479 the degree of loss of MΦres is directly correlated to the degree of inflammation as well as
480 the number of MΦres present within the peritoneal cavity (18). Thus, targeted expansion
481 of MΦres prior to or during PD fluid treatment may provide an option to retain enhanced
482 numbers of MΦres during inflammatory events. In contrast, recent work suggests that the
483 apparent reduction in the number of MΦres detectable in the peritoneal wash is due to
484 the adherence of these cells to the peritoneum rather than an actual loss in numbers (25).
485 This is especially noteworthy in the context of peritoneal dialysis as it would bring these

486 cells in close contact to the main site of pathology. Moreover, targeted disruption of the
487 coagulation cascade reduced the apparent loss of MΦres and prevented their adherence
488 (25) potentially opening a window for therapeutic intervention. Currently neither approach
489 (increasing MΦres numbers or preventing the MDR) is likely suitable for use in human
490 patients due to systemic and off-target effects of the applied methodology. However,
491 both approaches indicate intriguing opportunities to develop novel techniques specifically
492 targeting peritoneal MΦres.
493
494 Independent of the therapeutic potential discussed above, analysing the immune cells
495 contained in the effluent of PD patients may yield a useful biomarker strategy. Changes in
496 the composition of myeloid cells as described by Liao et al. (11) as well as the
497 assessment of cellular activation markers as described in mice here, may allow risk-
498 based patient stratification. Patients likely to develop pathological sequelae of PD
499 treatment could then be prioritised for kidney transplant or transferred to haemodialysis
500 prior to PD failure or before overt pathological changes occur.
501
502 Lastly, our data comparing PBS injected animals to Physioneal 40 injection indicated a
503 limited impact of the composition of the PD fluid on the observed physiological changes.
504 This seems in contrast to the commonly held believe that the constituents of PD fluid, in
505 particular lactate, glucose-degradation-products (GDP) and low pH, are instrumental in
506 driving PD related pathology (69, 70). However, our data is in line with a recent report
507 investigating the use of so-called biocompatible PD fluid in patients. Despite considerably
508 lower levels of GDP and lactate in these solutions, the morphological changes observed
509 were very similar to those seen in patients utilising standard PD fluid and even an early
510 increase in microvascular density was detected (71). Of note, it has been suggested that
511 this adverse effect of biocompatible PD fluid may be due to an altered inflammatory
512 response (72). Thus, together with our data this suggests it may be more effective to
513 prevent pathological changes by targeting the elicited immune response to PD fluid
514 instillation, even in the absence of peritonitis.
515
516 Taken together we have shown here that repeated exposure of the peritoneal
517 microenvironment to PD fluid instillation in mice led to a gradual change in phenotype as
518 well as activation response of peritoneal resident cells. These changes were relatively
519 long lasting and resulted in a more vigorous response to inflammatory triggers. Thus,

520 targeting MΦres and preventing excessive inflammatory responses in PD patients may
521 pose an exciting novel approach to limit PD-related pathology.
522
523 **Materials and Methods:**
524
525 **Ethics Statement**
526 All animal experiments were performed in accordance with the UK Animals (Scientific
527 Procedures) Act of 1986 under a Project License (70/8548) granted by the UK Home
528 Office and approved by the University of Manchester Ethical Review Committee.
529 **Mice and in vivo treatments**
530 Eight to 13 week old male and female C57BL/6 mice were obtained from a commercial
531 vendor (Envigo, Hillcrest, UK) and maintained in groups of 4-6 animals in specific
532 pathogen–free facilities at the University of Manchester. Experimental mice were
533 randomly allocated to treatment groups using a computer-based randomization
534 technique and age and sex matched. Euthanasia was performed by asphyxiation in
535 carbon dioxide in a rising concentration. Animal numbers were based on initial power
536 calculations of key determinants derived from preliminary experiments.
537 Animals were injected i.p. with 500 µL peritoneal dialysis fluid (Physioneal 40, 3.86%
538 glucose, Baxter HealthCare Ltd., Compton, UK) for the indicated number of injections.
539 Injections were carried out daily or every other day for three or five days per week for up
540 to 4 weeks (maximum 14 injections per animal). Control animals were left untreated or
541 received an equal volume of PBS i.p. as indicated.
542 For the induction of inflammatory responses PD fluid treated mice received intra-
543 peritoneal injections of 400 µL 4% Brewer modified thioglycollate medium (BD
544 Biosciences, San Jose, CA) or sterile saline as control. The attenuated *Salmonella*
545 *enterica* serovar Typhimurium strain SL3261 (ΔaroA) (73) was cultured overnight at 37^0C in
546 a shaking incubator from frozen stock in Luria-Bertani broth with 50 µg/mL streptomycin.
547 The following morning, culture was diluted in fresh Luria-Bertani broth with 50 µg/mL
548 streptomycin and incubated at 37^0C in a shaking incubator to ensure the bacteria were in
549 the growth phase. CFU/mL was estimated by the OD600 reading. Animals were pre-
550 treated with 20 mg streptomycin 1 day prior to oral infection with ~1x10^8 CFU
551 *Salmonella* Typhimurium diluted in PBS. Infectious doses and peritoneal bacterial
552 burdens were enumerated by plating inocula or peritoneal exudate cells in 10-fold serial
553 dilutions in PBS on LB-Agar plates.

554 **Cell-isolation**

555 Peritoneal cavity exudate cells (PEC) were obtained by washing the cavity with 10 mL
556 lavage media comprised of RPMI 1640 (Sigma-Aldrich, Dorset, UK) containing 2 mM
557 EDTA and 1% L-Glutamine (Thermo Fisher Scientific, Waltham, MA). Erythrocytes were
558 removed by incubating with red blood cell lysis buffer (Sigma-Aldrich). Cellular content
559 was assessed by cell counting using Viastain AO/PI solution on a Cellometer ® Auto 2000
560 Cell Counter (Nexcelom Bioscience, Manchester, UK) in combination with multicolor flow
561 cytometry.

562 **Flow cytometry**

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

584 **Cell-culture experiments**

585 For in vitro stimulation of PD fluid-conditioned cells, whole PEC were counted as
586 described above and seeded to 96-well U bottom plates at 3x10^5 cells per well in RPMI
587 1640 containing 5 % foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100

588 µg/mL streptomycin and stimulated with lipopolysaccharide (LPS, 100 ng/mL; *Salmonella*
589 *enterica* ser. Typhimurium; Sigma-Aldrich) and recombinant murine Interferon γ (IFNγ, 20
590 ng/mL PeproTech EC Ltd.) or medium alone for 6 h or with murine recombinant
591 Interleukin–4 (rIL-4, 20 ng/mL, PeproTech EC Ltd.) for 24 h and analysed for MΦ
592 activation markers by flow cytometry.

593 **Apoptotic cell uptake assay**

594 Uptake of apoptotic cells by peritoneal MΦres was assessed as previously described (52).
595 Briefly, thymocytes were collected from naive animals by mincing thymi through 2 µm
596 gauze until completely homogenized. Erythrocytes were removed by incubating with red
597 blood cell lysis buffer (Sigma-Aldrich). Thymocytes were resuspended at 1x10^7 cells/mL
598 in complete DMEM and incubated in the presence of 0.1 µM dexamethasone (Sigma-
599 Aldrich) at 37 ºC for 18 h. This produced >90 % apoptosis, as assessed by Viastain AO/PI
600 staining measured on a Cellometer ® Auto 2000 Cell Counter (Nexcelom Bioscience).
601 Subsequently, apoptotic thymocytes were washed twice with PBS and resuspended in
602 PBS at 10^6 cells/mL containing 40 ng/mL pHrodo-SE (Thermo Fisher Scientific) and
603 incubated at RT for 30 min. Thereafter the cells were washed twice with PBS and
604 resuspended in RPMI containing 5 % foetal bovine serum, 2 mM L-glutamine, 100 U/mL
605 penicillin and 100 µg/mL streptomycin. Unstained apoptotic cells served as staining
606 control.

607 **Statistical analysis**

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

615

616 **Author contributions**

617 - Tara E Sutherland (Resources, Writing – Review & Editing, Funding Acquisition)
618 - Tovah N Shaw (Investigation, Writing – Review & Editing)
619 - Rachel Lennon (Resources, Writing – Review and Editing, Clinical advice)
620 - Sarah E Herrick (Conceptualization, Writing – Review and Editing)
621 - Dominik Ruckerl (Conceptualization, Formal Analysis, Validation, Investigation, Writing -
622 Original Draft Preparation, Writing – Review and Editing, Visualization, Project
623 Administration, Funding Acquisition)

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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

References:

1. Luyckx, V. A., M. Tonelli, and J. W. Stanifer. 2018. The global burden of kidney disease and the sustainable development goals. *Bull. World Health Organ.* 96: 414–422D.
2. Kramer, A., M. Pippias, M. Noordzij, V. S. Stel, A. M. Andrusev, M. I. Aparicio-Madre, F. E. Arribas Monzón, A. Åsberg, M. Barbullushi, P. Beltrán, M. Bonthuis, F. J. Caskey, P. Castro de la Nuez, H. Cernevskis, J. De Meester, P. Finne, E. Golan, J. G. Heaf, M. H. Hemmelder, K. Ioannou, N. Kantaria, K. Komissarov, G. Korejwo, R. Kramar, M. Lassalle, F. Lopot, F. Macário, B. Mackinnon, R. Palsson, Ü. Pechter, V. C. Piñera, C. Santiuste de Pablos, A. Segarra-Medrano, N. Seyahi, M. F. Slon Roblero, O. Stojceva-Taneva, E. Vazelov, R. Winzeler, E. Ziginskiene, Z. Massy, and K. J. Jager. 2019. The European Renal Association - European Dialysis and Transplant Association (ERA-EDTA) Registry Annual Report 2016: a summary. *Clin Kidney J* 12: 702–720.
3. van de Luijtgaarden, M. W. M., K. J. Jager, M. Segelmark, J. Pascual, F. Collart, A. C. Hemke, C. Remón, W. Metcalfe, A. Miguel, R. Kramar, K. Aasarød, A. Abu Hanna, R. T. Krediet, S. Schön, P. Ravani, F. J. Caskey, C. Couchoud, R. Palsson, C. Wanner, P. Finne, and M. Noordzij. 2016. Trends in dialysis modality choice and related patient survival in the ERA-EDTA Registry over a 20-year period. *Nephrol Dial Transplant* 31: 120–128.
4. Chui, B. K., B. Manns, N. Pannu, J. Dong, N. Wiebe, K. Jindal, and S. W. Klarenbach. 2013. Health care costs of peritoneal dialysis technique failure and dialysis modality switching. *Am. J. Kidney Dis.* 61: 104–111.
5. François, K., and J. M. Bargman. 2014. Evaluating the benefits of home-based peritoneal dialysis. *Int J Nephrol Renovasc Dis* 7: 447–455.
6. Bartosova, M., and C. P. Schmitt. 2019. Biocompatible Peritoneal Dialysis: The Target Is Still Way Off. *Front. Physiol.* 9: 2047–13.
7. Morelle, J., A. Sow, N. Hautem, C. Bouzin, R. Crott, O. Devuyst, and E. Goffin. 2015. Interstitial Fibrosis Restricts Osmotic Water Transport in Encapsulating Peritoneal Sclerosis. *J Am Soc Nephrol* 26: 2521–2533.
8. Johnson, D. W., Y. Cho, B. E. R. Livingston, C. M. Hawley, S. P. McDonald, F. G. Brown, J. B. Rosman, K. M. Bannister, and K. J. Wiggins. 2010. Encapsulating peritoneal sclerosis: incidence, predictors, and outcomes. *Kidney Int.* 77: 904–912.

669 9. Moinuddin, Z., A. Summers, D. Van Dellen, T. Augustine, and S. E. Herrick. 2014.
670 Encapsulating peritoneal sclerosis-a rare but devastating peritoneal disease. *Front. Physiol.* 5: 470.
671 10. Danford, C. J., S. C. Lin, M. P. Smith, and J. L. Wolf. 2018. Encapsulating peritoneal sclerosis.
672 *World J. Gastroenterol.* 24: 3101–3111.
673 11. Liao, C.-T., R. Andrews, L. E. Wallace, M. W. A. Khan, A. Kift-Morgan, N. Topley, D. J.
674 Fraser, and P. R. Taylor. 2017. Peritoneal macrophage heterogeneity is associated with different
675 peritoneal dialysis outcomes. *Kidney Int.* 91: 1088–1103.
676 12. Wang, J., Z.-P. Jiang, N. Su, J.-J. Fan, Y.-P. Ruan, W.-X. Peng, Y.-F. Li, and X.-Q. Yu. 2013.
677 The role of peritoneal alternatively activated macrophages in the process of peritoneal fibrosis
678 related to peritoneal dialysis. *Int J Mol Sci* 14: 10369–10382.
679 13. Li, Q., M. Zheng, Y. Liu, W. Sun, J. Shi, J. Ni, and Q. Wang. 2018. A pathogenetic role for M1
680 macrophages in peritoneal dialysis-associated fibrosis. *Molecular Immunology* 94: 131–139.
681 14. Minutti, C. M., L. H. Jackson-Jones, B. García-Fojeda, J. A. Knipper, T. E. Sutherland, N.
682 Logan, E. Rinqvist, R. Guillamat-Prats, D. A. Ferenbach, A. Artigas, C. Stamme, Z. C. Chroneos,
683 D. M. Zaiss, C. Casals, and J. E. Allen. 2017. Local amplifiers of IL-4Rα-mediated macrophage
684 activation promote repair in lung and liver. *Science* 356: 1076–1080.
685 15. Gordon, S., and A. Plüddemann. 2017. Tissue macrophages: heterogeneity and functions. *BMC*
686 *Biol.* 15: 53.
687 16. Zhao, Y., W. Zou, J. Du, and Y. Zhao. 2018. The origins and homeostasis of monocytes and
688 tissue-resident macrophages in physiological situation. *J. Cell. Physiol.* 233: 6425–6439.
689 17. Gundra, U. M., N. M. Girgis, D. Ruckerl, S. Jenkins, L. N. Ward, Z. D. Kurtz, K. E. Wiens, M.
690 S. Tang, U. Basu-Roy, A. Mansukhani, J. E. Allen, and P. Loke. 2014. Alternatively activated
691 macrophages derived from monocytes and tissue macrophages are phenotypically and functionally
692 distinct. *Blood* 123: e110–22.
693 18. Ruckerl, D., S. M. Campbell, S. Duncan, T. E. Sutherland, S. J. Jenkins, J. P. Hewitson, T. A.
694 Barr, L. H. Jackson-Jones, R. M. Maizels, and J. E. Allen. 2017. Macrophage origin limits
695 functional plasticity in helminth-bacterial co-infection. *PLoS Pathog* 13: e1006233.
696 19. Allen, J. E., and D. Ruckerl. 2017. The Silent Undertakers: Macrophages Programmed for
697 Efferocytosis. *Immunity* 47: 810–812.
698 20. Uderhardt, S., M. Herrmann, O. V. Oskolkova, S. Aschermann, W. Bicker, N. Ipseiz, K. Sarter,
699 B. Frey, T. Rothe, R. Voll, F. Nimmerjahn, V. N. Bochkov, G. Schett, and G. Krönke. 2012. 12/15-
700 lipoxygenase orchestrates the clearance of apoptotic cells and maintains immunologic tolerance.
701 *Immunity* 36: 834–846.
702 21. Wang, J., and P. Kubes. 2016. A Reservoir of Mature Cavity Macrophages that Can Rapidly
703 Invade Visceral Organs to Affect Tissue Repair. *Cell* 165: 668–678.
704 22. Barth, M. W., J. A. Hendrzak, M. J. Melnicoff, and P. S. Morahan. 1995. Review of the
705 macrophage disappearance reaction. *J Leukoc Biol* 57: 361–367.
706 23. Accarias, S., C. Genthon, D. Rengel, S. Boullier, G. Foucras, and G. Tabouret. 2016. Single-cell
707 analysis reveals new subset markers of murine peritoneal macrophages and highlights macrophage
708 dynamics upon Staphylococcus aureusperitonitis. *Innate Immun* 22: 382–392.
709 24. Jonjic, N., G. Peri, S. Bernasconi, F. L. Sciacca, F. Colotta, P. Pelicci, L. Lanfrancone, and A.
710 Mantovani. 1992. Expression of adhesion molecules and chemotactic cytokines in cultured human
711 mesothelial cells. *J Exp Med* 176: 1165–1174.
712 25. Zhang, N., R. S. Czepielewski, N. N. Jarjour, E. C. Erlich, E. Esaulova, B. T. Saunders, S. P.
713 Grover, A. C. Cleuren, G. J. Broze, B. T. Edelson, N. Mackman, B. H. Zinselmeyer, and G. J.
714 Randolph. 2019. Expression of factor V by resident macrophages boosts host defense in the
715 peritoneal cavity. *Journal of Experimental Medicine* 216: 1291–1300.
716 26. Davies, L. C., M. Rosas, S. J. Jenkins, C.-T. Liao, M. J. Scurr, F. Brombacher, D. J. Fraser, J. E.
717 Allen, S. A. Jones, and P. R. Taylor. 2013. Distinct bone marrow-derived and tissue-resident
718 macrophage lineages proliferate at key stages during inflammation. *Nat Commun* 4: 1886.
719 27. Misharin, A. V., L. Morales-Nebreda, P. A. Reyfman, C. M. Cuda, J. M. Walter, A. C.
720 McQuattie-Pimentel, C.-I. Chen, K. R. Anekalla, N. Joshi, K. J. N. Williams, H. Abdala-Valencia,

721 T. J. Yacoub, M. Chi, S. Chiu, F. J. Gonzalez-Gonzalez, K. Gates, A. P. Lam, T. T. Nicholson, P. J.
722 Homan, S. Soberanes, S. Dominguez, V. K. Morgan, R. Saber, A. Shaffer, M. Hinchcliff, S. A.
723 Marshall, A. Bharat, S. Berdnikovs, S. M. Bhorade, E. T. Bartom, R. I. Morimoto, W. E. Balch, J. I.
724 Sznajder, N. S. Chandel, G. M. Mutlu, M. Jain, C. J. Gottardi, B. D. Singer, K. M. Ridge, N.
725 Bagheri, A. Shilatifard, G. R. S. Budinger, and H. Perlman. 2017. Monocyte-derived alveolar
726 macrophages drive lung fibrosis and persist in the lung over the life span. *Journal of Experimental*
727 *Medicine* 214: 2387–2404.
728 28. Braga, T. T., M. Correa-Costa, R. C. Silva, M. C. Cruz, M. I. Hiyane, J. S. da Silva, K. R. Perez,
729 I. M. Cuccovia, and N. O. S. Camara. 2018. CCR2 contributes to the recruitment of monocytes and
730 leads to kidney inflammation and fibrosis development. *Inflammopharmacology* 26: 403–411.
731 29. Puranik, A. S., I. A. Leaf, M. A. Jensen, A. F. Hedayat, A. Saad, K.-W. Kim, A. M. Saadalla, J.
732 R. Woollard, S. Kashyap, S. C. Textor, J. P. Grande, A. Lerman, R. D. Simari, G. J. Randolph, J. S.
733 Duffield, and L. O. Lerman. 2018. Kidney-resident macrophages promote a proangiogenic
734 environment in the normal and chronically ischemic mouse kidney. *Sci Rep* 8: 13948.
735 30. Lee, S. H., H.-Y. Kang, K. S. Kim, B. Y. Nam, J. Paeng, S. Kim, J. J. Li, J. T. Park, D. K. Kim,
736 S. H. Han, T.-H. Yoo, and S.-W. Kang. 2012. The monocyte chemoattractant protein-1 (MCP-
737 1)/CCR2 system is involved in peritoneal dialysis-related epithelial-mesenchymal transition of
738 peritoneal mesothelial cells. *Lab. Invest.* 92: 1698–1711.
739 31. Chen, Y.-T., H. Hsu, C.-C. Lin, S.-Y. Pan, S.-Y. Liu, C.-F. Wu, P.-Z. Tsai, C.-T. Liao, H.-T.
740 Cheng, W.-C. Chiang, Y.-M. Chen, T.-S. Chu, and S.-L. Lin. 2019. Inflammatory macrophages
741 switch to CCL17-expressing phenotype and promote peritoneal fibrosis. *J Pathol* path.5350.
742 32. Lambie, M. R., J. Chess, A. M. Summers, P. F. Williams, N. Topley, and S. J. Davies. 2016.
743 Peritoneal inflammation precedes encapsulating peritoneal sclerosis: results from the GLOBAL
744 Fluid Study. *Nephrol Dial Transplant* 31: 480–486.
745 33. Moriishi, M., H. Kawanishi, T. Kawai, S. Takahashi, T. Hirai, M. Shishida, H. Watanabe, and
746 N. Takahashi. 2002. Preservation of peritoneal catheter for prevention of encapsulating peritoneal
747 sclerosis. *Adv Perit Dial* 18: 149–153.
748 34. Bellón, T., V. Martínez, B. Lucendo, G. del Peso, M. J. Castro, L. S. Aroeira, A. Rodríguez-
749 Sanz, M. Ossorio, R. Sánchez-Villanueva, R. Selgas, and M. A. Bajo. 2011. Alternative activation
750 of macrophages in human peritoneum: implications for peritoneal fibrosis. *Nephrol Dial Transplant*
751 26: 2995–3005.
752 35. Chandrasekaran, P., S. Izadjoo, J. Stimely, S. Palaniyandi, X. Zhu, W. Tafuri, and D. M.
753 Mosser. 2019. Regulatory Macrophages Inhibit Alternative Macrophage Activation and Attenuate
754 Pathology Associated with Fibrosis. *The Journal of Immunology* 203: 2130–2140.
755 36. Han, Y., F. Y. Ma, G. H. Tesch, C. L. Manthey, and D. J. Nikolic-Paterson. 2013. Role of
756 macrophages in the fibrotic phase of rat crescentic glomerulonephritis. *AJP: Renal Physiology* 304:
757 F1043–53.
758 37. Nikolic-Paterson, D. J., S. Wang, and H. Y. Lan. 2015. Macrophages promote renal fibrosis
759 through direct and indirect mechanisms. *Kidney International Supplements* 4: 1–5.
760 38. Wang, Y., Y. P. Wang, G. Zheng, V. W. S. Lee, L. Ouyang, D. H. H. Chang, D. Mahajan, J.
761 Coombs, Y. M. Wang, S. I. Alexander, and D. C. H. Harris. 2007. Ex vivo programmed
762 macrophages ameliorate experimental chronic inflammatory renal disease. *Kidney Int.* 72: 290–299.
763 39. Ferenbach, D. A., T. A. Sheldrake, K. Dhaliwal, T. M. J. Kipari, L. P. Marson, D. C. Kluth, and
764 J. Hughes. 2012. Macrophage/monocyte depletion by clodronate, but not diphtheria toxin, improves
765 renal ischemia/ reperfusion injury in mice. *Kidney Int.* 82: 928–933.
766 40. Davies, L. C., M. Rosas, P. J. Smith, D. J. Fraser, S. A. Jones, and P. R. Taylor. 2011. A
767 quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations
768 after acute inflammation. 41: 2155–2164.
769 41. Miyanishi, M., K. Tada, M. Koike, Y. Uchiyama, T. Kitamura, and S. Nagata. 2007.
770 Identification of Tim4 as a phosphatidylserine receptor. *Nature* 450: 435–439.
771 42. Miyanishi, M., K. Segawa, and S. Nagata. 2012. Synergistic effect of Tim4 and MFG-E8 null
772 mutations on the development of autoimmunity. *International Immunology* 24: 551–559.

773 43. Li, J., B. Diao, S. Guo, X. Huang, C. Yang, Z. Feng, W. Yan, Q. Ning, L. Zheng, Y. Chen, and
774 Y. Wu. 2017. VSIG4 inhibits proinflammatory macrophage activation by reprogramming
775 mitochondrial pyruvate metabolism. *Nat Commun* 8: 1322.
776 44. Yuan, X., B.-H. Yang, Y. Dong, A. Yamamura, and W. Fu. 2017. CRIg, a tissue-resident
777 macrophage specific immune checkpoint molecule, promotes immunological tolerance in NOD
778 mice, via a dual role in effector and regulatory T cells. *Elife* 6: ra120.
779 45. Heng, T. S. P., M. W. Painter, Immunological Genome Project Consortium. 2008. The
780 Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol* 9:
781 1091–1094.
782 46. Haskó, G., B. Csóka, B. Koscsó, R. Chandra, P. Pacher, L. F. Thompson, E. A. Deitch, Z.
783 Spolarics, L. Virág, P. Gergely, R. H. Rolandelli, and Z. H. Németh. 2011. Ecto-5'-nucleotidase
784 (CD73) decreases mortality and organ injury in sepsis. *The Journal of Immunology* 187: 4256–
785 4267.
786 47. Rosas, M., L. C. Davies, P. J. Giles, C.-T. Liao, B. Kharfan, T. C. Stone, V. B. O'Donnell, D. J.
787 Fraser, S. A. Jones, and P. R. Taylor. 2014. The transcription factor Gata6 links tissue macrophage
788 phenotype and proliferative renewal. *Science* 344: 645–648.
789 48. Okabe, Y., and R. Medzhitov. 2014. Tissue-specific signals control reversible program of
790 localization and functional polarization of macrophages. *Cell* 157: 832–844.
791 49. Kitterer, D., S. Segerer, N. Braun, M. D. Alscher, and J. Latus. 2017. Gender-Specific
792 Differences in Peritoneal Dialysis. *Kidney Blood Press Res* 42: 276–283.
793 50. Bain, C. C., C. A. Hawley, H. Garner, C. L. Scott, A. Schridde, N. J. Steers, M. Mack, A. Joshi,
794 M. Guilliams, A. M. I. Mowat, F. Geissmann, and S. J. Jenkins. 2016. Long-lived self-renewing
795 bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities.
796 *Nat Commun* 7: ncomms11852.
797 51. Lopez-Castejón, G., A. Baroja-Mazo, and P. Pelegrin. 2011. Novel macrophage polarization
798 model: from gene expression to identification of new anti-inflammatory molecules. *Cell. Mol. Life*
799 *Sci.* 68: 3095–3107.
800 52. Miksa, M., H. Komura, R. Wu, K. G. Shah, and P. Wang. 2009. A novel method to determine
801 the engulfment of apoptotic cells by macrophages using pHrodo succinimidyl ester. *J Immunol*
802 *Methods* 342: 71–77.
803 53. Gautier, E. L., S. Ivanov, P. Lesnik, and G. J. Randolph. 2013. Local apoptosis mediates
804 clearance of macrophages from resolving inflammation in mice. *Blood* 122: 2714–2722.
805 54. Menzies, F. M., F. L. Henriquez, J. Alexander, and C. W. Roberts. 2010. Sequential expression
806 of macrophage anti-microbial/inflammatory and wound healing markers following innate,
807 alternative and classical activation. *Clin Exp Immunol*.
808 55. Zhang, S., C. C. Kim, S. Batra, J. H. McKerrow, and P. Loke. 2010. Delineation of diverse
809 macrophage activation programs in response to intracellular parasites and cytokines. *PLoS Negl*
810 *Trop Dis* 4: e648.
811 56. Ruckerl, D., and P. C. Cook. 2019. Macrophages assemble! But do they need IL-4R during
812 schistosomiasis? *Eur. J. Immunol.* 49: 996–1000.
813 57. Sica, A., M. Erreni, P. Allavena, and C. Porta. 2015. Macrophage polarization in pathology.
814 *Cell. Mol. Life Sci.* 72: 4111–4126.
815 58. Guillot, A., and F. Tacke. 2019. Liver Macrophages: Old Dogmas and New Insights. *Hepatol*
816 *Commun* 3: 730–743.
817 59. Ribatti, D. 2013. Mast cells and macrophages exert beneficial and detrimental effects on tumor
818 progression and angiogenesis. *Immunol Lett* 152: 83–88.
819 60. Wynn, T. A., and K. M. Vannella. 2016. Macrophages in Tissue Repair, Regeneration, and
820 Fibrosis. *Immunity* 44: 450–462.
821 61. Bain, C. C., and S. J. Jenkins. 2018. The biology of serous cavity macrophages. *Cell Immunol*.
822 62. Chen, Y.-T., H. Hsu, C.-C. Lin, S.-Y. Pan, S.-Y. Liu, C.-F. Wu, P.-Z. Tsai, C.-T. Liao, H.-T.
823 Cheng, W.-C. Chiang, Y.-M. Chen, T.-S. Chu, and S.-L. Lin. 2019. Inflammatory macrophages
824 switch to CCL17-expressing phenotype and promote peritoneal fibrosis. *J Pathol* path.5350.

825 63. Williams, J. D., K. J. Craig, N. Topley, C. Von Ruhland, M. Fallon, G. R. Newman, R. K.
826 Mackenzie, G. T. Williams, Peritoneal Biopsy Study Group. 2002. Morphologic changes in the
827 peritoneal membrane of patients with renal disease. *JASN* 13: 470–479.
828 64. Raby, A.-C., and M. O. Labéta. 2018. Preventing Peritoneal Dialysis-Associated Fibrosis by
829 Therapeutic Blunting of Peritoneal Toll-Like Receptor Activity. *Front. Physiol.* 9: 1692.
830 65. Song, L., G. Papaioannou, H. Zhao, H. F. Luderer, C. Miller, C. Dall'Osso, R. M. Nazarian, A.
831 J. Wagers, and M. B. Demay. 2016. The Vitamin D Receptor Regulates Tissue Resident
832 Macrophage Response to Injury. *Endocrinology* 157: 4066–4075.
833 66. Bacchetta, J., R. F. Chun, B. Gales, J. J. Zaritsky, S. Leroy, K. Wesseling-Perry, N. Boregaard,
834 A. Rastogi, I. B. Salusky, and M. Hewison. 2014. Antibacterial responses by peritoneal
835 macrophages are enhanced following vitamin D supplementation. *PLoS ONE* 9: e116530.
836 67. Lishko, V. K., B. Moreno, N. P. Podolnikova, and T. P. Ugarova. 2016. Identification of Human
837 Cathelicidin Peptide LL-37 as a Ligand for Macrophage Integrin αMβ2 (Mac-1, CD11b/CD18) that
838 Promotes Phagocytosis by Opsonizing Bacteria. *Res Rep Biochem* 2016: 39–55.
839 68. Campbell, S. M., J. A. Knipper, D. Ruckerl, C. M. Finlay, N. Logan, C. M. Minutti, M. Mack,
840 S. J. Jenkins, M. D. Taylor, and J. E. Allen. 2018. Myeloid cell recruitment versus local
841 proliferation differentiates susceptibility from resistance to filarial infection. *Elife* 7: 955.
842 69. Krishnan, M., P. Tam, G. Wu, A. Breborowicz, and D. G. Oreopoulos. 2005. Glucose
843 degradation products (GDP's) and peritoneal changes in patients on chronic peritoneal dialysis: will
844 new dialysis solutions prevent these changes? *Int Urol Nephrol* 37: 409–418.
845 70. Mortier, S., D. Faict, N. H. Lameire, and A. S. De Vriese. 2005. Benefits of switching from a
846 conventional to a low-GDP bicarbonate/lactate-buffered dialysis solution in a rat model. *Kidney Int.*
847 67: 1559–1565.
848 71. Schaefer, B., M. Bartosova, S. Macher-Goeppinger, P. Sallay, P. Vörös, B. Ranchin, K.
849 Vondrak, G. Ariceta, A. Zaloszyc, A. K. Bayazit, U. Querfeld, R. Cerkauskiene, S. Testa, C.
850 Taylan, J. VandeWalle, Y. Yap, R. T. Krmar, R. Büscher, A. K. Mühlig, D. Drozdz, S. Caliskan, F.
851 Lasitschka, S. Fathallah-Shaykh, E. Verrina, G. Klaus, K. Arbeiter, R. Bhayadia, A. Melk, P.
852 Romero, B. A. Warady, F. Schaefer, A. Ujszaszi, and C. P. Schmitt. 2018. Neutral pH and low-
853 glucose degradation product dialysis fluids induce major early alterations of the peritoneal
854 membrane in children on peritoneal dialysis. *Kidney Int.* 94: 419–429.
855 72. Blake, P. G. 2018. Is the peritoneal dialysis biocompatibility hypothesis dead? *Kidney Int.* 94:
856 246–248.
857 73. Hoiseth, S. K., and B. A. Stocker. 1981. Aromatic-dependent Salmonella typhimurium are non-
858 virulent and effective as live vaccines. *Nature* 291: 238–239.
859
860