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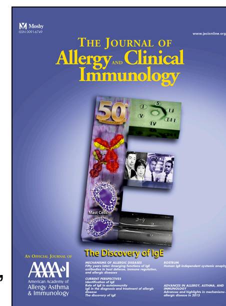

Mer-mediated eosinophil efferocytosis regulates resolution of allergic airway inflammation

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1 **Mer-mediated eosinophil efferocytosis regulates resolution of allergic**
2 **airway inflammation**

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25 **Abstract**

26 **Background:** Eosinophils play a central role in the propagation of allergic diseases including
27 asthma. Both recruitment and retention of eosinophils regulate pulmonary eosinophilia but
28 the question of whether alterations in apoptotic cell clearance by phagocytes directly
29 contributes to resolution of allergic airway inflammation remains unexplored.

30

31 **Objectives:** In this study we investigated the role of the receptor tyrosine kinase Mer in
32 mediating apoptotic eosinophil clearance and allergic airway inflammation resolution *in vivo*
33 in order to establish whether apoptotic cell clearance directly impacts upon the resolution of
34 allergic airway inflammation.

35

36 **Methods:** Alveolar and bone-marrow macrophages were used to study Mer-mediated
37 phagocytosis of apoptotic eosinophils. Allergic airway inflammation resolution was modelled
38 in mice using ovalbumin. To determine apoptotic cell clearance *in vivo*, fluorescently labeled
39 apoptotic cells were administered intratracheally or eosinophil apoptosis was driven by
40 administration of dexamethasone.

41

42 **Results:** Inhibition or absence of Mer impaired phagocytosis of apoptotic human and mouse
43 eosinophils by macrophages. Mer-deficient mice displayed delayed resolution of ovalbumin-
44 induced allergic airway inflammation together with increased airway responsiveness to
45 aerosolized methacholine, elevated bronchoalveolar lavage fluid protein levels, altered
46 cytokine production and an excess of uncleared dying eosinophils after dexamethasone
47 treatment. Alveolar macrophage phagocytosis was significantly Mer-dependent, with the
48 absence of Mer attenuating apoptotic cell clearance *in vivo* to enhance inflammation in
49 response to apoptotic cells.

50

51 **Conclusions:** We demonstrate that Mer-mediated apoptotic cell clearance by phagocytes
52 contributes to resolution of allergic airway inflammation, suggesting that augmenting
53 apoptotic cell clearance is a potential therapeutic strategy for treating allergic airway
54 inflammation.

55

56

57

Key Messages

- Mer drives clearance of apoptotic eosinophils, key cells in allergic airway inflammation.
- Absence of Mer leads to delayed resolution of inflammation and increased airway resistance in allergic airway inflammation.
- Augmenting apoptotic cell clearance is therefore a potential therapeutic strategy for treating allergic inflammation.

Capsule Summary

Defective apoptotic cell clearance is observed in allergic diseases including asthma, but whether this directly contributes to pathophysiology is unclear. Using Mer deficient mice, we demonstrate that impaired apoptotic cell clearance exacerbates allergic airway inflammation.

Key Words

Eosinophil, Apoptosis, Phagocytosis, MerTK, Inflammation Resolution, Allergic airway Inflammation, Airway Resistance.

Abbreviations

AC:	Apoptotic cells
AMs:	Alveolar macrophages
BALF:	Bronchoalveolar lavage fluid
BMDMs:	Bone marrow-derived macrophages
bmEos:	Bone marrow-derived eosinophils
DMEM:	Dulbecco's modified Eagle Medium
FCS:	Fetal calf serum
FLT3-L:	FMS-like tyrosine kinase 3 ligand
H&E:	Hematoxylin and eosin
IMDM:	Iscove's modified Dulbecco's medium
i.p.	Intraperitoneal

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91 i.t. Intratracheal
92 LPS: Lipopolysaccharide
93 Mer^{KD}: Mer-deficient/kinase dead
94 OVA: Ovalbumin
95 PAS: Periodic acid-Schiff
96 PBS: Phosphate-buffered saline
97 R_i: Resolution interval
98 Pros1: Protein S
99 RPMI: Roswell Park Memorial Institute 1640 medium
100 SCF: Stem cell factor
101 TAM: Tyro-3/Axl/Mer
102 TLR: Toll-like receptor
103 WT: Wild type
104
105
106
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110 ## Introduction

111 Eosinophils play a major role in the propagation of allergic airway diseases such as asthma[1,2].
112 During inflammation, eosinophils are recruited from the bone marrow and migrate to
113 inflamed tissue where they can release a range of cytotoxic eosinophil-derived products that
114 promote inflammation, tissue remodelling, airway hyperresponsiveness and organ
115 dysfunction[3].
116
117 Tissue presence of eosinophils is determined by both recruitment and retention within
118 inflamed sites. Eosinophil elimination from the lung can be regulated by transepithelial
119 migration and mucociliary clearance, or by apoptosis and subsequent phagocytosis by
120 macrophages, dendritic cells and airway epithelial cells, a process termed efferocytosis[4]. The
121 relative role and importance of eosinophil apoptosis and efferocytosis in the resolution of
122 allergic airway inflammation in humans remains controversial[5] but several lines of evidence
123 suggest that these pathways have relevance to allergic disease states. Prolonged eosinophil
124 longevity (with reduced apoptosis) associates with increasing asthma severity in humans[6],
125 while macrophages from individuals with severe or poorly controlled asthma have defective
126 efferocytosis[7,8]. In addition, we have recently shown that driving eosinophil apoptosis with
127 the flavone wogonin attenuates allergic lung inflammation in mice *in vivo*[9], suggesting that
128 modulation of eosinophil apoptosis is a *bona fide* target for treating allergic diseases. The
129 question of whether alterations in apoptotic cell clearance by phagocytes directly contributes
130 to resolution of allergic airway inflammation remains to be addressed.
131
132 Although the molecular mechanisms driving changes in eosinophil lifespan and clearance *in*
133 *vivo* remain poorly defined, it is known that glucocorticoids, the main treatment for asthma
134 and other allergic diseases, induce eosinophil apoptosis and upregulate macrophage
135 phagocytosis of apoptotic cells *in vitro*[10,11]. Glucocorticoid-augmented efferocytosis is
136 dependent upon Mer[11], a member of the Tyro-3/Axl/Mer (TAM) receptor tyrosine kinase
137 family[12]. There are two well-defined ligands for Mer, Protein S (Pros1) and Gas6, which can
138 bridge to phosphatidylserine exposed on apoptotic cells. The importance of TAM receptors
139 and their ligands in efferocytosis has been demonstrated using Mer-deficient (Mer^{KD}) and
140 triple TAM-deficient mice. These mice are characterised by impaired efferocytosis in
141 lymphoid tissues, diminished apoptotic germ cell removal by Sertoli cells in the testis and
142 defective pruning of the photoreceptors in the retina by the retinal pigment epithelial cells[13–

143 [15]. Previous studies have investigated the role of Mer in neutrophil-dominant lung injury
144 models (induced by lipopolysaccharide (LPS) and bleomycin)[16], while Axl downregulation
145 has been demonstrated in moderate-severe human asthma[17]. However, the potential role of
146 Mer in regulating eosinophil clearance and resolution of allergic airway inflammation
147 remains unexplored.

148

149 In the present study, we investigated Mer-mediated eosinophil efferocytosis and its role in
150 allergic airway inflammation resolution *in vivo,* to establish whether apoptotic cell clearance
151 directly impacts upon the resolution of allergic airway inflammation. Absence or inhibition of
152 Mer impaired phagocytosis of apoptotic human and mouse eosinophils by macrophages,
153 while Mer-deficient mice had delayed resolution of ovalbumin (OVA)-induced allergic
154 airway inflammation.

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157 # Methods

158 **Eosinophil isolation**

159 Human granulocytes were isolated from blood of healthy volunteers as described[19] (Lothian
160 Research Ethics Committee (#08/S1103/38; #15-HV-013)). Eosinophils were subsequently
161 isolated by anti-CD16^{+ve} microbeads (Miltenyi Biotec, #130-045-701) following
162 manufacturer's instructions with purity >95% as assessed by cellular morphology of Diff-
163 Quik stained cytocentrifuge preparations. Cells were cultured in Iscove's modified
164 Dulbecco's medium (IMDM; Gibco) with 10% autologous serum (37°C/5% CO_2).

165

166 Mouse bone marrow-derived eosinophils (bmEos) were generated from unselected bone
167 marrow progenitor cells using an extended 14 day version of a described protocol[20]. Briefly,
168 bone marrow cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI
169 1640; Gibco), supplemented with 20% FCS (fetal calf serum), 100 IU/mL
170 Penicillin/Streptomycin, 2mM L-glutamine, 25mM HEPES (Sigma), 1x nonessential amino
171 acids and 1mM sodium pyruvate (both Gibco), 50μM 2-mercaptoethanol, stem cell factor
172 (SCF; 100ng/mL, PeproTech) and FLT3-ligand (FLT3-L; 100ng/mL, PeproTech) for the first
173 4 days before switching to media containing IL-5 (10mg/mL, PeproTech) for the remainder
174 of the culture period. After 14 days, cells were >95% eosinophils as assessed by cellular
175 morphology and expression of Siglec-F by flow cytometry.

176

177 **Macrophage isolation**

178 Mouse bone marrow-derived macrophages (BMDMs) were generated as described[21]. Tibias
179 and femurs were flushed with Dulbecco's modified Eagle Medium (DMEM; Gibco) and red
180 blood cells lysed with ACK lysis buffer (Gibco) prior to passing through a 40μm cell strainer.
181 Cells were plated onto 15cm cell culture dishes (Corning) in DMEM with 20% FCS, 100
182 IU/mL Penicillin/Streptomycin and 20% L929 supernatant. Media were replaced after 3 days.
183 On day 6, differentiated macrophages were washed in phosphate-buffered saline (PBS;
184 Gibco) and detached using a cell scraper. Cells were plated at 0.7×10^6/mL in DMEM without
185 serum for 1 hour to allow adhesion before culturing in DMEM with 10% FCS ± 200nM
186 dexamethasone for 24 hours prior to experimentation, a widely established protocol to
187 enhance efferocytosis[12,22].

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189 Mouse alveolar macrophages (AMs) were obtained by lung lavage with 10mL PBS/0.5mM
190 EDTA. AMs were centrifuged at 350g for 5 minutes then resuspended in IMDM and
191 incubated at 150,000 cells per well in a 96 well plate. After 1 hour, culture media was
192 replaced with IMDM supplemented with 10% FCS prior to overnight incubation.

193

194 ***In vitro* phagocytosis assays**

195 The analysis of phagocytosis of fluorescently-labelled apoptotic cells was performed using a
196 modified previously described method[12]. Macrophages were stained with CellTrace Far Red
197 (Thermo Fisher Scientific) as per manufacturer's instructions prior to the addition of
198 apoptotic cells. Human eosinophil constitutive apoptosis was induced by overnight culture,
199 while apoptosis of mouse bmEos was induced by overnight culture with 1μM budesonide in
200 the absence of IL-5. Apoptosis was examined by Annexin-V and propidium iodide staining
201 by flow cytometry. Apoptotic eosinophils were labeled with pHrodo as per manufacturer's
202 instructions then washed and resuspended at $4x10^6$/mL (human eosinophils) or $5x10^6$ (mouse
203 bmEos) in IMDM and co-incubated with macrophages for 1 hour with 33nM Protein S
204 (Pros1) with or without 1μM BMS777607 (Selleck Chemicals) as per figure legends. After
205 co-incubation, macrophages were detached with 0.05% trypsin/0.53mM EDTA and
206 phagocytosis assessed by flow cytometry (BD LSR Fortessa, BD Biosciences)[23,24].

207

208 **Western Blotting**

209 Western blotting was performed as described[25,26]. Briefly, BMDMs were lysed in 0.1%
210 Nonidet P40 containing a protease inhibitor cocktail[26]. Lysates were separated on a 12%
211 Tris-HEPES Precise gel (Thermo Fisher Scientific) and transferred electrophoretically onto
212 polyvinylidene difluoride (PVDF) membranes (Merck Millipore). Membranes were blocked
213 with 5% non-fat milk (Marvel) in Tris-buffered saline (TBS)/0.1% Tween-20 before
214 incubation with primary antibodies directed against Mer (1:000; AF591, R&D Systems) and
215 β-actin (1:50,000; A1978, Sigma). This was followed by horseradish-peroxidase-conjugated
216 secondary antibodies (1:2500; Dako) and incubation with ECL prime (GE Healthcare). Blots
217 were exposed to light-sensitive film (MOL7016, SLS) and processed through an X-ray
218 developer (Ecomax Processor, Photo Imaging Systems Ltd.).

219

220 ***In vivo* model of allergic airway inflammation**

221 Experiments were performed in accordance with the UK Home Office Animals (Scientific
222 Procedures) Act 1996, following review by local ethics committee. Wild type control (WT;

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223 C57BL/6, Charles River Laboratories) and Mer^{KD} mice[27] (C57BL/6 background) were bred
224 and maintained in specific pathogen-free conditions. Genotypes were confirmed prior to
225 experimental procedures, with 6-8 week old mice used for *in vitro* experiments and 8-16
226 week old female mice used for *in vivo* experiments. OVA-induced allergic airway
227 inflammation was modelled as described[9]. Briefly, mice were sensitized by intraperitoneal
228 (*i.p.*) alum-precipitated (Alum Imject, Pierce Biotechnology) ovalbumin (OVA, Sigma; 20µg
229 of OVA and 50µL of alum per mouse) on days 1 and 10 and challenged on days 22, 23 and
230 24 by intratracheal (*i.t.*) OVA (50µg). Mice were culled and bronchoalveolar lavage fluid
231 (BALF) and lung tissue were acquired and processed as described[19].
232 BALF cells and lung interstitial inflammatory cells were incubated with combinations of
233 antibodies against CD45/CD11b/Ly6G/Siglec-F/F4/80, with flow cytometric analysis
234 performed in the presence of Flow-Check Fluorosphere counting beads (Beckman Coulter) to
235 allow quantification of cell numbers. The resolution interval (R_i; the time for eosinophil
236 numbers to decline to half-maximal numbers) was calculated as previously described[28].
237 BALF cytokines and mucus (MUC5AC) were quantified by ELISA (R&D Systems or Caltag
238 Medsystems) or by forward phase protein microarray and expressed as either relative to
239 lavage fluid control (PBS) or as relative expression in the Mer^{KD} mice compared to wild type
240 controls at day 7 post OVA challenge (as indicated in the figure legends).
241 Lungs from separate animals were fixed in 10% formalin (Sigma) prior to sectioning and
242 staining with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Histological
243 scoring of alveolar and interstitial inflammatory cell infiltration was quantified after analysis
244 by two independent, blinded observers, with quantification of airway mucus production
245 performed using the mucus-goblet index as described[9].
246 Airway responsiveness to aerosolized methacholine was assessed in anesthetised and
247 mechanically ventilated WT and Mer^{KD} mice after sensitization and challenge with OVA
248 (Buxco Research Systems, Wilmington, NC). Lung resistance was determined and expressed
249 relative to baseline values in the absence of methacholine with nebulized phosphate-buffered
250 saline given as vehicle. Airway resistance, in the absence of methacholine challenge, was
251 measured in OVA-naïve WT and Mer^{KD} mice.
252 In separate experiments dexamethasone (2mg/kg) was administered *i.p.* on day 3 post-OVA
253 to induce eosinophil apoptosis, prior to quantification of BALF apoptotic eosinophil number
254 by Annexin-V binding (of $CD45^{+ve}$/$CD11b^{+ve}$/$Ly6G^{-ve}$/Siglec-F^{+ve} cells).
255
256 ***In vivo* phagocytosis experiments**

257 Following isolation from peripheral blood human granulocyte apoptosis was induced by
258 overnight culture prior to labelling with CellTracker™ Green as per manufacturer's
259 instructions. Apoptosis was confirmed by Annexin-V and propidium iodide staining by flow
260 cytometry. A total of 100,000 labelled apoptotic human cells were administered *i.t.* to naïve
261 WT and Mer^{KD} mice. Mice were culled after 0 and 3 hours and BALF retrieved for analysis
262 of $CellTracker^{TM}$ Green positive alveolar macrophages ($CD45^{+ve}$/$CD11c^{+ve}$/$CD11b^{-ve}$ cells),
263 uncleared apoptotic cells (mouse $CD45^{-ve}$/CellTrackerTM $Green^{+ve}$ cells), recruited mouse
264 granulocytes ($CD45^{+ve}$/$Ly6G^{+ve}$ cells) and necrotic debris ($EpCam^{-ve}$/$CD11c^{-ve}$/$F4/80^{-ve}$ low
265 SSC/FSC events).

266

267 **Data analysis**

268 Data were analysed using Graphpad Prism (v5) with flow cytometry data analysed using
269 FlowJo software (Treestar). All data are expressed as mean ± SEM and analysed by Student's
270 *t*-test or analysis of variance (ANOVA) as appropriate with significance accepted at $P < 0.05$.

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Results

Mer deficiency delays the resolution of allergic airway inflammation

To investigate the role of apoptotic cell clearance in the resolution of allergic airway inflammation, wild type (WT) and Mer deficient (Mer^{KD}) mice were sensitized and challenged with OVA prior to acquisition of tissue on days 1, 3, 7 and 10 post-OVA challenge (Figure 1A). While both WT and Mer^{KD} mice had similar peak BALF eosinophil numbers at day 3 post-OVA (1.27±0.12 vs. 1.12±0.23 x10^{6}/mL), delayed resolution of eosinophilic inflammation was observed in the Mer^{KD} mice at day 7 (Figure 1B and 1C). This revealed a Δ change in BALF eosinophil numbers between day 3 and day 7 of 1.79x10^{5}/day in the WT mice vs. 0.63x10^{5}/day in the Mer^{KD} mice, with a resolution interval (R_i) that was prolonged by 2 days in the Mer^{KD} mice (6.5 days vs. 8.5 days). Interestingly, numbers of interstitial eosinophils were similar at day 7 (0.58±0.06 vs. 0.73±0.12 x10^{6}/mL; Figure 1D). Similarly, H&E stained lung sections demonstrated predominantly perivascular inflammation, with no significant differences between WT and Mer^{KD} mice (Figure 1E-G).

The functional consequences of the delayed inflammation resolution in the Mer^{KD} mice were explored by measuring airway responsiveness in anesthetized, mechanically ventilated mice in response to aerosolized methacholine. Mer^{KD} mice that had been sensitised and challenged with OVA had increased airway resistance (Figure 2A). Baseline airway resistance (in the absence of methacholine) was unaltered in naïve Mer^{KD} mice (Figure S1) confirming that the observed increase in airway resistance was not innate, but specific to the presence of allergic airway inflammation. Airway mucus production was similar in both WT and Mer^{KD} mice (Figure 2B-E), although BALF total protein was increased in Mer^{KD} mice (671.7±99.9 vs. 990.0±28.8 μg/mL, P<0.05), consistent with the increased inflammation observed (Figure 2F).

Enhanced cytokine production is not a major feature of allergic inflammation in Mer^{KD} mice

Given that signalling via TAM receptors, including Mer, acts to suppress pro-inflammatory cytokine production[29], we analysed BALF cytokines to examine whether raised levels of cytokines were present in the Mer^{KD} mice and contribute to excess inflammation. Alterations in BALF proteins and cytokines were investigated by analysis of 43 separate targets at four separate time points. This analysis revealed that the overall pattern of cytokine expression

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304 was similar between Mer^{KD} and WT mice (Figure S2) with 8 cytokines upregulated >10%
305 and 21 downregulated >10% in the Mer^{KD} mice at day 7 (Figure 2G). Despite increased
306 relative expression of MCP5 (CCL12), RANTES (CCL5) and MCP-1 (CCL2) in the BALF
307 of Mer^{KD} mice at day 7, these chemokines/cytokines were expressed at low absolute levels
308 (data not shown). Overall, we interpret these data as suggesting that enhanced cytokine
309 production was not the major mechanism behind the delayed resolution of allergic
310 inflammation observed in the Mer^{KD} mice.
311
312 **Mer inhibition or deficiency impairs phagocytosis of apoptotic eosinophils**
313 We next investigated the relevance of Mer-mediated apoptotic eosinophil clearance to the
314 observed delayed resolution of allergic airway inflammation in Mer^{KD} mice. Loss of Mer
315 expression on macrophages from Mer^{KD} mice was confirmed by Western blotting of bone
316 marrow-derived macrophages (BMDMs) and by flow cytometry of alveolar macrophages
317 (AMs; Figure S3A-C). WT AMs expressed both Mer and Axl, with Axl expression
318 unchanged on AMs from Mer^{KD} mice (Figure S3B&C). To assess macrophage capacity for
319 efferocytosis, BMDMs (Figure 3A) and AMs (Figure 3B) from WT and Mer^{KD} mice were co-
320 cultured with pHrodo-labelled apoptotic eosinophils (Figure S4). To ensure that Mer-
321 dependent efferocytosis was not limited by bridging ligand availability, exogenous Mer
322 ligand (Protein S; Pros1) was also added to these experiments. Although we observed a
323 significant component of Mer-independent phagocytosis of apoptotic eosinophils, around
324 30% of AM phagocytosis was Mer-dependent (Figure 3A&B). Furthermore, AMs treated
325 with BMS777607 (a c-Met inhibitor that inhibits Axl, Tyro3 and Mer) displayed substantial
326 inhibition of phagocytosis of apoptotic eosinophils (Figure S3D&E), consistent with the
327 expression of both Mer and Axl by AMs. Inhibition of BMDM efferocytosis by BMS777607
328 was less marked, consistent with low level expression of Axl by these cells (data not shown)
329 and previous data demonstrating that WT and $Axl^{-/-}$ BMDMs have similar rates of
330 efferocytosis[12].
331
332 **Mer augments apoptotic cell clearance *in vivo* to dampen inflammation**
333 To further investigate the role of Mer-mediated engulfment of apoptotic eosinophils *in vivo*,
334 the glucocorticoid dexamethasone was administered to OVA-sensitised and challenged mice
335 at the peak of inflammation (day 3 post-OVA) to induce eosinophil apoptosis[10] (Figure 3C).
336 BALF was acquired at 8, 16 and 24 hours post dexamethasone administration and eosinophils
337 analysed for evidence of cellular death by Annexin-V binding (Figure S5). This revealed a

338 time-dependent increase in the percentage of Annexin-V positive eosinophils seen in BALF
339 of Mer^{KD} mice (4.8±0.5% vs. 9.2±0.6 at 16h, $P<0.05$) (Figure 3D&E) consistent with a
340 compromised capacity for eosinophil clearance in the absence of Mer.

341 Furthermore, direct *i.t.* administration of labelled human granulocytes which had undergone
342 cell death (predominantly apoptosis; Figure S4B) to naïve WT and Mer^{KD} mice with tissue
343 acquisition 3 hours later revealed a significant increase in total BALF cells in the Mer^{KD} mice
344 (Figure 4A). Total BALF cell numbers in the WT mice 3 hours after apoptotic cell
345 administration were near identical to those of mice that had been administered PBS as a
346 control, indicating successful clearance of the dead cells in the presence of intact Mer-
347 mediated efferocytosis (Figure 4A). In contrast, increased total numbers of cells were
348 observed in the Mer^{KD} mice suggesting that the absence of Mer-mediated efferocytosis
349 resulted in either failed clearance of the administered apoptotic cells, or recruitment of
350 inflammatory cells in response to the apoptotic cells, or both. Indeed, alveolar macrophages
351 ($CD45^{+ve}/CD11c^{+ve}$ cells, Figure S6A) from Mer^{KD} mice were characterised by reduced
352 phagocytosis of the administered apoptotic cells *in vivo* (Figures 4B&C). Minimal
353 phagocytosis was observed in lung interstitial macrophages, which also express Mer, from
354 either WT or Mer^{KD} mice. This is consistent with their limited anatomical ability to access
355 the airway lumen[30] (data not shown).

356 In addition, an increased proportion of the administered apoptotic cells (mouse $CD45^{-}$
357 $^{ve}/CellTracker^{+ve}$) were recovered in BALF from Mer^{KD} mice (Figure 4D), highlighting the
358 importance of Mer in mediating apoptotic cell clearance within the airway lumen. In parallel,
359 an increased percentage of mouse neutrophils ($CD45^{+ve}/Ly6G^{+ve}$ cells) was observed in
360 BALF from Mer^{KD} mice (Figure 4E). This increase in neutrophils was not observed in BALF
361 recovered from Mer^{KD} mice immediately after administration of apoptotic cells (0 hours post
362 *i.t.* AC), confirming that neutrophils were recruited in a time-dependent fashion specifically
363 in the Mer^{KD} mice in response to apoptotic cells (Figure 4E). Lastly, a population of low
364 SSC/FSC events that did not express markers of alveolar macrophage or epithelial cell origin
365 ($EpCam^{-ve}/CD11c^{-ve}/F4/80^{-ve}$) was present in the BALF from Mer^{KD} mice at 3 hours post *i.t.*
366 AC. This population was minimal in the WT mice (Figure 4F and Figure S6B&C) at 3 hours
367 and minimal at 0 hours in the Mer^{KD} mice (data not shown). As uncleared apoptotic cells can
368 undergo necrosis and release damage-associated molecular patterns leading to the recruitment
369 of inflammatory cells[31], we hypothesised that the low SSC/FSC events were necrotic debris

370 from the instilled human granulocytes. Consistent with this, cytocentrifuge preparations of
371 flow-sorted low FSC/SSC events revealed only cellular debris, in comparison to sorted
372 $CD45^{+ve}$/$F4/80^{+ve}$/$CD11c^{+ve}$ events which clearly demarcated the AM population (Figure
373 S6D&E). Overall, these data demonstrate that Mer augments apoptotic cell clearance *in vivo*
374 to dampen inflammation in response to dying cells.

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376 **Discussion**

377 Restoration of tissue homeostasis following tissue injury or infection requires termination of
378 pro-inflammatory signalling and resolution of the inflammatory response. Control of the
379 resolution process is achieved through a combination of local production of pro-resolution
380 mediators and apoptosis of recruited inflammatory cells, together with their phagocytic
381 removal[2,32]. It is widely accepted that disruption of the processes underlying the timely
382 resolution of inflammation represents a significant contributory factor to the development of
383 many inflammatory diseases. One corollary of the pivotal role of dysregulated resolution of
384 inflammation in disease pathogenesis is that pharmacological modulation of the processes
385 underlying inflammation resolution represent an attractive strategy to attenuate ongoing
386 inflammation and accelerate the restoration of tissue homeostasis[33,34]. In support of this
387 suggestion induction of apoptosis of either neutrophils or eosinophils in mouse models of
388 sterile, infectious or allergic inflammation results in reduced inflammation in the airways and
389 accelerated resolution of inflammation[9,19,35].

390

391 Induction of granulocyte apoptosis during inflammation would exert beneficial effects by
392 directly reducing the overall tissue burden of granulocytes and by limiting the release of
393 cellular contents that contribute to further tissue damage and the development of persistent
394 inflammation. Moreover, there may be additional, indirect effects as a consequence of
395 phagocyte uptake of apoptotic cells. Both "professional" and "non-professional" phagocytes,
396 including airway epithelial cells, can mediate apoptotic cell clearance through multiple
397 molecular pathways[4,36]. Such functional redundancy is thought to reflect the importance of
398 effective apoptotic cell removal in both homeostatic and inflammatory processes.
399 Efferocytosis promotes the resolution process by modulation of phagocyte production and
400 release of anti-inflammatory lipids and cytokines together with suppression of pro-
401 inflammatory cytokine release[37,38]. In particular, the receptor tyrosine kinases Axl and Mer
402 mediate clearance of apoptotic cells and membranes by dendritic cells and macrophages. Axl
403 and Mer exhibit segregation in terms of both expression and activity in a variety of tissue
404 settings, suggesting that they may perform distinct, yet complementary physiological roles[12].
405 Expression of Axl is strongly induced by TLR-ligands and has been shown to play a major
406 role in immunosuppression during inflammation. In contrast, Mer is upregulated by liver X
407 receptor (LXR) ligands and glucocorticoids and is thought to function predominantly in tissue
408 homeostasis. However, antibody-mediated inhibition of Mer exacerbates inflammation

409 following LPS challenge in the lung and augmentation of Mer activity exerts protective
410 effects[16,39]. Together with evidence that resolution-phase macrophages express high levels of
411 Mer, these data suggest that Mer represents an important contributor to the process by which
412 inflammation normally resolves.
413
414 In this study, we have examined the role of apoptotic cell clearance in the resolution of
415 inflammation associated with airway allergy. We report a number of novel findings that
416 extend our understanding of the role of apoptotic cell clearance and Mer-mediated signalling
417 in inflammation and tissue repair. First, we have demonstrated that the onset of inflammation
418 in response to OVA challenge in the lung is similar in the absence of Mer, with equivalent
419 numbers of eosinophils present in the BALF of WT and Mer^{KD} mice at day 3. However, at
420 later time points (day 7) BALF eosinophils persist in the Mer^{KD} mice, together with increased
421 BALF protein levels. The presence of ongoing inflammation in the absence of Mer
422 demonstrate that Mer is an important contributor to the efficiency of resolving eosinophilic
423 inflammation in the airways. Yet, the cellular inflammation in Mer^{KD} mice challenged with
424 OVA returned towards baseline levels by day 10, suggesting that Mer-independent
425 mechanisms ultimately allow clearance of recruited eosinophils in Mer^{KD} mice. The lack of
426 an effect of loss of Mer upon lung histology and on the numbers of tissue eosinophils may be
427 due to different mechanisms involved in eosinophil clearance in the airways and in the
428 interstitial regions. One possibility is that eosinophils exhibit differential susceptibility to
429 apoptosis in these distinct micro-environments, with airway eosinophils being more sensitive
430 to undergoing pharmacological induction of apoptosis and subsequent phagocytic clearance
431 than interstitial eosinophils[9].
432
433 Second, contrary to expectation, we did not observe highly elevated pro-inflammatory
434 cytokine profiles in the Mer^{KD} mice at any of the time points examined during the course of
435 the OVA-induced inflammatory response. Although Mer has been reported to suppress
436 macrophage TNF production (for example following LPS-induced inflammation in the
437 peritoneal cavity or in the lung[27]) it is possible that there may be stimulus-specific effects and
438 that Mer does not act to counter-regulate a Th2-mediated inflammatory response.
439 Comparison of expression levels at day 7 revealed that some potentially important
440 chemokines, such as CCL12 and CCL5 (RANTES), that may act to recruit eosinophils were
441 present at elevated levels in Mer^{KD} mice. However, these chemokines were present at
442 relatively low levels in both WT and Mer^{KD} mice. Similarly, CCL11 and IL-5 that are

443 important for eosinophil recruitment and survival were expressed at roughly equivalent or
444 lower levels in Mer^{KD} mice at 7 days. We suggest that these changes in chemokine/cytokine
445 profiles in the Mer^{KD} mice are unlikely to account for the significant differences in eosinophil
446 numbers observed. Whether Mer-driven resolution of allergic inflammation is associated with
447 changes in the production of pro-resolving lipid mediators remains to be determined, but in a
448 model of sterile peritonitis Mer deficiency was associated with reduced levels of lipoxinA4
449 and Resolvin D1[40].

450

451 Third, the delayed resolution of inflammation we observe in Mer^{KD} mice was accompanied
452 by increased airway resistance, suggesting that the altered inflammatory response in the
453 absence of Mer has consequences in terms of lung function. Since airway resistance was
454 similar in naïve WT and Mer^{KD} animals, Mer is unlikely to represent a dominant factor in
455 regulating airway function under homeostatic conditions. However, perturbation of lung
456 homeostasis following injury or infection could highlight the role for Mer in regulation of
457 responses to airway challenge. We did not find any significant changes in airway mucus
458 production between WT and Mer^{KD} mice. It is possible that the differences in airway
459 inflammation and resistance we have observed do not impact upon mucus production, or that
460 mucus production is a less sensitive indicator of altered inflammation resolution.

461

462 To investigate the underlying mechanism of Mer in the process of inflammation resolution,
463 we directly tested whether induction of high levels of apoptosis in eosinophils would reveal
464 differences in the capacity for clearance of apoptotic cells in Mer^{KD} mice. In these
465 experiments, we treated animals with dexamethasone at the peak of BALF eosinophil
466 recruitment and tracked the extent of apoptosis present in BALF. We observed approximately
467 twice as many Annexin-V positive apoptotic eosinophils in Mer^{KD} mice when compared with
468 WT, consistent with a compromised capacity for eosinophil clearance in the absence of Mer.
469 Glucocorticoids also act to increase Mer expression and function in macrophages which
470 would further highlight the effect of Mer deficiency in this experimental model. Although our
471 experiments did not specifically examine the possibility that some dexamethasone-treated
472 eosinophils were progressing directly to necrosis *in vivo* without having first undergone
473 apoptosis (primary necrosis), this represents an important area for future study. Eosinophils
474 activated by inflammatory mediators can undergo primary necrosis more readily[41,42] and our
475 subsequent experiments demonstrate that apoptotic cells lose their membrane integrity *in vivo*
476 to become necrotic (secondary necrosis[43]), with this effect being marked in the absence of

477 Mer-dependent cell clearance. Our data demonstrates that approximately 25-30 percent of the
478 total capacity for apoptotic cell clearance of bone marrow-derived macrophages or alveolar
479 macrophages is Mer-dependent. Assuming that Mer mediates a similar proportion of
480 macrophage capacity *in vivo*, the extent of apoptosis occurring during resolution of an
481 inflammatory response may be sufficient to overwhelm the Mer-independent phagocytosis
482 component leading to enhanced necrosis and amplification of inflammation.

483

484 In this manuscript, we have identified a role for apoptotic cell clearance by Mer in allergic
485 airway inflammation, demonstrating a delay in resolution of inflammation in Mer^{KD} mice.
486 Together, our data demonstrate that apoptotic cell clearance by phagocytes directly
487 contributes to the resolution of allergic airway inflammation, suggesting augmentation of
488 apoptotic cell clearance as a potential therapeutic strategy for treating allergic inflammation
489 in humans.

490

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633 ## Figure legends

634

635 **Figure 1. Mer^{KD} mice have delayed resolution of allergic airway inflammation *in vivo*.**
636 (A) Schema of experimental protocol. (B) Bronchoalveolar lavage fluid (BALF) eosinophils
637 in wild type (WT) and (C) Mer^{KD} mice at 1, 3, 7 and 10 days post-ovalbumin (OVA) with
638 delta (Δ) change in eosinophil number between day 3 and day 7 shown (n=7-10). (D)
639 Interstitial eosinophils at day 7 in WT and Mer^{KD} mice (n=6-8). (E-F) Representative lung
640 sections stained with haematoxylin and eosin (H&E) at day 7 post-OVA from (E) WT and
641 (F) Mer^{KD} mice (scale bar 20μm, x100 original magnification). (G) Quantification of H&E
642 stained lung sections at 7 days post-OVA treatment (n=5). Data are expressed as mean ±
643 SEM, analyzed by 1-way analysis of variance (ANOVA) with Newman-Keuls Multiple
644 Comparison Test (B, C) or by Student's t-test (D, G), *p<0.05, **p<0.01, ***p<0.001.

645

646 **Figure 2. Mer^{KD} mice have exacerbated allergic airway responses.** (A) Airway
647 responsiveness to aerosolized methacholine was assessed in anesthetized and mechanically
648 ventilated mice at 7 days post-OVA with lung resistance expressed relative to WT baseline
649 (after nebulization of PBS without methacholine; n=4-5). (B-C) Representative day 7 lung
650 sections stained with periodic acid Schiff (PAS) from (B) WT and (C) Mer^{KD} mice (scale bar
651 20μm, x200 original magnification). (D) Quantification of mucus production at day 7 as
652 assessed by the mucus-goblet index (MGI) on PAS stained lung tissue sections (n=5). (E)
653 Bronchoalveolar lavage fluid (BALF) Mucin5AC (MUC5AC; a mucus glycoprotein) and (F)
654 total protein content were measured at day 7 post-OVA (n=6-8). (G) Cytokine array showing
655 day 7 cytokines, chemokines & proteins upregulated in the MerKD mice depicted in red,
656 those downregulated depicted in blue. Data are expressed as mean ± SEM, analyzed by 2-
657 way analysis of variance (ANOVA) (A) or by Student's t-test (D, E, F), *p<0.05, **p<0.01.

658

659 **Figure 3. Mer deficiency impairs phagocytosis of apoptotic eosinophils.** (A) Phagocytic
660 capacity of mouse bone marrow-derived macrophages (mBMDMs) from wild type (WT) or
661 Mer^{KD} mice was assessed after co-culture with apoptotic mouse bone marrow-derived
662 eosinophils in the presence of Protein S (Pros1) (n=4-5). (B) Phagocytic capacity of mouse
663 alveolar macrophages (mAMs) from WT or Mer^{KD} mice was assessed after co-culture with
664 apoptotic human eosinophils in the presence of Pros1 (n=4-5). (C) Schema of *in vivo*
665 experimental protocol. (D) Annexin-V binding of bronchoalveolar lavage fluid (BALF)

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666 eosinophils from WT or Mer^{KD} OVA-treated mice at 8, 16 and 24 hours post-dexamethasone
667 (Dex) treatment (n=4-7). (E) Representative flow cytometry plots (Annexin-V/Siglec-F) from
668 WT and Mer^{KD} mice showing the presence of Annexin-V^{+ve} eosinophils
669 (CD45^{+ve}/CD11b^{+ve}/Ly6G^{-ve}/ Siglec-F^{+ve}/Annexin-V^{+ve} cells) at 16 hours post-Dex. Data are
670 expressed as mean ± SEM, analyzed by Student's t-test (A, B) or 2-way analysis of variance
671 (ANOVA) with Bonferroni test (D), *p<0.05, ***p<0.001.
672
673 **Figure 4. Mer^{KD} alveolar macrophages have an impaired engulfment capacity causing**
674 **delayed apoptotic cell clearance *in vivo*.** CellTracker™ Green fluorescently labeled
675 apoptotic human cells (AC) or PBS control were administered intratracheally (*i.t.*) to naïve
676 wild type (WT) and Mer^{KD} mice, with bronchoalveolar lavage fluid (BALF) collected after 3
677 hours. (A) Total BALF cell count (B) percentage of alveolar macrophages
678 (CD45^{+ve}/CD11c^{+ve}/CD11b^{-ve}) phagocytosing labelled apoptotic cells and (C) representative
679 flow cytometry plots (SSC/CellTracker™ Green) from WT-PBS, or *i.t.* AC treated WT and
680 Mer^{KD} mice showing CellTracker Green positive (i.e. engulfing) alveolar macrophages. (n=3
681 WT-PBS; n=6-7 *i.t.* AC treated WT and Mer^{KD} mice). (D) Uncleared apoptotic cells (CD45^{-}
682 ^{ve}/CellTracker™ Green^{+ve} cells) in BALF after 3 hours (n=7-9). (E) Recruited mouse
683 granulocytes (CD45^{+ve}/Ly6G^{+ve} cells) in BALF (n=2-3). (F) Percentage of necrotic debris
684 present in BALF 3 hours after apoptotic cell administration (n=8-10). Data are expressed as
685 mean ± SEM, analyzed by Student's t-test, *p<0.05, ***p<0.001.
686
687

688 Supplementary Figure Legends:

689

690 **Figure S1. Lack of Mer has no effect on airway resistance in naïve mice.** Relative airway
691 resistance was assessed in anesthetized and mechanically ventilated naïve WT and Mer^{KD}
692 mice in the absence of methacholine and expressed relative to WT mice values. Data are
693 expressed as mean ± SEM, analyzed by Student's t-test (n=6-7).
694
695 **Figure S2. Time course of BALF cytokines and proteins in wild type and Mer^{KD} mice**
696 **post-ovalbumin.** BALF cytokines and proteins were measured by forward phase protein
697 array (n≥3 per genotype at each timepoint) with red depicting high expression and green low
698 expression.

699

700 **Figure S3. Confirmation of Mer expression on bone marrow derived and alveolar**
701 **macrophages from WT mice and absent expression in Mer^{KD} mice.** (A) Western blot of
702 lysates of BMDMs from wild type (WT) or Mer^{KD} mice. (B-C) Representative flow
703 cytometry histograms of AMs isolated from WT and Mer^{KD} mice showing (B) Mer and (C)
704 Axl expression. (D) Bone marrow-derived macrophages (mBMDMs) from WT or Mer^{KD}
705 mice were co-cultured with apoptotic mouse bone marrow-derived eosinophils in the
706 presence of Protein S (Pros1) with or without BMS777607 (BMS; c-met inhibitor to inhibit
707 Axl/Tyro3/Mer) (n=4-5). (E) Alveolar macrophages (mAMs) from WT or Mer^{KD} mice were
708 co-cultured with apoptotic human eosinophils in the presence of Pros1 with or without
709 BMS777607 (n=4-5). Data are expressed as mean ± SEM, analyzed by 2-way analysis of
710 variance (ANOVA) with Bonferroni test (E, F), $^{**}p<0.01$, $^{***}p<0.001$.

711

712 **Figure S4. Analysis of human granulocyte viability, apoptosis and necrosis.** (A)
713 Assessment of viable (Annexin-V^{-ve}/PI^{-ve}), apoptotic (Annexin-V^{+ve}/PI^{-ve}) and necrotic (PI^{+ve})
714 human eosinophils by Annexin-V/propidium iodide (PI) staining by flow cytometry prior to
715 incubation with mouse alveolar macrophages and (B) human granulocytes undergoing
716 constitutive apoptosis prior to intratracheal administration to mice. Example flow cytometry
717 plots and cumulative data shown (n=2-4). (C) Representative cytocentrifuge preparation of
718 aged human eosinophils, black arrow highlights an apoptotic eosinophil with typical cellular
719 shrinkage and nuclear condensation (scale bar 20µm, x1000 original magnification).

720

721 **Figure S5. Flow cytometry gating strategy to identify Annexin-V^{+ve} eosinophils.**
722 Representative flow cytometry plots showing the gating strategy used to identify Annexin-
723 V^{+ve} eosinophils (CD45^{+ve}/CD11b^{+ve}/Siglec-F^{+ve}/Ly6G^{-ve}/Annexin-V^{+ve} cells) within BALF
724 of OVA-challenged mice.

725

726 **Figure S6. Identification of alveolar macrophage phagocytosis of apoptotic cells,**
727 **uncleared human granulocytes and necrotic debris *in vivo*.** (A) Representative flow
728 cytometry plots showing the gating strategy used to identify alveolar macrophages
729 (CD45^{+ve}/CD11c^{+ve}/CD11b^{-ve} cells) within the bronchoalveolar lavage fluid (BALF) of
730 apoptotic cell-treated mice. (B-C) Representative flow cytometry plots demonstrating AM
731 population (red) and low FSC/SSC necrotic debris population (blue) in BALF from (B) WT
732 and (C) Mer^{KD} mice 3 hours after administration of apoptotic cells. (D-E) Cytocentrifuge

733 preparations of FACS sorted (D) low FSC/SSC events and (E) CD45^{+ve}/F4/80^{+ve}/CD11c^{+ve}
734 alveolar macrophages (scale bar 40µm, x1000 original magnification).
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Figure 1 Felton JM *et al.*

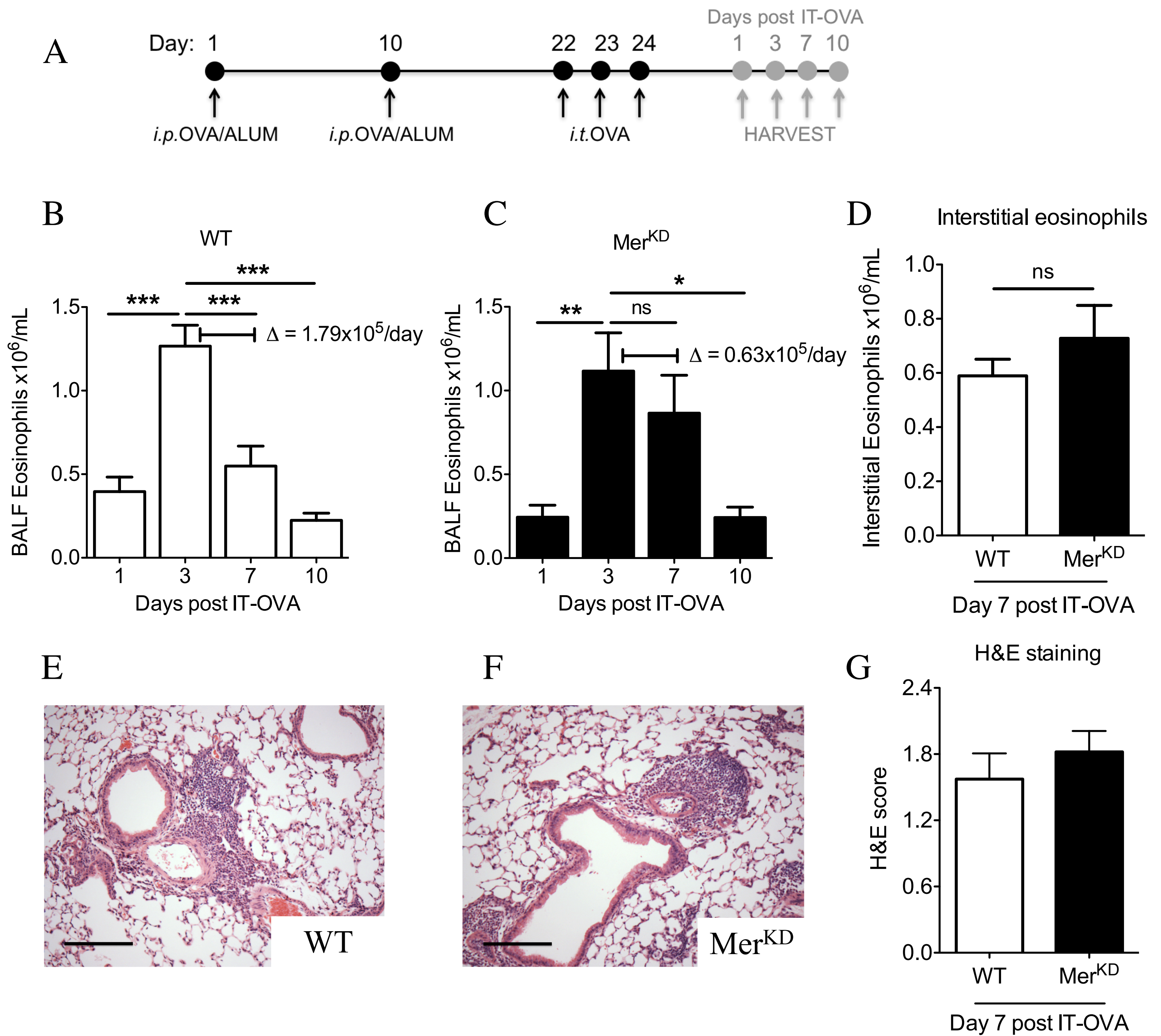

Figure 2

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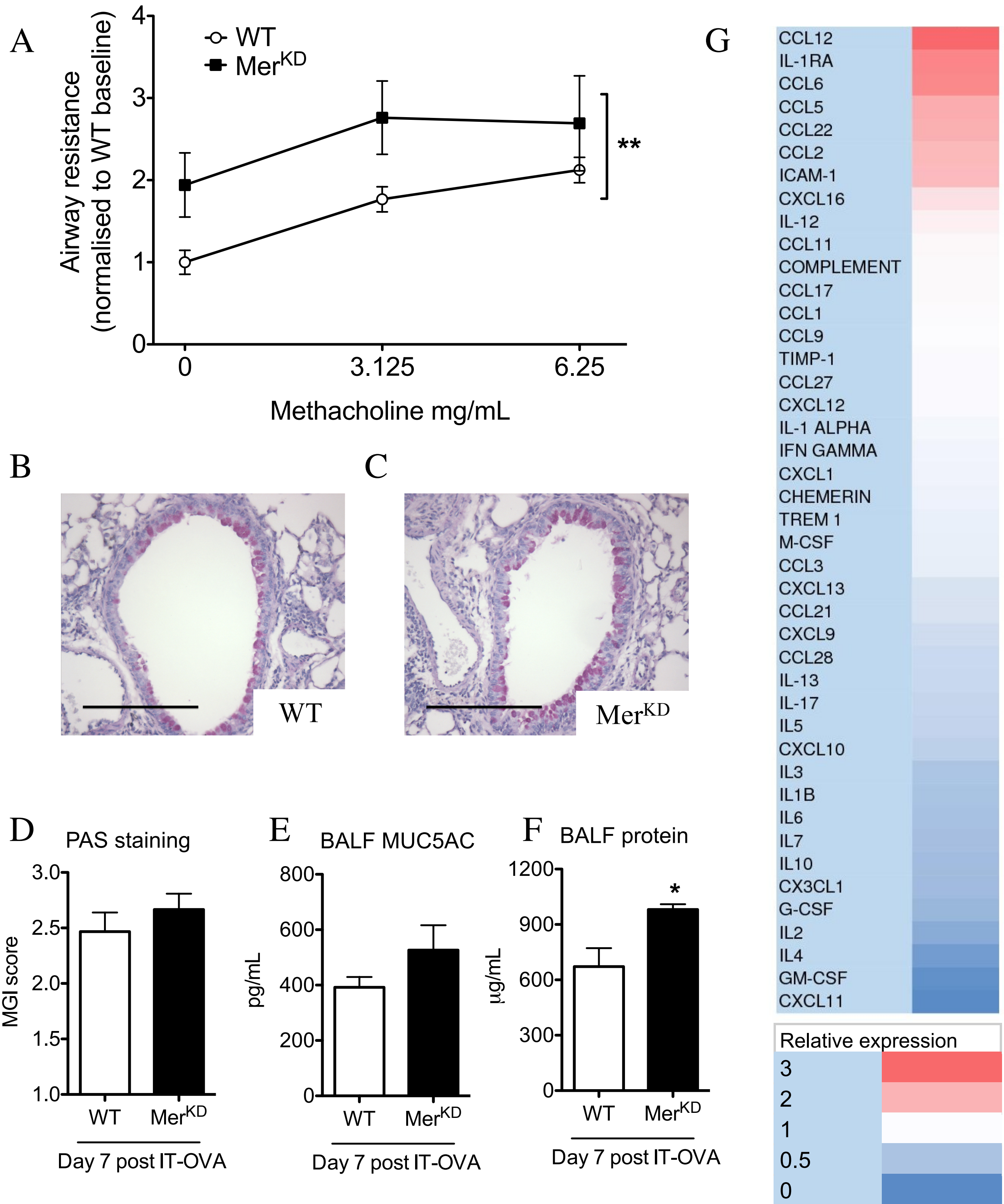

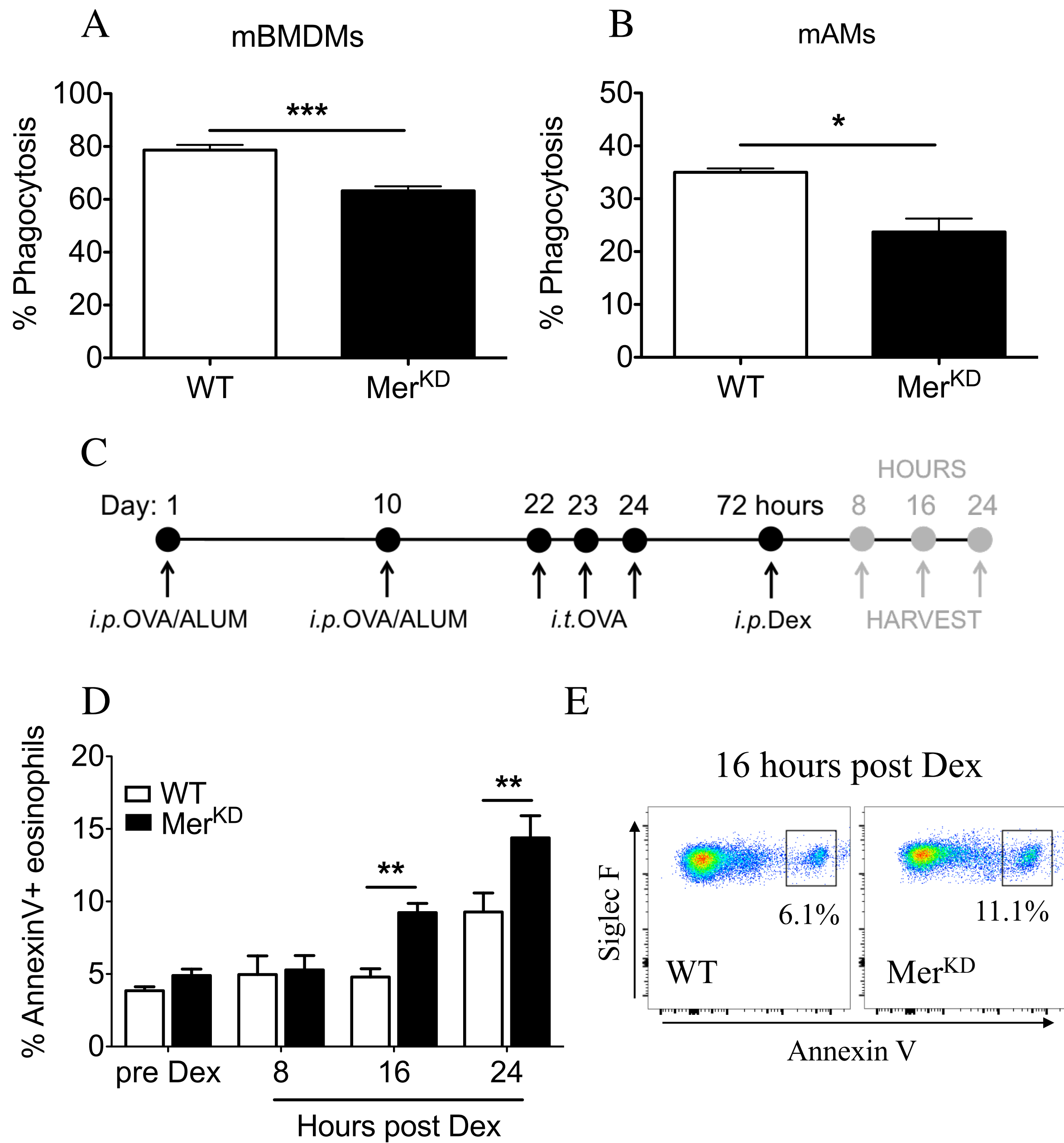
A
mBMDMs
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***
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Mer^KD
B
mAMs
% Phagocytosis
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WT
Mer^KD
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72 hours
HOURS
8
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i.p.OVA/ALUM
i.p.OVA/ALUM
i.t.OVA
i.p.Dex
HARVEST
D
% AnnexinV+ eosinophils
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WT
Mer^KD
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pre Dex
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6.1%
11.1%
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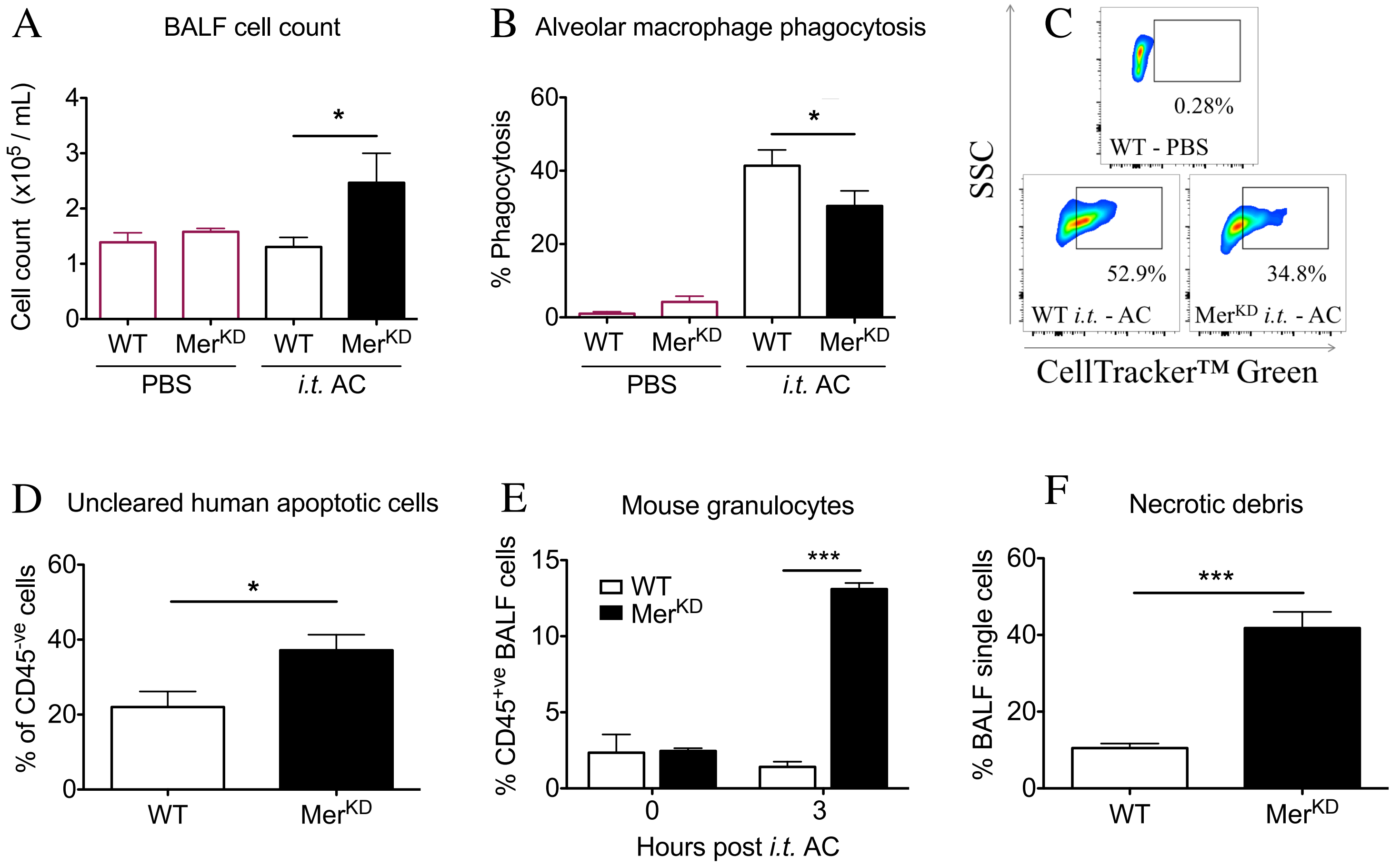
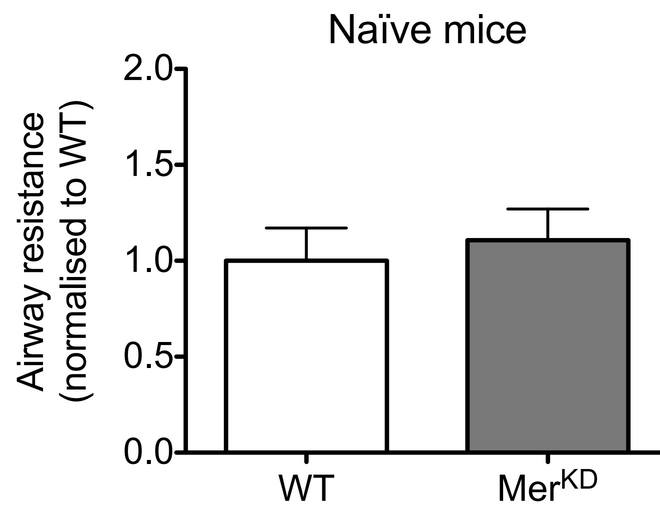
Annexin V

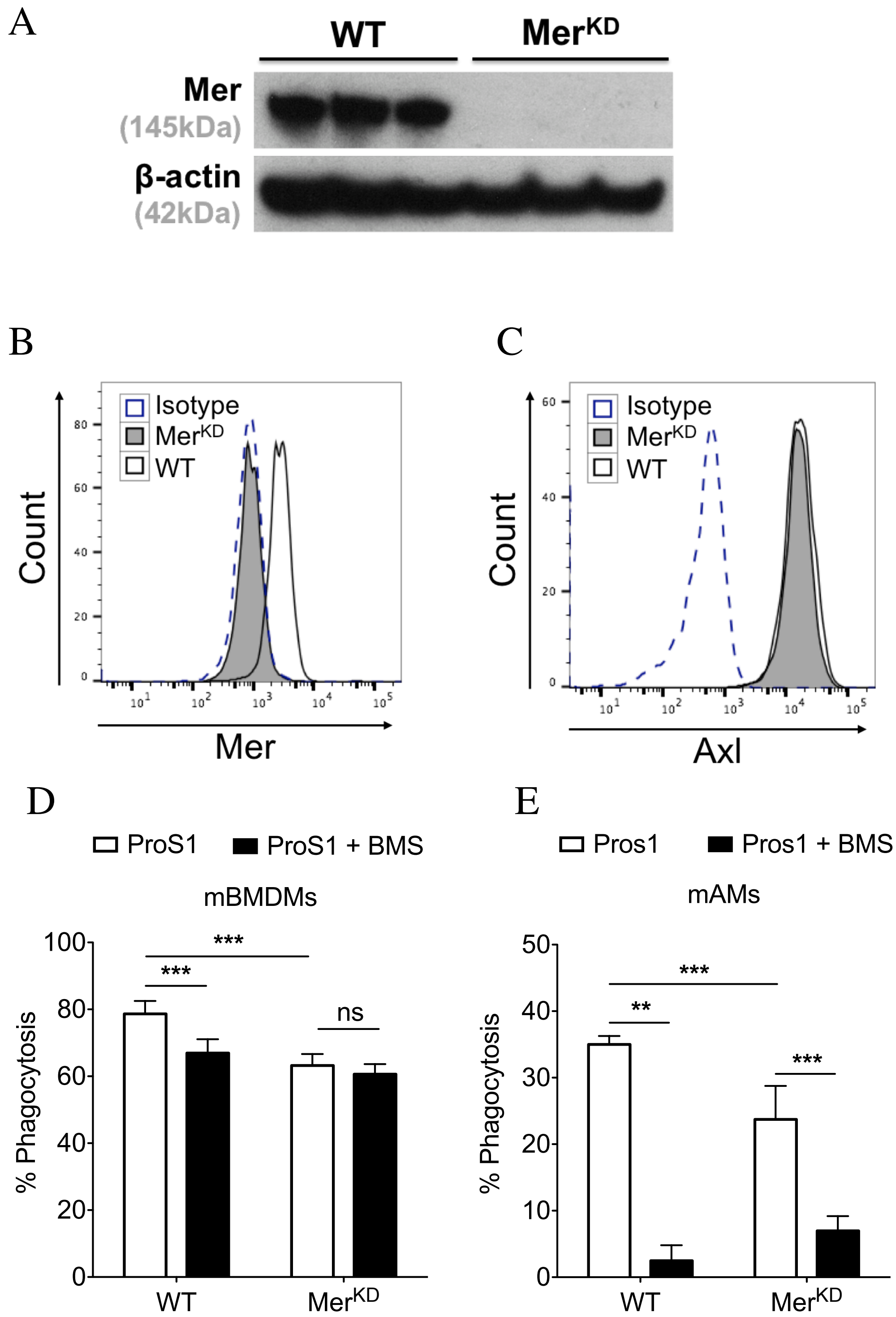
Figure 4 Felton JM *et al.*



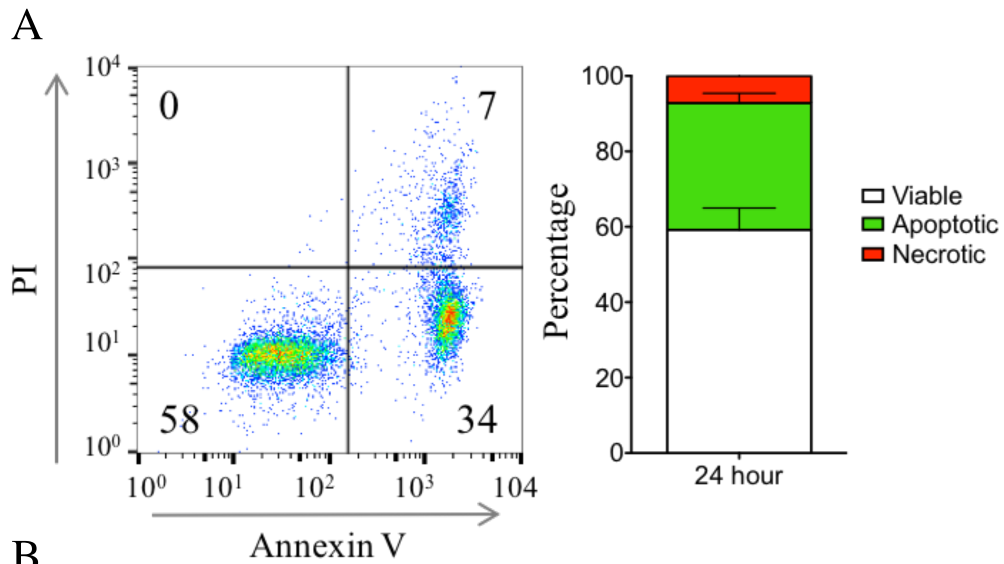
Supplementary Figure S1 Felton JM *et al.*



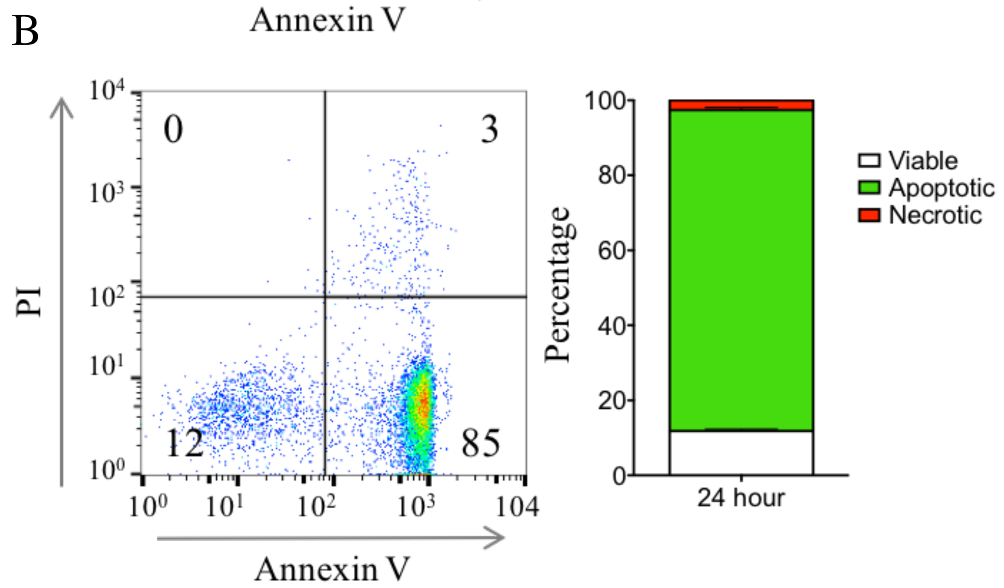
Supplementary Figure S2 Felton JM *et al.*


A
WT
MerKD
Mer
(145kDa)
β-actin
(42kDa)
B
Isotype
MerKD
WT
Count
Mer
C
Isotype
MerKD
WT
Count
Axl
D
ProS1
ProS1 + BMS
mBMDMs
% Phagocytosis
***
***
ns
WT
MerKD
E
Pros1
Pros1 + BMS
mAMs
% Phagocytosis
***
**
***
WT
MerKD

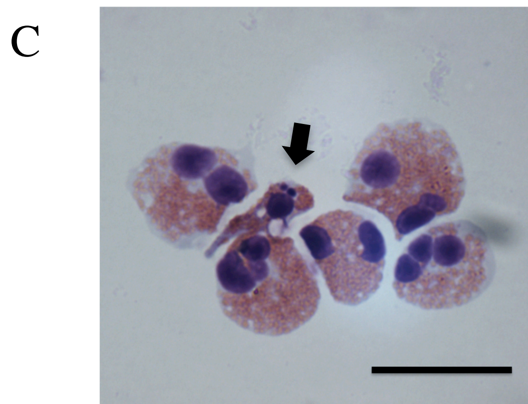
A



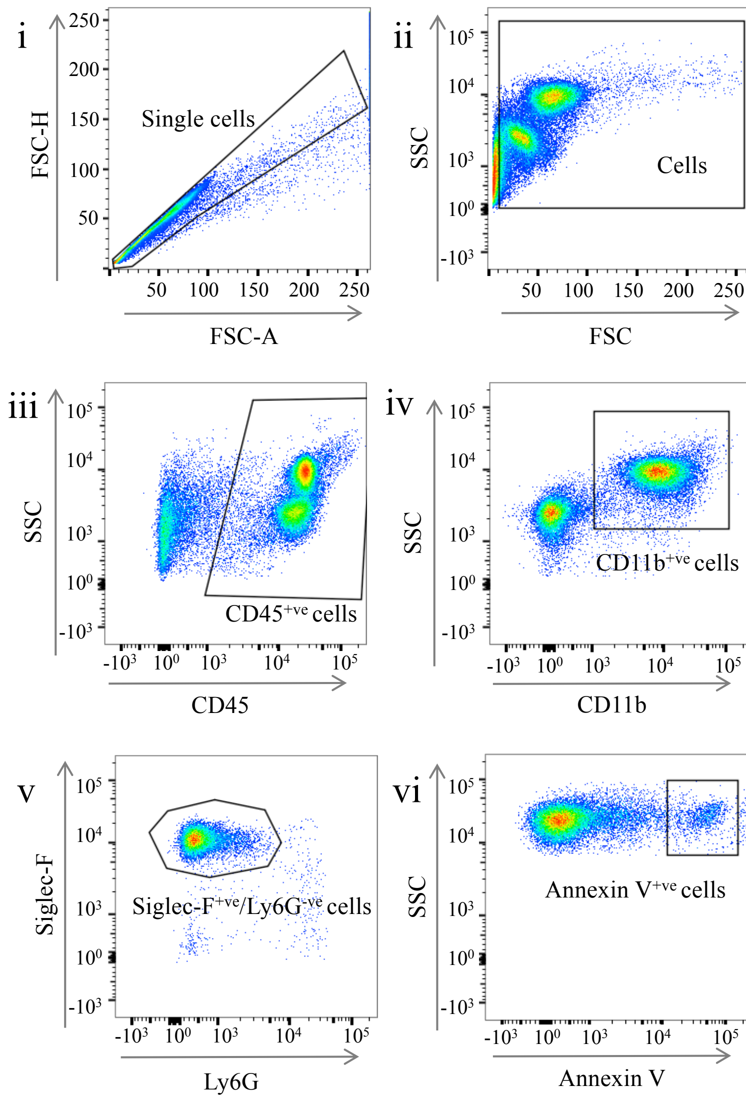
B



C

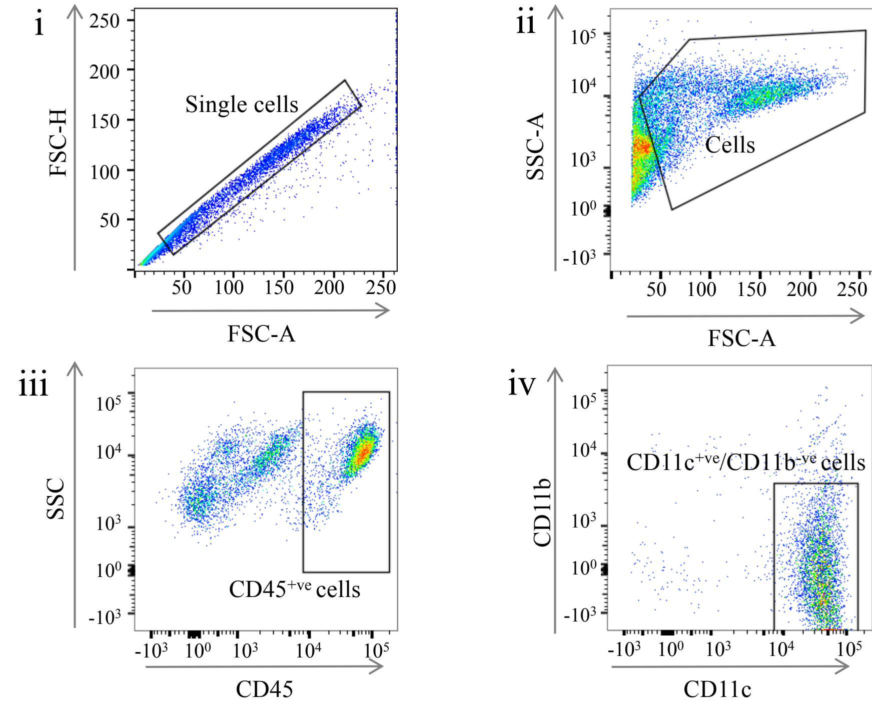


Supplementary Figure S5 Felton JM *et al.*

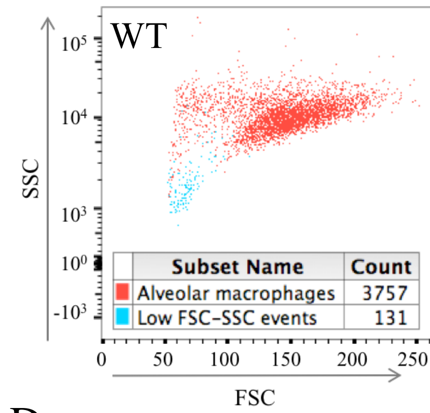


Supplementary Figure S6 Felton JM *et al.*

A

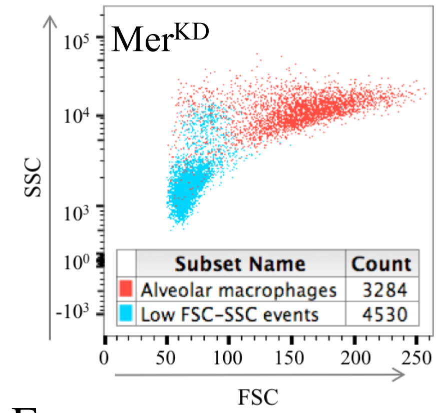


B



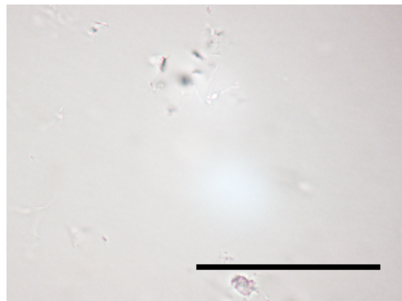
	Subset Name	Count
	Alveolar macrophages	3757
	Low FSC-SSC events	131

C



	Subset Name	Count
	Alveolar macrophages	3284
	Low FSC-SSC events	4530

D



E