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Tumour-derived GM-CSF promotes granulocyte immunosuppression in mesothelioma patients

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1 **Tumour-derived GM-CSF promotes granulocyte immunosuppression in**
2 **mesothelioma patients**

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19

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23 **Conflict of Interest**

24 The authors declare that they have no potential conflict of interest.

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26 **Abstract**

27 **Purpose:** The cross talk between tumour cells, myeloid cells, and T cells can play a
28 critical role in tumour pathogenesis and response to immunotherapies. Although the
29 aetiology of mesothelioma is well understood the impact of mesothelioma tumour cells
30 on the surrounding immune microenvironment is less well studied. In this study the effect
31 of the mesothelioma tumour microenvironment on circulating and infiltrating
32 granulocytes and T cells is investigated.

33 **Experimental Design:** Tumour tissues and peripheral blood from mesothelioma patients
34 were evaluated for presence of granulocytes, which were then tested for their T cell
35 suppression potential. Different co-cultures of granulocytes and/or mesothelioma tumour
36 cells and/or T cells were set up to identify the mechanism of T cell inhibition.

37 **Results:** Analysis of human tumours showed that the mesothelioma microenvironment is
38 enriched in infiltrating granulocytes, which inhibit T cell proliferation and activation.
39 Characterisation of the whole blood at diagnosis identified similar, circulating,
40 immunosuppressive $CD11b^{+}CD15^{+}HLADR^{-}$ granulocytes at increased frequency
41 compared to healthy controls. Culture of healthy-donor granulocytes with human
42 mesothelioma cells showed that GM-CSF upregulates NOX2 expression and the release
43 of Reactive Oxygen Species (ROS) from granulocytes, resulting in T cell suppression.
44 Immunohistochemistry and transcriptomic analysis revealed that a majority of
45 mesothelioma tumours express GM-CSF and that higher GM-CSF expression correlated
46 with clinical progression. Blockade of GM-CSF with neutralising antibody, or ROS
47 inhibition, restored T cell proliferation suggesting that targeting of GM-CSF could be of
48 therapeutic benefit in these patients.

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49 **Conclusions:** Our study presents the mechanism behind the cross-talk between
50 mesothelioma tumours and the immune micro-environment and indicates that targeting
51 GM-CSF could be a novel treatment strategy to augment immunotherapy in patients with
52 mesothelioma.

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53 **Translational Relevance**

54 The functional role of granulocytes and their cross talk with tumour cells and T cells in
55 human mesothelioma is not well understood. We demonstrated that GM-CSF is expressed by
56 mesothelioma tumour cells, and can polarize granulocytes to up regulate ROS production which
57 in turn suppresses the T cell proliferation and function. As GM-CSF plays a role in driving an
58 immunosuppressive granulocyte phenotype in mesothelioma, targeting GM-CSF could
59 represent an alternative therapeutic approach for these patients.

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60 **Introduction**

61 Malignant mesothelioma is an aggressive cancer arising from the mesothelial cells lining
62 the pleura, peritoneum and pericardium (1). The majority of patients present with
63 advanced stage disease and are not candidates for surgery. Although chemotherapy
64 improves outcome for these patients, the median overall survival is less than 24 months
65 (2). Immunotherapy approaches relying on T cell anti-cancer activity, such as peptide
66 vaccines and CAR T cells, have shown only limited efficacy suggesting that the
67 underlying immune microenvironment may play a role in muting the immune response
68 (3, 4).

69 Myeloid cells play an important role in the balance of pro- and anti- cancer T cell
70 responses. Murine models of mesothelioma have shown that monocytes, macrophages,
71 and dendritic cells may be modulated by the tumour microenvironment (5-7). However,
72 the functional role of granulocytes and their mechanism of action in human mesothelioma
73 is not well understood. Studies in mesothelioma have suggested the ratio between
74 peripheral blood or intra-tumoural neutrophils and lymphocytes correlates with
75 prognosis, indicating a key interaction between these cells in tumour pathogenesis (8). In
76 other cancers, secreted factors within the tumour microenvironment control the
77 differentiation of granulocytes. In turn this may promote inflammation within the tumour
78 microenvironment or lead to changes in the interaction with the adaptive immune
79 response. Here we investigate the mechanisms underlying the cross-talk between
80 mesothelioma tumour cells, granulocytes and T cells.

81

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82 **Materials and Methods**

83 **Patients and sample collection**

84 Heparinized blood samples were obtained from patients with malignant mesothelioma
85 (n=47) who were enrolled in IRB approved protocols at the National Cancer Institute,
86 Bethesda, USA and the University of Birmingham, UK before treatment (Table S1).
87 Written informed consent was obtained from all the patients and the study was conducted
88 in accordance with recognized ethical guidelines. Blood from healthy donors was
89 obtained from the NIH Blood Bank (n=30) and at the University of Birmingham, UK
90 (n=18), in heparin tubes. Patients with both histologically confirmed pleural (n=24) and
91 peritoneal (n=9) mesothelioma were included in this study and at the time of enrolment
92 had clinical and/or radiological evidence of disease. A number of patients had received
93 prior treatments including surgery and systemic chemo or immune-therapy (Table S1).
94 The transcriptomes of 87 mesothelioma tumours diagnosed between 1999 and 2013, held
95 within the R2: Genomics Analysis and Visualisation Platform (http://r2.amc.nl) were
96 analysed for CSF2 expression. Patients were aged from 28 to 81 years of age at
97 diagnosis. 56 patients had a history of asbestos exposure, 14 had no history, and 17 were
98 not known. Of the 87 patients' samples histologies were distributed as follows: 23
99 biphasic, 5 diffuse, 57 epithelioid, 2 sarcomatoid.

100

101 **Cell lines**

102 Human mesothelioma cell lines (ED(MSTO211)-H, AC-Meso Y9-Meso, MPM15,
103 MPM26, MPM30, MPM34, MPM43) purchased from the Aichi Cancer Research Centre

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104 Institute and Mesobank UK were cultured in RPMI-1640 (Invitrogen) with 10% heat-
105 inactivated fetal bovine serum, glutamine (1X), sodium pyruvate (1X) and Penicillin-
106 Streptomycin (RPMI 10%=R10%). The cell lines were cultured in a humidified
107 atmosphere at 37°C with 5% CO_2. All cell lines were verified by Northgene (UK) DNA
108 Short Tandem Repeat analysis within the last 6 months. All cell lines were tested of
109 mycoplasma and were negative). Cell lines were used for up to 5 passages.

110

111 **Flow cytometric analysis of whole blood and tumours**

112 Whole blood and fresh tumour samples from diagnostic surgery were processed within
113 12h of collection. 10 samples from patients with benign pleural pathologies of infectious
114 and inflammatory nature were also included as a comparison. Whole blood was either
115 lysed using ammonium chloride solution according to manufacturer's instructions
116 (Qiagen) or using a hypertonic ammonium chloride solution (150mM NH_4Cl, 10mM
117 $KHCO_3$, 0.1mM EDTA) for 10 minutes at room temperature (maintained at 21-23°C) at a
118 ratio of 1:9 (volume of sample: volume of lysing solution) prior to antibody staining.
119 Where indicated peripheral blood was separated using a Lymphoprep density gradient.
120 Tissue samples were digested using Type II collagenase (Worthington) for three hours at
121 37°C. Immune populations were identified by staining with anti- CD11b, anti-HLA-DR,
122 anti-CD13, anti-CD14, anti-CD15, anti-CD66b, and anti CD45 antibodies (BD
123 Biosciences) on ice or at room temperature for 30minutes. Cells were acquired using
124 FACS-Canto II (BD Biosciences) and Cyan (Beckman Coulter) and analysed either by
125 FCS Express 4 software (DeNovo Software) or FlowJo (Tree Star).

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126

127 **Isolation of granulocytes, T cells, and mesothelioma cells for functional assays**

128 The whole blood from healthy donors and patients were processed as described above.
129 We isolated the low density granulocytes from the peripheral blood mononuclear layer
130 and high density granulocytes from the layer of white cells on the red cell pellet,
131 following Lymphoprep centrifugation, by magnetic bead isolation using anti-CD15
132 microbeads (BD Pharmingen) and MACS LS separation columns (Miltenyi Biotech)
133 according to manufacturer's instructions. Cell purity was >98% as confirmed by flow
134 cytometry. Cell populations were similarly isolated from collagenase digested tumours
135 using MACS beads (anti-CD15 for granulocytes and anti-CD14 for monocytes), followed
136 by flow cytometric confirmation of purity. The dose of collagenase selected has
137 previously been established to not affect cell surface marker expression or cell viability.

138 For isolation of autologous T cells and myeloid cells from the whole blood the target
139 populations were enriched first using positive selection with CD45 magnetic beads
140 (Miltenyi Biotech), followed by staining with myeloid antibodies (above) and anti-CD3
141 antibody (Biolegend). Cells were sorted on Astrios (Beckman Coulter) using a 100µm
142 nozzle. DAPI was used as a viability marker to gate out the dead cells.

143

144 **Granulocyte polarisation**

145 To generate tumour conditioned media (TCM), cell lines or sorted patients' tumour cells
146 were plated (1.5 x 10^6 cells) and cultured for 72 hours. The conditioned media was
147 removed and filtered prior to use. Following lymphoprep isolation high density

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148 granulocytes were enriched by CD15 magnetic bead isolation as above, healthy donor
149 granulocytes were plated in R10% in 24 well plates, at concentrations of 1x 10^6 per well.
150 TCM was added as 25% of the total volume as indicated. Granulocytes were harvested
151 following 24 hours of culture, washed twice prior to use in suppression assays.
152 Granulocyte viability was confirmed to be >90% in all cases, by flow cytometry, before
153 further experimentation.

154

155 **Autologous T-cell proliferation assays**

156 Sorted $CD3^+$ T cells were labelled with 10μM carboxyfluorescein diacetate succinimidyl
157 ester (CFSE; Life Technologies) and cultured with sorted granulocytes at ratios of 1:0,
158 1:0.5, 1:1 in complete media at 37°C, 5% CO_2 for 4 days in the presence of 1:1 ratio of
159 anti-CD3/ anti-CD28 dynabeads (Invitrogen). Cells were stained with V450 anti-CD4
160 (Clone-RPA-T4; BD Biosciences) and APC-Cy7 anti-CD8 (Clone-RPA-T8; BioLegend)
161 and proliferation was determined by CFSE dilution. Unstimulated T cells were used as a
162 negative control. The effect of the addition of L-NMMA (0.5mM, NG-Methyl-L-arginine
163 acetate), nor-NOHA (0.5mM, N-Omega-hydroxy-nor-L-arginine) and iNAC (10mM) (all
164 from Sigma Aldrich) was similarly tested. The percentage of cells that diluted CFSE
165 (divided cells) was determined.

166

167 **Peripheral Blood Lymphocyte cell proliferation assay**

168 Peripheral blood lymphocytes (PBLs) (2 x 10^5) were cultured in 96 well flat bottom
169 plates with coated anti-CD3 antibody (3μg/mL) and anti-CD28 antibody (2μg/mL), in

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170 200µL R10%. Cells were incubated at 37ºC, 5% CO_2 for 4 days and then 1µCi/well ^{3}H-
171 thymidine (Perkin Elmer Life Sciences) was added for 12-16 hours. ^{3}H-thymidine
172 incorporation was measured using a TopCount reader (Perkin Elmer). The suppressive
173 ability of autologous or conditioned granulocytes was assessed by co-culturing purified
174 cells together with the PBLs. nor-NOHA (0.5mM), L-NMMA (0.5mM), iNAC (10mM;
175 Sigma Aldrich) was added to cells in culture. 25mM HEPES was added to the medium to
176 maintain the pH after iNAC addition. Data are expressed as a percentage of PBL
177 proliferation driven by antibody co-stimulation in the presence of MDSC, compared with
178 PBL proliferation in the absence of suppressive cells (100%).

179

180 **Reactive Oxygen Species assay**

181 Sorted granulocytes were stained with 2', 7'– dichlorofluorescein diacetate (DCFDA)
182 using DCFDA cellular ROS detection assay kit (Abcam) for 30 minutes at 37ºC. The
183 stained cells were analyzed on a BD FACS Calibur and Cyan (Beckman Coulter). Cells
184 stained with Tert-butyl hydrogen peroxide (TBHP), TCM polarized granulocytes were
185 also incubated with Phorbol 12-myristate 13-acetate (PMA) (concentration need to be
186 added) during the staining with DCFDA, this was used as a positive control.

187 Quantification of H_2O_2 production was measured using the Amplex Red Hydrogen
188 Peroxidase assay kit (Invitrogen). Following culture in mesothelioma conditioned media
189 for 24 hours, sorted granulocytes were washed twice in R10%, counted and plated in
190 Krebs–Ringer phosphate buffer, according to manufacturer's guidelines. Detection of

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191 H_2O_2 was carried out following 30 minutes of incubation at 37°C using a microplate
192 reader at 560nm.
193

194 **ELISA**

195 The concentrations of cytokines within conditioned media following culture with T cells,
196 mesothelioma cell lines ($1x10^6$/mL) or sorted tumour cells were quantified using a
197 competitive enzyme linked immunoassay according to the manufacturers' instructions.
198 The following molecules were tested GM-CSF (Biolegend), IL-13 (BD Biosciences), IL-
199 8 (Biolegend), IL-6 (Biolegend), G-CSF (R&D Systems), VEGF (R&D Systems),
200 Mesothelin (Biolegend).The concentration of IFN-γ in co-culture supernatants was
201 determined by Ready Set Go ELISA kit (eBioscience).
202

203 **RT-Q-PCR analysis**

204 RT-Q-PCR was used to detect NOX2 expression in cell line supernatant conditioned
205 granulocytes (0, 4, 8, 12, 24 hour time points). RNA was extracted using an RNeasy Mini
206 kit (Qiagen). cDNA was prepared using SuperScriptTM III Reverse Transcriptase
207 (Invitrogen, CA) following the manufacturer's instructions. RT-Q-PCR was done in
208 duplicate using FAST SYBR Green Master Mix (Applied Biosystems) and the Applied
209 Biosystems 7500 Fast Real-Time PCR system. Analysis of gene expression was
210 calculated according to $2^{-\Delta T}$ method and plotted as arbitrary units of mRNA relative to
211 GAPDH. Gene specific primer sequences were NOX2 (CAAGATGCGTGGAAACTA,

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212 F; TCCCTGCTCCCACTAACA, R) and GAPDH (CCAGCCGAGCCACATCGCTC, F;
213 ATGAGCCCCAGCCTTCTC, R) (Eurofins).
214

215 **Immunohistochemistry**

216 Mesothelioma sections, from diagnostic tumour biopsies (n=38), were deparaffinised in
217 Histoclear (National diagnostics) and ethanol, and rehydrated in 0.3% hydrogen peroxide
218 for 15 minutes. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0)
219 for 20 minutes in a microwave oven. Slides were cooled and washed prior to blocking in
220 5X Caesin (Thermofisher) for 30 minutes at room temperature. Sections were then
221 incubated over night with primary antibody, rabbit anti-GM-CSF (Novus Biologicals),
222 diluted in PBS. Sections were washed and secondary antibody (Universal ImmPRESS
223 antibody, Vector Laboratories) was added at room temperature for 30 minutes, followed
224 by further washing and addition of DAB substrate (ImmPACT DAB, Vector
225 Laboratories) for 5 minutes. After counterstaining with Harris haematoxylin (Sigma),
226 slides were dehydrated using ethanol and Histoclear and mounted using Omnimount
227 (National diagnostics). Slides were examined and photographed using a Nikon Eclipse
228 400 microscope.
229

230 **Statistical analysis**

231 Continuous parameter values were compared between two groups using an exact form of
232 a Wilcoxon rank sum test. Paired comparisons were performed using a Wilcoxon signed
233 rank test. Spearman correlation analysis was used to determine the correlation between

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234 age and MDSC parameters. The correlations are interpreted as follow: strong if $|r| > 0.70$;
235 moderately strong if $0.50 < |r| < 0.70$; weak to moderately strong if $0.30 < |r| < 0.50$; weak
236 if $|r| < 0.30$. All p-values are two-tailed and presented without adjustment for multiple
237 comparisons because all tests performed were considered to be exploratory.

238

239 **Results**

240 **Mesothelioma tumours modulate infiltrating myeloid cells to suppress T cell**
241 **responses**

242 The immune microenvironment in mesothelioma has been shown to have strong
243 prognostic implications, with infiltration by $CD8^{+}$ lymphocytes conferring a favourable
244 prognosis (9) and the association of peripheral blood granulocyte –to-lymphocyte ratio
245 with poorer prognosis (10-12). However our understanding of the biological cross-talk
246 between mesothelioma cells, granulocytes, and T cells in human patients is limited.

247 Interrogation of the transcriptomic profile of 87 mesothelioma tumours, held within the
248 R2: Genomics Analysis and Visualisation Platform (http://r2.amc.nl), suggested a
249 significant infiltration of immune cells in the tumour microenvironment (Fig. 1a). Flow
250 cytometric analysis of 18 digested, human mesothelioma tumours confirmed this data at
251 the cellular level within the tumour microenvironment (Mean: CD15= 8.6%, CD14=
252 4.8%, CD3=6.7%; Fig. 1b). Immuno-histochemical staining of a further 38 mesothelioma
253 tumours supported the findings, with identification of granulocytes in all samples at
254 diagnosis (Figure 1c). Characterisation of tumour-associated granulocytes by flow-
255 cytometry showed that they expressed CD11b, CD15, with low/absent CD33 expression,

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256 and low/absent HLA-DR expression (Fig. 1d). Murine model of mesothelioma recently
257 identified that the immune-regulatory transcriptome of granulocytes may be altered
258 within the tumour microenvironment with potential effects on surrounding T cells and
259 tumour cells.(13) To investigate the effects of granulocytes on T cells, $CD15^+$
260 granulocytes were sorted from mesothelioma tumours at the time of resection, and co-
261 cultured with T cells from healthy donors. Tumour derived granulocytes suppressed T
262 cell proliferation to a greater extent, compared to those from healthy donor blood or those
263 extracted from pleural tissue with benign pathologies (Fig. 1e). Similarly $CD14^+$
264 monocytes from mesothelioma tumours were sorted and also found to suppress T cell
265 proliferation ($p=0.0002$) (Supplementary Fig. 1a). Therefore the tumour
266 microenvironment is able to locally modulate infiltrating myeloid cells to inhibit T cell
267 proliferation.

268

269 **Mesothelioma creates a systemic immunosuppressive environment through**
270 **circulating granulocytes**

271 The effects of mesothelioma tumours on the immune system may be limited to the local
272 tissue microenvironment or could also lead to systemic alteration. To test this we
273 compared T cells from the blood of patients at diagnosis to those from healthy donors,
274 and observed that mesothelioma patients' T cells have a reduced proliferation capacity
275 compared to those in healthy donors (Fig. 2a). We have previously identified that
276 tumour-metabolism of arginine can create a systemic environment inhibitory to T cell
277 responses. As mesotheliomas are known to be arginine auxotrophs, we measured the
278 arginase activity of mesothelioma cell lines (14). No significant arginase activity was

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279 identified suggesting an alternative mechanism must be responsible (Supplementary Fig.
280 1b). As we identified immunosuppressive granulocytes infiltrating mesothelioma
281 tumours, we therefore hypothesised the T cell suppression may be due to the presence of
282 these circulating immunosuppressive myeloid cells.

283 To investigate the hypothesis, the frequency of granulocytic and monocytic cells was
284 characterised in the whole blood of healthy donors and mesothelioma patients (n=33) at
285 diagnosis (Table S2). There were significant increases in the percentage of HLA-DR-
286 granulocytes ($CD14^{-}CD15^{+}CD11b^{+}HLADR^{-}$) in the whole blood, compared to healthy
287 controls (p=0.013) (Fig. 2b). Subpopulation analysis revealed only a marginal difference
288 in HLA-DR- monocytes (CD14+CD15-CD11b+HLADR-) compared to healthy donors
289 (p=0.05) (Figure 2c) and no difference in the frequency of $HLA\text{-}DR^{+}$ monocytes
290 ($CD14^{+}CD11b^{+}HLADR^{+)}$ (median 7.0% versus 8.9%; p=0.14) or $HLA\text{-}DR^{+}$ granulocytes
291 ($CD15^{+}CD14^{-}CD11b^{+}HLADR^{+}$) cells (median 0.83% vs. 1.46%; p=0.29) between
292 healthy donors and mesothelioma patients. Consistent with reports of increased
293 granulocyte:lymphocyte ratios in mesothelioma patients, granulocytes were the most
294 predominant population overall (Median Frequency: $CD14^{+}$ Monocytes-: 0.198% vs
295 $CD15^{+}$ Granulocytes- 66.8%). Immunophenotyping revealed significant differences in
296 the relative expressions of granulocyte markers CD11b and CD66b compared to those
297 from healthy donors (Fig. 2d and Supplementary Fig. 1c).

298 As $CD15^{+}$ granulocytes are the major population of circulating myeloid cells in
299 mesothelioma patients and shared the same immunophenotype as tumour-infiltrating
300 granulocytes described above, their functional effects on T cells was examined further.
301 Co-culture of sorted whole blood granulocytes from patients decreased both autologous

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302 CD4$^+$ and CD8$^+$ T cell proliferation at ratios of 1:1 and1:0.5 (T cells:granulocytes) (Fig.
303 2e and Supplementary Fig. 1d) and activation (p=0.0078, Fig. 2f).

304 Immunosuppressive granulocytes may be methodologically identified in the PBMC layer
305 (classical G-MDSC; low-density) and on the red cell pellet (high-density) following
306 density gradient centrifugation of whole blood. Analysis of separated whole blood
307 identified a significant increase in the frequency of granulocytes within the PBMC layer,
308 however the frequency is extremely low (median <10%) with 90% of the granulocytes
309 lying on the red cell pellet (Figure 3a). This small population of granulocytes had only a
310 weak ability to suppress T cell proliferation (Fig. 3b). Only the high-density
311 granulocytes, which had increased frequency in the patients, had suppressive activity
312 (Figure 3b). Granulocytes from healthy donors had minimal effects on T cell proliferation
313 (Fig. 1e and Supplementary Fig. 2a) or IFN- γ release (Supplementary Fig. 2b). Therefore
314 granulocytes within the blood and tumours of mesothelioma patients share the same
315 immunophenotype and functional capacity to suppress T cell proliferation and activation,
316 thus extending the immunosuppressive microenvironment.

317

318 **Mesothelioma conditioned granulocytes suppress T cell proliferation by generation**
319 **of reactive oxygen species**

320 Granulocytes can impair T cell proliferation through a number of mechanisms including
321 arginine depletion, nitric oxide species or reactive oxygen species production, and release
322 of immunosuppressive cytokines (15-19). We examined iNOS and Arginase I expression
323 in patients' granulocytes and those from healthy donors identifying no significant

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324 differences in expression (Fig.3c). The addition of the Arginase or iNOS inhibitors, nor-
325 NOHA or L-NMMA respectively, to sorted patients' granulocytes did not rescue T cell
326 proliferation thus excluding these mechanisms. (Fig.3d). No evidence for
327 immunosuppressive cytokine release from these cells was identified in patient plasma by
328 ELISA (IL-10, IL-1β, IL-4, IL-13 Supplementary 2c). In a mesothelioma murine model
329 Reactive Oxygen Species have been demonstrated to suppress T cell responses (20).
330 Gating on granulocytes identified that mesothelioma patients' upregulate ROS, compared
331 to healthy controls ($p=0.03$; Fig. 3e, Supplementary Fig. 2d). Addition of the ROS
332 inhibitor iNAC, to sorted patients' granulocytes restored both autologous $CD4^+$ and $CD8^+$
333 T cells proliferation (Fig. 3f and Supplementary Fig. 2e) and IFN-γ release (Fig. 3g). We
334 confirmed that ROS production was reduced by the addition the inhibitor iNAC
335 ($p=0.031$, Fig. 3h). PDL1 is another mechanism that myeloid cells may use to modulate T
336 cells. There was no significant difference in the frequency of CD15+PDL1+ cells in the
337 blood or tumours of patients compared to those from healthy controls (Supplementary
338 Fig. 2f). Correlating the frequency of PDL1+CD15+ cells with CD3+ frequency revealed
339 no significant correlation in the blood ($p=0.4976$, $r=-0.3214$) (Supplementary Figure 2f),
340 but there was a significant correlation in the tumour ($p=0.0583$, $r=0.8286$)
341 (Supplementary Fig 2g and h). The findings suggest that in the tumour, granulocyte
342 PDL1 may be a secondary mechanism of modulating T cell numbers inside the tumour
343 microenvironment, but not peripherally.

344

345 **GM-CSF from mesothelioma tumour cells drives granulocyte ROS production**

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346 Although it is recognised that mesothelioma tumours release G-CSF, which may
347 contribute to granulocyte expansion and recruitment, the mechanism by which
348 granulocytes are polarised to upregulate ROS production and suppress T cells is unknown
349 (21, 22). Granulocytes produce reactive oxygen specifies through the activity of NADPH
350 oxidase enzyme (NOX2) expression. Consistent with this we demonstrated NOX2 is
351 expressed in patients' granulocytes and healthy donors (Fig. 4a). To examine the effect of
352 the mesothelioma microenvironment on granulocytes, healthy-donor derived
353 granulocytes were cultured in the conditioned supernatants of mesothelioma cell lines or
354 primary tumours. Conditioned supernatants led to an upregulation of NOX2 expression
355 over time (Fig. 4b), with accompanying increase in the production (Figure 4c,
356 Supplementary Fig. 3a and 3b) and release of reactive oxide species (Fig. 4d). PMA was
357 used as positive control for ROS induction in granulocytes upregulation, confirming the
358 mesothelioma specific mechanism (Supplementary Fig. 3c). The mesothelioma-
359 conditioned granulocytes showed a strong ability to suppress T cell proliferation (Figure
360 4e), which could be rescued by the addition of iNAC (Fig. 4f). LOX-1 has been reported
361 to be a marker for some granulocytic MDSCs (23). Conditioned media led to no change
362 in LOX-1 on healthy donor granulocytes (Supplementary 3d). Therefore mesothelioma
363 cells signal to granulocytes to modulate their function.

364 To identify the nature of the mesothelioma-granulocyte cross-talk, mesothelioma
365 conditioned supernatant was first boiled to denature all proteins. Boiled supernatant lost
366 the ability to polarise granulocytes to suppress T cell proliferation (Supplementary Figure
367 3e) consistent with the release of a soluble molecule from the mesothelioma cells.
368 Arginine depletion, a potential mechanism of polarisation due to mesothelioma arginine

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369 auxotrophism, similarly did not polarize healthy donor granulocytes to produce ROS
370 consistent with this finding (Supplementary 3f).

371 ELISAs for cytokines involved in granulocyte signalling were performed of supernatants
372 from mesothelioma cell lines and mesothelioma human primary tumour cells, identified a
373 number of key molecules were highly expressed – G-CSF, GM-CSF, IL-13, IL-6, IL-8,
374 VEGF, PGE2, Mesothelin, (Fig. 5a). In particular mesothelioma cells release IL-8
375 (neutrophil chemotactic protein; mean concentration 981pg/ml) and G-CSF (mean
376 concentration 283pg/ml) which are known to attract granulocytes into the tumour
377 microenvironment. No evidence of serum amyloid-A release from mesothelioma cells
378 was found. Interrogation of the R2 database confirmed a similar cytokine expression
379 profile from 87 human tumours (Fig. 5b). Culture of healthy-donor granulocytes with
380 individual recombinant cytokines identified above, showed that GM-CSF led to the
381 highest upregulation of ROS production (Fig. 5c) with associated upregulation in NOX2
382 expression (Supplementary Fig. 3g). Granulocytes treated with recombinant GM-CSF
383 inhibited T cell proliferation (Fig. 5d and 5e), and T cell proliferation was rescued by the
384 inhibitor (iNAC) or removal of reactive oxygen species with catalase (Fig. 5e). No
385 increases in plasma GM-CSF concentrations are identified in patients at diagnosis,
386 suggesting that the intra-tumoural release of GM-CSF drives the ROS upregulation in
387 granulocytes. (Supplementary Fig. 4a) Addition of anti-GM-CSF neutralising antibody to
388 mesothelioma cell line co-cultures inhibited ROS upregulation (Fig. 6a and 6b) and the
389 release of peroxide species in conditioned granulocyte supernatants (Supplementary Fig
390 4b) confirming mesothelioma-released GMCSF drives granulocyte ROS production and
391 T cell suppressive activity. The addition of anti-GM-CSF neutralising antibody rescued T

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392 cell proliferation, confirming the mechanism of mesothelioma polarisation of
393 granulocytes (Fig. 6c).

394 ROS production from conditioned healthy granulocytes correlated with the concentration
395 of GM-CSF in tumour conditioned media (r=0.438, p=0.0118) (Fig. 6d).
396 Immunohistochemistry of mesothelioma tumours confirmed that GM-CSF is expressed
397 within the tumour microenvironment of patients (Fig. 6e and Supplementary Fig. 4c) and
398 transcriptomic analysis of 87 primary tumour samples within the R2: database
399 demonstrated that GM-CSF is expressed in over 50 % of the samples, and does not
400 correlate with histological subtype (Fig. 6f).

401 In summary mesothelioma creates an immunosuppressive microenvironment locally and
402 systemically through the release of GM-CSF from tumour cells which induces
403 granulocyte ROS production to inhibit T cell function.

404

405 **Discussion**

406 Although the role of monocytes and macrophages in mesotheliomas has previously been
407 well documented in human tissue and murine models, granulocytes have received little
408 attention. In this study we focused on human mesotheliomas, identifying the mechanism
409 by which tumour cells modulate granulocyte function to suppress T cell responses. A
410 previous immune-histochemical study identified that high CD4 T cell counts or low
411 neutrophil counts within mesothelioma tumours are linked to better patient outcomes
412 (24). To evaluate the seemingly reciprocal relationship between granulocytes and T
413 lymphocytes we first confirmed that granulocytes make up a significant proportion of

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414 infiltrating immune cells, with relatively fewer T cells. To date granulocyte function in
415 mesotheliomas has almost exclusively been studied in murine cell line xenografts.
416 Murine granulocytes may be alternatively activated in mesotheliomas (N1 vs N2) or
417 characterised as granulocytic MDSCs (G-MDSC) (13, 20, 25). In all of these murine
418 cases the granulocytic cells express reactive oxide species – a well-established
419 mechanism of T cell suppression (26). We carefully considered whether our tumour-
420 infiltrating and circulating granulocytes could be G-MDSC according to recent guidelines
421 for nomenclature which define MDSCs based on immunophenotype, density, and
422 suppressive activity (27). In our mesothelioma patients both circulating and tumour-
423 associated granulocytes were $CD11b^{+}CD14^{-}CD15^{+}$/ $CD66b^{+}$, fitting with the G-MDSC
424 phenotype. However blood G-MDSCs are classically described as being low density
425 cells, following separation with density centrifugation. We showed that in mesothelioma
426 patients' blood, low density granulocytes are a minority population within the PBMC
427 layer and have minimal T cell suppressive activity – thus the two populations are distinct.
428 In addition no evidence of altered LOX-1 expression, a marker recently identified on low
429 density G-MDSCs, was found after mesothelioma conditioning of healthy donor
430 granulocytes (23). The most suppressive granulocytes are those of high density, and act
431 through ROS release. The intra-tumoural granulocytes we studied share the same
432 immunophenotype and suppressive mechanism. As discussed in the consensus
433 recommendations based on current technology there is no unique marker to distinguish
434 suppressive granulocytes from G-MDSC, particularly for intra-tumoural cells. Notably
435 the need to use cell density on separation as a method to define immune cell subsets is
436 extremely limited, and alternative methodologies will be developed for the future

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437 characterisation of these cells. Our findings highlight the plasticity of granulocytes in
438 humans and their place in regulating the tumour associated immune microenvironment
439 (13). Similar examples of human cancer-associated, immunosuppressive granulocytes, as
440 opposed to G-MDSC, have been identified in melanoma and non-small lung cancer,
441 driven through the release of tumour derived factors (15, 28).

442 Our analysis of the supernatants of mesothelioma cell lines and primary tumor tissue
443 revealed a cytokine profile consistent with granulocyte attraction and modulation within
444 the tumor microenvironment. A number of factors have been reported to modulate
445 granulocyte function in murine models of mesothelioma. In a murine model of
446 mesothelioma, prostaglandin inhibition reduced the number of granulocytic MDSCs (20).
447 TGF-beta within murine mesothelioma tumors also drives the expression of the
448 chemokines CCL3, CCL5, and CCL2 in pro-tumoral granulocytes (29). For humans no
449 direct mechanism of mesothelioma modulation of granulocytes has been shown although
450 the mesothelioma inducing mineral erionite can directly stimulate ROS production in
451 healthy donor-derived neutrophils (30). IL-8 (CXCL8) is a potent pro-inflammatory
452 cytokine and is primarily known for its chemotactic and activating action on neutrophils,
453 along with inhibition of normal neutrophil apoptosis (31-33). Our finding of moderate
454 levels of IL-8 released from mesothelioma cells, may contribute to the enhanced
455 granulocytes infiltration of mesothelioma tumours.. Targeting of IL-8 in models of
456 tumours such as fibrosarcoma and prostate carcinoma prevents the influx of host
457 neutrophils (34). IL-8 is also an autocrine growth factor in a number of cancer types (35-
458 37), including mesothelioma (38).

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459 We identified that meostheliomas can also release G-CSF, a second well established
460 cytokine that induces granulocyte infiltration. Notably G-CSF production by
461 mesothelioma is reported to confer a more aggressive phenotype (39-41).Although we
462 confirmed mesothelioma tumours release IL-8 and G-CSF or prostaglandins, these
463 factors had no impact in generating suppressive granulocytes. Instead we demonstrated
464 that GM-CSF is expressed by mesothelioma tumor cells, and can polarize granulocytes to
465 upregulate ROS productio . No differences in the effect of granulocyte derived ROS was
466 found on CD4+ versus CD8+ T cells.Establishment of cell lines from primary
467 mesotheliomas have reported significant production of GM-CSF (42) and this cytokine
468 can drive suppressive granulocyte activity in murine models for a number of solid
469 tumors (43-45). Although we identified GM-CSF was widely expressed in our samples
470 studied, the effects of prior therapies in our patient population, on GM-CSF expression is
471 unknown.

472 Clinically GM-CSF has been used as an alternative to G-CSF to support myeloid cell
473 recovery post-chemotherapy in mesothelioma patients (46, 47). No differences in
474 outcome were reported for the two growth factors, although the effects on immune
475 parameters are not available. Recombinant GM-CSF has also been administered
476 alongside a tumor vaccine in this patient group (48-50) and used alongside
477 immunotherapy approaches in neuroblastoma. In the mesothelioma studies GM-CSF was
478 administered to patients in all study arms, regardless of whether they received the
479 investigational tumour/peptide vaccines or not. Although responses are noted, it is not
480 possible to understand whether the cytokine had any effect on outcomes both within the
481 trial populations or compared to historical controls. It is possible that administration of

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482 GM-CSF may inhibit anti-tumor T cell responses, through the induction of G-MDSC,
483 contributing to the lack of clinically relevant T cell responses seen in these patients. In
484 two trials where GM-CSF was administered intra-lesionally to mesothelioma, neutrophil
485 infiltration and maturation was enhanced, however, this was not associated with tumor
486 responses in the majority of patients (51, 52). Indeed a Phase II clinical trial in
487 neuroblastoma demonstrated difference in prognosis if GM-CSF is administered
488 intravenously vs subcutaneously, which could impact the dose-dependent effects of this
489 cytokine on granulocyte phenotype (53, 54).

490 Preclinical studies which block GM-CSF have resulted in reversal of T cell inhibition by
491 MDSCs in the setting of pancreatic tumours, and improvements in phenotype in
492 inflammatory disease models (55, 56). Our data suggests that targeting the GM-CSF
493 pathway may be of benefit in mesothelioma. Clinically relevant approaches to target GM-
494 CSF have been focused on inflammatory diseases (57). Mavrilimumab (CAM-3001) is a
495 human anti-GM-CSF receptor-a antibody which has completed Phase I and II clinical
496 trials in the setting of rheumatoid arthritis (58). Our findings suggest that anti-GM-CSF
497 or anti-GM-CSF receptor antibodies could play a critical role in mesothelioma treatment,
498 particularly alongside T cell immunotherapies.

499

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683 **Figure Legends**

684 **Figure 1. Granulocytes in tumor tissue suppress T cell proliferation**. a)
685 Transcriptomic expression of CD14, CD15, CD3E, and Mesothelin in 87 mesothelioma
686 tumours from the R2: Genomics Analysis and Visualisation Platform (http://r2.amc.nl).
687 b) Percentages of $CD15^+$, $CD14^+$, and $CD3^+$ cells detected by flow cytometry in the
688 digested tumor tissue of 18 patients with confirmed mesothelioma. c) Representative
689 tumor sections from 6 patients (total stained = 38 tumours) demonstrating infiltration of
690 CD15 expressing cells within mesothelioma tumours. Images were taken at 20X
691 magnification. d) Immunophenotype of tumour-associated granulocytes by flow-
692 cytometry identified they expressed CD11b, CD15, with low/absent CD33 expression,
693 and low/absent HLA-DR expression. e) T cell proliferation from healthy donors is
694 suppressed following culture with $CD15^+$ granulocytes (representative 1:0.5 ratio) sorted
695 from mesothelioma tumours, compared to those cultured in complete media alone, with
696 $CD15^+$ granulocytes from the blood of healthy donors, or from pleural tissue with benign
697 pathologies.

698

699 **Figure 2. Granulocytes are elevated in peripheral blood of mesothelioma patients**
700 **and suppress T cell proliferation and activation** a) Sorted $CD3^+$ T cells from the blood
701 of mesothelioma patients (6 untreated, 2 with prior therapy) have reduced proliferative
702 capacity compared to those sorted from the blood of healthy donors. b) Increased
703 frequency of $CD15^+$ granulocytes in the peripheral blood of mesothelioma patients
704 (n=33) and healthy donors (n=30) at diagnosis. c) Marginal increased frequency of
705 $CD14^+HLADR^-$ monocytes in the peripheral blood of mesothelioma patients (n=33) and

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706 healthy donors (n=30). d) Expression of CD15, CD16, CD11b, and CD66b markers on
707 granulocytes of healthy donors and mesothelioma patients, as detected by flow
708 cytometry. e) Autologous $CD4^+$ and $CD8^+$ T cell proliferation is suppressed following
709 culture with $CD15^+$ granulocytes sorted from the blood of patients at diagnosis. T cells
710 and granulocytes were co-cultured at ratios of 1:0.5 and 1:1 respectively and compared to
711 T cells alone (1:0). f) T cell-derived IFN-γ release in culture supernatants is significantly
712 impaired following co-culture with $CD15^+$ granulocytes sorted from the blood of patients
713 at diagnosis.
714
715 **Figure 3. Granulocytes from mesothelioma patients suppress T cell proliferation**
716 **through ROS** a) The frequency of $CD11b^+CD15^+$ cells was compared in the whole blood
717 and PBMC layer following Lymphoprep separation, for 18 mesothelioma patients and 12
718 healthy donors. The majority of $CD15^+$ granulocytes lie on the red cell pellet following
719 lymphoprep separation. b) Healthy donor T cell proliferation is most suppressed
720 following culture in the presence of blood $CD15^+$ granulocytes from mesothelioma
721 patients which have been collected from the red cell pellet (High Density) after
722 Lymphoprep separation. Low density granulocytes isolated in the PBMC layer of the
723 same blood samples were comparatively less suppressive to T cell proliferation. c)QPCR
724 analysis of the expression of iNOS and Arginase in granulocytes sorted from healthy
725 donors or patients d) T cell proliferation is not restored by the addition of L-NMMA or
726 nor-NOHA to the cultures in the presence of $CD15^+$ granulocytes from patients. 2
727 representative patients are shown. e) Increased frequency of ROS^+ $CD15^+$ granulocytes in
728 the blood of patients from mesothelioma patients compared to healthy donors f)

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729 Inhibition of NOX2 activity with iNAC reversed the suppressive effect of granulocytes
730 on CD4 and CD8 T cell proliferation. g) Inhibition of NOX2 activity with iNAC restored
731 T cell activation, as measured by IFN-γ release into cell supernatants. h) Culture of
732 patients' granulocytes with iNAC reduced the intracellular production of ROS confirming
733 the known specificity of drug action.
734

735 **Figure 4. ROS generation by granulocytes is upregulated by the mesothelioma**
736 **microenvironment** a) Expression of NOX2 by qRT-PCR in $CD15^+$ cells from the blood
737 of healthy donors and mesothelioma patients. b) NOX2 expression in $CD15^+$
738 granulocytes is upregulated over time following co-culture with mesothelioma cell lines,
739 as assessed by qRT-PCR. c) ROS production is up-regulated in healthy-donor derived
740 granulocytes following culture in conditioned media from sorted mesothelioma malignant
741 cells or mesothelioma cell lines, compared to complete RPMI. ROS species are detected
742 by DCFDA staining and flow cytometry. d) Release of ROS from $CD15^+$ granulocytes is
743 upregulated after culture with mesothelioma cell lines or sorted mesothelioma malignant
744 cells as detected by hydrogen peroxide species, using a colorimetric assay. e) T cell
745 proliferation is significantly inhibited following culture with cell line conditioned- or
746 tumour conditioned- granulocytes. Comparison made with T cells cultured with
747 granulocytes conditioned by completed media alone. f) Treatment of mesothelioma cell
748 line conditioned granulocytes with iNAC prevents suppression of T cell proliferation
749
750

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751 **Figure 5: Mesothelioma cells release GM-CSF to up-regulate granulocyte ROS and**
752 **suppressive activity** a) Cytokine multiplex assay determined the cytokine profile of
753 tumor cell supernatants and cell line supernatants. Increased concentrations of GM-CSF,
754 IL-8, GCSF, VEGF, IL-6 and mesothelin are found. Low concentrations of prostaglandin
755 E2 and IL-13 were detected. b) Transcriptomic expression of GMCSF, GCSF, IL-6, IL-
756 13, IL-8, VEGF, and mesothelin in 87 mesothelioma tumours from the R2: Genomics and
757 Visualisation Platform c) ROS production (DCFDA staining) by healthy donor CD15$^+$
758 cells treated with detected cytokines to determine which were capable of enhancing ROS
759 production. GM-CSF increased ROS production most prominently. d) T cell proliferation
760 was significantly suppressed by granulocytes conditioned with recombinant GM-CSF,
761 compared to control granulocytes. Ratios of 1:1 and 1:0.5 T cells:granulocytes shown e)
762 Inhibition of granulocyte ROS production (iNAC) or accumulation (catalase) after
763 healthy donor granulocytes were conditioned with GM-CSF, restores T cell proliferation
764 compared to controls.
765
766 **Figure 6: Anti-GM-CSF neutralizing antibody can prevent granulocyte suppressive**
767 **function** a) The addition of anti-GM-CSF neutralizing antibody to mesothelioma cell line
768 conditioned media prevents granulocyte ROS expression, as measured by DCFDA
769 staining. Representative histograms for ED cell line shown. b) The addition of anti-GM-
770 CSF neutralizing antibody to mesothelioma cell line conditioned media prevents
771 granulocyte ROS expression. Geometric means for DCFDA staining shown following
772 flow cytometric detection. c) The addition of anti-GM-CSF neutralizing antibody to
773 mesothelioma cell line conditioned media, prevents granulocyte suppressive activity

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774 compared to granulocytes cultured in tumour-conditioned media alone. d) Correlation
775 between GM-CSF concentrations in tumour conditioned media and expression of
776 Reactive Oxygen Species in conditioned granulocytes, as measured by DCFDA mean
777 fluorescence intensity by flow cytometry. Linear correlation line shown. e)
778 Immunohistochemistry staining demonstrating the presence of GM-CSF in mesothelioma
779 tumor sections. Mesothelioma of epithelioid (first image), adenomatoid (second and third
780 images) and mixed/biphasic (fourth image) type demonstrated cytoplasmic positivity of
781 tumor cells in a diffuse pattern. Images taken at 20 X magnification. (f) Interrogation of
782 87 primary tumor samples within the R2: database demonstrated that GM-CSF is
783 expressed in over 50 % of the samples, and did not correlate with histological subtype.

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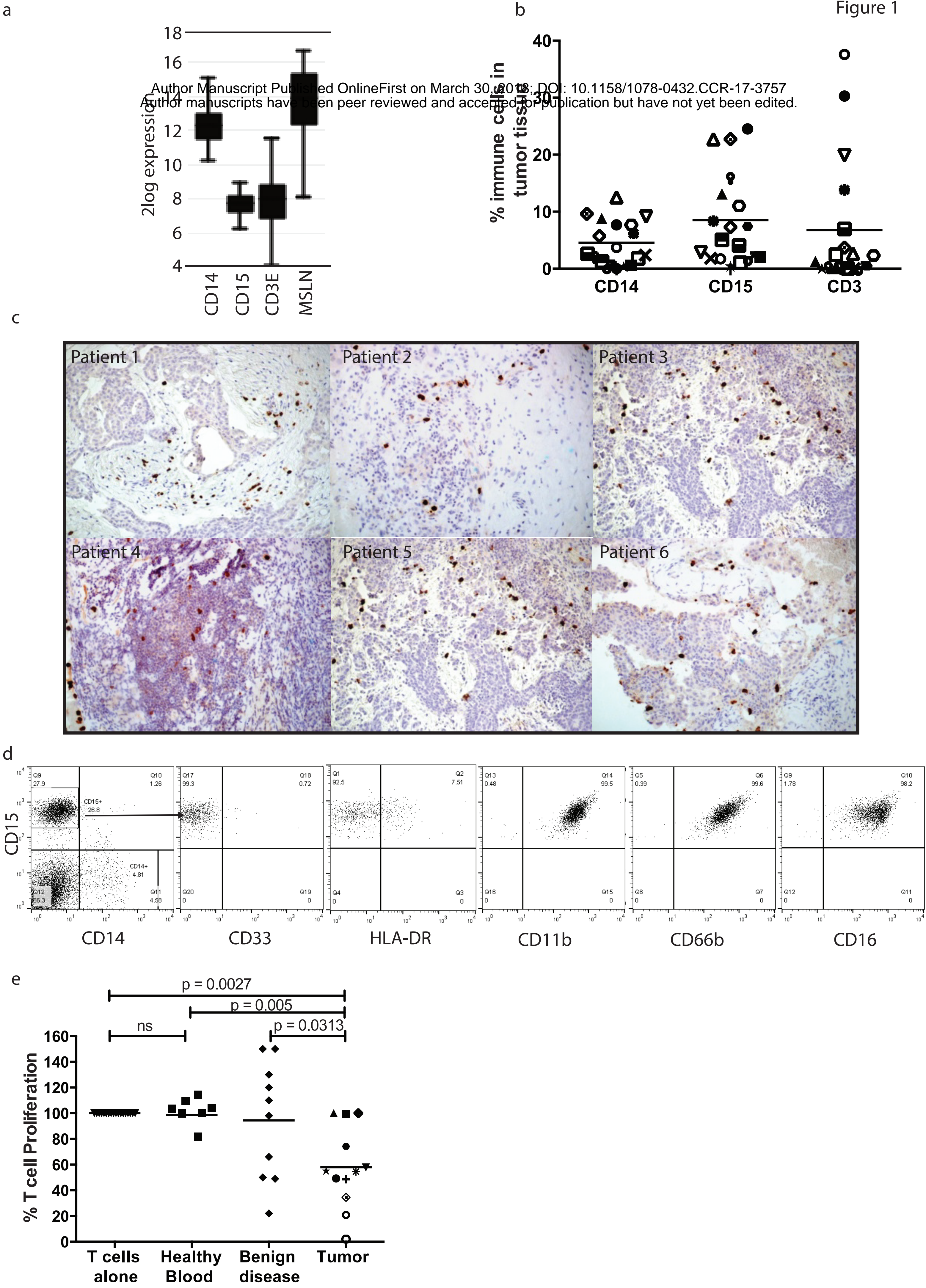
Figure 1



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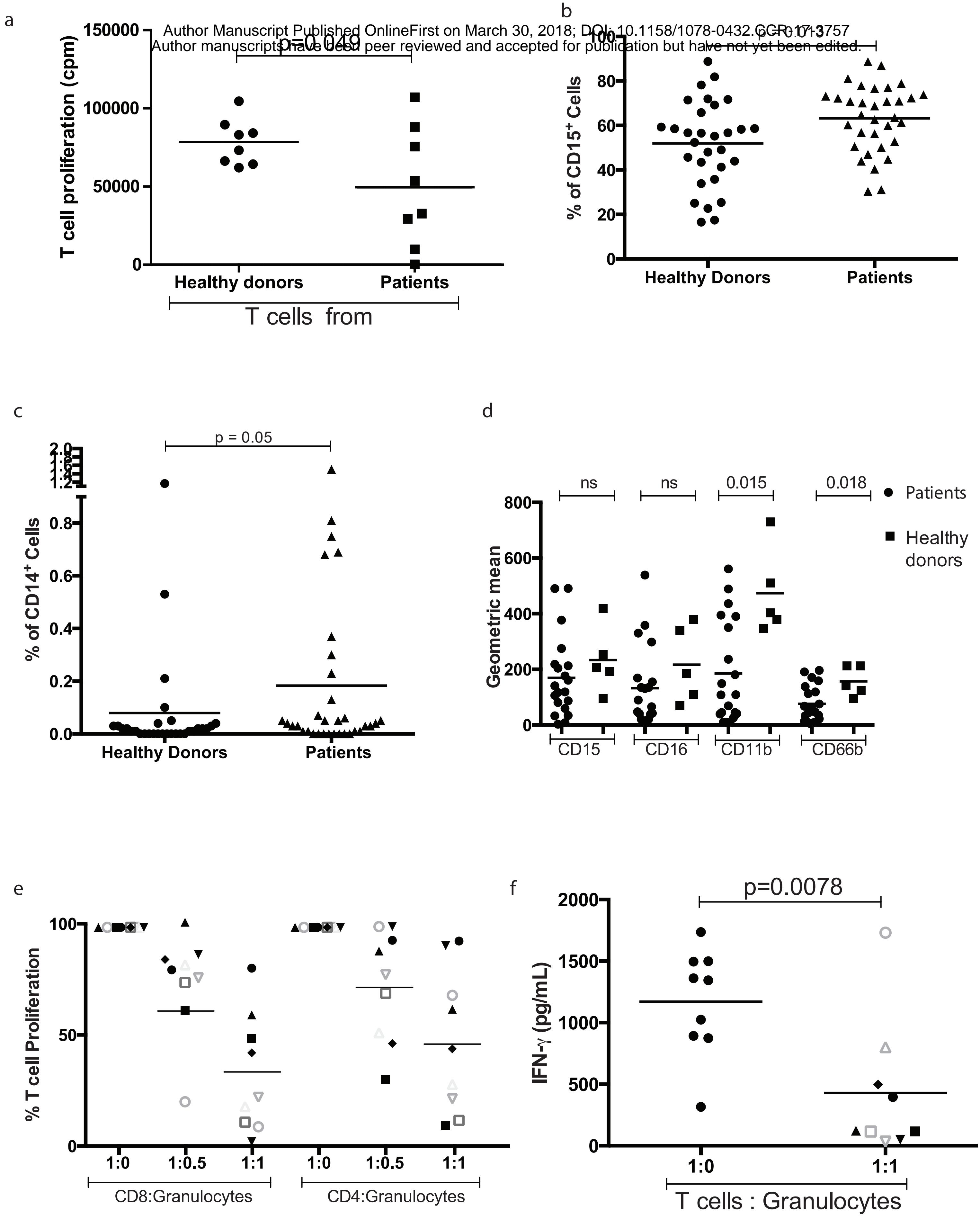

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Figure 3

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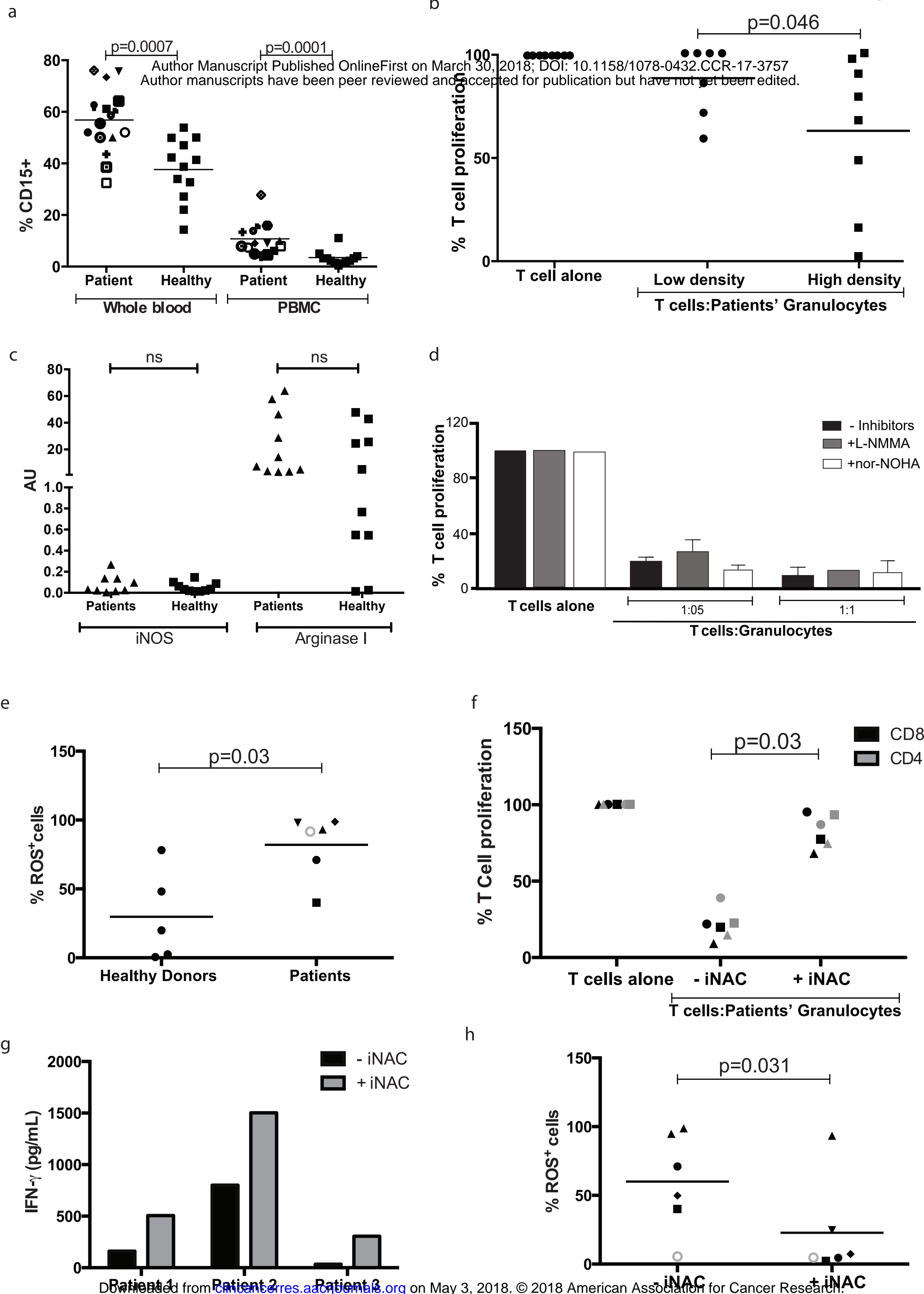

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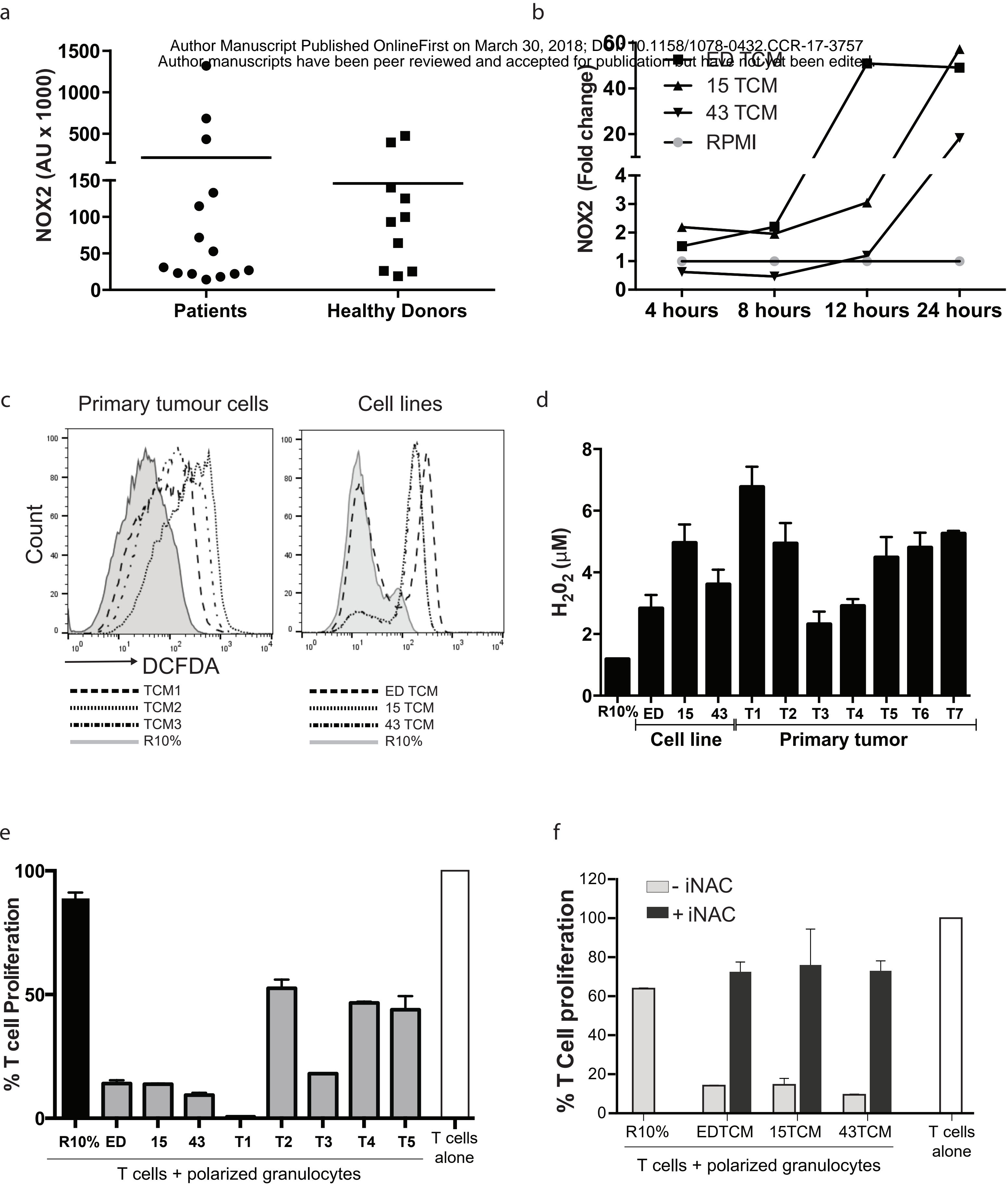

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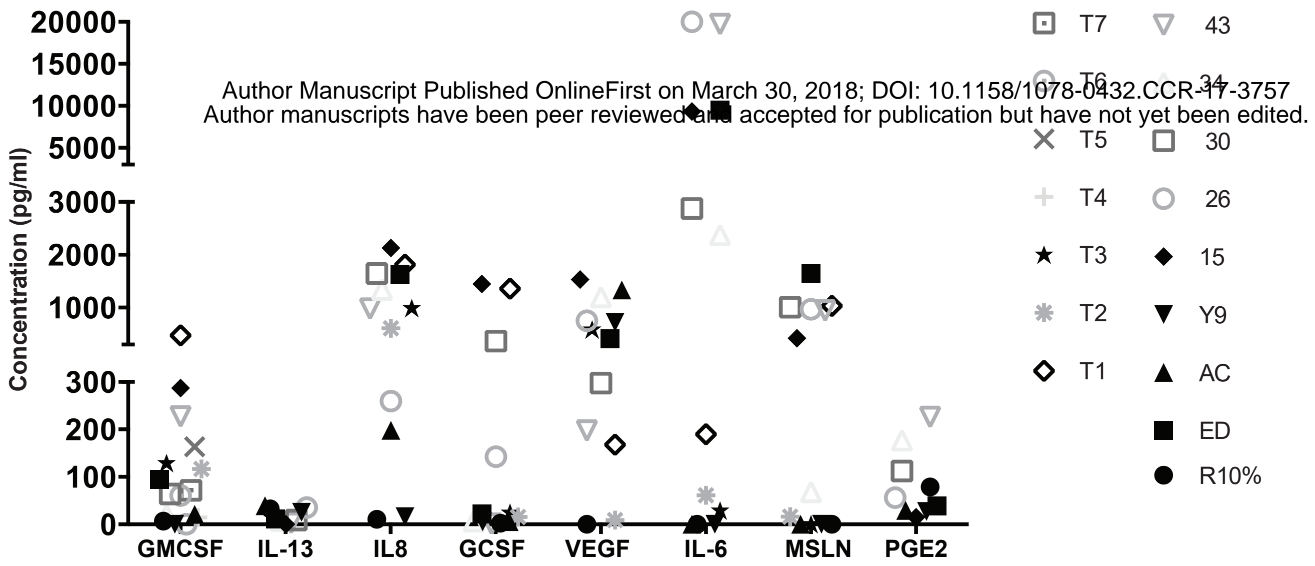
Figure 5

a



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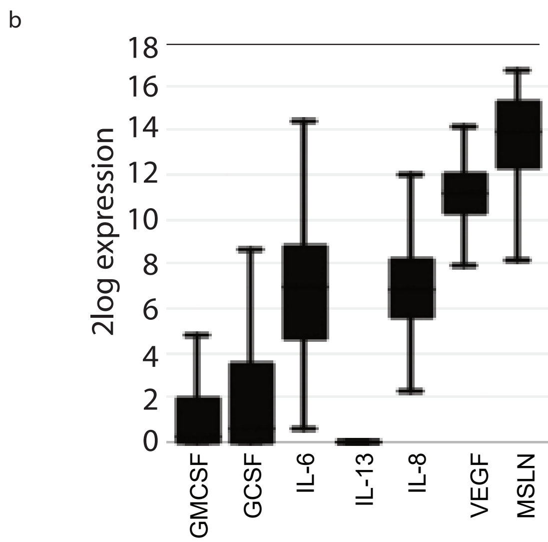

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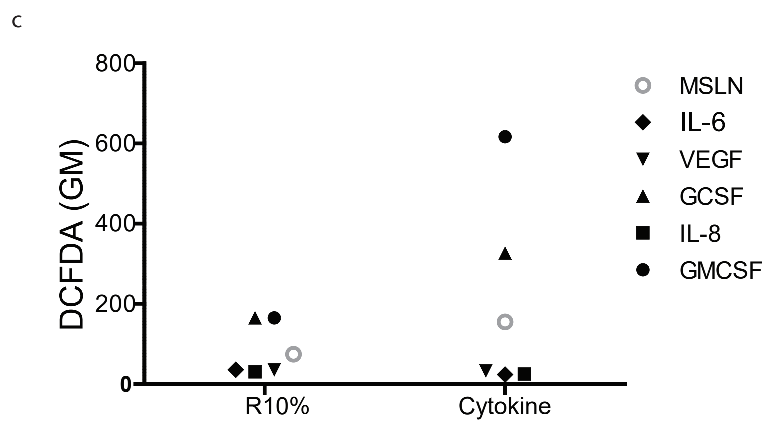

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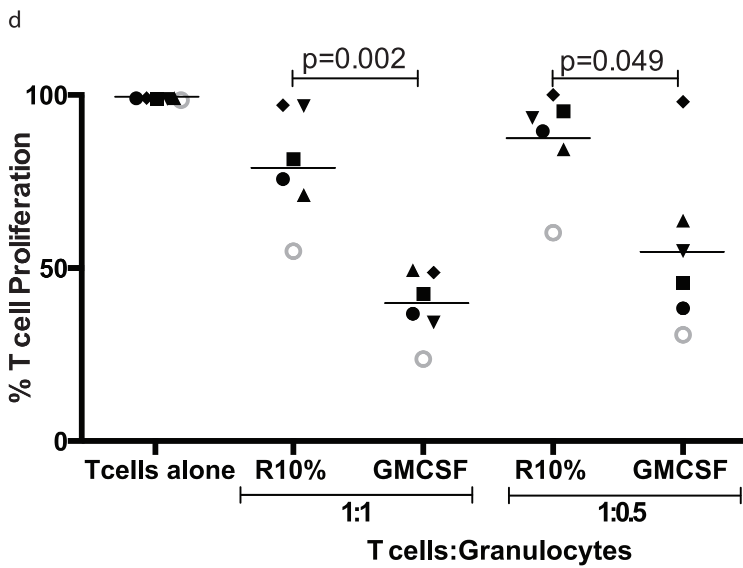

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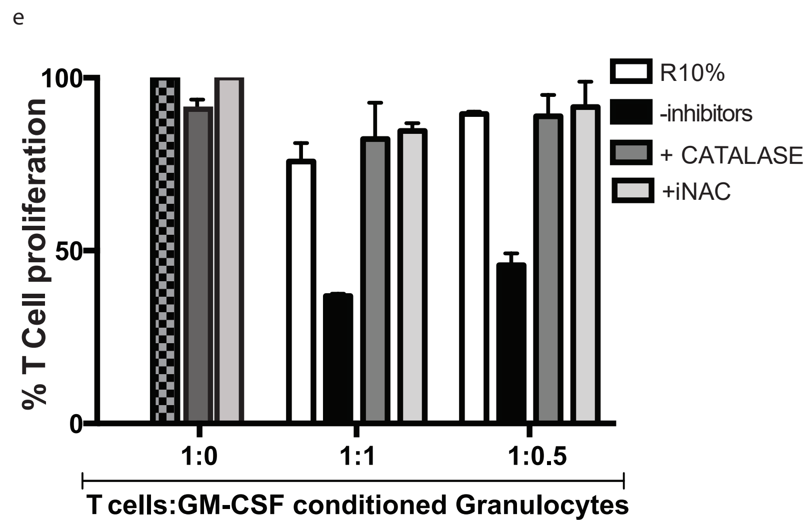

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Figure 6

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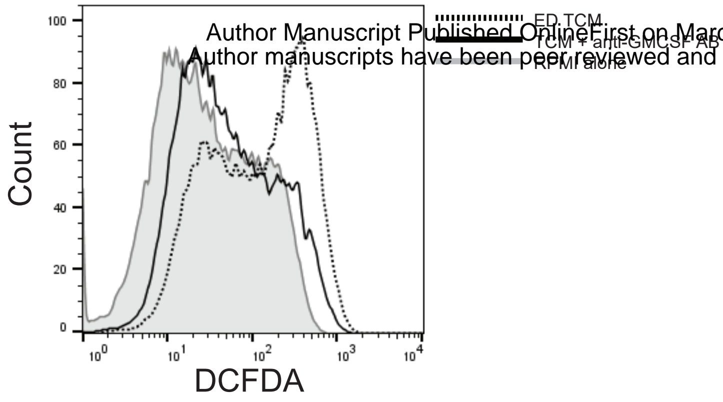

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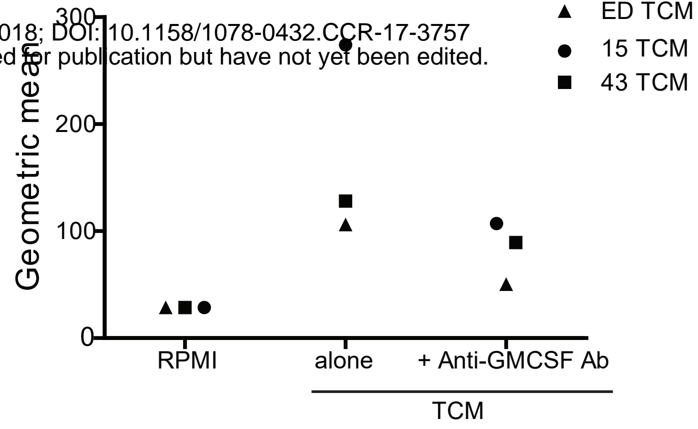

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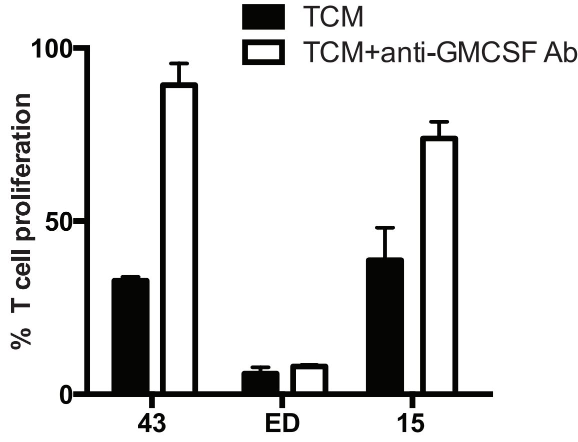

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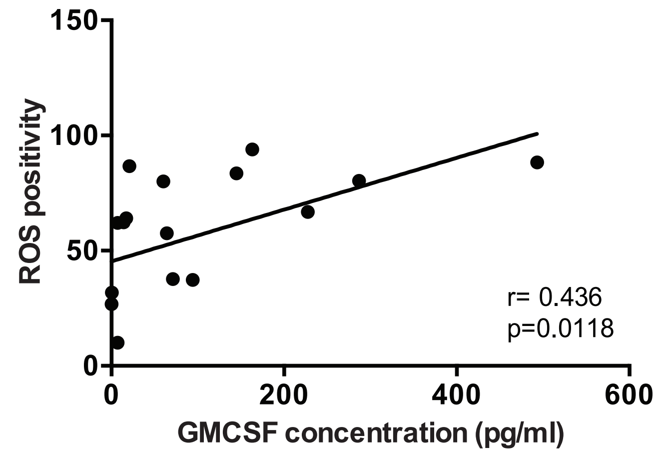

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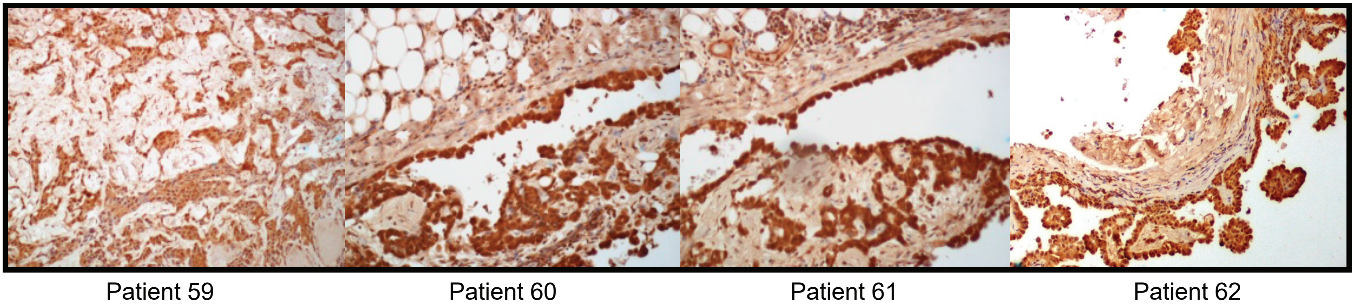

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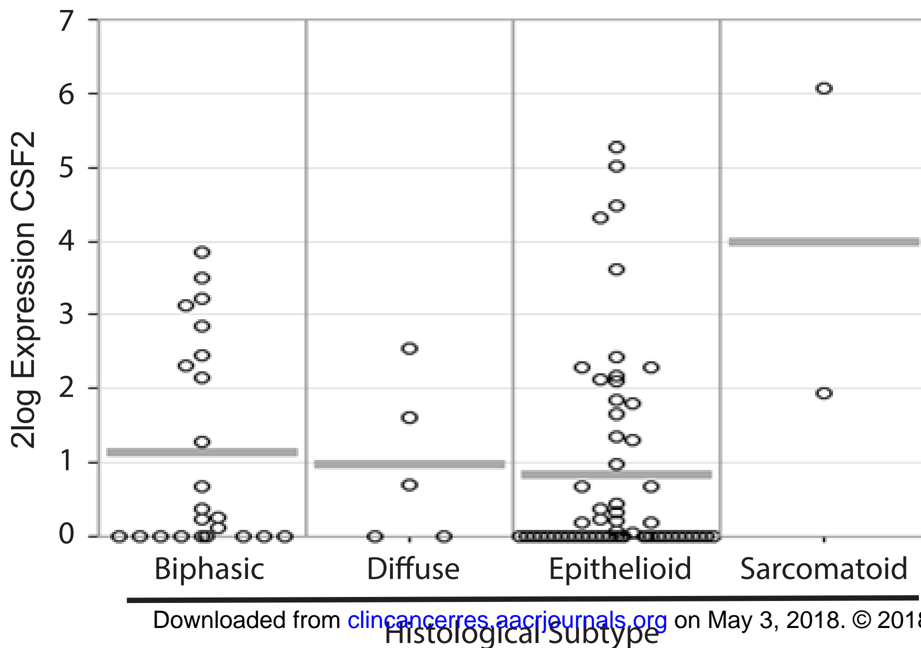

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Tumour-derived GM-CSF promotes granulocyte immunosuppression in mesothelioma patients

Swati Khanna, Suzanne Graef, Francis Mussai, et al.

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