

1 **The proteasome regulator PSME4 drives immune evasion and abrogates anti-tumor**
2 **immunity in NSCLC**

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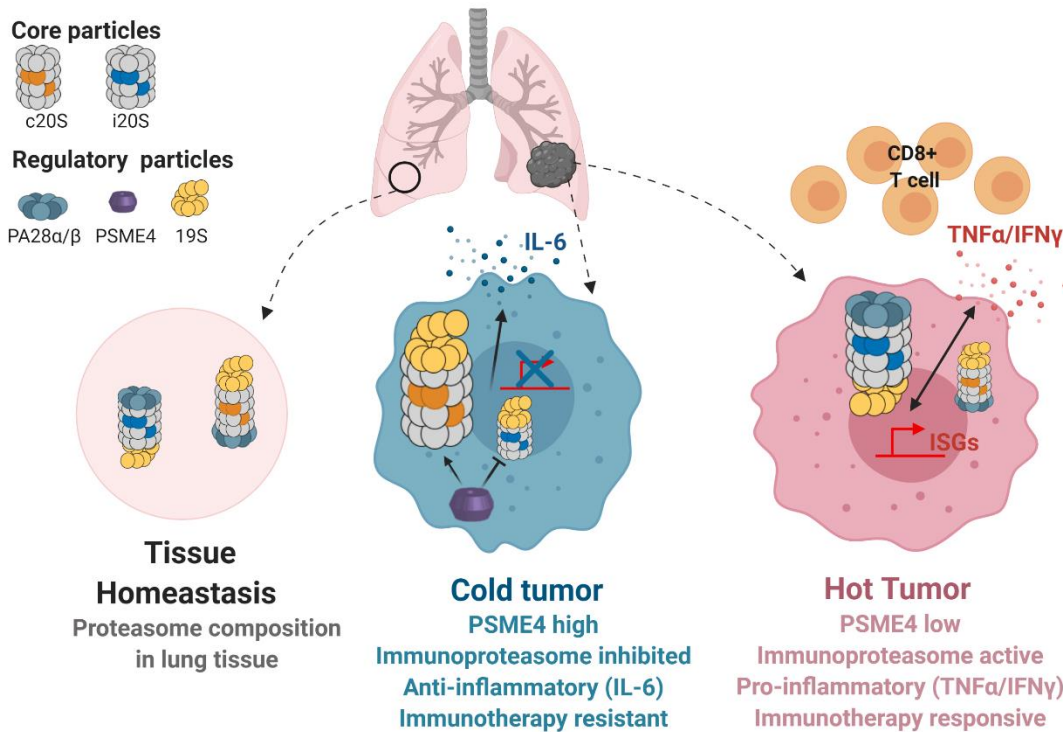
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36

37 Graphical Abstract



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39 Highlights

- 40 • Mapping the degradation landscape in Non-Small Cell Lung Cancer (NSCLC) uncovers
- 41 altered proteasome activity and composition
- 42 • Proteasome regulator PSME4 plays an anti-inflammatory role in NSCLC by attenuating
- 43 immunoproteasome activity
- 44 • PSME4 restricts tumor antigen presentation and cytokine secretion, defining a ‘cold’ tumor
- 45 environment
- 46 • PSME4 drives tumor immune evasion and is associated with resistance to immunotherapy

47 Summary

48 Protein degradation by proteasomes is important for the immune response against tumors. Antigens
49 generated by the proteasome promote immune cell infiltration into tumors and improve tumors’
50 responses to immunotherapy. For example, immunoproteasomes – a subset of proteasomes induced
51 by inflammatory signals – may improve the response of melanomas to immune checkpoint
52 inhibitors (ICI) by eliciting tumor inflammation. Yet, it is unclear whether and how protein
53 degradation by proteasomes impacts cancer progression and contributes to immune evasion and
54 resistance. Here, we profile the proteasome-cleaved peptides in lung cancers and find that PSME4
55 serves as a novel inhibitory regulator of the immunoproteasome, playing an anti-inflammatory role
56 in cancer. Biochemical assays combined with scRNA-seq, immunopeptidomics and in vivo
57 analyses demonstrate that PSME4 promotes an immunosuppressive environment around the tumor
58 and abrogates anti-tumor immunity by inhibiting antigen presentation and attenuating tumor
59 inflammation. Furthermore, we find that PSME4 expression is correlated with responsiveness to
60 ICI across several cancer types. Our findings suggest that PSME4-mediated regulation of
61 proteasome activity is a novel mechanism of immune evasion in non-small-cell lung carcinoma and
62 may be targeted therapeutically for restoring anti-tumor immunity.

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65 Introduction

66

67 Proteasomal degradation of proteins plays a key role in inflammation and antigen presentation.
68 Further, immunoproteasome upregulation has been associated with response to immune checkpoint
69 inhibitors (ICI; (Ayers et al., 2017; Harel et al., 2019; Kalaora et al., 2020; Riaz et al., 2017a;
70 Rousseau and Bertolotti, 2018; Spits and Neefjes, 2016; Tripathi et al., 2016)), yet whether and
71 how proteasomes may play a role in resistance to therapy remains poorly understood. Tumor
72 immunogenicity is promoted by the switch from constitutive proteasomes to immunoproteasomes
73 that are induced by inflammatory signals (Driscoll et al., 1993; Gaczynska et al., 1993; Salzman
74 et al., 1999; Winter et al., 2017). The canonical constitutive- and immunoproteasomes differ by
75 three catalytic subunits Psmb8, Psmb9 and Psmb10 that are encoding the proteins LMP7, LMP2
76 and MECL1, respectively. The immunoproteasome is induced by inflammatory cytokines (e.g.
77 IFN) and is basally expressed mainly in immune cells (Driscoll et al., 1993; Gaczynska et al., 1993;
78 Salzman et al., 1999). Previous studies uncovered an altered cleavage pattern by the
79 immunoproteasome, which is responsible for generating more hydrophobic peptides, that are
80 thought to be preferential for binding the Transporter associated with antigen processing (TAP) and
81 MHC class I presentation (Chong et al., 2018; Javitt et al., 2019a; Rock et al., 2016). Beyond
82 antigenicity, immunoproteasomes have also been suggested to increase cellular inflammation by
83 attenuating IL-6 secretion and promoting inflammatory cytokine secretion (Arima et al., 2011;
84 Ferrington and Gregerson, 2012; Kammerl and Meiners, 2016; Kitamura et al., 2011; Liu et al.,
85 2012; Muchamuel et al., 2009; Schmidt et al., 2018). However, whether and how tumorigenic
86 mechanisms modulate the immunoproteasome and constitutive proteasome functions to shape their
87 microenvironments remains poorly understood.

88

89 In addition to the core particle of the proteasome, proteasome composition and the degradation
90 landscape may be affected by specific regulatory particles such as the 19S regulatory complex
91 (PSMC1-6 and PSMD1-14), or the regulatory caps PA28 $\alpha\beta$ (PSME1-2), PA28 γ (PSME3) or
92 PA200 (PSME4) (Collins and Goldberg, 2017; Coux et al., 2020; Rousseau and Bertolotti, 2018)
93 that bind the catalytic core and affect gate opening and substrate selection. The 19S regulatory
94 complex been shown to be pivotal for binding of ubiquitinated species and substrate unfolding,
95 while the other regulatory subunits are suggested to have more specialized roles, including
96 mediating inflammatory responses, histone degradation, and the response to DNA damage
97 (Antoniou et al., 2012; Blickwedehl et al., 2012; Collins and Goldberg, 2017; Khor et al., 2006;
98 Mandemaker et al., 2018a, 2018b; Qian et al., 2013; Rousseau and Bertolotti, 2018; Ustrell et al.,
99 2002; Welk et al., 2016). The different catalytic cores, together with the diverse range of regulatory
100 subunits, introduce great potential diversity for generating hybrid proteasomes of the 20S core
101 particle with different combinations of regulatory caps, thereby altering protein cleavage (Fabre
102 et al., 2014, 2015; Morozov and Karpov, 2019; Raule et al., 2014; Toste Rêgo and da Fonseca, 2019).
103 However, it remains underexplored how the regulatory and catalytic subunits shape tumor-host
104 interactions.

105

106 Here, we analyzed, for the first time the degradation landscape in clinical samples of resected
107 tumors and found a distinct signature that was associated with NSCLC. Altered proteasome activity
108 in the cancerous tissue revealed an altered composition of proteasomes, with significant
109 upregulation of the regulatory cap, PSME4, which was associated with poor survival. Exacting
110 functional and biochemical assays together with single cell RNA analysis and *in vivo* work
111 surprisingly revealed that, PSME4-capped immunoproteasomes, which were not described before,
112 directly attenuate the catalytic activity of the immunoproteasome. We could further show that
113 PSME4-capped constitutive proteasomes exhibited an increase in caspase-like activity in NSCLC.
114 These marked opposing effects upon binding of PSME4 to the constitutive or immuno-
115 proteasomes, in turn, lead to decreased tumor immunogenicity and contribute to tumor growth *in*

116 *vivo*. Further, we show that PSME4-associated proteasomes play an anti-inflammatory role in
117 NSCLC, and that PSME4 promotes a ‘cold’ tumor signature associated with resistance to ICI across
118 cancer types. Collectively, our findings uncover new insight into the degradation landscape of
119 NSCLC and elucidate a causal role of PSME4 in shaping the tumor microenvironment to suppress
120 anti-tumor immunity. Together, our work introduces a novel paradigm by which proteasome
121 composition and heterogeneity should be examined in the context of cancer and response to
122 immunotherapy in NSCLC and beyond.

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124 **Results**

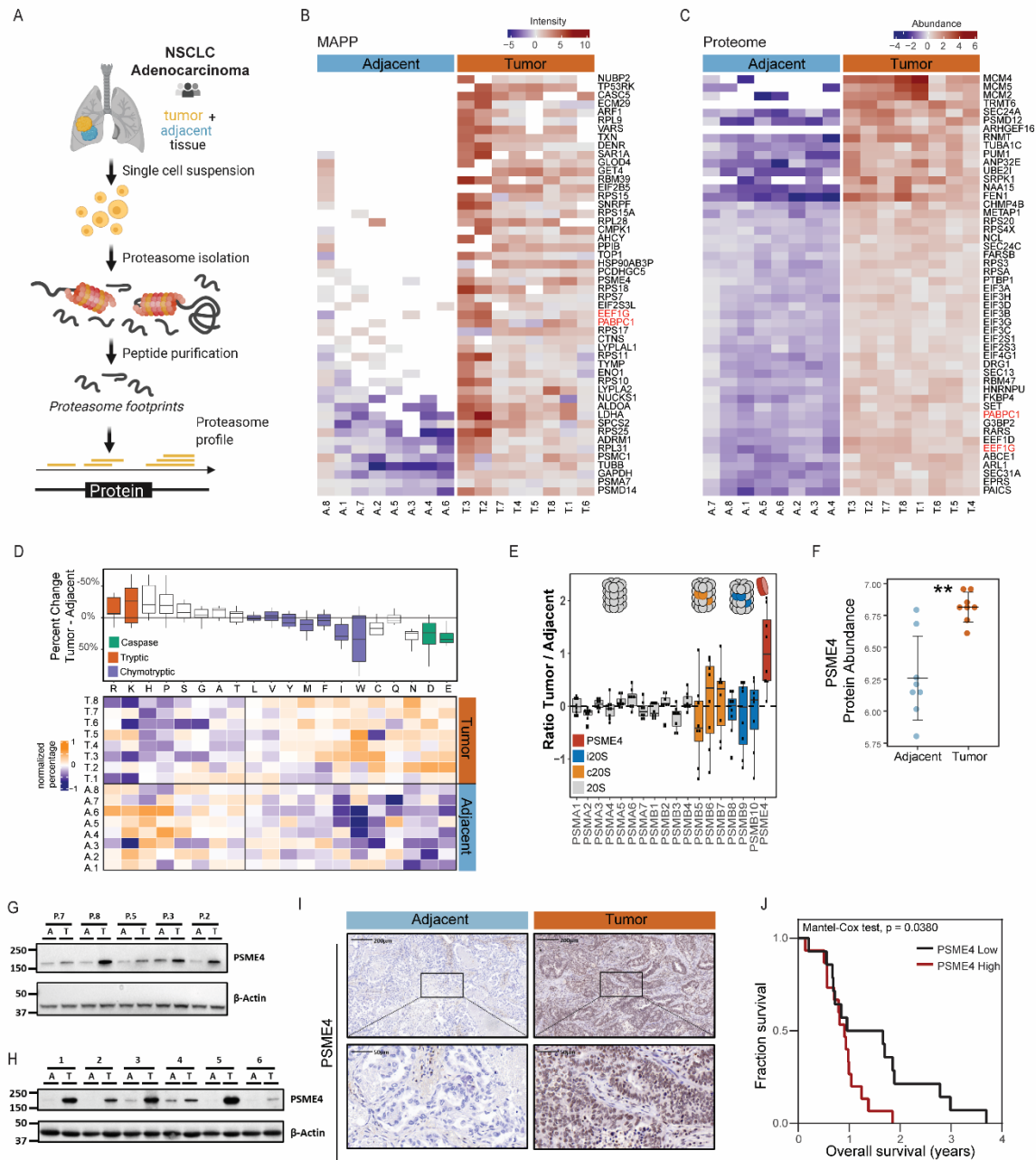
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126 **Proteasome profiling reveals an altered proteasome activity and distinct cleavage patterns in** 127 **lung adenocarcinoma**

128 To address these questions, we utilized our recently established proteasome profiling approach for
129 Mass spectrometry analysis of proteolytic peptides (Javitt and Merbl, 2019; Wolf-Levy et al., 2018)
130 (MAPP) to analyze tissue samples of non-small cell lung cancer (NSCLC) and adjacent tissue
131 controls from the same lungs, processed in parallel. We then isolated proteasomes from tumor
132 samples and analyzed the nascent peptides that were either ‘trapped’ inside or closely associated
133 with the proteasomes to detect patterns of degradation in single peptide resolution (Figure 1A).
134 Proteomic analysis of both protein abundance and degradation revealed that different tumors had
135 shared patterns of degradation that were distinguished from those of the adjacent control tissues
136 (Figures S1A-D). Detailed examination of the substrates highlighted cellular targets that were
137 readily detected in tumor tissues, some of which (e.g. CASC5; Figures 1B) are associated with
138 cancer and are not expected to be expressed in the healthy lung. Notably, some of the degradation
139 targets were not detected at all by the proteomic analysis, suggesting that they might be rapidly
140 degraded and therefore not accumulating in cells to reach a level that is detectable by mass
141 spectrometry analysis (Figures 1C, S2A-B).

142

143 Beyond specific degradation substrates, we found an increase in nuclear proteins that were
144 degraded in the cancerous tissues (Figures S3A-C). Further, we found significant alterations in the
145 cleavage pattern of substrates in the cancerous tissue (Figure 1D). Specifically, the carboxy
146 terminal residue of the degraded peptides, which is the site of proteasome cleavage, differed
147 between the tumor and adjacent samples (Figures. 1D). These observations suggested that
148 proteasome composition and function may be altered in NSCLC. We therefore examined the
149 protein abundance of different proteasome subunits. Proteasome complexes are comprised of the
150 core particle, which process the substrates, and regulatory caps, which control substrate
151 entry (Collins and Goldberg, 2017; Coux et al., 2020; Livneh et al., 2016; Motosugi and Murata,
152 2019; Rodriguez et al., 2007; Rousseau and Bertolotti, 2018). Notably, while subunits of the
153 proteasome core particle (e.g. 20S and i20S) were similar between the tumor and adjacent tissues,
154 the regulatory cap PSME4 (also known as PA200) was significantly upregulated in the cancerous
155 tissue (Figures 1E-F). We confirmed that PSME4 is upregulated in the cancerous tissue using
156 CPTAC proteomics (Gillette et al., 2020) and 2 additional patient cohorts (Figures 1G-H, S4A-C).
157 As previously reported (Khor et al., 2006) we found PSME4 was localized mainly to the nucleus
158 (Figure 1I, S4D-E). Notably, we showed that high PSME4 levels in lung cancer were associated
159 with poor prognosis of NSCLC patients (Mantel-Cox p value = 0.038, Figures 1J and S5A).
160 However, we did not observe any association in this cohort between PSME4 abundance and
161 NSCLC subtype, smoking or tumor stage (Figure S5A-E). Together, these results show that the
162 proteasome composition and proteasome-associated cleavages are changed in NSCLC and
163 suggested that PSME4 may impact tumor growth through modulating proteasome activity.



164
 165 **Fig 1. Proteasome profiling reveals an altered proteasome activity and distinct cleavage patterns in**
 166 **lung adenocarcinoma** (A) Schematic representation of the workflow. Analysis of lung adenocarcinoma and
 167 adjacent lung tissue was performed using standard whole cell extract proteomics to assess protein abundance
 168 and MAPP to study the proteasome composition and degradome of the sample (N=8 tumor and 8 adjacent
 169 sections). (B-C) The top 50 most tumor-associated proteins as identified by MAPP (B) or whole cell
 170 Proteomics (C) are shown for each of the tumor and adjacent samples. The proteins in both lists are marked
 171 in red. (D) MAPP identified peptides were divided based on their carboxy terminal residue. The percentage
 172 of peptides with each terminus was calculated for each sample and normalized across patient samples. Amino
 173 acids are annotated with their matching proteasome cleavage activity; rows are clustered using Pearson
 174 distances. The bars represent the percent change for each amino acid at the carboxy terminus comparing
 175 tumors and adjacent tissues. (E) The ratio between subunit abundance in the proteasome immunoprecipitated
 176 from tumor and adjacent tissue per patient. Colors and diagrams indicate the identity of the proteasome
 177 subunits. (F) PSME4 abundant in the pulldown from tumor samples and from adjacent tissues (**Wilcoxon

178 P = 0.0078). **(G-H)** Tumor (T) and adjacent (A) tissues from an Israeli (G) or German (H) cohort was
179 immunoblotted for PSME4. β -Actin was used as a loading control. **(I)** Immunohistochemistry of PSME4
180 in the tumor and adjacent tissues shows increased abundance in the tumor tissue with nuclear localization in
181 both. **(J)** Surviving analysis shows that patients having lung tumors with increased staining of PSME4
182 (immunohistochemistry) had significantly reduced survival compared to patients with lung tumors
183 expressing low levels of PSME4 (Mantel-Cox test $p = 0.0380$).

184

185 **PSME4 restricts immunoproteasome activity and reduces immunopeptidome diversity and** 186 **cellular inflammation**

187 Proteolytic activities of the constitutive proteasome and immunoproteasome are different (Driscoll
188 et al., 1993; Gaczynska et al., 1993; Salzman et al., 1999; Winter et al., 2017). We therefore
189 assessed whether the PSME4 level was associated with the function of both proteasome subtypes
190 in patient-derived tumor samples. Notably, the caspase- and chymotryptic-like cleavage activities
191 of the proteasome positively correlated, whereas tryptic-like cleavage negatively correlated with
192 the abundance of PSME4 (Figures 2A-B, S6A). This could not be explained by a general shift in
193 the amino acid composition across the degraded substrates (Figure 2C). PSME4 has been
194 previously shown to alter cleavage specificity of the constitutive proteasome (Blickwedehl et al.,
195 2012; Toste Rêgo and da Fonseca, 2019; Ustrell et al., 2002). However, it was not known if and
196 how PSME4 affected the activity of the immunoproteasome (Figure 2D). We found by biochemical
197 examination that PSME4 bound both constitutive proteasome and immunoproteasome in a human
198 lung cancer cell line (Figure 2E, S6B-D), suggesting that PSME4 can affect the activity of the two
199 proteasome subpopulations independently. We then assessed the effect of supplementing
200 recombinant PSME4 on the proteasome activity in vitro using model substrates for defined
201 constitutive cleavage sites. This system allowed us to decouple the effect of PSME4 on the
202 proteasome from potential transcriptional effects that have been reported in other
203 settings (Blickwedehl et al., 2012; Qian et al., 2013). We found that the caspase-like ($\beta 1$) activity
204 increased (Figure 2F, S7A-D) whereas the tryptic-like ($\beta 2$) activity decreased upon PSME4
205 supplementation (Figure 2G and S7E), corroborating our above results from clinical samples
206 (Figures 2A-B). Surprisingly, PSME4 had opposite effects on the constitutive proteasome $\beta 1$ and
207 its immunoproteasome counterpart $\beta 1i$ subunit, increasing the former and inhibiting the latter
208 (Figures 2H, S7F). In fact, PSME4 inhibited all immunoproteasome-associated activities ($\beta 1i$, $\beta 2i$
209 and $\beta 5i$) under inflammatory stimulation (Figures S7F-H), in which the immunoproteasome is
210 predominant. Thus, PSME4 is the first proteasomal subunit identified that inhibits the
211 immunoproteasome activity.

212

213 Proteasome-cleaved peptides serve as the major source of antigens presented by class I MHC. Both
214 the observed PSME4-mediated increase in caspase-like activity and decrease in tryptic-like activity
215 suggested generating peptides that are less favorable for MHC-I presentation. We therefore
216 hypothesized that PSME4 might counteract the generation of antigenic epitopes by the
217 immunoproteasome and decrease tumor antigenicity. To address this question, we performed HLA
218 immunopeptidomics of lung cancer cells with and without overexpressing PSME4 (Figure S8A).
219 We identified 463 peptides (10% of the identified peptides) that were significantly changed upon
220 PSME4 overexpression compared to the control (Figure S8B). As caspase-cleaved peptides do not
221 bind well to the MHC (Javitt et al., 2019a; O'Donnell et al., 2020), they are not highly presented
222 in either condition. Yet, the percentage of peptides with tryptic termini were decreased following
223 PSME4 overexpression (Figure 2I), corresponding to the PSME4-driven change in proteasome
224 activity (Figure 2B). Further, examination of the protein source of the presented peptides, we found
225 an enrichment of nuclear proteins in the immunopeptidome following PSME4 overexpression
226 (Figure S8C). Finally, when examining the distribution of amino acids across the differentially
227 presented peptides, we found that the peptides presented in the PSME4-overexpressing cells were

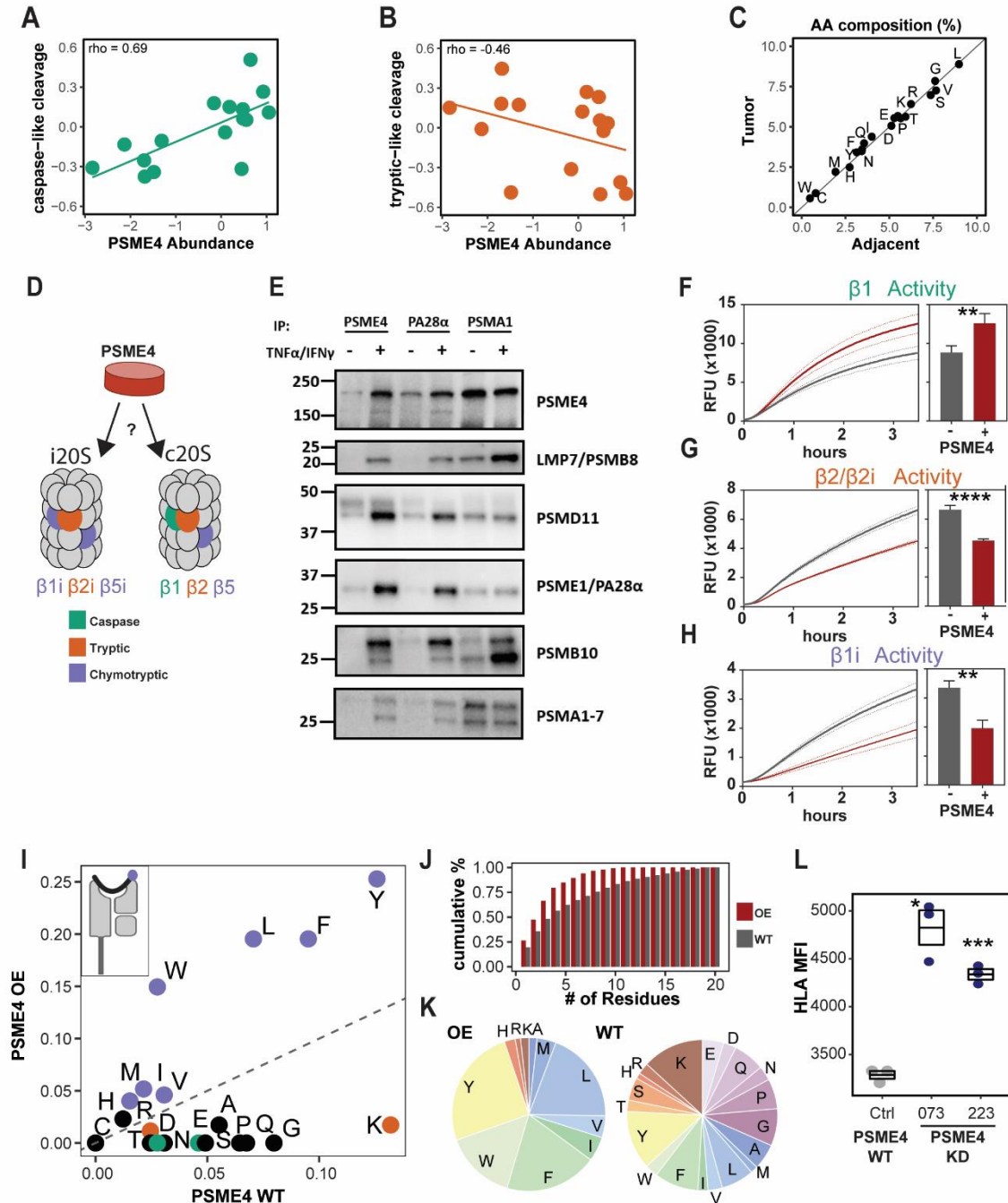
228 less diverse than those in the control (Figures 2J-K). Notably, only 4 residues in the overexpressing
229 cells, compared to 9 residues in the control, make up 80% of the carboxy-termini of the peptides
230 associated with MHC binding, indicating that PSME4 overexpression significantly restricts the
231 diversity of presented antigens (Figures 2J-K). As PSME4 was previously reported to be
232 incorporated in only a small percentage of proteasome complexes (Fabre et al., 2014; Morozov and
233 Karpov, 2019) we sought to examine whether depleting it would impact significantly the
234 immunopeptidome. In accordance, we could show that depleting PSME4 (Figures S9A-C)
235 significantly increased antigen presentation, as exhibited by 30% increase in surface HLA
236 molecules (Figures 2L and S10). Together these results suggest that PSME4 decreases the diversity
237 of the cellular immunopeptidome, attenuates antigen presentation and immunoproteasome activity.
238

239 **PSME4 reduces cellular inflammation and cytokine secretion**

240 Beyond antigenicity, the immunoproteasome has been previously implicated in modulating
241 inflammation by modulating cytokine secretion (Arima et al., 2011; Ferrington and Gregerson,
242 2012; Kammerl and Meiners, 2016; Kitamura et al., 2011; Liu et al., 2012; Muchamuel et al., 2009;
243 Schmidt et al., 2018). We therefore hypothesized that PSME4 might promote an
244 immunosuppressive environment by direct prohibiting tumor inflammation, in addition to
245 modulating antigenicity. To gain insight into the functional consequences of upregulation of
246 PSME4 in NSCLC, we stratified patient tumors from The Cancer Genome Atlas (TCGA) based on
247 their PSME4 expression levels and associated them with cellular functions (Figure S11A). We
248 found that PSME4-high tumors were enriched with pathways related to cell cycle and DNA
249 replication (Figure S11B), whereas PSME4-low tumors were associated with a number of immune
250 and inflammation-related pathways (Figure 3A). To examine whether there might be a causal
251 relationship between PSME4 and cancer inflammation, Proteomic analysis revealed that IFN
252 signaling pathways were enriched in the PSME4-depleted cells following inflammatory stimulation
253 (Figure 3B, S11C-D).
254

255 Furthermore, we found increased abundance of multiple components of the antigen presentation
256 machinery (e.g. TAP1; Figure 3B) in the PSME4 depleted cells in accordance with the observed
257 increase in surface HLA (Figure 2L). By contrast, KRAS signaling and epithelial to mesenchymal
258 transition (EMT) pathways, which have been previously associated with immunoproteasome-
259 deficient lung cancer (Tripathi et al., 2016) were also enriched here in control conditions with high
260 PSME4 (Figure S11D). Likewise, we found that depleting PSME4 significantly increased the
261 expression of IFN-inducible genes, such as IFIT1 and ISG15 (Figure 3C, S11E-F). Interestingly,
262 STAT1 and STAT2 were more abundant whereas STAT3 and STAT5 were reduced in PSME4-
263 depleted cells than in control cells (Figures 3D). STAT1 and STAT3 have previously been shown
264 to play opposing roles in cancer-related inflammation with STAT3 serving a pro-oncogenic
265 role (Avalle et al., 2012). Consistent with this, we also observed decreased IL6 expression upon
266 PSME4 depletion in two lung cancer cell lines (Figures. 3E, S11D-E). IL-6 is a hallmark of STAT3
267 signaling and serves an anti-inflammatory and pro-oncogenic role in tumor cells (Avalle et al., 2012;
268 Johnson et al., 2018). Thus, we hypothesized that the upregulation of PSME4 in lung cancer may
269 directly attenuate tumor inflammation and drive immune suppression in the microenvironment by
270 modulating IL-6 expression.
271

272 To determine the effect of PSME4 on cytokine secretion in the tumor microenvironment, we
273 utilized the KP1.9 cells derived from a *Kras*^{G12D};*Trp53*^{-/-} murine lung adenocarcinoma in an
274 orthotopic murine model. We established KP1.9 isogenic cell lines with either ectopic expression
275 (KP1.9^{PSME4_OE}) or knockdown (KP1.9^{PSME4_KD}) of PSME4 (Figures S12A-F). Depletion of PSME4
276 reduced its levels to be comparable with those of normal lung epithelial cells (Figure S12C). We
277 first examined the cytokine profile of these cell lines and found that TNF α , IFN γ and IL-17a were



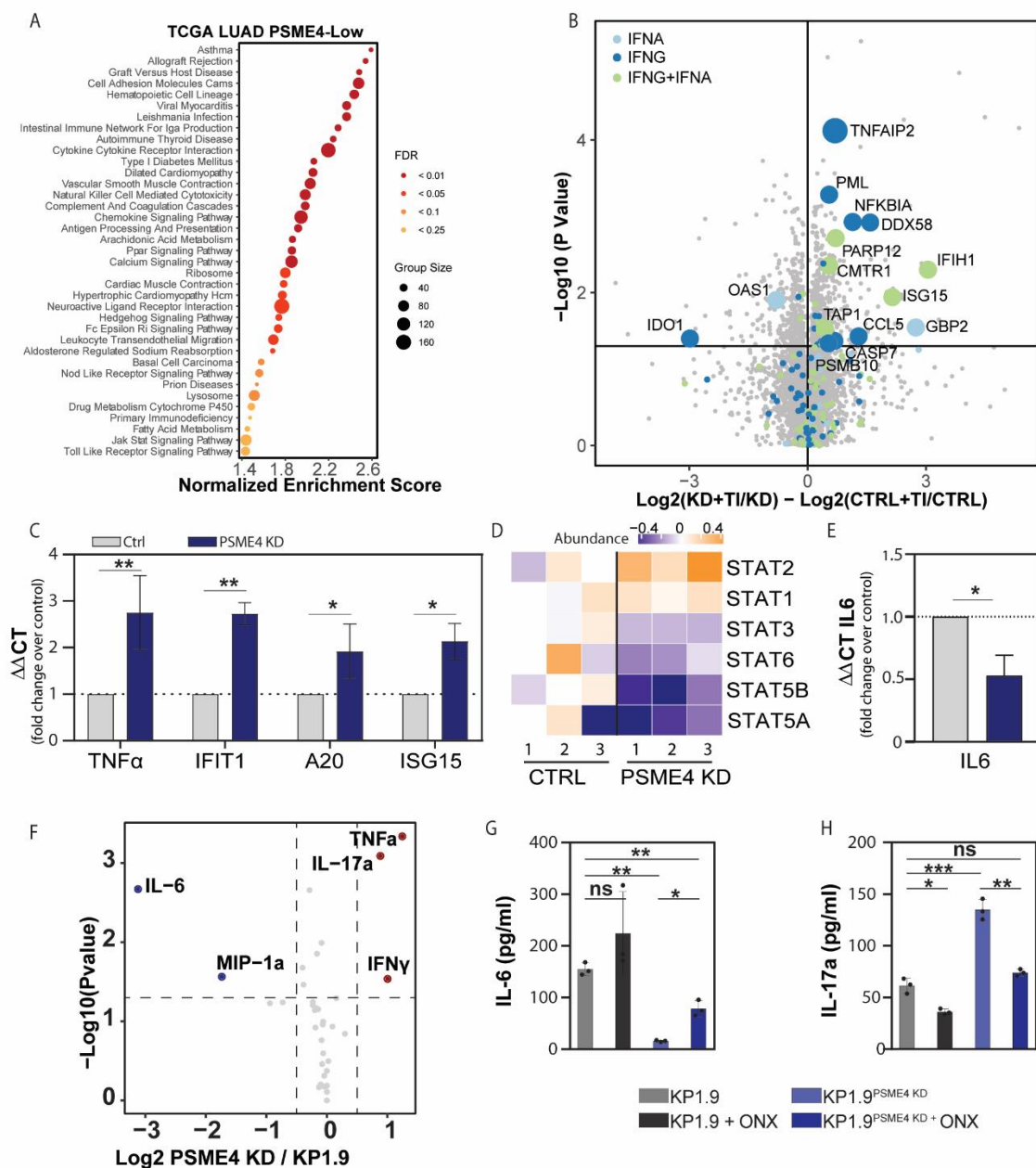
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Fig 2. PSME4 restricts proteasome activities and reduces immunopeptidome diversity. (A – B) The carboxy terminal residue of peptides was used to classify peptides based on the proteasome activity attributed to their cleavage. The abundance of PSME4 in the samples based on WC proteomics correlated with the caspase-like signature (A; spearman $\rho = 0.69$) and was anti-correlated with the tryptic-like signature (B; spearman $\rho = -0.46$). (C) The average amino acid percent composition for all the peptides identified by MAPP in the tumor samples (y-axis) is plotted against the corresponding adjacent samples. (x-axis). (D) Schematic representation of the constitutive proteasome (i20S) and immunoproteasome (c20S) with associated catalytic subunits. (E) Proteasome complexes were immunoprecipitated (IP) with the indicated antibody from A549 cells that were either treated with TNF α and IFN γ or left untreated and blotted for the indicated proteasome subunits. (F–H) Proteasome activity assays using the nLPnLD-AMC (F), RLR-AMC (G) or PAL-AMC (H) fluorogenic substrate to measure the activity of indicated subunits. Relative fluorescence (RFU) of the substrate

290 is shown across the 3.5 hours of the experiment (left) or at the endpoint (right). A549 lysates were untreated
291 (F), to measure the constitutive proteasome activity, or treated with TNF α and IFN γ (G and H), to measure the
292 immunoproteasome activity. Recombinant PSME4 was added to the lysate where indicated (red, paired T-test;
293 **P < 0.01, **** P < 0.0001). (I) The percentage of each amino acid in the carboxy terminus of the peptides
294 differentially presented on MHC molecules in the A549 cells overexpressing PSME4 (PSME4 OE) vs. empty
295 vector control (PSME4 WT) are shown in a scatter plot. Residues are colored based on the associated cleavage
296 activity: green - caspase, purple - chymotryptic and orange - tryptic; and a dotted line is shown for X=Y. (J)
297 The cumulative percentage of peptides explained by the number of different amino acids (from one to all twenty)
298 at the carboxy terminus (end position) in PSME4 WT (WT, grey) and PSME4 OE (OE, red) immunopeptidome.
299 (K) The percentage of each amino acid in the carboxy terminus of the peptides differentially presented on MHC
300 molecules in the A549 cells overexpressing PSME4 (PSME4 OE) vs. empty vector control (PSME4 WT). (L)
301 Median fluorescence intensity from flow cytometry analysis of MHC-I expression using a pan HLA-A/B/C
302 antibody (W6/32) for A549 cells with PSME4 knockdown (shA223 and shA073) or control (shCtrl). The HLA
303 mean fluorescent intensity (MFI) is significantly higher in the PSME4 knockdown compared to the control (*
304 P = 0.01, *** P = 0.0001).

305
306 Increased while IL-6 and MIP-1 α were decreased by the depletion of PSME4 (Figure 3F),
307 consistent with the observed changes in the human lung cancer cell line. Importantly, the
308 immunoproteasome has been previously implicated in modulating cytokine secretion, primarily in
309 the context of lymphocytes (Arima et al., 2011; Kitamura et al., 2011; Liu et al., 2012; Muchamuel
310 et al., 2009; Schmidt et al., 2018). For example, mutations in immunoproteasome subunits that limit
311 the proteasomal activity increase IL-6 secretion (Arima et al., 2011). We therefore hypothesized
312 that PSME4-mediated restriction of immunoproteasome activity in cancer cells might directly
313 increase tumor IL-6 secretion. Indeed, blocking immunoproteasome activity with ONX0914
314 partially rescued the loss of IL6 secretion from PSME4-depleted KP1.9 cells, suggesting that
315 modulation of immunoproteasome activity contributes to the effect of PSME4 on IL-6 expression
316 (Figures 3G, S12G-I). Furthermore, inhibiting the immunoproteasome almost completely
317 abrogated the increase in IL-17 secretion in PSME4-depleted cells (Figures 3H).

318
319 **PSME4 promotes an immunosuppressive tumor microenvironment**
320 As tumor inflammation and antigenicity are key to anti-tumor immunity, we examined the effect
321 of PSME4 *in vivo* by injecting mice with KP1.9, KP1.9^{PSME4 OE} or KP1.9^{PSME4 KD} (Figures 4A).
322 Notably, mice bearing KP1.9^{PSME4 OE} showed marked reduction in their body weight starting a
323 month from tumor cell injection (33 days), as compared to mice bearing KP1.9 tumors (Figure
324 S13A). Lungs of mice bearing KP1.9^{PSME4 OE} tumor were significantly larger, contained numerous
325 lesions whereas lungs of mice bearing KP1.9^{PSME4 KD} did not show any detectable lesions (Figures
326 4B-C, S13B-D). These effects were not driven by a change in the proliferation rate of tumor cells
327 (Figures S13E-F). Next, we analyzed the changes in the immune milieu by performing single cell
328 RNA sequencing (sc-RNA seq) of CD45+ cells 3 weeks after the injection of parental KP1.9 or
329 KP1.9^{PSME4 KD} tumor cells. This allowed us to examine the effect of PSME4 on the early immune
330 response to the tumor, without bias due to dramatically different levels of tumor burden (Figures
331 4D-E S14A-B). The overall distribution of cell types was similar among groups (Figure S14A).
332 However, the relative sizes of the alveolar and cycling macrophage clusters as well as *Ccr7*+
333 regulatory DCs, which have been previously shown to play an immunosuppressive role in NSCLC
334 (Lavin et al., 2017; Maier et al., 2020), in the lung of mice bearing KP1.9^{PSME4 KD} tumors were
335 smaller than in the lung of mice bearing KP1.9 tumors (Figures 4F-H). Interestingly, we
336 concurrently observed a decrease in the secretion of the macrophage recruiting chemokine MIP-
337 1 α /CCL3 (Baba and Mukaida, 2014) from the KP1.9 tumor cells upon PSME4 depletion (Figure
338 3F). We also observed an increase in plasmacytoid DCs (pDCs) in the mice bearing KP1.9^{PSME4 KD}
339 tumors (Figure 4I). While pDCs have been proposed to have both pro- and anti-inflammatory roles
340 in cancers (Li et al., 2017), here we see the expression of *Ii12a* and an enrichment for pathways
341 related to antigen presentation and processing (Figure 4J, S15A-B), suggesting that pDCs serve a



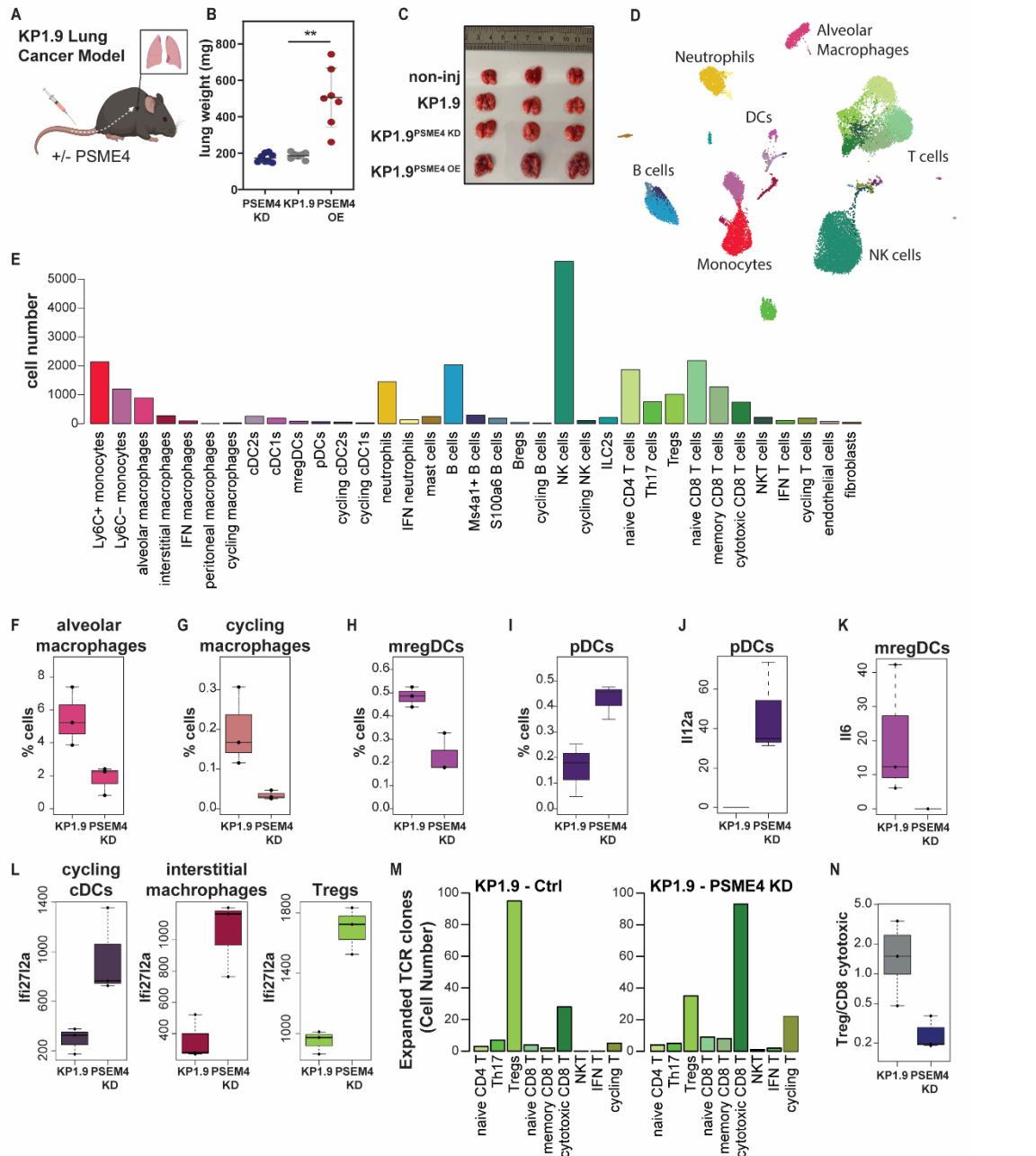
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 343 **Fig. 3 Depleting PSME4 increases cellular inflammation and cytokine secretion** (A) TCGA LUAD samples
 344 were stratified by PSME4 expression and PSME4- high samples were compared to PSME4- low (n = 230
 345 samples). The normalized enrichment score for KEGG pathways significantly overrepresented in PSME4-low
 346 samples are shown. Dot color indicates FDR threshold and size indicates number of genes in the pathway
 347 (Group Size). (B) The difference of the log₂ transformed ratio of protein abundances following stimulation with
 348 TNFα and IFNγ (TI) compared to unstimulated (UT) between PSME4 depleted A549 cells (shPSME4) and
 349 control (shCtrl) is plotted against the negative log₁₀ transformed p value for a student's t test comparing the
 350 two ratios. Proteins involved in IFN signaling are marked (light blue: response to IFN-alpha (GO: 0035455)
 351 dark blue: response to IFN-gamma (GO:0034341), or green: proteins in both lists). Points that significantly
 352 differed between the two conditions (student's t test P ≤ 0.05) and are annotated as IFN signaling pathway
 353 components are labeled with the gene name. (C) qPCR of genes downstream to IFNγ activation (TNFα, IFIT1,
 354 A20 and ISG15) following stimulation with TNFα and IFNγ. A549 cells expressing shPSME4 are compared to
 355 those expressing shCtrl (paired T test **P < 0.01 and *P ≤ 0.05). (D) Abundance of STAT proteins from
 356 shPSME4 -or shCtrl-expressing cells stimulated with TNFα and IFNγ. (E) qPCR of IL6 in A549 cells

357 expressing shPSME4 (blue) or shCtrl (grey) following stimulation with TNF α and IFN γ (students' T test *P \leq
358 0.05). (F) The log₂ transformed ratio of the level of secreted indicated cytokines between PSME4-deficient
359 (KD) and control (KP1.9) KP1.9 cells following stimulation with TNF α and IFN γ is plotted against the negative
360 log₁₀ transformed p value. (G-H) The amount of IL-6 (G) or IL-17a (H) secreted from KP1.9 or PSME4-
361 deficient KP1.9 cells following stimulation with TNF α and IFN γ , with or without the immunoproteasome
362 inhibitor ONX-0914 (ONX). Cytokines are measures in pg per ml (student's T test (ns P>0.05, *P<0.05, **P <
363 0.01, ***P < 0.001).

364
365 pro-inflammatory role in this context. This is in contrast to the mature DCs enriched in
366 immunoregulatory molecules (mregDCs), which are decreased in KP1.9^{PSME4_KD} and express higher
367 levels of *Il6* (Figure 4K, S15C-D). Likewise, the increased secretion of IFN γ upon PSME4
368 depletion was accompanied by an increase in the expression of IFN response gene *Ifi272a* in several
369 cell types, including interstitial macrophages, cycling dendritic cells and Tregs (Figure 4L). Finally,
370 to examine if there is a skew towards particular cell fates, we characterized the T cell populations
371 and T cell receptors (TCR). We reasoned that TCR clones that were not unique were result of
372 expansion (Figures. 4M, S16A-B). We found that cytotoxic T cell and regulatory T cell populations
373 had considerably expanded (Figures S17A-B, S18A-D). There were more cytotoxic CD8 T cells in
374 the KP1.9^{PSME4_KD} milieu whereas Tregs expanded in the KP1.9 bearing mice, with a significant
375 difference in the Treg/CD8 T cell ratio between the two groups (Figure 4N). Together, our results
376 suggest that upregulation of PSME4 may both drive immune evasion by reducing cytotoxic T cell
377 activity and play an active role in tumorigenesis by promoting an immune-suppressed
378 microenvironment *in vivo*.

379
380 To further validate the changes observed by scRNA-seq, we analyzed the lung of tumor-bearing
381 mice and stained for tumor infiltrating lymphocytes (TILs, Figure 5A, S19A-D). Here too, we
382 found that KP1.9^{PSME4_OE} tumors exhibited a decrease in the CD8⁺ T cell/Treg ratio (Figures 5A-B).
383 Moreover, our analysis revealed a significant increase in exhausted PD1⁺ CD8⁺ T cells (Figure 5C
384 and S19A) and a decrease in IFN γ in tumor infiltrating lymphocytes (TILs) in KP1.9^{PSME4_OE} tumor-
385 bearing mice (Figure 5D-E), suggesting decreased tumor inflammation. Furthermore, we observed
386 a decrease in naïve CD8⁺ T cells (CD62L⁺, CD44⁻; Figures 5F-G, S20B-C) suggesting that
387 KP1.9^{PSME4_OE} exhibit reduced inflammation and T cell activation. To determine whether the
388 PSME4-induced effect on tumor growth was indeed immune-mediated, we repeated the experiment
389 using RAG1-deficient mice, which lack lymphocytes. In contrast to WT mice where the KP1.9-
390 bearing mice lost weight more quickly than the KP1.9^{PSME4_KD}, no differences in weight loss or
391 survival between mice bearing PSME4-deficient and those bearing KP1.9 tumors (Figure S21)
392 were observed, further corroborating that the PSME4-driven changes in tumor progression is
393 mediated by lymphocytes *in vivo*. To further consolidate this point we tested the ability of
394 splenocytes isolated from tumor-bearing mice to kill the tumor cells. We found that splenic
395 lymphocytes from mice bearing KP1.9^{PSME4_OE} tumors exhibited reduced propensity to kill KP1.9
396 cells (Figures 5H S22A). By contrast, splenic lymphocytes from mice bearing KP1.9^{PSME4_KD} tumor
397 demonstrated killing even better than those from mice bearing KP1.9 tumors (Figure 5H). These
398 results indicated that the anti-tumor activity we observed indeed depended on loss of PSME4.
399 Accordingly, we found a significant increase in a Th17-associated cytokine signature (IL-22, IL-
400 17a and IFN γ), which is associated with promoting CD8 cell anti-tumor responses(Knochelmann
401 et al., 2018), in the secretome of splenic lymphocytes from mice bearing KP1.9^{PSME4_KD} tumors
402 (Figures 5I-K, S22B-D). These findings were in agreement with our observation that depletion of
403 PSME4 induces the secretion of IL-6, a key cytokine regulating the Treg / Th17 balance by
404 inhibiting Treg and promoting Th17 differentiation (Kimura and Kishimoto, 2010).

405



406

407 **Fig. 4 PSME4 promotes an immunosuppressive tumor environment** (A) Schematic of orthotopic lung
 408 tumor models by tail vein injection of KP1.9 cells overexpressing (KP1.9^{PSME4} OE) or deficient of (KP1.9^{PSME4}
 409 ^{KD}) PSME4 to C57/B6 mice (B) The lung weight of mice bearing parental, PSME4-overexpressing (OE), or
 410 PSME4-deficient (KD) KP1.9 tumors (**P = 0.0020). (C) Photograph of representative lungs from mice not
 411 injected with tumor cells (non-inj) 41 days after the injection of parental, PSME4 overexpressing (OE), or
 412 PSME4 deficient (KD) KP1.9 cells. (D) Uniform manifold approximation and projection visualization
 413 (UMAP) showing 35 cell populations across the three replicates from mice bearing KP1.9 (Ctrl) or
 414 KP1.9^{PSME4} ^{KD} tumors. (E) Bar graph showing total number of cells per cell type identified across the three
 415 replicates from mice bearing KP1.9 (Ctrl) or KP1.9^{PSME4} ^{KD} tumors (KD). (F-I) Boxplot showing relative
 416 abundance of alveolar macrophages (F), cycling macrophages (G), mature DCs enriched in
 417 immunoregulatory molecules (mregDCs) (H), and plasmacytoid DCs (pDCs) (I) in the lung of mice injected
 418 with KP1.9 (n = 3) or KP1.9^{PSME4} ^{KD} (PSEM4KD) (n = 3) cells. Boxplot shows 25th to 75th percentiles with
 419 the 50th denoted by a line; whiskers show 1.5× interquartile range, or maximum or minimum if smaller. (J,

420 **K)** Boxplot showing pseudobulk tpm expression of *Il6* in mregDCs (J) or expression of *Il12a* in pDCs (K) in
421 the lung of mice injected with KP1.9 (n = 3) or KP1.9^{PSME4 KD} (PSEM4KD) (n = 3) cells. **(L)** Boxplots
422 showing pseudobulk tpm expression of *Ifi2712a* in cycling cDCs, interstitial macrophages and regulatory T
423 cells in the lung of mice injected with KP1.9 (n = 3) or KP1.9 PSEM4KD) (n = 3) cells. **(M)** Barplot showing
424 number of TCR clones which were expanded (at least 2 cells sharing the same TCR clone) in the different T
425 cell populations in a representative samples from mice injected with KP1.9 or KP1.9 PSEM4 depleted
426 (PSEM4KD) cells. **(N)** Ratio of number of expanded (i.e. having non-unique TCR) Tregs to cytotoxic CD8
427 ratio in each sample.

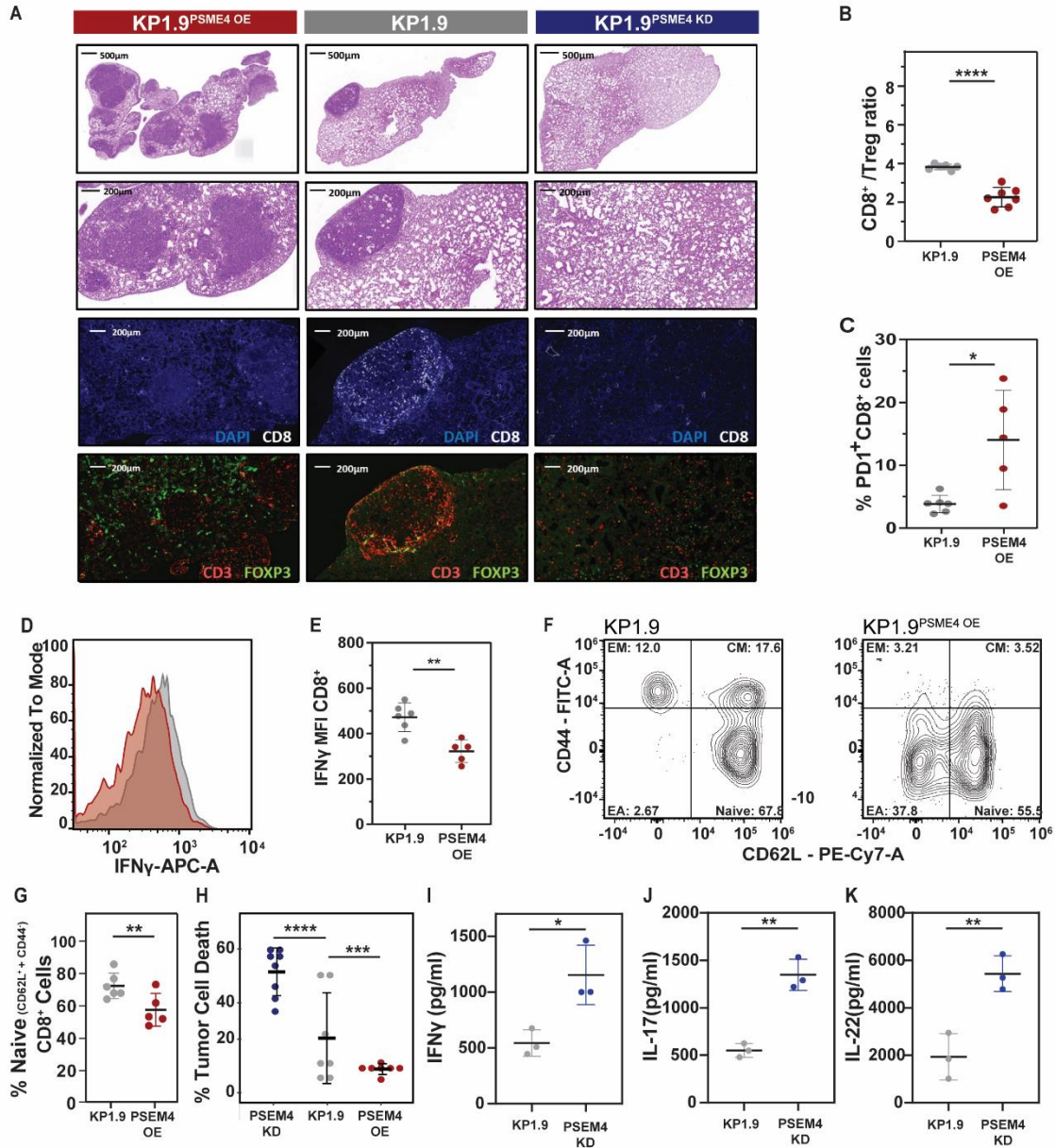
428

429 **PSME4 drives a ‘cold’ tumor signature in NSCLC and is associated with reduced response** 430 **to ICI across multiple cancer types.**

431 Our findings that PSME4 plays an anti-inflammatory role in cancer raise the intriguing possibility
432 that it promotes immune evasion in cancer and may be associated with resistance to
433 immunotherapy. Indeed, NSCLC with high levels of PMSE4 had reduced signature of T cell
434 infiltration and inflammation (Figures 6A, S23). PSME4 and immunoproteasome levels vary
435 greatly among different types of cancer (Figures. 6B and S24A). Therefore, we analyzed RNA-seq
436 data from the CPI1000+ cohort (Litchfield et al., 2021), comprising 6 different patient cohorts of
437 three different cancer types treated with ICI (Van Allen et al., 2015; Hugo et al., 2016; Mariathasan
438 et al., 2018; McDermott et al., 2018; Riaz et al., 2017b; Snyder et al., 2014) and found that PSME4
439 varies greatly among individual tumors and cancer types and that tumors with high expression of
440 PSME4 were less likely to respond to immunotherapy (Figures. 6C-D). This was true within each
441 cancer type examined separately, and when response rate was measured across the CPI1000+
442 cohort (ref) (Figure S24B). Specifically, melanoma, in which the immunoproteasome has been
443 reported to be upregulated and associated with response to therapy(Harel et al., 2019; Riaz et al.,
444 2017a), expressed PSME4 lowly on average whereas renal and bladder cancers exhibited a wide
445 range of expression for both the immunoproteasome and PSME4 (Figure 6C). Since we showed
446 that PSME4 attenuates immunoproteasome activity, we hypothesized that the ratio between
447 PSME4 and the immunoproteasome levels, and not their absolute level, may be a better measure to
448 examine response to ICI. Furthermore, we found that in the PSME4-low cellular state, PSMB10 is
449 the immunoproteasome subunit that is most increased (Figure 3B), so we chose it to categorize
450 immunoproteasome levels in the cohorts. Notably, the ratio of PSME4 to PSMB10 yielded a more
451 significant association to the response to ICI than either subunit alone (Figure 6E, S24B-C). To
452 control for the potential bias of data pooling, we also calculated the effect size across the cohorts
453 by combining the individual effect sizes of each biomarker in each cohort (Figure 6F and S25A-
454 B). PSME4 , PSMB10 and the ratio between them all had strong effect sizes on responsiveness,
455 even in comparison with tumor mutational burden, previously shown to be one of the strongest
456 classifiers of responsiveness to ICI (Litchfield et al., 2021). Furthermore, we showed that PSME4
457 did not strongly correlate with other biomarkers from the cohort and that is still had a significant
458 contribution to a general linear regression model of responsiveness even when the other significant
459 biomarkers, such as TMB were included (Figure S25A; PSME4 - P = 0.0194).

460

461 To demonstrate that the stratification of patients by the ratio of PSME4 and PSMB10 expression
462 translates to NSCLC we validated the signature we observed in the ICI1000+ cohort in a 27 patient
463 NSCLC cohort (Kim et al., 2020). Indeed, we saw that NSCLC patients with low levels of PSME4
464 in the tumor had a higher ICI response rate, consistent with the findings in Melanoma, Bladder and
465 Kidney cancer, albeit not significantly due to the small size. To strengthen this observation, we
466 utilized an Ex-Vivo Organoid Culture model (EVOC(Kamer et al., 2021)), which contain tumor
467 tissue, infiltrating lymphocytes and stroma cells (Figure 6G, S26A-B). We assessed the expression
468 of PSME4 and PSMB10 as well as stained for CD8⁺ T cell infiltrates and PDL1 from a section of
469 the resected tumor (Figure S26A-B). In parallel, we tested the IFN γ levels following treatment with
470 either Durvalumab alone (α PD-L1) or together with Ipilimumab (α CTLA-4) in the EVOCs. IFN γ



471
 472 **Fig 5. PSME4 abrogates anti-tumor immunity.** (A) H&E staining of lungs from mice bearing KP1.9,
 473 KP1.9^{PSME4 OE}, or KP1.9^{PSME4 KD} tumors (top) and immunofluorescence staining of the tumor sections with
 474 indicated antibodies (bottom). (B) The ratio of CD8 T cells to Tregs in the spleens of mice bearing KP1.9^{PSME4}
 475 OE or KP1.9 tumors (****P < 0.0001). (C) The percent of PD1 positive CD8 T cells in the lung of the mice
 476 bearing KP1.9^{PSME4 OE} or KP1.9 tumors (*P = 0.0440). (D) A histogram of IFN γ stained CD8 T cells of mice
 477 bearing KP1.9 (grey) or KP1.9^{PSME4 OE} (pink) tumors. (E) The median fluorescent intensity (MFI) of IFN γ
 478 stained CD8 T cells in the lung of mice bearing KP1.9^{PSME4 OE} or KP1.9 tumors (E; *P = 0.0017). (F-G) The
 479 percent of different subsets of CD8-positive lymphocytes (naïve, early activated, effector memory [EM] or
 480 central memory [CM], which are CD62L and/or CD44 positive, in the lung of mice bearing KP1.9^{PSME4 OE} or
 481 KP1.9 tumors. (H) The percent of KP1.9 cells co-cultured with splenic lymphocytes from mice bearing
 482 KP1.9, KP1.9^{PSME4 OE} or KP1.9^{PSME4 KD} tumors that is dead (****P < 0.0001, ***P = 0.0021). (I-K) The
 483 amount (pg/ml) of IFN γ (I) , IL-17 (J) or IL-22 (K) secreted from splenic lymphocytes from mice bearing
 484 KP1.9 or KP1.9^{PSME4 OE} tumors (*P < 0.05, **P < 0.01).

485 levels (Kamer et al., 2021) were then used to define a response-like signature in the EVOCs.
486 Notably, we found that tumors with responder hallmarks had significantly lower PSME4/PSMB10
487 ratios compared to non-responders (Figures 6H-I, S26C-F) and that neither PSME4 nor PSMB10
488 individually associated with the response (Figures S26H-I). This result did not merely reflect
489 immune cell infiltration as both PSME4 and PSMB10 were expressed in the epithelial tissue, and
490 PSMB10 was more highly expressed in the lymphocyte infiltrates, consistent with the known
491 expression of the immunoproteasome in immune cells (Figure 6I).

492

493 **Discussion**

494

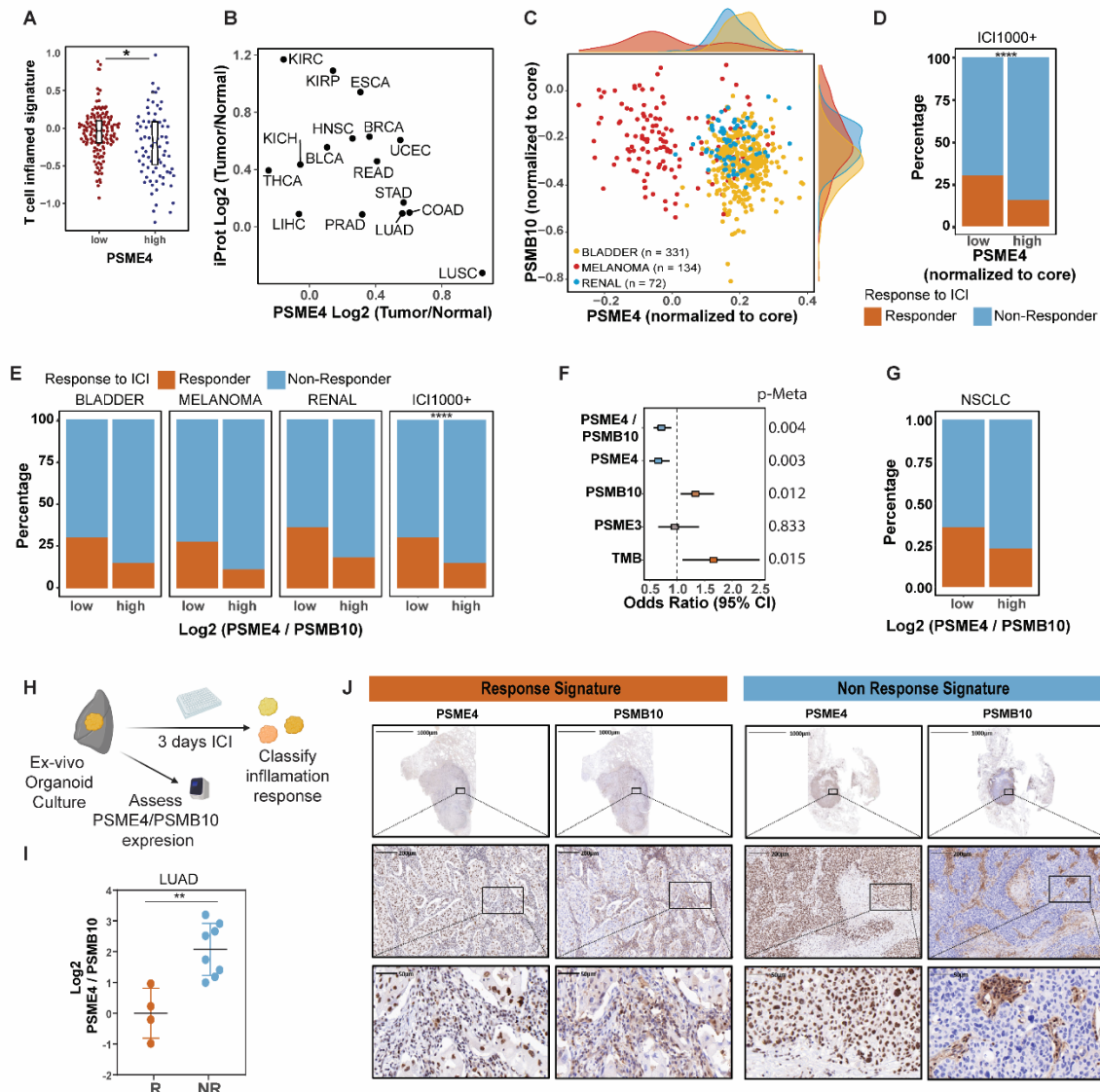
495 We show that altered proteasome composition and function shape tumor-host interactions in
496 NSCLC and that PSME4-capped proteasome plays an anti-inflammatory role in cancer by
497 attenuating immunoproteasome activity. The attenuated immunoproteasome activity in turn leads
498 to reduced antigenicity by restricting immunopeptidome diversity and altering surface HLA
499 presentation as well as reducing cellular inflammation in an IL-6 dependent manner. While label-
500 free quantitative MS analysis of cell lines suggested that <5% of 20S proteasomes bear PSME4
501 regulators (Fabre et al., 2014; Morozov and Karpov, 2019), our results suggest that this frequency
502 is increased in many cancer types. As PSME4 is associated with DNA damage and histone
503 organization, we speculate that it may be upregulated to offer a protective mechanism to cope with
504 the associated proteotoxic stress and DNA damage (Ahmed, 2019; Kammerl et al., 2019; M. Cantin
505 and V. Richter, 2012; Srinivas et al., 2019). Further, as PSME4 is known to play a nuclear role and
506 was suggested to modulate transcription (Jiang et al., 2021) it will be intriguing to comprehensively
507 examine the effect its upregulation has on the transcriptional landscape, which is beyond the scope
508 of our study. Nevertheless, the dominant effect PSME4 has on the degradation landscape, and the
509 altered catalytic activity of the immunoproteasome even in cell-free systems, strongly suggest that
510 PSME4 exerts its effect also by directly modulating proteasome activity, post-translationally.

511

512 Moreover, we found PSME4 to be the first known example of a regulator differentially modulating
513 the activities of the constitutive proteasome and immunoproteasome. PSME4 is one of a small
514 number of markers of resistance to ICI, as opposed to markers of responsiveness such as the
515 immunoproteasome, TMB, infiltrate signatures and others. As such, it highlights the potential of
516 targeting PSME4 expression or its binding to proteasomes as a novel therapeutic approach in
517 treating NSCLC and in sensitizing response to ICI. Because PSME4 levels are tumor-type specific,
518 such approaches are expected to differ between cancer types. Together, our results highlight the
519 degradation landscape and the balance between different proteasome compositions as an
520 understudied factor in pathogenesis of human diseases in general and cancer proteostasis in
521 particular. This novel proteasome-dependent mechanism of immune evasion allows tumors to
522 abrogate anti-tumor immunity is of particular interest as it provides another layer to the
523 understanding of tumor proteostasis control.

524

525



526
 527 **Fig 6 Responsiveness to ICI is reduced in PSME4-high tumors across multiple cancer types.** (A) T-cell
 528 inflammation signature was calculated for the LUAD samples in CPTAC stratified according to the PSME4
 529 expression level. (B) The ratio of PSME4 expression in the tumor versus normal tissue from the indicated
 530 TCGA cohort is plotted against the ratio of the mean of the immunoproteasome (iProt) subunits PSMB8-10
 531 and PSME1-2 in the tumor versus normal tissue. (C-E) The expression of PSME4 normalized to the core
 532 proteasome subunits is calculated for bladder cancer, melanoma and renal tumors across the Hugo,
 533 Mariathasan, McDermot, Riaz, Snyder, and Van Allen cohorts. PSME4 is plotted against PSMB10 (C).
 534 PSME4 normalized to the core proteasome subunits (D) or the ratio of PSME4 and PSMB10 expression (E)
 535 was used to stratified patients (upper and lower 50% of samples). The percentage of responders and non-
 536 responders to ICI was counted for each group per cancer type and across the CI1000+ cohort (χ^2 test $D^{****}P$
 537 = $B^{****}P = 0.000096$, $E^{****}P = 0.000016$). (F) The effect size (calculated as the log₂ odds ratio for
 538 response versus no response) and significance of the proteasome subunit biomarkers and Tumor Mutational
 539 Burden (TMB) in meta-analysis across all cohorts incorporating the effect sizes and standard errors from
 540 each individual cohort. The meta p values (p-Meta) are shown on the right. (G) The ratio of PSME4 and
 541 PSMB10 expression was used to stratified patients who responded to ICI in the NSCLC Kim cohort. (H)
 542 Workflow of the Ex-Vivo Organoid Culture System (EVOC). A fraction of each resected tumor was assessed
 543 for PSME4 and PSMB10 expression using quantitative PCR and the rest was treated for 3 days with immune

544 checkpoint inhibitors (ICI). Response-like signature to ICI was classified by IFN γ expression. **(I)** The ratio
545 of PSME4 to PSMB10 expression determined by qPCR in patient tumors with EVOC ICI responder or non-
546 responder signature (** P = 0.0056). **(J)** Immunohistochemistry for PSME4 and PSMB10 of sections with
547 an ICI response-signature and an ICI non-response-signature EVOC samples.

548 **Materials and Methods**

549

550 **Purification of proteasome complexes.**

551 Lung Adenocarcinoma tumors and adjacent tissues were mechanically disrupted and passed
552 through a 70 µm cell strainer 93070 (SPL). Cells were lysed with 25 mM HEPES, pH 7.4, 10%
553 glycerol, 5 mM MgCl₂, 1 mM ATP, and 1:400 protease-inhibitor mixture (Calbiochem), then
554 homogenized through freeze–thaw cycles and passed through a needle. The lysates were cleared
555 by 30-min centrifugation at 21,130g at 4 °C. Lysates were treated with 2 mM 1,10-phenanthroline
556 (Sigma), cross-linked with 0.5 mM DSP (Thermo Fisher Scientific) for 30 min at room temperature,
557 and quenched in 100 mM Tris-HCl, pH 8, 5 mM L-cysteine for 10 min at room temperature. For
558 immunoprecipitation, the lysates were then incubated with Protein G–Sepharose beads (Santa
559 Cruz) with antibodies to PSMA1 and eluted with 100 mM Tris-HCl, pH 8, 8M urea and 50 mM
560 DTT for 30 min at 37 °C. Subsequently, 1% trifluoroacetic acid (TFA) was added. Aliquots of each
561 elution fraction were analyzed by SDS–PAGE to evaluate yield and purity.

562 **Purification and concentration of proteasome peptides.**

563 A critical step in our procedure is the separation of peptides from the proteins eluted in the
564 proteasome pulldown. MAPP analyzes endogenously cleaved peptides, whereas the proteasome
565 complex and associated proteins are physically excluded. Immunoprecipitated proteasomes and
566 their encompassed peptides were loaded on C18 cartridges (Waters) that were prewashed with 80%
567 acetonitrile (ACN) in 0.1% TFA, then washed with 0.1% TFA only. After loading, the cartridges
568 were washed with 0.1% TFA. Peptides were eluted with 30% ACN in 0.1% TFA. Protein fractions
569 were eluted with 80% ACN in 0.1% TFA.

570 **Mass spectrometry sample processing**

571 Protein fraction after proteasome purification; Samples were loaded onto 3 kDa molecular weight
572 cut-off spin columns. Volume was reduced to 25µL by centrifugation at 14,000g for 10min. 175µL
573 8M urea was added and centrifuged at 14,000g for 10min. Filters were reversed and centrifuged to
574 extract the proteins. Proteins were reduced with 5 mM dithiothreitol (Sigma) for 1hr at room
575 temperature and alkylated with 10 mM iodoacetamide (Sigma) in the dark for 45 min at room
576 temperature. Samples were diluted to 2M urea with 50mM ammonium bicarbonate. Proteins were
577 then subjected to digestion with trypsin (Promega; Madison, WI, USA) overnight at 37°C at 50:1
578 protein:trypsin ratio, followed by a second trypsin digestion for 4 hr. The digestions were stopped
579 by addition of trifluoroacetic acid (1% final concentration). Following digestion, peptides were
580 desalted using Oasis HLB, µElution format (Waters, Milford, MA, USA). The samples were
581 vacuum dried and stored in -80°C until further analysis.

582 Total proteomics; Lysates in 5% SDS in 50 mM Tris-HCl were incubated at 96 °C for 5 min,
583 followed by six cycles of 30 s of sonication (Bioruptor Pico, Diagenode, USA). Proteins were
584 reduced with 5 mM dithiothreitol and alkylated with 10 mM iodoacetamide in the dark. Each
585 sample was loaded onto S-Trap microcolumns (Protifi, USA) according to the manufacturer's
586 instructions. In brief, after loading, samples were washed with 90:10% methanol/50 mM
587 ammonium bicarbonate. Samples were then digested with trypsin for 1.5 h at 47 °C. The digested
588 peptides were eluted using 50 mM ammonium bicarbonate; trypsin was added to this fraction and
589 incubated overnight at 37 °C. Two more elutions were made using 0.2% formic acid and 0.2%
590 formic acid in 50% acetonitrile. The three elutions were pooled together and vacuum-centrifuged
591 to dry. Samples were kept at -80 °C until analysis.

592 HLA immunopeptidomics was performed as described previously (Kalaora et al., 2020). In brief,
593 cell-line pellets were collected in triplicate from 2x10E8 cells. Cell pellets were lysed on ice with
594 a lysis buffer containing 0.25% sodium deoxycholate, 0.2 mM iodoacetamide, 1 mM EDTA, 1:200
595 Protease Inhibitor Cocktail (Sigma-Aldrich), 1 mM PMSF and 1% octyl-β-D glucopyranoside in
596 PBS. Samples were then incubated in rotation at 4°C for 1 hour. The lysates were cleared by
597 centrifugation at 48,000 g for 60 minutes at 4°C and then passed through a pre-clearing column
598 containing ProteinA Sepharose beads. HLA-I molecules were immunoaffinity purified from

599 cleared lysate with the panHLA-I antibody (W6/32 antibody purified from HB95 hybridoma cells).
600 Affinity columns were washed first with 400 mM NaCl, 20 mM Tris-HCl and then with 20 mM
601 Tris-HCl pH 8.0. The HLA-peptide complexes were then eluted with 1% trifluoroacetic acid
602 followed by separation of the peptides from the proteins by binding the eluted fraction to Sep-Pak
603 (Waters). Elution of the peptides was done with 28% acetonitrile in 0.1% trifluoroacetic acid. The
604 peptides were dried by vacuum centrifugation.

605 **Liquid chromatography mass spectrometry**

606 Peptide fraction; ULC/MS grade solvents were used for all chromatographic steps. Each sample
607 was loaded using split-less nano-Ultra Performance Liquid Chromatography (10 kpsi nanoAcquity;
608 Waters, Milford, MA, USA). The mobile phase was: A) H₂O + 0.1% formic acid and B) acetonitrile
609 + 0.1% formic acid. Desalting of the samples was performed online using a reversed-phase
610 Symmetry C18 trapping column (180 μm internal diameter, 20 mm length, 5 μm particle size;
611 Waters). The peptides were then separated using a T3 HSS nano-column (75 μm internal diameter,
612 250 mm length, 1.8 μm particle size; Waters) at 0.35 μL/min. Peptides were eluted from the column
613 into the mass spectrometer using the following gradient: 4% to 35%B in 120 min, 35% to 90%B in
614 5 min, maintained at 90% for 5 min and then back to initial conditions.

615 The nanoUPLC was coupled online through a nanoESI emitter (10 μm tip; New Objective;
616 Woburn, MA, USA) to a quadrupole orbitrap mass spectrometer (Q Exactive Plus, Thermo
617 Scientific) using a FlexIon nanospray apparatus (Proxeon).

618 Data was acquired in data dependent acquisition (DDA) mode, using a Top10 method. MS1
619 resolution was set to 70,000 (at 400m/z), mass range of 375-1650m/z, AGC of 3e6 and maximum
620 injection time was set to 100msec. MS2 resolution was set to 17,500, quadrupole isolation 1.7m/z,
621 AGC of 1e5, dynamic exclusion of 40sec and maximum injection time of 150msec.

622 **Mass spectrometry data analysis.**

623 Raw data were analyzed in MaxQuant software (version 1.6.0.16) with the default parameters for
624 the analysis of the proteasomal peptides, except for the following: unspecific enzyme, LFQ
625 minimum ratio count of 1, minimum peptide length for unspecific search of 6, maximum peptide
626 length for unspecific search of 40, and match between runs enabled. A stringent false discovery
627 rate (FDR) of 1% was applied for peptide identification. For the analysis of tryptic digests, the
628 default parameters were set, apart from a minimum peptide length of 6. Masses were searched
629 against the human proteome database from UniprotKB (last update September 2018).

630 **Proteomics processing and label-free quantification.**

631 Peptides resulting from MaxQuant were initially filtered to remove reverse sequences and known
632 MS contaminants. For MAPP peptide fraction we removed antibody and proteasome peptides as
633 contaminants. To decrease ambiguity, we allowed peptides that had at least two valid LFQ
634 intensities out of sample replicates, and we included razor peptides, which belong to a unique
635 MaxQuant 'protein group'. MAPP protein intensities were inferred with MaxQuant. For graphical
636 representation, intensities were log transformed, and in Python v3.6, zero intensity was imputed to
637 a random value chosen from a normal distribution of 0.3 s.d. and downshifted 1.8 s.d. For clinical
638 cohorts, zero intensity was imputed to half the minimum. The presence of missing values reflects
639 both technical and biological variation in the samples. In cases of matched samples ratios were
640 calculated per pair and paired t tests were used. Otherwise, ratios were calculated based on the
641 median of each group and a non-paired student t test was used. The protein fraction of the MAPP
642 analysis was normalized to the mean of the core proteasome subunits (PSMA1-7 and PSMB1-4) to
643 control for efficiency. For comparison of WC proteomics and MAPP data, proteins were ranked by
644 their signed pvalue: the sign of the fold-change between conditions multiplied by the negative log
645 10 transformed p value. Proteins which had positive (tumor-increased) values were binned into 10
646 groups of equal size. Any proteins which were detected in MAPP but not detected or defined as
647 adjacent -associated in WC proteomics were termed not-detected. The proteins in MAPP or WC
648 proteomics with the top 50 signed p values were presented in heatmaps in Figure 1. For the
649 immunopeptidomics, values from a peptide that was not detected in a treatment pair were not

650 included in the analysis. The binding of peptides was predicted by netMHC based on A549
651 haplotypes as described previously (Javitt et al., 2019b). Any peptide with a binding rank greater
652 than 5 was considered as a contaminant for analysis of the differentially presented peptides.

653 **TCGA and CPTAC analysis.**

654 The cancer genome atlas (TCGA) data was mined using the xenaPython package in Python 3.6.
655 The results shown in this analysis are in whole or part based upon data generated by the TCGA
656 Research Network: <http://cancergenome.nih.gov/>. The full lung carcinoma cohort (both lung
657 adenocarcinoma [LUAD] and lung squamous cell carcinoma [LUSC] designations, n = 1128, 3A
658 and S10) or the LUAD cohort alone (n = 576, S2) as indicated. For the cross-cancer proteasome
659 ratio the GDC pan cancer dataset was used. PSME4 high tumors in Figure 6 and Figures. S24-25
660 were defined as those with above average expression of PSME4. Only cancer types with 10 or more
661 normal controls were used. Ratios presented are the difference between the means of the normal
662 and tumor groups. Data used in this publication were generated by the Clinical Proteomic Tumor
663 Analysis Consortium (NCI/NIH). The CPTAC LUAD cohort (n = 213) was used. In cases where
664 ratios between the adjacent and tumor samples are presented only those samples with matching
665 controls are used. Otherwise, all the tumor and normal (adjacent control) samples were utilized.
666 The T cell inflammation signature was based on Spranger et al. (Spranger et al., 2015; Trujillo et
667 al., 2018).

668 **Immune checkpoint inhibitor response analysis.**

669 Data reprocessing of six cohorts of ICI treated patients across three cancer types (Van Allen et al.,
670 2015; Hugo et al., 2016; Mariathasan et al., 2018; McDermott et al., 2018; Riaz et al., 2017b;
671 Snyder et al., 2014) was performed as described previously (Litchfield et al., 2021). The expression
672 of different proteasome subunits was examined across the samples. Where noted, proteasome
673 subunit expression was normalized to the core proteasome subunits (PSMA1-7 and PSMB1-4) for
674 each sample. Patients were then stratified by the expression of different subunits (50 percent highest
675 and lowest expressers) and this was correlated with response to ICI which was defined as in
676 (Litchfield et al., 2021). To avoid data pooling a meta-statistics approach was used as described in
677 (Litchfield et al., 2021). Likewise, to confirm the independence of different biomarkers a general
678 linear regression model was used. The model was first generated for 35 biomarkers and then
679 iteratively reduced to only the markers which significantly contributed to the classification. The
680 signatures were confirmed in an independent cohort of NSCLC (Kim et al., 2020).

681 **Statistical analysis and data visualization.**

682 Statistical analyses were performed in R v 3.6.2 and GraphPad Prism v 7.04. In R data was
683 visualized using the complexheatmap (Zuguang Gu et al., 2016) and ggplot (Wickam, 2016)
684 packages. Defaults were used unless otherwise noted. Protein annotation and gene ontology
685 analysis were performed with the gene-set enrichment analysis and protein subcellular localization
686 was extracted from the Human protein atlas (Thul et al., 2017; Uhlen et al., 2017; Uhlén et al.,
687 2015) with “uncertain” or “approved” localization reliabilities excluded.

688 **Antibodies and plasmids**

689 Rabbit anti PSME4 HPA060922 1:1000 WB, 1:300 IF (Sigma), Rabbit anti PSMD11 ab99414
690 1:1000 WB, Mouse anti proteasome 20s PSMA1-7 subunits ab22674 1:1000 WB, Rabbit anti
691 PSMB10/MECL1 ab183506 1:1000 WB, Mouse anti β Actin ab170325 1:5000 WB (Abcam),
692 Rabbit anti PA28 α /PSME1 9643 1:1000 WB, Rabbit anti PSMB8/LMP7 13635 1:1000 WB
693 and Rabbit anti PA28 γ 2412 1:1000 WB (Cell Signaling).

694 human HLA-A,B,C-PE (W6/32, Biolegend 311406), FC, 1:20; mouse CD45.2-PerCP/Cy5.5 (104,
695 eBioscience 45-0454-82), FC, 1:200; mouse CD3e-Super Bright 436 (145-2C11, eBioscience 62-
696 0031-82), FC, 1:100; mouse CD4-APC/Cy7 (GK1.5, Biolegend 100414), FC, 1:200; mouse CD8a-
697 Super Bright 702 (53-6.7, Biolegend 100748), FC, 1:200; mouse CD279 (PD-1)-PE (RPM1-30,
698 eBioscience 12-9981-81), FC, 1:100; mouse IFN γ -APC (XMG1.2, eBioscience 17-7311-82), FC,
699 1:160; mouse/human CD44-FITC (IM7, Biolegend 103005), FC, 1:200; mouse CD62L-PE/Cy7
700 (MEL-14, Biolegend 104418), FC, 1:6000; mouse FoxP3-Pe (FJK-16s, eBioscience 12-5773-82),

701 FC, 1:200; mouse H-2Db-FITC (KH95, Biolegend 111505), FC, 1:200; mouse H-2kb-PE (AF6-
702 88.5.5.3, eBioscience 12-5958-82), FC, 1:500; Zombie Aqua (Biolegend 423102), FC, 1:300;
703 Propidium Iodide (Sigma Aldrich P4170), FC, 1 ug/mL; CFSE (Biolegend 423801), FC, 5 uM.
704 Mouse anti alpha-6, produced from hybridoma was a kind gift from Keiji Tanaka. IgG1k isotype
705 antibody (BioLegend, BLG-400139) was used as a control of MAPP method.
706 Goat anti Mouse 488 A11029 1:400, Goat anti mouse 647 A31571 1:400 (Invitrogen), Goat anti
707 Rabbit 647 AB150075 1:400 (Abcam), Goat anti mouse HRP 115-035-205 1:5000, Goat anti
708 Rabbit HRP 111-035-003 1:5000 (Jackson labs).
709 MISSION shRNA targeting mouse or human PSME4 or RFP were obtained from Sigma
710 (TRCN0000176569, TRCN0000178428, TRCN0000158223, TRCN0000157073).
711 pcDNA3.1_PSME4 (Welk et al., 2019) was transfected into A549/KP1.9 cells with Lipofectamine
712 2000 (Thermo Fisher).

713 **Cell culture and drug treatments**

714 A549 were originally obtained from ATCC grown in DMEM and used as model systems for cell
715 biology studies including western blot, biochemistry, and imaging. KP1.9, kindly provided by
716 Alfred Zippelius, were grown in Iscove's MDM. Cells were routinely tested for mycoplasma
717 contamination. Media were supplemented with 10% fetal bovine serum, 1%
718 Penicillin/streptomycin and L-glutamine (2mmol/l) (Biological industries), unless otherwise
719 indicated, at 37 °C with 5% CO₂.

720 For TNF- α /IFN- γ activation cells were allowed to seed overnight, and subsequently treated with
721 TNF- α (PeproTech, human; 300-01A or mouse; 315-01A) and IFN- γ (PeproTech, human; 300-02
722 or mouse; 315-05) at 20 ng/ml and 10 ng/ml, respectively, for 24h.

723 Cell death assessment was done by trypan blue staining and counted with Countess II™ automated
724 cell counter (Thermo Fisher) and by Cell titerGlo assay (Promega). Proliferation of mammalian
725 cells were measured by KIT8 assay (Sigma).

726 **Proteasome cleavage reporter assay**

727 Cells were lysed with 25 mM HEPES, pH 7.4, 10% glycerol, 5 mM MgCl₂, 1 mM ATP, and 1:400
728 protease-inhibitor mixture (Calbiochem), then homogenized through freeze-thaw cycles and
729 passed through a needle. The lysates were cleared by 30-min centrifugation at 21,130g at 4 °C to
730 remove cell debris. Protein concentration of the supernatant was determined by a NanoDrop
731 spectrophotometer (Thermo Fisher Scientific) by measuring the absorbance at 280 nm.

732 Proteasomal activity in cell fractions was determined by cleavage of the fluorogenic precursor
733 substrate Suc-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC), Ac-Pro-Ala-Leu-AMC (Ac-PAL-
734 AMC), Z-Leu-Leu-Glu-AMC (Z-LLE-AMC), Ac-Arg-Leu-Arg-AMC (Ac-RLR-AMC), Ac-Ala-
735 Asn-Trp-AMC (Ac-ANW-AMC) (Boston Biochem), Z-Leu-Leu-Glu- β NA (Z-LLE- β NA), Ac-
736 Nle-Pro-Nle-Asp-AMC (Ac-nLPnLD-AMC) (Bachem). 10 μ M substrate was added to 10 μ g total
737 protein/well, and incubated in a reaction buffer (50 mM Hepes, pH 7.5, 1 mM DTT, 5 mM MgCl₂
738 and 2 Mm ATP). When using recombinant proteins such as PSME4 [purified as described in(Toste
739 Rêgo and da Fonseca, 2019)] or PSME1 (AB-206168, abcam) 1 nM of each protein was add to 100
740 ul final reaction. Fluorescent increase resulting from degradation of peptide-AMC at 37°C was
741 monitored over time by means of a fluorometer (Synergy H1 Hybrid Multi-Mode Microplate
742 Reader, BioTek) at 340 nm excitation and at 460 nm emission, using the proteasome inhibitor,
743 MG132 (Calbiochem) as background. Resulting product curves were followed for up to 3.5 hours.
744 Each value of fluorescence intensity represents a mean value obtained from three independent
745 experiments.

746 **Immuno-blotting**

747 Cells were lysed in STET buffer (50 mM Tris-HCl, pH 8.7.5, 150 mM NaCl, 2 Mm EDTA 1%
748 Triton-X and 1:400 protease-inhibitor mixture (Calbiochem)). Protein concentration was assessed
749 using BC Assay Protein Quantitation Kit (Interchim). 20 μ g of total protein was separated by SDS-
750 PAGE on 4-20% gradient ExpressPlus PAGE M42015 (GenScript) and transferred onto PVDF
751 membranes using iBlot™ 2 Gel Transfer Device (Thermo Fisher Scientific). The membranes were

752 blocked in 5% milk prepared in TBS-0.1% Tween and incubated in primary antibodies overnight
753 at 4°C followed by washing and incubation with secondary antibody. Blots were developed using
754 the ChemiDoc XRS+ Imaging System (Bio-Rad) and band intensities were quantified with imageJ
755 analyzer software.

756 **Immuno-precipitation**

757 Cells lysed in 25 mM HEPES, pH 7.4, 10% glycerol, 5 mM MgCl₂, 1 mM ATP, and 1:400
758 protease-inhibitor mixture (Calbiochem), then homogenized through freeze–thaw cycles and
759 passed through a needle. The lysates were cleared by 30-min centrifugation at 21,130g at 4 °C to
760 remove cell debris. Pre-cleared cell lysates were prepared by incubating with Protein A/G
761 conjugated agarose beads for 30 min at 4°C with slow rotation. 1 mg Pre-cleared cell lysates were
762 then added to 20 µL of fresh Protein A/G conjugated agarose beads along with 4 µg of antibodies
763 (as described). The mixture was incubated over-night at 4°C with slow rotation followed by three
764 1 ml washes with chilled PBS buffer. The target conjugates were then eluted from beads by mixing
765 protein loading dye (with reducing agents) and heating for 5 min at 95°C followed by denaturing
766 Western blot.

767 **Gel Filtration**

768 Gel filtration experiments were performed using Superose® 6 Increase 10/300 GL size exclusion
769 column (GE Healthcare) ÄKTA pure protein purification system (Cytiva). The running buffer used
770 was 25 mM HEPES, pH 7.4, 10% glycerol, 5 mM MgCl₂ and 1 mM ATP. The column was
771 calibrated using gel filtration molecular weight standard (Bio-Rad). The following standards were
772 used for calibration: Thyroglobulin (670 kDa, Rs=8.6 nm), γ-globulin (158 kDa, Rs=5.1 nm),
773 Ovalbumin (44 kDa, Rs=2.8 nm), Myoglobin (17 kDa, Rs=1.9 nm), Vitamin B12 (1.35 kDa), and
774 Dextran blue (2 MDa).

775 A 0.1 ml protein sample at a final concentration of 0.4 mg/ml was filtered and chromatographically
776 analyzed using a flow rate of 0.5 ml/min. Absorbance was monitored at 280 nm, elution volumes
777 were determined from UV chromatogram. The partition coefficient, K_{av} , was calculated from the
778 elution volume of the sample, V_e , and total bed volume, V_t , using the expression: $K_{av} = (V_e -$
779 $V_0)/(V_t - V_0)$. Calibration curves and equations were established.

780 **Surface HLA staining in flow cytometry**

781 A549 and KP1.9 cells were treated with hTNFα/hIFNγ (as described above) or mTNFα/mIFNγ (as
782 described above), respectively, for 24 hr. Cells were harvested and stained with Live/Dead Aqua
783 staining kit from BioLegend as per manufacturer's protocol. Approximately 1x10⁶ cells were
784 washed, blocked with 2% FCS for 5 min and stained with anti-HLA-A/B/C-PE (A549) or anti-
785 MHCI-Kb-PE/MHCI-Db-FITC (KP1.9) in 100ul for 30 min. Cells were washed and acquired.

786 **Tissues fixation, processing and embedding**

787 Tissues were fixed in 4% paraformaldehyde for one week. Dehydration of the tissue was done by
788 serial immersion in increasing concentrations of alcohol (70 to 100%) and removal of the dehydrant
789 with Xylene. The tissue was embedded in paraffin, sectioned at 4 µm and mounted on microscope
790 slides. The slides were placed in an oven for one hour at 60°C or overnight at 37°C before
791 immunohistochemically staining.

792 **Immunohistochemistry (IHC) and Immunofluorescence staining**

793 H&E stains were performed on an automated device according to manufacturer's instructions. IHC
794 stains were performed on a Benchmark XT staining module (Ventana Medical Systems Inc.; USA)
795 using iVIEW DAB Detection Kit (760-091, Ventana Medical Systems Inc.) or Ultra VIEW
796 Universal DAB Detection Kit (760-500, Ventana Medical Systems Inc.; USA). Antibody details
797 are described above. Following immunostaining, sections were counterstained with hematoxylin
798 (Ventana Medical Systems Inc.), rinsed in distilled water and dehydrated manually in graded
799 ethanols. Finally, the sections were cleared in xylene and mounted with Entellan (Surgipath
800 Medical Industries Inc) on glass slides.

801 The immunofluorescent staining was performed at Molecular Cytology Core Facility of MSKCC
802 using Discovery XT processor (Ventana Medical Systems). Sections were stained with antibodies

803 for CD31 (endothelial cell marker), CD3 (T cells), CD8 (T cells) and FoxP3 (Tregs). Scanning of
804 slides was done by PANNORAMIC SCAN II (3DHISTECH).

805 **IHC staining PSME4**

806 Paraffin embedded mouse and human tissues were cut in 3 µm thick sections using the Hyrax M55
807 microtome (Zeiss). Tissue sections were incubated for one hour at 60 °C in order to melt paraffin,
808 deparaffinized by incubating two times in xylene for 5 min and rehydrated in a descending alcohol
809 series (100 %, 90 %, 80 % and 70 % (v/v)) for 1 min. To block endogenous protease activity and
810 to permeabilize sections for nuclear staining they were incubated in a methanol/hydrogen peroxide
811 (80 %/1.8 % (v/v)) solution for 20 min. Tissue sections were rinsed in Milli-Q® water and heat-
812 induced antigen retrieval was performed in citrate buffer pH 6 using a decloaking chamber (Biocare
813 Medical). After washing with TBST unspecific binding sites were blocked for 30 min with Rodent
814 Block M (Biocare Medical). The slides were washed again in TBST and incubated with anti-
815 PSME4 antibody (sc-135512, Santa Cruz) diluted in Antibody Diluent (DAKO) for 1 h at RT. After
816 extensive washing in TBST sections were incubated with MACH 2 Rabbit AP-Polymer (Biocare
817 Medical) for 30 min at RT. Sections were rinsed again in TBST and incubated in Vulcan Fast Red
818 AP substrate solution (Biocare Medical) for 10 min. Tissue sections were washed in TBST and
819 MilliQ® water and hematoxylin counterstaining (Carl Roth) was performed to visualize nuclei.
820 After repeated washing in TBST, sections were dehydrated in ethanol and xylene and mounted
821 using Entellan mounting medium (Merck Millipore). Slides were imaged using the MIRAX
822 scanning system (Zeiss).

823 Stainings were analyzed by an expert clinical pathologist blinded to the sample identity. Tumor
824 staging was done as described previously (Klotz et al., 2019). Semiquantitative scores for PSME4
825 expression were obtained by defining the percentage of PSME4 positively stained tumor areas
826 multiplied by the intensity of staining as graded between 1 (weak) to 3 (strong). Scores were
827 dichotomized into high (>80) and low (<80) expressing tumors with the score 80 representing the
828 median of all samples and PSME4 expression scores were correlated with survival of the patients
829 for this cohort.

830 **q-PCR analysis**

831 RNA was extracted using Direct-zol™ RNA MiniPrep R2051 (ZYMO research) mRNA levels
832 were ascertained by RT using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) real
833 time quantitative PCR using sybr-green (Kapa Biosystems) and the StepOnePlus™ Real-Time PCR
834 Systems (Life Technologies) using the following primers:

<u>Primers for real time PCR</u>			
mPSME4	F	CGGTTGGCTGAACAAGTTAATG	
mPSME4	R	CTCAGGTCTCATAAGTGCAAGG	
hPSME4	F	ATTTGGAGTTACCCTGGAGACC	
hPSME4	R	GCAGCTTTTCACGAGTGTTTTG	
hPSMB10	F	TCCTTCGAGAAGTCCAAAGA	
hPSMB10	R	ATCGTTAGTGGCTCGCGTATC	
hTNFa	F	CCACGTCGTAGCAAACCACC	
hTNFa	R	TTGAGATCCATGCCGTTGGC	
hISG15	F	GAG AGG CAG CGA ACT CAT CT	
hISG15	R	CTT CAG CTC TGA CAC CGA CA	
hIFIT	F	GATCAGCCATATTTCAATTTGAATC	
hIFIT	R	GAAAATTCTCTTCAGCTTTTCTGTG	
hIL6	F	TTCTCCACAAGCGCCTTCGGTC	
hIL6	R	TCTGTGTGGGGCGGCTACATCT	
hA20	F	GCGTTCAGGACACAGACTTG	
hA20	R	TTCATCATTCCAGTTCAGGATATC	
hRPS18	F	ATTAAGGGTGTGGGCCGAAG	
hRPS18	R	TGGCTAGGACCTGGCTGTAT	
hGAPDH	F	CAACGGATTTGGTCTGATTG	
hGAPDH	R	GATGACAAGCTTCCCCTTCT	
mGAPDH	F	AAGGTCATCCCAGAGCTGAA	
mGAPDH	R	CTGCTTACCACCTTCTTGA	

835

836 All values were normalized to the mRNA abundance of housekeeping genes (RPS18 or GAPDH
837 in Human; GAPDH in Mouse). Each primers pair was calibrated using the Absolute Quantification
838 program with increasing concentrations of cDNA.

839 **EVOC preparation**

840 Resected tumors were treated for 3 days, in an Ex Vivo Organ Culture (EVOC) with ICI and
841 responders were classified based on their IFN γ expression as described (Kamer et al.). In short,
842 IFN γ levels were tested following treatment with Durvalumab alone (α PD-L1), or combined with
843 Ipilimumab (α CTLA-4) in the EVOCs. In cases where IFN γ was significantly enriched by at least
844 1.9-fold induction the EVOCs were designated as ‘responder’, while the rest were termed ‘non-
845 responder’. This was recently shown to be a correlate for responsiveness to ICI in patients
846 (Kamer et al.).

847 **Orthotopic lung cancer model**

848 Mouse experiments were conducted according to approved experimental procedures (Approval
849 numbers 04400520-2 and 04990620-1). Male C57Bl/6J mice (Envigo, Israel) or RAG1 -/- (Jackson
850 Laboratory) at the age of 8-10 weeks were injected i.v. with 2×10^5 cells in 200 μ L PBS (Biological
851 Industries, Israel). Mice were observed for adverse effects and weights noted twice a week.
852 Peripheral blood was collected from the tail vein. At the end of experiment, mice were sacrificed
853 by CO₂ asphyxiation and tissues collected to cold PBS.

854 **Tissue processing and flow cytometry staining**

855 Peripheral blood was washed and red blood cells removed by ACK lysis buffer (150 mM NH₄Cl,
856 10 mM KHCO₃, 0.1 mM EDTA in 0.1x PBS-/-) and washed in flow cytometry buffer (PBS-/-,
857 0.5% BSA, 2mM EDTA).

858 Spleens and lungs were weighted before further processing. The left lung lobe was used for
859 histological analysis of tumor development (hematoxylin and eosin) after fixation in 4%
860 formaldehyde (Biolabs, Israel). Right lung lobes were minced and digested with 5 mL digestion
861 buffer: Collagenase 4 (200U/mL, Worthington Biochemicals), DNase I (100 ug/mL, Sigma
862 Aldrich) in PBS++ supplemented with 2 mM CaCl₂ for 20 minutes at 37°C under shaking. Single
863 cell suspensions of spleen and lungs were obtained by straining cells through 100 μ m strainers and
864 washed in cold flow cytometry buffer. Red blood cells were removed by ACK lysis buffer, washed
865 with flow cytometry buffer and strained again.

866 For intracellular cytokine and transcription factor stainings, cells were incubated for 4 hours with
867 Brefeldin A (Biolegend) and Monensin (Sigma Aldrich). First, viability staining was done with
868 Zombie Aqua (Biolegend) according to the protocols. Cell surface staining was performed in flow
869 cytometry buffer in 100 μ L at appropriate dilutions. When needed, cells were fixed and
870 permeabilized with the FoxP3 transcription factor kit from eBioscience (Thermo Fisher Scientific)
871 and stained for FoxP3 and IFN γ according to the manufacturers protocol. Samples were acquired
872 on an Attune Nxt with Autosampler and analyzed in FlowJo (V10.7.1, Becton, Dickinson and
873 Company). Exemplary gating strategies are provided Figures S10, S20 and S22)

874 **Cytotoxicity assay**

875 10,000 KP1.9 WT cells were stained with CFSE (5uM; BioLegend), at a cell concentration of
876 1×10^6 /ml), plated onto a 96-well plate and allowed to attach overnight. The following day,
877 splenocytes were isolated from naïve mice, or from mice bearing KP1.9-WT, -PSME4 KD, or -
878 PSME4 OE tumors. Red blood cells were lysed using ACK buffer, cells were washed in PBS, and
879 100,000 splenocytes were plated on top of the tumor cells in RPMI medium + beta-mercaptoethanol
880 (50uM). Co-culture was maintained for 7 days. On day 7, cell supernatants were collected onto a
881 new 96-well plate, and the attached tumor cells were detached with Trypsin containing Propidium
882 Iodide (PI). After cell detachment, the supernatant was used to resuspend the detached cells, and
883 the entire sample was acquired.

884 **Cytokine and chemokine secretion**

885 Tumor microenvironment; 10,000 KP1.9 WT cells were plated onto a 96-well plate and allowed to
886 attach overnight. The following day, splenocytes were isolated from naïve mice, or from mice
887 bearing KP1.9-WT, -PSME4 KD, or -PSME4 OE tumors. Red blood cells were lysed using ACK
888 buffer, cells were washed in PBS, and 100,000 splenocytes were plated on top of the tumor cells in
889 RPMI medium + beta-mercaptoethanol (50uM). Co-culture was maintained for 48h or 7 days. The
890 medium was collected at 48h and 7days by centrifugation. Cytokines and chemokines concentration
891 in the tumor microenvironment were determined by 36-Plex Mouse ProcartaPlex (ThermoFisher,
892 EPX360–26092–901) as per manufacturer’s instructions. Samples were acquired on MAGPIX
893 System (Luminex) and analysed by MILLIPLEX Analyst V5.1 Flex.
894 Tumor cells secretion; 10,000 KP1.9 shCtrl or KP1.9 PSME4 KD cells were plated onto a 96-well
895 plate and allowed to attach overnight. The following day cells were treated with mTNF α and mIFN γ
896 mix for 24h in the last 6h ONX-0914 (1 μ M) (APExBIO, A4011) was added to desired wells. Cells
897 were washed twice and incubated for 24h in fresh medium. The medium was collected flowed be
898 centrifugation. Cytokines and chemokines secretion were determined by 36-Plex Mouse
899 ProcartaPlex (ThermoFisher, EPX360–26092–901) as per manufacturer’s instructions. Samples
900 were acquired on MAGPIX System (Luminex) and analysed by MILLIPLEX Analyst V5.1 Flex.

901

902 **Single cells RNA-Seq of CD45+ cells from lung tissues**

903

903 **CD45+ cell isolation from lung tissues**

904

904 C57Bl/6J mice were injected with KP1.9 shCtrl or shPSME4 cells (0.25x10⁶ cells, i.v.). After 21
905 days, lungs were perfused manually with 3 mL cold PBS w/o Mg/Ca through the right ventricle of
906 the heart and harvested (4x shCtrl, 4x shPSME4) into cold PBS. The lung tissue was cut into pieces
907 and processed using a GentleMACS Octo Dissociator (program 37C_m_LDK_1, Miltenyi Biotec)
908 with the mouse lung dissociation kit (130-095-927, Miltenyi Biotec) according to the
909 manufacturer’s protocol. After the run, cells were strained through a 70 μ m mesh and washed with
910 FACS buffer (0.5% BSA, 2 mM EDTA in PBS without Mg/Ca). Red blood cells were lysed with
911 ACK buffer for 4 minutes, the reaction stopped by addition of cold FACS buffer. Finally, cells
912 were resuspended in FACS buffer with FC block (anti-CD16/CD32, 1:500, Biolegend 101330) and
913 incubated for 5 minutes. Lung cells from shCtrl or shPSME4 were stained with four differently
914 labeled CD45 antibodies (APC, PE/Cy7, FITC, violetFluor 450) for 30 minutes at 4C. Cells were
915 washed with FACS buffer twice, and the shCtrl or shPSME4 samples unified for parallel sorting
916 of the four lung populations. Propidium iodide was added fresh (1 μ g/mL) to exclude dead cells,
917 and cells were sorted on BD FACSAria III and BD SORP FACSAria II sorters running on BD
918 FACSDiva software (V8.0.1) using a 85 μ m nozzle. A gating strategy is provided in the
919 supplementary single cell documentation. Finally, cells were washed twice (PBS w/o Mg/Ca,
920 0.04% BSA) and counted on a Neubauer chamber with trypan blue, and three samples from each
921 condition were chosen (high cell count and viability >90%) for further processing.

Target	clone	color	Company	lot	Cat#	dilution
CD45.2	104	APC	eBioscience	2265360	17-0454-82	300
CD45.2	104	Pe/Cy7	eBioscience	4330432	25-0454-82	300
CD45.2	104	FITC	Biolegend	B250186	109806	300
CD45	30-F11	violetFluor 450	Tonbo	C0451092017753	75-0451	300

922

923

923 **Single cell RNA-seq using Chromium 10x genomics platform**

924

925

926

927

Single cell RNA-seq libraries were prepared at the Crown Genomics institute of the Nancy and
Stephen Grand Israel National Center for Personalized Medicine, Weizmann Institute of Science.
Cells were counted and diluted to a final concentration of approximately 1000 cells/ μ L in PBS
supplemented with 0.04% BSA. Cellular suspension was loaded onto Next GEM Chip K targeting

928 6000 cells and then ran on a Chromium Controller instrument to generate GEM emulsion (10x
929 Genomics). Single-cell gene expression libraries as well as single cell V(D)J libraries were
930 generated according to the manufacturer's protocol using the Chromium Next GEM Single Cell 5'
931 Reagent Kits v2 (Dual Index) workflow. Final libraries were quantified using NEBNext Library
932 Quant Kit for Illumina (NEB) and high sensitivity D1000 TapeStation (Agilent). Libraries were
933 pooled according to targeted cell number, aiming for at least 5,000 reads per cell for V(D)J libraries
934 and 20,000 reads per cell for gene expression libraries. Pooled libraries were sequenced on a
935 NovaSeq 6000 instrument using an SP 200 cycles reagent kit (Illumina).

936 **Raw reads processing**

937 Raw sequencing bcl files were converted to fastq files, aligned to GRCm38 mouse genome, unique
938 molecular identifiers (UMIs) were quantified using Cell Ranger suite (v 6.0.0). V(D)J libraries were
939 mapped to GRCm38 VDJ reference provided by 10x Genomics (v 5.0.0), and clones were
940 identified, counted and summarized with Cell Ranger suite (v 6.0.0). Filtered count matrices were
941 imported into R for further processing.

942 **Clustering and cell type identification**

943 In gene expression libraries low quality cells with more than 10% reads mapping to mitochondrial
944 genome were removed. The Seurat R package (version 4.0.3) was used to normalize and scale
945 expression values for total UMI counts per cell (Stuart et al., 2019). Due to complexity and
946 variability in the immune cell population we performed stepwise clustering (Grün et al., 2015). A
947 detailed clustering strategy is shown in supplementary single cell documentation. First, 2000 highly
948 variable genes were identified using vst method. Dimensionality reduction was done with principal
949 component analysis, with the first 30 principal components used for nearest neighbor graph
950 construction and clustering. For each cluster based on their marker expression we identified
951 whether it belongs to one of the groups: non-immune, B cells, T cells, ILCs, macrophages and DCs,
952 cycling cells and other. Subsequently we performed clustering within each group individually as
953 described above, for each cluster we identified markers (Seurat FindMarkers function) and removed
954 clusters containing doublets, i.e. clusters that did not have any uniquely expressed genes and
955 contained markers specific for of at least 2 abundant clusters. For example, clusters with high
956 expression of Ncr1 (NK cell marker) and Cd79b (B cell marker) was considered doublets.

957 Finally, for each cluster we identified markers (Seurat FindMarkers function) and annotated them
958 through comparison with markers in literature and ImmGen database(Heng et al., 2008; Maier et
959 al., 2020; Rosser and Mauri, 2015)

960 **Functional analysis of cell populations**

961 Gene Ontology analysis was performed using g:Profiler2(Raudvere et al., 2019) with default
962 settings, and multiple hypothesis testing adjustment using all mouse genes as background control.
963 The log₁₀ FDR-adjusted P values were plotted as barplots.

964 **Differential expression analysis**

965 To compare gene expression of each cell type cluster between conditions, we calculated pseudobulk
966 by adding reads from all cells within each cluster in a sample. We then used DESeq2 with default
967 parameters to determine differential expression(Love et al., 2014).

968 **Statistics and reproducibility**

969 All experiments were performed in three individual replicates unless otherwise mentioned. For
970 each experiment, all compared conditions were analyzed by MS at the same time. For the NSCLC
971 study, nine samples from tumors and adjacent tissue were analyzed, one was excluded for poor
972 technical quality. The samples were processed independently and analyzed by MS at the same time
973 to maintain comparability across samples and decrease batch effects.

974 **Ethics Statement**

975 Human lung tissues obtained from patients surgically treated for lung cancer were provided by the
976 Asklepios Biobank for Lung Disease, Gauting, Germany or obtained from the Israel National
977 Biobank for Research. Samples were obtained under the approval of the Ministry of Health IRB
978 approval for the Israel National Biobank for Research, protocol no. 118-2018, or the ethics

979 committee of the Ludwig-Maximilians University Munich according to national and international
980 guidelines (project number 333-10).

981 For the ex-vivo organoid cultures, all patients provided written informed consent for the use of
982 blood samples and tumor specimens for research. This study was approved by the Sheba Medical
983 Center ethics committee.

984 **Code availability**

985 All the code used is available from the corresponding author upon reasonable request.

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996 **Author Contributions.**

997 AJ, MDS and YM conceived, designed and interpreted experiments and wrote the manuscript. AL-
998 E and SM provided feedback on the manuscript. MDS, MR, AU, HW-L, AE-L proteomics
999 preparation and SM for in vitro work. MPK and IC in vivo and immunofluorescence. AAK, MPK
1000 and AJ performed and analyzed the scRNAseq experiment. AJ and KL analyzed the ICI cohort.
1001 IK, EBD and OZ conducted the EVOG and immunohistochemistry. VW, ESP, ML, and IK analysis
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