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Poly(I:C) primes primary human glioblastoma cells for an immune response invigorated by PD-L1 blockade

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28

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36

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42

43 **Abstract**

44

45 Prognosis of glioblastoma remains dismal, underscoring the need for novel therapies.
46 Immunotherapy is generating promising results, but requires combination strategies to unlock
47 its full potential. We investigated the immunomodulatory capacities of poly(I:C) on primary
48 human glioblastoma cells and its combinatorial potential with programmed death ligand (PD-
49 L) blockade. In our experiments, poly(I:C) stimulated expression of both PD-L1 and PD-L2 on
50 glioblastoma cells, and a pro-inflammatory secretome, including type I interferons (IFN) and
51 chemokines CXCL9, CXCL10, CCL4 and CCL5. IFN-β was partially responsible for the
52 elevated PD-1 ligand expression on these cells. Moreover, real-time PCR and chloroquine-
53 mediated blocking experiments indicated that poly(I:C) triggered Toll-like receptor 3 to elicit
54 its effect. Cocultures of poly(I:C)-treated glioblastoma cells with peripheral blood mononuclear
55 cells enhanced lymphocytic activation (CD69, IFN-γ) and cytotoxic capacity (CD107a,
56 granzyme B). Additional PD-L1 blockade further propagated immune activation. Besides
57 activating immunity, poly(I:C)-treated glioblastoma cells also doubled the attraction of $CD8^+$
58 T cells, and to a lesser extent $CD4^+$ T cells, via a mechanism which included CXCR3 and CCR5
59 ligands. Our results indicate that by triggering glioblastoma cells, poly(I:C) primes the tumor
60 microenvironment for an immune response. Secreted cytokines allow for immune activation
61 while chemokines attract $CD8^+$ T cells to the front, which are postulated as a prerequisite for
62 effective PD-1/PD-L1 blockade. Accordingly, additional blockade of the concurrently elevated
63 tumoral PD-L1 further reinforces the immune activation. In conclusion, our data proposes
64 poly(I:C) treatment combined with PD-L1 blockade to invigorate the immune checkpoint
65 inhibition response in glioblastoma.

Introduction

Glioblastoma is the most common malignant primary brain tumor and carries an extremely poor prognosis.[1] The current standard of care consists of maximal surgical resection followed by chemoradiotherapy and eventually chemotherapy alone.[2] Nevertheless, tumor recurrence is nearly inevitable, contributing to a median survival of only 14.6 months and a five-year survival of less than 5.5 %.[1,2] In order to control this devastating disease, new treatment modalities are urgently required.

Immunotherapy is being intensively investigated due to its ability to specifically target cancer cells and mediate long-term surveillance. The recent clinical success of immune checkpoint inhibitors in melanoma and lung cancer has accelerated research on employing these pathways to relief immunosuppression observed in cancer, including in glioblastoma.[3,4] Immune checkpoints are molecules that dampen immune responses in order to prevent autoimmunity.[5] However, they are commonly hijacked by tumors to escape immunity, which is a hallmark of cancer.[6,7] Since tumor-infiltrating lymphocytes (TILs) are encountered in glioblastoma and expression of programmed death ligand 1 (PD-L1) has been observed,[8,9] it has been proposed that this immune checkpoint pathway plays a role in controlling the antitumor immune response in glioblastoma.[4,8] Preclinical glioblastoma mouse models have shown encouraging results with immune checkpoint blocking antibodies and several clinical trials investigating their therapeutic benefit in glioblastoma are ongoing.[4,10-12] Nevertheless, combination therapy is key to improving cancer treatment efficacy and preclinical studies that help guide rational choices of combination strategies remain highly warranted.

Alleviation of existing immunosuppression in combination with boosting immune activation has been postulated as an immunotherapeutic strategy to pursue for eliciting effective immune

90 responses at the tumor site. Polyriboinosinic-polyribocytidylic acid, or poly(I:C), is considered
91 an immunostimulant with high potential to boost cancer immunotherapy.[13] This synthetic
92 mimetic of viral double-stranded RNA binds to either the endosomal Toll-like receptor 3
93 (TLR3), or the cytoplasmic receptors melanoma differentiation-associated protein 5 (MDA-5)
94 or retinoic acid-inducible gene 1 (RIG-I).[13,14] As such, poly(I:C) activates several immune cell
95 types, including dendritic cells (DC) and natural killer (NK) cells, leading to antitumor
96 responses.[15] Furthermore, poly(I:C) also influences tumor cells, resulting in inhibition of
97 proliferation and induction of apoptosis.[15,16] Several clinical trials are/have been testing
98 poly(I:C), including in glioblastoma.[15]

99 Since both immune checkpoint inhibitors and poly(I:C) are being investigated in a clinical
100 setting, we examined in this study how poly(I:C) treatment of primary human glioblastoma
101 cells affects their immunomodulatory capacity in order to assess its potential for combination
102 therapy with immune checkpoint ligand blockade.

103

104 **Results**

105

106 *Poly(I:C) modifies the immunological profile of primary glioblastoma cells*

107 Primary glioblastoma cells were treated with increasing concentrations of poly(I:C) in order to
108 assess its influence on the expression of immune checkpoint ligands PD-L1 and PD-L2 in
109 glioblastoma. Poly(I:C) significantly upregulated the expression of both PD-1 ligands on
110 glioblastoma cells (Fig. 1A). PD-L1 was upregulated to a greater extent than PD-L2 and this in
111 a dose-dependent manner. The upregulation of PD-L1 was correlated with the upregulation of
112 PD-L2 ($R = 0.726$, $p < 0.05$; Fig. 1B), but only for the latter the basal expression level was also
113 correlated with expression after treatment with 10 µg/ml poly(I:C) ($R = 0.855$, $p < 0.01$; Fig.
114 1B). Furthermore, poly(I:C) also significantly induced expression of both PD-1 ligands on PD-
115 $L1^-$ and PD-$L2^-$ primary glioblastoma cells, resulting in 95 % and 74 % of the cells becoming
116 PD-$L1^+$ and PD-$L2^+$, respectively (Fig. 1C). Of note, poly(I:C) appeared to be as potent as
117 interferon (IFN)-γ in upregulating and inducing the PD-1 ligands. These results show that
118 poly(I:C) is an efficient stimulator of PD-L1 and PD-L2 expression on glioblastoma cells.

119 Poly(I:C) is recognized as a potent inducer of type I IFN.[15,17] Hence, we investigated whether
120 poly(I:C) could initiate a pro-inflammatory secretome in primary glioblastoma cells. Poly(I:C)
121 significantly induced the secretion of vast amounts of IFN-β, as well as IFN-α to a lesser extent
122 (Fig. 2). On the other hand, IFN-γ was not secreted following poly(I:C) treatment (Fig. 2). In
123 addition, we also observed a significant increase in secretion of interleukin (IL)-15 (three-fold),
124 while the secretion of transforming growth factor (TGF)-β was significantly decreased by 26
125 % (Fig. 2). Finally, we assessed the secretion of poly(I:C)-inducible chemokines capable of
126 attracting lymphocytes. The secretion of C-C motif chemokine ligand 4 (CCL4), CCL5 and C-
127 X-C motif chemokine ligand 10 (CXCL10) was significantly invigorated upon poly(I:C)

128 exposure, while also CXCL9 secretion was elevated (Fig. 2). These data show that exposure to
129 poly(I:C) converts the secretome of glioblastoma cells towards an immunostimulatory profile.

130

131 *Poly(I:C) mediates its effect via TLR3*

132 Poly(I:C) can mediate its effect via the endosomal receptor TLR3 or the cytoplasmic receptors
133 MDA-5/RIG-I (reviewed in reference 15). We assessed their adaptor molecules, TLR adaptor
134 molecule 1 (TICAM1) for TLR3 and mitochondrial antiviral signaling protein (MAVS) for
135 MDA-5 and RIG-I, in order to evaluate which receptor pathway in primary glioblastoma cells
136 is activated by poly(I:C). RNA was isolated from glioblastoma cells following a 24-hour
137 treatment with poly(I:C). The quantitative real-time PCR (qRT-PCR) data revealed a significant
138 upregulation of TICAM1, but not of MAVS (Fig. 3A). The enhanced expression of the TLR3
139 downstream molecule TICAM1 indicates that poly(I:C) mediates its effect in glioblastoma cells
140 via the endosomal TLR3-TICAM1 axis. Next, we validated the involvement of TLR3 by
141 blocking its signaling using chloroquine, an inhibitor of TLR3 signaling.[18] Indeed, the addition
142 of chloroquine significantly reduced the poly(I:C)-mediated upregulation of both PD-L1 and
143 PD-L2 (Fig. 3B-C). These data suggest that the effect of poly(I:C) is, at least primarily,
144 mediated via TLR3-TICAM1 signaling.

145

146 *Poly(I:C) triggers de novo production of PD-L1 and PD-L2 in primary glioblastoma cells*

147 Since intracellular PD-L1 has been observed,[8,9,19] we investigated whether poly(I:C) induced
148 translocation of intracellular PD-1 ligands rather than their *de novo* synthesis. qRT-PCR data
149 showed significantly elevated mRNA of both PD-L1 and PD-L2 following poly(I:C) treatment
150 (Fig. 4A). In particular, PD-L1 mRNA levels were enormously elevated, which supports our
151 flow cytometric data. Elevated transcription of PD-L1 was correlated with PD-L2 transcription

152 (R = 0.707, $p < 0.05$; Fig. 4B). Next, we used immunohistochemistry (IHC) to reveal the cellular
153 location of the PD-1 ligand proteins before and after poly(I:C) treatment. Whereas naïve
154 glioblastoma cells were only faintly positive for PD-L1, poly(I:C)-treated glioblastoma cells
155 presented a strong positive staining for PD-L1 protein (Fig. 4C). In contrast to naïve
156 glioblastoma cells, poly(I:C)-treated glioblastoma cells displayed cytoplasmic PD-L1 with
157 membrane accentuation. Moreover, the increase in PD-L1 protein observed following poly(I:C)
158 treatment was greater than the amount of PD-L1 protein which was visually present in the
159 cytoplasm of naïve glioblastoma cells. A similar observation regarding PD-L2 protein was
160 made in naïve versus poly(I:C)-treated glioblastoma cells (Fig. 4D). These data indicate that
161 the heightened membrane expression of PD-1 ligands on glioblastoma cells following treatment
162 with poly(I:C) is mainly derived from *de novo* produced protein.

163

164 *The effect of poly(I:C) on the expression of PD-L1 and PD-L2 is partially mediated by IFN-β*

165 We described the release of type I IFN (Fig. 2), which have been attributed the capacity to
166 upregulate the expression of PD-L1.[20] In a series of blocking experiments, we investigated
167 whether IFN-α and IFN-β were involved in induction and upregulation of PD-L1 and PD-L2
168 following poly(I:C) treatment. IFN-β, in contrast to IFN-α, significantly contributed to the
169 upregulation and induction of both PD-1 ligands (Fig. 5A-B). Whereas IFN-β contributed
170 similarly to upregulation and induction of PD-L2 by poly(I:C), it was less involved in induction
171 of PD-L1 compared to its upregulation. IFN-α was involved in the induction of neither PD-L1
172 nor PD-L2, but contributed to the upregulation of both ligands in three and four out of five
173 primary glioblastoma cell lines, respectively, although not statistically significant (Fig. 5A-B).
174 These results indicate that the effect of poly(I:C) on PD-L1 and PD-L2 is partially mediated via
175 downstream-secreted IFN-β.

176

177 *Poly(I:C) modifies the immunomodulatory capacity of primary glioblastoma cells*

178 The observed poly(I:C)-induced pro-inflammatory secretome by and immunosuppressive PD-
179 1 ligand expression on primary glioblastoma cells may have opposing effects on immune cells.
180 Therefore, we investigated the overall immunomodulation of primary glioblastoma cells
181 following treatment with poly(I:C) by setting up cocultures with peripheral blood mononuclear
182 cells (PBMC), in which we focused on lymphocytes as effector immune cells. Based upon the
183 activation marker CD69, the number of activated $CD8^+$ T and $CD4^+$ T cells as well as NK cells
184 was significantly higher in cocultures with poly(I:C)-treated glioblastoma cells compared to
185 naïve glioblastoma cells (Fig. 6A). A similar significant increase could be observed for the
186 degranulation marker CD107a (Fig. 6A). These observations were validated by cytokine
187 analyses, which showed a significant increase in IFN-γ as well as granzyme B (Fig. 6B).
188 Secretion of TNF-α was not elevated in cocultures with poly(I:C)-treated glioblastoma cells
189 (Fig. 6B), neither was IL-1β release (data not shown). Given primary glioblastoma cells did not
190 release IFN-γ in response to poly(I:C) (Fig. 2) and granzyme B is restricted to lymphocytes,[21]
191 these results point towards both phenotypic and functional immune cell activation following
192 poly(I:C) treatment of glioblastoma cells.

193

194 *Poly(I:C)-treated primary glioblastoma cells attract effector lymphocytes*

195 Using a transwell migration assay, we investigated whether lymphocytes are attracted to
196 poly(I:C)-treated primary glioblastoma cells. $CD8^+$ T cells were significantly more attracted to
197 poly(I:C)-treated glioblastoma cells by nearly two-fold, whereas also $CD4^+$ T cells migrated
198 significantly more to this condition (Fig. 7A). On the other hand, chemoattraction of NK cells
199 was not significantly altered, although two out of six PBMC donors presented a considerably

200 enhanced migration (Fig. 7A). Of note, considering attraction of $CD8^+$ T cells to poly(I:C)-
201 treated glioblastoma cells was favored over $CD4^+$ T cells, poly(I:C) treatment of primary
202 glioblastoma cells also stimulated a more favorable CD8:CD4 T cell ratio (Fig. 7B). Since T
203 cells showed an enhanced migration, we next examined the chemokine receptor profile of the
204 migrated T cells in both conditions. CXCR3 and CCR5 were significantly downregulated on
205 both $CD8^+$ and $CD4^+$ T cells in the poly(I:C) condition (Fig. 7C). These data indicate that
206 poly(I:C)-treated glioblastoma cells stimulate lymphocyte attraction via a mechanism that
207 involves CXCR3 and CCR5.

208

209 *PD-L1 blockade reinforces the immune activation triggered by poly(I:C)-treated primary*
210 *glioblastoma cells*

211 Poly(I:C)-treated primary glioblastoma cells activate immune cells, despite elevated tumoral
212 PD-1 ligand expression. We examined the effect of additional PD-L1 or PD-L2 blockade on
213 immune activation. PD-L1 blockade significantly propagated the immune activation
214 established via poly(I:C) treatment of glioblastoma cells, as shown by IFN-γ secretion (Fig.
215 8A). Furthermore, additional PD-L1 blockade also significantly increased granzyme B release
216 and $CD8^+$ T cell proliferation (Fig. 8B-C). In contrast, additional blockade of PD-L2 had a more
217 limited effect on enforcing immune activation. IFN-γ secretion and $CD8^+$ T cell proliferation
218 were only stimulated in up to half of the patients (Fig. 8A and 8C). Similar to PD-L1 blockade,
219 also additional PD-L2 blockade resulted in a modest, but significant elevated granzyme B
220 release (Fig. 8B). These data suggest that the combination of PD-L1 blockade with poly(I:C)
221 can strengthen immune responses against glioblastoma.

222

223 **Discussion**

224

225 Inevitable progression and ultimate fatality underscore the urgency of new treatment modalities
226 for glioblastoma. Recent breakthroughs in immunotherapy and improved understanding of
227 tumor immunology have encouraged the belief for immunotherapeutic success in glioblastoma.
228 The present study demonstrates that poly(I:C) induces an immunostimulatory profile in primary
229 human glioblastoma cells, despite elevated expression of PD-L1 and PD-L2, which is in part
230 due to secreted IFN-β. This situation enables immune activation which is invigorated by
231 additional PD-L1 blockade. Moreover, attraction of lymphocytes, in particular $CD8^+$ T cells,
232 creates a tumor micro-environment (TME) primed for antitumor responses.

233 We show that both PD-L1 and PD-L2 are present at low levels on a subset of naïve primary
234 human glioblastoma cells. Basal expression of PD-L1 on glioblastoma cells has been
235 described.[8,9,19] We are the first to show PD-L2 expression on human glioblastoma cells as well
236 as induction and upregulation of PD-L1 and PD-L2 on glioblastoma cells due to poly(I:C). This
237 is in concordance with PD-L1 upregulation observed in neuroblastoma cells.[22] Of note, another
238 study demonstrated downregulation of PD-L1 on hepatoma cells *in vivo*,[23] however, it was not
239 determined whether poly(I:C) mediated this directly or via bystander cells. The positive
240 correlation observed between the transcriptional activities of PD-L1 and PD-L2 is supported by
241 a recent Genome Atlas analysis of glioblastoma samples.[24]

242 Next, we investigated how this effect of poly(I:C) on primary human glioblastoma cells was
243 mechanistically driven. Since intracellular PD-L1 has been described,[8,9,19] this could have
244 served as a possible source of the elevated membrane expression observed following poly(I:C)
245 treatment. However, IHC analysis revealed that the increase in membrane PD-L1 and PD-L2
246 expression originated foremost from *de novo* protein, as we could not detect sufficient

247 intracellular amounts pre-exposure. Such *de novo* protein production is akin to the IFN-γ-
248 mediated mechanism of PD-L1 upregulation in breast cancer cell lines[25] and is supported by
249 the increased transcription of PD-L1 and PD-L2 genes that we observed in poly(I:C)-treated
250 primary glioblastoma cells.

251 Poly(I:C) is known to be an efficient inducer of pro-inflammatory cytokines, in particular of
252 type I IFN.[15] In this regard, we demonstrated that poly(I:C) indeed drastically modified the
253 secretome of primary human glioblastoma cells. Our data show that poly(I:C) induces the
254 release of both IFN-α and IFN-β by primary glioblastoma cells, which is in contrast to findings
255 of Glas et al.[16] The observed upregulation of TICAM1 but not MAVS suggests that this effect
256 is mediated via the endosomal TLR3-TICAM1 pathway, as it has been shown that TLR3 and
257 TICAM1 become upregulated following engagement with poly(I:C).[26] Indeed, upon addition
258 of chloroquine, a TLR3 inhibitor,[18] the poly(I:C)-mediated upregulation of membrane PD-L1
259 and PD-L2 was drastically reduced. Moreover, it has been reported that the TLR3-TICAM1
260 pathway is required for IFN-β release by tumor cells in response to anthracyclines.[27] Subsequent
261 para-/autocrine activation of IFN-α and -β receptor subunit 1, IFNAR, led to tumor cell-
262 mediated release of CXCL10 which ultimately recruited immune cells.[27] These data support
263 our findings that poly(I:C) induces the secretion of type I IFN by primary glioblastoma cells,
264 but also of chemokines CXCL9, CXCL10, CCL4 and CCL5, either directly or via a para-
265 /autocrine circuit. Furthermore, to our knowledge, we are the first to report on the receptor
266 signaling pathway activated by poly(I:C) to augment PD-L1 and PD-L2 expression.

267 IFN-γ has most often been described to stimulate PD-L1 expression,[19] however, also type I IFN
268 have been attributed this capacity.[17,20] Our data indicate that IFN-β contributes to both induction
269 and upregulation of PD-1 ligands on primary human glioblastoma cells via the aforementioned
270 para-/autocrine signaling, whereas IFN-α was not significantly involved in our experiments.
271 This difference in contribution may possibly be a reflection of the difference in magnitude of

272 secretion following poly(I:C) treatment, which was 23-fold higher for IFN-β. Nonetheless,
273 others identified IFN-β as the most potent PD-L1 stimulant of both type I IFN in melanoma.[20]
274 Our data suggest that the effect of poly(I:C) on PD-1 ligand expression on primary glioblastoma
275 cells is propagated via poly(I:C)-induced release of IFN-β.

276 Poly(I:C) generated a type I IFN response by primary human glioblastoma cells, which plays
277 an important role in antitumor immunity,[17] but also the release of other cytokines was
278 modulated. Of note, we found elevated secretion of IL-15 and reduced secretion of TGF-β by
279 poly(I:C)-treated primary human glioblastoma cells. IL-15 is a highly potent immune-activating
280 cytokine, which potentiates the antitumor functions of several immune cell types, including NK
281 cells, γδT cells, and DC.[28-30] We are, to our knowledge, the first to report on IL-15 secretion by
282 glioblastoma cells in particular and its production by human cancer cells following poly(I:C)
283 treatment in general. Two murine studies demonstrated therapeutic efficacy of IL-15 in
284 glioblastoma, dependent on activation and recruitment of lymphocytes.[31,32] Moreover, type I
285 IFN have been shown to induce IL-15 secretion as well as expression of IL-15 receptor-α, which
286 allows transpresentation of IL-15.[33] TGF-β is the most powerful tumor-promoting and
287 immunosuppressive cytokine and actively plays a role in glioblastoma.[34] We report a decreased
288 secretion of TGF-β by primary glioblastoma cells following poly(I:C) treatment, which is in
289 concordance with findings in neuroblastoma cells.[22] Reduction of TGF-β release has been
290 demonstrated by IFN-α in wound healing[35] and IFN-β in adenoviral cancer therapy,[36] which
291 might provide a possible explanation for our observation. Altogether, the modified secretome
292 of poly(I:C)-treated primary glioblastoma cells points towards an immunomodulatory shift
293 towards activation.

294 We demonstrated that poly(I:C)-treated primary human glioblastoma cells indeed activate
295 lymphocytic populations, i.e. $CD8^+$ and $CD4^+$ T cells as well as NK cells, and stimulate their
296 degranulation. Overall, this resulted in an augmented IFN-γ and granzyme B secretion, that

297 could be completely assigned to the PBMC. Boes et al. also observed increased activation of
298 CD8$^+$ and CD4$^+$ T cells in coculture with poly(I:C)-treated neuroblastoma cells, based on
299 membrane immunophenotyping.[22] When in addition PD-L1 was blocked, we found immune
300 activation was even more profound. Hence, these data indicate that poly(I:C) treatment of
301 primary glioblastoma cells invigorates immune activation, despite elevated expression of
302 immunosuppressive PD-1 ligands, and that this immune activation can further be propagated
303 by additional PD-L1 blockade. This is in line with peripheral solid tumor murine models, where
304 the combination of poly(I:C) with blockade of the PD-1/PD-L1 axis was more beneficial than
305 either treatment individually.[37,38] It will be interesting to investigate whether this also happens
306 with brain tumors, given their unique localization. In this view, therapeutic efficacy of
307 ipilimumab against brain metastases indicates the feasibility of antibody-mediated immune
308 checkpoint blockade in the brain.[8]

309 Tumor-infiltrating effector lymphocytes are key to efficacious immunotherapy,[39] which
310 warrants strategies for their trafficking. We showed that poly(I:C)-treated primary human
311 glioblastoma cells enhance attraction of CD8$^+$ effector T cells, and to a lesser extent of CD4$^+$
312 helper T cells. This is noteworthy, since tumor-infiltrating CD8$^+$ T cells are inversely correlated
313 with tumor grade[40] and are positively associated with overall survival.[41] Moreover, higher
314 attraction of CD8$^+$ T cells in comparison to CD4$^+$ T cells also drives a more favorable CD8:CD4
315 T cell ratio, which has been shown to be lower in malignant compared to benign brain
316 malignancies.[42] In this context, dense CD8$^+$ T cell infiltration has been shown to correlate with
317 improved survival in brain metastases.[43] We observed an increased CD8:CD4 T cell ratio,
318 however, this remained rather low due to the use of healthy PBMC. This is in concordance with
319 Kmiecik et al., who observed far more CD4$^+$ than CD8$^+$ T cells in the healthy donor peripheral
320 blood in contrast to a more balanced CD8:CD4 TIL in glioblastoma.[44] Hence, stimulating the
321 attraction of CD8$^+$ T cells may be indicative of improving the prognosis of glioblastoma

322 patients. Furthermore, blockade of the PD-1/PD-L1 axis has been reported to primarily affect
323 $CD8^+$ T cells, whereas CTLA-4 blockade primarily acts on $CD4^+$ T cells.[45] Indeed, Nagato et
324 al. identified $CD8^+$ T cells as mediators of the therapeutic efficacy of poly(I:C) plus PD-1/PD-
325 L1 blockade in peripheral solid tumor models,[38] although Bald et al. ascribed prominent roles
326 to $CD4^+$ T cells and NK cells also.[37] We observed a substantial release of CXCL9, CXCL10,
327 CCL4 and CCL5, chemokines which strongly attract lymphocytes. Furthermore, lymphocytes
328 migrating to supernatant derived from poly(I:C)-treated primary glioblastoma cells presented a
329 downregulation of corresponding chemokine receptors CXCR3 and CCR5. This observation
330 suggests that, based upon the process of receptor sequestration,[46,47] ligands of both receptors
331 are involved in the improved T cell chemotaxis. In this view, CXCL10-mediated intracranial
332 infiltration of $CD8^+$ T cells was observed following intramuscular injections with stabilized
333 poly(I:C) in a glioblastoma mouse model,[48] which suggests that poly(I:C) enables immune
334 infiltration in glioblastoma, even when administered peripherally. Our data suggest that
335 poly(I:C)-stimulated tumoral secretion of CXCL9, CXCL10, CCL4 and CCL5 could have
336 participated in the enhanced T cell trafficking.

337 Immune checkpoint blockade prolongs survival in patients with pre-existing lymphocytic
338 infiltrates and failure to PD-L1 treatment has been correlated with the absence of intratumoral
339 $CD8^+$ T cells.[12,49] TILs are encountered in the majority of glioblastoma, however mostly
340 sparse/moderate and rather at the perivascular and invasive areas than within the tumor tissue.[8]
341 Nduom et al. postulated that low tumoral PD-L1 expression in glioblastoma may be a reflection
342 of a relatively low $CD8^+$ T cell infiltrate, which might be an indication of non-responsiveness
343 to PD-1 blockade in glioblastoma.[9] Another indication may be the incapacity of TILs to readily
344 invade the tissue, rendering the tumor “immune-excluded”.[50] In this case, the tumor should
345 advance in the cancer-immunity cycle for an optimal response to immune checkpoint blockade,
346 e.g. by increasing immunological infiltration.[50] Poly(I:C) is capable of activating cellular

347 antitumor immunity in immune cell-poor tumors, as demonstrated by a beneficial outcome in
348 conjunction with PD-1 blocking therapy in immune cell-poor melanoma.[37] This supports data
349 of us and others that poly(I:C) attracts effector lymphocytes, in particular $CD8^+$ T cells.[38,51] At
350 the same time, poly(I:C) repolarizes tumor-resident microglia and macrophages towards an M1
351 active profile and matures DC.[51,52] Hence, poly(I:C) primes the TME for battle, but it also
352 stimulates the expression of PD-L1 and PD-L2. However, this provides an additional target and
353 blockade of the PD-1/PD-L1 axis may relieve the brake in order to propagate immunity. Indeed,
354 a murine glioblastoma DC vaccination study required combinatorial anti-PD-1 treatment to
355 unleash an antitumor response.[53]

356 In this study, we focused on the lymphocytic compartment of immune cells. However, it is well
357 known that the majority of the immune infiltrate in glioblastoma consists of tumor-associated
358 microglia and macrophages (TAMs), which are polarized towards an immunosuppressive and
359 tumor-supportive state.[44] Pyonteck et al. showed that repolarization of TAMs via CSF-1R
360 inhibition led to an antitumor response in glioma[54] and this treatment option is currently under
361 investigation in clinical trials in glioblastoma, alone or in combination with PD-1 blockade.[55]
362 Interestingly, also poly(I:C) is capable of reverting these tumor-residing microglia to an
363 antitumor state.[52] Moreover, poly(I:C) abrogated the immunosuppressive functions of myeloid-
364 derived suppressor cells in a murine breast cancer model, leading to an enhanced T cell influx
365 and enhanced survival.[56] Recently, it has been shown that PD-1 on TAMs suppresses their
366 phagocytic functions and antitumor immunity.[57] On the other hand, PD-1 ligand expression by
367 TAMs may contribute to immunosuppression.[58] Hence, PD-L1 blockade could restore
368 antitumor functions of and inhibit immunosuppression by TAMs. Therefore, we speculate that
369 *in vivo*, treatment with poly(I:C), in particular in combination with PD-L1 blockade, may have
370 a more profound effect than what we observed *in vitro* with lymphocytes.

371 The predictive value of PD-1, PD-L1 or PD-L2 as biomarker for blocking therapy is still under
372 investigation,[8,9] although tumoral PD-L1 expression has been confirmed as a significant, but
373 not absolute, predictive marker in several solid malignancies.[59] A robust assay for evaluation
374 of the blocking efficacy *in vitro* has yet to be recommended. We and others have observed no
375 evidence of enhanced cytotoxicity *in vitro*[60] and only few papers in the enormous field of
376 immune checkpoint blockade actually have. Furthermore, Beavis et al. have shown *in vivo* that
377 the antitumor effect of combined PD-1 and adenosine receptor A2 blockade was largely
378 independent on perforin, despite elevated granzyme B^+ $CD8^+$ T cells, but lost in IFN-γ knockout
379 mice.[60] Therefore, the effect of immune checkpoint blockade may be primarily IFN-γ-mediated,
380 possibly amongst other cytokines. We propose analysis of IFN-γ secretion in *in vitro* tumor
381 cell-immune cell cocultures as a read-out of immune activation mediated by immune
382 checkpoint blockade. This outcome can be assessed in a quick and reliable manner using IFN-
383 γ ELISA.

384 In conclusion, poly(I:C) shifts the immunomodulatory profile of primary human glioblastoma
385 cells towards immune activation via the TLR3-TICAM1 pathway, despite a stimulated
386 expression of immune checkpoint ligands PD-L1 and PD-L2, which is partially dependent on
387 autocrine/paracrine secreted IFN-β. Poly(I:C)-treated primary glioblastoma cells stimulate the
388 attraction of lymphocytes, in particular $CD8^+$ T cells, while also enhancing lymphocytic
389 activation. Immune activation is further invigorated by additional blockade of PD-L1. Both
390 poly(I:C) and immune checkpoint blockade are being investigated in clinical trials. We propose
391 the combinatorial treatment to strengthen their therapeutic efficacy and generate a profound
392 immune response. Data in other murine cancer models look promising, but do not have to
393 account for the blood-brain barrier as encountered in glioblastoma. Therefore, we plan to
394 investigate this combination strategy in *in vivo* glioblastoma models as validation of this *in vitro*
395 proof of concept.

396 **Material and Methods**

397

398 *Ethics statement*

399 Human tumor tissue was obtained from residual material following standard surgery of patients
400 diagnosed with glioblastoma, performed in Antwerp University Hospital and AZ Nikolaas, and
401 provided by Biobank@UZA (Belgium; ID: BE71030031000). The use of these tissues for this
402 study was approved by the local Ethics Committee of the University of Antwerp (Belgium;
403 reference number: 13/46/454). Characteristics of the primary tumor tissues are shown in Table
404 1. PBMC were isolated from adult volunteer whole blood donations (supplied by the Red Cross
405 Flanders Blood Service, Belgium).

406

407 *Primary human cell culture*

408 Primary human glioblastoma cell cultures were established from patient-derived tumor tissue
409 samples.[61] Tumor specimens were cut into small pieces and cultured at 37 °C and 5 % CO_2 in
410 Dulbecco's Modified Eagle Medium (DMEM; Gibco, 10938) supplemented with 10 % fetal
411 bovine serum (FBS; Gibco, 10270), 2 mM L-glutamine (Gibco, 25030), 100 U/ml penicillin
412 and 100 µg/ml streptomycin (Gibco, 15140). Following outgrowth, cells were harvested using
413 0.05 % trypsin-EDTA (Gibco, 25300) and filtered over a 40 µm cell strainer (Falcon, 352340)
414 prior to subculturing. All cultures were confirmed as *Mycoplasm* free following MycoAlert
415 analysis (Lonza, LT07-218).

416 PBMC were isolated from peripheral whole blood using Ficoll-Paque PLUS density gradient
417 centrifugation (GE Healthcare, 17-1440) and frozen in FBS containing 10 % dimethyl sulfoxide
418 (Merck, 1.02931.0500) in liquid nitrogen (Air Liquide). For experiments, PBMC were thawed

419 one day in advance in Roswell Park Memorial Institute medium (RPMI; Gibco, 52400)
420 supplemented with 10 % FBS, 2 mM L-glutamine, 1 mM sodium pyruvate (Gibco, 11360), 500
421 U/ml penicillin, 500 µg/ml streptomycin and 1.5 KU/ml DNase I (Sigma, D4263).

422

423 *Flow cytometric tumor phenotyping*

424 The response to poly(I:C) was analyzed by incubating the primary human glioblastoma cells
425 with 1, 10 and 100 µg/ml poly(I:C) (InvivoGen, tlrl-pic), or 5000 IU/ml IFN-γ (ImmunoTools,
426 11343536) as positive control. After 24 hours, supernatant was collected for cytokine analysis.
427 Primary glioblastoma cells were harvested and stained for 15 minutes with phycoerythrin-
428 conjugated antibodies against PD-L1 (BD Biosciences, 557924), PD-L2 (BD Biosciences,
429 558066), or isotype control (BD Biosciences, 555749). Additionally, dead cells were excluded
430 using propidium iodide (Life Technologies, P3566). Expression of PD-L1 and PD-L2 on viable
431 primary glioblastoma cells was measured on a BD FACScan flow cytometer (Becton
432 Dickinson). Data analysis was performed using FlowJo v10 software (TreeStar). Representative
433 flow cytometry profiles are shown in Supplementary Figure S1. Difference in mean
434 fluorescence intensity (ΔMFI) was calculated to evaluate target upregulation ($\Delta MFI = MFI_{target}$
435 $- MFI_{isotype}$), while the overton subtraction tool in FlowJo was used to determine the percentage
436 of positive cells (overton subtraction on: $histogram_{target} - histogram_{isotype}$).

437 Blocking experiments were performed similarly with one additional step. In order to investigate
438 the involvement of TLR3, the primary glioblastoma cells were incubated for one hour with 50
439 µM chloroquine (Abcam, ab142116) prior to the stimulation with 10 µg/ml poly(I:C). For the
440 assessment of the involvement of type I IFN, pre-incubation was performed with 10 µg/ml
441 blocking antibody against IFN-α (InvivoGen, maba-hifna-3), IFN-β (InvivoGen, mabg-hifnb-
442 3), or corresponding isotype control (InvivoGen, maba2-ctrl and mabg2a-ctrlm). The effect of

443 blocking on PD-L1 and PD-L2 upregulation by poly(I:C) was calculated using the following
444 formula: relative upregulation = ($MFI_{poly(I:C)+blocking}$ – $MFI_{unstimulated+isotype}$) / ($MFI_{poly(I:C)+isotype}$ –
445 $MFI_{unstimulated+isotype}$). The blocking effect on PD-L1 and PD-L2 induction by poly(I:C) was
446 calculated accordingly using overton data.

447

448 *Cytokine analysis*

449 Secretion of cytokines and chemokines by primary human glioblastoma cells in response to
450 stimulation with poly(I:C) was analyzed using electrochemiluminescent detection on a
451 SECTOR3000 (MesoScale Discovery/MSD) using Discovery Workbench 4.0 software (MSD).
452 The human cytokine panel included IFN-β (MSD, K151ADB-1), TGF-β (MSD, K151IUC-1),
453 CXCL9 (MSD, N45IA-1), CCL5 (MSD, K151BFB-1) and IFN-α, IL-15, CXCL10 and CCL4
454 (MSD, K15067L-1). Additionally, IFN-γ secretion was analyzed using ELISA (eBioscience,
455 88-7316) with Nunc-Maxisorp flat-bottom 96-well plates (eBioscience, 44-2404). The ELISA
456 was read on an iMark Microplate Absorbance Reader (Bio-Rad) using Microplate Manager 6
457 Software (Bio-Rad). Immune activation in cocultures of primary glioblastoma cells and PBMC
458 was assessed using ELISA for secretion of IFN-γ (eBioscience) and granzyme B (R&D
459 Systems, DY2906 with DY008) and using electrochemiluminescence for TNF-α secretion
460 (MSD, K15067L-1). Standards and samples were measured in duplicate. All cytokine secretion
461 assays were performed according to the manufacturer's instructions.

462

463 *RNA isolation and quantitative real-time PCR*

464 Primary human glioblastoma cells were incubated with 10 μg/ml poly(I:C) for 24 hours. RNA
465 was isolated from the cell pellet using the miRNeasy Mini Kit (Qiagen, 217004) according to
466 the manufacturer's instructions, including a DNase treatment (Qiagen, 79254). A NanoDrop

467 1000 spectrophotometer (Thermo Fisher Scientific) was used to determine the RNA

468 concentration.

469 RNA levels were analyzed by qRT-PCR on a LightCycler 480 (Roche) using the Power SYBR

470 Green RNA-to-CT 1-Step Kit (Thermo Fisher Scientific, 4389986) according to the

471 manufacturer's instructions. The custom-made primers (Biolegio) are shown in Table 2. The

472 threshold for primer efficiency was set at [90-110%]. Determination of the number of

473 housekeeping genes and data analysis were performed using qbase+ software (Biogazelle). A

474 melt curve analysis was included in order to detect non-specific amplification.

475

476 *Immunohistochemical tumor phenotyping*

477 Primary human glioblastoma cells were incubated with 10 µg/ml poly(I:C) for 24 hours. Next,

478 the harvested cells were fixed for at least one hour in 4 % buffered formaldehyde (Chem-Lab

479 NV, CL02.0644.5000) and embedded in paraffin in order to acquire cell blocks, which were

480 cut into 5 µm-thick sections. The sections were baked in an oven for 0.5 to 1 hour at 60 °C in

481 order to facilitate attachment and to soften the paraffin. Staining was performed as previously

482 described with adaptations.[62] IHC for PD-L1 was performed on an Omnis instrument (Dako)

483 with EnVision FLEX TRS Low pH antigen retrieval buffer at 95 °C for 30 minutes (Dako

484 Omnis, GV80511-2). The IHC staining protocol from the manufacturer's datasheet of PD-L1

485 (clone 28-8, Abcam, ab205921) was slightly adapted (20-minute incubation with 1:50 diluted

486 antibody). The EnVision FLEX+ DAB detection kit (Dako Omnis, GV80011-2) was used

487 according to the manufacturer's instructions, with the addition of the EnVision FLEX+ Rabbit

488 LINKER (Dako Omnis, GV80911-2). Sections were counterstained with hematoxylin (Dako

489 Omnis, GC808) for 5 minutes as part of the automated staining protocol. IHC for PD-L2 was

490 performed on a Benchmark Ultra XT autostainer (Ventana Medical Systems Inc.) with CC1

491 antigen retrieval buffer at 97 °C for 52 minutes (Ventana, #950-124). The UltraView DAB
492 detection kit (Ventana, #760-051) was used according to the manufacturer's instructions. The
493 IHC staining protocol from the manufacturer's datasheet of PD-L2 (clone # 176611, R&D
494 Systems, MAB1224) was slightly adapted (12-minute incubation with 1:1500 diluted
495 antibody). Sections were counterstained with hematoxylin II (Ventana, #79-2208) as part of the
496 automated staining protocol. After the BenchMark staining, PD-L2 IHC slides were washed 1
497 minute in reagent buffer and 1 minute in distilled water. After the staining process with either
498 antibody, the sections were dehydrated in graded alcohol, cleared in xylene, mounted with
499 Quick-D Mounting Medium (Klinipath, 7280) and coverslipped. Images were taken using a
500 BX51 microscope (Olympus) equipped with an DP71 digital camera (Olympus). CellSens
501 Dimension Software (Olympus) was used for image acquisition and processing.

502

503 *Coculture experiments*

504 One day prior to coculturing, primary human glioblastoma cells were incubated with 10 µg/ml
505 poly(I:C), and frozen PBMC were thawed. After 24 hours, supernatant was collected and the
506 primary glioblastoma cells were harvested, resuspended in their supernatant and seeded at
507 $20x10^3$ cells per tube. Next, $800x10^3$ PBMC in DMEM were added to the respective tubes, and
508 the cocultures were incubated for 24 or 96 hours. After 24 hours, lymphocytes were stained for
509 immunophenotyping; after 24 and 96 hours, supernatant was collected for cytokine analysis of
510 granzyme B and IFN-γ, respectively.

511 Blocking experiments were performed similarly with one additional step. Prior to PBMC
512 addition, the primary glioblastoma cells were incubated for one hour with 3 µg blocking
513 antibody against PD-L1 (eBioscience, 16-5983), PD-L2 (eBioscience, 16-5888), or isotype

514 control (eBioscience, 16-4714). Following the addition of PBMC, the coculture contained 10
515 μg/ml blocking antibody and was maintained for 96 hours.

516

517 *Flow cytometric immunophenotyping*

518 Immunophenotyping of lymphocytes was performed on the cocultures in the immune
519 activation, migration and proliferation assays. For assessment of lymphocytic activation after
520 24 hours of coculture, the cells were stained for 15 minutes with an immune activation panel
521 consisting of activation marker CD69 (BD Biosciences, 557756), degranulation marker
522 CD107a (BD Biosciences, 555800), lineage markers CD3 (BD Biosciences, 560835), CD8
523 (Molecular Probes, MHCD0828) and NKp46 (BD Biosciences, 558051), and LIVE/DEAD
524 Fixable Aqua Dead Cell Stain (Molecular Probes, L34957). CD107a and the corresponding
525 isotype antibody were added to the cocultures from the beginning. For assessment lymphocyte
526 migration and the chemokine receptor profile, the panel consisted of chemokine receptors
527 CXCR3 (BD Biosciences, 550967) and CCR5 (BD Biosciences, 557752), lineage markers CD3
528 (BD Biosciences, 560176), CD8 (Molecular Probes) and CD56 (BD Biosciences, 345811), and
529 the LIVE/DEAD viability stain (Molecular Probes). In these experiments, an isotype panel
530 harvesting corresponding species- and isotype-matched antibodies at identical concentrations
531 was included to serve as controls (BD Biosciences, 555748, 555749 and 560167 for immune
532 activation, and 345818 and 557907 for chemokine receptors), along with the lineage markers
533 and the viability stain. For the proliferation assay, the panel consisted of lineage markers CD3
534 (BD Biosciences, 560176) and CD8 (BD Biosciences, 345774), and the LIVE/DEAD viability
535 stain (Moelcular Probes). Samples were analyzed on a BD FACSAria II flow cytometer (Becton
536 Dickinson). Data were acquired using FACSDiva software (BD Biosciences) and analyzed
537 using FlowJo v10 software. Representative flow cytometry profiles are shown in
538 Supplementary Figure S1.

539

540 *Chemotaxis assay*

541 Transwell chemotaxis assays were performed using 24-well transwells with a 5 µm pore size
542 polycarbonate membrane (Corning, 3421). After 24 hours, supernatant of poly(I:C)-treated and
543 naïve primary human glioblastoma cells was collected and transferred to the 24-well plate.
544 Next, the transwell was placed in the well and $1 x 10^6$ PBMC were added to the transwell insert.
545 Migrated cells were collected from the lower compartment after three hours of incubation at 37
546 °C with 5 % CO_2. Following staining, cell counting and phenotyping was performed on a BD
547 FACSAria II using a fixed volume and a continuous flow rate. Migration due to poly(I:C)
548 treatment of primary glioblastoma cells was calculated as: relative migration = (number of
549 migrated cells towards $supernatant_{poly(I:C)-treated}$ / number of migrated cells towards
550 $supernatant_{naïve}$).

551

552 *Proliferation assay*

553 The proliferation assay was set up similarly to the coculture experiment described earlier. Three
554 days prior to coculturing, PBMC were thawed, seeded at $1 x 10^6$ PBMC/ml and stimulated with
555 2 µg/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich, P8139) and 50 IU/ml IL-2
556 (Immunotools, 11340025). One day prior to coculturing, primary human glioblastoma cells
557 were incubated with 10 µg/ml poly(I:C). The next day, supernatant from the primary
558 glioblastoma cell cultures was collected and the tumor cells were harvested, resuspended in
559 their supernatant and seeded at $20 x 10^3$ cells per tube. One hour prior to coculturing, PD-L1 or
560 PD-L2 on the primary human glioblastoma cells was blocked as described earlier. On the same
561 day, the PBMC were labelled with 5 µM 5,6-carboxyfluorescein diacetate succinimidyl ester
562 (CFSE; Molecular Probes, C34554) according to the manufacturer's instructions. Next,

563 200x10^3 PBMC in DMEM were added to the respective tubes, which were then incubated at 37
564 °C with 5 % CO_2. After six days, $CD8^+$ T lymphocytic proliferation was asses by quantifying
565 the percentage of CFSE-diluted $CD8^+$ T lymphocytes on a BD FACSAria II. The proliferation
566 percentages were normalized to the isotype control, which was set at 0, and the effect of
567 blocking antibodies was subsequently evaluated relative to this value.

568

569 *Statistics*

570 Prism 7.03 software (GraphPad) was used for data comparison and artwork. Statistical analysis
571 was performed using SPSS Statistics 24 software (IBM). Paired-wise non-parametric tests were
572 performed to compare medians between two (Wilcoxon signed ranks test) or multiple groups
573 (Friedman test with Dunn-Bonferroni post-hoc testing). Correlations were investigated using
574 the Pearson's correlation coefficient. Median values are depicted. Differences were predefined
575 to be statistically significant when $p < 0.05$.

576

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578

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587

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789

790 **Figure Legends**

791

792 **Figure 1. Poly(I:C) stimulates PD-L1 and PD-L2 expression on primary human**
793 **glioblastoma cells.** (A and C) Poly(I:C) upregulates (A; ΔMFI) and induces (C; % positive
794 cells) expression of both PD-L1 and PD-L2 on primary glioblastoma cells; n = 8; Friedman test
795 with Bonferroni correction; concentrations are denoted in µg/ml poly(I:C) and U/ml IFN-γ. (B)
796 Plots showing correlations between PD-L1 and PD-L2 upregulation as well as between the
797 expression levels at basal or poly(I:C) treatment conditions for each protein. Treatment with 10
798 µg/ml poly(I:C) was selected for analysis; Pearson's correlation coefficient. Bar represents
799 median. Symbols depict primary glioblastoma cells derived from different glioblastoma
800 patients. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

801

802 **Figure 2. Poly(I:C) drives an immunostimulatory secretome of primary human**
803 **glioblastoma cells.** Poly(I:C) enhances the release of type I IFN, IL-15 and chemokines by
804 primary glioblastoma cells, while TGF-β secretion is inhibited. IFN-γ secretion is not induced;
805 n = 6-8; Friedman test with Bonferroni correction. 10 µg/ml poly(I:C) and 5000 IU/ml IFN-γ
806 were used. Bar represents median. Symbols depict primary glioblastoma cells derived from
807 different glioblastoma patients. Grey symbols represent data points above the detection limit;
808 these were capped at the detection limit for visualization and analysis purposes since differences
809 were apparent. **, $p < 0.01$; ***, $p < 0.001$.

810

811 **Figure 3. Poly(I:C) mediates its effect on PD-L1 and PD-L2 expression in primary human**
812 **glioblastoma cells primarily via the TLR3-TICAM1 pathway.** (A) Poly(I:C) upregulates
813 mRNA expression of TLR3 adaptor molecule TICAM1 but not MAVS, the adaptor molecule

814 for the cytosolic receptor pathway, in primary human glioblastoma cells. mRNA expression
815 following poly(I:C) treatment is shown relative to the basal, naïve condition (dashed line at 1);
816 n = 8; Wilcoxon Signed Ranks test. (B) Patient-specific presentation of the proportional PD-L1
817 and PD-L2 expression in the naïve setting (white) versus poly(I:C) treatment with (light grey)
818 and without (dark grey) the TLR3 inhibitor chloroquine. TLR3 inhibition clearly diminishes
819 the effect of poly(I:C) on both PD-1 ligands. (C) Relative upregulation (ΔMFI) of PD-L1 and
820 PD-L2 by poly(I:C) when TLR3 signaling is blocked with chloroquine. Data are normalized to
821 naïve (baseline) and poly(I:C) treatment (100%, dashed line) as to show the percentage of
822 upregulation by poly(I:C) not accounted for by TLR3; n = 5; Wilcoxon Signed Ranks test. Bar
823 represents median. Symbols depict primary glioblastoma cells derived from different
824 glioblastoma patients. CQ: chloroquine; *, $p < 0.05$; **, $p < 0.01$.

825

826 **Figure 4. Poly(I:C) stimulates PD-L1 and PD-L2 expression on primary human**
827 **glioblastoma cells via enhanced protein production.** (A) Poly(I:C) upregulates mRNA
828 transcription of PD-L1 and PD-L2 in primary human glioblastoma cells. mRNA expression
829 following poly(I:C) treatment is shown relative to the basal, naïve condition (dashed line at 1);
830 n = 8; Wilcoxon Signed Ranks test. (B) Plot showing the correlation between PD-L1 and PD-
831 L2 mRNA expression following poly(I:C) treatment relative to naïve cells; Pearson's
832 correlation coefficient. (C-D) Poly(I:C) increases both membrane and intracellular expression
833 of PD-L1 (C) and PD-L2 (D) on primary human glioblastoma cells; n = 6; representative areas
834 per specimen are shown. Bar represents median. Symbols depict primary glioblastoma cells
835 derived from different glioblastoma patients. -, no poly(I:C); +, 10 µg/ml poly(I:C); *, $p < 0.05$;
836 **, $p < 0.01$.

837

838 **Figure 5. The poly(I:C)-mediated effect on PD-L1 and PD-L2 expression on primary**
839 **human glioblastoma cells is in part via autocrine or paracrine signaling of downstream-**
840 **secreted IFN-β.** (A-B) Relative protein upregulation (A; ΔMFI) and induction (B; % positive
841 cells) of blocking IFN-α or IFN-β additionally to poly(I:C) treatment, as determined by flow
842 cytometry. Data are normalized to naïve (baseline) and poly(I:C) treatment (100%, dashed line)
843 as to show the portion of upregulation or induction by poly(I:C) not accounted for by IFN-α or
844 IFN-β. IFN-β, but not IFN-α, is significantly involved in the upregulation and induction of PD-
845 L1 and PD-L2 on primary glioblastoma cells; n = 5; Wilcoxon Signed Ranks test. Bar represents
846 median. Symbols depict primary glioblastoma cells derived from different glioblastoma
847 patients. *, $p < 0.05$.

848

849 **Figure 6. Poly(I:C)-treated primary human glioblastoma cells invigorate immune**
850 **activation.** (A) Poly(I:C)-treated primary human glioblastoma cells increase the number of
851 activated ($CD69^{+}$) lymphocytes and stimulate degranulation ($CD107a^{+}$ lymphocytes); n = 6,
852 Wilcoxon-Signed Ranks test. (B) Poly(I:C)-treated primary human glioblastoma cells increase
853 immune activation and cytotoxic potential as shown by elevated release of IFN-γ and granzyme
854 B, respectively. TNF-α secretion was not altered; n = 5-6, Wilcoxon-Signed Ranks test. Bar
855 represents median. Symbols depict different PBMC donors each cocultured with primary
856 glioblastoma cells derived from different glioblastoma patients. -, no poly(I:C); +, 10 µg/ml
857 poly(I:C); *, $p < 0.05$.

858

859 **Figure 7. Poly(I:C)-treated primary human glioblastoma cells demonstrate elevated**
860 **attraction of lymphocytes.** (A) The migration of T cells towards primary human glioblastoma
861 cells is enhanced by tumor priming with 10 µg/ml poly(I:C). Data is normalized to and shown

862 relatively to migration to naïve primary human glioblastoma cells (dashed line at 1); n = 6;
863 Wilcoxon Signed Ranks test. (B) Preferential attraction of $CD8^+$ T cells leads to increased
864 CD8:CD4 T cell ratios following poly(I:C) treatment of primary glioblastoma cells; n = 6;
865 Wilcoxon Signed Ranks test. (C) Expression of chemokine receptors CXCR3 and CCR5 is
866 downregulated on T cells that migrated towards supernatant derived from poly(I:C)-treated
867 primary human glioblastoma cells; n = 6; Wilcoxon Signed Ranks test. Bar represents median.
868 Symbols depict different PBMC donors each cocultured with primary glioblastoma cells
869 derived from different glioblastoma patients. -, no poly(I:C); +, 10 µg/ml poly(I:C); *, $p < 0.05$.

870

871 **Figure 8. PD-L1 blocking invigorates the immunostimulatory effect of priming primary**
872 **human glioblastoma cells with poly(I:C).** (A and C) Additional blocking of PD-L1, but not
873 PD-L2, further invigorates the immune response, as measured by IFN-γ secretion (A) and $CD8^+$
874 T cell proliferation (C); n = 5-6; Wilcoxon Signed Ranks test. (B) Additional blockade of either
875 PD-L1 or PD-L2 results in a modest but significant increase in granzyme B release; n = 6;
876 Wilcoxon Signed Ranks test. Bar represents median. Symbols depict different PBMC donors
877 each cocultured with primary glioblastoma cells derived from different glioblastoma patients. -
878 , no compound; +, 10 µg/ml blocking antibody, isotype antibody or poly(I:C); *, $p < 0.05$; **,
879 $p < 0.01$.

880

881 **Tables**

882

883 **Table 1. Characteristics of primary human glioblastoma tumor tissue specimens.**

Sex	Age	Diagnosis	Location tumor	MGMT[†]
♂	64	Recurrent[‡]	Occipital (R)	-
♀	74	Newly diagnosed	Frontal (R)	+
♀	85	Newly diagnosed	Temporofrontal (R)	-
♀	51	Newly diagnosed	Frontal (R)	Unknown
♀	62	Newly diagnosed	Temporal (R)	-
♀	59	Newly diagnosed	Frontal (R)	-
♂	50	Newly diagnosed	Temporal (L)	-
♂	62	Newly diagnosed	Temporal (R)	+

884 All patients were diagnosed with glioblastoma multiforme (World Health Organization grade
885 IV astrocytoma). [†], methylguanine methyltransferase (MGMT) promotor hypermethylation; [‡],
886 patient received treatment prior to resection of the recurrent tumor. L, left; R, right.

887

888 **Table 2. Primers for quantitative real-time PCR.**

Gene	Forward primer	Reverse primer
GAPDH	5’-TGCACCACCAACTGCTTAGC-3’	5’-GGCATGGACTGTGGTCATGAG-3’
MAVS	5'-CACAGCAAGAGACCAGGATCG-3'	5'-GCCGCTGAAGGGTATTGAAG-3'
PD-L1	5'-CTGTCACGGTTCCCAAGGAC-3'	5'-GGTCTTCCTCTCCATGCACAA-3'
PD-L2	5'-GTACATAATAGAGCATGGCAGCA-3'	5'-CCACCTTTTGCAAACTGGCTGT-3'
RPL13A	5’-CCTGGAGGAGAAGAGGAAAGAGA-3’	5’-TTGAGGACCTCTGTGTATTTGTCAA-3’
SDHA	5’-ACTCAGCATGCAGAAGTCAATGC-3’	5’-ACCTTCTTGCAACACGCTTCCC-3’
TICAM1	5'-GGCTTTCAGGGTGGTCAGAA-3'	5'-GACCGTAGCAATACCCCCAC-3'

889 For the qRT-PCR reaction, 200 mM of each primer and 20 ng RNA were added to the reaction
890 mix. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L13a
891 (RPL13A) and succinate dehydrogenase complex flavoprotein subunit A (SDHA) served as
892 housekeeping genes as determined using qbase+ software.