



**Bacterial sepsis increases survival in metastatic melanoma:
Chlamydomphila pneumoniae induces macrophage polarization
and tumor regression.**

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For Review Only

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5 **Bacterial sepsis increases survival in metastatic melanoma:**

6 ***Chlamydomonas pneumoniae* induces macrophage polarization and tumor regression**

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30 Introduction

31 The initiative of the current study was the unexpected complete tumor regression of a
32 patient with stage IV cutaneous metastatic melanoma, who suffered multifactorial sepsis
33 syndrome during BOLD chemotherapy (**Suppl. Fig. 1.**). After targeted antibiotic treatment
34 and combined complication-free chemotherapy, the patient's physical condition improved
35 and-unexpectedly- the metastases disappeared. The patient has been asymptomatic and
36 metastasis- free ever since the end of BOLD therapy. A significant decrease in the volume of
37 the previously palpable axillary and abdominal metastases was observed already when BOLD
38 was interrupted due to sepsis. For a timeline of events, see **Table 1.**

39 Molecular genetics research in the last decade helped the development of BRAF
40 inhibitors and immunooncological agents, which brought about a significant improvement of
41 the life expectancy of melanoma patients. Once the gold standard (Avril *et al.*, 2004),
42 Dacarbazine-based chemotherapies are still approved and widely applied in melanoma therapy,
43 but their efficacy is known to be relatively low (Garbe *et al.*, 2011). In the light of this, the
44 fact that clinical improvement was observed quite early during the chemotherapy suggested
45 other factors behind the outcome, and the concurrent sepsis seemed to offer a potential
46 explanation.

47 It has long been recognized that cancer patients might recover following bacterial
48 infections (Wiemann and Starnes, 1994; Hobohm, 2001). The hypothesis was that fever and
49 TNF- α induced by the infectious agents caused the tumor regression, but this could not be
50 reproduced by TNF- α administration or hyperthermia (Nauts *et al.*, 1946; Tsung and Norton,
51 2006). It has been observed that an attenuated form of *Listeria monocytogenes* can infect
52 cancer cells, but not normal cells, and this phenomenon resulted in a potentially effective
53 experimental cancer therapy. (Quispe-Tintaya W *et al.* 2013)

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3 54 Vaccination with intracellular pathogens like Bacillus Calmette-Guerin or Vaccinia
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5 55 virus significantly decreased the incidence of melanoma (Krone *et al.*, 2005). However, a
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7 56 convincing explanation is still missing.

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10 57 While it is generally accepted that anti-tumor immune mechanisms overlap with anti-
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12 58 bacterial immune responses (Chen *et al.*, 2007; Adams, 2009;), the exact mechanism induced
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14 59 by microbes is not understood.

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16 60 As immune responses appear to be decisive factors also in the outcome of melanoma
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18 61 (Ridnour *et al.*, 2013; Shimanovsky *et al.*, 2013), we hypothesized that sepsis, by triggering
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20 62 polarized, “joint” anti-bacterial and anti-tumor immune responses, could induce tumor
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22 63 regression. This hypothesis was tested in our experimental model.

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25 64 To clarify the role of the adaptive immune system in the anti-tumor immune
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27 65 mechanisms induced by *C. pneumoniae* (CP, successfully identified in the primary melanoma
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29 66 after our patient recovered from sepsis-**Suppl. Fig. 1f**), lung metastases (LM) were induced in
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31 67 immunocompetent C57BL/6 mice or immunodeficient NSG mice. Animals were then CP- or
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33 68 mock-treated (Suppl. Materials and methods). To assess the effects of treatment, histological,
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35 69 immunological and molecular analyses were done.

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41 **Results**

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43 72 In immunocompetent, CP- treated animals, the number of LMs significantly decreased
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45 73 (P=0.003) (**Fig. 1a**), while the survival (**Fig. 1t**) significantly increased (P=0.04) compared to
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47 74 mock treatment. This was not observed in immunodeficient mice, and the treated animals did
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49 75 not develop fever (33.2 °C±1.0 mock vs. 34.8 °C±0.5 CP) or high plasma levels of TNF-α
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51 76 either, which is against the “fever hypothesis” (Wiemann and Starnes, 1994; Hobohm, 2001).

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54 77 Histological analysis of slices from the lungs of mock-treated melanoma-bearing
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56 78 immunocompetent mice showed a high number of LMs, with frequent intra-tumor necrosis

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3 79 (**Fig. 1e**). In contrast, fewer and smaller foci of regressive LMs were observed in the CP-
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5 80 treated immunocompetent animals (**Fig. 1f**). Moreover, in this group, a high number of tumor-
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7 81 infiltrating mononuclear histiocytes and lymphoid cells were identified in the LMs. The LMs
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9 82 did not exhibit significant intratumor immune reactions in the immunodeficient mice,
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11 83 regardless of treatment type (**Fig. 1g, h**). Markedly increased immune reaction in the lungs of
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13 84 the CP-treated mice was also verified by immunolabeling of the cell surface activation
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15 85 markers CD11b and CD80 (**Fig. 1i-l**). Immune cell invasion was not detected after mock
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17 86 treatment - the immune cells were concentrated in the marginal zones of the tumors (**Fig. 1i,**
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19 87 **k**). In contrast, after CP treatment, marked infiltration by activated lymphocytes was seen in
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21 88 the internal tumor stroma (**Fig. 1j, l**); differences were significant (**Fig. 1m, n**) ($P=0.0001$).
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25 89 To assess macrophage polarization, M1 (anti-tumor) or M2 (pro-tumor) macrophage -
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27 90 specific cytokine and chemokine transcriptome profiling was done (Mantovani *et al.*, 2004).
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29 91 Macrophage markers were detected with Q-PCR from pooled lung samples 2, 4 and 12 hours
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31 92 after mock or CP treatment. Four hours after CP application, markedly increased levels of M1
32
33 93 - specific mRNA transcripts for CCL2, CCL3, IL6, CXCL10, CCL7, CD80, CXCL11,
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35 94 CXCL9, IL23, and TNF α were detected. In line with this, the mRNA expression of most M2-
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37 95 specific markers decreased. Interestingly, the levels of some important M2 markers
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39 96 (CXCL13, IL1Ra) were actually increased (**Suppl. Fig. 2a, b**). Upon CP administration, the
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41 97 quantity of M1-specific cytokine and chemokine mRNA was significantly increased
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43 98 ($P=0.014$) after 4 hours, in comparison to M2- markers.
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47 99 Alteration in the expression pattern of COX-1 and COX-2 is one of the key markers of
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49 100 macrophage polarization (Martinez *et al.*, 2006; Mantovani *et al.*, 2013). Western blot
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51 101 analysis revealed that 12 hours after CP treatment, protein expression of the M2-specific
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53 102 COX-1 decreased by half, whereas the protein expression of the M1-specific COX-2
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55 103 increased more than two fold (**Suppl. Fig. 2c, d**).
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3 104 Two hours after CP treatment -but not after mock treatment- CXCL1 melanoma
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5 105 growth factor immunoreactivity become undetectable (**Suppl. Fig. 3a, b**). To assess whether
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7 106 this *in vivo* phenomenon was due to a direct CP: CXCL1 interaction, equal amounts of
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9 107 recombinant CXCL1 were incubated *in vitro* (in the presence of protease inhibitors) with
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11 108 increasing quantities of CP. CXCL1 levels were determined by Western blotting. CP depleted
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13 109 CXCL1 in a dose-dependent manner, suggesting a strong and direct binding by CP (**Suppl.**
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15 110 **Fig. 3c, d**).

111 Discussion

112 Our results seem to indicate that CP treatment does indeed induce a complex anti-tumor
113 response. We showed that CP treatment can suppress LM formation in immunocompetent
114 (but not in immunodeficient) mice. M1-type macrophage polarization was demonstrated,
115 which is associated with anti-tumor effects (Sica *et al.*, 2008). The anti-tumor immune
116 polarization/activation was further supported by the profound enrichment of CD80 and
117 CD11b expressing immune cells in the lungs CP- treated animals (Prebeck *et al.*, 2001). Of
118 special importance, the melanoma growth factor CXCL1 was completely depleted by CP,
119 both *in vivo* and *in vitro*.

120 As (i) CXCL1-induced NF- κ B activity was shown to facilitate melanoma transformation by
121 allowing melanocytes to escape apoptosis; and (ii) κ B- α Δ N (super-repressor of NF- κ B)
122 reduced tumor growth and metastatic potential of melanoma cells (Dhawan *et al.*, 2002), we
123 consider it a possible scenario that not only the CP induced M1 type macrophage polarization
124 but the induced CXCL1 depletion could significantly contribute to the tumor regression.
125 Evidently, results of the animal study strongly support our assumption about the role of sepsis
126 in the observed outcome; however, these data cannot exclude the role of the BOLD therapy.
127 The conclusion one can safely draw at this point is that sepsis, in the context of BOLD,

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3 128 resulted in a dramatic improvement, otherwise not seen in uncomplicated therapy, which
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5 129 suggests that the occurrence of sepsis was an event of key importance.
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13 132 **Competing financial interests**

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16 133 The authors declare no competing financial interests.
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195 **Table 1: Timeline of Clinical Case Report**

day	
-360	The patient herself detected a bleeding nevus-like lesion on the back and an enlarged axillary lymph node; no steps were taken.
120	Hospital visit. X-ray, mammography and abdominal doppler seems to be negative, axillary lymph node biopsy was proposed. The patient was temporarily lost from follow up.
0	Hospital visit for abdominal pain, gastritis was diagnosed and a gastric polyp was removed. Tumor masses were discovered in the retroperitoneal lymph nodes (15-20 mm), spleen (67 mm) and bladder (40x68 mm). Another tumor was detected in the brain by CT (40 mm).
4	The intracranial tumor mass was removed surgically and diagnosed as amelanotic melanoma metastasis.
24	Cranial radiotherapy was initiated.
30	Leukocytosis, fever. Amoxicillin+clavulanic acid treatment.
32	Radiotherapy completed.
35	BOLD (bleomycin, oncovine, lomustine and dacarbazine) chemotherapy initiated.
37	On the 3rd day of chemotherapy, it was suspended because of vomiting and fever. The gastric fluid contained <i>Escherichia coli</i> and <i>Candida albicans</i> . <i>Clostridium difficile</i> toxin was also detected. Fluconazole and ceftriaxone (later metronidazol) treatment was initiated.
52	CVC was removed because of putative <i>Pseudomonas aeruginosa</i> infection. This was later confirmed by blood test.
59	The primary tumor was excised and analyzed (Melanoma malignum, Br 1.52 mm, C1. III., pT2b).
77	BOLD, 2nd treatment cycle. Decrease of axillary and abdominal metastases was detected.
120	BOLD, 3rd cycle. Further improvement of the axillary and intra-abdominal metastases was recorded. No intra-abdominal lymphadenomegalia, a single liver metastasis and shrinking splenic metastasis was detected.
162	BOLD, 4th cycle. Complete remission of the axillary and abdominal metastases was observed.
210	BOLD, 5th cycle. Complete remission of the axillary and abdominal metastases was observed.
255	BOLD, 6th cycle. The patient is asymptomatic and PET/CT-verified metastases free.
>1500	The patient is asymptomatic and PET/CT-verified metastases free.

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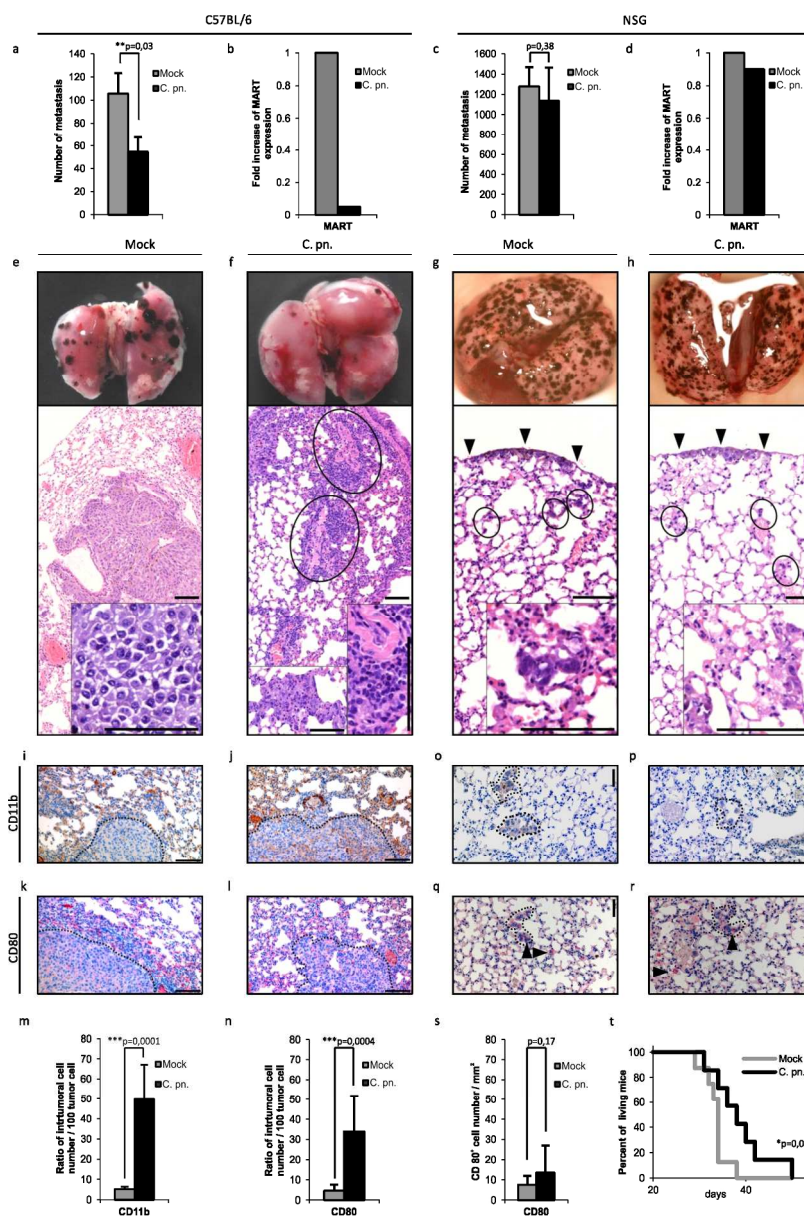
197

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3 198 **Figure 1:**

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6 199 *C. pneumoniae* treatment results in melanoma metastasis regression and increases
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8 200 survival of animals as well as of CD11b⁺ and CD80⁺ immune cell infiltration of tumor
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10 201 tissues

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13 202 Number of lung metastases (a, c), melanoma antigen-MART (b, d) and survival rate (t) of
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15 203 mock or *C. pneumoniae* (*C. pn.*) treated immunocompetent C57BL/6 and immunodeficient
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17 204 NSG mice. Representative images and HE-stained histological sections of dissected lungs of
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19 205 mock (e) and *C. pneumoniae* (f) treated immunocompetent mice, as well as of mock (g) and
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21 206 *C. pneumoniae* (h) treated immunodeficient (NSG) animals. Note that the subpleural
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23 207 metastasis formation is extensive in diameter but not in thickness in NSG mice (arrowheads).
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25 208 Scale bars, 100 μ m. (e) Trophical necroses indicating high tumor burden. Insert: atypical
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27 209 tumor cells and regions of necrosis. (f) Circles and right insert, foci of regressive metastases,
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29 210 left insert: areas of residual pneumonitis after *C. pneumoniae* treatment. (g, h) In both mock
30
31 211 and *C. pneumoniae* treated NSG mice, miliary metastases were developed subpleurally
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33 212 (arrowheads) and intraparenchymally (circles) without significant inflammatory reactions
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35 213 (inserts: higher magnification of intraparenchymal metastases). Immunohistochemistry of
36
37 214 CD11b (i, j, DAB, brown) and CD80 (k, l, Fast red, red) on lungs of mock (i, k) or *C.*
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39 215 *pneumoniae* (j, l) treated C57BL/6 mice. Dashed lines indicate tumor border. (i-r) Arrows
40
41 216 indicate infiltrating immune cells. Intratumoral number of CD11b⁺ (m) and CD80⁺ (n) cells
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43 217 determined as a ratio of 100 tumor cells in C57BL/6 mice. CD80⁺ cells in NSG lungs counted
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45 218 by square millimeter (s). Data are given as mean \pm SD.
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Number of lung metastases (a, c), melanoma antigen-MART (b, d) and survival rate (t) of mock or C. pneumoniae (C. pn.) treated immunocompetent C57BL/6 and immunodeficient NSG mice. Representative images and HE-stained histological sections of dissected lungs of mock (e) and C. pneumoniae (f) treated immunocompetent mice, as well as of mock (g) and C. pneumoniae (h) treated immunodeficient (NSG) animals. Note that the subpleural metastasis formation is extensive in diameter but not in thickness in NSG mice (arrowheads). Scale bars, 100 μ m. (e) Tropical necroses indicating high tumor burden. Insert: atypical tumor cells and regions of necrosis. (f) Circles and right insert, foci of regressive metastases, left insert: areas of residual pneumonitis after C. pneumoniae treatment. (g, h) In both mock and C. pneumoniae treated NSG mice, miliary metastases were developed subpleurally (arrowheads) and intraparenchymally (circles) without significant inflammatory reactions (inserts: higher magnification of intraparenchymal metastases). Immunohistochemistry of CD11b (i, j, DAB, brown) and CD80 (k, l, Fast red, red) on lungs of mock (i, k) or C. pneumoniae (j, l) treated C57BL/6 mice. Dashed lines indicate tumor border. (i-r) Arrows indicate infiltrating immune cells. Intratumoral number of CD11b+ (m) and CD80+ (n)

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cells determined as a ratio of 100 tumor cells in C57BL/6 mice. CD80+ cells in NSG lungs counted by square millimeter (s). Data are given as mean \pm SD.
173x257mm (300 x 300 DPI)

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Supplementary Discussion:

The blots of the Proteome Profiler (Suppl. Fig. 3a, b) in the upper left lower left and lower right positions are the so-called assay controls, they merely indicate that the test was technically successful. The rest of the proteins –which could be relevant in the anti-tumoral immune response-are as follows (from left to right and from top to bottom): C5a, CD54, CXCL1, MCP-1, IL-16, IL-1Ra, CCL5.

- **C5a** is a complement protein that has been implicated in tumorigenesis. C5a accelerates tumor progression, can directly activate myeloid-derived suppressor cells, stimulate angiogenesis and cell migration. C5a increases VEGF level, prevents the activation of apoptotic caspase 3 and DNA fragmentation, and may function as an anti-apoptotic molecule (Kim *et al.*, 2005; Gunn *et al.*, 2012). We found decreased levels of this protein after treatment.

- **CD54** (ICAM-1) decreased in our in vivo model and expresses with a dose- and time-dependent increase in human malignant melanoma cells on stimulation of TNF-alpha. Inhibition of ICAM-1 expression on melanoma cells reduces the metastatic ability of the melanoma cells, indicating an important role of ICAM-1 in metastasis (Miele *et al.*, 1994). B. Cava *et al.* described that metastasis reduction of B16 cells is correlated to the reduction of plasma gelatinolytic activity and to the decrease of cells expressing CD44, CD54, and integrin- β_3 adhesion molecules.

- **CXCL1** is a melanoma growth factor and known as M2 marker. Our results suggest that the depletion of CXCL1 could play a role in the reduction of metastasis formation.

- **MCP-1** (decreased in our model) is produced by a variety of tumors, including B16F1 and plays an important role in tumor progression, especially in angiogenesis (Kim *et al.*, 2005; Koga *et al.*, 2008). Tumor cell-activated macrophages release TNF α , which facilitates the

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3 MCP-1 production of tumor cells. Thus, disruption of tumor-stromal cell interaction may
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5 inhibit tumor progression by reducing the production of tumor-promoting proinflammatory
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7 mediators, such as MCP-1 (Yoshimura *et al.*, 2015).
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10 - **IL-16**: it is a pleiotropic cytokine that functions as a chemoattractant, hence a modulator of
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12 T cell activation. The cytokine function is exclusively attributed to the secreted C-terminal
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14 peptide, while the N-terminal product may play a role in cell cycle control. Caspase 3 is
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16 reported to be involved in the proteolytic processing of this protein
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18 (<http://www.cancerindex.org/geneweb/IL16.htm>). IL-16 appears in the literature remarkably
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20 scarcely in connection with cancer, and we could not detect alteration in its level either;
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22 therefore, we do not know the relevance of this protein to the discussed observation.
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26 - **IL-1Ra** (moderately decreased or unchanged in our model) is the receptor antagonist of IL-1
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28 and it has been described as pleiotropic (Aubie *et al.*, 2015; Di Mitri *et al.*, 2014). Although
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30 IL-1Ra has been described to inhibit subcutaneous B16 melanoma growth *in vivo* (McKenzie *et*
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32 *al.*, 1996) we did not observe significant changes in its level upon treatment; therefore, similar
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34 to IL-16, the relevance of this finding is still unknown.
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38 - **CCL5** (decreased in our model) is a chemokine with tumor supportive properties (Adler *et*
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40 *al.*, 2003; Sugawara *et al.*, 2008). In rectal cancer, significant decrease of CCL5 was
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42 associated with a favorable response to chemoradiation therapy (Tada *et al.*, 2014). Moreover,
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44 Mdr2 and CCR5 (CCL5 receptor) double knock-out mice exhibited significant decrease in
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46 tumor incidence and size of hepatocellular carcinoma (Barashi *et al.*, 2013). Finally, CCL5
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48 was found to enhance cytotoxicity of regulatory T cells against CD8⁺ cells (Chang *et al.*,
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50 2012).
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Supplementary Materials and methods

The mouse model

B16F1 melanoma cells (ECACC, Salisbury, UK) were administered intravenously (1×10^5 cell/100 μ l) to 6-8 week old female immunocompetent C57BL/6 or immuno-deficient NOD/Scid IL2rg null (NSG) mice (Charles River Laboratories, Budapest, Hungary). One week after the tumor cell administration, mice were treated with *C. pneumoniae* strain CWL-029 (VR-029, ATCC, Wesel, Germany) propagated in Hep2 cells (CCL-23, ATCC, Wesel, Germany) (Mantovani *et al.*, 2004). *C. pneumoniae* and the mock control (processed Hep2 cells) were heat-inactivated at 90°C for 30 minutes. Mice were mildly sedated with sodium pentobarbital (7.5 mg/ml) and treated intranasally with 1×10^6 IFU *C. pneumoniae* 7, 9, 11, 14, and 16 days after tumor implantation. In the case of immune-deficient mice, since physical conditions of NSG mice deteriorated extremely rapidly, animals were euthanized at day 14 after the third *C. pneumoniae* treatment. The special advantages of this model are: (i) with intravenous injection of melanoma cells, visible lung tumor metastases develop within 7 days after injection without significant spreading into other organs; and (ii) *C. pneumoniae* is a lung-specific intracellular pathogen with a significant invasion rate even to the lung metastases. Two hours after the 1st inhalation (day 7), 4 hours after 2nd, 12 hours after 3rd and 24 hours after 5th inhalation, 3 animals/group were anaesthetized and their lungs were harvested for protein, mRNA and histological analysis. The remaining mice received the 4th (day 14) and the 5th (day 16) treatments and were followed for survival. At the end-point, the animals were euthanized, their lungs were removed and 3 independent persons counted the number of surface metastases in a blind fashion.

All animal experiments were performed in accordance with national (1998. XXVIII; 40/2013) and European (2010/63/EU) animal ethics guidelines. The experimental protocols were approved by the Animal Experimentation and Ethics Committee of the Biological

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3 Research Centre of the Hungarian Academy of Sciences and the Hungarian National Animal
4 Experimentation and Ethics Board (clearance number: XVI./03521/2011.). Informed consent
5 was obtained from all subjects.
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9 10 11 *Survival*

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13 For the survival experiments, groups of mice (n=15) were treated as described 5 times after
14 melanoma implantation. Kaplan-Meier survival curves were analyzed by a log-rank statistical
15 test and $p \leq 0.05$ was regarded as statistically significant. The body temperatures of 3
16 animals/group were measured using an AMA Digital AD 15 TH thermometer 2 hours after
17 the 1st treatment and 4 hours after the 2nd inhalation (day 7 and 9). All animal experiments
18 were authorized by the institutional and national animal welfare committees.
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29 *Cytokine and Chemokine Expression Analysis by Quantitative Real-time PCR*

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31 Total RNA was purified using a NucleoSpin RNA II RNA isolation kit (Macherey-Nagel,
32 Düren, Germany); first-strand cDNA was synthesized and Q-PCR reactions were performed
33 of M1 type (CCL2, CCL3, CD86, IL12, IL6, IL10, CXCL16, CCL7, CD80, CXCL11,
34 CXCL9, IL23, TNF α) and M2 type (CD163, CXCL13, TGF β , IL1Ra, CD23, CCL1, CCL22,
35 IL4, CCL17, CCL24, CD150, IL10, CXCL1) markers on pooled samples (n=3) on a
36 RotorGene 3000 instrument (Corbett Research) with gene-specific primers and SYBR Green
37 protocol to monitor gene expression. Each individual Ct value was normalized to the average
38 Ct values of four internal control genes (Δ Ct values). The final relative gene expression ratios
39 (fold change) were calculated as comparisons of Δ Ct values ($\Delta\Delta$ Ct values). Non-template
40 control sample was used for each PCR run to check the primer-dimer formation. Primer
41 sequences are available upon request.
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3 *C. pneumoniae* detection from the primary tumor of patient by PCR

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5 DNA was extracted from the formalin fixed paraffin-embedded (FFPE) samples using
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7 Nucleospin® FFPE DNA kit (Macherey-Nagel, Düren, Germany) according to the
8
9 manufacturer's instruction. For experiments involving a human subject were performed in
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11 accordance with National and Regional Ethics Committee VI-R-039/01840-2/2012;
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13 25363/2012/EKU; 448/PI/2012. The experimental protocols were approved by the National
14
15 and Regional Ethics Committee (clearance number: MCC-INTER-001.)
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21 *Histology, immunohistochemistry*

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23 Lung specimens were fixed in 4% buffered formaldehyde; then routine HE histology as well
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25 as standardized immunohistochemistry tissue microarray were performed using anti-CD11b
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27 (clone M1/70; R&D Systems, Minneapolis, MN) and CD80 (B7-1; R&D Systems,
28
29 Minneapolis, MN) antibodies.
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34 *Cytokine and chemokine detection by proteome profiling*

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36 Expression levels of different cytokines in pooled lung specimens were determined using
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38 Mouse Cytokine Array Panel A (R&D Systems, Minneapolis, MN), according to the
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40 manufacturer's instructions.
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45 *Western blot analysis of COX-1 and COX-2*

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47 To determine COX-1/COX-2 balance, Western blot analysis was performed using the lung
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49 lysates. Samples of total proteins were resolved on NuPAGE 4-12% Bis-Tris Gel, and then
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51 transferred to a nitrocellulose membrane. The membrane was incubated with anti-COX-1
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53 (1:250, R&D, Minneapolis, MN) mouse monoclonal antibody and anti-COX-2 (1:200, R&D,
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55 Minneapolis, MN) goat polyclonal antibody. After overnight incubation, the membranes were
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3 washed with 1 x TRIS Buffer supplemented with 0,05% Tween20 (Sigma, St. Louis, MO)
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5 and incubated for one hour with peroxidase conjugated anti-mouse (1:1000, R&D,
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7 Minneapolis, MN) and anti-goat IgG (1:10000, Sigma, St. Louis, MO) and developed using
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9 Odyssey Fc chemiluminescence detection system (LiCor Bioscience, Lincoln, NE).
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12 13 14 *Western blot analysis of CXCL1*

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16 Recombinant mouse CXCL1 protein (0.5 µg, R&D Systems, Minneapolis, MN) was mixed
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18 and incubated (37°C, 30 min) with different 10-fold (10-10000) dilutions of *C. pneumoniae*
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20 solutions (3.6 µg-0.00036 µg). CXCL1 protein amounts were then detected by Western blot
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22 analysis using an anti-CXCL1 antibody (1:1000, R&D Systems, Minneapolis, MN).
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25 26 27 *Statistical analysis*

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29 Kaplan-Meier survival curves were analyzed by a log-rank statistical test and $p \leq 0.05$ was
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31 regarded as statistically significant. Analyses of other data were performed using two-tailed
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33 Student's *t* test.
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Supplementary Figure 1:**Data obtained with the patient: Complete melanoma metastasis regression verified by PET-CT and retrospective PCR analysis-based detection of *C. pneumoniae***

(a, b) Ultrasonography; high tumor burden in the abdominal cavity. (c, d) CT and MRI scans; preoperational brain metastasis in the temporooccipital lobe and postoperational tumor-free brain status, respectively. (e) PET-CT scans; complete tumor regression in the body shortly after the septic event and BOLD treatment. (f) Retrospective detection of *C. pneumoniae* (*C. pn.*)-specific genes by RT-PCR. 16S rRNA: a housekeeping gene of *C. pn.* GroEL: Heat shock protein 60 of *C. pn.*, a group I chaperonin expressed on the surface of elementary bodies. MOMP: Major Outer Membrane Protein gene of *C. pn.* A-D: FFPE samples from different sections of primary melanoma; -C: PCR negative control (uninfected Hep2 cells); +C: PCR positive control (Hep2 cells infected by *C. pn.* strain TW183).

Supplementary Figure 2:***C. pneumoniae* treatment induces M1 type macrophage polarization**

(a) Relative alterations in the levels of individual M1 type and M2 type cytokine/chemokine specific mRNA transcripts in lung samples of *C. pneumoniae* (*C. pn.*) vs. mock-treated tumor-bearing C57BL/6 mice, as determined by real-time PCR. (b) Mean values of relative M1 and M2 cytokine mRNA expressions; at 4 hours after treatment, M1 and M2 levels are significantly different (two-tailed t-test). (c) Representative Western blot. Expressions of COX-1 and COX-2 were determined in lung samples 12 hours after *C. pneumoniae* or mock treatment of melanoma-bearing C57BL/6 mice (d) Densitometry analysis. Intensity of immunoreactive bands of COX-1 and COX-2 were determined and then normalized to that of

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3 vinculin. Data are presented as fold increase compared to values of the mock-treated group
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5 (regarded as 1). A minimum of three experiments yielded similar results.
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8 **Supplementary Figure 3:**

9 10 **CXCL1 is depleted by *C. pneumoniae* both *in vivo* and *in vitro***

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14 Assessment of cytokine/chemokine protein levels (Proteome profiler) in lungs of melanoma-
15 bearing C57BL/6 mice 2 hours after mock (a) or *C. pneumoniae* (b) treatment. Squares
16 indicate CXCL1 which disappeared 2 hours after *C. pneumoniae* treatment (c) Representative
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18 Western blot. Recombinant mouse CXCL1 protein (0.5 µg) was *in vitro* incubated with 500
19 IUFU/µl (1x) *C. pneumoniae*, (*C. pn.*) or its 10x, 100x, 1,000x, and 10,000x dilutions and
20 then Western blotting was performed. (d) Densitometry analysis of immunoreactive bands
21 shown in panel c. Values of the control (ctrl, vehicle treated) group were regarded as 1. Two
22 experiments yielded similar results.
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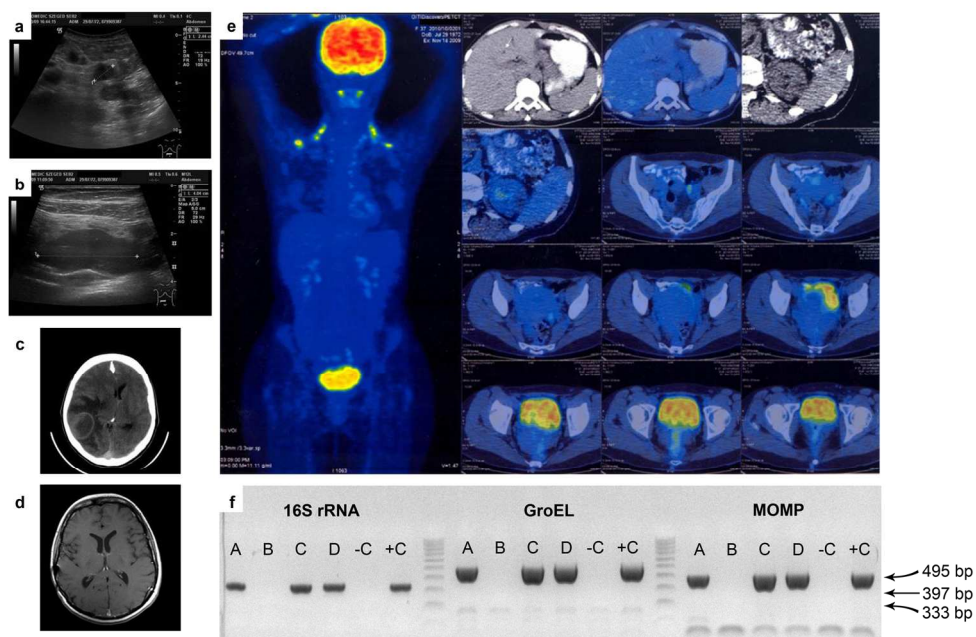
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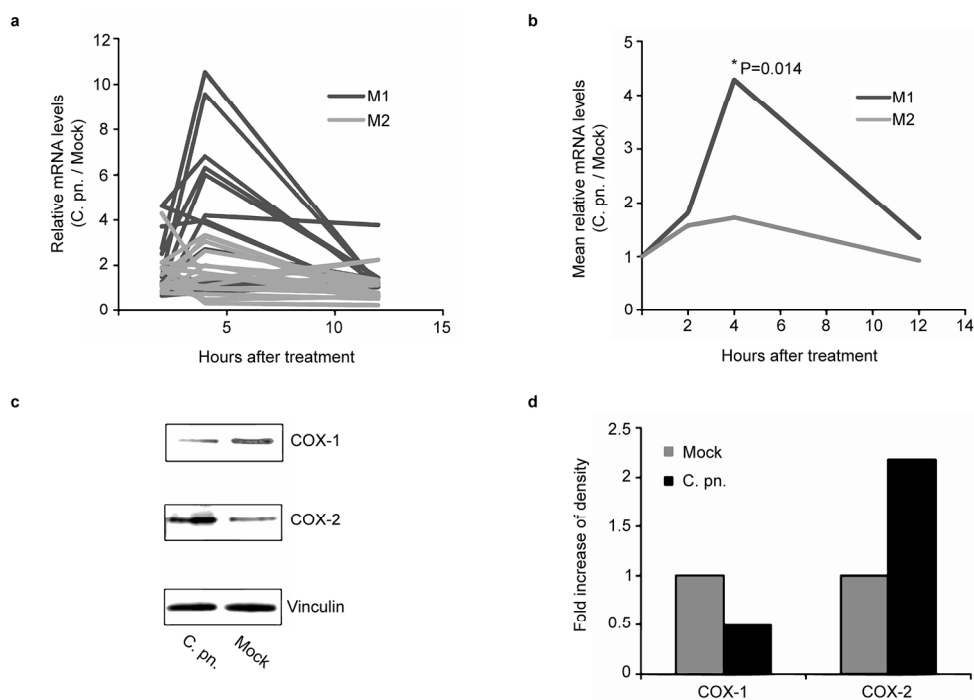
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Data obtained with the patient: Complete melanoma metastasis regression verified by PET-CT and retrospective PCR analysis-based detection of *C. pneumoniae*

(a, b) Ultrasonography; high tumor burden in the abdominal cavity. (c, d) CT and MRI scans; preoperational brain metastasis in the temporooccipital lobe and postoperational tumor-free brain status, respectively. (e) PET-CT scans; complete tumor regression in the body shortly after the septic event and BOLD treatment. (f) Retrospective detection of *C. pneumoniae* (*C. pn.*)-specific genes by RT-PCR. 16S rRNA: a housekeeping gene of *C. pn.* GroEL: Heat shock protein 60 of *C. pn.*, a group I chaperonin expressed on the surface of elementary bodies. MOMP: Major Outer Membrane Protein gene of *C. pn.* A-D: FFPE samples from different sections of primary melanoma; -C: PCR negative control (uninfected Hep2 cells); +C: PCR positive control (Hep2 cells infected by *C. pn.* strain TW183).

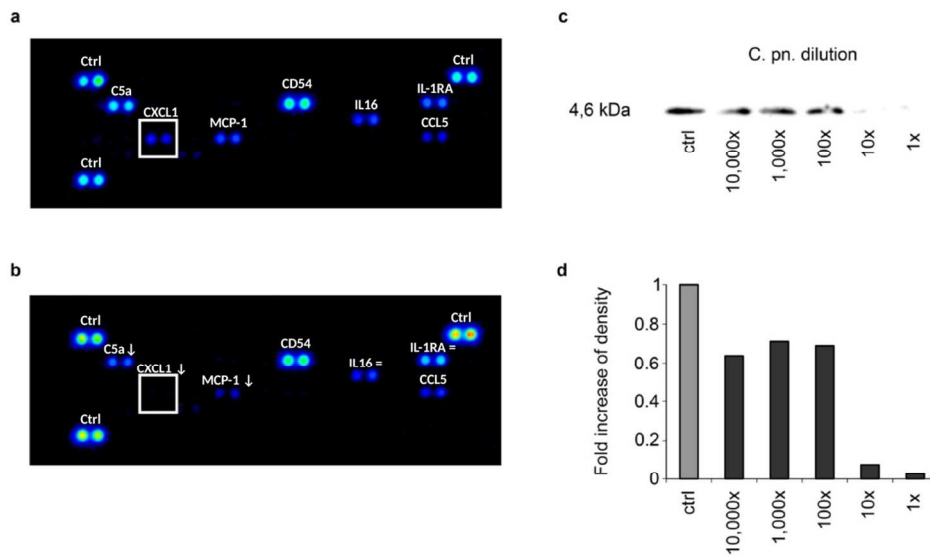
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C. pneumoniae treatment induces M1 type macrophage polarization

(a) Relative alterations in the levels of individual M1 type and M2 type cytokine/chemokine specific mRNA transcripts in lung samples of *C. pneumoniae* (*C. pn.*) vs. mock-treated tumor-bearing C57BL/6 mice, as determined by real-time PCR. (b) Mean values of relative M1 and M2 cytokine mRNA expressions; at 4 hours after treatment, M1 and M2 levels are significantly different (two-tailed t-test). (c) Representative Western blot. Expressions of COX-1 and COX-2 were determined in lung samples 12 hours after *C. pneumoniae* or mock treatment of melanoma-bearing C57BL/6 mice (d) Densitometry analysis. Intensity of immunoreactive bands of COX-1 and COX-2 were determined and then normalized to that of vinculin. Data are presented as fold increase compared to values of the mock-treated group (regarded as 1). A minimum of three experiments yielded similar results.

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CXCL1 is depleted by *C. pneumoniae* both in vivo and in vitro

Assessment of cytokine/chemokine protein levels (Proteome profiler) in lungs of melanoma-bearing C57BL/6 mice 2 hours after mock (a) or *C. pneumoniae* (b) treatment. Squares indicate CXCL1 which disappeared 2 hours after *C. pneumoniae* treatment (c) Representative Western blot. Recombinant mouse CXCL1 protein (0.5 μ g) was in vitro incubated with 500 IUFU/ μ l (1x) *C. pneumoniae*, (*C. pn.*) or its 10x, 100x, 1,000x, and 10,000x dilutions and then Western blotting was performed. (d) Densitometry analysis of immunoreactive bands shown in panel c. Values of the control (ctrl, vehicle treated) group were regarded as 1. Two experiments yielded similar results.

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22 Békésszentandrás,
23 Hungary

24 **Corresponding author's name:** Krisztina Buzas, Ph. D.
25 address: Temesvári krt. 62., H-6726 Szeged, Hungary
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10/29/2015

Prof. Barbara A. Gilchrist
editor-in-chief
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Dear Prof. Gilchrist,

We apologize for the inconvenience we caused. Indeed, it was our mistake that we mixed up the figure legends in the submission process, and we also agree that the arrows in the mentioned figure might be misleading.

We have corrected these mistakes, but no other changes (either in formatting or content-wise) have been made as compared to the version that had previously been accepted by the Reviewers.

The following corrections have been made:

1. The Figure legends that belongs to Supplementary Figure 3. has been corrected to reflect which is actually in the figure.
2. The arrows in panel b. have been removed from CD54 and CCL5, as requested.

We would also like to express our gratitude for Your kind patience and for having provided us this extra resubmission opportunity.

Sincerely,

Krisztina Buzas

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