**Zárójelentés**

Az OTKA által támogatott, **84064** azonosító számú
**"Bakteriális fertőzés és kemoattraktáns kezelés hatása attétek visszafejődésében"** című
project kutatásának lezárására.

**Bevezetés:** A melanoma malignum incidenciája az elmúlt 10 évben drámaian megnövekedett. Bár korai stádiumban a kezelés sebészeti beavatkozással jó hatásfokú lehet, a 3-4-es stádium, a recurrens illetve az extenzív melanoma kezelése nem kecsegtet sok sikerrel. Az előrehaladott állapotokban az ellenanyag alapú illeteve a különböző jelátviteli folyamatokat gátló kísérleti terápiák kerültek előtérbe az utóbbi években, de sajnos ezekkel is többnyire csak átmeneti és igen rövidtávú-néhány hónapos-sikerek érhetőek el.
Évszázados anekdotikus esetleírásokat találhatunk olyan daganatos gyógyulásokról, amelyeket bakteriális fertőzéseknek tulajdonítottak. Ebben a témában több sikeres próbálkozást tudhatott magáénak William Coley sebész, a mechanizmus magyarázatával azonban a tudomány azóta is adós maradt.

**Munkahipotézis:** arra a korábbi megfigyelésre alapozva, hogy az enyhe megbetegedést okozó *C. pneumoniae* egy M1 típusú makrofág polarizációs markernek minősülő kemokin termelődését indukálja, azt feltételeztük, hogy a melanoma malignum tüdőáttétei *C. pneumonia* hatására visszafejlődhetnek.

**Célkitűzés:** *C. pneumonia* kezelést követően tanulmányozni akartuk a kísérleti állatokban generált melanoma tüdőáttéteinek változását és a lejátszódó immunfolyamatokat.

**Az eredeti munkatervben megadott célkitűzéseknek megfelelő bontásban eredményeink a következőek:**

**1. év**

**CÉLKITŰZÉS 1. A tumort infiltráló makrofágok qualitative összehasonlítása bakteriális kezelést követően és kezelés nélkül.**

(A hivatkozott ábrák a csatolt közleményre utalnak)

Összehasonlító szövettani elemzést végeztünk a Mock kezelt kontrol állatok és az inaktivált *C. pneumoniae* kezelt egerek tüdejében bekövetkező változásokkal kapcsolatban.
A bakteriális kezelést kapott állatok esetében, amennyiben azok immunkompetens állatok voltak, a tumort infiltáló makrofágok száma jelentősen megnövekedett (Fig. 2). Míg a kontrol állatok esetében az immunsejtek megrekedtek a daganatok marginális zónájában, addig a *C. pneumoniae* kezelést kapott egereknél az immunsejtek képesek voltak behatolni a tumorszövetbe. Felületi markerek immunhisztokémiai jelölése alapján arra az eredményre jutottunk, hogy az infiltráló makrofágok CD11b és a CD80 pozitivitást mutatnak, amely M1 polaritású, anti-tumorális tulajdonságú sejttípusnak felelhet meg (Fig. 3).

Mindezeken a szövettani jellemzőkön kívül a *C. pneumoniae* kezelést kapott állatok túlélési idejei szignifikáns hosszabbodást mutattak ($p=0.04$). Ezzel összhangban a tüdőben található tumorok száma is szignifikánsan csökkent ($p=0.03$).

**2. év**

**CÉLKITŰZÉS 2. M1/M2 markerek expressziójának quantitatív analízise tumoros egerekből nyert mintákban.**

Munkánk során közel 30 M1és M2 polarizációs markert vizsgáltunk. Olyan citokinek és kemokinek időfüggő megjelenését vizsgáltuk, amelyek a szakirodalmi adatok alapján leírják a tumor asszociált makrofágok elköteleződésének irányát.
A kezeléseket követően az állatok tüdejéből 2, 4 és 12 óra elteltével vettünk mintákat Q-PCR analízisek elvégzéséhez. A csatolt közleményben a Fig. 4a ábra mutatja be a PCR termékek relatív szintjét a Mock kezelt kontrol mintákhoz hasonlítva.
A kezelések után 4 órával vizsgált mintákban az M1 kemokinek és citokinek szintjének átlagos növekedése szignifikáns eltérést mutatott(P=0.014) az M2 markerekéhez képest.

**3. év**

**CÉLKITŰZÉS 3. Polarizált makrofágok citokin és kemokin szekréciós profiljának vizsgálata.**

A 2. pontban ismertetett kinetikát követve olyan jellemző fehérjék kimutatását terveztük, amelyek segítségével behatárolhatóak a lejátszódó immunfolyamatok. A makrofágok polarizációját többek között a COX1 és COX2 aránya jellemzi. Az M2 polarizációt mutató COX1 szintjét a *C. pneumoniae* a detektálhatósági határ alá csökkentette a kezelést követő 4 órával, a COX2 kimutatása sajnálatos módon nem sikerült (Fig. 4b).
Bár a Proteome Profiler Assay nem minden vonatkozásában felelt meg a polarizáció meghatározására, a munkánk egyik legérdekesebb és minden vonatkozásában új eredményét ennek segítségével detektáltuk először. A CXCL1 oncoprotein melanoma növekedési faktorként ismert. A *C. pneumonia* kezelést követő 2 órán belül ellenanyag alapú kimutatási technikákkal a melanoma növekedési faktor szintje a detektálhatósági határ alá került (Fig. 5). Eredményeinket Proteome Profiler, Wester blot assay és dot blot mószerekkel is megerősítettük.

**Eredményeink disszeminációja:**

I. A kutatásunk eredményeiből **2 db *in extenso* közlemény** készült. Az első 2012-ben megjelent (10. pont). A második publikáció New England Journal of Medicine-re formázva, a csatolt dokumentumban megtalálható.

Mivel a szabadalmaztatási folyamat még ezidáig nem zárult le, ezért a kézirat beküldését el kellett halasztani. A kézirat feltöltése a szabadalmi bejelentést követően a lehető legrövidebb időn belül megtörténik.

***Az OTKA Főtitkárával való telefonos egyeztetés alapján tisztelettel kérem a bírálókat, a jelentés kezelése kapcsán a szabadalmi oltalom érvényesüléséig az újdonságrontást elkerülendő, kezeljék a jelentés adatait ennek megfelelően bizalmasan.***

*Annamaria Marton, Csaba Vizler, Edina Gyukity-Sebestyen, Maria Harmati, Gabriella Dobra, Katalin Nagy, Janos Minarovits, Erno Duda, Agnes Zvara, Laszlo Puskas, Robert Katona, Vilmos Tubak, Valeria Endresz, Zoltan Kis, Istvan B. Nemeth, Judit Olah, Lajos Kemeny, Krisztina Buzas:* ***Interactions between bacteria and tumoral microenviroment increase survival in human and in experimental metastatic melanoma model*** *(Formázva a NEJM követelményrendszerének megfelelően)*

II. A **szabadalmi bejelentés** előkészítése a Danubia Szabadalmi és Jogi Iroda Kft. közreműködésével történik. Cím: "Antitumor bacterial composition", aktaszám: 113570-3405A

**III. Eredményeink publikálása:**

A 2. és a 4. sorszámmal megjelölt konferenciákon munkánkkal első díjakat nyertünk az adott szekciókban, erről szóló dokumentumokat a nyomtatott formában beküldendő jelentéshez csatoltam.

1. *COST WG3 Pathophysiology of EVs meeting in Budapest, 2013* (meghívott előadó)
   Maria Harmati, Annamaria Marton, Robert L. Katona, Edina Gyukity-Sebestyen, Okay Saydam, Tanja Kalič, Istvan Nagy, Balazs Horvath, Csaba Vizler, Krisztina Buzas ***Interactions between melanoma cells and their microenviroment***

2. Annamaria Marton, Csaba Vizler, Erzsebet Kusz, Erno Duda, Janos Minarovits, Laszlo Puskas, Agnes Zvara, Attila Borics, Zoltan Hegedus, Edina Gyukity-Sebestyen, Robert Katona, Katalin Nagy, Istvan Nemeth, Lajos Kemeny, Krisztina Buzas: ***Gram negative bacteria induce anti-cancer immune response and regression of melanoma***, http://www.emds2012.eu/documents/EMDS_2012_Debrecen_Submitted_abstracts.pdf, 2012

3. Annamaria Marton, Csaba Vizler, Robert Katona, Viktoria Temesfoi, Erno Duda, Janos Minarovits, Zsuzsa Szathmary, Zsolt Szegletes, Laszlo Siklos, Erzsebet Kusz, O.M. Zack Howard, Krisztina Buzas: ***Melanoma cell derived exosomes transform macrophage and dendritic cell functions in vitro***, http://www.firnweb.com/FIRNProgram2011.pdf, 2011

4. Annamaria Marton, Csaba Vizler, Erzsebet Kusz, Erno Duda, Janos Minarovits Laszlo Puskas, Agnes Zvara, Attila Borics, Zoltan Hegedus, Edina Gyukity-Sebestyen, Robert L. Katona, Katalin Nagy, Judit Olah, Istvan Nemeth, Lajos Kemeny, Krisztina Buzas: ***Gram negative bacteria induce anti-cancer immune response and regression of melanoma metastases in vivo mouse model***, http://www.eado.org/activities/eado-congress/8, 2012

5. Annamaria Marton, Csaba Vizler, Erzsebet Kusz, Viktoria Temesfoi, Robert Katona, Vilmos Tubak, Gergely Maroti, Katalin Nagy, Janos Minarovits, O.M. Zack Howard, Erno Duda, Krisztina Buzas: ***Mesenchymal stem cell and immune cell functions altered by melanoma cell derived exosomes in vitro***, Scientific Program 18-21 April 2012, Gothenburg Sweden, 2012

6. Annamaria Marton, Csaba Vizler, Robert Katona, Viktoria Temesfoi, Erno Duda, Zsuzsa Szathmary, Zsolt Szegletes, Laszlo Siklos, Erzsebet Kusz, O.M. Zack Howard, Ferenc Banati, Sandor Spisak, Bela Molnar, Janos Minarovits, Krisztina Buzas: Melanoma cell derived exosomes alter immune cell functions *in vitro*, Acta Microbiologica et Immunologica Hungarica, 2011

7. Annamaria Marton, Maria Harmati, Robert L. Katona, Edina Gyukity-Sebestyen, Okay Saydam, Tanja Kalič, Gabor Decsi, Zsofia Tarnai, Erno Duda, Janos Minarovits, Katalin Nagy, Istvan Nagy, Balazs Horvath, Csaba Vizler, Anna Borsodi, Krisztina Buzas: ***The effect of***

***tumor cell derived exosomes on mesenchymal stem cells***, http://www.ici2013.org/pdf/uploads/abstracts-book.pdf, 2013

8. M. Harmati, A. Marton, R.L. Katona, E. Gyukity-Sebestyen, E. Kusz, Z. Tarnai, E. Duda, J. Minarovits, K. Nagy, I. Nagy, B. Horvath, C. Vizler and K. Buzas: ***Effect of tumour cell-derived exosomes on immune- and mesenchymal stem cells***, http://www.journalofextracellularvesicles.net/index.php/jev/article/view/20826/26894, 2013

9. Marton A, Gyukity-Sebestyen E, Harmati M, Katona R, Decsi G, Tarnai Z, Duda E, Minarovits J, Nagy K, Nagy I, Horvath B, Vizler C, Buzas K: ***Melanoma derived exosomes alter mesenchymal stem cell profile and support metastasis formation***, JOURNAL DER DEUTSCHEN DERMATOLOGISCHEN GESELLSCHAFT 11:(Suppl. 7.) p. 101. 1 p. (2013), 2013

10. Marton A, Vizler C, Kusz E, Temesfoi V, Szathmary Z, Nagy K, Szegletes Z, Varo G, Siklos L, Katona RL, Tubak V, Howard OM, Duda E, Minarovits J, Nagy K, Buzas K.: ***Melanoma cell-derived exosomes alter macrophage and dendritic cell functions in vitro.***, Immunology Letters, 2012

11. Marton Annamária, Vizler Csaba, Katona Róbert, Kusz Erzsébet, Duda Ernő, Minárovits János, Tubak Vilmos, Németh István, Borics Attila, hegedűs Zoltán, Endrész Valéria, Faludi Ildikó, Kemény Lajos, Buzás Krisztina: **Bakteriális indukció melanoma tüdőáttéteinek regresszóját idézi elő: makrofág polarizációs hatások vizsgálata *in vitro* és *in vivo* modellrendszerekben.** Immunológiai Szemle, 2011

IV. 2013. szeptemberében az NIH, National Cancer Institute-ban egy szemináriumon keretében ismertettem az OTKA által támogatott projektet és az eredményekből következő lehetséges gyakorlati alkalmazásokat. A Cancer and Inflammation Program egyik vezető senior kutatójának, Dr. Joost Oppenheimnek véleményét csatoltam.

V. Munkánk során alkalmunk nyílt nem kizárólag a 84064 kutatás címében meghatározott témát, de ahhoz szervesen kapcsolódó másik területet, az exosomák tulajdonságait is tanulmányozni.
Ennek publikációs eredményei a felsorolt közlésekben láthatóak. A csoportunkhoz csatlakozó hallagtók közül exosoma témában OTDK első helyezéséről elért oklevél a kiegészítő dokumentumok között szintén megtalálható.

VI. Részletes állatkísérletes protokollt dolgoztunk ki, amelyet a becsatolt kéziratban ismertettünk.

Annamaria Marton, Csaba Vizler, Edina Gyukity-Sebestyen, Maria Harmati, Gabriella Dobra, Katalin Nagy, Janos Minarovits, Erno Duda, Agnes Zvara, Laszlo Puskas, Robert L. Katona, Vilmos Tubak, Valeria Endresz, Zoltan Kis, Istvan B. Nemeth, Judit Olah, Lajos Kemeny, **Krisztina Buzas**

# Interactions between bacteria and tumoral microenviroment increase survival in human and in experimental metastatic melanoma model

## Background

Immune response seems to be a decisive factor in the outcome of melanoma malignum (**Shimanovsky A et al. 2012; Ridnour LA et al. 2013**). Anti-tumor immune effector mechanisms overlap with anti-bacterial immune responses. It has been recognized for over 100 years that cancer patients might recover following bacterial infections (**Wiemann B, Starnes CO.,1994; Maletzki C, et al, 2012**). Interestingly, a clinical study showed that febrile infections or vaccinations with *Bacillus Calmette Guerin* or vaccinia virus in early childhood significantly decrease melanoma incidence (**Krone B. Et al, 2003).**

Our studies were prompted by a case of unexpected remission in a grade IV metastatic melanoma patient suffering sepsis during regular BOLD chemotherapy. After antibiotic treatment and complication-free chemotherapy, her physical conditions markedly improved, and her metastatic lesions also disappeared. The patient has been completely asymptomatic and PET/CT-verified metastasis free from 2009 to this date. Based on statistics, the complete recovery suggested an adjuvant effect of sepsis.

*Chlamidophyla pneumoniae (C. pneumoniae)* has not previously been implicated in tumor regression. As a lung-specific intracellular pathogen, it seemed to be especially suitable for therapy of lung metastasis. Mice bearing melanoma lung metastases were treated with inactivated *C. pneumoniae*. The lung metastasis numbers significantly decreased, while the survival of the animals significantly increased. Studies of cytokine profile of the treated mice suggested that the tumor rejection might be caused by re-education of the tumor-infiltrating immune cells by the infection.

Although the presence of *C. pneumoniae* was not initially investigated in the patient, it was retrospectively verified by real time and nested RT-PCR from paraffin embedded primary tumor tissue dissected after recovery from the sepsis. Our results suggested that *C. pneumoniae* might be responsible for the unexpected tumor remission in humans and in experimental animals also, and attest the therapeutic potential of this approach.

## Clinical case report

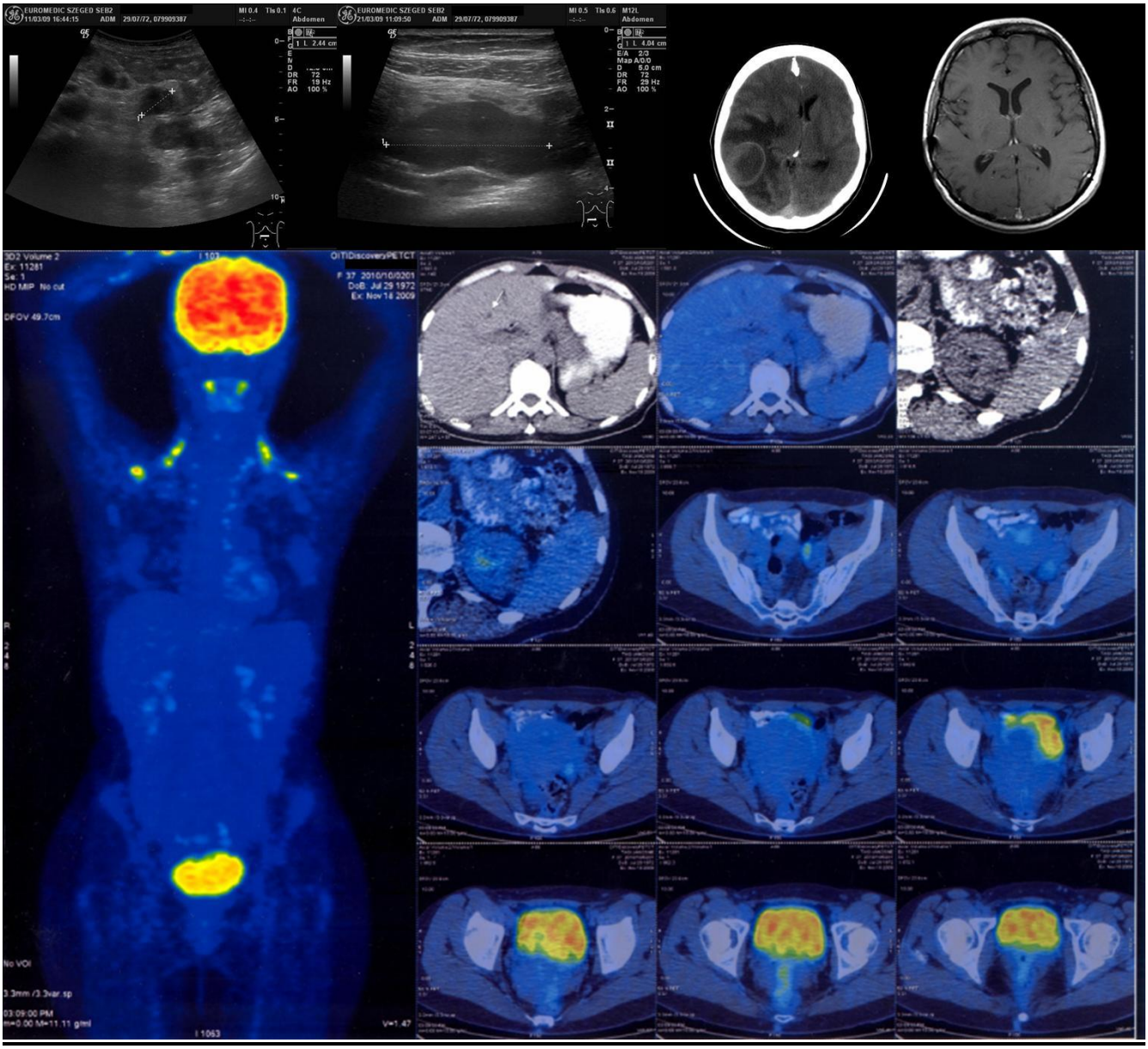
For the timeline of the events see Table 1.

**Table 1. Timeline of unexpected complete tumor regression in a 37-year-old woman with stage IV metastatic melanoma**

| 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, vincristine, lomustine and dacarbazine) chemotherapy initiated. |
| 37 | On the 3rd day of chemotherapy, it was suspended because of vomiting and fever. The gastric fluid contained *Escherichia coli* and *Candida albicans*. *Clostridium difficile* toxin was also detected. Fluconazole and ceftriaxone (later metronidazol) treatment was initiated. |
| 52 | CVC was removed because of putative *Pseudomonas aeruginosa* 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 lympadenomegalia, 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. |

***Fig 1a: Complete melanoma metastasis regression verified by PET-CT***

*Ultrasonography (pictures left up) showed high tumor burden in the abdominal cavity. CT and MRI scans (pictures right up) present the preoperational brain metastasis in the temporooccipital lobe and postoperational tumor-free brain status, respectively. PET-CT scans showed complete tumor regression in the body shortly after the septic event and BOLD treatment.*



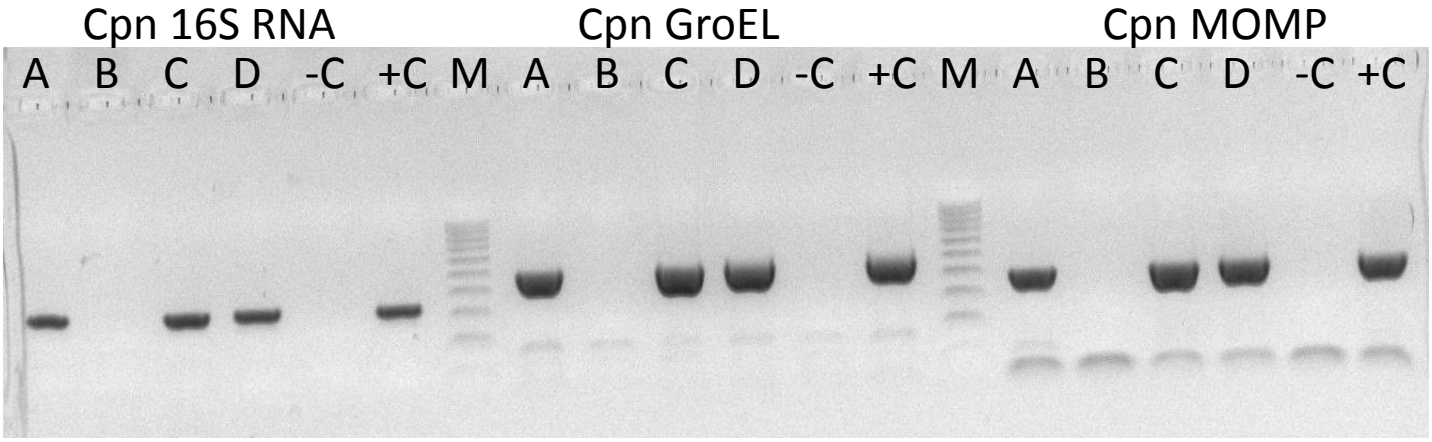



***Fig 1b. Retrospective PCR analysis verified the presence of C. pneumoniae in the formalin fixed paraffin-embedded (FFPE) sample.*** *A-D FFPE sample originated from different sections of primary melanoma; -C, PCR negative control; +C, PCR positive control. C. pneumoniae has been detected from A, B and D specimens.*

## Materials and methods

### *In vivo* mouse model

B16F1 melanoma cells (DTP, DCTP Tumor Repository, Frederick, MD, USA) were administered intravenously ($1x10^5$ cell/100 µl) to 6-8 week old female C57BL/6 and NSG mice. One week after the tumor cell administration the mice were treated with *C. pneumoniae* strain CWL-29 propagated in HEp2 cells (Burian et al, 2003). *C. pneumoniae* and the mock control were heat-inactivated at 90˚C for 30'. Mice were mildly sedated with sodium pentobarbital (7.5 mg/ml) and treated intranasally with $1\times10^6$ IFU *C. pneumoniae* 7, 9, 11, 14, and 16 days after tumor implantation. Two hours after the 1st inhalation (day 7), 4 hours after 2nd inhalation, 12 hours after 3rd inhalation and 24 hours after 5th inhalation, 3 animals/group were anaesthetized, their lungs removed and homogenized in RNA Later, Cell Lysis Buffer containing protease inhibitor, while the other part was frozen for histology. 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. For the survival experiments, groups of mice (n=15) were treated as described 5 times after melanoma implantation. Kaplan-Meier survival curves were analyzed by a log-rank statistical test and $p \leq 0.05$ was regarded as statistically significant. The body temperatures of 3 animals/group were measured using an AMA Digital AD 15 TH thermometer two hours after the 1st treatment and four hours after the 2nd inhalation (day 7 and 9). All animal experiments were authorized by the institutional and national animal welfare committees.

### Quantitative PCR (Q-PCR) of cytokines and chemokines.

Total RNA was purified using a NucleoSpin RNA II RNA isolation kit and Q-PCR reactions were performed on pooled samples (n=3) on a RotorGene 3000 instrument with gene-specific primers and SybrGreen protocol to monitor gene expression. Each individual Ct value was normalized to the average Ct values of four internal control genes (ΔCt values). The final relative gene expression ratios (fold change) were calculated as comparisons of ΔCt values (ΔΔCt values). Non-template control sample was used for each PCR run to check the primer-dimer formation. Primer sequences are available upon request.

### *C. pneumoniae* detection from the primary tumor of patient by PCR

DNA was extracted from the formalin fixed paraffin-embedded (FFPE) samples using Nucleospin® FFPE DNA kit according to the manufacturer's instruction. REDTaqReadyMix PCR Reaction Mix with $MgCl_2$ was used in the conventional PCR with a primer pair of *C. pneumoniae* specific 16SRNA, GroEL and MOMP **(FEMS Immunol Med Microbiol ]] (2008) 1–11)**. Positive results were confirmed with *C. pneumonia* specific TaqMan real time PCR **(Brittain-Long et al, Journal of Clinical Virology 41 (2008) 53–56) that** was applied by using LightCyclerTaqManMaster Kit and LightCycler 2.0.

### Histology

Lung specimens were fixed in 4% buffered formaldehyde and a standardized immunohistochemistry tissue microarray was performed; visualization was made by fast red and DAB. Slides were finally counterstained by hematoxilin, the red or brown reactions were evaluated under light microscope.

### Cytokine and chemokine detection by proteome profiling

Lung specimens were homogenized in cell lysis buffer. Equal protein loading into pooled samples were used to simultaneously detect the relative levels of different cytokines according to the manufacturer's instruction of Mouse Cytokine Array, Panel A.

**Western blot analysis of CXCL1**

Recombinant mouse CXCL1 protein was mixed with heat-inactivated *C. pneumoniae* stock and the 10, 100, 1000 and 10000 times diluted solutions were incubated for 30 minutes at 37 °*C*. We added to each mixture a constant amount of CXCL1 protein (0.5 μg) and different dilutions of *C. pneumoniae* solutions (3.6 μg-0.00036 μg). After SDS-PAGE, and transfer onto PVDF membranes CXCL1 was detected by anti-CXCL1.

**Results**

**1. *C. pneumoniae* treatment reduced the number of lung metastases and increased survival**

Immunocompetent C57BL/6 mice and multiply immunodeficient NOD/Scid IL2rg null mice were injected intravenously with B16F1 cells. One week later, the mice bearing established lung metastases were treated with *C. pneumoniae* suspension or mock control. In treated immune-competent, but not immunodeficient mice i) the number of lung metastases significantly decreased ($P \leq 0.003$, 3 independent experiments); ii) the survival significantly increased ($P \leq 0.04$, 3 independent experiments); iii) up to 10% of the mice showed complete tumor regression; these animals remained tumor free for the duration of the study. The treated animals did not have fever (33.2 ºC±1.0 control vs. 34.8 ºC±0.5 treated). All animals died in the absence of *C. pneumoniae* treatment.

**2. Histological analysis detected tumor regression and immune cell infiltration in the treated mice**

Lungs from the control melanoma-bearing group showed abundant metastases replacing large quantities of normal lung tissue (Fig. 2a). Tropical necrosis (asterisk) was noticeable, indicating high tumor burden. Many areas of residual pneumonitis was identified in the *C. pneumonia*-treated mice compared tithe Mock treated melanoma-bearing group (Fig. 2b) (circles and right bottom insert). Moreover, only foci of regressive metastases were seen. High number of tumor infiltrating mononuclear histiocytes and lymphoid cells are notably present in regressive metastases (Fig. 2b left bottom insert) compared to Mock treated sample (Fig. 2a insert). In both Mock treated (Fig. 2c) and *C. pneumoniae* treated (Fig. 2d) NSG melanoma groups, metastases (circles) were sub-pleural (arrowheads) and intra-parenchymal (circles) without a significant inflammatory reaction (inserts).

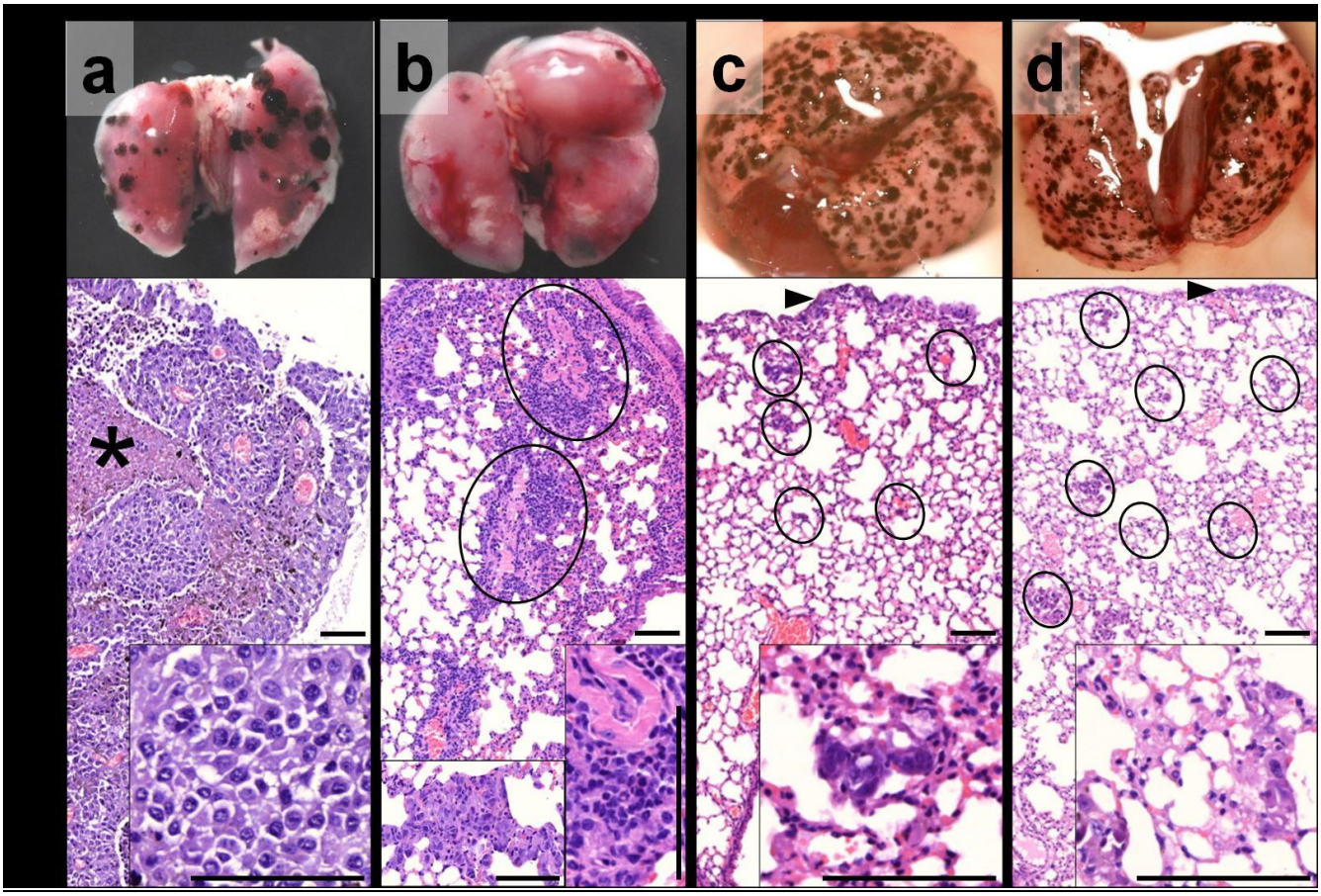



***Figure 2. C. pneumoniae treatment resulted melanoma metastasis regression in vivo.***
*Pictures and histological slides of the dissected lungs of control Mock treated melanoma-bearing group (a), in C. pneumoniae treated group (b), Mock treated (c) and C. pneumoniae treated (d) NSG melanoma groups. /HE slide staining; OM 50x, 200x and 400x; all bars represent 100µm/*

According to immunohistochemistry of cell surface activation markers, there was a markedly increased immunoreaction in the *C. pneumoniae*-treated melanoma-bearing animals. Moreover, as shown in Figure 2., high levels of tumor-infiltrating activated macrophages, dendritic cells and lymphocytes were detected within the tumor stroma, compared to the Mock treated group. Marked increases of CD11b and CD80 positive cells was detected in the *C. pneumoniae* groups (Fig 3).

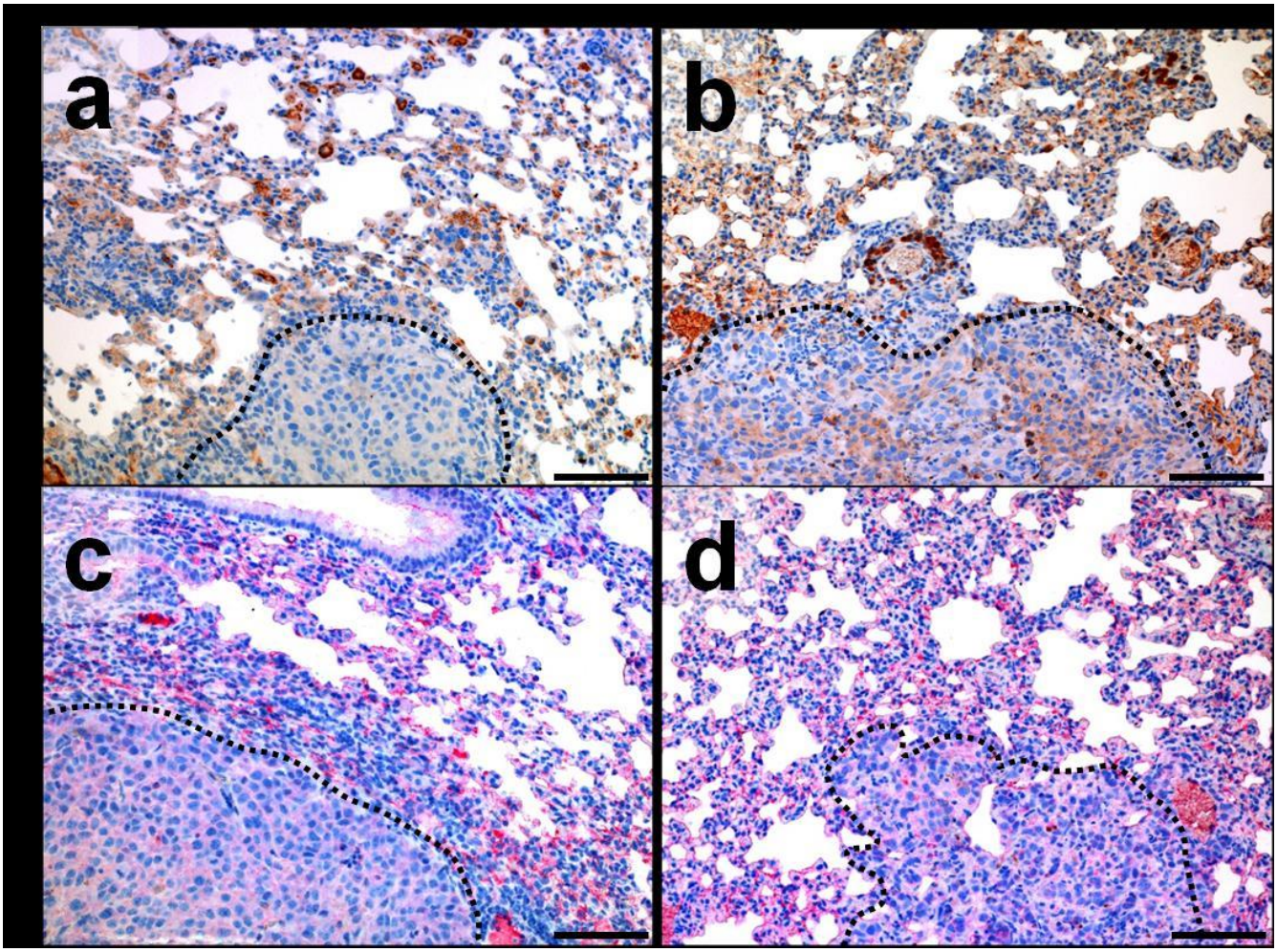



***Figure 3.: C. pneumoniae treatment increased the CD11b+ and CD80+ immune cell infiltration of tumor tissue***

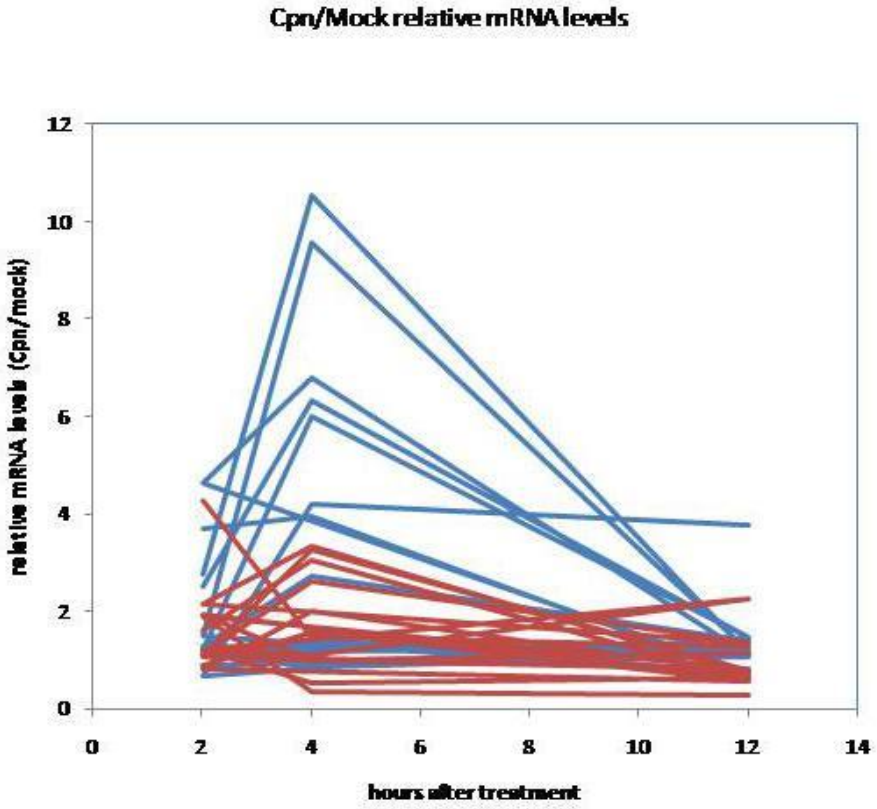
*CD11b (a, b) and CD80 (c, d) immunohistochemistry in control Mock treated group (a, c), in C. pneumoniae treated group (b, d). Immunohistochemistry; a, b: DAB-visualized brown-, c, d: Fast red-visualized red colorimetric reaction; OM 200x; bars represent 100 μm; interrupted lines indicate tumor border.*

### 3. *C. pneumoniae* treatment skewed cytokine and chemokine gene transcription favoring M1 type macrophage profile

We mapped cytokine and chemokine transcriptome profile tumor bearing mice with and without bacterial treatment. M1 and M2 type macrophage markers were detected with Q-PCR from pooled samples (3 animals/pool), after 2, 4 and 12 hours of treatment in the lung tissue. Messenger RNA level markedly increased for CCL2, CCL3, IL6, CXCL10, CCL7, CD80, CXCL11, CXCL9, IL23, TNFα-markers of M1 type macrophage polarization-after 2, 4 or 12 hours of bacterial treatment. For CD163, CCL1, TGFβ and IL10, decreased mRNA levels were detected, while other important M2 markers, such asCXCL13 and IL1Ra are increased.

The average mRNA content of M1 cytokines and chemokines significantly increased (P=0.014) after 4 hrs in comparison to M2 type macrophage markers (Figure 4). These results support our hypothesis that M1 type macrophage polarization plays a role in tumor regression.

Cox 1 and Cox 2 are co-expressed in the airways of mice; and their balance is one of the main markers in M1/M2 polarization. Cox 1/Cox 2 alterations were checked using Western blots. No changes were detected after 2 and 4 hrs for Cox 1, produced by tumor supportive M2 type macrophages, however, it was markedly reduced after 12 hours of *C. pneumoniae* treatment. No changes were detected in Cox2 levels. (Fig 4b)


Cpn/Mock relative mRNA levels
relative mRNA levels (Cpn/mock)
hours after treatment
0
2
4
6
8
10
12
14

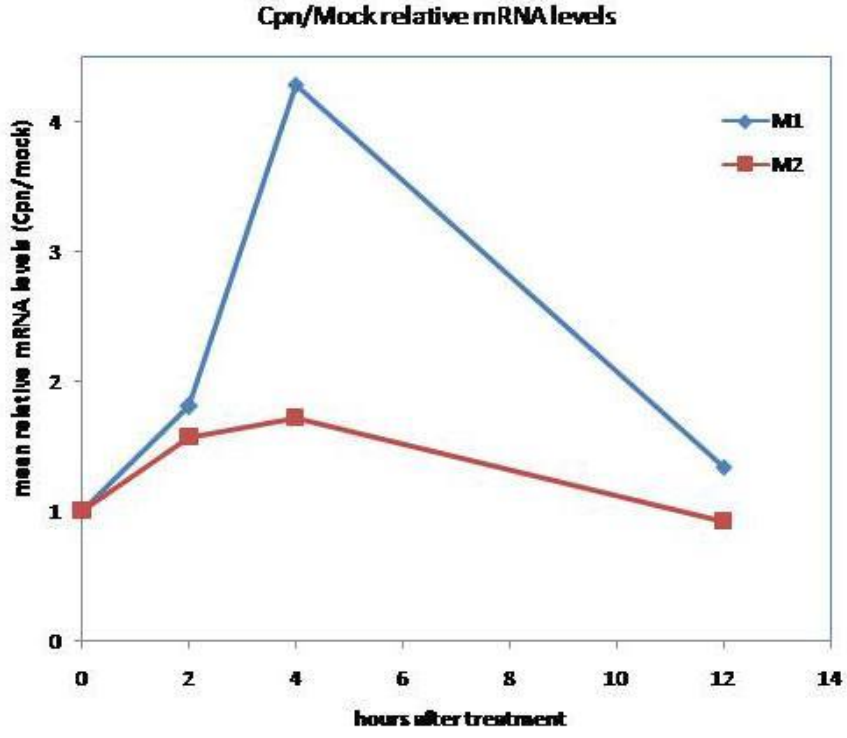

Cpn/Mock relative mRNA levels
mean relative mRNA levels (Cpn/mock)
hours after treatment
M1
M2
0
1
2
3
4
0
2
4
6
8
10
12
14

***Figure 4a. The relative amount of M1 type cytokine and chemokine transcripts increased in the** C. pneumoniae **treated mice.** a) Relative changes in the quantities of individual M 1type/M2 type cytokine and chemokine mRNA-s in C. pneumoniae treated vs. Mock treated tumor-bearing mice. The mRNA levels were determined by real-time PCR. b) Mean values of relative M1 and M2 cytokine mRNA levels. The difference at 4 hours was significant (two-tailed t-test, p=0.014).*

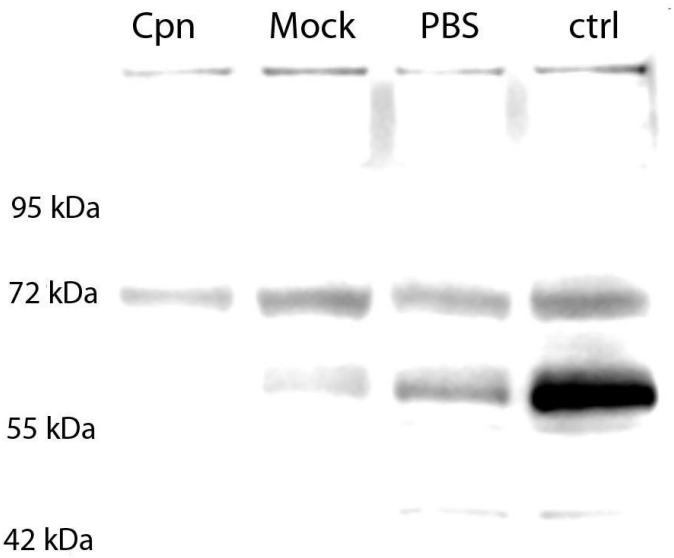
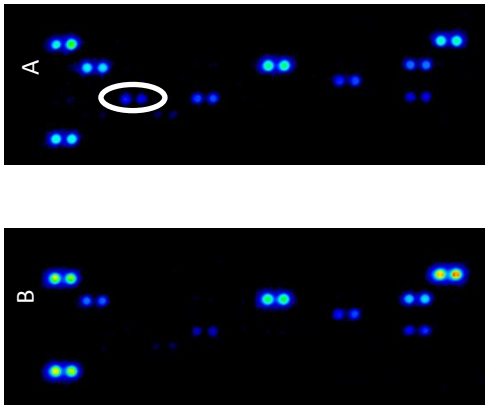



***Figure 4.b.: M2 type marker Cox1 level decreased in the lung of treated tumor bearing mice.** Cox1 immunoblot analysis of heat inactivated C. pneumoniae treated (Cpn), Mock treated (Mock) and PBS-treated melanoma bearing animals and untreated group (ctrl) after 4 hours of C. pneumoniae treatment.*

**4. The oncoprotein CXCL1 was depleted by *C. pneumoniae* treatment *in vivo***

Cytokine and chemokine protein level changes were investigated using Proteome Profiler array (R&D, ARY006). Interestingly, after 2 hours of treatment, the CXCL1 immunoreactivity completely disappeared from the lungs of experimental animals. To test the cause of this phenomenon, constant amounts of recombinant CXCL1 were mixed and incubated with increasing quantities of heat inactivated *C. Pneumoniae* in the presence of protease inhibitors. Our results suggest that the bacteria strongly bind this protein (Figure 5).



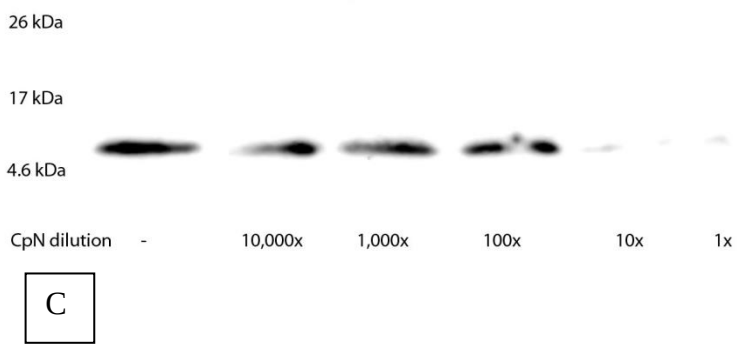



***Figure 5.: Oncoprotein CXCL1 was depleted by heat inactivated C. pneumoniae***
*Cytokine and chemokine detection by proteome profiling after 2 hours of control Mock treated (A ) and heat inactivated C. pneumoniae treated (B) melanoma-bearing mice. The circle indicates the presence of CXCL1. The signal disappeared 2 hours after C. pneumoniae treatment.*
*(C) SDS-PAGE and Western blot analysis of recombinant mouse CXCL1 protein after incubation with heat inactivated C. pneumoniae 500 CFU/µl (1x), and 10x, 100x, 1000x, 10000x dilution, respectively.*

**Discussion**

Life expectancy of stage IV melanoma patients is very poor even today. Dacarbazine-based (DTIC) mono-, or polychemoterapy remains the gold standard despite the low efficacy (Rigel, Avril, Schadendorf). The BOLD (**b**leomycin, **o**ncovin, **l**omustin, **d**acarbazine) regimen was originally reported to induce only 9% complete remission, and even this success rate was not consistently confirmed (Seigler, Prudente, Lakhani). In a sytematic metaanalysis, metastatic melanoma patients receiving polychemotherapy, including BOLD, showed 12.5 months mean survival and 20 months maximum survival (Garbe). Since our patient has had 45 months disease-free survival to date (Sept of 2013), it seems unlikely that short term BOLD therapy alone accounted for the disease-free survival.

The alteration in cytokine and chemokine profile of *C. pneumoniae* treated mice supports our hypothesis that alternative polarization of the immune response by infection or sepsis might induce tumor regression. In our mouse model we detected a switch from M2 to M1 profile, although we did not detect the whole spectrum of ”classical” M1 type macrophage polarization (**Mantovani, Sica**, **2012**). *C. pneumoniae* recognized by TLR2 and TLR4 was shown to induce the costimulatory molecules CD40, CD80 and CD86 on macrophages (**Prebeck S, 2001**); as well as the CD11b cell surface molecule (**Bachmaier K, 1999**). We could also detected enrichment of the CD80 and CD11b expressing cells in the lungs of treated animals.

While there is increasing evidence that immune stimulation by various microbial agents could support anti-tumor immune responses, the exact mechanisms are not known yet **(Trinchieri, 2013).** *Streptotoccus pyogenes* and *Serratia marcescens*, genetically modified Clostridiums, and *Salmonella typhi* has earlier been shown to induce tumor remission. The most plausible explanation of the effect is fever induction triggered by massive tumor necrosis factor production (**Hobohm, 2001)**; surprisingly, neither was present in our model.

Interestingly, in our experimental system we were able to deplete the melanoma growth factor CXCL1 with the *C. pneumoniae* extract used in the *in vivo* experiments, providing a possible explanation for the anti-melanoma effect. It has been demonstrated previously that melanoma cells express high levels CXCL1. CXCL1-induced NF-κB was facilitating transformation by allowing melanocytes to escape from apoptosis; the super repressor of NF-κB (IκB-α ΔN) was overexpressed in immortalized murine melanocyte clones **(Dhawanadn Richmond, 2002).**

According to these data, the survival benefit associated with *C. pneumoniae* treatment might be related to the development of anti-cancer immunity and/or disappearance of a melanoma growth factor CXCL1.

Our experimental animal model and retrospective demonstration of the presence of *C. pneumoniae* in our patient with complete remission might suggest that this pathogen may have been responsible for unexpected cases of tumor remission. Our study not only offers a feasible explanation for the beneficial effects of bacterial infection in cancer patients, but also offers a hope that this paradigm could be translated eventually into a clinical practice.

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