Tumor Progression Is Associated with Increasing CD11b$^+$ Cells and CCL2 in Lewis Rat Sarcoma

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Abstract. *Tumor models are essential for basic anticancer research and development of novel therapies. In this study, we used a rat sarcoma model in which subcutaneous tumor develops after D6 cell inoculation. The aim of the current study was to analyze changes in haematological parameters, immune cell sub-populations and cytokine profiling during tumor growth, after tumor excision and after second inoculation of D6 cells. Tumor progression was found to be associated with an increased number of leukocytes and increased proportion of CD11b$^+$ cells in peripheral blood. Serum concentration of chemokine (c-c motif) ligand 2, L-selectin and intra cellular adhesion molecule-1 also increased with growing tumor. However, the proportion of CD4$^+$, CD8$^+$ and MHC II$^+$ cells decreased with growth of tumors. After tumor excision, all these parameters returned to pre-inoculation levels and did not change even after a second inoculation of D6 cells. Moreover, absence of secondary tumors after second inoculation of D6 cells gives an insight into development of antitumor immunity stimulated by primary tumor.*

During its development, tumor induces immunological changes that can be associated with its progression or regression. The myeloid cells of innate immunity *e.g.* granulocytes, dendritic cells, macrophages/monocytes and natural killer cells, are important components of precancerous and malignant tissues. They release different kinds of cytokines, serine proteases, reactive oxygen species and other bioactive mediators that contribute to cancer development by regulating tissue remodelling and angiogenesis (1-4). On the other hand, tumor cell progression is mainly influenced by the number and activation state of Cluster of differentiation 8 (CD8$^+$) cytotoxic T-lymphocytes (CTLs) which can directly target and destroy the tumor cells. Normally, CTLs are not enough alone to kill tumor cells. They require T-helper type 1 cells (Th1, CD4$^+$) to mature and exert their activity. Th1 cells are also necessary for CTL clonal expansion and establishment of memory cells. CD4$^+$ and CD8$^+$ lymphocyte variation, both in number and in function, can play a critical role in cancer evolution (5-7). The shift of Th1 toward Th2 phenotype and the reduction in number and function of CTLs can lead to the cancer progression. This shift is dependent on and is regulated by cytokines/chemokines secreted either by immune cells or tumor cells themselves. Interferon γ (IFN-γ) and interleukin 2 (IL2) regulates development of Th1 cells, whereas IL4 and IL5 support Th2 activity (8). One of the important C-C family chemokines is monocyte chemoattractant protein-1 (MCP-1), also known as chemokine (c-c motif) ligand 2 (CCL2). In contrast to other chemokines of the C-C family which trigger Th1 phenotype, CCL2 acts as an inducing factor for the development of Th2 phenotype. Equilibrium between tumor-promoting and-suppressing chemokines plays a key role in the development of tumor and metastasis (9,10). In the context of developing antitumor immunity, the role of CCL2 was evident in many tumor models which are mediated through infiltration of lymphocytes (11). The role of adhesion molecules in cancer biology has long been investigated. High concentrations of soluble adhesion molecules *e.g.* intracellular adhesion molecule-1 (ICAM-1) and L-selectin, were found to be associated with many tumor models. The dynamic of these molecules shows a tendency for their increased expression with increasing tumor size and metastatic formation (12, 13).

A rat sarcoma model was established in our laboratory from a spontaneously developed neoplasm in the Lewis rat female (14, 15). Various cell clones with different biological characteristics were derived from the initially isolated R5-28 parental cell lines. Three clones named C4,

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C7 and D6 were characterized in detail by Holubova *et al.* (16), *In vivo*, tumors developed from C4 cells showed spontaneous regression in more than half of inoculated rats while C7 and D6 cells produced progressing tumors only. Immunological variation during their development was also noted.

An *in vivo* tumor model can help to better-define and follow the immune system activity under controlled conditions and at established time points. In the present study, we used D6 sarcoma cells to investigate the changes in the immune cell proportions and concentration of different protein molecules during tumor progression, after tumor ablation, and after second inoculation of D6 sarcoma cells.

Materials and Methods

Animals. Inbred rats of Lewis strain (8 weeks old) were purchased from the Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i., Prague. Animals were randomly divided into two groups: experimental (12 rats) and control (8 rats). Experimental animals were inoculated with D6 sarcoma cells. Peripheral blood was collected from the tail vein each week from the beginning (week 0) to the end of experiment (week 19). All rats were then euthanized by halothane inhalation followed by decapitation. All experiments were performed in accordance with the Project of Experiment number 098/2011, approved by the Animal Science Committee of the Institute of Animal Physiology and Genetics AS CR, following the rules of the European Convention for the Care and Use of Laboratory Animals.

Cell culture and inoculation. The D6 sarcoma cells were cultured *in vitro* using high-glucose Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS) (Lonza, Basel, Switzerland), 10 IU penicillin/5 μg streptomycin per 1 ml, 10 μM HEPES, 1 mM L-glutamine and 1 mM non-essential amino acids (all from Sigma-Aldrich) in 5% CO_2 at 37˚C. For inoculation, growing D6 cells (reaching almost monolayer) were washed with phosphate buffer saline (PBS), treated with 0.2% trypsin Ethylenediaminetetraacetic acid (EDTA) followed by gentle washing twice with PBS. Cell suspensions were injected subcutaneously on the dorsal side at a dose of 5×10^5 cells in 0.2 ml PBS into experimental animals. Control rats were injected with 0.2 ml PBS only. D6 sarcoma cell inoculation was performed twice during the whole experiment. The first inoculation was carried out at week 0 and the second inoculation at the 11th week of the experiment (*i.e.* six weeks after tumor excision). Tumor development was observed at regular intervals and tumor size before their excision was measured by manual Vernier calliper. Tumor volumes were determined using a modified ellipsoid formula $V=0.5\times (\text{length}\times\text{width}^2)$ (17).

Tumor excision. Tumor excision of experimental rats was performed under total anaesthesia during the 5th week after D6 cell inoculation. A combination of xylazine (Rometar 2%; Spofa a.s., Prague, Czech Republic; diluted 1:19 with physiological solution, used 0.2 ml per rat) and ketamine (Narketan 10%; Spofa a.s.; at 0.3 ml per rat) was applied intramuscularly as anaesthetic. After the excision of tumor, Vetalgin (Intervet International GmbH, Unterschleißheim, Germany; diluted 1:4 with physiological solution, used 0.1 ml per rat) and Pendepon Compositum [Biotika, a.s., Slovenská Ľupča, Slovak Republic; 0.1 ml (30,000 IU) per rat] were injected intramuscularly as analgesic and antibiotic, respectively.

Haematological parameters. Peripheral blood was taken from the tail vein into K3-EDTA tubes (Vacuette, Kremsmünster, Austria), just before inoculation of D6 cells, and at regular time intervals (once a week) during tumor progression, after excision of tumor and after the second inoculation of D6 cells until the end of experiment. Blood was diluted 1:1 with physiological solution and basic haematological parameters, namely the number of leukocytes, platelets and erythrocytes; haemoglobin concentration; and haematocrit, was assessed using an ABC Vet analyser (ABX Hematology, Montpellier, France).

Blood smear staining. Blood smears were stained by applying May-Grünwald solution (3 min). Then slides were washed briefly with distilled water and Giemsa-Romanovski solution (diluted 1:40 with distilled water) was applied (30 min). The stained blood smears were washed with tap water and dried at room temperature. This staining was done to assess which population(s) is/are responsible for increase in white blood cell (WBC) number.

Detection of immune cell proportions by flow cytometry. Peripheral blood was also used to measure the proportions of different immune cell types by flow cytometry. Lysed blood (EasyLyse; Dako, Glostrup, Denmark) was washed twice (5% foetal bovine serum in PBS with 0.1% aside). Cell suspension was stained (4˚C, 30 min) with mixtures of two labelled mouse anti-rat antibodies (anti-cluster of differentiation (CD)11b/fluorescein isothiocyanate (FITC) plus anti-CD4/phycoerythrin (PE); anti-CD11b/FITC plus anti-CD8/PE; anti-CD11b/FITC plus anti-major histocompatibility complex (MHC) class II RT1B/PE) for detection of T-lymphocytes ($CD4^+$ cells, $CD8^+$ cells), MHC II^+ cells (MHC II^+ $RT1B^+$ cells) and $CD11b^+$ ($CD11b^+$ cells) followed by the addition of propidium iodide to the cell suspension for detection of dead cells. The antibody to CD11b/FITC was diluted 1:300, all other antibodies were diluted 1:30 with washing solution (all antibodies used were produced by (AbD Serotec, Kidlington, UK). The stained cell suspensions were analysed by FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (Tree Star; Ashland, Oregon, USA) to assess the proportions of different lymphocyte subpopulations and granulocytes.

Immunohistochemistry. Tissue samples were taken from the excised tumors, frozen in liquid nitrogen and stored at –80˚C. Serial cryosections (8 μm) were prepared using a Leica CM 1850 cryostat (Leica Instruments GmbH, Wetzlar, Germany) to study distribution and localization of $CD11b^+$ cells using immunofluorescence technique. Cryosections were fixed with cooled acetone (–20˚C, 15 min) followed by washing with PBS (three times, 5 min each). Blocking of non-specific staining was achieved by incubation with 10% rat serum (1 h). FITC-conjugated mouse anti-rat CD11b antibody (AbD Serotec) diluted 1:100 with 10% goat serum was applied to the sections in a refrigerator overnight. Thereafter, tissue sections were washed with 0.2% bovine serum albumin (BSA) in PBS (three times, 5 min each) followed by nuclei staining for 10 min with diluted (1:500 with PBS) 4', 6-diamidino-2-phenylindole (DAPI). Finally, sections were washed with PBS and then briefly with distilled water. The stained sections were embedded in Mowiol

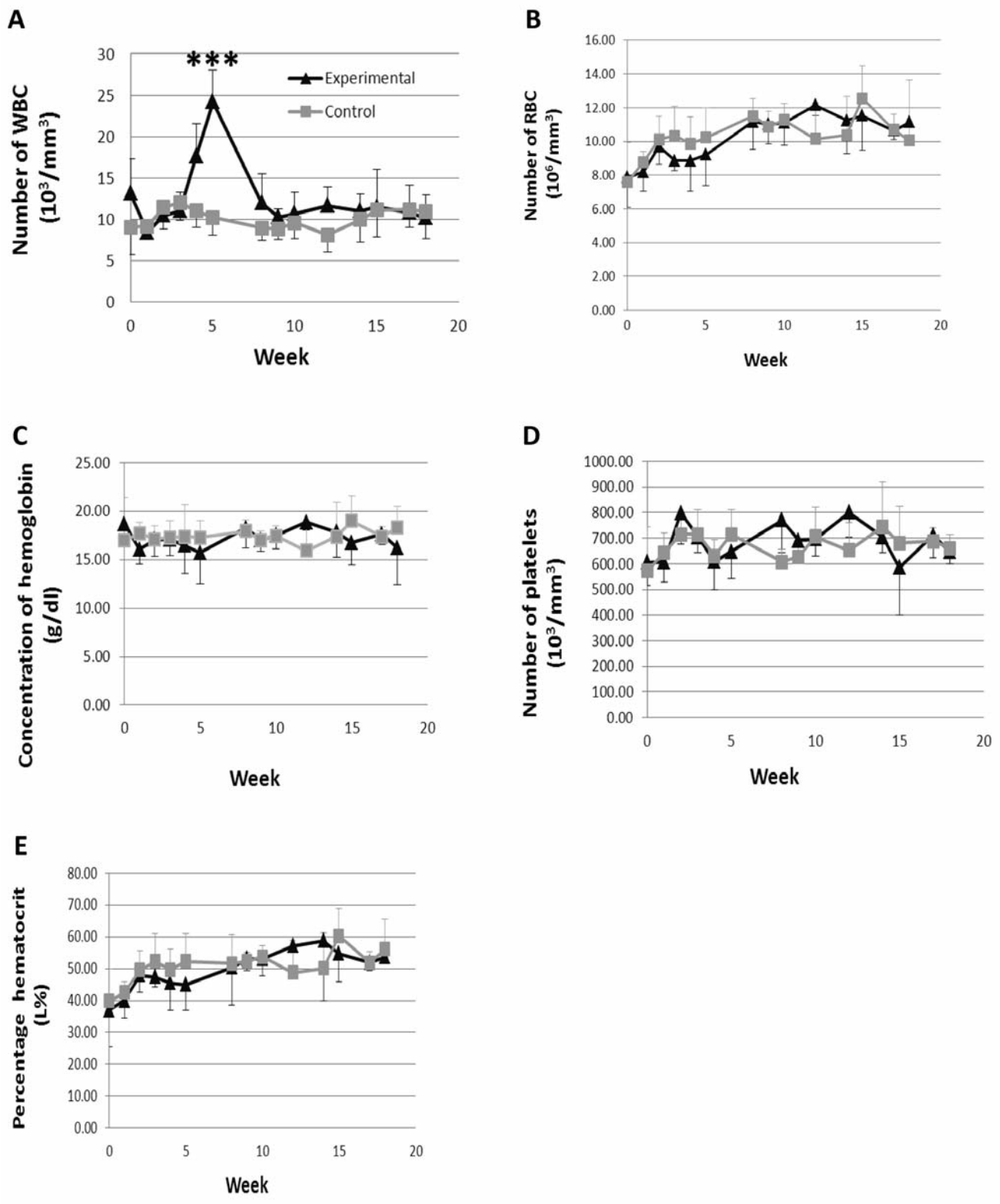

Figure 1. *Changes of haematological parameters with growing tumor and after its subsequent excision. Basic haematological parameters were measured during this experiment. Subcutaneous inoculation of D6 sarcoma cells took place at week 0 and week 11; tumor excision was carried out at week 5. In experimental rats, a very significant increase in white blood cells (WBC) number (A) was observed at the 5th week (p=0.001) compared to control rats. Other measured parameters did not show any significant changes.*

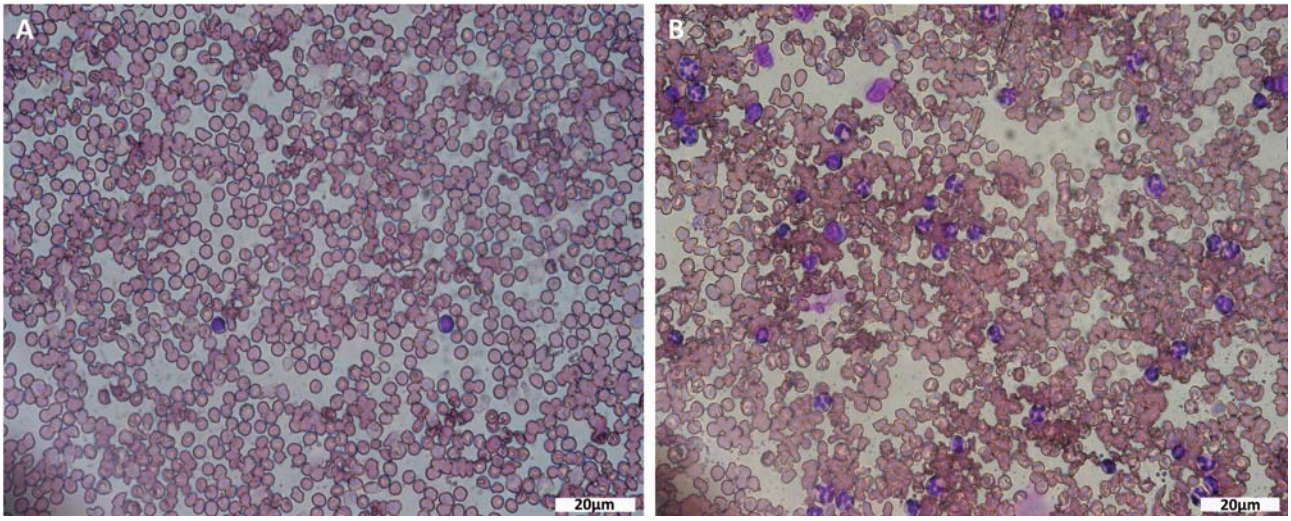

Figure 2. *Haematoxylin-eosin staining of blood smears. As compared to control animals (A), a very high number of neutrophil granulocytes (with segmented nuclei) were observed in peripheral blood of experimental animals (B) at the time of tumor excision (i.e. 5th week after D6 cell inoculation).*

(prepared according to the technical datasheet no. 777; Polysciences, Inc., Warrington, PA, USA) with n-propyl gallate (at a final concentration of 5 mg/ml; Sigma-Aldrich) as an anti-fading reagent. A BX51 fluorescence microscope (Olympus, Tokyo, Japan) with an Infinity 2 CCD Monochrome Microscopy Camera (Lumenera Corp., Ottawa, ON, Canada) and QuickPhoto Micro 2.3 software (Promicra s.r.o., Prague, Czech Republic) in pseudo-colour mode were used to observe distribution and localization of $CD11b^+$ cells in the stained tissue section.

Cytokine study. Rat serum isolated from peripheral blood was stored at –80˚C to study different protein molecules including cytokines. Protein profiling was performed to detect ICAM-1, IFNγ, IL-1β, IL-6, IL-10, leptin, L-selectin, CCL2, tissue inhibitor of metalloproteinase-1 (TIMP-1) and tumor necrosis factor-α (TNF-α) by quantibody array (Quantibody® Rat Cytokine Array 2; Ray Biotech Inc., Norcross, GA USA). The technique utilized for quantification of the 10 protein molecules was applied according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was carried out using software package NCSS 2001 (NCSS, Keysville, UT, USA). After normality testing, for non-normally distributed data the non-parametric Mann-Whitney *U*-test was used for evaluation of differences among the experimental and control groups in all studied parameters. Wilcoxon signed-rank test was used for pairwise comparisons at different time points. Differences were considered significant at $p \leq 0.05$.

Results

Characterization of subcutaneous tumors after inoculation of D6 sarcoma cells. Subcutaneous tumor started to appear macroscopically during the third week after D6 cell inoculation. Tumors were progressive and grew very fast after their appearance. They were freely movable at the time of excision. The average tumor size at the time of excision was 13,940.5 mm^3 (SD±4,189.2 mm^3). The tumors were encapsulated and the capsule was highly vascularized. Almost all excised tumors contained a necrotic area and most of the tumors had an internal cavity which was associated with internal haemorrhage. Since tumors of D6 sarcoma cells grow very rapidly after their appearance, three experimental rats exhibited penetration of subcutaneous tumors into the abdominal cavity so that it was impossible to remove the whole tumor. Two experimental rats died shortly after tumor excision due to postoperative complications.

In order to determine whether primary tumor can induce antitumor immunity, we again subcutaneously inoculated D6 sarcoma cells into the tumor-resected animals. The important finding was that no tumor appeared until eight weeks (*i.e.* experiment termination time) after the second inoculation of D6 cell in seven experimental rats.

Leukocytosis with growing tumor. In experimental animals, among all analyzed haematological parameters, only the WBC population was found to increase with growing tumor size as compared with control rats. The most significant changes were observed at the 5th week ($p=0.001$). Moreover, significant differences were also observed at different time intervals between the experimental animals (Table I). After tumor excision, the WBC count became almost normal in the subsequent three weeks. No significant changes were found in WBC count after the second inoculation of D6 sarcoma cells (Figure 1). No other measured parameter exhibited any significant change and their values were stable throughout the experiment with negligible changes.

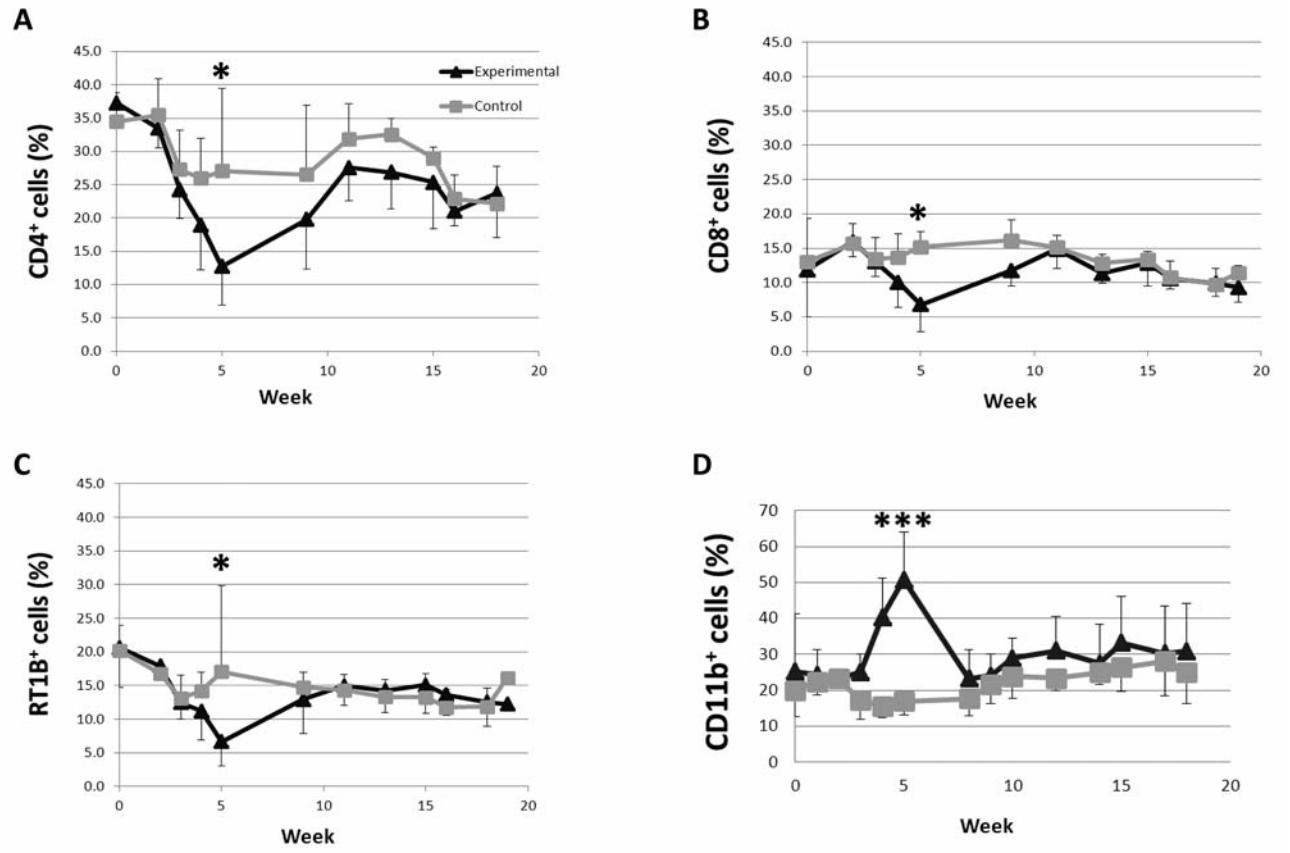

Figure 3. *Changes in leucocyte sub-populations with growing tumor and after its subsequent excision. The percentage of T-lymphocytes cluster of differentiation 4 ($CD4^+$), $CD8^+$, MHC II^+ cells, and $CD11b^+$ cells in peripheral blood of experimental (black) and control rats (grey) were assessed by flow cytometry. Subcutaneous inoculation of D6 sarcoma cells took place at week 0 and week 11; tumor excision was carried out at week 5. Differences between experimental and control rats were significant at ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$.*

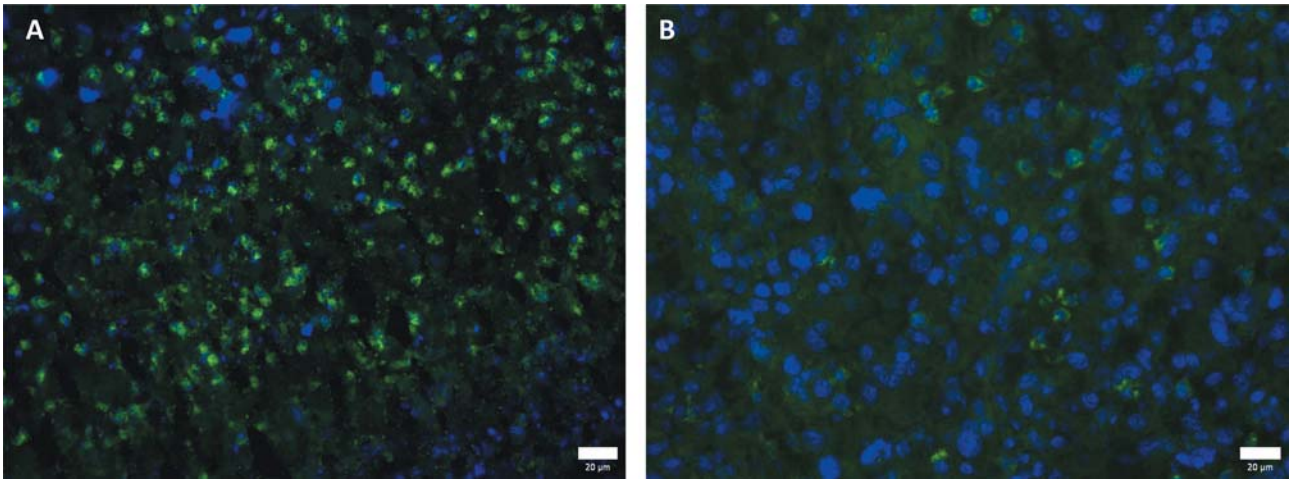

Figure 4. *Infiltration of cluster of differentiation 11b ($CD11b^+$) cells in tumor tissue. The immunohistochemical analysis of tumor cryosections showed very high infiltration with $CD11b^+$ cells (green) which were accumulated mainly in necrotic areas (A). On the contrary, a low number of these cells were evenly dispersed in the healthy, non-necrotic tumor tissue (B). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue).*

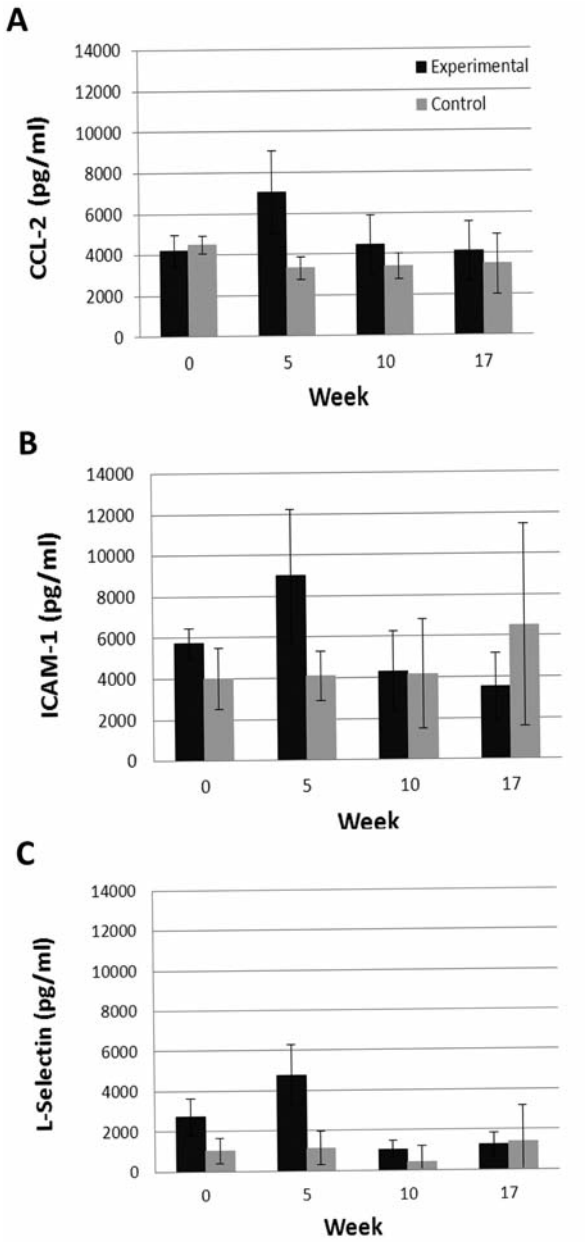

Figure 5. *Cytokine analysis by quantibody array. Among all 10 analyzed proteins, the concentrations of chemokine (c-c- motif) ligand 2 (CCL2) (A), intracellular adhesion molecule-1 (ICAM-1) (B) and L-selectin (C) showed clear increase which correlated with tumor growth. After tumor excision at the 5th week, their levels returned almost to the pre-inoculation values (week 0).*

Blood smear staining was carried-out to detect which population of WBC is responsible for the high increase in this cell type (ascertained by haematological analyser) with growth of tumor. It was observed that the number of neutrophil granulocytes (with segmented nuclei) in the peripheral blood of experimental rats greatly increased with increasing tumor size (Figure 2). After tumor excision, the number of neutrophil granulocytes returned to a normal low level found before the first inoculation of D6 cells.

Dynamics of leukocyte sub-populations during tumor progression and after tumor excision. $CD4^+$ and $CD8^+$ T-lymphocytes, MHC II^+ cells and $CD11b^+$ cells in peripheral blood were monitored by flow cytometry at weekly intervals. It was found that the proportion of $CD4^+$, $CD8^+$ and MHC II^+ cells significantly decreased with growing tumor size (Figure 3A, B, C). At the time of tumor excision (week 5), the proportions of these cell types were statistically lower ($p \leq 0.05$) in the experimental group than in the control group. After tumor excision, the proportions of all three of these lymphocyte sub-populations returned to the pre-inoculation values. There were no changes found in the proportion of these cell types after the second inoculation of D6 cells. Significant differences in the proportion of $CD4^+$, $CD8^+$ and MHC II^+ cells (as well as $CD11b^+$ cells) between various time points in experimental animals are summarized in Table I.

On the contrary, the proportion of $CD11b^+$ cells exhibited a different trend from that of other immune cells whereby an increase was observed with the growth of tumor. After tumor excision, the proportion of $CD11b^+$ cells became almost normal and it did not change after the second application of D6 sarcoma cells (Figure 3D). There was a significant difference ($p=0.001$) in the proportion of $CD11b^+$ cells between experimental and control animals just before tumor excision. Immunohistochemical analysis showed that $CD11b^+$ cells were unevenly distributed in the excised tumors. Very dense accumulation of these cells was found in necrotic areas with damaged cell structure (Figure 4A). On the contrary, in normal tumor tissue, $CD11b^+$ cells were sparsely dispersed (Figure 4B).

Changes in CCL2, L-selectin and ICAM-1 concentration. Our objective was to determine which cytokines or chemokines as well as soluble adhesion molecules were involved in tumor progression and to study their behaviour after subsequent tumor excision and after second inoculation of D6 cells. Assays of these molecules were performed on blood serum at the chosen time schedule. Concentrations of CCL2, ICAM-1 and L-selectin clearly increased with growing tumor size. After tumor excision at the 5th week, concentrations of these protein molecules returned to the normal, lower pre-inoculation levels. Concentrations were maintained at these values even after the second inoculation of D6 cells performed in the 11th week (Figure 5). The observed changes were significant for CCL2 and ICAM-1 between week 0 and week 5 ($p=0.05$) and for L-selectin between week 5 and week 10 ($p=0.02$). Concentrations of the other seven cytokines analyzed were at or below the limit of detection of the quantibody array kit.

Table I. *Statistical differences between studied parameters at different time points. Statistical analysis was carried-out by software package NCSS 2001 (NCSS, Keysville, UT, USA) to calculate significant differences between studied parameters. All significant differences shown are between experimental animals for different time points, except for the last column (5th week) that shows significant differences between experimental and control animals.*

	Experimental week						
Cells	0-3	0-5	3-5	5-13	5-18	9-13	5th
$CD4^+$	<0.01	<0.001	<0.01	<0.01	<0.01	<0.01	<0.05
$CD8^+$	<0.05	0.06	0.01	<0.05	<0.05	NS	<0.05
MHC II^+	<0.01	<0.001	<0.01	<0.05	<0.05	NS	<0.05
CD11b+	NS	<0.05	<0.05	<0.05	<0.05	NS	0.001
WBC	NS	0.01	0.01	0.01	0.01	NS	0.001

NS: Non-significant difference; WBC: white blood cells ; CD: cluster of differentiation ; MHC II^+: major histocompatibility complex II

Discussion

CD11b is one of the surface markers of myeloid cells such as neutophils, monocytes and dendritic cells. Some evidence suggests that neutrophils play an important role in carcinogenesis. They exhibit high functional plasticity and can develop pro-tumor and antitumor activity (18). As far as the pro-tumor function, neutrophils are directly involved in tumor progression, metastasis and angiogenesis (19, 20). Hypoxic conditions may appear during tumor growth. Myeloid cells migrate into the hypoxic areas of the tumor where they help in neovascularisation that is urgently needed for tumor cell survival (21, 22). Angiogenesis supported by neutrophils is mediated by secretion of vascular endothelial growth factor (VEGF), macrophage inflammatory protein-1α (MIP-1α), and MIP-2 (23). In the present study, we observed increasing percentage of $CD11b^+$ cells in the peripheral blood with the growth of tumor. We suppose that these $CD11b^+$ cells are neutrophils as we also found an increasing number of neutrophils with segmented nuclei in blood smears with tumor growth. The excised tumors were well-vascularized on the surface only and variable necrotic areas were found inside. The reason for this feature could be a shortage of oxygen *i.e.* hypoxia, due to fast tumor growth. Immunohistochemistry of tumor sections revealed a markedly uneven distribution of $CD11b^+$ myeloid cells. They were highly concentrated in necrotic areas and on the border between necrotic and normal tumor tissue. On the contrary, normal tumor tissue was only very slightly infiltrated with $CD11b^+$ cells. The increasing number of $CD11b^+$ cells in blood was probably associated with their pro-tumor function. These cells infiltrated into the subcutaneous rat sarcomas where they secreted positive signal(s) for the promotion of angiogenesis and tumor growth.

Tumor infiltration with myeloid cells is directed by C-C chemokines which may be secreted by tumor cells (24). CCL2 is a potent chemotactic factor for myeloid cells (10). In many cancer types (*e.g.* in prostate, breast, colorectal and Lewis lung carcinomas and melanoma), various ligands for CCL2 are produced that attract myeloid cells and maintain their suppressive activity (25, 26). CCL2 also participates in tumor neovascularisation by affecting macrophage infiltration (27, 28). In the Lewis rat sarcoma model, our observation showing a significant increase in the serum concentration of CCL2 at the time of tumor excision in comparison with the pre-inoculation level indicates that tumor infiltration by $CD11b^+$ myeloid cells was mediated by CCL2. Moreover, CCL2 also promoted survival of $CD11b^+$ myeloid cells in peripheral blood (29). Similarly, it is possible that in our study the increase in percentage of $CD11b^+$ cells was promoted by increased CCL2 concentration.

Apart from recruiting and directing leukocyte movement, several lines of evidence indicate that CCL2 might influence T-cell immunity. Impaired cell-mediated immunity associated with a switch of T-helper cells from Th1 to Th2 phenotype was observed in advanced cancer. The Th2 response is immunosuppressive and leads to tumor development. The polarization of Th2 response is mediated by various cytokines, including CCL2. CCL2 enhances IL-4 secretion by T-cells indirectly or directly by activating IL-4 promoters that help in the development and maintenance of Th2 response. This leads to immune suppression and development of tumor. A high level of CCL2 increases Th2 response. In contrast to the other chemokines of the C-C family, which trigger the Th1 phenotype upon their interaction with chemokine (c-c motif) ligand 5 on T-helper cells, CCL2 acts as a potent factor in the polarization of Th0 cells toward the Th2 phenotype (10, 30). It was observed *in vitro* that the parental R5-28 sarcoma cell line (15), as well as its D6 clone (16), secreted a high concentration of CCL2 into cell culture medium. Using D6 cells in the Lewis rat model *in vivo*, we found that subcutaneous sarcomas developing from the cancer cell inoculation rapidly grew. The reason for this tumor progression could be immune suppression that may be supported by an increasing concentration of serum CCL2.

With growing tumor size, we also found increasing concentration of two adhesion molecules, L-selectin and ICAM-1, in serum. Based on currently available data, soluble adhesion molecules contribute to cancer progression (31). Therefore they should not be regarded as unrelated and non-functional by-standers in tumor progression. The significant increase in serum L-selectin concentration was detected in patients suffering from non-Hodgkin's lymphoma (32). On the contrary, concentration of serum L-selectin significantly decreased in patients with malignant melanoma and the serum ICAM-1 concentration significantly increased with disease

progression (12). Immunologically, soluble adhesion molecules not only block their counter ligands on immune-competent cells, but also allow tumor cells to escape from surveillance by cytotoxic T-cells and natural killer cells (33). We suppose that the increase of L-selectin and ICAM-1 in serum is directly associated with tumor progression in our rat sarcoma model.

Effective $CD8^+$ T-cell-mediated cytotoxic killing may play a crucial role in the control of cancer development. However, as has been reported in patients with lung cancer, colorectal cancer and melanoma, the number and particularly the function of $CD8^+$ cells were limited due to the tumor milieu (34-36). Using flow cytometry, we observed a clear decline in the population of $CD8^+$ cells with tumor growth and their return to normal levels after tumor excision. This phenomenon has also been observed in patients with pancreatic cancer, in which both the number and function of $CD8^+$ T-cells decreased when compared with healthy donors. After tumor resection, $CD8^+$ T-cell numbers increased significantly (37). To establish effective immune response against tumor cells, the activation of various immune cell types, including $CD8^+$, $CD4^+$ and MHC II^+ cells, is necessary (38-40). Since we observed a decreased proportion of these cell types, this suggests that the tumor growth in our Lewis rat sarcoma model is associated with broad immunosuppression. The given outcome regarding the effect of primary tumor excision in our model can be further used for study of this issue at the molecular level.

In the present study, we characterized changes in the different immune cell subpopulations as well as in the concentration of the CCL2 and some adhesion molecules during tumor progression and after tumor excision followed by second inoculation of D6 rat sarcoma cells. We conclude that an increase in the population of $CD11b^+$ cells, along with alterations in CCL2 and the adhesion molecules L-selectin and ICAM-1, could play an important role in tumor progression. Moreover, failure of tumor development after second inoculation provides a clue towards induction of antitumor immune memory caused by the primary tumor.

Acknowledgements

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References

1 Okamoto R, Ueno M, Yamada Y, Takahashi N, Sano H, Suda T and Takakura N: Hematopoietic cells regulate the angiogenic switch during tumorigenesis. Blood *7*: 2757-2763, 2005.

2 De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M and Naldini L: Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell *3*: 211-226, 2005.

3 Nozawa H, Chiu C and Hanahan D: Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci USA *33*: 12493-12498, 2006.

4 Takakura N: Role of hematopoietic lineage cells as accessory components in blood vessel formation. Cancer Sci *7*: 568-574, 2006.

5 Prado-García H, Avila-Moreno F and López-González JS: Cytotoxic T-lymphocytes in cancer and autoimmunity. Rev Invest Clin *5*: 629-939, 2004.

6 Caserta S, Borger JG and Zamoyska R: Central and effector memory CD4 and CD8 T-cell responses to tumor-associated antigens. Crit Rev Immunol *2*: 97-126, 2012.

7 Lakshmi Narendra B, Eshvendar Reddy K, Shantikumar S and Ramakrishna S: Immune system: a double-edged sword in cancer. Inflamm Res 9: 823-834, 2013.

8 Toomer KH and Chen Z: Autoimmunity as a double agent in tumor killing and cancer promotion. Front Immunol *5*: 116, 2014.

9 Conti I and Rollins BJ: CCL2 (monocyte chemoattractant protein-1) and cancer. Semin Cancer Biol *3*: 149-154, 2004.

10 Deshmane SL, Kremlev S, Amini S and Sawaya BE: Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res *6*: 313-326, 2009.

11 Nakasone Y, Fujimoto M, Matsushita T, Hamaguchi Y, Huu DL, Yanaba M, Sato S, Takehara K and Hasegawa M: Host-derived MCP-1 and MIP-1α regulate protective antitumor immunity to localized and metastatic B16 melanoma. Am J Pathol *1*: 365-374, 2012.

12 Yamada M, Yanaba K, Takehara K and Sato S: Clinical significance of serum levels of soluble intercellular adhesion molecule-1 and soluble L-selectin in malignant melanoma. Arch Dermatol Res *6*: 256-260, 2005.

13 Dymicka-Piekarska V, Guzinska-Ustymowicz K, Kuklinski A and Kemona H: Prognostic significance of adhesion molecules (sICAM-1, sVCAM-1) and VEGF in colorectal cancer patients. Thromb Res *4*: 47-50, 2012.

14 Morávková A, Málek O, Pokorná E, Strnádel J, Hradecký, J and Horák V: Immune characterization of Lewis rats inoculated with K2 sarcoma cell line and newly derived R5-28 malignant cells. Folia Biol-Prague *6*: 159-165, 2005.

15 Strnádel J, Kverka M, Horák V, Vannucci L, Usvald D, Hlučilová J, Plánská D, Váňa P, Reisnerová H, and Jílek F: Multiplex analysis of cytokines involved in tumour growth and spontaneous regression in a rat sarcoma model. Folia Biol-Prague *6*: 216-219, 2007.

16 Holubova M, Leba M, Sedmikova M, Vannucci L and Horak V: Characterization of three newly established rat sarcoma cell clones. In Vitro Cell Dev Biol Animal *10*: 610-618, 2012.

17 Euhus DM, Hudd C, LaRegina MC and Johnson FE: Tumor measurement in the nude mouse. J Surg Oncol *4*: 229-234, 1986.

18 Brandau S, Moses K and Lang S: The kinship of neutrophils and granulocytic myeloid-derived suppressor cells in cancer: Cousins, siblings or twins? Semin Cancer Biol *23*: 171-182, 2013.

19 Gregory AD and Houghton AM: Tumor-associated neutrophils: new targets for cancer therapy. Cancer Res 7: 2411-2416, 2011.

20 Cools-Lartigue J, Spicer J, Najmeh S and Ferri L: Neutrophil extracellular traps in cancer progression. Cell Mol Life Sci *21*: 4179-4194, 2014.

21 Lee HW, Choi HJ, Ha SJ, Lee KT and Kwon YG: Recruitment of monocytes/macrophages in different tumor microenvironments. Biochim Biophys Acta *2*: 170-179, 2013.
22 Zhu G, Tang Y, Geng N, Zheng M, Jiang J, Li L, Li K, Lei Z, Chen W, Fan Y, Ma X, Li L, Wang X and Liang X: HIF-α/MIF and NF-ϰB/IL-6 axes contribute to the recruitment of $CD11b^+Gr\text{-}1^+$ myeloid cells in hypoxic microenvironment of HNSCC. Neoplasia *2*: 168-179, 2014.
23 Gong Y and Koh DR: Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an *in vivo* corneal model. Cell Tissue Res *2*: 437-448, 2010.
24 López-Lago MA, Posner S, Thodima VJ, Molina AM, Motzer RJ and Chaganti RS: Neutrophil chemokines secreted by tumor cells mount a lung antimetastatic response during renal cell carcinoma progression. Oncogene *14*: 1752-1760, 2013.
25 Zollo M, Di Dato V, Spano D, De Martino D, Liguori L, Marino N, Vastolo V, Navas L, Garrone B, Mangano G, Biondi G and Guglielmotti A: Targeting monocyte chemotactic protein-1 synthesis with bindarit induces tumor regression in prostate and breast cancer animal models. Clin Exp Metastasis *6*: 585-601, 2012.
26 Boelte KC, Gordy LE, Joyce S, Thompson MA, Yang L and Lin PC: Rgs2 mediates pro-angiogenic function of myeloid derived suppressor cells in the tumor microenvironment via up-regulation of MCP-1. PLoS One *6*: e18534. doi: 10.1371/journal.pone.0018534, 2011.
27 Wang S, Xu M, Li F, Wang X, Bower KA, Frank JA, Lu Y, Chen G, Zhang Z, Ke Z, Shi X and Luo J: Ethanol promotes mammary tumor growth and angiogenesis: the involvement of chemoattractant factor MCP-1. Breast Cancer Res Treat *3*: 1037-1048, 2012.
28 Arendt LM, McCready J, Keller PJ, Baker DD, Naber SP, Seewaldt V and Kuperwasser C: Obesity promotes breast cancer by CCL2-mediated macrophage recruitment and angiogenesis. Cancer Res *19*: 6080-6093, 2013.
29 Roca H, Varsos ZS, Sud S, Craig MJ, Ying C and Pienta KJ: CCL2 and interleukin-6 promote survival of human $CD11b^+$ peripheral blood mononuclear cells and induce M2-type macrophage polarization. J Biol Chem *49*: 34342-34354, 2009.
30 deSchoolmeester ML, Little MC, Rollins BJ and Else KJ: Absence of CC chemokine ligand 2 results in an altered Th1/Th2 cytokine balance and failure to expel Trichuris muris infection. J Immunol *9*: 4693-4700, 2003.
31 van Kilsdonk JW, van Kempen LC, van Muijen GN, Ruiter DJ and Swart GW: Soluble adhesion molecules in human cancers: sources and fates. Eur J Cell Biol *6*: 415-427, 2010.
32 Haznedaroglu IC, Benekli M, Ozcebe O, Savaş MC, Güllü IH, Dündar SV and Kirazli S: Serum L-selectin and P-selectin levels in lymphomas. Haematologia (Budap) *1*: 27-30, 2000.
33 Liu CM, Sheen TS, Ko JY and Shun CT: Circulating intercellular adhesion molecule 1 (ICAM-1), E-selectin and vascular cell adhesion molecule 1 (VCAM-1) in head and neck cancer. Br J Cancer *2*: 360-362, 1999.
34 Rosenberg SA, Sherry RM, Morton KE, Scharfman WJ, Yang JC, Topalian SL, Royal RE, Kammula U, Restifo NP, Hughes MS, Schwartzentruber D, Berman DM, Schwarz SL, Ngo LT, Mavroukakis SA, White DE and Steinberg SM: Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific $CD8^+$ T-cells in patients with melanoma. J Immunol *9*: 6169-6176, 2005.
35 Harlin H, Kuna TV, Peterson AC, Meng Y and Gajewski TF: Tumor progression despite massive influx of activated CD8(+) T-cells in a patient with malignant melanoma ascites. Cancer Immunol Immunother *10*: 1185-1197, 2006.
36 Zhang Y, Huang S, Gong D, Qin Y and Shen Q: Programmed death-1 upregulation is correlated with dysfunction of tumor-infiltrating $CD8^+$ T-lymphocytes in human non-small cell lung cancer. Cell Mol Immunol *5*: 389-395, 2010.
37 Yu X, Ji S, Xu J, Yao W, Qu B, Zhu W, Xu W, Zhang B and Xu Y: $CD8^+$ T-cells are compromised in human pancreatic cancer. Transl Med *2*: 105. doi:10.4172/2161-1025.1000105, 2012.
38 van de Berg PJ, van Leeuwen EM, ten Berge IJ and van Lier R: Cytotoxic human CD4(+) T-cells. Curr Opin Immunol *3*: 339-343, 2008.
39 Melief CJ: "License to kill" reflects joint action of CD4 and CD8 T-cells. Clin Cancer Res *16*: 4295-4296, 2013.
40 Haabeth OA, Tveita AA, Fauskanger M, Schjesvold F, Lorvik KB, Hofgaard PO, Omholt H, Munthe LA, Dembic Z, Corthay A and Bogen B: How do CD4(+) T-cells detect and eliminate tumor cells that either lack or express MHC class II molecules? Front Immunol *5*:174, 2014.

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