Original Contribution

THE ROLE OF CD68 POSITIVE TAM IN DEVELOPMENT OF NON-SMALL CELL LUNG CANCER

Pl. Minkov[1*], M. Gulubova[1], E. Obretenov[2], J. Ananiev[1]

[1]Department of General and Clinical Pathology, Medical Faculty, Trakia University, Stara Zagora, Bulgaria
[2]Department of Thoracic Surgery, Medical Faculty, Trakia University, Stara Zagora, Bulgaria

ABSTRACT
Non-small cell lung cancer (NSCLC) is a leading cause of cancer related mortality. Main factors of development of NSCLC are many immune cells involved in tumor microenvironment. Infiltration with CD68 positive tumor associated macrophages (TAM) in the tumor stroma or in the tumor islets is associated with prognosis and stage of the disease for a long time. Investigating immunohistochemically 40 patients with NSCLC we discovered that 25% of patients with metastasis in lymph nodes had low infiltration with TAM in the tumor border, while 35.7% of the patients without metastasis- the infiltration was high ($x2=5.19$; $p=0.023$). We also discovered that all patients in stage I and 66.7% of the patients in stage II had low infiltration with TAM in the tumor border, while 100% of those in stage IV had high infiltration in the border ($x2=5.44$; $p=0.02$). In addition, we observed that 52.9% of the patients in T3-4 the infiltration of the tumor border with TAM was high, while only 16.7% of those in T1-2 had high infiltration ($x2=2.92$; $p=0.087$).
In investigated by our team lung cancers, clear associations were established between CD68 positive cells density in the tumor border and stage of the disease.

Keywords: NSCLC, TAM, CD68, prognosis

INTRODUCTION
Lung cancer is the most common cause for cancer related mortality. In more than 85% of the cases of lung cancer non-small cell lung cancer (NSCLC) is diagnosed (1). Many factors are involved in progression and development of the NSCLC like: genetics, factors of the environment, anti- tumor immunity, tumor microenvironment and others. The central place in the processes of tumorigenesis and anti- tumor immunity is taken by the tumor microenvironment. The escape from the immune response by the tumor is achieved in different ways, from escape of anti- tumor immunity to induction of immune tolerance, which both ease the tumor growth and progression. Main mechanisms are inhibition of the antigen presentation, inhibition of the anti- tumor immune cells, and active recruitment of immune cells by the tumor itself (2, 3).

_____

*Correspondence to:*Plamen Minkov, MD, Department of General and clinical pathology, Medical faculty, Trakia University Armeiska Str.11, Stara Zagora, 6000, E-mail: minkov_plamen@abv.bg*

The tumors are infiltrated by many different immune cells. The infiltration of the tumors with specific immune cells is associated with good as well as bad prognosis, depending on the type of the cells (4). The consideration, that the defining the type, concentration and localization of the different immune cells in the tumor microenvironment could be used as predictive biomarker for progression and prognosis of the cancer is known for a long time (5).

Key component of the tumor microenvironment are the tumor associated macrophages (TAM) (6, 7). This group of immune cells plays an important role as immune regulator in the tumor microenvironment and is also potential target for anti- cancer therapy (8). Tumor associated macrophages demonstrate as anti- cancerous, as well pro- cancerous abilities. Which one of their abilities will be demonstrated depends on their specific polarization. There are two types of TAM, M1 type as consequence of classically activated TAM and M2 type as consequence of alternative activation of TAM (9).

Macrophages polarized as M1 phenotype are also known as inhibitory type macrophages because of their stimulation of Th1 mediated immune response (10). Substances like interferon-γ, lipopolysaccharides, TNF-α and GM-CSF can induce M1 polarization and the end result is tumoricidal and bactericidal effect (9, 11, 12).

Macrophages polarized as M2 phenotype are also known as "heeling" type macrophages, because of their role in Th2 mediated immune response (10, 11). The M2 type macrophages are activated by substances like IL-4, IL-10, IL-13 and prostaglandin E2 (12-14). The activated M2 macrophages induce Th2 mediated immune response and as end result processes of tissue reparation, remodeling, angiogenesis, and tumor progression are implemented (12, 13, 15).

The dual nature of TAM in the processes of tumor progression and development is established in vitro as well as vivo studies in different types of cancer. In these studies many markers for qualification of different types of TAM were used (CD68, CD163, CD204, HLA-DR and others). It was also payed attention to the location and density of TAM in tumor border, in the tumor islets, or in both places (16-20).

The role of TAM in progression and development of NSCLC remains unclear because of some discrepancies between the results of the present studies. It is not fully determined the association between the degree of infiltration with TAM and the prognosis and stage of NSCLC.

We aimed to investigate the degree of infiltration with CD68 positive cells in a group of patients with NSCLC and to correlate the data with clinical- morphological and overall survival data.

MATERIALS AND METHODS
Biopsy materials taken from 40 patients after lung resection in Clinic of Thoracic surgery of University Hospital in Stara Zagora were investigated. The biopsies were made in the years between 2007 and 2017. The group is made up by 37 men and 3 women, the age diapason was between 24 and 77 years (mean 62.8 years). Retrospectively, clinical ad histological data was gained and are presented in **Table 1**.

Table.1.*Themainclinicalandhistologicalparameters of thepatients(n=40)*

Parameter	Number (%)
Clinicaldata	*(n=40)*

Gender
   Male
   Female
	
	37 (92.5)
	3 (7.5)

Age (years)
   median
   (range)
	
	62.8
	(24-77)

T stage
   T1-2
   T3-4
	
	6 (15)
	34 (85)

N stage
   N0
   N1-3
	
	12 (30)
	28 (70)

Metastases
   No
   Yes
	
	33 (82.5)
	7 (17.5)

Clinical stage I-II III-IV	 21 (52.5) 19 (47.5)
Hystological type AC SCC	 15 (37.5) 25 (62.5)

The biopsy tissue is investigated by routine histochemical methods and immunohisto-chemically. After fixation in 10% buffered formalin, the tissue was included in paraffin and was cut in 4μm than deparaffining was made. Next step was inhibition of endogenous peroxidase for 5 min with blocking reagent following protocol for immune histochemical

investigation with respective antibody. Next process was antigen revealing in buffer solution for 20 min in 95 'C. After that the cuts were washed 3 times in PBS and were incubated with primary antibody for an hour. This was followed by another 3 times washing and incubation for 20 to 40 min with detection system. At the end, after another wash up and incubation, twice for 5 min in DAB the probes were stained with Mayer's hematoxylin.

Immunohistochemical investigation was made with monoclonal mouse anti-human antibody CD68 (KP1, DAKO) in solution 1:100, and detection system EnVision™ FLEX+, Mouse, High pH, (Link) (K8002, DAKO).

Cell counting
The CD68 positive cells were reported in two locations: tumor border and tumor nests in so called *hot spots* of five fields of vision in magnification x320, 0.74mm^2. The degree of infiltration with cells was calculated on 1 mm^2.

Statistical analysis
The data of the investigations was statistically processed with SPSS for Windows Version 16 (SPSS, Inc., Chicago, IL, USA). For comparison of the frequency of the investigated parameters χ2-test was used. For tracing of the survival of the patients – Kaplain- Meier analysis was used, which find the differences between curves by log- rank test. All analyses considered for reliable had level of significance $p<0.05$.

RESULTS
After immunohistochemical analysis, the gained data was correlated with clinical and morphological characteristics of the patients.

For this purpose the patients were divided in four groups: with low or high degree of infiltration with TAM in the tumor nests or border. Statistically significant association was found according to number of the CD68 positive cells in the tumor nests, the presence of lymph metastasis (N) and clinical stage. A tendency according to degree of infiltration and tumor size (T) was also established.

In 25% of the patients with lymph node metastasis, low degree of TAM infiltration in the tumor border was observed, while in 35.7% of the patients without metastasis the infiltration of the border was high grade (x2=5.19; p=0.023). We also found that 100% of the patients diagnosed in clinical stage I and 66.7% of the patients in clinical stage II had low grade infiltration with TAM in the border, while in 100% of the patients in clinical stage IV were with high grade infiltration of the tumor border (x2=5.44; p=0.02).

In addition, in 52.9% of the patients in T3-4 the infiltration of the tumor border with TAM was high, compared with only 16.7% of the patients in T1-2 with high infiltration of the border (x2=2.92; p=0.087).

Available data for survival was found for 31 patients but after analysis, no statistically significant correlation with degree of infiltration was established (**Figures 1,** 2).

No others significant correlations were found in analysis of the distribution and the grade of infiltration of the investigated cells and others parameters.
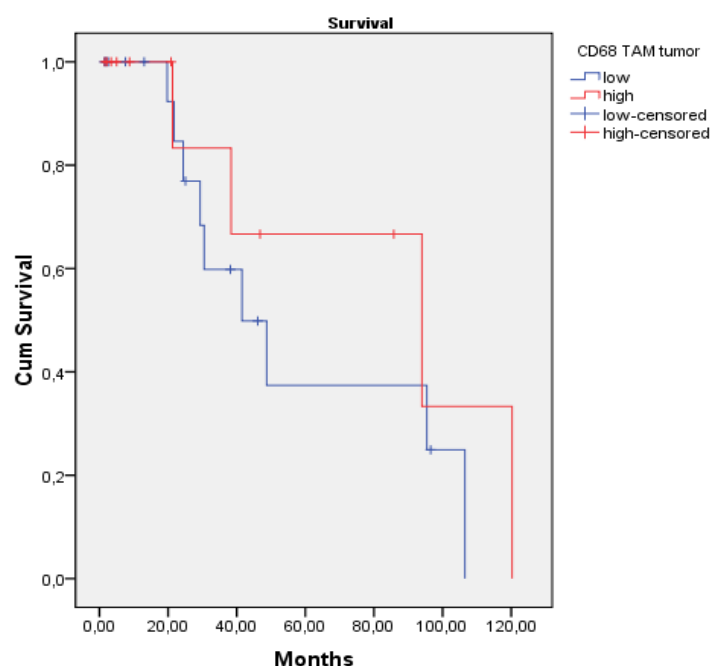
Figure 1. Associationbetweensurvivalanddegree of infiltrationwith CD68 intumornests.
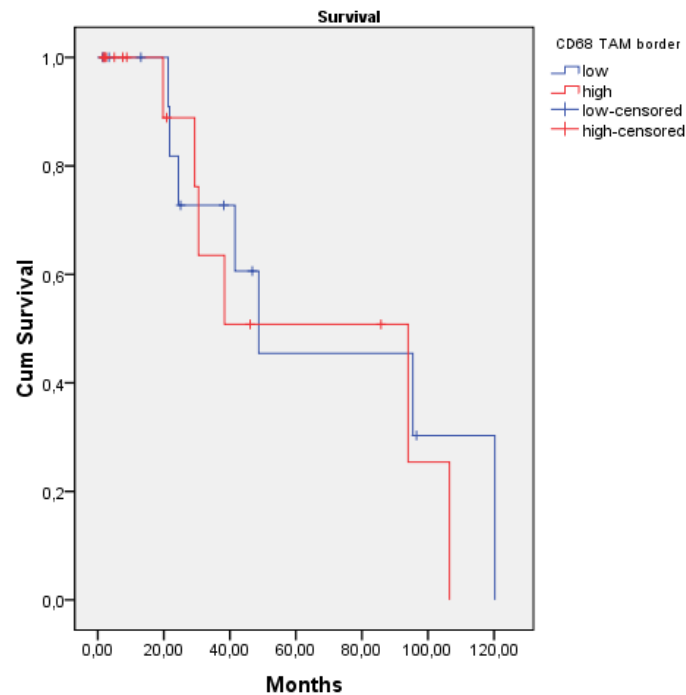
Figure 2. Associationbetweensurvivalanddegree of infiltrationwith CD68 intumorborder.

DISCUSSION

The most commonly used marker for investigation of TAM is CD68. In reviewing the available data in the literature, we found that in general there are nine studies which aimed to investigate the significance of the infiltration of NSCLC with CD68 positive cells, by paying attention of the distribution of the CD68 positive cells in the tumor border or islets (16, 19, 21- 27).

The sum of the infiltrating TAM in the tumor stroma and tumor nests fails to be associated with prognosis or severity of the disease in NSCLC, unlike in other tumors. Such association was missing after analysis of our results. Examined separately, the number of TAM infiltrating the tumor stroma and tumor nests respectively were established as informative about stage and prognosis of NSCLC. Having this in mind, in our study we divided the patients in categories according to the presence of low or high grade of infiltration with CD68 positive cells of tumor border or islets. The high grade infiltration of the tumor stroma with CD68 positive cells as well as low grade infiltration of the tumor islets indicates bad prognosis and advanced disease (19-22, 24, 26). All this data is confirmed by our results, where all patients with high degree of infiltration of the tumor border were in IV clinical stage. The presence of advanced disease could be explained by the different impact of CD68 positive cells depending on their distribution.

It is known that CD68 is a marker expressed on the surface of many different cells as monocytes, macrophages, fibroblasts, endothelial cells and even some cancer cells (28). Therefore, if CD68 is used as marker for detection of macrophages the results could be misleading.

Although, in our section was not found correlation between degree of infiltration in the tumor islets and prognosis or stage of disease, such correlation was observed in some studies. Patients with NSCLC and high grade infiltration with CD68 positive cells in tumor islets have better prognosis, and the CD68 positive cells were proven mostly as M1 type. Exactly the opposite, worse outcome is observed when the high grade of infiltration is found in the tumor stroma, where the dominant type of CD68 positive cells was M2 type. In support of this it was proven that 70% of TAM in the tumor islets is M1 type and also that TAM in tumor stroma most commonly are M2 type (20, 29). The difference in the distribution of M1 and M2 types of TAM is associated with prognosis and severity of disease and is totally correlating with their functions. The reverse polarization of CD68 positive TAM in tumor stroma or islets is definitive for pro- or anti- tumor capabilities that these cells realize depending on their localization.

Some studies investigated the tumor border/islet ratio of CD68 positive cells. The conclusions pointed that high border/stroma

236

ratio is associated with better prognosis (19, 23, 30, 31).

In conclusion, there is no established correlation between the total count of CD68 positive cells and histological type or TNM stage in patients with NSCLC. Lack of association is found between histological type and concentration of CD68 positive cells in the tumor islets, although intensively infiltrated tumors were mostly in III- IV clinical stage. High degree of infiltration in the tumor border with CD68 positive cells is definitively associated with low grade of differentiation and presence of lymph metastasis (16, 19, 23, 24, 32).

REFERENCES

1. Siegel RL, Miller KD, Jemal A. CancerStatistics, *CA Cancer J Clin.* 2017Jan;67(1):7-30, 2017.
2. Dunn GP, Old LJ, Schreiber RD.ThethreeEs of cancerimmunoediting. *Annu Rev Immunol.* 22:329-60, 2004.
3. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immuno selection and immunosubversion. *NatRevImmunol*. Oct;6(10):715-27, 2006.
4. Talmadge JE, Donkor M, Scholar E. Inflammatory cell infiltration of tumors:Jekyll or Hyde. *Cancer MetastasisRev.* Dec;26(3-4):373-400, 2007.
5. Pagès F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G, Lagorce C,Wind P, Marliot F, Bruneval P, Zatloukal K, Trajanoski Z, Berger A, Fridman WH,Galon J. Insitucytotoxicandmemory T cellspredictoutcomeinpatientswithearly-stagecolorectalcancer. *J ClinOncol.* Dec 10;27(35):5944-51, 2009.
6. Noy R, Pollard JW. Tumor-associatedmacrophages: frommechanisms to therapy. *Immunity.* Jul 17;41(1):49-61, 2014.
7. Chanmee T, Ontong P, Konno K, Itano N. Tumorassociatedmacrophagesasmajorplaye rsinthetumormicroenvironment. *Cancers (Basel).* Aug 13;6(3):1670-90, 2014.
8. Ruffell B, Coussens LM. Macrophagesandtherapeuticresistanceincan cer. *CancerCell.* 2015 Apr 13;27(4):462-72.
9. Mantovani A, Locati M. Tumor-associatedmacrophagesas a paradigm ofmacrophageplasticity, diversity, andpolarization: lessonsandopenquestions. *ArteriosclerThrombVascBiol*.Jul;33(7):1478-83, 2013.
10. Mills CD, Ley K. M1 and M2 macrophages: thechickenandtheegg of immunity. *JInnateImmun.* 6(6):716-26, 2014.
11. Mills CD. M1 and M2 Macrophages: Oracles of Health andDisease. *CritRevImmunol.* 32(6):463-88, 2012.
12. Biswas SK, Allavena P, Mantovani A. Tumor-associatedmacrophages: functionaldiversity, clinicalsignificance, andopenquestions. *SeminImmunopathol.* Sep;35(5):585-600, 2013.
13. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, BiswasSK, Allavena P, Mantovani A. Macrophage polarization in tumour progression. *SeminCancerBiol.* Oct;18(5):349-55, 2008.
14. Liu L, Ge D, Ma L, Mei J, Liu S, Zhang Q, Ren F, Liao H, Pu Q, Wang T, You Z. Interleukin-17 and prostaglandin E2 are involved in formation of an M2macrophage-dominan tmicroenviron-ment in lungcancer. *J ThoracOncol.* Jul;7(7):1091-100, 2012.
15. Guo C, Buranych A, Sarkar D, Fisher PB, Wang XY. Therole of tumor-associatedmacrophagesintumorvascularizati on. *VascCell.* Dec 6;5(1):20, 2013.
16. Pei BX, Sun BS, Zhang ZF, Wang AL, Ren P. Interstitialtumor-associatedmacro-phagescombinedwithtumor-derivedcolony-stimulating factor-1 andinterleukin-6, a novelprognosticbiomarkerinnon-smallcell-lungcancer. *JThoracCardiovascSurg.* Oct;148(4):1208-1216.e2, 2014.
17. Carus A, Ladekarl M, Hager H, Pilegaard H, Nielsen PS, Donskov F.Tumor-associatedneutrophilsandmacrophagesinnon-smallcelllungcancer: noimmediate-impactonpatientoutcome. *LungCancer.* Jul;81(1):130-7, 2013.
18. Hirayama S, Ishii G, Nagai K, Ono S, Kojima M, Yamauchi C, Aokage K, HishidaT, Yoshida J, Suzuki K, Ochiai A. Prognosticimpact of CD204-positive macrophagesinlungsquamouscellcarcinoma :possiblecontribution of Cd204-positivemacrophages to the tumor-promotingmicroenvironment. *J ThoracOncol.* Dec;7(12):1790-7, 2012.
19. Dai F, Liu L, Che G, Yu N, Pu Q, Zhang S, Ma J, Ma L, You Z. Thenumberandmicrolocalization of tumor-associatedimmunecellsareassociatedwithpat ient'ssurvivaltimeinnon-smallcell-lungcancer. *BMC Cancer.* May 20;10:220, 2010.
20. Ohri CM, Shikotra A, Green RH, Waller DA, Bradding P. MacrophageswithinNSCLCtumourisletsarepredominantly of a cytotoxic M1 phenotypeassociated-withextendedsurvival. *EurRespir J.* Jan;33(1):118-26, 2009.

21. Zhang B, Yao G, Zhang Y, Gao J, Yang B, Rao Z, Gao J. M2-polarizedtumor-associatedmacrophagesareassociatedwithpoorprognosesresultingfromacceleratedlymph angiogenesisinlungadenocarcinoma. *Clinics* (SaoPaulo. 66(11):1879-86, 2011.

22. daCostaSouza P, Parra ER, Atanazio MJ, daSilva OB, Noleto GS, Ab'Saber AM, deMoraisFernezlian S, Takagaki T, Capelozzi VL. Differentmorphology, stageandtreatmentaffectimmunecellinfiltrati onandlong-termoutcomeinpatientswithnon-small-celllungcarcinoma.*Histopathology.* Oct;61(4):587-96, 2012.

23. Feng PH, Yu CT, Wu CY, Lee MJ, Lee WH, Wang LS, Lin SM, Fu JF, Lee KY, Yen TH.Tumor-associatedmacro-phagesinstage IIIA pN2 non-smallcelllungcancerafterneoadjuvantchemot herapyandsurgery. *Am J TranslRes*. Oct 11;6(5):593-603.eCollection 2014, 2014.

24. Kim DW, Min HS, Lee KH, Kim YJ, Oh DY, Jeon YK, Lee SH, Im SA, Chung DH, KimYT, Kim TY, Bang YJ, Sung SW, Kim JH, Heo DS. High tumour islet macrophage infiltration correlates with improved patient survival but not with EGFR mutations, gene copy number or protein expression in resected non-small cell lung cancer. *Br J Cancer.* Mar 25;98(6):1118-24, 2008.

25. Chen JJ, Yao PL, Yuan A, Hong TM, Shun CT, Kuo ML, Lee YC, Yang PC.Up-regulation of tumor interleukin-8 expression by infiltrating macrophages: its correlation with tumor angiogenesis and patient survival in non-small cell lung cancer. *ClinCancerRes.* Feb;9(2):729-37, 2003.

26. Takanami I, Takeuchi K, Kodaira S. Tumor-associatedmacrophageinfiltration-inpulmonaryadenocarcinoma: associationwithangiogenesisandpoorprogno sis.*Oncology.* 57(2):138-42, 1999.

27. Eerola AK, Soini Y, Pääkkö P. Tumourinfiltratinglymphocytesinrelation totumourangiogenesis, apoptosisandprognosisinpatientswithlargece lllungcarcinoma. *LungCancer.* Nov; 26 (2): 73-83, 1999.

28. Gottfried E, Kunz-Schughart LA, Weber A, Rehli M, Peuker A, Müller A,Kastenberger M, Brockhoff G, Andreesen R, Kreutz M. Expression of CD68 innon-myeloidcelltypes. *Scand J Immunol.* May; 67(5):453-63, 2008.

29. Ma J, Liu L, Che G, Yu N, Dai F, You Z. The M1 form of tumor-associatedmacrophagesinnonsmallcelllung-cancerispositivelyassociatedwithsurvivaltim e. *BMC Cancer.* Mar 25;10:112, 2010.

30. Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, Aokage K, Saijo N,Nishiwaki Y, Gemma A, Kudoh S, Ochiai A. Predominantinfiltration of macrophagesand CD8(+) T Cellsin-cancernestsis a significantpredictor of survivalinstage IV nonsmallcelllungcancer. *Cancer.* Sep15;113(6):1387-95, 2008.

31. Cota GF, deSousa MR, Fereguetti TO, Rabello A. Efficacy of anti-leishmaniatherapyinvisceralleishmaniasisa mong HIV infectedpatients: a systematicreviewwithindirectcomparison. *PLoSNeglTropDis.* May 2;7(5):e2195, 2013

32. Welsh TJ, Green RH, Richardson D, Waller DA, O'Byrne KJ, Bradding P.Macrophageandmast-cellinvasion of tumorcellisletsconfers a markedsurvival-advantageinnon-small-celllungcancer. *JClin Oncol.* Dec10;23(35):8959-67, 2005.