Peer Review File

Article Information: http://dx.doi.org/10.21037/tlcr-20-1241

Reviewer A

1. Please discuss the effect of expression of PD-1 or PD-L1 in tumor-associated macrophages and the possible of of anti-PD-1 or anti-PD-L1 in TAM. There are many articles in this field, like
PD-1 expression by tumor-associated macrophages inhibits phagocytosis and tumor immunity (Nature. 2017 May 25; 545(7655): 495–499.)
Please include these articles.

Reply 1: Dear reviewer, many thanks for your helpful comment. A section discussing the role of PD1/PDL1 pathway is added to the manuscript.

Changes in the text:

#PD1/PDL1 immune checkpoints:

Programmed Cell Death Protein 1 (PD1)/PD1 Ligand (PDL) pathway plays an important part in establishing an immunosuppressive environment in the tumor and the evasion of cancer cells from anti-cancer immune responses. Of relevant clinical importance, PD1/PDL1 inhibition using blocking antibodies is being used for the treatment of many cancer types including melanoma, renal cell carcinoma, NSCLC, SCLC, squamous cell skin cancer, and triple-negative breast cancer (129). Tumor-infiltrating macrophages may express both PD1 and PDL1 (130). The anti-tumor effects of PD1 and PDL1 blocking antibodies were reversed following TAM depletion by anti-CSF1R antibody in a mouse model of colon cancer (CT26 cell line) (131); Hence, TAMs are significantly involved in the immunosuppressive potentials of PD1/PDL1 pathway in cancerous microenvironment. The majority of $PD1^+$ tumor macrophages display M2 phenotype. $PD1^+$ macrophages show defective phagocytic function in comparison with $PD1^-$ macrophages (131). Interferon-Sensitive Responsive Element (ISRE), STAT1, and STAT2 are pivotal for PD-1 expression in TAM (132).

TAMs are the most abundant $PDL1^+$ immune cells in human NSCLC stroma (133). While PDL1 is mostly recognized for its inhibitory effects on T cells through interacting with PD-1 (129), it seems that the main function of PDL1 in TAM is skewing macrophage polarization towards an immunosuppressive state. There is evidence that PDL1 affects TAM function through downregulating Akt/mTOR pathway (134). Additionally, TAMs that cross-present cancer antigen can evade immune destruction by T cells in a PDL1 dependent manner (135). Pyruvate Kinase (PK) M2 isoform and

Secreted Phosphoprotein 1 (SPP1) are the intracellular regulators of PDL1 expression in TAM (136, 137). To sum up, PD1 and PDL1 proteins are involved in regulating macrophage polarization.

PD1/PDL1 pathway has clinical implications in NSCLC. PDL1 expression, both in cancer cells and TAMs, was inversely associated with survival in early-stage NSCLC (138). The high density of $PDL1^+$ TAM in tumor tissue predicted better survival in NSCLC patients who were treated with PD1/PDL1 inhibitors (133). PD1/PDL1 immune checkpoint inhibitors such as Pembrolizumab, Atezolizumab, and Nivolumab, either as single-agent or in combination with chemotherapy, have shown clinical efficacy in $PDL1^+$ NSCLC (139-142). In conclusion, clinical application of PD1/PDL1 pathway has come of age for NSCLC. #

2. Add a table with possible drugs in this field, like direct targeting TAM, transferring M2 to M1, or and so on.

Reply 2: thanks for your comment. Table 2 that summarizes the molecular pathways that have been employed for TAM targeting and the potential drugs that have been used in clinical trials so far is added.

Reviewer B

In their manuscript Sahar sadat Sedighzadeh et al. focused on TAM targeting strategies in lung cancer and a discussion of macrophage polarization pathways. The work seems to accurately describe the selected topic.

A few minor remarks:

1. In the introduction, there is no mention of the subclasses of macrophages M2. Now it is known that M2 macrophages are divided into M2a, M2b, M2c, and M2d subcategories. Further in the work there are only references to these macrophage subclasses (Yaun et al. line 137-140). In the introduction, I will also suggest describing the occurrence of these subclasses.

Reply 1: Dear reviewer, many thanks for your helpful comment. We elaborated the role of M2 macrophage subtypes in the introduction. In addition, figure 1 depicts the molecular pathways that are involved in the induction of M2 subtypes and cytokines and biomarkers which are related to them.

Changes to the text:

#M2 phenotype consists of four well-defined subtypes including M2a induced by IL-4 and IL-13; M2b induced by immune complexes and IL-1 receptor agonists; M2c induced by IL-10, Transforming Growth Factor-Beta (TGF-β), and glucocorticoids; and M2d induced by IL-6, Leukemia Inhibitory

Factor (LIF) and adenosine. Although M2 macrophage subtypes have anti-inflammatory and immunoregulatory roles in common, they also exhibit different functions. For example, M2a subtype is involved in tissue fibrosis, M2b subtype contributes to tumor progression, M2c subtype is responsible for tissue remodeling, and M2d subtype promotes angiogenesis (21). Some common markers for M2 macrophages are arginase, CD206, CD204, and CD163. M2 macrophages are well-adapted to inflammatory response inhibitors and tumor progression (17, 22) (figure 1). #

2. Additionally, please quote and discuss the original works that uses the BALF bronchoalveolar lavage fluid, which reflects well the changes taking place in the tumor microenvironment and macrophages are the main population in this material.

Reply 2: thank you for your insightful comment. We added some studies that have examined BALF macrophages in lung cancer.

Changes to the text:

#Despite the fact that alveolar macrophages are increased and constitute the main cellular component in the bronchoalveolar lavage fluid of lung cancer patients (31, 32), they may show diminished phagocytic abilities (33) and reduced expression of markers characteristic of M1 macrophages such as HLA-DR, CD83, and ICAM-1 (33, 34). In addition, alveolar macrophages in lung cancer patients secrete lower levels of inflammatory cytokines such as IL-1 and TNF-α (33). #

English language and style are ok.

Therefore, I propose to publish this review for future use in clinical practice.

Reviewer C

The authors review role of tumor-associated macrophages in lung cancer. They mentioned tumor-associated macrophages and lung tumor growth, main pathways involved in macrophage polarization in cancers, role of miRNAs in macrophage polarization, macrophage immunotherapy in cancer, and macrophage and drug resistance.

This manuscript deals with an interesting and important topic.

Comment1: It is necessary to explain whether TAMs can be a therapeutic target for lung cancer, especially non-small cell lung cancer, compared to other cancers.

Reply 1: dear reviewer, many thanks for your comment. We added a paragraph and expressed our judgment regarding the future of macrophage therapy in NSCLC based on the current available preclinical and clinical evidence.

Changes to the text:

#To conclude regarding the possible roles of TAM targeting in NSCLC, as TAMs are abundant in the tumor microenvironment of NSCLC and plays important parts in its progression, targeting TAM, either

by eradicating them or re-educating them towards anti-cancer phenotype, will be a rationale strategy that can be translated to clinical application. #

Comment 2: In addition, explanation about role of M2 subtypes is needed.

Reply 2: Many thanks for your helpful comment. We elaborated the role of M2 macrophage subtypes in the introduction. In addition, figure 1 depicts the molecular pathways that are involved in the induction of M2 subtypes and cytokines and biomarkers which are related to them.

Changes to the text:

#M2 phenotype consists of four well-defined subtypes including M2a induced by IL-4 and IL-13; M2b induced by immune complexes and IL-1 receptor agonists; M2c induced by IL-10, Transforming Growth Factor-Beta (TGF-β), and glucocorticoids; and M2d induced by IL-6, Leukemia Inhibitory Factor (LIF) and adenosine. Although M2 macrophage subtypes have anti-inflammatory and immunoregulatory roles in common, they also exhibit different functions. For example, M2a subtype is involved in tissue fibrosis, M2b subtype contributes to tumor progression, M2c subtype is responsible for tissue remodeling, and M2d subtype promotes angiogenesis (21). Some common markers for M2 macrophages are arginase, CD206, CD204, and CD163. M2 macrophages are well-adapted to inflammatory response inhibitors and tumor progression (17, 22) (figure 1). #

Comment 3: It is also necessary to create a section related to the therapeutic limitations of TAMs regulation.

Reply 3: many thanks for your insightful comments. We added a section that discusses the therapeutic limitations of TAM therapy.

Changes to the text:

#Therapeutic limitations of macrophage regulation:

TAM targeting for cancer treatment is challenged by some obstacles including predisposition to infection, organ dysfunction, the need for multiple dosing, and the presence of redundant pathways. Macrophages are cardinal in protecting body from invading pathogens. TAM targeting strategies that are based on eradicating macrophages may increase susceptibility to infections. On the other hand, tissue-resident macrophages such as liver Kupffer cells and brain microglial cells are ubiquitous throughout the body and are significantly involved in maintaining organ homeostasis and their depletion may give rise to serious organ dysfunction. One possible solution for overcoming these problems is to find novel targets that are specifically upregulated by macrophages in cancerous microenvironment (218). Potential candidate molecules and pathways for TAM targeting may be present in diverse cell populations beyond TAM. For instance, CCR2 and CXCR4 are also expressed by lymphocytes (219, 220). Alterations of immune and non-immune cellular functions caused by unintended influence on cells that share the target with TAM may result in complications. This issue

also necessitates finding of targets specific to TAM (218).

The optimal dosing and frequency of TAM targeting therapeutics have not been determined. One special difficulty related to TAM repolarization is that TAM phenotype and function may return to its primary immunosuppressive state after the drug is withdrawn from tumor microenvironment (221).

The ultimate functional status of TAM is controlled by the complex interactions of several microenvironmental and intracellular regulators. The presence of redundant pathways of macrophage polarization and other immunosuppressive cells in tumor microenvironment may explain the observation that not all the tumors respond to TAM targeting strategies. Simultaneous interference with multiple contributors of immunosuppression and cancer progression either by combinatorial approaches or using agents with multiple relevant targets may enhance TAM targeting efficacy (164). #

Reviewer D

This is a narrative review of TAM particularly focused on polarization and therapeutic implications. In the section" Tumor-associated macrophages and lung tumor growth", the confusing results are well organized with proper consideration. The references cited in this manuscript are appropriate and the conclusions derived from these are interesting. This is a well written and useful contribution, which I think is suitable for publication in Journal of TLCR

Reply 1: dear reviewer, many thanks for your kind comments.

Reviewer E

In the manuscript entitled, "Narrative review of tumor-associated macrophages in 1 lung cancer: regulation of macrophage polarization and therapeutic implications", the authors wrote a comprehensive review in providing evidences regarding roles of macrophages in tumor microenvironment and discussing helpful researches targeting tumor associated macrophages (TAMs) using lung cancer as a model. The authors have done appreciable works to organizing related topics into a review. Some suggestions are list below hoping to improve the current manuscript.

1. Macrophages release cytokines or factors via exosomes, which is an emerging new topic. The authors shall describe more studies in this aspect.

Reply 1: dear reviewer, many thanks for your helpful comment. We added a section that

discusses the roles of exosomes in macrophage polarization.

Changes to the text:

#Role of extracellular vesicles in macrophage polarization:

Extracellular vesicles are membrane-bound structures that originate from cells and can be endocytosed by other cells. These vesicles contain macromolecules such as DNA, RNA, and proteins and the transfer of macromolecules between cells by the means of extracellular vesicles provides an intricate mode of intercellular communication and tissue and systems-level homeostasis. Intriguingly, neoplastic cells utilize extracellular vesicles for regulating the functions of other malignant cells, tumor microenvironmental cells, cells related to hematopoiesis, and distant organ niche. The main categories of animal extracellular vesicles are exosomes (30-120 nm), microvesicles (100-1000 nm), and apoptotic bodies (800-5000 nm) (154). Cancer cell-derived extracellular vesicles can promote M2 macrophage polarization. Non-coding RNAs including miRNA-103a, miRNA-25-3p, miRNA-130b-3p, miRNA-425-5p, and miRNA-301a-3p were present in lung cancer cell (CL1-5, NCI-42087, H1792, and H1437), colon cancer cell (HCT116), and pancreatic cancer cell (PANC1)-derived extracellular vesicles. These miRNAs targeted and decreased the level of PTEN which resulted in M2 macrophage polarization through STAT3 activation (155-157). Exosomes acquired from hypoxic epithelial ovarian cancer cells (SKOV3) harbored miRNA-21-3p, miRNA-125b-5p, miRNA-181d-5p, and miRNA-222-3p which degraded SOCS in macrophages and subsequently increased phosphorylated STAT3, a process which culminated in M2 macrophage polarization (158, 159). In addition, STAT6 upregulation mediated by cGAS/STING pathway is reported to be involved in cancer cell-derived extracellular vesicle-induced M2 macrophage polarization (160). Exosomes derived from DLD-1 colon cancer cell line contained miRNA-145 which polarizes TAMs towards M2 phenotypes by downregulating histone deacetylase 11 (161).

In a study by Chen *et al*, extracellular vesicles derived from lung cancer cell lines (HCC827, LLC, A549, and H460) were taken up by macrophages and promoted M2-like phenotype in TAMs. These TAMs produced IL-1β which enhanced lung cancer cell stemness and survival. It was revealed that lung cancer cell-derived microparticles contained non-coding RNAs that could stimulate TLR3 activation which subsequently increased pro-IL-1β expression in a NF-κB and MAPK-dependent pathway. Simultaneously, extracellular vesicles uptake by macrophages increased cytosolic calcium concentration and mitochondrial ROS production. ROS activated NLRP3 inflammosome which is required for IL-1β activation (162). In another study, the E3 Ubiquitin ligase TRIM59 was delivered to macrophages by lung cancer cell line (H1299 and A549)-derived exosomes. TRIM59 promoted ubiquitination and proteasomal degradation of ABHD5, a hydrolase enzyme which is involved in lipid

metabolism. Downregulation of ABHD5 activated NLRP3 inflammosome and resulted in enhanced IL-1β production (163). Collectively, these studies demonstrate the cardinal role of extracellular vesicles in regulating macrophage polarization. #

2. The authors may add more descriptions on inhibition of IL-6 and/or IL-6R in relation to macrophage polarization and treatment.

Reply 2: Many thanks for your comment. We added some additional studies in this regard.

Changes to the text:

#Anti-IL-6 receptor monoclonal antibody abrogated the ability of triple-negative breast cancer cell (MCF-10A) to induce M2 phenotype in macrophages (86).

A phase I clinical trial showed acceptable safety profile of Tocilizumab in combination with chemotherapy and IFN-α2b in recurrent epithelial ovarian cancer (89). A small phase II randomized clinical trial on patients with high-risk smoldering multiple myeloma showed moderately increased progression-free survival with Siltuximab treatment (43 patients) in comparison with placebo (42 patients) (90). #

3. There are common downstream signals with distinct roles in responding to GM-CSF and M-CSG. The authors shall give some examples and clarify the underline mechanisms.

Reply 3: thanks for your insightful comment. We added some explanations of the different outcomes of common signaling pathways in GM-CSF and M-CSF induced macrophage polarization.

Changes to the text:

#Although M-CSF and GM-CSF receptors share some common signaling pathways, they result in different transcriptional regulation and ultimate functional changes. For instance, both M-CSF and GM-CSF activate Ras/MAPK pathway in macrophages to upregulate the expression of Scavenger Receptor (SR)-A; However, M-CSF-mediated upregulation of SR-A is caused by binding of AP-1 to the enhancer of SR-A gene but GM-CSF increases the expression of SR-A through a different enhancer region (95). While IRF5 is implicated in GM-CSF-induced M1 macrophage phenotype, IRF4 orchestrates M2 macrophage polarization by M-CSF (96). #

4. The authors just briefly mentioned the manipulation of TLR pathways of macrophages for therapeutic aims at the last paragraph of the TLR section. Some examples and research articles shall be discussed.

Reply 4: thank you for your helpful comment. We added some studies that investigated the clinical applications of TLR modulators.

Changes to the text:

#The combination of Motolimod (a TLR8 agonist) with PEGylated liposomal Doxorubicin did not prolong overall survival or progression-free survival of recurrent ovarian, fallopian tube, or primary peritoneal carcinoma over placebo and PEGylated liposomal Doxorubicin in a phase II randomized clinical trial (126). EMD1201081 (a TLR9 agonist) and Cetuximab did not have superior efficacy than Cetuximab monotherapy in patients with recurrent or metastatic squamous cell carcinoma of head and neck revealed by a phase II randomized clinical trial (127). A phase I dose-finding trial enrolling previously-treated NSCLC patients showed acceptable safety and moderate overall response (15%) of IMO-2055 (a TLR9 agonist) with Erlotinib and Bevacizumab (128). #

5. The authors shall draw a figure describing using macrophages as a carrier for anti-cancer drugs delivery.

Reply 5: thankful to you! We added a new figure (figure 3).

6. Table 2 includes some microRNAs and their targets in macrophage polarization. The authors shall at least give two or three studies in main text to describe the detailed mechanisms involved.

Reply 6: thank you for your correct notion. We added some of those studies.

Changes to the text:

#Namely, miRNA-125b decreases the level of IRF4 by targeting it. IRF4 is a key signaling molecule for the induction of M2 phenotype. In line, miRNA-125b overexpression in macrophages potentiates their antigen-presentation capabilities, priming of T cells, and cancer cell killing (148). The overexpression of miRNA-720 reprograms TAMs toward M1 phenotype by targeting GATA3 (149). (Table 1)#

7. Minor comments: (1) lines 320-323, delete them due to redundancy, (2) line 338, shall be “study”, not stud, and (3) Ref 162 is a bit old. Several newer refs can be also cited.

Reply 7: we really appreciate your detailed review of the manuscript. We changed the text accordingly. We also added some more recent studies in the field.

Changes to the text:

#Pilot clinical trials of CD40 agonist antibodies in combination with chemotherapy or immune checkpoint inhibitors have shown acceptable safety profile and preliminary response in a variety of solid tumors including NSCLC (202-205). #