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Challenges and Exploration for Immunotherapy Targeting Cold Colorectal Cancer

Responses to the editor and reviewers' comments:

We are very grateful for your insightful and constructive comments. We agree that responding to these comments would make the paper considerably clearer and stronger. We have carefully revised the manuscript according to your suggestions. Our detailed responses to each of your comments can be found below (reviewers' comments are underlined). All changes have been highlighted in **blue** in the revised manuscript.

Reviewer #1: Minor revision

1. Reviewer wants to suggest to include systematic review (SR) and meta-analysis (MA) articles on immunotherapy and other therapeutic regimes in colorectal cancer as MA are evidence based results.

Response: We thank you for reminding us this important point. And we have y added these related references in the manuscript as Reference [9], [71], [81].

2. In the discussion on response of immunotherapy, it is suggestive to include the efficacies and responses related to the status of immunohistochemical expression of immune check point proteins such as PD-1, PD-L1, CTLA4 etc.

Response: We thank you for reminding us this important point.According to your suggestion, we have added the related contents in the manuscript in the Combined with targeted therapy(Lines 3 to 5 from the bottom)

3. For the reader's understanding, it is suggestive to explain some of the processes in the flow chart or diagrams.

Response: Thank you for your careful reading of our paper. According to your suggestion, we have redrawn Figure 1. to clearly show classification based on

immunological characterizations.

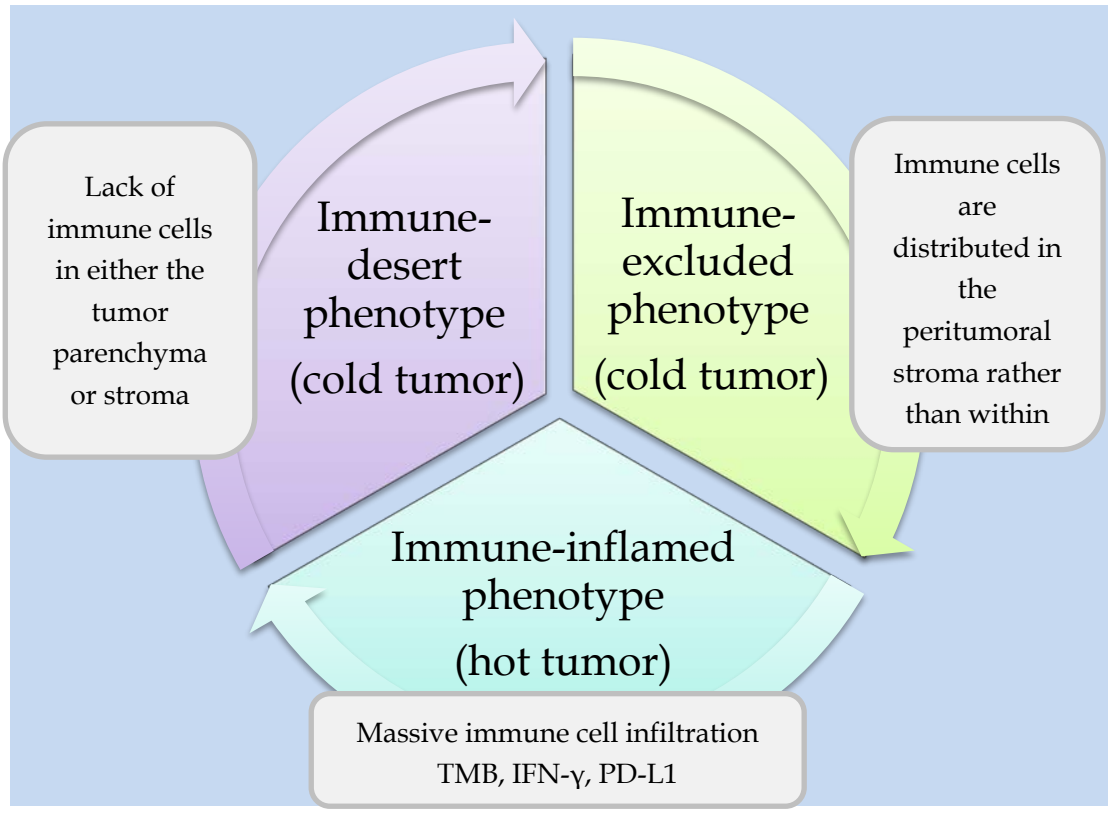

Figure 1. Immune-excluded phenotype: T cells cannot homie from the tumor stroma to the interior of the tumor; Immune-desert phenotype: Anticancer immune initiation error, including insufficient antigen release, insufficient antigen presentation, T cell initiation and activation disorders. Abbreviations: TMB: Tumor mutation burden; IFN-γ: Interferon- γ; PD-L1: Programmed cell death 1 ligand 1

4. References need to be cleaned up. For example, reference 57 and 58 overlapped. Please check all references.

Response: Thank you for your significant reminding. We are very sorry for the mistake and we correct the above errors and make an effort to correct errors.

Reviewer #2: Major revision

1. How about the anti-CTA therapy using anti--MAGE-A4 or NY-ESO for colon cancer?

Response: Truly thank you for pointing out this. Cancer/testicular antigens (CTAs)

are a class of normally expressed tumor antigens that are limited to male germ cells in the testes and are not found in adult somatic tissue. Because tumor tissue contains a variety of CT antigen epitopes, can be recognized by HLA, causing specific immune response against tumors, so CT antigen has become the most clinically valuable tumor-specific antigen in tumor immunotherapy, among which MAGE-A family and NY-ESO-1 are the most prominent, both overexpression can induce a strong T cell response, with the ability to induce humoral immune response in CRC patients, showing the potential to become a promising biomarker for CRC. MAGE-A for immunotherapy is still in its infancy, and MAGE-A antigenic peptides as vaccines still have certain limitations, because the specific cytotoxic T cells induced by MAGE-A can recognize only the complex of antigen peptides bound to MHC-I molecules, which greatly limits the application of tumor immunotherapy. Studies have confirmed that the unique immunogenicity of NY-ESO-1 can induce the production of autoantibodies, exert the killing effect of the immune system against target tumor cells, and its expression level is related to the specific B cell and T cell immune response, which provides a basis for us to further formulate NYESO-1-specific immunotherapy, NY-ESO-1 has different degrees of expression in a variety of tumor tissues, but the expression rate is low, which greatly reduces its application in tumor immunotherapy. Therefore, how to improve the expression rate of NY-ESO-1 in tumor cells has become a major problem in the application of NY-ESO-1 for tumor immunotherapy.

I have revised the contents of this part in the Combined with tumor vaccine, MAGE-A and NY-ESO-1, tumor-associated antigens with strong antigenicity and specificity in the cancer testis antigen (CTA) family, are highly expressed in a variety of tumor tissues, including CRC, and are expressed at low levels in normal tissues[111-112]. Therefore, they can be specifically recognized by the body's immune system and exert antitumor effects. MAGE-A and NY-ESO-1 can induce a robust T cell antitumor response, and their related antibodies can be used as alternative biomarkers in vaccine therapy research[113-114]. At

present, research on tumor vaccines based on MAGE-A4 and NY-ESO-1 has been carried out successively, and the results show that these vaccines have broad application prospects[114-115].

111 **Long YY**, Wang Y, Huang QR, Zheng GS, Jiao SC. Measurement of serum antibodies against NY-ESO-1 by ELISA: A guide for the treatment of specific immunotherapy for patients with advanced colorectal cancer. *Exp Ther Med* 2014; **8**: 1279-1284 [PMID: 25187840 DOI: 10.3892/etm.2014.1913]

112 **Saito T**, Wada H, Yamasaki M, Miyata H, Nishikawa H, Sato E, Kageyama S, Shiku H, Mori M, Doki Y. High expression of MAGE-A4 and MHC class I antigens in tumor cells and induction of MAGE-A4 immune responses are prognostic markers of CHP-MAGE-A4 cancer vaccine. *Vaccine* 2014; **32**: 5901-5907 [PMID: 25218300 DOI: 10.1016/j.vaccine.2014.09.002]

113 **Van Der Bruggen P**, Zhang Y, Chaux P, Stroobant V, Panichelli C, Schultz ES, Chapiro J, Van Den Eynde BJ, Brasseur F, Boon T. Tumor-specific shared antigenic peptides recognized by human T cells. *Immunol Rev* 2002; **188**: 51-64 [PMID: 12445281 DOI: 10.1034/j.1600-065x.2002.18806.x]

114 **Scanlan MJ**, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. *Cancer Immun* 2004; **4**: 1 [PMID: 14738373]

115 **Baumgaertner P**, Costa Nunes C, Cachot A, Maby-El Hajjami H, Cagnon L, Braun M, Derré L, Rivals JP, Rimoldi D, Gnjatic S, Abed Maillard S, Marcos Mondéjar P, Protti MP, Romano E, Michielin O, Romero P, Speiser DE, Jandus C. Vaccination of stage III/IV melanoma patients with long NY-ESO-1 peptide and CpG-B elicits robust $CD8^+$ and $CD4^+$ T-cell responses with multiple specificities including a novel DR7-restricted epitope. *Oncoimmunology* 2016; **5**: e1216290 [PMID: 27853637 DOI: 10.1080/2162402X.2016.1216290]

2. Is inflammation involved in the pathogenesis of colon cancer?

Response: Thank you for the comment. I should have explained that Inflammation is an important risk factor for the development of CRC, and the severity of inflammation is directly related to CRC risk. There is also increasing evidence that systemic inflammatory factors have a wide range of effects on the pathogenesis of colorectal cancer (CRC), and the reason why chronic inflammation may produce oxidative stress-induced DNA damage, thereby activating tumor promoting genes and inactivating tumor suppressor genes, further promoting the occurrence of colorectal cancer. The study found that NF-κB is a key regulator of innate immunity and inflammation, and its constitutive activation is associated with various types of cancer, which enhances tumor cell invasion by regulating genes that promote metastasis and angiogenesis, indicating a certain link between inflammation and the pathogenesis mechanism of cancer. It has been hypothesized that effective control of inflammation can reduce the incidence of inflammation-related colorectal cancer to some extent.

3. Do you think the colon cancer "hot tumor"?

Response: We totally understand the reviewer's concern. In the original manuscript we explain this problem in the abstract and in Part 2 and I am sorry that these parts were not clear in the original manuscript. I should have explained that CRC can be divided into dMMR/MSI-H and pMMR/MSS two types, dMMR/MSI-H because of DNA repair function defects, has a high tumor mutation load, can produce more neoantigens, and then increase immune cell infiltration, so that tumors are more easily recognized by the immune system, and then activate the opportunity to exert killing function, they often indicate that the efficacy of immunity is better, so this part of the MSI -H/dMMR people sensitive to immunotherapy belong to colorectal cancer " hot tumor".

2 **Weiden J**, Tel J, Figdor CG. Synthetic immune niches for cancer immunotherapy. *Nat Rev Immunol* 2018; **18**: 212-219 [PMID: 28853444 DOI: 10.1038/nri.2017.89]

3 **Russell SJ**, Barber GN. Oncolytic Viruses as Antigen-Agnostic Cancer Vaccines. *Cancer Cell* 2018; **33**: 599-605 [PMID: 29634947 DOI: 10.1016/j.ccell.2018.03.011]

4 **Jansen CS**, Prokhnevska N, Master VA, Sanda MG, Carlisle JW, Bilen MA, Cardenas M, Wilkinson S, Lake R, Sowalsky AG, Valanparambil RM, Hudson WH, McGuire D, Melnick K, Khan AI, Kim K, Chang YM, Kim A, Filson CP, Alemozaffar M, Osunkoya AO, Mullane P, Ellis C, Akondy R, Im SJ, Kamphorst AO, Reyes A, Liu Y, Kissick H. An intra-tumoral niche maintains and differentiates stem-like CD8 T cells. *Nature* 2019; **576**: 465-470 [PMID: 31827286 DOI: 10.1038/s41586-019-1836-5]

4. How do you think the combined therapy with anti-PD-1/PD-L1 and chemotherapy for colon cancer?

Response: Thank you for pointing out this problem in manuscript. According to the revised content, we have carefully taken the reviewers comments into account. I have revised the contents of this part as follows. Although chemotherapy drugs may kill immune cells that fight the tumor immune response, there is growing evidence that chemotherapy can activate the body's immune response through multiple mechanisms. At present, a series of studies have been carried out to explore the effect of chemotherapy combined with immunoantitumor therapy, which has a broad application prospects from the relevant results, but how to design the most reasonable plan to achieve better combination effects, such as the best combination time and drug dose, is still a hot topic for further exploration in the future research.