Age-Related Differences in Molecular Profiles for Immune Checkpoint Blockade Therapy

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Immune checkpoint blockade (ICB) therapies have significantly improved the prognosis and shown considerable promise for cancer therapy; however, differences in ICB treatment efficacy between the elderly and young are unknown. We analyzed the studies enrolled in the meta-analysis using the deft approach, and found no difference in efficacy except melanoma patients receiving anti–PD-1 therapy. Similarly, higher treatment response rate and more favorable prognosis were observed in elderly patients in some cancer types (e.g., melanoma) with data from published ICB treatment clinical trials. In addition, we comprehensively compared immunotherapy-related molecular profiles between elderly and young patients from public trials and The Cancer Genome Atlas (TCGA), and validated these findings in several independent datasets. We discovered a divergent age-biased immune profiling, including the properties of tumors (e.g., tumor mutation load) and immune features (e.g., immune cells), in a pancancer setting across 27 cancer types. We believe that ICB treatment efficacy might vary depending on specific cancer types and be determined by both the tumor internal features and external immune microenvironment. Considering the high mutational properties in elderly patients in many cancer types, modulating immune function could be beneficial to immunotherapy in the elderly, which requires further investigation.

Keywords: immune checkpoint blockade, age, immune profiles, therapy efficacy, immunotherapy

INTRODUCTION

Immune checkpoint blockade (ICB) therapies that enhance antitumor activity of T lymphocytes through blocking immune checkpoints (e.g., PD-1/PD-L1, CTLA-4) have been proven to dramatically improve patient survival in multiple cancer types (1, 2). In most cases, the incidence and mortality of malignancies are associated with increasing age and hence called aging diseases (3). Elderly patients are the major population that requires ICB treatments. However, the immune system changes with age, characterized by altered immune cells and decreased adaptive immunity (4, 5). This process, known as “immunosenescence”, might potentially affect immune responses, but
the exact role in ICB immunotherapy remains unknown. Although a few large-scale meta-analyses have been conducted (6–8), debate continues about whether there are differences in immunotherapy efficacy between the elderly and young. Wu et al. claimed that elderly patients receiving ICB treatment had better efficacy than young patients based on a meta-analysis of random clinical trials (6), while two other studies detected no differences in efficacy (7, 8). The underlying cause of the conflict is that age-dependent changes in intratumoral immune populations and response to immunotherapy in the tumor microenvironment remains unclear (9).

Many previous studies have sought to identify biomarkers to predict immunotherapy response. Tumor mutation burden (TMB) is positively correlated with tumor neoantigen load, and high TMB tends to increase the ability of T cells to recognize and kill tumor cells (10, 11). Several clinical trials have demonstrated the relationship between high TMB and ICB benefit (12, 13). PD-L1 is expressed on various cell types, including tumor cells and immune cells, and its expression can help to generate an immunosuppressive tumor microenvironment (14–16). Patients with PD-L1 overexpression are more likely to respond to anti–PD-L1 treatment. Cytolytic activity (CYT) correlates with neoantigen load and amplifications in regions containing immunosuppressive genes, and has predictive value for immunotherapy (17). A T cell-inflamed gene expression profile (GEP) including IFN-γ-responsive genes related to antigen presentation, cytotoxic activity, chemokine expression, and adaptive immune resistance can serve as an independent predictor of response to anti–PD-1 therapy (18–22). Additionally, there are some reports on other biomarkers (23, 24), including protein or mRNA expression of immune checkpoints (e.g., PD-1, CTLA-4), immune cells (e.g., CD4+CD8+ T cell), aneuploidy score, and BCR/TCR richness. In this study, we comprehensively analyzed age-associated differences in these immune-related molecular biomarkers to better understand age effects on ICB treatment efficacy.

### MATERIALS AND METHODS

#### Effects of Age on ICB Treatment in Clinical Trials

We systematically searched PubMed, Embase, MEDINE, and Web of Science for eligible clinical trials, published before Mar 1st, 2021. The search terms included "PD-1," "PD-L1," "CTLA-4," "immune checkpoint inhibitor," "atezolizumab," "avelumab," "durvalumab," "ipilimumab," "nivolumab," "pembrolizumab," and "tremelimumab." Two reviewers independently performed initial research by screening titles and abstracts of retrieved articles. Trials enrolled in our study should meet the following criteria (1): randomized controlled trials concerning cancer therapy (2); participants treated with PD-1 inhibitors, PD-L1 inhibitors, CTLA-4 inhibitors, or their combination compared to placebo or other anti-cancer drugs (3); trials with the hazard ratio (HR) of overall survival (OS) by using 65 as a cut-off age. After integrating our searching results with 2 published meta-analysis studies (6, 8), a total of 34 clinical trials about ICB treatment in 9 different cancer types (16 trials in NSCLC; 6 trials in melanoma; 3 trials in colorectal cancer; 3 trials in GEJC; 2 trials in RCC; 1 trial in SCLC, urothelial, TNBC and mesothelioma) were included in our study (see Supplementary Table S1 for full details). The deft approach (25) was used for assessment of age effect on the immunotherapy efficacy within each trial, and fixed-effect model (FEM) meta-analysis was applied to combine these estimates among trials. These interactions represent immunotherapy efficacy differences, in which hazard ratio (HR) >1 and HR <1 indicate OS advantage in the young and elderly, respectively.

#### Analysis of Molecular Profile in Patients With ICB Treatment

We comprehensively analyzed molecular profile in ICB treatment-related studies, including melanoma, lung cancer, bladder cancer, renal cell carcinoma, and other cancer types (see Supplementary Table S2 for full details). The profiling comprises of some biomarkers reported in these studies that are potential to alter treatment responsiveness, such as TMB, gene mutation (BRCA2, BRAF, PBRM1), neoantigen, CYT, GEP, gene expression of immune checkpoints (PD-1, PD-L1, PD-L2, CTLA-4), and protein expression of PD-L1. For survival analysis, HR and 95% CI were calculated using Cox proportional hazards model, and Kaplan-Meier survival curve was generated. Fisher’s exact test was used for comparison of individual gene mutation and benefit percentage between the elderly and young, and two-sided Mann-Whitney-Wilcoxon (MWW) test for other molecular features.

#### Analysis of Molecular Profile in Patients From The Cancer Genome Atlas (TCGA)

We performed a comprehensive analysis of molecular profiling across 27 cancer types in TCGA with ≥20 patients in both the elderly and young groups (see Supplementary Table S3 for full details). Data of mutation, gene expression, and protein expression were accessible from TCGA (https://portal.gdc.cancer.gov/). Immune checkpoint genes with known co-stimulatory or co-inhibitory effects were summarized by Auslander et al. (26). Six immune cells populations were evaluated based on Tumor Immune Estimation Resource (TIMER) (27) (http://cistrome.dfci.harvard.edu/TIMER/). T cell-inflamed gene expression profile (GEP) was calculated according to the signature from Ayers et al. (19). Immune cytolytic activity (CYT) was defined as the geometric mean of GZMA and PRF1 expression (17). Aneuploidy score, T cell receptor/B cell receptor (TCR/BCR) richness, and neoantigen load were derived from Thorsson et al. (https://gdc.cancer.gov/about-data/publications/panimmune) (28). To control for possible confounding factors, including gender (categorical), race (categorical), histologic type (categorical), pathologic stage (categorical), tumor purity (continuous), and smoking history (categorical), between the elderly and young, propensity score (PS) matching analysis was performed (29). Patients in the elderly and young group were matched for 6 factors using PS
calculated with the nearest neighbor method. The covariate balance was then checked to assess the adequacy of the propensity model. After balanced, we compared the molecular profile between the elderly and young. P value < 0.05 was considered statistically significant.

Analysis of Other Data Sets for Independent Verification

To verify above age-biased differences, we obtained TMB in three liver cancer projects (LICA-FR, LINC-JP, LIRI-JP) and one renal cell cancer project (RECA-EU) from the International Cancer Genome Consortium (ICGC) (https://dcc.icgc.org/). Two independent datasets containing gene expression data of lung cancer (GSE19804) (30) and endometrial cancer (GSE17025) (31) were analyzed as well. We performed PS matching analysis as described above and considered P < 0.05 as significance.

RESULTS

Pooled Estimate of Overall Survival (OS) From Meta-Analysis

To determine whether different ICB treatment efficacy exists due to age, we extracted and summarized survival data of the elderly and young from 34 clinical trials (Supplementary Table S1, Supplementary Figure S1). The deft approach was used to calculate HRs specific to each trial, and fixed-effect model for the pooled HRs. We found no significant difference concerning OS (HR=0.95; 95% confidence interval (CI), 0.88–1.02; p= 0.504, Figure 1) after pooling the 34 trials. Among 6 melanoma clinical trials, 5 of them showed OS advantage in elderly patients. Interestingly, we observed a particularly clear inconsistency of benefit in NSCLC patients that 9 out of 16 trials showed OS advantage in young patients, while 7 other trials displayed OS advantage in elderly patients.

Figure 1

Clinical outcomes in elderly and young patients receiving ICB treatment. The squares and vertical lines represent trial-specific HRs and 95% CIs. Red squares and orange background color indicate OS advantage in elderly patients and blue squares and green background color in young patients. The diamonds show the pooled estimate from fixed-effect meta-analysis. NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; GEJC, gastric or gastroesophageal junction carcinoma; SCLC, small cell lung cancer; HNC, head and neck cancer; TNBC, triple-negative breast cancer; MESO, mesothelioma.
advantage in elderly patients. It indicated that merely pooling different trials may not yield a convincing result. Moreover, we performed subgroup analyses according to the types of cancer, ICB treatment, and control choice. We observed insignificant pooled HR for ICB treatment vs. docetaxel (0.92; 95% CI, 0.80–1.05; p= 0.202, Supplementary Figure S2A) and ICB treatment vs. placebo (0.98; 95% CI, 0.84–1.15; p= 0.529, Supplementary Figure S2B). Similarly, the pooled HR for anti–CTLA-4 trials (1.04; 95% CI, 0.84–1.29; p= 0.219, Supplementary Figure S2C) remained insignificant, whereas anti–PD-1/PD-L1 trials (0.94; 95% CI, 0.86–1.02; p= 0.098, Supplementary Figure S2D) showed a trend toward better OS in the elderly. Moreover, different from anti–CTLA-4 trials (1.02; 95% CI, 0.8–1.31; p= 0.042, Supplementary Figure S2E), the pooled HR for anti–PD-1 trials (0.559; 95% CI, 0.358–0.871; p= 0.010, Supplementary Figure S2F) showed significant OS advantage in elderly patients among the melanoma subgroup.

**Age-Bias of Potential Molecular Biomarkers in ICB Treatment**

To explore the possible mechanism underlying age-associated immunotherapy responsiveness, 7 eligible ICB treatment datasets with molecular profile for each patient were analyzed after screening (18, 26, 32–36). We found a diverse survival pattern concerning OS between the elderly and young patients with ICB treatment (Figure 2A). Elderly patients with colorectal cancer (COAD) had better OS than young patients (p=0.044) (Supplementary Figure S3A). Compared with young patients, OS tended to be better in elderly patients with HNSC and NSCLC. In addition, we observed a significantly higher response rate in elderly melanoma patients from 3 studies (26% vs. 9%, p=0.019; 27% vs. 8%, p=0.012; 64% vs. 32%, p=0.037) (Supplementary Figures S3B–D). As regards molecular markers (Figures 2B, 3A), tumor mutation burden (TMB) was significantly higher in elderly patients with melanoma (p<0.001, p=0.015, Supplementary Figures S3E, F), clear cell renal cell carcinoma (KIRC) (p=0.044, Supplementary Figure S3G), and pan-cancer (p<0.001, Supplementary Figure S3H) than young patients. Similarly, significantly higher neoantigen load was observed in elderly patients with melanoma (p=0.016, Supplementary Figure S3I). In contrast, elderly melanoma patients seemed to have lower CTLA-4 (p=0.001, Supplementary Figure S3J) and PD-1 (p=0.033, Supplementary Figure S3K) expression than young patients. Other biomarkers showed no significant difference between the elderly and young.

**A Diverse Age-Bias of Immunologic Characteristics in Multiple Cancer Types**

No significant difference on confounding factors including gender, race, histologic type, pathologic stage, tumor purity, and smoking history between the elderly and young was observed by using PS matching analysis (Supplementary Figure S4). After controlling for these factors, we found that elderly melanoma patients had more TMB (p=0.002, Figure 3B, Supplementary Figure S5A) and neoantigen load (p=0.011, Figure 3B and Supplementary Figure S5B), which was consistent with immunotherapy data sets. Similarly, several other cancer types showed elderly-bias towards TMB, neoantigen load, and aneuploidy score (Figure 3B and Supplementary Figures S5C–F). In addition, elderly patients exhibited higher gene expression...
FIGURE 3 | Comparison of immune features between elderly and young patients from TCGA. (A) Overview of age-associated immune features comprising TMB, CYT, neoantigen, aneuploidy, BCR/TCR richness, immune cells, and checkpoints. (B) Differences in molecular biomarkers reported in immunotherapy-related studies, including TMB, CYT, GEP, neoantigen and protein PD-L1, and some potential biomarkers, including aneuploidy, BCR and TCR richness. (C) Differences in mRNA expression level of 34 immune checkpoints, including both stimulatory and inhibitory immune checkpoints. (D) Differences in 6 types of immune cells, including B cell, CD4+ T cell, CD8+ T cell, neutrophil, macrophage, and myeloid dendritic cell. Propensity score with the nearest neighbor method was used for comparison of immune features between the elderly and young. Sample size for each cancer type was displayed in Supplementary Table S3.
of immune checkpoints (Figure 3C) and higher levels of immune cells (Figure 3D) in some cancers, including esophageal carcinoma (ESCA), brain lower grade glioma (LGG), and prostate adenocarcinoma (PRAD). Protein PD-L1 significantly elevated in elderly patients in COAD (p=0.028, Figure 3B, Supplementary Figure S5G). By contrast, young-biased immune traits appeared in several cancers as well. For instance, a few biomarkers significantly elevated in young patients with breast invasive carcinoma (BRCA), including PD-L1 (p=0.049, Figure 3B, Supplementary Figure S5H), TCR richness (p=0.005, Figure 3B and Supplementary Figure S5I), 9 out of 34 immune checkpoints (Figure 3C), and 5 out of 6 immune cells (Figure 3D). Interestingly, young-bias existed in both stimulatory (CD27, ICOS, and so on) and inhibitory (BTLA, CTLA-4, and so on) immune checkpoints. Except macrophage, other five types of cells (B cell, CD4+ T cell, CD8+ T cell, neutrophil, and myeloid dendritic cell) showed young-bias.

### Verification of Age-Bias Using Independent Data Sets

Examination of several independent datasets was performed for validating the results from TCGA. Consistent with the elderly-bias on TMB in liver hepatocellular carcinoma (LIHC) (Figure 3B), significantly higher TMB was observed in three independent datasets (LICA-FR, p=0.001; LINC-JP, p<0.001; LIRI-JP, p<0.001) (Figures 4A–C). As for kidney cancer, TMB was significantly elevated in elderly patients with kidney renal clear cell carcinoma (KIRC) and kidney renal papillary cell carcinoma (KIRP) from TCGA (Figure 3B) and in elderly patients from another independent dataset (RECA-EU, p=0.015) (Figure 4D). Regarding uterine corpus endometrial carcinoma (UCEC), we observed a young-bias pattern concerning immune checkpoints (CTLA-4, ICOS, and so on) and CYT (p=0.013) and validated it in an independent dataset (Figure 4E). Similarly, young-biased immune checkpoints (BTLA, CD28, and so on) and CYT in

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**Figure 4** Differences in immune biomarkers between elderly and young patients in independent datasets. (A–D) Boxplots showing the differences in TMB between elderly and young patients with liver cancer (a, LICA-FR; b, LINC-JP; c, LIRI-JP) and renal cell cancer (d, RECA-EU). Boxplot center line, bounds of box and whiskers represent median, inter-quartile range and outliers. (E) Checkpoints, GEP, and CYT between elderly and young patients with UCEC (GSE17025, elderly=26, young=60) and BRCA (GSE42568, elderly=36, young=68).
BRCA were observed in TCGA and validated in another independent dataset (Figure 4F).

**DISCUSSION**

Accumulating evidence indicates that differences in the immune function between the young and elderly are closely associated with the pathogenesis of infections, autoimmune diseases, and malignancies and the response to vaccine, whereas the role in cancer immunotherapy is still ambiguous (37–39). The changes in the immune function of the elderly, also called immunosenescence, refer to a natural process occurring with age and leading to a decline in immune function (40). Due to this, young patients with tumors seem to be more likely to benefit from immunotherapy. However, in this study we compared survival differences between the elderly and young via the deft approach in clinical trials from meta-analysis (6, 8), and found no significant differences in most trials. In contrast, elderly patients may have a significantly better OS in some cancers, especially melanoma. Totally 5 of 6 melanoma clinical trials exhibited OS advantage in the elderly. Additionally, we found that elderly patients with melanoma receiving anti–PD-1 therapy were associated with better OS. In melanoma, the response rate significantly elevated in the elderly compared with young patients based on 3 ICB trials (33, 41, 42). These findings were also consistent with the results reported in other studies (43–45).

Overall, these lines of evidence suggest that superior efficacy in the elderly may truly exist in some cancer types. Although a majority of current studies defined the cut-off age as 65 years old, a small part of them took 70 or 75 years old as cut-off value and observed the reduced efficacy in elderly patients (46, 47). This inconsistency may be explained by patients’ own physical conditions, as patients aged over 70 or 75 are more susceptible to other diseases, such as respiratory, cardiovascular and cerebrovascular diseases, which might correlate with reduced OS and affect the immune system to some degree as well.

A few studies have been conducted to investigate potential reasons for superior efficacy in the elderly, which mainly focused on the function of the immune system and immune cells in it (48–51). For example, Kugel et al. believed that fewer Tregs and more CD8+ T cells may be responsible for the better response to anti–PD-1 treatment in the elderly based on an animal and clinical research (45). After analysis of molecular profiling in patients receiving ICB treatment, we found that the elderly melanoma patients had more TMB and neoantigen than the young. However, it may be restricted by some limitations, including small sample size and effects of confounding factors. Therefore, we took the TCGA and other independent datasets as the validation cohorts, performed PS matching analysis to avoid effects of confounding factors, and finally obtained similar results. One other suggestion could be that different intrinsic tumor properties partly contribute to different prognosis and response rate between the elderly and young. After exposure to experimental and intrinsic mutagens for long periods, the formers have more mutations and neoantigens which can be targeted by the host immune system (52). In addition to melanoma, similar results were seen in many other cancer types, such as HNSC, LIHC, COAD, and so on, which further supported the existence of different tumor properties between the elderly and young. It is, however, not applicable to all cancer types, such as LUSC and LUAD, and no obvious age-bias on efficacy was observed in lung cancer patients. Due to limited amount of available clinical trials for analysis, except melanoma and lung cancer, the association between efficacy and different age-related tumor properties is yet unknown and deserves further study.

Certainly, both tumor antigens and a functional immune system are necessary to identify and kill tumor cells accurately and efficiently (53–55). There is a body of evidence that almost all measures of innate and adaptive immunity are different between the elderly and young (5, 49). A remarkable feature is aging-related degeneration of the thymus, where T cells differentiate, develop, and mature. It has been reported that few CD8+ naïve T cells with shrunken antigen receptor repertoire, which participated in the process of antigen recognition (56, 57), was possessed for neoantigen in the elderly, indicating the increased difficulty for the elderly patients to recognize tumor antigens. This is only speculation, however, as no data to date is available to directly confirm the impaired ability of recognizing tumor neoantigens in the elderly. Additionally, stem cell memory T cells (TSCM) can produce more terminally differentiated daughter cells expressing effector molecule, which is crucial for immunotherapy (58, 59); however, no significant effects of age on TSCM were observed throughout life (60, 61). The characteristics of immune system in humans and other species were not exactly the same, and most of the current knowledge about age-related differences in immune function came from animal studies, especially mouse models, which limited their application for clinical immunotherapy to some extent. In our study, a divergent age-biased immune profiling was observed in a pancancer setting across 27 cancer types. Among these cancer types, part of them showed young-biased or elderly-biased immune traits, while a substantial portion of them showed no obvious age-bias. Intriguingly, stimulatory and inhibitory immune checkpoints have essential roles in immune activation and suppression (62), while both of them showed consistent changes with age in some cancer types. Thus far, there is no clear evidence that the functions of ICB-treatment associated immune components are impaired in the elderly patients. We hypothesize that immunotherapy efficacy might be tumor-specific, which is determined by both the tumor itself and the tumor immune microenvironment. Further in-depth investigation is needed to figure out whether and how immune system changed with age, which might help to predict and improve immunotherapy efficacy in the elderly.

A limitation of our study is the common shortcomings of meta-analysis which have been discussed in the research community, such as lack of individual patient data and inconsistent selection criteria. Another limitation concerns incomplete clinical information of patients in some cancer types from public datasets. In spite of these limitations, the
study certainly adds to our understanding of differences in immuno-therapy efficacy and immune profiling between the elderly and young.

CONCLUSIONS

In conclusion, age-biased immune profiling of the tumor properties and immune features are observed via a comprehensive pan-cancer analysis, and the balance between them could be involved in determining the ICB treatment efficacy, which might be beneficial to immunotherapy of elderly patients in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

N-HS and J-DX conceived and designed the study. Q-JZ, J-CL, and L-BS were responsible for data acquisition and analysis. Q-JZ, RC, C-JJ, and XZ wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by Medical key talent of Jiangsu Province (grant ZDRC2A016609); Fellowship of China Postdoctoral Science Foundation (grant 2020M671393); Jiangsu Province Postdoctoral Research Support Project (grant 2020ZJ134); the National Natural Science Foundation of China (81871151, 81971377).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.657575/full#supplementary-material

REFERENCES

1. FAO. The State of World Fisheries and Aquaculture 2018: Meeting the sustainable development goals. Rome: Food and Agriculture Organization of the United Nations (2018).
2. Xu JL, Lu YH. China fishery statistics yearbook. Beijing: China Agriculture Press (2018).
3. Zheng LB, Wang J, Mao Y, Chen RN, Su YQ, Chen J, et al. A novel stimulator of interferon gene (STING) from Larimichthys crocea and their involvement in immune response to ectoparasite Cryptocaryon irritans infection. Fish Shellfish Immun (2017) 71:239–45. doi: 10.1016/j.fsi.2017.09.056
4. Pang HY, Chen LM, Horare R, Huang YC, Wu ZH, Jian JC. Identification of DLD, by immunoproteomic analysis and evaluation as a potential vaccine antigen against three Vibrio species in Epinephelus coioides. Vaccine (2016) 34:1225–31. doi: 10.1016/j.vaccine.2015.11.001
5. Wang XW, Ao QJ, Li QG, Chen XH. Quantitative detection of a marine fish iridovirus isolated from large yellow croaker, Pseudosciaena crocea, using a molecular beacon. J Virol Methods (2006) 133:76–81. doi: 10.1016/j.jvirom.2005.10.025
13. Gregory AE, Titball R, Williamson D. Vaccine delivery using nanoparticles. 

12. Mody KT, Popat A, Mahony D, Cavallaro AS, Yu CZ, Mitter N. Mesoporous silica nanoparticles (Richardson) reared in Xiangshan Bay, China. *Aquacult Rep* (2016) 3:220–4. doi: 10.1016/j.aqp.2016.04.004

10. Smith JD, Morton LD, Ulery BD. Nanoparticles as synthetic vaccines. 

8. Wang Y, Liu GL, Li DL, Ling F, Zhu B, Wang GX. The protective immunity against grass carp reovirus in grass carp induced by a DNA vaccination using single-walled carbon nanotubes as delivery vehicles. *Fish Shellfish Immun* (2015) 47:732–42. doi: 10.1016/j.fsi.2015.10.029

7. Liu L, Ge MF, Zheng XY, Tao Z, Zhou SM, Wang GL. Investigation of *Vibrio alginolyticus*, V. harveyi, and *V. parahaemolyticus* in large yellow croaker, *Pseudosciaena crocea* (Richardson) reared in Xiangshan Bay, China. *Aquacult Rep* (2016) 3:220–4. doi: 10.1016/j.aqp.2016.04.004

6. Chen Q, Yan QP, Wang KJ, Zhuang ZX, Wang XR. Portal of entry for pathogenic *Vibrio alginolyticus* into large yellow croaker *Pseudosciaena crocea*, and characteristics of bacterial adhesion to mucus. *Dis Aquat Organ* (2008) 80:181–8. doi: 10.3354/dae01933

5. Marrack P, Mckee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol* (2009) 9:287–93. doi: 10.1038/nri2510

4. Amirali P, Sandy BH, Frances S, Liu J, Qiao SZ, Lu GQ. Mesoporous silica nanoparticles for bioadhesion, enzyme immobilisation and delivery carriers. *Fish Shellfish Immun* (2011) 31:2801–18. doi: 10.1016/j.fsi.2011.02.024

3. Preety S, Lukasz O, James M. Biomaterials for nanoparticle vaccine delivery systems. *Pharm Res-DoRdr* (2014) 31:2563–82. doi: 10.1007/s11095-014-1419-y

2. Amrilli P, Sandy BH, Frances S, Liu J, Qiao SZ, Lu GQ. Mesoporous silica nanoparticles for bioadhesion, enzyme immobilisation and delivery carriers. *Fish Shellfish Immun* (2011) 31:2801–18. doi: 10.1016/j.fsi.2011.02.024

1. Wang ZH, Shao YN, Li CH, Zhang WW, Duan XM, Zhao XL, et al. RNA-seq analysis revealed ROS-mediated related genes involved in cadmium detoxification in the razor clam *Sinonovacula constricta*. *Fish Shellfish Immun* (2016) 57:350–61. doi: 10.1016/j.fsi.2016.08.051

18. Yan DW, Shi Y, Wang HW, Li GX, Li XS, Wang BB, et al. A single mutation at position 136 in the envelope protein of tentbus virus is responsible for virus tissue tropism and transmissibility in ducks. *J Virol* (2019) 92:e00427–18. doi: 10.1128/JVI.00427-18

17. Wang ZH, Shao YN, Li CH, Zhang WW, Duan XM, Zhao XL, et al. RNA-seq analysis revealed ROS-mediated related genes involved in cadmium detoxification in the razor clam *Sinonovacula constricta*. *Fish Shellfish Immun* (2016) 57:350–61. doi: 10.1016/j.fsi.2016.08.051

16. Kole S, Qadiri SSN, Shin SM, Kim WS, Lee J, Jung SJ. Nanoencapsulation of inactivated-viral vaccine using chitosan nanoparticles: evaluation of its protective efficacy and immune modulatory effects in olive flounder (*Paralichthys olivaceus*) against viral haemorrhagic septicemia virus (VHSV) infection. *Fish Shellfish Immun* (2019) 91:136–47. doi: 10.1016/j.fsi.2019.05.017

15. Pavlinic N, Jerina I, Hrnjak J, Hribar A, Drob D, Ciglar G. Characterisation of *Vibrio parahaemolyticus* and *V. alginolyticus*, and characteristics of bacterial adhesion to mucus. *Aquaculture* (2019) 452:263. doi: 10.1016/j.aquaculture.2019.11.013

14. Mahony D, Cavallaro AS, Mody KT, Xiong L, Mahony TJ, Qiao SZ, et al. Development of oral DNA vaccine based on chitosan nanoparticles for the immunisation against redfish body iridovirus in turbot (*Scophthalmus maximus*). *Aquaculture* (2016) 452:263–71. doi: 10.1016/j.aquaculture.2015.11.013

13. Gregory AE, Titball R, Williamson D. Vaccine delivery using nanoparticles. *Front Cell Infect Mi* (2013) 3:13. doi: 10.3389/fimmu.2013.00013

12. Mahboobeh E, Maryam H, Mohnen M, Golamreza H, Khalil A, Mohammad R, et al. Co-delivery of dual toll-Like receptor agonists and antigen in poly (lactic-co-glycolic) acid polyethyleneimine cationic hybrid nanoparticles promote efficient in vivo immune responses. *Front Immunol* (2017) 8:1077. doi: 10.3389/fimmu.2017.01077

11. Harun NO, Costa MM, Secombes CJ, Wang TH. Sequencing of a second *Scophthalmus maximus* genome. *Biomaterials* (2015) 60:225–35. doi: 10.1016/j.biomaterials.2015.11.005

10. Smith JD, Morton LD, Ulery BD. Nanoparticles as synthetic vaccines. *Curr Opin Biotech* (2015) 34:217–24. doi: 10.1016/j.copbio.2015.03.014

9. Shaalan M, Saleh M, El-Mahdy M, El-Matbouli M. Recent progress in applications of nanoparticles in fish medicine: a review. *NanoMed-Nanotech* (2016) 12:701–10. doi: 10.1016/j.nano.2015.11.005

8. Shaalan M, Saleh M, El-Mahdy M, El-Matbouli M. Recent progress in applications of nanoparticles in fish medicine: a review. *NanoMed-Nanotech* (2016) 12:701–10. doi: 10.1016/j.nano.2015.11.005

7. Shaalan M, Saleh M, El-Mahdy M, El-Matbouli M. Recent progress in applications of nanoparticles in fish medicine: a review. *NanoMed-Nanotech* (2016) 12:701–10. doi: 10.1016/j.nano.2015.11.005

6. Shaalan M, Saleh M, El-Mahdy M, El-Matbouli M. Recent progress in applications of nanoparticles in fish medicine: a review. *NanoMed-Nanotech* (2016) 12:701–10. doi: 10.1016/j.nano.2015.11.005

5. Shaalan M, Saleh M, El-Mahdy M, El-Matbouli M. Recent progress in applications of nanoparticles in fish medicine: a review. *NanoMed-Nanotech* (2016) 12:701–10. doi: 10.1016/j.nano.2015.11.005

4. Shaalan M, Saleh M, El-Mahdy M, El-Matbouli M. Recent progress in applications of nanoparticles in fish medicine: a review. *NanoMed-Nanotech* (2016) 12:701–10. doi: 10.1016/j.nano.2015.11.005

3. Shaalan M, Saleh M, El-Mahdy M, El-Matbouli M. Recent progress in applications of nanoparticles in fish medicine: a review. *NanoMed-Nanotech* (2016) 12:701–10. doi: 10.1016/j.nano.2015.11.005

2. Shaalan M, Saleh M, El-Mahdy M, El-Matbouli M. Recent progress in applications of nanoparticles in fish medicine: a review. *NanoMed-Nanotech* (2016) 12:701–10. doi: 10.1016/j.nano.2015.11.005

1. Shaalan M, Saleh M, El-Mahdy M, El-Matbouli M. Recent progress in applications of nanoparticles in fish medicine: a review. *NanoMed-Nanotech* (2016) 12:701–10. doi: 10.1016/j.nano.2015.11.005

0. Shaalan M, Saleh M, El-Mahdy M, El-Matbouli M. Recent progress in applications of nanoparticles in fish medicine: a review. *NanoMed-Nanotech* (2016) 12:701–10. doi: 10.1016/j.nano.2015.11.005
45. Zaharoff DA, Rogers CJ, Hance KW, Schlom J, Greiner JW. Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. Vaccine (2005) 23:2085–94. doi: 10.1016/j.vaccine.2005.11.034

46. Carroll EC, Jin L, Mori A, Wolf NM, Olezycka E, Moran HBT, et al. The vaccine adjuvant chitosan promotes cellular immunity via DNA sensor cGAS-STING-dependent induction of type I interferons. *Immunity* (2016) 44:597–608. doi: 10.1016/j.immuni.2016.02.004

47. Zhu WT, Zhang YQ, Zhang JC, Yuan GL, Wu QL, Zhang XX, Niu BY, Wu BY, et al. Astragalus polysaccharides, chitosan and poly(I:C) obviously enhance inactivated *Edwardsiella ictaluri* vaccine potency in yellow catfish *Pelteobagrus fulvidraco*. *Fish Shellfish Immun* (2019) 87:379–85. doi: 10.1016/j.fsi.2019.01.033

48. Mohamedi SA, Heath AW, Jennings R. A comparison of oral and parenteral routes for therapeutic vaccination with HSV-2ISCOMs in mice: cytokine profiles, antibody responses and protection. *Antivir Res* (2001) 49:83–99. doi: 10.1016/S0166-3542(00)00142-X

49. Zhou YX, Quan GL, Wu QL, Zhang XX, Niu BY, Wu BY, et al. Mesoporous silica nanoparticles for drug and gene delivery. *Acta Pharm Sin B* (2018) 8:165–77. doi: 10.1016/j.apsb.2018.01.007

50. Zhu JF. T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. *Cytokine* (2015) 75:14–24. doi: 10.1016/j.cyto.2015.05.010

51. Gour N, Wills-Karp M. IL-4 and IL-13 signaling in allergic airway disease. *Cytokine* (2015) 75:68–78. doi: 10.1016/j.cyto.2015.05.014

52. Croissant JG, Fatieiev Y, Almalik A, Khashab NM. Mesoporous silica and organosilica nanoparticles: physical chemistry, biosafety, delivery strategies, and biomedical applications. *Adv Healthc Mater* (2018) 7:1700831. doi: 10.1002/adhm.201700831

53. Wangkahart E, Secombes CJ, Wang T. Studies on the use of flagellin as an immunostimulant and vaccine adjuvant in fish aquaculture. *Front Immunol* (2018) 9:3054. doi: 10.3389/fimmu.2018.03054

54. Fifs T, Gamvrelis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. *J Immunol* (2004) 173:3148–54. doi: 10.4049/jimmunol.173.5.3148

55. Duan JC, Yu YB, Shi HQ, Tian LW, Guo CX, Huang PL, et al. Toxic effects of silica nanoparticles on zebrafish embryos and larvae. *PLoS One* (2013) 8: e74606. doi: 10.1371/journal.pone.0074606

56. Laing KI, Secombes CJ. Chemokines. *Dev Comp Immunol* (2004) 28:443–60. doi: 10.1016/j.dci.2003.09.006

57. Fredriksen BN, Saevareid K, McAuley L, Lane ME, Bogwald J, Dalmo RA. Early immune responses in Atlantic salmon (*Salmo salar L.*) after immunization with PLGA nanoparticles loaded with a model antigen and beta-galucan. *Vaccine* (2011) 29:8338–49. doi: 10.1016/j.vaccine.2011.08.087

58. Siegrist F, Ebeling M, Certa U. The small interferon-induced transmembrane protein 1a as an immunostimulant and vaccine adjuvant chitosan promotes cellular immunity via DNA sensor cGAS-STING-dependent induction of type I interferons. *Immunity* (2016) 44:597–608. doi: 10.1016/j.immuni.2016.02.004

59. Pereiro P, Forn-Cuni G, Figueras A, Novoa B. Pathogen-dependent role of turbot (*Scophthalmus maximus*) interferon-gamma. *Fish Shellfish Immun* (2016) 59:25–35. doi: 10.1016/j.fsi.2016.10.021

60. Lo D, Feng LL, Li L, Carson MJ, Crowley M, Pauza M, et al. Integrating innate and adaptive immunity in the whole animal. *Immunol Rev* (1999) 169:225–39. doi: 10.1111/j.1600-065X.1999.tb01318.x

61. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity* (2010) 32:593–604. doi: 10.1016/j.immuni.2010.05.007

62. Mu PF, Wang YH, Ao QJ, Ai CX, Chen XH. Molecular cloning and bioactivity of an IL-2 homologue in large yellow croaker (*Larimichthys crocea*). *Fish Shellfish Immun* (2018) 81:309–17. doi: 10.1016/j.fsi.2018.07.040

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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