Establishment of Inflammatory Tumors in A Mouse Model for The Evaluation of Tumor Progression

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Background of research

An estimated 13 million people will die from cancer in the next decade [1]. About 400 new cases are reported in Hong Kong each year, with a higher prevalence in males (3:1) and the elderly. Although genitourinary cancer is particularly prone to recurrence, early detection has a good prognosis. Furthermore, most cancer patients are highly susceptible to bacterial infections after chemotherapy, and using the wrong antibiotics may affect their prognosis and survival [2, 3]. Recent studies that revealed intracellular bacteria's role in cancer cells mediating chemotherapeutic drug resistance and metastasis [Science 2017 and Cell 2022] highlight the urgent need to understand bacteria-tumor interactions in the context of tumor progression. **Predicting treatment outcomes and selecting the best antimicrobial combination therapy is critical to treating cancer effectively.** Although tumor models are widely used in laboratory settings, most are not translational because immune cells and other tumour microenvironment elements are not considered, resulting in an oversimplified model component. These procedures are critical given the aging population and the need for regular checkups for genitourinary cancer patients.

Elevated Incidence of Systemic Infections in Individuals: Following chemotherapy, patients often become highly vulnerable to bacterial infections, and inappropriate antibiotic treatment may exacerbate their prognosis and diminish survival rates. Chronic inflammation resulting from bacterial colonization has been identified as a significant factor in the initiation and advancement of cancer. Several years ago, bacteria colonizing human pancreatic tumor cells was discovered, yet comprehensive knowledge regarding the repercussions of these interactions remains largely obscure. Bacterial infections are linked to cancer development, particularly in the digestive and urinary systems, where they can induce chronic inflammation or generate carcinogenic metabolites, fostering cancer-promoting effects. Clinical evidence indicates that Escherichia coli and Clostridium nucleatum possess carcinogenic potential in colorectal cancer patients. At the same time, Helicobacter pylori infection exhibits a strong correlation with the risk of gastric and pancreatic cancer.

Cancer Mortality: It has been verified that diverse forms and locations of bacterial infection and colonization occur in various tumor types and stages. Recent research indicates that the health status of cancer patients with systemic bacterial infections may be influenced, suggesting that circulating bacteria could impact tumor progression. With the introduction of antibiotics in cancer therapy, there has been a shift in the predominant bacterial pathogens from Gram-positive to Gram-negative bacteria in recent decades, and these bacteria exhibit increased antibiotic resistance [22]. Consequently, bacterial infections in cancer patients have emerged as a significant complication during chemotherapy. Gram-negative bacteria predominantly cause sepsis in most cancer patients, with an average bacteremia recovery rate of only 51.3% (ranging from 24.7% to 75.8%) observed from 2007 to 2014 in this patient cohort.

Animal Tumor Models: Immunocompetent tumor mouse models have been widely used, particularly in studying bacteria-tumor and immune-tumor interactions. These models, which retain a fully functioning immune system, allow for exploring the complex interplay between the tumour microenvironment, the immune response, and the potential influence of pathogens. They provide valuable insights into the mechanisms of tumor progression, immune evasion, and possible therapeutic strategies. One notable model is the MB49 bladder cancer model, which has been instrumental in understanding the intricacies of bladder cancer biology and testing novel immunotherapies. Other relevant models include the 4T1 breast cancer model and the B16F10 melanoma model, both widely used for their ability to mimic the immune response seen in human cancers. In this proposal, we will use the MB49 model as a proof of concept study, with plans to extend our work to other tumor models.

Complexity in tumor microenvironments: The tumor microenvironment (TME) is a complex population, including tumor cells and various immune cells. The tumor-associated components in TME are highly heterogeneous and could contribute to cancer development and poor prognosis.

The pathogens within the tumor inflammatory microenvironment can colonize different solid tumour regions. The infecting bacteria, which can be classified as intratumoral bacteria (IB; within tumors) and extratumoral bacteria (EB; outside tumors), are linked to cancer development, especially in the digestive and urinary systems, and can cause chronic inflammation and lead to cancer-promoting effects. Furthermore, components of the TME, such as tumor-associated macrophages (TAMs), influence tumor progression. The specific polarization and phenotypic transition of TAMs in the tumor microenvironment lead to two-pronged impacts that could promote or hinder cancer development and treatment. As such, tumour characterisation and inflammatory interactions in vivo and in vitro are highly warrented.

Previous related work by the team: The PI's team introduced microwell arrays designed to create consistent co-cultures of bacteria-tumor clusters under defined conditions (**Figure 1**). These microwell-based assays facilitated the development of 3D structured biofilms, providing a more accurate representation of in vivo biofilms than conventional 2D models. By analyzing CV intensity through OD595 absorbance values and examining the structure via scanning electron microscopy (SEM) images, it was confirmed that biofilm presence is specific to the CT model. Co-cultures were established using bladder cancer cell lines (UMUC3) and pathogenic Escherichia coli (UTI89). Uropathogenic E. coli (UPEC) infection causing urinary tract infection (UTI) has been demonstrated as a contributing factor to bladder cancer development. Tumor clusters maintained viability before the onset of disease. The cells were homogeneously dispersed in the growth medium and introduced into each microchannel at the optimal concentration. After 24 hours, they formed uniform clusters in the center of each microwell, ready for subsequent analysis. These clusters can undergo live/dead staining, immunostaining, crystal violet staining, and colony-forming unit (CFU) quantification to investigate tumor response (**Figure 2**). The spatial distribution of bacteria within the tumor seems to influence the tumor response, evident in the higher cluster adherence observed with extratumoral bacteria (EB) instead of intratumoral bacteria (IB).

Co-PI **Prof Chow's** team employs various tumor mouse models to study immune-cancer interactions. Using the MB49 bladder cancer model, the team validated the above findings from the microwell system that UPEC infection enhanced bladder tumor growth *in vivo* compared to uninfected tumor, demonstrating that bacterial infection exacerbates tumor progression. Moreover, tumors infected by UPEC lacking biofilm-formation capability (fimH KO) showed retarded growth as compared to wild-type (WT) UPEC. Two key biofilm genes, fimH and csgA, were confirmed to be expressed only in WT but not in KO UPEC. Furthermore, histopathological staining showed that WT UPEC infection led to more disorganized and loosely arranged bladder tissue, indicating increased invasiveness of the tumor cells (Figure 4). Together, these results demonstrated that the MB49 inflammatory bladder cancer model is a robust tool to validate and complement the microwell-based *in vitro* screening assays.

This research aims to establish a mouse model of inflammatory tumors with clinical relevance. This model is anticipated to enhance our comprehension of the influence of bacteria on tumor progression. Furthermore, we expect that this model will serve as a valuable tool for drug discovery in the biomedical and pharmaceutical sectors, specifically for developing antibacterial agents.

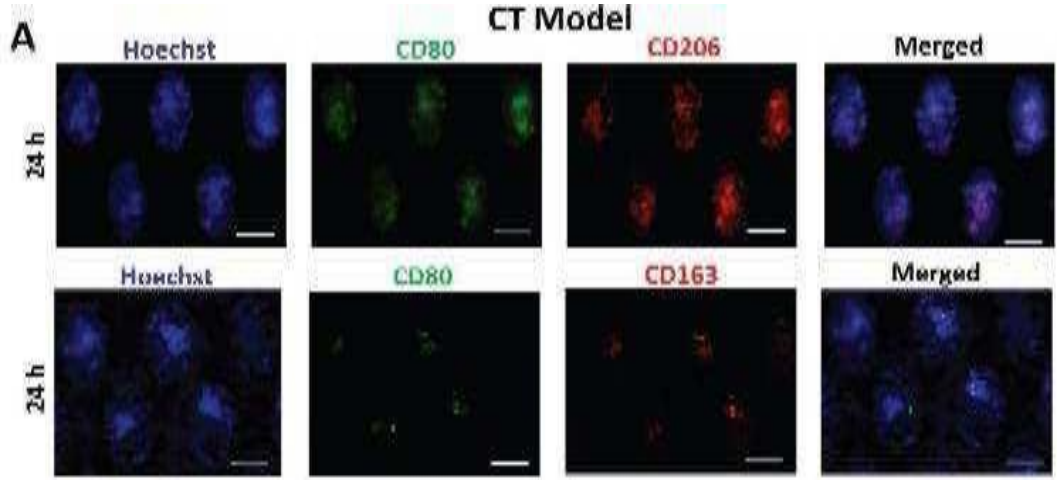

Figure 1: EB-based biofilms induced the transition of macrophages from M1 to M2 phenotypes. Representative fluorescent images of A) CT models after 24 h infection under MOI 1:1. Cells were stained with Hoechst (blue) and labelled for macrophage-specific biomarkers CD80 (green)/CD206 (red) and CD80/CD163(red): scale bar, 100 μm.

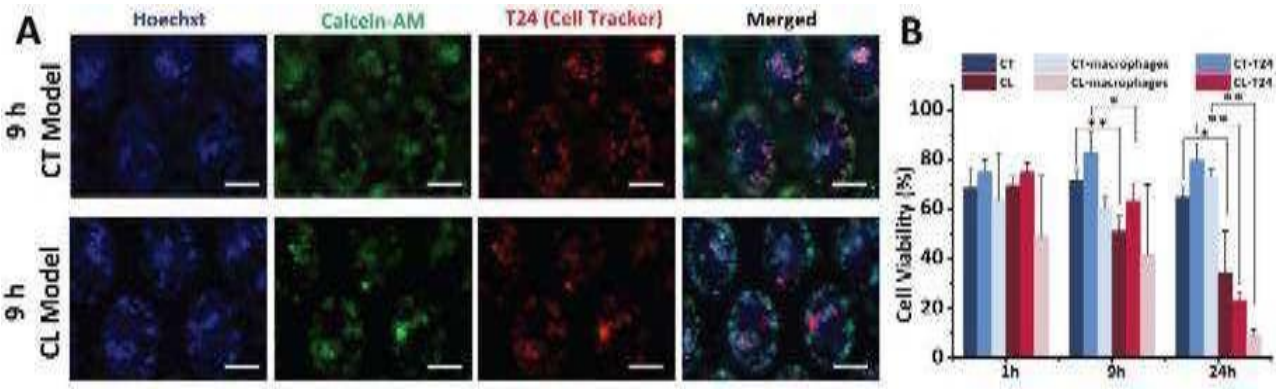

Figure 2: The presence of biofilm-induced M2 TAMs protected cancer cells from cell death and apoptosis. Representative images under A) cell viability assay in situ. Scale bar, 100 μm. B) Viability of M1 macrophages and T24 co-culture clusters at MOI 1:1 after infection at 1, 9, and 24 h. ***, $p < 0.001$; **$p < 0.01$; *, $p < 0.05$.

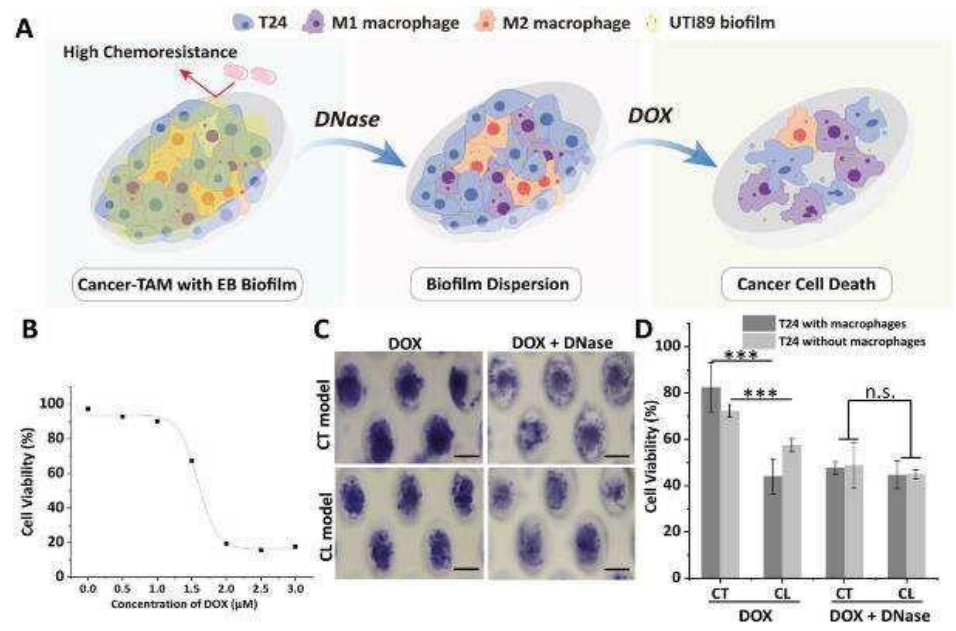

Figure 3: Combinational therapy of biofilm dispersing agent and anti-cancer drug enhances the therapy response of cancer cells. A) Schematics of screening procedures with DNase-DOX combinational treatment. B) Dose-response curve of DOX for cancer cell clusters. The corresponding DOX IC50 value of T24 clusters was 1.625 μm. C) Crystal violet (CV) staining of bacteria-infected clusters in CT and CL models in situ after treatment with single drug DOX or combinatorial treatment with DOX and 1× DNase. D) Cell viability in CT and CL models of macrophage-T24 culture or T24 without macrophages after treatment with single drug

DOX at IC50 concentration or combinatorial treatment with DOX and 1× DNase (T24: macrophage ratio: 2:1). ***p < 0.001; **p < 0.01; *p < 0.05; n.s., p ≥ 0.05, indicating no significant difference.

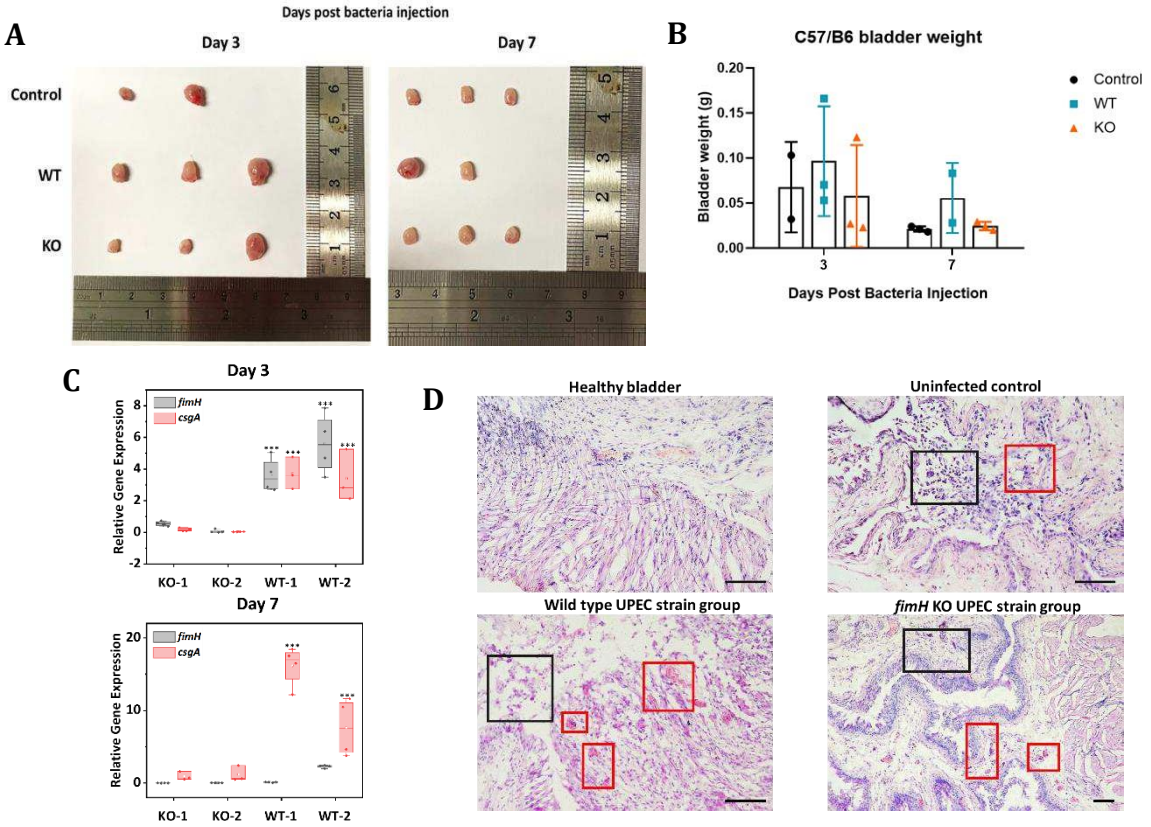

Figure 4: UPEC infection enhanced tumor growth *in vivo*. A) images of mouse bladders containing tumors after infecting with wildtype (WT) or fim knockout (KO) UPEC for 3 days and 7 days. B) weight of mouse bladders containing tumors after infecting with WT or KO UPEC for 3 days and 7 days. C) real-time quantitative PCR of biofilm genes in bladders containing tumors infected with WT and KO UPEC. D) H&E staining of bladder tissue from healthy bladder, uninfected bladder tumor, and bladder tumors infected with WT or KO UPEC.