RESEARCH LETTER

CD24 Is a Superior Immunotherapeutic Target to PD-1 in a Mouse Model of *Helicobacter*-Induced Gastric Cancer

Programmed death-1 (PD-1) antibody treatment has been recently approved for patients with advanced gastric cancer. Despite its success in several types of cancer, the efficacy of anti-PD-1 therapy is limited by many factors, such as tumor infiltration of effector lymphocytes and expression level of programmed death ligand-1 (PD-L1) which can be quantified as a combined positive score (CPS). An overexpression of PD-L1/PD-L2 has been identified in Epstein-Barr virus (EBV)–positive gastric adenocarcinoma, suggesting these patients may benefit from PD-1/PD-L1 blockade.[1] However, EBV-associated gastric cancer accounts for only 10% of all cases worldwide.[2] Accordingly, a meta-analysis for clinical trials with immune checkpoint inhibitors for advanced gastric cancer or esophagogastric junction tumors indicates that the objective response rate for anti-PD-1 treatment is 12% and the progression-free survival is 1.61 months.[3] The overall benefits from anti-PD-1/PD-L1 therapy for patients with gastric cancer remains unsatisfactory, and exploration of other immunotherapeutic targets is warranted.

The majority of clinically approved cancer immunotherapies target T-cell-mediated adaptive immunity. Until recently, various approaches targeting the innate immunity such as antagonist for the phagocytosis checkpoint CD47 have been developed.[4] CD24 is the most recent "don't eat me" signal identified in ovarian and breast tumor cells and suppresses phagocytosis via Siglec-10 expressed on macrophages.[5] However, the outcome of systemic inhibition of CD24 has not been fully explored in an immunocompetent mouse model of cancer.

As reported, CD24 overexpression is associated with poor survival of patients with gastric cancer, which implies its potential as a therapeutic target.[6] To investigate this issue, we first compared CD24 expression in normal and tumor tissues. Human gastric cancer samples exhibited a higher CD24 mRNA level than normal stomach tissues (Figure A1A). What is more, in a clinically relevant gastric cancer mouse model established by treatment with *Helicobacter* (*H.*) *felis* bacteria and chemical carcinogen N-methyl-N-nitrosourea (MNU), a higher percentage of gastric epithelial cells expressed CD24 in the tumor model than the normal control (Figure A1B). Interestingly, *H. felis*/MNU treatment strongly reduced PD-L1 expression in the mouse stomach (Figure A1C). Consistently, the expression level of PD-L1 in epithelial cells was also negligible (Figure 1B), which means this model can be considered CPS low.

It has been pointed out that antigen expression screening in patients for targeted therapies is essential to achieve the maximum therapeutic benefit of the treatment.[7] Thus, it can be speculated that the expression level of the inhibitory molecules is closely related to the responsiveness of these antibodies. Therefore, we further compared the expression levels of CD24 with the other two immune inhibitory molecules PD-L1 and CD47 in human gastric cancer samples and found that CD24 mRNA level was higher than either PD-L1 or CD47 (Figure 1A). In *H. felis*/MNU-induced gastric cancer in mice, membrane expression of CD24 protein in gastric epithelial cells was also more prevalent than PD-L1 and CD47 (Figure 1B). Together, CD24 serves as a potential therapeutic target and its abundance may be indicative for the suitableness of anti-CD24 therapy for gastric cancer.

To evaluate the therapeutic potential of systemic inhibition of CD24, we applied the CD24 blocking antibody in comparison with one of the most popular immunotherapeutic agents, PD-1 antibody in the *H. felis*/MNU-induced gastric cancer model (Figure 1C). Anti-CD24 treatment greatly repressed the tumor growth, while anti-PD-1 treatment showed no significant effect (Figure 1D and E). The pathological features including metaplastic and dysplastic grades were significantly reduced by CD24 antibody (Figure 1F). Moreover, anti-CD24 treatment did not change the ratio of $CD24^+$ cells in the stomach, suggesting that the therapeutic effect was not attributed to antibody-mediated depletion of CD24-expressing cells (Figure A2). Consistently, in a recent study using H/K-ATPase-IL-1β or gastrin-deficient mice, anti-PD-1 treatment also failed to inhibit the growth of established stomach tumors.[8]

We further analyzed the immune cells in the stomach tumors by fluorescence activated cell sorting. The ratios of $CD45^+$ cells in total cells were similar among all groups, while both anti-CD24 and anti-PD-1 treatments increased the ratios of $CD4^+$ and $CD8^+$ T cells and decreased the percentage of regulatory T cells in the stomach (Figure 2A–D), even though PD-1 antibody did not shrink the tumor. Then diving deeply into the T-cell population, we found no significant difference in the ratio of $CD69^+CD4^+$ T cells while there was a striking bump of $CD69^+CD8^+$ T cells after anti-CD24 or anti-PD-1 treatments (Figure A3), suggesting CD24 blockade also boosted the activity of $CD8^+$ T cells. Notably, neutrophils showed a significant upregulation in the stomach upon anti-PD-1 treatment, which might at least partly result in immune suppression and compromised antitumor effects mediated by T cells (Figure 2E) because tumor-associated neutrophils have been shown to foster gastric cancer progression.[9] Downregulation

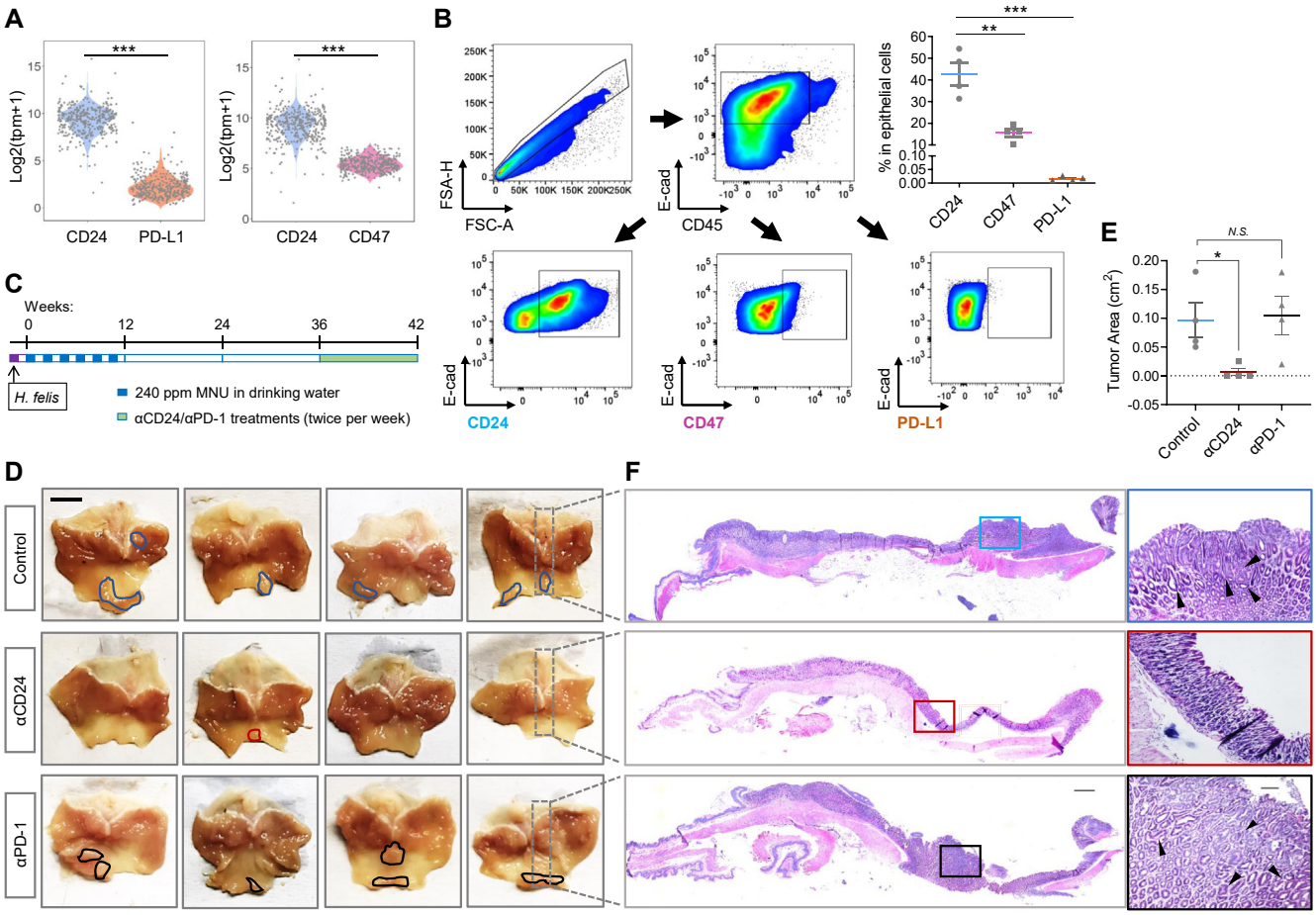

Figure 1. CD24 blockade effectively represses the gastric cancer progression in mice. (A) Comparison of mRNA expression levels for CD24 with PD-L1 or CD47 in human stomach adenocarcinoma (n = 375) from the TCGA database. (B) Fluorescence activated cell sorting analysis of CD24, PD-L1, and CD47 expression levels on the surface of gastric epithelial cells in *H. felis*/MNU gastric cancer mice. (C) Scheme of antibody treatments. (D) Macroscopic images of stomachs from *H. felis*/MNU mice treated with anti-CD24 (αCD24), anti-PD-1 (αPD-1), or control antibodies. Scale bar = 50 mm. (E) Quantification of stomach tumor areas in *H. felis*/MNU mice. (F) HE staining of the whole-stomach sections. Black arrowheads indicated invasive tumor cells in the stomach mucosa. Scale bars = 500 μm (left) and 100 μm (right). *$P < .05$, **$P < .01$, ***$P < .001$, *N.S.*, not significant.

of PD-L1 expression by *H. felis*/MNU treatment in the mouse stomach might represent another reason for the limited effectiveness of anti-PD-1 therapy (Figure A1C).

Given an identified role of CD24 in the inhibition of macrophage-mediated phagocytosis previously, we also investigated whether CD24 inhibition might influence the phenotype of tumor-associated macrophages (TAMs). A trending shift from M2-like to M1-like phenotype was observed in the CD24 antibody-treated *H. felis*/MNU mice (Figure A4A–E). This indicated that targeting CD24 might also contribute to polarization of the TAMs to M1 phenotype. Because it was observed that CD24 inhibition led to an enhanced phagocytosis of B cells by macrophages in vitro,[5] we also explored whether systemic blockade of CD24 in vivo would jeopardize B cells. Anti-CD24 treatment showed no obvious impact on the B cells from the spleens but significantly increased the ratios of B cells in both blood and stomach (Figure A4F). From the functional side, expression of the activation marker CD69 on B cells was not affected by CD24 antibody either (Figure A4G). Thus, systemic administration of CD24 antibody did not cause unwanted effects on the B cells; on the contrary, it promoted their presence in the stomach.

Driven by some unsatisfactory therapeutic results of PD-1 inhibitors in the clinic and the potential of phagocytosis checkpoint CD24 as an immunotherapeutic target,[5] our work went deeper into the therapeutic potential of anti-CD24 treatment in an immunocompetent, clinically relevant model of spontaneous gastric cancer and demonstrated that CD24 was a superior therapeutic target to PD-1 in this mouse model.

Given the critical role of innate immunocytes in priming adaptive immune responses, accumulating evidence has highlighted a dual role of CD47, the first identified phagocytosis checkpoint, in eliciting innate and adaptive antitumor immunity.[4,10] Consistently, our

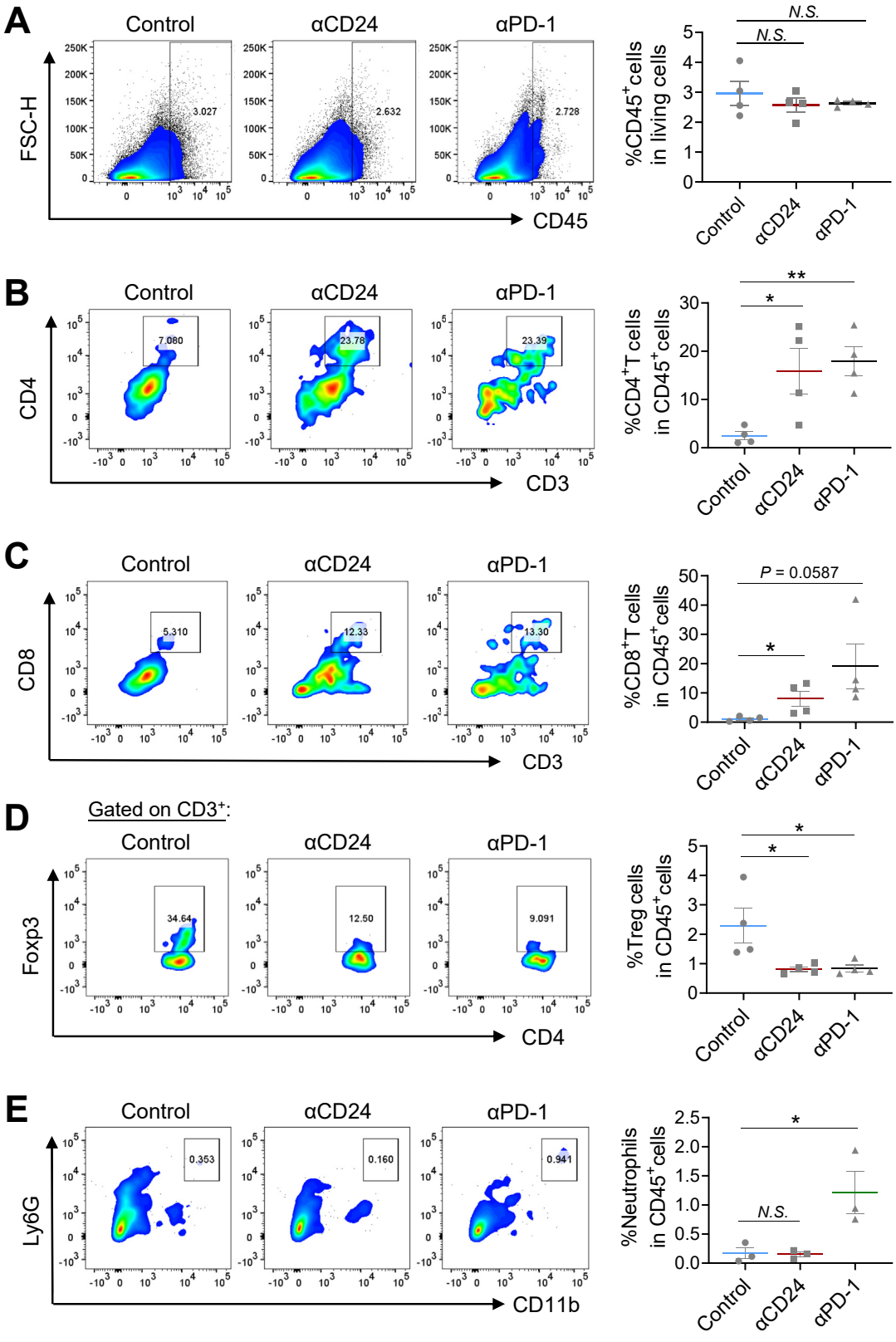

Figure 2. Analysis of tumor microenvironment upon anti-CD24 and anti-PD-1 therapies in *H. felis*/MNU-induced gastric cancer model. Fluorescence activated cell sorting analysis of immune cells including $CD45^+$ (A), $CD4^+$ T (B), $CD8^+$ T (C), regulatory T cells (D), and neutrophils (E) in the stomachs of *H. felis*/MNU mice treated with αCD24, αPD-1, or control antibodies. $^*P < .05$, $^{**}P < .01$, *N.S.*, not significant.

study also showed that systemic CD24 blockade coordinated both adaptive immunity via boosting tumor-infiltrating effector T and B lymphocytes and innate immunity via polarizing TAMs to M1 phenotype. Collectively, anti-CD24 therapy holds great promises for the treatment of gastric cancer by motivating both innate and adaptive immunity and could serve as an alternative or extra option for the nonresponders to anti-PD-1 therapy.

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Supplementary Materials

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Abbreviations used in this paper: **CPS, combined positive score; EBV, Epstein-Barr virus; MNU, N-methyl-N-nitrosourea; PD-1, programmed death-1; PD-L1, programmed death ligand-1; TAMs, tumor-associated macrophages**

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Ethical Statement:
The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

Data Transparency Statement:
The datasets used or analyzed and study materials will be available from the corresponding author on reasonable request.