Supplemental Material

Overcoming resistance of MMR-deficient tumors with high neutrophil levels to anti-PD-1 monotherapy by combination of anti-CTLA-4 and anti-PD-1 inhibitors

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Figs. S1 to S8
Supplemental figure S1: Gating strategy used for monitoring neutrophil population.
Flow cytometry analyses of blood neutrophils in 4T1 MMRDb tumor-bearing mice. Given that αLy6G antibody used for in vivo depletion of neutrophils was the same (clone 1A8) than the one used for blood immunomonitoring, the gating strategy 1 has been used for all experiments with neutrophil depletion. However, we validated that this immune population was Ly6Cmed Ly6Ghigh in mice treated with isotypes (strategy 2). The strategy 2 was used for all other experiments.
Supplemental figure S2: Monitoring neutrophils in blood after neutrophil depletion in 4T1 MMRD tumors-bearing mice

Longitudinal flow cytometry analyses of blood neutrophils of 4T1 MMRD8 tumors-bearing mice +/- depleted for neutrophil cells with αLy6G (1A8) antibody and subsequently treated with αPD-1 +/- αCTLA-4 or their respective isotypes (n=5-18 mice in each group). Data are shown as mean ± SEM. Symbol significance: * p≤0.05; ** p≤0.01; *** p≤0.001, **** p≤0.0001, + : treatment, - : isotype of treatment.
Supplemental figure S3: Longitudinal blood validation of Treg depletion using αCD25 antibody.

Longitudinal flow cytometry analyses of CD4+ and Treg (defined as CD4+ Foxp3+) cells in blood of 4T1 MMRDh tumor-bearing mice +/- depleted for Treg cells using αCD25 (PC-61.5) antibody and subsequently treated with αPD-1 or isotype (n=5-10 mice in each group). Data are shown as mean ± SEM. Statistical analyses were performed using Fisher LSD test for multiple comparisons. Symbol significance: * p≤0.05; ** p≤0.01; *** p≤0.001; **** p≤0.0001, +: treatment, -: isotype of treatment.
Supplemental figure. S4: Response rate to the αPD-1 + αCTLA-4 combination in 4T1 MMRD<sup>−/−</sup> syngeneic model

(A-B) Tumor growth kinetics and associated Kaplan Meier curves estimation of the indicated mice groups treated with αPD-1 + αCTLA-4 or its isotypes: 4T1<sup>MMRD<sup>−/−</sup></sup>, p30w, 4T1<sup>MMRD<sup>−/−</sup></sup>, p40w, 4T1<sup>MMRD<sup>−/−</sup></sup>, p30w, 4T1<sup>MMRD<sup>−/−</sup></sup>, p40w (n=5-26 mice/group). For tumor growth, data are shown as mean ± SEM and statistical analyses were performed using Mann Whitney test. For Kaplan Meier curves, statistical analyses were performed using Log-rank (Mantel-Cox) test. Symbol significance: * p≤0.05; *** ≤0.001, **** ≤0.0001.
Supplemental figure S5: Total regression of primary 4T1 MMRD希 tumors after immune checkpoint inhibitor treatment lead to a long-term T cell memory response.

Tumor growth kinetics during a 100-day period. Five mice bearing 4T1 MMRD希 tumors that underwent complete regression following αPD1 + αCTLA-4 antibodies treatment were rechallenged with either 4T1 parental cells (n=2) or 4T1 MMRD希 cells (n=3) more than 60 days after the first injection and tumor growth were monitored over 30-day period. Data are shown as mean ± SEM.
Supplemental figure S6: The αPD1 + αCTLA-4 combination treatment reshapes 4T1 MMRD^hi TME.

Flow cytometry analyses of tumor-infiltrating leukocytes in 4T1 MMRD^hi tumors resected 9 to 12 days after the first injection of αPD1 + αCTLA-4 antibodies or their isotypes (n=5-7 mice/group). The fraction of neutrophils (defined as CD11b^+ CD11c^− SiglecF^− Ly6G^hi Ly6C^int, among CD45^+), Cytotoxic T cells (CD8^+, among CD45^+), ICOS^+ cells (among CD4^+ and CD8^+), and PD-1^hi cells (among CD4^+ and CD8^+) are depicted. MMRD^hi tumors treated with isotypes (black), MMRD^hi tumors progressing under ICB treatment (blue) and MMRD^hi tumors responding to ICB treatment (pink) were analyzed separately. Data are shown as mean ± SEM. Statistical analyses were performed using Dunn’s test. Symbol significance: * p≤0.05; ** p≤0.01; *** ≤0.001, **** ≤0.0001.
Supplemental figure S7: 4T1 parental tumor-bearing mice remain refractory to the αPD-1 + αCTLA-4 combination after neutrophil depletion.

(A) Tumor growth kinetics of 4T1 parental tumor-bearing mice +/- depleted for neutrophil cells using αLy6G (1A8) antibody and subsequently treated with αPD-1 +/- αCTLA-4 (n=7 mice/group) (upper panel). (B) Longitudinal flow cytometry analyses of blood myeloid to lymphocyte ratio of 4T1 parental tumor-bearing mice +/- depleted for neutrophil cells using αLy6G (1A8) antibody and subsequently treated with αPD-1 +/- αCTLA-4 (n=7 mice in each group). Data are shown as mean ± SEM. Statistical analyses were performed using Mann Whitney test (Fig S7A) or Fisher LSD test (Fig S7B). Symbol significance: *** ≤0.001 and **** ≤0.0001.
Supplemental figure S8: Change of NLR two months after αPD(L)-1 treatment initiation can predict response or progression for patients with MMRD CRC or EC.

(A-B) Each arrow represents NLR at baseline (beginning of the arrow) and 2 months after αPD(L)-1 treatment initiation (end of the arrow) for one patient with CRC (A) or EC (B) who will progress (left panel), respond (middle panel) or have stable disease (right panel). Kaplan–Meier estimation of overall survival segregated by the percentage of NLR change after two months of αPD(L)1 treatment for colon (upper panel) and endometrium (lower panel) cancers are depicted.