Supplementary Figure 1. Flow cytometry gating for analysis of Tregs and PD1 expression in TILs. TILs were isolated as described in Material and Methods. Stained cells were first gated on lymphocytes based on the SSC-A and CD45, then CD4, CD8, CD25 and Foxp3, PD1, Granzyme B then CD69. Numbers indicate percentage of positive cells.

Supplementary Figure 2. Fractions of Tregs and resting dendritic cells in pre-nRT biopsies negatively correlated with prognosis of LARC. (A) Fractions of immune cell subsets in tumor samples inferred from gene-expression data using CIBERSORT. (B-C). Kaplan–Meier analysis of the correlation between Tregs (B) and resting dendritic cells(C) fraction and disease-free survival in patients of cohort 1. (D-E) Kaplan–Meier analysis of the correlation between Tregs (D) and resting dendritic cells (E) fraction and overall survival in patients in cohort 1. Using the log-rank test, statistical significance was set at $p < 0.05$.

Supplementary Figure 3. Several cytokines in pre- and post-nCRT serum correlated with prognosis. (A) Kaplan–Meier analysis of the correlation between GM-CSF level in pre-treatment serum and overall survival in patients of cohort 2. (B) Kaplan–Meier analysis of the correlation between IL-2 level in pre-nCRT serum and overall survival in patients of cohort 2. (C) Kaplan–Meier analysis of the correlation between IL-3 level in pre-nCRT serum and overall survival in patients of cohort 2. (D) Kaplan–Meier analysis of the correlation between IL-4 level in pre-nCRT serum and

overall survival in patients of cohort 2. (E) Kaplan–Meier analysis of the correlation between TNFα level in post-nCRT serum and disease-free survival in patients of cohort 2. Using the log-rank test, statistical significance was set at $p < 0.05$.

Supplementary Figure 4. The quantifications of CD8+, CD4+ T cells, Tregs and PD1+CD8+ or PD1+CD4+ T cells in unirradiated tumors from patients of cohort 3 were assayed using multiplex immunofluorescence staining. One group has one patient. Six to eleven fields for each patient are shown. Each point represents the count of each selected field.

Supplementary Figure 5. Determination of the optimal dosing schedule of anti-CD25 for depleting Treg cells in MC38 colon cancer-bearing mouse model. (A) The percentages of CD25-expressing CD8+, CD4+Foxp3-, CD4+Foxp3+ T cells in tumors from untreated C57BL/6 mice. (B) Representative dot plots show the percentages of CD4+Foxp3+ T cells in tumors from anti-CD25 treated and IgG control groups on day 3. CD4+Foxp3+ cells (%) 0, 1, 2, 5 and 8 days after the administration of 0.25mg anti-CD25 (C) and those on day 3 after the administration of various doses of anti-CD25 (D) in spleen and tumours from C57BL/6 mice. Assays were done by flow cytometry. Each experimental group consisted of three-four mice. Values are presented as means ± SD.

Supplementary Figure 6. Dose-fractionated RT combined with anti-CD25 enhances tumor growth control in a bilateral mouse model. (A) Schematic of the experimental setup. Yellow arrow: radiotherapy given (5 fractions of 2.3 Gy each). Timeline starts from original tumor implantation (day 0). Black arrows: drug treatments given. (B-C) Total irradiated and unirradiated tumor growth for MC38 tumors after the indicated treatment. Control: IgG (n=7); Radiotherapy: IgG +RT (n=8); Anti–CD25 (n=7); RT combined with anti–CD25 (n=8). Student's t test was used to compare tumor volume differences between treated groups and control groups, statistical significance was set at $p < 0.05$.

Supplementary Figure 7. Single dose RT combined with anti–CD25 changes the fractions of T cells in tumor or blood. (A) Proportion of CD44+ in CD3+ T cells in tumors. (B) Proportion of CD44+ in CD4+ T cells in tumors. (C) Proportion of CD44+ in CD8+ T cells in tumors. (D) Proportion of CD4+ T cells after gating on CD45+ cell in blood. (E) Proportion of CD8+ T-cells in blood. (F) Proportion of Tregs in blood. (G) Ratio of CD8+T cells/Tregs in blood. (H) Proportion of PD1+CD4+ T cells in blood. (I) Proportion of PD1+CD8+ T cells in blood. Student's t test was used to compare immune cell fraction differences between treated groups and control groups, statistical significance was set at $p < 0.05$.

Supplementary Figure 8. Dose-fractionated RT combined with anti-CD25 changes the fractions of CD4+, CD8+ and Tregs cells in irradiated and

unirradiated tumors and in blood. (A) Proportion of CD8+, CD4+ T cells and CD4+Foxp3+(Tregs), CD8+ and CD4+ T cells expressing Granzyme B and PD1+CD8+ T cells in irradiated tumors. (B) Representative dot plots show the percentages of CD4+Foxp3+ Tregs in irradiated tumors. (C) Representative dot plots show the percentages of CD8+ T cells expressing Granzyme B in irradiated tumors. (D) Proportion of CD8+, CD4+ T cells and Tregs in unirradiated tumors. (E) Proportion of CD8+, CD4+ T cells, CD8+CD69+ T cells and Tregs in blood. (F) Representative dot plots show the percentages of CD8+CD69+ T cells in blood. Dunnet t test was used to compare immune cell fraction differences between treated groups and control groups, statistical significance was set at $p < 0.05$.

Supplementary Figure 9. Determination of the optimal dosing schedule of anti-CTLA4 for depleting Treg cells in MC38 colon cancer-bearing mouse model. (A) Representative dot plots show the percentages of CD25+Foxp3+ T cells in tumors from anti-CTLA4 treated and IgG control groups on day 3. CD25+Foxp3+ cells (%) 0, 1, 2, 5 and 8 days after the administration of 0.25mg anti-CTLA4 (B) and those on day 3 after the administration of various doses of anti-CTLA4 (C) in spleen and tumor cells from C57BL/6 mice. Assays were done by flow cytometry. Each experimental group consisted of three-four mice. Values are presented as means ± SD.

Supplementary Figure 10. Representative dot plots show the percentages of CD4+, CD8+ T-cells(A), Tregs(B), PD1+CD8+/CD4+(C-D) and

CD44+CD4+/CD8+(E-F) T cells after gating on CD45+ cells analyzed by flow cytometry in the liver metastasis-derived cell suspensions corresponding to RT, RT+anti-CTLA4, RT+anti-CTLA4+anti-PD1 as indicated.

Supplementary Figure 11. RT combined with anti–CTLA4/anti–CTLA4+ anti-PD1 changes the fractions of TIL cells in irradiated tumors and blood. (A) Representative dot plots show the percentages of CD4+, CD8+ T-cells, Tregs, PD1+CD8+/CD4+ and CD44+CD8+/CD4+ T cells after gating on CD45+ cells analyzed by flow cytometry in the blood corresponding to IgG, RT, RT+anti-CTLA4, RT+anti-CTLA4+anti-PD1 as indicated. (B) Quantification of above cells in blood. (C) Representative dot plots show the percentages of CD4+, CD8+ T-cells, Tregs, PD1+CD8+/CD4+ and CD44+CD8+/CD4+ T cells after gating on CD45+ cells analyzed by flow cytometry in the irradiated tumor-derived cell suspensions corresponding to IgG, RT and RT+anti-CTLA4 as indicated. (D) Quantification of above cells in irradiated tumors. One-way analysis of variance and Student's t test were used to compare immune cell fraction differences between treated groups and control groups, statistical significance was set at $p < 0.05$.

Supplementary Table 1. Clinical characteristics of LARC patients of cohort 1.

Clinical features	Cohort 1-Number
Gender	
Male	**42**
Female	**39**
Age	
Mean± SD	**56.89 ± 11.84**
Lymphovascular invasion	
Positive	**10**
Negative	**71**
Pathologic T stage	
ypT0	**8**
ypT1-2	**21**
ypT3	**52**
Pathologic N stage	
ypN0	**53**
ypN1	**13**
ypN2	**15**
ypTNM stage	
ypCR	**7**
I	**17**
II	**29**
III	**28**
Tumor regression grade (TRG)	
Grade 1	**47**
Grade 2	**8**
Grade 3	**8**
Grade 4	**18**

Supplementary Table 2. Clinical characteristics of LARC patients of cohort 2.

Clinical features	Cohort 2-Number
Gender	
Male	**55**
Female	**35**
Age	
Mean± SD	**58.38 ± 9.51**
Lymphovascular invasion	
Positive	**2**
Negative	**88**
Pathologic T stage	
ypT0	**11**
ypT1-2	**28**
ypT3	**51**
Pathologic N stage	
ypN0	**54**
ypN1	**24**
ypN2	**12**
ypTNM stage	
ypCR	**9**
I	**23**
II	**21**
III	**37**

Supplementary Table 3. Response of the local irradiated site and distant unirradiated sites (Metastatic Site and Synchronous Extracolonic cancer) in cohort 3 were determined by TRG and MRI.

ID	Irradiation Site	Metastatic Site	Synchronous Extracolonic cancer	RECIST	Local TRG	Dis MRI
1	**Rectal**	**Liver**		**-66%**	**1**	**PR**
2	**Rectal**	**Liver**		**-46%**	**1**	**PR**
3	**Rectal**	**Liver**		**-31%**	**2**	**PR**
4	**Rectal**	**Liver**		**-21%**	**0**	**SD**
5	**Rectal**	**Liver**		**-32%**	**1**	**PR**
6	**Rectal**	**Liver**		**-78%**	**0**	**PR**
7	**Rectal**	**Liver**		**-30%**	**2**	**PR**
8	**Rectal**	**Liver (+)**		**+21%**	**2**	**PD**
9	**Rectal**	**Liver (+)**		**+21%**	**2**	**PD**
10	**Rectal**	**Lung**		**0**	**3**	**SD**
11	**Rectal**		**Esophagus**	**-0.27%**	**2**	**SD**
12	**Rectal**		**Prostate**	**-6%**	**1**	**SD**
13	**Rectal**		**Liver**	**-1%**	**1**	**SD**

SD: stable disease; PR: partial response. PD: progressive disease. Two patients were assigned a value of +21% for new lesions (+).

149 **Supplementary Table 4. Reactome pathway analysis of the protective factors for**
150 **overall survival**
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Pathway name	Entities pValue	Entities FDR
Metabolism of RNA	**1.89E-05**	**0.006099122**
Regulation of mRNA stability by proteins that bind AU-rich elements	**2.01E-04**	**0.021353506**
Cytokine Signaling in Immune system	**5.72E-04**	**0.048659623**
Signaling by Interleukins	**7.03E-04**	**0.049205398**
Host Interactions of HIV factors	**0.001142715**	**0.068562919**
Influenza Life Cycle	**0.002619159**	**0.073971983**
Downstream signaling events of B Cell Receptor (BCR)	**0.002945966**	**0.073971983**
mRNA Splicing	**0.003158388**	**0.073971983**
Interleukin-4 and 13 signaling	**0.004171803**	**0.09177966**

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