

Supplementary Data

**Circulating extracellular vesicles carrying Firmicutes reflective of the local immune status
may predict clinical response to pembrolizumab in urothelial carcinoma patients**

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Figure S1. Immunological status of previously reported peripheral and tumor tissue-infiltrated T cells. Characterization of tumor tissue-infiltrated (a) and peripheral T cells (b). (c) Classification of T cells based on the expression of surface immune checkpoint markers. Samples not used in this study are filled in black. (d) Number of tumor tissue-infiltrated T cells. (e) Frequency of tumor tissue-infiltrated CD8⁺ cells (left panel). Expression of immune checkpoint markers in CD8⁺ cells based on immunological classification (right panel). (f) Frequency of tumor tissue-infiltrated CD4⁺ cells (left panel). Expression of immune checkpoint markers in CD4⁺ cells based on immunological classification (right panel). (g) Frequency of peripheral CD8⁺ cells (left panel). Expression of immune checkpoint markers in CD8⁺ cells based on immunological classification (right panel). (h) Frequency of peripheral CD4⁺ cells (left panel). Expression of immune checkpoint markers in CD4⁺ cells based on immunological classification (right panel). Mann–Whitney test (Two-tailed). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Exact p values for (e): $p = 0.011$ (left panel), $p = 0.04$ (PD-1), $p < 0.0001$ (Tim-3), $p = 0.0007$ (CD25), $p = 0.0001$ (ICOS), $p = 0.003$ (4-1BB), $p = 0.0004$ (OX40); for (f): $p = 0.0026$ (left panel), $p = 0.36$ (CD45RA), $p = 0.24$ (PD-1), $p = 0.0005$ (Tim-3), $p < 0.0001$ (CD25^{high}), $p = 0.0004$ (ICOS), $p < 0.0001$ (4-1BB), $p < 0.0001$ (OX40); for (g): $p = 0.52$ (CD45RA), $p = 0.30$ (PD-1), $p = 0.21$ (Tim-3), $p = 0.41$ (CD25), $p = 0.017$ (ICOS), $p = 0.91$ (4-1BB), $p = 0.16$ (OX40); for (h): $p = 0.34$ (CD45RA), $p = 0.25$ (PD-1), $p = 0.71$ (Tim-3), $p = 0.0055$ (CD25^{high}), $p = 0.037$ (ICOS), $p = 0.29$ (4-1BB), $p = 0.90$

Figure S2. No significant difference in Bacteroidetes phylum or F:B (Firmicutes:Bacteroidetes) ratio among the immunologically categorized groups. (a) Percentage Bacteroidetes abundance based on immunological classification (Group I: 30, Group II: 20). (b) F:B ratio based on immunological classification. Mann–Whitney test (Two-tailed). (c) F:B ratio-based ROC analysis. Fisher's exact probability test. (d) Cumulative bar chart based on F:B ratio. Fisher's exact probability test

Figure S3. Cumulative bar chart based on abundance of peripheral Armatimonadetes (a), Elusimicrobia (b), and Nitrospirae (c). Fisher's exact probability test.

Figure S4. Expression of immune checkpoint markers in myeloid cells based on peripheral Firmicutes abundance (g) or presence/absence of AEN (h). for (g): $p = 0.80$ (CCR2), $p = 0.96$ (PD-L1); for (h): $p = 0.51$ (CCR2), $p = 0.54$ (PD-L1)

Figure S5. F:B (Firmicutes:Bacteroidetes) ratio-based prognosis analysis. (a) Cumulative bar chart based on the F:B ratio. Fisher's exact probability test. Progression-free survival ratio (b) and cancer-specific survival ratio (c) based on the F:R ratio

Fig. S1

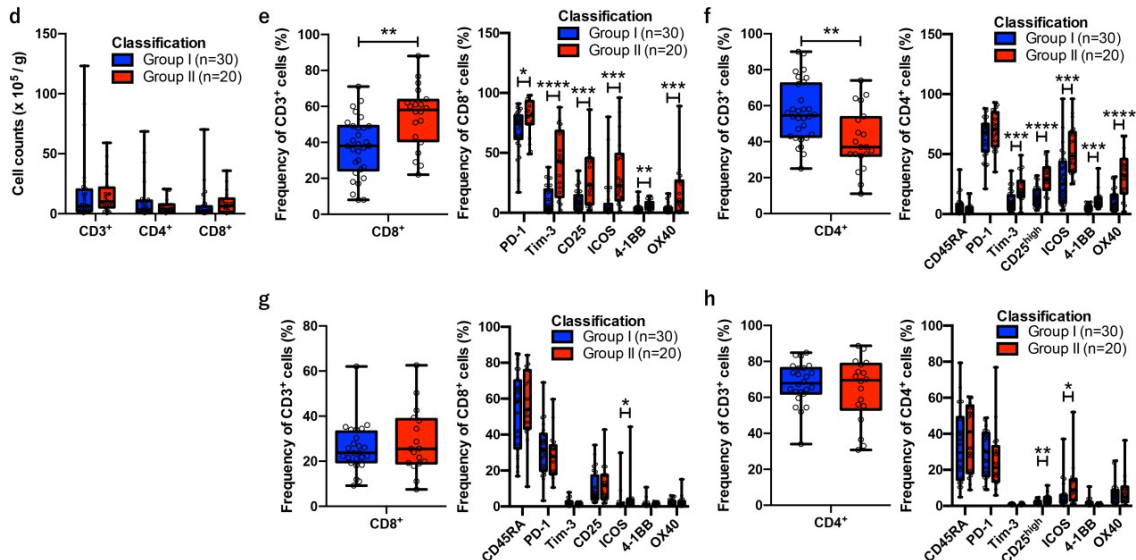
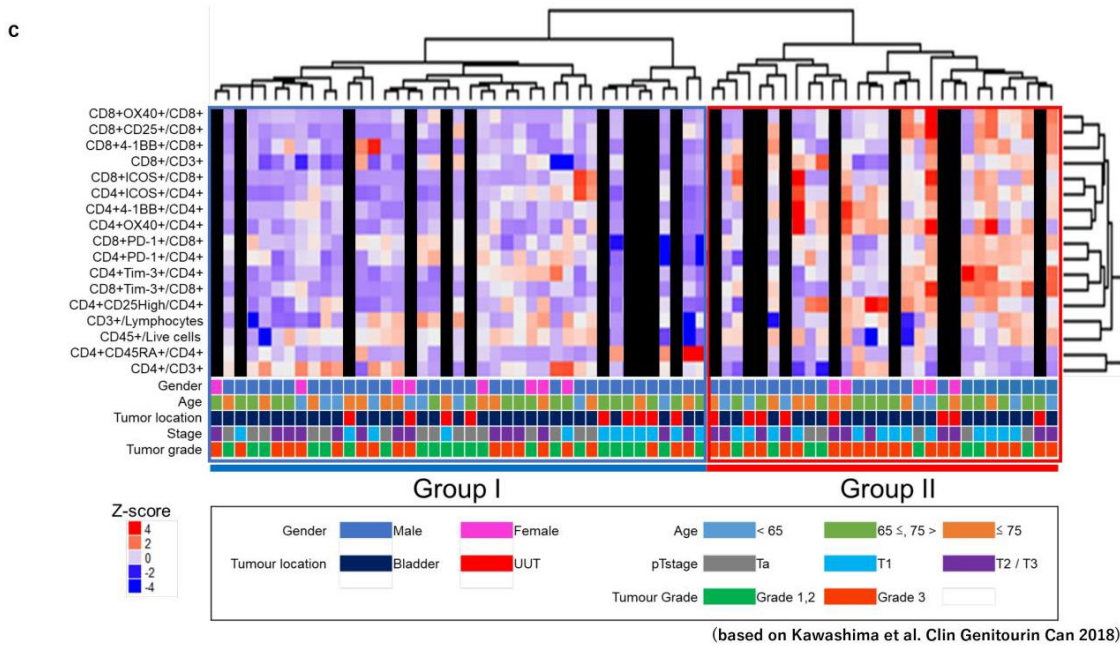
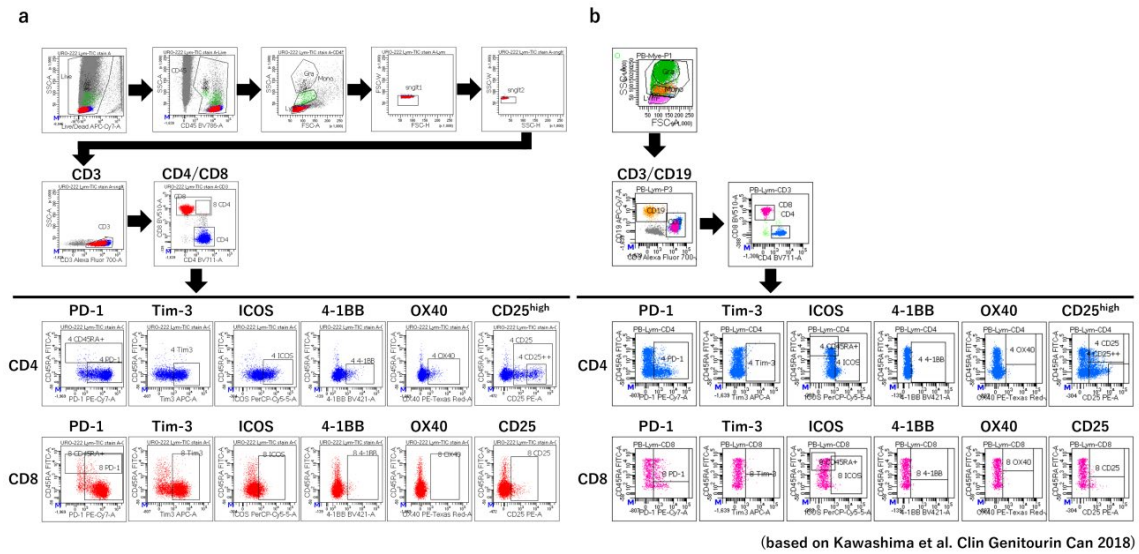


Fig. S2

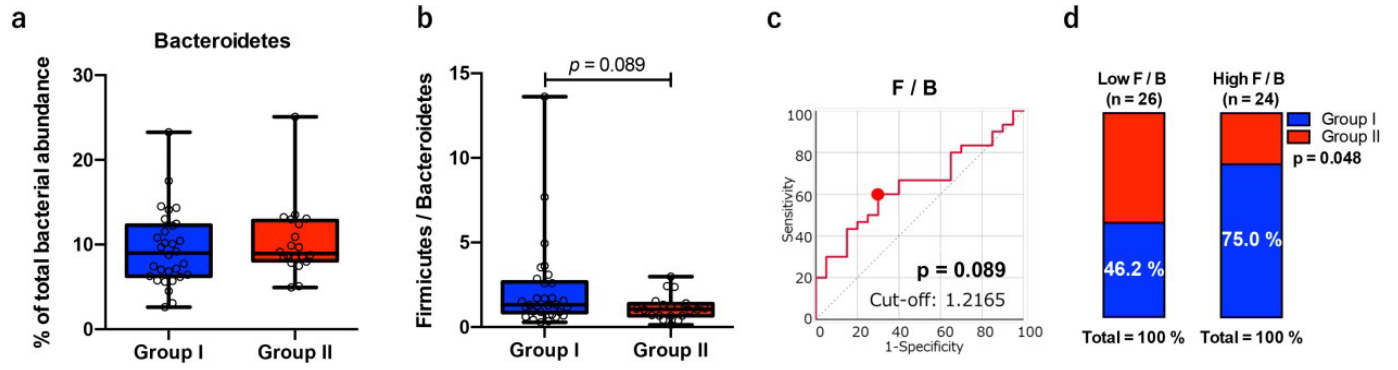


Fig. S3

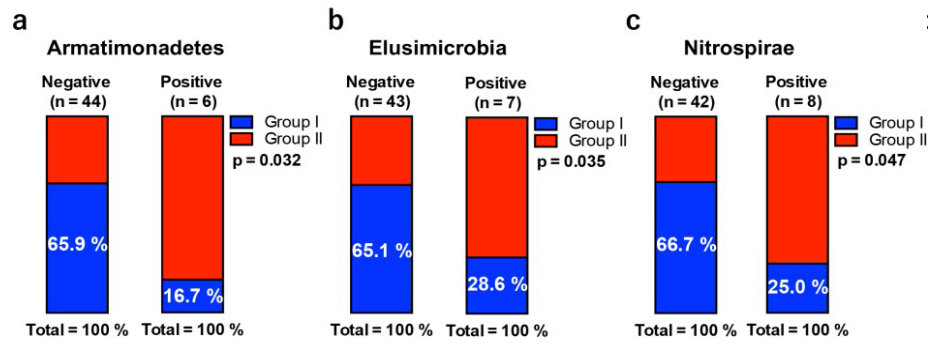


Fig. S4

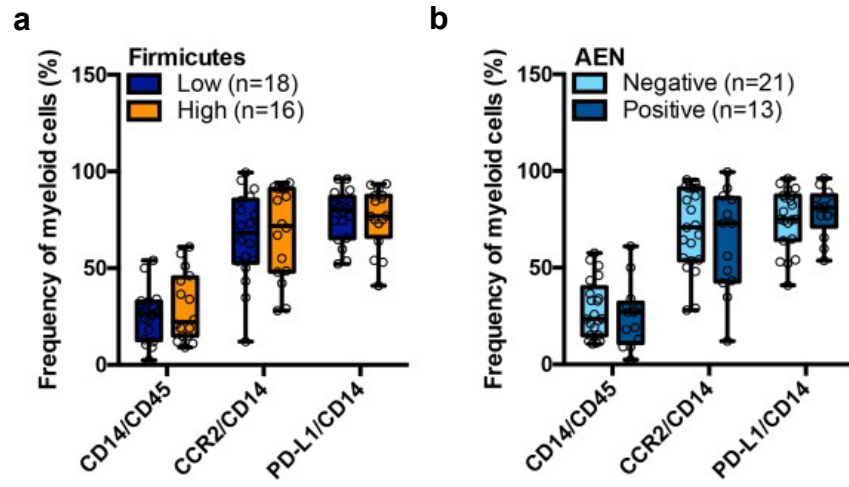


Fig. S5

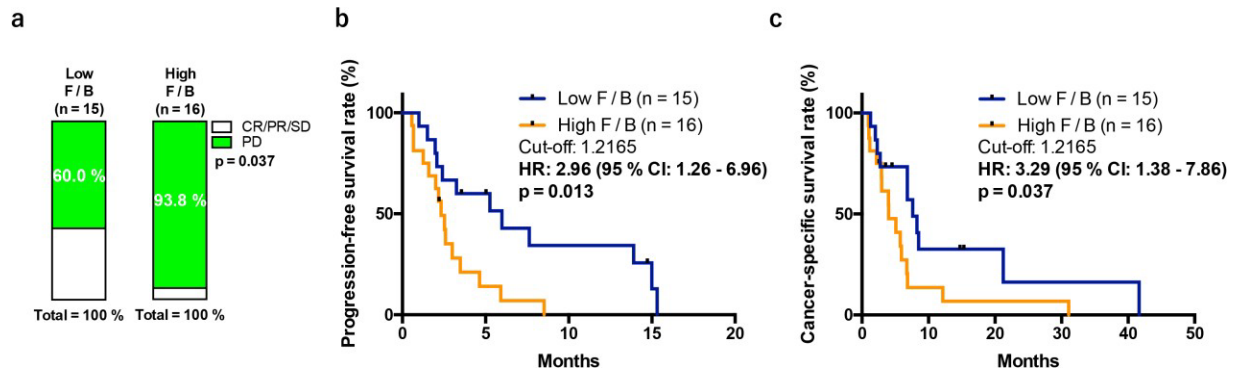


Table S1. Clinical characteristics of the patients enrolled in this study

Clinical specimens	Healthy Donor (n = 20)	Localized UC (n = 50)	Metastatic UC (n = 31)
Age (median) (years)	47–91 (70.5)	47–90 (70.5)	45–87 (73.0)
Sex			
Male	5	41	20
Female	15	9	11
Tumour location			
Bladder	N/A	50	9
Pelvis	N/A	0	7
Ureter	N/A	0	15
BMI (median) (kg/m ²)	17.9–27.8 (21.8)	17.9–37.6 (23.9)	14.9–27.4 (20.2)
Smoking status			
Current	0	12	2
Former	4	13	8
Never	16	25	21
Histological type			
UC only	N/A	49	31
UC with Sq	N/A	1	0
pT stage			
a	N/A	19	0
1	N/A	15	0
2 and more	N/A	16	31
Surgical treatment just before analysis			
TURBT or biopsy	N/A	50	18
Radical treatment	N/A	0	13
Systemic inflammatory disease			
No	N/A	46	0
Yes	N/A	4	31
Tumour grade (highest) ^a			
Grade 2	N/A	23	0
Grade 3	N/A	27	31
Tumour grade (highest) ^b			
Low grade	N/A	4	0
High grade	N/A	46	31
Immunological Classification			
Class I	N/A	30	N/A
Class II	N/A	20	N/A
NLR (median)	1.03–5.72(1.74)	0.61–16.60(2.39)	1.54–23.69 (5.66)
PLR (median)	0.48–1.71(0.72)	0.26–3.05 (0.81)	0.49–7.41 (1.50)
MLR (median)	0.11–0.44(0.20)	0.10–0.85 (0.23)	0.15–1.41 (0.52)
FAN score			
0, 1	20	47	14
2, 3	0	3	16

UC = urothelial carcinoma; N/A = not applicable; Sq = squamous; NLR = neutrophil-to-lymphocyte ratio; PLR = platelet-to-lymphocyte ratio; MLR = monocyte-to-lymphocyte ratio; FAN score = Fib-4 index, ALBI score and NLR Score. ^aBy the 1973 World Health Organization (WHO) grading system. ^bBy the 2004 WHO grading system.

