Supplementary information

Supplementary Tables

Suppl. Tab. 1

Specificity	*Clone*	*Fluorochrome*	*Vendor*
TIM-3	7D3	BV711	BD Biosciences
CD8	RPA-T8	BV510	BioLegend
CD57	HCD574	FITC	BioLegend
PD-1	EH12.2H7	PerCP-Cy5.5	BioLegend
BTLA	J168-570	PE-CF594	BD Biosciences
TCRαß	IP26	APC	BioLegend
CD45RA	HI100	AF700	BioLegend
CD38	HIT2	PE-Cy7	BioLegend
Integrin-beta7	BNI3	PE	Invitrogen
CCR7	G043H7	BV650	BioLegend
CD28	CD28.2	BV421	BioLegend
CXCR4*	12G5	BV421	BioLegend
CD4	SK3	APC/Fire 750	BioLegend
CD154*	24-31	APC-Cy7	BioLegend
live/dead		zombie yellow	BioLegend

Antibody panel for single cell index sorting and bulk T cell sorting
Reagents were used simultaneously in a 13-parameter panel and titrated individually to optimum concentrations before multicolor staining. *For patient 5, CXCR4 was used instead of CD28 and CD154 was used instead of CD4 antibodies.

Specificity	*Clone*	*Fluorochrome*	*Vendor*
human CD8	B9.11	APC	Beckman Coulter
mouse CD3	17A2	BV421	BioLegend
Live/dead		7AAD	BioLegend
T cell activation		GFP	

Antibody panel for 58$\alpha^-\beta^-$ cell lines expressing recombinant TCRs
For both tables: APC: allophycocyanin, BV: Brilliant Violet, FITC: fluorescein isothiocyanate, PerCP: peridinin chlorophyll, Cy: cyanine, PE: phycoerythrin, AF: Alexa Fluor, 7AAD: 7-Aminoactinomycin D, GFP: green fluorescent protein

Supplementary Table 2

		CD8⁻ d0		*CD8⁺ d0*		*CD8⁻ follow-up*		*CD8⁺ follow-up*	
Patient	*Days follow-up*	*Cells*	*Clones*	*Cells*	*Clones*	*Cells*	*Clones*	*Cells*	*Clones*
1	46	8,3E+05	2,383	2,8E+05	1,331	8,6E+05	1,283	1,3E+05	2,244
2	53	3,3E+05	2,180	5,7E+05	343	6,5E+05	2,792	5,6E+05	432
3	106	1,0E+06	1,464	1,0E+06	744	6,0E+05	1,757	5,0E+05	575
4	56	2,5E+06	2,924	4,7E+05	1,085	1,7E+06	2,756	4,1E+05	945
Mean	65	1,2E+06	2,238	5,8E+05	876	9,5E+05	2,147	4,0E+05	1,049

T cells sorted at d0 and follow up for TCRβ repertoire sequencing

From PBMC isolated at surgery and one follow-up time point, we sorted $CD8^+$ and $CD8^-$ T cells for TCRβ repertoire sequencing. Cells states the numbers of sorted cells per patient and population and clones the numbers of individual clones identified by repertoire sequencing. For sorting gates, see Supplementary Figure 6.

Supplementary Table 3

	Frequency in αβ T cells [%]		*Number of sorted cells*		*Number of cells with determined TCR sequences*	
Patient	*PD-1*$^+$	*TIM-3*$^+$	*PD-1*$^+$	*TIM-3*$^+$	*PD-1*$^+$	*TIM-3*$^+$
1	1.04	2.31	184	184	141	138
2	3.82	10.48	184	184	136	141
3	1.06	1.11	184	184	155	183
4	1.29	1.48	184	184	180	163

Suppl. Tab. 3 Absolute numbers and sequencing efficiency of rare-phenotype single T cells sorted from peripheral blood

184 cells with increased PD-1 and 184 cells with increased TIM-3 expression were sorted from PBMC isolated at d0. TCRαβ sequences were determined using single cell sequencing. For sorting gates, see Supplementary Figure 7.

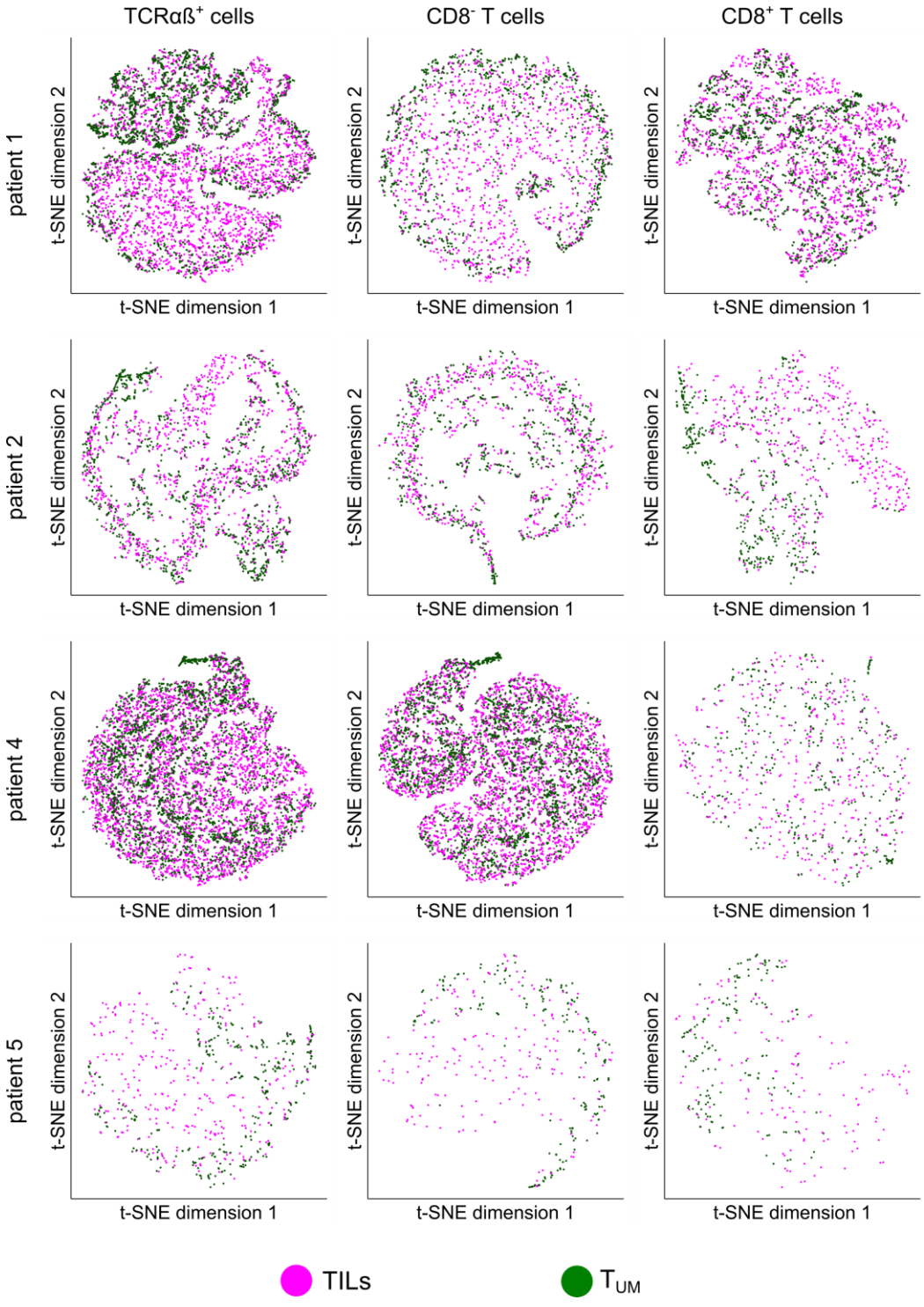
Supplementary Table 4

T cell line	*Corresp. patient*	*Number of recombinant T cells*	*Number of corresp. tumor tissue cells*	*Number of corresp. unaffected mucosa cells*	*HLA-mismatched patient, tissue*	*Number of cells from HLA-mismatched tissue*
58-1A4-1	4	40,000	39,875	7,442	2, UM	38,225
58-1A4-2	4	40,000	39,875	7,442	3, TU	36,250
58-1B4	4	40,000	39,875	7,442	3, TU	36,250
58-1C10	3	40,000	36,250	125,725	2, UM	38,225
58-11B1	2	40,000	26,400	38,225	3, TU	36,250
58-11B7	2	40,000	26,400	38,225	3, TU	36,250
58-13B10	2	40,000	26,400	38,225	3, TU	36,250

Suppl. Tab. 4 Co-incubation of TCR-recombinant cell lines with cells isolated from their corresponding tissues or tissues from HLA-mismatched controls
40,000 TCR-recombinant cells from each cell line were co-incubated with cells isolated from corresponding tumor tissue, corresponding unaffected mucosa, or tumor or unaffected mucosa tissue of an HLA-mismatched individual. Co-cultures were done in 96-well plates for 16 h in RPMI1640 containing 10 % fetal bovine serum at 37 °C and 5 % CO_2. All cell numbers represent cell numbers per co-incubation well. UM: cells isolated from unaffected mucosa, TT: cells isolated from tumor tissue

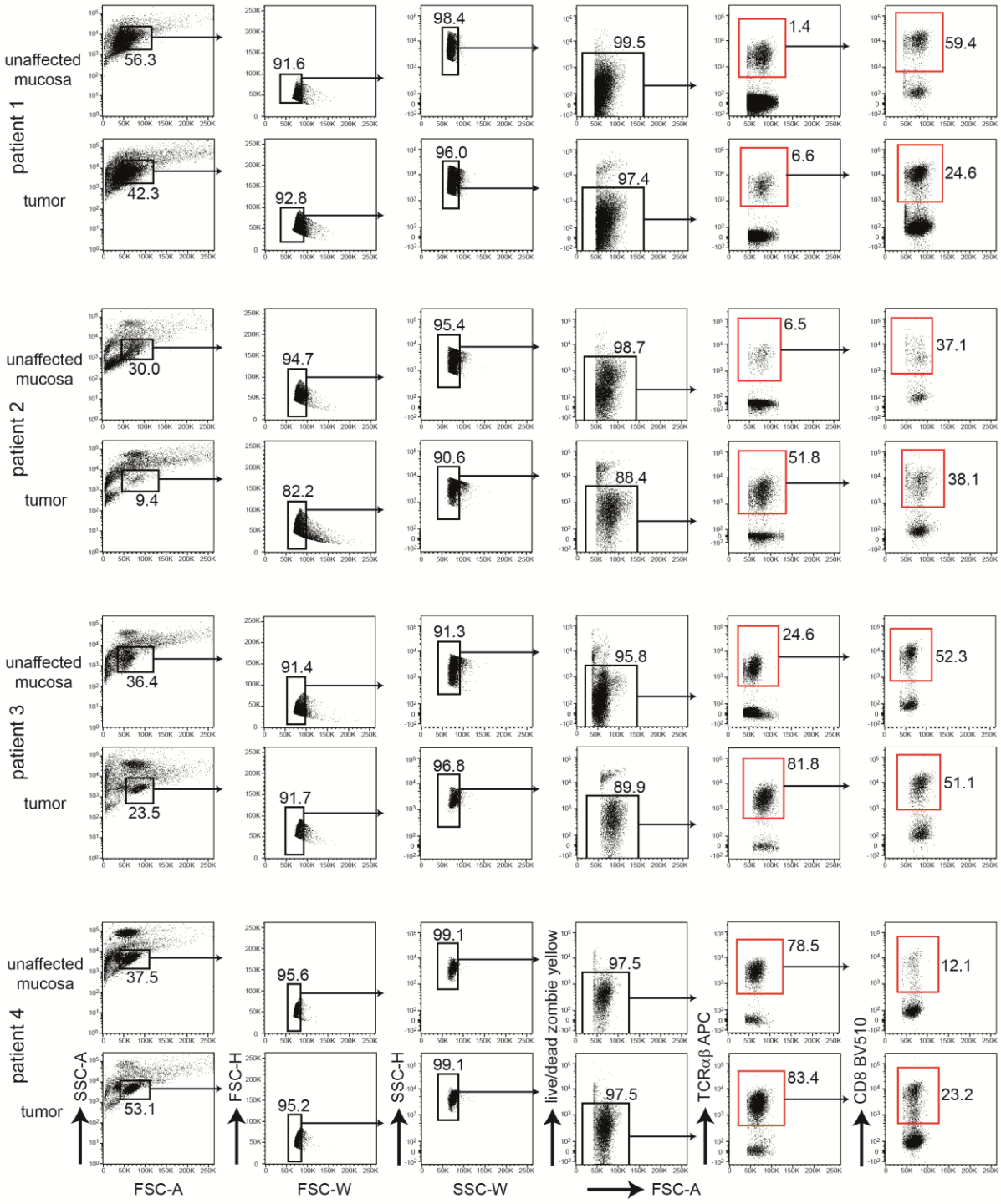
Supplementary Figures

Supplementary Figure 1



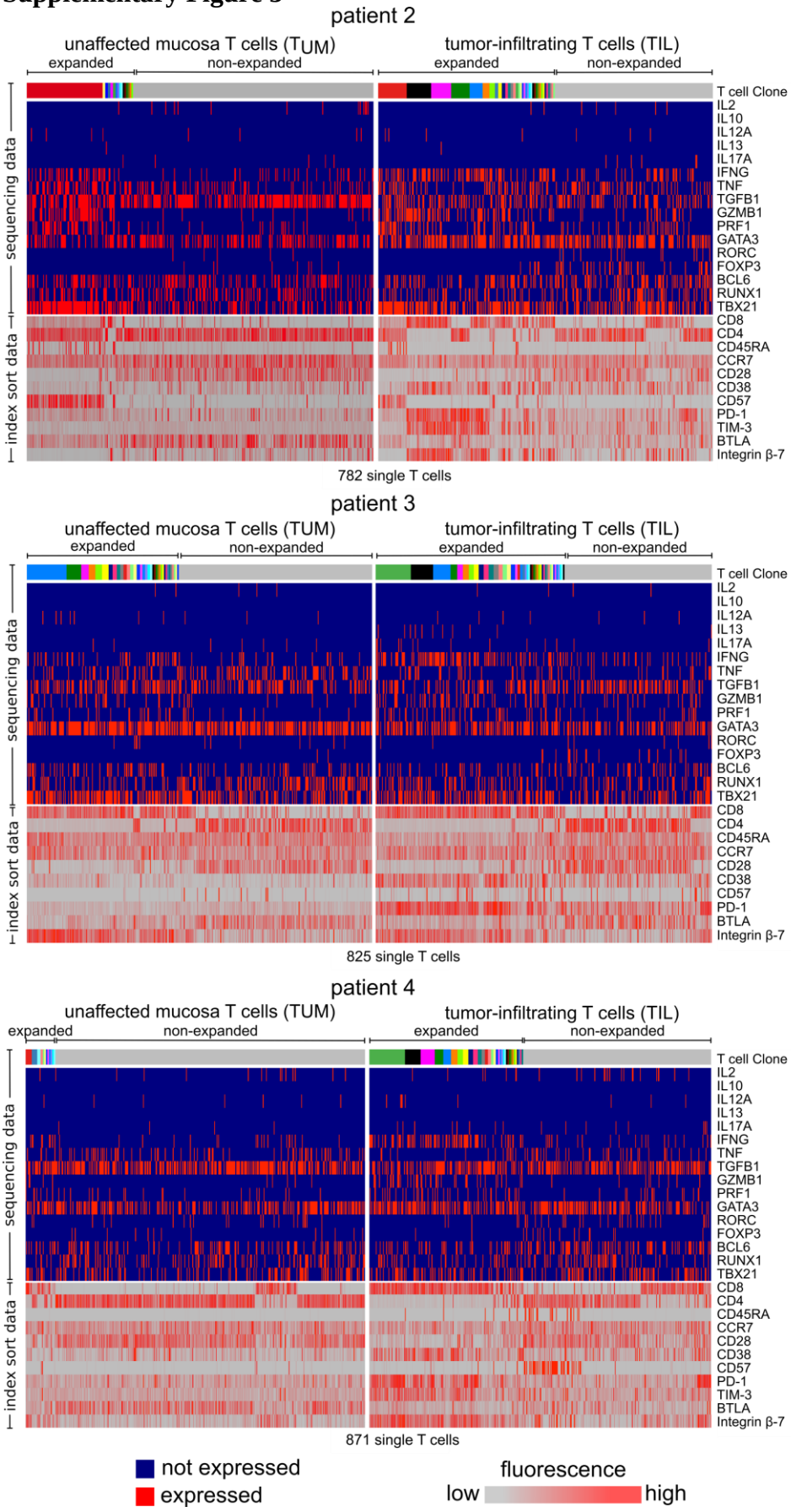
Suppl. Fig. 1: Immune phenotype compartments occupied by TILs and T_{UM}
Immune phenotype distances of single TILs and T_{UM} from 5 rectal cancer patients were visualized using t-stochastic neighbor embedding (t-SNE). TILs and T_{UM} are shown at a ratio of 1:1 after randomized selection from all recorded live TCRαβ$^+$, TCRαβ$^+$CD8$^+$, or TCRαβ$^+$CD8$^-$ cells.

Supplementary Figure 2



Suppl. Fig. 2 Gating strategy for TIL and T_{UM} index sorting
Cells were stained with a 13-parameter panel (Suppl. Tab. 1). FACS plots show sequential gating on single live lymphocytes, total TCRαβ$^+$, and TCRαβ$^+$ CD8$^+$ populations for cells isolated from tumor and unaffected mucosa of all four patients. The final gates for index sorting are highlighted in red. To select approximately equal proportions of single CD8$^+$ and CD8$^-$ T cells per sample, we sorted a variable number of CD8$^+$ cells (gate at the very right) in addition to total TCRαβ$^+$ cells.

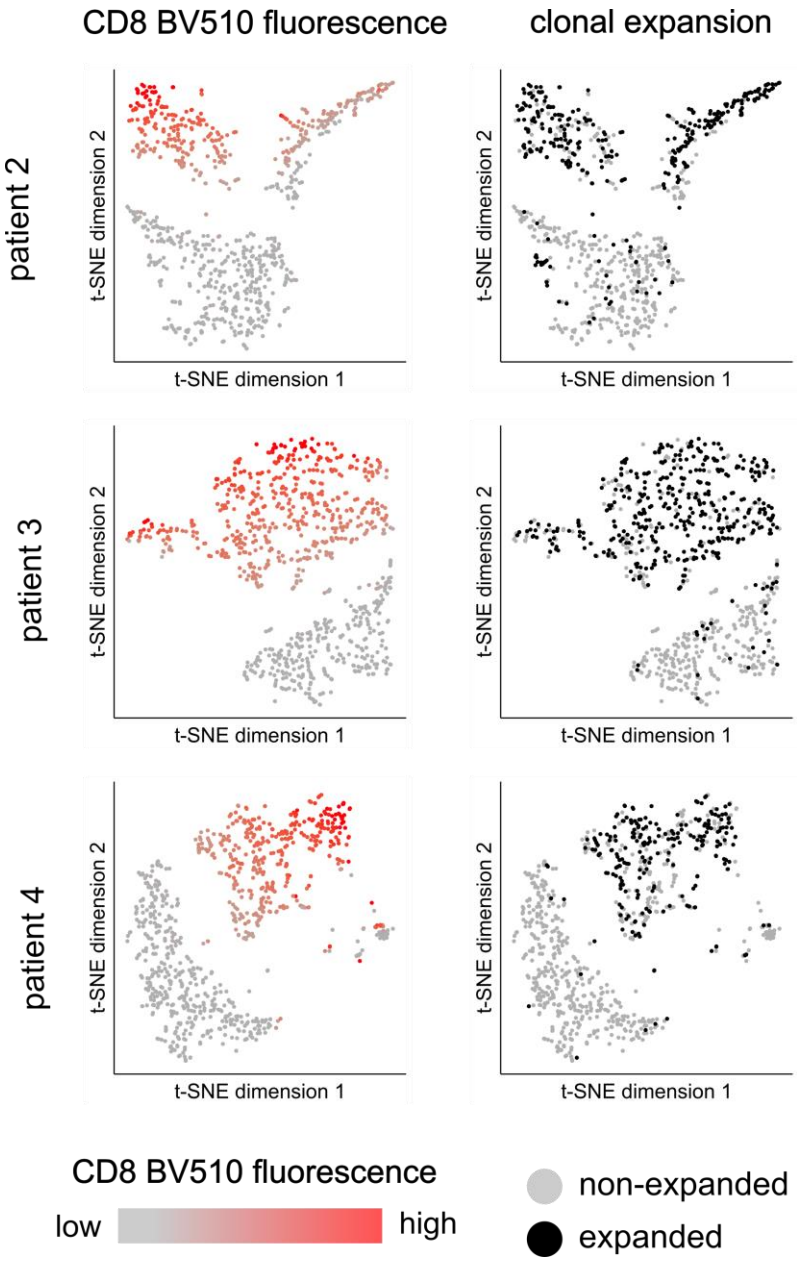
Supplementary Figure 3



Suppl. Fig. 3 Clonal TIL and T_{UM} expansion-associated phenotypes

Parallel next generation sequencing of TCRαβ, transcription factor, and cytokine genes from amplified cDNA of single T cells (Suppl. Fig. 2 for sorting gates). The sequencing and FACS data of single cells are arranged in columns with each column representing one single cell. The top bar indicates TCR sequences; adjacent columns with the same color in the top bar indicate single cells with identical complementarity determining region (CDR)-3 amino acid sequences in their TCRαβ genes. Clonal expansion was defined as the detection of at least two cells with identical TCRαβ sequences. The lower part of the heatmap is derived from the corresponding FACS index sort data and fluorescence intensities are color-coded from grey (lowest expression) to red (highest expression) for the indicated parameters. Patient 2 (384 TILs, 398 T_{UM}), patient 3 (407 TILs, 418 T_{UM}), patient 4 (437 TILs, 434 T_{UM})

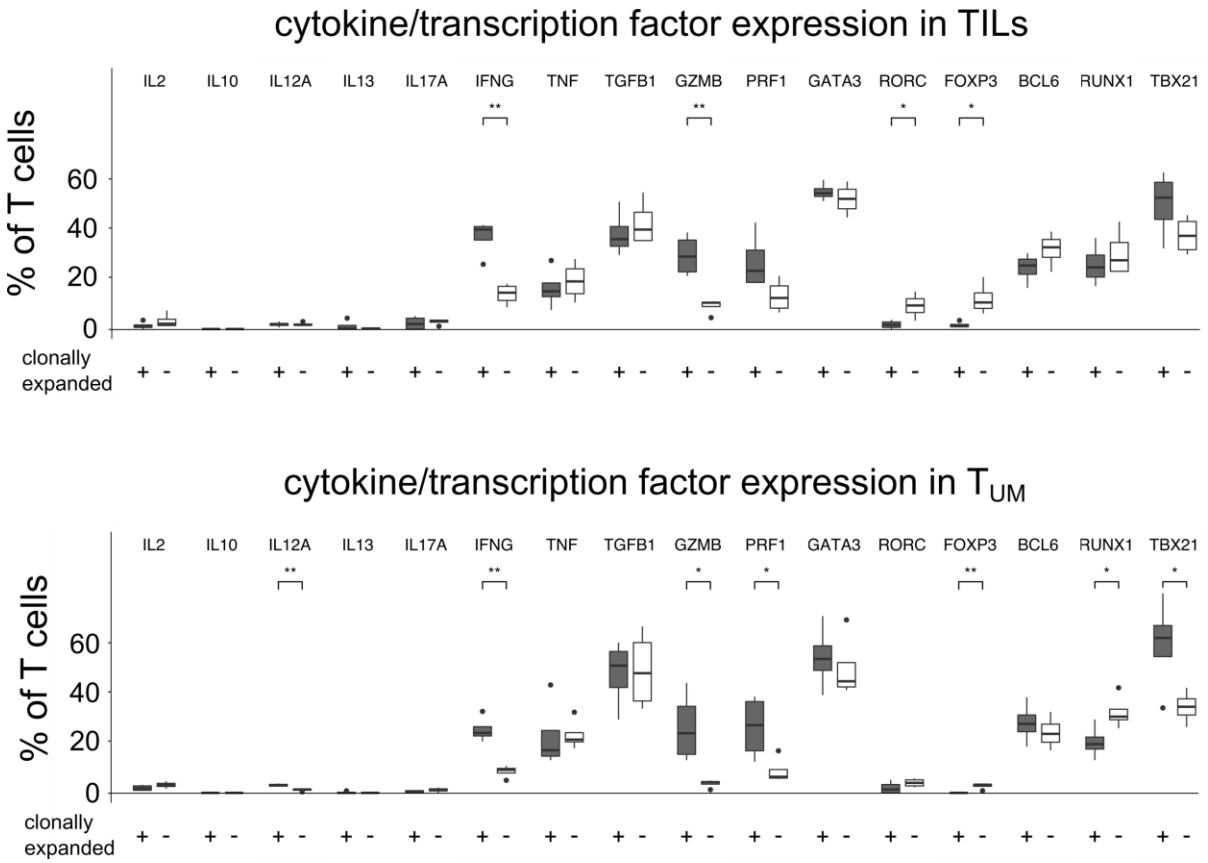
Supplementary Figure 4



Suppl. Fig. 4 Immune phenotype compartments occupied by clonally expanded TILs and T_{UM}

High-dimensional immune phenotypes of single TILs and T_{UM} (see Suppl. Fig. 3) were visualized with t-SNE. Cells were colored based on CD8 expression (left panels) or clonal expansion (right panels). Patient 2 (384 TILs, 398 T_{UM}), patient 3 (407 TILs, 418 T_{UM}), patient 4 (437 TILs, 434 T_{UM})

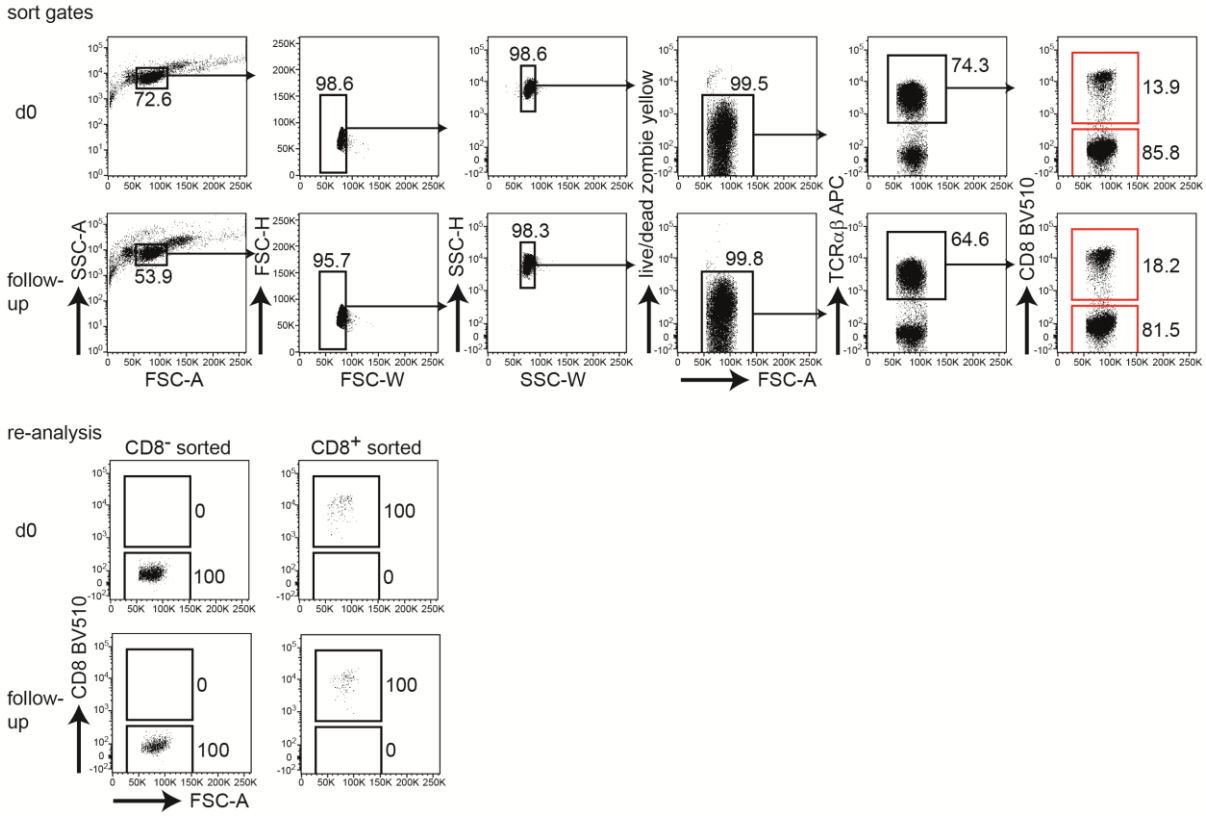
Supplementary Figure 5



Suppl. Fig. 5 Cytokine and transcription factor expression in clonally expanded vs. non-expanded TILs and T_{UM}

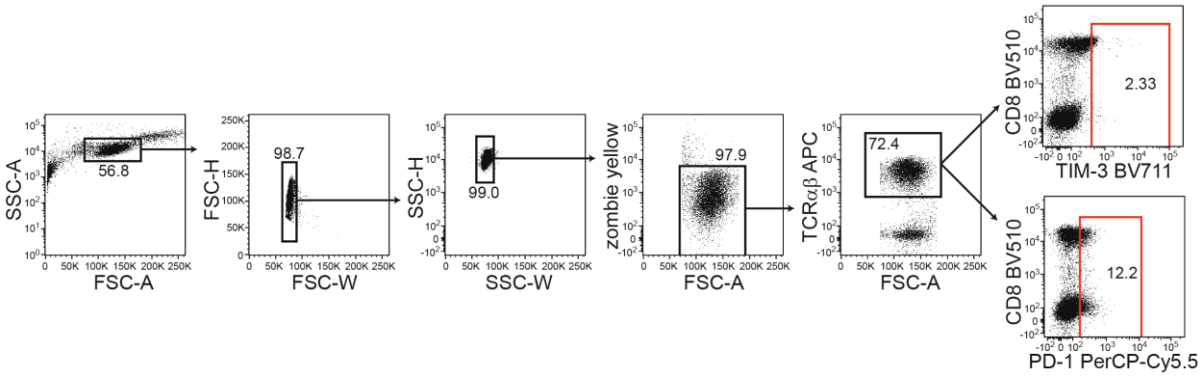
Cytokine and transcription factor expression were determined by single cell sequencing (Fig. 3 and Suppl. Fig. 3). Sequencing data from 4 independent experiments and patients. *p<0.05, **p< 0.01, determined by Student's t-test.

Supplementary Figure 6



Suppl. Fig. 6 Gating strategy and re-analysis of peripheral blood bulk CD8$^+$ and CD8$^-$ T cell sorting for TCRβ repertoire sequencing
PBMC from two different time points per patient were stained with a 13-parameter antibody panel (Suppl. Tab. 1). FACS plots show the sequential gating on single live CD8$^+$ and CD8$^-$ T cells from patient 3 as one representative example. The gates for sorting CD8$^+$ and CD8$^-$ populations are highlighted in red. Please see Supplementary Table 2 for detailed numbers of sorted cells from each of the four patients. Cells were sorted into RPMI1640 containing 2 % fetal bovine serum for subsequent re-analysis and DNA extraction for TCRβ repertoire sequencing.

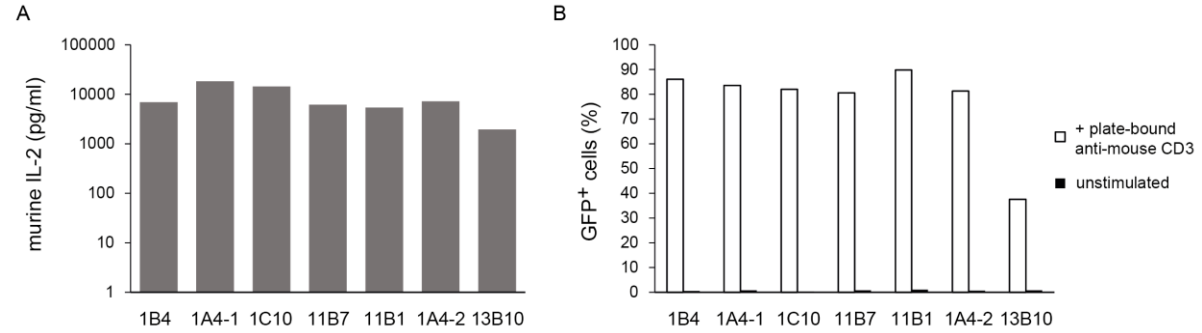
Supplementary Figure 7



Suppl. Fig. 7 Sort gates for single peripheral blood T cells with increased PD-1 and TIM-3 expression

To increase the chance of detection in peripheral blood, we sorted single cells with increased PD-1 and TIM-3 expression for subsequent single cell TCRαβ sequencing (see Suppl. Tab. 3 for detailed cell numbers and sequencing efficiency). The figure shows the gating strategy and sort gates (red) for patient 3 as one representative example.

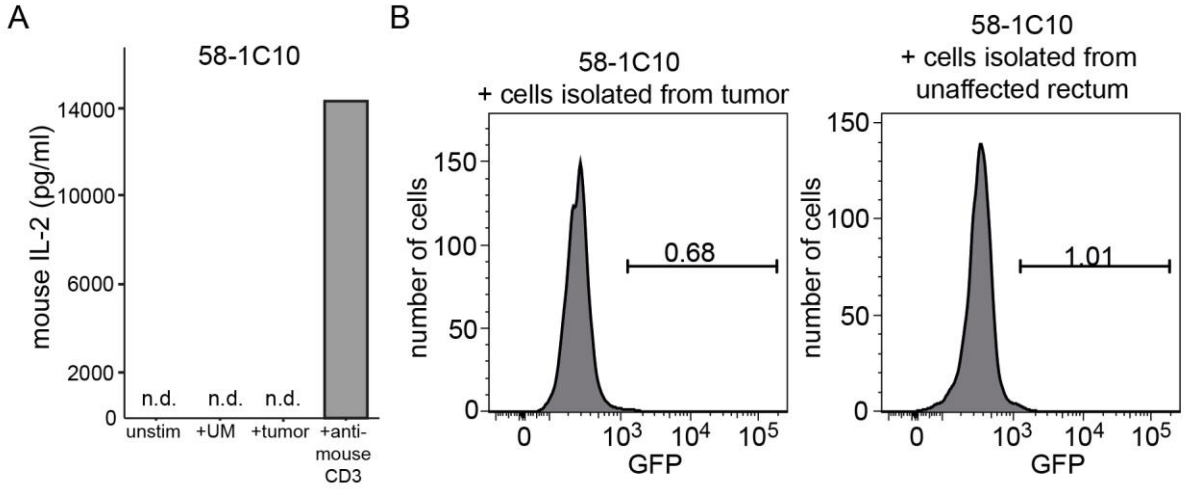
Supplementary Figure 8



Suppl. Fig. 8 IL-2 production and GFP expression of TCR-recombinant 58$\alpha^-\beta^-$ cell lines after stimulation with plate-bound anti-mouse CD3

58$\alpha^-\beta^-$ cells recombinantly expressing selected TCRs were stimulated with plate-bound anti-mouse CD3 as positive control in a total volume of 150 ul for 16 h. (A) Mouse IL-2 production measured by ELISA. (B) GFP expression detected with flow cytometry.

Supplementary Figure 9

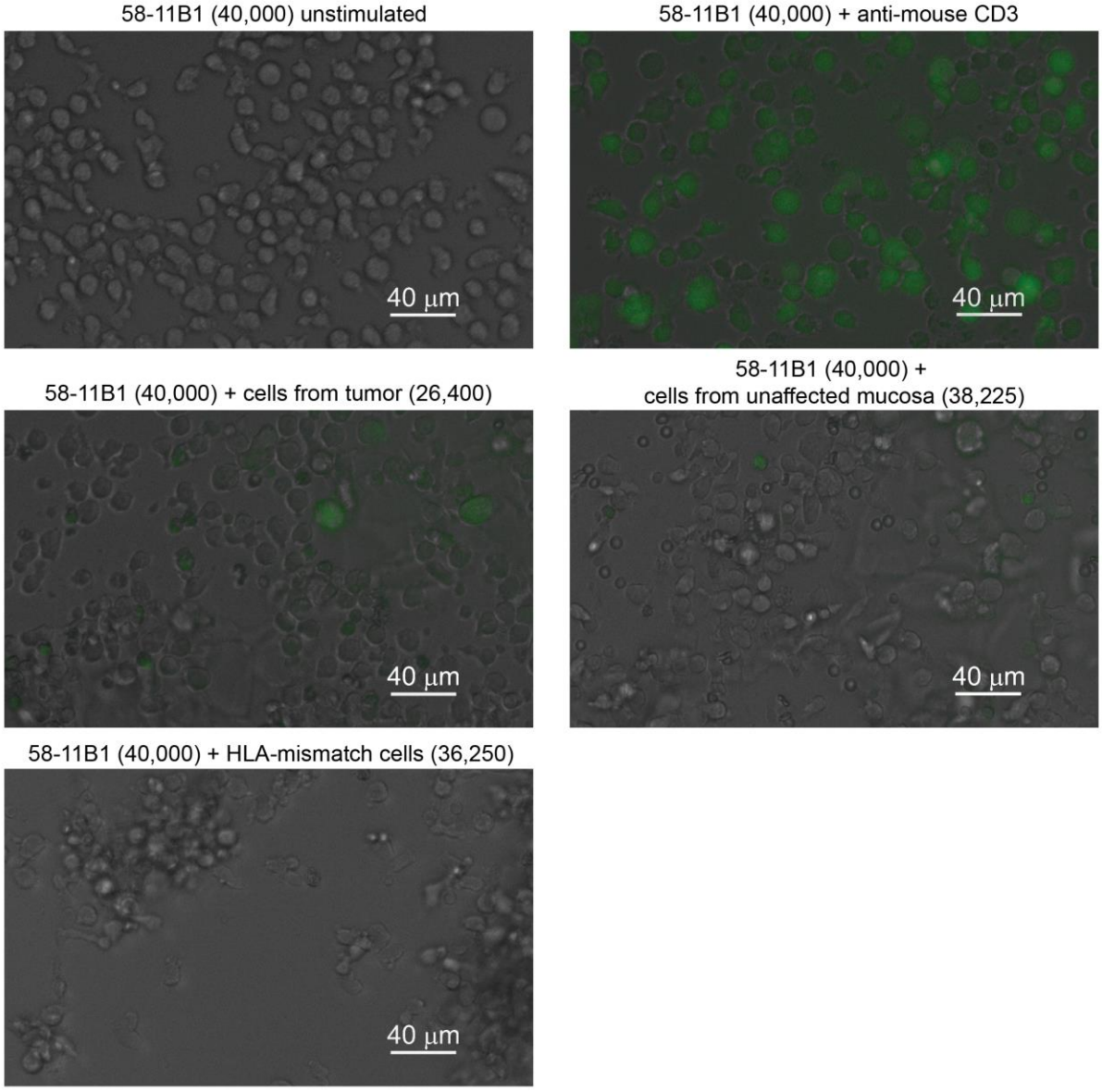


Suppl. Fig. 9 Co-incubation of TCR-recombinant cell lines with cells isolated from corresponding target tissue

58-1C10 cells as an example were left unstimulated, stimulated with plate-bound anti-mouse CD3 as a positive control, or co-incubated with cells isolated from their corresponding tumor or unaffected mucosa tissue. (A) IL-2 production by 58-1C10 after co-incubation with cells isolated from the corresponding tumor or unaffected mucosa tissues. (B) GFP expression of 58-1C10 measured by FACS. FACS plots were pre-gated on single live murine $CD3^+$ lymphocytes. For detailed cell numbers and co-incubation conditions, see Supplementary Table 4.

Supplementary Figure 10

A



Suppl. Fig. 10 continued

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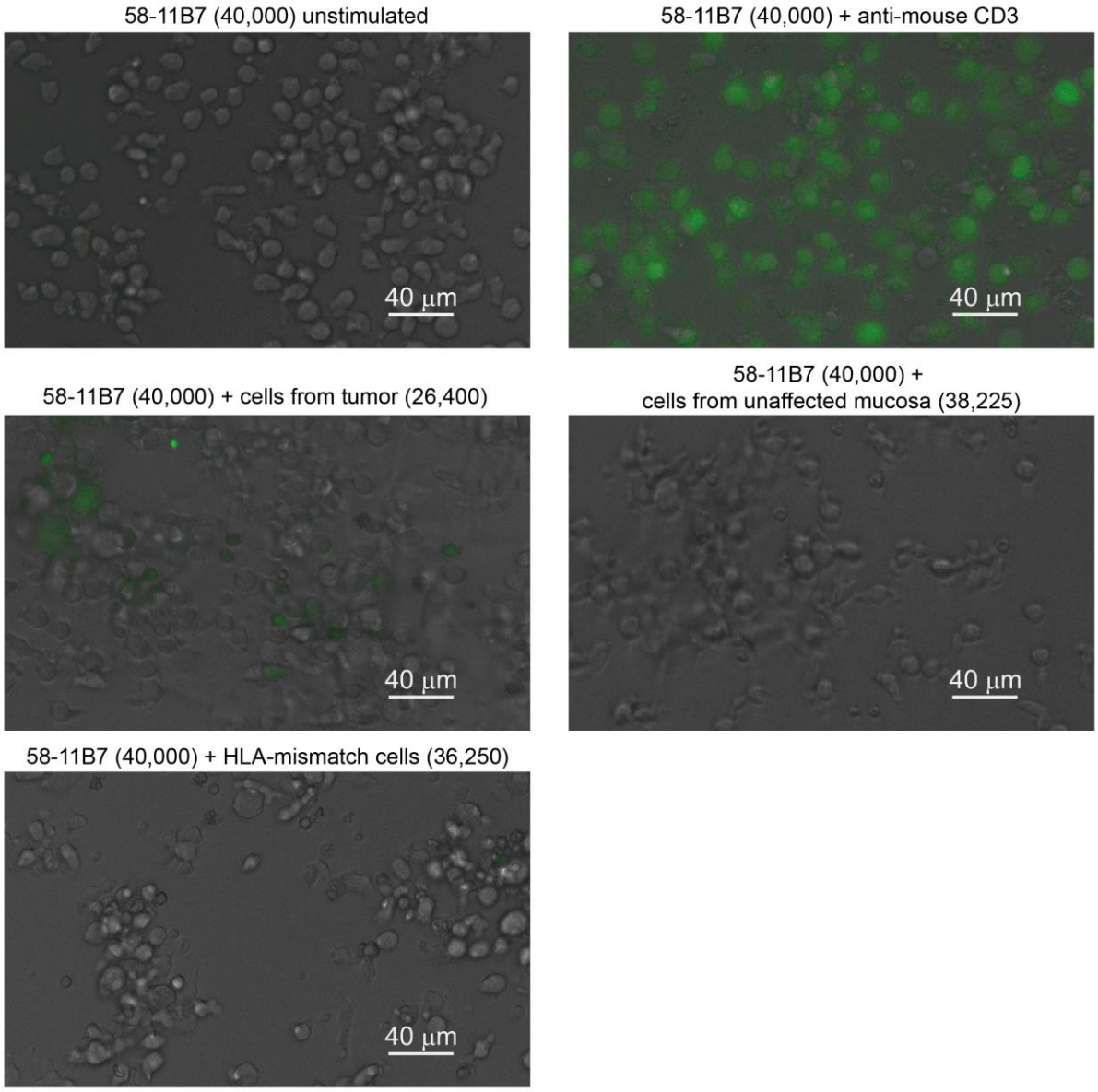

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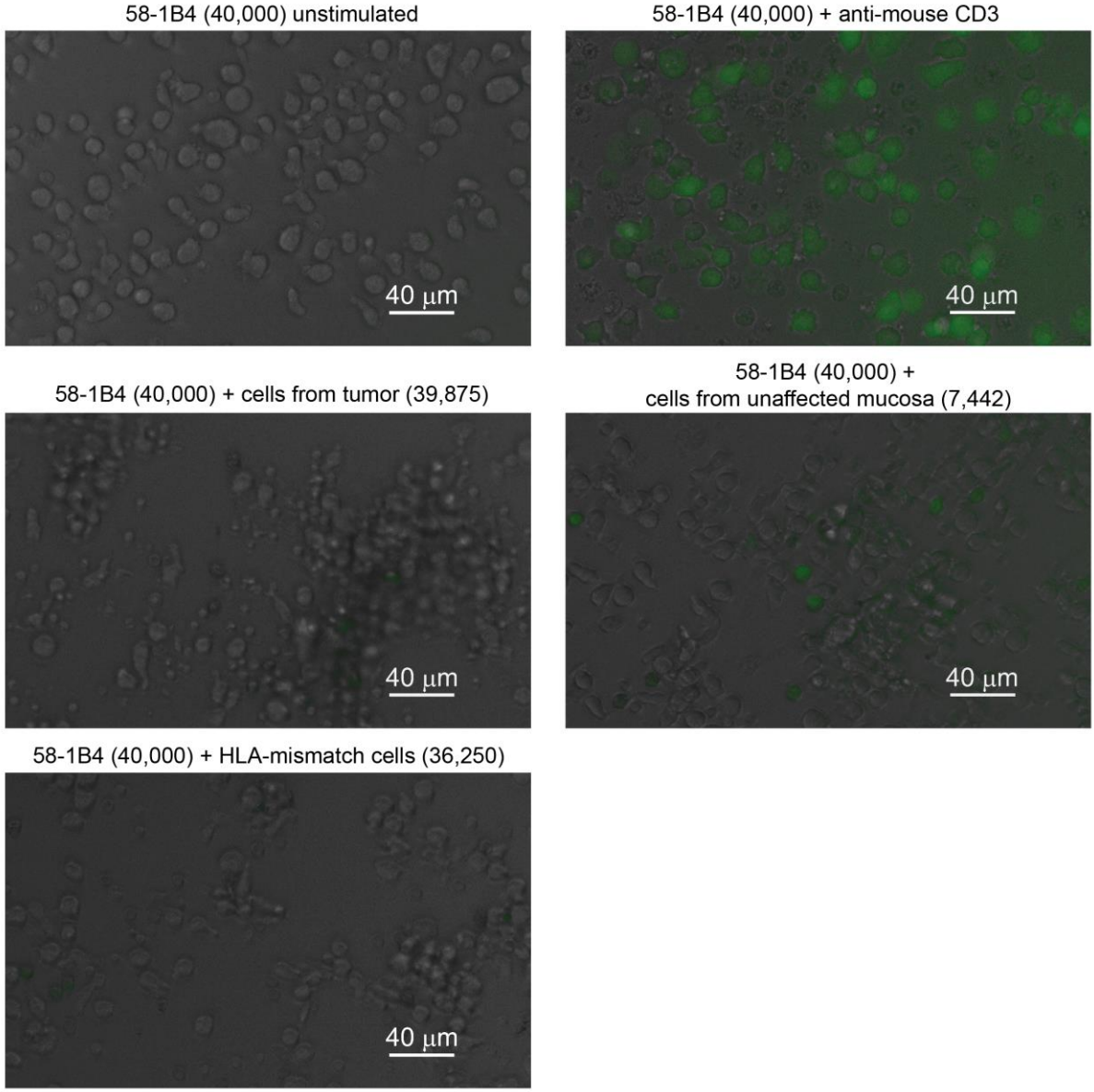
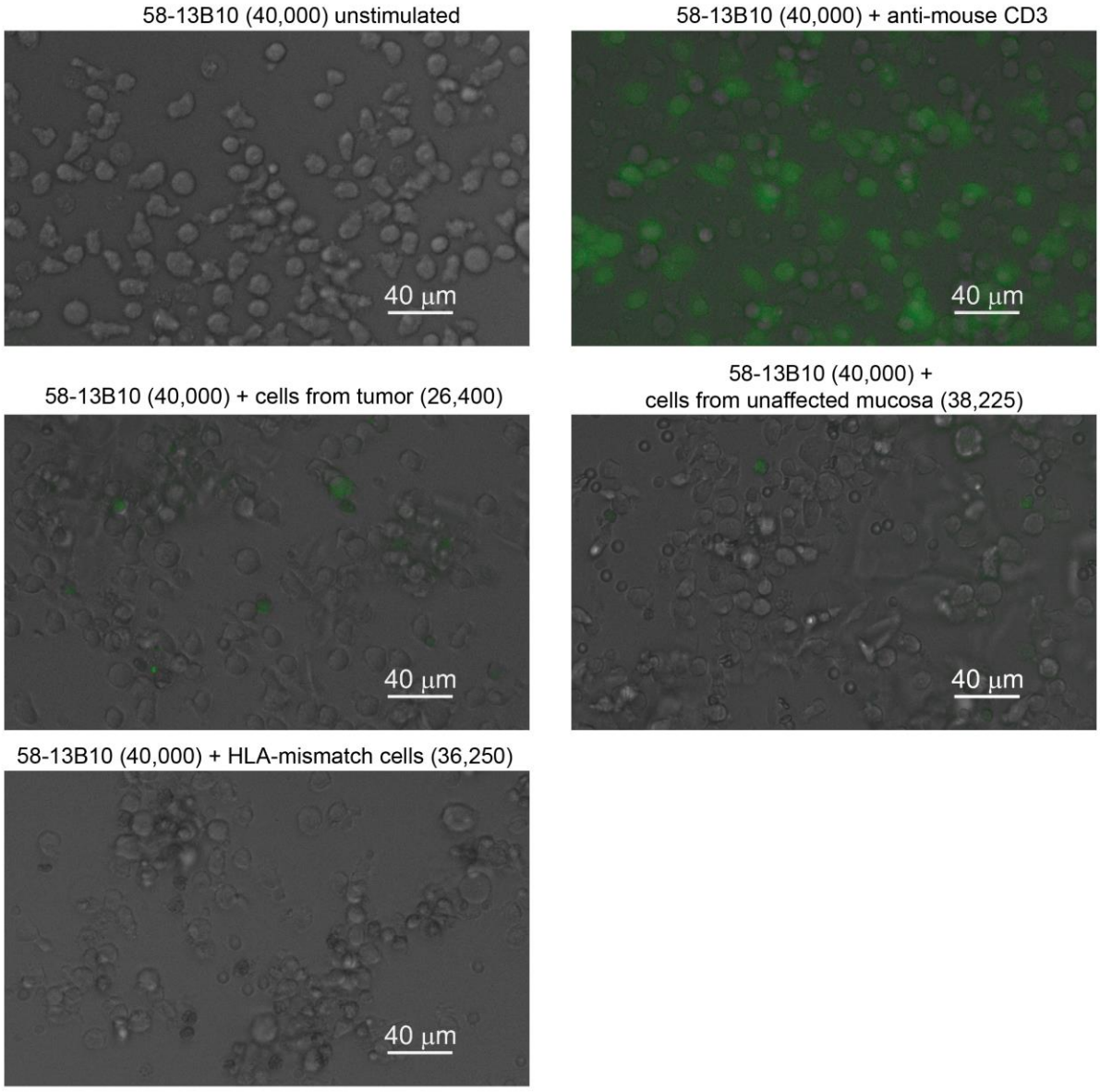


Suppl. Fig. 10 continued

B



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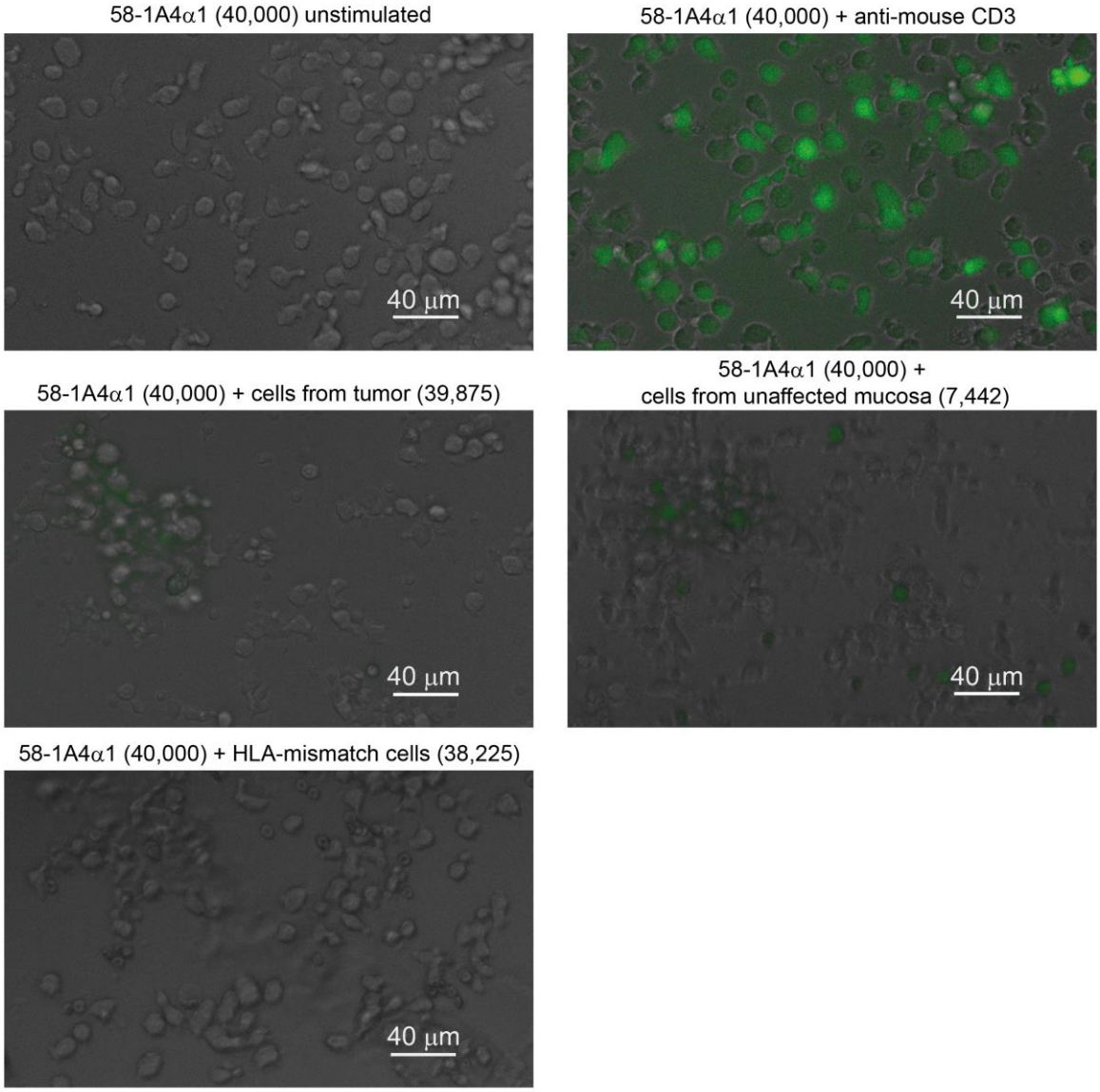

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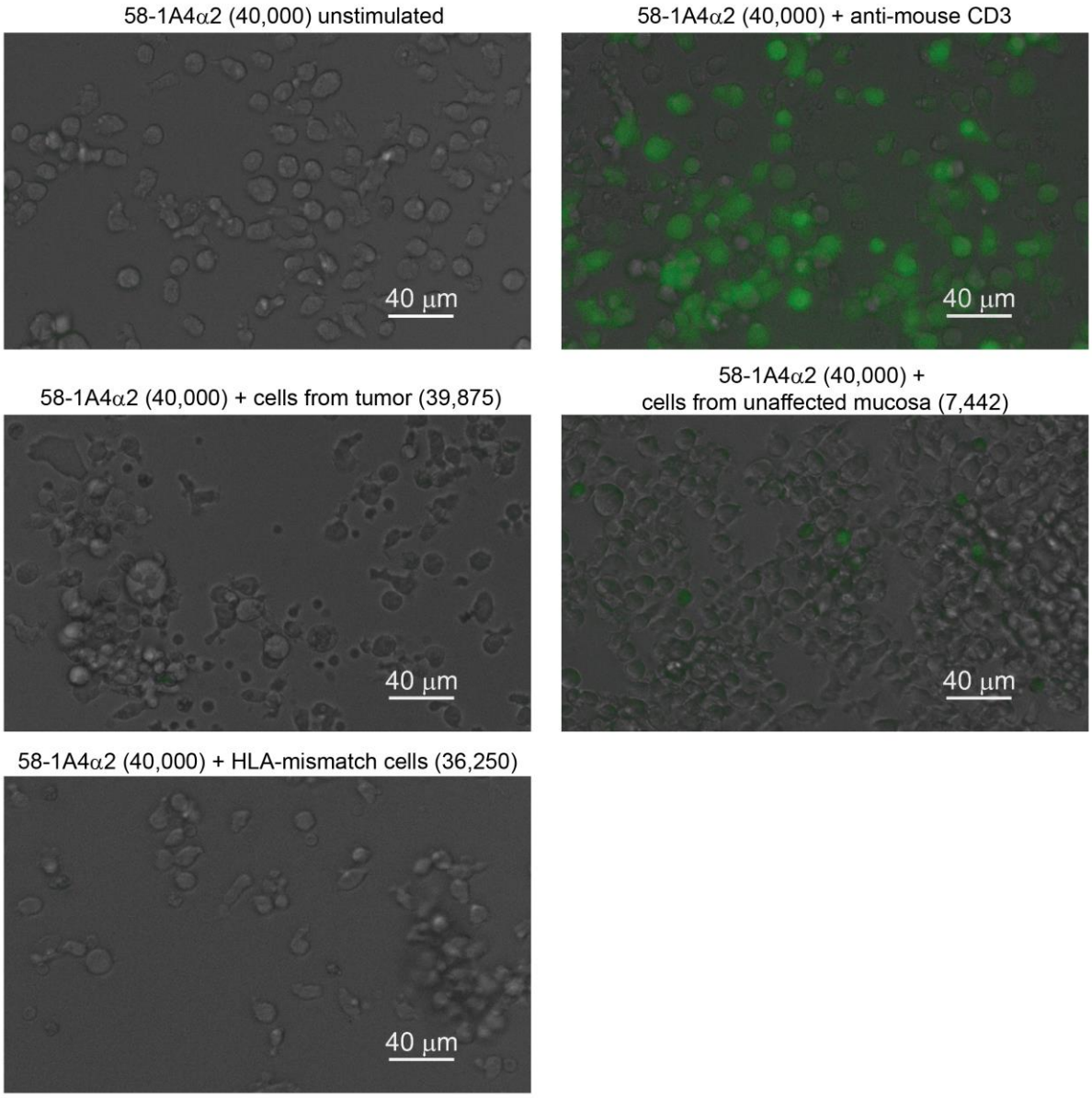

Suppl. Fig. 10 Co-incubation of TCR-recombinant cell lines with cells isolated from corresponding target tissue or cells isolated from tissue of an HLA-mismatched individual

$58\alpha^{-}\beta^{-}$ cell lines expressing selected recombinant TCRs were co-incubated with cells isolated from their corresponding tumor or unaffected mucosa tissue of origin, or with cells isolated from tissue of a different, HLA-mismatched individual. Absolute cell numbers for co-incubation are given in parentheses. For detailed cell numbers and culture conditions, see Supplementary Table 4. Fluorescence microscopy was used to screen the entire co-incubation wells for GFP^{+} cells that could not be distinguished from background with flow cytometry. Images from co-incubation with cells isolated from corresponding or HLA-mismatched tissues show single GFP^{+} cells if any were detectable in the entire well.