

# Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T Cells, Recurrence, and Survival in Epithelial Ovarian Cancer. *N Engl J Med* 2003;348:203-13.

**Supplementary Appendix 1. Antibodies Used for Immunostaining.**

Antibody	Target	Animal Source	Manufacturer	Clone
Anti-CD3	T cells	Mouse	BD Pharmingen	SK-7
Anti-CD3	T cells	Rabbit	Dako	
Anti-CD4	T-cell subgroup	Mouse	BD Pharmingen	SK-4
Anti-CD8	T-cell subgroup	Mouse	BD Pharmingen	SK-1
Anti-CD19	B cells	Mouse	BD Pharmingen	H1B19
Anti-CD57	Natural killer cells	Mouse	BD Pharmingen	NK-1
Anti-CD11c	Monocytes	Mouse	BD Pharmingen	S-HCL-3
Anti-CD45	All leukocytes	Mouse	Novocastra	X16/99
Anti-CD45RO	Memory cells	Mouse	BD Pharmingen	UCHL-1
Anti-CD83	Mature antigen-presenting cells	Mouse	BD Pharmingen	HB15a
Anti-MIG	Monokine induced by interferon- $\gamma$	Mouse	BD Pharmingen	B8-11
Anti-SLC	Secondary lymphoid-tissue chemokine	Rabbit	United States Biological	
Anti-MDC	Macrophage-derived chemokine	Goat	Santa Cruz Biotechnology	
Anti-VEGF	Vascular endothelial growth factor	Mouse	BD Pharmingen	G153-694
Anti-Ki-67	Proliferating cells, activated T cells, or both	Rabbit	Dako	
Anti-cytokeratin	Tumor cells and epithelial cells	Rabbit	Dako	
Anti-cytokeratin-8/18	Tumor cells and epithelial cells	Mouse	Novocastra	5D3

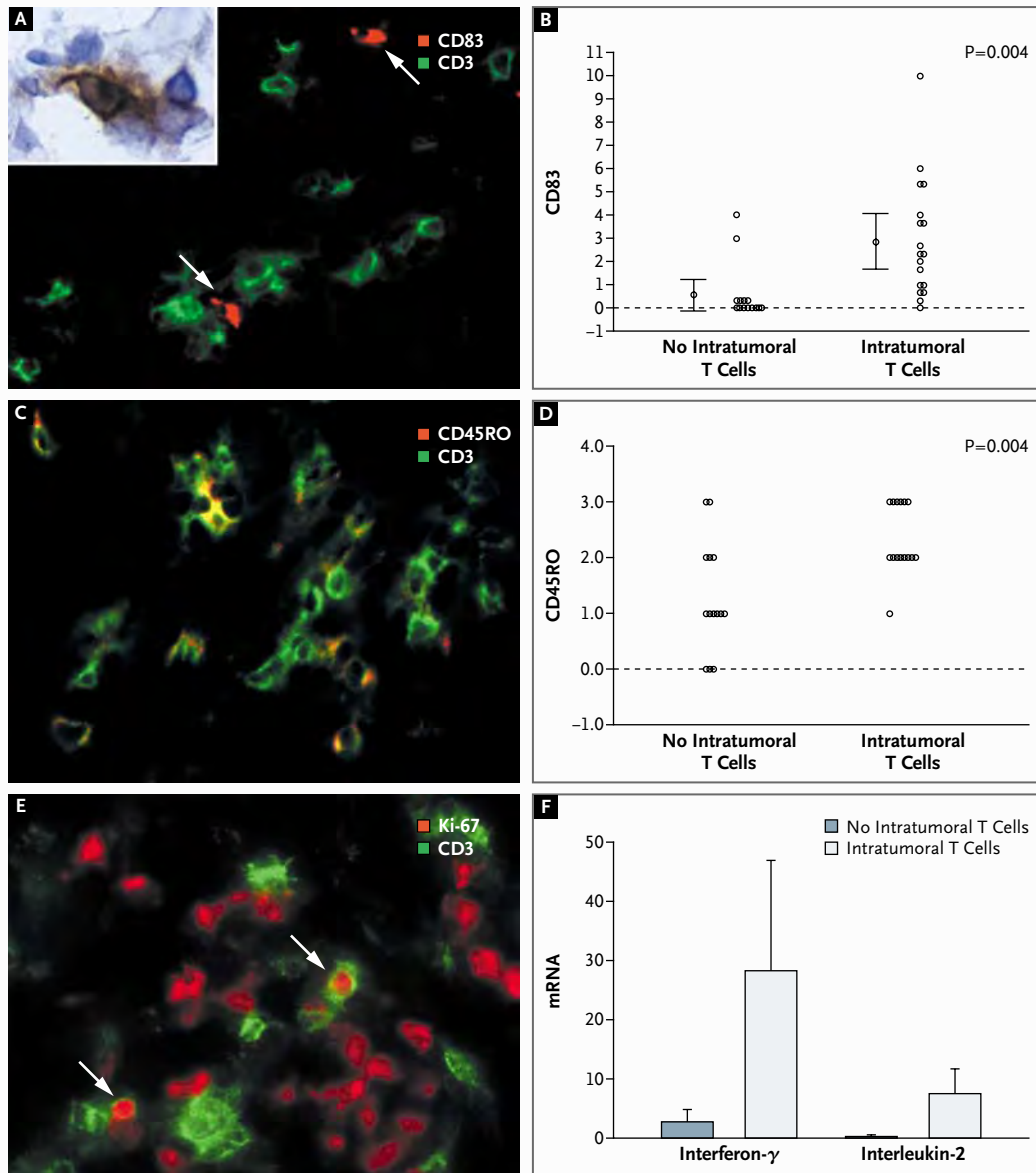
**Supplementary Appendix 2. Tests Performed on Tumor Specimens.\***

Test	Total No. of Tumor Specimens	No. of Specimens with Intratumoral T Cells	No. of Specimens without Intratumoral T Cells
Immunohistochemical analysis			
CD3	174	102	72
CD4	30	15	15
CD8	30	15	15
CD83	30	15	15
CD57	30	15	15
CD19	30	15	15
CD11c	30	15	15
CD45	30	15	15
CD3 and CD45RO	33	18	15
MIG	80	40	40
SLC	80	40	40
MDC	80	40	40
VEGF	80	40	40
Double immunofluorescence			
CD3–CD83	33	18	15
CD3–Ki-67	33	18	15
CD3–CD45RO	33	18	15
Real-time PCR			
CD3 $\epsilon$	26	16	10
Interferon- $\gamma$	26	16	10
Interleukin-2	26	16	10
Tumor necrosis factor $\alpha$	26	16	10
MIG	26	16	10
SLC	26	16	10
MDC	26	16	10
VEGF	26	16	10
MCP-1	26	16	10
I-309	26	16	10
SDF-1	26	16	10
VCAM-1	26	16	10
ICAM-1	26	16	10

\* MIG denotes monokine induced by interferon- $\gamma$ , SLC secondary lymphoid-tissue chemokine, MDC macrophage-derived chemokine, VEGF vascular endothelial growth factor, PCR polymerase chain reaction, MCP-1 monocyte chemoattractant protein 1, SDF-1 stroma-derived factor 1, VCAM-1 vascular-cell adhesion molecule 1, and ICAM-1 intercellular adhesion molecule 1.

**Supplementary Appendix 3. Amplification Primers Used for Real-Time Quantitative Polymerase Chain Reaction.**

Gene	Primer
Monokine induced by interferon- $\gamma$	GAC CTT AAA CAA TTT GCC CCA AG CAC ATC TGC TGA ATC TGG GTT TA
Macrophage-derived chemokine	TGC CGT GAT TAC GTC CGT TA TCT GAG GTC CAG TAG AAG TGT TTC A
Secondary lymphoid-tissue chemokine (exodus-2)	CCT CAA GTA CAG CCA AAG GAA GA TGC ACA TAG CTC TGC CTG AGA
I-309	CAG ACC AGA AGA CAT GCA GAT CA CCG CAA ATG AGA AGC AAC ATC
Stroma-derived factor 1	AAT TCT CAA CAC TCC AAA CTG TGC TGC ACA CTT GTC TGT TGT TGT TC
Monocyte chemoattractant protein 1	GCT CAT AGC AGC CAC CTT CAT T TGG TGA AGT TAT AGC AGC AGG TG
Interleukin-2	AAC TCA CCA GGA TGC TCA CAT TT TTA GCA CTT CCT CCA GAG GTT TG
Interferon- $\gamma$	TTG GCT CTG CAT TAT TTT TCT GTC CTG CCA GGA CCC ATA TGT AAA AG
Tumor necrosis factor $\alpha$	CCA GGC AGT CAG ATC ATC TTC TC AGC TGG TTA TCT CTC AGC TCC AC
Vascular endothelial growth factor	AAC CAT GAA CTT TCT GCT GTC TTG TTC ACC ACT TCG TGA TGA TTC TG
Vascular-cell adhesion molecule 1	CTT AAA ATG CCT GGG AAG ATG GT GTC AAT GAG ACG GAG TCA CCA AT
Intercellular adhesion molecule 1	ACG CTG AGC TCC TCT GCT ACT C GGG CAG GAT GAC TTT TGA GG
CD3 $\epsilon$	GTC CCA TGA AAC AAA GAT GCA GT CCA CCC ATT TCT TCA TTA CCA TC
Glyceraldehyde-3-phosphate dehydrogenase	CCT GCA CCA CCA ACT GCT TA CAT GAG TCC TTC CAC GAT ACC A



#### Supplementary Appendix 4. Activation of Immune Mechanisms in Association with Intratumoral T Cells.

In Panel A, double immunofluorescence with a monoclonal antibody against CD83 (Texas red; red, arrows) and with a polyclonal antibody against CD3 (fluorescein; green) demonstrates clustering of T cells around a CD83+ antigen-presenting cell. The inset shows immunohistochemical detection of a CD83+ cell with morphologic features of a dendritic cell, surrounded by small mononuclear cells with scant cytoplasm, identifying them as lymphocytes. Panel B shows quantification by image analysis of CD83+ cells detected by immunohistochemical analysis in tumors with T cells and those without T cells. There is a significantly higher number of CD83+ cells in tumors harboring T cells than in tumors without T cells. In Panel C, double immunofluorescence with a monoclonal antibody against CD45RO (Texas red; red) and a polyclonal antibody against CD3 (fluorescein; green) shows memory CD45RO+CD3+ T cells in a tumor containing T cells. Double-positive cells appear yellow due to colocalization of both CD45RO and CD3 on the cell surface. Panel D shows quantification by image analysis of CD45RO+ (memory) T cells detected by immunohistochemical analysis in the stroma of tumors with or without T cells. There are significantly more memory T cells in the stroma of tumors containing T cells than in the stroma of tumors without T cells. In Panel E, double immunofluorescence with a monoclonal antibody against CD3 (fluorescein; green) and a polyclonal antibody against Ki-67 (Texas red; red) reveals activated Ki-67+CD3+ T cells in a tumor containing T cells. Panel F shows the results of real-time quantitative PCR analysis of the levels of interleukin-2 and interferon- $\gamma$  messenger RNA (mRNA) in 16 ovarian carcinomas harboring intratumoral T cells and 10 ovarian carcinomas without intratumoral T cells. Higher levels of interleukin-2 mRNA ( $P=0.09$ ) and interferon- $\gamma$  mRNA ( $P=0.02$ ) were found in tumors containing T cells than in tumors without T cells. The presence of both cytokines indicates antigen-dependent T-cell activation and type 1 polarization. The y axis represents the relative expression of interferon- $\gamma$  and interleukin-2 in relation to glyceraldehyde-3-phosphate dehydrogenase.