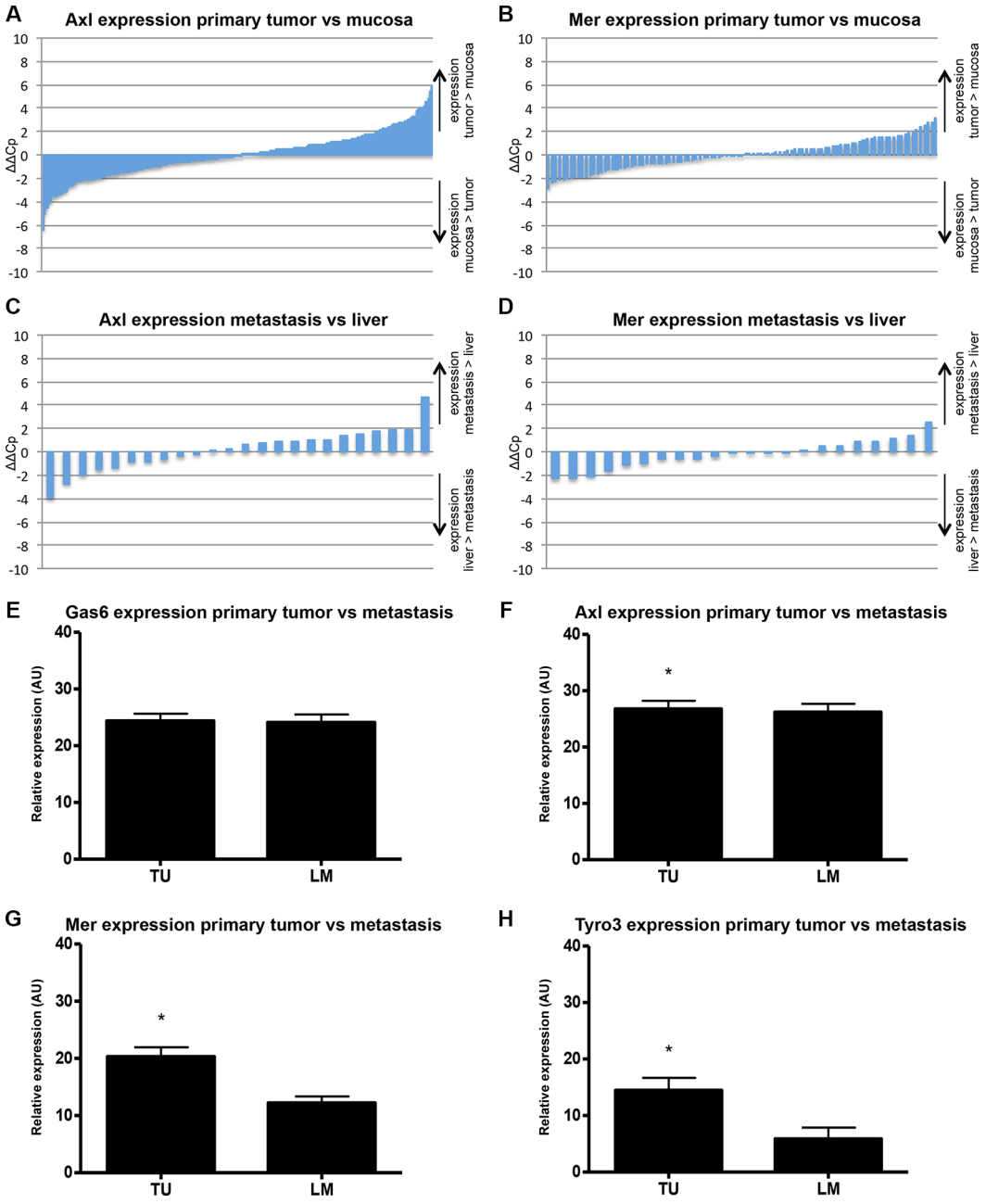
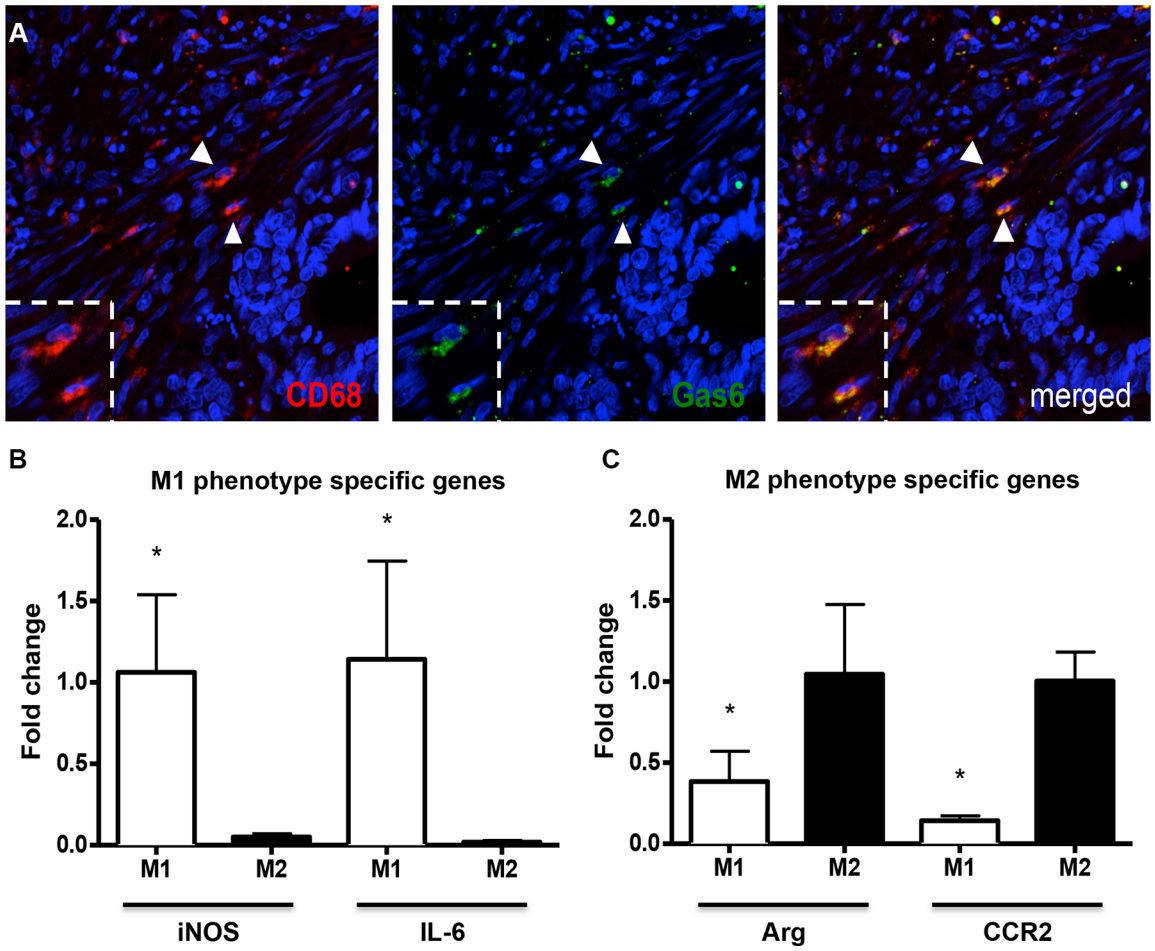
TAM receptors Tyro3 and Mer as novel targets in colorectal cancer

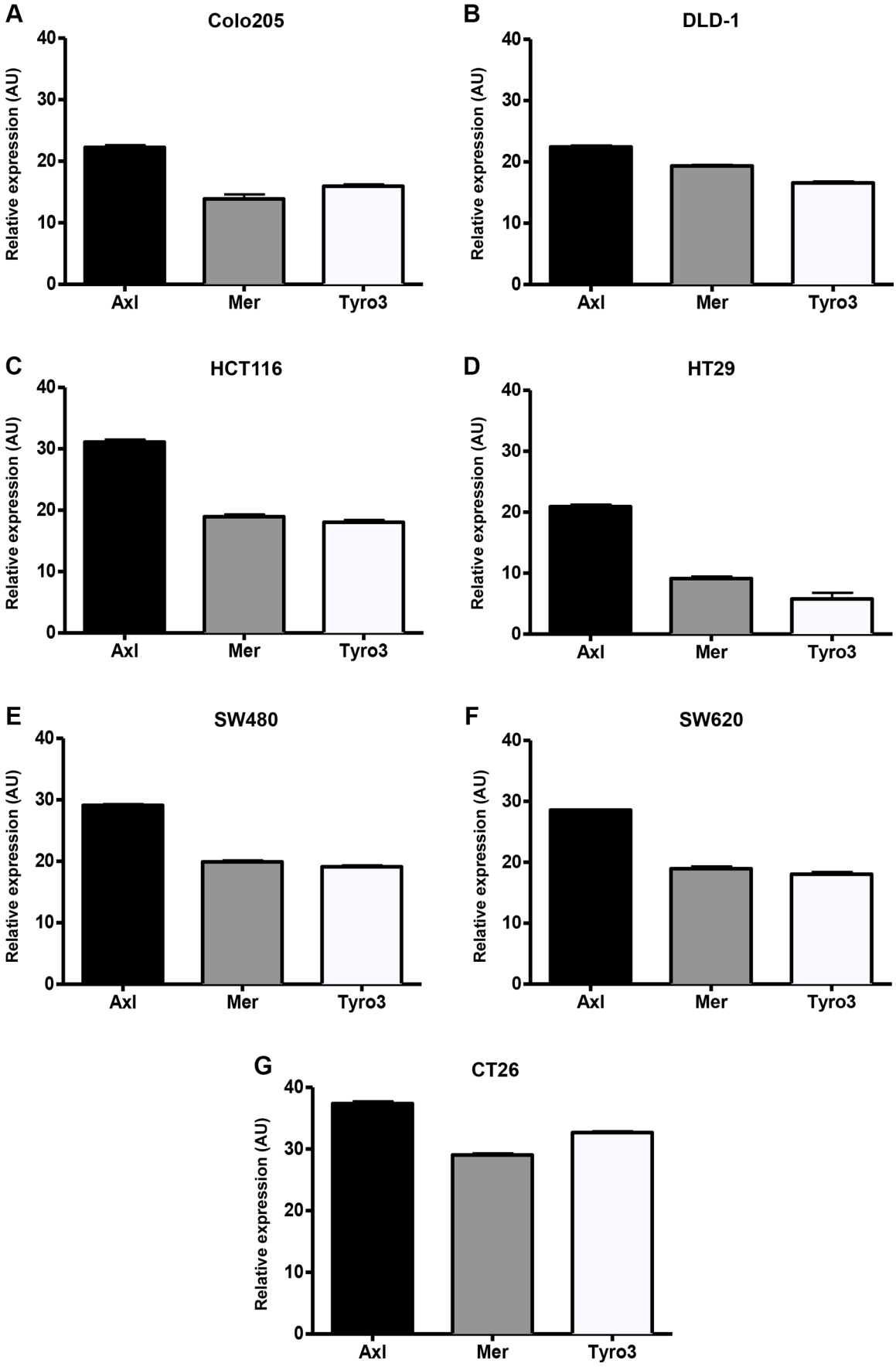
Supplementary Materials



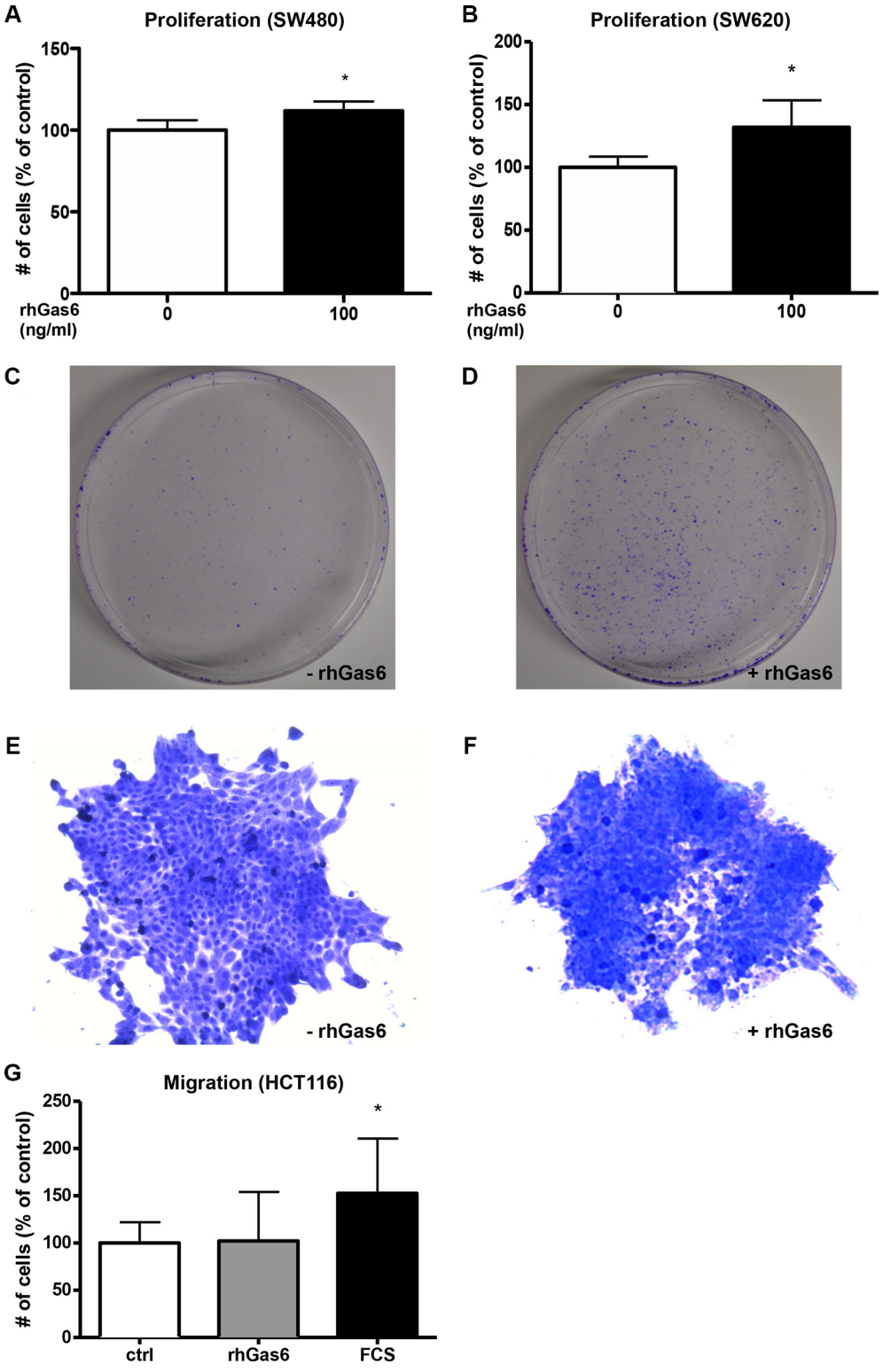
Supplementary Figure S1: *In vivo* mRNA expression analysis of human tissue samples. (**A**–**D**) Each bar represents one patient. Positive bars represent higher expression in tumor or metastasis compared to normal mucosa or normal liver tissue. Negative bars represent higher expression in mucosa or normal liver tissue compared to tumor or liver metastasis. (A) Axl mRNA expression in human CRC tumor samples and normal mucosa of the same patients respectively ($n = 200$; P = NS). (B) Mer mRNA expression in human CRC tumor samples and normal mucosa of the same patients respectively ($n = 103$; P = NS). (C) Axl mRNA expression in human CRC liver metastases and normal liver tissue of the same patients respectively ($n = 24$; P = NS). (D) Mer mRNA expression in human CRC liver metastases and normal liver tissue of the same patients respectively ($n = 24$; P = NS). (**E**–**H**) Average Gas6 (P = NS), Axl ($P < .05$), Mer ($P < .05$) and Tyro3 ($P < .05$) mRNA expression in tumor (TU; $n = 200$) and liver metastasis (LM; $n = 24$; AU = 50 – ΔCp).



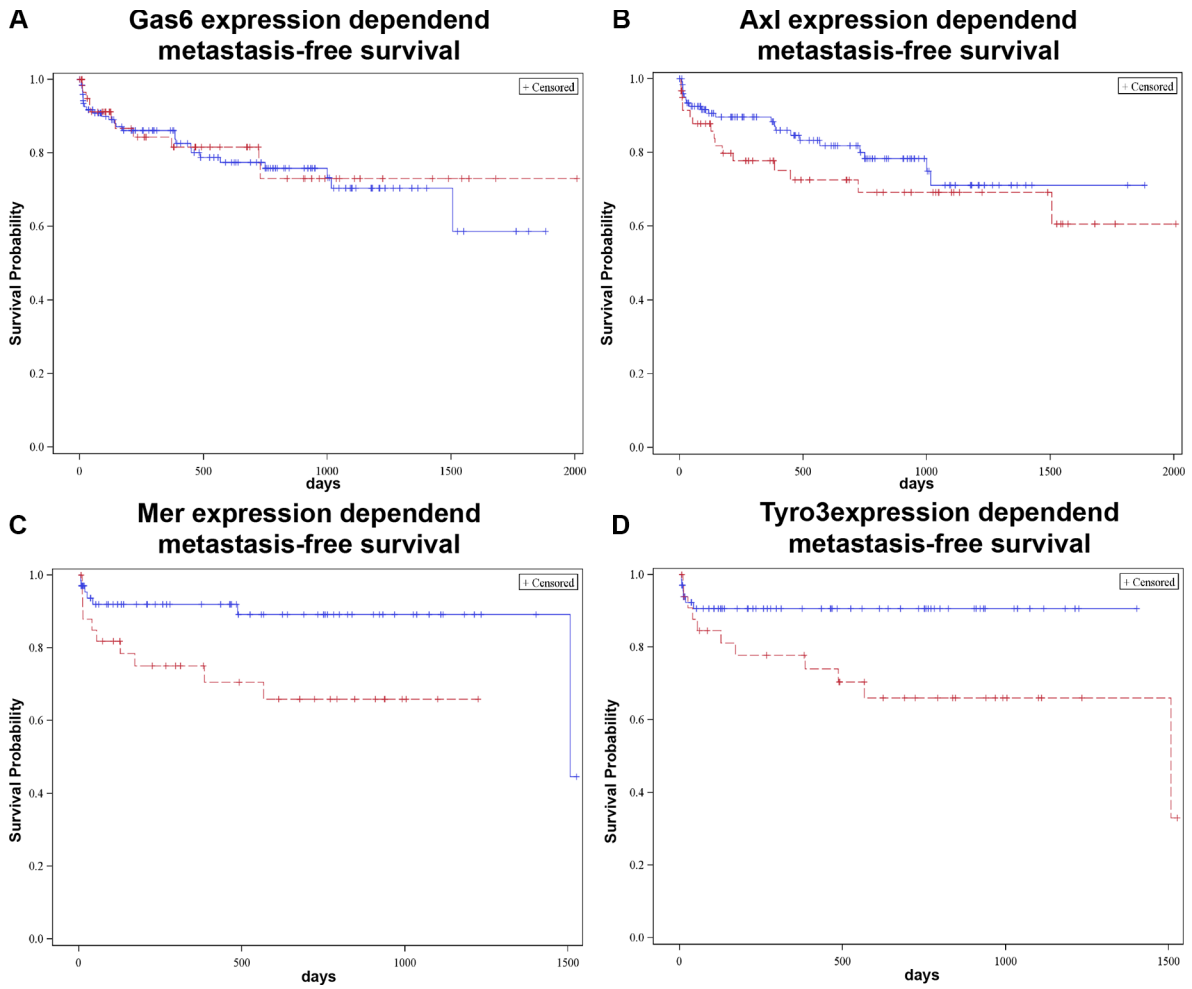
Supplementary Figure S2: Gas6/CD68 double immune staining and RT-PCR analysis for verification of macrophage M1 and M2 phenotype. (**A**) Exemplary immunofluorescence stainings for Gas6 (red) and CD68 (green) in human colorectal cancer samples showing co-localized expression in tumor infiltrating macrophages (orange). White arrows indicate double stained cells. (**B**, **C**) Macrophage cell line J774A.1 was used for verification of the differentiation towards an M1 or M2 phenotype by LPS (1 µg/ml) or M-CSF (10 ng/ml) treatment respectively. (A) mRNA expression of iNOS and IL-6 as two M1 phenotype specific genes is significantly higher in LPS (M1) compared to M-CSF (M2) treated macrophages ($n = 3$; $P < .05$). (B) mRNA expression of Arginase (Arg) and CCR2 as two M2 phenotype specific genes is significantly higher in M-CSF (M2) compared to LPS (M1) treated macrophages ($n = 3$; $P < .05$).



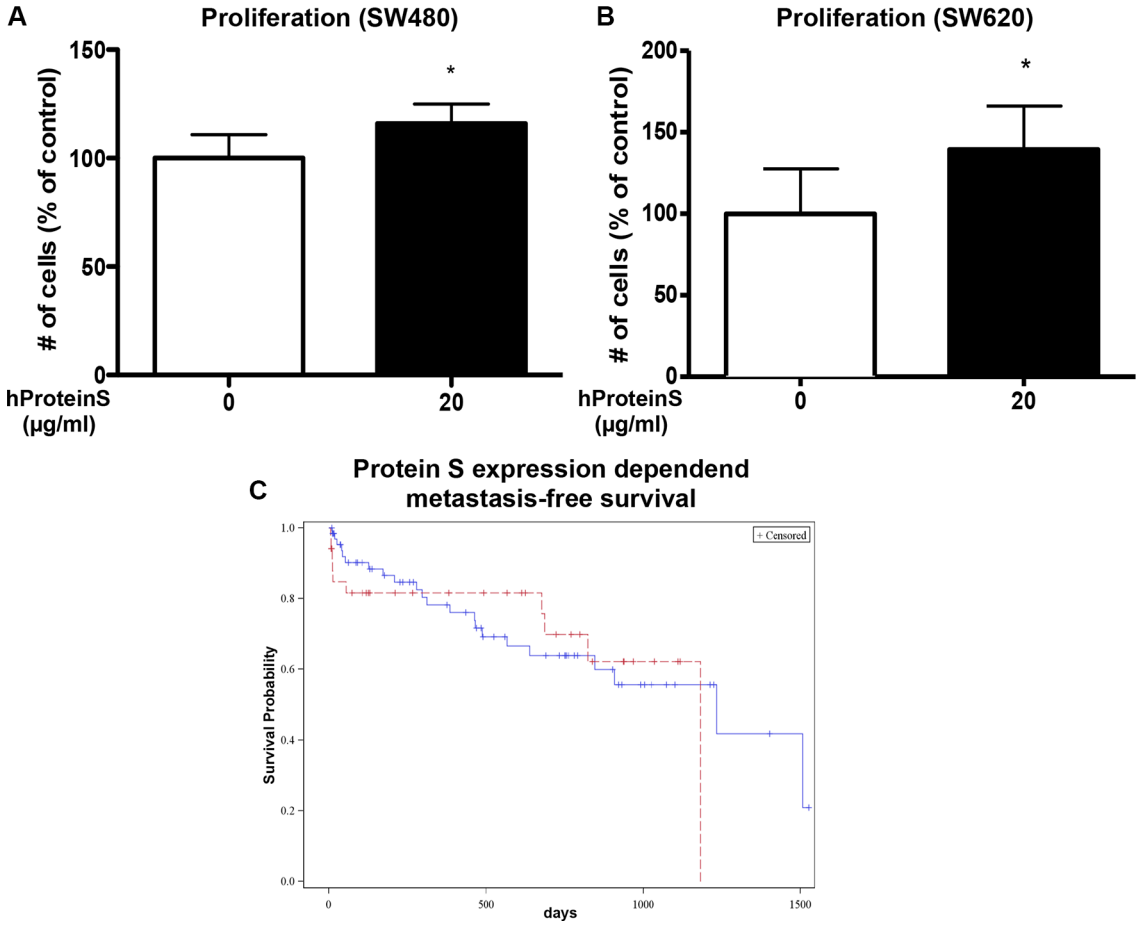
Supplementary Figure S3: TAM receptor expression in human and murine colorectal cancer cell lines. RT-PCR technique was used to analyze the TAM receptor expression levels of different human and murine colorectal cancer cell lines. (**A**–**F**) Axl, Mer and Tyro3 mRNA expression in human colorectal cancer cell lines (n = 3; AU = 50 – ΔCp). (**G**) Axl, Mer and Tyro3 mRNA expression in the murine colorectal cancer cell line CT26 (n = 3; AU = 50 – ΔCp).



Supplementary Figure S4: Gas6 induces proliferation, colony- and sphere-formation in human colorectal cancer cell lines *in vitro*. (**A**, **B**). Human recombinant Gas6 (rhGas6) induces proliferation of human colorectal cancer cell lines (SW480 and SW620) *in vitro* after 48 hours treatment ($n = 6$; $P < .05$). (**C**) Colony-forming assay; control treated colorectal cancer cells (HCT116). Only few colonies were formed after 10 days. (**D**) Colony-forming assay; colorectal cancer cells (HCT116) treated with 100 ng/ml recombinant human Gas6. Significantly more colonies were formed after 10 days compared to control treated cells. (**E**) Sphere-formation assay; control treated colorectal cancer cells (HCT116). Cancer cells were grown mainly in monolayer and were less likely to build spheres after 10 days. (**F**) Sphere-formation assay; colorectal cancer cells (HCT116) treated with 100 ng/ml recombinant human Gas6. Significantly more spheres were formed after 10 days compared to control treated cells. (**G**) Migration assay using a transwell method showing no influence of Gas6 concerning tumor cell migration after 24 hours treatment of human colorectal cancer cells (HCT116) with 100 ng/ml recombinant human Gas6 (rhGas6) *in vitro* ($n = 12$; P = NS). 20% FCS was used as a positive control showing significantly increased migration ($n = 12$; $P < .05$).



Supplementary Figure S5: Gas6 and TAM receptor expression correlated to patients metastasis-free survival. RT-PCR analysis was performed for Gas6, Axl, Mer and Tyro3 in colorectal tumor samples and normal mucosa of the patients listed in Table 1. The patient cohort was split in two groups comprising 33,3% of patients with the highest target gene expression (red) and the remaining 66,7% of patients (blue). Subsequently ΔΔCp values (Figure 1A, 1B and Supplementary Figure S1A, S1B) were correlated to patients metastasis-free survival. (**A**, **B**) Relative Gas6 and Axl mRNA expression is not associated with patients metastasis-free survival (n = 200; P = NS). (**C**, **D**) Relative Mer and Tyro3 mRNA expression in negative associated with patients metastasis-free survival (n = 103; P < .05).



Supplementary Figure S6: ProteinS induces proliferation *in vitro* but is not associated with patients survival *in vivo*. (**A**, **B**) WST-1 reagent (Roche) was used to measure cell viability *in vitro*. Human ProteinS (hProteinS) induces proliferation of human colorectal cancer cell lines (SW480 and SW620) *in vitro* after 48 hours treatment ($n = 6$; $P < .05$). (**C**) RT-PCR analysis was performed for ProteinS in colorectal tumor samples and normal mucosa of the patients listed in Table 1. The patient cohort was split in two groups comprising 33,3% of patients with the highest target gene expression (red) and the remaining 66,7% of patients (blue). Subsequently ΔΔCp values were correlated to patients metastasis-free survival. Relative ProteinS mRNA expression is not associated with patients metastasis-free survival ($n = 103$; P = NS)

Supplementary Table S1: Human primers for RT-PCR expression analysis in human tissue samples

Gene	Company	Sequence	Ref-No.
Gas6	Qiagen	-	QT00049126
Axl	Qiagen	-	QT00067725
Mer	Qiagen	-	QT00031017
Tyro3	Qiagen	-	QT00055482
Protein S	Qiagen	-	QT00011746
18s	Invitrogen	Fwd: AAA CGG CTA CCA CAT CCA AG Rev: CCT CCA ATG GAT CCT CGT TA	-

Primers were used for mRNA expression analysis in colorectal cancer tissue samples and normal mucosa as well as in colorectal liver metastasis and normal liver tissue respectively.

Supplementary Table S2: Patient characteristics for mRNA expression analysis of Gas6, Axl, Mer,Tyro3 and ProteinS

		n (%) or median (IQR)
	Total *n*	**200 (100)**
Gender		
	male	114 (57)
	female	86 (43)
Age (years)		65,13 (27–88)
Tumor size		
	T1	10 (5)
	T2	46 (23)
	T3	119 (59,5)
	T4	25 (12,5)
Lymph node status		
	positive	99 (49,5)
	negative	101 (50,5)
Distant metastases		
	positive	55 (27,5)
	negative	145 (72,5)
UICC		
	I	41 (20,5)
	II	51 (25,5)
	III	55 (27,5)
	IV	53 (26,5)
Tumor differentiation		
	high (G1)	1 (0,5)
	moderate (G2)	153 (76,5)
	poor (G3)	44 (22)
	N.N.	2 (1)
Tumor location		
	colon	101 (50,5)
	rectosigmoid	13 (6,5)
	rectum	86 (43)
Neoadjuvant therapy		
	Yes	46 (23)
	No	151 (75,5)
	N.N.	3 (1,5)
Adjuvant therapy		
	Yes	99 (49,5)
	No	101 (50,5)
Treatment		
	curative (R0)	155 (77,5)
	palliative (R1/R2)	45 (22,5)

Summary of patient characteristics used for mRNA expression analysis of the target genes Gas6, Axl, Mer, Tyro3 and ProteinS in colorectal cancer tissue and normal mucosa of each patient respectively (n = 200).

Supplementary Table S3: Patient characteristics for Gas6 and CD68 immunostainings

		n (%) or median (IQR)
Total *n*		**102 (100)**
Gender		
	male	62 (60,8)
	female	40 (39,2)
Age (years)		65 (21-88)
Tumor size		
	T1	3 (2,9)
	T2	21 (20,6)
	T3	65 (63,7)
	T4	13 (12,8)
Lymph node status		
	positive	47 (46,1)
	negative	55 (53,9)
Distant metastases		
	positive	22 (21,6)
	negative	80 (78,4)
UICC		
	I	20 (19,6)
	II	31 (30,4)
	III	28 (27,5)
	IV	23 (22,5)
Tumor differentiation		
	High (G1)	1 (1,0)
	Moderate (G2)	70 (68,6)
	Poor (G3)	30 (29,4)
	N.N.	1 (1,0)
Tumor location		
	colon	49 (48)
	rectosigmoid	6 (5,9)
	rectum	47 (46,1)
Neoadjuvant therapy		
	Yes	36 (35,3)
	No	66 (64,7)
Adjuvant therapy		
	Yes	41 (40,2)
	No	61 (59,8)
Treatment		
	curative (R0)	82 (80,4)
	palliative (R1/R2)	20 (19,6)

Summary of patient characteristics used for immunohistochemical analysis of Gas6 and CD68 in human colorectal cancer tissue samples and normal mucosa of each patient respectively ($n = 102$).

Supplementary Table S4: Human and murine cell lines used for *in vitro* assays

ATCC NO.	Name	Species	Origin	Histolgy	Tumor source	Mutant Gene	Mutation
CCL-222	Colo205	human	Colon	adenocarcinoma	Metastasis, Ascites	BRAF KRAS PIK3CA TP53	wt wt wt mutant
CCL-221	DLD-1	human	Colon	adenocarcinoma	Primary tumor	BRAF KRAS PIK3CA TP53	wt mutant mutant mutant
CCL-247	HCT116	human	Colon ascendens	carcinoma	Primary tumor	BRAF KRAS PIK3CA TP53	wt wt mutant wt
HTB-38	HT29	human	Colon	carcinoma	Primary tumor	BRAF KRAS PIK3CA TP53	mutant wt mutant mutant
CCL-228	SW480	human	Colon	adenocarcinoma	Primary tumor	BRAF KRAS PIK3CA TP53	wt mutant wt mutant
CCL-227	SW620	human	Colon	adinocarcinoma	Lymph node metastasis	BRAF KRAS PIK3CA TP53	wt mutant wt mutant
CRL-2638	CT26	mouse	Colon	carcinoma	-	BRAF KRAS PIK3CA TP53	wt mutant wt wt
TIB-67	J774A.1	mouse	Monocyte, Macrophage	-	Ascites	-	-

Detailed information about the different cell lines including mutations was collected from previously published literature (Ahmed D et al., Oncogenesis, 2013).

Supplementary Table S5: Human and murine primers for RT-PCR expression analysis *in vitro*

Gene	Species	Company	Sequence	Ref-No.
Gas6	human	Qiagen	-	QT00049126
Gas6	mouse	Qiagen	-	QT00101332
Axl	human	Qiagen	-	QT00067725
Axl	mouse	Qiagen	-	QT00101353
Tyro3	human	Qiagen	-	QT00055482
Tyro3	mouse	Qiagen	-	QT00197659
Mer	human	Qiagen	-	QT00031017
Mer	mouse	Qiagen	-	QT00148561
18s	human	Invitrogen	Fwd: AAA CGG CTA CCA CAT CCA AG Rev: CCT CCA ATG GAT CCT CGT TA	-
18s	mouse	Invitrogen	Fwd: GTA ACC CGT TGA ACC CCA TT Rev: CCA TCC AAT CGG TAG TAG CG	-

Primers were used for baseline mRNA expression analysis of Gas6, Axl, Mer and Tyro3 as well as after 5-FU treatment *in vitro*.

Supplementary Table S6: Murine primers for RT-PCR expression analysis of M1 and M2 phenotype macrophages *in vitro*

Gene	Company	Sequence	Ref-No.
iNOS	Invitrogen	Fwd: CTC ACT GGG ACA GCA CAG AA Rev: GGT CAA ACT CTT GGG GTT CA	-
IL-6	Invitrogen	Fwd: CAA AGC CAG AGT CCT TCA GAG Rev: GCC ACT CCT TCT GTG ACT CC	-
Arginase	Invitrogen	Fwd: GTG TAC ATT GGC TTG CGA GA Rev: AGG TGA ATC GGC CTT TTC TT	-
CCR2	Invitrogen	Fwd: CCT GCA AAG ACC AGA AGA GG Rev: TAT GCC GTG GAT GAA CTG AG	-

Primers of M1 (iNOS, IL-6) and M2 (Arginase, CCR2) phenotype specific genes were selected to verify the differentiation of J774A.1 towards an M1 or M2 phenotype by LPS (1 μg/ml) or M-CSF (10 ng/ml) treatment respectively.

Supplementary Table S7: Human siRNAs for TAM receptor knockdown *in vitro*

Gene	Company	Sequence	Ref-No.
Axl	Invitrogen	Fwd: CCA GGA ACU GCA UGC UGA AUG AGA A Rev: UUC UCA UUC AGC AUG CAG UUC CUG G	HSS183343
Mer	Invitrogen	Fwd: CCA GAA CCA UGA GAU GUA UGA CUG U Rev: AUA GUC AUA CAU CUC AUG GUU CUG G	HSS116030
Tyro3	Invitrogen	Fwd: GCU GUG CCU CCA AAC UGC CUG UCA A Rev: UUG ACA GGC AGU UUG GAG GCA CAG C	HSS187439

Human colorectal cancer cell line HCT116 was transfected with TAM receptor siRNAs and proliferation was assessed *in vitro* using the Cell Proliferation Reagent WST-1 (Roche).