

Activated Eosinophils Predict Longer Progression-Free Survival under Immune Checkpoint Inhibition in Melanoma

Nadine L. Ammann, Yasmin F. Schwietzer, Christian Mess, Julia-Christina Stadler, Glenn Geidel, Julian Kött, Klaus Pantel, Stefan W. Schneider, Jochen Utikal, Alexander T. Bauer, Christoffer Gebhardt

Table S1. Quantification summary of Siglec-8, ECP, EPX and CD8 expressing cells within the human tissue samples represented on the nine TMAs.

Following the immunofluorescence staining, counts of Siglec-8+, ECP+ and EPX+ eosinophils, as well as CD8+ T cells, are listed for each tissue type (nevus, primary tumor, associated metastasis). Positive cells were normalized to the punch area (in mm²) and an average value was formed for each tissue type. The corresponding standard deviation is given in brackets.

Cell-marker	Tissue typ			Total amount of analyzed tissue samples
	Melanocytic nevus	Primary melanoma	Metastasis	
Siglec-8	N=73	N=104	N=167	N=344
cells/mm ² (SD)	6.8 (7.3)	28.4 (45.8)	28.0 (42.4)	23.6 (39.8)
ECP	N=73	N=105	N=151	N=329
cells/mm ² (SD)	13.9 (15.2)	67.3 (84.8)	61.9 (109.0)	52.8 (90.6)
EPX	N=77	N=106	N=146	N=329
cells/mm ² (SD)	2.2 (12.9)	11.2 (29.2)	22.9 (74.7)	14.3 (53.4)
CD8	N=77	N=108	N=177	N=362
cells/mm ² (SD)	35.2 (52.4)	183.1 (291.9)	243.3 (393.5)	181.1 (328.3)

Abbreviations: Siglec-8: sialic acid-binding Ig-like lectin 8; SD: standard deviation; N: number of tissue samples; ECP: eosinophil cationic protein; EPX: eosinophil peroxidase; CD: cluster of differentiation

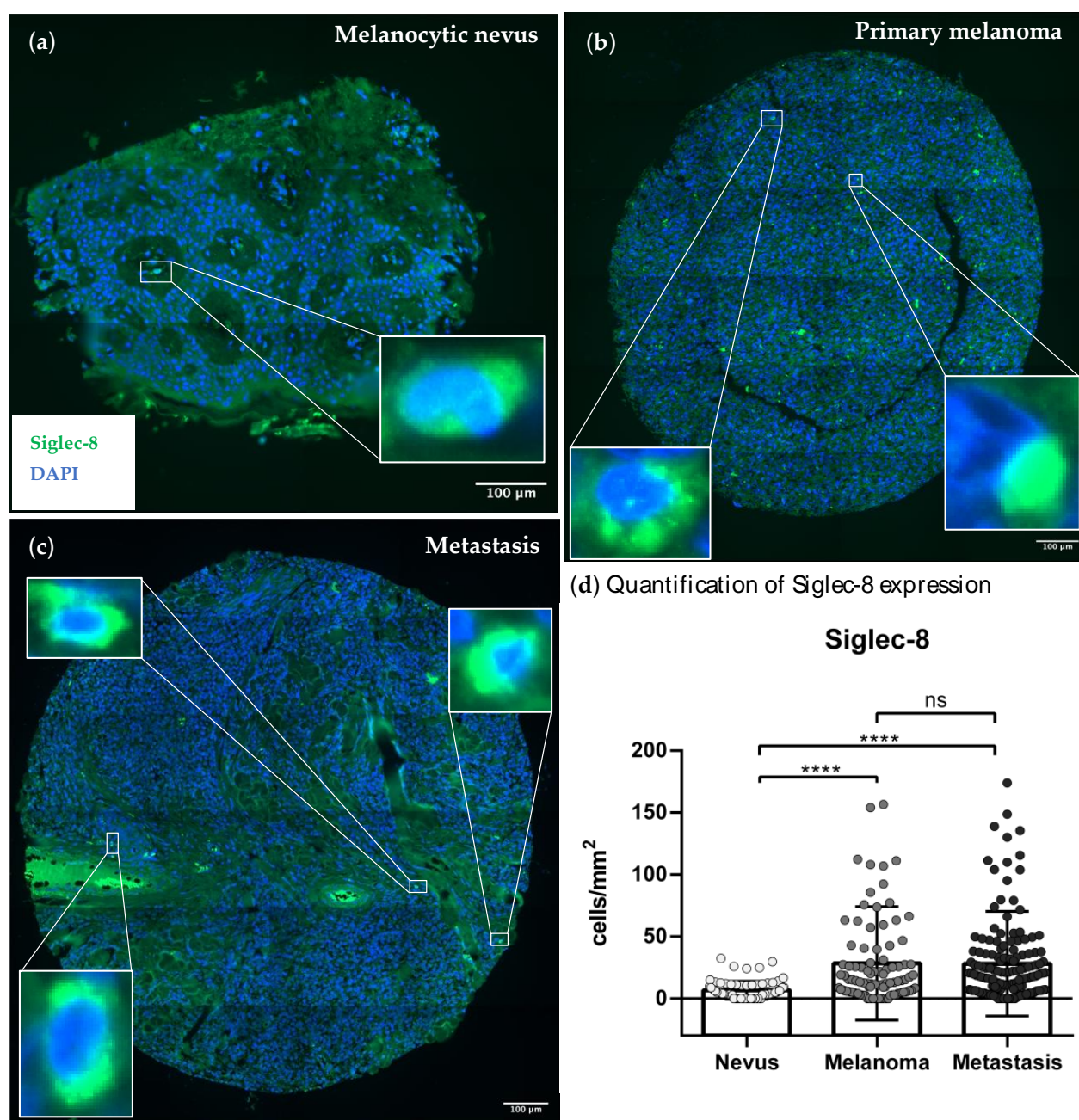


Figure S1. Representative expression patterns of Siglec-8⁺ eosinophils in human tumor tissue sections. Paraffin-embedded tissue samples of 73 melanocytic nevi, 104 primaries and 167 metastases from 118 melanoma patients were stained with immunofluorescent anti-Siglec-8 antibodies. (a) Exemplary image of a melanocytic nevi stained with anti-Siglec-8 antibodies; (b) Exemplary image of a primary melanoma stained with anti-Siglec-8 antibodies; (c) Exemplary image of an associated metastasis of melanoma stained with anti-Siglec-8 antibodies; (d) Comparison of Siglec-8 expression between nevi, primaries and metastases of melanoma patients. Abbreviations: Siglec-8: sialic acid-binding Ig-like lectin 8; ns: not significant; ****= $p < 0.0001$

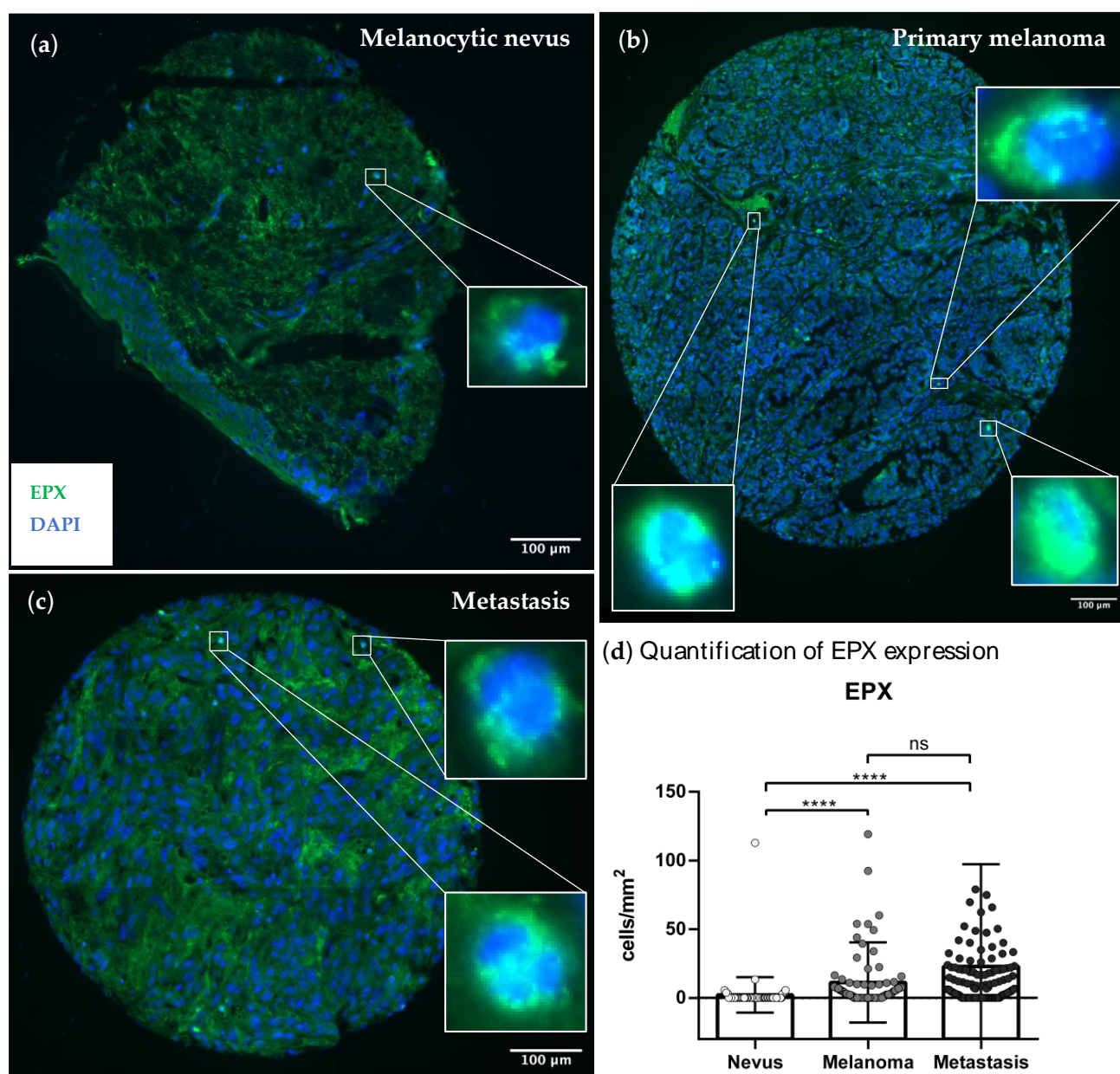


Figure S2. Representative expression patterns of EPX+ eosinophils in human tumor tissue sections. Paraffin-embedded tissue samples of 77 melanocytic nevi, 106 primaries and 146 metastases from 118 melanoma patients were stained with immunofluorescent anti-EPX antibodies. (a) Exemplary image of a melanocytic nevus stained with anti-EPX antibodies; (b) Exemplary image of a primary melanoma stained with anti-EPX antibodies; (c) Exemplary image of an associated metastasis of melanoma stained with anti-EPX antibodies; (d) Comparison of EPX expression between nevi, primaries and metastases of melanoma patients. Abbreviations: EPX: eosinophil peroxidase; ****= $p < 0.0001$

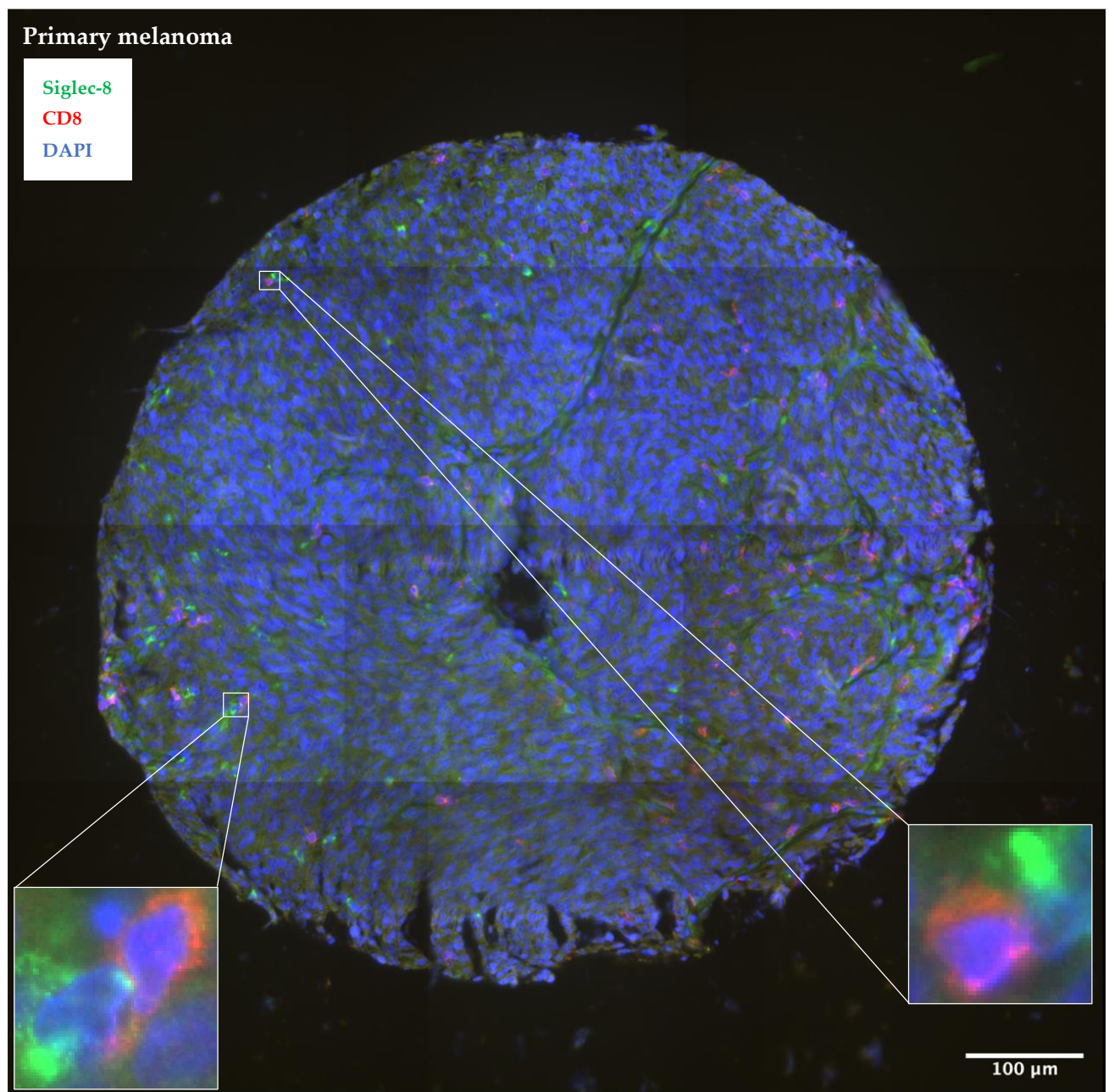


Figure S3. Primary melanoma with a high contiguous infiltration of Siglec-8 expressing eosinophils and CD8 expressing effector T-cells. Paraffin-embedded tissue samples of a metastasis is co-stained with immunofluorescent anti-Siglec-8 antibodies (marked green) and anti-CD8 antibodies (marked red). Nuclei were stained in blue with DAPI. Abbreviations: Siglec-8: sialic acid-binding Ig-like lectin; CD: cluster of differentiation; DAPI = 4',6-Diamidin-2-phenylindol

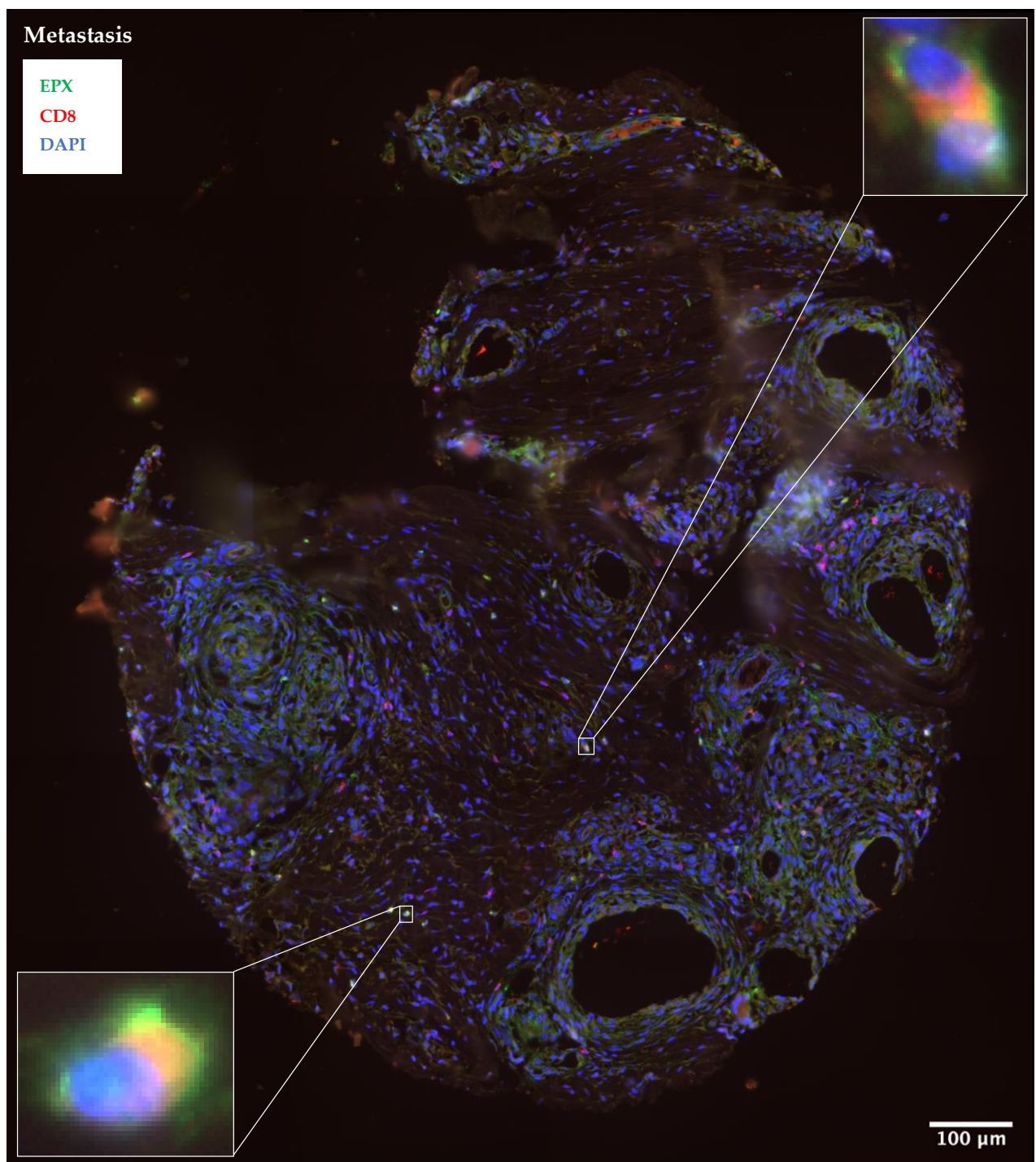


Figure S4. Melanoma metastasis with a high contiguous infiltration of EPX expressing eosinophils and CD8 expressing effector T-cells. Paraffin-embedded tissue samples of a metastasis is co-stained with immunofluorescent anti-EPX antibodies (marked green) and anti-CD8 antibodies (marked red). Nuclei were stained in blue with DAPI. Abbreviations: EPX: eosinophil peroxidase; CD: cluster of differentiation; DAPI = 4',6-Diamidin-2-phenylindol

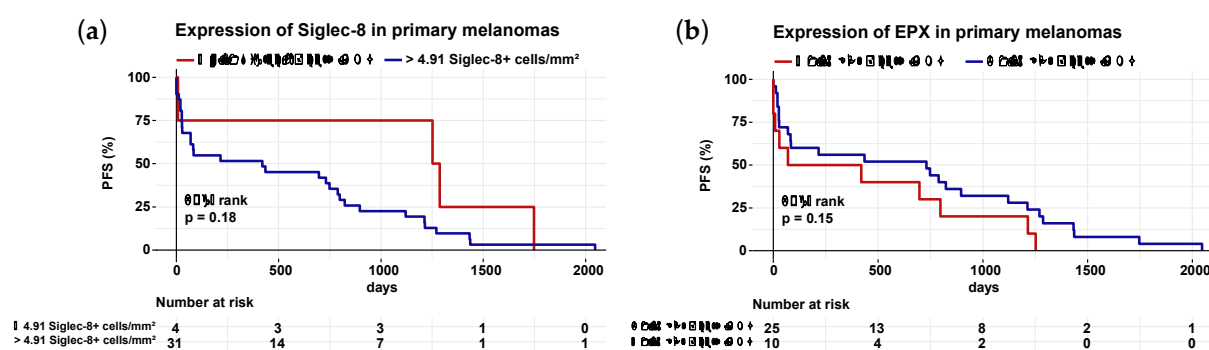


Figure S5. The number of Siglec-8 and EPX expressing eosinophils has no impact on PFS in primary melanoma. Kaplan-Meier survival curves for PFS of melanoma patients were stratified by the level (high vs. low infiltration) of Siglec-8+ and EPX+ eosinophils in the 106 primary melanoma samples. p -values were calculated by the two-sided log-rank test. (a) Survival analysis of melanoma patients with increased (>4.91 cells/mm²) Siglec-8 expressing cells in the primaries of melanoma versus those with low Siglec-8 expression; (b) Survival analysis for PFS of melanoma patients with increased (>1.76 cells/mm²) EPX expressing cells in the primaries of melanoma versus those with low EPX expression. Abbreviations: Siglec-8: sialic acid-binding Ig-like lectin 8; EPX: eosinophil peroxidase; PFS: progression-free survival; p : p -value

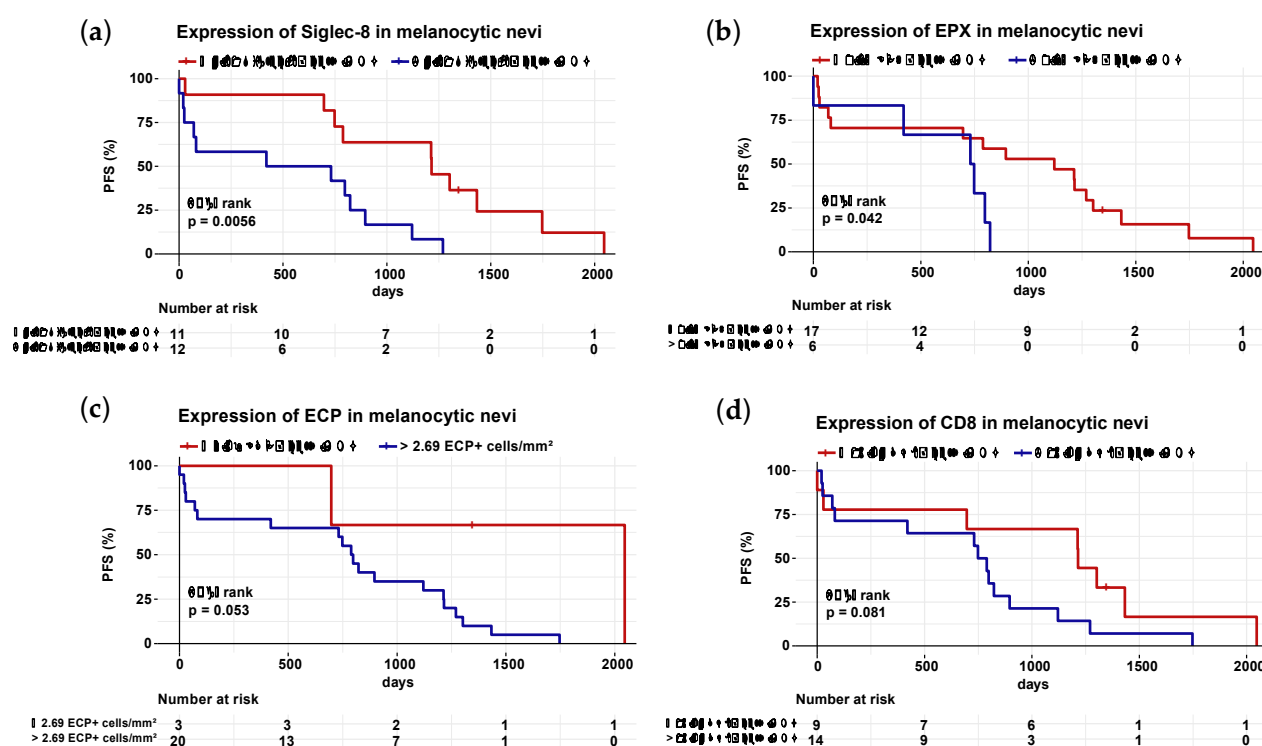


Figure S6. High numbers of Siglec-8, EPX, ECP expressing eosinophils and CD8 expressing effector T-cells are linked with impaired PFS in melanocytic nevi. Kaplan-Meier survival curves for PFS of melanoma patients were stratified by the level (high vs. low infiltration) of Siglec-8+, EPX+, ECP+ eosinophils and CD8+ T-cells in the 77 melanocytic nevi. p -values were calculated by the two-sided log-rank test. (a) Survival analysis of melanoma patients with decreased (<4.81 cells/mm²) Siglec-8 expressing cells in the melanocytic nevi versus those with high Siglec-8 expression; (b) Survival analysis for PFS of melanoma patients with decreased (<0.95 cells/mm²) EPX expressing cells in the melanocytic nevi versus those with high EPX expression; (c) Survival analysis of melanoma patients with decreased (<2.69 cells/mm²) ECP expressing cells in the melanocytic nevi versus those with high ECP expression; (d) Survival analysis for PFS of melanoma patients with decreased (<16.54 cells/mm²) CD8 expressing cells in the melanocytic nevi versus those with high CD8 expression. Abbreviations: Siglec-8: sialic acid-binding Ig-like lectin 8; EPX: eosinophil peroxidase; ECP: eosinophil cationic protein; CD: cluster of differentiation; PFS: progression-free survival; p : p -value

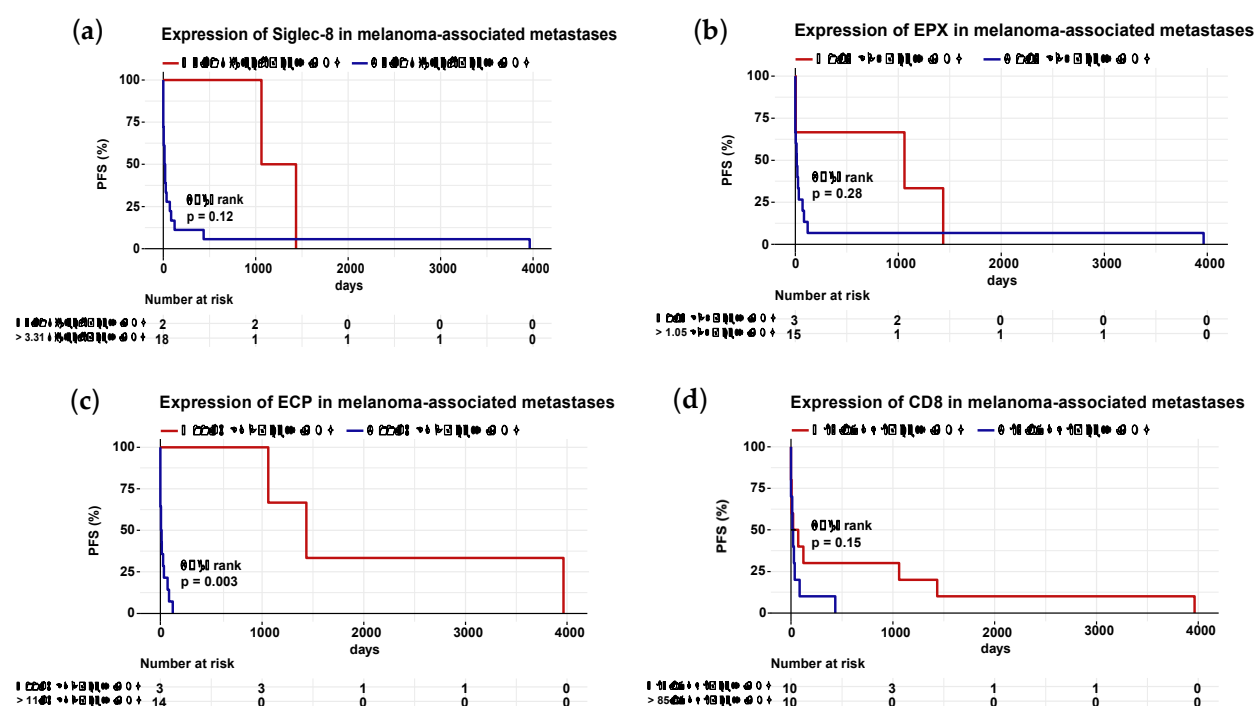


Figure S7. High numbers of Siglec-8, EPX, ECP expressing eosinophils and CD8 expressing effector T-cells are linked with impaired PFS in melanoma-associated metastases. Kaplan-Meier survival curves for PFS of melanoma patients were stratified by the level (high vs. low infiltration) of Siglec-8+, EPX+, ECP+ eosinophils and CD8+ T-cells in the 177 melanoma-associated metastases. *p*-values were calculated by the two-sided log-rank test. (a) Survival analysis of melanoma patients with decreased (<3.31 cells/mm²) Siglec-8 expressing cells in the melanoma-associated metastases versus those with high Siglec-8 expression; (b) Survival analysis for PFS of melanoma patients with decreased (<1.05 cells/mm²) EPX expressing cells in the melanoma-associated metastases versus those with high EPX expression; (c) Survival analysis of melanoma patients with decreased (<11.56 cells/mm²) ECP expressing cells in the melanoma-associated metastases versus those with high ECP expression; (d) Survival analysis for PFS of melanoma patients with decreased (<85.17 cells/mm²) CD8 expressing cells in the melanoma-associated metastases versus those with high CD8 expression. Abbreviations: Siglec-8: sialic acid-binding Ig-like lectin 8; EPX: eosinophil peroxidase; ECP: eosinophil cationic protein; CD: cluster of differentiation; PFS: progression-free survival; *p*: *p*-value

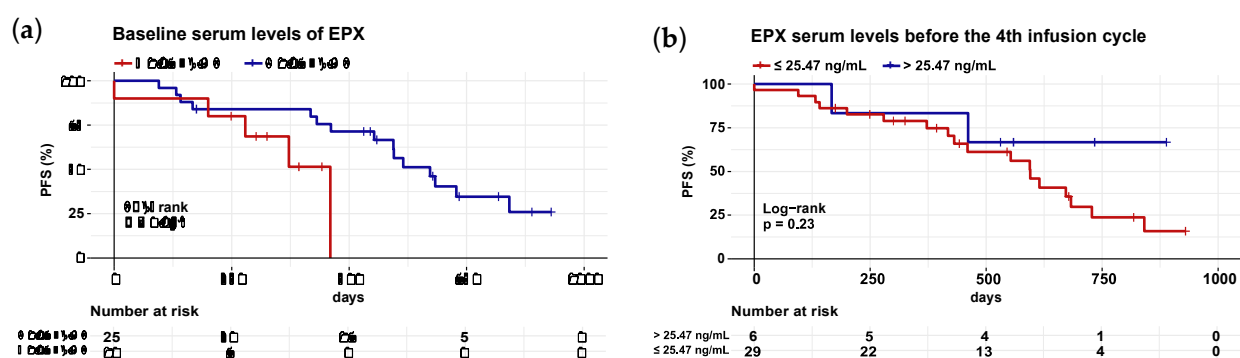


Figure S8. Elevated baseline EPX serum levels are associated with extended PFS in stage III and IV melanoma patients treated with ICI. Blood was taken before the start of ICI (baseline, BE) and shortly before the fourth infusion cycle of ICI (4th cycle, C4). Kaplan-Meier survival curves for PFS of advanced staged melanoma patients were stratified by the amount (high vs. low) of EPX serum levels. *p*-values were calculated by the two-sided log-rank test; (a) Survival analyses for PFS of advanced melanoma patients with elevated (>1.07 ng/mL) baseline EPX serum levels versus those with low EPX levels; (b) Survival analyses for PFS of advanced melanoma patients with elevated (>25.47 ng/mL) C4 EPX serum levels versus those with low EPX levels. Abbreviations: EPX: eosinophil peroxidase; PFS: progression-free survival; ICI: immune checkpoint inhibition; BE: baseline; C4: 4th infusion cycle; *p*: *p*-value

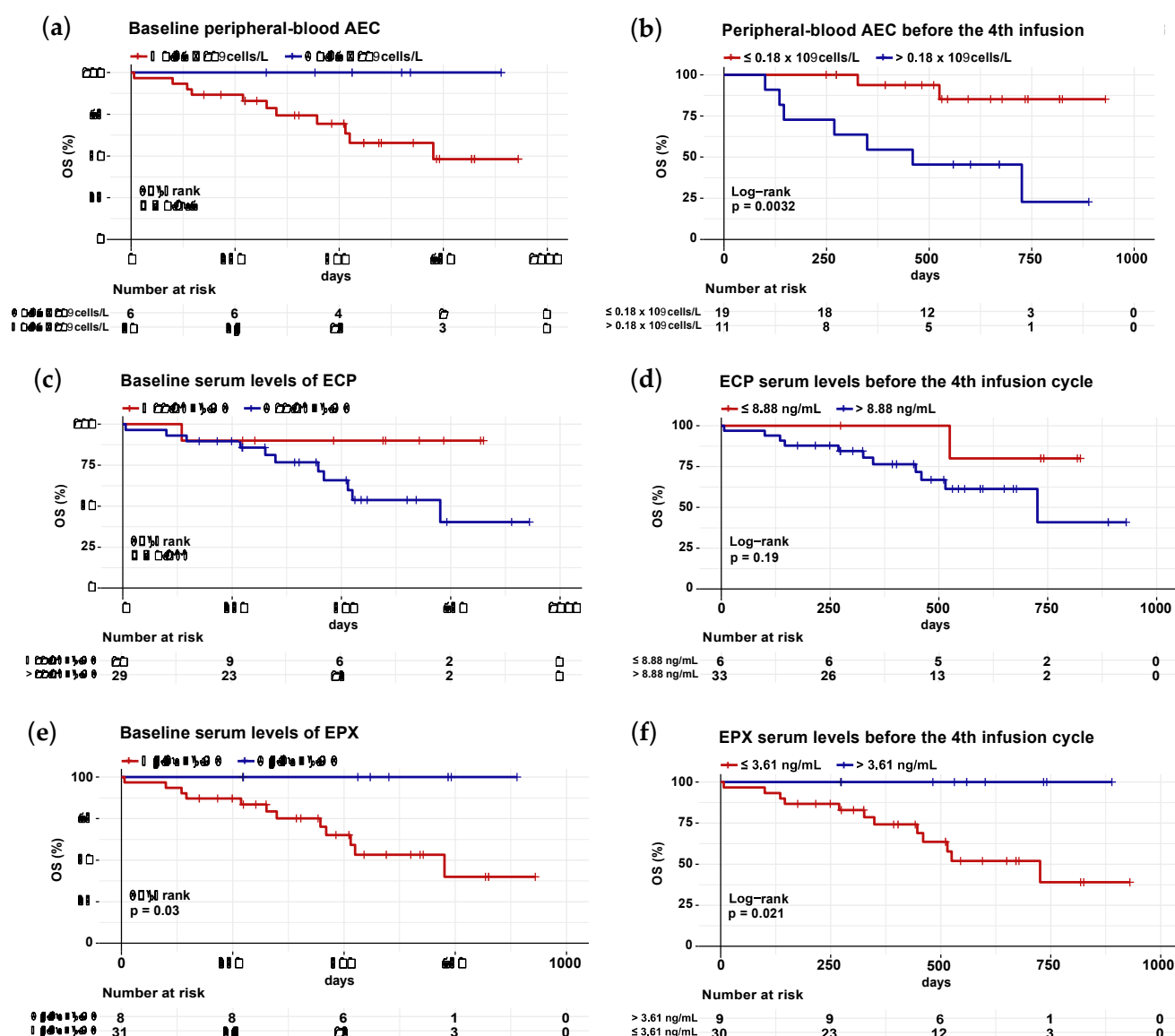


Figure S9. Impact of peripheral-blood AEC, ECP and EPX serum levels before and during ICI on OS in metastatic melanoma patients. Blood was taken before the start of ICI (baseline, BE) and shortly before the fourth infusion cycle of ICI (4th cycle, C4). Kaplan-Meier survival curves for OS of advanced staged melanoma patients were stratified by the amount (high vs. low) of peripheral-blood AEC, ECP and EPX serum levels. p -values were calculated by the two-sided log-rank test; (a) Survival analyses for OS of advanced melanoma patients with elevated ($>0.27 \times 10^9 \text{ cells/L}$) baseline AEC versus those with low peripheral-blood AEC; (b) Survival analyses for OS of advanced melanoma patients with elevated ($>0.18 \times 10^9 \text{ cells/L}$) C4 AEC versus those with low peripheral-blood AEC; (c) Survival analyses for OS of advanced melanoma patients with elevated ($>11.08 \text{ ng/mL}$) baseline ECP serum levels versus those with low ECP levels; (d) Survival analyses for OS of advanced melanoma patients with elevated ($>8.88 \text{ ng/mL}$) C4 ECP serum levels versus those with low ECP levels; (e) Survival analyses for OS of advanced melanoma patients with elevated ($>4.29 \text{ ng/mL}$) baseline EPX serum levels versus those with low EPX levels; (f) Survival analyses for OS of advanced melanoma patients with elevated ($>3.61 \text{ ng/mL}$) C4 EPX serum levels versus those with low EPX levels. Abbreviations: AEC: absolute eosinophil count; ECP: eosinophil cationic protein; EPX: eosinophil peroxidase; OS: overall survival; ICI: immune checkpoint inhibition; BE: baseline; C4: 4th infusion cycle; p : p -value

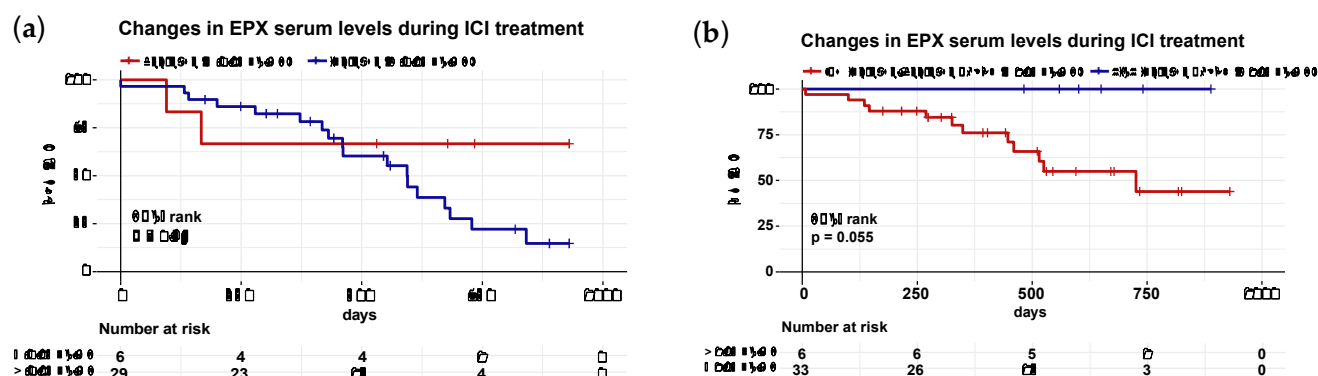


Figure S10. The progression of serum EPX levels under ICI has no significant impact on the PFS and OS of patients with metastatic melanoma. For the kinetic analyses, the level of EPX before the fourth infusion cycle of ICI (C4) was subtracted from the corresponding level before ICI was initiated (baseline). Kaplan-Meier survival curves for PFS and OS of patients with advanced melanoma were stratified by the extent of the difference between baseline and C4 values of EPX in the serum. *p*-values were calculated by the two-sided log-rank test; (a) Survival analyses for PFS of stage III and IV melanoma patients with decreasing (cut-off -0.15 ng/mL) EPX serum levels compared to patients with increasing EPX levels between baseline and C4; (b) Survival analyses for OS of stage III and IV melanoma patients with low increasing to decreasing (cut-off 1.05 ng/mL) EPX serum levels compared to patients with high increasing EPX levels between baseline and C4. Abbreviations: EPX: eosinophil peroxidase; ICI: immune checkpoint inhibition; C4: 4th infusion cycle; PFS: progression-free survival; OS: overall survival; BE: baseline; *p*: *p*-value

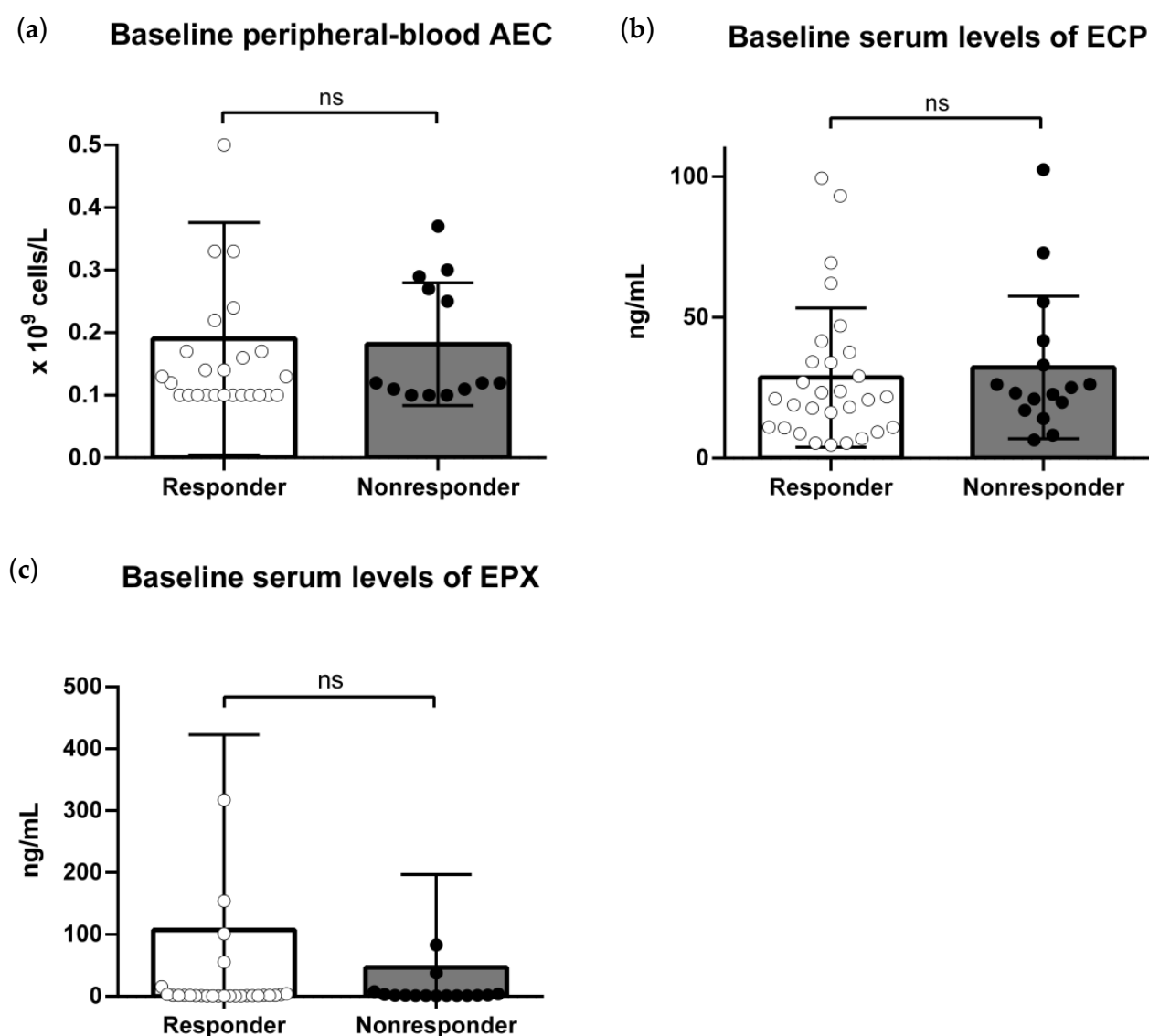


Figure S11. Responders and non-responders show no difference in their baseline levels of AEC, ECP and EPX. We classified the 45 metastatic melanoma patients into responders (13 patients with CR and 16 with PR) and non-responders (5 patients with SD and 11 patients with PD) based on their best response to ICI. Subsequently, baseline AEC in peripheral blood, as well as baseline ECP and EPX serum levels were compared within both groups via one-way analysis of variance (ANOVA). The p -values were calculated according to the Kruskal-Wallis test. (a) ANOVA analysis of baseline AEC in peripheral blood of responders and non-responders; (b) ANOVA analysis of baseline ECP serum levels among responders and non-responders; (c) ANOVA analysis of baseline EPX serum levels among responders and non-responders. Abbreviations: AEC: absolute eosinophil count; ECP: eosinophil cationic protein; EPX: eosinophil peroxidase; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; ICI: immune checkpoint inhibition; ANOVA: analysis of variance; ns: not significant

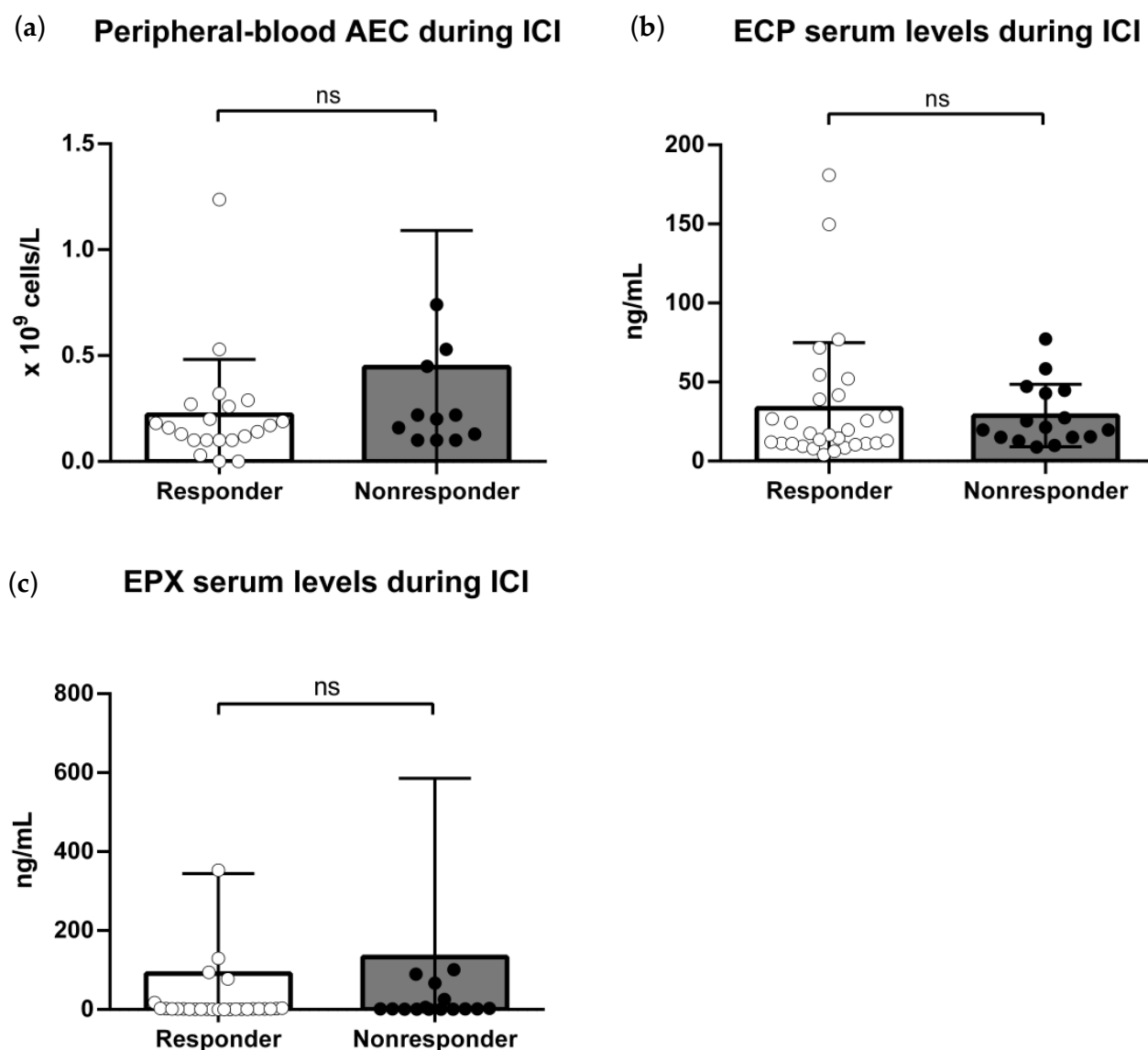


Figure S12. Responders and non-responders show no difference in their C4 levels of AEC, ECP and EPX. We classified the 45 metastatic melanoma patients into responders (13 patients with CR and 16 with PR) and non-responders (5 patients with SD and 11 patients with PD) based on their best response to ICI. Subsequently, C4 AEC in peripheral blood, as well as C4 ECP and EPX serum levels were compared within both groups via one-way analysis of variance (ANOVA). The *p*-values were calculated according to the Kruskal-Wallis test. (a) ANOVA analysis of C4 AEC in peripheral blood of responders and non-responders; (b) ANOVA analysis of C4 ECP serum levels among responders and non-responders; (c) ANOVA analysis of C4 EPX serum levels among responders and non-responders. Abbreviations: C4: 4th infusion cycle; AEC: absolute eosinophil count; ECP: eosinophil cationic protein; EPX: eosinophil peroxidase; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; ICI: immune checkpoint inhibition; ANOVA: analysis of variance; ns: not significant