SUPPLEMENTARY INFORMATION

CXCR4 inhibitors could benefit to HER2 but not to Triple-Negative breast cancer patients

Lefort S.[1,2], Thuleau A.[3], Kieffer Y. [1,2], Sirven P.[1,2], Bieche I.[4], Marangoni E.[3], Vincent-Salomon A.[5], and Mechta-Grigoriou F.[1,2]*

Running title: Inhibition of CXCL12/CXCR4 axis in breast cancers

Keywords: CXCR4, CXCL12/SDF-1, stroma, AMD3100, TN1403, PDX, HER2, TN

* Corresponding author: Fatima Mechta-Grigoriou Phone: +33 (0)1 56 24 66 53; Fax: +33 (0)1 56 24 66 50; E-mail address: fatima.mechta-grigoriou@curie.fr

SUPPLEMENTARY METHODS

Tumor dissociation and Flow cytometry analysis.

Tumors collected from PDX were immediately processed after sacrifice, and cut into small pieces in a petri dish. Tumor fragments were crushed with a 10ml needle piston. A first step of mechanical dissociation started in which 10ml of Cell Dissociation Buffer, PBS Based (Gibco, #13151-014) was added and the whole was incubated at 37°C for 30min. The fragments were mixed up and down every 10min. A second step of enzymatic dissociation followed, in which the remaining fragments were incubated in CO_2-Independent Medium (Gibco, #18045-088) with Collagenase I (2mg/ml, Sigma, #C0130), Hyaluronidase (2mg/ml, Sigma, #H3506) and DNase (25 µg/ml, Sigma), at 37°C for 30min. The fragments were again mixed up and down every 10min. After each 30min incubation period, the tumor pieces were filtered through a 40μm cell strainer (BD Biosciences, #352340). The released cells were centrifuged at 1200 r.p.m. for 3 min and stored in cold CO_2-Independent Medium with 30% FCS (fetal calf serum) at 4 °C. Dissociated cells were layered on a double ficoll gradient, 5ml of ficoll at 1.077g/ml density (Sigma-Aldrich, #Histopaque-1077) and 5ml of ficoll at 1.119g/ml density (Sigma-Aldrich, #Histopaque-1119) then centrifuged at 700g for 30min at room temperature. Cells from both interfaces were pooled and washed twice in CO_2-independent medium, finally cells were diluted in CO_2-independent medium with 30% FCS. Cell count and viability were assessed immediately after dissociation by Trypan blue (Gibco, #15250-061) exclusion on a hemocytometer. Flow cytometry was performed with directly conjugated antibodies according to standard techniques with analysis on LSRII flow cytometer (Becton Dickinson). The following antibodies were

used: anti-Human EpCAM (Biolegend, #9C4), anti-mouse Pan H-2 (Biolegend, #M1/42), anti-mouse CD45 (Invitrogen, #30-F11), anti-mouse Ly-6G (BD Biosciences, #1A8), anti-mouse F4/80 (Ebioscience, # BM8), and anti-mouse CD19 (BD Biosciences, #1D3). Data analyses were performed using FlowJo software. Granulocytes (Ly-6G high), B-lymphocytes (CD19 high), macrophages (F4/80 high) and monocytes (F4/80 med) were evaluated among the hematopoietic cell population (CD45 positive), which were negative for human EPCAM (tumor cells) and positive for Pan H-2 (HLA-I).

Complete blood count

The complete blood count was done on total blood, collected by cardiac puncture just after animal death. Complete blood count was evaluated using an automated hematology analyzer (MS9-3) from Melet Schloesing laboratoires.

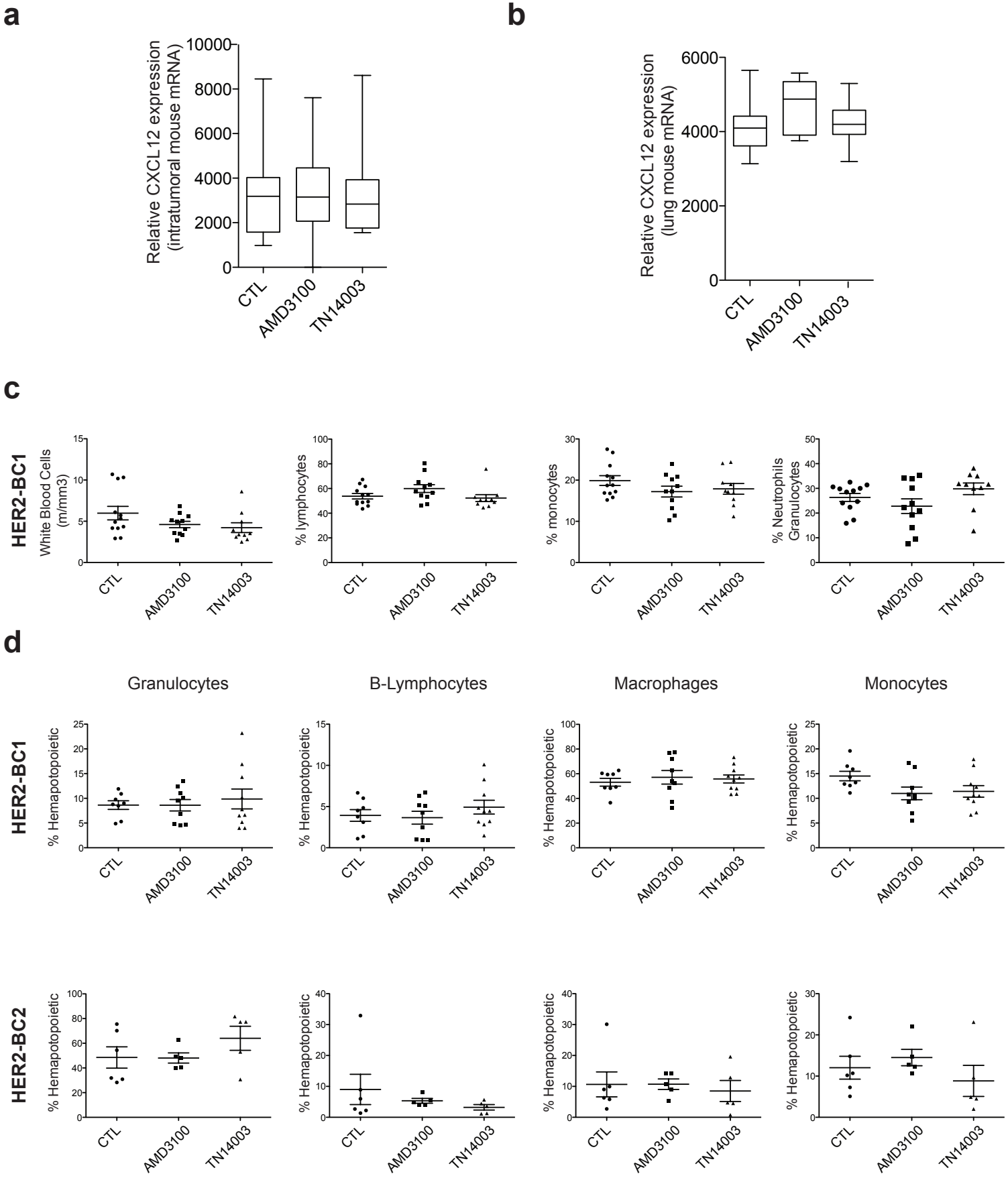
SUPPLEMENTARY FIGURES LEGENDS

Supplemental Figure 1. AMD3100 and TN14003 do not modulate CXCL12 expression rate or hematopoietic contents in HER2 PDX models (**a,b**) Box-and-whiskers plots showing CXCL12 mRNA levels in HER2 PDX tumors (**a**) and in lungs (**b**) of mice (N = 10 per group) treated with PBS (CTL), AMD3100 or TN14003. No significant differences (tested by Student t-test) were detected. (**c**) Complete blood count of HER2-BC1 mice treated with PBS, AMD3100 or with TN14003, as indicated. Data are means ± s.e.m (N = 10 per group) of total white blood cells (ml/mm^3) or expressed as percentage of lymphocytes, monocytes or neutrophils/granulocytes to total white blood cells. (**d**) Percentage of granulocytes, lymphocytes, macrophages, monocytes to hematopoietic (CD45+, Pan H2+, EpCAM-) cells in tumors from HER2-BC1 (Up) or HER2-BC2 (Down) mice treated with PBS CTL), AMD3100 orTN14003. Data are means ± s.e.m (N = 10 per group). No significant differences (tested by Student t-test) were detected.

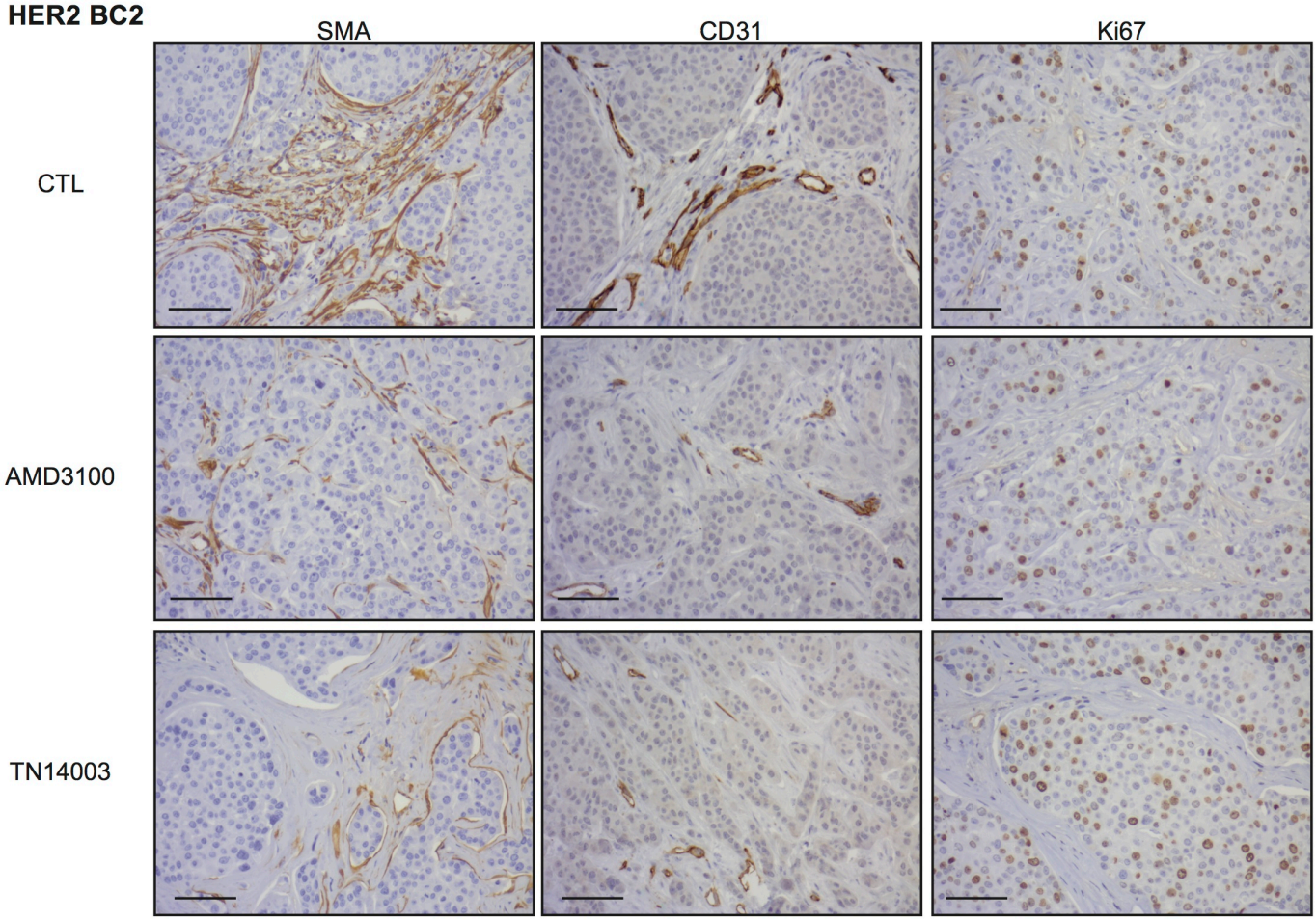
Supplemental Figure 2. CXCR4 inhibitors down-regulate angiogenesis and reactive stroma in HER2-BC2 PDX models (**a**) Representative views of SMA (Left), CD31 (Middle) and Ki67 (Right) IHC from tumors of HER2-BC1 PDX treated either with PBS (CTL), AMD3100 or TN14003, as indicated. Scale bar: 125 µm. (**b**) Scatter plots showing histological scores (HScores, see Methods) of stromal SMA (Left), CD31 (Middle) and Ki67 staining (Right) in tumors from HER2-BC2 PDX treated either with PBS (CTL), AMD3100 or TN14003, as indicated. Data are shown as means ± s.e.m (N = 5 per group). P-values are based on Student t-test.

Supplemental Figure 3. Inhibition of CXCR4 do not impede angiogenesis in TN-BC

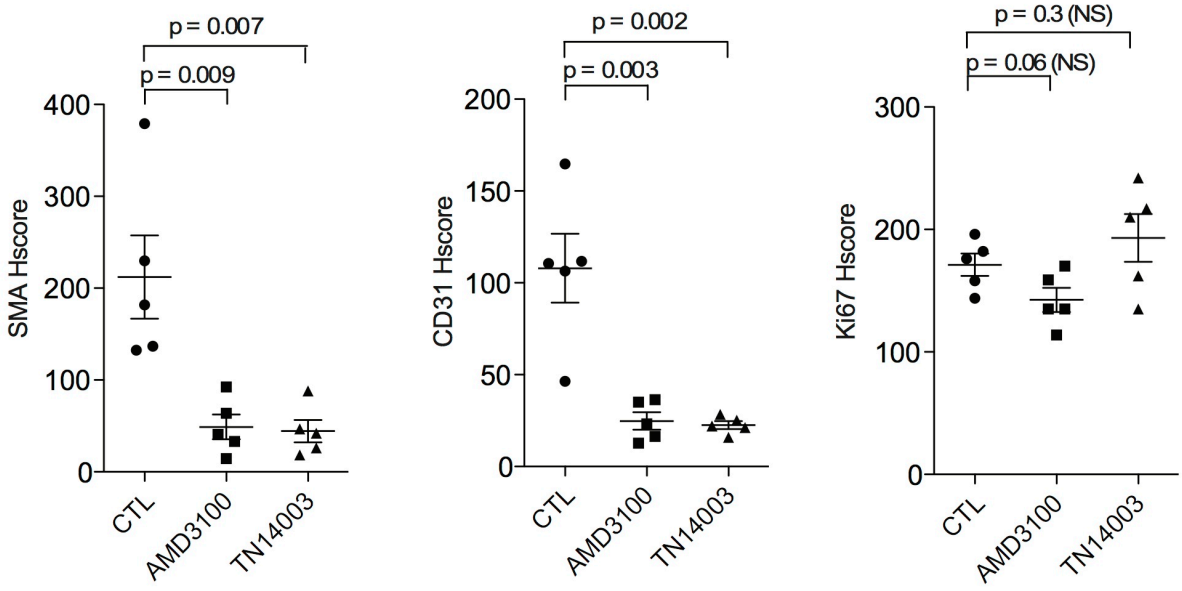
PDX models (**a**) Histological scoring of pulmonary metastases by a Pathologist from lungs of TN-BC1 PDX mice treated with PBS, AMD3100 or TN14003, as indicated. Data are means ± s.e.m (N ≥ 10 per group). P-values are from Student t-test. (**b-d**) Scatter plots showing histological scores (HScores, see Methods) of stromal SMA (**b**), Ki67 (**c**) and CD31 staining (**d**) in tumors from TN-BC1, TN-BC2 or TN-BC3 PDX models, treated either with PBS (CTL), AMD3100 or TN14003, as indicated. Data are shown as means ± s.e.m (N = 5 per group). P-values are based on Student t-test.

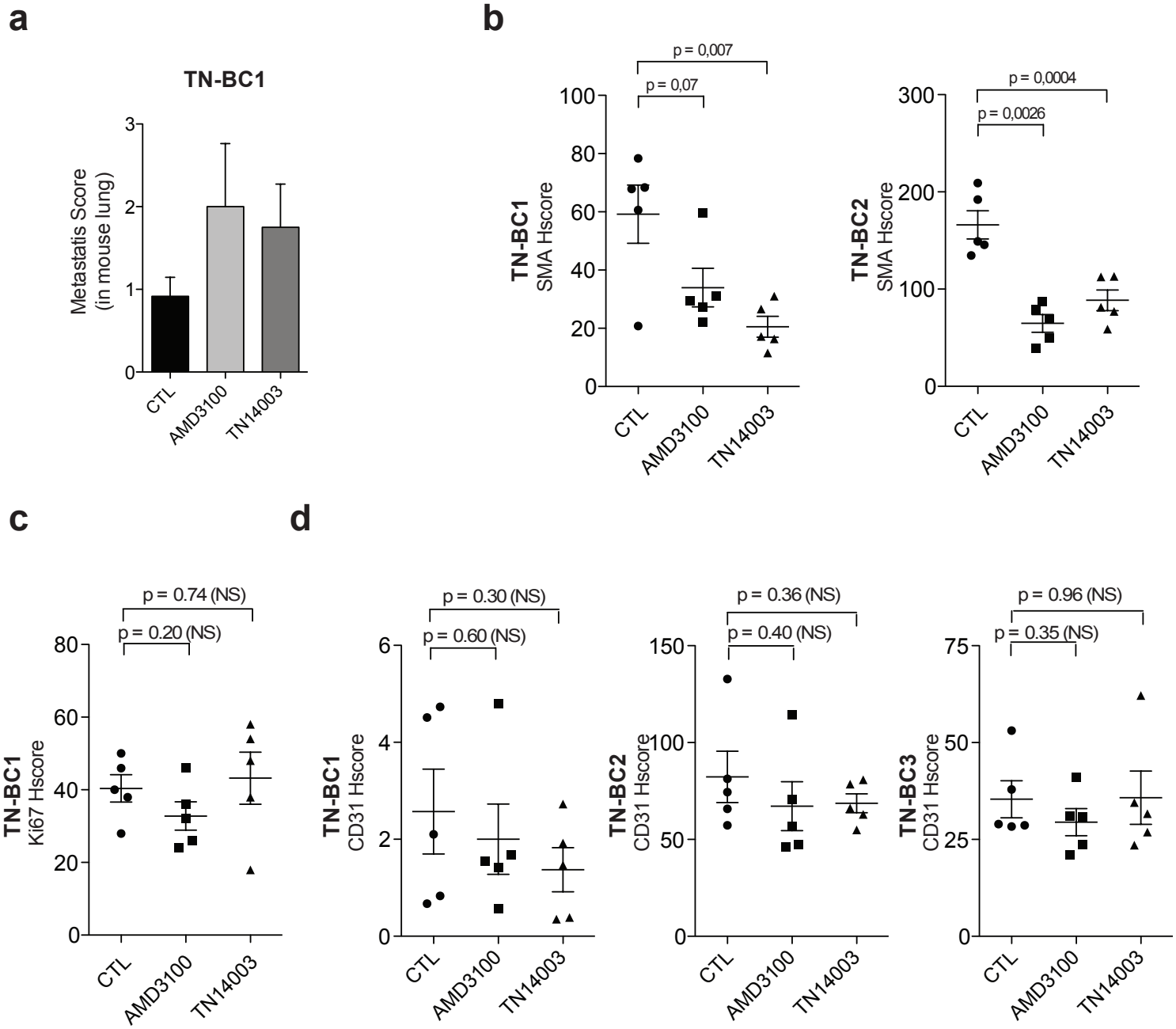

a
Relative CXCL12 expression (intratumoral mouse mRNA)
10000
8000
6000
4000
2000
0
CTL
AMD3100
TN14003
b
Relative CXCL12 expression (lung mouse mRNA)
6000
4000
2000
0
CTL
AMD3100
TN14003
c
HER2-BC1
White Blood Cells (m/mm3)
% lymphocytes
% monocytes
% Neutrophils Granulocytes
d
Granulocytes
B-Lymphocytes
Macrophages
Monocytes
HER2-BC1
HER2-BC2
% Hemapotopoietic
CTL
AMD3100
TN14003

a


HER2 BC2
SMA
CD31
Ki67
CTL
AMD3100
TN14003

b


p = 0.007
p = 0.009
SMA Hscore
400
300
200
100
0
CTL
AMD3100
TN14003
p = 0.002
p = 0.003
CD31 Hscore
200
150
100
50
0
CTL
AMD3100
TN14003
p = 0.3 (NS)
p = 0.06 (NS)
Ki67 Hscore
300
200
100
0
CTL
AMD3100
TN14003


a
TN-BC1
Metastatis Score (in mouse lung)
CTL
AMD3100
TN14003
b
p = 0,007
p = 0,07
TN-BC1 SMA Hscore
p = 0,0004
p = 0,0026
TN-BC2 SMA Hscore
c
p = 0.74 (NS)
p = 0.20 (NS)
TN-BC1 Ki67 Hscore
d
p = 0.30 (NS)
p = 0.60 (NS)
TN-BC1 CD31 Hscore
p = 0.36 (NS)
p = 0.40 (NS)
TN-BC2 CD31 Hscore
p = 0.96 (NS)
p = 0.35 (NS)
TN-BC3 CD31 Hscore