

# Humanized tumor mice—A new model to study and manipulate the immune response in advanced cancer therapy

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The immunological impact on antibody-based anticancer therapies remains incompletely understood due to the lack of appropriate animal models for *in vivo* analysis. Here, we present a novel humanized tumor mouse (HTM) model, generated by concurrent transplantation of human hematopoietic stem cells (HSCs) and human breast cancer cells in neonatal NOD-*scid* IL2R $\gamma^{\text{null}}$  mice. Five weeks after intrahepatic transplantation, a functional human immune system was developed in all organs, and, in addition, tumor cells were detectable in lung and bone marrow (early dissemination). After 3 months posttransplant, tumor-cell effusions and macroscopic tumors associated with liver or spleen were found. Furthermore, disseminated cells in different lymphoid and nonlymphoid organs were measurable. Tumor growth was accompanied by specific T-cell maturation and tumor cell-specific T-cell activation. In addition, Natural-Killer cell accumulation and activation were observed in HTM, which was further enhanced upon IL-15 treatment facilitating the possibility of immune cell modulation in, *e.g.*, antibody-dependent cellular cytotoxicity-based immunotherapeutic approaches. This novel mouse model makes it possible to combine transfer of MHC mismatched tumor cells together with human HSCs resulting in a solid coexistence and interaction without evidence for rejection. Overall, humanized tumor mice represent a powerful *in vivo* model that for the first time permits the investigation of human immune system-related target cancer therapy and resistance.

A significant body of evidence exists indicating that the immune system has the potential to contribute to the eradication of cancer cells.<sup>1</sup> Numerous attempts have been made for many years to specifically stimulate immune cells prompting a direct attack on cancer cells. However, the complexity of the immune system comprising a variety of cell types with differing functions as well as a vast number of regulatory mechanisms represents an obstacle to straightforward solutions.

Commonly used experimental models are based on co-incubation of immune effector cells<sup>2,3</sup> and target cells (*i.e.*, tumor cells). Those approaches suffer to some extent from artificial *in vitro* conditions and from an indirect read-out of effector cell activation by analyzing surrogate markers, *e.g.*, the cellular release of chromium or lactate dehydrogenase.

As for the utilization of *in vivo* models for the analysis of cancer biology, the fully murine transgenic MMTV-HER2 Balb/c mouse, engineered for spontaneous development of breast cancer,<sup>4</sup> has been proven to be a helpful tool in many studies. However, it is rather inadequate in answering questions specifically addressing the role of the human immune system in cancer defense, because the human and murine immune systems differ in both phenotype and function.<sup>5</sup>

Tumor-cell targeting, for instance, by humanized antibodies is widely used for the treatment of a variety of cancers.<sup>6,7</sup> Therapeutic success might be largely attributed to antibody-triggered immune defense, which, in turn, might be predominantly mediated by antibody-dependent cellular cytotoxicity (ADCC). However, resistance to antibody therapy is frequently observed in the clinic and insufficient immunological antitumor cell defense might play a key role in Trastuzumab therapy failure. Present mouse models are not adequate to evaluate the impact of the immune system, because ADCC differs significantly between men and mice.<sup>5,8,9</sup>

**Key words:** humanized tumor mice, immune-based therapies, breast cancer, NK cells, ADCC

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Typical strategies involving mouse models for the *in vivo* analysis of human tumor growth and tumor therapy are based on the use of athymic nude mice<sup>10</sup> or the use of completely immunodeficient animals, *e.g.*, NOD-/Shi-*scid* IL-2R $\gamma^{\text{null}}$  mice which are transplanted either without human immune cells or with human PBMCs<sup>11,12</sup>/MNCs<sup>13</sup> isolated from MHC mismatched donors. Such procedures are expected to cause not only an antitumor effect but even more likely xenograft-allograft rejection. Other strategies implanting, *e.g.*, human bone marrow together with tumor cells into immunodeficient mice were used as bone marrow metastases model showing homing of human cancer cells into the implanted human tissue but without full reconstitution of all human hematopoietic cell subsets.<sup>14</sup> The co-transplantation of human cancer cells with human complement enabled the study of complement-dependent cytotoxicity induction by therapeutic antibody treatment in NOG mice but again in the absence of the human immune system and its effector cells.<sup>15</sup>

Here, we present for the first time a humanized tumor mouse model (HTM) characterized by the development of a completely matured human immune system and the growth of cotransplanted human breast cancer cells forming solid tumors or tumor-cell effusions. This co-transplantation is an extension of the already existing humanized mouse model in which a fully functional human immune system develops upon CD34<sup>+</sup> cell transplantation and, which, in turn, served as a powerful *in vivo* tool for a variety of studies.<sup>16–20</sup> Nearly, all tested HTM (19/20), which were observed for more than 12 weeks, developed macroscopic tumors (associated to liver or spleen) or tumor-cell effusions. 12/16 HTM had also detectable tumor-cell dissemination into the lung, liver and spleen. Furthermore, immune modulating treatment with IL-15/IL-15Ra revealed in both humanized mice and in HTM increased NK cell numbers and activation. HTM enable more appropriate preclinical assessment of immune-based therapeutic antitumor strategies, *e.g.*, treatments with different cytokines (*e.g.*, IL-2, TNF, and GM-CSF), specific antibodies (inducing ADCC), new vaccination strategies or allogeneic or even autologous T-cell infusions.

## Material and Methods

### Breast cancer cell lines

BT474 or SK-BR-3 breast cancer cells were used for co-transplantation. The BT474 line was isolated by Lasfargues and Coutinho from a solid, invasive ductal carcinoma of the breast (ATCC number HTB-20). In contrast, the SK-BR-3 cell line, first described by Trempe and Old, was derived from a pleural effusion (ATCC number HTB-30). According to the editor's suggestion, authenticity of both cell lines was approved by the German Collection of Microorganisms and Cell Cultures (Göttingen, Germany).

### Mice

Mice [NOD-*scid* IL2R $\gamma^{\text{null}}$ ; (NSG)] used for the experiments were obtained from Jackson Laboratories and bred and kept

in a specialized pathogen-free facility at the University of Regensburg. For generation of humanized tumor mice, the animals were irradiated (1 Gy) and, 3 hr later, transplanted with  $3 \times 10^5$  human CD34<sup>+</sup> cells isolated from umbilical cord blood<sup>1</sup> using immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol together with  $3 \times 10^6$  BT474 or SK-BR-3 tumor cells. In all experiments, cells were cotransplanted into the liver of newborn mice. Animals were sacrificed and analyzed either at an early time point, *i.e.*, 5 weeks posttransplant, or at the age of 3–5 months.

### Mononuclear cell isolation from different tissues

Mononuclear cells were isolated from peripheral blood and different mouse tissues as previously described.<sup>17</sup> Liver and lung were cut into small pieces with scissors and were incubated in 5 ml RPMI medium-containing collagenase type 1 (2 mg/ml) and DNase (20  $\mu$ g/ml) for 30 min at 37°C before passing the suspension through 40- $\mu$ m nylon filters and centrifuged at 300g at 4°C for 10 min. The cell pellets were then resuspended in 5 ml of 40% Percoll/RPMI, underlayered with 5 ml 70% Percoll/RPMI, and centrifuged for 20 min at 800g. Cells were collected from the interface, washed twice with phosphate-buffered saline (PBS), supplemented with 5% FCS and centrifuged as described earlier.

### Flow cytometry analysis

Reconstitution with human immune cells and tumor cells was determined by flow cytometry using a LSR-II flow cytometer, which was run by the Diva software package (BD Biosciences, San Jose, USA). To reduce nonspecific binding, cells were incubated with mouse IgGs (10  $\mu$ g/ml, Sigma) on ice for 20 min before staining. Samples were stained using the following mAb: anti-CD3-FITC (clone HIT3a), anti-CD19-PE (clone HIB19), anti-CD25-FITC (clone M-A251), anti-CD33-PerCP-Cy5.5 (clone P67.6), anti-CD45-APC (clone HI30), anti-Her2-PE (clone Neu 24.7), anti-CD4-FITC (clone SK3) and anti-CD8-PE (clone HIT8a) from BD Biosciences. For further characterization, we used anti-CD3-PerCP (clone OKT3), anti-Foxp3-APC (clone 236A/E7), anti-CD45RA-PE-Cy7 (clone HI100) and anti-CD27-Biotin (clone O323) from eBioscience (San Diego, USA). For NK-cell staining, we used anti-CD56-FITC (clone MY3.1), anti-NKp46-PE (clone 9E2/NKp46), anti-CD16-PE-Cy7 (clone 3G8), and anti-CD3-APC (clone HIT3a) from BD Biosciences. Ki67 staining in tumor and ascites sections was done using the MIB-1 clone and bcl-2 staining using clone 124 (both Dako; Hamburg, Germany). Appropriate mouse immunoglobulin antibodies were used as isotype controls for all stainings.

### Immunohistochemistry and in situ hybridization analysis

Tissue specimens (spleen, liver, brain, thymus, lymph nodes and lung) were fixed with 4% formalin from which 4- $\mu$ m

<sup>1</sup>All pregnant women granted informed consent.

paraffin sections were prepared. Specimens were deparaffinized and pretreated by microwave heating for 30 min at 320 W in 0.1 M citrate buffer adjusted to pH 7.3. The immunostaining was automatically performed on a Ventana Nexes autostainer (Ventana, Tucson, USA) by using the streptavidin–biotin–peroxidase complex method and 3,3'-diaminobenzidine as chromogen. The autostainer was programmed based on the instructions given by the iView DAB detection kit (Ventana). The following antibodies were used: anti-CD3 (Thermo Fisher Scientific, Fremont, USA), clone SP; anti-CD56 (Leica Microsystems, Wetzlar, Germany) clone 1B6; Dako Glstrup, Denmark: anti-CD79a clone JCB117; anti-CK7 clone OV-TL-12/30; anti-CD45/LCA clone 2B11-PD7/26; anti-CD68, clone P6M1; anti-Her2 rabbit polyclonal A0485. All antibodies were tested for specificity using tumor mice without human immune cells and humanized mice without cotransplanted tumor cells. In general, the same positive controls as used in routine diagnostic were used in this study.

#### Soluble IL-15/IL-15R-alpha complex preparation and NK-cell activation

Human recombinant IL-15 and a recombinant fusion protein consisting of the ectodomain of the human IL-15 receptor-alpha-chain and the human IgG1 Fc (IL-15Ra/Fc) were purchased from R&D Systems (Minneapolis, USA). The IL-15/IL-15Ra/Fc complex was prepared as previously reported.<sup>21</sup> In brief, both IL-15 and IL-15Ra/Fc were resuspended in PBS solution and mixed together at a ratio of 1:6 (IL-15 to IL-15Ra/Fc). The mixture was incubated at 37°C for 30 min and then used for *in vivo* injection. For *in vivo* NK-cell stimulation, each mouse received 2.5 µg of IL-15/IL-15Ra/Fc complex i.p. in 200 µl of PBS once a week. All analyses of NK expansion were performed after 14 days in the treated mice. To study the upregulation of effector molecules in NK cells, bulk splenocytes were restimulated with K562 target cells at an effector to target ratio of 1:1 for 4 hr in the presence of 1 µg/ml monensin (BD Biosciences) *in vitro*. After surface staining for human CD3, NKp46, CD27 and CD56, the cells were washed, fixed, permeabilized and intracellularly stained for IFN-γ and Perforin followed by flow cytometry.

#### Tumor cell culture

For the enrichment of low number of cancer cells in organs of HTM, single-cell suspensions from liver, bone marrow and spleen were cultured in Dulbecco's minimal essential medium (DMEM) and supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were harvested after 2 or 14 weeks, respectively, by trypsinization and analyzed by flow cytometry or PCR. To evaluate the killing function of effector cells generated in HTM, cells from different organs were separated and co-cultured with tumor cells. Twenty-four hours later, tumor cells were harvested and stained with Annexin-V-APC (ImmunoTools, Friesoythe, Germany) and propidium iodide (PI). For *in vitro* restimula-

tion, SKBR3 cells were suspended at  $2 \times 10^6$ /ml DMEM and 100 µg/ml mitomycin C (Sigma-Aldrich), incubated for 1 hr at 37°C, washed three times and added into 96 well plates in a 1:2 ratio (tumor : spleen cells). Effector splenocytes obtained from untreated or IL-15/IL-15Ra-treated HTM were labeled with CFSE (5 µmol/ml; Invitrogen, Carlsbad, CA) before incubation with tumor cells in the presence of 50 IU/L human IL-2 for 4 days, and proliferation of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and CD56<sup>+</sup> NK cells was analyzed by the CFSE dilution. In addition, effector cells without CFSE labeling were incubated with tumor cells for 4 days, and cells and supernatant were analyzed using flow cytometry and ELISA (BD Bioscience: human IFN-γ BD OptEIA Elisa Set).

#### TCR analysis

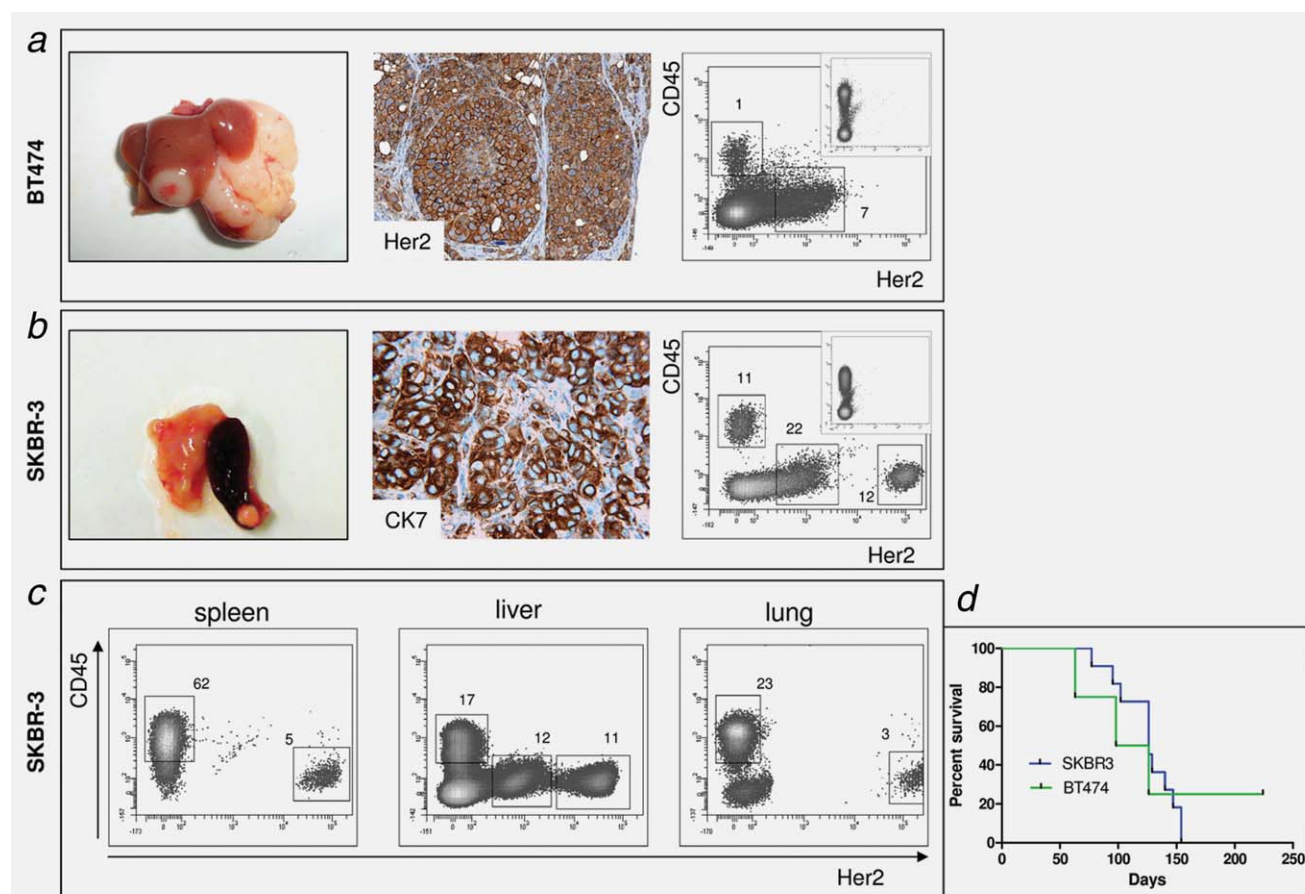
Human TCR alpha-chains and beta-chains containing the CDR3 region were amplified by RT-PCR using a panel of TCR Vα and TCR Vβ primers combined with a respective TCR constant region primer.<sup>22</sup> In brief, cells from spleen, lymph nodes, and liver were isolated as described earlier, washed in PBS and lysed in 200 µl TriReagent (Biozol, Eching, Germany). After isolation of total RNA and RT-PCR, a subfamily-specific TCR-PCR reaction was performed using a panel of 32 Vα and 36 Vβ primers as described followed by gel purification (NucleoSpin, Macherey-Nagel, Düren, Germany) and direct DNA sequencing of dominant TCR Vβ amplicons to assess clonal expansion of T cells.

#### Results

##### Humanized tumor mice develop solid human breast cancer tumors (BT474) and tumor cell effusion (SK-BR-3)

Transplantation of human CD34<sup>+</sup> hematopoietic stem cells (HSC) together with human breast cancer cell lines (BT474 and SK-BR-3) generated macroscopic tumor growth in the liver (BT474; Fig. 1a) or in the spleen (SK-BR-3; Fig. 1b) in addition to tumor-cell effusion into the peritoneal cavity (SK-BR-3). Histological analyses of the tumors revealed breast cancer cell origin proven by positive staining for Her2 (Fig. 1a), Cytokeratin-7 (CK7) (Fig. 1b) and also for Cytokeratin-18 (CK18; data not shown). Flow cytometric cell analysis upon tumor tissue disaggregation confirmed the histological results showing Her2<sup>+</sup> tumor cells and CD45<sup>+</sup> human immune cells in the tumors of the liver (Fig. 1a) and the spleen (Fig. 1b). Interestingly, the separation of tumor cells from the "spleen tumor" revealed two different subpopulations showing low- and high-Her2 receptor expression. In addition, tumor dissemination into other organs was verified by flow cytometry (Fig. 1c) and histological tissue stainings (data not shown), respectively (Table 1).

Using flow cytometry, a change in Her2 expression levels was detectable in different organs of HTM transplanted with SKBR-3 (Fig. 1b and 1c): 5/5 HTM showed two different populations in the liver (MFI high  $43245 \pm 6600$  SEM; MFI low  $1550 \pm 204$ ), 5/5 in the lung (MFI high  $40878 \pm 6375$ ; MFI low  $1684 \pm 174$ ) and 3/5 in the spleen (MFI high



**Figure 1.** Macroscopic, histological and flow cytometric analyses of tumors in HTM. Illustration of tumor growth *ex vivo* in liver (BT474; a) or spleen (SK-BR-3; b) 3 months after transplantation. Immunohistochemical staining for Her2 and Cytokeratin-7 in tumors of HTM (a + b; second column) in addition to flow cytometric staining of human hematopoietic cells (CD45<sup>+</sup>) and Her2<sup>+</sup> tumor cells isolated from single-cell suspension of tumor tissue in liver-associated tumor (a; BT474) and spleen-associated tumor (b; SK-BR-3). Her2<sup>+</sup> tumor cells are indicated by regions. Control liver and spleen staining of HTM without tumor are shown as inserts, respectively. Tumor-cell migration into other organs (spleen, liver and lung) of SK-BR-3 transplanted HTM is displayed in panel C, staining single-cell suspension of the organs for tumor cells (Her2<sup>+</sup>) in comparison with human hematopoietic cells (CD45<sup>+</sup>). Numbers indicate the percentages of corresponding populations. (d) Survival analysis was performed on day 226. HTM transplanted with SK-BR-3 had a median survival of 126 days (range, 77–154 days) and BT474-transplanted HTM 112 days (range, 63–224 days), respectively.

50950 ± 2902, MFI low 1273 ± 167). The phenomenon of different Her2 expression profile never appeared in BT474-transplanted mice even though both cell lines show similar homogeneous expression profiles before transplantation into the animals (MFI: 30,000).

Histological organ analysis revealed tumor-cell dissemination into the lung in BT474-transplanted HTM (3/4), into the brain (1/3) and into the kidney (1/4), whereas SK-BR-3 transplanted HTM did not show dissemination into the kidney (0/8) but into the spleen (7/11) and into the lymph node (2/2). Particularly, the lung seems to be a predominant dissemination organ (13/18) independent of the transplanted tumor cell line. Survival analyses were performed on day 226 revealing no significant differences between SK-BR-3 and BT474 transplanted HTM. HTM transplanted with SK-BR-3 had a median survival of 126 days (range, 77–154 days) and

BT474 transplanted HTM 112 days (range, 63–224 days), respectively (Fig. 1d).

HTM sacrificed 5 weeks *posttransplant* did not show macroscopic tumor growth (0/2). To expand cancer cells, which potentially disseminated into the bone marrow at this early stage of HTM development, we incubated cells isolated from the bone marrow and spleen for several weeks under conventional culture conditions. Afterward, we analyzed the cells by morphology using light microscopy, flow cytometry and PCR (Supporting Information Fig. 1). During the first 3 weeks of culture, hematopoietic cells died off and only tumor cells survived and started to proliferate. Only in the bone marrow (2/2) but not in the spleen (0/2), tumor cells were detectable by PCR analysis of human beta-actin, CK18 and specific MAGE-4 (but not MAGE 2) gene expression.<sup>23</sup> Thereby, the presence of BT474 cells in the bone marrow was confirmed,



Table 1. Tumor development in BT474 and SK-BR-3 transplanted humanized tumor mice (HTM) detected in histology and flow cytometry

Cell line	Tumor (T) Effusion (E)	Histology					Flow cytometry							
		Liver	Lung	Spleen	Tumor	Brain	Kidney	Effusion	Liver	Lung	Spleen	Tumor	bm	LN
BT 474 5 weeks	0/2 (T)	1/2	1/2	0/2	–	0/2	0/2	0/2	0/2	0/2	0/2	–	0/2	0/2
BT 474 > 3 months	4/5 (T)	2/4	3/4	0/4	3/3	1/3	1/4	0/5	0/2	0/2	0/2	1/2 (36%) <sup>1</sup>	0/2	0/2
SK-BR-3 > 3 months	15/15 (E)	3/11	9/12	7/11	11/11	1/4	0/8	5/5 (78.4% ± 11.5%) <sup>1</sup>	5/5 (11.5% ± 9.4%) <sup>1</sup>	5/5 (1.5% ± 0.4%) <sup>1</sup>	4/5 (1.9 % ± 1.3) <sup>1</sup>	7/7 (16.9% ± 9.6) <sup>1</sup>	2/5 (0.3% ± 0.05) <sup>1</sup>	2/2 (1.7% ± 0.6) <sup>1</sup>

<sup>1</sup>% of Her2+ tumor cells/live cells ± standard error of the mean.

which show the expression profile of the originally transplanted cell line (Supporting Fig. 1a–1c). Even those HTM that did not exhibit macroscopic tumors at the age of 5 weeks showed tumor-cell dissemination into the bone marrow. These cells seemed to be at a dormant state, because even in animals with high numbers of tumor cells in lung, liver, and spleen, 3 months posttransplant (Fig. 1c) did not show detectable levels of Her2<sup>+</sup> tumor cells in the bone marrow (Table 1 + Supporting Fig. 1d). Furthermore, immunochemical characterization elucidated proliferating (MIB-1) and nonapoptotic (bcl-2) tumor cells in different body regions as spleen, brain and effusion tumor cells isolated from peritoneal ascitis (Supporting Fig. 2). During therapy or immunomodulatory treatments, this specification can be used as additional tool for tumor-cell responsiveness.

### Hematopoietic reconstitution in all organs of humanized tumor mice

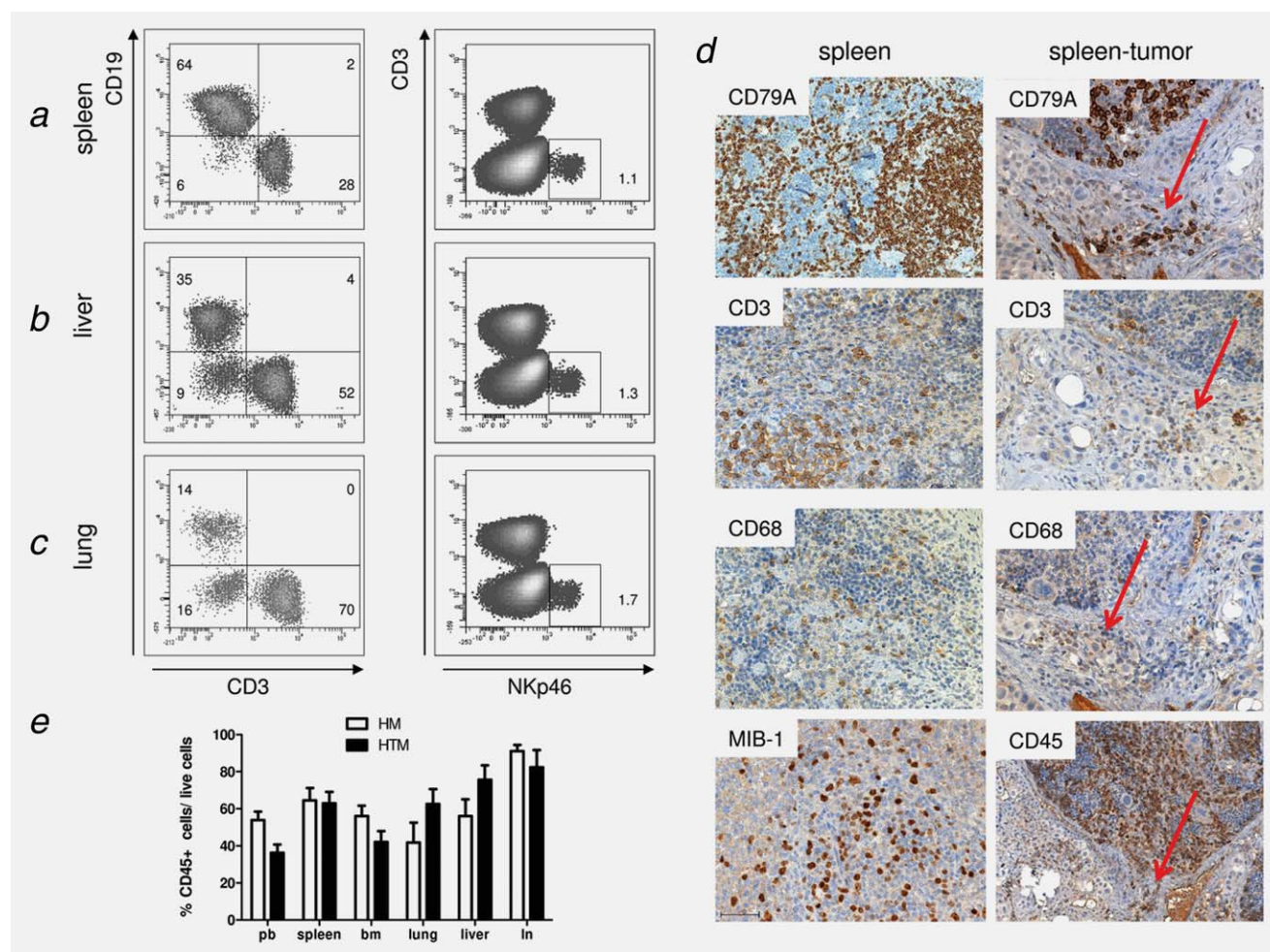
Human hematopoietic reconstitution levels and characterization of immune cell subsets were determined by flow cytometry (Figs. 2a–2c and 2e) and histology (Fig. 2d). Thereby, we characterized individual human immune cell subsets, *i.e.*, CD3<sup>+</sup> T, CD19<sup>+</sup> B (or CD79a<sup>+</sup>) cells, NKp46<sup>+</sup> NK cells, and CD68<sup>+</sup> macrophages in different organs. These immune cells also migrated into the peripheral area of the tumor (Fig. 2d, right column). By additional MIB-1 (Ki-67) staining proliferating cells in the center of B cell area in the spleen (Fig. 2d) were identified.

Looking for human engraftment in all organs of the HTM animals, we found equal levels of CD45<sup>+</sup> human immune cells in HTM compared to humanized mice (Fig. 2e) showing no negative impact of co-transplantation upon engraftment.

All flow cytometric data in Figures 2–5 represent HTM transplanted with SKBR-3, but similar results were obtained from BT474-transplanted HTM (data not shown).

### Human immune cell and tumor cell interaction in HTM without evidence for rejection

T cells developed in the thymus equally in HTM as in humanized mice showing a high concentration of premature double positive (CD4<sup>+</sup>/CD8<sup>+</sup>) T cells after 5 weeks and more differentiated single-positive phenotype (CD4<sup>+</sup> or CD8<sup>+</sup>) after 3 months (Fig. 3a, top panel). Over this period, T cells appeared premature in the thymus showing mainly no CD27 and CD45RA expression (Fig. 3a, right plot in upper panel). In HTM, CD3<sup>+</sup> T cells were found at higher levels (in terms of percentage) in the main tumor harboring organs, *i.e.*, lung and liver, and in addition in the lymphoid organs spleen and lymph node (Fig. 3b) when compared with humanized mice (Fig. 3c). In all organs, the CD4<sup>+</sup>/CD8<sup>+</sup> ratio significantly changed showing an increase of CD4<sup>+</sup> and a decrease of CD8<sup>+</sup> cells (Fig. 3c). All organs not only showed higher rates of CD4<sup>+</sup> T cells, but these cells were also characterized by a memory phenotype (CD45RA<sup>–</sup> CD27<sup>+</sup>). In contrast to naive humanized mice, significantly increased activation and



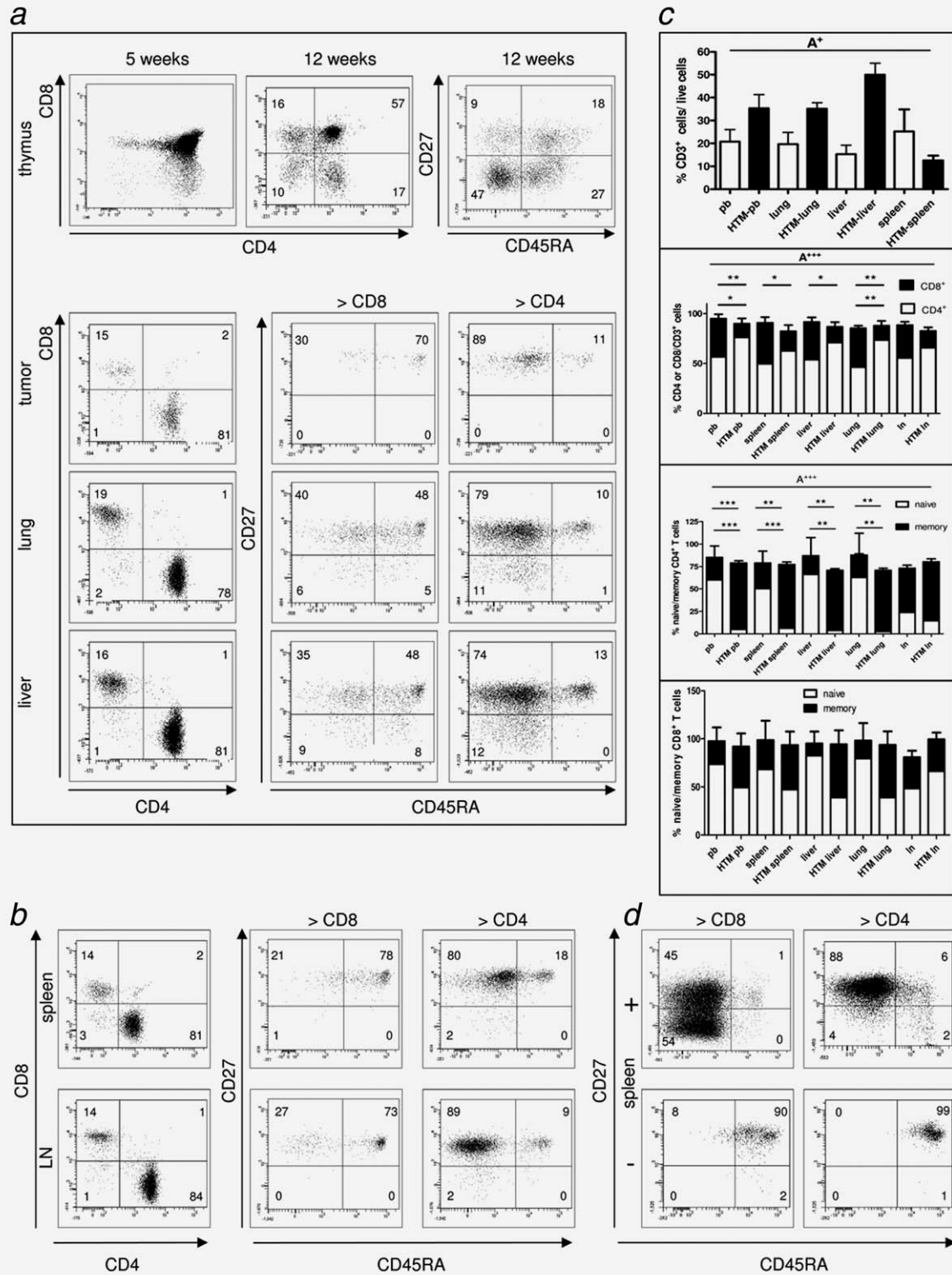
**Figure 2.** Distribution of hematopoietic cells in lymphoid and nonlymphoid organs. Flow cytometric analyses of human CD45<sup>+</sup> cells are stained for B cells (CD19), T cells (CD3) and NK cells (NKp46) in spleen (a), liver (b), and lung (c). (d) Immunohistological staining of spleen (left column) and spleen with associated tumor (right column) of human B cells (CD79A), human T cells (CD3), human macrophages (CD68), the leukocyte common antigen (CD45), and MIB-1 (Ki-67). (e) Comparison of engraftment levels (CD45<sup>+</sup> cells; no significances) in humanized mice and humanized tumor mice [peripheral blood (pb), bone marrow (bm), lung, liver, spleen and lymph nodes (ln)]. All data are collected from SK-BR-3 transplanted HTM in the age of 3–5 months posttransplant.

maturation of CD4<sup>+</sup> T cells were found in all organs of HTM except from lymph nodes (Fig. 3c). In the tumor, CD4<sup>+</sup> memory cells represented the main population, whereas only few CD8<sup>+</sup> T cells migrated into the tumor area (Fig. 3a). CD8<sup>+</sup> T cells showed no significant change of activation in HTM compared to humanized mice, but again, in lung and liver, the increase of memory CD8<sup>+</sup> T cells was most prominent (Fig. 3c). In comparison, humanized mice infected with *Leishmania major* showed high activation and maturation levels not only in CD4<sup>+</sup> but also in CD8<sup>+</sup> T cell populations, whereas naive humanized mice without infection maintained primarily naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 3d).

Further evaluation revealed the presence of regulatory T cells (Tregs) in all organs of humanized mice as well as of HTM (data not shown). These cells present a phenotype indicative for memory cells (CD45RA<sup>-</sup> CD27<sup>+</sup>).

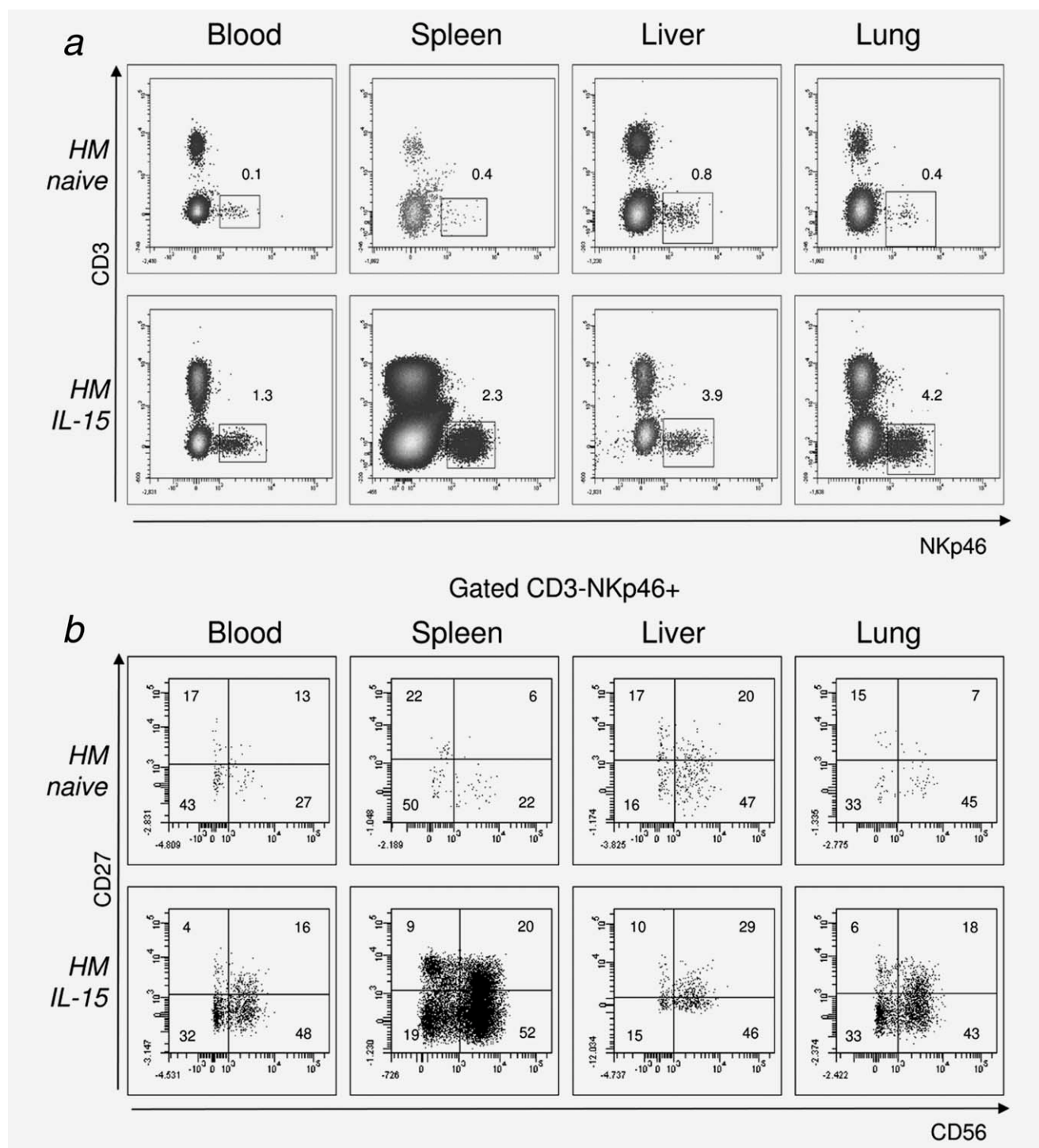
#### Increase of NK cell population in HTM which can be further enhanced with IL-15 treatment

Human NK cell numbers were higher in HTM than in control humanized mice as indicated by multiparametric flow cytometry (Figs. 4a, 4c and 4e). Importantly, further subset analysis of NKp46<sup>+</sup> NK cells revealed that CD56<sup>+</sup> as well as the recently described CD56<sup>-</sup> NK cells are present in both HTM as well as humanized control animals (Figs. 4b and 4d).<sup>24</sup> As the transpresentation of human IL-15 has recently been shown to be necessary for the development and differentiation of human NK cells *in vivo*,<sup>24,25</sup> we next treated humanized mice with human IL-15 precomplexed to human IL-15 receptor alpha *in vivo*. As expected, we found that IL-15/IL-15 receptor alpha treatment induces a significant expansion of human NK cells in all lymphoid and extra-lymphoid tissues analyzed (Fig. 4a). Further subset analysis revealed that, in comparison with untreated humanized mice,



**Figure 3.** Flow cytometric analyses of T-cell population, activation, and maturation in different organs in SK-BR-3 transplanted HTM. (a) Distribution of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (gating strategy: CD3<sup>+</sup> > CD4<sup>+</sup> or CD8<sup>+</sup>) as well as phenotypic classification of naive (gating strategy: CD3<sup>+</sup> > CD4<sup>+</sup>/CD8<sup>+</sup> > CD27<sup>+</sup> and CD45RA<sup>+</sup>) versus memory (gating strategy: CD3<sup>+</sup> > CD4<sup>+</sup>/CD8<sup>+</sup> > CD27<sup>+</sup> and CD45RA<sup>-</sup>) T cells in (a) thymus, tumor, lung and liver and (b) spleen and lymph nodes. (c) Percentage of human CD3<sup>+</sup> T cells, CD4<sup>+</sup>/CD8<sup>+</sup> of T cells and naive versus memory phenotype of CD4 and CD8 in humanized mice [peripheral blood (pb), bone marrow (bm), lung, liver, spleen and lymph nodes (ln)] compared to humanized tumor mice (HTM-organs). All data collected from SK-BR-3 transplanted HTM in the age of 3–5 months posttransplant. (d) Phenotypic analyses of naive versus memory T cells in a naive humanized mouse [bottom (–)] and *Leishmania major* infected humanized mouse [top (+)]. Significances between HTM and HM in all organs analyzed in two-way-ANOVA (A<sup>+</sup> =  $p < 0.05$ ; A<sup>++</sup> =  $p < 0.01$ ; A<sup>+++</sup> =  $p < 0.001$ ). Additionally, significances between the organs are marked with \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  analyzed with Bonferroni posttest.





**Figure 4.** IL-15 treatment in humanized mice and HTM induces NK cell proliferation and activation. Humanized mice (*a + b*) and SK-BR-3 transplanted HTM (*c + d*) were treated with IL-15/IL-15Ra as described in the material and methods section. Fourteen days later, cells were prepared from peripheral blood, spleen, liver, and lung and examined for the presence of NK cell subsets according to the expression of CD3 and NKp46 (*a + c*) and CD27 and CD56 in the CD3<sup>+</sup> NKp46<sup>+</sup> fraction (*b + d*) by multiparametric flow cytometry. Not treated humanized mice (*a + b*) and HTM (*c + d*) served as controls. Numbers indicate the percentages of corresponding populations.

(*e*) Percentage of CD56<sup>+</sup> (top), CD3<sup>+</sup> cells (second chart) and CD4/CD8 distribution (third chart) in humanized mice [pb, spleen, lung, liver and lymph nodes (ln)] compared to IL-15-treated humanized mice (organs + IL-15). Percentage of NKp46<sup>+</sup>CD3<sup>−</sup> cells in spleen, lung and liver of HTM with or without IL-15 treatment (fourth chart). All data collected from SK-BR-3 transplanted HTM in the age of 3–5 months posttransplant. Significances analyzed with two-way-anova (A\* =  $p < 0.05$ ; A\*\* =  $p < 0.01$ ).



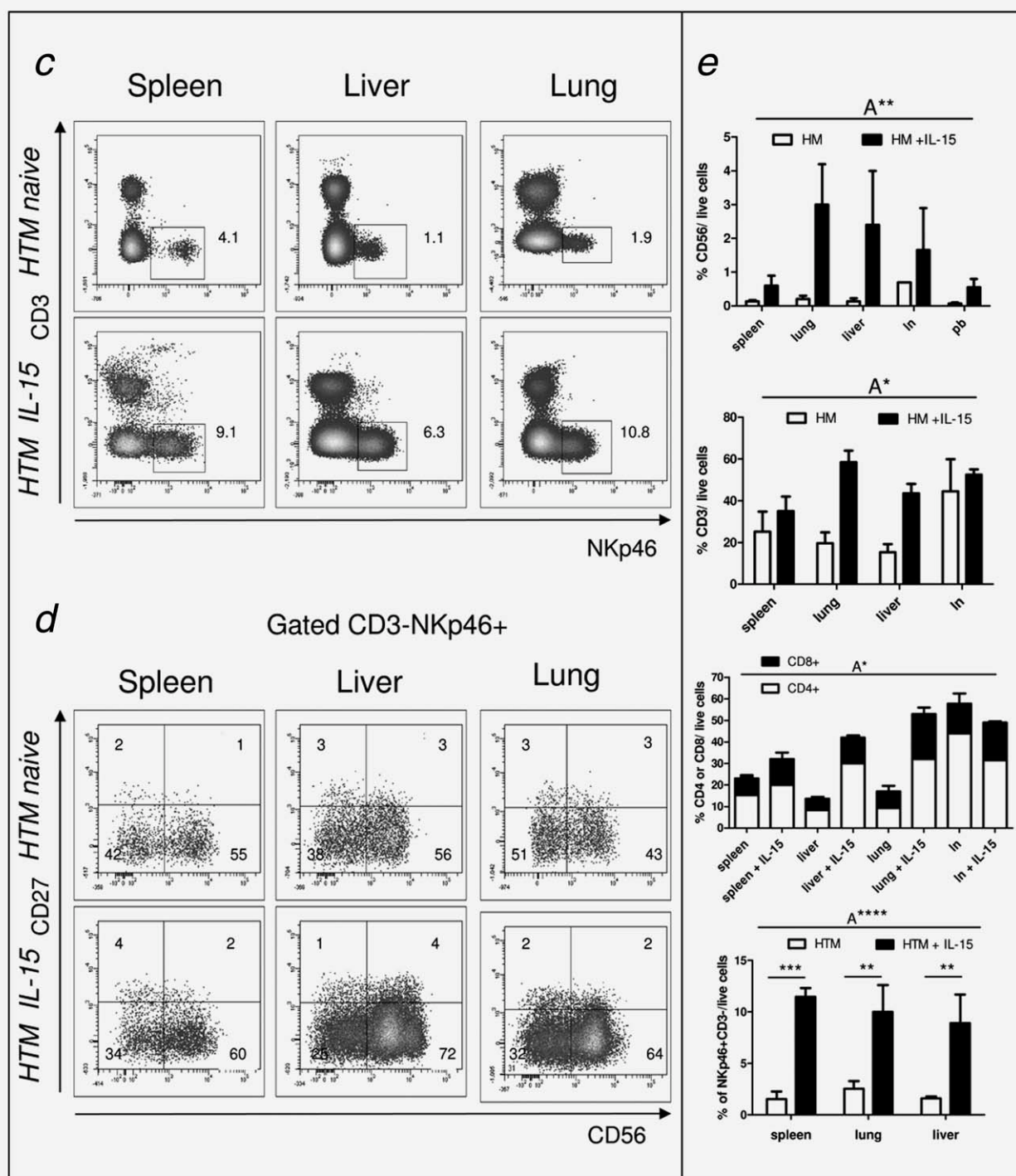
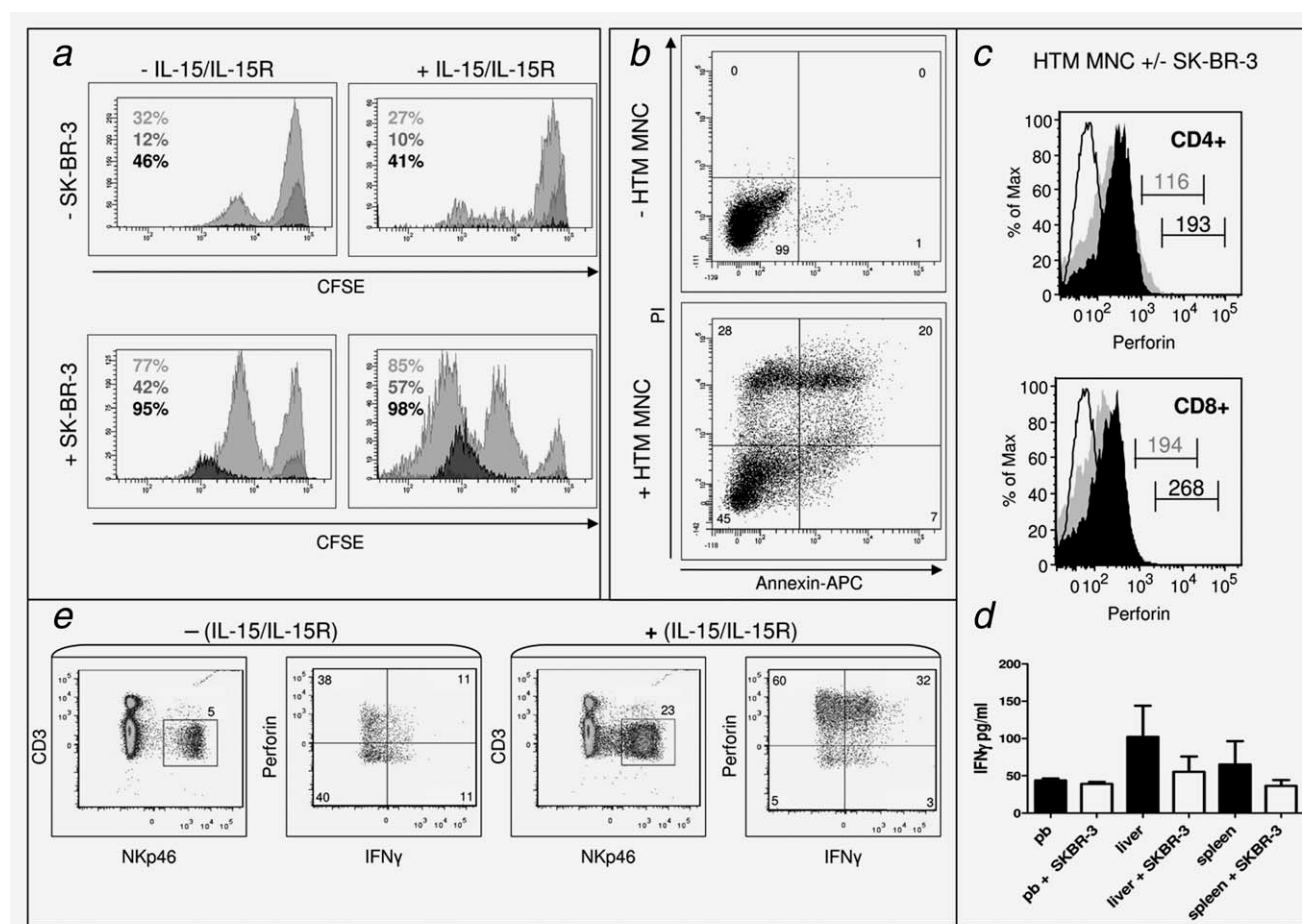


Figure 4. (Continued).

the pool of expanding NK cells consists mainly of mature CD56<sup>+</sup> NK cells, the majority of which were CD27 negative (Fig. 4b). This is in line with previous reports showing that human NK cells upregulate CD56 upon activation.<sup>26</sup> Importantly, we could also detect potentially resting CD56<sup>-</sup> NK cells in the IL-15-treated mice (Fig. 4b), indicating that in humanized mice, all subsets of NK cells properly differentiate

upon reconstitution with IL-15. The effects of the IL-15 reconstitution, however, were not only restricted to the NK-cell compartment as shown by the expansion of CD3<sup>+</sup> T cells (CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells) in extra-lymphoid organs of the IL-15-treated mice (Fig. 4e). Next, we tested our hypothesis that IL-15/IL-15 receptor alpha treatment significantly augments human NK cell numbers in humanized tumor



**Figure 5.** Functional studies of human NK and T cells in HTM. (a) Histogram of CD4<sup>+</sup> (light gray peak), CD8<sup>+</sup> (middle gray peak) and CD56<sup>+</sup> cells (dark gray peak) without (–) or with (+) SK-BR-3 *in vitro* restimulation of spleen cells isolated from HTM with or without IL-15/IL-15R *in vivo* treatment. Percentage of dividing cells is indicated in the respective panels. (b) Tumor cells without (top) and with spleen cells isolated from HTM (bottom) after 24 hr in culture stained for Annexin/PI. (c) After *in vitro* restimulation with SK-BR-3 cells, CD4 and CD8 T cells from the spleen cells (HTM) were stained for intracellular Perforin. Gray histogram in the absence and black histogram in the presence of SK-BR-3, transparent histogram representing isotype control. Bars indicate mean fluorescence intensity (MFI). (d) Supernatants of HTM organ cells restimulated as described in C were analyzed for IFN $\gamma$  production (in absence or presence of tumor cells). (e) Increase of CD3<sup>+</sup>NKp46<sup>+</sup> cells and their Perforin and IFN $\gamma$  production with or without IL-15/IL-15R *in vivo* treatment in HTM. All data are derived from SK-BR-3 transplanted HTM.

mice as well. For this purpose, we treated HTM with two doses of IL-15/IL-15 receptor alpha and analyzed frequencies of peripheral NK cells 2 weeks later. We found that humanized tumor mice treated with IL-15/IL-15 receptor alpha displayed a significant NK cell accumulation in the periphery when compared with untreated animals (Figs. 4c and 4e). Importantly, the majority of NKp46<sup>+</sup> cells featured an activated phenotype as indicated by expression of CD56 and absence of CD27, showing that the treatment with IL-15 can significantly enhance frequency of activated human NK cells in HTM (Fig. 4d).

#### Tumor-specific immune response of NK and T cells generated in HTM

After *in vitro* restimulation of spleen cells with SK-BR-3 cells, CD4<sup>+</sup> as well as CD8<sup>+</sup> T and NK cells increased prolifera-

tion, which was even more pronounced in cells isolated from *in vivo* IL-15-treated HTM (Fig. 5a). Annexin/PI staining of tumor cells after incubation with HTM spleen cells, which allows the direct evaluation of early and late apoptotic cells, revealed the induction of apoptosis in tumor cells if co-cultured with HTM effector cells (Fig. 5b). Further evaluation of the CD4<sup>+</sup> and CD8<sup>+</sup> T cells after co-cultivation indicated a slight increase of Perforin (Fig. 5c) but no change in IFN $\gamma$  production (Fig. 5d). In addition, TCR repertoire analysis revealed a less restricted polyclonal TCR pattern for the lymph node (28% restriction, *i.e.*, 8/35 specific TCR V $\beta$  PCR products were lacking) than for liver and spleen of HTM (42 and 46% restriction, respectively). Interestingly, comparing TCR repertoires in the aforementioned organs demonstrated that 17% of V $\beta$  chains (V $\beta$ 4, V $\beta$ 5S1, V $\beta$ 12, V $\beta$ 22 and V $\beta$ 24)

were absent likewise. Moreover, some TCR chains appeared to be dominantly expressed indicating clonal expansion. However, sequencing of dominant TCR V $\beta$  chains did not result in decipherable clonal sequence signals (data not shown). The functional analyses of NK cells in HTM revealed that these immune cells have effector competence as indicated by detectable IFN $\gamma$  and lower levels of Perforin (Fig. 5e, left panel). In addition, IL-15/IL-15R treatment in HTM not only increased the number of NK cells but also enhanced the effector competence, as indicated by upregulation of IFN $\gamma$  and Perforin levels (Fig. 5e, right panel).

## Discussion

In this study, we describe for the first time human chimeric tumor mice engineered by co-transplantation of CD34 $^{+}$  HSCs and two Her2 overexpressing breast cancer cell lines (BT474 and SK-BR-3, respectively). Interestingly, BT474 cells (originally isolated from a solid tumor) resulted in solid tumor formation and weak dissemination, whereas SK-BR-3 originally isolated from pleural tumor effusion induced tumor effusion accompanied by metastases in these mice. The HTM model is based on previously described humanized mice, which already represent a model particularly useful for pre-clinical evaluation of different therapy strategies.<sup>19,27</sup> Extending the humanized mouse model by co-transplantation of human stem cells together with human tumor cells provides a novel model that combines development of the functional human immune system together with a breast cancer tumor without induction of rejection by activated human immune cells. We observed, however, activation of the human immune system obviously triggered by the presence of the human tumor cells. This activation is indicated by CD4 $^{+}$  T cell and NK-cell expansion along with the production of Perforin and IFN $\gamma$ . In addition, a phenotype switch from a naïve CD4 $^{+}$  T cells (CD45RA $^{+}$ /CD27 $^{+}$ , typically found in non-manipulated humanized mice) toward a memory phenotype (loss of CD45RA expression) appears. In contrast to HTM, humanized mice infected with *Leishmania* do not only show CD4 $^{+}$  but also strong CD8 $^{+}$  cytotoxic T-cell activation and maturation due to the interaction of MHC-I on infected macrophages with CD8 $^{+}$  T cells (antigen presenting cell and T cell from the same individual; manuscript in preparation), which potentially does not occur in HTM (due to different origin of tumor and CD8 $^{+}$  effector cells). T cell activation in HTM is probably due to tumor-specific antigen interaction; however, the activation induced by mismatched MHC cannot be categorically excluded.

Human hematopoietic cells invaded tumor areas in HTM and flow cytometric analyses detected mainly CD4 $^{+}$  T cells and B cells but nearly no CD8 $^{+}$  T cells. As in humanized mice, HTM developed regulatory T cells in all organs representing the phenotype of a memory T cells (CD45RA $^{-}$ /CD27 $^{+}$ ).

Tumor specificity of this human immune cell activation was demonstrated by *in vitro* restimulation assays showing

proliferation of CD4 $^{+}$ , CD8 $^{+}$  and NK cells, IFN $\gamma$  and Perforin production, which results in killing of the co-cultured tumor cells. We found reconstitution of a nearly complete human TCR repertoire in spleen, lymph node and liver tissues of HTM by PCR analysis as likewise recently described for humanized mice by Shultz *et al.*<sup>28</sup> Therefore, HTM seems to be a promising model that allows qualitative and quantitative analyses of specific TCR sequences to assess the *in vivo* impact of various therapeutic strategies. Following determination of a TCR sequence directed against a tumor-associated antigen, the development of a specific T-cell response in a tumor microenvironment can easily be tracked *via* V $\beta$  chain-specific quantitative real-time RT-PCR.

Analyzing NK cells, we found an increase in frequency and activation in HTM when compared with humanized mice. To further enhance NK-cell frequency, we used the IL-15/IL-15R treatment for humanized mice as it has been previously described.<sup>24,25</sup>

In addition to frequency, the stimulation with IL-15 triggered NK-cell effector competence in humanized mice and in HTM indicated by the upregulation of IFN $\gamma$  and Perforin release. These NK cells comprise a substantial fraction of CD56 $^{+}$ CD27 $^{-}$  population that can be found in lymphoid as well as extra-lymphoid organs and express high levels of CD16 (Fc $\gamma$ R-III; data not shown). Hence immune modulation in HTM is feasible, which in turn offers all possibilities to study the therapeutic effect of, *e.g.*, anti-Her2 antibody treatment. Special immune responses such as ADCC by NK cells, which has been assumed to predominantly contribute to tumor cell eradication during antibody therapy,<sup>29</sup> can now be investigated. Clynes *et al.*<sup>30</sup> provided already pioneering work and showed that the activity of Trastuzumab on breast cancer xenografts was attenuated in knock-out mice lacking Fc $\gamma$ RIII receptor expression, which was strongly indicative for ADCC as a key player in the antitumor activity of Trastuzumab. This observation has been substantiated by Barok *et al.*<sup>31</sup> who demonstrated reduced antitumor activity by using Trastuzumab-F $_{(ab)2}$  instead of using the complete antibody. In 2009, Ito *et al.*<sup>11,13</sup> inoculated Hodgkin Lymphoma cells into NOD-/Shi-*scid* IL-2R $\gamma^{null}$  mice and cotransplanted already mature PBMCs from healthy human donors. The human PBMCs, however, did not develop in the mice and were MHC mismatched to the Hodgkin Lymphoma donor. Consequently, the PBMCs had to be expected to not exclusively show antitumor effects based on ADCC. It is however even more likely that they interact with murine tissue (GvHD) and MHC-mismatched tumor cells inducing inflammation, which insufficiently mimics a human immune response triggered by antibody therapy.

Shiokawa transplanted human NK cells 1 day after s.c. inoculation of an erythroleukemia cell line in NOD/SCID/ $\gamma c^{null}$  (NOG) mice and thereby also indicated the impact of ADCC in antibody-based therapy.<sup>32</sup> This again stresses the importance of NK cells as a keyplayer during antibody therapy. However, Shiokawa *et al.*<sup>32</sup> did not address the potential role of other immune cells, and, in contrast to the model we



present, the NK cells used by Shiokawa did not develop in mice. Extending all studies mentioned above, the co-transplantation of CD34<sup>+</sup> cells, as performed in this study, enables the co-evolution of human hematopoietic cells together with transplanted cancer cells in the presence of mouse tissue. We designed HTM by using breast cancer cell lines to guarantee reproducibility for future studies. The model, however, can potentially be extended to transplantation of primary tumor cells. A subsequent transplantation of tumor cells into humanized mice (instead of co-transplantation) has also been recently realized showing no general tumor rejection.<sup>13,33</sup> The co-transplantation of tumor cells together with human CD34<sup>+</sup> stem cells in neonatal mice, however, allows for simultaneous hematopoiesis and tumor growth avoiding rejection *ab initio*. These mice show human immune response without inducing immunological elimination of tumor cells, which mimics the situation in tumor patients. Therefore, HTM makes it possible to study *in vivo* processes such as human tumor growth, tumor burden, tumor-cell dissemination and metastasis (early on in lung and bone marrow) in association with a complete human immune system. Additional analyses of survival, proliferation and apo-

ptosis measurement *in vivo* and *in vitro* will assist therapy studies using HTM. Single cell-based flow-cytometric immunophenotyping of immune cells involved in ADCC facilitates the analysis of the cellular components contributing to antibody-based therapy. The potential to trigger ADCC-related tumor-cell defense by the treatment of HTM with, e.g., Trastuzumab in combination with cytokines like IL-1, IL-15 and IL-21<sup>34–36</sup> can now be elucidated *in vivo* under more “human like conditions” and might reveal new ways to overcome therapy failure in nonresponder patients.

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