Generation of a canine anti-EGFR (ErbB-1) antibody for passive immunotherapy in dog cancer patients.

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Supplementary Methods

Competitive flow cytometry

EGFR receptor status of P114, BT474 and A431 were assessed with Cetuximab (Erbitux®, Merck, Darmstadt, Germany) and Rituximab (MabThera®, Roche, Hertfordshire, United Kingdom) as isotype control, followed by staining with anti-human IgG AlexaFluor®488 (Invitrogen, Life Technologies, Grand Island, New York, USA). The assays were performed as described in the *Flow cytometry* method section of the original manuscript.

To address the difference in binding affinities of can225IgG towards human and canine EGFR, competitive flow cytometry was applied. Canine P114 cells were loaded with can225IgG at excessive concentrations (c=1µg/ml in 200 µl FACS buffer) for 30 min at 4°C. After two subsequent washing steps, cells were incubated with human recombinant EGFR (Acro Biosystems, Greater London, United Kingdom) in different concentrations for 30 min at 37°C to investigate competitive binding. Afterwards cells were washed again and stained with 200µl of 10µg/ml anti-dog IgG FITC antibodies for 30min at 4°C to detect remaining can225IgG at the cell surface. Upon two consecutive washing steps, cells were analyzed using the dual laser FACSCalibur™. 10.000 events of cells were recorded. For the data analysis, we additionally gated for single and living cells.

The same procedure was applied for human BT474 cells overexpressing human EGFR to compare disassociation kinetics.

Immunofluorescence

Specific binding of purified can225IgG antibodies was also confirmed by immunofluorescence. A431 cells were cultivated on cover glasses and fixed with paraformaldehyde (3.7%). Cells were stained with can225IgG and cetuximab, respectively. Rituximab and purified dog IgG Standard served as isotype controls. To visualize the bound antibodies, anti-dog IgG FITC (Jackson ImmunoResearch Europe Ltd., Suffolk, United Kingdom) and anti-human IgG AlexaFluor®488 (Invitrogen, Life Technologies, Grand Island,

New York, USA) were applied, respectively. In addition nuclei of cells were visualized by 4',6-diamidino-2-phenylindole (DAPI) staining. Pictures were acquired using an Axioplan 2 fluorescent microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Immunohistochemistry

Canine mammary tumor samples (previously fixed in buffered 10% formalin and paraffin embedded) were cut into serial 4-µm sections. Paraffin was removed with xylene and immunohistochemistry was performed using the FDA-approved *in vitro* diagnostic EGFR pharm Dx™ kit (DAKO, Glostrup, Denmark) for determination of EGFR-status. EGFR-overexpressing tumor samples were employed for staining with can225IgG and dog IgG Standard, respectively. Thus, antigen retrieval (citrate buffer), blockade of endogenous peroxidase (3% H_2O_2) and blocking of unspecific binding was performed using PBS supplemented with 3% normal goat serum, 2% BSA, 0.2% fish skin, 10% casein, 0.1% Triton X and 0.05% Tween 20. Subsequently, sections were incubated for 2h at RT with 0.2µg/ml biotinylated can225IgG or biotinylated dog IgG Standard. Bound antibodies were detected with Streptavidin-HRP (Vector Laboratories Inc., Burlingame, California, USA) followed by an amplification step with biotinylated Tyramide (self synthesized from 4-(2-Aminoethyl)phenol hydrochloride with 6-(Biotinamidocaproylamido)caproic acid N-hydroxysuccinimide ester (Sigma-Aldrich, St. Louis, Missouri), USA, which was again detected with Streptavidin HRP. Afterwards DAB-solution (DAKO, Glostrup, Denmark; 20µl Biphenyl-3,3',4,4'-tetrayltetraammonium tetrachloride -Chromogen per ml Substrate-buffer) was applied and counterstaining with hematoxylin was performed. Pictures were acquired on a Zeiss Axio Imager Z1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with the HistoQuest® software module (TissueGnostics, Vienna, Austria).