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Eradication of Canine Diffuse Large B-Cell Lymphoma in a Murine Xenograft Model with CD47 Blockade and Anti-CD20

Kipp Weiskopf^{1,2,3,*}, Katie L. Anderson^{4,5,6}, Daisuke Ito^{4,5,6,**}, Peter J. Schnorr^{1,2,3}, Hirotaka Tomiyasu^{4,5,***}, Aaron M. Ring^{1,2,3,7,****}, Kristin Bloink⁸, Jem Efe⁹, Sarah Rue⁹, David Lowery^{10,*****}, Amira Barkal^{1,2,3}, Susan Prohaska^{1,2,3}, Kelly M. McKenna^{1,2,3}, Ingrid Cornax^{5,11}, Timothy D. O'Brien^{5,11,12}, M. Gerard O'Sullivan^{5,11}, Irving L. Weissman^{1,2,3,†}, and Jaime F. Modiano^{4,5,6,12,†}

¹Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, California 94305, USA

²Ludwig Center for Cancer Stem Cell Research and Medicine, Stanford University School of Medicine, Stanford, California 94305, USA

³Stanford Cancer Institute, Stanford University School of Medicine, Stanford, California 94305, USA

⁴Department of Veterinary Clinical Sciences, University of Minnesota, St. Paul, Minnesota, 55108 USA

⁵Masonic Cancer Center, University of Minnesota, Minneapolis, 55455 Minnesota, USA

⁶Center for Immunology, University of Minnesota, Minneapolis, 55455 Minnesota, USA

⁷Department of Molecular and Cellular Physiology, and Department of Structural Biology, Stanford University School of Medicine, Stanford, California 94305, USA

⁸Elanco Animal Health US, Inc., 2500 Innovation Way, PO Box 708, Greenfield, IN 46140

⁹Genomics Institute of the Novartis Research Foundation, San Diego, CA 92121, USA

¹⁰Elanco Animal Health US, Inc., Greensboro, NC 27408, USA

¹¹Department of Veterinary Population Medicine, University of Minnesota, St. Paul, Minnesota, 55108 USA

Correspondence to: Jaime F. Modiano, Masonic Cancer Center, Univ. of Minnesota, 420 Delaware St., SE, MMC 806, Minneapolis, MN 55455. Tel.: 612-625-7436; Fax: 612-626-4915; E-mail: modiano@umn.edu; Kipp Weiskopf, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. Phone: 617-723-5500; Fax: 617-723-6439; kippw@alumni.stanford.edu.

[†]Equal contributions by authors

* Current Address: Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115, USA.

** Current Address: Japan Medical Strategy Immuno-Oncology, Bristol-Myers K.K, Tokyo, 160-0022, Japan

*** Current Address: Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, 113-8657, Japan

**** Current Address: Department of Immunobiology, Yale School of Medicine, New Haven, Connecticut 06520, USA

***** Current Address: Merial, Duluth, Georgia 30096, USA

Conflicts of Interest: KB is an employee of Elanco Animal Health (which acquired Novartis Animal Health in 2015). KW, PJS, AMR, ILW declare U.S. patent applications pertaining to CD47-blocking therapies assigned to Stanford University. KW, AMR, and ILW declare consulting and/or equity ownership in Forty Seven Inc. ILW is a Director of Forty Seven, Inc. SP and KMM are employees of Forty Seven, Inc. KW and AMR declare consulting and/or equity ownership in Alexo Therapeutics, Inc.

¹²Stem Cell Institute, University of Minnesota, Minneapolis, Minnesota, 55455 USA

Abstract

Cancer immunotherapies hold much promise, but their potential in veterinary settings has not yet been fully appreciated. Canine lymphomas are among the most common tumors of dogs and bear remarkable similarity to human disease. In this study, we examined the combination of CD47 blockade with anti-CD20 passive immunotherapy for canine lymphoma. The CD47/SIRP α axis is an immune checkpoint that regulates macrophage activation. In humans, CD47 is expressed on cancer cells and enables evasion from phagocytosis. CD47-blocking therapies are now under investigation in clinical trials for a variety of human cancers. We found the canine CD47/SIRP α axis to be conserved biochemically and functionally. We identified high-affinity SIRP α variants that antagonize canine CD47 and stimulate phagocytosis of canine cancer cells *in vitro*. When tested as Fc fusion proteins, these therapeutic agents exhibited single-agent efficacy in a mouse xenograft model of canine lymphoma. Since robust synergy between CD47 blockade and tumor-specific antibodies has been demonstrated for human cancer, we evaluated the combination of CD47-blockade with 1E4-cIgGB, a canine-specific antibody to CD20. 1E4-cIgGB could elicit a therapeutic response against canine lymphoma *in vivo* as a single agent. However, augmented responses were observed when combined with CD47-blocking therapies, resulting in synergy *in vitro* and *in vivo* and eliciting cures in 100% of mice bearing canine lymphoma. Our findings support further testing of CD47-blocking therapies alone and in combination with CD20 antibodies in the veterinary setting.

Keywords

Immunotherapy; canine; diffuse large B-cell lymphoma; CD47; CD20

Introduction

Due to high demand for cancer treatments by pet owners and a large clinical need, immunotherapeutic approaches are now being applied to veterinary settings (1). In the U.S. alone, the incidence of canine cancer is estimated to be over 4 million new cases per year, and cancer is believed to be the leading cause of disease-related mortality in dogs (2). Lymphoma is among the most common types of canine cancer, with incidence rates of approximately 20-107 cases per 100,000 dogs at risk (3). Humans exhibit a high degree of genomic similarity with dogs, and dogs share environments and exposures with their human owners (4). Many canine and human cancers appear to be conserved at the molecular level and in their clinical behavior, particularly lymphoma (2, 3). Veterinary studies represent an ethical use of animals in research, enabling the study of naturally occurring disease and provide accurate representations of tumor heterogeneity, the tumor microenvironment, and the course of illness progression. Thus, veterinary studies may be valuable ‘stepping stones’ in conceptual advancement towards understanding and treating human disease as well as ameliorating disease in valued companions (5).

A number of passive immunotherapies have become standard of care for human cancers, including CD20 antibodies for patients with non-Hodgkin lymphoma and other B-cell

malignancies. The CD20 monoclonal antibody (mAb) rituximab was the first mAb approved for the treatment of cancer and is responsible for increasing the 10-year survival of patients with non-Hodgkin lymphoma by over 15% (6). Expression of CD20 in dogs on normal and malignant B cells is conserved, supporting the promise of CD20 as a therapeutic target in canine lymphoma (7, 8). Recently, a canine-specific CD20 mAb was developed with canine Fc regions (1E4-cIgGB) that effectively depletes normal B cells in healthy dogs and is undergoing investigation for canine B-cell lymphoma (9).

Multiple mechanisms of action have been described for CD20 mAbs, but studies indicate a major *in vivo* mechanism is antibody-dependent phagocytosis by macrophages (10-14). The CD47/SIRP α axis is an immune checkpoint that limits the macrophage response to tumor-specific antibodies (11, 14-16). By binding to SIRP α , an inhibitory receptor on macrophages and other myeloid cells, CD47 transduces inhibitory signals that allow tumor cells to evade macrophage-mediated destruction (10, 11, 15, 17-21). As such, the combination of CD47-blocking agents and tumor-binding antibodies that bind to macrophage Fc receptors is highly effective in preclinical models of human lymphoma (10, 11). Many cancers express high CD47, and multiple CD47-blocking reagents are now under investigation in clinical trials for both solid and hematologic malignancies (clinicaltrials.gov identifiers NCT02216409, NCT02367196, NCT02663518, NCT02678338).

In this study, we investigated whether immunotherapeutic targeting of CD47 and CD20 could be applied to the canine system. We first characterized the canine CD47 and SIRP α homologs. Next, we identified a lead candidate that potently blocks canine CD47, induces macrophage phagocytosis of canine lymphoma cells, and eliminates canine lymphoma in xenotransplantation models. Last, we confirmed that CD47-blocking therapies augment the therapeutic response produced by anti-CD20 against canine lymphoma.

Materials and Methods

Cell lines and culture

The CLBL-1 canine diffuse large B-cell lymphoma cell line (22) was obtained from Dr. Barbara Rütgen (University of Vienna, Austria) in 2009 and was authenticated in 2015 by the Modiano lab using STR testing (DDC Medical). A GFP-luciferase⁺ CLBL-1 variant was generated by transfection with a *Sleeping Beauty* transposon system as described (23). Briefly, 1×10^6 CLBL-1 cells were transfected using a Nucleofector system, program DN-100 (Lonza) with 1 μ g of transposon-expressing pDNA vector along with 2 μ g of the GFP/luc vector pKT2/CLP-Luc-ZOG in 100 μ L of nucleofector solution SF (Lonza). CLBL-1 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) plus GlutaMAX (Invitrogen) supplemented with 20% fetal bovine serum (Omega Scientific or Atlas Biologicals), 100 U/mL penicillin and 100 μ g/mL streptomycin (Invitrogen). J774 cells were obtained from ATCC in 2012 and 2015 and authenticated in 2015 by the Modiano lab using STR testing (DDC Medical). J774 cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) with 10% fetal bovine serum (Atlas Biologicals).

Osteosarcoma lines OSCA-40 and OSCA-78 were derived in the Modiano lab in 2004 and 2008, respectively. They were authenticated in 2015 by the Modiano lab using STR testing

(DNA Diagnostic Center) and cultured as previously described (24). Hemangiosarcoma cell line COSB was re-derived by the Modiano lab in 2007 by xenograft passage of parental line SB. It was authenticated in 2015 by the Modiano lab using STR testing (DNA Diagnostic Center) with 1 out of 20 alleles differing from the parental line. Hemangiosarcoma cell line Emma was derived by the Modiano lab in 2008 and authenticated in 2015 by the Modiano lab using STR testing (DNA Diagnostic Center). COSB and Emma were cultured as previously described (25-27). Canine melanoma cell lines TLM1, CMGD2, and CMGD5 were derived by the Modiano lab in 1996, 2001, and 2001, respectively. They were authenticated in 2015 by the Modiano lab using STR testing (DNA Diagnostic Center). Canine melanoma cell lines were cultured as previously described (28, 29). Canine glioma cell lines Mac and Candy were provided by Dr. John Ohlfest (University of Minnesota) in 2009. They were cultured in neurobasal medium supplemented with N2 and B27 (Invitrogen) 10% fetal bovine serum, L-glutamine and Primocin (Invitrogen). They were passaged less than 20 times in the Modiano lab. Raji cells were obtained from ATCC between 2012-2015 and authenticated by the repository. Raji cells were cultured in RPMI 1640 plus GlutaMAX (Invitrogen) supplemented with 10% fetal bovine serum (Omega Scientific), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen).

Therapeutic agents

High-affinity SIRPα protein CV1 was produced as previously described (11). CV1-hIgG4 (11) and Hu5F9-G4 (30) were produced as previously described by expression in FreeStyle 293-F cells (Invitrogen) or CHO-K1 cells (Lonza). The native mouse antibody to dog CD20, 1E4, and chimeric mouse antibodies to CD20 on the canine IgGB background (IgG1 equivalent) Rtx-cIgGB and 1E4-cIgGB, were produced as described (9) and provided by Elanco Animal Health. For 1E4-cIgGB and Rtx-cIgGB ELISAs, binding to immobilized cCD20(ECD2)-hFc fusion protein was detected using a rabbit anti-canine IgG-HRP (US Biologicals #I1904-25Z) and Sure Blue TMB substrate (KPL) with absorbance measured at 650 nm in a Spectra Max (Molecular Devices).

Canine CD47/SIRPα sequences and analysis

Sequence accession numbers for CD47 sequences included canine CD47 (NP_001074190.1), human CD47 (NP_001768.1), mouse CD47 (BAA25401.1). Sequence accession numbers for SIRPα sequences included canine SIRPα (XP_005634938.1), human SIRPα variant 1 (PDB sequence ID 4CMM|A), human SIRPα variant 2 (PDB sequence ID 2UV3|A), C57BL/6 mouse SIRPα (XM_006498982.2), and NOD mouse SIRPα (31, 32). Multiple sequence alignment and phylogenetic analysis were performed using Clustal Omega (33). Sequence identity and similarity between CD47 and SIRPα variants were assessed by NCBI pBLAST alignment (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The human SIRPα secondary structure was previously described (32).

Canine CD47/SIRPα protein production

Canine CD47-Fc was constructed by inserting the canine CD47 extracellular domain (amino acids 1-116 of mature protein) into a modified pFUSE-hIgG4-Fc vector (Invivogen) with an IL-2 signal sequence and engineered Ser228Pro mutation (34). A C15G was introduced to the CD47 domain to facilitate purification as described for human CD47 (11, 35). Canine

CD47-Fc was expressed in FreeStyle 293-F cells (Invitrogen) and purified using HiTrap Protein A columns (GE Healthcare). Canine CD47-Fc was eluted using citrate-based (pH 3.0) or arginine-based (pH 4.0) buffers and immediately neutralized using 1.0 M Tris-HCl (pH 8.0).

Canine SIRP α tetramers were produced as described for mouse SIRP α (11). Briefly, the canine N-terminal domain of SIRP α (amino acids 1-118 of mature protein) was expressed in *E. coli* with an N-terminal maltose binding protein (MBP) tag followed by a rhinovirus 3C protease cleavage site and C-terminal biotin acceptor peptide (BAP) and 8 $\times$ histidine tag. The MBP-fusion protein was purified using Ni-NTA chromatography, and eluted products were digested with 3C protease to remove MBP, purified by an additional Ni-NTA chromatography step, and subjected to size exclusion chromatography with a Superdex-S75 column. The protein was biotinylated with BirA ligase. Tetramers were formed by incubation with Alexa Fluor 647-conjugated streptavidin. See Supplementary Materials for engineered protein sequence information.

Yeast surface display of CD47 and SIRP α variants

The extracellular domain of canine CD47, with the C15G mutation, was displayed on the surface of *S. cerevisiae* strain EBY100 as an N-terminal fusion to Aga2, leaving a free N-terminus as previously reported for human CD47 (36). The N-terminal domains of canine, C57BL/6, and NOD SIRP α were displayed on yeast by fusion to the C-terminus of Aga2 using the pCT302 vector as described for human and mouse SIRP α variants (11).

Binding and blocking of the canine CD47/SIRP α axis

Binding of canine CD47-Fc to canine and mouse SIRP α was assessed using yeast expressing canine or mouse SIRP α variants on their surface. Binding of canine CD47-Fc, Hu5F9-G4, and CV1-hIgG4 was detected using an Alexa Fluor 647-conjugated anti-human IgG (H+L) secondary antibody (Invitrogen). Blockade of canine SIRP α was assessed in a competition assay using varying concentrations of Hu5F9-G4 and CV1-hIgG4 combined with 50 nM Alexa Fluor 647-conjugated streptavidin canine SIRP α tetramers and incubated for 30 minutes on ice. Analysis was performed using an LSRFortessa with high-throughput sampler (BD Biosciences). Geometric mean fluorescence intensity was measured using FlowJo v9.4.10 (Tree Star), and data were fit to sigmoidal dose-response curves using Prism 5 (GraphPad).

Detection of CD47 expression on canine cancer cells

For studies using CV1-hIgG4, cells were incubated with CD47 antagonists, washed, and then binding was detected with an Alexa Fluor 647-conjugated anti-human IgG (H+L) antibody (Invitrogen). Wild-type human SIRP α allele 2 was produced as a fusion to human IgG4 (WT-hIgG4) as described (11) and was used as a staining control. Dead cells were excluded from the analysis using DAPI (Sigma Aldrich). Samples were analyzed on an LSRFortessa. For studies using directly conjugated Hu5F9-G4, 1×10^6 cells of each line were incubated with normal canine IgG and mouse IgG1 for 10 minutes on ice. Cells were subsequently labeled using Hu5F9-G4 conjugated to Alexa Fluor 488 or an isotype control antibody (human IgG4 conjugated to Alexa Fluor 488) and incubated on ice for 30 minutes.

Analysis was performed using a LSRII (BD Biosciences), and geometric mean fluorescence intensity was measured using FlowJo.

Macrophage differentiation and phagocytosis assays

Primary mouse macrophages derived from NSG mice were differentiated *ex vivo* in the presence of 10 ng/mL murine M-CSF (Peprotech) (11). Canine macrophages were derived from blood obtained from healthy companion dogs with approval from the University of Minnesota Institutional Animal Care and Use Committee (UMN IACUC, protocol #1304-30546A). Peripheral blood mononuclear cells were enriched by density gradient centrifugation over Ficoll-Paque Premium (GE Healthcare). Monocytes were purified on an autoMACS Pro Separator (Miltenyi) using anti-CD14 microbeads (Miltenyi) and differentiated to macrophages by culture for 7-10 days in IMDM plus GlutaMax (Invitrogen) supplemented with 10% autologous serum for each individual donor and 100 U/mL penicillin and 100 µg/mL streptomycin.

Evaluation of macrophage phagocytosis

Macrophage phagocytosis was evaluated as described (11). Briefly, 50,000 macrophages were co-cultured with 100,000 cancer cells labeled with either calcein AM or CFSE (Invitrogen). Phagocytosis in response to therapeutic treatments was evaluated after two hours of co-culture and analyzed by flow cytometry. NSG macrophages were identified by staining with PE/Cy7 or APC-anti-mouse F4/80 (eBioscience). Canine macrophages were stained with Tri-color-conjugated or PE-conjugated anti-CD14 clone TuK4 (Thermo Fisher, Miltenyi). Phagocytosis was evaluated as the percentage of macrophages engulfing fluorescent tumor cells using an LSRFortessa with high-throughput sampler or a LSR II and analyzed using FlowJo v9.4.10. Microscopic evaluation of phagocytosis was performed using an ImageStream MKII Flow Cytometer (Amnis).

In vitro cytotoxicity assays

Antibody-mediated direct cytotoxicity was evaluated in response to Rtx-cIgGB or 1E4-cIgGB. Cells were cultured with antibodies therapies for 72 hours, at which time viability was analyzed by MTS assay (Promega) according to the manufacturer's protocol. Complement-dependent cytotoxicity was evaluated by incubation of cells with antibodies against CD20 and rabbit complement (ImmunO, MP Biomedicals,) or heat-inactivated rabbit complement (56°C for 1 hour).

In vivo canine lymphoma xenograft models

Mice were maintained in a barrier facility and handled according to protocols approved by the Stanford University Administrative Panel on Laboratory Animal Care (protocol #26270) and UMN IACUC (protocol #1207A17291). Nod.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ (NSG) mice (37) were used for all *in vivo* experiments. Mice were engrafted with CLBL-1 lymphoma cells at approximately 6-12 weeks of age, and experiments were performed with age and sex-matched cohorts of 7-10 mice.

For a model of disseminated lymphoma, NSG mice were engrafted with 100,000 CLBL-1 cells or GFP-luciferase⁺ CLBL-1 cells intravenously via retro-orbital injections. Fluorescent

dissection microscopy and bioluminescence imaging was performed as described (11). Treatment with PBS or 250 µg of CV1-hIgG4 was initiated 24 hours or 6 days post-engraftment and continued daily for one to two weeks, as indicated. Orbital tumor scores were assessed on a three-point scale and scored as follows for visible tumor infiltration: 1 - undetectable or mild; 2 - moderate; 3 - high. Aggressive intra-abdominal lymphoma was modeled by injection of 50,000 cells intraperitoneally. In this model, systemic tumor dissemination leads to gross organomegaly and weight gain; therefore, tumor burden was assessed by weight gain over time. Treatment with PBS or antibodies against CD20 was initiated 3 days post-engraftment and continued twice per week until day 32 post tumor injection. For a localized model of canine lymphoma, 40,000 CLBL-1 cells were engrafted subcutaneously. Treatment was initiated 24 hours post-engraftment with anti-CD20 (250 µg/mouse, 3 days/week, continued until day 36) and CV1 (200 µg/mouse/day, continued until day 24). Tumor dimensions were measured to calculate volumes according to the ellipsoid formula ($\pi/6 \times \text{length} \times \text{width}^2$).

Pathological analysis of tumor specimens was performed by University of Minnesota Comparative Pathology Shared Resource by staining with hematoxylin-eosin, anti-CD20 (primary antibody: rabbit anti-human CD20 polyclonal antibody (Thermo Scientific)), detected with a rabbit EnVision+ Kit (Dako), anti-CD3 (primary antibody: rat anti-human CD3 mAb (Serotec), detected with a rat-on-mouse HRP polymer detection system (Biocare Medical)), and anti-CD79a (primary antibody (mouse anti-human CD79A monoclonal antibody (Biocare), detected with a mouse EnVision+ Kit).

Results

Molecular characterization of the *Canis familiaris* CD47/SIRPα axis

A homolog of canine CD20 has been identified, and a speciated antibody to canine CD20 has been developed (7-9). Therefore, we began our investigation by examining the canine CD47-SIRPα interaction. The existence of a canine CD47 homolog was supported by a prior serological study that examined antibody cross-reactivity (38). Additionally, a predicted homolog of canine CD47 was annotated in the canine genome (NM_001080721.1). However, no biochemical or functional analysis has been performed to validate this sequence. Alignment of the extracellular domain of the putative canine CD47 sequence with that of human and mouse showed the gene was more closely related to human CD47 with 66% amino acid identity and 79% amino acid similarity (Fig. 1A and B, Supplementary Table S1). A putative canine SIRPα homolog was predicted from the canine genome based on sequence similarity (XP_005634938.1), although no molecular validation has been performed. The CD47-binding domain of this gene product aligns with human and mouse SIRPα variants (Fig. 1C). As with canine CD47, the putative canine SIRPα homolog shares more similarity with human SIRPα variants than those of mouse (Fig. 1D). The CD47-binding domain of human SIRPα is highly polymorphic (31). Interestingly, the predicted canine SIRPα gene product shares 84-85% similarity with human SIRPα variants, whereas the similarity between human SIRPα variants 1 and 2 is 90% (Supplementary Table S2).

To define these predicted gene products as functional CD47 and SIRP α homologs, we assessed whether an interaction exists between the two proteins at the biochemical level. We cloned the N-terminal extracellular domain of the putative canine SIRP α gene and expressed it on the surface of yeast as a fusion to Aga2, a method previously used to evaluate the functions of human and mouse SIRP α (Fig. 2A) (11). Next, we produced the extracellular domain of canine CD47 as an Fc fusion protein (canine CD47-Fc). As hypothesized, we found that canine CD47-Fc binds to canine SIRP α with a dose-dependent relationship (Fig. 2B and C). This finding establishes the predicted genes as canine CD47 and SIRP α and confirms a biochemical interaction between the extracellular domains of the two proteins.

In order to validate the potential of CD47 blockade in xenograft models of canine lymphoma (22, 39), we evaluated whether canine CD47 could bind to mouse SIRP α . Allelic variants of mouse SIRP α have been described with different binding affinities for human CD47. In particular, the SIRP α allele from non-obese diabetic (NOD) mice interacts with human CD47, whereas there is minimal binding by the SIRP α allele from C57BL/6 mice (31). The preservation of this interaction across species is a major determinant for xenotransplantation of human tissues into immunocompromised mice, and it enables *in vivo* evaluation of therapeutic responses to CD47-blocking therapies. Therefore we investigated whether cross-reactivity exists between canine CD47 and mouse SIRP α . We expressed the mouse NOD and C57BL/6 SIRP α alleles on the surface of yeast and used canine CD47-Fc to assess binding. We found that, similar to human CD47 (31), canine CD47 exhibited much stronger binding to the SIRP α allele expressed by NOD mice versus that expressed by C57BL/6 mice (Fig. 2D and E). As with canine SIRP α , canine CD47-Fc binds to NOD SIRP α in a dose-dependent manner. Therefore, this finding identifies the NOD strain as the most appropriate background for evaluation of the canine CD47-SIRP α interaction.

Identification of therapeutic candidates that block the canine CD47/SIRP α axis

By cloning the canine CD47 and SIRP α genes, we were able to generate tools to investigate the ability of therapeutic candidates to bind and block canine CD47. As with the extracellular domain of canine SIRP α , we generated a construct that contains the extracellular domain of canine CD47 for display on the surface of yeast and tested the ability of two CD47-blocking therapeutics to bind and block canine CD47. The first was Hu5F9-G4, a humanized antibody to CD47. The second was CV1-hIgG4, a high-affinity SIRP α -Fc fusion protein (11). CV1-hIgG4 blocks both human and mouse CD47 (11); thus we hypothesized CV1-hIgG4 may also be a functional antagonist of canine CD47. To evaluate their binding to canine CD47, we incubated yeast cells expressing canine CD47 with varying concentrations of Hu5F9-G4 or CV1-hIgG4. We found that the agents differed in their ability to bind canine CD47 (Fig. 3A). CV1-hIgG4 demonstrated potent binding, with an EC₅₀ of approximately 741 pM (11). On the other hand, Hu5F9-G4 exhibited less potent binding by orders of magnitude and did not fully saturate the cells at the maximum concentration tested (10 μ g/mL).

To evaluate the interaction between canine CD47 and SIRP α further, we expressed and purified the N-terminal domain of canine SIRP α from *E. coli* (Supplementary Fig. S1A and B). We produced fluorophore-labeled canine SIRP α tetramers and confirmed that they could

bind to canine CD47 expressed on the surface of the yeast. Using this system, we generated an assay to investigate whether Hu5F9-G4 or CV1-hIgG4 could act as competitive antagonists to canine CD47. We incubated yeast expressing canine CD47 with varying concentrations of the CD47-blocking reagents in combination with the fluorophore-labeled canine SIRP α tetramers. In this competition assay, CV1-hIgG4 demonstrated potent antagonism of canine CD47 with an IC₅₀ of 2.92 nM (Fig. 3B) (11). Due to its weaker affinity for canine CD47, Hu5F9-G4 was unable to antagonize canine CD47 even at the highest concentration tested. These biochemical studies suggested high-affinity SIRP α reagents such as CV1 or bivalent CV1-hIgG4 could act as therapeutic candidates for blockade of canine CD47.

Canine cancer cells express CD47 on their surface

We next investigated whether canine lymphoma cells expressed CD47 on their surface. We found that CV1-hIgG4 binds to CLBL-1 cells with high affinity (Supplementary Fig. S2A). CD47 was similarly expressed on the surface of primary canine DLBCL samples and non-malignant leukocytes (Supplementary Fig. 2B). We also confirmed CD47 expression in each of 24 primary canine DLBCL samples using an available RNA sequencing dataset (40, 41), (Supplementary Fig. 2C). Since CD47 is highly expressed by human cancer cells (10, 11, 19-21), we also explored whether other canine cancer samples expressed CD47 on their surface. Additional samples included osteosarcoma, melanoma, and glioblastoma cell lines (Supplementary Fig. S2D and E). A range of CD47 expression was observed; yet, as with human cancer specimens, all samples tested expressed detectable surface CD47 (Supplementary Fig. S2D and E). This finding suggests that CD47 may allow canine cancer cells to evade the detection by the immune system and confirms the presence of the therapeutic target on multiple types of canine cancer.

Blockade of the canine CD47/SIRP α axis stimulates macrophage phagocytosis

To evaluate the functional efficacy of our CD47-blocking reagents against canine cancer, we tested the reagents in phagocytosis assays using CLBL-1 canine B-cell lymphoma cells (28). CLBL-1 cells were labeled with fluorescent dye calcein AM and cocultured with primary NOD-*scid*- $\gamma_c^{-/-}$ (NSG) (37) macrophages in the presence or absence of CD47-blocking therapies. Phagocytosis was measured by flow cytometry and evaluated as the percentage of calcein AM-positive macrophages, a population that exhibited phagocytic engulfment by microscopy (Fig. 3C, Supplementary Fig. S3). Compared to control treatment, Hu5F9-G4 was only able to induce moderate phagocytosis. However, treatment with CV1-hIgG4 produced maximal phagocytosis, corresponding with its greater ability to bind and block canine CD47 (Fig. 3C and D).

We then evaluated the ability of canine macrophages to respond to CD47-blocking therapies. Canine macrophages were differentiated *ex vivo* from purified monocytes in the presence of autologous serum. After 7-10 days, the cells were firmly adherent to tissue culture plates, exhibited a macrophage morphology, and expressed the macrophage marker CD14 (Supplementary Fig. S4A and B). Canine macrophages from three donors were cocultured with fluorescently labeled CLBL-1 cells and CD47-blocking therapies. Similar trends were observed as with NSG macrophages: CV1-hIgG4 produced a significant increase in

phagocytosis compared to no treatment or Hu5F9-G4 (Fig. 3E). Both therapies were comparable at inducing phagocytosis by canine macrophages when tested against human lymphoma cells (Supplementary Fig. S4C). Together, these *in vitro* findings led us to select CV1-hIgG4 as a lead therapeutic candidate for evaluation as monotherapy *in vivo*.

Effectiveness of CV1-hIgG4 against canine lymphoma in mouse xenograft models

To investigate the therapeutic efficacy of CD47 blockade *in vivo* against canine cancer, we established a xenotransplantation model of canine lymphoma (Fig. 4A). We injected 100,000 canine GFP-luciferase⁺ CLBL-1 lymphoma cells into the retro-orbital venous sinuses of NSG mice (37). The lymphoma cells efficiently engrafted and disseminated throughout the body. One day after engraftment, mice were randomized into two cohorts, and treatment was initiated with either vehicle control or 250 µg CV1-hIgG4. Treatment was administered daily for two weeks, and the mice were monitored for disease progression. Three weeks post-engraftment, nearly all mice in the control group developed early signs of morbidity, with evidence of disease burden around the retro-orbital injection site (Fig. 4B and C). In comparison, none of the mice treated with CV1-hIgG4 exhibited retro-orbital disease at this time. Mice were monitored for survival, and all mice in the control group developed rapid progression of their disease requiring humane euthanasia (Fig. 4D). In contrast, 50% of the mice treated with CV1-hIgG4 exhibited delayed progression of their disease, while the remaining 50% had no evidence of disease even after 200 days ($P = 0.002$).

As a model of more advanced disease, mice were engrafted with 100,000 GFP-luciferase⁺ CLBL-1 cells, and treatment was initiated 6 days post-engraftment, when bioluminescence imaging confirmed lymphoma cells had disseminated throughout the body of the animals (Fig. 4E). Mice were then randomized into treatment with either vehicle control or 250 µg CV1-hIgG4 daily. Mice were treated for 1 week, then bioluminescence imaging was performed to assess tumor burden. Even after this short course of intervention, mice treated with CV1-hIgG4 exhibited significantly less tumor burden than animals treated in the control group (Fig. 4F). However, treatment with CV1-hIgG4 did not significantly enhance survival in this model (data not shown).

Complement-dependent killing and macrophage phagocytosis by anti-canine CD20

Since previous studies have demonstrated synergy between CD47-blocking therapies and tumor-specific antibodies (10, 11), we next examined whether these principles might also apply to canine lymphoma. The chimeric antibody to canine CD20, 1E4-cIgGB, consists of the variable regions of mouse mAb 1E4, which binds canine CD20 extracellular domain 2 with high affinity (Supplementary Fig. S5A), and the canine IgGB Fc region (9). 1E4-cIgGB binds canine B cells *in vitro* (Supplementary Fig. S5B) and can deplete B cells in healthy dogs (9). Various independent mechanisms of action mediate B-cell depletion and the therapeutic effect of anti-CD20, including direct cytotoxicity, complement-dependent cytotoxicity (CDC), and antibody-dependent phagocytosis (ADP) (12, 42, 43). We examined the capacity of unmodified mAb 1E4 (mouse IgG2a) and chimeric 1E4-cIgGB to mediate these effects *in vitro*. Neither 1E4 nor 1E4-cIgGB exhibited direct cytotoxicity against CLBL-1 canine lymphoma cells (Fig. 5A), but both induced significant complement-mediated lysis of the target cells (Fig. 5B). Additionally, 1E4 significantly increased

phagocytosis of CLBL-1 cells in an ADP assay with IFN- γ primed mouse J774 cells as effectors (Fig. 5C).

Antitumor activity of anti-CD20 in a xenograft model of intra-abdominal canine lymphoma

The anti-canine CD20, 1E4-cIgGB, depletes B cells in healthy dogs, but it has not previously been investigated in dogs with naturally occurring lymphoma. Therefore, we evaluated the therapeutic efficacy of 1E4-cIgGB *in vivo* using intraperitoneal CLBL-1 xenografts in NSG mice (39, 44). In this aggressive model, CLBL-1 forms disseminated intra-abdominal tumors and grows systemically in visceral organs, leading to progressive weight gain until the tumor end point at 25-30 days. Mice were treated twice weekly with 1E4-cIgGB or a control antibody, Rtx-cIgGB, which consists of the variable domains of rituximab with a canine IgGB Fc region. Rituximab is specific for human CD20 and does not cross-react with the canine CD20 homolog (7) (Supplementary Fig. S5A). Weight gain was significantly decreased in the 1E4-cIgGB treatment group as compared to the Rtx-cIgGB treatment and the no treatment groups, indicating a therapeutic response (Fig. 6A). Engraftment of high-grade canine B-cell lymphoma was observed in the spleens of all mice (Fig. 6B and C). Nevertheless, histological analysis demonstrated an increase in the tumor necrosis scores from mice in the 1E4-cIgGB treatment group as compared to the Rtx-cIgGB treatment and the no treatment groups (Fig. 6D and E, Supplementary Table S3), suggesting that 1E4-cIgGB increased tumor cell death *in vivo*.

In vivo synergy of CD47 blockade with anti-CD20 immunotherapy for canine lymphoma

We next tested the hypothesis that CD47 blockade would enhance the effects of antibodies against CD20 to ablate canine lymphoma. We examined the capacity of this combination strategy to enhance phagocytosis of CLBL-1 canine lymphoma cells by primary NSG macrophages *in vitro*. CD47 was targeted using Hu5F9-G4 or monomeric CV1, which lacks a pro-phagocytic Fc region and therefore acts as a pure CD47-antagonist (11, 45-47). CV1 is not sufficient to induce phagocytosis by itself, but it augments phagocytosis when combined with a tumor-opsonizing antibody (11, 45-47). CD20 was targeted using the unmodified 1E4 mouse antibody or chimeric 1E4-cIgGB. CD47 blockade with monomeric CV1 or 1E4 alone were unable to elicit significant phagocytosis (Fig. 7A). However, the combination of CV1 and either 1E4-cIgGb or 1E4-mIgG2a elicited significant enhancement of phagocytosis that was greater than either therapy as a single agent. CV1 had no significant effect on proliferation of CLBL-1 cells (Supplementary Fig. S6), indicating CV1 did not cause growth inhibition via direct effects on tumor cells.

Subsequently, we examined this combination strategy in mice bearing subcutaneous CLBL-1 xenografts. Treatment was initiated 24 hours after tumor engraftment using anti-CD20 (250 μ g of Rtx-cIgGB or 1E4-cIgGB) three times per week and 200 μ g of monomeric CV1 daily per mouse. Treatment was continued for 24 days (CV1) or 36 days (anti-CD20). Subcutaneous tumors were visible within 2-3 weeks, and there was progressive tumor growth in mice receiving vehicle control treatment or Rtx-cIgGB. CD47 blockade and anti-CD20 therapy were well tolerated when administered alone or in combination, with no overt toxicity (Supplementary Fig. S7A and B). Both of these therapies significantly delayed tumor growth when administered alone, and remarkably no tumors were grossly visible by

day 21 in the group treated with the combination of CV1 and 1E4-cIgGB (Fig. 7B, Supplementary Fig. S7B). Consistent with this observation, CV1 and 1E4-cIgGB monotherapy prolonged survival of tumor bearing mice (Fig. 7C), but 17 of the 20 mice in these groups were sacrificed due to tumor progression by 60 days post-engraftment. In contrast, 100% (10/10) of the mice treated with monomeric CV1 in combination with 1E4-cIgGB survived until the experiment was terminated at day 68. All of the mice in the control groups and the 1E4-cIgGB monotherapy treatment group and eight of 10 mice in the CV1 treatment group had microscopic evidence of tumor in the subcutis and in lymphoid and visceral organs (Supplementary Fig. S7C). The derivation of the tumors from canine B-cell lymphoma cells was confirmed by immunohistochemistry (Fig. 7D). Infiltrating granulocytes and macrophages were repeatedly seen in the tumors and may have been associated with necrosis. The robust therapeutic activity of the CV1 and 1E4-cIgGB combination was underscored by the fact that none of the mice in this treatment group had any microscopic evidence of lymphoma in the subcutis or in any lymphoid or visceral organs examined (Supplementary Fig. S7C), suggesting complete eradication of disease by this combination strategy.

Discussion

Lymphoma is responsible for significant morbidity and mortality in pet dogs, bearing remarkable similarity to human non-Hodgkin lymphoma and providing clinically realistic opportunities to explore combinatorial strategies and dosing regimens that may translate to human disease. In this study, we explored the relationship between the CD47/SIRP α axis and anti-CD20 immunotherapy in canine B-cell lymphoma. Agents targeting CD47, an immune checkpoint, show great potential for multiple types of human cancer in preclinical models and are now under investigation in human clinical trials (10, 11, 19, 20, 30). We identified high-affinity SIRP α variants (11) as a class of therapeutics that potentially antagonize canine CD47. CV1-hIgG4 stimulated phagocytosis of canine lymphoma cells *in vitro* and produced cures in a significant proportion of animals when used as a single agent in a xenograft mouse model of canine lymphoma. Therefore, our data support the evolutionary conservation of structure and function of the CD47/SIRP α axis in the canine system.

Passive immunotherapies with tumor-specific antibodies have become the standard of care for many human cancers, particularly the use of antibodies to CD20 for B-cell lymphoma. Antibody therapies are now emerging for canine cancer (1). The CD47/SIRP α axis limits macrophage responses to therapeutic antibodies, and the combination of CD47-blocking therapies with anti-CD20 shows remarkable synergy for human non-Hodgkin lymphoma (10, 11). Therefore, we investigated the combination of CD47-blocking therapies with 1E4-cIgGB, a canine-specific anti-CD20 under development for canine lymphoma (9). We found that 1E4-cIgGB can act via multiple effector mechanisms, including CDC and ADP, suggesting favorable properties for passive immunotherapy of canine B-cell lymphoma. We observed a therapeutic effect using 1E4-cIgGB as a single agent in a model of aggressive, intra-abdominal canine lymphoma. Furthermore, when combined with CD47-blocking therapies, we observed synergy *in vitro* and *in vivo*. We found that the combination of CV1 monomer and 1E4-cIgGB elicited cures in 100% of mice bearing canine lymphoma,

whereas nearly all mice in the single agent groups exhibited evidence of disease. Therefore, our findings suggest that the combination with CD47-blocking therapies could augment the potential benefits of 1E4-cIgGB for canine lymphoma.

The CD47/SIRP α axis has been best characterized in the context of macrophage activation. We do not exclude the possibility that other SIRP α ⁺ myeloid cells might contribute to the efficacy observed in our models, as granulocytes and myeloid dendritic cells respond to CD47-blocking therapies (15, 48). Moreover, CD47-blockade can initiate antigen presentation that stimulates adaptive immune responses against tumors (48-50) and targeting CD47 might directly activate T cells or NK cells (51-53). Although lymphocytes are not necessary for a therapeutic response in xenograft models, they could yield greater efficacy in immunocompetent settings and will be important to evaluate in future veterinary studies.

We did not observe significant differences between CD47 expression on healthy canine leukocytes and primary lymphoma cells. However, CD47 expression alone is not sufficient to predict therapeutic efficacy or toxicity (46). In immunocompetent mice, a therapeutic window for targeting CD47 on tumors exists despite CD47 expression on normal cells (20, 48, 54). In nonhuman primates, therapeutic dosing of CD47-blocking agents can be achieved while limiting side effects (11, 30). IL10 appears to prevent macrophages from attacking healthy cells when the CD47/SIRP α interaction is disrupted (55); other factors that regulate the response to treatment likely depend on a balance between pro-phagocytic and other inhibitory signals, and the identification of these signals remains an active area of investigation.

Nevertheless, the strategy of combining CV1 with an opsonizing antibody is designed to maximize the therapeutic window for targeting CD47. In contrast to antibodies to CD47 or CV1-hIgG4, CV1 blocks CD47 without contributing a pro-phagocytic stimulus. CV1 does not stimulate phagocytosis by itself, but instead lowers the threshold for phagocytosis in the presence of an opsonizing antibody (11, 45-47). The combination of CV1 with 1E4-cIgGB enables targeting of CD47 without toxicity, since the anti-CD20 agent confers specificity.

The main toxicity from the use of CD47-blocking reagents is reduced red blood cell mass. Moderate anemia has been observed in mice and cynomolgus monkeys treated with anti-CD47 and high-affinity SIRP α -Fc fusion proteins (11, 20, 30). However, the anemia is transient and dosing strategies have been developed to reduce this toxicity (30). To reduce possible immunogenicity, chimeric SIRP α -Fc fusion proteins could be engineered by substituting the human Fc for an active or inactive canine Fc region (9). Nonetheless, immunogenicity may be reduced when used in combination with an agent such as 1E4-cIgGB that causes B cell depletion (9).

We focused on the CLBL-1 cell line as a model since it is the only known canine cell line that faithfully represents DLBCL, reproducibly forms tumors, and retains its phenotype in the xenotransplantation setting (39). Although primary canine DLBCL xenografts have been described (2), these tumors only form when implanted intraperitoneally into conditioned NSG mice (2) and their variability in growth makes therapeutic evaluation challenging. The CLBL-1 model demonstrated proof-of-concept for combined targeting of CD47 and CD20

at the biological level. Therefore, we believe the most informative and relevant next step of this research endeavor will be clinical studies in dogs with spontaneous disease.

We found that CD47 is widely expressed on multiple types of canine cancer. Therefore, as with the human CD47/SIRP α interaction, the principles established by this study may be generalizable to other types of canine cancer and other canine antibodies to tumors. A popular and successful strategy in immuno-oncology is to combine treatments; CD47-blocking therapies may thus complement emerging treatment modalities using passive immunotherapy for canine diseases (56). Furthermore, anti-CD20 is used for inflammatory or autoimmune disorders, and similar spectrums of disease exist in canine models that could also benefit from B-cell depletion in combination with CD47-blocking therapies. Overall, our findings indicate that CD47-blockade alone and in combination with anti-CD20 may be an effective strategy for treating canine lymphoma. These findings justify further evaluation in the veterinary translational setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The primary structures of CD47 and SIRPa are evolutionarily conserved

A Alignment of the extracellular domain of CD47 from humans and mice with the predicted canine CD47 protein (NM_001080721.1). Amino acids shaded in yellow represent substitutions relative to the human sequence. Numbers indicate amino acid position of mature human CD47 protein. **B** Phylogenetic tree showing evolutionary distances between the extracellular domain of human, canine, and mouse CD47. **C** Alignment of the N-terminal extracellular domain of SIRPa from humans and mice with the predicted canine domain (XP_005634938.1). Human SIRPa is polymorphic and the predominant two

variants are depicted. Mouse strains also exhibit allelic variation, with two alleles depicted (C57BL/6 and NOD) that differ in their binding affinity for human CD47. Amino acids shaded in yellow represent substitutions relative to the human SIRP α variant 1 sequence. The secondary structure and variable immunoglobulin domain loops are indicated by black boxes as previously described. Numbers indicate amino acid position of mature human SIRP α variant 1 protein. **D** Phylogenetic tree showing evolutionary distances between the N-terminal extracellular domain of human, canine, and mouse SIRP α alleles.

Figure 2. Canine CD47 and SIRPα biochemically interact and exhibit conservation of function

A Diagram showing yeast display strategy with canine or mouse SIRPα variants expressed as a fusion to Aga2p, which is displayed on the surface of yeast via disulfide bonds to Aga1p. Canine CD47-Fc was used to examine binding. **B** Representative histograms showing binding of canine CD47-Fc (3.16 μ M) to yeast displaying canine SIRPα on their surface as analyzed by flow cytometry. Canine CD47 exhibited pH sensitivity during purification, and protein eluted at pH 4.0 (blue) exhibited greater binding activity than that eluted at pH 3.0 (red). **C** Binding of varying concentrations of canine CD47-Fc to yeast

displaying canine SIRP α . Binding assessed as geometric mean fluorescence intensity (MFI) as measured by flow cytometry. Data represent mean $\pm$ SD of three independent titration series analyzed simultaneously as one experiment. **D** Representative histogram depicting binding of canine CD47-Fc to yeast displaying mouse SIRP α alleles from C57BL/6 or NOD mice. **E** Binding of varying concentrations of canine CD47-Fc to yeast expressing NSG SIRP α on their surface. Data represent mean $\pm$ SD for three independent titration series analyzed simultaneously as one experiment.

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Figure 3. CV1-hIgG4 is a potent antagonist of canine CD47

A Binding of Hu5F9-G4 and CV1-hIgG4 to the surface of yeast displaying the extracellular domain of canine CD47. Binding was detected with an anti-human IgG secondary antibody and evaluated by flow cytometry. The EC_{50} of binding for CV1-hIgG4 was 741 pM (56.33 ng/mL). Data represent mean $\pm$ SD of three independent titration series analyzed simultaneously as one experiment. **B** Blocking of canine CD47 on the surface of yeast. Yeast displaying canine CD47 on their surface were incubated with fluorophore-labeled canine SIRPα tetramers in competition with the indicated CD47-blocking reagents. Binding of canine SIRPα was inhibited by CV1-hIgG4 with an IC_{50} of 2.92 nM (405.2 ng/mL). No

appreciable blockade was observed with Hu5F9-G4 even at the maximal concentration tested (10 µg/mL). Data represent mean ± SD of three independent titration series analyzed simultaneously as one experiment. **C** Phagocytosis assays performed using NSG macrophages and CLBL-1 canine lymphoma cells labeled with calcein AM. Cells were treated with anti-CD47 (10 µg/mL) or CV1-hIgG4 (5 µg/mL). Phagocytosis was evaluated by flow cytometry as the percentage of F4/80⁺ macrophages that engulfed lymphoma cells and became positive for calcein AM fluorescence as previously reported. Representative flow cytometry plots with the percentage of phagocytic macrophages (blue) indicated for each treatment. **D** Summary of NSG phagocytosis assays showing mean ± SD for three independent samples analyzed as one experiment. Data are representative of at least two independent experiments using CV1-hIgG4. **E** Summary of canine macrophage phagocytosis assays showing mean ± SD for three independent canine blood donors each performed in triplicate and analyzed simultaneously as one experiment. ** $P < 0.01$, *** $P < 0.01$, **** $P < 0.0001$ for the indicated comparisons by one-way ANOVA with Tukey corrections for multiple comparisons.

Figure 4. CV1-hIgG4 is effective as a single agent for the treatment of canine lymphoma *in vivo*
A Representative images of canine lymphoma distribution on day 16 post-intravenous engraftment of 100,000 GFP-luciferase⁺ CLBL-1 cells. Top images show non-tumor bearing NSG mice, bottom images show organs 16 days post-engraftment with 100,000 CLBL-1 cells. For each organ, images are shown for white light and 488 nm excitation (GFP). Scale bar represents 5 mm. **B-D** NSG mice were engrafted with CLBL-1 canine lymphoma cells via the retro-orbital sinus. After 24 hours, mice were randomized and treated with daily injections of vehicle control (PBS) or 250 μ g CV1-hIgG4 daily for two weeks ($n = 7-8$ mice

per cohort). All mice were treated simultaneously and analyzed as one experiment. **B** Representative images of mice one week after discontinuing treatment. Tumor involvement was observed in the orbits of control mice but not mice treated with CV1-hIgG4. PBS control animals exhibit representative orbital tumors scores of 1 (top), 2 (middle), 3 (bottom). **C** Quantification of orbital tumor scores. Points represent measurements from individual mice, bars represent mean. **D** Survival of mice bearing disseminated CLBL-1 canine lymphoma. Black arrow indicates the last day of treatment. **E-F** NSG mice were engrafted with GFP-luciferase⁺ CLBL-1 canine lymphoma cells and treatment was initiated on day 6 post-engraftment. All mice were treated simultaneously and analyzed as one experiment. **E** Bioluminescence imaging of mice six days post-engraftment with GFP-luciferase⁺ CLBL-1 cells revealing disseminated tumor burden prior to initiating treatment. **F** Summary of total flux measured by bioluminescence imaging after one week of daily treatment with vehicle control or CV1-hIgG4. Points represent measurements from individual mice ($n = 7$ mice per cohort), bars represent mean $\pm$ SD. ns = not significant, $*P < 0.05$, $**P < 0.01$ by two-tailed t-test; $***P = 0.0002$ by Mantel-Cox test.

Figure 5. Anti-canine CD20 (1E4) exhibits anti-tumor activity *in vitro*

A Direct cytotoxicity assay using CLBL-1 cells cultured with 10 μ g/ml mIgG2a, 1E4-mIgG2a, or 1E4-cIgGB for 72 hours. Live cell numbers were analyzed by MTS assay as indicated by optical density (OD) measured at 490 nm. Experiments were performed in triplicate. **B** Complement-dependent cytotoxicity assay using CLBL-1 cells cultured with 10 μ g/ml mIgG2a, 1E4-mIgG2a, or 1E4-cIgGB in the presence of rabbit complement or heat-inactivated rabbit complement for 3 hours. Viability was normalized to the number of cells in the complement only condition using the MTS assay. Bars are the mean $\pm$ SD for three independent experiments each performed in triplicate. ns = not significant, **** $P < 0.0001$ by two-way ANOVA with Tukey corrections for multiple comparisons. **C** Antibody-dependent phagocytosis assay using CFSE-labeled CLBL-1 cells that were co-cultured with IFN- γ primed J774 cells in the presence of 10 μ g/ml control mIgG2a or 1E4-mIgG2a for 2 hours. Cells were harvested and stained with APC-conjugated F4/80 antibody and analyzed by flow cytometry. Representative flow cytometry plots are shown with the percent of F4/80⁺ J774 cells that engulfed CFSE⁺ tumor cells per total F4/80⁺ population (left). The mean $\pm$ SD for three independent experiments each performed in triplicate is shown above the plots. Phagocytosis was normalized to the no treatment condition (right). Bars represent the mean change $\pm$ SD. ns = not significant, * $P < 0.05$ by one-way ANOVA with Tukey corrections for multiple comparisons.

Figure 6. 1E4-cIgGB reduces intra-abdominal canine lymphoma burden

NSG mice (n = 4 for no treatment cohort, n = 12 for antibody treated cohorts) were injected intraperitoneally with 50,000 CLBL-1 cells and treated with the indicated antibodies (350 µg/mouse, twice per week) beginning three days post tumor inoculation. All mice were treated simultaneously and analyzed as one experiment. **A** Change in weight of mice on day 27 relative to day 5. Points represent individual mice, bars represent mean ± SD. ns = not significant, **P* < 0.05 by one-way ANOVA with Tukey corrections for multiple comparisons. **B** Representative photomicrographs of tumor engraftment in spleens of NSG mice as stained

with hematoxylin-eosin (HE). Asterisks indicate representative areas of tumor involvement. Magnification 100× (upper panels) and 400× (lower panels). **C** Immunohistochemical analysis of CD20 expression of a representative spleen from the Rtx-cIgGB treated group. **D** Photomicrographs of histological specimens (HE) depicting representative tumor necrosis scores. Magnification 20× (upper panels) and 100× (lower panels). **E** Summary of tumor necrosis scores among the treatment groups. Points represent measurements from individual mice, bars represent mean $\pm$ SD. ns = not significant, * $P < 0.05$ by one-way ANOVA with Tukey corrections for multiple comparisons.

Figure 7. CD47 blockade synergizes with anti-canine CD20 to ablate canine lymphoma *in vivo*

A Phagocytosis assay using CFSE-labeled CLBL-1 cells co-cultured with primary NSG macrophages in the presence of the indicated therapies for 2 hours. Cells were harvested and stained by APC-conjugated anti-F4/80 antibody and analyzed by flow cytometry. Phagocytosis was determined by the percent of macrophages that had engulfed a tumor cells, thereby becoming CFSE⁺. Data represent mean $\pm$ SD from three replicates analyzed simultaneously as one experiment. ns = not significant, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ by two-way ANOVA with Tukey corrections for multiple comparisons. **B** NSG mice

(n = 9-10 per cohort) were injected subcutaneously with 40,000 CLBL-1 cells and treated with the indicated therapies (anti-CD20: 250 µg/mouse, 3 days/week; CV1: 200 µg/mouse/day) beginning 24-hours post tumor inoculation. Tumor volume measurements over time are depicted, with points indicating measurements from individual mice and bars indicating mean values. All mice were treated simultaneously and analyzed as one experiment. ns = not significant, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ by two-way ANOVA with Tukey corrections for multiple comparisons. **C** Survival of tumor-bearing mice 60 days engraftment. ** $P < 0.01$, *** $P < 0.001$ by Mantel-Cox test with Bonferroni corrections for multiple comparisons. **D** Immunohistochemistry of a representative tumor stained for H&E, CD3, CD20, and CD79a.