**SUPPLEMENTARY DATA**

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

**AUTHORS:** Kipp Weiskopf, Katie L. Anderson, Daisuke Ito, Peter J. Schnorr, Hirotaka Tomiyasu, Aaron M. Ring, Kristin Bloink, Jem Efe, Sarah Rue, David Lowery, Amira Barkal, Susan Prohaska, Kelly M. McKenna, Ingrid Cornax, Timothy D. O’Brien, M. Gerard O’Sullivan, Irving L. Weissman, and Jaime F. Modiano

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**SUPPLEMENTARY TABLES**

**Supplementary Table S1. Similarity between CD47 variants across species.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **CD47 Homology** | | | | |
| Variant | Comparison | Identities | Positives | Gaps |
| Canine CD47 | Human CD47 | 66% (75/114) | 79% (91/114) | 1% (2/114) |
| Canine CD47 | Mouse CD47 | 58% (65/112) | 72% (81/112) | 0% (0/112) |
| Human CD47 | Mouse CD47 | 61% (69/114) | 75% (86/114) | 1% (2/114) |

**Supplementary Table S2. Similarity between SIRPα variants across species.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **SIRPα Homology** | | | | |
| Variant | Comparison | Identities | Positives | Gaps |
| Canine SIRPα | Human SIRPα variant 1 | 78% (93/119) | 85% (102/119) | 0% (1/119) |
| Canine SIRPα | Human SIRPα variant 2 | 75% (88/118) | 84% (100/118) | 0% (0/118) |
| Canine SIRPα | Mouse SIRPα C57BL/6 | 68% (80/118) | 79% (94/118) | 1% (2/118) |
| Canine SIRPα | Mouse SIRPα NOD | 68% (78/114) | 82% (94/114) | 0% (0/114) |
| Human SIRPα variant 1 | Human SIRPα variant 2 | 89% (106/119) | 90% (108/119) | 0% (1/119) |
| Human SIRPα variant 1 | Mouse SIRPα C57BL/6 | 65% (77/119) | 78% (93/119) | 0% (1/119) |
| Human SIRPα variant 1 | Mouse SIRPα NOD | 66% (76/115) | 80% (93/115) | 0% (1/115) |
| Human SIRPα variant 2 | Mouse SIRPα C57BL/6 | 66% (79/119) | 83% (99/119) | 1% (2/119) |
| Human SIRPα variant 2 | Mouse SIRPα NOD | 68% (78/114) | 86% (99/114) | 0% (0/114) |
| Mouse SIRPα C57BL/6 | Mouse SIRPα NOD | 84% (98/116) | 89% (104/116) | 1% (2/116) |

**Supplementary Table S3**. **Summary of tumor necrosis scores following treatment with the indicated therapies.** Data depict percent of mice, with number of mice per cohort indicated in parentheses.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Tumor necrosis score** | | | | |
|  | 0 | 1 | 2 | 3 |
| No treatment | 75% (3/4) | 25% (1/4) | 0% (0/4) | 0% (0/4) |
| Rtx-cIgGB | 64% (7/11) | 18% (2/11) | 18% (2/11) | 0% (0/11) |
| 1E4-cIgGB | 11% (1/9) | 44% (4/9) | 22% (2/9) | 22% (2/9) |

**SUPPLEMENTARY FIGURE LEGENDS**

**Supplementary Fig. S1.** **Recombinant production and evaluation of canine SIRPα protein.** **A** Coomassie-stained SDS-PAGE gel monitoring the purification of *Canis familiaris* SIRPα (cfSIRPα). CfSIRPα was expressed in *E. coli* with an N-terminal maltose binding protein (MBP) tag and C-terminal biotin acceptor peptide (BAP) and 8x histidine tags. The MBP fusion protein was first purified by Ni-NTA chromatography and MBP was removed by digestion with 3C protease. Cut cfSIRPα was purified from MBP with a second Ni-NTA column and then biotinylated using BirA ligase. **B** Size exclusion chromatograph of biotinylated cfSIRPα. Biotinylated cfSIRPα was further purified using an S75 gel filtration column to remove soluble protein aggregates and small molecular weight contaminants. Protein absorbance (UV280) is plotted as a function of volume eluate (mL).

**Supplementary Fig. S2. CD47 expression on canine cancer samples.** **A** Binding of CD47 antagonists to CLBL-1 canine lymphoma cells over varying concentrations. Binding was detected by flow cytometry using an Alexa Fluor 647-conjugated anti-human IgG secondary antibody, and data represent geometric mean fluorescence intensity (Geo. MFI) of binding. WT-hIgG4 indicates wild-type human SIRPα allele 2 as a fusion to human IgG4 as previously described ([1](#_ENREF_1)). Data represent mean ± SD of three independent titration series analyzed simultaneously as one experiment. **B** CD47 surface expression on non-malignant leukocyte subsets from healthy canine donors (n = 3) and primary canine lymphoma cells (n = 3). Values were measured by flow cytometry and are depicted as mean fluorescence intensity (MFI). Points represent values from individual donors, with mean ± SD depicted. CD47 expression was detected using Hu5F9-G4 conjugated to Alexa Fluor 488. Expression for all samples analyzed simultaneously in a single experiment. **C** CD47 and CD20 mRNA expression in primary B cell lymphoma samples. The fragments per kilobase per million reads (FPKM) values for CD47, CD20, and PSMB2 were extracted from next generation RNA sequencing data for enriched primary DLBCL cells from 24 dogs ([2](#_ENREF_2), [3](#_ENREF_3)). Expression was normalized to expression of the housekeeping gene, PSMB2. **D** Binding of CD47 antagonists to OSCA-40 canine osteosarcoma cells. Cells were incubated with 10 μg/mL Hu5F9-G4 or approximately 5 μg/mL WT-hIgG4 or CV1-hIgG4. Binding was detected as described above. **E** CD47 surface expression across various canine cancer samples. Lymphoma samples examined included a primary canine B-cell lymphoma sample and CLBL-1 cells. Solid tumor specimens examined included osteosarcoma (OSCA-40, OSCA-78), hemangiosarcoma (COSB, Emma), melanoma (TLM1, CMGD2, CMGD5), and glioblastoma (MAC, CANDY). Expression of CD47 was detected by flow cytometry. Cells of each line were stained using Hu5F9-G4 conjugated to Alexa Fluor 488 or an isotype control antibody. Representative histograms display binding with isotype control (black lines) or Hu5F9-G4 conjugated to Alexa Fluor 488 (blue lines).

**Supplementary Fig. S3. Microscopic visualization demonstrates macrophage engulfment of CLBL-1 cells.** CLBL-1 cells were labeled with calcein AM and co-cultured with NSG macrophages in the presence of vehicle control (PBS) or CV1-hIgG4. After 2 hours of co-culture, macrophages were stained with APC-F4/80 and phagocytosis was evaluated by imaging flow cytometry using an Amnis ImageStreamX MKII. For each treatment,three independent replicates were pooled for imaging analysis performed in one experiment. **A** Plots showingthegated F4/80hi macrophage population, with subpopulations of calcein AM mid (blue) and calcein AM high (pink) macrophages depicted. Percentages indicate percentage of total macrophage population. **B** Representative image of macrophages without engulfed material (calcein AM negative). **C** Representative image of non-engulfed calcein AM-labeled CLBL-1 cell. **D** Representative images of calcein AM high macrophages following PBS control treatment, which predominantly contained macrophages with adherent calcein AM-positive material. **E** Representative images of calcein AM high macrophages following CV1-hIgG4 treatment, which predominantly contained macrophages with engulfed calcein AM-positive CLBL-1 cells. Some macrophages exhibited phagocytosis of more than one lymphoma cell (bottom panel). **F** Representative images of calcein AM mid macrophages following CV1-hIgG4 treatment, which contained macrophages with engulfed or adherent calcein AM-positive material. **B-F** Scale bar represents 10 μm.

**Supplementary Fig. S4. Canine macrophages respond to CD47-blocking therapies *in vitro*.** CD14+ monocytes were purified from canine blood samples and differentiated ex vivo to macrophages by culture in 10% autologous canine serum. **A** Representative images of canine macrophages by phase contrast microscopy. Arrow indicates occasional giant cells that were observed in culture. 200× magnification. **B** Representative histogram showing expression of CD14 on the surface of canine macrophages. **C** Macrophages were co-cultured with GFP+ human Raji lymphoma cells in the presence of vehicle control (PBS) or the indicated CD47-blocking reagents. Phagocytosis was evaluated by flow cytometry as the percentage of CD14+ canine macrophages that engulfed GFP+ lymphoma cells. Phagocytosis was evaluated in response to the indicated CD47-blocking therapies. Data represent mean ± SD for one canine blood donor performed in triplicate. ns = not significant, \*\*\*\*p<0.0001 by one-way ANOVA with Tukey corrections for multiple comparisons.

**Supplementary Fig. S5. 1E4 antibody binds canine CD20 extracellular domain 2. A** Binding of 1E4 to canine CD20 extracellular domain 2 as assessed by ELISA. 1E4-cIgGB binds canine CD20 extracellular domain 2 expressed as a human Fc fusion protein (cCD20 ECD2-hFc), whereas Rtx-cIgGB or secondary antibody alone do not. The EC50 is indicated for 1E4-cIgGB binding. Data represent mean ± SD for three independent titration series analyzed simultaneously as one experiment **B** Analysis of 1E4-cIgGB binding to canine CLBL-1 lymphoma cells. Binding was detected using a secondary anti-cIg antibody conjugated with FITC. Shaded area: no antibody, black line: secondary antibody alone, red line: 1E4-cIgGB plus secondary antibody.

**Supplementary Fig. S6. CV1 does not inhibit proliferation of CLBL-1 cells *in vitro*.** CLBL-1 cells were cultured without treatment or in the presence of saturating concentrations of CV1 (1 μM). Cells concentrations were counted daily. Data represent mean ± SD for three independent replicates. Data are representative of two independent experiments. ns = not significant by two-tailed t test.

**Supplementary Fig. S7. Combination therapy with CD47-blockade and anti-CD20 antibodies produces *in vivo* efficacy with no overt toxicity. A** NSG mice (n = 9-10 per cohort) were injected subcutaneously with 40,000 CLBL-1 cells and treated with the indicated therapies (anti-CD20 antibodies: 250 µg/mouse, 3 days/week; CV1: 200 µg/mouse/day) beginning 24-hours post tumor inoculation. Body weights of mice (g) were measured over the course of treatment. **B** Tumor growth curves over time. Tumor volumes were measured on the same day for all groups and are shown staggered for clarity. Points indicate measurements from individual mice, and bars indicating mean values. Trend lines indicate median growth over time during indicated period. Dropout is due to mortality (see Fig. 7C). **C** Analysis of tumor burden by microscopic dissection on the final day of the experiment (day 68 post-engraftment). With the number of tumor-free mice and total number of mice indicated for each cohort. All mice were treated simultaneously and analyzed as one experiment. p <0.0001 by chi-square (χ2 = 40.35, df =4).

**SUPPLEMENTARY MATERIALS**

**Engineered Protein Sequences:**

Canine CD47-Fc (for binding analysis): this construct contains an IL-2 signal sequence followed by the N-terminal domain of canine CD47 (C15G) fused to human IgG4 (S228P).

MYRMQLLSCIALSLALVTNSGSQLIFNITKSVEYTAGNESVIIPCFVNNVEATNINEMYVKWKFRGKDIFTFDGAVQKTTHGDKFKSTKIVPQKLLNGIASLEMSKEEAVVGNYTCEVTELSREGETIIELKYRIVSWAAAPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSPGK

MBP-Canine SIRPα-BAP (for tetramer production): this construct contains MBP with a rhinovirus 3C protease site followed by the N-terminal domain of canine SIRPα, then a biotin acceptor peptide sequence and an 8x-histidine tag.

MKIKTGARILALSALTTMMFSASALAKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNNNNNNNNNNLGLEVLFQGPGSEAELQVIQPEKSVSVAAGETATLRCTLTSLIPVGKVEWFRGTGPGRELIFHFKGGHFPRVTNVSDSTKRNNTDFSIRISNITPADTGTYYCVKFQKGNPDVELKSGPGTLTSLIPVGKVEWFRGTGPGRELIFHFKGGHFPRVTNVSDSTKRNNTDFSIRISNITPADTGTYYCVKFQKGNPDVELKSGPGTLVTVSAKPSAAAGGGLNDIFEAQKIEWHEHHHHHHHH

Canine CD47-aga2p (for yeast display): this construct contains the aga2p signal sequence then the N-terminal domain of canine CD47 (C15G) fused to a Myc tag then aga2p.

MQLLRCFSIFSVIASVLAQLIFNITKSVEYTAGNESVIIPCFVNNVEATNINEMYVKWKFRGKDIFTFDGAVQKTTHGDKFKSTKIVPQKLLNGIASLEMSKEEAVVGNYTCEVTELSREGETIIELKYRIVSGSEQKLISEEDLGGGGSGGGGSGGGGSQELTTICEQIPSPTLESTPYSLSTTTILANGKAMQGVFEYYKSVTFVSNCGSHPSTTSKGSPINTQYVF

Aga2p-NOD SIRPα (for yeast display): this construct contains the aga2p signal sequence then aga2p followed by a Factor Xa protease site, a rhinovirus 3C protease site, then the N-terminal domain of NOD SIRPα and a Myc tag.

MQLLRCFSIFSVIASVLAQELTTICEQIPSPTLESTPYSLSTTTILANGKAMQGVFEYYKSVTFVSNCGSHPSTTSKGSPINTQYVFKDNSSTIEGRTRSGGGGSGGGGSGGGGSLEVLFQGPSGGSATRTEVKVIQPEKSVSVAAGDSTVLNCTLTSLLPVGPIRWYRGVGQSRQLIYSFTTEHFPRVTNVSDATKRSNLDFSIRISNVTPEDAGTYYCVKFQRGSPDTEIQSGGGTEVYVLAKPSAAASGGEQKLISEEDLLEI

Aga2p-C57BL/6 SIRPα (for yeast display): this construct contains the aga2p signal sequence then aga2p followed by a Factor Xa protease site, a rhinovirus 3C protease site, then the N-terminal domain of C57BL/6 SIRPα and a Myc tag.

MQLLRCFSIFSVIASVLAQELTTICEQIPSPTLESTPYSLSTTTILANGKAMQGVFEYYKSVTFVSNCGSHPSTTSKGSPINTQYVFKDNSSTIEGRTRSGGGGSGGGGSGGGGSLEVLFQGPSGGSKELKVTQPEKSVSVAAGDSTVLNCTLTSLLPVGPIRWYRGVGPSRLLIYSFAGEYVPRIRNVSDTTKRNNMDFSIRISNVTPADAGIYYCVKFQKGSSEPDTEIQSGGGTEVYVLAAASGGEQKLISEEDLLEI

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