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hu128.1, a humanized antibody targeting transferrin receptor 1, blocks erythroleukemia growth in xenograft mouse models

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Transferrin receptor 1 (TfR1), also known as CD71, is a transmembrane glycoprotein required for the cellular uptake of iron through its interaction with iron-bound transferrin (Tf).¹ In general, TfR1 is expressed at low levels in many types of normal cells;¹ however, TfR1 is expressed at high levels in rapidly proliferating cells and cells with a high need of iron, like erythroid progenitor cells that require large amounts for heme synthesis.^{1,2} TfR1 is overexpressed in multiple malignancies and is often associated with poor prognosis.¹ In fact, TfR1 has been considered as a universal cancer marker candidate.³

Erythroleukemia, or acute erythroid leukemia (AEL), is a rare and highly aggressive subtype of acute myeloid leukemia [AML (2-5% of AML cases)] with very poor prognosis, primarily characterized by immature erythroid progenitor cell (erythroblast) proliferation in the bone marrow.^{4,5} Erythroid progenitors express the highest levels of TfR1 in the body² which, combined with the vital role of TfR1 in cancer cell pathology and its extracellular availability, make TfR1 an attractive target for antibody-mediated therapy of this malignancy. A previous study identified a human anti-TfR1 IgG1 antibody (H7) that blocks Tf binding to TfR1 (a neutralizing antibody) and showed antitumor activity in nude mice bearing human erythroleukemic (ERY-1) cells implanted subcutaneously (s.c.).⁶

We previously developed mouse/human chimeric IgG1 and IgG3 antibodies (ch128.1/IgG1 and ch128.1/IgG3) and a humanized IgG1 (hu128.1) version targeting human TfR1 that do not interfere with ligand binding [Tf or hemochromatosis protein (HFE)], and thus, are non-neutralizing antibodies.⁷⁻¹¹ The ch128.1 antibodies show relevant antitumor efficacy in xenograft models of the human B-cell malignancies multiple myeloma (MM) and AIDS-related non-Hodgkin lymphoma (AIDS-NHL).^{1,8,9,11} The hu128.1 antibody also shows antitumor activity in xenograft models of human MM and AIDS-NHL.^{10,11} Here we evaluate the efficacy of hu128.1 in xenograft mouse models of erythroleukemia using human ERY-1 cells xenografted in SCID-

Beige mice. This is the first study to explore the efficacy of ch128.1 or hu128.1 antibodies in a mouse model of any form of AML.

Cell line: The human erythroleukemic cell line ERY-1¹² was a kind gift from Michel Arock (Pitié-Salpêtrière University Hospital, Paris, France). Cells were grown in RPMI medium (Thermo Fisher Scientific, Inc., Waltham, MA) containing 10% fetal bovine serum (FBS: Novus Biologicals, LLC, Centennial, CO) and penicillin/streptomycin (Thermo Fisher Scientific, Inc.) in 5% CO₂ at 37°C.

Recombinant antibodies: The humanized antibody (hu128.1) contains the human γ 1 and κ constant regions and targets human TfR1. We used the hu128.1 variant H6L7, which shares the binding properties of ch128.1/IgG1 and exhibits superior thermal stability whose development and expression in CHO cells was previously described.^{10, 11} A mouse/human chimeric IgG1/ κ antibody specific for the hapten dansyl (5-dimethylamino naphthalene-1-sulfonyl chloride) was produced in murine myeloma cells as described^{9, 11} and used as an isotype (IgG1) control.

Flow cytometry: ERY-1 cells (5×10^5) were incubated with 2 μ g of hu128.1 or the IgG1 isotype control in RPMI medium containing 10% FBS (media) for 1 hour on ice. To demonstrate TfR1 binding specificity, one sample was incubated with 2 μ g hu128.1 in the presence of 5 μ g soluble TfR1 (sTfR1, Sino Biological US, Inc., Wayne, PA). Cells were washed with media and were then incubated with a phycoerythrin (PE)-conjugated goat F(ab')₂ anti-human κ antibody (Southern Biotech, Birmingham, AL) for 30 minutes on ice. Cells were washed in autoMACS Running Buffer (Miltenyi Biotec, Gaithersburg, MD) and fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) diluted in PBS. Samples were resuspended in autoMACS Running Buffer and analyzed on a BD LSRFortessa™ X-20 SORP (BD Biosciences,

San Jose, CA). Histograms were created in FSC Express Version 3 (De Novo Software, Pasadena, CA).

In vivo efficacy studies: All animal research was approved by the UCLA Chancellor's Animal Research Committee (Institutional Animal Care and Use Committee) and was conducted in accordance with guidelines for the housing and care of laboratory animals of the National Institutes of Health and Association for Assessment and Accreditation of Laboratory Animal Care International. Female C.B-17 SCID-Beige mice (7-10 weeks old) were obtained and housed in the Defined Flora Mouse Core Facility in the Department of Radiation Oncology at UCLA. Mice were challenged s.c. (right flank) with 2×10^6 ERY-1 cells in Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific, Inc.). Mice were treated intravenously (i.v.) via the tail vein with 400 μg hu128.1 or the IgG1 negative control antibody in phosphate-buffered saline (PBS; Thermo Fisher Scientific, Inc.) on Day 2 or three times when tumors became palpable (~3-5 mm in diameter). In some cases, mice were treated with buffer alone as a negative control group. For Day 2 treatments, mice were randomized into treatment groups. For treatments that started after tumor growth, mice were put into groups with equal tumor size distribution. Tumor growth was monitored using a caliper and tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$. Survival was determined as the time from tumor challenge to the time tumor reached 1.5 cm in diameter. For the disseminated disease model, half of the mice were exposed to 3 gray (Gy) whole-body, sublethal irradiation (GammaCell40 irradiator ^{137}Cs , Best Theratronics, Ltd., Ottawa, ON, Canada), while the other half were left non-irradiated. The next day, mice were randomized into treatment groups and challenged i.v. with 5×10^6 ERY-1 cells via the tail vein. Mice were then treated with 100 μg hu128.1 or PBS alone. Survival was determined as the time from tumor challenge to development of hind-limb paralysis or moribundity. Tumor growth rate graphs and Kaplan-Meier survival plots were generated in GraphPad Prism, Version 9 (GraphPad Software, Inc., La Jolla, CA). Differences in survival were determined using the log-

rank test in GraphPad Prism and differences in tumor growth rates were determined using the rate-based T/C method.¹³ Antibody dosing was based on previous studies using ch128.1/IgG1 in xenograft mouse models of AIDS-NHL and MM using SCID-Beige mice.⁹⁻¹¹

The hu128.1 antibody binds ERY-1 cells and this binding was abrogated by incubation with soluble TfR1 [Figure 1], indicating that hu128.1 binds to TfR1 on the surface of ERY1 cells. Since ERY-1 cells have been previously inoculated s.c. into mice,⁶ we first evaluated the ability of hu128.1 to block s.c. tumor growth. When animals are treated with a single 400 µg dose on Day 2 after tumor implantation (a low-tumor burden model), the antibody completely blocked tumor growth [Figure 2A]. The antibody (given in three 400 µg doses every 2-3 days) slows the growth in a high-tumor burden model [Figure 2A]. However, despite the fact that s.c. tumors did not regrow after cessation of treatment, all animals succumbed to the disease due to the development of systemic disease. This result indicates that the ERY-1 cells are metastatic when implanted s.c. into SCID-Beige mice. In a second independent study, a single 400 µg dose completely blocked tumor growth compared to mice treated with the negative control IgG1 antibody [Figure 2B]. The antibody also slowed the growth in a high-tumor burden model [Figure 2B]. In 4 out of the 5 hu128.1-treated mice, tumors completely regressed. Out of these 4 mice, 2 had tumors regress, but later regrew s.c. tumors (one on Day 59 and the other on Day 79). Two remained tumor-free locally, but one of those developed hind-limb paralysis and was euthanized on Day 100. The last mouse remained tumor-free and survived for the duration of the study (to Day 160).

Since the above studies indicated that the ERY-1 cells had metastatic potential, we administered the cells i.v. and evaluated the therapeutic efficacy of hu128.1 against disseminated disease. We also wanted to determine the effect of whole-body irradiation prior to tumor implantation on ERY-1 tumor growth. Therefore, half of the mice were irradiated with 3 Gy

whole-body irradiation on the day prior to the challenge with ERY-1 cells. Two days after tumor challenge, mice were treated with 100 µg hu128.1. We found that irradiation is not required for tumor engraftment. However, irradiated mice developed disease faster with a median survival of 30 days compared to 40 days in non-irradiated mice [Figure 3], which is consistent with better bone marrow engraftment of malignant cells due to the irradiation. Treatment with hu128.1 prolonged survival in both cases; however, non-irradiated mice treated with hu128.1 showed a stronger antitumor response and never developed disease during the course of the study [Figure 3]. This is the first demonstration that ERY-1 cells can be used in a disseminated mouse model of erythroleukemia when cells are administered i.v.

In summary, our results show that the hu128.1 antibody provides protection against xenografted ERY-1 cells in SCID-Beige mice in both the local and disseminated tumor models. As expected, this protection is stronger when the tumor burden is lower. Previous studies with the ch128.1 antibodies used in xenograft models of MM in SCID-Beige mice show that the Fc region of the antibody is critical for the antitumor activity.^{1,8,9} Additionally, previous *in vitro* studies show that some malignant B cells are sensitive to treatment with a ch128.1 antibody through the mistrafficking and degradation of TfR1 leading to iron starvation, which is not observed in cells that are resistant.¹ However, the ch128.1 antibodies have antitumor activity in mouse models using both, cells that are sensitive to these iron starvation effects as well as those that are not sensitive.¹ Further studies are needed to explore the antitumor mechanism of hu128.1 observed here. Moreover, since hu128.1 does not cross-react with mouse TfR1,¹⁴ toxicities of hu128.1 treatment in our studies could not be evaluated. Additional studies in relevant animal models are required to evaluate potential toxicities of hu128.1 treatment. Studies using the neutralizing anti-TfR1 human IgG1 antibody (H7) in a scFv₂-Fc format that cross-reacts with mouse TfR1 demonstrated protection against ERY-1 s.c. tumors with no apparent toxicity.⁶ Furthermore, studies using the neutralizing anti-TfR1 human IgG1 antibody PPMX-T003,

previously known as JST-TFR09, showed only moderate anemia in non-human primates¹⁵ and in healthy human volunteers¹⁶, leading to an ongoing Phase I Clinical Trial in patients affected with the chronic erythroid myeloproliferative neoplasm polycythemia vera (NCT05074550). Taken together, these results suggest that hu128.1, a non-neutralizing antibody, has potential as a therapy for erythroleukemia. Further studies are warranted to explore this possibility.

References

1. Candelaria PV, Leoh LS, Penichet ML, Daniels-Wells TR. Antibodies Targeting the Transferrin Receptor 1 (TfR1) as Direct Anti-cancer Agents. *Front Immunol.* 2021;12:607692.
2. Richard C, Verdier F. Transferrin Receptors in Erythropoiesis. *Int J Mol Sci.* 2020;21(24):9713.
3. Essaghir A, Demoulin JB. A minimal connected network of transcription factors regulated in human tumors and its application to the quest for universal cancer biomarkers. *PLoS One.* 2012;7(6):e39666.
4. Alexander C. A History and Current Understanding of Acute Erythroid Leukemia. *Clin Lymphoma Myeloma Leuk.* 2023;23(8):583-588.
5. Cervera N, Guille A, Adelaide J, et al. Erythroleukemia: Classification. *EJHaem.* 2023;4(2):450-453.
6. Neiveyans M, Melhem R, Arnoult C, et al. A recycling anti-transferrin receptor-1 monoclonal antibody as an efficient therapy for erythroleukemia through target up-regulation and antibody-dependent cytotoxic effector functions. *MAbs.* 2019;11(3):593-605.
7. Rodriguez JA, Helguera G, Daniels TR, et al. Binding specificity and internalization properties of an antibody-avidin fusion protein targeting the human transferrin receptor. *J Control Release.* 2007;124(1-2):35-42.
8. Leoh LS, Kim YK, Candelaria PV, Martinez-Maza O, Daniels-Wells TR, Penichet ML. Efficacy and Mechanism of Antitumor Activity of an Antibody Targeting Transferrin Receptor 1 in Mouse Models of Human Multiple Myeloma. *J Immunol.* 2018;200(10):3485-3494.

9. Daniels-Wells TR, Candelaria PV, Leoh LS, Nava M, Martinez-Maza O, Penichet ML. An IgG1 Version of the Anti-transferrin Receptor 1 Antibody ch128.1 Shows Significant Antitumor Activity Against Different Xenograft Models of Multiple Myeloma: A Brief Communication. *J Immunother.* 2020;43(2):48-52.
10. Penichet ML, Daniels-Wells TR, Candelaria PV, Almagro JC. Compositions and methods for transferrin receptor 1 targeting. International Patent Application No. PCT/US2020/059532. Filed on November 6, 2020. Publication Number: WO/2021/092482A1, Publication Date: May 14, 2021.
11. Daniels-Wells TR, Candelaria PV, Kranz E, et al. Efficacy of Antibodies Targeting TfR1 in Xenograft Mouse Models of AIDS-Related Non-Hodgkin Lymphoma. *Cancers (Basel).* 2023;15(6):1816.
12. Ribadeau Dumas A, Hamouda NB, Leriche L, et al. Establishment and characterization of a new human erythroleukemic cell line, ERY-1. *Leuk Res.* 2004;28(12):1329-1339.
13. Hather G, Liu R, Bandi S, et al. Growth rate analysis and efficient experimental design for tumor xenograft studies. *Cancer Inform.* 2014;13(Suppl 4):65-72.
14. Helguera G, Jemielity S, Abraham J, et al. An antibody recognizing the apical domain of human transferrin receptor 1 efficiently inhibits the entry of all new world hemorrhagic Fever arenaviruses. *J Virol.* 2012;86(7):4024-4028.
15. Shimosaki S, Nakahata S, Ichikawa T, et al. Development of a complete human IgG monoclonal antibody to transferrin receptor 1 targeted for adult T-cell leukemia/lymphoma. *Biochem Biophys Res Commun.* 2017;485(1):144-151.

16. Ogama Y, Kumagai Y, Komatsu N, et al. Phase 1 Clinical Trial of PPMX-T003, a Novel Human Monoclonal Antibody Specific for Transferrin Receptor 1, to Evaluate Its Safety, Pharmacokinetics, and Pharmacodynamics. *Clin Pharmacol Drug Dev.* 2023;12(6):579-587.

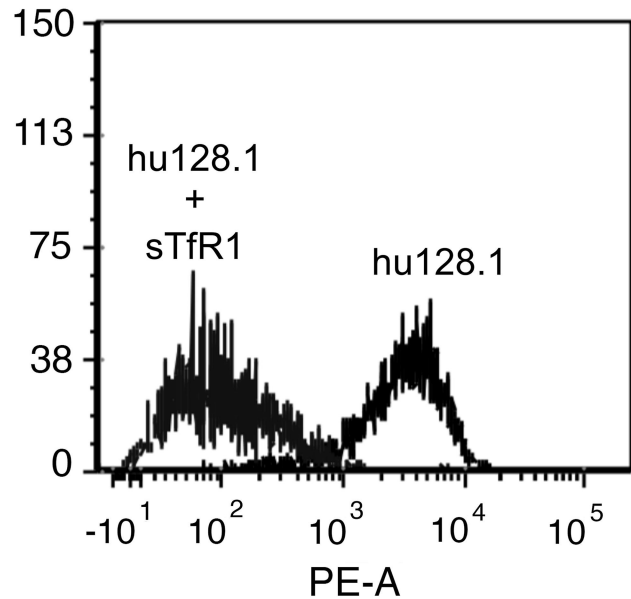
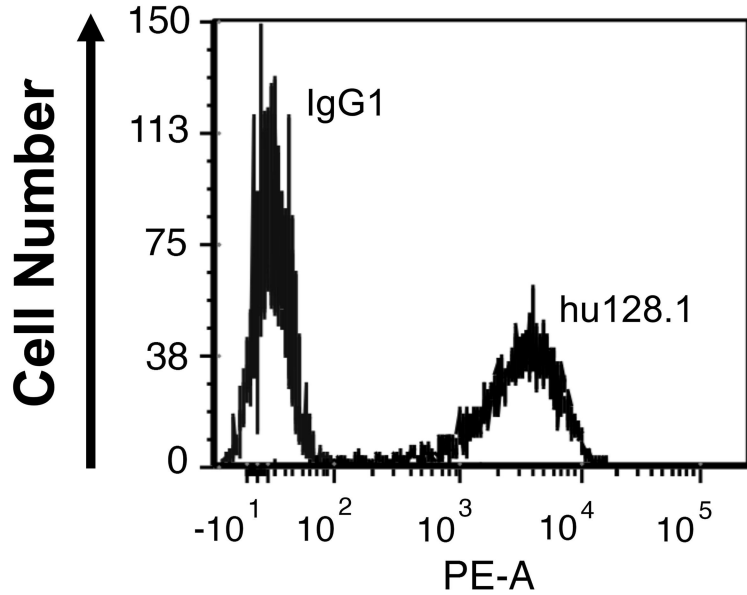
Figure Legends

Figure 1. Humanized 128.1 antibody (hu128.1) binding to transferrin receptor 1 (TfR1) on the cell surface. Flow cytometry analysis showing binding of hu128.1 to ERY-1 cells that express TfR1. Binding was detected using a phycoerythrin (PE)-conjugated goat F(ab')₂ anti-human κ antibody. The decrease in binding of hu128.1 in the presence of excess soluble TfR1 (sTfR1) is also shown (right panel).

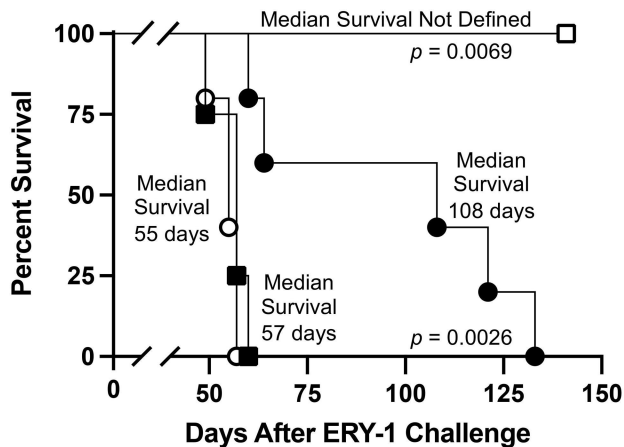
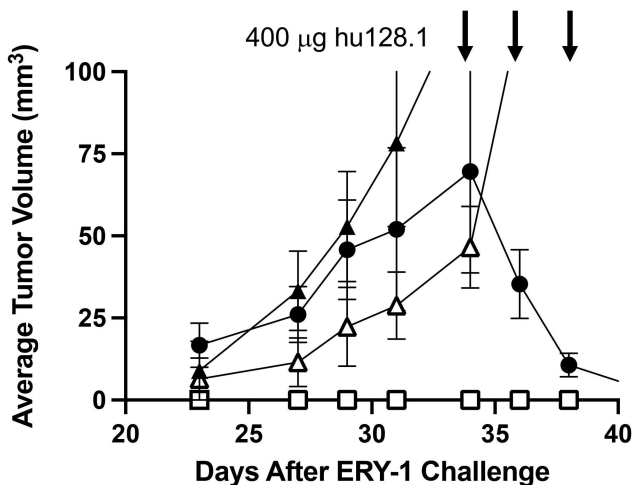
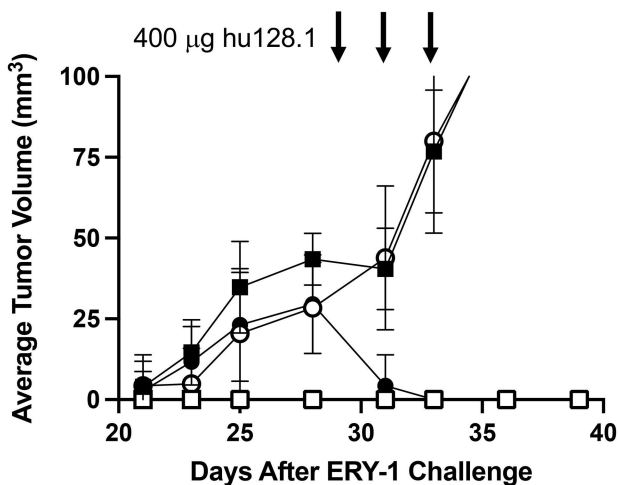
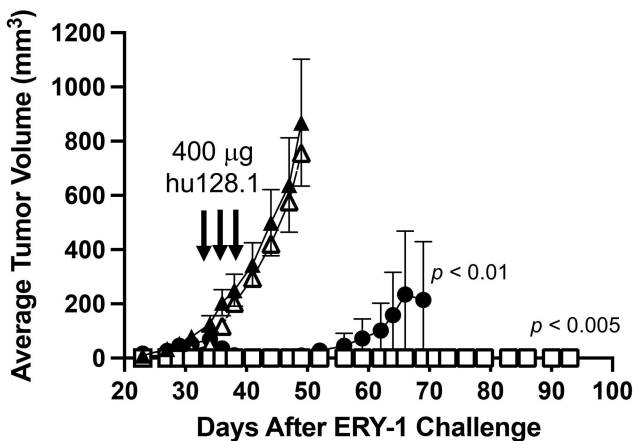
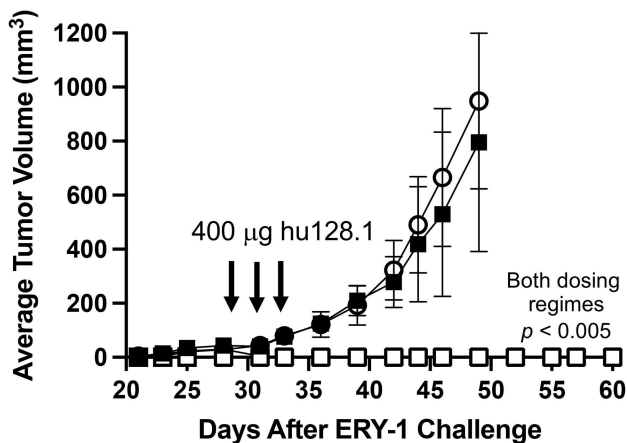
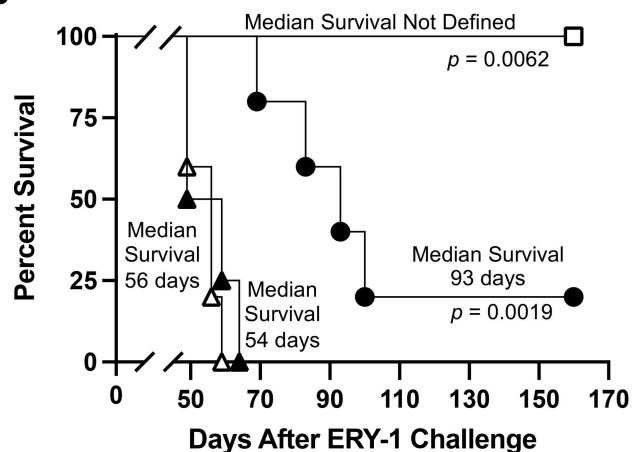
Figure 2. Efficacy against local (subcutaneous) ERY-1 tumors. SCID-Beige mice were challenged subcutaneously (s.c.) with 2×10^6 ERY-1 cells. A) Mice were treated intravenously (i.v.) with 400 μ g hu128.1 once on Day 2 or 3 times starting when tumors were palpable (Days 29, 31, and 33). The negative control group was treated with buffer on the corresponding days. B) Mice were treated i.v. with 400 μ g hu128.1 or the IgG1 isotype (negative) control antibody once on Day 2 or 3 times starting when tumors were palpable (Days 34, 36, 38). Top panels show the Kaplan-Meier survival plots with the median survival and *p*-values (log-rank test compared to the corresponding negative control group) indicated for each group. Middle panels show tumor growth curves as the mean \pm standard error of the mean (SEM) for each group with the *p*-values (rate-based T/C method compared to the corresponding negative control group) indicated for each group. Growth curves only show the time frame when all animals in the group were alive. Bottom panels show an enlarged area of the tumor growth curve in the middle panel from the beginning of the study (Days 20-40) allowing for a clear observation of the initial tumor growth and regression following treatment.

Figure 3. Efficacy of hu128.1 in a disseminated model erythroleukemia. On the day prior to ERY-1 cell challenge, half of the mice were irradiated with 3 Gray (Gy) whole-body irradiation to

facilitate bone marrow engraftment. The other half were left non-irradiated. The next day, all SCID-Beige mice were challenged with 5×10^6 ERY-1 cells i.v. Two days later mice were treated i.v. with 100 μ g hu128.1 or buffer alone as the negative control group. The Kaplan-Meier survival plot with the median survival and p -values (log-rank test compared to the negative control group) for each group are shown.

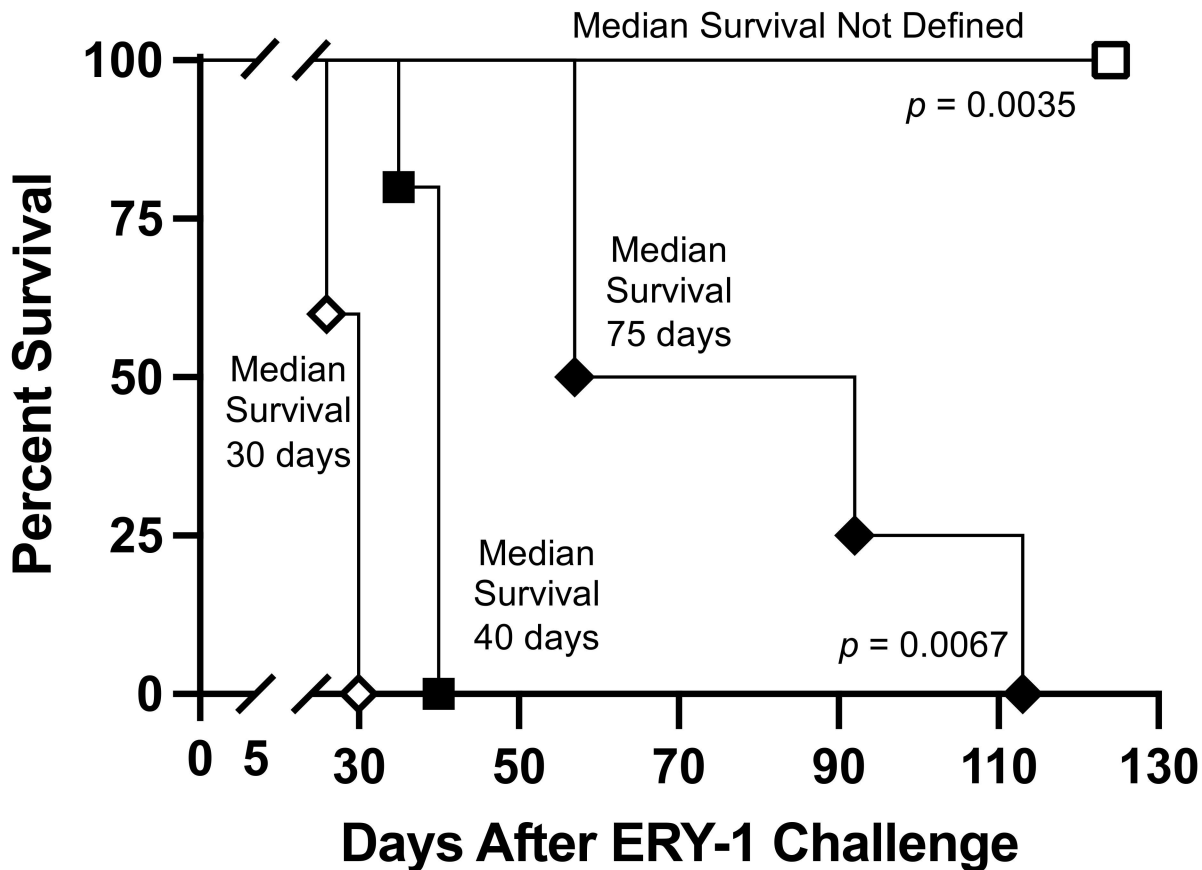


Fluorescence Intensity

A**B**

- Buffer (Day 2) $n = 4$
- hu128.1 (Day 2) $n = 4$
- Buffer (Days 29, 31, and 33) $n = 5$
- hu128.1 (Days 29, 31, and 33) $n = 5$

- ▲ IgG1 (Day 2) $n = 4$
- hu128.1 (Day 2) $n = 4$
- △ IgG1 (Days 34, 36, and 38) $n = 5$
- hu128.1 (Days 34, 36, and 38) $n = 5$



■ Buffer (Day 2) $n = 5$

□ hu128.1 (Day 2) $n = 5$

◇ Buffer (Day 2; irradiated) $n = 5$

◆ hu128.1 (Day 2; irradiated) $n = 4$