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**Studies of the Tumor Microenvironment in Pathogenesis of Neuroblastoma**

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<b>13. SUPPLEMENTARY NOTES</b>									
<b>14. ABSTRACT</b>  The NBL-Tag neuroblastoma mice were crossed with B-cell deficient mice in order to determine the effect of B-cell activity on tumor growth characteristics. Homozygous deficient B-Cell mice and heterozygous NBL-Tag mice were established. The NBL-Tag/B-cell deficient mice lacked B-cells as expected using flow cytometry analyses and immunohistochemistry. However, the lack of B-cells did not alter the growth patterns of NBL-Tag tumor formation as imaged by MRI. Studies using anti-B cell therapy were also conducted. The efficacy of the anti-mouse B-cell antibody was tested in wildtype C57B6 mice tissues (blood, spleen, and peritoneal cavity lymphocytes) and demonstrated superiority of the antibody to eliminate B-cells when given in the peritoneum. NBL-Tag mice given this antibody starting at 4 weeks of age did not show any difference in their growth characteristics. The anti-B cell antibody combination with chemotherapy in established tumors showed superiority compared with anti-Ragweed antibody control. Further studies are underway to elucidate the effect of macrophages on tumor formation and growth.									
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## **Introduction:**

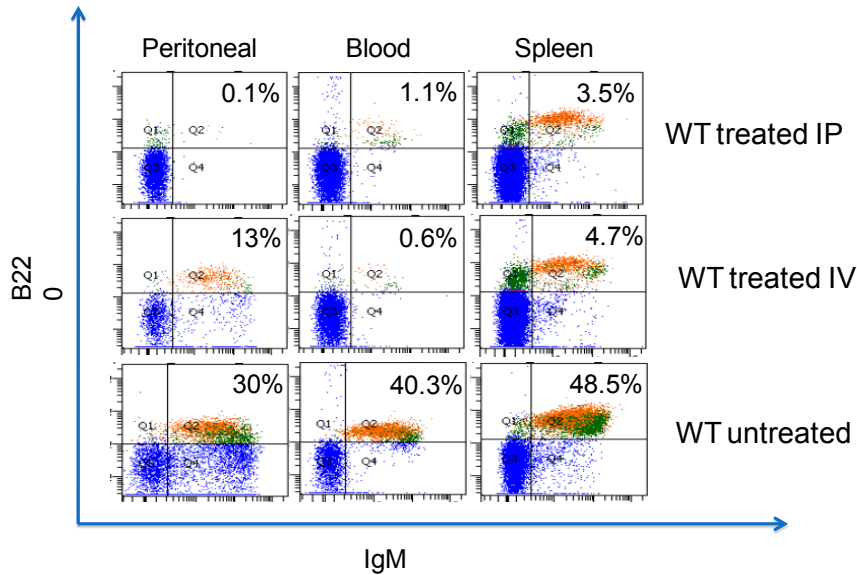
Neuroblastoma is the most common extra cranial solid tumor of childhood, and 45% of patients have high-risk tumors, nearly all of which are metastatic (stage 4) when diagnosed. Seventy-one percent of metastatic (stage 4) neuroblastomas (mNBL) lack amplification of the MYCN oncogene. An intriguing observation about survival of patients with MYCN non-amplified mNBL (mNBL MNA) is the extreme variation based on age at diagnosis. Children diagnosed less than 18 months of age have greater than 90% overall survival (O.S.), while those diagnosed after 18 months of age have only 45% O.S. even with improvements in therapy during the past 20 years. Recently, our group has identified several inflammation-related genes correlating with age at diagnosis and outcome in this group of tumors. Our characterization of a recently described 100% penetrant transgenic murine neuroblastoma model (NBL-Tag) established lack of MYCN amplification using comparative genomic hybridization (aCGH). Remarkably, tumors are not detected by MRI until 13 weeks of life, but they then grow rapidly with liver and bone marrow metastasis by 20 weeks and demise by 28 weeks. Tumor growth coincides with IL6 becoming detectable and increasing in blood, and tumors exhibit high expression of IL-4, IL-6, and IL-10 and are infiltrated by TAMs (CD11b+, F4/80+) and B lymphocytes (B220+,CD19+/IgM+). Immunofluorescence analyses demonstrate immunoglobulin deposition within the tumors. These data identify the NBL-Tag mice as the only known transgenic murine model for aggressive human mNBL MNA. Importantly, the pro-inflammatory microenvironment of the NBL-Tag tumors mimics that observed in human neuroblastoma. Our specific aims will allow us to identify the significance of a pro-inflammatory tumor microenvironment on neuroblastoma pathogenesis.

## **Body:**

In Task 1 of our work we have sought to determine the effects on tumor microenvironment and tumorigenesis of NBL-Tag mice crossed with RAG1 deficient mice (lack lymphocytes). In Task 2 we sought to determine the effects on tumor microenvironment and tumorigenesis of NBL-Tag mice after depletion of CD20 positive cells using mouse anti-CD20 antibody before and in early and late stages of tumor development. Both of these tasks have now been completed.

Our initial hypotheses was that decreasing level of expression of IgK levels, which we had shown to correlate with poor survival, could be accomplished by the use of our murine model of neuroblastoma (NBL-Tag) and mice that are B-lymphocyte deficient (Task 1). We hypothesized that immunocomplex deposition in the NBL-Tag tumors promotes a microenvironment favoring tumor growth. We also assessed effects of chemotherapy and a monoclonal antibody directed against CD20, a B-lymphocyte marker, on the growth of tumors and survival of NBL-Tag mice (Task 2).

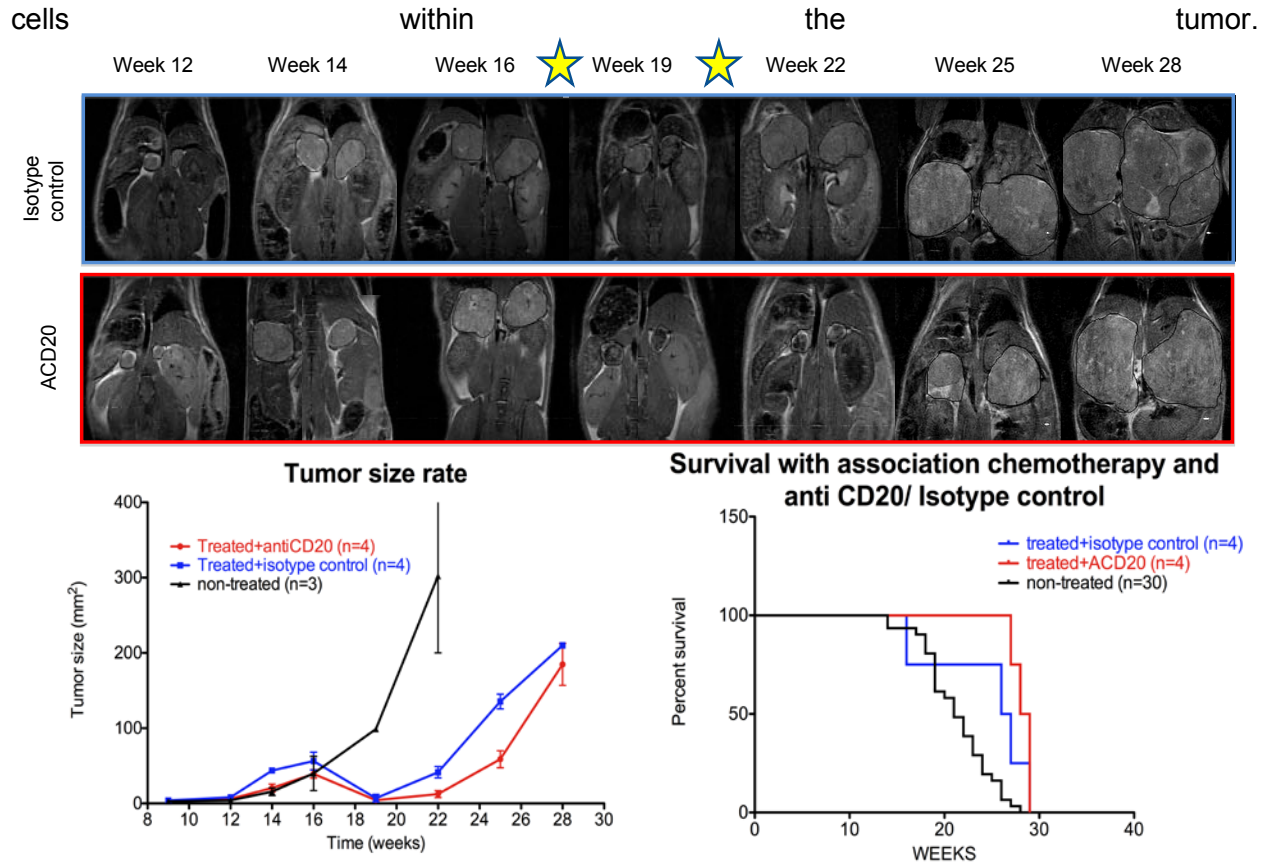
In brief, presence of B-lymphocytes in tumors was investigated by flow cytometry and Immunofluorescence (IF). Growth and survival of NBL-Tag mice were assessed after treatment with chemotherapy (two 5-day cycles of cyclophosphamide 110mg/kg/day and topotecan 0.4mg/kg/day), chemotherapy and mouse anti-mouse-CD20 antibody (10 mg/kg once per week), or chemotherapy and anti-Ragweed isotype antibody. Tumor growth was assessed by MRI. The efficacy of the anti-mouse-CD20 antibody was tested using intravenous (i.v.) or interperitoneal (i.p.) injection in wild type mice (Fig 1).



**Figure 1. Mouse anti-mouse CD20 Antibody (clone5D2 – kindly provided by Genetech) was administered as IV or IP in three C57B6. Representative flow cytometry analysis of B-cell population in the peritoneal cavity, blood and spleen are demonstrated. The IP treatment was more effective in removing B-Cells from all three compartments than IV treatment.**

All NBL-Tag mice developed bilateral primary tumors at 12-13 weeks of life with liver metastasis by 22 weeks of life. The median survival of NBL-Tag mice was 24 weeks. There was significant decrease in tumor size after each cycle of chemotherapy. Animals receiving chemotherapy and anti-CD20 therapy had significant decrease in the rate of tumor re-growth compared to the control group ( $p < 0.001$ ; Fig 2). Flow cytometry analyses of NBL-Tag mice treated with anti-CD20 combination confirmed absence of B-lymphocytes in blood and peritoneal cavity, and showed an increase in the number of CD11b+/Ly6G+ myeloid cells in NBL-Tag tumors (See CHLA's Poster Presentation attached as appendix 1).

Our group has also crossed mice deficient in B-lymphocyte (homozygous trait) with NBL-Tag mice. Ten animals were analyzed for this study, with similar number of control NBL-Tag animals. The MRI analysis of 5 NBL-Tag mice deficient in B-cells showed similar patterns of growth compared to those with normal population of B-cells (data not shown). Flow cytometry analyses showed lack of B-cells in tumors of double crossed NBL-Tag mice. Further, flow cytometry data are currently being analyzed to determine the percentage of other hematopoietic



**Figure 2.** MRI images of NBL-Tag mice treated with cyclophosphamide at 16 weeks and 19 weeks of age with either anti-mouse CD20 antibody or isotype control (upper panel). There was a statistically significant difference between the regrowth of tumor after treatment with anti-mouse CD20 antibody compared to isotype control (left lower panel). However there was no statistically significant difference observed in the overall survival of mice treated with anti-mouse CD20 antibody or isotype control (right lower panel).

#### KEY RESEARCH ACCOMPLISHMENTS:

- Anti-CD20 antibody treatment delays neuroblastoma regrowth after chemotherapy in a murine MYCN non-amplified model of neuroblastoma.
- Genetic ablation of B-lymphocytes does not alter the growth characteristics of the murine MYCN non-amplified neuroblastoma.

#### REPORTABLE OUTCOMES:

##### Abstracts:

Title: "Evaluation of Chemotherapy and Anti-CD20 therapy in a Transgenic Murine Model of Neuroblastoma Lacking MYCN Amplification." Sidonie Bonne, Michael Hadjidaniel, Long Hung, Sakunthala Muthgounder, Shawn Pronold, Michael Sheard, Hiroyuki Shimada, Shahab Asgharzadeh. Children's Hospital Los Angeles Annual Poster Session 2012.

Animal models: A cross between NBL-Tag mice and B-lymphocyte knockout mice were created and described..

**CONCLUSION**

NB-Tag is a robust mouse model of metastatic NBL-NA tumors recapitulating human disease. Our preliminary results reveal anti-tumor effect of treatment with anti-mouse CD20 and lesser effect of isotype control. However the transgenic B-lymphocyte experiments do not directly implicate B-cells in the neuroblastoma pathogenesis in this model. The observations that antibody treatment may delay tumor regrowth will be further examined using *in vitro co-culture* assays. We are continuing our work on Task 3 using

**REFERENCES**

None

**APPENDICES**

PDF version of the abstract presented at the CHLA Poster Session.



# Evaluation of Chemotherapy and Anti-CD20 therapy in a Transgenic Murine Model of Neuroblastoma Lacking MYCN Amplification.

Sidonie Bonne, Michael Hadjidaniel, Long Hung, Sakunthala Muthgounder, Shawn Pronold, Michael Sheard, Hiroyuki Shimada, Shahab ASGHARZADEH

## Abstract

**BACKGROUND:** In metastatic neuroblastomas MYCN non-amplified (mNBLMNA) Ig Kappa is a poor prognostic markers in patients older than 18months . To further investigate this condition we generated a transgenic mouse model of neuroblastoma lacking MYCN amplification (NBTag) and we identified intratumoral Ig deposition similar to human features. This new model is the only mNBLMNA model available to study contributions of microenvironment to neuroblastoma tumorigenesis in immune competent mice. We investigated *in-vivo* the role of B-lymphocytes in promoting a recurrence and growth of tumors. **METHODS:** Cohorts of transgenic mice were monitored for tumor growth (MRI) and survival. Presence of B cells in tumors was investigated by flow cytometry and Immuno-Fluorescence of frozen tumor sections. Then we investigated *in-vivo* a therapeutic regimen including standard chemotherapy (2 cycles of 5 days each, cyclophosphamide 110mg/kg/day, topotecan 0.4mg/kg/day) or in association with either a mouse specific antiCD20 antibody (mIg2a 5D2, 10 mg/kg/Week) or a control isotype (Rag IgG 1428). **RESULTS:** In NBTag mouse model, bilateral primary tumors occur in all mice by 12 weeks of life. Metastasis affecting preferentially the liver were noted in all mice by 24 weeks of life and tumor growth results in demise by 28 weeks. There is a significant tumor growth inhibition after two cycles of chemotherapy that is further enhanced when associated with anti-CD20 (p<0.001). Efficacy was noted in metastasis appearance as well. Flow cytometry analyses of lymphocytes in blood and peritoneum cavity after anti-CD20 confirmed absence of B-cells and an increase in myeloid precursor. **CONCLUSION:** NBTag is a robust animal system recapitulating human condition and is therefore suitable for use in preclinical trial and for fine characterization of tumor microenvironment in mNBLMNA. We present herein promising results of a combined therapy resulting in delaying tumor recurrence and regrowth associated with a longer survival.

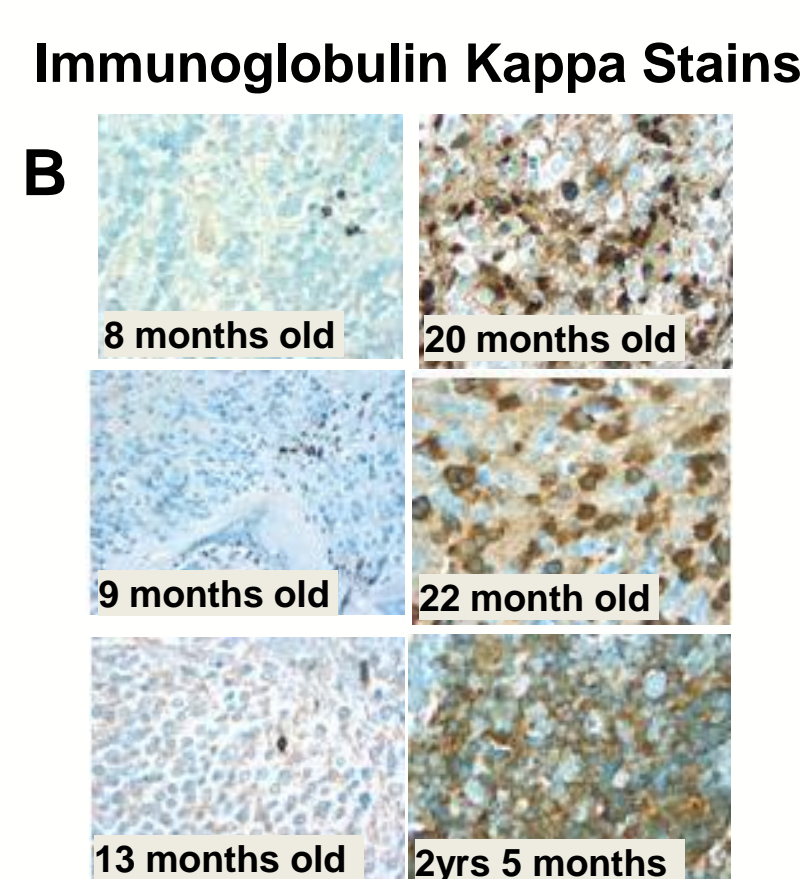
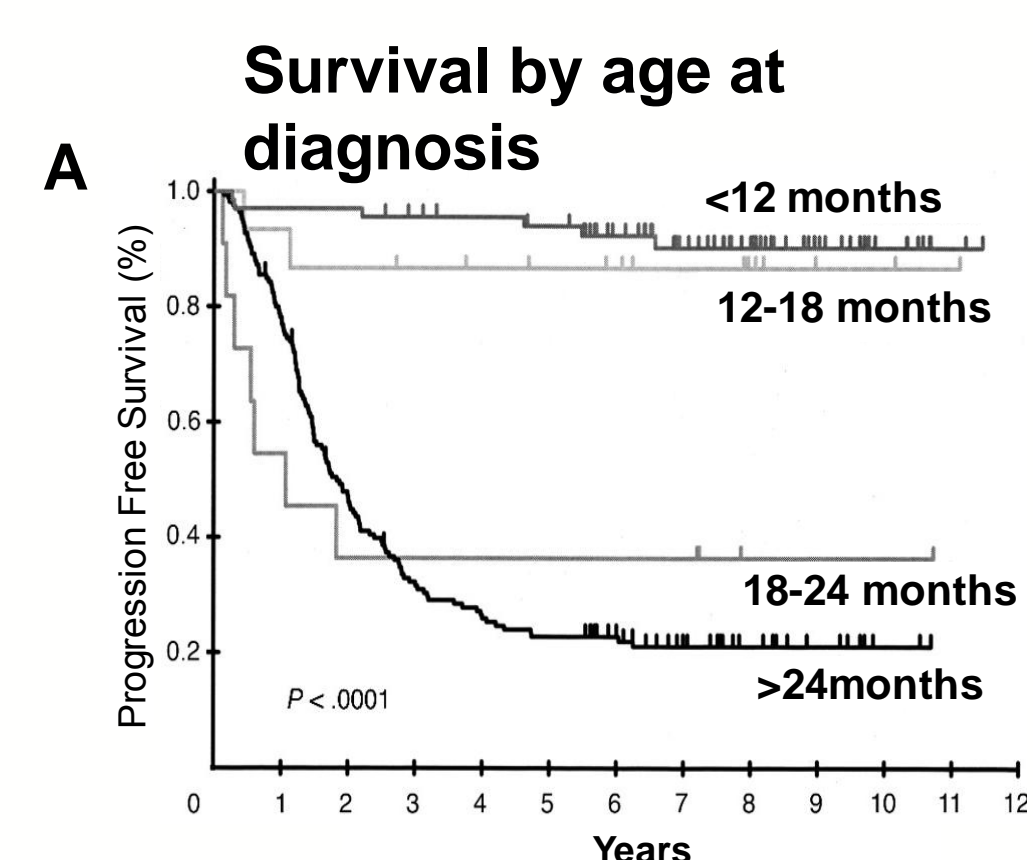
## Background

Over 50% of high risk neuroblastoma patients, the most common extracranial solid tumor of childhood, develop a fatal disease following intensive chemoradiotherapy. Majority of high-risk cases lack amplification of the MYCN oncogene (NBL-NA). Age at diagnostic in metastatic neuroblastoma MYCN non-amplified (mNBL-NA) is a risk criteria. Preliminary study using gene expression profiling of stage 4 (metastatic) NBL-NA tumors identified increased expression of Ig Kappa (IGKC) as poor prognostic marker in children diagnosed ≥18 months of age. We hypothesize that tumor associated B lymphocytes (TAB) play an important role in creating a protumor microenvironment and that Ig deposition plays a role in malignant phenotype of tumor. We investigated *in-vivo* the role of B-lymphocytes in promoting recurrence and growth of tumors lacking MYCN amplification.

**Aim 1: monitoring the response to chemotherapy in a mouse model of MYCN non amplified neuroblastoma (NBL-Tag).**

**Aim 2: Assessing the role of lymphocytes B on promoting tumor recurrence after induction chemotherapy using a new mouse specific anti-CD20.**

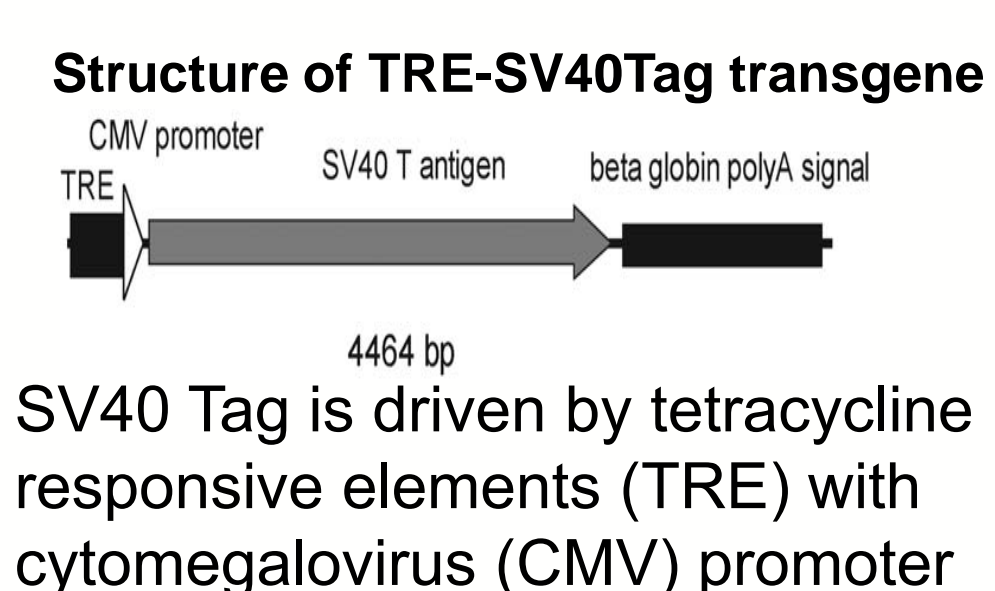
## MYCN non Amplified Neuroblastoma



**A:** Kaplan-Meier analysis of children with metastatic neuroblastoma lacking MYCN amplification reveals survival dependent on age of the child at diagnosis with those older than 18 months of age having worse outcome. **B:** IHC analysis of primary tumor these of children confirms strong staining of IGKC only in tumors of children diagnosed older than 18months old

## A new transgenic mouse-model for Neuroblastoma: NBL-Tag

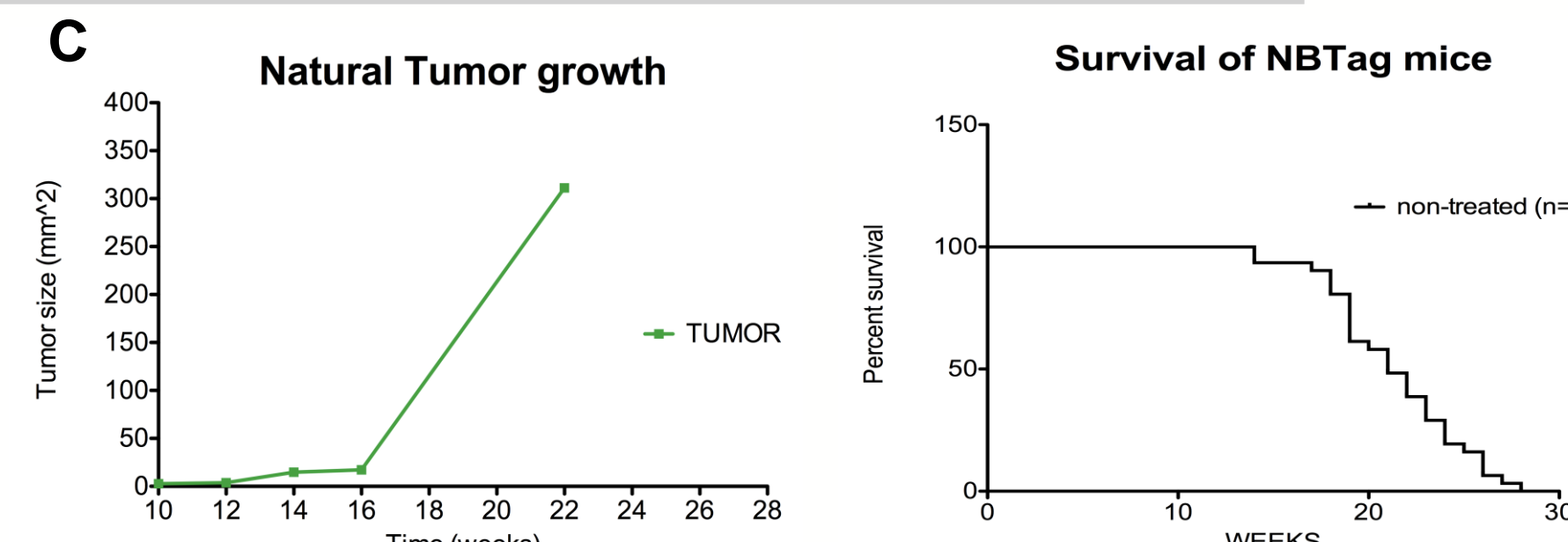
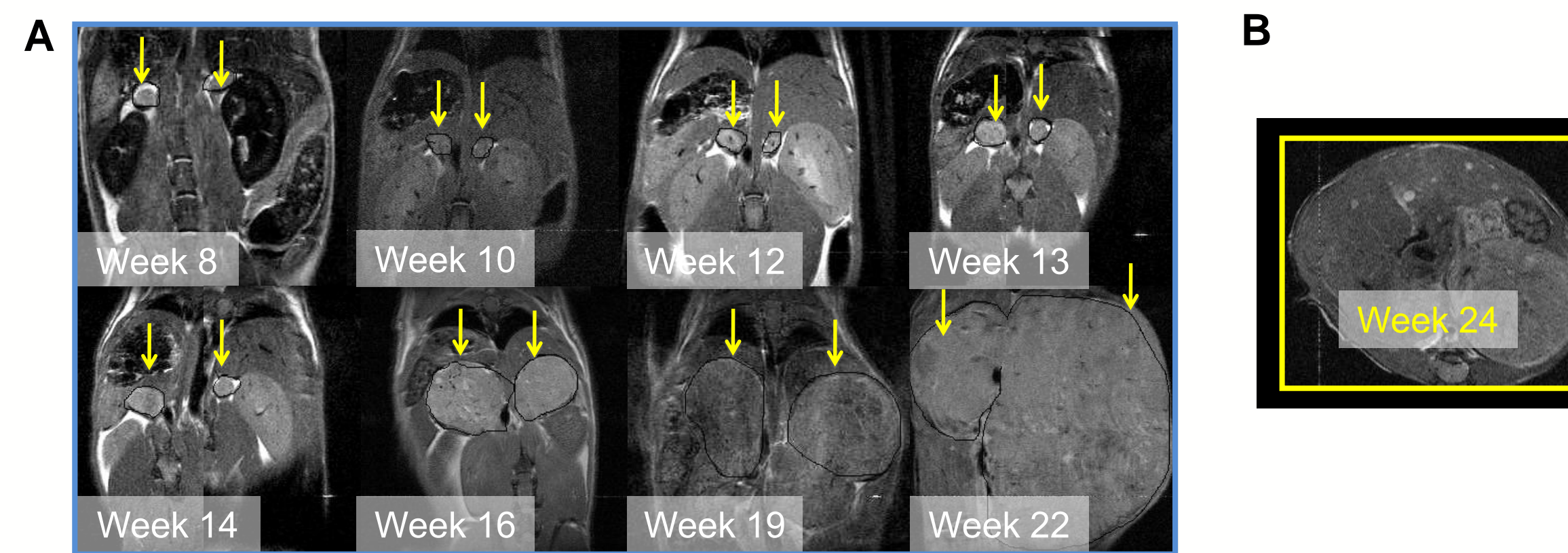
**A transgenic model of neuroblastoma identified in 2008** - Japanese investigators described an unexpected murine neuroblastoma model after attempting to create a tre-inducible SV40 large T antigen transgenic animal. Mice in this fully penetrant model develop bilateral adrenal tumors, positive for Tyrosine Hydroxylase and high levels of catecholamines.



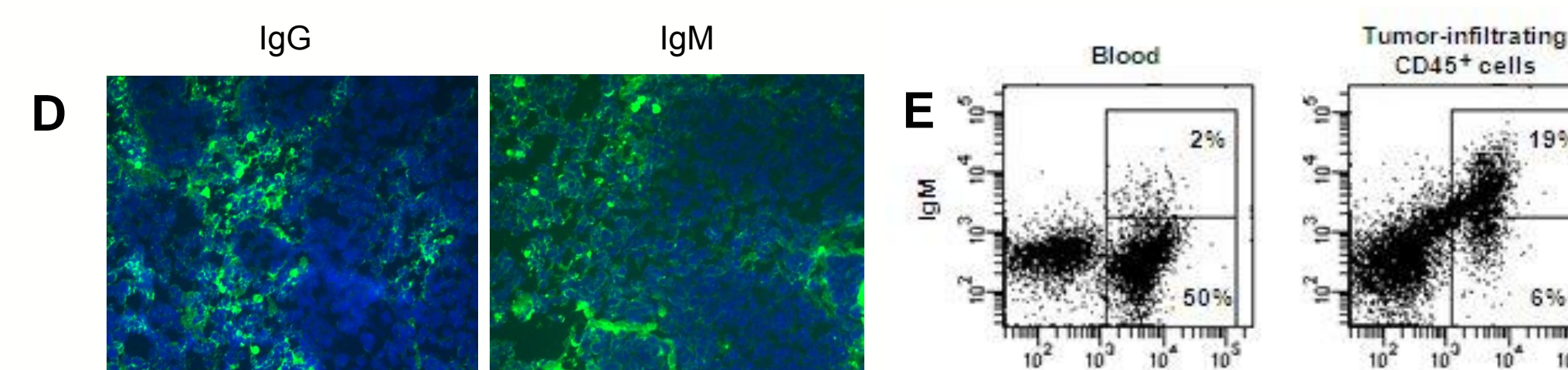
SV40 Tag is driven by tetracycline responsive elements (TRE) with cytomegalovirus (CMV) promoter

## Characterization of NBL-Tag transgenic mice

### Natural growth rate of Neuroblastomas in untreated NBL-Tag



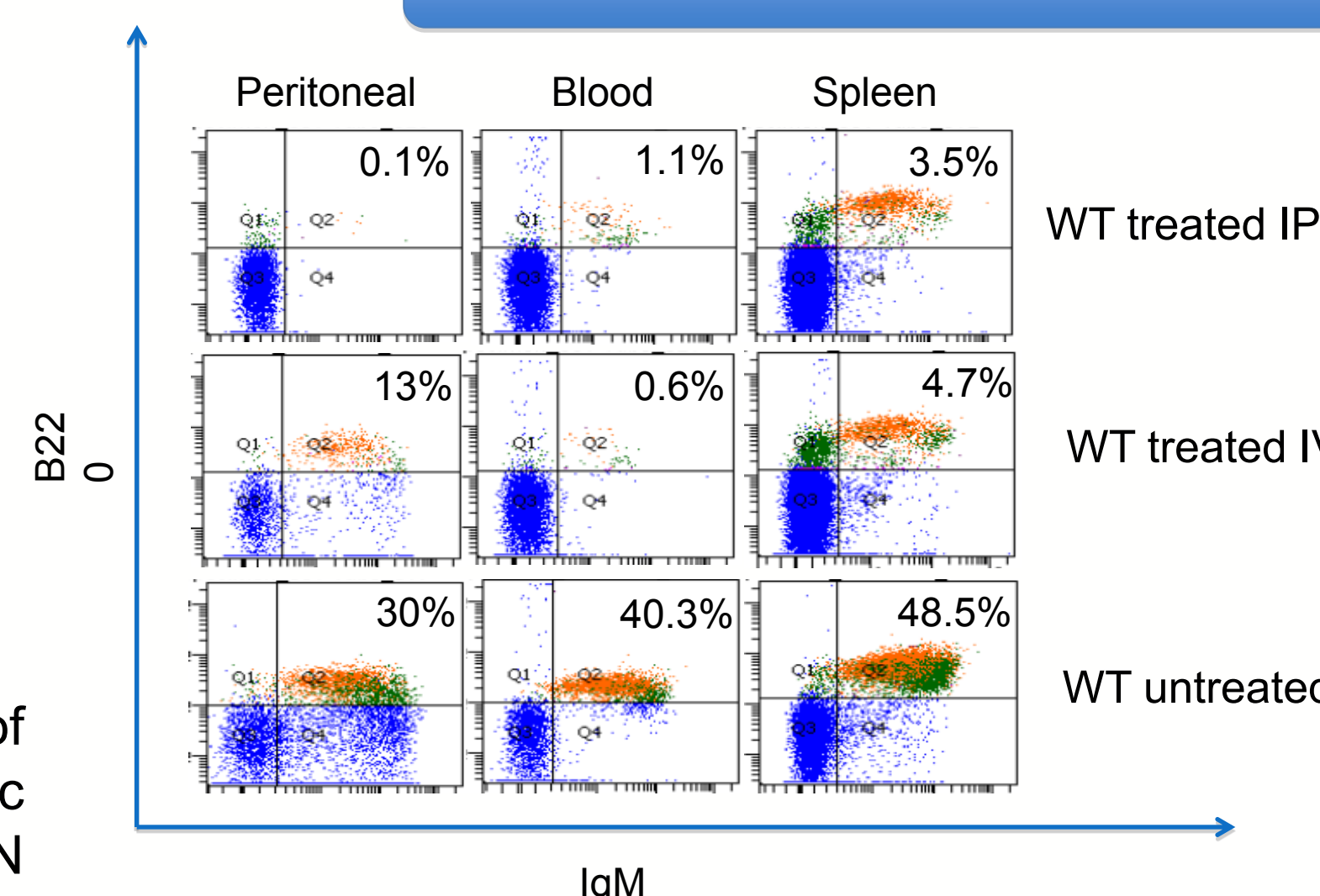
### Ig deposition & B-cell infiltration in NBL-Tag tumors



**A-B-C:** Tumors are 100% penetrant, start their growth only at 12 weeks (A) and metastasize in the liver by 24 weeks of life (B). **D:** NBL-Tag tumors were stained with rabbit anti-mouse IgM, IgG deposition. We identified intratumoral Ig deposition similar to what has been found in human neuroblastoma. **E:** FACS analysis of NBL-Tag blood and single cell suspension reveals presence of CD19+, IgM+ cells (matures B-cells) within the tumor.

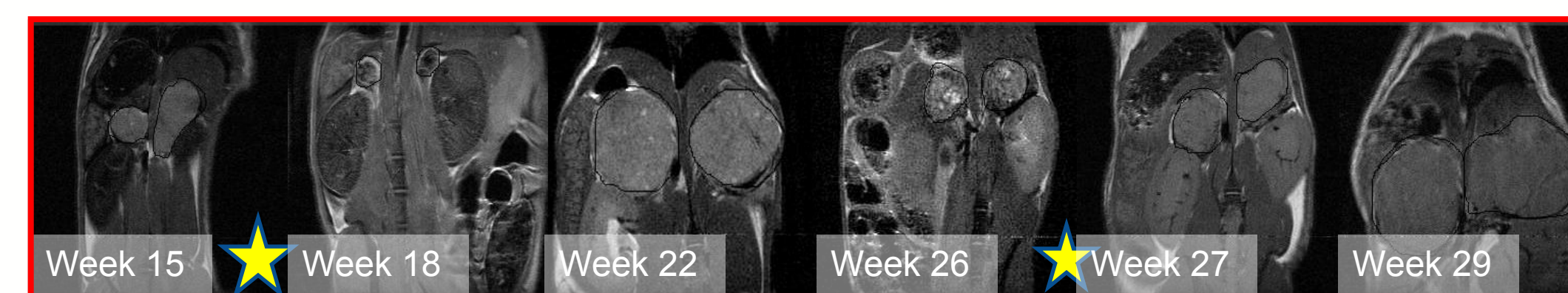
## A new mouse anti-mouse antibody

### Anti-CD20 gets rid of B-cells in WT controls

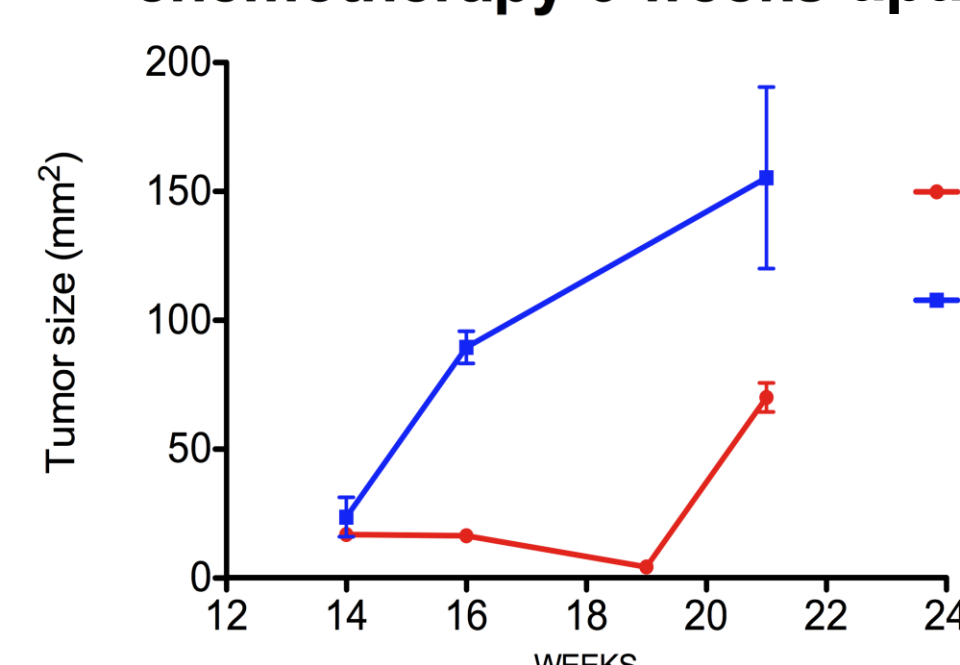


**GENENTECH** provides us with a mouse anti-mouse CD20 Antibody (clone5D2). After treatment with this ACD20 for a week, FACS analysis of peritoneal cells, blood and spleen show we almost totally get rid of B cells for Wild type mice treated IP or IV.

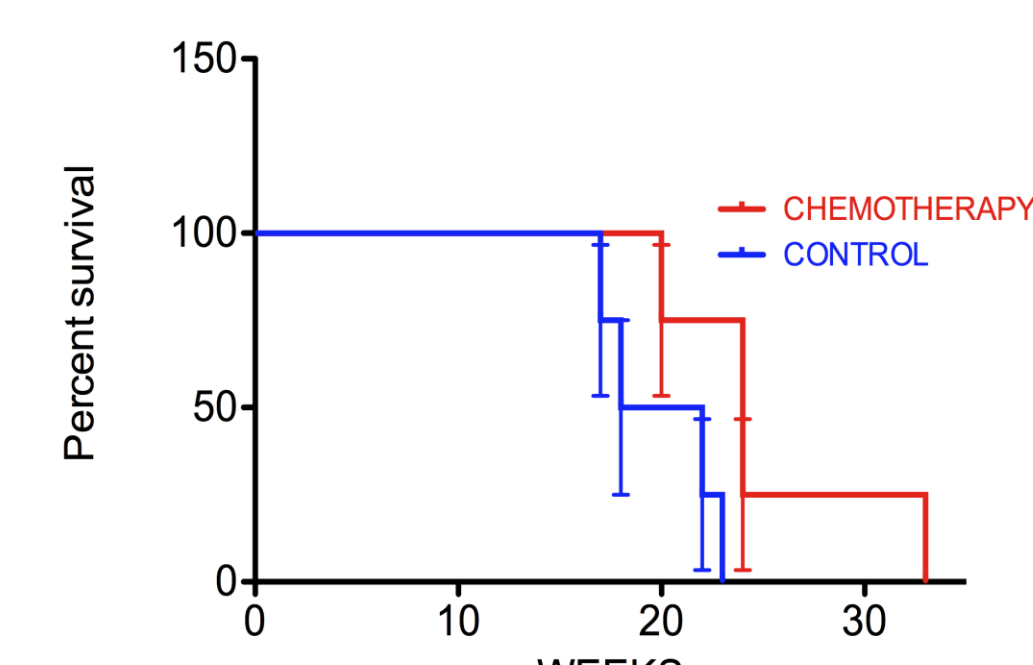
## Anti-tumor activity of standard chemotherapy



### Tumor growth rate with standard chemotherapy 6 weeks apart



### Survival with standard chemotherapy

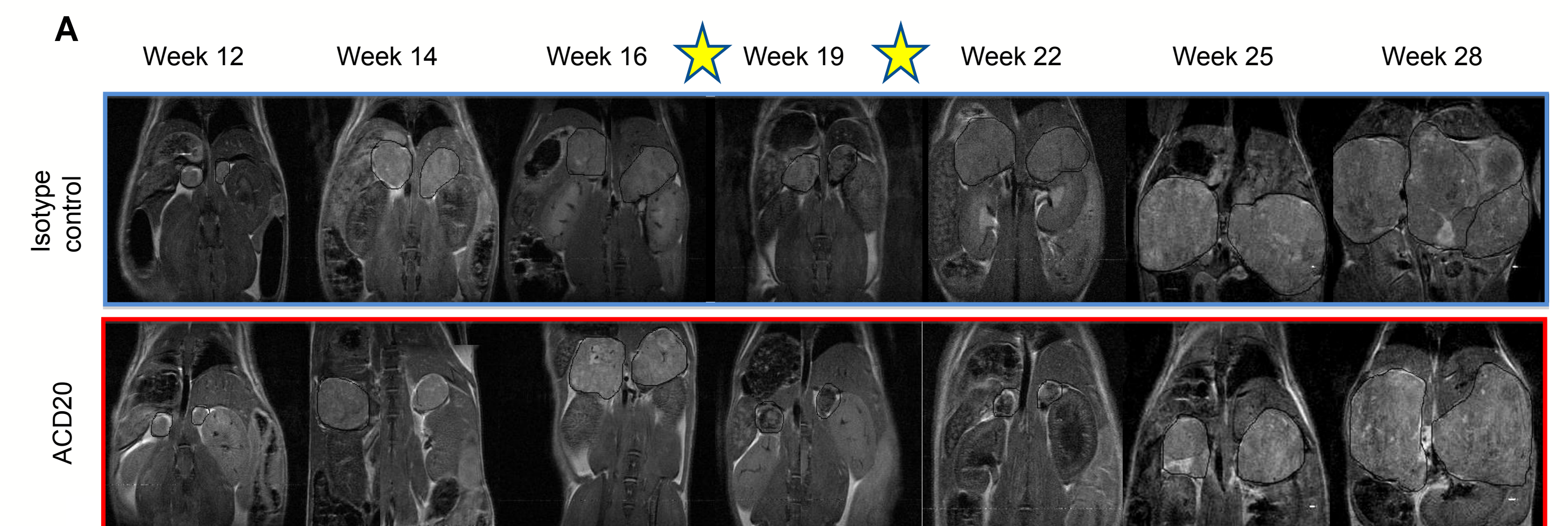


We investigated *in-vivo* a therapeutic regimen with standard chemotherapy (2 cycles of 5 days each, cyclophosphamide 110mg/kg/day, topotecan 0.4mg/kg/day). NBL-Tag appeared to live longer and there is a significant inhibition of tumor growth after two cycles of chemotherapy.

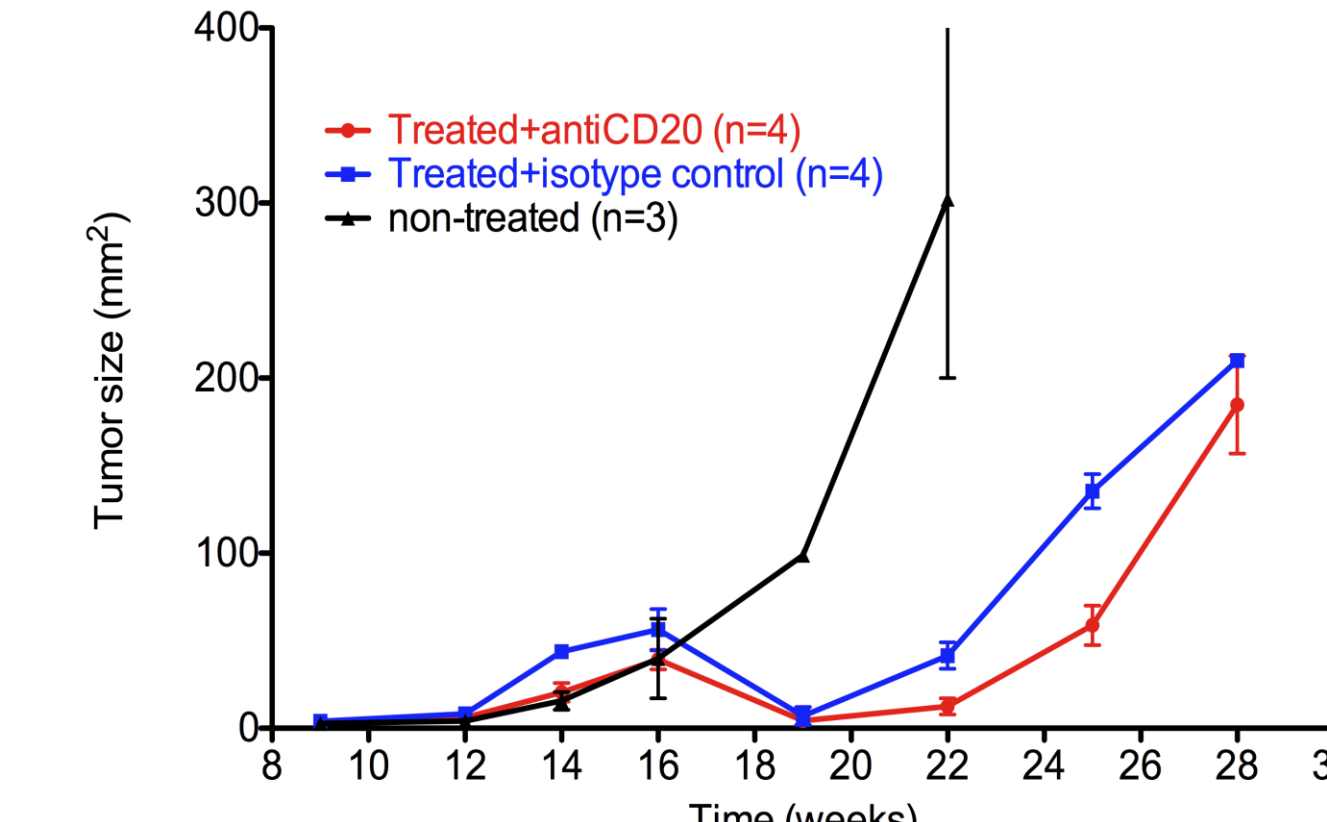
## Effect of combination with the Anti-CD20

### Tumor response to standard chemotherapy + anti-CD20

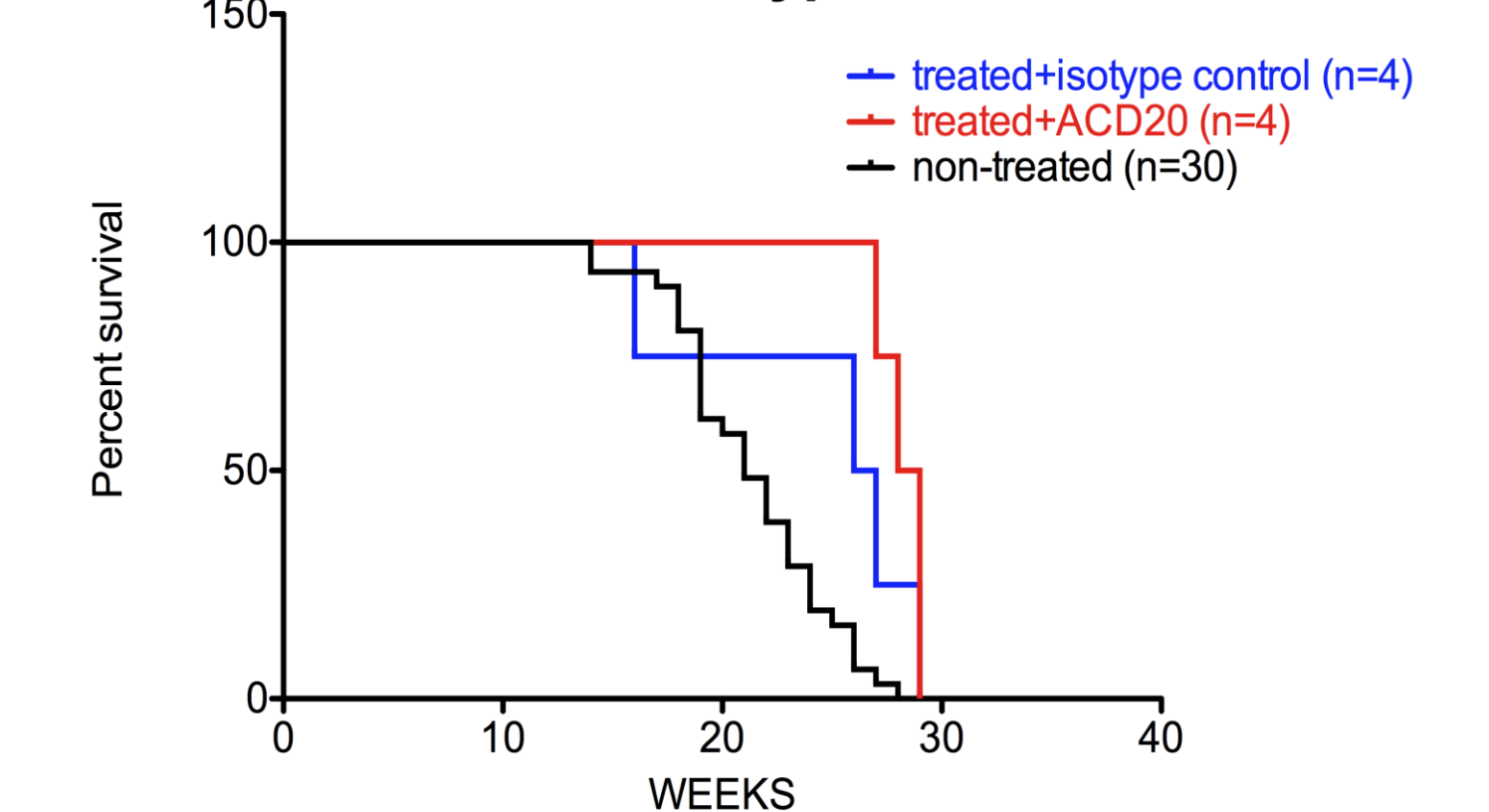
We investigated *in-vivo* a therapeutic regimen including standard chemotherapy (2 cycles of 5 days each, cyclophosphamide 110mg/kg/day, topotecan 0.4mg/kg/day 21 days apart) in association with either a mouse specific antiCD20 (mIg2a 5D2, 10 mg/kg/Week) or a control isotype (Rag IgG 1428)



### Tumor size rate

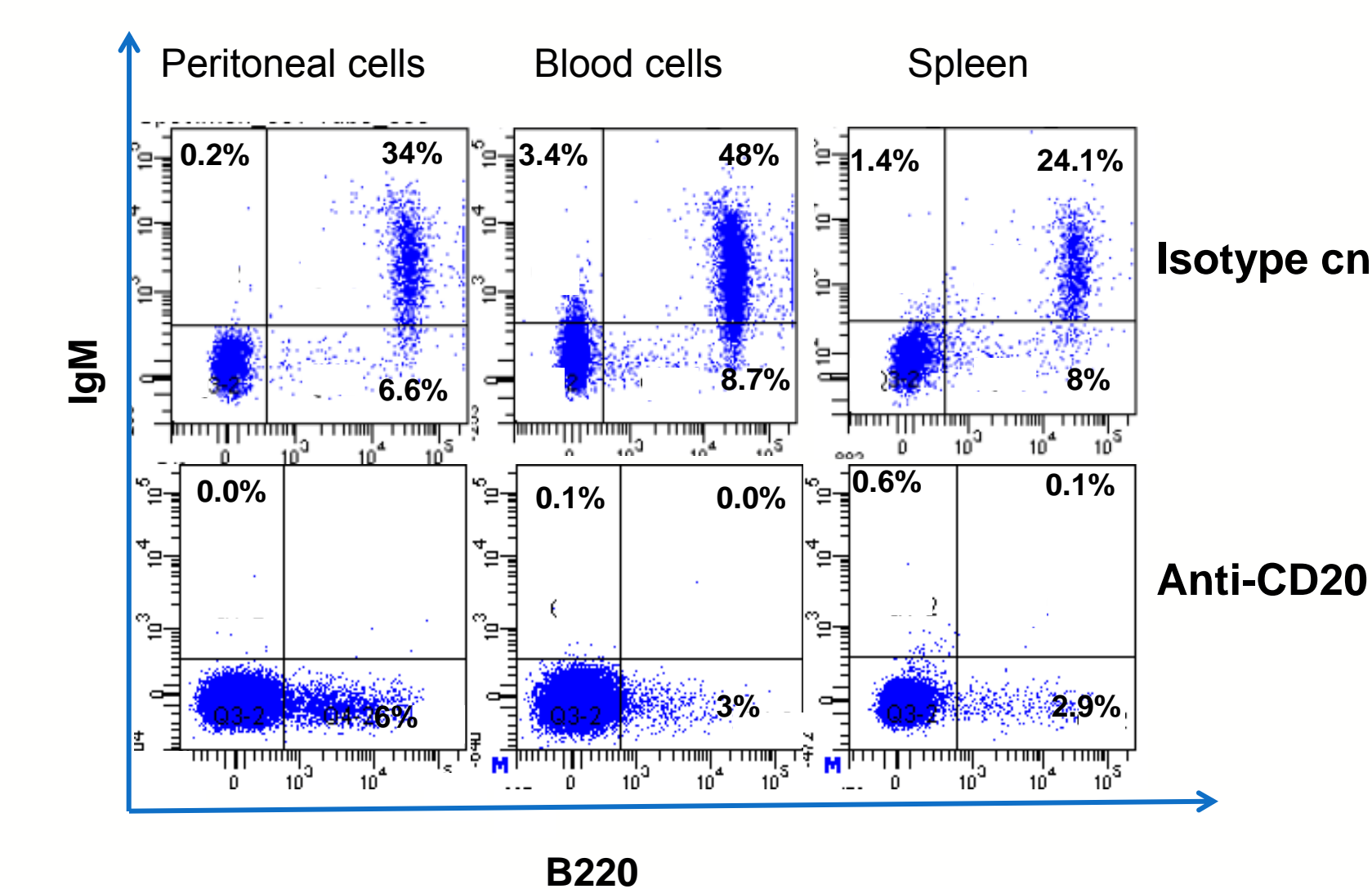


### Survival with association chemotherapy and anti CD20/ Isotype control



**A-B:** Tumor growth was followed by MRI: there is a significant inhibition of tumor growth after two cycles of chemotherapy that is further enhanced when associated with anti-CD20 (p<0.001). **C:** the overall survival is better with ACD20 and we noted an absence of metastasis in that group at death.

## Anti-CD20 gets rid of B-cells in NBL-Tag treated mice



FACS analyses of immune cells show 0% B220 and IgM cells within the blood and the peritoneum cells and spleen of treated mice versus present in the isotype control treated group.

## Conclusion

- 1.NBTag is a animal system recapitulating human condition and is therefore suitable for use in preclinical trial and for fine characterization of tumor microenvironment in mNBLMNA.
2. We present herein promising results of a combined therapy resulting in delaying tumor recurrence and regrowth associated with a longer survival.
- 3.This new model is the only mNBLMNA model available to study contributions of microenvironment to neuroblastoma tumorigenesis in immune competent mice.

## REFERENCES

Iwakura H, Int. J. Oncology 2008