

## Background

Tumor Necrosis Factor Receptor Super Family 25 (TNFRSF25), also known as Death Receptor 3 (DR3), is preferentially expressed by activated and antigen-experienced T-cells.<sup>1</sup> TNFRSF25 is a potent costimulatory molecule, similar to OX40, 4-1BB and GITR. PTX-35 was developed as a humanized, affinity matured, IgG2 mAb against TNFRSF25 for use with current cancer immunotherapy options, including cancer vaccines. All pharmacology and IND-enabling PK and toxicology has been completed for PTX-35. Previous proof-of-concept studies were completed elsewhere.<sup>2, 5</sup> Pharmacology studies described here were completed with a surrogate antibody, mouse-IgG1-PTX-35 (mPTX-35). Regulatory T-cell expansion studies were completed with adoptively transferred OVA-specific, TCR-transgenic, CD8+ T-cells (OT-1). Human PTX-35 was tested in 28-day mouse, 2-week and 8-week non-human primate (NHP), toxicology studies. Human, mouse, and monkey, in vitro, tissue-cross reactivity tests were also performed to check species cross-reactivity. Human PTX-35 was also tested in a human PBMC stimulation assay, in vitro, to check for impact on proliferation and cytokine release. TNFRSF25-engagement in mice expanded antigen specific CD8+ T-cells when given in the context of vaccination (6 and 19-fold increase in CD8+ T-cells in blood over vaccination alone at peak and boost, respectively), and Tregs were expanded in vaccine absence in FIR animals (2-fold increase in CD4+ Foxp3+ T-cells in blood). The MABEL and NOEL, in FIR mice, using mPTX-35, was determined to be 0.01 mg/kg and 0.001 mg/kg, respectively. The 2-week PTX-35 treatment of cynomolgus monkeys confirmed findings in mice, that treatment results in the expansion of Teff and Treg cell subsets. Intravenous bolus injection once every 2-weeks over an 8-week period was well tolerated giving a NOAEL of 100 mg/kg. Species cross-reactivity was observed for mouse, human and monkey tissues. No adverse events were recorded for the 28-day mouse toxicology study. Testing PTX-35 in a human PBMC, anti-CD3 proliferation assay, in vitro, showed that TNFRSF25-engagement provided the necessary costimulation to drive cellular division at sub-optimal concentrations of anti-CD3 (2-fold increase), providing the necessary in vitro, human proof-of-concept data, which was consistent across four different human blood donors. PTX-35 is a potent costimulatory agonist targeting a novel pathway that can work in concert with cancer vaccines. Due to the ability of PTX-35 to possibly stimulate both pro-inflammatory and anti-inflammatory pathways, depending on treatment context, therapeutic modulation can provide numerous opportunities for cancer and inflammatory diseases.<sup>3, 4, 6</sup>

## Mechanism of Action

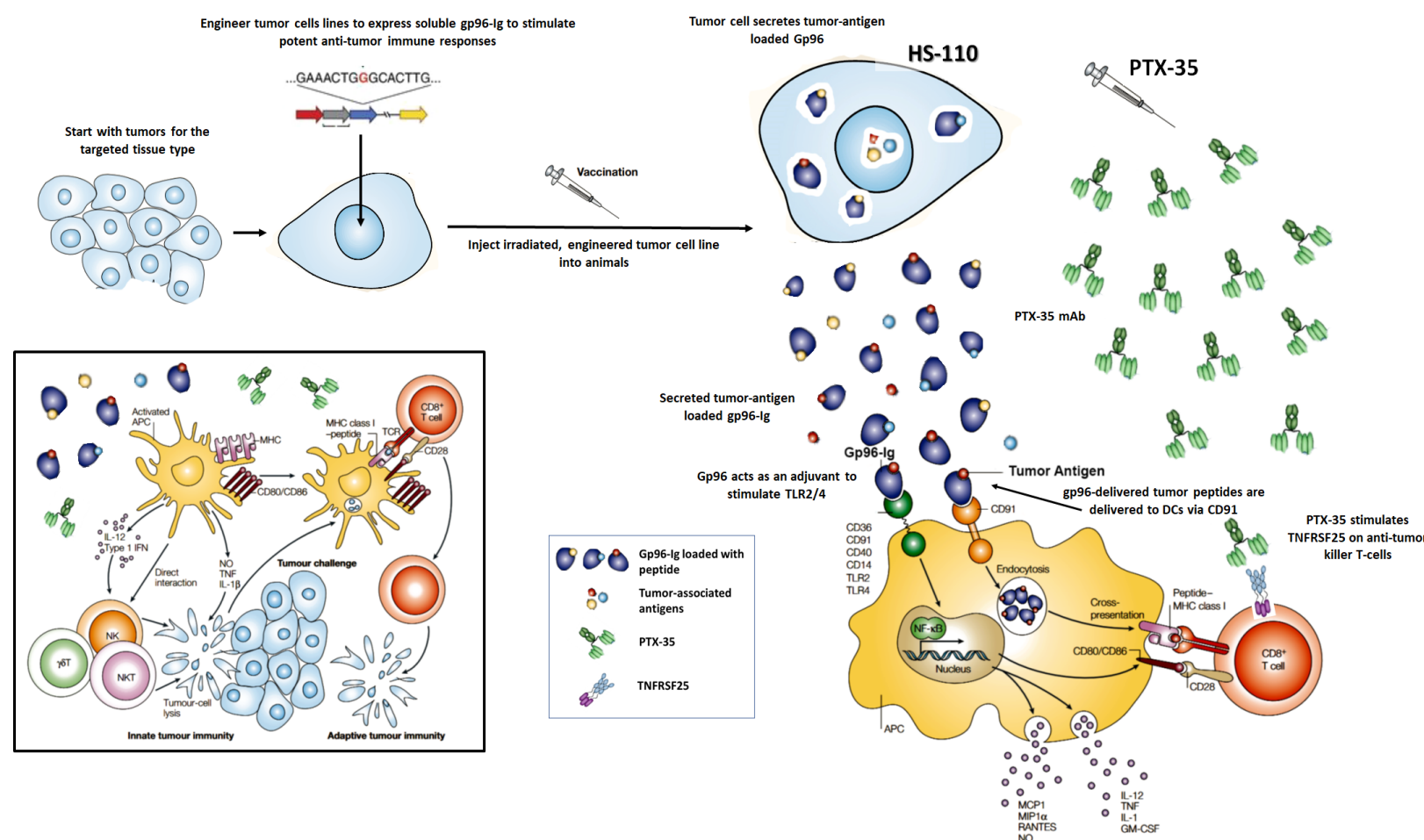


Figure 1: TNFRSF25 (DR3) Engagement by PTX-35 Synergizes with Cancer Immunotherapy  
Tissue-matched, allogeneic tumor lines are engineered to express soluble heat-shock protein, gp96, with the KDEL endoplasmic reticulum retention sequence replaced by an IgG1 Fc chain (HS-110). Since gp96 is responsible for folding endogenous proteins, many of which are destined for MHC-I loading; sequestered tumor peptides are delivered to antigen presenting cells (APCs) for CD8+ T-cell cross-presentation. Gp96 is a danger associated molecular pattern (DAMP) that stimulates inflammatory cascades via TLR2/4 stimulation and via uptake by scavenger receptor CD91. With the addition of a costimulatory TNFRSF25 agonist antibody, PTX-35, anti-tumor CD8+ T-cell responses are further enhanced. Activation of DCs by tumor-antigen loaded gp96 and CD8+ T-cell costimulation by PTX-35 can enhance the innate and adaptive arms of the immune system and synergize with antigen-driven immunotherapy to generate potent tumor-killing effector cells and long-term memory. *Figure originally adapted from Nichitta CV, Nature reviews Immunology, Vol-3, May 2003*

## Generation of Anti-TNFRSF25 Agonist Ab, PTX-35

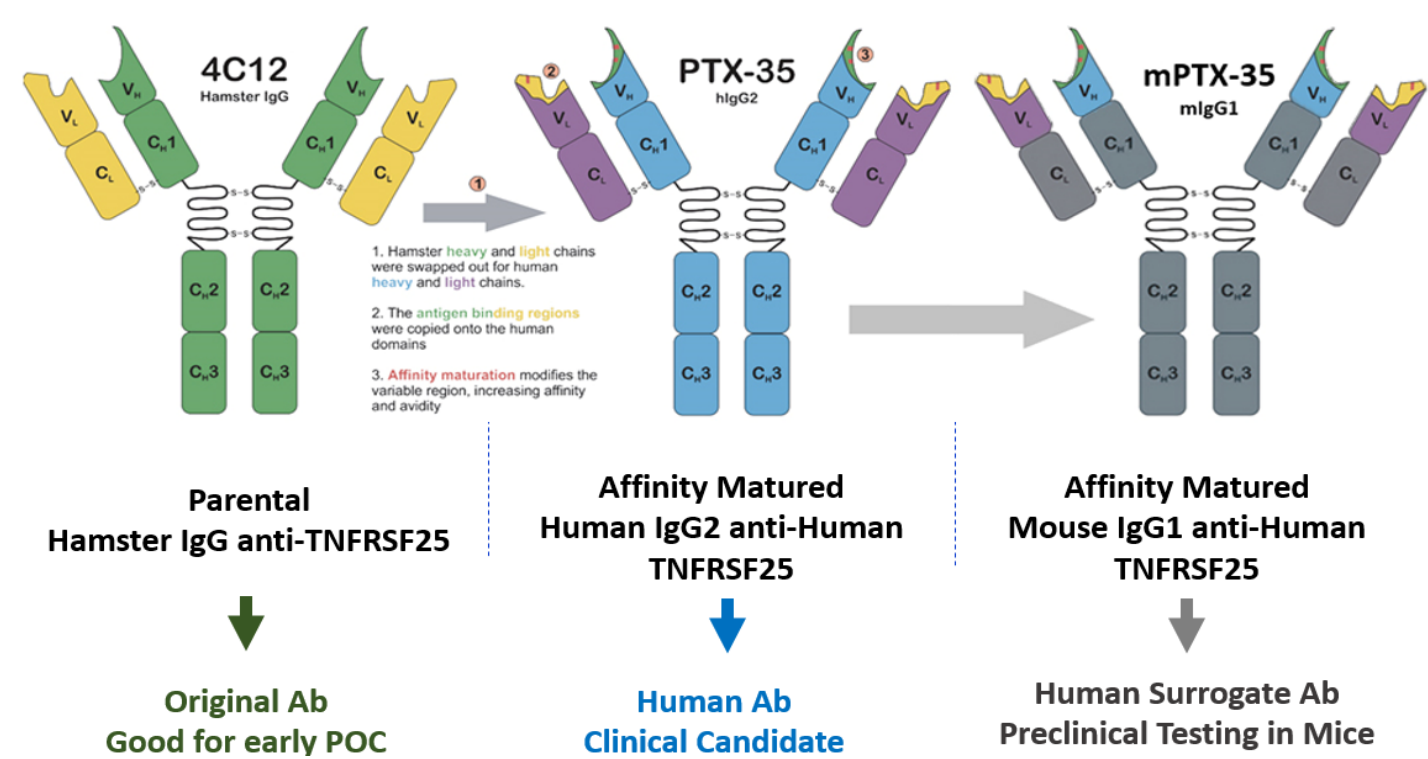


Figure 2: Generation of Human and Mouse Surrogate, Anti-TNFRSF25 mAb, PTX-35  
PTX-35 was developed by a humanization process of transferring the complementarity binding regions (CDRs) of the IgH and IgL chains from hamster antibody, 4C12, onto a human IgH and IgL chain framework backbone. PTX-35 was then matured by amino acid substitution to increase its affinity and strength of binding by modification in the variable binding regions. Mouse PTX-35 (mPTX-35) was generated by placing the human PTX-35 variable domains onto a mouse IgG1 backbone, to allow for proper in vivo Fc-Fc-gamma-R cross-linking and TNFRSF25 signaling.

## In Vitro Target Engagement & Affinity

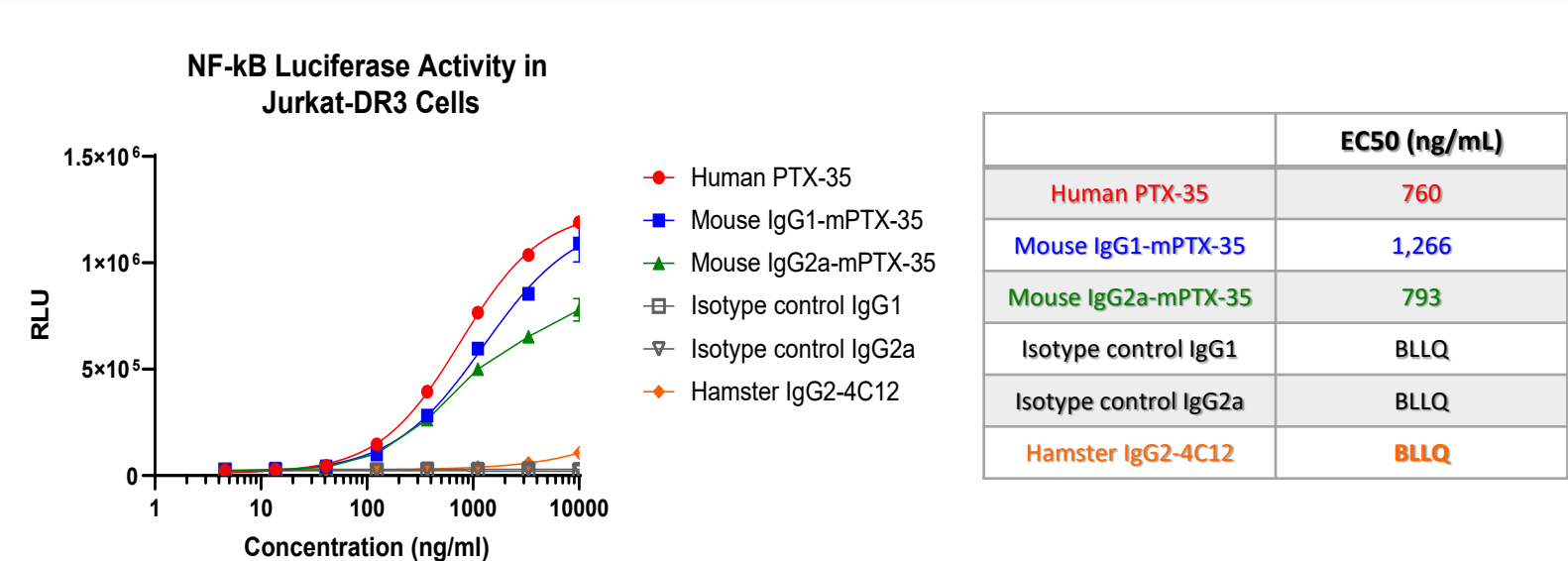


Figure 3: TNFRSF25/DR3-Relative Binding Affinity Determination in Jurkat Cells  
Serial dilutions of the human and mouse surrogate PTX-35 antibodies were made and dispensed into 96-well assay plates. 4C12, the precursor to PTX-35, and the IgG1 and IgG2a isotype controls were also tested. Jurkat-DR3 cells were then added to each of wells containing the antibody dilutions. The Jurkat-DR3 cells are a human T cell line that express the luciferase gene under the control of the NF- $\kappa$ B promoter that is activated upon stimulation of the death receptor 3 (DR3) signaling pathway. After incubation at 37°C for 6 hours, relative light units (RLU) were determined through use of the Bio-Glo<sup>TM</sup> luciferase assay system (Promega). BLLQ = Below lower limit of quantitation

## Costimulation Proof-of-Concept – Primary Human PBMCs

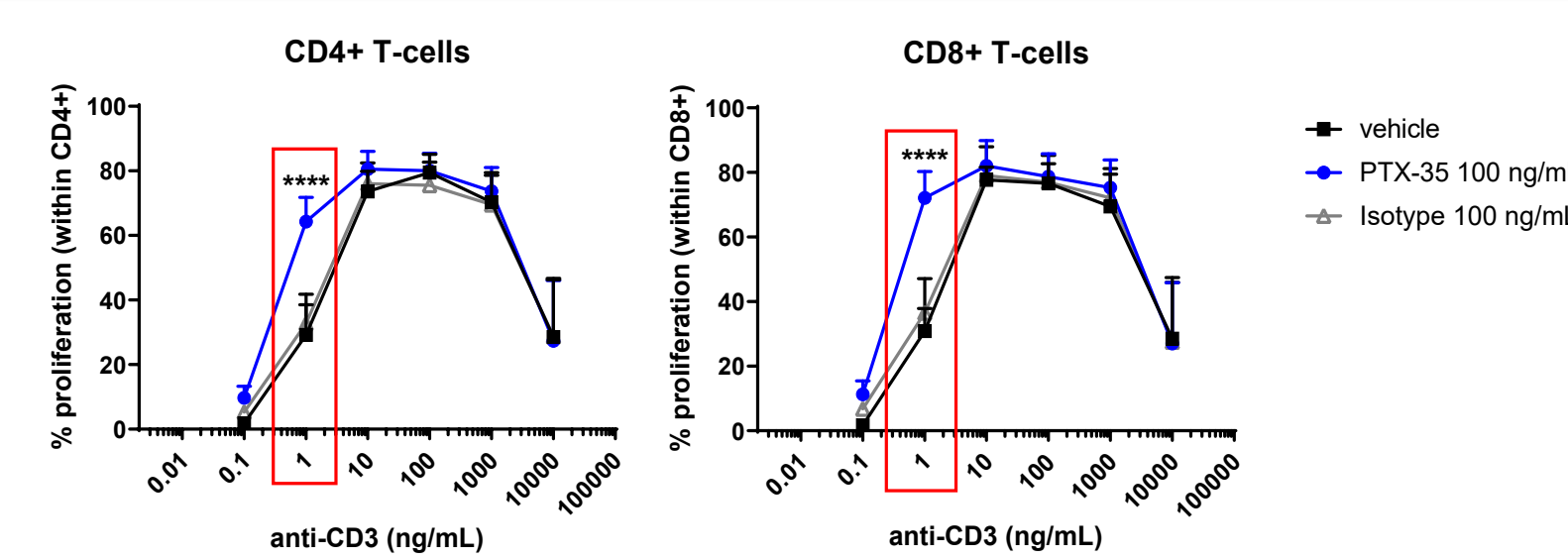


Figure 4: Plate-Bound Anti-CD3 Stimulated Human PBMCs in the Presence of PTX-35  
CFSE-labelled PBMC were stimulated with serial dilutions of plate-bound anti-CD3 for 72 hours in the presence of serial dilutions of test substance (PTX-35) or isotype control (hIgG2). Cells were then stained for viability, CD4 and CD8; and CFSE dilution was assessed by flow cytometry with gating on CD4 versus CD8 within the viable cell population. Data show the proportion of CD4+ and CD8+ T cells that divided and are presented as mean  $\pm$  SEM (n=4 biological replicates). \*\*\*\*p<0.0001 as determined using a repeated-measures two-way ANOVA with Dunnett's multiple comparison test comparing PTX-35 to isotype control.

## TNFRSF25-Agonism Synergizes with Immunotherapy

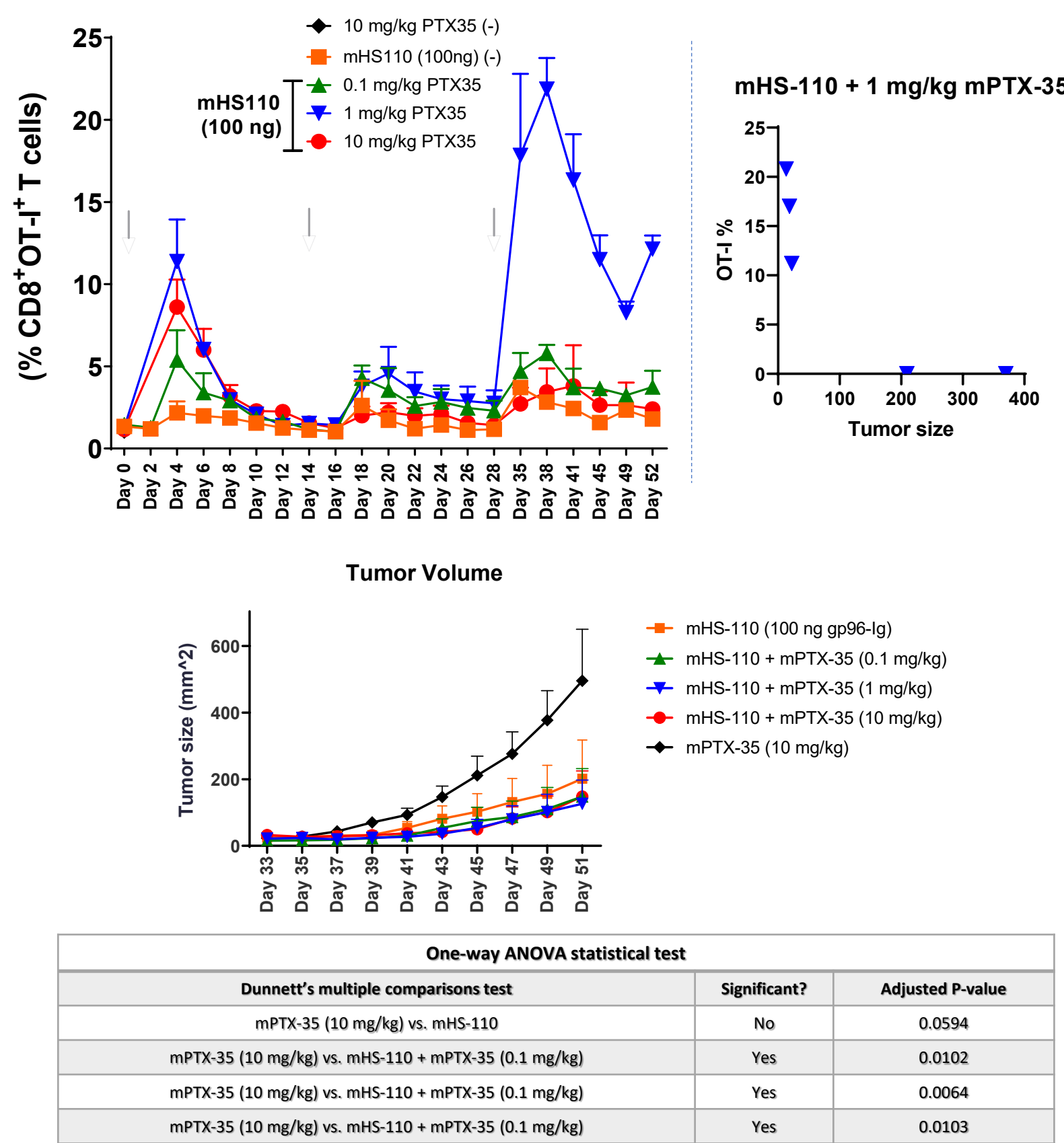


Figure 5: Synergistic Anti-Tumor and T-cell Activity of TNFRSF25 Agonism Combined with Antigen-Driven Cancer Immunotherapy  
(A) Purified, CD8+, OVA-specific, transgenic T-cell receptor, eGFP-OT-1 cells were transferred into C57BL/6 mice two days prior to challenge with vaccines and mPTX-35. Arrows indicate challenge dates; graph shows expanded OT-1 populations in the peripheral blood over time as determined by flow cytometry. (B) On day 28, B16F10-OVA tumor cells were implanted s.c. and growth was measured over time. (C) Tumor volume was matched against OT-1 expansion showing correlation.

## In Vivo, MABEL & NOEL Determination

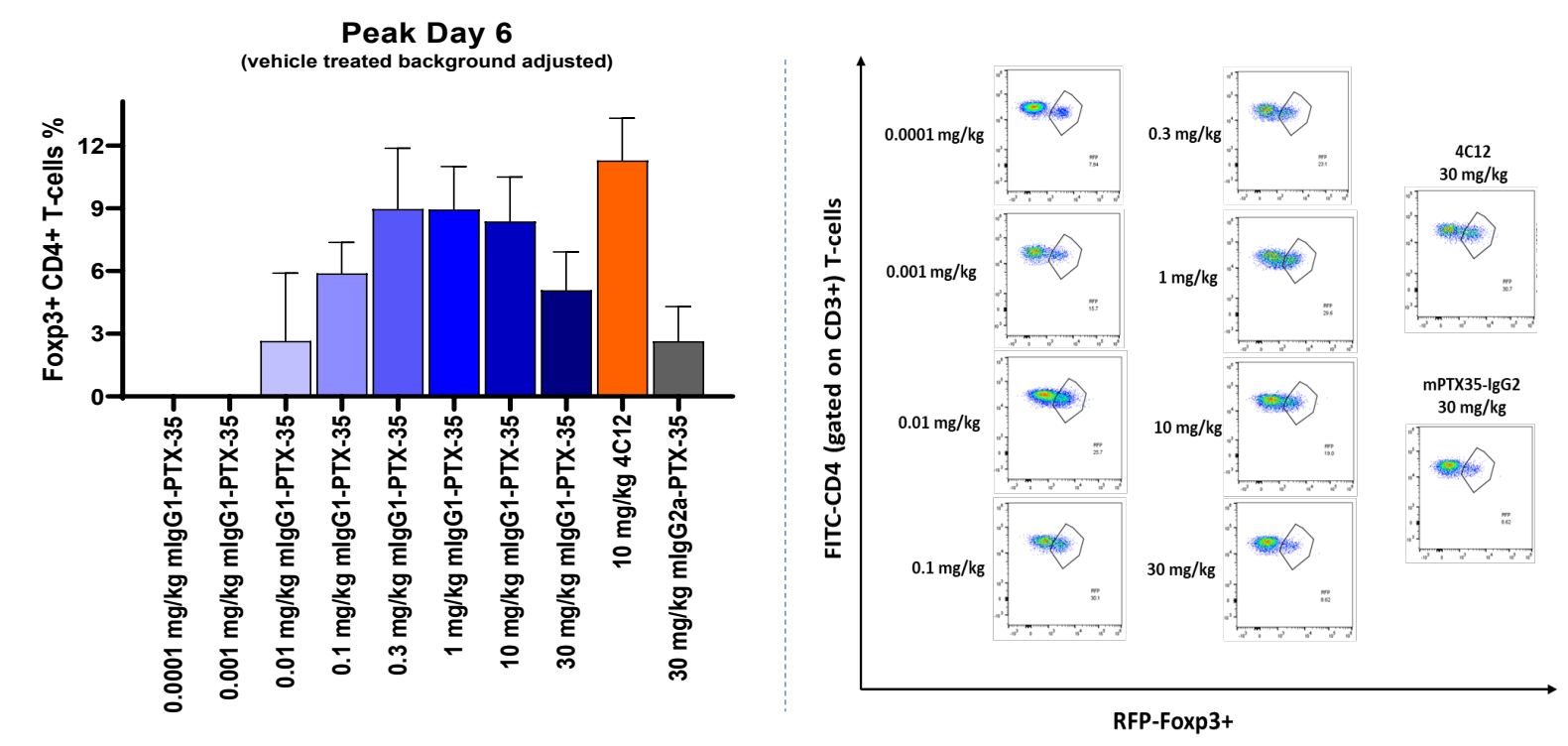


Figure 6: MABEL & NOEL Determination to Estimate Optimal Immunological Dose (OID) for Phase 1 Studies  
Using RFP-Foxp3 (FIR) transgenic mice, TNFRSF25 engagement in the absence of inflammation will drive the expansion of regulatory T-cells. Different concentrations of mouse PTX-35 was provided i.p., as a single injection, to FIR mice and regulatory T-cell expansion tracked in the peripheral blood over time by flow cytometry. The minimum anticipated biological effect level (MABEL) and no observed effect level (NOEL) were determined to be 0.01 mg/kg and 0.001 mg/kg, respectively. With a pharmacological active dose (PAD) range of 0.1 to 10 mg/kg.

## Pharmacodynamics in Non-Human Primates

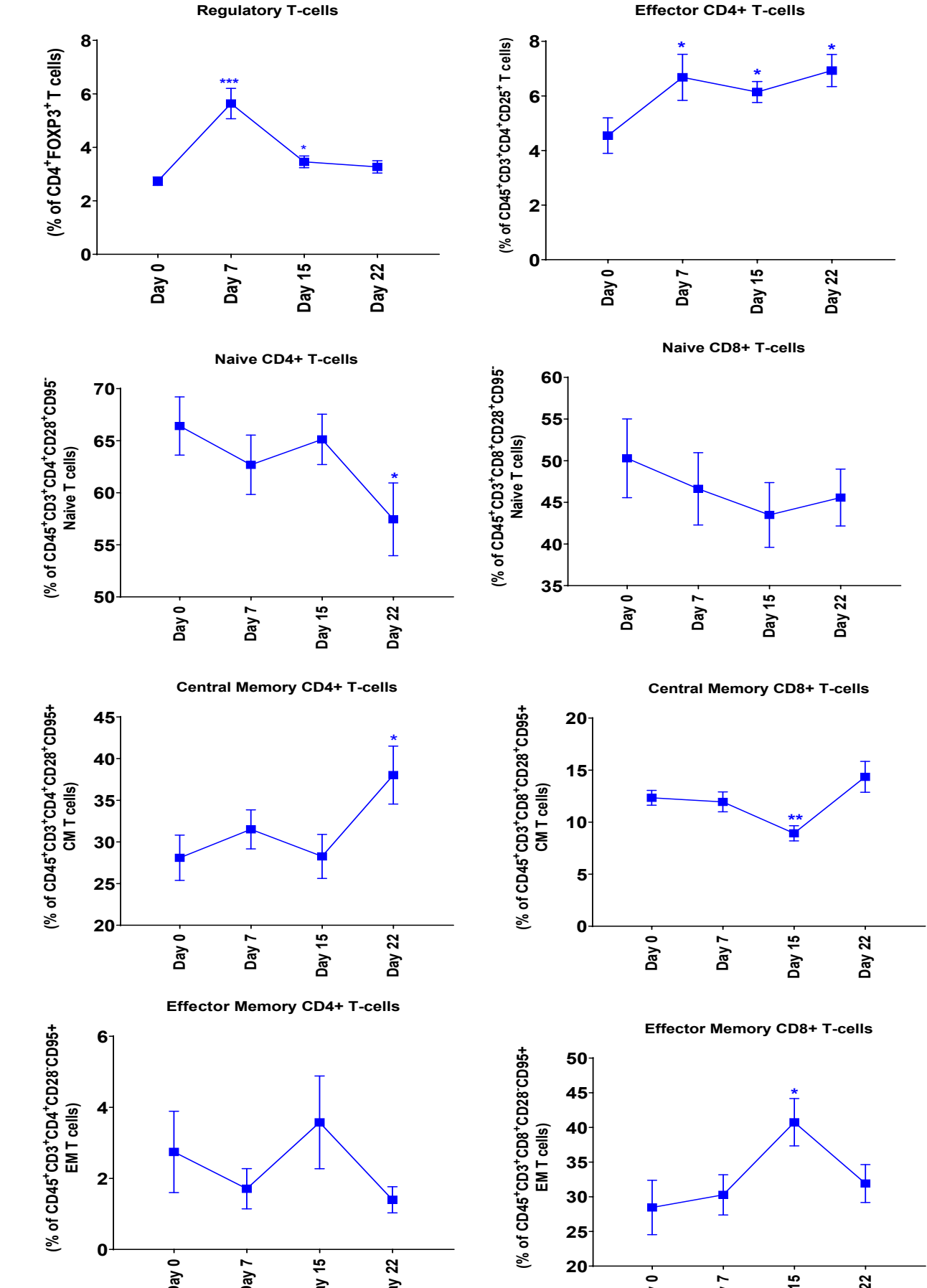


Figure 7: PTX-35 Expands Regulatory & Conventional T-cells in NHPs  
Human PTX-35 was tested in non-human primates (NHPs) in a dose ranging fashion by i.v. bolus injection at 1, 10, 50 and 96 mg/kg/dose, once every 14 days for a total of 2 doses. All doses resulted in plasma concentrations greater than 50-fold above the EC50, thus all groups were analyzed together. Monkeys were dosed on day 0 and 15. Graphs show flow cytometry of stained peripheral blood leukocytes, mean  $\pm$  SEM, statistics were Mann-Whitney, \*p<0.05, \*\*p<0.01.

## TNFRSF25-Agonism Does Not Increase Tumor Tregs

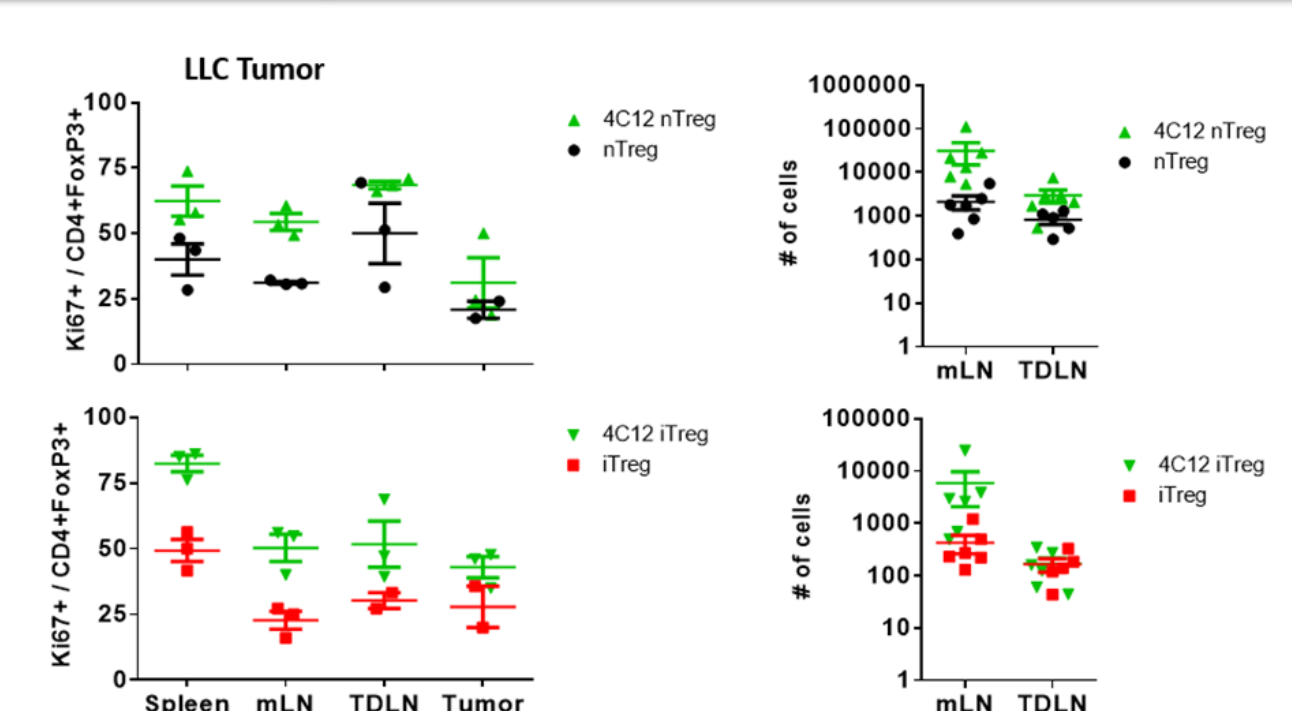


Figure 8: TNFRSF25-Agonism Does Not Increase Tumor Tregs  
Lewis lung carcinoma (LLC) tumors were established in mice, then treated with TNFRSF25-agonist, 4C12, alone. Spleen, mesenteric lymph node (mLN), tumor draining lymph node (TDLN) and tumor were analyzed for proliferating (Ki67) Foxp3+ CD4+ regulatory T-cells by percent and absolute number. Natural (nTreg) and induced (iTreg) cells were analyzed.

## Key Safety Findings - Summary

| Type                                                                                                                                  | Measured Parameter                              | Value                                                                           |
|---------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------|---------------------------------------------------------------------------------|
| Pharmacology                                                                                                                          | MABEL                                           | 0.01 mg/kg                                                                      |
|                                                                                                                                       | NOEL                                            | 0.001 mg/kg                                                                     |
|                                                                                                                                       | PAD                                             | 0.1 – 10 mg/kg                                                                  |
|                                                                                                                                       |                                                 |                                                                                 |
| Safety Pharmacology                                                                                                                   | Human PBMCs stimulated with anti-CD3 and PTX-35 | No increase in any deleterious cytokines, thirty-five different analytes tested |
| Tissue cross-reactivity                                                                                                               | Cytoplasmic and membrane                        | Human, monkey, mouse                                                            |
| Toxicology & TK                                                                                                                       |                                                 |                                                                                 |
| 28-day DRF in mouse                                                                                                                   | NOAEL                                           | 200 mg/kg                                                                       |
| i.v. bolus                                                                                                                            | C <sub>max</sub>                                | 2,440 ug/mL                                                                     |
|                                                                                                                                       | T <sub>max</sub>                                | 24 hrs                                                                          |
|                                                                                                                                       | AUC <sub>(0-216 hrs)</sub>                      | 390,000 hr*ug/mL                                                                |
| 2-week DRF in NHP                                                                                                                     | NOAEL                                           | 96 mg/kg/dose                                                                   |
| i.v. bolus                                                                                                                            | C <sub>max</sub>                                | 2,590 ug/mL                                                                     |
|                                                                                                                                       | AUC <sub>(0-360 hrs)</sub>                      | 261,500 hr*ug/mL                                                                |
| 8-week in NHP*                                                                                                                        | NOAEL                                           | 100 mg/kg                                                                       |
| i.v. bolus                                                                                                                            | C <sub>max</sub>                                | 2,660 ug/mL                                                                     |
|                                                                                                                                       | AUC <sub>(0-336 hrs)</sub>                      | 298,000 ug*hr/mL                                                                |
| *KLH immunization challenge demonstrated that PTX-35 had no deleterious effect on immunity or the ability to mount an immune response |                                                 |                                                                                 |

## Summary & Conclusions

- PTX-35 is a potent TNFRSF25 agonist mAb with a favorable safety profile targeted for combinations with antigen-driven cancer immunotherapy
- PTX-35 can expand conventional and regulatory T-cells in mouse, monkey and human without deleterious cytokine release or impact on mounting successful immune responses
- Five dose levels of PTX-35 will be explored in humans using traditional 3+3 design based on DLTs until OID is reached

## Acknowledgments & Contact Info

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## References

- Schreiber TH, Podack ER. Immunobiology of TNF $\alpha$  and TNFRSF25. Immunologic Research, December 2013, Volume 57 (issue 1-3), pp. 3-11
- Schreiber T.H., Levy R.B., and Podack E.R.; (2010). Therapeutic Treg expansion in mice by TNFRSF25 prevents allergic lung inflammation. J Clin Invest.; 120(10):3629-3640
- Melero L, Hirschhorn-Cymerman D, Morales-Kastresana A, Sanmamed M.F., and Wolchok J.D.; (2013). Agonist Antibodies to TNFR Molecules That Costimulate T and NK Cells. Clin Cancer Res.; 19(5); 1044-53
- Slebiada T.J. et al.; (2011). Triggering of TNFRSF25 promotes CD8+ T-cell responses and anti-tumor immunity. Eur J of Immunol 41 (9), 2606-2611
- Nishikii H. et al.; (2016). DR3 signaling modulates the function of Foxp3+ regulatory T cells and the severity of acute graft-versus-host disease. Blood 128 (24), 2846-2858