

# Pembrolizumab Enhances the Anti-Leukemia Activity of Antigen Specific Cytotoxic T Lymphocytes

Zara Italia<sup>1</sup>, Kathleen Ready<sup>1</sup>, Mao Zhang, MPH<sup>1</sup>, Maria R. Khouri, Pariya Sukhumalchandra, BS<sup>1</sup>, Anne V. Phillips, PhD<sup>1</sup>, Na Qiao, PhD<sup>1</sup>, Celine Kerros, PhD<sup>1</sup>, Lisa St. John, PhD<sup>1</sup>, Alexander A. Perakis, BS<sup>1</sup>, Daniel Ghaisariee<sup>1</sup>, Karen Clise-Dwyer, PhD<sup>1</sup>, Elizabeth Mittendorf, MD, PhD<sup>2</sup>, Naval G. Daver, MD<sup>3</sup>, Jeffrey J. Mollidrem, MD<sup>1</sup>, Qing Ma, PhD<sup>1</sup> and Gheath Alatrash, DO, PhD<sup>1</sup>

<sup>1</sup>Department of Stem Cell Transplantation & Cellular Therapy, The University of Texas MD Anderson Cancer Center, Houston, TX; <sup>2</sup>Department of Surgical Oncology, Dana-Farber/Brigham and Women's Cancer Center, Boston, MA, USA; <sup>3</sup>Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX

## Background

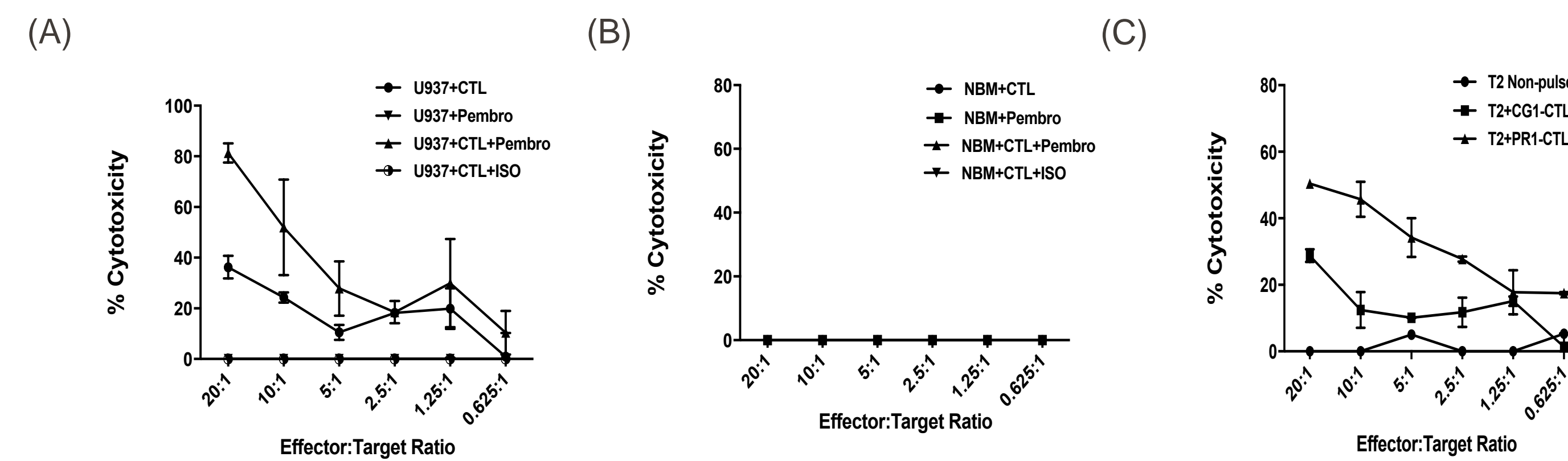
We have previously demonstrated that targeting leukemia-associated antigens, specifically PR1 and CG1, with antigen specific cytotoxic T lymphocytes (CTL) is a promising immunotherapeutic option. However, the immune response generated with this approach oftentimes fails to fully eradicate the underlying malignancy. The expression of immune inhibitory molecules, such as PD-L1 and its ligand PD1, by leukemia cells and leukemia-specific T cells, respectively, could be a contributor to the failure of cellular immunotherapy and allogeneic stem cell transplantation. It is, therefore, critical to investigate the role of immune modulation in combination with targeted cellular immunotherapy in the setting of acute myeloid leukemia (AML). Here, we specifically tested whether the anti-PD-1 agent, pembrolizumab (pembro), enhances the efficacy of CTLs that target the leukemia antigens PR1 and CG1.

## Experimental Design

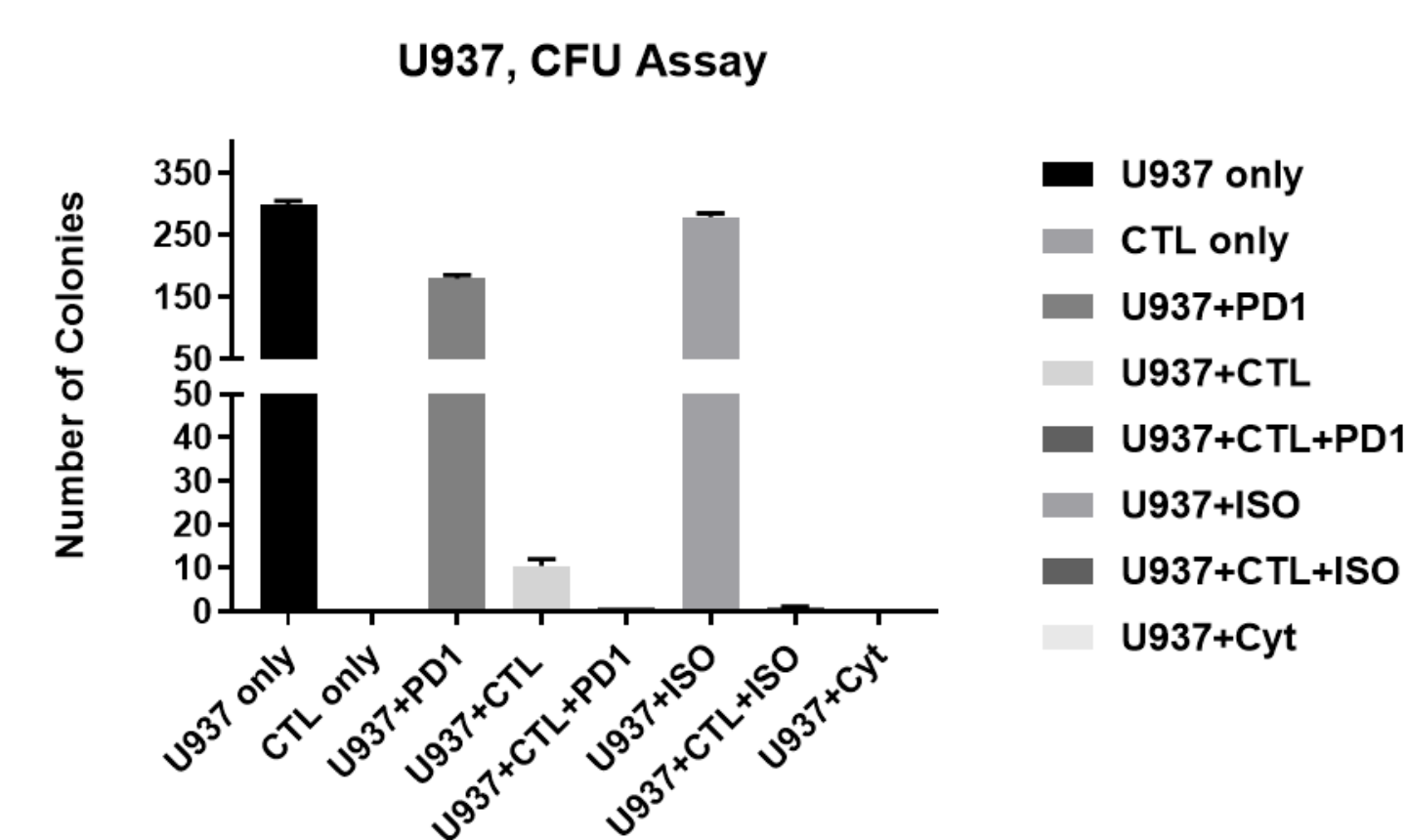
- CG1- and PR1-CTL:** To expand CG1 and PR1-specific CTLs, dendritic cells (DCs) were matured from adherent monocytes and then used as professional antigen presenting cells. Normal PBMC isolated from buffy coats were adhered to a six-well plate at 37° C in Macrophage Serum Free Medium. Lymphocytes from the same donor were separated and cocultured with 40 µg/mL of CG1 and PR1. Cells were then stimulated with interleukin (IL)-7 (10 ng/mL) and IL-2 (10 ng/mL) over 5 days. Adherent monocytes were matured into monocyte-derived DCs through the addition of granulocyte macrophage colony-stimulating factor (GM-CSF) (100ng/mL), IL-4 (50ng/mL), and tumor necrosis factor-α (25 ng/mL). After 5 days, DCs were detached from the six-well plates, cocultured with CG1 and PR1 peptides at 40 µg/mL and combined with the expanded lymphocyte population. Lymphocytes were expanded by coculture with mature DCs and stimulated with IL-7 (10 ng/mL) and IL-2 (25 ng/mL) for an additional 7 days. On day 14 of stimulation, cells were harvested and used in *in vitro* and *in vivo* assays.

- Cytotoxicity assay:** A standard cytotoxicity assay was used to determine specific lysis. Target cells, including T2, AML and normal bone marrow (NBM) cells were stained with calcein-AM for 15 min at 37° C. Stained cells were then washed three times in RPMI 1640, resuspended at  $2.0 \times 10^5$  cells/mL, plated onto a Terasaki plate, and cocultured with CG1-/PR1-specific CTLs at increasing effector: target ratios. After a 4-h incubation period, trypan blue was added to each well to quench fluorescence of dead cells. Fluorescence was measured using a Cytation 3 Imaging Reader. Positive control T2 cells were cultured overnight with soluble CG1 or PR1 (10 µg/mL), washed in RPMI 1640, and resuspended at  $1.0 \times 10^5$  cells/mL.

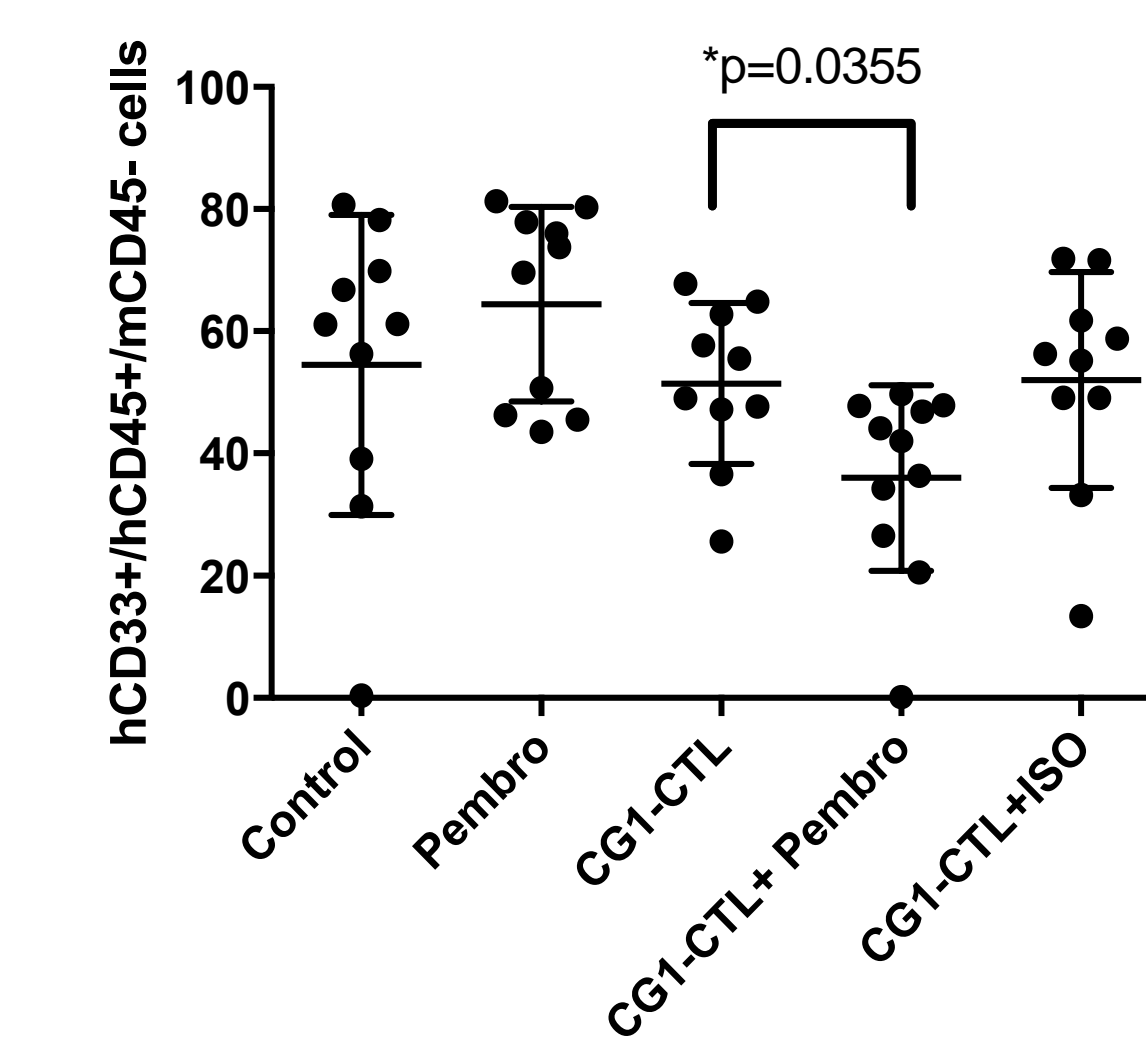
- Xenograft mouse model:** Sublethally irradiated (250 Rad) NOD scid gamma mice (NSG mice) were engrafted intravenously with 0.2-0.4 million HLA-A2-transduced U937 leukemia cells (U937-A2). On day 2, mice in the treatment groups were injected with CTLs (0.2-1 million). On day 3, mice were treated with pembro (100 µg/mouse) or isotype antibodies (100 µg/mouse). Mice were sacrificed on day 21. Marrow was processed into a single cell suspension and stained with human (h) CD45, hCD3, hCD33, and mouse CD45 antibodies. Samples were analyzed using flow cytometry. Treatment groups were divided into 5 groups: Control (only U937 cells), pembrolizumab only, CG1-CTL only, CG1-CTL+isotype, and CG1-CTL+pembrolizumab.



**Figure 1. Pembrolizumab enhances killing of AML by CG1- and PR1-specific cytotoxic T lymphocytes (CTLs).** (A, B) Peptide specific-CTL were co-cultured with target U937 cells or normal bone marrow (NBM) at various effector:target ratios. Cytotoxicity was determined by measurement of intracellular calcein-AM in live cells. (A) The addition of pembro to U937 cells co-cultured with CTL resulted in a two-fold increase in cytotoxicity compared to cells treated with CTL alone. (B) Addition of pembrolizumab had no effect on the killing of NBM by CTL. (C) T2 cells were loaded with calcein-AM, and then cocultured with CTLs for 4 hours at various effector:target ratios. T2 cell lines were pulsed with CG1- or PR1- peptides, or left without peptide pulsing (non-pulsed), as positive and negative controls, respectively. CTL=Cytotoxic T lymphocytes; pembro=pembrolizumab; ISO=Isotype.

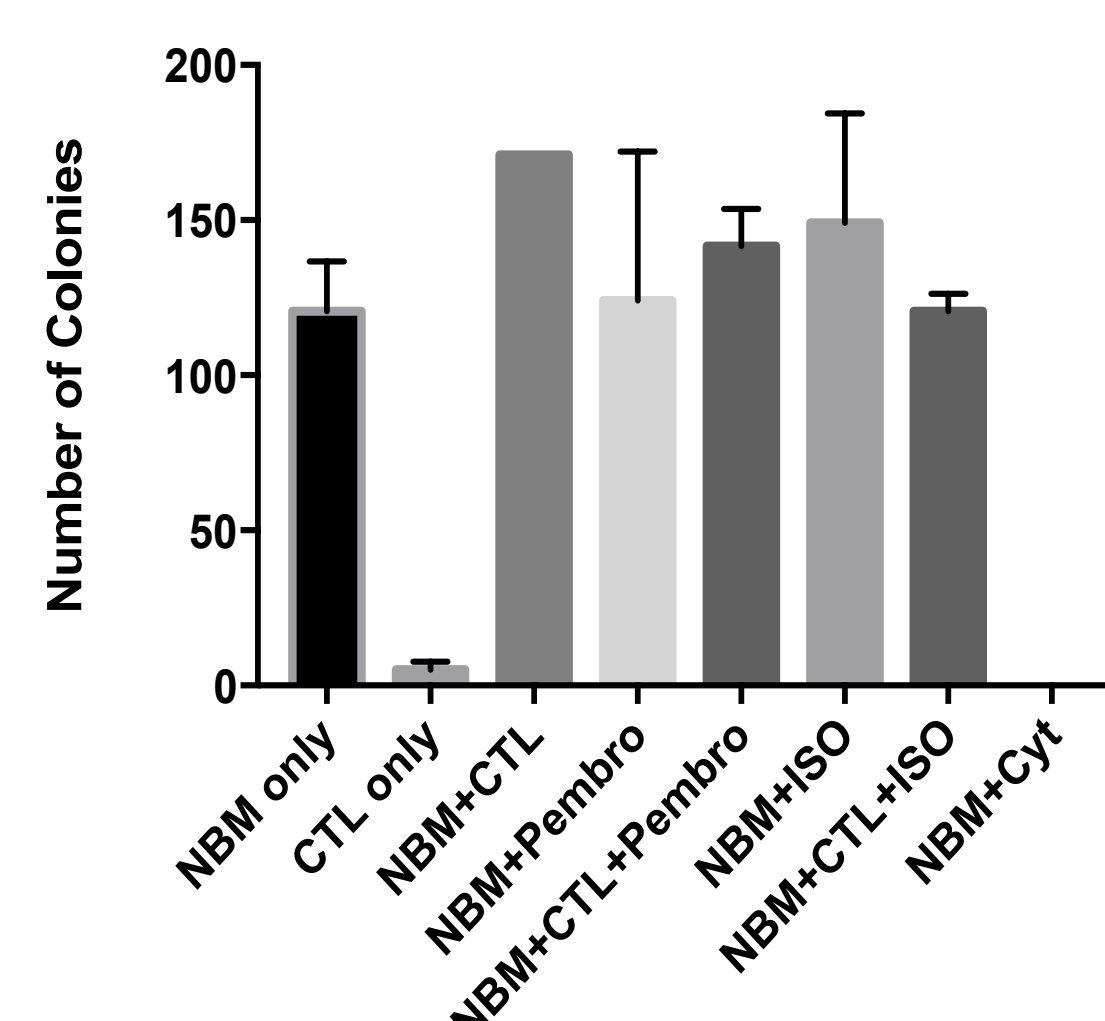


**Figure 2. Pembrolizumab enhances CTL activity against AML in CFU Assay.** Colony formation was detected in the U937+CTL condition, whereas colony formation in the U937+CTL+pembro condition was not observed. This highlights the role of pembrolizumab in enhancing CTL activity against leukemia cells. Cytarabine (Cyt) served as the positive control. CTL=Cytotoxic T lymphocytes; pembro, PD1 =pembrolizumab; ISO=Isotype.



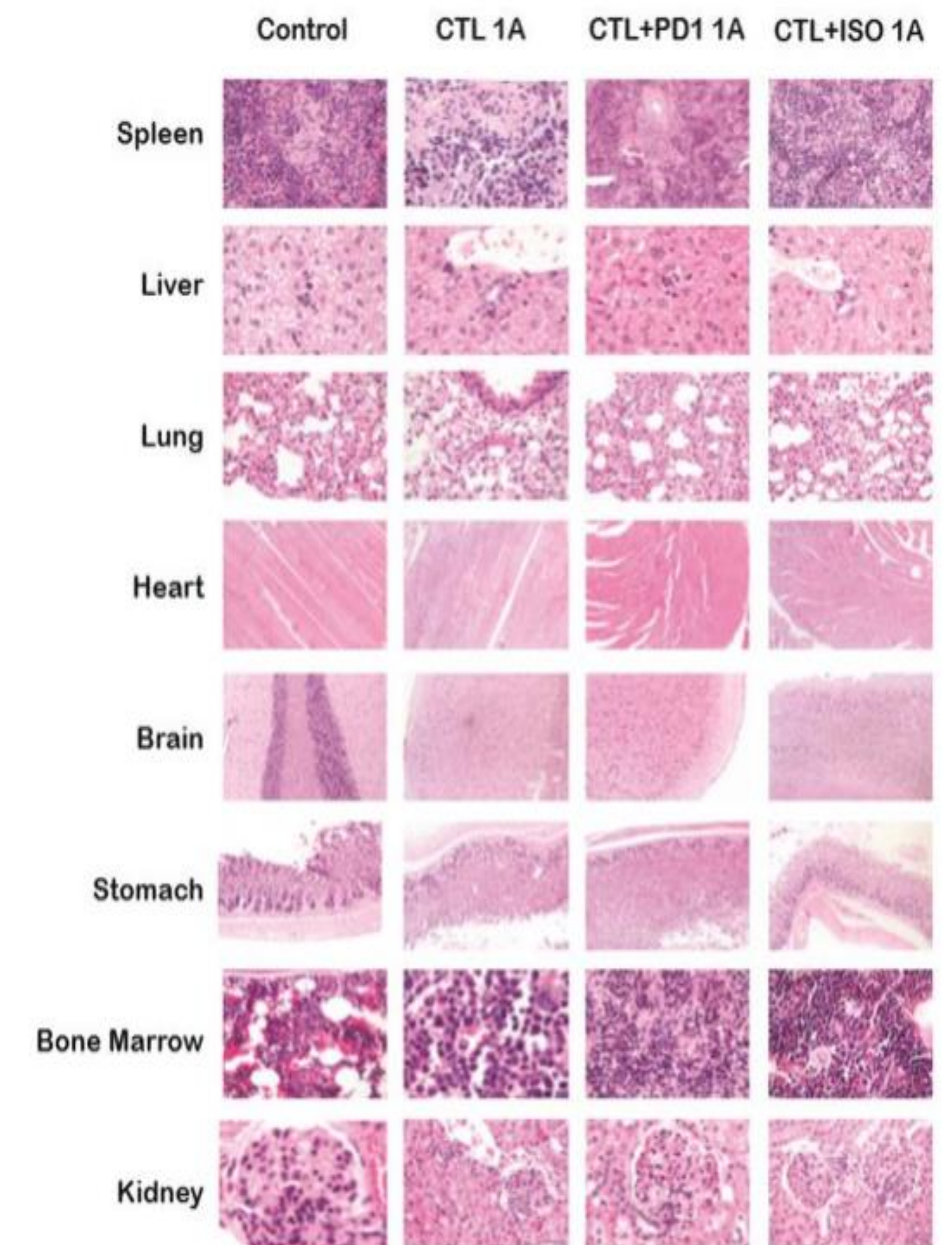
**Figure 3. Pembrolizumab enhances CTL activity *in vivo*.**

(A) Irradiated NSG mice were injected intravenously with U937 leukemia cells ( $0.2-0.4 \times 10^6$ ) on day 0. Mice were treated with CG1-CTL ( $0.2-1 \times 10^6$ ) +/- pembrolizumab or isotype (ISO) antibody or were left untreated. Mice were sacrificed for all groups when any mouse became moribund or during week 3. The results are expressed as percentage of CD33<sup>+</sup>/CD3<sup>-</sup> cells from viable hCD45<sup>+</sup>/mCD45<sup>-</sup> population within the bone marrow ( $p=0.0355$ ). CTL=Cytotoxic T lymphocytes; pembro=pembrolizumab; ISO=Isotype.



**Figure 4. Pembrolizumab does not alter CTL specificity.**

PR1- and CG1-CTL were co-cultured with normal bone marrow (NBM) for 14 days. Colonies were counted as a measure of hematopoiesis. Cytarabine (Cyt) was used as a control. CTL=Cytotoxic T lymphocytes; pembro=pembrolizumab; ISO=Isotype.



**Figure 5: Pembro does not alter CTL specificity to normal tissue.** Antigen-specific CTLs (about  $0.2 \times 10^6$  to  $1.0 \times 10^6$ ) were administered to mice twice weekly *i.v.* via tail vein. Mice were then treated with pembrolizumab 5 µg dose and frequency or isotype antibody or were left untreated. After two weeks, mice were sacrificed, and tissues were fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin prior to histologic examination. Data show equivalent effects on normal tissues between all groups

## Summary of Key Findings

- The addition of pembrolizumab to antigen-specific CTL resulted in an increase in cytotoxicity compared to cells treated with CTL alone (Figure 1).
- Colony formation confirmed enhanced killing of AML by CTL following the addition of pembrolizumab (Figure 2).
- In vivo* experiments showed enhanced killing of AML in mice treated with CTL+ pembrolizumab (Figure 3).
- In vitro* CFU assays using HLA-A2+ healthy donor bone marrow showed no change in the specificity of the antigen specific CTL following the addition of pembrolizumab. Specifically, we show a similar number of bone marrow colonies in the groups that were treated with pembrolizumab and no pembrolizumab (Figure 4).
- Pembro does not alter CTL specificity to normal tissue. (Figure 5).

## Conclusion

Pembrolizumab enhances killing of AML by CG1- and PR1-CTL. This strategy could prove beneficial in the setting of adoptive T cell therapy and allogeneic stem cell transplantation for AML.