

**Introduction**

- Beyond Cancer studies have shown that ultra-high concentration gaseous nitric oxide (gNO) induces an anti-tumor response in CT26 tumor-bearing mice.
  - Most of the CT26 tumor-bearing mice, whose primary tumors were treated with 20,000 and 50,000 parts per million (ppm) gNO, rejected a secondary tumor induction (63% and 89% respectively).
- In the current study we assessed the mode of action following a single gNO treatment.
- The effect on the primary tumor was assessed 14 days post treatment. The level of immune cells was assessed in the blood and spleen 21 days post treatment (figure 1).

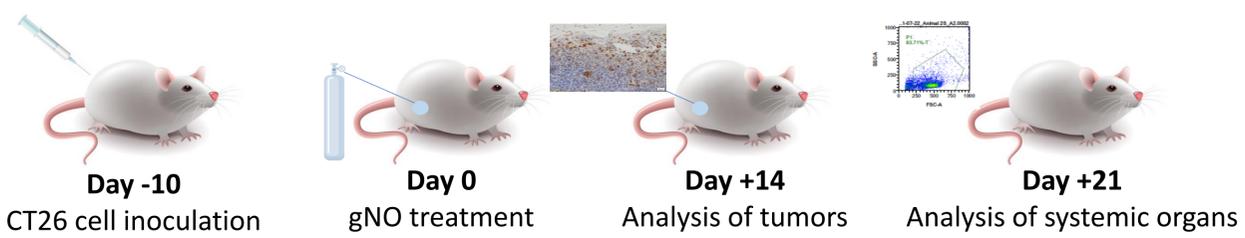


Figure 1. Assay scheme

**Results – The level of inflammation and lymphocyte Infiltration to the tumor 14 days post treatment**

- Treatment with 50,000 ppm gNO resulted in higher levels of inflammation, lymphocytes (figure 2A) and T-cells, B cells, macrophages and dendrocytes infiltration (figure 2C) compared to both nitrogen and 20,000 ppm NO, as viewed histologically.
- T cells penetrate to the tumor mass in the gNO treated groups, as opposed to the nitrogen treated group in which T cells are present at the margins of the tumor (figure 2B).

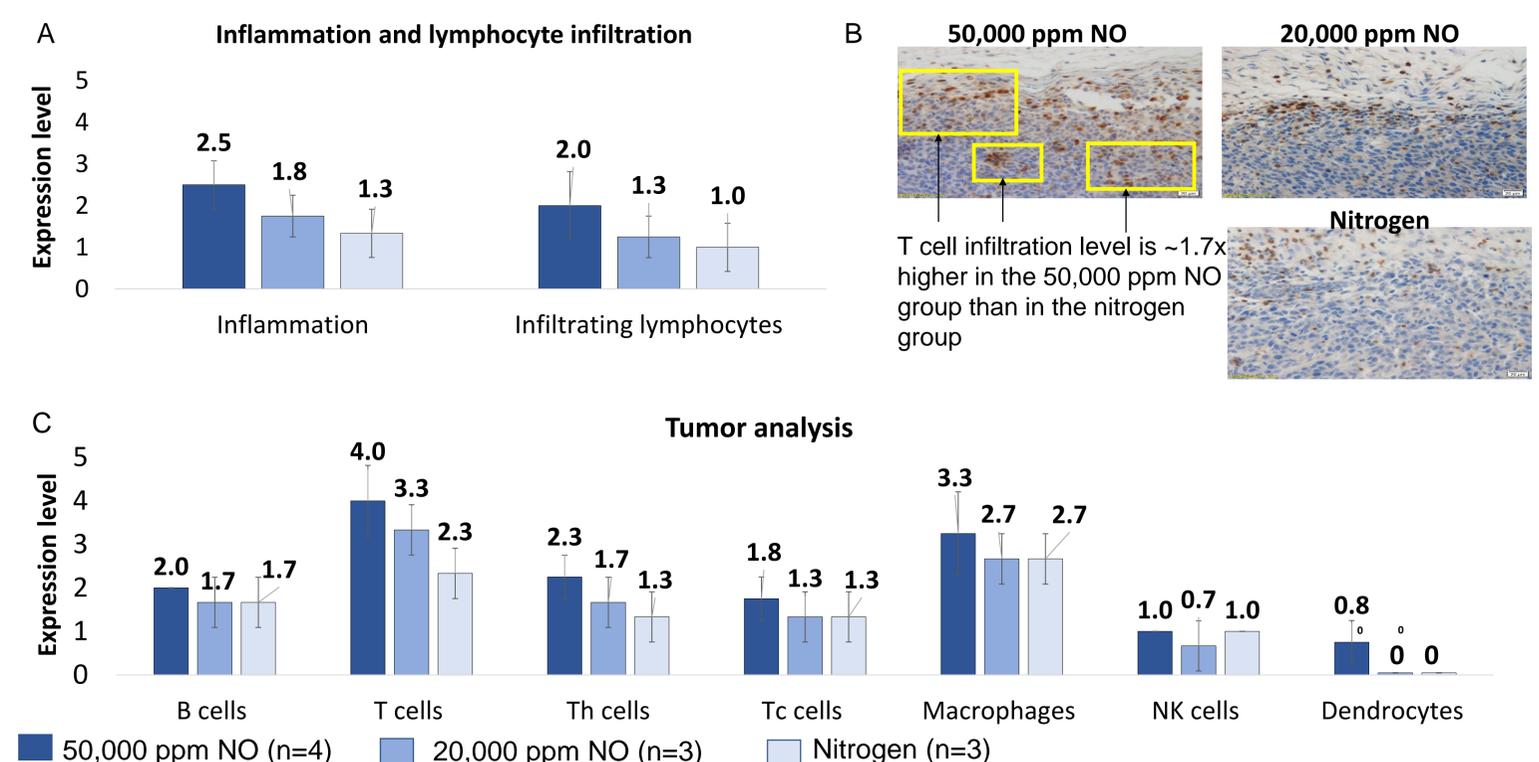


Figure 2. Histological and immunohistochemical analysis of gNO-treated tumors 14 days post treatment

**Results – Systemic immune cells in the blood and spleen and 21 days post NO treatment**

- Intra-tumoral injection of ultra-high doses of NO at 20,000 and 50,000 ppm gNO led to increased levels of IFN $\gamma$  T (table 1 and figure 3A) and B cells (figure 3B) and a decreased level of MDSCs (figure 3C) in the spleen.
- Elevated T cells and B cells were detected in the blood following NO treatment (figure 3D&E).

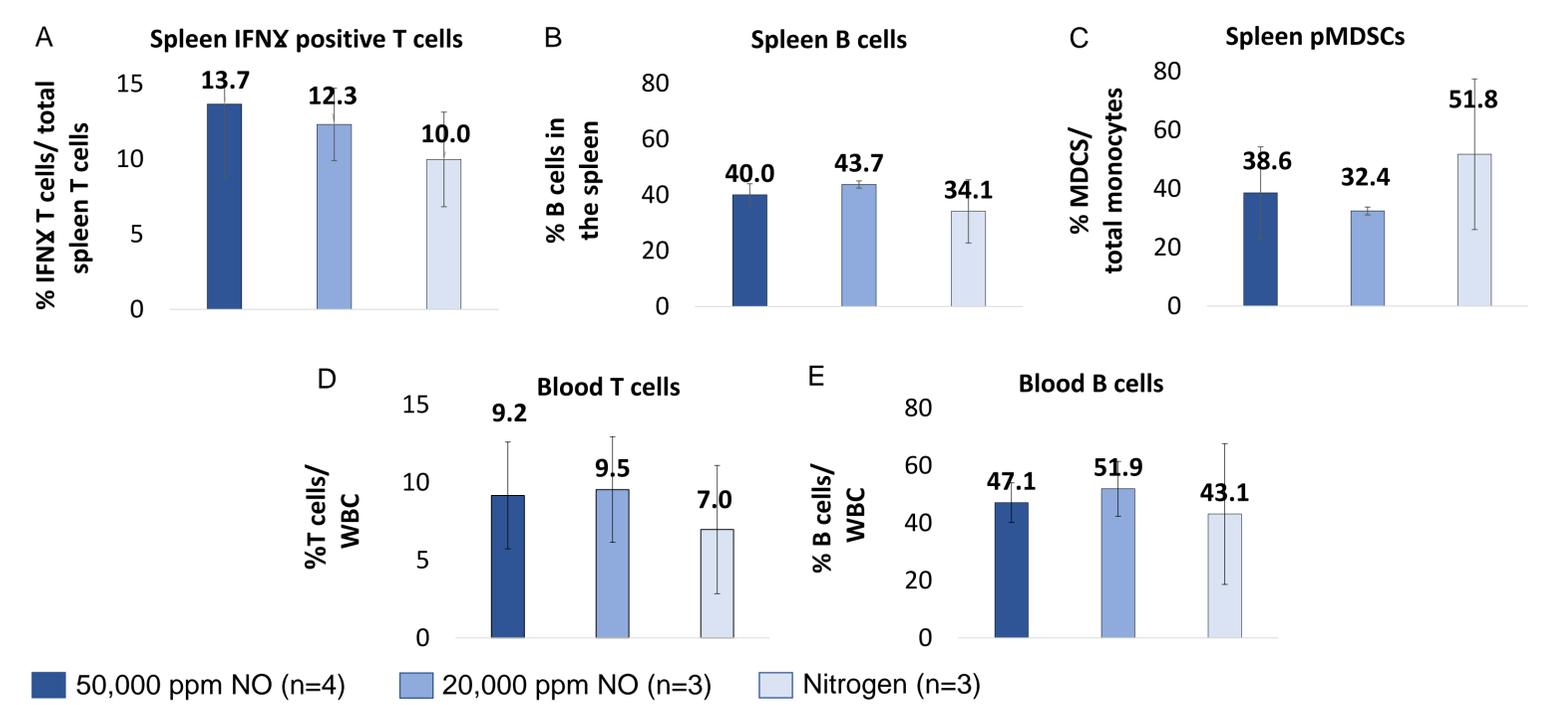


Table 1	%CD4+IFN $\gamma$ + T/T helper	%CD8+IFN $\gamma$ + T/T cytotoxic
50,000 ppm NO (n=4)	5.1 $\pm$ 2.2	36.1 $\pm$ 9.6
20,000 ppm NO (n=3)	4.5 $\pm$ 0.7	32.1 $\pm$ 5.0
Nitrogen (n=3)	3.7 $\pm$ 1.0	28.9 $\pm$ 5.1

Figure 3. Flow cytometry analysis of spleen and blood samples 21 days post treatment. Table 1. IFN $\gamma$  positive helper and cytotoxic T cells in the spleen 21 days post treatment

**Conclusions**

- Intra-tumoral injection of ultra-high concentrations of NO at 20,000 and 50,000 ppm gNO led to increased recruitment of lymphocytes and T cells, B cells, macrophages and dendrocytes to the primary tumor.
- Increased T cells and B cells were detected in the spleen and blood 21 days post NO treatment.
- Decreased pMDSCs were detected in the spleen 21 days post NO treatment.
- The data suggest that gNO induced innate and adaptive immune cell populations, and the reduction of immune suppressor cells, are indicative of an anti-tumor immune response that underlies the rejection of secondary tumors in gNO-treated mice.