# Downregulation of T Cell Activity by Cyclophosphamide-Induced Myeloid Derived Suppressor Cells is Reversed by the Glutathione Disulfide Mimetic NOV-002

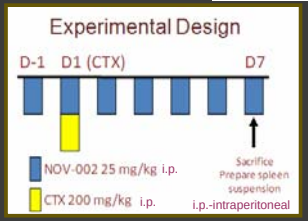
**C. Marcela Díaz-Montero,[1] Min Xu,[2] Osama Naga,[2] Chris Pazoles,[3] Alberto J. Montero[1]**

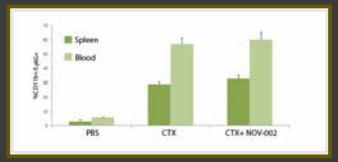
**University of Miami,[1] Miami, FL, Medical University of South Carolina,[2] Charleston, SC, Novelos Therapeutics,[3] Newton, MA**

## ABSTRACT

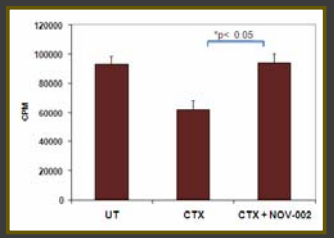
NOV-002 is a unique glutathione disulfide mimetic that in early phase clinical trials has been shown to significantly enhance the anti-tumor activity of various types of cytotoxic chemotherapy in many different solid tumors, e.g. non-small cell lung cancer, ovarian cancer, and breast cancer. NOV-002 has been shown to have direct immunomodulatory effects in patients, e.g. increases in circulating activated CD4+ T lymphocytes and NK cells. This is consistent with other data showing that small changes in intracellular lymphocyte GSH/GSSG levels have profound effects on T-lymphocyte function and survival. We have previously shown that cyclophosphamide (CTX) at doses used as part of standard adjuvant therapy for breast cancer leads to transient increases in myeloid derived suppressor cells (MDSC), and that CTX-derived MDSC have suppressive effects similar to tumor derived MDSC. Although the exact mechanism of action of NOV-002 is unknown, one hypothesis is that NOV-002 improves T-cell activation through a direct action on MDSC by decreasing production of reactive oxygen species (ROS). To test this hypothesis we utilized a model of CTX-induced MDSCs. A single intraperitoneal injection of CTX (4mg) results in transient lymphopenia. The recovery phase is characterized by a surge of MDSCs in both peripheral blood and spleen. Our results showed that NOV-002 restored MDSC downregulation of T cell activation in response to antigenic stimulation. In our model, CTX-induced MDSCs showed decreased downregulatory capabilities after in vivo treatment with NOV-002. Although the overall levels of circulating MDSCs remained unchanged, NOV-002 treated MDSCs had decreased ROS production, even after blockade of glutathione synthesis with buthionine sulfoximine. Moreover, NOV-002 treated MDSCs showed lower expression levels of iNOS. In conclusion, one immunomodulatory effect of NOV-002 could be through alteration of GSH/GSSG levels in MDSCs resulting in decreased generation of ROS and hence decreased suppressive capabilities.

## Effect of NOV-002 on MDSC mediated suppression of *in vitro* T cell activation



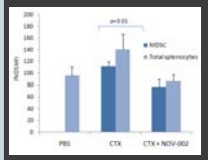




Levels of myeloid derived suppressor cells (MDSC) which are CD11b+/Ly6G+ were determined at day 7 post-CTX injection in spleen and blood by flow cytometry. Treatment with NOV-002 had no significant effect on CTX-induced mobilization of MDSCs.



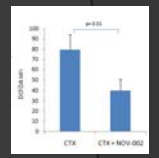

Splenocytes from treated mice (as indicated) were co-cultured with purified Pmel cells. Pmel cells are transgenic for a TCR specific for a peptide present in the gp100 protein. Pmel cells were activated with gp100 peptide and cell proliferation was measured by thymidine incorporation. Pmel cells co-cultured with splenocytes from CTX treated mice treated demonstrated a 40% decrease in their proliferative capacity when compared with untreated controls. The decrease in proliferative capacity was absent when pmel cells were co-cultured with splenocytes from mice treated with CTX and NOV-002.

## NOV-002 decreases iNOS expression and ROS production in CTX-induced MDSC



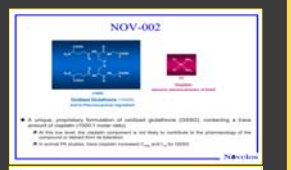

Splenocytes from mice treated as indicated were stained with mAbs against Ly6G, CD11b, and iNOS. Intracellular expression levels of iNOS was measured in MDSCs (Ly6G+/CD11b+) and total splenocytes. iNOS levels are expressed as the average (n=6 from two independent experiments) Mean Fluorescence Intensity (MFI) ± SDEV. There was a significant decrease in the levels of expression of iNOS in both MDSCs and total splenocytes after the addition of NOV-002 to CTX treatment. iNOS expression in MDSCs from PBS treated animals was not shown since the frequency of circulating MDSCs are very low (<5%).

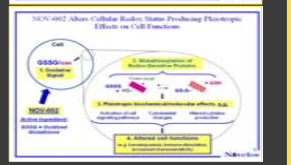
Generation of Reactive Oxygen Species (ROS) was determined by flow cytometry using the fluorescent probe CM2-DCFDA. Fluorescence was detected on the Ly6G+/CD11b+ population and is expressed as MFI. Treatment of mice with NOV-002 significantly reduced ROS levels in CTX-induced MDSC.




## NOV-002 Background

- The active ingredient in NOV-002 is **oxidized glutathione**
- Changes in the ratio of oxidized: reduced glutathione controls cellular redox state and can regulate protein function by the reversible formation of mixed disulfides between protein cysteines and glutathione (= **glutathionylation)**.
- Protein glutathionylation by NOV-002 results in pleiotropic effects on cell functions including cell signaling pathways, cytoskeletal architecture and cytokine production and is associated with hematopoiesis, immune stimulation and increased chemosensitivity of tumor cells.
- NOV-002, in combination with standard chemotherapy, is also the subject of an ongoing pivotal Phase 3 trial in advanced non-small cell lung cancer and two phase 2 trials: (i) in combination with doxorubicin-cyclophosphamide and docetaxel as part of neoadjuvant treatment of breast cancer; and (ii) in combination with carboplatin in platinum refractory ovarian cancer.



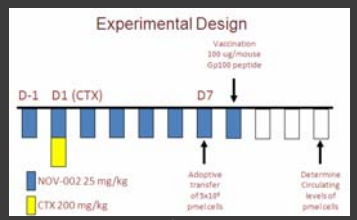



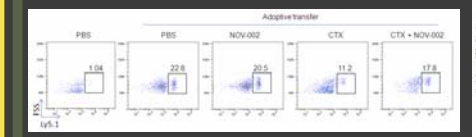

**References**

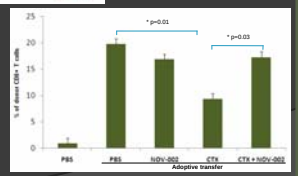
Townsend, DM et al. *Cancer Res* 2008; 68 (8): 2870-2877.

Townsend, DM et al. *Exp Opin Invest Drugs 2008; 17 (7): 1075-1083*

## Effect of NOV-002 on MDSC mediated suppression of *in vivo* T cell activation




On day 3 after vaccination, peripheral blood was collected, and analyzed by flow cytometry for the presence of donor Pmel cells, vis-à-vis donor Ly5.1 expression. Dot plots on the left show data from a representative animal. PBMCs were stained with anti- CD8 and anti-Ly5.1 mAbs. Data shows the percentage of donor (Ly5.1+) Pmel cells gated on the CD8+ fraction. Figure on the right shows the average of the percentage of donor CD8+ T cells in each experimental group ± SDEV. A significantly lower frequency of donor Pmel cells in the animals treated with CTX was observed. The addition of daily NOV-002 to CTX treatment, however restored frequencies of activated Pmel cells to levels similar to PBS controls.

## SUMMARY

•Recovery from CTX-induced lymphopenia results in MDSC accumulation, which in turn are able to directly suppress T cell activation.

•Daily NOV-002 administration resulted in a significant reversal of MDSC-induced suppression of T cell activation. This was observed:

- •*Ex vivo* following antigen-dependant *in vitro* activation of Pmel cells co-cultured with MDSCs from NOV-002 treated mice
- •I*n vivo* following vaccination after adoptive transfer of naïve Pmel cells in mice treated with NOV-002.

•MDSCs from mice treated with NOV-002 showed decreased iNOS expression levels, which also correlated with significantly decreased ROS production. Since a major mechanism of MDSC mediated T-cell suppression is through ROS accumulation, NOV-002 appears to have a direct immunomodulatory effect on MDSC by decreasing ROS.