# Modulating *In Vivo* T-cell Activation: 15 color Immunophenotyping, Cytokine Analysis and Cellular Redistribution

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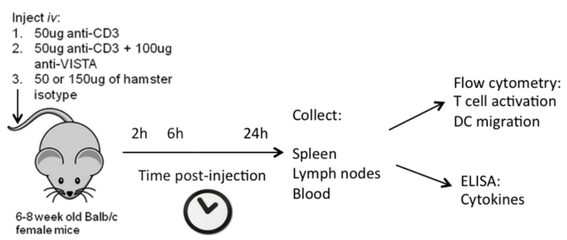
## Abstract

Understanding the mechanisms for modulating T-cell activation and inhibiting activated dendritic cell migration is important in our attempt to control key aspects of T cell regulation, from how to most effectively combat immune response dysregulation to the suppression of transplant rejection. The efficacy of models for T-cell activation and suppression can vary *in vivo* vs. *in vitro*. In this application, modulation of T-cell specific activation was achieved *in vivo* in a murine model through the injection of anti-CD3ε low-endotoxin, azide-free (LEAF) antibody with or without LEAF purified anti-PD-1H/VISTA co-injected. Using a 15 color flow cytometric assay, the kinetics of activation were monitored through multiple cell surface markers, early to late, and changes in cellular distribution from spleen to draining lymph nodes were compared. Dynamics of cytokine production were also monitored using ELISA assays.

## Introduction

Selective modulation of T-cell activation has implications ranging from transplant rejection to cancer immunotherapy. Employing low-endotoxin azide-free (LEAF™) antibodies *in vivo* can offer a convenient model for intervening in the T-cell activation pathway. Anti-CD3ε (clone 145-2C11) injection, for example, has been shown extensively to selectively stimulate T-cells *in vivo*. Anti-VISTA (V-domain Ig suppressor of T-cell activation), a PD-1 homolog, on the other hand, has demonstrated T-cell selective suppression in models of autoimmunity and immune surveillance. However, there is limited published data that profiles anti-VISTA activity. In this application, we apply a 15 color flow cytometry panel to assess the kinetics of T-cell and dendritic cell activation, the cellular distribution of dendritic cells for comparison with total cytokine content in serum at each time point.

**Figure 1.** *In Vivo* Protocol



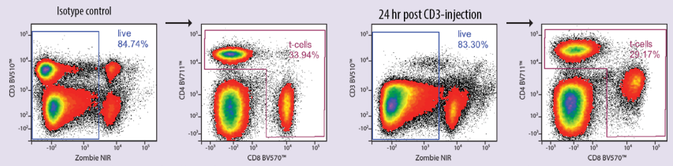

Three conditions were compared, anti-CD3ε injection alone, anti-CD3 and anti-VISTA co-injection and the isotype injected negative control. Three animals were included for each condition and time point, 2, 6 and 24 hrs. Spleen, draining lymph nodes and serum were collected and cells were analyzed unfixed by flow cytometry.

**Figure 2.** Flow Cytometric Immunophenotyping Panel

| | | | |
|---|---|---|---|
| Zombie NIR™ | CD3 BV510™ | CD4 BV711™ | CD8 BV570™ |
| CD19 BV650™ | CD49b APC | CD62L Alexa Fluor® 488 | I-A/I-E Alexa Fluor® 700 |
| CD44 BV785™ | CD69 PerCP/Cy5.5 | ICOS PE/Cy5 | CD11c PE/Cy7 |
| PD-L1 BV421™ | PD-1 BV605™ | FasL PE | |

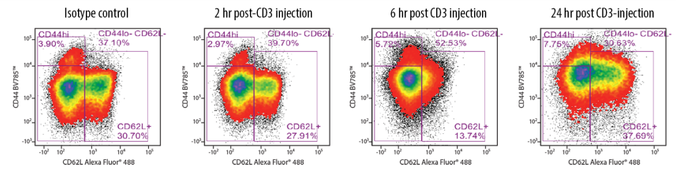
Immunophenotypic panel used to monitor cellular changes and distribution between time points and treatments. FMO (fluorescence minus one) controls were used to determine the gating strategy for every sample and parameter.

**Figure 3.** Anti-CD3ε injection caused cell surface CD3 down-regulation



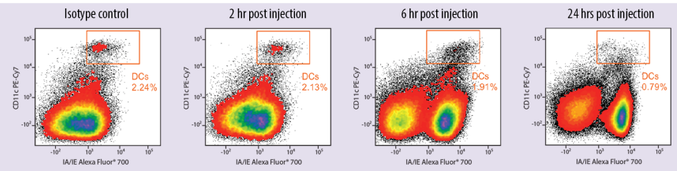

The injection of anti-CD3ε (145-2C11) resulted in a time dependent down-regulation of cell surface CD3, in this instance detected with the 17A2 clone against CD3 to avoid competitive binding of identical epitopes (Fig 3). CD3 down-regulation did not, however, affect the CD4 vs CD8 distribution of total live cells.

**Figure 4.** Total T-cell activation in lymph node



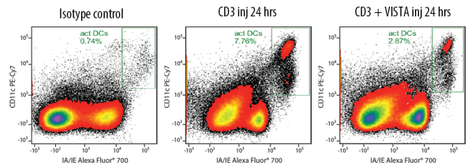

Total T-cell activation monitored by CD44 vs. CD62L also wasn't affected by CD3 down regulation. CD62L expression indicates a naïve T-cell. Down-regulation of CD62L and an increase in CD44 expression indicates a transition towards an activated, effector memory T-cell subset. At 2 hours, the CD62L down-regulation in both spleen and lymph node (Fig 4) ramps up, peaking at 6 hours and then shows some recovery at 24 hours. CD44 expression steadily increases through 6 hours, peaking at 24 hours.

**Figure 5.** Anti-CD3ε injection induces Dendritic Cell depletion in the spleen



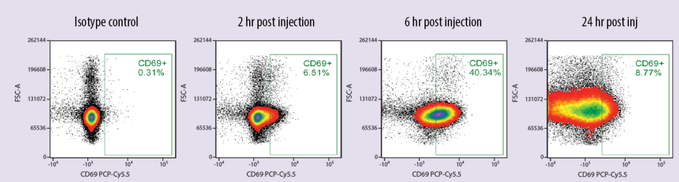

In the spleen, the resident DC population was characterized by an MHCII$^+$/CD11c$^+$ phenotype. Over the time course of the treatment, the dendritic cells showed significant changes in distribution, especially at 24 hrs. This correlates with a decrease in the spleen and a simultaneous increase in the lymph nodes (Fig 5). There was no statistically significant effect of the VISTA co-injection on splenic DC percentage.

**Figure 6.** Anti-VISTA modulates accumulation of Dendritic Cells in draining lymph nodes




However, there was a marked change in MHCII expression in lymph node DCs. There was also a significant effect on the CD3ε/ VISTA co-injected animals versus CD3ε alone treatment at 6 hrs (p=0.02) and 24hrs (p=0.002) (Fig 6.)

**Figure 7.** Anti-CD3ε-activated T-cells as gated in Figure 3 increase CD69 expression in lymph node




CD69 and ICOS were used to indicate early and late activation respectively. CD69 demonstrated very quick response to CD3ε stimulation with significant changes (p=<0.000006) in expression over time (Fig 7). However, anti-VISTA treatment did not significantly affect CD69 expression in the spleen or lymph node.

**Figure 8.** Increased expression of ICOS in splenic T-cells upon anti-CD3ε activation as gated in Figure 3

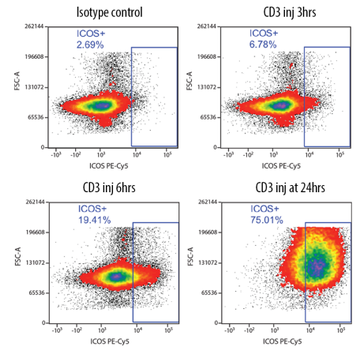
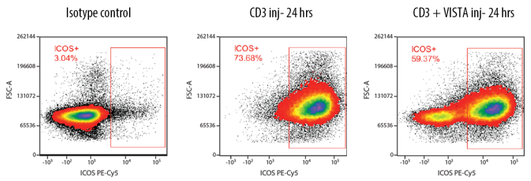
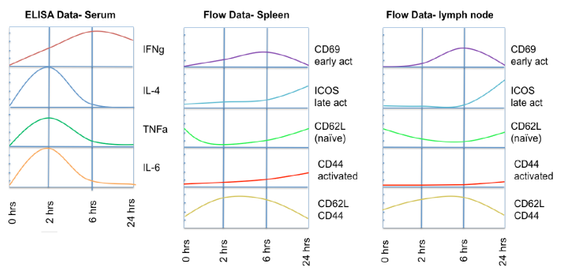



**Figure 9.** Anti-VISTA limits the number of activated ICOS$^+$ cells in the lymph nodes




ICOS expression was significantly increased upon anti-CD3ε exposure, in both anti-CD3ε plus anti-VISTA vs anti-CD3ε alone treatment conditions. Splenic ICOS expression was strongly significant (p=<0.00001) over the duration of CD3 activation (Fig. 8) and significant (p=0.003) in the lymph nodes at 24 hours between the anti-CD3ε plus VISTA injection vs. CD3ε alone treatment conditions (Fig. 9).

**Figure 10.** Graphic representation of the kinetics of cytokine expression and activation markers




ELISA on whole serum samples were analyzed for IFN-γ, IL-4, TNF-α and IL-6. Expression of IL-4, TNF-α and IL-6 peaked very fast after CD3ε injection and leveled back off to basal levels at 24 hours. The expression of these cytokines correlated with a decrease in CD62L expression. IFN-γ expression, on the other hand, was delayed, only peaking after 6 hours (Fig 10). Anti-VISTA co-injection did not have a significant effect on cytokine expression.

## Summary

CD3 *in vivo* activation is a very practical, accessible method of achieving T-cell specific activation. We have demonstrated that anti-VISTA antibody does suppress some indicators of T-cell activation, namely dendritic cell abundance in the lymph node and late-stage markers for activation like ICOS. It is possible that our method of co-injection limited the potential impact of VISTA on activation. It would be beneficial in the future to more extensively profile anti-VISTA at different delivery schedules, doses of anti-VISTA and more refined time points.