In-Vitro Inhibition of P38 Abrogates GITR-Induced Proliferation of Murine iTregs

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Accessibility
Phosphoproteomics Analysis of T cell Subsets treated with GITR Agonist Reveals a Unique Role for p38 in the Regulation of iT<sub>reg</sub> Function

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Abstract

Immunotherapy, involving the modulation of immune checkpoint receptors to enable an effective antitumor response, represents a major breakthrough in cancer therapy. This breakthrough has brought about a renewed focus to define and understand the immune mechanisms that contribute to antitumor immunity. Triggering the co-stimulatory receptor glucocorticoid-induced TNFR-related protein (GITR) on T cells has been identified as a promising cancer immunotherapy, yet the signaling events by which GITR agonism induces antitumor immunity are not well understood (Knee et al., 2016). GITR is expressed on activated CD4$^+$ and CD8$^+$ T cells (Ward-Kavanagh, 2016), and is constitutively expressed on CD4$^+$/CD25$^+$/FoxP3$^+$ regulatory T cells (T$_{regs}$) (Shimizu et al., 2002). While GITR appears to act as a conventional co-stimulatory receptor in effector CD4$^+$ and CD8$^+$ T cells, the role of GITR on CD4$^+$/CD25$^+$ T$_{regs}$ remains controversial. Joetham et al. found that GITR stimulation resulted in loss of T$_{regs}$ suppressive phenotype, while Liao et al., observed that GITR ligation enhanced natural T$_{regs}$ proliferation and maintained their suppressive phenotype. These contradictory findings demonstrate a need to understand more about the signaling events occurring in T$_{regs}$ as a result of triggering GITR. The goal of this research was to create a model system for elucidating and comparing GITR signaling events in induced-T$_{regs}$ (iT$_{regs}$), CD4$^+$ T effector (T$_{eff}$) cells, and CD8$^+$ cytotoxic T cells. Phospho-proteomics was then leveraged to identify differential signaling events among the T cell subsets. We used the GITR agonist monoclonal antibody (mAb) DTA-1 to stimulate the T cell subsets and
confirmed GITR signaling via NFκB pathway activation and phospho-JNK induction. The phospho-proteomics data identified many more unique phosphorylated protein sites among the subsets than those shared among the T cell subsets. We observed phospho-p38 MAPK induction in the iTreg subset but not in the CD4+ T eff cells or CD8+ cytotoxic T cells. We also observed additional phosphorylated proteins induced by GITR triggering in the p38 pathway in the iTreg subset including phospho-MKK3, phospho-MKK6, phospho-MAPKAPK-2, and phospho-ATF-2. This phospho-p38 MAPK induction is functionally important as inhibition of p38 abrogated GITR-induced proliferation of iTregs. These data demonstrate that GITR agonism induces distinct phospho-proteome profiles between T cell subsets, and this differential signaling leads to unique responses that will need to be considered when targeting GITR with immunotherapeutic strategies.
Dedication

I dedicate this work to my daughters Ellie and Isla. Your curiosity and love for the natural world is inspiring and I hope it only gets stronger. I love you both.
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Chapter I.

Introduction

According to the CDC, cancer is a leading cause of death in the United States, second only to heart disease (U.S. Cancer Statistics Working Group, 2018). The modulation of immune checkpoint receptors to enable an effective antitumor response represents a major breakthrough in immunotherapy. These advances have brought about a renewed focus to define and understand the immune mechanisms and underlying signaling events that contribute to antitumor immunity. A deeper understanding of these signaling events has the potential to reveal new and needed immunotherapeutic opportunities (Yang, 2015), as current immunotherapy strategies have only been shown to work in a portion of patients and only in the context of certain cancers (Postow et al., 2018).

One immune checkpoint receptor that is currently being pursued for its potential as an immunotherapeutic target for cancer is Glucocorticoid-induced TNFR-related protein (GITR). GITR is a co-stimulatory receptor expressed on activated CD4+ and CD8+ T cells (Ward-Kavanagh, 2016), and is constitutively expressed on CD4+/CD25+/FoxP3+ regulatory T cells (Tregs) (Shimizu et al., 2002). The GITR ligand (GITRL) is expressed primarily by antigen presenting cells such as DCs, macrophages, and B cells, and can be upregulated in endothelial cells by Type I IFN (Azuma, 2010) (Nocentini & Riccardi 2009). Signaling through GITR reduces the threshold of TCR activation in CD8+ T cells (Ronchetti et al., 2007), and enhances effector CD4+ and CD8+
T cell proliferation and cytokine production (Watts, 2005). Furthermore, GITR agonism using therapeutic monoclonal antibodies (mAb) can induce an antitumor immune response in syngeneic mouse models, including CT26, MethA, and B16-F10 (Knee et al., 2016). In addition, combined anti-GITR and anti-PD-1 mAb therapy induces antitumor immunity in a murine ID8 ovarian cancer model that is refractory to anti-PD-1 monotherapy (Lu et al., 2014). Due to this success in animal models, there are now GITR agonist monoclonal antibodies and GITRL fusion proteins in Phase 1 clinical trials (Acúrcio et al., 2018).

Even though clinical trials involving GITR agonism are underway, the signaling events by which GITR stimulation induces potent antitumor immunity are not well understood. This is most apparent with regards to GITR agonism in Tregs. It has been demonstrated that antibody-mediated depletion of Tregs in the murine tumor environment, using the GITR agonist antibody DTA-1, plays a key role in its antitumor efficacy (Coe et al., 2010). Another study suggests that in addition to the antibody mediated depletion of Tregs, impaired functionality of persisting Tregs as a result of GITR agonism is likely contributing to the effectiveness of DTA-1 therapy (Mahne et al., 2017). While GITR appears to act as a conventional co-stimulatory receptor in effector CD4+ and CD8+ T cells, the agonistic effects of GITR antibodies on CD4+/CD25+ Tregs are controversial. Joetham et al., (2012) found that GITR stimulation resulted in loss of natural Treg suppressive phenotype in vitro and in vivo. In contrast, Liao et al., (2010), observed that GITR ligation enhanced natural Treg proliferation and maintained their suppressive phenotype. These contradictory findings demonstrate a need to understand more about the signaling events occurring as a result of triggering GITR.
Cancer is a group of diseases that all involve cells in the body abnormally and uncontrollably growing and dividing, caused by genetic changes or damage to DNA (“What is Cancer,” 2015). Cancer cells can form tissue masses called tumors, which can influence their surroundings to secure nutritional requirements and to evade and/or suppress the immune system. The tumor and surrounding area that it interacts with is termed the tumor microenvironment (TME). The TME consists of tumor cells, immune cells, endothelial cells, and other cell types; and can influence tumor cell survival and progression (Wu & Dai, 2017). The TME contributes to tumor cell survival and progression in various ways. For example, altered vasculature architecture in the tumor leads to areas of hypoxia, which can confer resistance to apoptosis and increased survival to cancer cells. Also, cancer cells and cancer-associated cell types can provide signals that advance tumor growth and suppress the immune system.

In the TME, both innate as well as adaptive immune cells can be found interacting with cancer cells (Wu & Dai, 2017). These interactions influence the behavior and progression of the tumor, and occur through cytokine signaling and other secreted molecules, as well as direct cell contact. Examples of innate immune cells found in the TME are tumor-associated macrophages (TAMs), dendritic cells (DCs), myeloid derived suppressor cells (MDSCs), natural killer cells (NKs), and neutrophils. TAMs can differentiate into M1 subtype macrophages, which promote antitumor conditions, but they can also differentiate into M2 subtype macrophages, which have been shown to promote tumor growth and produce suppressive cytokines that can lead to immune
suppression (Wu & Dai, 2017). Likewise, MDSCs have also been described exerting T cell-suppressing effects in the TME (Gabrilovich et al, 2012).

Among the adaptive immune cells that can be found in the TME are various subsets of T cells, including CD8\(^+\) T cells, different CD4\(^+\) T helper cells (T\(_{H1}\)), T\(_{regs}\), and B cells (Hui & Chen, 2015). CD8\(^+\) T cells, or cytotoxic T lymphocytes (CTLs), are critical in anti-tumor immunity through direct killing of cancer cells and, along with the CD4\(^+\) T helper subset T\(_{H1}\)1, the production of inflammatory cytokines like interferon-\(\gamma\) (IFN-\(\gamma\)) (Sabharwal et al., 2018). More recently, CD4\(^+\) T\(_{H9}\) cells were demonstrated to induce potent antitumor responses in CD8\(^+\) CTLs via the recruitment of DCs to the tumor (Yong et al., 2012). CD4\(^+\) T\(_{H2}\) cells have a more complicated role in tumor immunity, in that they can recruit important antitumor macrophages and eosinophils, but they can also inhibit antitumor immunity by secreting IL-4 and IL-10. Finally, in the case of T\(_{regs}\), their presence in the TME suppresses antitumor immune cell responses through the secretion of anti-inflammatory cytokines like IL-10, and competition for nutrients and cytokines like IL-2, an essential cytokine for effector T cell maintenance. Indeed, high numbers of tumor infiltrating T\(_{regs}\) are associated with poor disease prognosis (Moreno Ayala et al., 2019).

It is clear that the immune system is equipped with the machinery necessary to clear tumors, if only the immunosuppressive and evasive maneuvers that tumors employ can be overcome. Some cancer cells avoid immune surveillance by simply being weakly immunogenic, and in other instances they can employ active measures to inhibit antitumor immune cells through the secretion of cytokines like TGF-\(\beta\) (Hanahan and Weinberg, 2011). These observations have made possible great opportunity for cancer
treatment in the form of immunotherapy; the enabling of an effective antitumor response through the modulation of immune cells or tumor cells.

**Immune Checkpoints**

Immune checkpoints are comprised of both stimulatory and inhibitory pathways that work to maintain a balance between necessary immune activation and ensuring self-tolerance. These pathways can be exploited by tumors, resulting in immune suppression and evasion (Baumeister et al., 2016). Immunotherapy seeks to overcome immune suppression and evasion by tumors through modulating the mechanisms that are preventing the recognition and clearance of the tumor cells by the immune system.

Early successes in immune checkpoint therapy have focused primarily on blocking inhibitory receptors. A pioneering discovery occurred when cytotoxic T lymphocyte-associated protein 4 (CTLA-4) was demonstrated to mediate inhibitory signals in activated T cells (Walunas et al., 1994). CTLA-4 can outcompete CD28 for their shared ligands, CD80 and CD86 (Sansom and Hall, 1993). This results in the inhibition of T cell proliferation and activation. James Allison theorized that blocking CTLA-4 in the context of cancer might allow for T cell proliferation and activation to increase beyond normal levels, as first described in Walunas et al., 1994. Indeed, he went on to show that CTLA-4-specific blocking antibodies led to lasting tumor regression in a mouse syngeneic tumor model (Chambers et al., 2001). Another groundbreaking example, PD-1 and its ligand, PD-L1, are key suppressors of T cell effector function through mechanisms that are non-redundant to CTLA-4. PD-L1 expressed on tumor cells and hematopoietic cells engages with PD-1 on the surface of T cells and inhibits their
effector function via the dephosphorylation of signaling events downstream of the T cell receptor (TCR) and CD28 (Freeman et al., 2000). T cells expressing multiple inhibitory receptors such as CTLA-4 and PD-1 often become exhausted, a term used to describe T cells that have lost their effector function due to chronic exposure to inhibitory signals (Wherry, 2011). Antibody blockade of the PD-1/PD-L1 axis results in reversal of T cell exhaustion enabling an antitumor immune response (Ribas and Wolchok, 2018). The FDA-approved PD-1 and PD-L1 blocking antibodies have been a major breakthrough in cancer treatment with FDA approval in 21 tumor types. However, these breakthrough immunotherapies only work in a subset of patients and in a subset of cancers. Preventing antigen-specific T cells from being suppressed by blocking inhibitory checkpoints is only half of the equation. Targeting co-stimulatory molecules in order to directly drive activation of antitumor immune cells has also shown promising results (Assal et al., 2015). In addition, combined therapies that have non-redundant inhibitory or stimulatory pathways are being pursued to overcome acquired resistance to a single therapy, many of these being combinations with PD-1 blockade (Mahoney et al., 2015).

GITR

GITR is one of the co-stimulatory molecules that has shown promising antitumor results. Unlike PD-1 and CTLA-4, GITR activation using agonist antibodies or recombinant GITRL directly potentiates CD8+ T cell responses in the form of increased proliferation, induction of IL-2 and Interferon-γ (IFN-γ), and the upregulation of CD25 (Knee et al., 2016). Recent studies have shown interesting roles for GITR stimulation in conventional CD4+ effector cells as well; namely skewing cells to IL-9-producing T cells.
(Th9), which leads to increased antitumor CTL responses through enhancement of DC functionality (Kim et al., 2015).

In Tregs, the role of GITR stimulation remains unclear. In early reports, GITR triggering using a rat IgG2b,λ anti-mouse agonist monoclonal antibody clone DTA-1, inhibited Treg suppression, as determined by an in vitro suppression assay (Shimizu et al. 2002). Later research has described Tregs derived from DTA-1-treated mice as being just as suppressive as Tregs from control mice, again using an in vitro suppression assay (Coe et al., 2010). It is also possible that GITR triggering in Teff cells drives resistance to Treg suppression. In research by Stephens et al., (2004), co-culture experiments were performed with different combinations of CD4+CD25+ Tregs and responder CD4+CD25− T cells from wild-type and GITR−/− mice treated with agonist anti-GITR Ab. GITR engagement on responder CD4+CD25− T cells, and not engagement on CD4+CD25+ Tregs was required to abrogate suppression. However, loss of Treg suppressive function after GITR triggering has been demonstrated in other research; Joetham et al., (2012) found that c-Jun N-terminal kinase activation was associated with loss of natural Treg suppressive phenotype after ligation of GITR in both in vitro and in vivo assays using the GITRL or anti-GITR DTA-1 mAb. In contrast, Liao et al., (2010), observed that ligation of GITR, using an Fc-GITRL fusion protein, enhanced natural Treg proliferation while maintaining their suppressive phenotype. Elucidating the signaling events that are activated among the T cell subsets following GITR stimulation may aid in our understanding of how GITR agonism effects these key cell types during an antitumor immune response. It is important to note, however, that in vitro assays, as proposed herein, may potentially miss the in vivo mechanism of action.
GITR is a TNF superfamily member. TNF family receptors are expressed on many immune cells and play diverse roles in immunity (Ward-Kavanagh, 2016). GITR is a transmembrane receptor that does not have intrinsic enzymatic activity, but GITR stimulation has been shown to recruit TNFR-associated factor (TRAF) proteins to the receptor’s cytoplasmic domain for NF\(_{\kappa}B\) signaling and MAPK signaling. There is evidence of differential TRAF recruitment among T cell subsets, and downstream signaling is poorly understood (Knee et al., 2016). In a yeast two-hybrid system using transfected GITR and TRAFs; TRAF1, TRAF2, and TRAF3 were shown to interact with GITR (Esparza and Arch, 2005). Upon further investigation an immunoprecipitation experiment in primary lymphocytes demonstrated GITR interaction with TRAF2, but TRAF2 was found to dampen activation of NF\(_{\kappa}B\) (Esparza and Arch, 2005). In CD4\(^+\) T cells, downstream of GITR, TRAF5 is engaged in NF\(_{\kappa}B\), p38, and ERK but not JNK signaling (Esparza et al., 2006). Kim et al., (2015) reported a role for TRAF6 in GITR-mediated conversion of CD4\(^+\) T cells to Th\(_9\) cells. Finally, in a CD8\(^+\) T cell model, GITR-induced NF\(_{\kappa}B\) signaling was positively regulated by TRAF2 and TRAF5, but TRAF1 was dispensable (Snell et al., 2010). NF\(_{\kappa}B\) signaling induced by GITR agonism has been demonstrated in the form of I\(\kappa\)B\(\alpha\) degradation, phospho-NF\(_{\kappa}B\) p65 induction, and Phospho-NF-\(\kappa\)B p105 induction among other signaling proteins in this pathway (Kim et. al., 2015). Sabharwal et al., (2018) saw induction of MAPK signaling events, phospho-ERK1/2 and phospho-JNK, after DTA-1 treatment in CD8\(^+\) T cells. JNK, but not ERK or p38, were activated by GITRL in T\(_{\text{regs}}\) cells in research by Joetham et al., (2012). In a CD4\(^+\) T cell model, anti-GITR stimulation induced P38, ERK, and JNK activation (Esparza and Arch, 2005), and Tang et al., (2015) observed GITRL-induced activation of
p38 and STAT3 in activated CD4⁺ T cells in support of Th17 cell differentiation.

**p38 MAPK Pathway and its role in Tregs**

The p38 MAPK signaling pathway can be activated by various stimuli including cytokines, growth factors, lipopolysaccharide (LPS), and ultraviolet (UV) light. P38 MAPKs, along with the JNK family pathway, are referred to as Stress-Activated Protein Kinases (SAPKs) due to their responsiveness to environmental stresses, and play an important role in modulating apoptosis, cell cycle, protein translation, DNA repair, and immune function (Whitmarsh, 2010). Downstream of TNF receptor family members, the MAP3K member TAK1 can be linked to TRAF6 by one of the adaptor proteins TAB1, TAB2, or TAB3 resulting in the phosphorylation of kinases MKK3, MKK4, and MKK6 (Huang et al., 2009). P38 is then activated by the highly selective pathway kinases MKK3 and MKK6, and to some extent MKK4, by direct phosphorylation of the Threonine-Glycine-Tyrosine motif. Many downstream targets/substrates of p38 have been identified ranging from transcription factors like ATF-2, STAT1, and c-Jun, to the protein kinases MAPKAP-K2 and MAPKAP-K3 (Cuenda and Rousseau, 2007).

P38 MAPK signaling plays important roles in Tregs. Inhibition of p38 in Tregs, using the p38-specific inhibitor SB203580, abrogates TNF-induced expansion and the upregulation of FoxP3 of mouse Tregs in *in vitro* and *in vivo* experiments (He et al., 2018). In another study also utilizing SB203580, p38 inhibition blocked the conversion of naïve T cells into induced-Tregs by TGF-β (Huber et al., 2008). SB203580 binds to the ATP-binding pocket on p38, thereby inhibiting its catalytic activity (Kumar et al., 1999).
It is clear that a more thorough understanding of the signaling events by which GITR activation induces effective antitumor immunity has the potential to reveal additional therapeutic opportunities. The experiments described herein can contribute to this greater understanding. The hypothesis for this study was that GITR agonism induces differential signaling events in T_{regS}, CD4^{+} T_{eff} cells, and CD8^{+} cytotoxic T cells resulting in unique transcriptional regulation and phenotypes. To test this hypothesis, a model system of the T cell subsets was developed and validated, and a phospho-proteomics approach was used to identify key signaling events downstream of GITR that may be unique to each T cell subset.

The technical goal of phospho-proteomics analysis was to detect GITR-regulated phosphorylation events occurring on tyrosine, threonine, and serine residues. Protein phosphorylation is a reversible post translational modification (PTM) that can activate or deactivate a multitude of receptors and enzymes that play a role in cellular processes (Ardito et al., 2017). Phosphorylation events are executed by kinases, and dephosphorylation is performed by phosphatases. The phosphorylation of a protein is more often associated with its activation; however, dephosphorylation can also be activating. For example, the dephosphorylation of nuclear factor of activated T cells (NFAT) family members leads to their translocation into the nucleus thereby allowing for interactions with other transcription factors and the subsequent upregulation of T cell activation-associated genes (Wu et al., 2006). These were important considerations used during the interpretation of the phospho-proteomics data.
The established anti-GITR agonist monoclonal antibody (mAb), DTA-1, was used to study GITR signaling. DTA-1 is a rat IgG2b,λ anti-mouse mAb antibody that was generated by immunization against CD4^+CD25^+ mouse T_{regs} and has demonstrated agonist activity (Shimizu et al. 2002). Work also by Shimizu et al. (2002), demonstrated that crosslinking of DTA-1 is not required to induce GITR signaling as determined by plate-bound and soluble antibody treatment comparisons. In support of this finding, murine GITRL crystallization revealed a dimeric molecule as opposed to the classical TNFR ligand trimeric structure, in which case the latter would have suggested a role for mediating antibody cross-linking (Chattopadhyay et al., 2008). With regards to receptors of the classical TNF superfamily interacting with the trimeric ligand assembly, agonist antibodies typically require Fcγ receptor-binding or oligomerization to induce agonist activity (Wajant, 2015). This unique GITR:GITRL interaction was also observed by Zhou et al., (2008) and the GITRL dimer was found to be biologically active.

The phospho-proteomics approach to identify signaling events taking place in GITR stimulated treatment groups of the T cell subsets requires 5 mg of protein (cell pellet) per condition, approximately 1x10^8 cells, for complete phospho-proteome coverage. CD4^+ T cell and CD8^+ cytotoxic T cell subsets are readily expandable to meet phospho-proteomics sample amount requirements. However, T_{regs} present a challenge in culture and expansion, namely the difficulty in maintaining expression of the subset defining transcription factor, FoxP3. In order to have enough FoxP3-expressing T_{regs} for proteomics analysis, an in vitro-induced T_{reg} model was utilized as described in Shi et al., (2018) to achieve cell numbers suitable for their proteomics studies investigating IL-2 signaling events in T_{regs}. Research by Kretschmer et. Al, (2005) demonstrated that mouse
naïve T cells can be converted into stable FoxP3-expressing T_{regs}, termed iT_{regs}. The use of iT_{regs} cells is a study limitation and may miss key differences between natural T_{reg} cells. In addition, in vivo versus in vitro considerations may lead to differences. However, in work by Haribhai et al., (2011), iT_{regs} and natural T_{regs} had closely matched mRNA transcripts and iT_{regs} were found to be a nonredundant subset playing an important role in the expansion of TCR diversity in the context of the regulatory response. The protocol used in the current study was optimized from Dr. Hao Shi, St. Jude Children’s Research Hospital (Shi et al., 2018) and Dr Il-Kyu Kim, College of Pharmacy, Seoul National University (Kim et al., 2015).

Specific Aim I: Model System Development and Validation

In Specific Aim I, murine T cell models of iT_{regs}, CD4^{+} T_{eff}, and CD8^{+} cytotoxic T cells for studying GITR signaling were generated. Validation of the models consisted of confirming suitable purity of the respective T cell subsets, confirming and optimizing anti-GITR DTA-1 agonist mAb treatment activity, as well as optimizing cell culturing conditions to achieve enough material for proteomics analysis. The overall purpose was to determine what treatment conditions would be used for phospho-proteomics analysis, to find readouts that could be used to validate the proteomics data, and to ensure that sample prep amounts could meet the requirements for phospho-proteomics analysis.
Specific Aim II: Phospho-Proteomics Analysis and Data Interpretation

T cell subsets treated with anti-GITR mAb DTA-1 or isotype control stimulation were analyzed by phospho-proteomics to identify signaling events resulting from GITR triggering. The phospho-proteomics analysis consisted of a phospho-tyrosine (pY) antibody-based enrichment or an immobilized metal affinity chromatography (IMAC) approach followed by liquid chromatography (LC)/ mass spectrometry (MS) to identify and quantify the modified peptides. IMAC enrichment utilizes positively charged Fe3+ to capture negatively charged phosphopeptides. The pY approach was used to enrich phospho-tyrosine peptides and the IMAC approach was used to enrich phospho-serine, phospho-threonine, as well as phospho-tyrosine modifications. IMAC and pY antibody-based strategies enrich different pools of phospho-tyrosine peptides (“PTMScan® Services,” 2019). In a recent study profiling drug response in human gastric carcinoma, Stokes et al., (2015) observed 3.6% overlap between IMAC and pY-enriched phosphopeptides. Selected protein phosphorylation events identified in the proteomics data were then confirmed via western blot. A gene ontology enrichment analysis was performed based on phosphorylation events regulated by GITR, as used by Weber et al., (2019), to evaluate the associated pathways and their functional implications.

It is clear that a greater understanding of the signaling events and mechanisms of action of promising immunotherapies is key to ensuring their success, and this investigation will help us better understand how GITR agonism drives T cell signaling for effective antitumor immunity. Presented herein, are unique observations of differential signaling between the three T cell subsets induced by GITR-stimulation, and
a follow-up study demonstrating a functional implication of one such unique signaling observation.
Chapter II.

Materials and Methods

Animals

6-10-week-old C57BL/6 mice were used to generate the T cell subsets for this study. Spleens and lymph nodes were dissected in a pathogen free animal facility at Cell Signaling Technology, Inc, and processed and cultured in the tissue culture environment. Animal protocols are approved by animal care oversite at the organization.

*In-vitro* T Cell Differentiation, Expansion, and anti-GITR Treatments

For initial splenocyte DTA-1 treatments, 6-10-week-old C57BL/6 mouse spleens were disaggregated, cultured in RPMI with 10% FBS and 20 ng/mL hIL-2 (Cell Signaling Technology), and stimulated with mouse magnetic beads coated with anti-CD3/CD28 (Gibco) for 7 days. The magnetic beads were added to cell cultures at a 1:1 ratio. The anti-CD3/CD28 magnetic beads were removed at day 7 by magnetic pelleting with three washes with RPMI to recover cells from bead pellet. Cells were rested for 24 hours in RPMI with 10% FBS and 20 ng/mL hIL-2 before anti-CD3/CD28 re-stimulation and treatments with DTA-1 at 2, 5, or 10 µg/mL for 30 min., or LTF-2 rat IgG2b isotype control for 30 minutes at 10 µg/mL (both from Bio X Cell).

Induced-T\textsubscript{reg}, CD4\textsuperscript{+} T\textsubscript{eff} cell, and CD8\textsuperscript{+} cytotoxic T cell subsets originated from 6-10-week-old C57BL/6 mouse spleens and lymph nodes that were disaggregated and
underwent leukocyte separation using ficoll (MilliporeSigma) centrifugation. Each subset was cultured in Click’s Medium (FUJIFILM Irvine Scientific) with 10% FBS, 1X Penicillin/Streptomycin, 1X Sodium Pyruvate, 1X GlutaMAX (all from ThermoFisher), 60 µM β-Mercaptoethanol (MilliporeSigma), and additional subset specific conditions. To generate iTregs, naïve CD4+ T cells were enriched using the Miltenyi mouse naïve CD4+ T cell isolation kit following the manufacturer’s protocol, and cultured with 5 ng/mL TGF-β1, 100 U/mL hIL-2 (both from Cell Signaling Technology), 10 µg/mL mouse anti-IFN-γ, and 10 µg/mL mouse anti-IL-4 (both from Biolegend). CD4+ T_{eff} cells were enriched using the Miltenyi CD4 (L3T4) mouse kit following the manufacturer’s protocol and were cultured with 10 ng/mL hIL-2 and 4 ng/mL mouse IL-12 (ThermoFisher). Mouse IL-12 was used to drive the effector CD4+ T cell phenotype, influencing general upregulation of effector cytokines (Vacaflores et al., 2016) and cell survival (Yoo et al., 2002). CD8+ cytotoxic T cells were enriched using the Miltenyi CD8α+ T cell isolation kit following the manufacturer’s protocol and were cultured with 10 ng/mL hIL-2. T cell subsets were plated at 1x10^6 cells/mL in 10 cm plates and were stimulated with plate-bound mouse anti-CD3/CD28 (Biolegend) for 3 days, then split into plates with no anti-CD3/CD28 for an additional 2 days before GITR stimulation experiments (Figure 2B). During the course of the 5 days in culture and during GITR stimulation, each T cell subset’s specific media conditions were maintained throughout, as described above. T cell subsets were treated at Day 5 of culturing with 5 µg/mL DTA-1 for 5 min., 15 min., 30 min., 60 min., 24 hr., 48 hr., and 72 hr. or with 5 µg/mL LTF-2 rat IgG2b isotype control for 15 min., 30 min., 48hr., and 72 hr.
Western Blotting

Cell extracts were generated by direct lysing of cell pellets at treatment end points in 1X sodium dodecyl sulfate (SDS) Loading Buffer with 1X dithiothreitol (DTT) reducing agent (41.7 mM) (Cell Signaling Technology, Inc.). Samples were boiled for 5 min. and then sonicated 3 times for 15 sec. using a probe sonicator before being stored at -20°C. For SDS-PAGE, 5 μL of cell extract was loaded per lane using 4-20% mini gels (Bio-Rad). Transfer of proteins onto nitrocellulose membranes was completed using a semi-dry transfer apparatus (Bio-Rad) and membranes were blocked for 1 hr. in 5% nonfat dry milk in 1X Tris Buffered Saline with Tween (TBS-T). Nitrocellulose membranes were then incubated with primary antibodies overnight at 4°C in 5% ultra pure bovine serum albumin (BSA) in 1X TBS-T or in 5% nonfat dry milk in 1X TBS-T. The membranes were washed 3 times for 5 min. using 1X TBS-T and were incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) and were washed again 3 times for 5 min. in 1X TBS-T before imaged using a ChemiDoc Touch instrument (Bio-Rad). All western blot primary and secondary antibodies were from Cell Signaling Technology, Inc.

Flow Cytometry

Enrichment of iT_{reg}, CD4^+ T_{eff} cells, and CD8^+ cytotoxic T cells was performed as previously described. T cell subsets were analyzed by flow cytometry at day 5 in culture (Figure 2). After fixation and permeabilization using the FoxP3/Transcription Factor Fixation/Permeabilization Kit according to the manufacturer’s protocol, subsets were
stained with FoxP3 (3G3) Mouse mAb (APC Conjugate), CD8α (2.43) Rat mAb (violetFluor™ 450 Conjugate), CD3 (17A2) Rat mAb (PE Conjugate), CD4 (RM4-5) Rat mAb (PE-Cy7® Conjugate), and appropriate isotype controls (all from Cell Signaling Technology, Inc.). In a separate experiment, T cell subsets underwent fixation and permeabilization and were stained with GITR (E9O9H) mAb or IgG isotype control and Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) was used as a secondary antibody. Flow analysis of GITR expression in each of the subsets was performed on the same day on the same run. For the proliferation studies, T cell subsets were stained with ViaFluor SE Cell Proliferation Dye (BioTium) according to the manufacturer’s protocol at the start of the proliferation assay, were treated as described in “Proliferation Assays” and as shown in Figure 13A and Figure 14A, and analyzed by flow cytometry at the experiment end points. The cytometer used for T cell subset validation and GITR expression analysis was the Beckman Coulter Gallios. The cytometer used for the proliferation assays in 96-well plate format was the FACS Calibur (BD Biosciences). Flow data were analyzed with FlowJo Software.

Phospho-Proteomics

The IMAC and pY antibody enrichment phospho-proteomics experiments were run independently and used independent sample preparations (n=2). A minimum of 5 mg of protein material (~1x10^8 cells) per condition was used for IMAC and again for pY analysis. iTregs, CD4^+ T_{eff} cells, and CD8^+ T cells were treated with 5 µg/mL DTA-1 at 5 and 15 min. or 5 µg/mL LTF-2 rat IgG2b isotype control for 15 min. (9 samples in total). Samples were then prepared for mass spectrometry following the manufacturers protocol,
PTMScan® Phospho-Tyrosine Rabbit mAb (P-Tyr-1000) Kit (Cell Signaling Technology, Inc.). A schematic of the approach is shown in Figure 6.

Phosphoproteome Sample Preparation

Samples were lysed in urea lysis buffer (20 mM HEPES [pH 8], 9 M urea, 1 mM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol-phosphate), were sonicated 3x for 15 sec., were centrifuged at 20,000 x g for 15 min. at room temperature and were then quantified using a Bradford Assay. Samples were then reduced at 55°C with 4.5 mM dithiothreitol for 30 min, followed by alkylation with 1/10 volume of iodoacetamide solutions for 15 min. at room temperature in the dark. For protease digestion, samples were diluted 1:4 with 20 mM HEPES (pH 8.0) and the proteins were digested overnight with 10 µg/ml trypsin-TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone). Digested peptides were acidified with 1% trifluoroacetic acid (TFA) and desalted over 360-mg Sep Pak Classic C18 columns (catalog no. WAT051910; Waters, Milford, MA). The peptides were eluted with 40% acetonitrile in 0.1% TFA, followed by drying under vacuum. Enrichment of phosphorylated peptides was then performed using PTMScan Fe-IMAC magnetic beads to enrich Ser/Thr/Tyr-modified peptides or phospho-Tyr-specific antibodies to enrich an additional pool of phospho-Tyr modified peptides (both from Cell Signaling Technology).

LC-MS/MS

The LC-MS/MS analysis was performed at Cell Signaling Technology, Inc. using the Orbitrap-Fusion Lumos, ESI-HCD. Peptides were loaded directly onto a 50 cm x 100 µm PicoFrit capillary column packed with C18 reversed-phase resin. The columns were
developed with a 150-minute linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nL/min. MS parameters were set to the following; MS Run Time 168 min., MS1 Scan Range (300.0 – 1500.00), Top 20 MS/MS (Min Signal 500, Isolation Width 2.0, Normalized Coll. Energy 35.0, Activation-Q 0.250, Activation Time 20.0, Lock Mass 371.101237, Charge State Rejection Enabled, Charge State 1+ Rejected, Dynamic Exclusion Enabled, Repeat Count 1, Repeat Duration 35.0, Exclusion List Size 500, Exclusion Duration 40.0, Exclusion Mass Width Relative to Mass, Exclusion Mass Width 10ppm). MS/MS spectra were evaluated using SEQUEST (Eng et. al., 1994) and the Core platform from Harvard University. Searches were performed against the most recent update of the Uniprot Mus musculus database with mass accuracy of +/-50 ppm for precursor ions and 0.02 Da for product ions. Results were filtered with mass accuracy of +/- 5 ppm on precursor ions and presence of the intended motif. A total of 9 samples were injected twice on the instrument for each enrichment strategy for a total of 36 LC-MS/MS experiments. This allowed for the maximal number of modified peptides to be identified, as well as allow for an assessment of technical reproducibility on the instrument. A manual review on a subset of the quant data was performed to ensure accuracy before the final reports were generated. A maximum % coefficient of variation (CV) was calculated from replicate injections, standard deviation of the replicate peak area measurements divided by the average peak area and convert to percentage. %CV is a measurement of variation in intensity between replicate injections and was used as a tool to gauge reproducibility of observed intensities.
Gene Set Enrichment Analysis

A gene set enrichment analysis (GSEA) was performed with the phospho-proteomics data for each of the T cell subsets using the observed changes regulated by GITR for the 5 μg/mL DTA-1 15 min. treatment condition. This condition was chosen as it represents the highest number of observed significant changes across the T cell subsets and offered the best opportunity for identifying GITR-regulated pathways. Genes corresponding to phospho-peptides that were both significantly upregulated as well as significantly downregulated (2.5 fold change) were included in the analysis. This is due to the goal of including as much pathway activity as possible without assuming that only upregulation of a site can be interpreted as activation. Some modified peptides mapped to more than one protein due to shared sequence identity, and it was not possible to rule out proteins in these instances. Therefore, all protein IDs were included in this analysis.

The GSEA was performed using custom scripts in R (R Core Team, 2017; https://www.R-project.org/) and leveraging the Molecular Signatures Database (MSigDB), a joint project of UC San Diego and Broad Institute (Subramanian et al., 2005), (Liberzon et al. 2011).

Proliferation Assays

A 3-day proliferation assay was initially performed on iTregs, CD4+ T_{eff} cells, and CD8+ T cells. Cells were stained with ViaFluor 488 SE Proliferation Dye (BioTium) at day 5 in culture for each subset, and subset specific media conditions were maintained during the proliferation assay. T cell subset specific culturing conditions are described in
a previous section. In summary, T cells were pelleted by centrifugation, the culture medium was aspirated, and cells were resuspended at 1×10^6 cells/mL in PBS containing 1 μM proliferation dye and incubated for 15 min. at 37°C. An equal volume of culture medium (described in cell culturing methods) was then added and cells were incubated for an additional 5 min. at 37°C. Labeled cells were then pelleted and resuspended in an equal volume of fresh culture medium and were incubated at 37°C for another 15 min., allowing the dye to react with intracellular proteins. Labeled cells were then pelleted a final time and resuspended in fresh culture medium at 1×10^6 cells/mL containing the appropriate T cell subset conditions. Cells were then plated in a 96-well plate at 1×10^5 cells per well, set up to allow for all conditions to be tested in triplicate, with and without plate-bound anti-CD3/CD28. T cell subsets were pretreated with the p38 inhibitor SB203580 (Cell Signaling Technology, Inc.) at 10 μM or vehicle control for 1 hour before treatment with 5 μg/ml DTA-1 or LTF-2 rat IgG2b isotype control for 3 days. SB203580 or vehicle control was administered about every 12 hours to respective treated groups. On day 3, cells underwent fixation and permeabilization using the FoxP3/Transcription Factor Fixation/Permeabilization Kit according to the manufacturer’s protocol, subsets were stained with FoxP3 (3G3) Mouse mAb (APC Conjugate), and T cell subsets were analyzed by flow cytometry.

Follow-up proliferation assays were then performed using only the iTreg subset. The iTregs were labeled with ViaFluor 488 SE Proliferation Dye at day 5 in culture as previously described. The iTregs were then plated in a 96-well plate at 1×10^5 cells per well, pretreated with SB203580 at 10 μM or vehicle control for 1 hour before treatment with 5 μg/ml DTA-1 or LTF-2 rat IgG2b isotype control for 4 days. SB203580 or
vehicle control was administered about every 12 hours to respective treated groups. On
day 4, the iTregs underwent fixation and permeabilization using the FoxP3/Transcription
Factor Fixation/Permeabilization Kit according to the manufacturer’s protocol, were
stained with FoxP3 (3G3) Mouse mAb (APC Conjugate), and were analyzed by flow
cytometry. In parallel, a dose-response experiment was performed using iTregs that were
stained with ViaFluor 488 SE Proliferation Dye at day 5 in culture and pretreated with
SB203580 at 1, 5, or 10 µM or vehicle control before treatment with 5 µg/ml DTA-1 or
LTF-2 rat IgG2b isotype control for 4 days. SB203580 or vehicle control was
administered about every 12 hours to respective treated groups at the appropriate µM
concentration. On day 4, the iTregs underwent fixation and permeabilization using the
FoxP3/Transcription Factor Fixation/Permeabilization Kit according to the
manufacturer’s protocol, were stained with FoxP3 (3G3) Mouse mAb (APC Conjugate),
and were analyzed by flow cytometry.

Statistical Analysis

GSEA analysis data were analyzed using Fisher’s exact test using R statistical
language (R Core Team, 2017; https://www.R-project.org/) and gene sets with a $P$ value
of $<0.05$ were considered statistically significant. All proliferation data with statistical
analysis were graphed and analyzed using a two-tailed Student’s $t$-test and results with a
$P$ value of $<0.05$ were considered statistically significant.
Chapter III.
Results

Specific Aim I: Model System Development and Validation

DTA-1 treatment induced IκBα degradation and NF-κB p65 phosphorylation in C57BL/6 mouse splenocytes independent of anti-CD3/CD28 stimulation.

DTA-1 has been used to trigger GITR in in vitro studies at concentrations ranging from 2 μg/mL as used in CD4+CD25+ Treg and CD4+CD25− T cell models (Stephens et al., 2004), to 10 μg/mL in a CD8+ T cell model (Snell et al., 2010). To validate GITR activation using the agonist mAb DTA-1 and to begin to optimize the concentration for the proteomics experiments, C57BL/6 mouse splenocytes were treated with different concentrations of DTA-1 (2 μg/mL, 5 μg/mL, and 10 μg/mL) for 30 min. with and without anti-CD3/CD28 re-stimulation (Figure 1). Re-stimulation here refers to the fact that the cells were stimulated initially with anti-CD3/CD28 to upregulate GITR expression, were “rested” without anti-CD3/CD28, and were then stimulated with anti-CD3/CD28 again as a condition with DTA-1. Importantly, DTA-1 treatment alone resulted in the degradation of IκBα and the induction of pNF-κB p65(Ser536) independent of anti-CD3/CD28 re-stimulation determined by western blot (Figure 1B). Phospho-SLP76 was used as a control to confirm anti-CD3/CD28 TCR stimulation (Wardenburg et al., 1998), and was induced only in anti-CD3/CD28 treated splenocytes (Figure 1B). Because DTA-1 treatment showed clear evidence of induction of NF-κB
signaling, it was decided that TCR stimulation with anti-CD3/CD28 would not be used in the context of GITR triggering for the proteomics samples, potentially allowing for a clearer interpretation of signaling events regulated by GITR alone. A working concentration of 5 µg/mL was chosen due to the strongest induction of pNF-κB p65(Ser536), no observed differences in IκBα degradation among the different concentrations, and an effort to conserve reagent instead of using a maximal concentration.

iTreg, CD4+ T eff cell, and CD8+ T cell subset enrichment and expansion resulted in >95% purity using expected expression of extracellular proteins as markers. The enrichment and expansion approaches used to generate iTreg, CD4+ T eff cell, and CD8+ T cell subsets are detailed in the Methods section. Multiple attempts to isolate and expand natural Tregs using the Miltenyi CD4+CD25+ Regulatory T Cell Isolation Kit were performed and resulted in the expansion of approximately 100-fold fewer cells than required for the phospho-proteomics analysis, as well as diminished FoxP3 expression at the end of the expansion (data not shown). Due to the limitations encountered with the expansion and maintenance of natural Tregs, an alternative approach to studying the Treg subset was employed using an in vitro-induced Treg model (iTreg). This model involved the enrichment of naïve CD4+ T cells and their subsequent polarization to iTreg cells by culturing with hTGF-β1, hIL-2, mouse anti-IFN-γ, and mouse anti-IL-4. Using the enrichment and cell culture methods described, we were able to reliably exceed 1x10^8 cells each for iTreg, CD4+ T eff cell, and CD8+ T cell subsets. The enriched T cell subsets were analyzed by flow cytometry at day 5 in culture to confirm expression, or lack
thereof, of unique subset markers (Figure 3). As expected, the iT_{reg} subset, first gated on CD3^{+} cells, demonstrated a 97.3\% CD4^{+}-expressing and 98.9\% FoxP3-expressing population of T cells. Of the enriched CD4^{+} T_{eff} cells gated on CD3, 98.8\% were CD4^{+}, with background CD8^{+} and FoxP3 expression detected, 0.54\% and 0.99\% respectively. Likewise, the CD8^{+} T cell population, again gated on CD3, were a 98.7\% pure CD8^{+} population with only background CD4^{+} and FoxP3^{+} expression detected, 0.30 \% and 0.32\% respectively.

GITR is most highly expressed in activated CD4^{+} T_{eff} cells, followed by iT_{reg}s and CD8^{+} T cells.

In a separate experiment, the enriched T cell subsets; iT_{reg}s, CD4^{+} T_{eff} cells, and CD8^{+} T cells were analyzed by flow cytometry to assess GITR expression. Each T cell subset underwent fixation and permeabilization, and were stained with anti-GITR or isotype control and analyzed by flow cytometry on the same day and the same flow run. All three subsets expressed GITR, with the highest expression level being on CD4^{+} T_{eff} cells (MFI: 39, 96.1\% positive), followed by iT_{reg}s (MFI: 14, 95.3\% positive), and then CD8^{+} T cells (MFI: 12.5, 92.4\% positive) (Figure 4). This was interesting due to the narrative in the literature highlighting regulatory T cells as the subset that expresses GITR at particularly high levels (Shimizu et al., 2002), (Mchugh et al., 2002). It is important to note that this characterization of GITR expression in T_{reg}s was initially focused on naturally occurring CD4^{+}CD25^{+}T_{reg}s, so this may explain the lower expression level in iT_{reg}s relative to the CD4^{+} T cell subset. Another possibility is that the CD4^{+} T_{eff} cell model used in this study simply represents particularly high GITR.
expression. Finally, this particular experiment was not repeated in triplicate to add confidence to GITR expression comparisons between subsets.

These flow data confirmed that one, the three T cell subsets were suitable T cell populations to represent the desired T cell subsets, and two, each T cell subset expressed GITR and therefore may be responsive to the GITR agonist antibody DTA-1.

DTA-1 induces NF-κB and JNK signaling in iTregs, CD4+ T eff cells, and CD8+ cytotoxic T cells

To confirm GITR triggering in each T cell subset and to optimize the DTA-1 treatment for an early GITR signaling timepoint, the three T cell subsets were treated with 5 µg/mL DTA-1 for 5, 15, and 30 min., or with LTF-2 rat IgG2b isotype control for 15 min., and then cell lysates were analyzed by western blot. Western blots were probed for NF-κB signaling components as used to validate the DTA-1 treatment in the splenocytes, and because NF-κB signaling downstream of GITR has been characterized in the literature (Knee et al., 2016). In the iTregs and CD4+ T eff cells, strong evidence of IκBα degradation occurred at the 5 and 15 min. respectively, and phospho-NF-κB p105(Ser933) was induced at the 5 min. timepoint (Figure 5). In the CD8+ T cells, phospho-NF-κB p105(Ser933) was induced by 5 min., but most significantly at 15 min. However, the clearest evidence of IκBα degradation in the CD8+ T cells was at 30 min. In addition, phospho-JNK induction was assessed in the T cell subsets due to reports in the literature describing its induction downstream of GITR in each of the T cell subsets investigated in this study (Esparza and Arch, 2005), (Joetham et al., 2012), (Sabharwal et al., 2018). Phospho-JNK was induced by GITR triggering in iTregs at 5 and
15 min. and completely absent by 30 min. In the CD4⁺ T eff cells and CD8⁺ T cells, Phospho-JNK was induced most significantly at 15 min. Even though IκBα degradation was most apparent in CD8⁺ T cells at 30 min., the 5 and 15 min. treatment timepoints were chosen to be carried out for the phospho-proteomics analysis. This was due to the fact that IκBα degradation occurs only after its phosphorylation (Chen et al., 1996), and because both phospho-NF-κB p105(Ser933) and Phospho-JNK were induced most significantly by 15 min. in all three subsets. Additionally, with a total sample number of 9, all conditions were able to be included in a single phospho-proteomics run (max of 10 samples).

Specific Aim II: Phospho-Proteomics Analysis and Data Interpretation

GITR activates unique phosphoproteome profiles in iTregs, CD4⁺ T eff cells, and CD8⁺ cytotoxic T cells

A phospho-proteomics analysis was performed on iTregs, CD4⁺ T eff cells, and CD8⁺ T cells treated with 5 μg/mL DTA-1 for 5 and 15 min., or 5 μg/mL LTF-2 rat IgG2b isotype control for 15 min. A total of 18,830 unique modified peptides were identified across the T cell subsets by IMAC enrichment and 771 unique modified peptides were identified by pY antibody enrichment (Table 1). The IMAC enrichment identified 16,750 phospho-serine-containing peptides, 3,276 phospho-threonine-containing peptides, and 330 phospho-tyrosine-containing peptides. Of the IMAC-enriched modified peptides, 5,645 were significantly upregulated or downregulated (fold change of ≥ 2.5 or ≤ -2.5) in DTA-1-treated sample with respect to the Isotype control in
one or more of the T cell subsets. Of the pY antibody-enriched peptides, 296 were significantly upregulated or downregulated in one or more of the T cell subsets. Of note, only 98 phospho-Tyr-containing upregulated or downregulated peptides were identified across all T cell subsets in the IMAC-enriched data set, arguing for the greater utility of the pY antibody enrichment approach to capture a more complete array of phospho-tyrosine modifications. What this means however, is that the vast majority of modified peptides that were enriched in the IMAC data set regulated by GITR, were phospho-Ser, phospho-Thr, or combinations of the two. This breakdown may not be out of the norm due to the fact that, in terms of PTMs, phosphorylation of tyrosine is more rare (Ardito et al., 2017).

When comparing the numbers of upregulated phospho-peptides between the T cell subsets, iTregs had the most increases across DTA-1 treatment timepoints at 2,466, followed closely by CD8+ T cells at 2,334, and the CD4+ Teff cells had 1,136. With regards to downregulated phospho-peptides as a result of GITR agonism, CD8+ T cells had the most at 2,334, followed by CD4+ Teff cells at 1,265, and iTregs had the fewest at 1,189. Importantly, most of the GITR-regulated peptides identified, were not shared between T cell subsets. Using the DTA-1 15 min. treatment timepoint, in which the highest number of GITR-regulated peptides were identified across the T cell subsets, the GITR-regulated phospho-peptides in each of the T cell subsets were plotted in a Venn diagram to visualize shared phospho-peptides (Figure 7). Surprisingly, only ~16% of the significantly upregulated IMAC-enriched phospho-peptides, and ~35% of the pY-enriched phospho-peptides were shared between two or more T cell subsets. Only ~18% of significantly downregulated phospho-peptides were shared between two or more T cell
subsets from both enrichment strategies. The iTreg and CD8+ T cell subsets stood out as the highest responding subsets to GITR triggering in terms of the number of significantly regulated phospho-peptides that were detected and CD8+ T cells had the most significantly downregulated phospho-peptides detected across enrichment strategies. Based on these data, GITR regulates far more unique phospho-peptides in the three T cell subsets than those that are shared between two or more subsets.

GITR-induced NF-κB signaling was confirmed in the phospho-proteomics data.

The identification of NF-κB signaling related subunits in the proteomics data was used as a starting point for validating the data package. The IMAC enrichment approach identified significant induction of phospho-IκBα(Ser32) in the 5 min. DTA-1-treated samples for each T cell subset (Figure 8A). A western blot was then performed on extracts of the T cell subsets treated with the same conditions as those that underwent proteomics analysis, and were probed for phospho-IκBα(Ser32) along with additional NF-κB pathway members phospho-NF-κB p65(Ser536), total NF-κB p65, phospho-NF-κB p105(Ser933), and total NF-κB p105 (Figure 8B). Phospho-IκBα(Ser32) induction was confirmed by western blot in the 5 min. DTA-1 treatment timepoints for each of the T cell subsets, and the induction was also absent or greatly reduced by the 15 min. timepoint. These data agree with the total IκBα data and contribute to a more complete picture of NF-κB pathway activity. Phosphorylation of IκBα is quickly followed by its proteasome-mediated degradation, resulting in the release of active NF-κB (Chen et al., 1996). The 5 and 15 min. DTA-1 treatment time points captured IκBα phosphorylation followed by its subsequent degradation in each of the T cell subsets. Induction of both
phospho-NF-κB p65(Ser536) and phospho-NF-κB p105(Ser933) were observed by western blot and were most significantly induced at the 5 min. DTA-1 treatment timepoints in each of the T cell subsets. Data from 3 separate experiments showed similar results. Taken together, NF-κB signaling represents a GITR-regulated pathway shared among all three T cell subsets and served as a data point for validating the proteomics data package.

GSEA identified a larger number of significantly upregulated gene sets in iTregs

As an approach to interpret the pathways regulated by GITR triggering in the T cell subsets, GSEAs were performed based on the significantly upregulated and downregulated phospho-peptides. For each T cell subset, gene IDs corresponding to the modified peptides were analyzed using custom scripts in R to identify significant enrichment within a given gene set comprising a pathway. The analysis was run using the phospho-proteomics results from the 5 µg/mL DTA-1 15 min. treatment timepoint, in which the highest number of GITR-regulated peptides were identified across the T cell subsets. The IMAC enrichment results and pY antibody enrichment results were analyzed separately. The IMAC GSEA resulted in 66 significantly enriched gene sets in the 15 min. DTA-1-treated iTregs, 21 in the CD4+ T_{eff} cell subset, and 28 in the CD8+ cytotoxic T cell subset (Tables 2, 3, and 4). The pY GSEA resulted in 7 significantly enriched gene sets in the 15 min. DTA-1-treated iTregs (Table 5), with no significantly enriched gene sets from the CD4+ T_{eff} cell subset or the CD8+ cytotoxic T cell subset. The low number of or lack of significant results in the pY GSEA could be explained by a lower number of significantly regulated peptides identified in that data set relative to the
IMAC data set. GSEA results from the CD4+ T<sub>eff</sub> and CD8+ cytotoxic T cell subsets were each comprised of 12 different E2 factor (E2F) family of transcription factor hits. E2F family transcription factors play important roles in cell cycle/division control, DNA repair, and apoptosis (Dimova and Dyson, 2005). A couple of standout gene sets were identified in the CD8+ cytotoxic T cell subsets; NFKAPPAB_01, underscoring NF-κB signaling induced by DTA-1, as well as GSE21360PRIMARY_VS_QUATERNARY_MEMORY_CD8_TCELL_DN, which called to mind work by Snell et al., (2012) where GITR signaling was demonstrated to promote the survival of CD8+ memory T cells. In the iT<sub>reg</sub> IMAC GSEA, results were overwhelmingly related to DNA regulation and chromatin remodeling. The iT<sub>reg</sub>pY GSEA however returned some interesting results including two instances of MAPK pathway related enrichment, IL-12 signaling pathway enrichment, and BIOCARTA_TCYTOTOXIC_PATHWAY, a reference to cytotoxic T cell-related surface molecules. These data identified more pathways regulated by GITR in iT<sub>regs</sub> than the other T cell subsets, suggesting more responsiveness to GITR stimulation. MAPK pathway signaling was also identified in the GSEA in the iT<sub>regs</sub> and therefore may be more modulated in this subset.

GITR triggering induces p38 signaling in iT<sub>regs</sub>, but not in CD4+ T<sub>eff</sub> cells or CD8+ T cells

MAPK signaling events were investigated because we observed differential signaling there in the phosospho-proteomic data packages (Table 6). The IMAC and pY phospho-proteomics fold change data were then compiled for the three main MAPK pathway families, ERK, JNK, and p38 to view GITR-regulated changes across the T cell
subsets (Table 6). To complement this data, a western blot experiment was run using the same DTA-1 conditions in each of the T cell subsets (Figure 9B). Upregulation of phospho-JNK was observed across all T cell subsets in the proteomics data and was confirmed in an earlier western blot experiment (Table 6, Figure 5B, Figure 9B).

Phospho-ERK was induced by DTA-1 treatment in iTregs and in CD4+ T_{eff} cells, but not in the CD8+ T cells (Table 6, Figure 9B). Interestingly, phospho-p38 was significantly induced by DTA-1 treatment in iTregs but not in CD4+ T_{eff} cells or CD8+ T cells (Table 6, Figure 9). The phospho-proteomics data indicated a 4.5-fold increase in phospho-p38 (Tyr182) at 15 min. after 5 ug/ml DTA-1-treatment in iTregs.

To further support and characterize p38 signaling as a result of GITR stimulation in iTregs, compared to CD4+ T_{eff} cells and CD8+ T cells, upstream and downstream signaling events in the p38 pathway were assessed in the phospho-proteomics data and by western blotting. In the phospho-proteomics IMAC data, phospho-TAB2 was significantly upregulated in iTregs at 5 and 15 min. DTA-1 treatment timepoints, went undetected in CD4+ T_{eff} cells, and was not significantly changed in CD8+ T cells. This was confirmed by western blot along with significant induction of phospho-TAK1, phospho-MKK3, phospho-MKK4, and phospho-MKK6, all specific to DTA-1-treated iTregs (Figure 10). The adaptor protein TAB2 can associate TAK1 with TRAF6 or another TRAF family member, leading to the activation of TAK1. TAK1 can then directly phosphorylate MKK3, MKK4, and MKK6. P38 can then be activated by the kinases MKK3, MKK4, and MKK6 by direct phosphorylation (Huang et al., 2009).

Downstream of p38, upregulated phosphorylation of MAPKAP-K2 (a direct target of p38) was observed in the IMAC phospho-proteomics data, and confirmed by western
blot, specifically in the iT\textsubscript{reg} subset at 5 and 15 min. DTA-1 treatment timepoints (Figure 11). Additional transcription factor targets were identified that are downstream of p38 that were either unique or enhanced with regards to their upregulation in iT\textsubscript{reg} as compared to CD4\textsuperscript{+} T\textsubscript{eff} cells and CD8\textsuperscript{+} T cells. Western blot analysis showed that phospho-ATF-2 and phospho-c-Jun were strongly induced in iT\textsubscript{reg}s at both 5 and 15 min. DTA-1 treatment timepoints with minimal induction in CD4\textsuperscript{+} T\textsubscript{eff} cells and CD8\textsuperscript{+} T cells (Figure 11). Interestingly, phospho-c-Jun(Ser73) was weakly induced in CD4\textsuperscript{+} T\textsubscript{eff} cells and CD8\textsuperscript{+} T cells, but strongly induced in iT\textsubscript{reg}s. Notably, induced phosphorylation of c-Jun(Ser63) was only observed in iT\textsubscript{reg}s. These data support the finding that GITR triggering induces p38 signaling differentially in the iT\textsubscript{reg} subset.

To see if GITR agonism resulted in any sustained or possibly delayed MAPK signaling after 24 or 48 hr., iT\textsubscript{reg}s, CD4\textsuperscript{+} T\textsubscript{eff} cells, and CD8\textsuperscript{+} T cells were treated with 5 µg/mL DTA-1 for 24 and 48 hr. or IgG isotype control for 48 hr. and cell extracts were analyzed by western blot (Figure 12). Phospho-p38 was most strongly induced in iT\textsubscript{reg}s at the 24 hr. DTA-1 treatment and was also evident at 48 hr. Phospho-p38 was also induced at the 24 and 48 hr. timepoints in CD4\textsuperscript{+} T\textsubscript{eff} cells and in the CD8\textsuperscript{+} T cells, however, it was relatively weakly induced compared to the iT\textsubscript{reg}s. Phospho-JNK was induced only in the iT\textsubscript{reg} subset. Phospho-ERK was induced in iT\textsubscript{reg}s, but seemed to be downregulated in CD4\textsuperscript{+} T\textsubscript{eff} and CD8\textsuperscript{+} T cell subsets. Although activity in all three main MAPK family members was detected, the far stronger phospho-p38 induction observed specifically in iT\textsubscript{reg}s, shows that this pathway has a degree of sustained activity, and supports further investigating the role of GITR-induced p38 signaling in iT\textsubscript{reg}s.
In-vitro inhibition of p38 blocks GITR-induced proliferation of iTregs

Unique p38 signaling induced by GITR triggering was observed in iTregs in the phosphoproteomics data and was confirmed by western blot. P38 signaling plays an important role in iTreg proliferation and the potentiation of a suppressive phenotype as supported in the literature (He et al., 2018) (Huber et al., 2008). We hypothesized that GITR triggering activates p38-MAPK signaling in iTregs and contributes to induction of iTreg proliferation. Therefore, following GITR stimulation, the p38 inhibitor, SB203580, would be hypothesized to inhibit iTregs proliferation. In CD4+ T eff cells and CD8+ T cells, we should not see the same effect of P38 inhibition as our data shows weak evidence of GITR-induced p38 phosphorylation as well as a lack of activity in other upstream and downstream p38 pathway members.

To test this hypothesis, a 3-day proliferation assay was performed on the three T cell subsets treated with DTA-1 or isotype control and treated with the p38-specific inhibitor SB203580 or vehicle control with and without plate-bound anti-CD3/CD28 stimulation during the course of the 3-day proliferation. Subsets were stained with the proliferation dye ViaFluor 488 SE at the start of the experiment, then subsets were analyzed by flow cytometry at the proliferation end point. In the data presented, lower MFI indicates a greater extent of T cell proliferation. SB203580 was used at 10 µM to inhibit p38 per manufacturer’s datasheet as well as in work by (Huber et al., 2008) and (He et al., 2018). In the iTregs, DTA-1 treatment alone induced highly significant proliferation compared to IgG. In the iTregs, DTA-1 treatment did not induce additional proliferation in the presence of anti-CD3/CD28; however, the anti-CD3/CD28 induced a
high level of proliferation, leaving little window to see an effect (Figure 13). The DTA-1-induced proliferation in iTregs was partially inhibited by SB203580 treatment. In the CD4$^+$ T$_{eff}$ cells, DTA-1 did not induce proliferation. In the CD8$^+$ T cells, DTA-1 induced significant proliferation in the absence of anti-CD3/CD28. However, like in the CD4$^+$ T$_{eff}$ cells, SB203580 treatment alone in the CD8$^+$ T cells led to more proliferation than DTA-1 treatment. TCR stimulation with anti-CD3/CD28 in each subset likely induced maximal proliferation masking the effects of DTA-1. In the CD4$^+$ T$_{eff}$ cells, overall proliferation was similar with and without anti-CD3/CD28 stimulation. These results support the hypothesis that GITR triggering activates p38-MAPK signaling in iTregs and contributes to induction of iT$_{reg}$ proliferation regarding GITR-induced p38 signaling and proliferation in iTregs, as well as suggest a different role for GITR stimulation in the context of p38 signaling in the CD4$^+$ T$_{eff}$ cells and CD8$^+$ T cells.

To confirm these results in the iT$_{reg}$ subset, a follow-up proliferation study was conducted. The proliferation assay follow-up study consisted of a 2-day proliferation assay with anti-CD3/CD28, and a 4-day assay with no TCR stimulation to allow for a more optimized timepoint to analyze DTA-1-induced proliferation. A 4-day dose-response experiment was also run in parallel to better characterize the effects of p38 inhibition and add confidence to the model system. iTregs were stained with the proliferation dye ViaFluor 488 SE at the start of the experiment, then were analyzed by flow cytometry at the proliferation end points. In the 2-day proliferation assay, DTA-1 did not induce significant proliferation in the context of anti-CD3/CD28, however, the anti-CD3/CD28 again induced a high level of proliferation potentially masking the effect of DTA-1 (data not shown). In the 4-day proliferation assay with no anti-CD3/CD28,
DTA-1 treatment induced highly significant proliferation in the iTregs and was completely abrogated by the p38 inhibitor SB203580 (Figure 14). These results were confirmed in a SB203580 dose-escalation study. iTregs were treated with IgG isotype control or 5 µg/ml DTA-1, and 1 µM, 5 µM, or 10 µM SB203580 or vehicle control, and were allowed to proliferate for 4 days before being analyzed by flow cytometry. SB203580 treatment abrogated DTA-1-induced proliferation in iTregs in a dose dependent manner, with the highest concentration of 10 µM resulting in no significant difference in proliferation compared to the IgG isotype control (Figure 15). These experiments showed that in-vitro inhibition of p38 blocks GITR-induced proliferation of iTregs, and is an example of GITR-mediated signaling that influences phenotype and is T cell subset specific.
Chapter IV.

Discussion

Significance of Results

A proteomics approach was used to characterize and compare the phosphoproteomes regulated by GITR in iTregs, CD4+ T eff cells, and CD8+ cytotoxic T cells. The initial hypothesis was that GITR agonism induces differential signaling events in iTregs, CD4+ T eff cells, and CD8+ cytotoxic T cells resulting in unique transcriptional regulation and phenotypes. This hypothesis was based around contradictory findings in the literature related to the effect of GITR agonism on Tregs, as well as a lack of defined signaling downstream of GITR in these subsets (Knee et al., 2016). The data presented herein demonstrate that GITR triggering induces unique phosphoproteomes in the three T cell subsets using the GITR agonist mAb DTA-1 at 5 µg/mL at 5 and 15 min. As illustrated in Figure 7, using the 15 min. DTA-1 timepoints at which the highest number of GITR-regulated phospho-peptides were identified across T cell subsets, only a minority of these sites were shared in two or more subsets. This difference supports the idea that GITR signaling will have different downstream consequences in the different T cells subsets. Also, it is important to note that these data represent GITR triggering with no concurrent TCR stimulation. The T cell subsets had been activated for 3 days previously with anti-CD3/CD28 to upregulate GITR but were rested for 2 days with no anti-CD3/CD28 prior to DTA-1 stimulation. This underscores the notion that, insofar as GITR is thought of as a co-stimulatory receptor, co-TCR stimulation is not needed to
observe hundreds of GITR-regulated phosphorylation events. No in-vivo experiments were conducted in this study, but it would be reasonable to hypothesize that the extent of GITR signaling observed in the absence of concomitant TCR signaling in-vitro, would contribute to relevant biology in-vivo. This is certainly not to downplay the role for GITR concomitant co-stimulation. Afterall, in CD8+ T cells, the proliferative response to CD28 co-stimulation is significantly impaired when GITR is absent (Ronchetti et al., 2007).

Comparing relative GITR expression among the in vitro stimulated T cell subsets studied herein, the CD4+ T_{eff} cells had the highest expression, followed by i{T}_{reg}, and then CD8+ T cells (Figure 4). However, iT_{reg}, and CD8+ T cells were the highest responding cells based on the number of GITR regulated phospho-peptides identified (Table 1). CD4+ T_{eff} cells play an important role in GITR-mediated antitumor immunity as highlighted in work by Kim et al., (2015), however, in studies like these, the functional consequences of GITR stimulation were assessed in the context of APCs or the use of anti-CD3. An interesting and important follow-up to my study would be to build a more complete picture of GITR-regulated signaling events by leveraging a phosphoproteomics analysis to compare signaling events regulated by GITR alone and those regulated by GITR in the context of simultaneous CD3/CD28 stimulation.

A GSEA was performed based on the GITR-regulated phospho-peptides identified in the phosphoproteomics analysis (Tables 2-5). Gene IDs corresponding to the modified phospho-peptides were analyzed using custom scripts in R to identify significant enrichment within a given gene set. The gene sets represent a wide range of cellular pathways and processes, from gene sets comprised of T Helper cell surface
molecules, to those comprising a functional signaling pathway, or comprised of genes with a shared transcription factor binding site. For a given signaling pathway, an assumption is made that the modification of peptides, as detected in this study, correlates with the expression of the components in that pathway. This is one important reason why gene set candidates need to be confirmed. The analysis was run using the phospho-proteomics results from the 5 µg/mL DTA-1 15 min. treatment timepoint, in which the highest number of GITR-regulated peptides were identified across the T cell subsets. The analysis highlighted NF-κB activation, which was confirmed in all three T cell subsets by western blot analysis (Figure 8), and identified significant upregulation of MAPK signaling in the iT_{reg} subset providing additional rational for the further investigation of MAPK family signaling events differentially regulated by GITR. Interesting gene set results beyond NF-κB and MAPK pathway signaling were identified in this study and require careful validation and further investigation. Of note, in CD4^{+} T_{eff} and CD8^{+} cytotoxic T cell subsets, both returned 12 E2F related hits. E2F family transcription factors are best known for their roles in cell cycle and differentiation (Cuitiño et al., 2019). In the CD8^{+} cytotoxic T cell subset treated with DTA-1, the gene set GSE21360_PRIMARY_VS_QUATERNARY_MEMORY_CD8_TCELL_DN was significantly enriched. This gene set represents genes downregulated in quaternary memory cells vs. primary memory CD8^{+} T cells. Quaternary memory cells were defined in this study as highly pure memory CD8^{+} T cell populations with a defined history of antigen exposures out to 4 encounters. Although this gene set was aimed at distinguishing between memory CD8^{+} T cell repeat antigen exposures, it is still representative of a core memory CD8^{+} T cell signature (Wirth et al., 2010). GITR
signaling promotes the survival of CD8+ memory T cells, so further investigation of the
enriched phospho-peptides, corresponding to those identified in this gene set, may aid in
creating a better understanding of the underlying signaling mechanisms leading to long-
lived T cell memory (Snell et al., 2012). In the iT\textsubscript{reg} subset treated with DTA-1 pY
GSEA, the gene set BIOCARTA\_TCYTOTOXIC\_PATHWAY was significantly
enriched by GITR triggering. This is an interesting result due to the fact that it may allow
for an opportunity to further study the effect that GITR agonism has on T\textsubscript{reg} suppressive
function. A complicating aspect of investigating these gene set results, is that the GSEA
consisted of combining genes corresponding to both upregulated and downregulated
phospho-peptides. This was done to avoid the assumption that only upregulated
phospho-peptides corresponded to activated pathways. What this means, however, is that
for a given pathway gene set result, the individual genes should be referenced back to the
phospho-peptide data for the relevant T cell subset(s) and interpreted at the site level to
validate the type of regulation implied by the pathway. The GSEA input did not
distinguish between upregulated and downregulated sites. When the analyses were
attempted using the up or down regulated sites separately, no significant gene sets or
pathways were identified.

From the phospho-proteomics data, p38-MAPK pathway signaling induced by
GITR was identified specifically in the iT\textsubscript{reg} subset and confirmed by western blot
(Figures 9-11). In T\textsubscript{regs}, inhibition of p38 abrogates TNF-induced proliferation (He et al.,
2018), and blocks the conversion of naïve T cells into iT\textsubscript{regs} by TGF-β (Huber et al.,
2008). Based on these data and supporting literature, the following hypothesis was
formed; GITR triggering activates p38-MAPK signaling in iT\textsubscript{regs} and contributes to
induction of iTreg proliferation. To test this hypothesis a 3-day proliferation assay was performed on DTA-1 or IgG isotype-treated iTregs, CD4+ T eff cells, and CD8+ cytotoxic T cells in the presence or absence of the p38 inhibitor SB203580. Indeed, p38 inhibition abrogated proliferation induced by GITR triggering in iTregs (Figures 13). This was confirmed again in a follow-up experiment using a longer proliferation timepoint to more optimally observe GITR-induced proliferation, as well as in an SB203580 dose-response experiment (Figures 14-15). In CD4+ T eff cells and CD8+ T cells, the same effect of p38 inhibition is not expected to be observed as our data showed weak evidence of GITR-induced p38 phosphorylation as well as a lack of activity in other upstream and downstream p38 pathway members. In the CD4+ T eff cells and CD8+ cytotoxic T cells, treatment with SB203580 actually led to more proliferation than DTA-1, suggesting that p38 signaling is likely playing a different role in these subsets, and in the context of DTA-1 treatment. However, it would be prudent to repeat the optimized 4-day proliferation study in CD4+ and CD8+ effector cells to further confirm the role of p38 and GITR stimulation on proliferation compared to Tregs.

When probing for possible GITR-regulated signals downstream of p38 signaling by western blot, differential c-Jun phosphorylation was observed in the iTreg subset. Phospho-c-Jun (Ser73) was induced the strongest by DTA-1 in iTregs, but was also induced in CD4+ T eff cells and CD8+ cytotoxic T cells. However, phosphorylation of c-Jun at (Ser63) was only observed in iTregs (Figure 11). C-Jun is a component of the transcription factor activator protein 1 (AP-1) and interacts with other AP-1 family members like activating transcription factor 2 (ATF-2), which was also induced by DTA-1 treatment in the iTreg subset. Activation of c-Jun has multiple biological results from
cell differentiation and proliferation to apoptosis (Angel and Karin, 1991). The functional consequences of differential phosphorylation of Ser63 and Ser73 on c-Jun are not well studied, but in a model of nitric oxide (NO)-induced apoptosis, phosphorylation of Ser63 on c-Jun was found to be responsible for the observed c-Jun dependent transcriptional activity but not phosphorylation at Ser73 (Li et al., 2004). It would be reasonable to hypothesize that the GITR-induced phosphorylation of c-Jun (Ser63) specific to the iT_{reg} subset drives unique transcriptional activity in iT_{regs}. It is also possible that the GITR-regulated transcription factors observed in this study are downstream of another MAPK family member and/or are influenced by crosstalk between the MAPK family members. Further investigation is required to identify a role for p-c-Jun (Ser63) in the context of GITR-regulated pathways in iT_{regs}.

Conclusion

The study herein demonstrated that GITR regulates unique phosphoproteomes in iT_{regs}, CD4^{+} T_{eff} cells, and CD8^{+} cytotoxic T cells. One implication of this is that T cell subsets respond differently to GITR stimulation and that this may lead to different phenotypes. In addition, simultaneous TCR stimulation is not needed to observe hundreds of GITR-regulated phospho-peptides in each of the T cell subsets. It is reasonable to hypothesize that similarly regulated signals may be occurring in vivo in a context like tumor immunity. If this is the case, GITR triggering should be appreciated beyond its role as a co-stimulatory receptor during TCR stimulation, and opportunities to leverage GITR stimulation for therapeutic ends should take into consideration the diverse phenotypes induced among unique T cell subsets and how this might affect the
therapeutic efficacy of anti-GITR treatments. Further investigation will be required to understand the potential contribution of GITR stimulation on T cells that have not received recent TCR stimulation. T\textsubscript{regs} in this scenario may be expected to have similar responses to those observed in this study due to their well documented constitutive expression of GITR (Nocentini et al., 2000). However, CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells require TCR stimulation to upregulate GITR, taking from 1-3 days for expression as was used in this study, and it is unclear what level of signaling through GITR would be detected without first inducing its upregulation in these subsets (Ronchetti et al., 2015). In \textit{vivo}, since GITR agonism drove T\textsubscript{reg} proliferation, one could hypothesize that GITR stimulation in the absence of sufficient TCR stimulation may result in an enhanced T\textsubscript{reg} compartment.

This study also demonstrated that inhibition of p38 reduces GITR-induced proliferation of iT\textsubscript{regs}. This is an example of a unique phenotypic change regulated by GITR and was specific to the iT\textsubscript{reg} subset. Although a functional suppression assay was not performed with the iT\textsubscript{regs} and a population of responder cells to understand the direct readout of anti-GITR stimulation and p38 inhibition, the GITR-induced proliferation of the iT\textsubscript{reg} subset and the literature describing the importance of p38 activity in support of iT\textsubscript{reg} suppressive function, point to GITR stimulation enhancing iT\textsubscript{reg} suppressive function. Inhibition of p38 can lead to loss of regulatory T cell function (Adler and Steinbrink, 2008), so GITR’s enhancement of p38 signaling may support the suppressive phenotype. This will need to be investigated further in a suppression assay.

Further implications of this study underscore p38 as a target for the modulation of T\textsubscript{reg} suppressive function. Overcoming the immunosuppressive TME remains an
important challenge for cancer immunotherapy, and T_{reg} are a central actor in the regulation of immunosuppression (Moreno Ayala et al., 2019). Replicating the antitumor efficacy of GITR therapy in humans will likely require an agonist antibody approach with a functional Fc to mediate depletion of T_{reg} in the tumor microenvironment. In this scenario, the goal would be to achieve agonist activity on the CD4^{+} and CD8^{+} T cells while iT_{reg} would be depleted from the tumor microenvironment. Using GITRL as a therapy would exclude the benefit of antibody-mediated depletion of iT_{reg} in the tumor microenvironment. Otherwise, the potential enhancement of T_{reg} overall numbers, and perhaps suppressive function, may not allow for the tipping of the scale in favor of antitumor immunity. In the context of GITRL therapy, perhaps p38 could be investigated as a combination therapy that may have the potential to overcome the problem of induced T_{reg} proliferation without hampering potentiation of CD8^{+} and CD4^{+} effector cells. In addition, p38 phosphorylation could be investigated as a biomarker for GITR triggering specific to T_{reg}.

Finally, it is clear that GITR signals in multiple ways and causes phenotypic changes in iT_{reg}. Therefore, GITR expression on iT_{reg} is more than just a “sink” to outcompete ligand binding with other T cell subsets. The unique GITR-regulated phosphoproteomes in the different T cell subsets observed in this study, underscores the importance of understanding GITR signaling to help interpret, anticipate, and modulate GITR therapy and combination strategies. The role of p38 signaling in GITR-induced proliferation of iT_{reg} is one example.
Study Limitations

The use of an *in vitro* induced-T\textsubscript{reg} model system is an imperfect model for regulatory T cells and may not represent the signaling events in natural T\textsubscript{regs}, and therefore may have less biological relevance. It is possible that at a later DTA-1 treatment timepoint, the phosphoproteomes may look more similar across these subsets, although the limited western blot experiments assessing MAPK signals do indicate that there are at least some sustained signaling differences among the T cell subsets (Figure 12).
Appendix I

Tables

Table 1. Summary of DTA-1 5/15 min. IMAC and pY Enrichment Results

<table>
<thead>
<tr>
<th></th>
<th>IMAC</th>
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<tr>
<td>Total Number of Detected Peptides</td>
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<td>771</td>
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<tr>
<td>Total Number of Significant FC (+ and -)</td>
<td>5,645</td>
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iTags

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CD4+ T eff cells

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CD8+ T cells

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Summary of IMAC and pY-enriched phospho-peptide data from T cell subsets treated with 5 µg/mL IgG isotype control for 15 min. or 5 µg/mL DTA-1 for 5 min. or 5 µg/mL DTA-1 for 15 min. Data are from two replicate LC-MS/MS runs per sample and were filtered to reflect nonredundant phosphorylation sites. The number of significant increases and decreases are shown for each T cell subset at 5 and 15 min. DTA-1 treatment timepoints relative to IgG isotype control. Significant fold change increases are defined as the number of upregulated phospho-peptides ≥ 2.5 fold change and the number of decreases are defined as the number of downregulated phospho-peptides ≤ -2.5 fold change.
Table 2. iTregs + DTA-1 15 min. IMAC GSEA Results

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A GSEA was performed on the significant phospho-peptide fold changes for each of the T cell subsets with a customized script in R software. Upregulated and downregulated sites were combined, and the associated gene names were used to run the analysis. When multiple gene names were associated with a unique site that had a significant fold change, all genes were included in the analysis. Gene sets are sorted by ascending p-value (including gene sets where p < 0.05).
Table 3. CD4+ T\textsubscript{eff} cells + DTA-1 15 min. IMAC GSEA Results

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<td>E2F1DP2_01</td>
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<td>E2F4DP2_01</td>
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<td>E2F_02</td>
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<td>E2F1_Q6_01</td>
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<td>GGGTGGRR_PAX4_03</td>
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<td>E2F_Q6_01</td>
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A GSEA was performed on the significant phospho-peptide fold changes for each of the T cell subsets with a customized script in R software. Upregulated and downregulated sites were combined, and the associated gene names were used to run the analysis. When multiple gene names were associated with a unique site that had a significant fold change, all genes were included in the analysis. Gene sets are sorted by ascending p-value (including gene sets where p < 0.05).
A GSEA was performed on the significant phospho-peptide fold changes for each of the T cell subsets with a customized script in R software. Upregulated and downregulated sites were combined, and the associated gene names were used to run the analysis. When multiple gene names were associated with a unique site that had a significant fold change, all genes were included in the analysis. Gene sets are sorted by ascending p-value (including gene sets where p < 0.05).
A GSEA was performed on the significant phospho-peptide fold changes for each of the T cell subsets from the pY-enriched data with a customized script in R software. Upregulated and downregulated sites were combined, and the associated gene names were used to run the analysis. When multiple gene names were associated with a unique site that had a significant fold change, all genes were plugged in to the analysis. These data represent the 15-minute DTA-1 treatment timepoint for iTregs. No significant gene sets were identified using the CD4+ Teff or the CD8+ T cell data. Gene sets are sorted by ascending p-value (including gene sets where p < 0.05).
Table 6. MAPK Pathway Members Detected After IMAC and pY Enrichment

<table>
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This table shows ERK, JNK, and p38 MAPK family members that were detected during the phospho-proteomics analysis of GITR-treated iTregs, CD4+ T<sub>eff</sub> cells, and CD8+ T cells. The Normalized Fold Change represents the fold change for the DTA-1 treatment timepoint indicated over IgG Isotype control. A hyphen means that the given peptide was not detected in the T cell subset. Fold changes highlighted in green represent significant increases ≥ 2.5 FC, and fold changes highlighted in red represent significant decreases ≤ -2.5 FC. In the peptide column, an * indicates the phosphorylated peptide. The enrichment approach that was used to identify the modified peptide is shown in the last column.
Appendix II.

Figures

Figure 1. DTA-1 treatment induced IκBα degradation and NF-κB p65 phosphorylation in C57BL/6 mouse splenocytes independent of anti-CD3/CD28 stimulation.

(A) Schematic of the experimental procedure. C57BL/6 mouse splenocytes were stimulated with mouse magnetic beads coated with anti-CD3/CD28 and cultured with IL-2 for 7 days. Anti-CD3/CD28 magnetic beads were removed for 24 hours before the anti-CD3/CD28 re-stimulation and DTA-1 or IgG isotype control treatments started. (B) Western blot analysis of C57BL/6 mouse splenocytes treated with DTA-1 or IgG isotype control with and without mouse anti-CD3/CD28 re-stimulation for 30 min.
Figure 2. Experimental scheme for the enrichment and validation of iT<sub>regs</sub>, CD4<sup>+</sup> T<sub>eff</sub> cells, and effector CD8<sup>+</sup> T cell subsets.

(A) Spleen and lymph nodes were dissected from 6-10-week-old C57BL/6 mice and were disaggregated and underwent T cell subset specific purification and enrichment and/or polarization. (B) Schematic of the experimental procedure timeline to generate iT<sub>regs</sub>, CD4<sup>+</sup> T<sub>eff</sub> cells, and CD8<sup>+</sup> cytotoxic cell subsets.
Figure 3. iT<sub>reg</sub>, CD4<sup>+</sup> T<sub>eff</sub> cells, and CD8<sup>+</sup> T cells express expected subset markers. Spleen and lymph nodes were dissected from 6-10-week-old C57BL/6 mice and were disaggregated and underwent T cell subset specific purification and enrichment and/or polarization. At day 5 in culture for each subset, cells were analyzed by flow cytometry for expression of CD3, CD4, CD8α, and FoxP3.
Figure 4. iTregs, CD4+ T eff cells, and CD8+ T cells express GITR. Spleen and lymph nodes were dissected from 6-10-week-old C57BL/6 mice and were disaggregated and underwent T cell subset specific purification and enrichment and/or polarization. At day 5 in culture for each subset, cells were fix/permed and the Flow analysis for GITR expression was performed on the same day. (A) Histograms showing GITR expression in iTregs, CD4+ T eff cells, and CD8+ T cells at day 5 in culture. The Y axis is normalized to peak height and plotted on a % scale in order to more easily compare GITR expression across the T cell subsets. (B) Column plot showing MFI of IgG Isotype and αGITR stained T cell subsets. (C) Column plot showing GITR MFI fold change over IgG isotype MFI.
Figure 5. The GITR agonist antibody DTA-1 induces NF-\(\kappa\)B and JNK-MAPK signaling in iT\(_{\text{regs}}\), CD4\(^{+}\) T\(_{\text{eff}}\) cells, and CD8\(^{+}\) T cell subsets. Spleen and lymph nodes were dissected from 6-10-week-old C57BL/6 mice and were disaggregated and underwent T cell subset specific purification and enrichment and/or polarization. (A) Schematic of the experimental procedure timeline to generate iT\(_{\text{regs}}\), CD4\(^{+}\) T\(_{\text{eff}}\) cells, and CD8\(^{+}\) T cell subsets and treat them with either IgG isotype control or DTA-1. (B) Western blot analysis of NF-\(\kappa\)B subunits and JNK MAPK in the indicated T cell subsets.
Figure 6. Phospho-Proteomics Analysis Schematic

Extracts of DTA-1 or Isotype treated iTregs, CD4+ T_{eff} cells, and CD8+ T cells were digested to peptides, purified over reversed phase columns, enriched with pY or IMAC, run in LC-MS/MS, database searched/score filtered, and finally quant data was generated. (A) Schematic of the phospho-proteomics approaches. pY and IMAC enrichments were performed on independent sample preps.
Figure 7. Venn Diagrams of DTA-1-regulated phosphorylation events at 15-minute treatment timepoint. (A) Venn diagrams showing the number of unique IMAC-purified phospho-peptides that had elevated phosphorylation ≥ 2.5 fold (left), and reduced phosphorylation ≤ -2.5 fold change (right). (B) Venn diagrams showing the number of pY antibody-purified phospho-peptides that had elevated phosphorylation ≥ 2.5 fold change (left), and reduced phosphorylation ≤ -2.5 fold change (right). The relative size of the T cell subsets in the diagrams corresponds with the number of unique phospho-peptides detected. A is scaled for IMAC-purified peptides and can be compared across increases and decreases, and B is scaled for pY-purified peptides and can be compared across increases and decreases.
Figure 8. Phospho-proteomics IMAC data confirms DTA-1-induced NF-κB signaling in all T cell subsets

Phospho-proteomics IMAC data confirms DTA-1-induced NF-κB signaling in all T cell subsets via detection of phospho-IκBα (Serine 32) and agrees with western blot data. (A) Mass spectrometry measured average abundance (intensity) of the indicated phosphorylated peptide detected in the T cell subsets. (B) Western blot analysis of NF-κB signaling pathway members specifically highlighting the detected induction of pIκBα (Ser32) (red boxes) by both phospho-proteomics and western blotting approaches. Column layout of samples in A corresponds with western blot lanes in B to easily compare detected phospho-induction by both techniques. Western blots were repeated in triplicate with similar results.
Figure 9. GITR triggering induces p38 phosphorylation in iTregs but not in CD4+ T eff cells and CD8+ T cells.

(A) Mass spectrometry measured average abundance (intensity) of the indicated phosphorylated peptide detected in the T cell subsets. (B) Western blot analysis of the three main phospho and total MAPK signaling pathway members. Phospho-p38 is highlighted specifically in red boxes to easily compare phospho-proteomics and western blotting approaches. Column layout of samples in A corresponds with western blot lanes in B to easily compare detected phospho-induction by both techniques. Relative fold change of p-p38 is shown normalized to IgG control for each subset. WB signal intensity was quantitated in Image Lab™ software. Western blots were repeated in triplicate with similar results.
Figure 10. GITR triggering induces signaling upstream of p38 phosphorylation in iT<sub>regs</sub> but not in CD4<sup>+</sup> T<sub>eff</sub> cells and CD8<sup>+</sup> T cells.

(A) Mass spectrometry measured average abundance (intensity) of the indicated phosphorylated peptide detected in the T cell subsets. pTAB2(Ser372) was not detected in the CD4<sup>+</sup> T<sub>eff</sub> cell population. (B) Western blot analysis of phospho and total proteins upstream of p38: TAB2, TAK1, MKK3, MKK4, and MKK6. pTAB2 is highlighted specifically in red boxes to easily compare phospho-proteomics and western blotting approaches. Column layout of samples in A corresponds with western blot lanes in B to easily compare detected phospho-induction detected with each technique.
Figure 11. GITR triggering induces signaling downstream of p38 phosphorylation in iTregs differentially compared to CD4+ T eff cells and CD8+ T cells.

(A) Mass spectrometry measured average abundance (intensity) of the indicated phosphorylated peptide detected in the T cell subsets. Phospho-MAPKAPK-2 (Thr208) was not detected in the CD8+ T cell population. pMAPKAP-2 (Thr222) is the human phospho site that the antibody product is named for, and (Thr208) is the mouse site. (B) Western blot analysis of phospho and total proteins downstream of p38. Column layout of samples in A corresponds with western blot lanes in B to easily compare detected phospho-induction detected with each technique.
Figure 12. GITR triggering induces sustained p38 phosphorylation in iTregs. (A) A schematic of the experimental procedure timeline to generate iTregs, CD4\(^+\) T\(_{\text{eff}}\) cells, and CD8\(^+\) T cell subsets and treat them with either IgG isotype control or DTA-1. (B) Western blot analysis of the three main phospho and total MAPK signaling pathway members, p38, JNK, and ERK.
Figure 13. DTA-1 induces proliferation in iTregs in the absence of CD3/CD28 stimulation and is partially blocked by the p38 inhibitor SB203580.

iTregs, CD4+ T eff cells, and CD8+ T cells were stained with ViaFluor 488 SE Proliferation Dye, were plated with and without anti-CD3/CD28 and were pretreated with SB203580 (10 μM) or vehicle control for 1 hour before treatment with IgG isotype or DTA-1 (5 μg/ml) for 3 days. SB203580 was administered every 12 hours to respective treated groups. (B) Column graphs showing statistical comparison of average MFI of the proliferation dye of the indicated treatment groups. iTregs were gated on FoxP3 and CD4+ T eff cells and CD8+ T cells were gated on lymphocyte population. Data represent triplicate experiments with standard error plotted. Statistical significance is shown relative to IgG unless noted otherwise.
Figure 14. *In-vitro* inhibition of p38 subverts GITR-induced proliferation of iTregs. iTregs were stained with ViaFluor 488 SE Proliferation Dye and were pretreated with SB203580 (10 μM) or vehicle control for 1 hour, then with IgG isotype or DTA-1 (5 μg/ml) for 4 days. SB203580 was administered every 12 hours to respective treated groups. (A) Schematic of iTreg enrichment, treatment, and proliferation study. (B) Histograms showing flow cytometry analysis of ViaFluor 488 SE Proliferation Dye gated on FoxP3+ cells. (C) Column graph showing statistical comparison of average MFI of the proliferation dye of the indicated treatment groups (n=4).
Figure 15. The p38 inhibitor, SB203580, abrogates DTA-1 induced proliferation of iTregs in a dose-dependent manner.

iTregs were stained with ViaFluor 488 SE Proliferation Dye and were pretreated with SB203580 (1, 5, or 10 µM) or vehicle control for 1 hour, then with IgG isotype or DTA-1 (5 µg/ml) for 4 days. SB203580 was administered every 12 hours to respective treatment groups at the indicated concentration. (A) Histograms showing flow cytometry analysis of ViaFluor 488 SE Proliferation Dye gated on FoxP3+ cells. (B) Column graph showing statistical comparison of average MFI of the proliferation dye of the indicated treatment groups with standard error shown (n=4). Statistical significance is shown relative to IgG Isotype control.
This illustration represents a possible GITR-regulated signaling cascade activating p38-MAPK and leading to proliferation in iT\textsubscript{reg}. In iT\textsubscript{reg}, the agonist mAb DTA-1 stimulates GITR and may be recruiting a TRAF family member protein(s) to the cytoplasmic domain. The adaptor protein TAB2 can link TAK1 with TRAF6, thereby enabling the phosphorylation of MKK3, MKK4, and MKK6. MKK3, MKK4, and/or MKK6 can directly phosphorylate p38, after which p38 can then interact with many different substrates, with one of the functional consequences being the induction of proliferation. GITR-induced iT\textsubscript{reg} proliferation can be abrogated by the p38 inhibitor SB203580 (dark red rectangle). Phosphorylated proteins that were detected via phospho-proteomics and/or western blot are shown in blue, and other interactions found in the literature are shown in white.
References


