# UNDERSTANDING & OPTIMIZING HUMAN T REGULATORY CELL FUNCTION IN PATIENTS WITH AUTOIMMUNITY AND/OR UNDERGOING TRANSPLANTATION

by

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## Abstract

$CD4^+FOXP3^+$ T regulatory cells (Tregs) are potent suppressors of inflammatory immune activity. Cellular therapy with Tregs is a promising way to induce antigen specific tolerance in transplantation and autoimmunity, as it would allow the reduction of nonspecific immunosuppression. Currently, Tregs are being tested in clinical trials; however, outstanding questions regarding stability, specificity, and longevity of transferred cells remain. The aim of this research was to better understand the potential plasticity of Tregs, develop novel methods of creating antigen specific Tregs, and determine the optimal signals for Tregs to persist after transfer. To better understand the pathological conversion of Tregs to inflammatory cells, I examined the phenotype of Tregs in systemic sclerosis, a Th2-biased disease. I found that Tregs in patient skin and blood had acquired Th2-cytokine and homing marker expression, respectively, and that both tissue-localized and homing cells express the receptor for IL-33, which was expressed in patient skin. This work suggests that sub-populations of Tregs have the capacity to become pathogenic upon encountering tissue-specific inflammatory signals. Next, in order to create antigen specific Tregs, I developed a novel chimeric antigen receptor (CAR) against HLA-A2 and tested its function. A2CAR-Tregs were highly activated and proliferative in response to HLA-A2, but they retained their suppressive capacity and expression of the transcription factors FOXP3 and Helios, and the effector molecules CD25 and CTLA-4. A2CAR-Tregs were also superior to polyclonal Tregs at preventing xenoGVHD in mice, even at low doses. Thus, A2CAR-Treg cell therapy is a promising new technology to create potent Tregs for antigen-specific transplant tolerance. Finally, to optimize Treg activity and persistence in patients, I developed a series of CARs containing different co-stimulatory domains. Four TNFR

superfamily domains were cloned, from 4-1BB, OX40, GITR and TNFR2, and three signalling domains were cloned from the B7/CD28 superfamily, from ICOS, PD-1, and CTLA-4. 4-1BB, OX40, ICOS, and PD-1 containing CARs were expressed on the surface of cells. These tools will provide valuable information in future research on Treg survival *in vivo*. Collectively, these studies have provided insights to improve both the safety and efficacy of Treg cell therapy.

## Preface

### Chapter 1

Versions of chapter 1 have been published:

MacDonald KG, Orban PC, Levings MK. T regulatory cell therapy in transplantation: stability, localization and functional specialization. Curr Opin Organ Transplant. 2012 Aug;17(4):343-8.

and:

Vent-Schmidt J, Han JM, MacDonald KG, Levings MK. The role of FOXP3 in regulating immune responses. Int Rev Immunol. 2014 Mar;33(2):110-28.

I performed literature review and wrote the majority of the text represented in my thesis. Levings MK reviewed, edited, and oversaw manuscript preparation.

### Chapter 3

A version of chapter 3 has been published:

MacDonald KG, Dawson NA, Huang Q, Dunne JV, Levings MK, Broady R. (2015). Regulatory T cells produce profibrotic cytokines in the skin of patients with systemic sclerosis. J Allergy Clin Immunol. 135(4):946-955.

I conducted the experiments on fibroblasts, sorted Tregs, and expanded T cells, analyzed all data, and wrote most of the manuscript. Patient skin and data were collected by Dunne, JV, and cultured and stained by Huang, Q. Chemokine staining and immunofluorescence were performed by Huang Q and Dawson NA. Levings MK and Broady R supervised the project and reviewed the manuscript.

**Chapter 4**

I was the primary contributor to Chapter 4. I planned, executed, and analyzed *in vitro* experiments. Orban PC planned and assisted with cloning lentivirus constructs. Hoppli R and Huang Q monitored and sacrificed mice with my supervision. All work was supervised by Borady R, Orban PC, and Levings MK

This work has been submitted as:

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**Chapter 5**

I conceptualized, planned, and executed chapter 5. I designed the lentiviral constructs. I cloned the co-stimulatory domains with the assistance of Huang Q. Orban PC planned and created co-inhibitory constructs. Huang Q performed transient transfection of co-inhibitory domains. All work was supervised by Broady R, Orban PC, and Levings MK.

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## List of abbreviations

ACA: anticardiolipin antibody
Allo: allogeneic
AML/RUNX1: acute myeloid leukemia 1 protein/Runt-related transcription factor 1
ANOVA: Analysis of variance
APC: antigen presenting cell
ATCC: American Type Culture Collection
BLIMP-1: B lymphocyte-induced maturation protein-1
CAR: chimeric antigen receptor
CEA: Carcinoembryonic antigen
CCR: chemokine (C-C motif) receptor
CD: cluster of differentiation
CLA: Cutaneous lymphocyte antigen
CPD: cell proliferation dye
CRTH2: chemoattractant receptor-homologous molecule expressed on Th2 cells
Ct: cycle threshold
CTLA-4: cytotoxic T-lymphocyte-associated protein 4
CXCR: CXC chemokine receptors
DAPI: 4',6-diamidino-2-phenylindole
DLCO: diffusing capacity of lung for carbon monoxide
DNA: Deoxyribonucleic acid
EAE: Experimental autoimmune encephalomyelitis
FcγR: Fc gamma receptor
FOXO1: forkhead box O1
FOXP3: forkhead box protein 3
FVC: forced vital capacity
FVD: Fixable Viability Dye
GARP: glycoprotein A repetitions predominant
GATA: GATA binding protein 3
GITR: glucocorticoid-induced TNFR family related gene
GLY: glycine
GVHD: Graft versus host disease
HER2: human epidermal growth factor receptor 2
HLA: human leukocyte antigen
HSCT: Hematopoietic stem cell transplantation
ICS: intracellular cytokine stain
ICOS: Inducible T-cell COStimulator
IFN: interferon
IL: interleukin
IMDM: Iscove's Modified Dulbecco's Medium
IRF: interferon regulatory factor
LAP: Latency Associated Peptide
LEF1: Lymphoid enhancer-binding factor 1
mAb: monoclonal antibody

MFI: mean fluorescence intensity
MHC: Major histocompatibility complex
MOG: Myelin oligodendrocyte glycoprotein
mRSS: modified rodnan skin score
NOD: Non-obese diabetic
NSG: NOD/SCID IL-2R$\gamma^{null}$
PBMC: Peripheral blood mononuclear cell
PD: Programmed cell death
PI3K: Phosphoinositide 3-kinase
PMA: phorbol 12-myristate 13-acetate
rH: recombinant human
RNA: Ribonucleic acid
ROR: RAR-related orphan receptor C
RT-PCR: Reverse transcription polymerase chain reaction
SATB1: special AT-rich sequence-binding protein-1
SCFV: Single-chain variable fragment
Scl-70: anti-topoisomerase
SEM: standard error of the mean
SSc: Systemic Sclerosis
TBET: T-box expressed in T cells
TBX21: T-box 21
TCR: T cell receptor
Th: T helper
Tconv: T conventional cells
TGF: transforming growth factor
TM: transmembrane
TNF: Tumor necrosis factor
TNFR: Tumor necrosis factor receptor
TNP: 2,4,6-trinitrophenyl
Tr1: Type 1 regulatory T cells
Tregs: T regulatory cells
TSDR: Treg specific demethylation region
xeno: xenogeneic

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*For Mathilda,*

*who never knew how science could help her*

*or how she would help science*

# Chapter 1: Introduction

## 1.1 T helper cells and the adaptive immune response

$CD4^+$ T helper cells develop in the thymus from lymphocyte progenitors generated in the bone marrow [1]. During development, T helper cells generate a T cell receptor (TCR) that recognize antigen presented in the context of MHC (called HLA in humans). T helper cells express the co-receptor CD4, which restricts antigen recognition to peptides presented in the context of class II MHC. Class II MHC is largely, although not exclusively, expressed by professional antigen presenting cells (APCs) such as dendritic cells and macrophages [2]. Once activated by APCs, $CD4^+$ T cells begin proliferating, differentiating to produce cytokines, and traffic to sites of inflammation. The combination of cytokines secreted by $CD4^+$ T cells directs both tissues and other immune cells to mount a response specific for the problem at hand, thus earning them the name "helper". For example, T helper cells have traditionally been characterized as either Th1 cells, which produce IFN-γ and boost the cytotoxic, $CD8^+$ T cell-driven antiviral response, or as Th2 cells, which produce IL-4 to help the antibody and B-cell-mediated parasite clearance [3]. Further understanding of $CD4^+$ T cells in immune responses has led to the discovery of additional subsets including but not limited to Th17 cells and T regulatory cells.

$CD4^+$ T cell differentiate into functional subsets after receiving three signals. "Signal 1" is activation of the TCR when antigen is presented in the context of class II MHC. Although the α and β chains provide antigen specificity, the CD3ζ, γ, δ, ε chains of the TCR complex contain motifs that are phosphorylated by the kinase LCK early in TCR signalling. This allows the recruitment and phosphorylation of ZAP70, which in turn activates linker for activation of T

cells (LAT) [4]. LAT forms a signalsome that propagates the TCR signal through multiple pathways, including PI3k, MAPK, NFκB, and calcium signalling/NFAT activation [5]. These activated pathways result in T cell proliferation, cytoskeletal rearrangement and migration, and expression of functional molecules.

Despite its fundamental role, TCR engagement alone can be insufficient to fully activate T cells. An additional signal, termed "signal 2", must often be provided to T cells along with signal 1[6]. Signal 2 is generally also provided by APCs, usually in the form of ligands that are upregulated by environmental signals to interact with co-stimulatory molecule on T cells [7]. CD28 is the prototypic co-stimulatory molecule, as it is constitutively expressed on T cells, and its ligation synergizes with TCR activation to boost downstream signalling pathways after interacting with CD80 and CD86 on dendritic cells. Many immunoglobin-like and TNF-like co-stimulatory molecules have been described, and some have been found to have co-inhibitory activity to negatively regulate TCR signalling. Thus signal 2 is an important modulator of TCR signalling to determine the degree of activation after antigen recognition.

The final signal to determine T helper cell differentiation is signal 3. Signal 3 refers to the cytokine signals provided to the T cells during and after activation. Cytokines can be produced by APCs during TCR engagement or by other immune or non-immune cells in the microenvironment shortly after activation. Signalling through cytokine receptors then activates STAT molecules, which in turn up-regulate lineage defining transcription factors. For example, IFN-γ promotes STAT1 and TBET for Th1 development, while IL-4 activates STAT6 and thus GATA-3, which promotes Th2 development. IL-23 and TGFβ induce RORC and FOXP3, respectively, to generate Th17 or Treg cells [8, 9]. As a final result of integrating TCR, co-receptor,

and cytokine signalling, epigenetic remodelling occurs to solidify T helper lineage commitment (**Figure 1.1**).

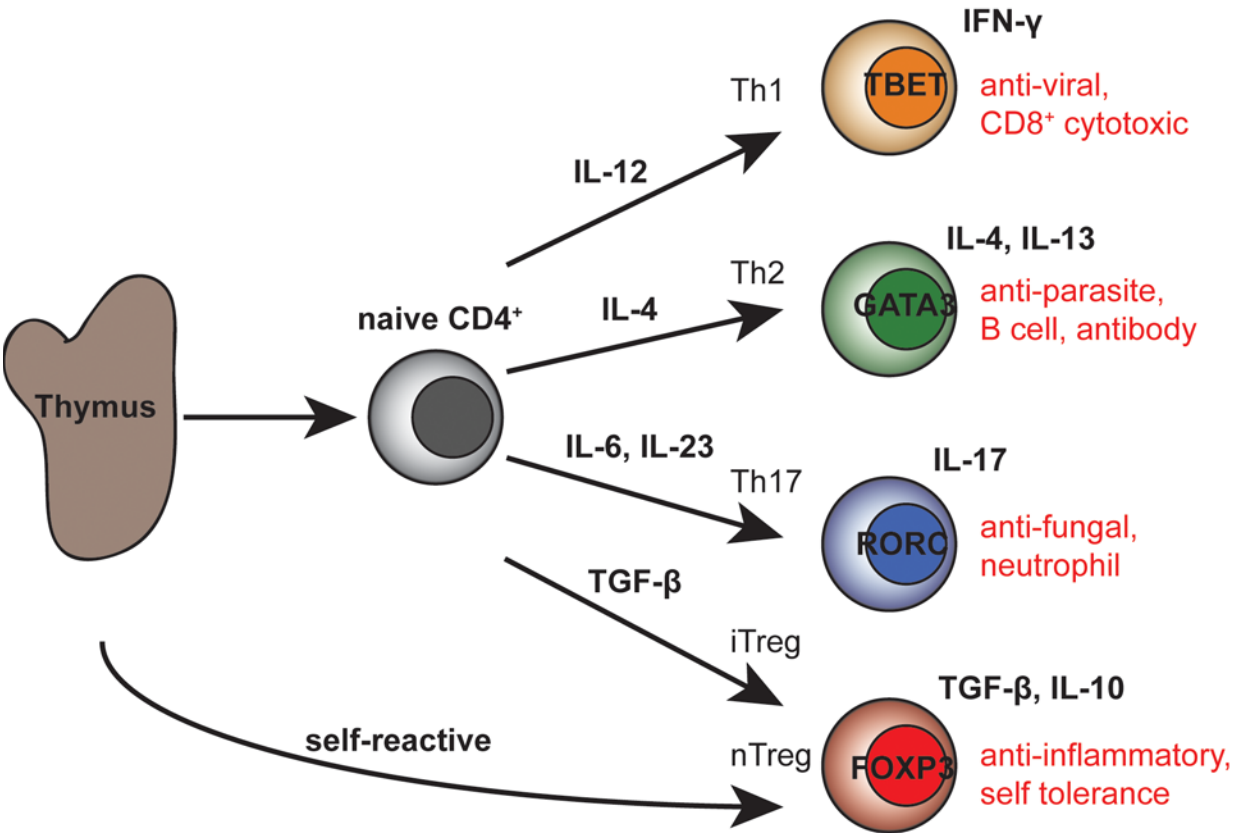



**Figure 1. 1** *Differentiation of CD4$^+$ T helper cell subsets.* CD4$^+$ T cells develop in the thymus then enter into circulation. Upon antigen encounter, T cell receptor and co-stimulatory ligation activate naïve CD4+ cells and cytokines provide signals to differentiate into helper lineages defined by master transcription factors and maintained by epigenetics. T helper subsets have distinct downstream roles in resolving infection and inflammation.

## 1.2 T regulatory cells

T regulatory cells (Tregs) are a subset of CD4$^+$ T helper (Th) cells that are defined by constitutively high expression of the transcription factor FOXP3. FOXP3$^+$ Tregs can be generated in two different ways: 1) those that leave the thymus as a naïve Treg (CD45RA$^+$), also known as natural Treg or tTreg, which are highly reactive to self-antigen, and 2) those that are

derived in the periphery when $CD4^+$ cells are activated under tolerogenic conditions, also known as an induced Treg ($CD45RO^+$) or pTreg. In both cases, constitutive, high FOXP3 expression is required for Treg suppression and is epigenetically maintained by demethylation of the Treg specific demethylated region (TSDR), which faithfully discriminates between $FOXP3^+$ activated T effector cells and true Tregs in humans [10]. High FOXP3 expression represses inflammatory cytokines, such as IL-2 and IFN-γ, and drives expression of key Treg molecules CTLA-4 and CD25. When Tregs are activated in an antigen-MHC dependent manner, they suppress many types of immune cells ranging from dendritic cells, which initiate an immune response, to the downstream effectors such as $CD4^+$ and $CD8^+$ T cells. The immunosuppressive function of Tregs is critical for tolerance to self-antigens, as demonstrated by the analysis of humans who lack fully functional Tregs and suffer from a variety of autoimmune syndromes [11, 12]. These patients, who have deleterious mutations in FOXP3, ultimately suffer from lethal autoimmunity.

### 1.2.1 FOXP3 driven mechanisms of suppression

FOXP3 functions to express key molecules for Treg mediated suppression, while also specifically repressing inflammatory cytokines. However, the importance of FOXP3 is demonstrated in scurfy mice, which lack the FOXP3 gene: even though these mice develop a Treg epigenetic signature, FOXP3 is still necessary to prevent inflammation[13]. Ectopic expression of FOXP3 has shown that FOXP3 directly induce critical suppressive molecules [14], such as CD25 and CTLA-4, in transcriptional complexes with NFAT [15] and AML/RUNX1 [16]. $FOXP3^+$ Tregs also express high levels of these and other target genes [17, 18].

CD25 is a canonical Treg surface marker that allows purification of Tregs but also plays a functional role. As the high-affinity chain of the IL-2 receptor, CD25 may allow Tregs to consume IL-2 at a greater rate than Tconv, which also express CD25 after activation but at lower levels, and thus reduce local concentrations of this growth factor [19]. However, CD25 also appears to have an important role in stabilizing Treg phenotype. FOXP3 loss was found to occur only in $CD25^{lo}$ populations of Tregs in fate-mapping [20]and transfer studies [21], suggesting that high CD25 expression and IL-2 signaling results in the most stable population of Tregs. Indeed, a sensor for IL-2 signaling in the TSDR that stabilizes FOXP3 expression was recently described [22].

CTLA-4 is also a critical molecule for Treg suppressive activity downstream of FOXP3. Although systemic knockout results in lethal inflammation due to expression by activated Tconv [23 24], Treg-specific knockouts also result in lymphoproliferative disease and systemic inflammation [25], suggesting a non-redundant role for CTLA-4 in Tregs. A large portion of CTLA-4 function is dependent on the extracellular domain [26], as CTLA-4 appears to outcompete CD28 for binding to co-stimulatory molecules CD80 and CD86 on dendritic cells [27]. However, internalization of CTLA-4 also appears to play a critical role in suppression as this allows CTLA-4 expressing cells to physically remove CD80/86 from dendritic cells and degrade these molecules [28]. This is consistent with the increase in CD80/86 expression observed in CTLA-4 knockout Treg mice. Whether CTLA-4 intracellular signaling plays a role in Treg phenotype has yet to be resolved [29].

### 1.2.2 Epigenetic maintenance of the Treg lineage

The activity of all transcription factors, including FOXP3, is dependent on the epigenetic landscape within a cell. Epigenetic modifications are changes to chromatin that affect its

packing, and thus the accessibility of genes to transcription factors for expression [30]. Modifications can occur at both the histone and DNA level. DNA modification, consisting primarily of methylation, is considered a more passive and stable process and occurs only at cytosine residues that are immediately followed by guanidine (CpG). Epigenetic differences between Tregs and other Th cell lineages are found both the level of DNA methylation [31] and histone modification [32]. It is the faithful propagation of epigenetic states that allow T helper lineages to be maintained despite numerous rounds of cell division.

A key component of constitutive FOXP3 expression is de-methylation of the FOXP3 gene, particularly the Treg-specific-de-methylated region (TSDR) [33] [33]. TSDR demethylation is an active process that occurs during thymic development of nTregs [34]. The role of this region is more apparent in human cells, where FOXP3 can be transiently increased after TCR activation but expression is not sustained [35]. Bona fide Tregs remain distinguishable because Tconv are heavily methylated at the TSDR [36]. Importantly, TSDR demethylation does not occur during *in vitro* differentiation of Tregs but can occur *in vivo*, suggesting that increasing FOXP3 alone is not sufficient to re-capitulate a Treg at the epigenetic level [10].

Although there is a clear role for epigenetic control of FOXP3 expression, Tregs also have distinct patterns of epigenetic modifications that contribute to the Treg phenotype[13]. Hypomethylation in Tregs is critical for the expression of FOXP3-independent transcription factors like Eos and Helios, but not of FOXP3 controlled genes such as IFN-γ and IL-2. FOXP3 also appears to bind enhancer regions made accessible through epigenetic remodelling driven by other transcription factors, and to promote Treg phenotype by altering the transcriptional complexes at these sites of open chromatin. For example, AP-1 and NFAT appear to play a critical role in priming the chromatin for FOXP3, with FOXO1 as a potential placeholder in the

complex until FOXP3 can replace it and drive expression or repression of Treg specific genes [37]. Thus, epigenetic remodelling must occur in order for FOXP3 to access genes critical for Treg development.

### 1.2.3 Treg cell therapy

Since Tregs play a major role in peripheral tolerance, considerable effort has been expended in developing ways to exploit their immunoregulatory properties in the context of transplantation. Transplantation is particularly well suited to interventions designed to induce tolerance, such as regulatory cell-based therapies, since the exact time of initiation of the undesirable immune response is known, and these cells are likely more effective at preventing alloantigen-directed immune attack than reversing it once it has begun. Indeed, proof of concept studies in animal models have shown that co-delivery of Tregs with stem cell or solid organ transplants prevents donor-specific immunity and promotes long-term tolerance[38].

The initial evidence that Tregs could be of therapeutic value was that re-constituting Treg-deficient mice with $CD4^+$ $CD25^+$ cells could rescue them from lethal autoimmunity [39, 40]. Since then, many Tregs have been shown to be effective at inducing tolerance in many settings despite a variety of Treg sources, isolation and expansion protocols, and diseases. Early cell transfer models in mice, where memory $CD4^+$ $CD25^+$ cells isolated from a previously tolerized mouse were able to prevent mismatched skin graft and heart rejection, suggested that Treg transfer could prevent allo-immunity and promote transplant tolerance [41-43]. Mouse naïve Tregs stimulated *in vitro* were also found to have suppressive activity *in vitro* and *in vivo* [44, 45]. Many transplant studies with both mouse and human cells have confirmed that Tregs are potent suppressors of allograft responses, particularly when primed with alloantigen [46-52]. Polyclonal

Tregs have also been used to treat graft-versus-host-disease (GVHD) after bone marrow transplantation in mice, even without pre-stimulation with alloantigen [53, 54]. Treg cell transfer has also been shown to promote tolerance to autoantigens in autoimmune disease, further supporting the rationale for using Treg cell therapy to induce tolerance [55-62]. Based on the success of these pre-clinical models, trials are currently underway in humans to treat both type 1 diabetes[63] and GVHD[64-66].

### 1.2.4 Aspects of Treg biology to consider for successful cell therapy

The potential for Treg cell-based therapies to dramatically change the immunological outcomes of transplantation is clear. As we have learnt more about the cellular and molecular biology of these cells, however, it has also become clear that many questions about how these cells work must be answered before we can optimize their therapeutic application. For example, Will Tregs remain phenotypically and functionally stable once they are transferred? Can we enhance Treg activity by directing the activity of stable $FOXP3^+$ cells? How long will transferred Tregs survive and can we expand their lifespan to achieve long-term tolerance? Here I will briefly review our current understanding of some aspects of Treg biology and how it relates to cell therapy in transplantation (**Figure 1.2**).

To be useful for cell therapy, transferred Tregs must maintain FOXP3 expression and be potently immunosuppressive *in vivo*. Initial fate mapping studies in mice suggested that up to 20% of Tregs could lose FOXP3 expression, particularly at sites of inflammation [67]. However, two subsequent fate-mapping studies argued that fully-committed natural Tregs are remarkably stable, and that any apparent instability may be related to the presence of uncommitted cells, or

peripherally-induced Tregs which may be less stable than thymically-derived cells [20, 68]. Such fate mapping assays are obviously not possible in humans, but single cell cloning has been used to investigate the stability of FOXP3 expression in man[69]. Like earlier fate mapping studies in mice, significant heterogeneity was observed in the Treg pool, with some cells losing FOXP3 and suppressive capacity. Notably, loss of the Treg phenotype was much less likely to occur in naïve Tregs, supporting the notion that $CD25^{hi}CD45RA^{+}$ T cells are likely the most robust cells for cell therapy applications [70]. These data also call into question the therapeutic applicability of induced Tregs in humans as these are much less likely to remain $FOXP3^{+}$ when transferred *in vivo* [71]. However, restricting the population of Tregs, while important for FOXP3 stability, also dramatically reduces cell numbers.

In order to establish long-term tolerance, adoptively transferred Tregs must either survive and expand in the recipient, and/or be able to convert recipient alloantigen-specific T cells to a regulatory phenotype [72], a process known as infectious tolerance. Infectious tolerance is known to depend on TGF-β production from Tregs [73], and on-going presence of recipient "infected" Tregs is required to prevent allograft rejection [74]. Data on the longevity of transferred Tregs in humans is limited. In a recent trial of Treg therapy in hematopoietic stem cell transplantation, the transferred cells were no longer detected in circulation after 2 weeks, but it is not known whether the cells migrated to tissues or died [66]. Lombardi *et al* have recently used Single Photon Emission Computed Tomography to image adoptively transferred Tregs in mice, and reported that 24 hours after intravenous injection the cells were primarily localized in the spleen [75]. In humans micro/PET computed tomography fusion has been used clinically to track infused T cells and a similar approach could be used to track Tregs [76]. The question of how long *in vitro* cultured T cells can persist after transfer *in vivo* has been studied extensively in patients

receiving adoptive T cell therapy for cancer. Recent studies have shown that expanded $CD8^+$ T cells can expand and remain in the blood for at least 6 months [77]. It is possible that T cell longevity is related to the strength of *in vitro* stimulation, a serious consideration given the very potent activation conditions that are currently used to expand human Tregs.

Since Tregs depend on exogenous IL-2, finding ways to make more IL-2 available to infused cells might help to support their engraftment and function. One approach is to couple IL-2 with anti-IL-2 antibodies which increases the half-life of the cytokine and targets its biological activity to specific immune cell subsets. For example, when coupled to the JES6-1A12 antibody, IL-2 complexes can increase endogenous Treg numbers and prevent the rejection of allogeneic pancreatic islets [78]. An alternative approach is to use low dose IL-2, which lacks the toxicity and immunostimulatory effects of the higher doses of IL-2 used to treat cancer patients [79]. This approach has been shown to increase the number and longevity of Tregs in patients with chronic graft versus host disease and cause a coincidental reduction of inflammatory symptoms [80] and reduction in acute GVHD [81]. The tolerability of low dose IL-2 and its ability to promote Treg expansion in an inflammatory setting suggests that it may be an ideal adjuvant to adoptive Treg cell therapy. Importantly, co-stimulation of Tregs by molecules such as CD28 significantly enhance their IL-2 sensitivity, suggesting that modulating co-stimulation of Tregs could also increase the efficacy of low-dose IL-2 treatment [82]. This could be particularly important in autoimmunity, where patient Tregs are relatively insensitive to IL-2 treatment [83]

A final pressing question is how immunosuppression may affect the longevity and function of transferred cells. There is a general consensus that calcineurin-targeted immunosuppression will likely be detrimental to Tregs and there is much interest in tailoring immunosuppression to use drugs such as sirolimus, anti-thymocyte globulin and/or

mycophenolate mofetil to preserve their viability *in vivo* [38]. Notably, despite enhancing FOXP3 and suppressive capacity [84], sirolimus also inhibits the expansion of Tregs so it may not be ideal for promoting the survival of adoptively transferred cells [85]. However, sirolimus provides insight into unique Treg signalling pathways that may be exploited: mTOR activity, which is inhibited by sirolimus, is reduced in Tregs [86, 87]. Reduced mTOR activity is likely related to dampened signalling of the upstream PI3K pathway, both of which are necessary for thymic Treg development [88]. Peripheral Treg induction can also be enhanced by blocking these pathways [89]. However, some PI3K activity does appear necessary for Treg suppressive activity [90], although high activity reduces suppression [91]. Further study of how to modulate Treg signaling pathways pre- and post-transfer could significantly enhance the efficacy of Treg cell therapy.

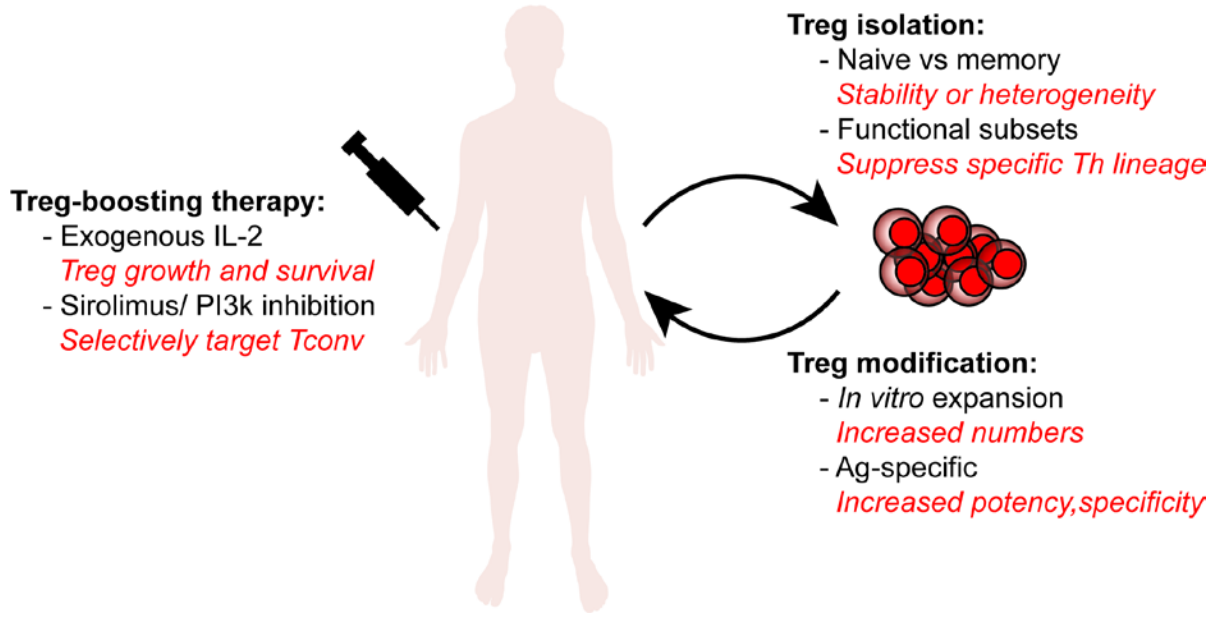



**Figure 1. 2** *Aspects of Treg biology to consider in cell therapy for transplant tolerance.* Cell therapy with Tregs may induce long-term tolerance without immunosuppressive drugs but only if the transferred cells are safe and effective. The heterogeneity of Tregs offers the opportunity to choose a subset of Tregs that may be more targeted. Tregs may also be modified in vitro to increase numbers or potency and specificity. Once returned to the patient, Tregs may be supported through low-dose IL-2 or by choosing immunosuppressive drugs that target signaling pathways that are already dampened in Tregs.

### 1.3 Th-like Treg subsets

Traditionally, transcription factors that play a major role in T cell lineage development have been termed “master regulators”. Their presence in a cell was seen as sufficient to promote development into a particular subset, such as FOXP3 for Tregs, TBET in TH1 cells, and ROR-γt in TH17 cells [92, 93]. Recently, a more nuanced understanding of how cell-intrinsic interactions can modulate the phenotype and function of a cell in normal and disease settings has emerged. In the case of Tregs, the relative levels of FOXP3 and transcription factors from other T cell lineages seem to determine whether a cell is suppressive, where it traffics to, and its potential for plasticity between lineages [93, 94]. An emerging concept is that the fine-tuning of Treg activity requires the adoption of inflammatory Th cell transcription factor networks [95]. Expression of proteins such as TBET and ROR-γt in Tregs, however, may not only play a critical role in Treg suppression, but also lay the groundwork for autoimmunity if the balance between FOXP3 and transcription factors with inflammatory potential is lost.

#### 1.3.1 The role of TBET in Tregs

The Th1 cell lineage is characterized by the master transcription factor TBET (encoded by *TBX21*), which drives the expression signature cytokines and chemokine receptors, such as IFN-γ and CXCR3, respectively. Initiation of a Th1 transcriptional program in a naïve $CD4^+$ T cell is sufficient to repress FOXP3 and prevent the development of induced Treg [96]. However, despite being a classical Th1 cytokine, IFN-γ is reported to be critical for Treg-mediated protection of allografts [97] and graft-versus-host disease [98]. Exposure of Tregs to IFN-γ and/or IL-12 (a classical Th1-polarizing cytokine) results in the development of a Th1-like state in Tregs, which begin to express TBET and CXCR3, but remain suppressive and can traffic to sites of

Th1-mediated inflammation [99]. Similarly, exposure of Tregs to IL-27 up-regulates CXCR3 expression and enables suppression of Th1 cell responses [100]. Suppressive, Th1-like Tregs have also been isolated from human peripheral blood on the basis of CXCR3 expression [95].

The development of Th1-like Tregs may be a precarious state, however, and under certain conditions, position these cells to become pathogenic and contribute to autoimmunity. For example, IFN-γ producing, less-suppressive Tregs were isolated from the blood of multiple sclerosis patients; their suppressive capacity could be partially rescued through blocking Th1 cytokines *in vitro*, or by treatment with IFN-γ *in vivo* [101]. Similarly, IFN-γ producing Tregs with reduced suppressive capacity were found in humans with autoimmune diabetes [102]. Thus although the ability of Tregs to adopt a Th1 phenotype is likely a mechanism for immune homeostasis, it may also allow for the potential of plasticity towards a pathogenic rather than immunosuppressive function.

### 1.3.2 The relationship between Th2 cells and Tregs

Th2 cells are primarily defined by the transcription factors GATA-3 and IRF4, and production of cytokines such as IL-4, IL-13 and IL-5. The transcriptional programs of Tregs and Th2 were initially found to oppose one another. Th2 polarization mediated by IL-4 was shown to block FOXP3 induction in induced Treg through direct binding of GATA-3 to the FOXP3 promoter [96, 103]. Conversely, FOXP3 induction in memory Th2 cells results in down-regulation of GATA-3, IRF4, and Th2 cytokines [104]. However, Tregs from mouse models with attenuated FOXP3 expression are skewed towards the Th2 lineage [105], and human $FOXP3^+$ memory cells preferentially convert to a Th2 phenotype upon loss of FOXP3, even in the absence of IL-4

signalling [70]. These data indicate that there is transcriptional cooperation between Th2 and Treg networks, not just competition.

Further evidence for shared Treg and Th2 programming comes from the observations that FOXP3 is required for IRF4 expression [106], and over-expression of FOXP3 upregulates GATA-3 expression [107]. In addition, IRF4 deletion in Tregs results in uncontrolled Th2 responses in mice [106], and Tregs lacking GATA-3 cannot control peripheral inflammation under homeostatic conditions due to the essential role for GATA-3 in binding the FOXP3 promoter and inducing its transcription [108]. Additionally, Tregs genetically deficient for GATA-3 fail to home to barrier tissues and are not protective in mouse models of colitis [109]. Moreover, it has been shown that FOXP3 and GATA-3 directly interact in transcriptional complexes to modulate the expression of a subset of FOXP3 controlled genes, and that GATA-3-mediated regulation of these genes is necessary for Tregs to control Th2 responses [110].

As with Th1-like Tregs, Th2-like Tregs appear to play a critical role in the suppression of Th2 responses. For example Th2-induced Tregs are able to specifically suppress Th2-mediated allergic inflammation in airways [104]. However, as for Th1-like Tregs, the ability of Tregs to adopt Th2 transcriptional networks carries some risk, as Th2-like Tregs may become dysregulated and begin to contribute to inflammation. For example, Th2-like Tregs have been identified in allergic asthma induced by respiratory syncytial virus infection in mice. This disease results in GATA-3 and IL-13 expression in Tregs and abrogated suppressive ability [111], highlighting the tenuous balance between tolerance and immunity. Whether Th2-like Tregs can contribute to human disease is unknown.

### 1.3.3 Cross-talk between FOXP3 and ROR-γt

Th17 cells are defined by expression of the transcription factor ROR-γt, the cytokine IL-17, and chemokine receptor CCR6 [94]. As with Th1 and Th2 cells, Th17 cell development was initially thought to be mutually exclusive with the development of Tregs [112]. Original studies found that FOXP3 competes with RUNX1 for interaction with ROR-γt, with high expression of FOXP3 resulting in inhibition of the Th17 cell differentiation [113]. This interaction between FOXP3 and ROR-γt, which occurs via exon 2 of FOXP3, can also directly suppress expression of IL-17 and IL-22 expression [114, 115]. By corollary, Th17 development can be enhanced if ROR-γt binds and represses the *FOXP3* promoter [116], or if expression of FOXP3 is lowered in Tregs [117, 118]. This competition between FOXP3 and ROR-γt is biologically important as co-expression of FOXP3 and ROR-γt poises the cell to go down either lineage and take on a suppressive or inflammatory role [119].

In humans, Th17-like Tregs are found in both health and disease [93, 94], and there is ongoing debate about the origin and function of these cells. Some data suggest that these cells are derived from Tregs that have acquired IL-17 production and are not suppressive [120, 121]. However, our recent data indicate that proliferation and cytokine production in Th17 cells is intrinsically regulated by FOXP3 [122], suggesting that $FOXP3^{+}IL\text{-}17^{+}$ cells could also originate from Th17 cells.

Although the precise origin of $FOXP3^{+}IL\text{-}17^{+}$ cells in humans is not yet known, animal studies have clearly shown that Treg and Th17 transcriptional programs can overlap. For example, $FOXP3^{+}ROR\text{-}\gamma t^{+}$ cells from non-obese diabetic mice are suppressive *in vivo and in vitro* [119]. Moreover, similar to Th1 and Th2-like Tregs, Th17-like Tregs require Th17 characteristics to home to, and suppress, Th17 responses [95, 123].

### 1.3.4 FOXP3-independent transcriptional regulation of Tregs

A wide variety of transcription factors compete and cooperate with FOXP3 to fine-tune Treg activity. In addition to the major Th cell lineage defining transcription factors discussed above, Treg cells may also co-opt transcription factors that are not associated with the development of a particular T cell lineage. For example, Blimp-1, contributes to the suppressive function of Tregs *in vivo*: Blimp-1-deficient Tregs are unable to prevent DSS colitis [124] due to a loss of IL-10 production [125]. In addition, IRF4 was found to be critical for Blimp-1 induction and optimal Treg effector activity, highlighting parallel roles for transcription factors expressed in Tconv cells in regulating the specialization of Treg phenotype and function. It is likely that many other transcription factors contribute to the Treg gene signature by interacting with FOXP3 in large transcriptional complexes. For example, Eos, IRF4, Satb1, Lef1 and Gata-1 seem to have a redundant ability to establish the Treg gene signature and FOXP3 occupancy of Treg genes [126].

As the molecular phenotype of Tregs is explored in more detail, it is increasingly apparent that many transcription factors that contribute to the development of the Treg lineage independently from FOXP3. For example, as discussed above, FOXO1 maintains the Treg phenotype by repressing specific genes [127]. Helios is another transcription factor that is known to contribute to the Treg phenotype in a FOXP3-independent manner [128]. Further understanding of the transcription factors that modulate Treg activity in both a FOXP3-dependent and -independent manner will provide a more complete picture of the role of FOXP3 in establishing Treg development and function.

### 1.3.5 Implications of Th-like Treg subsets for cell therapy

One approach to harness the heterogeneity of Tregs for cell therapy is to select subpopulations of Tregs expressing subsets of chemokine receptors that will control the trafficking of adoptively transferred cells to specific sites of inflammation. For example, Tregs which express the Th1 associated transcription factor TBX21, express CXCR3 and traffic to sites of Th1 mediated inflammation to suppress Th1 cells [129]. Similarly, Tregs have been shown to "imitate" Th2 or Th17 cells in order to suppress their responses [123, 130]. The pertinence of this concept has recently been demonstrated in humans, in that CXCR3-expressing Tregs were shown to be more potent than $CXCR3^-$ Tregs in suppressing IFN-γ production in mixed lymphocyte reactions [131]. These data suggest that if a Th cell lineage and its chemokine receptor profile are identified as playing a dominant role in rejection, then a sub-population of Tregs might be selected by the trafficking receptors they express, which could allow therapeutic suppression of the relevant branch of the immune system at the relevant site, while leaving the protective effects of the other branches intact. However, this strategy is not without risks; the transfer of populations that mimic Tconv in order to suppress them may also pre-select for cells with an innate pre-disposition for plasticity, as Tregs appear to exist on a continuum with other Th subsets that is dependent on the balance of lineage defining transcription factors and the epigenetic pre-disposition of the cell (**Figure 1.3**). Thus, further understanding the factors that drive plasticity in Tregs is necessary before including or excluding specific subsets in clinical trials.

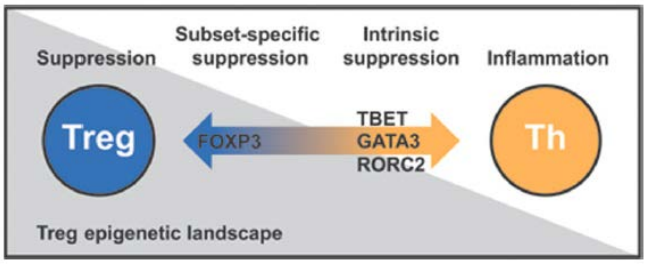



**Figure 1. 3** *Cell intrinsic factors that contribute to Treg lineage stability and plasticity.* T helper cells exist on a continuum of inflammation where distinct subsets represent the most common state, but intermediate states or plasticity can occur as well. With high levels of FOXP3 and a Treg epigenetic signature, Tregs will have the canonical features associated with their lineage. Upregulation of Th transcription factors allows Tregs to mimic and suppress individual subsets, but this opens the door for pathogenic Treg activity as the Treg epigenetic signature is lost. Th cells may also upregulate FOXP3 in order to intrinsically regulate their inflammatory activity, and if a Treg epigenetic signature is acquired, these cells may also become iTregs.

## 1.4 Antigen specific Tregs and chimeric antigen receptors

An alternative approach to boost the activity of Tregs for cell therapy is to increase the numbers of cells that will be active after infusion. Only 1-10% of Tregs are expected to be alloreactive [132], and this number is likely even lower in autoimmunity. Thus, at least 90% of transferred Tregs will receive no *in vivo* stimulation. Unsurprisingly, studies have shown that antigen-specific Tregs are much more potent. Because of their increased efficacy, antigen specific Tregs potentially could be infused in lower numbers, enabling the selection of desirable subsets of Tregs for cell therapy. For example, naïve Tregs or Th-like subsets could be sorted and made antigen specific for applications where lineage stability or Th mimicking are important, respectively.

Antigen-specific Tregs can be generated by various methods. Tregs can be isolated *ex vivo* from diabetic mice to prevent disease in NOD recipients[56, 133-136] or Tr1 cells can be cloned by pulsing with food antigen from patients with inflammatory bowel disease to reduce clinical symptoms[137]. Alternatively, Tregs can be engineered with a transgenic TCR cloned from CD8 cells to confer specificity to joint-antigens in arthritis[138] or prevent skin or heart transplant

rejection [52, 139]. Human Tregs have also been re-directed with a transgenic TCR specific for model antigens [140, 141] and for a clotting factor targeted in haemophiliacs[142]. In transplantation, alloantigen stimulation is also possible. Expanding alloantigen-specific Tregs offers superior protection for hair and skin allografts in mice [47, 48, 143] and reduce GVHD pathology [144]. Human Tregs can similarly be stimulated and expanded via alloantigen [145] or alloreactive Tregs can be sorted from co-cultures [50] to protect skin allografts. Regardless of the method of generation, antigen-specific Tregs provide better immunosuppression than polyclonal.

An alternative strategy for generating antigen specific Tregs is to transduce them with a chimeric antigen receptor (CAR). CARs are synthetic proteins created by combining an extracellular antigen recognition domain with a transmembrane domain and intracellular T cell activation domains (**Figure 1.4**)[146]. Proof-of-concept work exists for CAR function in T cells for cancer immunotherapy, although refinement is ongoing[147]. The potential that CARs can boost Treg cell therapy the same way they boost cell therapy for cancer is exciting; introducing antigen specificity for targeting could reduce generalized immunosuppression while activating a larger proportion of Tregs, and including appropriate T cell signaling domains could increase the suppressive activity and longevity of transferred Tregs. Early work with CAR Tregs has been promising, although largely limited to mouse models and model antigens[148-151]. Further work is required to determine appropriate antigen choice and optimal signaling domains for CAR Tregs.

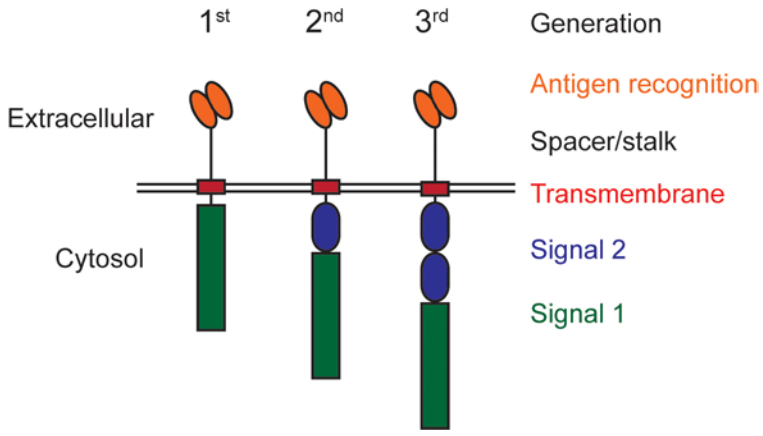



**Figure 1. 4** *Chimeric antigen receptor components.* All CARs consist of a minimum of three domains: an antigen recognition domain (orange), a transmembrane domain (red), and a T cell activation domain (green). An extracellular spacer region is often added to facilitate antigen binding, and second and third generation CARs contain one or two co-stimulatory domains (blue) respectively. Together, these components allow CARs to re-direct the antigen-specific activation of T cells.

### 1.4.1 Antigen choice and targets of current CARs

Chimeric antigen receptors are commonly made antigen-specific via the inclusion of a single-chain variable fragment at their 5' end, composed of the complimentarity determining regions of an antibody, with all the advantages and challenges of single chain antibodies. One advantage is that Scfvs are not MHC-restricted and thus can target any antigen that can generate an antibody response. This broadens the array of epitopes that CAR T cells can react to. For example, the surface protein CD19, which is expressed on B cells and some malignancies arising from B cells, has been successfully targeted by CAR T cell therapy in lymphoma patients. Other cell-surface antigens that have been targeted include, but are not limited to, CD20 (B cell lymphomas), Her2 (breast cancer), CEA (colorectal cancer), GD2 (neuroblastoma) and mesothelin (mesothelioma). Importantly, the use of a single chain antibody does not exclude targeting proteins that would normally be presented in MHC. For example, a melanoma peptide in the context of HLA-A1 has been targeted with a CAR [152].

One important challenge in selecting a CAR target is the tissue distribution of antigen. Although many over-expressed molecules can be identified in cancer cells, CAR T cells cannot discriminate between antigen expressed on healthy vs malignant tissues. This is seen in CD19 CAR T cell therapy, where patients who have had their lymphoma eliminated also experience a total loss of $CD19^+$ B cells[77, 153]. Cross reactivity with carbonic anhydrase IX expressed in the bile ducts caused liver toxicity in the treatment of renal carcinoma [154]. More dramatically, a patient given HER2 CAR T cells experienced a fatal reaction that was likely caused by low-level HER2 expression in the lungs [155]. Thus while scFv incorporation into a CAR provides considerable options for targeting T cell activity, target distribution is a critical consideration when choosing CAR antigens.

Because CAR Tregs are not targeting a particular cell type, i.e. a cancer cell as in CAR T cell therapy, and because their off-target effect are less likely to be lethal compared to cytotoxic T cells, the choice of target antigens for Tregs may be broader. For example, an entire tissue may be targeted rather than a protein restricted to a cancer cell. One such example is MOG-CAR Tregs, which protect mice against EAE[150]. Although MOG is a model antigen that drives expansion of autoreactive cells, it is not clear that MOG-CAR Tregs are reacting to antigen in the same way as pathogenic T cells. Rather, MOG-CAR Tregs traffic to the CNS where they are activated and retained, potentially suppressing due to local anti-inflammatory cytokine production and IL-2 consumption. Similarly, targeting an antigen in the colon via a CEA-CAR in Tregs allowed for potent suppression of CEA-CAR Tconv after trafficking to the site of antigen[151]. Whether CAR Tregs simply need to show up at the site of inflammation or if antigen specificity needs to be more targeted is unknown. Understanding the mechanism of suppression induced by CAR stimulation will be critical to inform CAR Treg antigen choice in the clinic.

### 1.4.2 T cell activating domains in CARs

Early chimeric antigen receptors were designed with a single cytoplasmic domain for T cell activation to provide signal 1 and are referred to as "first generation" (sometimes also "T bodies" or "chimeric receptors")[146]. These constructs contain either CD3ζ or the FcγR to provide activation signals via immunoreceptor tyrosine-based activation motifs, or ITAMs. ITAMs consist of a repeated YXXL sequence which can be phosphorylated on the tyrosine (Y) residue to induce signalling and activation downstream of receptor ligation[156]. First generation CARs were capable of inducing PI3k signalling and cytolytic activity in response to antigen. Promisingly, they also persisted in HIV patients at low levels for extended periods of time [157]. However, primary T cell lines and *in vivo* experiments showed limited cytokine production, proliferation, longevity, and efficacy of first generation CARs[146]. Strikingly, the most effective clinical trial with a first generation CAR achieved less than a 20% remission rate in neuroblastoma, and the efficacy was correlated with persistence of CAR T cells[158]

Second generation CARs were developed to address the limitations of first generation CARs by incorporating the cytoplasmic portion of a co-stimulatory molecule in addition to CD3ζ or FcγR[159, 160]. CD28, OX40, 4-1BB, ICOS, and others have been selected to provide signal 2 in addition to signal 1. As with endogenous TCR activation, greater signalling and activation was observed in the presence of co-stimulation after CAR ligation[159, 161]. Increased proliferation and survival were observed *in vivo*[162], and most importantly, second generation CARs were found to have much greater longevity when compared head to head with first generations CARs in a clinical trial[163]. Currently, the most successful clinical trials have been performed with second generation CARs[77, 153].

Despite their improved activity, second generation CARs have shown limitations in solid tumor malignancies and thus third generation CARs, containing a second co-stimulatory domain, have also been developed[164]. Third generation CARs are not as thoroughly tested as second generation CARs, however they are entering clinical trials and data comparing second and third generation CAR efficacy and safety will emerge.

The impact of signalling domains on CAR Tregs is largely unknown. Early studies used FcγR in CAR Tregs, with minimal Treg activation and proliferation observed, although localization to the site of antigen did occur[148, 149]. Subsequent studies of CAR Tregs using a second generation CAR containing CD3ζ and CD28 show convincing data that CAR Tregs proliferate in addition to trafficking in response to antigen[151]. These data suggest that second generation CARs are also more potent in CAR Tregs, although no direct comparison has been made in Tregs. Given the distinct signalling pathways found in Tregs, further study is warranted to understand which co-stimulatory domains in the context of a second generation CAR will be optimal for use in Tregs.

### 1.4.3 Considerations for additional CAR components

In addition to the antigen recognition and T cell activation domains, CARs have several other components that may vary [146]. One is the signal peptide, which directs insertion of the CAR into the endoplasmic reticulum, and can be chosen from a variety of proteins. CARs containing ScFv may vary in their antibody design (eg human vs mouse, rigid vs flexible linker). The stalk region or spacer, which elevates the single chain above the cell membrane, can be composed of different elements as well, such as CD8 or IgG. The importance of the length of the stalk appears to vary with each epitope [165]. Various transmembrane domains have also been used including

CD3ζ, CD8 and the corresponding TM domains from co-stimulatory molecules, and other TM domains. Interestingly, CD3ζ TM containing CARs appear to associate with the endogenous TCR and increase CAR signaling via a covalent interaction [166]. Many groups have independently developed functional CARs by trial and error, as it appears difficult with our current level of knowledge to predict the effects of including different components in a CAR. Direct comparisons of the impact of different components on CAR expression and function will be needed to rationally design future CARs and expand their therapeutic potential. This work will also need to be extended to Tregs, as no systematic comparison of the impact of CAR components on Tregs has been performed. Two Treg-specific questions about CAR design to address are 1) whether immunogenic components will be an issue in Tregs, since transgenic Tregs may be privileged from immune attack due to their suppressive capacity, and 2) whether the lower proliferative rate of Tregs will allow CAR expression to accumulate to higher levels on the cell surface, reducing the necessity for refinement of CAR components to increase expression and efficacy,

## 1.5 Conclusion

There is substantive evidence that therapeutic delivery of Tregs could be an ideal way to induce tolerance to transplanted organs and indeed there are already many trials planned to test this approach. To date, the majority of data to support this approach has come from animal models and involved transfer of large numbers of polyclonal cells into a lymphopenic environment in the absence of immunosuppressive agents. In considering translation of the approach to patient treatment, it is immediately evident that the experimental designs in mouse models bear little resemblance to what confronts transplant clinicians. While generation of

clinically relevant numbers of Tregs has become feasible, it is not clear if they will maintain their ability to survive and possibly expand *in vivo*. Additional questions remain regarding generation of antigen specific cells to increase the function of Tregs post-transfer and the stability of various sub-populations of Tregs.

### 1.6 Synopsis of research questions

As Treg cell therapy continues to move towards the clinic, questions about the safety and efficacy become critical. Because we have greater understanding of both the heterogeneity of Treg subpopulations and the core features that are necessary for Treg functions, future approaches will use this knowledge to refine cell therapy beyond infusion of bulk polyclonal Tregs. Thus, my thesis aims to answer three questions: are there cytokine or tissue environments that drive Treg inflammatory cytokine production in addition to the Th1 and Th17-like Tregs observed in humans? Can antigen specific Tregs be engineered while maintaining Treg phenotype and function? Finally, which engineered signals might impart optimal suppression and survival of transferred Tregs?

While Th1 and Th17-like Tregs have been described in humans, it was unclear whether Tregs could contribute to Th2-mediated inflammation. My *hypothesis* is that Th2-like Tregs will be found in chronic inflammation dominated by Th2 cytokines and my *rationale* is that plastic Tregs have been observed in Th1 and Th17 mediated autoimmune diseases. In Chapter 3, I describe observations in patients with systemic sclerosis, a Th2-driven disease. The phenotype of Tregs in the skin and blood was examined for inflammatory cytokine production and homing marker expression. Additionally, the role of IL-33, a Th2-polarizing cytokine, in contributing to Treg dysfunction was investigated.

Antigen specific Tregs are a highly potent option for Treg cell therapy, however cell numbers are limiting particularly when combined with stringent sorting parameters to avoid Treg subpopulations that can contribute to inflammation. My *hypothesis* is that allo-antigen specific Tregs can be generated by via a novel CAR and my *rationale* is that chimeric antigen receptors have been used to re-direct T cells in cancer immunotherapy. In Chapter 4 I created a novel CAR to activate Tregs in response to HLA-A2. Because this strategy has not been validated in human Tregs, I tested the maintenance of Treg phenotype after transgene transduction and stimulation. I also tested Treg suppressive function *in vitro* and *in vivo*.

The long term persistence and *in vivo* stability are necessary for successful cell therapy with Tregs. Currently, Tregs persist for a short time in patients. My *hypothesis* is that optimizing signals given to CAR Tregs will enhance their longevity and my *rationale* is that the type of co-stimulation in CARs has been shown to be important for persistence of cells *in vivo*. Genetically engineered cells have been shown to persist for much longer, which may be due to strong co-stimulatory signals included in the transgene. Co-stimulation also affects cell phenotype but the effects of co-stimulatory domains in CARs on Tregs has not been studied. In my final chapter, Chapter 5, I describe a series of constructs created in parallel to determine the optimal co-stimulatory domain to genetically engineer Tregs.

# Chapter 2: Material and methods

## 2.1 Patient samples (chapter 3)

**Subjects and sample collection.** Written informed consent was obtained according to protocols approved by the University of British Columbia Clinical Research Ethics Board. The clinical characteristics of subjects with SSc are described in **Table 3.1**. Controls were age and sex-matched. Blood was collected and PBMCs were cryopreserved. 2 x 4mm punch biopsies of affected mid forearm skin were obtain from SSc patients. For control skin, biopsies were isolated from skin discarded during plastic surgery. One 4 mm punch biopsy was fixed in formalin and embedded in paraffin, and the second was cultured as described below.

**Skin biopsy culture.** Biopsies were minced into 1mm pieces and placed on cellfoam matrices (Cytomatrix). The explants were cultured in IMDM (Stemcell Technologies) supplemented with 20% FCS (NorthBio), 1% streptomycin (Invitrogen), 1% glutamax (Invitrogen), 100U/ml IL-2 (Chiron) and 20 ng/ml IL-15 [167, 168]. In some cases 20 ng/ml rhIL-33 (Stemcell Technologies) with or without 20 ng/ml rhIL-4 (eBioscience) was added. After 3 weeks, non-adherent cells were collected for cytokine analysis and adherent cells were cultured in IMDM supplemented with 10% FCS for 2-6 weeks to obtain primary fibroblasts.

**T cell enrichment and expansion**. Where indicated, cells were isolated with EasySep Human $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies) from cryopreserved PBMCs or T cells from skin matrix culture. In some cases the cells were polyclonally expanded as described using

a protocol that preserves Treg function [169]. Expanded cells were rested for 24 hours without IL-2 before analysis.

**Flow cytometry.** PBMCs or non-adherent cells from skin explants were stimulated with 10 ng/ml PMA and 500 ng/ml ionomycin, in the presence of brefeldin A (10ug/ml), (all Sigma) for four h. Surface staining was performed with antibodies for CD3, CD8, CD4, and Fixable Viability Dye (FVD), followed by intracellular staining for FOXP3, IL-4, IL-13, IL-17 and/or IFN-γ. For analysis of homing markers, unstimulated PBMCs were stained with antibodies to CD3, CD4, CD8, FOXP3, CLA, CCR4, CCR6, CCR10 and CXCR3, as well as FVD. For ST2 staining, $CD4^+$-enriched cells were stained for FVD, CD4, FOXP3 and ST2. Samples were read on a BD LSR II Flow cytometer (BD Biosciences) and results analyzed using FlowJo Software version 8.7 and 10.0.6 (Tree Star). All PBMC data are gated as $CD3^+CD4^+CD8^-FVD^-$.

**Cytometric bead array.** $CD4^+$ cells were pre-enriched from cryopreserved PBMCs and stained with FVD, CD4, CD25, CD127, CLA and sorted into 4 populations: circulating Tregs, skin homing Tregs, circulating Tconv, and skin homing Tconv. Purity was checked by FOXP3 staining post-sort, and cells were stimulated with 1:1 ratio (beads:cells) of αCD3/CD28 beads for 72 hours. Supernatants were collected and IL-17, IFN-γ, and IL-4 concentration was determined by Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences) and analyzed by FCAP Array Software v1.01 (Soft Flow).

**Immunofluorescence.** Five-micrometre thick sections were deparaffinized and rehydrated by heating at 55 to 65 °C for 10 min, then cleared with xylene and rehydrated through an ethanol

gradient to water. After heat-induced antigen retrieval in sodium citrate buffer (pH=6.0), immunostaining was carried out using antibodies against human CD3 at 1:200 (Dako, polyclonal) and human IL-33 at 1:200 (R&D, polyclonal). Secondary antibodies were Alexa Fluor 488-conjugated donkey anti-rabbit IgG, and Alexa Fluor 568-conjugated donkey anti-goat IgG (Invitrogen). Tissues were mounted using ProLong gold antifade plus DAPI (4′,6′-diamidino-2-phenylindole) (Invitrogen) for DNA staining. Sections were captured with an Olympus BX61 Fluorescent upright microscope equipped with RETIGA Exi Fast1391 camera (Qimage) and InVivo software (version 5.0). Image data were processed, maintained in 12 bit format, and analyzed with ImagePro software.

**Fibroblast gene expression analysis**. Primary fibroblasts were stimulated with TNF-α, IL-1β, IFN-γ (10ng/ml each) or medium alone. RNA was isolated after 18-24 hours using with EZNA Total RNA kit I (Omega Bio-Tek) and converted to cDNA with qScript cDNA SuperMix (Quanta Bioscience). Levels of cytokine and 18s mRNA were assessed using PerfeCTa SYBR Green FastMix (Quanta Bioscience). Samples were run on a ViiA7 thermocycler (Applied Biosystems) with standard cycling parameters, including disassociation curve analysis. Δct for cytokines was calculated by normalizing to 18s; ΔΔct values shown were calculated by dividing Δct values by Δct of the control media alone condition.

**Statistics.** Statistical analysis was performed using Prism 6 software (GraphPad). Data relating to cytokine, chemokine receptor, and ST2 expression in blood, and cytokine and ST2 expression in skin had log-normal distribution, so results were transformed using base 10 logarithm prior to analysis with 2-tailed t-tests. IL-33 production by fibroblasts and matrix-polarization results were

analyzed using a 2-way ANOVA with Sidak's multiple comparisons test. P values <0.05 were considered significant, *<0.05, **<0.01, ***<0.001.

## 2.2 CAR generation and testing (chapters 4 and 5)

**A2 CAR generation.** Variable regions were cloned from the anti-HLA-A2 BB7.2 hybridoma (ATCC) using published methods [170] and converted into a single chain antibody (scFv). The ScFv was fused to a myc epitope tag in the extracellular region to enable cell surface detection by flow cytometer, transmembrane and intracellular domains of CD28, followed by CD3ζ as described [146](A2-CAR). The A2-CAR and control CAR specific for HER2 were cloned into a bi-directional lentivirus vector, and viral particles were produced as described [171]. Surface expression was determined via transient transfection of HEK 293T cells with Lipofectamine® 2000 (Life Technologies).

**Co-stimulatory domain swapping.** CARs were generated by cloning new sequences in the place of the CD28 domain and/or CD28 transmembrane domain. Transmembrane and cytoplasmic sequences were determined using UniProt [172]. All domain swapping was done in the HER2-CAR and lentivirus vector described above. Signal peptides were evaluated with SignalP 4.1.

**Treg sorting, transduction, and expansion.** Peripheral blood was obtained from healthy volunteers who gave written informed consent according to protocols approved by the University of British Columbia Clinical Research Ethics Board and Canadian Blood Service. $CD4^+$ T cells were isolated from HLA-$A2^-$ donors via RosetteSep (Stemcell) and enriched for $CD25^+$ cells

(Miltneyi) prior to sorting into live $CD4^{+}CD45RO^{lo}CD45RA^{hi}CD25^{hi}$ Tregs and $CD4^{+}CD45RO^{lo}CD45RA^{hi}CD25^{lo}$ T conventional (Tconv) using a FACSAria II (BD Biosciences). Sorted T cells were stimulated with artificial antigen-presenting cells (aAPCs) loaded with αCD3 mAbs as described [173] in 1000U/ml or 100U/ml of IL-2, for Tregs or Tconv, respectively. At day 2, cells were transduced with lentivirus at a multiplicity of infection of 10 virus particles : 1 cell. At day 7, $\Delta NGFR^{+}$ cells were purified with magnetic selection (Miltenyi) then restimulated with aAPCs as above and expanded for 7 days. To test effects of A2-mediated expansion, Tregs were re-stimulated with irradiated (10Gy) K562.64 cells [174], K562.64.HLA-A2 cells, or K562.64.HER2 cells [derived from K562.64 cells transduced with a lentivirus encoding HER2 and GFP] at a 1:2 (K562 : T cell) ratio for two weeks in the presence of 1000U/ml IL-2.

**Flow cytometry.** For phenotypic analysis, cells were stained for fixable viability dye (FVD) and surface markers before fix/perm with FOXP3/Transcription Factor Staining Buffer Set (eBiosciences) and staining for intracellular proteins. For analysis of cytokine production, cells were stimulated with 10 ng/ml PMA and 500 ng/ml ionomycin, in the presence of brefeldin A (10ug/ml), (all Sigma) for 4h. Samples were read on an LSRFortessa (BD Biosciences) and results analyzed using FlowJo Software version 8.7 and 10.0.6 (Tree Star).

**TSDR analysis.** DNA from frozen T cell pellets of male donors was isolated with the DNeasy Blood and Tissue Kit (Qiagen) and bisulfite converted with EZ Direct kit (Zymo Research). PCR of Bis DNA was performed with Human FOXP3 kit (Epigen DX) and prepared for pyrosequencing using PyroMark buffers (Qiagen), then run on a Biotage PyroMark Q96 MD pyrosequencer (Qiagen). Results were calculated with Pyro Q-CpG software (Biotage).

**Proliferation, activation, suppression, cytokine production.** To assess proliferation and activation, T cell lines were labelled with CPD670 or 450 (eBiosciences) and stimulated with K562.64 cells loaded with αCD3 and α28 mAbs (1μg/ml, each, 1 hour prior incubation), K562.64.HLA-A2 cells, or at a 1:2 (K562 : T cell) ratio. Staining of activation markers CD154 was performed in culture for 6 hours prior to analysis. Suppression was assessed with allogeneic HLA-$A2^-$ PBMCs labelled with cell proliferation dye (CPD, eBiosciences) and stimulated via αCD3/28 coated beads (Invitrogen) at a 1:8 or 1:16 bead to PBMC ratio for 96 hours. HLA-$A2^+$ PBMCs were added to assays where indicated. % suppression of $CD8^+$ cells was calculated using division index (100-[(DI PBMCs+Test)/(DI PBMCs)]*100).

To measure cytokine production, T cell lines were stimulated with the indicated K562 cells (1 K562 : 2 T cells) for 48 hours. Supernatants were collected and cytokine concentration was determined by Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences) and analyzed by FCAP Array Software v1.01 (Soft Flow).

***In vivo* experiments.** Animal protocols were approved by the UBC Animal Care Committee. 8-12 week-old female NOD/SCID IL-2R$\gamma^{null}$ (NSG) mice received whole-body irradiation (150cGy, RS-2000 Pro Biological System) 1 day pre-injection of $1x10^7$ HLA-$A2^+$ PBMCs with or without $1x10^7$ or $0.5x10^7$ of the indicated type of Tregs. Saline-injected mice served as controls. GVHD was scored based on weight, fur texture, posture, activity level and skin integrity, with 0-2 points per category as described[175, 176] Peripheral blood from saphenous vein

was centrifuged and plasma was aspirated and frozen at -80°C. Erythrocytes were lysed and leukocytes stained for flow cytometry using mAb.

**Statistics**. Analysis was performed using Prism 6 software (GraphPad). P values <0.05 were considered significant, *<0.05, **<0.01, ***<0.001. Significance of survival was determined by Log-rank (Mantel-Cox) test. Significance of GVHD onset was determined by ordinary one-way ANOVA with Tukey's multiple comparison's test. Significance of survival was determined by Log-rank (Mantel-Cox) test. All other significance was determined by 2-way ANOVA with Sidak's multiple comparisons test.

# Chapter 3: T regulatory cells produce pro-fibrotic cytokines in the skin of patients with systemic sclerosis

## 3.1 Introduction

Systemic sclerosis (SSc) is a severe autoimmune connective tissue disease characterized by vasculopathy, inflammation and fibrosis. The accumulation of collagen and other extracellular matrix proteins typically starts in the skin and can progress to internal organs, leading to their failure [177]. SSc is commonly divided into diffuse SSc, characterized by fibrosis of the skin and internal organs, and limited SSc, with little effect on internal organs. The etiology of SSc is unknown, but, like most autoimmune diseases, it likely results from a combination of environmental events and genetic predisposition, culminating in a failure to regulate tissue regeneration. Because of the lack of knowledge about the pathogenesis of SSc, there are currently no disease specific treatments and survival rates are poor [178].

Inflammatory lesions in SSc contain immune cell infiltrates, including activated T cells [179]. Since T cells are often found in proximity to collagen-producing fibroblasts, it has been hypothesized that T cell-derived cytokines may trigger activation of adjacent fibroblasts, leading to excess extracellular matrix deposition. Extensive research into the possible contribution of different subsets of T helper (Th) cells in this process has led to inconsistent results. Studies using histology and T cell clones found a possible role for Th2 cells [180-184], consistent with the fact that cytokines characteristic of Th2 cells, IL-4 and IL-13, are pro-fibrotic. Increased Th1, Th2 and/or Th17 cells have also been reported [185-189], leading to the possibility that many different types of Th cells may be involved at different stages and locations of the disease.

The normal and pathological functions of Th cell subsets are counterbalanced by the activity of T regulatory cells (Tregs) [190], with strong evidence that changes in circulating Tregs are associated with autoimmunity in humans [191]. The role of Tregs in SSc has also been investigated, with reports of decreased [192, 193], increased [194], or equal [195, 196], proportions of Tregs in the blood of individuals with SSc compared to controls. Using histology to count $FOXP3^+$ cells in skin lesions, Tregs are reported to be numerically deficient [192], but nothing is known about their phenotype and/or function at the site of pathology.

An emerging concept in autoimmunity is that Tregs can trans-differentiate into cells that produce cytokines characteristic of Th1 or Th17 cells, and, at least in animal models, directly contribute to pathology [197]. In humans, Tregs that produce IFN-γ and/IL-17 have been correlated with type 1 diabetes, multiple sclerosis, and juvenile idiopathic arthritis [102, 198, 199], although their origin(s) and role in affected tissues remains unclear. Here I investigated whether SSc is associated with the development of effector-cytokine producing Tregs and report the first example of tissue-localized trans-differentiation of Tregs into Th2-like cells in a human disease.

### 3.2 SSc patient characteristics

Patients were selected for inclusion based on either limited or diffuse SSc. Characteristics of our patient population are representative of SSc (**Table 3.1**).

| | **Limited SSc** | **Diffuse SSc** | **p-value between lcSSc and dcSSc** |
|---|---|---|---|
| Number | 15 | 34 | |
| Female : male | 12:3 | 24:10 | 0.7274 |
| Age at onset (yrs) | 51.7 ± 3.7 | 53.1 ± 2.4 | 0.7496 |
| Disease duration (yrs) | 5.7 ± 1.8 | 5.7 ± 1.1 | >0.9999 |
| Early (1-4 yrs) | 7 | 19 | 0.5430 |
| Late (5+ yrs) | 8 | 14 | |
| mRSS at inclusion | 8.33 ± 1.30 | 22.24 ± 1.57 | ****<0.0001 |
| Skin severity | 0.92 ± 0.11 | 2.08 ± 0.14 | ****<0.0001 |
| Lung severity | 1.66 ± 0.45 | 1.47 ± 0.25 | 0.7154 |
| FVC % predicted | 81.62 ± 7.36 | 74.32 ± 4.91 | 0.4166 |
| DLCO | 49.36 ± 6.14 | 55.07 ± 5.09 | 0.4790 |
| Interstitial Lung Disease | 5 | 16 | 0.5327 |
| Pulmonary Hypertension | 4 | 2 | 0.0624 |
| Maximum severity | 4.20 ± 0.67 [63] | 6.08 ± 0.39 [64] | *0.0232 |
| Scl-70 positive | 0 | 14 | **0.0023 |
| ACA positive | 4 | 0 | **0.0064 |
| Treatment | | | |
| + immunosuppression | 5 | 19 | 0.2165 |
| - immunosuppression | 10 | 15 | |

**Table 3. 1** *SSc patient characteristics.* Abbreviations: mRSS modified Rodnan skin score; FVC forced vital capacity; DLCO diffusing capacity of lung for carbon monoxide; Scl -70 anti-topoisomerase; ACA anticardiolipin antibody

### 3.3 Circulating Tregs from SSc patients do not produce Th1-, Th2- or Th17-cell associated cytokines

Since SSc has been associated with changes in Th1, Th2 and/or Th17 cells [180-189], I investigated whether circulating Tregs in subjects with SSc acquired the ability to produce IFN-γ, IL-4, IL-13 and/or IL-17. PBMCs from 36 patients and 20 controls were stimulated to induce cytokine production and analyzed by flow cytometry. I found that the proportion of circulating Tregs (defined as $CD4^+FOXP3^+$ cells) making Th-cell associated cytokines did not differ between subjects with SSc and controls (**Figure 3.1A**). There was also no difference in the proportion of $CD4^+$ T cells which were $FOXP3^+$: mean percent of $CD4^+FOXP3^+$ cells: (3.30±0.55% in normal blood *vs* 3.53±0.41% in SSc blood [p=NS]), consistent with some of the previous reports which enumerated Tregs in blood [195, 196]. Parallel analysis of cytokine production from $CD4^+FOXP3^-$ T conventional (Tconv) cells revealed that there were no significant differences in the proportions IFN-γ-, IL-4-, IL-13-, or IL-17-producing cells between patients and controls (**Figure 3.1B)**. Previous studies report a variety of conflicting findings related to changes in circulating Th1, Th2, and/or Th17 cells [185-189], but importantly, none specifically examined cytokine production in $CD4^+FOXP3^-$ T cells as I report here. Together, these data indicate that $CD4^+$ T cells in the blood of subjects with SSc do not have substantial alterations in effector cytokine production from Tconv cells or Tregs.

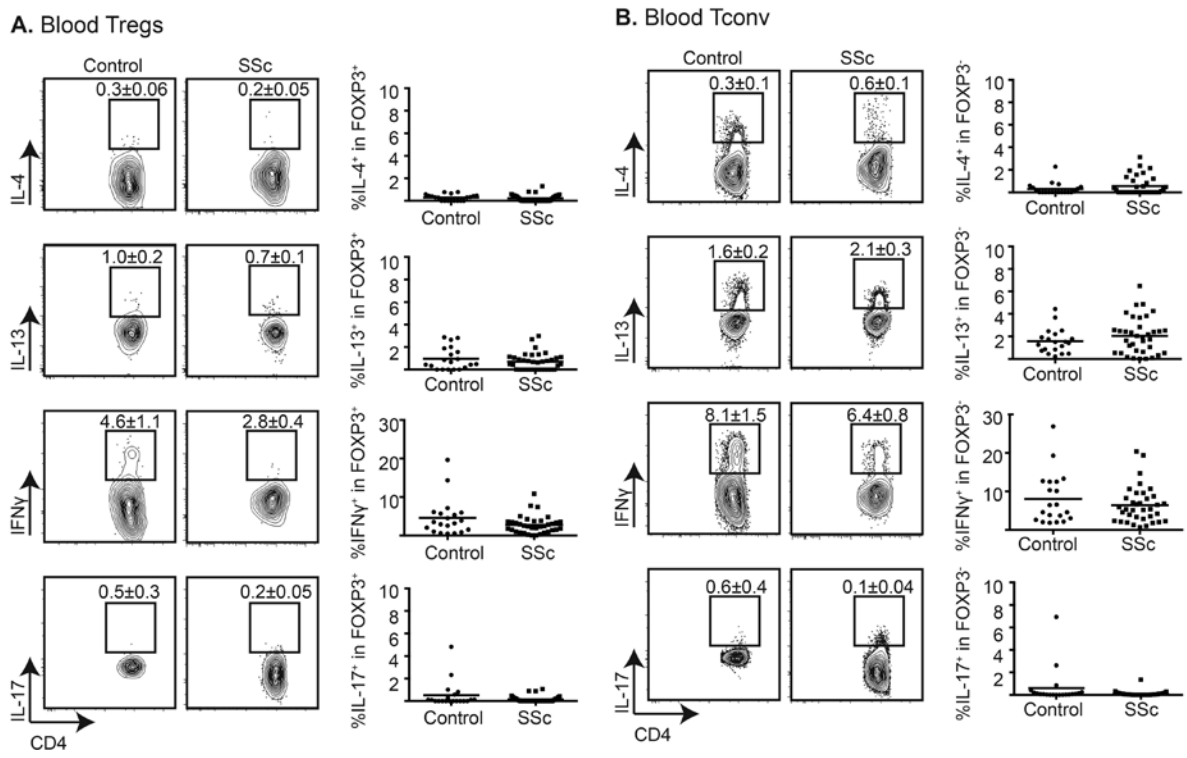



**Figure 3. 1** *Cytokine production profile of Tregs in the blood of SSc patients*. PBMCs from SSc patients or controls were stimulated with PMA and ionomycin, and expression of CD3, CD8, CD4, IFN-γ, IL-4, IL-13, IL-17, FVD, and FOXP3 were measured by flow cytometry. Shown is representative (left) and cumulative (right) data comparing the proportion of cytokine-producing (**A**) FOXP3$^+$ Tregs or (**B**) FOXP3$^-$ Tconv cells. Each symbol in the graph represents data one individual. Numbers in dot plots represent the average ± SEM, n=20 control and 36 for SSc subjects.

### 3.4 Tregs in the skin of SSc patients make Th2 cytokines

Since the skin is a major site of pathology in SSc, I next asked if skin-localized Tregs may be numerically and/or functionally impaired. Punch biopsies were obtained and skin resident T cells isolated from cultured explants [167, 168] were analysed by flow cytometry for expression of FOXP3 and cytokines. Consistent with previous reports [200, 201], control skin had a higher proportion of FOXP3$^+$ Tregs than blood (average percent of CD4$^+$FOXP3$^+$ T cells: 3.3±0.55% in blood, n=20, *vs* 23.7±4.5% in skin, n=13, p=0.0007). However, there was no

significant difference between SSc patients and controls in the proportion of skin-resident Tregs: 23.7±4.5% in controls (n=13) *vs* 30.3±2.8% in SSc (n=19) (p=NS). Although one previous study reported an increased proportion of Tregs in the skin of SSc patients with early disease [202], this study used histology, which is less quantifiable than flow cytometry.

Strikingly, the proportion of Tregs in the skin of SSc patients producing the Th2-cell-associated cytokines IL-4 and/or IL-13 was significantly increased (**Figure 3.2A)**. However, the mean fluorescence intensity of IL-4 or IL-13 in gated IL-4$^+$FOXP3$^+$ Tregs or IL-13$^+$FOXP3$^+$ Tregs was similar between controls and SSc patients (data not shown). Comparison of the overall proportions of Tregs simultaneously producing IL-4, IL-13 and/or IFN-γ revealed that significant differences between SSc patients and controls were restricted to Th2-like Tregs, since the proportion of cells making IFN-γ, with or without IL-4 and/or IL-13, was unchanged (**Figure 3.2B**). Also unchanged were the proportions of Tregs producing IL-10 (data not shown). The increase in production of Th2 cytokines by skin Tregs was not affected by disease duration, treatment, or subtype (data not shown). Analysis of cytokine production from skin-resident Tconv cells revealed that, as in the blood, there was no significant difference between patients and controls in the proportions of Th1, Th2 or Th17 cells (**Figure 3.2C**). These data show that the pro-fibrotic, Th2-cytokine rich environment of SSc skin could be driven by Tregs that have trans-differentiated into Th2-like Tregs rather than by conventional Th2 cells.

In humans, activated CD4$^+$ T cells may transiently express FOXP3 but we recently found that expression of FOXP3 can remain elevated in human Th17 cells[122]. These data raised the possibility that activated Th2 cells may also express FOXP3 for longer than expected based on observations in Th1 cells. To examine this question I sorted enriched populations of Th1 and Th2 cells from blood using cell surface receptors: Th1 cells were sorted as CXCR3$^+$CCR6$^-$CRTH2$^-$

and Th2 cells were $CXCR3^{-}CCR6^{-}CRTH2^{+}$ [203, 204]. The sorted Th1 and Th2 cells were expanded, and their expected cytokine phenotype was confirmed on the basis of high expression of IFN-γ or IL-4 and IL-13, respectively. Expression of FOXP3 was then monitored at different times after activation. Similar to unpolarized T cells [35], both Th1 and Th2 cells transiently upregulated FOXP3 2-4 days after T cell receptor stimulation, but then rapidly down-regulated expression upon return to the resting phase (data not shown). Thus unlike Th17 cells, conventional Th2 cells do not express high and sustained levels of FOXP3, supporting the interpretation that the $FOXP3^{+}$ T cells in the skin which produce Th2 cytokines are Tregs. In further support of this possibility, skin-resident $FOXP3^{+}$ cells expressed additional Treg-associated markers. Specifically, $FOXP3^{+}$ cells from the skin of control and SSc subjects expressed levels of CD25 and CTLA-4 that were higher, and levels of CD127 that were lower, compared to $FOXP3^{-}$ (data not shown). Collectively, these data argue against the possibility that the IL-4 and IL-13-expressing $FOXP3^{+}$ cells are activated or resting Th2 cells.

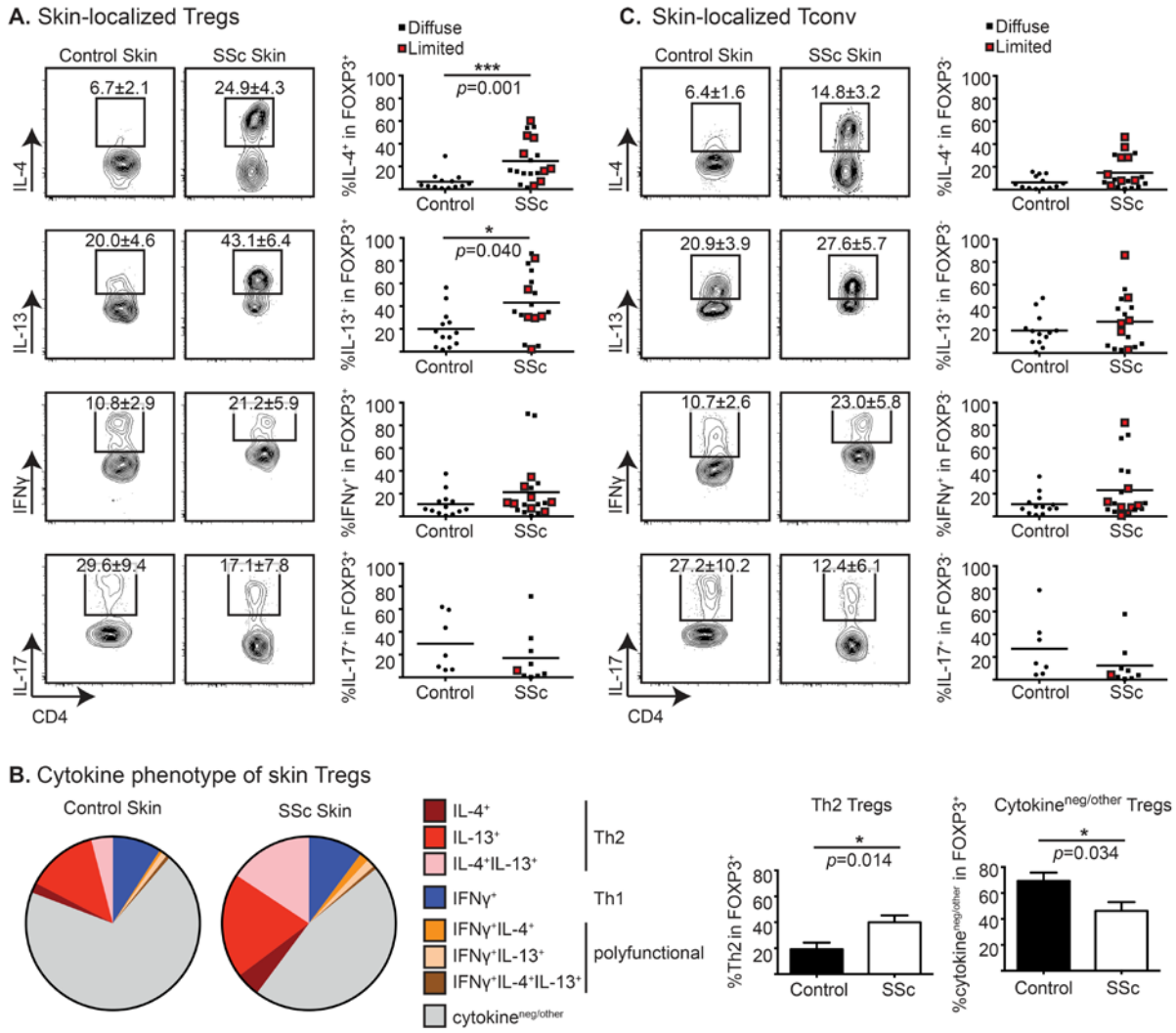



**Figure 3. 2 .** *Tregs in the affected skin of SSc patients produce Th2 cytokines*. 4mm punch biopsies from the affected skin of subjects with limited or diffuse SSc, or skin from normal controls, were cultured as explants. After 3 weeks non-adherent cells were collected, stimulated with PMA and ionomycin, and stained for CD3, CD8, CD4, IFN-γ, IL-4, IL-13, FOXP3, and FVD. Shown is representative (left) and cumulative (right) data comparing the proportion of cytokine-producing (**A&B)** $FOXP3^+$ Tregs or **C)** $FOXP3^-$ Tconv cells. Each symbol in the graph represents data from one individual. Numbers in dot plots represent the average ± SEM, n= 13 control and 19 for SSc subjects.

### 3.5 SSc patients have an increase in circulating Tregs expressing Th2-cell-associated chemokine receptors

In addition to lineage defining cytokines, fully differentiated Th cell subsets and their precursors can be identified by expression of unique patterns of chemokine receptors. Specifically, Th1 cells are characteristically $CXCR3^+$, Th2 cells are $CCR4^+CCR6^-CXCR3^-$, and Th17 cells are $CCR4^+CCR6^+CXCR3^-CCR10^-$ [204]. Interestingly, human Tregs can be similarly subdivided on the basis of these chemokine receptors into subsets of cells that mirror Th cell lineages, yet retain their suppressive function [95]. Accordingly, I revisited the phenotype of circulating Tregs in SSc patients, measuring the proportions of cells expressing Th cell-associated patterns of chemokine receptors. I did not use CRTH2 in this sorting strategy because, whereas only terminally differentiated TH2 cells express CRTH2 [205], CCR4 is also expressed by cells with the potential to become Th2 cells. Indeed after stimulation and expansion, $CCR4^+CCR6^-CXCR3^-$ T cells have a Th2 cytokine production profile very similar to T cells sorted on the basis of CRTh2. Regardless of whether patients had limited, diffuse, early or late SSc, or were taking immunosuppression, there was a significant increase in the proportion of circulating Tregs expressing Th2-associated homing molecules, with a parallel decrease in Tregs expressing chemokine receptors characteristic of Th1 or Th17 cells. Similar analysis of Tconv cells for expression of Th cell associated chemokine receptors revealed no significant differences (data not shown), indicating that the increased proportion of circulating Th2-like cells is specific to Tregs.

We next asked whether the capacity of Th2-like Tregs or Tconv cells to home to the skin differed between healthy individuals and subjects with SSc. By analysis of cutaneous

lymphocyte antigen (CLA) expression, a skin homing marker [206], I found that within the skin-homing subset, there is a significant increase of Th2 markers on SSc Tregs compared to Tconv cells, while this difference is not significant in healthy controls (**Figure 3.3B**). Although SSc Tregs had increased proportions of Th2-cell associated and skin homing markers, consistent with the lack of cytokine production detected by intracellular staining (**Figure 3.1A**), sorted $CLA^+$ or $CLA^-$ Tregs also secreted negligible amounts of cytokines (**Figure 3.3C**). Therefore, individuals with SSc have an increase in the proportion of circulating Tregs, but not Tconv cells, which co-express Th2-lineage markers and are poised to migrate to the skin, but do not yet produce Th2-cell associated cytokines.

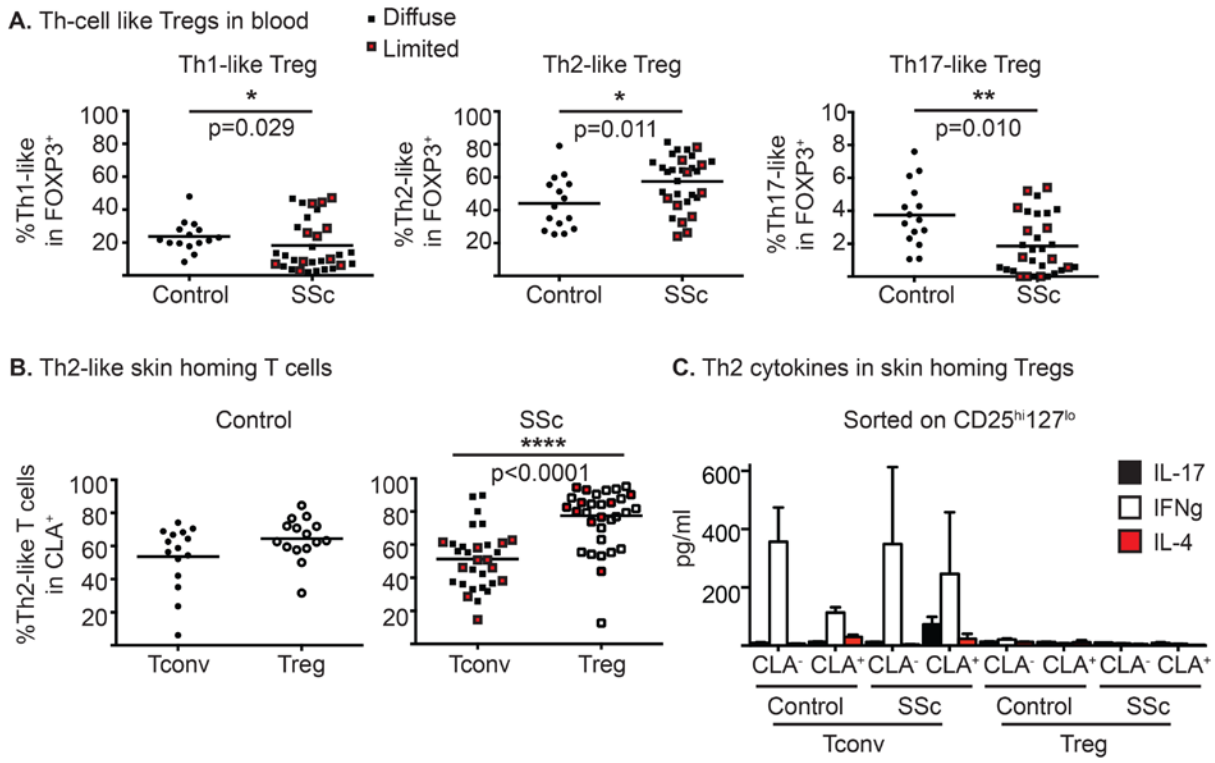



**Figure 3. 3** *The blood of SSc patients has an increased proportion of skin-homing Th2-like Tregs*. PBMCs from SSc patients and controls were stained for CD3, CD4, CD8, CXCR3, CCR4, CCR6, CCR10, CLA, and FOXP3, and analyzed by flow cytometry. **A)** The proportions of Th1 ($CXCR3^+$), Th2 ($CXCR3^-CCR4^+CCR6^-$) and Th17-like ($CXCR3^-CCR4^+CCR6^+CCR10^-$) cells within $FOXP3^+$ cells were determined. **B**) The proportions of Th2-like ($CXCR3^-CCR4^+CCR6^-$) cells within $CLA^+FOXP3^+$ or $CLA^+FOXP3^-$ were determined. Each symbol in the graph represents data one individual. n=15 control and 31 for SSc subjects. C) Skin-homing ($CLA^+$) and circulating ($CLA^-$) Tregs ($CD25^{hi}127^{lo}$) and Tconv ($CD25^{lo}127^{hi}$) were sorted from PBMCs and stimulated for 72 hours. IL-17, IFN-γ, and IL-4 production was measured in supernatants by CBA. Graphs show mean ± SEM (n=2 for control and 2 for SSc subjects).

### 3.6 IL-33 stimulates Tregs in the skin to produce Th2 cytokines

The increase in Tregs producing Th2 cytokine specifically in the skin, but not the blood, of SSc patients raises the question: what factors drive skin-localized Tregs to become Th2-like cells? Human Tregs can differentiate into IFN-γ-or IL-17-producing cells upon exposure to classical Th1 or Th17 polarizing cytokines, respectively [101, 120, 207], but whether or not Tregs can

be similarly induced to produce Th2 cytokines is unknown. Since immune cells are found in proximity to fibroblasts in skin affected by SSc [208, 209], I asked if non-immune cells could be a source of Th2-polarizing cytokines. When fibroblasts from SSc patients were stimulated with inflammatory cytokines (IL-1β and TNF-α) known to be upregulated in SSc [177, 178, 210], expression of *IL33* mRNA was significantly upregulated (**Figure 3.4A**) compared to similarly stimulated normal control fibroblasts. Expression of mRNAs for other Th2-polarizing cytokines, including *IL4*, *IL13* and *TSLP*, were neither consistently expressed in fibroblasts, nor upregulated upon exposure to inflammatory signals (data not shown).

IL-33 is a Th2-cell inducing cytokine associated with fibrosis and SSc [210-213]. Although I found no effect of IL-33 on cytokine production by blood Tregs (data not shown), culture of normal skin biopsies in the presence of IL-33, resulted in a significant increase in the proportion of IL-13-producing Tregs (**Figure 3.4B**). Addition of IL-33 did not result in a change in the production of IL-4 or IFN-γ. To further explore a possible role for IL-33 in the local differentiation of Th2-like cells, immunofluorescence staining was performed on skin biopsies from controls and SSc patients. The dermis of SSc skin was highly enriched in IL-33$^+$ cells [214], and CD3$^+$ T cells were consistently found in close proximity to the IL-33$^+$ cells (**Figure 3.4C**). Thus, Tregs homing to skin affected by SSc would be exposed to IL-33, consistent with the possibility that this cytokine contributes to their differentiation into Th2-like cells in tissues.

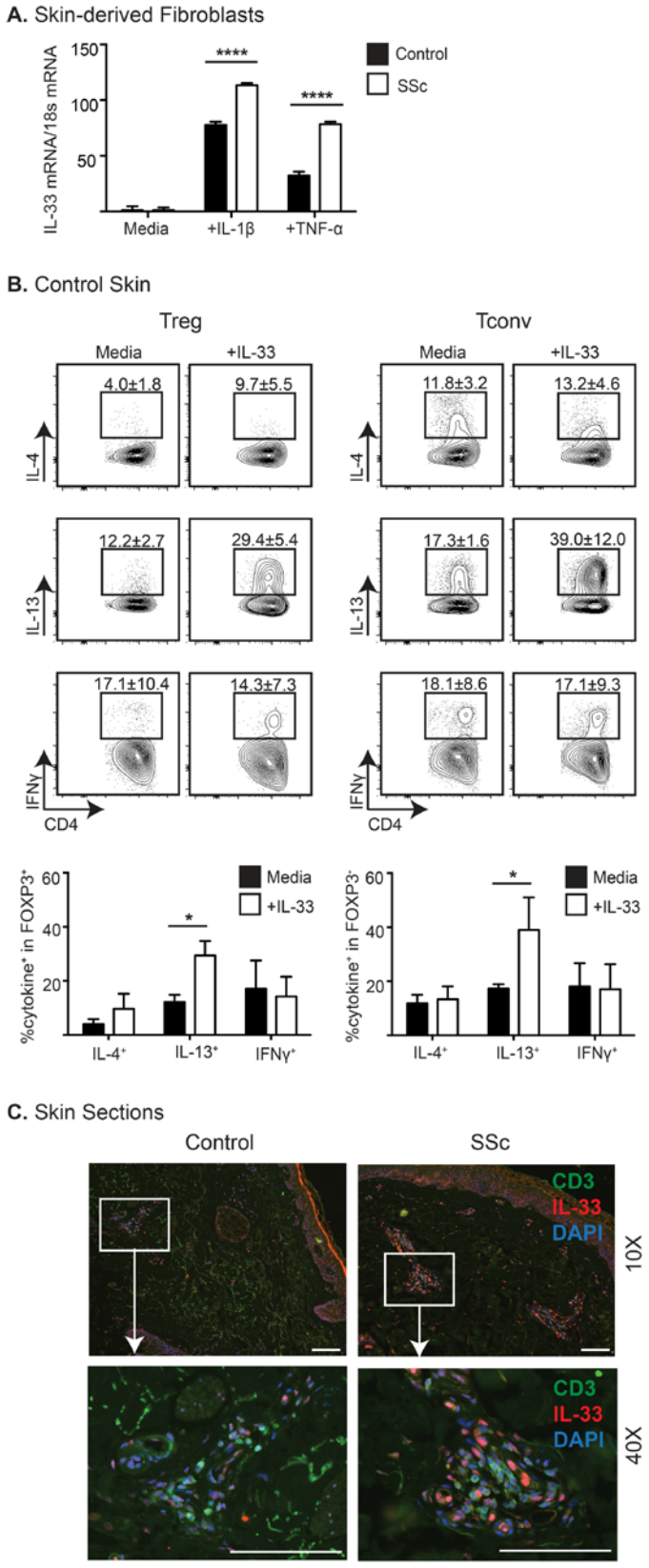
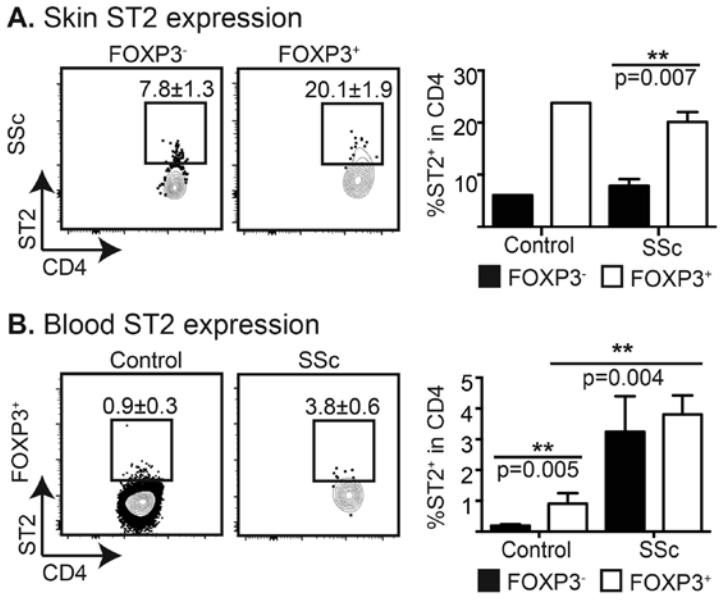



**Figure 3. 4** *IL-33 stimulates the production of IL-13 from skin Tregs.* **A**) Fibroblasts from the skin of SSc patients (n=3) or controls (n=2) were stimulated with or without IL-1β or TNF-α, for 24 hours and qPCR was used to measure levels of *IL33* mRNA relative to 18s. Shown is the fold change relative to the amount in control fibroblasts cultured in media. **B**) Skin biopsy explants from normal controls were cultured in the presence or absence of rhIL-33. After 3 weeks, the non-adherent cells were collected, stimulated with PMA and ionomycin, and analyzed by flow cytometry for CD3, CD8, CD4, IFN-γ, IL-4, IL-13, and FOXP3. The top panel depicts representative plots and bottom panel shows the average ± SEM (n=3). **C)** Immunofluorescence staining was performed on µm thick sections of skin from controls or SSc subjects. Sections were co-stained for CD3 (green), IL-33 (red) and DAPI (blue). Top depicts representative data at 10x magnification, with white insert indicating the location of the image taken at 40x magnification (n=3 for control and n=3 for SSc). Scale bar represents 100µm.

### 3.7 Skin resident Tregs express ST2

To define whether IL-33 may act on Tregs via direct or indirect mechanisms, I examined the expression of ST2, the IL-33-specific component of the IL-33 receptor. The role of this receptor in enhancing the development of classical human Th2 cells is well known [215] and it has recently been reported that in mice colon or muscle resident Tregs can also express ST2 [216, 217]. Whether or not human Tregs can also express ST2 was unknown. Isolated $CD4^+$ T cells from skin biopsies were co-stained for FOXP3 and ST2, and as shown in **Figure 3.5A**, in both controls and SSc subjects, a significantly higher proportion of skin $FOXP3^+$ Tregs co-expressed ST2 compared to $FOXP3^-$ Tconv cells. By comparison, $FOXP3^+$ Tregs in the blood expressed significantly less ST2 than in the skin. Notably, although the proportions were small, both $FOXP3^+$ and $FOXP3^-$ cells from the blood of SSc subjects had increased expression of ST2 (**Figure 3.5B**), supporting the notion that in this disease circulating T cells are poised to become Th2 cytokine producing cells.




**3. 5** ***Skin localized Tregs express ST2.*** **A**) Expanded $CD4^+$ T cells from control (n=1) and SSc (n=3) skin biopsies were rested overnight then stained for FVD, CD4, FOXP3 and ST2. Representative plots show ST2 expression on $FOXP3^-$ and $FOXP3^+$ cells from SSc skin, graph shows averaged data ± SEM for SSc (n=3). **B**) $CD4^+$ enriched T cells from control (n=6) and SSc (n=4) PBMCs were stained as in (**A**). Representative plots show expression of ST2 on $FOXP3^+$ cells from controls and SSc Subjects. Graph shows average ± SEM (n=4-6).

## 3.8 Discussion

The role of Th cell subsets in SSc has remained controversial and unclear for many years. By parallel analysis of Tregs and Tconv cells in blood and skin, I found that the most profound changes occur in Tregs that are localized in affected tissue. Combined analysis of Th cell lineage and skin homing markers, and cytokine production, supports a model where circulating Tregs in SSc patients are enriched for cells which are "poised" to traffic to the skin and produce Th2 cytokines. Once in lesional skin, Tregs would be exposed to Th2-polarizing factors, including IL-33, resulting in a pathological differentiation signal to differentiate into cells producing Th2 effector cytokines. Localized production of pro-fibrotic cytokines from Tregs is a previously unknown aspect of SSc that is likely to be an important factor in disease pathophysiology.

Classically, Tregs do not produce cytokines that are characteristic of other Th cell lineages. This phenotype is thought to be due to the repressive function of FOXP3, which switches off transcription of many cytokines [190]. Recently, however, there is growing evidence that Tregs in the blood of patients suffering from autoimmune diseases such as type 1 diabetes or multiple sclerosis, can acquire the ability to produce cytokines such as IFN-γ and IL-17 [198]. Mouse models have shown that acquisition of effector cytokine production by Tregs can have an important role in disease pathogenesis [218]. In SSc patients, I found no evidence for this so-called "T cell plasticity" in blood: Tregs were present in normal proportions in PBMCs and did not produce significant amounts of cytokines associated with Th cell lineages. Thus, in contrast to other autoimmune diseases, neither a defect in the number nor in the quality, at least as relates to cytokine production, of circulating Tregs seems to have a major role in the pathogenesis of SSc.

The vast majority of research on human Tregs has been carried out using blood, but there is a growing appreciation for the role of Tregs in tissues [219], and for their environmental sub-specialization [197]. Specifically in skin, data from mice and humans show that Tregs traffic to this tissue, where they maintain immune homeostasis [220] and limit inflammation during antigen recall [221]. My study shows the importance of parallel analysis of blood and affected tissues for understanding the role of local versus systemic Treg dysfunction in autoimmune disease.

The transcriptional programs of Tregs and Th2 cells were initially thought to oppose one another; for example, ectopic expression of FOXP3 suppresses IL-4 [171] and conversely IL-4 can block Treg induction [103]. However, there are also commonalities in Treg and Th2 transcriptional programming [106, 107, 190] with recent evidence for the development of pathological Th2-like Tregs in allergic asthma induced by respiratory syncytial virus infection in mice [111]. Hence expression of FOXP3, IL-4 and IL-13 are not mutually exclusive, and plasticity of Tregs towards Th2-like cells may have an important role in other diseases associated with over production of these cytokines.

One caveat to many studies on human Tregs is the fidelity of FOXP3 as a reliable marker of this lineage as activated Tconv cells can also express FOXP3 [35]. Although I was unable to sort sufficient numbers of $FOXP3^+$ cells from skin biopsies to carry out analysis of the Treg specific demethylation region, several lines of evidence argue against the possibility that Tregs producing IL-4 and/or IL-13 in SSc skin are activated Th2 cells. First, the overall proportion of $FOXP3^+$ T cells does not differ between controls and SSc patients; if these cells were activated Th2 cells, a larger proportion of $FOXP3^+$ cells would be expected in SSc skin. Second, $FOXP3^-$ T cells in cultures of skin explants are distinguishable from *bona fide* Tregs on the basis of intensity of FOXP3 expression [200], and expression of Th2 cytokines was not restricted to $FOXP3^{low}$ cells

(data not shown). Third, I found that human Th2 cells do not express high and sustained levels of FOXP3. Since the skin explants do not have exogenous T cell receptor stimulation, any *in vivo* expression of activation-induced FOXP3 should have returned to baseline. Finally, $FOXP3^+$ cells in the skin express high levels of CD25 and CTLA-4, and low levels of CD127, similar to blood Tregs and as other groups have reported for skin Tregs [200, 222]. Although I cannot exclude the possibility that some of the $IL\text{-}4^+/IL\text{-}13^+$ $FOXP3^+$ T cells in the skin may be activated Th2 cells, it seems unlikely that the striking increase in IL-4- and IL-13-expressing $FOXP3^+$ T cells could be solely ascribed to the loss of Tregs and the acquisition of activated Th2 cells.

SSc patients have long been known to have high levels of IL-4 and IL-13 in serum and tissue [179, 180], the cellular origin of these cytokines is unclear. My data show that Tregs could be a previously unrecognized source of Th2 cytokines in fibrotic tissues. A question remains regarding the functional effects of these Th2-like Tregs on immune and non-immune cells. Since IL-4 and IL-13 are both pro-fibrotic cytokines, and IL-13 is required for IL-33-driven fibrosis [223], it is very likely that Th2-like Tregs would enhance, rather than resolve, skin fibrosis. In terms of the effects of Th2-like Tregs on immune cells, I found that CD25 and CD127 could not be used to reliably sort homogenous population of $FOXP3^+$ cells and that overall the biopsies from fibrotic SSc skin contained insufficient Tregs to test in classical *in vitro* suppression assays. Regardless of whether or not Th2-Tregs affect T cell proliferation, however, the effect of Th2 cytokines on non-immune cells likely contributes to the pathological response.

Since circulating Tregs expressing Th2-associated chemokine receptors express very little IL-4 [95] or IL-13, the full differentiation of Th2-like Tregs may only occur in response to specific environmental cues. In SSc, IL-33 could be one such cue since it increases expression of IL-13 from Tregs without affecting FOXP3 expression. In support of a role for IL-33, this

cytokine is known to be elevated in the serum of SSc patients and is associated with the development of IL-13 dependent fibrosis [211, 212, 223]. Interestingly, a significant proportion of skin-localized Tregs express ST2, consistent with emerging data on the importance of this molecule in tissue-specific immune regulation[217]. Whether $ST2^+$ Tregs traffic to the skin and expand due to local signals, or $ST2^+$ Tregs are induced in the skin is unclear. In mice, IL-33 can act indirectly via dendritic cells to expand $ST2^+$ Tregs [224]. Further work will be required to define the direct versus indirect mechanisms of action of IL-33 on skin localized human Tregs.

Collectively, my data provide the first evidence for the differentiation of human Tregs into Th2-cytokine producing cells, and for their environmental specialization, providing a new paradigm for how Treg plasticity could contribute to autoimmunity. My results also highlight the importance of examining Tregs at the site of affected tissue, and raise the possibility that developing strategies to block this local transdifferentiation of Tregs could be a new approach to treating SSc.

# Chapter 4: Generation of alloantigen-specific T regulatory cells by expression of a novel chimeric antigen receptor targeting HLA-A2

## 4.1 Introduction

The essential role of T regulatory cells (Tregs) in preventing autoimmunity and controlling responses to allo-antigens is well established. Multiple Treg-based cell therapy approaches are now being tested in the clinic, with early promising results in prevention of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HSCT) [64-66] or improvement in c-peptide levels in type 1 diabetes[63]. The results of these phase I trials indicate that Treg cell therapy seems to be well tolerated and possibly efficacious, but there may be a transient risk of generalized immunosuppression [225].

Data from animal studies indicate that the potency and specificity of Treg cell therapy can be significantly enhanced by the use of antigen-specific cells. Models of autoimmunity have shown that antigen specific Tregs are superior to polyclonal Tregs in reducing disease: Tregs isolated from pancreatic lymph nodes or pulsed with islet antigen were significantly better at preventing or curing type 1 diabetes than were polyconal Tregs [56, 133-136], and Tregs expressing an auto-antigen-specific transgenic T cell receptor (TCR) reduced CNS inflammation in EAE compared to polyclonal Tregs [226]. Antigen specificity even confers enough potency to compensate for sub-optimal Tregs generated by TGF-β induction *in vitro* [227] or transgenic FOXP3 expression[138], resulting in comparable suppressive capacity to natural Tregs. Similarly, alloantigen-specific Tregs enriched by alloantigen-stimulated expansion or expression of a TCR transgene are more effective than polyclonal cells at preventing rejection of organ and tissue

grafts [47, 48, 52, 139, 143]. Although limited, there is some evidence that Tregs expanded with allo-antigens effectively prevent GVHD [144] and that *in vivo* induction of antigen-specific Tregs promotes acceptance of hematopoietic allografts without GVHD [228]. Humanized mouse models have shown similar results, where allo-antigen expanded Tregs are more potent suppressors of skin graft rejection than polyclonal Tregs [50, 145].

An alternate approach to generating antigen-specific T cells is the use of chimeric antigen receptors (CARs), in which T cells are genetically engineered to express an extracellular single-chain antibody fused to intracellular signaling domains, bypassing the need for MHC-peptide presentation and enabling efficient generation of antigen-specific cells [229, 230]. Tregs expressing CARs for model antigens have been tested [148-151, 231], leading us to hypothesize that this approach could also be used in the context of transplantation. Here I describe a new approach to generate potent alloantigen-specific Tregs using a new CAR targeting HLA-A2. By targeting HLA-A2 with a CAR, the goal is to activate large numbers of Tregs in the transplanted tissue to provide potent and lasting immunosuppression only in the presence of the alloantigen.

## 4.2 Construction and validation of specificity of an HLA-A2 specific CAR

We aimed to generate a new CAR specific for HLA-A2 as this is a commonly mismatched antigen in transplantation with a prevalence of ~ 50% in Caucasians [232] and HLA-A mismatching is associated with poor GVHD outcomes [233]. As detailed in the Methods, I generated lenti viral vectors encoding the HLA-A2 specific CAR by cloning and sequencing the heavy and light chain variable regions of the BB7.2 mAb and fusing the resulting scFv to CD28 and CD3ζ in the classical second generation CAR structure [234] (**Figure 4.1A&B**). A lenti vector

encoding a well characterized HER2-specific CAR (HER2-CAR) [146]served as a negative control. Surface expression of the A2-CAR was confirmed by transient transfection of 293T cells and flow cytometric staining for the extracellular myc epitope (**Figure 4.1C**). mAbs re-formatted to scFv may have reduced antigen binding or specificity depending on their components [235, 236]. I therefore used tetramers made from HLA-A2 or HLA-A24 to stain transfected 293T cells. As shown in **Figure 4.1D**, only cells expressing the A2-CAR bound to HLA-A2 tetramers, confirming the expected antigen specificity.

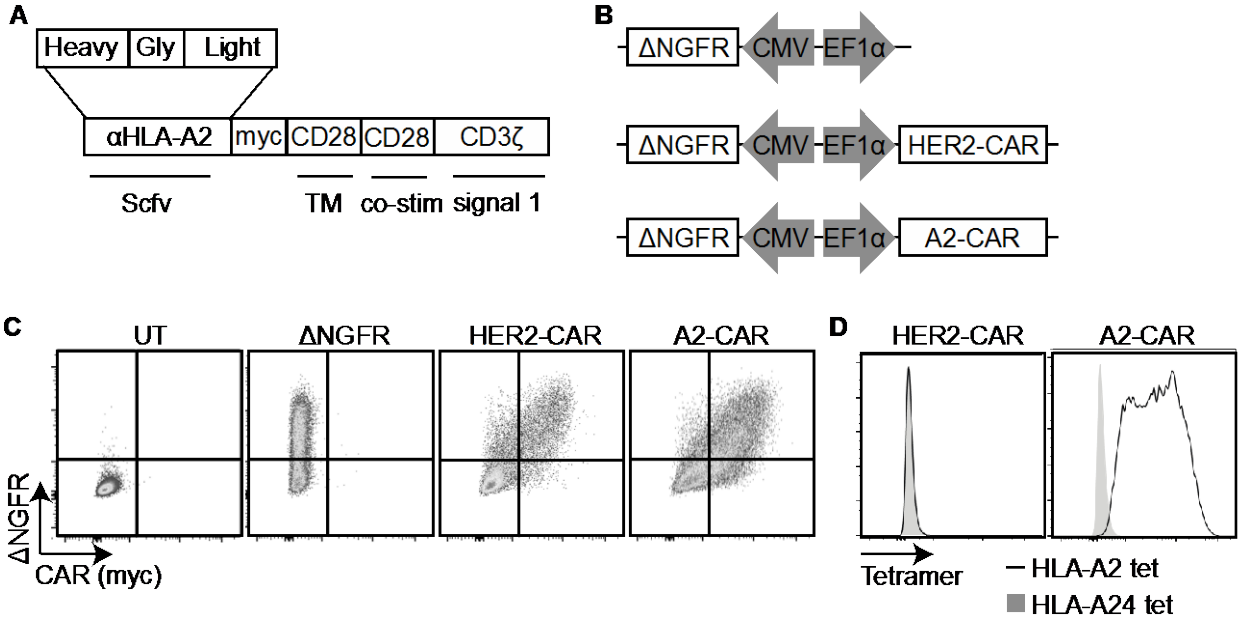


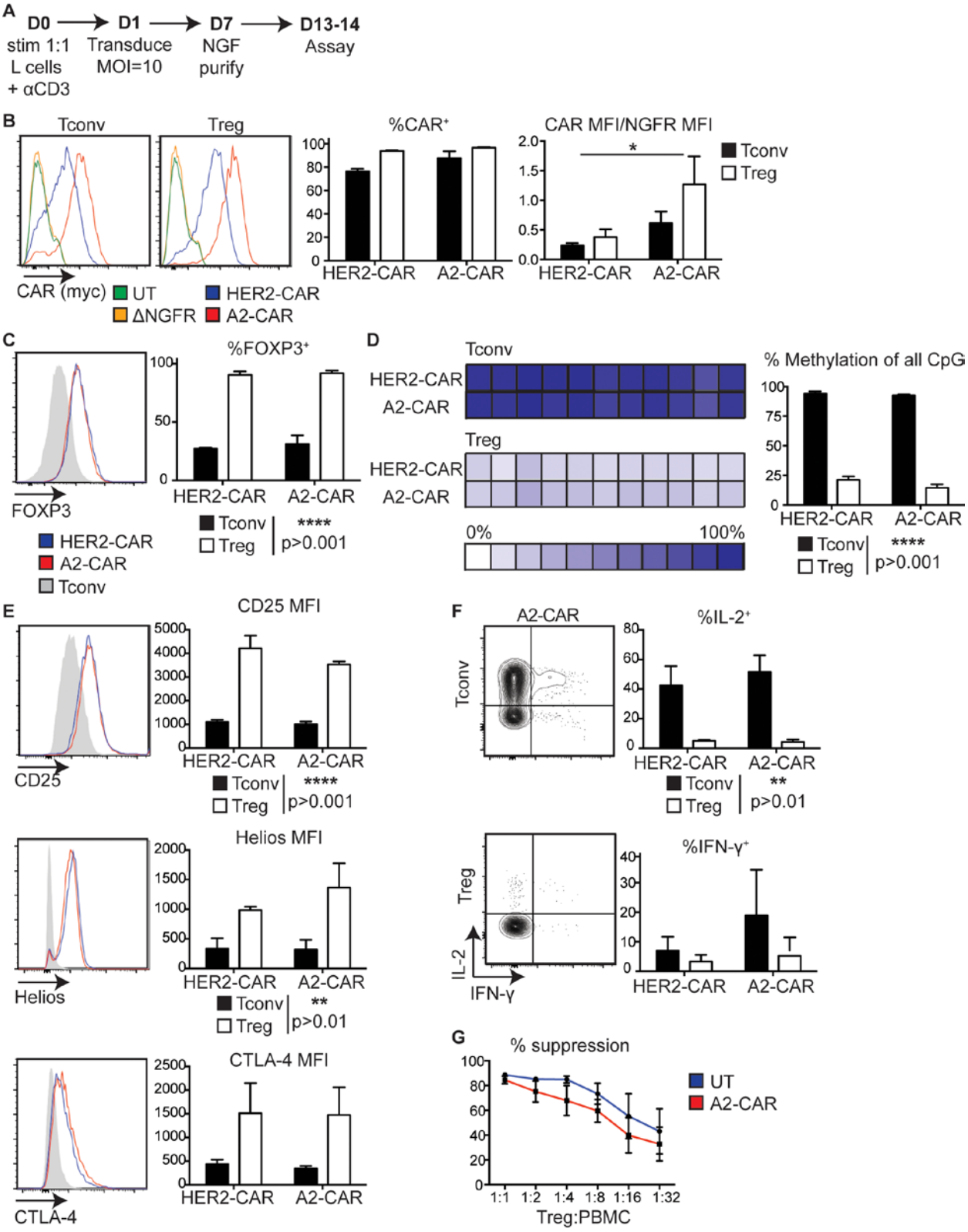

**Figure 4. 1.** *Construction, expression, and antigen specificity of an HLA-A2 specific CAR.* **A**) Schematic of domains in the A2-CAR (*Gly*=glycine serine linker; *myc*=myc tag; *ScFv*=single chain antibody; *TM*=trans-membrane). **B**) Schematic of bi-directional lentiviral vectors which encode the truncated nerve growth factor receptor as a selectable marker under a minimal CMV promoter and CARs specific for HLA-A2 of HER2 under the EF1α promoter. **C**) 293T cells were transfected with an empty vector (ΔNGFR), a HER2-specific CAR, or the A2-CAR. Surface expression was confirmed by detection of the myc-epitope tag by flow cytometry. **D**) 293T cells expressing the HER2- or A2-CAR were stained with HLA-A2 or HLA-A24 tetramers. Data are representative of 2 independent experiments.

### 4.3 Tregs expressing CARs remain phenotypically stable

Tregs were sorted a CD25hiCD45RA$^+$ cells as this population is known to be the most homogeneous with the highest expansion potential [237]. As outlined in **Figure 4.2A**, Tregs (or control Tconvs) were stimulated, transduced and purified as ΔNGFR$^+$ cells, then expanded for an additional 6 days. At the end of these 14 days of culture, high expression of A2-CAR or HER2-CAR on both Tregs and Tconvs was confirmed with flow cytometry (**Figure 4.2B**). Interestingly, while similar proportions of Treg and Tconv populations were positive for CAR expression, on a per-cell basis A2-CAR expression appeared to be higher in both cell types than the HER2-CAR. To exclude the possibility that A2-CAR cells had a greater number of lentiviral vectors inserted in their DNA, CAR intensity was normalized to the expression of ΔNGFR. A2-CAR expressing cells were still found to have significantly more myc expressed on a per-cell basis than HER2-CARs, suggesting that the A2-CAR is highly expressed.

While surface expression of the A2-CAR is desirable, high expression of CARs has been linked to antigen-independent CAR activation [238]. Furthermore, there is only one previous report of CAR expression in human Tregs and these cells were bead sorted on CD25, which enriches but does not purify Tregs[231]. In fact, their low FOXP3 purity and co-production of IFN-γ and IL-10 is suggestive of a Tr1 or memory T cells population. Given my high A2-CAR expression levels and lack of phenotyping of human Tregs transduced with a CAR, it is important to exclude the possibility that high expression of a CAR in Tregs might result in loss of the expected Treg phenotype. As shown in **Figure 4.2C**, A2-CAR expressing Tregs had significantly higher expression of FOXP3 than Tconvs and preserved high demethylation of the Treg specific demethylation region (TSDR) of the FOXP3 locus (**Figure 4.2D**). High expression of other canonical Treg markers, including CD25, Helios, and CTLA-4 was also preserved in A2-CAR

expressing Tregs (**Figure 4.2E**). In comparison to CAR-expressing Tconv cells, A2-CAR Tregs also did not contain a significant proportion of IL-2- or IFN-γ- producing cells (**Figure 4.2F**). Finally, to test if A2-CAR Tregs preserved their *in vitro* suppressive function when activated via their endogenous TCR (i.e. not the A2-CAR), HLA-A2- PBMCs were stimulated with αCD3/CD28 mAbs with or without decreasing ratios of A2-CAR Tregs. As shown in **Figure 4.2G**, A2-CAR Tregs suppressed the proliferation of CD8 T cells in a dose dependent manner. Thus expression of a second generation CAR comprised of CD28 and CD3ζ does not alter the phenotype or function of Tregs.


A
D0
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D7
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Treg
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%IFN-γ+
G
% suppression
UT
A2-CAR
1:1
1:2
1:4
1:8
1:16
1:32
Treg:PBMC

**Figure 4. 2** *Expression of CARs in Tregs does not affect their phenotype or function*. **A**) Schematic diagram outlining the protocol to transduce naive human Tregs with lentiviral vectors encoding CARs. **B**) Extracellular expression of CARs was assessed by staining for the myc epitope tag and proportion of $CAR^+$ cells is summarized. CAR MFI was determined relative to ΔNGFR MFI on the same cell. **C**) Expression of FOXP3 was assessed at day 14; representative data on the left and averaged data (n=3) on the right. **D**) TSDR methylation was determined by pyrosequencing. Top panel shows data from each CpG residues, with averaged data combining all CpGs is below (n=4). **E**) Tregs or Tconvs were analyzed for the indicated markers; representative staining (left) and average mean fluorescence intensity (right, n=6). **F**) Cells were re-stimulated with PMA/ionomycin then stained for IL-2 and IFN-γ. Representative data on the (left) and averaged data on the right (n=3) **G**) Suppressive capacity of transduced Tregs when stimulated through endogenous TCR was assayed by titrated Treg ratios with HLA-$A2^-$ PBMCs in the presence of αCD3/28 coated beads. Division index of $CD8^+$ cells was determined after 96hours (n=3). Data are expressed as average ± SEM.

### 4.4 A2-CAR-mediated stimulation causes potent activation and proliferation of Tregs

We next investigated how stimulation via the A2-CAR affected the phenotype and function of Tregs. A2-CAR expressing Tregs, or Tconv as a control, were left unstimulated, or CAR stimulated with K562 cells expressing HLA-A2, or TCR stimulated with K562 cells loaded with αCD3/CD28 mAbs for 24 hours. Compared to cells stimulated via the endogenous TCR, CAR stimulation resulted in up-regulation of CD69 and CD154 activation markers in both Tregs and Tconvs (**Figure 4.3A**). In comparison to TCR-activated Tregs, A2-CAR-mediated stimulation also caused significantly greater upregulation of latency-associated peptide (LAP) and GARP, the inactive form of TGF-β and one of its receptors, respectively. Despite similar activation to A2-CAR Tconv in response to HLA-A2, A2-CAR Tregs do not produce inflammatory cytokines when co-cultured with HLA-A2 expressing cells while Tconv produce significant amounts of IFN-γ, TNF-α, IL-6, IL-4, and IL-2. (**Figure 4.3B**). After 4 days, antigen-driven proliferation was measured by dilution of cell proliferation dye, revealing that CAR-mediated stimulation broke the usual anergic phenotype of Tregs and stimulated proliferation in the absence of exogenous IL-2 (**Figure 4.3C**), To test how A2-CAR stimulation affects the *in*

*vitro* suppressive function of Tregs, HLA-$A2^+$ and HLA-$A2^-$ PBMCs were co-cultured with a titrated dose of A2-CAR or HER2-CAR (for TCR stimulation only) expressing Tregs, and all cells were stimulated with αCD3/28 beads. This was done to mimic a scenario where A2-CAR Tregs are receiving activation signals via both their endogenous TCR and their CAR because it is unlikely that A2-CAR Tregs would receive CAR stimulation alone in an allogeneic setting. A2-CAR stimulation in addition to TCR stimulation had no impact on the suppression of HLA-$A2^-$ PBMCs, as both conditions resulted in a dose-dependent reduction in proliferation (significant relative to Tconv, data not shown) (**Figure 4.3D**). The proliferation of HLA-$A2^+$ PBMCs was also inhibited; however, the suppressive capacity of A2-CAR Tregs relative to A2-CAR Tconv could not be assessed to due to lysis of HLA-$A2^+$ PBMCs by A2-CAR Tconv (data not shown). Importantly, Tregs receiving concurrent CAR and TCR stimulation are just as suppressive as Tregs stimulated through their TCR alone (HER2-CAR Tregs). This is true for PBMCs expressing target antigen (HLA-$A2^+$) and those that do not stimulate through the CAR (HLA-$A2^-$), which is promising since both donor and recipient will be present and require suppressing in transplantation.

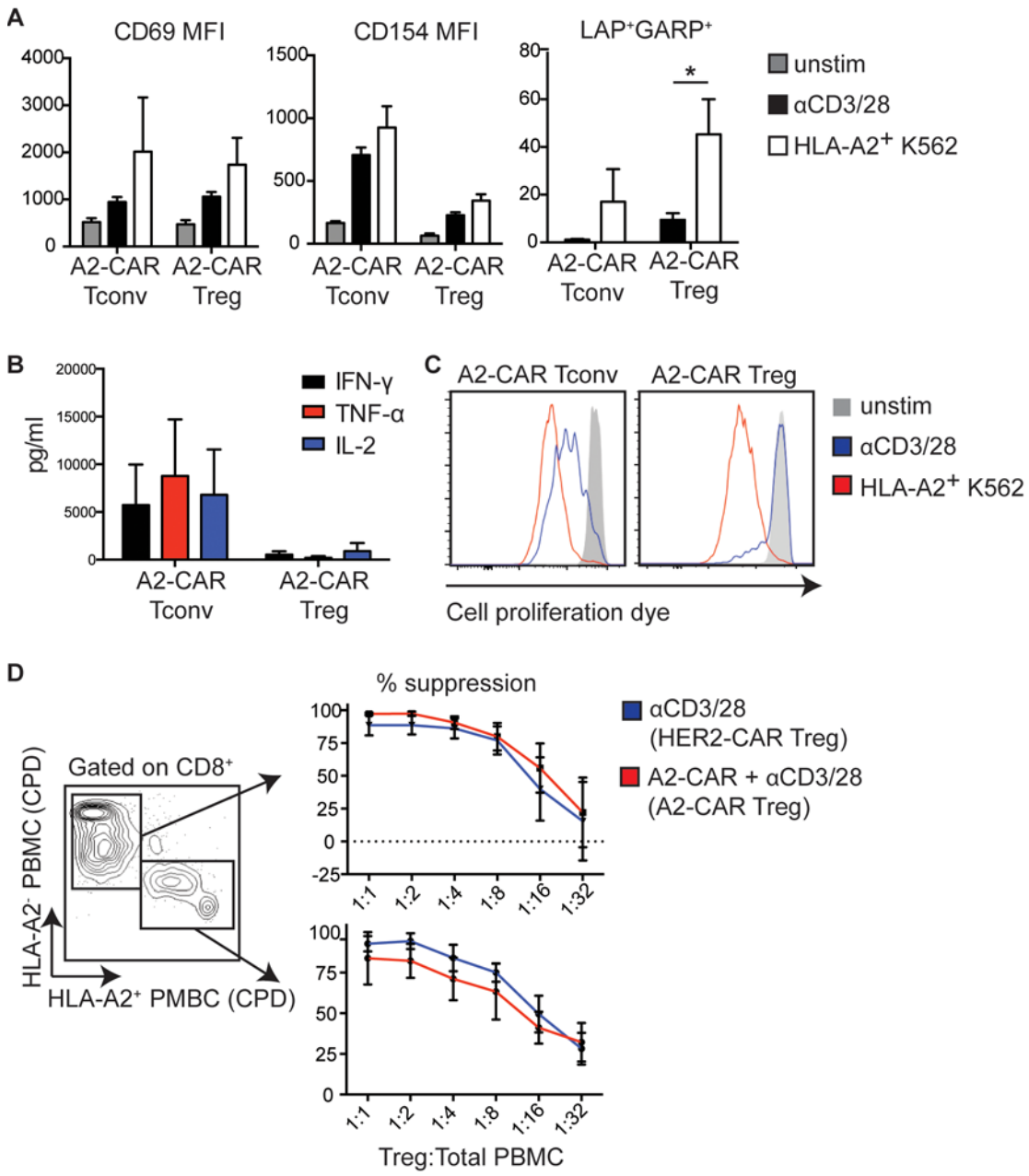


**Figure 4. 3**. *Stimulation through the A2-CAR causes Treg activation and proliferation* . A2-CAR Tregs or Tconvs were left unstimulated, or stimulated with K562.64 cells loaded with αCD3/28 mAbs or expressing HLA-A2. **A**) After 1 day, T cells were assayed for expression of activation markers. LAP/GARP gates were set on unstimulated **B**) After 2 days amounts of cytokines in supernatants were measured by cytometric bead array **C**) After 4 days proliferation of A2-CAR T cells was measured by dilution of cell proliferation dye. **D**) Suppressive capacity of transduced Tregs when stimulated through endogenous TCR and CAR was assayed by titrated Treg ratios with 1:1 HLA-A2$^{+}$ to HLA-A2$^{-}$ PBMCs in the presence of αCD3/28 coated beads. Division index of CD8$^{+}$ cells was determined after 96hours. n=3 for all samples. Data represents average ± SEM.

### 4.5 A2-CAR-mediated expansion maintains functional Tregs

How long-term CAR-mediated stimulation of Tregs might affect their phenotype and/or function is unknown. To ask this question, I analyzed the phenotype of A2-CAR-expressing Tregs (generated as in **Figure 4.2A**) that were expanded for 2 weeks either by stimulation through their endogenous TCR with K562.64 cells loaded with αCD3/28 mAbs, or with K562 cells expressing HLA-A2. Cells were rested 24 hours before analysis to ensure that they are in a resting state. Consistent with the finding that CAR-mediated stimulation was more potent than TCR stimulation in short term activation assays (**Figure 4.3C**), CAR-stimulation resulted in a significantly higher fold expansion and increased viability of Tregs compared to TCR-expanded cells (**Figure 4.4A**), although Tregs expanded through their CAR have more limited proliferation than A2-CAR expanded Tconv (data not shown). Both TCR and CAR-stimulated Tregs cells remained > 85% $FOXP3^+$ and had similar levels of FOXP3 on a per-cell basis (both $\%FOXP3^+$ and MFI significantly higher than Tconv). However, there was a slight down-regulation of the A2-CAR MFI in CAR-expanded cells, likely due to receptor internalization, as the overall proportion of $CAR^+$ cells was similar after TCR or CAR expansion (**Figure 4.4B**). Tregs expanded through their CAR also maintained high expression of CD25 and Helios, with an increase in CTLA-4 expression, although no changes in these markers were significant (**Figure 4.4C**). Finally, CAR-stimulated expansion of the A2-CAR Tregs did not affect their function, as judged by their ability to suppress $CD8^+$ T cell proliferation (**Figure 4.4D**). In fact, CAR expanded Tregs appear to have superior suppressive capacity to TCR expanded cells, possibly because of the poor viability of the latter.

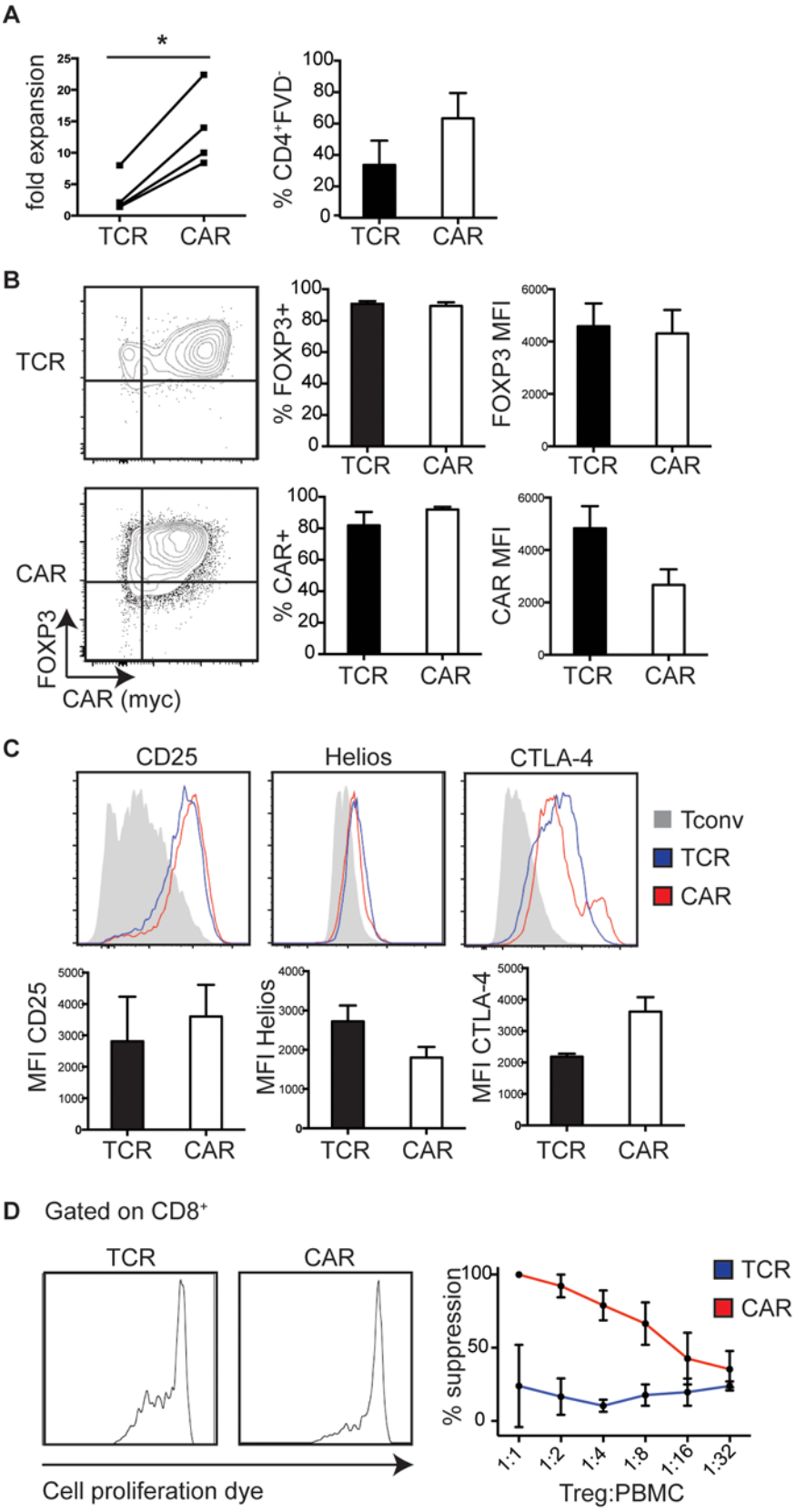


**Figure 4. 4.** *CAR expanded Tregs are phenotypically stable and are more suppressive than TCR expanded Tregs.* A2-CAR Tregs were stimulated with K562.64 cells loaded with αCD3/28 mAbs (TCR) or expressing HLA-A2 (CAR) and expanded 2 weeks with IL-2. **A**) Fold expansion based on cell counts at D0 of re-stimulation and D14, and viability of CAR and TCR expanded Tregs. **B**) FOXP3 purity and CAR expression representative plots left and summary right. **C**) Treg marker expression of TCR-expanded (blue) and CAR expanded (red) Tregs (all Tconv controls in grey). **D**) Suppressive capacity of CAR-expanded A2-CAR Tregs when stimulated through endogenous TCR was assayed by titrated Treg ratios with HLA-A2$^-$ PBMCs in the presence of αCD3/28 coated beads. Representative histograms show CD8 proliferation at 1:16 ratio (n=3). Data are expressed as ± SEM.

### 4.6 A2-CAR Tregs are superior to polyclonal Tregs at preventing XenoGVHD mediated by HLA-A2$^+$ T cells

In order to test the functional capacity of A2-CAR Tregs *in vivo*, I used a mouse model in which human PBMCs engrafted into immunodeficient NSG mouse cause xenogeneic GVHD [85]. 1 x10$^7$ PBMCs from an HLA-A2$^+$ donor were injected into irradiated NSG mice with or without the indicated type of Treg at a 1:1 or 2:1 ratio (i.e. 1 x10$^7$ Tregs or 5 x10$^6$ Tregs). Mice were monitored for up to 7 weeks by clinical score, weight, and blood draws. Consistent with previous reports, control Tregs expressing the HER2-CAR, which would be stimulated via their endogenous TCR by xenogeneic antigens, significantly improved survival of mice when infused at a 1:1 ratio (**Figure 4.5A**). In contrast, mice receiving A2-CAR Tregs at either a 1:1 or 1:2 ratio were significantly more protective. A2-CAR Tregs improved survival (**Figure 4.5A**) and delayed onset of xenoGVHD (**Figure 4.5B**). Even if xenoGVHD developed, the mice lost less weight (**Figure 4.5C**) and disease progression was slower (**Figure 4.5D**).

I also drew blood from the mice weekly to monitor engraftment of human T cells and survival of the infused Tregs. As shown in **Figure 4.5E**, in comparison to mice receiving HER2-CAR Tregs, mice injected with A2-CAR Tregs had a significantly reduced absolute number of human HLA-A2$^+$CD45$^+$ cells per μl of blood. The decrease in circulating HLA-A2$^+$ cells corresponded with expansion and persistence of A2-CAR Tregs in mouse blood, likely reflecting *in vivo* antigen stimulation (**Figure 4.5E**). Notably, the A2-CAR Tregs remained FOXP3$^+$, and were detectable in circulation for twice as long as the HER2-CAR Tregs. As seen *in vitro* (**Figure 4.4B**), cells that received CAR-stimulation *in vivo* also had a lower MFI of CAR expression, but they also had a higher FOXP3 MFI (**Figure 4.5G**). This is likely because it was necessary to compare A2-CAR and HER2-CAR Tregs at D7 while both populations were still

detectable in the blood but the A2-CAR Tregs are clearly still activated. Together this suggests that strong antigen-specific activation of A2-CAR Tregs has resulted in greater expansion and suppression of xenoGVHD *in vivo*.

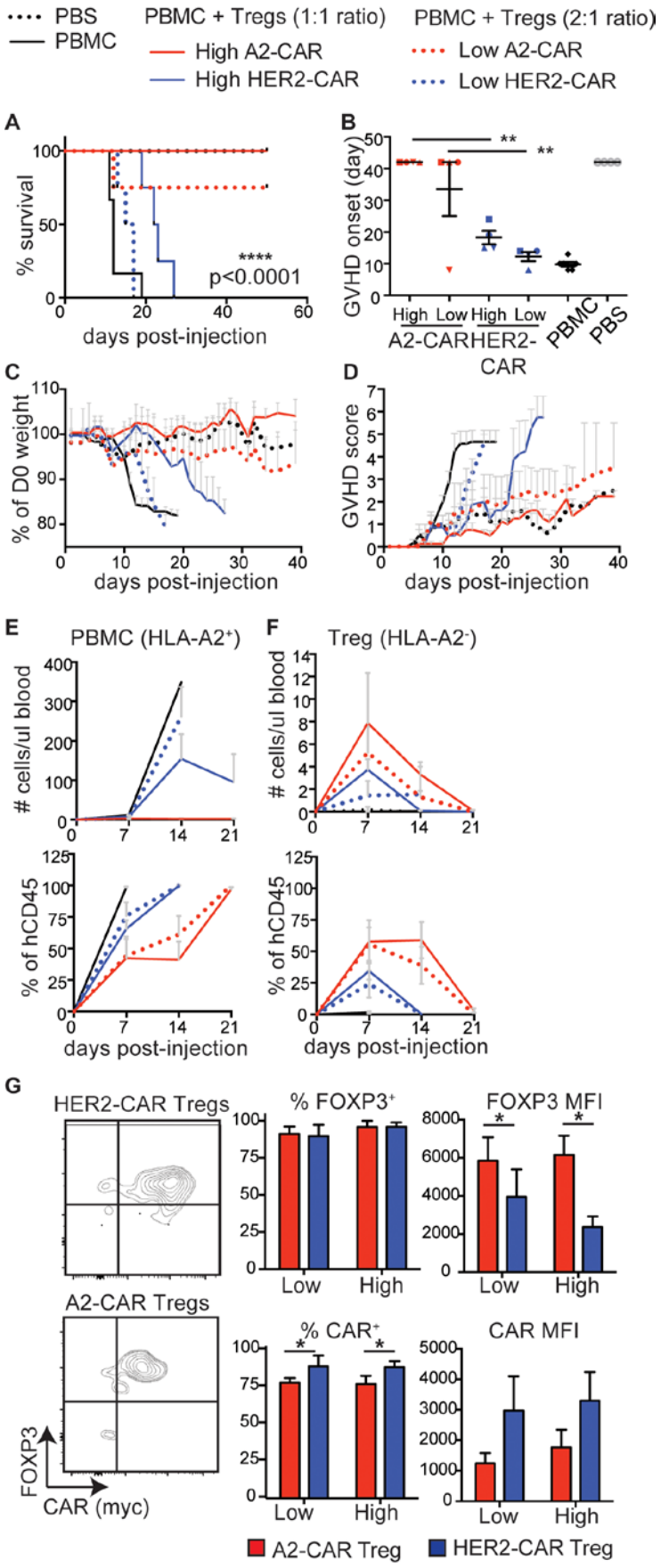


**Figure 4. 5**. *HLA-A2 CAR Tregs are superior to polyclonal Tregs at prevening xeno-GVHD.* Irradiated NSG mice were injected with 10M HLA-A2+ PBMCs alone or with 10M (1:1) or 5M (1:2) A2CAR or HER2CAR Tregs. Blood was monitored every 7 days (flow cytometry and CBA) and spleen engraftment was analyzed at endpoint (flow cytometry and ICS). **A**) Survival curve. **B**) Day of GVHD onset. **C**) % of weight at start of experiment. **D**) GVHD score. **E**) Engraftment in numbers and proportion of PBMCs (HLA-A2$^+$) and **F**) Treg (HLA-A2$^-$) .G) Representative plots and summary data for proportions and per-cell expression of FOXP3 and CAR in blood of A2-CAR (red) and HER2-CAR (blue) mice. Data are expressed at the ± SEM

## 4.7 Discussion

In this study, I show that the specificity of human Tregs can be redirected towards a transplant-relevant antigen using a CAR. Expression of an HLA-A2 specific CAR in Tregs enabled antigen-specific activation and proliferation that was stronger than that stimulated by the endogenous TCR. Despite strong CAR-mediated activation and/or expansion, A2-CAR Tregs retained high expression of FOXP3 and other Treg markers, demethylation of the TSDR, and had preserved suppression function *in vitro*. Adoptive transfer experiments revealed that A2-CAR Tregs which received *in vivo* CAR stimulation were significantly better than Tregs which received stimulation through the endogenous TCR at preventing xeno-GVHD. Collectively these data shown that human Tregs redirected to recognise a specific alloantigen are more effective at preventing GVHD than Tregs not thus directed, supporting the rationale to use CAR modified Tregs in the clinic.

Proof-of-concept work in mouse models demonstrated the feasibility of re-directing Tregs with a chimeric antigen receptor. A TNP-specific chimeric receptor with a CD28 transmembrane domain and the FCyR signaling domains was protective against TNP induced colitis whether expressed in T cells derived from a transgenic mouse line [148] or by retroviral transduction [149]. However, these papers leave some questions unanswered: the authors claim that antigen stimulation is sufficient to expand chimeric Tregs, however, in thymidine-based suppression assays chimeric Tregs alone do not appear to proliferate when pulsed with antigen. Additionally, antigen-specific stimulation is not compared to poly-clonal Treg stimulation but rather unactivated WT Tregs. Lastly, little Treg phenotyping was done beyond looking at FOXP3 and cytokines. Given the conflicting data and the dissimilarity of their constructs with

current CARs, the most important observation from their work appears to be that chimeric receptors result in homing to antigen *in vivo*. Antigen-driven homing was observed in another mouse CAR, where MOG-CAR cells trafficked to the CNS and reduced EAE symptoms and inflammatory cytokines [150]. Although this model used a CAR that was comparable to the one used here (i.e. contained both CD3γ and CD28 cytoplasmic domains), the Tregs were generated via FOXP3 transduction and thus the limited phenotyping done is not comparable to *ex vivo* human Tregs.

The structure of my CAR and the cell population studied is directly comparable to work done with tumor-antigen CEA CAR Tregs. The CEA CAR contains signalling domains from CD3γ and CD28, as with my CAR, and initiated proliferation in murine Tregs, as I observed in my human cells[151]. Convincing suppression *in vitro* and CEA-CAR Treg efficacy at both high and low doses is also similar to my data. However, the role of endogenous TCR stimulation was not addressed in this paper as Tregs and Tconv are both activated through their CAR, thus making it impossible to compare CAR-Treg activation to normal Treg activity. Additionally, although CEA is a human antigen, it is associated with colon cancer and thus not a desirable antigen to target in human Treg trials. Finally, the CEA CAR has been tested in human $CD25^+$ cells [231] but as expected with bead sorted Tregs, FOXP3 purity is low and no other Treg molecules are checked. Although these cells expressed IL-10 after CAR ligation they also expressed IFN-γ, which is strongly suggestive of memory or Tr1 cell contamination. Thus, my work overcomes two major challenges before CAR Tregs can be considered for the clinic: the A2-CAR represents the first human CAR that targets an antigen useful for inducing suppressive activity; and I have shown that pure, FACS-sorted human Tregs retain phenotypic and functional characteristics despite CAR expression and activation.

Tregs are known to have distinct intracellular signaling pathways, with a specific requirement for low levels of PI3K activity to retain high FOXP3 expression [239]. The A2-CAR used here uses a second-generation format with domains from CD28 and CD3ζ, both of which are necessary for Treg activation [240], suppressive capacity [241, 242], and homeostasis [243, 244]. There is evidence that unrestrained TCR signaling may be deleterious for Treg phenotype and function [91, 245, 246]. My data show that combined signaling via CD28 and CD3ζ breaks the classic anergic phenotype of Tregs in the absence of exogenous IL-2, but without compromising expression of FOXP3 or lineage stability, as evidenced by stable demethylation of the TSDR. Importantly, even following A2-CAR mediated expansion for two weeks, A2-CAR Tregs retained their expected phenotype and function, demonstrating that long-term exposure to the antigen does not compromise lineage stability. This is particularly important given the high expression levels of my CAR and the unavoidable co-stimulation of the CAR and TCR in an allogeneic setting. However, A2-CAR Tregs maintained their suppressive capacity *in vitro* after dual stimulation, and *in vivo* when injected into mice with allogeneic PBMCs. Thus CAR stimulation imparts sufficient signals for Treg activation and proliferation without destabilizing Treg phenotype and function.

An important finding in my work is that although CAR expression confers antigen specificity, it does not abrogate the function of the endogenous TCR. This is observable in my data, where TCR stimulation pre-, post- and concomitantly with CAR stimulation results in normal suppressive activity. Some groups have attempted to harness TCR specificity to create T cells with bi-specificity against both tumor and viral antigens [247-250] [251]. In all cases, transduction of virus-specific cells with a CAR allowed T cells to lyse both CAR-targeted tumor cells and virus-infected cells. Dual functionality is consistent with my assays, where CAR and TCR

stimulation at the same time does not affect suppression. Although the TCR specificity of my Tregs has not been investigated, it is likely that they are against self-antigens because I have sorted on the naïve, thymically-derived Treg population[252]. In an allogeneic HSCT setting, antigen-independent activation of the TCR by mismatched HLA may increase the proportion of cells activated by their TCR [145, 253]; indeed, this is counted on as the source of Treg activation in polyclonal infusions. However, I do not see any interference from CAR stimulation on TCR mediated suppressive activity, thus there should not be increased risks of undesirable immunosuppression from CAR stimulation over polyclonal Treg infusion.

# Chapter 5: Optimizing the structure of CARs for function in Tregs

## 5.1 Introduction

T cell receptor (TCR) signaling is a fundamental requirement for the activation and proliferation of T cells. Each $CD4^+$ T cell has a unique TCR that is generated in the thymus, allowing T cells to recognize a wide variety of antigens when they are presented in class II HLA on the surface of antigen presenting cells (APCs). Because TCR activation is critical, it has been termed "signal 1" of the required signals for T cell activation[254]. However, TCR engagement alone is insufficient to drive T cell activity. Given the resources consumed by adaptive immune responses and the potential for pathogenicity, it is not surprising that additional signals are required to overcome the anergy induced by TCR activation alone[6]. Co-stimulatory ligands, expressed by APCs under inflammatory conditions, interact with co-stimulatory molecules expressed by T cells and provide the "signal 2" required for activation of T cells. Co-stimulation can lower the threshold necessary for TCR activation, upregulate survival genes, and increase proliferation. Because a large number of co-stimulatory molecules have been described, it is likely that they have divergent as well as overlapping effects on T cell biology. For example, ICOS can provide proliferation and survival signals in the absence of CD28, however, CD28 ligation blocks Th17 development whereas ICOS ligation is permissive [255]. How co-stimulatory molecules alter T cell responses, on their own and in combination, is an active area of study with implications for cancer and autoimmunity.

The importance of co-stimulation is also apparent from work to engineer tumor antigen-specific T cells with chimeric antigen receptors (CARs). First-generation CARs attempted to

drive antigen-specific T cell activity by directly linking an antigen-recognition domain to the intracellular portion of the CD3ζ chain of the TCR. Although these constructs were able to drive some T cell activity, they had largely disappointing results when used to treat patients[146]. Addition of a CD28 domain to CARs, termed a "second generation", resulted in increased IL-2 production from Jurkat cell lines [159]and primary T cells, and increased proliferation, survival and cytotoxicity of T cells [161]. Strikingly, second generation CARs containing CD28 and CD3 ζ had greater *in vivo* expansion and persistence compared to first generation CARs when used in the same patient [163]. Subsequent work on second generation CARs compared the effects of replacing CD28 with domains from other co-stimulatory molecules. For example, CARs containing 4-1BB were found to have increased *in vivo* expansion compared to CD28 containing CARs [162]. Indeed, patient studies with 4-1BB CARs seem to have better clinical responses, although at this point no direct comparison has been done so it is not clear if this is due to differences in CAR components or procedures in different centers. In fact, the lack of standardized comparisons of CAR constructs is a recognized problem in the field.

While comparisons of co-stimulatory domains in CARs are limited, there is a complete lack of information regarding optimal CAR design for Tregs. Co-stimulation is also critical for Treg activity [256] and different co-stimulatory domains appear to influence cytokine production, Treg generation, and maintenance. Therefore the domain structure of CARs may be able to skew Tregs into cells with specific phenotypes and/or function. In order to understand the optimal signals in CARs for Treg function and longevity, a series of constructs that differ only in co-stimulation must be tested in parallel. Here I describe a series of such constructs and the insights they provide for generation of future CAR constructs.

## 5.2 Known roles of co-stimulatory domains in Tregs

To determine which co-stimulatory intracellular signalling domains to test in CAR designed for use in Tregs, I selected a series of co-stimulatory receptors to test: four were derived from the TNFR superfamily (**Table 5.1**), and 4 were derived from the B7/CD28 superfamily (including CD28, **Table 5.2**). As summarized in these tables the function of all 8 co-stimulatory molecules has been studied in mouse and/or human Tregs. However, the exact intrinsic role of these domains in Tregs has not been compared, nor have they been compared in CARs. Based on the observed importance of these domains in Treg biology or co-stimulation, I selected these domains for inclusion in novel CARs in the place of CD28.

| Marker | Species | Observation |
|---|---|---|
| **4-1BB** | **Mouse** | Increases Treg proliferation [257] and viability [258] |
| | | Upregulates TGF-β, CD25, and increases expansion [257] |
| | | Stimulation increases Treg proliferation *in vitro* [259] |
| | | FOXP3 drives 4-1BB expression [17] |
| | | Upregulated on active Tregs [260] |
| | | Ligation reduces EAE and increases Treg proliferation [261] |
| | **Human** | Activated human Tregs express 4-1BB rapidly enough to sort from Tconv [262] |
| **GITR** | **Mouse** | Expressed on Tregs and necessary for suppression [260, 263] |
| | | KO mice have a defect in Treg development [264] |
| | | Depletion results in multi organ inflammation and accelerates diabetes [265] |
| | | Ligation has a positive effect on Treg suppressive capacity [266] |
| | | Nec for thymic development, ligation increases 25 [267] |
| | | Ligation expands Tregs [268] |
| | | Stimulation is required for *in vivo* Treg suppression [269] |
| | | Promotes IL-10 and Tr1-like phenotype [270] |
| | **Human** | Less mRNA in new onset diabetes children [271] |
| | | Reduced on diabetic patient Tregs [272] |
| **OX-40** | **Mouse** | Depletion removes protection of allografts [273] |
| | | Necessary for thymic development, ligation increases CD25 [267] |
| | | Ligation increases survival, proliferation, and suppression [274] |
| | | Upregulated on active and resting Tregs [260] |
| | | KO Tregs are less sensitive to IL-2 [275] |
| | | Ligation in the absence of inflammation drives Treg accumulation and protects against EAE [276] |
| **TNFRII** | **Mouse** | Protects mice against diabetes [277] due to increased proliferation, and CD25, FOXP3 upregulation [278] |
| | | TNF boosts Treg numbers and function [279] |
| | | Colitis not prevented when Tregs lack TNFRII due to FOXP3 loss [280] |
| | | KO not suppressive *in vivo* or *vitro* [280] |
| | | Treg proliferation, survival, less and rescue of wt tregs but not TNFRII ko in EAE [281] |
| | | Necessary for thymic development, ligation increases CD25 [267] |
| | **Human** | Tregs upregulate FAS, OX40, 4-1BB, survival genes, and NFkB after ligation but Treg core signature is unchanged [281] |

**Table 5. 1** *Known roles of TNFR superfamily co-stimulatory domains in Tregs*

| Marker | Species | Observation |
|---|---|---|
| **CD28** | **Mouse** | Necessary for thymic development, stimulation upregulates FOXP3, CD25, and CTLA-4 [282, 283] |
| | | Blocking/depleting CD28 accelerates transplant rejection [284] |
| | | Strong stimulation blocks iTregdue to lck interaction [285] |
| | | CD28 ligation induces iTregs through IL-2 generation [286] |
| | **Human** | Stimulation expands Tregs [56, 287] |
| **ICOS** | **Mouse** | Blockade on IL-10 producing Treg accelerates diabetes [288] |
| | | Induces IL-10 [289] |
| | | Expression on Tregs is associated with cytokine production (IL-10, IFN-γ, IL-17) but better suppressive activity in atopic dermatitis model [290] |
| | | Increases survival, IL-10, and *in vivo* suppression [291] |
| | | KO in Tregs accelerates diabetes [292] |
| | | ICOSL on pDCs promotes IL-10 from Treg [293] |
| | | Drives Tr1 development [289] |
| | | Induces IL-$10^{+}$$FOXP3^{+}$ nTregs develop in thymus [294] |
| | | Increases Treg sensitivity to IL-2 to protect from diabetes [295] |
| | **Human** | Important for CTLA-4 expression [296] |
| | | Less mRNA in new onset diabetes children [271] |
| | | TNF boosts Treg numbers and function [279] |
| | | Colitis not prevented when Tregs lack TNFRII due to FOXP3 loss[280] |
| | | KO not suppressive *in vivo* or *vitro* [280] |
| | | Treg proliferation, survival, less and rescue of wt tregs but not TNFRII ko in EAE [281] |
| | | Necessary for thymic development, ligation increases CD25 [267] |
| | **Human** | Tregs upregulate FAS, OX40, 4-1BB, survival genes, and NFkB after ligation but Treg core signature is unchanged [281] |
| **PD-1** | **Mouse** | Induction of Tregs [297] through the inhibition of PI3K via PTEN |
| | | Induction of Tregs via increased sensitivity to TGF-β [298] |
| | | Expressed on Tregs [299] |
| | | Upregulated on Tregs after TCR stimulation [300] |
| | | Blocking reduces Treg ability to protect allografts [301] |
| | | Decreases stat5 phosphorylation in Tregs [302] |
| | | Inhibits peripheral Treg proliferation [303] |
| | **Human** | Expressed on melanoma patient Tregs and blocking reduced suppress capacity *in vitro* [304] |
| **CTLA-4** | **Mouse** | High on resting and active Treg [260] |
| | | Blocking on Tregs worsens colitis [305] |
| | | Treg intrinsic role in lymphoproliferative disease [306] |
| | | Cytoplasmic tail unhibits TCR signal and limits Treg proliferation [26] |
| | | KO increases Tregs numbers but decreases function in diabetes [307] |
| | | Non-redundant role with IL-10 and TGF-β in Treg suppression [308] |
| | | Modulation of signalling important for Treg induction in gut [309] |
| | | Regulation of CD28 necessary for thymic Treg induction [310] |
| | **Human** | Less mRNA in new onset diabetes in children [271] |
| | | Germ line mutation results in increased Treg numbers but decreases suppressive function [311] |

**Table 5. 2** *Known roles of B7/CD28 superfamily co-stimulatory domains in Tregs*

## 5.3 Signaling properties of selected domains

Each of the selected molecules has been reported to interact with the PI3K pathway, which is critical for Tconv activation but distinct in Treg [240](**Figure 5.1A**). For example, PI3K activity has been found to be necessary for Treg survival [90] but loss of control of this pathway also results in loss of Treg phenotype [246 245]. Because Tregs appear to require an optimal amount of PI3K signal [239] it was important to choose molecules with a range of PI3K modulation. As the founding member of the CD28/B7 superfamily, CD28 has a well-characterized role in augmenting PI3K activity. However, family members do not necessarily activate PI3K. Conflicting results have been described for CTLA-4 [29], with both PI3K activation and inhibition described. ICOS is known to activate PI3K and PD-1 is well characterized as inhibiting this pathway. All TNFR superfamily members are also described to have a positive effect on PI3K signaling as well [312]. In addition to PI3K activation, each of these proteins has been described to activate one or more alternative signaling pathways, such as MAPK and NFkB, although their role in Tregs is not well defined.

In order to determine the amino acid sequence to include in CARs, the transmembrane and cytoplasmic domains of these proteins were determined using Uniprot [172] (**Figure 5.1B**). Although several post-translational modifications and protein-interaction motifs have been described, the precise function of each component of the cytoplasmic tails has not been defined in Tregs. Additionally, current second generation CAR constructs contain the entire cytoplasmic region. Thus, I decided to include the entire cytoplasmic domain of these co-stimulatory molecules in order to capture both the effects on PI3K and other signaling pathways that are activated by these proteins. For example, these alternative pathways are likely what drives the

tolerogenic phenotype of ICOS stimulated cells despite the positive effect of PI3K on survival and proliferation.

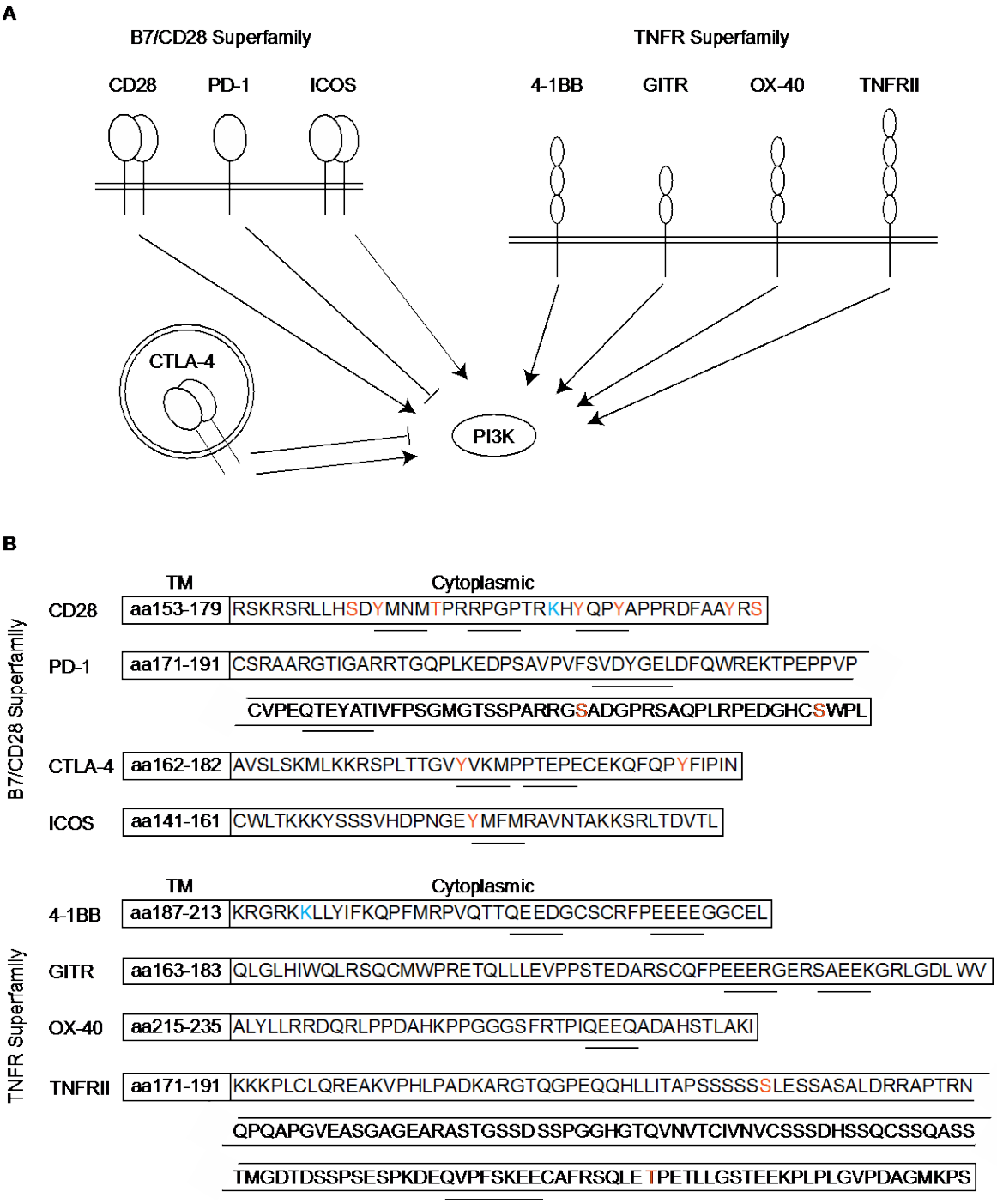


**Figure 5. 1.** *Properties of B7/CD28 and TNFR superfamily members* **A**) Influence of molecules on PI3K and other pathways involved in ligation of B7/CD28 and TNFR superfamily members.

**B**) Intracellular regions of proteins for inclusion into CARs. Phosphorylation sites are orange, and ubiquitinylation sites are blue. SH2 and SH3 protein interaction domains are underlined in B7/CD28 superfamily members and TRAF interaction motifs are underlined in TNFR superfamily members.

## 5.4 Construction and expression of TNFR superfamily CARs

Because 4-1BB and OX-40 have been successfully incorporated into previous CARs [146], I began by designing constructs containing co-stimulatory molecules from the TNFR superfamily. 4-1BB, GITR, OX-40, and TNFRII were selected based on their role in enhancing survival and proliferation (**Table 5.1**). Once designed, all CAR constructs were cloned into the lentivirus vector (**Figure 5.2A**). GITR and TNFRII have not been incorporated into previous CARs.

To test the surface expression of the new CAR constructs, all lentivirus vectors were transiently expressed in 293T cells. Surface staining with anti-myc revealed that the 4-1BB CAR and OX-40 CAR expressed efficiently on the surface of cells (**Figure 5.2B**). GITR and TNFRII, however, had minimal to no surface expression. Intracellular staining for myc revealed that these constructs were efficiently expressed within the cells, suggesting a solubility or trafficking problem, rather than a problem with the sequence of the construct.

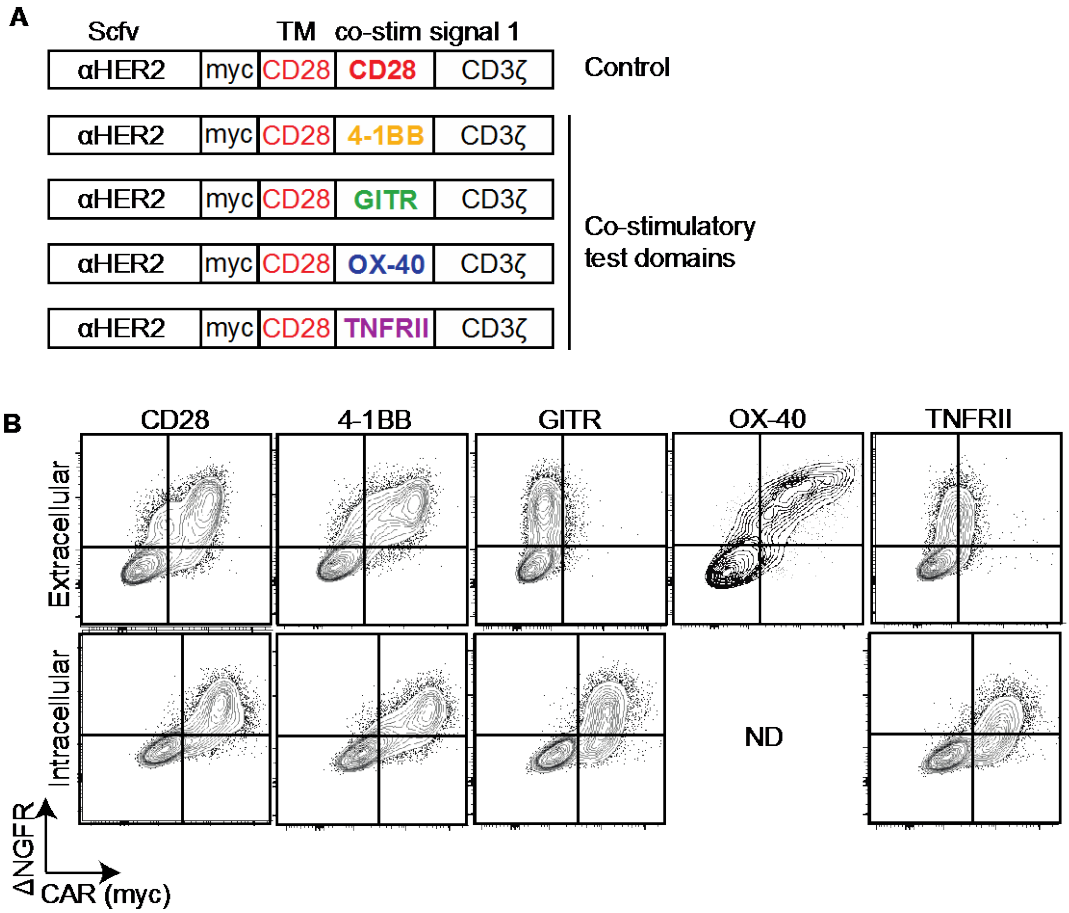


**Figure 5. 2.** *Construction and expression of TNFR superfamily CARs*. **A**) Schematic of domains in CARs (*myc*=myc tag; *ScFv*=single chain antibody; *TM*=trans-membrane). **B**) 293T cells were transfected with the control CD28-CAR, 4-1BB-CAR, GITR-CAR, OX-40-CAR and TFNRII CAR. Surface expression was confirmed by detection of the myc-epitope tag by flow cytometry. Intracellular staining was performed by myc staining after fixation and permeabilization. Gates were set on UT and ΔNGFR controls.

## 5.5 Construction and expression of B7/CD28 superfamily domains

Due to the lack of surface expression of 50% of my CARs, I decided to expand the repertoire of domains for efficient side-by-side comparison. In generating my next series of constructs, I selected several domains from the B7/CD28 superfamily known for their role in Treg biology: ICOS, for IL-10 production, PD-1 for Treg development, and CTLA-4 for TGF-β.

Additionally, because the capacity of novel CARs to traffic to the surface of the cell is unpredictable, I decided to include a second strategy for incorporating new co-stimulatory domains into a CAR. Both versions of CTLA-4 contained a point mutation, Y165G , to reduce internalization[313]. Although OX-40 and 4-1BB have been reported in functional CARs containing a CD28 transmembrane domain, more recent CARs including ICOS co-stimulation have also used the ICOS transmembrane domain [314]. Therefore, I generated a series of constructs containing either cytoplasmic domains downstream of the CD28 transmembrane sequence, or downstream on their native transmembrane domains in order to test the role of transmembrane source on CAR trafficking and solubility (**Figure 5.3A**). As with my first series of constructs, I then cloned these CARs into the pCCL lentiviral vector.

Surface expression of CAR constructs was again determined in 293T cells. The ICOS containing CAR only expressed on the cell surface when its native TM domain was used. In contrast, the CTLA-4 containing constructs did not express on the cell surface regardless of whether the native TM domain was used or not. On the other hand, PD-1 containing CARs were efficiently expressed on the cell surface with either the native or CD28 TM domain (**Figure 5.3B**).

Because CTLA-4 did not express even with its native TM domain, an additional attempt was made to rescue extracellular expression of this CAR. I considered the possibility that the choice of signal peptide may also affect cell surface expression. Specifically, the CARs tested above all contained a moderate efficiency signal peptide; it was possible that an Ig-derived signal peptide could more efficiently direct insertion of the CAR into the endoplasmic reticulum and thus better expression on the surface of the cell (i.e. since the protein has an antibody-derived ScFv at the amino terminus). The original generic signal peptide was replaced with an Igκ signal

peptide, and both constructs were expressed transiently in 293T cells. Although CAR expression was detected by intracellular staining for both constructs (data not shown), no detectable surface expression was observed even with the new signal peptide (**Figure 5.3C**).

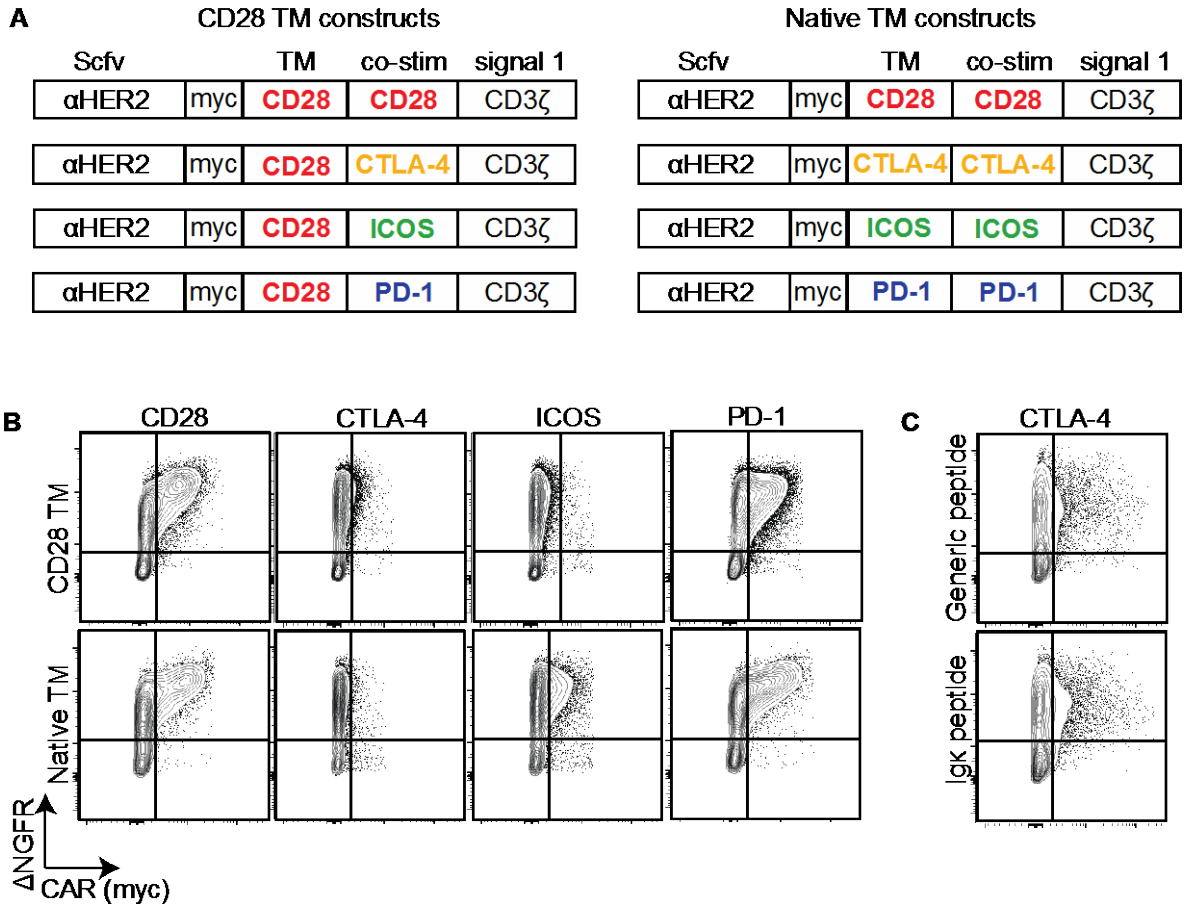


**Figure 5. 3.** *Construction and expression of B7/CD28 superfamily CARs*. **A**) Schematic of domains in CARs (*myc*=myc tag; *ScFv*=single chain antibody; *TM*=trans-membrane). **B**) 293T cells were transfected with the control CD28-CAR, CTLA-4-CAR, ICOS-CAR, and PD-1-CAR either with CD28 TM (above) or native TM (below). Surface expression was confirmed by detection of the myc-epitope tag by flow cytometry. Gates were set on UT and ΔNGFR controls. **C**) Surface expression of CTLA-4-CAR with a generic (above) or Igκ signal peptide (below).

## 5.6 Discussion

While the importance of including co-stimulatory domains in CARs to provide a signal 2 is well established, the biological effects and clinical implications of current CAR variations are not clear. Here I describe the creation of a variety of new CAR constructs that vary in only two components: co-stimulatory domains and transmembrane domains (as necessary for expression). These tools set the stage for a comprehensive study to define how the inclusion of different co-stimulatory domains affects the biology and function of CARs in Tregs.

To date knowledge about how CARs with co-stimulatory domains other than CD28 work is restricted to effector T cells and largely focused on 4-1BB. Use of 4-1BB in CARs was found to be superior to CD28 at inducing IL-2, killing, proliferation, and survival *in vitro* [315]. Similarly, 4-1BB CARs mediated more potent *in vivo* tumor killing in mouse models than CD28 [316]. This superior efficacy of 4-1BB for achieving T cell activity and persistence appears to bear out in clinical trials: of the CARs tested in B cell malignancies, the 4-1BB containing CAR appears to have the most promising clinical results [146]. However, some success has been achieved with CD28 containing CARs[234]. Importantly, many variations exist between clinical trials, including other aspects of these constructs and treatment regimen, and *in vitro* CAR studies making it difficult to directly compare data.

Recent work suggests that modifying co-stimulatory domains can be used as a strategy to tailor the function of T cells. For example, CARs containing ICOS stimulate T cell activation and proliferation while also skewing to a TH17 phenotype, suggesting a substantial impact of co-stimulation on CAR T cell biology [314]. PD-1 and CTLA-4 CARs have also been described as having an inhibitory effect on cytotoxicity, however, these CARs did not contain CD3ζ and were

not tested in Tregs. GITR and TBFRII containing CARs have not been described. Thus, there is a need to further study the biological effects of different co-stimulatory domains in CARs, and this is most readily achieved when similar constructs are compared side-by-side. It is also important to consider that the effects of co-stimulatory molecules may be different in Tregs than in Tconv. For example, on a Treg epigenetic background, ICOS may induce IL-10 instead of IL-17.

A major limitation in the generation of novel CARs is the lack of surface expression. By generating a number of constructs in parallel, here I am able to draw some conclusions about factors that may be affecting solubility of the CARs. One possibility is that larger domains, such as that in TNFRII may reduce expression. However, the good intracellular expression of myc in all cases indicates that CARs are expressed regardless of size, and not all small domains were expressed on the surface. Another possibility is that an inappropriate trafficking signal results in arrest of the CAR in the cytoplasm. But when I attempted to rescue CTLA4-CAR expression with a more efficient signal peptide, from Igκ, this was insufficient to induce surface expression, suggesting that inefficient insertion into the endoplasmic reticulum was not the problem.

Another factor that could contribute to cell-surface expression is the native biology of the components included in the CAR. For example, CTLA-4 is normally rapidly endocytosed after surface expression. Indeed, I used the point mutation Y165G to specifically attempt to abrogate internalization[313] but this was not sufficient to induce surface expression, suggesting that other factors outweigh the impact of the expression characteristics of certain domains. Altogether, this indicates that poor surface expression of CARs is determined by unfavorable intra- or inter-molecular interactions in the cell. Importantly, these interactions are difficult to predict but rescuing surface expression is possible in some cases by including the native TM domain of the

co-stimulatory molecule, as with ICOS. However, and ICOS containing CAR with a CD8 transmembrane domain but different antigen specificity expresses on the surface of cells [317]. Thus, it is likely that new intra- and inter- molecular interactions are being created along with every new combination of domains in CARs, which are impossible to predict until expression is tested.

# Chapter 6: Conclusion

## 6.1 Significance and considerations of Th2-Tregs in SSc

In chapter 3 of my thesis, I aimed to describe a novel aspect of Treg plasticity. To do this, I examined Tregs in the blood and skin of SSc patients because this is a Th2-driven disease. I observed Th2 homing marker expression in the blood of patients and Th2 cytokine expression in the skin. Thus I have demonstrated that human Tregs can convert to a Th2-like phenotype, which has not been previously described in humans. Finally, I observed that IL-33 is more highly expressed in the skin of SSc patients, and that Tregs express the receptor for this Th2-polarizing cytokine and can convert to IL-13 producers in healthy skin when exposed to IL-33. Treg plasticity in response to IL-33 could be a novel mechanism for loss of tolerance.

Healthy humans have a diversity of homing markers on Tregs, which is thought to allow Tregs to suppress specific Th subsets by mirroring them [95]. Tregs particularly seem to share the Th2 homing marker CCR4: this allows trafficking to the skin [318] and within the skin [319] to control inflammation. Nearly all Tregs in human blood express the skin homing molecules CCR4 and CLA, both of which are functional and do not affect suppression, but underlines the importance of Treg migration to the skin in humans [320]. Indeed, an increased proportion of $CLA^+$ Tregs results in better protection of skin allografts [321]and reduced skin GVHD after HSCT [322]. Thus under normal conditions Tregs appropriate Th2 homing markers to maintain immune homeostasis. However, I found the expression of Th2 homing markers was significantly increased in the blood of SSc patients, both in bulk Tregs and those homing specifically to the skin. Conversely, Th1- and Th17 homing markers were significantly downregulated on SSc blood Tregs. Despite this enrichment for Th2-homing markers, there was no increase in cytokine

production by circulating Tregs, suggesting that while these cells are primed, full functional specialization occurs in the skin. One experiment to test the polarization of Tregs in SSc skin could be to transplant patient biopsies on to NSG mice, then observe the cytokine profile of immune cells that enter circulation. If cytokine production is potentiated in skin, the Tregs that leave the skin may be enriched for cytokine production. Alternatively, labelled, healthy peripheral blood Tregs may be injected into NSG mice bearing SSc skin grafts after the mice have been treated with anti-CD52 antibody to deplete circulating cells derived from the graft. The labelled Tregs can then be analyzed for cytokine production by immunofluorescence.

Once Tregs migrate to the skin from blood they encounter new signals and adopt a skin Treg phenotype and maintain immune homeostasis in the tissue[318]. For example, skin-derived $FOXP3^+$ cells in mice make IFN-γ and IL-10 in response to antigen presented by dermal DCs and their depletion worsens inflammation [323]. Interestingly, Tregs in an atopic dermatitis model were less suppressive when isolated from skin-draining lymph nodes than Tregs that had not trafficked to the skin, but their depletion in this model also increased inflammation[324]. Data from human studies support the notion that Tregs traffic to the skin, where they maintain a core suppressive program but are also more heterogeneous than circulating Tregs. Once in the skin, human Tregs remain $FOXP3^+$ and have other Treg phenotypic markers and can expand to limit inflammation during antigen recall [221].

Under homeostatic conditions, human skin Tregs are suppressive but also proliferate in an antigen-specific manner due to stimulation from Langerhans cells [222]. When isolated from a skin-matrix culture system such as ours, Tregs from normal human skin have typical markers such as FOXP3 and suppress in a contact dependent manner [200]. The literature is conflicting in regards to the quantity of Tregs in inflamed skin during human disease. In some cases $FOXP3^+$

cells in the skin are found to be numerically equivalent between healthy controls and patients [325], [326]. More frequently, Tregs are found to be increased in the affected skin in psoriasis [327], [328], [329]and SSc [202], or decreased in lupus [330]and SSc [193]. One strength of my culture system compared to these previous studies is the use of flow cytometry, which allows the acquisition of tens of thousands of events resulting in a more robust quantification than by histology or immunofluorescence. I did not find a numerical defect in Treg skin, suggesting that uncontrolled inflammation in SSc is not a problem of Treg quantity but rather one of Treg quality. This could be tested by cloning Tregs from SSc skin biopsies and comparing the suppressive capacities of IL-13$^+$ and IL-13$^-$ clones.

Mouse models have implied that Tregs have an innate ability to convert to a Th phenotype under certain inflammatory conditions and contribute to disease if they encounter a signal that is strong enough to upregulate Th2 markers even while remaining FOXP3$^+$ Tregs, resulting in Th2-like or "plastic" Treg. This was found to be the case in a mouse model of asthma, where RSV was able to induce both GATA-3 and IL-13 in FOXP3$^+$ Tregs and decrease their suppressive ability [39]. Upregulation of GATA-3 through deletion of itch in Tregs also leads to IL-4 production by FOXP3$^+$ cells, loss of suppressive ability and even the induction of Th2 cells [331]. This is consistent with my data: FOXP3$^+$ cells, which do not express cytokines in the blood, express Th2 cytokines in SSc skin. High level of FOXP3 and IL-4/IL-13 expression in the absence of TCR stimulation is consistent with a Th2-like Treg phenotype. Indeed, SSc FOXP3$^+$ cells do not produce cytokines in the blood, which is consistent with a Treg phenotype and similar to healthy donor FOXP3$^+$ cells. However, these FOXP3$^+$ cells in the blood are also consistent with a Th2-like Tregs phenotype based on their chemokine receptor expression. Notably, these Th2-like Tregs are enriched within the skin-homing subset. Whether these cells

have an intrinsic Th2 bias, have previously received an extrinsic Th2 signal, or both is unknown; it is clear, though, that these FOXP3$^+$ Tregs must traffic to the skin to receive the final signal to induce them to fully trans-differentiate to a Th2 cytokine producing phenotype, and this phenomenon appears to be specific to scleroderma. This is consistent with other autoimmune diseases where Treg plasticity with Th1 and Th17 lineages has been implicated [198, 199]. To my knowledge, this is the first description of Th2-like, cytokine producing Tregs in human disease. An interesting avenue of research may be to isolate Tregs from the periphery and tissue of patients with other Th2-mediated diseases. For example, bronchiolar lavage from asthma patients may have Th2-like Tregs either at the cytokine or transcription factor level, as suggested by previous mouse models.

The striking increase in Th2-cytokine producing cells in the skin of SSc patients but not the blood begs the question: what factors drive FOXP3$^+$ cells in the skin to produce IL-4 and IL-13? Addition of IL-4 to Tregs or skin cultures was insufficient to induce Tregs to produce Th2 cytokines. IL-33, a more recently described Th2-inducing cytokine [332, 333], is associated with fibrosis [334, 335 223], primarily mediated by IL-13[223]. Furthermore, IL-33 has been found in the serum of early SSc patients and correlates with fibrosis [211, 212]. Our data shows an increase in IL-13 but not IL-4 from FOXP3$^+$ Tregs when skin cultures are treated with IL-33. Interestingly, mouse IL-33 promotes allograft survival and prevents colitis through Th2 and Treg expansion [336-338]) by directly acting on ST2$^+$ Tregs [339]. Similar findings have not been described for humans, and we were unable to find any direct effect of IL-33 on sorted Tregs. IL-33 may be acting indirectly on skin Tregs through other cell types: IL-33 acts though ST2 on mast cells, basophils, eosinophils, dendritic cells, macrophages and Th2 cells to stimulate the release of IL-4, 5, 6, 13, and CCL2 [340-342]. Both IL-33 and ST2 have been found to be upregulated in SSc

fibroblasts and a broad range of skin immune cells [214], suggesting that IL-33 acts indirectly on skin Tregs in SSc. We confirmed that IL-33 and T cells are in proximity in SSc skin by immunofluorescence. Additionally, we found that SSc fibroblasts produce IL-33 after inflammatory cytokine activation, as has previously been described for healthy dermal fibroblasts [333]. Importantly, Tregs in the skin of both control biopsies and SSc biopsies express the IL-33 receptor, suggesting that IL-33 could have a direct role on Tregs. Levels of ST2 on circulating Tregs were also higher in SSc, further supporting their "primed" state to respond to cytokines when they enter the skin. Taken together, it appears that IL-33 may play a role in the progression of fibrosis in SSc through the Th2 polarization of skin Tregs. Future work to determine the precise effects of IL-33 on Tregs will need to be performed. For example, cell types present in the skin may contribute to full Treg polarization in the presence of IL-33, including DCs. Peripheral blood DCs may be generated and treated with IL-33 to test if healthy peripheral blood Tregs can become IL-33 producers. If ST2 can be induced in Tregs, the phenotypic and functional characteristics of Th2 Tregs can be examined in more detail, i.e. mRNA analysis of lineage defining transcription factors and suppression assays. Tregs sorted from SSc peripheral blood may also be treated with IL-33 in the presence of APCs and assayed as with healthy Tregs. If Th2 Tregs cannot be generated from peripheral blood, large sections of skin may be obtained from plastic surgery patients, from which the sorting of skin Tregs and APCs may be optimized. Isolation of these cell types would allow further analysis of their contribution to the polarization of Th2-like Tregs.

Chapter 3 of my thesis provides several important considerations for future work. First, this work confirms the role of Th2 cytokines in driving SSc, and reinforces the need to consider this Th subset in other fibrotic disorders. Also, the difference in Th2 chemokine vs cytokine

expression in the skin and blood highlights the importance of looking at different phenotypic markers in different tissue compartments in order to fully capture the diversity of Treg subtypes. Future studies should consider the source of Tregs to determine which markers will be most informative. Importantly, I have described a pathogenic role for ST2 and IL-33 expression in humans. IL-33 has a positive effect on mouse Tregs and is used to prevent harmful inflammation in mouse trials, thus it is important to describe a potential negative role for this cytokine. Additionally, the immunophenotyping described in my work may have prognostic value, which further studies could elucidate. Altogether, my thesis work has provided important insights into SSc and potential Treg plasticity.

## 6.2 Significance and considerations of A2-CAR Tregs

In chapter 4 of my thesis, I set out to create antigen specific Tregs that would be more effective for cell therapy. My goal was to create a chimeric antigen receptor to specifically activate Tregs in the presence of allogeneic cells. Given the current limitations of Treg cell numbers, this work sought to determine whether CAR stimulation can efficiently activate Tregs and drive expansion and proliferation without destabilizing Tregs. Both *in vitro* and *in vivo* work was carried out to determine the utility of CAR technology in Tregs.

In humans, clinically-applicable approaches to generate alloantigen-specific Tregs are currently limited to *in vitro* enrichment of alloreactive T cells following stimulation with allogeneic antigen presenting cells (APCs). For example, alloantigen-specific Tregs can be sorted on the basis of alloAPC-stimulated expression of CD69 and CD71[50], or expanded with CD40-stimulated allogeneic B cells[145]. Cells generated via these methods are more potently suppressive *in vitro* than polyclonal Tregs, and protect skin allografts better than polyclonal

Tregs. However, with both these approaches cell numbers may be limiting in the context of HSCT, as the frequency of alloantigen-specific cells decreases with increasing MHC-matching and indeed may not be feasible at all given the poor ability to detect alloantigen-specific responses in the HLA-matched setting[145, 253]. Tregs generated by these methods must be isolated and cultured in advance of transplant in order to obtain sufficient numbers and potency, but this process requires multiple rounds of sorting and prior knowledge of who the donor is. CAR transduction overcomes both of these problems, as A2-CAR Tregs are robustly reactive to allogeneic donors based on a single HLA mismatch and does not require access to donor tissue. An additional consideration is that extensive *in vitro* expansion may lead to loss of FOXP3 in Tregs[343] and, based on data from $CD8^+$ T cells, decreased telomere length and hence *in vivo* survival [344]. The capacity of A2-CAR Tregs to expand *in vivo*, as observed in the blood of mice, suggests that the *in vitro* culture period of these cells may be reduced to increase the *in vivo* longevity of Tregs. This could be determined by injecting HLA-A2 transgenic mice with A2-CARTregs and monitoring their expansion and phenotype over time, and if *in vivo* expansion is successful, decreasing numbers of A2-CAR Tregs may be injected at earlier time points. Decreasing manipulation and converting A2-CAR Treg generation to good manufacturing practices will require additional study.

Long term persistence may be key for Treg cell therapy, since depletion of Tregs results in a return of inflammation in tolerized mice [74]. In human trials, Treg numbers are increased within 14 days of infusion of autologous cells [63]. Allogeneic cells can be tracked by flow cytometry, and were observed in GVHD for up to 14 days, with a peak at 2 or 4 days after infusion[66]. In CAR T cell therapies, transferred cells are detectable in the blood for several weeks [345] with an average of 30 days [346]. An additional advantage of CAR therapy is the ability to track

transduced T cells by flow cytometry and/or RT-PCR. The sensitivity of PCR is much greater than flow cytometry and revealed persistence of CAR T cells in patients up to three years after infusion [77]. Although I observed A2-CAR Tregs *in vivo* for twice as long as polyclonal Tregs, they were not detectable by flow cytometry after two weeks. Several factors may have affected the *in vivo* persistence of these cells. First, it is possible that A2-CAR Tregs traffic into tissues and out of the blood. Alternatively, PBMC engraftment, which was potently inhibited by A2-CAR Tregs, may be required to prolong Treg persistence: the lack of circulating PBMCs may have reduced HLA-A2 antigen levels below the threshold required for Treg proliferation and survival signals. Similarly, a reduction in PBMC numbers also would result in no IL-2 production, which is likely required for long-term *in vitro* survival of Tregs, regardless of CAR stimulation. Lastly, it is possible that these cells simply had a limited life span after *in vitro* expansion as observed in cancer immunotherapy[344]. Strategies for supporting Treg survival post-transfer are currently being studied and will be an important area of study as Tregs cell therapy is expanded in the clinic. For example, exogenous IL-2 injections, use of immunosuppressive drugs like sirolimus that target signaling pathways already dampened in Tregs, or depletion of auto-reactive cells to create a niche for Tregs, could enhance Treg engraftment during Treg cell therapy [347].

Of critical importance for extending the use of CARs to Tregs is the functional ability of CAR-stimulated Tregs: do these cells remain suppressive? And how do they suppress? Thus far, preliminary mouse work is promising[148-150], as is one study on relatively impure $CD25^+$ Tregs[231]. Tregs use multiple mechanisms to suppress inflammatory responses [348]. These include, but are not limited to: reduction in co-stimulation by DCs via CTLA-4; competition for the T cell growth-factor cytokine IL-2 via CD25; and expression of anti-inflammatory cytokines such as

IL-10 and TGF-β. CAR Tregs generated via my protocol express and retain high levels of CTLA-4 and CD25. Interestingly, CTLA-4 expression and IL-2 repression appear to be sufficient in re-capitulating Treg function [349]. Although an immediate reduction in inflammation is a critical part of Treg activity, the conversion of inflammatory cells to an anti-inflammatory phenotype is necessary for long-term tolerance. This conversion, termed infectious tolerance, is partially mediated by anti-inflammatory cytokines [350]. Although minimal levels of IL-10 were detected after stimulation with HLA-A2, CAR Tregs upregulate high levels of LAP and GARP, two surface bound proteins that hold and prime membrane-bound TGF-β for release [351, 352]. Altogether, it appears that CAR Tregs are able to suppress through a variety of mechanisms. Given that the CAR we have generated reacts to allo-antigen, it is challenging to assess CAR stimulation alone when targeting HLA in a human system with TCR sufficient cells. However, CAR Tregs retain suppressive capacity when stimulated through both their CAR and TCR *in vitro* and *in vivo*, or when stimulated through their TCR after CAR expansion *in vitro*. It is possible that in addition to TGF-β production and killing, CAR Tregs may be more potent than polyclonal Tregs simply because high levels of proliferation result in much larger numbers of Tregs after CAR stimulation. Future work to determine Treg mechanisms of suppression will include transwell suppression assays to determine if contact is required for suppression. If suppression is at least partially contact dependent, suppression assays will be performed with DCs to determine if A2-CAR activation causes Tregs to downregulate co-stimulatory molecules on APCs, such as CD80 and CD86, as is suggested by CTLA-4 expression. Suppression assays may also be performed while blocking CTLA-4 or membrane-bound TGFβ- to determine the contribution of these molecules. Cytotoxicity may also be assessed as a mechanism of suppression by determining the level of caspase-3 activation in HLA-$A2^+$ cells co-cultured with

A2-CAR Tregs. If suppression is at least partially contact independent, suppression assays may be performed with blocking antibodies for Treg suppressive cytokines, such as soluble TGF-β and IL-10, or by supplementing with pro-inflammatory factors removed by Tregs, such as ATP and IL-2. Additionally, Treg functional markers, such as CTLA-4, CD25, CD39 and others, should be assayed at the end of suppression assays to confirm that these molecules are upregulated after CAR stimulation. Regardless of mechanism, here I have demonstrated the feasibility of re-directing Tregs to a transplant-relevant antigen with a chimeric antigen receptor, and shown that CAR Tregs remain functionally and phenotypically stable *in vitro* and *in vivo*.

An outstanding question from our model of xenoGVHD is the effect of A2-CAR Tregs on the graft-versus-leukemia, or GVL effects. When HSCT is performed for hematological malignancies Tconv in the graft are important for killing malignant cells but they also drive GVHD. While a reduction in GVHD is desirable, a loss of GVL would increase the risk of lethal disease recurrence and thus new strategies that prevent GVHD while preserving GVL are highly desirable. Treg cell therapy is an attractive option for HSCT patients, as the immunosuppressive activity of Tregs appears to limit GVHD without affecting GVL in mouse models[46, 58, 353]. Similarly, humanized mouse models show successful treatment of GVHD by Tregs while Tconv remained able to clear leukemic cell lines[354]. Importantly, no increase in leukemia relapse has been observed in human Treg trials for GVHD[65, 66]. Currently no research exists on the effect of antigen-specific Tregs on GVL. One intriguing possibility with A2-CAR Tregs is that they may provide potent GVHD protection in HLA-$A2^+$ recipients without abrogating GVL due to down regulation of HLA-A2 by cancer cells[355]. However, there was a significant reduction in engraftment of HLA-$A2^+$ PBMCs in our GVHD model, suggesting that loss of GVL is a possibility. Reduced engraftment is likely due to excessive Treg activity because PBMC

engraftment is also reduced by polyclonal Tregs. Experiments should be performed to determine the outcomes of this delayed engraftment and whether the effects could be mitigated. For example, GVHD experiments should be carried out in mice engrafted with a leukemic cell line to determine whether reduced PBMC engraftment by A2-CAR Tregs is reducing GVL activity. A2-CAR Tregs may also be titrated down to lower numbers to achieve the same engraftment as polyclonal Tregs so that their ability to prevent GVHD without GVL loss can be directly compared. The potential effects of CAR Tregs on GVL will need to be examined in future studies.

Here I have demonstrated the feasibility of re-directing Tregs to a transplant-relevant antigen with a chimeric antigen receptor, and shown that CAR Tregs remain functionally and phenotypically stable *in vitro* and *in vivo*. This work provides proof-of-concept that CAR Tregs may be effective in treating GVHD and may provide an exciting new clinical option for Treg cell therapy. Additionally, the A2-CAR could be used in Treg cell therapy for any transplant where an HLA-A2 mismatch occurs. Outstanding questions do remain: what is the mechanism of CAR-Treg suppression? And will CAR-Tregs be useful in GVHD, where the GVL effect must be preserved? Future work will be performed to address these issues. Overall, this work supports the use of CARs to create antigen specific Tregs to increase the potency of cell therapy.

### 6.3 Significance and considerations of co-stimulatory domain swapping in CARs

Chapter 5 of my thesis describes the conception, design, and generation of multiple CAR constructs in order to better understand the role of co-stimulation in optimal Treg CAR design. I found that CAR expression is highly variable depending on different factors: some co-stimulatory domains require their native TM, whereas others appear to express regardless of the

TM. Some constructs did not express on the cell surface even after switching out multiple components, most notably CTLA-4. In addition to bringing valuable insights to CAR design, these constructs will be used in future experiments to directly compare the influence of unique signaling and TM domains. This will be very useful to the field, since standardization is currently lacking and clinical results with CARs are variable. Finally, the optimal signalling domain to reinforce Treg phenotype can now be studied. The work presented in my thesis represents a critical step forward in optimizing CAR Tregs for the clinic.

Although the effects of co-stimulatory domains in CARs on Treg phenotype are unknown, we can expect and look for certain aspects of Treg biology based on previous work. Co-stimulatory domains are known to provide positive signals for T cell survival. Although early work suggested that co-stimulatory ligation reduces Treg suppressive capacity this was likely due to the pro-survival effects on Tconv and not intrinsic negative signals in Tregs, since knockout mouse models have shown the importance of these molecules in Tregs [356]. In fact, co-stimulation seems to have similar beneficial effects for Tregs as Tconv. 4-1BB is known to increase Treg proliferation [257], and stimulating 4-1BB or OX-40 on Tregs increases both their proliferation and viability [258]. Depletion of OX-40 expressing Tregs ablates their ability to protect allografts [273]. Similarly, work suggesting that TNF-α boosts Treg numbers to reduce the rate of diabetes seemed contradictory[277], since TNF-α is a pro-inflammatory cytokine. However, TNFRII is highly expressed on Tregs and ligation along with TCR stimulation results in Treg proliferation and upregulation of FOXP3 and CD25[278]. Of the co-stimulatory molecules chosen for analysis here, only GITR has had a long-appreciated role in Treg function [357]. GITR knockout mice were observed to have a defect in Treg development [264]and GITR ligation has a positive effect on Treg suppressive capacity [266]. Given the biology of these co-stimulatory

domains, it is desirable to see how they can promote Treg viability and longevity in the context of CAR stimulation. However, only two of these domains expressed on the surface of cell lines: OX-40 and 4-1BB. These domains have been described in previous CARs so it is not surprising that they expressed in my system. GITR and TNFRII, on the other hand, have not been previously described and did not express on the surface of cells. The reasons for this are unknown.

In addition to co-stimulation, CARs that promote an anergic or anti-inflammatory cell type may help to reinforce Treg phenotype and make more stable cells for Treg cell therapy. In fact, previous work has shown that CTLA-4 and PD-1 in the context of a CAR can provide an inhibitory signal to Tconv [289]. Tregs are known to express high levels of CTLA-4, which is necessary for their suppressive abilities. However, most of the CTLA-4 biology has been focused on its extracellular role as a competitive inhibitor of CD28[6]. CTLA-4 is likely to have cell intrinsic signaling, since some studies have shown positive and negative effects of CTLA-4 on the PI3K pathway. However, it has also been implicated in cell motility. Studies have yet to reveal the cell-intrinsic role of CTLA-4 for Tregs. The intrinsic signaling of PD-1 is better understood. PD-1 is upregulated by cancer cells in order to provide anergic signals to the attacking T cell. Importantly, this results in induction of Tregs [297] through the downregulation of the PI3K pathways through induction of the phosphatase PTEN[298]. Thus, a PD-1 containing CAR may strongly reinforce a Treg phenotype and prevent conversion to an inflammatory phenotype. Finally, ICOS, although co-stimulatory in the induction for survival and proliferation genes, may play a role in reinforcing Treg phenotype as well. ICOS has been reported to induce IL-10 production, which is a potent inhibitor of inflammation and important for infectious tolerance. However, stimulation via an ICOS containing CAR can result in Th17 cytokine

production [314]. Although the effects of these domains on Treg phenotype has yet to be tested, their role in reinforcing Treg phenotype may be useful for long-term tolerance in cell therapy, or they may induce particular aspects that are useful for disease-specific therapy. For example, if the ICOS-CAR promotes IL-10 production, this construct may be most useful for inflammatory bowel disease because of the important role for IL-10 in gut tolerance [137, 358].

There are two major questions to address based on my work generating CARs with different co-receptor domains. First, further work needs to be done to explore the contribution of various domains to successful CAR expression on the surface of the cell. This could include swapping out the HER2 ScFv for the anti-HLA-A2 ScFv to determine whether the single chain can rescue surface expression. Non-expressing constructs could also be observed using fluorescence microscopy to determine where the constructs are trafficking to by staining for both the CAR and various organelles. If the factors that reduce surface expression can be identified, future CAR design may be more successful. The second major question to address is the biological differences induced by the unique signalling of each co-stimulatory and co-inhibitory domain. The next steps to do this would be to transduce Treg and Tconv cells with expressed constructs and perform phenotypic assays: 1) proliferation assays after membrane labelling and CAR ligation; 2) phosphoflow for pAkt in the PI3k pathway after myc crosslinking; 3) cytometric bead array analysis of cytokines in supernatants after CAR crosslinking; and 4) long term effects of various CAR constructs on long term *in vitro* and *in vivo* Treg survival, phenotype, and function.

The final data chapter of my thesis addresses the need for Treg-specific understanding and potential downstream signaling effects of different co-stimulatory domains in CARs. Currently, CAR design has been optimized in Tconv for cytotoxicity in cancer immunotherapy. I

proposed creating a series of CAR constructs that differed only in co-stimulatory domains that could be used to determine the optimal design for functional and stable Tregs.

## 6.4 Overall conclusion

The aim of my thesis was to better understand factors that can contribute to Treg stability, specificity, and longevity. My data provide insight into how Tregs may become pathological under certain inflammatory conditions in tissues by observing skin-localized Tregs in SSc; describes a novel method to re-direct the antigen specificity of Tregs for allogeneic transplantation using a CAR for HLA-A2; and provides a series of lentiviral constructs for the advancement of CAR Treg cell therapy. Together these findings represent a significant contribution to the understanding and optimization of Tregs for cell therapy and in autoimmunity.

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