CTLA-4 induced signal transduction in regulating differentiation and plasticity of Tc17 cells

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Abbreviations

ADAP	adhesion- and degranulation-promoting adaptor protein
AhR	aryl hydrocarbon receptor
APCs	antigen presenting cells
B7-1	CD80
B7-2	CD86
BCR	B cell receptor
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
CFSE	carboxyfluorescein succinimidyl ester
ChIP	chromatin immunoprecipitation
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T lymphocyte associated antigen 4
d	day
DAG	diacyl glycerol
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Eomes	eomesodermin
ER	endoplasmic reticulum
et al.	et alii
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
GADS	GRB2-related adaptor protein
GRB2	growth factor receptor-bound protein 2
h	hours
HIF	hypoxia-inducible factor
i.p.	intraperitoneally

i.v.	intravenously
ICOS	inducible T cell activation costimulator
IFN	interferon
IL	interleukin
IRF	interferon regulatory factor
ITAMs	immunoreceptor tyrosine-based activation motifs
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
LC-MS	liquid chromatography–mass spectrometry
MAPK	mitogen-activated protein kinase
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
NK	natural killer
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed death-1
PFA	para-formaldehyde
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristoyl acetate
pMHC	peptide loaded MHC
PRRs	pattern recognition receptors
qPCR	quantitative PCR
ROR	related orphan receptors
SEM	standard error of mean
SHP2	SH2 domain containing protein tyrosine phosphatase

SLP76	SH2 domain-containing leukocyte protein of 76 kDa
SOCS	suppressor of cytokine signaling
STAT	signal transducers and activators of transcription
TAP	transporter associated with antigen presentation
TCR	T cell receptor
TGF	transforming growth factor
TGN	trans-Golgi network
Th	T helper
TLR	Toll like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
TRIM	T cell receptor-interacting molecule
ZAP-70	zeta associated protein of 70 kDa
β_2M	β_2-microglobulin

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1. Introduction and background

1.1 The immune system

Humans and other mammals live in an environment that is heavily populated by both pathogenic and non-pathogenic microbes, and that containing a vast array of toxic or allergenic substances, which impair body's balance and result in illness. The pathogenic microbes challenge the host by a very broad selection of pathogenic mechanisms by which they replicate, spread and threaten normal host functions. The immune system is a powerful protective mechanism of the host to control and usually eliminate these pathogenic organisms and harmful toxins from the body. In addition, the immune system also recognizes abnormal mutant cells and protects against cancer by eliminating them. To accomplish this task, the immune system uses a vast variety of cells and proteins produced by them. An important feature of the immune system is to recognize structural features of the pathogen or toxin that mark it as distinct from normal and abnormal cellular components and between 'self' and 'non-self'. This property of the immune system is essential to protect the host from their harmful effects without damaging the normal host tissues. Impairment of the immune system to recognize and tolerate these self-antigens may lead to development of autoimmune disorders causing inflammation and destruction of specific tissues and organs.

The immune system is divided into two broad types which are closely linked: The innate immune system and the adaptive immune system.

1.1.1 The innate immune system

The innate immunity constitutes the first line of host defense and is named for being present from birth and without having to learn through exposure to an invader like the adaptive immunity. However, the components of the innate immunity provide an immune response to all foreign invaders in an almost similar way. Unlike acquired immunity, it has no memory of the foreign antigen encounters and does not provide any ongoing protection against future infection. Moreover, the innate immune response which begins to act within minutes of encounter with a pathogen, has a critical role in controlling infections before the onset of initial adaptive immune response, which may take about 4-7 days (1, 2). The innate immune system responds to common structures shared by vast majority of pathogens, termed as PAMPs (pathogen-associated

molecular patterns). These PAMPs are recognized by PRRs (pattern recognition receptors) like TLRs (Toll-like receptors). If the pathogens evade or bypass the innate immune response an adaptive immune response is required (3, 4).

The cellular components of the innate immune system include: macrophages, mast cells, neutrophils, eosinophils, basophils, natural killer cells and dendritic cells (5). The complement system, also known as the complement cascade, displays a mechanism that complements other aspects of the innate immune response as well as adaptive immune responses. The complement system consists of a variety of proteins, which once activated initiate the complement cascade functions that include: opsonization, chemotaxis, cell lysis and agglutination. Cytokines produced by the immune cells are the messengers of the immune system. Cytokines and the complement system also have important properties like enhancing the adhesive molecules of vascular endothelium, causing circulating leukocytes to stick to the endothelial cells of the blood vessel wall and migrate between them to the site of infection (6, 7). The innate immune system plays a crucial role in the initiation and subsequent direction of adaptive immune responses. Changes in the innate immune cells that have taken up pathogens provide signals that synergize in activating lymphocytes of the adaptive immunity (4).

1.1.2 The adaptive immune system

Unlike the innate immune response the adaptive immune response is not quick and only based on identification of general threats. The adaptive immunity, upon initial exposure to different pathogens takes time to learn the best way to attack each pathogen. However, thereafter the adaptive immunity generates a memory of the immune response to the specific pathogen by remembering their antigen and subsequent responses to that specific antigen are quicker and more effective than the initial responses. The cellular components of the adaptive immune system are composed of T and B lymphocytes. Both these cell types are identified by the expression of unique receptors termed TCR (T cell receptor) and BCR (B cell receptor).

T cells when not appropriately directed have the potential to do enormous damage to the healthy tissues by responding to the self antigens opposed to foreign antigens. Hence T cells are screened extensively and undergo positive and negative selection for self tolerance in the thymus as soon as a T cell receptor is formed and expressed on the cell surface of a T cell progenitor. This tolerance mechanism that operates in the thymus before maturation and circulation of T cells is

referred to as ‘central tolerance’. However the central tolerance mechanism alone is insufficient as not all antigens that T cells need to be tolerant are expressed in the thymus. Hence, additional tolerance mechanism referred to as ‘peripheral tolerance’ exist, which screens the mature circulating T cells reactive to self antigens which are not thymically expressed. Central (Bone marrow) and peripheral mechanisms are also implicated in B cell tolerance, which is important in preventing the development of antibody responses to self antigens (2).

1.2 B cells

B cells function mainly by secreting antibodies and are capable of recognizing a wide variety of foreign antigens, including proteins, polysaccharides and lipids. Upon first encounter with an antigen, B cells quickly initiate the primary response and divide in order to become plasma cells (effector B cells) or memory B cells. Memory B cells remember the antigen and plasma cells secrete antibodies which identify the freely circulating pathogens (8).

1.3 T cells

T lymphocytes or T cells, which regulate a wide range of immunological processes by cytokine secretion and expression of immunomodulatory molecules, are the major effectors of the cellular adaptive immune responses. T cells are specifically categorized by the receptors and co-receptors expressed on surface of the cell. They recognize a specific antigen via TCR which is composed of α and β chains (αβ T cells). A minor subpopulation of T cells possesses a TCR composed of γ and δ chains (γδ T cells) (9). These γδ T cells represent an atypical type of T cells which operate as a bridge between innate and adaptive immune responses (10).

The majority of classical T cell compartment is composed of αβ T cells, which express the TCR consisting of heterodimer α and β chains that together bind to the peptides of the specific antigen. These peptides are presented to the T cell by MHC (major histocompatibility complex) molecules which are expressed on the surface of APCs (antigen presenting cells) or target cells. The formation of stable TCR:antigen:MHC complexes helps in recognizing the antigen and in activating the T cells. The α and β chain of the TCR is associated with the CD3 complex (CD3γ, CD3ε, and CD3δ) and with a homodimer of ζ-chain which are localized on the cytoplasmic side of the T cell. Both the CD3 complex and ζ-chain participate in T cell signal transduction upon antigen recognition (11). Additionally, binding of the co-receptors CD4 and CD8 to the MHC

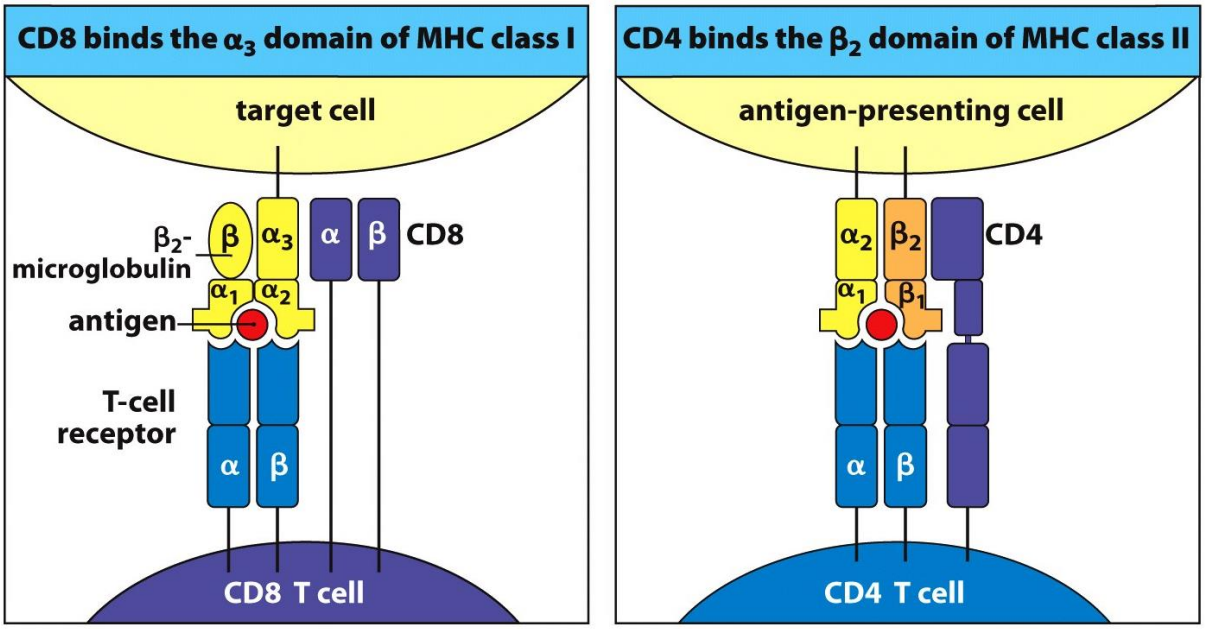

Figure 1.1: Molecules involved in the formation of stable MHC:antigen:TCR complex.

Interaction between target cell and CD8$^+$ T cells (left panel) and APCs and CD4$^+$ T cells (right panel). (The immune system, 4thed.Garland Science, 2014).

molecules is necessary for proper T cell activation, as it greatly enhances the sensitivity to the antigen and intracellular signaling. The co-receptor CD4 recognizes and binds to class II MHC molecules, whereas the co-receptor CD8 binds to class I MHC molecules (Fig. 1.1). The CD4$^+$ and CD8$^+$ T cells have very different functions. CD4$^+$ T cells express the CD4 co-receptor and are known as T helper (Th) cells, whose main function is to secrete cytokines, which orchestrate the adaptive immune response by helping other cells function properly. T cells expressing the CD8 co-receptor are often referred to as cytotoxic T lymphocytes (CTLs), as CD8$^+$ effector T cells can kill virus-infected cells or tumor cells by recognizing foreign antigens or tumor specific antigens within the MHC class I molecules (2).

1.4 T cell activation

The process of T cell activation involves a coordinated sequence of molecular events. It is initiated by the TCR upon recognition of the antigen presented by MHC molecules on APCs. This leads to the formation of an immunological synapse which facilitates the transduction of intracellular signaling pathways through the CD3 complex. These intracellular signaling

pathways regulate transcription and secretion of cytokines, cell survival, proliferation and differentiation.

The antigen is presented as peptide fragments loaded onto MHC molecules on the surface of APCs. These peptide fragments are generated inside the APCs by the degradation of foreign protein antigens. MHC proteins bind to these fragments, carry them to the surface of the cell and present them to the TCR expressed on T cells. MHC proteins are classified into two main structurally and functionally distinct classes: class I MHC proteins, and class II MHC proteins. While stimulation of $CD4^+$ T cells is dependent on recognition of exogenous peptides whose presentation is restricted to class II MHC molecules, $CD8^+$ T cells get activated only upon recognition of intracellular pathogenic peptides, which are strictly presented by class I MHC molecules. Class II MHC molecules recognized by $CD4^+$ T cells are normally found on B lymphocytes, dendritic cells and macrophages. In contrast, class I MHC molecules are expressed by all nucleated cells. Class I MHC molecules consist of a transmembrane glycoprotein comprising a heavy α chain and a small extracellular protein called β_2M (β_2-microglobulin).The heavy α chain is folded into three extracellular globular domains (α_1, α_2, α_3) with β_2M contributing as a fourth domain. The α1 and α2 domains form a helix, which binds antigenic peptides with length of approximately 8–10 amino acids (12). The peptides presented by class I MHC molecules are proteins degraded by cytosolic proteasomes. These peptides derived from proteasomal degradation are translocated into the ER (endoplasmic reticulum) by TAP (transporters associated with antigen presentation) to access class I MHC molecules. In the ER, the class I molecules MHC fold and assemble, while peptide binding is an integral part of the assembly process, as peptide inserts itself deep into the class I MHC peptide-binding groove. Without peptides, nascent class I MHC molecules are stabilized by ER resident chaperone proteins such as calreticulin and ERp57. The interaction between TAP and class I MHC molecules in the ER is associated with tapasin, which acts as a bridging molecule between the two. Upon association of peptides to class I MHC molecules, the chaperones and TAP are released and fully assembled peptide–class I MHC complexes are recruited into cargo vesicles for transport to the Golgi apparatus from where they are trafficked to the cell surface. Conversely, peptides and class I MHC molecules that fail to associate in the ER are returned to the cytosol for degradation (13–15). The peptide loaded onto the class I MHC complex expressed on the surface of APCs is then recognized by the TCR on $CD8^+$ T cells, which play a critical role in elimination

of pathogen infected cells. Using this surveillance system, effector $CD8^+$ T cells play a crucial role in controlling infections by viruses, intracellular bacterial pathogens and also exert potent antitumor activity. Mice models represent a valuable tool for investigating the dynamics of $CD8^+$ T cell responses against viral and bacterial infections and tumors. Murine models of Lymphocytic choriomeningitis virus, Listeria monocytogenes and B16 melanoma have played an especially important role in this aspect (16–18).

The signaling pathway mediated by the TCR has been well characterized. Within minutes after detection of an antigen-MHC complex, the kinase Lck (lymphocyte-specific protein tyrosine kinase) is recruited to the immunological synapse, which then phosphorylates the TCR ζ- chain and the ITAMs (immunoreceptor tyrosine-based activation motifs). The phosphorylated ITAMs serve as a binding site for ZAP-70 (Zeta associated protein of 70 kDa), which is then activated by ITAM bound Lck. Upon activation, ZAP70 phosphorylates the downstream adaptor molecule LAT (linker for activation of T cells), which can recruit and bind to various signaling molecules to form a multiprotein complex, called LAT signalosome. This complex constitutes of various signaling molecules including PLCγ1 (phospholipase Cγ1), SLP76 (SH2 domain-containing leukocyte protein of 76 kDa), GRB2 (growth factor receptor-bound protein 2), GADS (GRB2-related adaptor protein), ITK (interleukin-2-inducible T cell kinase), ADAP (adhesion- and degranulation-promoting adaptor protein), NCK1 (non-catalytic region of tyrosine kinase adaptor protein 1) and VAV1. Following TCR activation this complex propagates signals leading to activation of the MAPK (mitogen-activated protein kinase) and phosphatidylinositol lipid signaling pathways resulting in Ca^{2+} influx. The activation of these signaling pathways leads to the coordinated nuclear mobilization of transcription factors that are crucial for expression of genes essential for T cell growth and differentiation. One of the outcomes of the signals transduced by a TCR trigger is the transcription of IL-2 (Interleukin-2), a potent T cell growth factor (19–21).

A second set of signals (signal 2) followed by the antigen specific signal generated by TCRζ-CD3-CD4/CD8-p56lck complexes is required for an optimal immune response. This important second set of signals is provided by surface molecules like CD28, ICOS (inducible T cell activation costimulator), CTLA-4 (cytotoxic T lymphocyte associated protein 4), PD-1 (programmed death-1) which are expressed as cell surface molecules on T cells. Considering the diverse action of these molecules one gets confused with the term “costimulatory” molecules.

Positive costimulatory molecules such as CD28 enhance the activating signal induced by the TCR, whereas PD-1 functions as a negative regulator of T cell activation. Moreover, other molecules such as CTLA-4 may induce different responses depending on the nature and setting of the TCR and pMHC (peptide loaded MHC) ligation (22–24). As new molecules providing signal 2 are discovered, the signaling events mediated by these molecules in different T cell subsets remains of major interest.

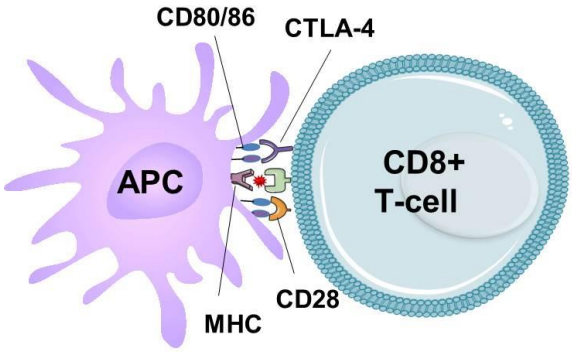

Figure 1.2: Schematic illustrating the interaction of co receptors CD28 and CTLA-4 on CD8$^+$ T cell with CD80 and CD86 on APCs.

The interaction of these co receptors with CD80/86 controls the output of immune response. Red: antigen. Green: TCR

1.5 CD28

The primary costimulatory event in T cell activation involves the interaction of CD28 expressed on T cells with the B7 ligands B7.1 (CD80) and B7.2 (CD86) present on APCs (24). In addition to the stimulatory effect mediated by TCR:pMHC interaction, binding of CD28 with CD80 and CD86 provides a second signal required for efficient T cell activation. In addition to sustaining T cell activation and proliferation, CD28 transduced signals mediate a number of events including the expression of cytokines and cytokine receptor and upregulation of anti-apoptotic molecules such as Bcl-XL (25). Co-stimulatory signals provided by CD28 also reduce the threshold required for T cell activation by permitting activation to occur with fewer TCR:pMHC interactions (26, 27). Studies using genetically modified mice lacking these co-stimulatory molecules have illustrated their role in T cell activation. Mice deficient in CD28 receptor failed to mount sufficient immune responses (28, 29).

The cytoplasmic domain of CD28 consists of YMNM motif, which when tyrosine phosphorylated following TCR activation interacts with the SH2 domain of PI3K (30). The kinase PI3K phosphorylates PIP2 (phosphotidylinosotol (3,4)-bisphosphate) converting it to PIP3 (phosphotidylinosotol (3,4,5)-trisphosphate), which allow recruitment of proteins containing PH (pleckstrin homology) domain to the plasma membrane. PDK1 (phosphoinositide-dependent protein kinase 1) is thus recruited and phosphorylated, which in turn activates PKB/Akt (protein kinase B) (31, 32). Activation of PKB enables phosphorylation of BAD, hence promoting T cell survival (33). CD28 also signals by recruitment of GRB2 to the aspergine residue of YMNM motif. GRB2 in turn interacts with SOS (son of sevenless) and VAV, which results in downstrean activation of MAPK and JNK pathway (34).

1.6 Cytotoxic T lymphocyte associated antigen 4

While the signals provided by costimulatory molecule CD28 upon interaction with B7 ligands has been clearly elucidated, the role of another co-receptor, CTLA-4, which also binds to the B7 ligands (35, 36) is more controversial. CTLA-4 is found to have similar homology to CD28, but has much higher affinity for B7 ligands than the later (Fig. 1.2) (37, 38). However, unlike CD28, CTLA-4 is not expressed on the surface of naive T cells, but is rapidly expressed upon T cell activation (39, 40). A majority of CTLA-4 is localized in several intracellular compartments: the TGN (trans-Golgi network), endosomes, and lysosomes. Even though when CTLA-4 is optimally expressed on the surface of T cells following activation, the surface expression represents only small amounts of intracellular CTLA-4. The surface expression of CTLA-4 is a highly dynamic process and a tight regulation of CTLA-4 surface expression is necessary for an efficient and controlled immune response. Briefly explaining the mechanisms regulating CTLA-4 surface expression; newly synthesized CTLA-4 resides in the TGN, where it associates with the transmembrane adaptor TRIM (T cell receptor-interacting molecule) and/or the clathrin adaptor AP-1. Following T cell activation, the transmembrane adaptor TRIM promotes the release of CTLA-4 to the surface of T cell towards the TCR engagement site. CTLA-4 externalization to the cell surface is also dependent on PLD (phospholipase-D) and GTPase and ARF-1 (ADP ribolyzation factor-1) activity. The pool of intracellular CTLA-4 is exported to the surface before being returned to the endocytic vesicles. This process of CTLA-4 trafficking has been shown to involve a region of its cytoplasmic tail known as Y_{201}VKM motif. The clathrin adaptor molecule

AP-2 binds to this Y_{201}VKM motif of CTLA-4 and mediates its internalization (Fig. 1.3) (22, 41, 42). This entire process of CTLA-4 trafficking is thought to be controlled by phosphorylation of the Y_{201} residue within the YVKM motif, since mutation of the Y_{201} residue prevented the interaction of AP-2 resulting in increased surface expression of CTLA-4 (43, 44).

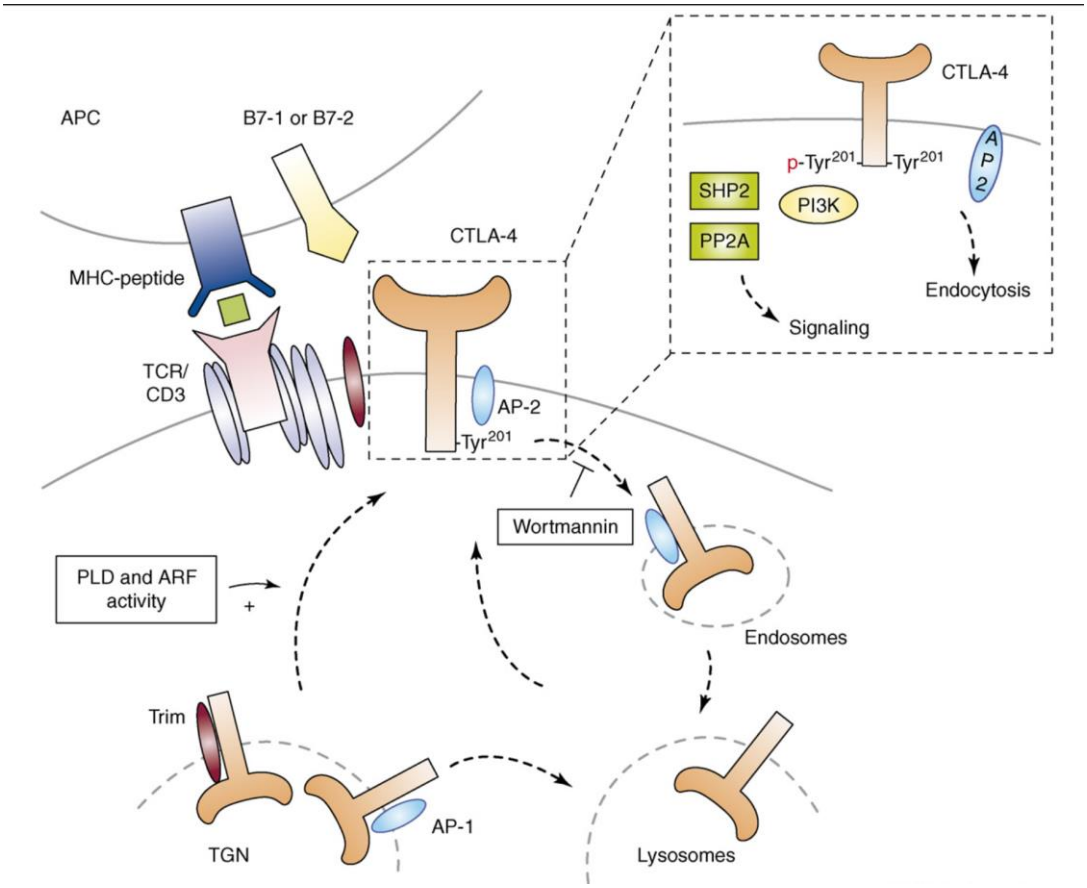

Figure 1.3: Model describing the regulation of CTLA-4 surface expression and internalization.

Schematic summary of the mechanism involved in the trafficking of CTLA-4. (Valk et al. Trends in Immunology, 2009)

The mechanism by which CTLA-4 suppresses T cell responses remains controversial and multiple mechanisms of how CTLA-4 mediates inhibition of T cell responses have been suggested.

The development of massive lymphoproliferative disorder in CTLA-4 knockout mice is perhaps the strongest evidence supporting CTLA-4 as an inhibitor of T cell proliferation (45, 46). One

model of mechanism is that co-stimulatory molecules CD28 and CTLA-4 compete to bind to the same ligands on dendritic cells led to the suggestion that this should have functional significance. Even though both molecules bind to the same ligands, it has been shown that CTLA-4 binds to these ligands with greater avidity and affinity than that of CD28 (47). Hence it was thought that CTLA-4 could sequester the B7 ligands away from CD28 and thus acts as an inhibitory counterpart to stimulatory effects of CD28. It was also proposed by others that soluble anti-CTLA-4 mAb (monoclonal antibodies) block the interaction of CTLA-4 with B7 ligands resulting in an enhanced T cell proliferation (48). However, this mechanism of extrinsic effects of CTLA-4 by sequestration of B7 ligands alone may not be sufficient for down regulation of T cell responses, since it has been shown that transgenic mice lacking the cytoplasmic domain of CTLA-4 still suffer from lymphoproliferative disorders (49).

Recently a number of studies have suggested that CTLA-4 is able to function cell extrinsically and expression of CTLA-4 within a population of T cells is enough to confer the ability of these cells to suppress proliferation of cells not expressing CTLA-4. One possible way of this mechanism is through the action of Tregs (regulatory T cells) which constitutively express CTLA-4. Initial studies provided an increasing evidence for the role of CTLA-4 in Tregs, as an antibody mediated blockade of CTLA-4 in Tregs inhibits their suppressive effect (50–52). Additionally, it was also shown using CTLA-4 knockout regulatory T cells that CTLA-4 is a clear requirement for Treg function (53, 54). Even though the exact role of CTLA-4 mediated suppression by Tregs is not completely understood so far, recent studies using CTLA-4 deficient Tregs showed CTLA-4 dependent modulation of dendritic cells (55). Also others have showed that CTLA-4 was able to capture B7 ligands by a process of trans-endocytosis and remove them from the surface of the cells (56) suggesting a possible explanation of how CTLA-4 could exert its inhibitory function. However, it was demonstrated by others that inhibitory effects of CTLA-4 are not completely dependent on B7 ligands, as a B7 non-binding CTLA-4 mutant inhibited T cell proliferation and cytokine production in otherwise CTLA-4 deficient T cells (57).

Another suggested mechanism of action of CTLA-4 is that it acts as a negative regulator through intracellular signaling events via its cytoplasmic tail. Although it has been shown that CTLA-4 suppresses the production of IL-2 and its receptor, as well as causing cell cycle arrest, the exact signaling methods employed by CTLA-4 remain unclear (58). It has been shown that the tyrosine phosphatase SHP2 associates with the YVKM motif within the cytoplasmic domain of CTLA-4.

This is initiated when the Y_{201} becomes phosphorylated and is thought to suppress T cell responses by dephosphorylation of neighboring TCR and hence down regulating T cell activation (59, 60). However, this mechanism remains controversial as there is some evidence that the mutation of the Y_{201} residue and hence inhibiting phosphorylation does not diminish suppressive effect of CTLA-4 on T cell activation (61).

The cytoplasmic tail of CTLA-4 has also been shown to interact with PI3K. The PI3-kinase is recruited by CD28 leading to activation of Akt (62, 63). This gave rise to the hypothesis that binding of CTLA-4 to PI3K reduces the later's availability to CD28, thus suppressing the positive signaling pathways involved in driving T cell activation (62). However, the significance of this interaction in suppressing the T cell immune responses remains contentious, since inhibition of PI3K has little effect on the function of CTLA-4 (25).

It has also been reported that signals mediated by CTLA-4 may be dependent on binding of phosphatase PP2A (protein phosphatase 2 A) to its cytoplasmic tail (64, 65). PP2A is thought to interact with the cytoplasmic tail of CTLA-4 and prevent its inhibitory function. Upon activation of T cells PP2A becomes dissociated from CTLA-4, thus freeing CTLA-4 to act as an inhibitor of T cells (62). However, there are controversial studies about PP2A suppressing the inhibitory effects of CTLA-4 as one study reported that mutating PP2A binding site increased the inhibitory capacity of CTLA-4 (64) while the others have reported that wild type as well mutant CTLA-4 lacking the PP2A binding site were functionally able to inhibit T cell responses (66). Hence, the exact mechanism of how PP2A regulates CTLA-4 activity is not completely understood so far. Even though a lot has been written about the inhibitory signals originating from CTLA-4, there is still much uncertainity to the exact nature and the functional importance of such signals in different T cell subsets.

1.7 Cytokines

Along with costimulatory receptors, receptors for cytokines are also present on the surface of T cells. Cytokines are group of small secreted proteins, peptides and glycoproteins which are known to regulate important cellular responses. Cytokine is the general term for these proteins; a cytokine can be more specifically defined as lymphokine (cytokines made by lymphocytes), chemokine (cytokines with chemotactic activities), monokine (cytokines made by monocytes),

and interleukin (cytokines made by one leukocyte and acting on other leukocytes). The cytokines produced by the cells may act on the same cell (autocrine action), or on nearby cells (paracrine action) and regulate T cell functions such as differentiation and proliferation synergistically or antagonistically (67). Many of these cytokines signal through cytokine receptors and JAK-STAT pathways and may also activate the MAPK pathway. The term cytokine milieu appears frequently in the literature, which is in reference to the composition of cytokines that are present in the surrounding environment. The differentiation of T cells into different effector cell types is often determined by the cytokine milieu that is present locally. Thus, cytokines shape the nature of the T cell immune response in addition to the TCR and costimulatory receptors signaling.

Cytokine signal delivery relies on several intermediary factors like the structure of the cytokine receptor, the availability of downstream signaling pathways through the cytokine receptor(s) and additional regulators of the downstream cytokine signaling pathways [e.g., SOCS (suppressor of cytokine signaling) molecules]. In addition, most cytokines have complex functions that may contribute to distinct immunomodulatory roles *in vivo* (68).

1.8 STAT signaling in response to cytokines

A majority of cytokines predominantly transmit signals via STAT (signal transducer and activator of transcription) molecules. Although TCR and costimulatory-mediated signaling relies on the several rounds of signal amplification through a variety of kinases; the cytokine-receptor interactions with respective cytokines program their function by directly phosphorylating the transcription factors STATs. The discovery of different STATs and their activation by different cytokines suggested that there are many STATs and each one is activated by different cytokines promoting a distinct outcome. Interferons promote STAT1 and STAT2 activation, resulting in the transcription of interferon-inducible genes. IL-6 and other gp130-utilizing cytokines could induce the activation of STAT3 and thus promote induction of inflammatory cytokines (69, 70). IL-12 induces the activation of STAT4, which in T cells is shown to promote the differentiation of the IFN-γ producing effector T cell phenotype (71). IL-2, an important cytokine for survival of T cells, induces the activation of STAT5, resulting in the transcription of pro-survival genes, as well as Foxp3, aiding in the development of regulatory T cells (72). The cytokine IL-4 induces the activation of STAT6, which enhances the transcription of GATA3 and IL-4 itself and is critical in the development of Th2 and Tc2 cells (73, 74).

Cytokines upon interaction with cytokine receptors enhance tyrosine phosphorylation of STAT proteins which induces the homodimerization of STAT molecules. This phenomenon of homodimerization would result in nuclear accumulation of these STAT homodimers. The nuclear translocated STAT homodimers could then bind their respective target sequence, recruit coactivators and effect transcription. However, the identification of different STAT protein activation by a majority of cytokines further complicates the understanding of cytokine mediated STAT signaling. The type of STAT activation can be heavily dependent on cell type, activation or differentiation state, the type of receptor expressed and the timing and the dose of cytokines. The discovery of activation of several STATs by a particular cytokine raised the question of whether the STAT proteins that are being activated in parallel induce separate transcriptional events, or are working in a synergistic manner promoting an alternative transcriptional outcome (75). Nevertheless, a majority of these problems have been addressed, at least partially, by using cells deficient in a particular STAT or cytokine receptor. For instance, in addition to STAT4, IL-12 has been shown to activate STAT3 and STAT5, but only cells deficient in STAT4 lack sensitivity to IL-12 (76–78). Even though distinct STAT proteins are activated in response to a single cytokine, the predominantly activated STAT may influence the activity of other STATs. For instance studies have suggested that relative abundance of activated STAT3 and STAT1 proteins may influence the activity of each other (79, 80). Studies have also suggested that closely related STATs can bind to the same DNA motif, but one may positively regulate and the other may negatively regulate gene transcription. For instance STAT5 associates with the IL-17 promoter and displaces the positive transactivating factor STAT3 (81, 82). In conclusion, a cytokine may activate a number of STATs, but the relative amount of predominantly activated STATs defines the specific type of T cell differentiation.

1.9 T cell differentiation

T cell immune responses are divided into different categories: generation of “helper” T cells, generation of “cytotoxic” T cells and modulation of immune responses by Tregs. A broad generalization of T cells separates helper function to $CD4^+$ T cells and cytotoxic functionality to $CD8^+$ T cells. Helper $CD4^+$ T cell immune responses generate cytokines and chemokines that either activate the specific function (cytokines) of neighboring cells or recruit (chemokines) new immune cell subsets to the site of pathogen encounter (8). Whereas $CD8^+$ T cells are also capable

of producing a diverse array of cytokines, however, their function appears to be mostly focused on the elimination of pathogen-infected host cells by cytotoxic means. The cytotoxic function of $CD8^+$ T cells is most commonly accomplished by the delivery of cytotoxic granules into the cytosol of the infected cell (2). Even though these are the canonical functions of $CD4^+$ and $CD8^+$ T cells, the different subsets of these cells have numerous exceptions.

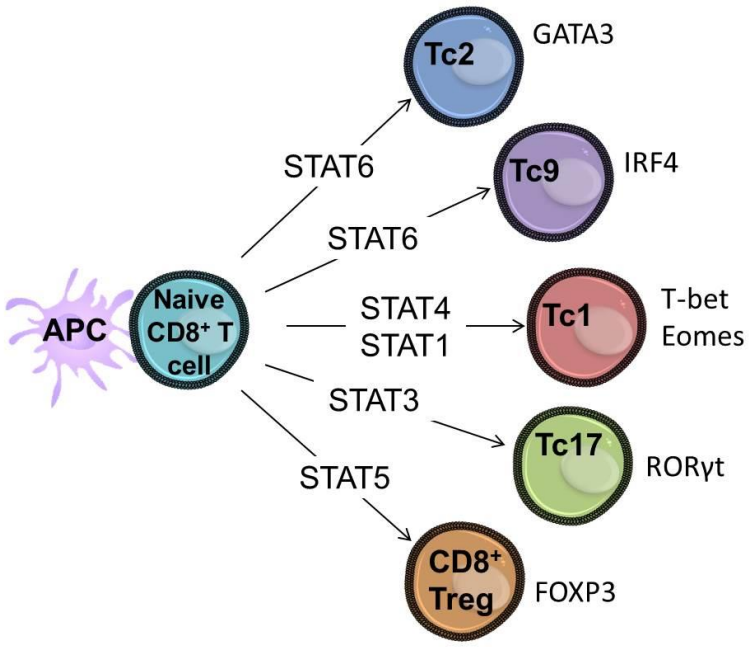

Figure 1.4: Schematic illustration of the differentiation of $CD8^+$ T cells.

The picture represents the distinct $CD8^+$ T cell subsets and their master regulators.

The coordination of complex signaling networks between the innate and adaptive immune systems enables efficient host defense against invading pathogens. Upon interaction with the antigen presented by APCs, $CD4^+$ T cells can differentiate into a variety of effector subsets classified as Th1, Th2, Th9, Th17, Tfh (follicular T helper cells), and Treg cells. Along with $CD4^+$T cells, $CD8^+$ T cells constitute an important wing of adaptive immune responses contributing to clearance of intracellular pathogens and providing long-term protection. Therefore, in a similar manner to that of $CD4^+$ T cells, also $CD8^+$ T cells under particular stimulatory conditions acquire the expression of IL-4, IL-5, IL-9, IL-13, IL-17 or suppressive

activity and are classified as Tc1, Tc2, Tc9, Tc17 or $CD8^+$ Treg cells, which thereby influence immune responses (Fig. 1.4) (83).

1.10 Cytotoxic $CD8^+$ T cells

Cytotoxic T lymphocytes (also called Tc1 cells) are the best characterized effector $CD8^+$ T cell subpopulation that are specialized in the eradication of intracellular pathogens and even cancer. These cytotoxic T cells produce robust amounts of IFN-γ and TNF-α. Additionally, the $CD8^+$ T cells activated and polarized into Tc1 cells generate large amounts of secretory vesicles, which when released in close contact to antigen presenting cells, directly lyse the neighboring target cell or the pathogen infected cell. The lytic activity of Tc1 cells is mediated through the cytotoxic molecules of perforin and granzyme protein families contained within the vesicles. Since cytotoxic $CD8^+$ T cells recognize the antigen presented on the ubiquitously expressed class I MHC molecules, $CD8^+$ T cells can interact virtually with most of the cells in the body. Pathogen infected cells presenting antigen in the form of peptide/class I MHC complexes on their surface are identified by the $CD8^+$ T cell and directly lysed by interactions of their pathogen peptide/MHC complex with the TCR of $CD8^+$ T cells. Engagement of the TCR directs the secretory lytic vesicles to the region of target cell interaction, and promotes the release of cytotoxic molecules into the synapse between the cells, thereby lysing the antigen presenting cell. In the same way, $CD8^+$ T cells can lyse cancerous cells upon encounter of tumor-associated antigens (84–86). CTLs are mainly programmed through T-bet (T box transcription factor). In addition, another T-box transcription factor Eomes (Eomesodermin) also co-operates with the former and supports the differentiation of Tc1 cells. The cytokines IL-12 and IFN-γ are known to amplify the Tc1 differentiation program through action of STAT4 and STAT1 (87).

1.10.1 The effect of CTLA-4 on Cytotoxic T lymphocytes

CTLA-4 was shown to be expressed on $CD8^+$ T cells by 2 to 6 fold higher frequency than $CD4^+$ T cells and is retained at the cell surface for longer times (Fig. 1.5), indicating that CTLA-4 might play a prominent role in $CD8^+$ T cells. Interestingly, genetic inactivation of CTLA-4 enhanced the cytotoxic activity of CTLs. CTLA-4 was shown to significantly down regulate the effector molecules IFN-γ and granzyme B after antigen specific primary stimulation.

Furthermore, CTLA-4 selectively inhibited T-box transcription factor Eomes and therefore downregulated the effector molecule production by Tc1 cells (88, 89).

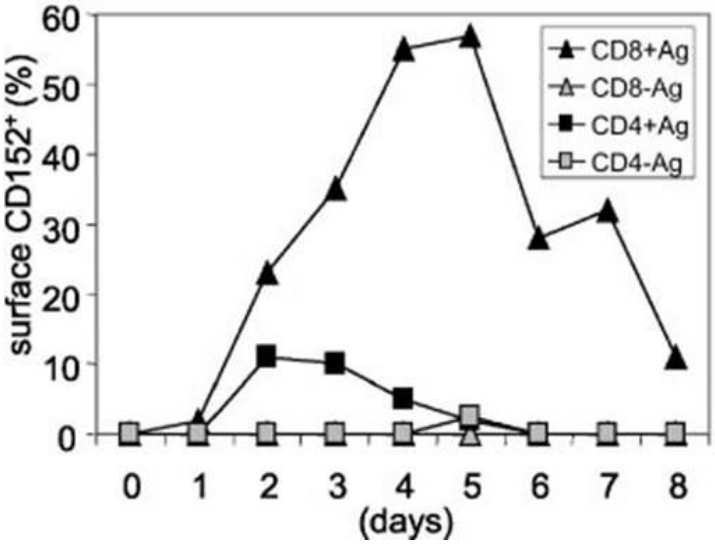

Figure 1.5: Kinetics of CTLA-4 surface expression on $CD4^+$ and $CD8^+$ T cells.

CTLA-4 surface expression was measured in TCR transgenic $CD8^+$T cells at indicated time points after antigen-specific primary stimulation with OVA-peptide presented on APCs. Symbols filled in dark display percentage of CTLA-4 positive T cells in cultures stimulated with antigen (Ag) whereas gray filled symbols represent CTLA-4 positive T cells in unstimulated cultures.(Pandiyan et al. Journal of Immunology, 2007).

1.10.2 CTLA-4 blockade immunotherapy

With the strong evidence revealing that blockade of CTLA-4 could enhance T cell responses, the application of anti-CTLA-4 monoclonal antibodies quickly followed to enhance tumor immunotherapy. A significant number of studies have investigated blockade of CTLA-4 in boosting anti-tumor immunity. The initial studies have displayed reduction in growth of colon carcinoma and fibro sarcoma upon treatment with anti-CTLA-4 mAb (90). Additionally, blockade of CTLA-4 was able to reduce growth of established tumors and protect against a second tumor challenge. Ensuring these initial results further studies extended the role of CTLA-4 blockade in treatment of prostate cancer (91, 92), melanoma (93, 94), ovarian carcinoma (95) and mammary carcinoma (96). A number of studies were performed combining anti-CTLA-4 mAb with depletion of $CD4^+CD25^+$ regulatory T cells (97), or low-dose chemotherapy (98) to

further enhance elimination of tumors that are resistant to an anti-tumor response. These studies revealed the importance of CTLA-4 blockade on $CD8^+$ T cells in providing protective immune responses.

1.11 Tc17 cells

1.11.1 Generation and differentiation of Tc17 cells

Similar to $CD4^+$ T cells, the $CD8^+$ T cells can also differentiate into IL-17 producing cells called Tc17 cells, depending on activation conditions and the cytokines in the microenvironment (99). The development of Tc17 cells is initiated by the cytokines TGF-β (transforming growth factor β) and IL-6 (Fig. 1.6) (100, 101). IL-6 signals mainly through the JAK/STAT pathway, whereas TGF-β signals primarily through the SMAD pathway. Binding of IL-6 to IL-6R causes JAKs to activate members of the STAT family by phosphorylation. IL-6 is thought to primarily activate STAT3, which is known to be crucial in governing differentiation of IL-17 producing T cells as cells deficient in STAT3 displayed complete loss of IL-17. IL-6 activated STAT3 promotes the expression of IL-21 and IL-23R, which in a STAT3 dependent manner amplify their own transcription, and stabilize IL-17 production (99, 102–104). The development of Tc17 cells is also orchestrated by the STAT3- regulated lineage-specific transcription factor RORγt, which initiates the transcription of IL-17A, IL-17F and IL-23R. Accordingly, loss of function in RORγt impairs Th17 differentiation *in vitro* and *in vivo* (105, 106). The transcription factor IRF4 is also demonstrated to be important for differentiation of IL-17 producing T cells by positive regulation of RORγt (107–109). IL-17 producing T cells display significant suppression of IFN-γ and IL-2 expression as well as reduced downstream-activation of STAT1 and STAT5, which are known to curtail differentiation of IL-17 producing T cells. In the nucleus, STAT3 and STAT5 compete for binding to the IL-17 promoter, leading to gene activation or silencing, respectively (81, 82). As STAT1 and STAT3 inhibit each other, relatively higher expression of STAT3 than STAT1 is required for the optimal activation of the transcriptional machinery for Tc17-related genes (79, 80). In comparison to Tc1 cells, Tc17 cells express very low amounts of typical CTL expressed granzyme B, and Eomes. Consistent with the impaired expression of molecules characteristic for CTLs, these cells have greatly impaired killing activity as compared with CTLs (99) and have been considered to promote tumor progression (110, 111) .

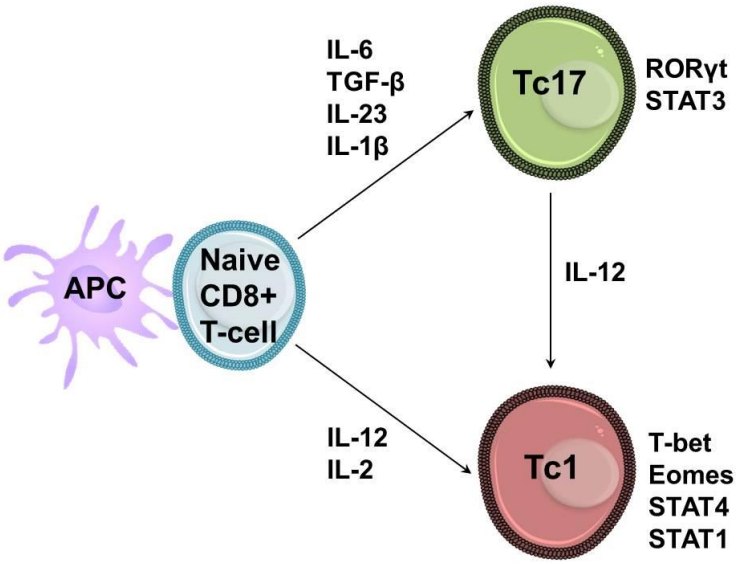

Figure 1.6: Classical differentiation of Tc1 and Tc17 cells and plascticity of Tc17 cells.

The scheme represents molecules involved in the differentiation of Tc1 and Tc17 cells and plasticity of Tc17 cells.

1.11.2 The relationship between Tc1 and Tc17 cells

Previous studies have demonstrated that $CD8^+$ T cells lacking both, Eomes and T-bet, fail to differentiate into functional Tc1 cells. Upon LCMV (Lymphocytic chloriomeningitis virus) infection, $CD8^+$ T cells deficient in Eomes and T-bet develop Tc17 characteristics with low cytotoxic activity causing progressive inflammation (112). However, the function of these Eomes and T-bet double-deficient Tc17 cells differ from regular Tc17 cells, since other studies show a protective function of Tc17 cells in viral infections in mice and humans. Tc17 cells have been shown to be protective against lethal influenza infection in mice and provoke a strong influx of neutrophils into the lungs (113) and also against vaccinia virus infection in mice (114). In humans, increased Tc17 frequencies were shown to correlate with control of disease progression in hepatitis C virus infection (115). Likewise, in a mouse melanoma model, Tc17 cells displayed enhanced anti-tumor immunity by starting to produce IFNγ, suggesting that Tc17 cells display microenvironment-dependent lineage plasticity and convert to a Tc1-like phenotype (116).

However, apart from gaining Tc1 characteristics like expression of IFNγ, certain Tc17-related characteristics such as increased persistence of survival *in vivo* are retained by the converted Tc17 cells, which clearly distinguish them from conventional Tc1 cells (117). With this in mind, factors that determine Tc17 lineage plasticity or stability, though not fully understood so far, remain of major interest.

2. Objective

$CD8^+$ T cells constitute an important branch of the adaptive immune system in higher vertebrates, contributing to effective elimination of intracellular pathogens and viruses thus providing long-term protection. The majority of these functions are fulfilled by the very well characterized $CD8^+$ T cell subpopulation the cytotoxic T lymphocytes (also known as Tc1 cells). It is demonstrated that Tc1 cells are able to kill infected cells and secrete cytokines such as IFN-γ and TNF-α mediated by transcription factors T-bet and Eomes. However, there is growing evidence that, in a similar manner to the subpopulations of $CD4^+$ T cells, $CD8^+$ T cells under particular conditions can differentiate into Tc1, Tc2, Tc9, Tc17 or $CD8^+$ T regulatory fate and thereby influence immune responses. The differentiation process of $CD8^+$ T cells is dictated by antigen strength and co-stimulatory molecules like CD28, CTLA-4, PD-1, ICOS etc., which are expressed on the surface of T cells. CTLA-4, the first target with reported effectiveness in immune checkpoint therapy is expressed on the surface of activated $CD8^+$ T cells. Blockade of CTLA-4 on $CD8^+$ T cells is demonstrated to be of particular importance in enhancing effector functions of Tc1 cells, owing to their enhanced ability to control tumor progression and secretion of the cytokines IFN-γ and TNF-α by selectively enhancing T-box transcription factor Eomes. However, the role of CTLA-4 in regulating the differentiation and cytotoxicity of IL-17 producing $CD8^+$ T cells (Tc17 cells) which are known to have reduced cytotoxic potential is not completely understood. Taking this into consideration, the aim of this study was to determine the effect of CTLA-4 in differentiation of Tc17 cells, particularly the molecular mechanism by which CTLA-4 regulates Tc17 differentiation and their plasticity. The resulting findings are intended to provide insights into the regulatory framework of Tc17 immune responses in order to extend the possibilities of Tc17 based immune therapies.

3. Materials and methods

3.1 Materials

3.1.1 Devices

AutoMACS pro	Miltenyi Biotech, Germany
Centrifuge, Biofuge fresco	Heraeus, Germany
Centrifuge, Multifuge 3SR	Heraeus, Germany
Incubator	Binder, Germany
NanoSpectrophotometer	Implen, Germany
pH-meter	WTW, Germany
Pipettes	Eppendorf, Germany
Real time PCR detection system, CFX96	Biorad, USA
Sonicator, SONOPULS	Bandelin electronic, Germany
Sterile hood, Herasafe	Thermo Fischer Scientific, USA
Thermal cycler, DNA engine	Biorad, USA
ThermoMixer	Eppendorf, Germany

3.1.2 Plastic articles and cell culture materials

The listed materials are from the following companies, unless otherwise stated: B. Braun (Germany), Eppendorf (Germany), Greiner Bio-One (Austria), Corning (USA),

2 ml, 1.5 ml, 0.5 ml microcentrifuge tubes, 15 ml and 50 ml conical centrifuge tubes, cell culture plates, petri dishes, cell strainers, cell culture flasks, syringes, PCR 96 well plate (Biozym Scientific, Germany).

All the chemicals and consumables used were supplied by Merck (Germany), Carl Roth (Germany), Sigmal Aldrich (USA) or Thermo Fischer Scientific (USA), unless otherwise stated.

3.1.3 Buffers

Erythrocyte lysis buffer	10 mM $KHCO_3$
	155 mM NH_4Cl
	0.1 mM EDTA (pH 7.5)
Pepscan Dilution Buffer	50 mM HEPES-KOH (pH 7.5)
	10 mM $MgCl_2$
	10 mM $MnCl_2$
	Complete protease inhibitor cocktail (Roche, Switzerland)
	Phosphatase inhibitor cocktail (Roche, Switzerland)
Buffers for ChIP Assay	
L1 buffer	50 mM Tris (pH8.0)
	2 mM EDTA (pH 8.0)
	0.1 % NP40
	10 % Glycerol
L2 Buffer	50 mM Tris (pH 8.0)
	5 mM EDTA
	1 % SDS
Wash Buffer	20 mM Tris (pH 8.0)
	2 mM EDTA
	0.1 % SDS
	1 % NP40
	500 mM Nacl
Elution Buffer	1 X TE
	2 % SDS

3.1.4 Mice and cell line

All animal experiments were performed under license approved from the Landesverwaltungsamt Sachsen-Anhalt in Halle. C57BL/6JRj mice were obtained from Janvier labs, Ly5.1, CTLA-$4^{+/+}$

and CTLA-4$^{-/-}$ OT.1 (118) mice were bred under pathogen free conditions following institutional guidance, at the central animal facility of University hospital, Magdeburg (Germany). Sex and age matched mice were used for all experiments. All mice have been backcrossed for more than 15 generations to the C57BL/6JRJ strain. Efforts were put to minimize stress and suffering of the animals used for *in vivo* experiments. Animals used for the experiments were killed by cervical dislocation. Phoenix cells were maintained in DMEM, 10 % FCS (fetal calf serum), 1 % P/S (Penicillin/Streptomycin), 1 % glutamine, 25 mM HEPES, 5 µg/ml Plasmocin (Invivogen, USA). The OVA-transfected B16 tumor cell line (119) were maintained in RPMI 1640, supplemented with 10% heat-inactivated FCS, 1% P/S, 25 mM HEPES, 1 mM Sodium pyruvate, 50 µM 2-ME and 1 mg/ml G418.

3.1.5 Antibodies and inhibitors

Table 3.1: Mouse antibodies used for flow cytometry

anti-CD25 (7D4)	BD Biosciences, USA
anti-CD44 (IM7)	Biolegend, USA
anti-CD45.2 (104)	BD Biosciences, USA
anti-CD62L (MEL14)	Biolegend, USA
anti-CD69 (H1.2F3)	BD Biosciences, USA
anti-CD8α (53–6.7)	Biolegend, USA
anti-CD8α (53–6.7)	BD Biosciences, USA
anti-CD90.2 (53-2.1)	BD Biosciences, USA
anti-CTLA-4 (UC10-4B9)	eBioscience, USA
anti-Eomes (Dan11mag)	eBioscience, USA
anti-IFN-γ (XMG1.2)	BD Biosciences, USA
anti-IL-17 (Tc11–18H10)	BD Biosciences, USA
anti-IL-17 (Tc11–18H10.1)	Biolegend, USA
anti-IL-23R (078-1208)	BD Biosciences, USA
anti-pSTAT1 (Tyr701) (58D6)	Cell signaling, USA
anti-pSTAT3 (Tyr705) (D3A7)	Cell signaling, USA
anti-pSTAT5 ((Tyr794) (SRBCZX)	eBioscience, USA

anti-RORγt (AFKJS-9)	eBioscience, USA
anti-STAT1	Cell signaling, USA
anti-STAT3	Cell signaling, USA
anti-STAT5	Cell signaling, USA
anti-T-bet (4BIO)	Biolegend, USA
anti-TCR Vα2 (B20.1)	BD Biosciences, USA
anti-TNFα (MP6-XT22)	BD Biosciences, USA
donkey anti-rabbit IgG (Poly4064)	Biolegend, USA

- S31-201 a chemical probe inhibitor, which selectively blocks STAT3 phosphorylation, dimerization, DNA binding, and STAT3-dependent transcription was purchased from Merck Millipore, Germany.

3.1.6 Primers

Sequences of primers used in quantitative real time RT-PCR (qPCR)

HPRT (Forward: 5´-CTC CTC AGA CCG CTT TTT GC-3´ and Reverse : 5´-AAC CTG GTT CAT CAT CGC TAA TC-3´), T-bet (Forward: 5´-TCA GGA CTA GGC GAA GGA GA-3´ and Reverse : 5´-TAG TGG GCA CCT TCC AAT TC-3´), Eomes (Forward: 5´-TGA TAG TGT TGC AGT CTC TG-3´ and Reverse : 5´-CAA TCT GAT GGG ATG AAT CG-3´), SOCS3 (Forward: 5´-GTT GAG CGT CAA GAC CCA GT-3´ and Reverse : 5´-GGG TGG CAA AGA AAA GGA GG-3´), SOCS1 (Forward: 5´-CGC CAA CGG AAC TGC TTC TTC-3´ and Reverse : 5´-TCA GGT AGT CAC GGA GTA CC-3´), RORα (Forward: 5´-TCT CCC TGC GCT CTC CGC AC-3´ and Reverse : 5´-TCC ACA GAT CTT GCA TGG A-3´), RORc (Forward: 5´-TGC AAG ACT CAT CGA CAA GG-3´ and Reverse : 5´-AGG GGA TTC AAC ATC AGT GC-3´), AhR (Forward: 5´-ACC AGA ACT GTG AGG GTT GG-3´ and Reverse : 5´-TCT GAG GTG CCT GAA CTC CT-3´) Runx-1 (Forward: 5´-GAA GAA CCA GGT AGC GAG ATT CAA C-3´ and Reverse : 5´-TGG CGG ATT TGT AAA GAC GG-3´), IRF-4 (Forward: 5´-TCT TCA AGG CTT GGG CAT TG-3´ and Reverse : 5´-CAC ATC GTA ATC TTG TCT TCC AAG TAG-3´), IL-23R (Forward: 5´-TGA AAG AGA CCC TAC ATC CCT TGA-3´ and Reverse : 5´-CAG AAA ATT GGA AGT TGG GAT ATG TT-3´), IL-21 (Forward: 5´-CAT CAT TGA CCT CGT

GGC CC-3′ and Reverse : 5′-ATC GTA CTT CTC CAC TTG CAA TCC C-3′), IL-17f (Forward: 5′-CTC CAG AAG GCC CTC AGA CTA-3′ and Reverse : 5′-AGC TTT CCC TCC GCA TTG ACA-3′), IL-17a (Forward: 5′-CCC ATG GGA TTA CAA CAT CAC-3′ and Reverse : 5′-CAC TGG GCC TCA GCG ATC-3′) and HIF-1α (Forward: 5′-CGG CGA AGC AAA GAG TCT G-3′ and Reverse : 5′-ATA ACT GAT GGT GAG CCT CAT AAC-3′).

Sequences of primers used for ChIP qPCR

IL-17A (Forward: 5′- GAG ACA GAT GTT GCC CGT CA-3′ and Reverse : 5′- TTT CTT GTT TGC GCG TCC TG-3′)

3.2 Methods

3.2.1 $CD8^+$ T cell differentiation *in vitro*

Spleens and lymph nodes of CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ OT.1 mice were collected to obtain naive $CD8^+$ T cells ($CD8^+$ $CD62L^{high}$). Naive $CD8^+$ T cells were isolated to a purity of ≥ 98 % by magnetic beads separation using AutoMACSpro (Miltenyi Biotec, Germany). For antigen (Ag) specific activation naive $CD8^+$ T cells were stimulated with 1 µg/ml of LPS-free SIINFEKL ($OVA_{257-264}$) peptide (Invivogen, USA) and CD90-depleted splenocytes from C57BL/6JRJ mice at a ratio of 4:1. For Ab specific agonistic stimulation, $CD8^+$ T cells isolated from spleen and lymph nodes of C57BL/6JRJ mice were used. Cells were stimulated with plate bound immobilized anti-CD3 (3µg/ml), anti-CD28 (0.25 – 4µg/ml) (Biolegend, USA) and anti-CTLA-4 (10µg/ml) (4F10) or Isotype (BD Biosciences, USA) antibodies. All cells were cultured in serum free x-vivo 15 medium (Lonza, Switzerland). For Tc17 differentiation the cells were conditioned with 2 ng/ml TGF-β, 10 ng/ml IL-6 (R&D systems, USA), 25 ng/ml IL-23, 5 ng/ml IL-1β (Biolegend, USA) and 10 µg/ml anti-IFN-γ XMG.1.2 (DRFZ, Berlin). For Tc1 differentiation, the cells were conditioned with with 5 ng/ml IL-12 and 1 ng/ml IL-2 (Biolegend, USA). To determine Tc17 plasticity, primary stimulated Tc17 cells were re-stimulated with fresh plate bound immobilized anti-CD3 (Biolegend, USA) in the presence or absence of anti-CTLA-4 and were conditioned with 5 ng/ml IL-12 and 1 ng/ml IL-2.

3.3.2 Flow cytometry, surface, and intracellular staining and integrated MFI (iMFI)

The cells were harvested and stained with the indicated surface markers in PBS/0.2 % BSA. Prior to intracellular cytokine analysis, the cells were re-stimulated with PMA, ionomycin under Brefeldin A treatment for 4 h. Intracellular staining was performed after the cells were fixed with 2% paraformaldehyde (Morphisto, Germany) in PBS for 20 min on ice and permeabilized in 0.5% saponin (Sigma-Aldrich, USA) in PBS/BSA. The transcription factors were measured by fixing the cells with 4% formaldehyde (Merck, Germany) in PBS for 10 min at 37°C, followed by permeabilization in ice-cold 90% methanol (Carl Roth, Germany) in H2O for 30 min. The cells were then stained in PBS/BSA. Variations in FACS analyses were corrected by normalizing the measurements and considering the MFI of unstimulated cells as the basal level. All cytometric analyses were performed using a FACS-Canto II™ (BD Biosciences, USA) and FlowJo™ software (FlowJo LLC, USA). Labeling of cells with CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester, purchased from Molecular Probes, USA) was performed as follows: $1x10^7$ cells/ml were washed with PBS and stained with CFSE (5 µM) for 5 min on ice in the dark, and the reaction was stopped by adding RPMI-1640 medium with 10% FCS. iMFI is a multiplication of MFI and frequency of cytokine producing cells, which gives a value of total functional quality of cytokine producing cells (120).

3.2.3 Quantitative real-time RT-PCR

RNA was isolated from Tc17 cells at the indicated time points using the NucleoSpin RNA/Protein Isolation Kit (Macherey-Nagel, Germany) and was reverse transcribed using the Applied Biosystems Reverse Transcription Kit. The cDNAs were stored at –20°C. Gene expression was analyzed using Fermentas Maxima™ (Thermo Scientific, USA) SYBR Green qPCR Master Mix on a CFX96™ Real-Time PCR detection system (Bio-Rad, USA). HPRT was used as a control. Primer pairs for quantitative real-time PCR (qPCR) were purchased from TIB MOLBIOL, Germany (primer sequences are shown in materials).

3.2.4 Plasmid preparation and production of retroviral supernatants

The retroviral vectors containing MSCV-IRES-GFP (MIG), RORγt-IRES-GFP (107) and IRF4-IRES-GFP have been described before (121). Competent *E. coli* cells (JM109, Promega, USA)

were thawed and specific recombinant plasmid was added to the cells; this mixture was then incubated on ice for 30 minutes, heat shocked for 45 seconds at 42°C, and then immediately placed on ice for 2 minutes. 250 µl of SOC medium was then added to the cells, and the culture was shaken for one hour at 37°C. The transformation reactions were plated on to LB agar plate containg ampicillin overnight at 37°C. Colonies were then picked from the plates and grown in liquid cultures overnight. Thereater the plasmids were purified using plasmid DNA purification kit (Macherey Nagel, Germany).

Eighteen hours before transfection $2x10^6$ phoenix cells were plated on a 100 mm culture dish. A DNA mix containing 10 µg gag-pol, 3 µg eco-env and 10 µg plasmid of interest was prepared in 900 µl Opti-MEM. 69 µl of FuGene was directly added to the DNA mix and votexed for 1 sec. The mixture was incubated for 15 min at room temperature. The phoenix cell medium was then replaced with 4.1 ml fresh medium and the DNA-FuGene mix was added drop by drop to the culture dish. After 24 h, media were exchanged with 10 ml fresh medium. 16 h later, first virus supernatant was collected and 6 ml fresh medium was added to the cellls followed by second virus supernatant collection after 8 h. The virus supernatants were filtered through 0.45 µm filters and stored at -80°C.

3.2.5 Retroviral transduction

CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ naive OT.1 $CD8^+$ cells were primed in Tc17 conditions. 24 and 36 h later Tc17 cells were infected twice with retrovirus by adding medium containing virus to the cells and centrifuging at 2000rpm and 33°C for 90 min. 12 h after second virus infection, cells were washed and recultured under Tc17 conditions and 48 h later the cells were analysed for GFP and IL-17 expression by flow cytometry.

3.2.6 Chromatin Immunoprecipitation (ChIP)

Tc17 cells were stimulated with either the STAT3-inducing cytokines IL-6 and IL-23 or the STAT5-inducing cytokine IL-2 for 30 min, and the protein-DNA complexes were crosslinked with 1% formaldehyde for 5 min and quenched by adding 125 mM Tris (pH7.5). Cells were then lysed for 5 min in L1 buffer which keeps the nuclear membrane intact. The nuclei from cells were collected by centrifugation, resuspended in L2 lysis bufer and lysed by sonication using one fifth of total power in 3 pulses (each pulse 12 sec). The protein-DNA complexes were

immunoprecipitated with magnetic µMACS™ Protein G MicroBeads (Miltenyi Biotech, Germany) coated with either an anti-STAT3 antibody or an anti-STAT5 antibody (Cell Signaling Technology, USA). The magnetic immune complexes were passed through a separation column placed in the magnetic field of a MACS Separator (Miltenyi Biotech, Germany) and washed three times using wash buffer. The labeled complexes were retained in the column, and the other proteins were efficiently washed away. The immunoprecipitated protein-DNA complex was eluted from the column using elution buffer (preheated to 95°C), and the protein-DNA crosslinks were reversed at 65°C overnight. The DNA was then purified from the sample, eluted (Invisorb, Genomic DNA KIT II, Stratec Biomedical, Germany), and analyzed by quantitative PCR (primer sequences are shown in materials) using a CFX96^{TM} Real-Time PCR detection system (Bio-Rad, USA). The Ct value for each sample was normalized to the corresponding input DNA (collected prior to immunoprecipitation) value.

3.2.7 Kinome array analysis

For kinome array, CTLA-4$^{+/+}$ and CTLA-4$^{-/-}$ Tc17 cells that had been cultured for 3 days were washed twice with PBS and lysed in complete cell lysis buffer (Cell signaling Technology). The protein concentration in the cell lysate was determined using a BCA assay (Pierce BCA protein Assay kit, Thermo Fischer Scientific) and adjusted to a concentration of 2 mg/ml using dilution buffer. A 10-µl activation mix containing 50% glycerol, 50 mM $MgCl_2$, 50 mM $MnCl_2$, 0.25 mg/ml PEG 8000, 0.25 mg/ml bovine serum albumin, and 2,000 µCi/ml [γ-33P]ATP (Hartmann Analytic, Germany) was added to 90 µl of cell lysate to ascertain kinase activity. The peptide arrays, which contain 1,024 different kinase pseudo-substrates in triplicate (Pepscan, Lelystad, Netherlands), were incubated with the activation mix and the cell lysate for 2 h in a humidified chamber at 37^{o}C. Subsequently, the arrays were washed with each of the following solutions: PBS containing 1% Triton X-100, 1% SDS in demineralised water and distilled water. The slides were air dried and exposed to a phosphoimaging screen for 72 h.

3.2.8 Data acquisition and analysis of PepChip array

The data on the phosphoimaging screen were acquired using a Phosphoimager (GE Healthcare Lifesciences) and quantified using ScanAlyze software (Leland Stanford Junior University, USA). Subsequently, the data were exported to a spreadsheet program (Microsoft Excel 2010; Microsoft Co., USA). The spot densities were corrected for the individual backgrounds to

diminish interarray variance. The variation between arrays and individual experiments was reduced by normalizing the data to the 99th percentile of the intensity of each array. The averaged spots were included in dissimilarity measurements using a ranking method to identify peptides with either significantly increased or decreased phosphorylation.

3.2.9 Pull-down experiments and mass spectrometric analysis

The peptides CSPLT TGV (p)YVKMPPTEPE**S**EKQFQPYFIPIN with the indicated tyrosine either phosphorylated or non-phosphorylated were used in the pull-down experiments in order to profile the phosphorylation-dependent interaction partners. The serine in the sequence was introduced instead of cysteine in the original sequence in order to allow for cysteine-mediated covalent coupling to the beads. Thirty million CTLA4$^{-/-}$ CD8^{+} T cells were stimulated, harvested and lysed and the soluble fraction of the lysate was incubated with the peptide beads prior to tryptic on-bead digest. The digest was performed either in ^{18}O or ^{16}O water with swapped labels in the two replicate experiments regarding the phosphorylated or unphosphorylated peptide bait. LC-MS analysis was subsequently performed on an Orbitrap LTQ XL machine and data was analyzed by Mascot Distiller.

3.2.10 *In vitro* CD8^{+} T cell cytotoxicity assay

Syngenic T cell depleted splenocytes were labeled with either 5 μM CFSE (CFSE^{high} cells) or 0.25 μM CFSE (CFSE^{low} cells) in RPMI medium (5 min on ice) and washed twice. CFSE^{high} cells were pulsed with 1 μg/ml $OVA_{257–264}$-peptide (1 h at 37°C). CFSE^{low} cells were used as internal controls and were not pulsed with OVA. To the pre-differentiated CTLA-4$^{+/+}$ and CTLA-4$^{-/-}$ Tc17 cells (0.2x10^{6} each), a 1:1 mixture of OVA-pulsed (CFSE^{high}) and -unpulsed (CFSE^{low}) splenocytes were added at different ratios. At indicated time points, quantification of CFSE-labeled cells was performed and OVA-specific lysis was quantified.

3.2.11 Adoptive T cell transfer and melanoma model

CD45.1 (Ly.5.1) C57BL/6 mice received a subcutaneous (s.c.) injection into the right flank with 2x10^{5} B16-OVA melanoma cells in PBS. Approximately 10 days after the tumor cell injection, mice that had developed a substantial tumor (~100 mm^{3}) received an i.v. injection with either PBS or *in vitro* generated CD45.2-expressing CTLA-4$^{+/+}$ or CTLA-4$^{–/–}$ OT.1 Tc17 cells. Tumor

growth was then monitored on a daily basis. Mice with large tumors were humanely killed. Adoptively transferred $CD8^+$ $CD45.2^+$ cells were analyzed by flow cytometry as described above.

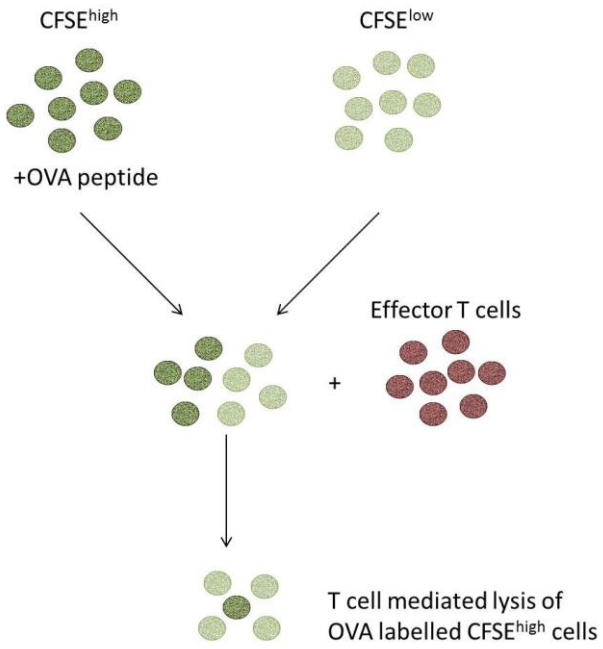

Figure 3.1: Illustration of in vitro cytotoxicity test.

T cell depleted splenocytes were stained with different concentrations of flourescent cell staining dye CFSE in two separate batches. The strongly stained cells ($CFSE^{high}$) were loaded with antigen wheresas the weakly stained cells ($CSFEl^{ow}$) were not. Subsequently, equal number of both the cells were mixed and cultured with pre-differentiated $CD8^+$ T cells. After 18 hours the antigen specific lysis of $CFSE^{high}$ cells was analyzed by flow cytometry.

3.2.12 Statistical analysis

Data were analyzed by Microsoft Excel 2010 (Microsoft Co., USA) and Prism 6 (Graphpad software Inc, USA). Data are presented as ± SEM. *P*-values are computed by unpaired Student's *t*-test or Mann-Whitney U-tests. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, n.s: not significant.

4: Results

4.1: CTLA-4 supports differentiation of IL-17 producing $CD8^+$ T cells

4.1.1: Genetic deletion of CTLA-4 decreases frequency of IL-17 producing $CD8^+$ T cells

To determine whether CTLA-4 is expressed on the surface of IL-17 expressing $CD8^+$ T cell (Tc17 cells) in a similar manner to Tc1 cells (89), naive $CD8^+$ $CD62L^+$ OT.1 T cells were stimulated with $OVA_{257–264}$ peptide and congenic APCs in Tc17 skewing conditions (as described in *Material and Methods*). Flow cytometric analysis revealed that $CD8^+$ T cells unambiguously express CTLA-4 on the cell surface upon antigen-specific stimulation independent of the Tc1 or Tc17 skewing conditions (Fig. 4.1 A). Expression of CTLA-4 on the cell surface was observed to be upregulated 48 h after beginning of stimulation (Fig. 4.1 B).

To evaluate the role of CTLA-4 in Tc17 differentiation, CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ OT.1 $CD8^+$ T cells have been stimulated under Tc17 conditions and the expression of IL-17 was measured at different time points following stimulation. Strikingly, CTLA-$4^{+/+}$ Tc17 cells displayed a higher frequency of IL-17 producers than CTLA-$4^{-/-}$ Tc17 cells on day 3 when CTLA-4 was observed to be strongly expressed on the surface of Tc17 cells (Fig. 4.2 A). On day 1, CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ Tc17 cells showed less than 2% frequency of IL-17 producers. Although it has been shown that CTLA-4 blockade leads to enhanced IFN-γ production (which inhibits Tc17 differentiation) in Tc1 cells (88), no distinct increase in the frequency of IFN-γ-producing cells was detected under optimal Tc17 conditions (Fig. 4.2 A top and bottom panel). To determine whether the difference in frequency of CTLA-4 mediated Tc17 differentiated cells is due to difference in proliferation, CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ OT.1 $CD8^+$ T cells were stained with CFSE before primary stimulation under Tc17 conditions. $CD8^+$ T cells stimulated under Tc1 condition were also set up in a similar manner to compare Tc17 and Tc1 cell proliferation mediated by CTLA-4. Interestingly, regular monitoring of these cells showed comparable proliferation (Fig. 4.2 B). In addition to the frequency of IL-17 producing cells the mean fluorescent intensity (MFI) of IL-17^{high} producers was higher for CTLA-$4^{+/+}$ Tc17 cells (7955 ± 110.3 vs. 6318 ± 83.89; $p < 0.0001$; n=6) suggesting that CTLA-4 likely controls the quality and quantity of Tc17 cells.

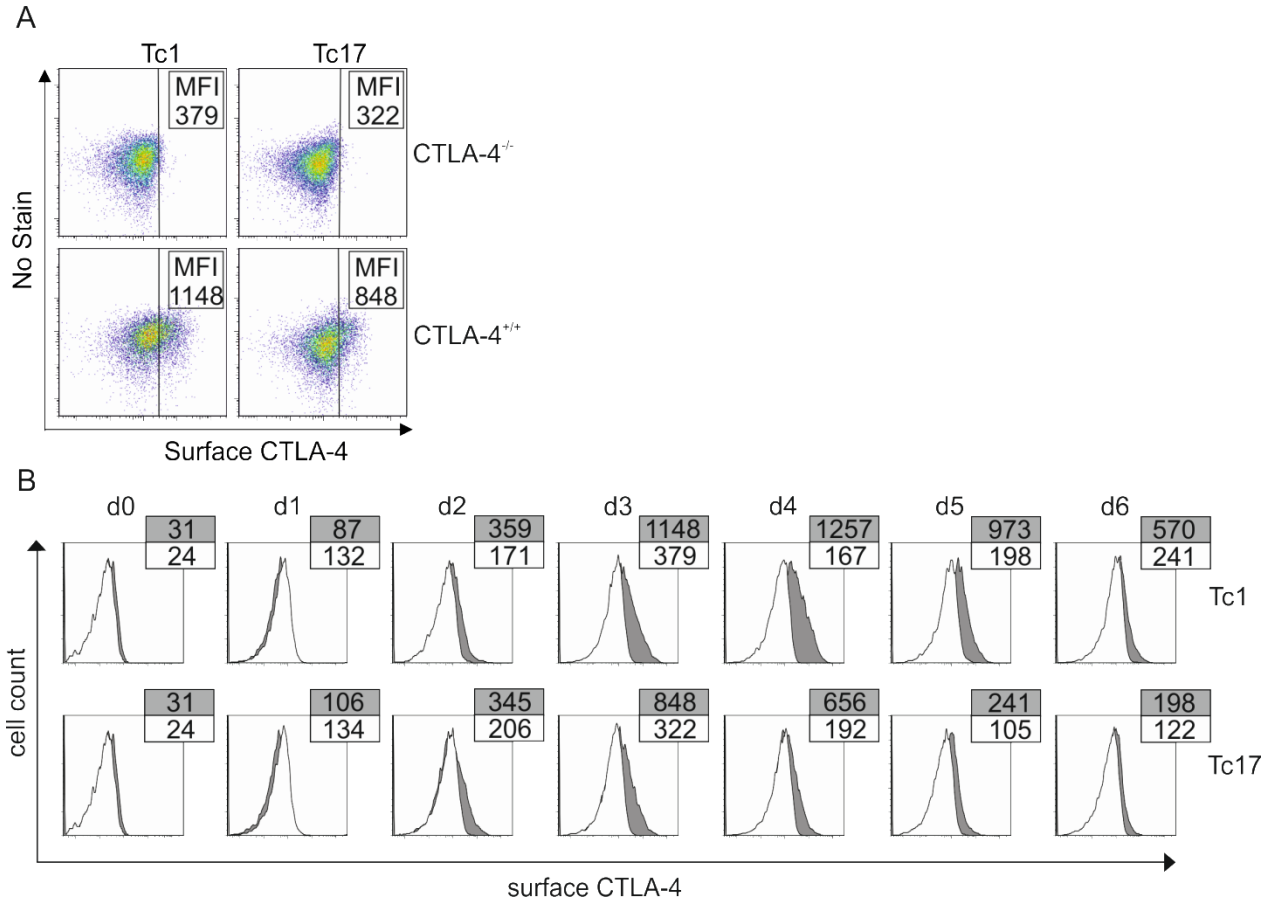

Figure 4.1: CTLA-4 surface expression on activated CTLA-4$^{+/+}$ CD8^{+} T cells.

Naive CTLA-4$^{+/+}$ (grey-filled histograms) and CTLA-4$^{-/-}$ (open histograms) OT.1 CD8^{+} T cells were activated antigen-specifically and cultured in Tc1 or Tc17 conditions. (A) Dot plot of CTLA-4 surface staining (day 3). (B) Kinetics of CTLA-4 surface expression at the indicated time points after primary antigen-specific activation of CTLA-4$^{+/+}$ Tc1 and Tc17 cells (grey-filled histograms). CTLA-4$^{-/-}$ Tc1 or Tc17 cells (open histograms) were used as controls. Data shown are from a single experiment representative of two independent experiments performed.

It has been reported that altered strength of TCR signals might affect T cell differentiation (122). Hence, even though a widely established experimental system was used (88, 89, 123), the experiments were repeated additionally with a wide range of $OVA_{257-264}$ peptide concentrations (1/ 0,1/ 0,01 µg/ml) which also displayed similar results, ensuring that the observed impact of CTLA-4 on Tc17 differentiation is not due to altered TCR avidity. Taken together these results indicate for the first time that CTLA-4 significantly enhances Tc17 differentiation.

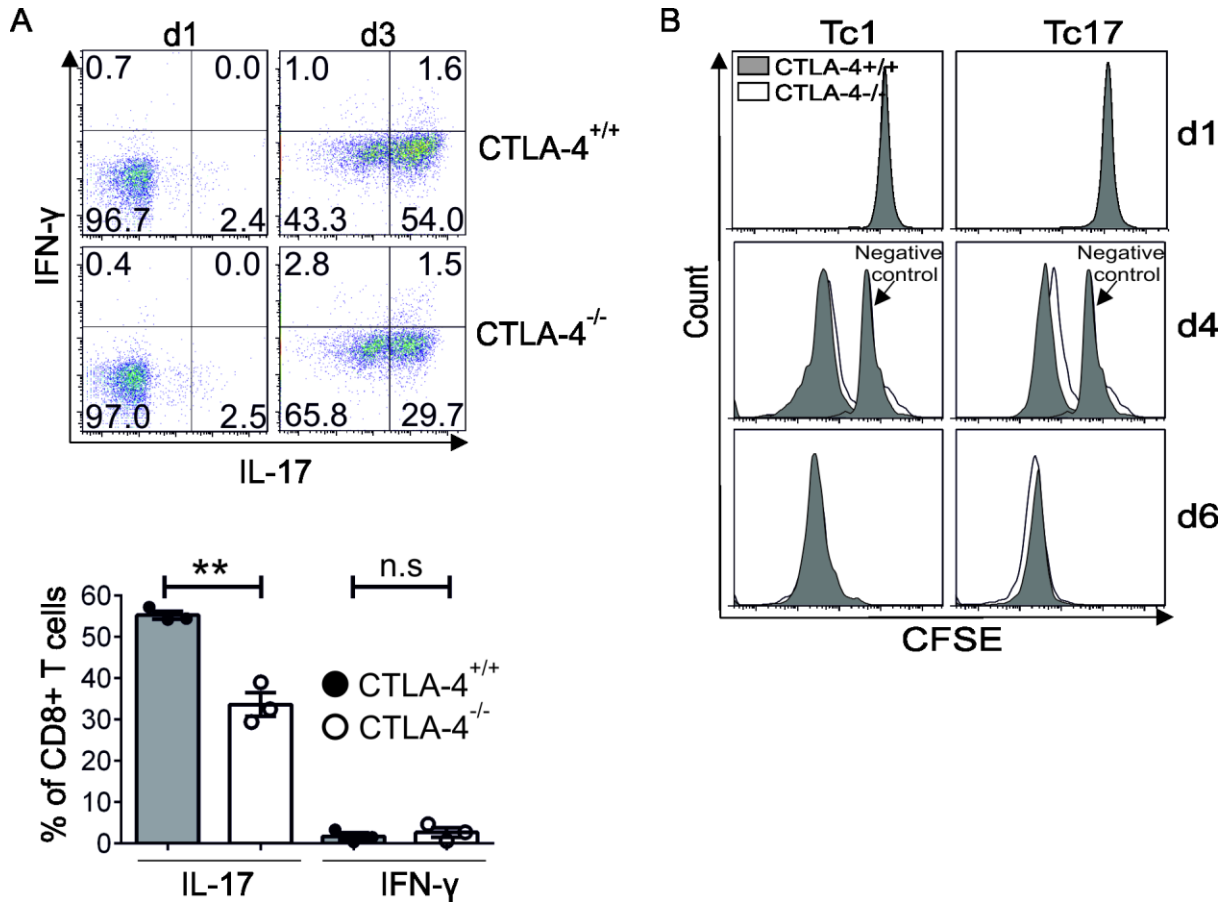

Figure 4.2: Role of CTLA-4 in Tc17 differentiation.

*(A) Naive $CD8^+$ T cells from CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ OT.1 mice were activated with the specific antigen $OVA_{257-264}$ in the presence of APCs under Tc17 conditions. IL-17 and IFN-γ expression in these cells was analyzed by flow cytometry at d1 and d3 after primary stimulation (top). Cumulative staining results of day 3 are shown on the bottom. The data are representative of three independent experiments. (B) Naive CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ OT.1 $CD8^+$ T cells were labeled with CFSE (5 μM) before priming them with APCs and culturing under Tc17 condition. Proliferation of CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ Tc17 cells was evaluated by CFSE dilution at the indicated time points. Tc1 cultures were also set up similarly, to be referred as a control. Unstimulated CTLA-$4^{+/+}$ or CTLA-$4^{-/-}$ OT.1 $CD8^+$ T cells were used as a negative control. Data are shown from a single experiment representative of two independent experiments. The error bars denote ± SEM. **P < 0.01, n.s.: not significant, unpaired t-test.*

4.1.2: CTLA-4 mediated cell intrinsic signaling enhance Tc17 differentiation

To exclude the possibility of sequestration of B7 ligands on APCs by CTLA-4 and thus downregulating CD28 signaling (Fig. 4.2 A top and bottom panels), an *in vitro* differentiation model was used, where $CD8^+$ T cells were stimulated with plate bound immobilised anti-CD3, anti-CD28 and anti-CTLA-4 antibodies providing an agonistic signal (58, 124). Interestingly, in a similar manner to APC stimulated cells, $CD8^+$ T cells from C57BL/6JRJ mice that were crosslinked with anti-CD3, anti-CD28 and anti-CTLA-4 (+agon. [Agonistic] αCTLA-4) displayed a 4-fold increase in the frequency of IL-17-producing cells (23.4%) compared to cells that were engaged with anti-CD3, anti-CD28, and isotype control antibody (αCD3) (4.8%) (Fig. 4.3 B) Although, with the increase in anti-CD28 concentration, the frequency of IL-17-producing cells enhanced; the cells that were treated with additional agonistic anti-CTLA-4 still displayed a significantly increased frequency of IL-17 producers (αCD3 32.6% vs +agon. αCTLA-4 43.3%) (Fig. 4.3 B). Similar results were obtained using CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ $CD8^+$ T cells which were stimulated with latex beads coated with anti-CD3, anti-CD28 and anti-CTLA-4 antibodies (Fig.4.3 A). In the absence of CD28 signals, CTLA-4 signaling still resulted in a 3-fold increase in the frequency of IL-17 producers on day 3 (Fig. 4.3 C, left and right panels). To determine the possibility of differences in activation, the expression of the activation-induced surface molecules CD44, CD25 and CD69 was analyzed. Similar expression of these molecules in the presence or absence of CTLA-4 signal excluded the possibility of differences in activation (Fig. 4.3 D). Collectively, these results indicate that CTLA-4 controlled IL-17 response is mediated by its own intracellular signaling mechanism.

4.2: mRNA expression of Tc17 hallmarks is curtailed in CTLA-4 deficient Tc17 cells

To further investigate the molecular mechanism underlying the distinct degrees of CTLA-4 mediated Tc17 differentiation, qPCR analysis was performed. Experiments were done using mRNA extracted from pooled Tc17 $CD8^+$ T cells, as well as only from purified IL-17^+ Tc17 cells that were selected using an IL-17 secretion assay. The mRNA expression of Tc17 supporting factors RORc, RORα, IL-17A/F and Runx-1 was observed to be significantly high in the whole CTLA-$4^{+/+}$ Tc17 population (not shown) as well as the purified IL-17-secreting CTLA-$4^{+/+}$ Tc17 cells (in comparison to IL-17-secreting CTLA-$4^{-/-}$ Tc17 cells) 3 days after stimulation.

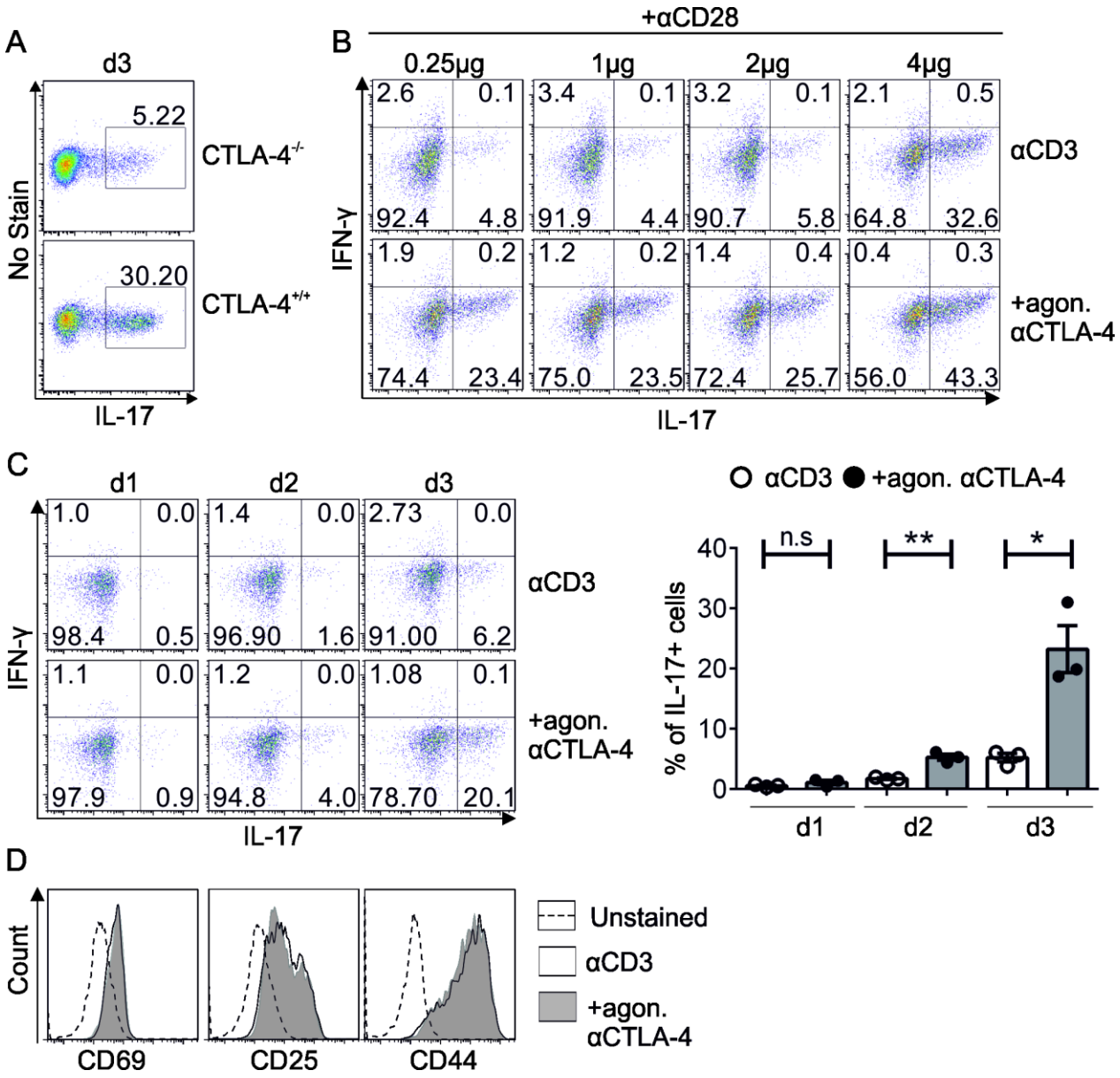

Figure 4.3: Analysis of the exclusive role of CTLA-4 in Tc17 differentiation.

(A) Tc17 cell cultures of CTLA-4$^{+/+}$ and CTLA-4$^{-/-}$ OT.1 CD8^{+} T cells were set up by using latex beads which were coated with anti-CD3 (0.75 μg/ml), *anti-CD28* (2.5 μg/ml) *and anti-CTLA-4* (8 μg/ml) *antibodies. 3 days after primary stimulation IL-17 expression was analyzed in these cells by flow cytometry. Data shown are from single experiment, representative of two independent experiments. (B) CD8^{+} T cells from C57BL/6JRJ mice were stimulated under Tc17 conditions by crosslinking the cells with plate-bound immobilized anti-CD3 and anti-CD28 in the presence (+agon. αCTLA-4) or absence (αCD3) of immobilized anti-CTLA-4. Three days after the primary stimulation, IL-17 expression in these cells was analyzed by flow cytometry. The data are from one representative experiment. (C) IL-17 and IFN-γ expression in CD3-stimulated cells in the presence or absence of CTLA-4 crosslinking was analyzed by flow cytometry every day until day 3. Cumulative staining results are shown on the right. The data are representative of three independent experiments. (D) CD8^{+} T cells from C57BL/6JRJ mice were cultured*

*as in (C) and analyzed for the surface expression of CD69, CD25 and CD44 on day 3 by flow cytometry. The data are from a single experiment that is representative of 3 independent experiments. The error bars denote ± SEM. **P < 0.01, *P < 0.05, n.s.: not significant, unpaired t-test.*

Interestingly, mRNA levels of the Tc17 supporting factors RORc, IL-21, IRF-4, and AHR were equally high in CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ Tc17 cells at early time points. These mRNAs together with the mRNA of IL-23R were rapidly down regulated in CTLA-$4^{-/-}$ Tc17 cells 3 days after stimulation. The mRNA expression of Tc1 supporting factor T-bet was barely detectable and was observed to be at similar low levels in CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ Tc17 cells. Conversely, mRNA expression of another Tc1 supporting factor Eomes had a tendency to appear slightly elevated only in CTLA-$4^{-/-}$ Tc17 cells at later time points (Fig. 4.4 A). In conclusion, IL-17-producing CTLA-$4^{+/+}$ Tc17 cells showed an upregulation of Tc17 program factors, and this might represent a higher degree of Tc17 differentiation compared to IL-17^{+} CTLA-$4^{-/-}$ Tc17 cells. In support of this possibility, prolonged higher frequency of RORγt expressing CTLA-$4^{+/+}$ Tc17 cells was also detected by flow cytometry (d2: 60% vs. 61%, d3: 40.30% vs. 20.70%; Fig. 4.4 B), whereas frequency of T-bet expressing cells remained at comparable low levels (d3: 1.36% vs.2.56%).

4.3: Role of RORγt and IRF4 in CTLA-4 mediated Tc17 differentiation

To determine if the reduced ability of CTLA-$4^{-/-}$ $CD8^{+}$ T cells to express RORγt or IRF4 is responsible for diminished Tc17 differentiation, retroviral transduction experiments were performed to overexpress RORγt and IRF4 in CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ Tc17 cells. Ectopic overexpression of RORγt promoted IL-17 production in CTLA-$4^{-/-}$ Tc17 cells, however, the percentage of IL-17 producing cells was also enhanced in CTLA-$4^{+/+}$ Tc17 cells. Thus, RORγt overexpression alone did not restore the generation of Tc17 cells in CTLA-$4^{-/-}$ cultures. Retroviral infection leading to overexpression of IRF4 alone was also not able to rescue IL-17 expression in the CTLA-$4^{-/-}$ phenotype (Fig. 4.4 C). Likely, the effects of CTLA-4 on single downstream transcription factors - at least of IRF4 and RORγt - do not govern enhanced degree of Tc17 differentiation.

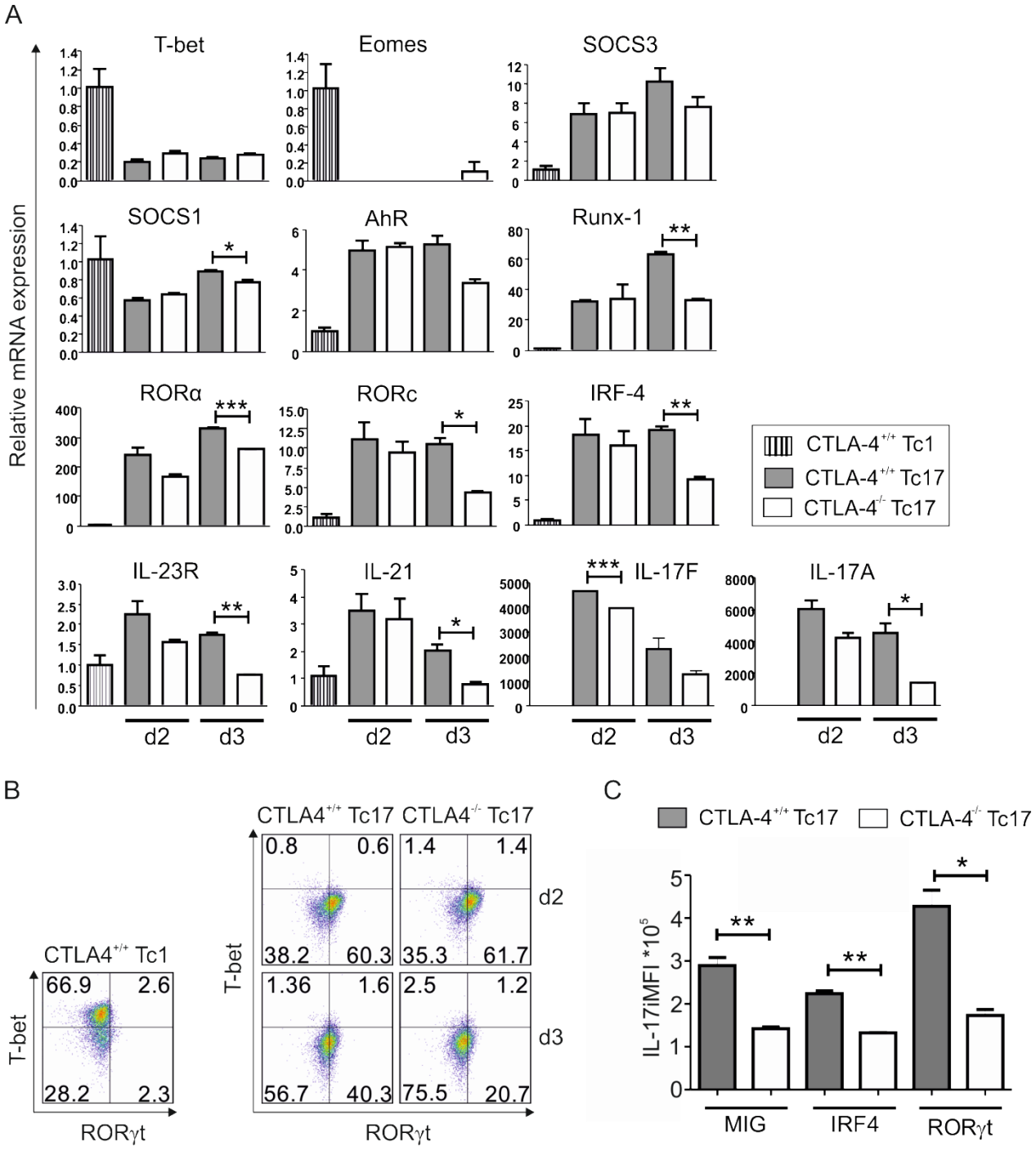

Figure 4.4: Impact of CTLA-4 on the Tc17 differentiation program.

(A) CTLA-4$^{+/+}$ (grey bars) and CTLA-4$^{-/-}$ (white bars) Tc17 cells were harvested at the indicated time points after primary activation in vitro, CTLA-4$^{+/+}$ Tc1 (white-striped bars) cells were harvested at day 3 after primary activation in vitro. Live IL-17^{+} Tc17 cells were isolated from CTLA-4$^{+/+}$ and CTLA-4$^{-/-}$ Tc17 cultures using an IL-17 cytokine secretion assay and enriched by FACS to a purity of >98%. Cell lysis, RNA extraction and RT cDNA synthesis of the enriched IL-17 producers and Tc1 cells were performed. The expression of the indicated genes, relative to the housekeeping gene HPRT, is shown as mean ± SEM

*duplicates from a single experiment representative of two performed. (B) CTLA-4$^{+/+}$ and CTLA-4$^{-/-}$ Tc17 cells were harvested on day 3 after primary activation and analyzed for expression of RORγt and T-bet by flow cytometry. Data are from a single experiment representative of three performed. (C) Role of RORγt and IRF4 in CTLA-4$^{+/+}$ and CTLA-4$^{-/-}$ Tc17 cells in inducing high IL-17. CTLA-4$^{+/+}$ and CTLA-4$^{-/-}$ naive CD8^{+} T cells were primed in Tc17 conditions and 24 and 36 h later infected twice with retrovirus overexpressing MSCV-IRES-GFP (MIG), RORγt-IRES-GFP and IRF4-IRES-GFP. 12 h after 2nd virus infection the cells were washed and restimulated again with IL-17 inducing cytokine milieu and 48 h later iMFI of GFP expressing IL-17 producing cells was analysed by flow cytometry as described in materials and methods. Data are shown as mean ± SEM, duplicates from a single experiment representative of two perfprmed. ***P < 0.001, **P < 0.01, *P < 0.05, unpaired t-test.*

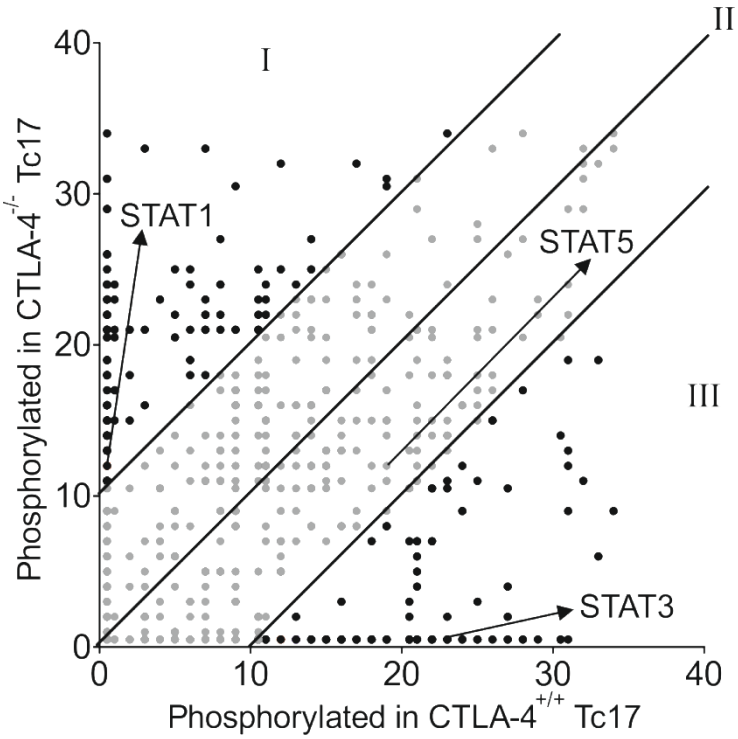

Figure 4.5: PepChip analysis of the effects of CTLA-4 in Tc17 differentiation.

Dot plot representing the phosphorylation status of the kinase-specific peptide substrates spotted on the PepChip array. Different kinase activities in the lysates from the CTLA-4$^{+/+}$ and CTLA-4$^{-/-}$ Tc17 cells are shown using a ranking method; each spot represents the extent of phosphorylation of a specific peptide substrate. Using a ranking method, a bisymmetric distribution of peptides is generated, in which phosphorylation was either significantly increased or decreased by CTLA-4 signaling. Peptide substrates that were phosphorylated in the absence of CTLA-4 signaling are reflected by an equivalent peptide with altered or unaltered phosphorylation in response to CTLA-4 signaling. The ranks of the differentially phosphorylated peptides of interest (STAT3, STAT5, and STAT1) are marked by arrows. Spots representing peptides with significantly decreased (I), increased (III), or unaltered (II) phosphorylation as a result of CTLA-4 signaling are shown.

4.4: PepChip analysis to determine the target molecules of CTLA-4 in Tc17 differentiation

To identify target molecules of CTLA-4 signal transduction, the lysates of CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ Tc17 cells (that had been stimulated for 3 days) were analyzed using a peptide array (PepChip) with different kinase consensus substrates spotted in triplicate. Analysis of the kinomic profile revealed substantial differences in the phosphorylation of kinase substrates in the presence and absence of CTLA-4 signal. Interestingly, tyrosine phosphorylation of the substrate representing STAT3 was increased in CTLA-$4^{+/+}$ Tc17 cells compared to CTLA-$4^{-/-}$ Tc17 cells. In contrast, the tyrosine phosphorylation of the substrate for STAT1, which is known to inhibit differentiation of IL-17 producing cells, was enhanced in CTLA-$4^{-/-}$ Tc17 cells. The phosphorylation of the consensus substrate for STAT5, which is also known to inhibit differentiation of IL-17 producing cells, was similar under both conditions (Fig. 4.5).

4.5: CTLA-4 enhances STAT3 activity in Tc17 cells

To support the results of PepChip analysis, the effect of CTLA-4 signaling in regulating the phosphorylation levels of the target proteins was analyzed by flow cytometry. As shown in figure 4.5 CTLA-4 signaling enhances phosphorylation of STAT3 and partially inhibits STAT1 phosphorylation without any effect on STAT5 (Fig. 4.6 A). Published data have shown that STAT5 competes with IL-17 enhancer STAT3 and directly binds to the IL-17a promoter region and functions as a direct transcriptional repressor of IL-17 (82, 125). Hence, the binding of both STAT proteins to the IL-17 promoter was evaluated using chromatin immunoprecipitation (ChIP) assays followed by quantitative PCR (qPCR). The absence of CTLA-4 signaling led to enhanced binding of STAT5 on the IL-17 promoter region. The evidence of STAT5 binding correlated with significantly less binding of STAT3 at the same IL-17 promoter region. In contrast, STAT3 binding to the IL-17 promoter was increased by 3-fold compared to STAT5 in the cells that were crosslinked with CTLA-4 antibodies (Fig. 4.6 B).

It has been previously described that STAT3 is necessary for the expression of Tc17 type master regulators RORα and RORγt. Also, differentiation of IL-17 producing cells was found to be greatly compromised in STAT3-deficient cells. Thus, the impact of CTLA-4-induced STAT3

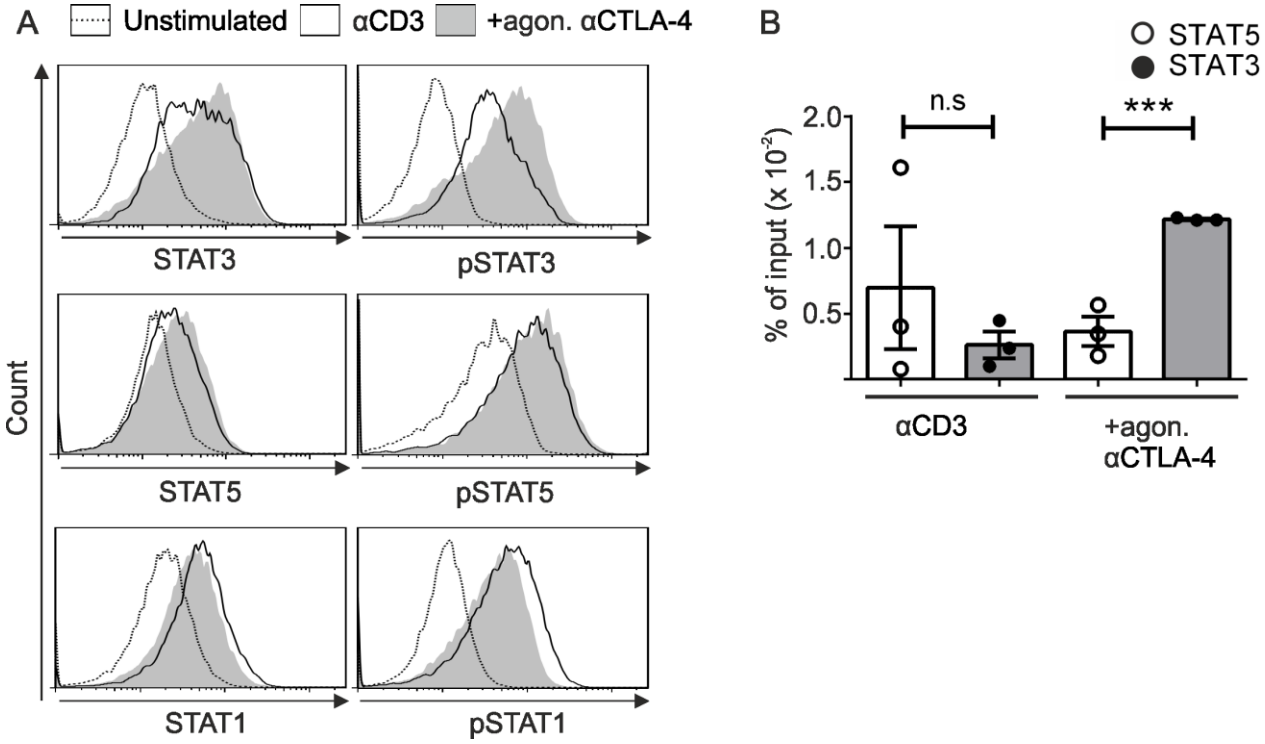

Figure 4.6: CTLA-4-regulated STAT phosphorylation determines Tc17 differentiation.

*(A) CD8$^+$ T cells from C57BL/6JRJ mice primed under Tc17 conditions with anti-CD3 in the presence or absence of additional CTLA-4 crosslinking for 3 days, followed by 10 min stimulation with IL-6+IL-23 (STAT3) or IFN-γ (STAT1) or IL-2 (STAT5). The cells were subsequently harvested, and analyzed for the expression of total and phosphorylated STAT3, STAT1 and STAT5. The data are from a single experiment that is representative of two independent experiments. (B) ChIP analysis of Tc17 cells that were stimulated with anti-CD3 in the presence or absence of additional CTLA-4 crosslinking for 3 days. Tc17 cells were stimulated with IL-6+IL-23 or IL-2 for 30 min, and protein-DNA complexes were crosslinked with formaldehyde and immunoprecipitated with anti-STAT3 or anti-STAT5. The bound DNA was purified and amplified by quantitative PCR with primers designed for the IL-17a promoter site. The results are presented relative to the input DNA. The data are representative of three independent experiments. The error bars denote ± SEM. ***P < 0.001, n.s.: not significant, unpaired t-test.*

activity on Tc17 differentiation was determined next by inactivating STAT3 with S31-201 a chemical probe inhibitor, which selectively blocks STAT3 phosphorylation, dimerization, DNA binding, and STAT3-dependent transcription. Flow cytometric analysis of CTLA-4 targets revealed that inactivation of STAT3 in cells crosslinked with CTLA-4 reduced the 2-fold enhanced expression of the Tc17 signature transcription factor RORγt (+agon. αCTLA-4 DMSO vs +agon. αCTLA-4 S31-201) (Fig. 4.7 B, left and right panels). Similarly, the 4-fold increase in

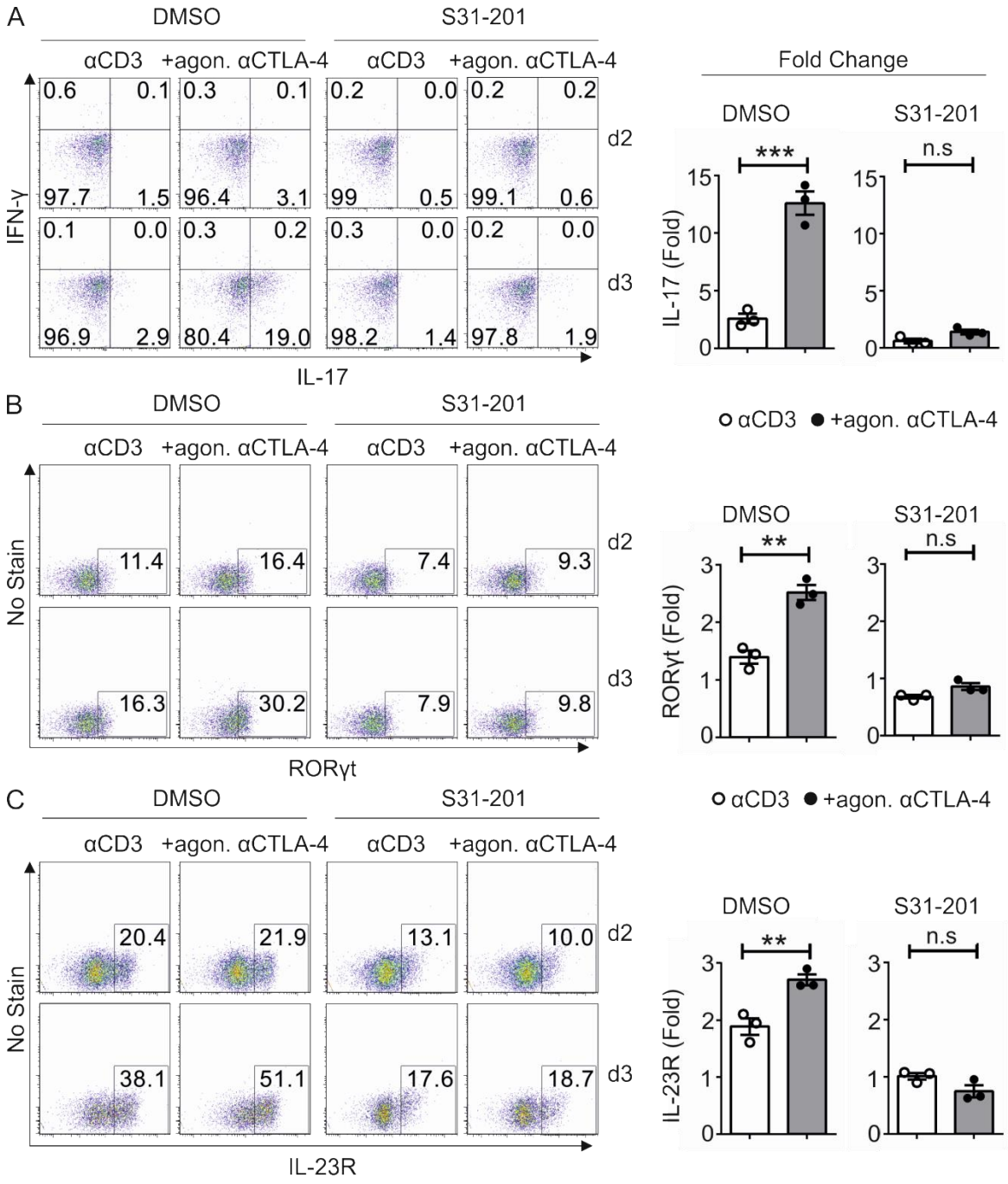

Figure 4.7: Impact of STAT3 on CTLA-4-mediated Tc17 differentiation.

*$CD8^+$ T cells from C57BL/6JRJ mice primed under Tc17 conditions with anti-CD3 in the presence or absence of additional CTLA-4 crosslinking were untreated (DMSO) or treated with the STAT3 inhibitor (S31-201) 24 h after stimulation. The expression levels of the Tc17 signature molecules IL-17, IFN-γ (A), RORγt (B), and IL-23R (C) were analyzed by flow cytometry at the indicated time points (left), and the d3 results are shown as the fold increase compared to the d2 control cells (right). The data are from a single experiment that is representative of three independent experiments. The error bars denote ± SEM. ***P < 0.001, **P < 0.01, n.s.: not significant, unpaired t-test.*

the expression of IL-17 was also significantly reduced upon STAT3 inactivation in cells crosslinked with CTLA-4 (Fig. 4.7 A, left and right panels). In addition, CTLA-4 mediated significant increase in IL-23R expression was reduced by more than half applying STAT3 inactivation (Fig. 4.7 C, left and right panels).

Taken together, these results suggest that CTLA-4 promotes STAT3 activation and binding to the IL-17 promoter region and thereby enhance the expression of IL-17 in Tc17 cells. Consequently, the data also show that CTLA-4 induces the expression of RORγt protein which is encoded as the STAT3-dependent target gene, along with IL-23R, which is essential for maintaining the Tc17 phenotype.

4.6 CTLA-4 delays Tc17 lineage plasticity following recall response *in vitro*

Even though it is well established that inhibiting CTLA-4 enhances Tc1 differentiation (88, 89), Tc17 cells lacking CTLA-4 signal did not acquire classical Tc1 features such as IFN-γ production after primary activation (Fig. 4.2, 4.3). Hence, the role of CTLA-4 in regulating the lineage plasticity of Tc17 cells to express Tc1 characteristics was then monitored. To evaluate the Tc1 conversion, primary differentiated Tc17 cells were re-stimulated with the Tc1 skewing cytokines IL-12 and IL-2 to determine the downstream targets of the Tc1 lineage. Initially, flow cytometric analysis showed that the CTLA-4-crosslinked cells more effectively retained their Tc17 profile, even under Tc1-skewing conditions, compared to the activated control cells (Fig. 4.8 A left and right panels). To further investigate the molecular mechanism underlying the distinct degrees of CTLA-4 mediated control of Tc17 plasticity, qPCR analysis was performed by using mRNA extracted from Tc17 cells restimulated under Tc1 environment. In addition to the reduced IL-17 expression observed by flow cytometry, a 2-fold decrease in the mRNA levels of the Tc17-supporting factors HIF-1α and IRF4 was observed in the activated control cells (αCD3) (Fig. 4.8 B). The mRNA expression of important Tc1-supporting transcription factor Eomes, but not that of T-bet, was elevated in the re-activated control cells that did not receive a CTLA-4 signal. Flow cytometric analysis of T-bet and Eomes proteins extended these mRNA findings (Fig. 4.8 C). In addition, similar results were observed with other Tc1-supporting molecules, such as IFN-γ (Fig. 4.8 B). Together, these results indicate that CTLA-4 signaling controls Tc17 lineage plasticity by inhibiting Tc1 differentiation and delaying downregulation of Tc17 supporting factors.

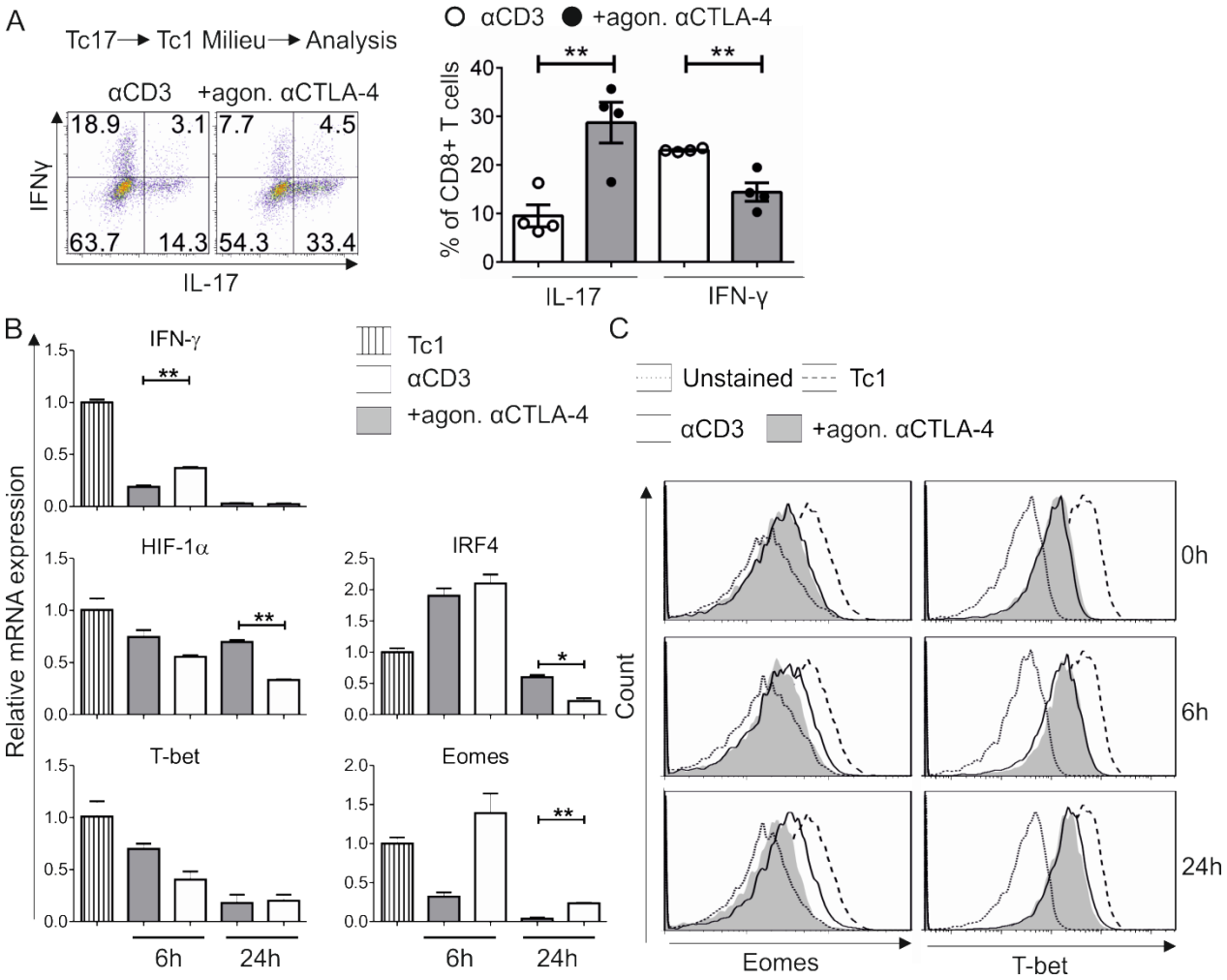

Figure 4.8: Role of CTLA-4 in regulating Tc17 stability and plasticity.

*(A) Tc17 cells that had been stimulated for 3 days (as in Fig.4.3 C) were washed twice with medium and stimulated again with fresh plate-bound immobilized anti-CD3 in the presence or absence of additional crosslinking with anti-CTLA-4 coupled with IL-12 and IL-2 cytokines. Twenty-four hours later, IL-17 and IFN-γ expression in these cells was determined using flow cytometry (left). Cumulative staining results are shown on the right. The data are representative of four independent experiments. (B) Tc17 cells were harvested on day 3 after primary stimulation (considered as 0 h before re-stimulation), re-activated as described above, and harvested at the indicated time points. The harvested cells were lysed, RNA was extracted, and RT was used to synthesize cDNA. The expression levels of the indicated genes relative to the housekeeping gene, HPRT, are shown as the mean ± SEM of duplicates from a single experiment that is representative of three experiments. (C) Tc17 cells were stimulated as in (B) and analyzed for the expression of Eomes and T-bet by flow cytometry at the indicated time points. The data are from a single experiment that is representative of two independent experiments. The error bars denote ± SEM. **P < 0.01, *P < 0.05, unpaired t-test.*

4.7 CTLA-4 induced STAT3 activity stabilizes Tc17 differentiation

In accordance with the recognized importance of STAT3 in CTLA-4-mediated Tc17 differentiation, the role of STAT3 in controlling CTLA-4-mediated Tc17 plasticity was evaluated next. Interestingly, unlike in primary stimulated cells there was no difference of STAT3 phosphorylation in Tc17 cells restimulated under Tc1 conditions, despite crosslinking with CTLA-4. Even though STAT3 and STAT5 phosphorylation remained similar with and without CTLA-4 crosslinking after re-stimulation with Tc1-inducing cytokines (Fig. 4.9 A), a 3-fold increase in STAT5 binding to the IL-17 promoter was observed by ChIP followed by qPCR in cells that were not crosslinked with CTLA-4 antibodies. In contrast, STAT3 binding to the IL-17 promoter was enhanced by 2-fold in the cells that were crosslinked with CTLA-4 antibodies (Fig. 4.9 B). It has been previously shown that the relative protein activation abundance of STAT1 versus STAT3 may reciprocally influence the activity of the other protein (79, 80). Consequently, the phosphorylation of STAT1 was measured in Tc17 cells restimulated with Tc1-inducing cytokines. Interestingly, a significant increase in STAT1 phosphorylation was observed in the cells that were not crosslinked with CTLA-4 (Fig. 4.9 A). The normalized pSTAT3 and pSTAT1 levels were used to compare the pSTAT3/pSTAT1 ratio. Tc17 cells crosslinked with CTLA-4 displayed a 2-fold increase in the pSTAT3/pSTAT1 ratio compared to control cells (Fig. 4.9 C).

As the phosphorylated tyrosine residue on cytoplasmic tail of CTLA-4 makes a potential binding site for SH2 domain containing STATs, affinity-purification mass spectrometry (AP-MS) was used in order to search for phosphorylation-dependent interaction partners. Therefore, synthetic peptides containing the YVKM motif (tyrosine-phosphorylated and non-phosphorylated) were immobilized on beads and incubated with lysates from anti-CD3/CD28 stimulated Tc17 cells. After several washing steps enriched proteins were eluted and quantified by nano LC-MS/MS using the ^{18}O-labelling method with the help of Dr. E. Krause and Dr. B. Kuropka (FMP Berlin). While the previously known binder p85 subunit of PI3K (63) significantly enriched to the phosphorylated YVKM with a $^{18}O/^{16}O$ (phosphorylated/nonphosphorylated peptide) ratio of > 100, neither STAT1 nor STAT3 showed direct association with the phosphorylated cytoplasmic tail of CTLA-4.

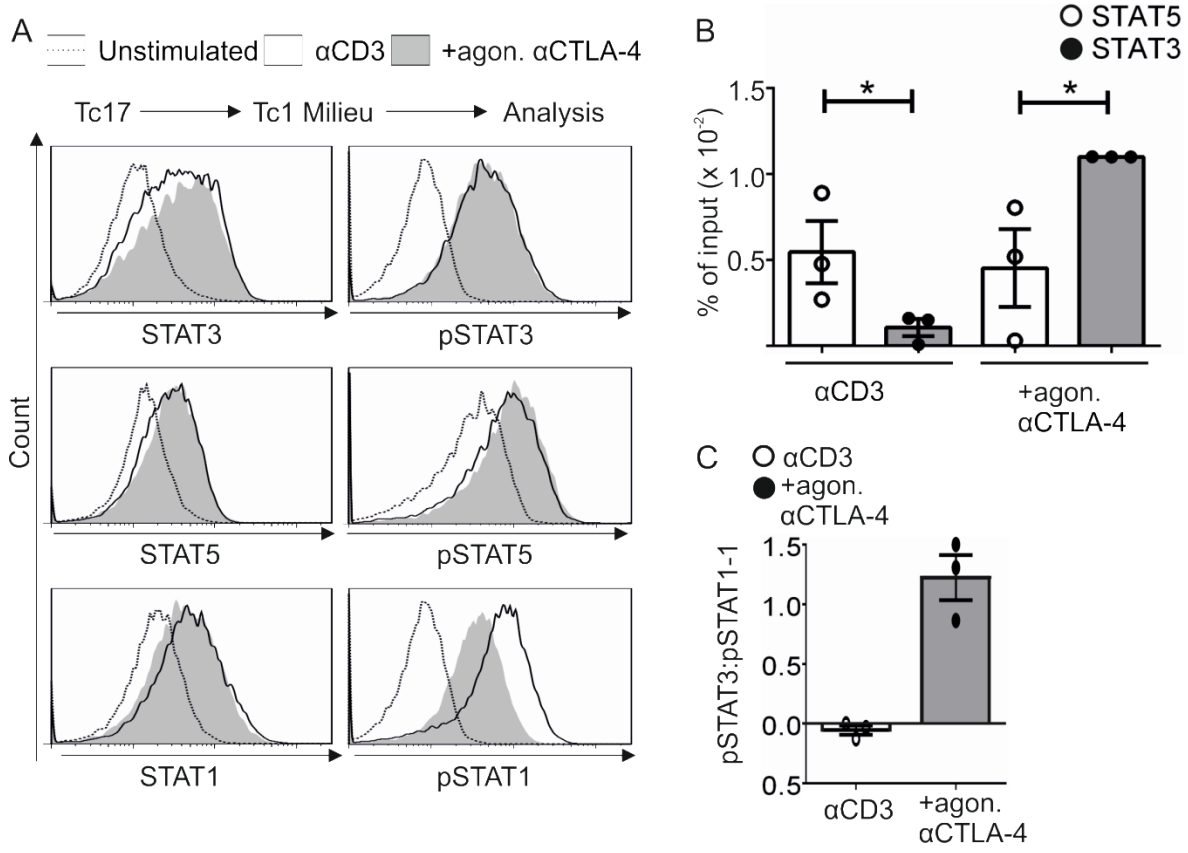

Figure 4.9: CTLA-4-enhanced STAT3 activity stabilizes Tc17 differentiation.

*(A) Tc17 cells were re-activated in a Tc1 environment for 24 h (as described in Fig. 4.8), followed by 10 min stimulation with IL-6+IL-23 (STAT3) or IFN-γ (STAT1) or IL-2 (STAT5). The cells were subsequently harvested, and analyzed for the expression of the total and phosphorylated forms of STAT3, STAT1 and STAT5. The data are from a single experiment that is representative of two independent experiments. (B) ChIP analysis of Tc17 cells that were re-stimulated with Tc1-inducing cytokines for 24 h. The re-activated Tc17 cells were stimulated with IL-6+IL-23 or IL-2 for 30 min, and the protein-DNA interactions were crosslinked with formaldehyde and immunoprecipitated with anti-STAT3 or anti-STAT5. The bound DNA was purified and amplified by quantitative PCR with primers designed for the IL-17a promoter site. The results are presented relative to the input DNA. The data are representative of three independent experiments. (C) STAT3 and STAT1 phosphorylation levels in the re-stimulated cells were normalized to those in unstimulated cells, and the pSTAT3/pSTAT1 ratio was compared among cells that were crosslinked with anti-CD3 in the presence or absence of CTLA-4. The data are representative of three independent experiments. The error bars denote ± SEM. *P < 0.05, unpaired t-test.*

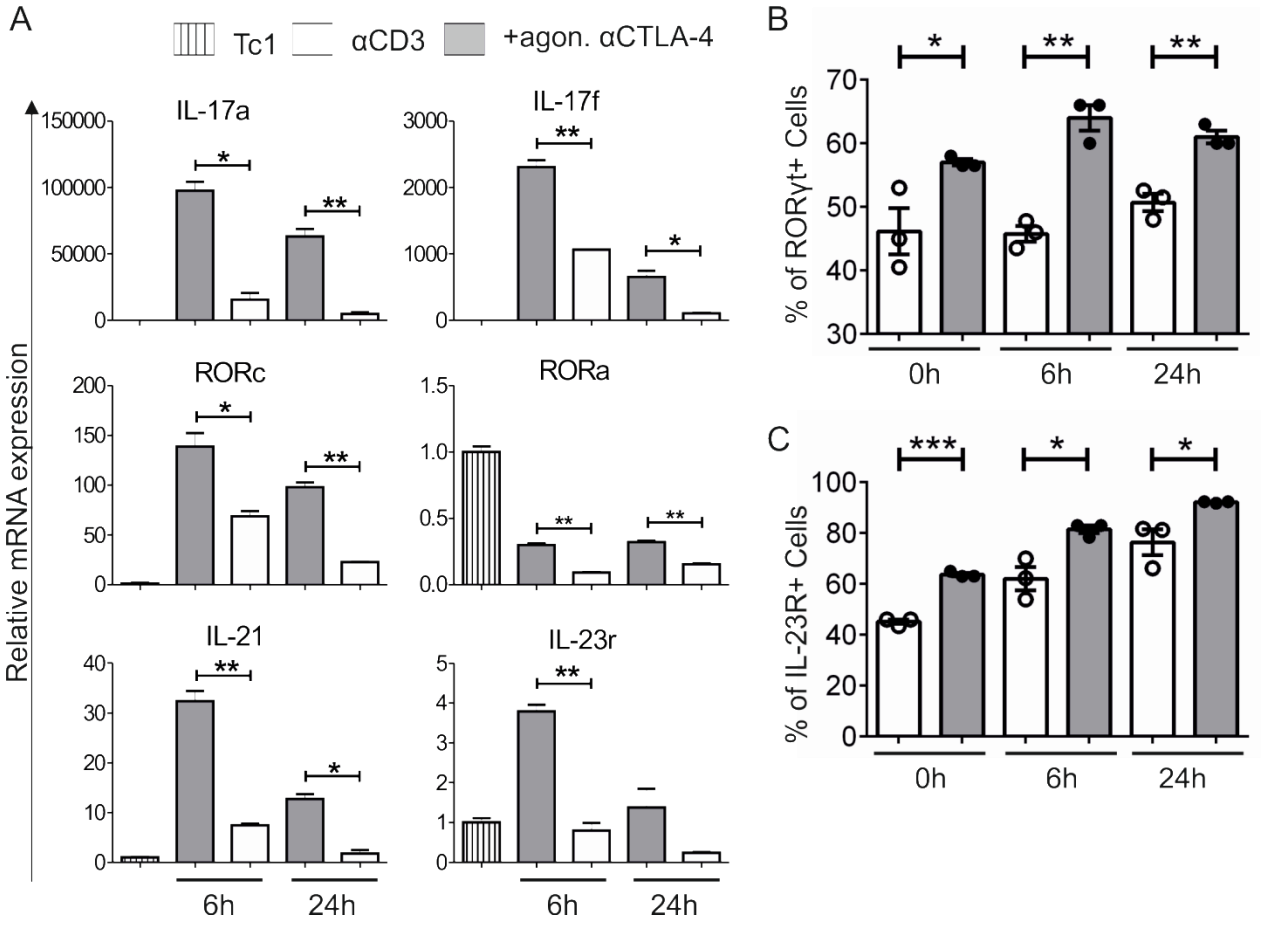

Figure 4.10: CTLA-4 delays downregulation of STAT3 dependent target genes

*(A) Tc17 cells were harvested on day 3 after primary stimulation (considered 0 h before re-stimulation), re-stimulated (as described in Fig. 4.8), and harvested at the indicated time points. The harvested cells were lysed, RNA was extracted, and RT was used to synthesize cDNA. The expression levels of the indicated genes relative to the housekeeping gene HPRT are shown as the mean ± SEM of duplicates from a single experiment that is representative of three experiments. (B, C) Tc17 cells were stimulated as in (A) and analyzed for the expression of (B) RORγt and (C) IL-23R by flow cytometry at the indicated time points. The data are representative of three independent experiments. The error bars denote ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05, unpaired t-test.*

To determine whether CTLA-4 enhanced STAT3 activity induces transcription of other STAT3 dependent target genes that helps to stabilize Tc17 differentiation, qPCR analysis was performed. The increased mRNA expression of the Tc17-supporting factors IL-17, RORc, IL-21 and IL-23R in the qPCR analysis (Fig. 4.10 A) correlated well with the relative increase in the amount of pSTAT3 in cells that were crosslinked with CTLA-4 antibodies (Fig. 4.9 C). In support of the

qPCR data, increased RORγt and IL-23R expression were also detected by flow cytometry in the cells that underwent CTLA-4 crosslinking (Fig. 4.10 B and C).

Collectively, these results indicate for the first time that CTLA-4 mediates an increase in the relative amount of pSTAT3 as compared to pSTAT1 and thus helps in stabilizing Tc17 differentiation. This effect is associated with enhanced RORc transcription and a reduced susceptibility for Tc17 lineage plasticity.

4.8: CTLA-4 deficient Tc17 cells efficiently controls tumor progression

Considering the above-reported induction of STAT3 activity by CTLA-4, it was hypothesized that CTLA-4-deficient Tc17 cells, which cannot efficiently upregulate STAT3 activity, strongly support tumor rejection *in vivo*. For this purpose, the capacity of adoptively transferred CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ Tc17 cells were analyzed to control the progression of pre-established melanoma by B16 $OVA_{257-264}$-expressing melanoma cells in a mouse model. To differentiate adoptively transferred congenic OT.1 CD45.2 $CD8^{+}$ T cells, recipient tumor-bearing mice were used on a Ly5.1 background. Tumor progression was measured for up to 6 days following adoptive transfer of Tc17 cells (Fig. 4.11 A). Tumor outgrowth was progressing dramatically in PBS-treated tumor-bearing mice. In mice receiving adoptively transferred Tc17 cells, tumor growth measurements clearly showed that CTLA-$4^{-/-}$ Tc17 cells significantly restricted tumor progression, whereas CTLA-$4^{+/+}$ Tc17 cells were not able to control the tumor growth (d2 to d6, Fig. 4.11 B and C left panel). CTLA-$4^{-/-}$ Tc17 cells indeed displayed a 2-fold higher efficiency in controlling tumor progression compared to CTLA-$4^{+/+}$ Tc17 cells (Figure 4.11 C right panel). Interestingly, in addition to enhanced anti-tumor activity, the *in vivo* re-stimulated CTLA-$4^{-/-}$ Tc17 cells displayed enhanced expression of Tc1-like characteristics; for example, a 4-fold higher frequency of IFN-γ/TNF-α double producers was observed in CTLA-$4^{-/-}$ Tc17 cells compared to CTLA-$4^{+/+}$ Tc17 cells (Fig. 4.11 D). These kind of double producers are well known to control infection and tumor progression in mice and humans (120, 126–128). Collectively, these results show that CTLA-4 deficiency *in vivo* enhances the functional and transcriptional plasticity of Tc17 cells and thus profoundly augments their anti-tumor activity.

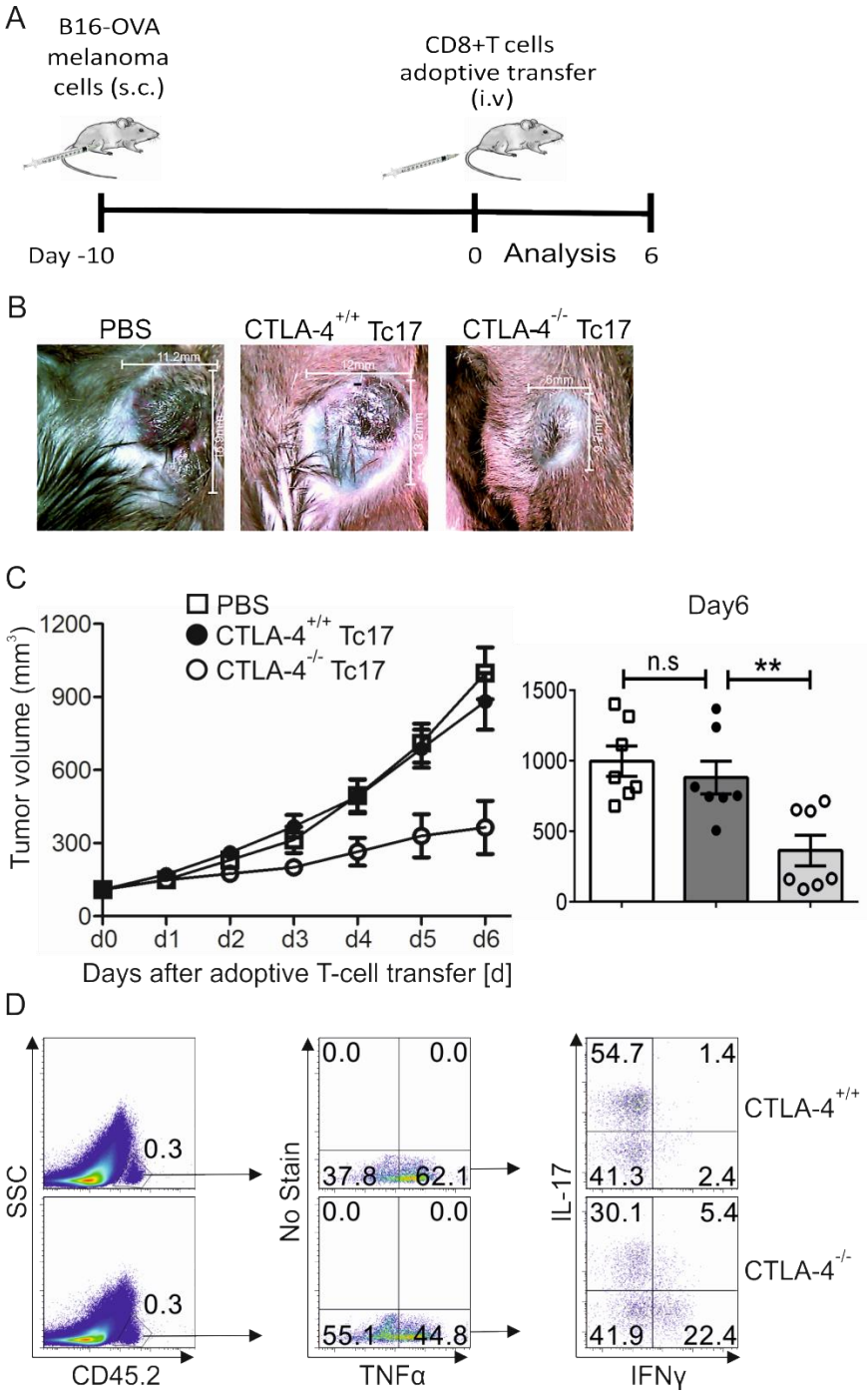

Figure 4.11: Response of CTLA-4$^{+/+}$ and CTLA-4$^{-/-}$ Tc17 cells in controlling melanoma.

(A) Schematic of the tumor experiment. Recipient Ly5.1 mice were s.c. injected with B16-OVA melanoma cells. Approximately 10 days later, when a visible tumor was present, CTLA-4$^{+/+}$ and CTLA-4$^{-/-}$ OT.1 CD8^{+} T cells that had been stimulated under Tc17 conditions for 3 days were adoptively transferred into the recipient mice through intravenous (i.v.) injection, and tumor growth was measured for the next 6 days. (B) Pictorial representation of tumor size in the recipient mice on day 6 after adoptive transfer with PBS or CTLA-4$^{+/+}$ or CTLA-4$^{-/-}$ OT.1 Tc17 cells. (C) Tumor growth in the mice receiving PBS or CTLA-

*$4^{+/+}$ or CTLA-$4^{-/-}$ OT.1 Tc17 cells was measured on a daily basis until day 6. Results represent ± SEM of seven mice per group from three separate experiments Cumulative bar graphs of tumor volume in the recipient mice on day 6 are shown on the right. Results represent ± SEM of seven mice per group from three independent experiments (D) Adoptively transferred CD45.2^{+} cells were surface stained ex vivo in the splenocytes of the tumor-bearing mice 6 days after the transfer of CTLA-$4^{+/+}$ or CTLA-$4^{-/-}$ OT.1 Tc17 cells and were analyzed for TNF-α, IL-17 and IFN-γ production by flow cytometry. The data are from one one representative experiment. The error bars denote ± SEM. **P < 0.01, n.s.: not significant, Mann-Whitney U-test.*

4.9: Blockade of CTLA-4 enhances cytotoxic activity of Tc17 cells

Recognizing the role of CTLA-4 in regulating the functional and transcriptional plasticity of Tc17 cells, it was then hypothesized that CTLA-4 may also regulate the cytotoxic potential of Tc17 cells. To determine the cytotoxic activity of CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ Tc17 cells an *in vitro* cytotoxicity assay was performed. T cell depleted splenocytes stained with CFSE at a concentration of 0.25 µM and 5 µM were considered as $CFSE^{low}$ and $CFSE^{high}$ cells respectively. $CFSE^{high}$ cells were loaded with OVA peptide and were considered as target cells. A mixture of equal amount of $CFSE^{low}$ and $CFSE^{high}$ cells were added to the pre-differentiated Tc17 or Tc1 cells (effector cells) in effector to target cell ratios of 1:1 and 3:1 (E/T ratio). 18 h after restimulation, about 20% increase in target cell specific lysis by CTLA-$4^{-/-}$ Tc17 cells was observed, compared to CTLA-$4^{+/+}$ Tc17 cells (Fig. 4.12 A). These results indicate that CTLA-$4^{-/-}$ Tc17 cells have superior cytotoxicity compared to CTLA-$4^{+/+}$ Tc17 cells.

Considering the previous reported observation that inactivation of STAT3 enhances the cytotoxicity of $CD8^{+}$ T cells (129, 130), the role of STAT3 in regulating cytotoxic potential of Tc17 cells which get a CTLA-4 signal was evaluated next. For this purpose, the expression of a degranulation marker CD107a, which is well known to determine cytolytic activity of $CD8^{+}$ T cells (131) was analysed in restimulated Tc17 cells. Flow cytometric analysis showed that the expression of CD107a was strongly diminished in CTLA-4-crosslinked Tc17 cells which were restimulated under Tc1 conditions (Fig. 4.12 B). Interestingly, upon STAT3 inactivation diminished expression of CD107a was significantly enhanced in CTLA-4-crosslinked Tc17 cells (Fig. 4.12 B). Collectively, these results indicate that CTLA-4 mediates an increase in the relative amount of pSTAT3 which helps to stabilize Tc17 differentiation and hamper cytotoxic activity of Tc17 cells.

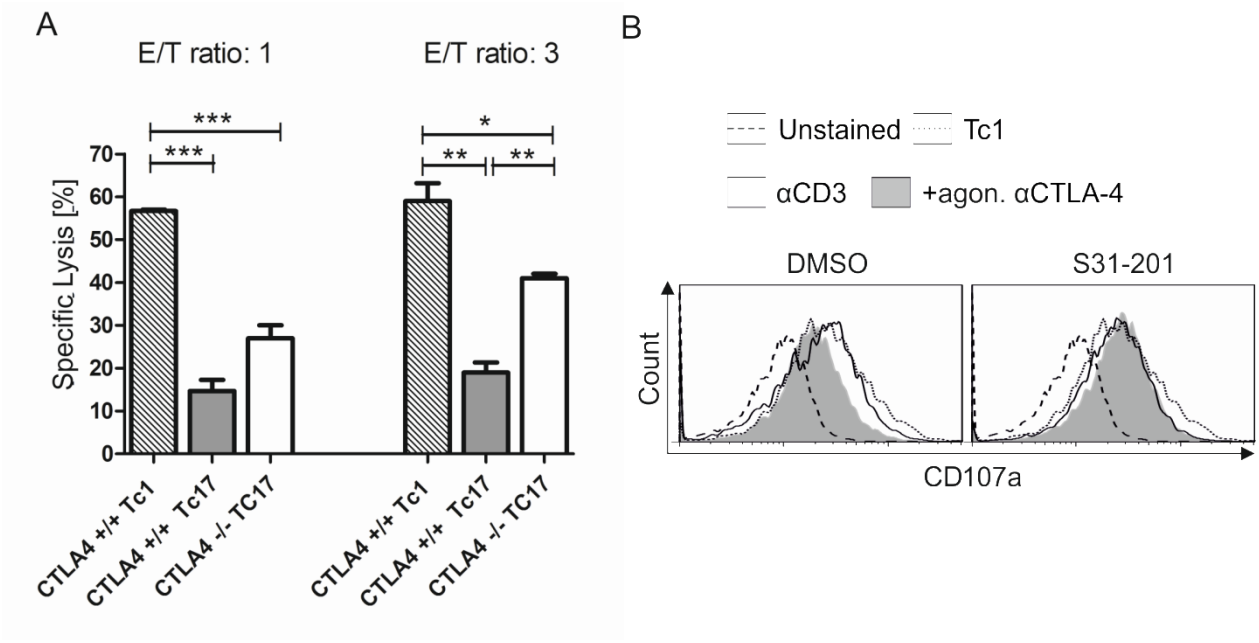

Figure 4.12: CTLA-4 regulates the cytotoxic activity of Tc17 cells.

*(A) In vitro cytotoxicity assay of primarily differentiated CTLA4$^{+/+}$ and CTLA4$^{-/-}$Tc17 cells. Naive CTLA-4$^{+/+}$ or CTLA-4$^{-/-}$ CD8^{+} T cells were cultured under Tc17 conditions for 3 days. T cell depleted splenocytes were stained separately with different concentrations of CFSE (5 µM and 0.25 µM) and named accordingly as CFSE^{high} and CFSE^{low}. Only the CFSE^{high} cells were labeled with OVA peptide and were considered as target cells. CFSE^{low} and CFSE^{high} cells (target cells) were pooled together at 1:1 ratio and a mixture of these cells were added to the pre differentiated CD8^{+} cells (effector cells) at indicated effector to target cell ratio [E/T ratio]. After 18 h of restimulation, frequency of cells with different CFSE intensity was analyzed by flow cytometry. The specific lysis of the target cells is shown. Data are shown as mean ± SEM, triplicates from a single experiment representative of two performed. (B) 3 day cultured Tc17 cells in the presence (S31-201) or absence (DMSO) of STAT3 inhibitor were re-stimulated as in Figure. 4.8 and analyzed for the expression of degranulation-associated surface molecule CD107a on CD8^{+} T cell. The data are from a single experiment that is representative of three independent experiments. The error bars denote ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05, n.s.: not significant, unpaired t-test.*

5. Discussion

CTLA-4 has been recently reported to inhibit Tc1 (CTL) type $CD8^+$ T cell responses (88, 89). However as the role of CTLA-4 in regulating other $CD8^+$ T cell subsets is not completely understood, the present study was designed to identify the role of CTLA-4 in regulating IL-17 producing $CD8^+$ T cells, known as Tc17 cells. This study is the first to report that CTLA-4 enhances Tc17 differentiation by regulating the third signal for $CD8^+$ T cell activation, namely, cytokine receptor signaling, affecting STAT molecules. It was also found that, CTLA-4 stabilizes Tc17 differentiation and increases the resistance of Tc17 cells to plasticity by inhibiting development of Tc1-like functionality *in vivo*. Taken together, this study provides new evidence for the differential capacity of CTLA-4 to control $CD8^+$ T cell immunity.

5.1 CTLA-4 delivers an intrinsic signal facilitating Tc17 differentiation

The initial point of investigation presented here, was the finding that surface expression of CTLA-4 on Tc17 cells was dramatically increased as in Tc1 cells (89). This finding suggested that CTLA-4 could play a central role in regulating Tc17 responses in a similar manner to Tc1 responses.

CTLA-4 has been shown to exert various cell-intrinsic as well as cell-extrinsic effects (56, 123, 132–134). The results of the work presented here demonstrate for the first time that CTLA-4 which is known to inhibit IFN-γ expression in $CD8^+$ T cells also enhances IL-17 expression when stimulated in a Th17 cytokine milieu (118). The finding is supported by the data showing that the effect was detectable starting from day 2 (not on day 1), when CTLA-4 surface expression was detectable (Fig. 4.2 A, 4.3 C). IFN-γ was observed to be similarly expressed at a very low level in CTLA-$4^{-/-}$ and CTLA-$4^{+/+}$ Tc17 cells indicating that the diminished IL-17 expression in CTLA-$4^{-/-}$ Tc17 cells had no co-relation with IFN-γ expression. As the cells used for these experiments were strictly sorted on a naive phenotype to a purity of greater than 98 %, the effect of CTLA-4 on Tc17 differentiation is most likely not due to difference in activation status. Additionally, the CTLA-$4^{-/-}$ mice not older than 7 weeks were used in these experiments as a source for $CD8^+$ T cells. Others have shown that $CD8^+$ T cells from CTLA-$4^{-/-}$ mice at this age are in naive stage (135). CTLA-$4^{-/-}$ Tc17 cells displayed reduced IL-17 production even with various Ag-concentrations indicating that the decreased IL-17 production of CTLA-$4^{-/-}$ Tc17

cells is independent of distinct Ag/TCR avidity (122). The distinct difference in IL-17 production was also consistent even in the absence of CD80 and CD86 binding to CTLA-4 (Fig. 4.3 A-C), indicating that the cell extrinsic effect of CTLA-4 on APCs which was demonstrated in Treg cells is inefficient in Tc17 cells (56, 136). Previously, it was shown by others that the IL-17 production in $CD4^+$ T cells is enhanced through blockade of CTLA-4-B7 interaction using specific monoclonal antibodies (137). If this enhancement of IL-17 production could be attributed to $CD8^+$ Tc17 cells, enhanced frequencies of $IL\text{-}17^+$ cells in CTLA-$4^{-/-}$ $CD8^+$ T cells would have been expected, but the opposite was the case. This enhanced IL-17 production by $CD4^+$ T cells under CTLA-4 blockade might be explained by the experimental setting which may produce side effects of using whole αCTLA-4 abs leading to crosslinking instead of blockade. Other data indicated that the altered CD28 signals affect Th17 differentiation (138). However, the distinct difference in IL-17 expression was observed even in the absence of CD28 signaling in cells crosslinked only with anti-CD3 and anti-CTLA-4 (Fig. 4.3 C). Similar expression of the activation-induced surface molecules CD44, CD25 and CD69 in cells crosslinked with or without CTLA-4 antibodies made it unlikely that the effects are due to differences in activation. Thus, the data together with the literature demonstrate that after its initiation by T cell activation, in the presence of IL-17 inducing cytokines CTLA-4 mediates – at least mainly - a cell-intrinsic effect that enhances differentiation of the Tc17 program.

5.2 CTLA-4 restricts cytotoxic function of Tc17 cells

Tc17 cells are known to be phenotypically unstable (117, 139). In this study CTLA-4 was observed to play a central role in controlling Tc17 stability *in vitro* and *in vivo* (Fig. 4.8A and 4.11D). *In vivo*, CTLA-$4^{-/-}$ Tc17 cells displayed a significant ability to control tumor progression (Fig. 4.11 B and C). Indeed, adoptively transferred CTLA-$4^{-/-}$ Tc17 cells gave rise to an increased number of IFN-γ/TNF-α co-producers (Fig. 4.11 D), which correlates well with tumor rejection (140). However, it needs to be further investigated whether the IFN-γ/TNF-α producing cells are transitioned from former IL-17 producing cells and if this transition is enhanced in CTLA-4 deficient Tc17 cells. Previous studies have shown that IL-12 plays a crucial role in the conversion of Tc17 cells towards IFN-γ-producers and consequently improved their anti tumor effects *in vivo* (141). Thus, the conversion of IL-17 producing cells into IFN-γ/IL-17 and IFN-γ/TNF-α

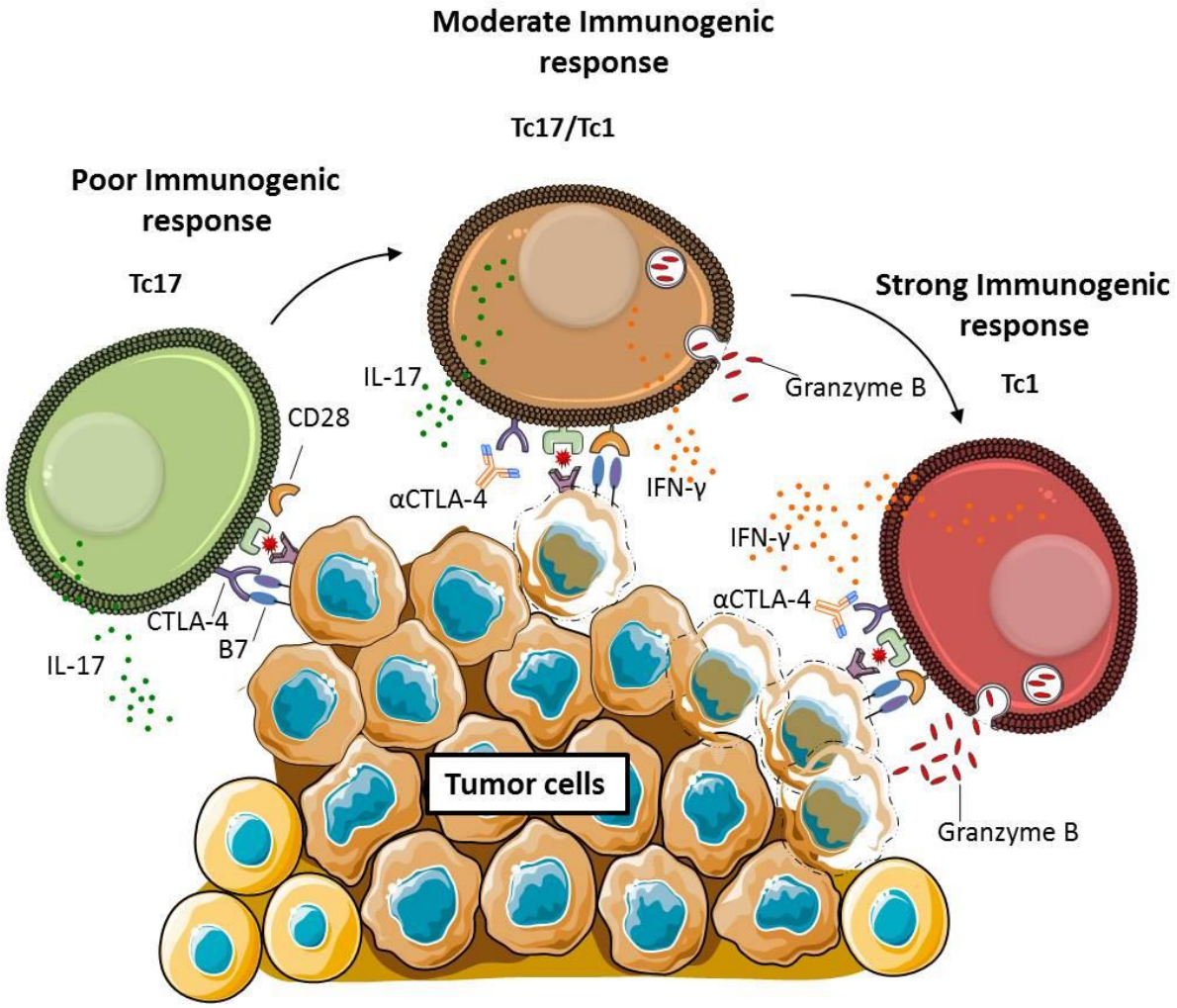

Figure 5.1: Model demonstrating influence of CTLA-4 on Tc17 plasticity and development of cytotoxic activity.

Blockade of CTLA-4 trigger the functional plasticity and anti-tumor activity of Tc17 cells by converting them to Tc1 like cells secreting IFN-γ and cytotoxic molecule granzyme B.

producers (Fig. 4.11 D) could be explained by different susceptibilities to IL-12 (139, 142, 143). However, similar phosphorylation of IL-12 induced STAT4, which is known to enhance IFN-γ production, indicated that CTLA-4 does not alter IL-12 signaling. Along with enhanced IFN-γ/TNF-α production, absence of CTLA-4 signal also displayed a significantly enhanced expression of otherwise repressed T cell cytotoxicity marker CD107a, correlating well with enhanced cytotoxic activity against tumors (131). It is likely that this mechanism occurs in response to anti-CTLA-4 therapy (Ipilimumab) in melanoma patients (126, 127).These findings

are in line with previous reports using multiple strategies that enhance the plasticity of Tc17 cells in tumor-bearing subjects and develop Tc1-like cytokine patterns that results in stronger anti-tumor immunity due to increased cell persistence and cytotoxicity (116, 141). Efforts to inhibit Tc17 cell activity are likely to result in a valuable strategy for treating cancer (144); indeed, CTLA-4 blockade is well known to augment the anti-tumor activity of T cells in patients with advanced melanoma (145).

Unwanted IL-17 production by CTLA-4 signaling may be the major contributor to the pathological effects of Tc17 cells, not only in the tumor environment but also in autoimmunity (146–148).

5.3 CTLA-4 divergently regulates the hierarchical differentiation of Tc17 and Tc1 cells

Previous reports suggest that the Tc17 program might be induced by default in the absence of Tc1 promoting conditions, which is characterized by the lack of the expression of T-bet and Eomes (112). In this study, CTLA-4-competent Tc17 cells display reduced expression of Tc1-related molecules and enhanced expression of Tc17-promoting molecules, such as IL-17 A/F, RORc, RORα, IRF-4, IL-21 and IL-23R. The rapid downregulation of mRNA levels of the Tc17 supporting factors RORc, IRF-4, IL-21 and IL-23R were observed starting from day 3 after stimulation (Fig.4.4 A), just when CTLA-4 appeared to be maximally expressed on the cell surface. Accordingly, Tc1 supporting factor Eomes appeared to be slightly elevated only in CTLA-$4^{-/-}$ Tc17 cells at later time points, implying that the Tc1 differentiation program is suppressed by CTLA-4 (88), thus giving space for the Tc17 default program (112). Reduced IL-21 production in CTLA-$4^{-/-}$ Tc17 cells shows the possibility that Eomes is not suppressed anymore (149), and thus, enhanced Eomes expression might downgrade the Tc17 master regulators transcription and Tc17 differentiation. However, this mechanism is unlikely, as CTLA-4 has no effect on T-bet and a combined suppression of T-bet and Eomes is necessary to induce Tc17 differentiation (112). Furthermore, mRNA expression of IL-23R was indeed enhanced by CTLA-4. Enhanced IL-23R could help in further enhancing the central role of CTLA-4 to sustainably enhance the properties of Tc17 cells (148, 150). SOCS3 has been reported to be mechanistically involved in the conversion of Tc17 cells (142), but was not significantly controlled by CTLA-4. The enhanced mRNA expression of Runx-1 in CTLA-$4^{+/+}$ Tc17 cells

might further stabilize Tc17 differentiation by prolonging the transcription of RORc. However, as mRNA and protein expression of T-bet were found to be similar in CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ Tc17 cells (Fig. 4.4 A and B), regulation of Runx1 by T-bet in CTLA-$4^{-/-}$ Tc17 cells is improbable (151). Intriguingly, CTLA-4-deficient Tc17 cells demonstrated strong similarities to IRF4-deficient $CD4^+$ T cells, which displayed an intrinsic downregulation of IL-17, IL-23R and RORγt mRNA as well (107, 152). However, forced overexpression of IRF4 could not rescue the diminished IL-17 production in the CTLA-$4^{-/-}$ Tc17 cells but surprisingly marginally reduced IL-17 expression in both the cell types. Thus, IRF4 is likely only one of the several players contributing to CTLA-4-mediated Tc17 differentiation (Fig. 4.4 C). In addition, recently, it has been shown that IRF4 is required for the development of effector $CD8^+$ T cells and forced expression of IRF4 in IRF4$^{-/-}$ $CD8^+$ T cells enhanced expression of IFN-γ and TNF-α (121). As IRF4 induces so many aspects of $CD8^+$ T cell differentiation, forced expression of IRF4 could just mimic a very strong differentiation signal which could overrun the Tc17 program. Ectopic overexpression of Tc17 master transcription factor RORγt was also not able to rescue the Tc17 phenotype on its own. Thus, the effects of CTLA-4 on single downstream transcription factor at least of IRF4 and RORγt cannot explain CTLA-4-enhanced degree of Tc17 differentiation.

5.4 CTLA-4-mediated effects on STATs stabilize Tc17 differentiation

Previous reports have shown differential regulation of IL-17 through STAT3 and STAT5 in $CD4^+$ T cells (81). The defective RORc, IL-21 and IL-17 expression by CTLA-4-deficient $CD8^+$ T cells are in accordance with the phenotype of STAT3-deficient T cells (105, 153, 154), indicating that the effect of CTLA-4 is likely by inhibiting a signaling knot upstream of RORγt, IL-23R, IL-17, and other molecules. The significantly enhanced phosphorylation of STAT3 in cells crosslinked with CTLA-4 antibodies illustrated that CTLA-4 signaling in $CD8^+$ T cells specifically enhances the expression of IL-17 and Tc17-related molecules in a STAT3-dependent manner. Accordingly, inactivation of STAT3 reversed the stimulatory effect of CTLA-4 on Tc17 differentiation by downregulating IL-17, RORγt and IL-23R. Additionally, identification of increased STAT3 binding to the IL-17 promoter compared to the IL-17 transcription repressor STAT5 in CTLA-4 competent Tc17 cells further supports the role of STAT3 in CTLA-4 mediated Tc17 differentiation (Fig. 4.6 B).

The CTLA-4-crosslinked cells re-stimulated under Tc1 environment exhibited prolonged expression of the Tc17 hallmarks and diminished upregulation of the Tc1 markers, implicating that CTLA-4 initiates Tc17 differentiation and inhibits Tc1-like factors. According to the data, this mechanism involves regulation of both both STAT1 and STAT3, whereas STAT5 is only indirectly involved. Enhanced IL-17 expression in re-stimulated Tc17 cells crosslinked with CTLA-4 made it tempting to speculate that CTLA-4 mediated prolonged Tc17 differentiation is due to stronger STAT3 activation. However, the identical phosphorylation of STAT3 mystified its importance in enhancing IL-17 expression by CTLA-4 competent Tc17 cells re-stimulated under Tc1 environment. Although STAT3 and STAT5 remained similarly activated independent of CTLA-4 signal, the ChIP assay revealed that CTLA-4 competent cells have more STAT3 binding to IL-17 promoter than STAT5, illustrating that enhanced IL-17 expression in CTLA-4 competent cells is because of the enhanced relative amount of activated STAT3 binding to the IL-17 promoter (Fig. 4.9 B). Previous studies have shown that cytoplasmic domain of CTLA-4 apparently binds to STAT5 (155). This encouraged the concept that CTLA-$4^{-/-}$ Tc17 cells might have enhanced translocation of STAT5 into the nucleus which competes with STAT3 to bind to the IL-17 promoter. However, this could be appropriate only in the case of CTLA-$4^{-/-}$ Tc17 cells but not by CTLA-4 blockade, as mutation of tyrosine residue in the CTLA-4 cytoplasmic tail did not abrogate its interaction with STAT5 (155).

The Tc17 program might be induced by default in CTLA-4 competent Tc17 cells by inhibiting of T-bet and Eomes transcription factors, which enhance Tc1 program (112). However, this is unlikely, as absence of both T-bet and Eomes would have been an obligatory prerequisite, but CTLA-4 only down-regulates Eomes (Fig. 4.8 B and C). Nevertheless, $CD4^{+}$ T cells deficient in STAT1 have been shown to strongly upregulate IL-17 production independent of T-bet and Eomes manipulation (80, 156). This finding suggests that the CTLA-4-mediated downregulation of STAT1 is likely an intrinsic inducer of IL-17 expression. Indeed, individuals with inactivating mutations of STAT1 have severely impaired capacity (often lethal in children) to mount immune responses against the intracellular pathogens like mycobacteria and viruses (157). Additionally STAT1 has been shown to enhance Eomes expression which is needed for granzyme B upregulation and cytotoxicity (158, 159). Thus, CTLA-4-mediated-inhibition of STAT1 activity undermines the cytotoxic capacity of Tc17 cells.

STAT3 has been shown in previous reports to stabilize Tc17 phenotype (99). In this study, regardless of whether CTLA-4-induced Tc17 cells are re-stimulated under Tc1 conditions, STAT3 binds to the IL-17 promoter relatively more than STAT5 (Fig. 4.9 B). However, similar levels of STAT3 and STAT5 phosphorylation were detected in Tc17 cells re-stimulated under

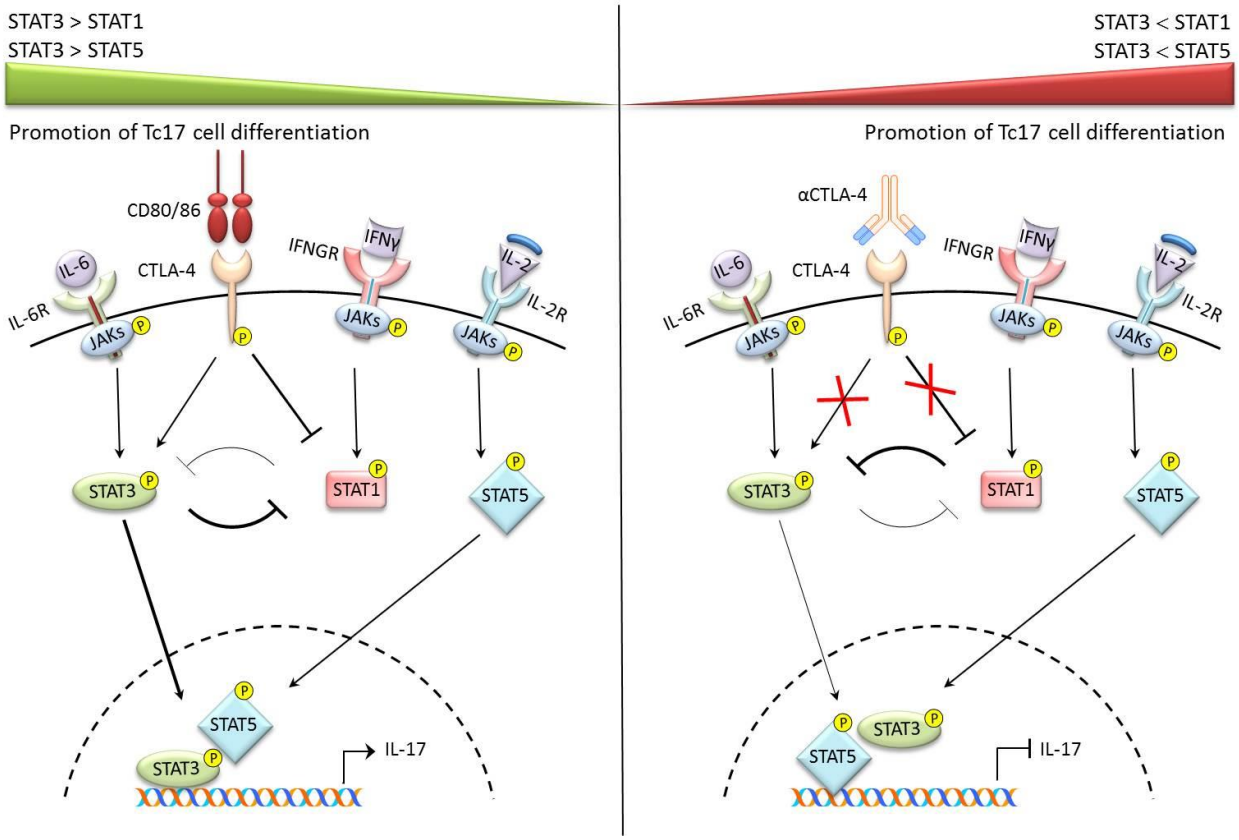

Figure 5.2: Model demonstrating the relationship between CTLA-4 and STATs.

Cell intrinsic signals provided by CTLA-4 inhibits activation of STAT1 and enhances the relative activity of STAT3, thereby augmenting the binding of STAT3 to the IL-17 promoter in competition with STAT5and thus enhancing IL-17 expression. Whereas, absence of CTLA-4 signaling enhances STAT1 activation thus decreasing the relative activity of STAT3, which is associated with enhanced binding of STAT5 to the IL-17 promoter than that of STAT3 causing inhibition of IL-17 expression.

Tc1 environment. The initial discrepancy is explained by the fact that STAT molecules regulate each other. STAT3 activation is enhanced in STAT1-null cells, and conversely, STAT1 activation is enhanced in STAT3-null cells (79, 160–163). Although, no difference in STAT3 phosphorylation was observed during re-stimulation in a Tc1 environment, STAT1 phosphorylation was significantly downregulated by CTLA-4 in these studies. Even though the

AP-MS experiments using ^{18}O-labelling revealed no direct association of CTLA-4 with STAT1, a close interaction could be possible, as upon T cell activation CTLA-4, IFNGR and STAT1 are expressed in a polarized manner in the immunological synapse (164). Thus, CTLA-4 could negatively regulate the IFN induced STAT1 phosphorylation by phosphatases such as SHP2 recruited to its cytoplasmic tail (165, 166). Additionally, CTLA-4 bound SHP2 may also negatively regulate phosphorylation of IFNGR bound tyrosine kinase Jak2 which is essential for activation of STAT1 (167). As STAT3 and STAT1 have antagonistic functions the ratio of pSTAT3/pSTAT1 determines the Tc17 cell phenotype (80). Therefore, the higher pSTAT3/pSTAT1 ratio in the CTLA-4-crosslinked Tc17 cells, which were re-stimulated in a Tc1 environment, explains the CTLA-4-mediated stability of Tc17 differentiation. The increased mRNA expression of the STAT3 target molecules IL-17, IL-23R, IL-21 and RORc in these cells further strengthens the role of the relative amount of pSTAT3 in stabilizing Tc17 differentiation. STATs can also have indirect effects on each other ; for instance, STATs regulate SOCS3, which in turn impedes Tc17 cell differentiation (168). However, SOCS3 mRNA expression was not distinctly regulated in the absence of CTLA-4 signals. The effect of the CTLA-4-STAT3 axis may also be mediated by RORγt expression, but RORγt overexpression on its own did not reverse the suppressed IL-17 production (118).

5.5 Conclusion

In summary, a CTLA-4-mediated cell-intrinsic effect enhances Tc17 cell differentiation and is associated with enhanced STAT3 activity. Consequently, the absence of CTLA-4 signal enhances the susceptibility of Tc17 lineage plasticity and the ability of Tc17 cells to convert to Tc1-like cells. Mechanistically, CTLA-4 maintains higher activity of STAT3 than those of STAT1 and STAT5, which are known to inhibit the generation of the Tc17 subset. The data further indicate that blockade of CTLA-4 signaling by Ipilimumab may enhance the plasticity of Tc17 cells, which are frequently observed in the tumor microenvironment, thus converting them to Tc1-like cells with increased cytotoxicity and survival. The use of Ipilimumab has been shown to improve overall survival rates in melanoma patients while improving T cell anti-tumor responses and this was associated with increased production of polyfunctional cytokines such as IFN-γ and TNF-α. Thus, blockade of CTLA-4 would have benefits not only in initiating the differentiation of cytotoxic $CD8^+$ T cell responses but also in mobilizing the cytotoxic activity of pre-existing tumor-specific Tc17 cells.

In addition to the reported regulation of Tc17 plasticity by STATs and its impact on tumor biology, these results also show that the surface molecule CTLA-4 regulates the third signal for T cell differentiation, namely, cytokine receptor signaling, which is an important feature in the decision-making process that determines the type of T cell differentiation. Although the main focus here was on Tc17 cells, this relationship between CTLA-4 and STAT1 or STAT3 is likely to impact other STAT1- or STAT3-producing lineages, such as Tc1 cells, T-helper subpopulations and Treg cells. Likely, this also influences various immune settings, making this pathway acutely relevant in the context of immune checkpoint therapy and cytokine-based drug design.

M.Sc., Aditya Arra

Titel: CTLA-4 induced signal transduction in regulating differentiation and plasticity of Tc17 cells

6. Summary

IL-17 producing $CD8^+$ T cells (Tc17 cells) are known to display reduced cytotoxic activity. However, Tc17 cells have been shown to provide immunity against tumors in mice by easily converting into Tc1-like cells in a pro-inflammatory environment, retaining their Tc17 characteristics, such as cell longevity. The inhibitory surface-molecule CTLA-4 plays a critical role in the regulation of T cell immune responses. As the blockade of CTLA-4 on Tc1 cells has been shown to enhance T cell cytotoxicity, there is a great interest in identifying novel mechanisms of action of CTLA-4 in regulating Tc17 cells to evoke effective cytotoxic anti-tumor responses. Using *in vitro* and *in vivo* models the molecular pathways underlying the CTLA-4–mediated differentiation and plasticity of Tc17 cells were investigated in the present work. *In vitro* studies using $CD8^+$ T cells lacking CTLA-4 signal displayed an intrinsic limitation to produce IL-17, indicating that CTLA-4 specifically promotes differentiation of Tc17 cells. Even though, Tc17 cells lacking CTLA-4 signaling have limited expression of IL-17 inducing transcription factor RORγt, a forced overexpression of RORγt did not rescue IL-17 expression in these cells. Interestingly, further studies demonstrated that Tc17 cells lacking CTLA-4 signaling have limited activation of STAT3 leading to compromised production of its target gene products such as IL-17, IL-21, IL-23R and RORγt. Upon re-stimulation with the Tc1 inducing cytokine IL-12, these cells displayed faster downregulation of Tc17 hallmarks and acquire Tc1 characteristics such as IFN-γ and TNF-α co-expression which are known to correlate with tumor control. The quality of CTLA-4-induced Tc17 cells was tested *in vivo*, utilizing OVA-expressing B16 melanoma cells. Upon adoptive transfer, unlike CTLA-$4^{+/+}$ the CTLA-$4^{-/-}$ Tc17 cells were highly efficient in the antigen-specific rejection of established OVA-expressing B16 melanoma *in vivo*. *Ex vivo* analysis and restimulation experiments under Tc1 conditions demonstrated a high-quality Tc17 differentiation program by CTLA-4 displaying delayed downregulation of Tc17-related molecules such as IL-17 and RORγt. Mechanistically, in primary and re-stimulated Tc17 cells, STAT3 binding to the IL-17 promoter was strongly augmented by CTLA-4.This was associated with less binding of STAT5 and reduced relative activation of STAT1 which is known to block STAT3 activity. Consistent with these findings, inhibition of CTLA-4-induced STAT3 activity reversed enhancement of signature Tc17 gene products, rendering Tc17 cells susceptible to conversion to Tc1-like cells with enhanced cytotoxic potential. Thus, CTLA-4 critically shapes the characteristics of Tc17 cells by directly and indirectly regulating the relative activity of different STATs, namely STAT3, STAT5 and STAT1. This provides a better understanding of the mode of action of CTLA-4 blockade immune therapy on tumor promoting Tc17 cells found in the tumor microenvironment and thus provide new perspectives to enhance cytotoxicity of anti-tumor responses.

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7. Zusammenfassung

Es konnte gezeigt werden, dass IL-17 produzierende $CD8^+$ T-Zellen (Tc17-Zellen), eine reduzierte zytotoxische Aktivität aufweisen. Außerdem ist bekannt, dass murine Tc17-Zellen in einem proinflammatorischen Milieu spontan in Tc1-ähnliche Zellen differenzieren, die die Immunität gegen Tumore vermitteln, dabei aber ihre Eigenschaften als Tc17-Zellen, wie Langlebigkeit, behalten. Das inhibitorische Oberflächenmolekül CTLA-4 spielt eine wichtige Rolle bei der Regulation der Immunantwort von T-Zellen. Die Blockade von CTLA-4 auf Tc1-Zellen führt zu einer verstärkten Zytotoxizität der T-Zellen. Um eine effektive zytotoxische Immunantwort auf Tumoren hervorzurufen, besteht daher ein großes Interesse an der Identifizierung von neuen Wirkungsmechanismen von CTLA-4 bei der Regulation von Tc17-Zellen.

Durch die Verwendung von *in vitro* und *in vivo* Modellen wurden in der vorliegenden Arbeit die molekularen Signalwege der CTLA-4 vermittelte Differenzierung und Plastizität von Tc17-Zellen untersucht. Durch *in vitro* Untersuchungen von $CD8^+$ T-Zellen, die keineCTLA-4 Signale haben, konnte eine intrinsische vermittelte Reduktion der Bildung von IL-17 gezeigt werden, was für eine CTLA-4 spezifische Differenzierungen von Tc17-Zellen spricht. Obwohl diese Tc17-Zellen eine verringerte Expression des an der IL-17 Produktion beteiligten Transkriptionsfaktors RORγt zeigten, führte eine Überexpression von RORγt in diesen Zellen nicht zu einer verstärkten Bildung von IL-17. Interessanterweise beobachteten wir bei weiteren Untersuchungen, dass in Tc17-Zellen ohne CTLA-4 Signal auch die Aktivierung von STAT3 reduziert war, was zu einer verringerten Transkription von STAT3 vermittelten Genprodukten wie IL-17, IL-21, IL-23R und RORγt führte. Durch eine Restimulation mit dem Tc1 induzierenden Zytokin IL-12 zeigten diese Zellen außerdem eine schnellere Herunterregulierung von Tc17-Zellmarkern sowie gleichzeitig eine Zunahme von Tc1-Zellmarkern, wie der Koexpression von IFN-γ und TNF-α, die mit der Anti-Tumor-Kontrolle korrelieren.

Die Qualität der CTLA-4 induzierten Tc17-Zellen wurde *in vivo* mit Hilfe von OVA-bildenden B16 Melanomzellen getestet. Durch adoptiven Zelltransfer transferierte CTLA-$4^{-/-}$ Tc17-Zellen waren dabei sehr effizient bei der antigen-spezifischen Abstoßung von OVA-bildenden B16 Melanomzellen *in vivo*, im Gegensatz zu CTLA-$4^{+/+}$ Tc17-Zellen. *Ex vivo* Analysen und Restimulationsexperimente unter Tc1-Bedingungen demonstrierten ein umfangreiches durch CTLA-4 gekennzeichnetes Tc17 Differenzierungsprogramm mit einer verzögerten

Herunterregulierung von Tc17 assoziierten Molekülen wie IL-17 und RORγt. In primären und restimulierten Tc17-Zellen war die STAT3 Bindung an den IL-17 Promotor durch CTLA-4 verstärkt. Gleichzeitig waren die Bindung von STAT5 und die relative Aktivierung von STAT1, einem bekannten Inhibitor der STAT3-Aktivität, reduziert. Im Einklang mit diesen Ergebnissen führte die Inhibition der CTLA-4 induzierten STAT3 Aktivität zu einer verminderten Bildung von Tc17 Genprodukten, wodurch Tc17-Zellen empfänglich für die Umwandlung in Tc1-ähnliche Zellen mit erhöhtem zytotoxischem Potential werden. CTLA-4 beeinflusst die Eigenschaften von Tc17-Zellen durch direkte und indirekte Regulation der relativen Aktivität verschiedener STAT-Moleküle, wie STAT3, STAT5 und STAT1.

Diese Ergebnisse führen zu einem besseren Verständnis über die Wirkungsweise einer CTLA-4 blockierenden Immuntherapie auf Tc17-Zellen im Tumormikromilieu und bieten neue Perspektiven zur Steigerung von Anti-Tumor-Antworten.

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9. Curriculum Vitae

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Conference	15^{th} German meeting on T-cells: subsets and functions, Marburg, Germany (2012).
Talk	CTLA-4 (CD152) enhances differentiation of Tc17 cells

Conference	43rd annual meeting of german society for immunology (DGfI), Mainz, Germany (2013).
Poster	CD152 (CTLA-4) unleashes a Tc17 differentiation program with sustainability
Conference	44th annual meeting of german society for immunology (DGfI), Bonn, Germany (2014).
Poster	Tc17 differentiation and plasticity is controlled by CTLA-4 (CD152)
Conference	4th European Congress of Immunology (ECI), Vienna, Austria (2015).
Poster	CTLA-4 (CD152) supports tumor progression by preventing plasticity of Tc17 cells
Conference	Immunology 2016, Seattle, USA.
Poster	Plasticity along with differentiation of Tc17 cells is controlled by CTLA-4

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10. List of Publications

Arra, A., H. Lingel, B. Kuropka, J. Pick, T. Schnoeder, T. Fischer, C. Freund, M. Pierau, and M. C. Brunner-Weinzierl. 2016. The differentiation and plasticity of Tc17 cells are regulated by CTLA-4-mediated effects on STATs. *Oncoimmunology*. 6(2): e1273300 **(IF: 7.64)**

Pick, J*., A. Arra*, H. Lingel, J. K. Hegel, M. Huber, G. Nishanth, G. Jorch, K.-D. Fischer, D. Schlüter, K. Tedford, and M. C. Brunner-Weinzierl. 2014. CTLA-4 (CD152) enhances the Tc17 differentiation program. *European journal of immunology*. 44(7): 2139–2152. **(IF: 4.17)**

*** These Authors contributed equally to this work**

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12. Declaration

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zum dem Thema

CTLA-4 induced signal transduction in regulating differentiation and plasticity of Tc17 cells

selbständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den

Aditya Arra