UNIVERSITY OF CALIFORNIA SAN DIEGO

Collective control of adaptive immunity by T cell co-inhibitory signaling

A Dissertation submitted in partial satisfaction of the requirements
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by

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The dissertation of Preston Dennett is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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2022

DEDICATION

This dissertation is dedicated to the collective of mostly unrecognized researchers who together produced the modern image of adaptive immunity.

EPIGRAPH

I believe it is safe to state that our understanding of immunological recognition is approaching some sort of asymptote, where future experiments are obvious, technically difficult to perform, and aim to achieve ever higher degrees of precision rather than revolutionary changes in our understanding. Thus, this is a good time to take stock of immunology, to catalog what is known, to ask how we arrived where we are, and to look ahead to where we might go.

Charles Janeway (1989)

...the immune system (like the brain) reflects first ourselves, then produces a reflection of this reflection, and that subsequently it reflects the outside world: a hall of mirrors...

The mirror images of the outside world however, do not have permanency in the genome. Every individual must start with self…

those who always seek exterior pressures (e.g., microbes) to account for the evolution of the sets of V genes, would do well to turn their vision towards the interiors of themselves, and there discover the mystery, perhaps never completely revealable, of the immune system.

Niels Jerne (1984)

Looking at what men have found out about arranging the musical intervals and forming precepts and rules in order to control them for the wonderful delight of the ear, when shall I be able to cease my amazement?…

But surpassing all stupendous inventions, what sublimity of mind was his who dreamed of finding means to communicate his deepest thoughts to any other person… by the different arrangements of twenty characters upon a page!

Galileo Galilei (1632)

Language is, at its core, a system that is both digital and infinite. To my knowledge, there is no other biological system with these properties...

Noam Chomsky (1991)

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LIST OF ABBREVIATIONS

Anti-Id	Anti-idiotypic antibody
APC	Antigen Presenting Cell
BAR	Biotinylation by Antibody Recognition
BSA	Bovine Serum Albumin
CAR	Chimeric Antigen Receptor
CNS	Central Nervous System
CST	Clonal Selection Theory
CTL	Cytotoxic Lymphocyte
CTLA4	Cytotoxic T Lymphocyte-associated Antigen 4
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
EM	Electron Microscopy
FACS	Fluorescence-Activated Cell Sorting
FAO	Fatty Acid Oxidation
GVHD	Graft vs. Host Disease
HSA	Human Serum Albumin
HSC	Hematopoietic Stem Cell
ILC	Innate Lymphoid Cell
irAEs	immune related Adverse Events
ITIM	Immuno-receptor Tyrosine-based Inhibitory Motif
ITSM	Immuno-receptor Tyrosine-based Switch Motif

LIP	Lymphopenia induced proliferation
LPS	Lipopolysaccharide
LRBA	LPS-responsive beige like anchor protein
MDSC	Myeloid Derived Suppressor Cell
MHC	Major Histocompatibility Complex
MS	Mass Spectrometry
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PBST	PBS + 0.1% Tween-20
PD-1	Programmed cell Death protein 1
PD-L1	Programmed cell death protein ligand 1
PEI	Polyethylenimine
PFA	Paraformaldehyde
pMHC	Peptide-loaded MHC
POI	Protein of Interest
PRR	Pattern Recognition Receptor
ROI	Region of Interest
RPMI	Roswell Park Memorial Institute Medium
RT	Room temperature (25ºC)
SARS2	SARS-Cov2
SH2	Src Homology region 2
TME	Tumor Microenvironment
Treg	Regulatory T cell

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ABSTRACT OF THE DISSERTATION

Collective control of adaptive immunity by T cell co-inhibitory signaling

by

Preston Dennett

Doctor of Philosophy in Chemistry

University of California San Diego, 2022

Professor Enfu Hui, Chair

Professor Ulrich Müller, Co-Chair

The ability of T lymphocytes to detect and eliminate cancer and viral infection is restricted by pathological subversion of the co-inhibitory receptor signaling pathways which normally function to restrain the immune response and preserve "self" tolerance. As a result, cancer treatments employing therapeutic antibodies to block the T cell co-inhibitory receptors PD-1 or CTLA4 have seen dramatic clinical success over the past decade. However, despite extensive efforts to improve and predict patient responses to these therapies, additional advances have been slow to materialize and severe autoimmune side effects remain common. To more effectively manipulate the curative potential of adaptive immunity to eliminate cancer and infectious disease, a greater understanding of how T cell co-receptor signaling operates from the level of single cells and molecules to that of the entire system is critical but remains incomplete. To advance understanding of T cell signaling at the molecular and cellular level, an APEX2 peroxidase-based proximity labeling approach is developed and applied to identify novel effectors and substrates of co-inhibitory immune receptors by mass spectrometry. To clarify how CTLA4 coordinates cellular and system level regulation of immune responses, an alternative mechanism is described in which CTLA4 acts in a cell intrinsic manner to regulate availability of the B7 co-stimulatory ligands on the surface of activated T cells. In this model, CTLA4 control of immune homeostasis is achieved in part by directing *cis*-endocytosis of T cell B7 ligands which are both endogenously expressed and inherently acquired via trogocytosis upon contact with activating APCs. Importantly, these APC-derived innate inflammatory signals targeted by CTLA4 are displayed to other T cells alongside co-acquired peptide antigen during an immune response. A model is presented in which this pathway thereby allows CTLA4 to exert dual cell intrinsic and extrinsic regulatory function to more efficiently operate at the interface between innate and adaptive immunity and between single T cells and the lymphocyte collective.

Chapter 1: Introduction

In the early 1900s Paul Ehrlich provided the first clear demonstration of "self" † / non-self discrimination by the vertebrate adaptive immune system by immunizing goats with xenogenic red blood cells (RBCs) to induce the production of anti-RBC antibodies in recipient animals.[1,2] Remarkably, these antibodies were able to bind and destroy donor goat-derived red blood cells while leaving the virtually identical "self" RBCs intact in recipient goats.[1] These results led Ehrlich to the prescient hypothesis that adaptive immunity must require some form of selective process to prevent destructive autoimmunity arising from "self" recognition. At the same time, Elie Metchnikoff had shown that in addition to this 'humoral' immunity mediated by antibodies, an innate mechanism of defense against pathogens was performed by specialized amoeboid-like cells or 'phagocytes' which he observed to rapidly migrate toward sites of injury to engulf and digest invading microbes and cellular debris, a process termed phagocytosis. Observing this process of acute inflammation even in primitive invertebrates, Metchnikoff argued for the primary role of this innate, cellular form of immunity mediated by phagocytosis.[3] It was later understood in vertebrates that these 'myeloid' phagocytic cells of innate defense can act as antigen presenting cells (APCs), responsible for presenting both "self" and foreign antigens to 'lymphoid' cells (lymphocytes) of the adaptive immune system.[4] Together this work established the first description of innate and adaptive immunity and ignited a debate over their relative centrality that arguably continues to the present day (section 5.4). Indeed, the extent and means by which myeloid innate immune cells (such as monocytes macrophages, and dendritic cells) impinge on lymphocytes of the adaptive immune system to determine (1) whether or not to

† Note: "Self" is considered to include homeostatic levels of environmental, ingested, and microbiota-derived antigens.

evoke an immune response (i.e. “self” /non-self discrimination) and (2) what type of response to induce (immune class regulation / T helper subset differentiation), remains contentious and unresolved.[2] A more coherent understanding of how this decision-making process occurs from the level of single cells to that of the system is likely to facilitate rational manipulation of adaptive immunity with potential to deliver more effective cures for cancer, autoimmunity, and infectious disease. This dissertation aims to provide further evidence toward addressing these fundamental questions in two parts:

First, in Chapter 3 the development and application of a proximity labeling proteomics method is described with the aim of identifying downstream effectors and substrates of co-inhibitory immune receptors by mass spectrometry (MS). By performing a spatially restricted biotinylation reaction in intact cells prior to streptavidin affinity enrichment, this approach enables comprehensive profiling of membrane-associated biomolecular condensates involved in immune cell signaling in the native cellular environment. This work aims to further our understanding of PD-1 and other co-inhibitory receptor signaling pathways that are essential for preventing autoimmunity and immunopathology but are invariably subverted for immune evasion in cancer and chronic infection.

Second, in Chapter 4 evidence is presented for an alternative cell intrinsic pathway by which the essential co-inhibitory immune receptor CTLA4 can exert regulatory function both in single T cells and at the level of the collective by coordinating control of co-stimulation within networks of activating lymphocytes. Specifically, a mechanism is described in which levels of the B7 co-stimulatory ligands CD80 and CD86 (CD80/86) expressed on the surface of activating T cells can be limited by CTLA4-mediated *cis*-endocytosis. In this model, a major regulatory target of CTLA4 is proposed to be the non-equilibrium pool of induced and exogenous CD80/86

ligands which are both endogenously expressed and inherently acquired upon contact with activating APCs and re-displayed among responding lymphocytes alongside co-transferred peptide antigen (pMHC) via trogocytosis during an immune response. It is proposed that this unusual mechanism of CTLA4 regulatory activity implies a more general theoretical framework in support of the historical view that core system-level features of adaptive immunity such as "self" tolerance and immune class regulation are principally mediated and regulated by the collective behaviors of lymphocytes themselves.

1.1. Innate immunity

Innate engulfment of non-self or "damaged self" via actin-dependent phagocytosis is an evolutionarily ancient phenomenon. Originating as a mode of nutrient acquisition, this process is thought to have been central in the origin of the eukaryotic cell itself.[5] As complex multicellular eukaryotes emerged, phagocytosis became additionally functionally expanded in specialized cell types for maintenance of the cellular collective, acquiring essential roles in homeostasis and defense against parasitic invaders.[6] As a result, all metazoans possess mechanisms of innate immunity mediated by specialized phagocytic cells.[7]

The division between innate and adaptive immunity arose in early vertebrates during the Cambrian period following the bifurcation of the myeloid and lymphoid lineages of leukocytes (i.e. "white blood cells"), which emerge during hematopoiesis.[8] While lymphocytes are the mediators of adaptive immunity, mechanisms of innate defense are predominately carried out by diverse populations of phagocytic myeloid cells including monocytes and macrophages and dendritic cells (DCs).[9] During tissue damage or infection, phagocytic cells act as initial mediators of acute inflammation, rapidly infiltrating sites of injury where they secrete and respond to various cytokines.[10] This process serves to protect the organism against foreign

pathogens and restore homeostasis through direct uptake of microbes and cellular debris.[10] Notably however, even in the absence of acute injury, the scale of organismal homeostasis achieved by specialized phagocytic cells is immense. For example in the human body, ~300 billion cells naturally undergo cell death each day, most of which are subsequently digested by phagocytes.[11] This process serves to position phagocytes as key sentinels and regulators of organismal composition, tissue injury, and regeneration. In vertebrates this information is communicated to lymphocytes via the process of antigen presentation.[9]

An antigen is defined as any substance that can be recognized by the adaptive immune system (i.e. any molecular structure or peptide that can be bound by an antibody or recognized by a T cell receptor).[2] Peptide antigens recognized by T cell receptors are displayed by both innate and adaptive immune cells in the context of major histocompatibility complex (MHC) molecules.[12] Extracellular proteins internalized by phagocytic antigen presenting cells (APCs) are proteolytically processed into peptides 13-17 amino acids in length and loaded onto class II major histocompatibility complex (MHC-II) molecules for presentation to CD4+ T cells (Section 1.2).[4] While MHC-II expression is restricted to immune cells, all nucleated cells express class I major histocompatibility complex (MHC-I) on the cell surface displaying peptides 8-9 amino acids in length, reflecting the current status of intracellular protein synthesis.[4] Display of non-"self" peptide in the context of class I MHC can be sufficient to induce targeted cell killing by cytotoxic CD8+ T cells (Section 1.2), therefore downregulation of MHC-I is a common strategy induced during viral infection and in cancer for immune evasion[13] This process is limited by a unique mechanism of innate immunity mediated by a specialized subset of lymphoid cells termed 'natural killer' cells (NK cells) which target and eliminate cells found to not display MHC-I, termed detection of "missing self."[14]

Myeloid dendritic cells (DCs) and macrophages of innate immunity and B cells of the adaptive immune system are the primary cell types responsible for displaying processed antigens to T cells in the context of MHC-II molecules and are therefore classed as "professional" antigen presenting cells (APCs).[4] Among these, DCs exhibit particularly extensive phenotypic heterogeneity that allows their classification into various subtypes.[15] While DCs have been shown to represent the major cell type responsible for priming naive T cells in vivo, the generality of this paradigm has been challenged more recently as macrophages and B cells also appear to have this capacity.[16–18] Additionally, it has recently been demonstrated that the highly abundant subsets of circulating and tissue resident myeloid cells termed 'polymorphonuclear granulocytes' (including neutrophils, mast cells, basophils, and eosinophils) are also capable of antigen presentation to lymphocytes, often upon acquisition of antigen from DCs via trogocytosis.[19–21] Similarly, more recently characterized subsets of innate lymphoid cells (ILCs) have also been shown to have antigen presentation capabilities both by endogenous expression and acquisition of pMHC-II via trogocytosis.[21,22]

To assess potential threats of ingested cellular debris, microbes, and antibody bound molecules, professional APCs utilize pattern recognition receptors (PRRs) to survey internalized antigens as well as the local environment for pathogen associated molecular patterns (PAMPs) and "danger" associated molecular patterns (DAMPs).[23,24] PAMPs consist of evolutionarily conserved structural features of pathogens including structural components of bacterial and fungal cell walls (e.g. lipopolysaccharide (LPS), chitin respectively), and viral capsids.[24–26] High evolutionary conservation of these structures facilitates innate recognition of PAMPs by germline encoded PRRs such as Toll like receptors (TLRs) which are expressed within endosomal compartments and on the cell surface of immune cells.[23] Similarly, DAMPs represent

endogenous molecules released by damaged or dying cells undergoing so-called "immunogenic cell death" which can also be recognized by TLRs and various other PRRs.[24] Recognition of PAMPs or DAMPs by APC-expressed PRRs induces APC maturation and cytokine production, triggering the upregulation of antigen processing pathways, MHC molecules, and co-stimulatory ligands such as CD80 and CD86 on the cell surface to promote T cell activation (section 1.2).[27] In this way myeloid cells of the innate immune system are thought to provide the critical inflammatory context of antigenic stimuli as they are presented to lymphocytes to play central roles in the induction and regulation of adaptive immune responses (referred to collectively as the PAMP/ "Danger" model of T cell activation, Section 1.6.4-5).[9,24,25]

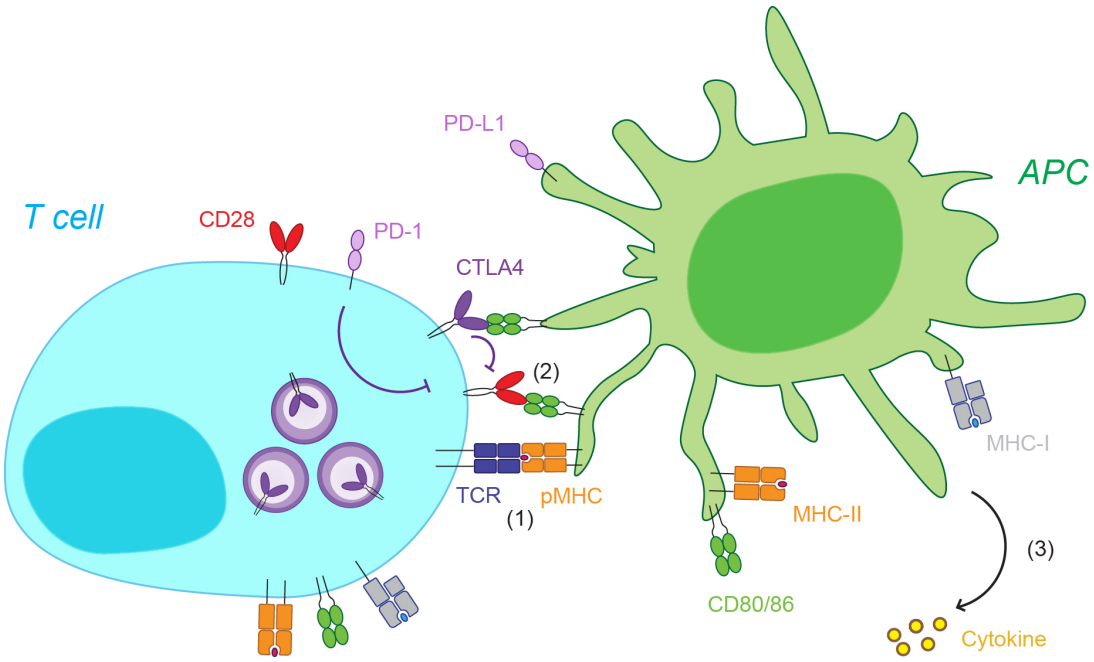


Figure 1 Adaptive immunity is mediated and regulated by T lymphocytes.

Current models of T cell activation involve 3 signals: (1) TCR recognition of peptide antigen (pMHC) in the context of class I or class II major histocompatibility complex (MHC) molecules. Signal 1 is either stimulatory or inhibitory for T cells depending on the presence or absence of signal (2), respectively. Signal (2) consists of co-stimulatory signaling delivered via T cell expressed CD28 engaging with CD80/86 ligands which are upregulated by APCs upon innate detection of pathogens or in response to signal (3), i.e. inflammatory cytokines. TCR/CD28 signaling is counteracted by the canonical co-inhibitory "immune checkpoint" receptors PD-1 (which binds PD-L1 or PD-L2) and CTLA4 which competes with CD28 for CD80/86 binding. PD-1 acts in a cell intrinsic manner while CTLA4 is thought to act cell extrinsically to restrain T cell proliferation, differentiation, and effector function (section 1.3).

1.2. Adaptive immunity

The human adaptive immune system consists of approximately 10^{12} lymphocytes, representing ~1% of the total body mass and, in terms of cell numbers, one order of magnitude greater than number of neurons in the human brain.[28] This network of cells is composed primarily of T and B lymphocytes, present in a ~5:1 ratio and undergoing continuous circulation between the blood and lymphatic system.[29] Each T and B cell clone expresses a unique antigen receptor on its surface that is randomly generated by somatic recombination.[30] Upon antigen recognition under activating conditions, these clonal populations undergo clonal proliferative expansion and acquire effector functions.[31,32] T cell receptors recognize short peptides 9-17 amino acids in length presented in the context of class I or class II major histocompatibility complex molecules (MHC).[12,33] In contrast, B cell receptors are membrane bound immunoglobulins (i.e. antibodies) unique to each B cell clone.[34,35] While the scale of T and B cell receptor diversity remains unknown, it is estimated to consist of millions of unique specificities producing a repertoire capable of recognizing virtually all possible molecular structures and protein compositions.[36]

This ability for universal recognition by the adaptive immune system necessitates mechanisms for preserving "self" tolerance to prevent autoimmunity. This is achieved by various mechanisms including negative selection of repertoire diversity against "self" antigens during lymphocyte development, positive selection for dominant "self"-specific regulatory T cells, and various co-inhibitory signaling pathways and cell-based suppressive mechanisms (i.e. 'suppressor T cells') among mature lymphocytes.[37–39] Additionally, antigen recognition alone is insufficient to induce full lymphocyte activation and instead promotes a transient state of hypo-

responsiveness termed “anergy.”[40] Full lymphocyte activation is thus said to require an essential “second signal.”[41,42] For B cells this additional signal requires antibody-recognized antigen to be internalized, proteolytically processed, and presented in the context of MHC-II on the B cell surface to stimulate recruitment of CD4+ T cell help.[43,44] For T cells the “second signal” is defined under the PAMP / Danger model as co-stimulatory signaling delivered via T cell expressed CD28 binding with CD80 or CD86 expressed by an activated antigen presenting cell (APC) (Section 1.2.3).[25,45]

1.2.1 T lymphocytes

T lymphocytes are categorized into several classes and subsets, including most broadly by expression of the CD4 and CD8 co-receptors which mediate binding to class II and class I MHC molecules respectively.[46] CD8+ effector T cells, termed ‘killer’ or cytotoxic or T lymphocytes (CTLs) recognize of intracellular antigens in the context MHC-I and are therefore primarily responsible for detecting and eliminating virally infected cells or tumor cells displaying mutated ‘neoantigens.’[47] CD4+ T cells recognize extracellular-derived antigens and upon activation differentiate into various context-dependent “helper” subsets.[48] These cells are commonly referred to as T helper (Th) cells due to their essential roles in providing “help” to facilitate the maturation of APCs, CD8+ T cell activation, and affinity maturation of antibodies in B cell responses.[49] Although some B cell and CD8+ T cell responses can initiate independently of CD4+ T cells, the formation of B and T cell memory has been shown to more strictly require CD4+ T cell help.[50–52]

1.2.2 CD4+ T cell subsets

CD4+ T helper (Th) cells are interchangeably referred to as ‘conventional’ CD4+ T cells (Tconv) to distinguish them from the essential immunosuppressive subset of regulatory CD4+ T

cells (Treg). Treg are required for immune homeostasis in jawed vertebrates and can be most generally defined by expression of the transcription factor FOXP3, the high affinity IL-2 receptor α chain (CD25), and high levels of the co-inhibitory receptor CTLA4 which is required for their suppressive function.[38,53–55] Conventional / helper CD4+ T cells are historically most broadly divided into Th1 and Th2 subsets reflecting their involvement in mediating cellular (i.e. predominately T cell-mediated) and humoral (i.e. largely antibody-mediated) immune responses, respectively.[56]

While the FOXP3+ Treg lineage commitment is reported to be the most stable, attempts to further classify CD4+ Th cell subtypes beyond Th1/Th2 based on cytokine production, cell surface markers, and lineage specific transcription factors has recently been confounded by high levels of plasticity and observed interconversion of Th fate commitments.[48] For example, Th17 and antigen-experienced Tconv cells have been shown to convert to a Treg phenotype in some *in vivo* contexts.[57,58] Interestingly, CTLA4 has been reported to be involved in the peripheral induction of both Treg as well as non-FOXP3 expressing suppressive CD4+ T cells ($FOXP3^-$/$IL\text{-}10^+$) by unknown mechanisms (section 4.4.2).[59,60] Similarly, in some contexts CTLA4 has been associated with inhibition of Th17 and Th2 but not Th1 subset differentiation, however the basis of this determination is poorly understood.[61,62]

During a Th2 response, immature B cells migrate to germinal centers (GCs) where they undergo somatic hypermutation targeted to the genetic locus encoding the variable region of the antibody receptor binding site.[63] Within GCs, high affinity B cell clones outcompete others for various signals orchestrated by helper CD4+ T cells to promote affinity maturation.[63,64] Interaction with CD4+ T cells is additionally required for B cell clones to undergo "class-switching" to produce antibodies with differing constant regions, which confer distinct

properties.[63,65] The formation of GCs as well as affinity maturation, and class switching are regulated by FOXP3+ CD4+ regulatory T cells (Treg) and follicular regulatory T cells (Tfr) via the essential T cell co-inhibitory receptor CTLA4, however precisely how this occurs mechanistically has remained unclear.[63,64,66]

1.2.3 T cell activation and priming

T cell activation canonically requires simultaneous delivery of two signals from a single APC*, consisting of recognition of peptide antigen by the TCR ('signal one'), and co-stimulatory signaling ('signal two').[25] Co-stimulatory signaling is provided via CD28, a transmembrane receptor constitutively expressed by resting T cells, binding to the B7 family ligands CD80 or CD86 (CD80/86) displayed on activated APCs.[45] While CD86 and to a lesser extent CD80 are also constitutively expressed by APCs, these ligands are highly upregulated upon activation by PRR-mediated detection of PAMPs, DAMPs (Section 1.2).[45] Receipt of signal one in the presence of signal two is sufficient to induce proliferative expansion of T cell clones and enhanced production of various cytokines, including the key T cell growth factor IL-2.[67] Importantly, significant levels of TCR signaling in the absence of sufficient co-stimulation via CD28 and/or inappropriate cytokine stimulation (sometimes referred to as 'signal three') can induce a temporary state of lymphocyte inactivation known as "anergy."[40,68]

*Notably, the requirement for co-stimulation to be provided by a single vs. potentially other 'bystander' APCs is a theoretical requirement of the current PAMP/Danger model of T cell activation (sections 1.6.4-6) which has been disputed.[69] This requirement arises based on the notion that APCs are thought to be critically responsible for making the determination of whether or not to induce an immune response against a particular displayed antigen by interpreting internalized PAMP / "Danger" signals via PRRs. In this dissertation it is proposed that data

presented in Chapter 4 are consistent with an alternative perspective favoring of collective information processing by responding lymphocytes as responsible for determining whether or not to induce an immune response (sections 5.2, 5.4, 5.6). This hypothesis uniquely allows 'bystander' co-stimulation as well as the potential for trogocytosis-mediated integration of co-stimulatory information onto the surface of T cells over time, independently of concurrent TCR stimulation (Section 4.4.5).

Following initial antigen recognition in the context of class I/II MHC ('signal one'), T lymphocytes are generally envisaged to form large stable immunological synapses with antigen presenting cells.[70] Early models of T cell differentiation describe T cell metabolic reprogramming, proliferative potential, and even memory function to be programmed during this 12-24 hour period of initial conjugation with an APC.[71] This programming is thought to occur as an integration of co-stimulatory and co-inhibitory signaling ('signal two') within a distinct "cytokine milieu" ('signal three') which has been shown to determine CD4+ T helper subset differentiation.[72,73] However, high levels of heterogeneity observed in the proliferative expansion and differentiation of single T cells has long cast doubt on the generality of this view.[48,70,74–76]

Additionally, in contrast to the more widely studied large symmetrical immune synapses which can form between stably conjugated T cells and APCs, numerous in vitro and in vivo imaging studies of the past decade have supported the notion that T cells often form highly transient functional interactions with multiple APCs.[70,77–79] This "kinapse" interaction mode may be facilitated in part by long membrane projections termed microvilli, which densely cover the T cell surface and have been shown to transmit information bi-directionally.[80,81] In support of the physiological relevance of T cell microvilli and the kinapse scanning mode, TCRs and co-stimulatory molecules have recently been shown to be concentrated within T cell microvilli.[82–84]

1.2.4 T cell differentiation and adaptive memory

The initial ~3-4 days of activation and expansion of T cell clones during initiation of an immune response, termed "T cell priming," occurs in the lymph nodes (LNs) where antigens are delivered via the lymphatics and/or transferred from migratory dendritic cells (DCs) and dispersed among LN resident DCs for display to lymphocytes.[85,86] Traditionally viewed as a single event in which stable long-term conjugation with a 'professional' APC mediates the full induction of a T cell response as described above, T cell priming in vivo increasingly appears to be more complex, involving serial interactions with multiple DCs as well as with other lymphocytes.[79,87,88] This process is followed by formation of homotypic cell clusters around activating APCs and swarming behaviors produce high levels of T-T interactions which have been proposed to be involved in collective information processing.[88–90] Indeed T-T interactions beyond initial contact with APCs were recently shown to be required for generation of CD8+ T cell memory as well as involved in mediating 'quorum'-like coordination of proliferation dynamics in the T cell population.[87,91]

Antigen clearance induces the contraction phase of an immune response during which the vast majority of clonally expanded lymphocytes die by apoptosis while some clonal populations persist as long-lived memory cells which rapidly expand in response to secondary antigen encounter.[92] CD8+ T cell differentiation is most widely reported to follow a linear differentiation pathway in which naïve T cells differentiate into effectors which then give rise to memory T cells.[92,93] While this model has been broadly supported for CD8+ T cells, the generation of various subtypes of memory CD4+ T cells is more ambiguous.[94,95] Indeed in both cases disparate observations have precluded greater understanding of the formation of the T cell memory pool under a single model, in part due to the observed plasticity in lineage commitment and difficulty

in defining T cell memory populations using protein surface markers detectable by flow cytometry.[96] In contrast, other models of T cell memory have emphasized the importance of naïve precursor frequency and interclonal competition among lymphocytes in memory commitment.[97–99]

Formation of both CD8+ and CD4+ T cell memory populations have been shown to be regulated by the co-inhibitory receptor CTLA4 by largely unknown mechanisms, with CTLA4 blockade associated with enhanced formation of CD8+ T cell memory.[100] Furthermore, somewhat paradoxically in terms of CTLA4 being commonly described as a molecular "brake" to restrain T cell proliferation, resting CD4+ memory T cells have been shown to possess high levels of intracellular CTLA4 while retaining potential for rapid proliferative expansion during secondary antigen exposure.[101–103]

1.2.5 Central and peripheral tolerance

The universality of antigen receptor diversity in the adaptive immune system creates a strict requirement for the preservation of "self" tolerance to avoid lethal autoimmunity. Two mechanisms by which this occurs are known as "central" and "peripheral" tolerance. Central tolerance is established during lymphocyte development, although this process is incomplete with relatively high levels of natural auto-reactivity observed in healthy human T and B cell repertoires.[104–106] These auto-reactive cells are typically described as having escaped clonal deletion due to 'failure' of central tolerance and highlight the requirement for mechanisms of peripheral tolerance to prevent autoimmunity.[39]

Central tolerance is established during lymphocyte development in the primary lymphoid organs, the bone marrow and the thymus.[39] T and B lymphocytes arise from a common lymphoid progenitor which differentiates from hematopoietic stem cells (HSCs) of the bone marrow.[107] B

cells are selected against "self" antigens through various stages of development and cycles of receptor editing.[108] T cells migrate to the thymus where they undergo negative selection against the almost complete diversity of endogenous peptide antigens expressed by an organism.[109] These peripheral self antigens are promiscuously expressed by medullary thymic epithelial cells (mTECs) by activity of the AIRE transcription factor and transferred by poorly understood mechanisms of 'antigen spreading', including trogocytosis, to various types of dendritic cells present in the thymus.[109,110] During T cell development, high affinity TCR interaction with a "self" peptide generally leads to clonal deletion or "diversion" toward an immunosuppressive regulatory phenotype while insufficiently low TCR affinity induces "death by neglect."[109] Interestingly, it was recently reported that AIRE deficiency induces ectopic CTLA4 expression in mTECs. mTEC expression of CTLA4 was associated with moderately reduced CD86 levels on thymic DCs and an impaired transfer of "self" antigens from mTECs to thymic DCs resulting impaired Treg development and organ specific autoimmunity (discussed in section 4.4.3).[111]

A principle mechanism of peripheral tolerance is the induction of T cell "anergy," defined as a prolonged state of hypo-responsiveness induced upon receipt of TCR stimulation ("signal one") in the absence of co-stimulatory signaling via CD28 engagement with CD80 or CD86 ("signal two").[40] CTLA4 has been shown to be involved in the induction of T cell anergy in vivo with anti-CTLA4 blockade treated or CTLA4-deficient T cells exhibiting resistance to anergy induction and anergic cells showing high levels of CTLA4.[101,112,113] However, precisely how CTLA4 is involved in regulating anergy induction is unclear (section 4.4.2). Additional mechanisms proposed to function in the preservation of peripheral tolerance include the combined action of various other co-inhibitory receptor signaling pathways ('immune checkpoints') including PD-1, (Section 1.2.6), as well as circulating and tissue-resident

immunosuppressive cell types including FOXP3+ regulatory T cells (Treg), innate lymphoid cells (ILCs), and myeloid-derived suppressor cells (MDSCs).[114–116]

1.3. Immune Checkpoints

T lymphocytes express co-inhibitory receptors on the cell surface termed 'immune checkpoints' that function to limit cell proliferation, differentiation and effector functions.[117] Together, these co-inhibitory molecules act to counter co-stimulatory signaling (e.g. CD28) and TCR signaling to restrain adaptive immune responses and preserve "self" tolerance to prevent immunopathology and autoimmune disease.[118] The archetypal examples of immune checkpoint receptors are cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death protein 1 (PD-1).[117] First characterized in the 1990s, CTLA4 and PD-1 have emerged as critical regulators of adaptive immunity.[119,120] The central importance of immune checkpoint pathways in maintaining immune tolerance is highlighted by the breakthrough clinical success achieved in cancer therapy by targeting these receptors and their ligands with blocking antibodies to prevent ligand / receptor interactions.[121,122] This approach, termed "immune checkpoint blockade" (ICB) is thought to re-invigorate an anti-tumor immune response by blocking inhibitory signals, leading to enhanced T cell activation and elimination of cancer cells.[117]

Remarkably, cancer immunotherapy using CTLA4 / PD-1 blockade alone or in combination can achieve lasting elimination of disease in ~10-30% patients on average.[117] However, the efficacy of ICB treatment varies widely between patients and cancer types and predictive biomarkers of therapeutic success have remained elusive as has the ability to predict and prevent immune-related adverse events (irAEs).[123,124] While anti-CTLA4 / anti-PD-1 combination therapy has significantly increased efficacy against some cancer types, up to 50% of patients experience irAEs. Although many irAEs are relatively mild, these also include

debilitating complications such as endocrinopathies which are observed in ~10% of patients receiving ICB therapy.[124–126] For example, anti-CTLA4 monotherapy causes autoimmune hypophystis in 3-17% of patients, a severe chronic inflammatory disorder of the pituitary gland.[126]

Inability to further expand therapeutic success of ICB therapies to broader subsets of patients and mitigate autoimmune side effects reflects a fundamental gap in our understanding of the vertebrate adaptive immune system. Indeed despite this clinical success, the precise mechanisms of action resulting in successful treatment and/or adverse events in vivo remain largely ambiguous and controversial.[127,128] This is due in part to a complex network of interactions among T cell co-inhibitory receptors and their respective ligands which can be expressed by a broad range of lymphoid, myeloid, and parenchymal cell types rendering the precise mechanism(s) of action difficult to discern in vivo.[118] Further complexity arises from the fact that PD-1 and CTLA4 each possess two unique ligands (PD-L1 / PD-L2 and CD80 / CD86, respectively).[129] Notably of these, CD80 and CD86 represent shared ligands of CD28 and CTLA4 while PD-L1 can additionally engage in poorly understood crosstalk via formation of a *cis* heterodimer with CD80 on the cell surface, thereby disrupting the CD80 homodimer.[130]

Beyond engagement with multiple ligands both in trans and in cis, immune checkpoint receptors and ligands additionally appear to be capable of exerting function on cell types which do not express these molecules following exogenous ligand / receptor acquisition via trogocytosis (section 1.5).[131] Despite this complexity, complete characterization of co-inhibitory immune receptor signaling at the molecular, cellular, and system level represents an urgent unmet need in fundamental research with demonstrated potential to transform cancer into a

manageable condition and generate more effective treatments and vaccination strategies for control of autoimmunity and infectious disease.

1.3.1 PD-1

PD-1 is a 50kDa type I transmembrane glycoprotein and key co-inhibitory immune receptor which is rapidly upregulated upon T cell activation to restrain T cell signaling and prevent tissue damage.[132,133] Upon engagement with its ligands PD-L1 or PD-L2, PD-1 functions by direct recruitment of the protein tyrosine phosphatase SHP2 to induce downmodulation of the TCR and CD28-associated phosphotyrosine signaling cascades (Figure 2).[133–135] To date, SHP2 is the only known effector of PD-1 mediated inhibition, yet several recent studies from both mouse models and cell culture assays have suggested that PD-1 retains inhibitory function in the absence of SHP2, and its paralog SHP1, potentially suggesting a role for unknown effectors.[136,137]

High levels of PD-1 during chronic viral infection and cancer have been associated with the acquisition of an "exhausted" T cell phenotype characterized by restricted proliferative capacity and reduced cytokine production that is generally referred to as 'dysfunctional.'[138] More recently however, T cell exhaustion is increasingly recognized to represent a heterogeneous spectrum of adaptive and pathological states which act to limit T cell function in order to re-establish immune homeostasis in settings of chronic infection and inflammation.[139,140] Because this exhausted T cell phenotype is also observed in the immunosuppressive tumor microenvironment (TME) of solid tumors, it has been proposed that anti-PD-1 blockade therapies act to reverse exhaustion and re-awaken anti-tumor immunity.[141] While T cell focused models of ICB efficacy have received significant support, a contributing role by other lymphoid cells including B cells and NK cells as well as myeloid cells has recently been implicated in therapeutic success.[142–144] Indeed, PD-1 and its ligands PD-L1 and PD-L2 are also expressed in

other lymphoid lineages (B cells, NK cells) as well as myeloid cells (macrophages, monocytes, granulocytes, and dendritic cells), and tumor cells, complicating definitive determination of the precise mechanism(s) of action immunotherapies targeting the PD-1 pathway in vivo.[144–146]

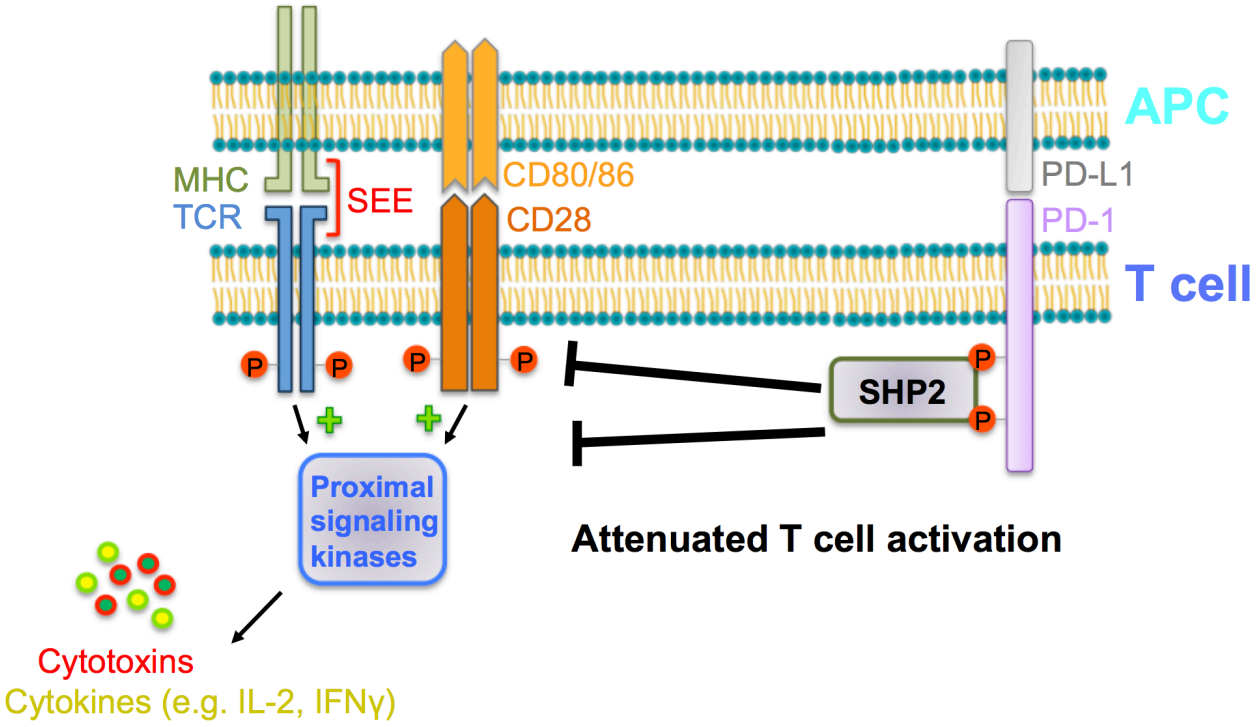


Figure 2 TCR / CD28 inhibition by PD-1 / PD-L1 interaction.

Depiction of key signaling components of the immunological synapse during T cell conjugation with an antigen-presenting cell (APC). Upon recognition of peptide MHC, the T cell receptor (TCR) induces phosphorylation and activation of proximal signaling kinases. The bacterial superantigen *Staphylococcus enterotoxin E* (SEE, shown in red), can be used to stimulate TCR in the absence of agonist peptide. Shown in the middle are co-stimulatory interactions between CD28 and its ligands CD80/86. Shown on the right are co-inhibitory PD-1:PD-L1 interactions, which lead to PD-1 phosphorylation at tyrosine motifs (ITIM and ITSM), recruitment of the tyrosine phosphatase SHP2, and inhibition of T cell function.

1.3.2 CTLA4

CTLA4 is a ~42kDa disulfide-linked homodimeric type I transmembrane glycoprotein which serves as an essential co-inhibitory immune receptor by restraining CD28 co-stimulatory signaling.[147] Although CD28 and CTLA4 share the same B7 family ligands CD80 and CD86, CTLA4 binds these molecules with significantly higher affinity and functions during T cell priming to restrain lymphocyte proliferation and differentiation.[148–150] While CD28 is constitutively expressed on resting T cells, CTLA4 expression is induced upon naïve CD4+ and CD8+ T cell activation following TCR recognition of an agonist peptide antigen (pMHC) ("signal one") in the presence of co-stimulation via CD28 ("signal two").[151]

The centrality of CTLA4 function in the adaptive immune system is most strikingly highlighted by the catastrophic lethal autoimmune phenotype observed in CTLA4 knockout mice, which exhibit uncontrolled lymphoproliferation and die of multi-organ lymphocytic infiltration and tissue destruction within ~2-3 weeks of age.[152] This phenotype can be rescued by homozygous deletion of either CD28 or both CD80 and CD86, generating mice that are severely immunocompromised but viable.[153,154] In humans, CTLA4 haploinsufficiency is extremely rare and heterozygous single nucleotide polymorphisms (SNPs) are also associated with severe autoimmune disease.[155] Similarly, biallelic mutations in factors involved in CTLA4 trafficking, such as LPS-responsive beige like anchor protein (LRBA) which protects CTLA4 from lysosomal degradation, have been associated with autoimmune disorders.[156] Importantly these and other autoimmune disorders, such as rheumatoid arthritis, can in some cases be controlled by treatment with the CTLA4 ectodomain alone fused with an immunoglobulin domain (CTLA4-Ig / "Abatacept") to block CD80/86.[157–159] Overall, while these data demonstrate that CTLA4 achieves its essential functions through CD28 antagonism mediated by CD80/86 binding, several

unusual features of CTLA4 biology have long defied mechanistic understanding and precisely how antagonism of the CD28 pathway occurs remains unclear.[160]

Expression of CTLA4 mRNA peaks ~24-36 hours post activation at which point it becomes detectable at the protein level on the T cell surface.[147] Unlike typical immune receptors, CTLA4 surface levels are generally extremely low and primarily localized within cytoplasmic vesicles which polarize toward the immunological synapse and fuse with the cell surface in response to TCR stimulation, in a manner regulated by TCR signal strength (Figure 3).[161,162] While CTLA4 vesicular trafficking is poorly understood, it is known to be mediated by the short (36aa) intracellular tail sequence of CTLA4 which is highly conserved in all vertebrates and 100% conserved in mammals.[163] CTLA4 undergoes cytoplasmic tail dependent and ligand-independent constitutive cycling to the cell surface followed by recycling and shuttling to endosomal and lysosomal compartments, overall exhibiting a ~95% intracellular localization at equilibrium.[164,165] It has been reported that CTLA4 is stabilized at the cell surface as a result of TCR-induced tyrosine phosphorylation of the endocytic "YVKM" motif present in the CTLA4 cytoplasmic tail region, which prevents its binding to the clathrin adaptor protein AP-2.[166] However, a conflicting report from Professor David Sansom and colleagues provided evidence that clathrin and dynamin-dependent CTLA4 cycling continues during TCR stimulation, in support of their "trans-endocytosis model" of CTLA4 function (section 1.3.2.2).[166,167]

CTLA4 exhibits differential expression in various T cell subsets, reaching significantly higher total and surface levels in CD4+ T cells compared with CD8+ T cells following activation.[168] Although generally absent from resting T cells, memory CD4+ T cells have also been shown to exhibit high levels of intracellular CTLA4, giving rise to the notion that the history of antigen experience by T cells may contribute to the size of the intracellular CTLA4

pool.[101] Most importantly, CTLA4 is constitutively expressed at high levels in the immunosuppressive subset of CD4+ FOXP3+ regulatory T cells (Treg) which have been viewed as the archetypal example of essential CTLA4-mediated suppressive function.[169] Indeed, neonatal FOXP3+ (Treg) specific CTLA4 knockout phenocopies the lethal autoimmune phenotype observed in global CTLA4 deficiency, albeit with delayed onset.[53]

While it has been shown that CTLA4 is essential for antigen-specific suppressive functions of Treg in numerous contexts in vivo, the precise role for CTLA4 in these cells remains unclear.[53,170–172] Definitive determination of the role for CTLA4 in Treg-mediated immunosuppression has been precluded partly by the complexity of Tregs themselves, which are known to mediate non antigen-specific "bystander" suppressive function through a variety of mechanisms which in some cases also appear to employ CTLA4.[172] Examination of the role for CTLA4 in Treg function is additionally limited by the requirement of CTLA4 for Treg development and homeostasis.[172] Indeed as discussed below, Treg-specific CTLA4 deletion in adult animals induces proliferative expansion of the Treg compartment which nevertheless exhibits defective suppressive function resulting in delayed onset and non-lethal but severe multi-organ autoimmune disease.[173]

1.3.2.1 CTLA4 control of B cell responses

Through control of helper CD4+ T cell proliferation and differentiation, CTLA4 also controls B cell antibody responses.[64] Global deletion of CTLA4 in adult mice followed by immunization was shown to induce expansion of T follicular helper cells (Tfh), a specialized subset of CD4+ T cells involved in stimulating germinal center B cells to generate high affinity antibodies, as well as T follicular regulatory cells (Tfr), a specialized subset of Treg involved in suppressing B cell responses.[174] Expansion of these cell populations was associated with

dysregulated antigen specific and total antibody production. Interestingly, this study reported that Tfr suppression of B cells was CTLA4-dependent but not associated with down-regulation of CD80/CD86 on B cells in GCs[174] Similarly a parallel study by Sakaguchi and colleagues showed that Treg specific reduction in CTLA4 expression enhanced Tfh cell and GC formation which was also associated with a loss of antigen specificity, proposed to be mediated by an outgrowth of auto-reactive clones.[171] Together these and related similar findings demonstrate that the absence of CTLA4 induces both cell-autonomous and non-autonomous proliferative expansion of CD4+ T cell subtypes and B cells with potential to enhance antigen specific as well as non-specific responses.[64] Importantly, despite expanded cell numbers CTLA4-deficient suppressive cell types such as Treg and Tfr exhibit defective inhibitory function.[171]

Not examined in the above studies is the recently described expression of CTLA4 by B cells themselves. The expression of CTLA4 and CD28 by B cells in some contexts may add an additional layer of complexity to CTLA4 control of costimulatory signaling and immune homeostasis. Indeed, B cells have been shown to de-repress CD28 during differentiation into antibody-producing 'plasma cells' (PCs) and CD138+ PCs were also recently shown to express CTLA4.[175,176] Intriguingly, a specialized subset of B cells termed B-1a cells, which emerge during murine fetal development and possess an immunoglobulin repertoire enriched for auto-reactivity, were found to require expression of CTLA4 to prevent spontaneous production of autoantibodies.[176]

1.3.2.2 Intrinsic vs. Extrinsic Models of CTLA4 function

Despite extensive reports of cell intrinsic inhibitory effects of CTLA4 expression, CTLA4 does not appear to recruit intracellular phosphatases to inhibit co-stimulatory signaling.[149,160] Indeed, no intrinsic CTLA4-mediated inhibitory signaling pathway has yet been

identified distinct from CD28 signal deprivation.[160] These findings have contributed to a more refined view of CTLA4-mediated cell intrinsic inhibitory signaling which is proposed to occur via direct competition with CD28 at the cell surface, termed "negative co-stimulation."[177] In this model, the dynamic interplay of CD28 and CTLA4 are thought to fine tune TCR signal strength to impact the differentiation landscape of activating T cells in a manner that may be uncoupled from CTLA4 control of cell proliferation.[177] This notion is supported by the numerous seemingly cell intrinsic effects of CTLA4 function described above such as its ability to control proliferative expansion of expressing cells, increase the threshold of TCR activation in CD8+ T cells and CD4+ memory T cells, as well as additional conflicting reports on CTLA4 ligand engagement enhancing T cell motility or adhesion.[160,178–183]

However, cell intrinsic models of CTLA4 regulatory function have been strongly opposed by the observation that CTLA4+ cells are able to dominantly control the auto-reactivity of CTLA4 deficient T cells in trans (i.e. in a cell extrinsic manner or non cell-autonomous manner).[149,160] This remarkable property of CTLA4-mediated regulatory function was demonstrated by the rescue of the lethal autoimmune phenotype associated with CTLA4 deficiency by reconstitution of bone marrow (BM) chimera mice (CTLA4-/-) with a mixture of CTLA4-/- and CTLA4+ cells. This ability of CTLA4+ cells to dominantly control the auto-reactivity of CTLA4-deficient cells in *trans* represents strong support for a primarily cell extrinsic regulatory function of CTLA4.[169,184,185]

A new model was developed to account for this surprising mode of action in which CTLA4 expressing T cells are thought to act in a purely cell extrinsic manner to control levels of co-stimulatory signals in the local environment. In this model, T cell expressed CTLA4 is proposed to extract and internalize bound CD80/86 from the surface of APCs upon cell-cell

contact via “trans-endocytosis” (TE).[186] The TE model addresses several unusual features of CTLA4 which appear optimized for ligand depletion, including its highly endocytic behavior and lysosomal trafficking which results in clear CTLA4-associated accumulation and degradation of exogenous CD80/86 by T cells in vitro.[186,187]

However despite clear evidence of CTLA4-mediated degradation of CD80/86 acquired from APCs in vitro,[167,186] the TE model has not been widely accepted and the physiological relevance of this phenomenon is controversial.[172,188] Indeed, despite efficient CD80/86 depletion from APCs during co-culture with Tregs in vitro, in vivo effect sizes are often surprisingly small or undetectable and direct observations of TE have been very limited, even when using Treg expressing transgenic TCRs.[90,189,190] Furthermore, it has been difficult to extend a purely extrinsic model of CTLA4 function beyond Treg to attempt to account for the observed inhibitory effects in lower CTLA4 expressing cells, such as activated CD8+ and conventional CD4+ T cells, as well as multiple other expressing cell types (including B cells, monocytes and DCs, and various tumor cells).[191]

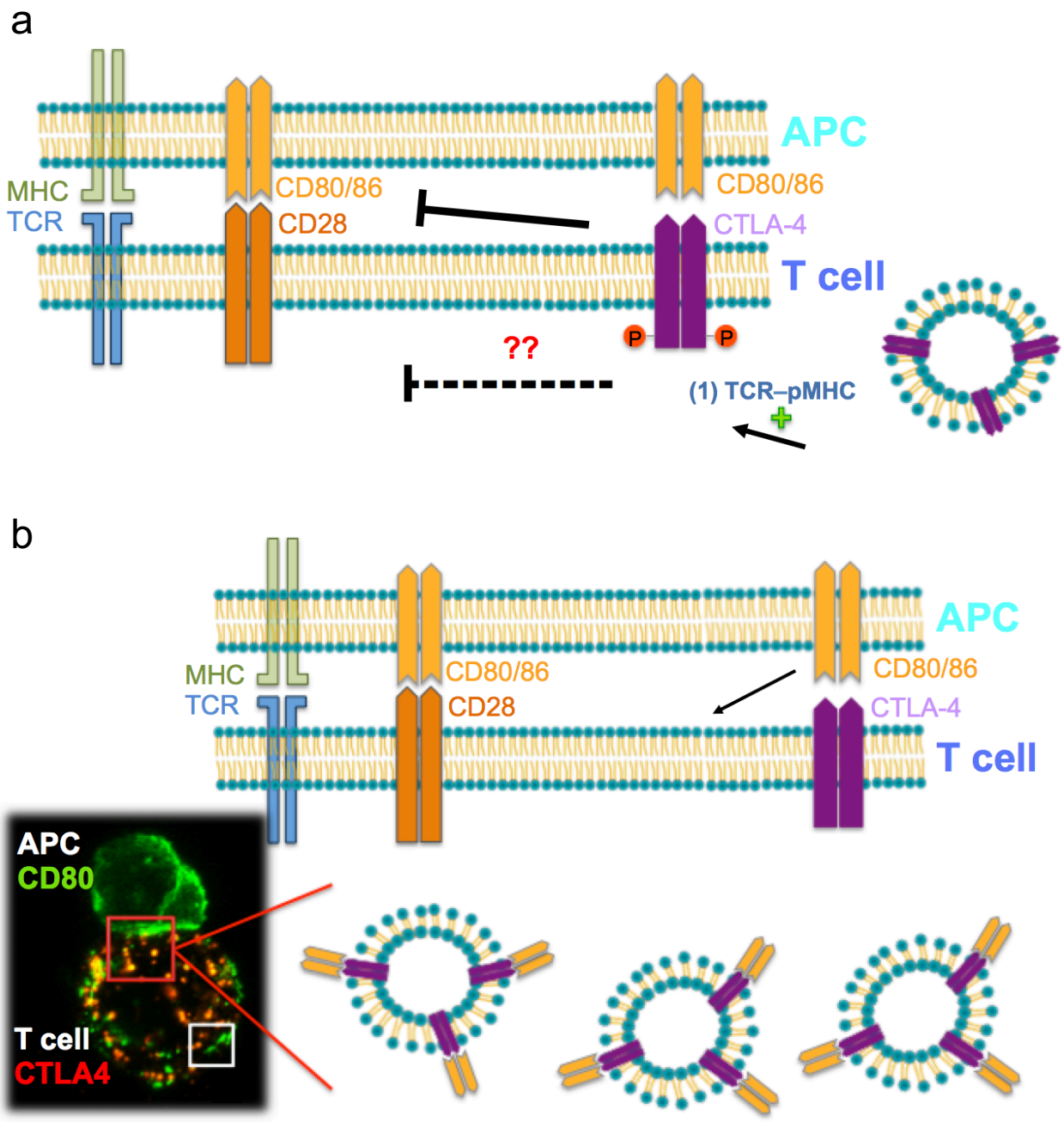


Figure 3. Models for CTLA4 mediated T cell inhibition

Depiction of T cell synapse with an antigen presenting cell (APC). The T cell receptor (TCR) recognizes peptide antigen in the context of MHC on the APC surface. In (a), CTLA4 competes with CD28 for engagement with APC expressed co-stimulatory molecules CD80/86. In (b), CD80/86 are shown to be internalized by CTLA4 where they are degraded. Confocal microscopy image shows exogenous CD80-mGFP accumulation within CTLA4-mCherry associated vesicles. White inset shows CD80-mGFP acquired via CTLA4-independent trogocytosis.

1.4. *Cis* interactions

"*Cis* interactions" can be most broadly defined as ligand-receptor interactions that occur on or within single cells. While generally characterized as occurring between endogenously co-expressed ligand / receptor pairs positioned adjacently on a planar membrane surface, under this broader definition the landscape of potential "*cis*" interactions appears to be increasingly complex. For example, in addition to true planar adjacent interactions among endogenously co-expressed molecules, activating lymphocytes are also known to inherently acquire surface membrane from conjugate cells upon contact via the process of trogocytosis (Section 1.5).[192] While the precise mechanism and dynamics of plasma membrane acquisition and re-display via trogocytosis are currently unclear, this process results the display of discrete exogenous membrane fragments held in apposition at the recipient cell surface followed by internalization and / or membrane fusion, with apparent possibilities for *cis* interactions and sustained signaling during each stage.[130,193–195] Additionally, T cells are densely covered in large 3D membrane projections (~0.1μm in diameter and up to 5μm in length) termed microvilli, which have recently been shown to act as key signaling hubs where TCR and co-stimulatory signaling components become concentrated.[80,82–84,196] Taken together, the emerging image of lymphocyte cell biology appears to offer multiple opportunities for an expanded definition of *cis* regulation. Specifically, in this context *cis* interactions can be considered to include local trans interactions with surface receptors / ligands displayed on acquired membrane fragments prior to membrane fusion, adjacent receptor / ligand pairs co-expressed on the plasma membrane surface co-expression or following membrane fusion after acquisition, and potentially long range *cis* interactions mediated by microvilli.

In the traditional adjacent / planar context, several functionally relevant *cis* interactions have been identified thus far, although their regulatory effects remain generally poorly understood. These include PD-1 / PD-L1 expressed on T cells and PD-L1 / CD80 expressed on APCs, which have both been shown to inhibit PD-1 signaling in T cells.[197–199] Additionally disruption of CD80 homodimers by PD-L1 binding in *cis* on APCs was reported to inhibit high-avidity CD80 engagement and depletion by CTLA4 while preserving CD28 interactions.[200] Furthermore, it was recently shown that CD80 depletion from APCs via CTLA4-mediated trogocytosis can produce a synergistic inhibitory effect of CTLA4 ligation by increasing levels of free surface PD-L1 on APCs to enhance PD-1 signaling in T cells.[188] In NK cells various HLA and H2 (MHC) molecules have also been shown to engage in *cis* with the NK cell receptors Ly49 and LILRs or PIRs following acquisition from target cells.[201,202] Similarly, inhibitory as well as activating *cis* interactions associated with 'cis-endocytosis' have also been described in cells co-expressing both Notch and Notch ligands, offering some precedent for the cis-endocytosis model of CTLA4 function described in Chapter 4.[203,204]

1.5. Trogocytosis

Trogocytosis, from the Greek *trogo* meaning "to gnaw" or "to nibble" can be most generally defined as a cellular ingestion process distinct from phagocytosis in that (1) it occurs stepwise via "nibbling" rather than in a single engulfment event, and (2) is uniquely associated with functional re-display of acquired membrane fragments and associated exogenous surface molecules.[131] This phenomenon was first observed in parasitic amoeba which employ trogocytosis for both target cell ingestion as well as for immune evasion (by re-displaying acquired "self" pMHC molecules) and is particularly widespread among immune cells.[205–207] The potential immunological significance of trogocytosis was first recognized in the 1970s following

observations of host-derived MHC molecules by genetically distinct donor T cells.[208,209] However despite a long history of in vivo observations, the functional importance of trogocytosis in immune cell function has not been widely accepted.

In support of its potential fundamental importance as a mode of intercellular communication, trogocytosis appears to be conserved from amoeba to complex multicellular eukaryotes and is commonly exploited for immune evasion by eukaryotic, bacterial, and viral pathogens as well as in cancer.[210] In vivo functional impacts of trogocytosis have been documented in an array of eukaryotic pathogens, including the malaria parasite and toxoplasmosis as well as in numerous viral infections such as influenza and Epstein Barr Virus (EBV) where surface protein transfer is subverted to facilitate immune evasion and expansion of viral tropism.[211,212] For example, during NK cell attack of EBV infected cells, trogocytosis mediated transfer of the viral entry receptor CD21 from infected cells to NK cells was observed to cause NK cells themselves to become permissive to infection by EBV, despite not expressing CD21 endogenously.[212,213] Similar mechanisms have been proposed for the expansion of HIV tropism via trogocytosis mediated CD8+ T cell acquisition of the CD4 co-receptor to enable HIV infection.[214]

1.5.1 Lymphocyte Trogocytosis

Described as an "inherent property" of lymphocyte activation, T cells and B cells engage in contact-dependent trogocytosis during cell-cell interactions resulting in rapid (minute timescale) acquisition and transient re-display of conjugate cell membranes bearing functional surface receptors.[131,192,215] Although the precise mechanism(s) of lymphocyte trogocytosis remain unknown, membrane acquisition is known to be facilitated by specific receptor / ligand interactions between target and recipient cells.[216] Although trogocytosis is to some extent

inherently bidirectional, T cells appear to exhibit a strong bias for unidirectional acquisition of CD80/86 and pMHC that is greatly enhanced by CD28 expression and / or TCR activation.[217,218] Indeed, transfer of CD28 / CTLA4 or TCR from T cells to APCs via trogocytosis has not been reported. Interestingly, DCs and various myeloid cells commonly engage in bidirectional and serial transfer of pMHC and other surface molecules both among other myeloid cells and to lymphocytes via trogocytosis as well as release of extracellular vesicles upon activation.‡[131,219–221] Notably, while ligand / receptor interactions enhance specific transfer, trogocytosis invariably involves passive co-transfer and re-display of 'bystander' surface molecules as well.[131,222]

It is currently not entirely clear the extent to which antigen-dependent vs. antigen-independent interactions mediate T cell trogocytosis in vivo. It has been demonstrated that trogocytosis can occur in an antigen-independent manner via CD28 binding to CD80/86 but acquisition is further enhanced upon antigen recognition by the TCR as well as in subsequent contacts after T cell activation.[131,217,218] Functionally, T and B cell acquisition of APC-derived membrane fragments has been observed to endow lymphocytes with the capacity to act as APCs.[131] This process has been shown to regulate CD4+ T cell proliferative expansion, CD8+ T cell priming and memory, the polarization of specific T helper subsets, and the recruitment of T cell help to CD8+ T cells and B cells.[194,215,223–231]

Additional examples of immune cell trogocytosis of particular functional and clinical relevance is that of immune checkpoint receptors such as PD-1 and its ligands PD-L1 / PD-L2 as well as TIM-3 which was recently identified as a regulator of trogocytosis.[232] In these studies, natural killer cell acquisition of PD-1 was shown to be involved in immune evasion in leukemia,

‡ Notably, contact-dependent membrane exchange via trogocytosis is difficult distinguish from activation-induced EV secretion / capture and display which has been described in both T cells and APCs in vivo. In many cases these processes likely share mechanistic origin and functional impacts (e.g. via polarized secretion upon activation).[221,360,361,401]

PD-L1/PD-L2 acquisition by monocytes for immune evasion in Hodgkin lymphoma, and antigen-specific acquisition of PD-L1 from DCs by CD8+ T cells was observed to trigger fratricide of PD-1+ CD8+ T cells.[131,233,234] More recently, trogocytosis-mediated pMHC acquisition has gained attention as a critical limitation of chimeric antigen receptor (CAR) T cell therapies which rely on engineering T cells to express a specific antigen receptor to target and eliminate tumor cells. These approaches have been highly successful in treatment of certain hematological malignancies, particularly B cell lymphomas, however, antigen specific interactions of CAR-T cells with target B cells has been observed to trigger trogocytosis, antigen transfer, and subsequent fratricidal killing by T cells, limiting therapeutic effectiveness (discussed section 5.3).[235,236] Representing a unique antigen-specific marker of T cell activation, other recent publications have employed trogocytosis for ligand discovery of 'orphan' TCRs and as a readout for successful targeting by in vivo generated CAR T cells.[237,238]

1.6. Current models of immune system regulation

1.6.1 Early Theories of Antibody Formation

The universality and specificity of the immune response was strikingly revealed in 1901 when Paul Ehrlich and colleagues reported that goats immunized with xenogenic red blood cells (RBCs) were able to produce antibodies which specifically recognized and destroyed RBCs from the donor goat while leaving recipient goat RBCs intact.[1] These results led Ehrlich to recognize that the remarkable ability of the adaptive immune system to specifically recognize subtle divergence in the molecular features of foreign antigens while preventing self-recognition must require some form of learning process by which "self" non-self discrimination could be achieved. A chemist influenced by Darwin's Theory of Evolution by Natural Selection, Ehrlich proposed the first "Selective Theory" of adaptive immunity on the basis of these observations.[239]

In his use of analogy to the chemical side chains of organic dyes that he had pioneered for histology, Ehrlich was also among the first to articulate "Receptor theory" in his hypothesis that immune cells must contain a very large number of antibody receptors, what he called "differing side chains," the best of which would then be selected by antigen to stimulate further production.[239]

Also in the early 1900s, Karl Landsteiner showed that specific antibodies could be produced against –ortho, –meta, and –para positioning of a single carboxyl group on an aromatic amine, highlighting the universality and specificity of the adaptive immune system at the atomic scale.[2] In absence of a plausible mechanism for genetically encoding such an apparently inconceivable scale receptor diversity, Linus Pauling and others proposed alternative "Instructive" theories of antibody formation in which antibodies were said to be initially amorphous and only acquired their highly specific shape upon interaction with an antigen.[239] Viewed to more satisfyingly incorporate the emerging principles of molecular biology and protein folding, Pauling's Instructive model remained the dominant framework until 1955 when Niels Jerne proposed the first modern Selective Theory of antibody formation, following his identification of antigen-specific 'natural' antibodies in unimmunized rabbits.[240]

Similarly influenced by Darwin, Jerne's "Natural Selection" Theory proposed that the complete diversity of unique antibody specificities pre-existed in circulation and were selected by antigen. Upon binding, the antibody/antigen complex was envisaged to be taken up by phagocytic cells which then induced production of further identical copies of this antibody by unknown mechanisms.[240] This suggestion that antibody internalization by phagocytes could somehow induce expression of the corresponding gene in an antibody producing cell was the most sharply criticized aspect of the Jerne's model, seeming to imply specific information

transfer from protein to DNA. This contradicted the emerging understanding of the Central Dogma of molecular biology at this time, leading James Watson to famously tell Jerne that his theory "stinks."[241] Notably, even after the biology of antigen presentation by phagocytes was clarified to resolve Watson's criticism, Jerne would retain the more unorthodox view that the specific variable region binding sites of antibody molecules "idiotopes" were capable of encoding and transmitting information to regulate the adaptive immune system at the network level (section 1.6.8).[242]

1.6.2 Clonal Selection Theory

Jerne's "Natural Selection" Theory immediately catalyzed development of Clonal Selection Theory (CST), which remains the core foundation of modern immunology. First outlined briefly in a 1957 review article by David Talmage, CST arose from a specific modification of Jerne's more abstract proposal to suggest that each individual antibody producing cell displays a unique antibody receptor on its cell surface.[243] Upon stimulation, these "precursor cells" would be induced to proliferate and secrete soluble forms of the antibody receptor which could then be observed to accumulate to high levels in serum as specific immunoglobulins. Within this framework it was then proposed that the mystery of "self" tolerance despite the universality of antigen receptor diversity could be resolved most simply if antibody precursor cells required time to fully mature to be responsive to antigen.[244] Importantly, it was envisaged that precursor cell interaction with antigen prior to this mature stage, such as during neonatal development, would instead induce clonal elimination.

Peter Medawar had earlier provided the first experimental confirmation of this central tenet Clonal Selection Theory by inoculating neonatal mice with cells from a normally non-compatible transplant donor and observing that this treatment to rendered the mice tolerant to

transplantation later in life.[245] For this work Medawar shared 1960 Nobel Prize with MacFarlane Burnet, who most famously formalized CST. In 1958, Joshua Lederberg was the first to demonstrate that clonal B cells produced one specific antibody.[34] Lederberg later made the important mechanistic advance that self-reactive B and T lymphocytes were deleted during development in the bone marrow and thymus respectively as part of a learning process termed "central tolerance." Notably, Lederberg uniquely maintained the view that such an antigen-dependent learning process should continue in some form throughout life.[246]

1.6.3 "Second Signals" of T cell activation: 'Quorum' and co-stimulation models

By the late 1960s it became clear that several experimental results involving the induction and breaking of immune tolerance in animals could not be explained by CST alone and that a "second signal" was required for full activation of lymphocytes in addition to antigen recognition (i.e. "signal one").[41,247,248] This original 'second signal' model stated that coordinated recognition of linked antigens[§] among responding lymphocytes (i.e. 'Quorum') was required for full activation, whereas antigen recognition by single lymphocytes alone led to inactivation.[249] This model, first proposed by Bretscher and Cohn in 1968, was ultimately validated in its description of the "second signal" requirement for CD4+ T cell help to stimulate the induction of B cell antibody responses, most CD8+ T cell responses, and T cell memory (section 1.2).[32,250,251] Notably however, this model also predicted a similar requirement for CD4+ T cell "help" for full activation of CD4+ T cells themselves, with equally compelling supporting evidence.[247,252–254]

The two principal challenges to 'Quorum'-based second signal models of CD4+ T cell consist of the so-called "scarcity problem" and the "priming problem."[247] First, the "scarcity problem" is simply that it seems statistically improbable that at least two rare antigen-specific

[§] i.e. derived from a single molecule

CD4+ T cells (estimated frequency ~10^{-6}) can efficiently form a ternary complex with an activated APC during priming to mediate antigen-specific cooperation in the induction of an immune response.[247,255] Secondly, the "priming problem" raises the question that if cooperation among activated CD4+ T cells is required for full CD4+ T cell activation, then how does the first antigen-specific CD4+ T cell become activated? In a modern version of this framework, the "wo step / Two signal model of lymphocyte activation / inactivation", Bretscher has proposed solutions to both problems by permitting limited CD4+ T cell proliferation following activation by APC displaying pMHC-II in the presence of co-stimulation (to reduce scarcity) followed by interaction of at least two antigen-specific CD4+ T cells with a specific B cell (to eliminate priming problem and enhance non-self specificity of immune responses).[247] In section 5.5 I will suggest that a model which incorporates the functional implications of widespread trogocytosis among activating lymphocytes may also help to mitigate both the 'priming' and 'scarcity' problems.

Following the description of T cell proliferation induced by co-stimulation in the form of CD28 / B7 interactions (section 1.2.3) it appeared to many that the molecular nature of the essential "second signal" of CD4+ T cell activation had been identified and its original meaning, as antigen-linked cellular cooperation, was widely lost.[42] As was noted at the time however, this solution to the "priming problem" (i.e. by naming the essential "second signal" of CD4+ T cell activation as co-stimulatory signaling) came at an enormous theoretical cost, relegating foundational control over CD4+ T cell activation, and therefore "self" tolerance, to myeloid APCs and their germline encoded PRRs responsible for controlling B7 expression (section 1.1).[42] As a result, Bretscher and Cohn and many others have long maintained that cellular cooperation mediated by recognition of linked antigens (i.e. "Quorum") remains essential for full CD4+ T

cell activation.[88,248,249,256,257] This framework accounts for diverse contemporary and particularly historical observations made at the level of the system, including the induction and breaking of immune tolerance by linked-antigens and also incorporates a mechanism of immune class regulation (section1.6.7).[247,248,253,258]

1.6.4 PAMP / Pattern Recognition Theory

In Charles Janeway's 1989 essay "Approaching the Asymptote? Evolution and Revolution in immunology," he famously contradicted the so-called "Landsteinerian fallacy" (section 1.6.1)– i.e. the notion that adaptive immunity "had evolved to recognize all non-self substances."[259] Janeway proposed that rather than "self" / non-self discrimination as originally envisaged, the immune system was instead primarily oriented toward recognition of infectious entities as indicated by the presence of co-stimulatory signaling on APCs. Janeway went on to accurately predict that control of co-stimulation would be mediated by a class of so-called "Pattern Recognition Receptors" (PRRs) expressed by innate immune cells (APCs) which recognize evolutionarily conserved features of microbial pathogens (section 1.1).[25,259] Exposing what he called "immunologists' dirty little secret," Janeway correctly observed that adjuvant consisting of bacterial cells or other factors acting to stimulate innate immune cells had been present (either inadvertently or deliberately) in most immunization experiments carried out up to that point.[259] The revolutionary impact of Janeway's "Pattern Recognition Theory" was immediately recognized and contributed to broad mainstream support of the notion that mechanisms of innate immunity are responsible for initiating and instructing adaptive immune responses.[260]

1.6.5 The Danger model

While Janeway's view retained a "self" / non-self dichotomy (non-infectious "self" /

infectious non-self), the modern understanding of the adaptive immune system has largely developed within the context of Polly Matzinger's further modification Janeway's proposal, known as the "Danger" model. This framework was and remains far more theoretically controversial, criticized in part even by Janeway himself, yet has been enormously successful in extending Janeway's PRR theory to include the majority of what is currently known about the complex interplay of innate and adaptive immune responses.[260–263] Specifically, Matzinger recognized that the PRR/PAMP model alone could not account for key features of adaptive immunity which occurred in the absence of pathogens, including organ transplant rejection and elimination of cancer.[**24,263] To address this issue, Matzinger made the more radical proposal that "self" / non-self discrimination by the adaptive immune system actually does not occur and whether or not to induce a response is instead determined by the presence or absence of 'danger' in the form of endogenous alarm signals.[24] According to this view, (hereafter referred to collectively as the PAMP/ "Danger" model), both "self" / non-self discrimination and immune class regulation are thought to be primarily instructed by innate immune cell control of co-stimulatory signaling (the modern "second signal") and by the particular inflammatory environment of diseased / damaged tissues themselves ("signal three").[260]

1.6.6 Comparison and current view: "Innate control of adaptive immunity"

An unexpected benefit of the combined PAMP / "Danger" paradigm arose from its apparent theoretical weakness in accounting for the robustness of peripheral tolerance. Specifically, the intuitive recognition that according to the PAMP/ "Danger" model, inflammation would seem to carry a much higher risk for autoimmunity than is commonly

[**]The danger model was initially used to explain why the adaptive immune system did not eliminate cancer. Once it was discovered that T cells in fact can and do efficiently eliminate tumor cells, the danger model was adapted to explain why, highlighting the remarkable plasticity of this framework (Discussed in section 5.4, 5.12).[263]

observed gave support to the earlier notion that preservation of immune tolerance is an ongoing process and likely therefore motivated characterization of the activities of various co-inhibitory signaling pathways and dominant immunosuppressive cells.[37,45,264,265] This feature of the PAMP/"Danger" framework likely also contributed to mainstream support following the re-discovery of the controversial class of regulatory "suppressor" T cells during the 1990s.[266]

Overall, the PAMP / "Danger" paradigm has resulted in the modern foundational understanding of peripheral tolerance and immune class regulation as largely controlled by mechanisms of innate immunity.[260] This framework consists of a greatly expanded molecular "second signal" incorporating the dynamic interplay of multiple co-stimulatory and co-inhibitory pathways, a third signal consisting of networks of pleiotropic cytokines released by activating and suppressive APCs, and more recently the inclusion of potential regulatory effects of the organismal microbiome and associated metabolites.[260,267,268] Additionally, somewhat loosely but vitally incorporated into this picture of adaptive immunity is dominant immune suppression and regulation of tissue homeostasis and regeneration by regulatory T cells, which occurs in part via poorly understood CTLA4-dependent mechanisms.[269,270] Together this framework has produced a remarkably complete description of adaptive immune responses. Notably however, despite this apparent success and near-completeness, longstanding historical and modern refuting data continue to challenge the fundamental validity of the theoretical foundations of this perspective (section 1.7, 5.4).[254,258,263]

1.6.7 Implications: Current models of immune class regulation – the Cytokine Milieu and DC subtype hypotheses

The most generally accepted models of immune class regulation (i.e. T helper subset polarization)[††] consist of the "cytokine milieu" and DC subtype hypotheses, which arose as natural predictions of the PAMP/ "Danger" framework.[264] In these models, the nature of the particular "Danger" reflected by distinctive cytokine profile within the local environment and / or of the specific initiating DC subtype respectively are critically responsible for determining T helper subset polarization of an adaptive immune response.[271] While it is clearly demonstrated that specific cytokines and specialized subsets of antigen presenting can drive differentiation of specific T helper subtypes, how this relates to CD4+ T cell determination at the system level of immune responses is less clear.[258] Indeed recent studies have demonstrated a surprising level of heterogeneity in T helper subset differentiation, not only interclonally but also for T cells bearing identical TCR specificities (i.e. intraclonal heterogeneity) during immune responses.[272] Furthermore, T helper subtype commitment increasingly appears to display a high level of plasticity rather than stable fate commitments as originally envisaged (section 1.2.2).[48]

Quorum[‡‡] models: Threshold and Cytokine Implementation Hypotheses

An alternative model for Th1/Th2 polarization was proposed by Peter Bretscher in 1974, termed the "Threshold Hypothesis," is able to account for a large number of system level

[††] Note: Here, T helper subset 'determination' and 'polarization' refer to the nature of the adaptive immune response at the system level rather than differentiation of individual cells, e.g. historically as predominately cellular (Th1) vs. 'humoral' i.e. antibody-mediated (Th2).

[‡‡]The Threshold / Cytokine Implementation hypotheses, and the "Two step / Two signal model of lymphocyte activation / inactivation" are hereafter referred to collectively as Bretscher's "Quorum" framework. Importantly, "quorum" in this sense is used to refer to a hypothetical minimum number of cooperating individual lymphocytes required to induce a response of a certain type, which is related to but somewhat distinct from the more common usage in terms of bacterial or lymphocyte "quorum behavior" which generally refers to population density-dependent coordination of gene expression and the resulting emergent functions of cellular collectives.[459]

observations.[258] In this model, immune class regulation is determined by the extent of cooperation among antigen-specific CD4+ lymphocytes.[273] This perspective can provide quantitative explanations for diverse immunological phenomena, with low levels of cooperation tending to polarize responses toward a cell-mediated (Th1) type whereas high levels of cooperation mediate sufficient coordination with B cells to drive a humoral (Th2) response.[258] Specific examples described by this framework include: the relationship between minimally vs. highly "foreign" antigens and their propensity to induce Th1 vs. Th2 responses, respectively (due to differential degrees of CD4+ T cell cooperation), and the role of antigen dose in this decision-making process, (wherein low vs. high doses of antigen in immunization can be observed to polarize immune responses toward Th1 or Th2 type respectively).[258] This model further accounts for the observation that immune responses tend to evolve toward Th2 polarization over time, (reflecting expansion of antigen-specific 'precursor' CD4+ T cells and resulting enhanced B cell cooperation).[258] Importantly, this model leads to specific predictions which have been extended to successful vaccination strategies and experimental perturbations such as transient CD4+ T cell depletion which was found to be capable of changing a Th2 or mixed Th1/Th2 response toward Th1 polarization.[258] These findings have significant clinical relevance as Th1 responses are difficult to elicit by current vaccination protocols but highly desirable for controlling disease such as cancer and chronic infection (e.g. patients with a sustained Th1 response against HIV do not develop AIDS).[2]

A related proposal, termed the "Cytokine Implementation hypothesis" provides an alternative perspective to the current understanding of specific cytokines as 'instructive' for CD4+ T cell polarization in immune responses.[258] Briefly, this model states that rather than being instructive, elaborated cytokines often serve to promote 'coherence' of an immune response.[258]

The conceptual basis for this hypothesis is the observation that major cytokines produced by a particular T helper subtype (e.g. IL-4 by Th2 cells) tend to be produced by and enhance coherent differentiation of the producing subtype, while inhibiting opposing subtypes.[274] This framework is consistent with the emerging understanding, based on single-cell TCR sequencing, that despite individual CD4+ T cell differentiation trajectories likely being determined by the 'history' of cell experience and the environmental context of activation, an additional selective process appears to be responsible for determining the proliferative expansion of relevant Th subtypes which otherwise exhibit interclonal and even intraclonal heterogeneity in differentiation as discussed above.[258,272,275]

1.6.8 Idiotypic Network Theory

Following his role in the development of Clonal Selection Theory (CST), Niels Jerne went on to further predict that a more general theory of adaptive immunity, which would include but supersede CST, would be needed for a more complete understanding of network-regulation at the level of the entire system.[242] Contrary to the modern view, in which lymphocytes are most commonly analogized with patrolling "soldiers" or rather, according to Matzinger's revision, as "policemen" – (i.e. inactive at steady-state but constantly patrolling for foreign threats or awaiting "danger") — Jerne proposed instead that the adaptive immune system was primarily "self"-focused and dynamically active even in the absence of foreign antigen.[276] In this, Jerne was responsible for development of a cognitive model of adaptive immunity, proposing that the system exhibits a mainly suppressive "eigen behavior" arising from internal network interactions that allow it to develop a dynamic sense of self-awareness and yet remain open to outside stimuli in a manner similar to the central nervous system.[242] Beginning this line of thought with his studies of abundant circulating immunoglobulins ("natural antibodies") in unimmunized animals,

Jerne predicted that such a network would arise between "self"-specific binding interactions among the repertoire of antigen receptor diversity and an additional layer of unique specificities, which recognize these antigen-binding sites themselves.[242]

To facilitate discussion of these interactions, Jerne coined various terms including "epitope" and "idiotope."[277] Epitope was defined as it remains in current usage, as the part of an antigen to which an antibody specifically binds, whereas an idiotope was defined as the unique set of epitopes composing the variable region of an antibody molecule. Thus anti-'idiotypic' (anti-Id) antibodies represent antibodies specific for the variable region of another antibody molecule (i.e. 'anti-antibodies' directed toward epitopes within the antigen binding site).[277] These anti-idiotypic antibodies can compete for antigen binding to the original induced antibody and in some cases can act as a molecular mimic of the original antigen.[278,279] Jerne envisaged that this dynamic harmony among primarily internally focused idiotypic network interactions first encodes and maintains a memory of "self" and that this same process, in its disruption and return to equilibrium, allows the system to learn from experience and encode new memories similar to the human brain.[276] Since Jerne's first proposal of this concept in the 1970s, the induction of anti-idiotypic (anti-Id) antibodies and their regulatory consequences during infection, autoimmune disease, and cancer have been documented in 1000s of publications and Jerne was awarded the Nobel prize for his development of Network Theory in 1984.[242]

Despite an enormous body of literature spanning several decades and continued interest and successful application by clinicians, consideration of Idiotypic Network Theory has been largely abandoned in modern immunology and is generally described disparagingly as a "failed paradigm".[280,281] Indeed the notion that Network theory represents an anachronistic and superfluous phenomenological abstraction is ostensibly supported by the observation that it

seemed to lack explanatory power for the more basic functioning of adaptive immune responses, which were more readily understood under CST and the PAMP / "Danger" model that came to replace Network theory as the dominant paradigm in immunology in the early 1990s.[280,281]

Relevance of Network Theory: A potential mechanism for SARS2 immunopathology

Clinically, anti-Id antibodies have been successfully employed as antigen mimics for vaccination and as therapeutics for autoimmune disease and cancer.[282–284] As a notable contemporary example of the urgent clinical relevance of this phenomenon, Harvard Medical School professor and clinician Dr. Dan Longo and UC Davis professor William Murphy recently proposed an anti-idiotypic mechanism for chronic SARS-Cov2 (SARS2) induced immunopathology or "long COVID." In this model, anti-SARS2 spike protein ('S protein') antibodies are hypothesized to induce the production of anti-idiotypic (i.e. anti-anti-S) antibodies which act as a molecular mimic of a pathological structure in the SARS2 spike itself.[285] Specifically, SARS2 spike induced anti-Ids are hypothesized to engage in auto-reactive binding to host factors normally targeted by the SARS2 spike protein for viral entry such as ACE2 or neuropilin-1, thereby triggering chronic vascular and/or neurological autoimmune pathology.[285] They argue that such a mechanism could account for the similar clinical manifestations of rare COVID vaccine side effects and acute and long COVID immunopathologies such as autoimmune myocarditis, thrombocytopenia, vasculitis, nerve damage and neuropsychiatric syndromes.[286–288] In support of this concept, anti-idiotypic antibodies against murine coxsackievirus B3 have previously been shown to bind myocyte antigens to induce autoimmune myocarditis.[289] Additionally, it was recently observed that auto-antibodies against the SARS2 entry receptor ACE2 could indeed be detected in plasma of patients hospitalized with COVID-

19, implicating formation of anti-Ids against anti-SARS2 spike protein antibodies induced by infection (discussed in section 5.13).[290]

Network Theory: Future outlook and a primordial role for trogocytosis?

In pursuit of a more general theory of adaptive immunity, some have continued to advocate for consideration of idiotypic networks and in these efforts a major goal is to unify the B cell / antibody-based idiotypic interactions described by Jerne with those of T lymphocytes.[291,292] These interactions are similarly thought to consist of TCR recognition of peptides derived from variable regions of antibodies as well as TCR variable regions as means for learning and maintaining "self" tolerance, regulating immune responses, and encoding immune memory.[291,292] In support of this concept, it has been shown that TCR degradation products (i.e. TCR 'idiotopes') can be presented on class I MHC to allow for regulatory CD8+ T cells to control pathogenic CD4+ T cells.[293,294] While research in Network Theory remains highly esoteric and marginalized, these efforts may become increasingly relevant in stimulating progress toward system level understanding and control, particularly light of the increasing public health burden of autoimmune disease and the potential idiotypic etiological basis of SARS2 spike-induced immunopathology outlined above.[285]

In discussion section 5.10.2 I will describe a hypothesis that the continued existence of a more primitive set of "idiotypic-like" network interactions among T cells via trogocytosis-mediated acquisition and display of TCR-specific and associated antigens may contribute to some of the difficulty in detecting and manipulating the idiotypic network described by Jerne.

1.7. Summary and Perspective

Two critical determinations are made by the adaptive immune system: (1) whether or not to induce a response upon antigen encounter (i.e. "self" / non-self discrimination) and (2) what

type of response to induce.[2] Because CD4+ T cell "help" has been shown to be required for both the induction of B cell antibody responses as well as most sustained CD8+ responses and T cell memory, the mechanism of CD4+ T cell activation and helper subset differentiation can be considered central to both determinations.[259] According to the predominant modern view of adaptive immunity under the PAMP/ "Danger" paradigm, both CD4+ T cell activation and helper subset polarization are principally determined in response to co-stimulatory signaling and the local characteristic 'cytokine milieu' provided by specialized subsets of myeloid antigen presenting cells (APCs) of the innate immune system (section 1.6.7).[260]

While co-stimulation and the nature of the local activating environment can clearly determine T cell proliferation and differentiation, how this scales to system level behavior in adaptive immunity is less clear.[275,295] Indeed, despite its enormous descriptive power and continued broad support, the foundational theoretical validity of the "Danger" model of T cell activation remains inconsistent with numerous historical and modern observations made at the level of the system.[88,248,258,296] These include most critically the induction and breaking of immune tolerance through recognition of linked antigens as well as the differential Th1 / Th2 polarization of immune responses in the absence of corresponding changes in 'Danger' signals.[254,297] Indeed, many aspects of Th1 / Th2 polarization can instead be predicted and manipulated on the basis of antigen abundance and CD4+ T cell precursor frequency.[§§258,275,298] Together these and other recent findings support a role for an additional layer of regulation by networks of responding lymphocytes.[87,91,215,227,299]

[§§] A hypothesized role for trogocytosis-mediated antigen competition in mitigating the effects of heterogeneous antigen-specific CD4+ precursor frequency is discussed in section 5.5

1.8. Dissertation Hypotheses

In Chapter 4 an alternative mechanism is described for the essential T cell co-inhibitory receptor CTLA4 acting via *cis*-endocytosis to deplete the B7 co-stimulatory ligands (CD80/86) from the surface of activated T cells in a manner promoted by TCR signaling. In this model, a major regulatory target of CTLA4 inhibitory function is proposed to be the non-equilibrium pool of T cell CD80/86 which are both endogenously expressed and inherently acquired via trogocytosis upon contact with activating antigen presenting cells (APCs). During an immune response these APC-derived inflammatory signals are displayed by responding T cells alongside co-transferred peptide antigens (pMHC), enabling antigen presentation among T cells. In light of a potential role for CTLA4 in regulating the functional effects of trogocytosis mediated T-T antigen presentation via *cis*-endocytosis, several hypotheses summarized below are suggested for further study.

Most directly, it is proposed that CTLA4-mediated *cis*-endocytosis of APC-derived CD80/86 facilitates efficient cell intrinsic and TCR-dependent regulation of "extrinsic" co-stimulatory signals as they are integrated and displayed by recently activated T cells alongside co-acquired peptide antigen. By permitting fine TCR-dependent control over co-stimulatory signaling, this mechanism seems to offer several advantages for CTLA4-dependent collective control of lymphocyte proliferation and differentiation. Most broadly, the outcomes of this process are hypothesized to become critical at the level of the system by impinging upon the ability of individual activating T cells to establish a coherent 'Quorum' of similarly responding clones. The presence or absence of quorum among lymphocytes and the role of trogocytosis and CTLA4 in this determination is proposed to constitute alternative molecular and cellular basis for "self" tolerance and class regulation by the adaptive immune system (section 5.2, 5.4, 5.6).

The primary opposition to previously proposed 'Quorum' models of T cell activation consist of the so-called "scarcity" and "priming" problems (section 1.6.3).[247] In Chapter 5 it is argued that both problems are mitigated in a framework that recognizes trogocytosis as a fundamental feature of T cell activation (section 5.3, 5.5). Similarly, it is proposed that rather than solely targeting APCs directly, consideration of CTLA4-dependent regulation of co-stimulatory signaling in the context of antigen presentation among responding T cells displaying APC-derived surface molecules can provide a synthesis of the current PAMP/Danger paradigm with a 'Quorum' framework of T cell activation and priming (section 5.4, 5.6). Specifically, information exchange via trogocytosis appears to allow for decentralized antigen-specific competition and cooperation among lymphocytes such that self-organizing responses to both specific and associated antigens can occur in parallel as antigen becomes dispersed and displayed by the network (section 5.5). In this respect, this view also seems to recapitulate elements of Jerne's Network Theory (section 1.6.8), as the unique specificities of T cell antigen receptors, as well as their homing to distinct tissue environments,[***] is predicted to produce a kind of dynamic contextual 'internal reflection' of specifically acquired and associated antigens, both in homeostasis and throughout the course of an immune response.[270] In this model, natural auto-reactivity observed in the repertoire of healthy humans represents less of a problem or "failure" of central tolerance but rather a feature of the network which is developed, and perhaps originally evolved, in the context of collective "self"-focused regulation (section 5.10).

It is further argued that this perspective does not necessarily conflict with increasingly sophisticated linear models of adaptive immunity but rather complements this understanding by revealing a highly subtle means for rapid information transfer and collective processing within

[***]Display of tissue-associated antigens is likely particularly relevant for the "self"-skewed TCR repertoire of regulatory T cells (section 5.7).[270]

the system (section 5.9.2). Indeed, collective communication among T cells via this ubiquitous phenomenon of transient "mirroring" of APC-derived surface molecules via trogocytosis should perhaps be expected to be largely functionally indistinguishable from initial activating T cell / APC interactions in vivo. It is hypothesized that this process nevertheless facilitates temporal integration of both antigenic and contextual co-stimulatory information onto the surface of activating lymphocytes where CTLA4 can efficiently exert antigen receptor-dependent regulatory function.

Distinct from purely *trans* interactions with APCs, a *cis*-endocytosis mechanism is uniquely able to distinguish CTLA4 regulation of T cell trogocytosis and generates several predictions that may expand CTLA4 biology to address several longstanding paradoxes and help clarify the elusive molecular basis of its complex coordination of lymphocyte collective behaviors. Despite its mechanistic subtlety, recognition of trogocytosis as a fundamental feature of lymphocyte activation produces otherwise unanticipated perspectives on poorly understood immunological phenomena. These include for example, additional support for a 'developmental' model of T cell differentiation and memory (section 5.11), an alternative framework for decoding the functional logic of surprisingly abundant but poorly understood *cis* interactions among co-expressed immune receptor / ligand pairs, and possible novel regulatory functions for other currently enigmatic immune checkpoint receptors such as LAG-3 and TIM-3, both of which were indeed recently shown to be respectively involved in mediating immune suppression and preventing anti-cancer immunity via trogocytosis (section 5.14).[232,300] Finally, it is proposed that this view, distinct from previous Quorum hypotheses of T cell activation and the PAMP/ "Danger" framework, has the unique advantage of incorporating principles of Network theory and a foundational role for immunosuppressive regulatory T cells in the maintenance of immune

tolerance, tissue homeostasis, and regeneration (section 5.10). From this perspective an alternative model is proposed to attempt to clarify the CTLA4-dependent mechanism of antigen-specific suppression by these cells (section 5.2 and 5.7).

Commonly suspected to represent an epiphenomenon arising from termination of the immune synapse or an aberration associated with T cell dysfunction, I will defend the view that trogocytosis instead represents a primordial mode of intercellular communication ubiquitously employed by immune cells.[192,207,213,217,222,301] In this context, a hypothesis is proposed that trogocytosis was intimately involved in the evolutionary origins of the adaptive immune system itself (section 5.10), with potential implications for evolutionary theory more broadly (section 5.10.3). Analogous to complex decision-making processes made at the level of the colony by social insects through environmental accumulation and local display of cuticular hydrocarbons among individuals (section 5.9.1), it is proposed that trogocytosis likely facilitated and accelerated the emergence of complex collective behaviors and functional specialization of lymphocyte subsets in early vertebrate evolution.[302] This unusual "reflective" mechanism is hypothesized to have allowed networks of lymphocytes bearing APC-derived surface molecules to begin to create dynamic internal images of both "self" and foreign stimuli to allow the early immune system to begin to dynamically "see itself" through what could be considered a primitive form of idiotypic interactions (section 1.6.8, 5.10.2). The fundamental importance of this phenomenon despite its inherent subtlety is perhaps best captured by the potential parallel with Janeway's revolutionary prediction of immune regulation by PRRs –specifically in as much as the equivalent proposal may hold true for trogocytosis-mediated network interactions, that "this earlier system persists today and accounts for many attributes of the immune system."[259]

Chapter 2: Materials and methods

2.1. Cell lines

Jurkat T cell and Raji B cell lines were obtained from ATCC. To generate cell lines expressing the fluorescently tagged fusion proteins shown in Table 1, 1 million cells were resuspended in 1ml of lentiviral supernatant collected from HEK 293T cells transfected with constructs indicated in Table 3. After 12 hours of incubation at 37ºC / 5% CO_2 cells were resuspended with fresh complete RPMI medium (supplemented with 10% FBS, 100U/mL penicillin, and 100µg/mL streptomycin). After culturing cells for 5 days, transgene expression was quantified by flow cytometry and positive cells were sorted by FACS if necessary as indicated.

Table 1. List of cell lines

Indicated are the transduced cell type, transgene, fluorescent label, and final cell line nomenclature.

Cell type	Parental line	Protein tagged	Promoter	Fluorophore	Cell line ID
Jurkat	WT	CTLA4	sffv	mCherry	CTLA4-mCherry high
Jurkat	WT	CTLA4	sv40	mCherry	CTLA4-mCherry low
Jurkat	WT	CTLA4	sffv	HaloTag +JFX549/646	CTLA4-Halo high
Jurkat	WT	CTLA4	sv40	HaloTag +JFX549/646	CTLA4-Halo low
Jurkat	CD28 KO	CTLA4	sffv	mCherry	CTLA4-mCherry high
Jurkat	CD28 KO	CTLA4	sv40	mCherry	CTLA4-mCherry low
Jurkat	CD28 KO	CTLA4	sffv	HaloTag +JFX549/646	CTLA4-Halo high
Jurkat	CD28 KO	CTLA4	sv40	HaloTag +JFX549/646	CTLA4-Halo low
Raji B	CD80/86 KO	CD80	sffv	mGFP	Raji-CD80-GFP
Raji B	CD80/86 KO	CD86	sffv	mGFP	Raji-CD86-GFP

2.2. Cell culture

Jurkat T and Raji B cells were cultured in complete Roswell Park Memorial Institute (RPMI) Medium (Corning) 10% fetal bovine serum (Omega) and 100units/mL Penicillin + 100µg/mL Streptomycin (HyClone). Cells were maintained in T25 or T75 flasks (VWR) and incubated at 37°C and 5% CO_2 with passaging every 2-3 days.

2.3. Protein purification

Recombinant ALFA-APEX2 was cloned into pET28 expression vector and transformed into BL21(DE3) strain of e coli and cultured overnight at 37ºC in 5mL. After overnight incubation bacterial culture was scaled to 1 L, cultured for 4 hours at 37ºC to OD=0.6 and induced with 0.1mM IPTG overnight at 16ºC under constant shaking. Bacterial culture was then collected by centrifugation at 5,000xg at 4ºC before addition of ice cold lysis buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 5% glycerol, 5 mM imidazole, 10 ug/ml DNAase, 1 mM PMSF (freshly added), 5 mM beta-mercaptoethanol (freshly added) and lysis by sonication. Lysate was then clarified by centrifugation at 20,000xg for 30 minutes at 4ºC. Clarified lysates were next incubated with 50% Ni-NTA beads for 3 hours at 4ºC prior to addition of wash buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 30 mM imidazole with 5 mM beta-mercaptoethanol). Samples were then eluted in 20 mM HEPES, pH 8.0, 150 mM NaCl, 0.1-0.5 M imidazole with 5 mM beta-mercaptoethanol). Eluates were concentrated using centrifugal filter (Amicon), and purified using Superdex 75 gel filtration column. Subsamples were taken at each step for analysis by SDS-PAGE.

2.4. Proximity labeling

For MS sample preparation 20-40 million Jurkat cells expressing PD-1 ALFA tag were conjugated at 1:1 ratio with 20-40 million Raji B cells expressing PD-L1 mCherry in the presence or absence of SEE (30ng/mL). Cell conjugates were then fixed in freshly prepared 2% PFA for 10 minutes at 37ºC either immediately ("t=0") or at 3 minutes, 7 minutes, 10 minutes, or 30 minutes post conjugation. Cells were then washed 3x in PBST and permeabilized using 0.5% Triton-X for 15 minutes at room temperature (RT) and blocked in 1% BSA in PBST for 2 hours rocking at RT. Samples were then incubated with 125nM recombinant ALFA-APEX2 overnight at 4ºC under constant inversion, protected from light. Following overnight incubation samples were washed extensively over 4 hours in PBST with buffer exchange every 30 minutes while protected from light. Proximity labeling reaction was performed by incubation with 2.5μM biotin tyramide (Fisher) for 5 minutes prior to addition of reaction buffer containing 0.003% H_2O_2, reaction was allowed to proceed for 2 minutes at room temperature before removing reaction buffer by centrifugation followed by aspiration and washing 3x in quenching solution as described previously.[303] Subsamples were taken and PD-1 associated biotin deposition at immune synapse was assessed by staining with streptavidin-647 (Jackson) and imaging by TIRF microscopy. Samples were next heated at 65ºC overnight in lysis buffer containing 2% SDS, and EDTA-free cOmplete protease inhibitor tablet (Roche) overnight to reverse formaldehyde crosslinking. Following lysis samples were diluted to 0.2% SDS final concentration, incubated with benzonase (EMD Millipore), and enriched overnight using streptavidin magnetic beads (Pierce) by incubation at 4ºC with constant inversion. Following overnight incubation beads were washed and eluted by boiling in the presence of 8% SDS in excess biotin as described previously or chemically cleaved by addition of DTT for cleavable biotin-SS-tyramide (Sigma).

2.5. Pharmacological treatments

For experiments using inhibition of CTLA4, cells were treated with 50-100µg/mL Ipilimumab (Selleckchem) at a cell density of 20 million/mL for 30 minutes at 4ºC. Recombinant anti-CD28 scFv (gift from Dr. Xiaozheng Xu), was used at (10-50µg/mL).

Table 2. List of antibodies

This table shows a list of antibodies, supplier, and dilution used for Flow Cytometry (FCS), Western Blotting (WB) or immunofluorescence (IF).

Antibody	Species	Fluorophore	Supplier	Clone	Catalog #	App.	Dilution
CD28	Mouse	APC	BioLegend		302912	FCS	1:200
CD80	Mouse	APC	BioLegend	2D10	305220	FCS	1:200
CD86	Mouse	APC	BioLegend		374207	FCS	1:200
CTLA4	Mouse	APC	BioLegend	L3D10	349908	FCS	1:200
PD-1	Mouse	APC	BioLegend	EH12.2 H7	329907	FCS	1:200
PD-L1	Mouse	APC	BioLegend		393609	FCS	1:200
CD28	Rb mono	N/A	CST	D2Z4E	38774S	IF	1:200
CTLA4	Mouse	N/A	SB Bio	BSB-88	BSB-2880 (ASR)	IF	1:100
CD80	Mouse	N/A	ProteinTech	1E2F10	66406-1	IF	1:200

2.6. Western Blotting

For Western blotting, cells were collected by centrifugation at 300xg and resuspended in 4X Laemmli buffer to a final concentration of 1X for cell lysis. 10% SDS-PAGE gels were cast and ran at 150V for 45 minutes in 1X TGS running buffer (Tris 25 mM, Glycine 192 mM, 0.1% SDS, pH 8.3). Proteins were blotted onto nitrocellulose membrane using wet transfer method in

BioRad transfer tank at 100V for 50 minutes in 1X transfer buffer (20% Methanol, 25mM Tris 192mM Glycine). Membranes were blocked in 5% BSA for 1 hour before probing with primary antibody diluted in 5% BSA in PBST (PBS-/-, 0.1% Tween-20) overnight. Membranes were then washed in (PBST) 3x, and HRP conjugated secondary antibody was diluted in 5% BSA in PBST and incubated for 1 hour. For biotinylation analysis membranes were blotted directly with streptavidin-HRP. Upon addition of luminol and H_2O_2 developing reagents (SuperSignal West Pico PLUS, Thermo Fisher) proteins were visualized by chemiluminescence.

2.7. Plasmids and cloning

Genes of interest were amplified by PCR from existing plasmids in the Hui Lab. Recombinant inserts were cloned using overlap PCR or Gibson assembly and ligated into the pHR plasmid vector linearized with NotI and MluI, or EcoRI as indicated in Table 3.

Table 3. List of plasmids

List of plasmids generated for use in this thesis. Left column shows the final plasmid identification, right shows plasmid vector backbone digested with indicated restriction enzymes.

Plasmid name	Backbone/restriction enzymes
pHR_hPD1_FLAG (3x)	pHR (MluI+NotI)
pHR_hPD1_mGFP-ALFA tag	pHR (MluI+NotI)
pHR_hPD1_mGFP-ALFA tag (2x)	pHR (MluI+NotI)
pET28a_10xHis-preSci_APEX2_ALFA Nb	pET28a
pHR_hPD1-(21aa)-ALFA tag	pHR (MluI+NotI)
pHR_hPD1-(21aa)-ALFA tag (2x)	pHR (MluI+NotI)
pHR_CTLA4 (Y139A)	pHR (MluI+NotI)
pHR_CD28 (Y122A)	pHR (MluI+NotI)
pHR-hPD1ΔCyt_ALFA (2x)	pHR (MluI+NotI)
pHR-PD1 FFmut_ALFA (2x)	pHR (MluI+NotI)
pHR-PD1 FF scrambled_ALFA (2x)	pHR (MluI+NotI)
pHR-hPD-1_extm-hCTLA4_mGFP_ALFA (2x)	pHR (MluI+NotI)
pHR-hPD1ΔCyt-mGFP_ALFA (2x)	pHR (MluI+NotI)
pHR-PD1 FFmut_mGFP_ALFA (2x)	pHR (MluI+NotI)
pHR_pSV40 CTLA4	pHR (MluI+NotI)
pHR_pSV40 CTLA4 (Y139A)	pHR (MluI+NotI)
pHR-hPD-1_extm-hCTLA4_ALFA (2x)	pHR (MluI+NotI)
pHR-hPD-1_extm-hCTLA4 (YF)_ALFA (2x)	pHR (MluI+NotI)
pHR_CTLA4 (Y139A) mCherry	pHR (EcoRI+NotI)
pHR_SV40_CTLA4 HaloTag	pHR (EcoRI+NotI)
pHR_SV40_CTLA4 (Y139A) HaloTag	pHR (EcoRI+NotI)
pHR_SV40_CTLA4 (Y139A) mCherry	pHR (EcoRI+NotI)
pHR_pSV40 CD28	pHR (EcoRI+NotI)

2.8. Lentivirus production

Lentivirus constructs were generated using the HIV-1 based pHR lentiviral vector digested with NotI and MluI or EcoRI (Section 2.6). For each cell line, 0.8 million HEK 293T cells were seeded onto 6-well plates (VWR) ~24 hours prior to transfection. At 70% confluence HEK 293T cells were transfected with 2.5µL (500ng) of each of the indicated plasmids: transfer

plasmid containing gene of interest (pHR), pMD2.G (VSV-G envelope protein), and psPAX2 (Gag, Pol, Rev, and Tat). Transfection was performed using 10μL Polyethylenimine (PEI) (1mg/mL stock solution) diluted 1:6 into 50μL DMEM. PEI-DMEM mixture was combined with the above plasmids and incubated at room temperature (RT) for 15 minutes prior to diluting in 1mL complete DMEM and applying to HEK 293T cells. Transfected cells were then cultured at 37ºC / 5% CO_2 for 12 hours prior to replacing medium with complete RPMI. Lentivirus was harvested by collecting supernatant 72 hours post-transfection. HEK 293T cells and debris were removed from lentiviral supernatant by centrifugation at 1000xg for 5 minutes. Viral supernatant was frozen in liquid N_2 and stored at -80°C.

2.9. Fluorescence activated cell sorting (FACS)

For FACS sorting cells were centrifuged at 300xg for 3 minutes and resuspended to 10 million / mL in FACS buffer (PBS -/-, 2% FBS). Viable cells were identified based on light scatter. In addition, single cell gating (FSC-A vs. FSC-W / SSC-W vs. SSC-A) was used to exclude doublets and cell aggregates. mGFP and mCherry expressing cells were sorted using 488nm laser excitation and 510-550nm emission, and 552nm excitation with 600-620nm emission, respectively, using a BD FACS Aria cell sorter (BD Biosciences).

2.10. Flow cytometry

Cells were centrifuged at 300xg and stained with ViaFluor 405 or 488 cell proliferation dyes (Biotium) to distinguish cell types after they are combined for the trogocytosis assay. Following trogocytosis assay and/or FACS sorting, cells were stained with indicated antibodies for 1 hour on ice at 4ºC. These cells were washed and resuspended using FACS buffer (PBS-/-, 2% FBS), yielding cell densities <10 million / mL prior to analysis. These cells were analyzed on a BD LSR Fortessa (BD Biosciences). Acquired datasets were analyzed using FlowJo software.

2.11. Light microscopy

For imaging experiments, cells were seeded on #1.5 glass bottomed 96-well plates (CellVis), coated with Poly-D-lysine (Core Bio). Cells were permeabilized in 0.1% Triton/PBS for 10 minutes at room temperature, blocked in 5% BSA/PBS for 1 hour, and incubated with indicated antibodies for 1-2 hrs. Images were acquired on a spinning disk confocal microscope (Nikon) using a 100x oil objective.

2.12. Image analysis

Image analysis was performed using Fiji, using a custom macro that uses in built Fiji plugins to: (i) segment cells, (ii) quantify signal intensity, and (iii) measure correlation between channels.

2.13. Electron microscopy

1 million Jurkat, Tconv, or Treg cells and 1 million Raji cells were collected per experiment. CD4+ T cells were incubated with 5nM of TMR-Halo ligand (Janelia Flour) in complete medium for 30 minutes at 37ºC for HaloTag labeling. Cells contact was then induced by centrifugation and cells were incubated for 5 or 15 minutes in a 37º water bath (short incubation was performed for figure 15 to preclude possible ligand internalization by "trans-endocytosis"). Cells were next resuspended in media and seeded at low density into 35mm glass bottomed gridded dishes (MatTek) and incubated for 15 mins prior to fixing in 4% (v/v) paraformaldehyde (Electron Microscopy Sciences) for 15 minutes at RT. For EM immunolabeling cells were incubated with CD80 antibody (1:200) for 2 hours at RT, followed by gold conjugated secondary antibody (EMS, 1:200) for 1 hour at RT. Cells were washed in PBS and incubated with 3',3'-diaminobenzidine (DAB). While in DAB solution, cells expressing HaloTag fusion proteins were subjected to laser excitation at 546nm for 1 minute to induce

oxidative polymerization of DAB proximal to the Halo ligand fluorophore. Cells were then post-fixed in 2.5% (v/v) glutaraldehyde (Electron Microscopy Sciences) for 30 minutes. Cells were washed (3x 5 minutes) in 0.1M sodium cacodylate buffer and post-fixed in 2% (w/v) reduced Osmium/0.1M cacodylate for 30 minutes. Cells were washed (5 x 1 minute) in glass-distilled ultrapure H_2O. Cells were dehydrated through an ethanol series; 20%, 50%, 70%, 90%, 100%, 100%, 100%, (v/v) for 3 minutes each. Ethanol was removed and Durcupan resin (Sigma) was poured over the cells and incubated for a total of 4 hours with constant rocking and 2 resin exchanges. Fresh Durcupan was then added and samples were incubated at 60ºC for 48 hours. The ROI was removed from the dish using a diamond saw and mounted onto an EM sample dummy-stub. 70 nm sections were cut using an EM UC7 microtome and a diamond knife (Diatome) and collected using 3mm copper slot grids (Luxel).

2.14. Statistical analysis

Numerical data was displayed using GraphPad Prism, and statistically analyzed using a one-way ANOVA or Student' T-Test. Significance is depicted using *, which indicates: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Chapter 3: Proteomic profiling of the PD-1 proximal signalosome

3.1. Introduction

PD-1 is co-inhibitory immune receptor that restrains T cell activation and effector function upon interacting with its ligands PD-L1 or PD-L2, expressed on APCs and target cells (section 1.3.1). Mechanistically, activation of PD-1 leads to the recruitment of the protein tyrosine phosphatase SHP2, which functions to de-phosphorylate both CD28 and TCR proximal signaling components, thereby collectively dampening TCR and co-stimulatory signaling. While SHP2 is considered the canonical effector of PD-1 inhibitory signaling, experiments in human and mouse T cells have indicated that additional unidentified factors may be involved in PD-1 function.

Several recent studies have sought to define the PD-1 interactome using traditional affinity purification methods coupled with mass spectrometry (AP-MS).[136,304,305] While these analyses have produced novel insights, AP-MS most reliably detects stable binding interactions that form in free-solution whereas T cell signaling occurs in a unique cellular environment in which signaling components rapidly accumulate in highly concentrated protein condensates termed 'signalosomes'.[306,307] High local concentration of recruited factors within these assemblies can allow for relatively low affinity interactions to contribute to signaling outputs.[308] However the formation and stability of these dynamic assemblies is promoted by restricted 2D diffusion at the membrane interface, a critical feature which is destroyed by cell lysis required for traditional AP-MS.[309]

More recently, spatially restricted proteomics methods known as proximity labeling have been developed to overcome traditional limitations of AP-MS enabling comprehensive proteomic profiling of biomolecular condensates in intact cells.[310] Proximity labeling approaches

typically involve fusing a promiscuous labeling enzyme such as a peroxidase (e.g. APEX2) or biotin ligase with a target protein of interest to induce local covalent deposition of a modified biotin substrate onto nearby proteins (Figure 4).[311,303] This technique thus allows for streptavidin enrichment of native protein-protein interaction networks and comprehensive profiling by mass spectrometry (MS).[303,312] A recently published proximity labeling method was therefore adapted to probe the composition of the PD-1 inhibitory signalosome in the native cellular environment. This approach was optimized to uncover novel regulators, effectors, and substrates of co-inhibitory receptor signaling, with the goal of identifying potential unknown effectors of SHP1/2-independent inhibitory functions of PD-1. Specifically, in this section the development of a nanobody-based proximity labeling approach is described which utilizes the recently developed ALFA-nanobody / ALFA-tag system to specifically recruit APEX2 to the PD-1 proximal signalosome in activated T cells.[313] This approach successfully identified known interactors of PD-1 and is amenable for proteomic profiling of additional immune co-receptors.

3.2. Results

Development of proximity labeling approach to define PD1 interactors

Proximity labeling using the soybean ascorbate peroxidase APEX2 typically relies on genetic fusion of the peroxidase directly to the protein of interest.[303] Upon addition of hydrogen peroxide and phenolic biotin substrate, APEX2 catalyzes the local generation of highly reactive phenoxyl radicals resulting in the biotinylation of proximal proteins that can then be enriched by streptavidin and identified by MS.[303] However, peroxidase fusions often perturb native protein function and can be particularly problematic for transmembrane proteins.[314] Additionally, recent technological advances in MS instrumentation and data analysis have made MS analysis of formaldehyde fixed samples feasible and highly efficient. Recently described proximity labeling methods have exploited this potential for antibody-based proximity labeling. In these approaches horseradish peroxidase (HRP) conjugated secondary antibodies can be used in the place of genetic peroxidase fusions.[315] I hypothesized that such an approach based on formaldehyde fixation and antibody labeling would be amenable to profiling the rapid kinetics of immune co-receptor signaling.

I therefore designed a recombinant fusion protein consisting of APEX2 fused with a recently described camelid nanobody, termed ALFA nanobody.[313] By tagging PD-1 with a short epitope "ALFA-tag" which is bound with picomolar affinity by the ALFA nanobody, this system allows for specific targeting of APEX2 to the PD-1 proximal signalosome (Figure 4).[313]

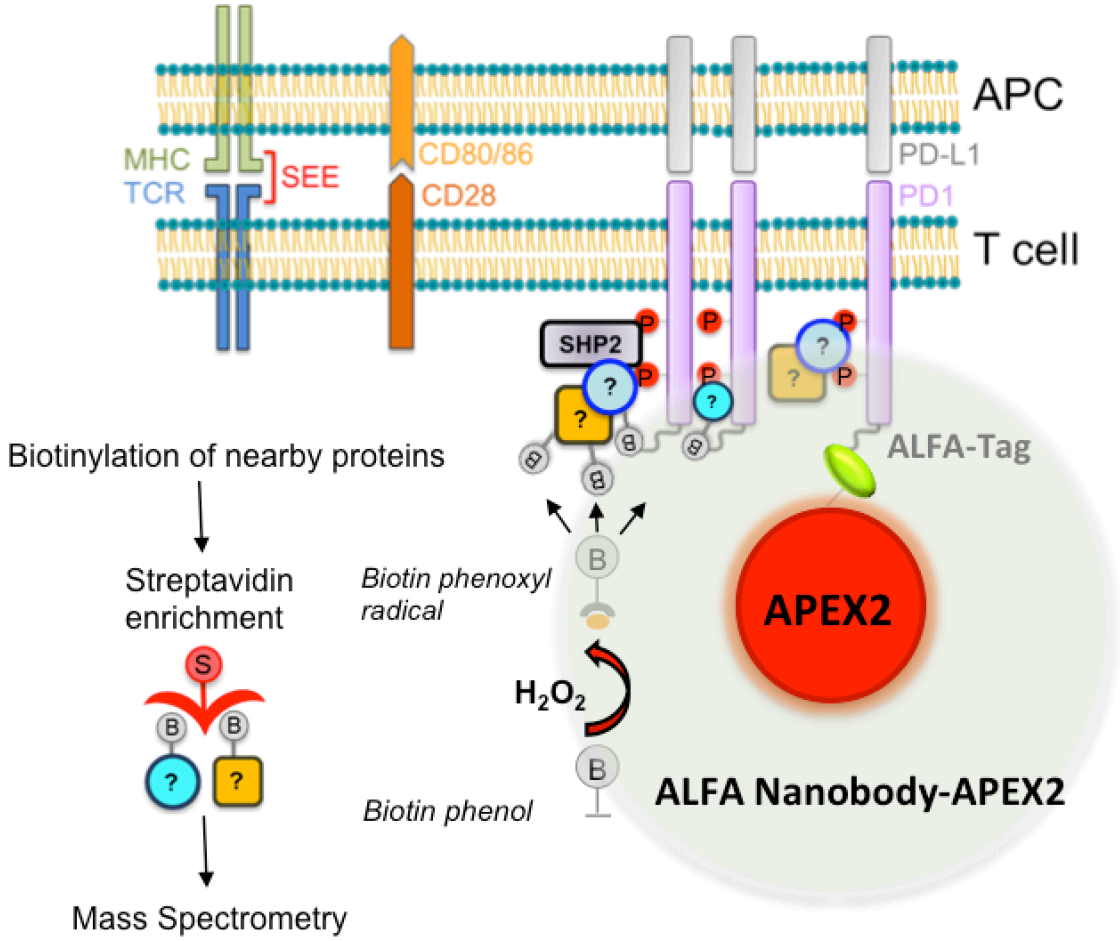


Figure 4. Nanobody-based proximity labeling of PD-1 during T cell activation:

Proximity labeling in fixed, permeabilized T cells during activation by bacterial 'superantigen' (SEE) pulsed artificial antigen presenting cells (APCs). APEX2 peroxidase conjugated nanobody locally generates highly reactive phenoxyl radicals from a modified phenolic biotin substrate in the presence of H_2O_2. Biotinylated proteins proximal to PD-1 are then enriched by streptavidin and identified by mass spectrometry.[303,315]

To produce recombinant ALFA-nanobody fused to APEX2, a bacterial expression vector was designed encoding APEX2 with a C-terminal fusion to ALFA-nanobody with a 6x histidine affinity tag. This construct was expressed in E coli and purified using a histidine-binding nickel (Ni-NTA) resin. After elution from the beads, monomeric APEX2-ALFA-nanobody (ALFA-Nb) was obtained using gel filtration chromatography (Figure 5). This APEX2-ALFA-Nb conjugate enables tethering of the APEX2 peroxidase to any ALFA tagged protein of interest, thus

allowing for versatile and reproducible proteomic profiling of protein interaction networks in fixed samples.

To confirm that the purified APEX2-ALFA-Nb is catalytically active, an in vitro colorimetric assay was performed to assess the ability of the APEX2 peroxidase to convert a colorless soluble 3,3' -diaminobenzidine (DAB) solution into an opaque brown precipitate via the well-known peroxidase catalyzed induction of oxidative polymerization of DAB in the presence of H_2O_2 (Figure 5D).[316]

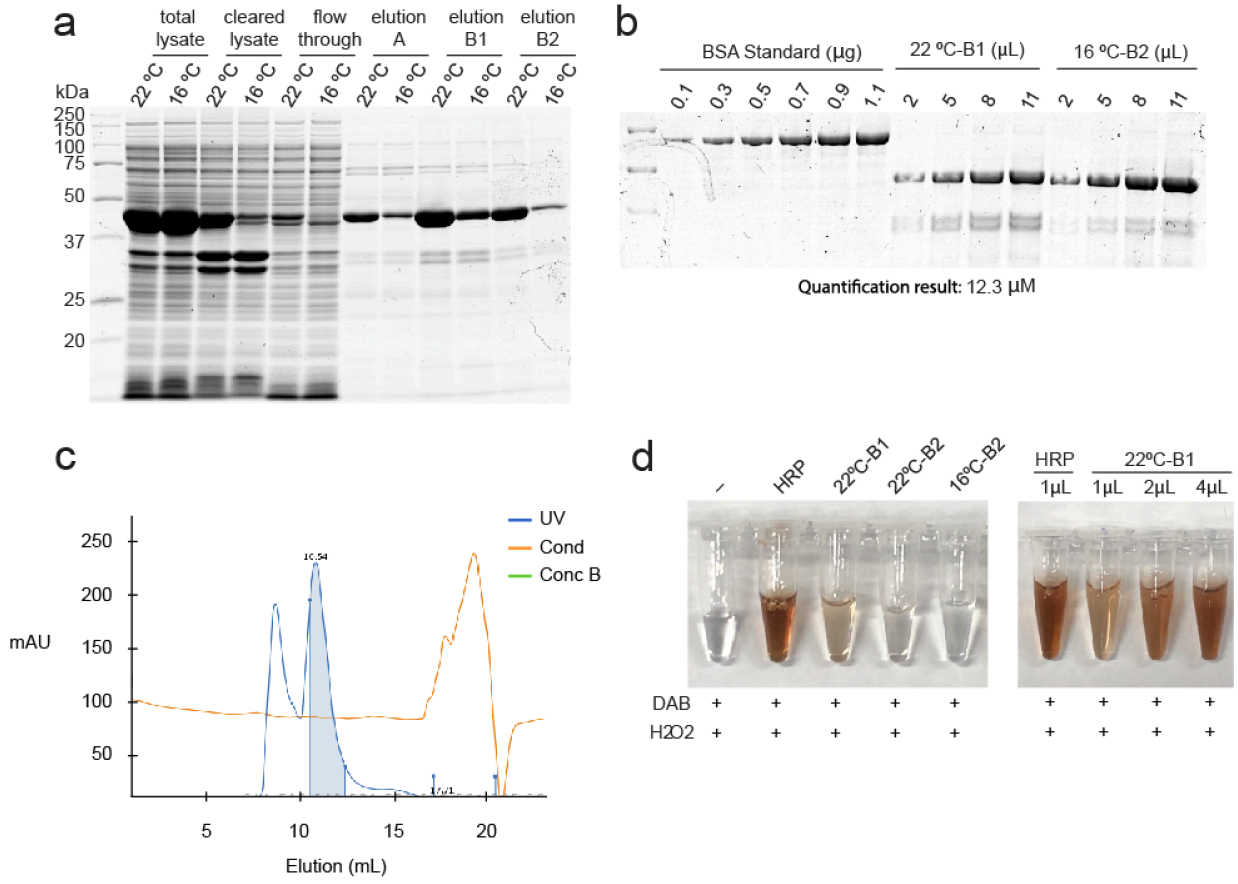


Figure 5. Purification and activity test of recombinant ALFA-APEX2 nanobody

(a) Coomassie blue stained gel depicting indicated fractions during purification of 6xHis-Tagged-ALFA-APEX2 nanobody from E. Coli. E. Cultures were induced with IPTG overnight at 16 or 22ºC, lysed and incubated with Ni-NTA agarose at 4ºC for 4 hours. After rinsing the unbound proteins, the bound protein was eluted with the indicated concentrations of imidazole. (b) Western blot depicting quantification of purified ALFA-APEX Nanobody using a BSA standard. (c) FPLC chromatogram of ALFA-APEX2.

elution from gel filtration column (Superdex 75iGL), highlighted peak shows monomeric fraction of recombinant ALFA-APEX2. (d) In vitro peroxidase activity determined by oxidative polymerization of diaminobenzidine (DAB) in the presence of H_2O_2. Images taken after 5 minutes incubation at RT.

To further test the enzymatic activity of the recombinant APEX2 and specificity of ALFA-nanobody binding in intact cells, immunofluorescence staining was performed to visualize biotinylated regions in the presence and absence of biotin tyramide and H_2O_2 after incubating with the recombinant ALFA-APEX2 nanobody fusion. Using fluorescent streptavidin to detect biotin, biotinylation was observed to be specifically enriched at the immune synapse only in the presence of biotin tyramide, validating successful recruitment of APEX2 to ALFA-tagged PD-1 (Figure 6A).

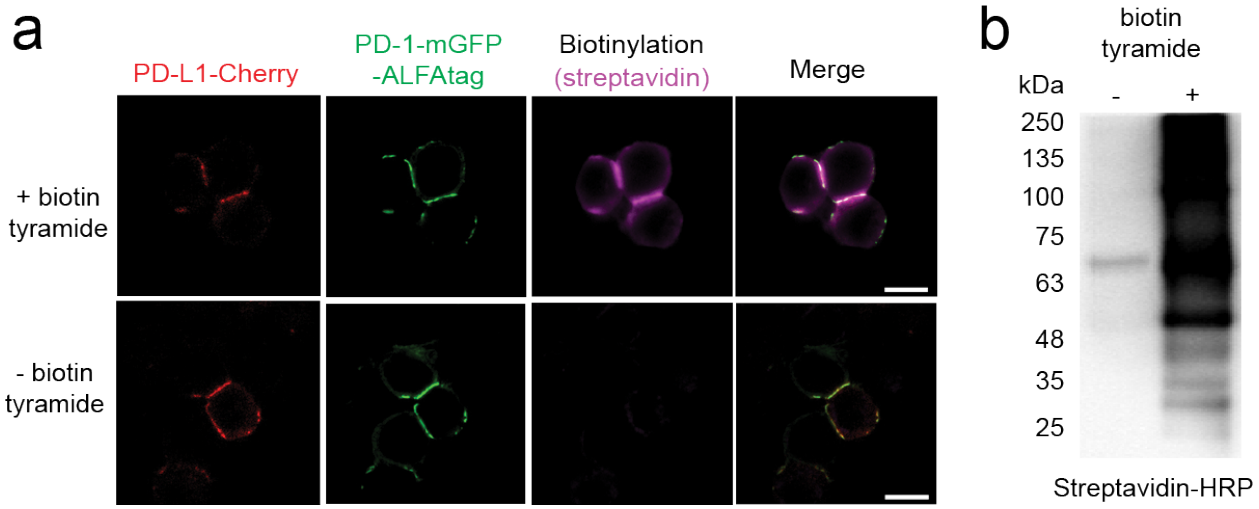


Figure 6. ALFA-APEX2 nanobody conjugate induces PD-1 proximal biotinylation at the immunological synapse

(a) Confocal microscopy of PD-1-mGFP-ALFA tag- expressing Jurkat T cells in conjugate with PD-L1-mCherry expressing Raji APCs after treatment with recombinant APEX2-ALFA-Nb, biotin tyramide, and H_2O_2. Streptavidin staining shows local deposition of biotin at the immune synapse. Scale 10µm. (b) Western blot showing biotinylation of Jurkat lysate in the presence or absence of biotin tyramide, as detected by streptavidin-HRP.

To define the PD-1 interactome using APEX2-ALFA-Nb, Jurkat T cell lines were engineered expressing PD-1 WT or a signaling-deficient mutant tagged with a C-terminal ALFA tag epitope. To activate PD-1 signaling and the assembly of the inhibitory signalosome, each Jurkat line was co-cultured with superantigen-loaded, PD-L1-expressing Raji B cells. Jurkat:Raji conjugates were then fixed in 2% formaldehyde at specified timepoints (2m, 5m, 15m) to capture the dynamic composition of the PD-1 signalosome during TCR activation. Cells were then permeabilized in 0.1% Triton-X and incubated with 125 nM ALFA-APEX2 nanobody overnight at 4ºC. Cells were then washed 3X in PBST (PBS-/-, 0.1% Tween-20) over 2 hours and incubated with 2.5 µM biotin tyramide for 5 minutes at RT. Biotin labeling reaction was then performed by addition of 0.003% (w/v) H_2O_2 and incubation for 2 minutes at RT prior to

centrifugation, aspiration of reaction buffer, and quenching by resuspending 3X in 10mM Sodium Azide, 5mM Trolox 10mM Sodium Ascorbate in PBST.[303]

Proteomic analysis using proximity-based labeling approach

For proteomic analysis, the labeling reaction was conducted as described in section 2.4. Cells were next lysed and enriched for labeled proteins using streptavidin coated magnetic beads (Pierce). These samples were then analyzed by mass spectrometry (MS) in collaboration with Dr. Jolene Diedrich and Professor John Yates at Scripps Research Institute. As a validation of this approach, SHP2 and PD-L1 were highly enriched as PD-1 interactors, in addition to the recently described PD-L1 regulator HIP1R (Figure 7). Additional PD-1 associated proteins identified include the adaptor protein Grb2, which was previously implicated in the formation of membrane-associated condensates in T cell signaling, and several isoforms of protein kinase C (PKC) reported to be involved in regulating T cell proliferation via poorly understood mechanisms (Table 4).[317,318] In support of the specificity of this approach, the most highly enriched candidate factor, PKCα, was previously reported to be the major PKC isoform involved in TCR downregulation.[319]

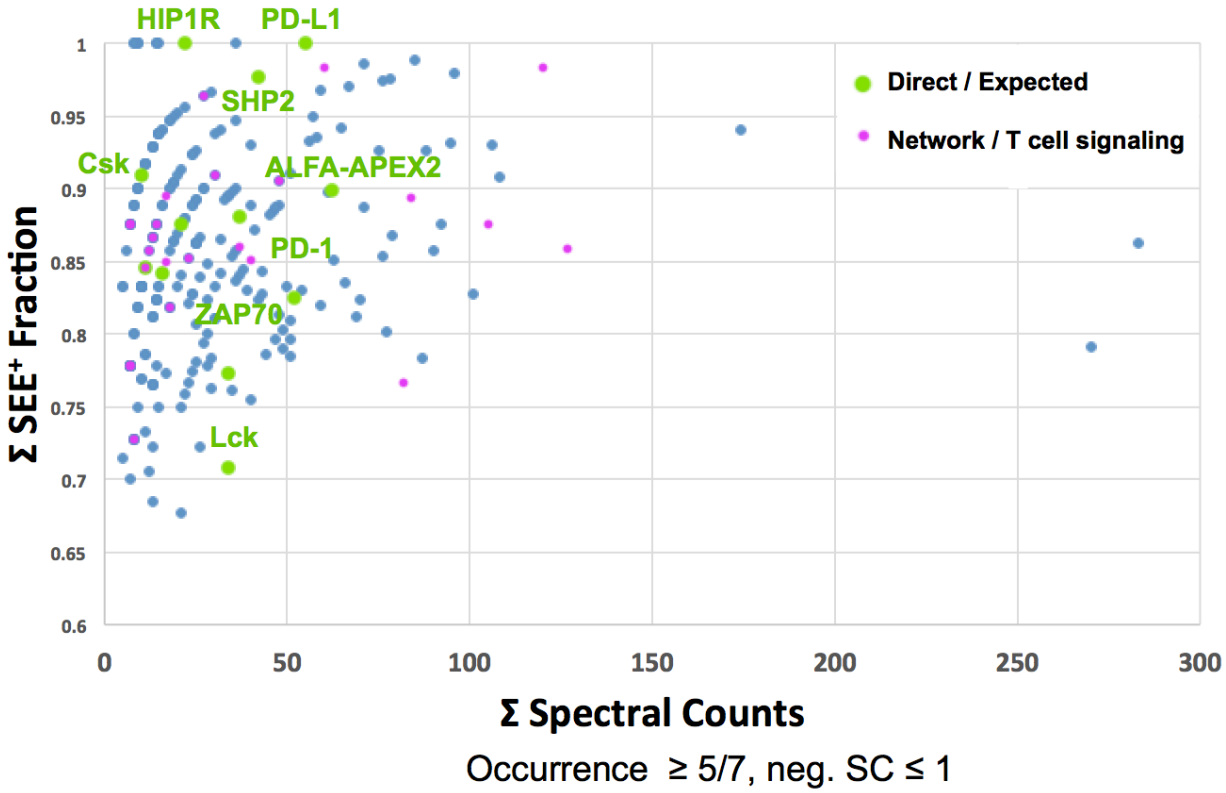


Figure 7. PD-1 interactors identified by proximity labeling-MS.

Proteomics data showing high enrichment of expected PD-1 interactome. Data is plotted as the sum of SEE+ "sample fractions" from n=7 analyzed samples quantifying spectral counts identified in the activated (APC conjugated) vs. no conjugation negative control conditions (y axis) (Note: "sample fraction" is defined here as spectral counts identified in a given SEE+ sample divided by the sum of spectral counts identified in all samples analyzed, including negative controls; e.g. ΣSEE+ fraction = 1.0 indicates a protein for which spectral counts were identified only in the SEE+ samples whereas 0.9 indicates a protein for which out of e.g. 10 total spectral counts from all experiments were identified, 9 of these were identified in the SEE+ condition). vs. sum of spectral counts mapped to each identified protein from all samples (x-axis). Analyzed proteins were filtered by occurrence in at least 5 out of 7 samples and data was combined to reflect 3, 5, and 7-minute timepoints post activation. Proteins for which > 1 spectral count was identified in 0 timepoint or negative control samples were excluded from analysis.

Biochemical and functional screening of candidate PD-1 interactors in intact T cells and cell-free reconstitution assays

Working in collaboration with Dr. Xiaozheng Xu, Jurkat cell lines co-expressing PD-1 mCherry and mGFP tagged candidate interactors (Table 4) were generated to validate novel PD-

1 interactions in intact T cells. These cells were incubated on supported lipid bilayers (SLB) functionalized with anti-CD3 and recombinant PD-L1 to promote T cell activation and PD-1 inhibitory signaling (Figure 8). We then visualized and quantified recruitment of the mGFP-tagged protein to PD-1 microclusters using TIRF microscopy. This assay did not yield any target proteins for further validation however, future optimizations and applications of the approach are described in the discussion section.

Table 4. Candidate PD-1 associated proteins

Description	YY/FF	Sum SC
Protein kinase C (PKC-α)	7.0	72
Growth factor receptor-bound protein 2 (GRB2)	4.2	62
Tyrosine-protein phosphatase non-receptor type 11 (SHP2)	2.6	27
AH receptor-interacting protein (AIP)	2.5	14
CDK5 regulatory subunit-associated protein 3	2.3	15
Coronin-1C	2.0	85
Guanine nucleotide-binding protein (GNB4)	1.8	93
Protein arginine N-methyltransferase 3	1.8	60
Serine/threonine-protein phosphatase 2A	1.6	132
Condensin complex subunit 2 (NCAPH)	1.6	46
Inosine-5'-monophosphate dehydrogenase 2 (IMPDH2)	1.6	62
Protein kinase C delta (PKC-δ)	1.5	25
Lupus La protein	0.5	171
Abl interactor 1	0.5	152
14 kDa phosphohistidine phosphatase (PHPT1)	0.4	83

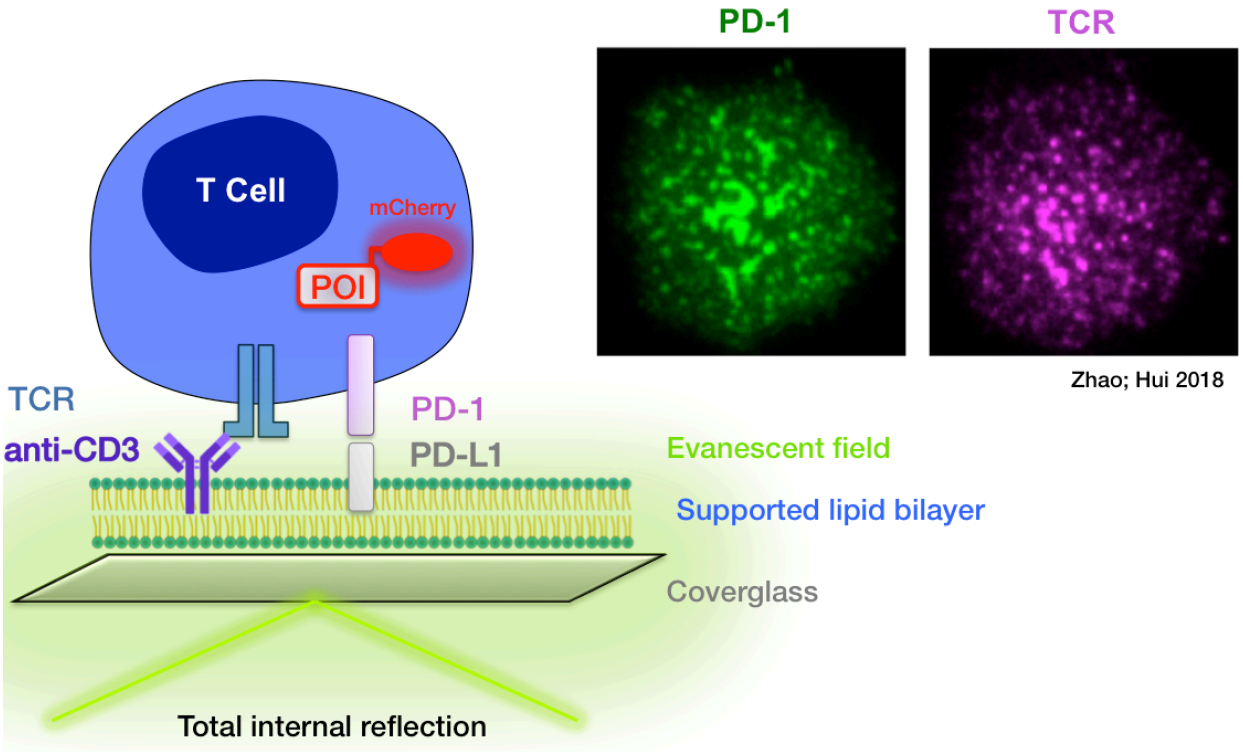


Figure 8. Validation of candidate PD-1 interactors

Depiction of supported lipid bilayer (SLB) total internal reflection (TIRF) microscopy assay. mCherry fusions of candidate proteins of interest (POI) identified by MS were imaged in live cells upon conjugation with SLB functionalized with recombinant PD-L1 and anti-CD3 antibody to induce TCR stimulation. Representative TIRF image.[198]

3.3. Discussion

This chapter describes a novel proximity labeling approach using APEX2 fused with the camelid ALFA nanobody for proteomic profiling of inhibitory co-receptor signalosomes. Based on the recently described biotinylation by antibody recognition (BAR) method, additional optimizations were made to improve data quality while reducing cost and sample input.[315] By using minimal paraformaldehyde (PFA) fixation, homemade reaction buffers, and a chemically cleavable biotin tyramide to enhance elution efficiency, cost and required sample input were reduced by >10 fold.

Preliminary data obtained using this technique reproduced the expected network of PD-1 interactors with high enrichment (Figure 7). Although novel inhibitory effectors of PD-1 were not identified, the obtained results indicate that this method is effective for generating robust spatial proteomics datasets of T cell co-inhibitory receptors. In future experiments, functional effects of candidate factors could be evaluated by genetic deletion or overexpression to evaluate impacts on co-inhibitory signaling in addition to the microscopy assay shown here.

Additionally, the modular design of this approach makes it possible to be readily expanded to profile the interactomes of additional more poorly characterized immune checkpoint receptors. Reduced sample input also makes this approach amenable to proteomic profiling of primary human T cells lentivirally transduced to express ALFA-tagged co-receptors or using peroxidase-conjugated antibodies to target endogenous proteins directly.[315] This approach is expected to be particularly useful for proteomic profiling of biomolecular condensates which are increasingly appreciated to mediate T cell signaling.[318] These dynamic low-affinity assemblies are unique to the intracellular environment and disrupted by cell lysis, rendering them inaccessible to characterization using classical biochemistry approaches.

3.4. Acknowledgements

Chapter 3 in full, is being prepared for submission publication with the following authors, Preston Dennett, Xiaozheng Xu, Jolene K. Diedrich, John Yates, Enfu Hui†. The dissertation author was the primary investigator/author of the manuscript.

Chapter 4: A cell intrinsic route for CD80/86 depletion by CTLA4

4.1. Introduction

CTLA4 is an essential co-inhibitory receptor induced upon T cell activation to restrain proliferative expansion of lymphocytes.[151] The strict requirement for CTLA4 expression in the maintenance of immune homeostasis is highlighted by the dramatic lethal autoimmune phenotype observed in neonatal CTLA4 deficient mice.[152] These animals die of catastrophic lymphoproliferative disease resulting in multi-organ lymphocytic infiltration and tissue destruction within ~4 weeks of age.[152] CTLA4 is known to achieve its essential functions by preventing the canonical T cell co-stimulatory receptor CD28 from accessing their shared ligands CD80 and CD86 (CD80/86 or 'B7') expressed by antigen presenting cells (APCs), however precisely how this occurs has remained a longstanding mystery in T cell immunology.[149] Although constitutively expressed by APCs at low levels, CD80/86 are rapidly upregulated in response to detection of pathogen associated molecular patterns (PAMPs) or endogenous 'danger' signals during the initiation of an immune response, representing the essential 'second signal' for T cell activation and proliferation in addition to antigen recognition.[24,25,45] The critical role of CTLA4 in opposing CD28 signaling to restrain T cell proliferation has been demonstrated genetically by the rescue of the lethal autoimmune phenotype of CTLA4-deficient mice by concurrent homozygous deletion of CD28 or both CD80 and CD86, generating mice that are viable but immunocompromised.[153,154] Underscoring the general role for CTLA4 in mediating immune suppression, autoimmune disorders such as rheumatoid arthritis and graft vs. host disease (GVDH) have been successfully treated using the CTLA4 ectodomain alone fused with an immunoglobulin domain (CTLA4-Ig) to block CD80/86 from CD28 binding.[159,320] While these results demonstrate that some key functions of CTLA4 can be recapitulated via global

reduction in CD28 signaling using the ectodomain alone, the endogenous function of CTLA4 appears to be more complex. Specifically, more subtle regulatory effects of CTLA4 in vivo are likely critically mediated by the short (36 amino acid) intracellular tail sequence which is 100% conserved in mammals and confers several of the unusual characteristic features of CTLA4 including its highly endocytic behavior resulting in primarily intracellular localization and lysosomal trafficking.[149,160,163]

While the notion of direct competition with CD28 at the surface of expressing T cells is consistent with the apparently cell intrinsic effects of CTLA4 expression in fine tuning T cell proliferation and differentiation throughout an immune response, several unusual features of CTLA4 biology have challenged straightforward cell intrinsic models.[177,321] This includes its primarily (~95%) intracellular localization within endocytic vesicles where CTLA4 undergoes constitutive ligand-independent cycling between the cell surface and lysosomal compartments.[165,166] Upon stimulation of the T cell receptor CTLA4 vesicles exhibit focal polarization to the cell surface where CTLA4 mediates high affinity/avidity CD80/86 binding.[161] For cells expressing high levels of CTLA4, ligand binding is ultimately associated with intracellular accumulation of exogenous CD80/86 inside CTLA4-vesicles and subsequent lysosomal degradation.[161,186] While not excluding intrinsic competition models, this apparent optimization for ligand depletion and degradation combined with evidence from mixed bone marrow (BM) chimera mice (Section 1.3.2.2) in which the presence CTLA4+ cells is sufficient to dominantly control auto-reactive CTLA4 deficient cells in *trans* to rescue lethal autoimmunity, have together been used to argue in favor of a completely cell extrinsic model of CTLA4 function.[160] In this model CTLA4 is proposed to act via trans-endocytosis (TE) to extract

and degrade CD80/86 from APCs to control levels of co-stimulatory ligands in the local environment.[160,322]

Interestingly, despite abundant supporting evidence that CTLA4 indeed accumulates exogenous ligands in vitro and down-regulates CD80/86 surface levels on APCs, the TE model and a purely extrinsic model of CTLA4 function has not gained universal support.[149,323] Indeed the research group of Shimon Sakaguchi recently proposed an alternative mechanism in which CTLA4-mediated CD80/86 ligand depletion from APCs did not require endocytosis but could instead be mediated by trogocytosis upon ligand binding.[188] Although occasionally conflated in the literature, trans-endocytosis and trogocytosis are mechanistically and functionally distinct processes. Trogocytosis is a cellular ingestion process which is evolutionarily conserved in eukaryotes and particularly prevalent among immune cells.[192,213] Characterized by contact-dependent acquisition of plasma membrane fragments, trogocytosis is most easily distinguished from *trans*-endocytosis in that it results in functional re-display of exogenous surface molecules on recipient cells while *trans*-endocytosis is associated with direct internalization. Despite being promoted by specific receptor / ligand interactions, trogocytosis is invariably associated with non-specific co-transfer of unbound 'bystander' surface molecules as well, which are similarly re-displayed alongside specifically acquired ligands.

Despite naïve T cells being CD80/86 negative, these co-stimulatory ligands are both endogenously expressed and inherently acquired from target APCs alongside specific and 'bystander' pMHC during T cell activation via trogocytosis.[91,217,218,324,325] While CD28 and TCR engagement have been shown to be sufficient to induce ligand trogocytosis, a potential role for CTLA4 in this process is unclear.[217,218,326] One obvious limitation of the TE model is that CTLA4 surface levels are extremely low in activated CD8+ and conventional CD4+ T cells and

CTLA4-dependent ligand depletion effects, even for high CTLA4-expressing Treg, are typically poorly detectable in vivo.[189,190] I therefore hypothesized that the non-equilibrium pool of nascently expressed and exogenously acquired CD80/86 on the T cell surface may represent a key regulatory target of CTLA4-mediated cell intrinsic ligand depletion during T cell activation via *cis*-endocytosis. Furthermore, due to the constitutively high levels of CD28 on resting T cells and rapid kinetics of CD80/86 acquisition via trogocytosis, I hypothesized that CD28-dependent ligand acquisition and re-display would occur upstream of CTLA4-mediated depletion from the cell surface in many contexts.[327]

To examine this possibility, an in vitro trogocytosis assay was established using Jurkat T cells expressing CTLA4 and Raji B cells expressing CD80-mGFP. This assay was applied to examine the relative contributions of CD28 and CTLA4 in ligand acquisition and depletion as well as the fate of acquired CD80 in the presence of CTLA4 in isolated single T cells. In this assay CD28 was shown to be primarily responsible for driving acquisition and display of the co-stimulatory molecules CD80/86 as well as peptide antigen (pMHC) via trogocytosis while CTLA4 was found to induce cell intrinsic depletion of acquired CD80/86 from the cell surface over time.

4.2. Results

CTLA4 and CD28 expressed on T cells promotes acquisition of CD80/86 and MHC from APCs via trogocytosis

It was previously shown that CD28 expression by T cells is sufficient to induce antigen-independent acquisition of CD80/86 ligands alongside various bystander surface molecules from APCs including peptide antigen (pMHC).[217,218] This phenomenon, termed trogocytosis, occurs with rapid kinetics and results in functional re-display of the exogenous APC-derived surface

receptors on the T cell plasma membrane.[192] Although CTLA4 is known to bind CD80 and CD86 with significantly higher affinity than CD28, the extent of CTLA4 contribution to ligand acquisition via trogocytosis is largely unknown, in part due to previous reports describing CTLA4 function in the context of the hypothetical related process of *trans*-endocytosis (Note on the disputed biological existence of *trans*-endocytosis of full length transmembrane proteins is discussed in section 4.4.1). Furthermore, the interplay of the opposing activities of CD28 and CTLA4 in ligand acquisition and depletion in the context of trogocytosis has not been determined. To investigate the relative roles of CD28 CTLA4 in T cell acquisition of CD80/86 from APCs, I established an in vitro trogocytosis assay. In this assay, Jurkat T cells expressing CTLA4 (WT) or a CD80/86 binding-deficient mutant (Y139A) were briefly co-cultured with Raji B cells as APCs expressing CD80-mGFP. To assess the role of T cell receptor (TCR) engagement, these experiments were performed in the presence or absence of the staphylococcus enterotoxin E (SEE) superantigen.[328] Cell contact and associated membrane transfer was induced by centrifugation and after 5 minute incubation at 37ºC, levels of acquired exogenous CD80-mGFP on the Jurkat cell surface were analyzed by flow cytometry using anti-CD80 antibody staining. This assay revealed that high levels of CD80-mGFP and pMHC were transferred from Raji B cells to Jurkat T cells upon cell-cell contact (Figure 9A), as described previously.[217,218] Ligand transfer was found to be mostly eliminated in the absence of CD28 expression while TCR stimulation induced by SEE slightly enhanced but was not necessary for CD80/86 and pMHC acquisition, consistent with previous findings (Figure 9B).[217]

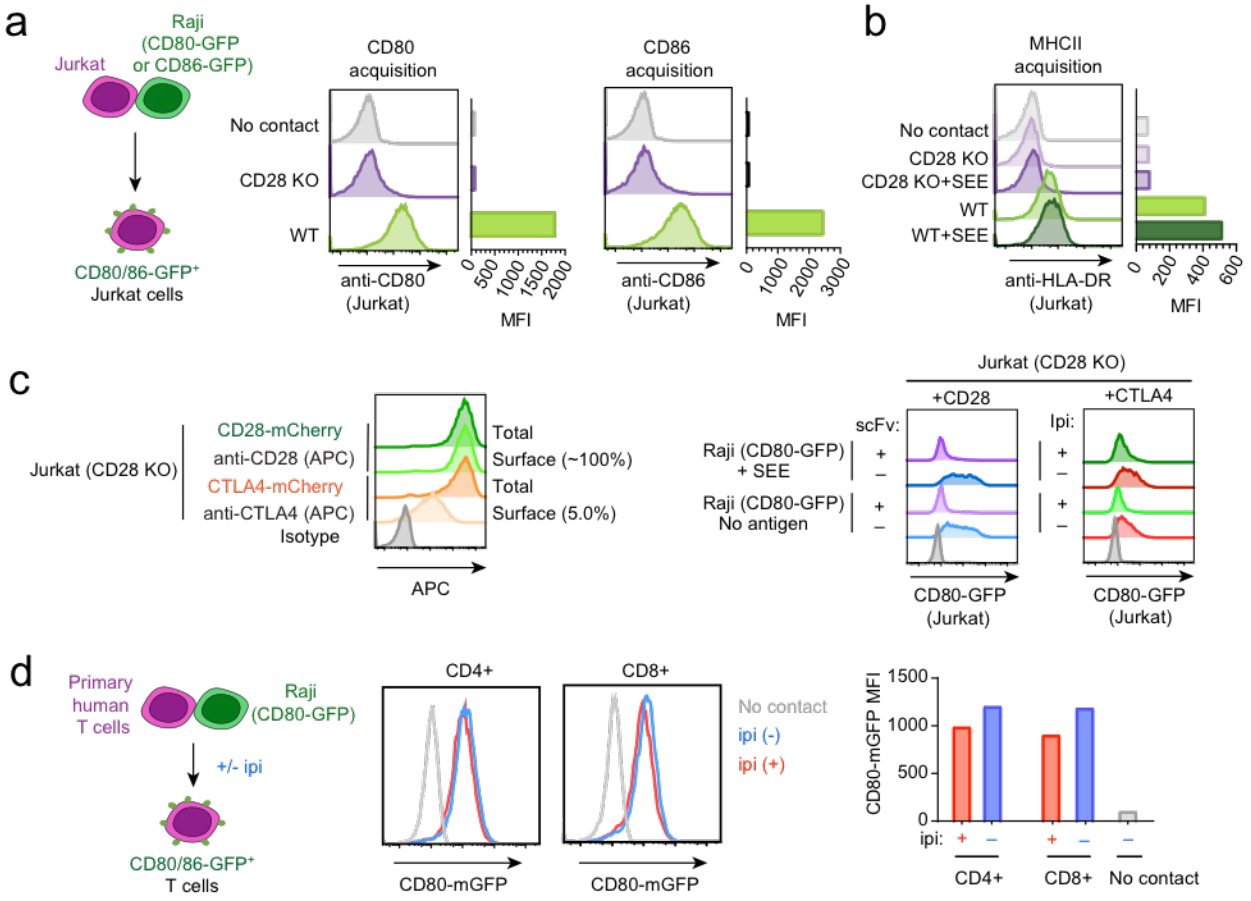


Figure 9. CD28 and CTLA4 promote acquisition and re-display of CD80/86 and MHC-II from Raji APCs

(a) Flow cytometry histograms showing surface CD80/CD86 levels on WT and CD28 KO Jurkat cells after 5-minute contact with Raji B cell expressing CD80-mGFP.

(b) Flow cytometry histograms showing surface MHC-II (HLA-DR) levels on WT and CD28 KO Jurkat after 5-minute contact with Raji B cell expressing CD80-mGFP.

(c) Flow cytometry histograms of CD28 KO Jurkat transduced to express equal total levels of CD28-mCherry or CTLA4-mCherry, plots show CD28 or CTLA4 total vs. surface levels by antibody staining in the presence or absence of 0.1% saponin, respectively. Right: Flow cytometry histograms show CD28-mCherry or CTLA4-mCherry CD80 levels quantified by mGFP signal after 5-minute conjugation with Raji B cells expressing CD80-mGFP in the presence or absence of 30ng/mL SEE and indicated anti-CD28 scFv or anti-CTLA4 blockade antibody.

(d) Flow cytometry histograms and quantification showing primary human CD4+ or CD8+ T cells conjugated with Raji B cells expressing CD80-mGFP in the presence or absence of ipilimumab (100µg/mL)

To further examine the relative contributions of CD28 and CTLA4 in this transfer process these data were next compared to CD28 deficient cell lines generated using CRISPR (CD28 KO). Here it was observed that in absence of CD28, CD80 transfer was largely abrogated (Figure 9A-B), irrespective of TCR engagement induced by SEE (Figure 9B). Next, working in collaboration with Dr. Xiaozheng Xu, these CD28 KO cells were reconstituted by lentiviral transduction to express equal total levels of CD28 or CTLA4 fused with mCherry (Figure 9C), hereafter referred to as Jurkat (CD28-mCherry) and Jurkat (CTLA4-mCherry), respectively. Upon comparing levels of CTLA4-mCherry and CD28-mCherry mediated CD80/86 acquisition, it was observed that similar to CD28, CTLA4 expression is also sufficient to induce ligand transfer (Fig 9C). Notably however, even at these supraphysiologically high levels of total CTLA4, the magnitude of CD80/86 transfer was substantially less than that observed in WT Jurkat cells expressing endogenous levels of CD28 alone. These results likely reflect the predominately (~95%) intracellular localization of CTLA4 at steady-state (Figure 9C). In this assay, low CTLA4 levels at the cell surface, even for cells expressing very high total levels, likely limit the ability of CTLA4 to induce comparable levels of CD80/86 acquisition, which is known to be facilitated by direct ligand / receptor binding interactions. It is notable however that considering only 5% of CTLA4 is present on the cell surface, CTLA4 appeared to drive higher levels of CD80/86 trogocytosis relative to CD28 on a per molecule basis, presumably due to its ~10-20 fold higher affinity for these ligands. Supporting the notion that CTLA4 engages in trogocytosis upon ligand binding rather than direct *trans*-endocytosis, both CD80/86 and 'bystander' MHC levels were observed to increase on the surface of Jurkat T cells expressing high levels of CTLA4. Together these data indicate that both CD28 and CTLA4 induce

trogocytosis upon CD80/86 binding with the contribution of each determined by their relative surface levels.

To examine the relative contribution of CD28 and CTLA4 in CD80 acquisition in a more physiological context, the trogocytosis assay was repeated using human primary CD4+ and CD8+ T cells in the presence or absence of the anti-CTLA4 blockade antibody, ipilimumab to block CTLA4/CD80 interactions. In this assay the blockade condition showed slightly reduced mGFP signal for both CD4+ and CD8+ T cells (Figure 9D) revealing an apparent ~10% contribution of CTLA4 in CD80-mGFP acquisition as quantified by GFP signal. Although accurate analysis of CD80/86 surface levels is confounded by the fact that human primary T cells endogenously express these molecules upon activation, similar results (~10-15% increase in CD80 signal in the absence of ipilimumab) were obtained by performing anti-CD80 surface staining of CD4+ and CD8+ T cells immediately following brief (5-minute) conjugation with Raji B cells expressing CD80-mGFP.[91,324] Additionally, the rapid kinetics of this increase in CD80 surface staining likely precludes endogenous upregulation in further support of the notion that CD28, and to a lesser extent CTLA4, binding induces CD80 acquisition and display on T cells via trogocytosis.

In summary, these experiments demonstrate that while both CTLA4 and CD28 are capable of promoting acquisition of CD80/86 ligands alongside ‘bystander’ pMHC via trogocytosis, CD28 is likely the dominant driver of ligand acquisition in many contexts, with 80-100% of transfer being CD28-mediated even in the presence of CTLA4 overexpression at supraphysiologically high levels.

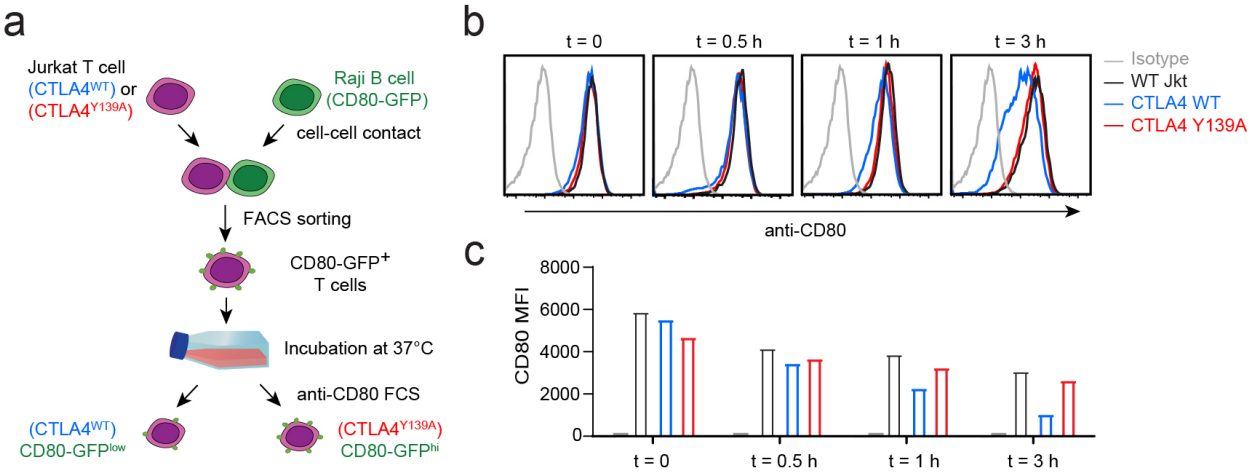


Figure 10. CTLA4 promotes elimination of acquired CD80 from the cell surface

(a) Schematic depicting experimental design. Jurkat cells expressing WT or Y139A mutant CTLA4 conjugated with Raji B cells expressing CD80-mGFP. After 5-minute conjugation, cell contacts were disrupted and CD80-mGFP+ Jurkat cells were isolated by FACS. CD80-mGFP+ Jurkat cells were then cultured in suspension ($0.5x10^6$ /mL) at 37ºC for 30 minutes, 1 hour, or, 3 hours prior to analysis of CD80 levels.

(b) Flow cytometry histograms indicating exogenous CD80 surface levels on indicated Jurkat at specified timepoints post-contact.

(c) Quantification of data shown in (b)

CTLA4 can act in a cell intrinsic manner to deplete exogenous CD80/86 from the T cell surface

After investigating the interplay of CD28 and CTLA4-mediated acquisition of CD80 / CD86 ligands via trogocytosis, the downstream fate of acquired CD80/86 was next examined, specifically the potential role of CTLA4 in depleting these molecules from the cell surface by acting in *cis*. Importantly, it has previously been proposed that CTLA4 can extract and internalize CD80/86 molecules from APCs directly via trans-endocytosis.[186] In these models, CTLA4 was proposed to act in a purely cell extrinsic manner by depleting CD80/86 from the surface of APCs that form stable contacts with a CTLA4-expressing T cells.[187] Whether CTLA4 can similarly act in a cell intrinsic or "pseudo-extrinsic" manner, by depleting acquired CD80/86

ligands from the surface of activating T cells themselves via "*cis*" endocytosis, has not been investigated.

To address this possibility, I followed the fate of acquired CD80 on Jurkat T cells expressing WT CTLA4 or a CD80/86 binding-deficient mutant (Y139A) by flow cytometry. To this end, I applied the trogocytosis assay described above (Figure 10A), and CD80-mGFP+/CTLA4+ double positive Jurkat T cells were subsequently purified by fluorescence activated cell sorting (FACS). After sorting, exogenous CD80-mGFP+ Jurkat cells were cultured at 37ºC and CD80 surface levels were quantified over time by antibody staining followed by flow cytometry analysis. In this assay, Jurkat cells expressing WT but not Y139A CTLA4 exhibited accelerated CD80 depletion from the cell surface (Figure 10B-C).

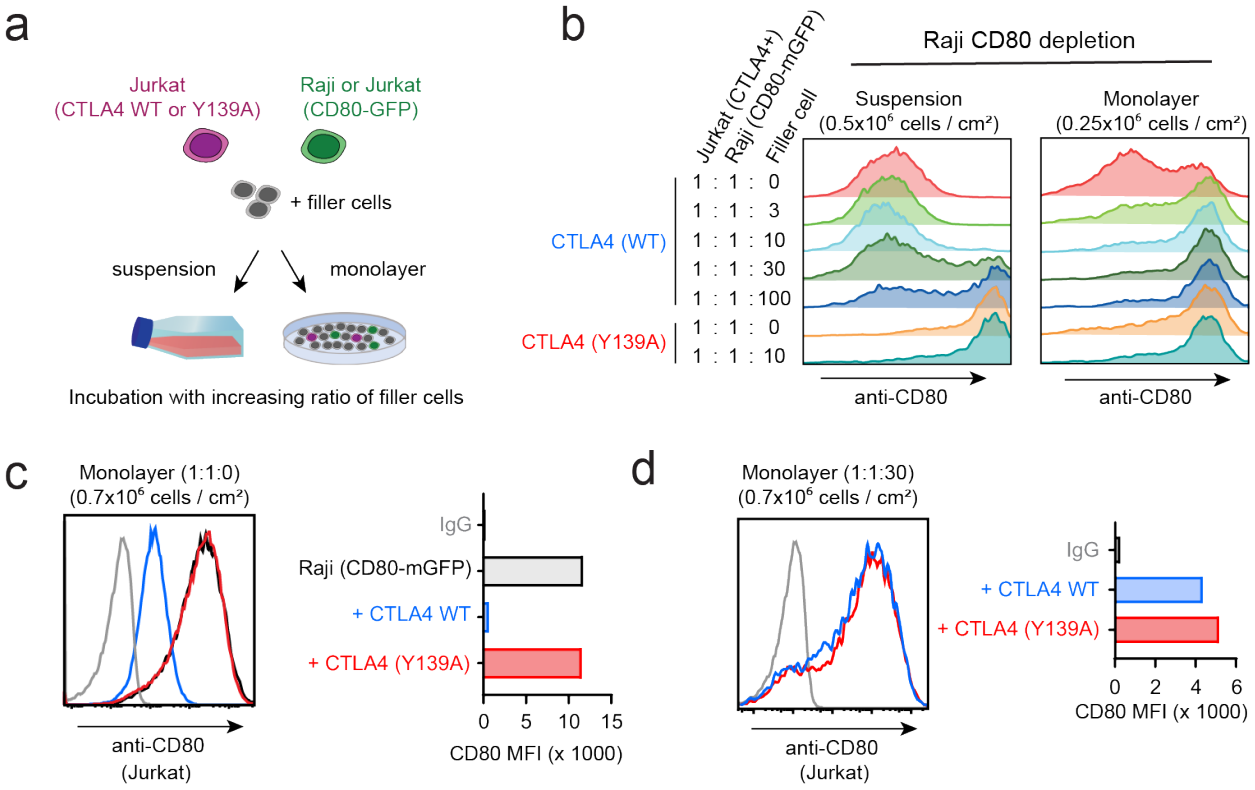


Figure 11. T cell monolayer culture in the presence of excess filler cells prevents trans depletion by CTLA4.

(a) Schematic of filler cell validation assay shows Jurkat cell expressing WT or Y139A mutant CTLA4 co-culture with Jurkat 'T-T Indicator' cell line expressing CD80-mGFP. Cells mixed with indicated ratios of CD28 knockout Jurkat 'filler cells' cultured for 12 hours at 37ºC either in suspension or as an adherent monolayer.

(b) Flow cytometry quantifying CD80 levels on T-T indicator cell line (Jurkat CD80-mGFP) after 12 hours incubation with WT or mutant CTLA4 expressing Jurkat co-cultured with indicated ratios of filler cells.

(c) Flow cytometry indicating CD80 levels on indicator cell line when co-cultured with CTLA4 WT or Y139A mutant in the absence of filler cells for 3h as an adherent monolayer.

(d) Flow cytometry indicating CD80 levels on Jurkat "T-T indicator" cell line when co-cultured with Jurkat expressing CTLA4 WT or Y139A mutant in the presence of 30-fold excess filler cells for 3 hours as an adherent monolayer.

Importantly, while these data support the notion that CTLA4 efficiently targets trogocytosed CD80/86 ligands for depletion, this result could also be facilitated by T-T interactions during culture, thereby enabling CTLA4 trans-endocytosis of CD80/86 as proposed previously for APCs.[186] To examine this possibility and test the hypothesis that CTLA4 may

additionally target T cell expressed CD80/86 ligands by *cis*-endocytosis, a filler cell monolayer assay format was employed to completely block T-T interactions during incubation. In this assay, the presence of a large excess (>30-fold) of CD28 knockout (CD28 KO) Jurkat 'filler' cells is sufficient to block T-T contacts among CD80-mGFP expressing Jurkat cells when cultured as an adherent monolayer (note CD28 KO Jurkat were shown to be relatively inert in ligand transfer and were therefore chosen as filler cells to reduce potential confounding effects of CD28-mediated trogocytosis, (Figure 9B). To validate this approach the trogocytosis assay was repeated (Figure 11A) and CTLA4-mediated trans depletion of CD80-mGFP expressed by Raji B cells was quantified in the presence of increasing filler cells when cultured in suspension or as an adherent monolayer (Figure 11B). Surprisingly, it was found that even in the presence of 100-fold excess filler cells, CD80-mGFP on Raji cells was substantially reduced following 12 hours of suspension culture at 37ºC with high CTLA4 expressing Jurkat cells, as quantified by anti-CD80 antibody staining and analysis by flow cytometry (Figure 11B, left). However, as expected performing the incubation as adherent monolayer was sufficient to completely block CTLA4-mediated trans CD80 depletion (Figure 11, right). This validation assay was therefore next repeated using Jurkat T cells expressing CD80-mGFP as a more accurate indicator of CD80 depletion mediated by T-T interactions. First, by performing monolayer culture of Jurkat cells expressing high levels of WT or Y139A mutant CTLA4 with Jurkat (CD80-mGFP) at 1:1 ratio in the absence of filler cells, robust trans depletion of CD80 by WT CTLA4 was confirmed (Figure 11C). Upon repeating this monolayer assay in the presence of 30-fold excess filler cells however, CD80 depletion was observed to be almost completely eliminated, with Jurkat (CD80-mGFP) largely equivalent CD80 levels irrespective of culture with Jurkat expressing WT or Y139A mutant CTLA4 (Figure 11D). Together these data support that T cell culture as an

adherent monolayer in the presence of excess filler cells is capable of eliminating confounding effects of CTLA4-mediated trans CD80-depletion in order to test the hypothesis that CTLA4 may additionally act in *cis* to deplete acquired CD80 from the cell surface.

Next, the trogocytosis assay described above was repeated (Figure 12A) and Jurkat (CTLA4 WT) or Jurkat (CTLA4 Y139A) displaying exogenous CD80-mGFP were isolated by FACS. Surface staining of CD80-mGFP was then quantified by flow cytometry after 30 minutes or 3 h of culture as an adherent monolayer in the presence of excess filler cells. As expected, high levels of CD80 acquisition via CD28-dependent trogocytosis was observed at early timepoints. Notably, however, CTLA4 WT but not CTLA4 Y139A Jurkat T cells were observed to rapidly eliminate acquired CD80 from the cell surface over time (Figure 12B). Similar results were obtained using ipilimumab treatment to block CTLA4 / CD80 interactions, resulting in preservation of CD80 on the T cell surface (Figure 16B). As any potential trans CTLA4 depletion activity via T-T interactions is blocked by the filler cell monolayer assay format, these data indicate that observed CD80 depletion depends on direct CTLA4:CD80 binding interactions occurring in a cell intrinsic manner.

Similar results were obtained using primary human CD4+ T cells transduced to express either WT or Y139A mutant CTLA4, although interestingly in primary cells intrinsic depletion by CTLA4 was more strictly antigen dependent (data not shown). Together, these experiments provide direct evidence that CTLA4 expressed by T cells is sufficient to deplete acquired CD80 via a cell intrinsic route which is antigen-independent in Jurkat T cells and largely antigen-dependent in primary human CD4+ and CD8+ T cells. Furthermore, by comparison of CD80 depletion in monolayer vs. suspension format, it was observed that the presence of T-T interactions in suspension culture did not increase CD80 depletion kinetics (Figure 12C). The

lack of any observed enhancement in the presence of trans contacts supports the notion that at least under these assay conditions (Jurkat T cells expressing very high levels of CTLA4 and displaying exogenous CD80-mGFP), *cis* and not *trans*-endocytosis represents the primary route of CTLA4-mediated CD80-mGFP depletion from the T cell surface.

Figure 12. CTLA4 promotes cell intrinsic depletion of trogocytosed CD80

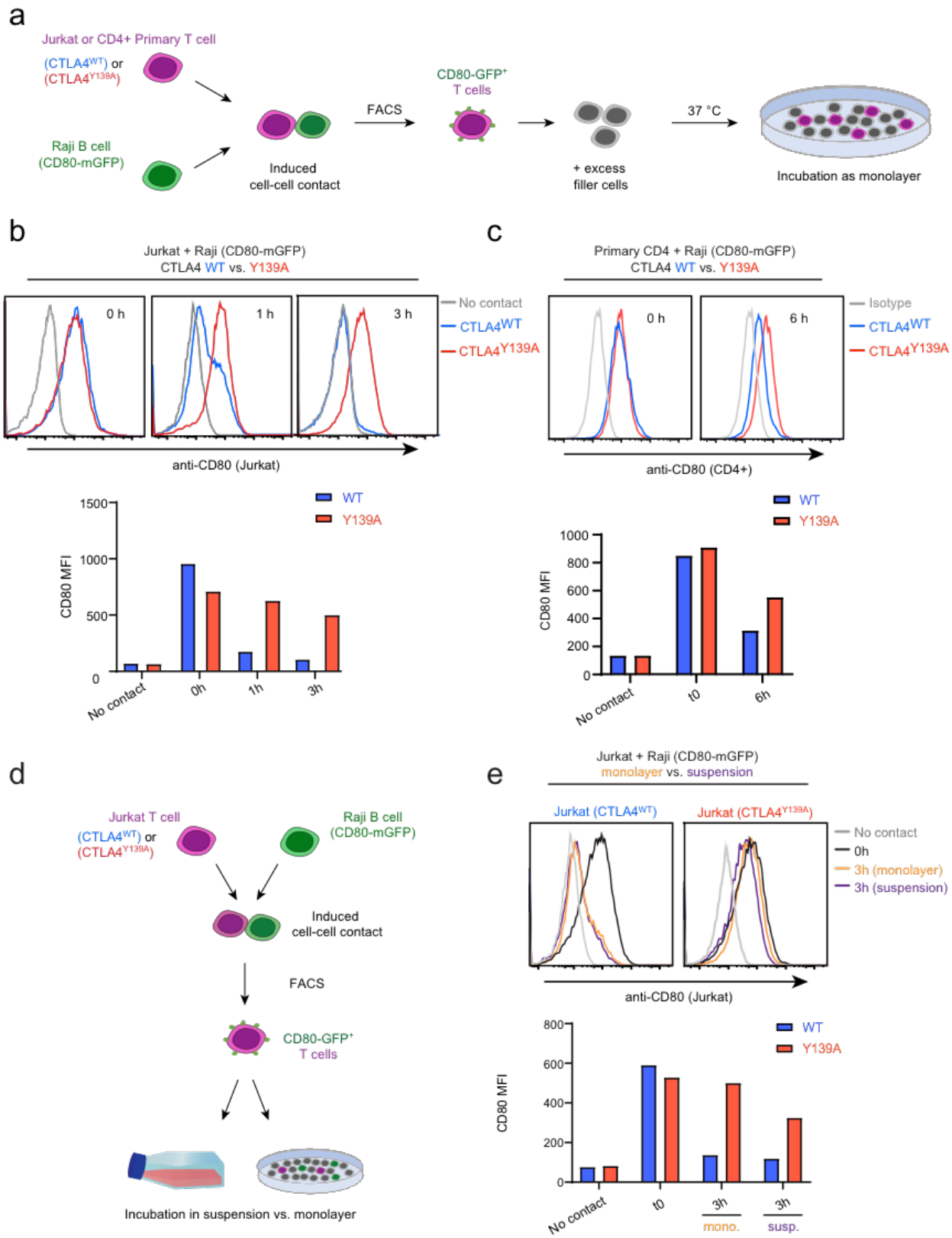
(a) Diagram indicating experimental design. Jurkat T cells were transduced to overexpress CTLA4 WT or Y139A mutant and conjugated with CD80-mGFP expressing Raji B cells for 5 minutes followed by FACS sorting to purify CD80-mGFP+ Jurkat or primary CD4+ T cells. Cells were then cultured as an adherent monolayer in the presence of 30-100-fold excess filler cells (Jurkat CD28 KO) to block T-T interactions,

(b) Flow cytometry histograms showing CD80 surface levels on indicated Jurkat T cells after 0, 1, or 3-hour culture at 37ºC as adherent monolayer in the presence of 30-fold excess filler cells.

(c) Flow cytometry histograms showing CD80 levels on isolated primary CD4+ T cells after 5-minute contact with Raji CD80-mGFP ('t=0') and after 6 hour culture as adherent monolayer in the presence of 100-fold excess Jurkat 'filler' cells.

(d) Diagram of trogocytosis assay comparison of monolayer vs. suspension culture.

(e) Flow cytometry histograms show quantification of exogenous CD80 surface levels in Jurkat cells expressing CTLA4 WT or Y139A mutant at 0 or 3 hours culture in monolayer vs. suspension format.


a
Jurkat or CD4+ Primary T cell
(CTLA4WT) or
(CTLA4Y139A)
Raji B cell
(CD80-mGFP)
Induced
cell-cell contact
FACS
CD80-GFP+
T cells
+ excess
filler cells
37 °C
Incubation as monolayer
b
Jurkat + Raji (CD80-mGFP)
CTLA4 WT vs. Y139A
0 h
1 h
3 h
No contact
CTLA4WT
CTLA4Y139A
anti-CD80 (Jurkat)
WT
Y139A
CD80 MFI
1500
1000
500
0
No contact
0h
1h
3h
c
Primary CD4 + Raji (CD80-mGFP)
CTLA4 WT vs. Y139A
0 h
6 h
Isotype
CTLA4WT
CTLA4Y139A
anti-CD80 (CD4+)
WT
Y139A
CD80 MFI
1000
800
600
400
200
0
No contact
t0
6h
d
Jurkat T cell
(CTLA4WT) or
(CTLA4Y139A)
Raji B cell
(CD80-mGFP)
Induced
cell-cell contact
FACS
CD80-GFP+
T cells
Incubation in suspension vs. monolayer
e
Jurkat + Raji (CD80-mGFP)
monolayer vs. suspension
Jurkat (CTLA4WT)
Jurkat (CTLA4Y139A)
No contact
0h
3h (monolayer)
3h (suspension)
anti-CD80 (Jurkat)
WT
Y139A
CD80 MFI
800
600
400
200
0
No contact
t0
3h
mono.
3h
susp.

To examine CD80 acquisition and depletion by CD28 and CTLA4 in isolated single cells, I next performed a confocal microscopy assay. First, the CD80 acquisition kinetics of primary human CD4+ T cells were examined by performing the trogocytosis assay described above using Raji B cells expressing CD80-mGFP. Before and after 5 minutes of cell contact, cells were fixed in formaldehyde and permeabilized in 0.1% saponin followed by anti-CD28 and anti-CTLA4 antibody staining. This data revealed initially uniform plasma membrane distribution of CD28 in resting CD4+ T cells (Figure 13A) followed by CD28 cluster formation and high co-localization with CD80-mGFP upon conjugation with Raji B cells (Figure 13B, upper). Synapse termination was additionally associated with high levels of CD28/CD80-mGFP co-localization (Figure 13B, lower). Consistent with flow cytometry data indicating a primary role for CD28 in mediating CD80 acquisition, both conditions displayed minimal CD80 association with CTLA4. Notably however, CTLA4 polarization to the cell surface is observed to increase over time in response to TCR stimulation. This assay therefore likely understates the potential role for CTLA4 in ligand acquisition in contexts where cognate antigen is abundant, such as homotypic T cell clusters surrounding activated DCs during an immune response.

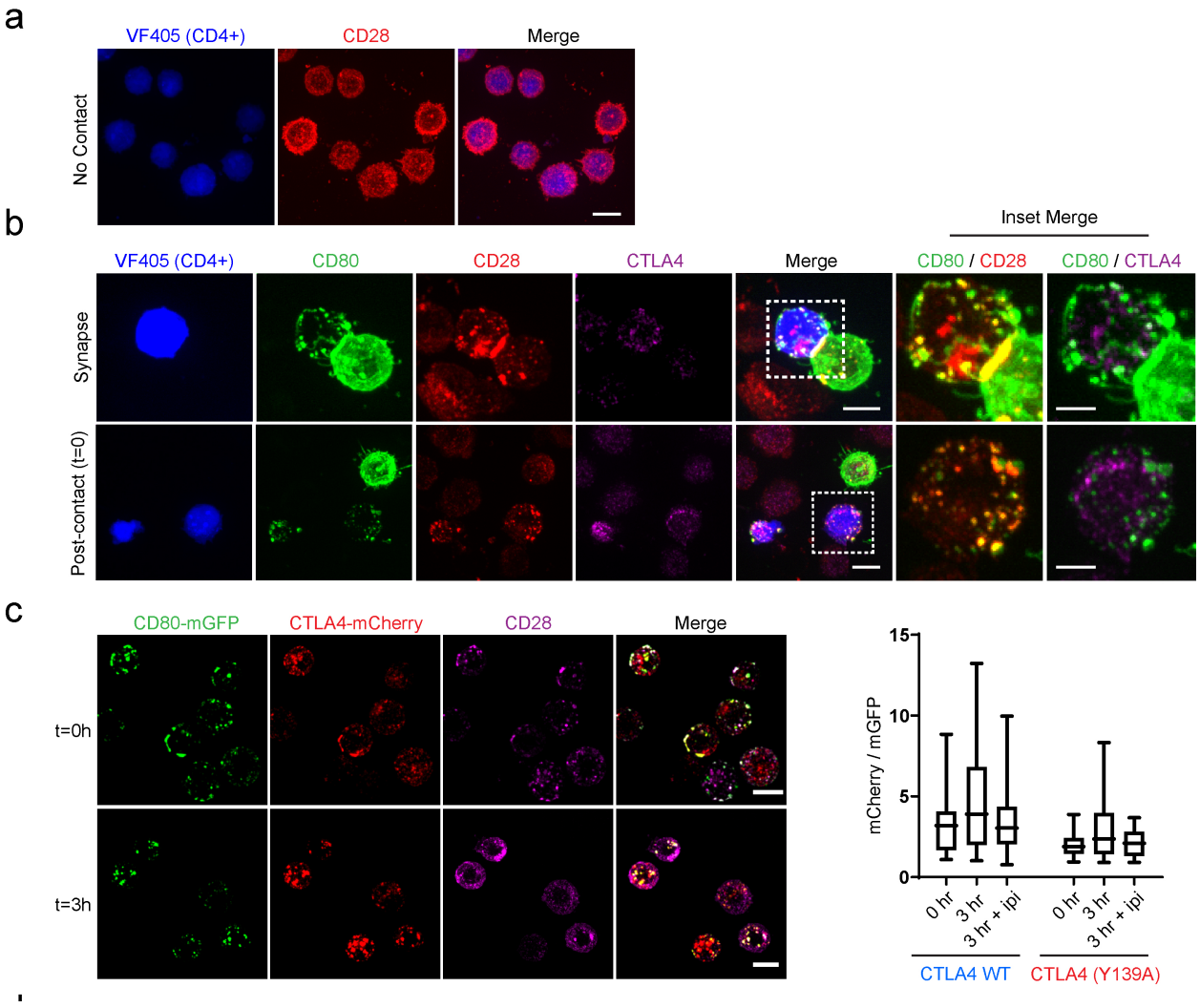


Figure 13. CD80-mGFP acquired by CD28 accumulates in CTLA4 associated vesicles.

(a-b) Confocal microscopy of human primary CD4+ T cells showing anti-CD28 and anti-CTLA4 antibody staining prior to (a) and post (b) conjugation with Raji B cells expressing CD80-mGFP. Scale 10µm, inset 5µm.

(c) Confocal microscopy of representative Jurkat cells expressing CTLA4-mCherry and stained with anti-CD28 antibody, fixed at 0h (upper) or 3h (lower) post contact with CD80-mGFP expressing Raji B cells. Quantification of imaging data shown at right. P=0.006 (WT 0h vs. 3h) P=0.12 (WT 0h vs 3h+ipi). Scale 10µm.

Because the vesicular localization of CTLA4 results in low efficiency antibody staining and poor fluorescence signal, Jurkat T cells expressing WT or Y139A CTLA4 fused with

mCherry were next generated to further examine co-localization of acquired CD80-mGFP with CTLA4-mCherry over time. First Jurkat (CTLA4 WT-mCherry) and Jurkat (CTLA4 Y139A-mCherry) were induced to acquire exogenous CD80-mGFP by co-culture with Raji B cells as described above. These cells were then cultured at 37ºC on cover glass in the presence of excess filler cells to prevent T-T interactions and in the presence or absence of 100μg/mL ipilimumab to block CTLA4/CD80 interactions. At 0 and 3 hours cells were fixed in formaldehyde and stained using anti-CD28 antibodies to quantify co-localization of CD80-mGFP with CD28 and CTLA4 over time. Confocal imaging of these cells again revealed initially high co-localization between acquired CD80 and CD28 following cell-cell contact followed by a gradual accumulation of CD80 in CTLA4-enriched vesicles over time in isolated single cells (Figure 13C). This process was inhibited by treatment with the anti-CTLA4 blockade antibody ipilimumab, supporting a CTLA4 binding-dependent mechanism. Together these data provide direct support for notion that CTLA4 directs *cis* capture of acquired CD80/86 from the surface of activating single T cells.[†††]

[†††]These experiments were incomplete at the time of generating this dissertation due to thesis laboratory funding constraints, representative results are shown in Figure 13C which were reproduced and quantified by Dr. Xiaozheng Xu (manuscript submitted for publication).

Figure 14. CD28 mediates rapid acquisition of APC-derived membrane fragments upon B7 binding.

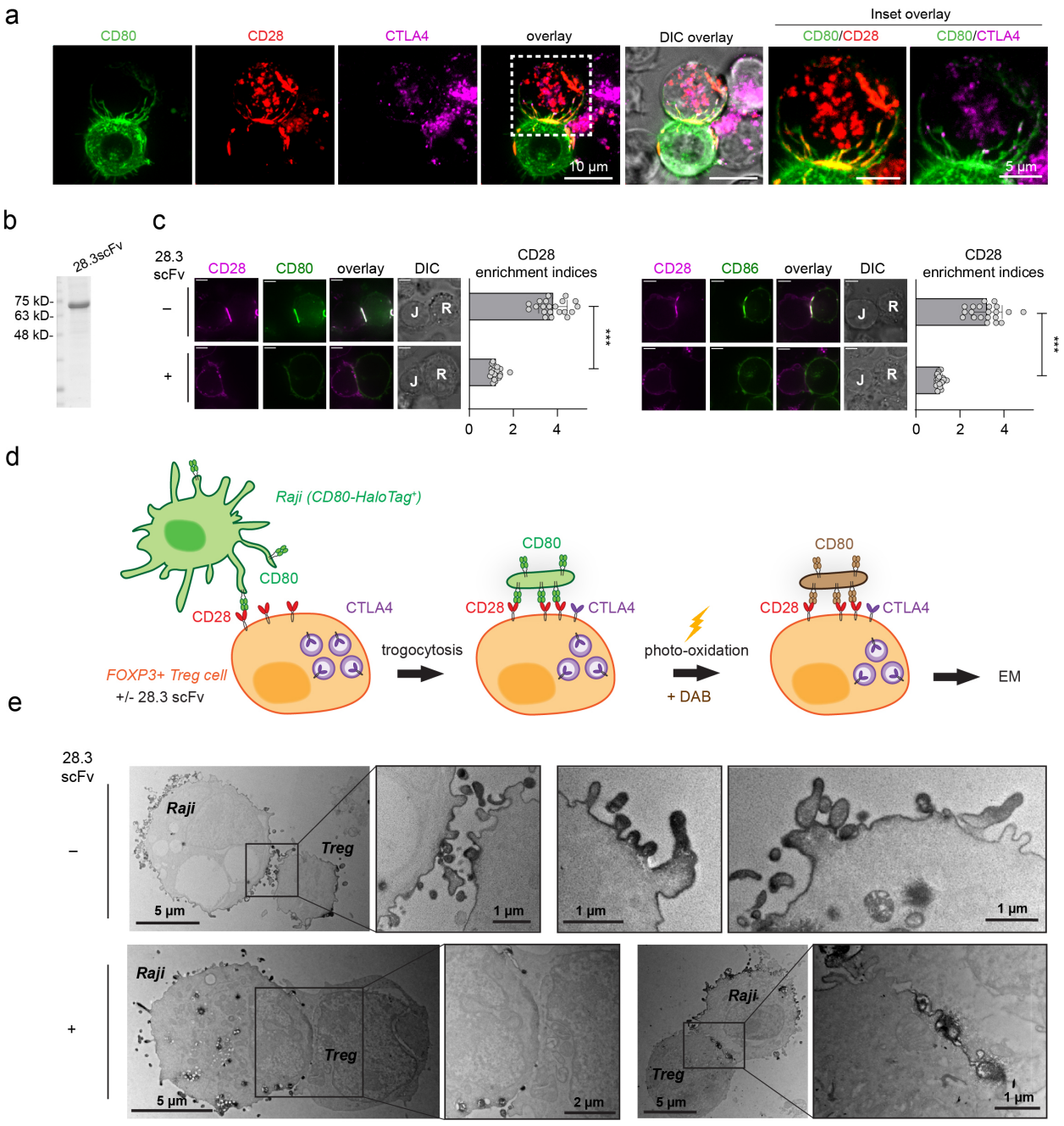
(a) Confocal microscopy of Jurkat T cells expressing CD28-mCherry and CTLA4-HaloTag (JFX-647) in conjugation with Raji B cells expressing CD80-mGFP. Insets show CD28 and CTLA4 co-localization with Raji derived microvilli. Scale: 5 μm.

(b) Coomassie brilliant blue stained SDS-PAGE of purified MBP-28.3scFv.

(c) Representative confocal microscopy of CD80-GFP/CD86-GFP and CD28-mCherry in a Raji (CD80-GFP$^+$):Jurkat (CD28-mCherry$^+$) conjugate (left) or Raji (CD86-GFP$^+$):Jurkat (CD28-mCherry$^+$) conjugate (right), with or without the presence of 28.3scFv. Scatter plots immediate right showing the synaptic enrichment indices of CD28. In DIC images, R denotes Raji, J denotes Jurkat. scale: 5 μm. n = 20 conjugates.

(d) Cartoon depicting EM labeling strategy of human regulatory T cells following conjugation with Raji B cells expressing CD80-HaloTag. Laser excitation at 549nm induces oxidative polymerization of electron-dense 3'3-diaminobezidine (DAB) proximal to CD80.

(e) Representative electron micrographs of human Treg cells (n=7) following acquisition of CD80-bearing plasma membrane fragments from Raji APC, indicated by DAB staining.



Interestingly, while performing these confocal imaging experiments it was observed that the Jurkat / Raji cell conjugation events frequently involved extensive contacts mediated by CD28 engagement with CD80 enriched in B cell microvilli (Figure 13B, Figure 14A). Notably, even in these cells which were lentivirally transduced to express high levels of CTLA4, co-

localization of CTLA4 with CD80 was substantially less than that observed with CD28 during membrane acquisition but was observed to gradually increase over time as CTLA4 becomes polarized to the cell surface as shown in Figure 13C. This result is consistent with the notion that CTLA4-mediated *cis*-endocytosis of B7 likely occurs downstream of ligand acquisition via trogocytosis in many contexts.

To further investigate the role for CD28 in mediating membrane acquisition from APCs upon B7 binding, I took advantage of a single-chain variable fragment (scFv) derived from CD28.3, an anti-CD28 antibody, in order to block CD28:B7 interactions. This reagent was generated and characterized by Dr. Xiaozheng Xu and shown to be effective in blocking CD28 binding with CD80 and CD86 during T cell conjugation with Raji APCs (Figure 14B-C). To visualize CD28-mediated trogocytosis by electron microscopy, I next generated Raji B cells expressing CD80-HaloTag to enable photo-oxidation of 3'3' diaminobenzidine (DAB) upon laser excitation of JF549 Halo ligand.[329] This technique induces local deposition of an electron dense DAB polymer to identify CD80 and associated plasma membrane fragments by EM (Figure 14D).

To examine CD28-mediated trogocytosis in a more physiological setting, purified human regulatory T cells (FOXP3+ / CD25 high / CD127 low) were co-cultured with Raji B cells (CD80-HaloTag) for 15 minutes in the presence or absence of anti-CD28 scFv (Figure 14D). Following cell fixation and photo-oxidation of DAB, samples were prepared for EM and imaged using a Zeiss Libra transmission electron microscope (TEM) in collaboration with Dr. Alice Sherrard. In the absence of anti-CD28 blockade treatment, electron micrographs revealed Treg acquisition of APC-derived membrane fragments as indicated by DAB staining (Figure 14E, upper). Interestingly, these results appear to show relatively rapid fusion of acquired APC

membrane fragments with recipient Tregs (Figure 14E, upper). Strikingly, in the presence of 28.3scFv to block CD28:B7 interactions, membrane transfer and fusion appeared to be largely abrogated (Figure 14E, lower). Together these results support a model in which CD28 mediates acquisition of exogenous membrane fragments via trogocytosis upon B7 ligand binding.

While the precise mechanism of trogocytosis remains unknown and can likely occur via multiple mechanisms mediated by distinct receptor / ligand interactions, these data support a role for functional membrane transfer via mechanical scission of fine membrane projections bearing co-stimulatory ligands upon CD28 binding. Indeed at least in this system B cell microvilli appear to be a significant source of functional membrane transfer (Figure 14A) and it is tempting to speculate that a similar mechanism may apply to T cell capture of membrane projections extended by activated dendritic cells during the initiation of immune responses.[330] This notion is consistent with the characteristic rapid kinetics and contact-dependent acquisition of exogenous surface molecules that characterizes trogocytosis.[131]

Figure 15. T cell trogocytosis and CTLA4-mediated depletion of CD80 visualized by EM.

(a) Confocal microscopy of immunological synapse between Raji B cell expressing CD80-mGFP (left) and Jurkat T cell expressing CTLA4-mCherry (right) Image shows CD80-mGFP accumulation in CTLA4-associated vesicles and CTLA4-independent acquisition of membrane fragments enriched in CD80-mGFP (white arrow). Scale 10μm. Correlated light electron micrograph (CLEM) of these cells appears to show active membrane transfer (red arrow).

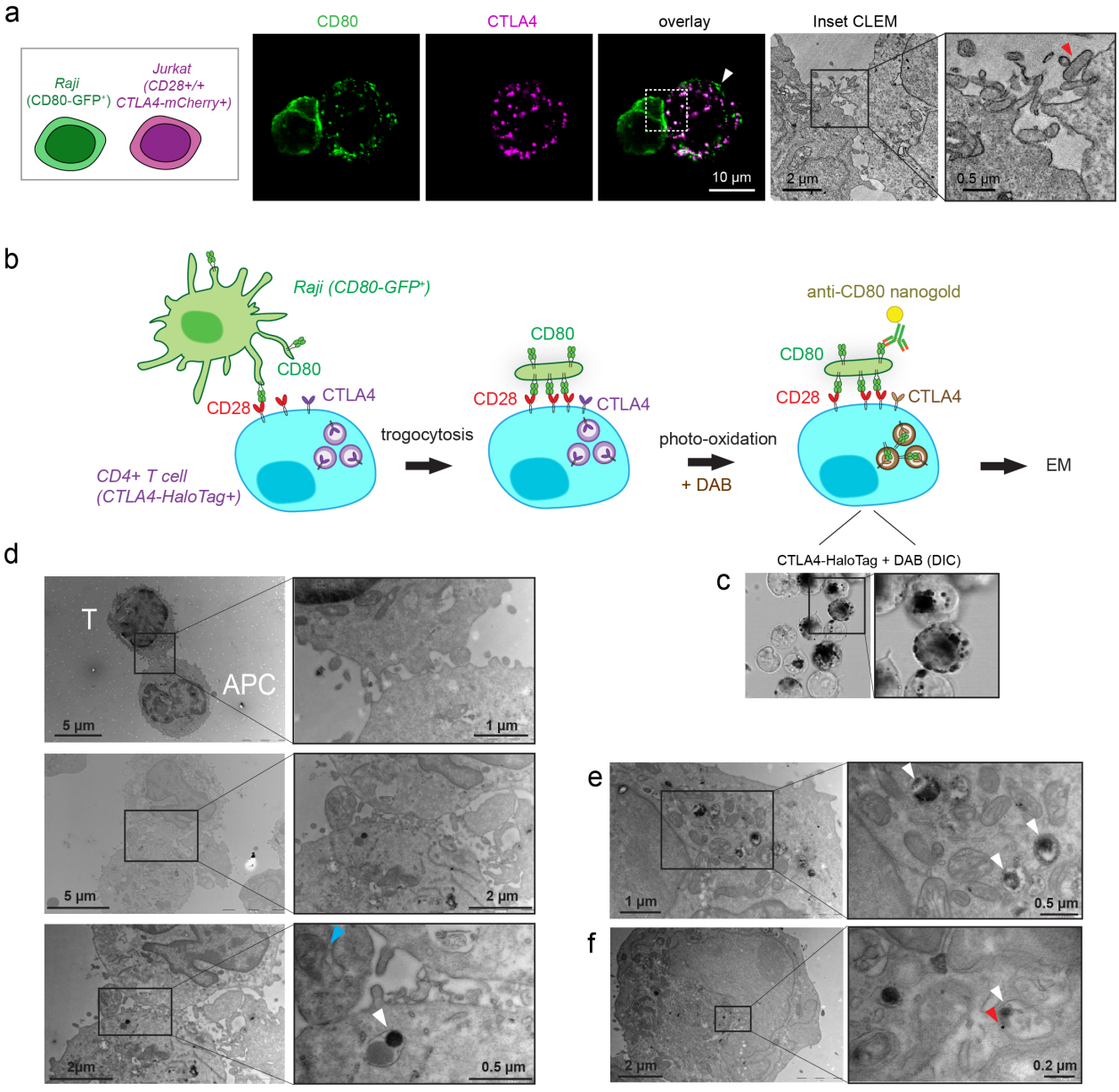
(b) Cartoon depicts EM labeling strategy to visualize CTLA4 in intact primary human CD4+ T cells using HaloTag CTLA4 fusion and 549nm excitation in the presence of 3,3'-diaminobenzidine (DAB) to induce oxidative polymerization and production of an osmiophilic (i.e. electron dense) precipitate. Bright field mage at right shows Jurkat T cells expressing CTLA4-HaloTag after photo-oxidation.

(c) Sample image of DAB precipitate induced by photo-oxidation of CTLA4-HaloTag and visualized by light microscopy (DIC) prior to sample preparation for EM.

(d) Panel shows electron micrographs of membrane transfer during conjugation of primary human CD4+ T cells with Raji B cells expressing CD80-mGFP. Lower right micrograph appears to show CTLA4 containing vesicle fusion with endosomal compartment (white arrow) and transfer of large membrane fragment potentially containing an intact mitochondrion (blue arrow), as recently reported.[464]

(e) Electron micrograph of photo-oxidized human primary CD4+ T cell expressing CTLA4-HaloTag shows CTLA4 vesicles in close association with mitochondria (white arrows).

(f) Electron micrograph of human primary CD4+ T cell showing CTLA4-HaloTag photo-oxidation (white arrow) and associated acquired CD80, indicated by gold particle (red arrow).


a
Raji
(CD80-GFP+)
Jurkat
(CD28+/+
CTLA4-mCherry+)
CD80
CTLA4
overlay
Inset CLEM
10 µm
2 µm
0.5 µm
b
Raji (CD80-GFP+)
CD80
CD28
CTLA4
CD4+ T cell
(CTLA4-HaloTag+)
trogocytosis
CD80
CD28
CTLA4
photo-oxidation
+ DAB
anti-CD80 nanogold
CD80
CD28
CTLA4
EM
CTLA4-HaloTag + DAB (DIC)
c
d
T
APC
5 µm
1 µm
5 µm
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2µm
0.5 µm
e
1 µm
0.5 µm
f
2 µm
0.2 µm

Working in collaboration with Dr. Alice Sherrard, we next sought to further clarify the molecular mechanism of CTLA4 mediated *cis* capture of exogenous CD80 by visualizing the immunological synapse by correlated light electron microcopy (CLEM). Here we observed extensive acquisition of APC-derived membrane fragments by Jurkat (Figure 15A) and primary human CD4+ T cells (Figure 15D). To visualize *cis* capture of acquired CD80 molecules, I generated human CD4+ T cell lines expressing CTLA4-HaloTag by lentiviral transduction. These cells enable precise subcellular localization of CTLA4-associated vesicles via photo-oxidation of diaminobenzidine (DAB) to induce local production of an electron dense precipitate upon excitation of TMR Halo ligand at 546nm (Figure 15C). Following CD4+ T cell conjugation with Raji B cells expressing CD80-mGFP, we next performed photo-oxidation of CTLA4-HaloTag in the presence of DAB as described above combined with anti-CD80 antibody staining using secondary antibodies conjugated with 1.4nm gold particles and subsequent silver enhancement to 5-10nm, to obtain dual labeling by EM. (Figure 15B). Resulting electron micrographs showed intracellular vesicles labeled with CTLA4 photo-oxidation (Figure 15D-F), and gold particles indicating the presence of CD80 within these intracellular vesicles (Figure 15F).

Cis-endocytosis of acquired B7 by CTLA4 limits co-stimulatory capacity of 'T-APCs' during subsequent T-T interactions

In collaboration with Dr. Xiaozheng Xu, we next sought to establish an in vitro cell-based assay to assess the potential physiological impacts of CTLA4-mediated intrinsic depletion of CD80/86 ligands. One scenario in which a *cis* depletion pathway might be particularly relevant in vivo would be the extent to which CTLA4 may act to limit levels of exogenous CD80/86 displayed alongside acquired antigen to other T cells in T-T interactions during priming.[87,215,227]

Indeed, homotypic T cell clusters have been shown to form around activating APCs in vivo and it has recently been proposed that these interactions may be involved in collective decision-making by lymphocytes.[87] Furthermore it has been demonstrated that acquisition of APC-derived membrane fragments endows lymphocytes with the ability to act as APCs.[215,218,227] While numerous examples of "T-APC" have been shown previously, the functional importance of this phenomenon is controversial and its regulation is poorly understood.[222] Considering that APC surface expression of CD80/86 is generally required as the essential "second signal" for T cell activation, I hypothesized that "*cis*" endocytosis of T cell expressed CD80/86 by CTLA4 may be involved in limiting the capacity of activated T cells to themselves act as APCs. Importantly, peptide antigen (pMHC) recognition in the absence of CD80/86 can induce a transient inactivated state known as T cell anergy in responding cells.[40] In this context it seems possible that a cell intrinsic pathway would potentially enable T cells expressing high levels of CTLA4 to deplete CD80/86 associated with acquired pMHC to generate antigen-specific tolerogenic stimuli (e.g. in immunosuppressive regulatory T cells).

To examine this possibility, the trogocytosis assay described above (Figure 16A) was modified to include conjugation of FACS sorted exogenous CD80/MHC-II expressing Jurkat "T-APCs" with unstimulated Jurkat cells, in the presence or absence of CTLA4 activity. Briefly, acquisition of CD80 and MHC-II onto Jurkat T cells expressing CTLA4 WT was first induced by co-culture with Raji B cells expressing CD80-mGFP. CD80-mGFP+ / pMHC-II+ Jurkat (CTLA4 WT) cells were then isolated by FACS and cultured as an adherent monolayer for 3 hours in the presence or absence of ipilimumab. Recombinant anti-CD28 scFv was additionally added during incubation to examine potential effects of CD28 on ligand depletion. Analysis of CD80 surface levels by flow cytometry showed that incubation with ipilimumab to block

CTLA4 but not anti-CD28 scFv blockade treatment prevented elimination of acquired CD80 (Figure 16B). Following incubation for 3 hours at 37ºC, CD80+ / pMHC-II+ double-positive Jurkat (CTLA4 WT) were then conjugated with unstimulated WT Jurkat cells in the presence of bacterial superantigen SEE (30 ng/mL) to quantify the ability of these Jurkat 'T-APCs' to activate other T cells. IL-2 production was then quantified by ELISA as a readout of unstimulated Jurkat T cell activation. As expected, higher levels of IL-2 production were induced by conjugation with Jurkat cells that were pre-incubated in ipilimumab and therefore retained exogenous CD80/86 alongside acquired pMHC-II on the cell surface (Figure 16C). On the contrary, significantly less IL-2 was produced upon conjugation with untreated Jurkat due to cell intrinsic CTLA4 activity which promoted elimination of CD80/86 prior to contact with unstimulated T cells. Collectively, these data support a model in which cell intrinsic CTLA4 activity can limit the surface lifetime of exogenous CD80/86 co-stimulatory ligands displayed alongside acquired antigen to other T cells.

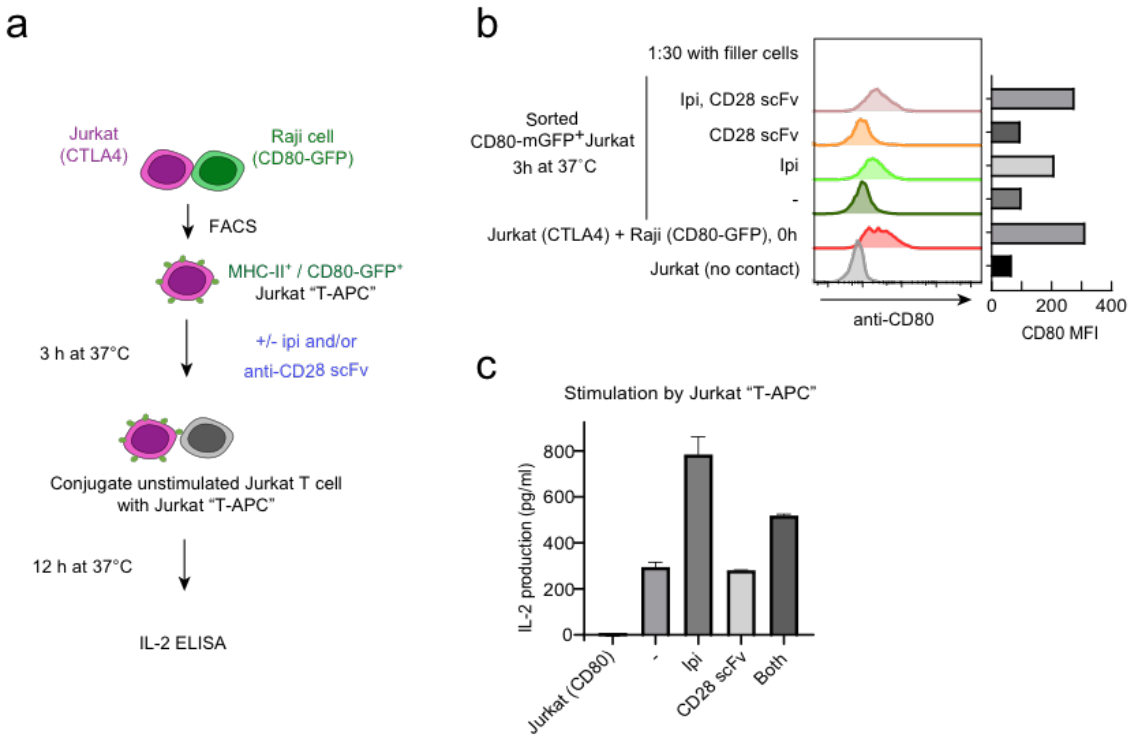

Figure 16. Cell intrinsic CTLA4 activity limits the co-stimulatory capacity of 'T-APCs'

(a) Jurkat T cells expressing CTLA4 were conjugated with Raji B cells, FACS sorted, and cultured as adherent monolayer with excess filler cells in the presence or absence of indicated antibodies.

(b) Flow cytometry indicating CD80 surface levels after 3 hours incubation in the presence of indicated blockade antibody treatments.

(c) Histograms show results of ELISA measuring IL-2 production by unstimulated WT Jurkat cells upon conjugation with T-APC following incubation with specified antibody blockade condition (upper) or upon conjugation with T-APC of specified genotype (lower).

4.3. Discussion

CTLA4 exerts complex control over lymphocyte proliferation and differentiation via cell intrinsic and extrinsic mechanisms but the precise mechanism has long remained elusive. In this chapter, evidence for a two-step mechanism was presented in which CTLA4 engages in *cis*-endocytosis of CD80/86 ('B7') from the T cell surface downstream of ligand acquisition via trogocytosis. Specifically, a simplified in vitro trogocytosis assay was applied using Jurkat T cells expressing CTLA4 and Raji B cells expressing CD80-mGFP to examine the relative

contributions of CD28 and CTLA4 in ligand acquisition and depletion as well as the fate of acquired CD80 in the presence of CTLA4 in isolated single T cells. In this assay T cells were observed to rapidly acquire and display B7 and 'bystander' pMHC following contact with Raji B cells as APCs. This effect was non-antigen specific and primarily driven by CD28 engagement with B7 molecules on APCs independently of TCR stimulation, as reported previously.[217] Using adherent monolayer culture conditions and excess filler cells to block T-T interactions, it was observed that acquired CD80 was efficiently bound and internalized by CTLA4 acting in a cell intrinsic manner. Ligand depletion by CTLA4 was largely dependent on TCR stimulation in primary human T cells but not Jurkat T cells, consistent with the notion that specific APC-derived B7 depletion can occur in *cis* in a manner promoted by TCR signaling. Importantly, this depletion effect was accelerated in the presence of high CTLA4 expression and blocked by ipilimumab treatment or in cells expressing a CTLA4 point mutant (Y139A) unable to bind B7, supporting a role for direct CTLA4 binding in *cis*-endocytosis of acquired B7 ligands.[331]

Mechanistically CTLA4 has been proposed to act via *trans*-endocytosis and degradation of CD80/86 from APCs to control B7 levels in the local environment.[186] A potential contribution by CTLA4 mediated *trans*-endocytosis was excluded in these B7 depletion assays by the use of adherent monolayer culture conditions in the presence of excess CD28 deficient 'filler' cells to block T-T contacts. Interestingly however, it was observed that the rate of CTLA4-dependent CD80 depletion from the cell surface was not significantly enhanced in the presence of *trans* contacts (Figure 12). This result is consistent with a relatively efficient cell intrinsic route of CD80 depletion mediated by CTLA4 acting in *cis* independently of *trans*-endocytosis (Comparison of proposed two-step model vs. *trans*-endocytosis and relevance is discussed below).

The kinetics of B7 ligand acquisition / depletion in the presence of CD28 / CTLA4 co-expression was further examined by visualizing this process in isolated single cells by confocal microscopy. In this assay, acquired CD80 was observed to initially strongly co-localize with CD28 at the cell surface and to a lesser extent with CTLA4, depending on relative expression level. Furthermore, acquired CD80 was observed to gradually accumulate in CTLA4-associated intracellular vesicles over time in a manner which was partially blocked by ipilimumab treatment. (partial blockade by ipilimumab is discussed in section 4.7). Together, these results are consistent with the proposed model in which CTLA4 can operate downstream of CD28-mediated acquisition of B7 ligands via trogocytosis to mediate depletion in a cell intrinsic manner.

Although both CD28 and CTLA4 were found to be capable of CD80/86 acquisition via trogocytosis, accumulation of CD80/86 on the T cell surface in the presence of high levels of CTLA4, as well as the late expression kinetics of CTLA4 during T cell activation, are consistent with a model in which B7 acquisition via CD28 can frequently occur upstream of CTLA4-mediated *cis*-endocytosis. While data presented here cannot rule out direct internalization of CD80/86 via CTLA4 *trans*-endocytosis in parallel, it is notable that CTLA4 expression alone was also sufficient to induce CD80/86 and MHC acquisition and display on the T cell surface (Figure 9C). This observation supports the notion that ligand capture by CTLA4 may similarly occur via a two-step mechanism in which ligand transfer occurs via trogocytosis upstream of subsequent internalization by *cis*-endocytosis (a process similarly described for "*trans*-endocytosis" of full length Notch ligands; see note on the disputed biological existence of *trans*-endocytosis below). Despite representing a somewhat subtle distinction from the current model of CTLA4 *trans*-endocytosis, TCR promoted *cis*-endocytosis of CD80/86 ligands from the

surface of activating T cells appears to create a unique regulatory opportunity for CTLA4 to exert dual intrinsic / extrinsic function within the lymphocyte population, potentially providing an alternative account for its elusive intrinsic and extrinsic effects in maintaining immune homeostasis.

The acquisition of exogenous pMHC and B7 molecules via trogocytosis during T cell activation has been previously shown to endow recipient T cells with the capacity to act as APCs ('T-APCs') both in vitro and in vivo.[87,110,194,218,224–227,229–231,299,332–337] Therefore, in collaboration with Dr. Xiaozheng Xu, we established an in vitro assay to assess T cell antigen presentation ability using IL-2 production. Following contact-dependent trogocytosis with Raji B cells, resulting Jurkat T cells displaying exogenous CD80 and pMHC-II were purified by FACS. As expected, these 'T-APCs' were shown to induce IL-2 production by unstimulated WT Jurkat cells in the presence of TCR crosslinking using SEE. Furthermore, the co-stimulatory capacity of these T-APCs was shown to be diminished by cell intrinsic activity of CTLA4 over time and could be preserved by ipilimumab treatment.

Although the use of Jurkat T cells and bacterial superantigen to induce TCR stimulation in this assay represents a highly artificial setting, these results provide a proof of principle in favor of cell intrinsic CTLA4-mediated regulation of T-T antigen presentation via trogocytosis. Notably antigen presentation in the absence of co-stimulatory ligands is well-established to induce a temporary hypo-responsive state known as T cell 'anergy.'[40] In this context, intrinsic B7 depletion by high levels of CTLA4 in the presence of co-acquired pMHC might be expected to generate 'anergizing' or 'tolerogenic' display of acquired antigens by these cells. This model may therefore have implications for understanding the mechanism of CTLA4-dependent antigen-specific suppression by regulatory T cells (section 5.7).

Importantly, although anergy induction by pMHC display in the absence of B7 is the simplest interpretation, interactions among activated lymphocytes are highly complex involving multiple ligand / receptor interactions, signaling pathways, and exchange of soluble mediators. With this in mind, it is difficult to predict the outcomes of T cell antigen presentation which are likely to be varied and context-dependent in vivo. Consistent with this notion and in tangential support to the model above, double negative (CD4- / CD8-) regulatory T cells were previously shown to induce suppression by acquisition of allo-antigens via trogocytosis to induce subsequent killing of antigen-specific CD8+ T cells.[334] More recently, pMHC-II trogocytosis by these cells was shown to be mediated by the poorly understood CD4 homolog LAG-3 in a mouse model of allergic asthma, further highlighting the complexity of mediators and regulators of T cell trogocytosis as a fruitful area for future research.[300]

Ongoing efforts will aim to gain further insights into the mechanism and precise membrane topology of CTLA4-mediated *cis* capture and the fate of internalized ligands using dual labeling and correlated light and electron microscopy (CLEM). Preliminary results show CTLA4 polarized to acquired membrane fragments and inside cytoplasmic vesicles containing CD80 indicated by gold labeling (Figure 15E). Intriguingly, electron micrographs also appear to frequently show CTLA4 vesicles containing exogenous CD80 in close association with mitochondria in primary human CD4+ T cells (Figure 15). This result is discussed below in the context of a potential role for trogocytosis-mediated accumulation of membrane lipids in mitochondrial fatty acid oxidation (FAO) which has recently been shown to be involved T cell differentiation and memory formation.[338–340] (section 5.11).

Together these results are consistent with the hypothesis that CTLA4 exerts dual intrinsic / extrinsic regulatory effects on lymphocyte populations in part by acting via a cell intrinsic

mechanism to bind and deplete acquired "extrinsic" co-stimulatory ligands from the cell surface as they are re-displayed during T cell activation. Additionally, T cell activation also induces corresponding endogenous expression of CD80/86 by T cells which could then be similarly regulated by TCR / CTLA4-dependent *cis*-endocytosis to form an integrative circuit reflecting APC-derived information (section 4.4.5). This easily overlooked mechanism for CTLA4 regulation of T cell CD80/86 may therefore play an underappreciated role in controlling T cell proliferation during an immune response. Finally, it is proposed that this process may also represent an alternative mechanism for antigen-specific suppression by regulatory T cells (section 5.7).

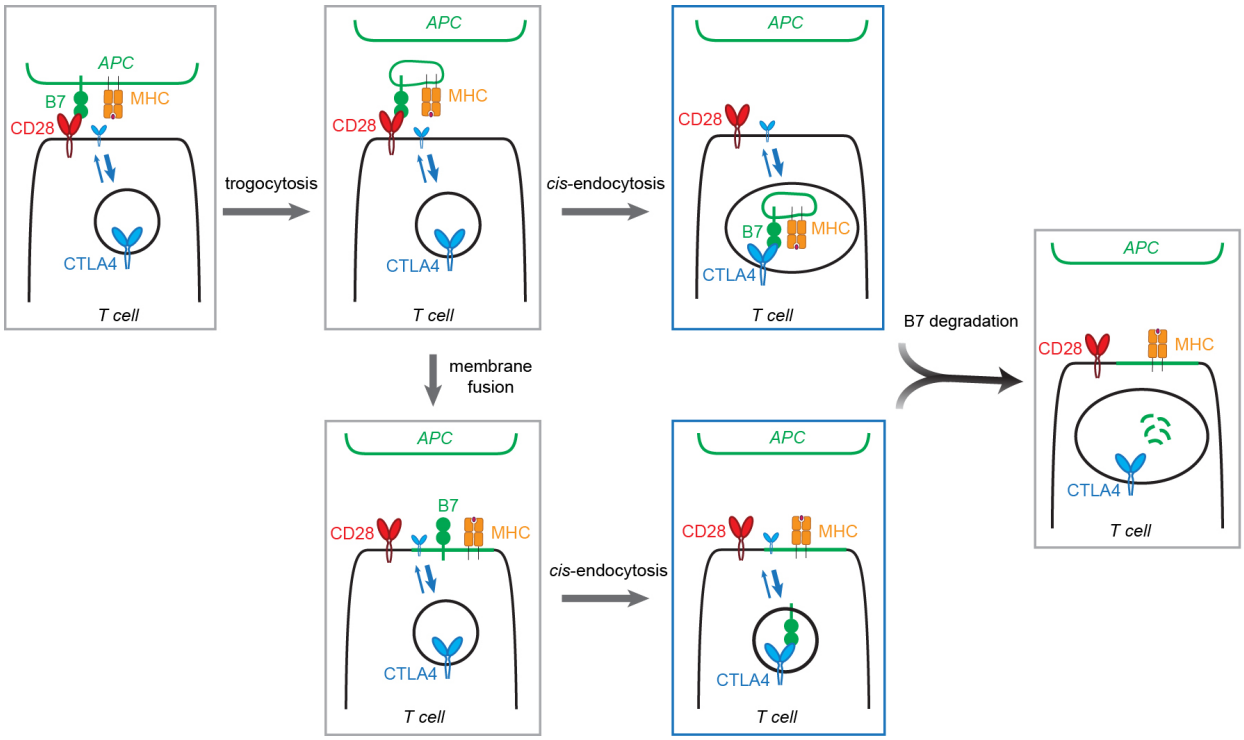

Figure 17. Model summary

Proposed model of CTLA4 mediated *cis*-endocytosis of B7 ligands acquired via CD28-dependent trogocytosis.

4.4. Specific implications and predictions of proposed model

The following subsections describe these specific findings in the context of previous publications in support of an alternative model of CTLA4 function in which a major regulatory target consists of T cell expressed CD80/86 acquired in the context APC-derived membrane fragments and displayed by T cells during an immune response (Figure 18). Unique predictions and interpretations from this perspective are also discussed.

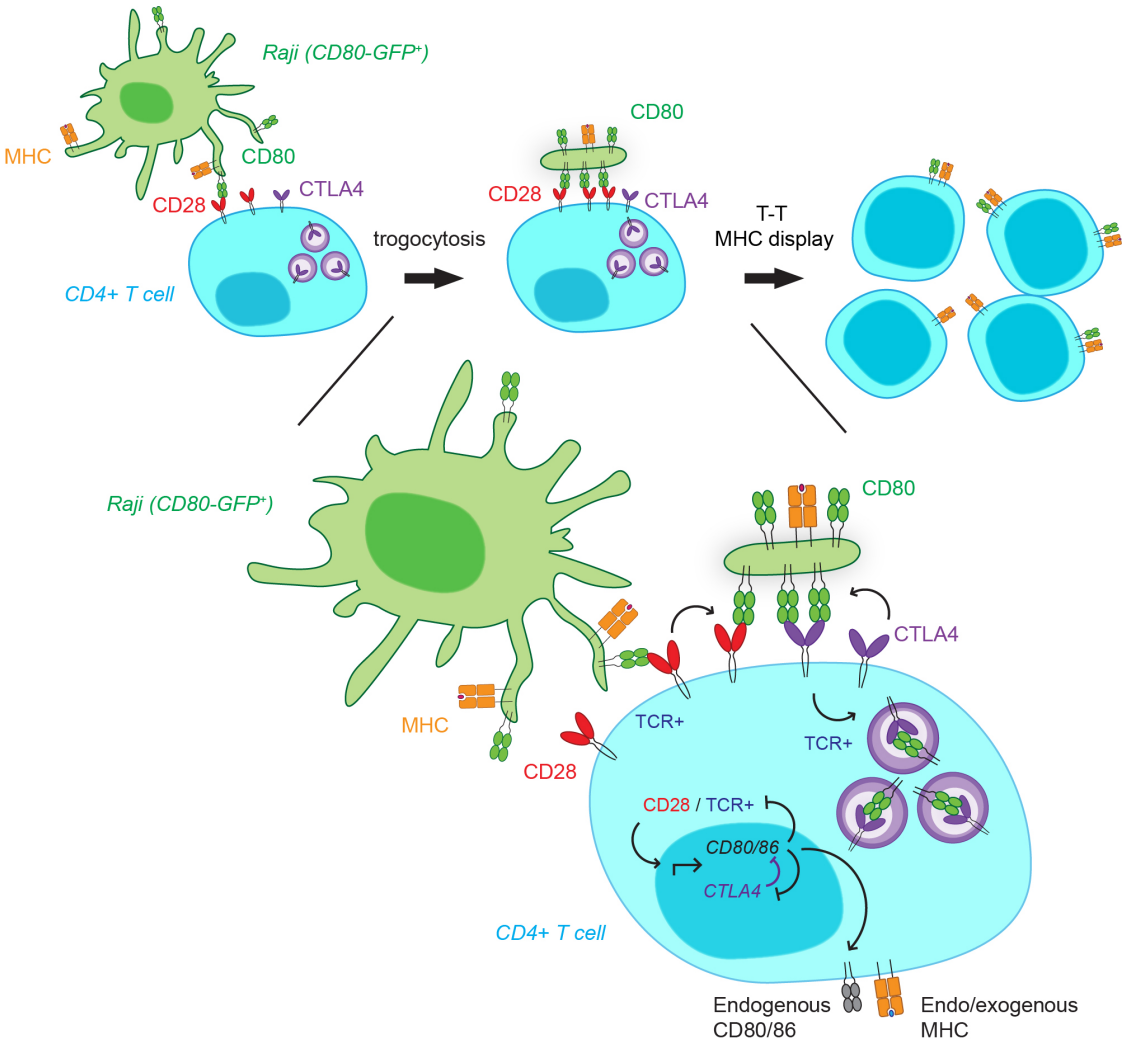

Figure 18. Model implications

(a) TCR-dependent polarization of CTLA4 to the cell surface at acquired membrane: non-equilibrium source of CD80/86 may facilitate cell intrinsic titration of trogocytosis-mediated sustained co-stimulatory signaling. (Discussion 4.4.5)

(b) *Cis* competition between CD28 and CTLA4 for acquired CD80/86 at the cell surface may liberate CD28 for further rounds of CD80/86 transfer to enhance pMHC acquisition by high CTLA4 expressing T cells. (Discussion 4.4.6, 5.7)

(c) High affinity binding to acquired CD80/86 by CTLA4 may prevent 'stealing' of acquired membrane fragments by lower CTLA4 expressing T cells. (Discussion 4.4.6)

(d) Cell intrinsic internalization of acquired CD80/86 by CTLA4 may promote tolerogenic display of acquired pMHC and inhibit pMHC transfer via CD28/B7 (Discussion 4.4.3, 5.7)

(e) CD28/CTLA4-mediated capture and internalization of membrane lipids via trogocytosis may impact T cell metabolism, differentiation, and memory. (Discussion 5.11)

4.4.1 Two step model of CTLA4 function (Trogocytosis + *cis*-endocytosis) vs. *trans*-endocytosis

The rapid kinetics of ligand acquisition via CD28-induced trogocytosis and resulting accumulation of exogenous B7 / pMHC on the surface of activating T cells observed in these in vitro assays and by others in vivo support a scenario in which CTLA4-mediated internalization in *cis* may efficiently operate downstream of ligand acquisition during T cell activation. Importantly, these data do not rule out and are indeed not mutually exclusive of a direct role for CTLA4-mediated *trans*-endocytosis of CD80/86 from APCs occurring in parallel. However, it is notable that CTLA4 alone was also observed to be sufficient to drive acquisition and display of both CD80 and non-specific 'bystander' pMHC, prior to subsequent specific depletion of CD80 from the cell surface as discussed above. This observation is unexpected in the context of a direct *trans*-endocytosis model but consistent with the proposed two-step trogocytosis-based model and several recent reports.

These include, for example, the recent discovery of exogenous CD80/86 as an activation marker displayed by human Treg in vivo which was identified using a multi-omics approach combining single-cell RNA sequencing and mass cytometry.[341] This study highlights the technical difficulty of examining trogocytosis-dependent regulatory interactions of acquired ligands in vivo, which are often concurrently endogenously expressed by recipient cells (section 4.4.5). An additional recent report utilized a biochemistry approach employing a separation-of-function mutant in which the endocytic tail sequence of CTLA4 was removed to further demonstrate that CTLA4-mediated endocytosis was not required for ligand depletion from APCs, which was instead mediated via trogocytosis.[188] Taken together, these results support the hypothesis that B7 / pMHC acquisition and display via trogocytosis can efficiently occur upstream of CTLA4-dependent internalization, even in high CTLA4 expressing human Treg cells. The inherent difficulty for in vivo studies to differentiate '*trans*-endocytosis' from a two-step mechanism involving rapid ligand acquisition via trogocytosis followed by more gradual '*cis*-endocytosis' by CTLA4 highlights the requirement for in vitro assays examining single cells to reveal this distinction. Despite the mechanistic subtlety however, a model in which CTLA4 mediates and regulates ligand acquisition and re-display via trogocytosis produces otherwise unexpected regulatory effects not predicted by the *trans*-endocytosis model.

Note on the biological existence of "*trans*-endocytosis" of full length transmembrane proteins: *Trans*-endocytosis was originally described for the Notch signaling pathway and refers to direct internalization of the Notch receptor extracellular domain upon binding-induced proteolytic cleavage.[342] Notably, early reports of "extraction" of full-length transmembrane Notch ligands via *trans*-endocytosis described in cell culture systems was apparently mediated either partly or entirely by *cis*-endocytosis following intercellular transfer of plasma membrane

via a process resembling trogocytosis and this process ultimately proved to be undetectable in *Drosophila* development.[343,344] Similarly, the other major example of "*trans*-endocytosis" of full-length transmembrane proteins in cell biology, that of Ephrin receptors, was recently identified to be mediated via a trogocytosis mechanism.[345,346] While trogocytosis + *cis*-endocytosis could be said to represent only a slight modification of the *trans*-endocytosis model of CTLA4 function, the notion that this distinction is irrelevant requires the assumption that the functional implications of ligand transfer and re-display via trogocytosis is irrelevant; at least for adaptive immunity, this assumption is increasingly difficult to hold (section 5.3).

4.4.2 CTLA4-dependent induction of in vivo anergy and FOXP3- / IL-10+ Treg

With respect to the controversial role of lower levels of CTLA4 in the induction of in vivo anergy or 'adaptive tolerance' in conventional CD4+ T cells, one possible mechanism for this to occur according to this model would be due high levels of CTLA4 induced in a proliferating T cell population which subsequently fails to establish quorum (i.e. a minimum number of linked antigen-specific cooperating T cells to induce an immune response). Elevated CTLA4 in these cells might be expected to raise the co-stimulation threshold for subsequent TCR activation in an intrinsic manner.[102,347,348] Furthermore, in the presence of persistent antigen stimulation and strong TCR signaling, sustained high levels of CTLA4 may also allow conventional CD4+ T cells to similarly act as tolerogenic 'T-APC' as described below for Treg. This scenario is also consistent with the CTLA4-dependent conversion of anergic (FOXP3-/IL-10+) CD4+ T cells to a suppressive phenotype distinct from Treg, which has been previously reported both in vitro and in vivo.[60,349] Intriguingly, and in support of this 'T-APC' model of antigen-specific suppression by CTLA4, conversion of these anergic cells to a suppressive phenotype was recently shown to require second administration of antigen while CTLA4 levels

remained high following anergy induction.[350] In summary, this mechanism may help to provide a more unifying view of the role for CTLA4 in the induction of in vivo anergy and in suppressive T cell subsets beyond FOXP3+ Treg as well.

4.4.3 High CD80/86 levels enhance non antigen-specific pMHC transfer via CD28

While numerous studies have reported trogocytosis-mediated acquisition and display of specifically recognized pMHC by TCR engagement in vivo, the data presented here highlight the potential for high levels of non antigen-specific pMHC acquisition driven by CD28 in the presence of high levels of CD80/86.[217,218] This phenomenon may have interesting implications in facilitating CD28-dependent dispersal and display of pMHC in inflammatory contexts where B7 ligands are abundant. Consistent with this notion, antigen-specific trogocytosis has been successfully utilized for identification of antigen-specific T cells both in vivo and in vitro while this approach was unable to identify antigen-reactive Tconv and Treg at sites of inflammation due to elevated levels of non-antigen specific pMHC accumulation.[237,238,351] This consideration may have important consequences for understanding the currently somewhat inexplicable robustness of peripheral tolerance under inflammatory settings (section 5.4) and the elusive mechanism of antigen-specific suppression by regulatory T cells (section 5.7).

A potentially interesting corollary of the observation that high CD80/86 levels appear to facilitate non-specific pMHC co-transfer via CD28 / B7 interactions would be that CTLA4-dependent binding and/or internalization of CD80/86 might be predicted to limit antigen dispersal. It seems conceivable that the outcome of this process would be a net ‘flow’ of antigen alongside co-stimulatory molecules and membrane lipids toward higher CTLA4-expressing cells in networks of activating lymphocytes and in inflamed tissues (section 4.4.6).

In tentative support of the hypothesis that B7 molecules promote pMHC transfer and CTLA4 may limit this process, an intriguing recent publication showed that ectopic CTLA4 expression by AIRE deficient medullary thymic epithelial cells (mTECs) (which are known to express CD80/86), prevented pMHC transfer to thymic DCs via an unknown mechanism.[111] Similarly, in a recent report describing marginal zone (MZ) B cells acquiring DC function via trogocytosis, a dramatic enhancement of B cell acquisition of pMHC-II from DCs was observed to correlate with greatly increased CD86 expression by DCs.[231] A potential role for CD86 in enhancing pMHC-II acquisition by MZ B cells was not examined by the authors however who showed support for an alternative mechanism based on direct acquisition of pMHC-II from cDCs mediated by complement component C3 binding to pMHC-II on cDCs and facilitating interaction with the complement receptor 2 (CR2) expressed by MZ B cells.[231] Importantly, this exchange was followed by subsequent MZ B cell priming of CD4+ T cells using the DC-derived pMHC-II.[231]

In the context of the hypothesis outlined above it is notable that although the model presented by the authors represents a more direct pathway, it may be difficult to exclude possible effects of pMHC-II acquisition mediated via CD4+ T cell expressed CD28 and subsequent serial transfer to MZ B cells as similar processes have been reported previously.[219] Similarly, the possibility for direct priming of antigen-specific CD4+ T cells by non-specific CD4+ T cells bearing DC-derived pMHC-II acquired via CD28 has been demonstrated.[218] While difficult to confirm in vivo, this potential for inversion of classically considered roles in antigen presentation and for non-specific antigen dispersal via CD28 / B7 interactions highlights the versatility of this perspective and the possibility for lymphocyte trogocytosis to subtly enhance the efficiency of immune cell interactions and the speed of intercellular communication during an immune

response (section 5.5). Finally, this potential for high levels of antigen dispersal and decentralized priming interactions seems to have far-reaching theoretical implications that may help account for the robustness of immune tolerance. In this view, "self" / non-self discrimination appears to be continuously 'crowd-sourced' among the repertoire, potentially facilitating higher-level encoding of a dynamic memory of "self" by the network (section 5.10).

4.4.4 CD28 is a major but not exclusive driver of B7 / pMHC acquisition via trogocytosis

Using an in vitro trogocytosis assay consisting of Raji B cells transduced to express CD80-mGFP as APC, endogenous CD28 expressed by T cells was observed to efficiently outcompete CTLA4 for CD80/86 engagement at the cell surface during transient cell-cell contacts. As a result, CD28 / B7 binding resulted in rapid acquisition and display of B7 and co-acquired non-specific 'bystander' pMHC-II molecules upstream of more gradual CTLA4-dependent depletion of B7 ligands from the cell surface over time. In these assays, strong TCR stimulation using bacterial superantigen resulted in only mild increase in ligand acquisition and this effect was largely CTLA4-independent. In further support of a primary role for CD28 in ligand acquisition, primary human CD4+ and CD8+ T cells were also observed to acquire CD80 from Raji B cells in a CTLA4-independent manner, with no effect of CTLA4 blockade treatment on acquired levels (Figure 9D). While CTLA4 is poorly expressed in conventional CD4+ and CD8+ T cells, similar results obtained using high CTLA4-expressing primary human Treg (data not shown) and induced 'iTregs' in a previous report.[326] Taken together, these data suggest CD28 is likely to be a major driver of B7 / pMHC acquisition via trogocytosis, at least in the presence of high levels of CD80/86 such as those examined here. (Implications of non-specific B7 / pMHC acquisition via CD28 in inflammatory settings is discussed in section 4.4.3).

While these results support a dominant role for CD28 in mediating B7 / pMHC

acquisition upstream of CTLA4 depletion in most settings (i.e. high CD28 and low surface CTLA4), they cannot rule out a significant contribution of CTLA4-induced trogocytosis in some contexts, particularly in high expressing cells (e.g. Treg) in the context of high levels of TCR-induced CTLA4 polarization to the cell surface. Indeed despite normally being restrained by 95% intracellular localization and low total levels, CD80 binding by CTLA4 actually appears to induce even greater levels of ligand acquisition when compared with CD28 on a per molecule basis, presumably due to the ~20-fold higher affinity/avidity of CTLA4 interactions with B7 ligands (Figure 9C).[161,352] Together these data demonstrate that both CD28 and CTLA4 induce contact-dependent B7 / pMHC acquisition via trogocytosis upon ligand binding and that the relative contribution of CTLA4 is likely dependent on TCR signaling which directly correlates with the magnitude of induction of CTLA4, its polarization to the cell surface, and cycling rate.[162,165]

4.4.5 Titration of trogocytosis-mediated sustained co-stimulatory signaling via CTLA4

It is understood but rarely discussed that the TCR, CD28, CTLA4, CD80/86 and IL-2 operate within intricately interconnected antagonistic positive feedback loops.[91] In this context, a link between TCR / CD28-mediated exogenous B7 acquisition, endogenous expression, and CTLA4-dependent intrinsic depletion may fit well within this circuit to facilitate extrinsic / intrinsic integration of T cell signaling. In this respect, a two-step mode of ligand acquisition via trogocytosis followed by *cis*-endocytosis may offer several otherwise unexpected benefits for regulation. Specifically, the maintenance of CTLA4 in a primarily intracellular (95%) localization with cell surface expression regulated by TCR / CD28 signaling would likely enable CD28 to have first access to any excess unbound B7 ligands at the cell surface prior to fusion of acquired membrane fragments and subsequent internalization, even in the case of acquisition

driven by CTLA4. This framework is consistent with the paradoxical strict requirement for CD28 signaling in Treg homeostasis despite expression of constitutively high levels of CTLA4.[353] As discussed below, this interplay of CD28 and CTLA4 competitive binding for exogenously acquired and endogenously expressed co-stimulatory ligands may also be expected to facilitate cell intrinsic integration of extrinsic co-stimulatory signals to fine-tune both homeostatic and activation-induced T cell proliferation.

In the context of B7 / pMHC trogocytosis, CTLA4 appears to engage in direct competition for B7 contained on acquired membrane fragments in a TCR-dependent manner as proposed previously for cell-cell contacts.[160] Importantly, unlike stable cell contacts, exogenous membrane fragments represent a non-equilibrium pool of B7 which is not subject to continuous replenishment from endogenous expression by APCs. As the magnitude of CTLA4 induction, polarization to the cell surface, and cycling rate are all intimately linked to the level of TCR/CD28 signaling, reciprocal regulation in this context would seem to facilitate efficient TCR-dependent titration of co-stimulatory signaling within a very narrow range.[162] For example, as levels of B7 ligands in the environment / bound at the cell surface (in the form of discrete fragments) begin to become limiting, the associated decrease in CD28/TCR signaling would induce parallel reduction in CTLA4 surface levels, increasing available B7 for CD28 binding. At the same time, TCR/CD28 induced endogenous expression of CD80/86 produces an additional layer of co-stimulatory 'buffering' capacity which is expected to be similarly antagonized by CTLA4 operating in this cell intrinsic context, a process which was indeed recently shown to regulate homeostatic proliferation of Treg.[354] While perhaps initially allowing intrinsic integration of 'extrinsic' B7 levels bound at the cell surface, endogenous expression likely becomes a more significant source of B7 as T cells become less dependent on APCs during

proliferative expansion.[91] In this scenario, endogenous expression and exchange of T-cell derived B7 is also likely to be a major target of *cis*-endocytosis by CTLA4 operating in the same integrative "nested antagonistic feedback loop" as recently described.[91]

Overall this mechanism is proposed allow CTLA4 to fine-tune cell proliferation in a cell intrinsic manner and also facilitate network integration as T cells simultaneously display acquired co-stimulatory and antigenic information to other cells while regulating intrinsic and extrinsic B7 ligand availability via the same TCR / CTLA4-dependent mechanism. Consideration of this pathway may therefore help to account for the extraordinary sensitivity of T cell responses to small changes in B7 levels and the complex intrinsic / extrinsic regulatory effects achieved by CTLA4, even when expressed at extremely low levels such as in activated CD8+ T cells and conventional CD4+ T cells.[177,190]

Finally, this perspective appears to have far-reaching implications for T cell biology. Classically, T cell activation is considered to require concurrent TCR / CD28 binding with pMHC and B7 displayed by a single activated APC.[47] This is a theoretical requirement however, based on the PAMP / Danger framework which places APC control over the inflammatory context of antigen presentation via its expressed PRRs at the basis of immune tolerance.[24] Interestingly, the existence of this requirement has not been demonstrated and has in fact been refuted experimentally, with 'bystander' APCs shown to be capable of delivering the essential co-stimulatory signal.[69] Because CD28 signaling requires TCR activation, in this context it seems plausible that pre-assembly of CD28 / B7 clusters onto a 'cis' interface via trogocytosis may represent a means for T cells to integrate co-stimulatory information onto the cell surface over time, possibly even prior to antigen recognition. Importantly however, upon fusion of acquired membrane fragments *cis* CD28 / B7 interactions at the cell surface may likely become

inhibitory via reduction of available CD28 for *trans* binding, as described for other *cis* interactions such as in the PD-1 and Notch signaling pathways.[199,355] In this context, *cis*-endocytosis of T cell intrinsic B7 by CTLA4 may be expected to relieve *cis* inhibition of CD28 and thereby confer a paradoxical advantage to high CTLA4 expressing T cells which is uniquely contingent on extrinsic B7 availability.

4.4.6 *Cis* competition by CTLA4 as a means to enhance CD28-dependent B7 / pMHC acquisition – an alternative mechanism for trans B7 depletion and network integration.

In addition to B7 / MHC acquisition mediated by direct CTLA4 / B7 binding in *trans* and associated trogocytosis under conditions of strong TCR stimulation, *cis*-competition by CTLA4 with CD28 for acquired B7 ligands at the T cell surface may additionally liberate CD28 for further rounds of B7 acquisition. Because individual CD28 / B7 interactions appear to act cooperatively to acquire excess CD80/86 in the form of large membrane fragments associated with pMHC molecules (Figure 9), this mechanism may provide an alternative route for high levels of CTLA4 expression to significantly enhance B7 / pMHC acquisition and produce a dynamic, integrative 'flow' of acquired ligand under inflammatory (i.e. high CD80/86) conditions where CD28 binding may otherwise become saturated. This process could provide a significant physiological benefit by facilitating pMHC accumulation and suppression by high CTLA4 expressing Treg in inflamed tissue environments, potentially resulting in a net transfer of B7 and pMHC toward the highest CTLA4 expressing cells. Such a phenomenon could also conceivably contribute the observed paradoxical clonal dominance of high CTLA4-expressing cells in vivo despite its common description as a molecular 'brake' on T cell activation and proliferation (Discussion 5.2).[356] Notably, this mechanism is consistent with the observation of

non-specific pMHC accumulation on infiltrating Treg in contexts of autoimmune inflammation described above.[351]

Importantly, this mechanism of CTLA4-mediated *cis* competition would not be expected to produce excess levels of B7 on the cell surface of CTLA4 expressing cells, but rather facilitate TCR-dependent control the available surface levels of B7 within a narrow range as discussed in section 4.4.5. Interestingly however, specific depletion of B7 molecules by CTLA4 may result in accumulation of co-transferred pMHC at the cell surface, unless pMHC trogocytosis similarly regulated by alternative mechanisms. (Potential for regulation of pMHC trogocytosis by LAG-3 is discussed in Section 5.13).

One related possible outcome of *cis*-acting CTLA4 on interclonal competition among lymphocytes would be that high affinity/avidity CTLA4 interactions at the cell surface might prevent subsequent 'stealing' of acquired membrane fragments by less activated clones expressing lower levels of CTLA4 or CD28. As extensive membrane transfer via serial trogocytosis has been shown to occur among activated CD4+ and CD8+ T cells and monocytes in vitro it seems plausible such a phenomenon could similarly promote a paradoxical clonal advantage of high CTLA4 expression during an immune response.[219]

4.4.7 Study limitations

Notably, complete blockade of CTLA4 depletion effects by ipilimumab was observed to be invariably incomplete relative to the B7-binding deficient CTLA4 point mutant (Y139A). Because this could be partly mitigated by use of high concentration of ipilimumab (100μg/mL) and extended incubation times (>1 hour), this result likely reflects the 90% intracellular localization of CTLA4 combined with the inherent difficulty in blocking high affinity '*cis'* interactions using a soluble antibody. This observation may be of clinical relevance as it seems

that the mechanism of CTLA4 activity may not have been successfully targeted therapeutically using blockade antibodies. Specifically, significant evidence has emerged that ipilimumab mediates its anti-cancer effects at least partly via antibody-dependent cellular cytotoxicity (ADCC) of immunosuppressive Treg.[357] In support of this hypothesis, the anti-CTLA4 blockade antibody tremelimumab which was modified to prevent ADCC, failed in stage III clinical trials against metastatic melanoma.[358] This technical limitation may therefore reflect the need for more effective modes of targeting the CTLA4 pathway.

Importantly, while similar data were obtained using primary human T cells and WT Raji cell donors, these assay conditions primarily examined transformed Jurkat T cell lines in the presence of Raji B cells transduced to express high levels of CD80/86-mGFP. These conditions enhance levels of CD28-dependent ligand transfer and may obfuscate a more substantial role for TCR engagement or that of other adhesion molecules in mediating trogocytosis of CD80/86 and/or pMHC.

Notably, flow cytometry based assays likely do not distinguish between CTLA4-mediated internalization of CD80 and CTLA4-binding induced blockade of CD80 epitopes detected by antibody staining at the cell surface. The rapid loss of CD80 levels detected by anti-CD80 staining in the presence of high levels of CTLA4 appears to be at least partly associated with the latter (i.e. high affinity CTLA4 binding at the cell surface). However confocal microscopy studies confirm that CTLA4 binding is associated with near-complete internalization of CD80 on slightly longer timescales (i.e. 1-3 hours).

An additional limitation of this study is the method of inducing ligand transfer via cell-conjugation followed by dissociation of cell conjugates by pipetting. In addition to high CD80/86

expression levels, it seems likely that this approach exaggerates ligand transfer by mechanical disruption of cell membranes and/or potentially reflects an artificial mode of membrane transfer which may even be distinct from lymphocyte trogocytosis observed in vivo. These possibilities were examined by confocal microscopy using unstimulated Jurkat T cells or primary human CD4+ and CD8+ T cells activated by anti-CD3/anti-CD28 bead stimulation and co-cultured with Raji B cells expressing CD80-mGFP in the absence of mechanical dissociation by pipetting. These cells similarly exhibited substantial amounts of contact-dependent membrane acquisition from Raji B cells which was CD28-dependent and independent of dissociation by pipetting. Importantly, the localization and morphology of acquired membrane fragments was identical to those observed using mechanical dissociation.

It is also worth noting that the mechanism of trogocytosis remains unknown and mechanical scission or release of fine membrane projections indeed seems likely to represent a key source of membrane transfer in vivo.[80,81,359] Furthermore, electron microscopy (EM) of trogocytosis+ primary human T cells revealed the expected topology of acquired membrane fragments (Figure 14-15) which was similarly observed by EM in a recent report.[194] Finally, regardless of the precise mode of membrane acquisition or effects of ligand overexpression, the goal of these assays was to simulate the process of contact-dependent membrane transfer, which is well-established to occur in vivo, for the purpose of assessing the potential impact of CTLA4 expression on acquired ligands in *cis*. This was successfully demonstrated and it seems plausible that these results can be extended across the range of relevant B7 expression levels and in various modes of acquisition that result in T cell display of exogenous B7 ligands at the cell surface.[81,110,221,360,361]

Finally, potential effects of trogocytosis on recipient cell signaling were not examined. In

addition to cell surface signaling discussed above, it is tempting to speculate that a cell intrinsic CTLA4 pathway could similarly be involved in regulating trogocytosis-mediated sustained intracellular signaling which has been previously reported to occur.[195,362]

4.5. Model summary

Overall it is proposed that this cell intrinsic mechanism for CD80/86 depletion by CTLA4-dependent *cis*-endocytosis may play an underappreciated role determining outcomes of interclonal competition among lymphocytes during an immune response. This is suggested to occur by enabling TCR / CTLA4-dependent regulation of proliferative expansion and antigen presentation by activating T cells. This pathway thereby provides an alternative mechanistic basis for the ability of CTLA4 to coordinate lymphocyte proliferation and immune homeostasis via ligand depletion despite its extremely low surface levels and subtle depletion effects in vivo. In this model, dual intrinsic / extrinsic effects are proposed to act in concert to limit the ability of individual activating lymphocytes to establish a coherent 'Quorum' of similarly responding clones.

While some of the scenarios outlined above represent more speculative outcomes of *cis* competition between CD28 and CTLA4 at the cell surface, most have been demonstrated in previous studies examining cell-cell contacts.[363,364] Interestingly however, this perspective seems to provide a broader description of CTLA4 function that is uniquely contained within a single mechanism. Specifically, at lower levels (e.g. in activated CD8+ T cells or conventional CD4+ T cells) CTLA4 is suggested act in a partly intrinsic and integrative manner by enabling TCR-dependent titration of acquired and expressed B7 ligand availability to limit cell proliferation induced by T cell activation and associated trogocytosis-mediated sustained signaling (Section 4.4.5). At higher CTLA4 levels (e.g. in Treg or possibly highly activated Tconv) this same

process may be expected to produce the observed dominant extrinsic suppressive mode of action and confer a paradoxical competitive advantage to expressing cells.[365] This is hypothesized to occur by allowing high CTLA4 expressing cells to act as a 'molecular sink' within the network to: prevent CD28 / B7 saturation in *cis* and thereby enhance CD28 *trans* binding and associated acquisition of B7 and co-transferred pMHC (section 4.4.6), accelerate internalization of acquired membrane lipids to potentially enhance mitochondrial fatty acid metabolism associated with T cell memory phenotype (section 5.11), and perhaps in parallel mediate direct impacts on T-T antigen presentation.

One unique prediction of this *cis* competition model would be that CD28 expression in Treg should exhibit a cooperative effect with CTLA4 to enhance trans depletion of B7 from APCs, a phenomenon which is otherwise thought to be exclusively CTLA4-dependent. (This result was subsequently confirmed by Dr. Xiaozheng Xu and included in the manuscript submitted for publication).

In terms of regulation of T cell antigen presentation it is proposed that TCR-dependent CTLA4 activity can function to limit the temporal duration that exogenous co-stimulatory information is displayed alongside co-acquired pMHC to similarly reactive clones among the repertoire during subsequent T-T interactions which have previously been shown to regulate T cell proliferation and differentiation during priming.[87,91,227,299] This mechanism would represent an efficient independent route for intrinsic control of 'extrinsic' co-stimulatory signals displayed in the local environment distinct from *trans* CTLA4 / CD28 competition or CTLA4-mediated depletion of CD80/86 from myeloid APCs that have thus far been considered in current models. Such a mechanism would seem to represent an intriguing blurring between "cis" and "trans" activity of CTLA4 and of cell autonomy itself in the context of membrane exchange within

networks of activating lymphocytes. Consideration of a cell intrinsic CTLA4 pathway in this context has the advantage of more directly / locally linking the strength of TCR signals with control of co-stimulation.

4.6. Acknowledgements

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Chapter 5: General Discussion

5.1. Theoretical implications Summary

Independent of the precise mechanistic details by which the CTLA4-mediated B7 *cis*-endocytosis pathway described in Chapter 4 might facilitate TCR/CTLA4-dependent control of cell proliferation and interclonal competition / cooperation among lymphocytes during an immune response, a perspective in which the canonical co-stimulatory / co-inhibitory receptor pair (CD28 / CTLA4) appear to be intimately engaged in mediating and regulating the effects of T cell trogocytosis has surprisingly broad functional and theoretical implications. First, the notion that CTLA4 may achieve its functions in part by regulating antigen presentation among T cells highlights a potentially fundamental role for T-T interactions and collective decision-making by lymphocytes in adaptive immunity which have been proposed previously but remain not widely considered.[87,227] This pathway therefore has the distinct benefit of providing additional evidence in favor of a reconsideration of a 'Quorum' framework of lymphocyte activation and immune class regulation (section 5.4) while uniquely incorporating the central role of co-stimulatory signaling provided by the PAMP/ "Danger" model (section 1.6.4) through a T cell intrinsic mechanism for fine TCR-dependent control of B7 ligand availability (section 4.4.5). Second, the apparent capacity of such a phenomenon to produce a dynamic "self" reflecting network appears to recapitulate the essence of Jerne's Network Theory (section 5.10). It is suggested that targeting of this temporally integrative and reflective process by CTLA4 provides regulatory access to a higher level of information to help achieve its essential functions in controlling immune homeostasis (section 5.2). Third, as the most bioenergetically 'expensive' macromolecule, the substantial accumulation of membrane lipids by T cells engaged in

trogocytosis mediated by CD28 and CTLA4 highlights the potential for a fundamental role for lipid metabolism to provide a relatively simple mechanism for rewarding outcomes of interclonal competition and cooperation during an immune response. This perspective may therefore have unexpected implications in relation to the recently established but poorly understood role for lipid metabolism, autophagy, and mitochondrial fatty acid oxidation in T cell activation, differentiation, and memory (section 5.11).[338,339,366] Finally, on the basis of the apparent mechanistic simplicity of the "self"-reflective and competitive network described above, the evolutionary conservation of trogocytosis in eukaryotes, and its ubiquity among immune cells, it is hypothesized that this process was likely directly involved in the evolutionary origins of adaptive immunity (section 5.10) and that this perspective may have implications for evolutionary theory more broadly (section 5.10.3). Specifically, trogocytosis is proposed to represent a primordial mode of intercellular communication which facilitated the emergence of highly integrated "self"-focused collective behaviors among primitive leukocytes, the associated development of dominant mechanisms for preserving "self" tolerance, and internal selective pressure for distinct mechanisms of somatic GOD to emerge twice in early vertebrates.[192,367]

5.2. A 'Quorum' model of CTLA4 regulatory function

Reconsidered in a 'Quorum' framework, the longstanding enigma of CTLA4-dependent antigen specific suppression by regulatory T cells and the apparently purely cell extrinsic rescue of CTLA4-deficient bone marrow chimeras by CTLA4+ cells in *trans* (Section 1.3.2.2, 5.7) may be more readily understood by the ability of a small number of dominant 'informed' individuals (i.e. Treg) to control collective behaviors. Indeed, similar of phenomena of collective 'leadership' have been described to emerge in embryonic development, metastatic cancer, and schooling fish, as in human societies (section 5.9).[368–371] Application of these universal principles

of complex collectives to the study of the immune system has been noted by others and upon first consideration, ostensibly supports the notion that the persistent mystery of CTLA4 function may simply represent the limits of reductionist approaches to understand the emergence of collective behaviors, which will ultimately require increasingly sophisticated computational methods.[88] To some extent, the truth of this assumption appears inevitable and it is of course terribly plausible that no further molecular bases for CTLA4-mediated regulation of network-level phenomena such as immune tolerance and class regulation will be identified, and are indeed not theoretically necessary. However, as these higher-order outputs of complex systems are known to be determined by the nature of local interactions among individual components, it then becomes extremely fortunate, from a cell biology perspective, that the network appears to be behaving very strangely, rapidly exchanging information in a highly unusual manner.

In this context, it seems plausible that the fundamental significance of this process of surface membrane exchange may have previously evaded powerful reductionist approaches in part because it appears to create such a unique example of an inversion in the basic assumption of reductionism, (i.e. that a system can be described by the sum of its components), instead providing a clear demonstration of individual components integrating and reflecting the dynamic context of the system itself. This sacrifice of cell autonomy may therefore provide a previously unappreciated means for network integration through local interactions among responding lymphocytes, offering new opportunities for self-organizing antigen-specific information processing and a greater capacity for auto-regulation despite the inherently diffuse nature of the system (Section 5.9). CTLA4 operating at this level may enable regulatory access to more information to facilitate its remarkable abilities in coordinating control of lymphocyte collective behaviors.

5.3. Theoretical significance of trogocytosis

The functional implications of trogocytosis in immune cell function have long been debated.[222] Despite extensive reports of functionally relevant effects, it has been difficult to reconcile this apparent gross violation of cell autonomy with the precision and efficacy of increasingly sophisticated reductionist models of immune networks. Decades of research in immunology exploiting specific cell identity markers displayed on the surface of immune cells and detected by flow cytometry, and more recently using single-cell RNA sequencing, have produced a remarkably complete description of adaptive immunity without considering —and indeed arguably thanks to ignoring— this seemingly chaotic process of surface membrane exchange (Discussed in section 5.9.2). In this context it appears to stretch credulity to consider the potential for a fundamental importance of trogocytosis, particularly when it generally appears to produce no "dramatic effects." That is, trogocytosis does not seem to generate the ostensibly expected chaotic departures from reductionist / linear models of immune cell function that ignore this phenomenon entirely. However, this intuition may prove to be misleading, as cell biology has recently reached a level of sophistication in which "dramatic effects" can be more subtle, manifest in the form of fine tuning and conferring robustness in the flow of information through complex systems.[‡‡‡] Regardless of the apparent success of our models based on more rigid notions of autonomy among immune cells, it has become empirically unequivocal that trogocytosis represents a potentially functional component of every immune response in vivo and indeed seemingly of every immune cell interaction, serving as a bona fide marker of T cell

[‡‡‡]This principle is perhaps best exemplified at the molecular level by the enigmatic small ubiquitin like posttranslational modification (SUMOylation) of nuclear proteins in eukaryotes which exert spatially localized, bidirectional, and subtle yet completely essential regulatory effects on virtually all nuclear processes including facilitating fate transitions and maintaining robustness of gene expression networks in the establishment and maintenance of cell identity.[461–463]

activation, with exogenous membrane acquisition correlating with the magnitude of TCR stimulation.[131,223,372,373] In light of this, we can therefore only speculate as to whether an adaptive immune system can even exist without this process of surface membrane exchange and should be willing to consider it as potentially fundamental (Section 5.10).

Resistance to the elevation of trogocytosis as a fundamental and primordial "language" of the innate and adaptive immune systems seems natural upon consideration that this process seems to violate almost all of the most core tenets of cell biology. These include Cell Theory (trogocytosis permits extreme violations of cell autonomy), the Central Dogma of molecular biology (trogocytosis enables information transfer directly via protein exchange, permitting cells to mediate and respond to signaling via proteins they do not themselves encode or express endogenously), and basic notions of Receptor Theory (ligand acquisition facilitates functional receptor/ligand interactions to occur in *cis*). Additionally, Clonal Selection Theory remains the foundational understanding of adaptive immunity, which by definition requires some degree of maintenance of cell autonomy. Nevertheless, in light of accumulating evidence over several decades and multiple recent major publications documenting the functional effects of trogocytosis in vivo, the current consensus appears to be trending toward a broader acceptance, wherein regulatory consequences of this process are acknowledged under certain special circumstances. However, these instances are almost invariably discussed under pathological contexts and/or in terms of the need for 'strict regulation' and 'careful control' of trogocytosis in order to preserve more rigid notions of cell autonomy in adaptive immunity and the underlying theoretical framework that implicitly excludes the functional implications of this process in normal physiology (section 5.4, 5.6).[188,194,231–233,374–376]

As a pertinent example, instances of CAR-T cell trogocytosis-induced fratricide as a cause for failure of cancer immunotherapy have been among the most readily incorporated into the mainstream literature (section 1.5.1).[235,236,377] It seems that this is likely due to these findings re-enforcing the common perception of trogocytosis as a potential artifact or sign of T cell dysfunction (in this case enhanced by the high affinity TCRs used for CAR-T cells). However in the opposing view, if this process of antigen-specific acquisition and display during T cell activation and associated auto-regulation by fratricidal CD8+ T cells is considered to represent a typical component of immune responses, such a phenomenon could have far-reaching implications. Specifically this process, for example targeted in some instance against CD4+ 'T-APCs' displaying acquired pMHC-I, would offer an alternative account for several decades of observations of the now-controversial and notoriously elusive class of "suppressor" CD8+ T cells, which have recently been implicated in the etiology of multiple sclerosis.[37,378–381] Additionally, this unusual phenomenon again seems to provide a route toward reviving the essence of Jerne's Network Theory for the system-level auto-regulation of T cell responses (section 1.6.8, 5.10).

Most generally it is tempting to speculate that in this apparent chaos and radical departure from the current theoretical framework of immunology there may be some analogy to the limits of classical mechanics in 20th century physics. This description was adequate for understanding and generating predictions about the properties of matter until it was examined too closely. At this point highly unusual behaviors began to emerge and new theories were required which seem irreconcilable and defy reductionist comprehension. It is particularly intriguing to consider that the present situation in immunology may even have a similar solution if, following Carlo Rovelli's Relational interpretation of Quantum Mechanics, networks of interactions can be said

to define the essence of physical systems themselves rather than the pre-determined properties of discrete component entities *per se*.[382]

5.4. CTLA4 regulation of T cell trogocytosis provides a synthesis of divergent models

The paradigm of lymphocyte activation based on the presence or absence of co-stimulatory signaling as described under the PAMP/ "Danger" model has been criticized for implying little safeguard to prevent autoimmunity arising against abundant peripheral "self" antigens under inflammatory conditions when B7 levels are high.[383] This theoretical concern is particularly pressing in light of the observation that natural auto-reactive T cells have been found to be more prevalent in the periphery of healthy humans and mice than previously thought, thereby rendering the mechanistic basis for the paradigmatic 'innate control' of peripheral tolerance in adaptive immunity somewhat ambiguous.[247,254,384] Similarly, efforts to scale models of T cell differentiation developed in the context of the PAMP/Danger paradigm to the level of the system in order to robustly manipulate immune class regulation therapeutically have been largely unsuccessful (section 1.6.7).[258] Regulatory T cells (Treg), which dominantly prevent immunopathology and autoimmunity while also regulating B cell responses have therefore become increasingly central in models of adaptive immunity.[64] By considering the essential function of these cells as both instructed by and principally involved in regulating myeloid APCs, Treg maintain a loose preservation of the current theoretical framework of immune tolerance and class regulation under the PAMP/"Danger" paradigm. Intriguingly however, the precise mechanism for antigen-specific suppression by regulatory T cells remains similarly ambiguous despite decades of research.[64,172,266]

It is well-established however, that antigen-specific (and some non-specific) suppression by Treg is mediated by constitutively high expression of CTLA4, which is similarly required for

Treg control of B cell responses.[64,169] Additionally, the primary alternative models of peripheral tolerance and class regulation beyond the PAMP/Danger model (mediated by antigen linked cellular cooperation or "Quorum" among lymphocytes and/or the selective expansion of CD4+ T cell subsets during an immune response), equally point to CTLA4 control of cell proliferation as the regulator of this process.[272,385] CTLA4 and its elusive control of co-stimulatory signaling and T cell proliferation is therefore conspicuously positioned at the nexus of several longstanding mysteries in immunology concerning the basis of peripheral tolerance and immune class regulation. In this context it is tempting to speculate that clarification of the molecular mechanism by which CTLA4 achieves coordinated control of lymphocyte proliferative responses may provide a unifying solution.

To this end it is proposed that the acquisition and display of APC-derived antigen and co-stimulatory ligands among lymphocytes via trogocytosis may provide an optimal regulatory target for CTLA4 to exert its complex functions at the interface between innate and adaptive immunity ("self"/ non-self discrimination) and between single cells and the responding lymphocyte collective (immune class regulation). This possibility may have been neglected from wider consideration for two major reasons: (1) this process is expected to be largely indistinguishable from direct regulation of APCs by CTLA4 expressing T cells in vivo which occurs in parallel, and (2) recognition of trogocytosis as a fundamental feature of T cell activation seems to be in conflict with most major theories in adaptive immunity and cell biology (section 5.3). In terms of the PAMP / "Danger" paradigm specifically, a major conflict arises as a result of the observation that this process of pMHC transfer among T cells would seem to place the co-stimulatory context of antigen presentation beyond the control of PRRs expressed by myeloid dendritic cells responsible for initial antigen processing and ostensibly the foundational

interpretation of immunogenic context (Section 1.6.4-5).[25,264] The otherwise somewhat intuitive notion of an additional layer of collective processing of antigenic and co-stimulatory information by lymphocyte antigen-receptors through interclonal competition (and cooperation)[§§§] facilitated by ubiquitous acquisition and re-display of APC-derived surface molecules via trogocytosis, therefore represents a radical departure from the current theoretical foundations of adaptive immunity.

It is important to note however, that the modern paradigm is itself a radical shift from the first ~100 years of research and theory in immunology which was experimentally and conceptually centered on lymphocytes following the early success of Ehrlich's 'humoralist' approach over Metchnikov's innate description of immunity (Chapter 1: Introduction).[259] In this sense, the modern paradigm can be said to represent an enormously successful 'Metchnikovian' revival, which was largely inspired by Janeway's revolutionary impact and arguably extended farther than he himself intended via Matzinger's "Danger" model (Section 1.6.5-6).[24,25,259] In this context, it is suggested that a partial return to the historically central view of lymphocytes as the regulators and mediators of immune tolerance and class regulation, (through the acquisition and collective interpretation of APC-derived information) may allow for a synthesis of several otherwise contradictory and/or forgotten frameworks that nevertheless clearly account for non-overlapping aspects of adaptive immunity (i.e. the PAMP/"Danger" paradigm, lymphocyte 'Quorum' models, and Jerne's Network Theory). Encouragingly, it seems that this compromise can be achieved simply by recognition of leukocyte trogocytosis as an integral feature of immune responses, an interpretation which itself appears unequivocal (Section 5.3). In light of these

[§§§] This notion of competition / cooperation is more similar to Jerne's Network description in that I suggest that this process is primarily "self" focused and dynamically occurring to greater or lesser extents among clonal populations both in unimmunized homeostasis and in response to inflammation during an immune response.

considerations it is therefore proposed that for the persistent problems of immune tolerance and class regulation, a greater appreciation for this fundamental mechanism of collective control and its regulation by CTLA4 may provide a solution.

5.5. T cell trogocytosis overcomes the 'scarcity' and 'priming' problems

The primary theoretical opposition to 'Quorum' models of lymphocyte activation and class regulation consist of the so-called "scarcity" and "priming" problems. In Bretscher's modern formulation of the original "second signal" model, termed the "two step, two signal model of CD4+ T cell activation / inactivation," he has addressed both problems (section 5.6, 1.6.3) to provide an account for how rare specific CD4+ T cells could cooperate with a specific B cell in the induction of an immune response.[247] While these explanations alone seem to provide a more complete account of adaptive immune responses than the current understanding, it is intriguing to consider that this framework may become more efficient (and perhaps at once both more robust and less rigid) in the context of CD4+ T cell and B cell cooperation facilitated by trogocytosis.

In this view, following antibody receptor-mediated internalization, a specific B cell displaying processed peptide antigens in the context of MHC-II would no longer be required to engage in direct conjugation with an antigen specific CD4+ T cell, at least not initially during "step one." Instead trogocytosis might be expected to facilitate more decentralized proliferative expansion of increasingly specific CD4+ T cell clones as this B cell processed pMHC-II becomes dispersed and displayed locally among the network of responding lymphocytes through antigen specific as well as non-specific (i.e. CD28 / CD80/86 mediated) pMHC exchange and T-T antigen presentation. In this scenario, T cell clones would be envisaged to compete for antigen and associated APC-derived membrane fragments via otherwise counterintuitive synergistic

activities of TCR affinity and CD28 expression with CTLA4 (section 4.4.6). Indeed, such a process of antigen-specific competition has been shown to reduce effects of the highly heterogeneous precursor frequency of antigen-specific CD4+ T cells in the generation of T cell memory.[386] Similarly, antigen-specific competition was shown to promote independence of ongoing and initiating immune responses, a key feature of adaptive immune responses, allowing for example, efficient recruitment of naïve clones against antigens appearing late in infection or upon challenge from a secondary infection.[387] Intriguingly, and consistent with the model presented here, this study also showed that these competitive effects were independent of differential access to APCs.[387] While the authors did not hypothesize T cell trogocytosis and T-T antigen presentation may be involved in this competitive process, this was proposed previously in a similar model by Julie Helft and colleagues in 2008.[227,386,387]

This decentralized view of antigen-specific interactions mediated by trogocytosis would similarly apply to the recruitment of CD4+ T cell help to prime other CD4+ T cells as well as cross priming of cytotoxic CD8+ T cell responses. In this framework, associative antigen recognition and coherence among populations of activating lymphocytes can be established by numerous clones responding in parallel as an immunogenic stimulus enters the network and both specific and associated "bystander" antigens become dispersed alongside co-stimulatory ligands via competitive exchange and display among activating T cells. Similarly, the "scarcity problem" has also been noted in previous models attempting to address the longstanding ambiguity around the conditional requirement for CD4+ T cell help in priming cytotoxic CD8 T cells.[299] Specifically, early studies maintained an "absolute requirement" for the formation of a ternary cell complex between an antigen-bearing DC, an antigen-specific CD4+ T cell, and a specific CD8+ T cell.[388,389] While later publications have proposed that CD8+ T cell-cross priming can

occur sequentially, (i.e. through subsequent CD8-DC contact following CD4+ T cell 'licensing' of DCs), a substantial "scarcity problem" seems to remain to facilitate rapid CD8+ T cell responses.[390] In support of the concept that trogocytosis makes this process far more efficient, CD8+ cross-priming by CD4+ T cells bearing DC-derived membrane fragments displaying MHC-I was demonstrated by Xiang and colleagues in 2005 and subsequently visualized in vivo.[228,299,389,391] However similar to related publications on the functional implications of DC-dressed "T-APCs", this phenomenon has not been incorporated into mainstream models of CD8+ T cell cross-priming.

5.6. CTLA4 model overview: original and modern "second signals" of T cell activation

The basis for peripheral tolerance and the elusive mechanism of CTLA4 regulatory activity are conspicuously linked at the level of CD4+ T cell activation, priming, and suppressive function. It seems plausible that the reason the fundamental importance of the distinction between the contemporary and original conceptions of the essential "second signal" of CD4+ T cell activation has been so subtle as to be easily ignored lies in the fact that Bretscher and Cohn's "second signal" (i.e. presence of antigen-linked "quorum" among lymphocytes) can indeed be induced by co-stimulation and is regulated by the opposing activities of CD28 and CTLA4.[247,249]

Similarly, in this perspective, the mechanism of CTLA4 regulatory function likely continues to defy mechanistic understanding as a result of the fact that it has primarily been examined within the framework of the modern molecular "second signal", (i.e. targeting CD80/86 at the surface of dendritic cells). This mechanistic focus follows the so-called "Copernican" turn under the "Danger" paradigm away from lymphocytes and toward innate myeloid APCs at the basis of adaptive immunity (section 5.4).[262] By placing innate control of co-stimulatory signaling by APCs at the theoretical foundations of peripheral tolerance and immune

class regulation, the modern framework implicitly excludes the possibility for the essential function of CTLA4 to be achieved by acting on the responding lymphocyte collective more directly. This emphasis may have been misdirected in part by the subtle nature of membrane transfer via trogocytosis however, which blurs the distinction between T cells and APCs in vivo. In this view, in contrast to antagonizing the modern molecular "second signal" of T cell activation as generally considered, CTLA4 appears instead to be involved in determining the presence or absence of Bretscher and Cohn's cellular "second signal" (i.e. antigen-linked cellular cooperation or "quorum" among lymphocytes) thereby providing an alternative basis for "self" / non-self discrimination and immune class regulation.[247,253,258,273] This is proposed to occur by CTLA4 acting at the interface between single cells and the local lymphocyte population to coordinate interclonal competition and cooperation through TCR-dependent integration of CD80/86 levels that are exogenously acquired and endogenously expressed upon contact with activating APCs. This view leads to a model in which CTLA4 regulatory function may be more completely understood by considering its ability to regulate the duration and inflammatory context in which both antigen and co-stimulatory information provided by APCs are "reflected" among the network of responding lymphocytes via trogocytosis.

5.7. CTLA4 function in regulatory T cells

A 'Quorum' model of CTLA4 function involving cell intrinsic regulation of trogocytosis may help account for some of its elusive regulatory effects at the network level (Section 5.2). One particularly prominent example would be in relation to the longstanding enigma regarding the ability of CTLA4+ Treg to rescue the lethal autoimmune phenotype observed in CTLA4 deficiency in an apparently purely extrinsic manner in mixed bone marrow (BM) chimera mice. In these animals, the presence of CTLA4+ Treg has been shown to be sufficient to dominantly

control auto-reactive CTLA4-deficient T cells in *trans* to restore immune homeostasis.[160] Importantly, although FOXP3+/CD25+ regulatory T cells can inhibit immune responses via multiple mechanisms, the essential role for CTLA4 in the suppressive function of these cells has been clearly demonstrated.[169] For example, while reconstitution of CTLA4-deficient mice with CTLA4+ (low) (FOXP3-/CD25-) conventional CD4+ T cells (Tconv) in mixed BM chimeras is only able to prevent lethal autoimmunity for a few weeks, forced overexpression of CTLA4 in Tconv in these animals has been shown to be sufficient to prolong life >12 months.[181,392] These results are consistent with other reports suggesting that CTLA4 is indeed responsible for dominant control of auto-reactivity by Treg and perhaps for mediating antigen-specific suppression by non-Treg suppressive cells as well (section 4.4.2).[53,171,176,350] Despite several decades of study however the basis for antigen-specific suppression by Treg and the role for CTLA4 in this process remains mechanistically ambiguous.[172]

Interestingly, one of the pioneers of suppressor T cell biology, Professor Ethan Shevach and colleagues recently proposed a trogocytosis-based mechanism for antigen-specific suppression by Treg in which capture of cognate pMHC was shown to be responsible for mediating specific suppression.[393] This model is well-supported by previous reports of TCR-specific engagement of pMHC leading to antigen depletion via trogocytosis, a process termed 'antigen grazing.'[394] However, while this process of antigen-specific depletion may be more efficient as Treg reach high densities as suggested in the original 'antigen grazing' model, this 'grazing' mechanism may over-emphasize the specificity of TCR-mediated trogocytosis considering that trogocytosis is known to be invariably associated with bystander pMHC acquisition as well.[131,195,217]

Importantly, this non-specific antigen transfer is likely to be particularly prevalent in the

presence of high levels of B7 molecules, as shown here (Chapter 4) and previously, where non-specific B7 / pMHC trogocytosis efficiently occurs via CD28 (Section 4.4.3).[218] Additionally, the reported lack of a CTLA4-mediated effect in Shevach's model seems contrary to the extensive support in the literature for a central role for CTLA4 in this phenomenon, as summarized above.[169,171] Finally, a significant limitation of this model is the apparent requirement for relatively low levels of target pMHC or high numbers of specific Treg to achieve sufficient TCR-specific pMHC depletion via trogocytosis. These requirements appear unlikely to be met for many relevant peripheral "self" antigens known to be targeted for suppression by Treg, which are generally highly abundant. Addressing this last criticism in a subsequent review article, the authors suggested that CTLA4-dependent depletion of B7 from APCs may operate as an auxiliary mechanism of suppression in these contexts where specific pMHC depletion via trogocytosis is insufficient.[395] While this seems plausible, this version of Shevach's model essentially represents a hybrid TCR trogocytosis / CTLA4 *trans*-endocytosis model of suppression that arguably resembles the current ambiguity where an antigen-specific mechanism for suppression mediated by CTLA4 in Treg (and a unifying mechanism for CTLA4 function more broadly) seems lacking. In this respect, a slight modification of the authors' proposed model to include cell intrinsic B7 depletion by CTLA4 following specific pMHC acquisition and subsequent tolerogenic antigen presentation via T-T interactions might provide a more unifying solution. Indeed, in the aforementioned review article the Shevach and co-author Akkaya suggest that acquired pMHC may be re-displayed by Treg to induce suppression in subsequent T-T interactions.[395] The data presented here, wherein high levels of CTLA4 are able to specifically deplete B7 from co-acquired pMHC at the cell surface in a TCR-dependent manner, seems to provide support for this latter scenario.

Notably, while preference toward suppression of specific antigens may be biased by TCR-specific recognition during the acquisition step via trogocytosis as suggested by Akkaya and Shevach, this acquisition-focused mechanism for suppression might be expected to lead to Treg becoming dysfunctional under inflammatory conditions where B7 ligands are highly abundant.[393] In these settings, which may be among the most relevant in vivo, Treg have been reported to accumulate antigen non-specifically via trogocytosis, findings which are consistent with the CD28-mediated B7 / pMHC acquisition mechanism shown here.[351] Although Haastert and colleagues observed this phenomenon under autoimmune conditions where Treg may have indeed been partly dysfunctional, Bahcheli and Piccirillo also found that Treg activation was associated with trogocytosis.[396] In this setting, in vitro suppression by Treg was actually positively correlated with the magnitude of trogocytosis and remarkably, the in vitro suppressive effect was similarly shown to occur in the absence of APCs via T-T interactions.[396] While the ability of in vitro suppressor assays to examine the relevant in vivo function of Treg has been questioned, this model is consistent with previous reports showing the positive correlation of T cell trogocytosis with activation of other T cell subsets including CD4+ and CD8+ T cells.[131,172,372] Overall, the reported association of non-specific antigen accumulation with suppressive function may be difficult to reconcile with Shevach's more precise mechanism of antigen-specific suppression mediated by TCR / pMHC recognition-induced trogocytosis.[351,396]

In this context it is tempting to speculate that rather than in the acquisition step, antigen-specific suppression may instead occur in the second step via TCR-mediated focal polarization of CTLA4 to sites of recognized antigens and associated B7 depletion. In this view of "*cis*" regulation by Treg expressed CTLA4, it seems conceivable that even in the presence of high levels of non-specific pMHC transfer associated with high CD28 / B7 engagement under

inflammatory conditions, the reportedly promiscuously binding and "self"-skewed TCR repertoire of Treg may even facilitate preferential B7 depletion from acquired "self" pMHC while preserving B7 associated with non-"self" pMHC at the cell surface.[395] Interestingly however, in either case, because the CTLA4-mediated intrinsic depletion of acquired B7 shown here was accelerated by but did not strictly require TCR engagement, this non-specific accumulation of 'bystander' pMHC on the Treg surface may additionally serve as a beneficial means to facilitate more gradual suppression of even unrecognized antigens through CTLA4-mediated B7 depletion over time. This process would be consistent with the reported role of CTLA4 in mediating broad non-specific 'bystander' suppression by Treg as well.[397,398]

Importantly, irrespective of the efficiency of a more speculative "self" / non-self 'filtering' mechanism via CTLA4 *cis* regulation of B7 levels at the Treg cell surface described above, in the context of the "Quorum" framework described in section 5.2, complete accuracy by Treg is likely much less strictly required to achieve effective suppression. In this view, Treg are responsible for preventing 'Quorum' of auto-reactive T cells, not for depleting all potentially autoreactive "self" pMHC and associated B7 individually. In this context it again may actually prove beneficial for Treg to engage in non-specific promiscuous sampling and display of local pMHC via trogocytosis, which are strongly numerically biased toward "self" in abundance.

An additional advantage of this perspective is the consideration that Treg may equally effectively utilize CTLA4 to suppress responding auto-reactive T cells in *trans*. As these cells are also known to acquire and display both specific and associated pMHC via TCR / CD28 engagement, auto-reactive T cells can therefore be expected to be particularly enriched in "self" pMHC at the cell surface. In this way, auto-reactive 'T-APCs' displaying the evidence of their transgression in the form of excess acquired "self" pMHC and elevated B7 may similarly be

targeted by Treg. This could occur through 'stealing' of displayed "self" pMHC via the high levels of CTLA4 expressed by Treg followed by subsequent tolerogenic re-display as suggested above (section 4.4.6), as well as by direct TCR recognition and targeted suppression in *trans* analogous to current models of APC de-commissioning and tolerogenic conversion by Treg.[399,400] Notably, although the direct de-commissioning of an APC is likely to be a highly efficient route for Treg-mediated suppression, de-commissioning of associated auto-reactive 'T-APCs', or their direct conversion to a suppressive phenotype by Treg acting via T-T contacts might be expected to be largely indistinguishable processes in vivo which are likely synergistic.

5.8. On *cis* vs. *trans* B7 depletion by CTLA4

Functional *cis*-interactions between transmembrane receptors and ligands is an emerging theme in immune cell signaling.[197–200] While typically described in the context of endogenously co-expressed ligand / receptor pairs positioned adjacently on a planar membrane interface, the ubiquitously observed phenomenon of functional plasma membrane exchange among immune cells via trogocytosis, and through various forms of extracellular vesicles, is likely to facilitate more complex regulatory interactions and sustained signaling in "*cis*."[131,195,221,225,401] Despite being largely experimentally intractable to detect or examine in vivo, this expanded set of *cis* interactions –most broadly defined as ligand / receptor interactions occurring on or within single cells—likely facilitates more information-dense interactions among responding lymphocytes. Notably, collective behaviors of complex systems are principally determined by the nature of local interactions among individuals.[402] I therefore hypothesized that collective information processing via membrane exchange, and its regulation by CTLA4, may represent an underappreciated element of system level phenomena in adaptive immunity.

Importantly, the benefit of a cell intrinsic CTLA4 pathway is not that *cis* CD80/86 depletion is necessarily more substantial than *trans* depletion. One of the key implications of recognition of trogocytosis as a fundamental feature of lymphocyte activation is that, the line between "*cis*" and "*trans*" (and between activating APC and responding T cells) appears to become intriguingly blurred within clusters of activating lymphocytes during an immune response. Within this complex environment, a *cis* pathway represents a special case where trogocytosed CD80/86 can be experimentally distinguished as a regulatory target of CTLA4, as distinct from *trans* depletion of CD80/86 expressed by APCs or by T cells themselves. By allowing fine TCR-mediated control of co-stimulatory ligands in the context of acquired antigen, a *cis* depletion pathway also leads to some unique predictions in support of the concept that this easily overlooked process may allow CTLA4 to more efficiently regulate interclonal competition and cooperation within networks of responding lymphocytes. Indeed, analogous to becoming more experimentally tractable for experimentalists, a *cis* pathway may similarly aid activating T cells themselves in maintaining cell autonomy in the midst of extensive membrane exchange by offering a means for fine control and integration of both intrinsic and extrinsically derived co-stimulatory signals via TCR-dependent CTLA4 induction and polarization (Section 4.4.5-6).

Overall, this integrative process is likely occurring in *cis* and in *trans* on both endogenously expressed and exogenously acquired CD80/86 ligands displayed alongside endogenously processed and exogenously acquired pMHC. The relative contribution of each pathway is expected to depend on the highly interconnected variables of relative CTLA4 / CD28 expression, levels of antigen and co-stimulatory ligands in the local environment, and the strength of TCR signaling (Section 4.4.5-6).

5.9. Collective Behavior in lymphocytes, social insects, and human societies

Density dependent deprivation of CD28 signaling by increasing activity of CTLA4 has recently been shown to control CD8+ cell expansion in a population intrinsic manner analogous to quorum regulation**** by bacteria.[91] This work provides strong support for the notion that key elements of adaptive immunity can be mediated through collective self-regulation by lymphocytes. While robust control of lymphocyte proliferative expansion and contraction is arguably the key function of CTLA4, analogy with bacterial quorum regulation in response to population density may neglect potential for more complex computations performed by networks of responding lymphocytes in a CTLA4-dependent manner. Despite unifying principles often observed in the collective behaviors of complex systems across domains of life, the emergence of a dynamic sense of "self", learning, and memory within adaptive immune networks appears to strain the bacterial analogy. A perhaps more fitting parallel has long been recognized in the similar processes which emerge from neural networks as a result of information exchange among densely interconnected systems of neurons and glia in the human brain.[276] In this section I will argue that the mechanistic basis for how similarly 'information-dense' phenomena of "self" awareness, learning, and memory can be achieved in the inherently diffuse adaptive immune system represents one of the last great mysteries of biology and propose a tentative solution. I will suggest that despite extensive parallels with bacterial quorum sensing, this problem is rather on the order of, and of the same nature as, the so-called "hard" problem of consciousness.

The study of cellular and animal collective behaviors has shown that within complex systems lacking central control, highly robust and self-regulating network behaviors can emerge which are determined by local interactions among individuals.[403] The study of these phenomena,

**** "Quorum regulation" generally refers to coordinated regulation of gene expression and emergence of novel functions upon reaching a minimal "quorum" population density, detected on the basis of secreted soluble factors.[459]

from collective movement of birds and schools of fish, to network properties of the nervous system and metastatic cancers, has revealed that local regulatory principles observed in one system are often generally scalable and applicable to others.[369–371,403] For example, principles which emerged from the study of light detection and predator evasion in schools of fish have been successfully applied to the modeling of democratic decision making in human societies, revealing otherwise unexpected predictions. In this context, the study of collective behavior among lymphocytes may be further illuminated by exploring additional examples of decentralized network control provided by nature, particularly those composed of highly sophisticated individuals. One example which may be particularly revealing is that of social insects.[87]

5.9.1 Social insects, Lymphopenia-induced autoimmunity, and the TCR affinity paradox

Several decades of work by Deborah Gordon has revealed a remarkable mechanism for task allocation by harvester ants. Similar to other ant species, the behavior of individuals within these colonies is observed to be restricted toward carrying out a specific task according to the dynamic needs of the colony.[404] Categories of tasks identified by Gordon include: scouts, foragers, nest maintenance / repair workers, "midden" workers, and a 'reserve' group of inactive relatively sedentary ants* (Note on sedentary ants below). Using harvester ant task allocation as a model for collective decision-making, Gordon noted high levels of plasticity in response to specific environmental perturbations.[404] For example, a nest disturbance rapidly induces allocation of more nest maintenance workers and equally, the addition of food outside the nest induces larger numbers of foragers, etc.[404] Remarkably, Gordon demonstrated a relatively simple mechanism for the determination of task allocation, which occurs at the level of the colony yet was entirely dependent upon local interactions among responding ants. Specifically, these

coordinated behaviors were found to not be dependent on pheromones or scent trails but rather mediated through antennal contacts during local interactions in which ants were found to display and exchange cuticular hydrocarbons which accumulate on ants and convey information about the recent history of individuals (i.e. regarding the local environment and what task each ant had previously been engaged in).[404]

As general principles of collective behavior have frequently been observed to be applicable across divergent systems, this phenomenon of collective decision-making achieved at the level of the colony through accumulation and exchange of information in the form of cuticular hydrocarbons among ants provides a clear proof of principle for the potential for coordination of collective behaviors among lymphocytes via trogocytosis in adaptive immunity. Such a mechanism may also provide more suitable framework to account for one of the more intractable longstanding mysteries of T cell signaling, the so-called "low affinity / high sensitivity" paradox of antigen recognition by the TCR. Briefly, it has been observed that T cells can induce a response in the presence of 0.01% foreign pMHC.[2] How this level of sensitivity and specificity could be achieved despite the relatively low affinity and fast off-rate of TCR/pMHC binding has motivated extensive characterization of the TCR/MHC signaling complex to the level of single molecules yet continues to defy mechanistic explanation.[405,406] Consideration that an additional layer of computation occurs at the level of the T cell collective may offer a cellular solution to this molecular conundrum that similarly incorporates the otherwise unsettling heterogeneity of T cell responses which have been recently observed using single cell technologies.[272] Such a solution would be similar to that employed by schools of fish to avoid predation, where it has been demonstrated that it is the collective but not necessarily individual fish which coordinates extremely robust movements to evade attack – indeed, in this and other

examples the stochastic variation of responses actually represents an essential feature of the robustness of the collective.[371]

*Note on sedentary ants: Intriguingly, it is tempting to speculate that this population of sedentary ants may have some conceptual relevance to the poorly understood phenomenon of the induction of autoimmunity in the context of lymphopenia (e.g. such as that observed in SARS2-associated immunopathology) (Section 5.13).[407] Specifically, it has been similarly shown in both schooling fish and in human societies that a 'buffer' population of disinterested individuals tends to reduce "extremism" (in this case, i.e. autoimmunity) and promote more robust democratic decision-making by the collective.[408]

5.9.2 Reconciling reductionism with T cell trogocytosis and CTLA4 function

Trogocytosis appears to offer a spectacularly odd example of a molecular "physicalization" of information exchange and integration between the individual and the collective. This section will attempt to reconcile this apparent chaotic loss of individual cell identity with the clear success of reductionist approaches in describing adaptive immunity by analogy with behavioral trends in human societies. In these terms, in both immunology and consumer marketing surveillance the primary goal is to, understand, predict, and manipulate trends from the level of individual members of a population to that of the entire collective. In this pursuit, consideration of trogocytosis in both modeling approaches and clinical interventions in immunology may offer unique advantages to gain novel insights and to perform targeted perturbations of system level function. Such considerations might be expected to provide an analogous level of additional information as would be obtained for example in the study of an emerging fashion trend if consumer surveillance became sufficiently advanced to include individual members of a human population transiently exchanging fashion accessories during

interactions with near-neighbors, subsequently receiving local positive or negative reinforcement, and ultimately joining, exiting, or actively opposing an emerging trend.

This unusual analogy highlights that networks composed of sophisticated individuals can engage in unexpected violations of reductionist principles that become particularly prominent at the smallest scales and at the origins or "edges" of otherwise more homogenous population trends. It further serves as a *reductio ad absurdum* against the so-called "priming" problem in "Quorum" models of CD4+ T cell activation (section 1.6.3) by highlighting the decentralized manner in which information can move through complex systems to initiate self-organizing collective behaviors in parallel (section 5.5). For example, the search for the origin of a particular fashion trend would not realistically be expected to encounter a "priming problem" of the first participant as it is more intuitively understood that such phenomena emerge collectively.

Importantly, this conceptually and experimentally intractable loss of autonomy in decision-making by individuals when joining or opposing emerging population trends is not mutually exclusive with the ability for robust reductionist examination of such trends once formed. This is exemplified by the success of flow cytometry and of single cell technologies in the description of immune responses while preserving potential for the exchange of exogenous surface molecules via trogocytosis to serve fundamental importance in information exchange and processing by the network. Indeed, this process seems to represent a rare example of collective behavior taking physical form at the level of the individual, a phenomenon which is not theoretically necessary but nevertheless exists and may therefore provide an invaluable source of additional insight into network behavior and its control. In this context, the ability for CTLA4 to exert regulatory function at this reflective interface may offer a mechanistic and molecular route toward a more cellular and system level perspective of the nature of the adaptive immune

system, redefined in terms of the collective behaviors of lymphocytes.

5.10. Network Theory and the evolutionary origins of adaptive immunity

Niels Jerne's Network theory, for which he was awarded the Nobel Prize in 1984, is arguably the most complete attempt to construct a general theory of adaptive immunity.[242] Network Theory provides an elegant albeit unsettlingly sophisticated description of how the universality of antigen receptor diversity could manifest an ability for the adaptive immune system to essentially become internally self-aware in order to achieve learning, memory, and homeostasis via internally reflective network interactions. In his construction of Network Theory Jerne recognized the power of the enormous repertoire of antigen receptor diversity, the requirement for the adaptive immune system to achieve a dynamic balance of "self"-focused regulation to avoid catastrophic autoimmunity, and its inherent capacity to achieve this via interconnected adaptive recognition of somatically generated variable regions themselves (i.e. formation of idiotypic networks) (section 1.6.8).

Interestingly, despite its ability to describe how, like the brain, the adaptive immune system creates an internal image of "self" to interpret non-self and establish memory in network-encoded pattern, Jerne's theory does not easily address how such a complex system could have first arisen in early vertebrate evolution. This was unlikely a pressing concern during the time that Network Theory was developed however, as until fairly recently it was widely held that the key innovation for the vertebrate adaptive immune system emerged in a single evolutionary "big bang" event. This event was considered to be the invasion of the Rag transposon into the immunoglobulin locus of an ancestral jawed vertebrate which was responsible for conferring the enzymatic machinery to enable rearrangement of antigen receptor genes in the process known as somatic Generation Of Diversity (somatic GOD).[409] However, this solution recently became

untenable upon the discovery of a completely distinct mechanism of somatic GOD in primitive jawless vertebrates (agnathans).[410] Intriguingly, although utilizing different machinery and alternative receptors, the agnathan adaptive immune system possesses similar organizational features, including 3 lineages of lymphoid cells analogous to T cells, B cells, as well as innate cells similar to gamma delta T cells or NK cells.[411] The identification of a conserved genetic program to give rise to these cells has demonstrated that these features emerged in a common ancestor of jawed vertebrates and agnathans, prior to the somatic GOD events.[412] In light of the apparent absence of adaptive immune systems in all other metazoans, including sophisticated invertebrate species, skepticism has been raised that pathogens alone could have provided sufficient selective pressure to give rise to this complex network of lymphocytes and the subsequent emergence of mechanisms for somatic GOD twice in early vertebrates, rendering the origins of adaptive immunity highly enigmatic.[367,413,414]

A major strength of Janeway's paradigm shift over Jerne's Network theory was the facile extension of the PAMP hypothesis to the evolutionary origins of immunity.[259] It would therefore seem that if the proposal presented here is to represent any further clarification in terms of Network theory, it should similarly extend to the origins of the system. To this end a scenario is proposed in which a more primitive form of 'idiotypic-like' network interactions may have arose via trogocytosis in early vertebrates to facilitate specialization of lymphocyte subsets and dominant mechanisms of "self" tolerance, both previously identified as major evolutionary bottlenecks prerequisite for the emergence of somatic GOD and adaptive immunity.[367]

While it seems probable that trogocytosis plays a role facilitating 'true' idiotypic interactions through dispersal and presentation of pMHC bearing T cell idiotopes (e.g. to potentially induce clone-specific regulatory 'suppressor' cells during clonal expansion, or to

coordinate interclonal repertoire 'training' in thymic development) (Section 1.6.8 and 4.4.3), it is tempting to speculate that trogocytosis may also be itself sufficient to independently produce 'idiotypic-like' interactions.[293] Specifically, it seems that the acquisition and re-display of both TCR-specific and associated 'bystander' peptide antigens during T cell activation inherently produces its own form of "internal image" of activating stimuli. This process thereby incorporates antigen and tissue context and could be expected to produce a network of cooperative, competitive, and suppressive (regulatory) T-T interactions for each T cell activation event, seemingly resembling a more primordial form of idiotypic network interactions. Indeed, this sacrifice of cell-autonomy by immune cells via trogocytosis appears to provide a relatively simple mechanism to facilitate rapid communication, network integration, and increased capacity for "self"-focused regulation at the system level. Importantly, this process would be expected to occur even prior to the generation of antigen receptor diversity by early lymphocytes bearing non-clonally distributed polymorphic antigen receptors. In this way, trogocytosis may have enabled the network of varying specificities of the ancestral adaptive immune system to begin to dynamically 'see itself' to facilitate the evolution of more complex auto-regulatory control.

5.10.1 Network hypothesis for the origins of adaptive immunity

Trogocytosis is a phylogenetically ancient phenomenon that is conserved in eukaryotes, being first discovered in parasitic amoeba (section 1.4).[213] As the origins of trogocytosis therefore predate the bifurcation of the myeloid and lymphoid lineages and the somatic GOD events, it seems plausible to extend the notion of trogocytosis-mediated network interactions to attempt to provide insights into the origins of adaptive immunity. Indeed, it was previously been proposed by Joly and Hudrisier that trogocytosis may have played a fundamental role in facilitating the evolution of the first immune cells in primordial multicellular collectives:[192]

> *"trogocytosis may have developed initially as a symbiotic arrangement: leukocytes may be 'feeding' off other cell types in return for undertaking the defense of the organism against pathogens. Because lipids are the most energetically demanding components to generate, fragments of plasma membrane acquired by lymphocytes could contribute substantially to their metabolic balance, thereby increasing their capacity to proliferate."*

An extension of this concept is here proposed that trogocytosis became further specialized in early vertebrates to facilitate the formation of an inherently "self"-examining symbiotic relationship between myeloid cells and lymphocytes. Analogous to complex system-level decision making and task allocation observed in colonies of harvester ants via contact-dependent exchange of cuticular hydrocarbons (section 5.9.1), trogocytosis may have enabled increasingly information-dense interactions among immune cell networks to facilitate functional specialization and collective decision-making by lymphocytes, even prior to the emergence of antigen receptor diversity. This model is consistent with the otherwise difficult to explain existence of a common ancestor of jawed vertebrates and agnathans in which lymphocyte subsets arose prior to the somatic GOD events.[412,415] Additionally, fratricide and cannibalism among early lymphocyte subsets displaying specific and co-acquired antigens seems intrinsically capable of producing an internally balanced system, ultimately favoring the emergence of more complex regulatory machinery for mediating dominant forms of immune tolerance, such as the CTLA4 pathway.

In terms of the traditional pathogen-oriented view of the origins of adaptive immunity, competition for consumption of bio-energetically "expensive" membrane lipids among lymphocytes may have provided a relatively simple mechanism to facilitate lymphocyte proliferation-driven amplification of innate immune responses initiated by phagocytic myeloid cells acting as APCs. Importantly, by acquisition and re-display of both foreign and numerically abundant co-acquired "self" antigens, such a system might be expected to have an inherent

tendency for self-restraint mediated by fratricidal (e.g. CD8 suppressor cells) and/or suppressive regulatory (FOXP3+ CD4) interactions. However, in terms of the origins of immune tolerance, it is intriguing to consider that in contrast to the traditional pathogen-oriented view, such a system engaged in intercellular communication and integration via trogocytosis would likely be primarily "self"-focused in both its origins and function. Specifically, this would be expected due to the extreme numerical abundance of predominately "self" antigens displayed by phagocytes engaged in tissue homeostasis. In this view, 'niche' competition among primordial lymphocyte collectives for the complete diversity of accessible "self" antigens may have represented a major driver for antigen receptor diversity rather than defense against pathogens, as seemingly predicted by Jerne (see epigraph).

In the context of this relatively simple mechanism for the generation of a "self"-reflective network, multiple rounds of whole genome duplication and increasing body size along with more complex predation behaviors during early vertebrate evolution likely served to increase the scope of "self" antigen diversity while favoring the emergence of increasingly sophisticated network behavior and ultimately, clonal immune responses. In this primitive idiotypic network view of the origins of adaptive immunity in jawed vertebrates, it is speculated that non-specific antigen transfer and display mediated by cellular adhesion molecules, non-clonally distributed antigen receptors, and the MHC / B7 / CD28 / CTLA4 system arose first and subsequently facilitated the emergence of RAG-mediated somatic recombination mechanisms for generating variable antigen receptors.

5.10.2 Specific role for CTLA4 in a direct synthesis of 'Quorum' models with Network Theory

In a 'Quorum' or Network theory framework respectively, either individual clonal specificities or the idiotypic network interactions between them can be considered to represent the essential 'nodes' of the adaptive immune system. Information exchange between this nodal network is collectively responsible for encoding complex "self" awareness and memory at the level of the system in a manner similar to the emergence of cognition and memory from neural networks. In this context it is intriguing to consider that in both cases, (i.e. for either network 'nodes' as clonal specificities themselves or as idiotypic interactions), binding-dependent trogocytosis of specific peptide epitopes or TCR idiotopes respectively, and the associated co-transfer of linked 'bystander' antigens would seem to produce a characteristic contextual 'blurring' around the edge of each node such that it might begin to partly merge with nearby (i.e. 'idiotypically cross-reactive') network nodes that share this expanded set of 'idiotypic'-like specificities mediated by binding-dependent trogocytosis. In this view, an ancestral form of 'idiotypic'-like interactions via trogocytosis seems to inevitably remain and enshroud any emergent more sophisticated idiotypic network,[††††] potentially conferring additional robustness and perhaps contributing to its difficulty to be detected and manipulated.

This perspective may restore some diffuse biological 'messiness' to Network theory and overcome intuitive aversion of cell biologists to the more mathematically precise 'symmetric' Idiotypic Network models proposed previously.[416] Notably, one potential paradox that emerges from any such highly interconnected "self"-focused idiotypic networks is excessively stable equilibria resulting in a loss of responsiveness to external antigen.[417] An elegant mathematical

[††††] The goal of this argument is to illustrate that this 'idiotypic-like' network mediated by trogocytosis remains, independently of the prevalence of 'true' idiotypic interactions.

and biological solution to this fundamental issue in modeling approaches is the incorporation of a highly destabilizing exponential growth term to reflect cell proliferation.[417] In this context, CTLA4-dependent regulation of co-stimulation and cell proliferation appears to represent a particularly potent mode of network control.

5.10.3 Summary and implications in evolutionary theory and regenerative medicine

Representing a uniquely striking manifestation of the blurring between the individual and the collective, examination of intercellular communication via trogocytosis may provide new insights into the evolution and regulation of collective information processing in complex systems. This section presented a model in which trogocytosis is proposed to have contributed to the generation of a competitive and cooperative "self" focused interaction network capable of producing dominant mechanisms of immune tolerance to facilitate the emergence of mechanisms of somatic GOD twice in early vertebrates. Although speculative, this view may have some potential to reveal fundamental features of adaptive immune system function. For example, this perspective highlights CD28/B7 interactions as a potentially ancestral mediator of antigen transfer and emphasizes its role in competition among lymphocytes via exchange of membrane lipids. This view would lead to the prediction that the currently neglected observations of non-specific pMHC and lipid acquisition via CD28/B7 engagement such as those described in Chapter 4 (section 4.4.3) may be of fundamental importance in the control of T cell activation and differentiation (Section 5.11). While otherwise difficult to test experimentally, this model predicts that widespread functional trogocytosis is likely to be similarly observed in the adaptive immune system of jawless vertebrates.

Although the "self"-oriented view of the origins of adaptive immunity described above might be criticized as Lamarckian, this may be another feature that this network process shares

with social insects. For example, in the evolution of collective behaviors in harvester ants, selection is said to occur at level of the colony.[418] This definition appears to include the effectiveness of emergent cooperative solutions arising from unique combinations of variation among individuals over time. In this context it is intriguing to consider more broadly the potential for similar processes in which selection may act on irreducible "emergence" rather than individual system components. This perspective may be particularly essential for understanding the case of early vertebrates as Darwinian evolution first began to essentially re-invent itself internally in the form of somatic GOD and clonal immune responses.[367] In this view, it seems conceivable for a dynamic interplay to emerge between genetic variation at the population level and selective pressure within the early immune system itself. Specifically, as ant colonies are selected for their ability to most efficiently derive emergent solutions to problems of resource allocation in changing environments, the adaptive immune system may have been similarly selected for its solutions to the dynamic regulation of tissue homeostasis and inflammation.‡‡‡‡ This selective process occurring within populations of early vertebrates might be expected to more efficiently facilitate the emergence of intricate network solutions such as the domestication of transposable elements for the production of variable antigen receptors.

The most far-reaching implication of this perspective is that a relatively simple integrative process by single cells may have facilitated the formation of a sufficiently densely interconnected network to result in the eventual emergence of a unique form of self-awareness.[367] This phenomenon seems to provide a striking demonstration that this type of awareness, involving a dynamic sense of self, learning, and memory, is implicit in highly

‡‡‡‡ In this analogy each individual in a population of early vertebrates could be considered to represent a 'colony' of leukocytes which are selected for emergent solutions over the lifetime of the organism. Importantly, the effectiveness of system-level outputs is expected to vary according to the unique combinations of variation comprising the collective while these emergent outputs are nevertheless irreducible to genetic variation of individual components themselves.

sophisticated network arrangements of matter. More speculatively, it seems that such emergent systems of collective control (from gene regulatory networks, nervous systems, or complex behaviors in social insects) may have an underappreciated Lamarckian tendency for enhancing their own emergence and sophistication in a manner that scales with the interrelated variables of environmental niche and network complexity –and the ability for natural variation in these systems to give rise to more or less optimal 'solutions' among members of the population.[419] In this sense, in contrast to more conventional Darwinian evolutionary notions of blind force (i.e. random mutation and natural selection over vast timescales), this example seems to provide an unexpected source of optimism for a more cooperative image of network evolution in the origins and development of life. From the scale of single molecules (e.g. effector proteins engaging in collective 'swarming' behavior within biomolecular condensates to derive emergent signaling outputs (Chapter 3)), to cooperation of cellular collectives, these processes appear to provide an underappreciated unit of selection which may produce an inherent tendency toward a kind of emergent 'intelligent' optimization of complex systems with their environment.

Regardless of the precise evolutionary mechanisms involved however, it is tempting to speculate that the additional regulatory capacity for control of tissue homeostasis and regeneration by primitive 'regulatory' lymphocytes may have been involved in alleviating selective pressure for lifelong maintenance of greater degrees of plasticity in cell identity that may have otherwise been required to facilitate robust wound healing in early vertebrates. In this context it seems conceivable that a novel mechanism for tissue homeostasis and regeneration regulated by lymphocytes may have been a significant factor in the emergence of more restricted cellular differentiation programs in the development of more sophisticated body plans during the adaptive radiation of vertebrates in the Cambrian 'explosion.' This model would thereby provide

an alternative "self"-focused account (as opposed to explanations based on enhanced defense against pathogens) for the conspicuously concurrent emergence of adaptive immunity and the rapidly expanded phenotypic diversity of vertebrates –two pivotal events in the history of life on Earth long been hypothesized to be somehow related.[367] Similarly, this ancestral evolutionary tradeoff to regulatory T cells in controlling tissue regeneration and homeostasis, and its further sophistication in tetrapods (particularly in mammals), may be somehow related the progressively limited regenerative capacity of these animals as well as the recently discovered role for Treg in its regulation.[270,420,421] Although highly speculative, this alternative perspective of the evolutionary origins and function of Treg may therefore have significant implications for the advancement of regenerative therapies using these cells.

Whether trogocytosis functioned to produce primitive "idiotypic"-like interactions to facilitate the emergence of complex collective behaviors and mechanisms for dominant "self" tolerance to favor the somatic GOD events may remain speculative. However, such trogocytosis-mediated interactions indeed appear to exist and produce dynamic, "self"-focused 'internal images' of immunogenic stimuli in the adaptive immune systems of modern vertebrates. This perspective may therefore prove useful in unifying Network Theory with second signal models of T cell activation to stimulate further progress toward a more general theory of adaptive immunity.

5.11. T Cell Differentiation and Memory

While linear models of memory T cell differentiation in which effectors give rise to memory cells have maintained broad support, others have proposed an alternative "developmental" model in which less differentiated memory cells become established during priming and these cells give rise to effectors.[92,99] In this context, a perspective which

incorporates a potentially ancestral (section 5.10) CD28 and CTLA4-mediated process of proliferative competition among lymphocytes for antigen, co-stimulatory ligands, and associated membrane lipids via trogocytosis seems to favor a developmental model of T cell differentiation. The hypothesis that clones more successfully competing for antigen during an immune response will be relatively metabolically rich from lipid accumulation via trogocytosis is consistent with the preferential requirement for lipid metabolism in Treg, as well as CD4+ and CD8+ memory T cells.[99,366,422,423] Indeed, preferential lipid metabolism is a recently established feature of T cell "stemness" and memory phenotype while transition to aerobic glycolysis is associated with CD4+ and CD8+ T cell activation and differentiation.[99,366,422] While a role for accumulation of membrane lipids via trogocytosis has not previously been considered in this context, such a mechanism may also provide a direct mechanistic route for the poorly understood immunomodulatory properties of various bioactive lipids.[424]

In further support of the notion that CD28 / CTLA4 mediated trogocytosis and lipid internalization is associated with increased lipid metabolism and T cell memory formation, CD28-induced fatty acid oxidation (FAO) has been reported to be involved in 'mitochondrial priming' of memory CD8+ T cells, endowing these cells with 'latent mitochondrial respiratory capacity'.[338] Intriguingly, it was additionally shown that fatty acids needed for CD8+ T cell memory formation are produced by cell intrinsic lysosomal lipolysis.[339] Although the relevant inducer of lysosomal lipolysis remains unknown, a recently proposed source is indeed membrane phospholipids degraded by autophagy, a process which was recently shown to promote mitochondrial FAO in cancer cells.[425,426] In further support of this hypothesis, autophagy pathway components were recently shown to be unexpectedly required for CD8+ and CD4+ T cell memory formation.[427,428]

A similar unexpected implication of an antigen / lipid competition-focused model may be counterintuitive metabolic benefits of lipid internalization mediated by the highly endocytic co-inhibitory receptor CTLA4 which is expressed at high levels in Treg and memory T cells. These most successful competing clones expressing high levels of CTLA4 might be expected to accumulate more significant quantities of membrane lipids resulting in reprogramming of cellular metabolism while acquiring phenotypic features of memory cells and dividing more slowly. Such a mechanism is consistent with the emerging paradigm of T cell differentiation consisting of early segregation into slowly proliferating central memory precursor cells (CMPs) and rapidly dividing non-CMPs.[429] Interestingly, this model would also provide an alternative framework to conceptualize the dynamic plasticity observed in T cell memory vs. effector lineage commitment wherein contributions from CD28 / CTLA4 mediated lipid accumulation might be expected to shift according to the dynamics of interclonal competition and cooperation throughout an immune response.[386,387]

Finally, the inherent directionality of trogocytosis-mediated pMHC acquisition and associated CTLA4 polarization to acquired membrane fragments may additionally provide novel regulatory opportunities for asymmetric division to impact T cell memory and effector differentiation. Indeed, asymmetric division during the initiation of immune responses has been observed previously and segregation of trogocytosed molecules to the 'distal pole complex' opposite of APC conjugation site was reported by Osborne and Wetzel in 2012, a phenomenon which the authors similarly hypothesized might be involved in asymmetric division.[362,430] In separate but related examples, roles for display of acquired pMHC in memory formation and recall has been proposed and demonstrated in several cases.[229,230,336,431,432]

5.12. Potential criticisms and response

It could be argued that the intimate connection between co-stimulatory signaling provided by APCs and the establishment of quorum among lymphocytes can be understood within the current PAMP/Danger model and related frameworks. Indeed "quorum" models of T cell function have recently been proposed without raising larger objections to the current theoretical foundations of adaptive immunity.[91,257] Notably however, as the basis for determining both whether and what kind of immune response is induced, the current paradigm can be readily extended to explain almost any experimental result (section 1.6.5).[263] This is likely in part due to the validity of much of this perspective and of its core postulate (i.e. the centrality of co-stimulation / 'inflammation' more broadly, and its regulation by innate myeloid cells in coordinating lymphocyte proliferation and function). Yet despite being a highly fruitful approach, the unfalsifiable plasticity and open-endedness of the PAMP/ "Danger" paradigm is strong justification for a close evaluation of its limitations (section 1.6.6). This is best achieved by examining where this perspective contrasts with foundational theoretical principles in immunology, particularly as revealed by early experiments which by necessity relied on system level outputs, and additionally the accumulation of conflicting modern observations.

In this regard, the cited work supporting the centrality of cooperative antigen recognition among lymphocytes appears compelling, unequivocally accounting for experimentally defined models of the induction and breaking of immune tolerance and class regulation which cannot be easily explained by differences in "Danger", distinctive cytokine milieu, or specialized DC subtypes.[247,254,258] Together it can be argued that the 'Quorum' framework of lymphocyte activation and class regulation proposed by Bretscher and others has produced the most complete model of adaptive immunity that is scalable from the cellular to the system level. It is therefore

striking that this perspective has been almost entirely neglected from mainstream consideration.[258] The clear "physicalization" of collective self-regulation in the form of widespread functional trogocytosis among lymphocytes and its regulation by CTLA4 may therefore represent one of the few remaining examples of empirical evidence still available in further support of this now radical shift in perspective (section 5.4). Extending beyond previous 'Quorum' models, I suggest that this restoration of lymphocytes at the foundation of adaptive immunity through an unusual engagement in auto-regulation of information provided by APCs via trogocytosis also highlights a propensity for "self"-focused 'reflective' regulation at the network level. This perspective recapitulates Jerne's Network theory and more readily incorporates the evolutionary origins and function of regulatory T cells as well as the two somatic GOD events in early vertebrate evolution (section 5.10). In this way the theoretical value of providing a more efficient means for TCR-dependent control over co-stimulatory signaling via trogocytosis appears immense, offering a route toward synthesis of otherwise contradictory historical and contemporary observations within a single framework (section 5.4).

This perspective could similarly be criticized for neglecting the clearly demonstrated roles of extraordinarily sophisticated networks of phagocytic myeloid cells in controlling adaptive immunity. On the contrary, this framework does not exclude the critical functions of myeloid APCs in initiating and regulating adaptive immune responses. Rather, in this view, myeloid APCs of innate immunity can be viewed as providing the crucial and intricate "shape" of the impression which is dynamically made onto the adaptive immune system by both foreign and "self" antigens over time. In this sense it could even be conversely argued, perhaps less controversially, that networks of lymphocytes comprising the adaptive immune system simply represent a means for the collective of myeloid APCs to amplify, process, and encode memory of

their own signaling responses. The model proposed here advocates only for the consideration that an additional and essential layer of computation exists which is performed cooperatively by lymphocytes and that this "self"-reflective process is regulated by CTLA4 to coordinate system level phenomena such as tolerance, class regulation, and adaptive memory.

Most specifically, the central argument I hope to advance is that ubiquitous trogocytosis among leukocytes facilitates a continuous internal 'reflection' of evolving "self" and non-self stimuli in what is essentially an act of cognition processed collectively by network interactions among the repertoire (section 5.10.2). I suggest that the fundamental importance of this otherwise highly subtle form of collective auto-regulation is manifest most strikingly by the elusive behavior of its essential regulator, CTLA4.

5.13. Clinical implications

Recognition of trogocytosis as a fundamental feature of lymphocyte activation and means for network-level regulation may offer new avenues to understand several longstanding enigmas that are of urgent importance to human health. Notably, the CD4 homolog, LAG-3 represents a particularly attractive candidate for regulation of pMHC trogocytosis and T cell antigen presentation.[433] Therapeutic targeting of this immune checkpoint receptor is the third most advanced in clinical development behind PD-1 and CTLA4 but its precise function remains surprisingly poorly understood.[434]

In the context of the hypothesized intimate role of trogocytosis in the evolutionary origins and subsequent regulatory development of adaptive immunity (section 5.10), it is tempting to speculate that analogous to the opposing role of CTLA4 following gene duplication and divergence from CD28, LAG-3 may also be engaged in some form of integrative cell intrinsic regulation via pMHC trogocytosis. This hypothesis is consistent with the conspicuously similar

features that LAG-3 shares with CTLA4, such as its primarily intracellular localization, rapid trafficking to the cell surface during activation, binding to MHC-II with much higher affinity than its competing co-expressed receptor CD4, its role in restraining homeostatic proliferation, and its involvement in mediating suppression by Treg, all achieved via a longstanding enigmatic mechanism.[434] In support of a role for LAG-3 in T cell trogocytosis it was indeed recently shown that LAG-3 was involved in mediating pMHC-II acquisition to induce immune suppression by a specialized subset of 'double negative' (CD4- / CD8-) regulatory T cells in a mouse model of allergic asthma.[300] Additionally, it was recently reported that a subset of intra-islet CD8+ T cells in a mouse model of type 1 diabetes exhibit LAG-3-dependent restraint against autoimmune destruction of beta cells in the pancreas via an unclear mechanism potentially mediated by LAG-3 inhibition of T cell epitope spreading.[435] Intriguingly, and in tentative support of the hypothesis outlined above regarding potential for exogenous pMHC-II display regulated by LAG-3, these CD8+ T cells were otherwise found to be "phenotypically, transcriptionally, epigenetically, and metabolically" similar to other 'exhausted' intra-islet CD8+ T cells, with LAG-3 depletion nevertheless enhancing auto-reactive effector function.[435] Although type 1 diabetes is thought to be predominately mediated by infiltrating CD8+ T cells, a role for CD8+ T cell 'licensing' by CD4+ T cells has been suggested previously and CD4+ / CD8+ (as well as CD8+ / CD8+) crosstalk via trogocytosis and T-T priming has been demonstrated.[336,436,437]

Another implication of this perspective with respect to immunotherapy is the basis for the observed synergy of immune checkpoint blockade with chemotherapy.[438] This effect is generally understood under an updated version of the PAMP/ "Danger" paradigm to be mediated by cancer cells undergoing so-called 'immunogenic cell death' induced by chemotherapeutic drugs.[263] This mode of cell death is thought to result in the release of DAMPs into the tumor

microenvironment, APC activation, and ultimately enhanced T cell priming against mutated tumor 'neo-antigens.'[438,439] Similar to other descriptive models generated in the context of the "Danger" framework, much of this hypothesis has been demonstrated and is broadly supported yet arguably remains seemingly incomplete, with relatively limited predictive power or clinical success. In the context of a 'Quorum' / Network-oriented perspective of adaptive immunity, one somewhat counter-intuitive and relatively unexplored consideration for the basis of checkpoint inhibitor synergy with traditional cancer treatments would be chemotherapy / radiation-induced lymphopenia. Iatrogenic lymphopenia in cancer patients is typically associated with immune suppression and poor prognosis yet in some cases can paradoxically promote potent tumor-specific immune responses.[438,440–443] Indeed acute lymphopenia, and specifically the associated homeostatic proliferation which subsequently restores the peripheral lymphocyte pool, has long been associated with spontaneous induction of autoimmunity in some contexts for reasons that remain poorly understood.[444]

Because tumor cells are invariably recognized, to some extent, as "self" by the adaptive immune system, it can be argued that what is required for successful cancer treatment is the precise and limited induction of 'autoimmunity.' In terms of the 'Quorum' / Network framework described here, it seems likely that the lymphopenic environment represents a highly permissive state that favors escape from the normally densely interconnected network interactions created by the diverse repertoire which otherwise maintains dominant suppression of autoreactive cells in dynamic equilibrium (See note on sedentary ants in section 5.9.1).[445,446] It is in this context that homeostatic proliferation may result in rare "self" antigen-specific T cells reaching quorum to drive an anti-"self" autoimmune response.[447]

Interestingly, these considerations may also have relevance for understanding and treating SARS-Cov2 immunopathology. Notably, lymphopenia has been associated with acute SARS-Cov2 infection and more recently, a striking total absence of naïve T and B cells was observed in patients suffering from the multi-symptom chronic 'long COVID' syndrome.[448] While chronic COVID syndrome remains poorly understood, its etiology appears most readily explained by a constellation of extraordinary properties of the SARS2 spike glycoprotein itself, which include a unique furin cleavage site that enables broad infectivity, a short (~20aa) motif resembling the staphylococcal enterotoxin B superantigen (SEB), an immunodominant T cell epitope consisting of a short peptide motif that is conserved in the surface proteins of several microorganisms including the malaria parasite *plasmodium malariae,* an ability to directly bind CD4 to facilitate productive infection of CD4+ T cells, and additional binding to neuropilin-1 as a host entry factor (expressed by subpopulations of regulatory T cells).[449–454] In this context, network level dysregulation of CD4+ T cell help associated with acute lymphopenia, T cell hyper-activation and 'exhaustion' by the superantigenic character of the SARS2 spike protein likely represent major contributing factors to various combined immuno-deficient and/or autoimmune manifestations of SARS-Cov2 spike protein exposure.[455,456] Perhaps most intriguingly, this model would include the hypothesis for induced dysregulated production of anti-idiotypic auto-antibodies (e.g. anti-ACE2, anti-neuropilin-1) which was recently proposed to account for the striking similarities in clinical manifestations of rare SARS2 spike-based vaccine injuries and 'long COVID' chronic syndromes involving apparently similar vascular and neurologic immunopathology (section 1.6.8).[285,287,290,457,458]

5.14. Theoretical Summary

Charles Janeway's 1989 proposal of the PRR theory of immune regulation stated, "*I raise the possibility that the second signals arose prior to the development of specific antigen recognition… more as positive initiators of immunity than as late adaptations to avoid autoimmunity.*" Interestingly, Janeway seems to refer to both the historic and the modern conception of "second signals" of T cell activation (i.e. antigen-linked cellular cooperation and costimulatory signaling, respectively), the latter of which he theoretically formalized in this essay with such revolutionary success that the former was largely forgotten.[259] This distinction may remain essential for understanding adaptive immunity however, and has been only masked by the ability of the latter ('inflammation') to induce the former ('quorum') among lymphocytes. This division is proposed to represent the interface where CTLA4 exerts regulatory control between the innate and adaptive immune systems and between single T cells and the leukocyte collective.

In this framework it is similarly hypothesized that the connection between these "second signals" arose from the beginning. Specifically, that in these earliest stages, even prior to somatic GOD, determination of the presence or absence of 'Quorum' among lymphocytes was facilitated by trogocytosis, which is similarly enhanced by inflammation but nevertheless remains inherently "self"-focused via continuous display of antigen-receptor specific and associated antigens. This re-emphasis on "self" and lymphocyte encoded antigen receptors seems to support a final 'Jernian' modification of Janeway's enormously successful pathogen-oriented paradigm. I attempted to argue that this slight shift in perspective may have significant implications for developing a more complete understanding of the induction, regulation, and formation of memory in adaptive immune responses. Finally, it is proposed that this perspective uniquely

provides an alternative account for the evolutionary origins of the adaptive immune system which incorporates an essential role for regulatory T cells in dominant "self" tolerance, tissue homeostasis, and regeneration.

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