THIOL-MEDIATED REDOX MODULATION OF THE ADAPTIVE IMMUNE RESPONSE

By

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To my wife, Ying Liu and my son, Chen Yan

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PREFACE

This thesis is composed of five chapters. The introduction (chapter 1) is a review article about our current understanding of thiol-mediated immunoregulation (Yan Z. & Banerjee, R. 2010. Redox remodeling as an immunoregulatory strategy. *Biochem.* 49(6):1059-1066). Chapter 2 is a copy of publication by Yan Z. et al. (Yan Z., Garg S.K., Kipnis J., Banerjee, R. 2009. Extracellular redox modulation by regulatory T cells. *Nat. Chem. Biol.* 5:721-723). In this chapter, all authors contributed to the experimental design, data analysis and manuscript writing. Zhonghua Yan and Sanjay K. Garg performed the experiments. Contents of chapter 3 have been submitted for publication: Yan Z., Garg S.K., Banerjee, R. Regulatory T cells interfere with glutathione metabolism in dendritic cells and T cells. In this chapter, Zhonghua Yan and Ruma Banerjee designed research, analyzed data and wrote the paper; Zhonghua Yan and Sanjay K. Garg performed the experiments. Contents of chapter 4 were published in the following paper: Garg S.K.*, Yan Z.*, Vitvitsky V., Banerjee, R. 2010. Differential dependence on cysteine from transsulfuration versus transport during T cell activation. *Antioxid. Redox Signal.* (Epub ahead of print). *These authors contributed equally. In this chapter, all authors contributed to research design, data analysis and paper writing; Zhonghua Yan and Sanjay K. Garg performed the experiments.

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AβH, L-aspartic acid β-hydroxamate
ACV, acivicin
AIDS, acquired immunodeficiency syndrome
AP1, activator protein 1
APC, antigen presenting cell
Bcl-2, B cell leukemia/lymphoma 2
BSO, buthionine sulfoximine
CBS, cystathionine β-synthase
CMFDA, chloromethylfluorescein diacetate
CSE, γ-cystathionase
CTLA-4, cytotoxic T lymphocyte antigen 4
DC, dendritic cell
FACS, fluorescence-activated cell sorting
Foxp3, forkhead box P3
γ-GCS, γ-glutamylcysteine synthetase
GGT, γ-glutamyltranspeptidase
GM-CSF, granulocyte macrophage colony-stimulating factor
GRB2, growth factor receptor bound protein 2
GSH, glutathione
GSSG, glutathione disulfide
HIV, human immunodeficiency virus
HPLC, high performance liquid chromatography
ICAT, isotope-coded affinity tag
IDO, indoleamine 2, 3-dioxygenase
LAT, linker for activation of T cells
LCK, leukocyte-specific protein tyrosine kinase
LPS, lipopolysaccharides
MRP1, multidrug resistance protein 1
MHC, major histocompatibility complex
NAC, N-acetyl-cysteine
NADPH, nicotinamide adenine dinucleotide phosphate

Ncf1, neutrophil cytosolic factor 1
NF-κB, nuclear factor-κB
Nrf-2, nuclear factor erythroid 2-related factor-2
PBS, phosphate buffered saline
PDTC, pyrrolidine dithiocarbamate
PFA, paraformaldehyde
PLC γ1, phospholipase C γ1
PMA, phorbol 12-myristate 13-acetate
PPG, propargylglycine
Ref-1, redox factor 1
ROS, reactive oxygen species
SAS, sulfasalazine
t-BuOOH, tertiary butyl hydroperoxide
TCR, T cell receptor
Teff, $CD4^+CD25^-$ effector T cell
TGFβ, transforming growth factor beta
Tn, naïve $CD4^+CD25^-$ T cells
TNF, tumor necrosis factor
Treg, $CD4^+CD25^+FoxP3^+$ regulatory T cell
Trx, thioredoxin
ZAP-70, zeta-chain-associated protein kinase 70

ABSTRACT

THIOL-MEDIATED REDOX MODULATION OF THE ADAPTIVE IMMUNE RESPONSE

By

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T cell activation and proliferation requires a reducing microenvironment that is provided by antigen presenting cells especially dendritic cells (DCs). Naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) suppress proliferation of $CD4^+CD25^-$ effector T cells (Teffs) by mechanisms that are not well understood. Here, we have demonstrated that inhibition by Tregs of DC-induced extracellular redox remodeling is a component of the Treg immunosuppressive mechanism. We showed that the mechanism of redox remodeling during T cell activation involved secretion of glutathione (GSH) by dendritic cells and its subsequent cleavage to cysteine. Extracellular cysteine accumulation resulted in a lower redox potential, which is conducive to proliferation, and changed the net redox status of exofacial protein domains. Suppression of DC-dependent Teff cell proliferation by Tregs was correlated with a significant diminution in extracellular cysteine concentration and was abrogated by addition of exogenous cysteine. We demonstrated that Treg-mediated redox perturbation was antigen-dependent, antigen-

nonspecific and cytotoxic T-lymphocyte antigen 4-dependent. Tregs used multiple strategies for extracellular redox remodeling including modulation of GSH metabolism in DCs and competitive uptake of cysteine. By interfering with the extracellular cysteine pool, Tregs not only decreased the intracellular GSH levels in Teffs, but also blocked GSH relocalization into the cytoplasm, thus inhibiting T cell activation and proliferation.

The synthesis of GSH, a major cellular antioxidant with a critical role in T cell proliferation, is limited by cysteine. We have evaluated the contributions of the x_c^- cystine transporter and the transsulfuration pathway to cysteine provision for GSH synthesis and antioxidant defense in naïve versus activated T cells and in the immortalized T lymphocyte cell line, Jurkat. We showed that the x_c^- transporter while absent in naïve T cells, was induced after activation. We also demonstrated the existence of an intact transsulfuration pathway in naïve and activated T cells and in Jurkat cells. The flux through the transsulfuration pathway increased in primary T cells in response to oxidative challenge by peroxide. Inhibition of the transsulfuration pathway in both primary and transformed T cells decreased cell viability under oxidative stress conditions.

Chapter 1

General Introduction

1.1 Regulation of T cell activation by dendritic cells

The immune system is composed of a variety of organs, cells and molecules that protect an organism from infections by pathogens and from the growth of tumors. It is typically divided into two categories, the innate immune system and the adaptive immune system. Innate immunity provides the first line of defense against pathogens in a non-specific manner. Components of the innate immune system include the skin and the mucous membranes, physiological conditions such as temperature and pH, phagocytic cells such as tissue macrophages, and inflammation. Adaptive immunity only exists in vertebrates, reacting to antigens with a high degree of specificity and immunological memory. B cells, T cells and the molecules they produce, including antibodies and cytokines, are the main agents of adaptive immunity. The adaptive immune response is initiated by the innate immune system via the process of antigen presentation. These two immune systems interact and collaborate, increasing the effectiveness of the immune response (*1*).

T cells derive their name from the thymus, the organ in which they mature and are distinguished by the presence of T cell receptors (TCRs). The latter recognize antigens bound to the major histocompatibility complex (MHC) molecules on antigen presenting cells (MHC class II) or target cells (MHC class I). Like most immune cells,

T cells are initially derived from hematopoietic stem cells in the bone marrow where progenitor T cells are formed. They migrate to the thymus via the bloodstream to mature via positive and negative selection processes (*2*). "Naïve" T cells released from the thymus have yet to encounter an antigen and are in the G0 stage of the cell cycle. They circulate through the vascular system to secondary lymphoid tissues such as lymph nodes where they may encounter antigen-MHC complexes and in the process, become activated. Activated T cells play important roles in cell-mediated functions in the adaptive immune system and their dysfunction is manifest in a number of immune diseases (*3*).

Antigen presenting cells such as dendritic cells (DCs), macrophages, and B cells express MHC class II molecules and co-stimulatory molecules on their membranes, and specialize in presenting antigens to naïve $CD4^+$ T cells. DCs are the most potent professional antigen presenting cells (*4*) and originate from hematopoietic stem cells in the bone marrow. Precursor DCs are released from the bone marrow and circulate in the bloodstream to different tissues where they reside as immature DCs until they encounter antigens. Once internalized, antigens are processed and then displayed on MHC class II molecules and the resulting mature DCs migrate to lymph nodes where they interact with and activate antigen-specific T cells, which subsequently proliferate and differentiate into effector T cell subsets (*2, 5, 6*).

Classically, three sets of signals in the immune synapse are recognized to be essential for priming naïve T cells: (i) specific engagement of the TCR by an antigen-MHC class II complex, (ii) interactions between co-stimulatory molecules, CD28 on T cells and CD80/86 on the antigen presenting cells, and (iii) secretion of cytokines (Fig. 1.1A). These signals result in the activation, survival, and differentiation of T cells. In

addition to these signals, T cell activation and proliferation requires a reducing microenvironment that is achieved by cysteine secretion from antigen presenting cells (*7, 8*). Cysteine is the major low molecular weight thiol compound in the reducing milieu (*7, 9*). It is the limiting amino acid for glutathione (GSH) synthesis. In T cells, GSH is shown to be important for the proliferative response of T cells to mitogens and antigens (*10*). Extracellular cysteine concentration significantly affects intracellular GSH levels, viability and DNA synthesis of T cells. However, cysteine is easily oxidized to cystine in the oxygenated extracellular space. The concentration of cysteine in the plasma (10-25 μM) is much lower than of cystine (100-200 μM) (*11*). Naïve T cells can not take up cystine efficiently due to the low expression of the x_c^- cystine transporter. However, antigen presenting cells such as DCs possess the x_c^- cystine antiporter, which can take up cystine from the extracellular space in exchange for glutamate. The cystine imported by DCs is processed via an elaborate pathway to furnish extracellular cysteine that can be utilized by T cells. Interaction of antigen presenting cells with T cells further increases cysteine production and also induces secretion of thioredoxin 1 (Trx1). In this way, antigen presenting cells supply T cells with a reducing extracellular milieu necessary for T cell proliferation and function (*7*).

The physiological relevance of redox remodeling by antigen presenting cells for T cell activation is demonstrated by the hyporesponsiveness of T cells from normal gut, which results from the inability of mucosal macrophages to provide a reducing microenvironment and contrasts with the presence of this capacity in peripheral blood monocytes in the same organism (*12, 13*). Under conditions of chronic mucosal inflammation as seen in inflammatory bowel disease, ulcerative colitis, and Crohn's

disease, recruitment of peripheral blood monocytes results in sustained antigen-driven responses of T cells in the gut and is believed to be important in the etiology of these diseases (*13*).

1.2 Mechanisms of suppression by regulatory T cells

Discriminating self from non-self is a primary function of the immune system, and regulatory T cells play a cardinal role in maintaining self-tolerance and preventing autoimmunity by mechanisms that remain to be fully elucidated (*14-16*). To achieve self-tolerance, T cells are "educated" in the thymus and autoreactive T cells are destroyed. However, a small fraction of self-reactive T cells escape from the thymus into the periphery, and if left unchecked, can cause autoimmune diseases (*17*). Naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T cells, which comprise about 5%-10% of total $CD4^+$ T cell population, suppress autoreactive T cells to maintain immune tolerance (*18*). Sakaguchi and coworkers made the groundbreaking discovery of this distinct T cell subpopulation in 1995 and demonstrated that depletion of the $CD25^+$ population from the $CD4^+$ T cells induced autoimmunity when T cells were transferred to the immunodeficient nude mice (*14*). In contrast, transfer of the $CD4^+CD25^+$ T cells together with the $CD4^+CD25^-$ T cells prevented autoimmune diseases. Besides the role of regulatory T cells in controlling autoimmunity, they also play important roles in controlling anti-microbial, anti-tumor responses and transplantation immunity (*18*). Mutations in the transcriptional regulator, Foxp3, which is preferentially expressed in regulatory T cells (*19*), results in multiorgan autoimmune diseases and is fatal (*20*). Foxp3 is the master regulator for regulatory T cell development in the thymus and for the

suppressive function of regulatory T cells (*20*). The co-receptor, cytotoxic T lymphocyte antigen 4 (CTLA-4) (*21*), expressed preferentially on regulatory T cells, interacts with CD80/CD86 (Fig. 1.1B), i.e., the same ligand that binds CD28 expressed on naive T cells. However, CTLA-4 interacts with CD80/CD86 with much higher affinity and suppresses induction of CD80/86 expression by antigen-specific T cells, consequently limiting the capacity for activating naïve T cells (*22*). Regulatory T cells mature in the thymus, migrate to lymph nodes and are activated by self or nonself antigen-presenting cells. The homing receptors on regulatory T cells enable them to traffic to sites of infection to control immune responses (*15*). Regulatory T cells also suppress the activation and proliferation of B cells, DCs and natural killer cells by mechanisms that remain to be fully elucidated (*23*).

Some of the strategies used by regulatory T cells for mediating their suppressive effects (*15, 16*) are shown in Fig. 1.2 and include: (i) secretion of inhibitory cytokines viz. TGFβ, IL-35 and IL-10 (*24-26*), (ii) cytolytic suppression by secretion of the proteases granzyme-A or granzyme-B (*27, 28*). (iii) metabolic disruption e.g. by direct transfer of cAMP to effector T cells (*29*) or by secretion of pericellular adenosine (*30*), which inhibits effector T cell functions and enhances induced regulatory T cell generation, (iv) suppression of DC maturation and/or function (*31*) by induction of indoleamine 2,3-dioxygenase, which catalyzes the rate-limiting step in tryptophan catabolism and creates in turn, a shortage of this essential amino acid for effector T cells (*32*), and (v) by interfering with extracellular reductive redox remodeling by DCs during T cell activation (*8*). The panoply of suppressive strategies identified to date for regulatory T cells raises questions about their integration and relative importance in vivo. In the proposed

"hierarchical" model, one or few master mechanisms govern regulatory T cell suppressive functions in various physiological settings (*16*). Alternatively, in the "contextual" model, the microenvironment and tissue compartment govern the suppressive strategy that is deployed, resulting in the differential contribution of a given mechanism in different disease models (*16*). Given the importance of regulatory T cells in controlling autoimmunity and inflammation, and their influence on tumor and microbial immunity, elucidation of the mechanisms by which these cell types exert their effects have important implications for therapeutic target identification and development of intervention strategies.

1.3 Redox potentials in the intra- and extra-cellular compartments

The cytoplasmic and extracellular redox potentials are vastly different and influence the structure, stability, and function of the macromolecules that reside in each compartment. Within the intracellular compartments, several redox buffers exist e.g. Trx, GSH, and cysteine, and the relative concentrations of their oxidized versus reduced species sets the ambient redox poise for the system. Interestingly, the individual redox systems appear to be under kinetic control, are not in equilibrium with each other, and independently regulate the redox status of their client redox partners (*33, 34*). Quantitatively, GSH is the major intracellular redox buffer and is found at concentrations ranging from 0.5-10 mM in mammalian cells (*35*). The intracellular GSH/GSSG (glutathione disulfide) redox potential in dividing cells is estimated to range from -260 mV to -230 mV and is progressively more oxidized in cells undergoing differentiation/growth arrest (-220 mV to -190 mV) or apoptosis (-170 mV to -150 mV)

(*33*) (Fig. 1.3). The redox potentials for the $Trx1_{red}/Trx1_{ox}$ couple in cytoplasm and nuclei are ~-280 mV and -300 mV, respectively, while the redox potential of mitochondrial $Trx2_{red}/Trx2_{ox}$ couple is estimated to range from -360 mV to -340 mV (*34*).

Extracellularly, the cysteine/cystine couple represents the major thiol/disulfide redox buffer. Plasma cystine and cysteine concentrations are reported to be 100-200 μM and 10-25 μM, respectively and a redox potential of ~-80 mV for this couple has been estimated for plasma in healthy humans (*11*). Paralleling the changes in the intracellular GSH/GSSG redox potential, increasing extracellular cysteine/cystine potentials are associated with cells undergoing proliferation (<-80 mV), differentiation/growth arrest (~-80 mV) or apoptosis (0 mV to -80 mV) (Fig. 1.3). An age-dependent increase in the extracellular redox potential has been reported, which is also influenced by lifestyle choices such as smoking and by diseases such as AIDS (*33*). In contrast to the intracellular compartment, the GSH concentration in the extracellular space is very low (2-4 μM in human plasma). A major fate of secreted GSH is cleavage to its component amino acids, glutamate, cysteine, and glycine. The cysteine thus released is a major source of extracellular cysteine and cystine, which is formed rapidly in the oxidizing milieu of this compartment (*36*).

Although dynamic regulation of the extracellular redox potential, which is linked to intracellular metabolism, has an important bearing on cell function, it is less well-studied and appreciated than intracellular redox control and its perturbations in pathological states. Reactive cysteines on proteins can be reversibly oxidized to sulfenic acids or form disulfide bonds, which can induce changes in their structure and function and elicit downstream effects in redox signaling pathways (*37, 38*). Disulfide bonds on

ectodomains of membrane proteins and in secreted soluble and matrix proteins form a dynamic scaffold that can be reorganized by their shuffling or by their reduction (*39*). It has been proposed that a general loosening of the extracellular disulfide crosslink scaffold might precede cell division (*40, 41*). Cancer cells typically have higher membrane thiol levels in comparison to nontransformed cells, and it is speculated that this might facilitate higher proliferative rates (*42*). The redox status of specific membrane proteins influence their transport or receptor activity (*33*). For instance, CD4 (cluster of differentiation 4), a glycoprotein found on the surface of helper T cells that is used as a receptor by HIV-1 for gaining entry, has a redox sensitive disulfide bond in one of its four immunoglobulin-like domains (D2). T cell activation shifts the equilibrium from the disulfide to the dithiol state (*43*). Locking the dithiols in the D2 domain by chemical modification blocks HIV-1 entry, indicating that a redox-linked conformational change in CD4 is critical for viral penetration into T cells (*43*).

1.4 T cell induced extracellular redox remodeling by dendritic cells

DCs are the key regulators in the adaptive immune system. Not only can they initiate the primary immune responses, but they also induce the immune tolerance and regulate responses of different T cell subsets depending on the context (*5, 6*). Upon interaction with T cells, DCs release cytokines as well as other low molecular weight metabolites such as cysteine, glutamate and serotonin to modulate T cell immunity. Serotonin released by DCs binds to the serotonin receptors on T cells, decreasing cAMP levels, thus promoting T cell activation (*44*). DC-T cell interaction results in accumulation of glutamate, which acts on metabotropic glutamate receptor 1 and 5 to

modulate T cell activation (*45*). DCs can also take up cystine and release cysteine, Trx, and GSH into the extracellular space, thereby modulating the extracellular redox environment for optimal T cell function (*7, 46*).

The physiological relevance of extracellular reductive modeling during an adaptive immune response is supported by the dramatic increase in free thiols in lymphoid tissue following immunization (*47*). Under these conditions, enhanced nonprotein thiol staining is observed both inside cells and in the extracellular space. In contrast, Peyer's patches from the gut shows virtually no staining for nonprotein thiols under these conditions, consistent with the antigenic hyporesponsiveness of this intestinal microenvironment (*12, 13*).

The magnitude of extracellular cyteine accumulation during activation of T cells increases with time and with the DC to T cell ratio and requires sustained contact between DCs and T cells. Increased cell surface thiols on T cells is correlated with increased production of the cytokine, IL-2, in vitro and enhanced proliferation in vivo (*48*). Naïve T cells require cysteine for GSH synthesis. However, cysteine is the least abundant of all amino acids in circulation (*49*) and naïve T cells are unable to import cystine efficiently due to the low expression of the cystine transporter, x_c^- (*50*), thus creating a metabolic dependence on antigen presenting cells to meet their cysteine needs. Antigen presenting cells possess the x_c^- antiporter that uses the glutamate gradient to drive import of cystine, which is subsequently converted to cysteine in the reducing intracellular milieu and is ultimately secreted into the extracellular space. Evidence has been accumulating that extracellular cysteine plays an important role in modulation of T cell functions (*49, 50*). Small variations in extracellular cysteine concentration strongly

influence T cell functions even in a background of high cystine concentration (*9, 51*). Thus, antigen presenting cells such as DCs and macrophages can regulate T cell activation and proliferation by modulation of cysteine supply.

In addition to stimulating cysteine secretion, the interaction between antigen presenting cells and T cells results in the appearance of extracellular Trx1 (*7*). Trx1 is secreted by several cell types via a nonclassical leaderless secretory pathway under conditions of oxidative stress and inflammation (*52*). Secreted Trx1 does not appear to play a role in direct reduction of extracellular cystine leading to cysteine accumulation during T cell activation (*8*). Extracellular Trx1 interacts in a redox-sensitive manner with the TNF receptor superfamily member 8 (*53*) and exhibits proinflammatory effects by stimulating cytokine release and proliferation of lymphocytes (*52, 54*).

The pathway for extracellular cysteine accumulation during co-culture of DCs and naïve T cells has been mapped recently (*8*). In principle, two metabolic routes could be considered to lead to enhanced cysteine accumulation outside the cell (Fig. 1.4): (i) the transsulfuration pathway (blue), which provides an avenue for conversion of methionine to cysteine, and (ii) import of cystine into the cell where it is rapidly reduced to cysteine and converted to GSH, which is subsequently secreted and degraded by the ectoenzymes γ-glutamyltranspeptidase and a dipeptidase. Metabolic labeling and pharmacological inhibition studies have established the involvement of the convoluted metabolic pathway originating in cystine and culminating in GSH-derived cysteine as the source of extracellular cysteine provided by DCs (*8*). This pathway demonstrates the dynamic interplay between the intra- and extra-cellular compartments for redox homeostasis via interconnected but independent redox nodes, i.e., GSH and cysteine.

The extracellular cysteine/cystine redox potential for DCs in culture is ~-80 mV, a value that is consistent for cells experiencing growth arrest (*8*). Naive T cells in culture that have not received activation signals are fated to undergo apoptosis and exhibit an extracellular cysteine/cystine redox potential of ~-45 mV. In contrast, when naïve T cells receive activation signals during co-culture with DCs, a more reducing extracellular environment reflected in a redox potential of -110 mV (at 36 h), results. This redox potential change is consistent with conditions that are conducive for T cell proliferation (*8*).

In addition to triggering intracellular signaling pathways, engagement of DCs and T cells during activation leads to dynamic changes in the redox status of exofacial proteins in both cell types. A 30 mV potential shift is expected to lead to a 10-fold change in the ratio of reduced:oxidized cysteines in proteins. Indeed, enhanced cell surface labeling of protein thiols with the fluorescent dye, Alexa-maleimide, is seen during co-culture of DCs and T cells (by ~4 and 8-fold respectively) as visualized by confocal microscopy and quantified by FACS analysis (*8*).

1.5 Redox signaling during T cell activation

Paralleling reductive remodeling of the extracellular redox poise with consequent effects on the exofacial protein thiol status and intracellular redox metabolism, is the initiation of a flurry of redox-active signaling across the immune synapse. The timing and balance between oxidative and reductive responses to engagement of antigen presenting cells and T cells are important for modulating activation, proliferation, and apoptosis of T cells. At low levels, ROS (reactive oxygen species) e.g. H_2O_2 and $O_2^{\bullet -}$, are considered to

be mitogenic and their downstream effects are commonly mediated via changes in protein phosphorylation and/or activation/inhibition of transcription factors (*55*). Crosslinking of the TCR and the co-stimulatory molecule, CD28, results in enhanced intracellular H_2O_2 production that is needed for NF-κB activation and IL-2 and IL-2 receptor α chain gene transcription (*56*) and is consistent with an important role for ROS in the immediate early events during activation. Significant sources of ROS include membrane-bound NADPH-dependent oxidase, lipoxygenase, and the mitochondrial respiratory chain (Fig. 1.5A, red arrows). However, sustained pro-oxidant conditions inhibit T cell proliferation and promote apoptosis (*57*).

During activation, increased ROS levels launch an antioxidant response that is relayed via signaling pathways in antigen presenting cells and in T cells and result in activation of protein tyrosine kinases (e.g. Fyn, Src, and Lck in T cells (Fig. 1.5B)), oxidative inhibition of protein tyrosine phosphatases e.g. SHP1 and activation of transcription factors e.g. NF-κB (*58*). The NF-κB pathway regulates the expression of various inflammatory genes including cytokines, chemokines, and costimulatory molecules. We speculate that as a consequence of an initial increase in ROS levels by mechanisms that are not clear, NF-κB is activated in DCs and stimulates GSH biogenesis (via activation of γ-glutamylcysteine ligase (*59*)) (Fig. 1.5A, red arrows). Increased GSH synthesis is both an autocorrective reaction to oxidizing conditions and initiates the next response phase, i.e., an antioxidant wave (Fig. 1.5A, blue arrows). We hypothesize that the NF-κB signaling pathway is important for stimulating extracellular cysteine accumulation (*8*). The combined effect of these cellular responses would be the initiation of an antioxidant response leading to a reductive milieu both in the intra- and extra-

cellular space that is conducive to T cell proliferation. The importance of plasticity in redox remodeling during T cell activation is supported by the observation that deficiency of Ncf1 encoding neutrophil cytosolic factor 1 (or P47phox), the activating protein in the NADPH oxidase complex, results in a reduced capacity for reactive oxygen species genesis, increased cell surface thiols, and enhanced T cell autoreactivity in an arthritis model (*48*).

GSH serves as an important proliferative signal in T lymphocytes (*60*) and is required for cell cycle progression from the G1 to S phase (*61*). It is needed for the activity of ribonucleotide reductase and therefore, for DNA synthesis (*62*). Furthermore, the activities of telomerase (*63*) and of key transcriptional factors, e.g. NF-κB and AP1 (*64*), and cell cycle proteins e.g. Id2 and E2F4 (*63*) are redox regulated. GSH is concentrated in the nucleus during the early phase of cell proliferation and becomes more evenly distributed in confluent cells (*65*). GSH regulates nuclear protein function via glutathionylation and protects DNA from oxidative damage during the key stage of replication (*65*). ROS can either activate or inactivate specific redox-sensitive targets at cell cycle checkpoints, thus deciding cell fate (*66*). Overexpression of the B cell leukemia/lymphoma 2 (Bcl-2) protein results in increased synthesis and nuclear sequestration of GSH and decreased sensitivity to apoptosis (*67*).

Redox signaling cascades are also elicited in T cells upon activation. For instance, a 10-30% decrease in intracellular GSH in peripheral T lymphocytes completely abrogates T cell receptor-stimulated calcium signaling (*68*). The adaptor protein linker for activation of T cells (LAT) (Fig. 1.5B), a membrane protein that plays a central role in signal transduction during T cell activation, is also influenced by the intracellular redox

status (*69*). Marked diminution in intracellular GSH level as seen under chronic oxidative stress conditions causes a conformational change in LAT, apparently via formation of an intramolecular disulfide bond, and results in its displacement from the membrane (*69*). This cytoplasmic relocalization results in failure to phosphorylate in response to T cell activation and derails the signal transduction cascade that leads eventually to expression of IL-2 and other genes. This redox-sensitive conformational displacement is associated with the hyporesponsive phenotype of synovial T cells in rheumatoid arthritis because of their depleted antioxidant capacity resulting from the chronic inflammation associated with this disease of the joints (*69*).

In summary, redox responsive signaling networks during T cell priming involves dynamic and spatially regulated changes in the intra- and extra-cellular compartments and comprises both small molecules (e.g. ROS and redox-active metabolites) and proteins. Redox signaling has several important implications for T cell biology (*55*). Hypoxic conditions as encountered in poorly oxygenated tumors might limit the efficiency of T cell priming and contribute to their anergic phenotype in this environment. Alternatively, a pro-oxidant environment resulting from ROS production by active neutrophils might facilitate the priming of T cells or if overwhelming, impair signaling and lead to abrogation of cell inhibitory signals normally delivered by autoantigens. Additionally, redox signaling appears to influence T cell commitment to the Th1, Th2, and regulatory T cell phenotypes (*70, 71*).

1.6 In vivo studies and therapeutic implications

The redox status of secondary lymphoid organs such as lymph nodes and spleens are more reducing than non-lymphoid organs (*47*). The nonprotein thiol content in

lymphoid tissues is reported to increase in response to immunization with DCs, B cells, and macrophages, contributing to the reductive remodeling (*47, 72*). It is speculated that the reducing microenvironment might protect lymphoid organs from oxidative stress during T cell activation and antibody production (*73, 74*). However, low levels of ROS are essential for the onset of the immune response. In vivo treatment of mouse models with catalytic antioxidants (manganese porphyrin derivatives) causes inefficient $CD4^+$ T cell activation and proliferation by inhibiting generation of ROS in antigen presenting cells (*75*). The catalytic antioxidants inhibit DNA binding by NF-κB and subsequent production of proinflammatory cytokines (*76*). Redox modulation by catalytic antioxidants also suppresses $CD8^+$ T cell functions such as proliferation and lysis of target cells (*77*).

The x_c^- cystine transporter, which transports cystine using the glutamate gradient, plays an important role in redox-based immunoregulation. Under normal conditions, lamina propria macrophages are unable to transport cystine and secrete cysteine because they lack the x_c^- transporter (*13*). In inflammatory bowel disease, local recruitment of peripheral blood monocytes which exhibits high expression of the x_c^- transporter leads to extracellular cysteine accumulation and hyperreactivity of lamina propria T cells (*13*). Furthermore, lymphoma cells, which cannot import cystine like naïve T cells, depend on tumor-associated somatic cells such as activated macrophages and DCs for their cysteine supply. Inhibition of the x_c^- transporter by sulfasalazine inhibits growth of lymphoma cells and tumor progression (*78*). Overexpression of the x_c^- transporter in lymphoma cells greatly increases intracellular and extracellular cysteine levels, protecting cells from oxidative stress induced cell death (*79*).

Redox modulation as a strategy for immunoregulation has been used in several diseases. HIV infects and kills $CD4^+$ T cells, leading to a significant decrease in functional $CD4^+$ T cells in AIDS. HIV infected individuals have lower cellular and plasma GSH levels compared with healthy controls, which correlates with low T cell numbers and deficient function. Administration of N-acetyl cysteine, a cysteine precursor, restores intracellular GSH levels and has shown benefits for HIV-infected individuals (*80*). Sulfasalazine is used in the treatment of T cell mediated autoimmune diseases such as inflammatory bowel disease and rheumatoid arthritis. It decreases proliferation of autoreactive T cells by inhibiting the x_c^- cystine transporter thereby perturbing the redox environment (*81*).

Since regulatory T cells play a central role in suppression of various immune responses, manipulation of their function is an important strategy for immune intervention. Enhancing regulatory T cell function in autoimmunity, allergy, transplantation, and pregnancy disorders can diminish unwanted immune responses. On the other hand, attenuating regulatory T cell function in cancer and microbial infection may be desirable (*82*). The recent identification of a novel immunosuppressive strategy deployed by regulatory T cells, which impacts the intra- and extra-cellular redox environments during T cell activation (*8*), illuminates a new therapeutic target.

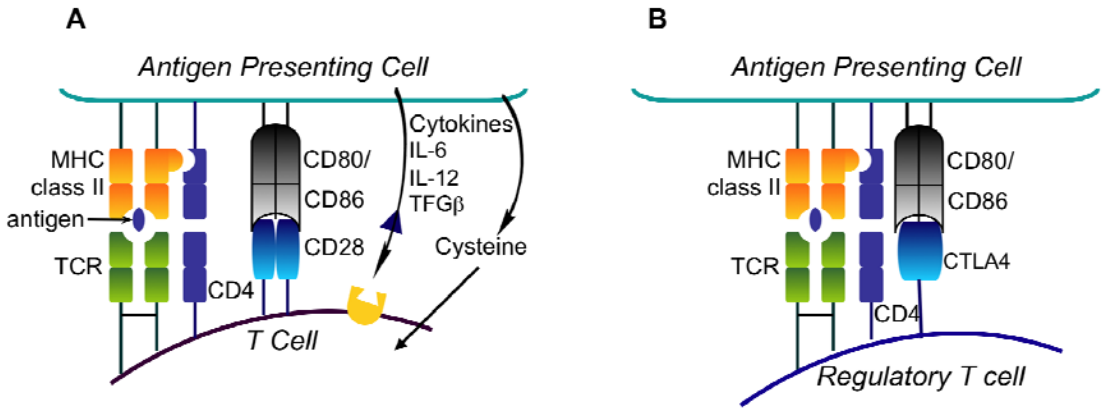

Figure 1.1 Molecular interactions in the immune synapse. (A) Signals required for $CD4^+$ T cell activation and proliferation include: (i) TCR-antigen•MHC complex interaction, (ii) interaction of CD28 on T cells and CD80/CD86 on the antigen presenting cells, (iii) secreted cytokines such as IL-6, IL-12 and TGFβ and (iv) a reducing microenvironment shaped mainly by extracellular cysteine accumulation. (B) Interaction of regulatory T cells with antigen presenting cells. Regulatory T cells constitutively express high levels of CTLA-4, which interacts with CD80/CD86 on antigen presenting cells, thus inhibiting their presentation capacity for interactions with effector T cells.

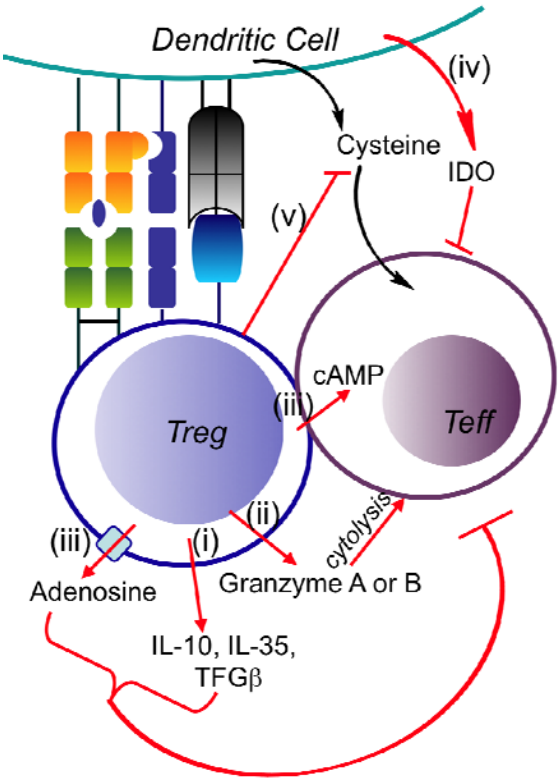

Figure 1.2 Mechanisms used by regulatory T cells for suppressing autoreactive effector T cells. Regulatory T cells suppress the function of effector T cells via the following mechanisms: (i) secretion of inhibitory cytokines such as TGFβ, IL-10 and IL-35, (ii) cytolysis by granzyme A or granzyme B, (iii) disruption of important metabolites including cAMP and adenosine, (iv) inhibition of DC function via the CTLA4-dependent induction of indoleamine 2,3 dioxygenase (IDO) and (v) modulation of the extracellular redox microenvironment. The red arrows denote the actions of regulatory T cells.

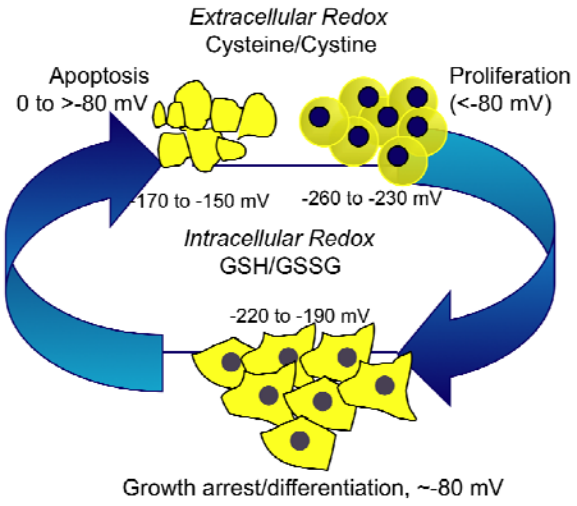

Figure 1.3 Correlated changes between cell cycle progression and the extra- and intra-cellular redox potentials. The GSH/GSSG couple represents the major intracellular redox buffer. The redox potential of the intracellular GSH/GSSG couple becomes more oxidized when cells progress from proliferation (-260 mV to -230 mV) to differentiation/growth arrest (-220 mV to -190 mV) to apoptosis (-170 mV to -150 mV). The cysteine/cystine couple is the main extracellular thiol/disulfide pool. Changes in the extracellular cysteine/cystine redox potential follows the same pattern, i.e., it is most reduced during proliferation (<-80 mV) and becomes increasingly oxidized during differentiation/growth arrest (~-80 mV) and apoptosis (0 mV to -80 mV). This figure is adapted from reference (*33*).

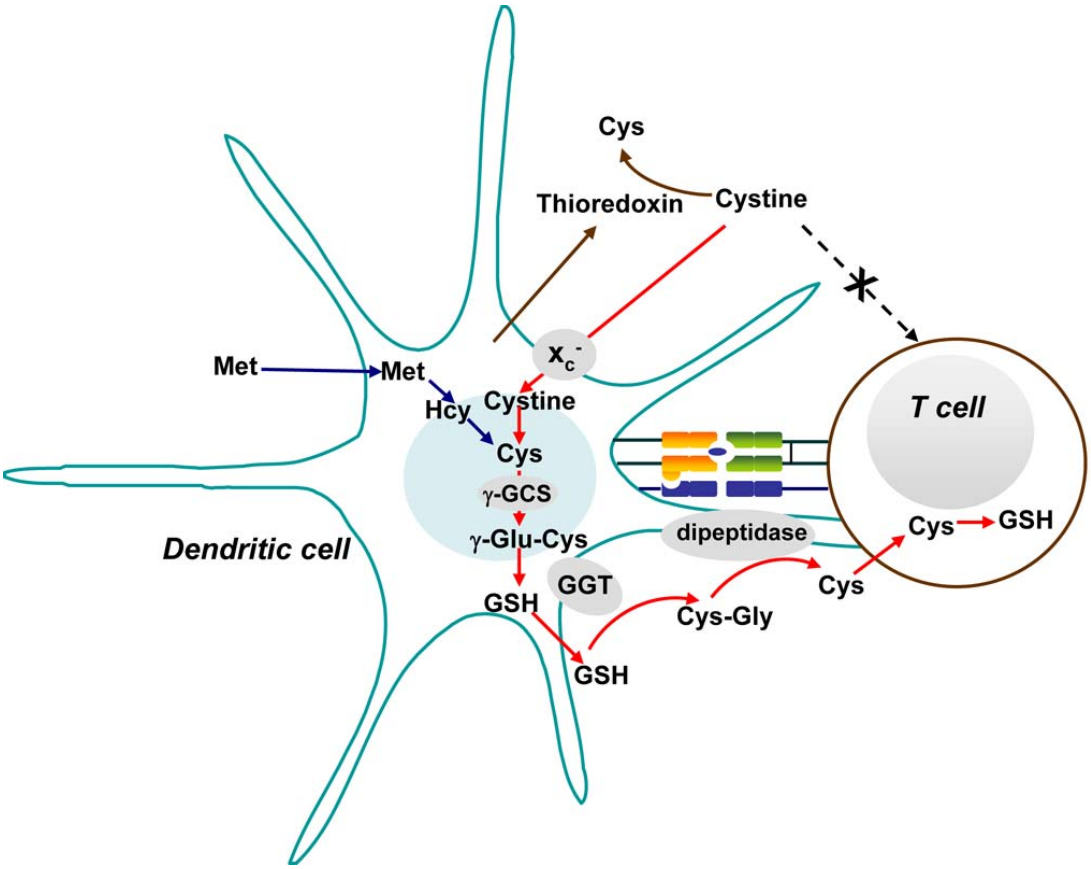

Figure 1.4 Mechanism of redox remodeling by DCs. The possible sources of extracellular cysteine that accumulates during DC and T cell co-culture include: (i) increased flux through the transsulfuration pathway leading to enhanced synthesis of cysteine from methionine, (ii) direct reduction from cystine catalyzed by extracellular thioredoxin. (iii) x_c^--dependent import of cystine, its subsequent intracellular conversion to GSH, which is exported and degraded by the ectoenzyme, γ-glutamyltranspeptidase and a membrane-bound dipeptidase to furnish cysteine. The extracellular accumulation of cysteine results in a reducing microenvironment for T cell activation and proliferation and also provides T cells with cysteine needed for the synthesis of GSH.

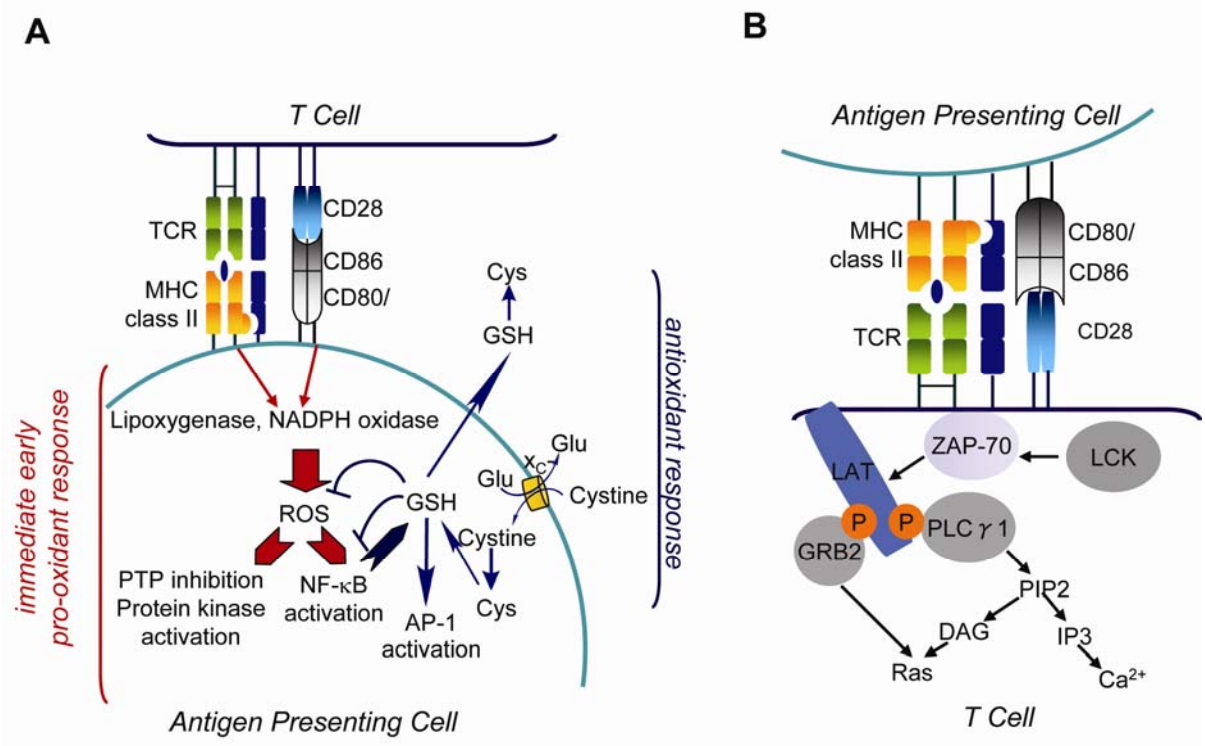

Figure 1.5 Redox signaling responses in DCs and T cells during T cell activation. (A) Redox signaling in DCs. The TCR-antigen•MHC complex interaction and the co-stimulatory signal results in an immediate early pro-oxidant response in DCs with ROS production e.g. by lipoxygenase and NADPH oxidase. Low levels of ROS act as signaling molecules to inhibit protein tyrosine phosphatases (PTPs) and activate protein kinases. ROS also activates the NF-κB pathway, which stimulates the expression of γ-glutamylcysteine ligase, thus increasing GSH synthesis. GSH activates the AP1 signaling pathway and initiates an antioxidant response. We postulate that system x_c^--dependent cystine uptake, GSH export and degradation into extracellular cysteine are stimulated as part of this response. (B) TCR signaling in T cells. Stimulation of T cells by DCs via the TCR results in phosphorylation and activation of ZAP-70 by leukocyte-specific protein tyrosine kinase (LCK). ZAP-70 directly phosphorylates the adaptor protein LAT and causes the assembly of multiprotein signaling complexes. Recruitment of the growth factor receptor bound protein 2 (GRB2) and phospholipase C γ1 (PLCγ1) to LAT leads to activation of downstream Ras and calcium signaling pathways.

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Chapter 2

Extracellular Redox Modulation by Regulatory T Cells

2.1 Abstract

We demonstrate that the mechanism of redox remodeling during mouse T cell activation involves secretion of glutathione by dendritic cells and its subsequent cleavage to cysteine. Extracellular cysteine accumulation results in a lower redox potential, which is conducive to proliferation, and changes the net redox status of exofacial protein domains. Regulatory T cells inhibit this redox metabolite-signaling pathway, which represents a previously unrecognized mechanism for immunosuppression of effector T cells.

2.2 Introduction

T cell activation and proliferation require a reducing microenvironment in the immune synapse that is provided by professional antigen presenting cells, especially dendritic cells (DCs) (*1*). The mechanisms underlying extracellular redox remodeling by DCs and redox communication during activation of $CD4^+CD25^-$ effector T cells (Teff) remain unclear but results in accumulation of extracellular cysteine (Cys_{ex}) (*1*). The proliferative response of activated Teff cells requires glutathione (GSH), an abundant intracellular antioxidant (*2*). The synthesis of GSH is limited by the availability of Cys and Teff cells are inefficient at transporting cystine (*3*), the predominant form of this amino acid in the extracellular milieu, thus creating a metabolic dependence on DCs. Naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg), which constitute <10% of total $CD4^+$ T cells maintain peripheral tolerance by suppressing autoimmune T cells (*4, 5*). The mechanism of suppression by Treg cells is complex and is incompletely understood (*5, 6*). In this study, we have tested the hypothesis that inhibition by Treg cells of DC-induced extracellular redox remodeling is a component of the Treg immunosuppressive mechanism. We demonstrate that the increase in $[Cys]_{ex}$ upon co-culture of DCs with naïve $CD4^+CD25^-$ T cells (Tn) results from the system x_c^--dependent import of cystine, its conversion to glutathione, which is subsequently exported and degraded by the ectoenzyme, γ-glutamyltranspeptidase (GGT) and a membrane bound dipeptidase, to furnish Cys. Suppression of DC-dependent T cell proliferation by Treg cells is correlated with a significant diminution in $[Cys]_{ex}$ and is abrogated by addition of exogenous Cys. The decrease in the extracellular redox potential during DC-T cell interaction and T cell activation is associated with an increase in the levels of exofacial

surface thiols in both DCs and T cells, which is diminished in the presence of Treg cells. This study elucidates the pathway of thiol release by DCs in response to T cell stimulation, demonstrates that Treg cells interfere with the process of extracellular redox remodeling and identifies a novel mechanism for immunomodulation.

2.3 Results and Discussion

Co-cultivation of Tn cells with immature DCs results in a time- and ratio-dependent increase in $[Cys]_{ex}$ (Fig. 2.1a), which is paralleled by extracellular cystine consumption (Fig. 2.2) as previously seen during antigen-specific activation of T cells (*1*). In subsequent studies, a 1:4 ratio of DC:T cells was used. When DC and T cells from syngenic and allogenic mice were employed, a substantial difference in Cys_{ex} accumulation was not observed (data not shown). While intracellular redox homeostasis is important for the operation of cellular processes (*7*), the Cys/cystine redox couple is a quantitatively significant determinant of the extracellular redox potential, which plays a critical regulatory role in cell proliferation, differentiation and apoptosis (*8, 9*). It is estimated to be ~-80 mV for DC cultures (Fig. 2.1b), which is expected to promote growth arrest/differentiation (*8, 10*). Co-culture of DCs with Tn cells decreased the redox potential to a more reducing value (-110 mV), consistent with remodeling of the extracellular potential to a value conducive to cellular proliferation (*8*).

Sustained contact between DCs and T cells appears to be required for $[Cys]_{ex}$ accumulation and was not observed in a transwell culture system (Fig. 2.3) and further accumulation of Cys was not observed following removal of T cells after co-culture with DCs for 24 h (not shown). DC-T cell contact may result in the activation of various signal

transduction pathways in DCs, which, in turn, could regulate Cys_{ex} accumulation. Among them, the NF-κB pathway, which is activated in mature DCs, plays an essential role in effective antigen presentation and T cell activation (*11, 12*). To assess whether this signaling pathway plays a role in extracellular redox remodeling, DCs were pretreated with inhibitors of NF-κB activation, pyrrolidine dithiocarbamate (PDTC) or BAY 11-7082, for 2 h, and then co-cultured with Tn cells. $[Cys]_{ex}$ was significantly diminished by these inhibitors (Fig 2.1c). Lipopolysaccharides (LPS), which induces DC maturation via the NF-κB pathway (*12*), also results in Cys_{ex} accumulation, which was abrogated by PDTC (Fig. 2.1c), thus supporting a role for the NF-κB pathway in stimulating Cys_{ex} release by DCs.

To distinguish between the alternative pathways that might contribute to Cys_{ex} accumulation (Fig. 2.1d), various approaches were employed. First, the contribution, if any, of the transsulfuration pathway to Cys_{ex} was assessed. However, radiolabel incorporation from [^{35}S]-methionine into total Cys_{ex} and GSH_{in} was not enhanced (Table 2.1) and inhibition of the transsulfuration pathway with the suicide inactivator, propargylglycine (PPG), failed to inhibit Cys_{ex} accumulation (Fig. 2.4) during DC-Tn cell co-culture. Furthermore, co-cultivation of cells in medium lacking cystine resulted in failure of $[Cys]_{ex}$ accumulation (data not shown), suggesting that extracellular cystine is the major source of Cys_{ex}. Inhibition of GSH synthesis with butathionine sulfoximine (BSO) and inhibition of GSH export with MK-571 significantly suppressed $[Cys]_{ex}$ accumulation (Fig. 2.4), indicating a role for GSH_{in} synthesis and transport by DCs for provision of Cys_{ex} needed for T cell function.

To examine the contribution of cystine transporters and GSH metabolism to Cys_{ex} accumulation, we inhibited the x_c^- and X_{AG} cystine transporters and GGT with sulfasalazine, L-aspartic acid β-hydroxamate (AβH) and acivicin respectively (Fig. 2.1d). Inhibition of the x_c^- but not the X_{AG} transporter suppressed Cys_{ex} accumulation by ~25% (Fig. 2.1e), diminished incorporation of [^{14}C]-cystine into GSH in DCs by ~20% and decreased the $[GSH]_{in}$ by ~25% (Fig. 2.5). The expression of xCT, the catalytic subunit of the x_c^- transporter (*13*), was induced in DCs co-cultured with T cells (Fig. 2.1f), indicating a role for this high affinity cystine/glutamate antiporter in DC-dependent redox remodeling. Inhibition of GGT with acivicin decreased $[Cys]_{ex}$ by >35% (Fig. 2.1e), consistent with the model that secreted GSH is a source of $[Cys]_{ex}$. When acivicin and sulfasalazine were co-administered, the $[Cys]_{ex}$ was reduced by ~70%.

Extracellular thioredoxin (Trx_{ex}), which accumulates during DC-T cell co-culture, has been proposed to function in cystine reduction under these conditions (*1*). However, the source of electrons for the operation of this catalytic cycle is not known. Secreted Trx was also observed during DC-T cell co-culture in our study, but not when either cell type was cultured alone (Fig. 2.6). If Trx_{ex} does indeed play a role in increasing $[Cys]_{ex}$, then inhibition of cystine import should not affect Cys_{ex} accumulation. However, $[Cys]_{ex}$ decreased in sulfasalazine-treated cultures (Fig. 2.1e) although Trx_{ex} levels were unchanged (Fig. 2.6). Together with the observation that secreted thioredoxin reductase, which reduces Trx, is not detected when DCs and T cells are co-cultured (not shown), our data are consistent with an alternative function of Trx_{ex} in T cell activation as suggested recently (*14*).

An increase in $[GSH]_{in}$ is correlated with the onset of T cell proliferation and Cys is the limiting reagent for GSH synthesis (*3, 15*). Increased $[Cys]_{ex}$ during co-culture of DC and T cells is associated with increased $[GSH]_{in}$ in T cells (Fig. 2.1g), which was significantly inhibited by sulfasalazine and acivicin respectively.

Next, we investigated whether Treg cells can perturb Cys_{ex} accumulation induced by DC-Tn cell interaction. In contrast to Tn cells, co-culture of Treg cells with DCs did not affect the $[Cys]_{ex}$ (Fig. 2.7). However, co-culture of DCs, Tn and Treg cells resulted in significantly decreased $[Cys]_{ex}$ compared to co-cultures of DC and Tn cells, and was sensitive to the ratio of DC:Tn:Treg cells (Fig. 2.8a). LPS stimulation of DCs increased the $[Cys]_{ex}$ and was suppressed by Treg cells (Fig 2.8b). Enhanced Cys_{ex} accumulation was observed during co-culture of LPS-activated DCs and Tn cells, which was inhibited by Treg cells (Fig 2.9). Furthermore, $[GSH]_{in}$ in Teff cells was significantly decreased under these conditions (Fig. 2.8c, d), indicating that Treg cells perturb intracellular redox homeostasis in Teff cells by interfering with extracellular redox remodeling.

Since the outcome of a successful activation event is T cell proliferation, a process that is suppressed by Treg cells, we assessed the effect of Treg cells on T cell proliferation in the presence and absence of exogenous Cys. Lower $[Cys]_{ex}$ was correlated with inhibition of T cell proliferation as measured by $[^3H]$-thymidine incorporation (Fig. 2.8e) or a mitochondrial activity (Fig. 2.10) assay. This inhibition was alleviated by provision of Cys_{ex} at concentrations seen under DC-T cell co-culture conditions (Fig. 2.8c and Fig. 2.10). Exogenous Cys did not significantly change the proliferation of Teff cells and as expected, did not induce Treg proliferation (Fig. 2.11).

The extracellular redox environment influences the equilibrium between oxidized and reduced thiols on exofacial membrane proteins (*16*) and a 30 mV potential change should lead to a 10-fold change in the ratio of reduced:oxidized Cys in proteins. The surface thiol status of T cells is responsive to reactive oxygen species levels that in turn, influences T cell functions and the susceptibility at the organismal level, to autoimmune diseases and to HIV infection (*17-19*). Hence, we investigated changes in the exofacial thiol status during T cell activation using Alexa-maleimide for thiol group labeling coupled with confocal microscopy and fluorescence-activated cell sorting (FACS) analysis (Figs. 2.8f-j). Co-culture conditions enhanced cell surface labeling on DCs and T cells by ~4 and 8-fold respectively (Fig. 2.8f-h). Hence, redox remodeling of the extracellular compartment during T cell activation affects the redox status of membrane proteins on both cell types, which might be important for signaling in the immune synapse and/or for supporting downstream effector functions. In comparison to Tn cells, co-culture of DCs with Treg cells induced a minor increase in surface thiol labeling on DCs and Treg cells (Fig. 2.12). Addition of Treg cells to DC-Tn co-cultures suppressed cell surface thiol labeling on T cells and to a lesser extent, on DCs (Fig. 2.8i,j). Similarly, Treg cells decreased surface thiol labeling in LPS-stimulated DCs. Hence, interference by Treg cells in redox remodeling of the extracellular microenvironment appears to have a more pronounced effect on T cells compared to DCs.

The ability of Treg cells to modulate metabolite signaling between DC and T cells has precedence. Thus, Treg cells induce indoleamine 2, 3-dioxygenase in DCs, which catalyzes the oxidative catabolism of tryptophan (*20*). Furthermore, TGF-β, an inhibitory cytokine involved in Treg-mediated suppression (*21*), is activated by oxidation and

inactivated by free thiols (*22*), demonstrating the need for the appropriate redox microenvironment for its function.

In summary, we have elucidated the mechanism of extracellular redox remodeling by DCs during T cell activation, which is needed for their subsequent proliferation, and demonstrated that Treg cells interfere with this process. T cells are inefficient at transporting cystine and depend on antigen presenting cells for the provision of Cys (*3, 23*), the least abundant of all amino acids in circulation (*24*). However, Cys release by DCs clearly has other consequences including shifting the ambient redox poise to be more reducing, promoting cell proliferation and changing the redox status of many cell-surface proteins, which await identification. Many exofacial membrane protein domains and extracellular proteins are Cys-rich and their oxidation state and function are influenced by the redox potential of the extracellular compartment (*16, 25*). Subtle changes in the extracellular redox status may cause profound functional changes in redox sensitive proteins, shaping the outcome of DC-T cell interaction. The ability of Treg cells to intervene in this process represents a previously unrecognized mechanism for immunosuppression of autoreactive T cells.

2.4 Materials and Methods

2.4.1 Mice

BALB/c mice (7-10 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained in pathogen-free animal facilities at the University of Michigan. All procedures for animal handling were performed in accordance with the protocols approved by the University of Michigan.

2.4.2 Cell purification and culture

DCs were isolated from bone marrow by a previously described method (*26*), with some modifications. Briefly, bone marrow cells from the BALB/c mice were depleted of red blood cells and plated at 2 x 10^6 cells/ml for 7 days in recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF, 20 ng/ml, BD Biosciences) and recombinant murine interleukin 4 (IL-4, 20 ng/ml, BD Biosciences) in DMEM supplemented with 100 μg/ml penicillin and streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol (2-ME), 1 mM pyruvate, 1:100 nonessential amino acids, and 10% heat-inactivated fetal bovine serum. The culture medium was changed and the floating cells were discarded every two days.

Tn ($CD4^+CD25^-$) and Treg ($CD4^+CD25^+$) cells were purified from mouse lymph nodes by magnetic-activated cell sorting using an AutoMACS sorter (Miltenyi Biotec) as described (*27*). Flow cytometry analysis of the purity of Treg cells and Teff cells shows > 90% $CD4^+CD25^+$ with > 85% expressing $Foxp3^+$ and > 95% $CD4^+CD25^-$, respectively (Fig. 2.13). For activated Teff cells, purified cell populations were cultured in 24-well plates (1 ml) supplemented with 0.5 μg/ml anti-CD3 and irradiated splenocyte feeders (1:3).

DCs (5 x 10^5 /well) were co-cultured in 24 well plates with or without naïve Teff cells at 1:1, 1:2 and 1:4 ratio for 48 h at 37°C in a 5% CO_2 incubator in RPMI medium supplemented with 100 μg/ml penicillin and streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 2.5% heat-inactivated fetal bovine serum in the presence of anti-CD3 antibody (1 μg/ml). Sulfasalazine (SAS, 500μM), acivicin (ACV, 250 μM), L-Aspartic acid β-hydroxamate (AβH, 400 μM), propargylglycine (PPG, 2.5 mM) and

butathionine sulfoximine (BSO, 200 μM) were added to the co-culture to inhibit the x_c^- cystine transporter, γ-glutamyltranspeptidase (GGT), X_{AG} cystine transporter, γ-cystathionase and γ-glutamylcysteinyl synthetase, respectively. For the inhibition of the NF-κB pathway, DCs were pretreated with 200 μM pyrrolidine dithiocarbamate (PDTC) or 10 μM BAY 11-7082 for 2h and then co-cultured with naïve Teff cells in fresh medium. In transwell experiments, DCs were co-cultured either directly with naïve Teff cells or with naïve Teff cells placed in transwell chambers (Millicell, 0.4 μm pore size; Millipore, Bedford, MA). For the Treg experiments, DCs were co-cultured respectively with Treg cells (1:1), naïve Teff cells (1:4) and naïve Teff and Treg cells together (1:4:1, 1:4:2), or DCs were treated with lipolysaccharide (LPS, 100 ng/ml) in the absence and presence of Treg cells for 48h. For activated Teff cells, co-cultures were performed in 24 well plates at a DC: Teff cell ratio of 1:4 in DMEM medium supplemented with 100 μg/ml penicillin and streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 1 mM pyruvate and 1:100 nonessential amino acids.

2.4.3 Measurement of thiols and metabolic labeling

Extracellular cystine and Cys concentration was measured using the HPLC method as described (*28*). To measure the extracellular total GSH and homocysteine by mass spectrometry, culture supernatants were reduced by 10 mM dithiothreitol for 15 min followed by alkylation with 5 mM N-ethylmaleimide (NEM) for 30 min at room temperature. GSH, homocysteine and Cys were used as external standards and 50 μM NEM-derivatized 3-D_2-Cys was used as an internal standard. The samples and standards were analyzed by multiple reaction monitoring (MRM) mass spectrometry using the 4000 Q TRAP® LC/MS/MS System from Applied Biosciences.

Intracellular GSH concentration and [^{35}S]-methionine incorporation into GSH was measured as described previously (*29*). Results were normalized to the protein concentration. Alternatively, L-[^{14}C]-cystine was employed to follow its contribution to the intracellular GSH. For this, 5 µl/ml L-[^{14}C]-cystine (0.02 mCi/ml, PerkinElmer) was added to the culture medium to obtain a final activity of 0.1 µCi/ml and the samples were processed as described for radiolabeled methionine.

2.4.4 Western blot analysis

Supernatants from DCs cultured with activated T cells in the presence and absence of sulfasalazine were analyzed by Western blot to detect the extracellular thioredoxin as described (*1*). Rabbit polyclonal antibody against human thioredoxin (courtesy of Dr. Vadim Gladyshev, University of Nebraska) was employed for detection of thioredoxin.

To detect the xCT expression levels, DCs were cultured with and without naïve Teff cells for 36h and harvested in lysis buffer. Aliquots of cell lysates (20 µg) were boiled and loaded on to a 10% SDS polyacrylamide gel and electroblotted to a PVDF membrane. Expression of xCT was investigated by immunoblotting with the polyclonal xCT antibody (1:2000) (Abcam) and the secondary horseradish peroxidase-linked anti-rabbit IgG antibody (1:2500). Blots were developed by using the chemiluminescent horseradish peroxidase system as per the vendor's protocol (Sigma).

2.4.5 T cell proliferation assay

Naïve Teff cells (2 x 10^5) were cultured with varying numbers of Treg cells for 72 h in flat 96-well plates with APCs (either DCs (5 x 10^4) or irradiated splenocytes (6 x 10^5)) and 1 µg/ml anti-CD3. Cys (50 µM and 100 µM final concentration) was added every 24 h during the culture. T cell proliferation was assayed by measuring incorporation of [^{3}H]

thymidine (1 μCi/ml, PerkinElmer) during the last 6 h of culture. Alternatively, proliferation was measured by using the "CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay" kit (Promega) according to the manufacture's instructions.

2.4.6 Flow cytometry and confocal microscopy

Naïve Teff cells cultured with or without DCs for 1h and 48h were washed with ice-cold media. Alexa-maleimide (ALM)-488 (Invitrogen) was added to the cell suspension at a final concentration of 5 μM and incubated for15 min on ice. Cells were washed three times with cold PBS, fixed in 2% paraformaldehyde and then analyzed by flow cytometry using the FACSCalibur instrument (BD phamingen). Data were analyzed using the Flowjo software (Treestar). DCs cultured with or without naïve T cells for 1h and 48h were washed with ice-cold media and then stained with 5 μM ALM-488 for 15 min on ice. Cells were washed three times with cold PBS and detached by incubating with accutase (Phoenix flow systems) for 10 min. After centrifugation, DCs were fixed in 2% paraformaldehyde and analyzed by flow cytometry. For the Treg experiments, DCs were co-cultured respectively with naïve Teff cells (1:4) and naïve Teff and Treg cells together (1:4:2), or DCs were treated with LPS (100 ng/ml) in the absence and presence of Treg cells for 36h. Cell surface thiols on DCs and T cells were determined as described above.

For the confocal microscopy experiments, DCs were cultured with or without naïve Teff cells in 8-well chamber slides (Labtek) for 36h. Since naïve Teff cells can not survive for 36h, they can not be used as a control for activated Teff cells. We thus used activated Teff cells cultured with or without DCs for 16h to detect the cell surface thiol change on Teff cells. DCs and Teff cells were washed with ice-cold media and then stained with 5μM ALM-594 for 15 minutes on ice. Cells were washed three times with

cold PBS, fixed in 4% paraformaldehyde and then mounted. The samples were examined using an Olympus FV500 confocal microscope and photographed.

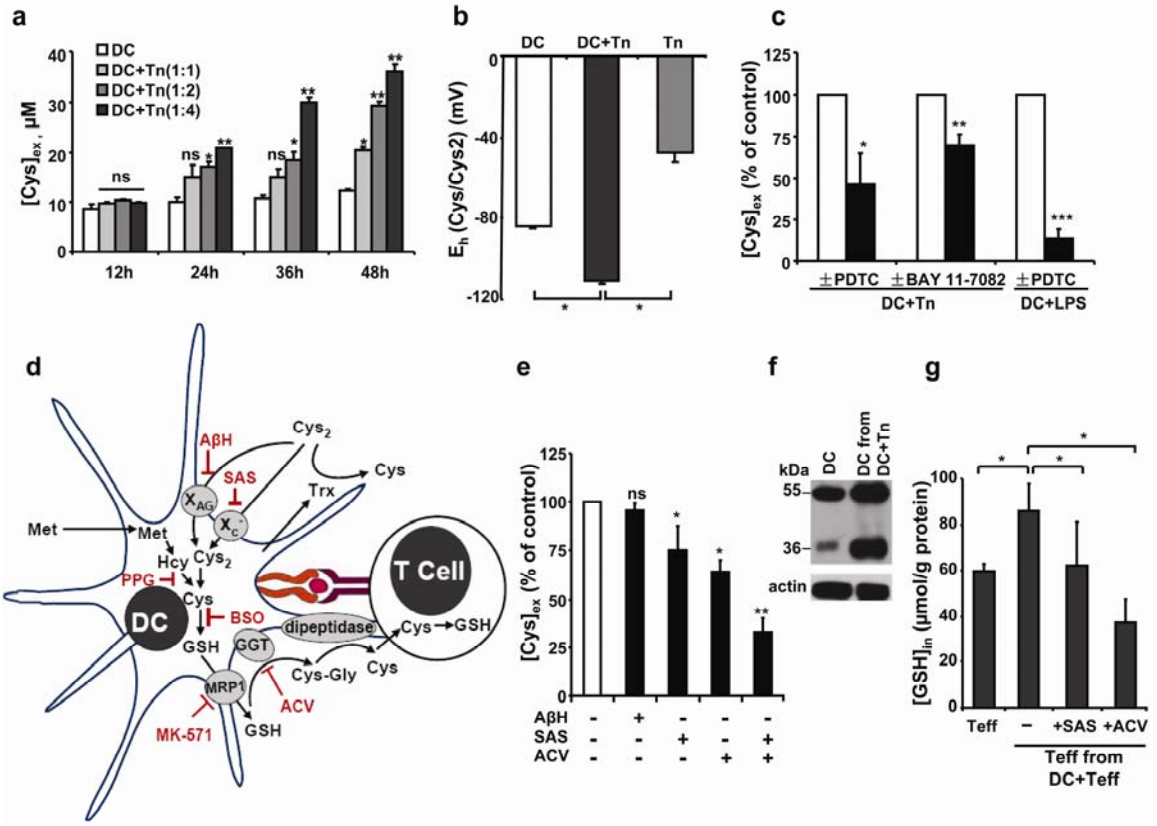

Figure 2.1 Mechanism of Cys_{ex} accumulation. (a) DCs were co-cultured with Tn cells at a 1:1, 1:2 or 1:4 ratio for different durations, and the $[Cys]_{ex}$ was measured. **(b)** Changes in the extracellular Cys/cystine redox potential at 36 h. The extracellular Cys/cystine redox potential was calculated according to the Nernst equation: $E_h = E_o + RT/2F \ln([cystine]/[Cys]^2)$, using E_o=-250 mV (pH=7.4). **(c)** Effect of NF-κB pathway inhibitors on $[Cys]_{ex}$. **(d)** Cys metabolism during DC-T cell interaction and the effects of inhibitors. Propargylglycine (PPG), butathionine sulfoximine (BSO), sulfasalzine (SAS), L-aspartic acid β-hydroxamate (AβH), acivicin (ACV) and MK-571 inhibit γ-cystathionase, γ-glutamylcysteinyl synthetase, the x_c^- and the X_{AG} transporter, γ-glutamyltranspeptidase, and the multidrug resistance protein 1 (MRP1) respectively. **(e)** Inhibition of $[Cys]_{ex}$ by various inhibitors. DCs were co-cultured for 36 h with Tn cells (1:4) ± 400 µM AβH, or ± 500 µM SAS, or ± 250 µM ACV or ± SAS+ACV. $[Cys]_{ex}$ is expressed as a percent of the concentration in untreated DC-T cell co-culture medium. The data represent the mean ± SD of at least 4 experiments with different batches of cells. **(f)** xCT expression in DCs cultured ± Tn cells for 36 h. xCT migrates as 35 and 55 kDa bands. **(g)** $[GSH]_{in}$ in Teffs cells co-cultured with DCs (1:4) for 16 h ± SAS or ACV. Representative data from one of three independent experiments are shown for **a, b, c, f, g**. (*, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$; two tailed Student's t-test).

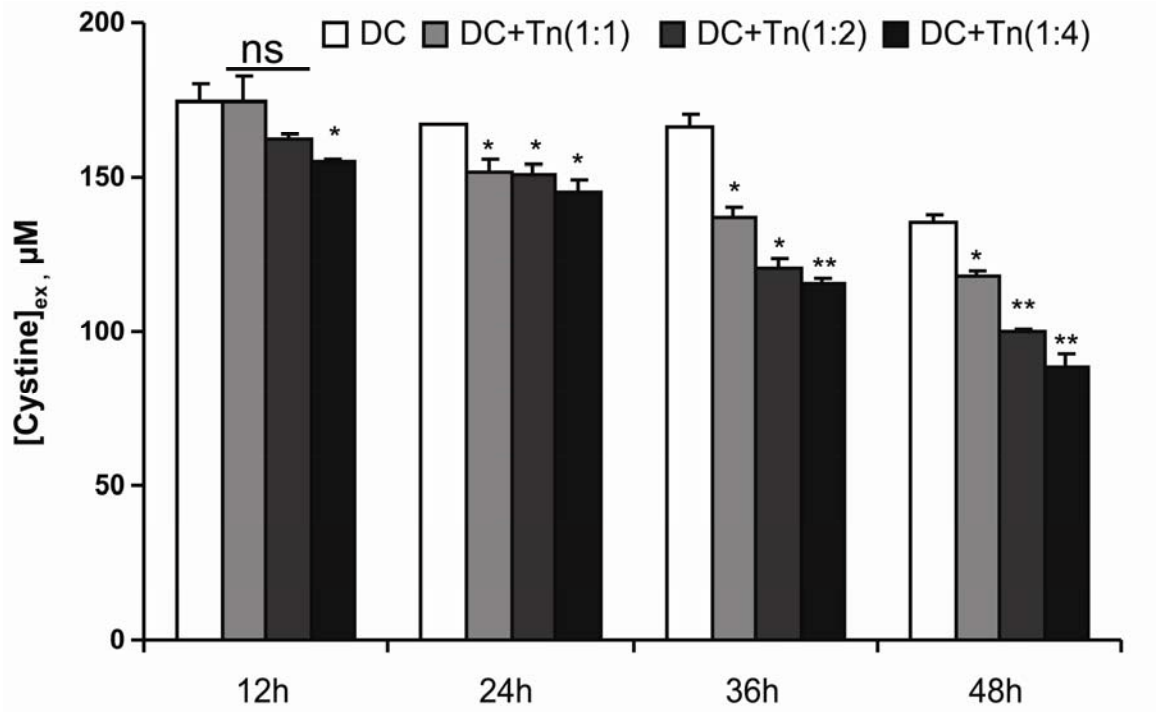

Figure 2.2 Extracellular cystine consumption during DC and Tn cell co-culture. DCs were co-cultured with Tn cells at a 1:1, 1:2 and 1:4 ratio for different durations, and the cystine concentration in the conditioned medium was determined. Data shown are the mean ± SD and are representative of 3 independent experiments. Student's t-test revealed a significant consumption of cystine from the media by DCs, which was both DC:T cell ratio- and time-dependent. (*, $p<0.05$; **, $p<0.005$).

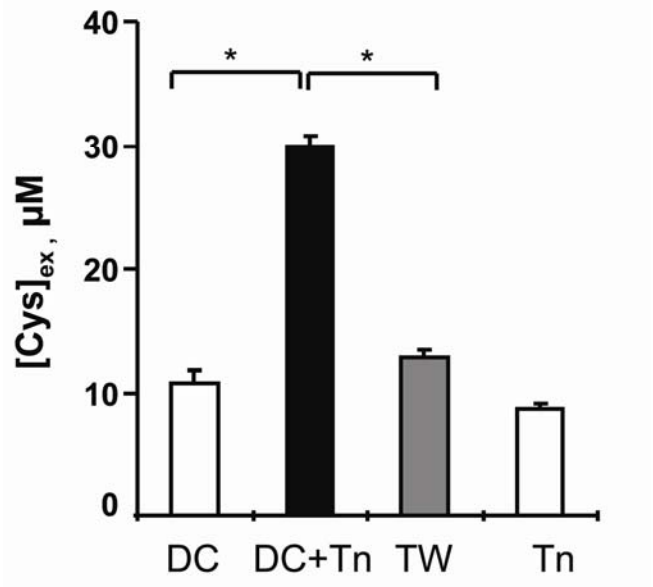

Figure 2.3 Effect of transwell culture on Cys_{ex} accumulation. DCs were co-cultured with Tn cells (1:4) (black bar) or with Tn cells (1:4) placed in the upper chamber of a transwell (TW) system (grey bar) for 36 h. Representative data (mean ± SD) from one of three independent experiments are shown. Student's t-test revealed a significant increase in Cys accumulation only when DCs were in direct contact with Tn cells. (*, $p<0.05$).

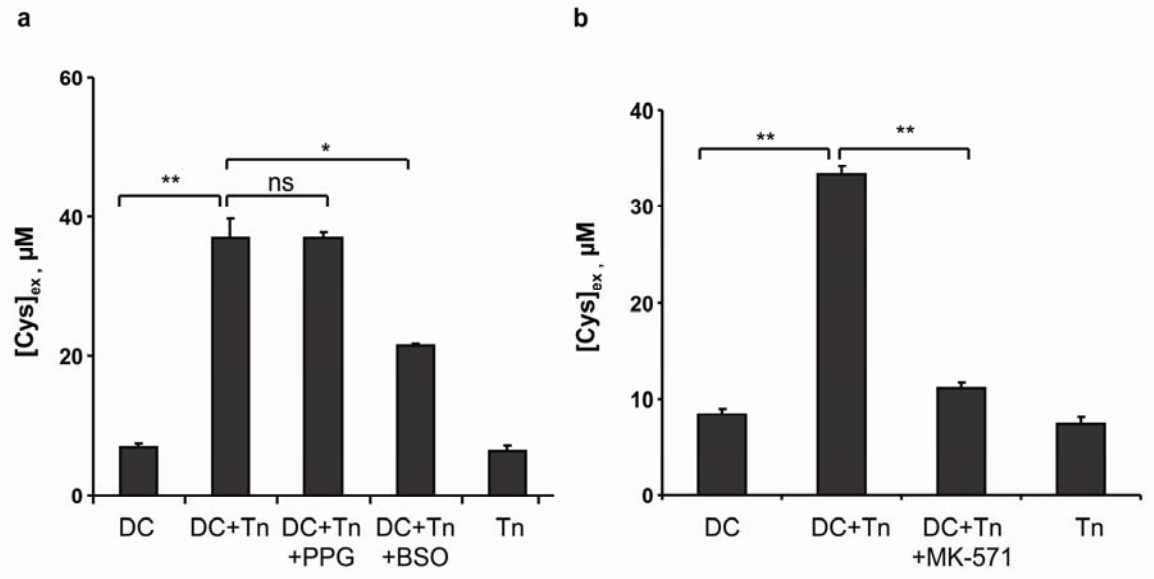

Figure 2.4 Inhibition of GSH synthesis and GSH export but not the transsulfuration pathway suppresses Cys_{ex} accumulation. DCs were co-cultured with Tn cells (1:4) for 36 h in the absence or presence of **(a)** 2.5 mM propargylglycine (PPG) or 200 μM buthionine sulfoximine (BSO) or **(b)** 50 μM MK-571. PPG, BSO, and MK-571 inhibit γ-cystathionase, γ-glutamylcysteinyl synthetase, and the multidrug resistance protein 1 respectively. Representative data (mean ± SD) from one of two independent experiments are shown. Student's t-test revealed significant inhibition of DC-T cell-induced Cys_{ex} accumulation in the presence of either BSO or MK-571 but not PPG. (*, $p<0.05$; **, $p<0.005$).

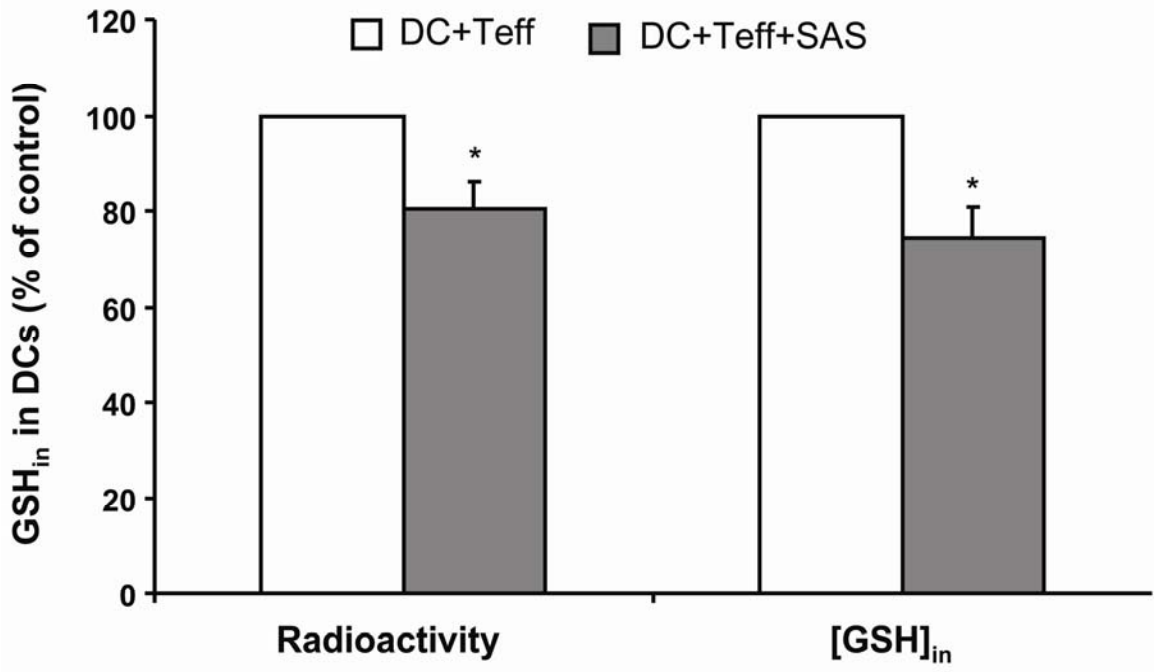

Figure 2.5 Inhibition of the x_c^- transporter decreases $[GSH]_{in}$ and GSH radioactivity incorporation in DCs. DCs were co-cultured with Teff cells (pre-activated $CD4^+CD25^-$ T cells) (1:4) ± 500 μM sulfasalazine (SAS) for 16 h. L-[^{14}C]-cystine was added to the culture medium to a final activity of 0.1 μCi/ml at the beginning of the experiment and its incorporation into GSH_{in} and the $[GSH]_{in}$ in DCs were measured. Results are expressed as a percent of untreated co-culture control. Data are the mean ± SD of two independent experiments. Student's t-test revealed significant inhibition of radioactivity incorporation and a decrease in $[GSH]_{in}$ in the presence of SAS. (*, $p<0.05$).

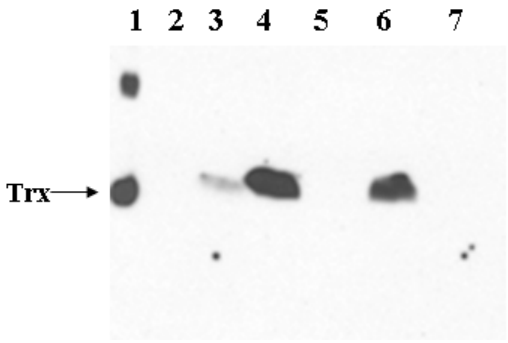

Figure 2.6 Inhibition of Cys_{ex} accumulation by SAS does not significantly affect Trx secretion. Medium from cell cultures treated ± 500 μM SAS were examined by Western blot analysis for the presence of Trx. 1: Trx standard; 2: DC medium; 3: T cell medium; 4: DC-T co-culture medium; 5: DC culture medium + SAS; 6: DC-T co-culture medium + SAS; 7: T cell medium + SAS.

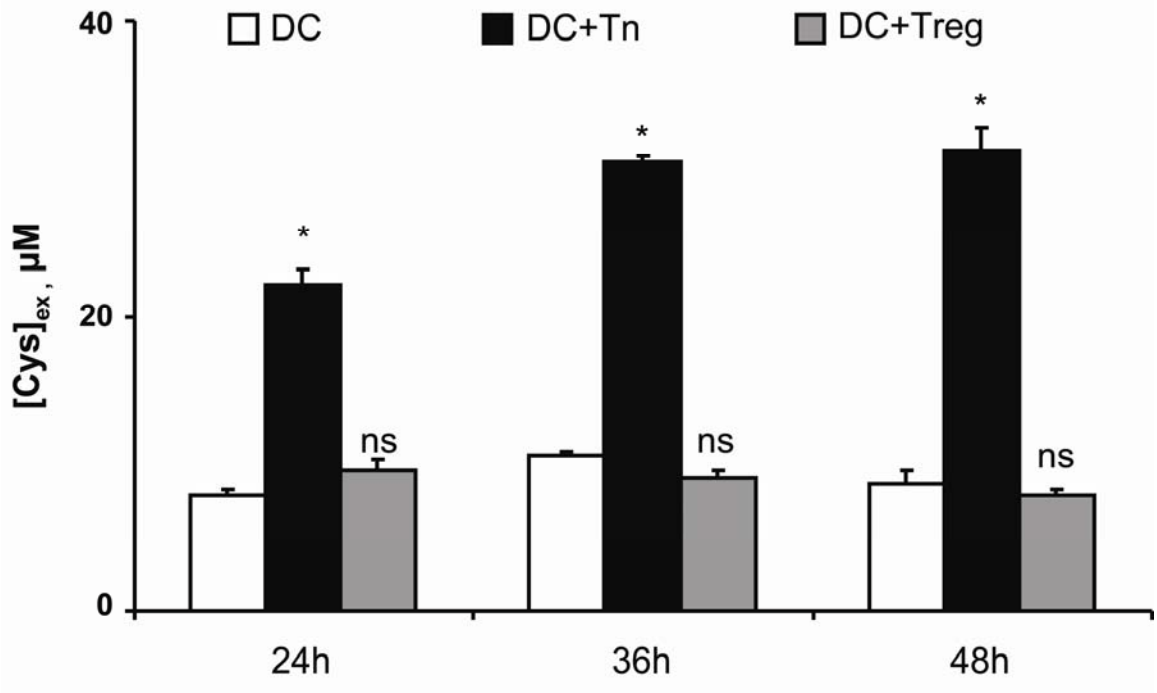

Figure 2.7 Co-culture of DCs with Tn cells, but not with Treg cells results in Cys_{ex} accumulation. DCs were co-cultured with either Tn cells (1:4) or with Treg (1:4) cells in the presence of 1 µg/ml anti-CD3 antibody. (*, $p<0.05$; ns, not significant).

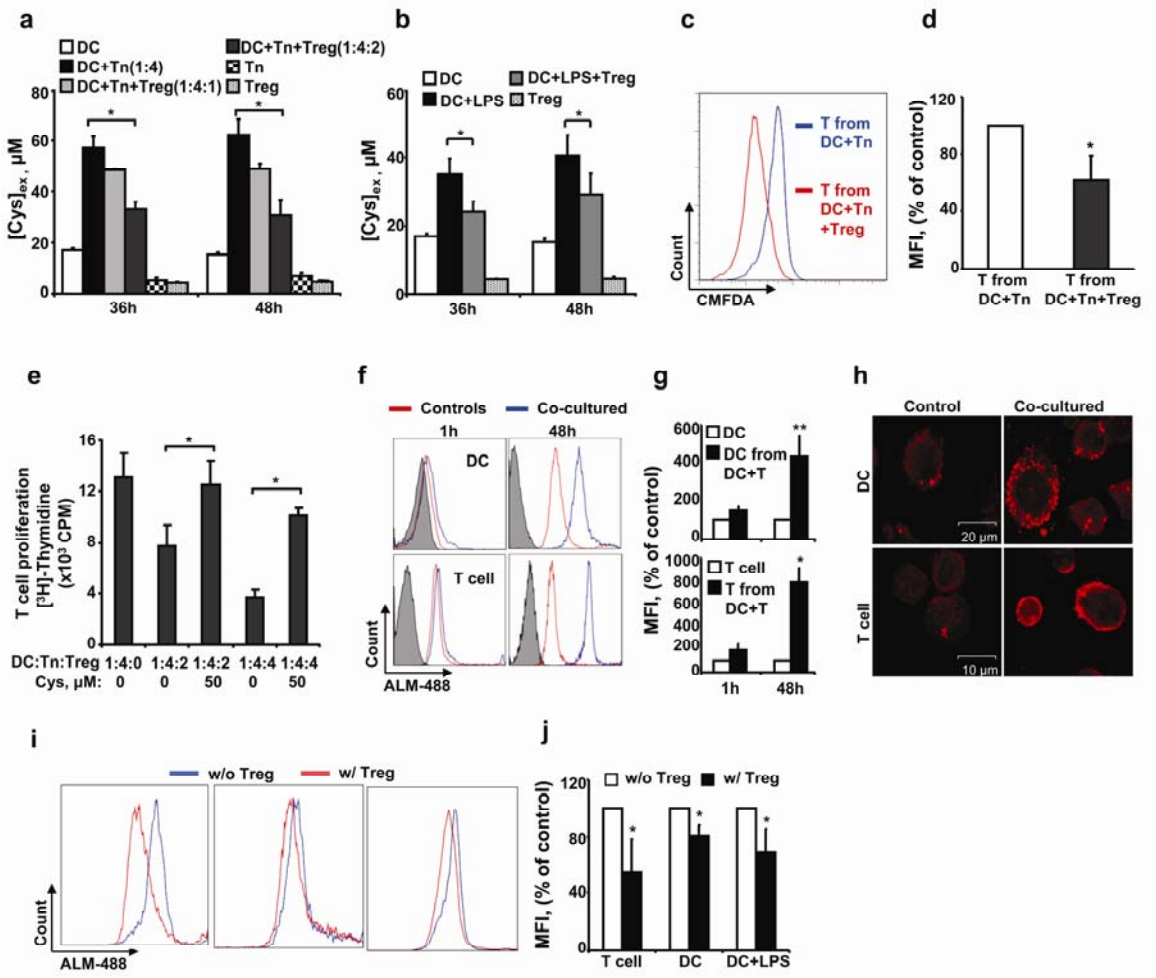

Figure 2.8 Treg-mediated extracellular redox remodeling. (a) $[Cys]_{ex}$ during co-culture of DCs with Tn cells (1:4) or with Tn+Treg cells (1:4:1 or 1:4:2). **(b)** $[Cys]_{ex}$ in DC+LPS±Treg (1:2). **(c)** Treg-mediated suppression of $[GSH]_{in}$ in Teffs measured by labeling with chloromethylfluorescein diacetate (CMFDA) and **(d)** quantitative analysis. Student's t-test revealed a significant reduction in DC+T cell- and DC+LPS-induced $[Cys]_{ex}$ (panels a, b, n=4) and GSH_{in} labeling in T cells (panel d, n=2) in the presence of Treg cells. **(e)** Teff proliferation ± Tregs and with the addition of Cys to the medium as measured by the $[^3H]$-thymidine incorporation assay. Student's t-test revealed significant inhibition of proliferation of Teffs by Tregs, which was abrogated by addition of exogenous Cys (n=3). **(f)** Cell surface thiol levels on DCs and T cells as a function of co-culture using Alexa-maleimide 488 (ALM-488) staining followed by FACS analysis. **(g)** Quantification of the mean fluorescence intensity (MFI) data shown in panel f (n=4). **(h)** Confocal microscopy using Alexa-maleimide 594 staining shows an increase in cell surface thiol levels on DCs and T cells as a function of co-culture as compared to single culture. **(i)** Tregs suppress surface thiol levels on DCs, T cells and LPS-activated DCs. **(j)** Quantitative analysis of FACS data shown in panel i (n =3). Data represent the mean ± SD of independent experiments (n as indicated in each section). (*, $p<0.05$; **, $p<0.005$; two tailed Student's t-test).

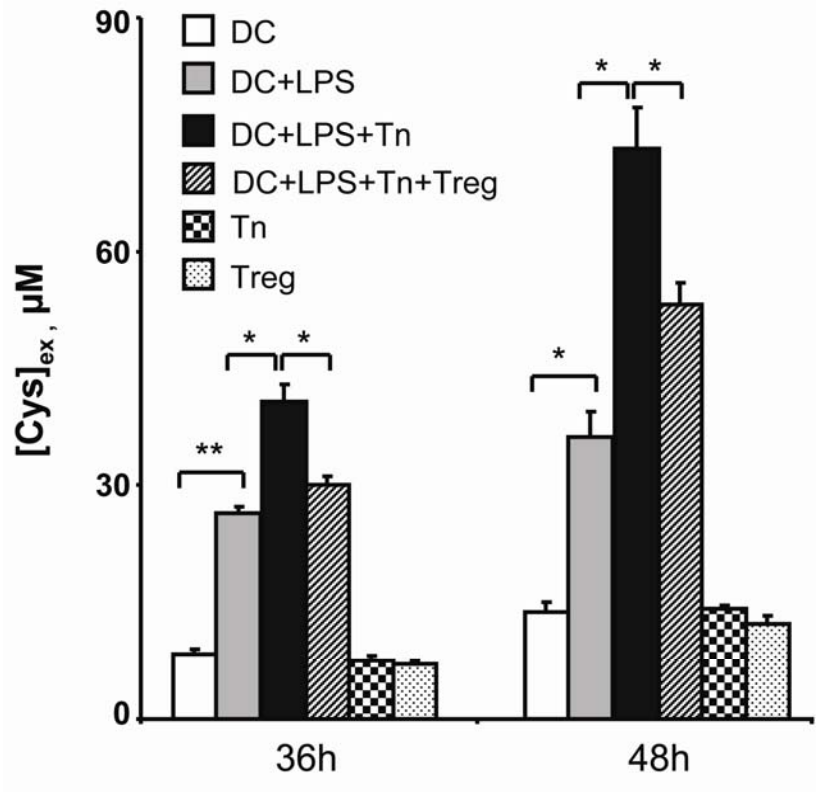

Figure 2.9 Treg cells suppress Cys_{ex} accumulation induced during co-culture of LPS-activated DCs and Tn cells. LPS-stimulated DCs were co-cultured with Tn cells in the absence or presence of Treg cells (1:4:2). Representative data (mean ± SD) from one of two independent experiments are shown. Student's t-test revealed significant inhibition of Cys_{ex} accumulation by Treg cells. (*, $p<0.05$; **, $p<0.005$).

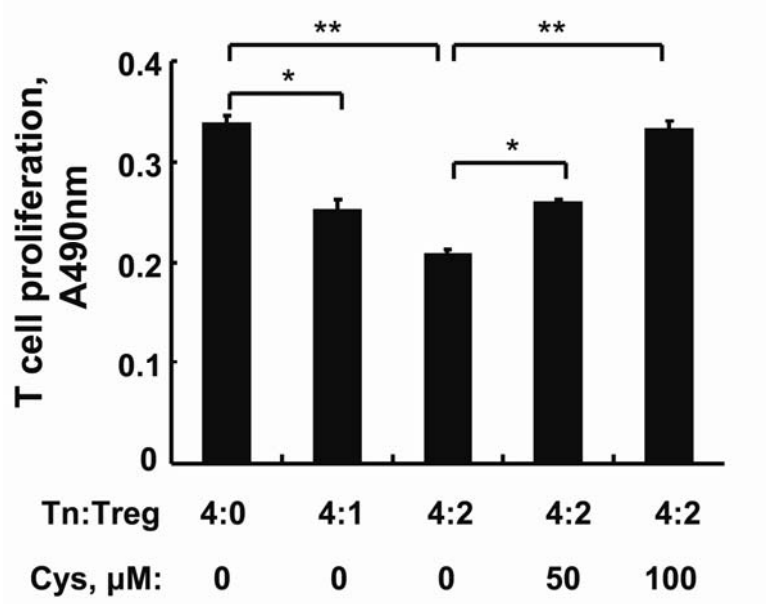

Figure 2.10 Exogenous Cys abrogates Treg-mediated suppression of T cell proliferation. T cell proliferation in the absence and presence of Treg cells and with the addition of Cys to the medium was measured by the "CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay". Data represent the mean ± SD (n=3). Statistical analysis was performed using Student's t-test (*, $p<0.05$; **, $p<0.005$).

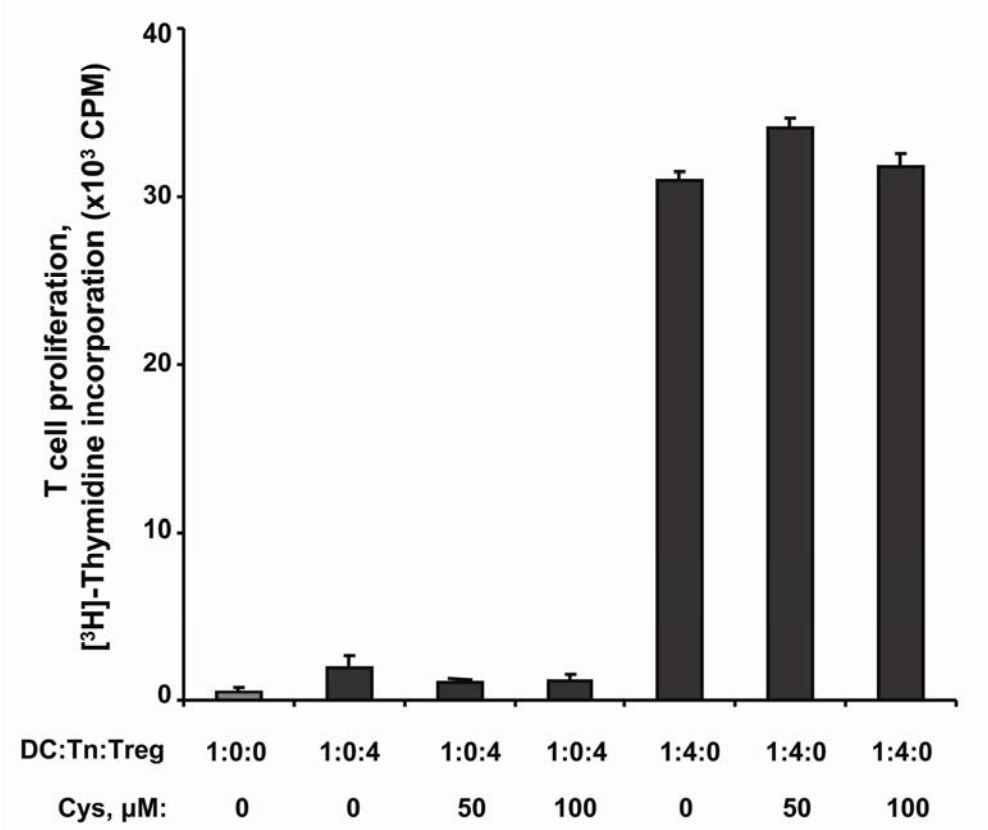

Figure 2.11 Exogenous Cys does not change Teff and Treg cell proliferation. DCs were co-cultured with either Tn or with Treg in the presence of different concentrations of exogenous Cys. Teff and Treg cell proliferation was measured by the [^{3}H]-thymidine incorporation assay. Data represent the mean ± SD of two independent experiments performed in duplicates.

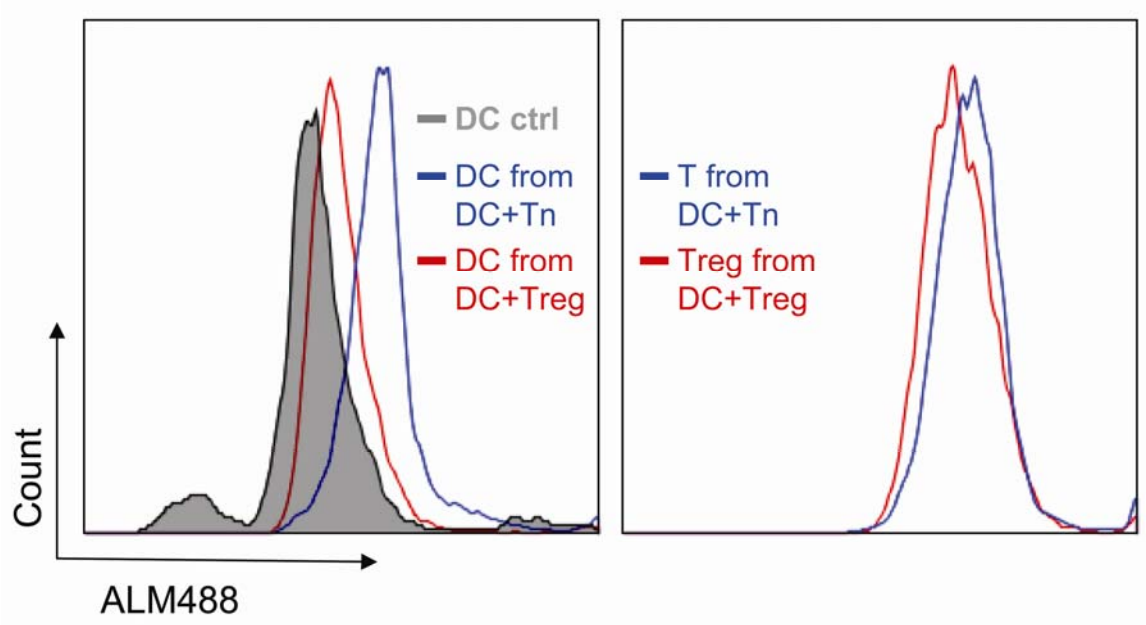

Figure 2.12 Treg cells cause relatively small changes in cell surface thiols on DCs (left panel, red line) and on T cells (right panel, red line). DCs were co-cultured with Tn or Treg cells at a 1:2 ratio for 36 h. Cell surface thiol levels on DCs and T cells were determined by FACS analysis. As a control, the effect of Tn cells on DCs cell surface thiols (blue line, left panel) is also shown. Results shown are representative of two independent experiments.

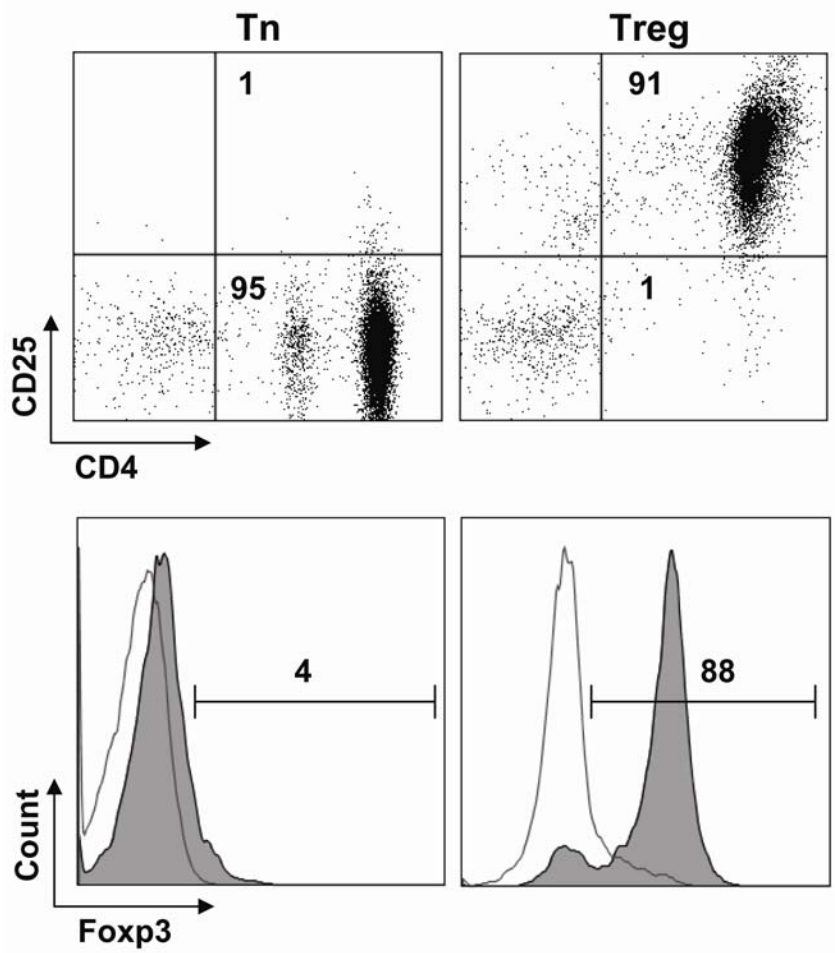

Figure 2.13 Purity of Treg and Tn cells. Tn cells and Treg cells were stained with anti-CD4-Alexa 647, anti-CD25-PE and anti-Foxp3-FITC and analyzed by flow cytometry. The numbers inside the boxes indicate percentage of $CD4^+CD25^-$, $CD4^+CD25^+$ and $Foxp3^+$ cells.

Table 2.1

Incorporation of [^{35}S]-Met into intracellular GSH and extracellular Cys.

	DCs	DCs from DC-T co-culture
$[GSH]_{in}$ (μmol/g protein)	112 ± 12.1	89.4 ± 22.1
GSH radioactivity (dpm/protein)	53.4 ± 9.2	40.7 ± 11.3
^{35}S-incorporation into GSH[a] (dpm/μM)	2.13 ± 0.30	2.22 ± 0.28
$[tCys]_{ex}$ (μM)	198.3 ± 8.9	149.5 ± 40.3
tCys radioactivity (dpm)	198.5 ± 20.5	142.0 ± 25.5
^{35}S-incorporation into tCys (dpm/μM)	1.00 ± 0.06	1.04 ± 0.10

[a] ^{35}S-incorporation in DCs was determined as the ratio of GSH_{in} (or $tCys_{ex}$) radioactivity to $[GSH]_{in}$ (or $[tCys]_{ex}$) concentration at 16 h in DCs cultured alone or with activated Teff cells. Representative data from one of two independent experiments are shown.

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Chapter 3

Regulatory T Cells Interfere with Glutathione Metabolism in Dendritic Cells and T Cells

3.1 Abstract

Naturally occurring $CD4^+CD25^+Foxp3^+$ Tregs suppress proliferation of Teffs by mechanisms that are not well understood. We have previously demonstrated a novel mechanism of Treg suppression, i.e., interference with extracellular redox remodeling that occurs during activation of T cells by DCs. In this study, we demonstrate that Treg-mediated redox perturbation is antigen-dependent but not antigen-specific, is cytotoxic T-lymphocyte antigen 4 (CTLA-4)-dependent and requires cell-cell contact. Tregs decrease expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis, leading to lower intracellular GSH synthesis in lipopolysaccharide-stimulated DCs. We have previously shown that DC-derived GSH is a major source of extracellular cysteine that accumulates during DC-dependent T cell activation. Additionally, Tregs might contribute to restricting extracellular cysteine accumulation by a competitive uptake mechanism; they partition cysteine more proficiently to the oxidation product, sulfate, while Teffs divert more of the cysteine pool towards protein and GSH synthesis. Tregs appear to block GSH redistribution from the nucleus to cytoplasm in Teffs, which is abrogated by the addition of exogenous cysteine. Together, these data provide novel insights into modulation of sulfur-based redox metabolism by Tregs leading to suppression of T cell activation and proliferation.

3.2 Introduction

A fundamental property of the immune system is to distinguish self from non-self and this education begins at the thymus where autoreactive T cells are recognized and eliminated. However, a small fraction of autoimmune T cells escape to the periphery and cause damage to host tissues. Tregs, by mechanisms that are not well understood, are able to inhibit autoimmune T cells to maintain self-tolerance and immune suppression (*1-3*). Tregs also play important roles in anti-tumor responses as well as transplantation immunity. Dysregulation of Treg function has been shown to be involved in different kinds of immunological diseases ranging from the digestive to the central nervous system (*4, 5*).

Tregs deploy various strategies to mediate their suppressive activity including: i) secretion of immunosuppressive cytokines such as TGF-β and IL-10; ii) cytolysis by granzyme secretion; iii) metabolic disruption e.g. by adenosine; iv) suppression of DC function e.g. via induction of indoleamine 2, 3-dioxygenase (IDO) (*6-8*) and (v) perturbing DC-dependent extracellular redox remodeling leading to restricted extracellular cysteine (Cys_{ex}) availability for naïve T (Tn) cells (*9*). A key role for CTLA-4, a co-receptor expressed preferentially on Tregs, is implicated in the Treg suppression mechanism (*1, 8, 10*). CTLA-4 interacts with CD80/CD86 on antigen presenting cells (APCs) and transduces an intracellular inhibitory signal to APCs. Thus, one strategy for Treg-dependent immunosuppression is via down-regulation of APC function (*1, 11*).

In addition to the T cell receptor (TCR)-antigen:major histocompatibility complex (MHC) class II interaction, costimulatory signals and cytokines, T cell activation and proliferation also requires a reducing microenvironment that is shaped mainly by APCs,

especially DCs (*9, 12, 13*). Upon stimulation by T cells, DCs increase uptake of cystine via the x_c^- cystine transporter, and by a convoluted metabolic route involving the γ-glutamyl cycle, furnish Cys_{ex}, resulting in a relatively more reducing redox potential that is conducive to T cell proliferation (*2, 9*). Furthermore, cysteine is needed by T cells for synthesis of GSH, which provides reducing power for DNA synthesis (*14*) and for cell cycle progression from the G1 to S phase (*15, 16*). Although extracellular cystine is relatively abundant, naïve T cells are inefficient at transporting the oxidized form of the amino acid and depend on DC-derived cysteine to meet their metabolic needs (*17*). DCs, by controlling the Cys_{ex} level, are able to affect intracellular GSH levels and subsequent redox signaling pathways in T cells (*2*).

The physiological relevance of redox remodeling is demonstrated by the dramatic increase in non-protein thiols in lymphoid tissues following immunization (*18*). Additionally, Payer's Patches from the gut show very low thiol staining because resident APCs from lamina propria lack the x_c^- transporter for cystine. However, under inflammatory conditions as in inflammatory bowel diseases, infiltration of peripheral APCs with high x_c^- transporter expression allows Cys_{ex} accumulation, promoting activation and hyperreactivity of lamina propria T cells (*19*). We have demonstrated that Tregs suppress Cys_{ex} accumulation, and that this is correlated with suppression of T cell activation and proliferation (*9*). However, the mechanism by which Tregs interfere with the redox signaling crosstalk between DCs and Teffs is unknown.

In this study, we demonstrate that the Tregs decrease Cys_{ex} levels in an antigen-dependent, antigen-nonspecific and CTLA-4-dependent manner. We show that Tregs use multiple strategies for extracellular redox remodeling including modulation of DC and

Teff GSH metabolism and competitive uptake of cysteine. Together, these studies provide the first mechanistic insights into how Tregs influence redox metabolism in DCs and consequently, in Teffs.

3.3 Materials and Methods

3.3.1 Animals

DO11.10 TCR transgenic mice were a generous gift from Dr. Nicholas Lukacs (University of Michigan) and were bred at our animal facility. BALB/c mice and CD1 mice (7-10 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME). All procedures for animal handling were performed in accordance with the protocols approved by the University's Committee on Use and Care of Animals.

3.3.2 Cell preparation and cell culture conditions

DCs were obtained from bone marrows of the DO11.10 mice and induced by recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 (R&D systems), as described previously (*9, 20*). Immature bone marrow-derived DCs were harvested at day 7 and used in T cell co-cultures as APCs.

Treg ($CD4^+CD25^+$) and Tn ($CD4^+CD25^-$) cells were isolated from mouse spleen and lymph nodes by magnetic-activated cell sorting using an AutoMACS sorter (Miltenyi Biotec) as described (*9, 21*). For activation of Teffs, purified Tn cells were cultured in 24-well plates (1 ml) supplemented with 1 μM $OVA_{323-329}$ antigen and irradiated splenocyte feeders (1:3) or DCs (2:1). The same conditions were employed for activation of Tregs, except that 20 ng/ml IL-2 (R&D systems) was also added to the medium.

DCs (5 x 10^5 /well) were co-cultured in 24 well plates with Tn cells (1:4) with or without Tregs (1:4:2) for 48 h at 37˚C in a 5% CO_2 incubator in RPMI medium supplemented with 100 μg/ml penicillin and streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 2.5% heat-inactivated fetal bovine serum in the presence of either 1 μM $OVA_{323-329}$ antigen or anti-CD3 antibody (1 μg/ml). Alternatively, DCs were treated with LPS (100 ng/ml) in the absence and presence of Tregs and 1 μM $OVA_{323-329}$ for 48 h. Anti-CTLA-4 antibody (100 μg/ml) was added to the DC+Tn+Treg or DC+LPS+Treg co-culture to block the function of CTLA-4. In transwell experiments, DCs were co-cultured with Tn cells and Tregs were either placed in direct contact with DC:Tn (at a ratio of 1:4:2) or the upper chamber of the transwell (Millicell, 0.4 μm pore size; Millipore, Bedford, MA) at the same ratio.

3.3.3 Measurement of thiols and disulfides by HPLC

Concentrations of extracellular cystine, cysteine, GSH and GSH_{in} concentrations were measured using the HPLC method as described (*9, 22*). The GSH_{in} values were normalized to protein concentration determined in the Bradford assay with bovine serum albumin as standard. The extracellular cysteine/cystine redox potential (E_h) was calculated using the Nernst equation: $E_h = E_o + RT/2F \ln([\text{cystine}]/[\text{cysteine}]^2)$, where E_0 = -250 mV at pH 7.4.

3.3.4 T cell proliferation assay

Tn cells (4 x 10^4) were cultured with varying numbers of Tregs for 72 h in round bottom 96-well plates with DCs (1 x 10^4) in the presence of either 1 μM $OVA_{323-329}$ or 1 μg/ml anti-CD3 antibody. When needed, 50 μM cysteine was added every 24 h during

the culture. T cell proliferation was assayed by measuring incorporation of [^{3}H]-thymidine (1 μCi/ml, PerkinElmer) during the last 16 h of culture.

3.3.5 Western blot analysis

DCs from control, DC+LPS, DC+LPS+Treg and DC+LPS+Treg+anti-CTLA-4 antibody-treated cultures were harvested and lysed in lysis buffer on ice. Aliquots of cell lysates (20 μg) were boiled and loaded on to a 10% SDS polyacrylamide gel and electroblotted onto a PVDF membrane. Antibodies against γGCS (Lab Vision) and β-actin (Sigma) were used to monitor expression of the proteins and detected using the Dura chemiluminescent horseradish peroxidase system (Pierce) as per the vendor's protocol.

3.3.6 Metabolic labeling

Activated Teffs and Tregs (2 x 10^{6} each) were cultured in cystine-free RPMI medium supplemented with 50 μM cysteine, 100 μg/ml penicillin and streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 2.5% heat-inactivated fetal bovine serum. [^{35}S]-cysteine (PerkinElmer, 1 μCi/ml) was added to the cell culture media for 24 h. T cells were collected and suspended in 125 μl PBS for the following analysis: 1) 25 μl for protein normalization: mixed with 25 μl of lysis buffer; 2) 50 μl for GSH analysis: mixed with 50 μl of metaphosphoric acid solution (16.8 g/liter metaphosphoric acid, 5 M NaCl, and 5 mM EDTA); Intracellular GSH radioactivity was measured and quantified as described previously (*9*); 3) 50 μl for taurine analysis and protein radioactivity measurement: mixed with 50 μl 10% trichloracetic acid (TCA). Proteins were precipitated with 10% TCA and dissolved in 1 M NaOH to measure radioactivity. Supernatants were used for taurine analysis using the HPLC method as described (*23*).

Medium samples were collected for the measurement of cysteine and sulfate radioactivity. To measure the radioactivity associated with Cys_{ex}, HPLC fractions containing cysteine were collected and measured by scintillation counting. For sulfate measurement, $BaCl_2$ (100 mM final) was added to the medium to precipitate sulfate. The pellet was dissolved in 1 M NaOH and the radioactivity was analyzed by scintillation counter. The radioactivity of sulfate in the control medium was subtracted from the final values.

3.3.7 Confocal microscopy and fluorescence analyses

Tn cells were cultured with DCs for 0, 6, 24, 36, 48 and 72 h in the presence of 1 μM $OVA_{323-329.}$ In the Treg experiment, Tn cells were pre-stained with 2 μM PKH26 (Sigma), a fluorescent dye that labels membranes, to distinguish them from Treg cells under co-culture conditions. When used, Tregs were added at a 1:4:2 ratio of (DC:Tn:Tregs) in the presence of 1 μM $OVA_{323-329}$ for up to 48 h. Cysteine (50 μM) was added every 24 h during the culture. At the conclusion of the experiment, T cells from different culture conditions were separated from DCs and labeled with 10 μM CMFDA for 30 min at 37˚C. Following removal of the staining solution, cells were incubated for another 30 min at 37˚C in 1 ml prewarmed serum-free medium. Hoechst dye (2 μg/ml, Invitrogen) was added during the last 5 min to stain nuclei. Cells were then washed with PBS and fixed with 4% paraformaldehyde. Confocal images were acquired using an Olympus FV500 confocal microscope. The following excitation wavelengths were employed for the individual dyes: 360 nm for Hoechst (emission: 480 nm), 551 nm for PKH26 (emission: 570 nm) and 492 nm for CMFDA (emission: 517 nm).

For quantitative analysis of the GSH fluorescence intensity, the nuclear perimeter was defined by the area of Hoechst staining and the cell perimeter from the bright field

image of the cell seen by light microscopy as described previously (*24*). The cytoplasmic volume was the difference in the whole cell volume and the nuclear volume. The nuclear/cytoplasm GSH ratio was represented as the total CMFDA intensity (calculated using the ImageJ software) in each compartment.

3.3.8 Statistical analysis

Comparison between groups was done using two-tailed Student's *t*-test. *P* values <0.05 were considered to be statistically significant.

3.4 Results

3.4.1 Treg-mediated redox remodeling is antigen-dependent, but antigen-nonspecific

We have previously used BALB/c mice as a source of immune cells and shown that Tregs suppress Cys_{ex} accumulation with anti-CD3 antibody stimulation (*9*). Although this mode of T cell activation is widely used to mimic antigenic stimulation, the absence of antigen activation limits biological interpretation (*25*). We therefore evaluated the antigen-dependence of Treg-mediated redox remodeling using DO11.10 $OVA_{323-329}$-specific TCR transgenic mice. DCs, Tn and Tregs were purified from DO11.10 mice and co-cultured in the presence of either $OVA_{323-329}$ antigen or anti-CD3 antibody. As shown in Fig. 3.1A, the magnitude of Cys_{ex} accumulation was similar regardless of whether $OVA_{323-329}$ antigen or anti-CD3 antibody stimulation was utilized. Furthermore, the magnitude of decrease in Cys_{ex} accumulation by Tregs was also similar under both conditions (Fig. 3.1A). DCs in culture hold the extracellular cysteine/cystine redox potential at ~-80 mV (Fig. 3.1B), consistent with the growth arrest/differentiation stage of these cells. Co-culture of DCs with Tn in the presence of either $OVA_{323-329}$ antigen or

anti-CD3 antibody stimulation resulted in a reductive shift to ~-120 mV, consistent with conditions favoring T cell proliferation (*2, 26*). However, addition of Tregs to the DC-Tn cell co-culture caused an oxidative shift to ~-100 mV. We have previously shown that addition of exogenous cysteine at levels seen under DC-Tn co-culture conditions abrogates Treg-mediated suppression of T cell proliferation induced by anti-CD3 antibody activation (*9*). Similarly, with OVA stimulation, addition of 50 μM cysteine also alleviated inhibition of T cell proliferation by Tregs (Fig. 3.1C). In subsequent experiments, DCs, Tn and Tregs from DO11.10 mice were used with $OVA_{323-329}$ antigen stimulation.

Activation of Tn cells requires antigen presented by MHC class II molecules to be recognized by the TCR. To evaluate whether the Treg effect on Cys_{ex} is antigen-specific, DCs and Tn cells from DC11.10 mice and Tregs from different mouse strains were used. Tregs from DO11.10 mice suppressed Cys_{ex} levels to ~70% as compared with DC-Tn co-culture, as expected (Fig. 3.1D). Similarly, Tregs from BALB/c mice and CD1 mice also suppressed Cys_{ex} accumulation to a similar extent, indicating that Treg-mediated redox remodeling is antigen-nonspecific.

3.4.2 Treg-mediated redox remodeling is contact and CTLA-4 dependent

We have previously shown that DC-T cell contact is required for Cys_{ex} accumulation (*9*). However, in principle, Treg suppression of Cys_{ex} accumulation could occur simply via competition for cysteine released by DCs. To address this possibility, Tregs were cultured in the upper chamber of a transwell set-up while DCs and Tn cells were in the lower chamber. Under these conditions, Tregs were unable to suppress Cys_{ex} accumulation (Fig. 3.2A). Furthermore, Cys_{ex} levels were not diminished when Tregs

pre-activated by DCs and IL-2 were employed. These results suggest that cell-cell contact is required for Treg mediated redox remodeling, consistent with the view that Tregs suppression of T cell proliferation in vitro is cell-contact dependent (*1, 8*).

Tregs constitutively express high levels of CTLA-4, which competes with CD28 on Teffs and transmits inhibitory signals to cells (*1, 10, 27-29*). To investigate the role of CTLA-4 in Treg-mediated redox remodeling, we used anti-CTLA-4 antibody. As shown in Fig. 3.2B, anti-CTLA-4 antibody abrogated the effect of Tregs on Cys_{ex} accumulation (Fig. 3.2B). As a control, anti-CTLA-4 antibody added to a DC-Tn cell co-culture did not interfere with $[Cys]_{ex}$ levels. These results demonstrate that diminution in Cys_{ex} by Tregs is dependent on co-stimulatory molecules e.g. CTLA-4.

3.4.3 Tregs interfere with GSH metabolism in DCs

In principle, Tregs can interfere with the redox crosstalk between DCs and Teffs in at least three ways: 1) modulation of DC function that leads to lower cysteine production, 2) competition for the Cys_{ex} pool, and 3) modulation of Teff function by limiting cysteine availability. First, we investigated whether Tregs inhibit cysteine secretion by DCs using LPS stimulation to exclude the effects of Teffs on DCs in the experiment. We have demonstrated that LPS, like Teffs, stimulates Cys_{ex} accumulation (*9*) and that Tregs suppress this effect in a CTLA-4-dependent fashion (Fig. 3.3A). We found that Tregs, in addition to decreasing Cys_{ex} levels, also decreased $[GSH]_{ex}$, a response that was abrogated by anti-CTLA-4 antibody (Fig. 3.3B). This was paralleled by an increase in $[GSH]_{in}$ in LPS-stimulated DCs which was inhibited by Tregs in a CTLA-4-dependent manner (Fig. 3.3C). Western blot analysis revealed significant up-regulation of the catalytic subunit of γ-GCS in response to LPS stimulation, attenuation of this response by

Tregs and abrogration of the Treg effect by anti-CTLA-4 antibody (Fig. 3.3D). In contrast, Tregs did not alter the expression of other key proteins involved in mediating Cys_{ex} accumulation viz., the x_c^- transporter and γ-glutamyltranspeptidase. These results suggest that one mechanism by which Tregs decrease Cys_{ex} accumulation is by inhibiting GSH synthesis in DCs.

3.4.4 Cysteine uptake and utilization by Tregs and Teffs

To determine whether competition by Tregs for the Cys_{ex} pool represents an additional mechanism of regulating Cys_{ex} levels, we used a metabolic labeling approach with [^{35}S]-cysteine. After 24 h, the radioactivity associated with the cysteine pool was not significantly different in the Treg versus Teff medium (Fig. 3.4A). This suggests that cysteine consumption by Tregs might constitute one mechanism for reducing Cys_{ex} during activation of T cells.

However, for a competition to be a plausible mechanism, Tregs, which are nonproliferating, must show net consumption of the imported cysteine to support its continuous influx. To address this issue, we determined the fate of cysteine imported by Teffs and Tregs. The four major intracellular cysteine sinks are protein, GSH, taurine and sulfate (*30*) and the incorporation of [^{35}S]-cysteine into these pools in activated Treg and Teff cells was assessed. As shown in Fig. 3.5A, Teffs showed considerably higher incorporation of radioactive cysteine into protein (50%) and GSH (25%) compared to Tregs. This is consistent with activated Teff cells being fated to proliferate while Tregs are anergic. In contrast, a greater proportion (50%) of cysteine was oxidized to sulfate by Tregs compared to Teffs (Fig. 3.4A). Almost no incorporation of radioactivity into taurine was seen in the intra- or extra-cellular pools in either Teffs or Tregs. A very

similar pattern of radioactivity distribution was observed when Teffs and Tregs were activated by DCs instead of irradiated splenocytes (Fig. 3.4B). Collectively, these results reveal that cysteine is preferentially utilized for protein synthesis by Teffs and is catabolized to sulfate by Tregs.

3.4.5 Tregs block GSH relocalization in Teffs during T cell activation

Next, we investigated how restricted cysteine availability in the presence of Tregs affects mobilization of the GSH_{in} pool in activated Teffs. Previous studies have shown that GSH concentrates in the nucleus during early stages of cell proliferation but later, redistributes approximately equally between the nuclear and cytoplasmic compartments (*24*). We imaged changes in GSH_{in} localization during T cell activation and found that GSH co-localized with nuclear DNA during the early stages of T cell activation (Fig. 3.5A). However, 24 h later, GSH appeared to be more diffusely distributed between the cytoplasmic and nuclear compartments. In order to quantitate this change, we compared the ratio of the nuclear:cytoplasmic GSH fluorescence observed by confocal microscopy analysis and found that it decreased ~2-fold over a 24 h activation period and was unchanged over the next 48 h (Fig. 3.5B). To examine the effect of Tregs on GSH localization in Teffs, we pre-stained Teffs with PKH-26 (red) to distinguish Teffs from Tregs. In the presence of Tregs, GSH relocalization in Teffs appeared to be inhibited (Fig. 3.5C). Addition of 50 μM cysteine to the DC-Tn-Treg culture restored GSH relocalization. The nuclear/cytoplasmic GSH ratio for Teffs from the DC-Tn-Treg co-culture was similar to that of Tn cells (Fig. 3.5D). Exogenous cysteine abrogated the effect of Tregs on the nuclear:cytoplasmic GSH ratio. While these data suggest that Tregs stall relocalization of GSH_{in}, we note several limitations with the analysis and therefore,

interpretation of the results. First, the large nuclear volume of T cells makes accurate determination of the fluorescence intensity in the cytoplasmic compartment difficult. Second, during activation, the volume of Teff cells increase making the comparative estimation of nuclear:cytoplasmic total GSH fluorescence more complicated.

3.5 Discussion

While the requirement for a reducing microenvironment to support T cell activation and proliferation has been known for quite a while (*12, 31*), redox modulation has emerged as an immunosuppressive strategy only recently (*2, 9*). We have previously described this novel suppression mechanism whereby Tregs cause an oxidative shift in the extracellular redox environment during DC-dependent T cell activation (*9*). In this study, we demonstrate that Tregs deploy multiple strategies for inhibiting DC-dependent extracellular redox remodeling (Fig. 3.6).

Activation of Teffs requires interaction between the TCR and a specific antigen presented by MHC class II molecules. However, the antigen specificity for the suppressive action of Tregs is controversial. While several studies suggest Tregs suppress in an antigen-restricted manner in vitro and in vivo (*32-34*), others report that Tregs do not require specific antigen-TCR interaction for suppression of Teff proliferation (*35, 36*). We demonstrate that while remodelling of the extracellular environment by Tregs is antigen-dependent it is not antigen-specific. These results are consistent with the observations of Vignali and coworkers who have shown that Tregs can suppress Teffs derived from mouse strains with distinct antigen specificities (*35*). Hence, Tregs might

harbor constitutive suppressive activity and mediate redox remodeling via a bystander suppression mechanism.

Recent studies by Sakaguchi and coworkers demonstrate an essential role for CTLA-4 in immunosuppression by Tregs (*1, 10*). Foxp3, a transcription factor needed for Treg function regulates CTLA-4 expression. Addition of anti-CTLA-4 antibody or disruption of the CTLA-4 gene in Tregs blocks Treg suppression and causes a variety of autoimmune diseases (*10, 37*). We showed that Treg-mediated redox remodeling is both contact- and CTLA-4-dependent. Administration of anti-CTLA-4 antibody blocked the Treg effect on Cys_{ex}, GSH_{ex}, GSH_{in} and γ-GCS expression, suggesting that CTLA-4 plays an important role in several aspects of redox remodeling by Tregs. By interacting with CD80/CD86 on DCs, Treg-derived CTLA-4 triggers several signaling pathways in DCs including activation of IDO expression and induction of the Foxo3 transcription factor (*27-29*). Tregs down-regulate CD80/CD86 costimulatory molecules on DCs to restrain the maturation and antigen presenting capacity of DCs (*38, 39*).

We found that GSH synthesis in LPS-stimulated DCs is down-regulated by Tregs, which is consistent with lower GSH extrusion by DCs and consequently, lower Cys_{ex} generation via the γ-glutamyl cycle. The regulatory crosstalk between Tregs and APCs has been extensively investigated. Since Foxo3 is activated via CTLA-4 signaling and Foxo3 is a sensor and regulator of redox signaling (*29, 40*), a potential link between this transcriptional factor and the γ-glutamyl cycle merits investigation.

The maintenance of GSH_{in} levels requires the availability and transport of cysteine, the amino acid that limits GSH synthesis. Since cysteine is easily oxidized to cystine in the oxidizing extracellular milieu, the concentration of cysteine in the plasma is much

lower than of cystine (*41*). However, the cystine transporter activity in naïve T cells is very low compared to the cysteine transporter activity (*42*), thus making them dependent on APCs to meet their cysteine needs (*2, 9, 12*). DCs express the x_c^- cystine transporter, and import cystine efficiently, stimulating Cys_{ex} accumulation during T cell activation (*9, 12*). This response is inhibited by Tregs at multiple levels including cysteine consumption and its preferential oxidation to sulfate. However, the in vivo significance of this competitive mechanism for diminishing Cys_{ex} is uncertain since Teff cells outnumber Treg cells, which represent only ~5-10% of total $CD4^+$ T cells (*3*).

GSH plays essential roles in T cell functions including DNA synthesis (*15, 43*). Perturbation of GSH_{in} levels and the GSH/GSSG redox status dramatically affects T cell proliferation, DNA synthesis and cytotoxic T cell activity (*44*). GSH_{in} levels also substantially influence T cell signal transduction pathways such as tyrosine phosphorylation, AP1 and nuclear transcription factor κB (NFκB) (*45*). Overexpression of B cell leukemia/lymphoma 2 (Bcl-2) results in increased GSH levels by recruiting GSH to the nucleus to prevent apoptosis (*46*). GSH is concentrated in the nucleus during the early stage of cell proliferation and distributed more evenly when cells reach confluency. GSH regulates nuclear protein functions via glutathionylation, which also protects protein thiols from further oxidation (*24*). Although our analysis of cytoplasmic versus nuclear GSH pools is limited by the technical challenges discussed above, at least qualitatively, the data suggest that GSH is concentrated in the nucleus in naïve T cells but is more diffusely distributed in T cells undergoing activation and proliferation. Progression through the cell cycle is correlated with metabolism-linked redox changes (*47*) and inhibition of GSH relocalization dynamics by Tregs might represent one

mechanism of inhibition of Teff proliferation. While the significance of GSH relocalization from the nuclear to the cytoplasmic compartment for Teff proliferation is not known, it is interesting that the inhibitory effect of Tregs on GSH_{in} dynamics is alleviated by exogenous cysteine added at a concentration seen under DC-T cell co-culture conditions.

In summary, our study reveals that Tregs deploy multiple strategies for perturbing the extracellular redox environment during T cell activation, affecting both DCs and Tn cells. Elucidation of the signaling pathways that connect Treg engagement and the redox metabolic responses uncovered in this study might allow identification of potentially novel therapeutic targets for modulating Treg functions.

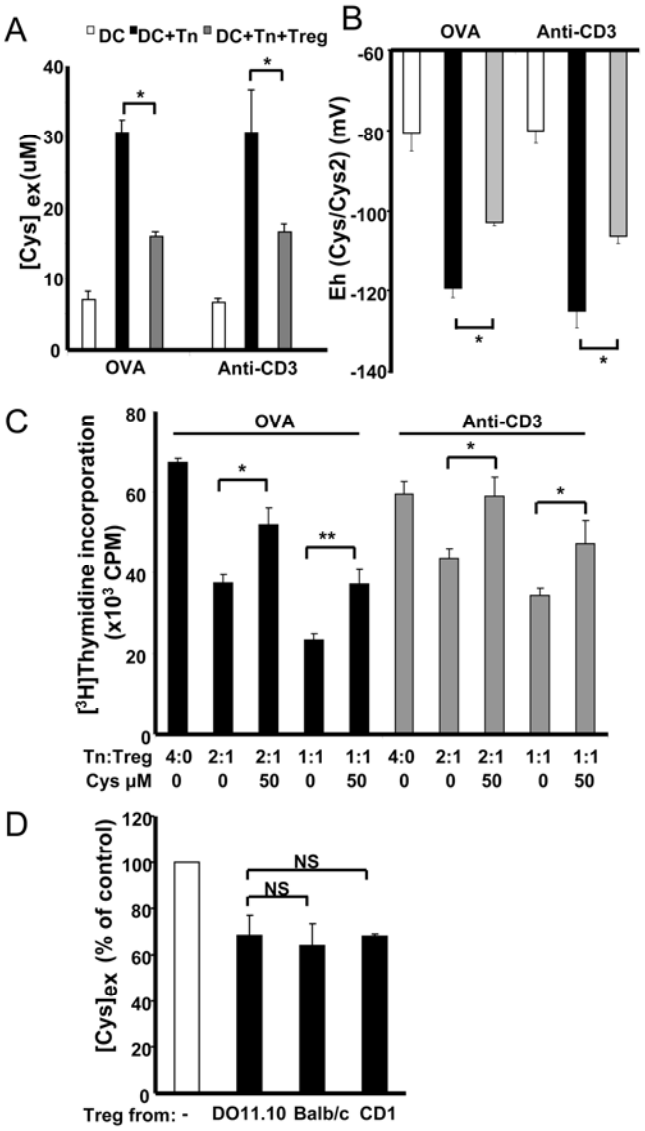

Figure 3.1 Antigen-dependent but antigen-nonspecific Treg-mediated redox remodeling. DCs were co-cultured with Tn cells (1:4) or with Tn+Treg cells (1:4:2) in the presence of 1 µM $OVA_{323-329}$ antigen or 1 µg/ml anti-CD3 antibody. (*A*) Tregs suppressed Cys_{ex} accumulation regardless of $OVA_{323-329}$ antigen or anti-CD3 antibody activation. (*B*) Tregs increased the extracellular cysteine/cystine redox potential in an antigen-dependent manner. (*C*) Addition of exogeous cysteine abrogated Treg suppression of Teff proliferation, which was antigen-dependent. (*D*) Treg suppression of Cys_{ex} accumulation is not antigen specific. DCs were co-cultured with Tn cells (1:4 ratio, both from DO11.10 mice) in the presence of 1 µM $OVA_{323-329}$ antigen. Tregs from DO11.10, BALB/c and CD1 mice were added separately to the DC-Tn co-culture, and the $[Cys]_{ex}$ was measured. $[Cys]_{ex}$ is expressed as percentage of the concentration in DC-Tn cell co-culture medium. Data represent the mean ± SD of four *(A, B)* and two *(C, D)* independent experiments each performed at least in duplicates. * $P<0.05$; ** $P<0.005$; NS, not significant; two-tailed Student's *t*-test.

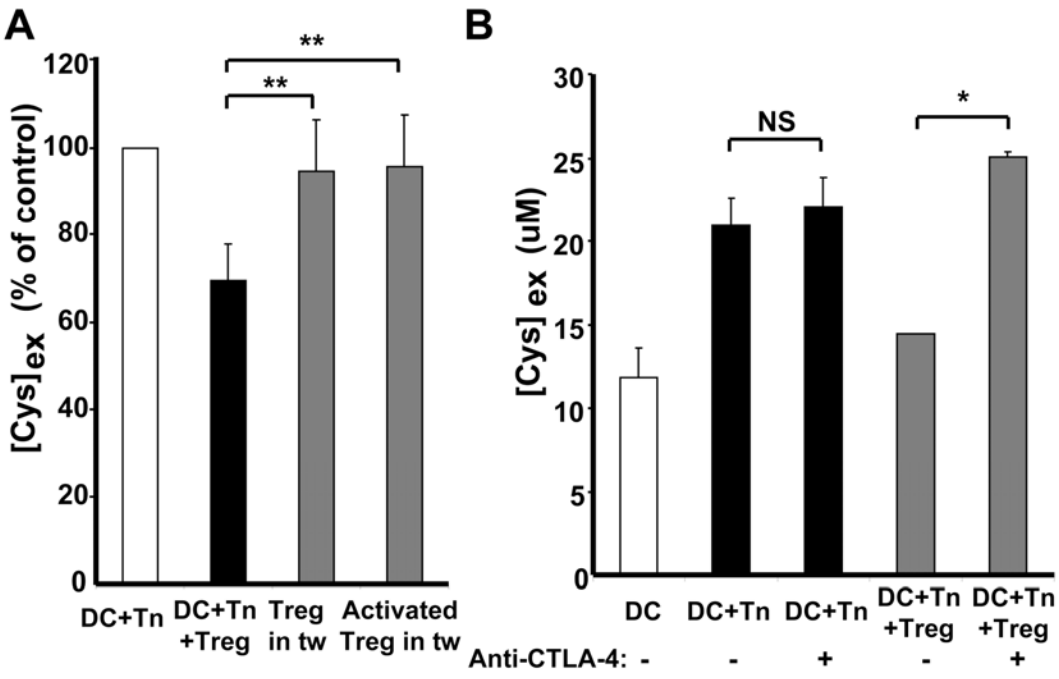

Figure 3.2 Contact and CTLA-4 dependent redox remodeling by Tregs. *(A)* DCs were co-cultured with Tn+Treg cells (1:4:2) or with Tregs placed in the upper chamber of a transwell (tw). In another experiment, Tregs were first activated by DCs in the presence of 1 μM $OVA_{323-329}$ and IL-2 (20ng/ml) and then cultured in the transwell. $[Cys]_{ex}$ is expressed as a percent of the concentration in untreated DC-Tn cell co-culture medium. (*B*) DCs were co-cultured with Tn cells (1:4) or with Tn+Treg cells (1:4:2) ± anti-CTLA-4 antibody (100 μg/ml). Data represent mean ± SD and are representative of 3 independent experiments performed in duplicate. * $P<0.05$; ** $P<0.005$; NS, not significant; two-tailed Student's *t*-test.

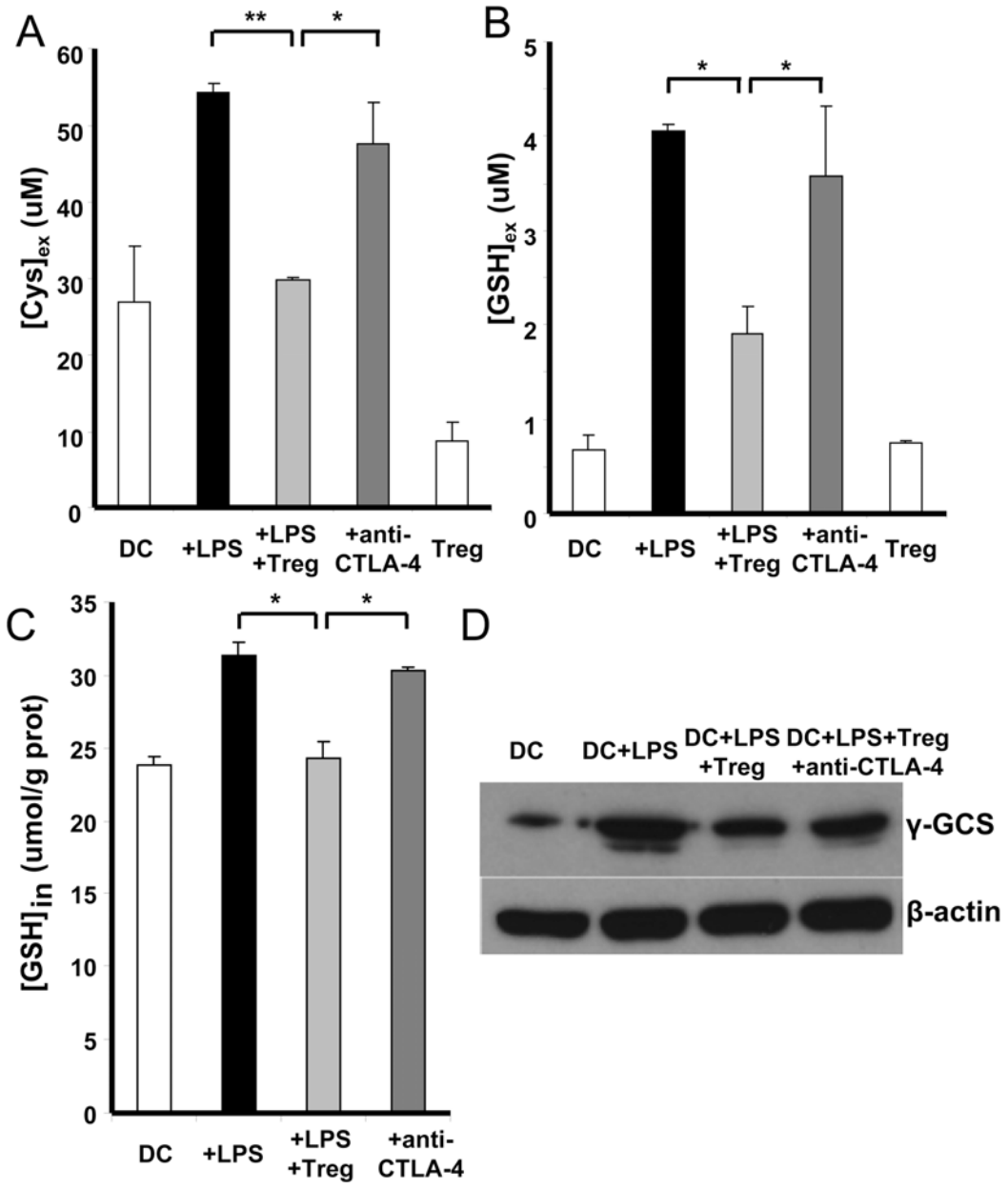

Figure 3.3 Tregs interfere with GSH synthesis by DCs. DCs were treated with LPS (100 ng/ml) and cultured ± Tregs (1:2) and ± anti-CTLA-4 antibody (100 μg/ml). $[Cys]_{ex}$ *(A)* and $[GSH]_{ex}$ *(B)* were measured by HPLC. *(C)* $[GSH]_{in}$ was measured by HPLC and normalized to protein concentration. *(D)* Western blot analysis of the expression of γ-GCS in DCs. β-actin was used as a control for equal loading. Data represent mean ± SD *(A, B and C)* and are representative of 3 independent experiments *(A-D)*. * P<0.05; ** P<0.005; two-tailed Student's *t*-test.

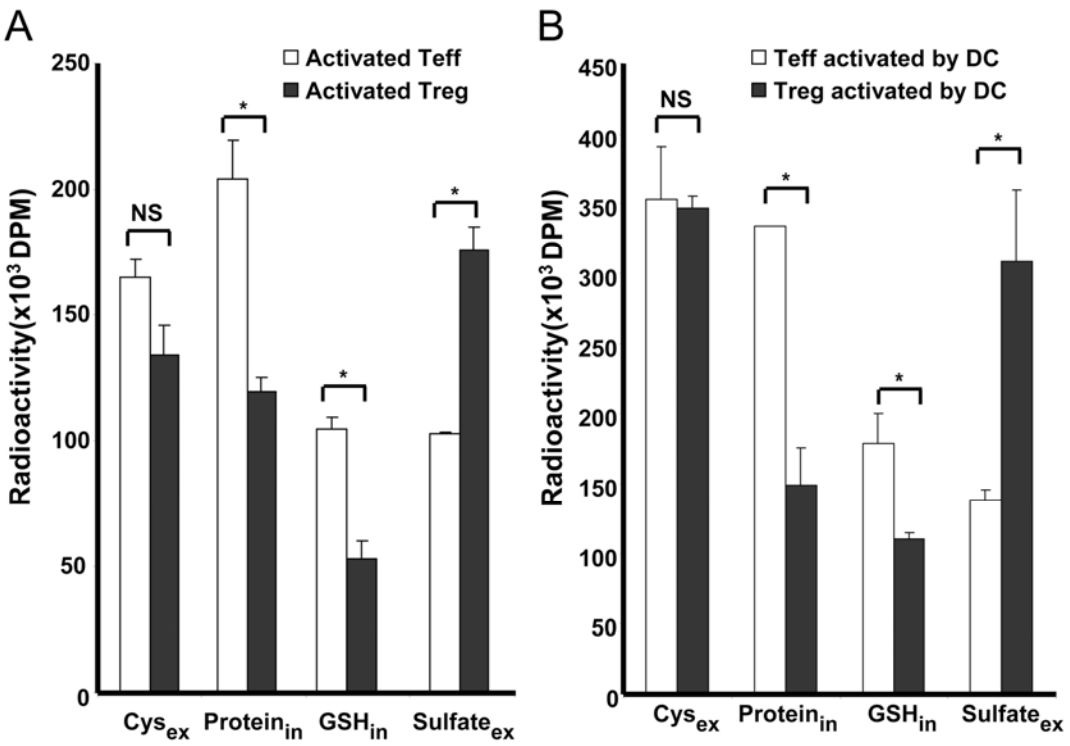

Figure 3.4 Fate of cysteine in Tregs and Teffs. *(A)* Tn and Tregs were activated by irradiated splenocytes (1:3) in the presence of 1 μM $OVA_{323-329}$ antigen for 72 h. IL-2 (20 ng/ml) was added for Treg activation. Activated Teffs and Tregs (2 x 10^6 each) were incubated with [^{35}S]-cysteine (1 μCi/ml) in T cell medium containing 50 μM cysteine for 24 h. [^{35}S]-cysteine remaining in the medium and its incorporation into intracellular protein and GSH and extracellular sulfate were determined. *(B)* Tn and Tregs were activated by DCs (2:1) in the presence of 1 μM $OVA_{323-329}$ antigen for 48 h and separated from DCs. [^{35}S]-cysteine and cystine in the medium and its incorporation into protein, GSH and sulfate were determined. Data are representative of three *(A)* and two *(B)* independent experiments. * $P<0.05$; NS, not significant; two-tailed Student's *t*-test.

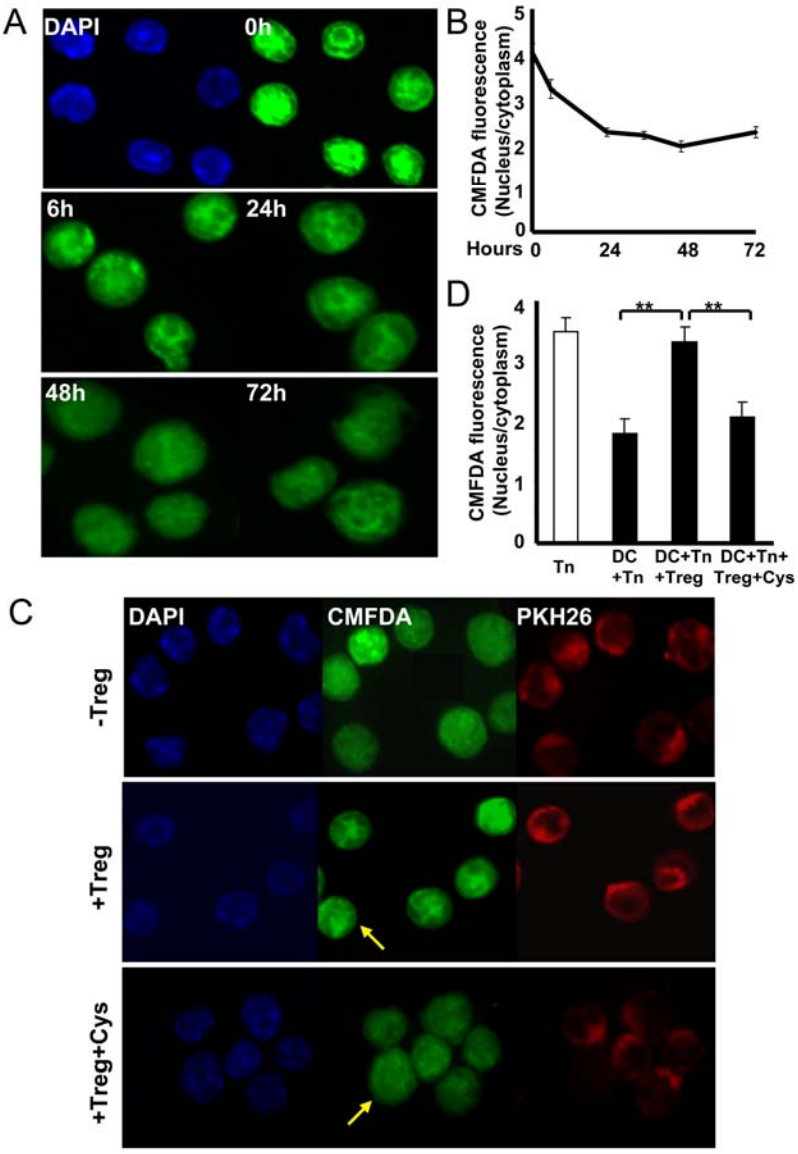

Figure 3.5 Tregs block relocalization of nuclear GSH into cytoplasm in Teffs during T cell activation. *(A)* Confocal microscopy images of GSH localization in T cells during 72 h of T cell activation. T cells were co-cultured with DCs for 0, 6, 24, 36, 48, 72 h and separated from the co-culture by gentle pipetting and centrifugation. Cells were then stained with CMFDA for GSH (green), Hoechst 33342 (blue) for nuclei and imaged by confocal microscopy. *(B)* Quantification of the nuclear:cytoplasmic GSH ratio from experiment shown in panel A (n=4). The nuclear:cytoplasmic GSH ratio is represented as the total CMFDA intensity in each compartment. *(C)* PKH-26 pre-labeled Tn cells (red) were co-cultured with DCs in the presence or absence of Treg cells (1:4:2) ± 50 μM cysteine for 24 h. T cells were separated and stained with CMFDA (green) and Hoechst 33342 (blue) to label GSH and nuclei, respectively. Yellow arrows indicate Tregs. *(D)* Quantitative analysis of the nuclear:cytoplasmic GSH ratio shown in panel C (n=3). * $P<0.05$; ** $P<0.005$; two-tailed Student's *t*-test.

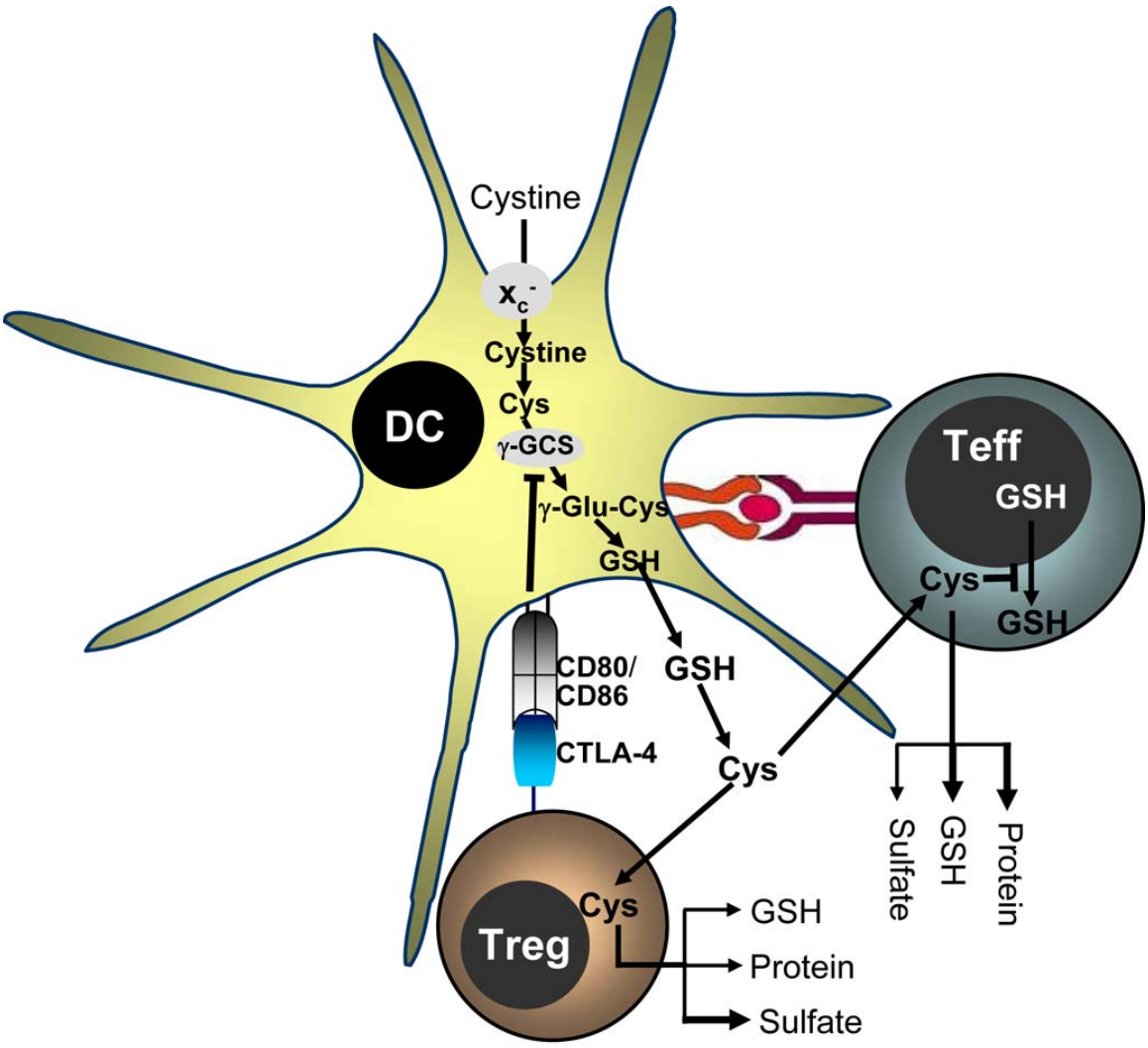

Figure 3.6 Model showing mechanism of Treg-mediated redox remodeling. Interaction of CTLA-4 on Tregs with CD80/86 on DCs triggers signaling in DCs that inhibits GSH_{in} synthesis. Tregs also compete with Teffs for cysteine uptake under in vitro conditions. These two processes decrease the Cys_{ex} pool. Limiting the cysteine pool not only decreases the $[GSH]_{in}$ in Teffs, but also blocks GSH relocalization into the cytoplasm, thus inhibiting T cell activation and proliferation.

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Chapter 4

Differential Dependence on Cysteine from Transsulfuration versus Transport during T Cell Activation

4.1 Abstract

The synthesis of glutathione, a major cellular antioxidant with a critical role in T cell proliferation, is limited by cysteine. In this study, we have evaluated the contributions of the x_c^- cystine transporter and the transsulfuration pathway to cysteine provision for glutathione synthesis and antioxidant defense in naïve versus activated T cells and in the immortalized T lymphocyte cell line, Jurkat. We show that the x_c^- transporter while absent in naïve T cells, is induced after activation, releasing T cells from their cysteine dependency on antigen presenting cells. We also demonstrate the existence of an intact transsulfuration pathway in naïve and activated T cells and in Jurkat cells. The flux through the transsulfuration pathway increases in primary but not in transformed T cells in response to oxidative challenge by peroxide. Inhibition of the transsulfuration pathway in both primary and transformed T cells decreases cell viability under oxidative stress conditions.

4.2 Introduction

T cells play important roles in innate and adaptive immune responses and their dysfunction due to excessive sensitivity to self-antigens or to deficiency, are associated with pathologies. Redox modulation has emerged as a key strategy in regulation of T cell functions (*1, 2*). Glutathione (GSH), a major cellular antioxidant and a cysteine reservoir, is an important component of redox signaling pathways and plays an essential role in T cell function. An increase in intracellular GSH levels is needed for the proliferative response of T cells to mitogens and antigens (*3*). Perturbation of intracellular GSH levels and the GSH/GSSG redox status dramatically affects DNA synthesis, T cell proliferation and the cytotoxic T cell response (*4, 5*).

Low cysteine levels allow activation of NFκB-dependent transcription in the early G1 phase of the cell cycle whereas in the late G1 and S phases, IL-2-dependent cell proliferation is correlated with higher cysteine/glutathione levels (*6*). GSH depletion restricts cell cycle progression from the G1 to S phase (*7*). Cysteine, the limiting amino acid for GSH synthesis, can be obtained from metabolism through the transsulfuration pathway, or via transport. The ASC system imports cysteine whereas the x_c^- antiporter uses the transmembrane glutamate gradient to drive import of cystine into the cell where it is subsequently reduced to cysteine (Fig. 4.1). The x_c^- cystine transporter is composed of two subunits, the transmembrane xCT light chain, which houses the transporter activity and the regulatory extracellular 4F2 heavy chain (*8*). In the extracellular compartment, cysteine exists predominantly in its oxidized form and the concentration of plasma cysteine (10-25 μM) is significantly lower than of cystine (100-200 μM) (*1*).

Naïve T cells reportedly lack the x_c^- transporter and are thus metabolically

dependent on antigen presenting cells (APC) for meeting their cysteine needs during activation and proliferation (*9-11*). Endowed with the x_c^- transporter, APCs take up cystine, and using a convoluted metabolic route involving conversion to GSH followed by its secretion and cleavage, furnish extracellular cysteine for uptake by T cells (*11*). GSH levels in APCs influence T cell response patterns with low GSH favoring a Th1- over a Th2-associated cytokine secretion pattern (*12*). The contribution if any, of the transsulfuration pathway, which converts methionine to cysteine, to redox metabolism in naïve T cells is not known. Methionine is an essential amino acid that is converted via the methionine cycle to homocysteine. Two enzymes in the transsulfuration pathway, cystathionine β-synthase (CBS) and γ-cystathionase, convert homocysteine to cysteine, and play a quantitatively significant role in supplying cysteine needed for GSH synthesis in several cell types (*13, 14*).

In the present study, we have evaluated changes in the transsulfuration pathway and the x_c^- system during transformation of naïve T cells to the activated state. We demonstrate using metabolic labeling and pharmacological inhibition studies, the presence of an intact transsulfuration pathway in both naïve and activated T cells and in Jurkat cells. We find that the expression of xCT is induced upon T cell activation weaning T cells off their metabolic dependence on APCs. Under oxidative stress conditions, the transsulfuration pathway is upregulated in naïve but not transformed T cells while its inhibition enhances cellular susceptibility to death in both naïve and transformed cells.

4.3 Materials and Methods

4.3.1 Mice and cell lines

Male BALB/c mice (7-10 weeks) were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in pathogen-free animal facilities at the University of Michigan. The University's Committee on Use and Care of Animals approved the protocol for animal handling used in this study. Jurkat cells were obtained from ATCC (Manassas, VA).

4.3.2 Isolation and preparation of murine primary cells

$CD3^+$ T lymphocytes were prepared from lymph nodes and spleen that were harvested and mashed. T cells were enriched by negative selection on T cell columns (R&D Systems, MN) as per the vendor's protocol (*15*). For in vitro activation, as isolated naïve T cells were suspended in RPMI media containing 2.5% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1x penicillin/streptomycin (Invitrogen), 50 μM 2-mercaptoethanol (T cell medium) and incubated with either anti-CD3 + anti-CD28 antibodies (eBiosciences) or with anti-CD3 antibody + dendritic cells or irradiated splenocytes (as APC, 1:4 ratio) for 48-72 h. Following incubation, activated T cells were separated from APCs by gentle pipetting and T cells were collected by low speed centrifugation at 200 x *g* for 10 min at 4˚C. Activation of T cells was assessed by monitoring the activation markers CD25 and CD69 by flow cytometry (Fig. 4.2). Numbers inside panels indicate the percentage of naïve and activated $CD3^+$ T cells positive for CD25-FITC and CD69-PE.

Dendritic cells were obtained from femurs and tibias removed from adult male mice and the bone marrow were flushed out with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS). Red blood cells were lysed with ACK buffer (Lonza, Walkersville, MD).

Bone marrow cells were plated at 2-5 x 10^6 cells/ml in a 250 ml flask (total 15 ml) in DMEM supplemented with 100 µg/ml penicillin/streptomycin, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 1 mM pyruvate, 1:100 nonessential amino acids, and 10% heat-inactivated FBS (dendritic cell medium). Recombinant murine granulocyte macrophage colony-stimulating factor and recombinant murine interleukin 4 (R&D systems), at 20 ng/ml each, were added on day 0. On days 2 and 4, the culture medium was replaced with dendritic cell medium supplemented with cytokines, and floating cells were discarded. On day 7, cells were collected with accutase (eBioscience) to harvest adherent cells and centrifuged, the pellet was resuspended in fresh dendritic cell medium and used in T cell co-cultures as APCs.

4.3.3 Cell culture conditions

Jurkat cells were cultured in DMEM media containing 10% FBS, 2 mM L-glutamine and 1x penicillin/streptomycin (Jurkat media). To activate Jurkat cells, phorbol 12-myristate 13-acetate (PMA) was added to the culture medium at a final concentration of 100 ng/ml for 48 h. PMA was washed out and cells were incubated with fresh culture medium at the start of the experimental treatment. Naïve or pre-activated T cells were cultured in T cell medium and at the indicated time points, an aliquot of the medium was collected for extracellular cysteine, cystine and glutamate measurement, rapidly frozen and stored at -80˚C until further use. To measure the intracellular thiol concentration and to estimate flux through the transsulfuration pathway, naïve or activated T cells were incubated with 2 µCi/ml L-[^{35}S]-methionine (specific activity of 1175 Ci/mmol, Perkin Elmer) in the presence or absence of 2.5 mM propargylglycine (PPG) for 6 or 12 h. The final concentration of L-[^{35}S]-methionine was 2 nM and final specific activity, 20

μCi/μmol. To measure the effect of oxidative stress on the transsulfuration flux, the same experiments were repeated except in the presence of 20 μM and 100 μM t-BuOOH for naïve and activated T cells respectively. At the indicated time points, cells were washed with ice cold PBS, harvested and frozen at -80°C until further use.

4.3.4 Metabolite analysis

Samples for analysis of extracellular cysteine, cystine and glutamate were prepared by mixing conditioned media with an equal volume of metaphosphoric acid solution and the precipitated proteins were sedimented by centrifugation at 13,000 x *g* for 10 min at 4ºC. Protein-free extracts were alkylated with monoiodoacetic acid, derivatized with 2,4-dinitrofluorobenzene solution and analyzed via HPLC as previously described (*13, 15*). The concentration of metabolites in the control medium was subtracted from the final values. Intracellular GSH and [^{35}S]-methionine incorporation into GSH were quantified as described previously (*13, 15*). The intracellular values were normalized to the protein concentration in each sample.

4.3.5 Western blot analysis

T cells (naïve or activated T cells) and Jurkat cells were harvested and lysed on ice as described previously (*14*). Antibodies against methionine synthase, CBS, xCT (Novus), γ-glutamylcysteine ligase (γGCL, Lab Vision) and actin (Sigma) were used to monitor expression of the respective protein antigens and detected using the Dura chemiluminescent horseradish peroxidase system (Pierce) as per the vendor's protocol.

4.3.6 [^{14}C]-Cystine uptake assay

Naïve or activated T cells (2 x 10^6) were incubated with 0.1 μCi/ml, [^{14}C]-cystine (250 mCi/mmol, Perkin Elmer,) in the presence or absence of 500 μM sulfasalazine (SAS)

for the indicated times. The final concentration of [^{14}C]-cystine was 400 nM and the final specific activity was 0.5 μCi/mmol. Cells were collected, washed 3 times with ice-cold phosphate-buffered saline and then lysed with 400 μl of 500 mM NaOH. Scintillation cocktail was added and total intracellular [^{14}C] radioactivity was recorded. Data were normalized to cell number.

4.3.7 Cell viability assay

Cells were incubated with the indicated concentrations of tertiary butyl hydroperoxide (t-BuOOH) in the presence or absence of 2.5 mM PPG for 12-14 h. Cell viability was determined by incubating the cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (0.5 mg/ml) followed by washing, solubilizing in dimethylosulfoxide and reading the optical density at 553 nm.

4.3.8 Statistical analyses

Comparison between groups was done using the Student's *t* test. $P < 0.05$ was considered to be statistically significant.

4.4 Results

4.4.1 The x_c^- transporter is induced during APC-independent activation of T cells

While T cells depend on APCs for their cysteine needs (*9, 11*), it is known that T cells can be activated ex vivo in an APC-independent fashion, i.e., by antibodies to the T cell receptor, CD3 and to the co-stimulatory molecule, CD28. This raises the obvious question as to how cysteine needs are met under these activation conditions. To assess the involvement of the x_c^- transporter in cystine uptake, we used Western blot analysis to monitor expression of the xCT subunit in naïve and anti-CD3/anti-CD28 activated T cells.

As reported previously (*16*), xCT expression was not detectable in naïve T cells, but was highly induced in activated T cells (Fig. 4.3a).

To evaluate whether induction of xCT is correlated with a functional difference in cystine transport in activated but not naïve T cells, we measured the kinetics of cystine consumption. During incubation with anti-CD3 and anti-CD28 antibodies, T cells progressively consume increasing amounts of cystine and accumulate cysteine in the extracellular compartment (Fig. 4.3b), consistent with the induction of the x_c^- transporter. Cysteine accumulation plateaued after 48 h at which time extracellular cystine concentration was very low.

4.4.2 The x_c^- transporter is induced during APC-dependent activation of T cells

Next, we examined whether x_c^- expression is also induced in T cells activated in the presence of dendritic cells and anti-CD3. We found that xCT was strongly induced after 48 h of incubation (Fig. 4.4a). We focused the remainder of the study on APC-activated T cells.

4.4.3 Cystine/glutamate exchange is mediated by the x_c^- transporter

We compared the kinetics of [^{14}C]-cystine uptake in naïve versus activated T cells. Under these conditions, activated T cells showed a time-dependent increase in intracellular radiolabel accumulation (Fig. 4.4b), which was sensitive to inhibition by SAS, an inhibitor of x_c^- (Fig. 4.4c). In contrast, negligible uptake of radioactivity was observed with naive T cells. Since, x_c^- is an antiporter that exchanges cystine stoichiometrically with glutamate, we assessed extracellular glutamate levels in the conditioned media from activated T cells cultured following their separation from APCs. Under these conditions, activated T cells showed a time-dependent increase in

extracellular glutamate levels, which was sensitive to SAS treatment (Fig. 4.4d,e). Naïve T cells do not export glutamate and the basal concentration in the culture medium (~100 μM) is unchanged over a 24 h incubation time (Fig. 4.4d).

4.4.4 The endogenous transsulfuration pathway is a source of cysteine for GSH in naïve T cells

Since naïve T cells do not express x_c^-, we wondered whether the endogenous transsulfuration pathway is a source of cysteine needed for GSH and other biosynthetic purposes in these cells. To test this, we measured the transfer of radioactivity from [^{35}S]-methionine to GSH in the presence and absence of PPG, a suicide inhibitor of γ-cystathionase, the second enzyme in the transsulfuration pathway (Fig. 4.1). The transsulfuration pathway is clearly intact in both naïve and activated T cells as evidenced by the incorporation of radiolabel from [^{35}S]-methionine into GSH and its inhibition by PPG (Fig. 4.5a). Radiolabel incorporation in naïve cells was inhibited to ~50% and ~33% of the untreated values at 6 h and 12 h respectively by PPG. In activated T cells, accumulation of radioactivity in GSH was less sensitive to PPG inhibition.

The 100% increase in [^{35}S]-labeling of GSH in naïve T cells between 6 and 12 h was accompanied by an ~40% increase in GSH concentration during the same time period (Fig. 4.5b). The GSH concentration was higher at 6 h in activated compared to naïve T cells and increased only marginally (~12%) at 12 h. We examined the expression of some key regulatory enzymes in sulfur metabolism: methionine synthase, CBS and γ-glutamyl-cysteine ligase in naive and activated T cells. While methionine synthase expression was unchanged, activation was accompanied by increased expression of CBS and γ-glutamyl-cysteine ligase (Fig. 4.5c).

4.4.5 T cells activate the transsulfuration pathway upon peroxide challenge

Mammalian cells are known to upregulate flux through the transsulfuration pathway upon oxidative stress conditions and, in the short term, increase GSH concentration by ~40% (*17, 18*). We assessed whether the transsulfuration pathway responds similarly in naïve and activated T cells to peroxide challenge. For this, naïve or APC-activated T cells were incubated with [^{35}S]-methionine and challenged with the indicated concentration of t-BuOOH in the presence or absence of PPG for 6 h and 12 h (Fig. 4.6). t-BuOOH treatment induced a 50% increase in radiolabel incorporation in naïve T cells both at 6 and 12 h after stimulation, which was completely inhibited by PPG (Fig. 4.6a). Activated T cells showed a modest increase (~25%) in radiolabel incorporation into GSH at 6 h and a more sizeable increase (~65%) after 12 h of t-BuOOH exposure (Fig. 4.6b). These results demonstrate that T cells activate the transsulfuration pathway in response to peroxide-induced oxidative stress.

4.4.6 Transformed T cells do not activate the transsulfuration pathway upon peroxide challenge

Jurkat cells are commonly used as a model for T cells in laboratory experiments. Since the metabolism of transformed cells is often distinct from that of the primary cells from which they are derived, we investigated whether Jurkat cells have an intact transsulfuration pathway that is responsive to oxidative stress conditions. A time-dependent increase in radiolabel incorporation from [^{35}S]-methionine into GSH that was sensitive to PPG inhibition confirmed the presence of an intact transsulfuration pathway in these cells (Fig. 4.7a). The intracellular GSH concentration initially increased (50%) between 3-12 h of incubation after which it stabilized (Fig. 4.7b). The sensitivity of the

intracellular GSH pool to PPG, which diminished to ~50% and ~30% of control values at 12 and 24 h respectively, indicated that between 50-70% of the cysteine used for GSH synthesis is derived via other routes. The presence of the xCT subunit of the cystine transporter as detected by Western blot analysis (not shown) suggests that Jurkat cells can derive cysteine from imported cystine. Unlike primary T cells, the transsulfuration pathway in Jurkat cells is unresponsive to peroxide stress as evidenced by the lack of increased radiolabel incorporation (Fig. 4.7c) and decreased intracellular GSH upon t-BuOOH exposure (Fig. 4.7d).

PMA-activated Jurkat cells also showed a time-dependent increase in radiolabel incorporation from [^{35}S]-methionine into GSH (Fig. 4.7e) albeit the extent of incorporation was considerably lower than in unactivated cells (Fig. 4.7c). As with resting Jurkat cells, the transsulfuration pathway in activated Jurkat cells was unresponsive to oxidative stress, at least for the first 12 hours following peroxide treatment. Thereafter, a ~45% decrease in radiolabel incorporation into GSH was seen between 12-24 h (Fig. 4.7e) although the intracellular GSH pool size decreased only marginally over the same time period (Fig. 4.7f). This suggests that cysteine derived from some other source, possibly transport, might be responsible for maintaining intracellular GSH concentrations under these conditions.

4.4.7 Transsulfuration pathway blockade increases T cell susceptibility to oxidative stress-induced cell death

Since the transsulfuration pathway is important in many cell types for cysteine provision especially under oxidative stress conditions, we compared the viability of T cells exposed to varying concentrations of t-BuOOH in the presence and absence of PPG

(Fig. 4.8). Both naive T cells and Jurkat cells are significantly more susceptible to peroxide-induced cell death (LD_{50}= 35 μM and 42 μM respectively) than activated T cells (LD_{50}= 240 μM). Inhibition of the transsulfuration pathway increased the sensitivity of all three cell types (LD_{50}= 21 μM, 32 μM and 150 μM for naïve, Jurkat and activated T cells, respectively).

4.5 Discussion

The requirement for a reducing extracellular microenvironment for T cell activation and proliferation has been suspected from their dependence on exogenous reductant such as β-mercaptoethanol added to the culture medium (*19*). The basis of this dependence has been ascribed to the inability of naïve T cells to efficiently transport cystine, the oxidized form of the amino acid that is relatively abundant in circulation. More recent studies have demonstrated that APCs, especially dendritic cells, provide the extracellular reducing milieu to facilitate an immune response (*9, 11*). In the present study, we demonstrate that during APC-independent activation of T cells by anti-CD3 and anti-CD28 antibodies, cystine uptake and extracellular cysteine accumulation, hallmarks of APC metabolism during activation, are observed. This begs the obvious question as to what is the primary sulfur metabolic status of T cells, i.e., what pathways exist for provision of cysteine needed for GSH and other biosynthetic needs in naïve cells and what routes for cysteine acquisition are added upon activation. To address these questions, we have characterized the transsulfuration pathway in naïve and activated T cells, its contribution to GSH homeostasis and antioxidant capacity under oxidative stress conditions and the induction of the x_c^- transporter system that leads to APC independence. We also compare the sulfur

metabolic status of Jurkat cells, a transformed cell line that is widely used as an experimental model system for T cells.

Activation of T cells is accompanied by up-regulation of pathways of nutrient uptake, ATP production, macromolecule synthesis and programs T cells for proliferation (*20*). We show that xCT, the catalytic subunit of the x_c^- cystine transporter, is highly expressed in T cells activated in an APC-dependent or independent manner, but is not detectable in naïve T cells. Earlier studies have shown that the mRNA levels for xCT are very low in naïve T cells, whereas both xCT mRNA and protein are expressed in dendritic cells (*10, 11, 21*). Mice with homozygous disruption of the x_c^- transporter ($xCT^{-/-}$) exhibit redox imbalance in the plasma, with high cystine and low GSH in comparison to $xCT^{+/+}$ mice. Embryonic fibroblasts from $xCT^{-/-}$ mice fail to survive in culture unless supplemented with cysteine derivatives or antioxidants (*22*). Enhanced xCT activity in astrocytes increases GSH synthesis and protects neurons from oxidative stress (23). Expression of xCT is regulated by Nrf-2 (nuclear factor erythroid 2-related factor-2), which binds to the antioxidant response element (*24*) in response to oxidative stress. Although the mechanism of transcriptional induction of xCT during T cell activation awaits elucidation, it is possible that an initial pro-oxidant response during T cell activation leads to the transcriptional activation of xCT.

Clearly, in the absence of x_c^-, T cells must obtain cysteine needed for protein synthesis and for maintaining GSH at the levels seen in naïve cells from elsewhere. One such avenue is the transporter for cysteine. In T cells, cysteine uptake is mainly mediated by the sodium dependent ASC system (for alanine, serine and cysteine) (*25*). The cystine transport activity is extremely low in resting T cells compared to the cysteine transport

activity. When T cells are activated, both cystine and cysteine transporter activities are up-regulated, with the cystine uptake rate still being lower than the cysteine uptake rate (*25*).

Another route for provision of cysteine is the transsulfuration pathway whose presence in T cells is controversial. Human lymphoid cell lines from normal subjects but not from a cystathionuric patient with γ-cystathionase deficiency, were able to convert [^{35}S]-homocysteine to [^{35}S]-cysteine, consistent with the presence of the transsulfuration pathway (26). However, other studies using the PCR and flow cytometry techniques reported that T cells lack γ-cystathionase and concluded that these cells lack the transsulfuration pathway (*16, 27*). Using a sensitive metabolic radiolabeling method, we confirm the presence of an intact transsulfuration pathway in naïve and activated T cells as well as in Jurkat cells (Figs 4.5 and 4.6). The transsulfuration pathway is estimated to contribute ~50% of the cysteine in the GSH pool in hepatoma cells lines and in macrophages (*13, 14*). Our studies demonstrate the quantitative significance of the transsulfuration pathway to GSH homeostasis in T cells where inhibition by PPG caused a ~35%, ~25% and ~50% decrease in GSH concentration within 12 h in naïve, activated and Jurkat T cells, respectively. Furthermore, increased flux through the transsulfuration pathway is observed under oxidative stress conditions in primary T cells and represents an autocorrective response for rebuilding antioxidant capacity, i.e., regaining the GSH pool size compromised by oxidizing conditions. However, even in the absence of increased flux through this pathway, its importance to the cellular capacity for countering oxidative stress is exemplified by the decrease in LD_{50} for peroxide in all three cell types treated with the transsulfuration inhibitor, PPG (Fig. 4.8).

The transsulfuration pathway and the x_c^- transporter play important roles in several settings of intercellular communication. For example, the transsulfuration pathway is induced during monocyte differentiation and plays an important role in intracellular killing of mycobacteria (*14*). Inhibition of the transsulfuration pathway during mycobacterial infection allows bacteria to proliferate in the hostile environment of the host. Redox signaling between astrocytes and T cells endows astrocytes with a neuroprotective phenotype (*15*) and blockade of the x_c^- transporter is detrimental for this phenotype. Immunosuppression of auto-reactive T cells by regulatory T cells involves interference with extracellular cysteine accumulation by dendritic cells (*11*).

Since naïve T cells have the capacity for cysteine synthesis via the endogenous transsulfuration route, why then are they dependent on APCs for provision of cysteine during activation? We posit that the key role for APC-derived extracellular cysteine might be to create a reducing microenvironment needed to facilitate inter- and intra-cellular signaling. The cysteine/cystine redox couple is an important redox buffer and is considered to be an important indicator of the extracellular redox poise (*28*). Human cells in the culture maintain an extracellular redox potential of ~-80 mV, which is associated with growth arrest/differentiation. This value is similar to the plasma cysteine/cystine redox potential in young healthy adults (*29*). More negative redox potential values favor cellular proliferation whereas more positive values favor cell death. Hence, cysteine accumulation by APCs is instrumental for fashioning a reductive shift in the extracellular microenvironment that is conducive for subsequent T cell proliferation. In addition to remodeling of the extracellular redox potential, it is possible that flux through the transsulfuration pathway is insufficient for meeting the cysteine needs of T cells that

receive activation signals, which eventually leads to an increase in cell size in addition to triggering proliferation. The relative importance of APC-derived cysteine for extracellular redox remodeling versus supporting intracellular biosynthetic needs, awaits further investigation.

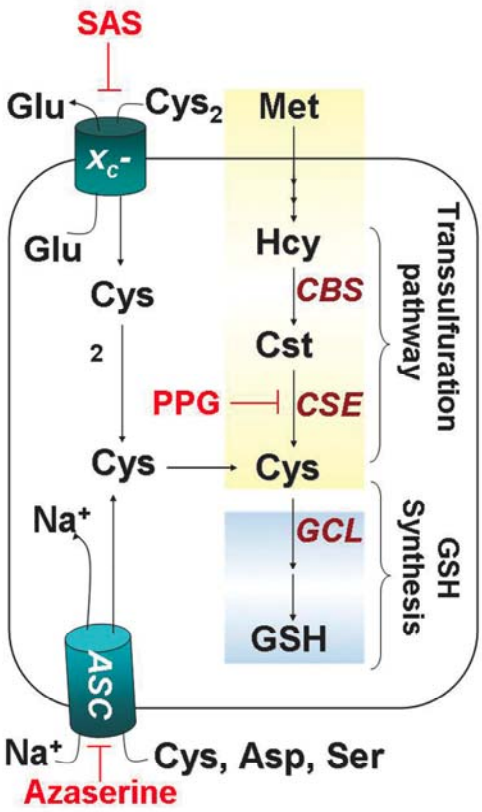

Figure 4.1 Glutathione homeostasis in T cells by the x_c^- transporter and the transsulfuraton pathway. PPG, SAS, and azaserine are inhibitors of γ-cystathionase, the x_c^- transporter and the neutral amino acid transporter ASC, respectively. CBS, CSE and GCL denote, cystathionine β-synthase, γ-cystathionase and γ-glutamylcysteine ligase, respectively. This figure was prepared by Sanjay K. Garg.

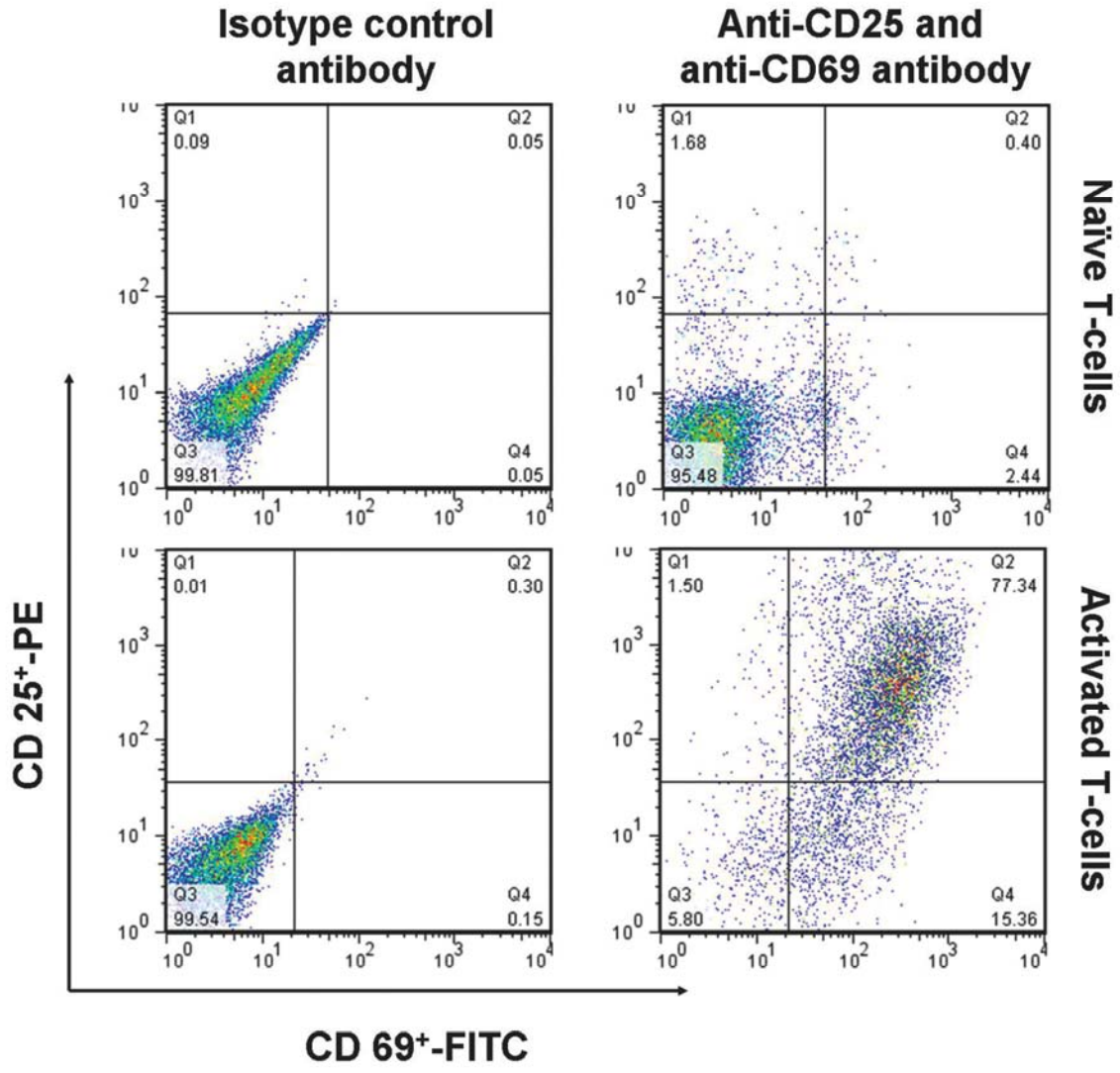

Figure 4.2 Upregulation of activation associated markers on T cells surface. Naïve and dendritic cell-activated T cells were stained with anti-CD25-PE and anti-CD69-FITC or the corresponding isotype controls and analyzed by flow cytometry. Numbers inside the boxes indicate percentage of $CD3^+$ naïve and activated T cells positive for CD25-FITC and CD69-PE.

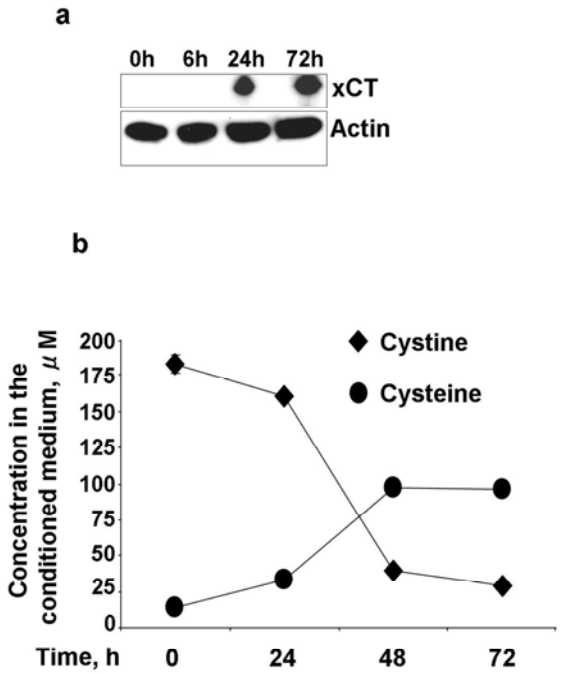

Figure 4.3 T cells induce the cystine transporter during activation. **(a)** xCT expression increases upon activation of T cells by anti-CD3/anti-CD28 antibodies. Cell lysate from either naïve T cells or T cells activated by anti-CD3/anti-CD28 antibodies for 6, 24 and 72 h were subjected to immunoblotting and probed with anti-xCT antibody. Actin is shown as an equal loading control. **(b)** Naïve CD3+ T cells were incubated with anti-CD3 and anti-CD28 antibodies and the concentration of cystine and cysteine were measured at the indicated time points. Data are representative of 2 (a) and 3 (b) independent experiments performed in triplicates (b). Zhonghua Yan and Sanjay K. Garg performed the experiments together in figure 4.3a. Sanjay K. Garg performed the experiments in figure 4.3b.

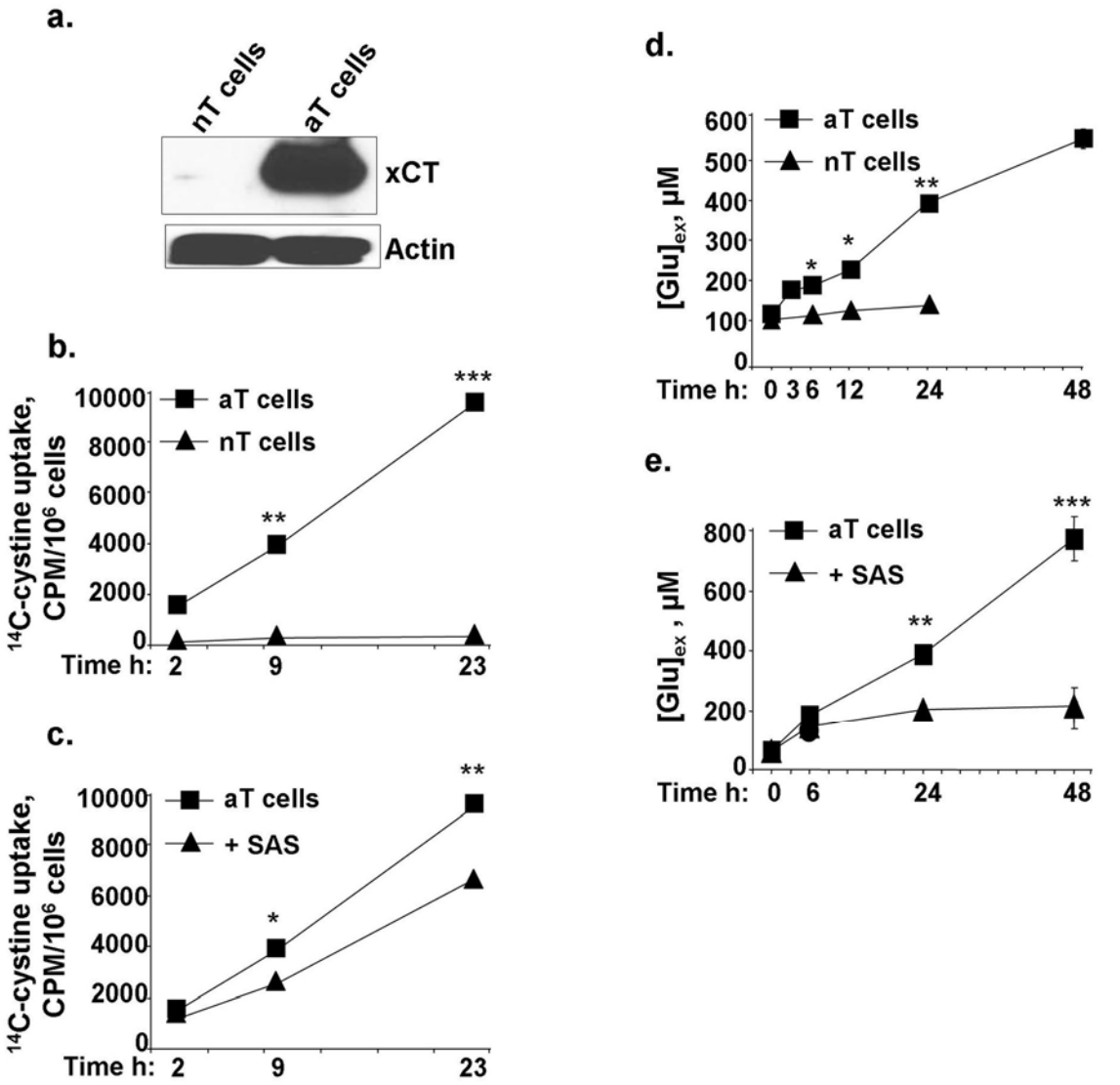

Figure 4.4 Activated but not naïve T cells mediate cystine/glutamate exchange via x_c^-. **(a)** xCT expression increases upon activation of T cells by dendritic cells. Cell lysate from either naïve T cells or T cells collected from a dendritic cell and T cell co-culture were subjected to immunoblotting and probed with anti-xCT antibody. Actin is shown as an equal loading control. Naïve **(b,d)** or activated **(b, c, d, e)** T cells were incubated with 0.1 µCi/ml [^{14}C]-cystine in the presence or absence of 500 µM SAS **(c, e)** for the indicated times. An aliquot of the medium at indicated times was removed for glutamate analysis **(d, e)** and cells were collected for intracellular radioactivity measurement **(b, c**). For this, cells were collected in a tube, washed 3 times with cold phosphate-buffered sailine and then lysed with 400 µl NaOH (500 mM). Scintillation cocktail was added and total intracellular [^{14}C] radioactivity was recorded and normalized to the cell number. Data are shown as mean ± SD and are representative of 2 (**a, b, e**) and 3 (**c,d**) independent experiments performed in duplicates. * $p \leq 0.05$, ** $p \leq 0.01$ *and* *** $p \leq 0.001$. Sanjay K. Garg performed the experiments in figure 4.4b-e.

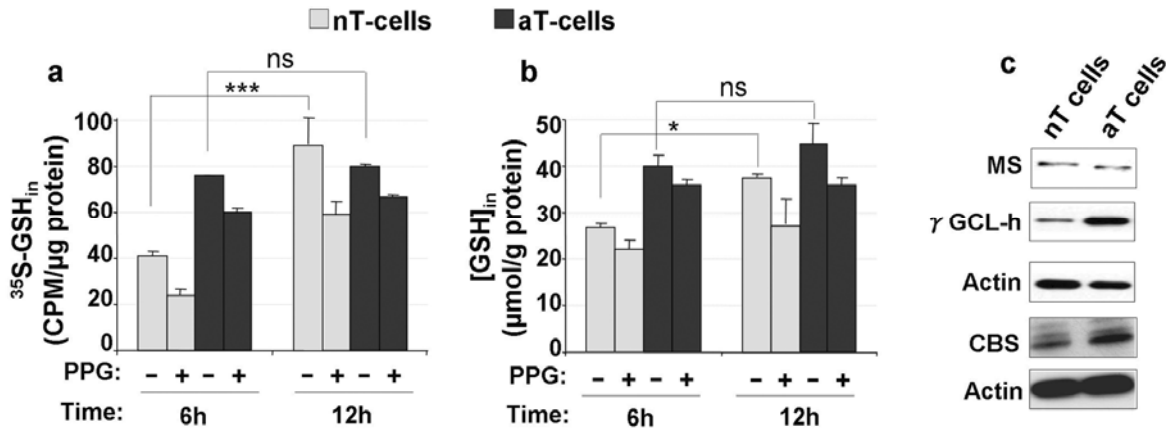

Figure 4.5 The transsulfuration pathway is intact in both naïve and activated T cells. Naïve (gray) and activated (black bar) T cells were incubated with 2 µCi/ml L-[^{35}S]-methionine in the presence or absence of 2.5 mM PPG for 6 and 12 h. At the indicated time, cells were harvested and radioactivity incorporation in GSH **(a)** and the intracellular GSH concentration **(b)** were measured and normalized to protein. Data are shown as mean ± SD and are representative of 3 independent experiments performed on different batches of cells. Statistical analysis using Student t-test revealed significant changes in intracellular GSH synthesis and radioactive labeling over time in both naïve and activated T cells and its inhibition by PPG. * $p \leq 0.05$, *** $p \leq 0.001$ *and ns=not significant.* Panel **c** shows the comparison of expression level of CBS, methionine synthase, γGCL (heavy subunit) between naïve and activated T cells via Western blot analysis. Zhonghua Yan and Sanjay K. Garg performed the experiments together in figure 4.5a, b. Sanjay K. Garg did the experiments in figure 4.5c.

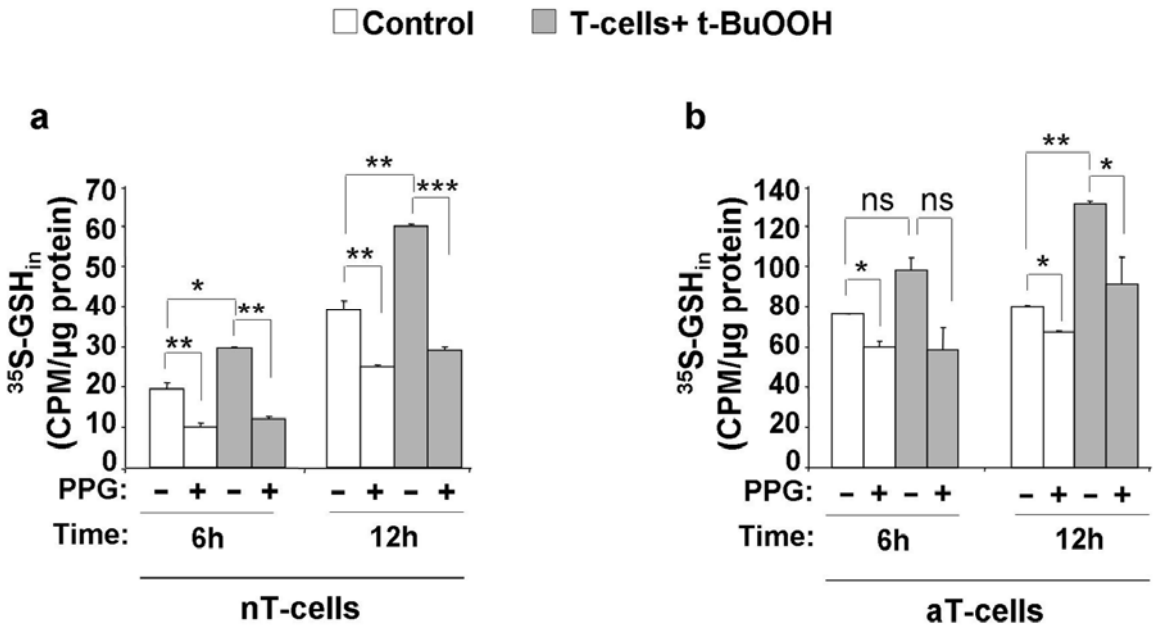

Figure 4.6 Peroxide stress activates the transsulfuration pathway in both naïve and activated T cells. Naïve T cells (**a**) ± 20 µM t-BuOOH (gray bar) were incubated with 2 µCi/ml L-[^{35}S]-methionine ± 2.5 mM PPG. Activated T cells (**b**) ± 100 µM t-BuOOH (gray bar) were incubated with 2 µCi/ml L-[^{35}S]-methionine ± 2.5 mM PPG. At the indicated time, cells were harvested and radioactivity incorporation into GSH was measured and normalized to protein concentration. Data are shown as mean ± SD and are representative of 3 independent experiments performed on different batches of cells. Statistical analysis using the Student t-test revealed significant changes in intracellular radiolabeling upon t-BuOOH treatment and inhibition by PPG. *$p \leq 0.05$, **$p \leq 0.01$, *and* ***$p \leq 0.001$. Zhonghua Yan and Sanjay K. Garg performed the experiments together in figure 4.6a, b.

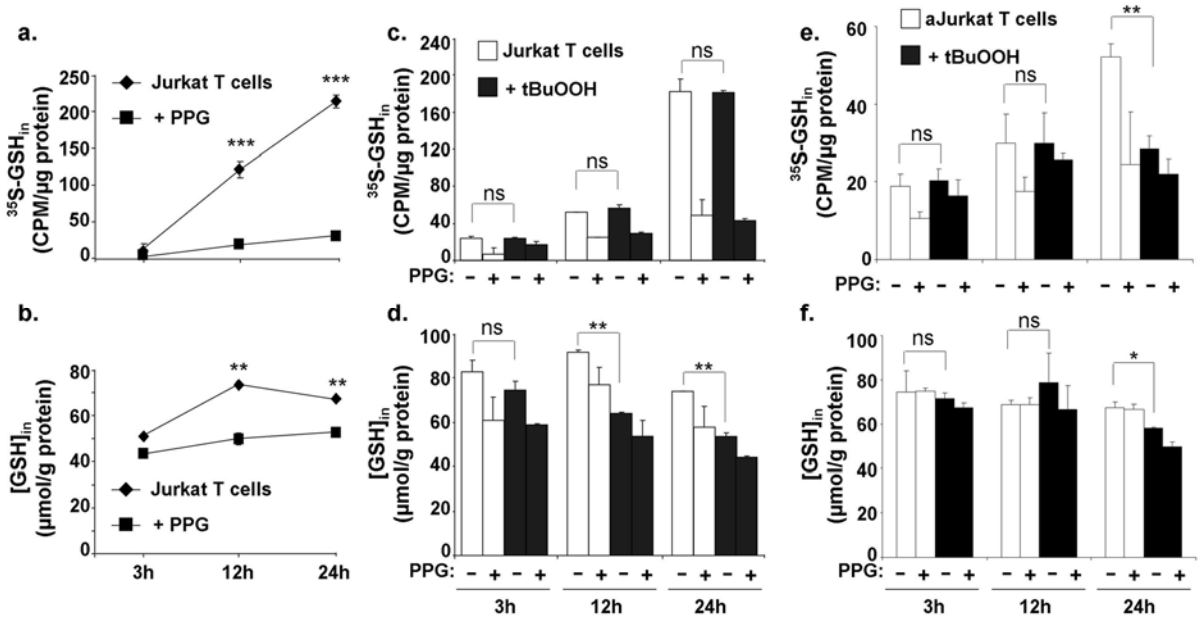

Figure 4.7 Jurkat cells have an intact transsulfuration pathway that is unresponsive to peroxide stress. Resting Jurkat cells (a-d) or PMA-activated Jurkat cells (e-f) were either untreated (a, b) or treated with 10 µM t-BuOOH (c, d, e, f) and incubated with 2 µCi/ml L-[^{35}S]-methionine ± 2.5 mM PPG as described under Methods. At the indicated time, cells were harvested and radioactivity incorporation into GSH (a, c, e) and the intracellular GSH concentration (b, d, f) were measured and normalized to protein concentration. Data are shown as mean ± SD and are representative of 3 independent experiments performed in duplicates. Statistical analysis using the Student's t-test revealed a significant time-dependent increase in intracellular GSH synthesis and radiolabeling in Jurkat cells and inhibition by PPG. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, *ns=not significant.*

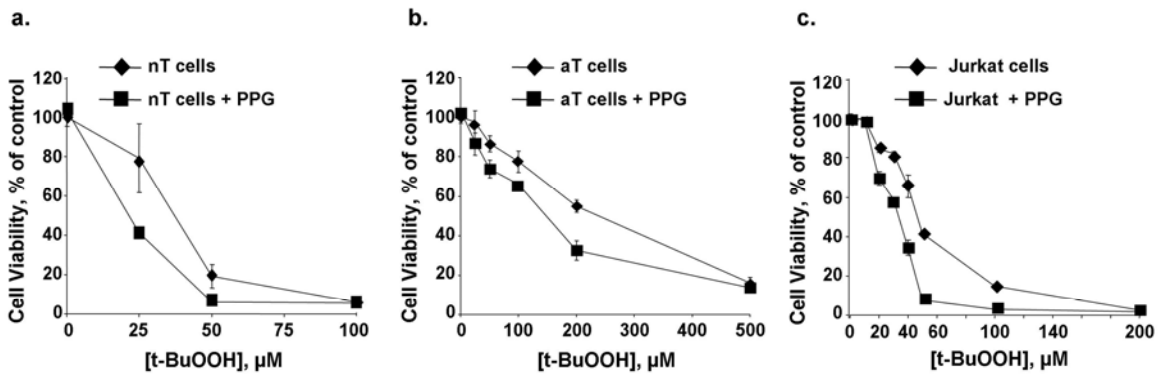

Figure 4.8 Blockade of the transsulfuration pathway enhances T cell susceptibility to oxidative stress-induced cell death. Naïve (**a**) or activated (**b**) T cells or Jurkat cells (**c**) were incubated with the indicated concentrations of t-BuOOH ± 2.5 mM PPG for 12-14 h. Cell viability was determined as described under Methods. Data are shown as mean ± SD and are representative of at least 2 independent experiments performed in duplicates.

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Chapter 5

Conclusions and Perspectives

5.1 Summary of findings

Activation and proliferation of T cells require a reducing extracellular microenvironment in the immune synapse that is provided by antigen presenting cells, especially dendritic cells. Stimulation of dendritic cells by T cells activates the NF-κB pathway in dendritic cells and induces an antioxidant response. It also enhances system x_c^--dependent cystine uptake, leading to increased GSH synthesis, export, and finally, degradation to cysteine outside the cell. Accumulation of extracellular cysteine supports GSH synthesis in T cells while also leading to a more reducing redox potential that is needed for T cell proliferation.

Naturally occurring regulatory T cells, a suppressor sub-population of T cells, prevent autoimmune diseases and maintain peripheral tolerance by suppressing self-reactive effector T cells. They also suppress beneficial immune responses to parasites, viruses, and tumors. However, their mechanism of suppression is still not fully understood. Here, we have demonstrated that inhibition by regulatory T cells of dendritic cell-induced extracellular redox remodeling is a component of the regulatory T cell suppression mechanism. Suppression of DC-dependent T cell proliferation by regulatory T cells is correlated with a significant diminution in extracellular cysteine concentration and is abrogated by addition of exogenous cysteine. We demonstrated that regulatory T

cell-mediated redox perturbation is antigen-dependent but not antigen-specific, is CTLA-4-dependent, and requires cell-cell contact. Regulatory T cells decrease expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis, leading to lower intracellular GSH synthesis in LPS-stimulated DCs. Additionally, regulatory T cells might contribute to restricting extracellular cysteine accumulation by a competitive uptake mechanism, and they partition cysteine more proficiently to the oxidation product, sulfate, while effector T cells divert more of their cysteine pool towards protein and GSH synthesis. By interfering with the extracellular cysteine pool, regulatory T cells not only decrease the intracellular GSH levels in effector T cells, but also block GSH relocalization into the cytoplasm, thus inhibiting T cell activation and proliferation. Furthermore, the decrease in the extracellular redox potential during T cell activation is associated with an increase in the levels of cell surface thiols in both DCs and T cells, which is diminished in the presence of regulatory T cells.

We also investigated the role of the x_c^- cystine transporter and the transsulfuration pathway in the regulation of GSH homeostasis in T cells and in the defense against oxidative stress. The x_c^- transporter is absent on naïve T cells, but up-regulated during T cell activation. We showed the existence of the intact transsulfuration pathway in naïve and activated T cells and the Jurkat T lymphoma cell line. The flux through the transsulfuration pathway increases in T cells under oxidative stress conditions. Inhibition of the transsulfuration pathway increases the vulnerability of T cells and Jurkat cells to oxidative cell death.

5.2 A general mechanism of Treg suppression

In this thesis, we report a novel mechanism of regulatory T cell suppression of effector T cell activation and proliferation by modulation of the extracellular redox environment (*1, 2*). In contrast to naïve T cells, co-culture of regulatory T cells with DCs does not affect extracellular cysteine concentration. However, regulatory T cells suppress cysteine accumulation in the extracellular compartment when added to co-cultures of DCs and naive T cells. As a consequence, both intracellular (diminished GSH levels in T cells) and extracellular (diminished cell surface thiol labeling on T cells and on DCs) perturbations in the redox status result (*3*). Remarkably, although regulatory T cells are known to mediate their suppressive functions by multiple strategies, provision of a single reagent, i.e., exogenous cysteine at concentrations seen under DC-T cell co-culture conditions, alleviates inhibition of T cell proliferation (*3*). This observation begs the question as to whether redox regulation serves as a master switch in the multipronged suppressive action of regulatory T cells.

We posit that the redox changes in the intra- and extra-cellular compartments influence one or more of the well known regulatory T cell suppressive mechanisms. For instance, the anti-inflammatory cytokine IL-10, has antioxidant properties (*4*), and TGFβ, a multifunctional cytokine, is redox regulated (*5*). Activation of latent TGFβ requires reductive cleavage of a disulfide bond that links it to the latency-associated peptide, but over-reduction leads to formation of inactive TGFβ monomers. Thus, activation and inactivation of TGFβ are subject to redox control, and dynamic changes in the extracellular redox milieu might be important for regulating TGFβ activity (*6*). Furthermore, granzyme A, the cytolytic T cell protease, cleaves redox factor 1 (Ref-1), which in turn enhances cell death (*7*). Regulatory T cells induce indoleamine 2, 3-

dioxygenase in DCs, which catalyzes the oxidative catabolism of tryptophan (*8*). The tryptophan metabolites generated from the kynurenine pathway by indoleamine 2, 3-dioxygenase, which inhibits T cell proliferation, are redox active (*9*). Subtle changes of the extracellular redox status may cause the functional changes of these redox sensitive proteins, which affects T cell activation and proliferation. Regulatory T cells need to modulate the extracellular redox status for these molecules to work in a proper redox microenvironment. Thus, redox control may be integral to regulatory T cell mediated suppression mechanisms and is more pervasive than previously recognized.

Furthermore, extracellular redox remodeling is utilized by other cell types such as myeloid-derived suppressor cells and B cells to modulate immune responses as well (*10*). Myeloid-derived suppressor cells are induced by tumor cells from myeloid progenitor cells to suppress the anti-tumor immunity (*11*). One mechanism they use to suppress T cell activation and function is by consuming cystine and limiting cysteine availability. These myeloid-derived suppressor cells express high levels of the x_c^- transporter to uptake cystine but do not export cysteine, which makes a cystine and cysteine deficient microenvironment inhibiting T cell proliferation (*10*). During B cell differentiation into plasma cells, ROS levels increase initially followed by an antioxidant response, including induction of antioxidant enzymes and accumulation of small molecular weight thiols (*12*). Thus, thiol-mediated redox modulation has emerged as a key regulatory strategy for the regulation of adaptive immune responses.

5.3 Future directions

It has long been known that T cell activation and proliferation require a reducing milieu, which is shaped primarily by the metabolic activity of antigen presenting cells, especially DCs. Interactions of DCs with naïve T cells stimulate cystine consumption and cysteine accumulation in the extracellular space, which produces an extracellular redox potential suitable for T cell proliferation (*3, 13*). A more reducing extracellular redox potential is reflected in the increased T cell surface thiol status (*3*). Isotope-coded affinity tag (ICAT) coupled to mass spectrometry is being employed to identify the redox-sensitive cell-surface thiols on T cells (*14*). Our preliminary study using Jurkat cells in the presence and absence of N-acetyl-cysteine (NAC) identified about 60 membrane proteins showing redox sensitivity. Examples of the potential redox sensitive membrane proteins that were identified in this preliminary study include CD5, CD45, and annexin A2. CD5, which contains several cysteine-rich domains, is highly expressed on T cells and upregulated upon strong activation (*15*). CD45 is shown to be a redox sensitive protein tyrosine phosphatase, controlling the production of cytokines (*16*). Annexin A2 is a substrate of Trx and involved in the processing of plasminogen (*17*). These redox-sensitive membrane proteins are potential targets for extracellular redox regulation during DC-T cell interaction. The effects of redox status change on the function of these redox sensitive proteins and on T cell biology, i.e., activation and proliferation, remain to be elucidated. Identification and characterization of redox sensitive proteins on DCs and T cells will shed light on potential pharmacological targets to modulate immune responses.

Accumulation of extracellular Trx1 has been observed under DC-T cell co-culture conditions. The role of extracellular Trx1 in T cell activation and proliferation is also an interesting topic to be studied. Despite the antioxidant role of intracellular Trx1 in

maintaining the cytosolic redox environment, a reductive role for extracellular Trx1 is quite controversial. Many studies suggest cytokine/chemokine-like functions for extracellular Trx1 in immune and cancer cells. Extracellular Trx1 exhibits proinflammatory effects by stimulating cytokine release and proliferation of lymphocytes (*18, 19*). It also exhibits a chemotactic effect, inducing migration of neutrophils, monocytes and T cells (*20*). However, several recent studies suggest the role of extracellular Trx1 in reducing disulfide bonds on cell surface membrane proteins. Matthias et al. showed that reduction of the CD4 glycoprotein by Trx1 secreted by effector T cells facilitates the entry of HIV-1 (*21*). Using the mechanism-based kinetic trapping method, CD30 was found to be the main interaction partner for extracellular Trx1 on the surface of lymphocytic cell lines (*22*). More recently, extracellular Trx1 was shown to activate the classical transient receptor potential (TRPC) channel by breaking the disulfide bridge in TRPC5 (*23*). Thus, the extracellular Trx1 accumulated in DC-T cell co-culture may change the redox status of T cell surface proteins such as CD4 and CD30, which influence T cell activation and proliferation. T cells play a central role in the pathogenesis of rheumatoid arthritis. Extracellular concentrations of Trx1 are highly elevated in the serum and synovial fluid of rheumatoid arthritis patients, which makes it a good biomarker for the disease activity of rheumatoid arthritis (*24*). However, the role of extracellular Trx in rheumatoid arthritis is unclear. We therefore can consider a role for extracellular Trx in regulating T cell surface thiol status, thus affecting T cell reactivity and arthritis development.

The greater availability of extracellular cysteine influences the intracellular antioxidant capacity within T cells since cysteine limits GSH biosynthesis. Consequently,

intracellular GSH levels rise and in turn, influence T cell signal transduction pathways and gene expression. The choreography of GSH localization and the GSH/GSSG redox potential changes during T cell activation, and their correlation with the onset and operation of signaling pathways and cell cycle progression await elucidation.

Modulation of the extracellular redox microenvironment induced by DC and T cell interaction by regulatory T cells, could be mediated by one or more mechanisms. For instance, by limiting cysteine availability, regulatory T cells deprive effector T cells of a building block needed for protein and GSH synthesis. Alternatively, by perturbing the redox environment, regulatory T cells can have both indirect effects by enhancing other suppressive mechanisms used by them (as discussed above) and direct effects on T cell activation and proliferation targets, which are sensitive to the redox potential and the redox status of key signaling proteins. Many questions remain to be addressed regarding how regulatory T cells inhibit reductive remodeling by DCs. For instance, how do regulatory T cells modulate the redox environment and redox signaling in vivo? Is there a connection between the mechanism for perturbing redox remodeling and Foxp3 expression, and what is the extent of cross-talk between the other suppressive mechanisms and redox remodeling? And finally, what is the physiological relevance of the redox remodeling mechanism in normal and disease states? The answers to these questions will help illuminate the biology of regulatory T cell suppressive mechanisms and identify potential therapeutic targets.

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