Free radicals and redox signalling in T-cells during chronic inflammation and ageing

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Abstract
During chronic inflammation and ageing, the increase in oxidative stress in both intracellular and extracellular compartments is likely to influence local cell functions. Redox changes alter the T-cell proteome in a quantitative and qualitative manner, and post-translational modifications to surface and cytoplasmic proteins by increased reactive species can influence T-cell function. Previously, we have shown that RA (rheumatoid arthritis) T-cells exhibit reduced ROS (reactive oxygen species) production in response to extracellular stimulation compared with age-matched controls, and basal ROS levels [measured as DCF (2′,7′-dichlorofluorescein) fluorescence] are lower in RA T-cells. In contrast, exposing T-cells in vitro to different extracellular redox environments modulates intracellular signalling and enhances cytokine secretion. Together, these data suggest that a complex relationship exists between intra- and extra-cellular redox compartments which contribute to the T-cell phenotype.

Introduction
The principal function of the immune system is to discriminate between self and non-self, and the effector T-cell arm (including TH1, TH2 and TH17; see Figure 1) is responsible, along with the B-cell arm of the immune system, for elimination of non-self. Removal of any self-reactive T-cells occurs early during development; however, Treg cell (regulatory T-cell)-mediated immunosuppression is also responsible for ensuring that self-tolerance is maintained [1]. Activation of T-cells typically leads to clonal growth and de novo protein synthesis; however, inappropriate T-cell function has been implicated during ageing and autoimmune conditions such as RA (rheumatoid arthritis).

It is becoming increasingly evident that the intracellular T-cell redox environment works co-operatively with the extracellular matrix and exofacial membrane proteins to influence T-cell function in health, ageing and disease. The present article explores how redox remodelling within and from the inside to the outside of the cell may be responsible for regulating T-cell responses and how it is influenced by age and chronic disease.

The importance of cysteine redox state in physiology
The major plasma antioxidant protein is albumin, and although the synthesis of some acute-phase proteins by the liver, such as fibrinogen, declines with age, it is unclear whether the liver synthetic rate and plasma concentration of albumin is reduced [2,3]. Nevertheless, albumin thiol content is decreased with age and this is paralleled by an increase in the cystine/cysteine ratio in plasma [4,5]. Consequently, the plasma compartment and extracellular face of cells tends to be more oxidizing during ageing and chronic inflammatory conditions with persistent phagocytic ROS (reactive oxygen species)/RNS (reactive nitrogen species) production such as RA [6]. In an attempt to maintain exofacial protein thiols, cystine is recycled by many cells, with cysteine exported out of the cell via cystine transporters; in association with exported cysteine, protein disulfide-isomerase catalyses the reduction of surface thiols [7,8]. Effective extracellular communication between cells is dependent on receptor-ligand interactions. Several receptors have essential thiol moieties which must be chemically reduced for effective function, including the adhesion molecule ligand VLA-4 (very-late antigen 4), highlighting the importance of close control of cellular redox state in immune cell recruitment [9].

Regulation of cellular redox state
The methionine transulfuration pathway produces cytosolic cysteine from methionine; however, the majority is delivered as cysteine by the cystine/glutamate antiporter. Cystine is converted into cysteine at the expense of GSH oxidation for use in intracellular protein and GSH synthesis, but also...
returns cysteine to the extracellular environment for maintenance of exofacial membrane redox [7]. Cellular redox balance is achieved via three major redox couples: NAD+–NADH, NADP+–NADPH and the cysteine-containing tripeptide GSH–GSSG [10]. Cellular GSH concentration is dependent on the activity of GCL (γ-glutamylcysteinyl ligase) and cysteine availability [11]; expression of the rate-limiting GCL subunit is coupled to cellular redox state through the Nrf2 (nuclear factor-erythroid 2-related factor 2)–KEAP1 (Kelch-like enoyl-CoA hydratase-associated protein 1) system, providing a mechanism for cellular adaptation to oxidative stress through de novo GSH biosynthesis.

T-cell thiol oxidative modification may arise from: (i) an increase in ROS/RNS production, e.g. from mitochondria, enzymes such as the NADPH oxidase isoforms and hypoxia/hyperoxia; (ii) lack of cysteine and lower rates of glutathione and thioredoxin synthesis; and (iii) reduced enzymic conversion of oxidized thioredoxin or GSSG back into reduced thioredoxin and GSH. These latter processes are normally catalysed by thioredoxin and glutathione reductases which require NADPH as a cofactor. In addition to the reduction of sulfenic and sulfonic acids to thiols, thioredoxin reductase can also repair nitrosylated proteins [12]. If repair is ineffective, any increase in oxidized GSSG or mixed disulfides formed between oxidized proteins and GSH is minimized by effective efflux via MRPs (multidrug-resistance-associated proteins). MRPs are also responsible for GSH transport and as such can manipulate the intracellular redox state [13]. In addition, organelles such as the mitochondrion and possibly the nucleus maintain active transport processes for GSH to preserve a local reducing environment against concentration gradients as required for active gene transcription and to minimize damage from ROS leakage during respiration [14,15]. Considering the potential for discrete redox compartments in the cell that must be maintained independently, Jones and colleagues have devised a model aimed to predict their importance and propose that thioredoxin-dependent antioxidant pathways contribute the majority of the cellular antioxidant defence due to peroxiredoxins and protein disulfides [16]. Indeed, normal human Treg cells express and secrete higher levels of thioredoxin 1, which may prevent uncontrolled immune reactions by favouring the survival of suppressor rather than effector cells [17]. The efficiency of thioredoxin 1 is likely to be of particular significance during chronic inflammation when ROS/RNS production by phagocytes will favour a more oxidizing extracellular environment [18].

Figure 1 | T-cell populations, cytokines and cross-talk in the regulation of the T-cell repertoire
The development and maturation of T-cells occurs in the thymus from a common lymphocyte progenitor produced in the bone marrow. The pathway of differentiation is influenced by cytokine signals in the thymus, e.g. precursor CD4+ cells mature to Treg cells in the presence of excess TGFβ (transforming growth factor β). Treg cells themselves express high levels of IL-10 and TGFβ, but unlike TH1 and TH2 cells respectively, produce very little IL-2 and IL-4. The Treg cells suppress T-cell functions via superoxide anion radical and thiol depletion in target cells in order to avoid chronic activation or autoreactivity of T-cells in the periphery. Adapted from [18] with permission.

T-cell activation alters the redox state
Early studies investigating redox involvement in T-cell function focused on how the intracellular redox state is influenced by binding of an external ligand or activating signals. The earliest studies used mixed lymphocytes, and, in this way, Lacombe et al. [19] reported that stimulation of a mixed lymphocyte population with the mitogen ConA (concanavalin A), which recognizes bi- and tri-antennary mannose-rich carbohydrates, triggered an increase in the GSSG/GSH + GSSG ratio [19]. Subsequently, Los et al. [20] identified that stimulating T-cell proliferation via CD28, an important T-cell receptor co-activating receptor, resulted in GSH loss and an associated increase in cytosolic ROS in a rapid 5-lipoxygenase-dependent manner (within 30 min). The activity of 5-lipoxygenase was essential for subsequent activation of NF-κB (nuclear factor κB), cytokine production and proliferation [20]. Subsequent reviews have summarized activation-induced oxidative modifications to receptor-coupled signal transduction machinery in T-cells that link increased ROS, loss of GSH, protein oxidation and NF-κB activation [21,22]. However, not all oxidative protein changes support T-cell proliferation. Cemerski et al. [23] suggest that oxidation of the C-terminal part of TCR (T-cell receptor)-ζ and the membrane-proximal domain of the accessory protein, p56-lck, contributes to loss of TCR function; this is also observed in T-cells from elderly subjects with the downstream consequence of reduced NF-κB activation [23]. The differing outcomes (increased or decreased NF-κB activation) in response to increased ROS may be dependent on the extent of redox disturbance [22].

In contrast with the examples cited above of receptor-activated increases in intracellular ROS, recent studies in
effector CTLs (cytotoxic T-lymphocytes) have shown that culture with IL (interleukin)-15 not only increased ROS and RNS, but also induced increases in expression of the antioxidant enzymes/molecules glutathione reductase, thioredoxin reductase 1, peroxiredoxin and superoxide dismutase. Growth in the presence of the cytokine IL-15 also inhibited apoptosis and supported retention of the memory CD8+ T-cell population. Therefore IL-15 promotes persistence not only due to increased levels of anti-apoptotic proteins, but also due to increased antioxidant levels supporting CTL function in controlling viral infection and tumour growth [25]. In addition to the intracellular antioxidant increase, an increased expression of cell-surface thiols was also observed in IL-15-cultured T-cells, raising the possibility of inside-out signalling.

**Inside-out signalling by T-cells**

How a change in intracellular oxidants may influence the extracellular environment is increasingly recognized as an important question, but one that has not been widely investigated in the context of T-cells. Nevertheless, as early as 1996, Lawrence et al. [26] demonstrated that T-cell activation triggers a later increase (after 16 h) in exofacial thiol content for B-cells and CD4+ cells involving de novo protein synthesis [26]. More recent studies indicate that Treg cells may inhibit naïve T-cell maturation; normally, the dendritic cells which present antigen also undertake extracellular redox remodelling by providing a source of extracellular cysteine available for naïve T-cells [7]. However, Treg cells mop up cysteine, thus preventing naïve T-cell proliferative responses that are dependent on reduced exofacial protein thiols. Further T-cell subset-specific responses which are cell-surface thiol-dependent are also observed following treatment with the autocrine lectin galectin-9. In contrast with TH1 cells, CD4+ TH2 cells are resistant to galectin-9 death owing to alternative glycosylation. On TH2 cells, galectin-9 binds cell-surface protein disulphide-isomerase, increasing retention of the isomerase on the cell surface and altering the redox status at the plasma membrane, resulting in increased cell migration through the extracellular matrix via β3 integrins [8].

In the unrelated colon cancer cell line Caco2, a systematic variation in extracellular thiol/disulfide redox state (Er) over a physiological plasma range (0 to −150 mV) was investigated for alterations in cell proliferation and measured as BrdU incorporation. BrdU incorporation increased as a function of redox state and was greatest in the most reduced environment (−150 mV). Addition of a growth factor [e.g. EGF (epidermal growth factor)] increased the rate of BrdU incorporation at more oxidizing redox conditions (0 to −80 mV) only, and cellular GSH was not significantly affected by variation in extracellular Er [27]. These data confirm the importance of extracellular redox state for growth and proliferative response to growth factors.

The aforementioned evidence that redox state influences functional outcome following T-cell activation invokes the possibility of using pharmacological means to modulate redox state and therefore influence outcome.

**Modulating T-cell redox state can influence cell function in vitro and in vivo**

Evidence is accumulating that local fluxes in redox state, possibly within discrete subcellular compartments, can influence cell function, regulating diverse processes from receptor–ligand engagement to transcription factor activation and gene transcription [28,29]. Moreover, an increase in the oxidized GSSG/GSH ratio intracellullarly is observed in several cell types with age and chronic disease, possibly reflecting a reduced ability to adapt after prolonged oxidative stress. For example, ageing T-cells are functionally hyporesponsive to the proliferative effects of ConA. This may reflect the 2.5-fold increase in memory T-cells with age, which typically show lower response to ConA measured as IL-2 production and proliferation [30]. Pgp-1hi (Pgp is P-glycoprotein) and Pgp-1lo T-cells from young mice proliferate equally well when stimulated by optimal doses of PMA, suggesting that the poor response to ConA does not reflect poor viability. The importance of redox modulation in this phenotype is supported by studies by Franklin et al. [31], who demonstrated that GSH augments proliferative responses to ConA to a greater degree in older rather than younger rats and that this was associated with an increase in transferrin receptor expression in older T-cells.

We have reported previously that a loss in intracellular GSH enhanced T-cell IL-2R (IL-2 receptor) expression in response to phytohaemagglutinin which is usually NFAT (nuclear factor of activated T-cells)- and AP-1 (activator protein 1)-dependent, but that cytotoxic effects of methotrexate were decreased [32]. Although these observations suggest that the intracellular redox state has a strong influence on cellular outcome, they are at odds with other reports which indicate augmented proliferation in the presence of increased glutathione. Using plumbagin, a thiol-depleting agent that results in increased cytosolic ROS, mitogen-induced T-cell proliferation and cytokine [IL-2/IL-4/IL-6/IFNγ (interferon γ)] production was suppressed; however, this effect was reversed by thiol antioxidants, but not by nonthiol antioxidants. These data suggest that thiol depletion, but not ROS, plays an important role in T-cell response, at least in plumbagin-treated cells [33].

One of the most elegant demonstrations of how redox change influences outcome has arisen from the Nef−/− mouse model which is deficient in NADPH oxidase. NOX2-derived ROS have been shown to suppress antigen-dependent T-cell reactivity and to reduce the severity of experimental arthritis in both rats and mice [34]. Failure to produce superoxide anion by NADPH oxidase prevents the normal Treg cell inhibition of effector T-cells. This is confirmed further by the use of N-acetylcysteine and diphenylene iodonium which abrogate native Treg cell inhibition of T-effectors [35]. Following up this work, it has been suggested that Treg cell-mediated redox change is antigen-dependent,
but not antigen-specific, is CTLA-4 (cytotoxic T-lymphocyte antigen 4)-dependent, and requires cell–cell contact. T cells support extracellular redox remodelling by lowering the capacity for GSH synthesis by dendritic cells at the level of GCL, the limiting enzyme for GSH synthesis. Another important difference between Treg cells and effector T-cells is the preferred utilization route for intracellular cysteine: Treg cells promote cysteine oxidation to the sulfinate, whereas effector T-cells divert more of the cysteine pool towards protein and GSH synthesis. Treg cells appear to block GSH redistribution from the nucleus to the cytoplasm in effector T-cells, which is abrogated by the addition of exogenous cysteine [36]. Treg cells are increased in inflammatory diseases such as RA, probably due to aberrant proliferation, apoptosis and trafficking as well as their greater resilience to oxidative stress compared with conventional T-cells; Treg cells express and secrete higher levels of thioredoxin 1, thereby maintaining surface thiols which associates with reduced apoptosis [17].

Given the importance of maintaining an appropriate redox balance for controlling appropriate responses to stimuli, we have investigated whether general antioxidant supplementation with ascorbic acid (vitamin C) would influence the T-cell proteome in vitro and in vivo [37,38]. Ascorbic acid supplementation lowered intracellular glutathione concentration significantly, and protein expression changes observed in vitro were related to increases in anti-apoptotic, glycolytic, immune and signalling functions [37]. In particular, T-cell phosphatidylinositol-transfer protein was elevated by ascorbic acid in vitro. Together with our previous study measuring the effect of ascorbic acid on protein oxidation where a decrease in protein carbonyl was only observed in subjects with low plasma ascorbic acid status, these findings do support the concept that it is possible to modulate T-cell protein expression by dietary intervention. However, benefit may only be observed in subjects with low plasma ascorbic acid content [39]. Further investigation is needed to examine the significance of ascorbate-induced loss of cellular glutathione.

A similar observation was reported by Wilankar et al. [40] who noted that a vitamin E (α-tocotrienol)-like molecule, GT (γ-tocotrienol) lowered intracellular glutathione in lymphocytes, an effect that could not be abrogated by thiol or non-thiol antioxidants [40]. GT was more effective in suppressing ConA-induced T-cell proliferation, activation of NF-κB, AP-1 and NF-κB-dependent gene expression and cytokine production compared with vitamin E in vitro and in vivo. However, transient exposure of lymphocytes to GT (4 h) resulted in higher survival and proliferation of lymphocytes in vitro and in vivo in syngeneic and allogeneic hosts due to NF-κB, AP-1 and mTOR (mammalian target of rapamycin) activation in lymphocytes upon transient exposure [40]. mTOR is a nutrient sensor and, together with PI3K (phosphoinositide 3-kinase), determines the repertoire of adhesion and chemokine receptors expressed by T-cells according to their maturation state; hence lymphocytes use PI3K and mTOR to match metabolism and trafficking [41].

**Influence of intracellular metabolism on T-cell redox**

T-cell growth and function in response to stimulation requires sufficient energy generation, therefore activated T-cells decrease lipid oxidation and undergo a rapid increase in glycolysis following increased expression of GLUT1 (glucose transporter 1). At the conclusion of an immune response, oxidative phosphorylation tends to be associated with glycolytic oxidative phosphorylation occurs, with a corresponding decrease in GLUT1 expression [42]. However, in chronic immunostimulatory conditions such as RA and SLE (systemic lupus erythematosus), metabolic switching occurs in favour of an oxidative phosphorylation metabolic phenotype [43], a switch that can be induced in CD8+ T-cells in vitro following multiple antigenic challenge and is associated with higher mitochondrial ROS production. Inhibition of fatty acid oxidation decreases NADPH levels and the GSH content and elevates intracellular ROS. These results suggest that modulation of fatty acid oxidation in addition to the pentose phosphate pathway controls cellular NADPH levels, which is an essential cofactor for the recycling of GSH from GSS [44]. Stimulated CD4+ T-lymphocytes can differentiate into effector T-cell or inducible Treg cell subsets with specific immunological roles. Effector T-cells and Treg cells require distinct metabolic pathways to support these functions. TH1, TH2 and TH17 cells express high surface levels of the glucose transporter GLUT1 and are highly glycolytic. In contrast, Treg cells express low levels of GLUT1 and have high lipid oxidation rates. These data demonstrate that CD4+ T-cell subsets operate distinct metabolic pathways which may be manipulated in vitro to control Treg cell and effector T-cell development in inflammatory diseases [42]. Chronic inflammation is associated with a systemic metabolic shift with an increase in circulating lipids, favouring the metabolic profile of Treg cells [45].

**Conclusion and limitations**

In association with the decline in the extracellular redox state with ageing, there is an increase in Treg cells. Treg cells produce superoxide and scavenge extracellular cysteine from TH cells, consequently reducing proliferative responses and cytokine production resulting in a hyporeactive TH cell population. Accurate measurements of thiol and superoxide flux in the absence of significant cell manipulation during purification are needed to consolidate existing findings.

Treg cells derive energy from oxidative phosphorylation and available lipid for metabolism is increased with age and inflammation. The importance of the link between metabolism and trafficking merits further investigation. This is paralleled by a reduction in naive T-cells and a reduced capacity to respond to new antigens. Maintaining a reducing environment within T-cells is essential to restore extracellular thiols on TH cells and increase sensitivity to antigens via the TCR. Therefore specifically targeting reducing equivalents...
to TH cells should be considered to bypass the scavenging effects of Treg cells.

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