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Glutathione and lymphocyte activation: a function of ageing and auto-immune disease

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SUMMARY

A decline in tissue and serum of glutathione (GSH) content and GSH-metabolizing enzymes with age has been implicated in the increasing susceptibility to carcinogens, disease and drugs which occurs with advanced age. Immunological senescence has been directly associated with increased incidence of cancer and infection with age. The auto-immune diseases of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) demonstrate depressed T-cell function together with B-cell hyperactivity. In addition, RA and SLE are chronic inflammatory conditions which have been associated with low serum and erythrocyte GSH concentrations when compared to normal. We hypothesized that augmentation of intracellular GSH concentrations in lymphocytes may enhance immune function in depressed immune states. Our data, using murine animal models for ageing (C57BL/6J) and the RA/SLE-like auto-immune diseases of the MRL/lpr mouse, indicate that intracellular glutathione of splenic lymphocytes does not decline with age or with a chronic inflammatory auto-immune disease. In contrast, immune responsiveness in splenic lymphocytes does decline. We can, however, augment both intracellular GSH concentrations and the immune response of splenic lymphocytes from animals of all ages as well as in those animals with the SLE-like auto-immune disease.

INTRODUCTION

The process of ageing is characterized by a decline in the immune system (Makinodan & Kay, 1980; Makinodan *et al.*, 1984). The sensescence of the immune system is responsible for the increased incidence of cancer and infection which occur with advanced age (Makinodan *et al.*, 1984). In immunological senescence, it is the T cell which undergoes the most significant change, with a decline in responsiveness to antigen and mitogen (Lui, Segre & Segre, 1982). The depressed T-cell function is believed to be due to an intrinsic intracellular defect which lowers responsiveness of the cell to foreign antigens (Makinodan & Kay, 1980; Makinodan *et al.*, 1984). Similarly, in the auto-immune diseases of rheumatoid arthritis (RA) and systemic lupus erythematous (SLE), patients demonstrate depressed T-cell function (Zvaifler, 1977) and B-cell hyperactivity (Carter, Bacon & Hall, 1981). Lymphocytes from RA and SLE patients demonstrate depressed responses to antigen and mitogen, presumably due to insufficient interleukin-2 (IL-2) production (Miyasaka *et al.*, 1984; Emery, Panayi & Nouri, 1984). Thus, the T-cell defect in RA and SLE, as in ageing, is linked to an inability to both produce and respond to exogenous IL-2 following mitogen or antigen challenge.

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Glutathione (GSH) is a tripeptide thiol which plays a significant role in the reduction of disulphides and in the protection of cells from the effect of radiation, oxygen intermediates and free radicals (Meister, 1983). In lymphocytes, GSH has been implicated in playing an important role in the initiation and progression of cellular activation (Chaplin & Wedner, 1978; Fishman *et al.*, 1981; Hamilos & Wedner, 1985). Depletion of intracellular GSH inhibits lymphocyte activation by mitogens (Hamilos & Wedner, 1985; Wellner *et al.*, 1982). Stohs and colleagues (Stohs, Lawson & Al-Turk, 1984) have recently examined GSH metabolism in the ageing process using a murine animal model. Their studies indicate that a decline in GSH levels of the lung, liver and whole blood of Swiss-Webster mice occurs with age. Stohs *et al.* (1984) suggested that a decrease in GSH content and GSH-metabolizing enzymes may contribute to the increased susceptibility to carcinogens, disease and drugs which occur with advanced age.

The process of inflammation is associated with response to oxygen intermediates and free radicals released from macrophages and neutrophils (Fantone & Ward, 1982). GSH is the cellular component responsible for the protection of cells from free radical damage (Meister, 1983). Thus, a chronic inflammatory process such as in RA and SLE would involve the utilization of GSH and potentially lower intracellular glutathione concentrations. Studies in RA patients have demonstrated low serum thiol levels when compared to normal individuals

(Lorber *et al.*, 1964). In addition, treatment of RA patients with thiol agents, such as D-penicillamine and gold salts, increases both serum and erythrocyte GSH concentrations (Munthe, Guldal & Jellum, 1979; Munthe, Kass & Jellum, 1981). Thus, in both ageing and the auto-immune diseases of RA and SLE, low intracellular GSH pools may be playing a role in defective T-cell function.

We have recently reported that modulation of intracellular GSH concentrations in murine splenic lymphocytes alters lymphocyte activation (Fidelus & Tsan, 1986). By using the cysteine delivery agent 2-oxothiazolidine-4-carboxylate (OTC), we could enhance intracellular GSH levels as well as mitogen responsiveness as measured by polyamine synthesis, and [^{3}H]thymidine ([^{3}H]TdR)-incorporation. In light of the findings of Stohs *et al.* (1984) in ageing and Lorber *et al.* (1964) in auto-immune diseases, we hypothesized that: (1) defective T-cell function that occurs with age and in auto-immune disease may, in part, be due to a decline in intracellular GSH concentration in the lymphocytes; and (2) that enhancement of intracellular GSH levels in lymphocytes from aged animals and animals with RA/SLE-like auto-immune disease may also stimulate the depressed immune response.

We therefore examined the intracellular GSH concentration of mitogen-induced proliferation of splenic lymphocytes of normal C57BL/6J mice at various ages, and the ability to enhance GSH concentrations and immune function using the thiol delivery agents OTC and 2-mercaptoethanol (2ME). In addition, lymphocytes were examined from MRLMp +/+ and MRL/lpr mice. The MRL mice bearing the lpr gene have the RA/SLE-like disease of which they die at approximately 6–7 months of age (Murphy & Roths, 1978; Andrews *et al.*, 1978). The studies reported here compare the augmentation of mitogen-induced T-cell proliferation of splenic lymphocytes from MRL/lpr mice to cells obtained from the congenic normal MRL +/+ of the same age.

MATERIALS AND METHODS

Reagents and chemicals

L-2-oxothiazolidine-4-carboxylate (OTC) was synthesized by the method of Kaneko *et al.* (1964) with a slight modification as described previously (Tsan *et al.*, 1985). Our product had a melting point of 171°. Element analysis revealed: C, 32·60%; H, 3·26%; N, 9·48%; S, 21·48% (calculated values for $C_4H_5NO_3S$: C, 32·64%; H, 3·42%; N, 9·52%; S, 21·79%). The sodium salt of OTC was used throughout the study.

DL-buthionine-S-R-sulphoximine (BSO) was obtained from Chemlog, South Plainfield, NJ. Glutatione, glutathione reductase (Type IV, EC 1.6.4.2), reduced nicotinamide adenine dinucleotide phosphate (NADPH), 5,5′-dithiobis-2-nitrobenzoate (DTNB), 2-mercaptoethanol (2ME), and Concanavalin A (Con A) were purchased from Sigma Chemical Co., St Louis, MO. [^{3}H]thymidine ([^{3}H]TdR) (73 μCi/mmol) was purchased from New England Nuclear, Boston, MA.

Medium

All cells were cultured in modified Eagle's Medium (MEM) supplemented with 5% fetal calf serum, 10 mM non-essential amino acids, 100 mM sodium pyruvate, 200 mM L-glutamine, 2·5 mM HEPES (Gibco, Grand Island, NY) plus 200 units/ml penicillin and 100 μg/ml streptomycin.

Cells

Splenic lymphocytes were obtained from C57BL/6J mice (Jackson Labs, Bar Harbor, ME) except for the 24-month-old mice which were obtained from Charles River (Wilmington, MA) under the NIA interagency agreement for studies on ageing. MRL/lpr and MRL/Mp−+/+ (MRL−+/+) mice were obtained from the breeding colony at Jackson Laboratory. Following cervical dislocation, spleens were removed, teased apart, and lymphocytes isolated by density-gradient centrifugation with Histopaque (Sigma). Cells (2×10^7/4 ml of media) were cultured in Costar 6-well plates for 18–20 hr at 37° in 5% CO_2/95% air atmosphere.

Measurement of total glutathione concentration

GSH analysis was carried out according to the methods of Tietze (1969) following extraction using the method of Burchill *et al.* (1978). Following culture of 2×10^7 cells/well in Costar 6-well plates, cells were harvested and pelleted at 400 **g** for 10 min. GSH was extracted using 10% trichloroacetic acid in 0·01 N HCl. After centrifugation, the TCA was extracted from the supernatant with diethyl ether. Aliquots of the supernate were assayed for GSH determination using the enzymatic assay of Tietze (1969).

Determination of lymphocyte activation as measured by DNA synthesis

[^{3}H]TdR incorporation as a marker of DNA synthesis was determined by incubating 2×10^5 cells in MEM plus supplements which were removed from the 6-well plates at the initiation of culture. Cells were incubated in 96-well plates (microtitre plates, Costar, Cambridge, MA) in a final volume of 200 ml and cultured for 48 hr. Each set of cells received an 18-hr terminal pulse with 1 μCi of [^{3}H]TdR. Cells were harvested and [^{3}H]TdR incorporation determined by standard liquid scintillation spectroscopy.

Ornithine decarboxylase (ODC) activity

Induction of ODC was determined as described previously (Fidelus, Laughter & Twomey, 1984). Cells were suspended at 1×10^7–2×10^7 in 4 ml of media in Costar 6-well plates. After incubation for 18–20 hr at 37° in a 5% CO_2 atmosphere, cells were centrifuged for 10 min at 400 ***g***. Cell pellets were sonicated in 50 mM Na_2HPO_4 (pH 7·2) containing 0·1 mM EDTA, 2·0 nM dithiothreitol, 5 mM NaF, 2 mM phenylmethylsulphonyl fluoride, and 60 μM pyridoxal phosphate. After centrifugation for 5 min at 15,000 r.p.m. at 4° in an Eppendorf centrifuge, aliquots (0·18 ml) of supernate were incubated with 0·02 ml of L-(^{14}C)-ornithine (40–60 mCi/mmol) for 60 min at 37°. Released $^{14}CO_2$ was collected on filter paper to which 0·02 ml of 2 N NaOH was added. The reaction was terminated with 1 M citric acid and the radioactivity of the filter paper counted by standard liquid scintillation spectroscopy (Packard, Downers Grove, IL).

RESULTS

In the investigation of Stohs *et al.* (1984), the GSH concentration of peripheral blood lymphocytes from Swiss-Webster mice

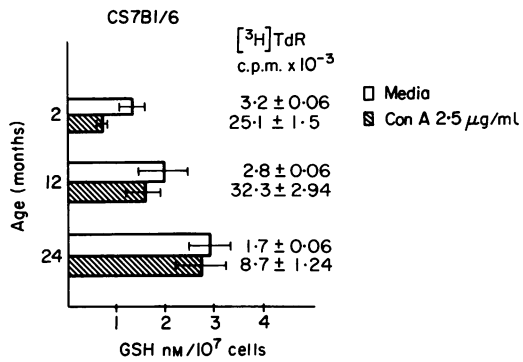

Figure 1. Intracellular GSH concentration in murine C57Bl/6 splenic lymphocytes at various ages in the absence or presence of Con A. The results are expressed as the mean ± SEM of six independent experiments. Lymphocyte activation was determined by [^{3}H]TdR incorporation after 48 hr of culture in the absence or presence of Con A. [^{3}H]TdR incorporation was expressed as the mean ± SEM of three independent experiments.

at various ages were examined. We, therefore, first needed to determine whether changes in intracellular GSH also occurred in lymphocytes obtained from the spleen. C57BL/6J mice were utilized in these studies at 2, 12 and 24 months of age. Splenic lymphocytes were examined for intracellular GSH concentrations following 18 hr of incubation in either media alone or with the mitogen Con A. Immune responsiveness was determined after 48 hr of culture by [^{3}H]TdR incorporation as an indication of cellular proliferation.

In our studies, intracellular GSH concentrations from C57Bl/6J splenic lymphocytes did not decline with age, but may in fact have slightly increased (Fig. 1). There was no statistically significant difference noted between the three age groups. Intracellular GSH concentrations varied greatly with increasing age (24-month-old mice). The mean elevation in intracellular GSH levels demonstrated in 24-month-old mice may, in part, be due to the difference in breeding and care of NIA C57BL/6J mice. Con A stimulation of splenic lymphocytes lowers intracellular GSH levels as demonstrated previously (Fidelus & Tsan, 1986), although the degree of change with mitogen stimulation was highly variable in an aged population. [^{3}H]TdR-incorporation by Con A-stimulated lymphocytes was suppressed in the 24-month-old mice compared to lymphocytes from 2- and 12-month-old mice. Thus, these data suggest that intracellular GSH concentrations of splenic lymphocytes from C57Bl/6J mice do not decline with age. Interestingly, in the Swiss-Webster mice examined by Stohs *et al.* (1984), there was no statistically significant change in GSH concentration between peripheral blood lymphocytes examined in 3-, 12- and 18-month-old mice. Their reported decline in intracellular GSH levels in peripheral blood lymphocytes could only be demonstrated when GSH levels were compared to 9-month-old animals. Our data also demonstrate that there is no direct correlation between intracellular GSH concentration and [^{3}H]TdR incorporation, suggesting that the decline in lymphocyte proliferation was not in response to declining intracellular GSH levels. This was directly demonstrated when splenic lymphocytes from 12- and 24-month old mice were compared for GSH concentration and mitogen-induced [^{3}H]TdR incorporation. Although there was no statistically significant change in GSH level, lymphocyte proliferation was severely depressed in the 24-month-old mice.

To further examine the role of intracellular glutathione in T-cell function, the murine model for auto-immune disease, MRL/lpr, and the congenic normal (MRL +/+), were investigated for GSH concentration and mitogen-induced lymphocyte proliferation. Splenic lymphocytes from 2-, 6- and 11-month-old MRL +/+ mice were compared to lymphocytes obtained from 2- and 6-month old MRL/lpr mice (Fig. 2). Intracellular GSH concentrations from MRL +/+ mice show no statistically significant change between 2 months and 11 months. Intracellular GSH concentrations in MRL/lpr splenic lymphocytes tend to be slightly higher overall than in MRL +/+ mice of comparable age, despite the active inflammatory process in MRL/lpr mice. In addition, there was no significant change with advanced age. The lack of significant alterations in intracellular GSH concentration occurring with age is in sharp contrast to the significant and rapid decline of mitogen responsiveness. Both MRL strains show a decline in Con A-induced [^{3}H]TdR incorporation with age, which occurs more rapidly than in the normal C57BL/6J mice. If one compares the 6-month-old MRL +/+ mice to the 6-month-old MRL/lpr mice there was a significant difference in [^{3}H]TdR incorporation following mitogen stimulation, while there was minimal change in intracellular GSH concentration. In fact, mean GSH concentrations for MRL/lpr splenic lymphocytes was slightly enhanced over MRL +/+ GSH levels. Thus, we conclude from Figs 1 and 2 that: (1) no appreciable change in intracellular splenic lymphocyte GSH concentration occurred with age; (2) intracellular GSH concentrations do not directly correlate with the ability to respond to mitogen stimulation; (3) the active inflammatory disease of the MRL/lpr mouse does not induce lower intracellular GSH concentrations in splenic lymphocytes when compared to their normal MRL +/+ counterpart.

We have previously shown that intracellular GSH concentrations in normal murine splenic lymphocytes can be enhanced with the cysteine delivery agents OTC or 2ME (Fidelus & Tsan, 1986). In addition, the enhancement in intracellular GSH level was also associated with an augmentation of mitogen-induced cellular proliferation. We therefore examined whether these

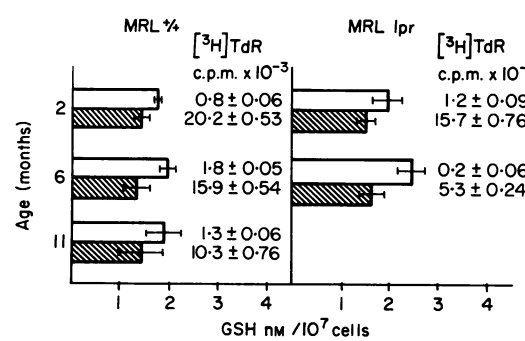

Figure 2. Intracellular GSH concentration in murine splenic lymphocytes at various ages in the absence [□ Media] or presence [■ Con A 2·5 μg/ml] of mitogen. GSH concentrations were expressed as the mean ± SEM of six to eight independent experiments. [^{3}H]TdR incorporation was determined after 48 hr of culture in the absence and presence of mitogen.

Table 1. The effect of OTC and 2ME on GSH concentrations in MRL splenic lymphocytes

	MRL +/+ (6 months)	MRL lpr (6 months)
Media	1·91 ± 0·13*	2·45 ± 0·15
OTC (10 mM)	2·56 ± 0·24	2·8 ± 0·24
2ME (5×10^{-5} M)	4·65 ± 0·24†	5·7 ± 0·24†
Con A (2·5 ug/ml)	1·34 ± 0·18	1·7 ± 0·12
Con A + OTC	2·17 ± 0·24‡	2·8 ± 0·18‡
Con A + 2ME	5·42 ± 0·76‡	5·1 ± 0·29‡
	$n=6$	$n=6$

* The results were expressed as nmoles GSH/10^7 cells (mean ± SEM) of six independent experiments.

†$P < 0·05$ or less versus control medium alone.

‡$P < 0·05$ or less versus Con A alone.

agents could in fact enhance the depressed immune responses of MRL/lpr splenic lymphocytes despite the lack of depression in intracellular GSH levels. In Table 1, intracellular GSH levels were compared in 6-month-old MRL +/+ and lpr mice in the presence and absence of mitogen, and cultured with the cysteine delivery agents OTC and 2ME. As demonstrated previously, both OTC and 2ME could enhance intracellular GSH concentrations in the presence or absence of Con A. 2ME was superior to OTC in its ability to enhance splenic lymphocyte intracellular GSH levels. Although GSH concentrations were slightly higher in MRL/lpr lymphocytes than MRL +/+ cells, Con A stimulation lowered GSH levels in both lymphocyte populations. Thus, although little change is seen in intracellular GSH levels with age, cysteine delivery agents can enhance intracellular GSH levels in splenic lymphocytes either in the presence or absence of mitogen.

Finally, we have established the ability to enhance intracellular GSH concentrations in splenic lymphocytes. Could we also augment the depressed immune responses which occur with age or with auto-immune disease? Two methods were used to evaluate immune responsiveness, [^{3}H]TdR incorporation (Table 2) and the induction of polyamine synthesis by the detection of ornithine decarboxylase activity (Table 3). [^{3}H]TdR incorporation, in the presence and absence of mitogen (Table 2), demonstrated that both OTC and 2ME could enhance [^{3}H]TdR incorporation in controls and Con A-induced proliferation in splenic lymphocytes from MRL at all ages. Similar in its ability to enhance intracellular GSH levels, 2ME was better than OTC in augmenting cellular proliferation. In severely depressed immune states such as that seen in the six-month-old MRL/lpr mouse, lymphocyte proliferation could be enhanced by 2ME to levels demonstrated by 2-month-old MRL/lpr mice examined in the absence of cysteine delivery agents.

When the induction of ODC by Con A was determined in the presence of either OTC or 2ME (Table 3), both cysteine delivery agents augmented Con A-induced ODC activity. Neither OTC nor 2ME stimulated ODC activity in splenic lymphocytes in the absence of Con A. These data demonstrate that the cysteine delivery agents OTC and 2ME can augment intracellular GSH levels in splenic lymphocytes as well as augment mitogen-induced lymphocyte activation. The enhancement in intracellular GSH levels and immune responsiveness occurs at any age and despite immunological status. Although intracellular GSH concentrations in murine splenic lymphocytes do not appreciably change with age or disease activity, one can augment these levels with cysteine delivery agents, and subsequently enhance lymphocyte activation.

DISCUSSION

There are several important issues suggested by these data. The first is the fact that although intracellular GSH levels of splenic lymphocytes do not appreciably change with age, the immune responsiveness of those lymphocytes does decline. Although Stohs *et al.* (1984) suggested that intracellular GSH levels declined with age, this observation is strictly based on comparing all GSH determination to those obtained from 9-month-old animals. If one actually compared GSH levels from 3-month-old to 18-month-old animals, there was no statistical difference. Even when comparing MRL strains, which age more rapidly and have a severe chronic inflammatory process, no appreciable difference in GSH levels in splenic lymphocytes was noted. Allowing for strain to strain variability and peripheral blood versus splenic lymphocyte GSH, changes in intracelullar GSH do not decline with age.

In contrast, immune responsiveness of splenic-derived lymphocytes does decline with age. Initial observation would suggest no link between intracellular GSH and immune responsiveness. However, our data using the cysteine delivery agents OTC and 2ME demonstrate that we can enhance intracellular GSH levels as well as augment lymphocyte proliferation at all ages investigated. Thus, intracellular GSH is associated with immune function. One possible explanation for this dichotomy is the suggestion of two intracellular pools of GSH. Gaetjens, Chen & Broome (1984) have suggested through their work on cellular proliferation in GSH-depleted cells that there is a cytoplasmic pool of GSH which makes up 90% of the total intracellular GSH which is not vital for cellular proliferation, and a second pool in the mitochondria which is smaller. The mitochondrial GSH pool is vital for cell growth. Several other investigators have also demonstrated the importance of mitochondrial GSH to cell growth (Palekar, Tate & Meister, 1975; Higashi *et al.*, 1977; Meredith & Reed, 1983). We would suggest that the mitochondrial GSH is necessary for lymphocyte activation and proliferation and that augmentation of mitochondrial GSH results in enhanced immune responsiveness. Similarly in ageing, changes in mitochondrial GSH levels may occur which do not affect lymphocyte activation, but the large source of cytoplasmic GSH which is unaffected with age, obscures detectable changes when total intracellular GSH levels are determined.

Many investigators have suggested that auto-immune diseases, like SLE and RA, which are associated with massive inflammatory disease may rapidly deplete intracellular GSH by free radical scavenging (Lorber *et al.*, 1964; Haataja, 1975). Therefore, one would expect significantly lower levels of intracellular GSH in MRL/lpr mice in which active disease is present. This does not appear to be the case in splenic lymphocytes and, in fact, intracellular GSH levels in splenic lymphocytes of MRL/lpr mice are slightly higher than MRL +/+ mice. It is interesting to note that Fujiwara & Kariyone

Table 2. The effect of OTC and 2ME on [^{3}H]thymidine incorporation

	[^{3}H]thymidine incorporation c.p.m. $\times 10^{-3}$			
	MRL +/+		MRL lpr	
	2 months	6 months	2 months	6 months
Media	0·46±0·02	0·4±0·01	1·17±0·17	0·23±0·01
OTC (10 mM)	1·16±0·9*	0·9±0·20	2·43±0·04*	0·35±0·08
2ME (5×10^{-5} M)	4·5±0·12*	1·8±0·05*	3·38±0·08*	0·62±0·07*
Con A (2·5 μg/ml)	20·2±0·5	15·9±0·54	15·7±0·76	5·3±0·24
Con A+OTC	26·2±1·3	25·1±0·12†	27·5±0·5†	6·8±0·18
Con A+2ME	40·6±2·0†	32·3±0·8†	36·8±0·29†	10·5±0·71†

The results are expressed as c.p.m. $\times 10^{-3}$ (Means±SEM) of three independent experiments for $2{\cdot}5 \times 10^5$ cells after 48 hr of culture.

* $P < 0{\cdot}05$ or less versus media control.

† $P < 0{\cdot}05$ or less versus Con A control.

Table 3. The effect of OTC and 2ME on ODC activity in MRL mice

	ODC*	
	MRL+/+ (2 months)	MRL lpr (2 months)
Media (control)	0·4±0·15†	0·5±0·01
OTC (10 mM)	0·6±0·39	0·25±0·21
2ME (5×10^{-5} M)	0·8±0·38	0·16±0·18
Con A (2·5 μg/ml)	41·1±2·4	33·1±1·9
Con A+OTC	96·6±5·3	47·0±2·4
Con A+2ME	228·0±30·0	92·2±0·5
	$n=3$	$n=3$

* ODC activity is expressed as pmoles $^{14}CO_2$ released/60 min/10^7 cells.

† Mean±SE of three independent experiments.

(1984) were able to induce immunoglobulin-secreting cells *in vitro* when culturing MRL/lpr splenic lymphocytes with 2ME. When similar cultures were established for the induction of immunoglobulin-secreting cells using OTC, no enhancement of plaque-forming cells were detected (unpublished observation). This suggests a difference in the mode of action for OTC and 2ME.

In conclusion, intracellular GSH levels of murine splenic lymphocytes do not significantly change with age or with active inflammatory disease. This is in contrast to the decline in the immune responsiveness of these cells. However, with the use of cystein delivery agents like OTC or 2ME, one can enhance both intracellular GSH concentrations and lymphocyte proliferation.

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