Am J Clin Exp Immunol 2012;1(2):113-123
www.ajcei.us /ISSN:2164-7712/AJCEI1208002

*Original Article*

# The cystine/glutamate antiporter regulates indoleamine 2,3-dioxygenase protein levels and enzymatic activity in human dendritic cells

Mildred L Mattox[1], June A D'Angelo[2], Zachary M Grimes[1], Edda Fiebiger[3], Bonny L Dickinson[1]

*[1]The West Virginia School of Osteopathic Medicine, 400 North Lee Street, Lewisburg, WV 24901; [2]The Research Institute for Children, Children's Hospital and Department of Pediatrics, Louisiana State University Health Science Center, New Orleans, LA 70118; [3]Division of Gastroenterology and Nutrition, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115*

Received August 18, 2012; Accepted September 10, 2012; Epub September 27, 2012; Published November 30, 2012

**Abstract:** Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme in the tryptophan-catabolizing pathway and a key regulator of peripheral immune tolerance. As the suppressive effects of IDO are predominantly mediated by dendritic cells (DCs) and IDO-competent DCs promote long-term immunologic tolerance, a detailed understanding of how IDO expression and activity is regulated in these cells is central to the rational design of therapies to induce robust immune tolerance. We previously reported that the cystine/glutamate antiporter modulates the functional expression of IDO in human monocyte-derived DCs. Specifically, we showed that blocking antiporter uptake of cystine significantly increased both IDO mRNA and IDO enzymatic activity and that this correlated with impaired DC presentation of exogenous antigen to T cells via MHC class II and the cross-presentation pathway. The antiporter regulates intracellular and extracellular redox by transporting cystine into the cell in exchange for glutamate. Intracellular cystine is reduced to cysteine to support biosynthesis of the major cellular antioxidant glutathione and cysteine is exported from the cell where it functions as an extracellular antioxidant. Here we show that antiporter control of IDO expression in DCs is reversible, independent of interferon-γ, regulated by redox, and requires active protein synthesis. These findings highlight a role for antiporter regulation of cellular redox as a critical control point for modulating IDO expression and activity in DCs. Thus, systemic disease and aging, processes that perturb redox homeostasis, may adversely affect immunity by promoting the generation of IDO-competent DCs.

**Keywords:** Indoleamine 2, 3-dioxygenase, human monocyte-derived dendritic cells, tryptophan, kynurenine, cystine/glutamate antiporter, glutathione, cystine, cysteine, peripheral immune tolerance, redox

## Introduction

The cystine/glutamate antiporter plays a pivotal role in controlling intracellular and extracellular redox through the uptake of extracellular cystine in exchange for intracellular glutamate [1-3]. Inside the cell, cystine is rapidly reduced to cysteine and functions both as an intracellular antioxidant and as the rate-limiting precursor for biosynthesis of the major cellular antioxidant glutathione [4]. Cysteine is also exported from the cell via the alanine-serine-cysteine transporter where it generates a reducing extracellular milieu [5-7]. This cystine/cysteine redox cycle is maintained by oxidation of extracellular cysteine to cystine for reuptake by the antiporter [6]. We previously demonstrated that blocking the function of the antiporter depleted intracellular glutathione and significantly impaired dendritic cell (DC) presentation of exogenous antigen to T cells via MHC class II and the cross-presentation pathway [8]. To explain this defect, we examined the possibility that the antiporter regulates the functional expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO). Our data show that blocking antiporter function resulted in a significant increase in IDO mRNA transcripts and IDO enzymatic activity in DCs matured by incubation with lipopolysaccharide (LPS) [9]. This finding is consistent with the concept that the outcome of DC–T cell interactions

may be productive priming or tolerance depending on the signals DCs receive from their cellular environment. Thus, these data raise the possibility that the cystine/glutamate antiporter, through regulation of cellular redox, may directly control the outcome of DC-T cell interactions by modulating IDO expression.

The immunosuppressive enzyme IDO has emerged as a major regulator of peripheral immune suppression and tolerance [10-13]. IDO catalyzes the initial and rate-limiting oxidative conversion of tryptophan to signaling metabolites termed kynurenines [14, 15]. Both tryptophan depletion and kynurenine accumulation create a highly immunosuppressive micromilieu that blocks T cell responses through several mechanisms including inhibition T cell proliferation, induction of T cell apoptosis and the expansion and stabilization of regulatory T cells (Treg) [12, 13, 16-19]. The suppressive effects of IDO are largely mediated by DCs [20, 21]. IDO-competent DCs control the development of peripheral tolerance in the context of various physiological and pathological conditions and promote long-term control of immune homeostasis by limiting exaggerated inflammatory responses to danger signals and assisting Treg effector function [12, 18, 22, 23]. IDO is therefore an attractive target for immune modulation and yet the molecular and biochemical mechanisms that control IDO expression and function at the transcriptional, translational and post-translational levels in health and disease are incompletely understood.

Here we studied the effect of antiporter blockade on extracellular tryptophan depletion and kynurenine accumulation, and examined a role for IFN-γ in antiporter-dependent control of IDO. We also tested whether the effect of the antiporter on IDO activity could be reversed by releasing antiporter inhibition or by normalizing redox with an antioxidant. Finally, we assessed whether the antiporter controls IDO enzymatic activity by regulating IDO protein levels. Our findings suggest that the cystine/glutamate antiporter, through control of cellular redox homeostasis, may directly influence the generation of IDO-competent DCs. This has implications for understanding how processes that dysregulate the redox equilibrium, such as systemic disease, cancer and aging, may impair adaptive immunity. The results of this study may also facilitate the development of new treatment avenues for blocking IDO activity in DCs to promote microbial clearance and antitumor immunity, or conversely, to stimulate IDO activity to prevent or attenuate autoimmunity, allergy and allograft rejection.

## Materials and methods

### *Materials*

*Escherichia coli 026:* B6 lipopolysaccharide (LPS; γ-irradiated; total impurities <5% protein), L-homocysteic acid, L-buthionine S,R-sulfoximine, diethyl maleate, cycloheximide, NaCl, Tris base, NP-40, SDS, sodium deoxycholate and goat anti-rabbit IgG-HRP were from Sigma-Aldrich (St. Louis, MI, USA). RPMI 1640, FBS, penicillin, streptomycin sulfate and amphotericin B were from Invitrogen (Carlsbad, CA, USA). Recombinant human IFN-γ was from Genway (San Diego, CA, USA). Cystine/cysteine-free medium was from MP Biomedicals (Solon, OH, USA). N-acetyl-L-cysteine and mouse anti-IDO monoclonal antibody were from EMD Millipore (Billerica, MA, USA). Complete Mini Protease Inhibitor tablets were from Roche (Indianapolis, IN, USA). Goat anti-mouse IgG-HRP antibody was from Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and rabbit anti-GAPDH monoclonal antibody was from Cell Signaling (Danvers, MA, USA).

### *Human monocyte-derived dendritic cells*

DCs were derived from monocytes purified from normal human buffy coats (purchased from The Blood Center, New Orleans, LA, USA) as described previously [8]. Briefly, human PBMCs were isolated from buffy coats by centrifugation on Ficoll-Paque (GE Healthcare, Uppsala, Sweden) and monocytes were purified by positive selection on CD14 Microbeads (Miltenyi Biotec, Auburn, CA, USA). DCs were derived by culturing monocytes ($10^6$ cells/ml) in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 10 U/ml penicillin, 10 μg/ml streptomycin sulfate, and 25 ng/ml amphotericin B) containing IL-4 (10 ng/ml; 290 U/ml; Peprotech, Rocky Hill, NJ) and GM-CSF (100 ng/ml; 560 U/ml; Leukine, Genzyme, Cambridge, MA) for 4 d in a humidified atmosphere at 37°C with 5% $CO_2$. Medium containing fresh cytokines was replenished on day 3 of culture.

*Quantification of IDO enzymatic activity*

IDO enzymatic activity in DCs was examined by quantifying the accumulation of kynurenine in cell-free culture supernatants by the colorimetric method or by quantifying kynurenine and tryptophan by HPLC. The colorimetric assay was described previously [9, 24]. Briefly, DCs were treated with or without LPS (1 μg/ml) for 4 h after which DCs were collected, washed and incubated in 6-well trays at a density of 1.25 x $10^6$ cells/ml in complete medium in the presence or absence of L-homocysteic acid (LHC; 2.5 mM) or in cystine/cysteine-free medium. Tryptophan (250 μM; Sigma-Aldrich) was added to wells as an IDO substrate. In other studies DCs were treated with N-acetyl-L-cysteine, cycloheximide or IFN-γ. Culture media was collected at 16 and 24 h and centrifuged to remove cells. Supernatants were extracted with 15% trichloroacetic acid (Sigma-Aldrich) and centrifuged to remove protein. Kynurenine in extracted supernatants was quantified by adding an equal volume of Ehrlich reagent (2% w/v 4-dimethylaminobenzaldehyde in glacial acetic acid; Sigma-Aldrich). The OD 480 nm was measured with a plate reader (BioTek Synergy II, Winooski, VT) and the kynurenine concentration was calculated by referral to a standard curve prepared with purified kynurenine (Sigma-Aldrich) diluted in TCA-extracted medium.

HPLC detection of tryptophan and kynurenine was performed using an established technique [25]. Samples of cell-free culture medium were shipped on dry ice to the Toxicology Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine where they were stored at -80°C. Standards were prepared by dissolving purified tryptophan and kynurenine in water. Samples and standards were injected onto a Phenomenex Synergi Fusion-RP 2.5 μ, 100 x 3.0 mm column and separated with methanol-water (27%-73%) with 0.05% trifluoroacetic acid at a flow rate of 0.3 ml/min. Tryptophan was detected by fluorescence with an excitation wavelength of 254 nm and an emission wavelength of 404 nm, and kynurenine was detected by UV at 366 nm. The background fluorescence due to phenol red in the culture medium was subtracted from the fluorescence signal in the samples as it interfered with the detection of tryptophan. Samples from two independent experiments were analyzed in duplicate and the Ave±StDev was calculated.

*mRNA measurements with nCounter*

The NanoString nCounter gene expression system (NanoString Technologies Inc., Seattle, WA, USA) was used to quantify individual mRNA transcripts as described previously [8]. Briefly, DCs were treated with LPS (1 μg/ml) for 4 h and then incubated in complete medium or in cystine/cysteine-free medium in the continued presence of LPS for 16 h. DCs (50,000 per condition) were lysed in RLT buffer (Qiagen, Valencia, CA, USA) supplemented with β-mercaptoethanol (Sigma). 10% of the lysates was hybridized for 16 h with the CodeSet and loaded into the nCounter prep station followed by quantification using the nCounter Digital Analyzer. The nCounter data was normalized against the positive spiked-in controls provided by the nCounter instrument as per the manufacturer's instructions. Transcript copy numbers were normalized to transcripts encoding the housekeeping genes GAPDH and HPRT1.

*IDO immunoblot*

IDO was detected in DC lysates by immunoblot. Briefly, DCs were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate) containing fresh protease inhibitors and equal quantities (25-30 μg) were separated on 10-20% SDS-PAGE gels and transferred to nitrocellulose. IDO was detected by enhanced chemiluminescence using a mouse anti-IDO monoclonal antibody and a goat anti-mouse IgG-HRP secondary antibody. Blots were stripped and probed for GAPDH with a rabbit anti-GAPDH monoclonal antibody and detected with a goat anti-rabbit IgG-HRP secondary antibody. Bands were quantified by densitometry using a Kodak Gel Logic 2200 imager (Rochester, NY, USA) and the IDO:GAPDH ratio was calculated.

*Multiplex cytokine bead assay*

IFN-γ was quantified in cell-free culture supernatants using Millipore's Milliplex Human 14-Plex Cytokine Detection System as per the manufacturer's instructions. Samples were analyzed in duplicate on a Becton Dickinson LSR II flow cytometer (San Jose, CA, USA).

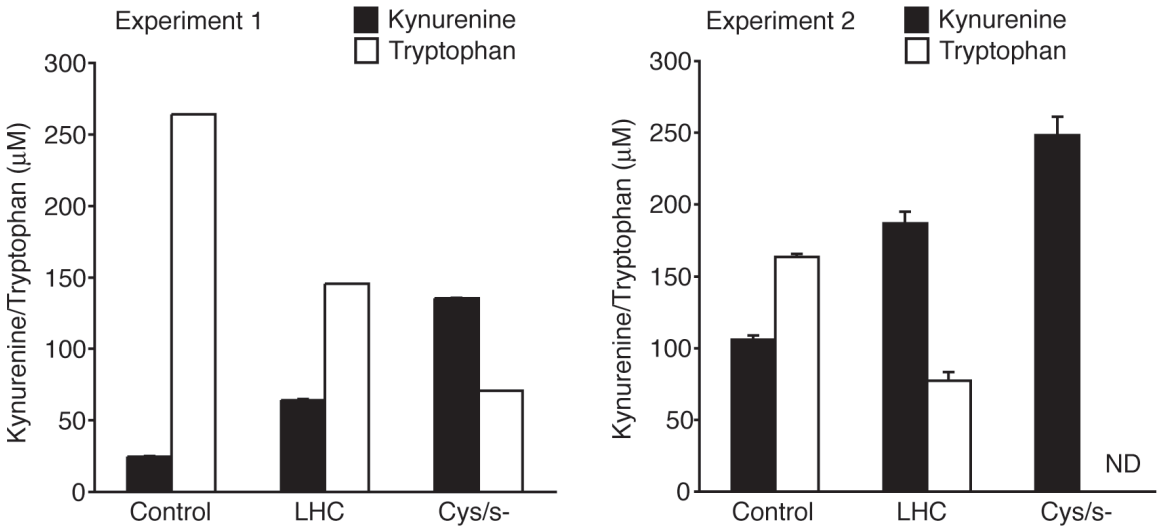


**Figure 1.** Blocking antiporter uptake of cystine stimulates IDO enzymatic activity. DCs were treated with LPS for 4 h and then cultured with LPS in complete medium, complete medium containing LHC, or in cystine/cysteine-free medium (Cys/s-) for 24 h. The concentrations of kynurenine and tryptophan in cell-free culture supernatants were quantified by HPLC. Data shown are two independent experiments with samples analyzed in duplicate (Ave±StDev). ND= none detected.

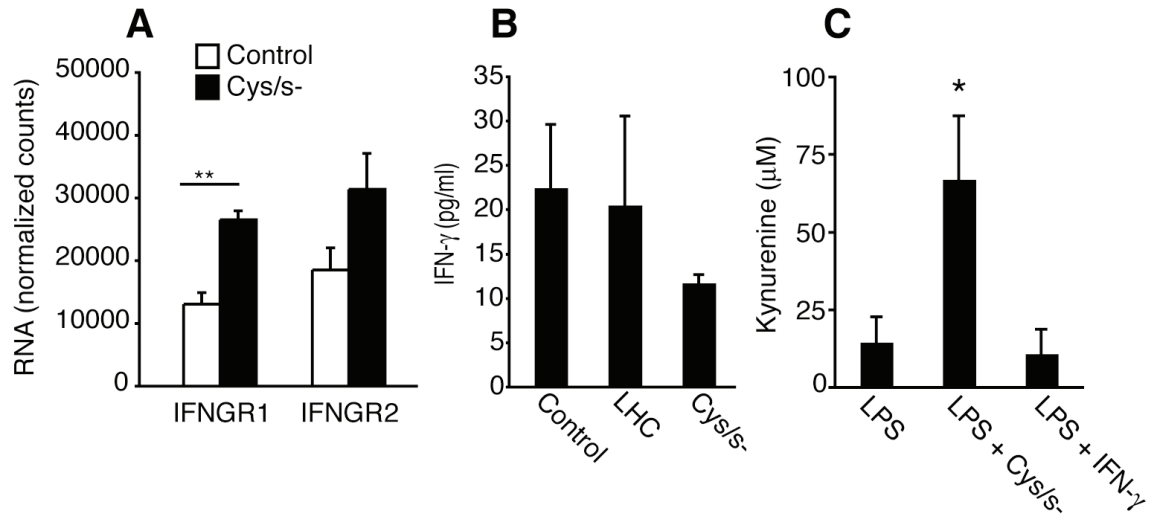


**Figure 2.** Antiporter regulation of IDO enzymatic activity is independent of IFN-γ. DCs were cultured in complete medium or in cystine/cysteine-free medium (Cys/s-) in the presence of LPS for 16 h and mRNA transcripts encoding IFNGR1 and IFNGR2 were quantified by digital mRNA profiling and normalized to HPRT1 and GAPDH transcripts. Data represent the Ave±SEM from three independent experiments with samples analyzed in triplicate (A). DCs were treated with LPS for 24 h and then cultured in complete medium, complete medium containing LHC, or in cystine/cysteine-free medium (Cys/s-) for 24 h. The concentration of IFN-γ in cell-free culture supernatants was quantified by cytometric bead array. Data are representative of two independent experiments with samples analyzed in duplicate (Ave±StDev) (B). DCs were treated with LPS for 4 h and then cultured with LPS in complete medium, in cystine/cysteine-free medium, or in complete medium containing IFN-γ (1 μg/ml) for 16 h. The concentration of kynurenine in cell-free culture supernatants was quantified by the colorimetric method. Data represent the Ave±SEM from three independent experiments with samples analyzed in triplicate (C).

*Cell viability*

DC viability was assessed by trypan blue exclusion. Because LPS-matured DCs are adherent to plastic, cells were gently scraped, transferred to tubes containing trypan blue and analyzed with a BioRad TC10 Automated Cell Counter (BioRad, Hercules, CA, USA). None of

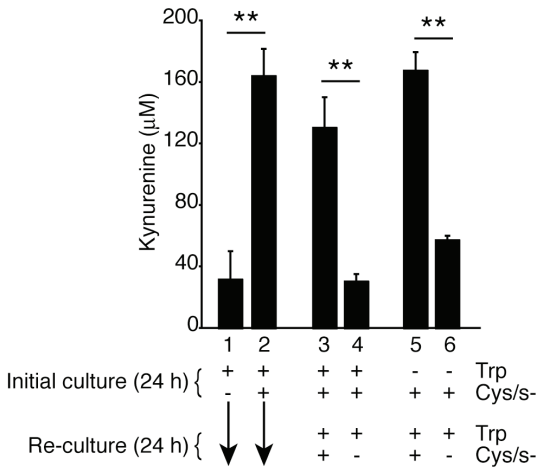


**Figure 3.** Antiporter control of IDO activity is reversible. DCs were treated with LPS for 4 h and then cultured with LPS in complete medium or cystine/cysteine-free medium in the presence or absence of tryptophan for 24 h. DCs were then maintained under those conditions or washed and re-cultured in complete medium or cystine/cysteine-free medium in the presence of tryptophan for 24 h. Kynurenine levels in cell-free culture supernatants were quantified by the colorimetric method. Data represent the Ave±SEM from three independent experiments with samples analyzed in triplicate.

the treatments result in significant cell death. The antioxidant NAC had no significant effect on cell viability (% viable cells Ave±StDev: control 91 ± 1, control + NAC 86 ± 5, LHC 84 ± 8, LHC + NAC 89 ± 6, Cys/s 87 ± 15, and Cys/s + NAC 86 ± 10; n=4). Similarly, neither BSO nor DEM had a significant effect on cell viability (control 91 ± 2, control + BSO 88 ± 6, and control + DEM 82 ± 12; n=3). Cycloheximide (2 μM) also had no significant effect on cell viability ( control 86 ± 10, control + CHX 85 ± 11, Cys/s 83 ± 14, and Cys/s + CHX 83 ± 11; n=3).

*Statistical analysis*

The Student's *t* test was used to determine statistical differences between treatment groups (*, $p < 0.05$; **, $p < 0.01$).

## Results

*The cystine/glutamate antiporter regulates the functional expression of IDO in human dendritic cells*

We recently showed that the cystine/glutamate antiporter regulates the functional expression of IDO in human monocyte-derived DCs [9]. To extend this observation, we used HPLC to assess IDO activity by quantifying changes in extracellular tryptophan and kynurenine following antiporter blockade. DCs were treated with LPS for 4 h to induce maturation and then incubated with LPS in complete medium, complete medium containing L-homocysteic acid (LHC), a potent competitive inhibitor of the antiporter, or in cystine/cysteine-free medium to prevent antiporter uptake of cystine. Consistent with our previous results, antiporter blockade resulted in the concomitant accumulation of extracellular kynurenine and depletion of extracellular tryptophan (**Figure 1**). The concentration of extracellular kynurenine quantified by HPLC was identical to that measured using a standard colorimetric assay (not shown). These data confirm that the antiporter regulates IDO enzymatic activity in DCs.

*Interferon-gamma does not play a role in redox modulation of IDO activity in DCs*

Interferon-gamma (IFN-γ) is one of the most potent inducers of IDO expression and activity in DCs and other immune cells [26, 27]. To determine whether IFN-γ plays a role in antiporter regulation of IDO activity, we first examined whether the antiporter modulates DC expression of the IFN-γ receptor. To do this, we used digital mRNA profiling to quantify transcripts encoding the IFN-γ receptor α and β chains (IFNGR1 and IFNGR2, respectively). DCs cultured in cystine/cysteine-free medium in the presence of LPS for 16 h exhibited a ~2-fold increase in both IFNGR1 and IFNGR2 mRNA transcripts relative to those DCs cultured in complete medium with LPS (**Figure 2A**). These data suggest that the antiporter may regulate DC expression of the IFN-γ receptor. To explore this further, we examined whether antiporter blockade could stimulate DC production of IFN-γ. DCs were treated with LPS for 24 h and incubated with or without LHC or cultured in cystine/cysteine-free medium for an additional 24 h after which IFN-γ was quantified in cell-free supernatants. In two separate experiments we found that DCs produced low (~ 20 pg/ml) levels of IFN-γ and that antiporter blockade did not significantly alter these levels (**Figure 2B**). To confirm this result, we examined the effect of exogenous IFN-γ on IDO activity. DCs were cultured with LPS in complete medium, cystine/cysteine-free medium, or in complete medium containing IFN-γ for 16 h and kynurenine levels

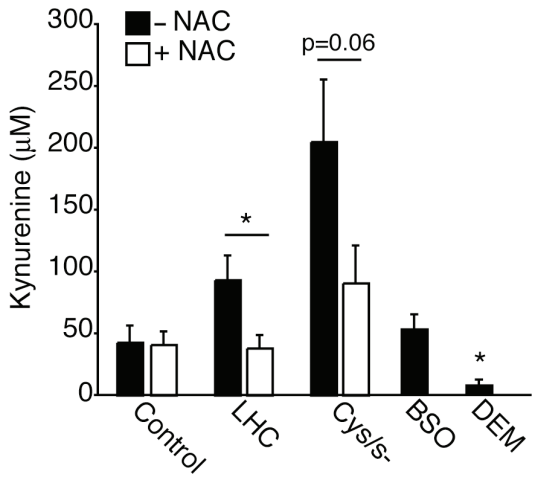


**Figure 4.** The antioxidant N-acetyl-L-cysteine interferes with antiporter regulation of IDO enzymatic activity. DCs were treated with LPS for 4 h and then incubated for 24 h with LPS in complete medium, complete medium containing LHC, DEM (40 μM), BSO (1 mM) or NAC (5 mM), or in cystine/cysteine-free medium in the presence or absence of NAC (5 mM). Data represent the Ave±SEM from three independent experiments with samples analyzed in triplicate.

were quantified in cell-free culture media. Consistent with the results of other studies, exogenous IFN-γ failed to induce robust IDO activity in mature DCs (**Figure 2C**) [26, 28]. Taken together, these data suggest that the mechanism by which the antiporter controls IDO activity in DCs does not depend on the action of IFN-γ.

*Antiporter control of IDO enzymatic activity is reversible*

We next examined whether the increase in IDO activity observed following antiporter blockade is reversible. To do this, DCs were treated with LPS for 4 h and then cultured with LPS in complete medium or in cystine/cysteine-free medium in the presence or absence of tryptophan. After 24 h DCs were either maintained in culture or washed and re-cultured for 24 h in complete medium or in cystine/cysteine-free medium containing tryptophan after which kynurenine levels were measured. Consistent with our previous data, incubation of DCs in cystine/cysteine-free medium induced IDO activity (**Figure 3**, compare bars 1 and 2). When DCs were initially cultured in cystine/cysteine-free medium with tryptophan and then re-cultured in complete medium containing tryptophan, IDO activity was decreased to baseline levels when compared with DCs re-cultured in cystine/cysteine-free medium containing tryptophan (**Figure 3**, compare bars 3 and 4). This suggests that IDO activity is readily suppressed when the antiporter is re-engaged by culturing DCs in complete medium (i.e., medium containing cystine). To test whether the presence of the IDO substrate tryptophan is required for antiporter modulation of IDO activity, DCs were cultured in cystine/cysteine-free medium in the absence of tryptophan and then re-cultured with tryptophan in cystine/cysteine-free medium. IDO activity was robust when DCs were re-cultured in the presence of tryptophan, suggesting that tryptophan is not required for the antiporter to up-regulate IDO activity (**Figure 3**, compare bars 3 and 5). As before, this increase in IDO activity was readily reversed when the DCs were re-cultured in complete medium in the presence of tryptophan (**Figure 3**, compare bars 5 and 6). We also found that IDO enzymatic activity was sustained as there was no significant difference in kynurenine levels between DCs cultured in cystine/cysteine-free medium for 24 h without tryptophan and then cultured for 24 h with tryptophan and those DCs cultured in cystine/cysteine-free medium in the presence of tryptophan for a full 48 h (i.e., IDO enzymatic activity is measurable even after 48 h; **Figure 3C**, compare bars 3 and 5). Finally, we verified that IDO activity is negligible in the absence of extracellular tryptophan, as kynurenine was undetectable in culture medium when DCs were cultured without tryptophan in complete medium, complete medium with LHC, or in cystine/cysteine-free medium (not shown). This set of data shows that the cystine/glutamate antiporter readily modulates the "on-off" status of IDO activity in DCs.

*Antiporter control of redox regulates of IDO enzymatic activity*

To test whether the antiporter regulates IDO expression and activity by controlling cellular redox, we examined the effect of the antioxidant N-acetyl-L-cysteine (NAC) on IDO activity in DCs following antiporter blockade. DCs were treated with LPS for 4 h and then incubated with LPS in the presence or absence of NAC in complete medium, complete medium containing LHC, or cystine/cysteine-free medium. NAC had no effect on the baseline level of IDO enzymatic activity in LPS-treated control DCs, how-

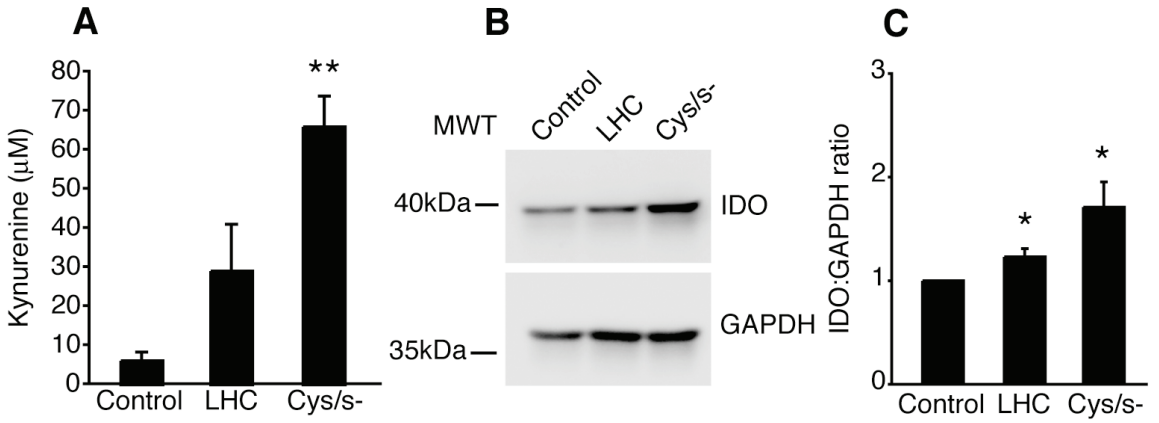


**Figure 5.** The antiporter regulates IDO protein levels. DCs were treated with LPS for 4 h and then incubated for 24 h with LPS in complete medium, complete medium containing LHC or in cystine/cysteine-free medium. IDO enzymatic activity was assessed by the colorimetric method. Data represent the Ave±SEM from five independent experiments with samples analyzed in triplicate (A). IDO and GAPDH protein levels were examined by immunoblot. One representative blot of five is show (B). IDO and GAPDH band intensity was quantified by densitometry and the IDO:GAPDH ratio was calculated. Data were normalized to controls (=1). Data represent the Ave±SEM from five independent experiments (C).

ever it potently reversed the increase in IDO activity observed when antiporter uptake of cystine was inhibited by culturing DCs in medium with LHC or in cystine/cysteine-free medium (**Figure 4**). These data suggest that the antiporter modulates IDO enzymatic activity by controlling cellular redox. NAC functions as an extracellular antioxidant but also may be transported into the cell and serve as a substrate for glutathione biosynthesis. Therefore, we examined whether the antiporter controls IDO expression by depleting intracellular glutathione. DCs were treated with L-buthionine S,R-sulfoximine (BSO) [29] to inhibit γ-glutamylcysteine synthetase, the rate-controlling enzyme for glutathione synthesis [29, 30], or with diethyl maleate (DEM) [31], which forms irreversible adducts with glutathione [32]. Neither BSO nor DEM treatment increased kynurenine levels in culture media suggesting that glutathione depletion alone does not induce IDO enzymatic activity (**Figure 4**).

*The antiporter controls IDO protein levels*

To examine whether the antiporter controls IDO enzymatic activity by regulating IDO protein levels in DCs, we compared the effect antiporter blockade on IDO protein levels by immunoblot. Consistent with our previous observations, IDO protein was undetectable in immature DCs and LPS treatment significantly increased IDO levels (not shown) [9]. IDO enzymatic activity was increased when antiporter function was inhibited with LHC and significantly increased following DC incubation in cystine/cysteine-free medium (**Figure 5A**) and this correlated with a significant increase in IDO protein levels (**Figure 5B** and **5C**). This suggests that antiporter blockade increases IDO activity by increasing IDO protein levels. To confirm this result, we blocked protein synthesis by treating DCs with cycloheximide (2 μM) and examined the effect on IDO enzymatic activity and protein. When LPS-treated DCs were cultured in complete medium with cycloheximide, both IDO enzymatic activity (**Figure 6A**) and protein levels (**Figure 6B** and **6C**) were reduced. Furthermore, cycloheximide significantly interfered with the increase in IDO enzymatic activity and protein levels when LPS-treated DCs were incubated in cystine/cysteine-free medium (**Figure 6A-C**). When tested at higher concentrations (10 and 50 μM), cycloheximide was toxic to the cells. Taken together, these data show that the antiporter regulates IDO enzymatic activity by controlling IDO protein levels post-transcriptionally.

## Discussion

Unraveling the mechanisms by which peripheral immune tolerance is regulated has direct relevance for the development of innovative clinical approaches to attenuate autoimmunity,

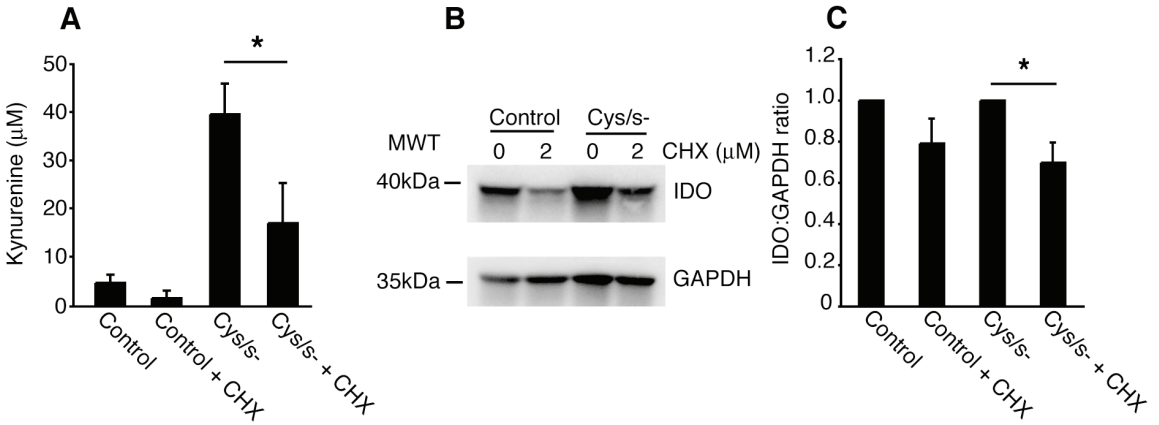


**Figure 6.** Antiporter regulation of IDO enzymatic activity depends on active protein synthesis. DCs were treated with LPS for 4 h and then incubated for 24 h with LPS in complete medium or cystine/cysteine-free medium in the presence or absence of cycloheximide (2 μM). IDO enzymatic activity was assessed by the colorimetric method. Data represent the Ave±SEM from five independent experiments with samples analyzed in triplicate (A). IDO and GAPDH protein levels were examined by immunoblot. One representative blot of three is show (B). Band intensity was quantified by densitometry and data were normalized to controls (=1). Data represent the Ave±SEM from three independent experiments (C).

allergy and transplant rejection. In our previous work we identified a novel role for the cystine/glutamate antiporter as a regulator of IDO activity in DCs. The goal of the current study was to test whether antiporter control of cellular redox could influence IDO enzymatic activity in DCs by modulating IDO protein synthesis. Our results suggest that perturbation of the intracellular or extracellular cystine/cysteine redox cycle maintained by the antiporter may directly regulate the functional expression of IDO.

To begin to define the process by which the antiporter controls IDO, we first confirmed that antiporter regulation of IDO enzymatic activity depended upon the presence of extracellular tryptophan. Antiporter blockade resulted in the concomitant increase in extracellular kynurenines and depletion of extracellular tryptophan as determined by HPLC. We next examined whether IFN-γ, the major inducer of IDO activity, participates in antiporter-dependent modulation of IDO activity in DCs. Although antiporter blockade increased mRNA transcripts encoding the alpha and beta subunits of the IFN-γ receptor, negligible quantities of IFN-γ were measured in the cultured supernatants of both control DCs and DCs cultured under conditions that inhibit antiporter activity. Furthermore, exogenous IFN-γ failed to induce IDO activity in both control DCs and in DCs in which antiporter function was inhibited. These data suggest that antiporter control of IDO expression occurs independent of IFN-γ. This is in line with the observation of other studies showing that prolonged incubation (48 h) of human monocyte-derived DCs with IFN-γ fails to induce IDO enzymatic activity [28]. Our findings are in contrast to that observed with human macrophages, where IFN-γ induces robust IDO activity, suggesting that IFN-γ may differentially regulate IDO in human DCs and macrophages [33].

Control of IDO enzymatic activity is complex and regulated at the transcriptional, translational and post-translational levels [34]. Therefore we next examined whether antiporter control of IDO enzymatic activity is reversible, sustained and dependent upon availability of its substrate tryptophan. Our data show that the increase in IDO activity observed following antiporter inhibition is rapidly reversed when the inhibition is removed. IDO activity is also sustained as significant levels of kynurenines were measured when DCs were cultured for 24 h in cystine/cysteine-free medium containing tryptophan, collected, washed and re-cultured under the same conditions for an additional 24 h. Furthermore, the ability of DCs to up-regulate IDO following antiporter blockade did not depend upon the availability of extracellular tryptophan; DCs cultured in medium devoid of cystine and tryptophan and then cultured in the same medium supplemented with tryptophan

exhibited IDO activity levels comparable to DCs cultured in cystine/cysteine-free medium in the continued presence of tryptophan for the full 48 h. This is an important observation because tryptophan is the least abundant of all the amino acids. These data suggest that IDO may be up-regulated even under conditions of limited tryptophan availability, thus rendering the IDO-competent DC poised to generate kynurenines when tryptophan becomes available.

A major function of the cystine/glutamate antiporter is to maintain the intracellular and extracellular redox milieu by sustaining intracellular glutathione levels and by generating a cystine/cysteine redox cycle, respectively. Studies show that exported glutathione and cysteine participate in redox remodeling in which sulfhydryl groups present in exofacial proteins on the cell surface and secreted proteins are maintained in their reduced state [35, 36]. Therefore we addressed the possibility that perturbation of intracellular or extracellular redox resulting from antiporter inhibition is the force driving up-regulation of IDO activity in DCs. In support of this idea, our data show that the antioxidant NAC reversed the increase in IDO enzymatic activity observed when antiporter function was inhibited. This is consistent with the observation that antioxidants inhibit IDO in IFN-γ activated human monocyte-derived macrophages [37]. NAC is often used to replete intracellular glutathione and we previously demonstrated that blocking antiporter activity both depleted intracellular levels of glutathione and decreased glutathione export from DCs [8]. Therefore we examined whether depleting intracellular glutathione alone could induce IDO activity. Neither BSO, which inhibits glutathione biosynthesis, nor DEM, which alkylates and inactivates glutathione, had a significant effect on IDO activity, as kynuneine levels were the same as that observed in control DCs. This raises the possibility that exofacial proteins or secreted proteins may function as extracellular redox sensors able to parlay changes in the external redox milieu into functional effects in DCs [38-40]. Our data suggest that one such effect may be to control IDO expression.

Finally, we show that the increase in IDO enzymatic activity observed following antiporter inhibition correlates with an increase in IDO protein levels and that this depends, in part, upon active protein synthesis. These data suggest that redox may control IDO enzymatic activity by controlling IDO protein synthesis rather than by protecting IDO from proteasomal degradation [41]. In summary, our data show that the antiporter, by controlling cellular redox, regulates IDO enzymatic activity in DCs by modulating IDO protein synthesis. This work extends our understanding of how IDO may be clinically manipulated for therapeutic benefit. Importantly, this study may also explain how IDO-competent DCs arise under pathophysiologic conditions characterized by imbalances in systemic redox as occurs in cancer, obesity, HIV, diabetes and aging, conditions that correlate with immune dysregulation and decline [32, 42-47].

## Acknowledgements

We thank Dr. Geraldine Magnin-Bissel for HPLC analysis of our samples (Research Scientist and Toxicology Lab Supervisor, Virginia-Maryland Regional College of Veterinary Medicine). The following WVSOM students contributed to this work: Brandon M. Morris, Kayleigh G. Pung, Evan S. Stern and Matthew J. Copeland. This work was supported with funds from The Research Institute for Children, The West Virginia School of Osteopathic Medicine, NIH grant R01AI075037 (E.F.) and Harvard Digestive Diseases Center grant P30 DK034-854.

**Address correspondence to:** Dr. Bonny L Dickinson, The West Virginia School of Osteopathic Medicine, 400 North Lee Street, Lewisburg, WV 24901. Tel: (304) 647-6370; E-mail: bdickinson@osteo.wvsom.edu

## References

[1] Sato H, Tamba M, Ishii T and Bannai S. Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. J Biol Chem 1999; 274: 11455-11458.

[2] Sato H, Shiiya A, Kimata M, Maebara K, Tamba M, Sakakura Y, Makino N, Sugiyama F, Yagami K, Moriguchi T, Takahashi S and Bannai S. Redox imbalance in cystine/glutamate transporter-deficient mice. J Biol Chem 2005; 280: 37423-37429.

[3] Jones DP, Go YM, Anderson CL, Ziegler TR, Kinkade JM Jr and Kirlin WG. Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control. FASEB J 2004; 18: 1246-1248.

[4] Rimaniol AC, Mialocq P, Clayette P, Dormont D and Gras G. Role of glutamate transporters in the regulation of glutathione levels in human macrophages. Am J Physiol Cell Physiol 2001; 281: C1964-1970.

[5] Christensen HN, Liang M and Archer EG. A distinct Na+-requiring transport system for alanine, serine, cysteine, and similar amino acids. J Biol Chem 1967; 242: 5237-5246.

[6] Banjac A, Perisic T, Sato H, Seiler A, Bannai S, Weiss N, Kolle P, Tschoep K, Issels RD, Daniel PT, Conrad M and Bornkamm GW. The cystine/cysteine cycle: a redox cycle regulating susceptibility versus resistance to cell death. Oncogene 2008; 27: 1618-1628.

[7] Angelini G, Gardella S, Ardy M, Ciriolo MR, Filomeni G, Di Trapani G, Clarke F, Sitia R and Rubartelli A. Antigen-presenting dendritic cells provide the reducing extracellular microenvironment required for T lymphocyte activation. Proc Natl Acad Sci USA 2002; 99: 1491-1496.

[8] D'Angelo JA, Dehlink E, Platzer B, Dwyer P, Circu ML, Garay J, Aw TY, Fiebiger E and Dickinson BL. The cystine/glutamate antiporter regulates dendritic cell differentiation and antigen presentation. J Immunol 2010; 185: 3217-3226.

[9] D'Angelo JA, Mattox ML, Fiebiger E and Dickinson BL. The Cystine/Glutamate Antiporter Regulates the Functional Expression of Indoleamine 2,3-Dioxygenase in Human Dendritic Cells. Scand J Immunol 2012 Oct; 76: 448-9. doi: 10.1111/j.1365-3083.2012.02743.x.

[10] Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, Brown C and Mellor AL. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 1998; 281: 1191-1193.

[11] Mellor AL, Keskin DB, Johnson T, Chandler P and Munn DH. Cells expressing indoleamine 2,3-dioxygenase inhibit T cell responses. J Immunol 2002; 168: 3771-3776.

[12] Belladonna ML, Grohmann U, Guidetti P, Volpi C, Bianchi R, Fioretti MC, Schwarcz R, Fallarino F and Puccetti P. Kynurenine pathway enzymes in dendritic cells initiate tolerogenesis in the absence of functional IDO. J Immunol 2006; 177: 130-137.

[13] Belladonna ML, Puccetti P, Orabona C, Fallarino F, Vacca C, Volpi C, Gizzi S, Pallotta MT, Fioretti MC and Grohmann U. Immunosuppression via tryptophan catabolism: the role of kynurenine pathway enzymes. Transplantation 2007; 84: S17-20.

[14] Yamamoto S and Hayaishi O. Tryptophan pyrrolase of rabbit intestine. D- and L-tryptophan-cleaving enzyme or enzymes. J Biol Chem 1967; 242: 5260-5266.

[15] Higuchi K and Hayaishi O. Enzymic formation of D-kynurenine from D-tryptophan. Arch Biochem Biophys 1967; 120: 397-403.

[16] Hwu P, Du MX, Lapointe R, Do M, Taylor MW and Young HA. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. J Immunol 2000; 164: 3596-3599.

[17] Orabona C and Grohmann U. Indoleamine 2,3-dioxygenase and regulatory function: tryptophan starvation and beyond. Methods Mol Biol 677: 269-280.

[18] Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, Marshall B, Chandler P, Antonia SJ, Burgess R, Slingluff CL Jr and Mellor AL. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science 2002; 297: 1867-1870.

[19] Hill M, Tanguy-Royer S, Royer P, Chauveau C, Asghar K, Tesson L, Lavainne F, Remy S, Brion R, Hubert FX, Heslan M, Rimbert M, Berthelot L, Moffett JR, Josien R, Gregoire M and Anegon I. IDO expands human CD4+CD25high regulatory T cells by promoting maturation of LPS-treated dendritic cells. Eur J Immunol 2007; 37: 3054-3062.

[20] Banchereau J and Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392: 245-252.

[21] Mellman I and Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. Cell 2001; 106: 255-258.

[22] Tan PH and Bharath AK. Manipulation of indoleamine 2,3 dioxygenase; a novel therapeutic target for treatment of diseases. Expert Opin Ther Targets 2009; 13: 987-1012.

[23] Mellor AL and Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol 2004; 4: 762-774.

[24] Takikawa O, Kuroiwa T, Yamazaki F and Kido R. Mechanism of interferon-gamma action. Characterization of indoleamine 2,3-dioxygenase in cultured human cells induced by interferon-gamma and evaluation of the enzyme-mediated tryptophan degradation in its anticellular activity. J Biol Chem 1988; 263: 2041-2048.

[25] Badawy AA and Morgan CJ. Rapid Isocratic Liquid Chromatographic Separation and Quantification of Tryptophan and Six kynurenine Metabolites in Biological Samples with Ultraviolet and Fluorimetric Detection. Int J Tryptophan Res 2010; 3: 175-86. Epub 2010 Dec 8.

[26] Jurgens B, Hainz U, Fuchs D, Felzmann T and Heitger A. Interferon-gamma-triggered indoleamine 2,3-dioxygenase competence in human monocyte-derived dendritic cells induces regulatory activity in allogeneic T cells. Blood 2009; 114: 3235-3243.

[27] Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G and Wachter H. Characteristics of interferon induced tryptophan metabolism in human cells in vitro. Biochim Biophys Acta 1989; 1012: 140-147.
[28] Von Bubnoff D, Scheler M, Wilms H, Fimmers R and Bieber T. Identification of IDO-positive and IDO-negative human dendritic cells after activation by various proinflammatory stimuli. J Immunol 2011 Jun 15; 186: 6701-9. Epub 2011 May 4.
[29] Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW and Potter DW. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Anal Biochem 1980; 106: 55-62.
[30] Kuppner MC, Scharner A, Milani V, Von Hesler C, Tschop KE, Heinz O and Issels RD. Ifosfamide impairs the allostimulatory capacity of human dendritic cells by intracellular glutathione depletion. Blood 2003; 102: 3668-3674.
[31] Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y and Figdor CG. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 2000; 100: 575-585.
[32] Kim HJ, Barajas B, Chan RC and Nel AE. Glutathione depletion inhibits dendritic cell maturation and delayed-type hypersensitivity: implications for systemic disease and immunosenescence. J Allergy Clin Immunol 2007; 119: 1225-1233.
[33] Hissong BD, Byrne GI, Padilla ML and Carlin JM. Upregulation of interferon-induced indoleamine 2,3-dioxygenase in human macrophage cultures by lipopolysaccharide, muramyl tripeptide, and interleukin-1. Cell Immunol 1995; 160: 264-269.
[34] Braun D, Longman RS and Albert ML. A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation. Blood 2005; 106: 2375-2381.
[35] Banerjee R. Redox outside the box: linking extracellular redox remodeling with intracellular redox metabolism. J Biol Chem 287: 4397-4402.
[36] Martner A, Aurelius J, Rydstrom A, Hellstrand K and Thoren FB. Redox remodeling by dendritic cells protects antigen-specific T cells against oxidative stress. J Immunol 2011 Dec 15; 187: 6243-8. Epub 2011 Nov 16.
[37] Thomas SR, Salahifar H, Mashima R, Hunt NH, Richardson DR and Stocker R. Antioxidants inhibit indoleamine 2,3-dioxygenase in IFN-gamma-activated human macrophages: posttranslational regulation by pyrrolidine dithiocarbamate. J Immunol 2001; 166: 6332-6340.
[38] Laragione T, Gianazza E, Tonelli R, Bigini P, Mennini T, Casoni F, Massignan T, Bonetto V and Ghezzi P. Regulation of redox-sensitive exofacial protein thiols in CHO cells. Biol Chem 2006; 387: 1371-1376.
[39] Skalska J, Brookes PS, Nadtochiy SM, Hilchey SP, Jordan CT, Guzman ML, Maggirwar SB, Briehl MM and Bernstein SH. Modulation of cell surface protein free thiols: a potential novel mechanism of action of the sesquiterpene lactone parthenolide. PLoS One 2009; 4: e8115.
[40] Yan Z, Garg SK, Kipnis J and Banerjee R. Extracellular redox modulation by regulatory T cells. Nat Chem Biol 2009; 5: 721-723.
[41] Orabona C, Pallotta MT, Volpi C, Fallarino F, Vacca C, Bianchi R, Belladonna ML, Fioretti MC, Grohmann U and Puccetti P. SOCS3 drives proteasomal degradation of indoleamine 2,3-dioxygenase (IDO) and antagonizes IDO-dependent tolerogenesis. Proc Natl Acad Sci USA 2008; 105: 20828-20833.
[42] Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K and Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. Biol Chem 2009 Mar; 390: 191-214.
[43] De la Fuente M, Hernanz A and Vallejo MC. The immune system in the oxidative stress conditions of aging and hypertension: favorable effects of antioxidants and physical exercise. Antioxid Redox Signal 2005; 7: 1356-1366.
[44] Richie JP Jr. The role of glutathione in aging and cancer. Exp Gerontol 1992; 27: 615-626.
[45] Hernanz A, Fernandez-Vivancos E, Montiel C, Vazquez JJ and Arnalich F. Changes in the intracellular homocysteine and glutathione content associated with aging. Life Sci 2000; 67: 1317-1324.
[46] Hernanz A, Fernandez-Vivancos E, Salazar RM and Arnalich F. Homocysteine and other thiol compounds in ageing. Biofactors 2000; 11: 47-49.
[47] Droge W. Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants. Exp Gerontol 2002; 37: 1333-1345.