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The Cystine/Glutamate Antiporter Regulates Dendritic Cell Differentiation and Antigen Presentation

June A. D’Angelo,* Eleonora Dehlink,† Barbara Platzer,‡ Peter Dwyer,§ Magdalena L. Circu,‡ Jone Garay,* Tak Yee Aw,† Edda Fiebiger,‡ and Bonny L. Dickinson*§

The major cellular antioxidant glutathione is depleted during HIV infection and in obesity. Although the consequence of glutathione depletion on immune function is starting to emerge, it is currently not known whether glutathione dysregulation influences the differentiation and maturation of dendritic cells (DCs). Moreover, the effect of glutathione depletion on DC effector functions, such as Ag presentation, is poorly understood. Glutathione synthesis depends on the cystine/glutamate antiporter, which transports the rate-limiting precursor cystine into the cell in exchange for glutamate. In this paper, we present a detailed study of antiporter function in DCs and demonstrate a role for the antiporter in DC differentiation and cross-presentation. We show that the antiporter is the major mechanism for transport of cystine and glutamate and modulates the intracellular glutathione content and glutathione efflux from DCs. Blocking antiporter-dependent cystine transport decreases intracellular glutathione levels, and these effects correlate with reduced transcription of the functional subunit of the antiporter. We further demonstrate that blocking antiporter activity interferes with DC differentiation from monocyte precursors, but antiporter activity is not required for LPS-induced phenotypic maturation. Finally, we show that inhibiting antiporter uptake of cystine interferes with presentation of exogenous Ag to class II MHC-restricted T cells and blocks cross-presentation on MHC class I. We conclude that aberrant antiporter function disrupts glutathione homeostasis in DCs and may contribute to impaired immunity in the diseased host. The Journal of Immunology, 2010, 185: 3217–3226.

Glutathione is the most prevalent low m.w. thiol in mammalian cells and the major determinant of cellular redox state (1–4). Glutathione plays a critical role in maintaining dendritic cell (DC) redox homeostasis and protecting DCs from oxidative stress, a state in which oxidants outnumber antioxidant defenses (5–11). The de novo synthesis of glutathione is regulated via the cystine/glutamate antiporter, which transports cysteine into the cell in its oxidized form in exchange for glutamate (12). Inside, the cell cysteine is readily reduced to cysteine and enters the glutathione biosynthetic pathway (12–17). The cystine/glutamate antiporter, also termed system xCT, is a heterodimer composed of xCT and CD98. The xCT L chain confers the specificity of amino acid transport, whereas the ubiquitously expressed CD98 H chain is common to other amino acid transport systems and is required for membrane expression of xCT (18, 19). In vitro, the antiporter also functions as a glutamate/glutamate exchanger to transport glutamate into the cell in exchange for the efflux of cellular glutamate. Glutamate can also be transported via the excitatory amino acid transporters (EAATs), and the two systems may be distinguished on the basis of their ionic requirements for transport. The cystine/glutamate antiporter is a chloride-dependent, sodium-independent transporter (20), whereas the EAATs are sodium-dependent transporters (21). The function of the cystine/glutamate antiporter has been well described in neutrophils, monocytes, and macrophages; however, its activity has not been rigorously characterized in DCs (22–24).

Currently, little is known about how glutathione homeostasis is maintained in DCs, and the effect of glutathione dysregulation on DC phenotype and function remains to be completely defined. DCs are highly specialized in their ability to process and present exogenous Ag to CD4⁺ Th cells and endogenous Ag to CD8⁺ T cytotoxic cells. DCs also present exogenous Ag in the context of MHC class I to CD8⁺ T cells, a process termed cross-presentation (25–27). Cross-presentation plays a pivotal role in generating CD8⁺ T cell responses against soluble, cell-associated, or pathogen-derived Ags that are not endogenously expressed by DCs (28). Among APCs, DCs are the only cells proficient at cross-priming (29). To present Ag, DCs must undergo functional maturation whereby the cell surface levels of MHC class II and costimulatory molecules required for T cell activation are increased and chemokine receptors that promote DC migration to lymph nodes are expressed (30, 31). The transition of an immature DC to the mature form is vital as only mature DCs can activate T cells; DCs arrested in an immature or semimature state induce T cell anergy, resulting in the development of tolerance (32–34). Whether the cystine/glutamate antiporter, by modulating the intracellular glutathione concentration, regulates DC maturation and Ag presentation has not been explored. In addition, nothing is known about whether glutathione regulates DC differentiation from monocyte precursors.
The goal of this study was to analyze antiporter function in DCs to provide critical insight into how the disruption of glutathione homeostasis may impact DC function. Our results highlight an important role for the cystine/glutamate antiporter in DC differentiation and Ag presentation. Disturbances in glutathione homeostasis are implicated in the etiology and/or progression of several human diseases including cancer and inflammatory, immune, metabolic, and neurodegenerative diseases (35). Thus, this study has relevance for understanding how glutathione depletion affects DC function in disease.

Materials and Methods

Materials

Escherichia coli 026:B6 LPS (gamma irradiated; total impurities < 5% protein), FITC-dextran (40,000 Da), t-homocysteic acid (LHC), N-threo-
β-hydroxyaspartic acid (THA), dimethylaminoalade (DMEA), and FITC-dextran were from Sigma-Aldrich (St. Louis, MO). Recombinant human IL-4 was from R&D Systems (Minneapolis, MN). Recombinant human GM-CSF (Leuken) was from Berlex Laboratories (Montville, NJ). RPMI 1640 media, FBS, penicillin, streptomycin sulfate, and amphotericin B were from Gibco (Grand Island, NY). Cystine/cysteine-free medium was from MP Biomedicals (Solon, OH). DL-threo-benzyloxyaspartate (TBOA) was from Tocris Bioscience (Ellisville, MO). The fluorophore-conjugated isotype control Ab and mouse mAbs to detect CD80, CD83, CD86, HLA-DR, HLA-
ABC, CD16, CD1a, CD14, CD62L, CD11c, and DC-specific intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)were from BD Pharmingen (San Diego, CA). Fluorophore-conjugated Abs to detect murine CD11c, CD14, CD8, Vα2, and Vβ5 were from BD Pharmingen.

Human monocyte-derived DCs

Human PBMCs were isolated from normal human buffy coats (purchased from the Blood Donor Center of Louisiana, New Orleans, LA) by centrifugation on Ficoll-Paque (GE Healthcare, Uppsala, Sweden). Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation (human CD14 Microbeads; Miltenyi Biotec, Auburn, CA). To derive DCs, monocytes (10^6 cells/ml) were cultured in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin). The fluorophore-conjugated Abs were detected by flow cytometry on a FACSCanto II flow cytometer (BD Biosciences). Data were collected using FACSDiva software (BD, Franklin Lakes, NJ). Human DCs and cells purified from the spleen, inguinal lymph nodes, and mesenteric lymph nodes of OT-I and OT-II mice were incubated for 5 min on ice with Fc block (BD Biosciences, San Jose, CA) in FACS buffer (Invitrogen) supplemented with 5% FCS. DCs were incubated with Abs for 15 min on ice, washed, and examined directly by flow cytometry on a FACS Canto II flow cytometer (BD Biosciences). Data were collected using FACS Diva software (BD Biosciences) and analyzed with FlowJo software. Gates were selected based on the staining pattern of DCs incubated with isotype control Abs. A total of 10,000 events were collected in each experiment.

Radiolabeled amino acid transport assays

The activity of the cystine/glutamate antiporter was measured as previously described with minor modifications (23). Briefly, DCs were equilibrated in transport medium (137 mM NaCl, 0.7 mM K\_2HPO\_4, 1 mM CaCl\_2, 1 mM MgCl\_2, 5 mM glucose, and 10 mM HEPES [pH 7.4]) at 37°C in 96-well plates. Then, t-\[^{14}\text{C}\]glutamate (18.5 μCi/ml; PerkinElmer, Waltham, MA) or t-\[^{14}\text{C}\]cystine (1.25 μCi/ml; PerkinElmer) was added to cells at a final concentration of 50, 100, or 200 μM, and the cells were incubated at 37°C for 5 min. Amino acid transport was stopped by transferring 96-well plates to an ice bath. DCs were then washed three times with ice-cold transport medium and lysed in 100 mM NaOH. Radioactivity in lysates was measured by liquid scintillation counting and normalized to the quantity of protein in lysates as determined using the Pierce Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL). To assess ionic dependence, assays were performed in transport medium containing 100 mM NaCl and varying concentrations of KCl, LiCl, or in the absence of LiCl, and NaCl. Data analysis was performed using GraphPad Prism software (GraphPad, La Jolla, CA).

Measurement of reduced glutathione and oxidized glutathione by HPLC

Reduced glutathione (GSH) and oxidized GSH (GSSG) were quantified in DC lysates and DC-conditioned medium by HPLC using an already established technique (36–38). DCs were incubated in complete medium in the presence or absence of LHC (2.5 mM) or incubated in cystine/cysteine-free medium for 24 h. Cell viability was confirmed using Invitrogen’s Live/Dead Cell Assay. None of the treatments resulted in significant cell death (nontreated immature DCs compared with LHC-treated immature DCs; p = 0.24; nontreated immature mature DCs compared with immature DCs incubated in cystine/cysteine-free medium; p = 0.88; nontreated mature DCs compared with LHC-treated mature DC; p = 0.76; and nontreated mature DCs compared with mature DCs incubated in cystine/cysteine-free medium; p = 0.43). At no time were reducing agents present in the medium. DCs were lysed in 5% trichloroacetic acid and centrifuged. Thiols in the acid supernatant were treated with iodoacetamide and derivatized with 1-fluoro-2,4-dinithrobenzene. GSSG and GSH were detected by reverse-phase ion exchange HPLC on a 250 × 4.6-mm Waters Spherisorb 10 μm NH\_2 column (Waters, Milford, MA). Proteins captured in the acid pellet were solubilized in 0.1 M NaOH, and protein was determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). The GSH and GSSG concentrations were determined by comparison with purified GSH and GSSG standards derivatized in the same manner. The GSH and GSSG concentrations were determined by comparison with purified GSH and GSSG standards derivatized in the same manner.
mRNA measurements with nCounter

The NanoString nCounter gene expression system (NanoString Technologies, Seattle, WA) was used to quantify individual mRNA transcripts using an approach similar to that described by Geiss et al. (39). Immature DCs were treated with or without 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 6 or 16 h. These treatments did not result in significant cell death (LPS-treated DCs incubated in complete medium compared with LPS-treated DCs incubated in cysteine/cysteine-free medium; p = 0.58). Then, 50,000 DCs per condition were lysed in RLT buffer (Qiagen, Valencia, CA) supplemented with 2-ME (Sigma-Aldrich). Ten percent 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 were normalized in two steps. First, we used the positive spiked-in controls provided by the nCounter instrument as per the manufacturer’s instructions. Second, we normalized copy numbers to the housekeeping genes GAPDH and HPRT1.

RT-PCR

RNA was extracted from DCs using RNAqueous-4PCR kit (Ambion, Austin, TX), according to the manufacturer’s instruction. RNA was treated with RNase-free DNase (Ambion) to remove DNA, and the RNA (1 μg) was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen), according to the manufacturer’s instruction. Then, xCT, EAAT1, EAAT2, and G3PDH cDNA were amplified by PCR using the Platinum PCR SuperMix (Invitrogen). PCR conditions were as follows: cDNA was denatured at 95˚C for 1 min, followed by 35 cycles of amplification at 95˚C for 15 s, 55˚C for 30 s, and 68˚C for 1 min. Final products were visualized at 67˚C for 7 min. The EAAT1, EAAT2, and xCT primers used were described previously (40, 41). The G3PDH primers used were as follows: 5′-GGA AAT CCC ATC ACC TTC CAG C-3′ (forward primer) and 5′-GTC ATACCA GGA AGT GAG CTT GAC C-3′ (reverse primer). Amplification products were resolved by electrophoresis on a 1.5% agarose gel.

T cell proliferation assays

Splenic DCs (4 × 10³) were cultured for 2 h or overnight (16 h) with LHC (10 mM) or in cysteine/cysteine-free medium. Following the incubation, DCs were seeded into round-bottom 96-well plates with 0–1000 μg chicken egg OVA (Sigma-Aldrich) for 3 h in the presence or absence of LHC (10 mM). Then, DCs were irradiated with 3000 rad, washed, and incubated with OT-I or OT-II T cells (4 × 10⁵) in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% glutamine, 10 mM HEPES, and 55 μM 2-ME. After 3 d in culture, cells were washed with 1 μCi [3H]thymidine (PerkinElmer) and incubated for 16 h, after which T cell proliferation was measured by liquid scintillation counting. To examine T cell proliferation, CFSE dilution, splenic DCs were cultured in medium or in medium containing LHC (10 mM) for 2 or 16 h. Then, DCs were pulsed with OVA (500 μg) for 3 h. OT-I cells were labeled with CFSE (Invitrogen) and washed with RPMI 1640 medium. DCs were then washed in PBS and cultured with CFSE-labeled OT-I cells at DC:T cell ratios of 1:1 and 1:3 in triplicate. After 3 d of culture, CFSE dilution of CD8α+ T cells was examined by flow cytometry.

Granzyyme B and IFN-γ ELISA

Granzyyme B and IFN-γ in culture supernatants were analyzed using the Ready Set Go ELISA kit (eBioscience, San Diego, CA), according to the manufacturer’s instructions.

Endocytosis assay

Endocytosis was measured as the cellular uptake of FITC-dextran and quantified by flow cytometry. DCs (2 × 10⁵ cells/sample) were treated with or without LHC (10 mM) for 2 or 24 h, washed, and incubated with FITC-dextran (1 mg/ml) in the presence or absence of LHC for 30, 60, and 90 min at 37 or 4˚C. After incubation, cells were washed three times with complete medium to remove excess dextran, and the uptake of FITC-dextran was determined by flow cytometry. A total of 10,000 cells/sample were analyzed.

Statistical analysis

For the amino acid transport assays, cell viability assays, T cell proliferation, endocytosis assay, ELISA, and the quantification of GSH and GSSG, differences between the means of experimental groups were analyzed by single-factor ANOVA (NS [p > 0.05]; a statistical difference between groups is denoted by # [p < 0.05]; ## [p < 0.01]; and a statistical significance between 37 and 4˚C controls or between groups as they relate to the nontreated control is denoted by # [p < 0.05; ## [p < 0.01]). The two-tailed t test was used to analyze statistical significances between treatment groups in the digital RNA profiling studies and in studies comparing cell surface expressed molecules by flow cytometry. Evaluations of donor data were performed with the Mann-Whitney U test using GraphPad Prism software (La Jolla, CA).

Results

DC maturation is associated with increased transport of cystine and glutamate

To elucidate a role for the cystine/glutamate antiporter in DC maturation and function, we used radiolabeled amino acid transport assays to compare the transport of glutamate and cystine in immature and mature human monocyte-derived DCs. Both immature DCs and DCs matured with LPS (hereafter referred to as mature DCs) internalized glutamate and cystine in a dose-dependent manner (Fig. 1A, 1B). Glutamate and cystine transport was significantly greater in mature DCs as compared with immature DCs, and transport did not occur via pinocytosis as transport was blocked at 4˚C (Fig. 1A, 1B). Glutamate uptake also did not depend on the activity of the Na⁺/H⁺ exchanger as DMA, an inhibitor of pinocytosis, did not block the uptake of glutamate by immature DCs (Fig. 1C). We next analyzed glutamate transport in DCs from 23 different donors. The data show that glutamate transport by mature DCs was significantly greater than by immature DCs (Fig. 1D). Statistical evaluation of DCs from 10 male (aged 33 ± 11; Ave ± StDev) and 6 female (aged 36 ± 19; Ave ± StDev) donors revealed that in this set of donors, age and biological sex had no significant effect on glutamate transport (data not shown). However, there was a trend for greater glutamate transport in immature and mature DCs from male donors than in DCs from female donors, and slightly higher levels of transport were observed in older donors than in younger donors.

Our results show that the activity of the cystine/glutamate antiporter is greater in mature DCs than in immature DCs. To explain this result, we examined whether DC maturation correlated with an increase in transcription of the functional subunit of the antiporter, xCT. Immature DCs were incubated with or without LPS for 4 h and then cultured for 6 or 16 h in complete medium. RNA transcripts encoding xCT were then measured by digital mRNA profiling. LPS induced an increase in xCT mRNA transcripts at both time points. We then examined whether blocking antiporter activity could affect xCT mRNA expression levels. When DCs were incubated with LPS for 4 h and then cultured in cysteine/cysteine-free medium for 6 or 16 h, we found that there was an increase in the level of xCT mRNA when compared with LPS-treated DCs incubated in complete medium (Fig. 2). These data show that transcription of xCT is increased both during DC maturation and when the activity of the antiporter is inhibited.

The cystine/glutamate antiporter is the major mechanism for glutamate and cystine transport in human DCs

To confirm that glutamate and cystine transport was dependent on cystine/glutamate antiporter activity, we compared glutamate and cystine transport in DCs treated with and without LHC. LHC is a potent competitive inhibitor of the antiporter that does not serve as a substrate for glutathione synthesis (42). Consistent with our previous results, glutamate and cystine transport was significantly greater in mature DCs than in immature DCs (Fig. 3A, 3B). LHC efficiently blocked both glutamate and cystine transport in both immature and mature DCs (Fig. 3A, 3B). Because the antiporter has similar affinities for cystine and glutamate (43), we next tested...
whether transport of cystine could be blocked in the presence of excess glutamate and vice versa. We found that excess cystine inhibited glutamate transport in immature and mature DCs (Fig. 3A). The reciprocal experiment showed that cystine transport was inhibited in the presence of excess glutamate (Fig. 3B).

Glutamate transport is also a function of the EAATs. To determine whether the cystine/glutamate antiporter or EAATs mediate the transport of glutamate in DCs, we examined DCs for the presence of EAAT1 and EAAT2 mRNA by RT-PCR. Both immature and mature DCs expressed xCT mRNA as well as transcripts for EAAT1 and EAAT2 (data not shown). To determine whether EAATs participated in glutamate transport in DCs, we measured transport in the presence or absence of sodium or chloride ions. We found that transport of glutamate in immature and mature DCs was chloride dependent and sodium independent (Supplemental Fig. 1A, 1B). This pattern of ionic dependence is a hallmark of the cystine/glutamate antiporter (42) and argues against a major role for the sodium-dependent EAATs in the transport of glutamate in DCs. To confirm this, we treated DCs with THA, a competitive antagonist transported in the place of glutamate (40), or TBOA, a nontransportable inhibitor specific for EAATs (44). In contrast to the block in glutamate transport when DCs were treated with LHC, THA and TBOA had no effect on glutamate transport in immature and mature DCs (Supplemental Fig. 2A, 2B). When THA or TBOA was used in combination with LHC, there was no additional inhibition in glutamate transport when compared with the effect of LHC alone (Supplemental Fig. 2A, 2B). These data show that the cystine/glutamate antiporter is the predominant mechanism for glutamate transport in DCs.

The cystine/glutamate antiporter regulates DC glutathione homeostasis

Next, we tested whether the cystine/glutamate antiporter could regulate intracellular glutathione levels in DCs. We first quantified GSH and GSSG in DC lysates by HPLC. Immature and mature DCs contained similar levels of GSH and GSSG (Fig. 4A, 4B), and thus, the GSH/GSSG ratio was also comparable between immature and mature DCs (Fig. 4C). Next, we treated immature and mature DCs with LHC (2.5 mM) for 24 h and examined the cellular GSH and GSSG content. Treatment with LHC did not affect DC viability when compared with nontreated DCs. LHC decreased the cellular GSH content by 24% in immature DCs and 36% in mature DCs (Fig. 4A). In contrast, LHC had no effect on immature or mature DC GSSG levels (Fig. 4B). The decline in cellular GSH with unchanged GSSG resulted in a decrease in the GSH/GSSG ratio in both immature and mature DCs treated with LHC (Fig. 4C). Similarly, when DCs were incubated in cystine/cysteine-free medium, GSH levels were reduced by 47% in immature DCs and by 49% in mature DCs (Fig. 4A). As with LHC, cystine/cysteine-free medium had no effect on DC GSSG levels (Fig. 4B) and thus decreased the GSH/GSSG ratio (Fig. 4C). This set of results suggests that a major function of the cystine/glutamate antiporter in DCs is to provide the cell with cystine to support the de novo biosynthesis of GSH. These data also show that blocking antiporter activity in DCs decreased the ratio of cellular GSH-to-GSSG, a hallmark of oxidative stress.

The cystine/glutamate antiporter controls the availability of glutathione for efflux from DCs

We next examined whether the cystine/glutamate antiporter regulates GSH efflux from DCs. In the extracellular compartment,
GSH scavenges extracellular reactive oxygen species (ROS), facilitates cysteine transport by neighboring cells, and reduces oxidized protein sulfhydryls (45, 46). We first measured GSH release from immature and mature DCs cultured in medium for 24 h. We also measured GSSG in the medium to determine whether GSH was oxidized following export from the cell. Immature and mature DCs exported similar quantities of GSH (Fig. 4D). The GSH detected in the medium was exported from DCs and not already present in the culture medium, because only low levels of GSH were detected in fresh, complete medium (∼12 pM GSH) and in cystine/cysteine-free medium (∼6 pM GSH). In contrast to GSH, only small quantities of GSSG were present in the medium of immature and mature DCs (Fig. 4D).

To examine a role for the antiporter in the regulation of GSH efflux, DCs were incubated with LHC or in cystine/cysteine-free medium for 24 h, after which GSH and GSSG were measured in the medium. LHC reduced GSH efflux from both immature and mature DCs by ∼35% (Fig. 4C, 4D). In contrast, LHC had no effect on GSSG concentrations in the medium of immature and mature DCs (Fig. 4D). Incubation of DCs in cystine/cysteine-free medium also reduced GSH efflux from immature DCs by ∼80% and from mature DC by ∼82% (Fig. 4D, 4E). Furthermore, the incubation of DCs in cystine/cysteine-free medium had no effect on the GSSG levels in the medium of immature and mature DCs (Fig. 4D). These data demonstrate that the cystine/glutamate antiporter controls the intracellular concentration of GSH and thus indirectly regulates GSH efflux from the cell by controlling the availability of GSH for export.

The cystine/glutamate antiporter regulates DC differentiation from monocyte precursors

We next tested whether the cystine/glutamate antiporter could regulate DC differentiation from peripheral blood monocytes that, like DCs, express a functional cystine/glutamate antiporter (47). Monocytes were cultured with or without LHC for 5 d in the presence of IL-4 and GM-CSF to induce differentiation into immature DCs (LHC treatment did not induce significant cell death when compared with nontreated cells; \( p = 0.64 \)). Then, DC expression of MHC class I and II, costimulatory molecules (CD80 and CD86), the activation marker CD83, and lineage markers were quantified by flow cytometry. LHC treatment significantly reduced the upregulation of MHC class I and II, CD80, and DC-SIGN (Fig. 5, Supplemental Fig. 3). In contrast, the expression of the other markers remained the same as that observed for immature DCs. In control experiments, LHC had no effect on the staining pattern of the isotype control Abs (data not shown). Taken together, these data suggest that the antiporter plays an important role in the development of DCs from monocytes.

FIGURE 3. Functional characterization of the cystine/glutamate antipporter in human DCs. IMDC and LPS-DC were incubated in medium for 30 min at 37˚C with or without LHC (10 mM) or in medium containing excess glutamate (2.5 mM) or cystine (1 mM) and then examined for transport of [\(^{3}H\)]glutamate (200 \( \mu \)M) (A) or [\(^{14}C\)]cystine (200 \( \mu \)M) (B). Data are mean values ± SEM of triplicate samples from two independent experiments. IMDC, immature DCs; LPS-DC, mature DCs.

FIGURE 4. The cystine/glutamate antiporter regulates human DC glutathione homeostasis. A–C, IMDC and LPS-DC were cultured for 24 h in complete medium, LHC-containing medium (2.5 mM), or cystine/cysteine-free medium. Cells were then lysed, and GSH and GSSG were measured in lysates by HPLC. Data were normalized to total cellular protein. GH (A), GSSG (B), and the GSH/GSSG ratio (C) are plotted (note differences in the y-axis scales between A and B). Data represent single measurements from four independent experiments. D and E, Glutathione efflux was measured by culturing IMDC and LPS-DC for 24 h in complete medium, medium containing LHC (2.5 mM), or cystine/cysteine-free medium. Cells were then removed by centrifugation, and GSH and GSSG in the medium were measured by HPLC. Data were normalized to total cellular protein. Data are plotted as GSH or GSSG in nanomoles per milligram of protein (D) and as GSH content relative to the nontreated IMDCs and mature DCs (= 100%) (E). Data represent single measurements from four independent experiments. IMDC, immature DCs; LPS-DC, mature DCs.
The cystine/glutamate antiporter does not regulate the phenotypic maturation of DCs

To determine whether the antiporter could regulate LPS-induced DC maturation, immature DCs were treated with or without LHC for 24 h and then incubated with or without LPS in the presence or absence of LHC for an additional 24 h, after which the cell surface expression of MHC class I and II, CD80, CD83, and CD86 was measured by flow cytometry. Consistent with the ability of LPS to induce DC maturation, LPS increased the surface expression of MHC class I and II, CD80, CD83, and CD86 relative to that observed in immature DCs (Fig. 6, compare white bars with black bars). When immature DCs were cultured for 24 h with LHC and then treated with LPS for an additional 24 h, we found that there was no change in the cell surface expression of MHC class II, CD80, CD83, or CD86 when compared with DCs treated with LPS alone (Fig. 6, compare dark gray bars with black bars). However, we did observe a change in MHC class I; LHC treatment decreased the level of MHC class I on the DC cell surface. LHC also had no effect on the phenotype of immature DCs (Fig. 6, compare white bars with light gray bars). Taken together, this set of data shows that the cystine/glutamate antiporter does not significantly regulate the phenotypic maturation of DCs.

The cystine/glutamate antiporter does not interfere with DC uptake of Ag

We next tested whether the cystine/glutamate antiporter could influence DC endocytosis as a parameter of functional maturation. DCs were treated for 2 or 24 h with LHC (10 mM) and then incubated with FITC-dextran for 30, 60, and 90 min at 37°C, after which FITC-dextran uptake was quantified by flow cytometry. Nontreated immature DCs actively took up FITC-dextran at each time point, and uptake was significantly reduced in the fully mature LPS-treated DCs (Fig. 7). When normalized to the maximum uptake of dextran by nontreated immature DCs at the 90-min interval (= 100%), DCs treated with LHC for 2 or 24 h exhibited the same rate of dextran endocytosis as the nontreated immature DCs. These data indicate that the activity of the antiporter is not required for DC uptake of Ag.

The cystine/glutamate antiporter regulates the presentation of exogenous Ag

To explore a role for the antiporter in Ag presentation, we examined murine DC presentation of OVA to T cells using the OT-I and OT-II system. T cells isolated from OT-I mice express a transgenic TCR that recognizes chicken OVA presented in the context of MHC class I. OT-II TCRs recognize OVA presented via MHC class II. Murine splenic DCs were treated with LHC for 2 or 16 h, and the presentation of exogenous OVA to OT-I and OT-II T cells was measured by [3H]thymidine incorporation. LHC potently reduced the ability of DCs to stimulate OT-I proliferation at both time points (Fig. 8A). When DCs were treated with LHC for 2 h, OT-I proliferation was reduced by 67, 58, and 91% relative to nontreated control DCs incubated with decreasing concentrations of OVA (1000, 100, and 10 μg) (Fig. 8A). At the 16-h time point, LHC reduced OT-I proliferation by 81, 77, and 48% relative to control DCs incubated with decreasing concentrations of OVA (Fig. 8A). Blocking antiporter transport of cystine also interfered with DC presentation of OVA to OT-II cells, although the effect was not as pronounced as that observed for the OT-I cells. When DCs were treated with LHC for 2 or 16 h and incubated with OVA (1000 μg), OT-II proliferation was reduced by 14 and 61%, respectively (Fig. 8C). When DCs were pulsed with lower concentrations of OVA, LHC more potently inhibited OT-II proliferation (Fig. 8C).

In addition to measuring T cell proliferation, we quantified the secretion of the CD8-specific effector molecule granzyme B from OT-I cells and IFN-γ from CD4+ OT-II cells by ELISA.
Although granzyme B is a marker for CD8+ T cells, we cannot exclude the possibility that it or IFN-γ was derived from other cells in the cultures. Consistent with the inhibitory effect of LHC on the ability of DCs to stimulate OT-I and OT-II proliferation, blocking antiporter activity reduced the levels of granzyme B and IFN-γ in the cocultures (Fig. 8B, 8D). Granzyme B was reduced 55 and 89% when DCs were treated with LHC for 2 h and pulsed with 1000 or 100 μg OVA, respectively. When DCs were treated with LHC for 16 h and pulsed with 1000 μg OVA, granzyme B production was reduced by 97% (Fig. 8B). Granzyme B was not detectable when DCs were treated with LHC for 2 h and pulsed with the lowest concentration of OVA or when DCs were treated for 16 h with LHC pulsed with 100 or 10 μg OVA (Fig. 8B). LHC also blocked IFN-γ production from OT-II cells, although the effect was less pronounced than that observed for granzyme B. IFN-γ secretion was not affected when DCs were treated with LHC for 2 h and pulsed with the highest concentration of OVA, and IFN-γ production was reduced 75% when DCs were treated with LHC for 16 h and pulsed with 1000 μg OVA (Fig. 8D). IFN-γ was not detectable when DCs were treated with LHC for 2 or 16 h and pulsed with 100 or 10 μg OVA (Fig. 8D).

These data show that the antiporter plays a fundamental role in regulating DC presentation of exogenous Ag to both class I and class II MHC-restricted T cells.

Finally, we confirmed these data in two separate experiments. In the first, we examined the effect of cystine/cysteine-free medium on murine splenic DC presentation of OVA to OT-I and OT-II T cells (Supplemental Fig. 4). As with LHC treatment, incubation of DCs

![Image](http://www.jimmunol.org/)

**FIGURE 8.** The cystine/glutamate antiporter regulates murine splenic DC Ag presentation to T cells. Murine splenic DCs were incubated for 2 or 16 h in complete medium or in complete medium containing LHC (10 mM) and then pulsed with OVA (0–1000 μg). DCs were cultured with OT-I or OT-II cells for 3 d, after which cocultures were spiked with [3H]thymidine and incubated overnight. T cell proliferation was measured by liquid scintillation counting (A, C), and granzyme B and IFN-γ were quantified in culture supernatants by ELISA (B, D). Data are from one experiment in which samples were analyzed in triplicate. nd, not detectable.

**FIGURE 9.** The cystine/glutamate antiporter regulates DC cross-presentation. Splenic DCs were incubated in complete medium or in complete medium containing LHC (10 mM) for 2 or 16 h and then pulsed with OVA (500 μg). DCs were then cultured with CFSE-labeled OT-I cells at a ratio of 1:1 (A) or 1:3 (B) for 3 d, after which T cell proliferation was measured by CFSE dilution by flow cytometry. Data are from one experiment in which samples were analyzed in triplicate.
in cystine/cysteine-free medium for 16 h reduced their ability to stimulate OT-I and OT-II T cell proliferation (Supplemental Fig. 4A, 4B, respectively). This block in T cell proliferation correlated with a decrease in T cell secretion of granzyme B and IFN-γ (Supplemental Fig. 4C, 4D, respectively). In the second experiment, we showed that LHC inhibited splenic DC presentation of OVA to CFSE-labeled OT-I T cells (Fig. 9, Supplemental Fig. 5). These data confirm that the antiporter plays a fundamental role in cross-presentation.

Discussion

The goal of this study was to examine the function of the cystine/glutamate antiporter in human and murine DCs to discern a role for the antiporter in the regulation of DC differentiation, maturation, and Ag presentation. We show that blocking antiporter transport of cystine had several important effects on DCs. Inhibiting antiporter activity reduced the quantity of intracellular glutathione available for export from DCs. The reduction in GSH efflux from DCs is an indirect effect of inhibiting the antiporter. To date, there is no evidence to support a role for the antiporter in GSH efflux from cells. Rather, it is likely that transporters, such as cystic fibrosis transmembrane conductance regulator, may be involved. Glutathione export is important because subtle changes in the extracellular redox status are known to regulate signaling in the immune synapse and shape the outcome of T cell activation (48). For example, extracellular cysteine accumulation results in a lower redox potential, which promotes T cell proliferation and modifies the redox status of proteins on the T cell surface.

Our data also show that antiporter activity is increased during LPS-induced maturation. Thus, the antiporter may play a role in regulating DC redox homeostasis during the maturation process. Microbial Ags, such as LPS, increase ROS in DCs by stimulating the activity of the NADPH oxidase (49). This raises the possibility that increased antiporter activity is critical for maintaining the intracellular glutathione balance necessary to adjust to the physiological changes that occur during DC maturation. Similarly, the antiporter may also function to maintain the intracellular redox status during DC Ag presentation in the lymph nodes, a microenvironment characterized by high rates of T and B cell proliferation, apoptosis, inflammation, and oxidative stress (50–52).

We also show that the antiporter plays a role in regulating DC differentiation from monocyte precursors. Blocking antiporter activity in monocytes prevented their differentiation into inflammatory DCs in the presence of IL-4 and GM-CSF. The most pronounced effect of LHC was on the expression levels of the Ag-presenting molecules MHC class I and II and DC-SIGN, a C-type lectin receptor, which participates in the activation of CD4+ T cells by mediating the transient adhesion of DCs to T cells (53). Whether this change in phenotype translates to an effect on DC function remains to be examined. In vivo, inflammatory DCs are generated from monocytes at inflammatory foci and function to induce robust Th1 immunity (54–56). Because inflammatory DCs have robust Th1-polarizing activity (54–56), the generation of fewer inflammatory DCs during infection may also significantly impair Th1 immunity in the setting of global glutathione depletion that characterizes the HIV-infected host.

In contrast to the role the antiporter plays in DC differentiation, antiporter activity was not required for DC maturation. Although oxidative stress is an important feature of DC maturation, we found that blocking antiporter activity did not impair LPS-induced maturation. LHC and cystine/cysteine-free medium decreased intracellular glutathione levels in immature DCs by 24 and 47%, respectively, but this did not affect LPS-induced upregulation of MHC class II, CD80, CD83, or CD86. Our results also show that the antiporter was the main mechanism for cystine and glutamate transport in human DCs and that the activity of the antiporter was increased during LPS-induced DC maturation. Although DC maturation correlated with an increase in antiporter activity, we did not observe a change in the GSH/GSSG ratio when mature DCs were incubated with LPS for 24 h. This result is in line with a previous report showing that GSH levels were the same in immature DCs and DCs matured in the presence of LPS and IFN-γ for 48 h (57). It is possible that the accumulation of ROS following LPS exposure induced a rapid and early depletion of intracellular GSH and that at later time points GSH homeostasis was restored by the action of the antiporter. Consistent with this, others have shown that a decrease in the GSH/GSSG ratio in DCs occurs as early as 4 h of incubation with LPS (58).

Finally, our data show that the antiporter played a central role in exogenous Ag presentation to both class I and class II MHC-restricted T cells. Although previous studies have examined the effect of glutathione depleting agents on murine DC Ag presentation, this study is the first to directly examine the effect of glutathione depletion on cross-presentation. In a previous report, for example, splenic APCs were treated with the thiol-alkylating agent diethyl maleate (DEM), which forms irreversible adducts with glutathione. APCs were treated with DEM, washed, and incubated with T cells in the presence of OVA for 72 h (59). Although glutathione depletion had no effect on the ability of the APCs to stimulate T cell proliferation, it interfered with T cell production of IFN-γ following Ag presentation. In that study, incubation of APCs with T cells and Ag in the absence of DEM would likely allow the intracellular glutathione levels in the APCs to normalize during the 72 h in coculture. This is in contrast to our study in which we observed a defect in both T cell proliferation and IFN-γ production. In our assay, DCs were pretreated with LHC and incubated with OVA for 2 or 16 h in the continued presence of LHC before they were irradiated, washed, and incubated with T cells. This eliminated the possibility that glutathione levels could be replenished in the DCs before they were incubated with Ag and T cells. Thus, we were able to demonstrate that glutathione depletion interfered with both exogenous Ag presentation to CD4+ T cells and cross-presentation to CD8+ T cells. Finally, in contrast to the clear defect in cross-presentation, we observed that OVA presentation on MHC class II molecules was relatively intact in DCs treated with LHC and pulsed with high concentrations of OVA. This suggests that blocking antiporter activity had little effect on Ag processing. The contribution of cystinosin, a lysosomal transmembrane protein involved in cystine export from the lysosomal compartment, was not examined in these studies. However, the studies presented in this paper as well as studies in human macrophages, which express both the cystine/glutamate antiporter and EAATs, make clear that the intracellular GSH level is controlled by the activity of transporters for cystine, glutamate, and glutamine (60). Elucidating the precise molecular mechanisms by which the antiporter controls Ag presentation in DCs will be an important area of future investigation.

In summary, the results of this study have significance for understanding how impaired DC function may contribute to the pathogenesis of type 2 diabetes and obesity as well as other diseases in which a perturbation of glutathione homeostasis plays a known role (8, 61–64).

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Disclosures

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References


