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Regulation of interleukin-4 signaling by extracellular reduction of intramolecular disulfides

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ABSTRACT

Interleukin-4 (IL-4) contains three structurally important intramolecular disulfides that are required for the bioactivity of the cytokine. We show that the cell surface of HeLa cells and endotoxin-activated monocytes can reduce IL-4 intramolecular disulfides in the extracellular space and inhibit binding of IL-4 to the IL-4Rα receptor. IL-4 disulfides were *in vitro* reduced by thioredoxin 1 (Trx1) and protein disulfide isomerase (PDI). Reduction of IL-4 disulfides by the cell surface of HeLa cells was inhibited by auranofin, an inhibitor of thioredoxin reductase that is an electron donor to both Trx1 and PDI. Both Trx1 and PDI have been shown to be located at the cell surface and our data suggests that these enzymes are involved in catalyzing reduction of IL-4 disulfides. The pro-drug *N*-acetylcysteine (NAC) that promotes T-helper type 1 responses was also shown to mediate the reduction of IL-4 disulfides. Our data provides evidence for a novel redox dependent pathway for regulation of cytokine activity by extracellular reduction of intramolecular disulfides at the cell surface by members of the thioredoxin enzyme family.

Introduction

Interleukin-4 (IL-4) is a hematopoietic cytokine that plays a central role in differentiation of naïve T-helper (Th) cells to Th2 lymphocytes, inhibiting development of Th1 lymphocytes and down-regulation of Th1-type cytokines such as IFN-γ [1,2]. It also regulates immunoglobin class switching to IgE and enhances IgE mediated responses by up-regulating IgE receptors on B-lymphocytes, mast cells, and basophiles [3,4]. IL-4 signaling is associated with the development of autoimmune diseases and allergic reactions [5]. It induces mucin gene expression responsible of airway obstruction in asthma [6] and several studies have shown benefits of inhibiting IL-4 signaling as treatment for allergic diseases [7,8].

The cellular redox state is an important modulator of immune responses and IL-4 signaling. Depletion of reduced glutathione (GSH) in antigen presenting cells inhibits Th1 lymphocyte differentiation, favors differentiation of Th2 cells and enhances their production of IL-4 [9]. Treatment of Th lymphocytes with GSH or the reducing agent *N*-acetylcysteine (NAC) induces differentiation of Th1 lymphocytes, decreases production of IL-4, and inhibits immunoglobulin class switching to IgE [10,11]. These effects have been suggested to be mediated by redox effects on intracellular signaling pathways down-stream to the binding of IL-4 to its cell surface receptor [11,12].

The cell surface is redox active and alterations in the redox state of protein thiols in cell surface receptors as well as their ligands have been shown to be important for several cellular processes [13,14]. For instance, entry of HIV-1 into T-lymphocytes is dependent on reduction of HIV-1 gp120 intramolecular disulfides in the extracellular space as well as disulfide exchange reactions in the cell surface CD4 receptor [15–17]. Protein disulfides isomerase (PDI) and thioredoxin 1 (Trx1) are present on the cell surface and these enzymes have been suggested to catalyze cell surface redox reactions [18–23]. IL-4 contains six cysteinyl residues that form three intramolecular disulfides [24,25]. Structural studies and site-directed mutagenesis show that these disulfides are important for structural integrity, receptor binding, and biological activity of the cytokine [26,27]. Considering the redox control of IL-4 signaling and the presence of enzymes on the cell surface that catalyzes reduction of protein disulfides reactions, we decided to test if IL-4 activity could be modulated by reduction of IL-4 disulfides in the

Abbreviations: DTT, dithiothreitol; GSH, reduced glutathione; IL-4, interleukin-4; IL-4R, interleukin-4 receptor; LPS, lipopolysaccharide; NAC, *N*-acetylcysteine; NEM, *N*-ethylmaleimide; PDI, protein disulfide isomerase; Th, T-helper; Trx1, thioredoxin 1; TR, thioredoxin reductase.

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extracellular space. Here we provide evidence that Trx1 and PDI reduce intramolecular IL-4 disulfides at the cell surface of HeLa cells and lipopolysaccharide-activated monocytes. IL-4 with reduced disulfides failed to bind the IL-4Rα receptor and the cytokine lost it biological activity. Our findings suggest that redox regulation of IL-4 signaling may occur in the extracellular space by reduction of IL-4 disulfides by cell surface enzymes.

Materials and methods

Cell culture. HeLa cells (American Type Culture Collection) were cultured in Dulbecco's minimal essential medium (DMEM, Gibco) supplemented with 10% fetal calf serum (Gibco), 1 mM glutamine, 10 U/ml penicillin G, and 10 μg/ml streptomycin sulfate. TF-1 cells (American Type Culture Collection) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 10 U/ml penicillin, 10 μg/ml streptomycin, 10 mM Hepes, 1 mM sodium pyruvate, and 2 ng/ml GM-CSF (Nordic Biosite, Sweden). Monocytes were isolated from buffy coats of healthy donors using the RosetteSep monocyte enrichment kit (StemCell Technologies) and Ficoll gradient separation (Ficoll-Hypaque, Amersham Biosciences). The purified monocytes (>95% $CD14^+$, $CD3^-$, $CD19^-$ cells) were either kept unstimulated or stimulated with 50 ng/ml lipopolysaccharide (Sigma) for 24 h. All cells were cultured at 37 °C and with 5% CO_2.

Detection of cell surface catalyzed IL-4 disulfide reduction. Cells were washed twice in Hank's balanced salt solution (HBSS) (Invitrogen). 500 ng/ml recombinant human IL-4 (Nordic Biosite) was added to the cells and incubated 15 min at 37 °C. The supernatants containing IL-4 in HBSS were removed and incubated with 2 μM NEM–biotin (Sigma) for 15 min to biotinylate IL-4 cysteinyl thiols. 96-Well Maxi-Sorp ELISA plates (Nunc) were coated with 1 μg/ml rat monoclonal anti-IL-4 antibody (clone 25D2, Endogen, USA) in carbonate buffer (pH 9.6) for 60 min at 37 °C. The wells were blocked with 1% bovine serum albumin in phosphate buffered saline (PBS), pH 7.4, for 1 h and also treated with 20 mM NEM (Sigma) for 30 min at 22 °C to alkylate any free thiol. The samples containing IL-4 in HBSS were diluted threefold in PBS and 0.05% Tween-20 (PBST) and transferred to the plates coated with the anti-IL-4 antibody. The plates were incubated at 22 °C for 60 min. The plates were washed with PBST and the biotinylated IL-4 was detected by 30 min incubation with streptavidin–alkaline phosphatase (Mabtech AB, Sweden) diluted 1:1000 in PBST supplemented with 1% BSA. The plates were washed four times in PBST and 1 mg/ml *p*-nitrophenyl-phosphate (Sigma) dissolved in 10% diethanolamine, pH 9.8, with 0.5 mM $MgCl_2$ was added. Absorbance at 405 nm was determined. Four individual experiments were performed for the HeLa cells with triplicate samples for each condition. Three individual experiments were performed for the monocytes (each experiment represents cells from different blood donors) with triplicate samples for each condition.

Inhibition of HeLa cell mediated IL-4 disulfide reduction by NEM or auranofin. HeLa cells were treated with 1 mM NEM for 5 min at 22 °C to alkylate cell surface thiols or treated with 500 μM auranofin (Sigma) for 5 min at 22 °C to inhibit TR1 activity. After washing the cells twice with PBS and then once with HBSS, IL-4 was added to the cells and the assay for IL-4 disulfide reduction was performed as described above. The data presented represent three individual experiments using triplicate samples for each condition in the individual experiments.

Reduction of IL-4 disulfides by Trx1 and PDI. 96-Well Maxi-Sorp plates were coated with 500 ng/ml (100 μl) IL-4 diluted in phosphate buffered saline (PBS), pH 7.4, for 60 min at 37 °C and the plates were blocked in PBST for 1 h at 37 °C. The wells were then incubated with combinations of 5 mM DTT, 1 μM Trx1 (IMCO, Sweden), 100 nM TR1 (Sigma), 1 μM PDI (Sigma) diluted in the assay buffer containing PBST and 200 μM NADPH. At indicated time points the wells were washed four times with PBST and then treated with 2 μM NEM–biotin diluted in PBST for 15 min at 22 °C to biotinylate IL-4 cysteinyl thiol. The plates were washed four times with PBST and biotinylated IL-4 was detected using streptavidin–ALP as described above.

IL-4-induced proliferation of TF-1 cells. TF-1 cells were washed with PBS and seeded at 10^5 cells/ml in 96-well plates (0.2 ml/well) in medium supplemented with 1 ng/ml IL-4, 1 mM NAC, or 5 mM NAC as indicated. The plate were incubated at 37 °C and 5% CO_2 for 72 h before 0.5 μCi ^{3}H-Thd were added to each well and the cells incubated for an additional 4 h. The cells were harvested using a Tomtec harvester (Wallac) and the incorporated ^{3}H-dThd determined using a Beta Plate Reader (Wallac).

Interaction between IL-4 and IL-4Rα. 96-Well Maxi-Sorp plates were coated with 2 μg/ml recombinant IL-4 receptor IL-4Rα (Nordic Biosite, Sweden) in PBS for 30 min at 22 °C. The plates were blocked with PBS and 2% BSA. 10 ng/ml IL-4 was incubated in the presence or absence of 5 mM DTT, 1 μM Trx1, 1 μM PDI, 100 nM TR, and 200 μM NADPH diluted in PBS for 15 min. The samples were then added to the IL-4Rα coated plate and incubated for 30 min at 22 °C. The wells were washed four times with PBST and then incubated with a mouse monoclonal anti-IL-4 antibody (1 μg/ml) diluted in PBST with 1% BSA for 30 min. The wells were washed with PBST and then incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody diluted 1:1000 in PBST with 1% BSA. After four washes in PBST the wells were exposed to substrate buffer and analyzed as described above.

Statistical analysis. Statistical significance was calculated using two-tailed student's *t*-test.

Results

Extracellular reduction of IL-4 intramolecular disulfides

IL-4 contains six cysteinyl residues that form three intramolecular disulfides [24,25]. We decided to investigate if these disulfides could be reduced by the redox active cell surface of human peripheral blood monocytes and the HeLa cancer cell line. A biotinylated derivative of the alkylating agent *N*-ethylmaleimide (NEM) was used to specifically bind protein thiols and detect the reduction of IL-4 disulfides. Recombinant IL-4 was incubated with the cells for 15 min and subsequently treated with NEM–biotin to label any IL-4 cysteinyl thiols formed. IL-4 was captured on ELISA plates coated with anti-IL-4 antibodies and biotinylated IL-4 was detected using a streptavidin–alkaline phosphatase conjugate (Fig. 1A). Native IL-4 does not contain any free thiols and treatment of IL-4 with NEM–biotin showed as expected only a weak background signal similar to the signal when no IL-4 was added to the assay. In contrast, the signal from NEM–biotin labeled cysteinyl thiols increased ≈fourfold when IL-4 was incubated with HeLa cells. Addition of HeLa cells increased the background signal level of the assay, but the signal from NEM–biotin labeled IL-4 cysteinyl thiols was greater than twofold higher than the background level ($p < 0.05$). Trx1 is a potent reductase that is known to reduce protein disulfides and addition of this enzyme was used as a control for the assay. Incubation of IL-4 with Trx1 resulted in a marked increase in NEM–biotin labeling that was >10-fold higher than background levels.

There was no increase in NEM–biotin labeled IL-4 when the cytokine was incubated with unstimulated monocytes (Fig. 1B). However, the signal for NEM–biotin labeled IL-4 increased twofold when LPS-activated monocytes were used for the experiments ($p < 0.01$). The increased labeling of IL-4 cysteinyl thiols with