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Ptaquiloside reduces NK cell activities by enhancing metallothionein expression, which is prevented by selenium

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ABSTRACT

Pteridium aquilinum, one of the most important poisonous plants in the world, is known to be carcinogenic to animals and humans. Moreover, our previous studies showed that the immunosuppressive effects of ptaquiloside, its main toxic agent, were prevented by selenium in mouse natural killer (NK) cells. We also verified that this immunosuppression facilitated development of cancer. Here, we performed gene expression microarray analysis in splenic NK cells from mice treated for 14 days with ptaquiloside (5.3 mg/kg) and/or selenium (1.3 mg/kg) to identify gene transcripts altered by ptaquiloside that could be linked to the immunosuppression and that would be prevented by selenium. Transcriptome analysis of ptaquiloside samples revealed that 872 transcripts were expressed differentially (fold change > 2 and $p < 0.05$), including 77 up-regulated and 795 down-regulated transcripts. Gene ontology analysis mapped these up-regulated transcripts to three main biological processes (cellular ion homeostasis, negative regulation of apoptosis and regulation of transcription). Considering the immunosuppressive effect of ptaquiloside, we hypothesized that two genes involved in cellular ion homeostasis, metallothionein 1 (Mt1) and metallothionein 2 (Mt2), could be implicated because Mt1 and Mt2 are responsible for zinc homeostasis, and a reduction of free intracellular zinc impairs NK functions. We confirm these hypotheses and show increased expression of metallothionein in splenic NK cells and reduction in free intracellular zinc following treatment with ptaquiloside that were completely prevented by selenium co-treatment. These findings could help avoid the higher susceptibility to cancer that is induced by *P. aquilinum*-mediated immunosuppressive effects.

1. Introduction

Bracken fern (genus *Pteridium*) is a ubiquitous plant known to cause toxicity syndromes in herbivores and cancer in animals and humans (Gil da Costa et al., 2012a; Vetter, 2009). Nevertheless, humans have used its crosiers and rhizomes as food in some regions of the world such as Brazil and Japan (Kamiyama et al., 1986; Shahin et al., 1999; Ulian et al., 2010), and this feeding pattern has already been associated with a greater prevalence of certain types of cancers (*e.g.*, esophageal, gastric) (Abnet, 2007; Sugimura, 2000). Similarly, concerns exist regarding the indirect consumption of bracken's toxins through consumption of milk from livestock that have fed on the plant (Alonso-Amelot and Avendano, 2002; Shahin et al., 1999). Environmental contamination could also be a problem, as has already been demonstrated for ptaquiloside in soil and water (Rasmussen et al., 2003). In fact, epidemiologic studies have attributed high rates of stomach cancer to people living in areas infested by bracken fern, for example, in the highlands of western Venezuela (Alonso-Amelot and Avendano, 2001) and in Gwynedd, North Wales (Galpin et al., 1990). More recently, meat was identified as another potential source of intoxication, as bracken toxins were detected in the skeletal muscle and liver of cattle fifteen days after bracken consumption had ended (Fletcher et al., 2011).

The main toxic agent found in *P. aquilinum* is ptaquiloside, which has been proven to be responsible for carcinogenic effects

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and a number of well-recognized toxicity syndromes in herbivores (Yamada et al., 2007). These toxic effects consist of a thiamine deficiency in horses and pigs, an acute hemorrhagic syndrome in cattle, retinal degeneration in sheep and a urinary bladder neoplasm of epithelial and mesenchymal origin and an epithelial tumor of the upper gastrointestinal tracts of ruminants. The complete description of the effects of bracken fern has been reviewed recently (Gil da Costa et al., 2012a).

Our previous studies showed that ptaquiloside is an immunosuppressor that caused a reduction in mouse splenic NK cell-mediated cytotoxicity and IFNγ production (Latorre et al., 2009). Moreover, we verified that selenium supplementation can prevent this reduction in NK-mediated cytotoxicity (Latorre et al., 2011). A greater incidence of chemical-induced preneoplasic lesions are noted in mice immunosuppressed with bracken fern (Caniceiro et al., 2011), and our findings may be of great relevance in avoiding the increased susceptibility to cancer caused by the plant.

The molecular mechanism underlying ptaquiloside-induced immunosuppression and its prevention by selenium are unknown. Thus, the objective of this study was to verify the mechanism of action of ptaquiloside-induced immunosuppression in splenic NK cells using gene expression microarray analysis. We performed transcriptome analysis in splenic NK cells from mice treated for 14 days with ptaquiloside (5.3 mg/kg) and/or selenium (1.3 mg/kg) to identify gene transcripts altered by ptaquiloside that could be linked to the immunosuppression and that would be prevented by selenium.

2. Materials and methods

2.1. Mice

Fifty eight sixty-day-old male C57BL/6J mice, bred in the Department of Pathology at the School of Veterinary Medicine and Animal Sciences, were used. The mice were housed in controlled temperature (22–25 °C), relative humidity (50–65%) and lighting (12 h/12 h light/dark cycle) conditions. Drinking water and standard diet (Nuvilab-CR1®, Nuvital Nutrientes LTDA) were provided *ad libitum*. All procedures were performed following the Guide for the Care and Use of Laboratory Animals NIH publication No. 85-23 (http://www.nap.edu/readingroom/books/labrats/) and were reviewed and approved by the Bioethics Committee of the FMVZ-USP (process #1061/2007).

2.2. Ptaquiloside

Ptaquiloside was purified from dried *P. aquilinum* crosiers using a previously described procedure (Oelrichs et al., 1995) that was later modified. In brief, ground plant material (100 g) was extracted using a Soxhlet apparatus with $CHCl_3$ (48 h) followed by a 1:1 mixture of $CHCl_3$/MeOH (48 h). The $CHCl_3$/MeOH extract was evaporated to dryness at 40 °C under reduced pressure (rotary evaporation). The residue was collected in H_2O (100 ml) and extracted twice with diethyl ether (100 ml) and then twice with n-butanol (100 ml). The n-butanol extract was concentrated under reduced pressure (rotary evaporator), and the residue was subjected to flash column chromatography [silica gel eluted using an EtOAc/MeOH gradient (0–12% MeOH)]. The collected fractions were monitored using apci-MS, and the fractions that contained ptaquiloside were combined and separated using flash chromatography with silica gel eluting with dichloromethane/MeOH (88/12). The fractions that contained ptaquiloside were combined and separated a final time using reverse phase HPLC (10 mm × 300 mm C18 column; gradient elution with H2O/MeOH; 30% MeOH – 95% MeOH for 20 min; UV detection at 220 nm). The purified ptaquiloside was assayed to be >98% using HPLC-apci-MS and NMR analysis. Ptaquiloside was used at a dose of 5.3 mg/kg for the *in vivo* experiments, as previously described (Latorre et al., 2011). For the *in vitro* studies, a concentration of 4.4 μg/ml of ptaquiloside was used. This concentration was determined by preliminary tests that demonstrated a reduction in NK cell cytotoxicity *in vitro*.

2.3. Selenium

Sodium selenite (Na_2SeO_3) (Labsynth, Brazil) was used as the source of selenium and will be described throughout this article as selenium. Importantly, none of the mice in this study were selenium deficient because they received standard diet (Nuvilab-CR1®, Nuvital Nutrientes LTDA) containing 0.05 ppm selenium. As in our previous work (Latorre et al., 2011), we used a dose of 1.3 mg/kg selenium for the *in vivo* experiments, based on the results of Albers et al. (2003), and a concentration of 0.1 mM for the *in vitro* studies. This concentration was determined by preliminary tests that demonstrated an increase in NK cell cytotoxicity *in vitro*.

2.4. In vivo experimental design

Mice were separated into four groups, with five mice per group, as follows: control (Co), ptaquiloside (Pt), ptaquiloside and selenium (PtSe), and selenium (Se). In general, experimental mice were treated by daily gavage for 14 days with ptaquiloside (5.3 mg/kg) and/or selenium (1.3 mg/kg). The Co mice received only water and were treated at the same time as the experimental mice. The body weight of each mouse was measured every 3 days for dose adjustment. On day 15 of the experiment, mice from all groups were killed with an overdose of CO_2 and splenic cell suspensions were then prepared to isolate NK cells (see below).

2.5. Isolation of splenic NK cells

Spleens were removed aseptically and made into a single-cell suspension. Briefly, for each mouse, the isolated spleen was gently squeezed by the distal end of a syringe into a plate of cold RPMI medium (Gibco). The erythrocytes present in the suspension were then lysed using sterile 0.4% ammonium chloride solution. Splenocytes were centrifuged at 1200 rpm (4 °C, 8 min), and the pelleted cells were then re-suspended in RPMI-complete medium (supplemented with 10% FBS, Gibco). To separate non-adherent from adherent cells, the samples were incubated on 6-well plates for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. Next, non-adherent cells were harvested and filtered through a 70 μm cell strainer. Untouched NK cells were isolated according to the manufacturer's protocol using an NK cell isolation kit, LS columns and a QuadroMACS cell separator system (Miltenyi Biotec, Inc.). The viability of the separated cells was verified using the Trypan Blue assay and was found to be 100%.

The percentage of splenic NK cell ($CD3^-$ $NK1.1^+$) recovery after the isolation procedure was evaluated using splenic cells from five mice that were processed with PE-labeled anti-CD3 (clone 17A2) and PerCP-Cy5.5-labeled anti-NK1.1 (clone PK136) antibodies (BD Pharmingen) in a FACSCalibur™ flow cytometer equipped with Cell Quest Pro® software (Becton Dickinson [BD] Immunocytometry System) and analyzed with FlowJo 7.2.6® software (Tree Star Inc, Ashland, KY) as demonstrated in Fig. 1A and B. The percentages of splenic NK cells presented in Fig. 1A and B represent the mean obtained from five mice.

2.6. RNA isolation and whole-genome gene expression profile

Isolated splenic NK cells from Co ($n = 5$), Pt ($n = 5$), PtSe ($n = 5$) and Se ($n = 5$) groups treated daily by gavage for 14 days were used. Total RNA was isolated with the RNAspin Mini RNA Isolation Kit and RNA integrity was assessed using a 2100 Bioanalyzer (Agilent). Double-stranded cDNA was synthesized from 200 ng total RNA using the Agilent One-Color Spike-Mix as positive controls and cDNA Master Mix (Agilent). cRNA was transcribed from the cDNA and labeled using the Quick Amp Labeling Kit (Agilent). Cyanine 3-labeled and amplified cRNA was purified using RNAspin mini columns (GE Healthcare) and the Cy3 concentration was evaluated using a NanoVue™ Plus spectrophotometer (GE Healthcare). Cy3-labeled cRNA (1.65 μg) was fragmented and hybridized to Whole Mouse Genome 4 × 44k arrays (Agilent) at 10 rpm/17 h at 65 °C. Hybridized arrays were washed with the Gene Expression Wash Buffer Kit (Agilent) and scanned using an Agilent Microarray scanner. Data were extracted using the Agilent Feature Extraction 9.5.3.1 software. Five slides with four arrays each (4 × 44k) were used, and one sample from each group (Co, Pt, PtSe and Se) was loaded onto each slide giving a total of five arrays per group.

2.7. Real-time quantitative PCR

Isolated splenic NK cells from Co ($n = 3$), Pt ($n = 3$), PtSe ($n = 4$) and Se ($n = 3$) groups treated daily by gavage for 14 days were used. Total RNA was extracted using an RNAspin Mini RNA Isolation Kit, following manufacturer's instructions and RNA integrity was assessed using a 2100 Bioanalyzer (Agilent). Real-time quantitative PCR of the *Mt2* gene and the reference *18s* gene was performed using the Verso™ 1-Step QRT-PCR Rox Kit (Thermo Scientific), following manufacturer's instructions, on the ABI Prism 7500 thermocycler (Applied Biosystems). Primers were designed using Primer-3 software (Rozen and Skaletsky, 2000) and were run in BLAST (Altschul et al., 1990) to verify the absence of local alignments with DNA or other RNA transcripts. The following primers were used: Mt2_F (CCGATCTCTCGTCGATCTTC), Mt2_R (GCAGGAAGTACATTTGCATTG), 18s_F (CCTGCGGCTTAATTTGACTC) and 18s_R (CTGTCAATCCTGTCCGTGTC). Finally, relative gene expression data were processed and analyzed according to Livak's method (Livak and Schmittgen, 2001).

2.8. In vitro experimental design

Splenic cell suspensions were prepared from six untreated mice, and non-adherent cells were separated as outlined above. Cultures of non-adherent cells were incubated on 24-well plates and treated as follows: untreated (Co), 4.4 μg/ml ptaquiloside (Pt), 4.4 μg/ml ptaquiloside + 0.1 mM selenium (co-incubation) (PtSe) and 0.1 mM selenium (Se). All treatments were incubated for 1 h at 37 °C in a humidified atmosphere with 5% CO_2. Following treatment, the cells were washed and

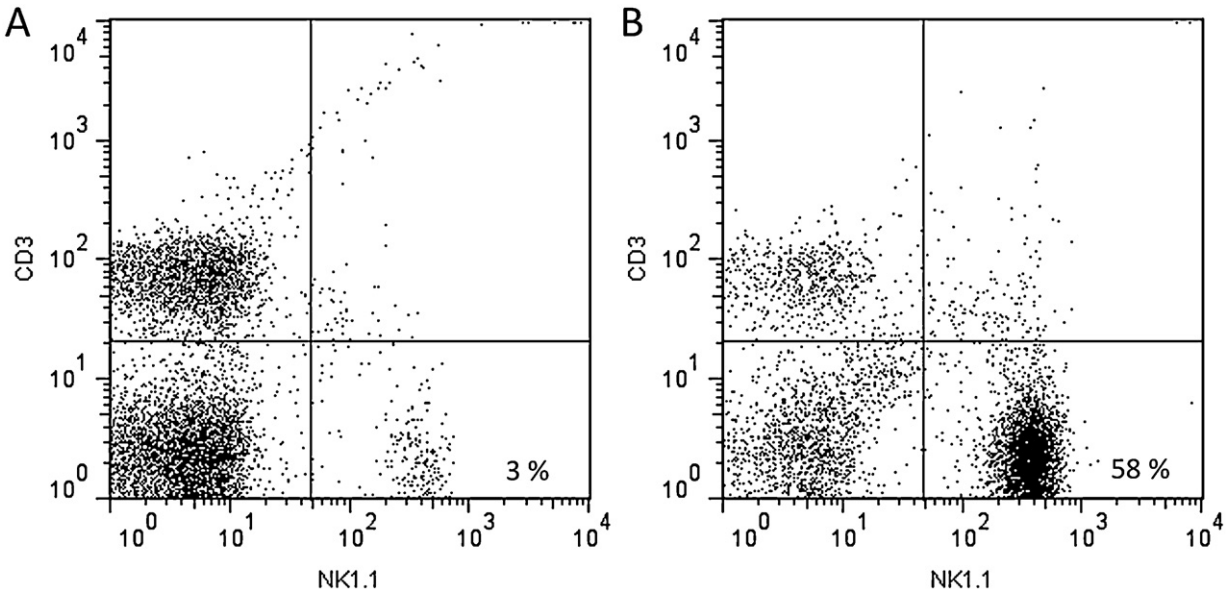

Fig. 1. Analysis of splenic NK cells recovery after the isolation procedure. (A) CD3 *vs*. NK1.1 dot plot showing the mean percentage of splenic NK cells ($CD3^-$ $NK1.1^+$) prior to the isolation procedure. (B) CD3 *vs*. NK1.1 dot plot showing the mean percentage of splenic NK cells ($CD3^-$ $NK1.1^+$) after the isolation procedure.

re-suspended in complete RPMI medium and then prepared for the detection and quantification of the proteins metallothionein 1 and 2 (Mt1 and Mt2) and free zinc (Zn^{2+}) as an indicator of their activities.

2.9. Detection and quantification of metallothionein 1 and 2 in splenic NK cells

Following *in vitro* treatment of cultures of non-adherent splenic cells, the cells were adjusted to 1×10^6 cells/50 μl and incubated with 0.5 μl Mouse BD Fc Block™ (clone 2.4G2, BD Pharmingen) for 5 min (to block the Fc-mediated adherence of antibodies) prior to staining with specific antibodies. These cells were then stained (simultaneously) for surface antigens (CD3 and NK1.1) for 30 min at 4 °C in the dark. The cells were then washed in 2 ml PBS, fixed and permeabilized with a Cytofix/Cytoperm Plus Kit (BD Biosciences) following the manufacturer's protocol. During the permeabilization step, the cells were stained intracellularly with the primary antibody (anti-metallothionein that cross reacts with Mt1 and Mt2, clone UC1MT, Abcam) for 30 min at 4 °C in the dark, then washed and stained with the secondary antibody (FITC-labeled goat polyclonal anti-mouse IgG, Abcam). Finally, the cells were washed free of unbound antibody and then resuspended in PBS for flow cytometry using a FACSCalibur™ flow cytometer equipped with Cell Quest Pro® software (Becton Dickinson [BD] Immunocytometry System). A total of 100,000 target cells were collected by flow cytometry, and the results were expressed as mean fluorescence intensity (MFI). Data analyses were performed with FlowJo 7.6.4® software (Tree Star Inc., Ashland, KY).

2.10. Quantification of free intracellular zinc in splenic NK cells

The free intracellular zinc concentration in NK cells was measured using the method proposed by Haase et al. (2006), with modifications. Following the *in vitro* treatments outlined above, the non-adherent cells were adjusted for 1×10^6 cells/well. FluoZin™-3 AM ester, dissolved in Pluronic® F-127 (1:1) (Molecular Probes), was then added to the cultures at a final concentration of 1 μM and the cells were incubated at 37 °C in a humidified atmosphere at 5% CO_2 for 30 min. The cells were then washed in PBS (5 min, 2000 rpm) and incubated with 0.5 μl Mouse BD Fc Block for 5 min (to block the Fc-mediated adherence of antibodies) prior to staining with specific antibodies. The cells were then stained (simultaneously) for surface antigens (CD3 and NK1.1) for 30 min at room temperature in the dark. Finally, the cells were washed free of unbound antibody and resuspended in PBS for flow cytometry using a FACSCalibur™ flow cytometer equipped with Cell Quest Pro® software (Becton Dickinson [BD] Immunocytometry System). A total of 100,000 target cells were collected by the flow cytometer, and the results were expressed as the mean fluorescence intensity (MFI). Data analyses were performed using FlowJo 7.6.4® software (Tree Star Inc, Ashland, KY). The concentration of intracellular labile zinc in nM, was calculated from the mean fluorescence intensity using the formula $[Zn^{2+}] = Kd \times [(F - F_{min})/(F_{max} - F)]$, where, as specified by manufacturer, the dissociation constant of FluoZin™-3 AM ester–zinc complex was 15 nM. F_{min} and F_{max} were determined using non-adherent splenic cells from a separate group of 4 mice. To determine F_{min}, the zinc specific chelator TPEN [100 μM] (Sigma) was added simultaneously with FluoZin™-3 AM ester, and to determine F_{max}, the ionophore Pyrithione [50 μM] (Sigma), plus $ZnCl_2$ [100 μM] (Sigma), was added simultaneously with FluoZin™-3 AM ester (data not shown).

2.11. Induction of overexpression of metallothionein 2 in the non-adherent splenic cells and the determination of its role in NK activity

Splenic cell suspensions were prepared from three untreated mice, and non-adherent cells were separated as outlined above. Briefly, 5×10^5 cells suspended in OptiMEM I (Invitrogen) were incubated with or without 0.2 μg of TrueORF™ vector containing a *Mus musculus* Mt2 cDNA (OriGene) mixture with 0.5 μl Lipofectamine (Invitrogen) per well at 37 °C in a humidified atmosphere at 5% CO_2, following the manufacturer's instructions. Six hours after incubation, the culture medium was replaced with RPMI supplemented with 10% FBS. Twenty-four hours after incubation, the cells were fixed and permeabilized using a Cytofix/Cytoperm Plus Kit and then stained intracellularly with the primary antibody anti-Myc (clone 9E10, OriGene) and with the secondary antibody PerCP-labeled rat anti-mouse IgG1 (clone X56, BD Pharmingen) for detection of the recombinant protein Mt2 containing Myc as an epitope (Supplementary Fig. S1). Next, splenic cell suspensions were prepared from the other six untreated mice, and the non-adherent cells were incubated or not with the TrueORF™ vector containing *M. musculus* Mt2 cDNA (OriGene) as described above. To verify the effect of overexpression of Mt in the NK cells, we quantified the free intracellular concentration of zinc after 24 h of incubation as described above. Furthermore, to evaluate the NK cytotoxicity (effector cell), we co-incubated these cells with the YAC-1 mouse lymphoma cell line as a target, as previously described (Latorre et al., 2011). Briefly, triplicate cell cultures from each treatment were incubated with 5×10^5 effector cells and 5×10^3 target cells stained with CFSE (ratio 100:1) for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2. The spontaneous death rate was determined by incubating YAC-1 cells alone in complete RPMI medium. Propidium iodide (PI) was then added, and the samples were acquired using flow cytometry. Overall, 5000 target cells were collected by flow cytometry (FACSCalibur™). The data were analyzed using FlowJo 7.6.4® software. The level of NK cell cytotoxicity was expressed as follows:

$$\text{Cytotoxicity}\,(\%) = \frac{\text{dead targets in samples}\,(\%) - \text{spontaneously dead targets}}{100 - \text{spontaneously dead targets}\,(\%)} \times 100$$

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2012.12.010.

2.12. Dichlorofluorescin oxidation assay

Reactive oxygen species (ROS) in NK cells were measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as proposed by Jyothi and Khar (1999), with modifications. Non-adherent splenic cells were isolated from a group of 6 untreated mice and treated *in vitro* as outlined above, but with different time intervals 15, 30, 60 and 120 min. These cells were adjusted to 1×10^6 cells/well and DCFH-DA (Sigma) was added to the cultures at a final concentration of 60 μM and the cells were then incubated at 37 °C for 30 min. The cells were then washed in PBS at 4 °C (5 min, 2000 rpm) and incubated with 0.5 μl Mouse BD Fc Block for 5 min (to block the Fc-mediated adherence of antibodies) prior to staining with specific antibodies.

The cells were then stained (simultaneously) for surface antigens (CD3 and NK1.1) for 30 min at 4 °C in the dark. Finally, the cells were washed free of unbound antibody and resuspended in PBS at 4 °C for flow cytometry using a FACSCalibur™ flow cytometer equipped with Cell Quest Pro® software (Becton Dickinson [BD] Immunocytometry System). A total of 100,000 target cells were collected by the flow cytometer, and the results were expressed as the mean fluorescence intensity (MFI). Data analyses were performed using FlowJo 7.6.4® software (Tree Star Inc., Ashland, KY).

2.13. Gene expression microarray analysis

The probe-level data from the gene expression microarray experiments were preprocessed using log2 transformation to mitigate the significant differences between them, preserving the small intensity variations and to soften the noise inherent in the data acquisition process. Next, box plots were used to verify the distribution of the data, and we observed that animals Co1 and Pt4 presented with many outliers. We substituted data from these mice with the mean of other mice from the same treatment group. Gene expression analysis was performed as previously described by Cui and Churchill (2003); thus, Student's *t*-tests were used to compared expression data between Pt-treated and Co mice, Se-treated and Co mice and PtSe-treated and Co mice. The *p* values for all comparisons were adjusted using a false discovery rate (FDR). A fold change of ±2.0 and an FDR corrected *p* value < 0.05 (FDR < 0.05) were used as the criteria for determining statistical significance using the Matlab's Bioinformatics Toolbox (http://www.mathworks.com/products/bioinfo/description3.html). The gene expression data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30629).

2.14. Gene ontology analysis

The statistically significant transcripts from all comparisons were uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource (http://david.abcc.ncifcrf.gov/home.jsp) where the Functional Annotation Clustering tool was applied to generate clusters of overrepresented Gene Ontology (GO) terms (Huang et al., 2009).

2.15. Statistical analyses

The data were analyzed in GraphPad Prism 5.00® software (GraphPad Software, Inc., San Diego, CA) using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons or Student's *t*-tests to compare two groups. Non-parametric data were compared using the Kruskal–Wallis test followed by Dunn's test, and percent data from two groups were compared using the Mann–Whitney test. Data are expressed as the mean ± SD, or as the median with range, and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Gene expression changes in splenic NK cells from mice treated with ptaquiloside and/or selenium

We performed transcriptome analysis on isolated splenic NK cells from 20 mice (5 mice/group) that were treated daily by gavage for 14 days with ptaquiloside and/or selenium to identify transcripts that were associated with ptaquiloside-induced immunosuppression. The gene expression profiles from the Pt, PtSe and Se experimental groups, compared with the control group, showed 872, 302 and 489 altered genes, respectively (Table 1). Of the up-regulated gene transcripts from all experimental groups, 123 were mapped to biological processes, from which we highlighted five, although, as shown in Fig. 2, none had high enrichment scores (≥1.3). The Pt and Se groups showed a very different pattern of distribution of differentially expressed genes in these 5 biological processes, whereas no particular biological process was favored in the PtSe group. The corresponding genes for each enriched GO term are listed in Table 2 and in Supplementary Table 1. When considering gene transcripts that could be related to the immunosuppressive effects of ptaquiloside, we did not find genes directly related to this effect, but selected the genes metallothionein 1 (Mt1) and metallothionein 2 (Mt2), which are involved in cellular ion homeostasis and act as zinc regulator that is essential to normal immune function. Ptaquiloside treatment increased the expression of these gene transcripts 2.9-fold ($p < 0.001758$) and 3.0-fold ($p < 0.025148$) compared with the control group, respectively.

Table 1
Significantly altered genes in splenic NK cells from mice treated daily by gavage, for 14 days, with ptaquiloside (5.3 mg/kg) and/or selenium (1.3 mg/kg).

Treatment comparison	No. of up-regulated genes	No. of down-regulated genes	Total
Ptaquiloside *vs.* control	77	795	872
Ptaquiloside + selenium *vs.* control	47	255	302
Selenium *vs.* control	30	459	489

Transcripts showing > 2 fold change and $p < 0.05$ compared with the control group.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2012.12.010.

Of the down-regulated gene transcripts, 1174 were mapped to a wide range of biological processes. Transcripts with enrichment scores ≥1.3 for at least one of these groups are presented in Fig. 3. These data showed no specific biological process that was associated with ptaquiloside-induced immunosuppression in NK cells. The corresponding genes for each enriched GO term are listed in Supplementary Table 2.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2012.12.010.

The differential gene expression detected by microarray analysis was validated using real-time PCR with RNA samples isolated from splenic NK cells from 13 separate similarly treated mice (at least 3 mice/group). The gene Mt2 that was up regulated in the Pt

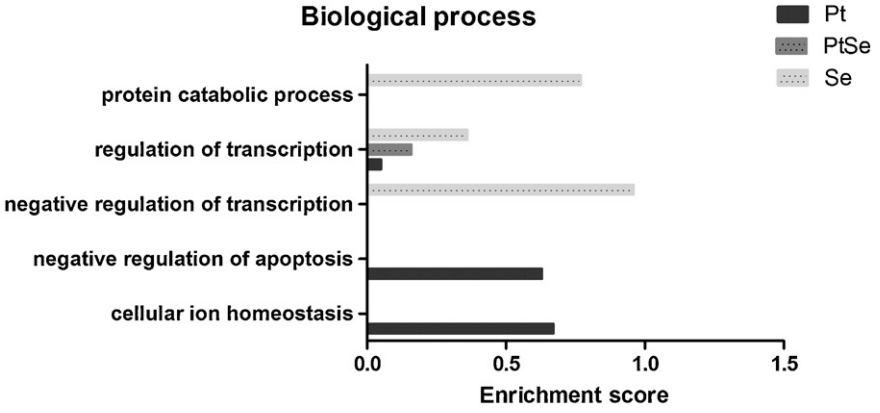

Fig. 2. The distribution of biological processes in the up-regulated gene transcripts from the experimental groups *vs.* the control group. One hundred and twenty three of 154 genes were mapped to five biological processes.

Table 2
The GO terms are over-represented among the genes whose expression was up regulated in the experimental groups *vs.* the control group. Each category biological process (GOTERM_BP_FAT) is represented by at least 3 genes.

Term	Pt	PtSe	Se
	Gene Symbol		
GO:0006873~cellular ion homeostasis	Mt1		
	Mt2		
	Olig2		
GO:0043066~negative regulation of apoptosis	Sycp3		
	Tsc22d3		
	Xrcc2		
GO:0045449~regulation of transcription	Ahrr	Ahrr	Ahrr
	Egr4	9030624G23Rik	
	Hic1	Hic1	Hic1
		Nrg1	Nrg1
	Olig2		Olig2
	Tsc22d3	Tfb1m	Zfp54
GO:0016481~negative regulation of transcription			Ahrr
			Nrg1
			Olig2
GO:0030163~protein catabolic process			Cacul1
			Dtl
			Gm6206

Genes whose expression was up regulated in 2 experimental groups at least are highlighted. Gene name and their GenBank accession number: Ahrr – aryl-hydrocarbon receptor repressor (NM_009644.2); Cacul1 – CDK2 associated, cullin domain 1 (NM_001172096.1); Dtl – denticleless homolog (Drosophila) (NM_029766.2); Egr4 – early growth response 4 (NM_020596.2); Gm6206 – predicted pseudogene 6206 (NC_000086.7); Hic1 – hypermethylated in cancer 1 (NM_001098203.1); Mt1 – metallothionein 1 (NM_013602.3); Mt2 – metallothionein 2 (NM_008630.2); Nrg1 – neuregulin 1 (NM_178591.2); Olig2 – oligodendrocyte transcription factor 2 (NM_016967.2); Sycp3 – synaptonemal complex protein 3 (NM_011517.2); Tfb1m – transcription factor B1, mitochondrial (NM_146074.1); Tsc22d3 – TSC22 domain family, member 3 (NM_001077364.1); Xrcc2 – X-ray repair complementing defective repair in Chinese hamster cells 2 (NM_020570.2); Zfp54 – zinc finger protein 54 (NM_011760.2); 9030624G23Rik – RIKEN cDNA 9030624G23 gene (NM_001256489.1).

group above was selected for validation. A significant difference was detected by real-time PCR analysis, as shown in Fig. 4.

3.2. Phenotypic confirmation of higher expression of metallothionein 1 and 2 induced by ptaquiloside in NK cells

To verify whether ptaquiloside also increases metallothionein 1 and 2 translation in NK cells, we incubated non-adherent splenic cells from six mice treated with ptaquiloside [4.4 μg/ml] and/or selenium [0.1 mM] *in vitro* for 1 h and then stained for surface antigens (CD3 and NK1.1) and intracellular metallothionein 1 and 2 (Mt). Unsurprisingly a higher intensity of expression of Mt 1 and 2 was observed in NK cells when they were treated with ptaquiloside (ANOVA, $p = 0.04$; $p < 0.05$, Co *vs.* Pt Dunnett's post-test, Fig. 5). In addition, PtSe group did not statistically differ from the Co group and did not contain the increase in Mt 1 and 2 observed in the Pt group.

3.3. Activity of metallothionein 1 and 2 in splenic NK cells

Because metallothionein 1 and 2 (Mt1 and Mt2) act as zinc regulators and the levels of free zinc correlate with its capacity to bind zinc ions, we measured the levels of free zinc ions in the NK cells to evaluate the activity of Mt1 and Mt2. For that, non-adherent splenic cells from the same six mice as used for analysis of Mt1 and Mt2 expressions (above) were used. Cells were treated with ptaquiloside [4.4 μg/ml] and/or selenium [0.1 mM] *in vitro* for 1 h and then stained for surface antigens (CD3 and NK1.1) and intracellular free zinc (Zn^{2+}, using FluoZin^{TM}-3 AM) because Mt1 and Mt2 are involved in the control of intracellular zinc homeostasis. As expected, we observed diminished intracellular Zn^{2+} in NK cells treated with ptaquiloside when compared with control-treated cells ($p = 0.0113$, Co *vs.* Pt, Student's *t*-test) and an increase in intracellular Zn^{2+} in cells co-treated with ptaquiloside and selenium compared with cells treated with ptaquiloside only

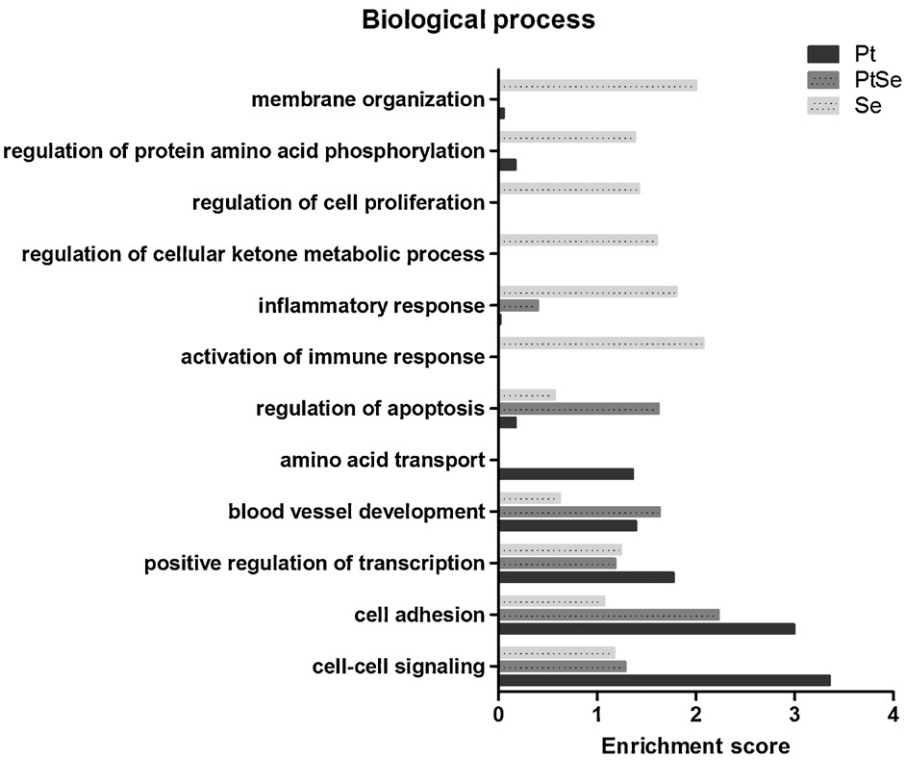

Fig. 3. The distribution of biological processes in the down-regulated gene transcripts from the experimental groups *vs.* the control group. One thousand one hundred and seventy four of 1509 transcripts were mapped to a wide range of biological processes; however, only transcripts with enrichment scores ≥1.3 for at least one of the groups are presented.

(Kruskal–Wallis, $p = 0.0044$; Dunn's post-test, $p < 0.01$, Pt *vs*. PtSe) (Fig. 6).

3.4. Overexpression of metallothionein 2 and its role in splenic NK cells

To verify whether the overexpression of metallothionein 2 could reduce the levels of free zinc ions in NK cells, we transfected these cells with a vector containing *M. musculus* Mt2 cDNA. Non-adherent splenic cells from six separated untreated mice were used. The cells were incubated with or without TrueORF^{TM} vector containing *M. musculus* Mt2 cDNA and then stained for surface antigens (CD3 and NK1.1) and intracellular free zinc (Zn^{2+}, using FluoZin^{TM}-3 AM). We then co-incubated these cells with YAC-1 (target cells) to verify the NK cytotoxicity. As expected, we observed diminished intracellular Zn^{2+} in the NK cells that overexpressed Mt2 ($p = 0.0343$, Student's *t*-test) and a consequently reduced NK cytotoxicity compared with those of the cells not overexpressing Mt2 ($p = 0.0260$, Mann–Whitney test) (Fig. 7A and B).

3.5. Intracellular ROS in splenic NK cells

Regarding several redox signals that can release zinc from metallothionein (Mt) and consequently increase the Mt expression to neutralize their oxidant activities, we evaluated whether ptaquiloside treatment increased the reactive oxygen species (ROS) in NK cells. For that, we used non-adherent splenic cells from six separate untreated mice. These cells were treated with

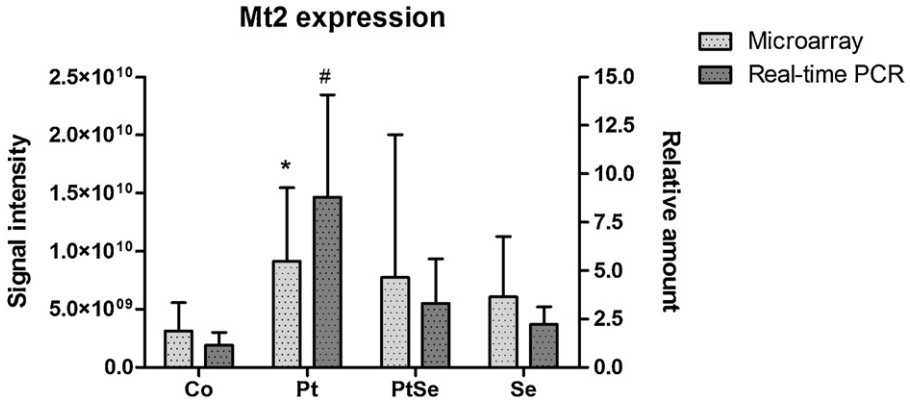

Fig. 4. Ptaquiloside treatment increases Mt2 expression in splenic NK cells that was inhibited by selenium co-treatment. Splenic NK cells were isolated from mice treated with ptaquiloside (5.3 mg/kg) and/or selenium (1.3 mg/kg) by daily gavage for 14 days. The differential gene expression detected using microarray analysis (5 mice/group) was validated by real-time PCR (at least 3 mice/group) using RNA samples isolated from splenic NK cells from independent experiments ($^{*}p < 0.025148$ *vs*. Co, Student's *t*-test; $^{\#}p < 0.0408$ *vs.* Co, Dunnett's post-test). Data are presented as mean ± SD.

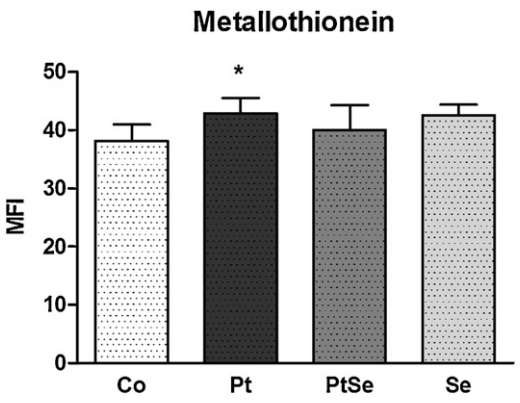

Fig. 5. Metallothionein 1 and 2 expressions in splenic NK cells treated with ptaquiloside and/or selenium for 1 h *in vitro*. We observed increased Mt1 and Mt2 expressions in NK cells treated with ptaquiloside (*$p < 0.05$, Dunnett's post-test). Data are presented as mean fluorescence intensity (MFI) ± SD, $n = 6$.

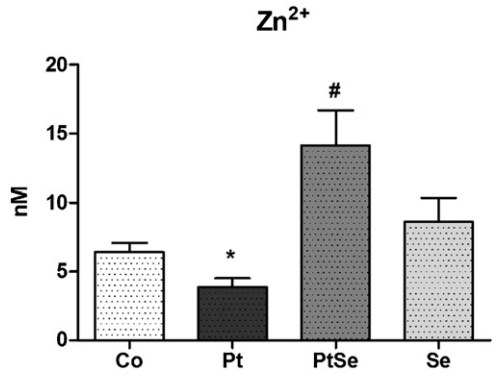

Fig. 6. The concentration of free intracellular zinc in splenic NK cells following treatment with ptaquiloside and/or selenium for 1 h *in vitro*. Note the reduction in zinc level in NK cells treated with ptaquiloside (*$p = 0.0113$, Co *vs.* Pt, Student's *t*-test) and the increase in zinc in cells co-treated with ptaquiloside and selenium (#$p < 0.01$, Pt *vs.* PtSe, Dunn's post-test). Data represent the mean and error of two independent experiments, ($n = 6$ mice/group/experiment).

ptaquiloside [4.4 μg/ml] and/or selenium [0.1 mM] *in vitro* for 15, 30, 60 or 120 min and then incubated with DCFH-DA to detect ROS. The cells were then stained for surface antigens (CD3 and NK1.1). We observed a significant reduction in DCF fluorescence in NK cells treated with selenium for 60 and 120 min compared with control-treated cells (Two-way ANOVA, $p = 0.0289$; Bonferroni post-test (60 min): Co *vs.* PtSe, $p < 0.001$; Co *vs.* Se, $p < 0.01$; Bonferroni post-test (120 min): Co *vs.* PtSe, $p < 0.01$; Co *vs.* Se, $p < 0.001$), but we did not observe any difference in cells treated only with ptaquiloside compared with control treated cells (Supplementary Fig. S2).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2012.12.010.

4. Discussion

Our findings showed for the first time that ptaquiloside-mediated immunosuppressive effects in splenic NK cells were associated with enhanced metallothionein expression that culminated in reduced free intracellular zinc. Moreover, we demonstrated that selenium co-treatment abolished these alterations in NK cells. These data corroborated our previous results, which revealed that selenium prevented and reversed ptaquiloside-induced immunosuppression (Latorre et al., 2011).

Ptaquiloside is known to cause DNA damage by acting as DNA-alkylating agent (Yamada et al., 2007). Previous studies have shown chromosomal aberrations in the lymphocytes of cows and humans who had consumed bracken fern (Lioi et al., 2004; Recouso et al., 2003), as well as in lymphocytes that had been treated *in vitro* with ptaquiloside (Gil da Costa et al., 2012b). This genotoxic effect might be responsible for the increased expression of genes associated with DNA damage repair and the negative regulation of apoptosis, such as Tsc22d3, Sycp3 and Xrcc2, observed in the splenic NK cells of mice treated with ptaquiloside (Table 2). Tsc22d3 has already been demonstrated to be expressed in splenic lymphocytes and is able to inhibit T cell apoptosis induced by treatment with anti-CD3 MAb (D'Adamio et al., 1997). The overexpression of Sycp3 in tumor cells was shown to activate the Akt pathway and increase the expression of antiapoptotic proteins (Kang et al., 2010). However, *Sycp3*$^{-/-}$ oocytes showed the inefficient repair of DNA double-strand breaks (Wang and Hoog, 2006) and deficient expression of Xrcc2 (which is important in DNA repair by homologous recombination), causing centrosome disruption and consequent mitotic catastrophe (Cappelli et al., 2011). These results confirmed the role of these genes in DNA damage repair.

Other noteworthy up-regulated genes following ptaquiloside administration in splenic NK cells included Mt1 and Mt2, which are members of the metallothionein family and can be indirectly related to the immunosuppressive effect of ptaquiloside. Metallothioneins are a family of small cysteine rich proteins that have a range of functions, including toxic metal detoxification and protection against oxidative stress, and with regard to their role in metal ion homeostasis, they can bind up to seven zinc ions and act as a zinc regulator (Sutherland and Stillman, 2011). In this manner, the cellular availability of free zinc ions correlates with the redox state of metallothioneins and their capacity to bind zinc ions (Maret, 2008). In this paper, we showed that ptaquiloside treatment increased

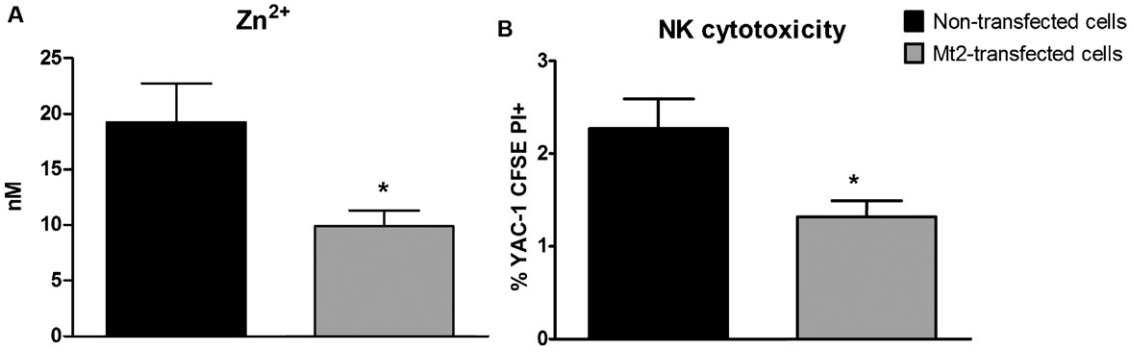

Fig. 7. Overexpression of Mt2 and its role in splenic NK cells. (A) Note the reduction in the free zinc level in the Mt2-transfected NK cells (*$p = 0.0343$, Student's *t*-test). (B) The NK cytotoxicity is also reduced in the cells overexpressing Mt2 (*$p = 0.0260$, Mann–Whitney test). The data represent the mean and the error, $n = 6$.

transcription and translation of metallothionein 1 and 2 in NK cells (Figs. 4 and 5) and reduced the concentration of free intracellular zinc ions (Fig. 6). Because zinc is essential for normal function of the immune system and decreased zinc levels have already been associated with impaired activity of different immune cells, including NK cells (Ibs and Rink, 2003), it is possible that the reduction in zinc levels observed here was the cause of the diminished NK cytotoxicity caused by ptaquiloside. In fact, this hypothesis was confirmed by the fact that overexpression of metallothionein 2 was induced by the transfection of *M. musculus* Mt2 cDNA in non-adherent splenocytes. The NK cells presented a reduction in the free intracellular concentration of zinc and a consequently diminished cytotoxicity (Fig. 7A and B).

In addition, we observed that selenium inhibited the higher expression of metallothionein (Fig. 5) and increased the free zinc concentration in NK cells co-treated with ptaquiloside (Fig. 6). Selenium compounds act as oxidants even in the reducing environment of the cytosol, and they react rapidly with zinc–sulfur clusters of metallothioneins to induce prompt release of zinc (Jacob et al., 1999). Therefore, NK activity can be recovered following selenium treatment even in the presence of ptaquiloside, due to the selenium-mediated increase in zinc level.

The mechanism underlying ptaquiloside-induced metallothionein expression in NK cells remains unknown. Considering metallothionein acts as an antioxidant, we could speculate that ptaquiloside treatment increases reactive oxygen species (ROS) in NK cells, which elevates metallothionein expression to effectively neutralize ROS activity (Sutherland and Stillman, 2011). However, the results of DCFH-DA oxidation assay rejected this hypothesis because the DCF fluorescence was not increased in the NK cells that were treated with ptaquiloside.

In conclusion, our findings are of vital importance because they could help avoid the higher susceptibility to develop cancer induced by the immunosuppressive effects of *P. aquilinum* that were revealed in our previous report (Caniceiro et al., 2011). Furthermore, selenium supplementation might help prevent some of toxic effects of ptaquiloside in humans who have been exposed directly or indirectly to it in areas infested by bracken fern, where the animal source foods, water and air are likely to contain ptaquiloside.

In summary, these results show for the first time that ptaquiloside-induced immunosuppression is associated with increased expression of metallothionein in NK cells and that selenium inhibited this alteration.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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