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Anti-inflammatory effects of zinc and alterations in zinc transporter mRNA in mouse models of allergic inflammation

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Lang C, Murgia C, Leong M, Tan L-W, Perozzi G, Knight D, Ruffin R, Zalewski P. Anti-inflammatory effects of zinc and alterations in zinc transporter mRNA in mouse models of allergic inflammation. *Am J Physiol Lung Cell Mol Physiol* 292: L577–L584, 2007. First published November 3, 2006; doi:10.1152/ajplung.00280.2006.—There is clinical evidence linking asthma with the trace element, zinc (Zn). Using a mouse model of allergic inflammation, we have previously shown that labile Zn decreases in inflamed airway epithelium (Truong-Tran AQ, Ruffin RE, Foster PS, Koskinen AM, Coyle P, Philcox JC, Rofo AM, Zalewski PD. *Am J Respir Cell Mol Biol* 27: 286–296, 2002). Moreover, mild nutritional Zn deficiency worsens lung function. Recently, a number of proteins belonging to the Solute Carrier Family 39 (ZIP) and Solute Carrier Family 30 (ZnT) have been identified that bind Zn and regulate Zn homeostasis. Mice were sensitized, and subsequently aerochallenged, with ovalbumin to induce acute and chronic airway inflammation. Mice received 0, 54, or 100 µg of Zn intraperitoneally. Tissues were analyzed for Zn content and histopathology. Inflammatory cells were counted in bronchoalveolar lavage fluid. Cytokine and Zn transporter mRNA levels were determined by cDNA gene array and/or real-time PCR. Zn supplementation decreased bronchoalveolar lavage fluid eosinophils by 40 and 80%, and lymphocytes by 55 and 66%, in the acute and chronic models, respectively. Alterations in Zn transporter expression were observed during acute inflammation, including increases in ZIP1 and ZIP14 and decreases in ZIP4 and ZnT4. Zn supplementation normalized ZIP1 and ZIP14, but it did not affect mRNA levels of cytokines or their receptors. Our results indicate that inflammation-induced alterations in Zn transporter gene expression are directed toward increasing Zn uptake. Increases in Zn uptake may be needed to counteract the local loss of Zn in the airway and to meet an increased demand for Zn-dependent proteins. The reduction of inflammatory cells by Zn in the airways provides support for Zn supplementation trials in human asthmatic individuals.

asthma; airway; lung

TO IMPROVE OUR UNDERSTANDING of the pathogenic mechanisms underlying asthma, it is appropriate to identify intrinsic factors that may influence airway inflammation. One such potential factor is the trace element, zinc (Zn). There is growing evidence linking asthma and Zn deficiency. Case-control studies in Scotland (33) and Spain (7) found that the combined risk of atopy, bronchial reactivity, and allergic-type symptoms were

four- to five-fold higher in individuals with low Zn intake compared with those with higher Zn intakes. Significant decreases in serum, plasma, or hair Zn levels have also been reported in some asthmatic individuals [reviewed in Murgia et al. (25)]. In addition, a negative relationship between wheezing and serum Zn-to-Cu ratio has been observed (31). Both asthma (12) and Zn deficiency (28) are thought to favor the T-helper-2 (Th2) inflammatory cytokine profile, and asthmatic individuals with systemic Zn deficiency may have further increases in Th2 proinflammatory cytokines.

Within cells, Zn occurs either tightly bound to metalloproteins or in more dynamic, free or loosely bound (labile) pools that are most susceptible to depletion in Zn deficiency (34). Labile Zn plays a role in general metabolism and gene expression, as well as in the regulation of secretion, programmed cell death (apoptosis), and signal transduction (36). Airway epithelial cells contain a high content of labile Zn, which is concentrated in the apical cytoplasm, just beneath the cilia (38). This region contains a number of Zn-dependent enzymes, including procaspase-3 (4), Cu-Zn-superoxide dismutase (Cu-Zn-SOD) (4), and the metalloproteinase, ADAM33 (14). Depletion of labile Zn renders airway epithelial cells highly susceptible to apoptosis induced by oxidants (4) or by the Fas death receptor pathway (2).

Features of human asthma can be replicated in animal models of allergic airway inflammation. In an acute model, Balb/c mice that have been sensitized, and then aerochallenged, with ovalbumin (OVA) develop a pronounced airway eosinophilia associated with airway hyperresponsiveness, mucous hyperplasia, damage to the airway epithelium, and collagen deposition (10). Chronic models have also been described in which smooth muscle hyperplasia and certain other features of airway remodelling are evident [reviewed in Fulkerson et al. (11)]. Previously, our laboratory has observed a marked loss of labile Zn in the airway epithelium of mice with acute airway inflammation (37). Moreover, using an acute model of allergic inflammation, mice fed moderately low (14 ppm) Zn diets were found to have significantly higher blood and airway eosinophilia (1.6-fold), more airway mucus cells (1.9-fold), and greater irritability to the bronchoconstrictor methacholine (up to 1.3-fold) than mice fed normal Zn (50 ppm) diets (37).

Zn homeostasis is achieved by the concerted actions of two families of proteins: Solute Carrier Family 39, which com-

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prises the ZRT/IRT-related (ZIP) proteins, are primarily involved in Zn uptake; Solute Carrier Family 30, which comprises the mammalian Zn transporter (ZnT) proteins, mediate intracellular Zn trafficking and Zn efflux (17). In addition, metallothioneins (MTs) participate in intracellular Zn homeostasis by binding Zn and mediating its transfer to cytosolic metalloenzymes (21). With the exception of MTs, studies concerning the characterization and expression of Zn transporters in the lung have been scarce. Here, we describe, for the first time, a study of Zn transporter gene expression in the lungs of mice before and after induction of allergic inflammation. In addition, given our laboratory's previous findings that Zn deficiency worsens allergic inflammation (37), we investigated whether Zn supplementation had beneficial effects on eosinophilia, airway mucus cells, and other histopathological features of allergic inflammation. Since inflammatory cells are recruited to the lung by specific chemokines and cytokines, including eotaxin, macrophage inflammatory proteins, and monocyte chemotactic proteins, we tested whether Zn supplementation affects the transcript levels of inflammatory mediators, as well as their receptors. The expression of MTs was also studied.

METHODS

Reagents

Unless otherwise mentioned, all reagents were purchased from Sigma-Aldrich Chemicals (St. Louis, MO), and reagents for RNA manipulation and reverse transcription were purchased from Qiagen (Clifton Hill, Victoria, Australia). Zn-free buffers were prepared as previously described (39).

Animals

Female Balb/c mice (aged 4–6 wk; pathogen free) were purchased from the University of Adelaide (Adelaide, Australia). Mice were fed a standard rodent chow (Joint Stock II Feed, Ridley Agricultural Products, Murray Bridge, South Australia, Australia), which contains 95 mg Zn/kg. For tissue collection, mice were anesthetized by an intraperitoneal overdose of pentobarbital sodium (50 mg/kg). All experiments were performed under the University of Adelaide and North Western Adelaide Health Service Animal Ethics Committees (M-54-2001/N-19-2001).

Analysis of Zn Transporter Gene Expression in Mouse Lung

Lung tissue was stabilized in RNeasy lysis buffer. RNA was isolated using an RNeasy minikit and digested with DNase, as per kit instructions. Equal amounts of RNA were pooled from the lungs of four to six mice within treatment groups as previously described (3, 24). In some cases, gene expression was also confirmed using RNA from individual mice (e.g., ZnT4, eotaxin, MT-I) (data not shown). cDNA was prepared from 800 ng of total RNA using an Omniscript RT kit with random decamers (1 μ M) and oligo(dT) primers (10 μ M). A one-eighth volume of the RT reaction was amplified using QuantiTect SYBRgreen PCR Kits. Gene sequences were obtained from GenBank, and primers were designed using the Web-based software Primer3 (30) and purchased from Geneworks (GeneWorks, Hindmarsh, South Australia, Australia). Accession numbers and primer sequences are provided as supplementary information in Table S1 (available online at the *American Journal of Physiology Lung Cell Molecular Physiology* Web site). The real-time assays were performed using a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as the housekeeping gene because its expression was more consistent than β_2 -microglobulin and GAPDH between treatment groups. Tissues isolated from mouse

brain, liver, and pancreas were used as positive controls. A sample without reverse transcriptase enzyme was included to check for genomic DNA contamination. Reaction for each primer pair was optimized with standard curves. The cycle thresholds (Ct) were obtained for test genes and for HPRT, and their differences were calculated (Δ Ct). Δ Ct values were transformed into absolute values and expressed relative to HPRT as described by Applied Biosystems (User Bulletin no. 2ABI).

Zn Supplementation and Induction of Allergic Airway Inflammation

Acute model. Mice were supplemented with 0, 54, or 100 μ g actual Zn (in the form of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) in 0.9% saline by intraperitoneal injection, twice a week, from days 0 to 33 or 36. Allergic airway inflammation was induced as previously described (37). Mice were killed over 2 days, on the day following their last nebulization.

Chronic model. Chronic allergic inflammation was induced according to McMillan et al. (22). Here, allergic inflammation was induced before Zn supplementation as per the acute model (days 0–34). On day 36, mice were split into four groups ($n = 6$) for Zn supplementation. From day 34 to day 52, mice were supplemented with either 0 or 54 μ g Zn twice a week and nebulized with either 0.9% saline (Sal-treated mice) or 1% OVA in 0.9% saline (OVA-treated mice) for 20 min, once a day (22). Body weights of the mice were measured every second or third day for the duration of the two protocols. On the day following the last OVA nebulization, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50–150 mg/kg body wt) before tissue collection.

Tissue Collection

Blood for serum Zn measurements was collected by cardiac puncture. The trachea was cannulated, and the lungs were lavaged with 1 ml of ice-cold Zn-free Hanks' balanced salt solution (HBSS; pH 7.4). Bronchoalveolar lavage fluid (BAL) was centrifuged (800 g, 5 min, 4°C), the cell pellet resuspended in 500 μ l of HBSS and retained for differential cell counts. Lung, liver, and spleen tissues were snap-frozen in liquid N_2 for Zn measurements or placed in RNAlater for RNA studies. Some lungs were inflated with 1 ml of 10% phosphate-buffered formalin (Sigma-Aldrich) for histological studies.

Measurement of Zn in Body Fluids and Tissues

Labile Zn in serum and cell-free BAL fluid was measured using a Zinquin fluorometric assay as previously described (39). Total Zn in the liver, lung, and spleen were determined by flame atomic absorption spectroscopy (303 atomic absorption spectrophotometer, Perkin-Elmer), as previously described (37).

Inflammatory Cell Infiltration into BAL Fluid

Total BAL cell viability was assessed by the exclusion of the vital dye, trypan blue (0.05%). Cell suspensions of 1×10^6 cells/ml were spun onto cytospin slides and stained with May Grunwald (Sigma-Aldrich). A differential cell count of 200 cells was performed in duplicates by two examiners, who were blinded to the treatment groups, using standard morphologic criteria.

Lung Histology

Paraffin-embedded sections were processed using standard procedures by staff at the Institute of Medical and Veterinary Science (Queen Elizabeth Hospital, South Australia, Australia). Sections were stained with hematoxylin and eosin for measurement of airway epithelial thickness, alcian blue/periodic acid-Schiff (PAS) for the identification of mucin-secreting cells, and Martius scarlet blue for identification of collagen. At least 10 images were captured from duplicate slides using a closed-circuit television video color camera mounted on an inverted Nikon TE-300 ($\times 20$ objective). Collagen deposition and airway epithelial thickness were measured by image

analysis (16) using VideoPro Image Analysis Software. Mucous hyperplasia was graded according to the method of Telmelkovski et al. (35).

Eosinophil Peroxidase Activity in BAL Fluid

Eosinophil peroxidase (EPO) content was measured using a colorimetric assay (6) to confirm the cell counts. An aliquot of lavage fluid was taken before centrifugation (i.e., contains cells in suspension), frozen at -80°C , and, on thawing, diluted in Zn-free HBSS. Thirty microliters of each dilution were mixed with 30 μl of OPD substrate solution [12 mM *o*-phenylenediamine (OPD), 0.11% hexadecyltrimethyl ammonium bromide, 0.005% H_2O_2 , in 0.05 M Tris-HCl pH 8.0]. The enzymatic reaction was stopped after 30 min at 22°C by the addition of 30 μl 4 N H_2SO_4 . Absorbance at 490 nm was measured on a plate reader (Titertek multiskan).

Pathway-Specific Gene Array

Only RNA from the acute mouse experiments were used in the cytokine gene experiments because the degree of inflammation, as determined by number of inflammatory cells in the BAL, was much greater than in the chronic model. For SuperArray analysis, 5 μg of RNA (pooled from the same four to six mice used in real-time experiments) were reverse transcribed with Biotin-16-dUTP (Roche Diagnostics Australia, Sydney, Australia) using a SuperArray TrueLabeling RT enzyme kit (SuperArray Bioscience, Frederick, MD) according to manufacturer's instructions. The cDNA probe mixture was hybridized to the Q Series Mouse Inflammatory Cytokines and Receptors cDNA array (GEArray MM-015, SuperArray Bioscience). The array was analyzed using CDP-Star substrate chemiluminescence and exposure to X-ray film. Data image files were analyzed according to GEArray Expression Analysis Suite provided by Superarray Bio-

science (<http://GEASuite.superarray.com>) and normalized for house-keeping gene signals. Results were confirmed by real-time PCR as described above.

Statistical Analyses

Data are expressed as means $\pm$ SE. Data were analyzed by one-way or two-way ANOVAs and Student's *t*-tests where appropriate using SPSS statistical software.

RESULTS

Body Weight and Growth Rates

The body weight and growth rates of the mice were unaffected by either Zn supplementation or airway inflammation in both the acute (end point: 18.26 ± 0.17 g; $n = 65$; overall mean $\pm$ SE) and chronic models (end point: 19.44 ± 0.26 g; $n = 37$, overall mean $\pm$ SE).

Inflammatory Cell Infiltration

In the BAL fluid of the OVA-challenged mice, there were significant and substantial increases in eosinophils (Fig. 1, A and B) and lymphocytes (Fig. 1, C and D), in both the acute and chronic models. There was also a significant increase in BAL EPO, confirming the eosinophilia (Fig. 1A). Total BAL macrophages increased significantly in the acute model (Fig. 1E) but not in the chronic model (Fig. 1F). The total number of eosinophils in BAL of OVA-treated mice was higher in the acute model of allergic inflammation ($1.63 \pm 0.15 \times 10^6$

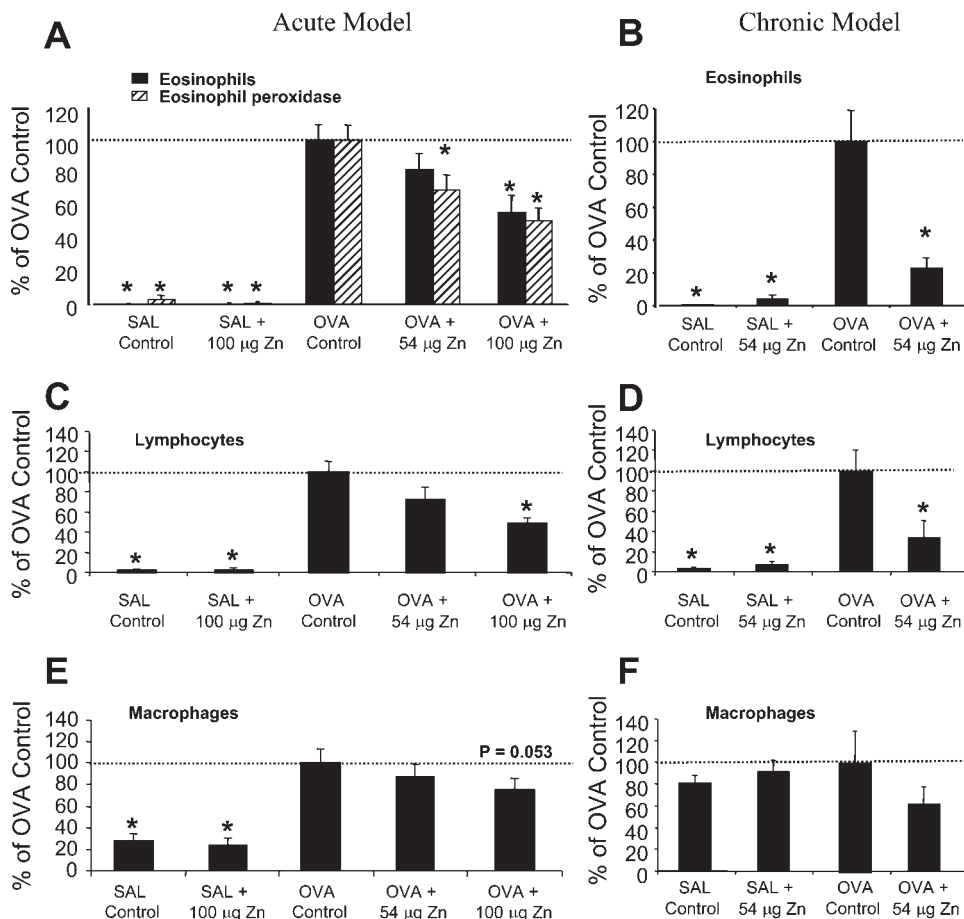


Fig. 1. Zn supplementation suppresses inflammatory cell infiltration in both acute and chronic models of allergic inflammation. Inflammatory cells (solid bars) were identified using standard morphometric criteria from stained cytopins of bronchoalveolar lavage (BAL) fluid collected from $n = 8-10$ (A, C, and E) and 4-6 mice (B, D, and F). Eosinophil peroxidase was measured using an enzymatic reaction with OPD substrate [12 mM *o*-phenylenediamine (OPD), 0.11% hexadecyltrimethyl ammonium bromide, 0.005% H_2O_2 , in 0.05 M Tris-HCl pH 8.0] to confirm the cell counts in A (cross-hatched bars). SAL, saline. Data are calculated as a percentage of that observed in the ovalbumin (OVA) controls. Data are means $\pm$ SE and were analyzed by 1-way ANOVA and *t*-tests, where appropriate. A and B: significant decrease in eosinophilia (A: $F = 3.991$, $P = 0.03$; B: $F = 16.115$, $P = 0.007$) and eosinophil peroxidase activity (A: $F = 10.37$, $P = 0.00$) in Zn-supplemented OVA-treated mice. C and D: significant decrease in the combined number of lymphocytes in Zn supplementation OVA-treated mice (C: $F = 6.974$, $P = 0.004$; D: $F = 5.37$, $P = 0.05$). E and F: macrophages are not significantly affected by Zn supplementation. *Significant difference from OVA controls (*t*-test, $P < 0.05$).

Table 1. Descriptive data: acute model of allergic inflammation with Zn supplementation

	Sal	Sal (100 µg Zn)	OVA	OVA (54 µg Zn)	OVA (100 µg Zn)
<i>Labile Zn in body fluids, µM</i>					
Serum‡ (n = 6–12)	15.6±0.9	24.1±1.1†	16.9±1.4	18.3±1.3	21.5±2.0†
BAL (n = 6–10)	0.44±0.05	0.38±0.07	0.43±0.03	0.37±0.02	0.41±0.04
<i>Total Zn in tissues, nmol/g wet lung</i>					
Liver‡ (n = 5–6)	381.8±14.6	483.3±37.3†	383.2±9.0	399.3±18.0	483.0±23.9†
Spleen (n = 5–6)	255.5±12.1	282.5±6.6	249.7±21.0	263.1±10.9	264.9±5.7
Lung (n = 5–6)	151.8±49.0	52.1±9.4	162.3±44.1	92.3±18.7	132.0±37.9
<i>Histopathology</i>					
Mucus score (n = 3–4)	0.03±0.03	0.01±0.01	1.6±0.6*	1.95±0.3	1.4±0.4

Values are means ± SE; n, no. of animals. Sal, saline; OVA, ovalbumin-treated mice; BAL, bronchoalveolar lavage fluid. *Significant difference between Sal and OVA control mice (*t*-tests, *P* < 0.05). †Significant effect of Zn supplementation relative to Sal or OVA controls as appropriate (*t*-tests, *P* < 0.05). ‡Zn supplementation significantly increased liver (*F* = 13.11, *P* = 0.00) and serum (*F* = 10.96, *P* = 0.00) Zn levels by 2-way ANOVA.

cells/ml) than in the chronic model ($0.39 \pm 0.094 \times 10^6$ cells/ml). Similarly, the numbers of other inflammatory cells were higher in the acute (lymphocytes: $0.55 \pm 0.07 \times 10^6$ cells/ml; macrophages: $0.23 \pm 0.03 \times 10^6$ cells/ml) than in the chronic model (lymphocytes: $0.14 \pm 0.028 \times 10^6$ cells/ml; macrophages: $0.16 \pm 0.05 \times 10^6$ cells/ml).

In both the acute and chronic models, Zn supplementation significantly decreased the number of BAL eosinophils by 40 and 80%, respectively (Fig. 1). In the acute model, EPO activity was similarly reduced by 40%. The reduction in the number of lymphocytes was 66 and 55% with Zn supplementation in the acute and chronic models, respectively (Fig. 1, C and D). Zn supplementation had no significant effect on macrophage numbers in the acute or chronic models.

Histopathology of the Lung

Mucous hyperplasia, as determined by increased PAS staining of mucopolysaccharide-containing goblet cells in airway epithelium, was a feature of the airways in OVA-challenged mice [acute (Table 1) and chronic (Table 2) models]. Airway thickness and collagen deposition were also studied in the chronic model, and both increased significantly in OVA-challenged mice (Table 2). In both the acute and chronic models, Zn supplementation had no effect on any of the histopathological features (Tables 1 and 2).

Zn levels in Serum, BAL, and Tissues

OVA treatment had no effect on labile Zn concentration in either serum or cell-free BAL fluid in both the acute and chronic models. There was also no effect on Zn contents of lung, liver, or spleen (Tables 1 and 2).

Labile Zn in BAL was unaffected by Zn supplementation in both the acute and chronic models. In the acute model, at the highest Zn dose (100 µg), serum labile Zn and total liver Zn content increased in both Sal and OVA mice (Table 1). Serum Zn did not increase significantly at the 54-µg Zn dose of Zn supplementation in either the acute or chronic models. Total Zn in the spleen and lung were both unaffected by Zn supplementation in both the acute and chronic models. The failure to detect alterations in Zn levels in the lung tissue with Zn supplementation may be due to technical limitations, because the Zn levels in the lung tissue samples collected were close to the lower detection limit of atomic adsorption. Note: labile Zn comprises only ~10% of total Zn in the body, and decreases in this labile pool may not be detected in atomic adsorption measures of total Zn.

Zn Transporter Expression

Transcript abundance, relative to the housekeeping gene HPRT, was measured by real-time RT-PCR. There was little

Table 2. Descriptive data: chronic model of allergic inflammation

	Sal	Sal (54 µg Zn)	OVA	OVA (54 µg Zn)
<i>Labile Zn in body fluids, µM Zn</i>				
Serum (n = 4–6)	13.7±1.2	11.4±3.0	11.9±3.6	10.0±3.7
BAL	0.43±0.02	0.44±0.02	0.44±0.01	0.41±0.03
<i>Total Zn in tissues, nmol/ml homogenate</i>				
Liver (n = 5–7)	36.9±1.4	32.07±2.6	33.72±5.9	33.9±2.1
Spleen (n = 5–7)	188.5±8.1	185.1±3.0	166.25±6.17	166.0±9.9
Lung (n = 4–7)	168.0±23.9	151.8±24.8	156.7±14.4	150.6±34.6
<i>Histopathology</i>				
Epithelial thickness (n = 4–5)	20.2±2.4	20.8±0.9	27.6±1.8*	27.3±3.2
Collagen thickness (n = 4–5)	6.3±0.9	9.0±1.2	9.7±0.8*	11.3±2.3
Mucus score (n = 4–5)	0.02±0.01	0.08±0.07	1.3±0.4*	1.1±0.2

Values are means ± SE. *Significant difference between Sal and OVA control mice (*t*-tests, *P* < 0.05).

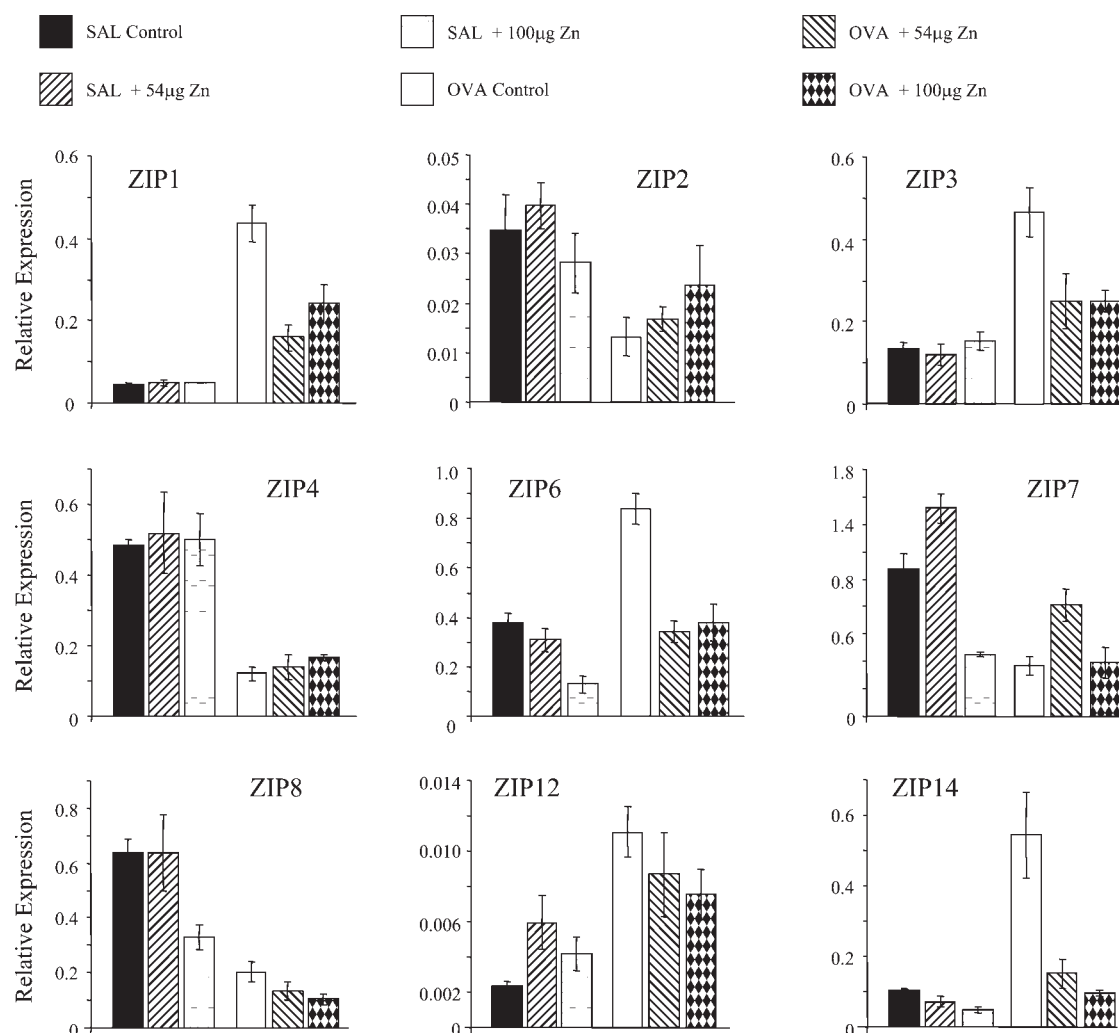


Fig. 2. Solute Carrier Family 39 (ZIP) gene expression is affected by airway inflammation and Zn supplementation. Zn transporter expression was determined by real-time PCR. Data are presented as relative expression of ZIP genes compared with hypoxanthine guanine phosphoribosyl transferase (HPRT). Data are means $\pm$ SE, where SE refers to assay precision, not sample variation.

expression of ZIP1, ZIP2, ZIP12, and ZIP14; moderate expression of ZIP3; and relatively high expression of ZIP4, ZIP6, ZIP7, and ZIP8 (Fig. 2). Among the ZnTs, there was little expression of ZnT7, moderate expression of ZnT1 and ZnT4, and relatively high expression of ZnT5 and ZnT6 (Fig. 3). ZnT2 expression was only just detectable; ZnT3, ZnT8, and ZnT10 were absent (data not shown).

Marked changes in expression of transporters were seen in the OVA-challenged mice. Of the ZIP transporters, increases were seen in ZIP1 (9.5-fold), ZIP3 (3.4-fold), ZIP6 (2.2-fold), ZIP12 (4.7-fold), and ZIP14 (5.3-fold) with allergic inflammation, whereas there were decreases in the expression of ZIP4 (4-fold), ZIP7 (2.9-fold), and ZIP8 (3.1-fold) (Fig. 2). Of the ZnT family, no changes were seen in expression of ZnT1, ZnT5, or ZnT7, whereas there was a decrease in ZnT6 (3.2-fold). ZnT4 mRNA decreased so dramatically in OVA-challenged samples, that transcript levels were undetectable (Fig. 3).

Zn supplementation was able to restore, at least in part, some of the alterations in Zn transporter gene expression induced by allergic inflammation (Figs. 2 and 3). For the following ZIP

proteins that were upregulated by inflammation, there was a significant decrease in inflammation-induced expression in Zn-supplemented mice. For example, in mice supplemented with 100 µg Zn, ZIP1, ZIP3, ZIP6, and ZIP14 expression decreased by 1.8-, 1.9-, 2.2-, and 1.9-fold, respectively (Fig. 2). Conversely, ZIP7 expression, which was downregulated by inflammation, significantly increased by 1.9-fold in OVA-treated mice supplemented with 100 µg Zn. The effects of Zn supplementation alone, in the absence of OVA-treatment, were varied. The expression of ZIP7 and ZnT7 increased in mice supplemented with 54 µg Zn, whereas expression of ZIP6, ZIP7, and ZIP8, decreased by 2.9-, 2.4-, and 1.9-fold, respectively, in mice supplemented with 100 µg Zn.

MT Expression

MT-I and -II mRNA levels increased in allergic inflammation by 11- and 34-fold, respectively (Fig. 4). Surprisingly, Zn supplementation did not enhance lung MT transcription in either the Sal- or OVA-challenged animals (Fig. 4).

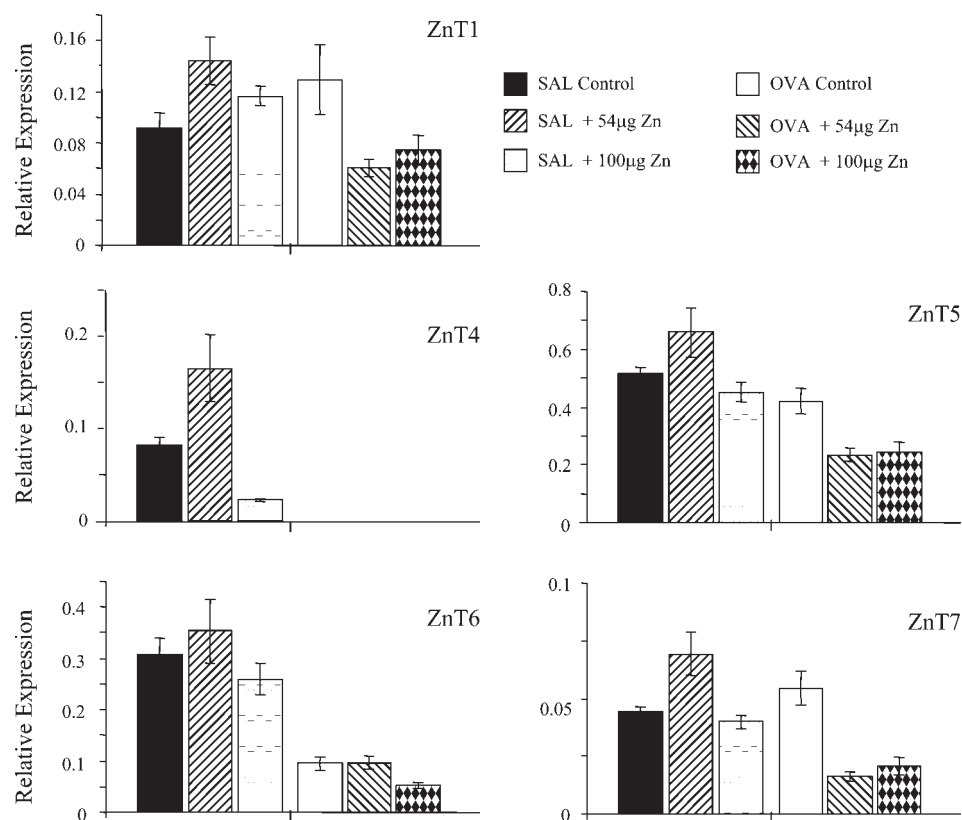


Fig. 3. Solute Carrier Family 30 gene expression is affected by airway inflammation and Zn supplementation. Zn transporter expression was determined by real-time PCR. Data are presented as relative expression of ZnT genes compared with HPRT. Data are means $\pm$ SE, where SE refers to assay precision, not sample variation.

Expression of Cytokines and Their Receptors in Acute Allergic Inflammation

The pathway-specific gene array MM-015 (SuperArray Bioscience, <http://www.superarray.com>) contains 96 cytokine and receptor cDNAs associated with inflammatory response. Expression analysis identified a group of genes that were induced by airway inflammation that included *ccl6* (up 2.2-fold), *ccl8* (6.7-fold), *ccl9* (6.0-fold), and *ccl11* (4.7-fold). Zn supplementation did not modulate the expression of any inflammatory cytokines or receptors represented in the array. Expression of chemokines induced by airway inflammation but not by Zn

supplementation was confirmed using real-time PCR (Fig. 5). Complete analysis of gene array is provided as supplementary data (Table S2).

DISCUSSION

In this study, we have demonstrated that multiple Zn transporter proteins are expressed in the mouse lung. We have also shown that Zn homeostasis is perturbed in allergic inflammation. Moreover, marked reductions in the number of inflammatory cells, including eosinophils, in the BAL were observed

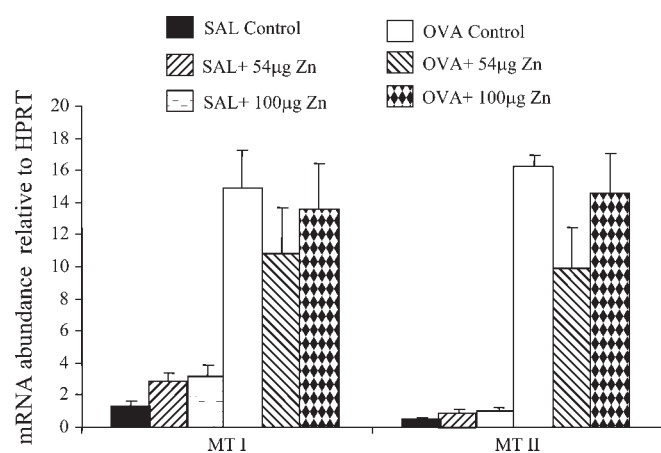


Fig. 4. Metallothionein MT-(I) and MT-II gene expression is affected by airway inflammation but not by Zn supplementation. Data are presented as relative expression of MT genes compared with HPRT. Data are means $\pm$ SE, where SE refers to assay precision, not sample variation.

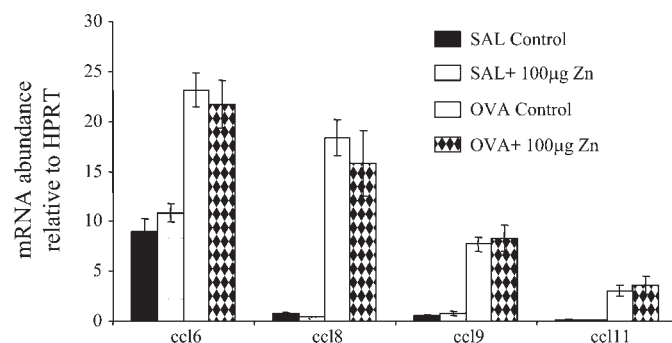


Fig. 5. Expression of inflammatory mediators induced by allergic airway inflammation. Changes in gene expression were identified using the cDNA gene array and confirmed by real-time PCR. Zn supplementation had no effect on inflammatory mediator gene expression. Data are presented as relative expression of target genes compared with HPRT. Data are means $\pm$ SE, where SE refers to assay precision, not sample variation. *Ccl6*, macrophage inflammatory protein-related protein-1 (NM_009139); *Ccl8*, a monocyte chemotactic protein (NM_021443); *Ccl9*, macrophage inflammatory protein-1 γ (NM_011338); *Ccl11*, eotaxin (NM_011330), an eosinophil chemotactic factor.

with Zn supplementation. This was accompanied by reversal of the inflammation-induced alterations in the expression of some Zn transporters.

There is clinical evidence linking asthma and Zn deficiency and as such, we hypothesized that proteins involved in maintaining Zn homeostasis might play a role in allergic inflammation (7, 25, 33). The changes we observed in the gene expression of the ZIP family of Zn transporter proteins during inflammation are mainly directed toward maintaining, or increasing intracellular Zn. ZIP1, ZIP3, ZIP6, ZIP12, and ZIP14 have been shown to have specific Zn influx activity [reviewed in Kambe et al. (17)], and the expression of all of these uptake transporter genes increased significantly with inflammation in our model (Fig. 2). Conversely, we observed a marked reduction in the mRNA levels of the epithelial Zn uptake protein, ZIP4, and of the less characterized proteins, ZIP7 and ZIP8. The down-regulation of ZIP4, ZIP7, and ZIP8 during inflammation may be explained by their subcellular localization. ZIP7 is thought to be involved in mobilizing Zn from the Golgi apparatus into the cytoplasm (15), and its downregulation could therefore indicate a need to reduce Zn efflux from the Golgi and to maintain intracompartamental Zn levels during inflammation. ZIP4 is located in the apical surface of intestinal cells (8), and may be lost during inflammation due to shedding of the airway epithelium.

Decreased gene expression of the vesicular transporter, ZnT4 and the Golgi uptake transporter, ZnT6, also occurs in the lung during allergic inflammation. Our laboratory has previously shown that both ZnT4 and Zn ions are localized in intracellular vesicles at the apical surface of airway (38) and kidney cells (29). ZnT4 protein levels also decrease in inflamed airways (Truong-Tran AQ, Murgia C, and Zalewski PD, unpublished observations). Because a ZnT4 mutation resulting in dramatically reduced expression leads to decreased Zn secretion by mammary and intestinal epithelial cells (1, 19, 26), it is possible that reduced expression of ZnT4 might exert a protective role in inflamed airway epithelium by increasing retention of intracellular Zn ions. Expression of ZnT1, ZnT2, ZnT5, and ZnT7, was unaffected by allergic inflammation. Because ZnT proteins are mostly responsible for intracellular compartmentalization, we suggest that ZnT1, ZnT2, ZnT5, and ZnT7, may play a crucial role in the delivery of Zn to the Zn-dependent proteins that are important for airway function, such as Cu-Zn-SOD, metalloproteinases, and alkaline phosphatase [reviewed in Murgia et al. (25)].

An increase in Zn uptake may be important to the lung during airway inflammation because Zn has antioxidant (13), anti-inflammatory (5), and cell survival-promoting properties (2, 38). In addition, our laboratory has previously shown that the amount of labile Zn decreases in the cells that line the airways during inflammation (37). Moreover, in inflamed airways, there is most likely an increased demand for labile Zn required for Zn-dependent proteins involved in inflammatory processes. Here, we observed a significant increase in MT-I and -II mRNA in inflamed lungs. MTs may deplete Zn in tissue and plasma (23) and thus, contribute to low Zn ion bioavailability in the inflamed airway epithelium. Having said this, it is possible that in inflamed airways the demand for Zn is so high, and Zn turnover so rapid, that a local state of labile Zn deficiency could still persist despite the observed increase in transcript levels of Zn uptake transporters.

Zn supplementation does reverse, at least in part, most of the alterations in Zn transporter gene expression associated with allergic inflammation. This reversal may result from lung cells responding to increased serum Zn. Alternatively, the reversal of the inflammation-induced alterations in Zn transporter expression could be due to the decrease in BAL inflammatory cells. Our gene expression analysis on the whole lung does not discriminate between cell types, and it is therefore possible that inflammatory cell mRNAs contribute to the gene expression patterns reported. Moreover, little is currently known about the regulation of Zn transporters, and it is possible that factors released by inflammatory cells might play a role in mediating Zn transporter expression in the lung. The inflammatory cytokine interleukin-6 has recently been shown to upregulate ZIP14 expression in the liver (20). Future studies now need to define which of the many cell types present in the lung contribute to the expression profiles observed here. In addition, functional protein studies are needed in both the normal and diseased lung.

Increases in eosinophils and their secreted products are known to correlate with disease severity in human asthmatic individuals (9). Here, Zn supplementation significantly decreased the number of eosinophils and other inflammatory cells in the BAL fluid of OVA-treated mice. Eosinophils located in the airway lumen are known to be highly activated (6) in this model, and they release a range of proinflammatory cytokines and cytotoxic proteins that further amplify the inflammatory response. Thus, by decreasing eosinophil number in BAL, Zn supplementation probably results in a less severe inflammatory response. Although evidence that Zn has anti-inflammatory properties is steadily growing [present study; reviewed in Shenkin et al. (32)], the mechanism/s involved are not clear. In this study, we tested whether Zn modulated the expression of a range of inflammatory cytokines and their receptors known to be important for recruiting inflammatory cells to the lung. However, the results of our gene array analysis led us to conclude that Zn supplementation does not regulate the gene expression, in our model, of any of the 96 inflammatory mediators or their receptors represented in the array. An alternative hypothesis is that Zn prevents migration of inflammatory cells from the circulation to the sites of inflammation by interfering with the adhesion of inflammatory cells to endothelial cells that line blood vessels; a step that is essential for propagating inflammation (27). Future studies should examine the effects of Zn, and the involvement of Zn transporters, in the migration of inflammatory cells from the airways. In addition to acting as an anti-inflammatory, Zn is thought to play a role in cell growth and repair [reviewed in Murgia et al. (25)]. In our study, Zn supplementation, whether given before the induction of allergic inflammation (acute model), or after (chronic model), had no effect on the histopathology of the airways, as measured by the thickening of the basement membrane, collagen deposition or mucus hyperplasia.

Clinical Prospective

Intervention with Zn supplements may be both preventative and therapeutic because first, maintaining airway Zn levels might lower the risk of developing asthma and second, restoring airway Zn levels in those who already have asthma may lessen the severity of inflammation and airway hyperrespon-

siveness. Zn treatment could be used in conjunction with existing asthma medications. Oral Zn supplements are already commercially available and have been used with variable success in another respiratory disease, cystic fibrosis (18). Other mechanisms that enable the targeting of Zn directly in the asthmatic lung may also be of benefit. As such, understanding the function and regulation of Zn transporters in the lung will be vital.

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SUPPLEMENTARY MATERIAL FOR

Anti-inflammatory effects of zinc and alterations in zinc transporter mRNA in mouse models of allergic inflammation

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[#]Equally contributing (alphabetical order)

Table S1. Gene specific primer sequences

Gene (Accession #)	Forward primer	Reverse primer
mBeta2 (NM_009735)	TACATACGCCTGCAGAGTTAAGC	AGGTTCAAATGAATCTTCAGAGC
mHPRT (NM_013556.1)	CTCATGGACTGATTATGGACAGG	TTAATGTAATCCAGCAGGTCAGC
mZnT1 (NM_009579)	TGGATGTACAAGTAAATGGGAATCT	GTCTTCAGTACAACCCTTCCAGTTA
mZnT2 (NM_011773)	CAGAAGGATTCTGGAAGTCACC	CGGGAAGACACCCAGAGG
mZnT3 (NM_011773)	GTCTCCCTCTGGATAGTCACTGG	GCATACTCTGCACCTGTAGATCC
mZnT4 (NM_011774)	TAGGTGGATACATGGCAAATAGC	AGTTCATATGGATGGTTCTCTGC
mZnT5 (NM_022885)	GCCTGTCAAGTTCTACTTCTGAGAC	TCTCGGTATGATATTAATCCCTCAA
mZnT6 (NM_144798)	TTAGAAGTCCTGGCTGTATTTGC	GAATAGAAAGCATCGTGAACAGG
mZnT7 (NM_023214)	CGCTTTCTCTTATGGGTATGTTAGA	TCTCTCGACTCCTTCTGAGAAAATA
mZnT8_2 (NM_172816)	TTCTCCATCTTACTCATGGAAGG	GTGACCAGATGTGTAGACTGTGC
mZnT10(NM_001033286)	TCTGAAGCACTCAATATCAGAGG	AGAATATGATAGCCGTGATGACC
mZIP1 (NM_013901)	CAGTGGAGACCTATTTGCTCTTTTA	CTACTTTTCTCTCACTCCAGACCAG
mZIP2 (XM_359330)	CATATGACTGCTGAAGCTCTGG	CGAGAAGAATTTCCCTTACTTCC
mZIP3 (NM_134135)	GCGTATTCTCTGGCTACATGC	TGAAGGTCTCCAGGTCTATAAAGG
mZIP4 (NM_028064)	ACTTTGTGGACTTTGTGTTTCAGG	GAGTATGGAGCTCAGAGTCTTGG
mZIP5 (NM_028051)	AGGACCTAGTGAGCAATCAGAGG	TTCTCCAAGATCCCTTTTGTTC
mZIP6 (NM_139143)	AAGTGAGAAGAAGGCAGAAATCC	GGAGAAGATGTAACAGAGCATCG
mZIP7 (NM_008202)	AACATAGCCATGGGACTTCTAGG	ATTCTACTGGGATGAGGAACAGC
mZIP8 (NM_026228)	CTAACGGACACATCCACTTCGA	CCCTTCAGACAGGTACATGAGCTT
mZIP12 (NM_001012305)	CATCTCATACCTCAGGTTCTTGG	ATTAATAATGGCAGACCCTTG
mZIP14 (NM_144808)	GACAATTACGTCTCCAAGTCTGC	CTTCTTGGAAGGAAGTGTCTCG
mEotaxin-1(NM_011330)	CTTCTATTCTGCTGCTCACG	TTGTAGCTCTTCAGTAGTGTGTTGG
mCc16 (NM_009139)	ATTCTTTATCCTTGTGGCTGTCC	GATCTGTGTGGCATAAGAGAAGC
mCcl8 (NM_021443)	ATCTACGCAGTGCTTCTTTGC	CACTGGATATTGTTGATTCTCTCG
mCc19 (NM_011338)	TTACAACTGCTCTTGGAATCTGG	GCAATCTGAAGAGTCTTGAAAGC
mMT-1 (NM_013602)	CAACGTCCTGAGTACCTTCTCC	AGGTGCACTTGCAGTTCTTGC
mMT-2 (NM_008630)	GCAAACAATGCAAATGTACTTCC	AAGTTGTGGAGAACGAGTCAGG

Table S2. Cytokines and receptors gene array (SuperArray MM0015) results

					sal	sal+	ova	ova+									
Position	UniGene	RefSeq Number	Symbol	Description	Group 4	Group 3	Group 1	Group 2	sal/ova	sal minus Blank	sal+ minus Blank	ova minus Blank	ova+ minus Blank	sal nor	sal+ norm	ova norm	ova+norm
1	Mm.6246	NM_007551	Blr1	Burkitt lymphoma receptor 1	4347.65	6497.17	4402.04	12199.02	0.99	-1119.09	2251.12	-5244.07	6254.35	-0.04	0.09	-0.18	0.20
2	Mm.89202	NM_011798	Xcr1	Chemokine (C motif) receptor 1	4162.29	5810.76	9025.27	11514.83	0.46	-1304.45	1564.71	-620.84	5570.16	-0.05	0.07	-0.02	0.18
3	Mm.234466	NM_009909	Il8rb	Interleukin 8 receptor, beta	5117.06	5908.52	10176.71	10135.50	0.50	-349.68	1662.47	530.60	4190.82	-0.01	0.07	0.02	0.14
4	Mm.12876	NM_009910	Cxcr3	Chemokine (C-X-C motif) receptor 3	5138.28	6807.44	10285.48	12033.67	0.50	-328.46	2561.39	639.37	6089.00	-0.01	0.11	0.02	0.20
5	Mm.1401	NM_009911	Cxcr4	Chemokine (C-X-C motif) receptor 4	9305.28	11493.97	14735.35	16831.72	0.63	3838.53	7247.91	5089.24	10887.05	0.14	0.31	0.18	0.35
6	Mm.274927	NM_009912	Ccr1	Chemokine (C-C motif) receptor 1	2425.74	6023.18	12231.75	10262.24	0.20	-3041.01	1777.13	2585.64	4317.57	-0.11	0.07	0.09	0.14
7	Mm.6272	NM_009915	Ccr2	Chemokine (C-C motif) receptor 2	3647.06	7717.89	13661.80	9658.00	0.27	-1819.68	3471.84	4015.69	3713.32	-0.06	0.15	0.14	0.12
8	Mm.57050	NM_009914	Ccr3	Chemokine (C-C motif) receptor 3	2681.43	3828.99	4290.93	5057.29	0.62	-2785.31	-417.06	-5355.18	-887.38	-0.10	-0.02	-0.19	-0.03
9	Mm.1337	NM_009916	Ccr4	Chemokine (C-C motif) receptor 4	2327.35	4990.90	2917.01	13696.30	0.80	-3139.39	744.85	-6729.10	7751.63	-0.11	0.03	-0.23	0.25
10	Mm.14302	NM_009917	Ccr5	Chemokine (C-C motif) receptor 5	2344.78	4324.61	8908.67	16883.97	0.26	-3121.96	78.56	-737.44	10939.30	-0.11	0.00	-0.03	0.35
11	Mm.8007	NM_009835	Ccr6	Chemokine (C-C motif) receptor 6	4442.17	3877.06	7271.82	12585.48	0.61	-1024.57	-368.99	-2374.29	6640.81	-0.04	-0.02	-0.08	0.21
12	Mm.2932	NM_007719	Ccr7	Chemokine (C-C motif) receptor 7	5670.98	6633.66	9172.97	16900.39	0.62	204.24	2387.61	-473.14	10955.72	0.01	0.10	-0.02	0.35
13	Mm.8000	NM_007720	Ccr8	Chemokine (C-C motif) receptor 8	6078.41	5716.91	8414.85	15326.17	0.72	611.67	1470.86	-1231.26	9381.50	0.02	0.06	-0.04	0.30
14	Mm.103813	NM_007721	Gpr2	G protein-coupled receptor 2	2833.69	5888.36	11516.22	8938.09	0.25	-2633.06	1642.31	1870.12	2993.42	-0.09	0.07	0.06	0.10
15	Mm.44065	NM_009987	Cx3cr1	Chemokine (C-X3-C) receptor 1	1560.66	5587.28	10614.19	7317.15	0.15	-3906.09	1341.23	968.08	1372.48	-0.14	0.06	0.03	0.04
16	Mm.240327	NM_008337	Ifng	Interferon gamma	1852.76	4599.17	4807.56	5467.63	0.39	-3613.98	353.11	-4838.55	-477.04	-0.13	0.01	-0.17	-0.02
17	Mm.874	NM_010548	Il10	Interleukin 10	2039.04	5950.39	1679.24	11270.47	1.21	-3427.71	1704.34	-7966.87	5325.80	-0.12	0.07	-0.28	0.17
18	Mm.26658	NM_008348	Il10ra	Interleukin 10 receptor, alpha	2277.60	3516.39	2771.26	13534.34	0.82	-3189.15	-729.66	-6874.85	7589.67	-0.11	-0.03	-0.24	0.25
19	Mm.4154	NM_008349	Il10rb	Interleukin 10 receptor, beta	4933.90	7102.19	6588.61	18450.55	0.75	-532.84	2856.14	-3057.50	12505.88	-0.02	0.12	-0.11	0.40
20	Mm.35814	NM_008350	Il11	Interleukin 11	5136.00	5424.45	9742.84	18689.33	0.53	-330.74	1178.40	96.73	12744.66	-0.01	0.05	0.00	0.41

21	Mm.193451	NM_010549	Il11ra1	Interleukin 11 receptor, alpha chain 1	6001.66	7216.21	10876.81	19171.70	0.55	534.91	2970.16	1230.70	13227.03	0.02	0.13	0.04	0.43
22	Mm.103783	NM_008351	Il12a	Interleukin 12A	2397.76	5547.10	10729.68	11385.03	0.22	-3068.99	1301.05	1083.57	5440.36	-0.11	0.05	0.04	0.18
23	Mm.239707	NM_008352	Il12b	Interleukin 12B	1137.45	7206.80	10696.72	7737.09	0.11	-4329.30	2960.75	1050.61	1792.42	-0.15	0.12	0.04	0.06
24	Mm.731	NM_008353	Il12rb1	Interleukin 12 receptor, beta 1	1236.83	5158.35	5304.81	7658.71	0.23	-4229.91	912.30	-4341.30	1714.04	-0.15	0.04	-0.15	0.06
25	Mm.188337	NM_008354	Il12rb2	Interleukin 12 receptor, beta 2	2774.08	5851.14	1271.22	8495.77	2.18	-2692.66	1605.09	-8374.89	2551.10	-0.09	0.07	-0.29	0.08
26	Mm.1284	NM_008355	Il13	Interleukin 13	2340.47	4966.83	2637.65	10676.43	0.89	-3126.28	720.78	-7008.46	4731.76	-0.11	0.03	-0.24	0.15
27	Mm.24208	NM_133990	Il13ra1	Interleukin 13 receptor, alpha 1	3271.19	5475.78	5075.80	14747.60	0.64	-2195.56	1229.73	-4570.31	8802.93	-0.08	0.05	-0.16	0.28
28	Mm.360560	NM_008356	Il13ra2	Interleukin 13 receptor, alpha 2	3451.64	5532.26	9678.11	16348.14	0.36	-2015.10	1286.21	32.01	10403.47	-0.07	0.05	0.00	0.34
29	Mm.4392	NM_008357	Il15	Interleukin 15	2898.96	7053.24	8462.06	16752.13	0.34	-2567.79	2807.19	-1184.05	10807.46	-0.09	0.12	-0.04	0.35
30	Mm.200196	NM_008358	Il15ra	Interleukin 15 receptor, alpha chain	1560.54	5512.97	10883.54	13242.38	0.14	-3906.21	1266.92	1237.43	7297.71	-0.14	0.05	0.04	0.24
31	Mm.10137	NM_010551	Il16	Interleukin 16	0.00	6624.28	11074.98	8371.91	0.00	-5466.74	2378.23	1428.87	2427.24	-0.19	0.10	0.05	0.08
32	Mm.5419	NM_010552	Il17	Interleukin 17	963.24	5247.62	4547.46	6765.72	0.21	-4503.50	1001.57	-5098.65	821.05	-0.16	0.04	-0.18	0.03
33	Mm.59313	NM_019508	Il17b	Interleukin 17B	2167.04	7510.00	0.00	9559.56	E	-3299.71	3263.95	-9646.11	3614.89	-0.12	0.14	-0.33	0.12
34	Mm.4481	NM_008359	Il17r	Interleukin 17 receptor	3039.14	6902.80	2341.83	9558.03	1.30	-2427.60	2656.75	-7304.28	3613.36	-0.09	0.11	-0.25	0.12
35	Mm.357587	NM_008360	Il18	Interleukin 18	3881.00	5664.16	4424.64	12776.11	0.88	-1585.74	1418.10	-5221.47	6831.44	-0.06	0.06	-0.18	0.22
36	Mm.15534	NM_010554	Il1a	Interleukin 1 alpha	3582.97	5823.90	9522.34	14734.14	0.38	-1883.78	1577.85	-123.76	8789.47	-0.07	0.07	-0.00	0.28
37	Mm.222830	NM_008361	Il1b	Interleukin 1 beta	1302.00	5250.74	8277.31	19474.67	0.16	-4164.75	1004.69	-1368.80	13530.00	-0.15	0.04	-0.05	0.44
38	Mm.896	NM_008362	Il1r1	Interleukin 1 receptor, type I	959.68	4954.51	8643.91	12800.13	0.11	-4507.07	708.46	-1002.20	6855.46	-0.16	0.03	-0.03	0.22
39	Mm.1349	NM_010555	Il1r2	Interleukin 1 receptor, type II	52.68	6890.35	10272.73	8094.42	0.01	-5414.07	2644.30	626.63	2149.75	-0.19	0.11	0.02	0.07
40	Mm.253664	NM_008365	Il18r1	Interleukin 18 receptor 1	1176.09	7936.63	5644.27	10448.78	0.21	-4290.66	3690.58	-4001.84	4504.10	-0.15	0.16	-0.14	0.15
41	Mm.14190	NM_008366	Il2	Interleukin 2	2759.38	8504.97	7851.72	8484.55	0.35	-2707.36	4258.92	-1794.39	2539.88	-0.10	0.18	-0.06	0.08
42	Mm.103794	NM_021380	Il20	Interleukin 20	3225.15	6609.66	6648.61	8190.67	0.49	-2241.60	2363.60	-2997.49	2246.00	-0.08	0.10	-0.10	0.07
43	Mm.157689	NM_021782	Il21	Similar to interleukin 21	1465.56	4944.48	4255.99	13707.39	0.34	-4001.19	698.43	-5390.12	7762.72	-0.14	0.03	-0.19	0.25
44	Mm.294870	NM_080837	D17Wsu104e	DNA segment, Chr 17, Wayne State University 104, expressed	2725.49	5918.61	10236.27	17124.83	0.27	-2741.25	1672.56	590.16	11180.16	-0.10	0.07	0.02	0.36
45	Mm.915	NM_008367	Il2ra	Interleukin 2 receptor, alpha chain	2108.87	5401.31	9482.94	20002.72	0.22	-3357.88	1155.26	-163.17	14058.05	-0.12	0.05	-0.01	0.45
46	Mm.35287	NM_008368	Il2rb	Interleukin 2 receptor, beta chain	1124.27	6958.57	9748.06	11662.82	0.12	-4342.48	2712.52	101.96	5718.15	-0.15	0.11	0.00	0.18
47	Mm.2923	NM_013563	Il2rg	Interleukin 2 receptor, gamma	2079.46	6439.75	10066.77	9518.56	0.21	-3387.29	2193.70	420.66	3573.89	-0.12	0.09	0.01	0.12

				chain													
48	Mm.276360	NM_021283	Il4	Interleukin 4	29.48	5820.11	3697.71	7259.68	0.01	-5437.27	1574.06	-5948.40	1315.01	-0.19	0.07	-0.21	0.04
49	Mm.4461	NM_010558	Il5	Interleukin 5	5935.18	13370.60	12615.95	9300.75	0.47	468.44	9124.55	2969.84	3356.08	0.02	0.38	0.10	0.11
50	Mm.3448	NM_008370	Il5ra	Interleukin 5 receptor, alpha	3614.08	4998.62	8452.25	9166.51	0.43	-1852.66	752.57	-1193.86	3221.84	-0.07	0.03	-0.04	0.10
51	Mm.1019	NM_031168	Il6	Interleukin 6	2278.28	3911.19	9517.52	13826.83	0.24	-3188.46	-334.86	-128.59	7882.16	-0.11	-0.01	-0.00	0.25
52	Mm.2856	NM_010559	Il6ra	Interleukin 6 receptor, alpha	3354.79	7388.21	12623.01	21520.54	0.27	-2111.95	3142.16	2976.90	15575.87	-0.07	0.13	0.10	0.50
53	Mm.4364	NM_010560	Il6st	Interleukin 6 signal transducer	8922.68	11918.15	14475.85	23063.69	0.62	3455.93	7672.10	4829.74	17119.02	0.12	0.32	0.17	0.55
54	Mm.3006	NM_008373	Il9	Interleukin 9	1786.92	4178.03	9559.07	6792.71	0.19	-3679.82	-68.02	-87.04	848.04	-0.13	-0.00	-0.00	0.03
55	Mm.384	NM_008374	Il9r	Interleukin 9 receptor	1880.82	5834.83	9243.56	7481.15	0.20	-3585.93	1588.78	-402.55	1536.48	-0.13	0.07	-0.01	0.05
56	Mm.87787	NM_010735	Lta	Lymphotoxin A	1491.78	6604.93	4054.37	6640.38	0.37	-3974.97	2358.88	-5591.74	695.71	-0.14	0.10	-0.19	0.02
57	Mm.1715	NM_008518	Ltb	Lymphotoxin B	3223.00	8155.96	8424.49	7258.67	0.38	-2243.74	3909.91	-1221.62	1313.99	-0.08	0.16	-0.04	0.04
58	Mm.3122	NM_010736	Ltbr	Lymphotoxin B receptor	4605.89	7665.82	10413.06	9677.46	0.44	-860.85	3419.77	766.95	3732.79	-0.03	0.14	0.03	0.12
59	Mm.2326	NM_010798	Mif	Macrophage migration inhibitory factor	3215.56	4786.83	10961.01	13251.41	0.29	-2251.19	540.78	1314.90	7306.74	-0.08	0.02	0.05	0.24
60	Mm.1283	NM_011329	Ccl1	Expressed sequence BF534335	676.75	4260.93	11712.35	15299.04	0.06	-4789.99	14.88	2066.24	9354.37	-0.17	0.00	0.07	0.30
61	Mm.4686	NM_011330	Ccl11	Small chemokine (C-C motif) ligand 11	3704.76	5677.40	16049.14	22421.13	0.23	-1761.98	1431.35	6403.03	16476.46	-0.06	0.06	0.22	0.53
62	Mm.867	NM_011331	Ccl12	Chemokine (C-C motif) ligand 12	3068.33	4618.54	10744.08	7591.61	0.29	-2398.41	372.49	1097.97	1646.94	-0.08	0.02	0.04	0.05
63	Mm.41988	NM_011332	Ccl17	Chemokine (C-C motif) ligand 17	1495.37	6197.00	9281.65	5886.22	0.16	-3971.37	1950.95	-364.46	-58.45	-0.14	0.08	-0.01	-0.00
64	Mm.379051	NM_011888	Ccl19	Chemokine (C-C motif) ligand 19	1291.22	5987.54	2922.39	5088.56	0.44	-4175.52	1741.49	-6723.72	-856.11	-0.15	0.07	-0.23	-0.03
65	Mm.290320	NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	2234.91	5588.95	9429.77	3801.08	0.24	-3231.84	1342.90	-216.34	-2143.59	-0.11	0.06	-0.01	-0.07
66	Mm.116739	NM_016960	Ccl20	Chemokine (C-C motif) ligand 20	4326.99	4528.53	9897.13	7193.98	0.44	-1139.75	282.48	251.02	1249.31	-0.04	0.01	0.01	0.04
67	Mm.348219	NM_011335	Ccl21a	Chemokine (C-C motif) ligand 21c (leucine)	5030.16	7358.81	11425.15	10961.59	0.44	-436.58	3112.76	1779.04	5016.92	-0.02	0.13	0.06	0.16
68	Mm.12895	NM_009137	Ccl22	Chemokine (C-C motif) ligand 22	2207.62	6605.75	12690.10	10998.14	0.17	-3259.13	2359.70	3043.99	5053.47	-0.11	0.10	0.11	0.16
69	Mm.31505	NM_019577	Ccl24	Chemokine (C-C motif) ligand 24	2388.39	5447.78	12186.88	8764.35	0.20	-3078.35	1201.72	2540.77	2819.68	-0.11	0.05	0.09	0.09
70	Mm.7275	NM_009138	Ccl25	Chemokine (C-C motif) ligand 25	1733.47	3064.15	13310.65	6529.33	0.13	-3733.27	-1181.90	3664.54	584.66	-0.13	-0.05	0.13	0.02
71	Mm.1282	NM_011337	Ccl3	Chemokine (C-C motif) ligand 3	1381.98	5864.34	8196.88	4862.47	0.17	-4084.77	1618.29	-1449.23	-1082.20	-0.14	0.07	-0.05	-0.03

72	Mm.244263	NM_013652	Ccl4	Chemokine (C-C motif) ligand 4	953.00	5527.60	2586.44	2145.02	0.37	-4513.74	1281.55	-7059.67	-3799.65	-0.16	0.05	-0.24	-0.12
73	Mm.284248	NM_013653	Ccl5	Chemokine (C-C motif) ligand 5	6756.76	11901.10	14318.05	5610.45	0.47	1290.02	7655.05	4671.94	-334.22	0.05	0.32	0.16	-0.01
74	Mm.137	NM_009139	Ccl6	Chemokine (C-C motif) ligand 6	5235.44	8268.48	17770.10	15783.29	0.29	-231.30	4022.43	8123.99	9838.62	-0.01	0.17	0.28	0.32
75	Mm.341574	NM_013654	Ccl7	Chemokine (C-C motif) ligand 7	1667.43	2714.29	11252.44	5643.52	0.15	-3799.31	-1531.76	1606.34	-301.15	-0.13	-0.06	0.06	-0.01
76	Mm.42029	NM_021443	Ccl8	Chemokine (C-C motif) ligand 8	3825.09	2643.87	21291.29	17501.85	0.18	-1641.66	-1602.18	11645.18	11557.18	-0.06	-0.07	0.40	0.37
77	Mm.2271	NM_011338	Ccl9	Chemokine (C-C motif) ligand 9	2701.15	3367.64	13541.20	13788.79	0.20	-2765.59	-878.41	3895.09	7844.12	-0.10	-0.04	0.14	0.25
78	Mm.877	NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10	3323.44	3990.31	14358.00	5724.31	0.23	-2143.30	-255.74	4711.89	-220.36	-0.08	-0.01	0.16	-0.01
79	Mm.131723	NM_019494	Cxcl11	Chemokine (C-X-C motif) ligand 11	2536.97	5227.44	8731.82	2269.72	0.29	-2929.77	981.38	-914.29	-3674.95	-0.10	0.04	-0.03	-0.12
80	Mm.10116	NM_018866	Cxcl13	Chemokine (C-X-C motif) ligand 13	526.98	6091.50	5573.52	0.00	0.09	-4939.77	1845.44	-4072.59	-5944.67	-0.17	0.08	-0.14	-0.19
81	Mm.358679	NM_019568	Cxcl14	Chemokine (C-X-C motif) ligand 14	5543.54	9454.33	9274.93	5975.30	0.60	76.80	5208.27	-371.18	30.63	0.00	0.22	-0.01	0.00
82	Mm.64326	NM_011339	Cxcl15	Chemokine (C-X-C motif) ligand 15	27532.60	26294.27	37793.59	41272.75	0.73	22065.85	22048.22	28147.48	35328.08	0.78	0.93	0.98	1.14
83	Mm.4979	NM_009140	Cxcl2	Chemokine (C-X-C motif) ligand 2	2373.42	1599.97	11785.63	6235.27	0.20	-3093.33	-2646.08	2139.52	290.60	-0.11	-0.11	0.07	0.01
84	Mm.4660	NM_009141	Cxcl5	Chemokine (C-X-C motif) ligand 5	4118.83	0.00	12055.48	7038.75	0.34	-1347.92	-4246.05	2409.37	1094.08	-0.05	-0.18	0.08	0.04
85	Mm.766	NM_008599	Cxcl9	Chemokine (C-X-C motif) ligand 9	3001.65	2303.44	11032.81	3639.99	0.27	-2465.10	-1942.61	1386.70	-2304.68	-0.09	-0.08	0.05	-0.07
86	Mm.190	NM_008510	Xcl1	Chemokine (C motif) ligand 1	3674.07	3924.38	10638.34	2588.35	0.35	-1792.67	-321.67	992.24	-3356.32	-0.06	-0.01	0.03	-0.11
87	Mm.103711	NM_009142	Cx3cl1	Chemokine (C-X3-C motif) ligand 1	5645.76	6971.78	8472.74	1754.06	0.67	179.02	2725.72	-1173.37	-4190.61	0.01	0.11	-0.04	-0.14
88	Mm.303231	NM_021704	Cxcl12	Chemokine (C-X-C motif) ligand 12	3054.56	5995.27	8996.55	4083.34	0.34	-2412.18	1749.21	-649.56	-1861.33	-0.08	0.07	-0.02	-0.06
89	Mm.341331	NM_009143	Sdf2	Stromal cell derived factor 2	4355.57	6714.98	10466.74	6942.46	0.42	-1111.18	2468.93	820.63	997.79	-0.04	0.10	0.03	0.03
90	Mm.137222	NM_031199	Tgfa	Transforming growth factor alpha	4108.54	5014.94	9924.30	4523.60	0.41	-1358.21	768.88	278.19	-1421.07	-0.05	0.03	0.01	-0.05
91	Mm.248380	NM_011577	Tgfb1	Transforming growth factor, beta 1	7701.45	7271.13	17353.11	10254.19	0.44	2234.71	3025.08	7707.01	4309.52	0.08	0.13	0.27	0.14
92	Mm.18213	NM_009367	Tgfb2	Transforming growth factor, beta 2	5056.11	179.84	9606.28	5765.21	0.53	-410.64	-4066.21	-39.83	-179.46	-0.01	-0.17	-0.00	-0.01
93	Mm.307887	NM_009368	Tgfb3	Transforming growth factor, beta 3	6307.96	6111.72	9938.55	7449.94	0.63	841.21	1865.66	292.44	1505.27	0.03	0.08	0.01	0.05

