

Decreased Selenoprotein Expression Alters the Immune Response during Influenza Virus Infection in Mice^{1–3}

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Abstract

Previous work from our laboratory demonstrated that host selenium (Se) deficiency results in greater lung pathology and altered immune function in mice infected with influenza virus. Because selenoproteins play a key role in determining the oxidant status of the host, we utilized a transgenic mouse line carrying a mutant selenocysteine (Sec) tRNA^{[Ser]Sec} transgene (*t-trspi*^{6A-}). The levels of selenoproteins are decreased in these mice in a protein- and tissue-specific manner. Male *t-trspi*^{6A-} and wild-type (WT) mice were infected with influenza and killed at various time points postinfection (p.i.). Lung mRNA levels for innate and pro-inflammatory cytokines increased with infection but did not differ between groups. However, at d 2 p.i., chemokine levels were greater in the *t-trspi*^{6A-} mice compared with WT mice. Additionally, IFN- γ was higher at d 7 p.i. in the *t-trspi*^{6A-} mice and viral clearance slower. Despite these immune system changes, lung pathology was similar in *t-trspi*^{6A-} and WT mice. ⁷⁵Se labeling experiments demonstrated that glutathione peroxidase (GPX)-1 and thioredoxin reductase, although greatly diminished in the lungs of *t-trspi*^{6A-} mice, were not altered as a result of infection. GPX-1 activity in the lungs of the *t-trspi*^{6A-} mice was ~82% of the WT mice. In addition, the GPX-1 activity in the lungs of Se-deficient mice was 125% less than in the *t-trspi*^{6A-} mice. These results suggest that although selenoproteins are important for immune function, there is a threshold of GPX-1 activity that can prevent an increase in lung pathology during influenza infection. J. Nutr. 137: 1466–1471, 2007.

Introduction

Influenza infection is a leading cause of morbidity and mortality. Annually, influenza infections are responsible for 3–5 million cases of severe illness and 250,000–500,000 deaths worldwide (1). In response to infection, the immune system produces proinflammatory cytokines, including TNF- α ⁴ and IL-6. These cytokines have a pyrogenic effect and are important for the activation of macrophage and the subsequent T-cell response. IFN- α and IFN- β are produced by influenza-infected cells to stop viral replication and to direct the subsequent T-cell response. During infection, chemokines, including macrophage inflamma-

tory protein (MIP)⁷-1 α and monocyte chemotactic protein (MCP)-1, are produced to recruit T-cells to the lung, which produce IFN- γ to reduce viral replication and clear the infection.

Influenza productively infects lung epithelial cells and abortively infects macrophage (2) and viral replication results in the production of both reactive oxygen species (ROS) and reactive nitrogen species (RNS) (3–7). ROS and RNS are produced by the epithelial cells' metabolic pathways and alveolar macrophage as part of the immune response to infection. Production of ROS and RNS results in an increase in nuclear factor κ B, which is a transcription factor that upregulates the expression of pro-inflammatory cytokines (7,8). Although ROS and RNS production are essential parts of the immune response to viral infection, these reactive species, along with infiltrating immune cells, are responsible in part for influenza-induced lung pathogenesis (9,10).

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³ Supplemental Figure 1 is available with the online posting of this paper at jn.nutrition.org.

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⁷ Abbreviations used: Ag, antigen; APC, antigen presenting cell; GPX, glutathione peroxidase; i⁶A, isopentyladenosine; mcm⁵U, methylcarboxymethyl-5'-uridine; mcm⁵Um, methylcarboxymethyl-5'-uridine-2'-O-hydroxymethylribose; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; p.i., postinfection; qRT, real time; RNS, reactive nitrogen species; ROS, reactive oxygen species; Se, selenium; Sec, selenocysteine; SeIP, selenoprotein P; TR, thioredoxin reductase; TRx, thioredoxin; *t-trspi*^{6A}, Sec tRNA^{[Ser]Sec} transgene; WT, wild type.

Selenium (Se) is an essential micronutrient in the diet of humans and other mammals. This trace element appears to be important for mounting immune responses, including immune responses to viral infections. In both animal and human studies, Se supplementation has been shown to increase T-cell proliferation and natural killer cell cytotoxicity (11,12), whereas deficiencies in Se have been demonstrated to result in more severe viral infections, including HIV and coxsackie virus (13–17). Influenza infection can induce the production of antioxidant enzymes in the lung, including the selenoproteins glutathione peroxidase (GPX)-1 and thioredoxin reductase (TR)1. One of our laboratories has demonstrated that Se deficiency during influenza infection can alter the expression of these antioxidant enzymes (18).

Several mouse models have been generated to examine the role of selenoproteins in health (19–23). These models have taken advantage of the fact that selenoprotein expression is unique in that this class of proteins is dependent on the presence of selenocysteine (Sec) tRNA^{[Ser]Sec} for their synthesis. Thus, by perturbing the expression of Sec tRNA^{[Ser]Sec}, the synthesis of different selenoproteins, or selenoproteins as a whole, can be modulated or depleted. In this study, we used a transgenic mouse line in which the transgene encodes a mutant Sec tRNA^{[Ser]Sec} wherein the expressed tRNA product lacks a highly modified nucleoside, isopentenyladenosine (i⁶A), at position 37 (19). As a consequence, the levels of numerous selenoproteins decrease in mice expressing Sec tRNA^{[Ser]Sec} without i⁶A (i⁶A⁻) in a protein- and tissue-specific manner. This includes selenoproteins important for their antioxidant properties, such as GPX-1 and TR1.

Previous studies from one of our laboratories have demonstrated that frank Se deficiency alters the immune response to influenza and coxsackie infections (P. Sheridan, M. Bailey, J. Sheridan, M. Beck, unpublished data; 24,25). In this study, we used the i⁶A⁻ transgenic mice to determine whether influenza infection altered the expression pattern of selenoproteins in the lung (site of infection) or other tissues and if altered expression of selenoproteins changes the immune response to influenza infection.

Materials and Methods

Materials. Se-75 (⁷⁵Se) (specific activity, 1000 Ci/mmol) was obtained from the Research Reactor Facility, University of Missouri and [³H]serine (specific activity, 29 Ci/mmol) from Amersham Biosciences. NuPage 10% polyacrylamide gels and See-Blue Plus2 protein markers were purchased from Invitrogen. All other reagents were commercial products of the highest grade available.

Mice. The mice used in this study were the same as those described elsewhere (19). Control mice encoding the wild-type (WT) Sec tRNA^{[Ser]Sec} gene (designated *trsp*) were in the same genetic background (FVB/N) as transgenic mice carrying a mutant Sec tRNA^{[Ser]Sec} transgene (designated *t-trspi⁶A⁻*) (19). The care of the mice was in accordance with the NIH institutional guidelines. Male mice were transported to the University of North Carolina animal facilities, which are fully accredited by the American Association for Accreditation of Laboratory Animal Care. The mice were housed 4 per cage and were maintained under protocols approved by the Institutional Animal Use and Care Committee. Mice were fed a commercially available nonpurified diet (Lab Diet 5P76, PMI Nutrition International) (26) and allowed to acclimate for 2 wk prior to influenza infection. Se-deficient mice were generated as previously described (18).

Influenza infection. Influenza A/Bangkok/1/79 (H3N2) was propagated in 10-d-old embryonated hens' eggs. The virus was collected in the allantoic fluid and titered by hemagglutination (27). For virus inoculation, mice were anesthetized with an intraperitoneal injection of ketamine (0.022 mg) and xylazine (0.0156 mg) and instilled intranasally

with 32 hemagglutination units of influenza virus in 0.05 mL of PBS. Mice were killed by rapid cervical dislocation on d 2, 3, 5, 7, and 14 postinfection (p.i.). Uninfected (d 0) mice served as controls.

Isolation, aminoacylation, and fractionation of tRNA and quantification of the Sec tRNA^{[Ser]Sec} isoforms. Total tRNA was isolated from mouse lungs, aminoacylated with [³H]Ser (19) and unlabeled amino acids in the presence of rabbit reticulocyte synthetases (28), and the resulting aminoacylated tRNA fractionated on a RPC-5 column (29) in the absence and subsequently in the presence of Mg²⁺ as described (19–21). The amount of Sec tRNA^{[Ser]Sec} expressed from *trsp* or from the mutant *t-trspi⁶A⁻* relative to the total Ser tRNA population and the distributions of the 2 Sec tRNA^{[Ser]Sec} isoforms, methylcarboxymethyl-5'-uridine (mcm⁵U) and methylcarboxymethyl-5'-uridine-2'-O-hydroxymethylribose (mcm⁵Um), have been detailed elsewhere (19–21).

Labeling of selenoproteins. Mice were injected intraperitoneally with 50 μCi of ⁷⁵Se/g and killed 48 h after injection. Plasma was collected and liver, lung, testes, spleen, cervical lymph nodes, brain, and cerebellum were excised, immediately frozen in liquid nitrogen, and stored at -80°C until ready for use. Tissues were homogenized, extracts electrophoresed along with molecular weight markers, and developed gels were stained with Coomassie Blue, dried, and exposed to a PhosphorImager as described [see (21) and references therein].

GPX-1 assay. GPX activity was measured by a coupled assay with yeast glutathione reductase using hydrogen peroxide as a substrate following previously published methods (30).

RNA extraction, RT, and real time-PCR. mRNA levels were determined by real time-PCR. Total RNA was isolated using the TRIzol method (Life Technologies), DNase-1 treated (Invitrogen) and reverse-transcribed with Superscript II First Strand Synthesis kit (Invitrogen) using oligo(dT) primers. Real time-PCR was performed using the TaqMan chemistry (Applied Biosystems) for IFN α, β, and γ; TNF-α; IL-6; MCP-1; MIP-1α; and the matrix gene of influenza. The levels of G3PDH were determined for all samples and used to normalize gene expression levels. All primers were designed using Primer Express 1.5 from Applied Biosystems. Because baseline values did not differ between *t-trspi⁶A⁻* and WT mice, data were expressed as fold of uninfected WT controls.

Pathology scores. The right lung was perfused with 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Pathology grading was performed semiquantitatively according to the relative degree of inflammatory infiltration as previously described (25).

TABLE 1 Distributions of Sec tRNA^{[Ser]Sec} isoforms in infected lung tissue of *t-trspi⁶A⁻* and WT mice¹

Genotype	Infection	Distribution ²		Total ³	mcm ⁵ Um/mcm ⁵ U ⁴	i ⁶ A ⁻⁵
		mcm ⁵ U	mcm ⁵ Um			
<i>t-trspi⁶A⁻</i>	<i>d</i>	%				
	0	63.9	36.1	2.4	0.5	16.9
	3	65.1	34.9	3.0	0.5	18.4
	5	68.9	31.1	3.6	0.5	16.9
WT	14	70.1	29.9	2.8	0.4	20.9
	0	46.5	53.5	4.7	1.2	0
	3	46.2	53.7	4.7	1.2	0
	5	45.6	54.3	5.1	1.2	0
	14	46.1	53.9	5.2	1.2	0

¹ Values are means, *n* = 3–4.

² Percentages of mcm⁵U and mcm⁵Um resolved by RPC-5 chromatography from the seryl-tRNA population.

³ Percentage of Sec tRNA^{[Ser]Sec} population relative to the total seryl-tRNA population.

⁴ Amount of mcm⁵U divided by the amount of mcm⁵Um.

⁵ Percentage of the i⁶A⁻ isoform relative to the total seryl-tRNA population.

Statistical analysis. Cytokine/chemokine mRNA levels are expressed as the fold of uninfected WT controls. Data were analyzed by the nonparametric Mann-Whitney U test to determine significant differences between the genotypes at each time point using JMP 5.1 statistical software. Differences were considered significant at $P < 0.05$.

Results

Sec tRNA^{[Ser]^{Sec}} analysis. The Sec tRNA^{[Ser]^{Sec} population consists of 2 isoforms that differ from each other by a single methyl group attached to the 2'-O-hydroxyl site on the ribosyl moiety at position 34 (designated Um34) [reviewed in reference (31)]. The nucleosides at position 34 are mcm⁵U and mcm⁵Um. The addition of Um34 and its synthesis is dependent on Se status (31). The distributions of the 2 isoforms, mcm⁵U and mcm⁵Um, and of the i⁶A⁻ isoform relative to the total seryl-tRNA population are shown (Table 1, columns 3, 4, and 7, respectively). The distributions of the mcm⁵U and mcm⁵Um isoforms in transgenic mice appeared to shift slightly in favor of mcm⁵U with increasing times of infection. However, the relative amounts of increase in mcm⁵U and decrease in mcm⁵Um were so low that these changes were not expected to alter selenoprotein expres-}

sion (31). As expected, the total amount of the Sec tRNA^{[Ser]^{Sec} population was reduced by about one-half to one-third in the lungs of *t-trspi⁶A⁻* mice as that found in the corresponding organ of control mice (19). The level of the i⁶A⁻ isoform varied slightly in the 4 different time points examined, but the range of variation was not unlike that found in earlier studies with this mutant isoform in different tissues and organs (19,22,23). The total amount of the Sec tRNA^{[Ser]^{Sec} population (column 5) and distributions of the 2 isoforms (columns 3 and 4) remained constant during the times of infection from d 0 until d 14 in control mice.}}

Selenoprotein expression. Selenoproteins identified in previous studies as TR1, GPX1, GPX4, and Sep15, and possibly selenoprotein W (14,19–22) and as selenoprotein P (SelP) and GPX3 (21), are designated by arrows on the sides of the liver and plasma panels (Fig. 1). The initial 4 lanes in each panel contained selenoproteins from control mice ranging from d 0 to 14 p.i. and they manifested very similar patterns of labeling. Although labeled selenoproteins were reduced substantially in transgenic mice (19,22,23), the pattern of labeling was similar in most cases, with the possible exception of SelP in plasma of transgenic

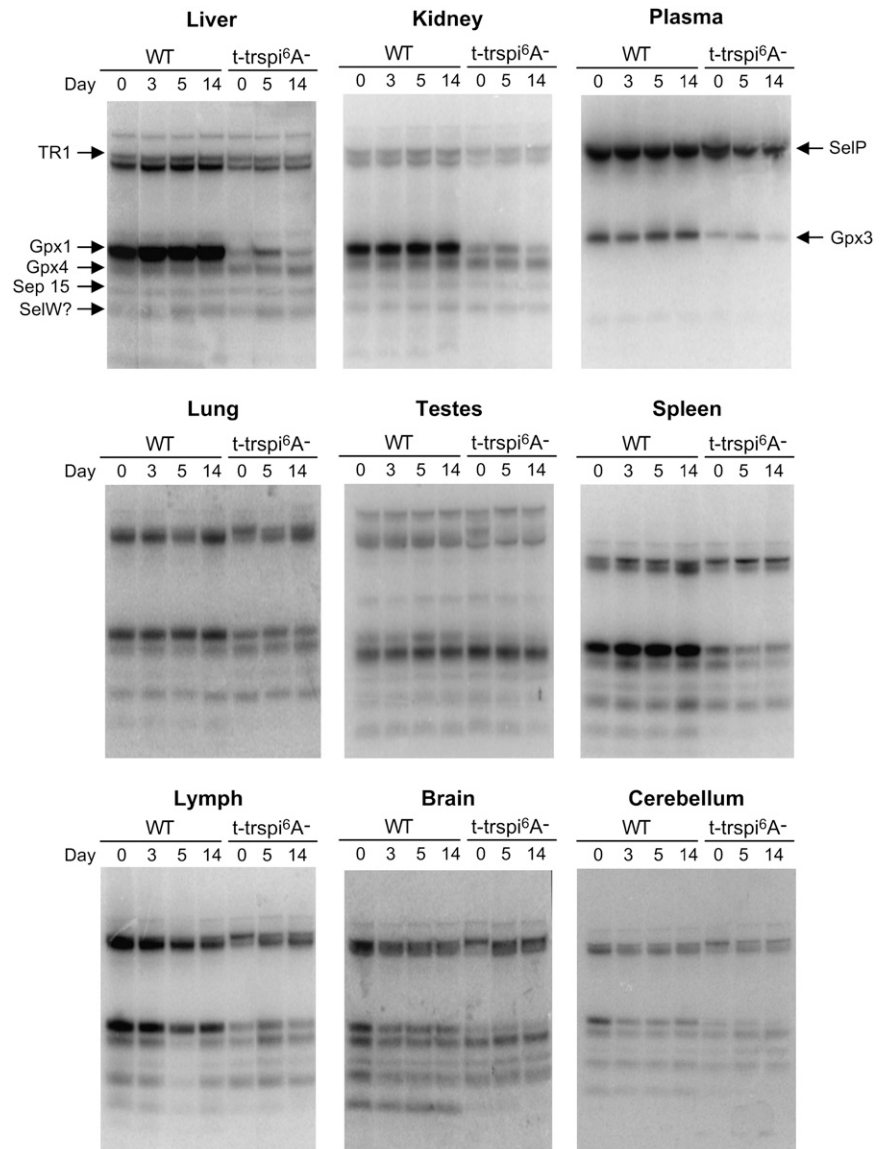


FIGURE 1 Selenoprotein analysis in organs of *t-trspi⁶A⁻* and control mice infected with influenza virus. Selenoproteins identified in previous studies are identified to the sides of the liver and plasma panels.

mice. SelP labeling appeared to decline in plasma at d 5 and 14, but this observation was not further explored, because SelP is principally synthesized in the liver and transported to other tissues in the plasma (21). The patterns of [⁷⁵Se] labeling of selenoproteins were similar in brain, cerebellum, liver, lymph nodes, plasma, spleen, and testes at the times analyzed during infection (d 0, 3, 5, and 14) in transgenic and WT mice. These observations suggest that the selenoprotein population is slightly perturbed by infection with influenza virus in transgenic or WT mice. Coomassie Blue-stained gels of total proteins from these organs appeared similar, demonstrating that virtually identical amounts of total protein were added to each lane (Supplemental Fig. 1).

GPX-1 activity. Not unexpectedly, GPX activity in *t-trspi*^{6A} lungs (1.25 ± 0.610 U/g tissue) was 82% less than that of the WT mice (6.99 ± 0.69 U/g tissue; $P \leq 0.01$). To determine how this decrease in GPX activity compared with the decrease in activity in the lungs of Se-deficient mice, we included lung samples from Se-deficient mice in the analysis. Interestingly, GPX activity in *t-trspi*^{6A} lungs was 125% higher than in Se-deficient mice (0.1 ± 0.06 U/g tissue; $P \leq 0.01$), suggesting that although the *t-trspi*^{6A} mutation decreases GPX activity, it is not as dramatic as in frank Se-deficient mice.

IFN- α , IFN- β , and pro-inflammatory cytokines are not altered in *t-trspi*^{6A} mice following influenza infection. The immune response to influenza is characterized by an early production of IFN- α/β and the pro-inflammatory cytokines TNF- α and IL-6. IFN- α , IFN- β , TNF- α , and IL-6 mRNAs were all increased in the lung following infection but did not differ between the 2 groups at any time point (Fig. 2).

MCP-1, MIP-1 α , and IFN- γ are increased in influenza-infected *t-trspi*^{6A} mice. Chemokines are produced to direct T-cells from the draining lymph node to the site of infection. Although both groups responded to infection by increasing chemokine gene expression, *t-trspi*^{6A} had increased MCP-1 ($P = 0.03$) and MIP-1 α ($P = 0.01$) expressions at d 2 p.i. At d 7 p.i., *t-trspi*^{6A} mice had greater IFN- γ expression than WT mice ($P = 0.03$) (Fig. 3).

Delayed viral clearance, but no increase in influenza, induced lung pathology in *t-trspi*^{6A} mice. Influenza-infected *t-trspi*^{6A} mice had higher levels of influenza replication at d 7 p.i., indicating an impaired ability to clear the infection from the lungs. Although lung pathology in both groups peaked at d 7 p.i., the groups did not differ in severity (Fig. 4).

Discussion

Following influenza infection, we examined the Sec tRNA^{[Ser]^{Sec} population and measured selenoprotein production by [⁷⁵Se]}

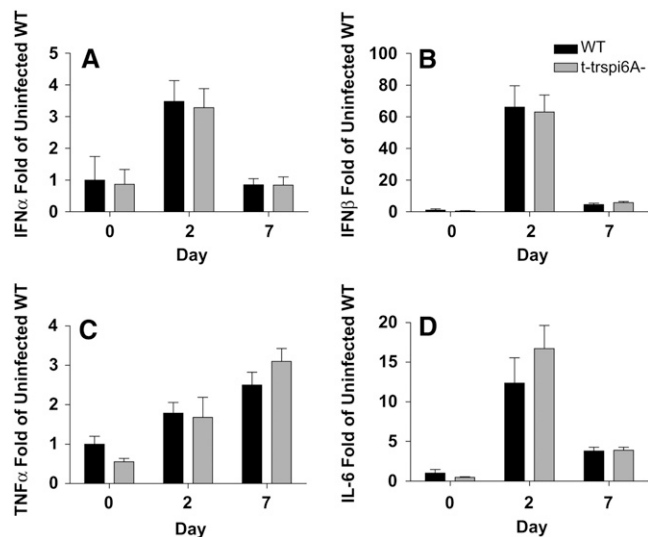


FIGURE 2 IFN- α , IFN- β , and pro-inflammatory cytokines in lungs of WT and *t-trspi*^{6A} mice following influenza infection. Values are means \pm SEM, $n = 6$. *Different from WT, $P < 0.05$.

labeling. Mutation of any modified base in Sec tRNA^{[Ser]^{Sec} results in a tRNA lacking Um34 and mimics a cell lacking the Um34 isoform (22,23). Interestingly, the 2 isoforms have different roles in selenoprotein synthesis, wherein mcm⁵U appears to support primarily the expression of housekeeping selenoproteins, whereas mcm⁵Um supports the stress-related selenoproteins (22,23). The slight variations observed in the Sec tRNA^{[Ser]^{Sec} population over the course of the infection period in *t-trspi*^{6A} and WT mice did not appear to be sufficient to cause major changes in selenoprotein expression. This was also demonstrated by a lack of appreciable change in the pattern of the [⁷⁵Se] labeling of selenoproteins in the various tissues examined, with the possible exception of SelP in plasma. It is not clear why SelP would decline in plasma, where it constitutes ~40% of the Se in this tissue (32). The other observed differences between transgenic and WT mice are most likely due to the differences in the selenoprotein population caused by the i⁶A mutant tRNA^{[Ser]^{Sec}. As expected, most selenoproteins decreased in the transgenic mice compared with WT mice in all tissues examined (19).}}}

A particularly interesting finding was that the pathology of the lungs of the transgenic mice did not differ from the WT mice. This is in contrast to Se-deficient mice, in which the lung pathology p.i. is greatly enhanced (18,25). A possible explanation for this finding is the level of GPX-1 activity. Although the transgenic mice had greatly diminished GPX-1 activity compared with the WT mice, the activity level was still 125% higher than Se-deficient mice. This suggests that there is a threshold of GPX-1 activity required to prevent the increase in lung pathology.

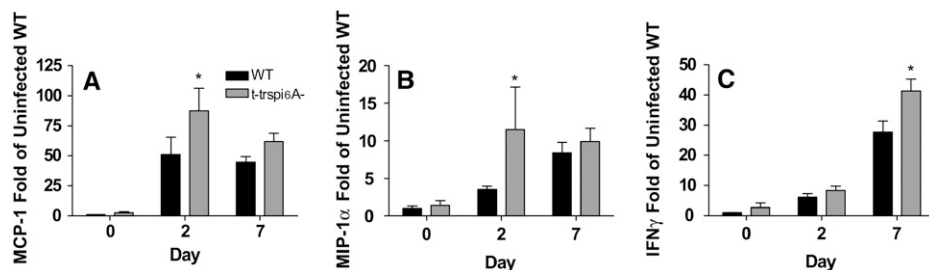


FIGURE 3 MCP-1, MIP-1 α , and IFN- γ in lungs of influenza-infected WT and *t-trspi*^{6A} mice. Values are means \pm SEM, $n = 6$. *Different from WT, $P < 0.05$.

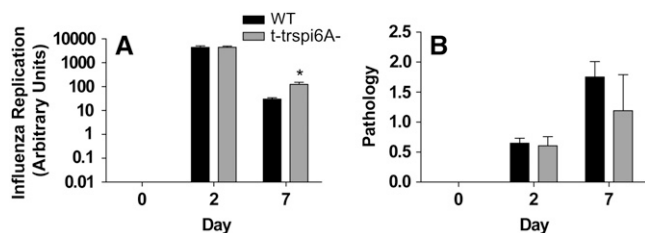


FIGURE 4 Viral clearance and lung pathology in WT and *t-trspi*^{6A-} mice. Values are means \pm SEM, $n = 6$. *Different from WT, $P < 0.05$.

However, other selenoproteins were also affected by a deficiency in Se and other selenoproteins were decreased in the transgenic mice. In addition, superoxide dismutase or catalase may have increased in the transgenic mice to compensate for diminished GPX-1 activity, as has been shown previously in Se-deficient mice (18).

Influenza infection is a potent inducer of IFN- α and IFN- β , as well as proinflammatory cytokines. In this study, the *t-trspi*^{6A-} influenza-infected mice mounted an innate immune response that was equal to the WT mice. The production of IFN- α and IFN- β is crucial for limiting the replication of influenza. Given that IFN production was similar in both groups at d 2 p.i., it is not surprising that viral replication was similar between both groups at this time point. There are currently no studies, to our knowledge, that examine the effect of either Se deficiency or supplementation on the production of IFN- α or IFN- β in vivo.

Although there were no differences in either TNF- α or IL-6 gene expression, there are published data that indicate that Se supplementation in vitro and in vivo may have the effect of either increasing or decreasing TNF- α production. In vitro studies in human umbilical vein endothelial cells demonstrated that sodium selenite decreased TNF- α production (33), whereas studies of splenic macrophages from mice given sodium selenite in water had increased basal levels of proliferation and increased TNF- α and IL-1 β protein production in response to LPS stimulation (34).

The cytokines and chemokines produced and the cells that are activated during the innate portion of the immune response are important for directing and shaping the subsequent adaptive immune response. The lungs of *t-trspi*^{6A-} mice had increased mRNA levels for both MIP-1 α and MCP-1 compared with WT mice. There are 2 possible mechanisms that may explain the increase in chemokine expression in *t-trspi*^{6A-} mice: 1) leukocytes from *t-trspi*^{6A-} do not respond to these chemokines; or 2) *t-trspi*^{6A-} mice have high levels of thioredoxin (TRx), which has been shown to inhibit leukocyte migration in response to other classical chemokines. In support of hypothesis 1, monocytes and neutrophils from Se-deficient mice with peritoneal plasmacytomas had impaired chemotactic responses to MCP-1 when compared with Se-adequate mice. The cells from the Se-deficient mice were able to respond to other chemokines, suggesting that the mechanism may be the downregulation of the receptor and not an inability to migrate (35). If the cells were not migrating to the site of chemokine expression, levels of chemokines may be increased. Studies from influenza-infected CCR2 knockout mice demonstrated that mice that were unable to respond to MCP-1 had increased levels of MIP-1 α and MCP-1 in the bronchial lavage fluid (36). Together, these data support a hypothesis that *t-trspi*^{6A-} have increased chemokine production, because the leukocytes fail to respond to the chemoattractant signal. Another possibility is that the *t-trspi*^{6A-} mice had increased levels of TRx as a result of decreased TR activity. TRx is released by

cells in response to oxidative stress (37,38) and has been demonstrated to be increased during some infections (39,40). TRx also functions as a chemoattractant (41). In vivo studies of leukocyte trafficking in TRx transgenic mice and in mice treated with TRx have revealed that TRx inhibits leukocyte trafficking in response to exogenous KC, MCP-1, RANTES, and LPS-induced inflammation (42). Together, these data indicate that chemokines and leukocyte trafficking are sensitive to manipulation by oxidative stress and Se and may be altering the immune response to influenza in the *t-trspi*^{6A-} mice. Further studies will be required to elucidate the exact mechanism.

Alterations in the redox regulation of either antigen (Ag)-presenting cells (APC) or T-cells during an Ag-specific response can alter the cell-mediated immune response (43–47). Conversely, catalytic antioxidants, such as GPX-1, decrease proinflammatory cytokine production and nuclear factor κ B (48,49). Additionally, catalytic antioxidants decrease the production of IFN- γ during in vitro T-cell stimulation by Ag. The mechanism proposed for decreased IFN- γ by antioxidants is the inhibition of the required pro-inflammatory cytokine and ROS production of T-cell activation (50). The increased production of IFN- γ by *t-trspi*^{6A-} mice may be related to increased production of ROS by APC as a result of decreased Se-containing antioxidant enzymes. A less likely explanation of these data suggests that IFN- γ levels remain high because influenza virus replication is still higher in the *t-trspi*^{6A-}. This is less likely, because we would expect higher levels of IFN- γ to more rapidly and effectively clear the virus.

The results presented here further our understanding of the importance of selenoproteins, particularly selenoproteins that function as antioxidants, in the response to viral disease. An increase in ROS due to a deficiency in GPX activity may be altering the bidirectional communication between APC and T-cells during Ag presentation, thereby affecting the immune response to infection. However, there appears to be a threshold effect of antioxidant protection. A decreased level of selenoproteins may alter the immune response, although not at a level low enough to induce changes in pathogenicity. Further studies are needed to determine the mechanism by which the *t-trspi*^{6A-} mutation affects the immune response.

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