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# Restraint stress alters lung gene expression in an experimental influenza A viral infection

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**Abstract**

In the present study the global effect of restraint stress on gene expression in the murine lung during an experimental influenza A/PR8 viral infection was examined. Gene expression profiling using high density oligonucleotide microarrays revealed that the expression of 95 genes was altered on day 3 post infection (p.i.), while 48 genes were altered on day 7 p.i. Restraint stress reduced and delayed the expression of specific cytokines, cell adhesion molecules and cell surface receptors indicating alterations in cell migration to the site of infection. Furthermore, mapping of the candidate genes to known pathways revealed that genes associated with host defense and immune responses, including chemotaxis and chemokine function, antigen presentation and processing, MHC class II receptor function and inflammation were the major pathways affected by restraint stress.


*Keywords:* Restraint; Stress; Influenza; Viral infection; Microarray; Gene expression

## 1. Introduction

The primary function of the immune system is to protect the host against infectious challenges. In response to viral infection, activation of innate and adaptive immunity is required to limit the initial spread of virus, and to ultimately terminate viral replication. However, psychological and physical stressors can lead to substantial alterations in anti-viral immune responses and viral pathogenesis (Sheridan et al., 1998; Bailey et al., 2003). As not all responses to stressors are immunosuppressive, the effect of stress on a viral infection depends upon a number of factors including the virulence of the virus and the nature of the stressor. In general, acute stressors have immunoenhancing or moderately immunosuppressive effects (Dhabhar and McEwen, 1997; Bailey et al., 2003). These effects may not dramatically alter the outcome of an infection, but they may skew the immune response and alter the formation of immunological memory. In contrast, repeated or chronic stress leads to more profound immune alterations that can have a significant impact on the health outcome of viral infections (Wonnacott and Bonneau, 2002; Sieve et al., 2004).

Previous studies from our laboratory have shown that repeated cycles of restraint stress (RST), during an influenza A/PR8 (A/PR8) viral infection in C57BL/6 mice, significantly increased plasma glucocorticoid levels compared to non-stressed, infected animals. Furthermore, lymphadenop-

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athy and the accumulation of mononuclear cells in the infected tissue were reduced in mice that underwent RST (Sheridan et al., 1991, Hermann et al., 1993, 1994). A role for stress-induced glucocorticoids in this context has been demonstrated as pharmacologic blockade of the type II steroid receptor with RU486 restored both the lymphadenopathy and the cell accumulation in the infected lungs (Hermann et al., 1995). RST also resulted in delayed natural killer (NK) cell recruitment to the lung parenchyma and reduced NK cytotoxicity during an A/PR8 infection (Hunzeker et al., 2004).

The present study focuses on RST-induced alterations in gene expression at the site of an experimental A/PR8 viral infection, the lung, by using a global approach with high density oligonucleotide microarrays. Since multiple cell types contribute to lung function and host defense, we chose to examine the effects of RST on the gene expression during infection in whole lung tissue. Therefore the alterations in mRNA levels are the combined result of changes in gene expression of resident cells and the gene expression of cells infiltrating the lung during the course of infection.

Previous data from our laboratory showed that mRNA expression levels of CCL2/MCP-1 and CCL3/MIP-1 alpha in A/PR8 virus-infected lungs increased by day 1 post infection (p.i.) and peaked around day 3 p.i. This rise in chemokine gene expression was paralleled by an immigration of NK cells into the lungs on days 1 and 3 p.i. By day 7 p.i. the percentage of NK cells returned to baseline (Hunzeker et al., 2004). However, virus-specific T cells continued to accumulate in the infected lung reaching peak numbers between 7 and 10 days p.i. (Baumgarth et al., 1994). Furthermore, it has been demonstrated that cytotoxic T cells ($CD8^+$) play an important role in viral clearance (Ada and Jones, 1986; Doherty et al., 1992; Mackenzie et al., 1989; Yap and Ada, 1978). Based on these findings, two time points during the infection were targeted for microarray analysis of gene expression in the lungs of A/PR8-virus infected mice: day 3 p.i. for innate responses and day 7 p.i. to assess adaptive responses.

## 2. Materials and methods

### 2.1. Animals

Male C57BL/6 mice, aged 6–8 weeks, were purchased from Charles River Laboratories (Wilmington, MA) and were housed 3–5 animals per cage in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). The animals were randomly assigned to the following experimental groups: non-stressed, influenza A/PR8 virus-infected controls on day 3 and day 7 (InfCTRL d3 and InfCTRL d7); food and water deprived, influenza A/PR8 virus-infected mice on day 3 and day 7 (InfFWD d3 and InfFWD d7) and restraint-stressed, influenza A/PR8 virus-infected mice on day 3 and day 7 (InfRST d3 and InfRST d7). An additional control group was comprised of non-stressed, non-infected mice (CTRL). All mice were kept under a 12:12 h light/dark cycle and had ad libitum access to food and water except during restraint stress or food and water deprivation cycles.

### 2.2. Infection

Mice were anesthetized by intramuscular injection with a mixture of ketamine (78.1 mg/kg) and xylazine (4.4 mg/kg) and infected intranasally with 16 hemagglutinating units (HAU) of influenza A/PR/8/34 (A/PR8) virus in 50 μl phosphate-buffered saline. The animals were sacrificed on day 3 and day 7 p.i. by cervical dislocation and the lungs were removed.

### 2.3. Restraint stress procedure

On 7 consecutive days, starting 1 day prior to infection, InfRST mice were placed in well ventilated plastic tubes for 16 h during their active phase within their home cages as previously described (Sheridan et al., 1991). During restraint, mice did not have access to food and water. To control for the impact of food and water deprivation on lung gene expression, InfFWD mice were allowed to move freely within their home cages, but food and water was removed during the same time period that InfRST mice were subjected to restraint stress. InfCTRL mice remained undisturbed in their home cages with ad libitum access to food and water.

### 2.4. Microarray sample preparation

cRNA target preparation for use with the Murine Genome U74Av2 GeneChip (Affymetrix, Santa Clara, CA) was performed according to the Affymetrix Expression Analysis Technical Manual and Roy et al. (2002). Briefly, total RNA from individual mouse lung tissue was isolated using TRIzol Reagent (GIBCO/BRL) and further purified with RNeasy columns (Qiagen, Valencia, CA). First strand cDNA synthesis was carried out with 10 μg total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and a T7-$(dT)_{24}$ primer (5′-GGCCAGTGAATTGTAATACGACTCATATAGGGAGGCGG$(dT)_{24}$-3′). Second strand synthesis was performed using *Escherichia coli* DNA polymerase, *E. coli* ligase and T4 DNA polymerase (Invitrogen, Carlsbad, CA). Double stranded cDNA was cleaned up by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. Biotin-labeled cRNA was prepared by in vitro transcription using the ENZO BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY). Fragmentation of the labeled cRNA and hybridization to the array were performed by the Davis Heart and Lung Research Institute Genetics/Microarray Core Lab, Ohio State University, Columbus, OH.

### 2.5. Data analysis

Data analysis was performed by using the Microarray Suite (MAS) 5.0 software (Affymetrix, Santa Clara, CA). Each microarray was subjected to a global scaling to a mean signal intensity of 1500. The presence or absence of a transcript probed by the array was determined with the absolute analysis algorithm using default detection $p$-value cutoffs. To determine the expression differences between the restraint-stressed animals and the non-stressed animals on day 3 and day 7 post infection each InfRST dataset ($n$=4 each day) was compared to its corresponding InfCTRL dataset ($n$=3 each day) using the MAS 5.0 comparison analysis at default settings (i.e. InfRST d3 vs. InfCTRL d3 and InfRST d7 vs. InfCTRL d7). In order to account for a possible influence of food and water deprivation during the restraint stress procedure, the InfFWD groups for day 3 and day 7 ($n$= 3 each day) were compared to their corresponding InfCTRL groups (i.e. InfFWD d3 vs. InfCTRL d3 and InfFWD d7 vs. InfCTRL d7). The median signal log ratio and the median fold change of all pairwise comparisons for the day 3 groups and the day 7 groups were calculated for every gene. For selection of differentially expressed genes in the InfRST vs. InfCTRL group the following criteria were applied: (1) at least 2-fold change in expression, (2) consistent changes in all pairwise comparisons and (3) $p$<0.05, Mann–Whitney $U$-test (SPSS for Windows, Version 11.5, SPSS, Chicago, IL). The corresponding genes were also selected from the InfFWD vs. InfCTRL group but without consideration of the above mentioned selection criteria. However, for genes where changes were indicated in the InfFWD vs. InfCTRL group, these changes were consistent in at least 5 out of 9 pairwise comparisons, but none of them was statistically significant (Mann–Whitney $U$-test, n.s.). The genes were grouped according to their biological function as derived from gene ontology annotations using the Netaffx analysis center (Liu et al., 2003) and the changes in gene expression are summarized in Table 1 for day 3 and Table 2 for day 7.

In addition, InfCTRL mice on day 3 and day 7 were compared to the uninfected CTRL group ($n$=3) as described above to assess alterations in gene expression upon an experimental influenza A/PR8 viral infection. However, this comparison is not the major focus of the present work and is only used to outline differences in activated signaling pathways in the InfRST animals versus the InfCTRLs (Fig. 1).

### 2.6. Mapping to pathways

To understand how restraint-induced changes were interrelated with respect to signaling pathways, the candidate genes were mapped onto known pathways using GenMAPP and MAPPFinder software (Dahlquist et al., 2002; Doniger et al., 2003) in combination with Gene Ontology annotations (Ashburner et al., 2000).

## 3. Results

### 3.1. Global analysis of lung gene expression

To compare the gene expression profile in the lungs of A/PR8 infected C57BL/6 mice that were exposed to repeated cyles of RST to non-stressed, infected animals, a high-throughput approach using high-density oligonucleotide microarrays was used. On days 3 and 7 p.i., the lung transcriptome of RST A/PR8-infected mice (InfRST) was compared to non-stressed, infected animals (InfCTRL). To account for a possible influence of food and water deprivation during the restraint stress procedure, the transcriptome of food and water deprived infected mice (InfFWD) was also compared to non-stressed infected mice (InfCTRL) on days 3 and 7 p.i.

On day 3 p.i., 95 genes were significantly altered at least 2-fold in the InfRST group compared to the InfCTRL group. These genes were grouped according to their biological function and are presented in Table 1 (expressed sequence tags (ESTs) and genes for which no description was available were excluded). Furthermore, the results from the comparison of the InfFWD group with the InfCTRL group is also shown in Table 1. This comparison assesses the impact of food and water deprivation on the alterations seen in the InfRST group. On day 7 p.i., gene expression of 48 genes was altered at least 2-fold in the InfRST group compared to the InfCTRL group and these genes are shown in Table 2 (again ESTs and genes for which no description was available were not included). Similar to day 3, the changes in the corresponding genes from the comparison of the InfFWD and the InfCTRL animals are shown. The majority of the changes on days 3 and 7 in the InfRST group were not present in the InfFWD group and therefore likely represent alterations that were induced by the stressor. Categorically, the biological functions that were affected by modulation of the expression of these genes include the immune response, cell growth, proliferation, differentiation and apoptosis as well as various metabolic activities. In addition, Tables 1 and 2 show several genes involved in various processes whose gene expression was altered also in the InfFWD mice. However, many of the changes were less pronounced, or were in the opposite direction, when compared to the InfRST group.

### 3.2. The impact of restraint stress on immune response

Chronic restraint decreased the gene expression of monocyte and lymphocyte attracting chemokines in the lungs of A/PR8 virus-infected mice (Table 1). In InfRST mice gene expression of CXCL12, CCL7/MCP-3 and CCL12/MCP-5 was decreased on day 3 p.i. compared to InfCTRL animals. Neutrophil-attracting calgranulin A and antiviral interferon-β were increased in the InfRST group on day 7 p.i. (Table 2), which is about 4–5 days later than in non-stressed, infected (InfCTRL) animals (data not shown). The gene expression of histocompatibility class II antigens was

Table 1
Restraint stress-induced alterations in gene expression in the lungs of influenza A infected mice on day 3 post infection

| Gene | Affymetrix accession no. | Median fold change in expression | |
|---|---|---|---|
| | | InfRST vs. InfCTRL | InfFWD vs. InfCTRL |
| *Immune response* | | | |
| histocompatibility 2, class II antigen A, alpha | 92866_at | −2.1 | nc |
| histocompatibility 2, class II antigen E beta | 94285_at | −2.1 | nc |
| periplakin | 102223_at | 2.2 | nc |
| Fc receptor, IgG, high affinity I | 102879_s_at | −2.5 | nc |
| leukocyte specific transcript 1 | 103571_at | −2.9 | nc |
| chemokine (C–X–C motif) ligand 12 (CXCL12) | 162234_f_at | −2.4 | nc |
| chemokine (C–X–C motif) ligand 12 (CXCL12) | 100112_at | −2.1 | nc |
| chemokine (C–C motif) ligand 12 (CCL12) | 93717_at | −3.4 | nc |
| chemokine (C–C motif) ligand 7 (CCL7) | 94761_at | −2.9 | nc |
| integrin alpha X | 104308_at | −2.2 | nc |
| macrophage scavenger receptor 1 | 94140_at | −2.1 | nc |
| complement component 3a receptor 1 | 103707_at | −2.8 | nc |
| interleukin 1 receptor, type II | 161689_f_at | 2.4 | nc |
| interleukin 1 receptor antagonist (IL-1ra) | 93871_at | −2.8 | nc |
| CD34 antigen | 97773_at | −2.1 | nc |
| CD44 antigen | 103005_s_at | −2.0 | −1.7 |
| CD72 antigen | 101878_at | −6.5 | 1.2 |
| *Cell growth, proliferation, differentiation, apoptosis* | | | |
| insulin-like growth factor binding protein 3 | 95082_at | 3.4 | nc |
| insulin-like growth factor binding protein 6 | 103904_at | 4.6 | nc |
| p53 apoptosis effector related to Pmp22 | 97825_at | 2.5 | nc |
| ecotropic viral integration site 2 | 98025_at | −2.4 | nc |
| schlafen 3 | 98299_s_at | −3.2 | nc |
| schlafen 4 | 92315_at | −2.1 | nc |
| granzyme A | 102995_s_at | −2.4 | nc |
| tenascin C | 101993_at | −2.5 | nc |
| cyclin G2 | 98478_at | 2.5 | 1.6 |
| transducer of ErbB-2.1 | 99532_at | 2.6 | 1.6 |
| brain derived neurotrophic factor | 102727_at | 2.1 | 1.6 |
| erythroid differentiation regulator | 98525_f_at | −2.4 | 1.6 |
| *Metabolism* | | | |
| aldehyde dehydrogenase family 1, subfamily A1 | 100068_at | 2.0 | nc |
| glycogenin 1 | 162262_f_at | −2.1 | nc |
| spermidine synthase | 92540_f_at | −2.2 | nc |
| branched chain ketoacid dehydrogenase E1, alpha polypeptide | 96035_at | 2.5 | nc |
| phospholipase A2, group XII | 104343_f_at | 2.1 | nc |
| glycine amidinotransferase (L-arginine:glycine amidinotransferase) | 96336_at | −4.3 | nc |
| chitinase, acidic | 160509_at | 2.9 | nc |
| stearoyl-Coenzyme A desaturase 1 | 94056_at | 2.2 | 1.6 |
| stearoyl-Coenzyme A desaturase 1 | 94057_g_at | 2.8 | 1.6 |
| ectonucleotide pyrophosphatase/phosphodiesterase 2 | 97317_at | 2.8 | 1.7 |
| dipeptidase 1 (renal) | 103644_at | 2.1 | 1.3 |
| carboxypeptidase E | 99643_f_at | 2.7 | 2.0 |
| latexin | 96065_at | −2.2 | −1.4 |
| carboxylesterase 3 | 101539_f_at | 3.1 | 1.9 |
| thioether *S*-methyltransferase | 97402_at | 2.1 | 1.5 |
| *Ion binding* | | | |
| selenium binding protein 1 | 100596_at | 2.5 | 1.5 |
| sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2 | 104375_at | 2.1 | 1.7 |
| *Cytoskeleton* | | | |
| microtubule-associated protein 1 light chain 3 | 160288_at | 2.4 | nc |
| tubulin, beta 5 | 94788_f_at | −2.1 | nc |
| gamma-aminobutyric acid (GABA(A)) receptor-associated protein-like 1 | 93011_at | 2.1 | nc |
| claudin 3 | 94493_at | 2.0 | nc |
| actinin, alpha 1 | 92280_at | 2.3 | 2.3 |

Table 1*(continued)*

| Gene | Affymetrix accession no. | Median fold change in expression | |
|---|---|---|---|
| | | InfRST vs. InfCTRL | InfFWD vs. InfCTRL |
| *Transport* | | | |
| solute carrier family 15 (H+/peptide transporter), member 2 | 103918_at | 2.3 | nc |
| solute carrier family 12, member 2 | 99500_at | 2.4 | 1.6 |
| ATPase, Na+/K+ transporting, alpha 1 polypeptide | 93797_g_at | 2.0 | 1.6 |
| ATP-binding cassette, sub-family A (ABC1), member 1 | 94354_at | 2.9 | 1.5 |
| solute carrier family 23 (nucleobase transporters), member 3 | 161149_r_at | 2.0 | 1.9 |
| *Electron transport* | | | |
| cytochrome b-245, beta polypeptide | 100300_at | −2.3 | nc |
| cytochrome P450, family 2, subfamily b, polypeptide 10 | 102701_at | 2.0 | nc |
| P450 (cytochrome) oxidoreductase | 99019_at | 2.6 | nc |
| *Others* | | | |
| tenascin C | 101993_at | −2.5 | nc |
| sciellin | 161132_at | 3.1 | nc |
| glycoprotein 49 B | 92217_s_at | −2.2 | nc |
| procollagen, type XVIII, alpha 1 | 101881_g_at | 2.4 | nc |
| transforming growth factor, beta induced, 68 kDa | 92877_at | −2.3 | nc |
| CDC like kinase 1 | 93274_at | 2.0 | 1.3 |
| CDC like kinase 4 | 101936_at | 2.0 | 1.7 |
| small proline-rich protein 1A | 160909_at | −3.2 | −2.0 |
| reduced expression 3 | 93021_at | 2.8 | 1.4 |
| acidic (leucine-rich) nuclear phosphoprotein 32 family, member A | 93372_at | 5.1 | 4.0 |
| DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5 | 93493_at | 2.1 | 1.9 |
| neural plakophilin-related arm repeat protein (fragment) homolog | 96187_at | 2.0 | 1.5 |
| plexin B2 | 96464_at | −2.0 | −1.4 |
| DnaJ (Hsp40) homolog, subfamily B, member 9 | 96679_at | 2.4 | 2.6 |
| trans acting transcription factor 3 | 96192_at | 2.0 | 1.9 |

Expressed sequence tags (ESTs) and genes for which no description was available are not shown. nc=no change.

about 2-fold decreased on day 3 p.i. and further decreased on day 7 p.i. in the InfRST group (Tables 1 and 2). Also immunoglobulin gene expression was decreased on day 7 p.i. In InfFWD mice those transcripts showed no changes or were increased as compared to InfCTRLs. The gene expression of T-cell specific cell surface markers was decreased on day 7 p.i. in the lungs of InfRST animals compared to InfCTRL mice (Table 2). This change is even more pronounced given that the expression of these genes was increased in the InfFWD group when compared to the InfCTRLs. Furthermore, $p56^{lck}$ and ZAP-70 tyrosine kinase gene expression were reduced about 3-fold in InfRST lungs compared to InfCTRL lungs, but increased almost 2-fold in InfFWD animals.

### 3.3. The impact of restraint stress on the inflammatory response pathway

The transcripts with the highest scores that were decreased in InfRST animals on day 3 and 7 p.i. were associated with the host defense and immune responses, chemotaxis and chemokine function, antigen presentation and processing, MHC class II receptor function, the inflammatory response, but also with extracellular matrix constituents and collagen (day 3) and oxygen transport (day 7). Fig. 1 is a graphical representation of our data from day 7 p.i. on the GenMAPP Inflammatory Response Pathway. Influenza A/PR8 infection activated inflammatory and immune response genes like interleukin (IL)-1β, IL-6, IL-10R and interferon-γ (Fig. 1A). RST markedly decreased the transcription of genes attributed to T cell mediated responses like IL-2Rβ, CD 28 or LCK and Zap-70 kinases in infected animals (Fig. 1B).

### 3.4. The impact of restraint stress on cell growth, proliferation, differentiation or apoptosis

In the lungs of InfRST mice gene expression of granzyme A (days 3 and 7) and granzyme B (day 7), which are important mediators of apoptosis produced by cytotoxic T cells and NK cells, was reduced as compared to InfCTRLs.

Gene expression of all four members of the *schlafen* (*slfn*) gene family in the lungs of non-stressed A/PR8-infected mice (InfCTRL) was increased on days 3 and 7 p. i. (data not shown). In contrast, restraint stress decreased the expression of *slfn* genes. When compared to InfCTRL, gene expression of *slfn* 3 and *slfn* 4 was 3.2-fold and 2.1-fold reduced in InfRST mice, respectively (Table 1).

### 3.5. The impact of restraint stress on metabolism

In InfRST mice the expression of genes associated with anabolic functions like glycogen synthesis (glycogenin 1), creatine synthesis (glycine amidinotransferase) and polyamine synthesis (spermidine synthase) were decreased on day 3 and day 7 p.i. However, stearoyl-Coenzyme A desaturase 1

Table 2
Restraint stress-induced alterations in gene expression in the lungs of influenza A infected mice on day 7 post infection

| Gene | Affymetrix accession no. | Median fold change in expression | |
|---|---|---|---|
| | | InfRST vs. InfCTRL | InfFWD vs. InfCTRL |
| *Immune response* | | | |
| S100 calcium binding protein A8 (calgranulin A) | 103448_at | 2.2 | nc |
| interferon beta, fibroblast | 94145_at | 2.0 | nc |
| histocompatibility 2, class II antigen A, alpha | 92866_at | −3.7 | nc |
| histocompatibility 2, class II antigen A, beta 1 | 100998_at | −3.7 | nc |
| histocompatibility 2, class II antigen E beta | 94285_at | −3.7 | nc |
| histocompatibility 2, class II, locus Mb1 | 98034_at | −3.4 | nc |
| histocompatibility 2, class II, locus Mb1 | 98035_g_at | −2.5 | nc |
| histocompatibility 2, class II, locus DMa | 93092_at | −2.5 | nc |
| Ia-associated invariant chain | 101054_at | −2.3 | nc |
| immunoglobulin heavy chain (J558 family) | 100583_at | −2.8 | 1.4 |
| immunoglobulin kappa chain variable 28 (V28) | 93086_at | −4.1 | 1.4 |
| immunoglobulin heavy chain 6 (heavy chain of IgM) | 93584_at | −2.4 | nc |
| T-cell receptor germline beta−chain gene constant region | 93105_s_at | −3.9 | 1.4 |
| T-cell receptor beta−chain mRNA, VDJ region, clone 7.9R.1F8 | 93106_i_at | −4.6 | 2.0 |
| thymus cell antigen 1, theta | 99057_at | −4.6 | 1.3 |
| CD3 antigen, delta polypeptide | 92683_at | −4.0 | 2.8 |
| CD8 antigen, beta chain | 162410_s_at | −5.7 | 1.7 |
| lymphocyte protein tyrosine kinase (p56-LCK) | 102809_s_at | −3.1 | 1.9 |
| tyrosine protein kinase ZAP-70 | 93662_s_at | −3.0 | 1.7 |
| *Cell growth, proliferation, differentiation, apoptosis* | | | |
| growth arrest and DNA-damage-inducible 45 alpha | 102292_at | 2.3 | nc |
| ephrin B2 | 160857_at | 2.5 | nc |
| granzyme A | 102995_s_at | −3.7 | 1.4 |
| granzyme B | 102877_at | −3.5 | nc |
| antigen identified by monoclonal antibody Ki 67 | 99457_at | −2.6 | nc |
| protein tyrosine phosphatase, non-receptor type 8 | 92356_at | −3.0 | 1.4 |
| *Metabolism* | | | |
| stearoyl-Coenzyme A desaturase 1 | 94056_at | 2.5 | nc |
| stearoyl-Coenzyme A desaturase 1 | 94057_g_at | 2.9 | nc |
| glycine amidinotransferase (L-arginine:glycine amidinotransferase) | 96336_at | −3.5 | nc |
| *Transcription regulation* | | | |
| mesenchyme homeobox 2 | 99937_at | 2.5 | nc |
| Kruppel-like factor 13 | 160617_at | −2.1 | −1.7 |
| *Others* | | | |
| apolipoprotein D | 93592_at | 4.8 | nc |
| coronin, actin binding protein 1A | 96648_at | −2.1 | nc |
| guanylate cyclase 1, soluble, beta 3 | 93954_at | 2.0 | nc |
| matrix metalloproteinase 8 | 94769_at | 2.6 | nc |
| actinin, alpha 1 | 92280_at | 2.1 | 1.7 |
| DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5 | 93493_at | 2.0 | 1.5 |
| DnaJ (Hsp40) homolog, subfamily B, member 9 | 96679_at | 2.4 | 1.7 |
| prolyl 4-hydroxylase, alpha 1 polypeptide | 104139_at | 2.2 | 1.5 |

and fatty acid synthase, both involved in fatty acid synthesis, were increased in InfRST and in InfFWD mice on day 3 p.i., but were only increased in InfRST animals on day 7 p.i.

### *3.6. The impact of food and water deprivation on lung gene expression*

Except for CD44 antigen on day 7 p.i., in InfFWD mice, expression of genes involved in the immune response was not altered, or the changes were in the opposite direction when compared to the InfRST group. Food and water deprivation resulted in altered expression of genes involved in metabolism and transport on day 3 p.i. (Table 1). However, these alterations were not observed on day 7 p.i.

## 4. Discussion

Chronic stress, experimentally induced by repeated cycles of restraint, induced substantial alterations in gene expres-

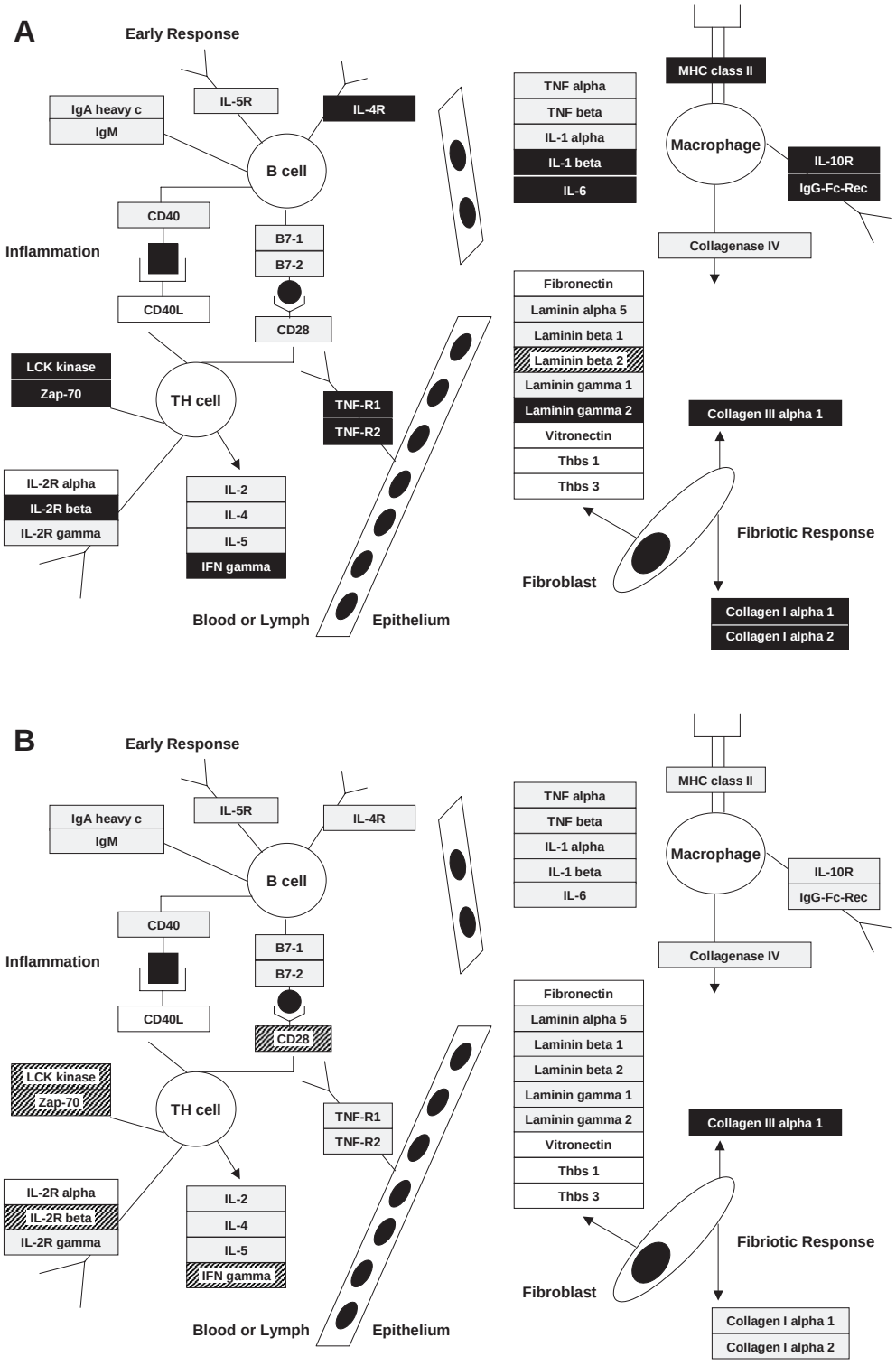


Fig. 1. GenMAPP Inflammatory Response Pathways integrating our expression data. (A) Alterations in gene expression caused by influenza A infection represented by the comparison of infected (InfCTRL) vs. uninfected (CTRL) mice. (B) Restraint-induced alterations in infected mice represented by the comparison of infected restraint mice (InfRST) vs. infected controls (InfCTRL). Boxes indicate increased (black) or decreased (hatched) expression as compared to the controls. Grey indicates that the selection criteria were not met, but the gene was represented on the array. White boxes indicate that the gene was not represented on the array.

sion in the lungs of influenza A/PR8 virus-infected mice. On day 3 and day 7 p.i., 95 and 48 genes were altered at least 2-fold in InfRST animals compared to InfCTRLs, respectively.

Influenza A/PR8 virus-infected epithelial cells and macrophages produce chemotactic, antiviral, and pro-inflammatory cytokines which stimulate the innate immune

response and recruit mononuclear cells for the clearance of the virus (Hennet et al., 1992). Restraint reduced the expression of monocyte and lymphocyte attracting chemokines on day 3 p.i. and delayed the increase in neutrophil-attracting and antiviral chemokines until day 7 p.i. (Tables 1 and 2). Furthermore, the expression of histocompatibility class II antigens on both days and immunoglobulins and T cell specific cell surface markers on day 7 p.i. was decreased in the lungs of InfRST animals. Together with the reduced expression of cell adhesion receptors CD34 (ligand for L-selectin) and CD44 on day 3 p.i., these observations suggest that RST reduced and delayed recruitment of monocytes/macrophages, neutrophils and lymphocytes to the site of infection. This is in line with recent studies showing a delayed migration of NK cells to the lung of influenza A/PR8 virus-infected mice following chronic restraint (Hunzeker et al., 2004). The RST-induced reduction of the mononuclear cell infiltrate in the lungs of A/PR8 infected mice was restored by treatment with the glucocorticoid receptor antagonist RU486, thus implying a role for glucocorticoids in this process (Hermann et al., 1995). The glucocorticoid receptor is highly expressed in human airway epithelium and bronchial vessel endothelium (Adcock et al., 1996) and modulatory effects of glucocorticoids on cytokine gene expression are well established (Refojo et al., 2001). Decreased cytokine production and therefore reduced recruitment of cells, that in turn are themselves producers of cytokines necessary for the activation of adaptive immune responses, may lead to a delay in the overall immune response to the pathogen and therefore increase the pathophysiology of this respiratory infection.

Apoptosis is an important mechanism for the elimination of virus-infected cells. Influenza A infection has been shown to increase gene expression of the apoptosis mediators perforin and granzyme A and B in activated $CD8^+$ cells in vivo (Johnson et al., 2003). However, destruction of lymphoid tissue and lymphoid depletion during an experimental influenza A infection, that ultimately leads to the death of the animal, appear to be the result of virus-induced apoptosis (Tumpey et al., 2000). Restraint decreased the expression of granzyme A and B in the lungs of InfRST mice. The reduced apoptosis might lead to less tissue damage and less respiratory difficulty, but at the same time might increase the viral load and therefore might delay viral clearance. Indeed, it recently has been shown that on day 5 p.i. restraint mice exhibited virus titers that were 20-fold higher than in infected control mice (Hunzeker et al., 2004).

Members of the *schlafen* (*slfn*) gene family have been implicated in growth regulation and T cell development (Schwarz et al., 1998). One interesting finding is the reduced expression of *slfn* 3 and 4 in InfRST animals. In addition, we found *slfn* 1, 2, 3 and 4 to be increased in non-stressed influenza A/PR8 infected animals when compared to non-infected controls. The stressor-induced alterations in *slfn* gene expression show the same direction as, for example, $p56^{lck}$ and ZAP-70 tyrosine kinase. Since both kinases play a role in the intracellular signaling during lymphocyte activation, *slfn* might be an interesting target for future studies. However, the role of the *slfn* gene family during infection has not been established yet.

The inherent aspect of food and water deprivation during restraint stress was not responsible for reduced expression of genes relevant to the immune response. Although food and water deprivation itself can have immunomodulatory effects (Good and Lorenz, 1992; Stefanski, 2001), basically no changes in gene expression of immune response related genes were observed on days 3 and 7 p.i. in InfFWD animals, except for T cell associated genes on day 7 p.i.. In contrast to the decreased expression of T cell related genes on day 7 p.i. in InfRST mice, food and water deprivation alone (InfFWD group) increased the expression of those genes (Table 2) and might indicate different regulation of T cell associated immune function in InfFWD and InfRST mice.

Alterations in the expression of genes involved in metabolism on day 3 p.i., that were observed in InfRST and InfFWD mice, were no longer observed in InfFWD on day 7 p.i. and probably represent metabolic adaptations to the limited availability of food and water. However, the comparison of InfRST and InfFWD groups (both vs. InfCTRL) clearly indicated decreased lung gene expression of genes involved in anabolic functions like glycogen synthesis, creatine synthesis and polyamine synthesis on day 3 p.i. that was attributed to chronic restraint only. It is known, that hormones like catecholamines and glucocorticoids, that are typically released in response to a stressor, trigger catabolic processes that are considered to restore internal homeostasis (Elenkov et al., 2000; Sapolsky et al., 2000).

Integration of the microarray data into known signaling pathways revealed that a large number of different genes were affected by chronic restraint. The majority of the altered genes were involved in the immune response to a viral pathogen. In summary, in chronically stressed, infected mice the expression of immune response genes was decreased and/or delayed. The results from this study therefore further support the notion that chronic stress can impair the host immune response to a viral infection and, in addition, may influence disease progression.

However, gene expression of lung resident cells as well as gene expression by infiltrating cells contributes to the overall disease outcome, and future studies will be necessary to separate these two aspects of the host response to viral infection during stress. Furthermore, posttranscriptional modifications represent an important regulatory mechanism as well and the future assessment of major gene products will be crucial for understanding the biological significance of stress-induced alterations in the immune response to an infectious challenge.

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