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Stress and the anti-influenza immune response: repeated social defeat augments clonal expansion of CD8⁺T cells during primary influenza A viral infection

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Abstract

Social disruption stress (SDR) prior to primary influenza A virus (IAV) infection augments memory to IAV re-challenge in a T cell-specific manner. However, the effect of SDR on the primary anti-viral immune response has not been elucidated. In this study, SDR-infected (INF) mice terminated viral gene expression earlier and mounted an enhanced pulmonary IAV-specific CD8⁺T cell response versus controls. Additionally, SDR-INF mice had a more pro-inflammatory lung profile prior to and during infection and an attenuated corticosterone response. These data demonstrate neuroendocrine modification of the lung microenvironment and increased antigen-specific T cell activation, clonal expansion and viral control in stress-exposed mice.

Keywords

psychosocial stress; glucocorticoid; mice; virus; influenza; T cell; immune response; HPA axis; sympathetic nervous system; tetramer; inflammation; neuroendocrine

1. Introduction

Neuroendocrine interactions influence the immune system via complex connections and feedback mechanisms (Sanders & Kohm 2002; Sternberg 2006; Webster Marketon & Glaser 2008). Environmental and behavioral cues are translated by the autonomic nervous system and the hypothalamic pituitary adrenal (HPA) axis into regulatory signals with immunological consequences. Immune cells, including lymphocytes, can both receive signals from hormonal and neuronal mediators and initiate signaling through the production

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of molecules such as cytokines. The respiratory tract contains rich vasculature and autonomic nervous system innervation, particularly within the lung, making it an excellent system in which to investigate stress-mediated changes in the immune system (Baile 1996; Hoyle *et al.*, 1998; Abelson *et al.*, 2010). These direct physical connections allow for ample exposure to stress-induced neural mediators and serum factors and may account for behavioral induced changes seen in the respiratory immune response. We recently reported that a mouse model of repeated social defeat, social disruption stress (SDR), leads to enhanced CD4⁺ and CD8⁺ memory T cell responses when the stressor is applied prior to a primary influenza A virus (IAV) infection and the memory and recall responses are tested (Mays *et al.*, 2010). Furthermore, some aspects of enhanced anti-viral immunity during the primary infection were transferable with the adoptive transfer of dendritic cells from socially-stressed mice to naïve mice prior to IAV infection (Powell *et al.*, 2011). However, it is not known at what point social stress affects the CD8⁺T cell populations during a primary anti-viral immune response, if changes observed in these cells have functional consequences, and how T cell subsets are affected.

Connecting the primary immune response to the establishment and function of immune memory is key for this study, because the response pattern of the primary CD8⁺T cell immune response ultimately shapes the pattern of protective immunological memory that will persist after resolution of the infection (Hou *et al.*, 1994; Badovinac *et al.*, 2004). In addition to laying a pattern for a future memory response during the primary infection, CD8⁺T cells are critical for virus eradication (Lin & Askonas 1981; Lukacher *et al.*, 1984; Epstein *et al.*, 1998). Consequently, stress-induced immune alterations, including these changes reported in anti-influenza memory cell populations after SDR, that are detected during memory responses likely stem from modification of the immune response during primary infection. Such stress-induced changes have yet to be investigated. Several possibilities exist, but the resulting enlargement of the memory CD8⁺T cell population was hypothesized to stem from either increases in clonal expansion of T cells during primary IAV infection in SDR-experienced mice, or a differential amount of effector cell contraction during the transition from primary IAV response to resting memory.

The present study was designed to characterize the impact of SDR on the primary antiviral immune response and to pinpoint changes that could lead to the previously observed SDR-induced anti-IAV memory enhancement (Mays *et al.*, 2010). This set of experiments focused on the IAV-specific adaptive immune response, specifically the CD8⁺T cell response, and associated environmental changes during a primary viral infection after exposure to SDR (SDR-INF) or no stressor exposure (INF). Earlier memory studies using the influenza A/PR/8/34 virus model focused on the NP366 subset of virus-specific CD8⁺T cells, as this is the immunodominant subset during memory in H2D^b mice for the influenza A/PR/8/34 virus. However, additional TCR subsets play an important role during the primary immune response (Chen *et al.*, 2001; Crowe *et al.*, 2003; Chen *et al.*, 2004). For the present study, we focused on two major, primary immunodominant epitopes, PA224-33 and NP366-74, which have been well-characterized by other groups (Doherty *et al.*, 2006). Unexpectedly, in this study there was a differential effect of SDR on the activation and expansion of two CD8⁺ T cell subsets during primary IAV infection, suggesting that stress may alter peptide liberation or processing by the immune system. SDR, furthermore, enhanced the overall anti-viral immune response in a manner that resulted in earlier cessation of viral gene expression and more pro-inflammatory and anti-viral cytokine expression. Taken together, this study demonstrates potent modulation of a primary murine IAV infection and the antiviral CD8⁺ T cell response by psychosocial stress.

2. Materials and Methods

2.1. Animals

Male C57BL/6 (6–8 weeks) mice were obtained from Charles River (San Diego, CA). Experimental animals were housed 3–5 per cage in an AAALAC (American Association of Accreditation of Laboratory Animal Care) accredited facility and had access to food and water *ad libitum*. Mice were allowed to acclimate to their surroundings for at least one week prior to any manipulation, and were maintained on a 12 hour light/dark cycle with lights on at 6.00. Intruders for the social disruption paradigm were C57BL/6 or CD-1 males that were pre-screened for aggressive behavior and were individually housed.

2.2. Social Disruption Stress

The social disruption stress paradigm has been previously characterized in detail (Avitsur *et al.*, 2001; Stark *et al.*, 2001). Briefly, mice underwent 6 consecutive cycles of SDR prior to infection. A single cycle of SDR consisted of placing an intruder into the cage of experimental mice from ~17.00 to 19.00 each day for 6 days. Sessions were monitored to ensure that the intruder repeatedly attacked and consistently defeated the resident mice. If the residents defeated the intruder, or if the intruder did not attack the residents, then the intruder was removed and replaced by a different intruder. During the stressor, neuroendocrine profiles and behavior were expected to be similar to those in previously published reports, which included increased anxiety-like behavior and a significant increase in circulating corticosterone and catecholamines (Avitsur *et al.*, 2001; Kinsey *et al.*, 2007). During SDR, the resident mice generally displayed submissive behaviors, including upright submissive posture, fleeing and crouching (Avitsur *et al.*, 2001). The non-stress control group (INF) was isolated from the stressor and had no visual, gustatory, or auditory exposure to SDR during the stress cycles for that individual experiment. Mice that were infected with IAV were all transferred, for regulatory reasons, to the same room of the animal facility in which SDR took place for other experiments. Overly aggressive intruders that were inflicting severe cutaneous wounds were removed and replaced with a different intruder. Less than 5% of mice needed to be removed from the study due to cutaneous wounding. All animal care procedures were according to guidelines established by the NIH Guide for the Care and Use of laboratory animals, and protocols were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee (ILACUC).

2.3. Virus and Infection

For the primary infection, mice were infected 14 or 36 hours after the conclusion of the last SDR cycle. Earlier experiments utilized the 36 hr gap, however, no immunological or physiological differences were noted between the two infection times, and the switch to a shorter time frame was made to facilitate a more efficient experimental timeline. Influenza A/PR/8/34 virus was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs, titrated using a hemagglutination assay, aliquoted and frozen at -70°C . Immediately prior to infection, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (78.1/4.4 mg/kg, Vedco, St. Joseph's, MO) diluted in sterile saline. For intranasal infection, 1 hemagglutinating unit (HAU) of influenza A/PR/8/34 virus suspended in 50 μl sterile PBS was instilled in the nares. This dose is sublethal, and results in mild clinical infection in C57BL/6 mice.

2.4. Corticosterone Analysis

On the indicated days post-infection, mice were sacrificed by decapitation and the trunk blood was collected. Care was taken to collect blood samples at the same time each morning with minimal manipulation immediately of mice prior to sampling. Serum was stored at

–70°C until analysis using the commercially available DA corticosterone assay (ICN Biomedicals Inc., Costa Mesa, CA) per manufacturer's instructions.

2.5. Isolation of Primary Cells

Mice were sacrificed via cervical dislocation. The apical lobe of each lung was flash frozen in liquid nitrogen for PCR analysis, and the remaining lung was digested for one hour on ice in HBSS and Type I collagenase (Worthington, Lakewood, NJ). Spleens and lungs were pulverized using a Model 80 Biomaster Stomacher (Seward, UK) in ice-cold HBSS. Red blood cells were lysed with buffer (0.16 M NH_4Cl , 10mM KHCO_3 , 0.13mM EDTA) and single cell suspensions were filtered through a nylon 70 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cell counts were determined using a Beckman Z2 Coulter Counter (Coulter Corp, Miami, FL).

2.6. MACs selection of CD8⁺T cells and Flow Cytometry

Specific lymphocyte subtypes were measured by immunofluorescent antibody staining and analysis using flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA). To examine activation markers on virus-specific CD8⁺T cells within the parameters of four-color flow cytometry, CD8⁺T cells were enriched by magnetic bead selection. After organs were processed to a single cell suspension, the MACs staining protocol was carried out per manufacturer's instructions for the CD8⁺T cell isolation kit (Miltenyi Biotec, Auburn, CA). After negative selection (all non-CD8⁺T cells were retained in separating column), cells were counted and adjusted to 2.0×10^7 cells/ml. An aliquot from each sample was stained for CD3, CD4 and CD8 to check for purity after MACs separation. Average post-sort CD8⁺ purity was 90.2%. This value was used to adjust the post-sort cell counts to give the total number of CD8⁺T cells per sample. Cells were stained for flow cytometry per standard FACs/tetramer protocol with modifications (Mays, *et al.*, 2010). Briefly, one million CD8⁺T cells were incubated with anti-CD16/CD32 for 10 minutes at room temperature, and then an optimized concentration of fluorochrome-conjugated antibody and tetramer was added for 45 min at 40°C. Samples were washed with FACS buffer and read on a FACSCalibur (BD Biosciences, San Jose, CA). From each preparation, 100,000 events within the lymphocyte gate were acquired. Appropriate compensation, isotype and negative controls were used to control for background and for instrument set up. An irrelevant tetramer for the herpes simplex virus (HSV) glycoprotein B 495–505 epitope (SSIEFARL) was used as a negative control for tetramer staining. Leukocyte subpopulations were identified and gated using forward versus side scatter characteristics. Cytotoxic T cells (CD8⁺, CD3e⁺ cells) were identified using directly-conjugated antibodies. Influenza-specific CD8⁺ cells (NP₃₆₆₋₇₄tetramer⁺, CD8⁺ cells) were identified by gating on the CD3⁺/CD8⁺ lymphocyte population and then selecting the CD3⁺/NP₃₆₆₋₇₄⁺ events. The tetramer probes consist of four complexed peptide/H2Db complexes labeled with commercially manufactured streptavidin-PE or APC and were conjugated by the NIH Tetramer Facility at Emory University. The NP₃₆₆₋₇₄ (influenza, ASNENMETM), PA224-36 (influenza, SSLENFRAYV) and GB495-505 (HSV, SSIEFARL) peptides were synthesized by the OSU Peptide Synthesis Facility. All antibodies were obtained from BD Biosciences (San Jose, CA). Results were analyzed using CellQuest software (BD Biosciences, San Jose, CA) and FlowJo (TreeStar, Ashland, OR).

2.7. IgG Enzyme-linked immunoassay (ELISA)

Blood samples were collected from the tail vein. Serum was separated and frozen until assayed by ELISA for IgG. Plates were coated with A/PR/8/34 virus overnight, blocked with 10% FBS then serially diluted samples (1:4) were assayed as previously described (Mays *et al.*, 2010). Antibody titers were determined by taking the reciprocal of the last dilution two

standard deviations above the mean O.D. of the negative serum control. The resulting titers were then log-transformed to a geometric mean titer for statistical analysis.

2.8. RNA extraction and Real-time PCR

RNA was isolated from the apical lobe of the lung in Trizol according to manufacturer's instructions (GibcoBRL, Rockville, MD). Reverse transcription and cDNA synthesis were carried out using a Promega kit (Madison, WI). Cytokine primer and probe sequences were developed with Primer Express Software from PE Biosystems (Foster City, CA.), and the influenza A matrix (M1) protein primers and probe were based on a previously published sequence (van Elden *et al.*, 2001). Primer/probe sequences were as follows: IFN α Forward (5'-GTCGGCAAAGAAATCAAGATGG) Reverse (5'-TCAATGGCAGAACTGTAGTCTTCG) and Probe (5'-CCTGACTTGTTTGAAGACCTAAAGAACTGTTACAGTGA); IFN β Forward (5'-GGCCTCAAAGGAAAGAATCTATAC) Reverse (5'-GTATTGCTTGGGATCCACACTCT) and Probe (5'-ATGAAAGACGGCACACCCACCTG); IFN γ Forward (5'-GATATCTCGAGGAAGTGGCAAAA) Reverse (5'-CTTCAAAGAGTCTGAGGTAGAAAGAGATAAT) and Probe (5'-TGGTGACATGAAAATCCTGCAGAGCCA); IL-12(p40) Forward (AGCTAACCATCTCCTGGTTTGC) Reverse (CACCTCTACAACATAAACGTCTTTC) and Probe (TGCTGGTGTCTCCACTCATGGCCA); Influenza matrix (M1) Forward (5'-GGACTGCAGCGTAGACGCTT) Reverse (5'-CATCCTGTTGTATATGAGGCCCAT) and Probe (5'-CTCAGTTATTCTGCTGGTGCACCTTGCCA). Cytokine, M1, and 18s probes were labeled at the 5' end with the reporter dye 6-carboxy-fluorescein (FAM) and the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3' end. Labeled probes were synthesized by PE Biosystems. Single-plex PCR reactions included primers, probe and Taqman Universal Master Mix (Applied Biosystems, Foster City, CA). The resulting change in fluorescence was measured using an Applied Biosystems 7700 Prism Sequence Detector (Applied Biosystems, Carlsbad, CA) and analyzed using Sequence Detector version 1.6. The relative amount of transcript was determined using the comparative CT (cycle threshold) method. The DCT value of the sample was calculated by subtracting the sample CT value from the sample's 18s CT. The DCT of the control group, unless otherwise noted, the uninfected, day 0 INF mice, was averaged to calculate the baseline DCT. This was subtracted from each sample's DCT to compute the DDCT value, which represents the fold-increase of gene expression over baseline. These are the values shown in the figures.

2.9. Statistical Analysis

All data were analyzed using Statview software (SAS Institute Inc., Cary, NC). Changes in body weight were analyzed using repeated measures analysis of variance (RM-ANOVA). Corticosterone levels, PCR and flow cytometric data were analyzed using two-factor ANOVA with stress (SDR) and day post-infection as the between subject factors, with individual t-tests when indicated by an *a priori* hypothesis including an expected biological function at a specific timepoint. When appropriate, a Fisher PLSD post hoc test was performed to assess differences between experimental groups. If data were non-parametric, a Mann-Whitney test was used. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. SDR exposure hastens viral clearance and modifies the basic responses to IAV infection

Influenza-related morbidity in the murine model is associated with sickness behavior. Sickness behavior during viral infection includes reduced motor activity, anhedonia,

(leading to weight loss) and reduction in social and grooming behaviors. Mouse body weight was measured daily throughout the primary IAV infection to monitor weight loss. SDR-INF mice (n=42) underwent 6 consecutive cycles of SDR and were infected 14 hours after the conclusion of the last cycle with A/PR/8/34 virus. INF mice (n=45) were concurrently infected with influenza A/PR/8/34 virus, and both groups were followed through 3-weeks post-infection (pi). Data are expressed as body weight (g) change per day. Mortality was not observed with the sublethal IAV dose. Throughout the primary influenza infection, no main effect was observed in body weight (Figure 1) between INF and SDR-INF mice (RM-ANOVA, $F(1)=0.45$, $p=0.50$), however a significant interaction between time and stressor was detected (RM-ANOVA, $F(21)=2.75$, $p\leq 0.0001$). This suggests that SDR alters the pattern of weight loss over time during primary IAV infection, though there is not a clear positive or negative effect on SDR-INF weight loss.

To assess whether SDR changed the immune control or clinical course of an IAV infection, viral replication was measured in the lungs during a primary IAV infection. The apical lobe of the lung was harvested at indicated points post-infection and used for real-time RT PCR analysis of influenza M1 gene expression. The influenza M1 gene codes for a protein that is part of the internal components of the virus, and is detectable in infected tissue during viral replication (Lamb & Krug 2001). Expression of influenza M1 RNA detected by real-time PCR served as a surrogate marker of influenza virus replication in lung tissue. Expression was calculated as fold-increase from day 1 (input virus) level at each point pi (Figure 2). There was a significant reduction in M1 gene expression in the SDR-INF group (10-fold less) when compared to the INF group at 7 days pi, indicating that viral replication was terminated earlier in SDR-INF mice ($p<0.05$, Mann-Whitney Rank Sum Test, $n=4-5$ mice/group/day). The peak expression (5 days pi, nonsignificant) and termination (7 days pi) of viral gene expression were queried independently as both are critical points during primary viral infection. This result was consistently observed in three similar experiments, however, the data shown in Figure 2 are from a single representative experiment. The nuances of this pattern are instructive for modulation of the anti-viral immune response due to SDR exposure, despite a lack of overall significance between stress groups over the entire time course (2-factor ANOVA, $F_{(1,31)}=2.563$, $p>0.05$).

A respiratory tract infection caused by an influenza virus induces a stress response in the host that is accompanied by activation of the HPA axis and leads to elevated levels of circulating corticosterone (Dunn *et al.*, 1989; Hermann *et al.*, 1994). In this manner, corticosterone levels may also be used as an adjunct measure of infection severity. To determine if SDR altered the IAV-induced HPA axis activation, serum corticosterone was measured in INF and SDR-INF mice (Figure 2). Pre-infection control mice had a circulating corticosterone concentration of 40 ng/ml, which agrees with previously published data on corticosterone levels at the nadir of the circadian rhythm (Hermann *et al.*, 1994). SDR-INF mice had ~80 ng/ml of serum corticosterone on day 0, though this difference was not statistically significant. Analysis revealed a significant stress-by-day interaction in serum corticosterone levels (2-factor ANOVA, $F_{(4,39)}=5.682$, $p<0.01$). In addition, Hermann *et al.* reported increased serum corticosterone in non-stressed mice at day 7 pi, so this critical time point was tested independently in the current dataset (Hermann *et al.*, 1994). Seven days after infection, IAV infection alone significantly increased the amount of serum corticosterone in the INF controls (T test, $p<0.05$), but did not significantly increase the serum corticosterone level of the SDR-INF group compared to baseline. Additionally, at day 7 there was a significant difference between stress groups (T test, $p<0.05$). The serum corticosterone concentration was 150 ng/ml in the INF mice at 7 days pi. In contrast, the corticosterone concentration was 60 ng/ml, or 40% of the concentration observed in the INF mice on the same day. Furthermore, the levels seen in the 7 dpi SDR-INF mice resembled

those measured in naïve controls. These data demonstrate that SDR prior to IAV infection attenuated the IAV-induced secretion of corticosterone.

As sympathetic nervous system activation has been shown to alter the development of the antibody response (Sheridan *et al.*, 1998; Sanders & Kohm 2002; Sanders *et al.*, 2003), we followed the development of the anti-IAV antibody response throughout the primary infection. Blood samples were taken from the tail vein at 2 and 3 weeks post-infection. An anti-influenza IgG ELISA was used to compare serum IgG antibody titers (Table 1). These data were used to confirm seroconversion of the mice, and also as a measure of the magnitude of the antibody response. Data are presented as the log-transformation of four-fold serial dilution of the serum samples. A 4-fold difference in antibody titer is considered biologically significant (Hobson *et al.*, 1972). In the log-transformed data presented in Table 1, a difference of >1 is a 4-fold change in antibody titer. IAV infection induced detectable levels of IgG antibody titers in the blood of the INF controls two weeks after the primary infection (Experiment 1). The IgG antibody titers rose between 2 and 3 weeks post-infection. SDR significantly reduced the IgG anti-influenza antibody titers after an IAV infection (ANOVA, $F_{(1,72)}=46.563$, $p<0.0001$). There was a greater than four-fold difference between the INF and SDR-INF groups at both 2 and 3 weeks pi. A similar pattern was observed in Experiment 2 in which SDR attenuated the IAV infection-induced IgG antibody titers (ANOVA, $F_{(1,62)}=17.223$, $p<0.001$). In this experiment, a 4-fold difference in titer was only observed at 2 weeks post-infection. However, there was still a 2-fold difference in antibody titers 3 weeks following the infection. These data suggest that SDR prior to an IAV infection led to a reduced titer of circulating anti-influenza IgG antibody.

3.2. SDR induced a pulmonary pro-inflammatory environment that was sustained throughout viral infection

In previous studies, CD11b⁺ myeloid cells from SDR mice have been shown to produce increased amounts of pro-inflammatory cytokines, including IL-6 and TNF- α upon TLR-ligation (Avitsur *et al.*, 2005; Powell *et al.*, 2009). These and other cytokines, such as the type I interferons (IFN α and β) and IFN γ , drive the anti-viral immune response. Thus, the expression of a panel of cytokines in lung tissue during a primary IAV infection was determined using a real-time PCR approach. In this experimental model, the protein levels of lung proinflammatory cytokines track closely with mRNA expression, as detailed in Curry *et al.* 2009, and Powell *et al.*, 2009, though we cannot rigorously exclude any post-translational modification of cytokines in this study. IFN α expression (Figure 3a) was significantly increased in SDR-INF mice throughout the infection ($F_{(1,31)}=7.190$, $p<0.05$). Near peak clonal expansion of the CD8⁺T cell response (day 7 pi), IFN α and β mRNA were significantly upregulated in the lungs of SDR-INF mice when compared with INF mice (Figure 3A, B). A Mann-Whitney Rank Sum Test was used to compare fold-increase data between stress groups during the critical points for individual cytokines IFN β and IL-12 (* $p<0.05$), though no overall significance was observed between stress groups during the infection (2-factor ANOVA: IFN β $F_{(1,31)}=0.702$, $p>0.05$; IL-12 $F_{(1,31)}=0.176$, $p>0.05$). Expression of IL-12 mRNA was significantly increased in SDR-INF mice at days 3 and 5 pi corresponding to the transition from the innate to the adaptive phase of the immune response, which is the critical time of action for IL-12. We found that, as expected, the Type I interferons were only actively expressed during the acute anti-viral response, as was IL-12. Exposure to SDR alone significantly increased lung mRNA expression of IFN γ , the upregulation of which persisted throughout a primary IAV infection (2-factor ANOVA, $F_{(1,43)}=14.83$, $p<0.001$). We noted in early studies a more sustained upregulation of pulmonary IFN γ mRNA expression in SDR-INF mice, and here report a longer time course for this cytokine than the others because its expression is sustained over a more protracted time period (Mays *et al.*, 2010). A panel of other cytokines and chemokines important

during IAV infection was also assayed. No differences were noted in the lung for IL-6, IL-15, MCP2 or MIP-1a, and no consistent differences in expression were noted for IL-10 and TNF α (data not shown). No other host defense mechanisms were assessed in this study.

3.3. SDR preferentially induced expansion of the NP366 but not PA224 subset of influenza-specific CD8⁺T cells

During resting memory, at 6 weeks pi, previous work from our group found a significant increase in the frequency of memory NP₃₆₆₋₇₄CD8⁺ T cells in lung tissue in SDR mice (Mays et al., 2010). The critical goal of the present study was the flow cytometric characterization of the primary CD8⁺T cell response using tetramer staining to enumerate virus-specific CD8⁺T cell populations. Work in the murine experimental influenza model has clearly shown important anti-IAV CD8⁺T cell responses are present in both the lung and spleen, and both organs were examined in this study (Doherty *et al.*, 2006). Cells from the spleen and lung were enriched for CD8⁺T cells and stained for flow cytometry. The peak CD8⁺T cell response occurred at 10 days pi in the lung, and 8 days pi in the spleen. Figure 4A shows the lung IAV-specific NP₃₆₆₋₇₄CD8⁺ T cell response, and illustrates a significant increase during clonal expansion in the SDR-INF NP₃₆₆₋₇₄CD8⁺ T cell population in the lung (2-factor ANOVA, $F_{(1,26)}=4.617$, $p<0.05$). Contraction of this cell population was observed after 10 days pi, and was similar between INF and SDR-INF NP₃₆₆₋₇₄CD8⁺ T cell populations (72% and 70%, respectively, by day 22 pi). Statistically significant changes were not observed in the spleen (Figure 4B). Taken together, these data suggest, first, that the alterations previously reported in the memory NP₃₆₆₋₇₄CD8⁺ T cell population (Mays *et al.*, 2010) occur during clonal expansion of the NP₃₆₆₋₇₄CD8⁺ T cell subset, and, second, that SDR enhanced the frequency of IAV-specific T cells at the site of infection in the lung tissue.

In the same manner as described for the NP₃₆₆₋₇₄CD8⁺ T cell population, another H2D^b restricted immunodominant subtype, the PA₂₂₄₋₃₃CD8⁺ T cell population, was assessed throughout the primary influenza infection. Figure 5 illustrates the expansion and contraction of D^bPA₂₂₄₋₃₃CD8⁺ T cells in lung (5A) and spleen (5B) tissues. No significant differences were seen between INF and SDR-INF mice for this population in lung tissue. Interestingly, in the spleen, SDR-INF mice had a significantly elevated D^bPA₂₂₄₋₃₃CD8⁺ T cell population at day 8 p.i (unpaired T test, $p<0.05$). As the peak of the splenic CD8⁺T cell response occurs near day 8 pi in an IAV infection, this day was tested independently using an unpaired T test. No significant increases were observed overall between INF and SDR-INF groups by ANOVA, nor was there a significant increase in SDR-INF clonal expansion for the PA₂₂₄₋₃₃CD8⁺ T cell subset, as was seen in SDR-INF lung tissue for the NP₃₆₆₋₇₄CD8⁺ T cell population (Figure 4A).

Activation markers on the CD8⁺T cell population were also analyzed during an IAV infection. CD8⁺T cells were isolated from the lung and stained for activation markers on day 0 and 9 days pi. The gating strategy for this experiment is detailed in Figure 6A–B. Significantly more CD8⁺T cells were found at day 9 pi in SDR-INF lung tissue (Figure 6C). A significant increase ($p<0.05$) was also seen in lung D^bNP₃₆₆₋₇₄CD8⁺ T cellularity (Figure 6D) on 9 day pi in SDR-INF mice. When activation markers on these cells were assessed, a significant increase in the number of lung CD25⁺ CD62L^{LO}NP₃₆₆₋₇₄CD8⁺ T cells was noted in the SDR-INF mice (Figure 6E), though the percent of activated D^bNP₃₆₆₋₇₄CD8⁺ T cells was not significantly different between INF and SDR-INF mice (Figure 6B). Functional CD8⁺T cells assays were not done on these samples. These data support and extend the observations from an earlier experiment, discussed in Figure 4, in which a significant increase in the lung D^bNP₃₆₆₋₇₄CD8⁺ T cell population during clonal expansion was found. Here, it is clear that an increase occurred in both the total CD8⁺T cell population (Figure 6C) and NP₃₆₆₋₇₄CD8⁺ cell population (Figure 6D), and that more highly-activated IAV-

specific effector cells were found in SDR-INF lungs at the peak of the CD8⁺T cell response (Figure 6E).

4. Discussion

This study was initiated to delineate when and how SDR alters the CD8⁺T cell immune response to primary IAV infection. We found that SDR alone altered the pulmonary cytokine milieu, and that epitope-specific CD8⁺T cell frequency was increased early in the infection during clonal expansion. Further changes in the lung pro-inflammatory gene expression profile were present following a pathogenic challenge. These findings extend the hyper-proinflammatory phenotype of SDR-experienced mice beyond the spleen and into non-lymphoid (lung) tissue. This is in agreement with Curry *et al.*, who reported an increase in lung IL-1 β expression after 2 cycles of SDR (Curry *et al.*, 2010). We do not anticipate that blockade of IFN γ would abolish these effects on T cells during IAV infection as it is not the only inflammatory factor induced by SDR, however we would expect alteration of the inflammatory milieu to potentially abrogate the effects seen in this study. Furthermore, as cytokines such as IL-6, which had been reported to be systemically upregulated following SDR, can be inflammatory or immunomodulatory, more complex regulation may be at hand (Avitsur *et al.*, 2005).

The HPA axis has a regulatory role in the attenuation of inflammation and return to homeostasis via glucocorticoid mediators. An interesting nuance of the SDR model is the development of functional GC resistance in CD11b⁺ and CD11c⁺ populations which has not been reported in other experimental models of repeated/chronic stress (Avitsur *et al.*, 2001; Powell *et al.*, 2009). Interestingly, in this study, SDR-INF mice did not experience the typical IAV-induced rise in circulating corticosterone (Hermann *et al.*, 1994). Corticosterone regulates cell trafficking to lungs during the anti-IAV immune response (Hermann *et al.*, 1995). Day 7, the time at which SDR suppressed the IAV-induced increase in serum corticosterone is a point during IAV infection in which CD8⁺T cells traffic to the infected lung tissue, so it follows that a decrease in corticosterone-mediated regulation of T cell trafficking at this time point could contribute to an increase in the influx of lung CD8⁺T cells during clonal expansion in SDR-INF mice. Alternate mechanisms, including direct or indirect effects of GCs on antigen presenting cells may be in play and should be investigated in future studies.

During IAV infection, SDR-INF mice terminated viral replication more rapidly in the lung than did INF mice during time points assessed by real-time RT PCR measurement of expression of the M1 influenza gene. Established work demonstrates the importance of the CD8⁺T cell in the termination of viral replication during a primary IAV infection (Lin & Askonas 1981; Lukacher *et al.*, 1984). Corresponding to the earlier termination of viral gene replication observed in SDR-INF mice, we also found increased numbers of virus-specific CD8⁺T cells in the lung tissue of SDR-INF mice, suggesting that these cells actively contributed to effective viral clearance in SDR-INF mice. When we followed the expansion and contraction of two CD8⁺T cell populations specific for two immunodominant epitopes of the A/PR/8/34 virus during a primary infection, we found significantly enhanced clonal expansion in the lung NP₃₆₆₋₇₄CD8⁺T cell subset, but not in the lung PA₂₂₄₋₃₃CD8⁺T cell subset. The approximate inverse was noted in the spleen profile, where a significant increase in the size of the PA₂₂₄₋₃₃CD8⁺T cell subset was measured at day 8 pi. Although it would be plausible to also see an SDR-effect in the post-effector phase CD8⁺T cell contraction, when the expansion and contraction of two immunodominant subtypes of CD8⁺T cells during a primary influenza infection were tracked, the data strongly supported an SDR-induced increase in clonal expansion rather than any event during the apoptosis-rich contraction phase. Enhanced expansion of the NP₃₆₆₋₇₄CD8⁺T cell population was confined to the lung.

Interestingly, this is the subtype that comprises 90–95% of a recall response to influenza A/PR/8/34 (Doherty *et al.*, 2006). In 2003, Crowe, *et al.* reported that NP366-74 and PA224-33 peptides are presented differentially by the innate immune system (Crowe *et al.*, 2003). When antigen was measured in cells recovered directly from influenza-infected C57BL/6 mice, NP366 was detected on both DCs and non-DCs, whereas efficient PA224 expression was restricted to the DC population (Crowe *et al.*, 2003). Other studies have shown more promiscuous presentation of PA224, but all agree that the NP366 epitope is more widely presented and cross-presented during IAV infection (Chen *et al.*, 2004). These studies suggest that during a primary infection, the equivalent NP₃₆₆- and PA₂₂₄-specific responses may result from the requisite stimulation of naïve CD8⁺T cells by DCs. During a secondary response, however, less stringent requirements for stimulation of CD8⁺T cells may result in an increased range of NP₃₆₆ presentation and the resulting shift in immunodominance (Crowe *et al.*, 2003; Chen *et al.*, 2004; La Gruta *et al.*, 2006). The amplification in clonal expansion described in the present study in the SDR NP₃₆₆₋₇₄CD8⁺T cell population occurred during the primary, not the recall, response. However, promiscuous antigen processing and presentation of the NP366-74 epitope, particularly in an environment where DCs are already activated by SDR (Powell *et al.*, 2011), could lend itself to preferential expansion of this population during a primary adaptive response. It is not known whether variation in inflammation alone may alter the CD8⁺T cell immunodominance hierarchy of an influenza infection, or if the present observation results from factors unique to neuroendocrine modulation of the immune response.

The model of social stress, SDR, used for the present study, however, is uniquely suited to activate the HPA axis, the SNS and other stress-reactive pathways in an ethologically relevant manner through disruption of an established social hierarchy and repeated physical defeat. Therefore, SDR may delineate differential regulatory mechanisms and relationships between the nervous, endocrine and immune systems versus those seen in other models of chronic stress. Similar to SDR, in many models of chronic stress, the DC also plays a central role and is frequently affected by both glucocorticoids and catecholamines with downstream and direct effects on CD8⁺T cells during an anti-viral immune response (Truckenmiller *et al.*, 2005; Freeman *et al.*, 2007; Elftman *et al.*, 2010). In SDR, as in all experimental systems, the nature of the pathogen under investigation plays a critical role in determining the direction of the inflammatory and adaptive immune responses. When a similar model of social stress was used in conjunction with a systemic lymphocytic choriomeningitis virus (LCMV) infection, the investigators also found no effect of SDR on CD8⁺T cell apoptosis, which is in agreement with the present work, however, in the LCMV model system, the stressor reduced the CD8⁺T cell response to LCMV and had no effect on virus clearance (Sommershof *et al.*, 2011). LCMV is a systemic infection with a very different tissue tropism and infectious course than IAV, which makes it difficult to directly compare the effect of SDR on immune parameters in each viral system. In a mouse model of a neurotropic virus infection, Theiler's murine encephalomyelitis virus, SDR prior to infection exacerbated disease by priming virus-induced neuroinflammation, however, the experience of the social stressor during infection had beneficial effects on clinical disease course and inflammation (Johnson *et al.*, 2004; Vichaya *et al.*, 2011). These differences underscore the importance of both the timing of the stressor and the cellular infectious profile of the viral agent used when comparing neuroendocrine regulation of replicating antigens.

Influenza A viruses and psychological stressors are long-standing hazards to general public health. Although the constantly drifting and shifting influenza virus is widely studied, the regulation and modulation of IAV infection by the neuroendocrine system is not clearly understood. Data presented in this study, and by others, demonstrate that components of the stress response interact potently with the anti-viral immune response to change the course of specific viral infections (Hermann *et al.*, 1993; Sloan *et al.*, 2006; Freeman *et al.*, 2007;

Mays *et al.*, 2010; Grebe *et al.*, 2010; Powell *et al.*, 2011). Given the importance of influenza viruses specifically to the global health scene, it is valuable to understand factors beyond the immune system that regulate the anti-IAV immune response.

While the present data are not the first to show that lung inflammation, including the upregulation of IFN γ gene expression, augments the murine immune response to IAV (Tuvim *et al.*, 2009), this is the first study to show that psychosocial stress contributes to lung inflammation which in turn augments the anti-IAV immune response. This study illustrates the complex interplay between the nervous, endocrine and immune systems and supports the study of stress biology in the context of viral immunology to aid in the improvement of vaccination strategies and anti-viral therapies.

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Figure 1.

Social disruption did not affect weight loss associated with an IAV infection. Mice were weighed at 24-hour intervals during IAV infection. SDR-INF mice (n=42, open boxes) underwent 6 days of SDR prior to infection, and were infected with a sub-lethal dose of IAV concurrently with INF (n=45, closed boxes) mice. Data are shown as body weight change (g) per day.

Figure 2.

IAV M1 gene expression was terminated earlier in SDR-INF mice, and SDR suppressed the virus-induced rise in circulating corticosterone. Mice underwent six cycles of SDR or were isolated from the stressor prior to infection with IAV. (A) On the indicated days post-infection, lungs were harvested and flash-frozen for real time PCR analysis of influenza matrix protein (M1) gene expression. Bars indicate the SEM of 4–5 mice per group. Data are representative of similar findings in at least three independent experiments; analysis was done on individual datasets. * $p < 0.05$, Mann-Whitney Rank Sum Test. (B) Mouse serum was sampled at various times during IAV infection, and corticosterone levels were assessed. Bars indicate SEM of 4–5 mice per group each day. The symbols # and * denote a significant (unpaired T test, $p < 0.05$) difference in corticosterone versus pre-infection, no SDR mice and between stress groups, respectively. Data are representative of two independent experiments.

Figure 3.

SDR increased expression of anti-viral and pro-inflammatory cytokine mRNA during the primary immune response. Mice underwent six cycles of SDR or were isolated from the stressor prior to infection with IAV. On the indicated day post-infection, lungs were harvested and flash-frozen for real time PCR analysis of cytokine gene expression for Type I interferons (A, B), IL-12 (C) and IFN γ (D). Bars indicate the SEM of 4–6 mice per group. Data from a representative experiment were compared across time using a two-factor ANOVA (main group effect, ** $p < 0.01$ or * $p < 0.05$), and a Mann-Whitney Rank Sum Test was used to compare fold-increase data between stress groups during the critical points for individual cytokines, * $p < 0.05$. Data are representative of similar findings in at least three independent experiments.

Figure 4.

Expansion and contraction of NP₃₆₆₋₇₄CD8⁺T cell population in (A) lung and (B) spleen tissues. INF (●) and SDR-INF(○) mouse lungs and spleens were sampled at various times during a primary IAV infection. Cells were harvested from lung or spleen tissue, enriched for CD8⁺T cells and stained for surface markers. During clonal expansion (0–10 dpi), of CD8⁺T cells in lung tissue, SDR>MEM by 2-factor ANOVA, $p<0.05$, $n=6/\text{group/day}$. No statistically significant differences were detected in the spleen.

Figure 5.

SDR altered splenic expansion but not contraction of the PA₂₂₄₋₂₃₃CD8⁺T cell population during a primary IAV infection. INF and SDR-INF mouse lungs (A) and spleens (B) were sampled at various times during a primary IAV infection. Cells were isolated from lung or spleen tissue, enriched for CD8⁺T cells and stained for flow cytometry. *SDR-INF>INF, $p < 0.05$, $n = 6/\text{group/day}$.

Figure 6.

Repeated defeat increased number of highly activated, virus specific CD8⁺T cells in lung tissue at the peak of the adaptive response to IAV infection. INF (●) and SDR-INF (○) mouse lungs were sampled at baseline and day 9 of a primary IAV infection. Cells were isolated from lung tissue, enriched for CD8⁺T cells and analyzed by flow cytometry. Sequential gates were set based on isotype and irrelevant tetramer staining. (A) Percent tetramer+ of total lung lymphocyte population after CD8⁺ MACs selection at 9 dpi. (B) Percent of lung tetramer+ population in the CD25⁺CD62L low population in lower right quadrant at 9 dpi. Surface staining included (C) total CD8⁺ T cells in lung tissue, (D) NP₃₆₆ tetramer-specific CD8⁺T cells, and (E) number of NP₃₆₆ cells with the activated phenotype CD62L_{LO}/CD25⁺NP₃₆₆₋₇₄CD8⁺. At day 9, CD8⁺T cells, NP₃₆₆₋₇₄CD8⁺T cells and CD25⁺/CD62L_{LO} NP₃₆₆₋₇₄CD8⁺ T cells were significantly elevated in the SDR-INF group when compared to INF mice, *p<0.05, unpaired T test, n=6/group/day.

Table I

Total serum anti-IAV IgG antibody titers after resolution of a primary IAV infection.

Experiment	Group	Week post-infection	
		2	3
Experiment 1	INF ^a	5.72 (+.29)	7.14 (+.20)
	SDR-INF	3.93 (+.13)	5.66 (+.30)
Experiment 2	INF ^b	5.12 (+.14)	5.06 (+.34)
	SDR-INF	3.82 (+.17)	4.47 (+.19)

SDR exposure prior to primary IAV infection resulted in reduced serum anti-IAV IgG titers following the resolution of the primary infection. The average anti-IAV IgG titer increased in both groups from week 2 to week 3. Data are presented as log-transformation of the serial four- fold serum dilutions; SEM in parenthesis.

^aSignificant week and group effect (ANOVA, $p < 0.001$)

^bSignificant group effect (ANOVA, $p < 0.001$)