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# A role for B cells in facilitating defense against an NK cell-sensitive lung metastatic tumor is revealed by stress

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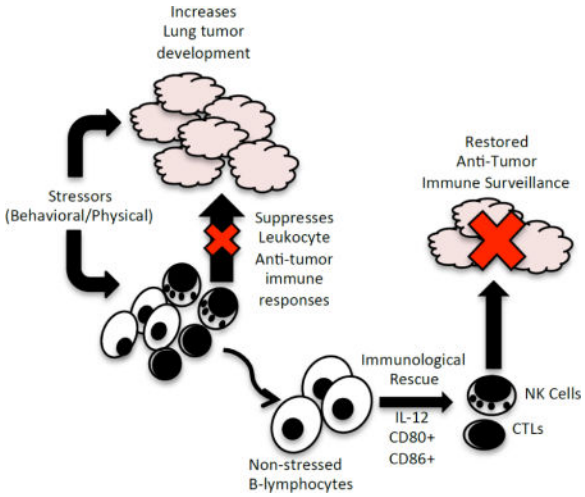
## Abstract

Stressors impair immune defenses and pose risks among cancer patients. Natural Killer cells are not the sole immune defense against tumor development. Utilizing an NK-sensitive tumor model, this study evaluated immune effects to stress and determined whether lung metastasis resulted from B cells' inability to augment tumorlytic function. Lung metastasis directly correlated with delayed lung B cell accumulation compared to NK, and T cells. Decreased interleukin-12 cytokine and $CD80^{+}$ molecule expression by B cells correlated with decreased tumor lysis and increased tumor development. Thus, tumor defenses in the lung given stress exposure can depend on the B cell function.

## Graphical abstract

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**Keywords**

Lung; metastasis; stress; immunity

## 1. Introduction

The metastatic potential of most tumors depends on their ability to escape the recognition and destruction by circulating and resident immune cell populations (Leber MF, 2009); (Teng MW et al., 2008). Stressors that impair the host immune defense system are believed to pose formidable risks among cancer patients, allowing for increase tumor spread and susceptibility to infectious disease (Giordano S et al., 2007); (Goossen GM et al., 2007); (Mazza JJ, 2010); (Wong A et al., 2010). Physical stressors such as surgery, chemotherapy and radiation have previously been shown to diminish the function of immune effector cells in cancer patients (Liasson P et al., 2009); (Eyrich M et al., 2009); Vallejo et al., 2003); (Andersen et al., 1998). Also, a large body of evidence suggests that psychological stress (e.g. depression, anxiety) associated with cancer diagnosis and therapies account for impaired immune status (Goodkin K (1995); (McGregor BA and Antoni MH 2009); (Nelson EL et al., 2008); (Steel J et al., 2004). Experimental animal models of stress and tumor development have substantiated such findings by demonstrating diverse effects of stress on tumor-immune defenses (Vegas O et al., 2006); (Azpiroz A et al., 2008). Such studies have spawned a growing interest in the field of psychoneuroimmunology (PNI) research, with the anticipated discovery of multidisciplinary therapies against cancer and other diseases.

Abrogation of natural killer cell function has been the primary focus of stress-induced immune suppression, resulting in cancer susceptibility. As early as 1978, Holden et. al., discovered that stressors could increase tumor growth in experimental animals, suppressing lymphoid proliferation and NK cell activity *in vitro* (Holden C, 1978). Subsequently, numerous human and animal studies highlighted the effect of stressors on NK cells and their association with tumor progression *in vivo* (Kanno J et al., 1997); (Staurenghi Ah et al., 1997). In recent years, attempts have been made to identify cellular and molecular mechanisms involved in NK suppression. For example, cancer-related stress was found to correlate with decreased NK cell toxicity (Witek-Janusek L et al., 2007); (Qiu YH, 2003); (Lang K et al., 2006); (Naliboff BD et al., 2008). Neuroendocrine mediators derived from activation of the central nervous system such as corticosteroids, catecholamines and opioids are found to impact NK cell function (Staurenghi Ah et al., 1997). Most recently, stress effects on NK-associated alterations on receptor-mediated activation and intracellular signaling pathways have been considered molecular targets, possibly explaining NK cell dysfunction (Kimura K et al., 2008); Oya H et al., 2000); (Varker KA et al., 2007).

NK cells are not the sole immune defense mechanism against tumor development, but instead require optimal collaboration with other host immune constituents. Notably is the complementary role of tumor-specific cytotoxic responses of $CD8^+$ T cells (Cuff S et al., 2010). In addition, dendritic cell-mediated tumor presentation plays an important role in directing tumor-specific adaptive T cell and humoral antibody responses (Chaux P, 1995); (Dumitriu IE et al., 2009); Lapteva N et al., 2009). Furthermore, in our investigation of

immune defense mechanisms against tumor development, we have previously reported that in addition to NK cells, B cells also participate in protection against the establishment and metastasis of tumors. Specifically, our results demonstrated that the extent of tumor size of a subcutaneous tumor as well as the propensity of experimental lung tumor metastasis was associated with decreased number of B cells in circulation and within the lung, respectively (Demetrikopoulos MK et al., 2000). Importantly, we found that antibody-mediated blockade of B cells rendered rats susceptible to increased lung metastasis similarly to rats administered antibody against NK cells (Quan N et al., 1999). These findings suggested that both NK and B cells could act in concert against *in vivo* tumor development.

To date, the mechanisms in which B cells protect against tumor development remains unclear. Our *in vitro* studies revealed a potential relationship between antitumor defenses and B cells by demonstrating a positive influence on IFN-γ production and tumor killing (Jones, HP et al., 2008). By contrast, B cells have also been found to promote tumor escape. Qin et al. demonstrated the ability of B cells to inhibit tumor resistance (Watt V et al., 2007); Qin Z, et al., 1998). Likewise, *in vitro* studies have revealed contradictory observations regarding their influence on NK cell activity and IFN-γ cytokine secretion (Michael A et al., 1989). Thus, in considering our findings and those of others, it is likely that B cells may play a pivotal regulatory role that determines the outcome of tumor development. Importantly, questions may be raised regarding yet undefined relationships of stress -related tumor development and the influences of B cell responses and other cellular immune constituents.

Utilizing an NK-sensitive tumor model of lung metastasis, the current study evaluated the immune effects related to a given stressor and determined whether progression of lung metastasis was in part a result of B cells' inability to provide its adjuvant effect on tumorlytic function. Stress -induced severity of lung metastasis was directly associated with a preferential delay in the accumulation of B cells within the lungs in response to tumor as compared to NK, $CD4^+$ and $CD8^+$ T cells, respectively. Importantly, we demonstrate that diminished IL-12 cytokine and $CD80^+$ co-stimulatory surface molecule expression by B cells resulted in a concomitant decrease in tumorlytic responses with increased lung tumor development given stress exposure. Based on our results, we believe that the dependency of B cells on antitumor immune responses has relevance in expanding the repertoire of tumor surveillance mechanisms beyond NK cellular responses, particularly in the context of stress exposures.

## 2. Materials and methods

### 2.1 Rats

One year old female specific pathogen-free Fischer 344 rats were used in the study. Rats were housed three to a cage and kept under sterile conditions (dark period was from 7:00 P.M. to 7:00 A.M. and food and water was provided ad libitum). Experiments were performed between the hours of 8:00 A.M. and 1:00 P.M. The Institutional Animal Care and Use Committee (IACUC) approved all studies performed in this study.

### 2.2 Tumor cell line

The MADB106 tumor, which is an adenocarcinoma derived from the pulmonary metastasis of a Fischer (344) rat, was used in all studies. The MADB106 cell line was maintained in complete culture media (RPMI 1640; Invitrogen, Corp, Grand Island, NY) supplemented with antibiotic/antimytotic cocktail (Gibco) and 10% fetal bovine serum (FBS) and expanded in sterile T75 tissue culture flasks at 37° C and 5.0% $CO_2$ culture conditions. To control for variability due to possible mutations, cells were limited to ten passages in culture prior to experimentation. Cells were harvested using 2% Na EDTA solution by mechanical disruption using a cell scraper to remove adherent cells from the surface of the culture flask. The tumor cells were suspended in sterile PBS and washed twice prior to their use.

### 2.3 Stress paradigm

Rats were subjected to aversive stimulation by means of inescapable electric foot shock or tail shock. For the foot shock, individual rats were placed in separate plexiglass opaque cabinets with a metal grid floor. Rats were subjected to inescapable electrical foot shocks for 30 min. For tail shock, individual rats were placed in a separate plexiglass apparatus as previously described. An electrical probe was attached three-quarters along the distal aspect of subjects' tail. Rats were subjected to 2 hrs of electrical tail shocks. As a control, additional rats remained in their home caged environment.

### 2.4 Sacrifice and tissue preparation procedures

Following foot shock and tail shock procedures, rats were immediately anesthetized by halothane inhalation and administered $2 \times 10^6$ tumor cells into the tail vein. Rats not subjected to either of these stressful procedures were simply removed from home cages in the colony and anesthetized and injected with tumor cells. At different time points after the tumor cell injection, rats were sacrificed. Under halathane anesthesia 1 ml of peripheral blood from the inguinal vein was collected and placed on ice in sterile hepranized tubes until analysis. The abdominal cavity and chest were opened and the spleen removed and placed on ice in sterile culture petri dishes containing RPMI wash media supplemented with 1% FBS. Lungs were also excised and infused with sterile cold PBS to flush out red blood cells and placed on ice in sterile petri dishes containing RPMI wash media supplemented with 1% FBS. To examine lung lesion development, lungs were excised with trachea attached an immediately infused with dye and placed in a dedying solution. Lung, spleen and blood tissue used for studies of lymphocytes in these compartments were processed immediately after sacrificed as described below.

### 2.5 Quantification of experimental lung metastasis

Rats were anesthetized with halothane, and tumor cells ($2 \times 10^6$) were administered intravenously by tail vein injection in a volume of 1.0 ml of PBS. At different time intervals after tumor-cell injection, animals were sacrificed to determine the extent of lung tumor development or to evaluate lymphoid responses. To quantify tumor development in lung, the method by Wexler et. al. was utilized (Wexler H, 1966). Briefly, lungs were removed and infused intratracheally with a 15% India ink solution. The lung tissue was then placed in a dedying solution (70% ethanol, 10% formaldehyde, 5% glacial acetic acid, and 15% distilled

water). After 24–48 hours, the lungs were counted. Lung lesion counts were determined blindly in which individuals performing counts were unaware of the experimental condition. The number of metastases on the surface of the lung was determined by counting the number of white nodules present on the tissue surface amidst normal lung tissue that appeared black. Each lobe from the lungs of every subject within an experimental group was inspected for continuity of tumor metastases. The ventral and dorsal surfaces of a single lobe chosen to be representative of the entire lung, was selected. The number of metastases on the entire lobe was counted.

### 2.6 Lymphocyte preparations

Mononuclear cells were isolated from the lungs and spleen as previously described (Kruisbeek AM, 2001). Lungs were perfused with sterile cold PBS not containing magnesium or calcium to remove contaminating blood cells and then separated into individual lobes and finely minced. The minced tissue was suspended in medium made up of RPMI 1640 (Gibco, Invitrogen, Corp, Carlsbad, CA) medium which contained 300 U/ml *Clostridium histolyticum* type I collagenase (Worthington Biochemical, Freehold, NJ), 50 U/ml DNase (Sigma, St. Louis, MO), 10% FBS (Gibco), HEPES, and antibiotic/antimytotic solution (Sigma). The tissues were then incubated at 37° C for 90–120 min. After incubation, the mixture was passed through a 250-μm nylon mesh to remove undigested tissue. Lymphocytes were purified from the remaining cell suspension by density gradient centrifugation using Lympholyte Rat (Accurate Chemicals, Westbury, NY). Following centrifugation, lung lymphocytes were washed twice by resuspension in RPMI wash media (Invitrogen, Grand Island, NY.) containing 1% FBS. After washing, cells were suspended in wash media and placed on ice for further analysis.

To obtain spleen cells, spleens were mashed spleens through a 0.2 μm nylon mesh to produce a cell suspension. Red blood cells were removed using ACK lysis buffer. Briefly, spleen cell suspensions were pelleted and resuspended in 5 mls of ACK lysis buffer for 10 min on ice. After incubation, 20 ml of wash media was added and spleen cells were washed twice in 20 ml of wash media. After washing, cells were suspended in 20 ml of wash media and placed on ice for further analysis.

To obtain cells from blood, the blood was collected via inguinal vein and placed in tubes containing heparin (10 units/ml). Blood lymphocytes were prepared by gradient centrifugation separation by overlaying whole blood samples on histopaque solution (Sigma). Isolated blood lymphocytes were collected from the histopaque gradient and washed twice in 10 ml of wash media. After washin g lymphocytes were resuspended in 10 mls of wash media until further analysis. Trypan blue staining and hemotcytometer counting method was used to determine the total number of viable lymphocytes isolated from each tissue.

### 2.7 Immunofluorescent characterization of lung, spleen and blood lymphocyte populations

Two-color immunofluorescent staining was performed to identify B, NK and T cell populations ($CD4^+$ and $CD8^+$) using, respectively, FITC-labeled anti-rat, CD45RA mAb (OX33), PE-labeled anti-rat NKR-P1 mAb (3.2.3), PE-labeled anti-rat CD4 (OX-4) and

FITC-labeled anti-rat CD8 (OX8 mAb). All antibodies were purchased from BD ParMingen, San Diego, CA. As controls, cells were also stained with antibody-matched fluorescently-labeled isotype controls to quantify autoflourescence of cell populations. Briefly, $1 \times 10^5 - 1 \times 10^6$ cells (depending on availability) extracted from lung and spleen per tube were incubated with purified 2.4G2 mAb (BD, PharMingen) for 25 min at 4° C to reduce non-specific binding of FcII/IIRs prior to fluorescent Ab staining. The cells were then incubated for 30 min in dark at 4° C with fluorescent Ab (1 ug/ml). Following staining, cells were washed twice in staining buffer ($Mg^{2+}$ free, $Ca^{2+}$-free PBS with 1% fetal bovine serum (FBS) (Invitrogen,) and resuspended in staining buffer for immediate analysis. Immunophenotyping of whole blood was performed by staining 100ul of whole hepranized blood with specific fluorescent antibodies (1ug/ml) for 30 min in dark at 4° C. Following staining, red blood cells were lysed with ACK lysis buffer and subsequently washed twice in staining buffer. Blood lymphocytes were immediately analyzed by flow cytometry techniques.

Lung, spleen and blood cells were analyzed on a FACScan (Becton-Dickinson) using the consort program. Positively stained cells were determined by subtraction of autofluorescence from isotype-matched control samples to determine positive staining. The proportion of each cell population was expressed as the percentage of the number of stained cells within the gated population based on forward and side scatter viable cell discrimination. Total cell numbers of different cell types showing specific staining were expressed as the total number of isolated lymphocytes from each respective tissue multiplied by the percentage of stained cells for each target population.

### 2.8 Positive selection of B cells

For separating B cells from the total lymphocyte population, which was done in some experiments with respect to lung lymphocytes, magnetic bead separation method was utilized as follows. Single cell suspensions were incubated for 2 hours in Petri dishes to deplete adherent cells from the population. The remaining cells (in suspension) were collected and washed twice in wash buffer (PBS containing 0.1% FBS). Briefly, total lung cells (devoid of adherent cells) were incubated with biotinylated anti-CD45RA antibody (1 ug per $10^7$ total cells) for 30 min at 4° C. After the incubation period, anti-$CD45RA^+$ labeled cells were then incubated for an additional 20 min with an optimal concentration (as specified by manufacturer) of strepavidin magnetic beads (Dynal, Brown Deer, WI). Labeled cells ($CD45RA^+$ B cells) were twice passed over column (LS columns, Dynal) attached to a magnetic apparatus (Dynal) for two successive rounds of column separation. The eluent containing unbound non -B cells was collected in sterile 3 ml tubes and placed on ice until further analysis. The bound B cells were recovered by removing the column from the magnetic apparatus, and eluting B cells into 3 ml sterile collection tubes by rinsing the column with 1 ml labeling buffer three times. After collection, B cells and non-B cells were pelleted and resuspended in 1 ml of wash media containing 1% FBS in the presence or absence of protease cocktail (Dynal) for 3 min (37° C) for detachment of strepavidin magnetic beads, respectively. All cell preparations were used for subsequent experimentation. The purity of B cells (≥ 98% purity) and negative cell fractions (≤ 1 % contaminating B cells) were confirmed by flow cytometry.

### 2.9 Determination of tumor-lytic response

Two independent experiments were performed to assess the cytolytic response against MADB106 tumor cells.

Experiment 1-Tumor ($2e^6$) or PBS was administered into the tail vein of non-stressed rats. Donor non-B cells were prepared from the lungs of naïve rats. Two hours later experimental and naïve rats were sacrificed and B cell and non-B cell populations were separated from total lung lymphocytes as described previously. An equal proportion of lung B cells from tumor or PBS-injected rats, which was predetermined by the percentage of B cells found in the lungs of PBS-treated mice (based on flow cytometery analysis), was co-cultured with non-B cells from the lungs of naïve donor rats in the presence of labeled MADB106 tumor cells at effector:target cell ratio. The percent of lysed tumor cells was determined as described below.

Experiment 2- Prior to tumor or PBS administration by tail vein injection, rats were either subjected to tail shock (see methods above) or remained in home cages. 2 hrs later, B cell and non-B cell populations, that were segregated from total lung lymphocytes as described above were cultured in the presence of MADB106 tumor cells representing four conditions; condition 1- B cells from non-stressed and non- B cells from non-stressed rats, condition 2- B cells from stressed and non-B cells from stressed rats, condition 3- B cells from stressed and non-B cells from non-stressed rats and condition 4- B cells from non-stressed and non-B cells from stressed rats. The proportion of lung B cells from each experimental condition was equal to the pre-determined percentage of B cells (flow cytometery analysis), found in the lungs of tumor-injected rats subjected to stressed rats.

In both experiments, cytolytic determination was measured using a fluorescent-based cytolytic assay known to be sensitive to small lymphocyte numbers (Ostler T et al., 2001). Prior to culture, $3 \times 10^6$ cells/ml of MADB106 tumor cells were flourescently labeled with PKH-26 dye (0.5 uM) (Sigma, ST. Louis, MO) at 28°C for 3 min. After staining, an equal volume of 1% bovine serum albumin (BSA) in RPMI 1640 medium was added to the cell suspension and incubated for an additional 1 min. Tumor cells were then washed twice by centrifugation with 5% FBS RPMI 1640 medium. The viability and fluorescence spectra overlap between propidium iodine (P.I.) and PKH-26 intensity of MADB106 cells was determined by flow cytometry prior to the execution of assay. Lymphocyte populations representing each experimental culture condition was incubated with MADB106 tumor target cells in 75×12 mm polypropylene tubes at selected effector: target-cell ratios (12.5:1, 25.0:1 and 50.0:1) in triplicate. After gentle centrifugation, cells were incubated for 90 minutes at 37 °C and 5% $CO_2$. After the incubation period, 0.5 pM final concentration of (PI) was added to each tube and placed on ice until analyzed using flow cytometery methods. Lytic responses were analyzed on a FACScan (Becton-Dickinson) using the consort program. Tumor lysis was expressed as the percentage of $PKH\text{-}26^+$ $PI^+$ staining of the gated tumor cell population minus $PI^+$ staining of MADB106 tumor cells cultured in the absence of lymphocytes.

### 2.10 Real time RT-PCR

Total RNA was prepared from fractions of lung lymphocytes described above; that is, RNA was prepared from purified i.e. B cells from lung and non-B cells from lung using RNA RNeasy mini-column Total RNA purification as described by the manufactures instructions (Qiagen RNeasy Minikit, Qiagen,). To eliminate any contaminating DNA, 10ul of DNase stock solution (Qiagen, 1500 Kunitz of solid DNAase I dissolved in 550ul of RNAse-free water) was added to 70ul buffer (Qiagen) and gently mixed by inversion. The solution was then pipetted directly onto the mini-column, and incubated at room temperature for 15 min. 350ul of wash buffer (Qiagen) was added to column and centrifuged (8,000 rpm) and the flow-through was discarded. Total RNA was eluded in wash buffer and stored at $^{-}80°$ C until use. cDNA was generated with random hexamers using ImProm-II Reverse Transcription System (Promega,). Real time RT-PCR amplification of cDNA samples was performed using SYBR green technology (Roche, Indianapolis, IN) on a BIO-RAD thermocycler (BIO-RAD, Hercules, CA). To ensure that cytokine-specific gene products were generated from selected primers, melt curve analysis was performed to exclude primer dimer formation and correct cytokine PCR product size was confirmed by gel visualization. Quantification of target cytokine gene expression was determined using standard curve semi-quantitative methods. Specifically, 10-fold dilutions ($10^1 – 10^7$) of cDNA samples were amplified with each target cytokine and housekeeping gene. The difference in cytokine gene expression between experimental samples was expressed as fold difference in target gene expression derived from the ratio of target gene/housekeeping gene based on mRNA levels extrapolated from standard curves of target and housekeeping genes determined by BIO-RAD gene analysis software (BIO-RAD).

### 2.11 Statistical analysis

Differences between control and experimental groups were assessed by one-way ANOVA. When an ANOVA was conducted, a comparison of each individual experimental condition with the control condition was then conducted using Dunnett’s test.

## 3. Results

### 3.1 Differences in lung tumor development correspond with intensity of stress event

We examined the effect that introducing rats to foot shock (mild stressor) and tail shock (intense stressor) would have on the propensity of lung tumor formation. Mild stress provoked a significant increase in lung tumor development as compared to rats under non-stressed conditions. A further significant increase in lung lesions was found when rats were exposed to intense stress (figure 1A and B). Thus, the extent of lung tumor development corresponded with the extent of the stress event.

### 3.2 Stress blocks lymphoid recruitment to the lungs in response to tumor

An early immune response to tumor is believed to deter the establishment and metastatic spread of several human and experimental tumors (Prendergast GC, 2008); (Rush et al., 1999); (Barlozzari T et al., 1985). We determined how stress exposure impacted the recruitment of lymphocyte populations to the lung, blood and spleen in response to tumor

injection. For comparison, an additional group of non -stressed rats were assessed after having received PBS. A significant increase in lymphocyte infiltrate into the lungs was observed within 1 hr and 2 hr following the administration of tumor cells. The increase in lymphocyte infiltrate was significantly attenuated by mild (foot shock) and intense stress within 1 hr and was sustained by intense stress (tail shock) 2 hr after tumor injection. Moreover, mild and intense stress significantly reduced the number of lymphocytes present in the lungs of tumor-injected rats compared to non-stressed rats devoid of tumor 1 hr after tumor injection. By 6 hr, the number of total lung lymphocytes was comparable between all experimental conditions.

In spleen, tumor administration of non-stressed rats resulted in a significant increase within 1hr followed by a significant decrease at 2 hr. By 6 hr cell numbers in non-stressed tumor-injected rats were comparable with PBS-treated rats. Whereas mild and intense stress significantly enhanced the number of spleen cells 2 hr after tumor injection, a significant reduction in spleen cells was demonstrated in response to intense stress at 1 hr and 6 hr among tumor-injected rats

Lymphocyte numbers were significantly reduced in blood within 1 hr and 2 hr among non -stressed and stressed rats after tumor injection compared to PBS-treated rats. By 6 hr following tumor injection, the number of total blood lymphocytes among rats subjected to mild and intense stress remain ed significantly lower compared to their non-stressed counterparts, which recovered in number comparable to PBS non-stressed rats (Figure 2).

### 3.3. Stress preferentially diminishes B cells' early accumulation in the lung of rats following tumor infection

Our previous published data demonstrates a propensity for NK and B cells to promote resistance against pulmonary tumor development in a syngeneic host (Quan N, et al., 1999). In the current study, we examined the distribution of B, NK, $CD4^+$ and $CD8^+$ lymphocytes present in the lung 1 hr, 2 hr and 6 hr following tumor injection among subjects previously exposed to mild stress, intense stress or no stress. Within 1 hr, a significant increase in all lymphocytes populations (B, NK, and T cells [$CD4^+$ and $CD8^+$] were found in the lungs following administration of tumor cells. Mild and intense stress significantly suppressed the infiltration of all lymphocyte populations to the lungs compared to non-stressed rat counterparts. However, whereas intense stress sustained the suppression of all lymphocyte populations within 2 hr following tumor injection compared to their non-stress counterparts, only B cells were significantly suppressed in response to mild stress. By 6 hr following stress and tumor injection, lymphocyte subpopulations in the lungs were comparable between all experimental groups (Figure 3).

We also examined whether stress-associated changes in the distribution of lung lymphocyte populations could be explained by preferences in their re-distribution in peripheral compartments such as the blood and spleen. In spleen, stress-dependent alterations in B cells were not observed following tumor injection. NK cells were significantly lower in rats exposed to intense stress at 1 hr compared to non-stressed tumor-injected rats and 6 hr compared to all experimental conditions. $CD4^+$ T cells were significantly increased in response to tumor administration at 1 hr, which was significantly attenuated by intense

stress. By 6 hr, $CD4^+$ T cells were significantly depressed by intense stress. Intense stress exposure resulted in a significant depression of $CD8^+$ T cells compared to mild stress and no stress counterparts within 1hr after tumor injection. No significant difference in $CD8^+$ T cells was observed 2 hr after tumor injection. By 6 hr, a significant increase in $CD8^+$ T cells was observed in response to tumor injection. Intense stress significantly attenuated such an increase (Supplementary Figure 1).

B, NK, $CD4^+$ T and $CD8^+$ T cells were significantly reduced within the blood of rats within 1 hr after tumor injection compared to PBS-treated rats. A further significant reduction by B cells was observed in response to mild stress 1 hr after tumor injection whereas other populations were unaffected compared to their tumor-injected counterparts. B, NK and $CD4^+$ T cells remained significantly reduced within 2 hr following tumor injection independent of stress exposure compared to PBS-treated rats. Blood $CD8^+$ T cell numbers however, were significantly reduced in response to intense stress only compared to PBS-treated rats. By 6 hr all lymphocyte populations in the blood of non-stressed tumor-injected rats were comparable to non-stressed PBS-treated rats. While both mild and intense stress sustained a significantly lower number of B cells in blood for a duration of 6 hr following tumor injection. Mild stress maintained a significantly reduced number of B and NK cells in blood compared to non-stressed tumor-injected counterparts and intense stress resulted in a significant reduction in all populations compared to their non-stressed tumor-injected counterparts (Supplementary Figure 2).

### 3.4 Pulmonary B cells from tumor-injected rats promote tumor lysis

The potential of pulmonary B cells to impact antitumor defenses is supported by our *in vitro* findings that pulmonary B cells can facilitate tumor lysis and IFN-γ cytokine production (Jones HP et. al. (2008). In the current study, B cells were isolated from the lungs of PBS-treated or tumor-injected rats 2 hr after intravenous injection. Isolated lung B cells from PBS-treated or tumor-injected rats were co-incubated with lymphocytes devoid of B cells isolated from PBS-treated rats to assess innate lysis of MADB106 tumor cells. As shown in figure 4, co-culture of B cells isolated from the lungs of tumor-injected rats resulted in significantly higher tumor lysis at each E:T ratio, suggesting that B cells acquiring an activated status in response to tumor during the initial stages of immune surveillance have the capacity to promote tumor killing.

### 3.5 Stress alters pulmonary B cells' ability to enhance tumor lytic function

We determined whether B cells isolated from the lungs under stress conditions could alter B cell's influence on tumorlytic function. B cells were isolated from the lungs of tumor-injected rats subjected to intense stress or no stress, and were incubated with either total lung lymphocytes devoid of B cells from corresponding stressed or non -stressed rats in the presence of MADB106 tumor cells. Co-incubation of lung B cells from stressed subjects with non-B cells from stressed subjects reported the lowest tumor killing capacity (figure 5b). In contrast, lung B cells from non-stressed subjects with non-B cells from non-stressed subjects resulted in the highest tumorlytic response (figure 5a). Interestingly, incubation of stressed B cells with non-stressed non-B cells led to diminished tumor lysis as compared to when non-stressed B cells were cultured with non-stressed non-B cells (figure 5a).

Moreover, an increase in tumor lysis was observed when non-stressed B cells were incubated with stressed non-B cells as compared to stressed B cells incubated with stressed non-B cells (figure 5b). Thus, tumor lytic capacity was found to depend on the source of B cells indicating that stress had a negative impact on B cell's ability to facilitate tumor lytic responses.

### 3.6 Altered IL-12 and CD80 mRNA expression by lung B cells corresponds with diminished tumorlysis

IL-12 cytokine is a key modulator of cytotoxic lymphoid activation, including the activation of NK and $CD8^+$ T cells (Sabel MS et al., 2007) and is expressed by B cells (Sugimot K et al., 2007). Induction of IL-12 and other cytokine responses also depends on the up-regulation of CD80 co-stimulatory molecule that provides a secondary signal upon antigenic processing and presentation (Sugimot K et al., 2007). In this study, rats exposed to foot shock and tail shock were injected with tumor or PBS as control. After 2 hr incubation period, B cells were isolated from the lungs and IL-12 and CD80 mRNA expression by purified B cells and Non-B cells were measured using quantitative real time RT-PCR techniques. As shown in figure 6, a significant induction of IL-12 mRNA expression was present in lung B cells of non-stressed rats injected with tumor as compared to PBS-treated rats. In contrast, IL-12 mRNA expression levels in B cells isolated from rats that received both foot shock and tail shock did not differ from PBS-treated rats, suggesting that stress abrogated the induction of IL-12 in response to tumor. B cells isolated from non-stressed subjects that were subsequently administered tumor demonstrated a significant increase $CD80^+$ mRNA expression (figure 6). These findings suggest potential mechanisms through which stress exposure alters B cell function.

## 4 Discussion

Associations of stress and cancer prognosis, as it relates to alterations in immune status, have primarily focused on defining the functional responses of NK cells (Lutgendorf SK et al., 2005). Utilizing an experimental model of lung tumor metastasis, that is known to be highly sensitive to NK cell activity, we have demonstrated that in addition to the NK cell action, B cells also apparently influences defense against lung tumor development (Demetrikopoulos MK et al., 2000); (Quan N et al., 1999). Our current findings raise the potential that diminished antitumor responses observed given stress exposure could be a consequence of altered B cell defense mechanisms. This study sought to determine whether stress -induced exacerbation in lung tumor development would be proportional with differences in B cell responses, thereby substantiating their role in lung tumor resistance akin to NK cells. Our results demonstrated that stress exposure affects the propensity of B cells to accumulate in the lung in a time and intensity-dependent manner resulting in greater tumor development. Furthermore, we found that pulmonary B cells exposed to stress exhibited dampened IL-12 cytokine and CD80 co-stimulatory surface molecule mRNA expression, corresponding with a deficiency in their ability to support tumor lysis.

The extent to which a given stressor may impact tumor progression has been shown to involve numerous factors including: stress type, intensity, temporal stress exposure and

individual characteristics (e.g. gender, genetic variations) (Zozulya AA et al., 2008); (Reiche EM et al., 2005); (Bleiker EM and van der Ploeg HM 1999). Initial studies demonstrated whether implementation of different stress protocols (type and duration) would predict susceptibility to lung tumor development. Consistent with previous studies (Mazur-Kolecka B et al., 1994); (Sklar LS et al., 1981), varying intensity and duration resulted in distinct outcomes in tumor lesion formation as evident by a greater number of lung lesions that developed depending on the intensity and duration of stress exposure.

The variation of timing and intensity of stressors on tumor progression and its potential influence on immune regulation remains unclear, particularly with respect to pulmonary B cell activity. We have previously reported that the extent of lung tumor development is depended on the action of NK and B cells during the immediate period after the injection of tumor cells in the lung (Quan N et al., 1999). Our current findings indicate that varying stress, resulting in differences in severity of lung lesion development is related to the temporal accumulation of total lung lymphocytes. Specifically, when subjects were exposed to two different stress applications, the extent of pulmonary lesion score was directly proportional to intensity and duration of stress that corresponded with the extent to which the infiltration of lymphoid cells was abrogated in response to tumor injection. These findings reinforce the importance of early immune surveillance necessary for pulmonary tumor clearance (Lalialis LV et al., 2008). Interestingly, different stressors had distinct effects on the distribution of lung B, NK, $CD4^+$ and $CD8^+$ T cells in response to tumor. As shown in figure 1, whereas an impaired accumulation of all cell-types tested was observed in response to both stressors during the initial hour following stress and tumor injection, the persistent abrogation of B cells (between 1 hr and 2 hr) given mild and intense stress suggest a preferential inhibitory effect on B cells may be associated at least in part with the extent of initial lung tumor formation. In addition to our findings in lung tissue, we also examined whether preferences in the distribution of lung cell-types could be explained by preferences in the re-distribution of immune cells in systemic compartments. For example, one might expect that the attenuation of B cells and other immune cells to the lung in response to tumor under stress conditions could be explained by the retention or sequestration of lymphoid cells in systemic compartments. Although stress -associated effects were observed in the distribution of lymphocytes in blood and spleen given stress exposure, a direct association was not apparent. Rather, these findings suggest a disassociation between the influence of stress on local and peripheral compartments with respect to the proportion of lymphocytes found at selected time points in response to stress exposures.

Our findings above suggest that a lack of anti-tumor responses could be explained by an insufficient accumulation of immune cells at the site of tumor (e.g. B cells and NK cells). The eradication of tumor cells depends on tumor lysis that is primarily the result of the activation and release of proteolytic enzymes such as perforin and granzyme by NK cells and cytotoxic T lymphocytes in close proximity with its tumor target. The prerequisites of this response include the recognition of tumor cells by NK and/or cytotoxic T lymphocytes through cellular receptor/ligand interactions (Long EO and Rajagopalan S, 2002); (Chavez-Galan L et. al., (2009) as well as the adjuvant effect of other immune cell responders through the release of soluble mediators (e.g. cytokine, chemokine, oxidative radicals) (Ralainirina N et al, 2007); Lin WW and Karin M, 2007); (de Visser Ke and Coussens LM, 2006);

(Biassoni R et al., 2002). In support, our previous published studies demonstrated that pulmonary B cells aid tumorlytic responses suggesting that the fate of tumor resistance at least in this tumor model is likely to depend on the pulmonary microenvironment. In support, Melamed, R et al., reported the ability of marginating pulmonary NK cells to have a higher propensity of tumor lysis in contrast to peripheral blood NK cells (Melamed R et al., 2010). In an attempt to link the stress-associated increase in lung tumor development to the functional response by B cells, the proportion of B cells pre-determined by the percentage of B cells found in the lungs of non-stressed tumor-injected rats and stressed tumor-injected rats in relation to the corresponding non-B cell lymphocyte populations were reconstructed *in vitro* to assess tumor lytic function. In this manner, we demonstrated that the different lytic capacity influenced by B cells representing each condition was not due to differences in the proportion of B cells with respect to NK cells and other lymphoid populations. In demonstrating that B cells are present in the lung during the initial hours following tumor exposure provide a higher tumorlytic potential than normal pulmonary B cells confirms our previous report of their antitumor response. We next determined whether stress exposure would have an impact on tumor lysis with the expectation that pulmonary B cells from stressed subjects would be deficient in augmenting the tumorlytic response of MADB106 tumor cells. The present results indicate that the level of tumorlytic activity is dependent on the nature of pulmonary B cells as the presence of stressed B cells significantly reduced the killing efficiency among non-stressed lymphocytes compared to co-culture with non-stressed B cells. Interestingly, non-stress B cells demonstrated a marked increase in tumorlytic activity in the presence of stressed non-B cells as compared to conditions where both stressed B and non-B cells were assessed for tumorlytic capacity. These findings reinforce the potential requirement for pulmonary B cells in optimal protection against lung tumor development, particularly under conditions of stress.

To date, the mechanisms behind B cell's ability to promote tumor killing remain unclear. Traditionally, B cells have been known to regulate tumor defense through antibody dependent cell cytoxicity (ADCC) by cross-linking of Fc receptors by NK cells (Sheeja K and Kuttan G, 2007); (Gazit R et al., 2007); (Kurai J et al., 2007). Furthermore, Guo et al. demonstrated B cells capable of inducing tumor-specific immune responses as antigen presenting cells (Guo Y et al., 1994); (Bennink JR et al., 1993); Peoples GE et al., 1993); (Pernis B and Weber DA, 1989). In the current study, we provide evidence that B cells isolated from the lungs of stressed rats have diminished IL-12 expression as compared to non-stressed tumor injected rats. As a potent stimulator of NK cells and cytotoxic T cells, the data suggest that B cells may augment tumor lytic responses through IL-12 mediation. This finding would support our previous studies that demonstrate the influence of lung B cells to modulate IFN-$\gamma$ cytokine production. In support Sugimoto, K et al., documented IL-12 expression by B cells (Sugmoto K et al., 2007). It is therefore consistent with the concept that IL-12 activation by B cells potentially provides an immunostimulatory effect toward NK cell and CTL responses that under stress conditions can be inhibited, allowing for increased tumor susceptibility. We also demonstrated the abrogation of CD80 co - stimulatory molecule expression by B cells from stressed subjects in response to tumor. Previous studies highlight the significance of co-stimulatory surface molecule expression for the activation of cytolytic function NK and $CD8^+$ T cells (Rao KL et. al., 1999). This finding

is also consistent with our previous findings, which demonstrate a preferential induction of $CD80^+$ expression by lung B cells in response to tumor (Jones HP et. al., 2008).

B cell-depleting antibodies such as rituximab and ocrelizumab have been used for targeting certain B cell lymphomas and other tumor malignancies (Sikder MA, Friedberg JW (2008)). Our current findings support the importance of B cell function within the lung compartment, particularly given stress exposure and offers opportunities for further testing of other B-cell targets whereby B-depleting antibodies will impact tumor malignancy. By increasing our understanding of the mechanisms through which stress may alter B cells, one may anticipate cancer intervention strategies that reduce the stress effects on B cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Highlights

- Intensity of aversive stress determines severity of lung metastasis
- Pulmonary B lymphocytes from tumor bearing rats support cell-mediated tumor lysis
- B lymphocyte response to lung tumor formation is preferentially suppressed given aversive stress
- B lymphocytes from non-stressed mice can rescue cell-mediated tumor lysis.
- Aversive stress decreases B-lymphocyte activation and IL-12 cytokine expression.

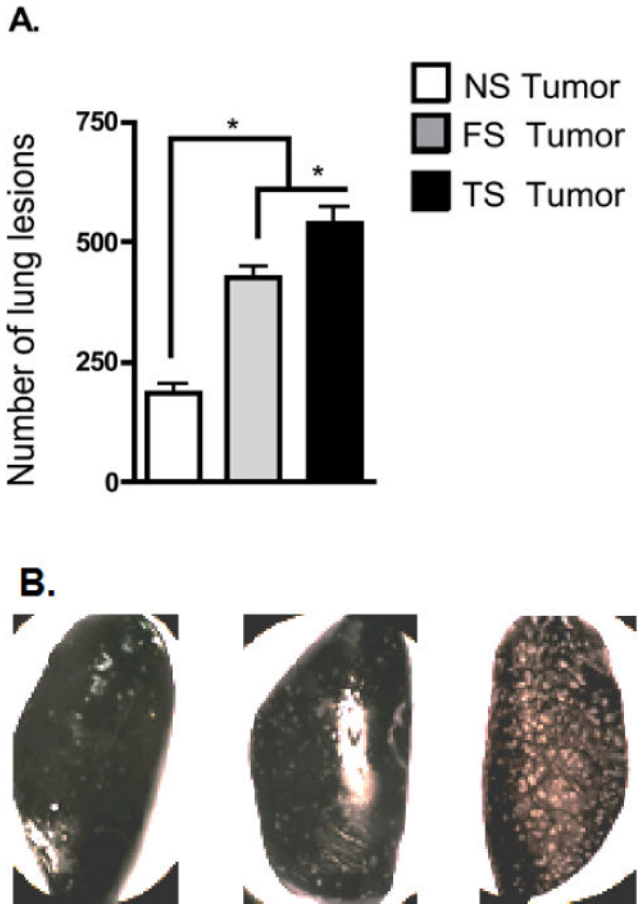


**Figure 1. Lung MADB106 tumor development in response to mild and intense stress**

MADB106 tumor cells were intravenously (i.v.) introduced by tail vein injection of 12-month-old female rats following exposure to foot shock (mild stress), tail shock (intense stress) or no shock. The number of tumor lesions on the outer surface of each lung lobe was determined macroscopically seven days after tumor administration. Bars graphs represent the mean (n=12–15 per experimental group) ± std. error. Asterisks indicate significant ($p \leq 0.05$) differences between indicated experimental groups (A). The gross lung tumor morphology is represented in each experimental group (B).

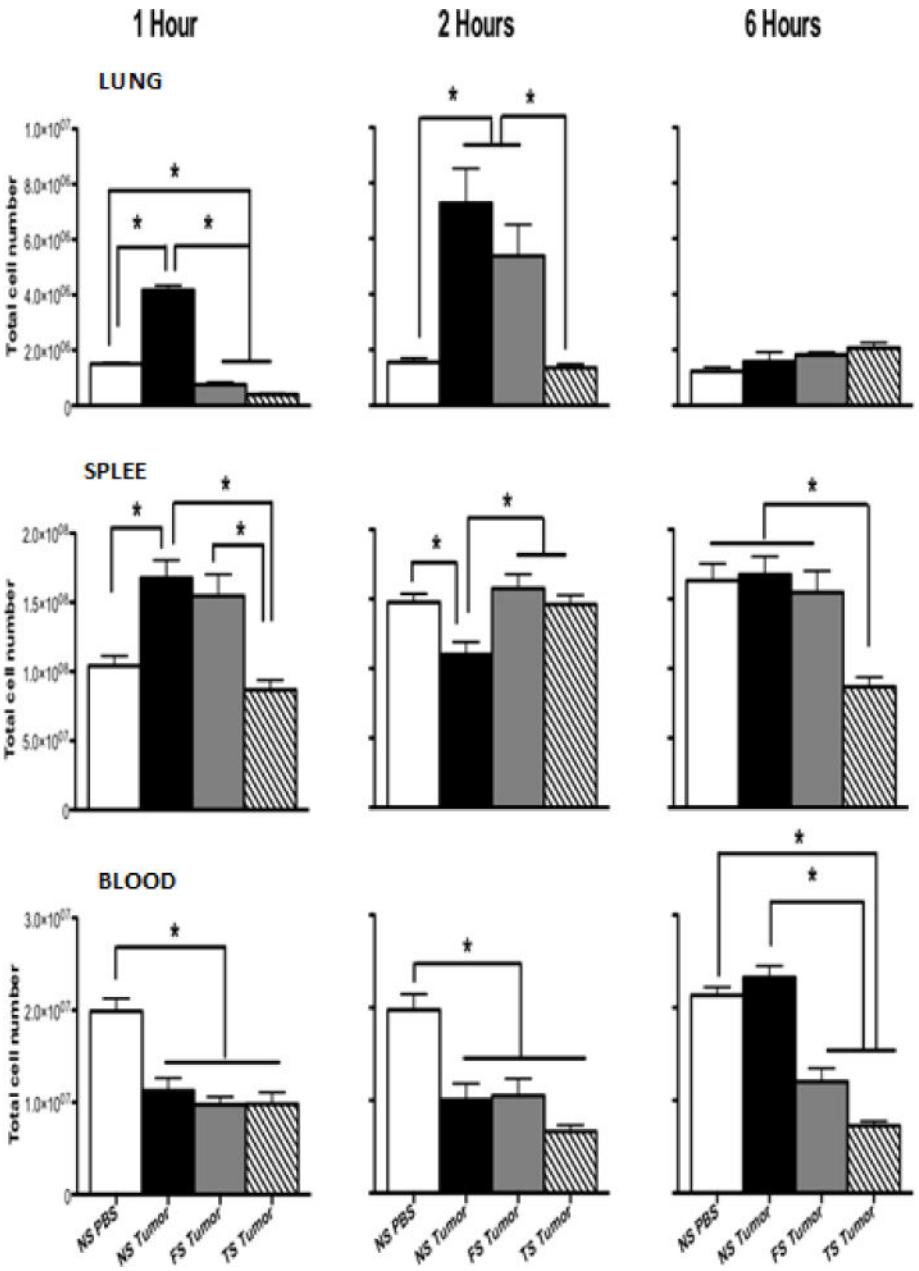


**Figure 2. Total lymphocyte distribution in rats following stress and tumor injection**

Total lymphocyte cells were determined in the lung, spleen and blood of rats at selected time intervals (1 hr, 2 hr, 6 hr) following stress exposure and i.v. tumor administration. For comparison, the number of total lymphocytes was determined from non-stressed mice i.v. administered phosphate buffered saline (PBS). Bar graphs represent the mean (n=8–10 per experimental group) ± std. error of the total number of representative phenotype of lymphocyte populations. Asterisks indicate significant ($p \leq 0.05$) differences between indicated experimental groups.

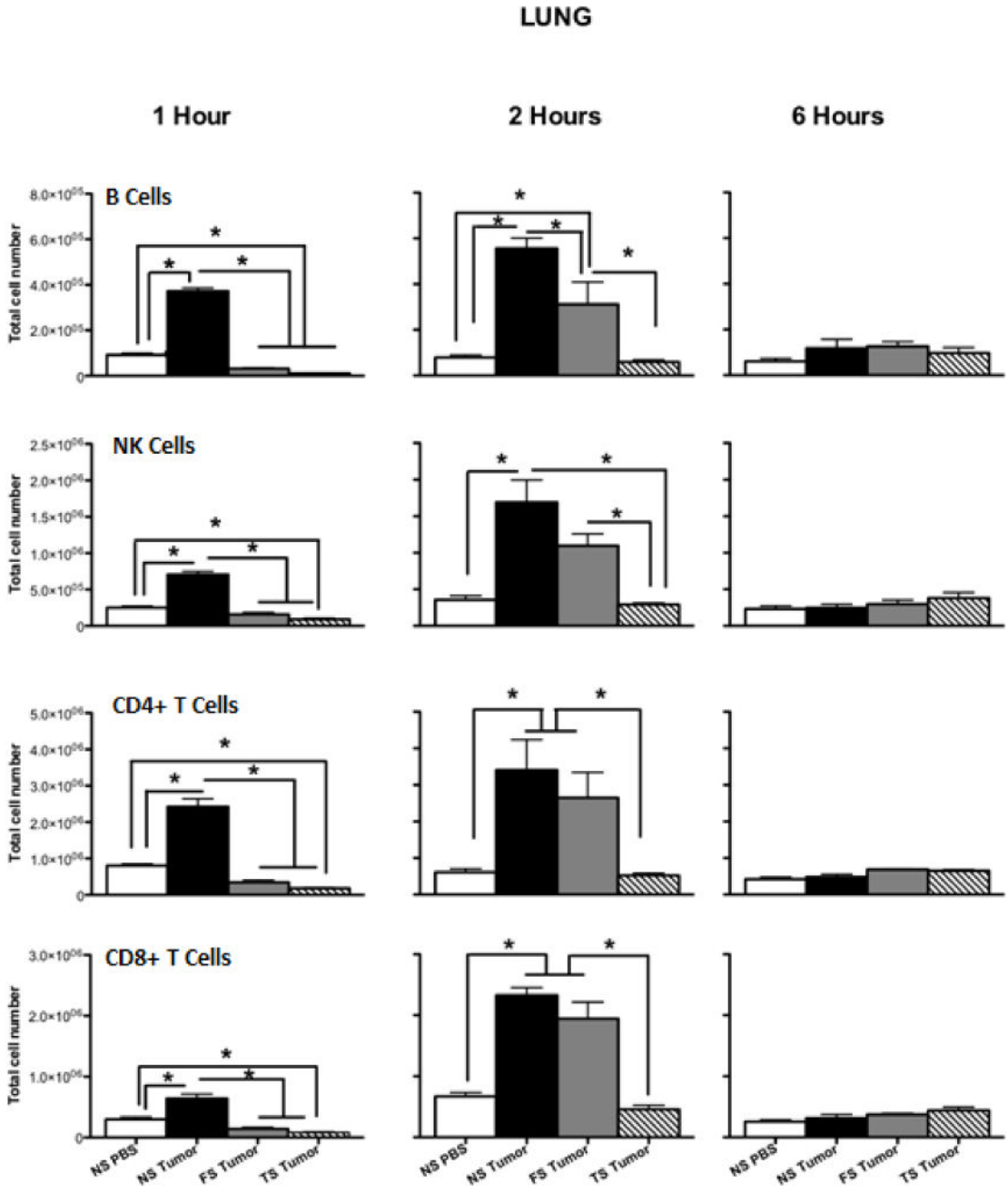


**Figure 3. Stress preferentially diminishes B cells' early accumulation in the lung of rats following tumor infection**

The phenotype (B, NK, $CD4^+$ and $CD8^+$) of lymphocyte cells were determined in the lung of rats exposed to mild and intense stress was determined at selected time intervals (1 hr, 2 hr, 6 hr) following stress exposure and i.v. tumor administration. For comparison, the number of total lymphocytes was determined from non-stressed mice i.v. administered phosphate buffered saline (PBS). Bar graphs represent the mean (n=8–10 per experimental group) ± std. error of the total number of representative phenotype of lymphocyte populations. Asterisks indicate significant ($p \leq 0.05$) differences between indicated experimental groups.

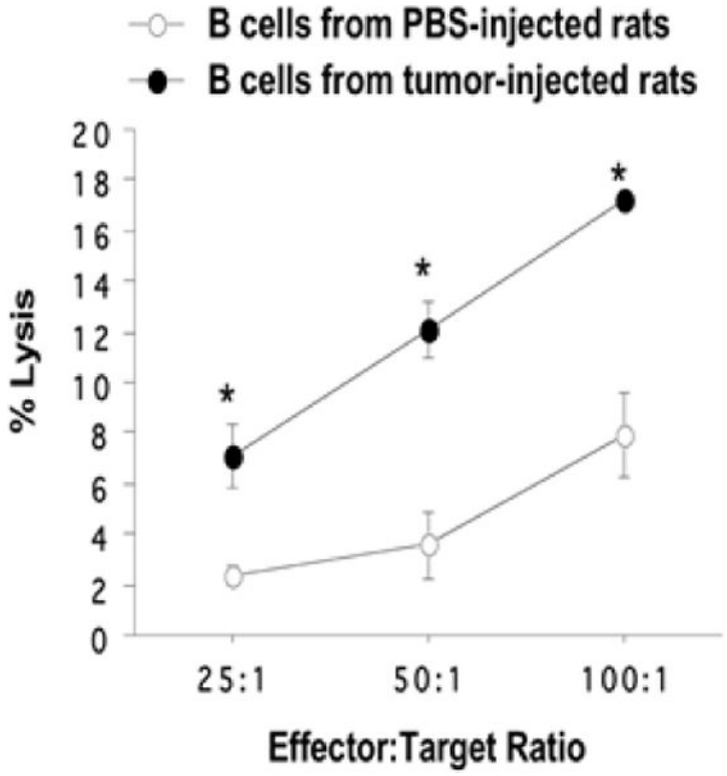


**Figure 4. Pulmonary B cells from tumor-injected rats promote tumor lysis**

2 hr after tumor administration, B cells isolated from the lungs were assessed for their ability to augment lymphocyte-mediated killing of MADB106 tumor cells as compared to B cells isolated from the lungs of PBS-treated counterparts. B cells representing each condition were incubated with total lung lymphocytes devoid of B cells from PBS-treated rats in the presence of MADB106 cells at designated effector to target (E:T) cell ratios. Data represents the mean (n=3) per E:T ratio ± std. error of two independent experiments. Asterisks indicate significant ($p \leq 0.05$) differences between indicated experimental groups at each E:T ratio.

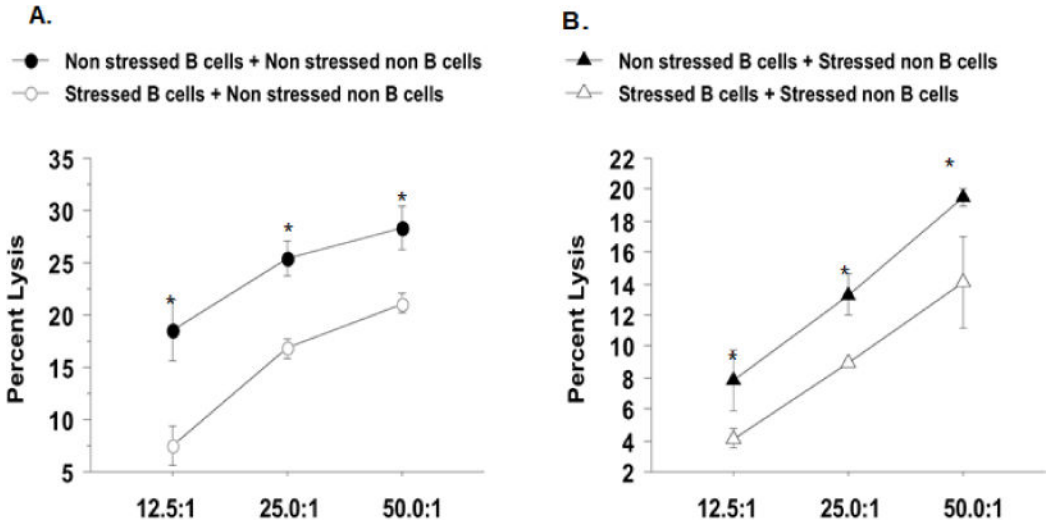


**Figure 5. Stress alters pulmonary B cells' ability to enhance tumor lytic function**

2 hr after tumor administration, B cells from the lungs of rats exposed to intense stress or no stress were incubated with tumor-bearing non-B cell lung lymphocytes representing the following experimental groups a) non-B cells from non-stressed rats and b) non-B cells from intense-stressed rats. The proportion of B cells to non-B cells to be cultured was pre-determined by flow cytometry assessment of the percentage of B, NK, CD4 and CD8+ T cells found in the lung 2 hr after tumor administration. Lymphocyte-mediated killing of MADB106 tumor cells was determined at designated effector to target (E:T) cell ratios. Data represents the mean (n=3) per E:T ratio ± std. error of two independent experiments. Asterisks indicate significant ($p \leq 0.05$) differences between indicated experimental groups at each E:T ratio.

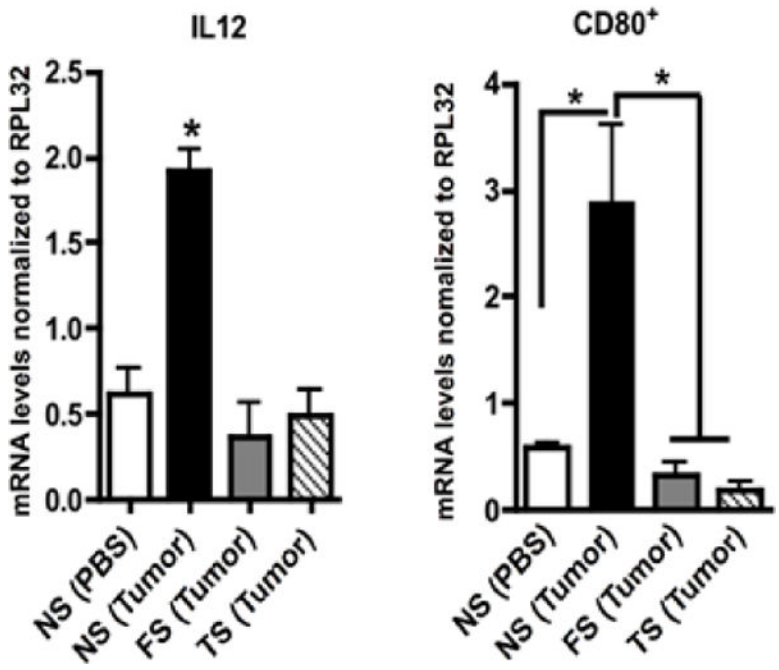


**Figure 6. Altered IL-12 and CD80 mRNA expression by lung B cells corresponds with diminished tumorlysis**

IL-12 cytokine and CD80 co-stimulatory surface molecule mRNA expression by lung B cells from non-stressed, mild stressed, and intense stressed rats 2 hr after tumor injection was determined by quantitative real time RT-PCR. Differences in IL-12 and CD80 mRNA expression levels were compared to B cells isolated from non-stressed PBS-treated rats. Data represents the mean n=3 of three independent experiments ± std. error. Asterisks indicate significant ($p \leq 0.05$) differences between indicated experimental groups at each E:T ratio.