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Pro-Inflammatory Cytokine Surge After Injury Stimulates An Airway Immunoglobulin A Increase

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Abstract

Background—Injury stimulates an innate airway IgA response in severely injured patients, which also occurs in mice. Tumor necrosis factor- α (TNF- α) and interleukin-1 beta (IL-1 β) stimulate the production of polymeric immunoglobulin receptor (pIgR), the protein required to transport immunoglobulin A (IgA) to mucosal surfaces. Blockade of TNF- α and IL-1 β eliminates the airway IgA response to injury. IL-6 stimulates differentiation of B cells into IgA secreting plasma cells at mucosal sites. We investigated the local and systemic kinetics of TNF- α , IL-1 β , and IL-6 after injury in mice. We also hypothesized that injection of exogenous TNF- α , IL-1 β , and IL-6 would replicate the airway IgA response to injury.

Methods—Experiment 1: Male Institute of Cancer Research (ICR) mice were randomized to uninjured controls (n = 8) or to surgical stress with laparotomy and neck incisions with sacrifice at 1, 2, 3, 5, or 8 hours after injury (n = 8/group). Bronchoalveolar lavage (BAL) and serum levels of TNF- α , IL-1 β , and IL-6 were analyzed by ELISA. Experiment 2: Male ICR mice were randomized to uninjured controls (n = 6), Injury (surgical stress that was similar to expt 1 except the peritoneum was left intact, n = 6), or Cytokine injection with intraperitoneal injection of recombinant TNF- α , IL-1 β , and IL-6. Animals were sacrificed at 2 hours after injury and nasal airway lavage and bronchoalveolar lavage IgA were analyzed by ELISA.

Results—Experiment 1: BAL TNF- α , IL-1 β and IL-6 levels increased in bimodal pattern after injury at 3 h and 8 h vs controls (p<0.05). Serum IL-6 did not increase at 3 h, but did show a significant increase by 5 h vs control (p<0.05). Serum levels of TNF- α and IL-1 β did not change. Experiment 2: Both Injury and combination TNF- α , IL-1 β and IL-6 cytokine injection significantly increased IgA levels in airway lavage (BAL+NAL) compared to control (p<0.01 for both).

Conclusions—Airway levels of TNF- α , IL-1 β , and IL-6 increase in a bimodal pattern after injury with peaks at 3 and 8 hours that do not correspond to serum changes. The peak at 8 hours is consistent with the known increase in airway IgA after injury. Intraperitoneal injection of a combination exogenous TNF- α , IL-1 β , and IL-6 replicates the airway IgA increase after injury. This effect is not seen with individual cytokine injections.

INTRODUCTION

Pneumonia is a major cause of morbidity in critically ill patients. Severely injured trauma patients often require intensive care unit admission and mechanical ventilation rendering them at particularly high risk for ventilator associated pneumonia (VAP).¹⁻³ VAP is a leading cause of death due to nosocomial infections and also results in prolonged ICU stays and costs.⁴⁻⁵ Because of this high risk for VAP after trauma, multiple studies have attempted to elucidate risk factors in this patient population. One postulated risk factor is the impairment of the immune response that occurs following injury.^{6, 7} This impaired immune response appears related to an intense pro-inflammatory reaction that occurs in the lungs following injury.⁸⁻¹¹ Pro-inflammatory cytokine profiles correlate with the development of VAP.^{3, 9} However, the majority of investigations into pro-inflammatory cytokines look at systemic and not localized responses.

One area critical to prevention of pneumonia is the mucosal immune system of the lung.^{12, 13} The mucosal immune system involves multiple components, but the major strategic defensive molecule is immunoglobulin A (sIgA), which binds to airway pathogens, preventing mucosal adherence and allowing for pathogen clearance.^{14, 15} Recently, our lab observed an effect of injury on the respiratory mucosal immune response. In severely injured humans, acute increases in airway IgA occurred within thirty hours of injury. This response is reproducible in a mouse model; significant peaks in airway IgA occur 8 hours after a controlled injury and return to baseline by 24 hours.¹⁶

Several known factors affect IgA concentrations at mucosal surfaces. A final common step in the expression of IgA at the mucosal surface is transport of IgA from the lamina propria across the epithelial layer to the mucosal surface. This step is dependent on a transport protein called polymeric immunoglobulin receptor (pIgR), a multi-domain membrane-spanning protein located on the basolateral membrane of epithelial cells.¹⁷ At this site, pIgR binds free IgA and transports it to the apical surface via transcytosis. Enzymatic cleavage releases IgA into the lumen. A part of the pIgR protein, secretory component, remains attached to the IgA molecule.¹⁸ The combined IgA with the secretory component from pIgR identifies it as secretory IgA (sIgA). The pIgR receptor is therefore consumed in a 1:1 ratio with successful transport of IgA to the mucosal border.¹⁹ Experimentally, pro-inflammatory cytokines released by injury stimulate pIgR production *in vitro*. Tumor necrosis factor-alpha (TNF- α) activates the nuclear factor kappa B (NF κ B) transcription-activating pathway to increase pIgR production²⁰⁻²² while Interleukin-1beta (IL-1 β) increases pIgR concentrations in a dose dependent manner.²³ Our lab previously showed that anti-TNF- α antibody stops the airway IgA increase following injury while anti-IL-1 β antibody impairs this response.²⁴ Another important pro-inflammatory cytokine, IL-6, regulates B cell terminal differentiation into plasma cells at mucosal sites, resulting in increases in sIgA concentrations.^{25, 26} Furthermore, production of IL-6 is stimulated by TNF- α and IL-1 β .²⁷

Because TNF- α and IL-1 β blockade abrogate the airway IgA response to injury in our mouse model, we investigated the role of pro-inflammatory cytokines in this process. We characterized the expression of three inflammatory cytokines, TNF- α , IL-1 β , and IL-6, after injury and hypothesized that exogenous administration of these pro-inflammatory cytokines could replicate the airway IgA response seen after injury.

MATERIALS AND METHODS

Animals

Male five-to-seven-week-old Institute of Cancer Research mice were purchased from Harlan (Indianapolis, IN) and housed in the Animal Research Facility of the William S. Middleton

Memorial Veterans Hospital, an American Association for Accreditation of Laboratory Animal Care accredited conventional facility. Mice were allowed to acclimatize for 1 week with free access to standard chow diet (PMI Nutritional International, St. Louis, MO) and water, under controlled conditions of temperature and humidity with a 12:12 hour light:dark cycle.

Experiment 1: Effect of Injury on TNF- α , IL-1 β , and IL-6 levels in bronchoalveolar lavage and serum

Animals were anesthetized with an intraperitoneal ketamine (100 mg/kg) and acepromazine (5 mg/kg) mixture. The skin was disinfected using 75% ethanol and 2 wounds were then created. First, a 3.0-cm celiotomy incision was made and the small intestine was gently eviscerated and immediately returned to the peritoneal cavity. The wound was closed in 2 layers with 3 simple interrupted 4-0 silk sutures per layer. Second, a 1.5-cm ventral neck incision was made and blunt dissection carried down to the pretracheal plane. This wound was closed with a single layer of 2 simple interrupted 4-0 silk sutures. This limited injury was chosen since it had been approved by the Animal Care Committee for use in all prior cannulation experiments. It induced an obvious physiologic response in animals, but it lead to recovery within 24 hours.

Animals were sacrificed at 1, 2, 3, 5, and 8 hours after injury ($n = 8$ for 1, 2, 3, and 8 h; $n = 7$ for 5 h) by exsanguination from a left axillary artery transection. Prior to sacrifice, awake animals received additional anesthesia (up to half of the original dose) until the righting reflex was lost. One group of animals ($n = 8$) was sacrificed without injury to provide baseline cytokine values (0 h).

Blood was collected from the left axillary artery transection site for the serum sample. For the bronchoalveolar lavage (BAL) specimen, a tracheotomy was created and 1 mL of normal saline was injected with an 18-ga catheter distally and aspirated.

TNF- α , IL-1 β , and IL-6 quantitative analysis

Concentrations in pg/mL of TNF- α , IL-1 β , and IL-6 were measured in BAL and serum using solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay) for the respective cytokines (BD Biosciences, Bedford, MA). Briefly, separate 96-well plates were coated with 100 μ L per well of either the anti-mouse TNF- α , IL-1 β , or IL-6 in a 1:250 dilution in 0.1 M sodium carbonate coating buffer (pH 9.5) and incubated overnight at 4°C. Plates were washed 3 times and blocked with 200 μ L of Phosphate-Buffered Saline (PBS) with 10% Fetal Bovine Serum (FBS) for 1 h at room temperature. One hundred microliters of BAL, serum or cytokine standard (BD Biosciences, Bedford, MA) were added, and the plates were incubated for 2 h at room temperature. The diluent was PBS with 10% FBS. Plates were washed 5 times, and 100 μ L of a 1:250 dilution of the secondary antibody, either biotinylated anti-mouse TNF- α or IL-1 β was added and incubated 1 h at room temperature. After washing 5 times, Streptavidin-horseradish peroxidase (SAv-HRP) conjugate was added, and the mixture incubated 30 min at room temperature. For IL-6, a 1:250 dilution of the secondary antibody was also used; however, this was mixed with the SAv-HRP, done in one step, and allowed to incubate for 1 h. Plates were then washed 7 times, and 100 μ L of the substrate solution (tetramethylbenzidine and hydrogen peroxide) was added; the mixture was then incubated for 30 minutes at room temperature in the dark. The reaction was stopped by adding 50 μ L of 2N H₂SO₄, and the absorbance was read at 450 nm in a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The mass amounts of TNF- α , IL-1 β , or IL-6 were calculated by plotting their absorbance values on their respective standard curves, which was calculated using a four-parameter logistic fit with SOFTmax PRO software (Molecular Devices, Sunnyvale, CA).

Experiment 2: Ability of exogenous TNF- α , IL-1 β , and IL-6 injection to replicate respiratory IgA response after injury

Animals were randomized to receive injury ($n = 6$) via surgical stress or an intraperitoneal injection of recombinant TNF- α , IL-1 β , and IL-6 ($n = 8$). The surgical stress was identical to experiment 1 except that the peritoneum was left intact. Uninjured animals serving as controls ($n = 6$) provided baseline values. For the IP cytokine injection, recombinant mouse TNF- α , IL-1 β and IL-6 (Sigma-Aldrich, St. Louis, MO) solutions reconstituted in distilled water were prepared.

Prior to this experiment, several pilot studies determined appropriate timing of sacrifice and the combination of cytokines for injection. Due to the lack of significant changes with injections of TNF- α alone or in combination with IL-1 β or IL-6, we combined all 3 cytokines for injection. Animals were anesthetized with an intraperitoneal injection of a ketamine (100 mg/kg) and acepromazine (5 mg/kg) mixture. Following anesthesia, animals received injury ($n = 6$) as in experiment 1 or received an IP injection consisting of TNF- α (2 μ g), IL-1 β (1 μ g), and IL-6 (1 μ g) ($n = 8$). Two hours later animals were sacrificed as in experiment 1 while the uninjured animals ($n = 6$) were sacrificed to provide baseline values (0h).

A BAL specimen was collected as in experiment 1. A nasal airway lavage (NAL) specimen was also collected by directing the 18-ga catheter proximally and injecting 1 mL of saline that was collected as it exited the nose. The NAL specimens were not collected for cytokine levels since pilot studies found no detectable levels of TNF- α , IL-1 β , and IL-6 in NAL.

IgA quantitative analysis

Total IgA in the NAL and BAL samples was measured using a sandwich ELISA. 96-well plates (BD Biosciences, Bedford, MA) were coated with 50 μ L of α -chain-specific goat anti-mouse IgA (Sigma-Aldrich, St. Louis, MO) 10 μ g/mL in 0.1 M carbonate-bicarbonate coating buffer (pH 9.6), and incubated overnight at 4°C. Plates were washed 3 times and blocked with 100 μ L of 1% bovine serum albumin in Tris-buffered saline with 0.05% Tween-20 solution (TBS-Tween) for 1 h at room temperature. One hundred μ L of NAL, BAL, (diluted 1:2 and 1:5 respectively), or IgA standards (seven two-fold dilutions, from 1,000-7.8 ng/mL: Sigma-Aldrich, St. Louis, MO) were added, and the plates were incubated for 1 h at room temperature. The diluent was 5% non-fat dry milk in TBS-Tween. The plates were washed 3 times, and 100 μ L of a 1:500 dilution of the secondary antibody, goat anti-mouse IgA, α -chain-specific-horseradish peroxidase conjugate (Sigma-Aldrich, St. Louis, MO), was added, after which, the mixture was incubated for 1 h at room temperature. Plates were washed five times, and 100 μ L of the substrate solution (H₂O₂ and o-phenylenediamine) was added: the mixture was then incubated for 12 min at room temperature. The reaction was stopped by the addition of 50 μ L of 2N H₂SO₄, and absorbance was read at 490 nm in a Vmax Kinetic Microplate Reader (Molecular Devices). The mass amounts of IgA in the samples were calculated by plotting their absorbance values on the IgA standard curve, which was calculated using a four-parameter logistic fit with SOFTmax PRO software (Molecular Devices).

Statistical analysis

TNF- α , IL-1 β , and IL-6 data in experiment 1 and IgA data in experiment 2 from each treatment group were compared using analysis of variance (ANOVA) and the Fisher protected least significance difference (PLSD) test, with $\alpha = 0.05$ (Statview 5.0.1, SAS, Cary, NC). Numerical results are presented as mean $\pm$ standard error of the mean.

RESULTS

Experiment 1

Compared to control, there were statistically significant increases in bronchoalveolar lavage concentrations of TNF- α (76.6 ± 13.3 vs 30.5 ± 6.8 pg/mL, $p < 0.05$), IL-1 β (199.5 ± 38.3 vs 85.5 ± 15.8 pg/mL, $p < 0.05$), and IL-6 (98.1 ± 19.7 vs 43.3 ± 7.9 pg/mL, $p < 0.05$) 3 hours after injury. At 5 hours after injury, concentrations of all three cytokines approximated baseline control concentrations. Significant increases in concentrations of TNF- α (104.1 ± 15.8 vs 30.5 ± 6.8 pg/mL, $p < 0.05$), IL-1 β (261.4 ± 39.0 vs 85.5 ± 15.8 pg/mL, $p < 0.05$), and IL-6 (145.4 ± 25.3 vs 43.3 ± 7.9 pg/mL, $p < 0.05$) occurred again at 8 hours after injury compared to controls. This resulted in a bimodal pattern of increase of TNF- α , IL-1 β , and IL-6 after injury (Figure 1). Serum IL-6 concentrations increased significantly by 5 and 8 hours after injury compared to controls (673.8 ± 138.2 & 738.2 ± 155.2 vs 0.0 ± 0.0 pg/mL, $p < 0.05$), with no significant changes in TNF- α . IL-1 β remained non-detectable (nd) at all times.

Experiment 2

Figures 2–6 depict the results of the pilot studies done in preparation for experiment 2. There were no significant differences in airway (NAL + BAL) IgA concentrations between any groups in the 5 pilot studies. Previous work showed an airway IgA peak at 8 hours after injury and data from experiment 1 revealed high airway concentrations of TNF- α , IL-1 β and IL-6 at 8 hours after injury. Therefore, the first pilot animals received an IP injection of phosphate buffered saline (PBS) or four doses of TNF- α (0.2, 1.0, 2.0, 4.0 μ g) and sacrificed 8 hours after injection with comparison to uninjured controls ($n = 5$ /group). No significant increase in airway IgA occurred at 8 hours. A second pilot experiment sacrificed mice 2 hours after injection ($n = 5$ /group) based on the cytokine kinetics data. The 2-hour time period showed a non-significant trend toward an increase in airway IgA with 2.0 μ g of TNF- α . This dose was used in a third pilot study with sacrifice at 2 hours compared to PBS injection and 0-hour controls ($n = 6$ /group), but IgA levels did not significantly increase. Subsequently, results of a fourth pilot study with injections of 1) PBS, 2) 2.0 μ g TNF- α , 3) 0.2 μ g IL-1 β , or 4) 2.0 μ g TNF- α + 0.2 μ g IL-1 β ($n = 11, 8, 10$, & 11 /group respectively) with sacrifice at 2 hours after injection were compared to uninjured controls with no significant differences between groups. A fifth pilot experiment added IL-6 with injections of 1) 1.0 μ g IL-6, 2) 2.0 μ g TNF- α , or 3) 2.0 μ g TNF- α + 1.0 μ g IL-6 with sacrifice at 2 hours compared to uninjured controls with no significant differences noted.

Injury caused a significant increase in airway (NAL+BAL) IgA concentrations compared to controls (140.3 ± 20.5 ng/mL vs. 68.7 ± 8.3 ng/mL, respectively, $p < 0.01$). Injection of the three cytokines (TNF- α , IL-1 β , and IL-6) significantly increased airway IgA concentrations compared to controls (133.3 ± 15.7 vs. 68.7 ± 8.3 ng/mL, respectively, $p < 0.01$) (Figure 7). There were no significant differences in airway IgA concentrations between the injury and the cytokine injection groups.

DISCUSSION

Injury stimulates both systemic and mucosal immune defenses. Systemically, acute increases in TNF- α , IL-1 β , IL-6, and other cytokines upregulate the acute phase protein response, the metabolic rate, mobilization of amino acids from lean tissues and immunity.²⁸ The current work demonstrates that these pro-inflammatory cytokines regulate an acute airway mucosal immune response that requires all three cytokines to function.

The mucosal surfaces respond through both innate and specific immune defenses. The major strategic specific immune defense is secretory immunoglobulin A (sIgA), which binds to

pathogens to prevent bacterial adherence and counter infection.^{13, 29, 30} Recently, we described the effect of injury on respiratory immune responses in humans noting significant airway IgA increases within 30 hours of serious injury. This response was reproducible in an animal model after a limited surgical stress with neck and abdominal incisions with significant increases in airway IgA at 8 hours with return to baseline by 24 hours.¹⁶ In both the human and mice, TNF- α and IL-1 β significantly increase in bronchoalveolar secretions to levels significantly higher than levels in the systemic circulation suggesting a local rather than systemic-driven stress response.³¹ In addition, blockade of TNF- α with anti-TNF monoclonal antibodies eliminated the IgA response to injury while IL-1 β blockade inhibited it.²⁴ Because IgA prevents bacterial adherence and counters invasion by bacteria, the increase after injury is likely a protective mechanism to prevent post-injury infections. Since parenteral nutrition also inhibits this response in mice, these results are consistent with the increased incidence of pneumonia noted in seriously injured, parenterally fed trauma patients.^{24, 32}

Transport of IgA from the lamina propria depends upon both production and transport. IL-6 causes terminal differentiation of B-cells to IgA-secreting plasma cells at mucosal sites and is one of the cytokines, along with IL-4, IL-5, and IL-10, important in stimulating IgA production.^{33–35} Transport of IgA is dependent upon pIgR expressed on the basement membrane of epithelial cells. pIgR molecules bind to IgA released in the lamina propria and the pIgR-IgA molecule is transported through the cell to the lumen where it is enzymatically cleaved after transport; pIgR is consumed 1:1 with the IgA molecule.^{18, 19} We recently showed that pIgR and IgA tissue levels in mice remain constant as luminal IgA levels increase after injury.³⁶ This suggests an upregulation of pIgR production after injury as it is rapidly consumed during IgA transport. This likely occurs via the pro-inflammatory cytokines since TNF- α and IL-1 β stimulate pIgR transcription via the NF κ B pathway.^{20, 23, 37} This is consistent with the cytokine peaks at 3 and 8 hours in the lavage specimens.

The ‘trigger’ for this response is unclear but it does not appear to be a process driven by systemic release of cytokines. Multiple studies have described elevated plasma cytokine levels in both animals and humans after injury.^{6, 38} Typically, serum increases in TNF- α and IL-1 β occur rapidly following injury with a lagging compensatory increase in the anti-inflammatory cytokines.^{28, 39} These elevations correlate with the degree of tissue injury, the degree of surgical stress, and the risk of subsequent complications.^{40–42} We found no early increases in serum levels of these cytokines although there is the slight possibility that they were cleared from the circulation prior to our one hour time point. This is possible because of the short serum half-life of TNF- α and IL-1 β .²⁸ This work and previous work suggests that the pulmonary response is primarily a local response.

It is clear, however, that a systemic, non-pulmonary, inflammatory response remains capable of stimulating the respiratory response since intraperitoneal administration of these 3 cytokines, but not the individual cytokines, to anesthetized animals produced a rapid pulmonary IgA response. That exogenous cytokine injection which caused increases in IgA at 2 hours may represent a difference in kinetics compared to a normal physiologic cytokine response. Still, it is unlikely that a systemic signal maintains the response since concentrations in BAL specimens were significantly higher than the serum levels and we noted a bimodal increase in BAL pro-inflammatory cytokines at times when serum levels of TNF- α and IL-1 β were low or not detectable. Only IL-6 increased to significant levels in the serum in the kinetic study.

These data support the hypothesis that a localized pulmonary pro-inflammatory response explains the increases in airway IgA. The 8 hour peak of these cytokines after injury corresponds to the previously described 8 hour peak of sIgA occurring after this limited injury.¹⁶ The reason for the bimodal increase in airway TNF- α , IL-1 β , and IL-6 after injury remains unclear and may be due to other inflammatory processes such as increased lung permeability,

increased neutrophil accumulation, or increased myeloperoxidase activity.^{43, 44} Since our previous work showed that the airway response is gone by 24 hours, it seems likely that these processes would return to normal by this time.

Systemic TNF- α , IL-1 β and IL-6 interact with pulmonary tissue. The pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6, are involved in the localized airway response to injury. A distinct kinetic bimodal pattern occurs in airway concentrations without significant changes in serum levels of TNF- α and IL-1 β . However, exogenous TNF- α , IL-1 β , and IL-6 in combination replicate the post injury IgA airway increases suggesting a potential for systemic stimulation of this localized airway response.

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Figure 1.
BAL concentrations of pro-inflammatory cytokines after injury
* $p < 0.01$ vs 0h, ** $p < 0.05$ vs 0h

Figure 2.
Pilot 1, airway IgA levels 8 hours after injection

Figure 3.
Pilot 2, airway IgA levels 2 hours after injection

Figure 4.
Pilot 3, airway IgA levels 2 hours after injection

Figure 5.
Pilot 4, airway IgA 2 hours after injection

Figure 6.
Pilot 5, airway IgA 2 hours after injection

Figure 7.
Airway IgA levels after injury or cytokine injection
p<0.05 vs control for injury and cytokines