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Mice depleted of CD8$^+$ T and NK cells are resistant to injury caused by cecal ligation and puncture

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We previously showed that beta 2 microglobulin knockout mice depleted of NK cells by treatment with anti-asialoGM1 (β2MKO/αAsGM1 mice) are resistant to sepsis caused by cecal ligation and puncture (CLP). β2MKO mice possess multiple immunological defects including depletion of CD8$^+$ T cells. This study was designed to determine the contribution of CD8$^+$ T and NK cell deficiency to the resistance of β2MKO/αAsGM1 mice to CLP-induced injury. β2MKO/αAsGM1 mice and CD8 knockout mice treated with anti-asialoGM1 (CD8KO/αAsGM1 mice) survived significantly longer than wild-type mice following CLP. Improved long-term survival was also observed in wild-type mice rendered CD8$^+$ T/NK cell-deficient by treatment with both anti-CD8α and anti-asialoGM1. Blood gas analysis and body temperature measurements showed that CD8$^+$ T and NK cell-deficient mice have significantly reduced metabolic acidosis and less hypothermia compared to control mice at 18 h after CLP. CD8$^+$ T/NK cell-deficient mice also showed an attenuated proinflammatory response as indicated by decreased expression of mRNAs for IL-1, IL-6 and MIP-2 in spleen and heart. IL-6, KC and MIP-2 levels in blood and peritoneal fluid were also significantly decreased CD8$^+$ T/NK cell-deficient mice compared to controls. CD8$^+$ T/NK cell-deficient mice exhibited decreased bacterial concentrations in blood, but not in peritoneal fluid or lung, compared to wild-type controls. These data show that mice depleted of CD8$^+$ T and NK cells exhibit survival benefit, improved physiologic function and an attenuated proinflammatory response following CLP that is comparable to β2M/αAsGM1 mice.
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We previously demonstrated that β2 microglobulin knockout mice that were depleted of natural killer (NK) cells by treatment with anti-asialoGM1 (β2MKO/αAsGM1 mice) are resistant to systemic injury caused by cecal ligation and puncture (CLP).[1] Specifically, β2MKO/αAsGM1 mice exhibit improved survival, less metabolic acidosis, reduced hypothermia and better hemodynamic function compared to control mice following CLP.[1,2] These improvements in physiological function are associated with attenuation of the CLP-induced proinflammatory response.

β2MKO/αAsGM1 mice have multiple immunological defects including an absence of CD8$^+$ T, natural killer (NK) and natural killer T (NKT) cells as well as deficient expression of the class I major histocompatability complex (MHC-I) and CD1 molecules.[1,3–5] One of our goals is to determine which of these immunological alterations confers resistance to the CLP-induced sepsis syndrome. We previously showed that adoptive transfer of CD8$^+$ T and NK cells into β2MKO/αAsGM1 mice will re-establish CLP-induced mortality.[1] In addition, we showed improved post-CLP survival in mice that are deficient of both CD8$^+$ T and NK cells. Based on this observation, we hypothesize that CD8$^+$ T and NK cell depletion contributes to the resistance of β2MKO/αAsGM1 mice to CLP-induced injury. To test this hypothesis, we directly compared CLP-induced mortality in β2MKO/αAsGM1 mice and CD8$^+$ T/NK cell-deficient mice. Acid–base balance, temperature, bacterial clearance and proinflammatory cytokine production following CLP were also measured in CD8α knockout (CD8KO) mice or wild-type mice that were depleted of CD8$^+$ T cells by treatment with antibody against CD8α. NK cells were depleted by injection of anti-asialoGM1.

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Materials and methods

Mice

Female, 6–8-week-old C57BL/6J wild type, β2 microglobulin knockout (β2MKO, strain B6.129P-B2 m^{tm1Unc}) and CD8α knockout (CD8KO, strain B6.129S2-Cd8a^{tm1Mac}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). CD8KO mice are functionally devoid of CD8$^+$ T cells.[6] Antibody-mediated depletion of CD8$^+$ T cells was achieved by intraperitoneal (IP) injection of anti-CD8α (50 μg, Cedarlane Laboratories) 24 h prior to CLP. Analysis of splenic and hepatic CD8$^+$ T cell numbers by flow cytometry showed greater than 95% depletion of CD8$^+$ T cells at 24 h after administration of anti-CD8α. Selective depletion of NK cells was performed by injection of anti-asialoGM1 (50 μg IP, Cedarlane Laboratories, Hornby, Ontario, Canada) 24 h prior to CLP. Treatment of mice with anti-asialoGM1 causes greater than 95% depletion of splenic and hepatic NK cells.[1] NK and NKT cell depletion was achieved by injection of anti-NK1.1 (clone PK136, 100 μg IP, Cedarlane Laboratories) and resulted in greater than 95% depletion of hepatic and splenic NK and NKT cells as determined by flow cytometry. Control mice were treated with nonspecific IgG (50 μg IP, Sigma Chemical, St Louis, MO, USA). The Institutional Animal Care and Use Committee at the University of Texas Medical Branch approved all studies.

Cecal Ligation and Puncture

Mice were anesthetized with 2% isoflurane in oxygen via facemask. A 1–2 cm midline incision was made through the abdominal wall; the cecum was identified and ligated with a 3-0 silk tie 1 cm from the tip. Care was taken not to cause bowel obstruction. A single puncture of the cecal wall was performed with a 20-gauge needle. The cecum was lightly squeezed to express a small amount of stool from the puncture site in order to assure a full thickness perforation. The cecum was returned to the abdominal cavity and the incision was closed with surgiclips. Mice were presented to the surgeon in a blinded fashion to minimize experimental bias. Sham mice underwent anesthesia and midline laparotomy; the cecum was exteriorized, returned to the abdomen and the wound was closed with surgiclips. Measurement of arterial blood gases, temperature, cytokine levels and bacterial colony counts were performed at 16 h after CLP because mortality of wild type controls begins at that time point.

RNAse Protection Assay

Spleen, heart, ileum and lung were harvested and flash frozen in liquid nitrogen. Samples were stored at -80°C until used. Total RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). The RNAse protection assay was performed using the Riboquant system (B-D Pharmingen) as per the manufacturer's instructions. Briefly, radiolabeled RNA probes were synthesized from DNA template sets using T7 RNA polymerase, ^{32}P-UTP and pooled non-radiolabeled nucleotides. Isolated total RNAs (20 μg/sample) were hybridized with the purified riboprobes and subjected to RNAse digestion. Protected RNA species were separated on 5% polyacrylamide sequencing gels using 0.5 $\times$ Tris-borate-EDTA running buffer. Gels were run at 50 W constant power for 70 min and dried under vacuum, and the protected fragments were visualized using autoradiography.

Enzyme-Linked Immunosorbent Assay (ELISA)

Peritoneal fluid was harvested from mice by peritoneal lavage with 5 ml of sterile saline. Cytokine levels in peritoneal fluid were determined using an ELISA according to the manufacturer's protocol (eBioscience, San Diego, CA, USA). Briefly, standards or experimental samples were added to microtiter plates that were coated with capture antibodies to the cytokine of interest and incubated for 2 h. After washing, horseradish peroxidase-conjugated, cytokine-specific antibody was added to each well, incubated for 2 h, and washed. Substrate solution was added and incubated for 30 min, and the reaction was terminated by the addition of stop solution. Cytokine levels were determined by measuring optical density at 450 nM using a microtiter plate reader (Dynatech Laboratories, Chantilly, VA, USA).

Bioplex Assay

Multiple simultaneous cytokine measurements were made using the Bio-Plex System (BioRad, Hercules, CA, USA) as per the manufacturer's instructions. Briefly, blood or peritoneal fluid was incubated with spectrally addressed polystyrene beads coated with cytokine-specific monoclonal antibodies. After washing the beads, a second set of fluorochrome-labelled cytokine-specific antibodies were added. The beads were again washed and cytokine levels were determined by measuring fluorescent signal following laser excitation.

Measurement of Temperature and Acid-Base Balance

Body temperature was measured by insertion of a rectal temperature probe prior to induction of anesthesia with 1.5–2.5% isoflurane in 100% oxygen via facemask. After induction of anesthesia, arterial blood for blood gas measurements was obtained by laceration of the carotid artery under

direct visualization using a surgical microscope. Blood was harvested using heparinized syringes and blood gas measurements were performed using iStat cartridges (iStat Corporation, East Windsor, NJ, USA).

Microbiology

Bacterial counts were performed on aseptically harvested blood, peritoneal fluid and lung. All fluid and tissue harvesting was performed under 2% isoflurane anesthesia. Blood was obtained by carotid laceration after aseptic preparation of the neck. Peritoneal fluid was harvested by injection of 5 ml sterile saline into the peritoneal cavity after aseptic preparation of the abdominal wall followed by aspiration of peritoneal fluid. Lung tissue was harvested under aseptic conditions after midline thoracotomy. After weighing, both lungs were homogenized in sterile saline using a sterile glass tissue homogenizer. Samples were serially diluted in sterile saline and cultured on tryptic soy agar pour plates. Plates were incubated (37°C) for 48–72 h and colony counts were performed. Anaerobic conditions were achieved using an anaerobic chamber and the BBL GasPak Plus Anaerobic system (Becton Dickinson, Sparks, MD, USA).

Statistics

All data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Survival curves were compared using the log-rank test. The mean and standard error of the mean were calculated in experiments with multiple data points. Data from multiple group experiments were analyzed using one-way analysis of variance (ANOVA) followed by a *posthoc* Tukey test to compare groups. Paired data were analyzed using a paired *t*-test. A value of $P<0.05$ was considered statistically significant.

Results

Mice Depleted of CD8+ T and NK Cells Exhibit Improved Survival after CLP

The survival of β2MKO/αAsGM1 mice and CD8 knockout mice treated with anti-asialoGM1 (CD8KO/αAsGM1 mice) was directly compared (Figure 1). Wild-type mice depleted of CD8+ T and NK cells by treatment with anti-CD8α and anti-asialoGM1 were also studied. Control wild-type mice showed a median survival time of 42 h and 100% mortality by 48 h after CLP. β2MKO/αAsGM1 and CD8KO/αAsGM1 mice exhibited 58 and 52% long-term survival, respectively. In all, 28% long-term survival was observed in wild-type mice depleted of CD8+ T and NK cells by treatment with anti-CD8α and anti-asialoGM1. All mice that survived beyond 96 h did not exhibit mortality out to 14 days post-CLP. Survival was significantly ($P<0.05$) improved in all experimental groups compared to wild-type controls. No significant difference in survival rate was observed when comparing β2MKO/αAsGM1, CD8KO/αAsGM1 and anti-CD8α/anti-asialoGM1 mice. Mice that survived out to 14 days after the CLP procedure were euthanized and abdominal contents were examined. The cecal ligature was intact in all mice and the cecum distal to the ligature appeared necrotic as indicated by a white–gray appearance compared to the pinkish-brown look of the adjacent bowel. All survivors had white, milky fluid in the peritoneal cavity. No other gross abnormalities were noted.

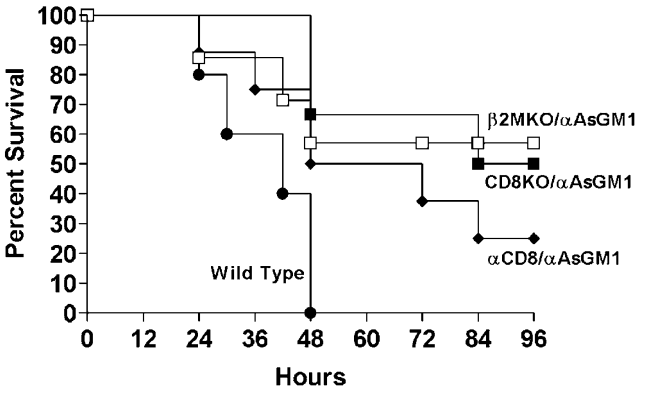

Figure 1 Wild-type mice treated with nonspecific IgG (wild type), β2 microglobulin knockout mice treated with anti-asialoGM1 (β2MKO/αAsGM1), CD8α knockout mice treated with anti-asialoGM1 (CD8KO/αAsGM1) and wild-type mice treated with anti-CD8α and asialoGM1 (αCD8/αAsGM1) underwent CLP. Survival was monitored every 12 h. $n=8$–12 mice/group.

The contribution of NKT cells to CLP-induced mortality was assessed by injection of mice with anti-NK1.1 (Figure 2). Treatment of mice with anti-NK1.1 results in depletion of both NK and NKT cells, whereas anti-asialoGM1 causes specific NK cell diminution.[7] Wild-type control mice exhibited 100% mortality by 30 h after CLP. Treatment of wild-type mice with anti-asialoGM1 or anti-NK1.1 did not significantly change CLP-induced mortality compared to control wild-type mice. CD8KO mice showed 100% mortality by 72 h after CLP, which was significantly ($P<0.05$) different from wild-type control mice. Long-term survival rates of 40 and 37%, respectively, were observed in CD8KO mice treated with anti-asialoGM1 or NK1.1. The overall survival rate was significantly ($P<0.05$) higher in CD8KO mice treated with anti-asialoGM1 or NK1.1 compared to wild-type or CD8KO mice treated with nonspecific IgG.

Mice Depleted of CD8+ T and NK Cells Exhibit Less Hypothermia and Metabolic Acidosis than Wild-Type Mice after CLP

Rectal temperature and acid-base balance were assessed in CD8KO/αAsGM1 mice 16 h after CLP

(Figure 3). Rectal temperature was significantly lower in wild-type mice treated with nonspecific IgG or anti-asialoGM1 compared to sham control mice. CD8KO mice treated with nonspecific IgG also exhibited decreased rectal temperature. However, CD8KO mice treated with anti-asialoGM1 did not have significant hypothermia and rectal temperature in these mice was significantly higher than in untreated wild-type mice.

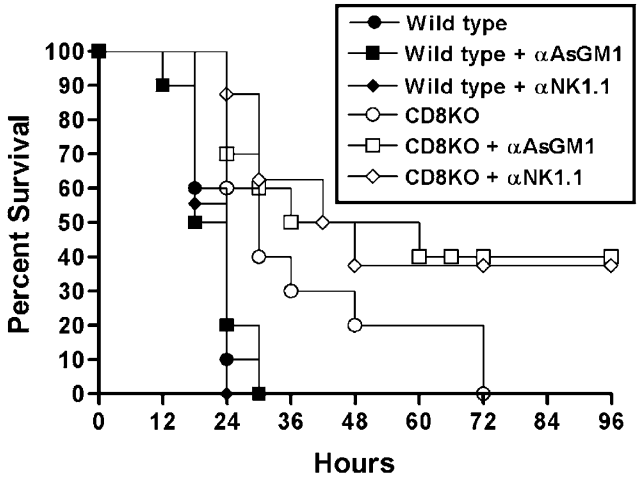

Figure 2 Wild-type and CD8KO mice treated with nonspecific IgG, anti-asialoGM1 or anti-NK1.1 underwent CLP. Survival was monitored every 12 h. $n = 8$–10 mice/group.

Assessment of arterial blood gases showed significant metabolic acidosis in wild-type mice treated with nonspecific IgG or anti-asialoGM1 as well as in CD8KO mice treated with nonspecific IgG. Arterial blood pH was significantly higher in CD8KO/αAsGM1 mice compared to wild-type mice but was not significantly different from sham controls (Figure 3). Results for blood bicarbonate and base deficit paralleled the findings for blood pH. Specifically, blood bicarbonate concentrations were significantly lower and base deficits were significantly higher in wild-type and CD8KO mice treated with nonspecific IgG compared to sham controls. These parameters were not significantly different in CD8KO/αAsGM1 mice compared to sham mice and were significantly improved compared to wild-type mice treated with nonspecific IgG.

Mice Depleted of CD8+ T and NK Cells Exhibit an Attenuated CLP-Induced Proinflammatory Response

The inflammatory response exhibited by wild-type and CD8KO mice after CLP was determined by

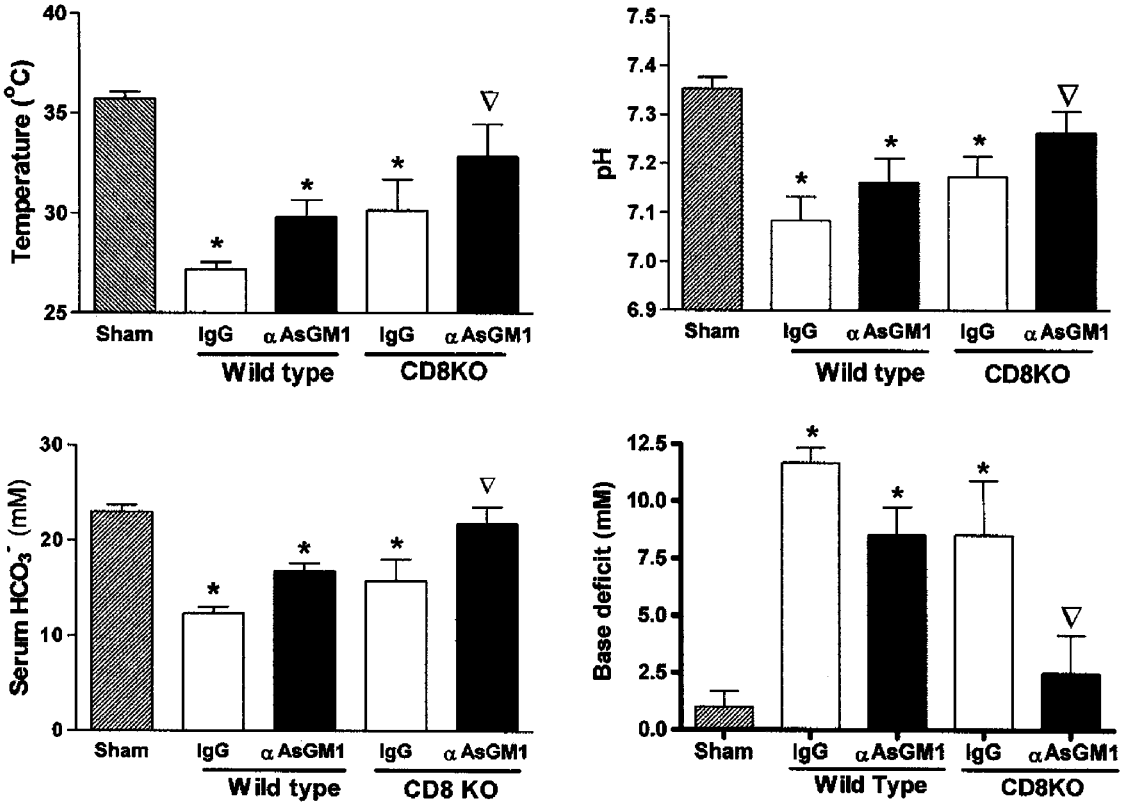

Figure 3 Rectal temperature and arterial blood gases were analyzed at 16 h after CLP in wild-type and CD8 knockout (CD8KO) mice treated with nonspecific IgG or anti-asialoGM1 (αAsGM1). Sham wild-type mice served as control. *Significantly ($P < 0.05$) different from sham. ▽Significantly ($P < 0.05$) different from wild-type mice treated with nonspecific IgG. Values represent the mean ± s.e.m. $n = 5$–7 mice/group.

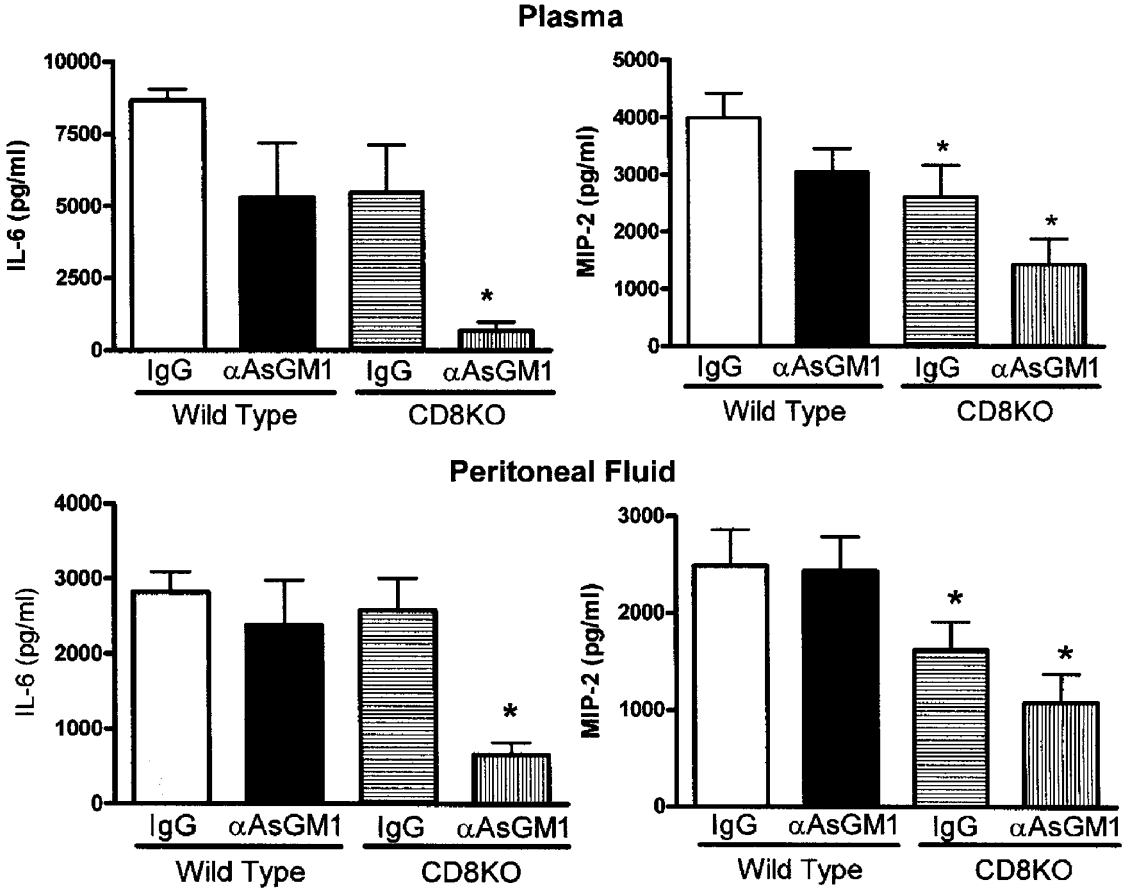

Figure 4 Peritoneal fluid and plasma were collected 16 h after CLP. Cytokine levels were determined using an ELISA. IgG = nonspecific immunoglobulin, αAsGM1 = anti-asialoGM1. Values represent the mean ± s.e.m. *Significantly ($P<0.05$) less than wild-type mice treated with nonspecific IgG. $n = 5$–7 mice/group.

measurement of cytokine concentrations in plasma and peritoneal fluid at 16 h after CLP. Levels of IL-6 and MIP-2 were measured 16 h after CLP (Figure 4). IL-6 concentrations in plasma and peritoneal fluid were significantly lower in CD8KO/αAsGM1 mice compared to wild-type mice treated with nonspecific IgG. Wild-type mice treated with anti-asialoGM1 and CD8KO mice treated with nonspecific IgG did not exhibit decreased IL-6 concentrations in plasma or peritoneal fluid compared to controls. Plasma and peritoneal fluid MIP-2 concentrations were significantly lower in CD8KO/αAsGM1 mice and CD8KO mice treated with nonspecific IgG compared to control wild-type mice. Wild-type mice treated with anti-asialoGM1 did not exhibit MIP-2 levels that were significantly different from control mice.

The CLP-induced proinflammatory response was also assessed in mice that were depleted of $CD8^+$ T cells by treatment with anti-CD8α (Figure 5). Wild-type mice treated with anti-CD8α plus nonspecific IgG or anti-asialoGM1 had lower plasma IL-6 levels than wild type mice treated with nonspecific IgG. In peritoneal fluid, IL-6 concentrations were only decreased in mice treated with both anti-CD8α and anti-asialoGM1. MIP-2 levels were significantly decreased in plasma and peritoneal fluid of wild-type mice treated with both anti-CD8α and anti-asialoGM1. Wild-type mice treated with anti-asialoGM1 alone or the combination of anti-CD8α and nonspecific IgG did not exhibit decreased MIP-2 concentrations in plasma or peritoneal fluid compared to control mice.

A more comprehensive analysis of plasma and peritoneal fluid cytokine levels in wild-type and CD8KO/αAsGM1 mice was performed using a bead array-based cytokine analysis system that allows for measurements of multiple cytokines in a single plasma or peritoneal fluid sample (Table 1). Among proinflammatory cytokines, IL-6 and IL-1β were decreased in the plasma and peritoneal fluid of CD8KO/αAsGM1 mice compared to wild-type controls 16 h after CLP. Plasma concentrations of TNFα and IL-1α were not different between groups but IL-1α was significantly lower in peritoneal fluid from CD8KO/αAsGM1 mice. Among cytokines that are commonly released by NK and Th1 cells after bacterial challenge and are characteristic of Th1 immune responses (type 1 cytokines), concentrations of IFNγ and IL-12 were very low in the plasma

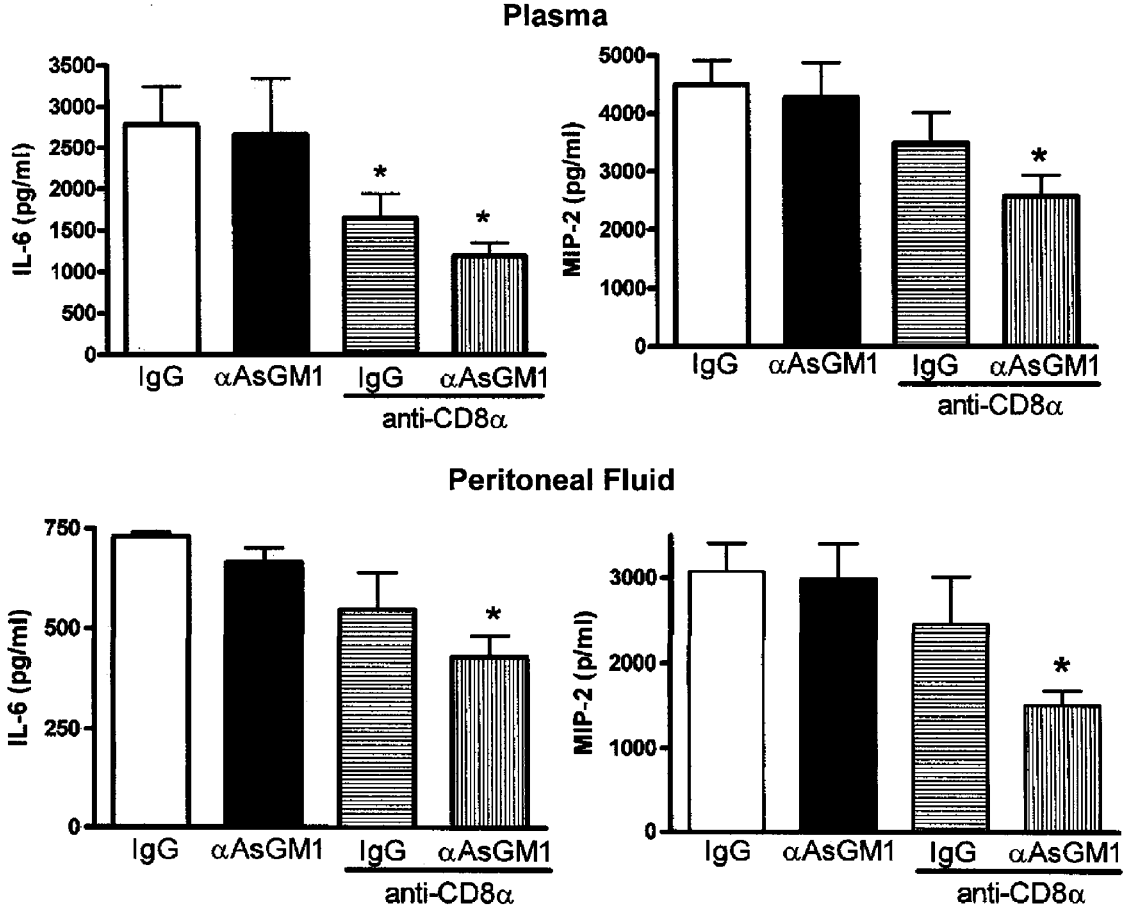

Figure 5 Peritoneal fluid and plasma were collected 16 h after CLP. Cytokine levels were determined using an ELISA. Values represent the mean ± s.e.m. IgG = nonspecific immunoglobulin, αAsGM1 = anti-asialoGM1. *Significantly ($P<0.05$) less than wild-type mice treated with non-specific IgG. $n = 5–7$ mice/group.

Table 1 Plasma and peritoneal cytokine concentrations 16 h after CLP

Cytokine	*Plasma*		*Peritoneal fluid*	
	Wild type	*CD8KO/αAsGM1*	*Wild type*	*CD8KO/αAsGM1*
Proinflammatory				
IL-6	5976 ± 553	684 ± 97*	1773 ± 254	461 ± 82*
IL-1β	46 ± 9	12 ± 3*	204 ± 31	85 ± 9*
IL-1α	83 ± 9	107 ± 11	76 ± 19	23 ± 3*
TNFα	16 ± 3	12 ± 3	28 ± 10	15 ± 2
Type 1				
IFNγ	21 ± 3	16 ± 5	16 ± 4	12 ± 2
IL-12	26 ± 5	16 ± 6	13 ± 6	10 ± 2
Type 2				
IL-10	7503 ± 1755	422 ± 79*	121 ± 39	14 ± 3*
IL-4	31 ± 5	21 ± 6	<1	<1
IL-5	<1	<1	11 ± 3	1 ± 1
Chemokines				
MIP-2	4271 ± 581	1394 ± 143*	1422 ± 212	443 ± 39*
KC	9987 ± 2441	1004 ± 153*	518 ± 38	82 ± 19*
MIP-1α	1072 ± 156	844 ± 113	121 ± 32	46 ± 7*
RANTES	126 ± 24	107 ± 9	20 ± 4	6 ± 2*

Cytokine concentrations as determined by bead array or ELISA at 16 h after CLP. Values are in pg/ml and represent the mean ± s.e.m. $n = 5–7$ mice/group. *Significantly ($P<0.05$) less than wild-type mice.

and peritoneal fluid of mice after CLP and were not significantly different between groups. Among anti-inflammatory and Th2 cytokines (type 2 cytokines), IL-10 was markedly increased in wild-type mice following CLP and was significantly lower in CD8KO/αAsGM1 mice. Concentrations of IL-4 and IL-5 in plasma and peritoneal fluid from wild-type and CD8KO/αAsGM1 mice were not significantly different between groups. The neutrophil chemoattractants MIP-2 and KC were significantly decreased in plasma and peritoneal fluid from CD8KO/αAsGM1 mice compared to wild-type controls. Plasma concentrations of the chemokines MIP-1α and RANTES were not different between groups but MIP-1α levels in peritoneal fluid were lower in CD8KO/αAsGM1 mice compared to wild-type controls.

The CLP-induced inflammatory response in wild-type and CD8+ T/NK cell-deficient mice was further assessed by measurement of proinflammatory cytokine expression in heart, spleen and ileum. Expression of mRNAs for IL-1β, IL-6 and MIP-2 16 h after CLP in wild-type and CD8KO/αAsGM1 mice was assessed by RNAse protection assay (Figure 6). Wild-type mice exhibited prominent expression of IL-1β, IL-6 and MIP-2 mRNAs in heart, spleen and ileum. Expression of mRNAs for these cytokines was decreased in the hearts and spleens of CD8KO/αAsGM1 mice. Interestingly, expression of these mRNAs in ileum adjacent to the ischemic cecum was not noticeably different between wild-type control mice and CD8KO/αAsGM1 mice except for MIP-2, which was lower in CD8KO/αAsGM1 mice compared to wild-type controls.

Expression of IL-1β, IL-6 and MIP-2 mRNAs were also measured in wild-type mice depleted of CD8+ T and NK cells by treatment with anti-CD8α and anti-asialoGM1 (Figure 7). These mice exhibited decreased cytokine expression in heart and spleen compared to wild-type mice treated with nonspecific IgG. Cytokine mRNA expression was comparable between groups in the ileum adjacent to the ischemic cecum.

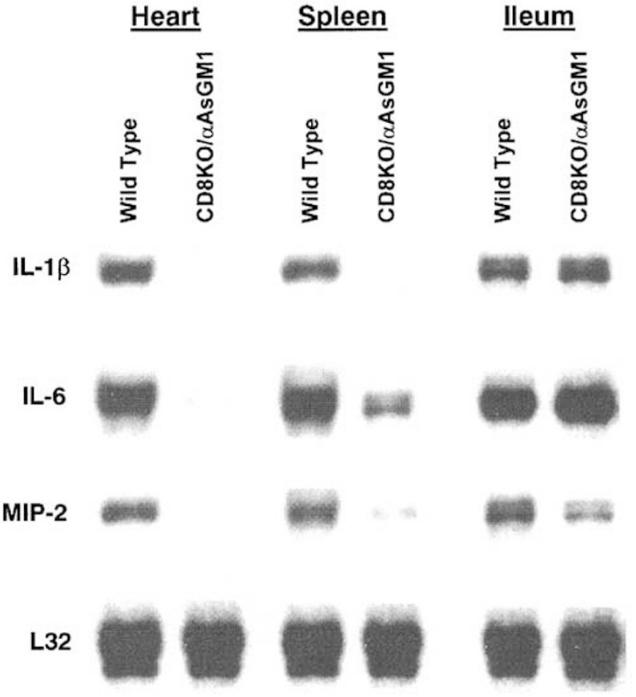

Figure 6 Cytokine mRNA expression was determined 16 h after CLP in wild-type mice treated with nonspecific IgG (wild type) and CD8 knockout mice treated with anti-asialoGM1 (CD8KO/αAsGM1) by RPA. Results are representative of at least three experiments.

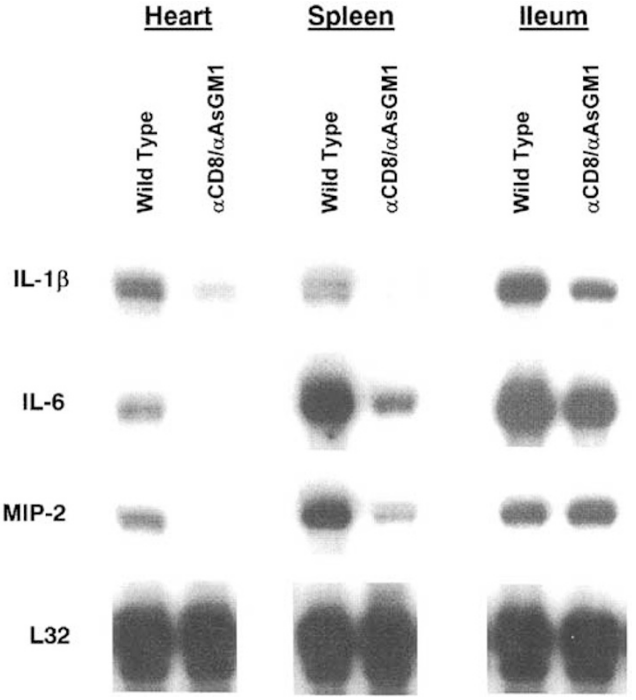

Figure 7 Cytokine mRNA expression was determined 16 h after CLP in wild-type mice treated with nonspecific IgG (wild type) and wild type mice treated with anti-CD8α and anti-asialoGM1 (αCD8/αAsGM1) by RPA. Results are representative of at least three experiments.

Bacterial Counts in Blood, but not Peritoneal Fluid or Lung, are Decreased in CD8KO/αAsGM1 Mice

Blood, peritoneal fluid and lung homogenates were cultured 16 h after CLP to measure viable bacterial (Figure 8). Colony counts for aerobes and anaerobes in peritoneal fluid and lung were not significantly different between CD8KO/αAsGM1 mice and wild-type controls. However, bacterial counts in blood were significantly lower in CD8KO/αAsGM1 mice.

Discussion

The findings presented in this report support the hypothesis that depletion of CD8+ T and NK cells is largely responsible for the resistance of β2MKO/

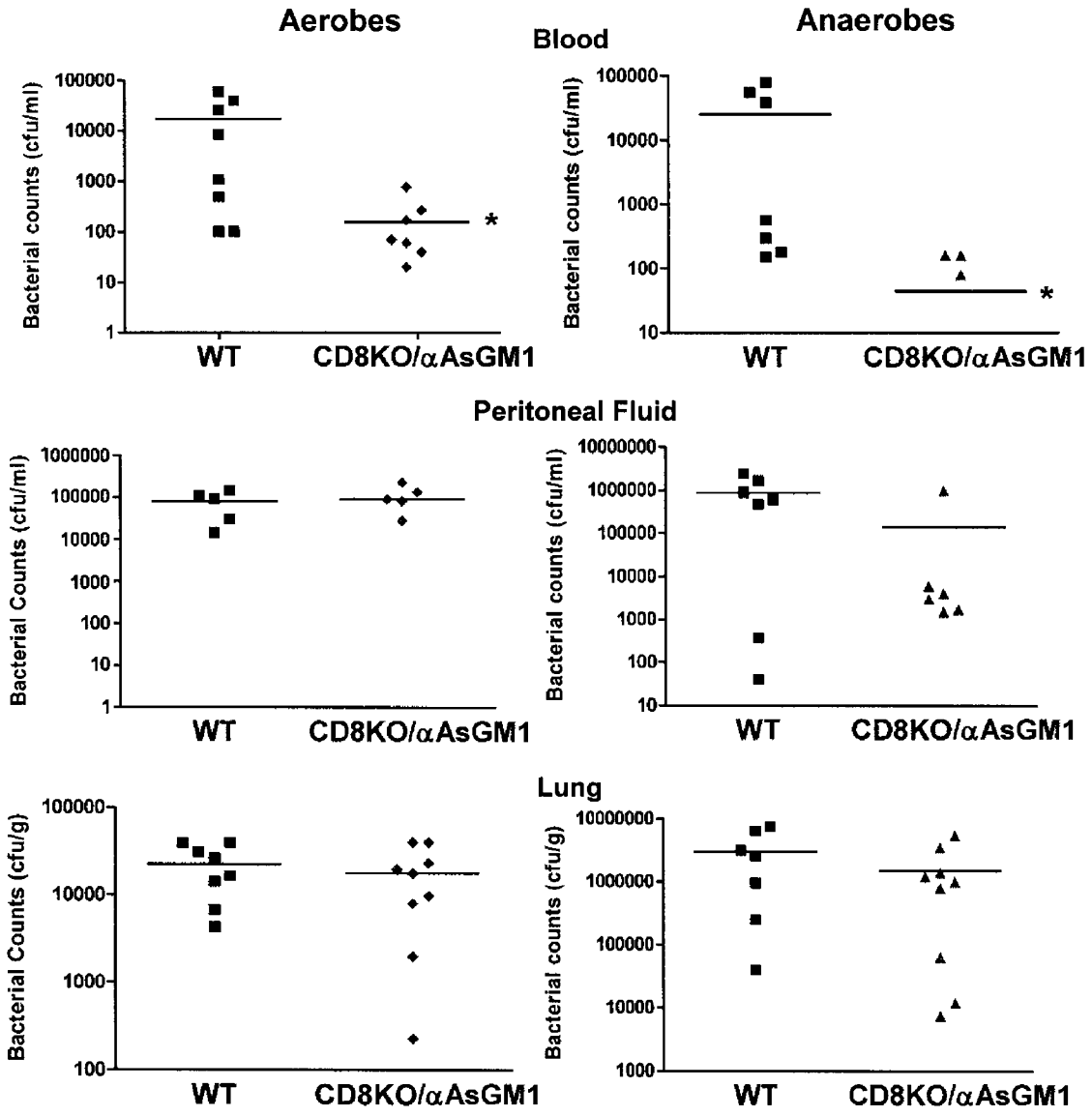

Figure 8 Bacterial burden in wild-type (WT) and CD8 knockout mice treated with anti-asialoGM1 (CD8KO/αAsGM1) after CLP. Blood, peritoneal fluid and lung were aseptically harvested 16 h after CLP. Aerobic and anaerobic bacterial colony counts were performed on serially diluted samples using tryptic soy broth plates. *Significantly ($P<0.05$) less than WT. $n=7$–8 mice/group.

αAsGM1 mice to CLP-induced mortality. Specifically, we show that CD8KO/αAsGM1 mice and wild-type mice treated with anti-CD8α plus anti-asialoGM1 have increased survival following CLP that is comparable to that observed in β2MKO/αAsGM1 mice. In a previous report, we showed that mice deficient in CD8+ T and NK cells exhibit improved survival compared to wild-type controls. The present study extends that finding by performing a direct comparison of post-CLP survival in β2MKO/αAsGM1 mice and CD8+ T/NK cell-deficient mice. The importance of CD8+ T and NK cell depletion to the resistance of β2MKO/αAsGM1 mice to CLP-induced injury is further supported by our previously published observation that adoptive transfer of CD8+ T and NK cells into β2MKO/αAsGM1 mice will re-establish CLP-induced mortality.[1] In addition, the alterations in temperature, acid–base balance and inflammation observed in mice specifically depleted of CD8+ T and NK cells in the present study closely approximate our previous observations in β2MKO/αAsGM1 mice.[1] Interestingly, depletion of both CD8+ T and NK cells is required for significant improvement in survival, acid–base balance, hypothermia and inflammation following CLP. Data presented in this report confirms our previous observation that depletion of NK cells alone does not improve CLP-induced mortality.[1] Nor did NK cell depletion decrease CLP-induced metabolic acidosis, hypothermia or inflammation. Specific depletion of CD8+ T cells improved survival time and decreased CLP-induced inflam-

mation but did not significantly affect acid–base balance or temperature. The improvement in survival and decrease in cytokine production observed in CD8+ T/NK cell-deficient mice was significantly better than in mice depleted of CD8+ T cells alone. CD8KO mice treated with anti-NK1.1 also exhibited improved survival compared to CD8KO mice treated with nonspecific IgG, but did not have improved survival compared to CD8KO mice treated with anti-asialoGM1. Treatment of mice with anti-NK1.1 causes depletion of NK and NKT cells, whereas anti-asialoGM1 treatment causes specific depletion of NK cells.[1,7] This observation suggests that NKT cells do not play a prominent role in CLP-induced injury.

Understanding the mechanisms by which CD8+ T and NK cell-depletion confers protection from CLP-induced injury is complicated by the complexity of the CLP model. It is likely that CLP represents a model of both infection and intestinal ischemia. The high bacterial counts in blood, peritoneal fluid and lung observed in this report and others[8,9] clearly demonstrates the functional importance of bacterial dissemination for CLP-induced mortality. We demonstrate significantly lower bacterial counts in blood at 16 h after CLP in CD8KO/αAsGM1 mice compared to wild-type controls. The significance of this finding is unclear given that bacterial burden in lung and peritoneal fluid was not different between groups. Most studies indicate that NK cells contribute positively to bacterial clearance mechanisms. Depletion of NK cells has been shown to impair bacterial clearance following CLP or challenge with other bacterial pathogens.[10–12] Few experimental studies have examined the importance of CD8+ T cells in mediating the host response to extracellular bacteria. A report by de la Calle-Martin *et al*[13] described recurrent bacterial respiratory tract infections in a patient with CD8+ T-cell deficiency due to a missense mutation in the CD8α gene. Therefore, it seems unlikely that depletion of CD8+ T and NK cells would improve bacterial clearance and resistance to the infectious component of CLP. Further studies will need to be performed using the CLP model as well as models of direct bacterial challenge to fully determine the effect of CD8+ T and NK cell depletion on the clearance of extracellular pathogens that are common causes of sepsis.

Intestinal ischemia is also likely to contribute significantly to CLP-induced pathology. However, the ischemic component of CLP has not been widely studied. The blood supply to the rodent cecum arises primarily from the superior mesenteric artery and runs from the base to the distal tip of the cecum.[14] Therefore, ligation of the cecum will disrupt blood flow to areas distal to the ligation site resulting in tissue ischemia. The functional importance of cecal ischemia in CLP-induced morbidity and mortality is supported by the studies of Singleton and Wischmeyer[15] in which the length of cecum ligated, rather than puncture size, was the major predictor of inflammation and mortality in rats following CLP. Other studies have shown that resection of the ischemic cecum will reverse CLP-induced mortality.[16] Our study shows that significant metabolic acidosis is associated with mortality in mice after CLP. The presence of metabolic acidosis indicates the existence of tissue ischemia and anaerobic metabolism. Whether this is due to cecal ligation or hypoperfusion secondary to sepsis-induced hypotension remains to be fully ascertained. However, taken together, these findings support the contention that cecal ischemia contributes to CLP-induced injury.

The functional role of CD8+ T and NK cells in mediating ischemia-induced injury in this model needs to be fully examined. However, some reports have demonstrated that CD8+ T cells participate in ischemia-associated injury in several settings. Granger and colleagues showed that CD8+ T cells contribute to neutrophil recruitment and intestinal injury during gut ischemia.[17,18] A role for CD8+ T cells in ischemia–reperfusion injury of the kidney has also been demonstrated.[19,20] Ayala and colleagues showed that blockade of Fas/FasL interactions will ablate CLP-induced injury.[21,22] CD8+ T and NK cells utilize the Fas/FasL pathway to induce cellular lysis and injury in a variety of scenarios.[23,24] The indirect evidence provided by this series of studies as well as the findings in the present study provide rationale to investigate the ischemic component of CLP-induced pathology and the contributions of CD8+ T and NK cells to this potential mechanism of injury.

Approximately 50% long-term survival was observed in mice depleted of CD8+ T and NK cells. Mice in this group that exhibited mortality died by 96 h post-CLP whereas long-term survivors were killed 14 days after the CLP procedure. Examination of abdominal contents showed that the cecal ligature was intact in all survivors and the cecum appeared necrotic. Our recent observations indicate that the ability of mice to respond to the infectious component of CLP-induced injury is likely a distinguishing factor between survivors and nonsurvivors in the CD8+ T and NK cell-depleted group. Specifically, CD8KO/αAsGM1 mice that are treated with imipenum for the first 72 h after CLP exhibit 100% survival compared to 50% survival in nonantibiotic-treated mice (unpublished observation). Antibiotic treatment does not confer survival benefit in wild-type mice exposed to CLP. This observation supports the notion that depletion of CD8+ T and NK cells confers resistance to the ischemic component of CLP-induced injury.

CD8KO/αAsGM1 mice exhibited markedly decreased production of proinflammatory cytokines and chemokines compared to wild-type control mice. Plasma and peritoneal fluid levels of IL-6, MIP-2 and KC were particularly attenuated in CD8KO/αAsGM1 mice. Previous studies have shown that elevated levels of IL-6 predict mortality

following CLP.[25,26] Increased plasma levels of MIP-2 and KC also correlate with poor outcome in rodent CLP models.[9] Our studies support the use of these cytokines as markers of lethality following CLP. Interestingly, concentrations of the type 1 cytokines IFN-γ and IL-12 were very low in both wild type and CD8KO/αAsGM1 mice. IFN-γ and IL-12 are normally elevated in response to systemic bacterial challenge.[27,28] The significance of low IFN-γ and IL-12 concentrations in blood and peritoneal fluid following CLP is not completely clear but further brings into question the functional role of bacteremia in CLP-induced death. However, Echtenacher *et al*[29] showed that neutralization of IFN-γ and IL-12 does not alter mortality in mice exposed to CLP. Their observation supports the contention that IFN-γ and IL-12 have minimal importance in the host response to CLP.

Overall, the present study indicates resistance of β2MKO/αAsGM1 mice to CLP-induced morbidity and mortality is likely due to depletion of CD8$^+$ T and NK cells. Deficiency of these cell types results in improved survival, decreased metabolic acidosis, less hypothermia and an attenuated proinflammatory response following CLP compared to wild-type controls. Additional mechanistic studies will be performed to fully understand the contribution of CD8$^+$ T and NK cells to CLP-induced mortality.

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