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Increased susceptibility to Candida infection following cecal ligation and puncture

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ABSTRACT

Secondary infection following septic insult represents a significant cause of morbidity and mortality in hospitalized patients. Sepsis induced immunosuppression is a major factor in the host's susceptibility to nosocomial infections and *Candida albicans* accounts for a growing number of these. Given the importance of improving our understanding of the immune response to sepsis and the increasing rates of *C. albicans* infections, we sought to develop a murine model of double injury consisting of primary peritonitis, i.e., cecal ligation and puncture (CLP), followed by a secondary challenge of *C. albicans*. As observed in previous work, after primary injury the immune profile of the host changes over time. Therefore, while keeping the mortality rates from the respective individual injuries low, we altered the timing of the secondary injury between two post-CLP time points, day two and day four. Mice subjected to *C. albicans* infection following CLP have significantly different survival rates dependent upon timing of secondary injury. Animals challenged with *C. albicans* at two days post CLP had 91% mortality whereas animals challenged at four days had 47% mortality. This improvement in survival at four days was associated with restoration of innate cell populations and as evidenced by stimulated splenocytes, increases in certain inflammatory cytokines. In addition, we show that susceptibility to *C. albicans* infection following CLP is dependent upon the depth of immunosuppression. Although at four days post-CLP there is a partial reconstitution of the immune system, these animals remain more susceptible to infection compared to their single injury (*C. albicans* alone) counterparts. Collectively, these studies demonstrate that immunosuppression following initial septic insult changes over time. This novel, two hit model of CLP followed by Candida provides additional insight into the immune compromised state created by primary peritonitis, and thereby opens up another avenue of investigation into the causes and possible cures of an emerging clinical problem.

1. Introduction

Nosocomial bloodstream infections (BSI) are on the rise in the United States. Among these BSI's, Candida species are the fourth most common, comprising 9.5% of total monomicrobial infections [1–3]. Recent studies have shown a 207% increase in the number of sepsis cases attributable to fungal infections [3]. Of these, *Candida albicans* accounts for nearly half of the mortality associated with systemic fungal infection [4]. Clinical reviews of patients with nosocomial candidemia show mortality rates ranging from 5% to 71% [5,6]. Other studies of patients with *Candida fungemia* found that one in four had polymicrobial bloodstream infections [2].

Possible causes for this increase have been attributed to the use of anti-neoplastic and immunosuppressive compounds, broad-spectrum antibiotics, the implantation of prosthetic devices and grafts, and extended ICU stays associated with long term catheter placement [3,7,8]. Similar to bacteria, *C. albicans* forms a biofilm on catheters and other devices, thereby creating resistance to therapy [8]. Importantly, patients with compromised immune systems as a result of trauma or disease, i.e., burns, neutropenia, and other infections, are susceptible to fungemia [3,7].

Over the last decade researchers have focused on trying to unravel the complex response of the immune system to sepsis. It is well known that sepsis leads to alterations in immunity which allow for increased susceptibility to secondary infection from organisms that normally do not infect immune competent hosts. In the early phase of sepsis, inflammatory cytokines, including IL-6, TNF-α, and IFN-γ, produce a relative hyper-inflammatory state [9–11]. In an effort to maintain homeostasis, the simultaneous production of anti-inflammatory cytokines, including IL-10, serve to balance this inflammatory state [9–13]. However, as the septic state progresses, anti-inflammatory cytokines dominate the cytokine milieu and a shift toward a dominant hypo-inflammatory

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state can develop. Animal studies as well as recent human studies highlight the importance of sepsis-induced immunosuppression and its role in mortality [14].

Given the importance of improving our understanding of the immune response to sepsis and increasing rates of *C. albicans* infections, we sought to develop a murine double injury model consisting of primary peritonitis, CLP, followed by a secondary injury of *C. albicans*. As observed in previous double injury studies, the timing of the secondary insult is a key factor in outcome [15]. Therefore, while keeping the mortality rates from the respective individual injuries relatively low, we altered the timing of the secondary injury between two time points, day two and day four. In addition, once the difference between an immune suppressed time point and an immune competent one was established, we sought, through acute studies, to define and describe the varying phenotypes.

2. Methods

2.1. Cecal ligation and puncture (CLP)

The CLP model of sepsis developed by Chaudry and modified for use in mice by Baker was employed as the initial septic insult [16,17]. Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) 8–10 weeks of age were used. The mice were anesthetized with isoflurane, a midline laparotomy was performed and the cecum identified. The distal one-third of the cecum was ligated and punctured once with a 30-gauge needle. This level of injury creates a prolonged infection with no mortality. The abdomen was closed and 1 ml of 0.9% saline mixed with 0.05 mg/kg of buprenorphine (Hospira, Lake Forest, IL) was administered subcutaneously (s.c.). A dose of Imipenem (25 mg/kg) was given s.c. 1 h post-CLP. Controls, i.e., sham-operated mice, were treated identically, except the cecum was neither ligated nor punctured. At one day post-CLP, 1 ml of 0.9% saline was given to all animals.

2.2. Microbiologic preparation

C. *albicans* strain MYA2430 (ATCC Manassas, VA) was cultured at 35 °C with constant agitation in Difco™ Sabouraud Dextrose broth (Becton, Dickinson and Co., Sparks, MD). The resultant growth was harvested, washed once in phosphate buffered saline, and re-suspended in sterile saline to a density of 0.3 A_{600nm}.

2.3. Candida (secondary injury)

Since intravenous challenge of *C. albicans* is a well established method of producing disseminated infection within mice and mimics the clinical scenario [4], we chose to use this route to induce secondary injury. At various time-points post CLP, mice were again anesthetized and given an intravenous injection (20 μl of 0.3 A_{600nm} *C. albicans*).

2.4. Experimental groups

Three groups were designed for both survival and acute studies. Two groups were given *C. albicans* at two or four days post-CLP. The third group, consisting of sham animals, received Candida at either two or four days post surgery. Survival studies were carried out for two weeks following Candida infection. Acute studies were designed to analyze immune status immediately before secondary injury (two and four days post CLP) and 48 h after secondary Candida injury (cultures done at six days).

2.5. Splenocyte cell counts

Splenocytes were harvested at the time of sacrifice. Total cell counts were obtained using Beckman Coulter Vi-Cell counter (Fullerton, CA). Mouse T and B cell populations were identified using fluorescein-labeled anti-mouse CD-4, CD-8, and CD-20 antibodies. For innate cell populations, DX5, CD11c, Gr1, and F480 antibodies were used for NK, dendritic cells, neutrophils, and macrophages, respectively. All antibodies (except F480-eBioscience, San Diego, CA) were obtained from BD Pharmingen San Diego, CA. Flow cytometric analysis (50,000 events/sample) was performed on FACScan (BD Biosciences, San Jose, CA).

2.6. Cytokine analysis

Splenocytes were cultured in the presence of CD3 and CD28 for 6 h prior to measurement. ELISAs were performed on supernatant from the stimulated splenocytes (R&D Systems, Minneapolis, MN). Individual assays were performed for IL-6, TNF-α, IL-10 and IFN-γ.

2.7. Blood and kidney fungal counts

Kidneys were harvested from all three groups at 48 h post-Candida infection and two groups at 6 days post-Candida infection (there were no two day survivors at this time point). Mice were anesthetized and blood was obtained by cardiac puncture and serially diluted for culture. For kidney harvest, the abdomen was opened, the left kidney removed and placed in a 15 ml disposable tissue grinder (VWR, Radnor, PA). Once thoroughly ground, the prep was serially diluted and placed on blood agar plates (Remel, Lenexa, KS). Colony counts were done at 24 h.

2.8. Statistical analysis

Student's t test was used for comparison of two groups, one-way ANOVA was used when three or more groups were compared. Chi square was used for survival studies.

3. Results

3.1. Survival from Candida dependent on timing of secondary injury

Survival from CLP alone was 100%. Candida infection alone caused a mortality of 11% (Fig. 1). Animals undergoing secondary injury with *C. albicans* two days post CLP had significantly greater mortality compared to both single injury animals and animals undergoing *C. albicans* infection four days post CLP (91% vs. 11% and 91% vs. 47%, respectively, $p < 0.0001$). In addition, four day animals had significantly increased mortality as compared to single injury animals (47% vs. 11%, $p < 0.002$).

3.2. Evaluation of splenocytes by flow cytometry reveals significant differences between two and four day animals

In order to further evaluate the immune status of mice undergoing secondary injury with *C. albicans*, we performed flow cytometric analysis to characterize the cellular composition of the spleen. Significant differences were seen in total splenocyte cell counts between animals two and four days post CLP, i.e., just prior to *C. albicans* secondary infection, (42×10^6 vs. 63×10^6, $p < 0.01$) (Fig. 2A). Animals two days post CLP had significant decreases in T and B lymphocyte counts as compared to sham animals (4.7×10^6 vs. 7.6×10^6 for CD4 T cells, 2.9×10^6 vs. 4.5×10^6 for CD8 T cells, 13.4×10^6 vs. 17.4×10^6 for B cells, respectively, $p < 0.05$) (Fig. 2A). Four day animals showed no significant

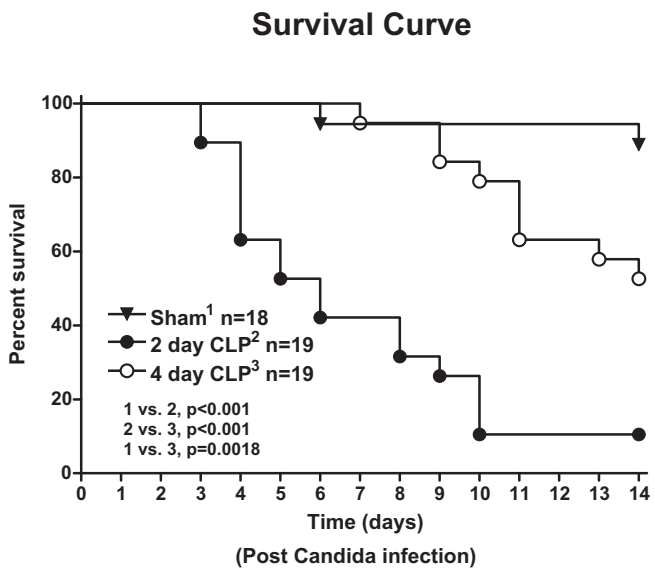

Fig. 1. Animals undergoing secondary injury with *C. albicans* at two days post CLP had significantly greater mortality compared to both single injury animals and animals receiving *C. albicans* four days post CLP, $p < 0.001$. Four day animals had significantly greater mortality than single injury animals, $p < 0.002$.

difference in CD8 T cells and B cells compared to sham animals, however they did have significantly less CD4 T cells (5.9×10^6 vs. 7.6×10^6, $p < 0.05$). Two and four day animals showed no differences in CD4 and CD 8 T cells, however two day animals had significantly less B cells (13.4×10^6 vs. 18×10^6, $p < 0.05$).

Analysis of neutrophils (CD11b+/Gr1+) and macrophages (CD11b+/F480+) revealed significant increases in the spleens of four day post CLP animals compared to both sham and 2 day animals (6.9×10^6 vs. 3.1×10^6 vs. 1.7×10^6, $p < 0.01$ and 3.33 vs. 2.38 vs. 1.24, $p < 0.01$, respectively) (Fig. 2B). Four day animals also showed significant increases in dendritic and NK cells compared to animals two days post CLP (2.8×10^6 vs. 2.0×10^6 and 2.6×10^6 vs. 1.8×10^6, respectively, $p < 0.05$) (Fig. 2B). Finally, two day animals had significantly decreased NK cells, neutrophils and macrophages compared to sham animals (1.8×10^6 vs. 3.0×10^6, $p < 0.01$, 1.7 vs. 3.1×10^6, $p < 0.05$ and 1.2×10^6 vs. 2.4×10^6, $p < 0.01$, respectively).

Evaluation of spleens 48 h post secondary injury (CLP/*C. albicans*) did not reveal any significant differences in overall splenocyte composition between two and four day animals (data not shown). However, there were significant losses in B cells, NK cells, dendritic cells and neutrophils in animals given Candida two days post CLP compared to single injury animals (data not shown). Animals receiving Candida four days post CLP only had significant decreases in their neutrophil population compared to single injury animals (data not shown).

3.3. Stimulated splenocytes show differences in cytokine production

TNF-α, IFN-γ, IL-6 and IL-10 production was measured in all three groups. TNF-α levels were significantly decreased in both two and four day groups (just prior to Candida infection) compared to sham mice (68 vs. 164 pg/ml, $p < 0.01$ and 88 vs. 164 pg/ml, $p < 0.05$, respectively). IFN-γ showed differences between the two CLP groups only, revealing less production in the two day animals (4073 vs. 10840 pg/ml, $p < 0.05$) (Fig. 3A). IL-6 was statistically higher within the four day group when compared to its two day counterpart (396 vs. 65 pg/ml, $p < 0.001$); in addition, the two day group produced significantly less IL-6 when compared to the shams (65 vs. 257 pg/ml, $p < 0.05$) (Fig. 3A). IL-10 levels were actually higher in the four day CLP group when compared to both two day group and shams (568 vs. 301 pg/ml, $p < 0.05$ and 568 vs. 133 pg/ml, $p < 0.001$, respectively) (Fig. 3A).

At 48 h post-Candida infection the same assays were performed. There were no significant differences in TNF-α production across all groups (data not shown). IFN-γ production remained low in two day animals, but was significantly greater in the 4 day group (132 vs. 1245 pg/ml, $p < 0.01$) (Fig. 3B). There was no significant difference between the four day and sham animals (1245 vs. 1011 pg/ml) (Fig. 3B). IL-6 remained significantly decreased in the two day group compared to sham animals, however there were no differences compared to the four day group (124 vs 451 pg/ml, $p < 0.05$ and 124 vs 372 pg/ml, $p < 0.05$) (Fig. 3B). IL-10 levels remained significantly elevated in the four day group compared to both two day CLP's and shams (812 vs. 253 pg/ml and 812 vs. 297 pg/ml, $p < 0.001$) (Fig. 3B).

3.4. Increased C. albicans in blood and kidney cultures from animals undergoing secondary injury at two days

Colony counts from cultures obtained 48 h after *C. albicans* were significantly different depending upon timing of secondary injury. Evaluation of blood cultures obtained 48 h after *C. albicans* infection revealed significantly more growth in two day animals compared to animals receiving secondary injury four days post CLP (20 vs. 0 CFU/ml, $p < 0.05$) (Fig. 4A). Consistent with the blood cultures, kidney cultures from two day animals revealed significantly more growth than their four day counterparts (1.2×10^5 vs. 6.7×10^3 CFU/ml, $p < 0.001$). Two day animals also had significantly more growth than animals undergoing *C. albicans* infection alone (1.2×10^5 vs 4.4×10^3 CFU/ml, $p < 0.001$) (Fig. 4A).

At six days post-Candida the same samples were taken from the surviving groups. Animals undergoing Candida infection four days post CLP had significantly greater growth in the kidney compared to animals receiving Candida infection alone (8.5×10^4 vs. 3.7×10^4 CFU/ml, $p = 0.013$) (Fig. 4B). Only one animal in the four day group had fungemia at this time point compared to no animals in the single injury group (data not shown).

4. Discussion

In this study we have shown that a systemic fungal infection, when given at varying time points following CLP, produces statistically significant mortality. Similar to previous studies, the timing of the secondary injury is a major factor [15]. Furthermore, our survival studies show that within the two day group, mice start dying as early as three days after Candida injection and continue to die throughout the study period, whereas the four day group does not experience its' first death until seven days post Candida.

Stimulated cytokines showed significant differences within the three groups. Of particular interest are the levels of IL-6, IFN-γ, and IL-10 among the two and four day groups prior to secondary infection. Higher levels of IL-6 and IFN-γ within the four day group are indicative of a potential Th-1 response to secondary challenge. However, IL-10, considered a marker of immune suppression shown to negatively correlate with survival in both humans and mice [18–21], is significantly higher in the four day group. Forty-eight hours after Candida infection, these groups continued to show significant differences in production of IL-6, IFN-γ, and IL-10. The ability of the cells in the four day group to produce equivalent amounts of pro-inflammatory cytokines (compared to single injury animals), at least in the early stages of secondary infection, may be indicative of the delayed death rates seen in our survival curves. However, the high levels of IL-10 at both points may be

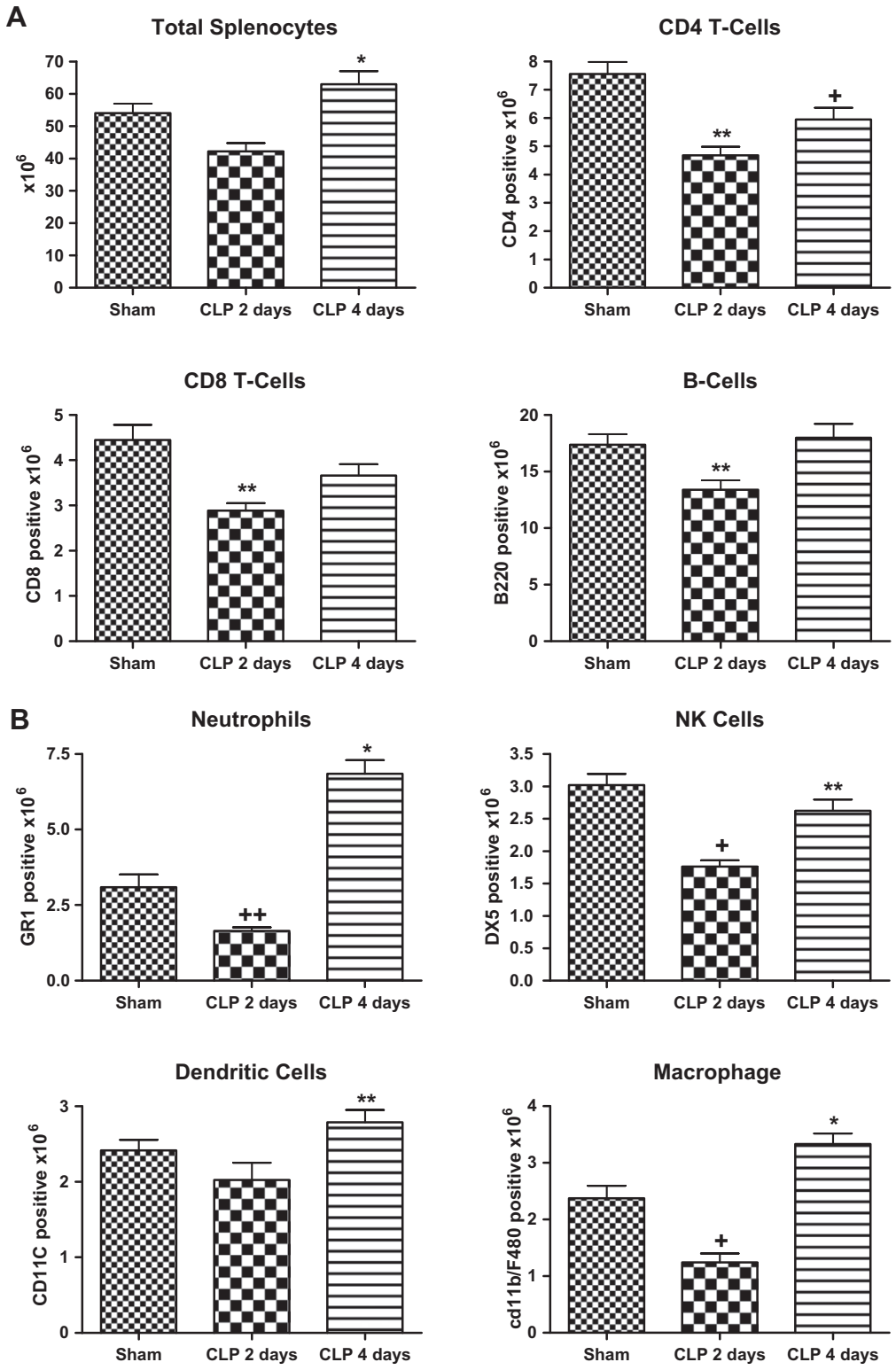

Fig. 2. Evaluation of splenocytes harvested from sham, two days post CLP (CLP 2 days) and four days post CLP (CLP 4 days) animals. (A) Significant differences were seen in total splenocyte counts between animals two and four days post CLP ($^{*}p < 0.01$). Animals two days post CLP did have significant decreases in both CD4 and CD8 T and B lymphocyte counts compared to sham animals ($^{**}p < 0.05$). Animals four days post CLP had significantly less CD4 T lymphocytes compared to sham animals ($^{*}p < 0.05$). $n = 6$ or 7 for all groups. (B) Analysis of neutrophils and macrophages revealed significant increases in the spleens of four day post CLP animals compared to both sham and 2 day animals ($^{*}p < 0.01$). Four day animals also showed significant increases in dendritic and NK cells compared to animals two days post CLP ($^{**}p < 0.05$). Two day animals had significantly decreased NK cells, neutrophils and macrophages compared to sham animals ($^{**}p < 0.05$, $^{+}p < 0.01$). $n = 6$ or 7 for all groups.

suggestive of the eventual mortality seen within this group. Of note, IL-10 has been shown to have immunosuppressive effects in regards to specific defense against C. albicans [22,23].

Flow cytometric analysis of splenocytes in animals two and four days post CLP shows significant loss of overall cell counts between groups. Previous studies have shown that this loss of both adaptive

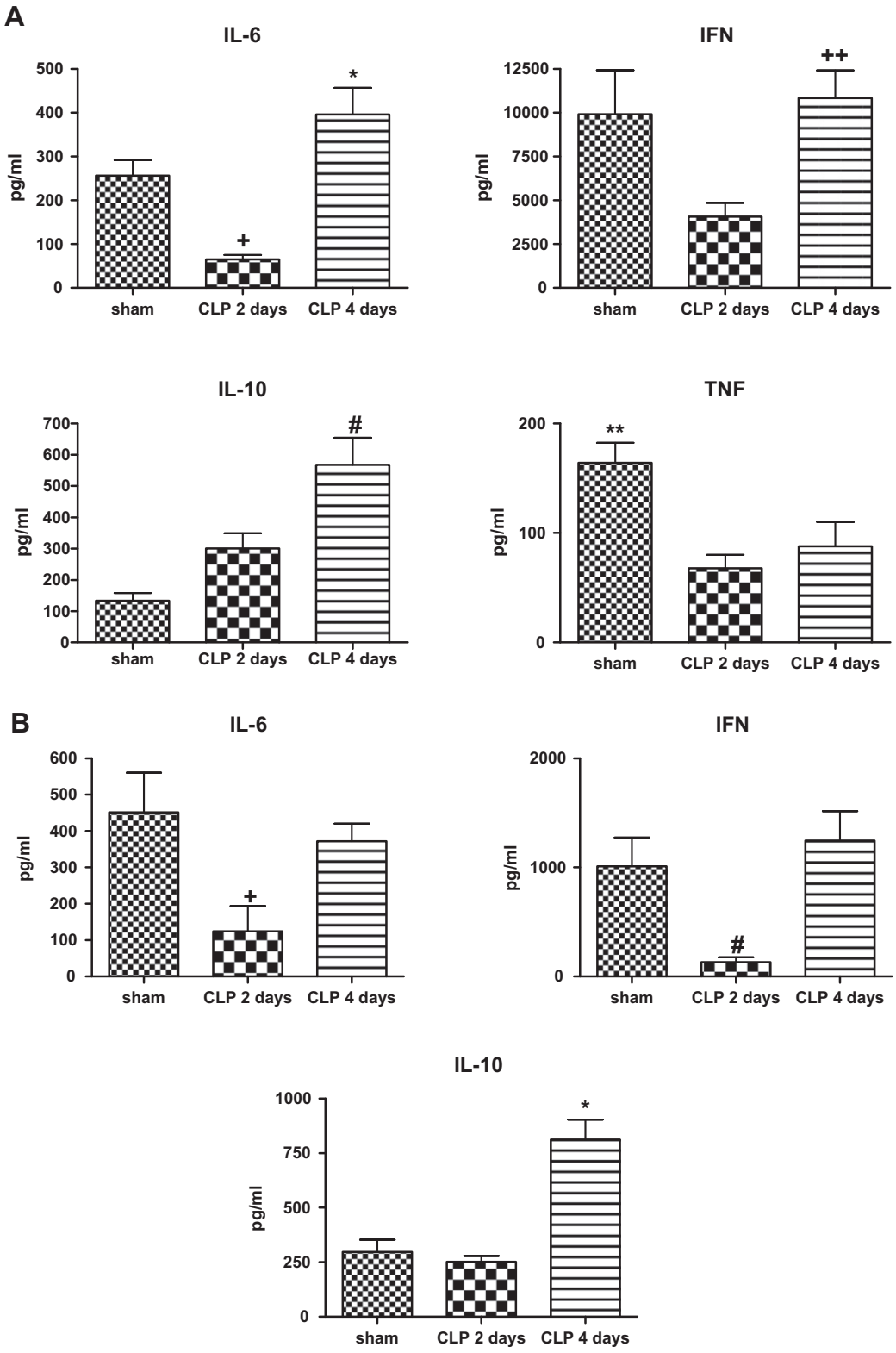

Fig. 3. (A) Evaluation of cultured splenocytes in the presence of CD3 and CD28 harvested just prior to secondary injury revealed significant differences in cytokine production. IL-6 production in animals four days post CLP was higher compared to two day animals ($^{*}p < 0.001$). In addition, the two day group produced significantly less IL-6 compared to shams ($^{+}p < 0.05$). TNF levels were significantly decreased from sham mice in both the two and four day groups ($^{**}p < 0.01$ and $^{**}p < 0.05$, respectively). IFN-γ showed differences between the two CLP groups only, revealing less production in the 2 day animals ($^{++}p < 0.05$). IL-10 levels were higher in the four day CLP's when compared to both two day CLP's and shams ($^{\#}p < 0.05$ and $^{\#}p < 0.001$, respectively). $n = 5$ for all groups. (B) IL-6 remained significantly decreased in the two group compared to sham animals ($^{+}p < 0.05$), however there was no differences compared to the four day group. IFN-γ production remained low in two day animals, but was significantly increased in the 4 day group ($^{\#}p < 0.01$). There was no significant difference between the four day and sham animals. IL-10 levels remained significantly elevated in the four day group compared to both two day CLP's and shams ($^{*}p < 0.001$). $n = 6$ or 7 for all groups.

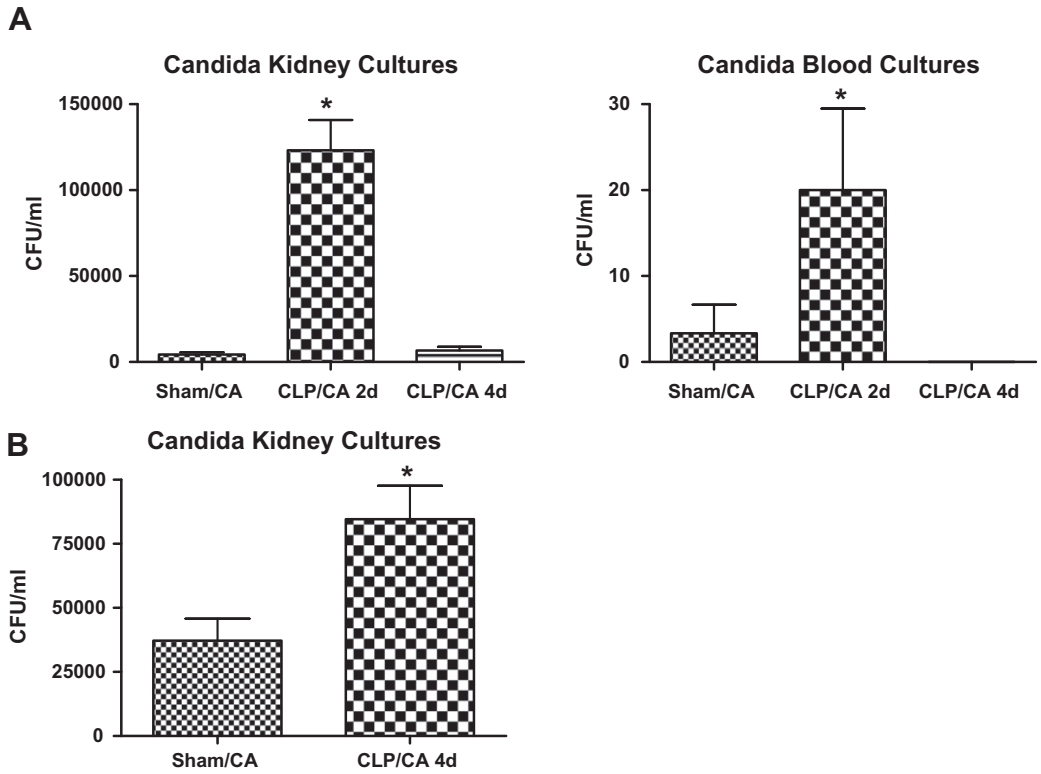

Fig. 4. (A) Evaluation of blood cultures obtained 48 h after *C. albicans* infection revealed significantly more growth in two day animals compared to four day animals ($^*p < 0.05$). Consistent with blood cultures, kidney cultures from two day animals revealed significantly more growth than four day animals ($^*p < 0.001$). Two day animals also had significantly more growth than animals undergoing *C. albicans* infection alone ($^*p < 0.001$). $n = 6$ or 7 for all groups. (B) At six days post-Candida four day animals had significantly greater growth in the kidney compared to animals receiving Candida infection alone ($^*p = 0.013$). $n = 6$ for all groups.

and innate immune cells plays a key role in the failure of the host to mount an effective immune response [11–15]. These studies have demonstrated the importance of T-cells in the clearance of Candida infections and the ongoing loss found in this model likely contributes to the differences in survival [24,25]. Other studies indicate that the innate system, particularly neutrophils and macrophages, are important in the clearance of fungal pathogens [26–29]. The relative neutropenia and decreased macrophages withing the two day group are likely another contributing factor to the differences in survival between two and four day animals.

Candida, when introduced outside of its' normally contained environment, tends to actively colonize the kidneys as well as the brain in both humans and mice [30,31]. The increased colonization of blood and kidneys in two day animals further demonstrates the failure of the immune system following a primary septic insult. Of interest, four day animals at 48 h post Candida show little growth in the blood and kidney and are equal to sham. However, cultures taken from this same group at six days post Candida provide evidence of the delayed inability of four day animals to clear infection, eventually leading to significant mortality.

These acute findings are further supported by the survival curve showing that four day animals have recovered enough immune function to mount an effective response for up to one week before succumbing to secondary infection. There are multiple explanations for the delayed mortality in the four day group as compared to sham. First, four day animals underwent CLP and although there is evidence that this infection is at least partially controlled, they continue to fight two distinct infections simultaneously as opposed to the single pathogen (Candida) of the sham mice. Second, with the exception of CD4 T-cells, four day animals have innate and adaptive cell numbers equal to if not greater than the sham group and are therefore able to mount a partial response as evidenced by similar levels of IFN-γ and IL-6 both before and after secondary injury. However, unlike the sham mice, they also produce significant amounts of IL-10 at these same time points. As mentioned earlier, high levels of IL-10 have significant immunosuppressive effects on the host in the context of Candida infection and are indicative of an ongoing immune dysfunction. Finally, cultures taken at the six day mark confirm the inability on the part of the four day group to control secondary infection.

From flow cytometry to stimulated cytokine values as well as culture counts, our acute data findings present the picture of a dysfunctional, but albeit evolving immunosuppressed state within the two double injury groups.

We have developed a novel murine model of secondary infection with CLP followed by *C. albicans*, potentially similar to what is found in patients. This study, when combined with our previous work, demonstrates that a variety of secondary injuries following murine CLP produce significant mortality [15,32]. These studies lend further credence to the hypothesis that sepsis can lead to a period of relative immunosuppression where levels of susceptibility change over time. The two hit model of CLP followed by Candida provides additional insight into the immune compromised state created by primary peritonitis, and thereby opens up another avenue of investigation into the causes and possible cures of an emerging clinical problem.

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