B cells enhance early innate immune responses during bacterial sepsis

Kindra M. Kelly-Scumpia,[1] Philip O. Scumpia,[4] Jason S. Weinstein,[5] Matthew J. Delano,[1] Alex G. Cuenca,[1] Dina C. Nacionales,[1] James L. Wynn,[6] Pui Y. Lee,[2] Yutaro Kumagai,[7] Philip A. Efron,[1] Shizuo Akira,[6] Clive Wasserfall,[3] Mark A. Atkinson,[3] and Lyle L. Moldawer[1]

[1]Department of Surgery, [2]Department of Medicine, and [3]Department of Pathology, University of Florida College of Medicine, Gainesville, FL 32610
[4]Division of Dermatology, Department of Medicine, University of California, Los Angeles, Los Angeles, CA 90095
[5]Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520
[6]Division of Neonatology, Department of Pediatrics, Duke University Medical Center, Durham, NC 27710
[7]Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University, Suita, 565-0871 Osaka, Japan

Microbes activate pattern recognition receptors to initiate adaptive immunity. T cells affect early innate inflammatory responses to viral infection, but both activation and suppression have been demonstrated. We identify a novel role for B cells in the early innate immune response during bacterial sepsis. We demonstrate that Rag1$^{-/-}$ mice display deficient early inflammatory responses and reduced survival during sepsis. Interestingly, B cell–deficient or anti-CD20 B cell–depleted mice, but not α/β T cell–deficient mice, display decreased inflammatory cytokine and chemokine production and reduced survival after sepsis. Both treatment of B cell–deficient mice with serum from wild-type (WT) mice and repletion of Rag1$^{-/-}$ mice with B cells improves sepsis survival, suggesting antibody-independent and antibody-dependent roles for B cells in the outcome to sepsis. During sepsis, marginal zone and follicular B cells are activated through type I interferon (IFN-I) receptor (IFN-α/β receptor [IFNAR]), and repleting Rag1$^{-/-}$ mice with WT, but not IFNAR$^{-/-}$, B cells improves IFN-I–dependent and –independent early cytokine responses. Repleting B cell–deficient mice with the IFN-I–dependent chemokine, CXCL10 was also sufficient to improve sepsis survival. This study identifies a novel role for IFN-I–activated B cells in protective early innate immune responses during bacterial sepsis.

CORRESPONDENCE
Lyle L. Moldawer:
moldawer@surgery.ufl.edu

Abbreviations used: CLP, cecal ligation and puncture; IFNAR, IFN-α/β receptor; IIC, innate inflammatory cytokine and chemokine; MZ, marginal zone; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor.

Inflammation is one of the earliest processes used during an innate immune response in an attempt to expel pathogens. Recognition of conserved pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors, such as Toll-like receptors (TLRs), on resident cells and recruited phagocytes initiates a signaling cascade that leads to a local inflammatory response, including the production of cytokines and chemokines, vasoactive peptides, complement, and reactive oxygen species (Takeda and Akira, 2005).

By activating APCs, TLR-driven inflammation also controls the development of adaptive immune responses (Akira et al., 2001; Schnare et al., 2001; Iwasaki and Medzhitov, 2004). Development and polarization of antigen-specific T and B cells is a well recognized function of PAMP-activated APCs (Iwasaki and Medzhitov, 2004; Pasare and Medzhitov, 2005). Recently, it was discovered that cells of the adaptive immune system provide additional signals to either restrict host inflammatory processes or contribute to protective inflammation and host defense. During viral infection, naive T cells were shown to limit mortality by dampening lethal innate immune system–mediated inflammation (Kim et al., 2007). Similarly, memory and effector T cells were found to suppress inflammasome-mediated inflammation by blocking activation of NALP1 and NALP3 (Guarda et al., 2009). More recently, however, memory T cells were shown to contribute to influenza-induced innate inflammatory cytokine and chemokine (IIC) production resulting in improved

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The Rockefeller University Press $30.00
J. Exp. Med. Vol. 208 No. 8 1673-1682
www.jem.org/cgi/doi/10.1084/jem.20101715

The Journal of Experimental Medicine

viral clearance (Strutt et al., 2010). These studies suggest that the adaptive immune system provides important feedback during acute inflammatory processes to provide host defense or limit pathology. However, little is known about how the adaptive immune system affects innate immunity during bacterial sepsis.

Bacterial sepsis is a scenario where pathological inflammation may lead to unwanted organ injury. In septic hosts, an exaggerated inflammatory response leads to sustained systemic inflammation, which contributes to failure to clear primary pathogens by causing defective innate and adaptive immune responses. Interestingly, Rag1$^{-/-}$ mice deficient in adaptive immunity demonstrate increased mortality after bacterial sepsis (Hotchkiss et al., 1999, 2000). Treatment of these mice with transgenic lymphocytes that are resistant to apoptosis was shown to improve survival (Hotchkiss et al., 1999), but which component of adaptive immunity regulates outcome is currently unknown.

In this study, we find, in response to bacterial sepsis, that mice devoid of an adaptive immune system actually demonstrate an attenuated and not an exaggerated inflammatory response. We find that deficiency of B cells, and not T cells, can completely replicate this phenotype and B cells contribute to inflammatory cytokine responses in vitro and in vivo. We also find that the mechanism of B cell activation in response to bacterial sepsis involves type I IFN (IFN-I) and redundant TLR signaling.

B cells produce inflammatory cytokines in vitro and in vivo and repletion of Rag1$^{-/-}$ mice with B cells improves survival, demonstrating that B cell function in the absence of T cell–dependent antibodies is important for sepsis outcome. Interestingly, mice deficient in B cells produce decreased levels of IFN-I–dependent cytokines. Treatment of Rag1$^{-/-}$ mice with the IFN inducible chemokine CXCL10 after sepsis initiation also improves outcome, and repletion of Rag1$^{-/-}$ mice with WT but not IFN-α/β receptor (IFNAR)$^{-/-}$ B cells restores IFN dependent and independent cytokine production. This study identifies a novel role for IFN-regulated B cells in modulating early innate immune responses during bacterial sepsis and identifies B cells as participants in a protective IFN-I–dependent circuit during sepsis.

RESULTS

Adaptive immune deficiency results in a decreased early inflammatory response and increased mortality during sepsis

As mice deficient in their adaptive immune system were previously shown to succumb more readily to bacterial sepsis (Hotchkiss et al., 1999, 2000), we first wished to determine how loss of the adaptive immune system was affecting early innate immune responses during bacterial sepsis. We confirm that Rag1$^{-/-}$ mice (mice deficient in B and T cells) are indeed highly susceptible to cecal ligation and puncture (CLP) lethality (Fig. 1 A; Fisher's exact test, P = 0.0001). Because the adaptive immune system has been shown to either augment or inhibit IIC production to viral challenge (Guarda et al., 2009; Strutt et al., 2010), we examined how loss of adaptive

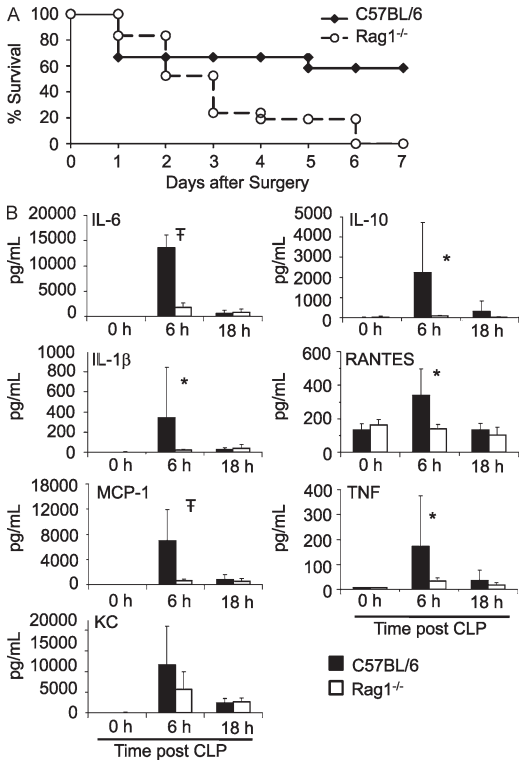

Figure 1. Adaptive immune effector cells are needed for survival to CLP-induced sepsis as well as the inflammatory response. (A) Rag1$^{-/-}$ mice (open circles; $n = 21$) and WT mice (closed diamonds; $n = 12$) were subjected to CLP and monitored for survival (P < 0.001). (B) Serum was collected at 0, 6, and 18 h after CLP from Rag1$^{-/-}$ mice (white bars) and WT animals (black bars), and cytokines were measured by Luminex multiplex analysis. Experiments in A were performed four separate times with $n \geq 6$ mice per group. The combined results from two independent experiments are shown. Experiments in B were performed two independent times with $n \geq 4$ per group. Shown in B are mean values + standard deviation. *, P < 0.05; ∓, P < 0.001 by Student's *t* test comparing WT with Rag1$^{-/-}$ mice.

immunity affects the systemic inflammatory response to sepsis. In WT mice, 6 h after CLP, the production of IIC in sera, including IL-1α, IL-1β, IL-6, IL-10, KC (IL-8 homologue), TNF, RANTES, and MCP-1, peaked before decreasing at 18 h (Fig. 1 B; IL1α not depicted). Of note, sepsis did not increase plasma cytokine concentrations not associated with a systemic inflammatory response, including IL-2, IL-3, IL-4, IL-7, IL-9, IL-15, IL-17, G-CSF, GM-CSF, and VEGF-A, in WT mice (unpublished data). Interestingly, IIC concentrations remained low in Rag1$^{-/-}$ mice at both 6 and 18 h after CLP and were significantly lower at 6 h (Fig. 1 B). These data suggest that cells of the adaptive immune system are contributing to early inflammation after bacterial sepsis.

B cell–deficient μMT$^{-/-}$ mice, but not TCR-deficient mice, display decreased IIC production and decreased survival after sepsis

To investigate whether T or B cells were contributing to IIC production in sepsis, we performed CLP in T cell–deficient TCR-α/β$^{-/-}$ and B cell–deficient μMT$^{-/-}$ mice.

Interestingly, we found that TCR-$\alpha/\beta^{-/-}$ mice display a modest but insignificant decrease in IIC production after sepsis (Fig. S1 A) and also do not display an increased susceptibility to sepsis-induced mortality when compared with WT mice (Fig. S1 B). Sepsis in $\mu MT^{-/-}$ mice, however, results in significant defects in IIC induction and increased mortality (Fig. 2, A and B), similar to $Rag1^{-/-}$ mice. These data suggest a novel and previously unrecognized role of B cells in regulating innate immune responses and, thus, the remainder of our investigations focused on the role of B cells in sepsis.

B cells can produce immunoregulatory cytokines, such as IL-6 or IL-12, upon TLR4 or TLR9 ligation (Hobbs et al., 1991; Yi et al., 1996; Sun et al., 2005), but whether they can contribute to IIC production in vitro has not been investigated in detail. We find that LPS stimulates $Rag1^{-/-}$ splenocytes to produce IL-6, and the addition of equal numbers of B cells results in a near doubling of IL-6 levels (Fig. S2), suggesting that B cells can contribute to cytokine production within a mixed cell population toward an inflammatory stimulus.

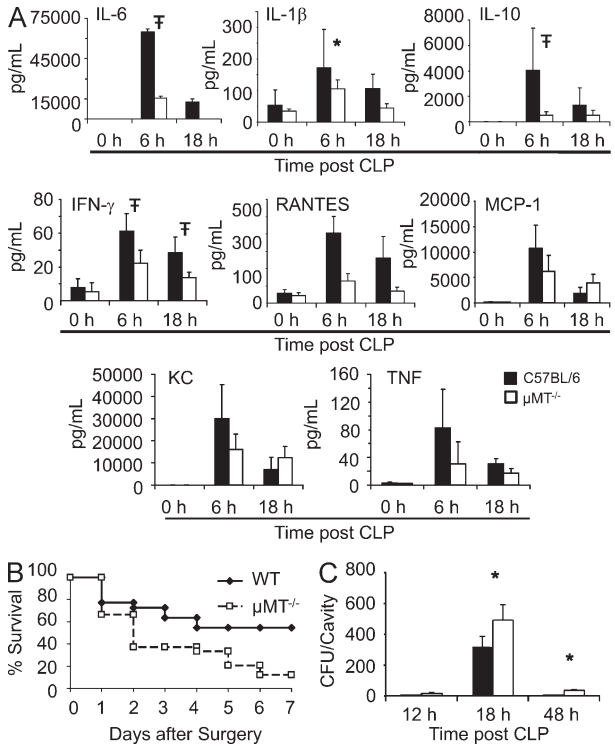

Figure 2. B cells are important for the cytokine response to CLP-induced sepsis and contribute to decreased bacteremia. (A) Serum was collected at 0, 6, and 18 h after CLP from $\mu MT^{-/-}$ mice (white bars) and WT animals (black bars), and cytokines were measured by Luminex multiplex analysis. The experiment was performed at least twice with $n \geq 4$ per group. Error bars represent standard deviation. (B) WT (closed diamonds; $n = 22$) and $\mu MT^{-/-}$ (open squares; $n = 24$) mice were subjected to CLP and monitored for survival ($P = 0.004$). Shown are the combined results of two independent experiments with $n \geq 10$ per group. (C) Bacterial counts were measured in the peritoneal washings of WT and $\mu MT^{-/-}$ mice 12, 18, or 48 h after CLP. The experiment was performed three independent times with an $n \geq 3$ per group. Shown in B and C are mean values + standard deviation. *, $P < 0.05$; ₸, $P < 0.001$ by students *t* test comparing WT with $\mu MT^{-/-}$ mice.

B cell depletion leads to decreased IIC production

To ensure that the lack of an inflammatory response in $Rag1^{-/-}$ and $\mu MT^{-/-}$ mice was a result of the absence of B cells and not the absence of antibodies, which can lead to inflammatory cytokine production by the cross-linking of FcγR on phagocytes, we chose to use a different model of B cell depletion. Anti-CD20 antibodies have been used in mice and humans to deplete CD20-expressing mature lymphocytes in leukemia/lymphoma as well as in autoimmune conditions. This results in the depletion of mature B cells that express CD20, but the majority of antibody production by plasma cells remains intact. Treatment of mice with 10 mg/kg anti-CD20 antibody resulted in ~70% depletion of $B220^+$ $CD19^+$ lymphocytes in the blood and spleen over isotype control antibody-treated mice 7 d after IP injection (unpublished data). Before CLP, 7 d after injection of the anti-CD20 or isotype control antibody, serum levels of total IgA and IgG_3 were increased in anti-CD20–treated mice, whereas no difference was detected in serum levels of IgM, IgG_1, or IgG_{2c} between the two groups. The only antibody that was significantly lower in anti-CD20–treated mice than isotype control-treated mice was IgG_{2b}. 18 h after CLP, levels of IgM, IgG_1, IgG_{2b}, and IgG_{2c} were similar between both groups, but levels of IgG_3 and IgA remained higher in anti-CD20–treated mice (Fig. 3 A). This suggests that if changes in cytokine levels exist, the difference can be attributed to the lack of or presence of B cells and not to differences in antibody levels between groups. Using a 10-cytokine Luminex array, we found that anti-CD20 treatment depletion of ~70% of mature B cells led to significant reductions in IL-6, IL-10, and MIP-1α concentrations 6 h after CLP (Fig. 3 B). When data from three separate experiments was combined, we found that IFN-γ, IP-10, MCP-1, and KC were also significantly lower in anti-CD20–treated mice, whereas TNF, IL-1β, and IL-12p70 levels were not different between groups (unpublished data). These data show that depletion of a large portion, but not all, of mature B cells in the face of near normal serum immunoglobulin levels is sufficient for a diminished early IIC response after sepsis. The fact that a few IICs did not differ between the groups suggests either that FcγR cross-linking of antibodies on phagocytes may also contribute to the production of certain cytokines or that the presence of the remaining fraction of undepleted mature B cells (30%) may be sufficient to allow the production of certain IICs.

Because lymphocyte IIC production was shown to contribute to viral clearance after influenza infection (Strutt et al., 2010), it may also contribute to bacterial clearance during sepsis. We next analyzed whether $\mu MT^{-/-}$ mice display defective bacterial clearance. At 18 and 48 h after sepsis, $\mu MT^{-/-}$ mice demonstrate an impaired ability to clear bacteria from the peritoneum (Fig. 2 C), indicating that the presence of B cells is required for optimal bacterial clearance during sepsis.

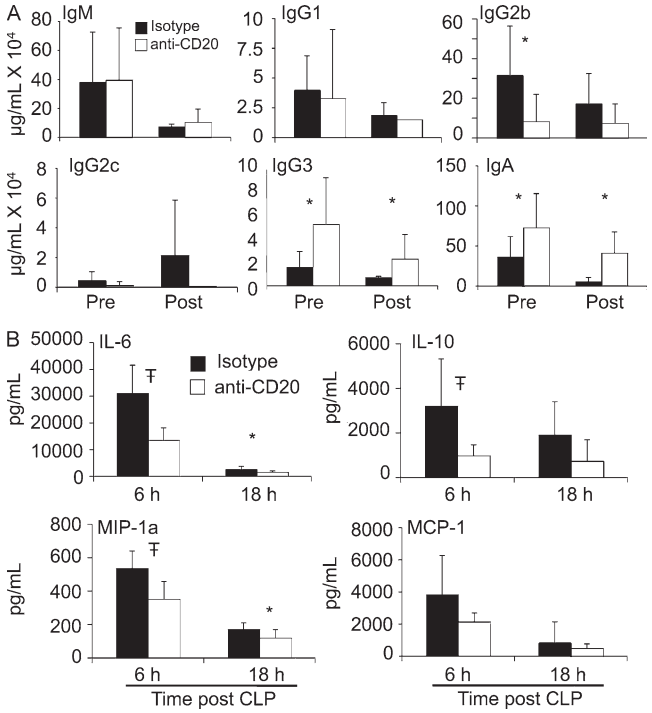

Figure 3. Depletion of B cells with anti-CD20 antibody decreases cytokine production after sepsis. C57BL/6 mice were treated intraperitoneally with 10 mg/kg anti-CD20 or isotype control antibody, and blood was obtained 7 d later, followed by CLP. 6 and 18 h after CLP, mice were bled again. (A) Before CLP (Pre) and 18 h after CLP (Post), serum was obtained and concentrations of IgM (top left), IgG1 (top middle), IgG2a (top right), IgG2c (bottom left), IgG3 (bottom middle), and IgA (bottom right) were quantified by ELISA. (B) 6 and 18 h after CLP, plasma was obtained and cytokine production was examined by Luminex. Experiments were performed three independent times with $n \geq 3$ per group. The data shown are from a single representative experiment with $n = 4$ per group. *, $P < 0.05$; ∓, $P < 0.001$ by Students *t* test CLP with isotype control versus CLP with anti-CD20 antibody treatment. Error bars represent standard deviation.

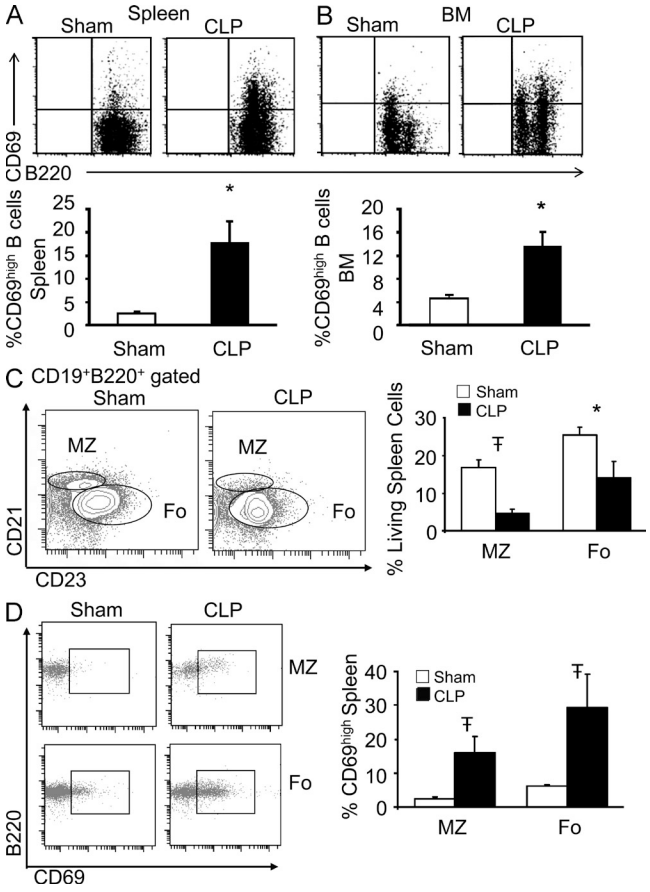

Figure 4. B cells are activated early after CLP. (A and B) Representative flow plots of the spleen (A, top left) and BM (B, top right) showing CD69 expression on $B220^+$ B cells after CLP. Graphs show the percentage of $B220^+CD69^+$ in the spleen (bottom left) and BM (bottom right). *, $P < 0.05$ by Students *t* test comparing Sham with CLP mice. $n = 4$ per group. (C) Representative flow diagrams showing the gating strategy for MZ ($B220^+IgM^+IgD^-CD21^+CD23^{lo}$) and follicular (Fo; $B220^+IgD^+IgM^{lo}CD23^{hi}$) B cells in the spleen 24 h after CLP or Sham treatment (left). The graph shows the total number of live MZ and follicular B cells in the spleen 24 h after Sham or CLP surgery (right). (D) Representative flow plots of activated ($CD69^+$) MZ and follicular B cells (left). The graph shows the percentage of activated B cells 24 h after Sham or CLP treatment (right). Experiments were performed three independent times with $n \geq 3$ per Sham group and an $n = 4$ per CLP group. *, $P < 0.05$; ∓, $P < 0.001$ by Students *t* test comparing Sham with CLP treatment. Error bars represent standard deviation.

Bacterial sepsis activates B cells

Because B cells are playing a pivotal role during sepsis, we next wished to examine how sepsis affects B cells. We find that as early as 24 h after CLP-induced sepsis, total $B220^+CD19^+$ cells display an increase in CD69 expression in the spleen, BM, and mesenteric lymph nodes (Fig. 4, A and B; and not depicted). This activation state decreases by day 3 after sepsis but again increases through 10 d (unpublished data).

Multiple B cell populations have different capacities to respond to antigens. Peritoneal B1 cells and marginal zone (MZ) B cells are responsible for most natural antibody production, which provides broad protection against a limited array of antigens such as LPS (Martin et al., 2001; McHeyzer-Williams, 2003; Yang et al., 2007). MZ B cells classically are the first B cells to encounter blood-borne pathogens, as they are located adjacent to splenic marginal sinuses where macrophages and dendritic cells trap antigens (Martin et al., 2001; McHeyzer-Williams, 2003). During *Staphylococcus aureus* infection or TLR stimulation, MZ B cells are also known to migrate to the T cell follicle (Lu and Cyster, 2002; Karlsson et al., 2003; Rubtsov et al., 2008) or they can develop into plasma cells to produce rapid but low-affinity IgM antibody (Martin et al., 2001; McHeyzer-Williams, 2003). Follicular B cells classically respond to T cell–dependent antigen and require a longer time to develop into plasma cells as they require T cell help. Not surprisingly, when examining peritoneal B1 cells, we find that the majority (>90%) of peritoneal B1a ($B220^+CD11b^+CD5^-$) and B1b ($B220^+CD11b^+CD5^-$) cells are depleted from the peritoneal cavity within 36 h after sepsis, concomitant with a dramatic increase in peritoneal neutrophil ($Gr\text{-}1^+CD11b^+$) influx (unpublished data). As peritoneal

B1 cells migrate to the spleen and lymph nodes after activation, we examined whether septic mice display an increase in splenic B1 cells or splenic B1 cell activation. We find a small (<50%) increase in $B220^+CD11b^+$ B1 cells in the spleen. However, these cells make up <10% of the total splenic B cell population and do not display an increased activation state, as determined by CD69 expression, and so were likely not contributing to the increased CD69 expression within splenic $CD19^+B220^+$ cells (unpublished data). When we analyzed the effects of sepsis on other B cell subtypes in the spleen, we find a reduction in both MZ ($B220^+IgM^+IgD^-CD21^+CD23^{lo}$) and follicular B cell populations ($B220^+IgD^+IgM^{lo}CD23^{hi}$), with a more pronounced depletion of MZ B cells, within 24 h (Fig. 4 C). The remaining MZ and follicular B cells demonstrate a seven- and fivefold increase in CD69 expression (Fig. 4 D), demonstrating that sepsis activates both cell populations, suggesting that B cell maturation progresses well during sepsis.

Because sepsis activates both MZ and follicular B cells, and either cell type can contribute to germinal center reactions or become antibody-secreting plasmablasts and plasma cells in the presence of TLR agonists or infections (de Vinuesa et al., 2000; McHeyzer-Williams, 2003; Song and Cerny, 2003; Rubtsov et al., 2008; Swanson et al., 2010), we next examined whether sepsis induces germinal center B cell and plasma cell development. Within 7 d, there is a dramatic increase in both $B220^+GL7^+IgD^{lo}$ germinal center B cells and $CD138^+IgD^{-/low}$ plasmablast/plasma cell formation (unpublished data). These data show that sepsis rapidly activates MZ and follicular B cells and induces both germinal cell reaction and plasma cell formation.

B cell activation during sepsis occurs in the absence of TLR pathways

We next wished to examine the pathways contributing to sepsis-induced B cell activation. Many pathways are activated during sepsis, including MyD88-dependent and -independent TLR signaling and IFN-I–dependent pathways which may result in B cell activation. In fact, B cell–intrinsic MyD88 signaling was shown to amplify early antibody responses (Meyer-Bahlburg et al., 2007). We first determined whether TLR pathways contribute to early B cell activation using $MyD88^{-/-}$ and $TRIF^{-/-}$ mice. We find that deficiency of either pathway had no effect on increased B cell CD69 expression (Fig. 5 A). These data suggest that TLR signaling is redundant in sepsis-induced B cell activation.

B cell activation during sepsis requires IFN-I

Because TLR signaling through MyD88 or TRIF was playing a partial or redundant role, we examined whether other immune pathways may be participating in sepsis-induced B cell activation. IFN-I is a pleiotropic cytokine that provides crucial signals to B cells. Recently, the TLR3 agonist double-stranded (ds) RNA was shown to amplify T cell–independent antibody production to NP-Ficoll by IFN-I–dependent activation of follicular B cells (Swanson et al., 2010). We next examined whether IFN-I participated in sepsis-induced B cell activation using mice deficient in $IFNAR^{-/-}$, the sole receptor responsible for IFN-I signaling. We find that CD69 expression is significantly decreased in mice deficient in $IFNAR^{-/-}$ compared with WT controls (Fig. 5 B), highlighting the role of IFN-I during sepsis-induced B cell activation.

Antibody-independent B cell functions are integral for sepsis outcome

The role of B cells in autoimmunity and infectious disease includes both antibody-dependent and antibody-independent functions (Chan et al., 1999; Leef et al., 2000; Lampropoulou et al., 2010). In $\mu MT^{-/-}$ mice, the production of the majority of immunoglobulins is severely impaired as a result of the deletion of the μ region of the immunoglobulin locus (Kitamura et al., 1991), but IgA and IgE secretion still occurs (Macpherson et al., 2000). As B cell production of antibodies against commensal organisms provides protective immunity in innate immunodeficient mice (Slack et al., 2009), and the CLP model induces sepsis using commensal microorganisms, we wished to determine whether antibody-dependent or -independent functions of B cells provide protective immunity during CLP-induced sepsis. Treatment of $\mu MT^{-/-}$ mice with sera from WT mice 2 h before sepsis from normal mice partially improves sepsis survival (Fig. 6 A).

To determine if an antibody-independent role of B cells contributes to survival during sepsis, $Rag1^{-/-}$ mice were given 10^7 $B220^+$ splenic B cells and were subjected to CLP 2 d later. Approximately 90% of these B cells are of the B2 or conventional B cell lineage. We find that treatment of

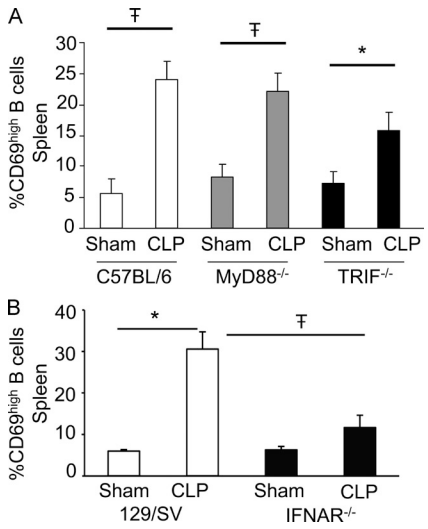

Figure 5. Early activation of B cells depends on IFN-I but not MyD88 or TRIF signaling, and T cell–independent antibody responses occur independently of MyD88. (A and B) Early activation of splenic B cells 24 h after CLP in WT $MyD88^{-/-}$ and $TRIF^{-/-}$ mice (A) or in 129/Sv or $IFNAR^{-/-}$ mice (B) as measured by the percentage of $CD69^+$ B cells. Experiments were performed two independent times with similar results with $n \geq 4$ mice per group. *, $P < 0.05$; ∓, $P < 0.001$ with Student's *t* test comparing Sham versus CLP animals in A or Tukey's post hoc analysis in B. Error bars represent standard deviation.

Rag1$^{-/-}$ mice with B220^{+} B cells also significantly improves sepsis survival (Fig. 6 B). Collectively, these data show that both T cell–independent B cell function and antibody production provide protection during sepsis.

Deficient chemokine response contributes to poor outcome in mice lacking B cells

Because it appears that other B cell functions, and not solely antibody production, contribute to survival after sepsis, we next examined whether the decreased cytokine and chemokine production directly contributed to the decreased survival in B cell–deficient mice. We previously found that IFN-I deficiency worsens sepsis outcome by impairing IFN-I–inducible chemokine production, resulting in impaired phagocyte-mediated host defense (Kelly-Scumpia et al., 2010). In this study, we find that B cells are activated by IFN-I, and μMT$^{-/-}$ mice produce decreased levels of IFN-I–inducible chemokines including CXCL10/IP-10, CCL3/MIP-1α, CCL5/RANTES, and CCL2/MCP-1 (Fig. 2 A and Fig. S3). This suggests that deficiency of B cells results in decreased IFN-I–dependent chemokine production, which may contribute to failure of innate immune-mediated bacterial clearance.

To confirm whether B cells are a relevant target of IFN-I and contribute to the overall IFN-I response, we performed a pilot study where Rag1$^{-/-}$ mice were given 10^7 B cells from 129/Sv or IFNAR$^{-/-}$ mice 48 h before sepsis and cytokines were examined. Interestingly, compared with WT mice, Rag1$^{-/-}$ mice given IFNAR$^{-/-}$ B cells possessed significantly lower levels of the IFN-I–inducible chemokines CXCL10/IP-10, CCL2/MCP1, and CCL3/MIP-1α 6 h after sepsis. This IFN-I–inducible response was partially restored when Rag1$^{-/-}$ mice were given B cells from 129/Sv mice, confirming that B cells contribute to the IFN-inducible cytokine response. Importantly, we find that the inflammatory cytokines TNF, KC, and IL-6, which are not regulated by IFN-I, also follow this similar pattern, indicating that IFN-I–activated B cells can also contribute to non–IFN-I inflammatory cytokine production (Fig. 7).

We previously showed that restoring the IFN-I–inducible chemokine CXCL10 was sufficient to restore bacterial clearance and survival after sepsis in IFNAR$^{-/-}$ mice. Given that IFN-I contributes to B cell activation in response to CLP, we examined whether CXCL10 can improve survival in μMT$^{-/-}$ mice. We find that, similar to results in IFNAR$^{-/-}$

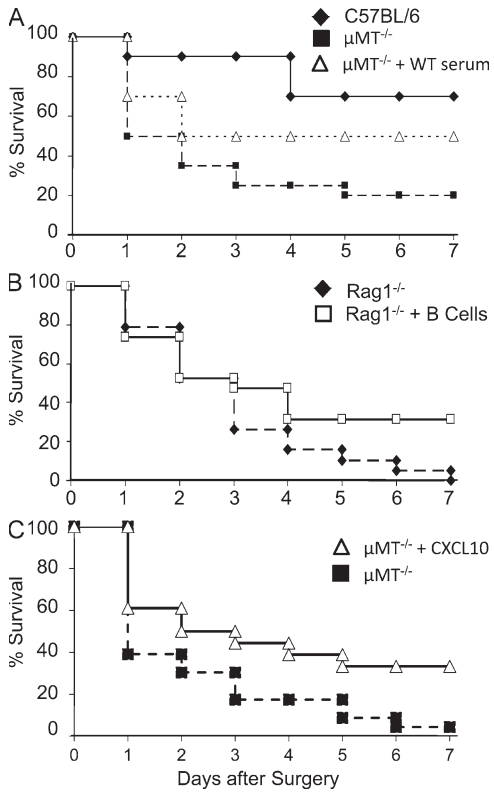

Figure 6. Antibody-dependent and -independent B cell function contributes to sepsis survival. (A) Blood was collected from untreated WT mice and spun down to collect sera. 2 h before CLP, 200 µl of untreated WT sera was injected intraperitoneally into μMT$^{-/-}$ mice. A survival study was performed comparing WT (closed diamonds, $n = 20$), μMT (closed squares, $n = 20$), and μMT + 200 µl WT sera (open triangles, $n = 20$; log rank test, P = 0.018). (B) B220^{+} B cells were isolated by magnetic bead separation from the spleens of WT mice and 10^7 cells were injected retroorbitally 48 h before surgery. A survival study was performed comparing Rag1$^{-/-}$ ($n = 19$) with Rag1$^{-/-}$ ($n = 19$) mice that received B cells isolated from WT animals (log rank test, P = 0.05). (C) 6 h after CLP, mice were injected with 100 ng mouse recombinant CXCL10 (IP-10) or sterile saline. Mice were monitored for survival. (Log rank test, P = 0.04; $n = 23$ for μMT$^{-/-}$ and $n = 18$ for μMT$^{-/-}$ + CXCL10). Data shown in A, B, and C are combined from two independent experiments with similar results.

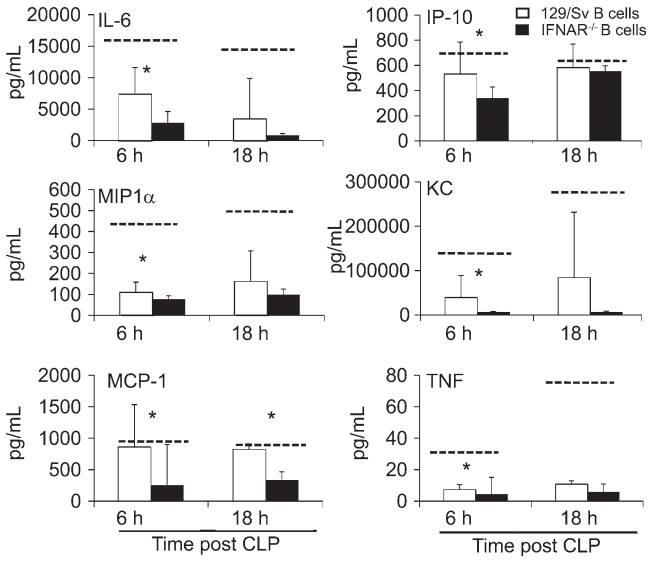

Figure 7. IFN-I signaling in B cells contributes to cytokine production in septic mice. 10^7 B cells from WT 129/Sv mice or IFNAR$^{-/-}$ mice were transferred into Rag1$^{-/-}$ mice. 2 d later, CLP was performed, and 6 and 18 h later, plasma was obtained and cytokine responses were evaluated by luminex analysis. Dashed lines represent cytokine concentrations in WT mice. *, P < 0.05 by one-way ANOVA with post hoc analysis by Tukey's test. The experiment was performed once with $n = 5$ per group. Error bars represent standard deviation.

mice, a single dose of CXCL10 2 h after CLP in $\mu MT^{-/-}$ mice improved survival to near WT levels (Fig. 6 C). This data identifies the B cell as a novel contributor to early IIC production resulting in improved innate immune responses and host defense after the onset of sepsis.

DISCUSSION

Cooperation of the innate and adaptive immune systems is necessary to provide protective immunity to pathogens. Classically, recruited phagocytes, including neutrophils and macrophages along with complement, control initial infection by producing free radicals and destructive enzymes, whereas dendritic cells and other APCs process antigens to present them to helper T cells, which coordinate appropriate adaptive immune responses. T lymphocytes mediate long-term cell-mediated immunity, whereas B cells mediate humoral antibody-mediated immunity. Both are responses that classically require several days to weeks to develop. Consequently, lymphocytes were thought of as bystanders during the acute phase of infections until instructed by the innate immune system to act during the later stages of infection. This model was challenged when studies demonstrated that T cell subsets regulate acute inflammatory responses to viral infection (Kim et al., 2007; Zhang et al., 2007). The exact mechanism of T cell control of IIC production by innate immune cells has not been fully elucidated but seems to involve two distinct mechanisms. Naive or effector T cells can attenuate pathological inflammation through viral infection or inflammasome activation, respectively, through direct cell-to-cell contact with APCs, likely through cell surface receptors (Kim et al., 2007; Guarda et al., 2009). Memory T cells, in contrast, can augment inflammatory responses to virus after direct recognition of antigen (Strutt et al., 2010). In this study, we demonstrate a previously unrecognized role of B cells as key participants in the early inflammatory cytokine response during bacterial sepsis. B cells are known to secrete cytokines (originally thought to stimulate autocrine) and T cell–independent immunoglobulin upon stimulation with TLR agonists such as LPS and CpG DNA in vivo and in vitro (Hobbs et al., 1991; Yi et al., 1996; Sun et al., 2005). These bacterial products can cross-link the B cell receptor and bind to TLRs simultaneously, thus generating signals needed for cytokine and antibody production simultaneously. During bacterial sepsis induced by CLP, multiple TLR agonists and other PAMPs are released locally and systemically that can directly activate multiple cell types including B cells. Without B cells, mice develop an inadequate inflammatory response and fail to clear bacteria, resulting in increased mortality. Our data suggests that both antibodies and a novel contribution of B cells to the early inflammatory cytokine response both contribute to the defects seen in $\mu MT^{-/-}$ and $Rag1^{-/-}$ mice.

Both TLR-dependent and TLR-independent signaling are integral to infection-induced antibody production (Pasare and Medzhitov, 2005; Gavin et al., 2006). In this study, we find that bacterial sepsis induces activation of follicular and MZ B cells. Interestingly, MyD88 or TRIF deficiency alone does not affect B cell activation. Not surprisingly, given the prominent role of IFN-I signaling in MZ and follicular B cell development and T cell–dependent and –independent activation (Lien et al., 2010; Swanson et al., 2010; Wang et al., 2010), we find that $IFNAR^{-/-}$ mice do display decreased B cell activation.

We previously found that $IFNAR^{-/-}$ mice succumb more readily to sepsis as the result of a failure to activate phagocytes, resulting in a failure to clear bacteria which can be overcome by treatment with the IFN-inducible chemokine CXCL10 (Kelly-Scumpia et al., 2010). Interestingly, mice deficient in B cells or both B and T cells demonstrate not only decreased inflammatory cytokine production during sepsis but also decreased production of IFN-I–inducible chemokines, including CCL5, CCL2, CCL3, and CXCL10, suggesting that B cells help produce the IFN-I which contributes to their own activation as well as the activation of neutrophils during sepsis. Similar to $IFNAR^{-/-}$ mice, when μMT mice are treated with an IFN-inducible chemokine, CXCL10, which they are deficient in, sepsis survival is improved. Collectively, our results show that IFN-I regulates activation of both innate and adaptive immune cells during sepsis and production of IFN-inducible chemokines and inflammatory cytokines by B cells contribute to clearance of bacteria during sepsis.

Both B and T lymphocytes produce long-lived specific immune responses to specific antigens and pathogens, which are responsible for immune memory. The contribution of lymphocytes to more rapid innate immune responses is now becoming recognized. Recently, T cells of the adaptive immune system were shown to dampen early inflammatory responses to either viral infections or viral (dsRNA) or inflammasome activators (Kim et al., 2007; Guarda et al., 2009). In this study, we used CLP, a model of polymicrobial sepsis which recapitulates many of the human responses to sepsis to determine whether the adaptive immune system contributes to innate immunity in response to severe bacterial infection. Using this model, it was previously found that mice without an adaptive immune system display diminished survival, which can be improved with repletion of transgenic lymphocytes overexpressing antiapoptotic proteins, but whether B cells are also protective was not examined (Hotchkiss et al., 1999). We find that B cells of the adaptive immune system can contribute to the early innate immune responses resulting in host defense to bacterial sepsis.

The role of T cells in sepsis has not been fully realized and, dependent on the model and the severity of the model of sepsis used, T cells have been shown to be protective, detrimental, or have no effect (Scumpia et al., 2006, 2007; Enoh et al., 2007, 2008; Busse et al., 2008; Stromberg et al., 2009). In more inflammatory models of sepsis, where shock and metabolic dyscrasias predominate, T cells demonstrate a detrimental role, whereas in models that develop only a mild to moderate shock phenotype and immunoparalysis predominates, modification of T cells to prevent apoptosis (Hotchkiss et al., 1999, 2000) or to co-stimulate T cells (Schwulst et al., 2006; Scumpia et al., 2007) has no doubt led to protection. Likely, the effects of T cells during sepsis lie on a spectrum

whereby the severity of the bacterial infection and the underlying strength of the host immune system contribute to how T cells participate in sepsis outcome. We chose to use a model of mild to moderate shock, whereby the mice develop a prominent immunoparalysis, because we feel this more closely approaches what is observed in humans clinically where patients require long ICU stays and are at risk for secondary infections. In this model, although TCR-deficient mice displayed a mild defect that could be investigated in future studies, we found that the predominant cell type of the adaptive immune system contributing to the early IIC response were B cells so we chose to further explore this novel finding.

Our studies show that B lymphocytes are an adaptive immune cell type needed to improve outcome and cytokine production and reduce bacteremia in response to sepsis. Using mice deficient in B and T cells ($Rag1^{-/-}$), mice deficient in $CD4^+$ and $CD8^+$ T cells (TCR-$\alpha/\beta^{-/-}$), and mice deficient in B cells ($\mu MT^{-/-}$), we show that there is a prominent effect of the loss of B cells on host innate immune responses through the attenuation of the early IIC response. $\mu MT^{-/-}$ mice failed to produce the usual increase in cytokines seen after sepsis and exhibited more peritoneal bacteremia. Survival of sepsis was partially restored by either the transfer of WT serum containing immunoglobulins or the transfer of B cells before their ability to produce significant levels of antibodies, suggesting that a B cell function separate from antibody production is participating in sepsis survival. Importantly, cytokine levels could be improved in $Rag1^{-/-}$ mice by the adoptive transfer of 10^7 WT $B220^+$ B cells that were able to respond to endogenous IFN-I and produce IFN-I–dependent chemokines but not by $IFNAR^{-/-}$ B cells. Similarly, administration of an IFN-I–dependent chemokine IP-10/CXCL10 to $\mu MT^{-/-}$ mice, a cytokine which these mice are deficient in, 2 h after sepsis initiation, was able to restore survival. These data suggest that the loss of these IFN-I–dependent chemokines produced by B cells may play a pathogenic role in patients taking potent immunosuppressive agents to prevent transplant rejection or for autoimmune disorders, rendering them susceptible to sepsis as a result of this inability to completely induce this protective host response.

MATERIALS AND METHODS

Mice. All experiments were approved by the Institutional Animal Care and Use Committees at the University of Florida College of Medicine. B6.129S2-Igh-6tm1Cgn/J ($\mu MT^{-/-}$) and B6.129P2-Tcrα^{tm1Mom}Tcrβ^{Tm1Mom} (TCR-$\alpha/\beta^{-/-}$ mice) backcrossed at least 10 generations to a C57BL/6J background, C57BL/6J.Rag1^{Tm1Mom} ($Rag1^{-/-}$), C57BL/6J-*Ticam1*^{Lps2}/J ($TRIF^{-/-}$), and C57BL/6 mice were purchased from The Jackson Laboratory. 129/Sv and $IFNAR1^{-/-}$ mice were purchased from B&K Universal and were a gift from W. Reeves (University of Florida College of Medicine, Gainesville, FL). $MyD88^{-/-}$ were a gift of A. Ayala (Brown University, Providence, RI).

Mouse infection models. For induction of polymicrobial sepsis, mice underwent CLP or a sham procedure, as previously described (Delano et al., 2007), to obtain a mortality of 10–20% in control mice by 7 d. In brief, a laparotomy was performed, the cecum was isolated, and ∼0.5 cm of cecum was ligated below the ileocecal valve and punctured through and through with a 27-gauge needle. Sham operation was performed by isolating the cecum without ligation or puncture. For CLP survival experiments, the model was modified to achieve ∼40–50% mortality by 7 d in WT mice by ligating ∼0.75 cm of cecum and puncturing the cecum with a 27-gauge needle.

In some experiments, mice were injected with 10 mg/kg anti-CD20 antibody (Biogen Idec) or IgG_1 isotype control antibody 7 d before surgery. Small amounts of blood were obtained from tail veins to ensure mice were bled from tail vein and flow cytometry was performed to ensure depletion of B cells occurred. Depletion of $B220^+CD19^+$ cells was consistently between 65 and 75%.

Cell purification and cell transfer experiments. For isolation of B cells, erythrocyte-depleted single cell suspensions from spleens of C57BL/6, 129/Sv, and $IFNAR^{-/-}$ mice underwent positive selection using the B220-positive selection kit (Miltenyi Biotec) as per manufacturer protocol. In brief, single cell suspensions were incubated with the B220 microbead cocktail for 30 min on ice at 10^7/10 µl of microbeads. Cells were washed twice and ran over LS columns using magnets (Miltenyi Biotec) to eliminate the negative fraction. The positive fraction was eluted off column. The purity of $B220^+$ cells was ∼90%. Of the contaminating cells, 2% or less each displayed the phenotype of $B220^-CD11b^+$, $B220^-CD4^+$, or $B220^-CD8^+$. Of the $B220^+$ cells, 95% of these cells were $B220^+CD19^+CD11b^-$ conventional B2 cells and 2 and 1.5% were $B220^+CD19^+CD11b^+$ and $B220^+CD19^+CD5^+CD11b^-$ B1 cells, respectively. Approximately 1% demonstrated the $B220^+CD19^-CD11c^{low}CD11b^-$ phenotype of plasmacytoid dendritic cells. After washing the purified cells, they were placed in sterile normal saline at a concentration of 10^8/ml, and 100 µl was injected retro-orbitally in lightly anesthetized mice.

Multiplex cytokine analysis. Assessments of cytokine profile were performed using a commercially available multiplexed kit (Milliplex 22-plex or 10-plex Mouse Cytokine/Chemokine MAP kit; Millipore). Simultaneous measurement of 10 or 22 cytokines/chemokines was performed. All assays were performed according to the manufacturer's protocols. Samples were diluted 1:1 in the assay buffer provided with the kit and at least two replicate wells were plated per sample. Cytokine concentrations were determined using BeadView software (Millipore).

Determination of bacterial counts. Peritoneal bacterial counts were determined by culturing 100 µl of serially diluted peritoneal washings on sheep's blood agar plates (Thermo Fisher Scientific) at 37°C in 5% CO_2. Plates were counted after 24 h of culture.

Flow cytometry. Immediately after being euthanized, blood, BM, and spleens were harvested and a single cell suspension was made. Cells were washed in PBS, pelleted, and subsequently stained for flow cytometry. Samples were acquired and analyzed on a LSRII flow cytometer (BD). At least 2×10^4 live (Sytox blue$^-$) cells were analyzed.

Statistics. Continuous variables were first tested for normality and equality of variances. Differences among groups were evaluated by Student's *t* test or one-way ANOVA with Tukey's post hoc analysis where stated. Differences in survival were determined by the log-rank test performed on Kaplan-Meier curves. Significance was determined at the 95% confidence level.

Online supplemental material. Fig. S1 shows that the lack of T cells had no effect on survival or the inflammatory response to CLP. Fig. S2 shows that repletion of B cells in $Rag1^{-/-}$ mice can increase cytokine responses. Fig. S3 shows that $\mu MT^{-/-}$ mice produce fewer IFN-1–inducible genes after CLP than WT mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101715/DC1.

The studies were supported by grants GM-40586 and GM-81923, awarded by the National Institute of General Medical Sciences. K.M. Kelly-Scumpia, M.J. Delano, and A.G. Cuenca were supported by a T32 training grant in burns and trauma (T32 GM-008721-13) awarded by the National Institute of General Medical Sciences.

A.G. Cuenca was also supported by an individual National Research Service Award (F32 GM-093665) awarded by the National Institute of General Medical Sciences.
The authors have no conflicting financial interests.

Submitted: 17 August 2010
Accepted: 13 June 2011

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