Published in final edited form as:
J Immunol. 2014 March 1; 192(5): 2305–2314. doi:10.4049/jimmunol.1302160.

Upregulation of GRAIL Is Associated with Impaired CD4 T-Cell Proliferation in Sepsis

Monowar Aziz, **Weng-Lang Yang**, **Shingo Matsuo**, **Archna Sharma**, **Mian Zhou**, and **Ping Wang**
Center for Translational Research The Feinstein Institute for Medical Research Manhasset, NY & Department of Surgery Hofstra North Shore-LIJ School of Medicine Manhasset, NY

Abstract

The loss of numbers and functionality of CD4 T-cells is observed in sepsis; however, the mechanism remains elusive. Gene related to anergy in lymphocytes (GRAIL) is critical for the impairment of T cell proliferation. We therefore aim to examine the role of GRAIL in CD4 T-cell proliferation during sepsis. Sepsis was induced in 10-week-old male C57BL/6 mice by cecal ligation and puncture (CLP). Splenocytes were isolated and subjected to flow cytometry to determine CD4 T-cell contents. CD4 T-cell proliferation was assessed by CFSE staining, and the expression of GRAIL in splenocytes was measured by immunohistochemistry, real-time PCR, and flow cytometry. The expressions of interleukin-2 (IL-2) and early growth response-2 (Egr-2) were determined by real-time PCR. As compared to shams, the numbers of CD4 T-cells were significantly reduced in spleens. Septic CD4 T-cells were less efficient in proliferation than shams. The IL-2 expression was significantly reduced, while the GRAIL expression was significantly increased in septic mice splenocytes as compared to shams. The siRNA-mediated knock down of GRAIL expression re-established the CD4 T-cell proliferation ability *ex vivo*. Similarly, the treatment with recombinant murine IL-2 to the septic CD4 T-cells restored their proliferation ability by downregulating GRAIL expression. Our finding reveals a novel association of the increased GRAIL expression with impaired CD4 T-cell proliferation, implicating an emerging therapeutic tool in sepsis.

Keywords

CD4 T-cells; CFSE; GRAIL; IL-2; Sepsis

Introduction

Sepsis occurs during severe infection which afflicts >750,000 people annually, leading to a huge economic burden in the United States (1). Although the patients with severe sepsis may die in the early phase due to a “cytokine storm” mediated by hyperinflammation, the majority of patients survives the initial insults and develops an immunosuppressive state that is manifested by the inability of the patients to eradicate the primary infection and/or the development of secondary infections (1, 2). An important mechanism of

Address for correspondence, proofs, and reprint requests to: Ping Wang, MD Director, Center for Translational Research The Feinstein Institute for Medical Research Professor and Vice Chairman for Research Department of Surgery Hofstra North Shore-LIJ School of Medicine 350 Community Dr., Manhasset, NY 11030 Tel: (516) 562-3411 Fax: (516) 562-2396 pwang@nshs.edu.

Author Contributions: MA, WLY conceived and designed the experiments. MA, SM, AS performed the experiments. MA, WLY, MZ analyzed the data. MA, WLY wrote the paper. PW reviewed the manuscript and supervised the whole project. All authors read and approved the final manuscript.

Conflict of Interest: The authors report no financial conflicts of interest.

immunosuppression is the extensive apoptosis-induced depletion of immune effector cells and alterations in myeloid cell functions (3-5). Recent studies indicated that the cells of the innate and adaptive immune systems suffer from multiple deficiencies in terms of cytokine production, proliferation and immune response following sepsis (3-6). Understanding the cellular and molecular mechanisms underlying the induction of immunosuppression following sepsis is critical for the development of therapies for patients following escaping from an initial hyperinflammatory episode.

CD4 T-cells are a sub-group of lymphocytes which play an important role in coordinating other cells of the immune system by releasing T-cell cytokines essential for their proliferation, differentiation and bactericidal functions against pathogens (7). An efficient activation of CD4 T-cells occurs from the simultaneous engagement of the T-cell receptor (TCR) and its co-stimulatory receptor, CD28 which give rise to release interleukin-2 (IL-2) that initiates proliferation, effector functions, and clonal expansion via IL-2 receptor (IL-2R)-mediated signaling (8). In the absence of robust activation initiated by TCR and CD28 signaling, CD4 T-cells fail to proliferate or to produce IL-2 and form a state of unresponsiveness following immunogenic stimulation, referred to as “anergy” (8-10). The development of anergy phenotype in CD4 T-cells depends on alteration of several gene expressions (8-11), which could reveal an unidentified link between CD4 T-cell malfunction in sepsis.

It has recently become evident that the post translational modification of proteins via ubiquitination plays an essential role in the regulatory mechanism of CD4 T-cell anergy (12). Gene related to anergy in lymphocytes (GRAIL), also known as ring finger protein-128 (RNF-128), has recently been identified as a novel E3 ubiquitin-protein ligase that induces and maintains anergy in CD4 T-cells (13-15). Recent studies revealed that the GRAIL expression can be correlated with the inhibition of CD4 T-cell proliferation and antigen-induced IL-2 transcription by disrupting the T-cell stimulatory signaling, indicating the anergy phenotype (16). In agreement, a report demonstrated that the T-cells from GRAIL knock-out mice are protective from anergy induction as they are hyperproliferative and produce higher amounts of cytokines than the wild-type cells in response to TCR stimulation (17). In quest of delineating the mechanisms, Nurieva *et al* revealed that the GRAIL-mediated T-cell unresponsiveness occurs due to the TCR-CD3 degradation (17). Ample evidences are now showing how the ubiquitin properties of GRAIL interacts with T-cells and antigen presenting cells (APC) receptors and cytoskeletal proteins and promote their degradation (18-22).

Despite successful elucidation of the function of GRAIL in CD4 T-cells for the development of oral tolerance (15), its role in acute inflammatory diseases remains to be elucidated. We, therefore, aim to find the novel link of GRAIL and CD4 T-cell unresponsiveness in context of its proliferation abnormalities during sepsis. Our results provide evidence showing that CD4 T-cells from septic mice exhibit defects in proliferation and immune responsiveness due to the upregulation of GRAIL expression.

Methods

Cecal ligation and puncture (CLP)

Male 10-week-old C57BL/6 mice (∼25 g) purchased from Taconic, Albany, NY were housed and fed a standard laboratory diet. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of The Feinstein Institute for Medical Research. Sepsis was induced in mice by following the CLP procedure as described previously (23). The mice were anesthetized by isoflurane inhalation, and the abdomen was shaved and washed with 10% povidone iodine. A 1-2-cm midline incision was performed to allow

exposure of the cecum and tightly ligated about 1.0 cm from the tip with a 3-0 silk suture. A through and through double puncture of the cecum was performed using a 22-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites and returned to the peritoneal cavity. The laparotomy site was then closed with 6-0 silk suture. Sham operated animals underwent the same procedure with the exception that the cecum was neither ligated nor punctured. The CLP animals were resuscitated with 1 ml of isotonic sodium chloride solution, containing PRIMAXIN (Merck & Co., Inc, Whitehouse Station, NJ) as antibiotic at a dose of 0.5 mg/kg BW via *s.c.* injection immediately after the surgery which reveals ∼80% of the survival at 72 h after CLP induction as described in our previously published report (23).

Isolation of splenocytes

The animals were anesthetized at different times after CLP or sham operation for the collection of spleens. Splenic cell suspensions were prepared by disruption using frosted glass slides in RPMI medium with 10% FBS, and the isolated cell suspensions passed through a 70 μm nylon (BD Falcon, Durham, NC). Red blood cell (RBC) lysis was conducted with splenic cell suspensions using RBC lysis solution (10 mM $KHCO_3$, 150 mM NH_4Cl, 0.1 mM EDTA, pH 8.0). After centrifugation at ×200 g for 10 min, the cell pellets were resuspended in RPMI medium with 10% FBS. Cells were then allowed to adhere on a 10-cm plate for 2 h to remove the adherent myeloid cells at 37°C in a 5% CO_2. The non-adherent cells, mainly the lymphocytes were removed by washing with pre-warmed RPMI medium, and used in subsequent studies.

Isolation of CD4 T-cells

CD4 T-cells from 10-week-old male C57BL/6 mice spleens were isolated by negative selection using mouse EasySep CD4 T-cell isolation kit (Stem Cell Technologies, Vancouver, Canada) which revealed at least 95% of the viable and pure CD4 T-cells (Data not shown).

Knock down of GRAIL expression by siRNA

CD4 T-cells isolated from the spleens of shams and 48 h CLP-induced sepsis mice were transfected with a mixture of four gene solution siRNAs (Rnf128, Gene ID: 66889) to knock down GRAIL expression by using the HiPerFect transfection reagent suitable for primary cell transfection by following the manufacturer's protocol (Qiagen, Valencia, CA). The target sequences for the mouse GRAIL siRNAs are: 1) CTCGAAGATTACGAAATGCAA, 2) CAGGATAGAAACTACCATCAA, 3) CTCTAATTACATGAAATTTAA, and 4) CAGGGCCTAGTTTACTATGAA. For the siRNAs transfection, a total of 5×10^5 CD4 T-cells/well were transfected with 75 ng each of these GRAIL siRNAs altogether using 3 μL of HiPerFect dissolved in serum free OPTIMEM medium (Invitrogen, Carlsbad, CA). After 3 h of transfection, an additional 400 μL of RPMI medium with 10% FBS was added into the cells, and then allowed to incubate at 37°C for 24 h, followed by the evaluation of transfection efficacy of gene knock down by flow cytometry and proliferation assay. A non-target negative control (NC)-siRNA (AATTCTCCGAACGTGTCACGT; Qiagen) was transfected into the CD4 T-cells which served as experimental control. After 24 h of GRAIL or NC-siRNA transfection, the percentages of the knock down of GRAIL expression in terms of its protein level over that of NC-siRNA-transfected control was evaluated by flow cytometry and expressed as histogram and bar diagram for mean fluorescence intensity (MFI).

Flow cytometric analysis

To examine the contents of CD4 T-cells, a total of 1×10^6 splenocytes were stained with PerCP/Cy5.5-CD4 antibody (Ab) (Biolegend, San Diego, CA) and analyzed in BD FACSVerse (BD Bioscience, San Jose, CA). For the assessment of intracellular GRAIL expression, 1×10^6 cells were first stained with PerCP/Cy5.5-CD4 Ab and then fixed and permeabilized with IntraPrep (Beckman Coulter, Fullerton, CA), followed by reacting with rabbit-anti-GRAIL primary Ab (Abcam, Cambridge, MA), for 45 min. After washing, the cells were stained with FITC-anti-rabbit-IgG (Southern Biotech, Birmingham, AL) and then subjected to acquisition by BD FACSVerse. Data were analyzed by FlowJo software (Tree Star, Ashland, OR) with 20,000 events per sample. Isotype controls and Fc blocker (anti-mouse CD16/32; clone: 93; eBioscience, San Diego, CA) were used for the samples.

Immunohistochemistry of GRAIL

Spleens were fixed in 10% formalin and embedded in paraffin. Tissue blocks were sectioned at a thickness of 5 μm and then deparaffinized, dehydrated, and incubated with rabbit anti-GRAIL Abs (Abcam, Cambridge, MA), followed by incubation with biotinylated anti-rabbit IgG (Southern Biotech). Staining was developed by Vectastain ABC reagent and a diaminobenzidine kit (Vector Laboratories, Burlingame, CA) and visualized under phase contrast light microscope (Nikon Eclipse Ti-S, Melville, NY).

Quantitative real-time PCR

RNA was extracted from splenocytes from shams and CLP-treated animals by the Trizol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA by using murine leukemia virus reverse-transcriptase (Applied Biosystems, Foster City, CA). A PCR reaction was carried out in 25 μl of final volume containing 0.08 μmol of each forward and reverse primer, cDNA, and 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems). Amplification was conducted in an Applied Biosystems 7300 real-time PCR machine under the thermal profile of 50°C for 2 min, 95°C for 10 min and followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Relative expression of mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method and the results were expressed as fold change in comparison to controls. The sequence of the primers used for this study were: IL-2 (NM_013693): forward: 5′-CCCACTTCAAGCTCCACTTC-3′, reverse: 5′-TTCAATTCTGTGGCCTGCTT-3′; GRAIL (NM_008594): forward: 5′- GGGAATTGAGGTGGATGTTG-3′, reverse: 5′-GTGGCTCATCTGCTCCTTGT-3′; Egr-2 (NM_008594): forward: 5′-CTCTACCCGGTGGAAGACCT-3′, reverse: 5′-ACCAGGGTACTGTGGGTCAA-3′;β-Actin (NM_007393): forward: 5′-CGTGAAAAG ATG ACCCAGATCA-3′, reverse: 5′-TGGTACGACCAGAGGCATACAG-3′.

Cell proliferation assay

CD4 T-cell proliferation was measured using a cell division tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR). A total of 1×10^6 splenocytes isolated from shams and CLP-treated mice were stained with the CFSE dye at 5 μM concentrations. The cells were incubated at 37° C for 10 min and then stopped the reaction by adding 4-5 volume of cold RPMI medium containing 10% FBS. After washing and resuspending the cells into the warm RPMI medium with 10% FBS, they were then plated in an anti-CD3/CD28 Abs (1 μg/mL of each Abs)-coated plates. After 72 h of incubation, the cells were stained with PerCP/Cy5.5-CD4 Ab and acquired for FACS analysis. Unstimulated CFSE-labeled cells served as a non-dividing control.

Cell cycle analysis

Cell cycle analysis was performed according the protocol as described previously (24). In brief, the cells (1×10^6) isolated from the spleens of shams and 24 h after CLP mice were harvested and fixed in 75% cold ethanol. The fixed cells were first incubated with PerCP/Cy5.5-CD4 Ab and then with PBS containing RNase (100 mg/ml) and propidium iodide (10 mg/ml), followed by the detection using a flow cytometer. Ten thousands events were collected per sample. Data acquisition and cell cycle analysis were performed using FlowJo software.

In vitro stimulation of recombinant mouse IL-2 (rmIL-2)

A total of 1×10^6 splenocytes were isolated from shams and CLP animals and then cultured in RPMI with 10% FBS in a 24-well plate coated with anti-CD3/CD28 Abs (1 μg/mL in each). The cells were then stimulated with rmIL-2 (R&D Systems, Minneapolis, MN) at a concentration of 10 ng/mL for 3 days for cell proliferation assay or 1 day for the assessment of GRAIL mRNA expression.

Statistical analysis

Data are expressed as means ± SE and compared by one-way ANOVA and Student-Newman-Keuls test. Student *t* test was used when two groups were compared. Differences in values were considered significant if $p < 0.05$.

Results

CLP decreases CD4 T-cell percentages and numbers in the spleen

After the induction of sepsis, cells were isolated from mice spleen and stained with anti-CD4 Ab. As shown in Figure 1, a gradual decrease in CD4 T-cells percentages and numbers were noticed in a time dependent manner, with the highest reduction occurred at 72 h after the initiation of CLP as compared to the shams by approximately 36% and 62% in terms of percentages (Figure 1A, B) and numbers (Figure 1C), respectively. Additionally, we have also noticed a decreasing trend in CD4 T-cells percentages and contents at the earlier time points of 5 h after sepsis induction, while the changes were insignificant as compared to shams (Figure 1).

CD4 T-cells from CLP mice exhibit impaired proliferation

To analyze the proliferative efficacy of CD4 T-cells, the cells were isolated from shams and CLP mice at different time points. The adherent mononeuclear cells were removed by allowing them to adhere in the plate for 2 h, while the non-adherent cells, which appeared to be the lymphocytes (data not shown), were stained with CFSE and stimulated *in vitro* with anti-CD3/CD28 ligands for at least 72 h. The cells were then stained with anti-CD4 Ab and analyzed by flow cytometry. CD4 T-cells isolated from septic spleens showed considerable decrease in their proliferation; however, a marked decrease in their proliferation was noted in cells isolated after 24, 48, and 72 h of CLP induction (Figure 2A). The quantitative analysis of proliferation index showed significant decrease in their efficacy in spleen-derived CD4 T-cells isolated from septic mice as compared the sham animals (Figure 2B).

Impaired cell cycle activity of CD4 T-cells from CLP mice

In order to assess the CD4 T-cell cycle profile in sham *vs.* septic animals, we isolated the cells from the spleens and then stained with propidium iodide and anti-CD4 Ab and then analyzed by flow cytometry. We noticed that the CD4 T-cells from septic mice showed 10-flod decrease in their G2 values as compared to the shams, suggesting that the cells from septic animals were incapable of cell division (Figure 3A). Conversely, the CD4 T-cells

from CLP-induced animals also showed an upregulation of sub G1 (<G1) values which indicated that a considerable number of cells were either in the resting stage or underwent apoptosis (Figure 3A). The quantitative analysis of the ratios of G2/G1 showed a significant reduction in their quantities in the septic CD4 T-cells as compared to the shams (shams: 0.04 ± 0.003 *vs.* CLP: 0.008 ± 0.002; $p<0.05$) (Figure 3B).

GRAIL is upregulated in CD4 T-cells following TCR stimulation

The immunogenic functions of CD4 T-cells are monitored by their ability to respond against TCR stimulation. To determine the status of GRAIL expression in CD4 T-cells under TCR stimulation, we isolated the splenocytes from shams and CLP animals and cultured in anti-CD3/CD28 Abs-coated plates for 24 h. The results showed that the expression of GRAIL in septic T-cells stimulated with anti-CD3/CD28 ligands were significantly induced as compared to the shams at its mRNA and protein levels (Figure 4A-C). Furthermore, to confirm its transcriptional regulation we aimed to assess its potent transcription factor, Egr-2 (18) in shams and CLP splenocytes treated with anti-CD3/CD28 Abs for 24 h, which revealed a significant induction of Egr-2 in septic T-cells as compared to the shams (Figure 4D). We therefore suggest that the decreased proliferation of CD4 T-cells in sepsis after treatment with its antigenic ligands occurs due to an upregulation of GRAIL through the increased expression of Egr-2.

GRAIL is upregulated in CD4 T-cells following sepsis

Considering GRAIL as an inducer of impaired CD4 T-cell proliferation during *in vitro* and *in vivo* tolerance (13, 15), we aimed to assess its expression in spleen tissues by immunohistochemistry and noticed an abundant expression of GRAIL in CLP-induced animals as compared to the shams (Figure 5A). From this immunostaining finding we have identified the localization of GRAIL predominantly seen at the germinal center where mainly the T- and B-cells are found (Figure 5A). We then isolated the cells from the spleen and after removing the adherent mononuclear cells the expression of GRAIL in non-adherent lymphocytes were determined, which showed significant upregulation of its mRNA levels in CLP-induced samples as compared to the sham animals (Figure 5B). To confirm the GRAIL expression in the CD4 T-cells, the splenocytes were reacted with the anti-CD4 Ab, as well as GRAIL Ab at the intracellular compartment and then analyzed its expression in CD4 T-cells by flow cytometry. The representative histogram (Figure 5C) revealed considerable upregulation of GRAIL protein in splenic CD4 T-cells isolated from different time points after CLP-induction than the shams. Correspondingly, the mean fluorescence quantification also revealed significant upregulation of its expression following CLP operation (Figure 5D).

Knock down of GRAIL expression restores CD4 T-cell proliferation ability *ex vivo*

In order to establish the link between upregulation of GRAIL and impaired CD4 cell proliferation in sepsis, we intended to knock down endogenous GRAIL expression in CD4 T-cells isolated from shams and 48 h CLP mice by GRAIL as well as NC-siRNAs transfection *ex vivo*, considering the fact that the optimum upregulation of GRAIL mRNA, as well as protein occurred at 48 h of sepsis (Figure 5). In order to optimize the protocol and evaluate the efficacy of GRAIL siRNAs, in an initial study we have transfected GRAIL and NC-siRNAs into the purified CD4 T-cells isolated from the sham mice spleen. As shown in Figure 6A and B, the transfection of GRAIL siRNAs in CD4 T-cells of the sham mice spleen was able to knock down the GRAIL expression of about 47% as compared to the non-target specific NC-siRNA-treated CD4 T-cells. As a result of the GRAIL knock down, the CD4 T-cells isolated from the sham as well was 48 h CLP mice showed remarkable restoration in their proliferation abilities under anti-CD3/anti-CD28-stimulated conditions

over that of NC-siRNA-treated CD4 T-cells in terms of reducing the G_0 population in sham (NC-si: 5.3% *vs.* GRAIL-siRNAs: 1.8%) and 48 h CLP (NC-si: 69.8% *vs.* GRAIL-siRNAs: 51.8%), while increasing the $G_{5\text{-}7}$ population in sham (NC-si: 21.8% *vs.* GRAIL-siRNAs: 69.7%) as well as in 48 h CLP (NC-si: 3.6% *vs.* GRAIL-siRNAs: 6.9%), indicating the reestablishment of cell division (Figure 6C, D). Hence, the collective evidences clearly demonstrate that the upregulation of GRAIL in sepsis inhibits CD4 T-cell proliferation which can be restored back to its functionally active state if the expression of GRAIL is abrogated by gene knock down strategy.

IL-2 restores septic CD4 T-cell proliferation via downregulating GRAIL

Considering IL-2 as a potent growth promoting cytokine for CD4 T-cells (25), we carried-out an *in vitro* study by comparing the CD4 T-cell's proliferation efficacy in shams against sepsis after treated with rmIL-2 in presence of TCR ligands. In order to perform this study, the cells were stimulated with anti-CD3/CD28 ligands in the presence or absence of rmIL-2 for three days and then the cell proliferation activity was assessed. According to Figure 7A, a noticeable increase in the proliferation of CD4 T-cells was found in shams as well as CLP animals if treated with rmIL-2 together with the TCR stimulatory ligands. As shown in Figure 7A and B, the G_0 population became reduced in both shams and CLP CD4 T-cells upon treatment with rmIL-2 (Sham: 15.6% *vs.* 6.8%; CLP: 48.6% *vs.* 36.3%), while the $G_{5\text{-}7}$ population was increased from 23.8% to 41.9% in shams and from 2.5% to 16.2% in CLP under rmIL-2 treated conditions, indicating the rapid cell division. In agreement with this finding we have also noticed significant downregulation of GRAIL mRNA under the stimulation with rmIL-2 in both shams as well as CLP splenocytes in presence of anti-CD3/CD28 Abs (Figure 7C). Based on these findings, we suggest IL-2 could be an effective inducer for promoting CD4 T-cell proliferation following sepsis via negatively regulating GRAIL expression.

Decreased expression of IL-2 in the splenocytes isolated from septic mice

To assess the status of IL-2 expression in sepsis, the cells were isolated from spleens and then performed PCR analysis, which showed significant reduction in its expression at various time points after the induction of sepsis in mice (Figure 8A).

Based on our studies, we conclude that the sepsis-mediated GRAIL upregulation induced CD4 T-cell malfunctions in terms of their proliferation and immunogenic response, which could be reversed by exogenous addition of rmIL-2 that negatively regulated GRAIL expression and restored CD4 T-cell proliferation in sepsis (Figure 8B).

Discussion

Sepsis is characterized by profound immune alteration as mediated by the cells of the innate and adaptive immune system (1, 4). In sepsis, strong immune activation is followed by a period of protracted immune suppression. During the immune suppressed phase, patients may have difficulty in eradicating the invading pathogens and are susceptible to life-threatening nosocomial infection. A growing body of evidences indicates that the immune suppression in sepsis arises due to the loss of effector T-cell numbers, as well as alteration in their functions (3, 6, 26-28). Two opposite cellular events, *i.e.*, increased apoptosis and decreased proliferation are known to contribute immunesupression; however, among them the impact of T-cell apoptosis has been largely documented in clinical and experimental sepsis (3, 26, 28-30). Hence, there is still an unmet need to elucidate the mechanism that attribute CD4 T-cell malfunction in terms of its defects in proliferation during sepsis.

Sepsis, initiated by the cecal ligation and puncture (CLP), has two distinct phases: 1) the initial hyperdynamic phase (<12 h); and 2) the late hypodynamic phase (>12 h) where among several characteristics a decreased content of T-helper cells is notable (31, 32). Although there are several commonly used double hit models induced by CLP followed by *P. aeruginosa* or *S. pneumoniae* infection to study the immunosuppressive phenomenon in terms of the unresponsiveness or the loss of CD4 T-cells are utilized (33-35), in our current single hit model we have also noticed remarkable loss of CD4 T-cell counts at the late phase of sepsis as compared to the early time points, *e.g.*, >5 h (Figure 1), which we think to exert an immunosuppressive environment to render the host susceptible to secondary infections at the late phase of polymicrobial sepsis created by CLP alone. In support of our current model, a recent report has also demarcated the stages of sepsis following CLP only, indicating the initial hyperimmune responsive phase from 0-12 h, followed by the immunosuppressive phase which starts at12 h and continues till 72 h, and then finally the resolution phase (36). Based on these evidences it is justifiable to adopt the current model which is not only suitable for studying the initial hyperdynamic phase, but also the immunosuppressive phenomenon that occurs at the late phase of sepsis. Nonetheless, studies to delineate the role of GRAIL for CD4 T-cell unresponsiveness in a double hit model by adopting CLP, followed by *P. aeruginosa* or *S. aureus* inoculation can be of further interest.

Currently, there are several reports showing the decreased numbers of CD4 T-cells in sepsis, while those findings were documented based on a single time point after disease initiation (3, 6, 37). Beside this, in murine double injury model of sepsis followed by pneumonia or in thermal injury-plus-sepsis which mimic clinically relevant secondary infection also contributed to a substantial depletion of lymph node CD4 T-cells via apoptosis (33, 34). In our current study, the CD4 T-cell quantification was carried-out at multiple time points after sepsis, which clearly revealed profound loss in their numbers during sepsis in a time dependent manner, hence providing valuable insights about the onset of immunosuppression. To reveal CD4 T-cell function in sepsis, we have noticed reduced expression of IL-2, a critical T-cell growth promoting factor, reflecting impairment in their proliferation during septic challenge. In agreement with this fact, Ayala *et al* also observed reduced expression of IL-2 upon *ex vivo* stimulation of septic CD4 T-cells with their corresponding ligands (38-40). The importance of IL-2 in sepsis was demonstrated in Ayala's report, where the treatment of recombinant IL-2 in septic animals exhibited significant survival benefits (38). It has been reported that decreased CD4 T-cell proliferation in human septic patients was evaluated by their ability to produce IL-2 and intracellular antigen Ki67 expression (29). However, in our study apart from the assessment of IL-2 levels as an indirect approach for T-cell propagation, we utilized a direct method to confirm abnormalities in CD4 T-cell proliferation in septic mice by using a non-radioactive CFSE dye followed by the quantitative determination of proliferation index which showed concordance with previous reports (29, 6).

Although CD4 T-cell anergy is one of the major impairments in immune suppression in sepsis, the intracellular mechanism remains enigmatic. A novel intracellular E3 ubiquitin ligase, GRAIL has recently been shown to induce CD4 T-cell anergy in experimental oral tolerance (15, 17). Studies showed that the mice deficient in GRAIL were not only able to break the tolerance but also improved proliferation efficacy in CD4 T-cells (15, 41). Moreover, the GRAIL deficient mice were capable of hypersecretion of IFNγ in Th1 cells, lowered IL-4 in Th2 cells and elevated IL-17 and IL-22 in Th17 cells, each of this transition of these cytokines may play major roles in sepsis (15, 17, 18). Although a number of studies concerning GRAIL-mediated T-cell anergy were conducted in *in vito* and *in vivo* tolerance models, there is still a need for adequate understanding for its role in acute inflammatory diseases. Although it has been shown the upregulation of GRAIL in lamina propira CD4 T-cells of chronic cases of ulcerative colitis patients (42), no such studies have been reported

in acute inflammatory disease conditions. In our acute disease model we for the first time studied GRAIL expression in splenic T-cells, which showed noticeable increase in their mRNA and protein levels after sepsis development. Since, we observed an abundant staining of GRAIL at the germinal centers as well as surrounded compartments where various populations of cells are located, we therefore propose to carry-out future studies to define its expression and functions in various cell types. Recently, a decent number of studies were performed on GRAIL's transcriptional, translational and post translational regulation utilizing several *in vitro* or *in vivo* tolerizing models (11, 13, 16, 18, 41). Considering calcium signaling as required for anergy induction in CD4 T-cells, studies with ionomycin showed it as an inducer of GRAIL expression via the activation of nuclear factor of activated T-cells (NFAT) pathway (18, 43, 44). Since the transcription factor, Egr-2, a known target genes of NFAT, is involved in the anergy induction (18, 45), we therefore implemented this idea to assess their expression in sepsis and found a marked induction of this gene, confirming a novel regulatory pathway with the involvement of this transcription factor for GRAIL upregulation in sepsis (Figure 4D). In quest of potential GRAIL regulators, considering IL-2 as a critical growth promoting cytokine of CD4 T-cells, we stimulated cells with recombinant IL-2 in presence of the T-cell stimulatory and co-stimulatory molecules, and noticed significant reduction of GRAIL expression in shams, as well as septic T-cells, therefore, suggested IL-2 as one of the effective negative regulators for GRAIL to reverse T-cell unresponsiveness in sepsis. Since sepsis is characterized by an alteration of pro- and anti-inflammatory cytokines (1), future studies using a wide range of cytokines will provide additional insights for GRAIL regulation under septic conditions. In consistent with this fact, the cells isolated from septic animals regain their proliferation ability upon exogenous treatment with IL-2 via down-regulation of GRAIL (Figure 7). Normal cell cycle functioning reflects the active stage of cellular physiology. In our current model, we noticed cell cycle impairment in terms of G2/M phase arrest in CD4 T-cells isolated from septic mice (Figure 3). In agreement with our findings, a recent study also demonstrated that GRAIL holds single positive thymocytes, naïve, memory and Treg cells in cell cycle arrest at the G1-S interphase (16, 18). Hence, we suggest that the occurrence of CD4 T-cell cycle arrest in sepsis could be linked to the upregulation of GRAIL.

Recently, the neutralizing antibodies against negative co-stimulatory molecules such as, anti-CTLA-4, and PD1, have been suggested to use as a therapeutic potential to reverse CD4 T-cell anargy and apoptosis in sepsis (46). Future studies to elucidate GRAIL function as downstream molecule in these events may provide additional insights towards anti-CTLA-4 or PD1-mediated therapeutic functions in CD4 T-cell restoration in sepsis. Moreover, IL-15 and IL-7 are now being used as an anti-apoptotic cytokine to prevent the loss of CD4 T-cells in sepsis (47, 48). The role of GRAIL in this event could be established by considering the recent hypothesis of GRAIL for controlling the cell cycle by regulating its elements. From this observation clinical importance and therapeutic potential of GRAIL could be established. Interestingly, the recent studies revealed that the GRAIL is up-regulated in $CD4^{+}CD25^{+}$ T regulatory cells (Treg) and are sufficient for the conversion of T cells to a regulatory phenotype (49). Tregs not only regulate the hyperinflammatory cellular activities, but also promote an immunosuppressive environment by an increased production of IL-10 and TGF-β (1-3, 39). It is now become an established fact that the Treg population in sepsis and trauma become increased and induces immune-tolerance in the host (27, 31, 50). From these observations, the higher levels of GRAIL in sepsis could be attained in part from the increased Treg populations. Apart from Tregs, studies of GRAIL in other T-cell subsets will provide greater impact in studying cellular proliferations and effector functions in sepsis.

In summary, we established a novel link between CD4 T-cell proliferation defect and the upregulation of GRAIL in sepsis. Based on our results, GRAIL could be an interesting candidate for diagnosis and prognosis of sepsis in terms of maintenance of the CD4 T-cell

anergy and persistence of immune suppression phenomenon. Finally, the identification of novel function of GRAIL in septic CD4 T-cells might provide an effective therapeutic target by maneuvering its expression for the restoration of efficient immune responses by CD4 T-cells.

Acknowledgments

We thank Christopher Colon and Herb Borrero at the flow cytometry facility of The Feinstein Institute for Medical Research for technical assistance and Dr. Asha Jacob for valuable discussion.

Grant Support: This study was supported by the National Institutes of Health (NIH) grants, RO1 GM057468, RO1 GM53008 (P.W.).

References

1. Aziz M, Jacob A, Yang WL, Matsuda A, Wang P. Current trends in inflammatory and immunomodulatory mediators in sepsis. J Leukoc Biol. 2013; 93(3):329–342. [PubMed: 23136259]
2. Hotchkiss RS, Monneret G, Payen D. Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. Lancet Infect Dis. 2013; 13(3):260–268. [PubMed: 23427891]
3. Hotchkiss RS, Tinsley KW, Swanson PE, Schmieg RE Jr, Hui JJ, Chang KC, Osborne DF, Freeman BD, Cobb JP, Buchman TG, Karl IE. Sepsis-induced apoptosis causes progressive profound depletion of B and CD4+ T lymphocytes in humans. J Immunol. 2001; 166(11):6952–6963. [PubMed: 11359857]
4. Muenzer JT, Davis CG, Chang K, Schmidt RE, Dunne WM, Coopersmith CM, Hotchkiss RS. Characterization and modulation of the immunosuppressive phase of sepsis. Infect Immun. 2010; 78(4):1582–1592. [PubMed: 20100863]
5. Delano MJ, Thayer T, Gabrilovich S, Kelly-Scumpia KM, Winfield RD, Scumpia PO, Cuenca AG, Warner E, Wallet SM, Wallet MA, O'Malley KA, Ramphal R, Clare-Salzer M, Efron PA, Mathews CE, Moldawer LL. Sepsis induces early alterations in innate immunity that impact mortality to secondary infection. J Immunol. 2011; 186(1):195–202. [PubMed: 21106855]
6. Carson WF 4th, Cavassani KA, Ito T, Schaller M, Ishii M, Dou Y, Kunkel SL. Impaired CD4+ T-cell proliferation and effector function correlates with repressive histone methylation events in a mouse model of severe sepsis. Eur J Immunol. 2010; 40(4):998–1010. [PubMed: 20127677]
7. Kasten KR, Tschöp J, Goetzman HS, England LG, Dattilo JR, Cave CM, Seitz AP, Hildeman DA, Caldwell CC. T-cell activation differentially mediates the host response to sepsis. Shock. 2010; 34(4):377–383. [PubMed: 20610943]
8. Fathman CG, Lineberry NB. Molecular mechanisms of CD4+ T-cell anergy. Nat Rev Immunol. 2007; 7(8):599–609. [PubMed: 17612584]
9. Mueller DL. Mechanisms maintaining peripheral tolerance. Nat Immunol. 2010; 11:21–27. [PubMed: 20016506]
10. Macián F, García-Cózar F, Im SH, Horton HF, Byrne MC, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. Cell. 2002; 109(6):719–731. [PubMed: 12086671]
11. Seroogy CM, Fathman CG. T-cell anergy: from phenotype to genotype and back. Immunol Res. 2003; 28(3):255–264. [PubMed: 14713718]
12. Mueller DL. E3 ubiquitin ligases as T cell anergy factors. Nat Immunol. 2004; 5(9):883–890. [PubMed: 15334084]
13. Anandasabapathy N, Ford GS, Bloom D, Holness C, Paragas V, Seroogy C, Skrenta H, Hollenhorst M, Fathman CG, Soares L. GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. Immunity. 2003; 18(4):535–547. [PubMed: 12705856]
14. Seroogy CM, Soares L, Ranheim EA, Su L, Holness C, Bloom D, Fathman CG. The gene related to anergy in lymphocytes, an E3 ubiquitin ligase, is necessary for anergy induction in CD4 T cells. J Immunol. 2004; 173(1):79–85. [PubMed: 15210761]

15. Kriegel MA, Rathinam C, Flavell RA. E3 ubiquitin ligase GRAIL controls primary T cell activation and oral tolerance. Proc Natl Acad Sci U S A. 2009; 106(39):16770–16775. [PubMed: 19805371]
16. Lin JT, Lineberry NB, Kattah MG, Su LL, Utz PJ, Fathman CG, Wu L. Naive CD4 t cell proliferation is controlled by mammalian target of rapamycin regulation of GRAIL expression. J Immunol. 2009; 182(10):5919–5928. [PubMed: 19414743]
17. Nurieva RI, Zheng S, Jin W, Chung Y, Zhang Y, Martinez GJ, Reynolds JM, Wang SL, Lin X, Sun SC, Lozano G, Dong C. The E3 ubiquitin ligase GRAIL regulates T cell tolerance and regulatory T cell function by mediating T cell receptor-CD3 degradation. Immunity. 2010; 32(5): 670–680. [PubMed: 20493730]
18. Whiting CC, Su LL, Lin JT, Fathman CG. GRAIL: a unique mediator of CD4 T-lymphocyte unresponsiveness. FEBS J. 2011; 278(1):47–58. [PubMed: 21078124]
19. Su LL, Iwai H, Lin JT, Fathman CG. The transmembrane E3 ligase GRAIL ubiquitinates and degrades CD83 on CD4 T cells. J Immunol. 2009; 183(1):438–444. [PubMed: 19542455]
20. Lineberry NB, Su LL, Lin JT, Coffey GP, Seroogy CM, Fathman CG. Cutting edge: The transmembrane E3 ligase GRAIL ubiquitinates the costimulatory molecule CD40 ligand during the induction of T cell anergy. J Immunol. 2008; 181(3):1622–1626. [PubMed: 18641297]
21. Ichikawa D, Mizuno M, Yamamura T, Miyake S. GRAIL (gene related to anergy in lymphocytes) regulates cytoskeletal reorganization through ubiquitination and degradation of Arp2/3 subunit 5 and coronin 1A. J Biol Chem. 2011; 286(50):43465–43474. [PubMed: 22016387]
22. Schartner JM, Simonson WT, Wernimont SA, Nettenstrom LM, Huttenlocher A, Seroogy CM. Gene related to anergy in lymphocytes (GRAIL) expression in CD4+ T cells impairs actin cytoskeletal organization during T cell/antigen-presenting cell interactions. J Biol Chem. 2009; 284(50):34674–34681. [PubMed: 19833735]
23. Miksa M, Wu R, Dong W, Komura H, Amin D, Ji Y, Wang Z, Wang H, Ravikumar TS, Tracey KJ, Wang P. Immature dendritic cell-derived exosomes rescue septic animals via milk fat globule epidermal growth factor-factor VIII. J Immunol. 2009; 183(9):5983–5990. [PubMed: 19812188]
24. Yang WL, Perillo W, Liou D, Marambaud P, Wang P. AMPK inhibitor compound C suppresses cell proliferation by induction of apoptosis and autophagy in human colorectal cancer cells. J Surg Oncol. 2012; 106(6):680–688. [PubMed: 22674626]
25. Bachmann MF, Oxenius A. Interleukin 2: from immunostimulation to immunoregulation and back again. EMBO Rep. 2007; 8(12):1142–1148. [PubMed: 18059313]
26. Tinsley KW, Grayson MH, Swanson PE, Drewry AM, Chang KC, Karl IE, Hotchkiss RS. Sepsis induces apoptosis and profound depletion of splenic interdigitating and follicular dendritic cells. J Immunol. 2003; 171(2):909–914. [PubMed: 12847261]
27. Wisnoski N, Chung CS, Chen Y, Huang X, Ayala A. The contribution of CD4+CD25+ T-regulatory cells to immune suppression in sepsis. Shock. 2007; 27(3):251–257. [PubMed: 17304105]
28. Remick DG. Pathophysiology of sepsis. Am J Pathol. 2007; 170:1435–1444. [PubMed: 17456750]
29. Roger PM, Hyvernat H, Ticchioni M, Kumar G, Dellamonica J, Bernardin G. The early phase of human sepsis is characterized by a combination of apoptosis and proliferation of T cells. J Crit Care. 2012; 27(4):384–393. [PubMed: 22824083]
30. Monneret G. How to identify systemic sepsis-induced immunoparalysis. Advances in Sepsis. 2005; 4:42–49.
31. Wang P, Chaudry IH. Mechanism of hepatocellular dysfunction during hyperdynamic sepsis. Am J Physiol. 1996; 270(5 Pt 2):R927–R938. [PubMed: 8928923]
32. Wesche DE, Lomas-Neira JL, Perl M, Chung CS, Ayala A. Leukocyte apoptosis and its significance in sepsis and shock. J Leukoc Biol. 2005; 78(2):325–337. [PubMed: 15817707]
33. Muenzer JT, Davis CG, Dunne BS, Unsinger J, Dunne WM, Hotchkiss RS. Pneumonia after cecal ligation and puncture: a clinically relevant “two-hit” model of sepsis. Shock. 2006; 26(6):565–570. [PubMed: 17117130]
34. Fazal N, Al-Ghoul WM. Thermal injury-plus-sepsis contributes to a substantial deletion of intestinal mesenteric lymph node CD4 T cell via apoptosis. Int J Biol Sci. 2007; 3(6):393–401. [PubMed: 17895960]

35. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. Nat Rev Immunol. 2013 [Epub ahead of print]. 10.1038/nri3552
36. Vachharajani VT, Wang X, Brown C, Liu T, Buechler N, Yoza BK, McCall CE. SIRT1 Inhibition: A Novel Treatment for Sepsis Induced Immunosuppression. J Leukoc Biol. 2013:S17. Supplemental.
37. Venet F, Pachot A, Debard AL, Bohé J, Bienvenu J, Lepape A, Monneret G. Increased percentage of CD4+CD25+ regulatory T cells during septic shock is due to the decrease of CD4+CD25- lymphocytes. Crit Care Med. 2004; 32(11):2329–2331. [PubMed: 15640650]
38. Chung CS, Xu YX, Chaudry IH, Ayala A. Sepsis induces increased apoptosis in lamina propria mononuclear cells which is associated with altered cytokine gene expression. J Surg Res. 1998; 77(1):63–70. [PubMed: 9698535]
39. Song GY, Chung CS, Chaudry IH, Ayala A. Immune suppression in polymicrobial sepsis: differential regulation of Th1 and Th2 responses by p38 MAPK. J Surg Res. 2000; 91(2):141–146. [PubMed: 10839963]
40. Ayala A, Song GY, Chung CS, Redmond KM, Chaudry IH. Immune depression in polymicrobial sepsis: the role of necrotic (injured) tissue and endotoxin. Crit Care Med. 2000; 28(8):2949–2955. [PubMed: 10966277]
41. Nurieva R, Thomas S, Nguyen T, Martin-Orozco N, Wang Y, Kaja MK, Yu XZ, Dong C. T-cell tolerance or function is determined by combinatorial costimulatory signals. EMBO J. 2006; 25:2623–2633. [PubMed: 16724117]
42. Egawa S, Iijima H, Shinzaki S, Nakajima S, Wang J, Kondo J, Ishii S, Yoshio T, Irie T, Nishida T, Kakiuchi Y, Yasumaru M, Yoshihara H, Kanto T, Tsujii M, Tsuji S, Hayashi N. Upregulation of GRAIL is associated with remission of ulcerative colitis. Am J Physiol Gastrointest Liver Physiol. 2008; 295(1):G163–G169. [PubMed: 18467499]
43. Heissmeyer V, Macian F, Im SH, Varma R, Feske S, Venuprasad K, Gu H, Liu YC, Dustin ML, Rao A. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. Nat Immunol. 2004; 5:255–265. [PubMed: 14973438]
44. Soto-Nieves N, Puga I, Abe BT, Bandyopadhyay S, Baine I, Rao A, Macian F. Transcriptional complexes formed by NFAT dimmers regulate the induction of T cell tolerance. J Exp Med. 2009; 206:867–876. [PubMed: 19307325]
45. Safford M, Collins S, Lutz MA, Allen A, Huang CT, Kowalski J, Blackford A, Horton MR, Drake C, Schwartz RH, Powell JD. Egr-2 and Egr-3 are negative regulators of T cell activation. Nat Immunol. 2005; 6(5):472–480. [PubMed: 15834410]
46. Chang KC, Burnham CA, Compton SM, Rasche DP, Mazuski R, Smcdonough J, Unsinger J, Korman AJ, Green JM, Hotchkiss RS. Blockade ofthe negative co-stimulatory molecules PD-1 and CTLA-4 improves survival in primary and secondary fungal sepsis. Crit Care. 2013; 17(3):R85. [PubMed: 23663657]
47. Unsinger J, McGlynn M, Kasten KR, Hoekzema AS, Watanabe E, Muenzer JT, McDonough JS, Tschoep J, Ferguson TA, McDunn JE, Morre M, Hildeman DA, Caldwell CC, Hotchkiss RS. IL-7 promotes T cell viability, trafficking, and functionality and improves survival in sepsis. J Immunol. 2010; 184(7):3768–3779. [PubMed: 20200277]
48. Inoue S, Unsinger J, Davis CG, Muenzer JT, Ferguson TA, Chang K, Osborne DF, Clark AT, Coopersmith CM, McDunn JE, Hotchkiss RS. IL-15 prevents apoptosis, reverses innate and adaptive immune dysfunction, and improves survival in sepsis. J Immunol. 2010; 184(3):1401–1409. [PubMed: 20026737]
49. MacKenzie DA, Schartner J, Lin J, Timmel A, Jennens-Clough M, Fathman CG, Seroogy CM. GRAIL is up-regulated in CD4+CD25+ T regulatory cells and is sufficient for conversion of T cells to a regulatory phenotype. J Biol Chem. 2007; 282:9696–9702. [PubMed: 17259178]
50. Ermann J, Szanya V, Ford GS, Paragas V, Fathmann CG, Lejon K. CD4+CD25+ T cells facilitae the induction of T cell anergy. J Immunol. 2001; 167:4271–4275. [PubMed: 11591749]

List of abbreviations

GRAIL	gene related to anergy in lymphocytes
RNF-128	ring finger protein-128
CLP	cecal ligation and puncture
CFSE	carboxyfluorescein diacetate succinimidyl ester
Egr-2	early growth response-2
TCR	T-cell receptor
APC	antigen presenting cells

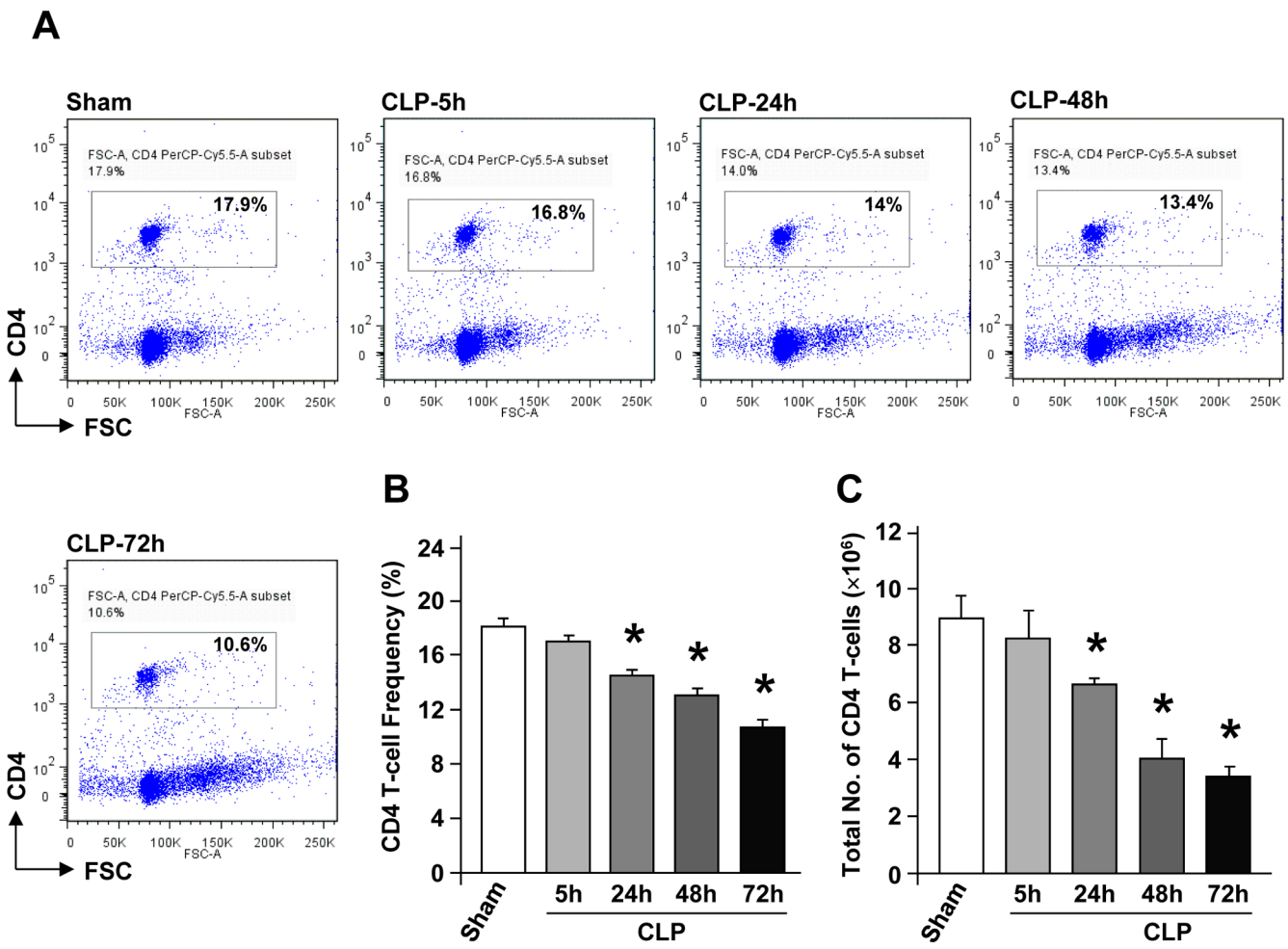

Figure 1. Decreased percentages and quantities of CD4 T-cells in sepsis

(A) A total of 1 × 10^6 cells isolated from spleens of sham and CLP-induced mice at 5, 24, 48, and 72 h time points were stained with PerCP/Cy5.5-CD4 Ab and then analyzed by flow cytometry and represented as dot blots. **(B)** Bar diagram indicating the percentages of the CD4 T-cells in sham *vs.* different time points of CLP are shown. **(C)** Total splenocytes were counted in a hemocytometer after harvested from euthanized sham and CLP mice by tryphan blue exclusion staining and then the total numbers of CD4 T-cells/mice were calculated from their percentages. No. of CD4 T-cells (×10^6) = Total no of cells/CD4 T-cells (%). Data are expressed as means ± SE (n=5 mice/group). *$P < 0.05$ *vs.* shams.

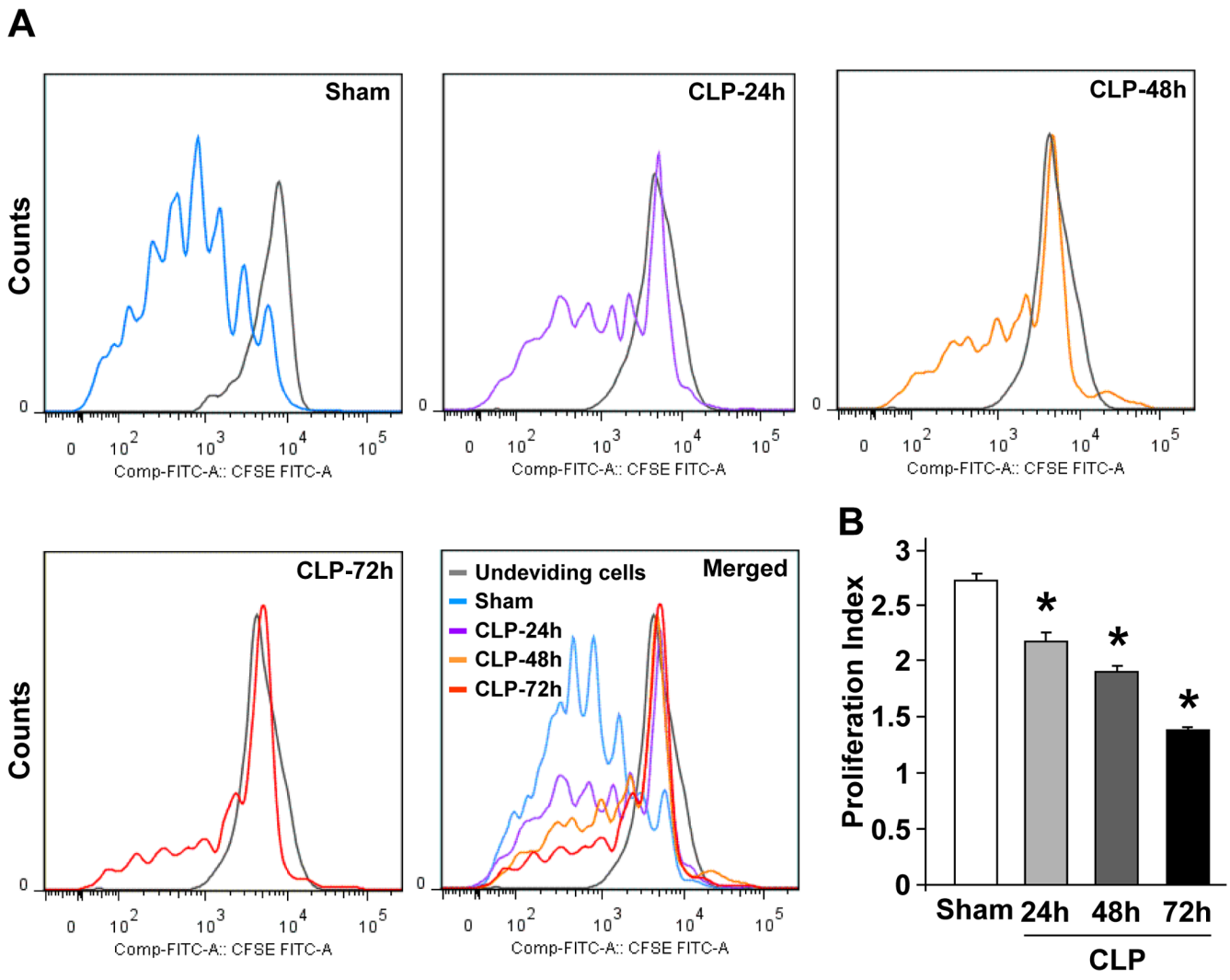

Figure 2. Impaired CD4 T-cell proliferation in sepsis

(A) Splenocytes were isolated from shams and CLP-induced mice at different time points and then a total of 1×10^6 cells were stained with CFSE and subjected to proliferation in a 24-well anti-CD3/CD28-coated (1 μg/mL) plates. Following 72 h of incubation, the cells were stained with PerCP/Cy5.5-CD4 Ab, and then flow cytometric acquisition was done. Representative histogram indicating the multiple peaks at successive generations of their divisions are shown. **(B)** CFSE cell proliferation data were quantitatively analyzed by FlowJo software and representated as bar diagram. Data are expressed as means ± SE (n=5 mice/group). *$P < 0.05$ *vs.* shams.

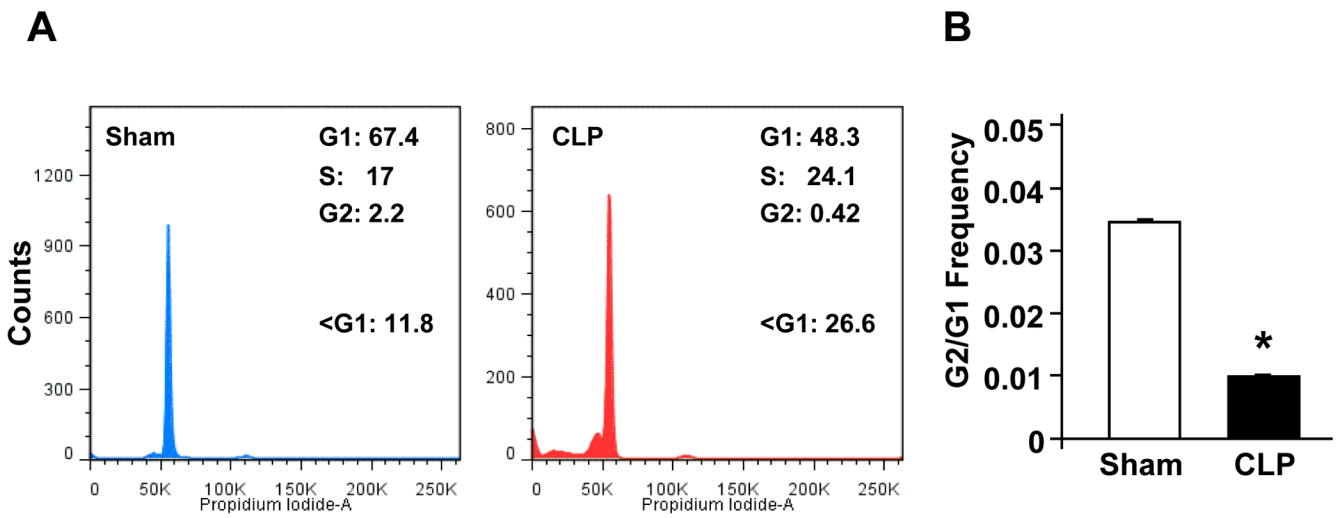

Figure 3. Impaired CD4 T-cell cycle profile in sepsis

(A) Cells (1×10^6) were isolated from spleens of shams and 24 h CLP-induced mice and then stained with anti-CD4 Ab and propidium iodide followed by data analysis using flow cytometry. **(B)** Data are analyzed by FlowJo software using the cell cycle tool and represented as G2/G1 values in the bar diagram. Results are expressed as means ± SE (n=5 mice/group). *$P < 0.05$ *vs.* shams.

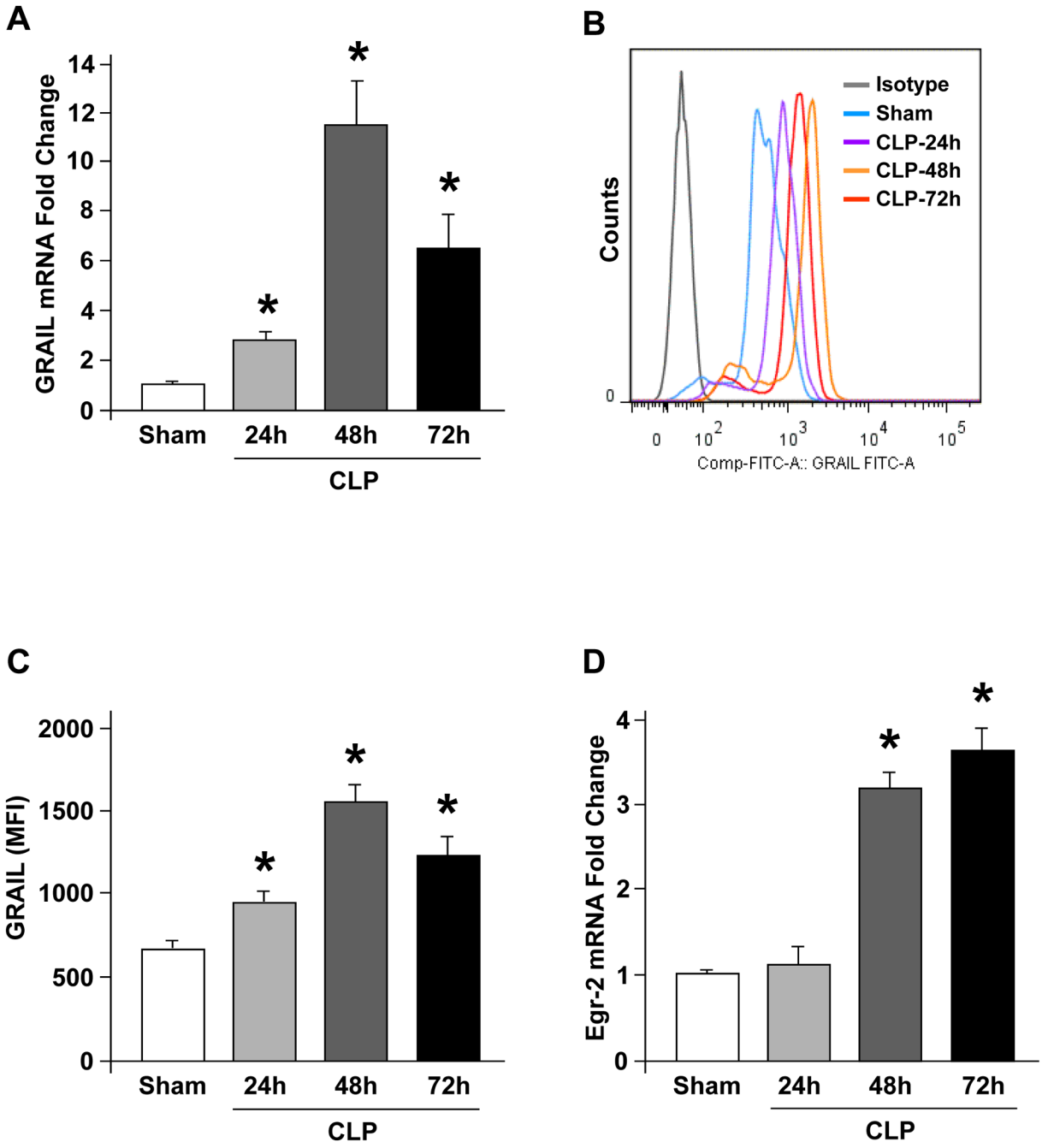

Figure 4. Upregulation of GRAIL in septic splenocytes following antigenic stimulation

(A) A total of 2×10^6 cells isolated from the spleens of sham and CLP-induced animals were stimulated with anti-CD3/CD28 (1 μg/mL) Abs for 24 h, followed by the realtime PCR using GRAIL primer. β-actin serves as an internal control for respective samples. **(B-C)** Shams and CLP animal's splenocytes (1×10^6) were isolated and *in vitro* stimulated with anti-CD3/CD28 (1 μg/mL) Abs for 24 h. Data was acquired using flow cytometry and the MFI between shams and CLP samples are shown in bar diagram. **(D)** Splenocytes (2×10^6) from shams and CLP-induced animals were stimulated with anti-CD3/CD28 (1 μg/mL) Abs for 24 h. The expression of Egr-2 was assessed by realtime PCR. β-actin acts as an internal

control. Data are expressed as means ± SE (n=5 mice/group). *$P < 0.05$ *vs.* Shams with anti-CD3/CD28 (+).

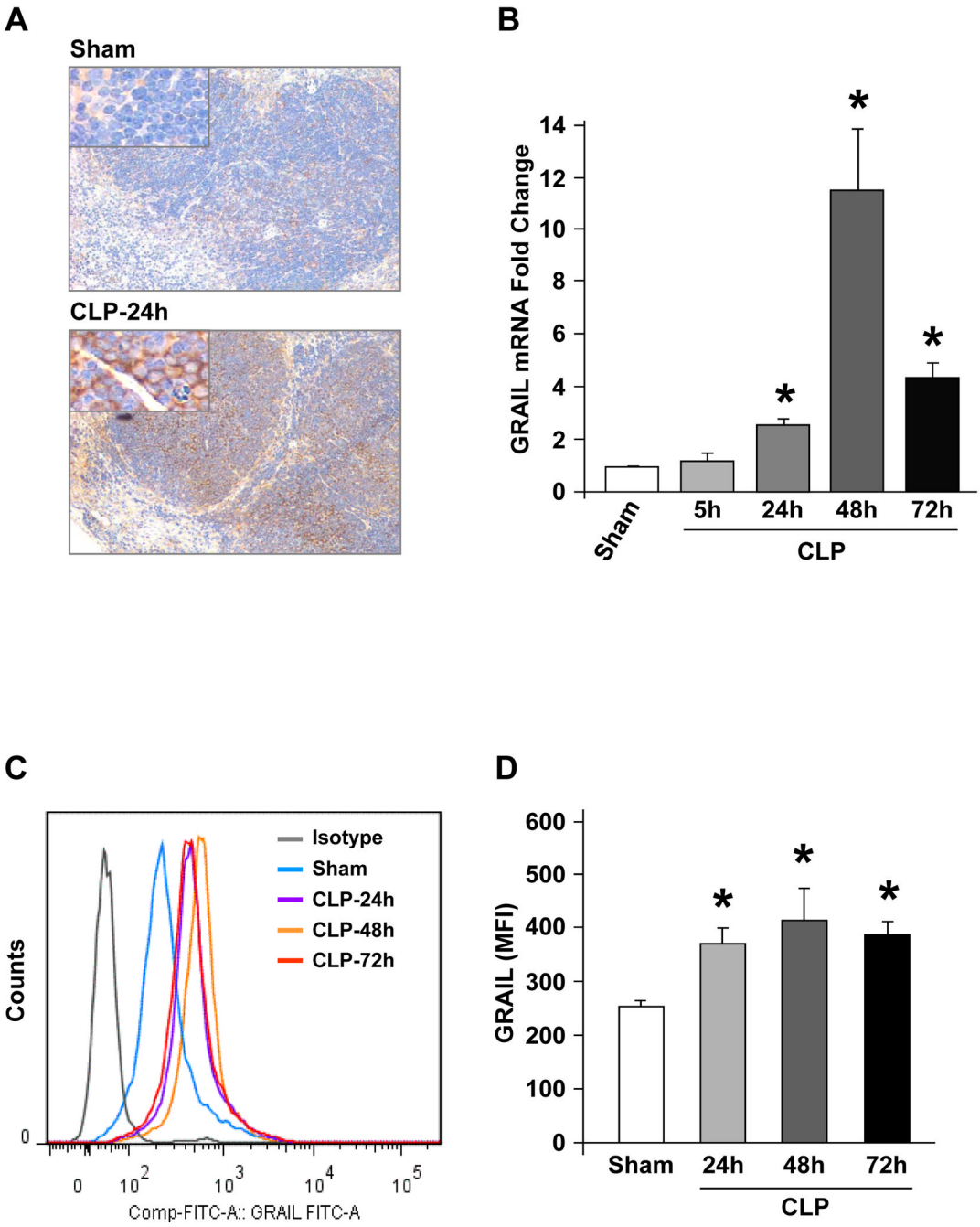

Figure 5. Upregulation of GRAIL in CD4 T-cells following sepsis

(A) After 24 h of CLP, spleen tissues were harvested from shams and septic mice and fixed in 10% formalin and embedded in paraffin. Tissue blocks were sectioned at a thickness of 5 μm and incubated with rabbit anti-mouse GRAIL Ab, followed by incubation with biotinylated anti-rabbit IgG. Staining was developed by Vectastain ABC reagent and a diaminobenzidine kit. Representative immunostaing images at original magnification of ×100 and ×200 (*inset*) are shown. **(B)** Cells were isolated from spleens of sham and CLP animals; a total of 2 ×10^6 cells were used to extract mRNA and subsequent realtime PCR analysis using GRAIL primer. The expression of β-actin serves as an internal control. The

data is expressed as fold induction in comparison to the shams. **(C)** Splenocytes (1×10^6 cells) from the shams and septic mice were stained for surface CD4 antigen and intracellular GRAIL using PerCP/Cy5.5-CD4 Ab, and rabbit anti-GRAIL primary Ab, respectively. A FITC-labeled anti-rabbit secondary Ab was used subsequently and then events were acquired in flow cytometer. **(D)** Bar diagram representing the mean fluoresce intensities of sham and septic samples are shown. Data are expressed as means ± SE (n=5 mice/group). *$P < 0.05$ *vs.* Shams.

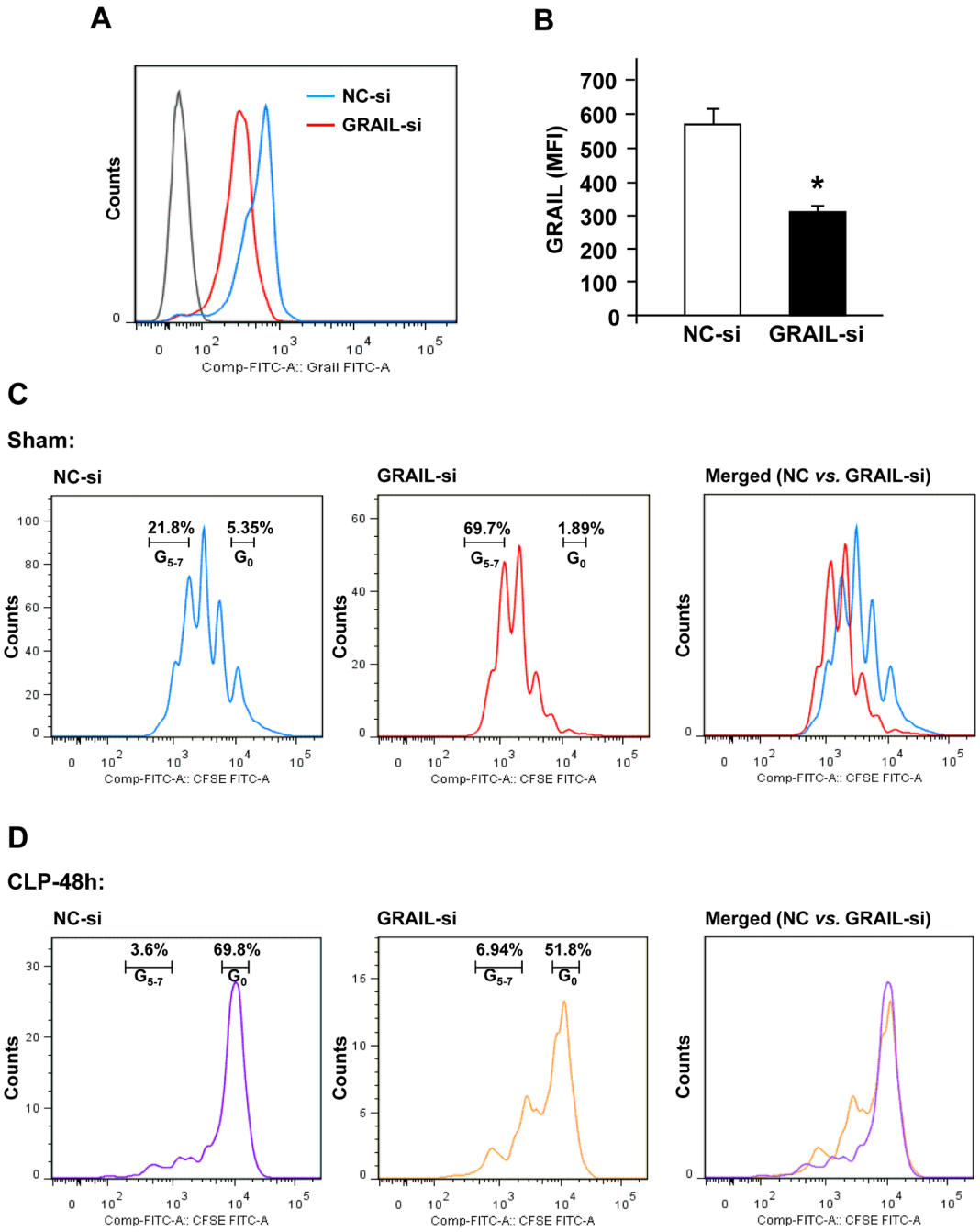

Figure 6. siRNA-mediated GRAIL knock down and assessment of CD4 T-cell proliferation

(A-B) To assess the GRAIL knock down efficacy, a total of 5×10^5 CD4 T-cells isolated from sham mice spleen were plated into the 24-well culture plates in 100 μL of RPMI + 10% FBS, and then transfected with 75 ng of each of the four siRNAs for GRAIL altogether or non-target specific NC-siRNA mixed with 3 μL of the HiPerFect in 100 μL of OPTIMEM medium. After 3 h of transfection, a total of 400 μL of RPMI + 10% FBS was added and incubated for 24 h at 37°C incubator. Afterwards, the cells were stained with the anti-GRAIL Ab as primary and FITC-anti-Rb secondary Ab by using the intra-cellular fixation and permeabilization reagent, and then subjected to flow cytometry analysis. Bar diagram representing the mean fluoresce intensities of NC-siRNA and GRAIL siRNAs are

shown. Data are expressed as means ± SE (n=3 mice/group). *$P < 0.05$ *vs.* NC-siRNAs. **(C-D)** After 24 h, a total of 5×10^5 GRAIL knocked down or NC-siRNA transfected CD4 T-cells were stained with CFSE (5 μM) and then plated into the anti-CD3/anti-CD28 (1 μg/mL of each Abs)-coated 24-well plates. The cells were then allowed to proliferate for 72 h and then acquired in flow cytometer followed by the assessment of the proliferation profile by FlowJo software. Anti-CD3/anti-CD28 unstimulated CFSE-labeled cells served as a non-dividing control. The histogram shows a marked increase in the percentages of $G_{5\text{-}7}$ frequency indicating the higher CD4 T-cell proliferation efficacy after *ex vivo* GRAIL siRNA transfection as compared to the negative control siRNA samples isolated from the spleens of shams as well as 48 h CLP animals. The statistical analysis using Student *t* test reveals significant increase in the $G_{5\text{-}7}$ population in GRAIL-siRNA-transfected 48 h CLP mice spleen CD4 T-cells than NC-siRNA treated controls (NC-si: 3.7 ± 0.07% *vs.* GRAIL-siRNAs: 8.6 ± 0.98%; $p<0.05$ as compared to NC-si; Data are expressed as means ± SE obtained from 3 mice).

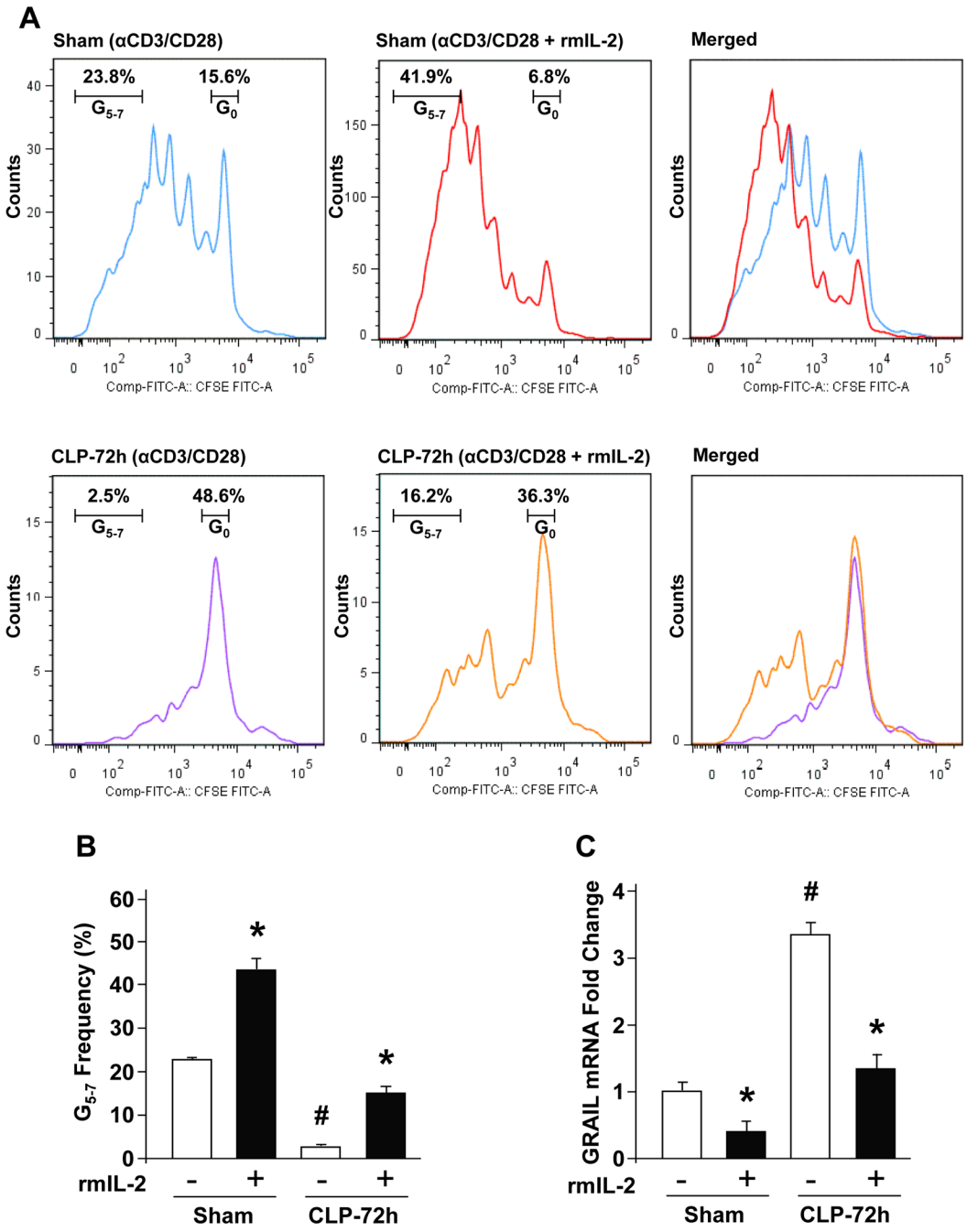

Figure 7. ***Ex vivo*** **stimulation of rmIL-2 induces septic CD4 T-cell proliferation via downregulating GRAIL expression**

(A) Each of the 1 × 10^6 sham and 72 h time point septic splenocytes were first stained with CFSE (5 μM) and then cultured for 72 h in presence of plate bound anti-CD3/CD28 (1 μg/mL) Abs and in presence or absence of rmIL-2 (10 ng/mL). The cells were then stained with PerCP/Cy5.5-CD4 Ab and subjected to flow cytometry analysis. Representative histograms indicating the CD4 T-cell proliferation is shown; G_0, generation 0 or undivided cells; G_{5-7} represents percentages of dividing cells. **(B)** The mean percentages of the G_{5-7} frequencies are shown in bar diagram as obtained from 3 mice in each group. **(C)** A total of 2 × 10^6 cells isolated from the spleens of sham and 72 h CLP mice were grown in plates coated with anti-

CD3/CD28 (1 μg/mL) Abs and in presence or absence of exogenous rmIL-2 (10 ng/mL). A realtime PCR assessment was done to evaluate the GRAIL and β-actin expression using respective primers. Data are expressed as means ± SE (n=3 mice/group). *$P < 0.05$ *vs.* Sham/CLP with anti-CD3/CD28 (+) and without IL-2 (-); #$P < 0.05$ *vs.* Shams with anti-CD3/CD28 (+) and without IL-2 (-).

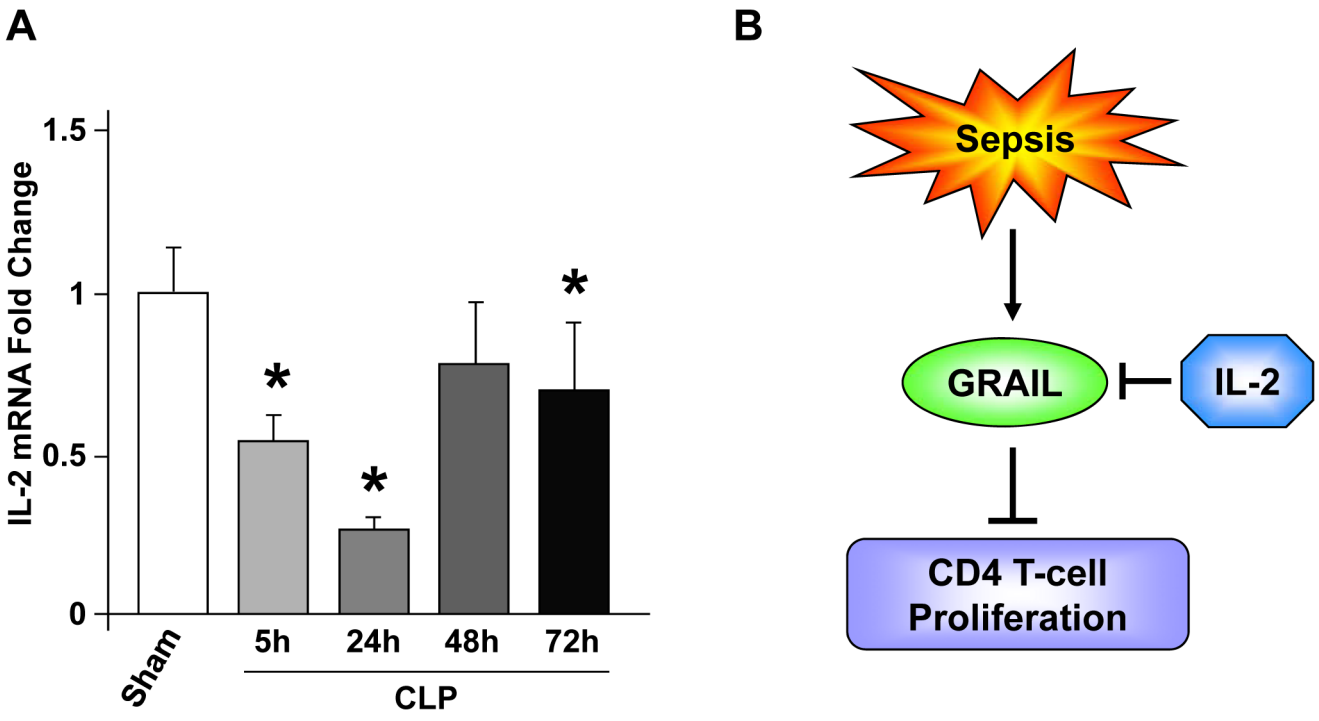

Figure 8. Dysregulation of *ex vivo* IL-2 expression in sepsis

(A) Cells were isolated from shams and CLP-treated animals at different time points and then grown in a 10-cm cell culture plate in RPMI + 10% FBS medium for 2 h. The adherent cells were excluded, while the non-adherent cells (2×10^6) were taken for the assessment of IL-2 by real-time PCR. The expression of β-actin serves as an internal control for respective samples. The data is expressed as fold induction in comparison to the shams. Results are expressed as means ± SE (n=5 mice/group). **P* < 0.05 *vs.* shams. **(B)** The hypothesis scheme showing the role of GRAIL in CD4 T-cell unresponsiveness in sepsis. Sepsis causes an upregulation of GRAIL which inhibits normal CD4 T-cell proliferation upon TCR stimulation. Conversely, the addition of exogenous rmIL-2 reverses the CD4 T-cell malfunction in context of its proliferation abilities by downregulating GRAIL expression.