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Alterations in Ag-specific naïve CD4 T cell precursors after sepsis impairs their responsiveness to pathogen challenge[1]

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Abstract

Patients surviving the acute stages of sepsis develop compromised T cell immunity and increased susceptibility to infection. Little is known about the decreased CD4 T cell function after sepsis. We tracked the loss and recovery of endogenous Ag-specific CD4 T cell populations after cecal-ligation and puncture (CLP)-induced sepsis, and analyzed the CD4 T cell response to heterologous infection during or after recovery. We observed that the sepsis-induced early loss of CD4 T cells was followed by thymic-independent numerical recovery in the total CD4 T cell compartment. Despite this numerical recovery, we detected alterations in the composition of naïve CD4 T cell precursor pools, with sustained quantitative reductions in some populations. Mice that had experienced sepsis and were then challenged with epitope-bearing, heterologous pathogens demonstrated significantly reduced priming of recovery-impaired Ag-specific CD4 T cell responses, both in magnitude of expansion and functional capacity on a per-cell basis, which also correlated with intrinsic changes in Vβ clonotype heterogeneity. Our results demonstrate the recovery of CD4 T cells from sepsis-induced lymphopenia is accompanied by alterations to the composition and function of the Ag-specific CD4 T cell repertoire.

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Introduction

CD4 T helper (Th) cells influence the function of a variety of innate and adaptive immune cells critical for the successful generation of a productive and protective immune response (1). For example, effective primary CD8 T cell responses (2, 3), the formation of functional CD8 T cell memory (4-7), efficient isotype switching in primary and memory B cell responses (8, 9), and the effector function of macrophages (10) all develop with the “help” of CD4 T cells. The ability of CD4 T cells to function in such an array of immunological settings is because effector CD4 T cells can take on different phenotypes (i.e., Th1, Th2, Th9, Th17, Tfh (1)), based on the cytokines and costimulatory molecules present at the time of Ag recognition. In turn, this plasticity enables CD4 T cells to drive a response that is best suited for the situation. Due to their importance in a broad variety of immune responses, perturbations in the CD4 T cell compartment can have dramatic consequences on the overall fitness of the immune system.

Sepsis strikes 750,000 Americans every year (11) with ~210,000 of these patients dying (12). Although sepsis has been defined as a systemic inflammatory response syndrome (SIRS) in the presence of a disseminated infection (13-15), it has become clear in the past decade that sepsis is not just the symptoms of a complicated infection. Instead, sepsis is now viewed as a syndrome stemming from the dysregulation of immune responses due to an invasive pathogen – a phenomenon that results in system-wide collateral damage (16). Sepsis-induced immune suppression is intricately related to the process of lymphocyte apoptosis that occurs after a septic event (17, 18). Sepsis-induced lymphopenia transiently creates a reduction in numbers of immune cells, including T cells. While the total T cell compartment recovers numerically after a septic event, it is unknown whether different Ag-specific T cell subpopulations can revert back to the antigenic diversity seen before sepsis, and whether changes in population diversity can affect the functionality of the immune system. Gross quantitation of CD4 T cells reveals that they are severely depleted during the acute stage of sepsis but gradually recover throughout the immunosuppressive phase of sepsis (19). However, there are knowledge gaps regarding the mechanism(s) driving this CD4 T cell recovery, the quality/functionality of the “recovered” CD4 T cell compartment, and the extent to which sepsis impairs Ag-specific CD4 T cell function in surviving animals.

In this study, we used peptide:MHC II (p:I-A^b) tetramer enrichment technology (20) to examine quantitative shifts within the endogenous naïve Ag-specific CD4 T cell repertoire at different time points after sepsis. Our findings suggest that the numerical restoration of the CD4 T cell repertoire after sepsis occurs via a peripherally-driven mechanism that is, in part, independent of Ag availability. And while the total CD4 T cell population recovers numerically, examination of individual Ag-specific populations revealed an asymmetric recovery in different Ag-specific precursor populations. Our results also suggest that, if inadequately recovered, Ag-specific CD4 T cell populations show impairments in expansion and function in response to pathogen challenge after sepsis. The implications of these findings within the context of long-term increased susceptibility to secondary infections (and the associated increased risk of mortality) will be discussed.

Materials and Methods

Mice

Euthymic and thymectomized C57BL/6 (B6) mice were purchased from The National Cancer Institute. Thy1.1/1.1 TCR-transgenic SMARTA (LCMV gp_{61-77}-specific) and SM1 (*S.typhimurium* $FliC_{447-460}$-specific) B6 mice were obtained from Drs. David Masopust and Marc Jenkins (University of Minnesota), respectively. All mice were housed in the same facilities for at least 4 weeks, regardless of their source. Animal procedures were performed according to National Institutes of Health guidelines and approved by the University of Minnesota Institutional Animal Care and Use Committee. In all *in vivo* experiments, groups consisted of four or more animals, and experiments were repeated at least two times with similar results before reporting.

Cecal ligation and puncture

Septic injury was induced by cecal ligation and puncture (CLP) (21). Briefly, mice were anesthetized and the abdomen was shaved, disinfected, and a midline abdominal incision was made. The distal third of the cecum was ligated with 4–0 silk suture and punctured once using a 25-g needle to extrude a small amount of cecal content. The cecum was returned to the abdomen, the peritoneum was closed via continuous suture, and the skin was sealed using surgical glue (Vetbond, 3M, St. Paul, MN). Saline (1 ml) was provided s.c. following the procedure for resuscitation, and Bupivicaine was administered at the incision site for postoperative analgesia. This level of injury was used to create a chronic septic state characterized by the loss of appetite and body weight, ruffled hair, shivering, diarrhea, and/or periorbital exudates, and with a 5-10% mortality rate. Sham-treated mice underwent the same procedure excluding cecal ligation and puncture.

BrdU incorporation and detection

To assess CD4 T cell proliferation, sham- and CLP-treated mice were given a BrdU pulse (2 mg in 0.2 ml/mouse i.p.; Sigma, St. Louis, MO) on d 6 after surgery. Blood was collected at the indicted times, followed by RBC lysis in ACK buffer. Cells were surface stained with PE CD4 (clone GK1.5; BioLegend), after which they were fixed with Cytofix/Cytoperm solution (BD Biosciences; San Diego, CA) and treated with DNase I (300 mg/ml in PBS; Sigma) for 1 h. BrdU was detected by intracellular staining with FITC-conjugated anti-BrdU (clone BU20A) or an IgG1 isotype (clone P3.6.2.8.1) mAb (both from eBioscience; San Diego, CA).

Adoptive cell transfers

SM1 or SMARTA TCR-tg CD4 T cells were obtained from the spleens of naïve SMARTA or SM1 mice. Contaminating memory phenotype ($CD44^{hi}$ $CD11a^{hi}$ $CD49^{hi}$) TCR-tg cells were consistently <5%. The purified cells were transferred to naïve B6 mice 1 day before sham or CLP surgery.

Experimental pathogens and infections

2W1S (EAWGALANWAVDSA)-expressing or $OVA_{323-339}$ (ISQAVHAAHAEINEAGR)-expressing, *ActAΔ L. monocytogenes* (attenuated Lm-2W1S or Lm-OVA, 10^7 PFU/mouse) was grown and injected i.v. as previously described (22). 2W1S-expressing *C.albicans* (*C. albicans*-2W1S) was derived from clinical isolate SC5314 (23). *C. albicans*-2W1S was grown to log phase (OD_{600} of 1.5) in YDAP medium, washed, and counted by hemocytometer before being resuspended at a concentration of 5×10^4 yeasts per i.v. challenge. Recombinant Lm-2W1S and *C. albicans*-2W1S were obtained from Drs. Marc Jenkins and Daniel Kaplan (University of Minnesota), respectively. For HSV-1 (KOS strain) and influenza A virus (strain ×31) inocula, frozen viral stocks were thawed and 2.5×10^4 PFU and 3000 EID_{50} units, respectively, was administered per mouse. Viral stocks were obtained from culture conditions that have been previously described (24-26). Intravenous challenges using 0.1 ml injection volumes were used for all of the inoculants described, except for IAV ×31 given intranasal using 0.02 ml aliquots per nostril. Infected mice were housed under the appropriate biosafety level.

Tetramers and peptides

I-A^b–specific tetramers containing 2W1S (EAWGALANWAVDSA), LCMV glycoprotein $(gp)_{66-77}$ (DIYKGVYQFKSV), $LLO_{190-201}$ (NEKYAQAYPNVS), $OVA_{323-339}$ (ISQAVHAAHAEINEAGR) peptides were obtained from Dr. Marc Jenkins. Biotinylated soluble I-A^b molecules containing HSV glycoprotein D $(gD)_{290-305}$ (IPPNWHIPSIQDA) or influenza A virus nucleoprotein $(NP)_{311-325}$ (QVYSLIRPNENPAHK) peptides (27, 28) covalently attached to the I-A^b beta chain were produced with the I-A^b alpha chain in *Drosophila melanogaster* S2 cells, then purified, and made into tetramers with streptavidin (SA)- phycoerythrin (PE; eBioscience) or SA-allophycocyanin (APC; Biolegend) as previously described (20, 29). Peptides used to elicit cytokine production or expand endogenous Vβ repertoire for quantification were synthesized by Bio-Synthesis (Louisville, TX).

Quantitation of endogenous Ag-specific CD4 T cell populations using p:I-A^b tetramer based-enrichment

To quantify the number of Ag-specific CD4 T cells within the spleens of sham- or CLP-treated mice, a tetramer-based enrichment protocol (29) using p:I-A^b tetramers was employed. Briefly, spleens were harvested for each mouse analyzed, a single-cell suspension was prepared, and APC- and PE-conjugated tetramers were added at a 1:400 dilution in tetramer staining buffer (PBS containing 5% BCS, 2mM EDTA, and 50μM Dasatinib, 1:50 normal mouse serum, and 1:100 anti-CD16/32 mAb). The cells were incubated in the dark at room temperature for 1 h, followed by a wash in 10 ml cold FACS Buffer. The tetramer-stained cells were then resuspended in 0.2 ml FACS Buffer, mixed with 0.05 ml of both anti-APC and –PE mAb-conjugated magnetic microbeads (Miltenyi Biotech), and incubated in the dark on ice for 30 min. The cells were washed and resuspended in 3 ml cold FACS Buffer and passed over a MACS separation column (Miltenyi Biotech) to enrich for the tetramer-specific cells. Columns were washed three times with 3 ml cold FACS buffer, before eluting the bound fraction with 5 ml cold FACS buffer. The resulting enriched

fractions were then stained with a cocktail of fluorochrome-labeled mAb (see below). Cell numbers for each sample were determined using AccuCheck Counting Beads (Invitrogen). Samples were then analyzed using an LSR II flow cytometer (BD) and FlowJo software (TreeStar Inc., Ashland, OR). The percentage of tetramer-positive events was multiplied by the total number of cells in the enriched fraction to calculate the total number of Ag-specific CD4 T cells in the spleen.

CD4 T cell assays

In vivo peptide stimulation was used to determine Ag-specific CD4 T cell function by intracellular cytokine production, as previously described (22, 30, 31). Briefly, infected mice were injected i.v. with 100 μg of the appropriate peptide. After 2 h, spleens were harvested in media containing 10 μg/ml brefeldin A. The resulting cell suspensions were fixed, permeabilized, and stained with anti-IFNγ and anti-TNF mAb. To specifically examine the function of Ag-specific Th17 cells sham and CLP-treated mice were infected 30 d after surgery with *C. albicans*-2W1S epicutaneously (23). On d 7 post-infection, the spleen and skin-draining (inguinal, brachial, axillary and cervical) LN were harvested, and dissociated into a single-cell suspension. The resultant cells were stimulated for 4 h with PMA (50 ng/ml) and ionomycin (1.5 μM) in complete RPMI media supplemented with monensin (1 μM). After stimulation, cell debris was filtered and the samples underwent tetramer enrichment as described. After tetramer enrichment and subsequent staining for cell surface markers, 0.1 ml aliquots from the enriched and flow-through fractions were suspended in fixation/permeabilization buffer (eBioscience) for 20 min at 4°C, and then stained for intracellular IFNγ and IL-17 accumulation overnight in permeabilization buffer (eBioscience). After staining, cells were resuspended in FACS buffer and 0.02 ml of counting beads (eBioscience) were added to each sample immediately before acquisition.

Flow cytometry

To assess the expression of cell surface proteins, cells were incubated with fluorochrome-conjugated mAb at 4°C for 30 min. The cells were then washed with FACS buffer (PBS containing 2% BCS and 0.2% NaN_3). For some experiments, the cells were then fixed with PBS containing 2% paraformaldeyhe. In procedures requiring intracellular staining, cells were permeabilized following surface staining using the transcription factor staining kit (eBioscience), stained for 1 h at 4°C with a second set of fluorochrome-conjugated mAb, and suspended in FACS buffer for acquisition. The fluorochrome-conjugated mAb used in both surface and intracellular stainings were as follows: Horizon™ V500 Thy1.2 (clone 53-2.1; BD Biosciences), Brilliant Violet™ (BV) 510 and FITC CD3 (clone 17A2; BioLegend), BV421 and BV605 CD4 (clone GK1.5; BioLegend), BV650 CD8 (clone 53-6.7; BioLegend), AlexaFluor®700 CD44 (clone IM7; BioLegend), APC and BV421 IL17A (clone TC11-18H10.1; BioLegend), APC and BV650 IFNγ (clone XMG1.2; BioLegend), PE-Cy7 IL2 (clone JES6-5H4; BioLegend), AlexaFluor®647 CD49d (clone R1-2; BioLegend), PerCP-Cy5.5 B220 (clone RA3-6B2; eBioscience), PerCP-Cy5.5CD11b (clone M1/70; eBioscience), PerCP-Cy5.5CD11c (clone N418; eBioscience), PerCP-Cy5.5 F4/80 (clone BM8; eBioscience), FITC FoxP3 (clone FJK-15S; eBioscience), FITC CD11a (clone M17/4; eBioscience), FITC TNFα (clone MP6-XT22; eBioscience), and PE-Cy7

CD11a (clone M17/4; eBioscience). FlowJo software (TreeStar) was used for analysis of samples acquired on an LSR II flow cytometer (BD).

Ag-specific TCR Vβ repertoire flow cytometry assay

Assessment of TCR Vβ repertoire diversity performed using a modification of a previously reported method (29, 32). Briefly, sham- or CLP-treated mice were injected with 50 μg of 2W1S peptide and 5 μg LPS on d 30 post-surgery. After another 3 d, splenic T cells were enriched for APC-2W1S:I-A^b tetramer-binding cells. The enriched population was subsequently divided into 2 equal aliquots and stained for surface markers along with 13 available TCR Vβ mAb multiplexed onto four separate flow cytometry detection channels, using directly conjugated antibodies and/or biotinylated mAb detected afterwards with Streptavidin-BV421 (Biolegend; San Diego, CA). The TCR Vβ mAb used were: FITC-conjugated mAb against mouse Vβ 2 (clone B20.6), Vβ 4 (clone KT4), Vβ 6 (clone RR4-7), Vβ 7 (clone TR310), Vβ 8.1/8.2 (clone KJ16-133.18) and Vβ 8.3 (clone 8C1); PE-conjugated mAb against Vβ 5.1/5.2 (clone MR9-4), Vβ 8.1/8.2 (clone MR5-2), Vβ 8.3 (clone 1B3.3), Vβ 9 (clone MR10-2) and Vβ 10 (clone B21.5); PerCP-eFluor®710 conjugated Vβ13 (clone MR12-3); and biotinylated mAb against Vβ 3 (clone KJ25), Vβ 4, V 5.1/5.2, V 6, V 10 and Vβ 14 (clone 14-2). Unless specified, mAb that detected the same TCR Vβ clonotype were from the same clone and vendor. All mAb used to detect Vβ clonotype distribution were purchased from either BD Biosciences, Biolegend, or eBioscience.

Statistical analyses

Data were analyzed using GraphPad Prism® (La Jolla, CA). Specific tests to determine statistical significance are indicated in the figure legends. Statistical signficance is indicated as follows: **** $p < 0.001$, *** $p < 0.005$, ** $p < 0.01$, * $p < 0.05$, and *ns*, no significance. Data scatter plots are presented as mean values ± SEM, and data shown as bar graphs are presented as mean ± SEM.

Results

Numerical recovery of CD4 T cells after sepsis occurs by a thymic-independent mechanism

Sepsis can be experimentally investigated using the cecal ligation and puncture (CLP) model (21), which is frequently used to assess the acute complications and mortality associated with severe septic events. The CLP model used in our studies induces a mild septic state resulting in ~10% acute mortality (Fig. 1A). This degree of injury creates immune defects similar to more severe models and permits the long-term study of immune system responses in septic mice (33-35). In addition, mice that experience this milder sepsis demonstrate the same symptomatology characteristic of severe experimental peritonitis, including cachexia, weight loss, piloerection, and lethargy. Consistent with previous data (19), we found a significant decrease in the total number of CD4 T cells in the spleen, inguinal LN, and blood 2 d after septic injury and a numerical recovery apparent by d 30 (Fig. 1B-C). These results led us to conclude that the attenuated CLP procedure that produces a mild septic insult can be used to interrogate the CD4 T cell loss and recovery in the context of sepsis.

CD4 T cell recovery is not usually dependent on thymic-derived T cells in models of experimentally-induced lymphopenia, since the export rate of naïve T cells from the thymus is not modulated by perturbations in the periphery (36). In contrast, work by Unsinger et al. suggested CD4 T cells did not homeostatically proliferate when transferred into CLP-treated recipients (17). These contradictory findings led us to examine the number of CD4 T cells in the blood of sham- and CLP-treated euthymic (WT) and thymectomized mice. CD4 T cell loss and recovery was similar in WT and thymectomized mice, with no statistical differences at any of the time points analyzed (Fig. 1C). Next, sham- and CLP-treate WT and thymectomized mice were given BrdU on d 6 after surgery to measure proliferation of the peripheral blood CD4 T cells. We found statistically higher frequencies of $BrdU^+$ CD4 T cells in CLP-treated mice versus shams, regardless of thymic presence or absence (Fig. 1D). Together, these results suggest that CD4 T cell recovery after sepsis occurs by a thymus-independent mechanism.

CD4 T cells that numerically recover after septic insult acquire an “Ag-experienced” phenotype

As naïve T cells homeostatically proliferate to fill lymphopenic niches, their surface phenotype changes to resemble T cells that have encountered their cognate Ag (37, 38). Importantly, this change in phenotype can be independent of cognate Ag recognition (39). We recently reported that the numerical recovery of CD8 T cells after septic injury is driven by homeostatic proliferation, indicated by an increased frequency of $CD11a^{hi}$ $CD44^{hi}$ CD8 T cells compared to sham mice (34) – even when cognate Ag was not encountered. Currently there is no canonical phenotype characterizing CD4 T cells that have undergone similar processes, but several phenotypes have been suggested. For example, CD11a and CD49d co-expression indicate an “Ag-experienced” CD4 T cell phenotype (40, 41). We noted that CD4 T cell recovery after sepsis was concomitant with an increased frequency of $CD11a^{hi}$ $CD49d^{hi}$ CD4 T cells among the total CD4 T compartment (Fig. 2A-B). CD44 and CD127 co-expression has also been suggested to be a phenotype for CD4 T cells after Ag-independent expansion (42), and we found CLP-treated mice had an increased frequency of $CD44^{hi}$ $CD127^{hi}$ CD4 T cells (Fig. 2A-B). To determine the extent to which these changes were dependent on TCR interaction with cognate Ag, we transferred TCR-transgenic Sm1 (transgenic epitope: $Flic_{427\text{-}441}$ from *S. enterica ser. typhimurium* flagellin (43)) and SMARTA (transgenic epitope: $gp_{61\text{-}77}$ from LCMV (44)) CD4 T cells into B6 mice prior to sham or CLP surgery. These are two disparately different pathogens, neither of which is normally found in SPF mice. We observed significantly increased frequencies of $CD11a^{hi}$ $CD49d^{hi}$ Sm1 and SMARTA CD4 T cells in the spleens of CLP-treated mice compared to sham-treated mice after 30 d (Fig. 2C). While Ag cross reactivity cannot be excluded, these phenotypic changes in TCR-transgenic CD4 T cells led us to conclude that the acquisition of an “Ag-experienced” phenotype was occurring without cognate Ag present in the septic host. Together, the data in Fig. 1 and 2 suggest lymphopenia-induced homeostatic proliferation plays a major role in the numerical recovery of the CD4 T cell compartment after sepsis.

Asymmetric recovery of Ag-specific naïve CD4 T cells after sepsis

The massive attrition of peripheral CD4 T cells and evidence supporting a peripheral mechanism of CD4 T cell recovery after sepsis led us to question the possibility of discrete changes within individual Ag-specific CD4 T cell populations. We used peptide:MHC II (p:I-A^b) tetramer-based enrichment ((20, 29); Fig. 3A) to quantify 6 different endogenous Ag-specific CD4 T cell populations of varying size, clonotype composition, and immunodominance. CLP-treated mice showed acute reductions on all populations examined on d 2 (consistent with the global lymphopenia) compared to sham-treated mice, but the numerical recovery of the different Ag-specific CD4 T cell populations 30 d post-CLP was asymmetric (Fig. 3B). Quantitatively, the 2W1S- and Listeria $LLO_{190-201}$-specific CD4 T cell populations were reduced in CLP-versus sham-treated mice, while the number of influenza A virus (IAV) $NP_{311-325}$-specific CD4 T cells increased in CLP-treated mice over sham mice. In contrast, LCMV gp_{66-77}–, HSV-1 $gD_{290-305}$–, and $OVA_{323-339}$–specific CD4 T cell populations recovered (i.e., no statistical difference between sham and d 30 CLP mice). It is important to emphasize that the changes in the individual Ag-specific CD4 T cell populations were not evident when examining bulk CD4 T cells in an Ag-independent manner (Fig. 1). These findings suggest that the loss of peripheral CD4 T cells after sepsis-induced lymphopenia is asymmetric between the individual Ag-specific CD4 T cell populations examined, and that recovery results in the overall changes in the composition of the naïve CD4 T cell pool in sepsis survivors.

Incomplete naïve precursor recovery after sepsis correlates with reduced proliferative capacity and cytokine production during Ag-specific CD4 T cell responses

The magnitude of an Ag-specific CD4 T cell response after priming directly correlates with the size of the precursor pool (29, 45). Seeing the numerical changes in Ag-specific CD4 T cell populations in CLP-treated mice, we examined the impact of septic injury on Ag-specific CD4 T cell responses after secondary heterologous pathogen challenge (Fig. 4A). To test this, we used an Ag-specific approach to track the CD4 T cell response to the model antigen 2W1S expressed in *Candida albicans* (*C. albicans*-2W1S)(23). This design allowed us to model an opportunistic super-infection that is common for sepsis survivors during convalescence (46). When CLP-treated mice were infected with *C. albicans*-2W1S on d 2 after surgery, CD4 T cell responses were significantly reduced compared to sham mice (data not shown), which was not surprising given the dramatic reduction in CD4 T cell numbers at this time point. When mice were inoculated with *C. albicans*-2W1S 30 d after surgery, we still saw a significant reduction in the peak 2W1S-specific CD4 T cell proliferative response in CLP-treated mice compared to sham-treated mice given the same infection (Fig. 4B-C). To see whether the pathogen used influenced the 2W1S-specific CD4 T cell response, sham- and CLP-treated mice were infected with 2W1S expressing attenuated *Listeria monocytogenes* (Lm-2W1S) 30 d after surgery. Assessing the CD4 T cell response to 2W1S or the endogenous $LLO_{190-201}$ epitope of Listeria listeriolysin-O revealed significantly reduced expansion for both Ag-specific CD4 T cell populations in CLP-treated mice compared to sham mice (Fig. 4D-E). We next examined the response in Ag-specific CD4 T cell populations that numerically recovered by 30 d after CLP surgery. After infection with recombinant attenuated Lm-OVA (Fig. 4F-G) or HSV-1 (Fig. 4H-I), we found the

quantitative expansion of $OVA_{323-339}$− or HSV $gD_{290-305}$–specific (47, 48) CD4 T cells was similar regardless of sham or CLP surgery. We also saw no statistical difference in the proliferative capacity of the NP_{311}-specific CD4 T cell population in sham- and CLP-treated mice after intranasal IAV infection (Fig. 4J-K), which was interesting since this Ag-specific CD4 T cell population numerically increased after sepsis-induced lymphopenia (see Fig. 3). However, the majority of the NP_{311}-specific CD4 T cells in uninfected CLP-treated mice adopted a memory phenotype (i.e., $CD44^{hi}$ - data not shown) at d 30 post-surgery. Recent data suggest that naïve CD8 T cells (cognate Ag inexperienced) give rise to more effector CD8 T cells than primary memory CD8 T cells when analyzed on a per-cell-basis after cognate infection (49). While it remains to be tested if the same phenomenon is true for naïve and memory CD4 T cell responses, these results suggest that the phenotype of the CD4 T cells (naïve vs. “memory”-like), in addition to the numbers of cells present, might contribute to the *in vivo* response to cognate Ag recognition. In summary, the data in Figure 4 shows that the proliferative capacity of an Ag-specific CD4 T cell population after sepsis correlates with the degree (reduced vs. complete or increased) of numerical recovery of its naïve precursor population, and the differences seen are intrinsic to the Ag-specific CD4 T cell populations examined and not due to the pathogen used.

We next examined the function of Ag-specific CD4 T cell populations that underwent a sepsis-induced numerical reduction (2W1S-specfic) or not (OVA_{323}-specific) in CLP-treated mice infected with Lm-2W1S or Lm-OVA 2 or 30 d after surgery via *in vivo* peptide restimulation (31) (Fig. 5A), which permits evaluation of cytokine production by an Ag-specific CD4 T cell population with almost no background (Fig. 5B). There was a persistent reduction in frequency and number of $IFN\gamma^+$ (Fig. 5C) or $TNF\alpha^+$ $IFN\gamma^+$ (Fig. 5D) 2W1S-specific CD4 T cells in CLP-treated mice compared to sham controls. In contrast, there was no difference in frequency or number of $IFN\gamma^+$ or $TNF\alpha^+$ $IFN\gamma^+$ $OVA_{323-339}$-specific CD4 T cells (Fig. 5E-G). Since CD4 T cells can adopt different effector phenotypes based on the pathogen encountered, we examined the function of total and 2W1S-specific CD4 T cells after an epicutaneous *C. albicans*-2W infection that primes for a Th17 repsonse (Fig. 6A) (23, 50, 51). There was no significant difference in the frequency and number of $IL\text{-}17A^+$ CD4 T cells from sham and CLP-treated mice after PMA/ionomycin stimulation (Fig. 6B-C). Significant reductions in the frequency and number of 2W1S-specific CD4 T cells from CLP-treated mice making IL-17A were seen, however, after stimulation (Fig. 6B & D). Together, these data demonstrate that (at least) for 2W1S-specific CD4 T cells, sepsis leads to fewer naïve precursors with a reduced capacity to proliferate and make effector cytokines (on a per cell basis) following antigenic stimulation.

Sepsis alters T cell receptor clonotype composition

Data in Fig. 1 and 2 suggest one means by which CD4 T cells recover from sepsis-induced lymphopenia is via homeostatic proliferation. However, homeostatic proliferation is limited in that it can only recreate the peripheral T cell pool from the available diversity. Given that changes have been detected in T cell repertoire diversity in septic patients (52), we analyzed TCRβ variable chain isotype (TCR Vβ) frequencies within the total and 2W1S-specific CD4 T cell populations to determine the extent to which sepsis affects clonotype diversity. Due to the technical limitation of analyzing naïve Ag-specific CD4 T cell populations of ≤200 cells,

we used a method to approximate clonotype diversity of an Ag-specific CD4 T cell population where excess peptide Ag was injected intravenously (29), and the resultant expansion of Ag-specific CD4 T cells was examined 3 d later via p:MHC II tetramer enrichment. To maximize Vβ identification within the Ag-specific population, we used TCR Vβ-specific mAb multiplexed into 2 flow cytometry panels (Fig. 7A). Similar to previous data (19), we did not detect significant changes in the TCR Vβ clonotypes when examined at the total CD4 T cell population level (Fig. 7B & C). While Vβ distribution of 2W1S-specific CD4 T cells in sham-treated mice was consistent with previous data (29), a skewing in a number of 2W1S-specific CD4 T clonotypes (specifically, Vβ 2, Vβ 5.1/5.2, Vβ 6, Vβ 8.1/8.2, and Vβ 10b) from CLP-treated mice was seen (Fig. 7B & D). These data show that an Ag-specific CD4 T cell population that is numerically truncated in a post-sepsis host after recovery also has altered clonal diversity (based on TCR Vβ usage), which could contribute to the reduction in subsequent function of Ag-specific CD4 T cells.

Discussion

Sepsis currently represents an unmet challenge in medicine. Despite modern intensive care practices, mortality from sepsis holds at 30-50% (53). Patients surviving a septic event often have suppressed immune function, a state that is thought to contribute to the increased susceptibility to (and mortality from) secondary nosocomial infections. A number of studies have examined the numerical and functional changes of various immune cell subsets after sepsis, but they have done so at the total population level. The goal of this study was to analyze the quantitative and qualitative changes in CD4 T cells, but at the level of Ag-specific populations, which permits a more rigorous and sensitive analysis of how these cells perform under various immunological settings. With this in mind, we have for the first time (to our knowledge) performed quantitative and qualitative analyses of multiple Ag-specific CD4 T cell populations in septic mice, before and after secondary heterologous infections. Our data demonstrate that a septic event induces deletion within each endogenous Ag-specific CD4 T cell population examined, and that this event is followed by a recovery of the Ag-specific repertoire in an irregular, or asymmetric, fashion. Moreover, our results show that sepsis-induced numerical changes to certain Ag-specific CD4 T cell populations can affect the function of the cells in question during the subsequent response to a pathogenic challenge.

Clearly defining the mechanism by which lymphocyte apoptosis occurs after sepsis is difficult, since no single intrinsic or extrinsic pathway dominates (54, 55). Similarly, there has been limited investigation into the mechanism(s) behind lymphocyte recovery after a septic event. The sole publication examining the process of CD4 T cell recovery following septic injury suggested that CD4 T cells did not undergo homeostatic proliferation during recovery from sepsis (19). This conclusion was largely reached by adoptively transferring a large number of TCR-tg CD4 (OT-II) T cells into septic mice 7 d after surgery. Even though these cells were introduced into a lymphopenic environment, it could be argued that since these CD4 T cells did not “experience” the septic event any T cell-intrinsic changes that occur during sepsis would not be present in these cells. Furthermore, it is clear that adoptive transfer experiments that utilize nonphysiologically large input numbers of TCR-tg T cells do not accurately recapitulate the endogenous Ag-specific T cell response (56). In contrast,

the similar recovery of CD4 T cell numbers in thymectomized and euthymic mice, along with the similar rates of BrdU incorporation in CD4 T cells of CLP-treated euthymic and thymectomized mice alike suggest CD4 T cells do indeed undergo homeostatic proliferation after septic injury. Unsinger et al. then went on to show increased frequencies of CD4 T cells in septic mice with "activated" ($CD69^{+}$) and "memory" ($CD44^{hi}$ $CD62L^{lo}$) phenotypes (19), leading them to suggest that the majority of activated and memory CD4 T cells arise from endogenous sources. Our data in Fig. 3 are consistent with these findings by Unsigner et al., but extend them to identify cells with phenotypes consistent with "homeostatic proliferation" for both endogenous CD4 T cells and adoptively transferred TCR-tg CD4 T cells (37, 42). It remains to be determined what the driving factor(s) is for the numerical recovery of CD4 T cells after sepsis. In addition to recovery by homeostatic proliferation, it is likely that some CD4 T cell populations respond directly to antigenic epitopes present in the proteins expressed by the various commensal bacterial species within the gut or to self-Ag, leading to a difference in functional potential compared to those CD4 T cells truly undergoing Ag-independent homeostatic proliferation. The cecum contains a high concentration of microbes that are a combination of Gram-positive and Gram-negative bacterial species, and the Ag expressed by these bacteria can be recognized by T cells and can drive effector responses – despite being commensal bacteria (57). It is tempting to speculate that the above-normal numerical recovery in the NP_{311}-specific CD4 T cell population in CLP-treated mice is due to direct antigenic stimulation as a result of cross-reactivity with some yet-to-be defined epitope expressed by the gut commensal bacteria, especially since the majority of NP_{311}-specific CD4 T cells in CLP-treated mice were also $CD44^{hi}$ (data not show). As a result, this population of cells could be considered a "memory" population with different functional characteristic, such as producing fewer effectors after influenza infection (49), over true "naïve" cells. The bacterial constituents of the gut microbiome are unique to each individual (especially humans), and can be strongly influenced by a variety of factors (58). Consequently, the extent of recovery and function of a particular Ag-specific T cell population after a septic event can be easily different as a result of the intestinal "health" of the individual, regardless of possible genetic similarities (e.g., same mouse strain from different vendors). As reagents become available to track CD4 T cell populations specific to Ag expressed by specific gut commensal bacteria, it will be interesting to investigate the potential impact of this component of polymicrobial sepsis on the recovery and function of such Ag-specific CD4 T cell populations in septic mice.

When examined at the bulk CD4 T cell level, our data are consistent with a number of other studies demonstrating sepsis-induced changes in the basic numerical and functional characteristics of CD4 T cells. It is important to emphasize that the sepsis-induced changes in the different Ag-specific CD4 T cell populations would not have been identified had we examined CD4 T cells as a whole. This includes the changes in naïve precursor numbers, proliferative capacity, and cytokine production by the different Ag-specific populations. While we recently showed that sepsis significantly deceases the Ag sensitivity of memory CD8 T cells (35), it remains to be determined to what extent Ag sensitivity is affected in naïve or memory Ag-specific CD4 T cell populations. In addition, TCR Vβ repertoire usage on CD4 T cells from sham- and CLP-treated mice was also investigated previously, where the authors found no skewing of the repertoire toward one particular Vβ subtype (19).

However, this conclusion was based on analyzing bulk CD4 T cells. Just as we only observed stochastic changes in the number of naïve CD4 T cells when we examined Ag-specific CD4 T cell populations using p:MHC II tetramers, alterations in Vβ repertoire usage after sepsis were observed when examining the endogenous 2W1S-specific CD4 T cell population. We realize that the method for examining Vβ repertoire usage required *in vivo* $2W1S_{56-68}$ peptide immunization to expand this Ag-specific population of cells, but it is important to emphasize that this technique results in an expanded T cell population that is reflective of the clonotype diversity of the naïve starting population (29). Even after the expansion, 2W1S:I-A^b tetramer enrichment and the multiplexed flow cytometry panel of Vβ-specific mAb were needed to complete the analysis of the endogenous 2W1S-specific CD4 T cell population. These results show the power of using these reagents and techniques to analyze small numbers of endogenous Ag-specific CD4 T cell populations.

Pools of Ag-specific CD4 T cell "precursors" are maintained in the periphery by frequent, low-level signals from self-Ag:MHC II and cytokines (most notably, IL-7 for naïve CD4 T cells (59) and IL-15 for naïve CD8 T cells (60)). The increased availability of these resources turns survival signals into mitogenic stimuli that restores T cell numbers through proliferative expansion in situations where T cell numbers drop acutely (61). With this in mind, there is considerable effort being spent identifying therapeutic strategies designed to enhance T cell recovery and function after sepsis, and the administration of agents that promote lymphocyte proliferation (e.g., IL-2, IL-7, and IL-15 (62-64)) or block the function of inhibitory molecules (e.g., PD-1 (65, 66) and CTLA-4 (67)) are producing encouraging results. For example, administration of IL-7 to septic mice shortly after sepsis induction can prevent T cell apoptosis and restore function (63, 64). Moreover, disruption of the PD-1:PD-L1 signaling pathway improves survival in animal models of sepsis (68, 69), and reverses T cell exhaustion in sepsis patients (66). Additional work is needed to determine the impact of such therapies at the level of Ag-specific T cell populations where, even if apparently subtle, physiologically meaningful changes may actually be present.

In the current study, we have assessed CD4 T cell function after sepsis primarily within the context of a "Th1" response, but it is important to emphasize that sepsis likely affects other CD4 T cell subsets needed for a variety of other immunological responses. For example, Th1 cells also provide necessary signals for B cell isotype switching (70), and ILβ-4 from Th2 cells also facilitates B cell isotype switching to IgG1 and IgE (71). Th17 cells are important in immunity to extracellular fungal and bacterial pathogens (72), and we saw a reduction in the Th17 response when using a secondary epicutaneous *C. albicans* after sepsis (Fig. 6). Thus, the loss or improper function of CD4 T cell responses is detrimental for immunity to a wide range of pathogens, especially those that frequently cause secondary infections in septic patients. In addition to the different "effector" CD4 Th subsets, $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{reg}) have been found at an increased frequency in septic patients, especially early after diagnosis (73-75). It was subsequently determined that the increased frequency of T_{reg} was the consequence of decreases in the effector CD4 T cell populations (76), suggesting that T_{reg} are more resistant to sepsis-induced apoptosis than conventional CD4 T cells (16). Regardless, the role of T_{reg} cells in the immunosuppression after sepsis has not been rigorously investigated and merits study. We also realize that the

sepsis-induced alterations in the Ag-specific CD4 T cell populations we examined could be due to CD4 T cell-intrinsic and/or -extrinsic factors. As CD4 T cells stimulation require Ag presentation from professional APC, changes in the number and/or function of DC could contribute to the observed modulation in CD4 T cell function (77-79).

The development and integration of a number of reagents and techniques over the last decade has permitted the characterization of Ag-specific endogenous naïve and memory T cell responses to a handful of experimental bacterial (such as *L. monocytogenes*) or viral (such as LCMV) pathogens in exquisite detail. These features have given us the ability to track the quantity and quality of endogenous CD4 T cells reactive to antigenic epitopes within these pathogens. Yet, one weakness of these (and other well-characterized) experimental pathogens is that they are typically not seen as nosocomial infection threats for septic patients. Most septic patients potentially face complications arising from secondary infection by the extracellular pathogens *Candida*, *Pseudomonas*, and *Staphylococcus* (46, 80, 81), and these pathogens have been used most often to examine alterations in animal survival in experimental models of sepsis. It is important to keep in mind that our use of Lm-2W for most of the studies was to take advantage of the wealth of information known regarding the infectivity/pathogenicity of this pathogen, the characteristic CD4 T cell response that it elicits, and the availability of reagents to critically investigate different aspects of this response. Moreover, while our analysis of sepsis-induced alterations in the responsiveness of 2W1S-specific CD4 T cells was largely performed after secondary infection with attenuated Lm-2W, we found the similar reductions in proliferative capacity of 2W1S-specific CD4 T cells after infection with *C. albicans*-2W (Fig. 4). These data serve as a starting point for future studies using pathogens that commonly plague sepsis patients once the reagents are developed to following CD4 T cell populations that are specific for Ag within these pathogens.

In summary, the data we presented here elucidates with detail how CD4 T cells are affected by sepsis. Our results reveal that after a septic event the recovery of individual Ag-specific CD4 T cell populations is skewed, which is an observation that is not evident when examining the bulk CD4 T cell pool. In addition, we show that an incomplete recovery of the Ag-specific T cell repertoire after sepsis stems from the attrition of Ag-specific TCR diversity, and that this phenomenon correlates with altered CD4 T cell responses long after sepsis. Ultimately, this study increases our collective understanding of why septic patients more easily acquire secondary infections.

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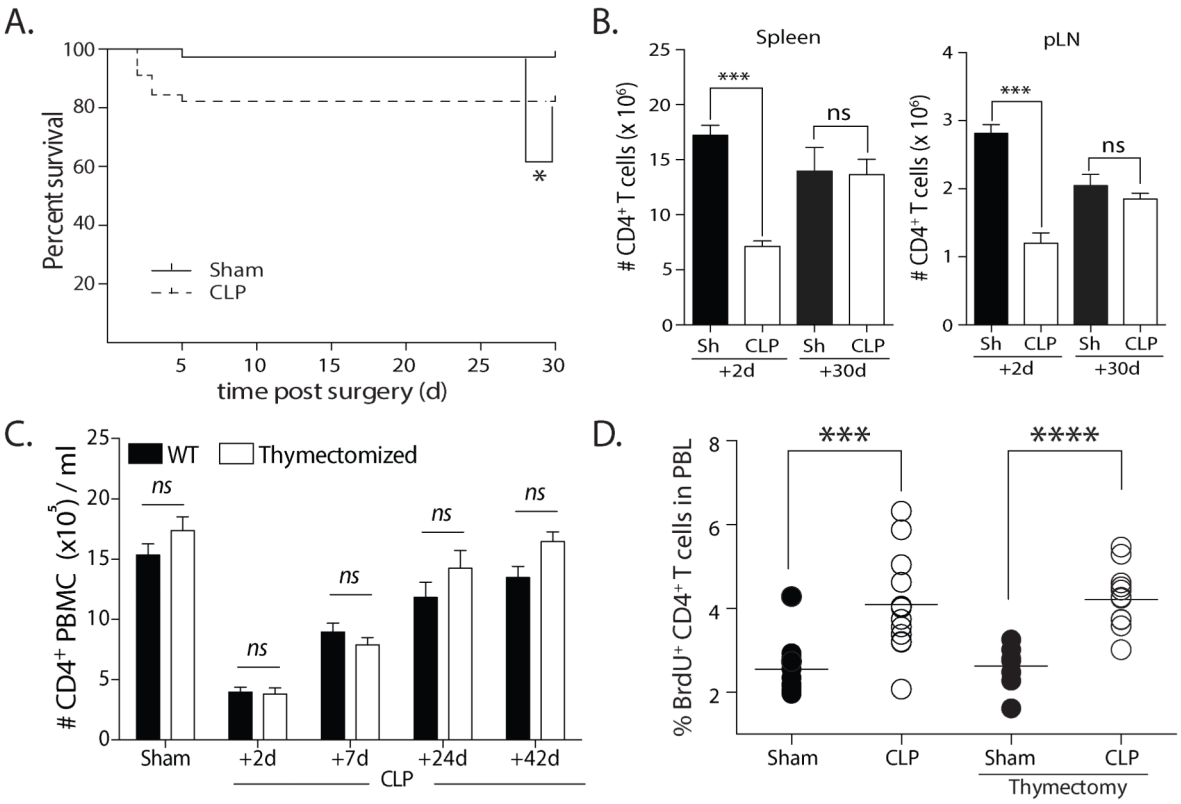

FIGURE 1. Numerical recovery of CD4 T cells after cecal ligation and puncture (CLP)-induced sepsis occurs by a thymus-independent mechanism

A. Kaplan-Meier survival curve of experimental cohorts after undergoing sham or CLP surgery. **B.** Number of CD4 T cells in spleen and inguinal lymph nodes (pLN) on d 2 and 30 after sham or CLP surgery. **C.** Thymectomized and euthymic mice underwent CLP surgery, and the number of CD4 T cells in the peripheral blood was measured over time. **D.** Thymectomized and euthymic mice underwent sham or CLP surgery. BrdU was injected i.p. 6 d later, and the frequency of peripheral blood CD4 T cells incorporating BrdU was determined 24 h later. Statistical significance was determined using Mann-Whitney U test (**A**) or one-way ANOVA (**B-D**) with multiple-testing correction using the Holm-Sidak method, and $\alpha = 0.05$, when deemed appropriate. **** $p < 0.001$; *** $p < 0.005$; * $p < 0.05$; and n.s. – not significant. Data shown are representative of at least 2 independent experiments of 4-5 mice/group in each experiment.

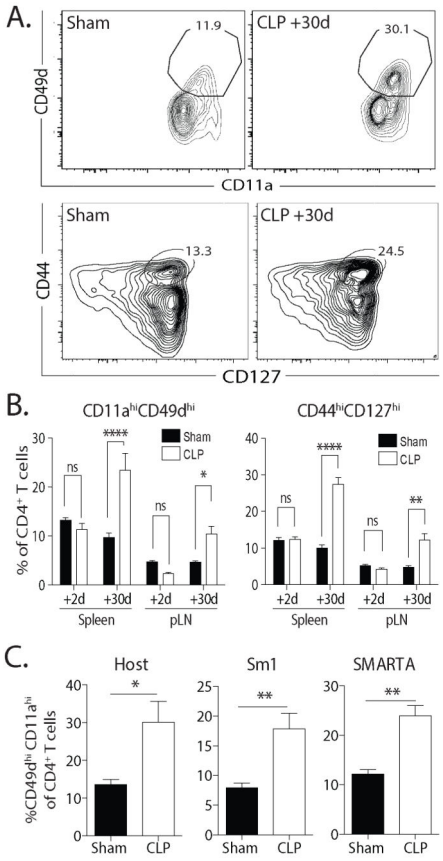

FIGURE 2. Numerical recovery of CD4 T cells after sepsis is accompanied by the acquisition of an "Ag-experienced" phenotype in an Ag-independent manner

A. The phenotype of CD4 T cells in the spleen was assessed 30 d after sham or CLP surgery. Representative flow cytometry plots depicting CD11a/CD49d and CD44/CD127 expression in CD4 T cells 30 d after sham or CLP surgery. **B.** Frequency of $CD11a^{hi}$ $CD49d^{hi}$ and $CD44^{h}$ $CD127^{hi}$ CD4 T cells 2 and 30 d after sham or CLP surgery. **C.** Salmonella $FliC_{447-458}$-specific Sm1 (CD90.1/CD90.1; 5×10^5/mouse) and LCMV gp_{61-77}-specific SMARTA (CD90.1/CD90.2; 10^6/mouse) CD4 T cells were adoptively transferred together into naïve CD90.2 B6 mice 1 d before sham or CLP surgery. After 30 d, the frequency of $CD11a^{hi}$ $CD49d^{hi}$ endogenous CD90.2/CD90.2 CD4 T cells and adoptively transferred TCR-tg CD4 T cells was determined. Statistical significance was determined using one-way ANOVA with multiple-testing correction using the Holm-Sidak method, and $\alpha = 0.05$, when deemed appropriate. **** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; and n.s. – not significant. Data shown are representative of at least 2 independent experiments of 4-5 mice/group in each experiment.

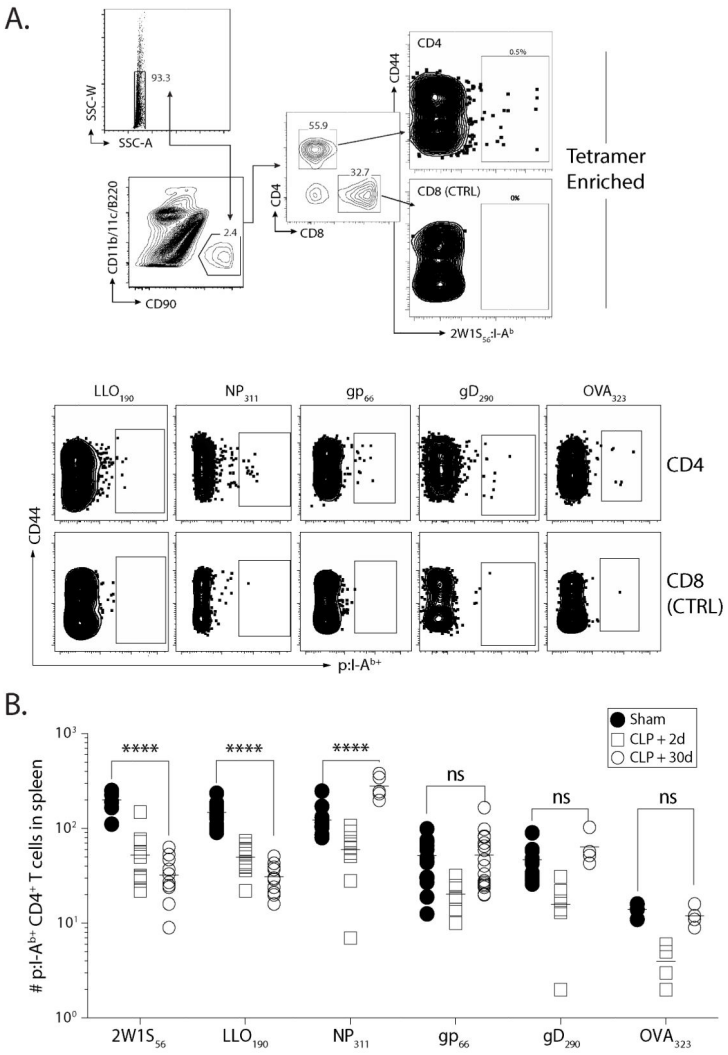

FIGURE 3. Numerical recovery of CD4 T cells after sepsis is accompanied by asymmetric changes in Ag-specific repertoire heterogeneity

Mice underwent sham or CLP surgery, and the number of Ag-specific CD4 T cells specific for 2W1S, *L. monocytogenes* $LLO_{190\text{-}201}$, influenza A virus $NP_{311\text{-}325}$, LCMV $gp_{66\text{-}77}$, HSV $gD_{290\text{-}302}$, and $OVA_{323\text{-}339}$ was determined 2 and 30 d later using p:I-A^b tetramer enrichment. **A.** Representative flow plots showing gating strategy used in tetramer-enriched cell fractions to detect the frequency of Ag-specific CD4 T cell populations. Shown is an example used to detect 2W1S:I-A^b-specific $CD4^+$ T cells. Gating for p:I-A^b-specific cells was determined using $CD8^+$ T cells as an internal negative control for tetramer binding. **B**. Number of Ag-specific, naïve CD4 T cell precursors across the 6 epitopes in sham- and CLP-treated mice 2 or 30 d after surgery. Statistical significance was determined using group-wise, one-way ANOVA with multiple-testing correction using the Holm-Sidak method, and $\alpha = 0.05$. **** $p < 0.001$; and n.s. – not significant. Data shown are the combined results from 2-4 independent experiments per population analyzed, with 3-5 mice/group in each experiment.

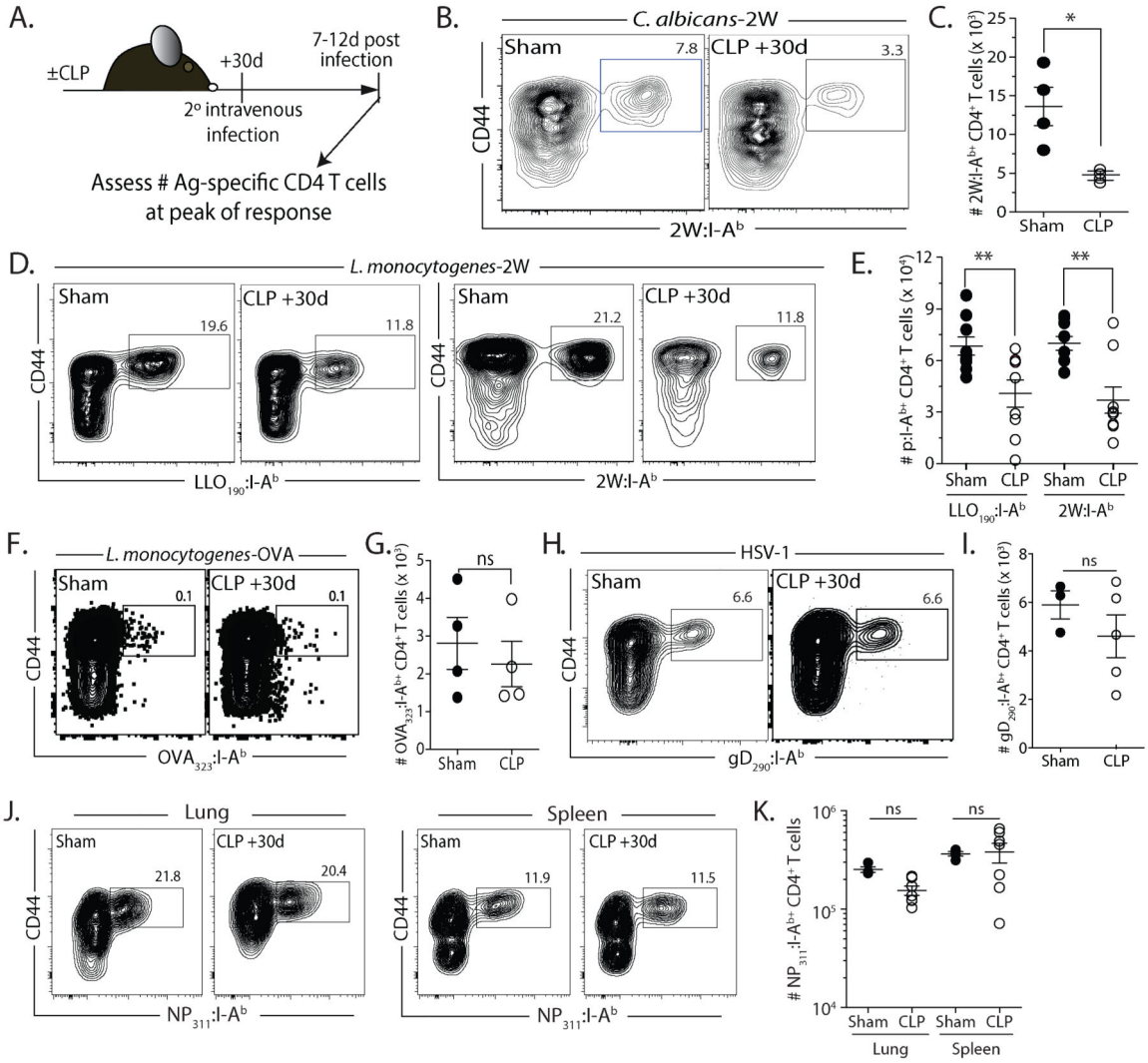

FIGURE 4. Expansion of epitope-specific populations correlates with precursor pool recovery after septic injury

A. Experimental design. Mice were infected with 2W1S-expressing *C. albicans* (*C. albicans*-2W; 5×10^4 yeasts in 0.1 ml i.v.), attenuated 2W-expressing *L. monocytogenes* (Lm-2W or Lm-OVA; 10^7 CFU in 0.1 ml i.v.), HSV-1 (2.5×10^4 PFU in 0.1 ml i.v.), or influenza A virus (×31; 3000 EID50 in 0.02 ml i.n.) 30 d after sham or CLP surgery. After another 7-12 d, the frequency and number of Ag-specific CD4 T cells was determined in the spleen. **B-C.** Representative flow plots showing the frequency (**B**) and number (**C**) of 2W1S-specific CD4 T cells in the spleens from sham- and CLP-treated mice 7 d after i.v. infection with *C. albicans*-2W. **D-E.** Representative flow plots showing the frequency (**D**) and number (**E**) of LLO_{190}- and 2W1S-specific CD4 T cells in the spleens from sham- and CLP-treated mice 7 d after i.v. infection with Lm-2W. **F-G.** Representative flow plots showing the frequency (**F**) and number (**G**) of OVA_{323}-specific CD4 T cells in the spleens from sham- and CLP-treated mice 7 d after i.v. infection with Lm-OVA. **H-I.** Representative flow plots showing the frequency (**H**) and number (**I**) of gD_{290}-specific CD4 T cells in the spleens from sham- and CLP-treated mice 9 d after i.v. infection with HSV-1. **J-K.**

Representative flow plots showing the frequency (**J**) and number (**K**) of NP_{311}-specific CD4 T cells in the lungs and spleens from sham- and CLP-treated mice 12 d after i.n. infection with ×31. Statistical significance was determined using group-wise, one-way ANOVA analyses followed by multiple-testing correction using the Holm-Sidak method, with $\alpha = 0.05$. ** $p < 0.01$; * $p < 0.05$; and n.s. – not significant. Data shown are the combined results from 2-4 independent experiments per pathogen tested, with 3-5 mice/group in each experiment.

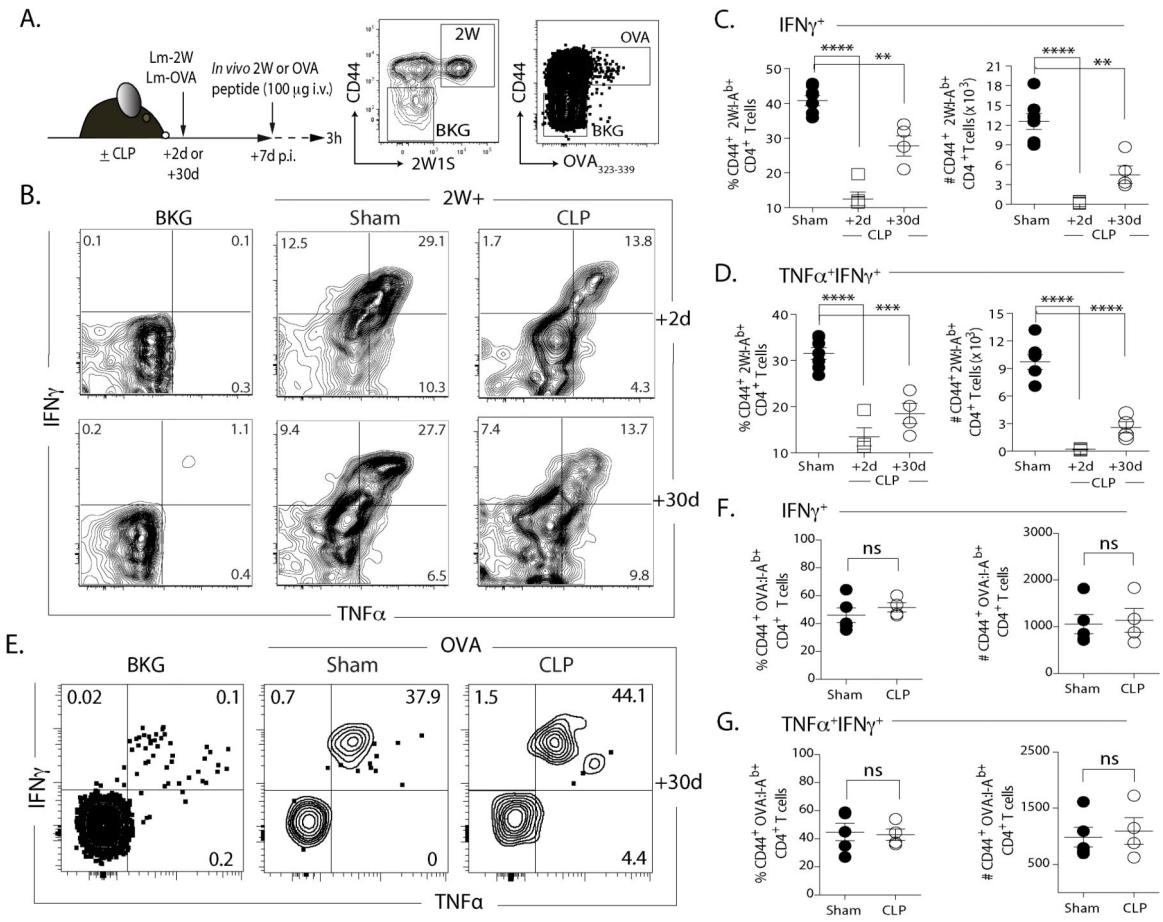

FIGURE 5. Inadequate recovery of precursor population correlates with inadequate CD4 T cell function after pathogen challenge

A. Experimental design. Mice were infected with attenuated 2W- or OVA-expressing *L. monocytogenes* (Lm-2W or Lm-OVA; 10^7 CFU in 100 µl i.v.) 2 or 30 d after sham or CLP surgery. After another 7d, the mice were injected i.v. with 100 µg $2W1S_{56\text{-}68}$ or $OVA_{323\text{-}339}$ peptide. Spleens were harvested 2 h later, and the frequency and number of $IFN\gamma^+$ or $TNF\alpha^+IFN\gamma^+$ $CD44^+2W{:}I\text{-}A^{b+}$ or $OVA_{323}{:}I\text{-}A^{b+}$ CD4 T cells was determined. **B.** Representative flow plots of intracellular IFNγ and TNFα detection in the $CD44^+2W{:}I\text{-}A^{b+}$ CD4 T cells after *in vivo* peptide restimulation. Plots show cells gated from internal control populations ($CD44^{lo}2W{:}I\text{-}A^{b-}$ CD4 T cells) denoted as background ("BKG") or $2W{:}I\text{-}A^{b}$-enriched CD4 T cells from sham- or CLP-treated mice. **C-D.** Frequency and number of $CD44^+2W{:}I\text{-}A^{b+}$-specific CD4 T cells in the spleen producing IFNγ (**C**) or TNFα and IFNγ (**D**). **E.** Representative flow plots of intracellular IFNγ and TNFα detection in the $CD44^+OVA_{323}{:}I\text{-}A^{b+}$ CD4 T cells after *in vivo* peptide restimulation. Plots show cells gated from internal control populations ($CD44^{lo}OVA_{323}{:}I\text{-}A^{b-}$ CD4 T cells) denoted as background ("BKG") or $OVA_{323}{:}I\text{-}A^{b}$-enriched CD4 T cells from sham- or CLP-treated mice. **F.** Frequency and number of $CD44^+$ $OVA_{323}{:}I\text{-}A^{b+}$-specific CD4 T cells in the spleen producing TNFα and IFNγ. Statistical significance was determined using group-wise, one-way ANOVA analyses followed by Holm-Sidak correction with $\alpha = 0.05$. **** $p < 0.001$;

*** $p < 0.005$; and ** $p < 0.01$. Data shown are the combined results of 2 independent experiments, with 3-5 mice/group in each experiment.

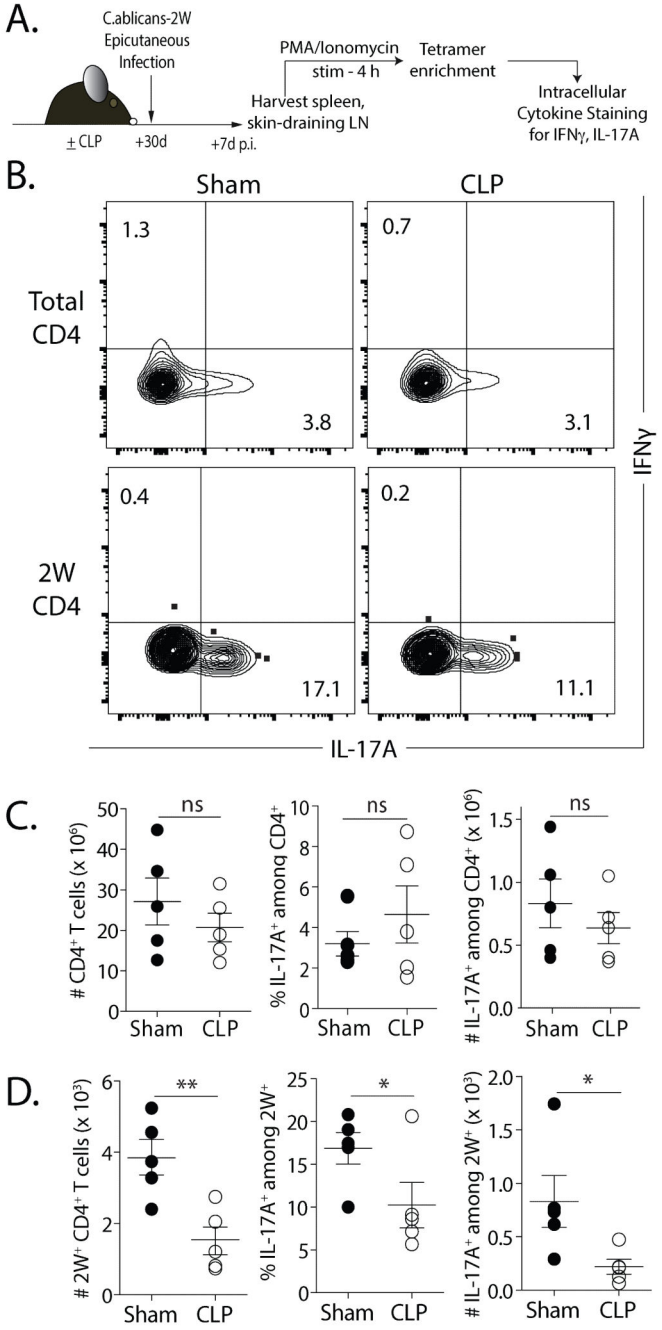

FIGURE 6. Ag-specific CD4 T cells have functional deficits in Th17-polarized responses

A. Experimental design. On d 30 after sham or CLP surgery, mice were infected epicutaneously with 2W1S-expressing *C. albicans* (*C. albicans*-2W; 10^8 yeasts in 0.05 ml). After 7 d, lymphocytes obtained from the skin-draining (inguinal, brachial, axillary and cervical) LN of infected mice were stimulated for 4 h with PMA/ionomycin. The stimulated samples were enriched for 2W1S-specific CD4 T cells and production of IFNγ and IL-17A was assayed by flow cytometry. **B.** Representative flow plots showing the gating strategy to identify IL-17A$^+$IFNγ$^-$ cells within bulk and 2W:I-A^b-specific CD4 T cells. **C-D.**

Frequency and number of IL-17A$^+$ in bulk (C) and 2W1S-specific CD4 T cells (D) in infected sham- or CLP-treated mice. Statistical significance was determined using Welch"s t-test. ** $p < 0.01$; * $p < 0.05$; and n.s. – not significant. Data shown are representative results from 2 independent experiments, with 5 mice/group in each experiment.

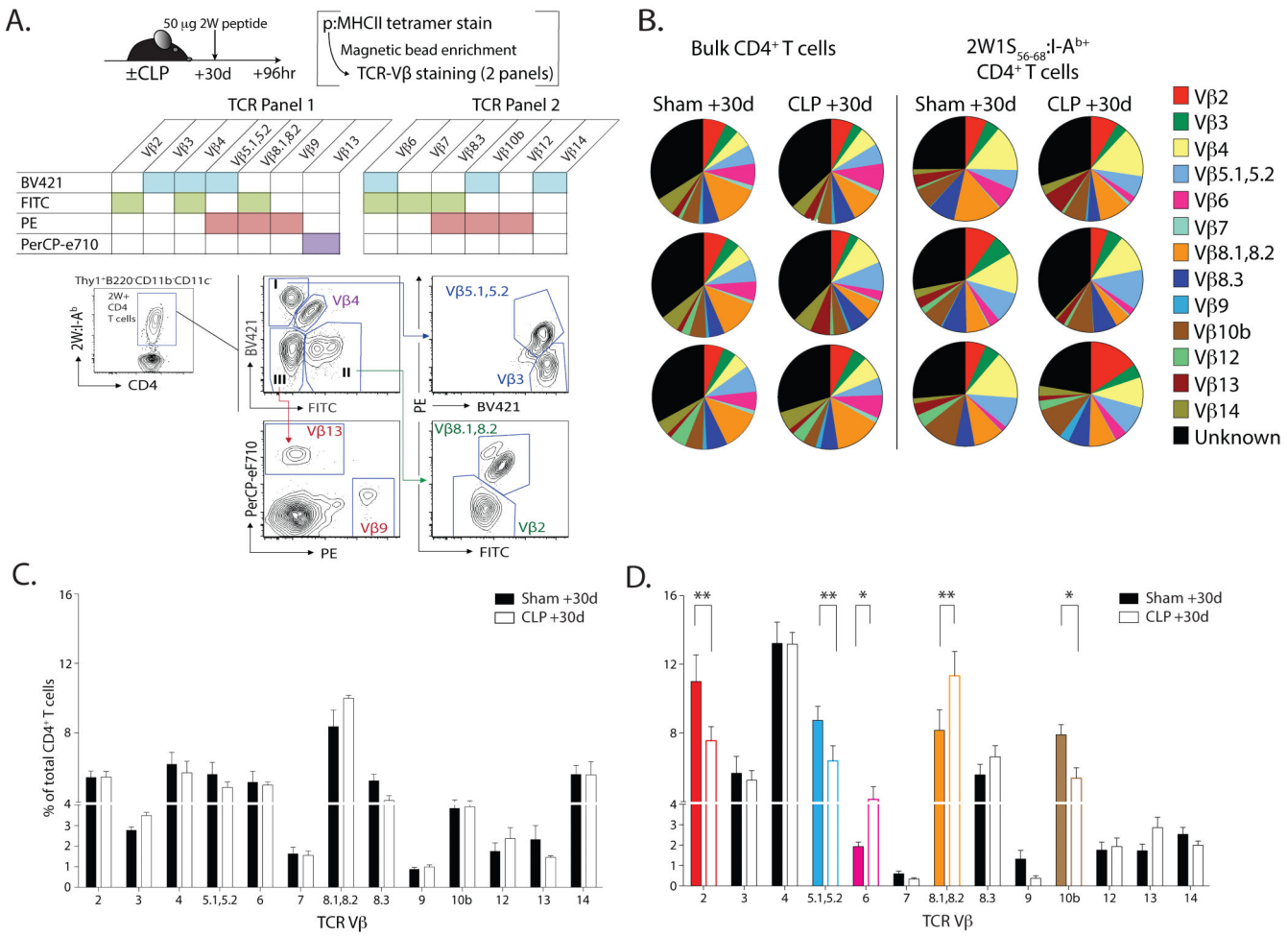

FIGURE 7. Sepsis alters the T cell receptor clonotype composition of Ag-specific CD4 T cell population

A. Experimental design. Mice were injected i.v. with 50 μg $2W_{56-68}$ peptide (along with LPS) 30 d after sham or CLP surgery. Splenocytes were harvested 4 d later and tetramer-enriched as previously described. The resultant sample was then used to determine the clonotype composition using two multiplexed flow cytometry panels consisting of the indicated murine TCR Vβ mAb. **B.** Representative flow plots showing the gating strategy to identify Vβ usage on 2W:I-A^b-specific CD4 T cells using TCR panel #1. **C.** Usage profile for TCR Vβ gene segments in total 2W:I-A^b-specific CD4 T cells from 3 representative individual sham- or CLP-treated mice. **D.** Averaged frequency of TCR Vβ of 2W:I-A^b-specific CD4 T cells in sham- or CLP-treated mice. Statistical significance was determined using group-wise, one-way ANOVA analyses followed by Holm-Sidak correction with $\alpha = 0.05$. ** $p < 0.01$; and * $p < 0.05$. Data are combined from 2 independent experiments, each having 5 mice/group.