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Alterations in Antigen-Specific Naive CD4 T Cell Precursors after Sepsis Impairs Their Responsiveness to Pathogen Challenge

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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–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 naive 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, with regard to both magnitude of expansion and functional capacity on a per-cell basis, which also correlated with intrinsic changes in Vβ clonotype heterogeneity. Our results demonstrate that 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. The Journal of Immunology, 2015, 194: 000–000.

CD4 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. CD4 T cells are able to function in such an array of immunological settings because effector CD4 T cells can take on different phenotypes [i.e., Th1, Th2, Th9, Th17, follicular Th (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. Because of 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 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 the numbers of immune cells, including T cells. Although 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 its immunosuppressive phase (19). However, there are knowledge gaps regarding the mechanism(s) driving this CD4 T cell recovery, the quality/functional diversity 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-MHCII) tetramer-enrichment technology (20) to examine quantitative shifts within the endogenous naive Ag-specific CD4 T cell repertoire at different time points after sepsis. Our findings suggest that the nu-
merical restoration of the CD4 T cell repertoire after sepsis occurs via a peripherally driven mechanism that is, in part, independent of Ag availability. Although 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 for mortality) are discussed.

Materials and Methods

Mice

Euthymic and thymectomized C57BL/6 (B6) mice were purchased from The National Cancer Institute. Thy1.1/1 TCR-transgenic (tg) SMARTA (lymphocytic choriomeningitis virus [LCMV] gp34,5-specific) and SM1 (Staphylococcus typhimurium FlcC25,26-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 ≥4 wk, regardless of their source. Animal procedures were performed according to National Institutes of Health guidelines and were 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, the abdomen was shaved and 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 extend a small amount of cecal content. The cecum was returned to the peritoneal cavity and the peritoneum was closed with 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 bupivacaine 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 CLP.

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 day 6 after surgery. Blood was collected at the indicated times, followed by RBC lysis in ACK buffer. Cells were surface stained with PE CD4 (clone GK1.5; BioLegend, San Diego, CA), after which they were fixed with Cytofix/Cytoperp 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 naive SMARTA or SM1 mice. Contaminating memory phenotype (CD44hiCD11aCD49d) TCR-tg cells were consistently <5%. The purified cells were transferred to naive B6 mice 1 d before sham or CLP surgery.

Experimental pathogens and infections

2W1S (EAWGALANAVWDSA), expressing ActAΔ Listeria monocytogenes (attenuated Lm-2W1S; 107 PFU/mouse) or OVA233–339 (ISQAVHAHAHAEINAGR)-expressing ActAΔ L. monocytogenes (attenuated Lm-OVA; 107 PFU/mouse) was grown and infected i.v., as previously described (22). 2W1S-expressing Candida albicans (Ca-2W1S) was derived from clinical isolate SC5314 (23). Ca-2W1S was grown to log phase (OD600 of 1.5) in YPD medium, washed, and counted by hemo- cytometer. Ca-2W1S was resuspended at a concentration of 5 × 106 yeast/ml i.v. challenge. Recombinant Lm-2W1S and Ca-2W1S were obtained from Drs. Marc Jenkins and Daniel Kaplan (University of Minnesota), respectively. For HSV-1 (KOS strain) and influenza A virus (IAV; strain X31) inocula, frozen viral stocks were thawed, and 2.5 × 106 PFU and 3000 EID50 units, respectively, was administered per mouse. Viral stocks were obtained from culture conditions that were described previously (24–26). Intravenous challenges using 0.1 ml injection volumes were used for all of the inoculants described, with the exception of IAV X31, which was given intranasally (i.n.) using 0.02 ml aliquots/nosritil. Infected mice were housed under the appropriate biosafety level.

Tetramers and peptides

I-Ab-specific tetramers containing 2W1S (EAWGALANAVWDSA), (FYVKPVYKSV), (LLQYQAYQFNVS) or OVA233–339 (ISQAVHAHAHAEINAGR) were obtained from Dr. Marc Jenkins. Biotinylated soluble I-Ab molecules containing HSVgp D (gD296–305) (IPPNWHISPQDA) or IAV nucleoprotein (NP)113–125 (QVYSLLRPENPAHK) peptides were obtained by Bio-Synthesis (Lewisville, TX). Peptides used to elicit cytotoxic production or expand endogenous Vβ repertoire for quantification were synthesized by Bio-Synthesis (Lewisville, TX).

Quantitation of endogenous Ag-specific CD4 T cell populations using p-I-Ab 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-Ab tetramers was used. Briefly, spleens were harvested for each mouse analyzed, a single-cell suspension was prepared, and allophycocyanin- and PE-conjugated tetramers were added at a 1:40 dilution in tetramer staining buffer (PBS containing 5% bovine calf serum [BCS], 2 mM EDTA, and 50 μM dapsulfobulin, 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 resuspended in 0.2 ml FACS Buffer, mixed with 0.05 ml each anti-CD4 and anti-PE mAb-conjugated magnetic MicroBeads (Miltenyi Biotec), 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 Biotec) 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 stained with a mixture of fluorochrome-labeled mAb (see later discussion). Cell numbers for each sample were determined using AccuCheck Counting Beads (Invitrogen). Samples were analyzed using an LSR II flow cytometer (BD) and FlowJo software (TreeStar, Ashland, OR). The percentage of tetramer+ 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-IFN-γ and anti-TNF mAbs. To specifically examine the function of Ag-specific Th17 cells, sham and CLP-treated mice were infected 30 d after surgery with Ca-2W1S epicutaneously (23). On day 7 postinfection, the spleen and skin-draining (inguinal, brachial, axillary, and cervical) lymph nodes (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 1640 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, 1-ml aliquots from the enriched and flow-through fractions were suspended in fixation/permeabilization buffer for 20 min at 4°C, and then stained for intracellular IFN-γ and IL-17 accumulation overnight in permeabilization buffer (both from eBioscience). After staining, cells were resuspended in FACS buffer with 0.1% 0.02 m sodium citrate, and 0.1% 0.02 sodium azide (Bell Easy) was 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% NaN3). For
some experiments, the cells were fixed with PBS containing 2% paraformaldehyde. In procedures requiring intracellular staining, cells were permeabilized following surface staining using the transfection factor staining kit (eBioscience), stained for 1 h at 4°C with a second set of fluorochrome-conjugated mAbs, and suspended in FACS buffer for acquisition. The following fluorochrome-conjugated mAbs were used for both surface and intracellular staining: Horizon V500 Th1.2 (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), Alexa Fluor 700 CD44 (clone IM7; BioLegend), allophycocyanin and BV421 IL-17A (clone TC11-18H10.1; BioLegend), allophycocyanin and BV650 IFN-γ (clone XMG1.2; BioLegend), PE-Cy7 IL-2 (clone JES6-5H4; BioLegend), Alexa Fluor 647 CD49d (clone R1-2; BioLegend), PerCP-Cy5.5 B220 (clone RA3-6B2; eBioscience), PerCP-Cy5.5 CD11b (clone M1/70; eBioscience), PerCP-Cy5.5 CD11c (clone N418; eBioscience), PerCP-Cy5.5 F4/80 (clone BM8; eBioscience), FITC Foxp3 (clone FJK-15S; eBioscience), FITC CD11a (clone M17/4; eBioscience), FITC TNF-α (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 was performed using a modification of a previously reported method (29, 32). Briefly, sham- or CLP-treated mice were injected with 50 μg 2W18 peptide and 5 μg LPS on day 30 postsurgery. Four days later, splenic T cells were enriched for allophycocyanin–2W1S:I-Ab tetramer-binding cells. The enriched population was subsequently divided into two equal aliquots and stained for surface markers along with 13 available TCR Vβ multiplexed onto four separate flow cytometry detection channels, using directly conjugated Abs and/or biotinylated mAbs detected afterward with Streptavidin-BV421 (BioLegend). The TCR Vβ mAbs used were FITC-conjugated mAb against mouse Vβ2 (clone B20.6), Vβ4 (clone KT4), Vβ6 (clone RR4.7), Vβ7 (clone TR310), Vβ8.1/8.2 (clone KJ16-133.18), and Vβ8.3 (clone 8C1); PE-conjugated mAb against Vβ3.1/5.2 (clone MR9-4), Vβ8.1/8.2 (clone MR5-2), Vβ8.3 (clone I3B3.3), and Vβ9 (clone MR10-2), and Vβ10 (clone B21.5); PerCP-eFluor 710–conjugated Vβ13 (clone MR12-3); and biotinylated mAb against Vβ3 (clone KJ25), Vβ4, Vβ5.1/5.2, Vβ6, Vβ10, and Vβ14 (clone 14-2). Unless otherwise specified, mAbs that detected the same TCR Vβ clonotype were from the same clone and vendor. All mAbs used to detect Vβ clonotype distribution were purchased from 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. 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 thymus-independent mechanism**

Sepsis can be investigated experimentally using the CLP model (21), which is used frequently 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 symptoms that are 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 LNs, and blood 2 d after septic injury, and a numerical recovery was apparent by day 30 (Fig. 1B, 1C). 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.

**FIGURE 1.** Numerical recovery of CD4 T cells after 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 LNs (pLN) on days 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 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. Data shown are representative of at least two independent experiments, with four or five mice/group in each experiment. *p < 0.05, **p < 0.005, ***p < 0.001, Mann-Whitney U test (A) or one-way ANOVA (B–D), with multiple-testing correction using the Holm–Sidak method, and α = 0.05, when deemed appropriate. ns, not significant.
CD4 T cell recovery is not usually dependent on thymic-derived T cells in models of experimentally induced lymphopenia, because the export rate of naive T cells from the thymus is not modulated by perturbations in the periphery (36). In contrast, work by Unsinger et al. (17) suggested that CD4 T cells did not homeostatically proliferate when transferred into CLP-treated recipients. These contradictory findings led us to examine the number of CD4 T cells in the blood of sham- and CLP-treated euthymic (wild-type [WT]) and thymectomized mice. CD4 T cell loss and recovery were similar in WT and thymectomized mice, with no statistically significant difference at any of the time points analyzed (Fig. 1C). Next, sham- and CLP-treated WT and thymectomized mice were given BrdU on day 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 sham, 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 naive 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, as indicated by an increased frequency of CD11ahiCD49dhi CD8 T cells compared with 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 coexpression 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 CD11ahiCD49dhi CD44hi CD4 T cells among the total CD4 T compartment (Fig. 2A, 2B). CD44 and CD127 coexpression also was suggested to be a phenotype for CD4 T cells that have undergone similar processes, but several phenotypes have been suggested. For example, CD11a and CD49d coexpression 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 CD11ahiCD49dhi CD44hi CD127hi CD4 T cells (Fig. 2A, 2B). To determine the extent to which these changes were dependent on TCR interaction with cognate Ag, we transferred TCR-tg Sm1 [tg epitope: FltC427–441 from *Salmonella*](https://www.jimmunol.org/article-pdf/0/0/0/0/0/0) and SMARTA [tg epitope: gp61–77 from LCMV (44)] CD4 T cells into B6 mice prior to sham or CLP surgery. These are two disparately different pathogens, and neither is normally found in specific pathogen–free mice. We observed significantly higher frequencies of CD11a^hi^CD49d^hi^ CD44^hi^ CD127^hi^ CD4 T cells in the spleens of CLP-treated mice compared with sham-treated mice after 30 d (Fig. 2C). Although Ag cross-reactivity cannot be excluded, these phenotypic changes in TCR-tg 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 Figs. 1 and 2 suggest that 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 naive 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 p1-A^+^ tetramer-based enrichment (20, 29) (Fig. 3A) to quantify six endogenous Ag-specific CD4 T cell populations of various size, clonotype composition, and immunodominance. CLP-treated mice showed acute reductions in all populations examined on day 2 (consistent with the global lymphopenia) compared with 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^+^ CD4 T cell populations were reduced in CLP-treated versus sham-treated mice, whereas the number of IAV NP^311–325^-specific CD4 T cells increased in CLP-treated mice over sham mice. In contrast, LCMV^gp61–77^-specific, HSV-1gD^290–305^-specific, and OVA^323–339^-specific CD4 T cell populations recovered...
(i.e., no statistical difference between sham and day-30 CLP mice). It is important to emphasize that the changes in individual Ag-specific CD4 T cell populations were not evident when examining bulk CD4 T cells specific for 2W1S, *L. monocytogenes* LLO190–201, IAV NP311–325, LCMVgp106–117, HSVgD290–302, and OVA323–339 was determined 2 and 30 d later using p1-Aα 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:1-Aα–specific CD4+ T cells. Gating for p1-Aα–specific cells was determined using CD8+ T cells as an internal negative control for tetramer binding. (B) Number of Ag-specific, naive CD4 T cell precursors across the six epitopes in sham- and CLP-treated mice 2 or 30 d after surgery. Data shown are the combined results from two to four independent experiments/population analyzed, with three to five mice/group in each experiment. ** ** ** **p < 0.001, group-wise, one-way ANOVA with multiple-testing correction using the Holm–Sidak method, and α = 0.05. ns, not significant.

**Incomplete naive 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 Ag (Ca-2W1S) (23). This design allowed us to model an opportunistic superinfection that is common in sepsis survivors during convalescence (46). When CLP-treated mice were infected with Ca-2W1S on day 2 after surgery, CD4 T cell responses were reduced significantly compared with 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 Ca-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 with sham-treated mice given the same infection (Fig. 4B, 4C). To determine whether the pathogen used influenced the 2W1S-specific CD4 T cell response, sham- and CLP-treated mice were infected with Lm-2W1S 30 d after surgery. Assessing the CD4 T cell response to 2W1S or the endogenous LLO190–201 epitope of *Listeria* listeriolysin-O revealed a significantly reduced expansion for both Ag-specific CD4 T cell populations in CLP-treated mice compared with sham-treated mice given the same infection (Fig. 4B, 4C). To determine whether the pathogen used influenced the 2W1S-specific CD4 T cell response, sham- and CLP-treated mice were infected with Lm-2W1S 30 d after surgery. 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infection (Fig. 4J, 4K), which was interesting because this Ag-specific CD4 T cell population increased numerically after sepsis-induced lymphopenia (Fig. 3). However, the majority of the NP311-specific CD4 T cells in uninfected CLP-treated mice adopted a memory phenotype (i.e., CD44hi; data not shown) at day 30 postsurgery. Recent data suggest that naive 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). Although it remains to be tested whether the same phenomenon is true for naive and memory CD4 T cell responses, these results suggest that the phenotype of the CD4 T cells (naive versus “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 Fig. 4 show that the proliferative capacity of an Ag-specific CD4 T cell population after sepsis correlates with the degree (reduced versus complete or increased) of numerical recovery of its naive precursor population, and the differences seen are intrinsic to the Ag-specific CD4 T cell populations examined and are 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 specific) or not (OVA323 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 with almost no background staining (Fig. 5B). There was a persistent reduction in the frequency and number of IFNγ+ (Fig. 5C) or TNFα+IFNγ+ (Fig. 5D) 2W1S-specific CD4 T cells in CLP-treated mice compared with sham controls. In contrast, there was no difference in the frequency or number of IFNγ+ or TNFα+IFNγ+ OVA323–339-specific CD4 T cells (Fig. 5E–G). Because 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 Ca-2W1S infection that primes for a Th17 response (Fig. 6A) (23, 50, 51). There was no significant difference

**FIGURE 4.** Expansion of epitope-specific populations correlates with precursor pool recovery after septic injury. (A) Experimental design. Mice were infected with Ca-2W1S (5 × 10^4 yeasts in 0.1 ml i.v.), Lm-2W1S 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 IA V (X31; 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 were determined in the spleen. 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 Ca-2W1S. Representative flow plots showing the frequency (D) and number (E) of LLO190-specific and 2W1S-specific CD4 T cells in the spleens from sham- and CLP-treated mice 7 d after i.v. infection with Lm-2W1S. Representative flow plots showing the frequency (F) and number (G) of OVA323-specific CD4 T cells in the spleens from sham- and CLP-treated mice 7 d after i.v. infection with Lm-OVA. Representative flow plots showing the frequency (H) and number (I) of gD290-specific CD4 T cells in the spleens from sham- and CLP-treated mice 9 d after i.v. infection with HSV-1. Representative flow plots showing the frequency (J) and number (K) of NP311-specific CD4 T cells in the lungs and spleens from sham- and CLP-treated mice 12 d after i.v. infection with X31. Data shown are the combined results from two to four independent experiments/pathogen tested, with three to five mice/group in each experiment. *p < 0.05, **p < 0.01, group-wise, one-way ANOVA, followed by multiple-testing correction using the Holm–Sidak method, with α = 0.05. ns, not significant.
in the frequency and number of IL-17A+ CD4 T cells from sham and CLP-treated mice after PMA/ionomycin stimulation (Fig. 6B, 6C). However, after stimulation, significant reductions were seen in the frequency and number of 2W1S-specific CD4 T cells from CLP-treated mice making IL-17A (Fig. 6B, 6D). Together, these data demonstrate that (at least) for 2W1S-specific CD4 T cells, sepsis leads to fewer naive precursors with a reduced capacity to proliferate and make effector cytokines (on a per-cell basis) following antigenic stimulation.

**Discussion**

Sepsis 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 examined the numerical and functional changes in various immune cell subsets after sepsis, but they did 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 performed, for the first time to our knowledge, 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, because 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. Although these cells were introduced into a lymphopenic environment, it could be argued that, because 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 use 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, suggests that CD4 T cells do indeed undergo homeostatic proliferation after septic injury. Unsigner et al. (19) then went on to show increased frequencies of CD4 T cells in septic mice with “activated” (CD69+) and “memory” (CD44+CD62Llo) phenotypes, 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. (19), 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 with 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<sub>311</sub>-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 because the majority of NP<sub>311</sub>-specific CD4 T cells in CLP-treated mice were also CD44<sup>hi</sup> (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 they 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 easily be 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 naive precursor numbers, proliferative capacity, and cytokine production by the different Ag-specific populations. Although we showed recently that sepsis significantly decreases the Ag sensitivity of memory CD8 T cells (35), it remains to be determined to what extent Ag sensitivity is affected in naive 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 investigated previously. The investigators found no skewing of the repertoire toward one particular Vβ subtype (19); however, this conclusion was based on the analysis of bulk CD4 T cells. Just as we only observed stochastic changes in the number of naive CD4 T cells when we examined Ag-specific CD4 T cell populations using p1-Aβ 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 2W1S56–68 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 naive starting population (29). Even after the expansion, 2W1S:I-Ab tetramer enrichment and the multiplexed flow cytometry panel of Vβ-specific mAbs 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

FIGURE 7. Sepsis alters the TCR clonotype composition of Ag-specific CD4 T cell population. (A) Experimental design. Mice were injected i.v. with 50 µg 2W1S56-68 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 used to determine the clonotype composition using two multiplexed flow cytometry panels consisting of the indicated murine TCR Vβ mAbs. Representative flow plots showing the gating strategy used to identify Vβ usage on 2W-I-Aβ-specific CD4 T cells using TCR panel 1. (B) Usage profile for TCR Vβ gene segments in bulk and 2W-I-Aβ-specific CD4 T cells from three representative individual sham- or CLP-treated mice. Averaged frequency of TCR Vβ of bulk (C) and 2W-I-Aβ-specific (D) CD4 T cells in sham- or CLP-treated mice. Data are combined from two independent experiments, each having 5 mice/group. *p < 0.05, **p < 0.01, group-wise, one-way ANOVA analyses followed by Holm–Sidak correction with α = 0.05.
and cytokines [most notably, IL-7 for naive CD4 T cells (59) and IL-15 for naive CD8 T cells (60)]. The increased availability of these resources turns survival signals into mitogenic stimuli that restore 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 to identify 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 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 immunological responses. For example, Th1 cells also provide necessary signals for B cell isotype switching (70), and IL-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 (Tregs) have been found at an increased frequency in septic patients, especially early after diagnosis (73-75). Subsequently, it was determined that the increased frequency of Tregs was the consequence of decreases in the effector CD4 T cell populations (76), suggesting that Tregs are more resistant to sepsis-induced apoptosis than are conventional CD4 T cells (16). Regardless, the role of Tregs 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 that we examined could be due to CD4 T cell intrinsic and/or extrinsic factors. Because CD4 T cell stimulation requires Ag presentation from professional APCs, changes in the number and/or function of dendritic cells 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 have permitted the characterization of Ag-specific endogenous naive and memory T cell responses to a handful of experimental bacterial (e.g., L. monocytogenes) or viral (e.g., 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 antigen 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-2W1S for most of the studies was to take advantage of the wealth of information 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, although our analysis of sepsis-induced alterations in the responsiveness of 2W1S-specific CD4 T cells was largely performed after secondary infection with attenuated Lm-2W1S, we found similar reductions in the proliferative capacity of 2W1S-specific CD4 T cells post-infection with Ca-2W1S (Fig. 4). These data serve as a starting point for future studies using yet-to-be-developed reagents for tracking CD4 T cell populations specific for Ag within pathogens that commonly plague sepsis patients.

In summary, the data presented in this article elucidate with detail how CD4 T cells are affected by sepsis. Our results reveal that the recovery of individual Ag-specific CD4 T cell populations after a septic event is skewed, which 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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