Bacterial Superantigens Expand and Activate, Rather than Delete or Incapacitate, Preexisting Antigen-Specific Memory CD8⁺ T Cells

Courtney E. Meilleur,¹ Christine M. Wardell,¹ Tina S. Mele,^{2,3} Jimmy D. Dikeakos,¹ Jack R. Bennink,⁷ Hong-Hua Mu,⁸ John K. McCormick,^{1,5,6} and S. M. Mansour Haeryfar^{1,2,4,5,6}

¹Department of Microbiology and Immunology, ²Division of General Surgery, Department of Surgery, ³Division of Critical Care Medicine and ⁴Division of Clinical Immunology and Allergy, Department of Medicine, and ⁵Centre for Human Immunology, Western University, and ⁶Lawson Health Research Institute, London, Canada; ⁷Viral Immunology Section, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; and ⁸Division of Rheumatology, Department of Internal Medicine, University of Utah School of Medicine, Salt Lake City

Superantigens (SAGs) released by common Gram-positive bacterial pathogens have been reported to delete, anergize, or activate mouse T cells. However, little is known about their effects on preexisting memory CD8⁺ T cell (TCD8) pools. Furthermore, whether SAGs manipulate human memory TCD8 responses to cognate antigens is unknown. We used a human peripheral blood mononuclear cell culture system and a nontransgenic mouse model in which the impact of stimulation by two fundamentally distinct SAGs, staphylococcal enterotoxin B and *Mycoplasma arthritidis* mitogen, on influenza virus- and/or cytomegalovirus-specific memory TCD8 could be monitored. Bacterial SAGs surprisingly expanded antiviral memory TCD8 generated naturally through infection or artificially through vaccination. Mechanistically, this was a T cell-intrinsic and T cell receptor β -chain variable-dependent phenomenon. Importantly, SAG-expanded TCD8 displayed an effector memory phenotype and were capable of producing interferon- γ and destroying target cells *ex vivo* or *in vivo*. These findings have clear implications for antimicrobial defense and rational vaccine design.

Keywords. *Staphylococcus aureus*; superantigen; staphylococcal enterotoxin B; *Mycoplasma arthritidis* mitogen; influenza; cytomegalovirus; T cell repertoire; TCD8; cytotoxicity; immunological memory.

Superantigens (SAGs) are potent exotoxins encoded by a number of bacterial pathogens, including *Staphylococcus aureus* [1, 2]. As unprocessed proteins, SAGs bind to major histocompatibility complex (MHC) class II molecules outside their peptide-displaying groove and to select T cell receptor (TCR) β -chain variable (V β) regions expressed by many T cells [3, 4]. Consequently, SAGs bridge MHC class II-expressing antigen-presenting cells and T cells to provoke aberrant T cell activation. The ensuing hyperinflammation and cytokine storm drive many clinical manifestations of SAG-mediated morbidities that vary in severity from self-limiting food poisoning to catastrophic toxic shock syndrome. SAGs may also be maliciously used as bioweapons, which is a matter of grave concern. Staphylococcal enterotoxin B (SEB) is designated as a category B priority bioterrorism agent by the Centers for Disease Control and Prevention [5].

At first glance, the ability of SAGs to turn on the immune system may appear puzzling if not counterintuitive to the survival of SAG-expressing bacteria. However, apart from our recent reports linking staphylococcal and streptococcal SAGs to niche adaptation in the host [6–8], it is believed that systemic exposure to SAGs creates a state of profound immunosuppression in those surviving the disproportionate inflammation [9–11]. Therefore, the host can be rendered vulnerable to the very bacteria that secrete SAGs and also prone to opportunistic infections caused by other microbes.

Activation-induced cell death and anergy resulting in physical and functional holes in the T cell repertoire, respectively, have been proposed to account for SAG-associated immunosuppression [10]. However, the evidence in support of such phenomena is experimental system-specific and based almost exclusively on findings obtained in mouse models [4, 10, 12, 13]. Moreover, while T cell anergy in the aftermath of an encounter with SAGs is relatively well documented in the CD4⁺ T cell compartment [14–16], only a precious little is known about how SAGs modulate mouse CD8⁺ T cell (TCD8) responses, especially those mounted by memory TCD8 [17–19]. Finally, whether/how SAGs manipulate human memory TCD8 is essentially unexplored. These are important questions because (1) viral infections that are normally dealt with by TCD8 are sometimes complicated by concomitant or secondary infections with SAG-producing bacteria, and (2) potential deletion of antiviral memory TCD8,

Received 2 August 2018; editorial decision 1 November 2018; accepted 7 November 2018; published online November 12, 2018.

Presented in part: 104th American Association of Immunologists Annual Meeting, Washington, D. C., May 12–16, 2017 (abstract #203.12); 30th Canadian Society for Immunology (CSI) Annual Meeting, Banff, Canada, April 7–10, 2017 (abstract #17); 29th CSI Annual Meeting, Ottawa, Canada, April 1–4, 2016 (abstract #28).

Correspondence: S. M. Mansour Haeryfar, PhD, Department of Microbiology and Immunology, Schulich School of Medicine and Dentistry, Western University, 1151 Richmond St, London, Ontario, N6A 5C1, Canada (Mansour.Haeryfar@schulich.uwo.ca).

The Journal of Infectious Diseases® 2019;219:1307–17

© The Author(s) 2018. Published by Oxford University Press for the Infectious Diseases Society of America. All rights reserved. For permissions, e-mail: journals.permissions@oup.com. DOI: 10.1093/infdis/jiy647

generated through vaccination or natural infections, may jeopardize one's ability to clear microbes, especially considering the gradual shrinkage of the thymus after puberty.

Here, we have addressed the questions presented above by monitoring antigen-specific TCD8 responses to influenza A virus (IAV) and cytomegalovirus (CMV) in a human peripheral blood mononuclear cell (PBMC) culture system simulating exposure to two SAGs of distinct bacterial origin. Our kinetic and functional analyses reveal that human memory TCD8 expressing SAG-responsive TCR Vβ families are surprisingly expanded, rather than deleted, by SAGs while retaining their cognate cytolytic activity. We also confirmed our findings using an in vivo nontransgenic mouse model.

METHODS

Ethics Statement

Peripheral blood specimens were collected from healthy donors who provided written informed consent according to a protocol approved by the Western University Research Ethics Board for Health Sciences Research Involving Human Subjects. Animal experiments were performed in compliance with Western University Animal Use Subcommittee and Canadian Council on Animal Care guidelines.

Bacterial SAGs

Recombinant SEB from *Staphylococcus aureus* (strain COL) was generated in *Escherichia coli* BL21(DE3) and purified via nickel column chromatography [20]. Native *Mycoplasma arthritidis* mitogen (MAM) was purified from *M. arthritidis* strain PG6 (ATCC 19611) as previously described [21]. We have ruled out the possibility of biologically meaningful endotoxin contamination in our SEB [22, 23] and MAM preparations [24].

Human PBMC Isolation and Culture

Eight healthy donors, four males and four females, were recruited (Table 1). PBMCs were isolated from heparinized blood through centrifugation in low-endotoxin Ficoll-Paque PLUS medium (GE Healthcare). An aliquot of PBMCs was

set aside for immediate tetramer staining, and the remainder was seeded in a 6-well plate at 2×10^6 cells/mL in Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM GlutaMAX-I, 0.1 mM minimal essential medium nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were left untreated or stimulated with 100 ng/mL SEB (or other staphylococcal/streptococcal SAGs) or with 20 ng/mL MAM at 37°C inside a humidified incubator containing 6% CO₂. After 3 days, cells were washed to remove residual SAGs and rested in the medium described above. The washing step was repeated on day 4, and cultures were halted or continued with medium replenishment until day 7 or day 11.

Tetramer Staining

Human PBMCs were stained for 30 minutes on ice with HLA-A*0201 tetramers loaded with GILGFVFTL or NLVPMVATV, which correspond to peptide epitopes of IAV (M1₅₈₋₆₆) and CMV (pp65₄₉₅₋₅₀₃), respectively [25, 26]. These epitopes will, hereafter, be referred to as M1₅₈ and pp65₄₉₅. Tetramer reagents were prepared by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Tetramer staining for each sample was followed by addition of a mouse anti-human CD8α monoclonal antibody (mAb; clone SK1; BD Biosciences) for another 30 minutes at 4°C. Cells were then washed and interrogated by a BD FACSCanto II cytometer after dead cell exclusion using 7-aminoactinomycin D staining and doublet exclusion. Data analysis was conducted using FlowJo software (Tree Star). Our gating strategy is depicted in Supplementary Figure 1.

51-Chromium (⁵¹Cr) Release Assay

HLA-A2⁺ T2 cells were provided by Dr Tania Watts (University of Toronto). They were pulsed overnight with 2 μM M1₅₈ peptide or with vehicle before they were labeled with 100 μCi of Na₂⁵¹CrO₄ for 1.5 hours at 37°C, washed, and used as target cells in cytotoxicity assays.

M1₅₈-specific TCD8 were isolated from day-11 PBMC cultures through staining with Alexa Fluor 647–conjugated M1₅₈:HLA-A2 tetramer, subsequent incubation with anti-Cy5/anti-Alexa Fluor 647 MicroBeads (Miltenyi Biotec), and magnetic cell sorting. Isolated TCD8 were >90% viable and were immediately employed as effector cells against ⁵¹Cr-labeled T2 target cells. Briefly, target cells were seeded at a concentration of approximately 10⁴ cells/well of 96-well U-bottomed plates together with effector cells in complete Roswell Park Memorial Institute medium. After incubation for 8 hours at 37°C and 6% CO₂, plates were spun at 456×g for 5 minutes before a 100-μL supernatant aliquot was harvested from each well. The ⁵¹Cr content of the samples was quantified using a γ-counter, and cognate TCD8-mediated cytotoxicity was calculated as follows: percentage of specific lysis = [(ER – SR)/(TR – SR)] × 100, where ER (experimental release) is determined from

Table 1. Sex, Age, and Recent Flu Vaccination Status of Healthy Donors Who Participated in This Study

Donor	Sex	Age, y	Seasonal Influenza Vaccination ≤3 mo Before Specimen Collection
1	Male	39	Yes
2	Female	28	No
3	Male	31	No
4	Female	25	No
5	Female	21	No
6	Male	33	Yes
7	Male	27	No
8	Female	24	No

wells receiving both effector and target cells, and SR (spontaneous release) and TR (total release) are obtained from wells containing target cells only and those containing target cells plus 0.5% Triton X-100, respectively.

TCR V β Profiling of Mouse Splenic T Cells

Female BALB/c mice were purchased from Charles River Canada, housed in our barrier facility, and used between 8 and 12 weeks of age.

Naive mice were euthanized, and the spleen from each was collected and homogenized to yield a single-cell suspension. Erythrocytes were eliminated via treatment with ammonium-chloride-potassium lysis buffer, and a cell strainer with 70- μ m-diameter pores was used to remove cell debris. We used a mouse V β TCR Screening Panel from BD Pharmingen. Splenocytes were suspended at 2×10^6 cells per mL of complete medium in T75 flasks, to which SEB (500 ng/mL) or phosphate-buffered saline (PBS) was added. After incubation for 3 days at 37°C, cells were washed and then were costained with an Alexa Fluor 647–labeled, anti-mouse CD8 α mAb (clone 53-6.7; eBioscience) and each of the FITC-conjugated anti-V β mAbs present in the screening kit. Cells were then washed again and analyzed cytofluorimetrically.

IAV and SEB Inoculations

The mouse-adapted Puerto Rico/8/1934 (H1N1) strain of IAV was propagated within the allantoic cavity of 10-day-old embryonated chicken eggs. To generate memory TCD8 against IAV, female BALB/c mice were given an intraperitoneal (i.p.) allantoic fluid inoculum containing ~600 hemagglutinating units of the virus. Sixty days later, mice randomly assigned to two experimental groups were injected i.p. with 50 μ g of SEB or with PBS. On day 3 after SEB challenge, memory TCD8 functions were evaluated.

Intracellular Interferon- γ (IFN- γ) Staining

Erythrocyte-depleted splenocytes were seeded at 2×10^6 cells/200 μ L/well of a U-bottomed microplate. Cells were left untreated or stimulated ex vivo for 5 hours at 37°C with 500 nM of TYQRTRALV, a synthetic peptide corresponding to the immunodominant H-2K^d-restricted epitope of the IAV nucleoprotein (NP_{147–155}; hereafter, “NP₁₄₇”) [27]. As an irrelevant (control) peptide, we used RPQASGVYM, the H-2^d-restricted immunodominant peptide of the lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP_{118–126}; hereafter, “NP₁₁₈”) [28]. LCMV is a natural mouse pathogen to which our animals had never been exposed. These peptides were procured or synthesized, purified by high-performance liquid chromatography, and analyzed by mass spectrometry by or under the supervision of the Research Technologies Branch, National Institutes of Health.

To retain IFN- γ among other soluble mediators within the endoplasmic reticulum of cognate TCD8, brefeldin A (Sigma-Aldrich) was present at 10 μ g/mL during the final 3 hours of

peptide stimulation. Plates were then spun, and pelleted cells were incubated for 20 minutes on ice with a 20- μ L aliquot/well of 2.4G2 hybridoma supernatant (Fc block). Next, cells underwent surface staining with an allophycocyanin (APC)–conjugated anti-CD8 α mAb (clone 53-6.7), fixed with 1% paraformaldehyde, permeabilized with 0.1% saponin, stained with a FITC- or PerCP-Cy5.5–conjugated anti-IFN- γ mAb (clone XMG1.2; eBioscience), and analyzed by flow cytometry.

The above staining protocol was also repeated with the addition of a FITC-conjugated anti-mouse TCR V β 8.1/8.2 mAb (clone MR5-2) or a mouse IgG2a isotype control during surface staining for CD8 α .

In Vivo Killing Assay

Syngeneic target splenocytes from naive BALB/c mice were prepared and split into two populations. The cognate target population was incubated for 45 minutes at 37°C with 500 nM of NP₁₄₇ and subsequently labeled with 2.5 μ M of carboxy-fluorescein succinimidyl ester (CFSE; Invitrogen) with gentle mixing at 37°C for 20 minutes. The irrelevant target population was coated with 500 nM of NP₁₁₈ and 0.5 μ M of CFSE. The CFSE reaction was stopped by the addition of cold fetal calf serum. CFSE^{high} and CFSE^{low} populations were then washed, mixed in equal numbers, and injected into the tail vein of naive BALB/c (control) mice or IAV-primed, SEB/PBS-challenged animals. Typically, each recipient was given 6×10^6 cells in 200 μ L sterile PBS. Four hours later, mice were euthanized, and the presence of CFSE-labeled target cells within their splenic cell preparations was analyzed using differential CFSE intensities to distinguish between cognate and control target cells. For each mouse, we acquired 2000 events within the CFSE^{low} gate. The specific lysis of NP₁₄₇-pulsed targets was calculated based on the following formula: % specific killing = $\{1 - [(\% \text{ NP}_{147}\text{-pulsed in primed mouse} / \% \text{ NP}_{118}\text{-pulsed in primed mouse}) / (\% \text{ NP}_{147}\text{-pulsed in naive mouse} / \% \text{ NP}_{118}\text{-pulsed in naive mouse})]\} \times 100$.

Statistical Analysis

Paired and unpaired Student *t* tests and Analyses of Variance (ANOVA) were performed as appropriate.

RESULTS

Human IAV-Specific Memory TCD8 Can Be Expanded by SEB

Several studies have demonstrated that bacterial SAGs cause memory T cell disappearance or hyporesponsiveness to cognate antigens in mice [19, 29, 30]. However, whether preexisting human memory TCD8 are deleted or anergized by SAGs is unknown. To address this question, we recruited eight HLA-A2⁺ healthy donors (Table 1) who had a detectable memory TCD8 population against M1₅₈, the HLA-A2–restricted immunodominant peptide epitope of IAV matrix protein 1 [26], in their peripheral blood (Figure 1). We focused on M1₅₈-specific TCD8 because of the conserved usage of the

V β 17 gene segments in their TCR [31], which can be targeted by SEB [32].

Using a culture system in which unfractionated PBMCs were left untreated or stimulated with SEB, to mimic the unbiased exposure of bulk immune cells to SAg in real-life scenarios, we observed a near-complete disappearance of tetramer-positive M1₅₈-specific TCD8 by day 4 post-SEB stimulation (Figure 1A–D). Much to our surprise, however, these cells reappeared as a dramatically enlarged population on day 7 and even more so on day 11 (Figure 1). The percentage of M1₅₈-specific TCD8 among total TCD8 was significantly elevated on both days 7 and 11 (Figure 1C). This was despite the fact that other TCD8 can also be expanded nonspecifically by SEB (Figure 1B), which inevitably results in underestimation of the impact of SEB on the M1₅₈-specific population as far as cell frequencies are concerned. Therefore, we additionally calculated the absolute

number of M1₅₈-specific TCD8 among a constant number of PBMCs (1 million) that were acquired for cytofluorimetric analyses at every time point for each donor (Figure 1D). While M1₅₈-specific TCD8 numbers declined on day 4, there were 6.2- and 9.2-fold increases, on average, on days 7 and 11, respectively. Upon further analyses, the expanded population displayed a CD45RO⁺CCR7⁺ phenotype consistent with an effector memory phenotype (Supplementary Figure 2), and predominantly used V β 17 in their TCR (mean $\pm$ SD): 67.4% $\pm$ 3.4%; n = 3).

The ultimate role of TCD8 in antiviral defense is to eliminate target cells displaying viral peptides bound to MHC class I molecules. Although T cell proliferation can be viewed as a functional readout, we were curious to know whether SEB-expanded M1₅₈-specific TCD8 retain their cognate cytolytic capacity. To this end, we magnetically sorted these cells out of day-11 cultures and used them against M1₅₈-pulsed and vehicle-pulsed

Figure 1. Staphylococcal enterotoxin B (SEB) stimulation of human peripheral blood mononuclear cells (PBMCs) expands preexisting M1₅₈-specific memory TCD8. *A*, PBMCs from 8 healthy donors were left untreated or stimulated with SEB (100 ng/mL) according to the depicted experimental protocol. *B–D*, Freshly isolated PBMCs and cells harvested from day-4, day-7, and day-11 cultures were stained first with Alexa Fluor 647–conjugated, GILGFVFTL (M1₅₈)–loaded HLA-A2 tetramers and then with a FITC-labeled anti-CD8 α monoclonal antibody. Representative cytofluorimetric plots from donor 1 are illustrated (*B*). The frequency (out of the total number of TCD8; *C*) and the absolute number (out of 1 million cells that were acquired by a cytometer; *D*) of M1₅₈-specific TCD8 were determined for each donor, and their fold changes over corresponding baseline (ie, day 0) values were calculated. Error bars represent standard errors of the mean. * P < .05 and ** P < .01, by the paired Student t test. The overall difference between the SEB and phosphate-buffered saline (PBS) treatment groups was also significant by analysis of variance (P = .0291 [*C*] and P = .0006 [*D*]).

T2 cells. At 1:1 and 0.25:1 ratios of effector cells to target cells, M1₅₈-specific TCD8 effectively lysed IAV peptide-coated target cells but not control target cells (Figure 2). For donor 7, who exhibited the most impressive expansion of M1₅₈-specific TCD8 (Supplementary Figure 3), we obtained enough cells to also set up a 5:1 ratio of effector cells to target cells, which resulted in higher (~63%) specific cytotoxicity (Figure 2).

Taken together, the above results indicate that SEB stimulation does not delete human IAV-specific memory TCD8. Instead, it triggers their expansion without impeding their cognate cytotoxic effector function.

SEB-Triggered Expansion of Antiviral Memory TCD8 Is Mechanistically Linked to Their TCR V β Makeup

We next asked whether SEB-induced expansion of M1₅₈-specific memory TCD8 was dependent on the type of TCR V β families they harbor and/or may have occurred in a bystander fashion. A subcohort of our donors had a detectable TCD8 population against pp65₄₉₅, an immunodominant peptide derived from the CMV lower matrix phosphoprotein pp65 [25], in addition to M1₅₈-specific TCD8. T cells recognizing pp65₄₉₅ use a diverse array of V β s in their TCR repertoire [33, 34]. This enabled us to simultaneously examine the effect of SEB on IAV- and CMV-specific memory TCD8 that have considerably different TCR V β usage characteristics. Tetramer staining revealed that while the M1₅₈-reactive population from all 3 donors were clearly expanded on days 7 and 11 (Figure 3A), pp65₄₉₅-specific cells did not undergo marked numerical changes (Figure 3B). In additional experiments, we found that staphylococcal and streptococcal SAGs that do not target TCR V β 17 fail to expand M1₅₈-specific TCD8 (Supplementary Figure 4).

Collectively, these results indicate that memory TCD8 populations are selectively, not globally, affected by SEB and that the TCR V β usage of TCD8 appears to dictate their responsiveness or lack thereof to SEB.

M1₅₈-Specific TCD8 Proliferate in Response to MAM

To ascertain whether human memory TCD8 can be expanded by more than one SAG (ie, SEB), we also used MAM, a SAG isolated from a fundamentally different bacterium (ie, *Mycoplasma arthritidis*) in terms of structure, genomic complexity, tissue target range, and pathogenicity [35]. Like SEB, MAM reacts with TCR V β 17 [36]—hence our justification for using this SAG as a tool.

The overall human T cell response to MAM is reportedly less robust than that to *S. aureus* enterotoxins [36, 37]. Nevertheless, day-7 and day-11 MAM-stimulated PBMC cultures reproducibly contained an enlarged M1₅₈-specific TCD8 population (Figure 4). This data set reveals that M1₅₈-specific T cell responses to MAM and SEB follow the same kinetic pattern and also reiterates our conclusion that antiviral memory TCD8 expansion by SAGs is TCR V β usage-dependent.

SEB Administration Expands Mouse Memory TCD8 Capable of Producing IFN- γ and Killing Target Cells In Vivo

In the next series of experiments, we attempted to extend our findings to an in vivo setting. Although mouse models are frequently used to study anti-IAV immunity, IAV is not a natural mouse pathogen. Therefore, to generate IAV-specific memory TCD8, we injected BALB/c mice intraperitoneally with IAV two months before administering a bolus dose of SEB. On day 3 after SEB administration, at which point T cell activation by this SAG reaches its peak, we enumerated IAV-specific splenic TCD8 by intracellular cytokine staining.

The above model offers several advantages. First, the TCD8 response to IAV is well-characterized in BALB/c mice and focused on a single immunodominant epitope, namely NP₁₄₇ [27, 38]. Second, NP₁₄₇-specific TCD8 primarily use TCR V β 8.1/2 [39], which is targeted by SEB in mice [40]. Third, unlike several other conventional wild-type mouse strains, BALB/c mice express MHC molecules with an adequate overall binding avidity for SEB [41]. Fourth, IAV inoculation of mice via the intraperitoneal

Figure 2. Staphylococcal enterotoxin B (SEB)-stimulated M1₅₈-specific TCD8 retain their cytolytic effector function. M1₅₈-specific TCD8 were magnetically isolated from day-11 peripheral blood mononuclear cell (PBMC) cultures and used at indicated ratios of effector cells to target cells against 51-chromium (⁵¹Cr)-labeled T2 cells that were pulsed with GILGFVFTL or treated with dimethyl sulfoxide (vehicle). Percent specific lysis of target cells was calculated in independent ⁵¹Cr release experiments using PBMC samples from 3 donors who exhibited the most substantial M1₅₈-specific T cell expansion upon SEB stimulation. Mean values ($\pm$ SDs) from triplicate (donors 1 and 6) or quadruplicate (donor 7) wells are shown. ** P < .01 and **** P < .0001, by the unpaired Student t test.

Figure 3. Staphylococcal enterotoxin B (SEB) stimulation of human peripheral blood mononuclear cells (PBMCs) expands M1₅₈- but not pp65₄₉₅-specific T cells. PBMCs from 3 donors who had detectable TCD8 populations against both M1₅₈ (A) and pp65₄₉₅ (B) were cultured in the presence or absence of SEB. Freshly isolated cells and those harvested from day-4, day-7, and day-11 cultures were stained first with Alexa Fluor 647–conjugated, GILGFVFTL (M1₅₈)–loaded (A) or NLVPMVATV-loaded HLA-A2 tetramers (B) and then with a FITC-labeled anti-CD8α monoclonal antibody. The absolute numbers (out of 1 million cells acquired by a cytometer) of M1₅₈- and pp65₄₉₅-specific TCD8 were determined for each donor, and their fold changes over baseline (ie, day 0) values were calculated. CMV, cytomegalovirus; IAV, influenza A virus.

route serves as a vaccination model in the absence of overt infection [42]. This allows for examination of the memory TCD8 compartment without complications otherwise arising from IAV-associated morbidity following intranasal infection.

A single injection of SEB was sufficient to raise NP₁₄₇-specific IFN-γ-secreting TCD8 numbers, with almost a 4-fold increase on average over control values (Figure 5A). This was particularly remarkable since our model did not include a boosting dose of IAV.

We stimulated naive BALB/c splenocytes with SEB in vitro and confirmed that Vβ8.1/2, which is predominantly used by NP₁₄₇-specific TCD8 [39], was indeed among TCR Vβ families that responded to SEB, as judged by their diminished detectability in a short-term culture (Figure 5B) resembling our human PBMC cultures. Furthermore, NP₁₄₇-specific cells in IAV-inoculated mice that later received either SEB or PBS maintained their TCR Vβ8 expression (Figure 5C).

Finally, using an in vivo killing assay, which we previously optimized for detection of TCD8-mediated cytotoxicity toward NP₁₄₇-coated targets [38], we demonstrated that SEB-expanded NP₁₄₇-specific T cells were potently cytolytic (Figure 5D). In vivo treatment with SEB enhanced TCD8-mediated destruction

of cognate target cells, whereas control targets pulsed with an irrelevant peptide (NP₁₁₈) remained present in the spleen of mice injected with either SEB or PBS.

In summary, the results presented above indicate that exposure to SEB augments IAV-specific responses in vivo by increasing the number of cytokine-producing and cytolytic TCD8.

DISCUSSION

Infections with SAg-producing bacteria are common in the clinic and may follow respiratory viral infections. A series of landmark articles by M. A. Blackman's group previously documented that IAV-specific mouse TCD8 responses may be unaffected, potentiated, or even hampered by staphylococcal SAg, depending on the timing, mode, sustenance, and route of SAg release and on the functional status of exposed TCD8 [17–19]. Whether human memory TCD8 generated through natural IAV infections or vaccination are impacted by SAg has been unknown.

In this investigation, short-term SEB stimulation of human PBMCs made preexisting IAV-specific memory TCD8 disappear. However, these cells became detectable in much greater numbers in continued cultures and retained their cognate

Figure 4. Exposure to *Mycoplasma arthritidis* mitogen (MAM) results in M1₅₈-specific memory T cell expansion. Peripheral blood mononuclear cells (PBMCs) from indicated donors were stained before and after stimulation with MAM (20 ng/mL). Freshly isolated cells and those harvested from day-4, day-7, and day-11 cultures were stained with Alexa Fluor 647–conjugated, GILGFVFTL (M1₅₈)–loaded HLA-A2 tetramers and a FITC-labeled anti-CD8 α monoclonal antibody. Cytofluorimetric plots are illustrated for each sample.

cytolytic activity. This finding is not in keeping with the reported ability of SAGs to eliminate or anergize T cells. It is likely that stimulation with SEB results in TCR downregulation, thus making memory TCD8 temporarily undetectable. SAG-induced TCR internalization has been reported before [10, 43] although much remains to be elucidated regarding its underlying molecular mechanism(s).

In our mouse model, SEB expanded memory TCD8 recognizing an IAV-derived immunodominant determinant (ie, NP₁₄₇), which were capable of producing IFN- γ and lysing target cells presenting H-2K^d:NP₁₄₇ complexes in vivo. Coppola and Blackman [17] previously reported reactivation of preexisting memory TCD8 by SEB. They did not examine epitope-specific responses but could generate ex vivo cytotoxicity against IAV-infected target cells after culturing CD8⁺CD62L^{lo}CD44^{hi} bulk memory cells from memory mice with SEB. Of note, our model simulates memory T cell generation as a consequence of vaccination. In contrast, in the model used by Coppola and Blackman, T cell memory was elicited via intranasal IAV

inoculation, which allows for viral propagation and may be viewed as an infection model. Therefore, it seems reasonable to predict vaccine- and infection-elicited memory TCD8 to be similarly affected by at least certain SAGs. It will be important to compare circulating and tissue-resident memory T cell responses to SAGs in future studies.

Monitoring the effects of SEB and MAM on IAV- and/or CMV-specific memory TCD8 in vitro or in vivo led us to conclude that SAGs typically expand T cells that express SAG-responsive V β s. SAGs' modus operandi in this context may imply that these toxins manipulate memory T cell responses with or without an accompanying cytokine storm. Full-blown toxic shock syndrome and cytokine storm can be encountered in the absence of SAG-producing bacteria in the circulation, thus yielding negative blood culture results. We previously demonstrated that Toll-like receptor 2 ligands embedded in the staphylococcal cell wall attenuate T cell responses to SAGs and prevent toxic shock syndrome [20]. Despite the T cell-autonomous mode of memory cell expansion by SAGs, it will be interesting

Figure 5. In vivo administration of Staphylococcal enterotoxin B (SEB) expands vaccine-elicited, V β 8.1/8.2⁺ memory TCD8 in mice without compromising their effector functions. **A**, BALB/c mice were inoculated intraperitoneally with influenza A virus (IAV) to generate memory TCD8 and, 2 months later, received SEB (50 μ g) or phosphate-buffered saline (PBS) intraperitoneally. Three days later, splenic NP₁₄₇-specific TCD8 were enumerated by intracellular cytokine staining. **B**, Splenocytes from naive BALB/c mice were cultured for 3 days in the presence or absence of SEB, and the frequencies of indicated T cell receptor (TCR) V β ⁺ cells among the total number of TCD8 were determined. **C**, The expression of TCR V β 8.1/8.2 by NP₁₄₇-specific TCD8 was also examined in IAV-primed mice in which PBS or SEB were injected. Open and filled histograms correspond to staining with an anti-mouse TCR V β 8.1/8.2 monoclonal antibody and isotype control, respectively. **D**, Cognate and irrelevant target cells were prepared by pulsing naive syngeneic splenocytes with TYQRTALV (the NP₁₄₇ peptide epitope of IAV) and RPOASGVYM (the NP₁₁₈ peptide epitope of LCMV), respectively. Target cells were labeled with 2 different doses of carboxyfluorescein succinimidyl ester (CFSE), washed, mixed in equal numbers, and injected intravenously into IAV-primed BALB/c mice that later received either SEB (50 μ g intraperitoneally) or PBS. Four hours later, mice were euthanized, and target cell populations were tracked on the basis of their differential CFSE staining intensities in each spleen. Histograms from representative mice are shown. Percent specific cytotoxicity was calculated as described in Methods. Each open and filled circle in panel **D** represents an individual mouse. Error bars in panels **A** and **D** represent standard errors of the mean. IFN- γ , interferon γ . ** P < .01 and *** P < .001, by the unpaired t test.

to explore indirect bystander effects, if any, of inflammatory cytokines on the magnitude and quality of antiviral memory responses. On the other hand, severe IAV infections may also, on their own, create cytokine storms with high morbidity and mortality rates [44], as was the case with the H1N1 pandemic flu of 2009. When severe influenza is followed by secondary infections with SAg-secreting bacteria, the impact of SAgS on memory TCD8 pools may influence clinical outcomes.

According to the doctrine of original antigenic sin [45, 46], which indicates that “first flu is forever” [47], the immune system tends to preferentially utilize its memory of earlier influenza infections when facing a new infection or vaccination. This concept is applicable not only to humoral immunity but also to TCD8 participation in antiviral responses [48]. This poses a challenge in designing influenza vaccines for inducing efficacious heterosubtypic immunity and also for elderly individuals with large memory T cell pools and a progressive decline in naive T cell output. Expanding functional memory TCD8 through nonspecific means (eg, SAgS) in a controllable manner may be a provocative yet tempting possibility. One should, however, keep in mind the findings of mouse studies in which CD4⁺ T cells are anergized by SAgS [15, 30, 49]. Helper CD4⁺ T cells play a pivotal role in orchestrating antibody responses, which cannot be compromised. Certain SAgS, such as MAM, a mitogen used in this work, can promote B cell differentiation and polyclonal immunoglobulin production in human PBMC cultures [50]. Much work lies ahead in exploring whether/how various SAgS target human memory CD4⁺ T cell responses. Our ongoing studies are also addressing the effects of SAgS on the epitope breadth of virus-specific primary and recall T cell responses.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We thank Dr Arash Memarnejadian, for his expert assistance with in vivo killing assays, Delfina Mazzuca for production and purification of SEB, and other members of the Haeryfar laboratory for helpful discussions.

Financial support. This work was supported by the Canadian Institutes of Health Research (CIHR-501100000032) (grant PJT-156295 to S. M. M. H.), the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, the Natural Sciences and Engineering Research Council of Canada (Alexander Graham Bell Canada Graduate Scholarship [doctoral] to C. E. M.), and the Ontario Ministry

of Training, Colleges, and Universities (Queen Elizabeth II Graduate Scholarship in Science and Technology to C. M. W.).

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Stach CS, Herrera A, Schlievert PM. Staphylococcal superantigens interact with multiple host receptors to cause serious diseases. *Immunol Res* **2014**; 59:177–81.
2. Tuffs SW, Haeryfar SMM, McCormick JK. Manipulation of innate and adaptive immunity by staphylococcal superantigens. *Pathogens* **2018**; 7:E53.
3. Dellabona P, Peccoud J, Kappler J, Marrack P, Benoist C, Mathis D. Superantigens interact with MHC class II molecules outside of the antigen groove. *Cell* **1990**; 62:1115–21.
4. White J, Herman A, Pullen AM, Kubo R, Kappler JW, Marrack P. The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* **1989**; 56:27–35.
5. Borchardt SM, Ritger KA, Dworkin MS. Categorization, prioritization, and surveillance of potential bioterrorism agents. *Infect Dis Clin North Am* **2006**; 20:213–25, vii–viii.
6. Kasper KJ, Zeppa JJ, Wakabayashi AT, et al. Bacterial superantigens promote acute nasopharyngeal infection by *Streptococcus pyogenes* in a human MHC Class II-dependent manner. *PLoS Pathog* **2014**; 10:e1004155.
7. Xu SX, Gilmore KJ, Szabo PA, et al. Superantigens subvert the neutrophil response to promote abscess formation and enhance *Staphylococcus aureus* survival in vivo. *Infect Immun* **2014**; 82:3588–98.
8. Zeppa JJ, Kasper KJ, Mohorovic I, Mazzuca DM, Haeryfar SMM, McCormick JK. Nasopharyngeal infection by *Streptococcus pyogenes* requires superantigen-responsive Vbeta-specific T cells. *Proc Natl Acad Sci U S A* **2017**; 114:10226–31.
9. Ivars F. Superantigen-induced regulatory T cells in vivo. *Chem Immunol Allergy* **2007**; 93:137–60.
10. Miethke T, Wahl C, Gaus H, Heeg K, Wagner H. Exogenous superantigens acutely trigger distinct levels of peripheral T cell tolerance/immunosuppression: dose-response relationship. *Eur J Immunol* **1994**; 24:1893–902.
11. Szabo PA, Goswami A, Memarnejadian A, et al. Swift Intrahepatic Accumulation of Granulocytic Myeloid-Derived Suppressor Cells in a Humanized Mouse Model of Toxic Shock Syndrome. *J Infect Dis* **2016**; 213:1990–5.
12. Kawabe Y, Ochi A. Programmed cell death and extrathymic reduction of Vbeta8⁺ CD4⁺ T cells in mice

- tolerant to *Staphylococcus aureus* enterotoxin B. *Nature* **1991**; 349:245–8.
13. McCormack JE, Callahan JE, Kappler J, Marrack PC. Profound deletion of mature T cells in vivo by chronic exposure to exogenous superantigen. *J Immunol* **1993**; 10:3785–92.
 14. Cauley LS, Cauley KA, Shub F, Huston G, Swain SL. Transferable anergy: superantigen treatment induces CD4+ T cell tolerance that is reversible and requires CD4-CD8-cells and interferon gamma. *J Exp Med* **1997**; 186:71–81.
 15. Eroukhmanoff L, Oderup C, Ivars F. T-cell tolerance induced by repeated antigen stimulation: selective loss of Foxp3- conventional CD4 T cells and induction of CD4 T-cell anergy. *Eur J Immunol* **2009**; 39:1078–87.
 16. Lussow AR, MacDonald HR. Differential effects of superantigen-induced “anergy” on priming and effector stages of a T cell-dependent antibody response. *Eur J Immunol* **1994**; 24:445–9.
 17. Coppola MA, Blackman MA. Bacterial superantigens reactivate antigen-specific CD8+ memory T cells. *Int Immunol* **1997**; 9:1393–403.
 18. Huang CC, Coppola MA, Nguyen P, et al. Effect of *Staphylococcus enterotoxin B* on the concurrent CD8(+) T cell response to influenza virus infection. *Cell Immunol* **2000**; 204:1–10.
 19. Huang CC, Shah S, Nguyen P, Altman JD, Blackman MA. Bacterial superantigen exposure after resolution of influenza virus infection perturbs the virus-specific memory CD8(+)-T-cell repertoire. *J Virol* **2002**; 76:6852–6.
 20. Chau TA, McCully ML, Brintnell W, et al. Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome. *Nat Med* **2009**; 15:641–8.
 21. Atkin CL, Wei S, Cole BC. The *Mycoplasma arthritidis* superantigen MAM: purification and identification of an active peptide. *Infect Immun* **1994**; 62:5367–75.
 22. Hayworth JL, Kasper KJ, Leon-Ponte M, et al. Attenuation of massive cytokine response to the staphylococcal enterotoxin B superantigen by the innate immunomodulatory protein lactoferrin. *Clin Exp Immunol* **2009**; 157:60–70.
 23. Shaler CR, Choi J, Rudak PT, et al. MAIT cells launch a rapid, robust and distinct hyperinflammatory response to bacterial superantigens and quickly acquire an anergic phenotype that impedes their cognate antimicrobial function: Defining a novel mechanism of superantigen-induced immunopathology and immunosuppression. *PLoS Biol* **2017**; 15:e2001930.
 24. Mu HH, Pennock ND, Humphreys J, Kirschning CJ, Cole BC. Engagement of Toll-like receptors by mycoplasmal superantigen: downregulation of TLR2 by MAM/TLR4 interaction. *Cell Microbiol* **2005**; 7:789–97.
 25. Kern F, Sural IP, Brock C, et al. T-cell epitope mapping by flow cytometry. *Nat Med* **1998**; 4:975–8.
 26. Morrison J, Elvin J, Latron F, et al. Identification of the nonamer peptide from influenza A matrix protein and the role of pockets of HLA-A2 in its recognition by cytotoxic T lymphocytes. *Eur J Immunol* **1992**; 22:903–7.
 27. Rötzschke O, Falk K, Deres K, et al. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* **1990**; 348:252–4.
 28. Whitton JL, Tishon A, Lewicki H, et al. Molecular analyses of a five-amino-acid cytotoxic T-lymphocyte (CTL) epitope: an immunodominant region which induces nonreciprocal CTL cross-reactivity. *J Virol* **1989**; 63:4303–10.
 29. Lee WT, Vitetta ES. Memory T cells are anergic to the superantigen staphylococcal enterotoxin B. *J Exp Med* **1992**; 176:575–9.
 30. Watson AR, Mittler JN, Lee WT. Staphylococcal enterotoxin B induces anergy to conventional peptide in memory T cells. *Cell Immunol* **2003**; 222:144–55.
 31. Lehner PJ, Wang EC, Moss PA, et al. Human HLA-A0201-restricted cytotoxic T lymphocyte recognition of influenza A is dominated by T cells bearing the V beta 17 gene segment. *J Exp Med* **1995**; 181:79–91.
 32. Thomas D, Dauwalder O, Brun V, et al. *Staphylococcus aureus* superantigens elicit redundant and extensive human Vbeta patterns. *Infect Immun* **2009**; 77:2043–50.
 33. Chen G, Yang X, Ko A, et al. Sequence and Structural Analyses Reveal Distinct and Highly Diverse Human CD8+ TCR Repertoires to Immunodominant Viral Antigens. *Cell Rep* **2017**; 19:569–83.
 34. Miconnet I, Marrau A, Farina A, et al. Large TCR diversity of virus-specific CD8 T cells provides the mechanistic basis for massive TCR renewal after antigen exposure. *J Immunol* **2011**; 186:7039–49.
 35. Cole BC, Atkin CL. The *Mycoplasma arthritidis* T-cell mitogen, MAM: a model superantigen. *Immunol Today* **1991**; 12:271–6.
 36. Friedman SM, Crow MK, Tumang JR, et al. Characterization of human T cells reactive with the *Mycoplasma arthritidis*-derived superantigen (MAM): generation of a monoclonal antibody against V beta 17, the T cell receptor gene product expressed by a large fraction of MAM-reactive human T cells. *J Exp Med* **1991**; 174:891–900.
 37. Cole BC, Washburn LR, Sullivan GJ, Ward JR. Specificity of a mycoplasma mitogen for lymphocytes from human and various animal hosts. *Infect Immun* **1982**; 36:662–6.
 38. Haeryfar SM, Hickman HD, Irvine KR, Tschärke DC, Bennink JR, Yewdell JW. Terminal deoxynucleotidyl transferase establishes and broadens antiviral CD8+ T cell immunodominance hierarchies. *J Immunol* **2008**; 181:649–59.
 39. Chen W, Antón LC, Bennink JR, Yewdell JW. Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. *Immunity* **2000**; 12:83–93.

40. Malchiodi EL, Eisenstein E, Fields BA, et al. Superantigen binding to a T cell receptor beta chain of known three-dimensional structure. *J Exp Med* **1995**; 182:1833–45.
41. Robinson JH, Pyle G, Kehoe MA. Influence of major histocompatibility complex haplotype on the mitogenic response of T cells to staphylococcal enterotoxin B. *Infect Immun* **1991**; 59:3667–72.
42. Rytelwski M, Meilleur CE, Yekta MA, et al. Suppression of immunodominant antitumor and antiviral CD8⁺ T cell responses by indoleamine 2,3-dioxygenase. *PLoS One* **2014**; 9:e90439.
43. Niedergang F, Hémar A, Hewitt CR, Owen MJ, Dautry-Varsat A, Alcover A. The *Staphylococcus aureus* enterotoxin B superantigen induces specific T cell receptor down-regulation by increasing its internalization. *J Biol Chem* **1995**; 270:12839–45.
44. Walsh KB, Teijaro JR, Wilker PR, et al. Suppression of cytokine storm with a sphingosine analog provides protection against pathogenic influenza virus. *Proc Natl Acad Sci U S A* **2011**; 108:12018–23.
45. FRANCIS T Jr. Influenza: the new acquaintance. *Ann Intern Med* **1953**; 39:203–21.
46. Monto AS, Malosh RE, Petrie JG, Martin ET. The doctrine of original antigenic sin: separating good from evil. *J Infect Dis* **2017**; 215:1782–8.
47. Viboud C, Epstein SL. First flu is forever. *Science* **2016**; 354:706–7.
48. Klenerman P, Zinkernagel RM. Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. *Nature* **1998**; 394:482–5.
49. Janik DK, Lee WT. Staphylococcal enterotoxin B (SEB) induces memory CD4 T cell anergy in vivo and impairs recall immunity to unrelated antigens. *J Clin Cell Immunol* **2015**; 6:1–8.
50. Crow MK, Zagon G, Chu Z, et al. Human B cell differentiation induced by microbial superantigens: unselected peripheral blood lymphocytes secrete polyclonal immunoglobulin in response to *Mycoplasma arthritidis* mitogen. *Autoimmunity* **1992**; 14:23–32.