Bacterial Superantigen Exposure after Resolution of Influenza Virus Infection Perturbs the Virus-Specific Memory CD8⁺-T-Cell Repertoire

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Heterologous viral infections have been shown to impact the preexisting memory CD8⁺-T-cell repertoire. Bacterial superantigens are products of common human pathogenic bacteria, including staphylococci and streptococci, that are potent T-cell-stimulatory molecules. In this report, we show that exposure to staphylococcal enterotoxin B, a bacterial superantigen, causes a selective functional deletion of cross-reactive influenza virus-specific CD8⁺ memory T cells. This perturbation of the memory repertoire can have a significant impact on viral clearance after secondary challenge.

Heterologous viral infections have been shown to perturb the memory CD8⁺-T-cell repertoire established to earlier infections both quantitatively and qualitatively. Specifically, the frequency of virus-specific memory CD8⁺ T cells for previous infections is generally reduced upon a second viral infection, but T cells that are cross-reactive with the heterologous virus are selectively retained (10, 20, 21).

Bacterial superantigens are potent immunostimulatory molecules expressed by common strains of gram-positive bacteria, including staphylococci and streptococci. Upon binding to the V β element of the T-cell receptor (TCR), superantigens activate T cells to proliferation, cytokine secretion, and/or cytotoxicity, followed by deletion or anergy (2, 16). Because superantigens are specific for the V β element of the TCR rather than the antigen combining site, they activate a high percentage of T cells. For example, staphylococcal enterotoxin B (SEB) activates murine V β 8⁺ T cells, which represent ~30% of total T cells in most mouse strains, including C57BL/6. Whereas the effects of superantigens on naive T cells have been well characterized, less is known about the effects on memory T cells. We have previously shown that SEB activates influenza virus-specific memory CD8⁺ T cells and drives them to cytolytic effector function (3). This demonstration that superantigens can activate memory CD8⁺ T cells prompted the question of whether superantigen administration to mice that had recovered from influenza virus infection 6 to 8 weeks earlier (influenza memory mice) would have an impact on the repertoire of memory T cells established to an earlier infection.

In the current study, the consequence of superantigen expo-

sure on the repertoire of CD8⁺ memory T cells was examined in a well-characterized murine influenza model. The memory response to influenza virus in C57BL/6 mice is directed largely toward a single, immunodominant epitope in the nucleoprotein molecule, NP₃₆₆₋₃₇₄, presented by D^b (1, 8, 23). We had previously shown that V β 8.3⁺ T cells represent a significant proportion of the response to NP₃₆₆₋₃₇₄/D^b in B6 mice (5, 8, 13). Since V β 8.3⁺ T cells are also SEB reactive, we employed this system to examine the long-term effects of SEB on both V β 8.3⁺ (SEB reactive) and total NP-specific memory CD8⁺ T cells.

Influenza memory mice were generated by intranasal infection of C57BL/6 mice with 240 hemagglutinating units (HAU) of A/HKx31 virus (x31 virus) (4). To assess the effect of bacterial superantigens on previously established memory CD8⁺ T cells, influenza memory mice were exposed to SEB. In some experiments, staphylococcal enterotoxin A (SEA), which specifically activates murine T cells expressing V β s 1, 3, and 11, was administered as a control. Endotoxin-free superantigens (Toxin Technology, Sarasota, Fla.) (26) and phosphate-buffered saline (PBS) were administered to influenza memory mice via surgically implanted Alzet miniosmotic pumps (Alza, Palo Alto, Calif.) that released 2 μ g of SEB or 0.1 μ g of SEA per h over a period of 7 days (14, 18). Preliminary studies showed that, as expected, the SEB pumps caused V β -specific expansion and deletion. However, because the pumps release low, sustained doses of SEB, there was a lower effective concentration and a sustained presence of SEB (data not shown). Because the goal of these experiments was to assess the long-term effects of superantigen exposure on the preexisting memory T-cell repertoire, the influenza memory mice were rested for 4 to 8 weeks after superantigen exposure prior to being analyzed to allow resolution of the acute in vivo response and to ensure that stable effects on the memory repertoire were being measured.

As a first step in assessing the effect of SEB exposure on the

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TABLE 1. SEB treatment of influenza memory mice causes significant reduction in the numbers of Vβ8.3⁺ NP⁺ memory CD8⁺ T cells in the spleen^a

Experimental treatment	No. of CD8 ⁺ T cells (10 ⁶)	No. of NP ⁺ T cells (10 ⁴)	No. of Vβ8.3 ⁺ NP ⁺ T cells (10 ⁴)
PBS	11.44 ± 3.57	103.4 ± 63.4	32.8 ± 23.9
SEB	10.63 ± 2.6	77.4 ± 37.8	8.90 ± 6.4 ^b

^a Influenza memory mice were surgically implanted with miniosmotic pumps containing PBS or SEB. Absolute numbers of spleen cells were determined by three-color surface staining.

^b Statistically significant difference ($P < 0.005$) versus PBS control as determined by Student's t test.

long-term influenza virus-specific memory CD8⁺-T-cell repertoire, the effect of SEB on the numbers of NP⁺ and Vβ8.3⁺ NP⁺ memory CD8⁺ T cells in the spleens of influenza memory mice before secondary challenge was assessed by three-color fluorescence-activated cell sorting (FACS) analysis (13). NP-specific T cells were identified with a tetrameric reagent, the H-2D^b glycoprotein complexed with the influenza virus NP₃₆₆₋₃₇₄ peptide conjugated with phycoerythrin (NP₃₆₆₋₃₇₄/D^b) (8). The data (Table 1) show that there was no significant effect of prior SEB exposure on either total CD8⁺ T cells or NP⁺ CD8⁺ T cells in the spleens of influenza memory mice. However, there was a statistically significant reduction in the Vβ8.3⁺ subset of NP⁺ T cells, which are SEB reactive.

Next, to assess the effects of SEB exposure on the repertoire of NP-specific CD8⁺ T cells elicited during the secondary recall response, memory mice were challenged with 60 HAU of A/PR8 virus 4 to 8 weeks after SEB exposure. A/PR8 virus bears the same internal proteins as x31, including the NP molecule, yet is serologically distinct and avoids neutralization of virus by cross-reactive antibodies. The effect of superantigen exposure on the proportion (Fig. 1) and absolute numbers (Table 2) of NP⁺ CD8⁺ and Vβ8.3⁺ NP⁺ CD8⁺ T cells elicited in the bronchoalveolar lavage (BAL) fluid was assessed 5 to 8 days after secondary challenge.

Analysis of individual mice showed that, as expected, there was an increase in NP⁺ cells between 5 and 7 days after viral challenge and that there was no major effect of either SEB or SEA on the absolute numbers or percentage of NP⁺ T cells in the BAL fluid. However, SEB but not SEA exposure caused a striking reduction in the Vβ8.3⁺ subset of NP⁺ T cells in the BAL fluid. In order to assess the functional impact of the SEB-induced reduction of the Vβ8.3⁺ subset of NP⁺ memory T cells, the recall cytolytic T-cell (CTL) response after secondary viral challenge with PR8 virus was monitored. T cells isolated from BAL fluid samples of secondarily challenged influenza memory mice that had previously been exposed to PBS, SEB, or SEA were tested for cytotoxic activity against NP₃₆₆₋₃₇₄-pulsed L-929D^b targets or L-929D^b targets alone in a standard 4-h ⁵¹Cr release assay (4, 13). The representative data (Fig. 2) show that, despite no significant effect on the total numbers of NP-specific T cells, there was a reduction in the NP-specific recall CTL response, indicated by a four- to eight-fold decrease in the NP-specific CTL activity at day 5 in the mice exposed to SEB. The effect was transient, however, in that there were no differences in the magnitudes of the CTL response assessed at 7 and 8 days postchallenge. Whether these

FIG. 1. Superantigen treatment reduces the proportion of virus-specific cells bearing cross-reactive TCRs in the BAL fluid of influenza memory mice after secondary viral challenge. Nonadherent cells isolated from the BAL fluid of influenza memory mice that had been treated with PBS, SEB, or SEA 1 to 2 months prior to secondary challenge were tested for total NP tetramer-positive cells (A) and Vβ8.3⁺ T cells among the NP tetramer-positive cells (B). The data were compiled from several experiments, and each data point represents analysis of an individual animal. Statistical significance (PBS versus SEB), determined by Student's t test: *, $P < 0.005$; **, $P < 0.05$; ***, $P < 0.000005$. Representative three-color FACS analysis of gated CD8⁺ T cells used to determine the proportion of Vβ8.3⁺ cells among NP tetramer-positive cells is also shown (C).

kinetic differences can be explained by a differential effect of SEB on effector and central memory cells (19) is under investigation.

The finding that SEB and SEA had little effect on the absolute percentage of NP⁺ cells in the BAL fluid of secondarily challenged mice suggested that the T-cell response had not been skewed toward a subdominant epitope. Analysis of the Vβ repertoire among both NP⁺ and NP⁻ cells in the BAL fluid with a panel of TCR Vβ-specific antibodies (13) showed that the substantial reduction in the major Vβ8.3⁺ subset of NP tetramer-positive cells was compensated for by an increase in

TABLE 2. SEB treatment of influenza memory mice causes a significant reduction in the numbers of Vβ8.3⁺ NP⁺ T cells elicited in BAL fluid upon secondary viral challenge^a

Treatment	Day 5			Day 7		
	No. of CD8 ⁺ T cells (10 ⁶)	No. of NP ⁺ T cells (10 ⁴)	No. of Vβ8.3 ⁺ NP ⁺ T cells (10 ⁴)	No. of CD8 ⁺ T cells (10 ⁶)	No. of NP ⁺ T cells (10 ⁴)	No. of Vβ8.3 ⁺ NP ⁺ T cells (10 ⁴)
PBS	0.45 ± 0.27	0.93 ± 0.47	0.32 ± 0.17	3.54 ± 3.64	128.4 ± 121.3	34.6 ± 33.6
SEB	0.57 ± 0.19	0.92 ± 0.66	0.07 ± 0.08 ^b	3.27 ± 1.42	132.7 ± 70.3	6.08 ± 5.8 ^c

^a Influenza memory mice were surgically implanted with miniosmotic pumps containing PBS or SEB. Absolute numbers of the indicated populations of cells in the plastic-nonadherent fraction of BAL cells was determined by three-color surface staining.
^b Statistically significant difference versus PBS control (*P* = 0.006) as determined by Students' *t* test.
^c Statistically significant difference versus PBS control (*P* = 0.049).

NP tetramer-positive cells bearing other TCR Vβ elements, as shown by a substantial increase in NP⁺ cells bearing Vβs 4, 11, and 13, with minor increases in T cells bearing other Vβs (Fig. 3A). As expected, there was no detectable perturbation of the repertoire of NP[−] CD8⁺ T cells (Fig. 3B). Taken together, the data demonstrate a Vβ-specific functional deletion of a subset of the influenza virus-specific memory CD8⁺-T-cell repertoire by SEB. This is consistent with previously described effects of heterologous viral infections (10, 20, 21) and thus broadens the spectrum of heterologous stimulation that may impact the preexisting memory CD8⁺-T-

cell repertoire. Differences between exposure to the superantigen described here and heterologous viral infections described previously (15, 16) must be borne in mind, however. In contrast to the effects described for heterologous viral infections, SEB exposure caused no major quantitative changes in CD8⁺ T cells specific for the immunodominant viral epitope in that there was little effect on the magnitude of NP⁺ CD8⁺ T cells in the influenza memory mouse spleen or attracted to the lung upon secondary challenge. The findings that SEB but not SEA affected Vβ8.3⁺ memory T cells and that the SEB effect was selective for the Vβ8.3⁺ subset of NP⁺ T cells strongly suggest a TCR-mediated mechanism. However, the mechanism has not been directly demonstrated, and an additional role for cytokines has not been ruled out. In order to determine whether the SEB-induced repertoire perturbations impacted protective immunity, viral clearances

FIG. 2. SEB-treated influenza memory mice have a delayed response to secondary viral challenge. Influenza memory mice (8 weeks after primary infection) were treated with PBS, SEB, or SEA. Eight weeks later, the mice were challenged intranasally with PR8, and nonadherent BAL fluid cells were assayed for CTL activity against NP₃₆₆₋₃₇₄ peptide-labeled targets (solid symbols) or target cells alone (open symbols) at days 5, 7, and 8 after secondary challenge. Three representative experiments are shown for analysis at day 5 (A), and single representative experiments are shown for analysis at days 7 and 8 (B). The data shown are representative of eight independent experiments.

FIG. 3. SEB treatment alters the TCR Vβ repertoire of NP⁺ CD8⁺ T cells elicited in the BAL fluid after secondary viral infection. The TCR Vβ repertoire of BAL fluid cells elicited 5 days after secondary viral challenge with PR8 was assessed by three-color FACS with a panel of antibodies specific for most of the murine TCR Vβ elements (13). Data are expressed as the percentage of CD8⁺ T cells expressing a particular TCR Vβ element among the NP tetramer-positive (A) or NP tetramer-negative (B) CD8⁺ T cells. The data shown are from a single, representative analysis of pooled data from five mice. The deletion of Vβ8.3⁺ NP⁺ T cells by SEB was consistently seen in three independent experiments, whereas the TCR Vβ expression of compensating T cells varied between experiments.

TABLE 3. SEB treatment of influenza memory mice causes delayed viral clearance after secondary viral challenge in thymectomized and aged mice^a

Day after secondary challenge	Treatment group	Virus titers (10 ³ PFU)
6	Sham thyx, PBS	2, 30, 31
	Sham thyx, SEB	1, 18, <1
	Thyx, PBS	110, 1, 13
	Thyx, SEB	50, 300, 420
7	Sham thyx, PBS	<1, <1, <1 ^b
	Sham thyx, SEB	<1, <1, <1
	Thyx, PBS	2, 3, <1 ^b
	Thyx, SEB	35, 34, 8 ^b
	Aged, PBS	<1, <1, 1 ^c
	Aged, SEB	150, 210, 140 ^c
8	Sham thyx, PBS	<1, <1
	Sham thyx, SEB	<1, <1
	Thyx, PBS	<1, <1
	Thyx, SEB	<1, <1

^a At the indicated times after secondary viral challenge with PR8, lungs were obtained for determination of virus titers. Thymectomized (thyx) and sham-thymectomized mice were infected with influenza virus strain x31 at least 3 weeks after thymectomy, and 6 to 8 weeks after infection, they were implanted with Alzet osmotic pumps containing PBS or SEB. Four to eight weeks after pump implantation, mice were challenged with PR8 virus. Aged mice were virally infected at 3 months of age with x31, implanted with PBS or SEB pumps at 9 months of age, and challenged with PR8 virus at 15 months of age. Each value represents analysis of an individual mouse. The data are from one representative experiment of two. Virus titers in the lungs of individual mice were determined by plaque assay on MDCK cells.

^b At day 7, virus titers between sham-thymectomized and thymectomized PBS- and SEB-treated mice were not statistically different.

^c Virus titers at day 7 between aged PBS- and aged SEB-treated mice were statistically different ($P < 0.05$).

after secondary challenge of PBS- and SEB-treated influenza memory mice were compared. Initial results showed no effect on viral clearance (data not shown), consistent with previous observations after depletion of all V β 8⁺ T cells by *in vivo* antibody treatment (5). These results are consistent with the idea that repertoire plasticity can effectively compensate for losses of major components of the repertoire (4, 6, 7). Recent studies showed concurrent primary responses during the recall response after secondary viral challenge (24). Therefore, the viral clearance experiments were repeated in mice that had been thymectomized prior to initial viral infection to eliminate the possible compensation of an SEB-induced defect in the memory response by naïve T cells that had emigrated from the thymus after SEB exposure.

Virus titers in the lungs of thymectomized and sham-thymectomized influenza memory mice that had been treated with PBS or SEB were assessed by a standard plaque assay (9) on days 6, 7, and 8 after secondary infection. The results (Table 3) show a delay in viral clearance in thymectomized but not sham-thymectomized SEB-treated influenza memory mice. In these experiments, thymectomy is predicted to impact the naïve rather than the memory repertoire, as persistence of memory T cells is independent of thymic output of naïve T cells (22). Therefore, these data suggest that the delayed viral clearance in SEB-treated thymectomized mice is a consequence of a reduced contribution of naïve CD8⁺ T cells to control of the viral challenge. This possibility can be directly

tested by adoptively transferring naïve CD8⁺ T cells into thymectomized mice prior to secondary challenge. However, the mice still cleared the virus, consistent with the plasticity of the memory repertoire.

Aged mice are thought to have an even more limited CD8⁺-T-cell repertoire (17). Therefore, we tested the effect of SEB exposure on the ability of aged mice to clear virus after secondary challenge. Analysis of aged mice at 7 days post-secondary challenge showed that SEB-treated mice had substantial amounts of virus in the lung, whereas PBS-treated age-matched controls had cleared the virus by day 7. Although a more thorough kinetics analysis is necessary, these data support the hypothesis that SEB-mediated effects on the preexisting memory repertoire are more profound in aged individuals, in whom the repertoire is predicted to be restricted and hence less plastic. We are currently characterizing the naïve and virus-specific CD8⁺-T-cell repertoire in aged mice.

Finally, the current experiments do not address the effect of SEB, thymectomy, or aging on virus-specific CD4⁺ T cells, which contribute both directly and indirectly to CD8⁺-T-cell recall responses (25). In this regard, it has been shown that CD4⁺ and CD8⁺ memory T cells are maintained differently (12), and in contrast to CD8⁺ memory T cells, memory CD4⁺ T cells have been shown to be nonresponsive to superantigen stimulation (3, 11, 15).

In conclusion, the present report shows that bacterial superantigen exposure profoundly affects the TCR repertoire of memory CD8⁺ T cells and the kinetics of the recall CTL response after secondary viral challenge. This effect is in contrast to our previous studies, in which we found no consequences of concurrent exposure to SEB and influenza virus on the generation of virus-specific memory CD8⁺ T cells (13). That bacterial superantigens have different effects on the generation and maintenance of memory cells is not surprising. Importantly, the data are consistent with the idea that, even when the CD8⁺-T-cell response to an immunodominant epitope is mediated by a limited repertoire of T cells, there is remarkable plasticity to the repertoire, and T cells bearing other TCRs can effectively compensate. There are limits to the plasticity, however, and in cases of severe repertoire perturbation, the response may be diverted to other, normally subdominant epitopes (4, 6) and may result in delayed viral clearance (7).

Immune individuals are probably exposed to bacterial superantigens with relatively high frequency, either in the context of bacterial infections or as isolated toxins as a consequence of food poisoning. Therefore, the ability of superantigens to perturb the repertoire of memory CD8⁺ T cells may be of significant clinical relevance, especially as the plasticity of the repertoire may diminish with age, making repertoire perturbations functionally more relevant in older individuals.

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