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Brief Definitive Report

Cross-linking of Major Histocompatibility Complex Class II Molecules by Staphylococcal Enterotoxin A Superantigen Is a Requirement for Inflammatory Cytokine Gene Expression

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Summary

Staphylococcal enterotoxin A (SEA) has two distinct binding sites for major histocompatibility complex (MHC) class II molecules. The aspartic acid located at position 227 (D227) in the COOH terminus of SEA is one of the three residues involved in its interaction with the DRβ chain, whereas the phenylalanine 47 (F47) of the NH_2 terminus is critical for its binding to the DRα chain. Upon interaction with MHC class II molecules, SEA triggers several cellular events leading to cytokine gene expression. In the present study, we have demonstrated that, contrary to wild-type SEA, stimulation of the THP1 monocytic cell line with SEA mutated at position 47 (SEA_{F47A}) or at position 227 (SEA_{D227A}) failed to induce interleukin 1β and tumor necrosis factor–α messenger RNA expression. Pretreatment of the cells with a 10-fold excess of either SEA_{F47A} or SEA_{D227A} prevented the increase in cytokine messenger RNA induced by wild-type SEA. However, cross-linking of SEA_{F47A} or SEA_{D227A} bound to MHC class II molecules with $F(ab')_2$ anti-SEA mAb leads to cytokine gene expression, whereas cross-linking with F(ab) fragments had no effect. Taken together, these results indicate that cross-linking of two MHC class II molecules by one single SEA molecule is a requirement for cytokine gene expression.

Bacterial superantigens (SAGs), including the *Staphylococcus aureus* products staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), and toxic shock syndrome toxin 1 (TSST1), have potent effects on the immune system and a pathophysiological role in toxic shock (1). Upon interaction with their natural receptors, the MHC class II molecules (2–4), SAGs activate a large fraction of T cells (5) and induce a variety of cellular events in MHC class II–expressing cells, such as cell–cell adhesion (6), inflammatory cytokine gene expression (7), and T cell–dependent B cell proliferation and differentiation (8). The crystal structure of the TSST1–DR1 and the SEB–DR1 complexes has been solved, and the residues involved in the interaction with MHC class II molecules are well defined (9, 10). The crystal structure of the SEA–DR complex is not yet available. However, it was shown that SEA has two distinct binding sites for HLA-DR molecules (11). The first site is located in the COOH terminus, whereas the second is located in the NH_2-terminal domain of SEA. Binding of SEA through its COOH terminus to HLA-DR is Zn^{2+} dependent, and this ion coordinates the interaction between histidine (H) at position 187 and 225, aspartic acid (D) at position 227 of the SEA, and the histidine 81 of HLA-DR molecules' β chain (12–15). The phenylalanine at position 47 (F47), located in the NH_2-terminal domain of SEA, is critical for the second SEA-binding site (11). Accordingly, our recent data indicate that SEA interacts through its NH_2 terminus with the DRα chain in a manner similar to SEB (Thibodeau, J., M. Dohlsten, I. Cloutier, C. Léveillé, P. Bjork, T. Kalland, W. Mourad, and R. P. Sekaly, manuscript in preparation). This finding led us to propose that the two binding sites on SEA may allow the cross-linking of two MHC class II molecules.

In the present study, we have used SEA mutants that bind either to the MHC class II α or β chain, to demonstrate that cross-linking two MHC class II molecules on the cell surface by a single SEA molecule is a requirement for inflammatory cytokine gene expression. This finding may be fundamental to the understanding of the mechanisms of action of bacterial SAGs.

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Materials and Methods

Generation of Recombinant SEA Mutants. Four different SEA mutants were generated as we have previously described: (*a*) The aspartic acid at position 227 was substituted by alanine (SEA_{D227A}); (*b*) the phenylalanine at position 47 was substituted by alanine (SEA_{F47A}); (*c*) double mutants in which F47 and D227 were substituted by alanines were generated ($SEA_{F47A/D227A}$); and (*d*) a control mutant in which the asparagine at position 128 was substituted by alanine was generated (SEA_{N128A}). Briefly, the SEA gene was cloned from *S. aureus* (16), and its sequence was found to be identical to a published one (17). The SEA gene was expressed in *Escherichia coli*, using the regulatory elements of staphylococcal protein A for transcription and translation and as a synthetic signal sequence for secretion (18). The SEA mutants F47A, D227A, and N128A and the double mutant F47A/D227A were obtained by alanine substitutions using oligonucleotide primers and PCR. A unique restriction enzyme site was introduced into each mutant to enable its identification. Each mutation was confirmed by sequencing the relevant portion of the gene. The SEA was then expressed in *E. coli* K12 strain UL635 and purified from the periplasmatic extract by affinity chromatography using immobilized anti-SEA antibodies. The purity of these mutants was confirmed by SDS-PAGE followed by Coomassie blue staining or immunoblot analysis with SEA-specific mAbs.

Cells. The THP1 monocytic human cell line was obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium containing 10% FCS and antibiotics. This cell line expresses very low levels of HLA-DR molecules, whereas it is completely negative for HLA-DQ and -DP (19). To induce high levels of MHC class II molecules, cells were stimulated with IFN-γ (50 U/ml) for 48 h. Before use, cells were washed twice with HBSS.

Flow Cytometric Analysis. Binding of SEA wild type (SEAwt) and mutants to IFN-γ–treated THP1 cells was determined by flow cytometric analysis. Cells were first incubated with 1 μg of SEAwt or SEA mutants (2×10^5 cells per sample). After 3 h of incubation, the cells were washed and resuspended in 100 μl of staining buffer containing 1 μg of anti-SEA mAb. 30 min later, the cells were washed, and rabbit anti–mouse IgG-FITC conjugate was added and incubated for 30 min. Washed cells were then analyzed by FACScan® (Becton Dickinson & Co., Cockeysville, MD). A total of 10^4 cells gated by light scatter on live cells were analyzed.

Binding Inhibition Assay. The capacity of the SEA mutants to inhibit binding of the ^{125}I-radiolabeled SEAwt to IFN-γ–treated THP1 cells was determined as we have previously described (20). Cells (5×10^5) were incubated for 3 h at 37°C with ^{125}I-labeled SEA (20 ng) in 200 μl of binding buffer (RPMI 1640 plus 2.5% FCS and 0.02% sodium azide). The cells were then pelleted through an oil cushion (84% silicon oil and 16% mineral oil), and their activity (cpm) was determined using a gamma counter. In the competitive tests, the cells were first incubated for 30 min at 37°C with cold SEAwt or SEA mutants. All tests were performed in triplicate, and SEM was <10% in all assays.

Northern Blot Analysis. Stimulation conditions for each experiment are detailed in the appropriate figure legends. RNA was purified according to the classical method (21), and 10 μg of RNA was loaded onto 1% agarose gels. The RNA was then transferred onto Hybond-N filter paper and was hybridized with random primer–labeled cDNA probes for IL-1β and TNF-α (22). Equal loadings of RNA were confirmed by hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. The messenger RNA (mRNA) hybridizing with the cDNA probes was visualized by autoradiography.

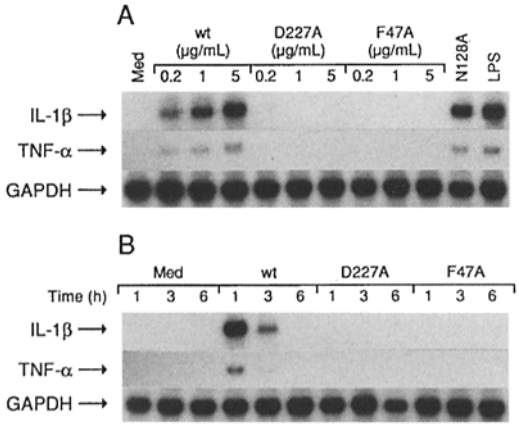

Figure 1. SEA_{F47A} and SEA_{D227A} failed to induce IL-1β and TNF-α mRNA expression in the THP1 monocytic cell line. (*A*) Dose response and (*B*) time-course of IL-1β and TNF-α mRNA expression in IFN-γ–treated THP1 monocytic cell line. Cells were treated with different concentrations (0.2–5 μg/ml) of SEAwt or SEA mutants for 1 h at 37°C, or with 5 μg/ml of SEAwt or SEA mutants for increasing periods of time. Cells were then harvested, RNA was extracted, and 10 μg of total RNA was loaded in each lane, subjected to electrophoresis, transferred to nitrocellulose filters, and hybridized with a probe for IL-1β followed by TNF-α and the housekeeping gene GAPDH as control for equal RNA loading.

Results and Discussion

Dimerization or even multimerization of receptors is commonly required for signal transduction (23). To assess the biological function of the two SEA-binding sites, we examined the capacity of SEA mutants to trigger inflammatory cytokine gene expression in THP1 cells. As previously demonstrated (24), stimulation of IFN-γ–treated THP1

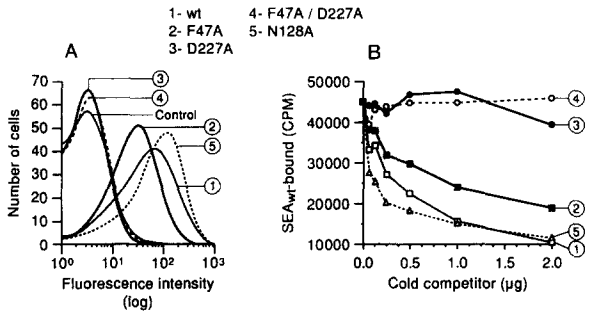

Figure 2. Binding of wild-type and mutated SEA to IFN-γ–treated THP1 as detected by flow cytometry (*A*), and the ability of SEA mutants to inhibit ^{125}I-labeled wild-type SEA binding to IFN-γ–treated THP1 (*B*). For flow cytometric analysis, cells were first incubated with SEAwt or SEA mutants, followed by anti-SEA mAb. Cells were then washed, rabbit anti–mouse IgG-FITC conjugate was added, and cells were incubated for 30 min. Washed cells were then analyzed by FACScan®. For binding inhibition assay, cells were incubated with ^{125}I-labeled SEA in the absence or the presence of cold SEAwt or SEA mutants. Cells were then pelleted through an oil cushion (84% silicon oil and 16% mineral oil), and their activity (cpm) was determined using a gamma counter. All tests were performed in triplicate, and SEM was <10% in all assays.

cells with SEAwt leads to signal transduction through MHC class II molecules, resulting in IL-1β and TNF-α gene expression. Fig. 1 *A* shows that stimulation of THP1 cells with SEAwt for 1 h induced a dose-dependent increase in the levels of IL-1β and TNF-α mRNA. Significant levels of both cytokines were observed at concentrations as low as 0.2 μg/ml. In contrast, mutants SEA_{F47A} and SEA_{D227A} failed to induce any detectable inflammatory cytokine gene expression at even the highest toxin concentrations used (5 μg/ml). SEA_{N128A} still has the same activity as the SEAwt (Fig. 1 *A*). Detailed time-course experiments revealed that SEAwt induces an early peak of both cytokines at 1 h, followed by a significant decrease after 2 and 3 h (Fig. 1 *B*). The failure of SEA_{F47A} and SEA_{D227A} to trigger cytokine gene expression can be explained by the inability of these mutants to interact with MHC class II molecules or by the absolute requirement for SEA interaction with the two sites on MHC class II molecules. To verify the first possibility, flow cytometric analysis and binding inhibition experiments were performed. Fig. 2 *A* indicates that SEAwt and SEA_{N128A} bound equally well to IFN-γ–treated THP1 cells. In contrast, binding of SEA_{F47A} was less efficient (at least twofold less), whereas binding of SEA_{D227A} or $SEA_{F47A/D227A}$ was almost undetectable. The specificity and characteristics of the binding were further addressed by determining the capacity of SEA mutants to inhibit the binding of ^{125}I-labeled SEAwt. Binding of ^{125}I-labeled SEAwt was completely inhibited by SEAwt and SEA_{N128A}. SEA_{F47A} also significantly inhibited the binding of ^{125}I-labeled SEAwt; however, a twofold excess of SEA_{F47A} was required to achieve

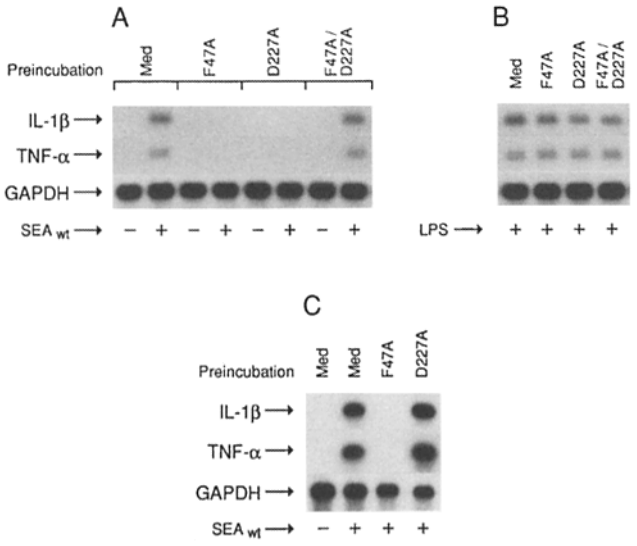

Figure 3. SEA_{F47A} and SEA_{D227A} inhibited SEAwt- (*A*) but not LPS-induced (*B*) IL-1β and TNF-α mRNA expression. (*C*) The inhibitory effect of SEA_{D227A} was completely abolished when cells pretreated with SEA_{D227A} were washed before their stimulation with SEAwt. Cells were first incubated with SEA_{F47A}, SEA_{D227A}, or $SEA_{F47A/D227A}$ (5 μg/ml) for 2 h at 37°C. Cells were then either stimulated directly (*A* and *B*) with SEAwt (0.5 μg/ml) or with LPS (1 μg/ml), or washed twice with HBSS (*C*), and then stimulated with SEAwt (0.5 μg/ml) for an additional 1 h. The reaction was stopped, total RNA was purified, and the levels of IL-1β and TNF-α mRNA were determined as described in the legend of Fig. 2.

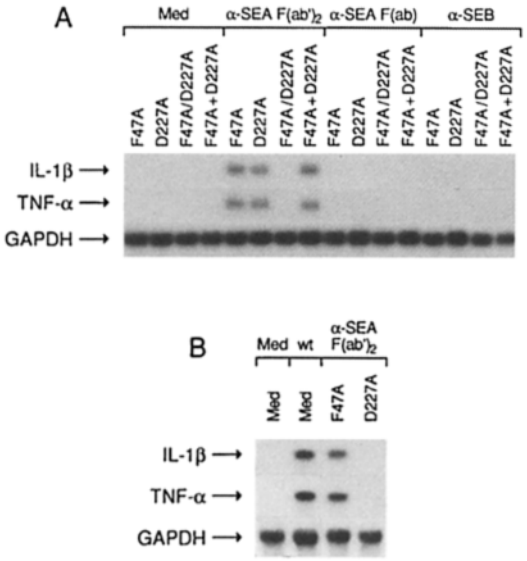

Figure 4. (*A*) Cross-linking of SEA_{F47A} or SEA_{D227A} bound to MHC class II molecules by $F(ab')_2$ anti-SEA–specific mAb is required for the induction of IL-1β and TNF-α mRNA expression. (*B*) The ability of $F(ab')_2$ anti-SEA mAb to cross-link SEA_{D227} and induce cytokine gene expression was completely abolished when SEA_{D227A}-pretreated cells were washed before the addition of $F(ab')_2$ anti-SEA mAb. Cells were first incubated with $SEA_{F47A/D227A}$, SEA_{F47A}, SEA_{D227A}, or both (5 μg/ml each) for 2 h at 37°C. $F(ab')_2$ anti-SEA mAb, F(ab) fragments of anti-SEA mAb, or an isotype control (anti-SEB) (10 μg/ml) were added directly to unwashed cells (*A*) or to twice HBSS-washed cells (*B*). 1 h later, the reaction was stopped, and IL-1β and TNF-α mRNA expression was determined by Northern blot analysis as described in the legend of Fig. 2.

the same magnitude of inhibition seen with SEAwt or SEA_{N128A}. In contrast, SEA_{D227A} and $SEA_{F47A/D227A}$ failed to affect SEAwt binding (Fig. 2 *B*). These results confirm those observed using Raji cells or DR1-transfected cells (11), and indicate that the failure of SEA_{F47A} to induce gene expression is not due to its inability to bind MHC class II molecules.

To verify the second possibility, we examined the effect of blocking one of those sites on the signal induced by SEAwt. Pretreatment of THP1 cells with a 10-fold excess of SEA_{F47A} or SEA_{D227A} but not $SEA_{F47A/D227A}$ completely inhibited the SEAwt-induced responses (Fig. 3 *A*). The same treatment failed to affect LPS-induced cytokine gene expression (Fig. 3 *B*), demonstrating the specificity of this effect. This inhibition is not the result of a negative signal, since it requires the continuous presence of SEA_{D227A} during SEAwt stimulation. Removal of the SEA_{D227A} excess by washing abolished its capacity to inhibit SEAwt-induced responses (Fig. 3 *C*). These results confirm our previous data (11, and Thibodeau, J., M. Dohlsten, I. Cloutier, C. Léveillé, P. Bjork, T. Kalland, W. Mourad, and R. P. Sekaly, manuscript in preparation), showing that SEA_{D227A} binds to DRα with low affinity (10^{-5} M, undetectable by cytometric analysis or binding inhibition assay), but it is still capable of preventing SEAwt from occupying the α chain binding site (Fig. 3 *C*). These results led to the hypothesis that SEA must cross-link two MHC class II molecules by occupying both MHC class II binding sites to efficiently

trigger THP1 activation. This is consistent with the absence of gene expression observed when both mutants were added simultaneously (Fig. 4 *A*).

Two possible models emerge to explain the results: First, multimerization of MHC class II molecules must occur in a specific topology involving a simultaneous interaction with both DRα and -β chains; second, multimerization of MHC class II molecules through either the α or β chain could lead to signal transduction. To differentiate between these two models, SEA mutants were added independently or in combination, and then cross-linked with $F(ab')_2$ or F(ab) forms of an SEA-specific mAb. Cross-linking of SEA_{F47A} or SEA_{D227A} with $F(ab')_2$ anti-SEA mAb leads to cytokine gene expression, whereas F(ab) fragments or the isotype control had no effect (Fig. 4 *A*). A similar response was obtained with cells stimulated with either mutant alone or with a combination of both. Cross-linking $SEA_{F47A/D227A}$ with $F(ab')_2$ anti-SEA mAb did not induce IL-1β and TNF-α gene expression. Accordingly, cross-linking of class II molecules, whether through the α or β chain, is sufficient for SEA-induced cytokine gene expression. The failure of F(ab) anti-SEA mAb to induce any response is not due to the inability of these fragments to bind to SEA, since Western blot analysis clearly demonstrates that F(ab) fragment recognized the SEA mutants in the same fashion as $F(ab')_2$ (data not shown). Once again, washing SEA_{D227A}-pretreated cells before their stimulation with $F(ab')_2$ anti-SEA abolished this response (Fig. 4 *B*). Altogether, these results indicate that the mere aggregation of MHC class II molecules by SEA is sufficient to induce signal transduction, a finding compatible with the fact that antibodies to several DR epitopes are also capable of eliciting similar events (22).

These results support the recent hypothesis that a single SEA molecule can cross-link two MHC class II molecules on the cell surface (11). Here we show that SEA cross-linking of MHC class II molecules leads to major effects on APCs, which in turn could enhance T cell activation. Cross-linking of class II molecules in a nominal antigen response leads to the up-regulation of costimulatory molecules in APCs (25). Similarly, the cross-linking suggested by the current studies also induces the expression of cytokines, which can modulate cellular immune responses (8). Considering the crystal structure of SEB (9), TSST1 (10), and SEA (26), it seems these three toxins bear a similar configuration, and in all cases their interaction with MHC class II molecules implicates dimerization of class II molecules or of the toxins. Indeed, in the SEB:DR1 cocrystal, the superdimer of class II dimers was present, and the presence of a TSST1 dimer was also observed in the TSST1:DR1 cocrystal. We hereby show that SEA can dimerize class II molecules by interacting with two different sites, which appears to be a prerequisite for their exquisite effects on T cells and on APCs.

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