Extracellular adherence protein (Eap) from *Staphylococcus aureus* does not function as a superantigen

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Abstract

Extracellular adherence protein (Eap) from *Staphylococcus aureus* has been reported to have strong anti-inflammatory properties, which make Eap a potential anti-inflammatory agent. However, Eap has also been demonstrated to trigger T-cell activation and to share structural homology with superantigens. In this study, we focused on whether Eap fulfilled the definition criteria for a superantigen. We demonstrate that T-cell activation by Eap is dependent on both major histocompatibility complex class II and intercellular adhesion molecule type 1, that cellular processing is required for Eap to elicit T-cell proliferation, and that the kinetics of proliferation resemble the profile of a conventional antigen and not that of a superantigen.

Keywords: Bare lymphocyte syndrome (BLS), extracellular adherence proteins, *Staphylococcus aureus*, superantigen, T-cell proliferation

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Staphylococcus aureus is an important cause of severe bacterial infections in humans [1]. Extracellular adherence protein (Eap) is a virulence factor expressed by the vast majority of *S. aureus* strains [2,3] and consists of four to six repeats. Eap has many functions, including involvement in adherence and internalization of the bacteria into eukaryotic cells [4,5], and anti-inflammatory activities mediated through binding to intercellular adhesion molecule type 1 (ICAM-1), resulting in impairment of neutrophil and T-cell recruitment [6,7]. Eap has also been shown to inhibit T-cell responses [8,9]. Taken together, the functional analyses of Eap have emphasized its anti-inflammatory properties, and consequently, the use of Eap as a potential anti-inflammatory agent has been proposed. However, Eap has furthermore been reported to have immunostimulatory properties, including activation of monocytes to secrete proinflammatory cytokines [10] as well as T-cell activation [8,9]. It has also been suggested that Eap interacts with immune cells in a superantigen manner, as the three-dimensional structure of Eap domains shows homology with the C-terminal domain of bacterial superantigens [11]. These starkly different functional properties of Eap prompted us to investigate the mechanism by which Eap activates human T-cells. We focused on the interaction between Eap and major histocompatibility complex (MHC) class II molecules and whether Eap fulfilled the definition criteria of a superantigen, including MHC class II dependence and no cellular processing.

To assess MHC class II dependency, a cell proliferation assay with human peripheral blood mononuclear cells (PBMCs) was used essentially as described elsewhere [8]. MHC class II molecules were blocked by pre-incubation for 20 min with either anti-human leukocyte antigen (HLA)-DR (Becton Dickinson) (20 μL/1 $\times$ 10^6 PBMCs) or anti-human ICAM-1 antibodies (CD54; R&D Systems) (8 mg/L/1 $\times$ 10^6 PBMCs). Anti-ICAM-1 antibodies were used because ICAM-1 is a known receptor for Eap [6,7]. PBMCs were then stimulated with Eap (9 mg/L) purified from *S. aureus* supernatants as described previously [5]. As positive controls, the superantigen toxic shock syndrome toxin 1 (TSST-1) (1.25 μg/L; Sigma) and phytohaemagglutinin (PHA) (2 mg/L, Sigma) were used. These concentrations were used in all of the experiments in this study. Proliferative responses were assessed after 72 h by [^{3}H]thymidine uptake. As previously reported [8], the proliferative response induced by Eap varied among different donors (Fig. 1a). Importantly, the response was almost completely abolished (98% inhibition) by pretreatment of the cells with anti-MHC class II antibodies ($p < 0.01$) (Fig. 1a), demonstrating that MHC class II is required for an optimal T-cell response by Eap. Also, anti-ICAM-1 antibodies inhibited Eap-induced proliferation, but to a lesser extent (55–82%; $p < 0.05$) (Fig. 1a), indicating that this receptor is also involved, most likely as a co-stimulatory molecule [12,13]. To ensure that the responses that we measured were Eap-specific, and not due to contaminating superantigens, experiments were conducted using recombinant Eap fragment encompassing repeats 1–3. Expression and purification of recombinant Eap repeats were performed as detailed

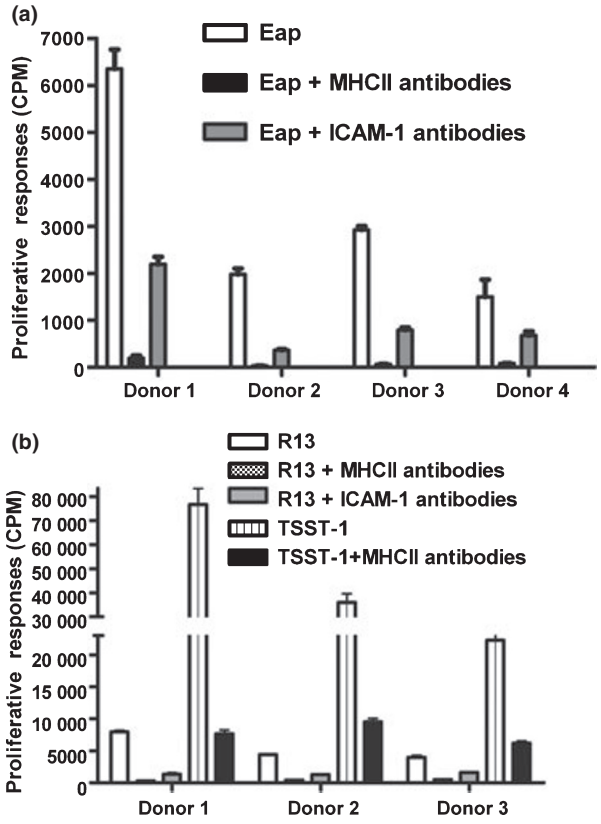

FIG. 1. Extracellular adherence protein (Eap)-induced T-cell activation is dependent on major histocompatibility complex (MHC) class II and intercellular adhesion molecule type 1 (ICAM-1). Peripheral blood mononuclear cells were pre-incubated with either anti-MHC class II antibodies or anti-ICAM-1 antibodies for 20 min prior to stimulation with Eap (9 mg/L) (a) or recombinant Eap fragment (R13) (30 mg/L) and toxic shock syndrome toxin 1 (TSST-1) (1.25 ng/mL) (b). T-cell proliferation was quantified by [^{3}H]thymidine uptake, and data are presented as mean counts per minute (CPM). Error bars indicate standard deviation. (a) and (b) show data from, respectively, four and three different donors. Statistical differences were determined by two-way ANOVA, and demonstrated significant inhibition of Eap responses by both anti-ICAM-1 ($p < 0.05$), anti-MHC class II ($p < 0.01$), and inhibition of TSST-1 by anti-MHC class II ($p < 0.05$).

elsewhere [14]. As shown in Fig. 1b, the recombinant Eap-repeat fragment also induced T-cell proliferation, and, similarly to what was found with native Eap, the response was inhibited by 92% and 71% by pre-blocking with anti-MHC class antibodies ($p < 0.01$) and ICAM-1 antibodies ($p < 0.05$), respectively. T-cell proliferation in the presence of TSST-1, the positive control, was inhibited by 73% and 90% by pre-blocking with anti-MHC class antibodies ($p < 0.05$) Fig. 1b.

To further assess MHC class II dependency, we used a co-culture system using T-cells together with metabolically inactive mitomycin C-treated BLS cells, untransfected or transfected with class II alleles. BLS cells originate from a patient with bare lymphocyte syndrome, and are deficient in class II expression. This model system has been used in several studies of streptococcal and staphylococcal superantigen-induced T-cell activation [15–17]. In this study, BLS cells transfected with the HLA class II alleles BLS-DQ3.2 or BLS-DR4 were cultured together with T-cells purified from healthy donors. Cells were prepared and cultured as previously detailed [17]. Mitomycin C-treated BLS cells (1×10^8 cells/L) were cultured with freshly isolated human T-lymphocytes (2×10^8 cells/L) in the presence or absence of Eap, TSST-1 or PHA. As shown in Fig. 2a, Eap failed to induce T-cell proliferation in this assay, whereas the superantigen TSST-1 induced marked T-cell responses when presented by the HLA class II-transfected cells. To investigate whether Eap required cellular processing in order to activate T-cells, monocytes (5×10^8 cells/L) were incubated for 3 h in the presence or absence of chloroquine (0.05 mM; Sigma) prior to incubation with freshly isolated lymphocytes (3×10^9 cells/L). The cells were stimulated with Eap, TSST-1 or a positive control antigen, CEF (cytomegalovirus, Epstein–Barr virus and influenza virus), a control peptide pool (NIH research & reference reagent) (2.5 mg/L). Chloroquine inhibits the acidification of early endosomes and lysosomes which result in an impairment of the antigen presenting process [18]. Chloroquine treatment resulted in a marked reduction of 40–92% in both Eap and CEF-induced T-cell responses, whereas the TSST-1 responses were equal in cultures with treated or untreated cells (Fig. 2b). The lack of response by Eap in this model indicates that Eap responses are MHC-restricted and require cellular processing in order to induce T-cell activation, just like a conventional antigen but unlike a superatigen.

Studies of the kinetics of T-cell activation [19] revealed that the proliferative response of Eap and CEF could be detected after 3 days of stimulation; the response peaked between days 5 and 7, and declined thereafter (Fig. 2c). On the contrary, TSST-1-induced and PHA-induced responses peaked at day 3 and decreased rapidly thereafter.

Thus, despite Eap's structural homology with superantigens [11], Eap does not act as a superantigen, which is in concordance with the report of Massey *et al.* [20]. However, considering Eap's potential use as an immunomodulatory agent, it is of importance to fully elucidate its interaction with human immune cells. Here, we demonstrate for the first time that Eap-induced T-cell activation is dependent on MHC class II, and that ICAM-1 also contributes to Eap-induced T-cell proliferation. In addition, we demonstrate that cellular processing is required for Eap to elicit T-cell proliferation, and that the kinetics of proliferation resemble the profile of a conventional antigen and not that of a superantigen.