

Use of Peptide-Major Histocompatibility Complex Tetramer Technology To Study Interactions between *Staphylococcus aureus* Proteins and Human Cells[∇]

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In this study, we report the use of peptide-major histocompatibility complex tetramer technology to study the interactions that occur between *Staphylococcus aureus* proteins and human leukocytes. We demonstrated that this technology can be used to study the activity of superantigens such as toxic shock syndrome toxin 1 and also found that despite similarities to known proteins (i.e., major histocompatibility complex [MHC] class II molecules and superantigens), the *S. aureus* Eap protein does not block MHC-T-cell receptor interactions and is not a superantigen. Instead, it has nonspecific cross-linking activity that is dependent upon having at least two of its six 110-amino-acid repeats.

The study of viral interactions with human cells has been greatly advanced by the use of peptide-major histocompatibility complex (pMHC) tetramer technology (1), but the use of this technology in the study of bacterial interactions is in its infancy. This technology is based on the construction of a protein complex consisting of recombinant major histocompatibility complex (MHC) molecules (either class I or class II) that present a specific antigen and have been tetramerized and fluorescently labeled. These molecules are used to specifically label T cells within a population that have receptors that recognize the antigen in question. Here, we evaluate the use of this technology in the study of proteins secreted by the major human pathogen *Staphylococcus aureus*. While *S. aureus* is a largely commensal organism living asymptotically in the nasal cavities of large proportions of the human population (15), it also causes infections that range widely in both the body site affected and the severity. Skin infections such as impetigo, folliculitis, and boils can be caused by *S. aureus*, as can life-threatening illnesses such as endocarditis, osteomyelitis, and septicemia (14). Despite its largely commensal lifestyle, this organism expresses a large number of factors such as toxins, adhesins, invasins, and modulins that contribute to its pathogenicity (14). These factors allow the bacterium to attach to and invade host tissues, causing widespread damage, and they also facilitate the evasion of the host's protective immune responses (6, 14).

Superantigens are proteins secreted by bacteria that act by nonspecifically cross-linking MHC class II (MHC-II) molecules to T-cell receptors (TCRs), resulting in the hyperactivation of the bound T cells and causing toxic shock (5, 10). In this study, we evaluated the use of pMHC technology to study this cross-linking

activity and found that the technology is sufficiently sensitive to enable the detection and enumeration of T cells that become cross-linked with MHC molecules upon exposure to the superantigen toxic shock syndrome toxin 1 (TSST-1).

Another protein with immunomodulatory activity secreted by *S. aureus* is the extracellular adhesion protein Eap (9), also known as the MHC analog protein Map (11). Eap is a member of the SERAM (secreted expanded-repertoire adhesive molecule) family (4), and a recent study has shown that at least 98% of *S. aureus* strains secrete a form of this protein (2). With some strain-to-strain variation, the Eap protein consists of four to six repeats of approximately 110 amino acids (2). It has been shown that, in vivo, Eap has anti-inflammatory activity mediated by its ability to block interactions between leukocytes and intercellular adhesion molecule 1 (3). Based on amino acid similarities to MHC-II molecules, it has been proposed that Eap may block MHC-TCR interactions (6, 11). In contrast to this putative blocking activity, a recent structural study has revealed homology between the individual repeats of Eap and the C-terminal halves of superantigens such as TSST-1 and staphylococcal enterotoxin B (7). This finding suggests that Eap may have superantigenic activity, that is, the ability to mediate the cross-linking of MHC molecules and TCRs (7). Here, we tested these two proposed activities of Eap by using pMHC technology and found that Eap neither blocks MHC-TCR interactions nor acts as a superantigen; instead, Eap has nonspecific cross-linking activity, for which at least two of its six repeats are required. While it is as yet unclear what the downstream effects of this cross-linking activity may be in vivo, we have shown that this pMHC technology is a valuable in vitro tool for the study of host-pathogen interactions.

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MATERIALS AND METHODS

Isolation of human PBMCs. Blood was collected from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation over Lymphoprep (Axis Shield). PBMCs

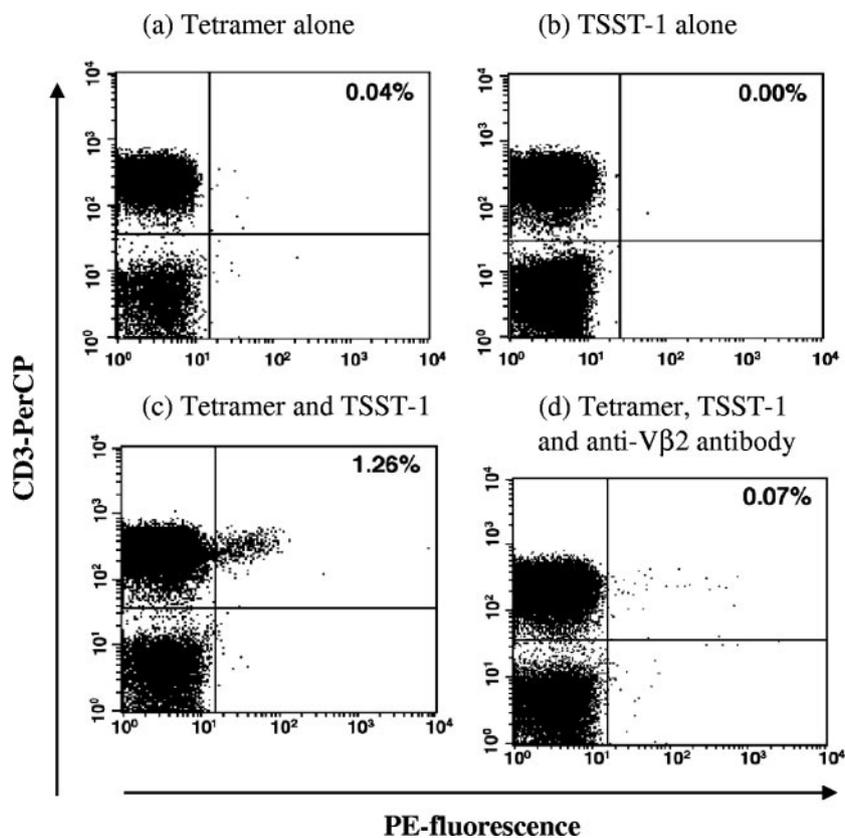


FIG. 1. pMHC tetramers can be used to study superantigen activity. Fluorescence-activated cell sorter (FACS) dot plots show results for PBMCs that were incubated with the indicated molecules. The plots represent 50,000 cells gated with live PBMCs. (a) The tetramer alone does not stain the cells; (b) TSST-1 alone does not cause cells to fluoresce; (c) TSST-1 causes a noncognate tetramer to stain a population of CD3⁺ cells (i.e., T cells); (d) anti-V β 2 antibodies block tetramer staining induced by the presence of TSST-1. PerCP, peridinin chlorophyll protein; PE, phycoerythrin.

were washed in RPMI medium supplemented with penicillin, streptomycin, and L-glutamine (Sigma-Aldrich).

Antibodies and MHC-II tetramers. Anti-CD3-peridinin chlorophyll protein (BD Biosciences), anti-CD4-fluorescein isothiocyanate (Beckman Coulter), and anti-CD4-allophycocyanin (BD Biosciences) were used in this study. Anti-V β 2 antibodies were purchased from Serotec and used at a concentration of 50 μ g/ml. The DRB1*0101 MHC-II tetramers p24.17-DR1 and HA307-DR1 were purchased from Beckman Coulter and are described elsewhere (16). Both were phycoerythrin conjugated and supplied at a concentration of 100 μ g/ml.

MHC-II tetramer staining. The human immunodeficiency virus (HIV) Gag p24-specific CD4⁺-T-helper-cell line Ox24-p24.17 and the influenza virus hemagglutinin-specific CD4⁺-T-helper-cell clone HA1.7 were maintained as described previously (16). For staining, 0.2×10^6 cells were washed and resuspended in phosphate-buffered saline–0.5% fetal calf serum. An MHC-II tetramer was added at a final concentration of 1 μ g/ml, and cultures were incubated at 23°C for 30 min. *S. aureus* strain Newman Eap was added at the same time as the MHC-II tetramer. Following tetramer staining, cells were washed and stained with antibody at 4°C for 20 min.

For MHC-II tetramer cross-linking experiments, 0.5×10^6 PBMCs were incubated with antibody in the presence or absence of Eap protein preparations or TSST-1 (1 μ g/ml; Sigma) and 1 μ g of the p24.17-DR1 tetramer/ml for 30 min at 23°C. The 1- μ g/ml concentration of TSST-1 was used to enable direct comparisons with the concentrations of Eap used; however, equivalent cross-linking was observed with concentrations of TSST-1 as low as 100 ng/ml. Stained cells were washed and fixed with 1% formaldehyde in phosphate-buffered saline before acquisition on a FACSCalibur flow cytometer (BD Biosciences). At least 30,000 events were acquired, and data were analyzed with CellQuest software (BD Biosciences). Each experiment was performed in triplicate and repeated three times with different donors. A representative data set is presented.

Native and recombinant Eap purification. Native and pseudo-Eap proteins were purified from strains Newman, mAHI2, and mAHI2(pCXEap) as de-

scribed previously (8). These strains were a generous gift from A. Haggar, J.-I. Flock, and M. Hussain. Recombinant forms of Eap repeat subunits (Eap19 and Eap10, also known as Map19 and Map10) were purified as described previously (12). Eap19 consisted of amino acids Gln20 to Gln240 and Eap10 comprised amino acids Gln20 to Gln130 of the mature peptide.

RESULTS AND DISCUSSION

pMHC tetramer technology can be used to study the cross-linking activity of superantigens. To determine whether pMHC tetramer technology can be successfully applied to the study of superantigens, we used the classic toxic shock syndrome superantigen TSST-1. PBMCs (0.5×10^6) were incubated with either TSST-1 (0.1 μ g/ml; Sigma) or an HIV-specific MHC-II tetramer (p24.17-DR1 [1 μ g/ml; Beckman Coulter]) (16) or both for 30 min at 23°C. Stained cells were washed and fixed before acquisition on a FACSCalibur flow cytometer (BD Biosciences). As the PBMCs used in this experiment were from healthy donors, the HIV-specific tetramer did not stain any cells (Fig. 1a), and we found that TSST-1 alone did not have any effect on cell fluorescence (Fig. 1b). When cells were incubated with both TSST-1 and the tetramer, a proportion of CD3⁺ cells (i.e., T cells) became labeled (Fig. 1c). TSST-1 binds predominantly to TCRs made with a V β 2 chain (13), and we found that the preincubation of the PBMCs with anti-V β 2 antibody (50 μ g/ml; Serotec) blocked TSST-1-

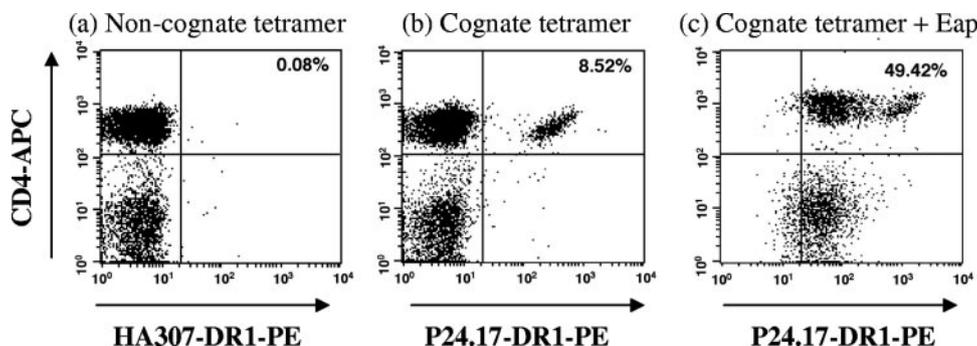


FIG. 2. Eap does not block TCR-pMHC-II interactions. Cells of an HIV Gag p24 CD4⁺-T-helper-cell line were incubated with HA307-DR1 (a noncognate tetramer), p24.17-DR1 (a cognate tetramer), or the p24.17-DR1 tetramer in the presence of Eap. The FACS dot plots show results for 30,000 events gated with live lymphocytes. The percentage of cells falling into the upper right quadrant of each plot is indicated. (a) The noncognate tetramer does not stain any cells; (b) the cognate tetramer stains a population of the cells; (c) the staining of cells (CD4⁺ and CD4⁻) by the cognate tetramer is increased in the presence of Eap protein. APC, allophycocyanin; PE, phycoerythrin.

mediated cross-linking of the tetramer to the CD3⁺ cells (Fig. 1d). This result verifies that MHC tetramer technology is sufficiently sensitive to use in the study of superantigen activity.

***S. aureus* Eap protein does not block MHC-TCR interactions.** Based on amino acid similarities to MHC-II molecules, it has been proposed previously that Eap may block MHC-TCR interactions (6, 11). To test this, we used a T-cell line harvested from an HIV-infected individual (the HIV Gag p24-specific CD4⁺-T-helper-cell line Ox24-p24.17) and a cognate

MHC-II tetramer (p24.17-DR1 at 1 μg/ml). When 0.5 × 10⁶ cells were incubated with a noncognate tetramer (HA307-DR1, an influenza virus-recognizing tetramer purchased from Beckman Coulter, at 1 μg/ml) as described above, no cells became labeled with the tetramer (Fig. 2a). When cells were incubated with the cognate tetramer (p24.17-DR1), a proportion of CD4⁺ cells corresponding to the helper T cells expressing HIV-recognizing TCRs became labeled (Fig. 2b). If Eap can block MHC-II-TCR interactions, we would expect this

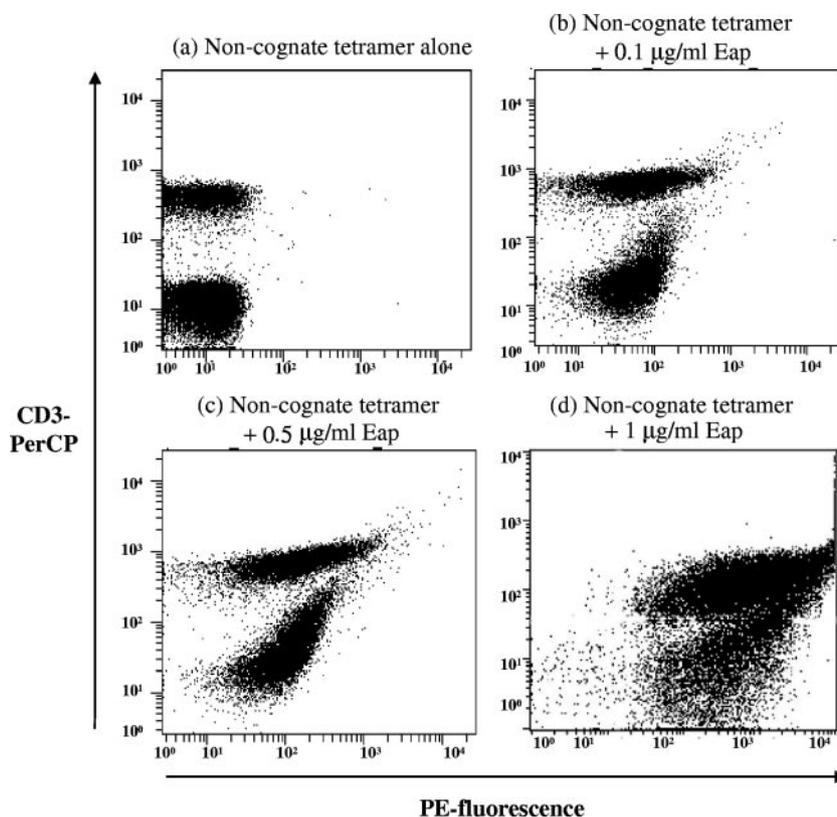


FIG. 3. Eap is not a superantigen. Dose-dependent effects on the cross-linking of pMHC tetramers to PBMCs by Eap are indicated. FACS dot plots show results for PBMCs that were incubated with the indicated molecules. The plots represent 50,000 cells gated with live PBMCs. (a) The noncognate tetramer alone does not stain the cells; (b) to (d) increasing the concentrations of Eap causes mass staining of PBMCs (CD3⁺ and CD3⁻) by a noncognate tetramer. PerCP, peridinin chlorophyll protein; PE, phycoerythrin.

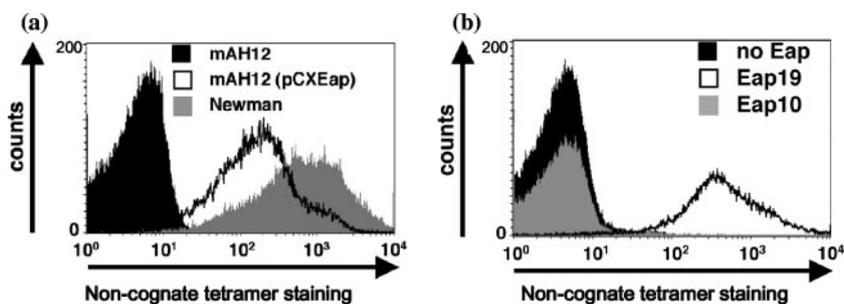


FIG. 4. At least two repeats of Eap are required to cross-link pMHC tetramers to PBMCs. FACS histogram plots show the nonspecific cross-linking of PBMCs and a noncognate tetramer following incubation with Eap protein preparations. (a) Native Eap was purified from the supernatants of the following *S. aureus* strains: Newman (wild-type *S. aureus*), mAH12 (Newman with the *eap* gene inactivated), and mAH12(pCXEap) (the complemented mAH12 strain with the *eap* gene present in multiple copies). (b) At least two Eap repeat units are required for cross-linking pMHC-II tetramers to PBMCs. The FACS histogram plot shows the nonspecific cross-linking of PBMCs and a noncognate tetramer mediated by Eap19, but not Eap10.

labeling to be inhibited. However, the preincubation of the T cells with a 1- μ g/ml concentration of native Eap protein (purified from the supernatant of *S. aureus* strain Newman) (8) resulted in a significant increase in the labeling of both the CD4⁺ and CD4⁻ T cells with the tetramer (Fig. 2c). This effect was also observed when the noncognate tetramer was used, suggesting that the result was not related to the HIV-specific cells or tetramer.

***S. aureus* Eap protein does not cross-link MHC and TCRs in a superantigen-like manner.** The ability to mediate cross-linking between MHC-II molecules and T cells is reminiscent of superantigens, and a recent structural study has revealed homology between the individual repeats of Eap and the C-terminal halves of superantigens such as TSST-1 and staphylococcal enterotoxin B, suggesting that Eap may have superantigenic activity (7). To compare the activity of Eap with that of a superantigen, we incubated PBMCs from healthy donors with an HIV-specific tetramer and a range of concentrations of Eap protein. Figure 3 shows dose-dependent, non-specific cross-linking of the tetramer to PBMCs (CD3⁺ and CD3⁻) by Eap. To ensure that the effects observed were Eap specific and not a result of other *S. aureus* proteins in the preparation, a pseudo-Eap protein purified from the supernatant of strain mAH12 (an *eap* knockout mutant of wild-type *S. aureus* strain Newman) and Eap purified from a variant of strain mAH12 carrying the *eap* gene on a multicopy plasmid [mAH12(pCXEap)] (8) were tested, and only preparations made using the strains containing an intact copy of the *eap* gene cross-linked the tetramer and PBMCs (Fig. 4a).

Although the ability to cross-link MHC-II molecules to cells is a feature of superantigens, Eap has none of the specific and subtle superantigenic features of TSST-1 (Fig. 1c). As further support of this lack of specificity, the incorporation of ligands to which Eap has been shown to bind (e.g., purified fibronectin, fibrinogen, and intercellular adhesion molecule 1 [10 μ g/ml]) reduced cross-linking of the tetramers to the PBMCs (data not shown). The effect was not specific to human PBMCs, as Eap also cross-linked the tetramers to Mac-T cells (bovine mammary epithelial cells) and Sho cells (murine fibroblasts).

Two or more repeats of Eap are required for cross-linking. As mentioned previously, the crystal structure of a single repeat of Eap suggests that the repeat resembles the C-terminal

half of a superantigen, i.e., it contains only one binding region (7). We hypothesized that cross-linking would require at least two Eap binding repeats, each with the ability to bind an individual molecule, thus cross-linking the molecules. To test this hypothesis, we purified two recombinant forms of Eap, one consisting of the first repeat and one consisting of the first two repeats (12). The two-repeat Eap19 protein (1 μ g/ml), but not the single-repeat Eap10 (at equimolar and 20-fold molar concentrations relative to the concentration of each Eap19 molecule [i.e., 0.5 and 10 μ g/ml]), cross-linked pMHC-II tetramers to PBMCs (Fig. 4b), verifying that two or more repeats of Eap are required for cross-linking. Preincubation with the single-repeat Eap10 protein (20 μ g/ml) for 30 min reduced the ability of Eap19 (1 μ g/ml) to cross-link pMHC-II tetramers to PBMCs by 74% ($P = <0.001$), suggesting that Eap10 saturates the binding sites, thereby preventing the cross-linking effect.

In this study, we developed pMHC tetramer technology to study the interactions between *S. aureus* proteins and human leukocytes. We showed that this technology is sufficiently sensitive to assay the cross-linking activity of the TSST-1 superantigen. We also studied some of the proposed activities of the Eap protein that have been inferred from sequence and structural similarities. We found that this protein does not have the ability to block MHC-TCR interactions; instead, it has non-specific cross-linking activity. This cross-linking activity is dependent upon the multirepeat nature of this protein, and while it is as yet unclear what the downstream effects of this cross-linking activity may be in vivo, we have shown that this pMHC technology is a valuable in vitro tool for the study of host-pathogen interactions.

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