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Intercellular and Intracellular Events Following the MHC-Unrestricted TCR Recognition of a Tumor-Specific Peptide Epitope on the Epithelial Antigen MUC1

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We examined the functional and molecular parameters involved in direct TCR recognition of a tumor-specific peptide epitope on the tumor Ag MUC1. This peptide epitope is tandemly repeated and recognized on the native molecule rather than processed and bound to the MHC. Even though the TCR was not MHC restricted, intercellular interactions found to facilitate this recognition included intercellular adhesion molecule-1/LFA-1, LFA-3/CD2, and class I/CD8. Intracellular parameters of MHC-unrestricted CTL activation were examined to compare the recognition of the MUC1 epitope presented on synthetic microspheres, with the recognition of the native epitope in the context of other molecules on the target cells. The epitope on microspheres induced a transient influx of Ca\textsuperscript{2+} that was not accompanied by detectable tyrosine phosphorylation of the \(\zeta\)-associated protein ZAP-70, whereas recognition of MUC1 epitopes on tumor cells caused a sustained Ca\textsuperscript{2+} influx and ZAP-70 phosphorylation. The transient influx of Ca\textsuperscript{2+} was not sufficient to cause translocation of the nuclear factor of activated T cells (NF-AT) into the nucleus or CTL proliferation. In contrast, recognition of the MUC1 epitope on tumor cells resulted in full activation of the CTL, nuclear translocation of NF-AT, and proliferation. MHC-unrestricted TCR triggering, therefore, involves similar intercellular and intracellular events that participate in the conventional, MHC-restricted Ag recognition. Direct recognition of the MUC1 peptide epitope by the TCR in the absence of presentation by the MHC induces a partial signal that is completed by further interactions of other receptor/ligand pairs on the surface of the CTL and their target cells.

common and therefore not well understood. Nothing is known about the T cell activation events that occur upon MHC-unrestricted recognition of Ag via the TCR, nor about the role that coreceptor and/or adhesion molecules play in this type of recognition. In this study, we have characterized for the first time, some of the cell surface and intracellular molecules that contribute to a productive MHC-unrestricted recognition of MUC1. We describe two types of MUC1-specific MHC-unrestricted CTL, one dependent on MHC class I/CD8 coreceptor engagement, and the other independent of this interaction. Both types are dependent on the additional interactions of adhesion molecules ICAM-1 and LFA-3, but differ in degrees. We further explored the activation of MHC-unrestricted MUC1-specific CTL in response to MUC1 epitopes expressed in the presence or absence of these molecules. We show that similar to the MHC-restricted peptide recognition, the MHC-unrestricted CTL recognition of the native MUC1 epitope on the surface of tumor cells induces sustained Ca\textsuperscript{2+} mobilization, phosphorylation of the ζ-associated protein ZAP-70, translocation of the nuclear factor of activated T cells (NF-AT)\textsuperscript{5} to the nucleus, and CTL proliferation. When these CTL were stimulated by MUC1 peptides on the surface of microspheres, in the absence of any other cell surface molecules, only a partial CTL response was observed characterized by a transient Ca\textsuperscript{2+} influx, lack of detectable ZAP-70 tyrosine phosphorylation, cytoplasmic NF-AT, and no proliferation. These data show that native MUC1 tandem repeat peptides can directly bind the TCR of MHC-unrestricted CTL and induce their activation, but this activation is only partial. When MUC1 tandemly repeated epitopes are recognized by CTL in the company of additional molecules on the surface of tumor cells, full CTL activation results.

Materials and Methods

Reagents and cell lines

The 100-amino acid-long MUC1 peptide ((GVSAPDTRPAFGSTAP PAH) × 5) was synthesized in PepTide Synthesis Facility, Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pennsylvania, employing F-moc chemistry, on an advanced Chemtech 200 synthesizer. The purified product was characterized by electron mass spectrometry and by ELISA using anti-MUC1 mAbs. Polylactide-coglycolide (PLGA) microspheres have been described previously (20). The MUC1 peptide or OVA peptide was conjugated to PLGA microspheres at Southwestern Research Institute (Birmingham, AL), in collaboration with Consta Corp. (Seattle, WA). The average number of MUC1 peptides per microsphere was 2.8 × 10\textsuperscript{6}. The breast tumor cell lines BT-20 (A24, A31; B15, 7; Bw4, 6) and CAMA-1 (A2; B3; B40/4701, B15) were previously described (16). The pancreatic tumor cell lines T3 M4 (A2; 7, B40/4701, B51/52/7801; Bw4, 6) and HPAF (A1, 2601; B5401/55/56/5901, B8; Bw6) were previously described (15). The MUC1-transfected cell line T2 was previously described (21). The lymphoblastoid cell line JY (A2; 2, B7, 7) was obtained from Dr. V. Engelhard, University of Virginia (Charlottesville, VA). The melanoma cell line DM-6 (A2; 2, B15; 44; Bw4) was obtained from D. Stingluff, University of Virginia.

Collection and culture of T lymphocytes

To generate MUC1-specific CTL lines, the tumor-draining lymph node from a patient with breast cancer was used as the source of the CD8\textsuperscript{+} T lymphocytes. Cells (10\textsuperscript{6}) were stained on ice for 1 h with Leu3a (anti-CD4) Ab (gift from Dr. E. Engelman, Stanford University, Stanford, CA) and 100-mm petri dishes (Nunc, LabTek, Naperville, IL) were precoated with 10 μg/ml goat anti-mouse IgG, γ-chain specific (Zymed Laboratories, San Francisco, CA) in 0.05 M Tris, pH 9.5, at room temperature for 1 h and washed. A quantity amounting to 5 × 10\textsuperscript{4} cells was added to each plate and incubated at 4°C for 1 h. The nonadherent CD8\textsuperscript{+} T cell-enriched population was collected, resuspended in AIM-V lymphocyte medium (Life Technologies) supplemented with 10% human AB serum (Gemini Bioproducts, Calabasas, CA) and 20 U/ml IL-2, at 1 × 10\textsuperscript{5} cells/ml, and stimulated with irradiated (6000R) allogeneic breast carcinoma cell lines BT-20 and CAMA-1 in an alternating fashion every 7 days. The CTL lines were cultured on tumor cells for 9 wk, and the CTL were further expanded on 10 ng/ml anti-CD3 mAb OKT-3 (American Type Culture Collection (ATCC), Rockville, MD) in the presence of irradiated allogeneic PBMCs and 10 ng/ml IL-2 for 2 wk, then switched to 0.1 ng/ml OKT-3 in the presence of PBMCs and 2 ng/ml IL-2 every 2 wk. MA MUC1-specific CTL became monoclonal, expressing one Vα and one Vβ chain, as evident by PCR amplification and sequencing (M. Alter et al., manuscript in preparation). The cultures were kept on OKT-3 for a period not exceeding 6 to 8 wk. Tyrosine phosphorylation, NF-AT translocation, and some of the Ca\textsuperscript{2+} influx measurements were performed on CTL that were in the second week after 0.1 ng/ml OKT-3 stimulation. To generate an allospecific (anti-HLA-A2) CTL line, peripheral blood of a donor with HLA type A21, 24; B7, 57 (A2 B7\textsuperscript{+}) was applied to a sodium diatrizoate-Ficoll gradient, lymphocyte separation medium (Organon Technica, Durham, NC), and the resulting mononuclear cell fraction was stimulated weekly with irradiated HLA-A2\textsuperscript{+} IV cells.

Cytotoxicity assays

Target cells were labeled for 1 h by incubating 1 × 10\textsuperscript{5} cells in 50 μCi of Na\textsubscript{2}\textsuperscript{14}C i o (Amersham, Arlington Heights, IL) at 37°C. Labeled cells were washed and seeded at 2 × 10\textsuperscript{4}/100 μl well in a 96-well V-bottom plate with varying numbers of effector T cells. The plates were centrifuged and incubated for 4 h in 5% CO\textsubscript{2} at 37°C. All determinations were performed in triplicates. Supernatants were harvested using a Skatron harvesting press (Skatron Instruments, Sterling, VA), and counted in a gamma counter (Co-Bra II, auto gamma; Packard Instrument, Downer’s Grove, IL). Maximum release was obtained by adding 100 μl of 1 N HCl to the labeled target cells. Spontaneous release was obtained by incubating the labeled targets with medium in the absence of T cells. Percentage of release was calculated using the following equation: percentage of release = (maximum release – spontaneous release)/(maximum release – spontaneous release). For blocking experiments with anti-CD3 mAb OKT-3 (ATCC), anti-CD58 (ATCC), anti-CD54 (ICAM-1) (PharMingen, San Diego, CA), anti-CD2 (LFA-2) (ATCC), anti-CD11a (LFA-1) (ATCC), and anti-class I mAb W6/32 (ATCC), 2 × 10\textsuperscript{4} labeled targets/100 μl were incubated for 30 min at 37°C in the presence of the indicated concentrations of Abs. T cells were then added at the indicated E:T ratio.

Fluorescence spectroscopy

A quantity amounting to 1 × 10\textsuperscript{5} T cells/ml was labeled for 20 min at room temperature in 50 μM of Calcium Green-1 acetoxyethyl (AM) ester solution (Molecular Probes, Eugene, OR) prepared according to the manufacturer’s instructions. Measurements were performed on a Perkin-Elmer LS50B (Perkin-Elmer, Norwalk, CT) luminescence spectrometer. A confocal microscope (CLSM) to quantify distribution and intensity of labeling. Images through the midplane of the cells were collected at 20-s intervals using a Nikon 20 or ×60 objective, 1024 × 1024 pixels, 512 illumination laser line, 535 primary dichroic, and 570 barrier filter (Zymed Laboratories) was then added at a 20 μg/ml concentration. The increase in fluorescence intensity was monitored over time relative to background.

Confocal microscopy

A quantity amounting to 5 × 10\textsuperscript{5} Calcium Green-1-loaded T cells was briefly centrifuged with 40 μg of MUC1- or OVA-conjugated PLGA beads and incubated for 30 min at 4°C on BT-20, HA5, or DM-6 tumor monolayers grown on sterile coverslips. T cells were observed under a Molecular Dynamics (Sunnyvale, CA) Multiprobe 2001 confocal laser microscope (CLSM) to quantify distribution and intensity of labeling. Images through the midplane of the cells were collected at 20-s intervals using a Nikon 20 or ×60 objective, 1024 × 1024 pixels, 512 illumination laser line, 535 primary dichroic, and 570 barrier filter (Zymed Laboratories) was then added under exactly the same conditions of laser intensity to ensure consistent sampling between conditions. Three-dimensional image stacks were also

\textsuperscript{5} Abbreviations used in this paper: NF-AT, nuclear factor of activated T cells; Ca\textsuperscript{2+}, intracellular calcium concentration; phenyl-GalNAc, phenyl-\textsubscript{N}-acetyl-\textsubscript{a}-galactosaminide; PLGA, polylactide-coglycolide.

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collected with no time delay, through the entire thickness of the cells, as rapidly as possible with a Z interval of 0.5 μm (15 images total).

**ZAP-70 immunoprecipitations and immunoblots**

T cells and stimulator cells were used at a ratio of 10:1. The T cells and stimulator cells or MUC1-conjugated microspheres were mixed and centrifuged briefly to facilitate contact. For T cell stimulations with anti-CD3 Ab, T cells were first stained on ice for 30 min with anti-CD3 mAb OKT-3 (ATCC) and washed, and the surface OKT-3 was cross-linked with goat anti-mouse Ab at 50 μg/ml (Zymed Laboratories). Stimulations were performed in microcentrifuge tubes at 37°C for the indicated times. The cells were lysed immediately in a final 1% Brij-97 (Sigma Chemical Co.), 150 mM NaCl, 10 mM Tris, pH 7.5, 1 mM Na3 VO4 (Sigma Chemical Co.), 10 μg/ml aprotinin (Sigma Chemical Co.), and 1 mM PMSF (Sigma Chemical Co.). ZAP-70 was immunoprecipitated from postnuclear lysates of 2 × 105 to 3 × 105 T cell equivalents by adding 1 to 1.5 μl of anti-ZAP-70 Ab (kind gift from Dr. A. Weiss, University of San Francisco, San Francisco, CA). The lysates were rotated with anti-ZAP-70 Ab for 24 h, followed by an overnight incubation with 100 μl protein G-Sepharose slurry (4 Fast Flow; Pharmacia Biotech, Uppsala, Sweden). The protein G-Sepharose beads were then washed twice in cold PBS/0.01% Brij-97/1 mM Na3 VO4 (Sigma Chemical Co.), and once in cold PBS. The immunoprecipitated proteins were eluted off protein G by boiling for 4 min in 75 μl 1 X SDS/sample buffer in the presence of 5% 2-ME (Kodak, Rochester, NY). The eluted proteins were loaded onto 12% SDS/polyacrylamide gels, electrophoresed, transferred onto nitrocellulose membrane (BioBlot, NC, Corning Costar, Corning, NY), and blocked in 5% nonfat powdered milk (Carnation). The membrane was probed with either anti-phosphotyrosine Ab (4G10) (Upstate Biotechnology, Placid Lake, NY) or anti-ZAP-70 mAb (Upstate Biotechnology) overnight at 4°C, followed by the secondary peroxidase-conjugated anti-mouse IgG (whole molecule) (Sigma Chemical Co.), and developed by enhanced chemiluminescence (ECL) method per manufacturer’s instructions (Amersham).

**Proliferation assays**

T cells were seeded at 2 × 10^3/100 μl/well in round-bottom 96-well plates (Costar, Cambridge, MA). Anti-CD3 mAb OKT-3 (ATCC) was immobi- lized to the wells at 30 μg/ml in sterile Dulbecco’s PBS overnight at room temperature following immobilization of goat anti-mouse IgG, γ-chain specific (Zymed Laboratories) at 30 μg/ml in sterile PBS (Sigma Chemical Co.), and once in cold PBS. The immunoprecipitated proteins were eluted off protein G by boiling for 4 min in 75 μl 1 X SDS/sample buffer in the presence of 5% 2-ME (Kodak, Rochester, NY). The eluted proteins were loaded onto 12% SDS/polyacrylamide gels, electrophoresed, transferred onto nitrocellulose membrane (BioBlot, NC, Comin Costar, Corning, NY), and blocked in 5% nonfat powdered milk (Carnation). The membrane was probed with either anti-phosphotyrosine Ab (4G10) (Upstate Biotechnology, Placid Lake, NY) or anti-ZAP-70 mAb (Upstate Biotechnology) overnight at 4°C, followed by the secondary peroxidase-conjugated anti-mouse IgG (whole molecule) (Sigma Chemical Co.), and developed by enhanced chemiluminescence (ECL) method per manufacturer’s instructions (Amersham).

**NF-AT translocation**

NF-AT translocation into the nucleus was observed by fluorescence microscopy, as previously described (22). T cells and stimulator cells, as indicated, at a ratio of 10:1, or T cells and 0.1 mg MUC1- or OVA-conjugated microspheres were incubated for 4, 18, 24, or 48 h at 37°C in sterile 96-well V-bottom plates. The cells were then centrifuged onto slides (CytoSpin 3; Shandon, Pittsburgh, PA) for 3 min at 250 rpm. The slides were fixed immediately in 0.5% paraformaldehyde (10 min), permeabilized in cold methanol (2 min), and rehydrated in PBS (10 min). The cells were stained with anti-NF-AT, mAb 7A6 (1:1,000) (kind gift from Dr. Gerald Crabtree, Stanford University), followed by anti-mouse biotin-conjugated goat anti-mouse IgG (H + L) rat-adsorbed Ab (1:1,000) (Caltag Laboratories, So, San Francisco, CA), and streptavidin-conjugated Cy-3-labeled Ab (1:12,000) (Jackson ImmunoResearch Laboratories, West Grove, PA). Hoesch dye was added for 30 s and washed. Separate red and blue sections were collected and overlaid for each field. Final image processing was performed using Adobe Photoshop (Mountain View, CA).

**Results**

The MHC-unrestricted CTL recognition of MUC1 is dependent to varying degrees on the expression of class I, CD54, and CD58 on tumor cells

Figure 1A shows the function of the clonal CTL line MA as an example of the function of all MUC1-specific MHC-unrestricted CTL we have derived, and compares it with the function of allo-reactive, HLA-A2-specific CTL. At 8 wk of culture, when MA was 100% CD8+, it killed MUC1+ breast tumor lines BT-20 and CAMA-1 and also MUC1+ pancreatic tumor cell line T3M4. The MUC1+ melanoma cell line DM-6 was not killed. The cytotoxicity of MA toward the MUC1+ tumors was blocked with anti-CD3 mAb (Fig. 1B). The reactivity of the HLA-A2-specific CTL against the same tumor targets was quite different, corresponding only to their HLA type. The MUC1+ HLA-A2+ melanoma cell line DM-6 was killed, as were the MUC1+ HLA-A2+ cell lines T3M4 and CAMA-1. CAMA-1 was killed at low levels in the experiment shown, but has been observed to be recognized at higher level in other experiments. The MUC1+ but HLA-A2+ (HLA-A24, 31) tumor cell line BT-20 was not killed (Fig. 1C). The HLA-A2-specific killing of this T cell line was blocked by the anti-HLA-A2 mAb MA2.1 (not shown). The cytotoxicity of the HLA-A2-specific T cell line toward the HLA-A2+ tumors was also blocked in the presence of anti-CD3 mAb (Fig. 1D).

We wanted to further show the lack of MHC restriction in MUC1-specific CTL function by the ability of MUC1-specific CTL to recognize and lyse T2 cells transfected with MUC1 cDNA (T2 MUC1) (Fig. 2). The advantage of using T2 is that it is a mutant cell line, deficient in TAP-mediated transport of peptides from the cytoplasm into the endoplasmic reticulum (ER) (23), and thus not expected to present MUC1 peptides efficiently. The disadvantage is that T2 MUC1 cells express a fully glycosylated MUC1 molecule (21) that does not express the unglycosylated epitope recognized by the MUC1-specific MHC-unrestricted CTL.
these experiments, three MUC1-specific CTL lines were used. For accessory role in MHC-unrestricted CTL recognition of this Ag. For molecules that do not present MUC1 peptides could still have an 3114 MUC-1-SPECIFIC MHC-UNRESTRICTED TCR SIGNALING

The dependence of MHC-unrestricted MUC1-specific CTL

effector function on CD54 and CD58. MUC1+ tumor cell target is CAMA-1. A, Differential dependence on anti-CD54 or anti-CD58 correlating with MHC dependency. The CTL (MHC dependent or independent)/mAb combination is indicated in the legend. B, Blocking effector function of two MUC1-specific CTL lines (MHC dependent (MA) or independent, as indicated) shown on the x-axis with anti-CD54 and anti-CD11a mAbs, as indicated in the legend at 50 μg/ml each. Data are representative of at least three independent experiments.

FIGURE 2. Cytotoxicity of the MUC1-specific CTL against muc-1-transfected T2 cells. MUC1-specific MA CTL were used. T2 MUC1 cells were treated with the O-glycosylation inhibitor phenyl-GalNAc every 24 h for a period of 72 h (72 h gi). BT-20 and HPAF are MUC1 tumor cells.

FIGURE 3. The dependence of MHC-unrestricted MUC1-specific CTL effector function on MHC class I. A, An MHC-independent CTL line. The x-axis shows the tumor cell targets, DM-6 (MUC1+) and T3M4, CAMA-1, and BT-20 (MUC1+). B, Two MHC-dependent CTL lines. On the x-axis are shown two MUC1-specific CTL lines, MA CTL shown on the left. The tumor cell target is MUC1+ CAMA-1. The percentage of specific killing is represented in A and B on the y-axis in the presence or absence of anti-class I mAb W6/32 or anti-CD8 mAb OKT-8 at 10 μg/ml, as indicated in the legend, and at an E:T ratio of 10:1. Data are representative of at least three independent experiments.

FIGURE 4. The presence of anti-CD54 or anti-CD58 mAb at different concentrations of 5, 25, and 50 μg/ml inhibited ~20%. In contrast, the same Abs were able to block the cytotoxic function of the MHC-dependent CTL (Fig. 4A). Dependence on CD11a and CD2, ligands for CD54 and CD58, respectively, expressed on the CTL, also showed correlation with the CTL dependence on MHC molecules. Figure 4B shows that anti-CD11a blocked the MHC-unrestricted cytotoxicity of the MHC-dependent MUC1-specific CTL, while no significant inhibition was observed for the MHC-independent MUC1-specific CTL. The presence of both each line, an E:T ratio was chosen in which ~50% maximal lysis was observed. One CTL line was equally cytotoxic against various MUC1+ tumors in the presence or absence of mAb to MHC class I molecules (Fig. 3A). The cytotoxicity of the other two CTL lines, including the MA CTL described above, toward the same MUC1+ tumor targets was inhibited significantly in the presence of mAb to class I molecules. The results for one of these targets, T3M4, are presented in Figure 3B. Inhibition of lysis was also seen in the presence of mAb to CD8, and the presence of both anti-class I and anti-CD8 mAbs had an additive blocking effect. These three CTL are good examples of two types of MHC-unrestricted MUC1-specific CTL we have observed consistently: MHC (and CD8) independent and MHC (and CD8) dependent.

Several different adhesion molecules are crucial in the MHC-restricted recognition of Ag by T cells (26), and we examined their role in MHC-unrestricted recognition of MUC1. We compared the MHC-unrestricted cytotoxic function of MHC-dependent and the MHC-independent MUC1-specific CTL for their dependence on CD54 (ICAM-1) and CD58 (LFA-3) molecules expressed on the tumor cell targets. The levels of these molecules were comparable on all tumor cell targets that were tested, as detected by FACS analysis. Dependence of the CTL-target interaction on these adhesion molecules corresponded well to their degree of dependence on MHC class I molecules. We found that the MHC-independent unrestricted CTL were also less dependent on CD54 and CD58 (Fig. 4A). The presence of anti-CD54 or anti-CD58 mAb at different concentrations of 5, 25, and 50 μg/ml inhibited ~20%. In contrast, the same Abs were able to block the cytotoxic function of the MHC-dependent CTL (Fig. 4A). Dependence on CD11a and CD2, ligands for CD54 and CD58, respectively, expressed on the CTL, also showed correlation with the CTL dependence on MHC molecules. Figure 4B shows that anti-CD11a blocked the MHC-unrestricted cytotoxicity of the MHC-dependent MUC1-specific CTL, while no significant inhibition was observed for the MHC-independent MUC1-specific CTL. The presence of both

(24). This epitope, however, can be generated by extensive treatment with phenyl-N-acetyl-α-D-galactosaminide (phenyl-GalNAc), a compound that inhibits elongation of the O-linked saccharide chains (25). We have published previously that treatment of many EBV-immortalized B cells transfected with MUC1 can sensitize them to killing by MUC1-specific CTL, and that untransfected parental cells treated the same way do not gain susceptibility to CTL lysis (16). In Figure 2, we show that following this treatment, specific lysis of T2 MUC1 cells at an E:T of 40:1 is indeed observed, although at lower levels than the specific lysis of the MUC1+ breast tumor cell line BT-20 and the MUC1+ pancreatic tumor cell line HPAF.

The question we next addressed was whether MHC class I molecules that do not present MUC1 peptides could still have an accessory role in MHC-unrestricted CTL recognition of this Ag. For these experiments, three MUC1-specific CTL lines were used. For
anti-CD11a and anti-CD54 almost completely blocked the cytotoxicity of the MHC-dependent CTL, but not the MHC-independent CTL (Fig. 4B). The same results were obtained with anti-CD2 Ab and the combination of anti-CD2 and anti-CD58 (not shown).

**MHC-unrestricted, MUC1-specific CTL influx Ca^{2+} in response to MUC1^+ tumor cells and MUC1 peptide conjugated to microspheres**

The observation that the effector function of some MHC-unrestricted CTL lines is critically dependent on different molecules expressed on the surface of tumor cells prompted us to examine the full extent to which a native MUC1 tandem repeat epitope can stimulate CTL in the absence of these molecules. We compared the signal-transduction events that take place following the MHC-unrestricted recognition of MUC1 expressed on tumor cells with the events that follow recognition of the MUC1 Ag alone. A synthetic 100-amino acid-long (five tandem repeats) MUC1 peptide was conjugated to the surface of 20 to 40 μm PLGA microspheres. These microspheres were stained with anti-MUC1 mAb, and were shown to express the relevant MUC1 immunodominant epitope PDTRP (not shown). As a control, microspheres conjugated with OVA were used. We first examined changes in intracellular calcium concentration ([Ca^{2+}]_i) in response to CTL stimulation. Figure 5A shows that the MUC1-specific, MHC-unrestricted clonal MA CTL undergo a large increase in [Ca^{2+}]_i, when incubated on a monolayer of the MUC1^+ breast tumor cell line BT-20. Similar results were obtained with a monolayer of the pancreatic MUC1^+ tumor cell line HPAF (not shown). The white pseudocolor indicates an increased level of [Ca^{2+}]_i. The CTL were found to form pseudopods over the tumor monolayer, a change in shape that is indicative of CTL activation. T cell shape has been shown to be sensitive to a rise in [Ca^{2+}]_i (27).

When MA CTL were incubated with MUC1-conjugated PLGA microspheres, they also underwent a similar influx of Ca^{2+}. Figure 5B shows a three-dimensional image reconstruction of a MUC1-specific CTL interacting with a large MUC1-conjugated microsphere. Several image slices on multiple planes were acquired as a representation of the Ca^{2+} influx at one time point, and they showed that the CTL had almost fused to the microsphere on one side.

**FIGURE 5.** MUC1-specific CTL influx Ca^{2+} in response to MUC1. A, MUC1-specific CTL MA showing Ca^{2+} influx in response to a monolayer of MUC1^+ BT-20 tumor cells at ×10 magnification. The data are representative of 35 separate fields. B, MUC1-specific CTL MA showing Ca^{2+} influx in response to a MUC1^+ microsphere. An enlarged three-dimensional reconstruction of the image at ×60 magnification is shown. The color scale on the right is for both A and B. It shows the range of fluorescence intensity from 0 to 255, in which white represents the highest fluorescence intensity of Calcium Green-1, and dark blue represents no fluorescence. The data were similar in two other independent experiments.

**FIGURE 6.** Ca^{2+} influx profile in MUC1-specific CTL in response to MUC1^+ tumor cell lines. Response of the MA CTL is shown. A, In response to cross-linking OKT-3 with anti-mouse IgG. B, In response to the MUC1^+ CAMA-1 cells. C, In response to the MUC1^+ BT-20 cells. D, In response to the MUC1^+ DM6 cells. The intensity of fluorescence expressed in arbitrary units is shown on the y-axis. Each tick mark represents 2 arbitrary units. The time of measurement in seconds is shown on the x-axis. Time t = 0 represents the beginning of measurement after a 5-s centrifugation of CTL with tumor cells, and immediate measurement after the addition of cross-linking Ab. Data are representative of at least three experiments.
We further examined the pattern of Ca\(^{2+}\) and their top are presumed to have interacted with the microsphere earlier, undergone a change in shape, in which the cell was now elongated MUC1 conjugated to microspheres, but a sustained Ca\(^{2+}\) in response to MUC1 IgG (not shown). Stimulation of MA CTL with MUC1 that began at 10 U or greater increase in fluorescence intensity was observed.

Cross-linking the TCR/CD3 complex on MUC1-specific MA CTL resulted in a slow stepwise sustained increase in fluorescence intensity that began at 50 to 60 s. Stimulation of MA CTL with the specificity that began at 10 s of measurement and continued only for 100 s, to microspheres shows a large increase in fluorescence intensity (Fig. 7A). This transient response was specific to MUC1-specific MA CTL, in as much as allo-specific CTL did not elicit a different experiment.

Along with increases in [Ca\(^{2+}\)], we measured another early parameter of T cell activation manifested by the tyrosine phosphorylation of the syk family protein tyrosine kinase ZAP-70 (28). MHC-unrestricted, MHC-dependent MA CTL were stimulated for different times with either the MUC1\(^+\) pancreatic tumor cell line T3M4, the MUC1\(^+\) melanoma cell line DM-6, or the MUC1\(^+\) microspheres. Figure 8A shows the tyrosine phosphorylation status of immunoprecipitated ZAP-70 molecules. Upon stimulation of MUC1-specific MA CTL with MUC1\(^+\) T3M4 tumor cells, there was a detectable increase in tyrosine phosphorylation of ZAP-70 (lane 3), compared with unstimulated CTL. The signal was not as strong as that detected after OKT-3 cross-linking (lane 2). No phosphorylated ZAP-70 was detected after stimulation with MUC1\(^+\)DM-6 melanoma cells either at 5 min (lane 7) or 1 min (not shown). This result shows that MHC-unrestricted recognition of MUC1 on tumor cells allows successful tyrosine phosphorylation of ZAP-70, a key player in signal transduction in MHC-restricted T cells. When MUC1 epistopes were presented to MA CTL at the surface of microspheres and in the absence of other signals from additional receptor/ligand interactions, no ZAP-70 phosphorylation was detected at either 1 min (lane 8) or 5 min (lane 9).

Immediate lysis of MA CTL following a brief 5-s centrifugation with MUC1-conjugated microspheres did not reveal ZAP-70 phosphorylation, and no phosphorylation was detected at 30 s either

Specific CTL undergo a transient Ca\(^{2+}\) influx in response to MUC1 conjugated to microspheres, but a sustained Ca\(^{2+}\) influx in response to MUC1\(^+\) tumor cells

We further examined the pattern of Ca\(^{2+}\) influx over a period of time. Measurements were made in microtitre plate wells by fluorescence spectroscopy. The background fluorescence was stable, and when ionomycin, a calcium ionophore, was added to the CTL, a 10-U or greater increase in fluorescence intensity was observed. Cross-linking the TCR/CD3 complex on MUC1-specific MA CTL resulted in a slow and sustained increase in fluorescent intensity that began at ~20 s (Fig. 6A). No such increase in fluorescence intensity was observed in the absence of cross-linking anti-mouse IgG (not shown). Stimulation of MA CTL with MUC1\(^+\) breast tumor cell lines CAMA-1 (Fig. 6B) or BT-20 (Fig. 6C) also resulted in a slow stepwise sustained increase in fluorescence intensity that began at 50 to 60 s. Stimulation of MA CTL with the MUC1\(^+\) melanoma cell line DM-6 showed no increases in fluorescence intensity over background levels (Fig. 6D).

Stimulation of MUC1-specific MA CTL with MUC1 conjugated to microspheres showed a large increase in fluorescence intensity that began at 10 s of measurement and continued only for 100 s, after which the fluorescence intensity declined to background levels (Fig. 7A). This transient response was specific to MUC1-specific MA CTL, in as much as allo-specific CTL did not elicit a change in fluorescence intensity upon the addition of an equal amount of MUC1\(^+\) microspheres (Fig. 7B). Similarly, the MUC1-specific CTL did not respond to OVA conjugated to microspheres (Fig. 7C), and neither did the control allo-specific CTL (Fig. 7D).

The MHC-unrestricted recognition of MUC1\(^+\) tumor cells, but not MUC1\(^+\) microspheres, results in tyrosine phosphorylation of ZAP-70

Along with increases in [Ca\(^{2+}\)], we measured another early parameter of T cell activation manifested by the tyrosine phosphorylation of the syk family protein tyrosine kinase ZAP-70 (28). MHC-unrestricted, MHC-dependent MA CTL were stimulated for different times with either the MUC1\(^+\) pancreatic tumor cell line T3M4, the MUC1\(^+\) melanoma cell line DM-6, or the MUC1\(^+\) microspheres. Figure 8A shows the tyrosine phosphorylation status of immunoprecipitated ZAP-70 molecules. Upon stimulation of MUC1-specific MA CTL with MUC1\(^+\) T3M4 tumor cells, there was a detectable increase in tyrosine phosphorylation of ZAP-70 (lane 3), compared with unstimulated CTL. The signal was not as strong as that detected after OKT-3 cross-linking (lane 2). No phosphorylated ZAP-70 was detected after stimulation with MUC1\(^+\)DM-6 melanoma cells either at 5 min (lane 7) or 1 min (not shown). This result shows that MHC-unrestricted recognition of MUC1 on tumor cells allows successful tyrosine phosphorylation of ZAP-70, a key player in signal transduction in MHC-restricted T cells. When MUC1 epitopes were presented to MA CTL at the surface of microspheres and in the absence of other signals from additional receptor/ligand interactions, no ZAP-70 phosphorylation was detected at either 1 min (lane 8) or 5 min (lane 9).

Immediate lysis of MA CTL following a brief 5-s centrifugation with MUC1-conjugated microspheres did not reveal ZAP-70 phosphorylation, and no phosphorylation was detected at 30 s either

![FIGURE 7. MUC1-specific CTL influx Ca\(^{2+}\) following interaction with 100-mer MUC1 peptide conjugated to PLGA microspheres, but not OVA conjugated to PLGA microspheres. Response to MUC1-conjugated microspheres by MUC1-specific MA CTL (A) and by allo-specific CTL (B). Response to OVA conjugated to microspheres by MUC1-specific MA CTL (C) and by allo-specific CTL (D). Intensity of fluorescence expressed in arbitrary units is shown on the y-axis. Each tick mark represents 2 arbitrary units. The time of measurement in seconds is shown on the x-axis. Time t = 0 represents the beginning of measurement after a 5-s centrifugation of CTL with microspheres. Similar results were obtained in another independent experiment.](http://www.jimmunol.org/)

![FIGURE 8. ZAP-70 tyrosine phosphorylation in MUC1-specific CTL upon stimulation with MUC1\(^+\) tumor cells, but not with MUC1\(^+\) microspheres. A. SDS-PAGE of ZAP-70 immunoprecipitates from 2 \times 10^7 MUC1-specific MA CTL equivalents immunoblotted with anti-phosphotyrosine mAb. Unstimulated MA CTL (lane 1); OKT-3 cross-linking at 5 min (lane 2); with MUC1\(^+\) tumor cells T3M4 at 1 min (lane 3), 5 min (lane 4), 15 min (lane 5), and 30 min (lane 6); with MUC1-negative melanoma cells DM-6 at 5 min (lane 7); with MUC1 conjugated to microspheres at 1 min (lane 8) and 5 min (lane 9). B. Immunoblot of the same gel as in A with anti-ZAP-70 Ab. C. Constitutively phosphorylated \(\zeta\)-chains coimmunoprecipitated with ZAP-70 using anti-ZAP-70 Ab, and immunoblotted with anti-phosphotyrosine mAb. Unstimulated MUC1-specific MA CTL (lane 1); OKT-3 cross-linking at 5 min (lane 2); with MUC1\(^+\) tumor cells T3M4 at 1 min (lane 3) and 5 min (lane 4); with MUC1-conjugated microspheres at 1 min (lane 5) and 5 min (lane 6); with MUC1-negative melanoma cells DM-6 at 5 min. Data are representative of three independent experiments.](http://www.jimmunol.org/)
C

no proliferation in response to the MUC1-conjugated microspheres. The proliferative response for MUC1-specific MA CTL is shown, and was measured by labeling with [3H]thymidine for 18 h at the end of a 3-day culture.

For MUC1-specific MA CTL proliferation in response to MUC1 conjugated to microspheres, whereas they did not respond to MUC1 tumor cells BT-20, CAMA-1 cells (CAMA-1), and CTL in the presence of irradiated CAMA-1 (T/CAMA-1). Consistent with this, NF-AT staining was predominantly cytoplasmic, excluded from the blue nuclear staining visualized by the Hoechst dye (Fig. 10, A and B). After stimulation with ionomycin and phorbol dibutyrate as positive controls, NF-AT staining was predominantly nuclear (Fig. 10, C and D). When CT1 were incubated with tumor cells, the allo-specific HLA-A2-reactive CTL translocated NF-AT in response to the HLA-A2+T3M4, but not to the HLA-A2-HPAF (Fig. 10, E and H). MHC-unrestricted MUC1-specific MA CTL translocated NF-AT to the nucleus in response to both MUC1+T3 M4 and HPAF (Fig. 10, F and I). When both CTL lines were tested in response to the HLA-A2+MUC1+ tumor DM-6, only the allo-specific CTL translocated NF-AT to the nucleus (not shown).

The MUC1 peptide alone conjugated to microspheres was not sufficient to cause NF-AT translocation when examined at three different time points, 4 h, 24 h, and 48 h after interaction with the CTL (24-h time point shown in Fig. 10I). No nuclear translocation of NF-AT was observed even in the layer of MUC1-specific MA CTL still in intimate contact with the MUC1-conjugated microspheres (4 h, Fig. 10J). As expected, no NF-AT translocation was observed in the control allo-specific CTL line in response to either MUC1 or OVA conjugated to microspheres (not shown).

Discussion

MHC-unrestricted recognition is a direct TCR recognition of an antigenic epitope that is neither processed by proteosomes nor presented within the groove of MHC molecules (30). Most of the described MHC-unrestricted αβ T cells have been specific for hapten, nonpeptide Ags, and carbohydrates. The MHC-unrestricted αβ T cell recognition of the tumor-specific Ag MUC1 is unique in that the epitope targeted on this Ag is a peptide sequence (15, 16, 31). Lack of MHC restriction has been described for Ags that have distinct properties such as multivalency, high level of expression, and an ordered conformational structure (8, 10, 12, 32). The MUC1 peptide epitope possesses all of these properties, in that it is tandemly repeated, it is overexpressed on the surface of tumor cells, and it has a rigid ordered structure (30, 19). The presence of this repeated rigid MUC1 epitope at high density is expected to result in the MHC-unrestricted recognition of MUC1+ tumor cells, but not MUC1+ microspheres, induces proliferation of MUC1-specific CTL.

To examine a downstream event in successful T cell activation, we chose MUC1-specific CTL proliferation in response to MUC1 conjugated to microspheres, or in response to the MUC1+ tumor cell lines (Fig. 9). The positive control was CTL proliferation in response to immobilized anti-CD3 (T/anti-CD3). B, Proliferation of irradiated DM-6 cells (DM-6), CTL in the presence of irradiated DM-6 (T/DM-6), irradiated BT-20 cells (BT-20), CTL in the presence of irradiated BT-20 (T/BT-20), irradiated CAMA-1 cells (CAMA-1), and CTL in the presence of irradiated CAMA-1 (T/CAMA-1). C, Proliferation of CTL in the presence of MUC1+ microspheres (T/MUC1 beads), and proliferation of CTL in the presence of OVA conjugated to microspheres (T/OVA beads). Data were reproduced in at least three independent experiments.

The MHC-unrestricted recognition of MUC1+ tumor cells results in NF-AT translocation to the nucleus, while recognition of MUC1+ microspheres does not.

NF-AT nuclear translocation is a consequence of MHC-restricted T cell activation, and has been shown to be necessary for proliferation and IL-2 production (29). We examined whether the different proliferative responses we observed would correspond to differential translocation of NF-AT to the nucleus. MUC1-specific and allo-specific CTL were stained with the anti-NF-ATc mAb 7A6. In unstimulated allo-specific and MUC1-specific MHC-dependent MA CTL, NF-AT staining was predominantly cytoplasmic, excluded from the blue nuclear staining visualized by the Hoechst dye (Fig. 10, A and B). After stimulation with ionomycin and phorbol dibutyrate as positive controls, NF-AT staining was predominantly nuclear (Fig. 10, C and D). When CT1 were incubated with tumor cells, the allo-specific HLA-A2-reactive CTL translocated NF-AT in response to the HLA-A2+T3M4, but not to the HLA-A2-HPAF (Fig. 10, E and H). MHC-unrestricted MUC1-specific MA CTL translocated NF-AT to the nucleus in response to both MUC1+T3 M4 and HPAF (Fig. 10, F and I). When both CTL lines were tested in response to the HLA-A2+MUC1+ tumor DM-6, only the allo-specific CTL translocated NF-AT to the nucleus (not shown).

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surface of microspheres. These results provide for the first time direct evidence that MHC-unrestricted CTL can be triggered by a peptide sequence in its native conformation and without its presentation by MHC molecules.

Upon further characterization of the Ca\(^{2+}\) influx in response to the MUC1 tandemly repeated peptide in isolation, we found that it was transient in nature and it did not result in NF-AT translocation to the nucleus or in CTL proliferation. The lack of NF-AT translocation to the nucleus, after a transient influx of \([\text{Ca}^{2+}]_i\), has been reported previously, in which successful NF-AT nuclear translocation was directly shown to be linked to the sustained nature of the \([\text{Ca}^{2+}]_i\) signal (22). We have demonstrated that MUC1-specific CTL do undergo a sustained \([\text{Ca}^{2+}]_i\) influx in response to MUC1 expressed at the surface of tumor cells. This prolonged \([\text{Ca}^{2+}]_i\) mobilization resulted in NF-AT translocation to the nucleus and subsequent CTL proliferation. Therefore, the MHC-unrestricted recognition of MUC1 also involves a regulated link between the \([\text{Ca}^{2+}]_i\) signal and downstream NF-AT activation.

FIGURE 10. NF-AT nuclear translocation in MUC-1-specific CTL in response to MUC1 expressed on tumor cells but not on microspheres. All stimulations shown were performed for 18 h, and the MUC1-specific CTL used were the clonal MA. Allo-specific CTL (A) and MUC1-specific CTL (B) unstimulated; allo-specific CTL (C) and MUC1-specific CTL (D) after ionomycin/phorbol esters; allo-specific CTL (E) and MUC1-specific CTL (F) after T3M4; allo-specific CTL (G) and MUC1-specific CTL (H) after HPAF; MUC1-specific CTL after stimulation with MUC1-conjugated microspheres (I); and MUC1-specific CTL in contact with MUC1-conjugated microspheres (J). NF-AT staining is represented by the red Cy-3 label, and nuclear staining is blue with the Hoechst dye. Cells were observed under oil immersion. Similar results were obtained in another independent experiment.
Our inability to detect ZAP-70 tyrosine phosphorylation despite the presence of a Ca\textsuperscript{2+} influx in response to the MUC1 peptide epitope alone at the surface of microspheres is indicative of partial signaling. Such partial signaling in T cells has been described by several groups (33, 34). In these reports, partial signaling was described postengagement of the TCR with altered peptide TCR ligands presented at the surface of APCs that provide the full range of costimulatory and accessory molecules. Partial signaling was manifested in the lack of detectable ZAP-70 phosphorylation, despite the presence of a transient Ca\textsuperscript{2+} influx (35). In the case of MHC-unrestricted recognition of MUC1, several possibilities may explain our inability to detect ZAP-70 phosphorylation in response to MUC1-conjugated microspheres. Because the Ca\textsuperscript{2+} influx in response to these microspheres was transient, it may be possible that the tyrosine phosphorylation of ZAP-70 was also of short duration. It is also possible that ZAP-70 does undergo tyrosine phosphorylation, but at levels below the sensitivity of immunoblotting. This is very likely, since when compared with the levels of ZAP-70 phosphorylation upon CD3 cross-linking, phosphorylation of ZAP-70 after stimulation with MUC1\textsuperscript{+} tumor cells appeared to be at a much lower level. Yet another, albeit less likely possibility is that when presented to CTL in isolation, MUC1 tandem repeat peptides may induce a Ca\textsuperscript{2+} influx through a pathway other than that initiated by ZAP-70 tyrosine phosphorylation. An important difference between our studies and studies with altered peptide ligands is that we compared the signaling elicited by the same MUC1 epitope in different contexts. Our results suggest that the same epitope can cause either a partial or complete signal through the TCR, depending on the participation or lack of participation of other accessory molecules. On the other hand, the same MUC1 epitope on tumor cells is surrounded by severely truncated saccharide chains (36), and such carbohydrates are absent on the synthetic 100-mer MUC1 peptide. We have in fact shown that the complexity of the saccharide chain surrounding the CTL epitope dramatically affects CTL effector function.\textsuperscript{5} It is thus possible that the presence of adjacent carbohydrate residues may contribute to either proper conformation of the MUC1 CTL epitope or the affinity of the TCR for the epitope.

Studies with altered peptide ligands have also reported a distinct pattern of \(\zeta\)-chain phosphorylation (33, 34). \(\zeta\)-Chain was constitutively phosphorylated in our CTL cultures because of their expansion on low levels of OKT-3 mAb. We consistently observed constitutive phosphorylation of the \(\zeta\)-chain by the appearance of both p21 and p23 forms, and thus could not address the effect that MUC1 recognition in isolation has on \(\zeta\)-chain phosphorylation. Furthermore, ZAP-70 and \(\zeta\)-chain have consistently coimmunoprecipitated even in unstimulated cells, indicating that in these CTL cultures ZAP-70 is already associated with the \(\zeta\)-chain. Therefore, a signal that phosphorylates ZAP-70 in association with the \(\zeta\)-chain is missing or weak in the MHC-unrestricted recognition of MUC1 epitope in isolation, but is present and strong when this epitope is recognized on the surface of tumor cells. We can speculate that the best candidate for this signal is the src protein tyrosine kinase \(lck\), which associates with the cytoplasmic tail of CD8 molecules (37) and phosphorylates ZAP-70 (28). These data emphasize that even though the recognition of MUC1 is MHC unrestricted, this recognition becomes more efficient in the presence of CD8 molecules (MHC dependency), as well as other accessory molecules. These molecules may augment the MHC-unrestricted signal through the TCR (38), allowing it to proceed to a full response. It should be emphasized that the MHC-unrestricted TCR signaling in this study was examined in MHC-dependent MUC1-specific CTL.

In another model of T cell activation, sustained signaling that productively results in T cell proliferation has been shown to require a prolonged occupancy of the TCR by its peptide/MHC ligand through the formation of stable T cell/APC conjugates (39–41). In these T cell/APC conjugates, continuous signaling through the TCR by serial engagements of up to 200 TCRs is maintained by the formation of a large changing area of membrane contact between the T cell and the APC. A number of adhesion molecules play a crucial role in maintaining the area of intimate contact between the two opposing membranes (42). Molecules such as CD8 and CD2 on T cells have been shown to have signaling capabilities on their own (43–45). A synthetic surface such as PLGA beads is devoid of these molecules, which may deprive CTL of prolonged conjugate formation and subsequent generation of costimulatory signals that allow a complete T cell response. Furthermore, the surface of PLGA beads is different in nature than the fluid bilipid membranes of cells. This may preclude rolling of CTL along the surface and achieving the minimal threshold level of TCR occupancy necessary for the end result of proliferation (46).

At the extracellular level of MHC-unrestricted recognition, we found that adhesion molecules such as LFA-1 and CD2 on the CTL are indeed important for proper effector function. We observed two patterns of dependence on these adhesion molecules correlating with the CTL dependence on MHC, and that may reflect the relative affinity of their TCRs. We had reported previously that human MUC1-specific CTL can kill either porcine or human muc-1-transfected target cells, depending on the density of MUC1 epitope expression and the simultaneous presence of MHC class I molecules (24). This dependence of MHC-unrestricted CTL on adhesion molecules was at first unexpected. However, as we show in this study, full CTL activation necessitates the presence of these molecules. Studies supporting our reported dependency of MUC1 recognition on adhesion molecules show that MUC1 is extensively sialylated, imparting negative charges on tumor cells (47). This hinders cell-cell interactions and CTL cytotoxicity (48, 49). Additional interactions of adhesion molecules at the cell surface are thus important to decrease repulsion between CTL and MUC1\textsuperscript{+} tumor cells, and to result in effective signaling through the TCR. Such interactions for overcoming cell surface net negative charges are just as important during MHC-restricted recognition of peptides (50).

In conclusion, the MHC-unrestricted recognition of the native MUC1 peptide involves the same events of T cell activation as the MHC-restricted recognition of a nominal peptide Ag. Only the initial recognition step differs in the MHC-unrestricted recognition, in which the MUC1 epitope directly engages the TCR rather than being presented as a processed peptide bound to MHC.

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References


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MUC-1-SPECIFIC MHC-UNRESTRICTED TCR SIGNALING


