

Monomorphic Molecules Function as Additional Recognition Structures on Haptenated Target Cells for HLA-A1-Restricted, Hapten-Specific CTL¹

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Hapten-specific T cells have been shown to recognize haptenated peptides with high avidity and, in some instances, with promiscuous MHC restriction. In this study, the impact of Ag density on MHC restriction of a CTL response specific to the trinitrophenyl (TNP) hapten was investigated. In this study, we demonstrate a novel recognition mechanism used by TNP-specific CD8⁺ CTL in the presence of high Ag doses. Although low levels of TNP epitopes on target cells allowed for HLA-A1-restricted CTL activity only, entirely MHC-independent target cell recognition became operative at high TNP loading. In both cases, recognition was mediated by the TCR. This MHC-independent recognition is target cell type restricted and critically involves in our model direct recognition of the ectonucleotidase family surface molecule CD39 by the CTL. *The Journal of Immunology*, 2001, 167: 2724–2733.

Hapten-specific T cell responses play an essential role in chemical- or drug-induced contact hypersensitivity. This inflammatory skin disease is considered to be primarily caused by Ag-specific CD8⁺ CTLs and tightly regulated in a complex interplay by CD4⁺ T cells (1–5).

The antigenic epitopes on hapten-modified cells recognized by T cells were enigmatic for a long time. This was particularly due to the ability of haptens, such as trinitrophenyl (TNP),³ to modify several cell surface proteins (6). Thereby, numerous hapten-carrier conjugates are generated that are potential neoantigens. It was originally anticipated that T cells recognize haptenated MHC molecules or MHC-associated cell surface molecules modified by haptens (7–9). Only more recently could it be shown that hapten-specific T cells recognize hapten-modified peptides presented on MHC molecules (10–14).

Nevertheless, T cell recognition of haptenated peptides has peculiar features. The avidity of T cells specific for TNP-modified peptides was reported to be 100-fold higher than the avidity of T cells specific for nonmodified peptides (13). Interestingly, MHC restriction analyses of hapten-specific T cells suggest that anti-hapten responses are less dependent on MHC recognition than anti-peptide responses. An explanation for this behavior might be the unique structure of hapten-modified peptides that results in longer, bulkier side chains extending from the peptide backbone, which may resemble additional contact sites between the Ag and the TCR.

The aim of this study was to analyze the role of Ag density on MHC restriction of a CTL response specific to TNP. We observed that a novel recognition mechanism is used by TNP-specific CD8⁺ CTL at high TNP loading of target cells. Although low levels of TNP epitopes on target cells allowed for HLA-A1-restricted CTL activity only, entirely MHC-independent target cell recognition became operative at high TNP loading. This MHC-independent recognition of haptenated target cells by these T cells does not seem to be promiscuous, but is cell type restricted. Differentially expressed cell surface molecules are obviously critical, and we provide evidence that one of such molecules that are directly recognized upon TNP modification by CTL is CD39, an ectonucleotidase family surface molecule.

Materials and Methods

Media, reagents, and chemicals

The cell culture medium RPMI 1640 (Life Technologies, Grand Island, NY) was supplemented with 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. FCS was replaced with 5% human AB serum (PAA Laboratories, Munich, Germany) for the cultivation of T cell lines. IL-2 was kindly provided by the Novartis Research Institute (Vienna, Austria).

NaF, 2,4,6-trinitrobenzene sulfonic acid (TNBS), PMA, ionomycin, monensin, brefeldin A, bromelain, chloroquine, subtilisin, and papain were purchased from Sigma Chemie (Deisenhofen, Germany). The 2,4-dinitrobenzene sulfonic acid was obtained from ICN Biomedicals (Eschwege, Germany). *O*-sialoglycoprotease from *Pasteurella hemolytica* was obtained from D. Sutherland (Oncology Research, Toronto Hospital, Toronto, Canada). Phosphoinositol-phospholipase C was from Immunotech (Marseille, France).

Antibodies

The following murine mAbs were generated in our laboratory: VIAP (calf intestine alkaline phosphatase specific), VIT3 (CD3), VIT4 (CD4), VIT8 (CD8), 6B6 (CD11a), VIM13 (CD14), 6F5 (CD43), and 1/47 (MHC-class II). MHC class I mAb W6/32, CD58 mAb TS2/9, β₂-microglobulin mAb BBM.1, and anti-TNP mAb 1B7-producing hybridomas were obtained from American Type Culture Collection (ATCC, Manassas, VA). The following mAbs were provided: CD3 mAb UCHT-1, CD14 mAb MEM18, and CD45R0 mAb UCHL1 were from An der Grub (Bio Forschungs, Kaumberg, Austria); CD8 mAb Campath8c was from G. Hale (Department of Pathology, University of Cambridge, Cambridge, U.K.). The CD3 mAb OKT-3 was purchased from Ortho Diagnostics (Raritan, NJ). The mAb HD37 (CD19) was provided by G. Moldenhauer (Division of Molecular Immunology, German Cancer Research Center, Heidelberg, Germany).

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³ Abbreviations used in this paper: TNP, trinitrophenyl; LCL, lymphoblastoid B cell line; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

CD56 mAb Leu19 was obtained from BD Biosciences (Mountain View, CA). Anti-TCR $\alpha\beta$ mAb BMA-031 was purchased from Immunotech. mAb MP9-20A4 against human TNF- α and mAb MP4-25D2 against human IL-4 were obtained from Caltag Laboratories (Burlingame, CA). The mAb against IFN- γ (GZ4) was purchased from Boehringer Mannheim (Mannheim, Germany). mAb A1 (CD39) was a gift of G. Aversa (Novartis Research Institute, Vienna, Austria), and mAb G20-10 (CD39) was obtained from the Fourth Workshop on Leukocyte Typing. MEM-61 (CD9) was provided by V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic). The mAbs were used in this study in functional assays at a concentration of 10 $\mu\text{g/ml}$, if not otherwise mentioned.

T cell preparation

PBMC were isolated from heparinized whole blood of normal healthy donors by standard density gradient centrifugation with Ficoll-Paque (Pharmacia, Uppsala, Sweden). Subsequently, T cells were separated by magnetic sorting using the MACS technique (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described (15), through negative depletion of CD14, CD16, CD19, CD33, and MHC class II-positive cells with the respective mAbs.

Cell lines

The following cell lines were used in this study: the EBV-transformed lymphoblastoid B cell lines (LCL) OM (HLA A1, 31; B7, 49; Cw7) and OTMA (HLA A3, 11; B7, 35; Cw4) were generated in our laboratory. The panel of HLA-typed EBV-LCL was a kind gift of the Department of Blood Group Serology (University of Vienna, Vienna, Austria). The cell lines K562, Daudi, Raji, Jurkat, THP-1, Rc-2a, and U-937 were obtained from ATCC.

TNP modification of cells

Cells were modified with TNP by treatment with TNBS, as described (15, 16). Briefly, cells were washed in PBS to remove serum proteins from the culture medium and resuspended in PBS at $1 \times 10^7/\text{ml}$. Then 1 ml TNBS solution (10 mM, pH 7.4) was added to 1 ml cell suspension. After shaking, the cells were incubated with TNBS at 37°C for 15 min. The reaction was stopped by adding 20 ml medium supplemented with 10% FCS. The cells were then centrifuged and washed three times with PBS to remove unbound TNBS as well as TNP-modified serum proteins. Although TNP modification did not affect cell viability as approved by propidium iodide staining and FACS analysis (data not shown), cells were always lost during this procedure and had to be readjusted to $1 \times 10^7/\text{ml}$. Modification of cells with DNBS was performed exactly as with TNBS.

Generation of T cell lines

Purified peripheral blood T cells ($1 \times 10^5/\text{well}$) were stimulated with TNP (5 mM)-modified autologous EBV-LCL ($5 \times 10^4/\text{well}$) that had been irradiated (3000 rad, ^{137}Cs source) in 200 μl complete medium plus 5% human AB serum in 96-well U-bottom plates (Costar, Cambridge, MA) for 7 days. T cells were then restimulated with TNP-modified OM cells ($2 \times 10^4/\text{well}$) and IL-2 (10 U/ml). Culture medium (100 μl) was replaced 3 days later with fresh medium containing IL-2 (10 U/ml). The outgrowing T cells were propagated then by weekly restimulation, and were used 8 wk later for functional studies.

Purified T cells of an allogeneic donor were used to establish an alloreactive CTL line directed against OM cells or other EBV-LCL. The procedure was essentially the same, except that untreated OM cells were used as stimulator cells.

Cytotoxicity assay

Cytotoxicity was measured in a standard 4-h ^{51}Cr release assay. Briefly, 2×10^6 target cells were resuspended in 100 μl PBS and labeled with ^{51}Cr (50 μCi) for 1 h at 37°C. After washing two times with medium, 5×10^3 target cells/well were added to triplicates of decreasing numbers of CTL in 96-well U-bottom plates. To determine the spontaneous release, only medium was added to the target cells in three wells, and to define the maximal release, 100 μl of a Triton X-100 solution (2%) was added to a separate triplicate of wells with target cells. The cells were then centrifuged, except when mAb inhibition studies were performed, and incubated at 37°C for 4 h. Released ^{51}Cr was then harvested with a filter harvesting system (Skatron, Oslo, Norway) and measured on a gamma counter (model 5000 Top-count Instrument; Packard, Meriden, CT). The percentage of specific lysis was calculated by the formula: (value of the probe - spontaneous release)/(maximal release - spontaneous release) \times 100.

For cold target inhibition assays, unlabeled inhibitor cells (cold targets) were seeded in plates at various cold to hot target ratios. Effector cells were

then added and incubated for 30 min at 37°C before labeled target cells (hot targets) were added. Cytotoxicity was assessed as described above.

Redirecited killing of target cells (THP-1) was induced by adding the CD3 mAb OKT-3 at 1 $\mu\text{g/ml}$. THP-1 cells were selected because they express Fc γ R and facilitate cross-linking of CD3/TCR complexes of the CTL by OKT-3.

Immunofluorescence analysis

For membrane staining, cells (5×10^5) were incubated for 30 min at 0–4°C with conjugated (FITC or PE) or unconjugated mAb. For stainings using unconjugated mAbs, FITC-conjugated F(ab') $_2$ of sheep anti-mouse Ig Abs (An der Grub) were used as a second step reagent.

Flow cytometric analysis was performed using a FACScan flow cytometer (BD Immunocytometry Systems, Palo Alto, CA).

TCR repertoire analysis

Fluorochrome-labeled anti-TCR mAbs used were against V α 2 (clone: F $_1$ FITC), V α 12.1 (6D6 FITC), V β 3.1 (8F10 FITC), V β 5.3 (W112 FITC), V β 6/7 (OT145 FITC), and V β 13.1/13.3 (BAM13 FITC; all obtained from Serotec, Oxford, U.K.); V β 1 (BL37.2 PE), V β 2.1 (MPD2D5 FITC), V β 5.1 (Immu157 FITC), V β 5.2 (36213 FITC), V β 7.1 (ZOE FITC), V β 8.1/8.2 (56C5.2 FITC), V β 9.1 (FIN9 PE), V β 11.1 (C21 FITC), V β 12.2 (VER2.32.1 FITC), V β 13.1 (Immu222 FITC), V β 13.6 (JU74.3 FITC), V β 14.1 (CAS1.13 FITC), V β 16.1 (TAMAYA1.2 FITC), V β 17.1 (E17.5F3 FITC), V β 18.1 (BA62.6 PE), V β 20.1 (ELL1.4 FITC), V β 21.3 (IG125 FITC), V β 22.1 (Immu546 FITC), and V β 23.1 (AF23 PE; all obtained from Immunotech). Anti-pan TCR $\alpha\beta$ PE (BMA031) was from Immunotech.

Depletion of predominant TCR-expressing T cells was achieved by coating of the respective mAbs onto beads (DynaL Biotec, Hamburg, Germany) and subsequent magnetic separation. Removal was examined by flow cytometry.

Determination of intracellular cytokines

The cytokine pattern of the TNP-reactive CTL was determined by the intracellular staining technique (15). Upon stimulation with PMA (100 nM) and ionomycin (1 μM) and in the presence of monensin (5 μM) for 18 h in 96-well U-bottom plates ($2 \times 10^5/\text{well}$), the cells were harvested and fixed for 20 min at room temperature by adding 100 μl FIX solution (An der Grub). Subsequently, cells were washed once with 4 ml PBS, resuspended in 100 μl PBS, and permeabilized by the addition of 100 μl PERM solution (An der Grub). Immediately, the indicated directly conjugated anti-cytokine mAbs were added and incubated for 20 min at room temperature. The cells were then washed twice, resuspended in PBS (200 μl), and analyzed by flow cytometry.

To determine Ag-induced cytokine production, TNP-specific CTL (5×10^5) were mixed with OM cells (1×10^6), either TNP modified or untreated, in the presence of monensin in siliconized tubes. The cells were centrifuged to allow conjugate formation and incubated again for 18 h, and cytokine production was assayed as described above.

TCR down-modulation

TCR down-modulation upon Ag contact was performed as previously described (17). TNP-specific CTL (5×10^5) were mixed with OM-LCL, OTMA-LCL, or K562 cells (1×10^6), either TNP modified or untreated, in siliconized tubes. The cells were centrifuged to allow conjugate formation and incubated for 6 h at 37°C. Thereafter, TCR cell surface expression density was assayed by CD3 mAb UCHT-1 reactivity and stained with PE-conjugated F(ab') $_2$ of goat anti-mouse Ig Abs (Caltag Laboratories). In selected experiments, OTMA-LCL cells were pretreated with A1 (CD39) for 30 min at 37°C.

Results

Characterization of the TNP-specific CTL line used in this study

Purified peripheral blood T cells from a healthy volunteer were stimulated with TNP-modified (5 mM) autologous EBV-transformed lymphoblastoid B cells called OM-LCL for 7 days, and subsequently restimulated weekly with TNP-modified OM-LCL cells in the presence of a suboptimal dose of IL-2 (10 U/ml), as described in detail in *Materials and Methods*.

After 2 mo, the outgrown T cells selectively recognized TNP-modified OM-LCL cells, but not untreated cells, and efficiently

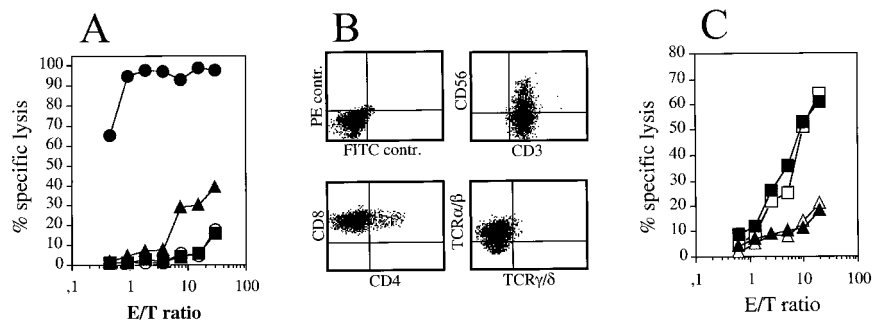


FIGURE 1. Characterization of the T cell line used in this study. *A*, T cells efficiently recognize and lyse TNP-modified (5 mM) OM-LCL cells (filled circles), but not untreated (open circles) or DNP-modified cells (filled squares). Recognition of TNP-modified cells is inhibited in the presence of the anti-TNP mAb 1B7 (filled triangles). The results are representative of three independent experiments. *B*, Immunophenotype of the TNP-specific T cell line after 2 mo of culture. *C*, Influence of TNP modification on the lytic capacity of alloreactive CTL. Alloreactive CTL directed against the EBV-LCL (A26, 29 B17, 18 Cw3) (squares), which do not lyse OM cells (triangles). The cytotoxic responses of the alloreactive CTL against TNP-modified target cells (filled symbols) were indistinguishable from the ones against untreated target cells (open symbols).

killed TNP-modified cells even at low E:T ratios (Fig. 1A). Treatment of OM-LCL cells with TNP does not facilitate lysis by alloreactive CTL (Fig. 1C), indicating that TNP modification is not a general bias toward increased sensitivity for CTL-mediated lysis. The TNP specificity was further supported by the observations that the anti-TNP mAb 1B7 strongly inhibited CTL recognition of TNP-modified target cells and that DNP-modified OM-LCL cells were not lysed (Fig. 1A).

Surface marker analysis revealed that >95% of the T cells obtained were CD8⁺ TCRαβ⁺ (Fig. 1B). The remaining cells were reproducibly CD8⁺/CD4⁺ double-positive T cells (TCRαβ⁺), whereas single CD4⁺ T cells or NK cells (CD3⁻CD56⁺) were not found. Analysis of the TCR repertoire indicated that this CTL line was not clonal with two dominant subpopulations (22 ± 3%) expressing TCR-αv12.1/-vβ13.3 and TCR-αv12.1/-vβ17, respectively. Upon stimulation with TNP-modified OM-LCL or PMA/ionomycin, the CTL produced IFN-γ and TNF-α, and only few IL-4-producing cells were found, which is typical for Tc1-type T cells (data not shown).

Recognition of TNP-modified cells is MHC class I independent

To analyze whether CTL recognition of haptenized cells was MHC class I restricted, we selected a panel of EBV-LCL-expressing mismatched MHC class I phenotypes and haptenized them with 5 mM TNP. As can be seen from Fig. 2A, all of the EBV-LCL tested were efficiently killed by the CTL upon TNP modification. Importantly, K562 cells were also recognized by these CTL upon TNP modification, whereas untreated or DNP-modified K562 cells were not lysed (Fig. 2B).

K562 cells do not express MHC class I molecules and, thus, are typical NK cell targets not recognized by classical CTL. In addition, K562 cells used in this study were also negative for β₂-microglobulin molecules and do not express MHC class II or CD1 molecule family members (data not shown). Thus, it is unlikely that nonclassical MHC class I molecules could substitute for MHC class I.

Two lines of evidence demonstrate that the CTL directly recognized TNP-modified cell surface proteins. First, broad proteolytic removal of cell surface proteins from K562 cells by bromelain, papain, or subtilisin treatment completely abolished CTL recognition (Fig. 2B). Second, pretreatment of K562 cells with inhibitors of endosomal processing (chloroquine), exocytosis of putative Ag-presenting molecules to the cell surface (brefeldin A), or metabolic energy (NaF) did not influence target cell recognition

(Fig. 2B). This makes it unlikely that TNP-modified cell surface molecules had to be processed for CTL recognition.

The ability to recognize TNP-modified target cells in an MHC-independent manner was not unique for CTL from donor OM, but also could be observed with TNP-specific CTL from two other donors (Fig. 2C).

CTL recognition of TNP-modified K562 cells is mediated by the TCR

The question arose as to whether the observed MHC class I-independent recognition of TNP-modified cells involves the TCR structure on T cells. To elucidate this issue, we used Ab inhibition studies and Ag-triggered TCR down-modulation as readout systems.

Results presented in Fig. 3A demonstrate that mAbs against TCRαβ or CD3 could significantly inhibit CTL recognition of TNP-modified OM-LCL cells or K562 cells. Interestingly, addition of the CD8 mAb Campath8c, known to block CTL target cell interaction by preventing CD8 binding to MHC class I (18), showed no such effect. mAbs against other cell surface molecules important for CTL target cell interaction such as CD11a (LFA-1) and CD58 (LFA-3) were inhibitory as expected.

Down-modulation of CD3 cell surface expression upon interaction with target cells pulsed with the appropriate Ag is a direct measure for TCR usage (17, 19). Accordingly, the CTL were cocultured with TNP-modified or untreated K562 cells, and the TCR cell surface expression density was analyzed. Results in Fig. 3B demonstrate that upon interaction with haptenized K562 cells, the TCR cell surface expression was down-modulated on the majority of CTL when compared with cocultures with untreated target cells.

Titration of TNP loading

Treatment of cells with TNP (5 mM) has been shown to result in massive haptenization so that a cell will carry up to 4×10^8 TNP molecules/cell on the surface (20, 21). In view of this peculiarly high antigenic density found on cells upon TNP modification, we were interested to determine how much hapten on a cell was needed to be recognized by CTL in a MHC class I-independent way.

For this purpose, we treated K562 and OM cells with titrated amounts of TNP and compared TNP loading and CTL recognition. Results presented in Fig. 4 demonstrate that clear differences exist between K562 and OM-LCL cells concerning the degree of haptenization required for CTL recognition. Whereas treatment of OM

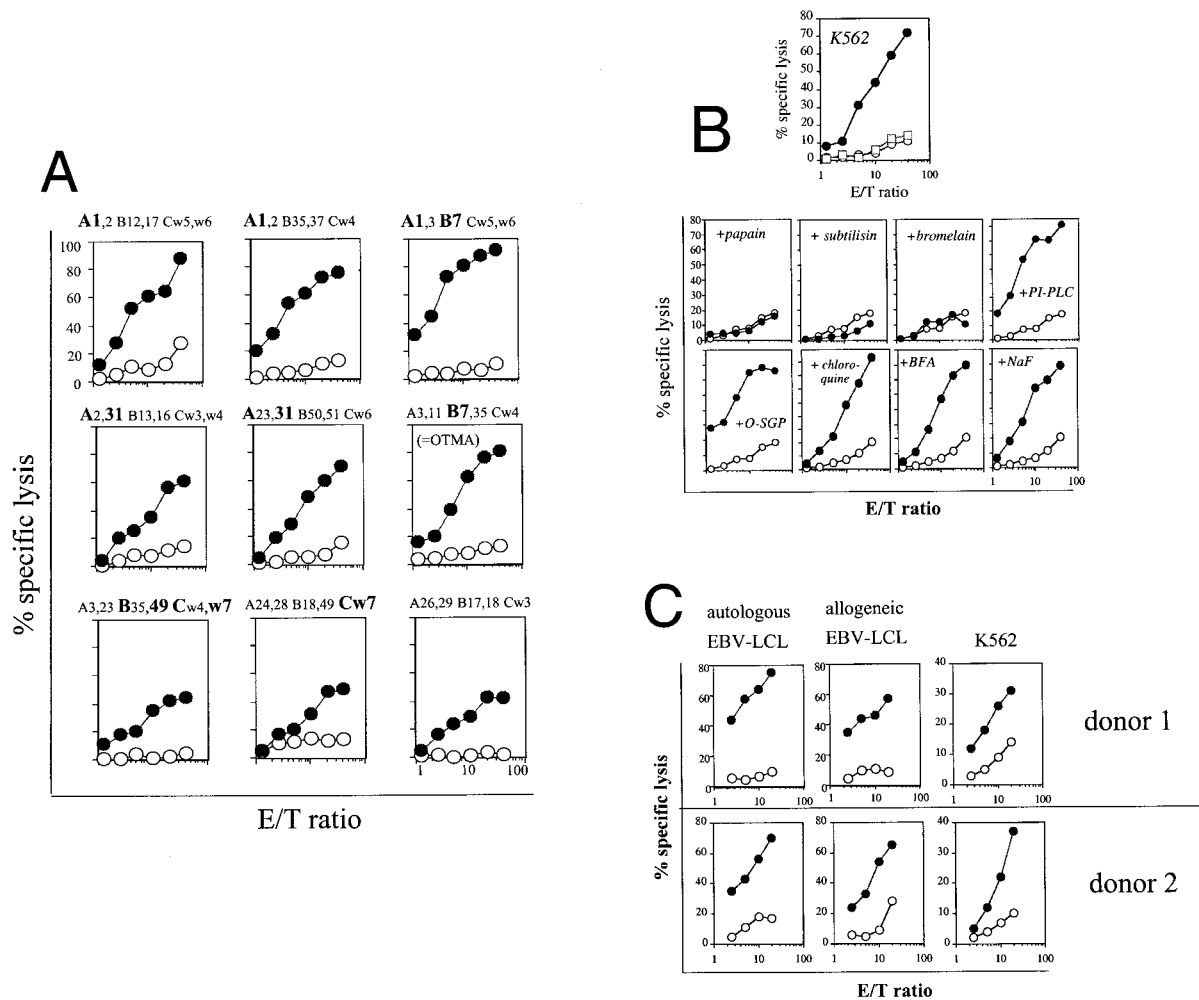


FIGURE 2. MHC-independent target cell recognition by the CTL. *A*, CTL recognition of target cells treated with TNP (5 mM) (filled circles) was not restricted to autologous OM cells, but was also observed with a panel of allogeneic EBV-LCL cells expressing mismatched MHC class I phenotypes; *B*, MHC-negative K562 cells, which were not recognized without TNP modification (open circles) or DNP modification (open squares). The results are representative of three independent experiments. Broad proteolytic removal of cell surface proteins from TNP-modified K562 cells (filled circles) with papain, subtilisin, or bromelain completely inhibits CTL recognition. Cleavage of GPI-anchored molecules or *O*-sialoglycoproteins with phosphoinositide phospholipase C or *O*-sialoglycoprotease from *Pasteurella hemolytica*, respectively, did not affect CTL recognition. Pretreatment of TNP-modified K562 cells with chloroquine, brefeldin A (BFA), or NaF did not prevent CTL recognition. The influence of the tested enzymes and inhibitors on CTL recognition of unmodified K562 cells was analyzed in parallel (open circles). *C*, The ability to recognize target cells in an MHC-independent manner was not restricted to donor OM, but could also be observed with TNP-specific CTL from two other donors (donor 1, HLA A1, 3 B8, 35 Cw4,w7; donor 2, HLA A1, A3 B7, 8 Cw7). The HLA haplotype of the allogeneic EBV-LCL was A34 B14 Cw8. CTL recognition of target cells treated with TNP (5 mM) (filled circles) and untreated target cell (open circles) was tested in parallel.

cells with TNP concentrations down to 1 μ M was sufficient for CTL recognition, K562 cells treated with TNP below 1 mM were no longer amenable to lysis (Fig. 4). In contrast to these differences, the anti-TNP mAb reactivity with the two cell lines was very similar over the entire dose range (Fig. 4). Because the binding of anti-TNP Abs to haptenized cells reflects the number of TNP molecules accessible to TCR recognition (19, 22, 23), we must assume that different mechanisms are involved in the recognition of TNP-modified K562 and OM-LCL cells by the CTL.

Low density TNP recognition is MHC class I dependent

Because autologous MHC class I-expressing OM-LCL cells, in contrast to MHC class I-negative K562 cells, were recognized by our CTL even when treated with low amounts of TNP, we wondered whether under these conditions CTL recognition was MHC class I dependent.

Results demonstrated in Fig. 5A indicate indeed that CTL recognition of OM-LCL cells treated with 10 μ M TNP could be

strongly inhibited with anti-MHC class I mAb W6/32 or CD8 mAb Campath8c (data not shown). These mAbs were ineffective when OM-LCL cells had been modified with 5 mM TNP. Moreover, CTL recognition of different EBV-LCL cells treated with 10 μ M TNP was restricted to HLA-A1-expressing target cells, meaning that HLA-unrelated target cells that were efficiently lysed upon modification with 5 mM TNP (see Fig. 2) were not lysed anymore (Fig. 5B).

MHC class I-dependent and -independent target cell recognition seem to be mediated by the same TNP-specific CTL

To elucidate whether MHC class I-dependent and -independent recognition of TNP-modified target cells was mediated by the same or different subpopulations of CTL within the TNP-specific CTL line from donor OM, we performed cold target inhibition experiments between OM-LCL cells treated with 10 μ M TNP (MHC-dependent recognition) and HLA-mismatched OTMA-LCL or K562 cells treated with 5 mM (MHC-independent recognition).

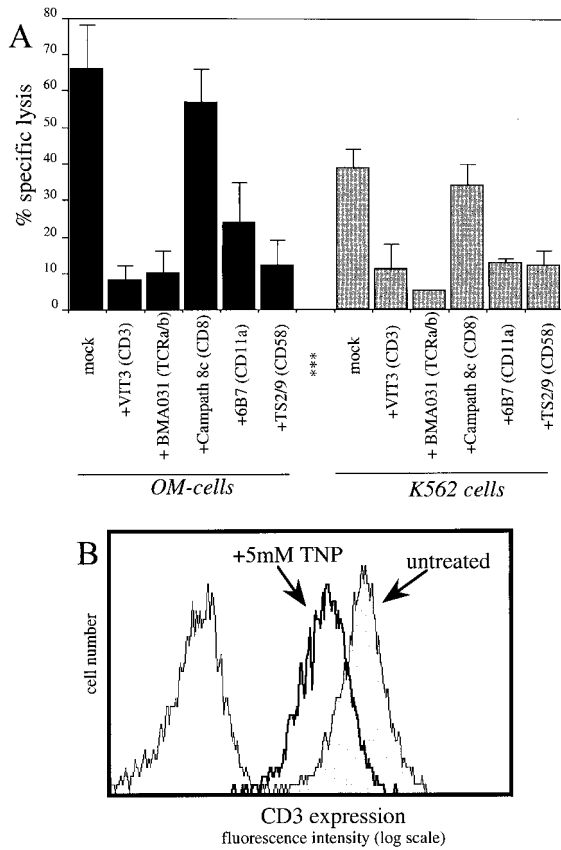


FIGURE 3. Target cell recognition is mediated by the TCR. *A*, Addition of mAbs against the TCR $\alpha\beta$ complex and adhesion molecules inhibits CTL recognition of TNP-modified autologous OM cells and also of MHC-negative K562 cells. The results demonstrate mean values \pm SD of four experiments. *B*, Down-modulation of CD3 cell surface expression on TNP-specific CTL upon cocultivation with TNP-modified K562 cells. Background fluorescence intensity was determined with the negative control mAb VIAP (calf intestine alkaline phosphatase specific) (thin line). The results are representative of three independent experiments.

Interestingly, these cold target cell inhibition experiments revealed strong cross-inhibition between target cells that were recognized by the CTL in an MHC-dependent way (OM-LCL) and target cells that were recognized in an MHC-independent way (OTMA-LCL, K562) (Fig. 6A).

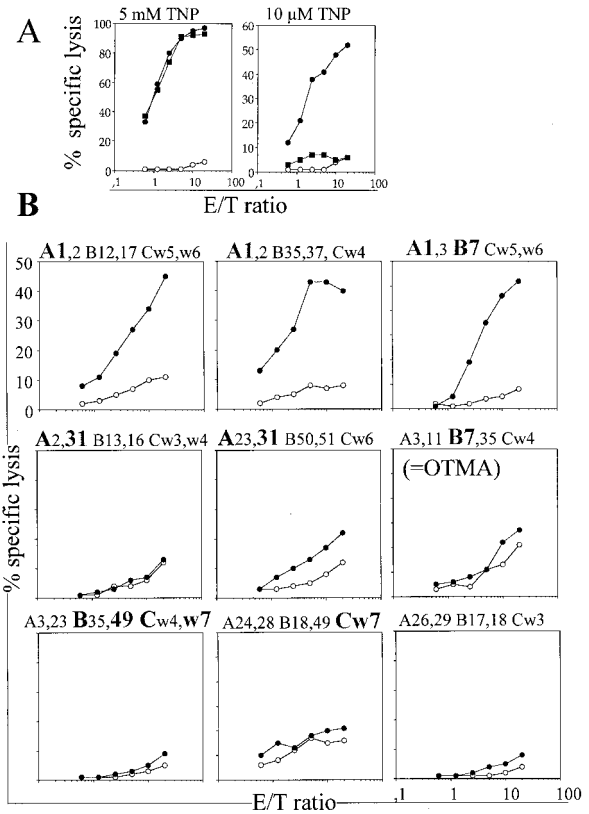


FIGURE 5. CTL recognition of low dose TNP-loaded OM cells is MHC class I dependent and HLA-A1 restricted. *A*, Autologous OM cells were modified with high (5 mM) or low (10 μ M) TNP and used as target cells. CTL activity against both kinds of target cells was evaluated in the absence (filled circles) or presence (filled squares) of mAb W6/32 against MHC class I molecules. CTL activity against untreated OM cells was tested for control (open circles). *B*, A panel of EBV-LCL cell lines with an overlapping or unrelated HLA phenotype in relation to OM cells (HLA: A1, 31; B7, 49; Cw7) was loaded with TNP (10 μ M) and used as target cells. HLA homologies of the different EBV-LCL cells with OM cells are bold.

Cross-inhibition was nearly as effective as cold target inhibition between OM-LCL vs OM-LCL (MHC dependent) and OTMA-LCL vs OTMA-LCL, or K562 vs K562 (MHC independent). Addition of untreated cells as cold targets had little inhibitory effects (Fig. 6A).

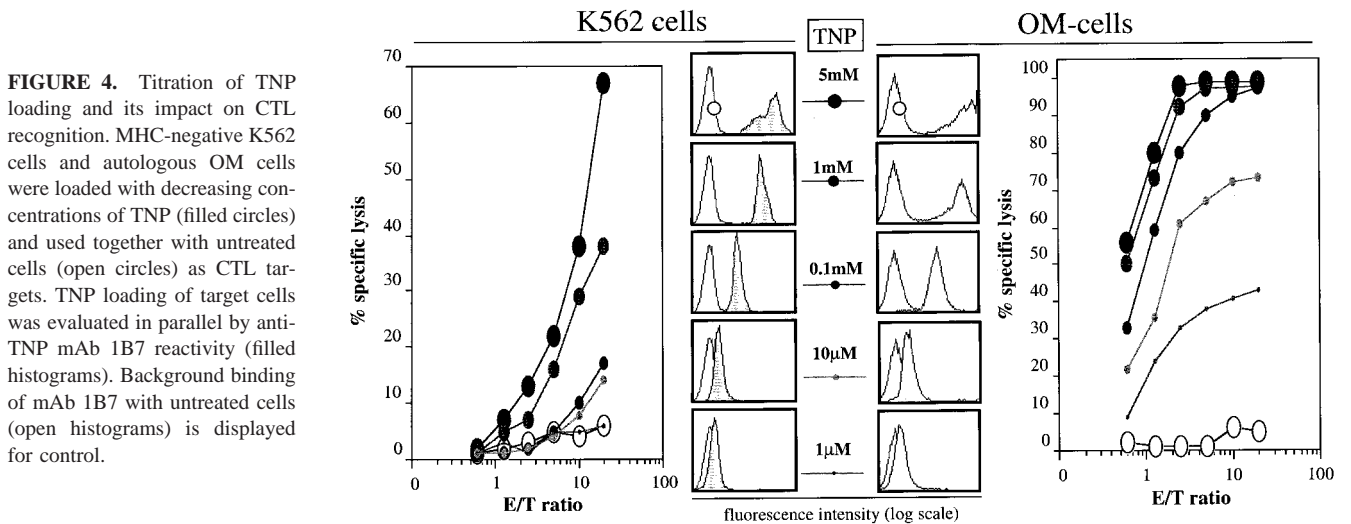


FIGURE 4. Titration of TNP loading and its impact on CTL recognition. MHC-negative K562 cells and autologous OM cells were loaded with decreasing concentrations of TNP (filled circles) and used together with untreated cells (open circles) as CTL targets. TNP loading of target cells was evaluated in parallel by anti-TNP mAb 1B7 reactivity (filled histograms). Background binding of mAb 1B7 with untreated cells (open histograms) is displayed for control.

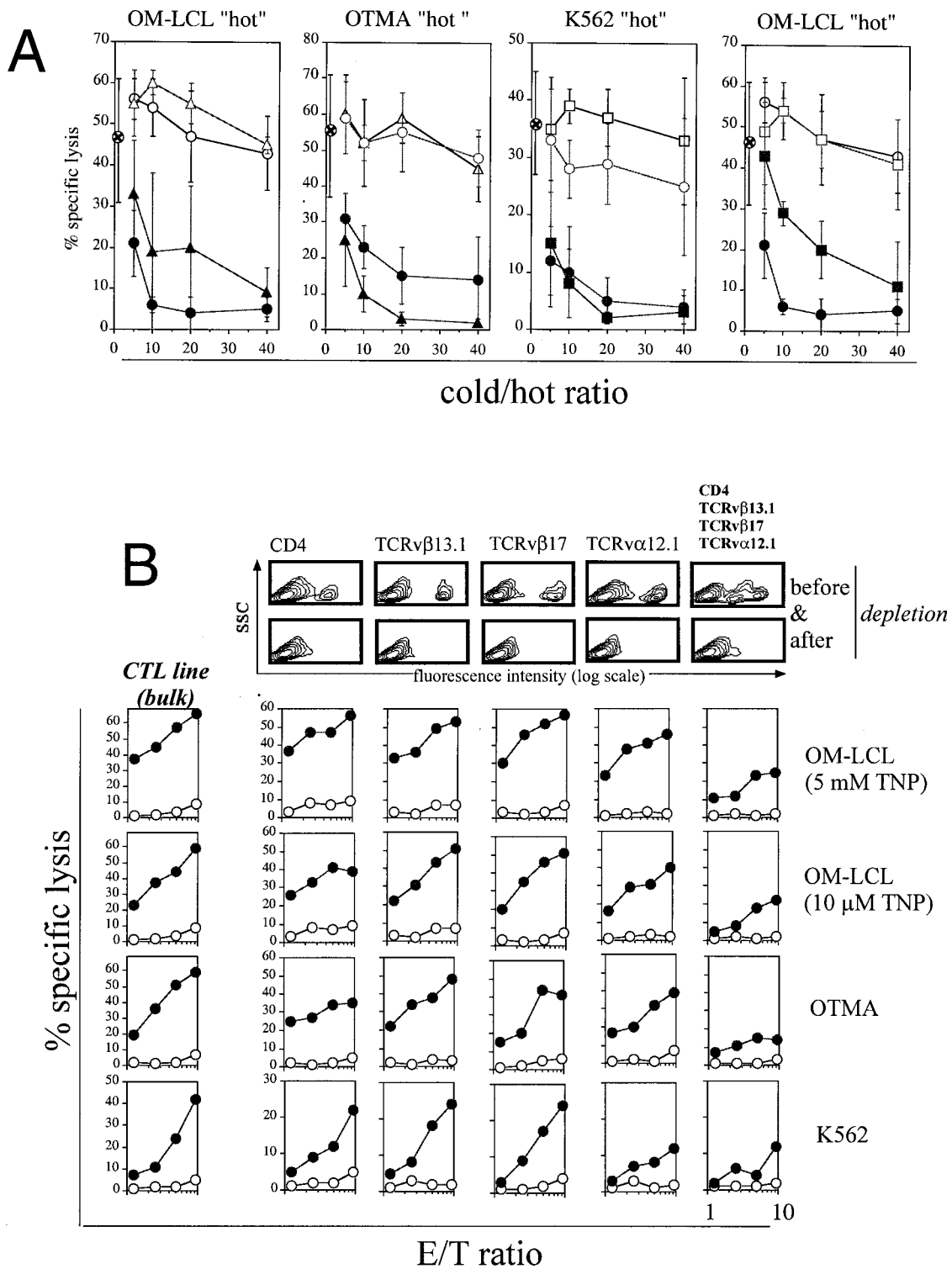


FIGURE 6. The majority of the TNP-specific CTL seems to be capable of recognizing target cells via the MHC-dependent and MHC-independent way. *A*, Cold target inhibition experiments. CTL activity against chrom-labeled (hot) OM cells loaded with 10 μM TNP (MHC I-dependent recognition) and K562 cells or OTMA cells loaded with 5 mM TNP (MHC-independent recognition) was investigated in the presence of the following cold targets: OM cells modified with 10 μM TNP (filled circles); OTMA cells modified with 5 mM TNP (filled triangles); K562 cells modified with 5 mM TNP (filled squares); untreated OM cells (open circles) or untreated OTMA cells (open triangles); untreated K562 cells (open squares). Results indicate the mean values ± SD of three experiments. The circles with a cross inside illustrate the level of lysis of the indicated hot target cells in the absence of cold targets. *B*, Depletion of the indicated subpopulations and analysis of the cytolytic activity of the remaining cells against four different target cells that were either TNP modified (filled circles) or untreated (open circles).

Because we were not able to generate (stable) cytolytic T cell clones from our TNP-specific CTL lines by several approaches, we depleted individual TCR-expressing subpopulations and an-

alyzed the recognition pattern of the remaining cells. The results of these experiments revealed that not the removal of individual populations, but the combined depletion of all four

major subpopulations significantly reduced the cytolytic potential of the OM-CTL (Fig. 6B). Importantly, this effect was seen with all target cell types analyzed reaching from MHC-dependent (OM-LCL treated with 10 μ M TNP) to MHC-independent recognition (OTMA, K562 treated with 5 mM TNP). Thus, the majority of the CTL was seemingly capable of recognizing TNP-modified target cells in an MHC-restricted as well as MHC-independent way.

MHC class I-independent recognition is restricted to certain cell types

The capability of TNP-specific CTL in recognizing TNP-modified target cells independent of MHC class I molecules indicated that probably any TNP-modified cells would be susceptible to lysis by this CTL line.

However, results presented in Fig. 7A demonstrate that not every TNP-modified target cell type could serve as CTL target.

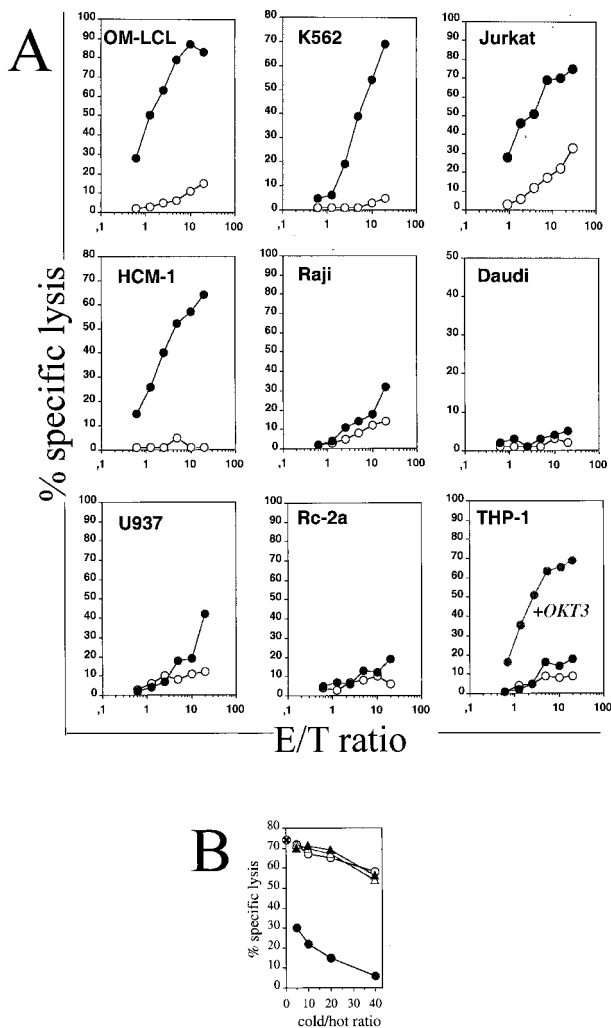


FIGURE 7. MHC-independent CTL recognition of TNP-modified target cells is cell type restricted. *A*, Different cell types were treated with 5 mM TNP (filled circles) and tested in parallel with untreated cells (open circles) as target cells. For THP-1 cells, CTL activity was further evaluated in the presence of CD3 mAb OKT3, to demonstrate the principal capability of the CTL to lyse THP-1 cells. *B*, CTL activity against chrom-labeled (hot) OM cells loaded with 5 mM TNP was analyzed in the presence of the following cold targets: OM cells modified with 5 mM TNP (filled circles); Daudi cells modified with 5 mM TNP (filled triangles); untreated OM cells (open circles); or untreated Daudi cells (open triangles).

Whereas all EBV-LCL tested ($n = 9$) as well as the T cell line Jurkat and the mast cell line HMC-1 were efficiently killed when TNP modified, the Burkitt cell lines Raji and Daudi as well as the myeloid cell lines THP-1, Rc-2a, and U-937 were not recognized by the CTL. The lack of CTL-mediated lysis was apparently not due to insufficient TNP loading as, e.g., Daudi cells were found to express even more TNP molecules on the surface than K562 cells after treatment with 5 mM TNP (data not shown). It is more likely that the CTL did not recognize certain target cells because of their lack of expression of appropriate antigenic epitopes upon TNP treatment. This is supported by the observation that TNP-modified THP-1 cells as well as untreated THP-1 cells (data not shown), which were not recognized by the CTL, were efficiently lysed by the CTL through redirected killing induced by the CD3 mAb OKT-3 (Fig. 7A). Moreover, TNP-modified Daudi cells did not inhibit CTL recognition of OM-LCL cells in cold target competition experiments (Fig. 7B).

CD39 molecules are directly recognized on TNP-modified EBV-LCL

The above finding suggested that the selective MHC-independent target cell recognition by the CTL was restricted by differentially expressed cell surface molecules. This difference was particularly striking among B cells. EBV-LCL were efficiently recognized upon TNP modification, whereas Burkitt lymphoma B cells such as Daudi were not. So we searched for cell surface molecules expressed on EBV-LCL, but not on Daudi cells.

A typical cell surface molecule that is expressed on EBV-LCL and not on Daudi cells is the B cell activation Ag CD39 (24) (Fig. 8A). When mAbs against CD39 were added to OTMA cells modified with 5 mM TNP (MHC-independent recognition), we observed a strong inhibition of lysis by the CTL (Fig. 8B). mAbs against CD9, which similar to CD39 is expressed on EBV-LCL but not on Daudi cells, showed no such effect. When CD39 mAbs were added to OM-LCL treated with 10 μ M TNP (MHC-dependent recognition), the mAbs failed to inhibit CTL-mediated lysis. In parallel experiments performed with alloreactive CTL, we also found no inhibitory effects with the CD39 mAbs. This indicates that only MHC class I-independent recognition of TNP-modified target cells, and not MHC-dependent, could be inhibited with CD39 mAbs, suggesting that TNP-modified CD39 plays a critical role in MHC-independent recognition of target cells by the CTL.

Moreover, pretreatment of TNP-modified OTMA cells (MHC-independent CTL recognition) with CD39 mAb A1 significantly inhibited CD3 down-modulation on the CTL, providing evidence that the CTL directly recognize TNP-modified CD39 molecules (Fig. 8C).

Interestingly, however, K562 cells do not express CD39, although they are specifically lysed upon TNP modification. Thus, there exist obviously additional cell surface proteins with similar structural and molecular features such as CD39, which are recognizable for hapten-specific CTL.

Discussion

Hapten-specific T cells are the essential mediators in allergic contact hypersensitivity, and it is now generally held that such T cells recognize hapten-modified peptides presented by MHC molecules (1, 25, 26). Yet it has been observed that MHC restriction of hapten-specific T cells is frequently promiscuous (13, 27–29). Because modification of cells with haptens generates numerous hapten-carrier conjugates, we analyzed in this study the impact of Ag density on MHC restriction of a CTL response specific to TNP. The results presented in this study demonstrate that T cell recognition of high dose haptentated target cells qualitatively differs from

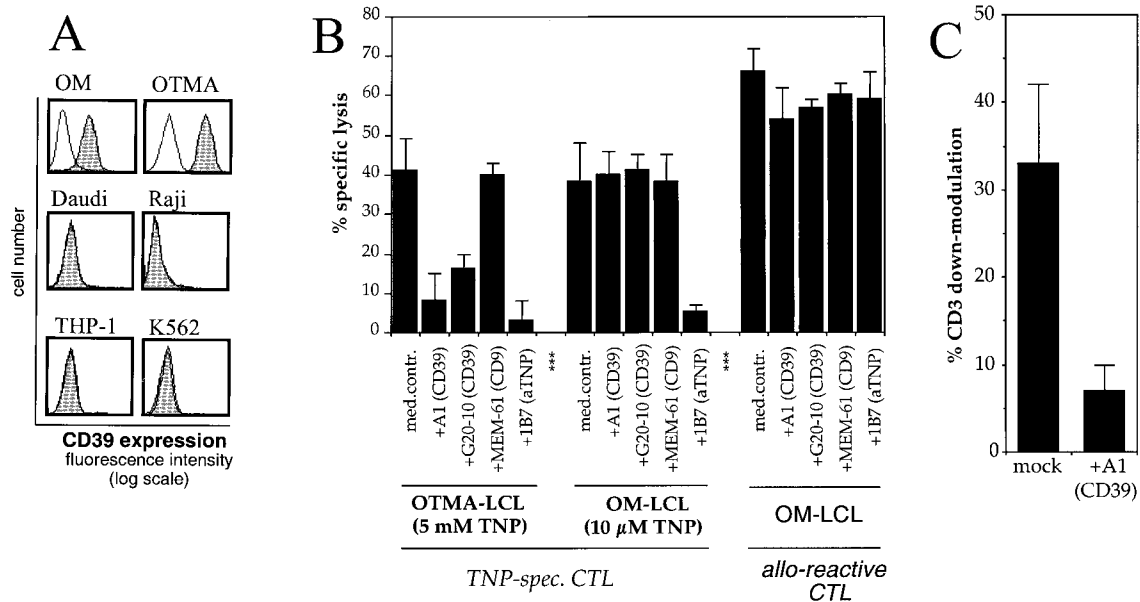


FIGURE 8. CD39 molecules are directly recognized on TNP-modified EBV-LCL by the CTL. *A*, Staining of OM, OTMA, Raji, Daudi, THP-1, and K562 cells with mAb A1 (CD39) demonstrates expression of CD39 on EBV-LCL, but not on Burkitt lymphoma cells (Daudi, Raji) or THP-1 and K562. *B*, Addition of CD39 mAbs strongly inhibits CTL recognition of OTMA cells loaded with high TNP doses (MHC-independent recognition), but not lysis of OM cells loaded with 10 μ M TNP (MHC I-dependent recognition) or lysis of untreated OM cells by an alloreactive CTL line. The results demonstrate mean values \pm SEM of three experiments. *C*, CD3 down-modulation on CTL upon coculture with OTMA cells loaded with 5 mM TNP (MHC-independent recognition) is inhibited by pretreatment of OTMA cells with CD39 mAb A1. The results demonstrate mean values \pm SD of three experiments.

that of low dose haptenated target cells in that it is MHC independent. However, under both conditions, target cell recognition by the TNP-specific, CD8⁺ CTL is mediated via their TCR $\alpha\beta$. The MHC-independent recognition property of the CTL became evident when we found that target cells expressing either mismatched MHC class I molecules or even no MHC molecules were recognized after high TNP loading. This MHC-independent recognition of haptenated cells by the CTL was not promiscuous, but cell type restricted. Thus, in addition to HLA-A1 molecules, which are critical for CTL recognition at low TNP loading, differentially expressed cell surface molecules seem to allow CTL recognition upon high dose TNP loading of target cells.

Hapten-specific T cells can detect antigenic structures in two ways. They can recognize the hapten only in context with a specific amino acid motif or independent of the carrier peptide (27). In the case of carrier-independent recognition, hapten-specific T cells frequently show little MHC restriction (13, 28, 29) and, in the presence of high antigenic valencies, even display promiscuous recognition properties for artificial carrier structures if they are modified with the appropriate haptens (30–33). For efficient recognition and lysis of target cells via the MHC-independent mechanism described in this study, loading with at least 1 mM TNP was required. On K562 cells, this treatment results in the expression of 6.8×10^8 TNP molecules per cell. This enormous amount is in agreement with previously reported values of $1\text{--}4 \times 10^8$ TNP groups for Chinese hamster ovary cells (20, 21) and $3\text{--}5 \times 10^7$ TNP groups for mouse lymphoma cells upon loading with 1 mM TNP (34). Moreover, Levy and coworkers (35) could demonstrate for TNP-modified splenic lymphocytes that $1\text{--}3 \times 10^6$ TNP groups/cell are accessible for anti-TNP Abs. Because the binding of anti-TNP Abs to haptenized cells inhibits T cell recognition (19, 22, 23), anti-TNP Ab reactivity is thought to reflect the number of TNP groups that should also be accessible for TCR. This particular high Ag density required for MHC-independent CTL recognition is suggestive of a carrier-independent mechanism (33, 36). Yet,

our CTL seem to recognize TNP in context with a specific amino acid background. This can be concluded from the observations that target cell recognition was restricted to certain cell types (EBV-LCL, K562, HMC-1). In contrast, Burkitt cell lines (Raji, Daudi) or several myeloid cell lines tested (THP-1, Rc-2a, U-937) were not recognized by the CTL despite high TNP loading. In addition, CTL recognition of TNP-modified target cells was not inhibited in the presence of very high amounts of TNP (up to 100 mM), when added in form of BSA, HSA, or fibrinogen conjugates (data not shown). It is thus tempting to speculate that qualitative antigenic differences, rather than merely high TNP valencies on a given target cell determine CTL recognition.

Hapten-specific T cells can express TCR with a remarkably high avidity. This can be concluded from old studies (30, 31) in which nuclear protein-specific T cells could be isolated by panning on nuclear protein-coated nylon fibers, and from binding studies with FITC-conjugated high polymeric Ficoll to a selected T cell hybridoma line (33). More recently, Franco et al. (13) could demonstrate that the avidity of hapten-specific T cells for TNP-modified peptides is 100-fold higher than the avidity of T cells specific for the nonmodified peptides. The reason for this seems to be based on the potential of hapten- or carbohydrate-modified peptides to interact extensively with side chains in the pocket formed by the complementarity-determining region 3 $\alpha\beta$ segments of TCR (37–40). This interaction will significantly strengthen contact formation between the antigenic structure and TCR, especially if multiple hydrogen bonds are established with the three NO₂ groups of the TNP moiety (13). The avidity between the TCR of our CTL and antigenic epitopes on certain cell surface molecules is obviously high enough to compensate for the lack of supportive MHC/TCR contact sites that are usually required for conventional peptide recognition (41).

Such appropriate antigenic epitopes are obviously generated in a sufficient number on CD39 molecules upon high dose TNP modification. CD39 is a 70- to 100-kDa cell surface glycoprotein with

an estimated loop-like extracellular structure that is caused by two transmembrane regions at the N- and C-terminal regions (42). The putative extracellular part of CD39 contains 26 lysine residues, which resemble potential TNP modification sites (26). Moreover, CD39 is expressed on the cell surface as homotetramer (43). Therefore, CD39 molecule complexes might resemble sites of high TNP concentrations on hapten-modified cells, which might explain their preferential recognition by the CTL. Evidence that TNP-modified CD39 molecules are directly recognized by our CTL was first obtained by mAb inhibition studies. mAbs against CD39 selectively inhibited MHC-independent recognition of our CTL, whereas HLA-A1-restricted recognition of OM-LCL cells or lysis of OM-LCL cell by an alloreactive CTL line was not affected. Second, the myeloid cell lines tested in this study (THP-1, Rc-2a, U-937) do not express CD39 molecules and were not recognized by our CTL. Third, pretreatment of OTMA cells (MHC-independent target) with CD39 mAb A1 strongly inhibited TNP-induced CD3 down-modulation. This demonstrates that loading of target cells with CD39 mAb inhibits recognition of the antigenic epitopes by the TCR.

CD39 was originally described as a B cell activation Ag (24, 44). The major function of CD39 seems to be its intrinsic ecto-ATPase activity (45, 46). Due to this enzymatic property, CD39 is considered to protect cells from exogenous ATP. Extracellular ATP released from dying cells or CTL is highly toxic for leukocytes. More importantly, CD39-deficient mice show disordered hemostasis and thromboregulation (47). Interestingly, ATPase activity is a characteristic marker of Langerhans cells (48), the crucial initiators of T cell responses in the skin. Langerhans cells indeed constitutively express CD39 (44). This suggests that Langerhans cells use CD39 as protection shield against extracellular ATP. One could thus speculate that chemical modification of CD39, such as with TNP, might resemble a danger signal for the immune system.

MHC class I-independent target cell recognition of CD8⁺, TCR $\alpha\beta$ ⁺ T cells is rare, but might be an important mechanism in diverse immune responses. It has been observed that tumor cell-specific CTL can directly recognize MUC-1 Ag (49–51). CD8⁺ T cells from allergic patients specific for a carbohydrate moiety present on pollen allergens were also found to recognize their Ag independent of MHC presentation (52). Whether CD39 is involved in contact hypersensitivity has not been investigated to date. However, the results of our study demonstrate that hapten-specific CTL can directly recognize CD39 molecules upon hapten modification.

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References

- Grabbe, S., and T. Schwarz. 1998. Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunol. Today* 19:37.
- Kolesaric, A., G. Stingl, and A. Elbe-Burger. 1997. MHC-class I⁺/II⁻ dendritic cells induce hapten-specific immune responses in vitro and in vivo. *J. Invest. Dermatol.* 109:580.
- Boulou, A., A. Cavani, and S. Katz. 1998. Contact hypersensitivity in MHC class II-deficient mice depends on CD8 T lymphocytes primed by immunostimulating Langerhans cells. *J. Invest. Dermatol.* 111:44.
- Kehren, J., C. Desvignes, M. Krasteva, M.-T. Ducluzau, O. Assossou, F. Horand, M. Hahne, D. Kägi, D. Kaiserlian, and J.-F. Nicolas. 1999. Cytotoxicity is mandatory for CD8⁺ T cell-mediated contact hypersensitivity. *J. Exp. Med.* 189:779.
- Kalish, R., and P. Askenase. 1999. Molecular mechanisms of CD8⁺ T cell-mediated delayed hypersensitivity: implications for allergies, asthma, and autoimmunity. *J. Allergy Clin. Immunol.* 103:192.
- Kaplan, G., H. Plutner, I. Mellman, and J. Unkeless. 1981. Studies on externally disposed plasma membrane proteins: trinitrobenzene sulfonic acid derivatization and immune precipitation. *Exp. Cell Res.* 133:103.
- Forman, J., E. Vitetta, D. Hart, and J. Klein. 1977. Relationship between trinitrophenyl and H-2 antigens on trinitrophenyl-modified spleen cells: H-2 antigens on cells treated with trinitrobenzene sulfonic acid are derivatized. *J. Immunol.* 118:797.
- Schmitt-Verhulst, A.-M., C. Pettinelli, P. Henkart, J. Lunney, and G. Shearer. 1978. H-2-restricted cytotoxic effectors generated in vitro by the addition of trinitrophenyl-conjugated soluble proteins. *J. Exp. Med.* 147:352.
- Ciavarrà, R., and J. Forman. 1981. Cell membrane antigens recognized by antiviral and anti-trinitrophenyl cytotoxic T lymphocytes. *Immunol. Rev.* 58:73.
- Ortmann, B., S. Martin, A. von Bonin, E. Schiltz, H. Hoschützky, and H. Weltzien. 1992. Synthetic peptides anchor T cell-specific TNP epitopes to MHC antigens. *J. Immunol.* 148:1445.
- Nalefski, E., and A. Rao. 1993. Nature of the ligand recognized by a hapten- and carrier-specific, MHC-restricted T cell receptor. *J. Immunol.* 150:3806.
- Romero, P., J. Casanova, J. Cerottini, J. Maryanski, and I. Luescher. 1993. Differential-T cell-receptor photoaffinity-labeling among H-2K^d restricted T-lymphocyte clones specific for a photoreactive peptide derivative: labeling of the α -chain correlates with J- α segment usage. *J. Exp. Med.* 177:1247.
- Franco, A., T. Yokoyama, D. Huynh, C. Thomson, S. Nathanson, and H. Grey. 1999. Fine specificity and MHC restriction of trinitrophenyl-specific CTL. *J. Immunol.* 162:3388.
- Steinbrink, K., C. Sorg, and E. Macher. 1996. Low zone tolerance to contact allergens in mice: a functional role for CD8⁺ T helper type 2 cells. *J. Exp. Med.* 183:759.
- Waclavicek, M., O. Majdic, T. Stulnig, M. Berger, R. Sunder-Plassmann, G. Zlabinger, T. Baumruker, J. Stöckl, C. Ebner, W. Knapp, and W. Pickl. 1998. CD99 engagement on human peripheral blood T cells results in TCR/CD3-dependent cellular activation and allows for Th1-restricted cytokine production. *J. Immunol.* 161:4671.
- Shearer, G. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur. J. Immunol.* 4:527.
- Valitutti, S., S. Müller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* 375:148.
- Salter, R., R. Benjamin, P. Wesley, S. Buxton, T. Garrett, C. Clayberger, A. Krensky, A. Normet, D. Littman, and P. Parham. 1990. A binding site for the T-cell co-receptor CD8 on the α 3 domain of HLA-A2. *Nature* 345:41.
- Preckel, T., M. Breloer, H. Kohler, A. von Bonin, and H. Weltzien. 1998. Partial agonism and independent modulation of T cell receptor and CD8 in hapten-specific cytotoxic T cells. *Eur. J. Immunol.* 28:3706.
- Denny, J., and M. Roberts. 1982. A new immunoreactive probe for the isolation and analysis of plasma membrane polypeptides. *J. Biol. Chem.* 257:2460.
- Raub, T., J. Denny, and M. Roberts. 1986. Cell surface glycoproteins of CHO cells: internalization and rapid recycling. *Exp. Cell Res.* 165:73.
- Pircher, H., R. Zinkernagel, and H. Hengartner. 1985. Inhibition of hapten-specific cytotoxic T cell recognition by monoclonal anti-hapten antibodies. *Eur. J. Immunol.* 15:228.
- Martin, S., B. Ortmann, U. Pflugfelder, U. Birsner, and H. Weltzien. 1992. Role of hapten-anchoring peptides in defining hapten-epitopes for MHC-restricted cytotoxic T cells. *J. Immunol.* 149:2569.
- Rowe, M., J. Hildreth, A. Rickinson, and M. Epstein. 1982. Monoclonal antibodies to Epstein-Barr virus-induced, transformation-associated cell surface antigens: binding patterns and effect upon virus-specific T-cell cytotoxicity. *Int. J. Cancer* 29:373.
- Martin, S., and H. Weltzien. 1994. T cell recognition of haptens, a molecular view. *Int. Arch. Allergy Immunol.* 104:10.
- Griem, P., M. Wulferink, B. Sachs, J. Gonzalez, and D. Gleichmann. 1998. Allergic and autoimmune reactions to xenobiotics: how do they arise? *Immunol. Today* 19:133.
- Weltzien, H., C. Moulon, S. Martin, E. Padovan, U. Hartman, and J. Kohler. 1996. T cell immune responses to haptens: structural models for allergic and autoimmune reactions. *Toxicology* 107:141.
- Kohler, J., U. Hartman, R. Grimm, U. Pflugfelder, and H. Weltzien. 1997. Carrier-independent hapten recognition and promiscuous MHC restriction by CD4 T cells induced by trinitrophenylated peptides. *J. Immunol.* 158:591.
- Moulon, C., D. Wild, A. Dormoy, and H. Weltzien. 1998. MHC-dependent and -independent activation of human nickel-specific CD8⁺ cytotoxic T cells from allergic donors. *J. Invest. Dermatol.* 111:360.
- Krawinkel, U., M. Cramer, T. Imanshi-Kari, R. Jack, K. Rajewsky, and O. Mäkelä. 1977. Isolated hapten-binding receptors of sensitized lymphocytes: receptors from nylon wool-enriched mouse T lymphocytes lack serological markers of immunoglobulin constant domains but express heavy chain variable portions. *Eur. J. Immunol.* 8:566.
- Weinberger, J., M. Greene, B. Benacerraf, and M. Dorf. 1979. Hapten-specific T-cell responses to 4-hydroxy-3-nitrophenyl acetyl. I. Genetic control of delayed type hypersensitivity by V_H and I-A-region genes. *J. Exp. Med.* 149:1336.
- Rao, A., S. Faas, and H. Cantor. 1984. Analogs that compete for antigen binding to an arsonate-reactive T-cell clone inhibit the functional response to arsonate. *Cell* 36:889.
- Siliciano, R., R. Colello, A. Keegan, R. Dintzis, H. Dintzis, and H. Shin. 1985. Antigen valence determines the binding of nominal antigen to cytolytic T cell clones. *J. Exp. Med.* 162:768.
- Hayle, A. 1980. Molecular requirements for trinitrophenyl recognition by anti-hapten cytotoxic T lymphocytes. *Cell. Immunol.* 49:408.
- Levy, R., G. Shearer, J. Richardson, and P. Henkart. 1981. Cell-mediated lympholytic responses against autologous cells modified with haptenic sulfhydryl reagents. I. Effector cells can recognize two distinct classes of hapten-reactive self sites on cell surface proteins. *J. Immunol.* 127:523.

36. Rao, A., W.-P. Ko, S. Faas, and H. Cantor. 1984. Binding of antigen in the absence of histocompatibility proteins by arsonate-reactive T-cell clones. *Cell* 36:879.
37. Harding, C., J. Kihlberg, M. Elofsson, G. Manusson, and E. Unanue. 1993. Glycopeptides bind MHC molecules and elicit specific T cell responses. *J. Immunol.* 151:2419.
38. Luescher, I., F. Anjuere, M. Peitsch, V. Jongeneel, J.-C. Cerottini, and P. Romero. 1995. Structural analysis of TCR-ligand interactions studied on H-2kd-restricted cloned CTL specific for a photoreactive peptide derivative. *Immunity* 3:51.
39. Kessler, B., O. Michielin, C. Blanchard, I. Apostolou, C. Delarbre, G. Gachelin, C. Gregoire, B. Malissen, J.-C. Cerottini, F. Wurm, et al. 1999. T cell recognition of hapten: anatomy of T cell receptor binding of a H-2kd-associated photoreactive peptide derivative. *J. Biol. Chem.* 274:3622.
40. Speir, J., U. Abdel-Motal, M. Jondal, and I. Wilson. 1999. Crystal structure of an MHC class I presented glycopeptide that generates carbohydrate-specific CTL. *Immunity* 10:51.
41. Garboczi, D., and W. Biddison. 1999. Shapes of MHC restriction. *Immunity* 10:1.
42. Maliszewski, C., G. Delespesse, M. Schoenborn, R. Armitage, W. Fanslow, T. Nakajima, E. Baker, G. Sutherland, K. Poindexter, C. Birks, et al. 1994. The CD39 lymphoid cell activation antigen: molecular cloning and structural characterization. *J. Immunol.* 153:3573.
43. Wang, T.-F., Y. Ou, and G. Guidotti. 1998. The transmembrane domains of ectoapyrase (CD39) affect the enzymatic activity and quaternary structure. *J. Biol. Chem.* 273:24814.
44. Kansas, G., G. Wood, and T. Tedder. 1991. Expression, distribution, and biochemistry of human CD39: role in activation-associated homotypic adhesion of lymphocytes. *J. Immunol.* 146:2235.
45. Wang, T., and G. Guidotti. 1996. CD39 is an ecto-(Ca²⁺, Mg²⁺)-apyrase. *J. Biol. Chem.* 271:9898.
46. Marcus, A., M. Broekman, J. Drosopoulos, N. Islam, T. Alyonycheva, L. Safier, K. Hajjar, D. Posnett, M. Schoenborn, K. Schooley, et al. 1997. The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. *J. Clin. Invest.* 99:1351.
47. Enjyoji, K., J. Sevigny, Y. Lin, P. S. Frenette, P. D. Christie, J. S. Esch 2nd, M. Imai, J. M. Edelberg, H. Rayburn, et al. 1999. Targeted disruption of cd39/ATP disphosphohydrolase results in disordered hemostasis and thromboregulation. *Nat. Med.* 5:1010.
48. Wolff, K. 1972. The Langerhans cell. In *Current Problems in Dermatology*. J. W. H. Mali, ed. Separatum, Basel, p. 79.
49. Barnd, D., M. Lan, R. Metzgar, and O. Finn. 1989. Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. *Proc. Natl. Acad. Sci. USA* 86:7159.
50. Takahashi, T., Y. Makiguchi, Y. Hinoda, H. Kakiuchi, N. Nakagawa, K. Imai, and A. Yachi. 1994. Expression of MUC1 on myeloma cells and induction of HLA-unrestricted CTL against MUC1 from a multiple myeloma patient. *J. Immunol.* 153:2102.
51. Noto, H., T. Takahashi, Y. Makiguchi, T. Hayashi, Y. Hinoda, and K. Imai. 1997. Cytotoxic T lymphocytes derived from bone marrow mononuclear cells of multiple myeloma patients recognize an underglycosylated form of MUC1 mucin. *Int. Immunol.* 9:791.
52. Corinti, S., R. De Palma, A. Fontana, C. Gagliardi, C. Pini, and F. Sallusto. 1997. Major histocompatibility complex-independent recognition of a distinctive pollen antigen, most likely a carbohydrate, by human CD8⁺ $\alpha\beta$ T cells. *J. Exp. Med.* 186:899.