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$CD8^+$ cells expressing high numbers of TCR per cell (TCR^{hi}) are important mediators of anti-tumor effects. To understand the relationship between TCR density and Ag affinity for TCR in the outcome of differentiation of CTL recognizing tumor Ag, we analyzed perforin induction in ovarian tumor-associated lymphocytes in response to the smallest possible changes in the atomic forces of interaction between Ag and TCR. Stimulating undifferentiated, apoptosis-resistant $CD8^+$ cells expressing high levels of E75-TCR (TCR^{hi}) with variants of the CTL epitope E75, HER-2 (369-377), induced their stepwise differentiation, first to IFN-γ^+ $Perf^-$, and then to TCR^{hi} IFN-γ^+ $Perf^+$ cells. Blocking caspase-9 activation at Ag stimulation also enhanced the generation of TCR^{hi} $Perf^{hi}$ cells, demonstrating that TCR density dictated the pathway of death activated by stimulation with the same agonist. Expansion and differentiation of TCR^{hi} $Perf^+$ CTL required an agonist of optimal CH_2 side chain length. Side chains one CH_2 shorter or longer than optimal were either less stimulatory or induced death of TCR^{hi} $Perf^+$ cells. Differentiation of TCR^{hi} $CD8^+$ cells can be finely tuned by side chains which induce small increments in the affinity of the Ag for TCR below the affinity which induce apoptosis.

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Subject terms: CH_2, methylene; γ-ABu, γ-aminobutyric acid; NVal, norvaline; NLeu, norleucine; NP, not pulsed with peptide, pMHC-I, peptide: MHC-I complex; J, joule, HER-2, HER-2/neu protooncogene; TAL, tumor-associated lymphocyte; Perf, perforin, MFI, mean fluorescence intensity; IFN-g= Interferon gamma, FS= Forward scatter;

Key words: CTL, tumor antigen, hydrophobic appendage, T cell receptor, tumor immunity, humans, breast cancer, ovarian cancer.

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Introduction

Human $CD8^+$ cells expressing higher-than-average numbers of TCR (TCR^{hi}) reportedly have high functional avidity for their tumor targets (1-3), as demonstrated by their ability to lyse targets pulsed with smaller amounts of exogenous Ag (10^{-7}–10^{-8} M) than do CTLs expressing fewer TCR. These observations suggest that high TCR density compensates for the low affinity of individual TCRs for self-Ag. TCR^{hi} CTLs, the most potent cytolytic effectors identified so far, are scarce in patients with cancer (1-3). In one study, some TCR^{hi} cells were shown to be insensitive to Ag or died at Ag concentrations of 10^{-6} M (3); in another study, TCR^{hi} cells expanded in response to Ag but required IL-7 and IL-10 for survival (2). These findings suggest that tumor Ag transmitted a negative signal to block the differentiation of those CTLs and that the cytokines used for CTL expansion apparently amplified the negative signal. In ligand: receptor interactions, when the concentration of the ligand is constant, the effects of the ligand on the cell depend on the density of its receptor. At constant ligand concentration and constant receptor density, the functional effects of the ligand change with its affinity for the receptor or the duration of the receptor engagement.

To elucidate the significance of TCR density in the differentiation of TCR^{hi} cells to cytolytic effectors we modified the affinity of the ligand (Ag) for the receptor (TCR) and simultaneously analyzed the responses of two polyclonal $CD8^+$ populations from the same individual that differed in the levels of TCR by one order of magnitude. Because the transition between mitosis and apoptosis in TCR^{hi} cells responding to tumor Ag takes place within a narrow range of Ag concentrations (5×10^{-6} M -10^{-7} M) (3), we modified the affinity of the Ag for TCR in the smallest possible changes (increments/decrements), using only atomic van der Waals forces from methylene (CH_2) groups appended to Gly. At only 2 kJ (0.5 kcal) per mole, van der Waals forces are the weakest forces between atoms; by comparison, the force involved in the formation of one hydrogen bond after the introduction of one hydroxyl group is 20 kJ per mole (4). Since longer and branched side-chains produce steric hindrance, and steric hindrance may offset gains from increments in van der Waals forces, we used only short linear CH_2 extensions.

We used CTLs isolated from tumor-associated lymphocytes (TALs) that recognize an epitope from the HER-2/*neu* protooncogene, which is present on normal epithelial cells but is also overexpressed in many epithelial cancers of the breast, lung, prostate, and ovary (5). Because HER-2 is a self-Ag, most T cells of high affinity for HER-2 epitopes are deleted (6), and the remainder recognize HER-2 peptides with low avidity (at about 10^{-6} M). Nevertheless, because the same HER-2 CTL epitopes are presented by substantial proportions of tumors, the CTL epitopes from HER-2 become significant for cancer therapy (7).

How undifferentiated TCR^{hi} cells respond to human tumor Ag and what is needed to induce their proliferation and differentiation to functional effector cells remain unknown. To identify the optimal agonist for inducing differentiation of TCR^{hi} cells, we constructed four variants of E75, the HER-2 (369-377) epitope for CTLs, by appending one, two, three, or four methylene groups to the glycine molecule at position 4 (Gly^4) to form a linear C-side chain. We then selected cells expressing high concentrations of TCRs for E75 (E75-TCR^{hi}) to evaluate the role of TCR density in CTL differentiation upon stimulation with the same ligands. The TCR^{hi} population which usually includes cells staining with Ag-tetramers/dimers with a mean fluorescence intensity

(MFI) higher than 10^2 was divided in two populations, one staining with Ag-pulsed HLA-A2: IgG dimers (dimers) with a MFI (TCR) between 10^2-10^3, and another which stained with Ag-pulsed dimers with a MFI (TCR) between 10^3-10^4. These populations were designated as TCR^{med} and TCR^{hi} respectively.

The ability of CTLs to synthesize perforin is critical to their ability to lyse target cells (8,9). Presumably cells with high-density TCR would need correspondingly high amounts of effector molecules such as perforin for maximum functionality. Perforin is undetectable in naïve CTLs but is up-regulated in response to signals from TCRs (8-10). Perforin also controls $CD8^+$ homeostasis independently of its role as an effector molecule (11-13). Large expansion of Ag-specific $CD8^+$ cells that produce IFN-γ in perforin-deficient mice resulted in lethal disease during viral infections (11,14). Most T cells with high affinity for self-Ag are deleted upon encountering that self-Ag; that deletion constitutes a mechanism for protection against autoimmunity (6,15). We sought to determine how to induce differentiation of such cells and to identify the factors controlling their differentiation and survival. The difficulty in addressing these questions is compounded in polyclonal human systems, not only because surviving TALs recognize Ag with low functional avidity because their perforin expression is impaired (16,17), but also a large number of TCRs with distinct affinities for Ag are present.

We found that:

(1)The variant G4.2 (i.e., E75 with two CH_2 groups appended at Gly^4) was more effective in inducing perforin of E75-TCR^{hi} cells than were variants with longer CH_2 side chains .
(2) differentiation of TCR^{hi} IFN-γ^- $Perf^-$ cells to TCR^{hi} IFN-γ^+ $Perf^+$ cells by G4.2 involved an intermediate step to TCR^{hi} IFN-γ^+ $Perf^-$ cells. TCR^{hi} IFN-γ^+ $Perf^-$ cells proliferated and differentiated in response to the variant G4.2 to $Perf^+$ cells, which mediated tumor lysis.
(3) E75-TCR^{med} IFN-γ^- $Perf^-$ cells primed with variants differentiated directly to TCR^{med} IFN-γ^+ $Perf^+$ cells.
(4) Higher TCR density was associated with greater resistance to differentiation to $Perf^+$ cells.
(5) The density of TCR in conjunction with the optimal affinity of the ligand, can avoid induction of death.

The ability to finely tune the affinity of Ag for TCR in relation to receptor TCR density should be important for novel strategies to eliminate tumor cells.

BODY

Materials and methods

Cells, Antibodies, and Cytokines. The ovarian TAL line TAL-1 was generated from heparinized ovarian ascites, collected under institutionally approved protocols. Lymphocytes from TAL-1 were cultured in t he presence of low concentrations of IL-2 (150-300 IU/ml) for 7 –14 days. Stimulation of these TAL-1 cells with 200–1,000 nM of any Gly^4 variant resulted in their secreting IFN-γ at levels increasing in tandem with the length of the CH_2 chain, indicating that the E75-TCR^+ cells in the TAL-1 population were functional and not tolerized. (not shown)

mAb used for detecting surface Ag and cytokines used for culturing T cells from TAL-1 are described elsewhere (18). APC-conjugated antibody to IFN-γ (IgG2a), PE-conjugated mouse (IgG2b) antibody to perforin (δG9), empty recombinant soluble dimeric human HLA-A2:IgG1 designated as "dimers", and all specific isotype Ig controls were obtained from BD Pharmingen (San Diego, CA). Proteasome and caspase inhibitors were obtained from R&D Systems (*Minneapolis, MN).*

Synthetic Peptides. The peptides used were E75 [HER-2 (369-377); KIFGSLAFL] (19) and its methylene-appended C-side-chain variants. Four CH_2 variants were generated by substituting the glycine at position 4 (Gly^4) with alanine and one of following synthetic amino acids: γ-aminobutyric acid, norvaline, or norleucine. (Advanced Chemtech, Louisville,KY) The abbreviations for the CH_2-extended E75 variants (G4.1, G4.2, G4.3, and G4.4) reflect the position (Gly^4) and number of the CH_2 group extensions (19). Another variant in which the alanine at position 7 (Ala^7) was replaced with norleucine was designated A7.3. All peptides were prepared by Dr. Martin Campbell (Peptide Synthesis Core Facility of The University of Texas M. D. Anderson Cancer Center). Amino acids were coupled in sequential format from the C-terminus using standard FMOC peptide chemistry on a Rainin Symphony Automated Peptide Synthesizer and purified by HPLC. The purity of the peptides ranged from 95% to 97%. Peptides were dissolved in PBS and stored at –20°C as aliquots of 2 mg/ml until use. Molecular modeling of peptide: HLA-A2 complexes was performed by Dr. Darrick Carter using as model the crystal structure of Tax peptide bound to HLA-A2 and also described in the previous year report.. (18)

T Cell : Peptide-HLA-A2 Dimer Association and Dissociation Assays. Expression of TCRs specific for HLA-A2 bound to the E75 peptide (E75-TCR^+ cells) was determined by using E75 dimers (dE75). dE75 were prepared as previously described(18) . Staining of cells was performed as described previously (19-21). Specific geometric (y^2) MFI (TCR) for each peptide was calculated by substracting the MFI (TCR) of cells stained with "empty dimers" (dNP) from the MFI (TCR) of cells stained with dE75, dG4.1, dG4.2, dG4.3 and dG4.4. To identify the changes in the affinity of variants for TCR, the y^2 (MFI) of TAL-1 cells stained with each peptide dimer, was determined immediately after staining (t_0) or two hours later (20-23). Empty dimers were prepared in the same conditions as dE75 dimers excepting that no peptide was added to the HLA-A2-IgG dimers. The increase or decrease in the MFI (TCR) induced by each CH_2 group extending the CH_2 chain relative to E75 was calculated by subtracting the MFI (E75-TCR) from MFI (TCR) of cells stained with each variant peptide (G4.1, G4.2, G4.3 and G4.4) and dividing the result by the number of CH_2 groups appended to each variant (1,2,3,4). These values were designated as MFI (TCR) per appended CH_2. Among cells staining positive with peptide bound to HLA-A2: IgG dimers, populations were considered to express TCR at low density (TCR^{lo}) if the geometric (y^2) MFI for cells staining with that dimer was between 10^1 and 10^2, at medium density (TCR^{mod}) if the MFI was between 10^2 and 10^3, and at high density (TCR^{hi}) if the MFI was between 10^3 and 10^4.

T-Cell Stimulation by the CH_2 Variants. Apoptosis was induced in TAL-1 by two successive stimulations with 10,000nM of each peptide pulsed on T2 cells. Surviving TAL-1 were then primed with 5,000nM of each peptide pulsed on irradiated T2 cells as described elsewhere (24, 25). Control cultures were stimulated with T2 cells that had been pulsed with peptide A7.3 . The death-resistant cells were then re-stimulated *in vitro* with irradiated T2 cells pulsed with each

peptide at a final concentration of 5 μM and expanded in IL-2. Expression of IFN-γ and perforin (Perf), markers of differentiation, was determined by using IFN-γ-APC–conjugated or perforin-PE–conjugated antibodies and matched PE/FITC/APC-conjugated isotype controls on dE75-stained and permeabilized cells (18). Fold expansion by each variant was calculated by dividing the number of E75-TCR^{hi}, TCR^{med} cells detected in each sample after stimulation by the number of E75-TCR^{hi}, TCR^{med} cells present before stimulation.

CTL Assays. E75-, A7.3-, and G4.1- to G4.4-stimulated TAL-1 were used as effectors in CTL assays. Ag recognition by the E75-variant-induced CTLs was determined as described elsewhere (26, 27). Recognition of E75 was considered specific when the mean specific lysis of T2 cells pulsed with E75 minus the standard deviation (SD) was at least 10% and was at least twice as high as the percentage of specific lysis of T2 cells that had not been pulsed with peptide, plus the SD (27) E75-specific tumor lysis was determined by subtracting the levels of SKOV3.A2 tumor lysis observed in the presence of T2-E75 cells from the levels of SKOV3.A2 tumor lysis observed in the presence of T2-NP cells (27). The tumor cells were then incubated with 10 μM MG132 (28, 29) for 30minutes, before and during labeling, then used as targets in CTL assays. High- and medium-avidity effector CTLs were distinguished in two ways-first by their ability to recognize E75 at concentrations at least two times lower than the E75-primed CTL (e.g., at 500 nM instead of at 1000 nM) and second by the ability of high-avidity CTLs to mediate an effector response (e.g., % specific lysis) that was (a) at least twice as high as the effector response at the same or lower Ag concentration and/or (b) at half the effector -to-target ratio of the medium-avidity CTLs.(30)

Caspase Inhibitors. The caspase inhibitors Z-IETD-fluoromethyl ketone-(fmk) (specific for caspase-8), Z-LEHD-fmk (specific for caspase-9), and EDVE-fmk (specific for caspase-3), have been reported to participate in perforin-mediated apoptosis (31). For these experiments, 2 x 10^6 G4.2-induced CTL were incubated with each caspase inhibitor at 37°C for 90 minutes, washed twice with PBS, and then stimulated with T2 cells pulsed with 5 μM of G4.2.

Statistical Analysis. Differences in the levels of IFN-γ, perforin, and cytolysis between were compared by using unpaired Student's *t*-tests from triplicate determinations. Differences were considered significant at $P < 0.05$.

KEY RESEARCH ACCOMPLISHMENTS

This study is related to Task1, Subtask 3, and Task 2, Subtask 2. This study expands and analyzes in more depth the function of HAB than originally proposed. There is additional work, incomplete, which is not presented here. A manuscript and a patent application also resulted from this work. They cannot be attached but they will be submitted separatedly.

Extending Peptide Side Chains with CH_2 Modifies the Affinity of E75 for TCR. Molecular modeling of the E75-HLA-A2 complex indicated that CH_2 extension in Gly^4 resulted in zigzag orientation of the CH_2 chain towards the solvent (**Figure 1A, B, C, D**). Peptide: HLA-A2 association and dissociation assays indicated that appending CH_2 groups did not increase the affinity of the variants for HLA-A2 over that for E75 and the stability of peptide: HLA-A2 complexes. (previous report) Peptide-HLA-A2 IgG dimer: TAL-1 association and dissociation

assays, done to determine how the CH_2 appendages affected the affinity of the peptide-HLA-A2 complex for TCR, showed that at t_0, TAL-1 stained more strongly with dG4.2, dG4.3, or dG4.4 than with dE75 or dG4.1. meaning the ligands G4.2, G4.3 and G4.4 had higher affinity for TCR (**Figure1E**). dG4.1 staining was weaker than dE75 staining. G4.1 dissociated faster than E75, G4.2 dissociated slightly slower than E75, while G4.3 and G4.4 dissociated slower than G4.2. The MFI (TCR) per CH_2 group appended followed a bell-shaped plot with a peak at G4.3. The overall specific affinity for TCR of each variant increased with addition of CH_2 groups but showed saturation at G4.4 (**Figure1E**). Changes in the MFI confirmed that each CH_2 group in the G4.1–G4.4 variants interacted with the TCR; moreover, each CH_2 group added to the chain affected the interaction of the existing CH_2 groups with the TCR and changed the affinity of the other groups for TCR and the stability of the TCR: peptide HLA-A2 complexes (**Figure 1E**). Results in **Figure 1F** show the average change in MFI (TCR^{hi}) and MFI (TCR^{med}) per added CH_2 group at t_0 (0h) and 2h later (2h). Both MFI (TCR^{hi}) and MFI (TCR^{med}) formed bell-shaped plots which peaked with G4.3 and G4.2 respectively. These results indicate that affinity of variants for TCR increased only within a range. In summary, except for the single-methylene-group variant G4.1, CH_2 extension increased the binding affinity of the variant for TCR^{hi} without increasing the binding affinity for HLA-A2.

Priming Apoptosis-Resistant TCR^{hi} IFN-γ^- $Perf^-$ TAL-1 with E75 or its Gly^4 Variants Induced Their Differentiation to IFN-γ^+ $Perf^-$ Cells. TAL- 1 contained significant proportions of *ex vivo* activated and differentiated T cells. TCR^{hi} and TCR^{med} cells were of similar size (mean forward scatter, 630), indicating that they were activated, not resting, cells and that the higher TCR density was not related to cell size. Representative results for $TCR^{hi\ and\ med}$ expression, cell size, and staining for perforin-positive ($Perf^+$) and IFN-γ^+-positive cells before apoptosis-induction, after the first stimulation and after the second stimulation are shown in **Figure 2, 3,** and **4**. To recapitulate the process of differentiation, we first deleted differentiated cells, i.e., TCR^{hi} and TCR^{med} $Perf^+$ IFN-γ^+ cells by stimulating TAL-1 twice with 10,000 nM E75, G4.1, G4.2, G4.3, or G4.4 pulsed on T2 cells to ensure that most (if not all) TCR^{hi} $Perf^+$ IFN-γ^+ cells were eliminated. E75 and all of the Gly^4 variants with higher affinity for TCR than E75 deleted most of the $TCR^{hi\ and\ med}$ $Perf^+$ cells (**Figure 5A**) and most of the IFN-γ^+ cells (also appendices). A7.3, the CH_2 position control peptide for G4.4, also induced deletion of TCR^{hi} $Perf^+$ cells (**Figure 2A**). Differences between the small numbers of surviving cells were not significant. The numbers of TCR^{hi} $Perf^+$ cells decreased insignificantly after stimulation with control T2 cells, which present only few endogenous self-peptides compared with unstimulated IL-2 cultured TAL-1.

Surviving cells were then primed with 5,000 nM Ag (i.e., half the concentration that induced apoptosis, which led to deletion of TCR^{hi} $Perf^+$ cells) , leaving only TCR^{hi} $Perf^-$ cells. To facilitate comparisons, the results are shown reported to 10^6 E75-TCR^+ cells (E75-TCR^{hi} cells **Figure 5B**) (E75-TCR^{med} cells **Figure 5C**). Only IFN-γ^+, $Perf^-$ TCR^{hi} cells were present. Although the majority of TCR^{hi} and TCR^{med}, $Perf^+$ died, some of the cells continued to express IFN-γ.

Priming with variants did not significantly increase the numbers of E75-TCR^{hi} cells. The ratio of E75-TCR^{med} to E75-TCR^{hi} which was 6:1 in control T2-NP cultures, remained the same (E75), or

increased in the cultures stimulated with A7.3 and G4 variants. E75-TCR^{hi} cells stimulated with E75, A7.3, and G4.1 expressed perforin, while cells primed with G4.2, G4.3 and G4.4 did not. In contrast, the majority of E75-TCR^{med} stimulated with G4.2, G4.3, and G4.4 expressed perforin, while few E75-TCR^{med} cells stimulated with E75, A7.3, and G4.1 expressed perforin. It is unclear whether the E75-TCR^{hi} Perf^{+} cells derived from the few Perf^{+} cells which survived apoptosis or from the Perf^{-} cells which differentiated in response to Ag-stimulation. It is evident that stimulation with variants had different effects than stimulation with wild-type E75, in perforin and IFN-γ induction, in E75-TCR^{hi} cells and E75-TCR^{med} cells. The results in **Figure 5B, C** indicate that CH_2 appendage was effective in inducing differentiation of both TCR^{hi} and TCR^{med} cells, but the effects differed, depending on the position of the appendage (G4 or A7) and the density of the TCR. We quantitated the effects of stimulation with variants of polyclonal populations only on the cells expressing E75-TCR. The effects of the variants on cells expressing specific TCR for the variants, or reacting with the variants with higher affinity than with E75 have not been determined. Therefore, it may not be excluded that e.g. E75-TCR^{med} cells are also G4.2-TCR^{hi} cells. The patterns of specific MFI (TCR) for variants-dimer complexes show parallels (**Figure 1E**) or inverse relationships (**Figure 1F**) with the resulting E75-TCR^{med} Perf^{+} cells (**Figure 5C**).

To identify the effects of restimulation in E75-TCR^{+} cells differentiation, all cultures were restimulated with T2 cells pulsed with the same amounts of priming peptide, as in vaccination studies. Results in **Figure 5D** show a significant increase in the numbers of TCR^{hi} cells, which was paralleled by a significant decrease in the numbers of E75-TCR^{med} cells. The decrease in E75-TCR^{med} cells ranged between 75-96% for cells stimulated by E75, G4.2, G4.3, and G4.4 respectively. E75-TCR^{med} cells increased by 45% in cultures restimulated with G4.1. In contrast, E75-TCR^{hi} cells increased almost eight-fold in cultures restimulated by E75 and G4.3, and by 15-20 fold in cultures restimulated with G4.1, G4.2 and G4.4. Because these populations are polyclonal, we cannot distinguish whether expansion of TCR^{hi} cells was due to a higher rate of division of only TCR^{hi} cells or to a 3-4 fold increase in the numbers of E75-TCR molecules per cell in populations of E75-TCR^{med} cells, or both. The increase in E75-TCR^{hi} cells in cells stimulated with G4.1, of lower affinity for TCR than E75, is surprising and suggests a process similar with homeostatic proliferation and differentiation induced by low affinity ligands.

G4.2 is the Most Effective in Inducing Differentiation TCR^{hi} Cells to Perf^{+} Cells. Perforin expression has been associated with terminal differentiation of CTL (32-34). If TCR^{hi} cells are partially differentiated, they would express perforin at restimulation; on the other hand, if they cannot differentiate, they would die at restimulation. To address this question, we examined perforin expression in primed and restimulated E75-TCR$^{hi\ and\ med}$ cells. **Figure 5A** shows that E75-TCR^{mod} cells primed with G4.2 and G4.4 expressed higher levels of perforin than cells primed with E75 and G4.3. The levels of perforin increased only slightly at restimulation. In contrast, E75-TCR^{hi} cells expressed very low levels of perforin at priming which increased at restimulation. The levels of perforin increased by 5-fold in cultures restimulated with E75 and G4.3 and by 7-fold in cultures stimulated with G4.1. G4.2 and G4.4 induced the highest levels of perforin in cells which lacked perforin after priming. We cannot exclude the possibility that some of the E75-TCR^{med} cells increased both the number of TCR molecules/ per cell and the amount of perforin per cell and become E75-TCR^{hi} cells. Of interest, many E75-TCR^{hi} cells,

restimulated by E75, G4.1 and G4.3 became apoptotic; the effects were stronger for G4.3 followed by E75 and G4.1 (data not shown).

CTLs Induced with G4.2 Lyse Tumor Cells. To address whether variant activated CTL had higher functional avidity for E75 than E75-activated CTL, we assessed the recognition of E75 by variant-induced CTLs. In the first experiment (**Figure 6B**) effectors were variant-primed cells, and only the TCR^{med} populations expressed perforin. Only G4.4-CTLs significantly recognized E75 (>10% lysis) at concentrations of 100 and 500 nM while lysis by G4.2 CTL was below the 10% cut-off level to be considered significant. The levels of perforin (**Figure 6A**), IFN-γ (G4.2: MFI (IFN-γ) = 25.9, G4.4 MFI (IFN-γ) = 18.23), E75-TCR (G4.2: MFI (y^2)= 212, G4.4 (MFI (y^2)= 158 were similar in G4.2 and G4.4 primed cells. Thus the results suggest a better "fit" between E75 and CTL primed by G4.4 resulting in higher functional avidity. E75-CTL did not recognize E75 at this concentration, but rather required 2,500 nM of the peptide for lysis to be detected (not shown).

To determine whether expression of perforin in E75-TCR^{hi} cells increased the functional avidity of the effectors, we repeated the assays with variant-restimulated cells at a lower E: T ratio (1: 2). (**Figure 6C**) E75-TCR^{hi} cells exceeded E75-TCR^{med} cells by 3-5 fold. Then, a lower E:T ratio should decrease the effects of TCR^{med} cells. We found that G4.2-CTL recognized E75 at a concentration of 50-100 nM. The functional avidity of G4.2-CTL for E75 was increased by a factor of at least four at re-stimulation, (from 7% lysis at 1:1 ratio to 19.5% lysis at 1: 2 ratio) the functional avidity of G4.4-CTL increased by a factor of two (from 15% lysis at 1:1 ratio to 20% lysis at 1: 2 ratio), whereas the functional avidity of G4.1 cells increased by a factor of ten from (3% lysis at 1:1 ratio to 16% lysis at 1:2 ratio). Therefore, G4.2-expanded TCR^{hi} $Perf^{+}$ cells of higher functional avidity for E75 than G4.4 and G4.1. The increase in functional avidity of the G4.2-CTL also paralleled the two-fold increase in perforin levels in cells expressing one log higher TCR levels. (**Figure 6A**) Death of G4.1 stimulated cells eliminated CTL which recognized E75 with higher affinity in this experiment. Specific lysis by G4.1-stimulated CTL for 500nM E75 decreased to 5% in a second experiment (not shown).

These results indicate that restimulation with G4.2 expanded better CTL of higher functional avidity for E75. These CTL were also more stable and survived longer than CTL stimulated with E75, G4.1, and G4.3.

To determine whether the higher functional avidity for E75 reflected a high functional avidity for tumor, we assessed E75-specific tumor lysis in E75-blocking experiments. In these experiments, SKOV3.A2 cells were treated or not treated with IFN-γ to activate Ag presentation. IFN-γ treatment increased the levels of HLA-A2 by a factor of three, as indicated by an increase in MFI for HLA-A2 from 114 to 342 (not shown). To verify that E75 was being processed endogenously, we treated targets with the proteasome inhibitor MG132 before adding the effectors. The IFN-γ-treated SKOV3.A2 cells were more sensitive to G4.2-CTL in the 4-hour CTL assay than were the untreated SKOV3.A2 cells (**Figure 6D**). Lysis of IFN-γ-treated tumor cells by G4.2-CTL had continued to increase at 20 hours, demonstrating that G4.2-CTL had high and stable functional avidity for E75. MG132 inhibited SKOV3.A2 lysis by 60% in the 4-hour CTL assay (**Figure 6D**), indicating that most of the E75 was being processed by proteasomes.

Activation by G4.2 in the presence of Caspase-9 inhibitor increases the numbers of TCR^{hi} Perf^{hi} cells. Induction of TCR^{hi} Perf^{+} cells raised the question of whether these cells were sensitive to Ag-induced apoptosis and, if so, how to avoid that Ag-induced cell death. To identify the pathway of preferential deletion by Ag of Perf^{+} cells, expressing high levels of perforin, the G4.2 cells used in the previous experiment were "rested" by culturing them in the absence of IL-2, treated with an inhibitor of caspase-8 or caspase-9, or remained untreated, and then stimulated with G4.2. IL-2 was added 24 hours later to avoid interference with TCR stimulation. Perforin expression was measured 48 hours later in E75-TCR^{hi} Perf^{+} cells, and as an internal control in E75-TCR^{med} Perf^{+} cells. Although separation of these cells at MFI (10^3) is arbitrary, from the levels of perforin, two populations were clearly distinguished in both TCR ^{hi} and TCR ^{med} cells: one population expressing MFI (Perf) < 100, and a second population expressing MFI (Perf) > 300.

Pretreatment with the caspase-8 inhibitor increased the number of E75-TCR^{hi} cells by only 15% and decreased their perforin level by 10% compared with cells stimulated with G4.2 in the absence of caspase-8 inhibitor, (**Figure 7A versus B**). By contrast, treating the G4.2 cells with the caspase-9 inhibitor doubled the number of TCR^{hi} cells and produced a 50% increase in perforin level per cell relative to stimulation with only G4.2 (**Figure 7C versus 7A**). These findings indicate that G4.2 induced death in TCR^{hi} Perf ^{hi} cells by activating caspase-9. In contrast, in E75-TCR^{med} cells, the caspase-8 inhibitor was more protective than the caspase-9 inhibitor, increasing the numbers of E75-TCR^{med} cells by 65% (**Figure 7E**) as opposed to only 25% for the caspase-9 inhibitor (**Figure 7F**) relative to the cells, which were not pretreated with caspase-inhibitor (**Figure 7D**). However the levels of perforin, in the Perf ^{hi} population, increased by 21% in the caspase-9 inhibitor-treated population, suggesting that caspase-9 is activated by TCR to delete cells expressing high levels of perforin.

REPORTABLE OUTCOMES

1. Hydrophobically-appended peptides (HA) corresponding to tumor Ag can be used to finely tune induction of differentiation in apoptosis-resistant and undifferentiated CD8^{+} cells.

2. HAB activate T cells expressing higher density (10 fold) of T cell receptor than average.

3. T cells expressing higher density of TCR than average can be divided in two populations: TCR^{hi} and TCR^{med} based on the levels of expression of TCR.

4. These two populations respond with different sensitivities to stimulation by HAB. TCR^{med} cells differentiate directly to IFN-γ^{+} Perf^{+} cells.

5. TCR^{hi} cells differentiate to cytolytic effectors sensitive to 50nM of exogenous pulsed peptide.

6. Differentiation and expansion of TCR^{hi} cells is not proportional with the increased hydrophobicity of the peptide. Increased hydrophobicity by the appendage does not directly correlate with immunogenicity.

7. The use of HAB, together with caspase-9 inhibitors and IL-2Rβ chain (CD122) blockers should be useful to expand TCR^{hi} cells in large numbers for adoptive biotherapies.

CONCLUSIONS

$CD8^+$ cells expressing large numbers of TCR are important for immunotherapy because they can recognize the small amounts of endogenous Ag presented by tumors (1-3). We report here several novel findings regarding the fine tuning of the affinity of the Ag in the differentiation of TCR^{hi} cells to high-functional-avidity, perforin-expressing cells.
These findings were obtained by simultaneous analysis of two human T-cell populations expressing a specific TCR for the same Ag that differed in the amount of TCR expressed per cell by one order of magnitude. The "classical" TCR^{hi} population (of MFI>10^2) (1-3) was separated in TCR^{hi} (MFI (TCR) >10^3) and TCR^{med} (MFI (TCR) <10^3). Although this separation was arbitrary, clear differences were observed among these populations with regard to their responses to CH_2 appended E75.

Expansion in TCR^{hi} cells was not associated with affinity of the Ag for TCR or with the stability of peptide-MHC: TCR complexes, as would be expected in classical models of TCR signaling. Responses at priming were weak and they increased at restimulation with G4.1, the agonist of lower affinity for TCR than E75 inducing stronger expansion than G4.2, G4.3, and G4.4.

Expansion, perforin and IFN-γ induction in the TCR^{med} cells paralleled the changes in affinity of the ligand for TCR. Exception made again G4.1. At priming G4.1-activated cells expanded to lower numbers than G4.3 and G4.4-stimulated cells. They were not deleted at re-encounter with G4.1 as it happened with G4.3-activated cells. Based on functional assays, G4.2 appeared the most effective among the CH_2 variants. G4.2-activated cells recognized Ag at lower concentrations than G4.4 and G4.1-activated cells. A tentative ranking of peptides presented in **Table I**, in the appended material indicates parallels between CTL lysis, the MFI (perforin), and survival of antigen activated cells.

Table I. *Ranking of CH_2 variants based on their ability to activate TCR^{hi} cells.*

Peptide	CH_2 added	TCR affinity	Proliferation Priming	Restimulation	Stability (Survival)	Perforin (MFI)	Cumulative Score	Score	CTL Lysis
		a	*b*	*c*	*d*	*e*	*a+b+d+e*	*a+c+d+e*	
E75	0	4	5	4*	4	4-5	17-18	16-17	5
G4.1	1	5	4	1	3	3	15	12	3
G4.2	2	3	3	2	1	1-2	8-9	7-8	1
G4.3	3	1	1	4*	5	4-5	11-12	14-15	4
G4.4	4	2	2	3	2	1-2	7-8	8-9	2

The highest rank (rank 1) was assigned to the agonists of higher TCR affinity, expanded more cells, the activated cells survived longer, etc.
See how the cumulative scores compare with CTL lysis.

The differences in ligand–receptor (TCR) affinity reflected differences in atomic forces induced in the CH_2 chain by the addition of single CH_2 groups. The average affinity for TCR per each CH_2 group appended was lower in G4.2 than in G4.4 at t_0 and was higher in the G4.3 variant than in the G4.2 or G4.4 variants. The fact that this difference corresponds to van der Waals forces of only 0.5–1 kcal per mole indicates the extraordinary sensitivity of TCR^{hi} and TCR^{med} cells to forces that are a full order of magnitude weaker than the forces generated by one hydroxyl group (5 kcal per mole). Unexpectedly, optimal affinity in these experiments corresponded to the linear extension of the side chain of E75 with two CH_2 groups, a variant not present in natural amino acids.

Our results indicate that expansion of TCR^{hi} $Perf^{hi}$ cells of high functional avidity for tumor Ag require fine-tuning of the Ag affinity for the TCR. Tolerance to tumor Ag is reportedly maintained at the level of effector expansion (35). Because expression of high levels of perforin in CTLs is followed by apoptosis when the TCR is re-stimulated by the ligand, approaches to protect, partially differentiated and differentiated CTL, such as the ones induced by G4.1 and G4.3, using caspase inhibitors, will ultimately result in higher numbers of effector TCR^{hi} $Perf^{+}$ cells than reactivation with wild-type agonists.

These results suggest that changes in ligand affinity by substitutions with natural amino acids will be unable to modulate the weak forces executing differential control of effector gene expression in TCR^{hi} cells. Differences of only two methylene groups (e.g., Gly vs γ-aminobutyric acid) are absent in natural amino acids; also, differences of one CH_2 group in linear chains of three to four CH_2 groups are not present in natural amino acids. The side chains of Val and Leu/Ile differ in one amino acid length and are branched. Differences in van der Waals forces from one CH_2 group in Ser and Thr should be masked by the 10-fold stronger forces from -OH groups. Synthetic amino acids are not genetically encoded; although some are generated in humans by enzymatic reactions during metabolism (e.g. γ-aminobutyric acid, a neuro-transmitter), they are not known to be incorporated into proteins. Expression of synthetic amino acids in proteins suggests that posttranslational modifications, such as methylation/

demethylation of a CTL epitope, may be taking place if the corresponding bacterial enzymes are present (36). The presence of norvaline and norleucine in bacterial proteins, in recombinant proteins, and in antibiotics, raises the possibility that TCR^{hl} cells specific for self-Ag are periodically activated and inactivated after interactions with bacterial or fungal pathogens.

In summary, our results provide a novel basis for possible control of proliferation and terminal differentiation of human anti-tumor CTLs that recognize self-Ag. The sensitivity to small changes in atomic force demonstrated by different responses to a 1- to 2-CH_2 difference in the Ag may be useful in the induction of anti-tumor responses.

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FIGURE LEGENDS

Figure 1. (A, B, C, D) Orientation of CH_2 chains appended at Gly^4 in the CTL epitope E75 as determined by molecular modeling of each peptide (G4.1-G4.4)-HLA-A2 complex in reference 18. **(E)** Extending the CH_2 side chain modified the affinity of the variant for TCR^{hi} and significantly less for TCR^{med} cells. **(F)** The MFI (TCR) of TCR^{hi} and TCR^{med} cells per appended CH_2 group follows a bell-shaped plot. Freshly isolated, unstimulated, TAL-1 were incubated with 15ul (5μM) final concentration of each peptide-dimer complex in the same experiment. All staining and flow cytometry analysis were performed in the same experiment. (î, ■) TCR^{hi} cells, (△,□) TCR^{med} cells (î,△) MFI (TCR) of cells at t_o, i.e. immediately after staining with peptide dimers. (■,□) 2h after staining with peptide dimers and incubation in PBS to facilitate dissociation from TCR.

Figure 2, 3, 4. Expression of TCR^{med} $Perf^+$, TCR^{hi} $Perf^+$, and of IFN-γ^+ cells in TAL-1 before and after stimulation E75 or its CH_2 variants. NP, negative control indicates that cells were stimulated with T2 cells which were not pulsed with peptide. IL-2 only indicates unstimulated TAL-1 cultured in IL-2. A7.3 indicate that cells were stimulated with cells which were pulsed with positive control A7.3 peptide. **Figure 2.** Expression of Ag-specific differentiated cells after induction of apoptosis by Ag. Compare expression of Ag-specific IFN-γ^+ $Perf^+$ cells, before apoptosis groups (T2-NP) and (IL-2 only) and after apoptosis. Groups. (T2-E75, T3-G4.1 and T2-G4.2). **Figure 3.** Induction of differentiation after priming of undifferentiated apoptosis-resistant cells. Compare lack of perforin expression and reduced IFN-γ expression in T2-NP-stimulated cells which died without stimulation and the cells stimulated with peptides. Compare the cells stimulated with G4 peptides in **Figure 2** and in **Figure 3**. **Figure 4. (A, B, C)** Control staining with empty dimers (dNP) and IgG2a-FITC conjugated. **(D, E, F)** Staining with dE75 and anti-perforin Ab. **(D)** E75-$TCR^{hi,\ med,\ and\ low}$ cells, **(E)** perforin –positive cells. **(F)** E75-TCR^+ $Perf^+$ cells. E75-TCR^+ cells were determined in the perforin–positive population. All cells were stimulated in the same experiment. All cells were stained in the same experiment.

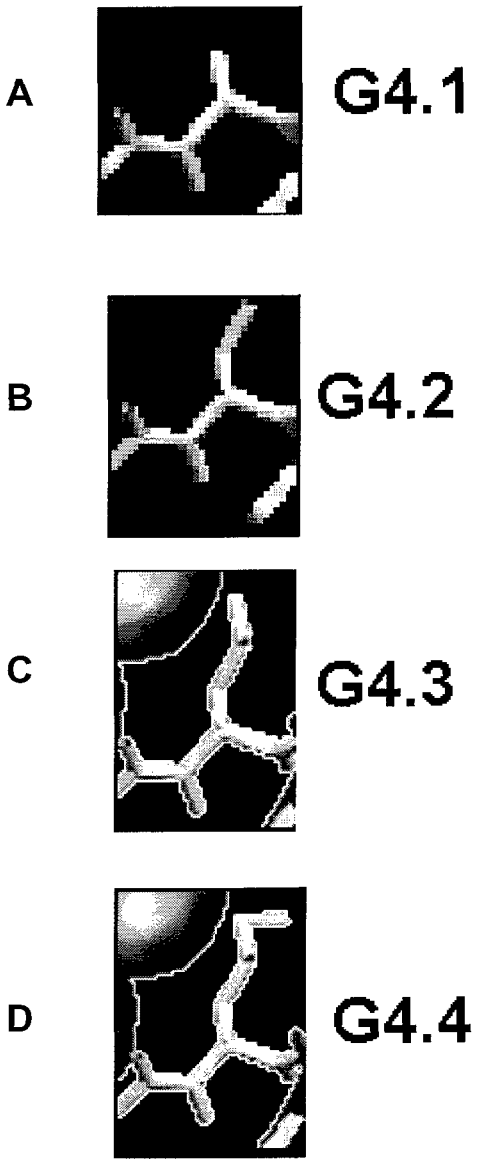
Figure 5. (A) Apoptosis-resistant TCR^{hi} and TCR^{med} do not express perforin. (PRE) shows that unstimulated TCR^{hi} and TCR^{lo} cells express perforin. The presence of $Perf^+$ and IFN-γ^+ cells in unstimulated and peptide stimulated cells is shown in the Appendix.
(B,C) Priming of apoptosis-resistant cells with E75 and its CH_2 variants, preferentially expands and differentiates E75-TCR^{med} cells. NP indicate control TAL-1 which were maintained in IL-2 and stimulated with "empty" T2 cells. (▨) $Perf^+$ cells; (▩) IFN-γ^+ cells. Results are expressed as per 10^6 E75-TCR^+ cells. **(D)** Restimulation of variant-primed cells with the same agonists results in expansion of E75-TCR^{hi} cells and contraction of E75-TCR^{med} cells; (□) E75-TCR^{hi} cells, (■) E75-TCR^{med} cells. Results in **A, B, C** are from one experiment, representative of two independently performed experiments (*) indicates significant differences in the E75-$TCR^{hi/med}$, IFN-γ^+ $Perforin^+$ cells activated by cytokines, APC and variants compared with E75.

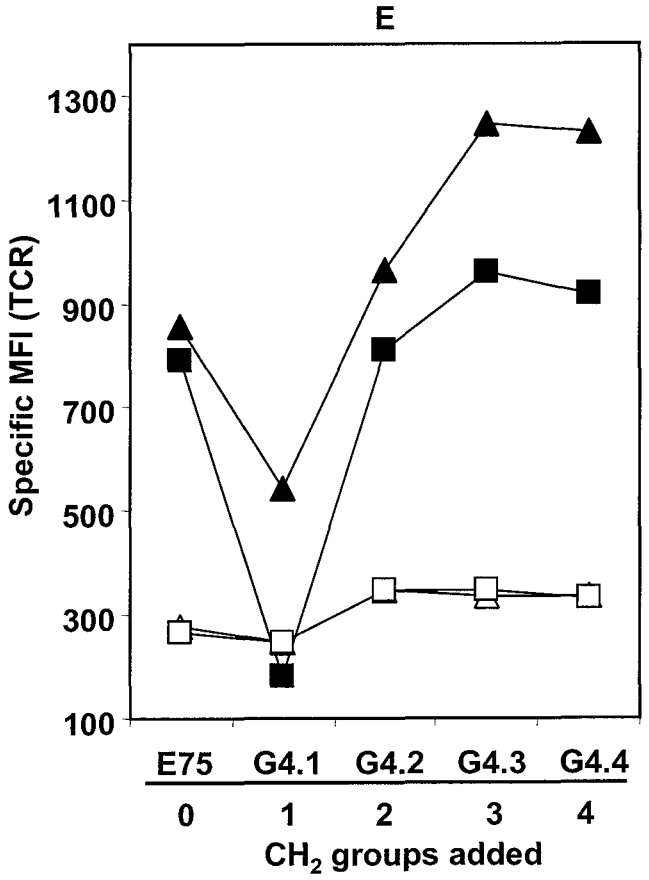
Figure 6. (A) Restimulation with E75 and its CH_2 variants results in higher levels of perforin in TCR^{hi} cells compared with TCR^{med} cells. (○) Primed TCR^{hi} cells; (□) restimulated TCR^{hi} cells; (●) primed TCR^{med} cells, (■) restimulated TCR^{med} cells; **(B, C)** E75-specific lysis by CTL after priming **(B)** and re-stimulation **(C)** of TAL-1 with peptides G4.1 (○), G4.2,(●), G4.3(△), and G4.4 (■). E : T ratios were 1 : 1 **(B)** or 1 : 2 **(C)**. Effectors indicate E75-TCR^{med} **(B)** and

E75-TCR^{med} (**C**) per tumor cell. Lysis by peptide primed TAL-1 containing TCR^{med} cells in 20h CTL assay of T2 cells pulsed with 100 and 500 nM E75, was E75-CTL = 0.0% and 0.0% and A7.3-CTL = 0.0% and 6.0 + 1.5%. In the same assay, lysis by, G4.1-primed CTL, G4.2-primed CTL, G4.3-primed CTL and G4.4-primed CTL of T2 cells pulsed with 100nM E75 was 16.23%, 23.74%, 24.53% and 26.49 % respectively. (**B**) Lysis by E75 and A7.3-reactivated CTL of T2 cells pulsed with 100 and 500nM E75 was, E75-CTL = 4.9+1.9% and 1.0 + 2.0%; A7.3-CTL 0.0 and 0.0%. The MFI TCR^{med} (TCR ± SD) was 175 ± 35, and the MFI (TCR^{hi}) was 3081 ± 370. (**D**) E75-specific lysis of SKOV3.A2 (HER-2^{hi}) tumor cells by G4.2-CTL. IFN-γ treatment of SKOV3.A2 cells increased the E75-specific tumor lysis by G4.2-CTL at the same low ratio of 1: 2. Tumor cells were treated with 100 IU of IFN-γ/ml for 20h, then labeled and used as targets in CTL assay. (**B, C, D**) Results show mean values of triplicate determinations in the same experiment + standard deviations.

Figure 7. Pretreatment of TAL-1 restimulated with G4.2 with the caspase-9 inhibitor, Z-LEHD-fmk, prior to reactivation by G4.2 increase the numbers of TCR^{hi} Perf ^{hi} cells TCR^{med} Perf ^{hi} cells. (**A-C**) TCR^{hi} cells; (**D-F**) TCR^{med} cells. The inserts in the lower left and right quadrants indicate the % Perf^{+} cells at numerator and MFI (perforin) at denominator, for cells shown in the upper left and upper right quadrants respectively .

Figure 1


A
G4.1
B
G4.2
C
G4.3
D
G4.4


E
Specific MFI (TCR)
1300
1100
900
700
500
300
100
E75
G4.1
G4.2
G4.3
G4.4
0
1
2
3
4
CH2 groups added

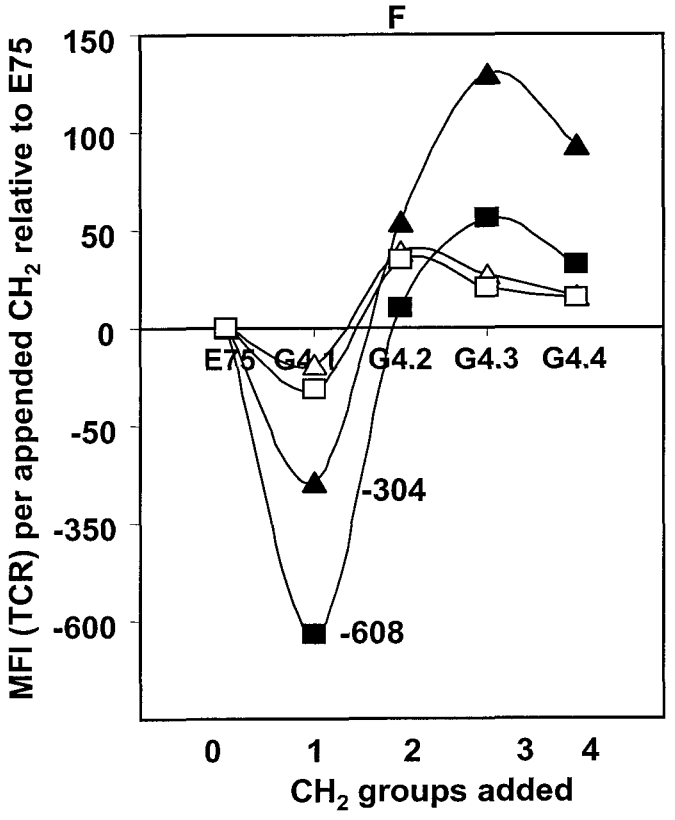

F
MFI (TCR) per appended CH2 relative to E75
150
100
50
0
-50
-350
-600
E75
G4.1
G4.2
G4.3
G4.4
-304
-608
0
1
2
3
4
CH2 groups added

Figure 2

Negative Control

Stimulated With

T2-NP

IL-2 Only

T2-E75

T2-G4.1

T2-G4.2

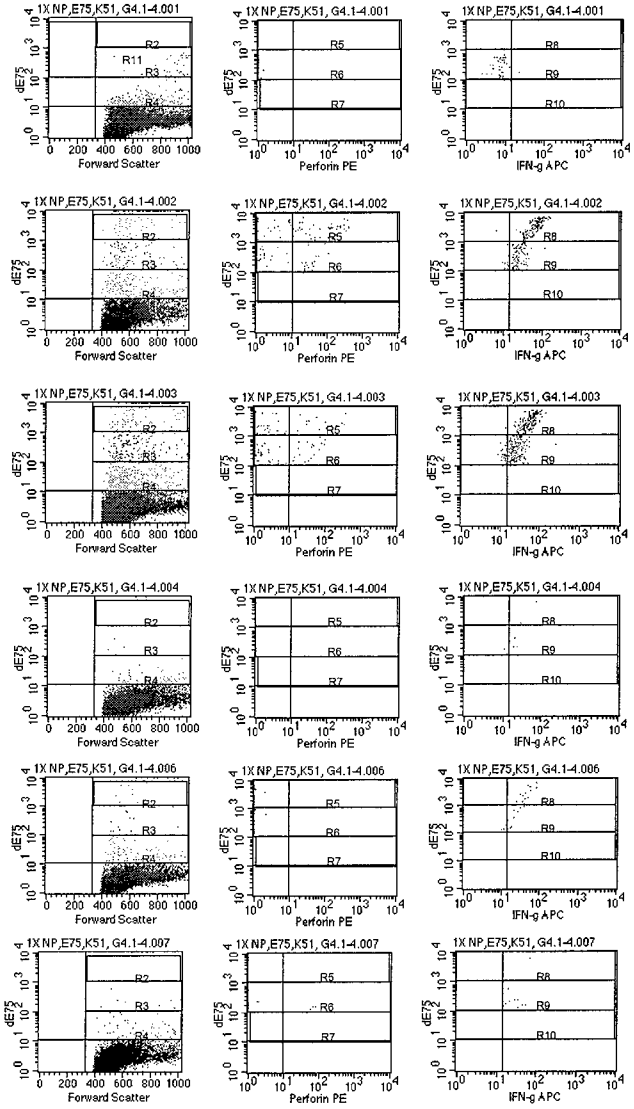


Figure 3

Negative Control

Stimulated With

T2-NP

T2-E75

T2-A7.3

T2-G4.2

T2-G4.3

T2-G4.4

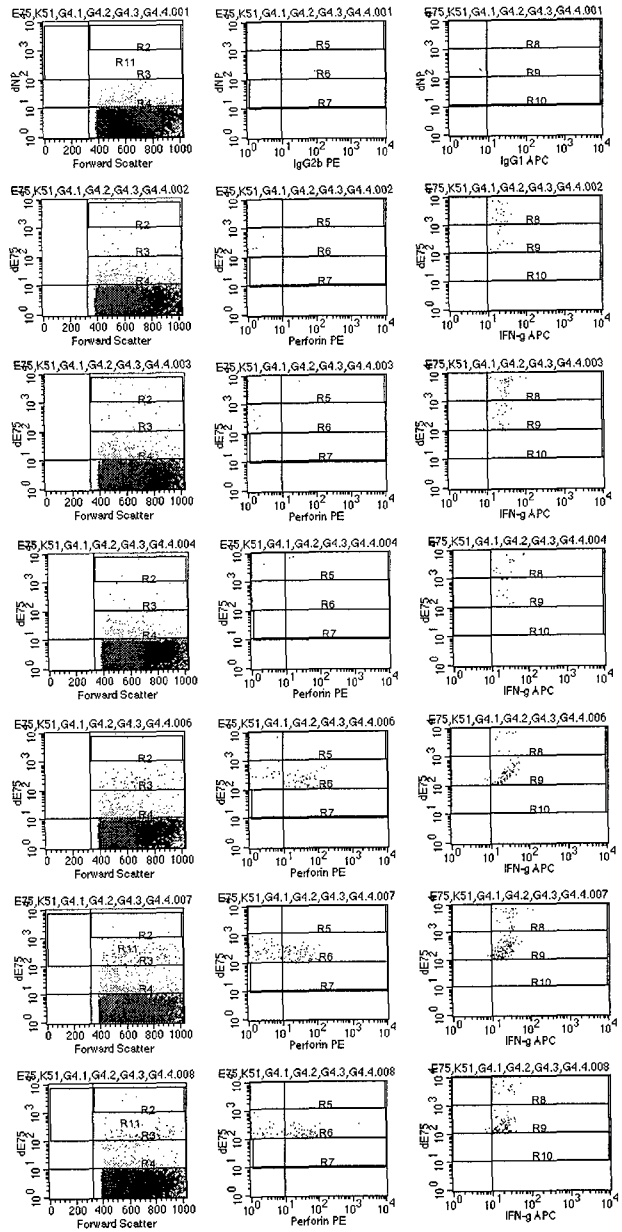


Figure 4

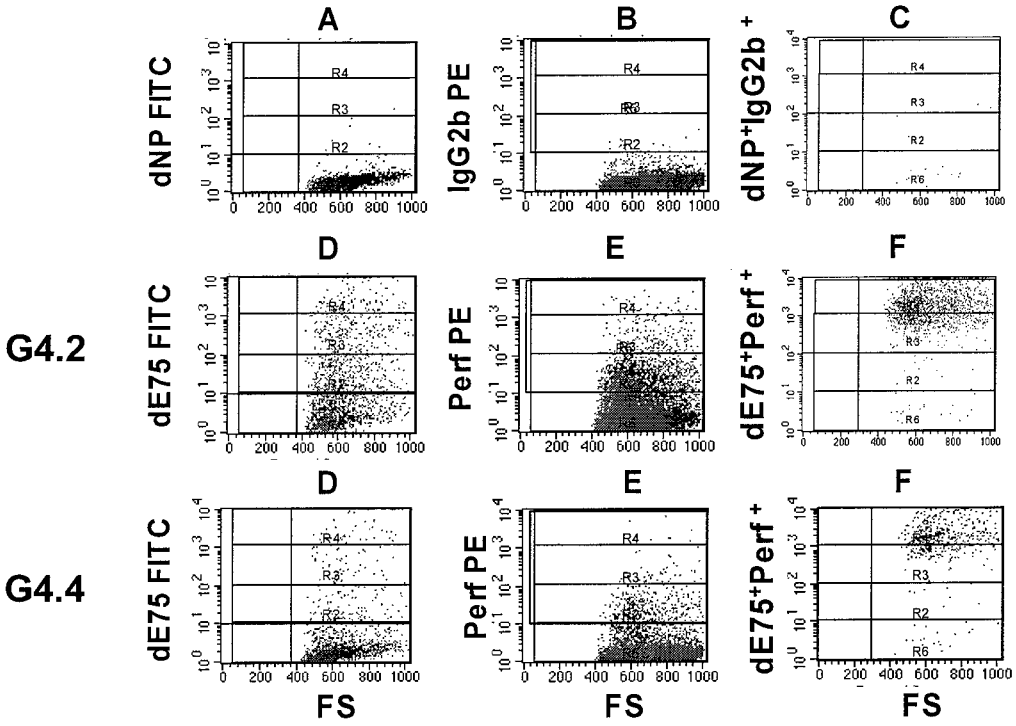
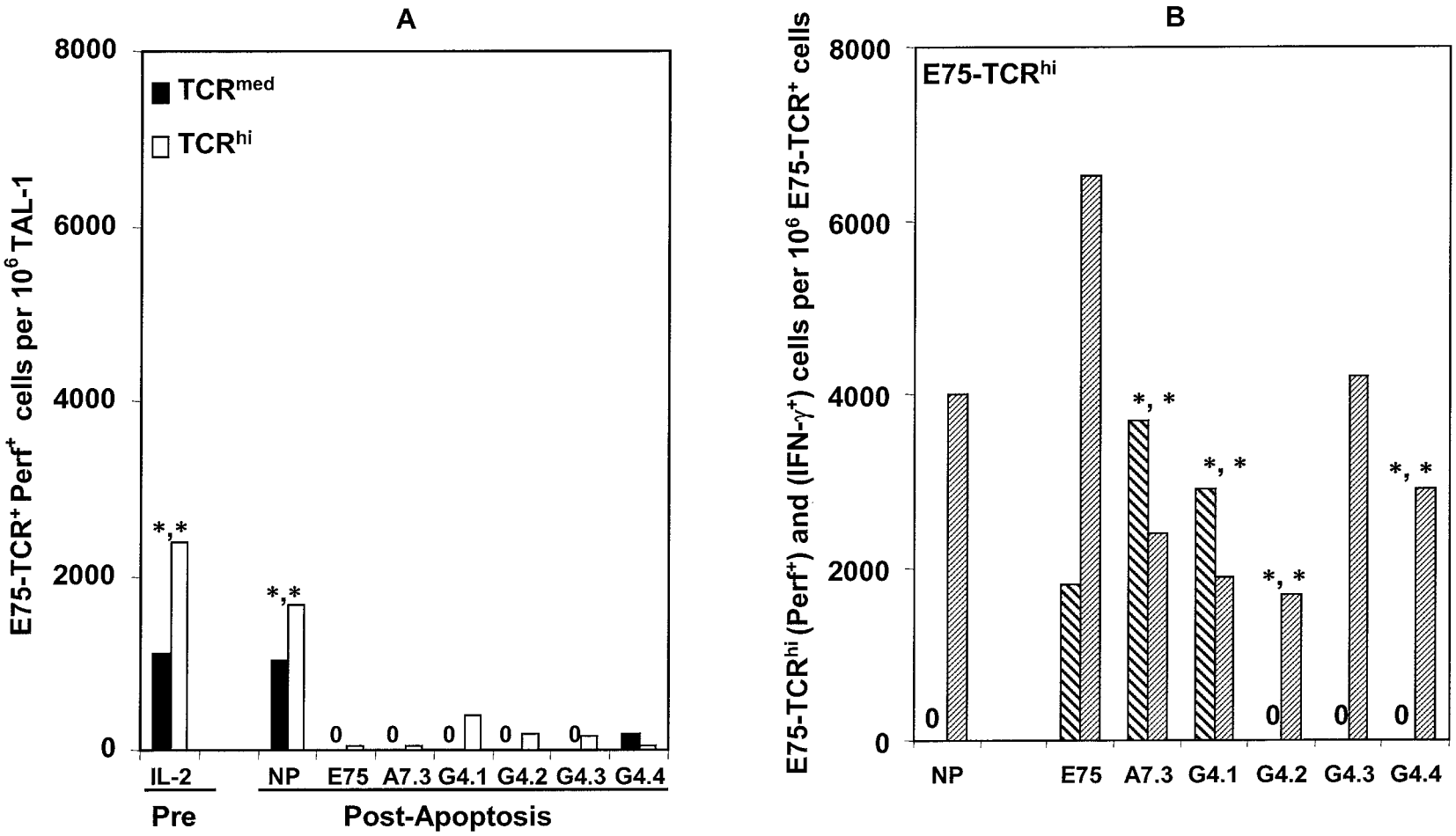

A
B
C
D
E
F
dNP FITC
IgG2b PE
dNP+IgG2b+
dE75 FITC
Perf PE
dE75+Perf+
G4.2
G4.4
R4
R3
R2
R6
FS

Figure 5


A
8000
6000
4000
2000
0
TCRmed
TCRhi
E75-TCR+ Perf+ cells per 10^6 TAL-1
,
,
0
0
0
0
0
IL-2
NP
E75
A7.3
G4.1
G4.2
G4.3
G4.4
Pre
Post-Apoptosis
B
8000
6000
4000
2000
0
E75-TCRhi
E75-TCRhi (Perf+) and (IFN-γ+) cells per 10^6 E75-TCR+ cells
0
*, *
*, *
*, *
0
0
0
NP
E75
A7.3
G4.1
G4.2
G4.3
G4.4

Kawano Figure 5

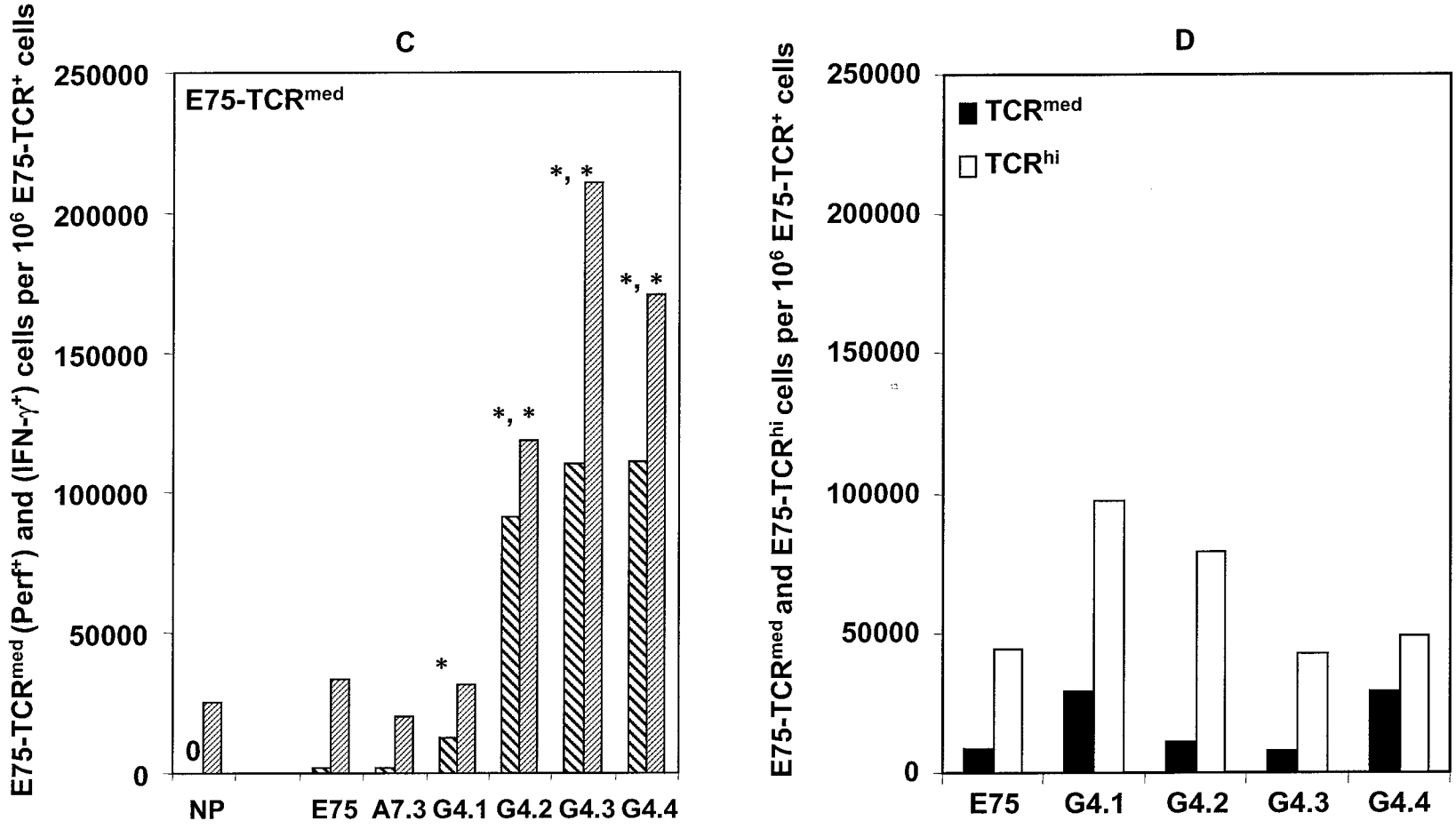


Figure 6

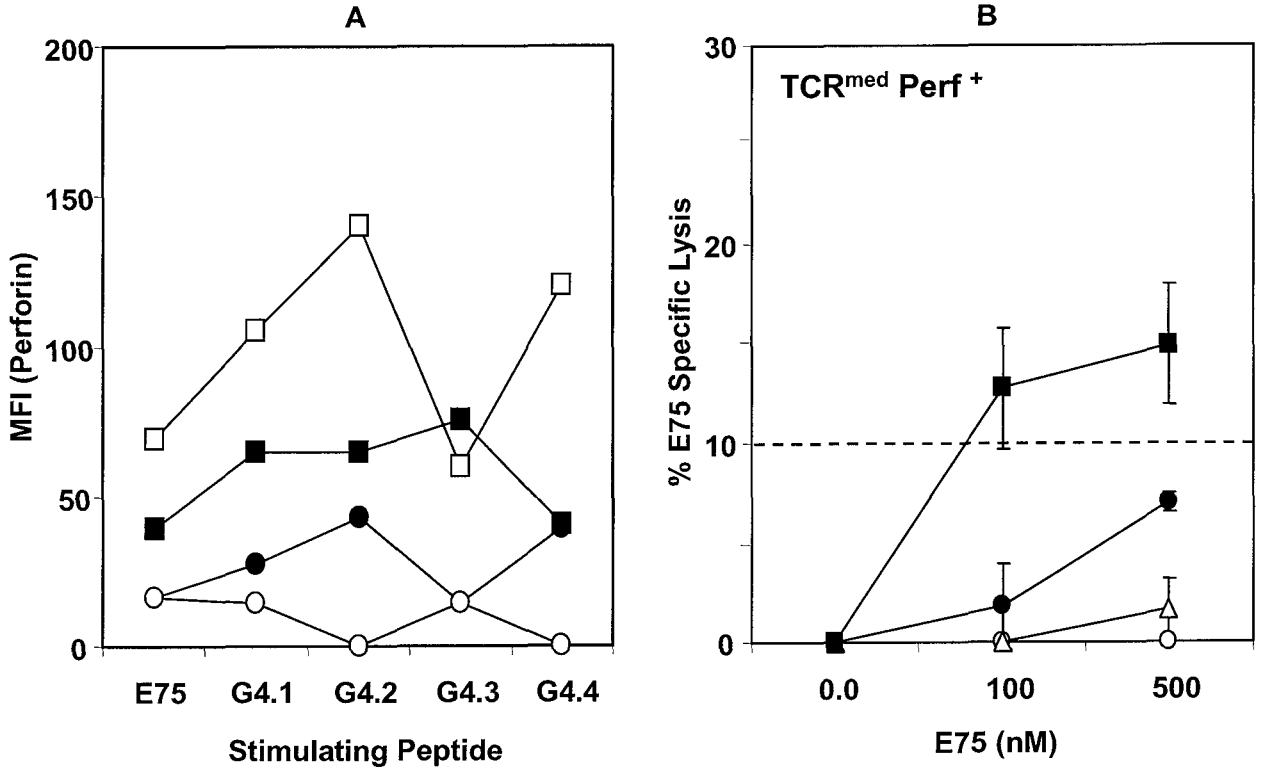

A
200
150
100
50
0
MFI (Perforin)
E75
G4.1
G4.2
G4.3
G4.4
Stimulating Peptide
B
30
20
10
0
% E75 Specific Lysis
TCRmed Perf +
0.0
100
500
E75 (nM)

Figure 6

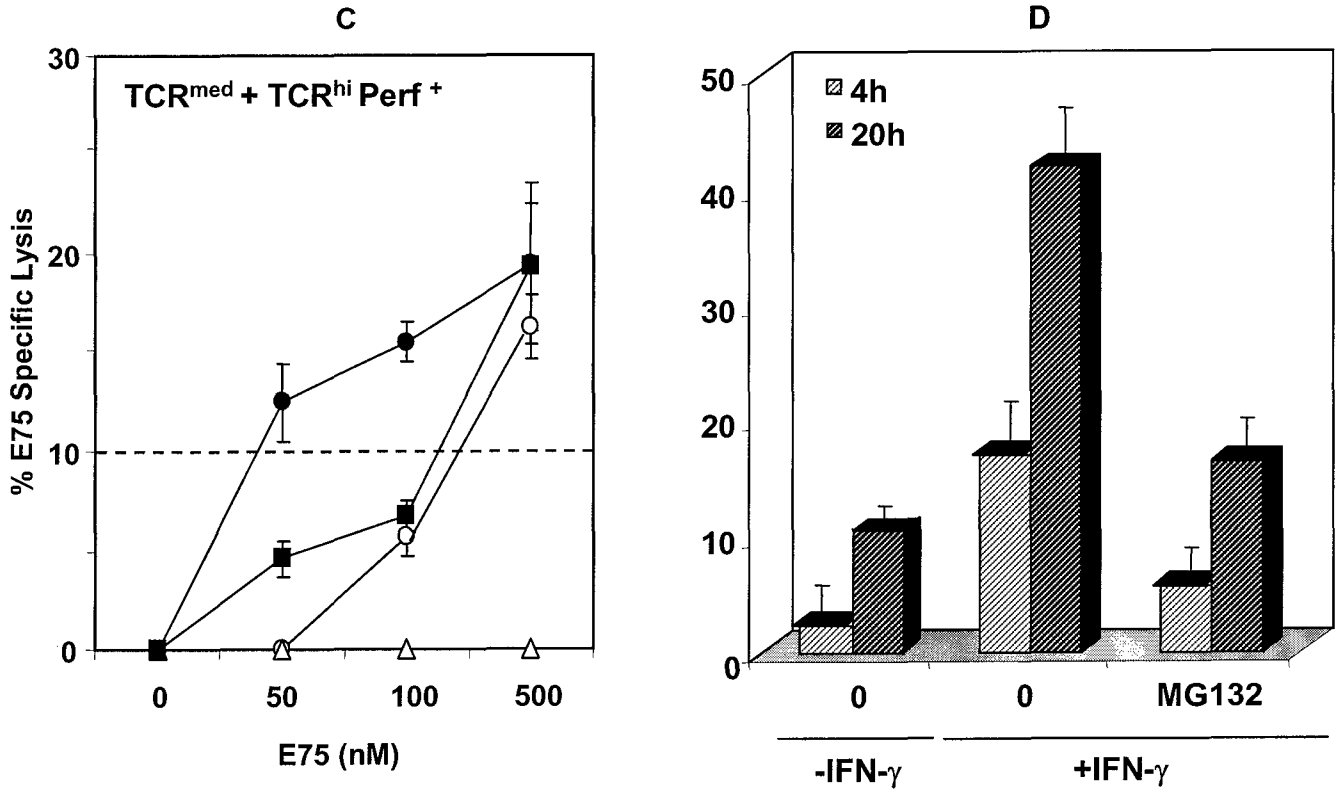
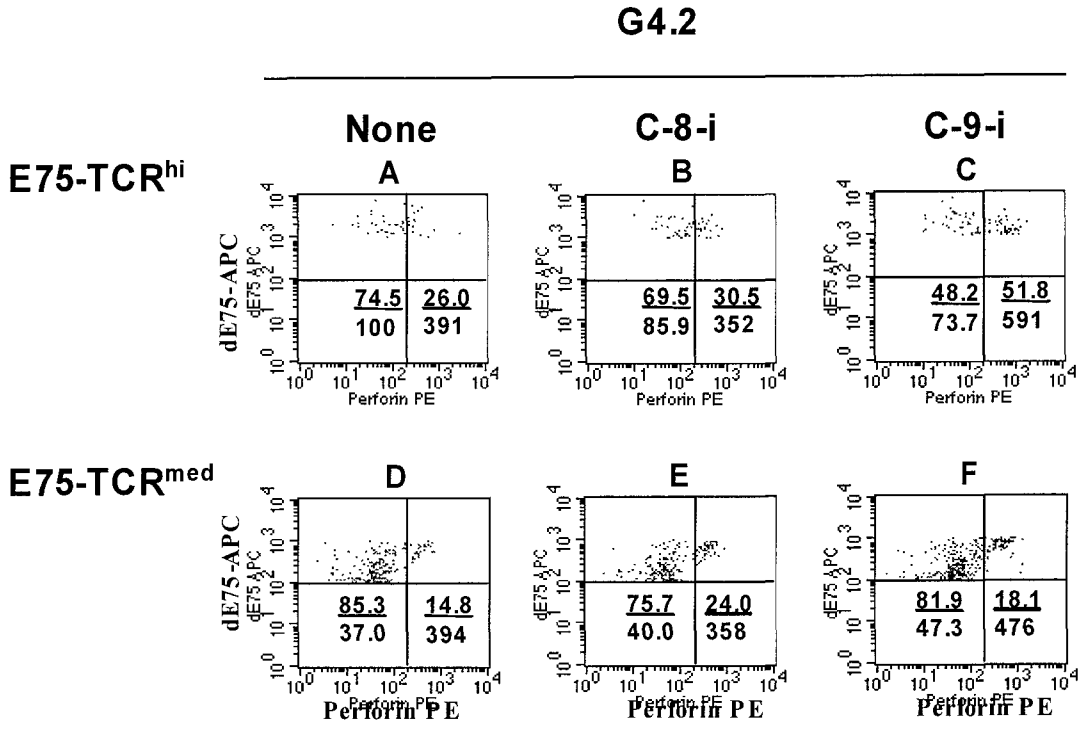

C
TCR^med + TCR^hi Perf +
% E75 Specific Lysis
30
20
10
0
0
50
100
500
E75 (nM)
D
4h
20h
50
40
30
20
10
0
0
0
MG132
-IFN-γ
+IFN-γ

Figure 7


G4.2
None
C-8-i
C-9-i
E75-TCR^hi
E75-TCR^med
A
B
C
D
E
F
dE75-APC
Perforin PE
74.5
26.0
100
391
69.5
30.5
85.9
352
48.2
51.8
73.7
591
85.3
14.8
37.0
394
75.7
24.0
40.0
358
81.9
18.1
47.3
476