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A Novel Postpriming Regulatory Check Point of Effector/Memory T Cells Dictated through Antigen Density Threshold-Dependent Anergy

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CTLs act as the effector arm of the cell-mediated immune system to kill undesirable cells. Two processes regulate these effector cells to prevent self-reactivity: a thymic selection process that eliminates autoreactive clones and a multistage activation or priming process that endows them with a license to kill cognate target cells. Hitherto no subsequent regulatory restrictions have been ascribed for properly primed and activated CTLs that are licensed to kill. In this study we show that CTLs possess a novel postpriming regulatory mechanism(s) that influences the outcome of their encounter with cognate target cells. This mechanism gauges the degree of Ag density, whereupon reaching a certain threshold significant changes occur that induce anergy in the effector T cells. The biological consequences of this Ag-induced postpriming control includes alterations in the expression of cell surface molecules that control immunological synapse activity and cytokine profiles and induce retarded cell proliferation. Most profound is genome-wide microarray analysis that demonstrates changes in the expression of genes related to membrane potential, TCR signal transduction, energy metabolism, and cell cycle control. Thus, a discernible and unique gene expression signature for anergy as a response to high Ag density has been observed. Consequently, activated T cells possess properties of a self-referential sensory organ. These studies identify a new postpriming control mechanism of CTL with anergenic-like properties. This mechanism extends our understanding of the control of immune function and regulation such as peripheral tolerance, viral infections, antitumor immune responses, hypersensitivity, and autoimmunity. The Journal of Immunology, 2007, 178: 2307–2317.

The fate of a specific T cell clone is determined in a secondary lymphoid organ where it is educated by a professional APC (pAPC)2 (1). Upon pAPC encounter, a constellation of events ensues, inter alia, costimulatory signaling and various interactions between membrane determinants in the immunological synapse, as well as cytokines secreted by the pAPC and the T cell that ultimately determine whether this T cell clone will undergo an activation process, tolerance, or apoptosis (2–4). Activation of a T cell effectuates transcriptional, biochemical, and morphological changes that enhance its proliferation, migration, and effector functions (5). Activated CTLs survive only days to weeks after elimination of the Ag source; however, a very small proportion of these T cells will transform and constitute subpopulations of memory T cells with distinctly different surface markers (6).

To date it is noted that properly activated memory CD8+ T cells will exert their cytotoxic effects on targeted pathogenic cells. Further TCR/MHC binding with a concomitant minimum Ag density is requisite to induce killing. No subsequent regulatory mechanisms were established as responsible for this cellular carnage. The minimal Ag densities required for proper CTL mediated killing were the focus of extensive investigation during the past decade (7, 8). Still, the influences of high Ag densities were not fully examined, specifically in the context of properly activated CTLs.

The fact that subdominant epitopes might be expressed at a higher copy number than immunodominant epitopes was demonstrated in vitro and resulted in confusion between the level of peptide presentation (Ag density) and the outcome of its presence (subdominance/imunodominance). The most abundant peptide was not necessarily the most immunogenic. This observation was mainly explained by parameters such as peptide affinity, complex stability, and TCR avidity (9).

The functional outcome of the exposure of CTLs to high Ag densities for long periods of time in cases of Ag persistence and chronic inflammation or repeated exposure to Ag was well characterized as a suppressive event and, in some cases, even as an apoptotic trigger to CTLs (10–12). T cells stimulation using a polyclonal stimulant such as Con A has been also demonstrated as an effective activator in low and medium doses but as a suppressive factor during exposure to high levels (13).

In this study we identify a new cell-localized mode of action that can render human activated memory cytotoxic CD8+ T cells anergic. In particular, this cytotoxic inhibitory phenomenon is elicited in a narrow range of Ag density. The molecular machinery for this Ag-induced postpriming control mechanisms entail changes in the expression of surface molecules that control synaptic activity, changes in cytokine profiles, and extensive alteration in gene expression profiles related to energy metabolism, membrane

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2 Abbreviations used in this paper: pAPC, professional APC; EGFR, epidermal growth factor receptor; MFI, mean fluorescence intensity; scFv, single-chain variable fragment.

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potential, impaired TCR signaling and function, and cell cycle arrest. The conglomeration of these results manifests a unique gene expression signature that confers anergy on memory T cells that encounter high Ag density on their target cells.

Materials and Methods

Cells

The T cell clones JKF6, D4F12, R6C12, and JR1E2, specific for the melanoma differentiation Ags, the MART122–35, gp100209–217, and gp100280–285-derived epitopes, respectively, were derived from tumor-infiltrating lymphocytes as previously described (10). These were expanded and activated by using 30 ng/ml mAB OKT-3, irradiated PBMCs from three different donors, and 500 IU/ml recombinant human IL-2. Cells were maintained in RPMI 1640 medium plus 10% human serum, 2 mM l-glutamine, 10 mM HEPES buffer, 50 μM 2-ME, and penicillin/streptomycin. Two T cell lines specific for EBV BMLF-1-derived peptide 280–288, the CMV pp65-derived peptide 495–503, and one T cell clone (R6C12) specific for the melanoma gp100 G209–217 peptide were stimulated in the presence of peptide-pulsed pAPCs and maintained in similar medium. The T cells were then analyzed for effector memory phenotype by flow cytometry with fluorescently labeled Abs and used for cytotoxicity assays. JY B lymphoblast cells were used as target APCs for CTLs using peptide pulsing at various peptide concentrations. They grow in RPMI 1640 plus 10% FCS. A431 and ATCA4 (A431 cells stably transfected with CD25) are human epithelial carcinoma HLA-A2-negative cells grown in RPMI 1640 plus 10% FCS. Human HLA-A2-positive normal fibroblasts were grown in DMEM plus 10% FCS.

Cytotoxicity assays

Three cell types were used as target cells. First, the EBV-transformed B cell line JY was incubated in presence of various peptide concentrations for 2.5 h at 37°C and subsequently washed. Second, cells of the HLA-A2-negative epithelial carcinoma cell lines A431 and ATCA4 were incubated with single-cell variable fragment (scFv)-HLA-A2-peptide fusion molecules (as previously described (11)). Third, HLA-A2-positive and -negative primary fibroblasts were infected with CMV at a multiplicity of infection of 1 and served as target cells 24, 60, 96, and 120 h postinfection. All cell types were targeted with [35S]methionine, washed, and incubated with appropriate CTLs. Relative lysis was calculated directly using the following: [specific lysis/maximal lysis] × 100. Maximal lysis was determined as the highest cytoxic activity of target cells at optimal Ag density. For CMV-infected cells, a CTL assay was performed as described above. Target cells were incubated in the presence of [35S]methionine before viral infection. Target cells in each assay originated from a single batch of infected cells. The lysis assay was 5-h long and was performed at time 0 and every 24 h until the 120-h time point as indicated in Fig. 1f.

Proliferation assay

Cell proliferation was measured by a [3H]thymidine incorporation assay. In brief, CTLs were cultured for 5 h in the presence of irradiated pulsed APCs presenting various peptide-MHC quantities. APCs were depleted using a CD19 EasySep kit (StemCell Technologies). CTLs (0.2 × 105) were plated (>98% CD3+ CD8+) in triplicate wells of a 48-well tissue culture plate for 24 h in the presence of 1 μCi of [3H]thymidine. After 24 h the cells were harvested and washed and [3H]thymidine incorporation was measured using a scintillation counter. Control CTLs were not exposed to pulsed APCs and served as a reference.

Ag density quantitation

The numbers of specific peptide-MHC complexes present on the surface of target cells were determined using two different strategies. For peptide-pulsed JY cells and CMV-infected primary fibroblasts, three TCR-like recombinant Fab Abs were used for the detection of the specific complexes on the target cell surface: H9 (CMV), CLA12 (MART-1), and 1A7(gp100), as previously described (12, 13). Briefly, HLA-A2-negative epithelial carcinoma cell lines we used a PE-labeled mAb, B7.2, directed toward the α3 domain of the HLA-A2-scFv fusion recombinant molecule. Binding was measured using a FACScaliber flow cytometer (BD Biosciences). To transform the florescent signal obtained by flow cytometry into the number of HLA-A2-peptide complex molecules/sites, we used the QuantibRITE PE kit (BD Biosciences) according to the manufacturer’s instructions. For JY APCs, the cells were pulsed with different peptide concentrations ranging from 500 μM down to 1 μM. In parallel experiments, CTLs were exposed to APCs pulsed with similar peptide concentrations and the cytotoxic activity was determined. Each peptide-pulsing experiment was repeated 20 times or more and followed by statistical analysis. By using the QuantibRITE PE kit (BD Biosciences) calibration beads we created a calibration curve of PE mean fluorescence intensities (MFI) for 0–10,000 PE molecules for each cytometer setting, and through the use of such a calibration curve we were able to convert each MFI resulting from the binding of our TCR-like Abs into a number of PE molecules that directly correlates to the number of HLA-A2-peptide complexes detected by the TCR-like Ab. This data representing the number of sites were correlated to the cytotoxic activity obtained by the appropriate CTLs at each peptide concentration used to pulse the target APCs. Fig. 1e shows that the peptide titrations correlated accurately with the fluorescent signal. Calculating the MFI for each peptide concentration created a range of ~1000 to a few dozen HLA-A2-peptide complexes on the surface of peptide-pulsed cells. The values in each spot is statistical significant toward nearby spots. The approach allowed us to detect as low as 10–20 specific peptide-MHC complexes on the surface of the target cell with statistical significance (α = 7.5%).

Cytokine secretion and T cell phenotype

The secretion of Th1/Th2 cytokines from CTLs exposed to various Ag densities over time was determined with a cytometric bead array immunoassay. IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were simultaneously measured by cytokometric bead array kit (BD Biosciences). For each cytokine, the manufacturer’s instructions. Cytokine secretion was measured in five independent assays and calculated using standard calibration curves. The phenotype of the CTLs was studied by using fluorescently labeled Abs (from Dako, Serotec, and BD Biosciences) and a FACS caliber cytometer (BD Biosciences), and analysis was done using CellQuest (BD Biosciences).

DNA microarray

The gene expression profile of the JKF6 memory T cell clone as a function of time postexposure to various Ag densities on target cells was analyzed using the Affymetrix human gene DNA array chip. Each time-Ag spot was composed of three independent biological assays. CTLs were incubated with pulsed JY APCs loaded with a peptide concentration that corresponds to an Ag density of 10, 100, and 800 sites per cell. CTLs were incubated for 4, 16, or 36 h with peptide-pulsed JY target cells that were subsequently depleted after incubation using CD19 EasySep (StemCell Technologies) magnetic beads. JY Depleted cultures contained ≥97.3% CD8+ T cells with no detectable contamination of CD19-positive JY target cells. All experiments were performed using Affymetrix Hu133A 2.0 oligonucleotide arrays as described (see URL1 at the end of this paragraph).

Data analysis

The probe sets contained in the Affymetrix Hu133A2 oligonucleotide array or oligonucleotide arrays were filtered using a MAS 5 algorithm. Treated and control samples were compared. The comparison generates a list of “active gene sets” of our TCR-like Abs into a number of PE molecules that directly correlates to the number of HLA-A2-peptide complexes detected by the TCR-like Ab. This data representing the number of sites were correlated to the cytotoxic activity obtained by the appropriate CTLs at each peptide concentration used to pulse the target APCs. Fig. 1e shows that the peptide titrations correlated accurately with the fluorescent signal. Calculating the MFI for each peptide concentration created a range of ~1000 to a few dozen HLA-A2-peptide complexes on the surface of peptide-pulsed cells. The values in each spot is statistical significant toward nearby spots. The approach allowed us to detect as low as 10–20 specific peptide-MHC complexes on the surface of the target cell with high statistical significance (α = 7.5%).
all treated sample as compared with the control samples (CTLs at time 0) for at least one time point. This list excluded up-regulated genes in all treated samples with signals $>20$ or detected as absent and down-regulated genes with baseline signals $<20$ and detected as absent in the control samples. For further filtering we used the probe sets changed by at least 2-fold (between signals) between the two treated samples at 36 h: 700 sites.

**FIGURE 1.** High Ag density induces hyporesponsiveness in memory activated CTLs. **A–D,** JY B lymphoblast APCs were pulsed with increasing concentrations (conc.) of melanoma differentiation Ags, the gp100$_{209-217}$ (**A**) and MART1$_{27-35}$ (**B**) peptides or the EBV BMLF-1$_{280-288}$ (**C**) and the CMV pp65$_{495-503}$ (**D**) derived peptides. Peptide-pulsed JY APCs were subsequently exposed to the appropriate HLA-A2-restricted and specific CTL clones/line. In each assay pulsing with an irrelevant HLA-A2-peptide was used as a negative control. **E,** JY APCs were pulsed with different MART1$_{27-35}$ peptide concentrations ranging from 500 nM down to 10 pM. The fluorescence intensity of binding of a TCR-like Ab, CLA12, which recognizes specifically HLA-A2/MART1$_{27-35}$ complexes was determined by flow cytometry and directly correlated with Quantibrite PE calibration beads that were used to create a calibration curve of MFI for 0–10000 PE molecules for each cytometer setting. The MFI resulting from the binding of the TCR-like Ab was converted into number of PE molecules that directly correlate to the number of HLA-A2-peptide complexes detected by the TCR-like Ab. Peptide titrations correlated accurately with the fluorescent signal. Calculating MFI for each peptide concentration created a range of ~1000 to a very few HLA-peptide complexes on the surface of peptide-pulsed cells. The SD of each spot is statistically significant toward its nearby spots. **F,** The cytotoxicity of a MART1$_{27-35}$ specific CTL clone acting against JY APCs pulsed at various peptide concentrations was correlated to the number of HLA-A2/MART1$_{27-35}$ complexes present on the target cell surface as detected by the HLA-A2/MART1$_{27-35}$-specific CLA12 TCR-like Ab. An irrelevant HLA-A2-restricted peptide was used as a negative control. **G and H,** A431 and ATAC4 human epithelial carcinoma HLA-A2-negative cells, which express EGFR and CD25, respectively, were incubated with single-chain HLA-A2/scFv fusion proteins to deposit variable amounts of HLA-A2/EBV (**G**) and HLA-A2/209 (**H**) peptide complexes, respectively, on the surface of the target cells. Targets were exposed to the appropriate EBV- and gp100$_{209}$-specific CTLs and lysis was measured. The number of HLA-A2/peptide complexes deposited on the target cell surface was determined using a PE-conjugated mAb BB7.2 that specifically recognizes HLA-A2 and PE calibration curves. **I,** Human HLA-A2 positive fibroblasts were infected with a CMV and the cytotoxicity of CMV pp65$_{495-503}$-specific CTLs was determined as a function of time postinfection and the number of HLA-A2/pp65 complexes (p-MHC com, peptide-MHC complexes) as determined by the TCR-like Ab H9 as described above.
Results

High Ag density induce hyporesponsiveness in memory activated CTLs

To study the role of Ag density on the response of activated memory CTLs we used properly activated human HLA-A2-restricted CD8+ T cell clones and lines with viral or tumor Ag specificity that originated from tumor infiltrating lymphocytes (15) or peripheral blood. These CTLs were established as effector memory CD8+ T cells by virtue of their characteristic phenotype, i.e., potent cytotoxic activity and cytokine secretions (IL-2, IFN-γ, and TNF-α) but lack of IL-10 or IL-4. They exhibit surface expression of the memory phenotype, i.e., CD3+, CD8+, TCRαβ+, CD45RA-, CD62L+, CD56+, CD85+, CD69low, and CD25low.

We exposed these memory CTLs to increasing Ag (peptide-MHC) densities using three experimental systems: 1) peptide-loaded pAPCs; 2) deposition of HLA-A2-peptide complexes on cells lacking endogenous expression of HLA-A2 through the use of an antibody-HLA-A2 fusion molecule (16); and 3) the use of viral-infected HLA-A2 target cells.

As shown in Fig. 1, A–D, the lytic activity induced by the CTL clones increased in a dose-dependent manner from low to intermediate concentrations of antigens peptide. However, further increase in peptide concentration significantly impaired the lytic activity of the CTLs, up to a 60% decrease from optimal lytic activity (Fig. 1, A–D). These results were observed with four different human HLA-A2-restricted, activated CD8+ T cell clones that recognize the melanoma differentiation Ags gp100-derived T cell epitope 209–217 (Fig. 1A) and 280–288 (data not shown) and MART-1 derived epitope 27–35 (Fig. 1B). Moreover, lytic activity was also tested in CTL lines that recognize virus-derived T cell epitopes. Fig. 1, C and D depict the results with two CTL lines originated from peripheral blood that recognize the EBV BMLF-1-derived peptide 280–288 (Fig. 1C) and the CMV pp65 derived-peptide 495–503 (Fig. 1D). The killing assays presented are 5-h cytotoxicity experiments, but similar results were observed with 2- and 16-h assays, indicating that CTL function is stably impaired.

Overall, these results show that the optimal peptide concentration, which is required to induce maximum killing, is approximately in the range of 1 × 10−7 M. The binding affinities of these peptides to HLA-A2 were similar and lay in the high range of the HLA Peptide Binding Predictions algorithm (http://bimas.dctr.nih.gov/molbio/hla_bind/). These results suggest that high Ag densities induce hyporesponsiveness in properly activated T cell lines and clones.

To directly quantify the specific Ag densities that mediate optimal cytotoxic activity or induce hyporesponsiveness of CTL activity, we used aliquots of TCR-like Abs previously developed in our laboratory (14, 17). TCR-like Abs bind to specific HLA-A2/peptide complexes per target (P22) and 100 sites per target (P21). Hierarchical clustering was performed using the DecisionSite for Functional Genomics (Spotfire). Genes were classified into functional groups using the GO annotation tool. Over-representation calculations were done using Ease (14). Functional classifications with an “Ease score” of <0.05 were marked as overrepresented.
with a peptide-specific, MHC-restricted manner but, in contrast to the low affinity of TCRs, these Abs exhibit a high affinity binding in the nanomolar range (12, 13). The titration of binding of such a TCR-like Ab is shown in Fig. 2. Fluorescently (PE) labeled secondary mAb and PE-calibration curves were used to enumerate the number of peptide-MHC complexes for each peptide-pulsing concentration as detected by the TCR-like Abs (Fig. 1E). As shown in Fig. 1F, for the MARTI27–35 HLA-A2-restricted CTL clone JKF6 these calibration curves enabled us to quantify the number of HLA-A2-peptide complexes required to achieve initiation, optimization, and inhibition of lysis by the appropriate CTL. Similar results were observed with other CTL specificities shown in Fig. 1, A, C, and D. This direct quantitation strategy reveals in all cases that very few complexes are required to initiate killing (10 complexes yielded >20–50% of cytotoxicity). An average of 80–120 specific HLA-peptide complexes on the surface of each target cell, termed the optimal Ag density, induced maximal CTL mediated killing; however, higher levels of Ag densities in the range of 500–700 HLA-A2-peptide complexes significantly reduced the lytic activity of the CTLs.

We applied a second experimental system that employs non-lymphoid cancerous epidermal cells rather than pAPCs. In this approach, the targeted deposition of HLA-A2-peptide complexes on target cells devoid of HLA-A2 expression is facilitated through a recently developed genetic fusion that was generated in our laboratory (18). In this fusion molecule, a cell targeting an scFv Ab fragment is fused genetically to a single-chain HLA-A2 molecule (Fig. 3A). These fusion proteins enabled us to coat HLA-A2 negative cell lines with different densities of HLA-A2 molecules bearing a specific peptide (Fig. 3B). HLA-A2-negative A431 epidermoid carcinoma cells expressing the epidermal growth factor receptor (EGFR) were coated with an anti-EGFR scFv HLA-A2 fusion that carries the HLA-A2-restricted EBV BMLF-1 derived peptide 280–288 (Fig. 1G), and ATAC4 cells (A431 stably transduced with CD25/Tac) were coated with an anti-Tac scFv HLA-A2 fusion that carries the melanoma Ag gp100-derived epitope 209–217 (Fig. 1H). These fusion molecules were effectively used to titrate and segregate low, optimal, and high Ag density cell colonies. Subsequently, HLA-A2 restricted lysis of these cohorts by the appropriate activated CD8+ CTLs was observed as shown in Fig. 1, G and H. Quantification of the number of anti-Tac scFv HLA-A2–209 and anti-EGFR scFv HLA-A2–EBV fusion molecules bound to the target HLA-A2-negative carcinoma cells at various concentrations was measured with the PE-labeled, HLA-A2-specific mAb BB7.2 and PE calibration curves.

The lytic activity for deposited HLA-A2-peptide complexes was similar to that of peptide-loaded pAPCs (Fig. 1, A–D), namely, CTL killing was initiated at 10–20 complexes, exhibited a peak of optimal lytic activity at intermediate HLA-peptide densities of ~100 complexes per cell, and was significantly inhibited by 40–60% at high Ag densities of >500 complexes per cell. These results were also observed with three other T cell clones and lines (data not shown). The pattern of inhibition of CTL lytic activity at high Ag densities was similar whether peptide-loaded pAPCs or HLA-A2 deposition on target cells was used.

We invoked a third experimental system in which normal human HLA-A2-positive fibroblasts were infected with CMV and the cytotoxic activity of the CMV-pp65-derived epitope 495–503-specific CTL line was determined. The number of pp65-derived HLA-A2/pp65495–503 complexes on the surface of the CMV-infected cells was determined using the TCR-like Ab H9 as described above. As shown in Fig. 1F, optimal CTL cytotoxic activity toward virus-infected cells was observed 96 h postinfection when the number of HLA-A2/pp65495–503 complexes was ~100 sites per cell. When the density of the pp65-derived HLA-A2-peptide complex was ~700 sites, CTL hyporesponsiveness was observed in a commensurate manner to that the other two experimental systems. In comparison with peptide pulsing or MHC deposition, this experimental model reflects the innate physiological processes in which intracellular viral derived peptides are translocated in the MHC complex to the cell surface and the density of such endogenously derived viral peptide–MHC complexes is increased as a function of time after infection.

Interestingly, the phenomenon of Ag density-induced nonresponsiveness is persistent. As shown in Fig. 4A, CTLs exposed initially to low or intermediate Ag density maintained their proper cytotoxic activity when they again encountered Ag at optimal density after 1 wk. However, the CTLs that were exposed initially to high Ag densities remained anergic. They exhibited 75% reduction in lytic activity compared with CTLs exposed to low or intermediate Ag densities. Two additional clones, D4F12 and R6C12, specific for HLA-A2/pp100, were examined for their ability to kill target cells 1, 3, and 7 days postexposure to various Ag densities. The results were similar to what was observed and shown in Fig. 4A; both lines demonstrated a similar significant reduction of 75–80% in their cytotoxic activity toward target cells after exposure to high Ag densities at day 0 compared with T cells, which encountered no Ag or were exposed to low or optimal Ag densities at day 0 (data not shown). Similar results were also observed for an EBV-specific T cell clone.
Impaired proliferation and cytokine secretion after exposure of activated CTLs to high Ag density

To further investigate the molecular mechanisms that control Ag-induced CTL hyporesponsiveness, we examined two major parameters in T cell biology: the secretion of cytokines and the expression of surface molecules associated with CTL function. As shown in Fig. 4D, we observed significant changes over time in the secretion pattern of Th1 cytokines IL-5, IL-2, and IFN-γ, but not TNF-α. As shown, the profile of cytokine release correlated with the killing pattern observed as a function of Ag density. Thus, CTLs exposed to optimal densities of peptide-MHC (~100 complexes) secrete a certain level of cytokines. However, once the threshold of peptide-MHC density has reached its upper limit, cytokine secretion decreased significantly, corresponding to a reduction in T cell function, to wit, anergy. In addition, as shown in Fig. 4, B and C, significant inhibition in CTL proliferation
and reduction in total RNA content was observed when exposed to high densities (500–700 complexes) but not to low or intermediate densities of Ag. To examine whether T cells underwent apoptosis after exposure to high Ag densities or if proliferation was impaired, we performed a viable cell count of CTLs 24, 48, and 72 h postexposure to pulsed APCs. T cells were incubated at an E:T ratio of 7:1 starting with 105 T cells per well. As shown in Fig. 4E, cell counts and cell viability, measured by staining with trypan blue, did not alter significantly between CTLs exposed to low or high Ag density compared with control cells that were not exposed to Ag, indicating that significant cell death occurring as a result of exposure to high Ag density was not observed. More profound was the extent of proliferation that was observed when CTLs encountered the optimal density of an Ag.

**Impaired expression of key functional molecules after the exposure of activated CTLs to high Ag density**

We studied the expression of key surface molecules that are associated with effector CTL function and immunological synapse formation as a function of time and Ag density. Exposure to increasing densities of Ag reveals significant and correlative alterations in surface molecule expression (Fig. 5). Major differences were observed in the expression patterns of CD8, CD3, CD45RO, and CD85 (Fig. 5A) as well as down-regulation of 70% in TCRαβ (supplemental video files). Moreover, as shown in Fig. 5, A–C, the expressions of CD8+ CD45RO and CD85 were palpably altered; CD8 and CD45RO surface expressions decreased in concordance with the increasing Ag density on the target cell and the time of exposure. The expression of CD85 (LIR1) was also altered upon the exposure of CTLs to increasing densities of Ag. In contrast, CD152 expression and staining with annexin V indicative of apoptosis were not altered (Fig. 5, D and E). Control CTLs that were incubated with nonpulsed APCs exhibited a similar phenotype to those exposed to low Ag density, i.e., ~10 complexes per cell (data not shown). The alteration in the expression of key functional molecules was both time and dose dependent and long lasting.

Fig. 6A summarizes the alterations in numerous experiments of the expression patterns of the three key surface markers as a function of time after exposure to increasing concentrations of peptide (Ag density). The percentage of CD8hiCD8lo, CD45Rohigh CD45Rolow, and CD85dimCD85− CTLs subpopulations is shown. Most pronounced are the results observed 48 h after encounter with high Ag densities in which there is a clear shift or conversion point between the “high” and “low” phenotype that occurs at a peptide concentration of ~1 × 10⁻⁷ M, which correlates to an Ag

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3 The online version of this article contains supplemental material.
density of ~100 peptide-MHC complexes per cell. A visual summary of these results can also be seen in the movies presented in the supplemental material (video files 1–5 of the supplemental data). These results demonstrate that high Ag densities engender changes in the expression patterns of key molecules that are required for proper T cell function and synaptic formation/interaction.

To examine whether the expression levels of key synapse molecules were altered for relatively long periods of time, we exposed CTLs to pulsed APCs for 24 h and subsequently isolated them.

**FIGURE 6.** The dynamics of the expression of key surface molecules after the exposure of activated CTLs to high Ag density. A. CD8$^{\text{high}}$CD45R0$^{\text{high}}$CD85$^{\text{dim}}$ homogenous CTLs were exposed to JY pulsed pAPCs. Two distinct subpopulations of CD8$^{\text{high}}$CD45R0$^{\text{high}}$CD85$^{\text{dim}}$ and CD8$^{\text{low}}$CD45R0$^{\text{low}}$CD85$^{\text{low}}$ phenotypes were created after exposure. The percentage of high and low subpopulations is shown as a function of time and Ag density on target cells. The high expression level population is marked with diamonds (♦) and the low expression level population with triangles (▲). A significant transition point marked with an arrow was observed when CTLs were exposed to peptide concentration (conc.) above $1 \times 10^{-7}$ M, corresponding to ~100 complexes/cell, most profoundly 48 h postexposure. B. Distribution of CD8$^{\text{high}}$ or CD8$^{\text{low}}$ subpopulations in CTLs 3 or 7 days after exposure to low, optimal, or high Ag density. The dominance of the CD8$^{\text{low}}$ subpopulation still persists 7 days after exposure to high Ag density.
These isolated T cells were cultured and 3 or 7 days postexposure were examined for CD8 and CD3 expression. The expression levels of both CD8 (Fig. 6B) and CD3 (data not shown) were significantly altered in a manner that was dose dependent and long lasting. Thus, the phenotype in which these CTLs are predominantly CD8low can persist even 7 days after their exposure to high Ag densities. These results, together with the data presented in Fig. 4A, showing that these cells also exhibit a 75% reduction in cytotoxic activity suggested that the hyporesponsive phenotype is a prolonged and long-lasting anergic status rather than a transient suppressive state or event.

We have shown that four critically important parameters in CTL function, namely proper cytotoxic activity, secretion of Th1 cytokines, clonal proliferation, and expression of key surface molecules intercept at an optimal Ag density of 100 complexes per target cell and that above a threshold of ~250 complexes per target they become hyporesponsive with concurrent down-regulation of the respective parameters.

Distinct gene expression signature indicative of anergy in CTLs exposed to high density of Ag

The fact that maximal alterations in the expressions of surface markers and cytokine secretion occurred after a relatively long period of time (maximal effect after 48 h with sustained effect of up to 7 days) suggested an alteration in gene expression. We therefore performed a comparative genome-wide microarray analysis (Affymetrix) of genes expressed by CTLs exposed to low, optimal, and high Ag densities as a function of time. A comparison between treated and nontreated samples identified a list of 5877 probe sets changed by at least 2-fold in one or more of the samples compared.
with time 0 (Fig. 7A). Comparing CTL samples exposed to high vs optimal Ag densities for 36 h identified 1070 probe sets that are differentially expressed by at least 2-fold between these two samples (Fig. 7B). The classification of these 1070 genes revealed four major categories of genes for which significant alternations in gene expression were observed between optimal and high density: energy metabolism, membrane potential, cell signaling, and apoptosis vs cell cycle control (Fig. 7C). A list of several key representative genes from this analysis that exhibit a major change in expression between optimal and high Ag density after 36 h of exposure to Ag is shown in Fig. 7D. The controls and normalizations for these DNA arrays experiments are summarized in the methods section of the supplementary data.

The gene analysis results of CTLs exposed to high Ag density revealed that the energy metabolism was marked by an inhibition of glycolysis indicative of the reduction of key glycolysis enzymes such as hexokinase and phosphofructokinase. Changes in membrane potential were reflected by alterations in ion channels expression. Severe hyperpolarization or depolarization of membrane potential and decreased calcium compliance might result from these alterations. Further impairments were observed in T cell signaling in general, including alterations in genes of the RAS, MAPK, and inositol 1,4,5-triphosphate pathways as well as alterations in TCR complex signaling. Gene expression profiles related to cell cycle control and apoptosis indicate a signature of cell cycle arrest with no marked shift toward apoptosis.

Overall, gene chip analysis presents a signature of an anergic T cell with ablated energy metabolism, altered membrane potential, impaired signaling, and cell cycle arrest. These data explains the severely impaired response of memory activated CTLs to high Ag density.

Discussion

The current literature describes two major mechanisms for the education of T cells to prevent autoreactivity, thymic selection through the deletion of self-reactive T cells, and a postthymic multistage process referred to as priming that is mediated by pAPCs in lymph nodes (19, 20). Two rival models are at the center of the priming process: the two-signal theories and the Ag localization, dose, time, and structure concept (21). The two-signal theory states that an Ag encounter (signal 1) with no additional co stimulation (signal 2) turns T cells off whereas signal 1, combined with signal 2, permits them with a license to kill (22). The Ag localization, dose, time, and structure model employs Ag parameters, i.e., that localization of infection and priming and the dose of the Ag combined with time kinetics and the structure of the Ag in determining whether a specific T cell clone will be activated. These two rival models share the assumption that immune reactivity is learned and that there is no unique antigenic structure discriminating self from non-self. The two-signal model postulates positive vs negative reactivity based on the presence or absence of costimulatory signals. It is recently becoming clear that this model might miss a few dimensions of the immune response (23–28). The geographical model proposed by Zinkelnagel et al. (29) claims that an Ag that is transported to secondary lymphoid organs in sufficient (but not excessive) amounts and for a sufficient time period (but does not persist) induces an effective immune response. In addition and complementary to the geographical model, our data suggest a third new control point that occurs at the peripheral in situ tissue level. Activated memory CD8+ CTLs possess properties of a self-referential sensory organ that is highly sensitive to Ag density. Killing is initiated at very low Ag density and reaches optimal killing activity at around 100 MHC-peptide complexes; however, once a certain upper threshold of ~250 MHC-peptide complexes is detected on the surface of the target cell a significant inhibition of CTL activity is observed that induces hyporesponsiveness or anergy of activated CD8+ T cell lines or clones. This hyporesponsive response is characterized by a significant long-term decrease in cytotoxic activity, inhibition of proliferation, and a substantially remodeled anergic T cell. Our findings may fit into the geographical model rather than into the two-signal theory because they enter another dimension in the context of proper and potent immune response. According to our findings, this may be the final regulatory checkpoint of such a model.

This work uncovers a control mechanism that functions as a negative regulator of CTL cytotoxic activity. Although the conservation of this phenomenon is yet to be studied in vivo, it is however well appreciated that many self-Ags are in general richly expressed (30); thus, one may hypothesize that this novel modality may serve to protect localized tissue from an errant auto reactive attack by essentially revoking its license to kill.

This induced anergy might explain, at least in part, peripheral tolerance, a safeguard mechanism that prevents autoreactivity when T cells sense high Ag densities that signal a dangerous autoreactive circumstance. Our results may correlate with the recent finding that agonist/endogenous peptide-MHC heterodimers can regulate T cell activation and sensitivity in CD4+ lymphocytes (31). According to these findings, which predict that CD8+ T cells also use self-peptide-MHC complexes in their response, initial killing at very low Ag densities (1–20 complexes) involves agonist/ endogenous heterodimer complexes. Optimal killing is achieved at ~100–250 complexes when most of the agonist/endogenous heterodimers are shifted into agonist homodimers as the number of agonist peptide-MHC complexes on the surface increase. However, the new safeguard control mechanism on CTL function, described herein, starts to operate when the number of agonist homodimers or larger MHC-peptide structures are generated due to high Ag density of >250 complexes (see schematic model in Fig. 8).

It has been recently demonstrated that the supramolecular complex composing the immunological synapse is composed of peripheral and central supramolecular activation clusters (32). The membrane surface inside the central supramolecular activation cluster was demonstrated to mediate the exocytosis of CTL lytic
granules. The central supramolecular activation cluster is mainly composed of TCR-MHC complexes and one can hypothesize that the physical limitations in this free surface due to steric interference by the high number of MHC-TCR interactions may reduce the ability of CTLs to perform target cell lysis. Our results also demonstrate that alterations in CTL function as a result of high Ag density occur at two time frames, namely short-term effects that result in impaired cytotoxic activity and long-term alterations that are affected by changes in cell proliferation, cytokine secretion, and the expression of cell surface functional molecules. These overall alterations, which culminate with the anergic function phenotype, may result from different checkpoints or regulatory mechanisms that exert their influence at various times through this complex process.

Most profound in this machinery are the anergic state expression signatures at high Ag densities compared with the full activation signature at the optimal density. Impairment in T cell function is due to major alterations in TCR signaling and function, membrane permeability, and alterations in energy metabolism and cell cycle control. Glycolysis was largely ablated, which indicates that energy metabolism may play a critical role in the control of function of T cells. It was recently suggested that T cell metabolic needs are governed by external signals (transcriptional and translation responses as well as costimulation) (33). Once T cells do not receive these signals they fail to increase their energy metabolism to meet the hyperbioenergetic demands of cell growth and are either deleted or rendered unresponsive to mitogenic signals. T cells exposed to high Ag densities are not subject to enhanced apoptosis but rather cell cycle arrest. Thus, these results further strengthen the anergic model for effector memory CTLs rather than deletion through Ag-induced cell death or apoptosis. The phenomenon of the Ag-induced hyporesponsiveness of properly activated CTLs exposed to high Ag densities presented herein may contribute to the understanding of immunodepression. It was shown previously in vitro that dominant epitopes are not necessarily presented at the highest copy number on the surface of target cells (9). In addition, it has been suggested that epitopes considered subdominant are major contributors to immunogenicity in HIV-1 (34). In this work we show that the display of large numbers of peptide-MHC complexes on target cells does not necessarily translate into a more potent CTL activity; on the contrary, it leads to suppression or unresponsiveness. Thus, we postulate that high Ag density of a particular epitope does not mean that it is an immunodominant epitope.

Taken in the context of the multitude of signaling and gene alteration events that occur in the high Ag density-induced anergic T cells, it is our hope that this work will further elucidate the underlying mechanism of T cell biology with ramifications leading to better understanding about the control of immune function and regulation, including immunomodulation, peripheral tolerance, control of viral infections, antitumor immune responses, hypersensitivity, and autoimmunity.

Disclosures

The authors have no financial conflict of interest.

References


