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Antigen-Dependent Integration of Opposing Proximal TCR-Signaling Cascades Determines the Functional Fate of T Lymphocytes

Ron Wolchinsky,* Moran Hod-Marco,* Kfir Oved,* Shai S. Shen-Orr,† Sean C. Bendall,‡ Garry P. Nolan,§ and Yoram Reiter*

T cell anergy is a key tolerance mechanism to mitigate unwanted T cell activation against self by rendering lymphocytes functionally inactive following Ag encounter. Ag plays an important role in anergy induction where high supraoptimal doses lead to the unresponsive phenotype. How T cells “measure” Ag dose and how this determines functional output to a given antigenic dose remain unclear. Using multiparametric phospho-flow and mass cytometry, we measured the intracellular phosphorylation-dependent signaling events at a single-cell resolution and studied the phosphorylation levels of key proximal human TCR activation- and inhibition-signaling molecules. We show that the intracellular balance and signal integration between these opposing signaling cascades serve as the molecular switch gauging Ag dose. An Ag density of 100 peptide–MHC complexes/cell was found to be the transition point between dominant activation and inhibition cascades, whereas higher Ag doses induced an anergic functional state. Finally, the neutralization of key inhibitory molecules reversed T cell unresponsiveness and enabled maximal T cell functions, even in the presence of very high Ag doses. This mechanism permits T cells to make integrated “measurements” of Ag dose that determine subsequent functional outcomes. The Journal of Immunology, 2014, 192: 2109–2119.

Cytotoxic T lymphocytes act as the effector arm of cell-mediated immune response to kill self cells that are virally infected or have undergone malignant transformation. Two processes prevent these effector cells from performing self-reactivity: a thymic selection process that eliminates autoreactive clones by negative selection (1) and a well-regulated multistage priming process that is necessary for the maturation of naive cells into effector CTLs (2–4). CTLs that have successfully surmounted these regulatory barriers elicit their response as effectors and differentiate into memory cells (5, 6). No subsequent regulatory restrictions have been reported for these properly primed and activated CTLs.

Anergy is a hyporesponsive state described in lymphocytes, in which a cell ceases to proliferate and to secrete crucial proinflammatory cytokines. Anergy has been categorized further into several distinct anergic states, such as clonal and peripheral (7). These anergic states differ in the types of cytokines inhibited, the requirement for Ag persistence, and the ability of the cells to recover from this hyporesponsive state (8). In addition, the various anergic states differ in the biochemical pathways responsible for their onset and maintenance; clonal anergy is initiated by blocking the Ras-MAPK pathway, whereas a defect in ZAP70 phosphorylation was shown to be involved in the peripheral anergy state (8).

Induction of T cell anergy was shown to be influenced by peptide–MHC (pMHC) levels in mice (9) and human T cells (10). High levels of pMHC on target cells generated unresponsiveness by enhancing TCR downregulation. This induction of T cell anergy using a supraoptimal Ag dose also was an extremely rapid process, because preincubation with peptide for as little as 30 min was sufficient to induce a complete unresponsive state (11).

We showed that primary human CTLs are also subjected to pMHC dose-dependent anergy (12), whereupon exceeding a threshold ~100 pMHC-I–specific complexes, a significant decrease in CTL proliferation, killing, and cytokine secretion was observed, as were alterations in the expression of key immunological synapse surface molecules. Significant changes in the gene-expression signatures in these CTLs exposed to supraoptimal pMHC doses also occurred, indicating an anergy-like phenotype in these memory CTLs (12).

A significant amount of work has been published on the triggering mechanisms of TCRs by Ag, as well as the minimal number of pMHC complexes required for initiation of T cell responses (13). However, much less attention has been paid to the effect of high Ag doses and their role in T cell function. For example, the molecular mechanisms underlying the switch from activation and optimal activity to anergy that is induced by Ag doses remain unknown.

In the current study, we explored the molecular mechanisms by which CTLs distinguish between low-to-optimal and supraoptimal levels of presented peptide–MHC class I (pMHC-I) complexes. We asked how T cells sense Ag dose and make the decision to become anergic after exposure to high Ag dose. We show that Ag-induced hyporesponsiveness is calcium independent and is distinct from currently described anergy molecular mechanisms. By evaluating the differential phosphorylation of 21 proteins involved in TCR signaling at low, optimal, and high pMHC-I Ag doses using phospho-flow and mass cytometry, we showed that the intracellular balance and signal integration between opposing activation- and...
inhibition-signaling cascades serves as the molecular switch gauging Ag dose. An Ag density of 100 pMHC complexes/cell was found to be the transition point between activation and inhibition cascades, whereas higher Ag doses induced an anergic functional state. Blocking of key inhibitory-signaling components reversed T cell unresponsiveness to high Ag doses.

Our results suggest that the cellular output of CTLs with respect to Ag dose on target cells is determined through the integration of relative phosphorylation levels of two functionally opposed proximal TCR-dependent signaling pathways.

Materials and Methods

Cells

The T cell clone JKF6, specific for the melanoma differentiation Ag MART-127–35, was derived from tumor-infiltrating lymphocytes, as previously described (14). These T cells were expanded and activated by using 30 ng/ml mAb OKT-3, irradiated PBMCs from three donors, and 500 IU/ml recombinant human IL-2. The cells were maintained in RPMI 1640 medium supplemented with 10% human serum, 2 mM L-glutamine, 10 mM HEPEs buffer, 50 μM 2-ME, and penicillin/streptomycin (CTL culture media). JY B lymphoblast cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and penicillin/streptomycin.

Peptides

MART-1 27–35 (ELAGIGILTV) and CMV pp65 495–503 (NLVPMVATV) were synthesized by Sigma.

Abs and reagents

CLA12 monoclonal TCR-like Ab was described previously (15). PD-1 (MH4) and IgGl isotype control (P3.6.2.1) Abs were purchased from ebioscience. Phospho-flow and mass cytometry time-of-flight (CyTOF) Abs are described in Supplemental Table IA and IB. Rabbit anti-LAT and rabbit anti-LAT (Y226) were purchased from Millipore, and anti-human Pan-Ras and rabbit anti-human RasGRP1 were purchased from Santa Cruz Biotechnology. Mouse anti-human GM130 was purchased from BD Biosciences, CFSE was purchased from Molecular Probes, and PKH26 was purchased from Sigma. Rabbit anti-SLP-76 (Y175) was a kind gift from Dr. Deborah Yablonksi (The Bruce Rappaport Faculty of Medicine).

Ag quantitation

The numbers of specific pMHC complexes present on the surface of target cells were determined by flow cytometry, as previously described (12) (Supplemental Fig. I). Briefly, a specific TCR-like clonotypic mAb that recognizes the MART-127–35 peptide, only when in complex with the HLA-A2 molecule, was used (16, 17). JY cells were pulsed with different peptide concentrations, ranging from 500 μM down to 100 pM. Specific binding of the TCR-like Ab was detected using PE-labeled anti-λ light chain mAb, and a QuantiBRITE PE kit (BD Biosciences) was used, according to the manufacturer’s instructions, to translate the measured fluorescent intensity into an absolute number of HLA-A2–peptide complex molecules/site. Each peptide-pulsing experiment was repeated 10 times.

Cytotoxicity assays

The EBV-transformed B cell line JY was labeled with [35S]methionine for 1.5 h. The cells were washed and incubated in the presence of various peptide concentrations for another 1.5 h at 37°C. The cells were washed of excess peptide and incubated with appropriate CTLs for 5 h. Relative lysis peptide concentrations for another 1.5 h at 37°C. The cells were washed of excess peptide and resuspended with appropriate CTLs for 5 h. Release was defined as (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100, with spontaneous release being the [35S]methionine released from target cells in the absence of effector cells and maximum release being the [35S]methionine released from target cells lysed with 0.05 M NaOH. Maximal lysis was determined as the highest cytotoxic activity of target cells at optimal Ag dose. In inhibition assays, the SHP1/2-specific inhibitor sodium stilbogluconate (SSG) was titrated at final concentrations in the range of 5–20 μM for 1 h prior to adding JY cells, followed by washing of CTLs and proceeding according to the standard cytotoxicity assay, as described above.

Real-time and gene arrays

CTLs were cultured with APCs pulsed with 100 pM, 100 nM, and 500 μM MART-1 peptide for 30 min or 6 h. CTLs were purified from the culture by negative selection, using CD19-conjugated magnetic beads (1 μg/1 × 10⁹ cells Ab, 10 μl beads/1 × 10⁹). Cells were incubated with beads for 30 min, after which they were supplemented with 1 ml PBS-0.1% BSA and 10 μl to magnet for 3 min to allow binding of the cells. Flow-through was centrifuged for 1 min to pellet the cells, and total RNA was extracted using an Aurum Total RNA Mini Kit (Bio-Rad). mRNA was converted to cDNA using a cDNA Reverse-Transcription Kit (Applied Biosystems). PCR amplification was performed with SYBR Green Master Mix (10 ng cDNA/reaction) using an ABI 7300 real-time PCR thermal cyclers (both from Applied Biosystems). The reactions were performed in duplicate; C threshold values were normalized to β2-microglobulin levels, and fold induction (n > 1) or reduction (n < 1) of each gene was calculated (ΔΔCt) with respect to the indicated controls (n = 3).

The gene-expression profile of the JKF6 T cell clone was analyzed using the Affymetrix human gene DNA array chip, as previously described (12).

Intracellular flow cytometry and data analysis

A total of 5 × 10⁶ JY cells were pulsed with MART-1 peptide in concentrations ranging from 500 μM to 100 pM in complete RPMI 1640 media for 1.5 h. The cells were washed of excess peptide and resuspended with CTL media. JY cells were added to 1 × 10⁶ CTLs and spun down at 100 × g for 10 s, followed by incubation at 37°C for 5 min. The cells were centrifuged at 1000 × g for 1 min, the supernatant was aspirated, and the pellet was resuspended in BD-Lyse solution for 15 min, according to the manufacturer’s instructions. The cells were washed thoroughly and resuspended in permeabilization solution, to which the following Abs were added: anti-human Lck, anti-human CD45, anti-human LAT, anti-human SLP-76, anti-human ZAP70, anti-human SHP-2, anti-human c-cbl, and anti-human CrkL. Anti-human CD19 was added to all tubes. The cells were incubated with the permeabilization-Ab solution for 20 min, after which the cells were washed and analyzed by an LSR II flow cytometer (BD Bioscience). Compensation was performed for each Ab.

The data were analyzed by gating out the B cells according to both cell size and positive CD19 staining. The T cells were analyzed for their median fluorescence intensity (MFI) per each detector. We first determined the maximal phosphorylation levels of CD3ζ (pY142) under saturated levels of anti-CD3 Ab. Activation of CTLs by ~1200 pMHC-I complexes/cell generated a similar phosphorylation intensity, allowing us to address this activation as the maximal activation of CTLs. Relative phosphorylation for each phospho-protein was determined as the maximum MFI × 100, whereas minimal and maximal MIFs were the lowest and highest MFI values in each experiment, respectively (500 μM and unpulsed APCs, respectively). Samples were analyzed using FCS express V3 (De Novo software) and Cytobank (https://cytobank.stanford.edu).

Mass cytometry staining and data acquisition

APCs were pulsed as previously indicated, washed, and cocultured with CTLs for 2, 5, or 10 min at 37°C. The cells were centrifuged at 1000 × g for 1 min, the supernatant was aspirated, and the pellet was resuspended in 1.5% paraformaldehyde for 10 min and then washed. Cells were quick-frozen and kept at −80°C until analysis. For mass cytometry analysis, procedures were carried out as previously described (18). Briefly, 1–3 million cells were thawed and surface stained for 30 min at ambient temperature with a mixture of metal isotope–conjugated Abs using predetermined concentrations (Supplemental Table I). Cells were washed twice with cell-staining media (PBS + 1% BSA) and permeabilized for 10 min with methanol on ice. Cells were washed twice with cell-staining media and stained with a mixture of intracellular Abs (Supplemental Table I) at ambient temperature for 30 min. All Ab-staining reactions were performed in exactly 100 μl final volume. After staining, the cells were washed once with cell-staining media and then resuspended in 1 ml 1:500 diluted 2000× iodide intercalator (DVS Sciences) suspended in 1.5% paraformaldehyde. Cells were incubated at maximum 20 min at ambient temperature or were stored overnight at 4°C. Finally, the cells were washed twice once in cell-staining media and twice in distilled water. For data acquisition, cell pellets were diluted in distilled water to the appropriate concentration to achieve an acquisition rate <500 events/s on the CyTOF instrument. CyTOF data were acquired and analyzed on the fly, using dual-count mode with noise-reduction on. All other settings were either default settings or optimized with tuning solution, as instructed by DVS Sciences. For data analyses, we gated on CD2⁺ CD8⁺ CD45RA⁻ cells to obtain a memory CD8⁺ population. We determined the level of phosphorylation of each phosphoprotein by obtaining the median level of the raw measured
values, and the relative (fold-change) phosphorylation was calculated with respect to the no-peptide condition. Qualitatively similar results were obtained when transforming the raw measures using an arcsin transformation. For the cluster analysis, we used a Pearson correlation to compute the distance between vectors of fold-change phosphorylation of each signaling molecule, computed across all doses and time points, with the fold-change determined in relation to the no-peptide condition at a given time point.

**Calcium flux measurement**

JY cells were stained with 1 μM PKH26 (Sigma) and pulsed with 500 μM, 100 nM, or 100 pM MART-1_{27–35} Peptide for 1.5 h. CTLs were stained with 1 μM CFSE and 1 μM Indo-1 for 45 min in CTL culture media at 37°C. All cells were washed thoroughly with media, resuspended in HBSS (Biological Industries), and incubated at 37°C. The analysis was performed using an LSR II flow cytometer equipped with a 355-nm excitation laser (BD). First, compensation between the necessary dyes was done. The basal ratio of bound/unbound calcium was established in CTLs, followed by the addition of JY cells at an E:T ratio of 2:1. Cells were analyzed continuously for 15 min. Ionomycin was added (1 μM) as positive control. The experimental results were analyzed using FlowJo software (TreeStar) by plotting CTLs and JY cells and gating the double-positive quadrant (i.e., the conjugates that formed after adding the APCs to the solution).

**Confocal microscopy**

JY cells were pulsed with various concentrations of peptide for 1–2 h in complete RPMI 1640 media at 37°C. JY cells were then added to CTLs at an E:T ratio of 2:1 and incubated for 5 min. The culture was centrifuged at 1000 × g for 1 min and resuspended in 1% formaldehyde for 10 min. The cells were washed twice to remove the fixative and blocked with PBS supplemented with 5% FCS, 0.15% Triton, or 0.05% saponin for 30 min at room temperature. Cells were stained with primary Abs suspended in Ab solution (PBS supplemented with 1% FCS, 0.1% BSA, and 0.15% Triton, or 0.05% saponin). Cells were washed twice with PBS supplemented with 0.1% BSA (w/v) and stained with secondary Abs: goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 568 suspended in Ab solution. Incubation with secondary Abs was for 20 min at 4°C in the dark, followed by the addition of DAPI (2 μg/mL) and incubation for 5 min at room temperature. Cells were washed, placed on slides, and analyzed using a Zeiss LSM 510 confocal microscope.

**Results**

**MART–specific CTLs are hyporesponsive following exposure to high Ag doses**

We demonstrated previously that CTLs exhibit hyporesponsiveness and anergic phenotype following an encounter with high numbers of pMHC-I complexes. This phenotype was shown to be persistent, where CTLs that encountered high Ag doses remained viable 7 d after Ag encounter, yet exhibited decreased lytic activity and cytokine secretion. Moreover, these studies used multiple CTL lines with viral and tumor specificities, expanding hyporesponsiveness to high Ag doses to more than a single T cell clone (12). Further studies also ruled out the possibility that, at high density of peptide, there is leaking of peptides or regurgitation of endocytically captured peptides that can bind to HLA molecules of the CTL themselves. First, before applying CTLs to peptide-pulsed target cells, the peptide is washed away so only the defined peptide levels, as measured by the HLA-A2–restricted peptide–specific Abs, are present on the cell surface. Second, we (19) showed previously that hyporesponsiveness to high Ag doses also was observed in assays in which the pMHC was covalently immobilized to a surface and, thus, CTLs cannot capture the peptides.

To investigate the underlying mechanism of this phenomenon, we first determined the extent of the Ag-dependent hyporesponsiveness in a primary cultured, MART-1_{27–35}–specific CTL clone. This clone was rapidly expanded as previously described (20, 21). The cytotoxic activity of this clone was tested using an [35S]–release assay; CTLs were challenged with [35S]–labeled target cells that were pulsed with MART-1_{27–35} peptide in a dose-dependent manner, and media radioactivity was measured after 5 h of coculturing (Fig. 1). Dose-dependent, Ag–specific cytotoxic activity against target cells was present until reaching ~100 pMHC-I/cell. Further increases in pMHC-I complexes on the surface of the target cells prompted a decrease in lytic activity; CTLs exhibited up to 50% inhibition in their killing efficiency (p ≤ 0.05) upon encountering ~1000 pMHC-I complexes/cell. These data confirm our previous findings that CTL-mediated killing possesses a nonmonotonic pattern, with an optimal killing activity at a dose of 100 pMHC-I complexes/target cell. Importantly, these observations were confirmed with various CTLs having tumor or viral specificity (12). The quantitative analysis of pMHC-I presentation was conducted, using a TCR-like mAb that specifically recognizes the Mart-1/HLA-A2 peptide complex, by flow cytometry (16, 17) (Supplemental Fig. 1).

**Ag–induced hyporesponsiveness in CTLs is not caused by impaired calcium signaling and is PD-1 independent**

Induction of anergy in T cells is a rapid process (11). We monitored the reduction in CTL-mediated killing of target cells presenting high pMHC doses after as little as 30 min (data not shown). Therefore, we hypothesized that the pMHC-I sensory mechanisms responsible for the induction of anergy are influenced by well-studied and rapid response mechanisms, such as alternations in intracellular calcium or the compartmentalization of key molecules. First, we explored the changes in intracellular calcium levels in these cells as a function of Ag dose. To this end, CTLs were stained with both the calcium indicator Indo-1 and CFSE. CTLs were cocultured with PKH26–stained target cells pulsed with MART-1_{27–35} peptide at concentrations yielding presentation of 0, 20, 100, or 1200 pMHC-I complexes/cell. Conjugates of CTLs with APCs were analyzed based on their double-positive CFSE^−PKH26^−staining properties, and intracellular calcium was determined (Fig. 2A). We found that CTL/target cell contact leads to a dose–dependent increase in the intracellular level of calcium in CTLs, as previously shown (22). Target cells pulsed with ~20 pMHC-I complexes induced minor changes in intracellular calcium, whereas CTLs encountering higher Ag doses of 100 or 1200 pMHC-I complexes/cell demonstrated a dose–dependent increase in intracellular calcium levels that was both linear and monotonic (Fig. 2B). These calcium-increase characteristics do not correlate with the nonmonotonic, pMHC-I–dependent killing of target cells, suggesting that the attenuation and inhibition of CTL responses to high Ag doses are not directly dependent on alterations in intracellular calcium.
Ag-DEPENDENT INTEGRATION OF TCR SIGNALING

Another path that has been associated with the initiation of tolerance is PD-1 (23, 24). To evaluate the role of PD-1 in Ag-induced hyporesponsiveness, JKF6 T cells were cocultured with target cells pulsed with MART-127–35 peptide in a dose-dependent manner. Killing was normalized to the maximal killing detected. A parallel ponsiveness in memory CD8+ T cells.

There was no significant increase in PD-1 expression in correlation with Ag-induced unresponsiveness. We concluded that PD-1 does not represent SEM. (C) PD-1 expression in JKF6 T cells. The cytotoxic activity of JKF6 T cell clone was tested using a [35S]-release assay: CTLs were challenged with [35S]-labeled target cells pulsed with MART-127–35 peptide in a dose-dependent manner. Killing was normalized to the maximal killed detected. A parallel “cold” coculture was mounted, in which CD8+ T cells were gated and analyzed for PD-1 expression. Filled squares indicate relative killing in each pMHC-I dose. Bars indicate the percentage of PD-1+ cells in the CD8+ population. (D) Confocal imaging of RasGRP1 intracellular localization. Peptide-pulsed JY cells were incubated with JKF6 CTLs for 5 min, followed by fixation and staining with anti-RasGRP1. Blue, DAPI; green: anti-human GM130 (Golgi marker); red: anti-RasGRP1. Numbers indicate the dose of pMHC-I presented on target APCs; two representatives from each treatment are displayed.

**FIGURE 2.** The effect of Ag dose on intracellular calcium and RasGRP1 localization. (A) Calcium flux increases as a function of Ag dose. PKH26-stained HLA-A2+ JY cells were deposited with high (1200 complexes), optimal (100 complexes), or low (20 complexes) MART-1 pMHCs or left untreated. A baseline ratio of calcium-bound Indo-1/unbound Indo-1 was acquired, followed by coincubation with the JY APCs (left arrow). CTL-APC conjugates (double-positive events) were gated and analyzed for violet/blue ratio for 10 min. The right arrow indicates the addition of Ionomycin. (B) Calcium-positive cells were plotted against the number of encountered pMHC-I complexes on APCs. Results are representative of three independent experiments. Error bars

Ag-induced hyporesponsiveness of CTLs is not dictated by the spatial location of Ras and RasGRP1

Previous studies demonstrated that pMHC engagement by TCRs of various affinities can be translated into distinct functional output related to anergy. More specifically, low-affinity binding of epithelial pMHC-I complexes by thymocytes results in the translocation of the Ras-pathway components to the Golgi in a positive-selection process, whereas high-affinity binding induced apoptosis by negative selection through the translocation of Ras-pathway components to the cytoplasm (25). Therefore, we examined the localization of Ras and RasGRP1 to the Golgi as a function of the number of pMHC-I complexes presented on target cells (Fig. 2D). JKF6 primary human T cells were cocultured with peptide-pulsed APCs for 5 min and subsequently analyzed by confocal microscopy. RasGRP1 and Ras displayed a diffused localization pattern across the cytoplasm. In contrast to the evidence indicating a role for these molecules in ligand discrimination by thymocytes during thymic selection, we found no correlation between Ag doses and the localization of Ras or RasGRP1 molecules in mature CTLs, suggesting that the Ag-induced hyporesponsiveness in response to high Ag doses is independent of the intracellular localization of Ras or RasGRP1.

**CTLs that bind target cells with high Ag dose exhibit no increase in short-term anergy or exhaustion-related genes**

Because CTLs that exhibit hyporesponsiveness due to binding of target cells with high-Ag dose are functionally anergic (12), we examined whether a CTL that encounters a high number of pMHC-I complexes on a target cell displays an elevated expression level of the following canonical anergy-related genes: EGR-2, EGR-3, Cbl-b, c-cbl, Itch, CREM, DGKα, and IKAROS (26). Real-time PCR experiments were performed 30 min and 6 h postexposure of CTLs to different Ag doses. The relative expression of cbl-b, c-cbl, and Itch in CTLs was similar after both 30 min and 6 h, regardless of pMHC-I dose encountered (Fig. 3A, 3B). The relative expression of the aforementioned molecules in relation to Ag doses also was analyzed at longer periods using Affymetrix gene chips, and changes in some genes were observed after 36 h (Fig. 3C). We calculated the ratio between gene expression in CTLs exposed to high Ag dose (1200 pMHC-I complexes/cell) and in CTLs exposed to an optimal Ag dose (100 pMHC-I complexes/cell) and found that it was ~1 for all anergy-related genes (Fig. 3C), suggesting that no significant upregulation of the analyzed genes occurred with an increased pMHC-I dose.

The absence of both calcium signal and the transcription of anergy-related genes suggests that the cells are exhausted following a high dose of pMHC-I complexes. To investigate this possibility, expression of the exhaustion-related transcription factors BATF and NFATc1 was evaluated at 36 h after the encounter with pMHC-I (27). Analysis of the expression of these transcription factors after encounter with high versus optimal Ag dose shows that, after 36 h, these factors are not overexpressed/upregulated in T cells that came into contact with high Ag doses (Supplemental Fig. 2). These
findings may suggest that Ag-dose–induced hyporesponsiveness is an additional distinct state in memory CTLs.

**Differential phosphorylation of proximal TCR-signaling molecules**

We then explored the phosphorylation pattern of key TCR-signaling molecules as a function of pMHC-I dose on target cells. To this end, we used the phospho-flow strategy (28, 29). Five TCR proximal tier–signaling molecules, Lck, CD3ζ, ZAP70, LAT, and SLP-76, were analyzed in addition to three key inhibitory molecules, c-cbl, SHP-2, and CrkL, which are associated with negative feedback on the TCR (see Supplemental Table IB for phosphorylation sites detected by phosphor-flow Abs). These molecules were selected because of their high proximity to the TCR itself and because of the minimal influence of generic downstream-signaling cascades, such as MAPK, on their function.

We first determined the maximal phosphorylation levels of CD3ζ (pY142) under saturation levels of anti-CD3 Ab. The level of phosphorylation that was measured in multiple experiments using saturating levels of anti-CD3 Ab (“maximum phosphorylation”) was similar to those that were present after exposing CTLs to target cells with ~1200 pMHC-I complexes/cell, allowing us to define this Ag dose as the maximal TCR-signaling capacity. CTLs were cocultured with target cells that presented increasing doses of pMHC-I complexes (Fig. 4A). Upon Ag encounter, all eight analyzed molecules exhibited a dose-dependent increase in phosphorylation (Fig. 4B). Relative phosphorylation was defined as measured phosphorylation (MFI units)/maximal phosphorylation (MFI units). The relative phosphorylation exhibited a monotonic increase as a function of increasing Ag levels. However, the pattern demonstrated by the activation- and inhibition-related molecules was different; the activation-related molecules exhibited a higher (step-like) phosphorylation pattern at lower Ag levels, whereas the inhibition-related molecules were activated with a more moderate slope and at higher Ag levels. For example, the phosphorylation of LAT and SLP-76 increased sharply to >60% of the maximal phosphorylation after encountering a pMHC-I dose corresponding to ~100 complexes/cell (Fig. 4B). Similarly, the relative phosphorylation of ZAP70, CD3ζ, Lck, and LAT increased rapidly by 8–20-fold upon encounter with optimal pMHC-I dose (Fig. 4C). Compared with that, the inhibitory-related molecules exhibited a phosphorylation pattern that was more moderate and lagged behind the activation-related molecules (Fig. 4B–D). Thus, all of the activation-related molecules, most profoundly LAT and Lck, exhibited a sharp, “switch-like” increase in phosphorylation as the pMHC-I dose approached the optimal Ag dose, which resembles a digital activation or switch. In contrast, c-cbl, SHP-2, and CrkL exhibited a much more gradual increase in phosphorylation under the same conditions. These different patterns created an Ag window in which the activation-related molecules already were largely activated, whereas the inhibitory-related molecules were not. This window corresponded to 100–250 pMHC-I complexes/target cell, and, at this point, CTLs also demonstrated their maximal cytotoxic and functional activity. At higher Ag doses, the inhibitory molecules were fully recruited, causing a substantial decrease in the functional performance of T cells (Fig. 4B).

We used a mass cytometry approach (CyTOF) to confirm and further elaborate the phospho-flow data. This strategy, in which heavy metal isotopes are used to label Abs and then labeled cells are analyzed by high-throughput mass spectrometry (18, 30), allowed us to simultaneously analyze 36 parameters, both membrane and intracellular, at a single-cell level. CTLs and target cells presenting predefined pMHC-I Ag doses were cocultured for 2, 5, or 10 min and analyzed for the phosphorylation state of both TCR–related–signaling proteins and other canonical-signaling proteins. After 2 min of coculture, the TCR-machinery proteins displayed a modest change in phosphorylation when CTLs encountered a low number of pMHC-I molecules (Fig. 5A, 5B). However, similarly to the phospho-flow data, a sharp increase (up to 6-fold) in the phosphorylation of activatory-related proteins, especially Lck and SLP-76, was observed when CTLs encountered high numbers of pMHC-I complexes. Similar “switch-like” behavior was demonstrated by other canonical pathway–signaling molecules, such as Akt, MAPK, Erk, and MEK (Fig. 5A, lower left panel), for which the level of phosphorylation greatly increased after moving from 50 to 250 pMHC-I complexes. The increase in the phosphorylation of the inhibition-related molecules SHP-2 and c-cbl occurred after 5 min, whereas SHP-2 showed a clear dose-dependent increase in its phosphorylation, with a marked increase when the T cells encounter a greater-than-optimal number of pMHC-I molecules. A significant decline in phosphorylation was observed 10 min following CTLs' encounter with target cells; a 2–10-fold decrease in phosphorylation of the various signaling molecules was revealed by mass cytometry analysis (Fig. 5A, 5B).

Clustering analysis based on pMHC-I dose across all three time points demonstrated positive correlation between activatory molecules, such as CD3ζ, Lck, SLP-76, and NF-κB, as expected. In contrast, these activation-related molecules exhibited a low correlation with c-cbl and SHP-2 as a result of the different dose-dependent patterns (Fig. 5C).

The differential phosphorylation data that were measured using both phospho-flow and the CyTOF revealed qualitative differences in the incremental fold change in phosphorylation of the proximal TCR activatory- versus inhibitory-related molecules. In light of these results, we hypothesized that this lag between the phosphorylation patterns of the activatory- and inhibitory-related molecules may explain the nonmonotonic nature of pMHC-I–dependent killing by CTLs. Based on our experimental data, we generated a simplified in silico model (Fig. 6A) that is composed of two modules, inhibitory and activatory, and their level of activity as a function of the Ag dose that is
FIGURE 4. Ag dose-dependent phosphorylation of major T cell–signaling components. HLA-A2+ JY cells were pulsed with increasing doses of MART peptide, washed, and cultured with MART-specific CTLs for 5 min (Materials and Methods). (A) Graphs show the gradually increasing phosphorylation of key TCR components in response to increasing Ag doses. Yellow and blue indicate increased and decreased phosphorylation, respectively. Numbers indicate the amount of pMHC-I complexes on target cells. Changes in phosphorylation of signaling proteins following activation of T cells were calculated using the inverse hyperbolic sine (arcsinh) of the MFI, with the first row of each lane used as phosphorylation reference, as previously described (50). (B) Relative phosphorylation was defined as the measured phosphorylation level of a specific protein divided by its maximal phosphorylation level under Ag saturation (see Materials and Methods). Relative phosphorylation was calculated after 5 min, and the average of eight independent experiments is shown. At 100 pMHC-I complexes, a significant elevation in relative phosphorylation is exhibited in ZAP-70, SLP-76, and LAT compared with CrkL, SHP-2, and c-cbl. Data are means; error bars represent SE. (C) The relative phosphorylation among activation molecules is significantly higher than that of inhibitory molecules at 100 pMHC complexes/cell. The activation-related molecules CD3, LAT, and SLP-76 show a significant increase in their relative phosphorylation compared with the inhibitory-related molecules SHP-2, c-cbl, and CrkL. Data are means; error bars represent SEM. (D) Dose-dependent incremental change in phosphorylation. pMHC dose-dependent incremental fold change in phosphorylation was determined by measuring the relative phosphorylation MFI of the indicated pMHC dose and dividing it by the MFI measured at previous lower dose of pMHC-I complexes. The transition from 50 pMHC-I complexes to 100 complexes generates a sharp increase in the phosphorylation of LAT, Lck, CD3, and ZAP-70. *p < 0.05 **p < 0.01, unpaired Student t test.

Killing of MART27–35-presenting target cells by SHP-1/2 inhibits CTLs

The results presented above suggest a model in which a signal received by the TCR is simultaneously conveyed via two modes of signal regulation: activatory and inhibitory. The summation of the signals delivered from these two branches ultimately determines the extent of the CTL’s effector function. To validate this model, we tested whether neutralizing the effect of key members in the inhibitory branch enable a dose-dependent monotonic killing pattern. Because SHP-1 is known to downregulate TCR signaling (31, 32), and our results suggested the involvement of SHP-2 in the inhibitory module, we targeted SHP-1/2 phosphatases by introducing the inhibitor SSG (33, 34) to CTLs. CTLs were treated with SSG and subsequently tested in a standard cytotoxicity assay (Fig. 5C). Both control and treated CTLs reached optimal killing of target cells at ~100 pMHC-I complexes/cell. However, at higher pMHC-I doses, SSG-treated CTLs continued to lyse APCs efficiently, whereas control CTLs lost a substantial fraction of their effector function (p < 0.05). Importantly, at pMHC-I doses > 1000 complexes/cell, the control CTLs were inhibited by 60%, whereas their SSG-treated counterparts did not exhibit any inhibition of killing function. These data strongly support our signal-integration model and suggest that inhibition of protein phosphatases, which partake in TCR signal attenuation, can alter, and even reverse, the Ag-induced hypo-responsive phenotype of these effector T cells (Fig. 7).

Discussion

In this study, we sought to decipher the molecular mechanisms by which CTLs sense the number of cognate pMHC complexes on the surface of target cells and translate this information into a non-monotonous functional output.
We show that the machinery for gauging Ag dose cannot be explained by known mechanisms, such as changes in intracellular calcium or spatial localization of signaling molecules. It uses signal-integration processes rallied from two opposing machineries, activatory and inhibitory, which function concomitantly at the proximal TCR signal-transduction cascade. Using phospho-flow and cytometry time-of flight strategies that measure phosphorylation levels of key proximal TCR activation- and inhibition-signaling–molecules, we found that the integration between activatory- and inhibitory-signaling modules serves as the molecular mechanisms to gauge Ag dose. Ag dose $\sim 100$ pMHC-I complexes/cell is optimal, yielding maximal $\delta$ between the activatory- and inhibitory modules, which translates into an optimal cellular output. Higher Ag doses cause the $\delta$ between the activatory and the inhibitory modules to disappear, resulting in induction of anergy-like hyporesponsiveness. This mechanism permits T cells to make integrated “measurements” of Ag dose that determine subsequent T cell functional outcomes.

The TCR was shown to be a highly sensitive apparatus, capable of sensing minute numbers of pMHC-I complexes. T cells have been described as an autonomous sensory organ that must independently detect, evaluate, and respond to any encounter with nonself (35). We previously identified a nonmonotonic effector function of CTLs, influenced by the number of pMHC-I molecules presented on target cells (12). When a lymph node–primed CTL encounters a target cell, it either elicits an effective effector function or may become hyporesponsive or anergic. These opposing outcomes can be dictated by the number of cognate pMHC-I complexes presented on the target cell. Upon encountering target cells presenting up to $\sim 100$ pMHC-I complexes, CTLs efficiently mount a cytotoxic reaction toward these cells, secrete inflammatory cytokines, and proliferate in a dose-dependent manner. When encountering target cells presenting pMHC-I complexes at higher
Materials and Methods

CTLs for 5 h. Cytotoxicity was assessed as described in Methods. The δ of the relative phosphorylation calculated for the activatory and inhibitory molecules, shown in arbitrary units. (C) SHP1/2 inhibition in CTLs exposed to high numbers of presented pMHC-I complexes abolishes Ag dose-induced unresponsiveness/anergy. HLA-A2+ JY cells were radioactively labeled, pulsed with various concentrations of MART-1, and cultured with SHP-1/2–inhibited JKF6 CTLs for 5 h. Cytotoxicity was assessed as described in Materials and Methods. Error bars represent SEM. *p < 0.05, paired Student t test.

Our current study demonstrates that signal integration may play an important role in functional decisions of T cells, leading to mechanistically distinct Ag-induced hyporesponsiveness phenotypes in CTLs.

Two major assumptions guided our work: 1) the decreased cytotoxicity and hyporesponsiveness are TCR dependent, inferred from the pMHC-specific nature of response by CTLs, and 2) Ag-dose input, and the decision made based on this calculation, occurs soon after the encounter with a target cell. The second assumption was based on the short time after which cellular output, expressed as diminished cytotoxicity at high pMHC complex numbers, is observed (11). Hyporesponsiveness is not likely to be explained by de novo transcription and expression of proteins in this short period of time; therefore, we focused on transcription-independent changes occurring minutes after the encounter between CTLs and cognate pMHC-I complex.

Anergy is generated as a result of incomplete activation of the T cell (42) or in an inhibitory environment (43). The latter form, commonly referred to as “adaptive tolerance,” was shown to cause desensitization of TCR signaling in an environment high in cognate Ag (44). Both types of anergy result in the loss of proliferation and IL-2 secretion, as well as alterations in effector functions (IFN-γ secretion, cytotoxicity) (8). Two extensively studied mechanisms responsible for the onset of anergy include a perturbation in the calcium-signaling pathway (lack of PLCγ activation or abrogation of intracellular increase in calcium) (8, 45) and upregulation of the PD-1 molecule. Our results show that hyporesponsive CTLs exhibit no abrogation in intracellular calcium levels following contact with high doses of pMHC-I complexes. In addition, these CTLs express low levels of PD-1 compared to expression levels described in other models (13, 24, 41). In fact, despite the decline in target cell lysis at high Ag doses, intracellular calcium levels constantly increase in a dose-dependent manner, suggesting the proper function of PLCγ. Calcium signaling was shown to facilitate cytoskeleton rearrangement and the secretion of lytic granules (46–49); the mechanisms associated with blocking these calcium-mediated processes in hyporesponsive CTLs have not been determined.

Our data suggest that, in this system of memory-activated CTLs, unresponsiveness is not the result of T cell exhaustion, which is a different state of unresponsiveness that exhibits a distinct set of

FIGURE 6. Integration of activatory and inhibitory signals. (A) An average of the relative phosphorylation of the activatory molecules (ZAP70, Lck, LAT, SLP-76) and the inhibitory molecules (SHP-2, c-cbl, CrkL) was calculated based on the experimental phospho-flow data obtained in 10 independent experiments. (B) The δ of the relative phosphorylation calculated for the activatory and inhibitory molecules, shown in arbitrary units. (C) SHP1/2 inhibition in CTLs exposed to high numbers of presented pMHC-I complexes abolishes Ag dose-induced unresponsiveness/anergy. HLA-A2+ JY cells were radioactively labeled, pulsed with various concentrations of MART-1, and cultured with SHP-1/2–inhibited JKF6 CTLs for 10 independent experiments. (B) Averages of the relative phosphorylation determined. (D) An average of the relative phosphorylation calculated based on the experimental phospho-flow data obtained in 10 independent experiments. *p < 0.05, paired Student t test.

FIGURE 7. Model for activatory and inhibitory signal summation by effector T cells. Upon interaction between a CTL and a target cell, Ag recognition by TCRs induces the phosphorylation of the ITAM of CD3 molecules, particularly the CD3ζ chains, by Lck kinase, leading to recruitment of ZAP70 and the subsequent activation of LAT and SLP-76. Simultaneously, inhibitory molecules are activated via phosphorylation. Below a threshold of 100 presented pMHC-I complexes, a relative activation of activatory molecules “outweigh” the net effect of the inhibitory molecules, and the intracellular balance and signal integration between these opposing signaling cascades is translated to an effector phenotype. When the number of presented pMHC-I complexes increases beyond this threshold, the relative phosphorylation of the inhibitory molecules increase, and the integration of signals generates an anergic phenotype.
genes and is dependent on PD-1 (27). Hebeisen et al. (50) recently reported a mechanism behind the regulation of responses to TCRs of different affinities. They also observed an increase in SHP-1 expression and activation that downregulates responses in CTLs bearing very high-affinity TCRs. They also reported increased expression of PD-1. Our findings, in which Ag-induced unresponsiveness is dependent on SHP-1 activation but is independent of PD-1 expression, may exemplify the differences between the mechanisms that may regulate responses to increased pMHC densities compared with increased TCR affinity.

Our results might suggest that calcium signaling does not play a direct role in the observed Ag-induced unresponsiveness, but they do not appear to exclude its involvement. Additional experimental data would be required to prove so.

In addition to the unimpaired calcium pathway, Ag-induced hyporessive CTLs do not exhibit diminished TCR phosphorylation or defects in the phosphorylation of proximal-signaling proteins conveying the signal from the receptor into the cell (ZAP70, LAT, SLP-76). Furthermore, our gene-expression analysis showed no altered expression of genes previously shown to be closely related to the onset of anergy (26). Last, the localization of Ras/RasGRP1 to the Golgi/plasma membrane was not affected by the number of cognate pMHC-I complexes encountered by CTLs. This mechanism was found to be of importance for inducing anergy through high-affinity binding by the TCR and the binding of a ligand presented in high numbers (25). Combined, our findings suggest that this Ag-dose induced hyporessiveness in CTLs is a distinct form of anergy, whose onset is mechanistically different from the currently described anergic phenotypes or mechanisms shared with thymic selection.

We did not detect any nonmonotonic behavior in any of the examined components. Therefore, we hypothesized that the nonmonotonic nature of the hyporessiveness stemmed from the balance between two separate machineries: one generating an activation signal and the second attenuating it. To investigate these relationships, we used both phospho-flow and CyTOF, which enabled us to perform multiparametric analysis of phosphorylated residues of key molecules within a heterogeneous cell population (18, 30, 51–53). The analyzed proteins encompassed the majority of the TCR pathway—the immediate TCR-interacting molecules associated with signal propagation or attenuation, as well as canonical downstream-signaling molecules. Following TCR triggering with suboptimal pMHC-I doses, proteins associated with TCR activation (Lck, CD3ζ, ZAP70, LAT, and SLP-76) are phosphorylated as expected. However, CrKL, c-cbl, and SHP-2 show a diminished increase in phosphorylation under these conditions. Upon crossing a threshold ~100 pMHC-I complexes, a sharp increase is induced in the phosphorylation of both TCR machinery proteins and the inhibitory cohort of proteins, resembling a digital switch. This switch-like phosphorylation is conveyed downstream and exhibited by members of canonical-signaling pathways. Thus, a pMHC-I dose-dependent lag in activation of the inhibitory molecules is generated; as the number of encountered pMHC-I increases, the phosphorylation of the inhibitory molecules increases, and the δ between the activatory and inhibitory modules diminishes, translating into a hyporessive state of the CTL. Abrogation of the negative signal by SSG, which is known to inhibit the key inhibitory phosphatases SHP-1 and SHP-2 via binding to their active site (33, 34), decreased the relative weight of signal attenuators and reversed the hypo-responsiveness state of CTLs. Of note, although SSG was shown to specifically inhibit SHP-1 and SHP-2, low levels of nonspecific binding to other phosphatases cannot be ruled out and might cause pan-phosphatase inhibition, which may result in a deficient cell activity. Our results show a marked increase in the killing activity of SSG-treated CTLs compared with their nontreated counterparts, despite possible nonspecific inhibitor activity. The induction of killing activity in SSG-treated CTLs, despite their exposure to high Ag doses, can be looked at as analogous to gain of function. This strongly supports our model, suggesting that inhibition of the negative-signaling module with all its constituents, and not the inhibition of a sole negative factor, affects the signal integrated by the CTL.

It was shown that T cell–activation molecules, such as Lck (54) and CD3ζ, are constitutively activated, but their effect on downstream-signaling molecules is inhibited by either sequestering the molecule through the signaling machinery (55) or by the constitutive attenuation by phosphatases (56). We suggest that the rapid increase in the relative activation of the activatory molecules (Fig. 4) is due to this already triggered state of the CTL's activatory machinery, which is kept at bay by constitutive attenuators. Activation violates the attenuation steady-state, and the cell is shifted to a preferred activated state. In addition, we and other investigators showed that Ag density, and not quantity, is the decisive factor in activation of a T cell and that the minimal number of pMHC-I complexes/TCR cluster required for the activation is approximately four (24, 57). Our results suggest that, in addition to this minimal activation requirement, maximal cluster activation might exist, as well. Thus, although low numbers of pMHC-I complexes result in few activated TCR clusters, which shifts the balance in favor of activation, a high number of pMHC-I complexes translates to increased density, which generates more activated TCR clusters until the balance between activatory and inhibitory signaling is shifted toward the induction of unresponsiveness.

A possible mechanism for this differential phosphorylation of the two modules might use differences in intracellular localization: although activatory molecules were shown to be membrane associated, the localization of SHP1/2 was shown to be at least partially compartmentalized (58). The translocation of such molecules may contribute to the lag observed in the relative phosphorylation increase. Alternatively, the affinities of the individual molecules operating in this signaling cascade may vary. Thus, the affinity of Lck for ZAP70 and of ZAP70 for SLP-76 and LAT might be higher, causing substantial substrate phosphorylation after T cell encounter with a few pMHC-I complexes. A lower affinity toward SHP1/2 and E3 ligases allows their activation only after an encounter with a higher number of MHC-I complexes, creating the lag in module activation. Such biochemical properties are yet to be determined.

The physiological significance of this study may be correlated with the findings of Michaeli et al. (59), who demonstrated that a tyrosinase-derived class I HLA-A2 peptide is presented at several thousand complexes/cell. In light of our results, such a dose induces anergy and tolerance, which might explain the low frequency of tyrosinase-specific CTLs in tumor-infiltrating lymphocytes isolated from melanoma patients (60). Given that memory CTLs require less high Ag doses, can be looked at as analogous to gain of function.

In summary, we suggest a model for the Ag-dose–sensory machinery of effector CTLs (Fig. 7). Upon interaction between a CTL and a target cell, TCR microclusters are formed, containing 10–100 TCRs (63, 64). Ag recognition by TCRs induces the phosphorylation of the ITAM of CD3 molecules, particularly the CD3ζ chains, by Lck kinase, leading to recruitment of ZAP70 and the subsequent activation of LAT and SLP-76. Simultaneously, inhibitory molecules are activated via phosphorylation, with the most prominent being the phosphorylation of SHP-1 by the same Lck kinase (56). The integration of these two opposing signaling modules yields the
nonmonotonic effector function and a corresponding functional phenotype of these CTLs (i.e., potent killing of target cells at optimal Ag doses and hyporesponsiveness at a high dose of pMHC-I complexes on target cells). Thus, we suggest that TCRs can sense Ag dose through the intracellular balance and signal integration between activatory- and inhibitory-related signals. This sensing machinery dictates the final functional outcome of these CTLs, whether optimal effector functions or hyporesponsiveness/anergy.

Disclosures

The authors have no financial conflicts of interest.

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**Supplementary Figure 1**: (A) Outline of peptide pulsing of JY cells and quantitation of presented pMHC-I complexes. The high affinity (∼50 nM) TCR-like Fab Ab CLA12 was incubated with JY APCs pulsed with MART-1_{27-35} peptide concentrations ranging from 500 μM to 100 pM. PE-labeled secondary mAb specific to the λ light chains of the TCR-like Ab was used to create a 1:1 ratio between the TCR-like Fab fragment and the PE-labeled secondary mAb. MFI was measured by flow cytometry under specific setting and compared to the MFI, under identical setting, of calibration beads with known predefined numbers of PE sites. Such calibration curve of PE-derived MFI for 0–10,000 PE-labeled molecules was generated using the QuantiBright PE kit (BD). The MFI measurements obtained for each peptide-loading concentration/dose was used to extrapolate the absolute number of specific HLA-A2-MART-1 complexes presented on the surface of the APC.
Supplementary Figure 2: Expression of exhaustion-related genes in hypo-responsive CTLs. Gene array analysis of anergy-related genes. Gene expression in CTLs was determined and the ratio between expression upon encountering high- and optimal antigen dose was calculated. Analysis was performed 36 h after encountering the target cells. Analyzed genes show no elevated expression at high pMHC-I doses.
Supplementary Figure 3: SLP-76 and LAT phosphorylation increase in response to pMHC-I dose on APCs (A). CTLs were cultured with peptide-pulsed APCs for 5 min. The heterogeneous cell culture was pelleted, lysed, and probed by Western blotting using anti-LAT, anti-phospho-LAT (pY226), and anti-phospho-SLP-76 (pY173) antibodies. Densities of the bands were quantitated by using NIH IMAGEJ software (B). To control for loading variability, values were normalized based on the dose of LAT bands. Results are average of 5 independent experiments. Error bars represent SEM. *, P <0.05 (paired-student’s T-test).
### Table 1a: Antibodies used in CyTOF analysis

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(CST – Cell Signaling Technologies)

### Table 1b: Antibodies used in FACS & Phospho-Flow analysis

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(Figure 4)