Title: HDAC6 regulates the dynamics of lytic granules in cytotoxic T lymphocytes.

Authors: Norman Núñez-Andrade1,2, Salvador Iborra3, Antonio Trullo4,5, Olga Moreno-Gonzalo1,2, Enrique Calvo6, Elena Catalán7, Gaël Menasche8, David Sancho3, Jesús Vázquez6, Tso-Pang Yao9, Noa Beatriz Martín-Cófreces1,2,#, Francisco Sánchez-Madrid1,2,#.

Affiliations: (1) Servicio de Inmunología, Hospital Universitario de la Princesa, UAM, IIS-IP. Madrid, 28006 Spain. (2) Laboratory of Intercellular communication, (3) Immunobiology of inflammation, (4) Microscopy and Dynamic Imaging Unit and (6) Proteomic Unit. Fundación CNIC, Madrid, 28029 Spain. (5) Center of Experimental Imaging, Ospedale San Raffaele, Milan, Italy. (7) Dept. Biochemistry and Molecular and Cell Biology, Universidad de Zaragoza, Spain. (8) Laboratory of Normal and Pathological Homeostasis of the Immune System, INSERM Unité Mixte de Recherche 1163, Paris France (9) Departments of Pharmacology and Cancer Biology Duke University, Medical Center, Durham, North Carolina 27710.

# These two authors contributed equally.

Contact information:
Francisco Sánchez-Madrid
Laboratorio de Comunicación Intercelular
Servicio de Inmunología
Hospital de La Princesa
Diego de León 62. 28006, Madrid. Spain
fsmadrid@salud.madrid.org
Phone: +34915202307
FAX: +34915202374
Abstract

HDAC6 is a tubulin deacetylase involved in many cellular functions related to cytoskeleton dynamics including cell migration or autophagy. In addition, HDAC6 affects antigen-dependent CD4+ T cell activation. In this study, we show that HDAC6 contributes to the cytotoxic function of CD8+ T cells. Immunization studies revealed defective cytotoxic activity in vivo in the absence of HDAC6. Adoptive transfer of wild-type or hdac6−/− CD8+ T cells to rag1−/− mice demonstrated specific impairment in CD8+ T cell responses against vaccinia infection. Mechanistically, HDAC6-deficient cytotoxic T lymphocytes (CTL) showed defective in vitro cytolytic activity related to altered dynamics of lytic granules, inhibited kinesin 1 – dynactin mediated terminal transport of lytic granules to the immune synapse and deficient exocytosis, but not target cell recognition, TCR activation or IFNγ production. Our results establish HDAC6 as an effector of the immune cytotoxic response by affecting the dynamics, transport and secretion of lytic granules by CTL.
Introduction

Cytotoxic T Lymphocytes (CTLs) are a specialized population of CD8+ T cells that provides defense against virus-infected cells and tumors. Naïve CD8+ T cells differentiate into CTLs upon antigen recognition, a process involving the synthesis and storage of cytotoxic mediators into lysosomal-derived lytic granules (LG) (Williams and Bevan, 2007). CTLs eliminate target cells by different mechanisms, including secretion of pro-inflammatory cytokines, e.g. TNFα or IFNγ (de Saint Basile et al., 2010), FAS-L ligation to its receptor as well as granule-mediated apoptosis upon cell-cell contact and immune synapse (IS) formation (Ritter et al., 2013). LG fuse with the plasma membrane and release granzymes, cathepsins and perforins (prf) (Lopez et al., 2013; Pardo et al., 2009). The IS acts as a focal point for exocytosis of LG. LG polarization towards the target cell depends on TCR engagement, driven by the relocation of the centrosome to IS. The LG degranulate at a secretory domain adjacent to the TCR-enriched region within the IS (de Saint Basile et al., 2010; Ritter et al., 2013).

Histone deacetylase 6 (HDAC6) is an ubiquitous, cytosolic protein from the class II HDACs family with X-linked inheritance, that binds to and deacetylates α-tubulin at lys40 (Hubbert et al., 2002; Valenzuela-Fernandez et al., 2008). HDAC6 also modulates other substrates, e.g. cortactin or hsp90. HDAC6 controls cell migration (Zhang et al., 2007), T-regulatory functions (de Zoeten et al., 2011) or CD4+ T cell activation (Serrerador et al., 2004). Consistently, HDACs inhibitors impair some immune functions (Mosley et al., 2006; Tsuji et al., 2015). However, the precise contribution in vivo, by using hdac6−/− mice, and the mechanisms involved remain unsolved. HDAC6 also functions as a scaffold protein in T cell migration (Cabrero et al., 2006) and the transport of misfolded proteins (Kawaguchi et al., 2003). In this report, we describe the impaired killing capacity of hdac6−/− CTLs. The molecular mechanism underlying this defect involves a scaffold role that positions HDAC6 as a protein that oversees the proper movement of LG, their transport to the IS and secretion towards the target cell.
Results and Discussion

**HDAC6 deficiency reduces the cytolytic capacity of CD8+ T lymphocytes.** We examined the ability of cytotoxic T cells from *hdac6*−/− mice to kill target cells *in vitro*. CD8+ T cells from wild-type (WT) and *hdac6*−/− mice expressing the transgenic OVA-specific TCR (OT-I) were *in vitro* activated and cultured to generate CTLs. Cell cytotoxicity was subsequently analyzed by survival of dye-labeled EL4 target cells pulsed or not with OVA257-264 peptide (SIINFEKL). *hdac6*−/− CTLs showed decreased killing activity (Fig. 1A), consistent with reduced expression of CD107a (lamp1) in *hdac6*−/− CTLs upon degranulation (Fig. 1B). Likewise, CTLs from OT-I mice treated with the HDAC6 inhibitor tubastatin A displayed a reduced killing ability (Suppl. Fig. 1A). We also detected decreased Prf1 secretion from activated (α-CD3+α-CD28 mAbs) *hdac6*−/− CTLs(Fig. 1C, left). Next, we assessed the secretion promoted by phorbol-12-myristate-13-acetate (PMA), to bypass TCR stimulation. Both CathepsinD and Prf1 decreased in supernatants from activated *hdac6*−/− CTLs (Fig. 1C, right). Altogether, our data demonstrate that *hdac6*−/− CTLs show reduced cytotoxic activity, and suggest that HDAC6 controls exocytosis.

We next tested IFN-γ production; the frequency of CTLs producing IFNγ and its secretion was unaffected in activated *hdac6*−/− CTLs (Fig. 1D-E), in contrast to what is described for ACY-1215, a recently described HDAC6 inhibitor, 10-fold more selective than for HDAC1/2/3 (class I HDACs) that shows slight activity against HDAC8 (Tsuji et al., 2015). Likewise, treatment of CTLs from OT-I mice with tubastatin A had no significant effect (Suppl. Fig. 1B). Importantly, T-cell signaling induced by anti-CD3/-anti-CD28 mAbs in *hdac6*−/− was comparable to control, as determined by PLCγ1 and erk1/2 phosphorylation (Fig. 1F). Likewise, the increase in intracellular calcium remained unchanged (Fig. 1G). As expected, tubulin acetylation at Lys40 was increased in *hdac6*−/− CTLs (Fig. 1F). These results suggest that the killing defect observed does not result from a general impairment of CTLs function.

**Defective *in vivo* and *ex vivo* killing in HDAC6 knockout mice.** The effector activity of *hdac6*−/− CD8+ T cells was tested *in vivo* following mouse immunization using SIINFEKL-pulsed dendritic cells. The *in vivo* killing activity against injected target cells (pulsed or not with SIINFEKL) was analyzed upon recovery by peritoneal lavage. Notably, *hdac6*−/− mice showed reduced specific killing of target cells (Fig. 2A, left
However, the frequency of SIINFEKL-specific CD8$^+$ T cells from the endogenous repertoire was not affected in $hdac6^{-/-}$ mice (Fig. 2A, right panel), suggesting that the cytotoxic function rather than the number of antigen-specific CTLs could underlie the defect. Next, we examined whether their decreased cytotoxic function resulted in an impaired ability to prevent morbidity/mortality during a viral infection. To restrict HDAC6 deficiency to CD8$^+$ T cells, we adoptively transferred $rag1^{-/-}$ mice with WT or $hdac6^{-/-}$ naïve CD8$^+$ T cells and subsequently challenged with a fully replicative vaccinia virus (VACV) WR strain. This infection model mimics the immunological and clinical features of smallpox vaccination in humans (Mota et al., 2011). CD8$^+$ T cell proliferation was comparable, or even increased (division 4) in $hdac6^{+/+}$ (Fig. 2B). $rag1^{-/-}$ mice passively-transferred with $hdac6^{-/-}$ CD8$^+$ T cells showed increased morbidity at days 9 and 11 post-infection (p.i.) (Fig. 2C). Virus titration from the lesion tissue demonstrated that $hdac6^{-/-}$ immune cells exerted less efficient virus clearance (Fig. 2D). Consistent with our findings on the lack of effect in endogenous antigen-specific CD8$^+$ T cell numbers, CTL expansion tracked at day 13 p.i. was not affected in $hdac6^{-/-}$ (Fig. 2E, left). The proportion of activated CD8$^+$ T cells (CD44$^{high}$) at early (5 d.p.i) and late stages (13-30 d.p.i) of the disease were similar for WT and $hdac6^{-/-}$ mice (tested in peripheral blood or spleen, respectively; Fig. 2E, right).

These in vivo results emphasize the role of HDAC6 in the CD8$^+$ T cell-dependent protection against VACV infection without affecting effector CD8$^+$ CTLs differentiation.

**HDAC6 drives the terminal transport of LG to the target cell.** CTL killing is limited to target cells (and not neighbor cells) by the confinement of secretion to the immune synapse established between the CTL and the target cell (de Saint Basile et al., 2010). Interestingly, cathepsinD and lamp1 (CD107a) intracellular co-localization was affected in $hdac6^{+/+}$ CTLs conjugated with target cells, pointing to the mislocalization of lytic mediators in these cells (Fig. 3A; images and middle graphs). The decreased secretion of lytic proteins from $hdac6^{+/+}$ CTLs suggested that HDAC6 regulates exocytosis of LG (Fig. 1C). Indeed, the translocation of centrosome to the contact area with the target cell was even more pronounced in $hdac6^{+/+}$ CTLs (Fig. 3A, right graph), in accordance with the effect described with the HDAC inhibitor Trichostatin A on the centrosomal polarization in CD4$^+$ T cells (Serrador et al., 2004). This suggests that the defective exocytosis may rely on the movement of LG themselves.
We thus monitored the dynamics of LG at the IS-subcortical cytoskeleton and their release by Total Internal Reflection Fluorescence microscopy (TIRFm). CTLs were loaded with a pH-dependent, lysosomal tracker that allows the visualization of the LG and settled on a stimulating surface to form an IS-like structure (Fig. 3B). These experiments revealed significant changes in the distribution of the LG and their dynamics, with a marked decrease in the number of LG detected at the IS-like in $hda\text{c6}^{-/-}$, suggesting alterations to the active transport of the granules from the centrosomal region to the plasma membrane. Indeed, the mean fluorescence intensity detected for $hda\text{c6}^{-/-}$ granules was lowered, which suggest a higher pH and therefore a different degree of maturation, although the LG displayed similar sizes in WT and $hda\text{c6}^{-/-}$ (Fig. 3C). The most remarkable difference pertained to the xy distribution of the LG, which was wider (diffusion surface) in the $hda\text{c6}^{-/-}$ CTLs, showing enlarged diffusion coefficient though maintaining similar duration times and path lengths (Fig. 3D). These data suggest that the LG from $hda\text{c6}^{-/-}$ CTLs are not properly targeted and/or that they dock deficiently at the IS.

Tubulin motors control the delivery of LG to the plasma membrane. Whereas dynein controls LG targeting to the centrosome (Burkhardt et al., 1993; Mentlik et al., 2010), kinesin-1/Slp3/Rab27a complex directs terminal transport to the plasma membrane for exocytosis (Kurowska et al., 2012). Dynactin may also be part of this complex, linking the cargo to kinesin-1 motor (Haghnia et al., 2007; Hendricks et al., 2010). We then hypothesized that HDAC6 regulates the movement and delivery of the LG at the IS through kinesin-1. Using a biochemical approach, we observed that HDAC6 formed a complex with kinesin-1 light chain (KLC1) upon triggering with anti-CD3/anti-CD28 mAbs (Fig. 3E). Moreover, interaction of the kinesin-activator complex dynactin subunits p150-glued and p50-dynamitin was impaired in $hda\text{c6}^{-/-}$ (Fig. 3F).

In summary, our data support a specific role for HDAC6 in the intracellular localization of lytic mediators and, particularly, in their exocytosis. Therefore, the catalytic and scaffold activities of HDAC6 may act at multiple levels in the control of cytotoxic-related pathways, making HDAC6 a potential candidate to target CTLs in specific diseases.
Materials and Methods

Mice. *hdac6*/* mice were generated through targeting of exons 10 to 13 (Gao et al., 2007). They were intercrossed in a C57BL/6 background to generate wild-type and knockout littermates. TCR(Vα2, Vβ5) transgenic mice (OT-I) were crossed to female *hdac6*/*+*/mice to generate WT and KO littermates; males were used for in vivo experimentation since *hdac6* is a X-linked gene. *rag1*/*−/−* mice were used for adoptive transfer experiments. These studies were performed according to the principles of the Declaration of Helsinki and approved by the local Ethics Committee for Basic Research at the CNIC and the Comunidad Autónoma de Madrid.

Cell culture. Cytotoxic cells were produced by culturing cells upon stimulation with SIINFEKL peptide (0.5 µM, 24h) or Concanavalin A (2.5 µg/ml, 36 h) and cultured in presence of IL-2 (50-100 IU/ml) for at least 7 days. All other cells were cultured and treated as described (Cascio et al., 2015; Martin-Cofreces et al., 2006; Sancho et al., 2008).

Immunoprecipitation, CTL signaling and immunoblotting. Experimentation was performed as described (Martin-Cofreces et al., 2012; Martin-Cofreces et al., 2008; Martin-Cofreces et al., 2006). KLC1 antibody was from Merck Millipore (Darmstadt, Germany; KLC), anti-HDAC6 from Assay Biotech (Sunnyvale, California, US) and anti-p50 and -p150 from BD Pharmingen (Franklin Lakes, New Jersey, US).

Measurement of intracellular variations in Calcium ions by flow cytometry. The method used for intracellular calcium influx is described (June and Moore, 2004). In particular, 5x10⁶ purified CD8⁺ CTLs generated in vitro were loaded with 2 µg.ml⁻¹ INDO-1 AM (Invitrogen Corporation) and stimulated with anti-CD3/anti-CD28 (BD Biosciences; Franklin Lakes, New Jersey, US) + anti-Armenian Hamster antibodies (Jackson Immunoresearch Laboratories; West Grove, PA, US. 6, 3 and 6 µg/ml, respectively).

In vitro degranulation assay. CD107a expression was monitored with anti-CD107a-Alexa647 antibody (BD Biosciences) in monensin-pretreated CD8⁺ OTI cells (5 mM) stimulated with SIINFEKL-pulsed EL4 (1 µM; 3 h, 37 °C). Cells were stained with anti-CD8-PE and anti-CD44-FITC, analyzed by FACS and data processed with FlowJo 7.6.5 (TreeStar Inc; Ashland, Oregon; US).
Confocal and Total Internal Reflection Fluorescence Microscopy analysis. Cell conjugates between CTLs and EL4 cells were allowed to form (15 min) and processed as described (Cascio et al., 2015; Martin-Cofreces et al., 2006) under a Leica SP5 confocal microscope (Leica Microsystems; Manheim, Germany) mounted on an inverted DMI6000 microscope fitted with a HCX PL APO 63x/1.40-0.6 oil objective. Images were processed using Imaris software (Bitplane; Zurich, Switzerland), Image J software (http://rsbweb.nih.gov/ij/) and assembled with Photoshop 6 software. 3D distance from the centrosomal centre of mass to the target cell-edge was measured by generating image masks from fluorescence with Imaris Software. TIRFm imaging was performed with a Leica AM-TIRF-MC-M system mounted on a Leica DMI-6000B microscope coupled to an Andor-DU8285_VP-4094 camera (Andor; Belfast, UK) fitted with a HCX-PL-APO 100.0x1.46 OIL objective as described (Baixauli et al., 2011; Martin-Cofreces et al., 2012). The laser penetrance used was 90 nm (561 nm laser). The LG mechanical properties were determined with a user-customized routine developed in Python. The software can be freely downloaded from: https://dl.dropboxusercontent.com/u/4050954/VesiclesAnalyser.zip. For more information, see the tutorial included.

Vaccinia virus (VACV) infection and virus titration. Tails were scarified with VACV (2x10^6 PFU/mouse) by gently scratching (x25) with a 28 1/2 G needle. For virus titration, tails were mechanical disaggregated (1ml of PBS), subjected to freeze-thaw cycles and sonication. Serial dilutions of the homogenates were added to monolayers of CV-1 cells seeded in 24 well plates. Cells were stained with Cristal violet 24 h later. We observed a detection limit of 5 PFU/tail, the number of plaques was multiplied by the reciprocal of sample dilution and converted to p.f.u./g of tissue.

In vitro and in vivo cytotoxicity assay. For in vitro experiments, EL4 target cells were incubated with 1 µM Cell Violet pulsed with 1 µM SIINFEKL or with 0.1 µM Cell Violet and no SIINFEKL, washed extensively, mixed (1:1), pooled with different dilutions of effectors, plated in a 96-well U-bottom plate for 5 h (37ºC) by triplicate and analyzed by FACS. Dead cells were excluded with Propidium iodide. The mean percentage of survival in antigen-loaded targets was calculated relative to antigen-negative internal controls in each sample. Specific lysis was calculated using the following equation: percentage specific lysis = 100 * (1 - (% Cell Violet 1µM/% Cell Violet 0,1µM)). All data were normalized to the basal specific lysis in absence of
effector cells. For in vivo assays, WT and hdac6−/− mice were immunized by i.p. injection of bone marrow dendritic cells pulsed with 1 µM of SIINFEKL and LPS (1 µg/ml) for 1 h. After 7 days, CD45.1 splenocytes were prepared as targets as described above and injected i.p. into recipients. Cells were recovered 24 h later by peritoneal lavage and in vivo killing measured (Hermans et al., 2004; Iborra et al., 2012; Sancho et al., 2008; Schulz et al., 2005).

**Statistical analysis.** Data were tested with GraphPad Prism software (La Jolla, California, US) for normality (D’Agostino-Pearson or the Kolmogorov-Smirnov test for small samples). Student’s t or Mann-Whitney test were used for normal or non-normal data, respectively and two-tailed ANOVA for grouped data (Bonferroni posttest).

**Acknowledgements.** We thank Manuel Gomez and Miguel Vicente for critical reading of the manuscript. Experimentation was performed at Cellomics and Microscopy Units (CNIC) and Flow Cytometry Core Unit (CNIO). This work was supported by MINECO [SAF2014-55579-R], CAM [INDISNET01592006], [BIOMID-PIE13/041; RD12/0042/0056] from IS Carlos III and FEDER and ERC-2011-AdG 294340-GENTRIS. CNIC is supported by MINECO and Pro-CNIC Foundation.

**Author contribution**

NNA, NBMC and FSM designed experimentation, made the figures and wrote the manuscript; NNA, SI, NBMC, OMG, JV, DS, GM and TSY and EC collected and/or analyzed data; AT developed the Quant Application.

**Conflict of interest**

Authors declare that they have no conflict of interest.
References


Figure 1. HDAC6 modulates the efficiency of target cell killing and degranulation of lytic mediators. (A) Graph showing in vitro cytotoxic assay for specific lysis of
SIINFEKL-pulsed EL4 target cells by OT-I-WT or -hdac6−/− CTLs. Mean ± SEM of specific lysis for 5 h are shown at the indicated target to effector ratios. All killing assay where performed by triplcate. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Unpaired T-test (n=5 mice for each genotype). Histograms, representative FACS profile. (B) Degranulation is shown as the percentage of CD107a+ cells detected by FACS in activated vs non-stimulated WT and hdac6−/− CD8+ CTLs. (C) Exocytosis of lytic mediators upon T cell activation. Left, Perforin content in culture cell supernatants was determined by ELISA. CTLs were stimulated with α-CD3+α-CD28+α-Armenian Hamster antibodies (5 h). Graph, Mean +/- SEM. Unpaired T test (n=6 mice for each genotype). Middle panel, Western blot showing exocytosis. PMA stimulation (1 h). Normalization was performed against cell fractions. Right graphs, Mean +/-SEM (3 independent experiments). (D) Graph, IFNγ secretion supernatants from anti-CD3/anti-CD28 mAbs activated WT and hdac6−/− CTLs by ELISA. (E) Percentage of IFNγ+ cells in activated WT and hdac6−/− CTLs (anti-CD3/anti-CD28 mAbs). (F) Effector CD8+ CTLs isolated from WT and hdac6−/− mice were activated (anti-CD3/anti-CD28 mAbs), lysed and blotted against PLCγ1 and erk1/2 phosphorylation and tubulin acetylation. (G) Calcium flux. WT and hdac6−/− purified CTLs pre-loaded with the INDO-1 AM probe were analyzed for Ca2+-free and Ca2+-bound by flow cytometry. Left graphs, time course ratiometric variation (left Y axis). Black line, Median function normalized to basal (0%) and total activation by ionomycin treatment (100%; right Y axis). Stimuli are indicated. A representative experiment is shown. Right graph, Mean of the increase in the ratio +/-SD (WT, n=7; hdac6−/− n=6. Mann Whitney test).
Figure 2. In vivo function of CTLs is impaired in HDAC6 knockout mice. (A) WT and hdac6⁻/⁻ were immunized against SIINFEKL peptide. 7 days after immunization,
cell violet-labelled, SIINFEKL-pulsed or not target cells (1 μM) were injected i.p., recovered through intraperitoneal lavage (24 h) and cell survival assessed by FACs (left panel). Relative percentage of CD8+ H-2Kb+ was determined to control avidity towards SIINFEKL (right panel; n=5 from 3 independent experiments). (B) Proliferation of CD8+ cells in VACV-OVA infected WT and hdac6−/− mice. (C) rag1−/− mice inoculated i.v. with 0.8x10^6 CD8+ naïve cells and infected with VACV-WR by tail scarification were weighted every 2 days. Graphs, means +/- SEM. (D) Titration of viral particles from scarified tails (13 d.p.i). The colonies of CV-1 cells infected in vitro with different dilutions of tail extracts were counted, and normalized to the tail tissue weight. (E) Percentage of CD8+ expansion and CD44 expression by FACs analysis in peripheral blood (5 d.p.i) and in spleen populations (13 and 30 d.p.i.). CD11b was used as negative control.
Figure 3. HDAC6 drives the terminal transport of LG to the target cell. (A) Confocal microscopy images of WT and hdac6−/− OT-I-derived CTLs conjugated with CMAC-loaded, OVA pre-pulsed target cell EL4 (500 nM SIINFEKL, Cyan) for 15 min. Green, CD107a. Red, CathepsinD. Magenta, α-tubulin. BF images correspond to a unique plane and fluorescence images, to maximal projections from Z-stacks. Middle graphs, Mander’s coefficient for co-localization of Lamp1 and CathepsinD. Mann-Whitney test (WT, n=39; hdac6−/−, n=35). Right graph, quantification of MTOC translocation (n>270, from 3 independent experiments); Unpaired T-test. (B)
Representative TIRFm images of Lysotracker red-loaded WT and $hdac6^{-/-}$ CTLs in anti-CD3/anti-CD28 mAbs-coated glass-bottom chambers. Video recording was initiated upon cell adhesion (37°C and 5% CO$_2$). Images were acquired for 5 min every 0.5 s. Bars, 4 µm. (C-D) Quantification for LG parameters at the IS-like was performed for each cell ($n=28$ for each genotype, 3 independent experiments). (E-F) Western blots showing immunoprecipitates from resting or activated WT and $hdac6^{-/-}$ CTLs (15 min) from a representative experiment out of three. Antibodies anti-KLC1 (E) or anti-p150 glued (p150) were used (F). Samples were blotted against indicated antibodies.