Villagra et al, A novel role of histone deacetylase 11 (HDAC11) as transcriptional regulator of interleukin 10 and immune tolerance

Supplementary Methods

Chromatin Immunoprecipitation. All steps were carried out at 4°C. RAW264.7 or PEM grown on 100 mm dishes were incubated for 10 min with 1% formaldehyde (Sigma, under gentle agitation. Cross-linking was stopped by the addition of 0.125 M glycine for 5 min. Then, cells were washed with 10 ml of PBS, scraped off in the same volume of PBS, and collected by centrifugation at 1,000*g* for 5 min. The cell pellet was resuspended in lysis buffer (50 mM HEPES [pH 7.8], 20 mM KCl, 3 mM $MgCl_2$, 0.1% NP-40, and a cocktail of protease inhibitors) and incubated for 10 min on ice. The cell extract was collected by centrifugation at 1,000*g* for 5 min, resuspended in a sonication buffer (50 mM HEPES [pH 7.9], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, and a cocktail of protease inhibitors), and incubated for 10 min on ice. To reduce the length of the chromatin fragments to approximately 500 bp (confirmed by electrophoretic analysis and PCR), the extract was sonicated using a Bioruptor™ from Diagenode. Eight pulses of 30 seconds with 30 seconds rest time were repeated six times at a frequency of 20 KHz. After centrifugation at 16,000*g*, the supernatant was collected, frozen in liquid nitrogen, and kept at -80°C. An aliquot was used for A260 measurements. Cross-linked extracts (6 U A260) were resuspended in a sonication buffer to a final volume of 500 µl. The samples were pre-cleared by incubation with normal mouse IgG plus protein A/G agarose (Santa Cruz Biotechnology) for 2 h at 4°C with agitation. After centrifugation at 1,000*g* for 5 min, the supernatant was collected and immunoprecipitated with specific antibodies as detailed for each experiment. The immunocomplexes (except from Flag/agarose conjugates) were recovered with the addition of 30 µl of protein-A agarose beads and subsequent incubation for 2 h at 4°C with agitation. The complexes were washed twice with sonication buffer

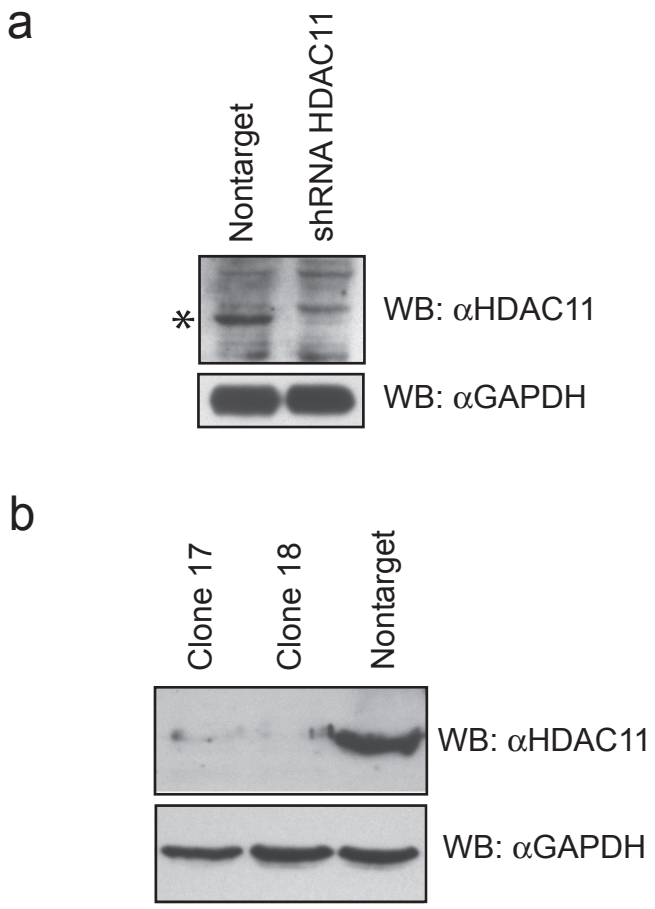
plus 500 mM NaCl, twice with LiCl buffer (20 mM Tris-HCl [pH 8.0], 250 mM LiCl, 1 mM EDTA, and 0.5% Triton X-100), and twice with TE buffer (2 mM EDTA and 50 mM Tris-HCl [pH 8.0]). The solution was incubated at each washing for 5 min at 4°C. The protein-DNA complexes were eluted by incubation with 100 µl of elution buffer (50 mM $NaHCO_3$ and 1% SDS) for 15 min at 65°C. After centrifugation at 1,000*g* for 5 min, the supernatant was collected and incubated with 10 µg of RNase A per ml for 1 h at 42°C. NaCl was added to the mixture to a final concentration of 200 mM and incubated at 65°C to reverse the cross-linking. The proteins were digested with 200 µg/ml of proteinase K for 2 h at 50°C. The DNA was recovered using Qiagen Quiaquick™ columns. The primers used were; *Il10* (proximal region) sense 5'-GCCCATTTATCCACGTCATT-3' and antisense 5'-CTGTTCTTGGTCCCCCTTTT-3', *Il10* (distal region) sense 5'-AACTCAGCCTGGAACTGACC-3' and antisense 5'-TCACCTGGGCAAGCAACTAC-3', *Il12* sense 5'-GTGGAGCCAAACAGGGAGGTA-3' and antisense 5'-GACGTCGAAATCCCAGTTTA-3'. All samples and Inputs were quantified using MyIQ single color real time PCR detection system (Bio-Rad) and iQ SYBR green Supermix (Bio-Rad). Single product amplification was confirmed by melting curve analysis and primer efficiency was near or close to 100% in all experiments performed. Quantification is expressed in arbitrary units, and target sequence levels were normalized to the input signal using the method of Pfaffl [36]. All ChIP experiments were repeated twice starting from the crosslinking, and final quantitative real time PCR was done in triplicates.

Plasmids: The 1.6 kb murine *Il12* promoter was obtained by PCR-amplification of genomic DNA from RAW264.7 cells with *pfx* (Invitrogen). The primers used for PCR

were 5'-CCGAGCTCGTATGTTGTCACAGCTTGTCTC-3' (sense), incorporating a restriction site for *Sac*I and 5'-AGAACTTACCTTGCTTTGCTG-3' (antisense). The PCR product was digested with *Sac*I and then cloned into the pGL3Basic vector digested with *Sma*I and *Sac*I to generate the plasmid p12-Luc. Similarly, the 1.6 kb murine *Il10* promoter was obtained from genomic DNA. The primers used were 5'-CCGAGCTCAGGGACCTTGTAGCTGCTCAG-3' (sense), incorporating a restriction site for *Sac*I and 5'-ATCTCGAGACCTCCTGTTCTTGGTCCCCC-3' (antisense), incorporating a restriction site for *Xho*I. The PCR product was digested with *Sac*I and *Xho*I and then subcloned into the pGL3Basic vector digested with *Sac*I and *Xho*I to generate the plasmid p10-Luc. A short version of the *Il12* promoter was generated by digestion with *Kpn*I and direct relegation. The chimeric construct *Il12/Il10* was generated by incorporation of the *Kpn*I fragment from *Il10* (distal promoter) into the short version of the *Il12* reporter gene digested with *Kpn*I. All constructs were confirmed by DNA sequencing. HDAC11-Flag carrying aminoacids 1 to 347 (1–347) and HDAC11-Flag carrying aminoacids 1 to 264 (1–264) were generated as follows: HDAC11 (IMAGE clone 3906049) was obtained from OpenBiosystems. The full-length cDNA, encoding amino acids 1-347, was PCR amplified, verified by sequencing, then inserted into pCDNA3-Flag, creating *Flag-HDAC11*. To prepare the 1-264 deletion mutant, *Flag-HDAC11* was digested with *Xho*I, which cuts once in the HDAC11 coding sequence and once in the vector. Following release of the fragment encoding amino acids 265-347, the resulting DNA fragment was re-ligated, producing *Flag-HDAC11* 1-264. Plasmids were purified by Qiagen maxipreps.

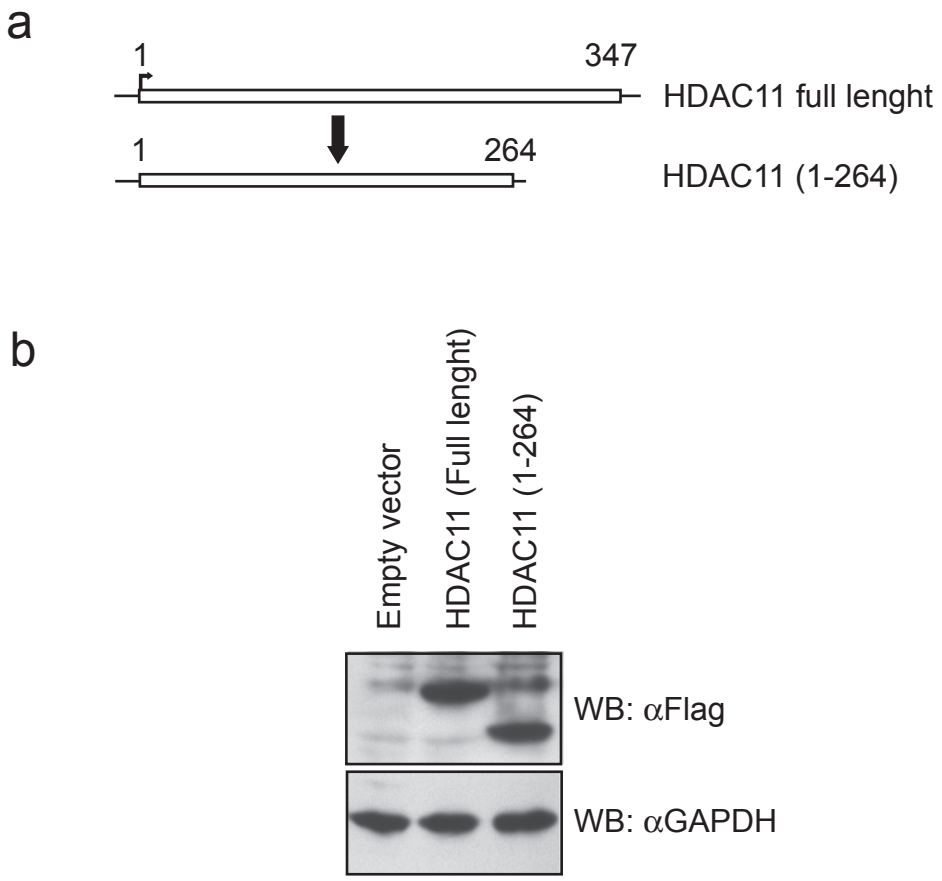
Tolerance model. Briefly, 2.5 x10^6 $CD4^+$ transgenic T cells specific for an MHC class II epitope of influenza hemagglutin (HA) were adoptively transferred into BALB/c mice. Two days later, half the mice were injected intravenously with a tolerogenic dose of HA-peptide$_{110-120}$ (275 mg), and two weeks later all of the animals were sacrificed. T cells were re-isolated from their spleens by centrifugation of splenocytes on a Ficoll gradient (Ficoll-Paque, Pharmacia Biotech) followed by passage through nylon wool. IL-2 and IFN-gamma production by clonotypic $CD4^+$ T cells in response to HA-peptide$_{110-120}$ presented by APCs was then determined

Supplement Figure 1



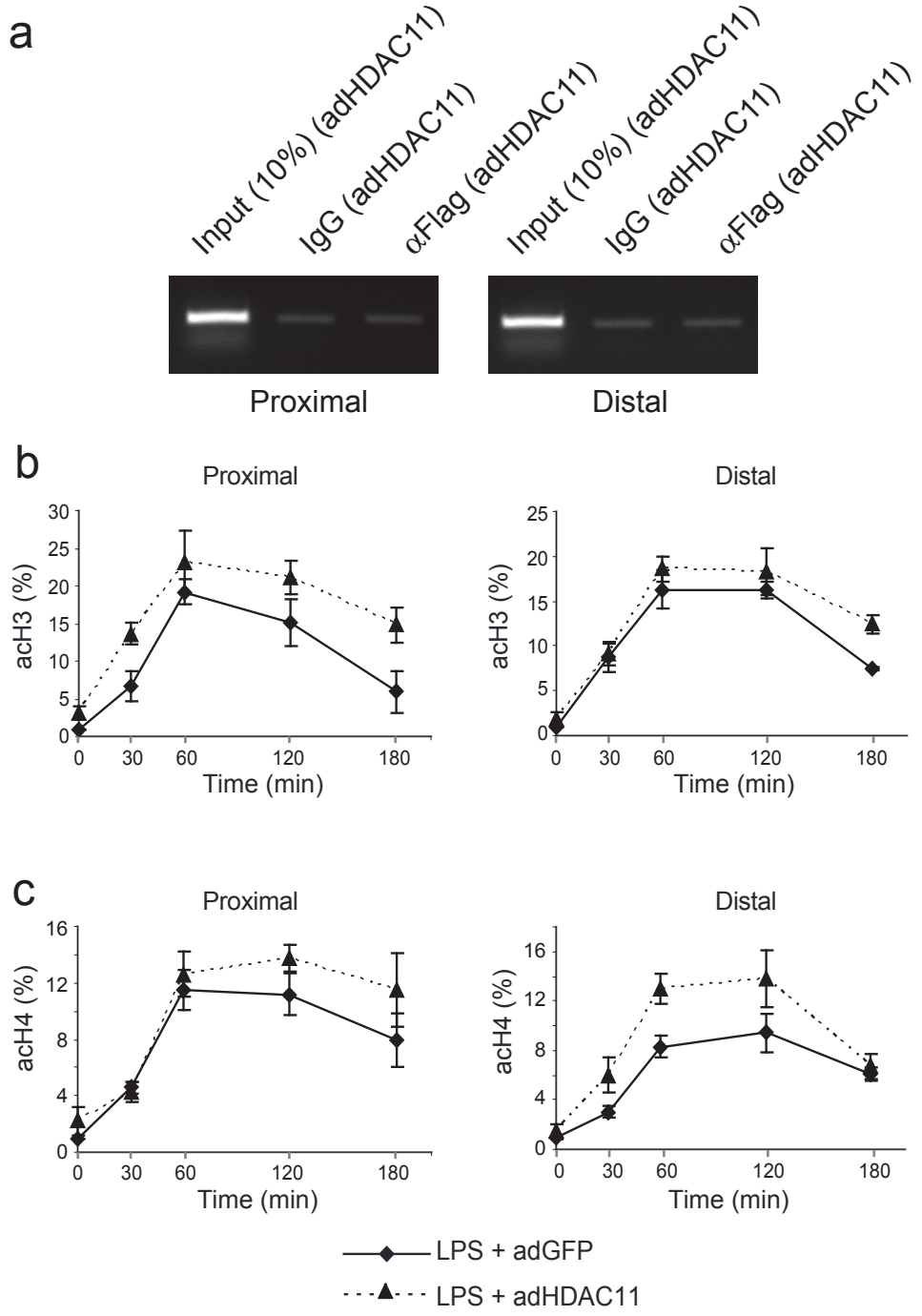
Supplemental Figure 1: Inhibition of HDAC11 protein expression in macrophages infected with shRNAHDAC11. (a) RAW264.7 cells were grown until 70% confluence and then hexadimethrine bromide (8 μg/mL) and lentiviral particles for shRNA HDAC11 or non-specific target particles were added to the cells. After 48 hours cells were harvested and a western blot analysis using a polyclonal anti-HDAC11 antibody (Ab18973) was performed. (b) Two clones were selected by their ability to grown in presence of 5μg/mL of puromycine, and evaluated by western blot using an anti-HDAC11 antibody (Ab 47036).

Supplement Figure 2



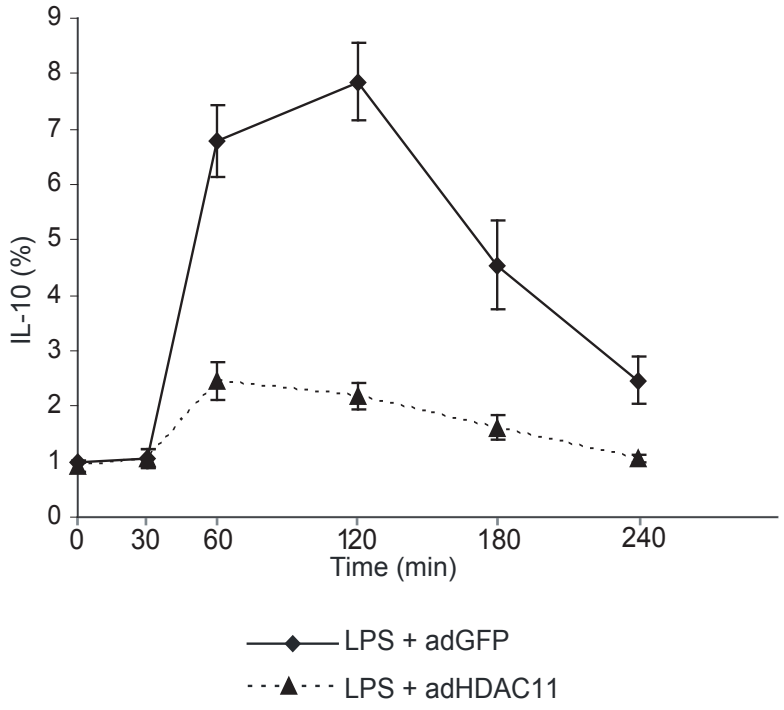
Supplemental Figure 2: HDAC11 (1-264) mutant with no deacetylase activity. (a) Schematic representation of HDAC11 coding region and the segments used to create the mutant version of HDAC11 with no deacetylase activity. (b) RAW264.7 cells were transfected by electroporation with plasmids coding for full length Flag-HDAC11, the deletion mutant (1-264) Flag-HDAC11 or empty vector. Cells were lysated and subjected to western blot using anti-Flag antibody to confirm protein expression.

Supplement Figure 3



Supplemental Figure 3: HDAC11 is not detected and does not inhibit H3 and H4 acetylation in the IL-12 promoter of LPS-treated macrophages. (a) RAW264.7 cells were infected with an adenovirus carrying HDAC11-Flag. After 24 hrs, cells were subjected to ChIP analysis using an anti-Flag antibody to evaluate HDAC11 binding to two different regions on the IL-12 promoter described as proximal or distal according to the transcriptional start. (b-c) RAW264.7 cells were infected with adenovirus coding for GFP of GFP-HDAC11. 24 hrs later cells were treated with LPS (1.0 ?g/mL) and subsequently harvested at baseline (time 0) or at 30, 60, 120 or 180 minutes after treatment. Cells were then subjected to ChIP analysis using the anti-hyperacetylated Histone 3 (b), or anti-hyperacetylated Histone 4 (c) antibodies.

Supplement Figure 4



Supplemental Figure 4: Kinetics of IL-10 mRNA expression in LPS-treated macrophages. RAW264.7 cells were infected with adenovirus coding for GFP of GFP-HDAC11. 24 hrs later, cells were treated with LPS (1.0 μg/mL) and subsequently harvested at baseline (time 0) or at 30, 60, 120, 180 or 240 minutes after treatment. Total RNA was isolated and quantitative RT-PCR was used to assess IL-10 mRNA expression.

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Supplementary Table I

RT-PCR oligos

	Forward	Reverse
Mouse *Il12p40*	5'-GCAACGTTGGAAAGGAAAGA-3'	5'-AAAGCCAACCAAGCAGAAGA-3'
Mouse *Il10*	5'-CAGGGATCTTAGCTAACGGAAA-3'	5'-GCTCAGTGAATAAATAGAATGGGAAC-3'
Mouse *HDAC11*	5'-TATCTGGAGAAGGTGGAGAG-3'	5'-ATGCAAGTTGAGGATGGAG-3'
Mouse *GAPDH*	5'-ATGGCCTTCCGTGTTCCTAC-3'	5'-CAGATGCCTGCTTCACAC-3'
Human *Il12p40*	5'-ATGTCGTAGAATTGGATTGG-3'	5'-CTAAGACCTCACTGCTCTGG-3'
Human *Il10*	5'-CAAGCTGAGAACCAAGACC-3'	5'-GCTTTGTAGATGCCTTTCTG-3'
Human *GAPDH*	5'-GAGAAGTATGACAACAGCCTCAA-3'	5'-AGTCCTTCCACGATACCAAAG-3'