Deacetylase Activity Is Required for STAT5-Dependent GM-CSF Functional Activity in Macrophages and Differentiation to Dendritic Cells

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ABSTRACT

Histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes play a major role in the transcriptional regulation of several genes. A part of their role in histone modification, HATs and HDACs modulate the activity of transcription factors and components of the basal transcriptional machinery. Here, we provide evidences that inhibition of deacetylase activity with Trichostatin A (TSA) blocks GM-CSF- but not M-CSF-dependent macrophage proliferation. This inhibition correlates with a block of *cicD1* and *c-myc* expression. TSA treatment also abolishes the GM-CSF-dependent MHC class II expression and generation of dendritic cells. Expression, phosphorylation and functional activity of STAT5a and STAT5b that mediates the signal transduction of these functions for GM-CSF were not affected. However, α-Polinerase II was not recruited to the promoter. The protection of apoptosis by GM-CSF that is mediated through PI3K/Akt pathway was not affected in the presence of TSA. Thus, these results demonstrate that HDAC is required for STAT5-dependent functional activities of macrophages as well as the generation of dendritic cells.

INTRODUCTION

Transcription is a highly regulated process that requires the orchestration and coordination of many factors involved in the production of transcription factors, in the alteration of their activities by post-translational modifications and in the chromatin remodeling. The binding of transcription factors to the promoter of a target gene and the recruitment of other regulators leads to localized changes in chromatin structure which, depending on the gene, could have a positive or negative effect on their transcription. In several genes, recruitment of proteins with histone acetyl transferase activity (HAT) promotes acetylation of histone proteins (particularly H3 and H4) leading to relaxed chromatin structure, binding of core transcription machinery to DNA and initiation of transcription. On the other hand, recruitment of histone deacetylases (HDACs) causes deacetylation of histones, chromosomal condensation and gene repression (1, 2). However, depending on the genes, deacetylation is also associated with activation of transcription according to the observation that histone hyperacetylation inhibits the expression of many genes (3). Moreover, some transcription factors need to be deacteylated in order to activate transcription, such as C/EBPβ, which activates Id-1 expression only when is deacetylated (4). In addition to histone acetylation, other histone and nonhistone protein modifications (acetylation, phosphorylation and methylation) are reported to regulate transcription (5-7).

Macrophages and are part of the innate immunity and they play a critical role in host defence mechanisms. Macrophages originate from undifferentiated stem cells and require specific growth factors (M-CSF, IL-3 and GM-CSF) for their generation (Metcalf, 1989). In the presence of growth factors and cytokines, macrophages may proliferate, differentiate to specific cell types depending on the tissue or become

activated and develop their functional activities. GM-CSF has been shown to have a profound influence on macrophage biology promoting the differentiation, proliferation and survival of these cells as well as many other functions including antigen presentation, chemotaxis, phagocytosis and release of reactive oxygen intermediates (8-10). In addition to macrophages, GM-CSF regulates some biological functions of other immune cells such as neutrophils, eosinophils, basophils and lympocytes (11) and is specially important for dendritic cell development and maturation (12). GM-CSF exerts its functions mainly through activation of Janus Kinase 2-signal transducer and activator of transcription 5 (JAK2-STAT5) signaling pathway. STAT5 belongs to STAT family of proteins which are important downstream effectors of cytokine signaling. STAT proteins are present in a latent form in the cytoplasm and migrate to the nucleus following cytokine-induced phosphorylation and dimerization. Once in the nucleus, STAT dimers bind to specific DNA binding sites and activate transcription of target genes. In addition, STAT activity is modulated through interaction with a variety of proteins (13) including cofactors and other transcription factors. Moreover, STAT family members interact with proteins with HAT activity (14-16) as well as with members of the HDAC family (4, 17) suggesting that STAT proteins could play a role in chromatin remodeling. In particular, transcriptional activity of STAT5 requires a histone deacetylase activity (18) and it has been suggested that administration of the deacetylase activity inhibitor Thricostatin A (TSA) may be beneficial in STAT5-associated cancers. However, little is known about the effects that TSA treatment could have on immune function.

In order to determine the effect of histone deacetylase activity on macrophages we treated bone-marrow derived macrophages with TSA. The STAT5-dependent activities such as GM-CSF-dependent proliferation and MHC class II gene expression

were reduced in TSA treated macrophages. Furthermore, TSA impairs differentiation of dendritic cells from bone-marrow. The expression of genes involved in proliferation, and differentiation was impaired in TSA-treated macrophages and dendritic cells. However, the apoptosis protection mediated by GM-CSF that use the PI3K/Akt signal transduiction was not affected by TSA. Together, these results indicate that histone deacetylase activity is essential for proper functionality of macrophages that depends of STA 5 as well as for generation of dendritic cells.

MATERIALS AND METHODS

Reagents

Recombinant murine GM-CSF, M-CSF and IFN-γ were purchased from Sigma Chemical (St Louis, MI, USA) and Trichostatin A was purchased from Tocris (Ellisville, MO, USA). The following antibodies were used: anti-Stat5a (R&D Systems, Minneapolis, MN, USA), anti-Stat5b (R&D Systems), anti-phospho-Stat5a/b Y694/Y699 (Upstate, Lake Placid, NY, USA), anti-RNA PolII (N20; Santa Cruz Biotechnology, California, USA), anti-MHC Class II (I-A) FITC conjugate (Chemicon, Temecula, CA, USA), anti-Cd11c PE conjugate (BD Pharmingen, San Diego, CA, USA) and anti-CD16/CD32 (BD Pharmingen). Peroxidase conjugated anti-rabbit (Jackson ImmunoResearch Labs, Cambridgeshire, UK) or anti-mouse (Sigma) were used as a secondary antibodies. All other chemicals were of the highest purity grade available and were purchased from Sigma Chemical. Deionized water further purified with a Millipore Milli-Q system A10 was used.

Cell Culture

Bone marrow-derived macrophages were isolated from 6-week-old Balb/c mice (Harlan Ibérica, Barcelona, Spain) as previously described (19). In some experiments bone marrow cells were differentiated to dendritic cells (12). For this, bone marrow cells were seeded at 10^6 cells/ml in 24-well tissue culture plates in 1ml of DMEM with 10% FCS containing 5ng/ml of GM-CSF. At day 2 and 4 the plates were shaken gently and 750 µl of the culture supernatant was collected and replaced by fresh medium with 5ng/ml of GM-CSF. In these steps weakly adherent granulocytes were eliminated. At day 6 and 8 we fed the plates aspirating 500µl of the culture supernatants (without

shaking) and adding back fresh medium with 5ng/ml of GM-CSF. At day 8 cells were also stimulated with 1µg/ml of LPS from *E.Coli* (Sigma) for 48 hours. At day 10 plates were shaken and the supernatant collected separating dendritic cells from adherent macrophages. Then cell surface staining was carried out using antibodies anti-CD11c and anti-MHC class II.

RNA Extraction and Real-Time RT-PCR

Cells were washed twice with cold PBS and total RNA was extracted with the EZ-RNA Kit as described by the manufacturer (Biological Industries, Israel). RNA was treated with DNAse (Roche, Basel, Switzerland) to eliminate contaminating DNA. For cDNA synthesis, 1µg of total RNA and M-MLV Reverse transcriptase RNase H Minus, Point Mutant, oligo$(dT)_{15}$ primer and PCR Nucleotide mix were used, as described by the manufacturer (Promega Corporation, Madison, WI). Real-Time PCR were performed using the SYBR Green PCR Core Reagents Kit (Applied Biosystems) as described the manufacturer with the exception that the final volume was 12.5µl of SYBR Green Reaction Mix. Real-time monitoring of PCR amplification was performed in the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Data were expressed as relative mRNA levels normalized to the β-actin expression level in each sample. The forward and reverse primers used to amplify mouse cDNAs are shown in Table 1 of Supplementary material.

A1, fw: 5'- AAGAGCAGATTGCCCTGGATGT-3' and Rv: 5'-CCAACCTCCATTCTGCCGTAT-3'; *Ciclin D1*, Fw: 5'-TGCTGCAAATGGAACTGCTTC-3' and Rv: 5'-CATCCGCCTCTGGCATTTT-3'; *c-Myc*, Fw: 5'-AACAGCTTCGAAACTCTGGTG-3' and Rv: 5'-

CGCATCAGTTCTGTCAGAAGGA-3'; *RelB,* Fw: 5'-CAAGAAGTCCACCAACACATC-3' and Rv: 5'-TTTTTGCACCTTGTCACAGAGC-3'; *IRF4*, Fw: 5'-TTCACAGTGAAAGACCTGCTGG-3' and Rv: 5'-AGGATGGTGCCACACTTTCCT-3'; *IRF1*, Fw: 5'-GAGATGTTAGCCCGGACACTTT-3' and Rv: 5'-CCATATCCAAGTCCTGACCCA-3'; *IAβ*, Fw: 5'-AAAGTGCGCTGGTTCAGGAAT-3' and Rv: 5'-TCATCTCCAGCATGACCAGGA-3'; *CIITA*, Fw: 5'-CACCCCCAGATGTGTATGTGCT-3' and Rv: 5'-ACGAGGTTTCCCAGTCCAGAA-3' *β-actin,* Fw: 5'-ACTATTGGCAACGAGCGGTTC-3' and Rv: 5'-AAGGAAGGCTGGAAAAGAGCC-3'. Annealing for all primers was performed at 60ºC for 30''.

Proliferation assay

Macrophage proliferation was measured as described (20). Cells were deprived of M-CSF for 16-18 hours and then 10^5 cells were incubated for 24 hours in complete medium in the presence of the growth factor. After this period, the medium was replaced with medium containing [^{3}H]-thymidine. After six additional hours of incubation, the medium was removed and the cells were fixed in ice-cold 70% methanol. After three washes, cells were solubilized and radioactivity was measured. Each experiment was performed three times and the results were expressed as the mean ± SD.

Apoptosis assay

Cell viability was assessed by particle counting using FACS (Coulter Multisizer II, Midland, Canada) and confirmed by trypan blue exclusion. Cell death was also assessed by FACS analysis using the rAnnex V-FITC kit (Bender MedSystems, Burlingame, CA) following manufacturer's instructions. Actinomycin D (Act D) was used as positive control of apoptosis. Each point was performed in triplicate and the results were expressed as the mean value ± S.D.

Cell surface staining

This assay was carried out using specific antibodies and cytofluorometric analysis as described (21). Cells were incubated for 15 minutes with 1µg/10^6 cells of anti-CD16/CD32 monoclonal antibody to block Fc receptors. Then cells were incubated for 1 hour with specific antibodies at 4ºC in the darkness. Cells were washed by centrifugation through an FCS cushion. Finally, they were fixed with PBS-2% paraformaldehyde. Stained cell suspensions were analyzed using an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL). Each experiment was performed three times and the results were expressed as the mean ± SD.

Protein analysis

Cells were lysed on ice with lysis solution (1% Triton X-100, 10% glycerol, 50 mM Hepes pH 7.5, 150 mM NaCl, protease inhibitors and 1mM sodium ortovanadate) (22). For immunoprecipitation assays, 150 µg of cell lysates were mixed with 75µl of 20% Protein-A-Sepharose (Sigma) and 2µl of specific antibodies anti-Stat5a and Stat5b to a final volume of 500µl. The reaction was carried out overnight at 4ºC in rotation. After three washes in cold washing buffer (PBS, 1% Nonidet P-40, 2mM sodium ortovanadate) pellets were heated to 95ºC in Laemmli SDS loading buffer and proteins

were separated in a SDS-PAGE gel and electrotransferred to nitrocellulose membranes. The membranes were blocked and incubated with primary and secondary antibodies as described by the manufacturer of each antibody used. ECL detection was performed using the EZ-ECL Kit (Biological Industries) and membranes were exposed to X-ray films (AGFA, Mortsel, Belgium).

Nuclear extract preparation and DNA-binding assay

Nuclear extracts were prepared from macrophages as described (23): cell pellets were washed twice with cold PBS buffer and resuspended in buffer A (10mM Hepes, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF, 0.01mg/ml aprotinin, 0.01mg/ml leupeptin, 0.086 mg/ml iodacetamide, 1mM sodium ortovanadate) and incubated on ice for 15 minutes. Cells were lysed adding 10% NP-40 and mixing by vortex. Nuclei were collected at the bottom of the tube by a 30 seconds centrifugation. After three washes with buffer A, nuclei were lysed with buffer B (20mM Hepes, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF, 5mM NaF, 0.01mg/ml aprotinin, 0.01mg/ml leupeptin, 0.086 mg/ml iodacetamide, 1mM sodium ortovanadate) at 4ºC for 30 minutes in rotation. Lysates were centrifuged 20 minutes at 4ºC at 13000 rpm and supernatants were stored at -80ºC until use.

Electrophoretic mobility sift assays were performed as described (24). Briefly, binding reactions were prepared with 6 µg of nuclear extracts and 20,000 cpm ^{32}P-labelled probe in the presence of 2 µg of poly(dI:dC) in a final volume of 15 µl containing 1X binding buffer (12 mM Hepes pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 0.12 mM EDTA, 0.3 mM PMSF, 0.3 mM DTT, 12% glycerol). An 8 minutes preincubation of extracts and poly(dI:dC) was performed. Then the radiolabeled probe was added and incubated for an additional 15 minutes at room temperature. Samples were loaded onto

4% acrylamide gel containing 5% glycerol and 0.25% TBE, and electrophoresed at 4ºC. Band-shift gels were dried and bands were visualized by autoradiography. For super-shift experiments, following the binding reaction, 2 µl of anti-Stat5a and anti-Stat5b antibodies were added and incubated for 30 min. Oligonucleotide used as probe in the assay were 5'-end labeled using T_4 Polynuclotide Kinase (USB Corporation, OH, USA). The probe was synthesized by Genotek (Barcelona, Spain) and correspond to a GAS-like element from the promoter of the bovine β-casein gene (5'-AGATTTCTAGGAATTCAAATC-3').

Chromatin immunoprecipitation (ChIP) assay

Cells ($20x10^6$) were cross-linked with 1% paraformaldehyde for 20 minutes at room temperature. After two washes with ice-cold PBS, cells were collected into 3 ml 0.1M Tris-HCl pH 9.4, 10 mM DTT with a scraper and were incubated for 15 minutes at 30ºC. Cells were centrifuged 5 minutes at 2000g at 4ºC and the pellets were washed sequentially by pipetting with ice-cold PBS, buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5) and buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5). 300 µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, 1mM DTT, 1mM PMSF, 0.01mg/ml aprotinin, 0.01mg/ml leupeptin, 0.086 mg/ml iodacetamide, 1mM sodium ortovanadate) was added and lysates were sonicated on ice using the Ikasonic U200S Control (Ika Labortehcnik) (15 pulses of 10 seconds, 30% cycle and 30% amplitude). Size of fragments obtained (between 200-1200 bp) was confirmed by electrophoresis. Soluble chromatin was collected after centrifugation of cell lysates at 14 000 rpm at 4ºC for 10 minutes and diluted to 1:10 in dilution buffer (1% Triton X-100, 2mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1, 1mM DTT, 1mM PMSF, 0.01mg/ml aprotinin,

0.01mg/ml leupeptin, 0.086 mg/ml iodacetamide, 1mM sodium ortovanadate). 1-5% of soluble chromatin was kept as imput control.

Soluble chromatin was pre-cleared with 20 µg of salmon sperm (Amersham Biosciences), 8 µg of unspecific IgGs, 20 µg of pre-immune serum and Protein-A-Sepharose at 50% overnight at 4ºC in rotation. After centrifugation, supernatants were collected and specific antibodies (Stat5a and Stat5b, 2µl) were added. A control immunoprecipitation was also performed with 1 µg of unspecific IgGs. Mixtures were incubated at 4ºC for 6 hours in rotation. After this period they were incubated overnight at 4ºC in rotation with protein-A-sepharose at 50%. Beads were collected and washed sequentially at 4ºC for 10 minutes with TSE I (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM TrisHCl pH 8.1), TSE II (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM TrisHCl pH 8.1) and buffer III (0.25 LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10mM TrisHCl pH 8.1). Beads were washed once with TE buffer by pipetting and immunoprecipitates were eluted three times (20 minutes incubation) with elution buffer (0.1 M $NaHCO_3$, 1% SDS). Reversion of cross-linking was performed overnight by heating samples and imput controls at 65ºC, and DNA was purified using the QIAquick Spin Kit (Qiagen).

For quantification, a real-time PCR was performed as described above using the primers showed in the Table 1 of supplementary material that correspond to Stat5 binding sites at Cis promoter (18). Amplification of c-jun coding region was used as a control of non-specific immunoprecipitation. Data were expressed as relative immunoprecipitation normalized to the c-jun amplification level in each sample.

forward, GTCCAAAGCACTAGACGCCTG, *reverse*, TTCCCGGAAGCCTCATCTT; and CAP site: *forward*, GTTCGCACCACAGCCTTTCAGTCC, *reverse*,

GTCCAGGGGTGCGAAGGTCAGG. Primers used were: *forward*, TGAAAGCGCAAAACTCCGAG; *reverse*, GCACCCACTGTTAACGTGGTT.

Statistical analysis

To calculate the statistical differences between the control and treated samples, we used the Student's paired *t* test. Values of $p<0.05$ or lower were considered significant.

RESULTS

Deacetylase activity is required for GM-CSF-dependent proliferation but not for protection from apoptosis.

GM-CSF is an important hematopoietic growth factor and immune modulator (11) and plays a critical role in regulating the generation and function of many immune cells. Regarding to monocytes and macrophages, GM-CSF promotes their differentiation, proliferation, survival and activation (8, 11, 25, 26).

To test if deacetylase activity has any functional implication in GM-CSF-dependent macrophage biology we first examined the proliferation of macrophages in the presence of the pharmacological HDAC inhibitor TSA. Cells were grown and differentiated in the presence of M-CSF for 7 days. After 18 hours of M-CSF-deprivation, macrophages became quiescent and proliferation was measured. When GM-CSF was added to the culture, thymidine incorporation increased in a dose-dependent manner (*Fig.1A*). Treatment with TSA resulted in a dramatically inhibition of GM-CSF-induced proliferation (*Fig 1A*). This experiment was repeated five times and differences between control and TSA-treated cells were found to be significant ($p<0.01$). Moreover, cell counting confirmed these results. To test whether this effect was specific or not, we analyzed M-CSF-dependent proliferation in the presence of TSA and not significant differences were found (*Fig.1B*). Both M-CSF and GM-CSF stimulate macrophage proliferation by activation of extracellular regulated kinases (ERK) (27, 28) indicating that TSA does not target this pathway. Because in some cell types such as macrophages STAT5 mediates the GM-CSF-dependent proliferation we hypothesize that one possible target of the inhibitory effect of TSA could be STAT5 activation. To test this hypothesis we examined the expression of STAT5 target genes

involved in proliferation in macrophages by real-time PCR. Treatment with 5ng/ml of GM-CSF increased the expression of *Cyclin D1* and *c-myc*, genes that are necessary for the cells to enter in the cell cycle. The induction of these genes was strongly inhibited by the addition of 20 nM of TSA (*Fig.1C*) indicating that TSA blocks GM-CSF-dependent proliferation by inhibiting STAT5-dependent gene expression.

In addition to stimulating cell proliferation, M-CSF and GM-CSF also promote cell survival (8). In the absence of a growth factor, macrophages undergo cell death by apoptosis, whereas treatment of macrophages with M-CSF or GM-CSF rescues these cells from cell death (29). We determined cell death using Annexin-V staining. The addition of M-CSF protected macrophages from apoptosis induced by starvation and this protection was not inhibited by TSA (*Fig.1D*). Interestingly, GM-CSF also protected the cells from apoptosis even in the presence of TSA (*Fig.1D*). These results indicate that the impaired proliferation observed in the presence of TSA is not due to an increased cell death. Also, this showed that histone deacetylation is necessary for the functional activities induced by GM-CSF that are STAT 5-dependent. Effectively, M-CSF and GM-CSF-dependent survival of macrophages are mediated by activation of phophatidylinositol 3-kinase (PI3K)/Akt pathway that is independent of the signal transduction pathway needed for proliferation (8). Together, these results demonstrate that TSA specifically inhibits the GM-CSF/STAT5 pathway in macrophages as no ERK-and PI3K-dependent functions were shown to be modified by TSA.

GM-CSF-dependent expression of MHC class II in macrophages requires a deacetylase activity.

A part of its role in innate immunity, macrophages play an important role in host defence as antigen presenting cells. Expression of MHC class II molecules at the cell

surface is critical for these function and therefore for activation of T cells. The major activator of MHC class II expression is IFN-γ (30), a cytokine secreted by antigen-activated Th1 lymphocytes that regulates MHC class II expression not only at transcriptional level but also at posttranscriptional level, including the regulation of translation and the half life of the protein (31, 32). In addition to IFN-γ, GM-CSF has a positive effect on antigen presentation capability (9) and induces the expression of MHC class II molecules (26). These observations led us to evaluate whether GM-CSF induces MHC class II expression in macrophages and if this induction is impaired in the presence of TSA. GM-CSF induced I-A expression at the cell surface of macrophages. Pre-treatment of these cells with TSA blocked this induction (*Fig.2A and 2B*). This induction of cell surface expression correlated with the increase of mRNA levels of I-Aβ in response to GM-CSF and its reduction in TSA pre-treated macrophages (*Fig.2C*) suggesting that TSA inhibits the expression of I-A genes at transcriptional level. This was confirmed because TSA do not modified the half live of mRNA of IA-β (data not shown), that is very stable (31, 33).To further investigate this we analyzed the expression of the class II transactivator (CIITA). The expression of this master regulator is related to both tissue specificity and quantitative expression of class II genes, and its absence causes a severe immunodeficiency (34). In bone marrow derived macrophages, GM-CSF increased the mRNA levels of CIITA (*Fig.2D*). Strikingly, TSA abolished CIITA expression in GM-CSF-stimulated macrophages confirming that reduced I-Aβ expression shown in TSA-treated macrophages is due to an impaired MHC class II transcription.

TSA blocks GM-CSF-dependent differentiation to dendritic cells

Dendritic cells (DCs) are antigen presenting cells crucial to both innate and adaptive immunity and they play a critical role in the induction and control of T-cell immunity and in the modulation of responses of B and NK cells. They originate *in vitro* from bone-marrow cultures in the presence of GM-CSF from MHC class II-negative precursors (35). To investigate the role of deacetylase activity in dendritic cell differentiation we examined the effect of TSA on bone-marrow GM-CSF-dependent differentiation to dendritic cells. After 8 days of culture in the presence of 5 ng/ml of GM-CSF we obtained a population of cells containing adherent macrophages and floating dendritic cells. The addition of 1 µg/ml of LPS to the culture promoted maturation of these cells and expression of MHC II (I-A) on cell surface. To quantify dendritic cell differentiation, FACS analysis was performed using CD11c and I-A as a specific markers of mouse dendritic cells (*Fig 3A).* After ten days of culture 70% of cells obtained were CD11c (+) and I-A (+). This percentage was dramatically reduced to 25% when TSA was present during the differentiation process (*Fig 3A, B*). A significant difference was found when we compared four independent experiments ($p<0.01$). This effect was specific of GM-CSF-dependent differentiation because M-CSF- dependent differentiation of macrophages was not affected by TSA (*Fig3C*). These results clearly demonstrate that TSA inhibits bone marrow cells differentiation to dendritic cells.

To study the mechanism by which TSA inhibits dendritic cell differentiation, we examined the expression of some of the genes involved in this process. Transcription factors of the IFN regulatory factor (IRF) family play critical roles in generation and function of dendritic cells. GM-CSF-dependent development of dendritic cells depends on IRF4 (36) as well as on IRF2 (37, 38). In addition, RelB, a member of the NF-κB family, has been reported to be important for the development of some subsets of

dendritic cells (39, 40). We have found that IRF4, IRF2 and RelB were expressed in mature dendritic cells. However, the presence of TSA during the differentiation process inhibited the expression of these genes (*Fig.4*). This effect was specific because the expression of other genes such as *Stat5a* or *IRF1*was not inhibited by TSA indicating that is not involved in DC development. These results suggest that TSA reduces GM-CSF-dependent DC development by inhibiting the expression of IRF4, IRF2 and RelB.

Deacetylase activity is needed for the recruitment of RNA polymerase II to the STAT5 target genes.

To determine the mechanism by which deacetylase activity regulates STAT 5-dependent gene expression we studied the GM-CSF signal transduction pathway. Once GM-CSF binds to its receptor, STAT 5a and STAT 5b become phosphorylated by JAK2 and migrate to the nucleus. TSA treatment of macrophages did not produce any change in STAT 5a and STAT 5b phosphorylation level (*Fig.5*). To determine the functional activity of the STAT 5 protein we measured their binding activity by EMSA. No effects were detected in TSA treated cells (*Fig.5B)*. Both STAT 5a and STAT 5b were present in the complexes as indicated by the super-shift observed when specific antibodies were added. The same complexes were present when we used nuclear extracts from TSA treated cells (*Fig.5B*). Moreover, *in vivo* DNA binding activity of STAT5 was analyzed by chromatin immunoprecipitation assays revealing that binding of both STAT5a and STAT5b to DNA *in vivo* was not affected by TSA treatment (*Fig.5C*). Therefore, these results indicate that TSA does not affect the GM-CSF-JAK2-STAT5 pathway in macrophages.

Because histone acetyl transferases and histone deacetylases play a crucial role in the formation of transcription preinitiation complex, we next tested the effect of TSA

in the recruitment of RNA polymerase II upon GM-CSF stimulation of macrophages. As revealed by chromatin immunoprecipitation analysis, RNA polymerase II was recruited to the promoter of the GM-CSF target gene *Cis* following 15 minutes of the cytokine stimulation. However, in cells treated with TSA this recruitment was prevented (*Fig 5D*). This demonstrates that TSA inhibits STAT 5-mediated transcription by blocking the recruitment of the basal machinery complex. Together, these results suggest that histone deacetylase activity is required to recruit RNA polymerase II and activate the transcription of STAT 5-target genes involved in proliferation and activation of macrophages.

DISCUSSION

Chromatin remodelling is an essential mechanism that regulates gene transcription. HATs and HDACs play pivotal roles not only in modifying histone tails but also in modulate the activity of some transcription factors. In this report, we have provided evidences to suggest that a deacetylase activity is required for proper functionality of macrophages and generation of dendritic cells. First, we have shown that TSA impair the proliferation of BMDM under GM-CSF stimulation. Other growth factors have been reported to regulate macrophage proliferation (27, 28). Among these, M-CSF seems to be the most specific and powerful inducer of macrophage generation and proliferation (41). Interestingly, M-CSF-dependent proliferation of macrophages was not inhibited by TSA supporting the idea that a deacetylase activity is only needed for GM-CSF-dependent proliferation. Because ERK1/2 signaling pathway has been shown to be involved in GM-CSF- and M-CSF-dependent macrophage proliferation (27, 28) we excluded that TSA could target this pathway. GM-CSF also activates the JAK2-STAT5 transduction pathway and STAT5 regulates the expression of *Cyclin D1* and *c-myc*, two well known proliferative genes (42, 43). Thus, we tested the expression of these genes in BMDM and we found that GM-CSF rapidly induced an increase in mRNA levels of *Cyclin D1* and *c-myc* and that TSA blocked this induction. These results demonstrate that TSA impair the GM-CSF-dependent proliferation of macrophages by inhibiting the expression of STAT5 target genes. Furthermore, GM-CSF protects macrophages from apoptosis induced by growth factor withdrawal by activation of the PI3K transduction pathway (8). According to that, we found that GM-CSF protected from apoptosis both macrophages treated and untreated with TSA supporting the idea that TSA exerts its effects in a STAT5 specific way.

In addition of its role in proliferation and survival, it has been shown that GM-CSF induces the expression of MHC class II in monocytes (26).We have shown that GM-CSF induces I-A expression in bone marrow-derived macrophages and this induction is due to an increase in CIITA mRNA levels. Once more, TSA abolished the expression of these genes revealing that a deacetylase activity is important for CIITA expression and antigen presentation capability of macrophages. As postulated (26) one possible mechanism by which GM-CSF induces CIITA expression could involved the binding of STAT5 to the IFN-γ-activated sequences (GAS) elements present in CIITA promoter. It may be also possible that GM-CSF activate STAT1, which can bind GAS elements. In any case, the mechanism by which TSA inhibits the expression of CIITA could be the same because a deacetylase activity is required for transcriptional activity of both STAT1 and STAT5 (17, 18, 44). In this line of evidence, we have found that TSA inhibited the recruitment of RNA Polymerase II to the promoter of a STAT5 target gene (*Cis*) in BMDM confirming that a deacetylase activity is required for the proper formation of the transcription preinitiation complex. Moreover, HDAC1 seems to be the responsible for this deacetylase activity because STAT1-, STAT2-, STAT3- and STAT5-HDAC1 interactions have been described in many cell types (Nusinzon, 2003; Xu, 2003 and Zhang, 2005). However, in our cell model, we were not able to detect any interaction between STAT5 and HDAC1 in co-immunoprecipitation assays and HDAC1 was not present in the promoter region of the *Cis* gene as revealed by ChIP experiments (data not shown). Nevertheless, we cannot exclude that other HDAC may be the responsible of this deacetylase activity.

Another important result of this work is the observation that TSA inhibits the generation of dendritic cell from bone marrow progenitors. Mouse DCs can develop from bone marrow cultures in the presence of GM-CSF (12) leading to the

differentiation of DCs expressing the DC markers CD11c, MHC molecules and co-stimulatory molecules. Our results demonstrate a 3-fold reduction in the number of CD11c+ IA+ cells (mature DCs). Again, this effect of TSA was specific as reflected by the fact that M-CSF differentiation of macrophages was not impaired in the presence of TSA. This result also suggests that the impaired DC development is not due to an unspecific toxicity of TSA in bone marrow progenitors. Consequently, when we assessed the expression of genes involved in DC development such as IRF2, IRF4 and RelB we found these genes were not expressed in mature DC when TSA was present during the differentiation process. The mechanism by which TSA inhibits the expression on these genes remains to be revealed. It has been reported that some STATs as well as members of the NF-κB family could bind to the IRF4 promoter in human monocyte-derived dendritic cells (45). However, although we found that STAT5 is present in the nuclear extracts of DC and can bind a β-casein probe we were not able to detect binding of STAT5 to the promoter of IRF4 (data not shown) suggesting that other transcription factors were involved in GM-CSF-dependent expression of IRF4. Among these, members of the NF-κB family of transcription factors are described to regulate the expression not only of IRF4 but also RelB (46). Strikingly, NF-κB activity has been shown to be inhibited by deacetylase inhibitors by a mechanisms that involves the suppression of proteasome activity (47, 48). To determine whether this mechanism is the responsible for TSA suppression of these genes in bone marrow-derived dendritic cells, further studies will be required.

Together, these results represent one of the few examples of the importance of HDAC activity in the development and function of dendritic cells and macrophages which are essential for the development of the innate and adaptive immunity. Because

TSA and other HDACs have some beneficial effects in some types of tumors (49) their ability to affect the immune response is an important result to be considered.

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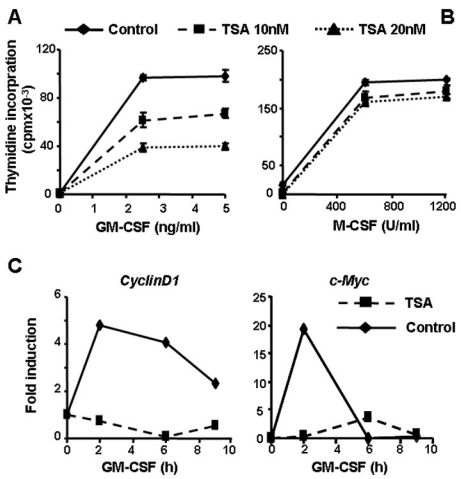
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TSA 20nM
B
Thymidine incorpration (cpmx10-3)
GM-CSF (ng/ml)
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C
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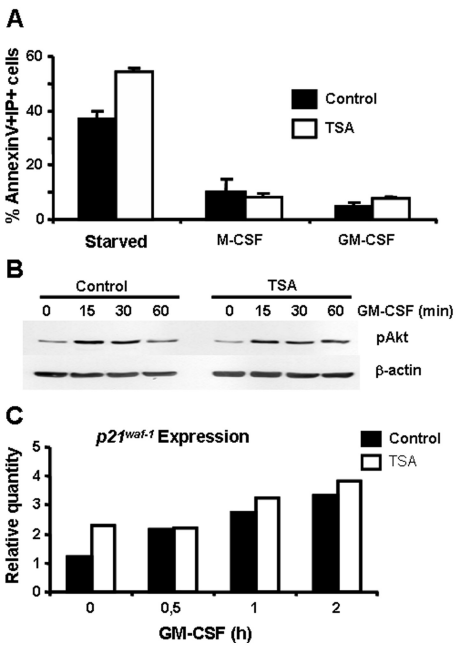
c-Myc
TSA
Control
Fold induction
GM-CSF (h)
GM-CSF (h)

Figure 1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% AnnexinV+IP+ cells
60
40
20
0
Control
TSA
Starved
M-CSF
GM-CSF
B
Control
TSA
0 15 30 60
0 15 30 60
GM-CSF (min)
pAkt
β-actin
C
p21waf-1 Expression
Control
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Relative quantity
5
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3
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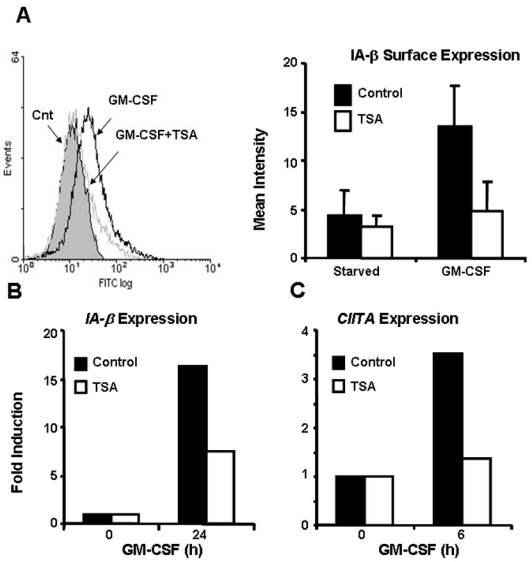
0
0
0,5
1
2
GM-CSF (h)

Figure 2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Cnt
GM-CSF
GM-CSF+TSA
Events
FITC log
IA-β Surface Expression
Control
TSA
Mean Intensity
Starved
GM-CSF
B
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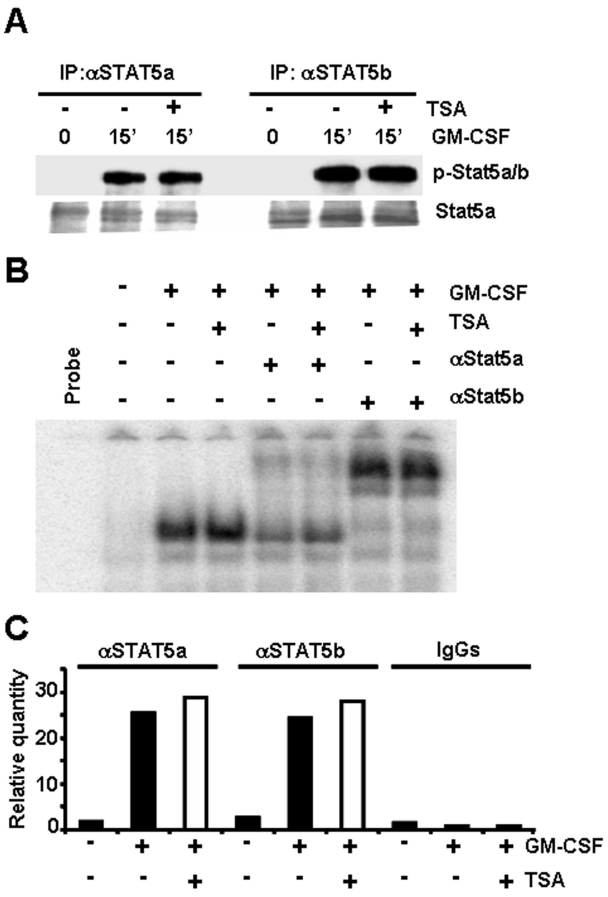
Control
TSA
Fold Induction
GM-CSF (h)
C
CIITA Expression
Control
TSA
GM-CSF (h)

Figure 3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15'
15'
GM-CSF
p-Stat5a/b
Stat5a
B
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TSA
αStat5a
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C
αSTAT5a
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IgGs
Relative quantity
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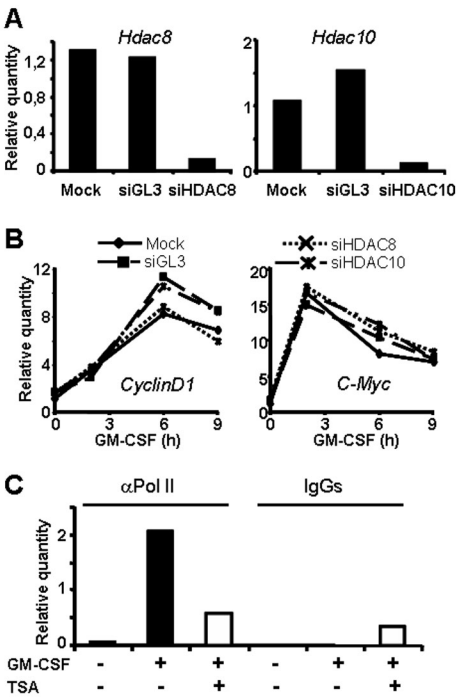
20
10
0
GM-CSF
TSA

Figure 4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Hdac10
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siHDAC8
Mock
siGL3
siHDAC10
B
Mock
siGL3
siHDAC8
siHDAC10
Relative quantity
12
8
4
0
20
15
10
5
0
CyclinD1
C-Myc
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GM-CSF (h)
C
αPol II
IgGs
Relative quantity
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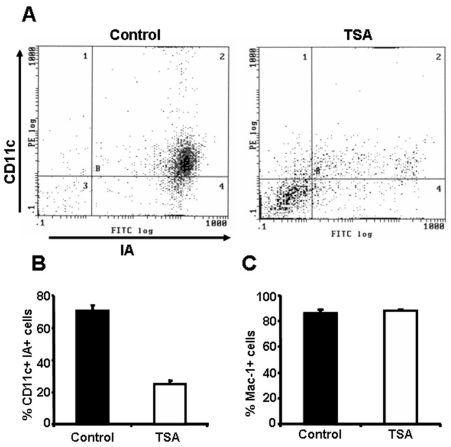
0
GM-CSF
TSA

Figura 5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TSA
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PE log
FITC log
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2
3
4
B
% CD11c+ IA+ cells
80
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Control
TSA
C
% Mac-1+ cells
100
80
60
40
20
0
Control
TSA

Figure 6

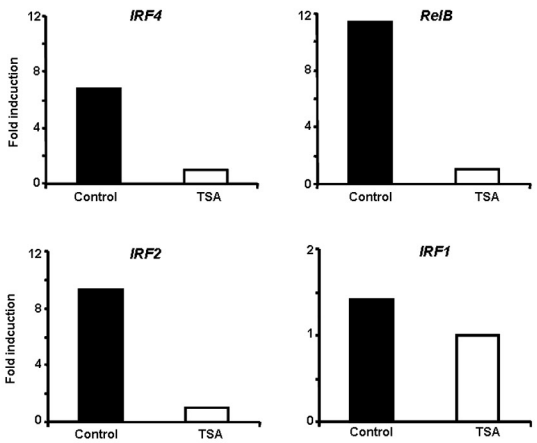

Figure 7