ARTICLE

Intracellular HSP70L1 inhibits human dendritic cell maturation by promoting suppressive H3K27me3 and H2AK119Ub1 histone modifications

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Epigenetic regulation has been attracting increasing attention due to its role in cell differentiation and behaviors. However, the epigenetic mechanisms that regulate human dendritic cell (DC) differentiation and development remain poorly understood. Our previous studies show that extracellular heat shock protein 70-like protein (HSP70L1) is a potent adjuvant of Th1 responses via stimulating DCs when released from cells; however, the role of intracellular HSP70L1 in DC differentiation and maturation remains unknown. Herein, we demonstrate that intracellular HSP70L1 inhibits human DC maturation by suppressing MHC and costimulatory molecule expression, in contrast to the adjuvant activity of extracellular HSP70L1. The stability of intracellular HSP70L1 is dependent on DNAJC2, a known epigenetic regulator. Mechanistically, intracellular HSP70L1 inhibits the recruitment of Ash1l to and maintains the repressive H3K27me3 and H2AK119Ub1 modifications on the promoter regions of costimulatory, MHC and STAT3 genes. Thus, intracellular HSP70L1 is an inhibitor of human DC maturation. Our results provide new insights into the epigenetic regulation of cell development by intracellular HSP70L1.

Keywords: HSP70L1; DNAJC2; Monocyte-derived dendritic cell; Histone modification; H3K27me3; H2AK119Ub1; H3K4me3

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INTRODUCTION

Dendritic cells (DCs) are potent and versatile antigen-presenting cells that are critical for immune responses and immune tolerance.[1,2] DCs are heterogeneous in ontogeny, tissue distribution, phenotypic and cytokine secretion patterns, and biological function.[3–5] Ontogeny studies have indicated that DCs are derived from hematopoietic stem cells that give rise to the four major subsets: conventional DCs (cDCs), Langerhans cells (LCs), monocyte-derived DCs (MoDCs), and plasmacytoid DCs (pDCs). The differentiation pathways of the four DC populations have been well characterized in terms of their progenitors or precursors, required cytokines, and key transcription factors.[6] Recently, epigenetic modifications, namely, DNA methylation, histone modifications, and chromatin remolding, have been shown to be important for immune cell differentiation via regulating transcription.[7,8] However, the roles and mechanisms of epigenetic regulation in DC ontogeny remain poorly understood.

Among the four DC subsets, only MoDCs are inducible under specific inflammatory conditions, while the other three subsets are generated under steady state conditions. cDCs and pDCs share common DC progenitors in bone marrow and are dependent on the presence of FMS-like tyrosine kinase 3 ligand for their development.[9] LCs originate from embryonic monocytes seeding into the skin before birth and require IL-34 for development; thus, in some aspects, they are similar to tissue-resident macrophages.[10,11] MoDCs are induced from monocytes, with inflammatory characteristics.[12] These inflammatory MoDCs have been proposed to be in vivo counterparts of in vitro derivatives of monocytes cultured with GM-CSF and IL-4, highly expressing CD11c and MHC-II.[13,14] When exposed to pathogen-associated molecular patterns or danger-associated molecular patterns (DAMP), all the four DC subsets become potent activators of adaptive immune responses.[15] However, the overactivation of DCs is typically harmful, leading to inflammatory injuries, such as autoimmune diseases. Therefore, studying the epigenetic mechanisms of the regulation of DC differentiation and maturation will help identify the mechanisms of DC-associated immune responses and homeostasis and lead to interventions that artificially regulate immune responses to appropriate intensities and durations to avoid immunological injury.

It is well established that Histone H3 lysine 4 trimethylation (H3K4me3) is associated with active gene transcription, whereas Histone H2A lys119 mono-ubiquitination (H2AK19Ub1) and Histone H3 lysine 27 trimethylation (H3K27me3) are associated with repressive gene transcription.[16–18] Emerging evidence has indicated that dynamic or inducible changes or dysfunction of these histone modifications regulate the differentiation and functional maturation of immune cells, thereby regulating immune responses or directing leukemia development.[19–24] Recent evidence has also demonstrated

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that these active and repressive histone modifications have both stage-specific effects on the promoter regions of transcription factors that direct DC subset commitment during differentiation and stimulus-dependent effects on the promoters of the genes that encode inflammatory signal molecules or phenotypes in DCs.[25–28] Thus, the identification of intrinsic regulators of the histone modifications required for the differentiation or maturation of specific DC subsets is critical for a complete understanding of DC-associated inflammatory responses and homeostasis. MoDCs are superior to the other three DC subsets in mediating inflammation and the artificial manipulation of DC-based immunotherapy because of their precursor number: monocytes represent 10–20% of peripheral blood mononuclear cells. Thus, the identification of intrinsic histone modification regulators of MoDC differentiation and maturation would provide new insights for evading MoDC-mediated inflammatory injury and designing MoDC-based immunotherapy.

Heat shock protein 70-like protein (HSP70L1) was first cloned from a human MoDC cDNA library by our group.[29] Under steady state conditions, intracellular HSP70L1 is one subunit of a heterodimer referred to as mammalian ribosome-associated complex (mRAC), which assists nascent polypeptide folding.[30] The other subunit of mRAC is the HSP40 homolog, DNAJC2.[31–33] DNAJC2 has recently been identified as an epigenetic regulator involved in versatile cell processes, including cell differentiation, senescence and quiescence, and nucleotide excision repair.[34–39] Our previous studies have validated that extracellular HSP70L1 is a strong adjuvant to stimulate Th1 responses via acting on MoDCs.[40] This DC-stimulatory activity relies on HSP70L1 binding to TLR2, TLR4, and CD91 on DCs;[41,42] thus, extracellular HSP70L1 is a DAMP. Furthermore, intracellular priming of DCs with recombinant adenovirus (Ad) carrying the fusion gene of *HSP70L1* together with the N terminal cancer antigen $CEA_{576\text{-}669}$ fragment also potentially stimulates anti-CEA specific $CD8^+$CTL responses.[43] To date, there is limited knowledge regarding the role of intracellular HSP70L1 in DC differentiation and maturation, particularly from the perspective of epigenetic regulation. In this study, we show that intracellular HSP70L1 is an intrinsic inhibitor of MoDC maturation. Mechanistically, intracellular HSP70L1 promotes H3K27me3 and H2AK119Ub1 histone modifications to inhibit the transcription of costimulatory and MHC-II genes. Therefore, our study provides new insight into the epigenetic regulation of immune cell differentiation by intracellular HSP70L1.

MATERIALS AND METHODS

Reagents

Fluorescence-conjugated Abs were obtained from Biolegend, including anti-HLA-DR (APC), anti-HLA-ABC (PerCP-Cy5.5), anti-CD80 (BV421), anti-CD86 (BV605), anti-CD40 (PE-Cy7), anti-CD54 (PE), anti-CD83 (FITC), and secondary Abs (Alexa Fluor 488 and Texas Red). Anti-HSP70L1 was obtained from Abcam. Antibodies specific for DNAJC2, (p)STAT3, (p)STAT5, (p)STAT6, (p)ERK1/2, (p)P38, (p)JNK1/2, H3K27me3, H3K4me3, H2AK119Ub1, GAPDH, Histone H3, and LaminA/C Abs were obtained from Cell Signaling. Anti-Ash1l was obtained from Santa Cruz. Human recombinant GM-CSF and IL-4 were obtained from R&D. Human CD14 immunomagnetic microbeads were obtained from MiltenyiBiotec. Whole cell, nuclear and cytoplasmic lysates were isolated using an M-PER™ Mammalian Protein Extraction Reagent kit or NE-PER™ Nuclear and Cytoplasmic Extraction Reagent kit, respectively (ThermoFisher Scientific). SiRNAs were synthesized by GenePharma, and primers were synthesized by Sangon; recombinant Ads carrying the gene of DNAJC2 or HSP70L1, respectively, were constructed by Hanbio (all in Shanghai, China). The sequences of the siRNA and primers are listed in supplementary Table 1.

Preparation of human MoDCs

Human peripheral $CD14^+$ monocytes were isolated using immunomagnetic microbeads and induced into MoDCs in the presence of hGM-CSF (50 ng/ml) plus hIL-4 (10 ng/ml) for 7 days. The day that monocytes were isolated was set as day 0. In some experiments, human MoDCs were transfected with recombinant Ad vectors at a MOI of 100 on day 1 or day 4 or with siRNAs at 20 nM on day 4. After an additional 36 or 48 h of Ad-mediated transfection or 48 or 72 h of siRNA transfection, MoDCs were collected for subsequent assays.

Quantitative real-time PCR

Quantitative PCR assays were performed with an SYBR RT-PCR kit (Takara) and LightCycler (Roche). Data were normalized to the *actin or gapdh* expression.

FACS

DCs were incubated with the antibody cocktails for 20 min at 4 °C after FcR blocking for phenotypic analysis of costimulatory and MHC molecules as previously described.[44] Data were obtained on Flow Cytometers (DxP Athena Cytekbio and LSRII BD Bioscience) and analyzed with Flowjo software.

Confocal microscopy analysis

MoDCs were fixed for 5 min at 21–23 °C with 2% paraformaldehyde and incubated with specific antibodies in the order of anti-DNAJC2, fluorescent dye-conjugated secondary antibodies, anti-HSP70L1 and fluorescent dye-conjugated secondary antibodies, or with anti-Ash1l and fluorescent dye-conjugated secondary antibodies in BD Perm/Wash buffer, for 120 min (for primary antibodies) or 40 min (for secondary antibodies) at 21–23 °C. After DAPI staining, the cell suspensions were spun onto glass slides using a cytocentrifuge 7620 (WESCOR) via centrifugation at 1000 rpm for 5 min for imaging.

Coimmunoprecipitation and western blot

Antibodies specific for DNAJC2 or HSP70L1 and control IgG were used for coimmunoprecipitation. The following antibodies were used for the western blots specific for DNAJC2, HSP70L1, (p)STAT3, (p)STAT5, (p)STAT6, (p)ERK1/2, (p)P38, (p)JNK1/2, GAPDH, Histone3 H3, H3K4me3, H3K27me3, H2AK119Ub1, and LaminA/C, respectively. Whole cell, nuclear and cytoplasmic lysates were prepared according to the manufacturers' instructions, and some lysates were first immunoprecipitated by specific or control Abs in some experiments. Protein extractions were loaded on SDS-PAGE, transferred to nitrocellulose membranes, and blotted as previously described.[45]

Chromatin immunoprecipitation-qPCR (ChIP-qPCR)

ChIP was performed as previously described.[46] The antibodies used for ChIP were specific for H3K27me3, H3K4me3, H2AK119Ub1, Ash1l, DNAJC2, and control Rabbit IgG, respectively. Immunoprecipitated DNA was purified using a PCR Purification Kit (Qiagen) and quantified by real-time PCR using an SYBR PCR kit and LightCycler. The PCR primer-pairs of the *CD40*, *CD86*, and *HLA-DR* promoter genes are listed in supplementary Table 1. Relative enrichment was first normalized to input chromatin and then calculated as folds of the control ChIP IgG. The results represent mean±s.d. of three independent PCR reactions from a single ChIP experiment that is representative of three independent experiments.

Statistical analysis

Statistical analysis was performed using Prism (GraphPad software) and Student's *t*-test, and significance was established at $p < 0.05$.

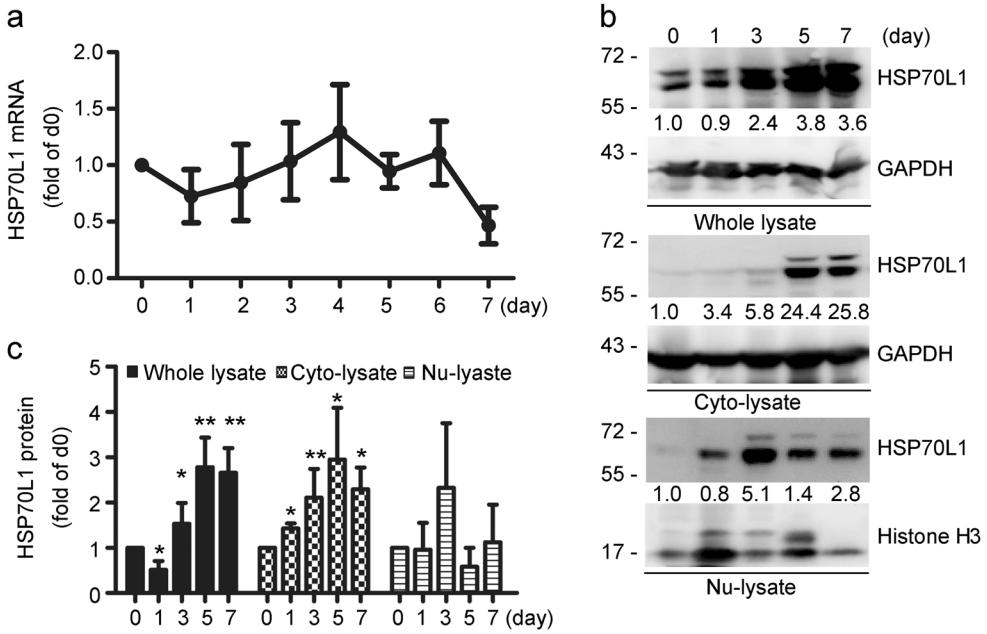

Fig. 1 HSP70L1 expression is dynamic during the differentiation of MoDCs. **a**–**c** Real-time PCR (**a**) and WB (**b**, **c**) analyses of the mRNA and protein amounts of HSP70L1 in whole cell, nuclear, or cytoplasmic lysates from MoDCs, respectively, at indicated times during the differentiation of monocytes into DCs induced by GM-CSF and IL-4 for 7 days. Data are normalized to *actin* (**a**), GAPDH or Histone H3 (**b**, **c**), and day 0 group (monocytes) is set as 1. Data are shown as the mean±s.e.m. of three to five independent experiments (**a**, **c**), and one representative result is shown (**b**)

RESULTS

Dynamic expression of HSP70L1 during human DC differentiation

To investigate the intracellular function of HSP70L1 in the differentiation of MoDCs, we first measured its expression during the course of differentiation. We found that the HSP70L1 mRNA transiently decreased at the early stage and then gradually increased until the late stage; however, it decreased again at the end of differentiation (Fig. 1a). The dynamic expression of the HSP70L1 protein in whole cell lysates was similar to its mRNA expression; cytoplasmic HSP70L1 did not exhibit an obvious decrease, although it was low at the early stage and progressively increased with the differentiation of MoDCs, while nuclear HSP70L1 was more varied at distinct stages of differentiation (Fig. 1b, c). Interestingly, cytoplasmic and nuclear HSP70L1 had distinct band-patterns. In addition to a low MW band of 58 KD, cytoplasmic HSP70L1 had a high MW band around 60 KD, while the nuclear HSP70L1 bands were more varied among distinct individuals: some HSP70L1 had only a 60 KD band or two additional MW bands around 72 KD (Supplementary Figure S1). The expression of HSP70L1 at high MW was upregulated from the middle to late stages of differentiation, which suggests that the post-translation modification (PTM) of HSP70L1 was dynamic during the differentiation of MoDCs and different between the cytoplasm and nucleus. In summary, HSP70L1 has temporally and spatially dynamic expression and PTMs during the differentiation of MoDCs.

HSP70L1 stability is dependent on DNAJC2

As HSP70L1 interacts with DNAJC2 to form mRAC, we subsequently measured their interaction in the cytoplasm and nucleus, respectively, by coimmunoprecipitation of lysates from the separated compartments. The immunoprecipitation of DNAJC2 or HSP70L1 from cytoplasmic lysates confirmed the interaction between DNAJC2 and HSP70L1 (Fig. 2a). The immunoprecipitation of nuclear DNAJC2 could co-precipitate HSP70L1, whereas the immunoprecipitation of nuclear HSP70L1 could not co-precipitate DNAJC2 (Fig. 2a). This difference might be due to the different modifications of HSP70L1 present in the nuclear and cytoplasmic HSP70L1-DNAJC2 complexes: the available anti-HSP70L1 antibody cannot efficiently precipitate the nuclear HSP70L1, which interacts with DNAJC2; moreover, nuclear HSP70L1 immunoprecipitated by this anti-HSP70L1 antibody was almost undetectable, which indicates its inefficiency when applying to immunoprecipitation of nuclear lysates.

We subsequently evaluated the effects of DNAJC2 on the HSP70L1 expression. We found that silencing DNAJC2 via siRNA downregulated the amounts of HSP70L1, and vice versa (Fig. 2b, c and Supplementary Figure S2a and S2b). Thus, DNAJC2 is critical for the HSP70L1 expression. We then measured the DNAJC2 expression during the differentiation of MoDCs. Similar to HSP70L1, the amounts of the DNAJC2 mRNA were initially reduced at the early stage, then increased from the middle to late stages, and decreased again at the end of DC differentiation (Fig. 2d). The dynamic expression of total DNAJC2 and nuclear DNAJC2 at the protein level was similar to its mRNA expression, while cytoplasmic DNAJC2 did not exhibit an obvious decrease at the early stage, although it progressively increased from the early to late stages and ultimately decreased at the end of MoDC differentiation (Fig. 2e and Supplementary Figure S2c). Confocal microscopy assays indicated that the colocalization of HSP70L1 and DNAJC2 was obvious at the middle stage and occurred mainly in the cytoplasm, while at the late stage, their nuclear locations were obvious, whereas their co-localization was reduced; these findings suggest that the two molecules had different nuclear functions at the late stage (Fig. 2f). Taken together, DANJC2 is critical for the stability of HSP70L1 and has similar expression dynamics as HSP70L1. They interact with each other but may also function independently at distinct stages of MoDC differentiation.

Intracellular HSP70L1 inhibits the maturation of MoDCs

To investigate the roles of intracellular HSP70L1 in the differentiation and development of MoDCs, we analyzed the differentiation and antigen-presenting function of MoDCs by siRNA-induced silencing and Ad-mediated overexpression. According to its expression pattern during differentiation, we transfected MoDCs with Ad carrying the *HSP70L1* gene (AdHSP70L1) on day 1 to

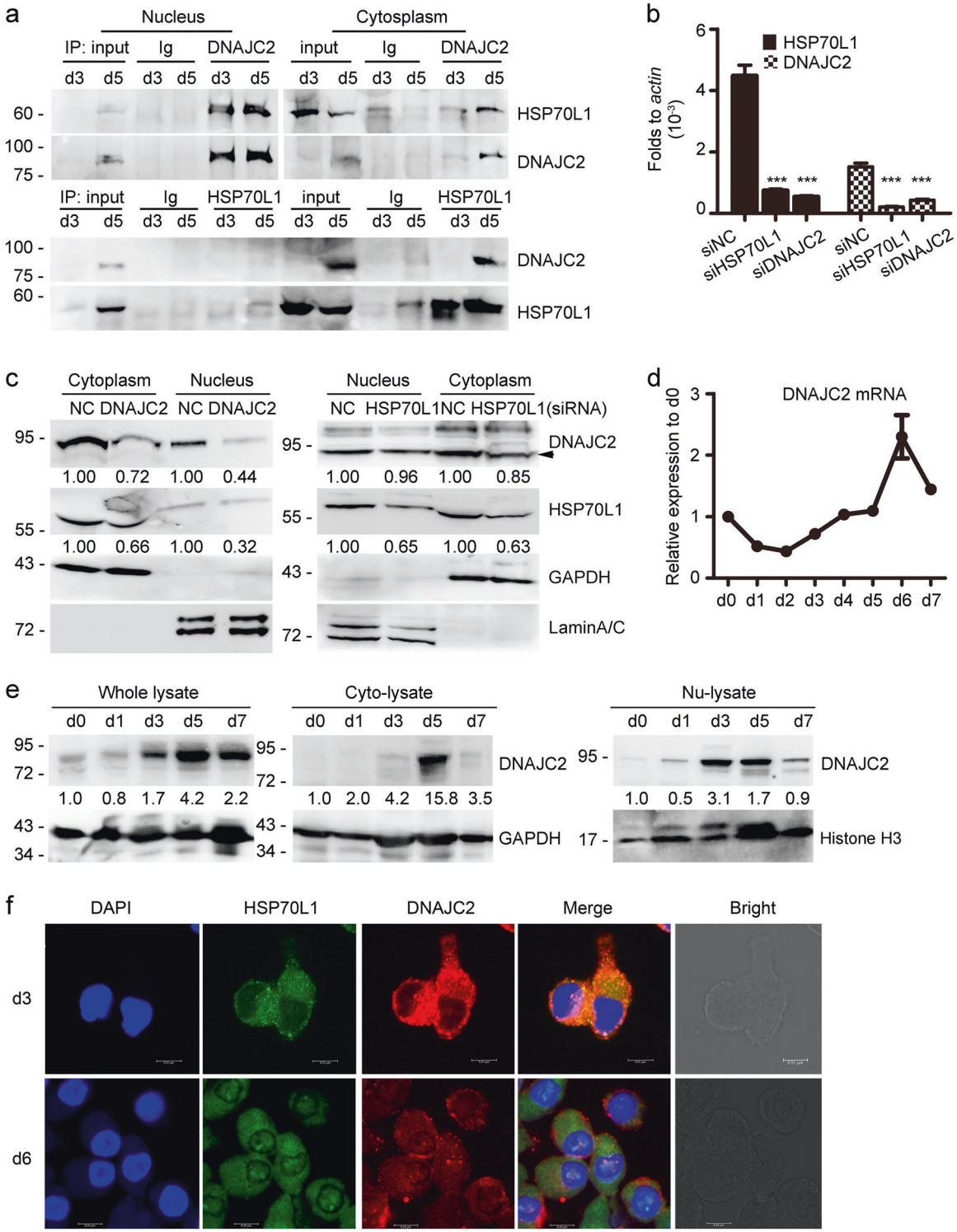

Fig. 2 HSP70L1 and DNAJC2 interact with and stabilize each other. **a** Coimmunoprecipitation analysis of the interaction between HSP70L1 and DNAJC2 at indicated time during the differentiation of MoDCs. **b**, **c** Real-time PCR (**b**) and WB (**c**) analyses of the expression of HSP70L1 and DNAJC2 in MoDCs transfected with siRNAs of control (siNC), HSP70L1 (siHSP70L1) or DNAJC2 (siDNAJC2) on day 4 for 48 h (PCR) or 72 h (WB), respectively. Data are normalized to *actin* (**b**), GAPDH (**c**, cytoplasmic lysate), or LaminA/C (**c**, nuclear lysate), and siNC group is set as 1 (**c**). **d**, **e** Real-time PCR (**d**) and WB (**e**) analyses of the expression of DNAJC2 at indicated time during the differentiation of MoDCs. Data are normalized to GAPDH (**d**, **e**, whole cell and cytoplasmic lysate) or Histone H3 (**e**, nuclear lysate), and day 0 group is set as 1. **f** Confocal microscopy analysis of the expression of HSP70L1 and DNAJC2 in MoDCs on days 3 and 6, respectively (TCS-SP8, Leica, Wetzlar, Germany). Bars are indicated in figures. Data are shown as the mean ±s.e.m. of three experiments (**b**, **d**). One representative result of two (**a**, **f**) or three (**c**, **e**) independent experiments is shown. ***$P < 0.001$ compared to siNC group (*t* test)

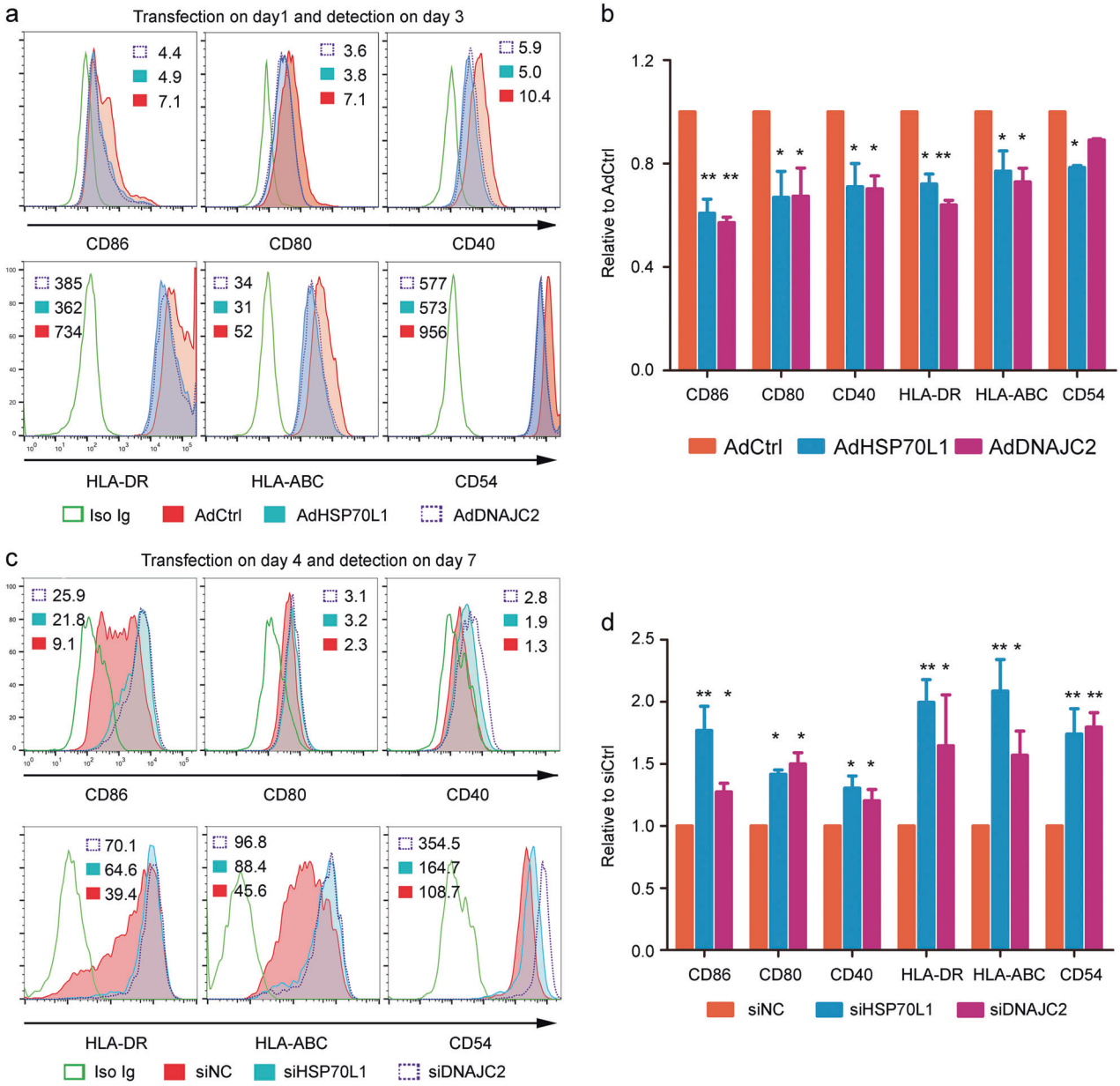

Fig. 3 HSP70L1 and DNAJC2 inhibit the maturation of MoDCs. **a**–**d** The expression of costimulatory and MHC molecules was detected using FACS on MoDCs transfected with control Ad (AdCtrl), AdHSP70L1, or AdDNAJC2 on day 1 (**a**, **b**) or with siRNAs of control (siNC), HSP70L1 or DNAJC2 on day 4 (**c**, **d**). Numbers in histograms (**a**, **c**) that represent one of three independent experiments are relative fluorescence intensities to isotype IgG staining (**a**, **c**), and data in (**b**, **d**) are shown as the mean±s.e.m of three experiments. *$p < 0.05$, **$p < 0.01$ compared to AdCtrl or siNC group (*t* test)

overexpress HSP70L1 at the early stage in the course of differentiation or transfected MoDCs with siRNA of HSP70L1 (siHSP70L1) on day 4 to silence its upregulation at the late stage. After 48–72 h of transfection, we analyzed DC and monocyte lineage markers, including CD11c and CD14, respectively, MHC, and costimulatory and adherent molecules. We found that CD14 was downregulated to undetectable expression, while CD11c was highly expressed; however, the expression of CD11c had no obvious changes on these MoDCs whether transfected with AdHSP70L1 at the early stage or siHSP70L1 at the late stage compared to the corresponding control group (Supplementary Figure 3), which indicates that HSP70L1 had no effect on the differentiation of monocytes into DCs. However, CD86, CD80, CD40, HLA-ABC, HLA-DR, and CD54 were significantly reduced on HSP70L1-overexpressing MoDCs once transfected with AdHSP70L1 at the early stage (Fig. 3a, b) and significantly upregulated on MoDCs once transfected with siHSP70L1 at the late stage (Fig. 3c, d). Consistently, DNAJC2 had similar effects as HSP70L1, likely due to their mutual stabilization of each other's expression (Fig. 3). Overall, intracellular HSP70L1 inhibits the functional maturation of MoDCs.

Intracellular HSP70L1 suppresses the activation of STAT3, STAT5, ERK, and P38 pathways

To ascertain the mechanisms that underlie the inhibitory effects of HSP70L1, several known pathways involved in MoDC development and maturation were evaluated, including the STAT and MAPK pathways. The transfection of MoDCs with AdHSP70L1 on day 1 mainly inhibited the activation of the STAT3 and STAT5 pathways via suppressing the expression of STAT3 or the

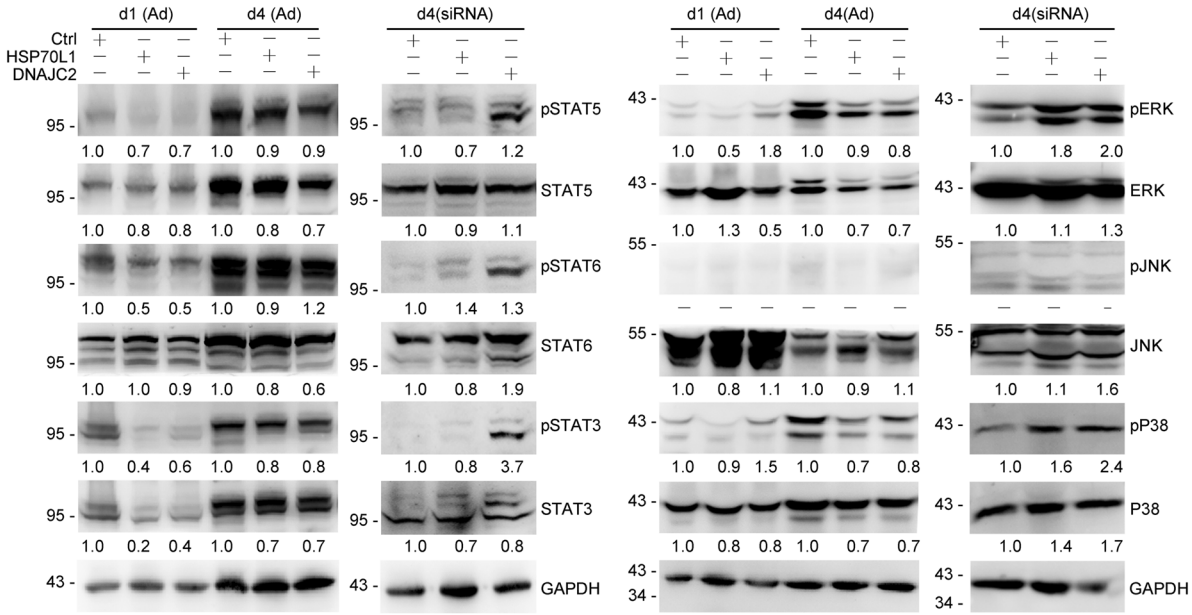

Fig. 4 HSP70L1 and DNAJC2 inhibit the activation of the STAT3, STAT5, ERK, and P38 pathways in MoDCs. The phosphorylation and constitutive expression of STAT3, STAT5, STAT6, ERK, P38, and JNK were detected after 36 h of transfection of MoDCs with AdCtrl, AdHSP70L1, or AdDNAJC2, respectively, on days 1 and 4, or 48 h of transfection of MoDCs with siNC, siHSP70L1, or siDNAJC2 on day 4. Gray intensities were normalized to GAPDH (constitutive expression) or corresponding non-phosphorylated transcription factors, and AdCtrl or siNC group was set as 1. Representative results of three independent experiments are shown

phosphorylation of STAT3 and STAT5, while other pathways had no obvious changes (Fig. 4 and Supplementary Figure S4a). The transfection of MoDCs with AdHSP70L1 on day 4 did not have a substantial effect on the previously described STAT pathways, whereas it mainly inhibited the phosphorylation of the ERK and P38 pathways (Fig. 4 and Supplementary Figure S4b). Consistently, silencing HSP70L1 at the late stage significantly enhanced the phosphorylation of ERK and P38 (Supplementary Figure S4c). DNAJC2 had both similar and different effects as HSP70L1 (Fig. 4 and Supplementary Figure S4a-c), which implies they have both common and different mechanisms. In summary, HSP70L1 inhibits the activation of the STAT3 and STAT5 pathways at the early stage and the ERK and P38 pathways at the late stage.

HSP70L1 regulates the trimethylation of Histone H3 at lysine 4 and 27

Histone modifications are tightly associated with cell differentiation and function and occur at the promoter and enhancer regions that impact the transcription of genes. To investigate whether the inhibition by HSP70L1 on the maturation of MoDCs was associated with the histone epigenetic modifications, we detected the effects of HSP70L1 on H3K4me3, H3K27me3, and H2AK119Ub1 in MoDCs transfected with AdHSP70L1 on day 1. The overexpression of HSP70L1 inhibited H3K4me3, enhanced H3K27me3 and had no obvious effect on H2AK119Ub1 (Fig. 5 and Supplementary Figure S5a and S5b). Similarly, so did as overexpression of DNAJC2 by transfection with AdDNAJC2, which implies they have common effects on Histone H3 modifications. Thus, HSP70L1 changes the trimethylation modifications of Histone H3 at lysine 4 and 27.

HSP70L1 promotes H3K27me3 and H2AK119ub1 modifications on the *CD40*, *CD86*, *HLA-DR*, *and STAT3* promoters

We subsequently investigated the modifications of H3K4me3, H3K27me3, and H2AK119Ub1 on the promoters of costimulatory, MHC and STAT3 genes that are critical for the binding of transcription factors.[20,25] ChIP assays indicated that H3K27me3 and H2AK119Ub1 were both increased on most regions of the *CD40*, *CD86*, and *HLA-DR* promoters, and H3K4me3 was increased

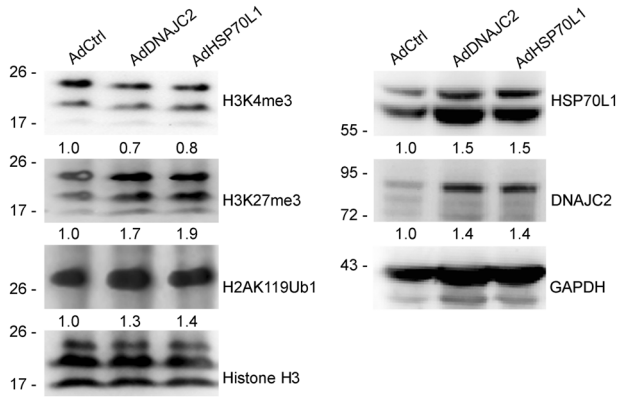

Fig. 5 Overexpression of HSP70L1 and DNAJC2 enhances H3K27me3 and inhibits H3K4me3 in MoDCs. WB analysis of the H2AK119Ub1, H3K4me3, and H3K27me3 modifications in MoDCs after 48 h of transfection with AdCtrl, AdDNAJC2, or AdHSP70L1 on day 1. Gray intensities are normalized to Histone H3 or GAPDH, and AdCtrl group is set as 1. Representative results of three (right panel) or six (left panel) independent experiments are shown

on the −576 bp region of the *CD40* gene and some regions of the *CD86* and *HLA-DR* promoters in MoDCs transfected with AdHSP70L1 at an early stage (Fig. 6a). In contrast to HSP70L1, the overexpression of DNAJC2 in MoDCs at the early stage mainly inhibited H3K4me3 on the promoter regions of the *CD40*, *CD86*, and *HLA-DR* genes and had no obvious effects on H3K27me3 and H2AK119Ub1 (Fig. 6a). Furthermore, HSP70L1 enhanced H3K27me3 on most regions of the *STAT3* promoter and both H2AK119Ub1 and H3K4me3 on the −1915 bp region (Fig. 6b), which supports the reduced expression of STAT3 in MoDCs transfected with AdHSP70L1. DNAJC2 also enhanced H2AK119Ub1 on the −1915 bp and −766 regions of the *STAT3* promoter, whereas it mainly inhibited H3K4me3 on the *STAT3* promoter (Fig. 6b). Interestingly, both HSP70L1 and DNAJC2 inhibited H2AK119Ub1 on the −1508 bp region of the STAT3 promoter.

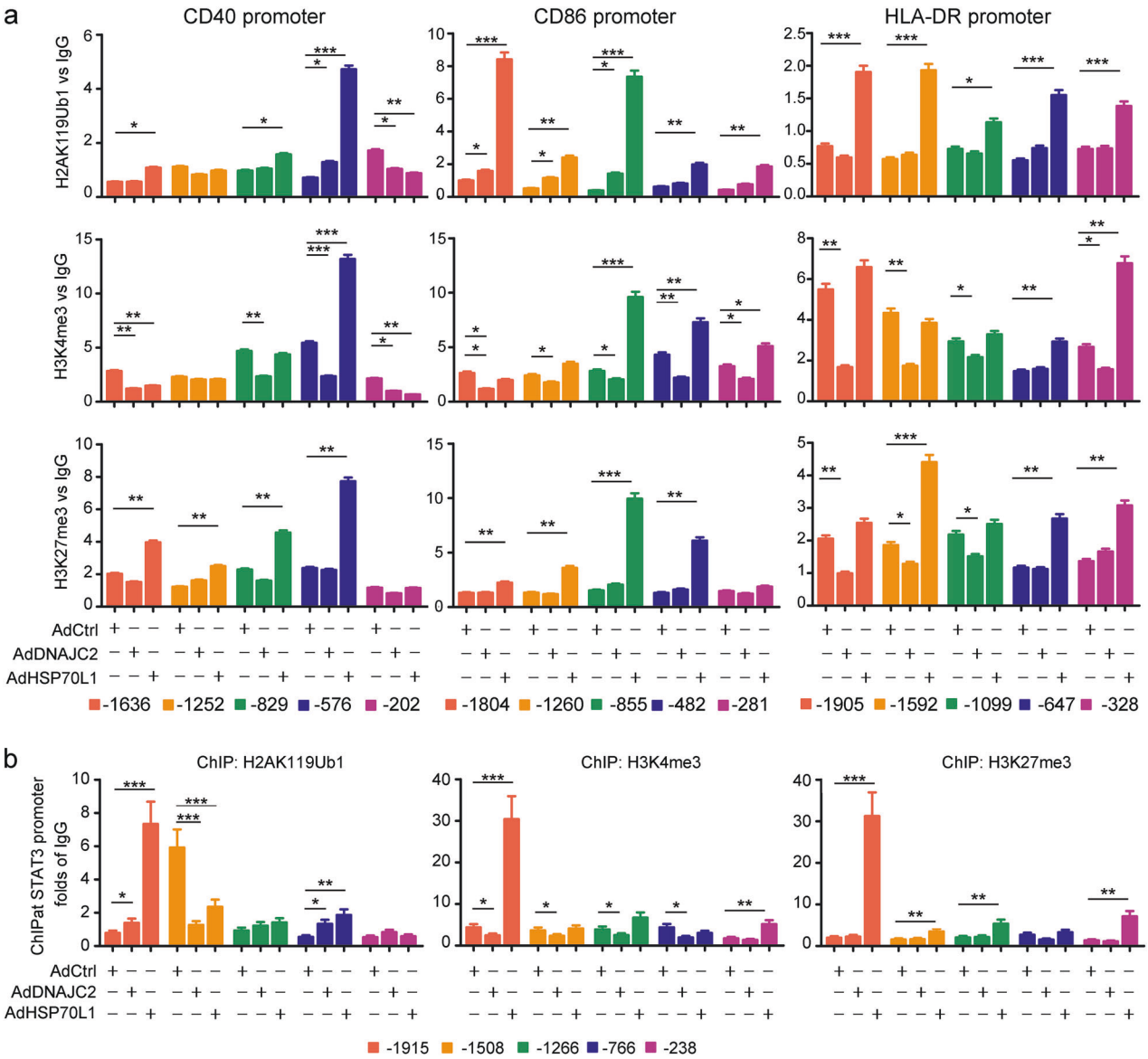

Fig. 6 Effects of the overexpression of HSP70L1 and DNAJC2 on the H2AK119Ub1, H3K4me3, and H3K27me3 modifications of the *CD40*, *CD86*, *HLA-DR*, and *STAT3* promoters in MoDCs at early stage. ChIP analysis of the H2AK119Ub1, H3K4me3, and H3K27me3 modifications on the *CD40*, *CD86*, *HLA-DR*, and *STAT3* promoter regions after 48 h of MoDCs transfected with AdCtrl, AdDNAJC2, or AdHSP70L1 on day 1. Data are normalized to input and then control IgG is set as 1. Data are shown as the mean ± s.d. of three determinants in one ChIP experiment. Representative results of three independent experiments are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to AdCtrl group (*t* test)

Thus, HSP70L1 mainly promotes the suppressive H3K27me3 and H2AK119Ub1 modifications on the promoters of the *CD40, CD86, HLA-DR*, and *STAT3* genes.

HSP70L1 inhibits the recruitment of Ash1l to the promoter regions of CD40, CD86, HLA-DR, and STAT3 in MoDCs

The mammalian Ash1 homolog, Ash1-like (Ash1l), is responsible for the trimethylation of H3K4 and H3K36.[19,21,47,48] Polycomb-repressive complex 2 (PRC2) catalyzes the trimethylation of H3K27, and PRC1 catalyzes the mono-ubiquitylation of H2Ak119 in an H3K27me3-dependent or independent manner.[49,50] DNAJC2 is a known antagonist of PRC1: it de-represses PRC1-silenced genes via displacing PRC1 from chromatin and then acting together with an H2A-specific deubiquitinase to catalyze H2AK119Ub1.[46,49] Therefore, to further understand how HSP70L1 affects histone modifications in MoDCs, we analyzed the recruitment of Ash1l and DNAJC2 on the promoter regions of *CD40*, *CD86*, and *HLA-DR* in MoDCs transfected with AdHSP70L1 at an early stage. The recruitment of Ash1l to the promoter regions of *CD40*, *CD86*, and *HLA-DR* in MoDCs was strongly inhibited by the overexpression of HSP70L1 at the early stage, and the abundance of DNAJC2 on the promoter regions of *CD40*, *CD86*, and *HLA-DR* was also clearly inhibited (Fig. 7a). Consistently, the transfection of MoDCs with AdDNAJC2 at the early stage also inhibited the recruitment of Ash1l and DNAJC2 to these promoter regions (Fig. 7a). Moreover, the transfection of MoDCs with AdHSP70L1 or AdDNAJC2 at the early stage also inhibited the recruitment of Ash1l to the *STAT3* promoter regions (Fig. 7b), potentially leading to the reduced active H3K4me3 or increased H3K27me3 and H2AK119Ub1 modifications on the *STAT3* promoter. Consistently, the overexpression of HSP70L1 inhibited the nuclear translocation and the expression of Ash1l (Fig. 7c, d). Thus, HSP70L1 inhibits the

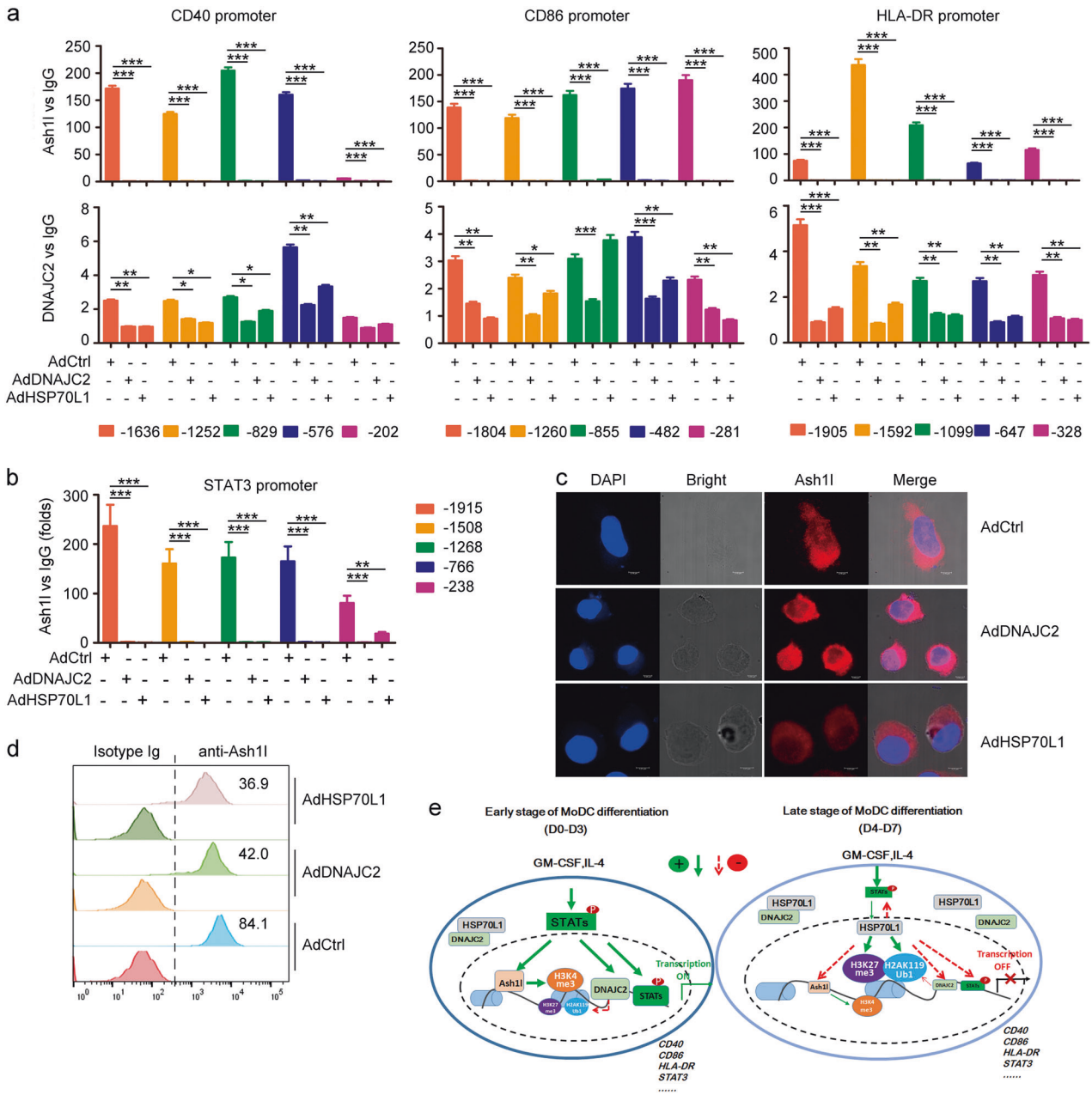

Fig. 7 Effects of the overexpression of HSP70L1 and DNAJC2 on the recruitment of Ash1l to the *CD40*, *CD86*, *HLA-DR*, and *STAT3* promoters in MoDCs. **a**, **b** ChIP analysis of the recruitment of Ash1l (**a**, **b**) and DNAJC2 (**a**) to the *CD40*, *CD86*, *HLA-DR*, and *STAT3* promoter regions after 48 h of MoDCs transfected with AdCtrl, AdDNAJC2, or AdHSP70L1 on day 1. Data are normalized to input, and control IgG is set as 1. Data are shown as the mean ± s.d. of three determinants in one ChIP. Representative results of three (**a**) or two (**b**) independent experiments are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to AdCtrl group (*t*-test). (**c**, **d**) Confocal microscopy (**c**) and flow cytometry (**d**) analyses of the expression of Ash1l in MoDCs after 48 h of transfection with AdCtrl, AdDNAJC2, or AdHSP70L1 on day 1. Numbers in histograms that represent one of two independent experiments are relative fluorescence intensities to isotype IgG staining (**d**). **e** A mechanistic scheme of intracellular HSP70L1 inhibiting the maturation of MoDCs

enrichment of Ash1l on the *CD40*, *CD86*, *HLA-DR*, and STAT3 promoter regions.

Taken together, the expression and post-translation modifications of HSP70L1 are dynamic in the course of monocytes differentiating into DCs, and its stability is dependent on its mRAC partner DNAJC2. Beyond its cytoplasmic mRAC-associated function, HSP70L1 can transfer into the nucleus, particularly from the middle to late stages of MoDC differentiation, and enhance the suppressive H3K27me3 and H2AK119Ub1 modifications on the promoters of costimulatory, MHC-II and STAT3 genes, potentially due to its inhibition of Ash1l recruitment, which thereby suppresses the transcription of these genes to block the further maturation of MoDCs (Fig. 7e).

DISCUSSION

Herein, we have demonstrated that intracellular HSP70L1 functions as an inhibitor in MoDC maturation via epigenetically regulating histone modifications. In the course of MoDC differentiation and development, the low expression of HSP70L1 at the early stage is associated with the removal of suppressive histone modifications, potentially triggered directly by GM-CSF and/or IL-4-induced signals,

which thereby initiate differentiation. During development, the progressively increased HSP70L1 expression sets a barrier to block overactivation of MoDCs by modulating their proper maturation. Thus, such finely tuned regulation of HSP70L1 expression at distinct developmental stages of MoDCs embodies a self-regulation feedback mechanism in cell differentiation.

Changes in Histone H3 modifications are associated with the development of MoDCs via regulating the on/off states of DC maturation-associated genes and the corresponding signaling genes, including transcriptional factors, activators, or regulators of signaling pathways, because some inhibitors, such as LSD1, PKMT G9a, and Ezh2, inhibit the expression of costimulatory and MHC-II molecules on MoDCs, as well as affect histone modifications and the activation of signaling pathways (data not shown). The overexpression of HSP70L1 does not widely inhibit the constitutive expression but rather the phosphorylation of STAT5,6, ERK and P38. The results indicate that HSP70L1 may be involved in the transcriptional regulation of kinases or other components that act in the JAK/STATs and MAPK pathways, or HSP70L1 regulates the JAK/STATs and MAPK pathways directly in the cytoplasm. Moreover, signaling pathways, such as JAK/STATs and MAPKs, are also expected to affect histone modifications. Thus, histone modifications and signaling pathways reciprocally regulate each other to form a complicated network in the development of MoDCs. Increased expression and PTMs of HSP70L1 should be the result of this interacting network from the middle stage, and with its accumulation, it enters into the nucleus and feedback inhibits the states of DC maturation-associated genes and signaling molecule genes to block the overactivation of MoDCs. However, we cannot determine whether HSP70L1-mediated epigenetic inhibition only occurs on the genes related to DC maturation. A genome-wide analysis of histone modifications in MoDCs at distinct stages of differentiation and with loss- or gain-of-function of HSP70L1 will provide a more detailed mechanistic explanation of its inhibitory effects on MoDC maturation from the perspective of histone modifications.

HSP70L1 is typically associated with DNAJC2 in the cytoplasm; however, their epigenetic regulatory mechanisms do not completely overlap with respect to histone modifications. HSP70L1 promotes the suppressive modifications of histone, such as H3K27me3 and H2AK119Ub1, whereas DNAJC2 mainly inhibits the active modification of histones, H3K4me3. One common effect might be dependent on the suppression of the recruitment of Ash1l to the promoter genes of costimulatory and MHC molecules. Acting as a histone methyltransferase that methylates H3K36 and H3K4, Ash1l has been reported to enhance or repress gene transcription, relying on the genomic contexts of its targeted genes.[51] In the present study, Ash1l is regarded as an activator of the maturation of MoDCs because both DNAJC2 and HSP70L1 strongly inhibit its abundance on the promoters of the costimulatory, MHC and STAT3 genes of MoDCs. The inhibition of Ash1l recruitment by HSP70L1 may contribute to the high levels of H3K27me3 and H2AK119Ub1 in MoDCs because H3K36me2 or H3K36me3 catalyzed by Ash1l is known to counteract PRC-mediated silencing.[52,53] DNAJC2 inhibits H3K4me3 potentially via directly reducing Ash1l abundance on the promoter regions of costimulatory and MHC genes. Further understanding of how HSP70L1 and DNAJC2 inhibit the development of MoDCs should focus on the mechanisms that inhibit the abundance of Ash1l and the recruitment of Ash1l to the promoter regions of costimulatory and MHC genes.

The codependence of HSP70L1 and DNAJC2 suggests that their interaction may contribute to their stabilization of each other. This interaction also exists in the nucleus, but at a low extent compared to their cytoplasmic interaction. The mechanism of HSP70L1 nuclear localization remains unclear; however, it may involve DNAJC2, as DNAJC2 is a known epigenetic factor that is recruited into the nucleus. Changes in post-translation modification may be involved in this process. Our finding that nuclear HSP70L1 has distinctly higher MW bands among individuals implies that the PTM of nuclear HSP70L1 is complicated and different from its cytoplasmic counterpart.

Interestingly, we found that transfection of AdDNAJC2 in MoDCs at the early stage of differentiation inhibited its localization on the promoter regions of costimulatory and MHC genes. First, this finding implies that inherent DNAJC2 is directly implicated in the differentiation of MoDCs via binding to the transcriptional regulatory regions of costimulatory and MHC genes, with active effects. Second, it implies that the stimulatory effect of DNAJC2 is low dose-dependent, whereas high levels of intracellular DNAJC2 are harmful. The high expression of intracellular DNAJC2, giving rise to epigenetic modifications, potentially impedes its binding to the promoter regions of costimulatory and MHC genes.

The co-occurrence of H3K4me3 and H3K27me3 on some promoter regions of *CD40*, *CD86*, *HLA-DR*, and *STAT3* genes of MoDCs transfected with AdHSP70L1 indicates a bivalent state of histone modification on these genes. Many developmental genes in embryonic stem cells have such co-occurrence of H3K4me3 and H3K27me3 histone modifications, referred to as bivalency, which is surmised as a poised state.[54] It has been shown that the bivalency is on the hypomethylated CpG-rich promoter regions and is transcription suppressive.[55] These hypomethylated CpG-rich promoters represent a switched state between a bivalent off and an H3K4me3-only on. This mode is indicative of a dually suppressive control mechanism in which H3K27me3 represses transcription and H3K4me3 represses DNA methylation via preventing the recruitment of DNA methyltransferases. Thus, costimulatory, MHC and STAT3 genes in MoDCs transfected with AdHSP70L1 at an early stage might represent such a case, with H3K4me3 reducing methylation in the CpG-rich promoter regions and H3K27me3 suppressing their transcription.

Our previous study has shown that intracellular $CEA_{576-669}$HSP70L1 promotes functional maturation. The structural changes caused by fusion of the $CEA_{576–669}$ fragment to the N-terminus of HSP70L1 could be responsible for the contrasting effects between $CEA_{576-669}$HSP70L1 and HSP70L1 because intracellular $CEA_{576-669}$HSP70L1 could not interact with DNCAJC2 (data not shown). Moreover, $CEA_{576-669}$HSP70L1 had no significant effects on the expression of intracellular HSP70L1 and its interaction with DNAJC2, which indicates that the stimulatory roles of $CEA_{576-669}$HSP70L1 in DC maturation were independent of the intrinsic HSP70L1-associated signaling pathways, and its stimulatory effect was confirmed to be dependent on the induction of IFN-β and the activation of the STAT1 pathway by our previous study.[43]

Overall, our results demonstrate a novel role of intracellular HSP70L1 in the differentiation and development of DCs from monocytes by controlling histone modifications of costimulatory and MHC genes, via common and distinct epigenetic mechanisms with its partner DNAJC2. Further investigations on how HSP70L1 enters the nucleus, the PTM of HSP70L1 and how nuclear HSP70L1 affects epigenetic modifications at the genome-wide level will provide further insights into the mechanisms of cell differentiation and development.

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ADDITIONAL INFORMATION

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