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Cystatin C regulates MHC II-peptide presentation and Erk-dependent polarizing cytokine production by bone marrow-derived dendritic cells

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ABSTRACT

Cystatin C is a ubiquitously expressed cysteine protease inhibitor that protects cells from either improper hydrolysis by endogenous proteases or pathogen growth/virulence by exogenous proteases. Although commonly used as a serum bio-marker for evaluating renal function, cystatin C is associated with many immunological disorders under various pathophysiological conditions. How cystatin C affects immune cells, especially dendritic cells (DCs), however, is far from clear. In this study, we found that pharmacological treatment with or genetic over-expression of cystatin C in bone marrow-derived DCs (BMDCs) reduced their capacity to stimulate $CD4^+$ T cell proliferation, despite increased antigen uptake. This reduced capacity corresponded with reduced MHC-II presentation due to diminished levels of the chaperon H2-DM in BMDCs. Instead of promoting proliferation, cystatin C promoted skewing of T cells towards pro-inflammatory Th1/Th17 differentiation. This was mediated by augmenting Erk1/2 MAP kinase phosphorylation in BMDCs, leading to secretion of polarizing cytokines, which in turn led to the Th deviation. Collectively, our study explained the cellular and molecular basis of how this protease inhibitor can regulate immune responses, namely by affecting BMDCs and their cytokine pathway. Our results might open up an avenue for the development of therapeutic agents for the treatment of cystatin C-related immunological diseases.

INTRODUCTION

Cystatin C is an alkaline type II cystatin with a molecular mass of 13,343 Da. It strongly inhibits the activities of papain-like cysteine proteases and legumain [1, 2]. It is ubiquitously expressed in nucleated cells and is secreted into various biological fluids including urine, blood, seminal fluid, saliva and cerebrospinal fluid [3], with physiological levels ranging from 0.5 mg/L in urine to 49 mg/L in seminal fluid [4].

~~Increased l~~Levels of cystatin C have been ~~reported~~ found to increase under certain inflammatory states e.g. in the synovial fluid ~~of patients with~~ during rheumatoid arthritis [5], in saliva ~~of patients with~~ during periodontitis [6] and in bronchoalveolar lavage fluid ~~from patients with~~ during emphysema [7]. As well as being an enzyme inhibitor, it is involved in signaling in multiple biological processes, such as protein turnover, regulation of phagocytosis, and activation of precursor proteins (e.g. enzymes and pro-hormone~~), major histocompatibility complex-II (MHC-II) mediated antigen presentation and apoptosis~~ [8, 9]. Clinically, increased cystatin C in the blood is a biomarker of disturbed glomerular filtration [10].

Recently, there has been growing evidence ~~suggests~~ that cystatin C is also involved in numerous immunological processes [8, 9, 11, 12]. Under certain pathological states, cystatin C can affect inflammation and apoptosis [13-15]. Mechanistically, it mediates immunomodulation not only by controlling the activity of cysteine proteases but also by other mechanisms [14]. For example, cystatin C may contribute to the proteolytic processing of pro-granzymes and other substrates in immune cells, Major Histocompatibility Complex-II (MHC-II) ~~MHC-II~~ mediated antigen presentation and maturation of dendritic cells (DCs) [8, 9], modulation of integrin function and formation of the skin barrier [16, 17]. Indeed, the association of cystatin C with various immune responses was so strong that the levels of extracellular cystatin C had diagnostic value ~~or were used~~ as a marker for disease prognosis in many inflammatory disorders [18].

The immunomodulatory functions of cystatin C may also be useful for treating infectious diseases. Cysteine proteases secreted by parasites not only facilitated their invasion by digesting host extracellular matrix, but also deviated~~s~~ the immune response to a Th2 response which ~~favors~~ favoured parasite proliferation [19]. Therefore, inhibition of these cysteine proteases led to a switch from the usual inefficient Th2 response to a curative Th1 response [20]. Similarly, fatal visceral leishmaniasis in BALB/c mice can be circumvented by co-administration of cystatin ~~together with~~ and interferon-γ (IFN-γ) [21]. This effect was correlated with up-regulation of nitric oxide (NO) generation and Th2 to Th1 conversion, leading to parasite elimination [21, 22].

Despite the remarkable effect of cystatin treatment on clearing pathogen infection by switching of Th responses, the major cellular targets affected by cystatin C eliciting this effect remain to be identified. DCs are the initiators of host specific immunity, and its unique cytokine profiles and antigen presentation ability ~~affect macrophages, monocytes, and most importantly,~~ can elicit T cell proliferation and Th1/Th2 differentiation. Interestingly, although cystatin C was expressed in all nucleated cells, it was preferentially expressed in immune cells, especially on DCs [13,23]. ~~It has been reported that~~ Although cystatin C has been reported to ~~can~~ inhibit the major cysteine protease cathepsin S in DCs to down-regulate MHC-II processing[24-26]. ~~O~~, other studies~~, however,~~ found no role of this protease inhibitor in MHC-II/antigen presentation in DCs [23, 27, 28]. Thus, the functions of cystatin C in DC-mediated immunity remained controversial.

In this study, we tested the effect of cystatin C on bone marrow-derived DCs (BMDCs) by pharmacological addition of recombinant protein or by genetic over-expression. We comprehensively examined BMDC function ~~and found that~~. ~~o~~On the one hand, we found that cystatin C ~~can~~ could down-regulate the MHC-II chaperon H2-DM resulting in diminished MHC II-peptide presentation~~, thereby suppressing DC-induced~~ and reduced T cell proliferation. On the other hand, cystatin C promotesd pro-inflammatory Th1/Th17 differentiation via Erk-dependent secretion of polarizing cytokines from the BMDCs. We hereby unveiled how cystatin C affecteds BMDCs and how such effects might influence immunological outcomes.

RESULTS

Cystatin C enhances the uptake of foreign antigens by BMDCs

A key feature of DCs is uptake of soluble and particulate antigens. Therefore, we examined the impact of cystatin C on this important function in BMDC. We sorted conventional DCs ($CD11c^{+}$ $MHCII^{hi}$ $CD11b^{lo}$) from the heterogenous GM-CSF cultures [29] , treated them with or without recombinant cystatin C and measured their ability to take up fluorescent chicken ovalbumin (OVA-FITC) as a soluble exogenous antigen by flow cytometry. Pre-treatment with cystatin C led to enhanced uptake of

OVA-FITC by the primary BMDCs compared to the uptake by untreated cells (Figure 1a). Moreover, this effect of cystatin C on primary BMDCs was supported by similar results from the BMDC line DC 2.4 cells[30], transduced with lentivirus encoding cystatin C with a GFP reporter (DC/CstC) or GFP alone (DC/GFP). The former had an almost two-fold increase of cystatin C production (Supplementary figure 1) and a greater capacity to take up phycoerythrin-labeled OVA (OVA-PE) (Figure 1b). The higher OVA-PE contents in DC/CstC were not caused by decreased protein degradation, as following the inhibition of intracellular lysosome protease activity by leupeptin, DC/CstC still contained more OVA than DC/GFP (Supplementary figure 2b), which occurred as early as 1 min after antigen encounter in the absence of leupeptin treatment (Supplementary figure 2a). ~~Apart from the~~ As well as uptake of OVA protein, we also compared the phagocytosis of apoptotic cells by the BMDCs. ~~Following~~ Either DC/GFP or DC/CstC were co-cultured ~~of the~~ with BODIPY-labeled apoptotic splenic cells ~~with either DC/GFP or DC/Cst C~~ for 2, 4 or 20 h under optimal physiological conditions~~,~~. ~~the apoptotic cells that were taken up by the BMDCs were examined for their BODIPY fluorescence~~ The level of uptake was assessed by flow cytometry after washing ~~off~~ away the apoptotic cells that had not been phagocytosed. We found that DC/Cst C had a greater ability to take up the apoptotic cells than that of DC/GFP (Figure 1c). Furthermore, confocal microscopy was used to distinguish between ~~in order to exclude the possibility that higher staining signals detected by FACS came from the~~ antigens that ~~were actually up-taken inside the cells rather than sticky on~~ was phagocytosed and antigen adhering to the cell surfaces~~, we examined the phagocytosis of the apoptotic cells by DCs under confocal microscopy~~. Again, we found that a substantial amount of the BODIPY labeled cells ~~ended up inside~~ were engulfed by the DC/Cst C (Figure 1d). Collectively, these results indicated that cystatin C could enhance the uptake efficiency of DCs.

Cystatin C weakens the ability of BMDCs to stimulate the proliferation of $CD4^+$ T cells

DCs are the most powerful antigen presenting cells to stimulate T cell

proliferation, and one of the few cell types that can stimulate naïve T cells. In order to investigate the influence of cystatin C on the DC-stimulated T cell proliferation, we sorted the $CD11c^{+}MHCII^{hi}CD11b^{lo}$ cells from the primary BM cultures, pulsed them with OVA protein, and incubated the BMDCs of C57BL/6 origin in the presence or absence of cystatin C, with T cells from lymph nodes and spleens of OT-II mice in 96-well round bottom plates. T cells had been labelled with ~~CSFE~~ CFSE to indicate proliferative cycles with each dye dilution. Primary BMDCs ~~could~~ stimulated the proliferation of antigen specific $CD4^{+}$T cells, whereas there was no expansion of T cells cultured alone (Figure 2a). Under the influence of cystatin C, however, the capacity of BMDCs to stimulate T cell proliferation was significantly compromised (Figure 2a). To verify this finding *in vivo*, we adoptively transferred purified CFSE-labeled $CD4^{+}$ OT-II cells into C57BL/6 mice, which were challenged one day later by injecting OVA-pulsed DC/GFP or DC/Cst C subcutaneously into the footpad. After 3 days, lymphocytes from draining LNs and spleens were examined by flow cytometry for CSFE dilution. Consistently, the cystatin C-overexpressing DC had a lower ability to stimulate the proliferation of $CD4^{+}$ T cells in both LNs and spleen ~~sites~~ (Figure 2b). The less proliferated T cells were not caused by different migration capacity of the DCs as they demonstrated similar CCR7 expression and numbers in the examined organs (Supplementary figure 3). Therefore, despite the enhanced uptake of antigen due to cystatin C, the inhibitory effect on T cell proliferation would seem counter-intuitive.

The reduced proliferation of $CD4^{+}$ T cells by cystatin C-conditioned BMDCs ~~was~~ is not due to NO production

Since it has been reported that cystatin C can induce activated macrophages to produce NO for the inhibition of T cell proliferation [21, 31], we investigated whether an analogous mechanism might explain why T cell stimulation was compromised by cystatin C-conditioned BMDCs. Therefore, the NO contents in the supernatants of the sorted DC/T cell co-cultures were measured by a Griess assay. T cells cultured alone did not produced NO at all, but in the co-cultures of BMDCs and T cells there

were limited amounts of NO detected. The addition of cystatin C in the co-culture, however, significantly increased the NO amounts (Supplementary figure 4a). To directly confirm that the NO came from cystatin C stimulated DC, not T cells in the co-culture, we incubated BMDCs and T cells separately with or without cystatin C for 16 hrs. We found that cystatin C treatment of BMDCs alone led to a two-fold increase of NO production (Supplementary figure 4b), whereas no difference was observed ~~between~~ in T cell samples regardless of cystatin C addition (data not shown). Collectively, these data suggested that NO from DCs could be the soluble factor that mediated the inhibitive effect of cystatin C on the DC-stimulated T cell proliferation. To confirm this, we utilized iNOS specific inhibitor L-NMMA to block NO production in the system. NO production by the BMDCs was reduced by L-NMMA in a dose dependent manner (Supplementary figure 4c), ~~but~~ without affecting DC viability ~~of DCs were not affected~~ (Supplementary figure 4d). We then added a suboptimal concentration of the L-NMMA into DC-T cell co-cultures to effectively bring the NO contents in both cystatin C pre-treated and non-treated co-cultures equally down to the levels of cystatin C non-treated co-cultures in the absence of the NO inhibitor (Figure 3a). Interestingly, in these same co-cultures, the diminished $CD4^+$ T cell proliferation by treatment of cystatin C was not affected by the addition of NO inhibitor (Figure 3b). We thus concluded that the cystatin C-stimulated NO production from BMDCs did not account for the reduced T cell proliferation.

Cystatin C reduces the expression of intracellular H2-DM, a chaperon that facilitates surface presentation of MHC-II

NO production could not fully explain the inhibitory effect of cystatin C-primed BMDCs. Therefore, we investigated other mechanisms. Beyond antigen uptake, DCs have to process and present antigen. First, we looked at MHC-II expression on BMDCs after cystatin C treatment. We found that cystatin C treatment significantly decreased the expression of MHC-II on both sorted primary $CD11c^+MHC\text{-}II^{hi}CD11b^{lo}$ BMDC (Figure 3c), and the cystatin C-overexpressing DC2.4 cells (Figure 3d) with or without LPS stimulation. To further investigate the mechanism behind the reduced

MHC-II expression, we examined the intracellular levels of MHC-II associated molecules. H2-DM, a molecular chaperon, is key to releasing endogenous CLIP from MHC-II α/β dimers to avail foreign peptide to assemble into the MHC-II groove for surface presentation [32]. Indeed, the membrane expression of MHC-II is reduced by reduced levels of H2-DM [33, 34]. Therefore, we investigated the possibility that cystatin C could affect intracellular H2-DM levels. Interestingly, we found that high expression of endogenous cystatin C resulted in decreased levels of intracellular H2-DM staining in DCs with or without LPS stimulation (Figure 3e). These results suggested that cystatin C could interfere with surface MHC-II-peptide biogenesis by down-regulating the intracellular levels of H2-DM in BMDCs.

Cystatin C augments the ability of BMDCs to drive Th1/Th17 differentiation

In addition to stimulating naïve T cells to proliferate, DCs can also induce them to differentiate into different effector helper cells. To investigate how cystatin C affects the differentiation of T cells by DCs, we co-cultured sorted primary BMDCs and $CD4^+$ T cells in the presence or absence of cystatin C for 4 days. The cytokines IFN-γ, IL-4, IL-17A and TGF-β in supernatants were detected by ELISA. Neither DCs nor T cells cultured alone produced much cytokines (Figure 4a). We could detect IFN-γ, IL-4 and IL-17A (but not TGF-β) in our co-cultures of DCs and T cells. Cystatin C treatment of primary BMDCs before co-culture led to a two to three-fold increase in secretion of Th1 and Th17 signature cytokines IFN-γ and IL-17A respectively, and a modest but significant decrease in secretion of the Th2 cytokine IL-4 (Figure 4a). This trend was also confirmed *in vivo* by qRT-PCR of the three cytokines in OT-II cells after footpad injection of OVA-pulsed DC/GFP or DC/CstC (Figure 4b). Moreover, this was correlated with qRT-PCR of the transcription factors corresponding to Th1, Th17 and Th2 (i.e. upregulation of T-bet, RORγT and down-regulation of GATA3 respectively) (Figure 4c). Collectively, these data suggested that cystatin C could augment Th1/Th17 immunity.

Cystatin C stimulates BMDCs to secret distinct T cell polarizing cytokines

Certain cytokines produced by DCs can induce T cell deviation. Therefore, we examined the intracellular cytokine secretion by BMDCs following the treatment of cystatin C and DC2.4 cells transduced with cystatin C. We found that the cystatin C treatment of BMDCs increased the intracellular staining of IL-12p35 and IL-6/IL-1β, the polarizing cytokines for Th1 and Th17 differentiation respectively, whereas that of IL-4, the polarizing cytokine for Th2, was decreased (Figure 5a). Similar results were observed in DC2.4 when cystatin C was over-expressed (data not shown). By qPCR of mRNA levels, we found that cystatin C-overexpressing DC2.4 had higher expression of IL-12p35/IL-6/IL-1β but lower expression of IL-4, compared with their control-transduced counterparts (Figure 5b). These results suggested that the cytokine profiles of DCs in response to cystatin C concord with the T cell cytokines driven by the cystatin C-conditioned DCs. Thus, these findings support our conclusion that cystatin C promotes the DC-induced T cell deviation into inflammatory Th1/Th17 immunity.

The unique T cell polarizing cytokine secretion by cystatin C-stimulated BMDCs is Erk1/2 MAP kinase dependent

Proteomic screen comparing DC transduced with and without cystatin C revealed that cystatin C can affect various proteins expression inside the cells (Supplementary table 2). To investigate the signaling pathway leading to the cystatin C-induced cytokine production, we investigated the impact of cystatin C over-expression on the phosphorylation/activation of both Erk1/2 mitogen-activated protein kinase (MAP kinase) and PI3 lipid kinase in DCs, as these pathways are known to be important regulators of cytokine secretion in immune cells[35, 36]. We found that the BMDC clone DC2.4 cells constitutively expressed basal levels of phosphorylation in Erk1/2, but not Akt, the direct downstream target of PI3 kinase following control vector transduction (Figure 6a). This limited Erk1/2 phosphorylation/activation could reflect the effect of endogenous cystatin C or simply the impact of virial transfection. When cystatin C was over-expressed in the cells, however, the Erk1/2 phosphorylation/activation was greatly enhanced while Akt phosphorylation remained

undetectable, suggesting a specific impact of cystatin C on Erk1/2 MAP kinase signaling pathway (Figure 6a). The specificity of cystatin C on Erk1/2 MAP kinase activation was further confirmed in the primary BMDCs by intracellular staining of the activated form of the protein; the levels peaked at 15 min post-cystatin C stimulation and declined at 30 min. No stimulatory effect was observed in p38 MAP kinase pathway at either 15 min or 30 min post stimulation, in contrast to the LPS positive control (Figure 6b). These results indicated that the Erk1/2 kinase pathway was specifically activated in BMDCs following cystatin C stimulation.

In order to verify whether the cystatin C-stimulated cytokine profiles were dependent on the activated Erk1/2 MAP kinase, we treated the transduced BMDC clones with or without PD98059, a specific inhibitor of Erk1/2 MAP kinase pathway for 24 hours before the cells were harvested and their cytokines expression at mRNA levels quantified by qPCR. Interestingly, we found that the polarizing effect of cytokine production from the DCs induced by cystatin C was abrogated i.e. the increase of IL-12p35 for Th1, IL-1β/ IL-6 for Th17, and decrease of IL-4 for Th2 differentiation reverted to their resting levels by the treatment of PD98059 (Figure 6c). Similar results were observed in primary BMDCs (data not shown). Interestingly, we found no significant change in cystatin C affected phagocytosis, NO production and MHC-II expression (Supplementary figure 5). These results indicated that Erk1/2 pathway specifically mediated the cystatin C-induced secretion of polarizing cytokines from BMDCs.

DISCUSSION

As a powerful endogenous cysteine protease inhibitor, cystatin C has been reported to possess immune modulatory functions, which ~~is~~ are reflected by ~~it's~~ the impacts on inflammatory cytokine secretion from macrophages and monocytes *in vitro* [20]. Administration of exogenous cystatin C can skew T cell differentiation *in vivo* from a ~~parasite permissive~~ Th2 response to a ~~anti-parasite~~ Th1 response for effective protection against *leishmaniasis* [21], ~~or~~ ~~suppresses~~ suppression of allergic responses [37], and ~~elicit strong host~~ immunity ~~for~~ to *T~~t~~richinella* ~~resistance~~ [38].

Alternatively, it can also up-regulate Treg or IL-10 and suppress inflammatory cytokine secretion to improve inflammatory bowel diseases[39], experimental colitis [40], as well as other mucosal inflammation[41]. However, the molecular mechanisms and cellular basis behind the immune regulatory functions of the cystatins are still far from clear. In the current study, we found that cystatin C has important effects on BMDC function that play important roles in both innate and adaptive immunity in terms of phagocytosis and effects on T cells especially their differentiation. Importantly, we revealed ~~that~~ at least two pathways by which BMDCs may be affected by cystatin C. Firstly, there was defective MHCII processing due to diminished H2-DM expression, which resulted in reduced T-cell proliferation. Secondly, increased Erk 1/2 phosphorylation in response to cystatin C led to polarizing cytokine secretion, which resulted in Th1/Th17 deviation.

As specialized antigen-presenting cells, DCs phagocytose exogenous antigens and digest them into polypeptides for selectively loading onto endogenous MHCII molecules to be recognized by $CD4^+$ T cells, which then proliferate and differentiate into a variety of effector cells to initiate immune responses. To mimic this *in vivo* scenario, FITC conjugated OVA protein was used as a foreign antigen to co-culture with the primary BMDCs to compare their phagocytotic capacities before and after cystatin C treatment in terms of their internalized FITC signals by FACS analysis. Interestingly, we found that cystatin C can increase the phagocytosis of OVA antigen by the BMDCs. A similar result was found in DC2.4 cells. Furthermore, not only the uptake of soluble antigen, but that of apoptotic cells as autoantigen were also found to be enhanced in the cystatin C-conditioned BMDCs (Figure1). To the best of our knowledge, this is the first demonstration of cystatin C affecting uptake by BMDCs.

The enhancement of innate immunity of the BMDCs in phagocytosis by cystatin C forebodes that the DCs will present more antigenic materials to stimulate adaptive immunity. Surprisingly, we found that cystatin C suppressed rather than increased the DC-induced T cell proliferation (Figure 2), a result in consistent with what others found in salivary cystatin from the soft tick to diminish the antigen-specific proliferation of mouse $CD4^+$ T cells [42]. Since it has been reported that cystatin C can

activate macrophages to increase the activity of iNOS and produce NO to inhibit the proliferation of T cells [21, 31], we first investigated this possibility by examining the NO contents in the DC/T cell co-culture system. In spite of the stimulating effect of the cystatin C on NO production from the DCs in the culture, NO inhibitor L-NMMA failed to rescue the suppressed T cell proliferation, although it effectively erased the differences of NO levels caused by cystatin C in the co-cultures (Figure 3a, b). The dose-dependent inhibition of NO concentration without affecting cell viability by L-NMMA indicated the specificity and fidelity of the inhibition assay. Therefore, we concluded that NO was not accountable for the compromised T cell proliferation in the DC/T cell co-culture in the presence of cystatin C.

It is well known that MHCII molecules on the DC surface are essential in presenting antigens to stimulate T cell proliferation. Consistent with their defective T cell stimulating capacity, the cystatin C pre-treated BMDCs was found to have decreased expression of MHCII (Figure 3c). Although it has long [24, 25], and more recently [26] been reported that cystatin C can inhibit the cleavage of MHC-II molecules by cathepsin S to reduce the expression of MHC-II molecules on the DC surface, others have demonstrated that cystatin C was not necessary for the cathepsin S mediated MHC-II processing and antigen presentation in mouse splenic DCs[27], or human cultured DCs [23, 28]. In searching for the other intracellular molecules that might also be subjected to cystatin C regulation, we found in the cystatin C-overexpressing DCs a decreased expression of H2-DM, one of the class II-like chaperone molecules catalyzing the MHC-II maturation and antigen presentation by exchange of CLIP fragment from MHCII groove for an antigen peptide [33]. Since the loss of H2-DM decreases the antigen loading of MHCII molecules [33, 43, 44], it is plausible that the down-regulation of intracellular H2-DM protein levels by cystatin C described herein could form a molecular basis to explain the compromised T cell proliferation. Genetic disruption of cystatin C in mice showed no measurable effect on MHCII-mediated antigen presentation by their splenic DCs [27]; however, this does not exclude a role of cystatin C in MHC-II processing, as during the life of the mouse, other cystatin family members might compensate for the loss of ~~Cyst~~ cystatin C. Since elevated levels of

cystatins around DCs can occur from endogenous production during inflammatory responses to microbial compounds[13-15], or from exogenous production by pathogens targeting host cysteine proteases[39, 40], the down-regulation of H2-DM by cystatin C could explain the suppressed immunity in some inflammatory diseases.

Our finding that alteration of T cell differentiation profiles by cystatin C-conditioned BMDCs *in vitro* is interesting (Figure 4). It agrees with previous reports that treatment with cystatin led to successful conversion of permissive Th2 to curative Th1 responses *in vivo* against parasite infections [20-22, 37]. In addition, we also found that cystatin C-conditioned BMDCs can induce T cells to secrete IL-17, which enable neutrophils to swallow and digest exogenous antigens, and play an important role in innate immunity and autoimmune diseases [45, 46]. Since cystatin C can directly stimulate the purified DCs to secrete polarizing cytokines, we contend that such cytokines are the molecular basis for the altered T cell differentiation.

The signaling pathway elicited by cystatin C in DCs is far from clear. Cystatin C by itself cannot induce TLR/MyD88 signaling in macrophages [47, 48]. We found that unlike many other stimuli, cystatin C did not activate many signaling pathways in DCs like PI3 lipid kinase and p38 MAP kinase; however, it did specifically activate Erk1/2 MAP kinase pathways (Figure 6a,b). Importantly, abolishment of Erk1/2 kinase activity in BMDCs by specific inhibitor cancelled their ability to secret T cell polarizing cytokines (Figure 6c), indicating this MAP kinase mediated the impact of cystatin C on T cell differentiation. The underlying mechanisms for Erk MAP kinase activation by cystatin are multifold. Some proposed inhibition of a regulatory protease that might degrade Erk upstream kinase such as MAP2K [49] as plausible explanation, whereas others proposed cystatin activates the IKK complex to phosphorylate p105, triggering its proteolysis. Consequently, tumor progression locus 2 (Tpl-2), a serine-threonine kinase, is liberated from p105, thereby inducing activation of the MEK/ERK MAPK cascade [48]. Whether cystatin C exerts its effect on Erk1/2 in BMDCs through inhibitor-dependent or independent function is currently unknown.

Cystatin C could regulate various immune responses by either enhancement of

host defenses as for parasite elimination[21, 38], or suppression of host immunity as for autoimmune disease treatments [26, 40, 50]. Our findings that cystatin C differentially affected BMDC function help to resolve the paradox of these reported cases, and suggest that different disease settings might render cystatin C to impact on the different functional aspects of DCs in terms of phagocytosis of foreign antigen, stimulation of T cell proliferation or direction of T cell differentiation, based on certain cellular environment. Importantly, we have identified the intracellular protein targets to mediate the cystatin C-induced effects on BMDCs. Since many small molecular mimetic drugs or pharmaceutical compounds against certain key molecules have been successfully developed for the treatment of diseases like cancer[51], viral infection[52], and type II diabetes[53], our findings could have great translational potential to interfere with various immune disorders associated with cystatin C in the clinic.

METHODS

Mice

Specific pathogen-free (SPF) C57BL/6 and OT-II TCR transgenic mice (C57BL/6 background) female mice, aged 7–11 week were purchased from Model Animal Research Center of Nanjing University, Nanjing, China, and housed in a SPF environment in the Anhui Normal University (ANU) Animal Facilities. All procedures conducted on mice were in accordance with the conditions specified and approved by the ANU Animal Experimentation Ethics Committee.

Cell cultures

The immature murine dendritic cell line DC2.4 and its transduced derivatives were cultured in RPMI1640 medium, supplemented with 100 U/ml penicillin, 100mg/L streptomycin, 2 mmol L^{-1} L-glutamine, and 10% FBS (GIBCO, Grand Island, NY, USA). Bone marrow (BM) cells were extracted from C57BL/6 mice and erythrocytes were removed by exposure to red blood cell lysis buffer (Biosharp, Hefei, Anhui, China). The remaining live cells were then cultured at a density of 1×10^6 mL^{-1} in DC medium containing 20ng mL^{-1} murine recombinant GM-CSF (Pepro Tech,

Rocky Hill, NJ, USA) with exchange of fresh media at day 3 and 5. The bone marrow-derived primary cultures were harvested at day 7 and $CD11c^{+}MHC\text{-}II^{hi}$ $CD11b^{lo}$ BMDCs were sorted by fluorescence-activated cell sorting (FACS) (Beckman Coulter MoFlo High-Performance Cell Sorter). For some experiments, BMDCs were exposed to chicken cystatin C (Sigma, St Louis, MO, USA) at 20μg mL^{-1} during the last 2 days of the cultures. All cells were incubated at 37°C with 5% CO_2.

Generation of cystatin C encoding lentivirus

The complete mouse *cst3* sequence was cloned into the EcoRI, BamHI restriction sites of pHBAAV-CMV-MCS-3flag-EF1-ZsGreen overexpressing lentiviral vector (Hanbio Biotechnology, Shanghai, China). Competent lentiviruses were packaged by transfection of 70-80% confluent 293T cells with the overexpressing lentiviral vector containing either *cst3* and *gfp* or *gfp* alone, together with pSPAX2 and pMD2G vectors using Lipofiter™ (Hanbio Biotechnology, Shanghai, China). Lentivirus was harvested at 48 and 72 h post transfection, centrifuged (2000 g, 10min, 4℃) to remove cell debris, and then filtered through a 0.45 mm cellulose acetate filter followed by ultracentrifugation at 82700 g, 4℃ for 120 min. The Lentiviral titer was then determined by counting fluorescence positive transduced 293T cells with a 3-fold dilution series of virus, and calculated as follows: titer（TU mL^{-1}）= cell number×% of GFP×MOI×dilution of viral×10^3.

Transfection of DCs and establishment of a stable cystatin C overexpressing DCs

For lentivirus infection, 2×10^4 DC2.4 cells in 200 μl complete RPMI1640 medium were cultured in 96-well plates. Next day when the cells were at 60–70% confluency, lentivirus endcoding *Cst3/gfp* or *gfp* only were added to the DCs at 60 MOI from viral stock of 2×10^8 pfu mL^{-1} and co-cultured with DC2.4 for 12h before the medium was changed with 200 μl new complete RPMI1640 medium. After 72 h, the transduced cells were selected by adding puromycin (Hanbio Biotechnology, Shanghai, China) at 2ug mL^{-1} in the following day. The cystatin C high-expressed

DC2.4 was assessed by qPCR and ELISA, and selected for subsequent experiments.

Flow cytometry

DCs were stained with varying combinations of mAbs against CD11c (N418-PE-Cy7), MHCII (M5/114-APC) (eBioscience, San Diego, CA, USA), CD11b (M1/70) (eBioscience, San Diego, CA, USA) with addition of propidium iodide (1μg mL^{-1}) to the final wash to stain dead cells. Cell analysis or sorting was performed on BD Canto II flow cytometer or Beckman Counter MoFlo flow cytometry sorter respectively. Flow Jo software was used to analyze the data.

Cell purification by MACS column

T cells were stained with CD4-PE conjugated primary antibody (eBioscience, San Diego, CA, USA). Then, the cells were extensively washed and labeled with anti-PE Micro Beads (Miltenyi Biotech, Bergisch Gladbach, Germany) before they were loaded on a MACS® Column. The magnetically labeled CD4-PE^+ cells were retained in the column while the unlabeled cells run through. After removal of the column from the magnetic field, the retained cells were eluted as the positively selected cell fraction with purity greater than 98%.

Analysis of uptake ability of DCs

To examine antigen uptake by DCs, 0.2×10^6 sorted $CD11c^+$MHC-$II^{hi}CD11b^{lo}$BMDCs were incubated at 37℃ for 4h with OVA-FITC (Beijing Boxi Technology Co. LTD, Beijing, China) (50μg mL^{-1}) in 500μl complete RPMI1640 medium. Alternatively, DC2.4 were incubated at 37℃ for 1 or 2h with OVA-PE (Shanghai Yuduo Biotechnology Co. LTD, Shanghai, China) (2 μg mL^{-1}) in 500μl complete RPMI1640 medium. After washing with PBS, the cells were analyzed immediately by flow cytometry. Cells incubated with OVA-FITC or OVA-PE at 4℃ were used as a negative control.

To obtain apoptotic cells as phagocytic substrates, freshly isolated splenic cells were killed by culturing with 12.5μM CCCP (Shanghai Xiangsheng Biotechnology

Co. LTD, Shanghai, China) (carbonyl cyanide m-chlorophenyl hydrazine)[15] for 4h, and then labelled with BODIPY as indicator. The BODIPY-labeled apoptotic spleen cells were then added to the DC2.4 in a 2:1 ratio (spleen cells: DC2.4) in complete RPMI 1640 medium. After 2h of incubation at 37℃, the mixed cells were harvested, dropped onto slides, and assessed by OLYMPUS FV1000 confocal fluorescence microscope.

Assessment of $CD4^+$ T cell proliferation and DC migration *in vivo*

3×10^6 purified $CD4^+$ T cells from OT-II mice were labeled with CFSE and adoptively transferred into B6 mice. 24 hours later, the mice were immunized with 1×10^6 OVA-pulsed DC/GFP or DC/Cst C. After 3 days, the indicated lymph nodes and spleen were harvested. The harvested samples were run through FACS and proliferated cells were calculated by the number of acquired $CFSE^{low}$ $CD4^+$ T cells (on FACS plot) × added BD calibrate APC beads / acquired bead number. For DC migration *in vivo*, 10×10^6 purified CFSE labeled DC/GFP or DC/CstC were injected into footpads. 24 hours later, the mice were sacrificed, and the indicated lymphoid organs were harvested. Single cell suspensions were made from the tissue and the CFSE positive cells were detected by flow cytometry.

Assessment of $CD4^+$ T cell proliferation *in vitro*

10×10^6 purified $CD4^+$ T cells from OT-II mice were labeled with 5μM CFSE in 1ml 0.1% BSA for 10 min at 37℃, and then the cells were washed three times. FACS sorted $CD11c^+MHCII^{hi}CD11b^{lo}$ BMDCs from C57BL/6 mice were pulsed with 1mg ml^{-1} OVA protein for 2 h before co-cultured with the CFSE-labeled $CD4^+$ T cells in 96-well round bottom plate at 1:10 ratio for 4 days *in vitro* before FACS analysis. Proliferated cells were calculated by the number of acquired $CFSE^{low}$ $CD4^+$ T cells (on FACS plot) × added BD calibrate APC beads / acquired bead number.

NO measurement

The NO contents in the supernatants of cell cultures were measured by a Griess

assay. In brief, 50μl supernatants were mixed with 50μl Griess reagent I and 50μl Griess reagent II, and absorbance was read at 540 nm. The NO concentrations were calculated using the standard curve.

Cytokine detection in cultures

For T cell cytokines, OVA-pulsed FACS-sorted $CD11c^{+}MHC\text{-}II^{hi}CD11b^{lo}$ primary BMDCs pre-treated with or without cystatin C were co-cultured with OT-II cells at 1:10 ratio for 4 days. Supernatants were collected and analyzed for IFN-γ, IL-4, IL-17A, by ELISA according to the manufacturer's instructions (R&D System, Minnesota, USA).

For DC cytokines, FACS-sorted $CD11c^{+}MHC\text{-}II^{hi}CD11b^{lo}$ BMDCs were re-suspended at 1×10^{6}/ml in fresh media in the presence or absence of cystatin C (20 μg mL^{-1}), and cultured for 20 h. Supernatants were collected forIL-6 by ELISA according to the manufacturer's instructions (R&D System, Minnesota, USA).

RNA isolation and qPCR analysis

Total RNA from purified cells was isolated by RNA extraction reagent Trizol (Sangon Biotech, Shanghai, China), and transcribed into cDNA using PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa, Beijing, China) according to the manufacturer's instructions. Real-time PCR with SYBR Green detection (SYBR Green Master Mix, SYBR$^{\circ R}$Premix ExTaqTMII, TaKaRa) was performed to quantify RNA expression using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Analysis was performed using CFX Manager Software (Bio-Rad). All data were expressed relative to HPRT as an internal control. Primers used are listed in Supplementary table 1.

Western blot

3×10^{6} DC2.4 were harvested, and immediately lysed on ice in cold lysis buffer containing 1% NP-40, protease inhibitor tablets (Roche, Basel, Switzerland), and 1mM Sodium orthovanadate (Beyotime, Shanghai, China) for 30 min. Cell debris

were spun down and protein extracts mixed with loading buffer before they were boiled at 95℃ for 5 minutes and separated by SDS-polyacrylamide gel electrophoresis. The total cellular proteins were transferred onto nitrocellulose membranes, and probed with antibodies specific for anti-phospho-Erk1/2 (Cell Signaling, Danvers, MA, USA), anti-phospho-Akt (Cell Signaling, Danvers, MA, USA), anti-β-actin (Santa Cruz Biotechnology, Delaware Ave Santa Cruz, CA, USA), and anti-β-tubulin (Multi Sciences, Hangzhou, Zhejiang, China).

Intracellular cytokine staining

GM-CSF cultured cells (5×10^5 mL^{-1}) were stimulated for 4 h at 37 °C with Golgi stop (BD Biosciences, San Jose, CA, USA) before the cells were harvested, washed, and stained with anti-mouse CD11c antibody (N418-PE-Cy7) (BioLegend, San Diego, CA, USA), anti-mouse MHCII antibody (M5/114-APC) (eBioscience, San Diego, CA, USA), and anti-mouse CD11b antibody (M1/70) (eBioscience, San Diego, CA, USA). Surface-stained cells were fixed (4% paraformaldehyde in PBS) and resuspended in permeabilization buffer (PBS, 0.5% bovine serum albumin, 0.5% saponin, 0.05% sodium azide). Permeabilized cells were incubated in the dark for 30 min at room temperature at 0.25 μg mL^{-1} per 10^6 cells with one of following antibodies: Rat Anti-Mouse IL-12 (p40/p70) (C15.6-FITC) (BD Biosciences, San Jose, CA, USA), Rat Anti-Mouse IL-4 (11B11-APC) (BD Biosciences, San Jose, CA, USA), Rat Anti-Mouse Erk1/2 (20A-PE) (eBioscience, San Diego, CA, USA), Rat Anti-Mouse p38 (36/p38-PE) (BD Biosciences, San Jose, CA, USA), or Rat Anti-Mouse H2DM (2E5A) followed by Goat anti-Rat Ig conjugated with APC (BD Biosciences, San Jose, CA, USA). Stained cells were washed twice in permeabilization buffer and resuspended in PBS permeabilization buffer. The signal intensity of interested molecules in $CD11c^+MHC\text{-}II^{hi}CD11b^{lo}$ BMDCs or DC2.4 was determined by flow cytometry.

Proteomic Study

$10x10^6$ DC2.4/CstC or DC2.4/GFP cells were lysed and 200 μg protein from each

sample were collected, in which the detergent, DTT and other low-molecular-weight components were removed using 200 µl UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0) by repeated ultrafiltration (Microcon units, 30 kD) facilitated by centrifugation (Bioprofile, Shanghai, China). The protein suspension was digested with 4 µg trypsin (Promega) in 40 µl 25 mM NH4HCO3 overnight at 37 °C, and the resulting peptides were collected as a filtrate. MS experiments were performed on a Q Exactive HF mass spectrometer that was coupled to Easy nLC (Thermo Scientific). The maximum injection time was set to for 50 ms for MS and 45 ms for MS/MS. Normalized collision energy was 28 and the isolation window was set to 1.2 Th. Dynamic exclusion duration was 60 s.

The MS data were analyzed using MaxQuant software version 1.6.1.0. and searched against the SwissProt Mouse database. The maximal two missed cleavage sites and the mass tolerance of 4.5 ppm for precursor ions and 20 ppm for fragment ions were defined for database search. Carbamidomethylation of cysteines was defined as fixed modification, while acetylation of protein N-terminal and Lysine, oxidation of Methionine were set as variable modifications for database searching. The database search results were filtered and exported with <1% false discovery rate (FDR) at peptide level, protein level, respectively.

Statistical analysis

The Mean value and Standard Deviation of independent experiments are shown in the graph, all experiments were repeated at least three times with similar results. Statistical differences between the groups were determined by using Student's *t*-test; *P*-values < 0.05 were considered statistically significant.

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Author Contributions: The work presented here was performed in collaboration with all authors. WZ, MZ, LS, FW, SC, YZ, SL and LL performed the experiments, collected and analyzed the data; SL and JH provided important reagents and facility; WZ and MZ prepared figure layout; YX designed the study and analyzed the data; YX, AL and YZ wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

FIGURE LEGENDS

Figure 1. The impact of cystatin C on antigen uptake by DCs. **(a)** $CD11c^{+}MHC\text{-}II^{hi}CD11b^{lo}$ BMDCs were sorted by flow cytometry as indicated before they were incubated with OVA-FITC (50μg/ml) for 4 hours and washed. OVA-FITC uptake was measured by flow cytometry. The endocytosis was assessed by comparing the differences of mean fluorescence intensity (MFI) between 37˚C and 4˚C ($\Delta MFI = MFI^{37\,℃} - MFI^{4\,℃}$). **(b)** DC2.4 cells transduced with CstC (DC/CstC) or GFP-containing empty vector (DC/GFP) were cultured with OVA-PE (2μg/ml) for 1

hour. OVA-PE uptake was measured by flow cytometry. **(c)** The DC/CstC or DC/GFP cells were cultured with BODIPY-labeled apoptotic splenic cells for 1 hour. BODIPY uptake was measured by flow cytometry. A representative histogram (right panels) and the summarized results (left panel) are shown. Error bars stand for mean ± SD from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired *t* test. **(d)** Z-stack confocal images of BOBPY-labeled apoptotic splenic cells (red) co-cultured with CFSE-labeled DC2.4 (green) in a 2:1 ratio in DC media, and incubated for 2 h at 37℃. Phagocytosis of apoptotic cells by the DCs was assessed by OLYMPUS FV1000 Confocal Fluorescence Microscope (Olympus Corporation). In horizontal and cross-section images note that parts of the splenic cells were localized inside, rather than overlapped outside the DCs. Representative data from one of the three similar experiments were shown.

Figure 2. Compromised DC-induced $CD4^+$ T cell proliferation in the presence of cystatin C. **(a)** Sorted $CD11c^+MHC\text{-}II^{hi}CD11b^{lo}$ BMDCs were treated with or without cystatin C, pulsed with OVA, and co-cultured with CSFE labelled $CD4^+$ T cells from OT-II mice for 3 days as described in Methods. The CFSE dilution of $CD4^+$ cells were examined by flow cytometry (right panels) and the proliferated T cells are shown plotted in the histogram (left panel). Bar graphs represent the mean ± SD from 3 independent experiments. ** $P < 0.01$, unpaired *t* test. **(b)** Purified $CD4^+$ T cells from OT-II mice were labeled with CFSE and adoptively transferred into C57BL/6 mice. 24 hours later, the mice were immunized with OVA-pulsed DC/GFP or DC/Cst C. After 3 days, the indicated lymph nodes and spleen were harvested and the proliferation of these cells was determined by CFSE dilution. A representative histogram (right panels) and the summarized results (left panel) are shown. Error bars stand for the mean ± SD from 4-6 mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired *t* test.

Figure 3. The compromised DC-induced $CD4^+$ T cell proliferation in the presence of CstC is not caused by NO production, but associated with reduced expression of

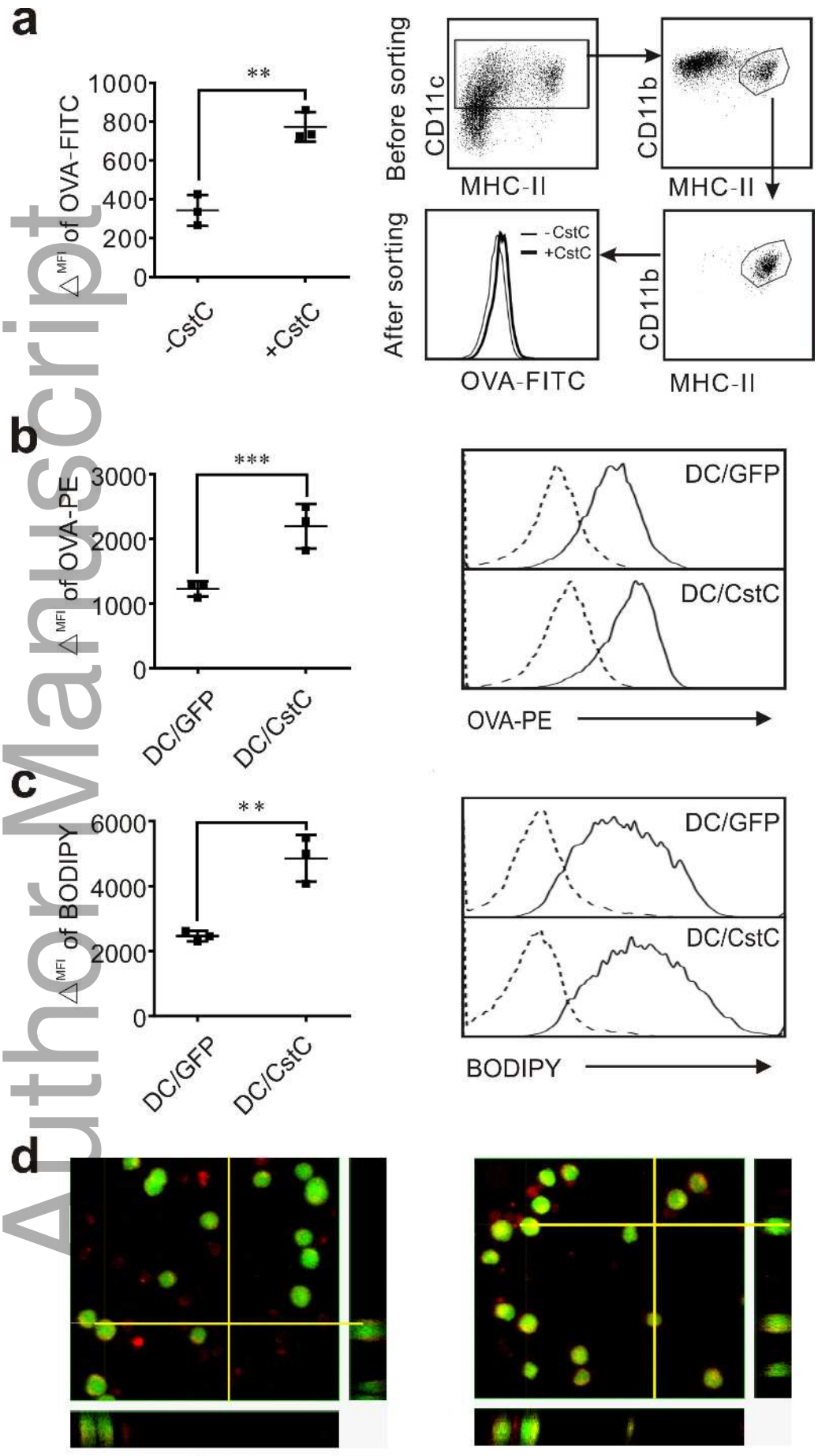
H2-DM in BMDCs. **(a)** After sorting, cystatin C pre-stimulated OVA-pulsed $CD11c^{+}MHC\text{-}II^{hi}CD11b^{lo}$ BMDCs were co-cultured with T cells from OT-II mice in the absence or presence of cystatin C or L-NMMA for 4 days. Their NO contents in the supernatants were examined by Griess assay. **(b)** As in (**a**), but the proliferation of $CD4^{+}$T cells was detected by flow cytometry, and shown in histogram. Data stand for the mean ± SD from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant, unpaired *t* test. **(c)** Sorted $CD11c^{+}MHC\text{-}II^{hi}CD11b^{lo}$ BMDCs were treated with cystatin C and/or LPS, harvested and their expression of MHC-II were detected by flow cytometry. **(d)** DC2.4 transduced with cystatin C or not were treated with or without LPS for 24 h, and their expression of MHC-II was detected by flow cytometry. **(e)** As in **(d)**, but the intracellular protein expression of H2-DM in DCs was detected by flow cytometry. A representative histogram (right panels) and the summarized results (left panel) are shown. Error bars stand for the mean ± SD from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired *t* test.

Figure 4. The impact of cystatin C on DC-induced T cell differentiation. **(a)** Sorted $CD11c^{+}$ $MHC\text{-}II^{hi}$ $CD11b^{lo}$ BMDCs were pulsed with OVA, and co-cultured with purified $CD4^{+}$T cells from OT-II mice for 3 days before the concentration of IFN-γ, IL-4, or IL-17A in the supernatants was assayed by ELISA. **(b & c)** DC/GFP or DC/CstC were pulsed with OVA protein and injected s.c into OT-II mice. 48 hours later, purified splenic $CD4^{+}$ T cells from the injected mice were assayed for mRNA levels of IFN-γ, IL-4, or IL-17A expression, or T-bet, GATA3, or RORγt expression. Data graphs represent the mean ± SD from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired t test.

Figure 5. The impact of cystatin C on cytokines expression of DCs. **(a)** The intracellular expression of IL-12p35, IL-1ß, and IL-4 in the gated $CD11c^{+}MHC\text{-}II^{hi}CD11b^{lo}$ BMDCs or concentration of IL-6 in the sorted $CD11c^{+}MHC\text{-}II^{hi}CD11b^{lo}$ BMDCs stimulated with or without cystatin C were

assayed by flow cytometer or ELISA respectively. **(b)** mRNA expression levels of IL-12p35, IL-12/IL-23p40, IL-1ß and IL-6 in DC/GFP or DC/Cst C were quantified by qPCR. Data graphs represent the mean ± SD from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired *t* test.

Figure 6. Cystatin C activatesed Erk MAP kinase pathway in DCs to affect T cell differentiation. **(a)** DCs transduced with or without cystatin C were lysed and the total cellular proteins separated by SDS-PAGE. p-Erk1/2, β-tubulin, p-Akt and actin were detected by western blot as described in Methods. **(b)** Freshly cultured BM cells in the presence of GM-CSF were left untreated, or stimulated with LPS for 15min, or with cystatin C for 15 or 30 min before they were stained for surface marker and intracellularly for phosphorylated form of Erk1/2 or p38. The activated Erk1/2 (upper panel) or p38 (lower panel) from $CD11c^{+}MHC\text{-}II^{hi}CD11b^{lo}$ BMDCs were examined. Representative data from one of the two similar experiments are shown. **(c)** DCs transduced with or without cystatin C were incubated in the presence or absence of PD98059 for 24 h before they were harvested and mRNA levels of the indicated cytokines in the cells were quantitated by qPCR. The results were calculated as relative expression of each gene examined over housekeeping gene Hprt. Bar graphs represent the mean ± SD from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, *ns* = not significant, unpaired *t* test.



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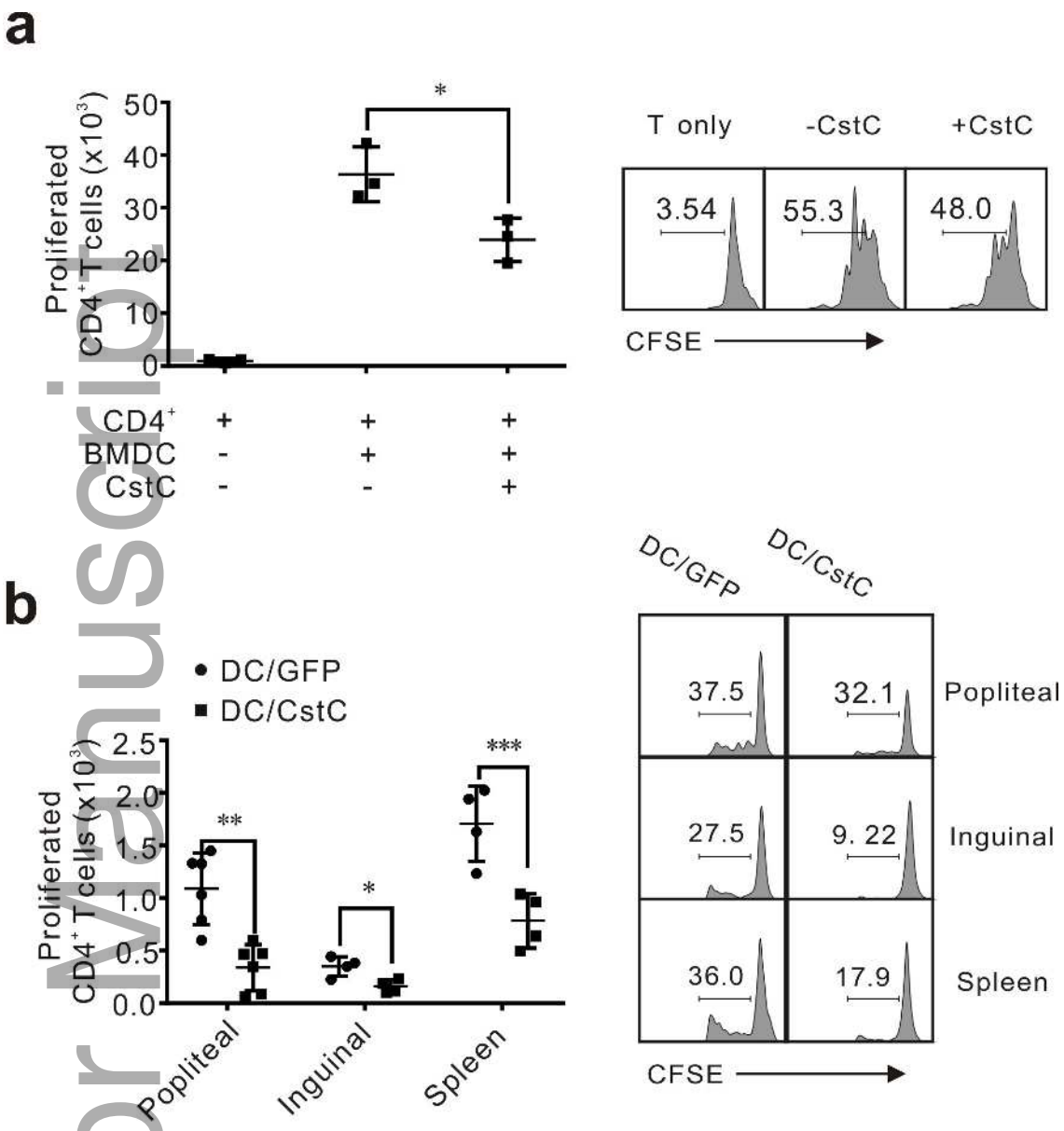

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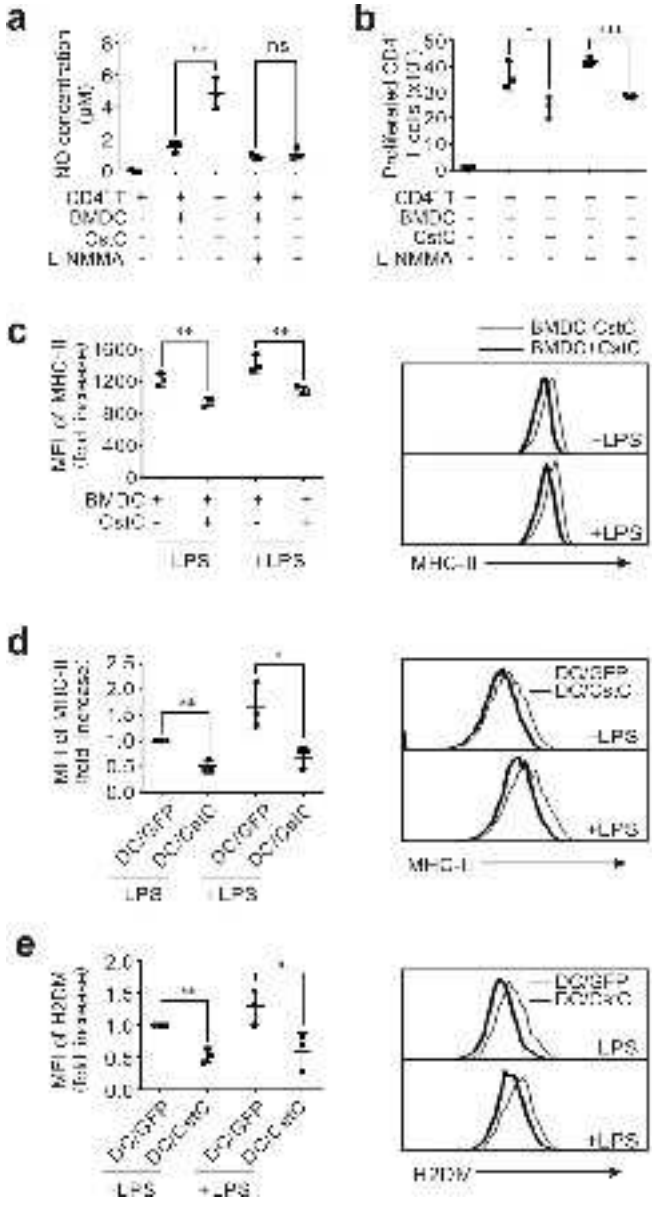

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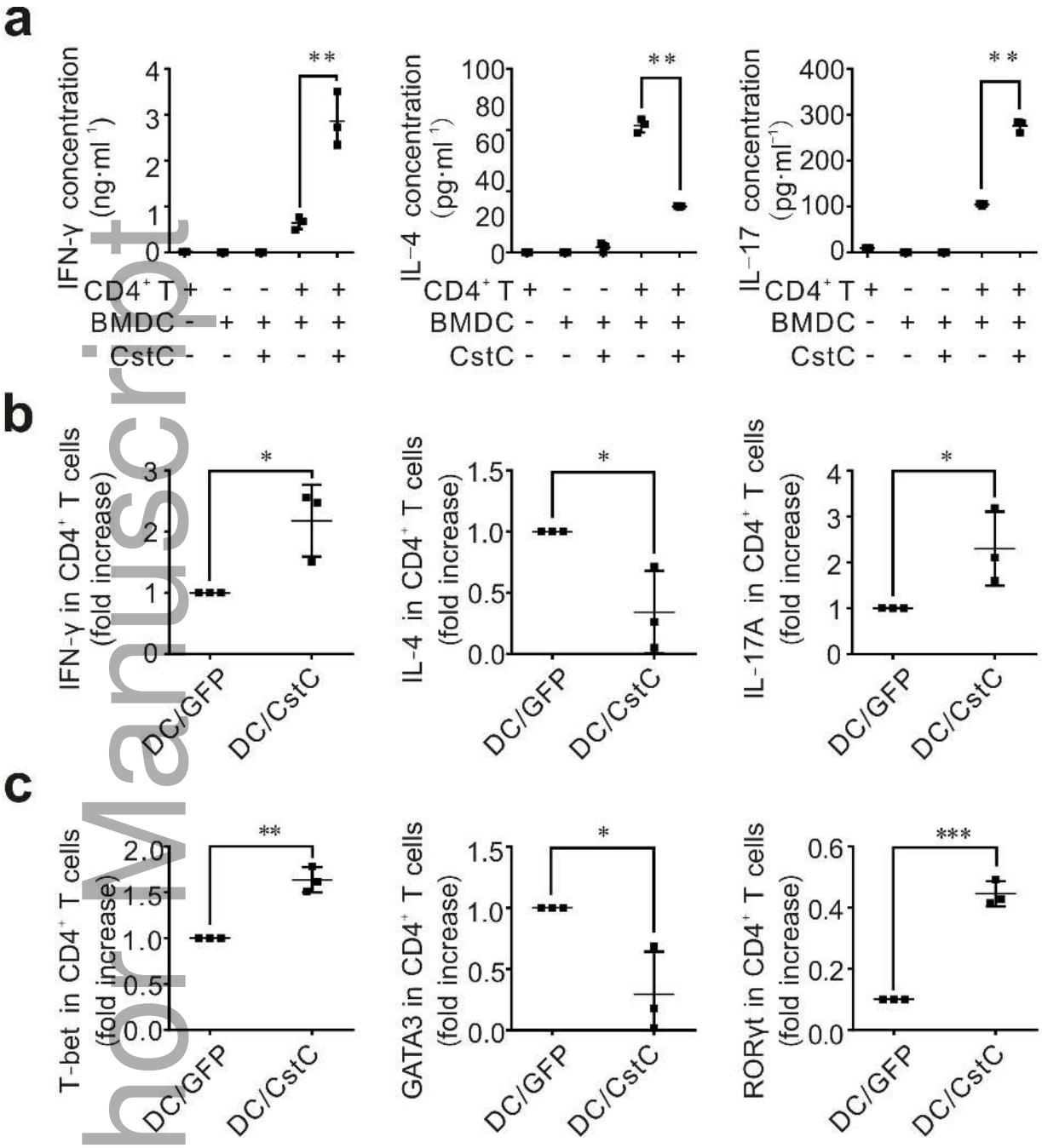

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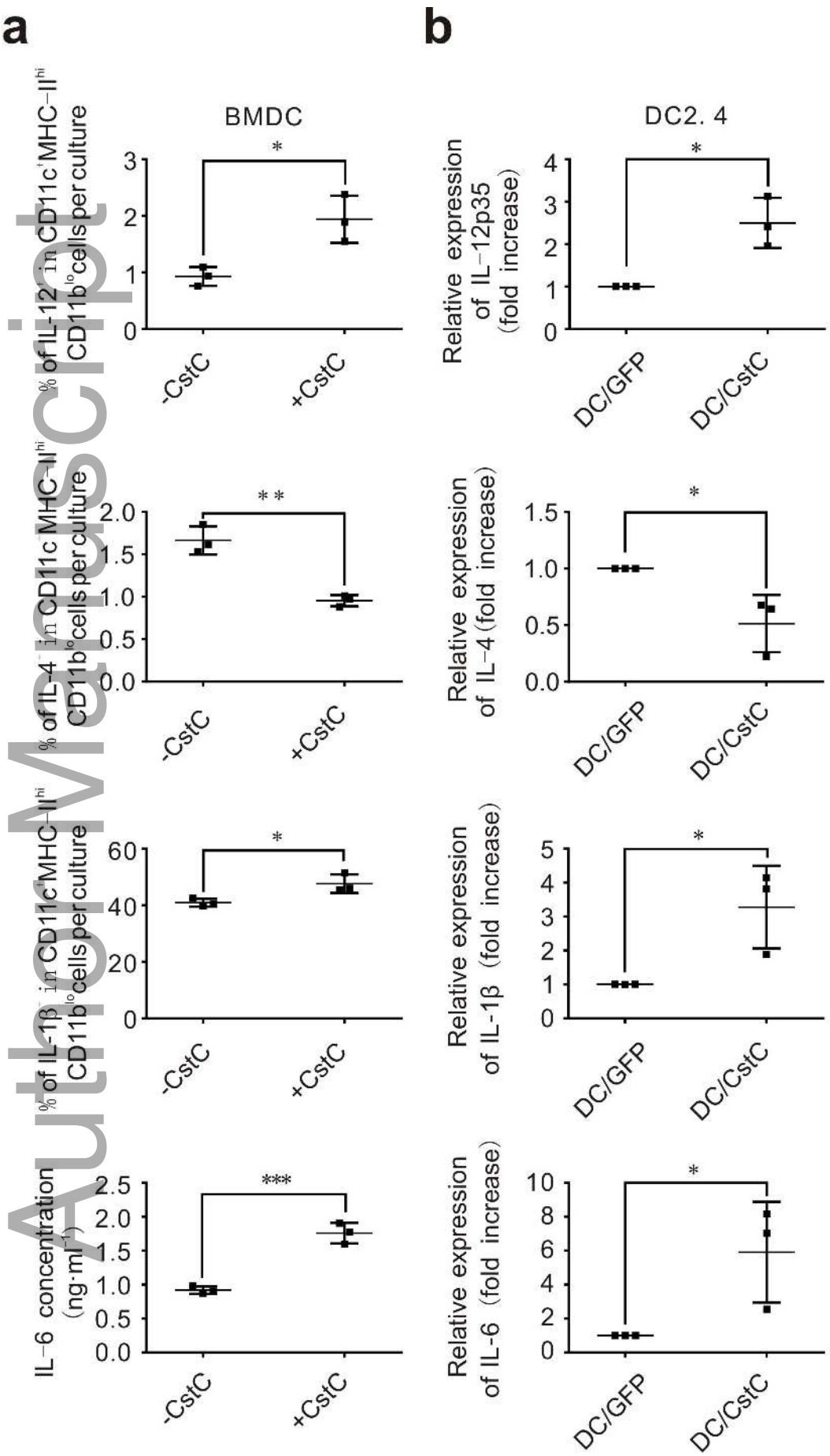

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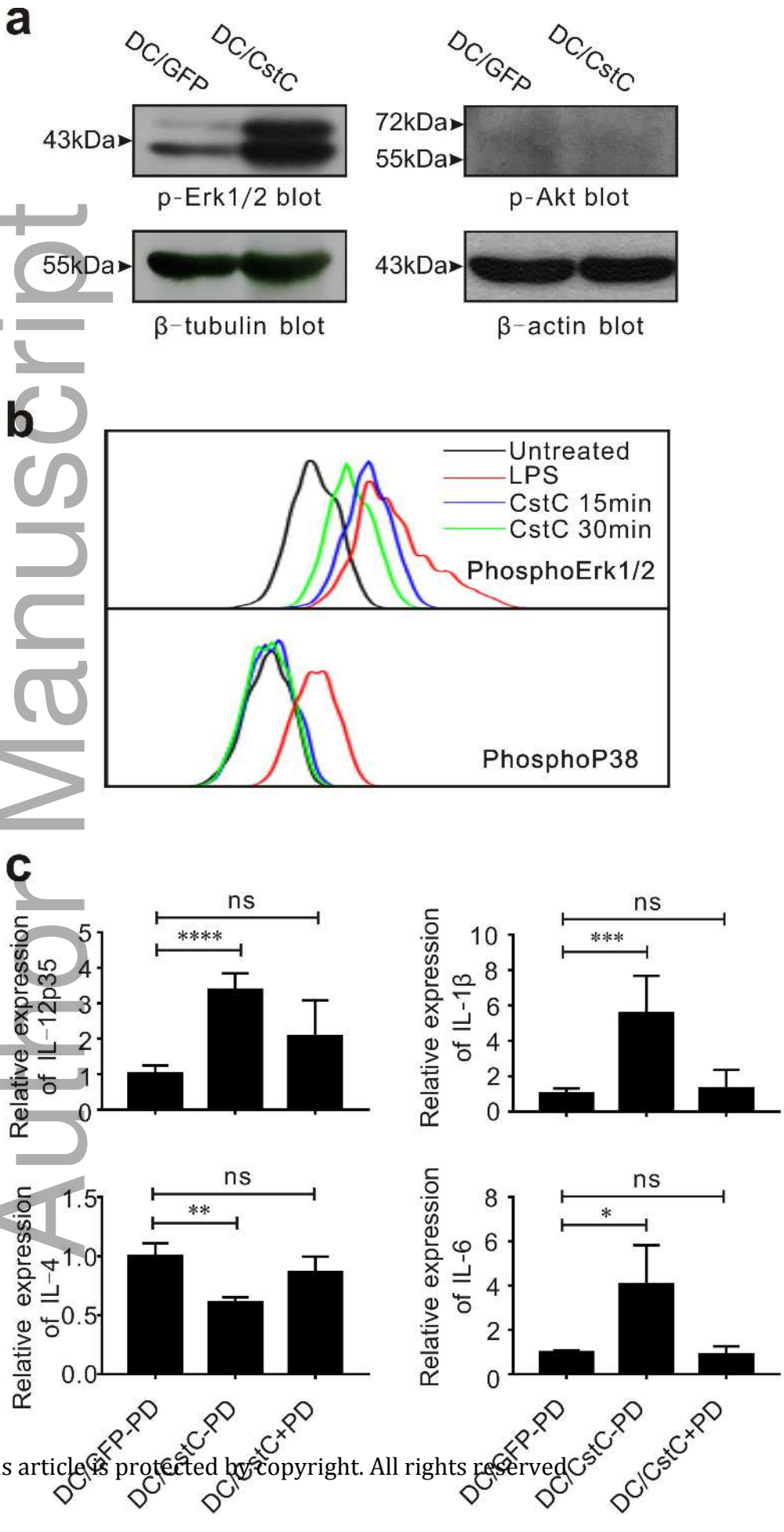

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