Curcumin ameliorates autoimmune diabetes. Evidences in accelerated murine models of type 1 diabetes

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Short tittle: Curcumin ameliorates autoimmunity in NOD

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ABSTRACT

Type 1 diabetes (T1DM) is a T-cell mediated autoimmune disease that selectively destroys pancreatic β–cells. The only possible cure for T1DM is to control autoimmunity against β–cell specific antigens. We explored whether the natural compound curcumin, with anti-oxidant and anti-inflammatory activities, might down-regulate the T-cell response that destroys pancreatic β cell to improve disease outcome in autoimmune diabetes. We employed two accelerated autoimmune diabetes models: 1) cyclophosphamide (CYP) administration to non-obese diabetic (NOD) mice and 2) adoptive transfer of diabetogenic splenocytes into NOD*scid* mice. Curcumin treatment led to significant delay of disease onset and in some instances prevented autoimmune diabetes by inhibiting pancreatic leukocyte infiltration and preserving insulin expressing cells. To investigate the mechanisms of protection we studied the effect of curcumin on key immune cell populations involved in the pathogenesis of the disease. Curcumin modulates T-lymphocyte response impairing proliferation and IFN–γ production through modulation of T-bet, a key transcription factor for pro-inflammatory Th1 lymphocyte differentiation, both at the transcriptional and translational levels. Also, curcumin reduces NF-κB activation in TCR-stimulated NOD lymphocytes. In addition, curcumin impairs the T-cell stimulatory function of dendritic cells with reduced secretion of pro-inflammatory cytokines and NO, low surface expression of coestimulatory molecules leading to an overall diminished antigen presenting cell activity. These *in vitro* effects correlated with *ex vivo* analysis of cells obtained from curcumin-treated mice during the course of autoimmune diabetes.

These findings reveal an effective therapeutic effect of curcumin in autoimmune diabetes by its actions on key immune cells responsible for β–cell death.

KEYWORDS

Inflammation; T-bet; T-lymphocytes; dendritic cells; NOD mouse

ABBREVIATIONS:

Ag	Antigen
APC	Antigen presenting cells
AUC	Area under the curve
CFSE	Carboxyfluorescein-diacetate succinimidyl ester
DC	Dendritic cells
Foxp3	Forkhead box 3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
NOD	Non obese diabetic
mAb	Monoclonal antibody
M	Mimotope
MFI	Mean fluorescence intensity
PMA/I	Phorbol 12-myristate 13-acetate / Ionomycin
Scid	Severe combined immunodeficiency

STZ	Streptozotocin
T-bet	T-box expressed in T-cells
T1DM	Type 1 diabetes
Th	T-helper
Treg	Regulatory T cell

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that arises from the selective and progressive loss of insulin-producing β cells by means of self-reactive T-lymphocytes [1, 2]. Treatment with insulin remains the most suitable therapy for T1DM patients. However, in many of them tight glycemic control is difficult to achieve leading to long-term vascular damage associated with kidney failure, heart disease, retinopathy and neuropathy [3]. Clinical manifestations of T1DM are only evident when more than 80% of the β cell mass has been destroyed [4]. It is possible to predict with a certain degree of accuracy those candidates that will progress to T1DM much before the appearance of clinical manifestations and prediction can be determined by measuring serum levels of autoantibodies in relatives of T1DM patients [5]. Thus, early therapeutic interventions would be beneficial to prevent T1DM. Considering the inflammatory nature of T1DM it is plausible to speculate that treatment with anti-inflammatory agents/drugs would be beneficial. Curcumin, a polyphenolic compound extracted from the rizhome of the spice plant *Curcuma longa*, has been extensively used for treatment of an ample spectrum of health problems. Curcumin possesses anti-depressant, anti-oxidative, anti-inflammatory and neuroprotective actions and acts through several intracellular mechanisms affecting multiple targets. Curcumin has been proved to be effective for the treatment of different forms of cancer, allergic reactions, asthma, Alzheimer´s disease and pathological disorders in which aberrant self-reactivity takes place such as inflammatory bowel disease, rheumathoid arthritis, experimental autoimmune encephalomyelitis and psoriasis [6-10]. The biosafety of curcumin has been proven exhaustively because of its use as a spice, colouring food agent and at higher doses in the Indian Ayurvedic medicine [11]. Also, curcumin inhibits the growth of tumour cells *in vivo* in athymic mice [12]. The best-known

mechanism is through its ability to modulate transcription factors such as NF-κB, AP-1, STAT and their down-stream signaling pathways [13, 14].

The hypothesis that administration of curcumin would ameliorate diabetes has been tested successfully in a murine model of insulin-resistance [15], and in a clinical trial on T2DM pre-diabetic patiens [16]. Its efficacy and safety has been previously tested in clinical trials [17, 18].

Curcumin has also been tested on a streptozotocin (STZ)-induced T1DM mouse model resulting in prevention of islet damage along with an *in vitro* protective effect on β cells, when cultured in the presence of inflammatory cytokines [19, 20]. Despite the importance of these studies using STZ to chemically induce β cell death, it is still unknown whether curcumin might be effective to prevent and/or ameliorate autoimmunity in an animal model that more closely resembles the human disease, particularly from the immunological standpoint. Herein, we report the therapeutic effect of curcumin and its putative mechanisms of action employing acute variants of the non-obese diabetic (NOD) mouse model [21].

MATERIALS and METHODS

Animals. The NOD, NOD*scid*, and NOD.BDC2.5 tgTCR (BDC2.5) mice (Jackson Laboratory, Bar Harbor, ME, USA) were bred under pathogen-free environment. BALB/c mice were purchased to FCEyN-University of Buenos Aires, Animal Facility. Studies were approved by the Animal Research and Care Committee (CICUAL #0001) FCEyN, University of Buenos Aires.

Accelerated models of T1DM and curcumin treatment. Cyclophosphamide (CYP)-induced diabetes was performed by injecting i.p. 200mg/kg body weight twice 14-days apart in female NOD mice. Adoptive transfer of diabetes was done in female NOD*scid* mice as described [22]. Curcumin (95%, Spectrum) was i.p. injected daily for 7-days and then, every other day until the end of the experiment. Diabetes was diagnosed when glycemia reached ≥300mg/dl in two consecutive readings (Optium Xceed®, Abbott Laboratories).

Histologic examination. The pancreata were fixed in 10% formaldehyde and embedded in paraffin. Insulin immunolabeling was performed on 7μm tissue sections with anti-human insulin mAb (BioGenex, Fremont, CA, USA). Sections were incubated with HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) and peroxidase activity developed with 3,3-diaminobenzidine (DAKO, Denmark), and counterstained with hematoxilin. At least 10 islets from each animal were scored for insulitis according to % of infiltration using the following criteria: 0, no insulitis; 1: <25%; 2: 25-50%; 3: 50-75%; and 4: >75%.

T-lymphocyte proliferation and ELISAs. Splenocytes were stained with carboxyfluorescein-diacetate succinimidyl ester (CFSE, Fluka-Sigma Aldrich, St. Louis, MO, USA) and cultured in RPMI 10% FBS and stimulated with PMA/Ionomicyn (Sigma Aldrich) or the

mimotope (Ac-MVLPLWVRME-NH_2), respectively. CD-4 T lymphocytes were stained for FACS analysis with biotinylated anti-CD4 (clone GK1.5) followed by streptavidin-allophycocyanin (eBiosciences, San Jose, CA, USA). Non-viable cells were excluded from analysis by 7AAD staining. ELISA kits (BD-Pharmingen, San Jose, CA, USA) were used to quantify IFN–γ and IL-4 in supernatants.

SDS-PAGE and Western blot analysis. $5x10^6$ splenocytes per well were cultured in 6-well plates, stimulated as described and curcumin was added prior to cell lysis. For phospho-NFκB detection, $2,5x10^5$ splenocytes were cultured in 96-well plates, stimulated with anti-CD3ε (10 μg/ml, eBiosciences, San Diego, CA, USA) and curcumin (10μM) or vehicle for 15, 30, 120 and 240 min. Lysis was done with 50mM sodium phosphate/1% v/v SDS/40mM 2-ME/2mM EDTA and loaded in 10% SDS-PAGE. Incubation with anti-T-bet Ab (Santa Cruz, Dallas, Texas, USA), anti-phospho-NFκB p65(Ser536) Ab (Cell Signalling Technology Inc., Danvers, MA, USA) and HRP-conjugated anti-mouse or anti-rabbit (Bio-Rad, hercules, CA, USA) were followed by ECL (Pierce Biotechnology, Rockford, IL, USA) detection.

Quantitative RT-qPCR. Total RNA was isolated using TriReagent (Sigma Aldrich). Reverse transcription was performed using MMLTV-RT (Promega, Madison, WI, USA) in the presence of RNAsin RNAse inhibitor (Promega) for 1h at 37°C followed by inactivation at 95°C. The following primers were used: mouse Foxp3, forward: 5´-CCCAGGAAAGACAGCAACCTT-3´ and reverse: 5´-TTCTCACAACCAGGCCACTTG-3´; mouse GATA-3: forward: 5´-CTACCGGGTTCGGATGTAAGTC-3´ and reverse: 5´-GTTCACACACTCCCTGCCTTCT-3´; mouse T-bet: forward:5'-GCCAGGGAACCGCTTATATG-3'and reverse: 5'-GACGATCATCTGGGTCACATTGT-3'.

Transient transfections and luciferase activity. EL4 T-cells (murine T-cell lymphoma) were transfected by electroporation [23, 24]. Luciferase activity was measured using the Luciferase measure kit (Promega). Cells were co-transfected with RSV-β-galactosidase expression vector for normalization. The murine IFN–γ promoter coupled to the luciferase reporter vector was obtained as described by Liberman *et al* [23], IFN–γ-Luc. T-bet binding sites coupled to the luciferase reporter vector is described in Liberman *et al* [24]. The murine T-bet expression vector is described in Liberman *et al* [23] [25]; pcDNA3-T-bet.

In vitro generation of dendritic cells. DC were generated from progenitor cells as described [26] and cultured in complete medium (RPMI 1640 from Invitrogen Life Technologies,10% FCS, glutamine, nonessential amino acids, sodium pyruvate, HEPES, 2-ME, and antibiotics) supplemented with GM-CSF and IL-4(1000 U/ml of each). DC were pre-treated for 2h with either 20μM curcumin or vehicle (DMSO) and then stimulated with 1μg/ml LPS (Sigma) and 50ng/ml IFN-γ (R&D, Minneapolis, MN, USA). After treatment, DC were analyzed by FACS. IL-12p70, IL-6 and TNF–α were evaluated using ELISA kits (BD-Pharmingen). Nitrite was measured as an indicator of NO production using Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diamine dihydrochloride in 2.5% phosphoric acid) at 570nm.

Flow cytometry and endocytosis assay. Cells were stained as described [26]. Dendritic cells were stained with: biotinylated anti-CD11c (clone HL3), phycoerythrin-conjugated-antiCD80 (clone 16-10A1), -antiCD86 (clone GL-1), -antiCD40 (clone 1C10) and –antiMHCII (clone M5/114.15.2), and streptavidin-allophycocyanin (eBiosciences). Isotype-matched mAb were used as negative controls. For endocytosis assay, DC ($1x10^6$ cells)

were incubated at 37°C for 1h with 10mg/ml FITC-dextran. Endocytosis was analyzed by FACS.

Mixed lymphocyte reaction. Splenocyte (BALB/c) were enriched in T-lymphocytes by nylon wool column. Cells were eluted with 10ml of warm RPMI 10% FCS and further diluted at a concentration of $2x10^6$ cells/ml in complete medium. NOD DC were co-cultured with the T-lymphocytes at 1:10, 1:20 and 1:40 ratio and cultured for 72h in complete RPMI and 1μCi/well [H^3]TdR was added for the last 18h. Cells were harvested and [H^3]TdR uptake was measured.

Crossed antigen presentation. . BDC2.5 mice were treated either with 25mg/kg curcumin or with vehicle (DMSO) i.p., daily for 7-days. Splenocyte suspensions were enriched in T-cell or APC after elution from nylon wool columns. The APC-enriched fraction (referred to as APC) was incubated with 1mg/ml mimotope and co-cultured with the T-lymphocyte fraction (referred to as T-cells, $2x10^5$) at 1:10; 1:20 and 1:40 ratio. APC from curcumin-treated animals were cultured with T-cells from vehicle-treated group and vice versa 72h and 1μCi/well [H^3]TdR was added the last 18h.

Statistical analysis. Results are presented as mean±SEM. Comparison between all means was done using ANOVA followed by Bonferroni´s multiple comparison test. Comparison between two means was performed by the Student´s t-test (one- or two-tailed). Incidence of diabetes between groups was compared by Kaplan Meyer analysis and the log-rank test. A $p<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Administration of curcumin prevents autoimmune diabetes in the cyclophosphamide-accelerated model of disease

Natural development of diabetes occurs in 60-80% of 12-30 week-old female NOD mice [21]. In order to overcome this asynchronously process, we employed CYP that induces rapid and synchronized diabetes in NOD mice [27]. Mice received curcumin injections i.p. since the day before the first CYP injection (at day 0, d0) daily during seven days and then, every other day until the end of the experiment (d60) (Fig. 1a). Vehicle-treated mice developed hyperglycemia between d20-24. Curcumin administration of 50; 25 and 5mg/kg led to 66.7% (n=6), 57.1% (n=8) and 41.2% (n=12) of diabetes-free mice at d60, respectively ($p<0.05$ vs. control, log-rank test) implicating that curcumin had a dose dependent effect on reduction of diabetes incidence. No statistical significant differences were detected between the highest doses of curcumin (25 and 50mg/kg of body weight). Therefore, we hereafter used curcumin 25mg/kg dose.

A glucose tolerance test was performed to assess the physiologic response of β cells. Curcumin-treated animals had a similar glucose clearance compared to non-diabetic, untreated age- and sex-matched mice (area under the curve: 12926 ± 698 (n=4) vs 13896 ± 599 (n=4), respectively). As expected, overtly diabetic mice had an elevated AUC: 19821 ± 2553 (n=4) in comparison to normoglycemic groups: the untreated and curcumin-treated mice ($p<0.05$). Administration of curcumin did not cause apparent toxicity in NOD mice.

Administration of curcumin delays disease onset employing the adoptive transfer model of autoimmune diabetes

We challenged the beneficial effects of curcumin using the adoptive transfer of disease into NOD*scid* as a model of T1DM [26, 28]. In this model, diabetes onset is more rapid (d20-30) and aggressive than the natural progression of the disease in NOD mice. Curcumin treatment significantly delayed the onset of T1DM (median= 49 days) in comparison to the control group (median= 29 days; $p<0.001$ vs. control, by log-rank test) (Fig. 2).

Curcumin inhibits pancreatic leukocyte infiltration

Islet infiltration (insulitis) initiates the destruction of β cells and eventually diabetes [29]. To investigate whether curcumin administration in NOD*scid* mice reconstituted with diabetogenic splenocytes had an effect on islet infiltration, we harvested pancreata for histological analysis after 20 or 35 days after adoptive transfer of disease. Islets from vehicle-injected NOD*scid* mice showed insulitis at d20 and this infiltration was aggravated at d35 (Fig. 3a). Insulitis augmentation correlated with a reduction of insulin staining, indicative of specific β cell loss. By contrast, the administration of curcumin prevented insulitis maintaining an intact pancreatic architecture and intense immune-staining of insulin (Fig. 3a). Quantification of islet infiltration is shown on Fig. 3c.

Also, we harvested pancreata from animals challenged with CYP and treated with curcumin. Histological analysis showed that curcumin reduced insulitis and preserved insulin expression in β cells (Fig. 3b).

Curcumin impairs polyclonal and Ag-specific T-lymphocyte pro-inflammatory responses

To define the mechanisms by which curcumin is involved in the prevention/delay of autoimmune diabetes, we examined its effect on Ag-specific T-cell responses *in vitro*. Mimotope(M)-stimulated BDC2.5-splenocytes [28] had a strong proliferative response *in vitro* and 10μM curcumin treatment resulted in a decrease in the proliferation of $CD4^+$T-lymphocytes as assessed by CFSE dilution (Fig. 4a). Similar effects were observed when stimulating with PMA/I (Fig. 4b). Th1-lymphocytes and their hallmark cytokine IFN–γ are central in T1DM pathogenesis [29]. Curcumin reduced M-stimulated IFN–γ release resulting in 18-fold less cytokine production ($p<0.01$ vs. M-stimulated splenocytes, Fig. 4c). Also, curcumin reduced IFN–γ release from PMA/I-stimulated splenocytes (Fig 4d). OVA stimulation did not affect cell proliferation or IFN–γ release (data not shown).

TBX21 (T-box transcription factor, also known as T-bet) is a key transcription factor that governs Th1 differentiation and controls the expression of IFN–γ [25]. Curcumin reduced Ag-specific expression of T-bet by splenocytes (Fig. 4e). The same effect was observed when NOD splenocytes were stimulated with PMA/I (Fig 4f).

Antigen stimulation of TCR signaling to NF-κB is required for T cell proliferation and differentiation of effector cells. To get insight into curcumin-induced signaling we determined whether it might inhibit NF-κB activation in NOD T lymphocytes. Plate-bound anti-CD3ε stimulation led to NF-κB activation in culture splenocytes and curcumin treatment impaired induction of phospho-NF-κB p65 (Ser536) as shown by Western blot analysis (Figure 4g).

Then, we asked whether curcumin might be able to modulate GATA-3, a master transcription factor involved in Th2 development. We did not detect splenocyte GATA-3

expression by Western blot possibly due to the strong Th1 bias of NOD splenocytes. GATA-3 expression levels were not modified in EL-4 T-cell line by curcumin (data not shown).

The transcriptional activity regulation of T-bet by curcumin was studied transfecting EL-4 T-cells with a reporter plasmid containing T-bet response elements and a -3447bp IFN–γ promoter cloned upstream of the luciferase gene (T-bet-RE-Luc and pIFN–γ-Luc) together with high expression levels of T-bet [23]. Curcumin inhibited T-bet transcriptional activity on its response elements (10 and 20μM vs. control, $p<0.01$ and $p<0.001$, respectively) and on the activity of IFN–γ promoter after PMA/I stimulation (10-20μM, $p<0.001$, Fig. 4h-i). Over-expressed T-bet levels were not changed by curcumin, confirming its effects exclusively at the transcriptional level (Fig. 4j).

Effect of long-term curcumin administration on immune splenocyte response *ex vivo*

We assayed whether the observed *in vitro* inhibitory effects of curcumin on T-lymphocytes also take place *in vivo*. The spontaneous IFN–γ secretion of splenocytes from curcumin-treated adoptively transferred mice was significantly lower than the control group (95.5% $p<0.05$, Fig. 5a). In line with this, we observed lower proliferation in splenocytes from curcumin-treated mice compared to those from the control group (56.9%, $p<0.05$, Fig. 5a). IL-4 and IL-10 secretion were undetectable (not shown). T-bet mRNA levels were significantly lower in curcumin-treated mice (52.8%, $p<0.01$) relative to controls (Fig. 5b). However, there were non-significant changes of FoxP3 ($p= 0.1$, CI:95%) and GATA-3 ($p=0.073$, CI:95%) spleen mRNA levels in curcumin-treated mice compared with the control group (Fig. 5b).

Curcumin down-regulates LPS/IFN–γ-induced maturation and function of dendritic cells

The importance of DC in the pathogenesis of autoimmune diabetes is well documented [30, 31]. DC were pre-treated with 20μM curcumin or alternatively, with vehicle for 2h and stimulated or not with LPS/IFN–γ for 24h. Curcumin pre-treatment of DC ($CD11c^+$) followed by LPS/IFN–γ stimulation exhibited a reduction in the percentage of cells expressing surface CD40, CD80, CD86 and MHC-II when compared with those stimulated with LPS/IFN–γ (Fig. 6a). The MFI of these molecules was also reduced by curcumin pre-treatment.

Curcumin strongly inhibited IL-12p70, IL-6 and TNF–α secretion 2- ($p<0.001$), 1.7- ($p<0.05$) and 3-fold ($p<0.001$) compared with LPS/IFN–γ stimulation. IL-10 secretion was not detectable (not shown). Curcumin inhibits inducible NO synthase in activated macrophages [32]. Consistent with its anti-oxidant capacity, curcumin significantly inhibited NO release in LPS/IFN–γ-stimulated DC ($p<0.05$, Fig. 6b).

Endocytosis is critical to mount an efficient immune response by DC. Analysis of curcumin-treated immature DC revealed a reduced ability of mannose receptor-mediated endocytosis (Fig.6c).

The modulatory effects of curcumin on DC maturation suggest that this agent might alter their function. Thus, we assayed a mixed leukocyte reaction using responder T-lymphocytes (BALB/c, $H2^d$) and DC (NOD, $H2^{g7}$). As expected, LPS-stimulated DC showed the strongest proliferative allogeneic T-cell response, whereas curcumin treatment of LPS-stimulated DC led to a significantly impaired proliferation of responder T-cells (Fig. 6d).

Ag-specific T-lymphocyte proliferation is diminished by curcumin action on both T-cell and APC *ex vivo*

We performed T-cell proliferation experiments to know whether the *in vitro* immunomodulatory effects of curcumin on both DC and T-lymphocytes also take place *in vivo*. This was addressed by means of a crosslinked Ag presentation assay in which BDC2.5 mice were treated either with curcumin or vehicle during 1-week. APC (from curcumin- or alternatively, vehicle-treated BDC2.5 mice) were pulsed with M and co-cultured with BDC2.5 T-lymphocytes (from curcumin- or alternatively, vehicle-treated mice). Fig. 7 illustrates a reduced T-cell proliferation in co-cultures where either APC or T-cells isolated from curcumin-treated animals were present. The proliferation was maximal when both cell populations came from the vehicle-injected mice. These data demonstrate that curcumin alters the antigen-specific T-cells response *ex vivo*.

DISCUSION

In the present work we challenged the well-known anti-oxidant and anti-inflammatory activities of curcumin in autoimmune diabetes employing the NOD mouse in which the immune system plays an essential role in the pathogenesis of disease, as occurs in T1DM [21]. The inhibition of pro-inflammatory cytokine-induced β cell death by curcumin and its beneficial effect on experimental models of diabetes by STZ administration has been previously reported [19]. Although, the administration of STZ in mice has been employed as a model of T1DM for long, this agent induces β cell death by chemical toxicity. In fact, necrotic β cells in STZ-injected mice have been detected as soon as 2-4h after its administration while leukocyte infiltration is evident only after 3-4 days [33]. We show that curcumin protected against the development of CYP-accelerated autoimmune diabetes in NOD mice, a model in which autoimmunity arises by reducing Treg lymphocyte number [21]. Moreover, in order to resemble the immunological activation state of an individual at the moment of T1DM diagnosis we employed the transfer of diabetogenic splenocytes to NOD*scid* mice [22]. Herein, we report for the first time that, in the context of these two accelerated models of disease, the administration of curcumin controlled islet-specific autoimmunity, delaying and in some instances completely stopping diabetes progression. Histological pancreata analysis showed that curcumin significantly diminished the presence of inflammatory cells characteristic of insulitis. In accordance, several studies have shown that curcumin inhibits inflammation and autoimmunity in animal models of atherosclerosis, multiple sclerosis, reumathoid arthritis, sepsis, psoriasis, Alzheimer´s disease, and experimental colitis [34-38].

The pathogenesis of T1DM is complex and involves the activation of APC such as, DC and macrophages, and the activation of Ag-specific Th1 cells. Pro-inflammatory cytokines

play a determinant role in autoimmune diabetes [39, 40]. We found that curcumin treatment resulted in a decrease of both T-cell proliferation and IFN–γ secretion induced by non-specific stimuli and importantly, in response to a diabetogenic peptide. These findings are in line with those described by others regarding curcumin inhibition of both T-cell differentiation and proliferation [7, 41].

T-bet is central in Th1 development [25]. Along with STAT4 and IL-12R signaling, T-bet directs histone posttranslational modifications and remodels *ifng* chromatin allowing efficient gene transcription [42]. Curcumin acts on IL-12-stimulated STAT4 signaling with impact on T-cell differentiation and proliferation [7, 41]. We found that curcumin inhibits T-bet expression levels in both non-specific and Ag-specific-stimulated T-lymphocytes. In addition, curcumin inhibited T-bet transcriptional activity on its response gene elements, affecting T-bet-induced IFN–γ promoter activity. These results may account for the observed curcumin-mediated IFN–γ secretion in splenocyte cultures and suggest that curcumin could play an immunomodulatory role in Th differentiation strongly inhibiting the Th1 inflammatory profile. In accordance, we found reduced mRNA T-bet levels in the spleens of curcumin-treated mice compared with controls. Meanwhile, GATA-3 and Foxp3 mRNA levels remained unchanged. Thus, curcumin did not exert major effects on Th2 and Treg subpopulations, suggesting that attenuation of diabetes might be due to regulation of effector T-cells at the initiation of autoimmunity. The significance of Th17 in T1DM is uncertain. Th17 cells transfer to NOD mice was found to induce diabetes only after *in vivo* conversion to Th1 [43]. Blockade of IL-17 did not prevent autoimmune diabetes [44]. Moreover, we did not observe abundant IL-17-producing splenocytes in NOD mice [40].

NOD mice possess several abnormalities regarding their immune system. Their APC have impaired ability to mediate tolerance induction (reviewed in [21]). NOD DC have

abnormally high antigen presenting stimulatory capacity governed by hyperactivation of NF-κB [45]. DC activation is a critical step for the induction of a strong immune response and within the signaling pathways involved in this process the nuclear translocation of the nuclear factor NF-κB p65 subunit plays an important role. In this respect, inhibition of NF-κB activation blocked maturation of DC [46]. It has been reported that treatment of immature DC with curcumin decreased stimulation-induced activation of NF-κB and repressed LPS-induced NF-κB promoter activity. Also, curcumin inhibited LPS-induced up-regulation of phosphorylation in mitogen-induced protein kinases (MAPKs), suggesting that curcumin inhibited NF-κB activation by suppressing MAPK intracellular signal [47]. It has also been found that curcumin modulate inducible NO synthase and IL-12 production through the reduction of NF-κB pathway in other immune cells such as monocytes and macrophages [32, 48]. Interestingly, this is consistent with our findings that curcumin inhibited NO, TNF–α and IL-6 secretion in LPS-stimulated DCs, also reported in earlier studies [7, 49]. IL-12p70 released by DC and macrophages drives differentiation of Th1 cells [50, 51]. Delayed onset and reduced incidence of autoimmune diabetes in recipient mice of DC with impaired IL-12p70 production has been reported [52]. Importantly, we show that curcumin-treated NOD DC reduced IL-12p70 release when stimulated *in vitro*. Taken together, the underlying impairment of DC maturation may account, in part, by the curcumin effect on the activation of NF-kB. Although, we cannot rule out the action of curcumin in other intracellular signal pathways [53]. The effect of curcumin in the MAPKs pathway remains to be determined in NOD-derived dendritic cells. Curcumin also reduced endocytosis and stimulatory capacity in an allogeneic T-lymphocyte response. These results are in accordance to what have been reported using DC from C57BL/6 mice [47] and human monocyte-derived DC [54].

APC from curcumin-treated mice exhibited a reduced ability to support a specific diabetogenic-peptide T-cell proliferation. The therapeutic effect achieved with curcumin may be due to the inability of APC to induce an optimal priming signal and/or to impair Th1 lymphocyte response, as demonstrated *in vitro*. Notably, when control-injected mice APC were employed as stimulators and T-cells from curcumin-treated animals as responders, a similar reduction in T-cell proliferation was observed, suggesting that curcumin *in vivo* acts on both APC and T-lymphocytes.

Activation of naïve T lymphocytes requires both antigen-specific signal through TCR-peptide-MHCII interaction and costimulatory signal by B7 ligand overexpressed on APC which bind to CD28 on their surface. NF-κB signaling is pivotal in controlling the proliferation of naïve T cells and the survival of T cells during antigen presentation [55]. There are several important molecules involved within the NF-kB signaling pathway and phospho-NF-κB p65 (Ser536) plays a key role in the activation of proliferation and gene transcription in T lymphocytes. Our results showed that curcumin exerted effective reduction of NF-κB activation in TCR-stimulated T lymphocytes of NOD mice.

Apart of the two signals consisting of TCR and CD28 on the T cell surface interacting with MHCII and B7 on APC, respectively, naïve Th activation requires a third signal [56]. This third signal consists of pro-inflammatory mediators produced by the innate immune response to boost the adaptive immune response and it is important for inducing, enhancing and prolonging the antigen-specific T cell response [57]. Interestingly, Tse et al. reported that modulation of a redox balance with the use of an antioxidant inhibited the generation of third signal from the innate immune response which leads to Ag-specific hyporesponsiveness [58]. In view of these evidences, we speculate that inhibition of the third signal is another mechanism that might take place with curcumin treatment

contributing to antigen-specific hyporesponsiveness in NOD accelerated models of diabetes. Indeed, the antioxidant activity of curcumin led to decrease NO and TNF-α secretion by stimulated DC.

Taken together, these evidences suggest that curcumin impairing the NF-kB signaling pathway at both T lymphocytes and APC, and inhibiting the third signal contributes to attenuation of immunity and subsequently ameliorates diabetes in accelerated murine models.

Recently, administration of curcumin was found to improve the management of peripheral complications associated with diabetes in experimental models [59] and in T2DM patients [16] with encouraging results regarding its anti-diabetic properties. Despite its poor bioavailability, the safety, tolerability and non-toxicity of curcumin with doses up to 12g/day are well established [16]. The present study highlights the ability of curcumin to modulate key immune cells involved in the attack against β cells and, demonstrates that this agent: 1) attenuates immunity, 2) delays diabetes onset and, in some instances, 3) blocks disease progression using aggressive NOD models. The potential therapeutic of curcumin in the naturally occurring diabetes in NOD mice remains to be established. We propose that curcumin in combination with immunomodulatory agents deserve to be investigated as therapy for T1DM.

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ACL and MJP contributed to the experimental work, analysis, interpretation of data and revised the manuscript critically.

DUALITY OF INTEREST

The authors declare that there is no duality of interest associated with this manuscript.

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FIGURE LEGENDS

Figure 1

Curcumin prevented diabetes in CYP-injected NOD mice. (a) Kaplan-Meier plot of cumulative diabetes incidence in mice treated with curcumin (Cur) 5mg/kg (n=12), 25mg/kg (n=8) and 50mg/kg (n=6) or vehicle (Veh, n=15). *$p<0.05$ vs vehicle, by log-rank (Mantel-Cox) test. (b) Glucose tolerance of diabetic vehicle-treated (Diab, CYP+vehicle) (n=4), non-diabetic curcumin-treated (Non diab, CYP+curcumin) (n=4) and non-diabetic untreated control mice (Non diab, No treatments) (n=4). Inset bar-chart shows quantification of AUC. Data are shown as mean±SEM. *$p<0.05$ vs diabetic mice, by ANOVA followed by Bonferroni.

Figure 2

Curcumin delayed the adoptive transfer of autoimmune diabetes.

Kaplan-Meier plot of cumulative diabetes incidence in NOD*scid* mice adoptively transferred with diabetogenic splenocytes and treated with curcumin 25mg/kg (n=11) or vehicle (n=5). ***$p<0.001$ vs vehicle, by log-rank (Mantel Cox) test.

Figure 3

Curcumin prevented insulitis. Immunostaining of β cells (light brown) from (a) curcumin- and vehicle-treated NOD*scid* mice at 20 and 35 days after adoptive transfer of diabetes. (b) CYP-challenged curcumin- and vehicle-tretated NOD mice. Representative islets are shown, 400x magnification. Insulitis quantification was done 20 (c) and 35 days (d) after adoptive transfer. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs veh by ANOVA, followed by Bonferroni.

Figure 4

Curcumin impaired Ag-specific and polyclonal T-lymphocyte responses *in vitro*. $CD4^+$T-lymphocyte proliferation was reduced by 10μM curcumin (thick line) when (a) BDC2.5-splenocytes were stimulated with M (dashed line) or (b) NOD splenocytes were stimulated with P/I (dashed line). Flow cytometric data shown CFSE/MFI of stimulated (normal font) and stimulated+curcumin (bold font) conditions. Shaded area in the histogram represents CFSE incorporation by non-stimulated T-lymphocytes and continuous thin line background staining. IFN–γ secretion of stimulated-splenocytes with (c) M or (d) PMA/I was diminished by 10μM curcumin. *$p<0.05$, **$p<0.01$ vs M or P/I alone, ANOVA followed by Bonferroni. Western blot of T-bet expression is shown for (e) M- or (f) PMA/I-stimulated splenocytes incubated or not with 10-20μM curcumin (normalized to GAPDH). Western Blot of (g) phospho-NF-κB p65 (Ser536) (p-p65) is shown for plate-bound anti-CD3ε stimulated NOD splenocytes treated or not with 10μM curcumin (Cur 10 or veh), normalized to loaded protein (ponceau). Representative of at least two independent experiments for each stimulation condition. T-bet transcriptional activity on (h) its response elements and (i) on IFN–γ promoter done with T-bet overexpression in EL-4 T-cells. Mean±SEM from 2-3 independent experiments are represented. **$p<0.01$, *** $p<0.001$, ANOVA followed by Bonferroni. (j) Western blot of T-bet is representative of two independent experiments.

Figure 5

Splenocyte response from adoptively transferred NOD*scid* mice treated with curcumin *in vivo*. (a) IFN–γ secretion and proliferation of splenocytes from vehicle- (n=3) or curcumin-treated (n=4) NODscid mice 35 days post-transfer. *$p<0.05$ by unpaired t-test; #$p<0.05$ by unpaired (one-tailed) t-test. (b) Spleen mRNA levels of T-bet, GATA-3 and Foxp3 of

vehicle-(n=6) or curcumin-treated (n=7) mice. **p<0.01 by unpaired t-test (CI: 95%). Mean ±SEM is shown.

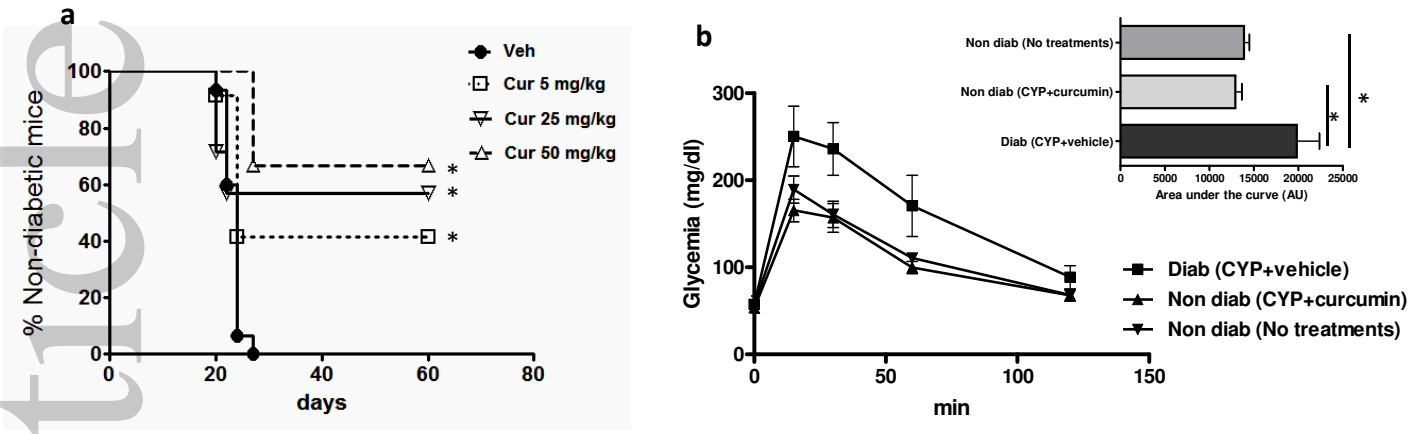
Figure 6.

Inhibition of LPS/IFN-γ-induced DC maturation and function by curcumin. (a) $CD11c^{+}$DC surface expression of coestimulatory molecules and MHC-II with the corresponding percentage and MFI (brackets) are shown for 20μM curcumin- (bold font, thick line) and vehicle-treated or LPS/IFN-γ-stimulated conditions (L/I, normal font, dashed line). Representative flow cytometric data from at least three independent experiments and MFI quantification from stimulated (L/I) vs curcumin-treated stimulated DC (L/I + Cur20) is shown, *p <0.05 by unpaired one-tailed Student t-test, n=3. (b) Pro-inflammatory cytokines and NO secretion *p<0.05, ***p<0.001 vs. LPS/IFN-g-stimulated DC, n=3 by ANOVA followed by Bonferroni. (c) Dextran-FITC uptake by CD11c+DC expressed by percentage and MFI (brackets) is shown for 20mM curcumin- (bold font, thick line) and vehicle-treated or LPS/IFN-γ-stimulated conditions (normal font, dashed line); shaded area represents DC autofluorescence. Representative FACS data of three independent experiments. (d) MLR shows a reduction in T-lymphocyte proliferation when co-cultured with LPS-stimulated curcumin-treated DC vs LPS-stimulated, **p<0.01, ***p<0.001, n=3, by ANOVA followed by Bonferroni for each T-cell:APC ratio.

Figure 7

Curcumin administration modulated splenic APC and T-cell responses. APC and T-cells were obtained from curcumin- and vehicle-treated BDC2.5 mice. APC were M-pulsed and co-cultured with T-cells. Impairment of T-cell proliferation was observed when APC from

curcumin-treated mice were co-cultured with T-cells from vehicle-treated mice and when APC from control group were co-cultured with T-cell from the curcumin-group. *$p<0.05$ and #$p<0.05$, respectively vs co-culture of APC and T-cells from vehicle-treated mice (by unpaired t-test for each APC:T-cell ratio).



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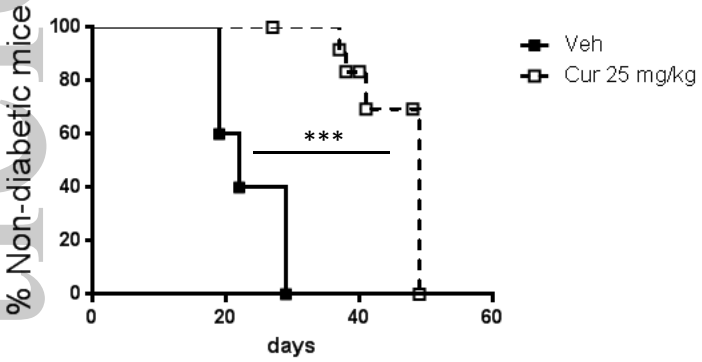

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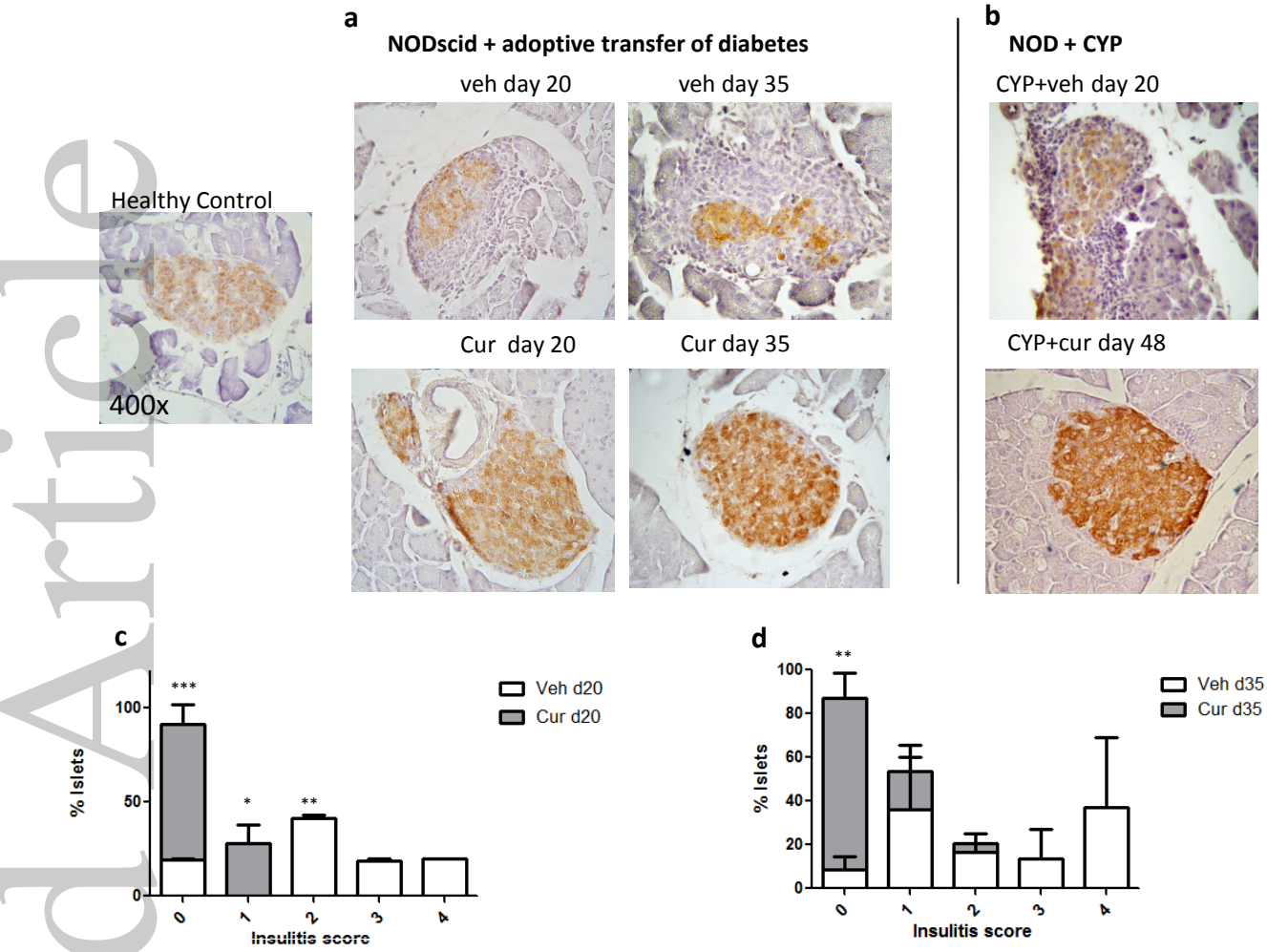

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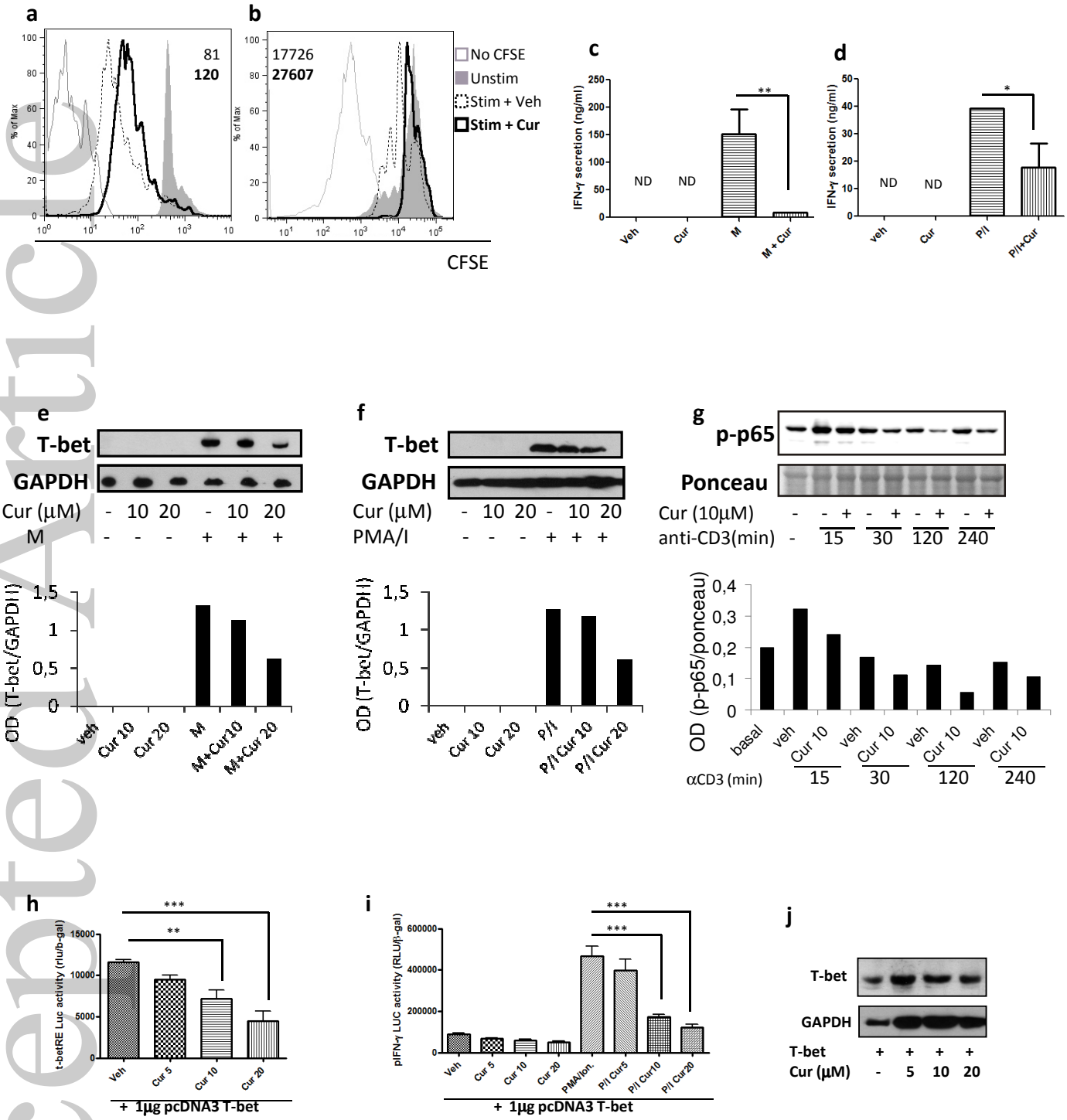

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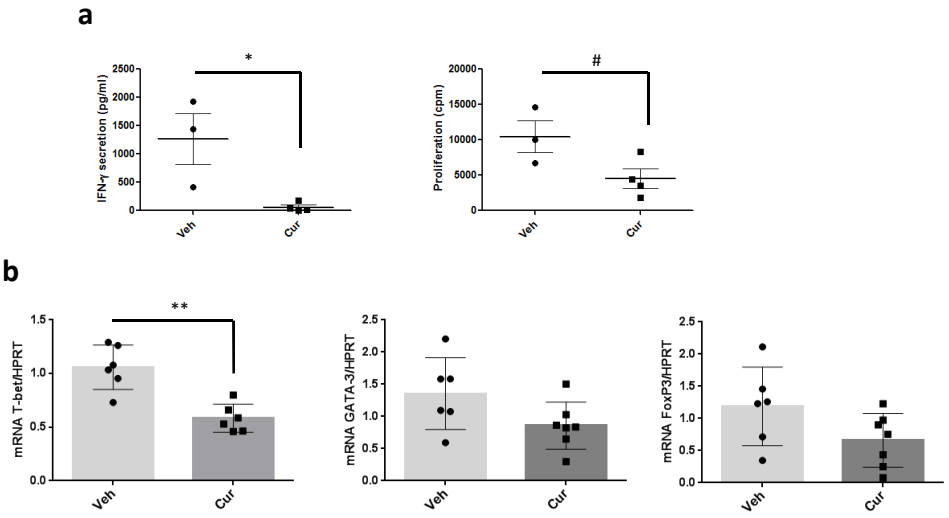

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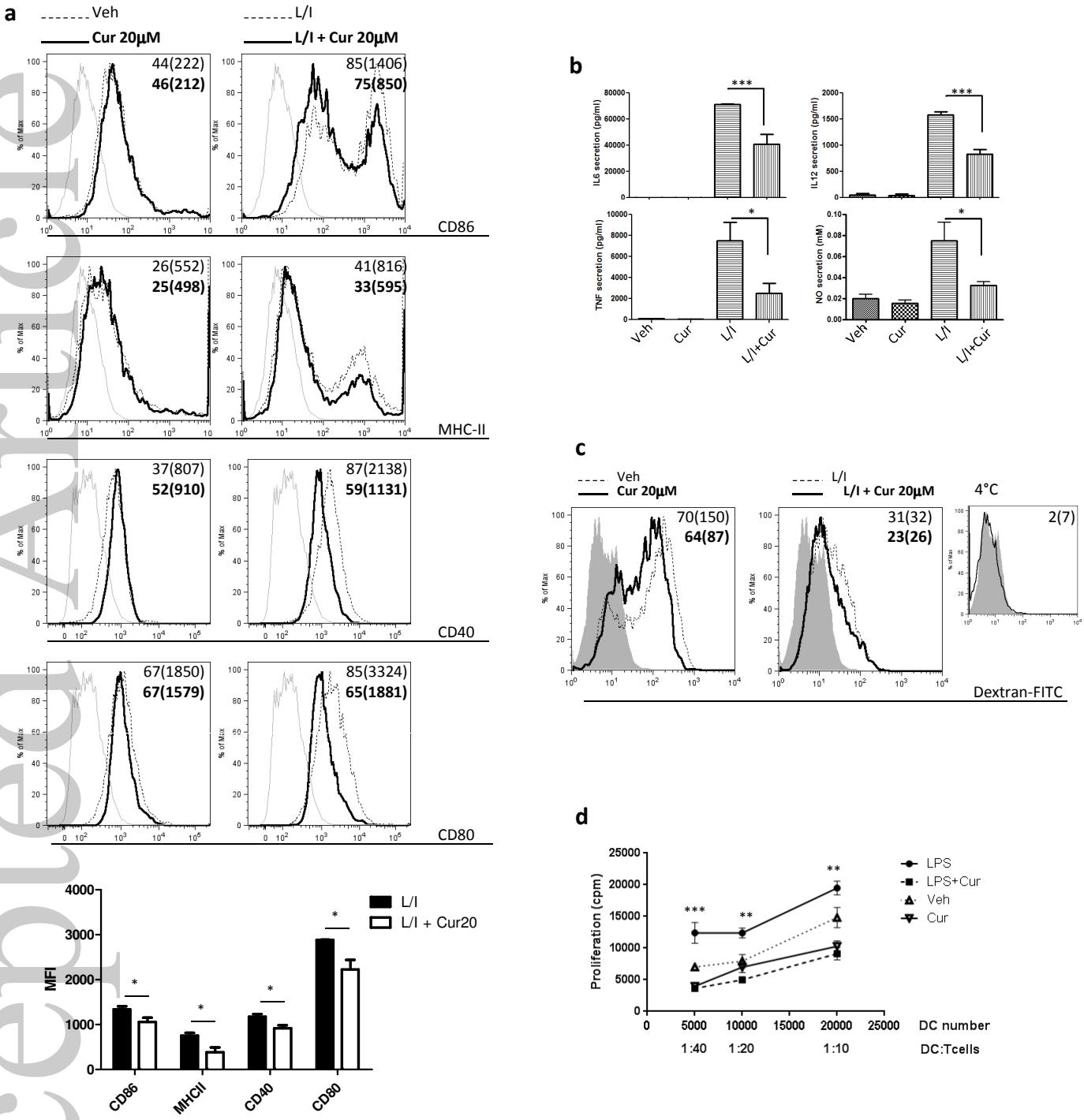

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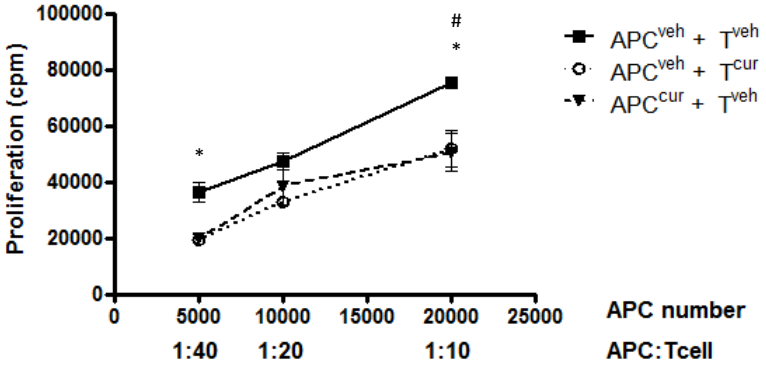

Figure 7.