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Glucuronoxylomannan promotes the generation of antigen-specific T regulatory cell that suppresses the antigen-specific Th2 response upon activation

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Abstract

T regulatory cells (Treg) have the capability to suppress the skewed immune response, but the generation of antigen (Ag)-specific Treg for therapeutic purpose is a challenge; the mechanism of Ag-specific Treg activation remains obscure. Here, we report that glucuronoxylomannan (GXM) is capable of promoting the development of human tolerogenic dendritic cells (DC). GXM-pulsed DCs increased the expression of forkhead box P3 (Foxp3) in naïve human $CD4^+CD25^-$ T cells *via* activating Fc γ receptor IIb and activator protein-1 and promoting the expression of transforming growth factor β in dendritic cells. Furthermore, the conjugated complex of house dust mite Ag, Dermatophagoides pteronyssinus (Der p) 1, and GXM-pulsed DCs to drive the naïve human $CD4^+CD25^-$ T cells to develop into the Der p 1-specific Tregs, which efficiently suppressed the Ag-specific Th2 responses. We conclude that GXM-conjugated specific Ag have the capacity to up-regulate the tolerogenic property of DCs and promote the generation of Ag-specific Tregs; the latter can be activated upon the re-exposure to specific Ag and suppress the skewed Ag-specific T helper (Th)2 responses.

Keywords: T regulatory cells • dendritic cells • allergy

Introduction

Apart from antigen (Ag)-presenting cells, dendritic cells (DC) also dictate T cell's phenotyping *via* providing given cytokines, such as DC-derived interleukin (IL)-12 induces T helper (Th)1 cells, IL-4 induces Th2 cells, whereas transforming growth factor (TGF)-β or IL-10 induces T regulatory cells (Treg) [1]. However, the underlying mechanisms of the induction of Treg generation, requiring cytokines, such as TGF-β, by DCs remains largely unknown. Costimulatory molecules, such as CD80 and CD86, play a crucial role in Th cell differentiation, but are not required by Treg development [2], whereas the inducible costimulator (ICOS)/ICOS ligand (ICOSL) is involved in Treg activities [3, 4]. T cells express ICOS [5]; ICOSL can be released by DC. Whether the interaction of ICOS/ICOSL assists the differentiation of Ag-specific Treg is an interesting topic and is worth being investigated. However, the regulation of the expression of ICOSL in DC remains unclear.

Previous reports [6] indicate that Treg functions are compromised in the body of patients with allergic diseases, which could be one of the mechanisms in disturbing the immune homeostasis in the body, such as causing the imbalance of Th1/Th2 response. Some investigators tried to counterbalance Th2 response by strengthening Th1 function. However, the outcome did not favour this speculation; it caused even more severe immune responses in the body [7].

The breaking down of mucosal tolerance is another pathogenic mechanism of allergic diseases. Tregs play a critical role in the maintenance of immune tolerance in the body [8]. Presumably, Ag-specific Tregs suppress activated Ag-specific Th1 or Th2 to prevent the unwanted immune responses. Indeed, the cumulative evidence indicates that Tregs have the capability to suppress the skewed immune responses such as Th1 or Th2 pattern immune inflammation [9]. Although mounting studies have described the generation

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of Treg [10, 11], the induction and activation of Ag-specific Treg are still an enigma that need to be further understood.

Two categories of Tregs are described in the body. One is the thymic-origin natural Tregs that are not expandable, which thus cannot be used for therapeutic purpose. Another is the inducible Tregs that can be generated from the peripheral naïve T cells. Several approaches have been tried in the generation of inducible Tregs, such as using TGF-β, IL-10 and zymosan to drive naïve T cells to become the Tregs [10–12]. Yet, the efficiency of the generation is to be improved. Ag-specific Tregs play an essential role in maintaining peripheral immune tolerance. Dysfunction of Treg in patients with allergic diseases has been proposed [13]. The goal of the classic therapeutic remedy in the treatment of allergic disease, the Ag-specific immune therapy, is to induce Ag-specific Tregs to suppress Ag-specific immune responses [14]. However, the mechanisms of Ag-specific Treg generation remain to be elucidated.

Glucuronoxylomannan (GXM) is the major polysaccharide component of the capsule of *Cryptococcus neoformans* and exerts potent immunosuppressive capacity [15, 16]. Previous reports indicated that GXM had the capability to increase the expression of TGF-β in macrophages [17]. TGF-β is important for Treg generation [18–21]. We thus suggested that GXM had immune regulatory function by promoting Treg generation. In the present study, we observed the conjugates of GXM and a specific Ag, Der p 1 (Der), in modulating DC's function and its downstream effect on the generation of Ag-specific Tregs. The suppressive effect on Ag-specific Th2 responses by the generated Tregs was also examined.

Materials and methods

Conjugation of GXM to Der

House dust mite Ag Der (Amersham, Shanghai, China) was conjugated to GXM (lipopolysaccharide [LPS]-free); GXM was isolated from the culture supernatant fluid of serotype D, strain B3501, following reported procedures [22] using N-succininmidyl (3-[2-pyridyl]-dithio) propionate (Pierce, Rockford, IL, USA) to conjugate the Der to GXM, according to reported procedures [23]. The conjugated Der–GXM was designated as gDer and purified by the Pharmacia fast protein liquid chromotography (FPLC) gel filtration.

$CD4^+$ T cell- and mononuclear cell-derived DC preparation

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by Ficoll-Hypaque (SigmaAldrich, Mississauga, ON, Canada) density centrifugation. $CD4^+$ T lymphocytes were purified by positive selection using an immunomagnetic cell sorting (MACS); the purity was assessed by flow cytometry (FACS; over 90%); the cells were cultured in RPMI1640 medium for 1 day and then used for further experiments. The study using human samples was approved by the research ethical committee at Shanxi Medical University (HR-02–2006).

For preparation of DC, PBMC were cultured with RPMI1640 medium supplemented with 20 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) (SigmaAldrich, Oakvall, ON, Canada) and 20 ng/ml IL-4 (SigmaAldrich, Oakvall). The media were changed on day 3. The cells were collected on day 6. $CD11c^+$ cells were isolated by MACS with a reagent kit (Meltenyi Biotec, Auburn, CA, USA). Sample cells were analysed by FACS. The purity was over 95%. The expression of CD80 and CD86 was at low levels that indicated that these DCs were at the immature stage. The DCs were used for further experiments.

Real-time quantitative reverse transcription polymerase chain reaction (qPCR)

Total RNA was extracted from the DCs using an RNeasy mini kit (Qiagen, Mississauga, ON, Canada). cDNA was synthesized using iScriptTMcDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada). The resulting cDNA was subjected to qPCR that was performed with a LightCycler using a SuperScript III Platinum SYBR Green Two-Step qPCR kit (Invitrogen, Burlington, ON, Canada). The amplified product was detected by the presence of an SYBR green fluorescent signal. The standard curve was designed with β-actin cDNA. The resulting amplicon was quantified with the standard curve. The primers and qPCR conditions included: TGF-β, forward: 5′-GGGACTATCCACCTGCAAGA-3′; reverse: 5′-CCTCCTTGGCGTAGTAGTCG-3′ (annealing temperature: 60°C, for 30 sec., 39 cycles. 239 bp; NCBI, NM_000660). β-actin: forward, 5′-GGACTTCGAGCAAGAGATGG-3′; reverse, 5′-AGCACTGTGTTGGCGTACAG-3′ (456 bp; NCBI, DQ407611).

Western blotting

Cellular and nuclear extracts were prepared. Equal amounts of protein (40 μg/well) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with biotin-labelled primary antibodies at a concentration of 1 μg/ml overnight at 4°C. Peroxidase-avidin was added at a concentration of 1 μg/ml to the membranes for 1 hr at room temperature. The membranes were then developed by ECL Plus Western blotting detection reagents (GE Healthcare Life Science, Baie d'Urfe, QC, Canada), and the signal was detected by an X-ray film.

ELISA

The levels of IL-2, IL-4, IL-10 and TGF-β1 in the extracts were assessed with commercial reagent kits (R&D Systems, Burlington, ON, Canada), following the manufacturer's instruction. The procedures of TGF-β1 ELISA were the same as for other molecules except that the samples needed acid activation; the procedures are detailed in the manufacturer's instruction. The levels of activated NF-κB and activator protein-1 (AP-1) were assessed with TransAM nuclear factor-κB (NF-κB) p65 and AP-1 family kits (Active Motif, Carlsbad, CA, USA), following the manufacturer's instructions.

Flow cytometry

The cells were fixed with 1% paraformaldehyde for 30 min. on ice. Cells used to detect the intracellular cytokines (such as IL-4 and interferon

[IFN]-γ) were permeabilized with a reagent kit (BD Bioscience, Mississauga, ON, Canada), following the manufacturer's instruction. The cells were then stained with fluorescence-labelled antibodies for 30 min. on ice and analysed with a flow cytometer (FACSarray, BD Bioscience). All the fluorescently labelled antibodies for the flow cytometry assay were purchased from BD Bioscience. Forward and side scatter were used to gate the positively stained cells. Data were analysed by the software FlowJo (Tree Star, Ashland, OR, USA).

Ag presentation assay

Ag presentation by gDer-pulsed DCs was analysed by reported procedures [24]. Briefly, gDer-pulsed DCs were seeded onto a 96-well microtitre plate (1×10^5 cells/well); the plate was incubated for 1 hr at 37°C. After washing with pre-warmed PBS three times, the cells were fixed with 1.0% paraformaldehyde for 5 min. on ice. The plate was then washed with PBS three times, and naïve $CD4^+$ T cells were added (1×10^5 cells/well). The plate was incubated at 37°C for 48 hrs and then centrifuged at $600 \times g$ for 10 min.; the supernatants were collected and subjected to IL-2 assay with ELISA.

RNA interference (RNAi)

Human siRNA of Fc γ receptor IIb (FcγRIIb), SHIP-1 and AP-1 was designed and synthesized by Invitrogen. The sequence of FcγRIIb was 5′-UUUGUUUGCCCUCUAGGGUTT-3′ (NM_001002273), SHIP-1 sequence was 5′-CGAGGCGGCCUGCGCGGCGTT-3′ (NM_001567) and c-Jun sequence was 5′-GGGCUCAGAGUUGCACUGATT-3′ (NM_002228); the control siRNA sequence was 5′-AAC GAA GCA ACT AAG CTC G-3′, which did not target any genes. Transfection was performed following the manufacturer's instruction. Briefly, siRNA was added to the culture of DCs (10^6 cells/ml) at a concentration of 60 pmol. Transfection was carried out with oligofectamine following the manufacturer's instruction. The efficiency of siRNA transfection was determined by Western blotting, which showed naïve DCs naturally express FcγRIIb, SHIP-1 and c-Jun that could be inhibited by treating the cells with the counterpart siRNA; the peak inhibitory effect was reached 48 hrs after transfection, which sustained for another 48 hrs and declined thereafter; the expression of FcγRIIb, SHIP-1 and c-Jun in these DCs returned to normal levels 5 days after transfection. The control siRNA did not affect FcγRIIb, SHIP-1 and c-Jun expression in DCs. The transcription efficiency was over 90%, and the inhibition was also over 90% and was reproducible in all experiments.

Chromatin immunoprecipitation assay (ChIP) assay

Fresh and GXM-stimulated cells were fixed by 1% formaldehyde at room temperature for 10 min. After washing, the cells were incubated with the lysis buffer for 20 min. at 4°C. The chromatin was sheared by sonication (Branson 250; Branson, Danbury, CT, USA) at a power setting of 2 and 30% duty cycle. The input samples were stored at −20°C until use. Other samples were precipitated by anti-acetyl histone H4 antibody (Upstate Biotechnology, Lake Placid, NY, USA). A part of the samples was used for the detection of acetyl histone H4 and activated AP-1 by immune blotting. DNA was isolated by phenol/chloroform extraction. One microlitre of immunoprecipitated DNA was used for each real-time PCR. The following primers specific for the TGF-β1 promoter −1458- to −1347-bp (122-bp) region were used: forward primer (5′-AGGCTGCTTAGCCACATG-3′) and reverse primer (5′-GTGGGAGGAGGGGGCAA-3′).

Assessment of Ag-specific T-cell proliferation

Isolated $CD4^+$ T cells were cultured in complete RPMI 1640 medium, which consisted of HEPES (2.5 mM/100 ml;), penicillin (100 U/ml;), streptomycin (100 μg/ml) and L-glutamine (2 mM) and was supplemented with 10% foetal calf serum (FCS; all the cell culture reagents were purchased from Fisher Scientific, Shanghai, China), in the presence or absence of specific Ag Der (10 μg/ml) and freshly prepared peripheral mononuclear cells (T cell:mononuclear cell = 10^6:10^5 cells/well) for 72 hrs. [^{3}H] thymidine (0.5 Ci/well) was added to the culture for the last 16 hrs. The [^{3}H] thymidine incorporation was assessed with a scintillation counter.

Statistic analysis

Data are presented as the means ± standard deviation (SD). Differences between the two groups were evaluated with the Student *t*-test; data among three or more groups were evaluated with ANOVA. A Bonferroni adjustment was applied to *post-hoc* group comparisons, when required. A $P < 0.05$ was accepted as a significant criterion.

Results

GXM promotes the development of tolerogenic DCs

DCs expressing high levels of TGF-β or IL-10 are termed as tolerogenic DCs, which play an important role in the generation of Tregs [1]. Previous reports indicate that GXM has the capability to modulate DC's function [23] and has immune regulatory capability [15]. In the present study, we observed that in the presence of GXM, the production of TGF-β1, but not IL-10 (data not shown), by DCs increased in a GXM-dose-dependent manner; GXM conjugated with Ag, Der, (gDer) also increased the expression of TGF-β in human DCs. The expression of TGF-β1 was detected in cellular extracts at the mRNA and protein levels. The elevation of TGF-β1 in the culture was detected 12 hrs after the addition of GXM, reaching the peak value at 24 hrs that sustained through the entire period of 72 hrs (Fig. 1). We did observe a trifle increase in TGF-β1 in DCs after stimulation with Der alone that did not reach the significant levels as compared with control group (data not shown).

FcγRIIb plays a role in GXM-induced immune regulation

It is reported that FcγRIIb mediates the effect of GXM in its immune suppressive function [15]; we thus considered that FcγRIIb might be involved in the increases in the production of

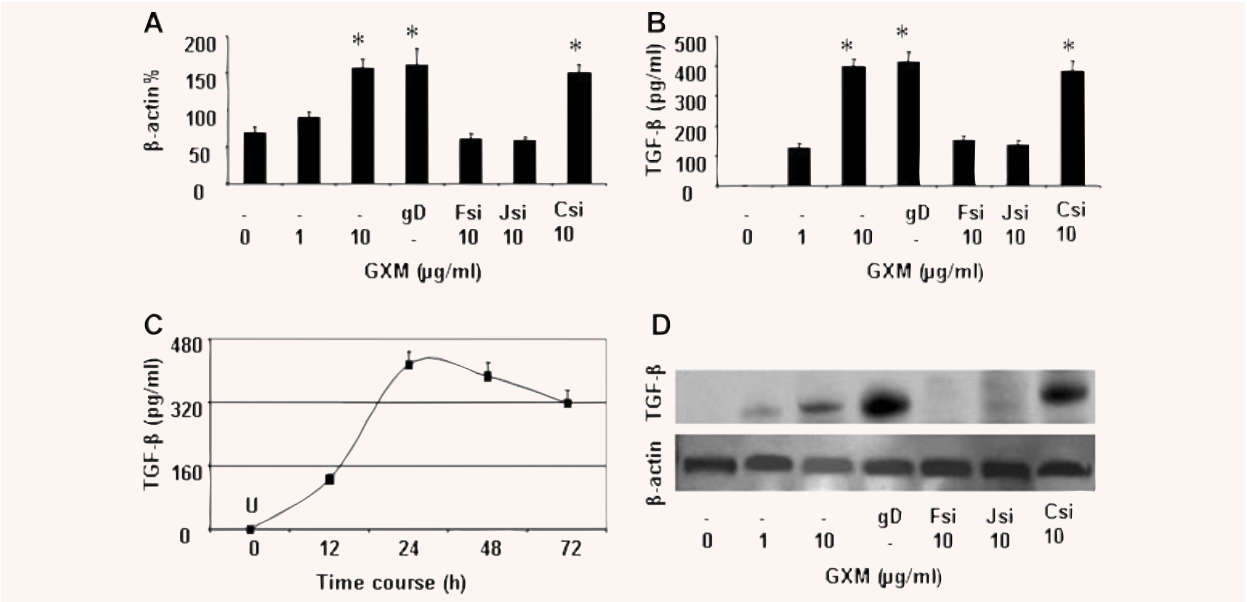

Fig. 1 GXM increases the expression of TGF-β1 in human DCs. DCs were generated from human peripheral mononuclear cells and cultured in the presence or absence of GXM at graded doses. Some DCs were pre-transfected with siRNA. The expression of TGF-β1 was assessed by qPCR, ELISA and Western blotting. (**A**) Bars indicate mRNA levels of TGF-β1 in cellular extracts (normalized as a percentage of β-actin). U, under the detectable level; gD, DCs were stimulated by gDer (10 μg/ml) instead of GXM; Fsi, FcγRIIb siRNA; Jsi, c-Jun siRNA; Csi, control siRNA. (**B**) Bars indicate levels of TGF-β1 in the supernatants. (**C**) Time course of TGF-β1 release by DCs (GXM dose = 10 μg/ml). (**D**) Western blotting bands show the protein of TGF-β1 in cellular extracts of DCs. Data are presented as the means ± SD from three separated experiments.

TGF-β1 in DCs by GXM. Indeed, the pre-treatment with RNAi of FcγRIIb, but not the control siRNA, abolished the increase in the production of TGF-β1 in DCs (Fig. 1).

SHIP-1 is a component in the downstream signal transduction pathway after FcγRIIb activation. To understand if SHIP-1 was involved in GXM-induced production of TGF-β in DCs, we assessed phosphorylated SHIP-1 in DCs after the stimulation of GXM. Indeed, the phosphorylated SHIP-1 increased in cellular extracts in a GXM-dose-dependent manner. The increase in phosphorylated SHIP-1 was blocked by pre-treatment with either FcγRIIb RNAi or SHIP-1 RNAi (Fig. 2A), but not with control siRNA.

Transcription factors AP-1 and NF-κB were proposed to play a role in TGF-β gene transcription [24]. To elucidate if AP-1 and NF-κB were involved in the increase in the GXM-induced TGF-β expression in DCs, the activated AP-1 and NF-κB in nuclear extracts were assessed with TransAM kits. As expected, the amounts of activated AP-1 and NF-κB increased in a time-dependent manner (Fig. 2B). The fact indicates that GXM stimulation activated AP-1 and NF-κB in DCs. To further confirm the role of AP-1 and NF-κB in TGF-β expression in DCs, genes of c-Jun (one of the main members of AP-1 family) or/and NF-κB were knocked down by RNAi prior to the exposure to GXM; the c-Jun siRAN transfection abolished GXM-induced TGF-β expression (Figs. 1 and 2D), whereas NF-κB RNAi did not, nor did the control siRNA. The fact indicates that GXM does increase NF-κB activity in DCs, but it is not indispensable in the process of GXM-induced TGF-β expression.

With ChIP assay, we further found that activated c-Jun was coprecipitated by anti-acetyl histone H4 antibody after the exposure to GXM. As shown by immune blotting, histone H4 acetylation increased in a time-dependent manner. Increase in activated c-Jun was also observed in parallel to the acetyl histone H4 (Fig. 2C). The ChIP assay also showed that TGF-β promoter DNA amplicon increased after the GXM stimulation in a GXM-dose-dependent manner. Pre-treatment with RNAi to knock down the genes of FcγRI, SHIP-1 or c-Jun abolished the increase in TGF-β promoter activation (Fig. 2D).

gDer modulates DC's function

One of the aims of this study was to generate Ag-specific Tregs. To elucidate whether GXM had the capability to modulate DC's function, we assessed the costimulatory molecule CD80 and CD86 in DCs after being stimulated by GXM, Der or gDer. The expression of CD80 and CD86 was detected in naïve control DCs at low levels; exposure to gDer, GXM or Der did not further increase the expression of CD80 and CD86 (Fig. 3A).

Since DCs play a critical role in the generation of Tregs, T-cell activation is involved in the process that may require certain costimulatory activities; we reasoned that there must have been other molecules fulfilling the costimulatory function in addition to CD80 and CD86. ICOS and ICOSL play a role in regulating Treg function

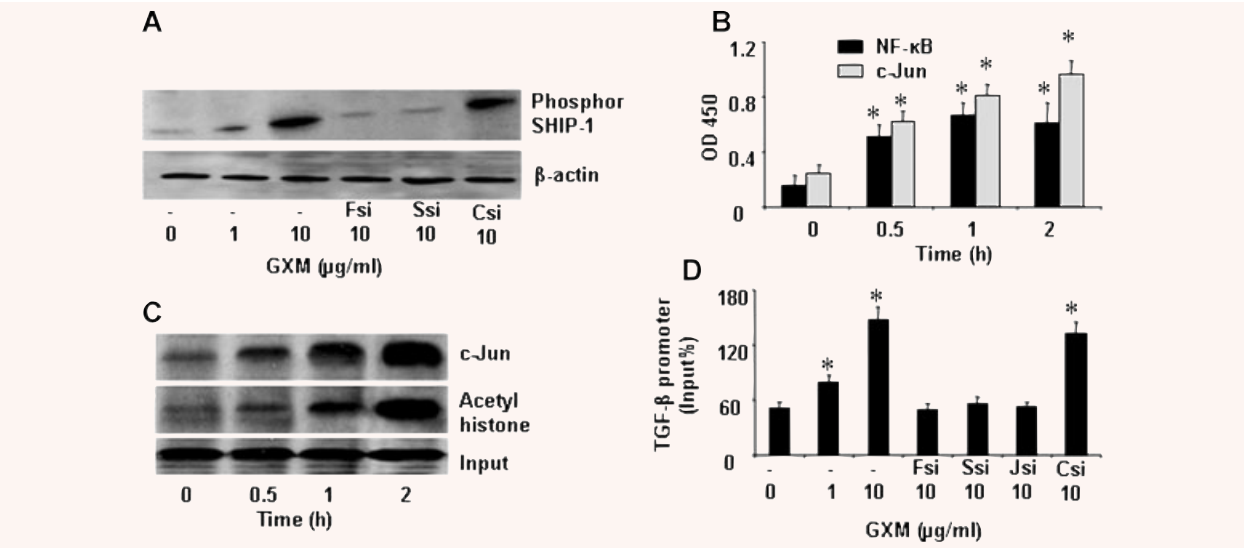

Fig. 2 GXM increases TGF-β promoter activity. DCs were treated with GXM at graded doses for 24 hrs. Some DCs were pre-transfected with siRNA. (**A**) Gel bands indicate phosphorylated SHIP-1 in DC cellular extracts that was detected by Western blotting. (**B**) Bars indicate the activated AP-1 in nuclear extracts that was assessed by a specific ELISA. (**C** and **D**) TGF-β promoter activation was analysed by ChIP assay. (**C**) Gel bands show anti-acetyl histone H4 antibody immunoprecipitated c-Jun and acetyl histone H4. (**D**) Bars indicate the amounts of amplified DNA products of TGF-β promoter that is normalized by input DNA. Fsi, FcγRIIb siRNA; Ssi, SHIP-1 siRNA; Jsi, c-Jun; Csi, control siRNA. Data were from three separate experiments.

[4]. T cells express ICOS [4]; activated DCs express ICOSL [25]. They might be involved in GXM-modulated DC's function. As shown by qPCR and Western blotting, the expression of ICOSL was markedly increased in DCs by exposure to gDer, GXM or Der, but not by media alone (Fig. 3B and C).

Der is an Ag that can be captured by DCs to be presented to T cells to initiate Ag-specific immune response. To elucidate if gDer-pulsed DCs also present Ag Der to T cells, we carried out a major histocompatibility complex (MHC) II-restricted Ag-presenting assay. As shown by ELISA, the levels of IL-2 in supernatant increased significantly in gDer-pulsed DC group as compared with naïve DC group (Fig. 3D). The results indicate that gDer-pulsed DCs have the Ag-presenting capability. On the basis of the fact that ICOS/ICOSL plays a role in the regulation and maintenance of Treg function [4], we inferred that the interaction of ICOS/ICOSL might be involved in the process of Ag-induced $CD4^+$ T-cell activation. Thus, a batch of $CD4^+$ T cells was treated with anti-ICOS antibody prior to the exposure to gDer-pulsed DCs. As expected, the IL-2 production by $CD4^+$ T cells was abolished (Fig. 3D). The results indicate that the interaction of ICOS/ICOSL was involved in gDer-pulsed DC-induced T-cell activation.

gDer-pulsed DCs increase Foxp3 expression in naïve human T cells

Expression of Foxp3 is regarded as a unique marker of Tregs [26]. Mounting evidence has indicated that Foxp3 is inducible in peripheral T cells [10, 26]. The remaining challenge is how to generate a large number of Ag-specific Tregs to be used in the treatment of allergic diseases. Prompted by previous report [19] that GXM had the capability of modulating the oral tolerance *via* modulating DC functions, we exposed isolated human naïve $CD4^+CD25^-$ T cells to the GXM-, Der- or gDer-pulsed DCs in the presence of IL-2 in culture media. As expected, the expression of Foxp3 increased significantly in the T cells after coculture with GXM-pulsed DCs for 72 hrs. The ratio of $Foxp3^+$ T cells reached 22.8 ± 2.9%. Considering that the ratio of cells was still low, which was far from the usable amounts, and that more coculture cycles might raise the ratio of $Foxp3^+$ T cells, we isolated the $CD4^+$ T cells from the coculture and re-exposed the cells to freshly prepared GXM-pulsed DCs for 1–3 more cycles. Indeed, further increases in the ratio of $Foxp3^+$ T cells were observed, which was over 60% $Foxp3^+$ T cells among $CD25^+$ T cells. Coculture with gDer-pulsed DCs further increased the ratio of $Foxp3^+$ T cells, which was over 90% $Foxp3^+$ T cells among $CD25^+$ T cells after the completion of four coculture cycles (Fig. 4). Der-alone-pulsed DCs did not increase $Foxp3^+$ T-cell generation (data not shown).

Activation of generated Tregs by specific Ag

It is proposed that Tregs have the capability to suppress the skewed immune responses by expression of suppressive molecules such as IL-10 and TGF-β [27], but the initiation factors for suppressive molecule expression in and release from Tregs

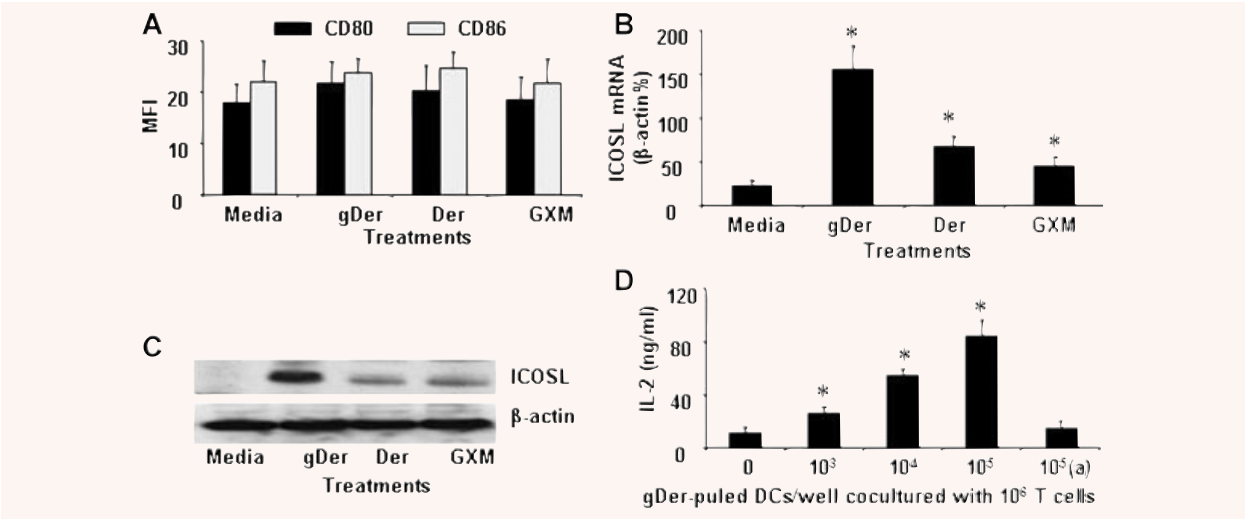

Fig. 3 gDer modulates DC's function. (**A–C**) Immature DCs were cultured with gDer, or GXM or Der for 24 hrs. (**A**) Bars indicate the expression of CD80 and CD86 in DCs that were assessed by FACS. MFI, medial fluorescence intensity. (**B**) Bars indicate ICOSL mRNA expression in DCs that was assessed by qPCR. (**C**) Western blotting bands show ICOSL protein in cellular extracts. (**D**) Ag presentation was assessed by IL-2 assay; the procedures are detailed in the text. Bars indicate the levels of IL-2 in the supernatants. (a), T cells were pre-treated with anti-ICOS antibody. Data are presented as the means ± SD. $*P < 0.05$, compared with the media group (**A–C**) or dose '0' group (**D**). Data were from three separate experiments.

remain obscure. As the Tregs were generated by coculture of naïve T cells with gDer-pulsed DCs, they might have become the Der antigen-specific Tregs and had the potential to be activated upon re-exposure to specific antigen, Der. Sample cells were analysed by FACS; the analysis showed that the ratio of $Foxp3^+$ T cells in isolated $CD25^+$ T cells was over 90%. Generated DCs (irradiated) were exposed to Der (10 μg/ml) for 30 min. at 37°C; after washing, the DCs were cocultured with isolated Tregs at graded ratios overnight. As shown by qPCR, ELISA and Western blotting, the expression of TGF-β in cellular extracts increased in response to coculture with Der-loaded DCs in a DC number-dependent manner. The surface expression of TGF-β was detected in Der-specific Tregs, which was markedly increased after the coculture with Der-pulsed DCs (Fig. 5).

Activated Tregs suppress Ag-specific Th2 response *in vitro*

We have found that the generated Ag-specific Tregs expressed the suppressive molecules, such as TGF-β, in response to re-exposure to specific Ag (Fig. 5). To elucidate if these Ag-specific Tregs have immune therapeutic potential, $CD4^+$ T cells were isolated from the peripheral blood of 15 patients with Der-positive skin test, high levels of Der-specific serum IgE antibody and typical clinical symptoms of perennial allergic rhinitis. Der-specific Tregs were prepared as described above. The Tregs and $CD4^+$ T cells were cocultured at graded ratio in the presence of irradiated DCs (T cell: DC = 10:1) and Der at a dose of 10 μg/ml for 72 hrs. As shown by [^{3}H]thymidine incorporation, T-cell proliferation increased significantly in response to specific Ag, Der, stimulation, which was efficiently suppressed by the presence of Der-specific Tregs in a cell number-dependent manner. ELISA results showed high IL-4 levels in the supernatant, which were suppressed by the coculture with Der-specific Tregs. In a separate experiment, with the same procedures, brefeldin A (10 μg/ml) was added to the culture for the last 3 hrs. The $CD4^+$ T cells were stained with anti-IL-4 antibody. The results showed that IL-4^+ T-cell population was significantly higher in patients with allergic rhinitis than in controls in response to re-exposure to specific Ag Der. The IL-4 response was significantly inhibited by the presence of Der-specific Tregs (Fig. 6). The results indicate that the generated Ag-specific Tregs have the capability to suppress the Ag-specific Th2 response, which has therapeutic potential in the treatment of allergic diseases.

Discussion

The present study revealed that GXM had the capability to promote the tolerogenic DC development *via* increasing the production of TGF-β by DCs. GXM-pulsed DCs enhanced the expression of Foxp3 in human $CD4^+CD25^+$ T cells to confer the regulatory feature on these cells manifesting the increases in the expression of the suppressive molecules, TGF-β. Furthermore, the study also showed that GXM conjugated with a common allergen, Der, generated Der-specific Tregs, which could be activated by re-exposure to Der. The activated Der-specific Tregs expressed the suppressive

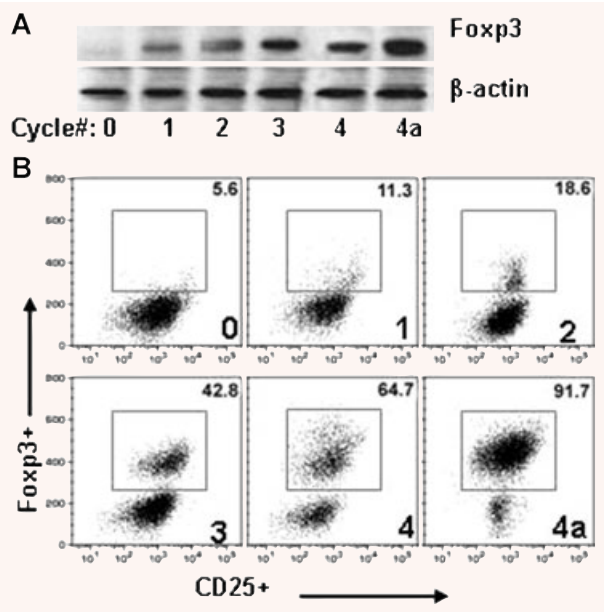

Fig. 4 Generation of Tregs. Human peripheral $CD4^+CD25^-$ T cells were cocultured with GXM- or gDer-pulsed DCs for 0–4 cycles. Foxp3 expression in T cells was detected by Western blotting and FACS. (**A**) Western blotting gel bands show Foxp3 protein in cellular extracts. 4a, $CD4^+CD25^-$ T cells were cocultured with gDer-pulsed DCs for 4 cycles. (**B**) FACS histograms. The gated cells are $Foxp3^+$ T cells. The number in the upper right indicates the percentage of $Foxp3^+$ T cells. The number in the lower right indicates the coculture cycles. Data were from four separate experiments.

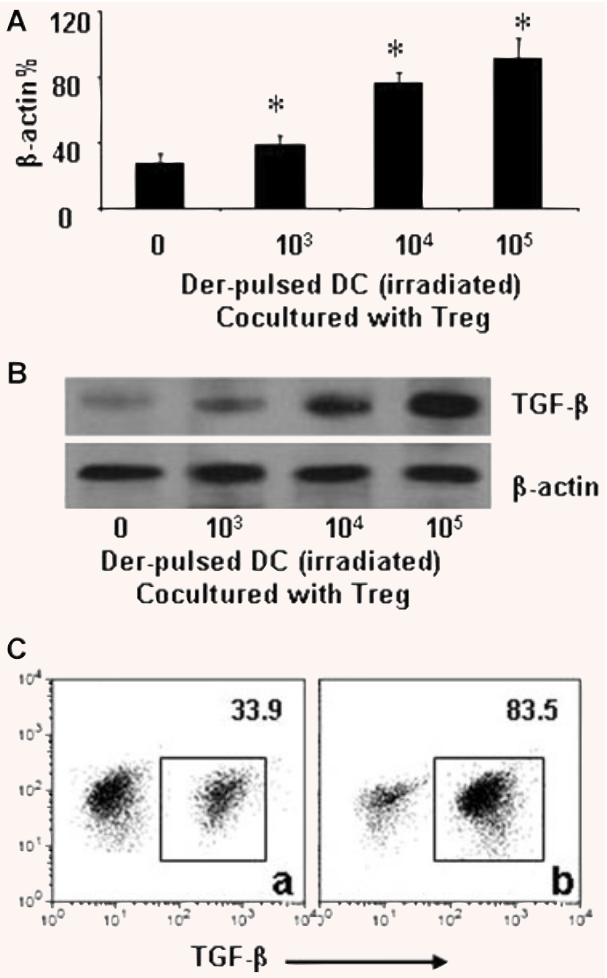

Fig. 5 Der-specific Tregs can be activated by specific Ag challenge. Generated Der-specific Tregs were cocultured with irradiated DCs at graded cell numbers (DCs had been pulsed by Der at 10 μg/ml for 30 min. overnight). (**A**) Bars indicate the levels of TGF-β mRNA in cellular extracts (percentage of β-actin; means ± SD. $*P < 0.05$, compared with dose '0' group). (**B**) Western blotting gels show TGF-β protein levels in cellular extracts. (**C**) FACS dot plots show TGF-β-positive staining (the gated cells) on the surface of Tregs before and after coculture with Der-pulsed irradiated DC (Treg:DC = 10:1). Data were from three separate experiments.

molecule TGF-β to suppress the Der-specific Th2 response in an *in vitro* experiment with $CD4^+$ T cells from patients with Der-specific allergic rhinitis.

Previous reports propose that GXM has immune suppressive function [28]. The major effect of GXM in its immune regulatory activity is suggested by modulating DC maturation [23], MHC II expression [28] and induction of apoptosis in target cells [28, 29]. In agreement with these studies, the present data demonstrate that GXM also promotes the development of tolerogenic DCs by enhancing TGF-β production in DCs. Tolerogenic DCs play a critical role in the development of immune tolerance in the body *via* the production of tolerogenic molecules, such as IL-10 or/and TGF-β. Our data show that GXM-pulsed human DCs have the capability to produce high levels of TGF-β, which can be released to the culture media or the microenvironment; the fact indicates that these DCs have the potential to generate the inducible extrathymic Tregs, in agreement with previous reports that indicate that TGF-β promotes the expression of Foxp3 in $CD4^+CD25^+$ T cells [10].

The signal transduction pathway of TGF-β expression in DCs was described in detail by a previous report [30]. In that study, the authors demonstrated that the stimulation of IL-1β induced TGF-β expression in DCs. Activation of NF-κB and AP-1 played a critical role in TGF-β gene transcription. The present data indicate that GXM activates FcγRIIb to induce SHIP-1 phosphorylation. The phosphorylated SHIP-1 activates NF-κB and AP-1 in DCs. The difference between Lee et al.'s report and our study is that we found that, although GXM induced NF-κB activation, blocking NF-κB by RNAi did not interfere with the GXM-induced TGF-β expression in DCs, but AP-1 antagonism did, whereas Lee et al. indicated that both NF-κB and AP-1 played important roles in TGF-β expression elicited by IL-1β. The fact reflects that different signal transduction

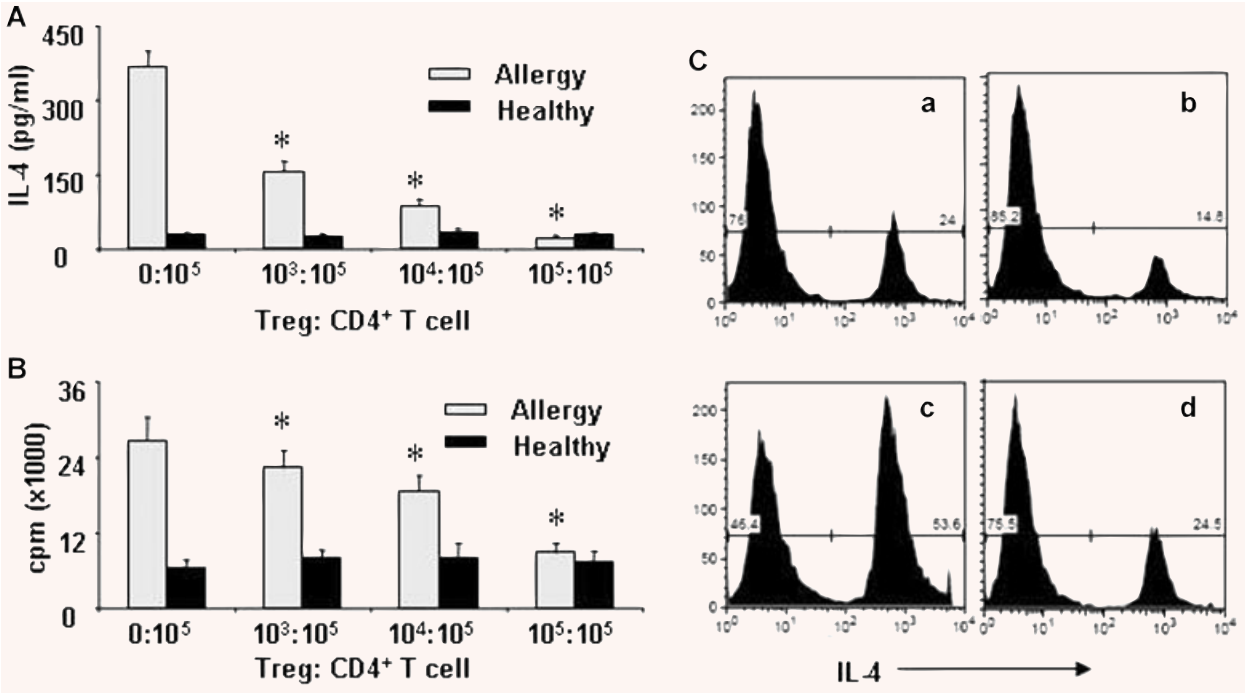

Fig. 6 Generated Tregs inhibit Ag-specific Th2 response. $CD4^+$ T cells were isolated from peripheral blood mononuclear cells by MACS from patients with Der-specific allergic rhinitis and healthy individuals. $CD4^+$ T cells were cocultured with generated Der-specific Tregs (both the donor cells for generation of Tregs and the $CD4^+$ T cells were obtained from the same individuals) for 72 hrs in the presence of irradiated DCs and specific antigen Der. (**A**) bars indicate levels of IL-4 in the supernatant. Allergy, samples were taken from patients with allergic rhinitis; Healthy, samples were taken from healthy individuals. (**B**) Bars indicate the cpm of $[^3H]$ incorporation. Data are presented as the means ± SD. $*P < 0.05$, compared with the $0:10^5$ group. (**C**) FACS histograms show IL-4^+ T cells (peaks on right side). The numbers indicate the percentage of cell populations. a and b, cells were collected from patients with allergic rhinitis; c and d, samples were collected from healthy individuals; a and c, cells were cultured with DC and specific antigen Der; c and d, cells were cocultured with Der-specific Tregs in the presence of DC and specific antigen Der. Data were from three separate experiments.

pathways exist in the induction of TGF-β expression in DCs. NF-κB is the transcription factor for many inflammatory molecules, such as IL-1, IL-6 and tumour necrosis factor (TNF)-α, and is involved in the inflammatory process; further studies are needed to address this difference.

It is accepted that tolerogenic DCs play a critical role in the generation of Tregs [31]. Several molecules have been recognized having the capability to generate Tregs, such as IL-10, TGF-β, retinoic acid and some immune suppressive agents such as rapamycin [10, 32–34]. These agents increase the levels of TGF-β in DCs to confer the capability on DCs to increase Foxp3 expression in $CD4^+$ or $CD8^+$ T cells, or render the target T cells to express TGF-β to fulfil the immune regulatory function. The present data demonstrate that DCs express high levels of TGF-β in response to the stimulation of GXM; the fact implicates that GXM has the capability to promote Treg T-cell development as a downstream effect.

It is proposed that the expression of Foxp3 is a unique identifier of $CD4^+CD25^+$ Treg; we thus used Foxp3 as an indicator to evaluate the effect of GXM in the induction of Treg. Although GXM increased the expression of TGF-β dramatically in DCs, the outcome of GXM-pulsed DCs and $CD4^+CD25^-$ T cells only yielded a little more than 20% cells expressing Foxp3 at the first coculture cycle. We reasoned the mechanism might be that the activated DCs were eliminated by T cells [35], which resulted in insufficient stimuli from DCs to T cells. The subsequent re-exposure to freshly prepared GXM-pulsed DCs proved the speculation that yielded a high ratio of $Foxp3^+$ T cells. The results indicate that multiple stimulation cycles are required in the generation of Tregs *in vitro*.

However, even though the coculture was carried out in as many as four cycles, the yielded $Foxp3^+$ T cells were only reached in some 60%; further coculture cycles did not further raise the ratio of $Foxp3^+$ T cells (data not shown). The reason might be the low production of costimulatory molecules from the GXM-pulsed DCs. In a separate experiment, we observed that gDer-pulsed DCs significantly increased ICOSL, which also plays an important role in the maintenance of Treg function [3]. The interaction of ICOS/ICOSL might assist Treg development. Indeed, Der-conjugated GXM (gDer)-pulsed DCs significantly increased the ratio of $Foxp3^+$ T cells, which was over 90%. Anti-ICOS antibody treatment

abolished the increase in the ratio of Foxp3$^+$ T cells. The results demonstrate that interaction of ICOS/ICOSL plays an important role in the generation of Tregs; this notion was also mentioned by a previous study [4].

There are two major categories of Tregs, thymus-derived Tregs and extrathymic Tregs, in the body [8, 10]. Since the natural Tregs are CD25$^+$ T cells, which category does the gDer-induced Tregs belong to? [8, 10]. The donor cells in the present study were naïve CD4$^+$CD25$^-$ T cells; we thus intend to regard that the induced Tregs in this study are extrathymic inducible Tregs. On the other hand, in a separate experiment, we employed RNAi to knock down TGF-β gene in DCs prior to the exposure to GXM; the subsequent coculture with naïve CD4$^+$CD25$^-$ T cells resulted in no Treg development (data not shown).

Mounting publications have demonstrated that Tregs have the capability to regulate the skewed immune response, which was assumed *via* expression of the suppressive molecules such as TGF-β [26]. However, the factors initiating the activation of Treg to express the suppressive molecules remain obscure. A unique finding of this study is that pulsed by gDer, the DCs have the capability to generate Ag-specific Tregs, which can be activated by re-exposure to specific Ag, Der, as shown in Figure 5. We regard that the response is specific because Der-pulsed DCs could induce the expression of TGF-β from Tregs, whereas irrelevant protein such as bovine serum albumin-pulsed DCs could not (data not shown). The expressed TGF-β can be converted to the surface of Tregs that have the potential to induce activated effector Th1 or Th2 cell's death *via* apoptosis pathway [36].

Tregs play a crucial role in the maintenance of immune tolerance in the body. Dysregulation of Treg causes various immune disorders. Application of Treg induction has been approved beneficial for organ or tissue transplantation [37]. On the basis of the suggestion that dysfunction of Treg is one of the pathogenic features of allergic diseases [6], different attempts using Treg to regulate the skewed Th2 response have been tried in both laboratory studies [38] and clinical practice [39]. The present study has provided further favourable information for this field that activation of Treg by specific Ag has high efficiency in the inhibition of Ag-specific Th2 responses.

In summary, using an immune suppressive molecule, GXM, conjugated with a specific Ag, Der, we successfully generated tolerogenic DCs and further generated Ag-specific Tregs. The generated Tregs could be activated by re-exposure to specific Ag, Der, to express the suppressive molecule TGF-β that efficiently inhibited the Ag-specific Th2 responses. We conclude that generated Ag-specific Tregs have therapeutic potential in the treatment of allergic diseases.

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