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Regulatory mechanisms of IL-2 and IFNγ suppression by quercetin in T helper cells

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ABSTRACT

Quercetin is a popular flavonoid compound that is biosynthesized by plants; it is suggested to modulate a variety of inflammatory responses of macrophages and T lymphocytes. Oral administration of quercetin in arthritic rats dramatically diminishes clinical signs of arthritis. Moreover, quercetin ameliorates experimental autoimmune encephalomyelitis, which is associated with Th1-mediated immune responses. Like quercetin inhibits macrophage-induced cytokine production, it also blocks IL-12-dependent JAK-STAT signaling in Th cells. Despite the anti-inflammatory effects of quercetin acting through Th cells, the regulatory mechanisms remain unclear. Here, we studied the function of quercetin in Th cells and found that quercetin suppressed both IFNγ and IL-2 production upon T cell receptor stimulation. Furthermore, we uncovered the regulatory mechanisms of quercetin involved in the inhibition of cytokine production during Th cell activation. The fact that quercetin-derived IFNγ suppression was blocked in T-bet-deficient Th cells demonstrated quercetin act through the modulation of T-bet expression. Whereas IL-2 inhibition by quercetin was independent of T-bet expression, quercetin diminished IL-2Rα expression, which is critical for positive regulatory loop of IL-2 autoactivation. Taken together, quercetin is suggested to repress both IFNγ and IL-2 cytokine production by independent mechanisms; T-bet-dependent IFNγ suppression and IL-2Rα-dependent IL-2 inhibition.

1. Introduction

Quercetin (3,3′,4′,5,7-pentahydroxyflavone dihydrate) is the most common flavonoid compound biosynthesized by plants as well as rutin (quercetin-3-rutinoside hydrate) [1]. It has been commonly absorbed by humans and also used as traditional therapeutic for many inflammatory disorders [2–11]. Muthian and Bright [12] reported that quercetin ameliorated experimental allergic encephalomyelitis associated with Th1 cell-mediated immune responses, by blocking IL-12 signaling through JAK-STAT activation. More recent reports demonstrated that quercetin suppressed clinical signs in rat arthritis by inhibiting generation of TNF-α and nitric oxide by macrophages [13–15] and that inhibition of TNF-α by quercetin

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Abbreviations: Th cells, T helper cells; T-bet, T-box protein expressed in T cells; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; IFN, interferon.

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was due to blockade of NF-kB recruitment to TNF-α gene promoter [16]. Like as macrophages play an important role in inflammatory responses by producing TNF-α and IFNγ, type I T helper (Th) cells produce a large amount of IFNγ, and inaugurate systemic and potent immune responses involved by cytotoxic T lymphocytes, unexpectedly contribute to chronic inflammation like autoimmune diseases [17–19]. Th1 cells were differentiated from CD4+ T precursor cells in response to antigen stimulation and unique cytokine circumstances, and distinguished from Th2 cells in the way that predominantly produce IFNγ [20].

For Th1 cell development, a specific transcription factor, T-bet is essential for inducing IFNγ gene expression in response to IL-12 and IFNγ [21–26]. T-bet plays a key role in Th1 cell differentiation by activating gene transcription through direct interaction to IFNγ gene promoter [25,27,28] and concomitantly suppresses Th2 cell differentiation by blocking GATA-3-mediated Th2 cytokine production [29]. Neurath et al. [30] reported while T-bet was active in Th1-mediated colitis (animal model of Crohn's disease); T-bet deficiency was protective from inflammation. It is believed that T-bet is integral to controlling the balance between pro and anti-inflammatory cytokines as well as between Th1 and Th2 cell differentiation.

Therefore, T-bet is strongly suggested to be a critical target for regulating inflammatory responses. Here, we studied whether quercetin influence on T-bet expression and also cytokine production in Th cells and found that quercetin repressed T-bet expression and both IFNγ and IL-2 cytokine production during Th cell activation. Furthermore, we uncovered the regulatory mechanisms of IL-2 and IFNγ suppression by quercetin in Th cells.

2. Materials and methods

2.1. Reagents

All cytokines and Abs were purchased from BD Pharmingen (San Diego, CA). Monensine, rutin (quercetin-3-rutinoside hydrate, R5143) and quercetin (3,3′,4′,5,7-pentahydroxyflavone dehydrate, Q0125) were obtained from Sigma–Aldrich, Inc. (St. Louis, MO) and dissolved in DMSO with concentration of 100 mM stock. Doxycycline was purchased from Sigma–Aldrich, Inc. Anti-T-bet mAb was from Santa Cruz Biotech, Inc. (Santa Cruz, CA).

2.2. Mice

Wild type C57BL6, T-bet-deficient mice and T-bet transgenic/deficient mice were housed in specific pathogen-free conditions at Ewha Womans University. T-bet transgenic/deficient mice were generated by injection of CD2-rtTA and TRE-T-bet genes into T-bet-deficient mice. All mice handling and experiments were done in accordance with Institutional Animal Care and Use Committee guidelines.

2.3. Isolation and activation of CD4+ T cells in vitro

Single cell suspensions were prepared form lymph node and spleen of wild type and T-bet-deficient mice and incubated with mouse CD4 micro beads for 30 min (Miltenyi Biotech, Auburn, CA). Positively isolated CD4+ T cells were re-suspended in complete medium with 2×10^6 cells/ml and seeded onto plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) Abs for 24 h in the absence or presence of rutin or quercetin. Recombinant human IL-2 (100 U/ml) was added up to enhance Th cell activation and proliferation. For T cell-specific restoration of T-bet, CD4+ T cells were isolated from T-bet transgenic/deficient mice and activated with TCR stimulation with 1 μg/ml of doxycycline in the presence or absence of quercetin.

2.4. Flow cytometric analyses of cytokines and cell surface protein

Activated Th cells were harvested and fixed with 4% paraformaldehyde solution. Cells were then rinsed twice with permeabilization buffer (0.1% saponin, 0.1% sodium azide, 1% FBS in PBS) and suspended with same buffer and incubated with specific Abs according to the instructor's manual (BD Pharmingen). After staining for 30 min on ice, cells were washed twice with FACS buffer (1% FBS in PBS), suspended and applied on a FACS Calibur (BD Biosciences). Cell populations were analyzed by CellQuest software (Tree Star, Ashland, OR).

2.5. ELISA

Activated Th cells were pelleted and supernatants were collected for ELISA. Supernatants were diluted 1–10 and incubated on capture Ab-coated ELISA plate. Biotinylated anti-cytokine Abs and phosphatase-conjugated streptavidin were sequentially incubated after plate washing and developed with phosphatase substrate. Color changes were read by ELISA plate reader (Molecular Devices, Palo Alto, CA). Purified and known concentrations of each cytokine, such as IL-2, IL-4 and IFNγ were incubated parallel with unknown samples for standard curves.

2.6. Isolation of total RNA and real-time PCR

Total RNA was isolated from the activated Th cells treated with vehicle and quercetin by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with DNase to remove any remnant genomic DNA. Two micrograms of total RNA was reacted using the Superscript first strand synthesis system (Invitrogen, Carlsbad, CA) for reverse transcription and quantitative real-time PCR using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The following primers were used: β-actin-FWD 5′-aagcaggagtatgacgagtccg-3′, β-actin-REV 5′-cggaactaagtcatagtccgcc-3′; T-bet-FWD 5′-cccccaaggaattgacagttg-3′, T-bet-REV 5′-gggaaactaaagctcacaaac-3′; CD25-FWD 5′-cgttgcttaggaaactcctgg a-3′, CD25-REV 5′-gctttctcgatttgtcatggg-3′; IFNγ-FWD 5′-agcaacagcaaggcgaaaa-3′, IFNγ-REV 5′-ctggacctgtgggttgttga-3′; IL-2-FWD 5′-ctcctgagcaggatggagaatt-3′, IL-2-REV 5′-cgcagaggtccaagtgtagct-3′; IL-4-FWD 5′-ggcattttgaacgaggtcaca-3′, IL-4-REV 5′-aggacgtttggcacatccat-3′; IL-12-FWD 5′-aatgttccagtgcctcaacc-3′, IL-12-REV 5′-ctagagtttgtctggccttctg-3′. The statistical significance of the difference was determined by Student's *t*-test. A p-value <0.001 was chosen as significant.

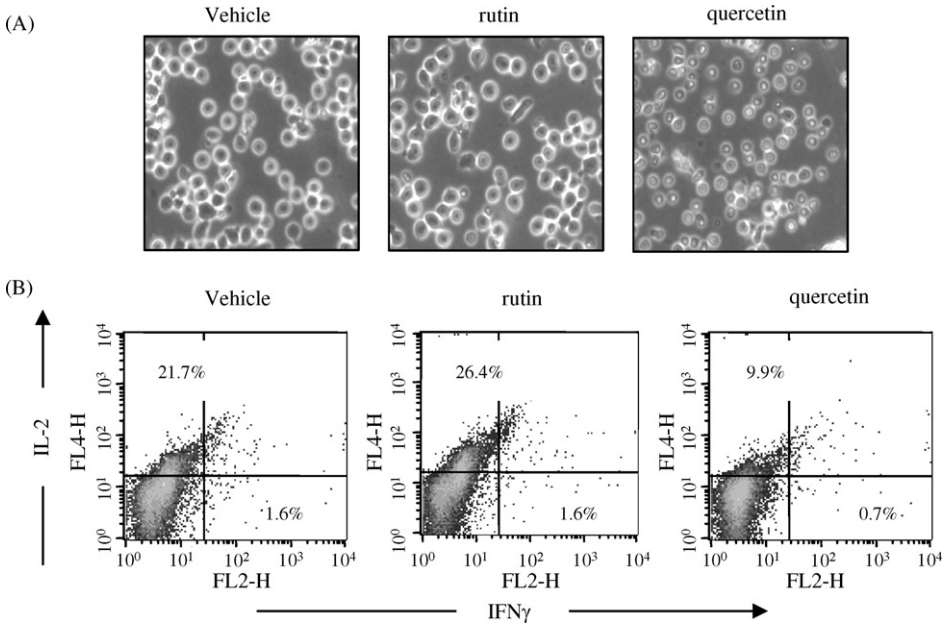

Fig. 1 – Effects of bioflavonoids on primary Th cell activation. Isolated CD4+ T cells were stimulated with anti-CD3 and anti-CD28 and co-treated with either rutin (40 μM) or quercetin (40 μM) for 24 h. (A) Activation of Th cells was observed by phase contrast microscope. (B) Monensine (2 μM) was added to the medium for 2 h before harvest for intracellular cytokine staining. Th cells were harvested, incubated with either anti-IL-2 Ab or anti-IFNγ Ab and acquired by FACS Calibur. Cytokine productions were analyzed by CellQuest program.

2.7. *Cell culture and transfection*

EL4 cells were maintained in complete RPMI1640 medium. Five million cells were electroporated with each 10 μg of T-bet expression vector and reporter gene that contains IFNγ gene promoter and grown for additional 48 h for luciferase assay (Promega Corp). For normalization of transfection efficiency, pCMV β-gal (Invitrogen) was co-introduced into the cells and β-galactosidase activity was determined by Galacto-Light (Tropix, Inc., Bedford, MA).

3. Results

3.1. *Quercetin negatively modulates Th cell activation*

Dietary flavonoid compounds, rutin and quercetin are noted for antioxidants to reduce the oxidative stress generated in inflammatory responses [1]. In particular, quercetin exerts anti-inflammatory activities by regulating inflammatory cytokine production mediated by macrophage and T lymphocytes [10,13,31]. We have tested the effects of rutin and quercetin on Th cell activation and Th cell-derived cytokine production. CD4+ T cells were isolated with 95% purity from lymph node and spleen and activated with plate-bound anti-CD3 and anti-CD28 for 24 h in the absence or presence of rutin or quercetin. Upon TCR activation, Th cells were morphologically enlarged and readily attached to the bottom of the culture plate as shown in Fig. 1A. While Th cells were normally activated in the presence of rutin, Th cell activation was blocked by quercetin (Fig. 1A). However, quercetin had no effect on cell morphology of the established Th cell lines, such as murine EL4 and human Jurkat T cells (data not shown). TCR stimulation increases cytokines productions such as IL-2 and IFNγ in primary Th cells. Increased IL-2 and IFNγ in activated Th cells were not changed by rutin, but significantly decreased by quercetin (Fig. 1B). It is likely that quercetin, unlike rutin attenuated primary Th cell activation and also cytokine production in Th cells upon TCR stimulation.

3.2. *Gene transcription of Th1 cytokines was selectively modulated by quercetin*

We also have measured accumulated cytokines in supernatants from activated Th cells cultured with either rutin or quercetin. Rutin did not affect IL-2 and IFNγ production, but quercetin decreased both cytokines in dose-dependent manner (Fig. 2A and B). On the other hand, IL-4 production was comparatively low, but not decreased by quercetin at all (Fig. 2B). Quercetin seems to selectively modulate IL-2 and IFNγ, Th1-like cytokines, not Th2 cytokines. As cytokine expression is prominently regulated at the level of gene transcription, mRNA levels of cytokines were assessed by quantitative real-time PCR. While rutin had no effect on gene transcription, it was clear that quercetin suppressed gene transcription of both IL-2 and IFNγ (Fig. 3A and B). In accordance with IL-4 level measured by ELISA in Fig. 2B, relative level of IL-4 transcripts was not a bit attenuated by quercetin, supporting the selective modulation of Th1-like cytokines by quercetin (Fig. 3B). A p value determined by Student's *t*-test showed no significant difference either on cytokines expressions between vehicle and rutin or IL-4 levels by quercetin treatment.

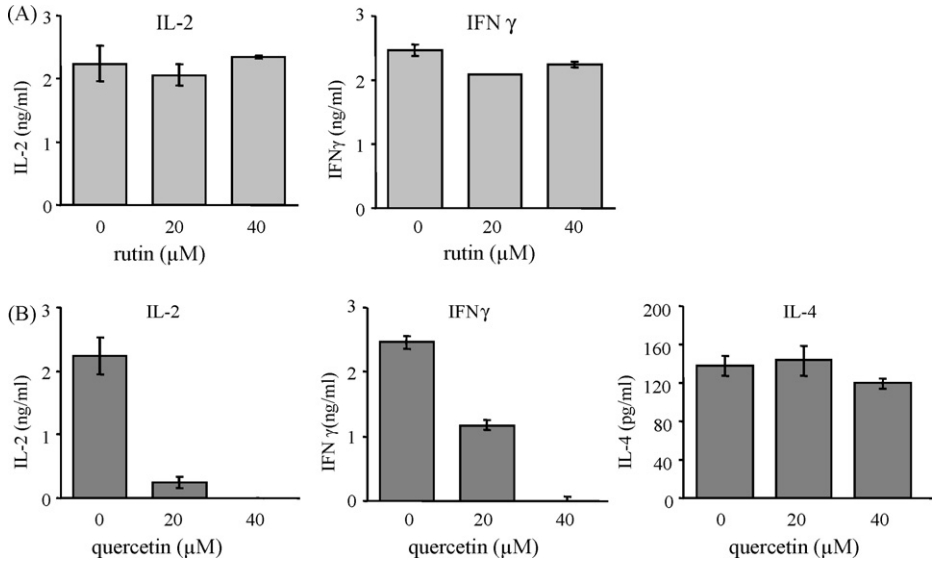

Fig. 2 – Effects of bioflavonoids on cytokine productions in activated Th cells. During Th cell activation, cells were treated with rutin (A) or quercetin (B) and supernatants were collected for cytokine production. Cytokines were measured by ELISA as previously described [44]. Three independent experiments were performed and reproduced and a representative result was presented.

3.3. *Quercetin significantly suppressed T-bet protein expression*

As quercetin selectively blocked gene transcription of Th1-like cytokines, we then assessed the function of quercetin on the expression of T-bet, a Th1-specific transcription factor. T-bet is a master transcriptional regulator of Th1 cells which predominantly produce IFNγ and IL-2, and modulates both Th1 and Th2 cell differentiation [25,26,29]. As we expected, rutin had no effect on either mRNA or protein level of T-bet (Fig. 4A and B). Surprisingly, quercetin magnificently decreased T-bet protein expression in dose dependent manner, but did not affect gene transcription of T-bet (Fig. 4A and C), insisting the mechanism of quercetin-mediated T-bet protein degradation. Consistent with the previous report [12], IL-12 gene transcription was inhibited by quercetin under the same condition (Fig. 4D).

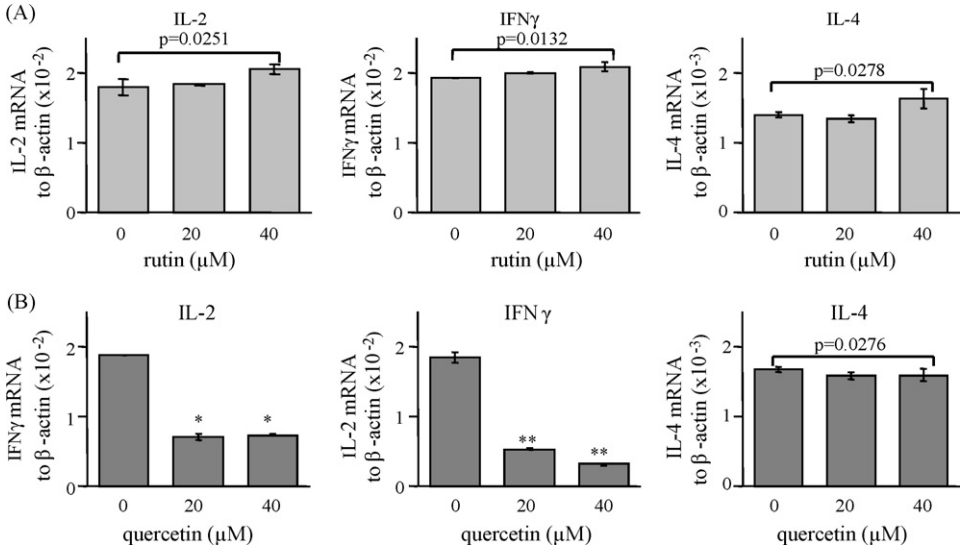

Fig. 3 – Function of quercetin on gene transcriptions of cytokines. TCR-stimulated Th cells were incubated with rutin (A) or quercetin (B) as indicated. Total RNA was harvested and used for the quantitative real-time PCR. Relative mRNA levels of cytokines were calculated from Ct values normalized by β-actin. Specific primers for cytokines were described in Section 2. A *P* value was determined by Student's *t*-test for the statistical significance. *$P < 0.001$ and **$P < 0.0001$ compared with control (0 μM).

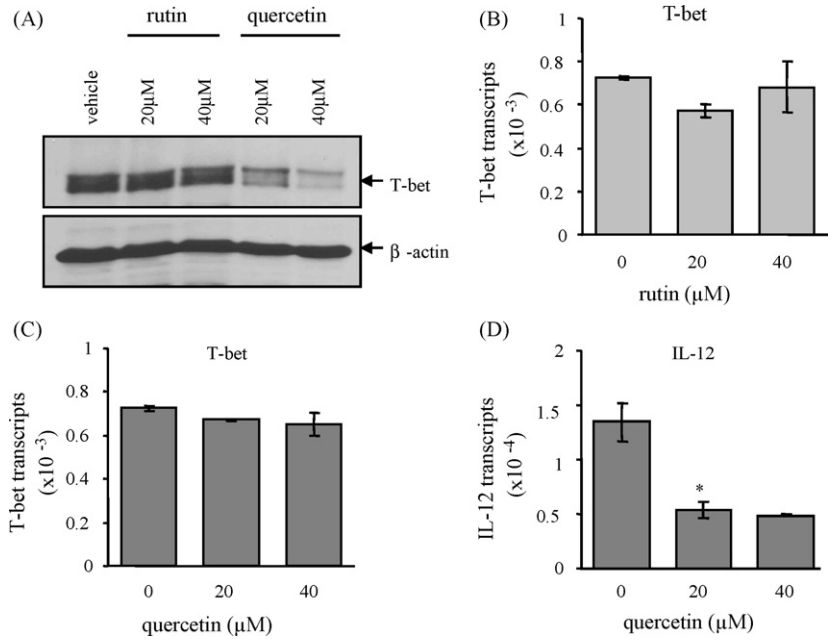

Fig. 4 – Decreased T-bet expression mediated by quercetin. (A) Total cell lysates were harvested from activated Th cells treated with either rutin or quercetin. Protein blots resolved by SDS-PAGE were incubated with monoclonal anti-T-bet Ab and subsequently incubated with anti-β-actin Ab after stripping. (B–D) Total RNA was harvested from rutin or quercetin-treated Th cells and analyzed for the mRNA levels of T-bet. Gene transcription levels of IL-12 were assayed in quercetin-treated Th cells using quantitative real-time PCR. One representative data was presented from three independent experiments.

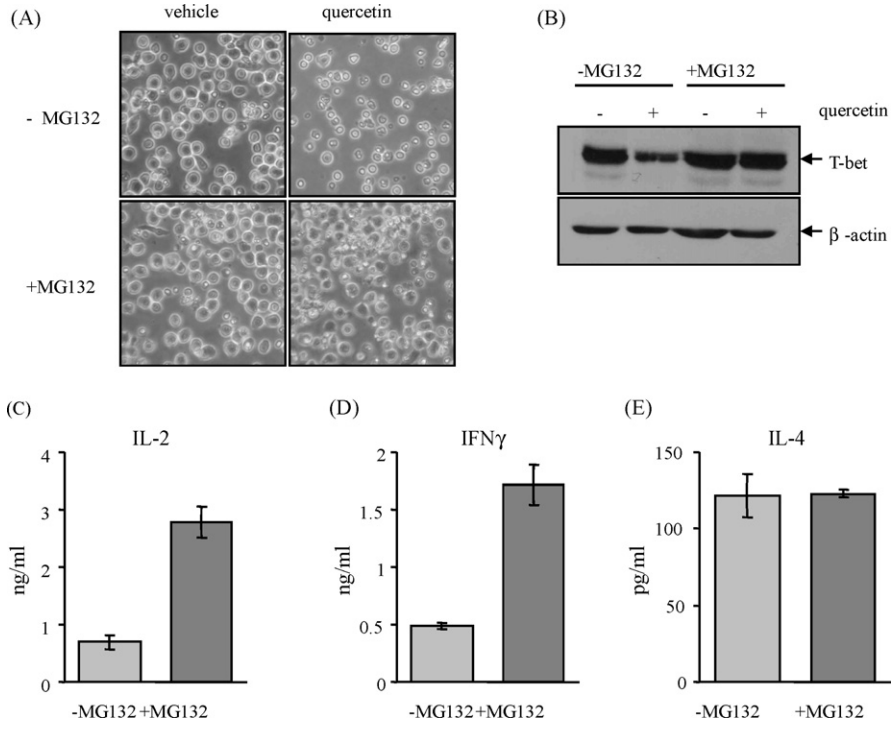

Fig. 5 – Inhibitory function of quercetin on T-bet expression was blocked by MG132. Th cells incubated with or without quercetin were subsequently treated with MG132 or not for 3 h before harvest. (A) Microscopic observations of Th cells by treatment of quercetin and/or MG132. (B) T-bet expression was determined by immunoblot assay. Cytokine productions of IL-2 (C), IFNγ (D) and IL-4 (E) were measured in supernatants by ELISA.

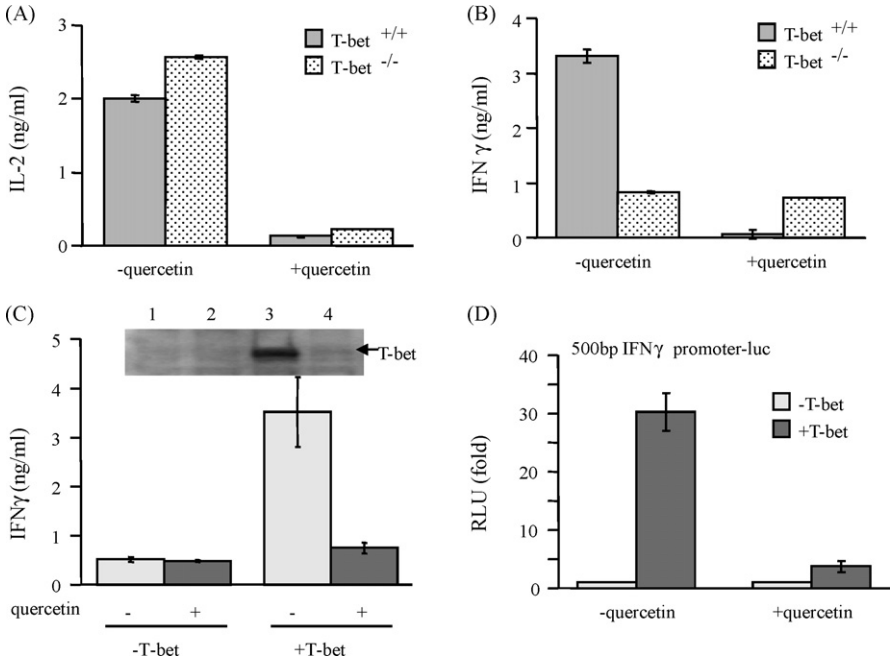

Fig. 6 – Quercetin required T-bet to suppress IFNγ. Single cell suspensions of CD4+ T cells were isolated from T-bet$^{+/+}$ and T-bet$^{-/-}$ mice and stimulated with anti-CD3 and anti-CD28 for 24 h. Quercetin was added to the cell culture medium and supernatants were collected for measurements of IL-2 (A) and IFNγ (B) by ELISA. (C) CD4+ T cells were isolated from T-bet transgenic/deficient mice and were incubated with dox (+T-bet) to induce T-bet expression or not (−T-bet). Restored expression of T-bet was determined by immunoblot (upper panel). IFNγ production was measured in supernatant by ELISA (lower panel). Numbers depict as follows: (1) -dox, -quercetin; (2) -dox, +quercetin; (3) +dox, -quercetin; (4) +dox, +quercetin. (D) EL4 cells were transfected with T-bet and IFNγ promoter-luciferase genes with 1:1 rationale. Luciferase activities were measured and normalized by β-galactosidase activities in the absence (−) or presence (+) of quercetin. Three independent assays were performed and one representative result was presented by fold induction.

3.4. *Quercetin-mediated T-bet protein degradation was inhibited by MG132*

In order to confirm the regulatory mechanism of quercetin on T-bet expression, quercetin was co-incubated with or without MG132, a proteasome inhibitor. As shown in Figs. 1A and 5A, primary Th cells were not activated upon stimulation on account of quercetin. However, co-treatment of MG132 seemed to block the function of quercetin on primary Th cell activation, since Th cells were reversed back to normal activation (Fig. 5A). Moreover, decreased expression of T-bet by quercetin was blocked by co-treatment of MG132, which was comparable to the level of control (Fig. 5B). It is likely that quercetin modulates T-bet protein degradation, instead of repressing T-bet gene expression. Meanwhile, reduced levels of Th1-like cytokines attenuated by quercetin were, in part, recovered by MG132 (Fig. 5C and D), but IL-4 production was scarcely affected by either quercetin or MG132 (Fig. 5E).

3.5. *T-bet was integral target for quercetin-mediated IFNγ suppression*

From the above results, T-bet was proposed to be an important target of quercetin to modulate IL-2 and IFNγ cytokine production. We then analyzed the effect of quercetin on the activation of T-bet-deficient Th cells comparing with wild type Th cells. T-bet-deficient Th cells were nicely activated and produced considerable amounts of IL-2 upon TCR stimulation. Quercetin suppressed IL-2 production in T-bet-deficient Th cells as much as in wild type Th cells (Fig. 6A), asserting that quercetin suppressed IL-2 irrespective of T-bet. As a consequence of T-bet function in IFNγ expression, T-bet-deficient Th cells produced impaired but fair amount of IFNγ as shown in Fig. 6B. Whereas IL-2 repression by quercetin was persisted even in the deficiency of T-bet (Fig. 6A), IFNγ suppression by quercetin was failed in T-bet-deficient Th cells (Fig. 6B). In order to confirm the T-bet-dependent IFNγ suppression by quercetin, we introduced T-bet transgenic/deficient mice, which re-expressed T-bet in a T cell-specific and doxycycline-inducible manner. Restoration of T-bet in Th cells was convinced by T-bet Western blot and increased IFNγ production (Fig. 6C). But, addition of quercetin rather decreased both T-bet expression and IFNγ production (Fig. 6C), insisted that IFNγ suppression by quercetin was mediated by modulation of T-bet expression. Furthermore, IFNγ promoter activity was increased by T-bet expression by 30-fold, but remarkably decreased by treatment of quercetin (Fig. 6D). These suggested that quercetin suppresses IFNγ production by modulating T-bet expression, but represses IL-2 production by separable mechanism from T-bet-dependent IFNγ suppression.

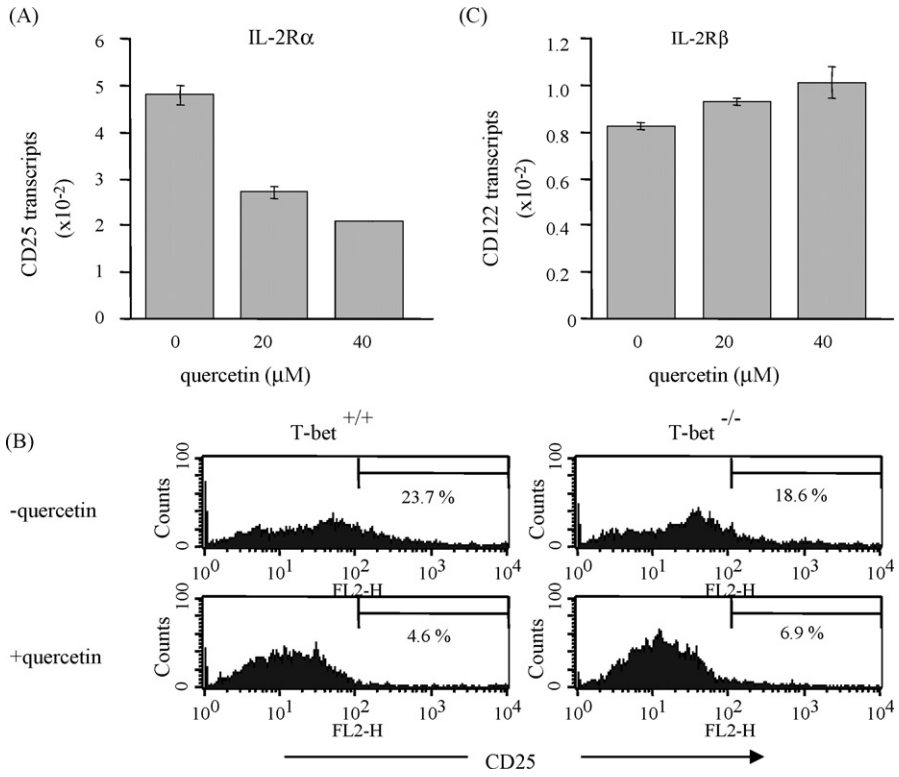

Fig. 7 – Modulation of IL-2/IL-2Rα by quercetin. Th cells isolated from T-bet$^{+/+}$ and T-bet$^{-/-}$ mice were activated by TCR stimulation in the co-presence of quercetin with amounts as indicated. (A) Total RNA was harvested from activated Th cells and used for real-time PCR. Relative levels of IL-2Rα (CD25) gene transcription normalized to β-actin were presented. (B) Activated Th cells were collected, fixed in 4% paraformaldehyde solution and incubated with PE-conjugated anti-IL-2Rα Ab. (C) Relative levels of IL-2Rβ were determined by real-time PCR and normalized to the levels of β-actin.

3.6. *Quercetin blocked IL-2Rα-mediated IL-2 expression*

As we found out that IL-2 regulation by quercetin was irrespective of T-bet, we inquired IL-2-specific signaling pathway. Upon TCR stimulation, IL-2 produced by Th precursor cells binds to IL-2 specific receptor (IL-2R) and integrates activation signals within the cells through JAK-STAT5 signaling cascade [32]. IL-2-mediated activation signals significantly increase a variety of gene expressions, including IL-2 and IL-2Rα (also known as CD25), which are involved in cell proliferation and differentiation of Th cells and also Th cell-mediated immune responses. As a result, IL-2 production was pivotally modulated by positive auto-regulatory mechanism of IL-2/IL-2R system. Therefore, we wondered if IL-2R expression was influenced by quercetin or not. As usual, IL-2Rα expression was increased in response to exogenous recombinant human IL-2 and TCR stimulation, but significantly blocked by co-treatment of quercetin (Fig. 7A and B). IL-2Rα expression was repressed by quercetin at both mRNA and protein levels and also reduced in a similar way in T-bet-deficient Th cells (Fig. 7B), convincing quercetin suppressed IL-2Rα-mediated IL-2 autoactivation signal, which is irrespective of T-bet as described in Fig. 6A. Meanwhile, IL-2Rβ (also known as CD122) expression was not influenced by quercetin (Fig. 7C). In summary, quercetin repressed IL-2, which was independent of T-bet function and mediated by blockade of IL-2Rα expression.

4. Discussion

Inflammatory responses are intentionally induced to combat invading pathogens, remove infected cells, and restore normal structure and function of tissues and critically mediated by a variety of cytokines. Modulation of cytokine production seems to be critical for regulating inflammatory diseases including cancer and has been studied in-depth in macrophages and T lymphocytes, major regulatory cells of innate and adaptive immune responses. Activation of macrophages upon exogenous pathogens secretes inflammatory cytokines like TNF-α and IFNγ, and derives Th1 cell lineage commitment, which prominently produces IFNγ and IL-2 as well [33]. Although appropriate Th1 responses are critically required for anti-tumor and anti-viral activities, constitutive overproduction of Th1 cytokines contributes to cell-mediated cytotoxic immune responses worsening autoimmune responses. Thus, optimal suppression of extravagant production of macrophages and Th1-mediated cytokines could be useful therapy for treating inflammatory disorders and autoimmune diseases [5,14,15].

Quercetin is a natural bioflavonoid compound found in many dietary fruits and vegetables and has been clinically shown to reduce the risks associated with high cholesterol levels and lower the incidence of prostate problems [34–38]. Previous results that quercetin modulate the production of inflammatory cytokines and nitric oxide in macrophages

strongly suggested quercetin would intensively modulate anti-inflammatory responses [14,16,39,40]. Recently, it has been published that quercetin also influences on functions of T lymphocytes. Although quercetin was suggested to differentially regulate Th1 and Th2 cytokines in PBMC by Nair et al. [41], Muthian and Bright [12] demonstrated quercetin inhibited IL-12-STAT4 signaling pathway in purified T lymphocytes and ameliorated experimental autoimmune encephalomyelitis (EAE), Th1-related autoimmune disease. Our finding that quercetin represses T-bet-dependent and independent IFNγ and IL-2 production also supports the fact that quercetin would be effective therapeutic compound for inflammatory and autoimmune diseases [5,8,15,36,42].

T-bet is known to be critical for both Th1 and Th2 cell differentiation [25,26]. T-bet directly binds to IFNγ promoter and enhancer and activates IFNγ gene transcription [27], but also interacts with GATA-3, a Th2-specific transcription factor and suppresses GATA-3-mediated Th2 cytokines productions [29]. Since T-bet-deficiency blocked the development of EAE by the reduction of IFNγ, T-bet could be a good target for developing new drugs treating autoimmune diseases. Surprisingly, T-bet expression was dramatically reduced by quercetin, which was the modulation of protein degradation mediated by ubiquitin–proteasome pathway. Although it is well known that T-bet expression was increased upon TCR stimulation and enhanced by IFNγ signaling cascade, the inhibitory mechanisms of T-bet expression remained unclear. Thus, further study on quercetin-mediated T-bet degradation would be informative to uncover the regulatory mechanisms of T-bet expression.

Quercetin also repressed IL-2 in Th cells, but the mechanism was distinguished from IFNγ suppression. Regardless of T-bet, quercetin blocked IL-2-mediated gene transcription of IL-2Rα, which is required for high affinity receptor complex for IL-2. Binding of IL-2 to IL-2R receptor integrates IL-2 signals into the cells through JAK-STAT5 activation and ultimately induces cell proliferation and differentiation. The inhibition of IL-2 results in the inhibition of T cell proliferation, which was in accordance with the effect of cell growth inhibition by quercetin [43]. Though the mechanism of quercetin to inhibit cell proliferation would be partly explained by the repression of IL-2 in immune cells including T lymphocytes, but regulatory mechanisms in other types of cells remained to be uncovered. Further studies on regulatory mechanisms of quercetin are required for the expanded clinical applications.

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