Miquelianin inhibits allergic responses in mice by suppressing CD4+ T cell proliferation

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Abstract

Background. Allergic disorders such as atopic dermatitis (AD), allergic rhinitis, asthma, and food allergy show a predominant immune response by type 2 helper T (Th2) cells. We previously revealed that Rosae multiflorae fructus extract ameliorates ovalbumin-induced allergic rhinitis symptoms in a mouse model and identified miquelianin (quercetin 3-O-glucuronide, MQL) as the active compound. However, it is unknown if MQL affects allergic diseases or regulates Th2 immune responses. In this study, we investigated the anti-allergic effects of MQL. Methods. To investigate anti-allergic effects of MQL, we used an ovalbumin (OVA)-induced Th2-dominant mouse model and assessed cytokine production, cell proliferation, and upstream signaling pathways. Results. Oral administration of MQL suppressed the production of Th2 cytokines and IL-2, and cell proliferation. Additionally, heme oxygenase-1 (HO-1) expression was increased by MQL in splenocytes. In ex vivo studies using splenocytes and CD4+ T cells from mice, MQL suppressed Th1- and Th2-related immune responses by inhibiting CD4+ T cell proliferation. Furthermore, MQL increased HO-1 expression by activating the C-Raf -ERK1/2-NRF2 pathway by inducing the generation of intracellular reactive oxygen species in CD4+ T cells. Finally, to verify the effects of MQL in vivo during an allergic disorder, we used a trimellitic anhydride-induced AD-like mouse model. Both topical treatment and oral administration of MQL ameliorated AD symptoms by suppressing Th2 immune responses, including serum IgE production Th2 cytokine production in draining lymph nodes. Conclusions. MQL can be used as a therapeutic agent for CD4+ T cell-mediated diseases such as allergic diseases.

Abbreviations

AD, atopic dermatitis; **APCs,** antigen-presenting cells;**Con A,** concanavalin A; **DC,** dendritic cells;**Dex,** dexamethasone; **DLNs** , draining lymph nodes;**ΦςεPI,** Fcε receptor I; **HO-1,** heme oxygenase-1;**IFN** , interferon**; Ig** , immunoglobulin**; IL,**interleukin; **Keap1** , Kelch-like ECH-associated protein 1;**MAPKs,** mitogen-activated protein kinases; **NAC,**N-acetylcysteine; **NRF2** , nuclear factor erythroid 2-related factor 2; **OVA,** ovalbumin; **PI,** propidium iodide;**STAT,** signal transducer and activator of transcription;**Th,** T helper; **TMA,** trimellitic anhydride;**TNF,** tumor necrosis factor

1. Introduction

Allergic diseases such as allergic rhinitis, asthma, food allergy, atopic dermatitis, allergic contact dermatitis, and anaphylaxis contribute to the rising cost of health care and cause a lower quality of life. Allergy is characterized by immune responses by antigen-specific type 2 helper T (Th2) cells that are involved in the development of antigen-specific immunoglobulin E (IgE) responses produced following class switching of B cells[1]. T cell receptors (TCRs) in naïve $CD4^+$ T cells recognize antigens upon co-stimulation by CD28 when contacting antigen-presenting cells (APCs). Antigen presentation to naïve $CD4^+$ T cells induces their differentiation into effector and memory T cells, and triggers clonal expansion. Activated T cells produce IL-2, which stimulates T cell proliferation, by autocrine or paracrine signaling[2,3]. Naïve $CD4^+$ T cells can differentiate into at least four subtypes including Th1, Th2, Th17, and induced regulatory T (iTreg) cells[4].

Among these subsets of $CD4^+$ T cells, Th2 cells are characterized by their production of interleukin (IL)-4, IL-5, and IL-13, and stimulate B cell class switching to produce antigen-specific IgE[5].

Heme oxygenases (HO) are essential enzymes that catabolize heme into iron (Fe), biliverdin, and carbon monoxide (CO). Three isoforms of HO exist: the inducible form (HO-1) and constitutive forms (HO-2 and HO-3). In particular, HO-1 is an inducible enzyme expressed in mammalian tissues and plays a cytoprotective role[6–8]. It is well established that oxidative stress activates the nuclear factor erythroid 2-related factor 2 (NRF2) pathway resulting in HO-1 expression. In turn, HO-1 plays an essential role in maintaining intracellular homeostasis against excess reactive oxygen species (ROS) generated by redox signaling[9], and protects cells against oxidative stress and inflammation[10,11]. In addition, HO-1 can regulate cell proliferation by arresting the cell-cycle by generating CO[12,13]. Previous studies investigating regulatory effects of HO-1 on T cells revealed that CO generated by HO-1 also suppresses $CD4^+$ T cell proliferation by inhibiting IL-2 production[14], and CO exposure suppresses proliferation and activation of T cells[15,16]. Phytochemicals obtained through fruit and vegetable consumption have protective effects against diverse diseases by inducing HO-1 expression by activating the NRF2 pathway[17]. Among these phytochemicals, quercetin, the aglycone of miqeulianin, has been reported to protect cells from oxidative stress and inflammation by generating HO-1[18,19], and recently, it was reported that quercetin suppresses T cell proliferation via HO-1 generation[20].

In a previous study, we revealed that *Rosae multiflorae* fructus extract ameliorates OVA-induced allergic rhinitis symptoms in a mouse model, and identified miquelianin as the active compound of *Rosae multiflorae*fructus[21]. Miqeulianin (quercetin 3-*O* -glucuronide, MQL), one of the flavonoids, is a quercetin glycoside. MQL has been reported to have antioxidant[22], antidepressant[23], and antiproliferative effects[24]. However, it is unknown if MQL affects allergic diseases or regulates Th2 immune responses. Thus, in this study, we examined the effect of MQL on a TMA-induced AD-like mouse model, and investigated the mechanisms by which MQL regulates Th2-related immune responses *in vitro* .

2. Materials and Methods

We provide full methods (detailed explanations of materials and methods) as supplementary materials in online supporting information as follows:

2.1. Materials

2.2. Animals

2.3. Induction of AD-like symptoms by TMA [25,26]

2.4. OVA-induced allergic mouse model

2.5. Culture of draining lymph node (DLN) cells and splenocytes

2.6. In vitro assay using mouse splenocytes immunized by OVA

2.7. $CD4^+$ T cell isolation and proliferation assay

2.8. HO-1 and NRF2 detection in $CD4^+$ T cells

2.9. Cell cycle analysis

2.10. Reactive oxygen species (ROS) detection

2.11. Measurement of cytokines and serum IgE using ELISA

2.12. Western blotting

2.13. Statistical analysis

3. Results

3.1. Effect of oral MQL administration on OVA-induced allergy

In this study, we examined whether oral administration of MQL affects Th2-related allergic immune responses using an OVA-induced mouse model of allergy. The mice were sensitized and immunized by OVA and orally administered MQL for 2 weeks. We investigated typical allergic responses such as production of IgE and Th2-mediated cytokines including IL-4, IL-5, and IL-13. We found that levels of serum IgE and splenic Th2 cytokines were significantly suppressed by oral administration of MQL**(Fig. 1B-E)** .

In our previous study, we showed that *Rosae multiflorae* fructus extract containing MQL suppressed Th2-related immune responses by inhibiting the proliferation of immune cells via up-regulation of HO-1 expression. Similarly, it has been reported that increased HO-1 expression in $CD4^+$ T cells suppressed proliferation by inhibiting IL-2 production[7]. Based on these studies, we measured IL-2 production and proliferation of splenocytes, and confirmed that these were significantly reduced in MQL-administered groups **(Fig. 1F and 1G)** . Furthermore, we found that splenocytes in MQL-administered groups had increased HO-1 expression compared to the sham group **(Fig. 1H)** .

3.2. Immunomodulatory effects of MQL on splenocytes from BALB/c mice immunized by OVA

BALB/c mice were immunized twice with OVA and alum, then splenocytes were isolated. Using this *ex vivo* system, we investigated whether MQL treatment regulates allergic immune responses by suppressing cell proliferation via increased HO-1 expression.

OVA-induced Th1- (IFN-γ) and Th2-related cytokines (IL-4, IL-5, and IL-13) were significantly suppressed by MQL treatment **(Fig. 2A-D)** . Next, to investigate whether the immunomodulatory effects of MQL were a consequence of decreased cell proliferation, we measured proliferation and IL-2 production. Notably, MQL treatment suppressed IL-2 production as well as splenocyte proliferation **(Fig. 2F and G)** . To further characterize these T cell subsets, we analyzed Th1- (STAT1) and Th2-associated transcriptional factor (STAT6), as well as HO-1. **Figures 2H and I** show that STAT6 and STAT1 phosphorylation were reduced by MQL treatment, whereas HO-1 expression was increased by MQL. Interestingly, however, the Th1-related cytokine IL-12 was increased by MQL treatment **(Fig. 2E)** . These results indicate that MQL treatment largely inhibits Th1- and Th2-related immune responses via reduction of cell proliferation induced by HO-1 up-regulation.

3.3. Effects of MQL on $CD4^+$ T cell proliferation and HO-1 expression

IL-2 is an essential factor for $CD4^+$ T cell proliferation and is produced following antigen recognition by TCR with co-stimulation by CD28. IL-2 then binds its receptor via paracrine and autocrine mechanisms and causes clonal expansion of $CD4^+$ T cells[3]. In this experiment, naïve $CD4^+$ T cells were isolated and stimulated with anti-CD3 (TCR stimulation) and anti-CD28 antibodies (co-stimulation). We then investigated the effects of MQL on $CD4^+$ T cell proliferation and IL-2 production, and found that MQL treatment suppressed both proliferation and IL-2 production in a dose-dependent manner **(Fig. 3A and B)** .

p21 (known as $p21^{WAF1/Cip1}$) is a cyclin-dependent kinase (CDK) inhibitor and arrests cell cycle progression in the G1 phase[27]. Furthermore, it was reported that up-regulation of HO-1 is able to increase = p21 levels, resulting in reduced cell proliferation[12]. Therefore, we investigated whether MQL affects the cell cycle in $CD4^+$ T cells, and observed that MQL treatment increased p21 levels and arrested the cell cycle at the G1 stage compared with untreated $CD4^+$ T cells **(Fig. 3C and D)** .

Nrf2 is a transcription factor of HO-1 that is sequestered in the cytoplasm by binding with Kelch-like ECH-associated protein 1 (Keap1). When Nrf2 is released from Keap1, it translocates into the nucleus then increases transcription of the HO-1 gene by binding to its antioxidant response element (ARE)[28,29]. We thus investigated Nrf2 translocalization following MQL and found that NRF2 nuclear translocation and consequent HO-1 were increased by MQL in $CD4^+$ T cells **(Fig. 3E and F)** . Collectively, these results demonstrate that MQL upregulates p21, arrests the cell cycle, and activates the NRF2-HO-1 pathway, resulting in reduced $CD4^+$ T cell proliferation.

3.4. Effects of MQL on MAP kinase signaling pathways

Next, to investigate MAP kinases upstream of MQL-induced HO-1 expression, we cultured $CD4^+$ T cells with U0126 (ERK inhibitor), SB20350 (p38 inhibitor), or SP600125 (JNK inhibitor) in the presence of MQL. As a result, MQL-induced HO-1 expression was most significantly reduced by U0126 treatment compared with the other inhibitors **(Fig. 4A)** . To explore this further, we measured ERK1/2, p38, and JNK activation in the presence or absence of T cell stimulation (anti-CD3/CD28 antibodies). Consistent with the previous finding, MQL dose-dependently increased the phosphorylation of ERK, but not p38 or JNK **(Fig. 4B and C)** .

The Ras-Raf-MEK-ERK signaling pathway plays a crucial role in gene expression related to cell growth, proliferation, differentiation, and survival[30,31]. Ras proteins are small GTPases that can activate Raf-MEK-ERK signaling[32]. GTP-bound Ras interacts with the Raf family (A-Raf, B-Raf, and C-Raf), inducing Raf dimerization and phosphorylation of C-Raf (also called Raf-1)[33,34]. It has also been reported that phosphorylation of C-Raf (Ser338) can induce ERK activation in lymphocytes such as T cells and B cells[35]. Therefore, we hypothesized that C-Raf could be the signal upstream of ERK, and indeed found that MQL treatment induced phosphorylation of C-Raf (Ser338) **(Fig. 4D)** .

3.5. Effects of MQL on ROS generation in $CD4^+$ T cells

Phytochemicals have been well-known as antioxidants that can directly scavenge ROS, and are used to protect against inflammation[36,37]. Whereas phytochemicals can generate appropriate intracellular ROS, resulting in up-regulation of HO-1 expression[38]. Furthermore, intracellular ROS have been linked to ERK1/2 as cAMT-mediated ROS production can induce ERK1/2 phosphorylation via Ras activation[39]. Thus, we investigated intracellular ROS generated by MQL in $CD4^+$ T cells using DCFH-DA or MitoSOX^{TM} Red, and found that MQL increased intracellular and mitochondrial ROS levels **(Fig. 5A and B)** . Next, to investigate effects of intracellular ROS generated by MQL on the C-Raf-ERK1/2 signaling pathway, $CD4^+$ T cells were cultured with MQL in the presence and absence of the antioxidant N-acetylcysteine (NAC). We found that MQL-induced C-Raf and ERK1/2 phosphorylation, as well as HO-1 expression, were suppressed by NAC treatment **(Fig. 5C and D)** . Taken together, these results suggest that MQL-induced ROS mediate Ras-Raf-ERK activation and HO-1 expression in $CD4^+$ T cells.

3.6. Effects of topical and oral MQL administration on TMA-induced AD symptoms

To evaluate the effects of MQL on Th2-mediated allergic diseases, we used a TMA-induced AD-like mouse model. Since AD symptoms have been known to occur through both external and internal factors, we administered MQL treatment either topically or orally. After topical treatment of MQL, ear thickness and infiltration of inflammatory cells were reduced compared to the sham group **(Fig. 6B and C)** . Furthermore, serum IgE levels were reduced in MQL10 group **(Fig. 6D)** . Next, to investigate the effects of MQL on T cell-mediated immune responses, we measured Th1 (IL-12 and IFN-γ) and Th2 (IL-13) cytokine production in the culture supernatant of DLNs and found that IL-13 and IFN-γ production were significantly reduced by MQL treatment, but IL-12 was not changed **(Fig. 6E-G)** .

Next, we investigated effect of oral MQL administration on TMA-induced AD. Similar to topical administration, oral MQL administration ameliorated AD symptoms such as ear swelling and infiltration of inflammatory cells in ear **(Fig. 6I and J),** and TMA-induced serum IgE production was decreased by MQL compared with the sham group**(Fig. 6K)** . In DLNs, oral administration of MQL reduced IL-13 production and increased IL-12 **(Fig. 6L and N)** . These results suggest that either topical or oral administration of MQL is a possible treatment to attenuate the symptoms of AD.

4. Discussion

To investigate effects of MQL on allergic immune responses, we used an OVA-induced allergic mouse model, and showed that MQL suppresses Th2-related immune responses such as serum IgE production and Th2 cytokine production by splenocytes. In our previous studies, we revealed that MQL was one of active compounds in *Rosae multiflorae* fructus extract, which can ameliorate asthma, rhinitis, and food allergies[21,40]. Furthermore, Nguyen *et al* . reported that *Rosae multiflorae* fructus has an anti-allergic effect by suppress-

ing antigen-specific T cell activation and proliferation *in vivo* [41]. Our results *in vivo* study also suggest that MQL suppresses splenocyte proliferation and IL-2 production, thereby inhibiting Th2-related immune responses. Therefore, we focused on $CD4^+$ T cells and investigated the anti-allergic mechanisms of MQL.

Th2-dominant responses, showing increased Th2 (IL-4, IL-5, and IL-13) cytokines and IgE production, are characteristic immune responses in patients with allergic diseases such as food allergy, asthma, and AD[42–44]. We previously reported some strategies to treat allergic diseases[45–47]. The first involves regulating the Th1/Th2 immune balance by suppressing excessively induced Th2-mediated immune responses or increasing Th1-related immune responses[48–50]. However, here we confirmed that MQL treatment does not increase the Th1-associated cytokine IFN-γ. Thus, we focused on T cell proliferation as reducing T cell clonal expansion and differentiation represents an alternative way to manage allergic diseases[2]. Mechanisms for suppressing T cell proliferation are typically inducing regulatory T cells (Treg) or up-regulating HO-1 expression. When we investigated the effects of MQL on Treg induction, MQL treatment did not increase $CD4^+Foxp3^+$ T cell populations (data not shown). Therefore, in the present study, we investigated the effects of MQL on Th2-dominant allergic immune responses and mechanisms involved in HO-1 expression.

HO-1 is known to have various physiological functions including a protective effect against oxidative stress[12,13]. In particular, it was reported that up-regulation of HO-1 in $CD4^+$ T cells suppresses their proliferation by inhibiting IL-2 production[7]. Similarly, our results showed that MQL suppresses $CD4^+$ T cell proliferation and IL-2 production induced by anti-CD3/CD28 antibody stimulation, while HO-1 expression was increased. Based on these results, we hypothesize that MQL may be able to suppress the $CD4^+$ T cell-mediated immune responses by inhibiting proliferation of $CD4^+$ T cells via up-regulation of HO-1. Furthermore, we speculate that HO-1 is a key regulator of $CD4^+$ T cell proliferation.

Up-stream signals of HO-1 have been reported by many previous studies. Notably, Nrf2, which is normally repressed by Keap1, translocates to the nucleus upon activation and binds to ARE sequences of the HO-1 gene. Especially, about the regulation of HO-1 on cell proliferation, activation of MAPKs (ERK, JNK, and p38) as up-stream signals of Nfr2 plays as factors, which are able to regulate cell proliferation. However, the specific MAPKs involved depend on the administered compounds. For example, it was reported that quercetin enhances Nrf2/HO-1 activity via both ERK and p38 signaling pathways[51,52] and suppresses T cell proliferation by enhancing HO-1 expression[20]. Quercetin-3-*O*-*β*- D-glucuronopyranoside, also known as isoquercitrin, can also up-regulate HO-1 expression via ERK pathways[53]. Although in a different cell type, in a recent study, Lee *et al.* revealed that MQL up-regulates HO-1 expression via the Nrf2 pathway in human hepatoma cells (HepG2) but does not activate nuclear factor-kappa B (NF-ϰB)[54]. In this study, we found that MQL activated Nrf2-HO-1 signals through phosphorylation of ERK in $CD4^+$ T cells. In experiments using MAPK inhibitors, we confirmed that U0126 (ERK inhibitor) most significantly suppressed HO-1 expression, and MQL strongly increased ERK phosphorylation in $CD4^+$ T cells. Although treatment with SB20350 (p38 inhibitor) suppressed HO-1 expression, it was relatively weak compared with the effect of U0126. Moreover, p38 phosphorylation was not detected following MQL treatment. Therefore, our results demonstrate that ERK activation is upstream of MQL-induced HO-1 expression in $CD4^+$ T cells.

Ras is a small GTP-binding protein with three isoforms (H-Ras, K-Ras, and N-Ras), and controls growth via ERK activation and other intracellular signaling pathways[55]. Membrane-bound Ras (GTP-bound active form) can activate Raf by recruiting Raf and promoting the formation of B-Raf /C-Raf complexes or homodimers. Activated Raf protein can then induce phosphorylation of ERK1/2[34,56]. Previous studies have reported that HO-1 expression can be up-regulated by activating the Ras-Raf-ERK signaling pathway[57,58]. Based on these findings, we investigated whether MQL-induced HO-1 up-regulation was associated with C-Raf phosphorylation. Our results showed that MQL could induce phosphorylation of C-Raf in $CD4^+$ T cells, indicating that C-Raf could be a potential up-stream signal of the ERK-Nrf2 signaling pathway.

ROS such as superoxide anion radicals ($\cdot O2^-$), hydroxyl radicals ($\cdot OH$), and hydrogen peroxide (H_2O_2) are constantly produced as a byproduct of mitochondrial oxidative metabolism. Excessively generated ROS, however, can induce oxidative stress, which underlies various diseases, while normal ROS levels can regulate cell growth by modulating proliferation and differentiation. Specifically, ROS activate cellular pathways

such as MAPK, JAK/STAT, Nrf2, and NF-κB signals[59–61]. Furthermore, intracellular ROS are known to induce HO-1 expression[62,63]. Consistent with our results, it was reported that intracellular ROS activates the Raf-MEK-ERK signaling pathways[64] and could induce ERK1/2 phosphorylation by activating Ras[39,65]. Importantly, flavonoids are antioxidant secondary phenolic metabolites naturally produced in fruits and vegetables, and are able to against oxidative stress[66]. However, the flavonoids can also increase intracellular ROS levels as prooxidants. For example, McNally e*t al* , revealed that curcumin increases HO-1 expression via ROS generation[67]. In addition, it has been reported that quercetin can also act as a prooxidant capable of producing intracellular ROS[37,68]. In this study, we found that MQL induces ROS production in $CD4^+$ T cells, which enhances HO-1 expression via Raf-ERK activation. Consequently, this leads to suppression of IL-2 production and $CD4^+$ T cell proliferation.

TMA as a hapten has been known to induce allergic AD by evoking Th2-dominant immune responses in mice[26,69–71]. Haptens act as antigens by generating hapten-protein complexes through conjugation with self-proteins then activate adaptive immune responses when recognized by APCs such as DCs[71]. Langerhans cells, which are DCs that reside in the epidermis, induce naïve $CD4^+$ T cell differentiation by presenting antigens after migrating into T cell-rich areas, such as the paracortex of the DLNs[72]. To characterize the effects of MQL on a TMA-induced AD-like mouse model, we administered MQL either orally or topically, since both oral and topical therapies are effective for treating AD. Considering that this was a disease model, we used a higher dosage of MQL (4 and 10 mg/kg) than in the OVA-induced Th2-dominant mouse model (MQL, 2 and 4 mg/kg). Our results revealed that AD symptoms such as ear swelling, tissue infiltration of inflammatory cells, and IgE production were ameliorated by MQL in both topical and oral treatments. Interestingly, serum IgE levels were strongly reduced by oral administration of MQL compared with topical administration, possibly because of IgE production involves a systemic immune response. However, in DLNs, which represent peripheral immune responses, IL-13 levels induced by TMA were dramatically reduced by topical treatment of MQL compared with oral administration. These results indicate that MQL may be effective as an oral or topical drug depending on the internal or external causes of atopic dermatitis. Further anti-proliferative effects of MQL were seen in allergic mouse models as Th2 (IL-13) and Th1 (IFN-γ) cytokines decreased, but not IL-12 (cytokine derived from APC). Fig. 6 shows that IFN-γ is suppressed by MQL topical treatment, but not IL-12, while oral administration of MQL did not affect IFN-γ production but increased IL-12 production. More evidently, IFN-γ/IL-12 ratios showed that MQL treatment decreases IFN-γ production compared with the TMA alone group as follows: Ratio of IFN-γ/IL-12 in the naïve group (oral, 1.3; topical, 1.7), TMA-induced AD group (oral, 2.4; topical, 8.9), MQL 4 group (oral, 2.3; topical, 6.5), and MQL 10 group (oral, 2.2; topical, 5.3). Therefore, we believe that MQL has the potential to be developed as a therapeutic agent for AD.

In conclusion, we demonstrated that MQL suppressed Th2-related immune responses by reducing $CD4^+$ T cell proliferation via up-regulation of HO-1 expression. Mechanistically, MQL increased HO-1 expression via activation of the Raf-ERK-Nrf2 pathway and by generating ROS in $CD4^+$ T cells. Furthermore, we verified that these effects of MQL on $CD4^+$ T cells leads to alleviated allergic diseases such as an atopic dermatitis *in vivo* . It is possible that MQL may provide clinical benefits on other allergic and $CD4^+$ T cell-mediated diseases. The application MQL on various diseases remains the focus of further studies.

Conflict of interest

No competing financial interests exist.

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Figure legends

Fig. 1**.** Effects of MQL on the OVA-induced allergic mouse model. (A) Experimental schedule of the OVA-induced allergic mouse model. Allergic immune responses in BALB/c mice were induced by i.p injection of OVA (10 μg) with alum (1 mg) on days 7 and days 14. MQL was orally administered daily from day 7 to day 21. The level of (B) serum IgE was analyzed by ELISA. Splenocytes were seeded to 5×10^6cells/mL and cultured in the presence of OVA (100 μg/mL) for 72 h. (C) IL-4, (D) IL-5, (E) IL-13, and (F) IL-2 cytokines in culture supernatant of splenocytes were measured by ELISA. (G) Cell proliferation was measured by MTT assay, and (H) HO-1 expression was detected in splenocytes of each group by Western blot**.** Results

are shown as mean ± SD. Asterisks (*) and (**) indicate significant differences, at $p < 0.05$ and $p < 0.01$, respectively, between the MQL-treated and non-treated groups.

Fig. 2. Immunomodulatory effects of MQL on splenocytes of BALB/c mice immunized by OVA. Allergic immune responses in BALB/c mice were induced by i.p injection of OVA (10 μg) with alum (1 mg) on days 7 and days 14. Splenocytes were seeded to 5×10^6cells/mL and cultured in the presence or absence of OVA (100 μg/mL) and MQL for 72 h. (A) IL-4, (B) IL-5, (C) IL-13, (D) IFN-γ, (E) IL-12, and (F) IL-2 cytokines in culture supernatant of splenocytes were measured by ELISA. (G) Cell proliferation was measured by MTT assay. (H) STAT1 and STAT6 phosphorylation and (I) HO-1 expression were detected by Western blot. Results are shown as mean ± SD. Asterisks (*) and (**) indicate significant differences, at $p < 0.05$ and $p < 0.01$, respectively, between the MQL-treated and non-treated groups.

Fig. 3. Effects of MQL on $CD4^+$ T cell proliferation and HO-1 expression. $CD4^+$ T cells isolated from splenocytes were seeded to 1×10^6 cells/mL and cultured with anti-CD3 (1 μg/mL) and anti-CD28 antibodies (1 μg/mL). $CD4^+$ T cells were cultured in the presence or absence of MQL for 48 h. (A) IL-2 in culture supernatant of $CD4^+$ T cells was measured by ELISA. (B) $CD4^+$ T cell proliferation was measured by MTT assay. (C) Expression of p21 was detected by Western blot, and (D) cell cycle of $CD4^+$ T cells was measured by flow cytometry. (E) HO-1 expression and (F) NRF2 nuclear translocation were detected in $CD4^+$ T cell cultured in the presence or absence of MQL by Western blot. Results are shown as mean ± SD. Asterisks (*) and (**) indicate significant differences, at $p < 0.05$ and $p < 0.01$ respectively, between the MQL-treated and non-treated groups.

Fig. 4. Effects of MQL on MAP kinase signaling pathways. (A) $CD4^+$ T cells were isolated from splenocytes and pre-treated with U0126 (5 μM), SB20350 (5 μM), or SP600125 (5 μM) for 1 h then treated with MQL for 24 h to detect HO-1 expression in $CD4^+$ T cells. (B) To analyze MAPK phosphorylation induced by MQL, $CD4^+$ T cells were pre-treated with MQL for 2 h before stimulation with anti-CD3 (1 μg/mL) and anti-CD28 antibodies (1 μg/mL). $CD4^+$ T cells were treated with MQL for 2 h without stimulation, and (C) MAPK phosphorylation and (D) C-Raf phosphorylation were detected by Western blot. Data are representative of three independent experiments.

Fig. 5. Effects of MQL on ROS generation in $CD4^+$ T cells. $CD4^+$ T cells isolated from splenocytes were cultured in the presence or absence of MQL for 2 h. Production of reactive oxygen species in $CD4^+$ T cells was detected by using (A) DCFH-DA and (B) MitoSox^{TM} Red and measured on a fluorescence plate reader or by flow cytometry. (C) $CD4^+$ T cells isolated from splenocytes were pre-treated with NAC (1 mg/mL) for 1 h then cultured for 2 h in the presence of MQL. Phosphorylation of ERK and C-Raf were detected by Western blot. (D) $CD4^+$ T cells were cultured with anti-CD3 (1 μg/mL) and anti-CD28 antibodies (1 μg/mL) for 24 h in the presence of NAC (1 mg/mL) and MQL. HO-1 expression in $CD4^+$ T cells was detected by Western blot. Results are shown as mean ± SD. Asterisks (*) and (**) indicate significant differences, at $p < 0.05$ and $p < 0.01$, respectively, between the MQL-treated and non-treated groups.

Fig. 6. Effects of topical and oral administration on TMA-induced AD symptoms. (A) Experimental schedule of the TMA-induced AD-like mouse model with topical treatment of MQL. Cells from DLNs were seeded to 1×10^6 cells/mL and cultured in the presence of Con A (2 μg/mL) for 48 h. An experimental AD-like lesion was induced on the shaved flank skin of BALB/c mice by sensitization with 5% TMA (50 μL) on day 0 then treated with 2% TMA (10 μL) on days 5-14. MQL (4 and 10 mg/mL, 10 μL) and Dex. (1 mg/mL, 10 μL) were administered topically. (B) Ear swelling was measured 24 h after TMA treatment. (C) Infiltrating inflammatory cells in the ear tissues were stained using hematoxylin and eosin. The levels of (D) IgE in serum and (E) IL-13, (F) IFN-γ, and (G) IL-12 cytokines in culture supernatant of DLNs were measured by ELISA. (H) Experimental schedule of the TMA-induced AD-like mouse model with oral administration of MQL. An experimental AD-like lesion was induced on the shaved flank skin of BALB/c mice by sensitization with 5% TMA (50 μL) on day 0 then treated with 2% TMA (20 μL) on days 5, 8, 11, 14, 17, 20, 23, and 26. MQL (4 and 10 mg/kg) and Dex. (1 mg/kg) were administrated by orally from day 5 to day 26. (I) Ear swelling was measured 24 h after TMA treatment. (J) Infiltrating inflammatory cells in the ear tissues were stained using hematoxylin and eosin. The levels of (K) IgE in serum and (L) IL-13, (M) IFN-γ, and

(N) IL-12 cytokines in culture supernatant of DLNs were measured by ELISA. Results are shown as mean ± SD. Asterisks (*) and (**) indicate significant differences, at $p < 0.05$ and $p < 0.01$, respectively, between the MQL-treated and non-treated groups of TMA-treated mice.

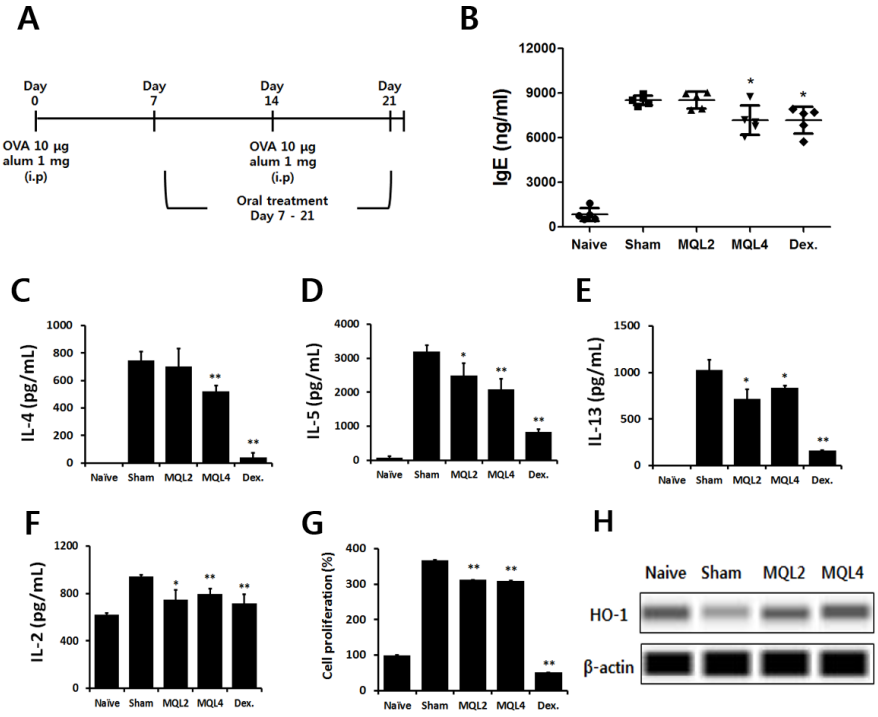


Figure 1

Figure 2

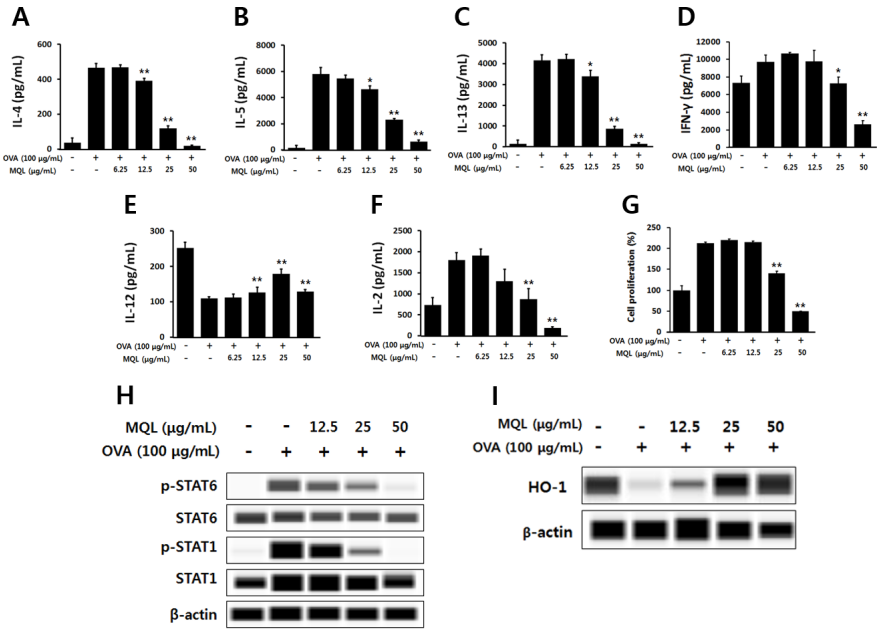
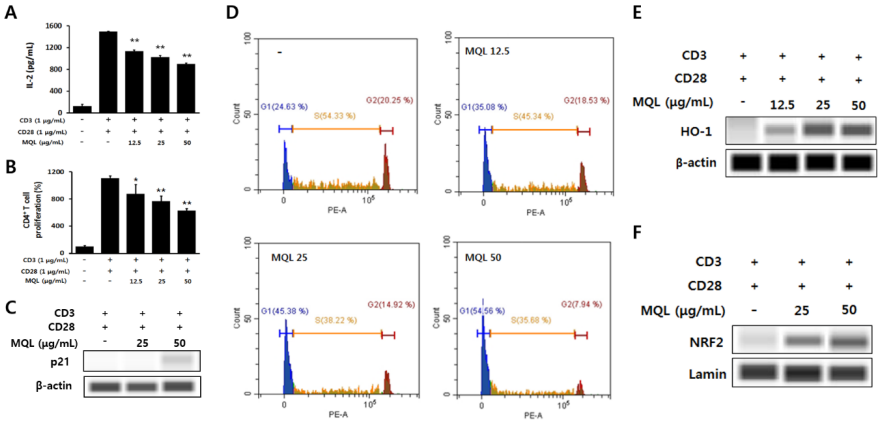


Figure 3



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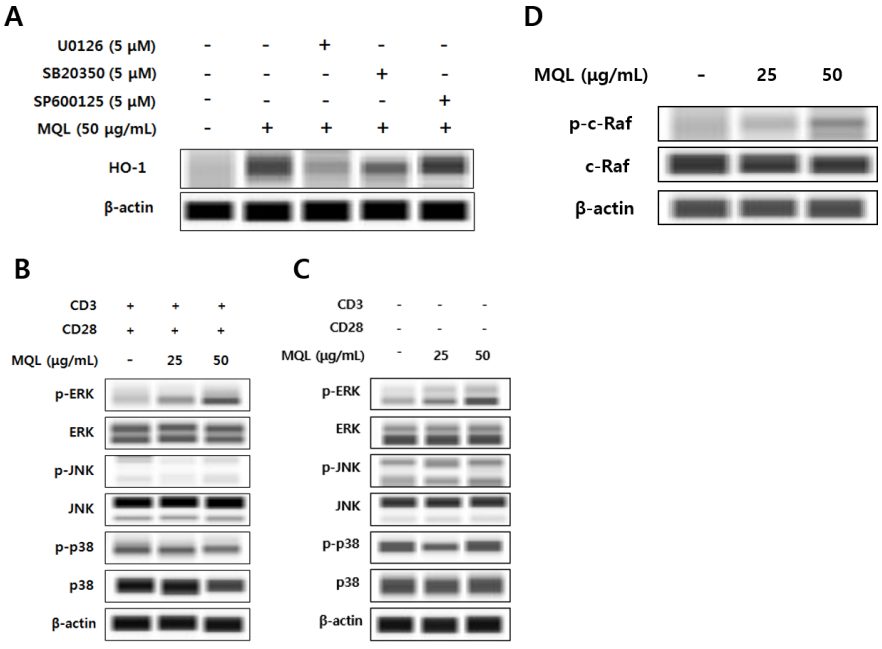

Figure 5

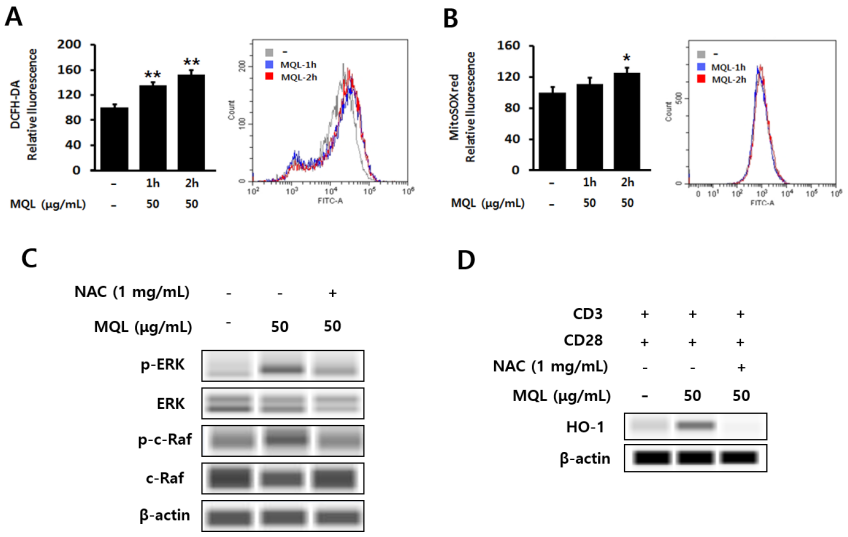

Figure 6