Statins inhibit chemotactic interaction between CCL20 and CCR6 *in vitro*: possible relevance to psoriasis treatment

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

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Psoriasis is a common and chronic inflammatory skin disease which is associated with IL-23/Th17 pathway. An increased expression of CC chemokine ligand 20 (CCL20) in psoriatic lesions can attract CC chemokine receptor 6 (CCR6)-expressing Th17 cells into the lesions. Lipid-lowering drugs, statins, possess other immune-modulating functions. We explored whether specific types of statins could inhibit CCL20 production in HaCaT cells. We also investigated whether statins could attenuate the chemotactic migration of CD4+ T cells toward CCL20.

We used enzyme-linked immunosorbent assay to evaluate CCL20 release from HaCaT cells stimulated by psoriasis-associated cytokines with or without statins. We performed fluorescence-activated cell sorting to investigate the level of surface CCR6 and CD45RO expression on human CD4+ T cells in various conditions. Functionally, the *in vitro* chemotaxis migration assay was performed and the number of migrating cells was analyzed using a flow cytometry.

We demonstrated that IL-1β, TNF-α, and IL-17A significantly increased CCL20 production from HaCaT cells in a dose-dependent manner. However, these increments were significantly inhibited by the addition of fluvastatin and simvastatin, but not by pravastatin. In the *in vitro* chemotaxis migration assay,

pretreatment with fluvastatin and simvastatin, but not with pravastatin, inhibited the chemotactic migration of human CD4+ T cells toward CCL20. However the level of surface expression of CCR6 on CD4+ T cells was not altered by statins.

Our results suggest that not all, but specific types of statins may be of benefit in managing psoriasis partially via interrupting the chemotactic interaction of CCL20/CCR6, the mechanism which eventually lessen the infiltration of lesional Th17 cells.

Key words: CCL20, CCR6, psoriasis, statins, Th17 cells

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I. INTRODUCTION

Psoriasis is a common and chronic inflammatory skin disorder characterized by epidermal hyperproliferation and infiltration of various type of immune cells, including T cells, dendritic cells, macrophages, and neutrophils.[1] Of recent, the pathogenesis of psoriasis is thought to be mediated by immunologic processes, with a particular interest in the IL-23/Th17 axis.[2,3] Discrete population of Th17 cells have been demonstrated in psoriatic plaques,[4] and myeloid dermal dendritic cells (DCs) isolated from psoriatic skin could efficiently differentiate naïve CD4+ T cells into both Th17 and Th1 phenotype.[5] Intradermal injection of IL-23, a pivotal cytokine in the induction and proliferation of Th17 cells, has been found to induce psoriasis-like skin inflammation in mice, and an increased level of IL-23p19 and IL-23p40 mRNA has been demonstrated in lesional skin of psoriasis leading to the successful use of ustekinumab, IL-23p40 monoclonal antibody, in treating psoriasis.[6-11]

Chemokines and chemokine receptors play a central role for the directional migration and tissue specific trafficking of leukocytes in both homeostatic and

inflammatory conditions.[12] A number of chemokines and chemokine receptors are expressed in psoriatic lesions and they seem to fundamentally contribute to the sequential and spatial recruitment of inflammatory cells in developing psoriatic lesions.[13,14] Recently, a particular interest in the interaction between CCL20 and CCR6 has arisen in the pathogenesis of psoriasis. CCR6 specifically responds to its sole chemokine ligand CCL20, and is expressed on virtually all human IL-17A- and IL-22-producing CD4+ T cells.[15-17] Both CCL20 and CCR6 are highly expressed in the psoriatic skin compared to non-lesional or normal skin and circulating peripheral blood mononuclear cells (PBMCs) from patients with psoriasis express higher CCR6 than those from healthy donors.[18] Harper and colleagues recently showed that psoriasis-associated cytokines, TNF-α and IL-17A, did markedly induce CCL20 release from human keratinocytes, resulting in the recruitment and maintenance of lesional Th17 cells in a positive feedback manner.[19] Of note, mice deficient in CCR6 fail to develop IL-23-induced psoriasis-like inflammation, indicating that the blockade of CCL20/CCR6 interaction may be a potential therapeutic target in psoriasis.[20]

Statins, 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors, are widely used to lower lipid levels and subsequently prevent atherogenesis.[21] Apart from its cholesterol-lowering effect, recent evidences have shown that statins have immune-modulating functions with positive effects in chronic inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, and Crohn's disease.[22,23] Among the anti-inflammatory mechanisms, statins can modulate inflammatory milieu by reducing the expression of chemokines and chemokine receptors, eventually inhibiting leukocytes migration.[23] In patients with coronary artery diseases, atorvastatin were found to significantly reduce expression of several chemokines and their receptors.[24] Grip and colleagues tested the effect of atorvastatin in patients with Crohn's disease and results revealed reduced C-reactive protein (CRP) and CXCL10 due to atorvastatin,

showing statin-mediated mucosal protective effects.[25,26]

So far, effects of statins on the CCL20/CCR6 chemotactic interaction have not been investigated. Therefore, we hypothesized that statins might inhibit CCL20/CCR6 interaction to confer a potential benefit in managing psoriasis. We explored whether statins could inhibit CCL20 production in cytokines-activated HaCaT cells and attenuate the chemotactic migration of CCR6+CD4+ T cells in response to CCL20.

II. MATERIALS AND METHODS

1. Reagents

Recombinant human IL-1β, TNF-α, IL-17A, IL-22, IFN-γ, and CCL20 were purchased from R&D Systems (Minneapolis, MN, USA). Fluvastatin, simvastatin, and pravastatin were the products of Calbiochem (La Jolla, CA, USA). The stock solutions of statins were made in DMSO. We purchased APC-conjugated mouse anti-human CCR6 and PE-conjugated mouse anti-human CD45RO antibodies from BD Pharmingen (San Jose, CA, USA). APC-conjugated anti-mouse CCR6 and PE-conjugated anti-mouse CD45RO antibodies were used for isotype controls (BD Pharmingen).

2. HaCaT cell cultures

The human keratinocyte cell line, HaCaT cell (kindly provided by Dr. Fusenig from German Cancer Research Center, Heidelberg, Germany), were cultured in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 10 U ml^{-1} penicillin and 10 μg ml^{-1} streptomycin (Gibco) at 37°C in a 5% CO_2 incubator. Before performing cytokine stimulation experiments, the growth medium was changed for serum-free RPMI 1640 and the experiments were initiated on 70% confluent HaCaT cells in 12-well plates. HaCaT cells were cultured either alone or with one of the following cytokines for 24 h; IL-1β, TNF-α, IL-17A, IL-22, and IFN-γ. According to a previous study, 2 μM of statins had minimal effects on the viability of HaCaT cells.[27] Thus, in some experiments, we added 2 μM statin in culture medium together with the cytokines.

3. Peripheral blood samples

PBMCs from blood samples of healthy volunteers (n=5) were isolated by gradient centrifugation with Ficoll-Plaque Plus (Pharmacia, Piscataway, NJ,

USA), washed with phosphate-buffered saline (PBS). CD4+ T cells were isolated by MACS CD4+ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Cell purity was confirmed by flow cytometry (> 95%). Isolated human peripheral CD4+ T cells were cultured in RPMI 1640 supplemented with 1% human plasma and stimulated with anti-CD3 (5 μg ml^{-1}) and anti-CD28 (2.5 μg ml^{-1}) (eBioscience Inc., San Diego, CA, USA). Some CD4+ T cells were pretreated with each statin (2 μM) for 24 h, and then used for the chemotaxis migration assay or FACS analysis.

4. Measurement of CC chemokine 20 production

Secreted CCL20 protein levels in cytokines-treated HaCaT cells supernatants were analyzed using Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions.

5. Chemotaxis migration assay

Cell migration was evaluated using the 24-well, 5-μm pore size polycarbonate Transwell system (Costar, Cambridge, MA, USA). Isolated CD4+ T cells from healthy donors were conditioned with statins as described above. Total of 10^6 CD4+ T cells were placed on the top of the Transwell in RPMI 1640 medium and incubated with or without the 500 ng ml^{-1} of recombinant human CCL20 in the bottom chamber for 3 h at 37°C. The number of migrating cells was counted using forward/side light scatter characteristics on flow cytometry. Chemotaxis indices were calculated as the ratio of the number of cells migrating toward CCL20 divided by the number of migrating cells in the negative control.[18]

6. Flow cytometric analysis

Cells were stained with the antibodies mentioned above. Briefly, isolated

CD4+ T cells were stained for cell surface molecules for 20 min at 4°C, washed with FACS buffer (PBS, 0.1% sodium azide and 2% fetal bovine serum) (BD Biosciences, San Jose, CA, USA) and resuspended in FACS buffer. Samples were acquired by an LSR-II flow cytometer (BD Biosciences) and analyzed with WinMDI ver 2.8 software (Bio-Soft Net). All incubation steps were done on ice. Appropriate isotype controls were used.

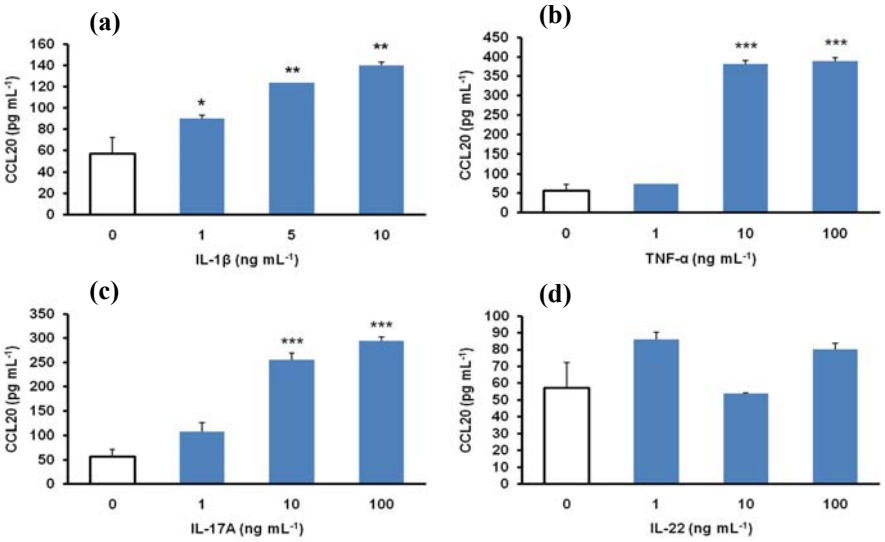
7. Statistical analysis

Statistical significance was calculated by Student's *t* test using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). All p values < 0.05 were considered significant.

III. RESULTS

1. Cytokines upregulated in psoriatic lesions, IL-1β, TNF-α, and IL-17A, increase CCL20 protein expression by HaCaT cells *in vitro*

Using HaCaT cells cultured as monolayers and psoriasis-related cytokines (IL-1β, TNF-α, IL-17A, IL-22, and IFN-γ), we found that IL-1β, TNF-α, and IL-17A significantly induced CCL20 protein at 24 h in a dose-dependent manner demonstrated by ELISA (Figure 1a-c). Compared with control group, 10 ng ml^{-1} IL-1β increased CCL20 release by 2.4 times, and both 100 ng ml^{-1} of TNF-α and IL-17A enhanced the chemokine production by 6.8 and 5.2 times, respectively. Optimal concentration for IL-1β was 10 ng ml^{-1} and those for TNF-α and IL-17A was both 100 ng ml^{-1} as previously described.[18,19] However, neither IL-22 nor IFN-γ increased CCL20 release by HaCaT cells (Figure 1d-e).



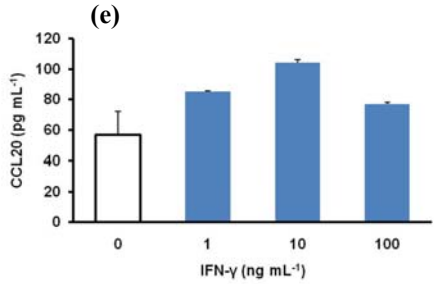
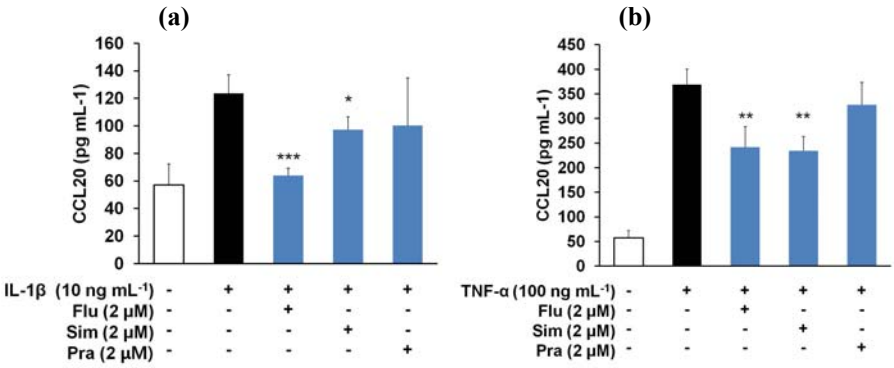


Figure 1. Effects of psoriasis-associated cytokines on release of CCL20 in HaCaT cells. Cells were incubated with medium alone or with various cytokines which are highly expressed in psoriatic plaques. At 24 h, CCL20 in the supernatant was analyzed by ELISA. CCL20 release was significantly induced by treatment with IL-1β, TNF-α, and IL-17A in a dose-dependent manner. Data are presented as mean ± SEM of four experiments conducted in duplicate (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus non-treated group).

2. Fluvastatin, simvastatin, but not pravastatin, can inhibit CCL20 release by HaCaT cells activated by IL-1β, TNF-α, and IL-17A

To study whether statins could inhibit CCL20 protein expression, HaCaT cells were co-cultured with indicated concentrations of psoriasis-related cytokines and 2 μM of fluvastatin, simvastatin, or pravastatin. Increased CCL20 protein expression by treatment with IL-1β, TNF-α, and IL-17A was significantly inhibited by fluvastatin and simvastatin (Figure 2a-c). Although CCL20 release was not induced by IL-22, fluvastatin and simvastatin also suppressed CCL20 production compared to control cells or cells stimulated by IL-22 (Figure 2d). However, fluvastatin and simvastatin did not significantly reduce CCL20 production in IFN-γ-treated cells (Figure 2e). In contrast to other statins, pravastatin had no effect on suppressing cytokines-induced CCL20 production. These results indicate that specific types of statins could reduce CCL20 production by HaCaT cells in psoriasis-oriented cytokines milieu.



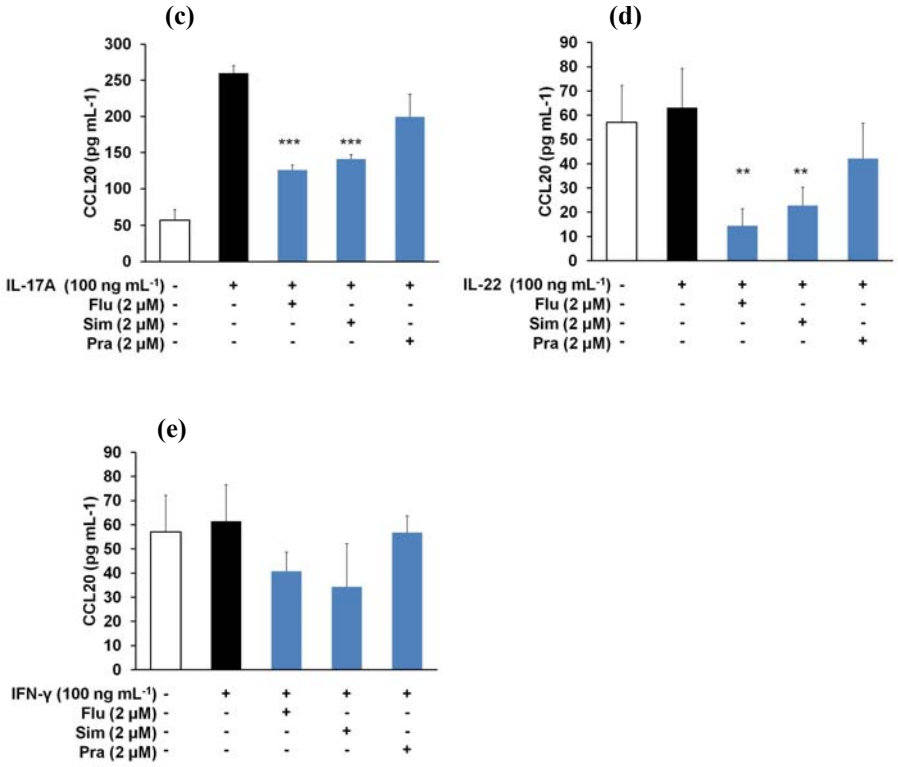


Figure 2. Effects of statins on release of CCL20 in HaCaT cells. Cultured cells were incubated with indicated concentrations of each cytokine and fluvastatin, simvastatin, or pravastatin. At 24 h, CCL20 release was analyzed by ELISA. Fluvastatin and simvastatin significantly inhibited CCL20 production by HaCaT cells. Data presented as mean ± standard deviation of four experiments conducted in duplicate (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus cytokine-treated group). (Flu: fluvastatin, Sim: simvastatin, Pra: pravastatin).

3. Fluvastatin, simvastatin, but not pravastatin, inhibit chemotactic migration of CD4+ T cells toward CCL20

The next question we addressed was whether statins could functionally inhibit the chemotactic migration of CD4+ T cells toward CCL20. The isolated human CD4+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies with or without the presence of 2 μM concentration of each statin. After 24 h of incubation, conditioned cells were placed on the chemotaxis chamber to enable them to migrate toward 500 ng ml^{-1} of CCL20, for 3 h. Flow cytometric analysis revealed that the number of migrating CD4+ T cells with stimulation was 30 times higher than control cells (Figure 3). Of note, both treatment with fluvastatin and simvastatin inhibited the chemotactic response of CD4+ T cells to CCL20, and fluvastatin more effectively reduced the chemotactic migration of CD4+ lymphocytes than simvastatin. However, the chemotaxis of CD4+ T cells to CCL20 was not suppressed by pravastatin.

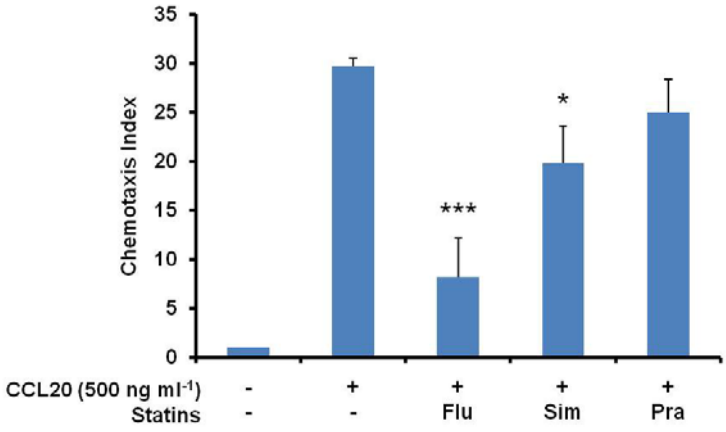


Figure 3. The inhibitory effect of statins on CD4+ T cells chemotactic migration to CCL20. Fluvastatin and simvastatin significantly inhibited chemotactic

migration of CD4+ T cells to CCL20. Data are presented as mean ± SEM of four experiments from different donors. Chemotaxis indices were calculated as the ratio of the number of cells migrating toward CCL20 divided by the number of migrating cells in the negative control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus CCL20-treated group). (Flu: fluvastatin, Sim: simvastatin, Pra: pravastatin).

4. Decreased chemotactic migration of statin-treated CD4+ T cells toward CCL20 was not mediated by CCR6 down-regulation

As treatment of fluvastatin and simvastatin inhibited the chemotaxis of CD4+ T cells toward CCL20, we wondered whether treatment with statins could down-modulate the surface expression of CCR6 on CD4+ T cells. CD4+ T cells were incubated under the same stimulating conditions described above. CD4+ T cell surface expression of CCR6 and CD45RO was confirmed by flow cytometric analysis (Figure 4 and 5). Compared with control cells, the percentage of CD4+CD45RO+CCR6+ T cells was not changed by antibody stimulation with or without statins (Figure 4a and b). Contrary to our expectation, surface CCR6 expression on CD4+ T cells was not changed in the presence of statins (Figure 5a-c).

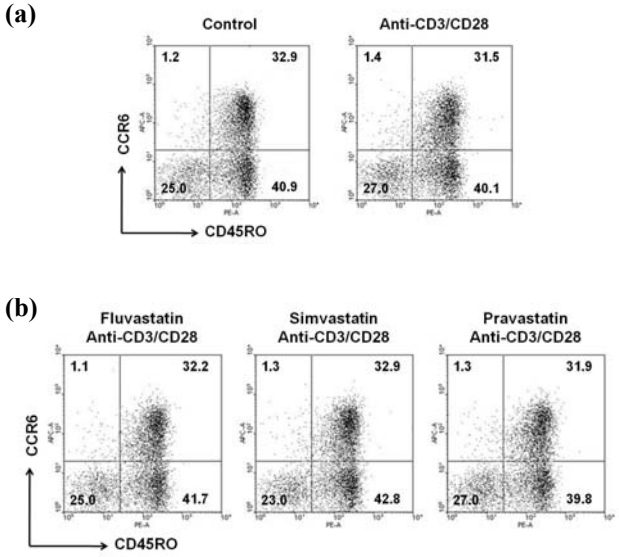


Figure 4. The percentages of CD4+CD45RO+CCR6+ T cells were not changed after stimulation with or without statins (2 μM) compared with control. Isolated human CD4+ T cells were stimulated with anti-CD3/CD28 antibodies with or

without statins supplement for 24 h. Flow cytometric results are representative of three separate experiments.

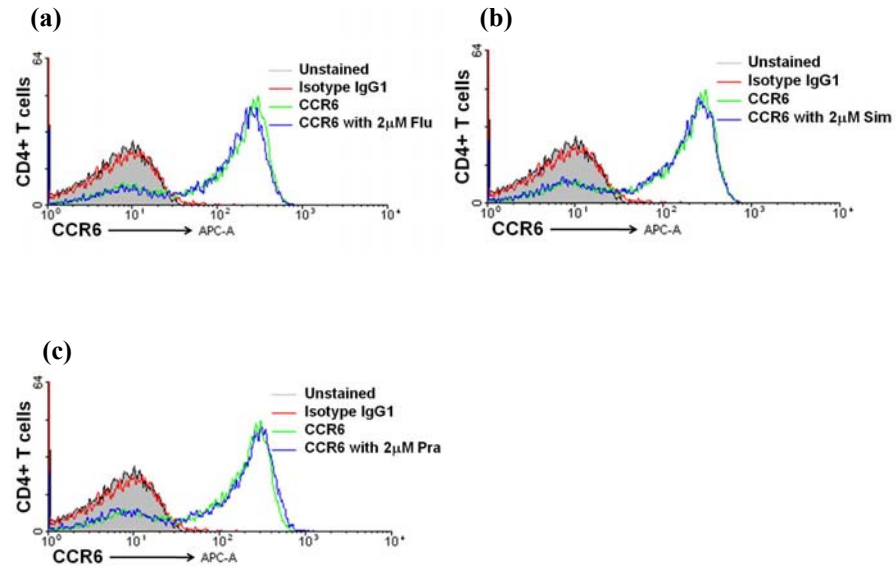


Figure 5. Surface CCR6 expression on human CD4+ T cells was not influenced by statins. Flow cytometric results are representative of three separate experiments. (Flu: fluvastatin, Sim: simvastatin, Pra: pravastatin).

IV. DISCUSSION

Psoriasis is a chronic inflammatory skin disease initiated and maintained mainly by pathogenic T cells.[28] The majority of lesional infiltrating T cells comprise of Th1 and Th17 type cells, and growing evidences have led to an interest in the Th17 subtype as genome-wide association studies indicate that Th17 differentiation-related genes which encode IL-23p40, IL-23p19, and IL-23R are associated with psoriasis.[29] In addition, the monoclonal antibody blocking both IL-12 and IL-23 is highly effective in psoriasis, and the treatment effects of narrow-band UVB therapy and anti-TNF antibodies are associated with the decreased expression of IL-17 signaling, but not with Th1 signaling.[30-32] Although those pathogenesis-based therapies are well tolerated overall, the long-term safety is an unresolved issue yet, thus new approaches are still needed with enhance efficacy, safety and convenience. Chemokine receptor CCR6 is found on all IL-17A-producing T cells, and it was described that inhibiting CCR6 could attenuate Th17-mediated joint inflammation in mice.[33] Of note, CC6-deficient mice were resistant to production of IL-23/IL-22-dependent psoriasis-like inflammation, suggesting the potential of CCR6/CCL20 inhibition as a therapeutic target in psoriasis.[20]

In the current study, we demonstrated that treatment with IL-1β, TNF-α, and IL-17A could enhance CCL20 release by HaCaT cells. Among the statins used, fluvastatin and simvastatin significantly inhibited CCL20 protein production by HaCaT cells stimulated by psoriasis-related cytokines, but the inhibitory effect was not found in pravastatin. In the *in vitro* chemotaxis migration assay, fluvastatin and simvastatin, but not pravastatin, efficiently suppressed chemotactic migration of CD4+ T cells toward CCL20; however, decreased chemotactic responses by statins were not associated with the alteration of surface CCR6 expression on CD4+ T cells.

Lipid-lowering drugs, statins, are currently used to reduce the risk of

ischemic heart disease and stroke.[34] The mechanisms of statins for primary prevention, however, have not only been attributed to their cholesterol-reducing effects, but also to their immune-modulating effects.[35] Statins modulate a broad range of pro-inflammatory immune mechanisms through the inhibition of small GTPases and other prenylated proteins involving posttranslational modification.[23] In our study, the enhanced protein expression of CCL20 by stimulated HaCaT cells was efficiently inhibited by fluvastain and simvastatin. Grip and colleagues demonstrated that atorvastatin reduced plasma levels of CXCL10 in patients with Crohn's disease with favorable clinical outcomes.[26] In cases of coronary artery disease, treatment with statins reduced macrophage inflammatory protein-1α and -1β that are associated with coronary artery inflammation.[24] In addition, CCL22 expression by IFN-γ-stimulated HaCaT cells, which plays an important role in allergic diseases by attracting Th2 cells, was inhibited by fluvastatin, suggesting fluvastatin as a possible therapeutic agent for atopic dermatitis.[27] Thus, it is likely that fluvastatin and simvastatin may contribute to reduce the recruitment of CCR6-expressing Th17 T cells into psoriatic lesions by inhibiting CCL20 secretion by lesional keratinocytes.

Statins can reportedly down-regulate chemokine receptors on monocytes that are crucial for other inflammatory diseases.[24,25] To assess the functional role of statins in the CCL20/CCR6 chemotactic interaction, *in vitro* chemotaxis migration assay and flow cytometry were performed. We have shown that treatment of CD4+ T cells with fluvastatin and simvastatin significantly inhibited the chemotactic migration toward CCL20; however, this effect did not go with the down-modulation of surface CCR6 expression. The RHO family of GTPases, of which CDC42, RAC, and RHO are best characterized, regulate actin and microtubule dynamics, and are central to cellular motility.[36,37] Recently a thorough proteomics study has revealed that the level of RAC involved in actin polymerization was increased in lipid rafts upon CCR6 activation.[38] As statins affect small GTPase function including RAC, it can be

postulated that the decreased chemotactic migration of CD4+ T cells toward CCL20 through our study is mediated by the inhibitory effects of statins on RAC GTPase.

Although all statins effectively lower cholesterol, it seems that they may have different anti-inflammatory functions possibly depending on their lipophilic properties.[39] Higher lipophilicity of fluvastatin compared to simvastatin or atorvastatin partially explain the inhibitory effect of fluvastatin and not of simvastatin nor atorvastatin on IFN-γ-induced CCL22 expression by HaCaT cells.[27] In our study, lipophilic fluvastatin and simvastatin demonstrated significant inhibitory effects on HaCaT cells or CD4+ T cells functions, but hydrophilic pravastatin did not. Furthermore, most lipophilic fluvastatin more effectively suppressed CCL20 production and the chemotactic response to CCL20 than did simvastatin. Collectively, our data suggest that specific types of statins may be of benefit to treatment of psoriasis as immunomodulators.

Currently, clinical studies on the potential benefits of statins in patients with psoriasis have not been properly investigated. One placebo-controlled study and case series reported the significant improvement in clinical severity after using simvastatin.[40,41] On the contrary, another case series demonstrated ambiguous effects of simvastatin with no significant improvement in disease severity.[42] According to our results, however, fluvastatin may present better effects compared to other statins in psoriasis patients partly by inhibiting CCL20/CCR6 chemotatic interaction. Further clinical research applying fluvastatin to a greater number of psoriasis patients is required.

The fact that patients with psoriasis face greater risk for comorbidities of metabolic syndrome and cardiovascular diseases compared with the general population is another rationale behind the use of statins in these patients.[43,44] Various types of statins are currently used to lower the risk of cardiovascular diseases and their accompanying risk factors.[34] Among the risk factors, high-sensitivity C-reactive protein (hsCRP) has recently been identified as a

strong predicting factor for cardiovascular events.[45] It was demonstrated that compared with normal control, higher hsCRP levels are found in patients with psoriasis and increased clinical severity is linked to increased hsCRP, suggesting a possibly higher cardiovascular morbidities in severe patients.[46] However, increased cardiovascular complications in psoriatic patients may be decreased by statins as in healthy populations with merely elevated hsCRP levels, statin significantly reduced the incidence of major cardiovascular events.[47] Thus, further studies are necessary to address the effects of statins in reducing metabolic complications among patients with psoriasis.

V. CONCLUSION

In conclusion, our results indicate that specific statins, but not all statins, inhibit the CCL20/CCR6 interaction, which plays an important role in psoriasis pathogenesis. CCL20 production by HaCaT cells and chemotactic migration of CD4+ T cells to CCL20 are most effectively inhibited by fluvastatin, suggesting that fluvastatin may be used clinically as a potential therapeutic drug for psoriasis patients. Future clinical trials and studies using an *in vivo* model of psoriasis will shed light on new therapeutic application of statins in the treatment and prevention of comorbidities in psoriasis.

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ABSTRACT (IN KOREAN)

CCL20과 CCR6의 상호작용에서 스타틴의 억제 효과에 대한 연구

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건선은 만성 염증성 피부 질환으로서 IL-23/Th17 경로가 발병에 중요한 역할을 한다. 건선 병변에는 케모카인 CCL20이 높게 발현되어 있으며 이는 CCL20에 대한 수용체인 CCR6를 발현하는 Th17 세포가 병변에 침윤되도록 한다. 고지혈증 치료제인 스타틴은 혈중 콜레스테롤을 낮추는 작용 외에 면역조절 작용이 있다. 이에 저자들은 스타틴이 HaCaT 각질형성 세포에서 분비되는 CCL20을 억제하는지, 또한 CD4+ T 세포의 CCL20으로의 화학적 이주를 억제하는지를 알아보고자 하였다.

HaCaT 각질형성세포에 IL-1β, TNF-α, IL-17A를 처리하였을 때 농도 의존적으로 CCL20의 단백질 분비가 증가하였다. 하지만 이러한 효과는 fluvastatin 혹은 simvastatin을 처리하였을 때 억제되었으며, pravastatin은 억제 효과가 없었다. 실험실적인 화학이주 분석 실험에서 인간 CD4+ T 세포는 CCL20으로의 화학이주 성향을 보였지만 이러한 화학이주는 fluvastatin 및 simvastatin에 의해 억제되었으며, pravastatin은 의미 있는 억제 효과가 없었다. 그리고 스타틴에 의한 CD4+ T 세포의 화학이주 억제 효과는 세포 표면의 CCR6 발현 감소와 관련이 없었다.

결론적으로 이번 연구로 모든 스타틴은 아니지만 특정한 스타틴이 CCL20/CCR6의 상호작용을 억제하여 Th17 세포의 화학 이주를 감소시킬 수 있음을 확인하였으며, 앞으로 fluvastatin의 치료적 효과를

평가하기 위해 fluvastatin을 이용한 건선 동물 모델 실험 및 건선 임상 연구가 필요할 것이라고 생각한다.

핵심되는 말: CCL20, CCR6, 건선, 스타틴, Th17 세포