Therapeutic effect of cell permeable peptide-conjugated methotrexate on mouse model of psoriasis induced by imiquimod

Dashlkhumbe Byamba

Department of Medical Science

The Graduate School, Yonsei University

Therapeutic effect of cell permeable peptide-conjugated methotrexate on mouse model of psoriasis induced by imiquimod

Directed by Min-Geol Lee

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Dashlkhumbe Byamba

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This certifies that the Doctoral Dissertation of Dashlkhumbe Byamba is approved.

Thesis Supervisor: Min-Geol Lee

Thesis Committee Member #1: Sang Kyou Lee

Thesis Committee Member #2: Dong Soo Kim

Thesis Committee Member #3: Jeon-Soo Shin

Thesis Committee Member #4: Kee Yang Chung

The Graduate School

Yonsei University

June 2012

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ABSTRACT

Therapeutic effect of cell permeable peptide-conjugated methotrexate on mouse model of psoriasis induced by imiquimod

Dashlkhumbe Byamba

Department of Medical Science
The Graduate School, Yonsei University

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Oral methotrexate (MTX) is an effective treatment for psoriasis being used over 50 years. However, common drug-related toxicities such as myelosuppresion, gastrointestinal irritation, nephrotoxicity, hepatotoxicity, and pulmonary fibrosis deter physicians from a prolonged prescription of MTX. These adverse effects may be minimized by administering MTX topically, using an effective drug delivery system. The aim of this work is to evaluate efficacy and safety of a cell permeable, protein transduction domain (PTD)-conjugated, MTX on imiquimod (IQM)-induced psoriasis-like skin inflammation in BALB/c mice. Topical application of cell permeable-MTX alleviated IQM-induced psoriasiform skin inflammation in mice. The cumulative PASI score of 1% PTD-MTX-applied mice was the lowest (5.35±0.29) followed by intraperitoneal (IP) MTX-injected (6.15±1.22), 0.1% PTD-MTX-applied (7.05±0.57), and IQM alone applied groups (8.15±0.58) in ascending order. Increased number of immune cells in the skin including epidermal MHC-II$^+$ cells and dermal MHC-II$^+$, CD11c$^+$, CD4$^+$ and IL-17A producing γδ TCR$^+$ cells in IQM-applied mice were normalized by both IP-MTX and cell permeable MTX. In addition, increased levels of IL-23, IL-17A and IL-22 in the skin were reduced by both IP-MTX and cell permeable MTX as well. However, altered composition of spleen cells induced by IQM was only affected by IP injection of MTX but not by cell permeable MTX. Besides, cell permeable MTX had no toxic

effect on the liver, kidney and myeloid cells, unlike IP-MTX.

In conclusion, topically applied, cell permeable MTX ameliorated IQM-induced psoriasiform skin inflammation without systemic immune suppressive nor toxic effects.

Key words: Psoriasis, imiquimod, MTX, protein transduction domain, cell permeable peptide

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I. INTRODUCTION

Oral methotrexate (MTX) is one of the potent and inexpensive therapeutics for psoriasis, and is being used since 1972 after approval by the FDA. Even though MTX is remarkably effective, because of the severe systemic side effects, MTX is only prescribed in moderate or severe cases of psoriasis with no history or abnormalities of liver, kidney diseases, chronic infection, systemic diseases and blood abnormalities. In order to minimize the systemic toxic effects of MTX people have been trying to use it locally or topically but most of those efforts have been unsuccessful[1]. The lack of clinical effectiveness of topical MTX in the treatment of psoriasis is thought to be related with poor penetration efficiency[1, 2].

Cell permeable peptides, commonly referred to as the protein transduction domain (PTD), are small cationic molecules that possess a potential to carry large molecules into cells. This property of PTD also enables skin non-penetrating medications to be used for therapeutic purposes[3, 4]. Thus PTD-conjugated MTX can be a promising tool for the treatment of psoriasis, possessing effective skin penetrating capacity and efficacy.

Psoriasis is a chronic inflammatory skin condition, and it causes burdens in mental, social and economical aspects of the patient's life which may lead to high

risks of morbidity, decreased quality of life, and reduced level of employment and income[5, 6]. Recent advance in understanding the immune pathogenesis of this disease led to the development of a new paradigm of psoriasis treatment with highly efficacious biologic therapeutics. However, although the biologics treatment possess very potent and rapid therapeutic efficacy, they may provoke serious adverse reactions, such as infection, malignancies and cardiovascular events[7]. Also, these treatments cost at least twenty million U.S dollars per year[8] that makes it impossible to use in developing countries. Therefore low cost, highly efficacious oral medication such as MTX is still a valuable therapeutic option. But due to drug-related toxicities, it needs considerations to change or modify to a less or non-toxic counterpart.

Ethiology of psoriasis is not known but disease develops in some people who have some susceptible genes upon encountering with environmental stressors. Upon encountering with danger signals, keratinocytes produce antimicrobial peptides (AMP) such as LL-37, cathelicidin, and proinflammatory cytokines such as IL-1, IL-6 and TNFα. AMP, LL-37, together with self DNA released by dead cells, activate cutaneous plasmacytoid dendritic cells (pDCs) to produce large amount of type I interferon (IFN). IFNα and proinflammatory cytokines produced by keratinocytes activate dermal DCs to migrate to draining lymph node, where they activate T cells[9]. Cytokines and chemotactic molecules such as AMP and chemokines produced by keratinocytes recruit myeloid DCs and T cells into skin. Those cells are the major contributor of IL-23/IL-17 axis that is essential in disease pathogenesis[10]. On the other hand IL-23 produced by myeloid DCs, and IFNγ, IL-17A and IL-22 produced by helper T cells (Th) subsets are key cytokines in psoriasis as they induce pathogenic T cell differentiation, chemotactic attraction and epidermal hyperplasia[11]. Recently, γδ T cells are emerging as another major source of IL-17 cytokine. Their number is increased in psoriatic lesional skin compared to healthy control[12]. As they produce IL-17A, they are also considered to be a pathogenic cell type in psoriasis.

Imiquimod (IQM)-induced psoriasis-like inflammation in human[13] and mice[14] has been reported. IQM is a ligand for toll-like receptor 7 (TLR7) and TLR8, and is

a potent immune activator. Psoriatic inflammation triggering effect of IQM is supposed to be related to the stimulation of TLR7 on pDCs and upregulation of type I interferon pathway[15]. Van der Fits et al.,[14] demonstrated that IQM induced psoriasis-like skin inflammation in mice. The histopathologic changes in the epidermis (acanthosis, parakeratosis, neoangiogenesis), and infiltration of $CD4^+$ T cells, $CD11c^+$ DCs and pDCs into dermis in mice were very similar to that of human psoriasis. Moreover, IQM-induced skin inflammation was immunologically mediated via the IL-23/IL-17 axis. More recently it has been reported that IQM application increased dermal IL-17-producing γδ T cells[12] and also IL-22 cytokine that was required for the induction of psoriasiform skin inflammation in mice[16].

The mechanism by which MTX exerts its therapeutic effect is not fully understood. However, it is believed that MTX has anti-proliferation and anti-inflammation actions. MTX directly inhibits DNA synthesis leading to mitotic suppression of hyper-proliferating keratinocytes[17]. Also, MTX inhibits certain cytokines, such as GM-CSF, IL-4, IL-13, TNFα and IFNγ produced by PBMC[18]. Induction of apoptosis in activated T cells[19,20] and inhibition of cell adhesion molecules such as cutaneous lymphocyte antigen (CLA), vascular E-selectin, CD103[21] and ICAM-1[22] are the other alternative explanations of its beneficial effect.

In this study, it was hypothesized that cell permeable-MTX can penetrate through the skin and show efficacy and tolerability on IQM-induced psoriasis-like skin inflammation in mice. The study revealed that IQM-induced psoriasiform skin inflammation in mice was alleviated by topical application of cell permeable-MTX, in respect to clinical manifestation, histological examination and immunological findings.

II. MATERIALS AND METHODS

1. Animals and approval of animal experiment.

Female BALB/C mice at 7 to 8 weeks of age were purchased and kept in specific pathogen free (SPF) condition after approval of animal experiment accomplishment by the Department of Laboratory Animal Medicine at Yonsei University College of Medicine.

2. Induction of psoriasiform skin inflammation in mice and administration of therapeutics

In order to examine the therapeutic effect of PTD-MTX, imiquimod-induced mouse model of psoriasis was used that was originally developed by Van der Fits *et al*.,[14]. Briefly, mice backs were shaved and the remaining hairs were completely removed with depilatory cream (Veet, Reckitt Benckiser, Cedex, France). Twenty five mice, each group containing 5 mice, were divided into 5 groups as follows: 1) disease free control group, and disease-induced group with 2) no treatment, 3) systemic MTX treatment (intra-peritoneal injection of MTX [I.P-MTX]), 4) topical treatment with 0.1% (w/w) and 5) 1% PTD-MTX application. Two days after the removal of the hair, commercially available IQM cream (5%) (Aldara; 3M Pharmaceuticals, UK) at a daily dose of 80 mg, containing 4 mg of active compound, was applied on the back skins and the right ears for 6 consecutive days. All therapeutics were started on day 6 and continued for 3 days. The treatment of IP-MTX (35mg/kg) was given once on day 6 and the topical therapeutic PTD-MTX (200 μL) at concentration of 0.1% and 1% were given twice on day 6 and 8. Control mice were applied only with a vehicle ointment (Petrolatum).

3. Scoring severity of skin inflammation

To score the severity of skin inflammation, a slightly modified scoring system was adopted that was developed based on the clinical Psoriasis Area and Severity Index (PASI). From day one to day 9, erythema, scaling, and thickness were scored

independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The level of erythema was scored using a scoring table with red taints. The thickness of the skin was measured with a vernier caliper (Mitutoyo, Tokyo, Japan) on the ear and the flank side without inducing any injury and scored as follows: none 30-45 μm , slight 45-60 μm, moderate 60-75 μm, marked 75-90 μm, very marked 90 μm and over. The cumulative score (sum of erythema, scaling, and thickening scores) was served as a measure of the severity of inflammation (scale 0–12).

4. Hematoxylin and eosin staining for histological evaluation

The ear and the back skin samples were collected from each experimental group. Six μm skin sections were prepared after fixation in formaldehyde for 24 hours and embedded in paraffin. Hematoxylin (Fisher Scientific, Pittsburgh, PA, USA) and eosin (Shandon, Pittsburg, PA, USA) staining was performed for elucidation of epidermal and dermal alteration. Images were captured using a DP71 digital camera attached to an Olympus BX41 microscope (Olympus optical Co Ltd, Tokyo, Japan).

5. Mouse skin single cell preparation and flow cytometric analysis

Mouse back lesional skins were cut and immersed in 80% ethanol for 1 min and then washed twice with PBS. To make single cells suspension, skin samples were incubated overnight at 4°C in 2.4 U/ml dispase-II (Roche Diagnostics GmbH, Mannheim, Germany) containing media. Epidermal layers were separated from the dermis with tweezers and the enzyme activities were neutralized with 2 mM EDTA. The epidermal sheets were cut into small pieces, incubated in 0.25% trypsin EDTA for 20 min at 37°C and filtered through a cell strainer with 40 μm porous nylon mesh (BD Falcon, Franklin Lakes, NJ, USA). The dermal sheets were washed twice with 5 mM EDTA containing PBS to inactivate the remaining dispase-II enzyme and cut into narrow strands. Thereafter the samples were incubated in 0.1% collagenase D (Roche Diagnostics GmbH) containing RPMI 1640 supplemented with 10% FBS, 0.1% gentamicin and 1% 1M HEPES buffer (Sigma Aldrich, St Louis, MO, USA) at

37°C for 2.5 hours. After that the samples were transferred to 15 ml conical tubes and vortexed vigorously for 3 min. The dermal single cells were obtained by filtering through 70 μm porous nylon cell strainers (BD Bioscience, San Jose, CA, USA). Single cells, either epidermal or dermal, were washed with PBS containing 1% FBS and 0.1% sodium azide (FACS buffer) and stained with fluorescence conjugated antibodies listed in Table 2 or appropriate isotype controls for 30 min at 4°C in the dark. Then the cells were washed twice with FACS buffer to wash unbounded antibodies. Fluorescence-labeled cells were detected by flow cytometry, LRS II (BD Bioscience). The data were analyzed using Flow Jo software (Tree Star, Ashland, Jackson, OR, USA).

Intracellular staining was performed after incubation of one million single cells with leukocyte activation cocktail which contained protein transport inhibitor (2 μL) at 37°C for 5 hours in 12 well plate. The stimulated cells were first stained for surface antigens, and then fixed and permeabilized with intracellular (IC) fixation buffer (eBioscience, San Diego, CA, USA), followed by staining for intracellular molecules such as IL-17A and IL-22. The fluorescence-labeled cells were detected and analyzed as mentioned above.

6. Spleen single cell preparation and flow cytometric analysis

Harvested spleens were weighed and minced on a mesh with the plunger of a syringe. Erythrocytes were lysed with RBC lysis solution (Sigma) and 10^6 cells were stained with fluorescence-conjugated antibodies listed in Table 1. The positive cells are detected by flow cytometry BD LSR-II. The data were analyzed by Flow Jo software.

7. RNA extraction and quantitative PCR

Samples from back skin (6 mm) were immersed briefly in RNAlater solution (Ambion, Austin, TX, USA) and snap-frozen in liquid nitrogen to prevent degradation of RNA. The tissues were ground with mortar and pestle in liquid nitrogen and transferred into hard tissue homogenizing tubes (Precellys, Bertin

Corporation, MD, USA) containing 2.8 mm ceramic beads. The samples were homogenized for 30 second in lysis buffer from QIAGEN kit. Total RNA was isolated from the tissues using an RNEasy fibrous tissue mini kit (QIAGEN Inc, CA, USA) following the manufacturer's recommendations. cDNAs were synthesized using transcriptor high fidelity cDNA synthesis kit (Roche Diagnostics) from 2 μg of total RNA. Quantitative PCR (qPCR) amplifications were performed with template cDNA (100 times diluted), primers and SYBR green mixture. Specific target sequences corresponding to murine GAPDH, IL-23p19, IL-17A, IL-17F and IL-22 were amplified by ABI PRIZM 7500 PCR machine (Applied Biosystems, Carlsbad, CA, USA). The sequences of primers are listed in Table 2. The gene expression amount was calculated comparing to GAPDH, a house keeping gene, by applying the $2^{-\Delta\Delta Ct}$ method, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{GAPDH}}$ and $\Delta\Delta Ct = \nabla Ct_{\text{induced}} - \nabla Ct_{\text{reference}}$

8. Protein extraction from skin samples and detection of cytokine concentration by ELISA

Samples from back skin (6 mm) were obtained and snap-frozen in liquid nitrogen. The tissues were weighed and protein extraction solution NP-40, containing 50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM PMSF, (Elpis Biotech, Daejon, Korea) (30 mg/ml) were added. The samples were homogenized for 30 sec in 2.8 mm ceramic beads containing hard tissue homogenizing tubes (Precellys, Bertin Corporation) by tissue homogenizer (Precellys, Bertin Corporation) in the presence of extraction buffer. IL-17A, IL-23 and IL-22 cytokine (eBioscience) concentrations in the skin tissue were detected by ELISA method according to the manufacturer's recommendation.

9. Complete blood cell count (CBC) and blood chemistry analysis

Mice were anesthetized with the mixture of Zoletil 10 mg/kg (Virbac S.A., Carros Cedex, France) and Rompon 1.5 mg/kg (Bayer Healthcare, Toronto, Canada) diluted in saline (1:10). Blood was collected from the heart using 1 ml syringe. CBC was performed using automatic hematology analyzer (HEMA VET

950, Drew Scientific Inc, Dallas, TX, USA). Plasma was obtained by centrifuging the blood at 2500 RPM for 5 min. Plasma levels of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), blood urea nitrogen (BUN), and creatinine (CRE) were measured by FUJI 4000 dry chemistry analyzer (FUJIFILM Medical Systems, NJ, U.S.A).

10. Statistical analysis

All quantitative data are presented as the mean ± SEM for n = 5 mice/group, unless otherwise indicated. Significance between groups was assessed by using two-tailed unpaired Student's t test. When comparisons were multiple, one-way ANOVA with a Kruskal–Wallis's test was used. A p value less than 0.05 was considered significant. *p*-values were indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Statistical analysis was performed with Microsoft Exel 2007 and GraphPad Prism software (Informer Technologies, San Diego, CA, USA).

Table 1. The list of antibodies used in flow cytometric analysis

Name of antibody	Clone	Manufacture
Rat anti-mouse MHC II (I-A/I-E)-PE	M5/114.15.2	eBioscience
Rat anti-mouse F4/80-Alexa Flour 488	BM8	eBioscience
Hamster anti-mouse CD11c-APC	N418	eBioscience
Rat anti-mouse CD3 APC-Cy7	17A2	BD Pharmingen
Rat anti-mouse CD8a-FITC	Ly-2, Lyt-2	BD Pharmingen
Rat anti-mouse CD4-FITC	GK1.5	BD Pharmingen
Rat anti-mouse CD45R/B220	RA3-6B2	BioLegend
Rat anti-mouse Gr1-Alexa Flour 700	RB6-8C5	BioLegend
Hamster anti-mouse gamma delta TCR-PE	GL3	eBioscience
Rat anti-mouse CD207 (Langerin)-FITC	eBioRMUL.2	eBioscience
Rat anti-mouse IL-17A-APC-Cy7	TC11-18H10	BD Pharmingen
Rat anti-mouse IL-22-PerCP-eFlour 710	1H8PWSR	eBioscience
Rat anti-mouse IL-23p40-PerCP-Cy5.5	C17.8	eBioscience

Table 2. PCR primers used for quantification of cytokines

Cytokines	Forward	Reverse
IL-17A	5’-TTT TCA GCA AGG AAT GTG GA	5’-TTC ATT GTG GAG GGC AGA C
IL-17F	5’-CAA GAA ATC CTG GTC CTT CG	5’-GAG CAT CTT CTC CAA CCT GAA
IL-22	5’-TTT CCT GAC CAA ACT CAG CA	5’-CTG GAT GTT CTG GTC GTC AC
IL-23p19	5’-CAC CTC CCT ACT AGG ACT CAG C	5’-TGG GCA TCT GTT GGG TCT
GAPDH	5’-TCC ACT GGC GTC TTC AC	5’-GGC AGA GAT GAT GAC CCT TTT

III. RESULTS

1. Topical application of IQM generated psoriasiform skin inflammation in mice

Consecutive application of IQM for 6 days on the back skin of BALB/c mice induced skin inflammation showing redness, thickness and scale, which was similar to human psoriasis (Fig. 1A). A modified scoring system that is based on PASI used in human psoriasis was applied to evaluate the severity of skin inflammation. The erythema appeared one day later, after applying IQM, and it reached a peak after 4 days. The both thickness and scaling were observed after 3 days, and increased gradually until day 6 (Fig. 1B). Histopathological examination detected increased epidermal hyperproliferation and altered differentiation of keratinocytes in IQM-induced psoriasiform skin inflammation (Fig. 1C). In flow cytometry analysis, increased numbers of antigen presenting cells, lymphocytes and other leukocytes were detected in IQM-applied mice skin. Epidermal MHC-II^+ cells (1.8 fold), dermal MHC-II^+ cells (2 fold), $CD11c^+$ cells (3.3 fold) and $CD4^+$ cells (7 fold) were increased in IQM-applied mice skin compared to those of the normal mice skin (Fig. 1D). All of these changes were mimicking the human psoriatic skin lesion and, therefore the IQM-induced mouse model of psoriasis was used throughout of this study.

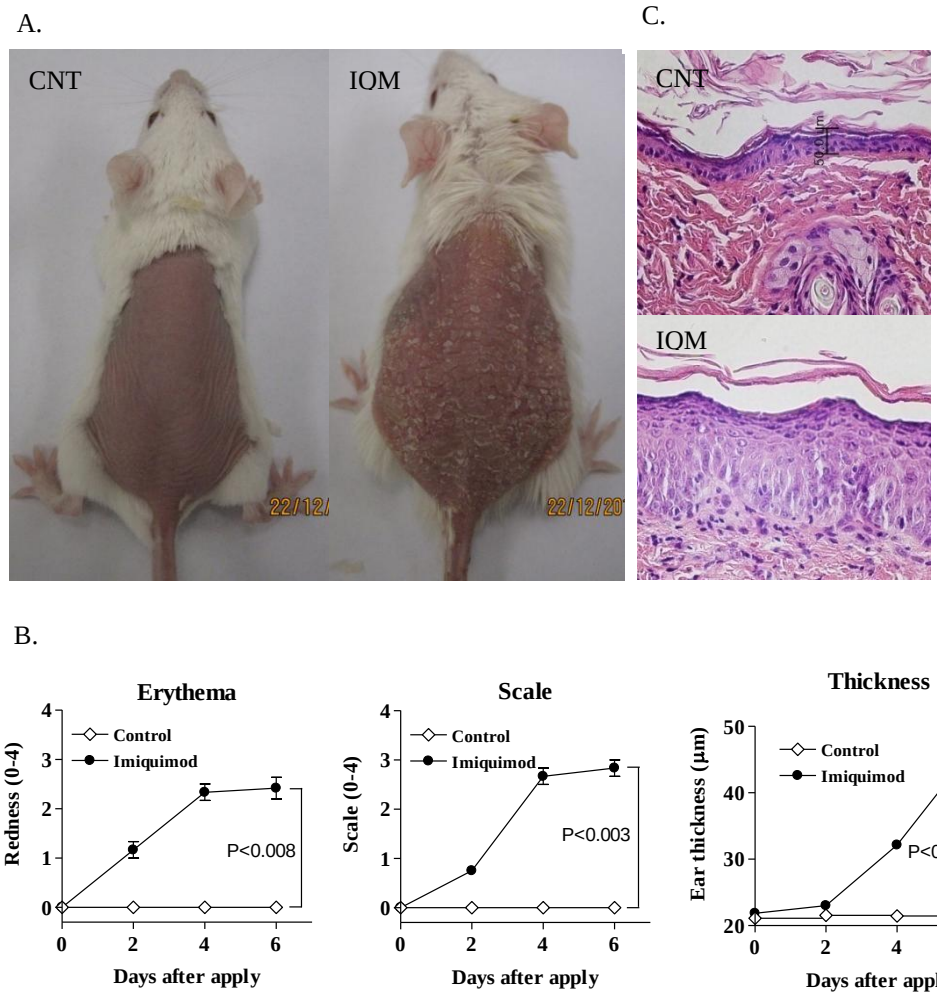


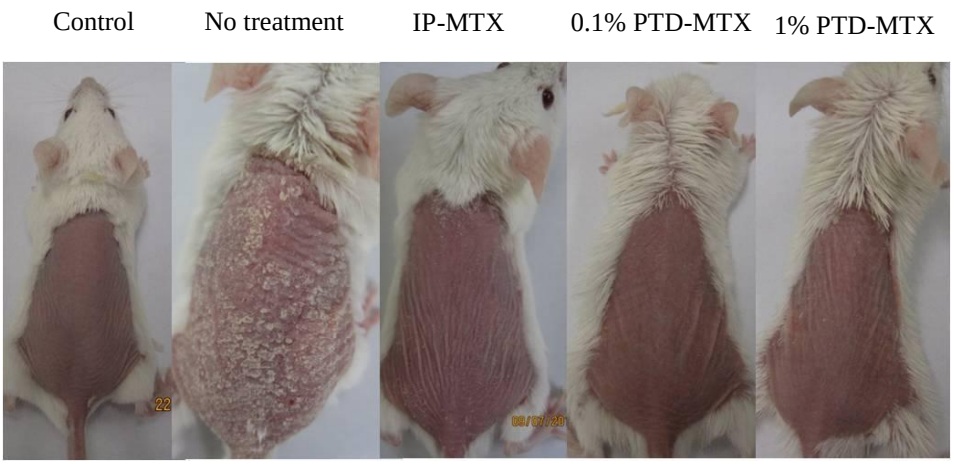
Figure 1. Imiquimod (IQM) induced psoriasis like skin inflammation in mice. (A) Pictures of IQM-applied BALB/c mice showing psoriasis-like skin changes including redness, thickness and scale after 6 days of application on the back (right side). But no changes were detected in the control mice (left side). (B) The level of severity was scored by psoriasis area severity index (PASI) system

and shown in X-Y plot. The connected closed circle indicates the redness, scale and thickness of IQM-applied mice, while connected open diamonds indicate that of control mice. (C) Figure of hematoxylin and eosin (H&E) staining showed epidermal hyperproliferation and altered differentiation of keratinocytes in IQM-applied mice (lower figure) but no obvious change was observed in the epidermis of control mice (upper figure). (D) In bar graph, epidermal and dermal cell numbers with (black) or without (light gray) IQM application are shown. The epidermal MHC-II$^+$ cells (2-fold), dermal MHC-II$^+$ cells (2-fold), CD11c$^+$ cells (3-fold), CD4$^+$ cells (3-fold) and γδ TCR$^+$ cells (1.8-fold) were increased by IQM-application. Each bar is an average of 5 independent samples. All of the increments were statistically significant (p<0.05). The significance between groups was determined by using two-tailed unpaired Student's t test.

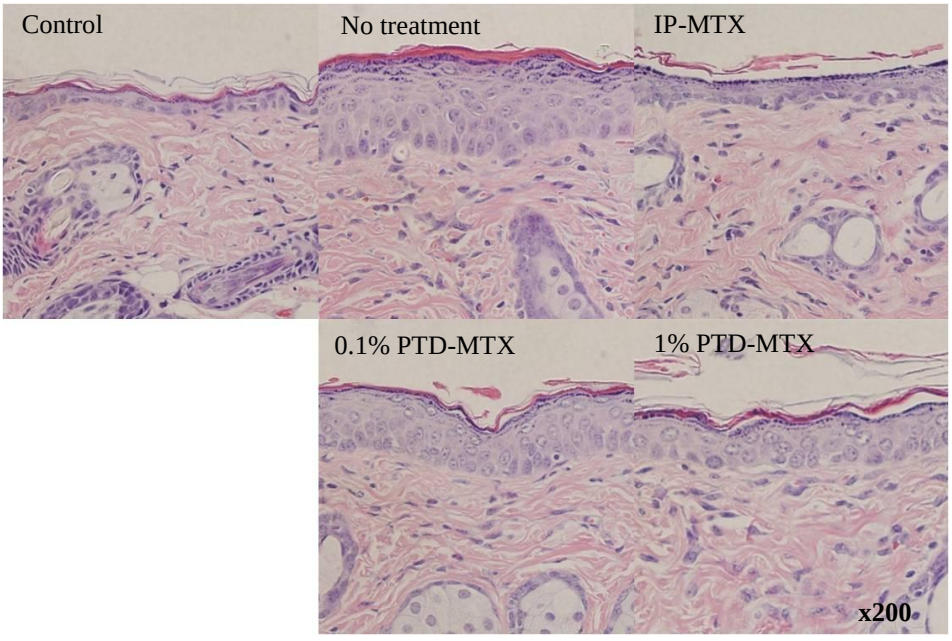
2. IQM-induced psoriasiform skin inflammation was ameliorated by systemic and topical MTX

After induction of psoriasiform skin inflammation, subsequent treatment with IP-MTX (systemic) and PTD-MTX (topical) were conducted for 3 days. Both treatments ameliorated the psoriasiform skin inflammation in macroscopic level (Fig. 2A). In histopathological evaluation, increased epidermal hyperproliferation of keratinocytes in IQM-applied mice was reduced completely by IP-MTX and almost completely reduced by 1% PTD-MTX within two days but mild reduction was observed in 0.1% PTD-MTX-applied mice (Fig. 2B). In severity examination, IP-MTX and topical 1% PTD-MTX reduced skin thickness, erythema within a day after administration. The cumulative PASI score of 1% PTD-MTX-applied mice was lowest (5.35±0.29) followed by IP- MTX (6.15±1.22), 0.1% PTD-MTX (7.05±0.57) and only IQM-applied mice (8.15±0.58) in ascending order (Fig. 2C).

A.


Control
No treatment
IP-MTX
0.1% PTD-MTX
1% PTD-MTX

B.


Control
No treatment
IP-MTX
0.1% PTD-MTX
1% PTD-MTX
x200

C.

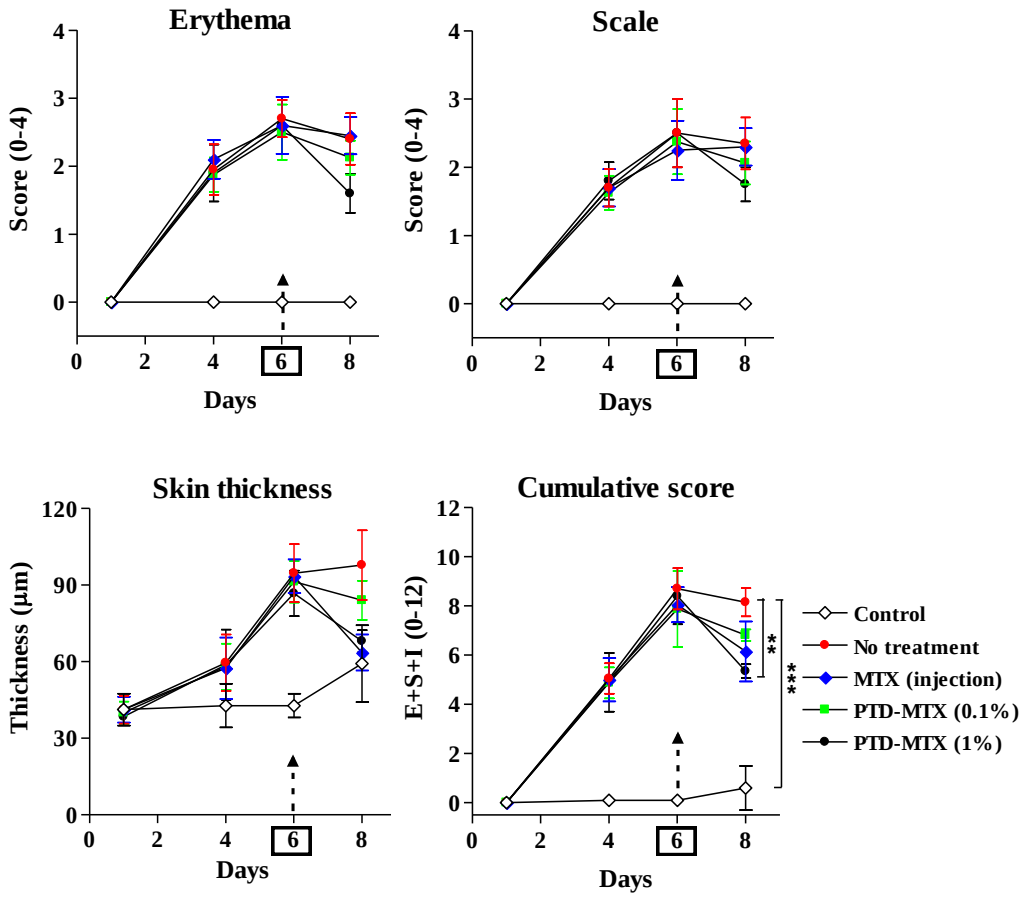


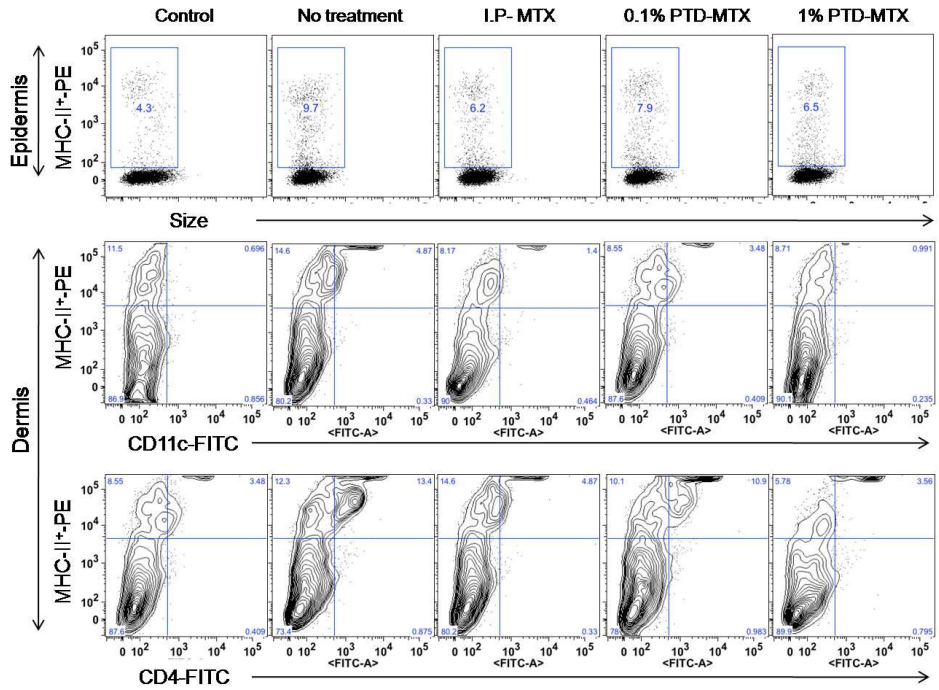
Figure 2. Topical application of PTD-MTX ameliorated IQM-induced psoriasiform skin inflammation (A) Macroscopic view of IQM-applied mice with or without treatment. IQM induced psoriasis-like skin inflammation showing redness, thickening and scale (no treatment) compared to control mice. Both intra-peritoneal injection of methotrexate (IP-MTX) (35 mg/kg) and topical application of protein transduction domain-conjugated (PTD)-MTX (0.1% and 1%) treatment alleviated the IQM-induced skin changes. (B) In histological examination, increased epidermal thickness in the IQM-applied mice were reduced completely

by IP-MTX and dose dependently reduced by PTD-MTX. (C) Severity of IQM-induced psoriasiform skin inflammation is represented by the PASI scoring system. IP-MTX and topical PTD-MTX treatment reduced IQM-induced redness, scale and thickness. The reductions were statistically significant ($p<0.05$) in 1% PTD-MTX group and IP-MTX group compared to no treatment group. The cumulative score was the lowest in 1% PTD-MTX group followed by IP-MTX group, 0.1% PTD-MTX group and no treatment group. The significance was assessed by one way ANOVA with Kruskal–Wallis's test. The photographs, shown here, are representative of 5 experimental subjects.

3. The number of infiltrating cells into skin in IQM-applied mice were normalized by systemic and topical MTX

Skin single cell suspension analysis by flow cytometric analysis revealed that MHC-II$^+$ cells in the epidermis were 4.66±1.0% in normal mice and 9.9±1.7% in IQM-applied mice. But this increment was reduced by IP-MTX (6.2±0.6%), 0.1% PTD-MTX (8.3±2.1%) and 1% PTD-MTX (6.8±0.6%). In the dermis of untreated mice, MHC-II$^+$ cells were 12.6±1.6% and increased to 24±6.3% by IQM application, but this increment was reduced to 12.8±2.9% by IP-MTX, 16.6±7.0% by 0.1% PTD-MTX and 11.4±2.9% by 1% PTD-MTX. A myeloid DC marker, CD11c$^+$ was also increased from 1.6±0.2% in normal skin to 5.4±0.4% in inflammatory skin by IQM-apply. This IQM-induced increase was normalized by IP-MTX (2.9±1.1%), and dose dependently reduced by 0.1% PTD-MTX (4.3±0.7%) and 1% PTD-MTX (1.9±0.7%). The CD4$^+$ T cells were increased 7-fold in IQM-applied mice compared to normal mice (12.7±1.4 vs 1.7±0.2%). But the increment was reduced partially by IP- MTX (3.1±0.9%) and completely by 1% PTD-MTX (1.8±0.7%) but not by 0.1% PTD-MTX (9.9±1.9%) (Fig. 3A and 3B).

A.



B.

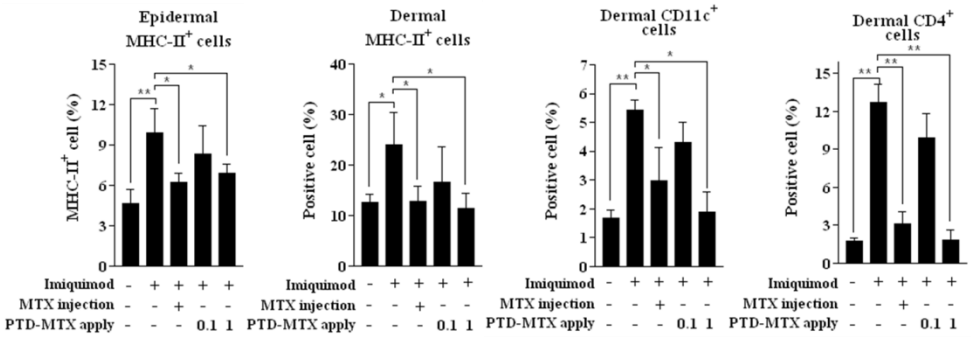


Figure 3. Both IP-MTX and PTD-MTX reduced the IQM-induced skin infiltrating immune cells in mice (A) Flow cytometric analysis of epidermal and dermal single cell suspension revealed that MHC-II$^+$ cells in the epidermis (upper row) were higher than in the control mice but this increment was reduced by IP-

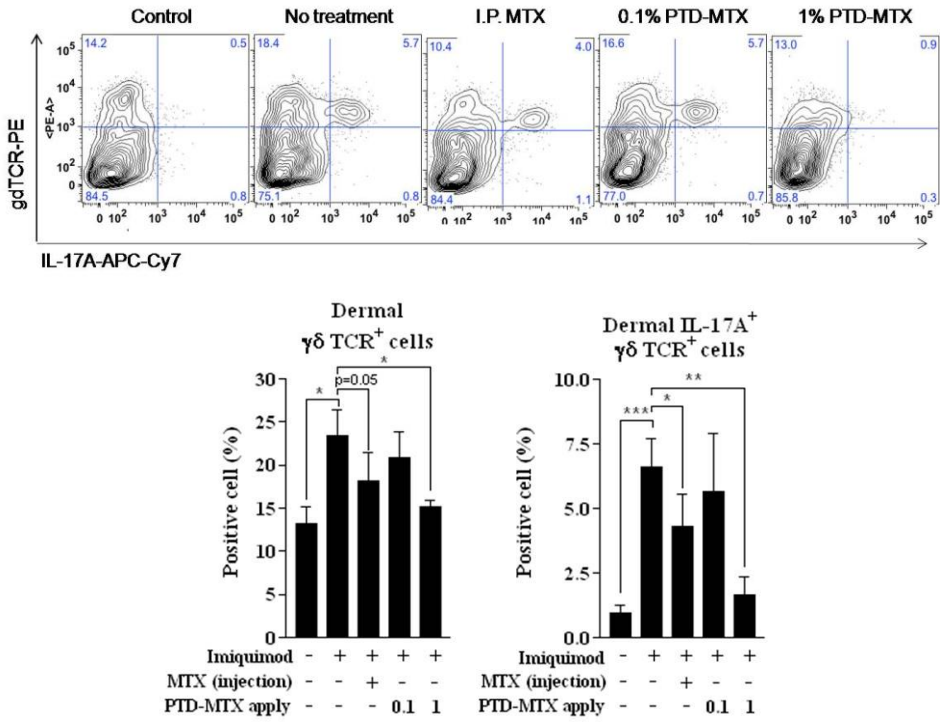
MTX and 1% PTD-MTX treatments for 3 days. Increased number of dermal MHC-II^+ cells (middle row), $CD11c^+$ (middle row) cells, and $CD4^+$ T cells (lower row) in IQM-applied psoriatic mouse skin were normalized by both IP-MTX and PTD-MTX. (B) The lower bar graphs represent the SEM of the 5 experimental samples.

4. IL-23/IL-17 axis contributing cytokines in the dermis of IQM-applied mice were downregulated by systemic and topical MTX

It has been demonstrated previously that IQM-induced psoriasiform skin inflammation is mediated with IL-23/IL-17 axis which is the main immunopathogenic factor for human psoriasis[10]. In addition, it has been known that γδ T cells are the major source of IL-17[42]. To examine whether systemic and topical MTX affect this axis, flow cytometric analysis of surface γδ T cell receptor and intracellular staining for intracellular IL-17A cytokine were performed. Gamma delta T cells comprised about 13.2±1.9% of dermal single cell suspension in normal mice but this proportion was increased to 23.4 ± 2.9% by application of IQM for 6 days. The IQM-induced increment of γδ T cells was reduced by systemic MTX (18.2 ±3.3%) and topical application of cell permeable MTX in a dose dependent manner; 1% PTD-MTX, 15.1±0.8% and 0.1% PTD-MTX, 20.8±2.9. IL-17A-producing γδ T cells barely existed in the dermal cell suspension in normal mice (0.9±0.3%) but those cells were increased by IQM application (6.6±1.1%). The increment of IL-17A-producing γδ T cells was partially reduced by systemic MTX (4.3±1.2%) but almost completely reduced by 1% PTD-MTX (1.6±0.7%) while 0.1% PTD-MTX barely affected to this increase (5.6±2.3%) (Fig. 4A). Protein levels of cytokines IL-17A and IL-22 in the dermal protein extracts were also examined. IL-17A was increased 2.2 fold and IL-22 was increased 2.4 fold in IQM-applied mice. This increment of IL-17A was reduced by 33% by IP-MTX, 24% by 0.1% PTD-MTX and 42% by 1% PTD-MTX. The increment of IL-22 was reduced by roughly 50% by IP-MTX, 40% by 0.1%-PTD-MTX and 45% by 1% PTD-MTX.

Next, mRNA levels isolated from the lesional skin of IQM-applied mice with or without treatments were examined. The level of mRNA encoding IL-23p19 was found to be increased 1.75 fold in the IQM-applied mice compared to that of the control mice but this increment in IQM-applied mice was decreased by systemic MTX, 0.1% PTD-MTX (1.5-fold) and 1% PTD-MTX groups (1.14-fold) (Fig. 4B)

A.



B.

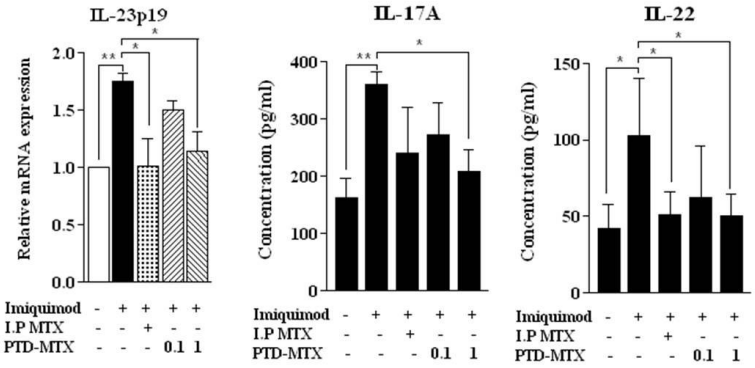


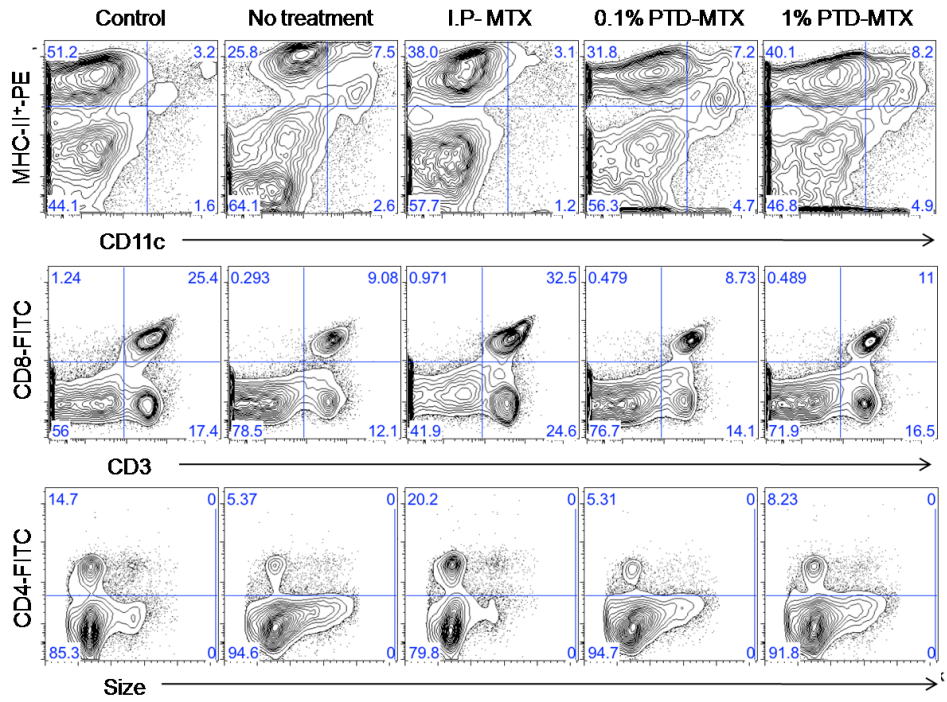
Figure 4. Increased level of IL-23, IL-17A and IL-22 cytokines in the dermis of IQM-applied mice were reduced by IP-MTX and PTD-MTX (A) Flow

cytometric analysis of dermal cell suspension showed that γδ T cells in IQM-applied mice were higher than that of the control mice. Among the γδ TCR^+ cells a proportion of cells were also IL-$17A^+$ in IQM-applied mice but not in the control mice. This population of cells was partially reduced by IP-MTX and 0.1% PTD-MTX but almost completely reduced by 1% PTD-MTX (upper row). In the bar graphs, an average of five experimental samples are shown (γδ TCR^+ in the right, γδ TCR^+ and IL-$17A^+$ cells in the left). (B) Protein levels of IL-17A and IL-22 in the skin protein extract, and mRNA level encoding IL-23p19 in skin tissue were increased by IQM-application. In the bar graph, IQM-induced increase of IL-17A and IL-22, and reduction of those cytokines by systemic MTX, 0.1% PTD-MTX and 1% PTD-MTX were shown.

5. Alterations of cellular composition in spleen by IQM were normalized by systemic MTX but not by topical MTX

IQM-application markedly altered splenic cell composition. Spleen cells; MHC-II$^+$ (1.3-fold), CD3$^+$ T cells (2-fold), CD4$^+$ T cells (2.1-fold), CD8$^+$ T cells (2.5-fold) and B cells (1.7-fold) were found to be decreased in the IQM-applied mice compared to that of the control mice. Inversely, spleen CD11c$^+$ cells were increased about 2-fold in IQM-applied mice compared to that of the control mice. These alterations in spleen were reversed only by IP-MTX but not by topical application of PTD-MTX, although CD45R/B220$^+$ cells which are not the main cell type in psoriasis were affected in 1% PTD-MTX applied mice. These results suggest that cell permeable MTX has no systemic immune inhibitory effect as the IP administration of MTX (Fig. 5A and 5B).

A.



B.

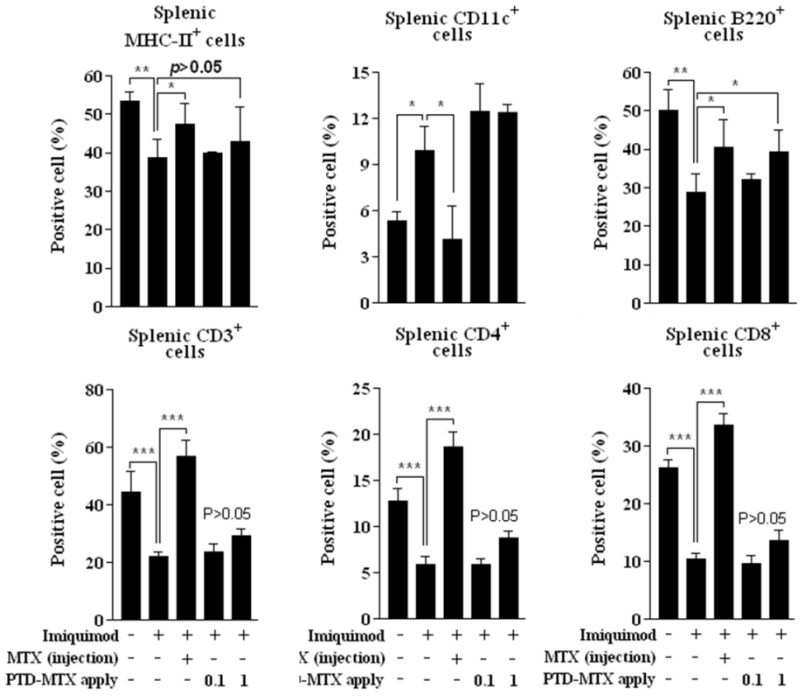


Figure 5. IQM-induced changes of spleen cell composition were normalized only by IP-MTX (A) One representative figure of flow cytometric analysis show that IQM-application decreased splenic cells, except $CD11c^+$, $MHC\text{-}II^+$, $CD3^+$, $CD4^+$, $CD8^+$ and $B220^+$ cells (second line). Most of these reductions, except $MHC\text{-}II^+$ cells and $B220^+$ cells, were partially reversed by IP-MTX but not by PTD-MTX, either 0.1% or 1% dose. (B) The bar graphs represent the average of the 5 experimental samples with their statistical significance.

6. Topical, cell permeable MTX, but not systemic MTX had no toxic effect on the liver, kidney and myeloid cells in IQM applied mice

Eight consecutive day application of IQM reduced the total white blood cell numbers. The proportion of neutrophils and monocytes were slightly increased but blood T cells were significantly decreased (Fig. 6A). IP injection of MTX as a systemic therapeutic agent decreased the total white blood cell count including myeloid lineage cells such as monocytes, neutrophils but not much affected to the proportion of lymphocytes. However, topical application of cell permeable-MTX had no toxic effect on the myeloid and lymphoid cells.

IQM application slightly elevated serum GOT but not GPT without statistical significance. IP-MTX on day 6 to 8 significantly increased serum GOT and GPT levels. However, PTD-MTX did not show any toxic effect on the liver in mice (Fig. 6B). Kidney toxicity was evaluated by measurement of creatinine (CRE) and blood urea nitrogen (BUN). BUN level was increased by IQM application, and IP-MTX significantly augmented the elevation of BUN. However, the level of serum CRE was no different among all groups (Fig. 6C).

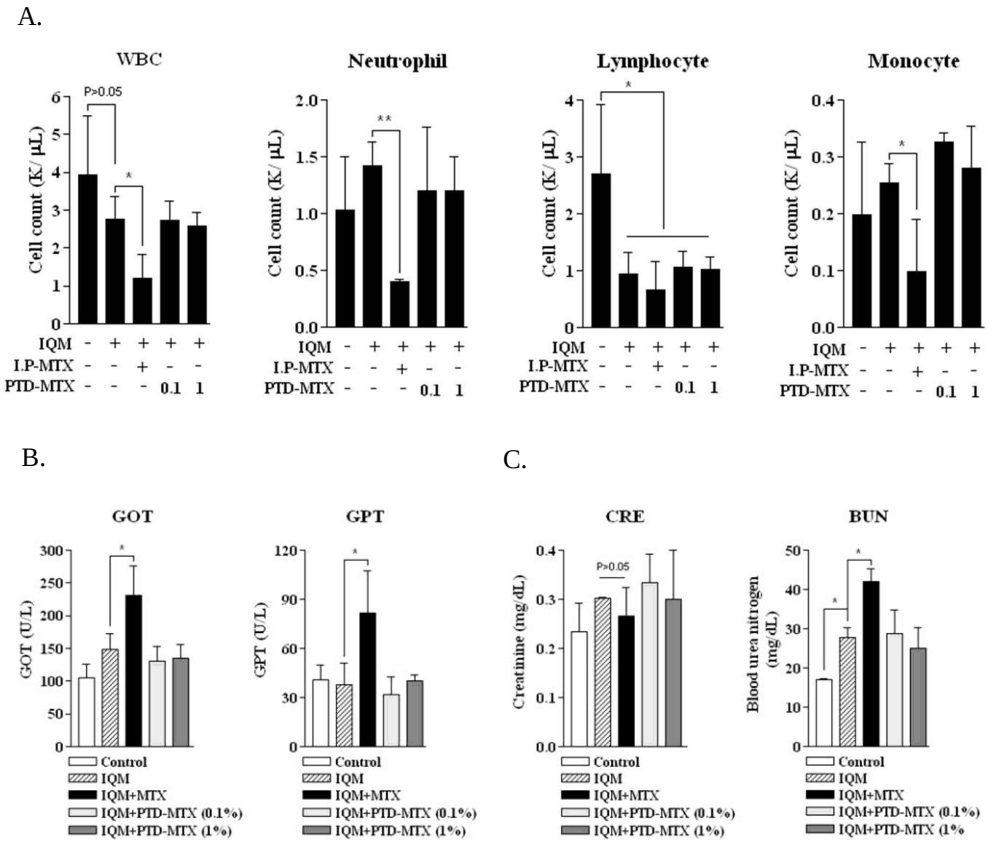


Figure 6. Topically applied, PTD-MTX had no toxic effects on liver, kidney and myeloid cells IQM was applied daily for 6 days and treatments were given for 3 days thereafter. After three days, mice were sacrificed to examine blood cell count, and plasma level of liver and kidney enzymes. (A) Six consecutive day application of IQM reduced total white cell and T cell counts but slightly increased neutrophil and monocyte counts. Treatment with IP-MTX reduced the number of total WBC, and also neutrophils and monocytes in IQM-applied mice. (B) IQM application for 6 days slightly elevated liver enzyme glutamate oxaloacetate transaminase (GOT) but not glutamate pyruvate transaminase (GPT), and kidney enzymes blood urea nitrogen (BUN) and creatinine (CRE) when measured after sacrifice. In the bar graph, it was shown that the levels of liver enzymes (GOT, GPT) and BUN were highly increased by IP MTX but not by PTD-MTX either 0.1% or 1% dose.

IV. DISCUSSION

This study was focused on the therapeutic and anti-inflammatory effect of cell permeable MTX in the mouse model of psoriasis. The results of this experiment indicate that topically administered cell permeable MTX was safe and it had favorable therapeutic efficacy when compared to the conventional systemic MTX. Previous efforts to administer MTX topically have been unsuccessful[1]. The lack of clinical effectiveness of topical MTX in the treatment for psoriasis is thought to be related to poor penetration across the skin barrier[2]. To solve this obstacle MTX was conjugated to cell permeable peptides to increase percutaneous delivery. This fusion form of MTX was detected not only in superficial but also in deeper tissues such as muscle and joints[23]. The cell permeable MTX in petrolatum base (0.1%, 1% w/w) showed remarkable clinical improvement, alleviation of histological alteration and suppression of immune cell infiltrate into epidermis and dermis in IQM-induced mouse model of psoriasis. IQM-induced increase of the epidermal MHC-II$^+$ cells, and the dermal MHC-II$^+$ cells, CD11c$^+$ myeloid DCs, CD4$^+$ and CD8$^+$ T cells were normalized with IP-MTX and topical cell permeable MTX. However, IQM-induced alterations of splenic cellular composition were reduced by IP-MTX but not by cell permeable topical MTX, indicating that topical MTX has no systemic immune inhibitory effect, unlike IP-MTX. Also topical, cell permeable MTX did not affect blood cell counts, plasma levels of liver enzymes and BUN levels in IQM-applied mice.

To minimize the systemic toxic effects of MTX several studies have been conducted to use it topically but most of those efforts have been unsuccessful. The main reason why previous efforts to deliver MTX topically failed to show significant clinical results seems to be related with inadequate penetration efficiency. To increase skin penetrating efficiency, various vehicles have been used such as MTX in vehicle N. This form inhibited epidermal DNA synthesis in hairless mice[17]. Although, topical use of 0.25% MTX in 12% sodium chloride and 12% carbamide cream increased serum and non-treated skin concentrations of MTX, very low excretion rate of MTX by urine and no clinical improvements were seen which lead

to a conclusion that the penetrated amount of MTX did not reach effective therapeutic concentration to induce clinical and histological improvements[24]. Another placebo controlled, double blind trial reported that complete and marked global improvement (erythema, scale and infiltration scores are multiplied by 5) was observed in 97.5% of the lesions on which 1% MTX in hydrophilic gel was applied twice daily for 8 weeks versus no improvement in the placebo group lesions[25]. This study suggested an evidence to use MTX topically, although most of the other studies showed unfavourable clinical effect. When the results of all the previous publications are taken together, although small amount of MTX could penetrate into skin, the lack of clinical effectiveness seem to be related with insufficient amount of drug in the dermis to show a therapeutic efficacy. Previously, it was confirmed that PTD-MTX very efficiently penetrated through skin and reached into the deep joint where it alleviated collagen induced arthritis (CIA) when applied percutaneously[23]. As the same formula of cell permeable MTX was used in this experiment the remarkable efficacy on the IQM-induced psoriasiform skin inflammation is thought to be due to the sufficient concentration of MTX that reached into the dermis.

Therapeutic action mechanism of MTX has not been fully elucidated yet, however, beneficial effect of MTX in psoriasis is supposed to be related with the direct inhibition of cell proliferation by blocking epidermal DNA synthesis[17]. Another mechanism of the therapeutic effect of MTX is an anti-inflammatory action. It is speculated that MTX increases extracellular adenosine level via inhibiting 5-aminoimidasole-4-carboxamide ribonucleotide (AICAR) transformylase[26]. The increased adenosine inhibits leukocyte accumulation in the inflammed site[27]. Moreover this anti-inflammatory effect is dependent on adenosine A_2 receptors[27, 28]. To examine whether MTX has an inhibitory effect on the immune cells and mediators involved in the pathogenesis of psoriasis, I performed several *in vitro* experiments. Interestingly, when keratinocytes, DCs, T cells and peripheral blood mononuclear cells (PBMC) from healthy donors were incubated with low to sublethal dose of MTX, at various time points, neither MTX itself nor cell permeable MTX exerted an inhibitory effect on their stimulation-induced molecules. For example, keratinocyte-produced CCL20, LPS-induced cytokine secretions from

DCs, CD3/CD28-induced proliferation of T cells, and chemotactic migration of PBMC toward keratinocyte conditioned media, were not affected by addition of MTX *in vitro* (data not shown). In some studies, it was reported that MTX inhibits some cytokines such as GM-CSF, IL-4, IL-13, TNFα and IFNγ produced by whole blood cells and PBMCs drawn from healthy subjects[18, 29] and also MTX inhibits intracellular IFNγ and IL-4 cytokines in activated $CD4^+$ T cells taken from patients with RA[30]. In contrast to these results, others could not detect any inhibitory effect on cytokine productions. IL-1 secretion from LPS-activated monocytes was not changed by MTX[29]. In line with this result, IL-1α, IL-1β and TNFα production from GM-CSF plus IFNγ-stimulated monocytes and macrophages[30] were not changed. However, an early study indicated that MTX inhibited some proinflammatory cytokine production by mononuclear cells taken from rheumatoid arthritis (RA) patients who responded well to MTX[31], but most of the other results were not consistently reproduced[32, 33]. Another explanation for the immune suppression of MTX is the induction of apoptosis in activated T cells which is supposed to be a mechanism of beneficial effect during psoriasis treatment[19]. This result was supported by another study which reported that MTX increased sensitivity to apoptosis in activated T cells via JNK pathway[20]. Inhibition of cell adhesion molecules such as cutaneous lymphocyte antigen (CLA), vascular E-selectin, CD103 and ICAM-1 is an alternative mechanism of MTX mediated immune regulation[21, 22]. Also, MTX treatment decreased CCR2 density on monocyte and $CD4^+$ T cells in RA[35]. In some recent reports, the therapeutic effect of MTX was attributed to decreased production of Th1 and Th17 cytokines and also possibly, at least in part, to increased production of Th2 cytokine or increased production of T reg cells in RA[36] but not in psoriasis.

In this study, epidermal $MHC\text{-}II^+$ cells (probably Langerhans cells) were found to be increased in IQM-applied psoriatic mice skin compared to control mice skin. This result is in line with the previous result[37] but differ in some aspects with other reports[38, 39]. It could be explained by the difference in early-onset and late-onset[40], and the difference in acute and chronic phase of the disease, or the difference between man and mice. Whatever the reason, this increase was reduced by systemic

MTX and topical MTX in a dose dependent manner. Dermal MHC-II$^+$, CD11c$^+$ and CD4$^+$ cells were significantly increased in IQM-applied mice skin which were in accordance with the previously reported data in human psoriasis[10, 11, 12]. These increased numbers of cells were reduced by both systemic and topical, cell permeable MTX in a dose dependent manner in our study. The results were consistent with the results in the human study in psoriasis, which showed that effective treatment of psoriasis with efalizumab and ethanercept[41] significantly reduces immune cell infiltrate and mediators. Cytokine IL-23 and Th17 cells are thought to be crucial in the pathogenesis of psoriasis. Recently, γδ T cells are highlighted as a major producer of IL-17A in a mouse model of psoriasis[12]. Those cells do not require TCR engagement and rapidly produce IL-17A, IL-17F in the early stage of inflammation. IL-23, together with IL-1 β, is an important cytokine in γδ T cell stimulation and expansion. To determine whether MTX affects these cells, γδ TCR$^+$ cells in the dermal cell suspension from IQM-applied mice with or without MTX treatment were examined by flow cytometry. Gamma delta TCR$^+$ cells increased in IQM-applied mice skin compared to the control mice skin. Among them some γδ T cells co-expressed intracellular IL-17A, but not IL-22, and those cells comprised about 7% in control group, 30% in no treatment group, 23% in IP-MTX treatment group, 27% in 0.1% PTD-MTX group and 10% in 1% PTD-MTX treatment group when it was examined at day 10. According to previous reports, γδ T cells comprise about 4% of human dermal leukocytes[11]. The frequency of γδ T cells in the dermis was significantly increased (around 40%) in patients with psoriasis compared to healthy controls[11]. Among them approximately 15% of γδ T cells in psoriatic lesions produced IL-17 upon IL-23 stimulation[12]. In agreement to previous reports the present study in mouse model of psoriasis also demonstrated that γδ T cells are important cell types in psoriasis. Moreover treatment with systemic MTX and topical MTX reduced the increased number of γδ T cells. To my best knowledge, this is the first study to report that both systemic and topical MTX reduce IL-17A producing γδ T cells in psoriasiform skin inflammation.

This study revealed that IQM-induced psoriasiform skin inflammation in mice was alleviated by topical application of cell permeable-MTX. The therapeutic

action mechanism was associated with the anti-inflammatory action of MTX. On top of the favorable therapeutic effect, the cell permeable MTX did not show any toxic effects in the mouse model of psoriasis.

V. CONCLUSION

The study revealed that IQM-induced psoriasiform skin inflammation in mice was alleviated by topically-applied cell permeable MTX in respect to clinical manifestation, histological features and immunological findings. In conclusion, topically-applied cell permeable MTX ameliorated IQM-induced psoriasiform skin inflammation without systemic immune suppressive or toxic effect.

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ABSTRACT (IN KOREAN)

이미퀴모드에 의해 유발된 건선양 피부염에서의 PTD-Methotrexate의 치료효과

<지도교수 이 민 걸>

연세대학교 대학원 의과학과

뱜바 다쉬룸베

건선은 전 세계적으로 1-3% 정도의 유병율을 보이는 흔한 염증성 피부 질환이다. 악화와 호전을 반복하는 만성적인 병의 경과로 건선 환자들의 삶의 질은 매우 저하되어 있다. 이러한 질환의 중요도로 인하여 현재 전세계적으로 건선의 새로운 치료법에 대한 연구가 활발히 이루어지고 있으며, 최근에는 많은 생물학적 제제가 개발되어 임상에 적용되고 있다. 그러나 이러한 새로운 약제들도 여전히 경제성과 안정성 면에서 기존의 고전적 약물을 충분히 대체하지는 못한다. 많은 고전적인 약물 중 Methotrexate (MTX)는 효과 및 경제성 면에서 건선 치료에 널리 이용되고 있다. 그러나 간독성을 포함한 여러 전신 부작용으로 인하여 장기적인 사용에 제한이 있으므로 국소도포 등의 새로운 약물전달체계의 개발이 필요하다. 이전 연구들에서 MTX의 국소도포를 이용한 시도는 있었으나 경피흡수의 제약으로 성공적이지 못했다. 따라서 본 연구에서 저자는 단백질전달펩타이드(PTD)를 이용한 국소 도포 MTX(PTD-MTX)의 건선에서의 치료효과를 관찰하였다. 본 연구에서는 BALB/c 쥐의 피부에 imiquimod (IQM)를 도포하여 유도한 건선피부병변 모델을 이용하여 실험을 진행하였다.

본 연구에서는 임상적, 조직학적 및 면역학적인 평가들을 시행하여

PTD-MTX에 치료효과를 검증했다. 면역학적인 분석방법으로 표피와 진피 및 비장의 단일세포부유액을 이용한 유세포분석을 시행하였고, 조직 내의 사이토카인의 단백 및 mRNA는 각각 ELISA과 real time PCR 기법으로 정량하였다. 먼저 PTD-MTX 도포가 IQM에 의해 유발된 건선양 피부변화를 임상적으로 호전시킴을 확인하였다. 임상적인 척도로 사용한 PASI 점수는 1% PTD-MTX를 처리한 군에서 가장 낮았고(5.35±0.29), 다음으로 복강내 MTX 주사한 군(6.15±1.22), 0.1% PTD-MTX를 처리한 군(7.05±0.57) 순으로 증가를 보였다. 면역학적 분석결과, 복강내 MTX를 주사한 군과 PTD-MTX를 처리한 군에서 표피의 MHC-II$^+$ 세포 , 진피의 MHC-II$^+$, CD11c$^+$, CD4$^+$ 및 IL-17A producing γδ TCR$^+$ 세포 등의 면역세포들이 감소되었다. 또한 IQM 도포 시 증가 되었던 IL-23, IL-17A 와 IL-22의 발현이 복강내 MTX를 주사한 군과 PTD-MTX를 처리한 군에서 감소됨을 확인하였다. 그러나 IQM에 의해 유도된 비장세포 조성의 변화는 복강내 MTX 주사에 의해서는 영향을 받았으나 PTD-MTX 도포는 비장세포 조성에 영향을 주지 않았다. 또한 PTD-MTX 도포군에서는 복강내 MTX 주사 군에서 발생한 간, 신장 및 골수 세포독성이 관찰되지 않았다.

이상의 연구결과를 토대로, PTD-MTX 국소도포법은 전신 면역억제나 장기독성 등의 부작용 없이 IQM에 의한 건선양 피부염의 치료에 효과적이어서, 안전하고 새로운 건선 치료 약물로 사용할 수 있을 것으로 생각한다.

핵심되는 말: 건선, 단백질전달펩타이드, 메토트렉세이트, 이미퀴모드