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Therapeutic Mode of Action of Methotrexate

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Objectives: Methotrexate (MTX) has been used in clinical practice for over a half-century and its action mechanism is believed to rely on the direct inhibition of DNA synthesis leading to suppression of cell proliferation. However, its anti-inflammatory action mechanism is not fully explained. In some autoimmune or overactive immune-related diseases such as psoriasis, it has been demonstrated that interleukin (IL)-23/IL-17/IL-22 pathway plays a key role in disease pathogenesis. In this study, we aimed to investigate the suppressive action of MTX on the IL-23/IL-17/IL-22 pathway in psoriasis. **Methods:** We made a model of psoriasis on mice using imiquimod (IMQ). The mice were divided into three groups: disease-free control group and disease-induced groups with no treatment and MTX-treatment. Clinical, histological and immunological parameters were evaluated among the groups. **Results:** Treatment with MTX decreased the psoriatic skin changes and the histological alterations induced by IMQ. MTX exerted its treatment effects via inhibition of the main players in the pathogenetic axis, the IL-23, IL-17A, F and IL-22, that were found to be increased in the diseased mice. Regulatory T cells expressing CTLA4 or GITR or PD1 molecules on their surface were not related to these decrements. **Conclusion:** The therapeutic action mechanism of MTX is related to the direct inhibition of the IL-23/IL-17/IL-22 pathway, but not the induction of inhibitory molecules or expansion of regulatory T cells.

Keywords: Methotrexate; Psoriasis; Interleukin-23; Interleukin-17; Regulatory T Cells

Introduction

Methotrexate (MTX) has been used for over a half-century, but its therapeutic action mechanism in autoimmune or overactive immune-related diseases has not been fully elucidated. The effects of MTX are speculated to be direct inhibition of DNA synthesis in actively proliferating cells [1], inhibition of certain cytokines produced by peripheral blood mononuclear cells [2], induction of apoptosis in activated T cells, and down-regulation of adhesion molecules on immune cells [3, 4].

The pathogenesis of autoimmune or overactive immune-related diseases such as inflammatory bowel disease, rheumatoid arthritis, and psoriasis is associated with IL-23 and IL-17 (also called IL-23/IL-17 axis), the former being mainly produced by myeloid dendritic cells and the latter being produced by the T helper 17 cell [5]. In human psoriasis, the IL-23/IL-17/IL-22 pathway is thought to be a key player in disease pathogenesis and it is also the main player for mouse psoriasiform skin inflammation [6, 7, 8].

On the other hand, it is also hypothesized that immune imbalance between regulator and activator can be one of the explanations of autoimmune or overactive immune diseases. It is implicated that $CD4^+$ and $Foxp3^+$ regulatory T cells (Treg) are responsible for this impairment. A study conducted by Sugiyama et al. reported that, however, the number of $CD4^+$ and $Foxp3^+$ cells in the peripheral blood of psoriatic patient is not different from that of in the healthy control, but the function of Tregs are impaired [9].

In this study, we aimed to determine if the therapeutic action of MTX is related to down-regulation of the IL-23/IL-17/IL-22 pathway, possibly via inducing some inhibitory molecules on Treg cells during autoimmune diseases such as psoriasis. Our study revealed that psoriasiform skin inflammation in response to imiquimod (IMQ) was decreased by the application of MTX via reduction of IL-23-producing I-A/I-E^+, $CD11c^+$, cells and IL-17A-, F, IL-22-producing $CD4^+$ and γδ T cells in the spleen and the draining lymph node. However, the therapeutic effect of MTX was not related to $Foxp3^+$ Tregs or other immune-suppressive molecules such as CTLA-4, PDL1, PDL2, and GITR expressed on Treg cells during psoriasiform skin inflammation in response to IMQ.

Materials and Methods

1. An animal model of IL-23/IL-17-related immune disease

To check the therapeutic effects of MTX, we induced psoriasiform skin inflammation in C57BL/6 or BALB/c mice, which was originally developed by Van der Fits et al. [10]. Briefly, the backs of all mice, either control or diseased groups, were shaved, and the remaining hairs were completely removed by a depilatory cream. The next day, mice were randomized into three groups with pre-coded cages, each containing four mice per group. The experimental design was as follows: (1) disease-free control group and disease-induced groups with (2) no treatment and (3) MTX-treatment. The mice in the control group were left untouched until day six. For mice in the diseased groups, IMQ cream (Aldara, 3M Pharmaceuticals, UK) was applied to the dorsal skin and the right ears for five days starting from day one. Treatment of MTX (25 mg/kg) was given intraperitoneally (IP) on day two and day four. All mice were sacrificed on day six.

2. Scoring severity of skin inflammation

To score the severity of skin inflammation, a modified scoring system that was developed based on the clinical psoriasis area severity index (PASI) score was adopted. The erythema, the scaling, and the 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 and scale was scored by careful observation by experienced researchers. The thickness of the skin on the IMQ-applied ear was measured with a vernier caliper (MitutoyoCorporation, Japan). The thickness of the ear skin was scored as follows: none = baseline (18-21 μm), slight = 0-25% increase from baseline, moderate = 25-50%, marked = 50-75%, very marked = 75-100%. The cumulative score (sum of erythema, scaling, and thickening scores) served as a measure of the severity of inflammation (scale 0–12).

3. Flow cytometric analysis of mouse spleen, draining lymph node and skin cells

On day 14 mice were sacrificed, and spleens, draining lymph nodes (dLNs) (from axillary, inguinal) and 2x3cm-sized back skins were harvested into ice cold phosphate-buffered saline. The spleens and the dLN were minced on a mesh with the plunger of a syringe in six well plates containing cold phosphate-buffered saline. Erythrocytes in spleens were lysed with RBC lysis solution (Sigma-Aldrich, USA). Single cells obtained from spleens and dLN were washed with phosphate-buffered saline containing 1% fetal bovine serum and 0.1% sodium azide (FACS buffer) and stained with the fluorescence-conjugated antibodies. Intracellular staining was performed as described in intracellular fixation buffer sheet (eBioscience, CA, USA), after incubation with leukocyte activation cocktail for four hours (BD Pharmingen, CA, USA). Data were analyzed using Flow Jo software (Tree Star, OR, USA). In some experiments, dermal single-cell suspensions were prepared as follows: the epidermal

sheets were discarded and the dermal sheets were incubated in 0.1% collagenase D (Roche Diagnostics GmbH, USA) in RPMI supplemented with 10% fetal bovine serum, 0.1% gentamicin and 1% 1 M HEPES buffer at 37°C for 2.5 hours. Then the samples were transferred to 15 mL conical tubes and vortexed vigorously for 3 minutes. Single dermal cells were obtained by filtering through nylon cell strainers with 40-μm pore size.

4. Statistical analysis

All quantitative data are presented as mean ±SD for the four mice in each group, unless otherwise indicated. Statistical significance was assessed by using two-tailed unpaired Student's t-test. For multiple comparisons, one-way ANOVA with Tukey Honest Significant Difference (HSD) test was used. A p-value <0.05 was considered to be significant.

Results

1. Effect of MTX on psoriasiform skin changes

It has been well documented that to apply a toll-like receptor 7 agonist IMQ on the skin is one of the easiest methods for inducing psoriasiform skin in a mouse. In this experiment, we adopted an IMQ-induced mouse model of psoriasis to demonstrate our hypothesis. Upon clinical evaluation, psoriasiform skin changes, including erythema, scale and thickness in response to IMQ were decreased by IP-MTX (Figure 1.a). Upon histopathological evaluation, the increased epidermal thickness in IMQ-applied mice was reduced by MTX (Figure 1.b). In the severity scoring, the cumulative PASI score was 8.65 ±0.58 in the diseased without treatment group (hereafter diseased group), 2.75 ±1.22 in diseased with MTX treatment group (hereafter MTX group) and 0 in the disease-free group (hereafter control group). Spleen

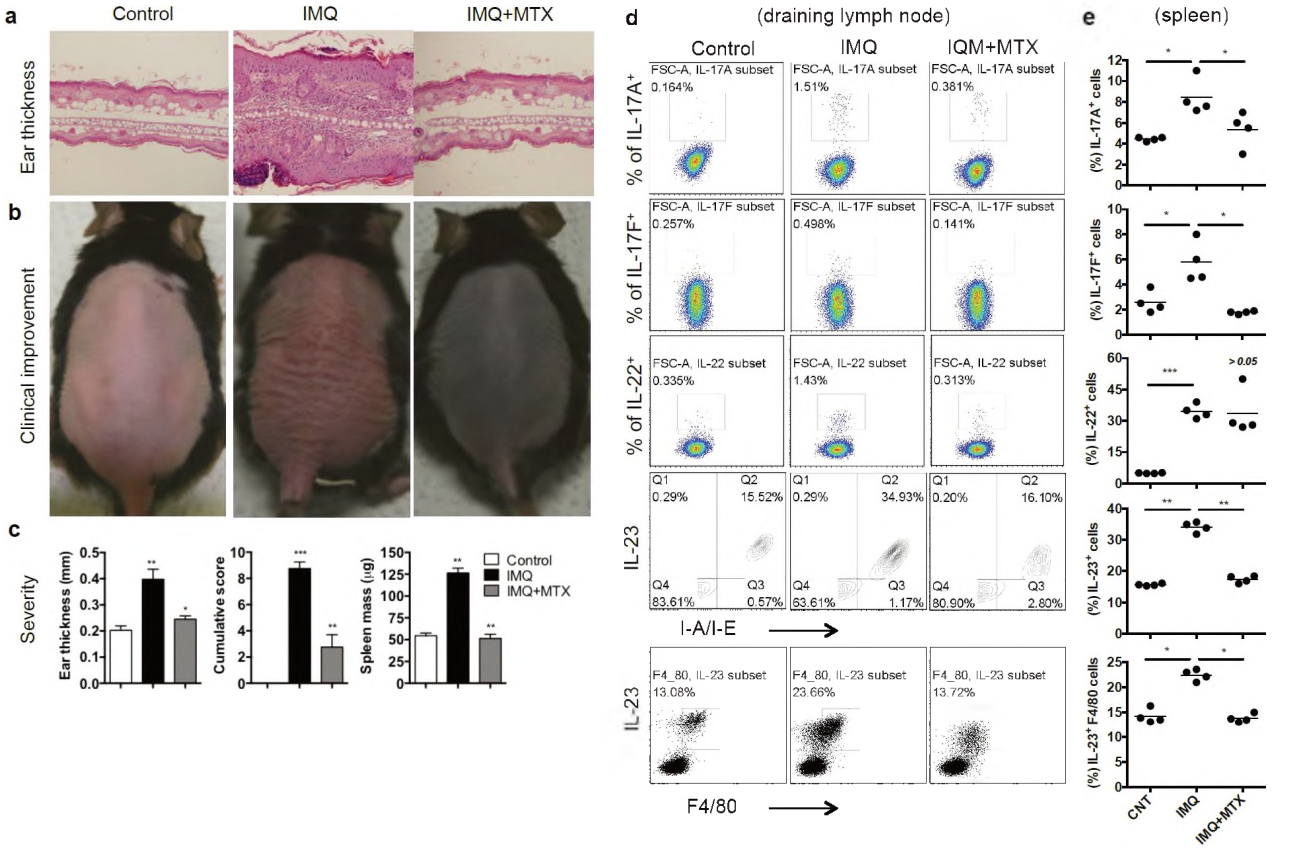

Figure 1. MTX reduced IMQ-induced psoriasiform skin changes via inhibiting IL-23/IL-17/IL-22 pathway (a) Cross section of ear tissue after application of IMQ with or without MTX treatment. (b) Psoriasiform skin change, showing redness, thickness and scale compared to the control mice. IP injection of MTX alleviated these skin changes. (c) Increased epidermal thickness in the IMQ-applied mice was reduced to baseline by IP-MTX. The severity of psoriasiform skin inflammation represented by cumulative PASI score was reduced by IP-MTX. Increased spleen mass in the IMQ-applied mice was reduced to baseline by IP-MTX. (d) IL-17A, F, IL-22, IL-23-producing cells were induced by topical application of IMQ in the draining lymph node and (e) the spleen. These increments were reduced by IP-MTX. The significance was assessed one-way ANOVA with Tukey's HSD test. The photographs shown here are representative of four experimental subjects. The p-values are indicated as follows: *p <0.05, **p <0.01, ***p <0.001.

Table 1. Percent positive cells in the dLN and spleen showing that MTX normalized the compositional alteration induced by IMQ

Group		Positive cells (%)							
		dLN				Spleen			
		CD3+	CD4+	CD8+	γδ TCR+	CD3+	CD4+	CD8+	γδ TCR+
Control mice		[a]72.7	70.6	24.3	0.82	50.0	18.7	7.7	0.89
		81.6	68.7	27.2	0.74	42.3	20.2	8.6	1.36
		73.3	58.8	37.1	0.69	44.6	18.6	8.0	0.85
		76.5	63.8	27.8	0.80	44.1	18.6	8.6	0.78
	Mean	76.0	65.4	29.1	0.76	45.0	19.0	8.2	0.97
	SD	4.07	5.29	5.55	0.06	3.40	0.78	0.45	0.26
IMQ-treated mice		79.8	51.2	43.2	2.76	30.1	10.5	3.8	1.68
		65.1	48.9	42.9	3.73	26.3	12.2	4.2	1.33
		72.4	52.8	41.7	3.54	31.7	11.5	4.2	1.33
		70.4	50.8	42.6	2.87	28.0	10.4	3.5	1.70
	Mean	72.0	50.9	42.6	3.22	29.0	11.1	3.9	1.51
	SD	6.09	1.60	0.65	0.48	2.58	0.86	0.34	0.21
IMQ+MTX-treated mice		76.5	61	32.6	2.19	45.0	16.2	6.6	1.55
		69.5	54.3	39.8	2.36	47.5	15.2	5.3	1.11
		76.6	54.7	39.8	1.90	42.3	17.4	8.4	1.70
		73.2	54.6	37.4	1.87	40.2	17.6	7.9	0.50
	Mean	74.0	56.1	37.5	2.08	43.0	16.6	7.0	1.21
	SD	3.36	3.24	3.39	0.24	3.50	1.12	1.39	0.54
		[b]ns	p <0.002	p <0.05	p <0.0001	ns	p <0.0001	p <0.001	ns
[c]CNT vs. IMQ		-	[f]**	**	***	-	***	**	-
[d]CNT vs. IMQ+MTX		-	*	*	**	-	*	ns	-
[e]IMQ vs. IMQ+MTX		-	ns	ns	**	-	***	**	-

[a]Percent positive cells in the dLN and the spleen [b]One-way ANOVA result [c]Difference between control group and IMQ-applied group by multiple comparison, Tukey's HSD [d]Difference between control group and IMQ-applied treatment group with MTX by multiple comparison, Tukey's HSD [e]Difference between IMQ-applied group and IMQ-applied treatment group with MTX by multiple comparison, Tukey's HSD [f]p-values were indicated as follows: *p <0.05, **p <0.01, ***p <0.001

Table 2. Proportion of IL-17A, IL-17F and IL-22-producing cells in dNL from IMQ-treated mice.

Cell type	Cells in dLN (%)					
	Mouse number				Average	SD
	1	2	3	4		
IL-17A positive cells	[a]0.93	1.49	0.8	0.2	0.9	0.5
γδ TCR positive	[b]87.0	80.7	89.0	90.0	86.7	4.2
Double negative	9.7	14.9	8.6	8.0	10.3	3.1
CD4 positive	2.3	2.9	1.5	0.8	1.9	0.9
IL-17F positive cells	3.4	4.3	2.8	2.5	3.3	0.8
γδ TCR positive	31.8	57.7	45	48.0	45.6	10.7
Double negative	30.7	19.7	25.7	24.7	25.2	4.5
CD4 positive	34.1	18.3	29.1	23.3	26.2	6.9
IL-22positive cells	1.5	0.9	1.0	1.3	1.2	0.3
γδ TCR positive	92	88	91.0	89.0	90.0	1.8
Double negative	6.8	5.8	5.8	6.8	6.3	0.6
CD4 positive	3	0.9	2	2.8	2.2	1.0

[a]Percentage of positive cells in dLN [b]Proportion of positive cells among the above population

mass was increased by 2.5-fold in the diseased group compared to the control group. This increment was normalized by the IP-MTX (Figure 1.c). To further confirm the therapeutic effect of MTX, single cell suspensions from the psoriasiform mice spleens and dLNs were analyzed by flow cytometry after MTX treatment or no treatment. The IMQ-applied mice showed a remarkable accumulation of IL-23-producing I-A/I-E$^+$, F4/80$^+$ cells and IL-17A, IL-17F, IL-22-producing γδ T cells. In the MTX group, these accumulated cytokine-producing cells, except IL-22, were reversed (Figure 1.d, e).

These results demonstrate that over-activated IL-23/IL-17/IL-22 pathway-related cells and soluble molecules are completely blocked by MTX. Thus, it was shown that MTX significantly decreased psoriasiform changes in skin that were related to inhibition of IL-23/IL-17/IL-22 pathway in mice. This result may also be applicable to other IL-23/IL-17 pathway-related immune diseases or be valuable for studying such diseases to use MTX as a positive control.

2. Mechanism of therapeutic efficacy of MTX

The therapeutic efficacy of MTX is mediated to block the distribution of CCR6-expressing γδ T cells induced by IMQ. Moreover the IL-23/IL-17/IL-22 pathway-contributing molecule CCR6, which is attracted by keratinocyte-derived CCL20, participates as another key player in the pathogenesis of psoriasis. CCR6 knockout mice showed no psoriasis-like alteration in response to IL-23 injection into the skin, but the alteration was observed in wild-type mice [11]. In some experiments, there were many CD3$^+$, CD4$^+$, CD8$^+$, γδ T cells and Foxp3+ cells that infiltrated into the dermis in response to IMQ when compared to that of control mice (data not shown). This phenomenon was mirrored in spleen samples except for γδ T cells and this composition alteration was not dramatic in dLN samples in the same group (Figure 2.a). The γδ T cells were found to be increased by 2-fold in the dermis, 1.5-fold in the spleen and 4-fold in the dLN in psoriasiform mice induced by IMQ (Table 1).

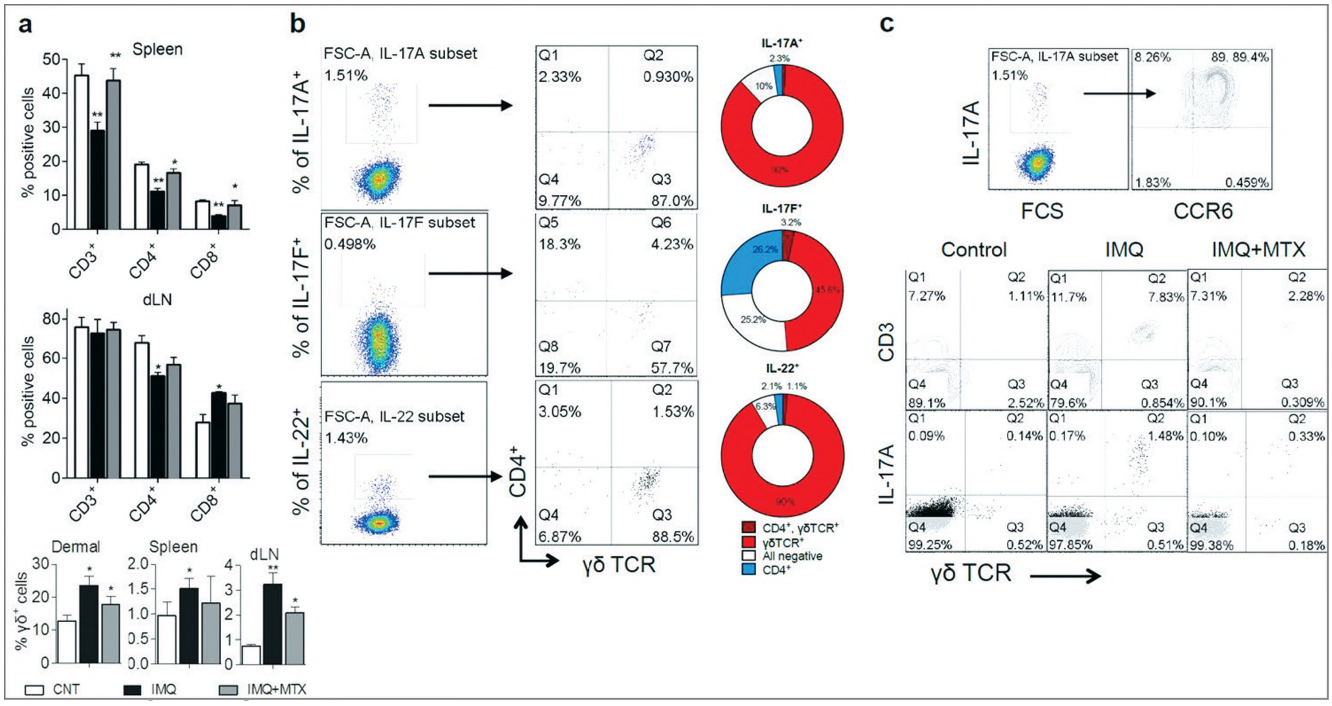

Figure 2. MTX blocked the distribution of CCR6-expressing γδ T cells induced by IMQ in psoriasiform mouse skin, spleen and dNL. (a) Flow cytometric analysis of single cell suspensions from skin, spleen, and dNL revealed that there were significant reduction of CD3$^+$, CD4$^+$ and CD8$^+$ cells in the spleen (upper panel) and a slight reduction of CD4$^+$ cells and a slight increment of CD8$^+$ cells in dLN (middle panel) in response to IMQ. These changes were dramatically reversed by MTX treatment except dLN cells. The γδ T cells were increased in all three organs in response to IMQ (lower panel). This increased distribution was blocked by MTX treatment. The bar graphs represent the SD of four experimental samples. The significance between groups was determined by using the two-tailed unpaired Student's t-test. The p-values are indicated as follows: *p <0.05, **p <0.01. (b) Upon FACS analysis over 90% of IL-17A and IL-22-producing cells in the dLN from IMQ-treated mice were γδ T cells. But about half of the IL-17F-producing cells in the same mice were γδ Tcells. (c) Moreover, IL-17A-producing cells were all CCR6 positive in dLN from IMQ-applied mice (upper panel) and those cells were reduced by MTX (lower panel).

We analyzed in detail wondering whether these cells are CD4+ or γδ T cells because, recently, it was shown that γδ T cells are a major source of IL-17 in a mouse model of psoriasis [6, 8]. In our study, when we gated the IL-17A+ and IL-22+ population, almost exactly the same number of the cells (90 ±4%) expressed γδ T cell receptor (TCR) molecules but no clear CD4+ population was detected. Among IL-17F-producing cells, over half were not γδ T cells (Figure 2.b). Our study also reproduced that γδ T cells were the main cell types that produce IL-17A and IL-22 cytokines but not absolutely IL-17F (Figure 2.b). Possibly, IL-17A and IL-17F-producing cells are not the same cells because there was another IL-17F-producing population that was neither CD4+ nor γδ T cells (Table 2).

Despite the results described above, the CCR6 chemokine receptor is highly expressed on the IL-17A, IL-17F, IL-22-producing cells including CD4+ and γδ T cells. Cells bearing this receptor were almost completely inhibited by MTX (Figure 2.c).

From these results it can be speculated that CCR6, because almost all γδ T cells express this molecule, may be responsible for the migration of IL-17A, F and IL-22-producing cells into the dermis and the dLN because the epidermis and lymphatic endothelial cells secrete the CCR6 ligand and CCL20 molecules. The beneficial effect of MTX is also related to the inhibition of the cells contributed in the CCL20/CCR6 pathway.

3. Mediation of MTX treatment effect

It is implied that the over-activation of immune cells might be related to functional disturbance of Tregs in psoriasis. To test whether MTX affects the regulatory arm of the immune system, we stained spleen and dLN cells with Foxp3 and various inhibitory molecules expressed on the Tregs. It was expected that the inhibitory molecules such as CD103, CTLA4, GITR and PD1 would be expressed less in the active stage of the disease compared to the MTX-induced healing stage. There was a parallel change of Foxp3+ cells together with an increase of IL-17A, F and IL-22-producing cells (Figure 3.a) in the spleens and the dLNs. We also examined the CD103, CTLA4, GITR and PD1 molecules, expecting to find functional aberration indicative

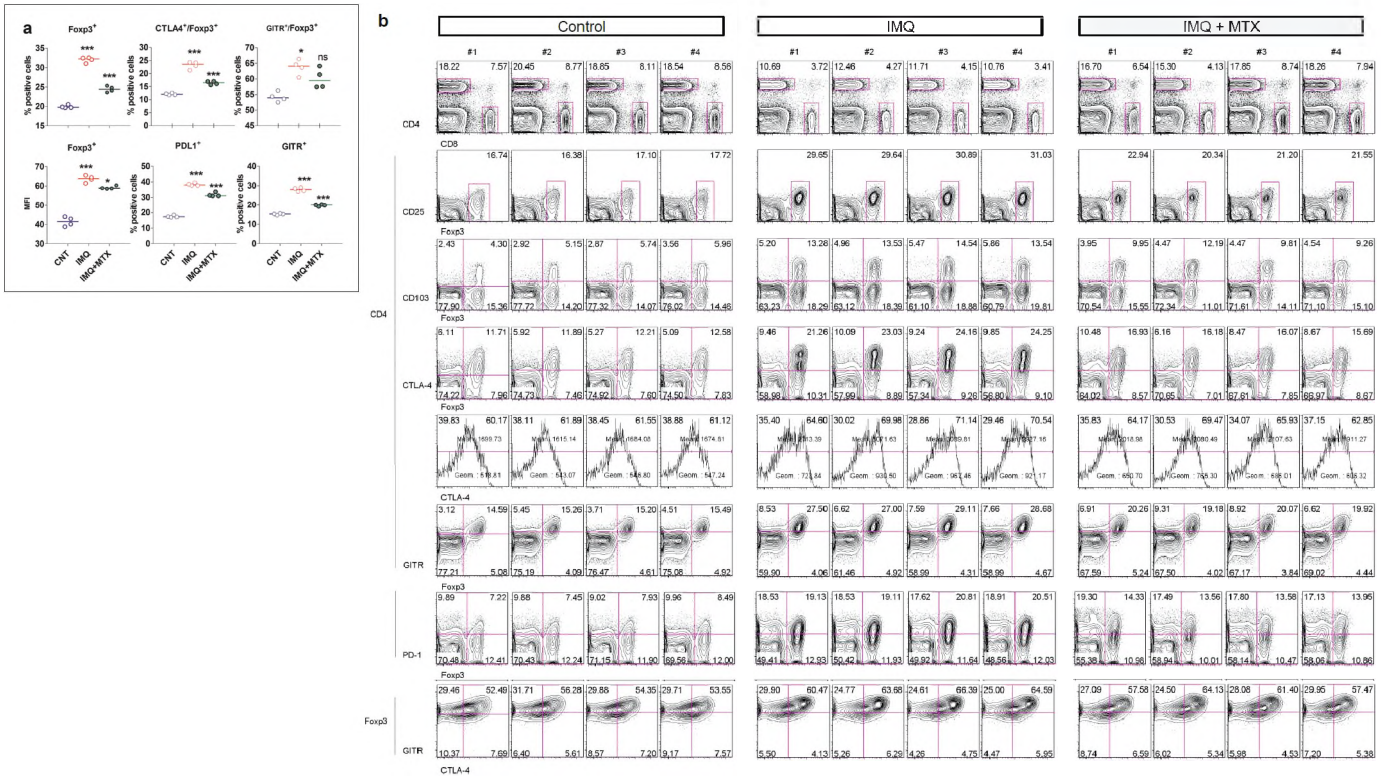

Figure 3. Therapeutic effect of MTX was not related to Foxp3. (a) Foxp3+ cells along with their inhibitory molecules were increased dramatically in response to IMQ. These increments were decreased by MTX significantly. The mean fluorescence intensity of Foxp3 was decreased less compared to the number of Foxp3+ cells. The p-values are indicated as follows: *p <0.05, **p <0.01, ***p <0.001. (b) One representative experiment of flow cytometric analysis from mouse spleen shows that IMQ application increased Foxp3-expressing cells and related molecules. All of these IMQ-induced changes were normalized by MTX.

changes on the Tregs. However, we could not find any trace to prove our hypothesis on mice in the control group, diseased group and MTX group (Fig. 3b).

It can be concluded that Treg cells may not be responsible for the disease resolution in psoriasiform changes induced by IMQ in the mouse and the treatment effect of MTX is not directly mediated by induction of Treg cells.

Discussion

This study examined the therapeutic action mechanism of MTX using a mouse model of psoriasis. Recent advances in the understanding of disease pathogenesis and the ease of psoriasis induction helped us to test the therapeutic mode of action of MTX. The pathogenesis of psoriasis is explained by over-activation of IL-23, IL-17A, IL-17F, IL-22-producing cells. Since these are the main players of the disease, it referred us to the IL-23/IL-17 pathway or the IL-23/IL-17 axis [5]. Another important player of psoriasis is CCR6/CCL20 expressing/secreting cells and pathway. This pathway is more prominent in a mouse model of psoriasis induced by IMQ [7]. In this study, the therapeutic mode of action of MTX was investigated by addressing how MTX affects the above axis.

The action mechanism of MTX in over-active or autoimmune diseases has not been fully elucidated. Its action mechanism has been explained by an anti-proliferative and anti-inflammatory effect on actively proliferating or over-activated immune cells. The anti-inflammatory effect of MTX has been shown to increase extracellular adenosine level [12]. The increased adenosine inhibits leukocyte accumulation into the inflamed site and this effect was dependent on adenosine A_2 receptors [13, 14].

In our study, we examined an inhibitory effect of MTX to the main player of psoriasis, the IL-23/IL-17/IL-22 pathway. Upon flow cytometric evaluation, single cell suspensions from the psoriasiform mouse spleens and dNLs contained numerous IL-23/IL-17/IL-22-producing cells. These cells were dramatically diminished by IP-MTX. In IMQ-induced psoriasiform skin inflammation, the main producer of the IL-23 was macrophages but the main producer of IL-17A, IL-17F, and IL22 cytokines were γδ T cells. In addition to the IL-23/IL-17/IL-22 pathway, CCR6, which is attracted by keratinocyte or lymphatic endothelial cell-derived CCL20, participated as another key player in the pathogenesis of autoimmune diseases like psoriasis. CCR6 knockout mice showed no psoriasis-like alteration in response to IL-23 injection into the skin but the alteration [11]. In our study, this chemokine receptor is highly expressed in the IL-17A, IL-17F, IL-22-producing γδ T cells in the skin, the spleen and the dLN in diseased mice. It is thought that this increment is due to the high secretion of CCL20 molecules from the epidermis, lymphatic endothelial cells [15], and stromal cells that attract CCR6 bearing γδ T cells in psoriasiform mice. Moreover, the increased distribution of CCR6-expressing cells was blocked by the treatment of MTX.

Lastly, Treg cells together with their inhibitory molecules were increased along with pathogenic cells in the IMQ-induced psoriasiform skin of mice. Treatment with MTX decreased this increment. This result indicates that Treg cells are not the main cell type responsible for disease resolution and the inhibitory molecules expressed on Treg cells were not related to disease healing stage as well. Rather, the therapeutic efficacy of MTX is associated with inhibition of IL-23/IL-17/IL-22 and CCL20/CCR6 pathways.

Our study revealed that MTX significantly inhibits the IL-23/IL-17/IL-22 pathway and distribution of CCR6 expressing γδ T cells in a mouse model of psoriasis. This experimental result is also applicable to other diseases whose pathogenesis is explained to be similar to psoriasis. However, our study clearly showed that MTX exerts its effect via inhibiting some immune cells and mediators in autoimmune or over-active immune diseases. Further study of its action on intracellular signaling molecules remains to be addressed. On the other hand, in the era of targeted therapy, older treatment choice for psoriasis, such as MTX is still a valuable option as it has similar beneficial effects like biologics.

Conflict of Interest

Authors state no conflict of interest.

Acknowledgments

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