一．中英文摘要

皮膚 T 細胞淋巴瘤屬於記憶型 T 淋巴球細胞所形成的腫瘤，主要影響的部位為人體的皮膚。雖然皮膚 T 細胞淋巴瘤在臨床上有許多不同的分型， 然而大部份的皮膚 T 細胞淋巴瘤都有一共同之特色，就是移行至皮膚的能力。皮膚 T 細胞淋巴瘤移行至皮膚的調控機轉大部分仍不明朗；過往的研究雖然顯示特定的趨化激素受體 (chemokine receptor) 於皮膚 T 細胞淋巴瘤中有提高之表現， 然而這些趨化激素受體是否真正可以調控皮膚 T 細胞淋巴瘤移行至皮膚仍屬未知。 在本研究中， 我們發現特定趨化激素受體 CCR4 在不同種類的皮膚 T 細胞淋巴瘤細胞株中有不同之表現: 來自蕈狀肉芽腫 (mycosis fungoides，惡性 T淋巴細胞絕大多數集中於皮膚) 的細胞株 (MJ) 相對於來自Sezáry syndrome (部分惡性 T淋巴細胞存在於週邊血液) 的細胞株 (Hut78) 有較明顯的 CCR4 之表現。我們進一步進行一系列的*in vitro* 功能測試 (趨化性， integrin 活化與黏著，血管內皮穿透)， 我們發現 具有較強CCR4 表現之 MJ 細胞株對於趨化激素CCL22 (CCR4 之ligand) 所引發之各種功能性表現都比對照組細胞株要明顯。這些結果顯示趨化激素受體CCR4 可能在皮膚 T 細胞淋巴瘤移行至皮膚的調控機轉中扮演重要角色。 Cutaneous T cell lymphoma (CTCL) is a clonal epidermotropic malignancy of memory T cells primarily involving the skin. The mechanisms governing CTCL skin homing, however, have not been fully clarified. It has been shown that certain chemokine receptors are up-regulated in CTCL cells, but it remains unanswered whether these chemokine receptors may play a critical role in the skin homing dynamics of CTCL. Using cell lines originally derived from patients with different subtypes of CTCL, we have shown higher CCR4 expression by the line (MJ) derived from the mycosis fungoides (MF), compared with the line from Sezáry syndrome (Hut78). In specific responses to CCL22 (a CCR4 ligand) treatments, MJ cells showed significant chemotactic migration, enhanced activation and adhesion of certain integrins (CD49d and CD29) *in vitro,* while the control cells (Hut78, CD4+CD45RO+ memory T cells, and Jurkat cells) did not. Furthermore, compared with Hut78 cells, MJ cells manifested significantly more transendothelial migration, in responses to treatments with either CCL22 or condition medium from dendritic cells *in vitro*. These results have provided further dynamic evidences, in line with multi-step cascade paradigm for leukocyte transendothelial migration, to support CCR4's critical role in the CTCL homing to skin.

二、緣由與目的

Cutaneous T cell lymphoma (CTCL) is a clonal epidermotropic malignancy of memory T cells (mTCs) primarily involving the skin. Mycosis fungoides (MF), the subtype of CTCL with the highest prevalence rate, typically presents as cutaneous patches or plaques which may progress to tumor stages, while Sézary syndrome (SS), the erythrodermic variant of CTCL, may develop either de novo or following certain stages of classic MF . With all the diverse clinical features, one common feature of CTCL is its nature of skin-homing, although to various extents in different subtypes. However, the current knowledge about the mechanisms regulating CTCL's trafficking to skin is still limited.

It has been suggested that, for certain typical inflammatory skin diseases (e.g. atopic dermatitis), in order to home to skin, different lines of T cells adopt a complicated multiple-step adhesion cascade (tethering, rolling, and adhesion), in which chemokines and their receptors play a pivotal role, to leave the blood flow and transmigrate through endothelial cells into skin . Importantly, certain chemokines and their receptors have been demonstrated to be highly expressed in inflammatory skin diseases, indicating their possible important roles during the process. For example, chemokine receptor CCR4 was found highly expressed by certain types of T cells in peripheral blood of patients with atopic dermatitis, while CCL17 (also known as thymusand activation-regulated chemokine, or TARC), one of the ligands for CCR4, was found up-regulated in lesional keratinocytes .

However, it remains elusive whether malignant clonal T cells of CTCL may also adopt the multi-step adhesion mechanisms to home to skin. Among few reports which have addressed this issue, Heald *et al*. have shown that T cells expressing cutaneous lymphocyte antigen (CLA), an adhesion molecule well known to mediate skin-homing of T cells, correlate with disease extent in SS and decline with clinical remission . Shiohara *et al*. have proposed that LFA-1 (CD11a/CD18) integrin expression by T cells may play a role in the epidermotropism of malignant T cells . By contrast, Hwang *et al.* reported a case of SS with down-regulated expression of LFA-1 (CD11a/CD18) but up-regulation of L-selectin (CD62L) . More recently, several reports have focused on the expression of chemokines and chemokine receptors by CTCL. Kallinich *et al.* have demonstrated differential expression pattern of chemokine receptors in T cells at different stages (patch/plaque versus tumor stages) , finding that chemokine receptors CCR4, CXCR3 and CXCR4 were highly expressed by MF T cells at early stages. Meanwhile, Kakinuma *et al.* showed that serum level of chemokine CCL17 (TARC), one of the ligands for CCR4, correlates with disease severity of MF, indicating CCR4 and its chemokine ligands may play a role in the pathogenesis of MF .

We hence postulated that CCR4, beyond expression by CTCL cells, may literally play a critical role in the skin homing dynamics of CTCL cells through multi-step adhesion cascade for leukocyte transendothelial migration. By using different CTCL cell lines (MJ and Hut78; derived from MF and SS respectively), we found there is differential expression of CCR4 expression by these lines (much more in MF-derived MJ than SS-derived Hut 78). In serial functional assays (in line with current adhesion cascade paradigm) to test whether CCR4 may play an important role in the CTCL migration, MJ cells showed more responses to CCR4 ligand (mainly CCL22, also known as macrophage-derived chemokine, or MDC), while other cells (Hut78, CD4+CD45RO+ mTCs, and Jurkat cells as controls) did not. Our results thus further support the postulation that CCR4 signaling may be functionally important in skin homing of CTCL.

三、結果與討論

Results

Expression of chemokine receptor CCR4 by different CTCL cell lines

Using qPCR and flow cytometry, we have measured and compared relative expression level of chemokine receptor CCR4 in two CTCL lines (MJ and Hut78), Jurkat cells (human leukemia T cell line), and CD4+RO+mTCs. By qPCR, higher expression of CCR4 was detected in MJ cells (~8.7-fold versus Hut78 cells by qPCR, Figure 1A), while Hut78 cells and other controls (Jurkat cells and CD4+RO+mTCs) showed relative low expression of CCR4 (Figure 1A). Flow cytometry assay (Figure 1 B-E) further demonstrated relative high expression of CCR4 by MJ cells (~87%) compared with Hut78 cells (~39%) and controls (Jurkat cells, ~43%; mTCs, ~42%). MJ cells originated from a patient with MF (the most typical CTCL subtype composed of predominantly skin-homing

malignant T cells), whereas Hut78 cells were from SS (with many malignant T cells remain in circulation instead of homing to skin), the differentialexpression of CCR4 by MJ and Hut78 cells appears to further substantiate CCR4¡¦s important role in CTCL skin homing.

MJ cells are more responsive to CCL22 (ligand for CCR4) in chemotaxis assay
To further determine whether CCR4 signaling may play a critical role in migration dynamics of MJ cells, we performed chemokine functional assay to compare MJ with Hut78 and other control cells. As shown in Figure 2A,MJ cells demonstrated more chemotaxis response to CCL22 (one of the tow ligands for CCR4), compared with Hut78 ($p < 0.01$) and the control cells (Jurkat and CD4+RO+mTCs). chemotaxis response of MJ cells to CCL22 is specific, since it could be negated by neutralizing Ab (Anti-CCL22) and PTX (Figure 2A). Moreover, CCL17 (another ligand for CCR4) also subject to chemotaxis assay; shown in 2B, CCL17 may also induce chemotaxis migration of MJ cells in a dose-dependent manner, but to a less degree, especially at higher concentration (100 ng/ml), in comparison with CCL22 ($p < 0.05$).

CCL22 treatment may enhance the activation of VLA-4 $\alpha 4\beta 1$ integrin (CD49d/CD29) on MJ cells
To address whether CCR4 signaling in MJ cells may induce subsequent multiple-stepadhesion cascade, which may lead to eventual transendothelial migration to skin, we investigated if CCL22 treatments may enhance integrin activation (i.e. conformation change to increase avidity), integrin adhesion (to its ligand), and ultimate transendothelial migration. cells were treated with CCL22 and then subject to flow cytometric assay to measure the expression of active form (with increased avidity) of αβintegrin by anti-CD49d and anti-CD29 mAbs able to detect active form of integrin as previously described . As shown in Figure 3A and B, MJ cells demonstrated enhanced expression (activation) of CD49d (Figure 3A) and (Figure 3B) after CCL22 treatment; this reaction is specific to CCR4-CCL22 interaction, as anti-CCL22 and PTX, but not isotype control, were able to attenuate the reaction. contrast, enhanced integrin activation was not detected in similar assays using Hut78, Jurkat, and CD4+RO+mTC cells (data not shown). Furthermore, was also able to enhance the expression of αβby MJ cells (3C). contrast, both chemokines (CCL22 and CCL17) were not able to induce enhanced expression of β_2 LFA-1 ($\alpha_L \beta_{2,}$ a/CD18) by flow cytometry (data not shown).

CCR4 signaling may enhance $\alpha 4\beta 1$ integrin adhesion to its ligand (VCAM-1) on MJ cells
To further determine whether interaction between CCR4 and CCL22 enhance in vitro adhesion of VCAM-1 (ligand for αβintegrin; usually expressed on ECs by stimulation) to MJ cells via αβintegrin (CD49d/CD29), MJ cells were subject to soluble biding assay by flow cytometry as aforementioned . As shown in 4A,following CCL22 treatment, CCR4 signaling by CCL22 treatment may specifically enhance adhesion of soluble VCAM-1, as this reaction could be blocked by either anti-CCL22 or PTX, but not isotype control Ab. Moreover, the CCL22-enhanced VCAM-1 adhesion is indeed integrin-dependent, as neutralizing mAb for CD49d, but not its isotype control, was able to inhibit the VCAM-1 adhesion reaction(Figure 4B). In contrast, soluble VCAM-1 adhesion was not enhanced in HuT78, Jurkat, and CD4+RO+mTC cells (data not shown) after treatment with CCL22. , CCL17 treatment was also able to enhance VCAM-1 adhesion to MJ cells, but to a less degree (~15% less than CCL2-ehanced binding, 4C). To further substantiate the results from soluble VCAM-1 biding assay, static cell adhesion assay using immobilized VCAM-1 was also performed. As shown in Figure 4D, CCL22 treatment may also enhance the binding of MJ cells to immobilized VCAM-1 (coated onto plate), as shown in Figure 4D (compared with non-chemokine treatment, $p<0.05$), which is comparable to Mn^{2+}induced reaction (a commonly used potent positive control for affinity regulation) .

CCR4-CCL22 interaction may enhance transendothelial migration of MJ cells
To determine whether CCR4-CCL22 interaction ultimately enhance CTCL migration through ECs, in vitro transendothelial migration assay to mimic the real skin milieu was set up by using skin-derived ECs (HMEC-1) and chemokine treatments (as described in Materials and methods). As shown in 5A, CCL22 treatment may enhance more migration of cells through ECs, as compared with control (Hut78, Jurkat, and)cells ($p< 0.05$, Figure 5A). This reaction was also CCR4-CCL22 and dependent,as it could be inhibited by neutralizing anti-CCL22 Ab, PTX (Figure 5A), and neutralizing for CD49d (Figure 5B), but not by isotype controls. On the other hand, treatments with CCL17 moderately enhanced the transendothelial migration of MJ cells, but to a less degree, in comparison with CCL22 (~1.7-fold above basal level with CCL17 versus ~4.0-fold with CCL22, $p< 0.05$; Figure 5B).

DCs may also enhance transendothelial migration of MJ cells by CCL22 secretion
It has been shown that DC is the major source of CCL22 in skin milieu , whereas CCL17 is mainly derived from keratinocytes (KCs) and ECs. In order to further substantiate our *in vitro* results (using exogenous chemokines CCL22 and CCL17 only), we next investigated whether human DCs and KCs may enhance transendothelial migration of CTCL via CCR4-CCL22 or CCR4-CCL17 interactions. High expression of CCL22 and CCL17, by DCs and HaCaT cells (immortalized KCs) respectively, was confirmed by ELISA assays (using CM from cultured cells) (Figure 5C). Note that CCL22 is predominantly produced by DCs, but not by HaCaT and HMEC-1 cells, whereas CCL17 is also found in DC- and HMEC-1-derived CM (Figure 5C). In transendothelial migration assay as shown in Figure 5D, DC-derived CM was able to significantly enhance transendothelial migration of MJ cells in a similar fashion as CCL22, which could also be blocked by neutralizing Abs (anti-CCL22 and anti-CD49d) and PTX, indicating that DC-enhanced transendothelial migration of MJ cells is also both CCR4-CCL22- and integrin-dependent. In contrast, HaCaT-derived CM could not significantly enhance MJ¡¦s transendothelial migration beyond basal level (~2.1-fold above basal level with HaCaT-derived CM, versus ~6.0-fold with DC-derived CM, $p< 0.05$; Figure 5D).

Cell survival of MJ cells in response to CCR4 signaling
As it has been well established that chemokine signaling may induce multiple effects, beside cell migration, on cancer cells , we investigated whether CCR4 signaling may also enhance cell survival of MJ cells. As shown in Figure 6, under nutrition deprivation (in 0.5% FBS), MJ cells showed enhanced survival ($p < 0.05$), compared with non-chemokine control, in the presence of CCL22 or CCL17, which is comparable to the effect induced by CXCL12, another chemokine well established to enhance tumor cell survival .

Immunoblotting for tracing CCR4 signal transduction route in response to CCL22

We further investigated MJ cells¡¦ signaling events in response to CCL22. As shown in Figure 7, in response to CCL22 treatment, the time-dependent accumulation of phosphorylated serine/threonine kinase, Akt (or PKB) was found in MJ cells. In contrast, the phosphorylation of another signaling effector molecule, Erk, was not found increased above basal level with the presence of CCL22 (data not shown).

DISCUSSION

As CCR4 has long been considered as an important mediator for CTCL skin homing, most evidences available to date to support this theory are based on expressional, rather than functional, studies. In light of the lack of sufficient functional evidences to support the critical role of CCR4 for the CTCL migration dynamics toward skin, herein we tried to address this issue and have demonstrated that CCR4 signaling, by CCL22 treatment, may specifically enhance chemotaxis, integrin activation, integrin adhesion, and overall transendothelial migration of MF-derived MJ cells, while SS-derived Hut78 cells and other control cells (CD4+RO+mTCs and Jurkat cells) did not respond to CCL22 as well. The present study not only confirms the critical role that CCR4 may play in the CTCL trafficking and skin homing, but also demonstrate that CCR4-mediated CTCL skin homing may be in accordance with the current lymphocyte trafficking paradigm (adhesion and transendothelial migration cascade) for inflammatory skin diseases.

CCL22 and CCL17 are the only two ligands for CCR4 and both have been shown to be expressed by various types of cells in skin milieu . We have been interested in whether there may be differences, between CCL22 and CCL17, with regards to their role and relative importance in mediating skin homing of CCR4-expressing CTCL. Using standard chemotaxis assays, we first tested CCR4 function of CTCL cells, in responses to treatments of both CCL17 and CCL22 respectively. As shown in Figure 2B, CCL22 was able to induce more chemotaxis of MJ cells than CCL17 (at 100 ng/ml). In integrein activation and adhesion assays, we also found that CCL22 appears to induce mildly more responses in MJ cells than CCL17. Moreover, CCL22, but not CCL17, could enhance transendothelial migration of MJ cells beyond basal level in vitro. These results are in line with several previous works that demonstrate CCL22 seems to be dominant over CCL17 in several CCR4-mediated reactions ; D'Ambrosio *et al.* have shown that CCL22 is much more powerful than CCL17 in the induction of integrin-dependent T cell adhesion to VCAM-1, which is in accordance with our results (Figure 4). Mariani et al. also have demonstrated that CCL22 is a more potent and rapid inducer of CCR4 internalization and recycling on Th2 cells . They also noted that, while incubation with CCL17 inhibited subsequent cell migration in response to CCL17 but not to CCL22, incubation of CCL22 inhibited subsequent migration in response to both CCL22 and CCL17, thus suggesting a hierarchy (CCL22 over CCL17) of cross-desensitization action between these two CCR4 ligands. This report appears to provide reasonable explanation to our results showing CCL22¡¦s superiority over CCL17 in the enhancement of basal transendothellial migration of MJ cells. Moreover, Cronshaw et al. have shown that CCL22 could induce a more lasting Akt phosphorylation in Th2 cells than CCL17 , which is also in line with our results of MJ cell CCR4 signaling (Figure 7).

In skin milieu, CCL22 has been found mainly expressed by DCs (both in epidermis and dermis), while CCL17 appears to be mostly produced by KCs and ECs , but also by DCs . Herein we have confirmed this specific pattern of chemokine expression *in vitro* (Figure 5C). Note that while CCL17 is expressed by all three types of cells (KCs, ECs, and DCs), CCL22 seems to be predominantly expressed by DCs. More importantly, in present study, we further demonstrate that conditioned medium from DCs has a superior effect to enhance transendothelial migration of MJ cells than KC-derived condition medium (Figure 5D), suggesting DC¡¦s pivotal role in the migration of CTCL cells toward dermis through vascular barrier. Given that CCL22 is a more potent (than CCL17) inducer of MJ¡¦s transendothelilal migration and DC¡¦s wide distribution (in both epidermis and dermis) and high motility, it is likely that DCs, rather than KCs or ECs, may play a more decisive role in mediating MJ¡¦s tranendothelial migration to skin dermis. Moreover, DCs may continue to impose major effects on CTCL cells after their migration into the dermis. It has also been found that CCL22 and CCR4 are involved in the formation of T lymphocyte-DC clusters in human inflamed skin . Berger *et al.* have demonstrated that CTCL cells can be reproducibly grown in culture for 3 months when cocultured with DCs , and further found that CTCL cells adopt phenotypes of T-regulatory (Treg) cells (expression of CD25/CTLA-4 and FoxP3) after the interaction with DCs . The fact that CTCL cells being primed (by DCs) into Treg-like cells may explain the immunosuppressive nature of CTCL, and is in accordance with the recent report from Curiel *et al.* demonstrating immune-suppressing Treg cells in ovarian cancer microenvironment have high CCR4 expression, and *in vivo* treatment with mAb to CCL22 (but not mAb to CCL17) decreased migration of Treg cells to tumors, thus further exemplifying the close relations between CTCL, Treg cells, cancer immunity, and CCR4-CCL22 interaction. Collectively, these evidences, including ours, may imply that soluble mediator secreted by DCs, especially CCL22, may play critical roles in many aspects of the skin homing process of CTCL cells, and also in subsequent survival and remodeling of CTCL cells in skin. More importantly, in line with the report from *et al.* , our findings may favor the selective targeting of CCL22, rather than CCL17, in the development of novel therapeutics against CCR4-mediated tumorigenesis, including CTCL.

Integrin-mediated firm arrest of CTCL cells on endothelial cells, activated by so called ¡§inside-out!··signaling of chemokine receptor , appears to be also important for CTCL skin homing. However, it has not been clearly characterized as for which integrins may play a relatively more important role in the transendothelial migration of CTCL cells. Previous report, nevertheless, does demonstrate that the expression of VCAM-1, the main ligand for α4 integrin, was up-regulated in the dermal endothelium of CTCL lesions . Herein we have demonstrated that α4 integrin, but not β2 integrin, may enhance the adhesion of MJ cells on VCAM-1 following CCR4 signaling *in vitro* (Figure 4). More importantly, we have shown herein that transenothelial migration of MJ cells, either enhanced by CCL22 or DC-derived conditioned medium, is dependent on α4 integrin (CD49d), indicating that in certain pathological condition like MF, certain set of chemokine signaling (CCR4/CCL22) and integrin type (α4) may have a predominant role in controlling integrin-mediated adhesion, as has been suggested by previous report .

We can¡¦t exclude that chemokines other than CCL22, may still play some roles in the tumorigenesis of CTCL. Indeed, previous report has noted that skin homing of Sézary cells may involve CXCR4 signaling . By qPCR, we have also found the expression level of CXCR4 by MJ cells is even higher in comparison with CCR4; however, while CCR4 is much discrepantly expressed by MJ and control cells (Hut78 and mTC), CXCR4 expression level (by qPCR) is similar between MJ cells and other CTCL cell lines (HH and Hut78), but much lower than that of +RO+T cells (~7-fold less), indicating a more specific role of CCR4 in CTCL pathogenesis. As for CCL17, given that its expression (production) is widely distributed in skin (from KCs, DCs, and ECs), it may still be able to contribute, to some extent, to the skin homing and tumorigenesis of CTCL (Figure 6). As suggested by Mariani *et al.* , CCL17 may still play a role to promote the arrest of rolling CTCL cells on ECs; meanwhile, the CCR4 on CTCL cells remains sensitive to CCL22 stimulation and capable of mediating subsequent transendothelial migration of CTCL toward dermis.

Using currently available CTCL cell lines, herein we have demonstrated the differential expression of CCR4 between MF-derived cells and SS-derived cells, and further shown the differential functional capacity of CCL22 and CCL17 in mediating CCR4-dependent adhesion and transendothelial migration cascades of MJ cells. Given the long-existing barriers in CTCL research caused by rare case number, difficulty to isolate CTCL cells, and lack of a proper animal model, these currently available CTCL cell lines may serve as legitimate tools for studies involving CCR4-mediating CTCL skin homing and tumorigenesis. More importantly, as CCR4 has become one of the main targets to develop novel therapies for cancer and viral infection , MJ cell line, with its differentially higher expression of CCR4 (versus Hut78 and mTC cells) and more sensitivity to CCL22 (versus CCL17), may well serve as a decent model for studies involving CCR4- or CCL22-targeting therapies.

附圖與說明

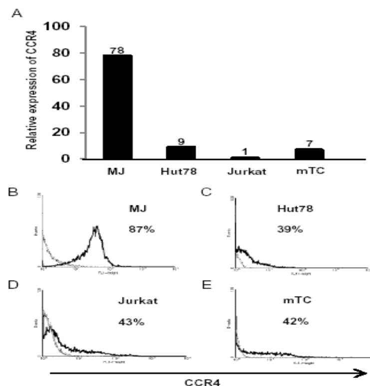

Figure 1. Relatively higher expression of CCR4 by CTCL MJ cells. (A) expression level of CCR4 (shown atop the black bar) in two CTCL lines (MJ and Hut78), Jurkat cells, and CD4+RO+mTCs were measured by qPCR. (B-E) Cells were stained with anti-CCR4 mAb (FITC-conjugated) and measured by flow cytometry.

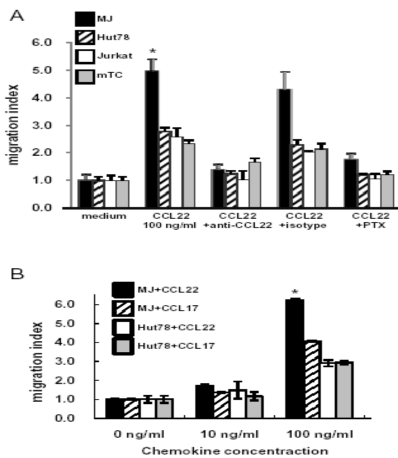

Figure 2. MJ cells show more responses to CCL22 in the chemotaxis assay. (A) of cells(MJ, Hut78, Jurkat, and mTC) were measured with exposure to medium only or CCL22 (100 ng/ml) in the presence of other agents (neutralizing anti-CCL22 Ab, isotype for anti-CCL22 Ab, and PTX) or not. * $p< 0.05$, MJ versus other types (Hut78, Jurkat, and mTC) of cells. (B) In another series of experiments, MJ and Hut78 cells were exposed to either CCL17 or CCL22 at 0, 10, or 100 ng/ml and chemotaxis migration was measured. * $p< 0.05$; MJ cells treated with CCL22 at 100 ng/ml versus other conditions (MJ with CCL 17 at 100 ng/ml or Hut with either CCL22 or CCL17 at 100 ng/ml).

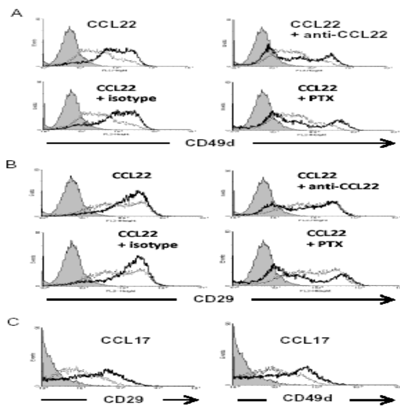

Figure 3. CCL22 treatment may enhance $\alpha\beta$integrin activation on MJ cells. (A and B) MJ cells were exposed to CCL22 treatment or not, plus the presence of other agents (neutralizing anti-CCL22 Ab, isotype for anti-CCL22 Ab, and PTX) or not, and then subject to flow cytometric analysis of α(CD49d) and β(CD29) integrin respectively. Gray region: istoype control Ab for anti-CD49d or anti-CD29. Dotted line: anti-CD49d or anti-CD29 Ab, without CCL22. Solid line: anti-CD49d or anti-CD29 Ab, with the presence of CCL22, plus other agents or not. (C) MJ cells were exposed to CCL17 treatment or not, and then subject to analysis of α(CD49d, right) and β(CD29, left) integrin respectively. Gray region: istoype control Ab for anti-CD49d or anti-CD29. Dotted line: anti-CD49d or anti-CD29 Ab, without CCL17 treatment. Solid line: anti-CD49d or anti-CD29 Ab, with CCL17 treatment. Results represent one of at least two experiments with similar results.

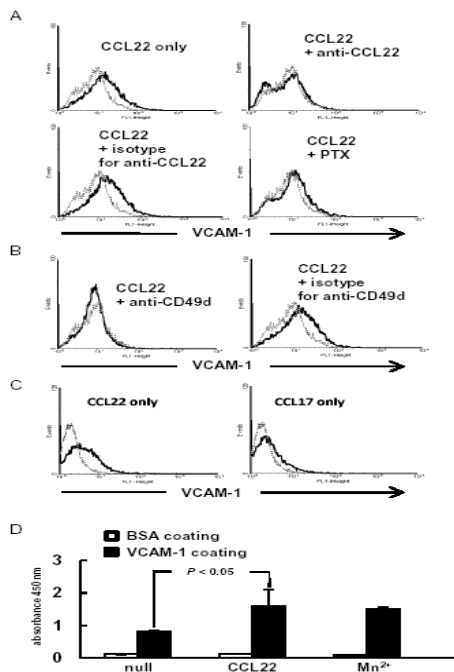

Figure 4. CCL22 treatment may enhance integrin-dependent adhesion to soluble or immobilized VCAM-1. (A) MJ cells were exposed to CCL22 treatment or not, plus the presence of other agents (neutralizing anti-CCL22 Ab, isotype for anti-CCL22 Ab, and PTX) or not, to test the dependence on CCL22 treatment, and then incubated with soluble VCAM-1 before flow cytometric analysis of VCAM-1 adhesion. Dotted line: no CCL22 treatment. Solid line: with the presence of CCL22, plus other agents (anti-CCL22 Ab, isotype control for anti-CCL22 Ab, or PTX) or not. (B) In another series of experiments, MJ cells were likewise exposed to CCL22 treatment or not, followed by VCAM-1 incubation and flow cytometric analysis as in (A), but with the presence of function-blocking anti-CD49d mAb or isotype Ab to test the dependence on αintegrin (CD49d). Dotted line: no CCL22 treatment. Solid line: with the presence of CCL22, plus neutralizing anti-CD49d Ab (or isotype for anti-CD49d Ab) or not. (C) In another series of experiments, MJ cells were exposed to either CCL22 or CCL17 treatment or not, and then subject to VCAM-1 incubation and flow cytometric analysis. Dotted line: no chemokine treatment. Solid line: with CCL22 or CCL17 treatment. (D) MJ cells were treated with CCL22 or not (null), and put onto either VCAM-1- or BSA-coated dishes before washing. Adhesion to VCAM-1 (or BSA) is reflected by the number of washing-resisting (adherent) cells, by commercial cell proliferation kit, shown as colorimetric reading at 450-nm absorbance.

Mn^{2+} was used in some groups (without CCL22) as a potent positive control for affinity regulation as previously described . Results represent one of at least two with similar results (A-D), and are shown as mean ±SD in (D).

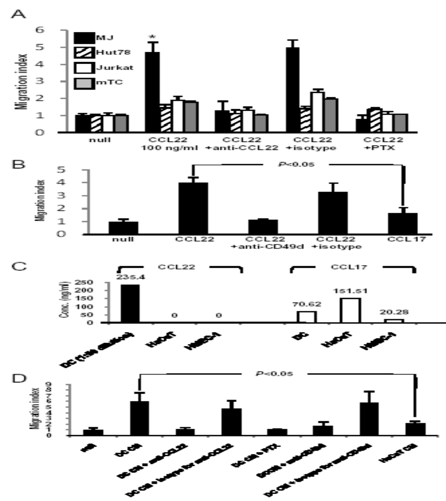

Figure 5. CCL22 and DC condition medium may enhance transendothelial migration of MJ cells. (A) Transendothelial migration cells(MJ, Hut78, Jurkat, and mTC) were measured with exposure to CCL22 (100 ng/ml), in the presence of other agents (neutralizing anti-CCL22 Ab, isotype for anti-CCL22 Ab, and PTX) or not. * $p < 0.05$, MJ versus other types (Hut78, Jurkat, and mTC) of cells. (B) In another series of experiments, MJ cells were likewise exposed to CCL22 treatment as in (A), but with the presence of neutralizing anti-CD49d Ab or isotype (for anti-CD49d Ab) to test the dependence on αintegrin (CD49d). (C) Expression of CCL22 and CCL17 by DC, HaCaT, and HMEC-1 cells were measured and compared by ELISA, using CM derived from respective types of cells. (D) In another series of experiments, MJ cells were exposed to DC- or HaCaT-derived CM, with or without the agents to test the CCL22-dpendence (neutralizing anti-CCL22 Ab, isotype for anti-CCL22 Ab, or PTX), and the agents to test αintegrin (CD49d)-dependence (the neutralizing anti-CD49d Ab or isotype).

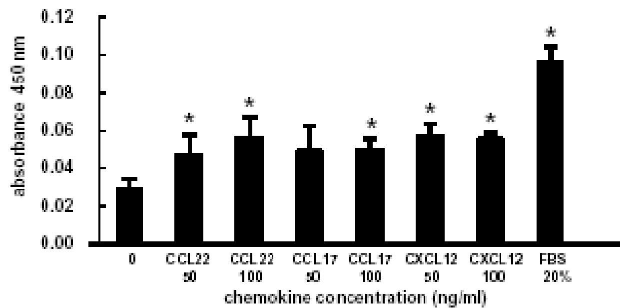

Figure 6. CCL22 and CCL17 mildly enhance cell survival of MJ cells under nutrition deprivation. Cells were seeded in 96-well plate with culture medium containing FBS at only 0.5 % and treated with CCL22, CCL17, or CXCL12 at 50- and 100- ng/ml concentrations in triplicate. Cells treated with PBS only or culture medium with 20% FBS respectively were used as controls. Cell viability was measured by colorimetric method with absorbance reading at 450 nm. * $p < 0.05$, versus non-chemokine control in low-serum condition.

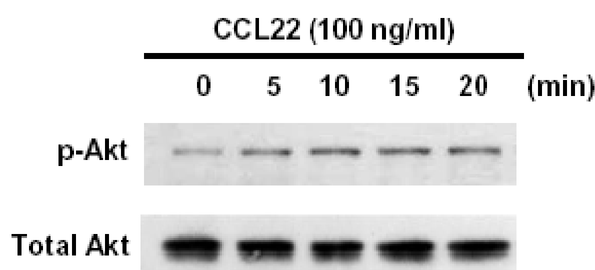

Figure 7. CCL22 treatment induces phosphorylation of Akt. MJ cells were treated with CCL22 (100 ng/ml) and cell lysate were subject to immunoblotting with various mAbs (anti-human/mouse/rat pan-Akt, affinity-purified rabbit anti-phospho-Akt (S473), anti-human/mouse/rat pan- ERK1/ERK2, and anti-phospho-ERK1/ERK2(T202/Y204) (R&D systems) at 4¢J overnight.

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四. 計畫成果自評

We have faithfully executed this granted project and current results rendered are very informative and indicative of significant clinical implication.

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