行政院國家科學委員會專題研究計畫　成果報告

T 淋巴球皮膚癌之皮膚趨向性研究

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行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：■個別型計畫　　□整合型計畫
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本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

執行單位：中國醫藥大學醫學系皮膚學科

中　華　民　國　　95　年　　10 月　　　日

一. 中英文摘要

T 淋巴球皮膚癌之本質為記憶型 T 淋巴球之癌化並以皮膚為主要侵犯部位. 雖然 T 淋巴球皮膚癌之臨床表現十分多樣，例如 mycosis fungoides (MF), Sezary Syndrome 以及非 MF 類; 但最重要之共同點為其惡性 T 淋巴球移行至皮膚之能力，然而其調控之機轉為何目前仍所知有限. 過往研究已發現，於一些典型之發炎性皮膚疾患，例如乾癬或異位性皮膚炎，疾病進展中之不同類型之正常 T 淋巴球移行至皮膚的過程中, T 淋巴球為了離開週邊血管之血流而進入皮膚，通常遵循著一套有許多黏合分子或趨化激素受體所參與調控之多種步驟的黏合 (adhesions) 過程，以使 T 淋巴球可以減緩其流動速度，進而與血管內皮細胞黏合，並穿過血管內皮細胞進入皮膚組織. 然而，是否 T 淋巴 球皮膚癌的惡性淋巴球也是遵循類似之模式而進入皮膚則仍不十分清楚. 一些相關研究曾發現某些黏合分子或趨化激素受體在 T 淋巴球皮膚癌的惡性淋巴球中的表現有其特異之處,可能對於 T 淋巴球皮膚癌的皮膚趨向性扮演某種角色. 然而，許多相關之重要問題仍有待研究，例如是否某些特定黏合分子或趨化激素受體在 T 淋巴球皮膚癌的惡性淋巴球之表現模式彼此有關? 是否趨化激素受體所引發之訊息傳導可以引發某些特定黏合分子之表現或是活化? 我們所進行的初步前驅實驗顯示某一特定 non-MF T 淋巴球皮膚癌惡性淋巴球細胞株 (HH CTCL cell line) 的惡性淋巴球確實有趨化激素受體 CCR4 之顯著表現，是否 CCR4 對於惡性 T 淋巴球的皮膚趨化性扮演重要之功能性角色仍有待進一步探討. 我們因此提出本計畫，進一步研究各種不同質性之 T 淋巴球皮膚癌是否有特定之黏合分子或趨化激素受體之表現或關聯，並更進一部探討是否特定趨化激素受體之訊息傳導會引發特定黏合分子之表現或是活化，以期進一步了解 T 淋巴球皮膚癌皮膚趨向性之調控機制.

Cutaneous T cell lymphoma (CTCL) is a clonal epidermotropic malignancy of memory T cells primarily involving the skin. With all its diverse manifestations including mycosis fungoides (MF), Sezary syndrome and non-MF CTCL, one foremost common feature of CTCL is its nature of skin-homing. However, little has been known about the mechanisms regulating CTCL's trafficking to skin. It has been suggested that for typical inflammatory skin diseases like psoriasis or atopic dermatitis, in order to home to skin, different lines of T cells engage multiple-step adhesion cascade pathways to leave the blood flow and transmigrate through endothelial cells into skin. Whether malignant clonal T cells of CTCL adopt similar mechanisms to home to skin, as seen in those inflammatory, non-malignant skin diseases, remains elusive. Several previous studies have demonstrated that aberrant expression of a subset of adhesions molecules including L-selectin and integrins, plus the preferential expression of certain chemokine receptors, may play roles for the skin homing of certain types of CTCL. However, some questions remain unanswered and we seek to address herein: is particular differential expression of some certain chemokine receptors and adhesions molecules by CTCL T cell correlated with each other to fit in the current multi-step paradigm? Are the chemokine/chemokine receptor interactions able to turn on the expression or activation of integrin on CTCL T cells? Our preliminary experiments showed that CCR4, CCR7 and CXCR4 were highly expressed by a non-MF CTCL cell line (HH), but only CCR4 was significantly expressed by HH CTCL when compared with CD4 T cells from healthy controls. This preliminary result does indicate that non-MF CTCL may also possess differential expression of certain chemokine receptors, like CCR4, which may play a role in the skin homing or other aspects of pathogenesis of non-MF CTCL. In order to further investigate the skin homing mechanisms for CTCL, herein we propose to investigate the expression status and the correlation/interaction of adhesion molecules (e.g. CLA, L-selectin, and integrins) and chemokine receptors in three different CTCL cell lines: human MJ, Hut78 and HH CTCL cell lines which represent MF, Sezary syndrome and non-MF CTCL respectively. Moreover, we will seek to explore whether signaling of certain chemokine receptors will lead to down stream up-regulation or activation of certain integrins on CTCL cells. Further understanding of these regulating mechanisms may have therapeutic implication of targeting certain molecules

essential for CTCL skin homing.

二、緣由與目的

Cutaneous T cell lymphoma (CTCL) is a clonal epidermotropic malignancy of memory T cells primarily involving the skin. The classification of CTCL is a subject of constant controversy and debates due to its striking variability in clinical course, histopatholoical features and molecular markers (Russell-Jones 2003). Mycosis fungoides (MF) and its variants represent the major components of CTCL. Classic MF typically presents with cutaneous patches or plaques which may progress to tumor stages; while erythrodermic variants of MF, in which patients develop generalized scaling bright red skin, may develop de novo or follow classic MF. Sezary syndrome is a subtype of erythrodermidc MF, with malignant clonal T cells comprising more than 5% of the total white blood count in the peripheral blood. Beside MF and its variant, other relatively rare non-MF CTCL variants include CD30+ or CD30- CTCL, small/large/mixed cell CTCL, and angiocentric/subcutaneous CTCL(Russell-Jones 2003).

With all this variability, one foremost common feature of CTCL is its nature of skin-homing. However, little has been known about the mechanisms regulating CTCL's trafficking to skin. To be more specific, whether malignant clonal T cells of CTCL adopt similar mechanisms to home to skin, as seen in other inflammatory, non-malignant skin diseases, remains elusive. It has been well-established that the migration of T cells from rapid blood flow to tissue is regulated by a complicated adhesion process. This typical multiple-step adhesion cascade (von Andrian and Mackay 2000), which leads to leukocyte trans-endothelium migration, involves at least three consecutive steps: (1) initial tethering and rolling mediated by primary adhesion molecules, (2) chemokine-mediated activation of integrins, and finally, (3) arrest mediated by activated integrins.

It has been suggested that for typical inflammatory skin diseases like psoriasis or atopic dermatitis, in order to home to skin, different lines of T cells engage the aforementioned adhesion cascade pathways to leave the blood flow and transmigrate through endothelial cells into skin. For examples, cutaneous lymphocyte-associated antigen (CLA), a glycoprotein adhesion molecule that mediates the initial tethering of T cells to cutaneous endothelium by binding to E-selectin (which is up-regulated in dermal microvasculare endothelial cell under inflammatory conditions), was found up-regulated in skin-homing T cells in several inflammatory dermatoses like psoriasis, atopic dermatitis, and allergic contact dermatitis (Robert and Kupper 1999). CLA is hence thought to be important, at least in part, for initiating T cell trafficking to skin in these inflammatory skin dermatoses. Importantly as well, certain chemokine and its receptor have been demonstrated to be highly expressed in inflammatory skin diseases, for example, chemokine receptor CCR4 was found highly expressed by CLA+ T cells in peripheral blood of patients with atopic dermatitis, while CCL17 (also known as TARC), the ligand for CCR4, was found up-regulated in lesional keratinocytes (Kakinuma, *et al* 2001). In the case of psoriasis, several chemokines, including CCL17 (TARC), CCL20 (MIP-3α), and CCL27 (CTACK) were found expressed by psoriasis lesions while lesional CLA+ T cells up-regulated their cognate chemokine receptor CCR4, CCR6 and CCR10 respectively (Campbell, *et al* 1999, Homey, *et al* 2002, Homey, *et al* 2000, Robert and Kupper 1999, Rottman, *et al* 2001). Thus, the T cell homing to skin seems to follow the multi-step paradigm in certain inflammatory dermatoses, in which CLA and chemokine/chemokin receptors may play important roles.

As mentioned earlier, it remains largely unclear whether the malignant T cells in CTCL also follow the classic multi-step cascade model to home to skin. Among few reports which had touched this issue, Heald et al (Heald, *et al* 1993) had shown that CLA+ T cells correlate with extent of disease in Sezary syndrome and decline with clinical remission. Shiohara et al (Shiohara, *et al* 1989) had 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 (Hwang and Fitzhugh 2001) reported a case of Sezary syndrome 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 chemokine/chemokine receptors by CTCL. Kallinich et al (Kallinich, *et al* 2003) demonstrated differential expression pattern of chemokine receptors in T cells at different stages (patch/plaque versus tumor stages). They found chemokine receptors CCR4, CXCR3 and CXCR4 were highly expressed by MF T cells at early stages, while MF tumor T cells were found to express CCR7 and down-regulate expression of CCR4 and CXCR3. Meanwhile, Kakinuma et al (Kakinuma, *et al* 2003) showed that serum level of chemokine CCL17 (TARC), one of the ligand for CCR4, correlates with disease severity of MF; they found patients at tumor stages had significantly higher level of serum CCL17 compared with patients at early stages (patch/plaque), indicating that CCR4/CCL17 may play a role in the pathogenesis of MF. Although these studies shed some light on the homing mechanism of CTCL to skin, some important questions remain unanswered yet as follows. **Is the particular differential expression of certain chemokine receptors and adhesions molecules by MF T cells correlated with each other to fit in the current multi-step paradigm? Are the chemokine/chemokine receptor interactions able to turn on the expression or activation of integrin on CTCL T cells? Do non-MF CTCL T cells also have similar differential expression pattern for some certain adhesions molecules or chemokine receptors?**

Herein we aim to investigate these questions as mentioned above. Given the difficulties of recruiting CTCL patients and collecting the malignant T cells, we had performed some preliminary studies using a CTCL cell line (HH) , which is the only one CTCL line available from local provider (NHRI cell bank at 新竹). HH line is originally from peripheral blood of a CTCL patient with aggressive ulcerative tumors instead of patch/plaque and erythroderma, hence being widely regarded as representative of non-MF CTCL. To determine whether certain chemokine receptors are differentially expressed by non-MF HH CTCL, we used quantitative real-time RT-PCR to perform a thorough screen for expression of chemokine receptors (CCR1-CCR10, CXCR1-CXCR6) by HH cells. We found CCR4, CCR7 and CXCR4 were highly expressed by HH cells. However, after further comparing the expression levels of these three chemokine receptors between HH cells and control CD4+ T cells from normal healthy donors, we found only CCR4 has significantly higher expression in HH CTCL.

Although CD4+ T cells may not be a perfect control, given that CTCL T cells are regarded as transformed memory T cells (CD4+ CD45RO+ memory T cell may serve as a better control (Hwang and Fitzhugh 2001), which we are now working on), this preliminary result does indicate that non-MF CTCL may also possess differential expression of certain chemokine receptor, like CCR4, which may also play a role in the skin homing or other aspects of pathogenesis of non-MF CTCL.

In order to further investigate the skin homing mechanisms for CTCL, herein we propose to investigate the expression profiles and the correlation/interaction of adhesion molecules (e.g. CLA, L-selectin, and integrins) and chemokine receptors in three different CTCL cell lines: human MJ, Hut78 and HH CTCL cell lines which represent MF, Sezary syndrome and non-MF CTCL respectively. Moreover, we will seek to explore whether signaling of certain chemokine receptors will lead to downstream up-regulation or activation of certain integrins on CTCL cells. Importantly, further understanding of these regulating mechanisms may have therapeutic implication of targeting certain molecules essential for CTCL skin homing.

三、結果與討論

Expression of chemokine receptors CCR4 by different CTCL cell lines Using quantitative real-time PCR (qPCR) and flow cytometry (Fig. 1), we have measured and compared relative expression level of chemokine receptor CCR4 in 3 different CTCL lines (MJ, Hut78, and HH), Jurkat cells (human leukemia T cell line), and CD4+CD45RO+ T cells. High expression of CCR4 was detected in MJ and HH cells, while Hut78 cells and other controls (Jurkat cells and CD4+CD45RO+ T cells) showed relative low expression of CCR4 (Fig. 1). Since MJ cells

represents MF characterized by skin-homing CTCL cells, while Hut78 was derived from SS patient (with less skin-homing nature), the differential expression of CCR4 by MJ and Hut 78 cells indicate its important role in CTCL skin homing.

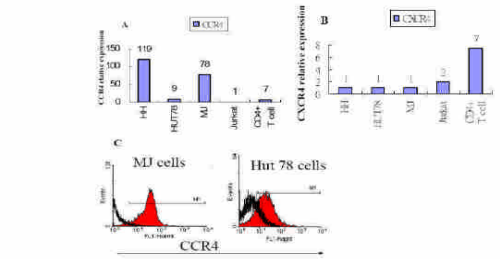

Fig. 1 Differential expression of CCR4 by CTCL cell lines

MJ (MF) cells are more responsive to CCL22 (ligand for CCR4) in chemotaxis assay in vitro To further test whether CCR4 signaling may play a critical role in regulating migration dynamics of MJ cells, we first performed chemokine functional assay to compare MJ with Hut78 and other control cells. As shown in Fig. 2, MJ cells demonstrated significant (**$p < 0.05$ versus isotype control**) chemotaxis response to CCL22 (ligand for CCR4, with abundant expression in skin **Ref**), whereas Hut78 and the control cells (Jurkat and CD4+CD45RO+ T cells) did not. The chemotaxis of MJ cells to CCL22 is specific, since it could be negated by neutralizing antibody (Anti-CCL22) and pertussis toxin (Fig. 2).

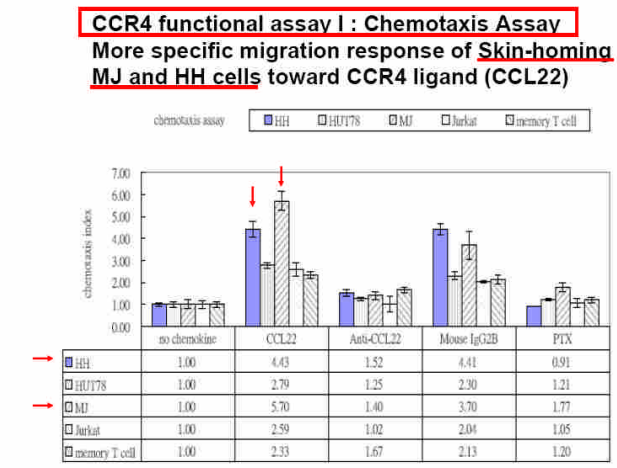

Fig. 2 MJ and HH CTCL lines showed more responses to CCL22 treatments in chemotaxis assays.

CCR4/CCL22 may activate integrin (CD29/CD49d) in MJ cells We next investigated whether CCR4/CCL22 signaling may induce subsequent multiple-step adhesion cascade including integrin activation, integrin adhesion, and transendothelial migration. MJ cells were subject to flow cytometric assay to measure the activation of integrin CD29/CD49d. As shown in Fig. 3, MJ cells demonstrated enhanced activation of CD29 and CD49d after CCL22 treatment; this reaction is specific to CCR4, as anti-CCL22 and pertussis toxin, but not isotype control, was able to block the reaction (Fig. 3). In contrast, integrin activation was not detected in similar assays using Hut78 , Jurkat, and CD4+CD45RO+ T cells (data not shown).

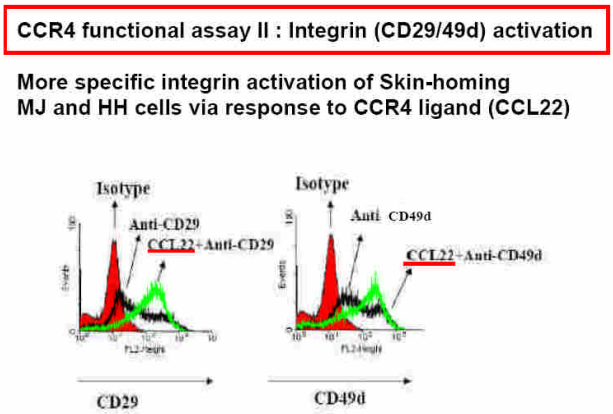

Fig. 3. MJ CTCL cell line showed specific integrin (CD29/49d) activation in responses to CCL22

CCR4/CCL22 may enhance integrin adhesion of MJ cells to integrin ligand (VCAM-1))To further determine whether CCR4/CCL22 interaction may enhance integrin adhesion to its ligand (on endothelial cells) in vitro, MJ cells were subject to flow cytometric adhesion assay as aforementioned. As shown in Fig. 4, CCR4/CCL22 signaling may specifically enhance integrin adhesion to VCAM-1, as this reaction could be blocked by either anti-CCL22., but not isotype control Ab. Moreover, the VCAM-1 adhesion is truly integrin-dependent, as neutralizing Ab for CD49d was able to reverse the VCAM-1 adhesion reaction (Fig. 4).

On the other hand, VCAM-1 adhesion was not enhanced in HuT78 Jurkat, and CD4+CD45RO+ T cells (data not shown) after treatment with CCL22.

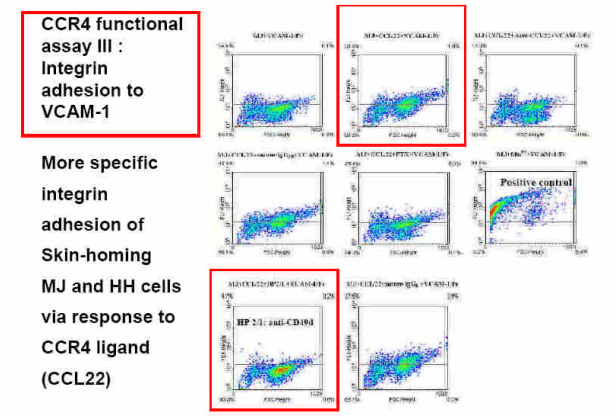

Fig. 4. MJ CTCL cell line showed specific integrin adhesion to VCAM-1 in responses to CCL22 treatments

CCR4/CCL22 may enhance transendothelial migration of MJ cells To determine whether CCR4/CCL22 interaction may enhance CTCL migration through endothelial cells in vitro, transendothelial migration assay was performed using skin-derived endothelial cells (HMEC-1). As shown in Fig. 5, there was higher numbers of migrated MJ cells through endothelial cells, as compared with control (CD4+CD45RO+ T cells) cells, whereas CCR4 signaling did not induce enhanced transendothelial migration of Hut78 cell. Likewise, this reaction was specific for CCR4, as it could be negated by neutralizing anti-CCL22 Ab and pertussi toxin, but not by isotype control.

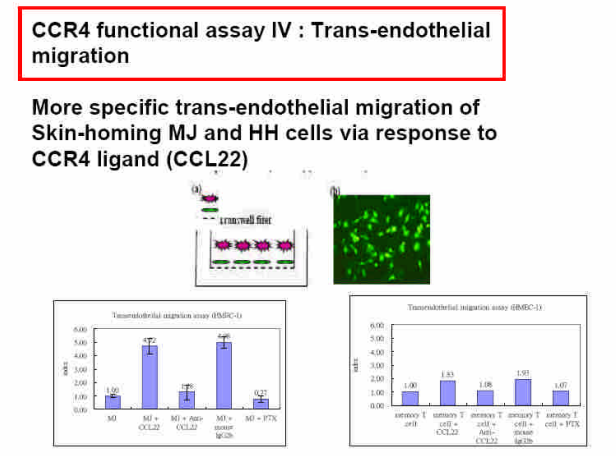

Fig. 5. MJ CTCL showed enhanced transendothelial migration in responses to CCL22, whereas normal memory T cells did not.

Discussion

Herein we have shown that CCR4 expression is more expressed by MJ and HH cells rather than Hut78 cells, at mRNA and protein levels. MJ cells showed more specific chemotaxis responses to CCL22 than control CTCL cells. In serial in vitro integrin activation and adhesion assays, MJ cells also showed more specific responses to CCL22 than other control cells. Importantly, MJ cells showed more specific transendothelial migration in responses to CCL22 than control cells. These results indicate that CCR4 may play important roles in skin homing of CTCL cells, possibly through leukocyte adhesion cascade paradigm.

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.

五、參考文獻

Campbell, J.J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., Andrew, D.P., Warnke, R., Ruffing, N., Kassam, N., Wu, L. & Butcher, E.C. (1999) The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. ***Nature,*** **400, 776-780.**

Heald, P.W., Yan, S.L., Edelson, R.L., Tigelaar, R. & Picker, L.J. (1993) Skin-selective lymphocyte homing mechanisms in the pathogenesis of leukemic cutaneous T-cell lymphoma. ***J Invest Dermatol,*** **101, 222-226.**

Homey, B., Alenius, H., Muller, A., Soto, H., Bowman, E.P., Yuan, W., McEvoy, L., Lauerma, A.I., Assmann, T., Bunemann, E., Lehto, M., Wolff, H., Yen, D., Marxhausen, H., To, W., Sedgwick, J., Ruzicka, T., Lehmann, P.

& Zlotnik, A. (2002) CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. ***Nat Med,*** **8, 157-165.**

Homey, B., Dieu-Nosjean, M.C., Wiesenborn, A., Massacrier, C., Pin, J.J., Oldham, E., Catron, D., Buchanan, M.E., Muller, A., deWaal Malefyt, R., Deng, G., Orozco, R., Ruzicka, T., Lehmann, P., Lebecque, S., Caux, C. & Zlotnik, A. (2000) Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis. ***J Immunol,*** **164, 6621-6632.**

Hwang, S.T. & Fitzhugh, D.J. (2001) Aberrant expression of adhesion molecules by Sezary cells: functional consequences under physiologic shear stress conditions. ***J Invest Dermatol,*** **116, 466-470.**

Kakinuma, T., Nakamura, K., Wakugawa, M., Mitsui, H., Tada, Y., Saeki, H., Torii, H., Asahina, A., Onai, N., Matsushima, K. & Tamaki, K. (2001) Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. ***J Allergy Clin Immunol,*** **107, 535-541.**

Kakinuma, T., Sugaya, M., Nakamura, K., Kaneko, F., Wakugawa, M., Matsushima, K. & Tamaki, K. (2003) Thymus and activation-regulated chemokine (TARC/CCL17) in mycosis fungoides: serum TARC levels reflect the disease activity of mycosis fungoides. ***J Am Acad Dermatol,*** **48, 23-30.**

Kallinich, T., Muche, J.M., Qin, S., Sterry, W., Audring, H. & Kroczek, R.A. (2003) Chemokine receptor expression on neoplastic and reactive T cells in the skin at different stages of mycosis fungoides. ***J Invest Dermatol,*** **121, 1045-1052.**

Robert, C. & Kupper, T.S. (1999) Inflammatory skin diseases, T cells, and immune surveillance. ***N Engl J Med,*** **341, 1817-1828.**

Rottman, J.B., Smith, T.L., Ganley, K.G., Kikuchi, T. & Krueger, J.G. (2001) Potential role of the chemokine receptors CXCR3, CCR4, and the integrin alphaEbeta7 in the pathogenesis of psoriasis vulgaris. ***Lab Invest,*** **81, 335-347.**

Russell-Jones, R. (2003) World Health Organization classification of hematopoietic and lymphoid tissues: implications for dermatology. ***J Am Acad Dermatol,*** **48, 93-102.**

Shiohara, T., Moriya, N., Gotoh, C., Hayakawa, J., Saizawa, K., Yagita, H. & Nagashima, M. (1989) Differential expression of lymphocyte function-associated antigen 1 (LFA-1) on epidermotropic and non-epidermotropic T-cell clones. ***J Invest Dermatol,*** **93, 804-808.**

von Andrian, U.H. & Mackay, C.R. (2000) T-cell function and migration. Two sides of the same coin. ***N Engl J Med,*** **343, 1020-1034.**