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乳癌腫瘤浸潤淋巴細胞分析：比較調節性 T 細胞、CD8+T 細胞與相關免疫標記的表現

Expression and Cell Markers of Regulatory T Cells and CD8+ T Cells in Tumor-Infiltrating Lymphocytes of Human Breast Cancer

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本論文係李朝樹君（P96421004）在國立臺灣大學臨床醫學研究所完成之碩士學位論文，於民國九十九年七月二十一日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

關鍵詞：乳癌，調節性 T 細胞，CD8+ T 細胞，腫瘤浸潤淋巴球，記憶 T 細胞

背景與目的

腫瘤細胞的生長需對抗來自宿主免疫系統的清除作用，在腫瘤組織微環境中有大量的浸潤淋巴球聚集（tumor infiltrating lymphocytes，TILs）。其中有一群 T 細胞具有調節免疫作用，具有 CD4+CD25+ 表現特徵，稱為調節性 T 細胞。調節性 T 細胞於細胞內高度表現 FOXP3 轉錄分子，其負向調節作用需靠細胞與細胞接觸的機制。許多的研究指出腫瘤細胞的免疫逃脫機制有調節性 T 細胞參與。T 細胞經過與抗原表現細胞接觸後受到刺激，naïve cell 會轉化成記憶 T 細胞，之後若經一定刺激可以再度被活化。記憶 T 細胞（memory T lymphocyte）以返回次級淋巴器官的能力與 effector 的功能區分為兩種類型：central memory cell（T_{CM}）及 effector memory cell（T_{EM}）。依據之前本實驗室對乳癌病患腫瘤浸潤淋巴球的測定，發現 TIL 中的 CD8+ T 細胞的比例會增加，而 CD4+ T 細胞的比例會減少。而增加的 CD8+ T 細胞顯著與乳癌疾病期別進展有相關。CD8+ T 細胞具有毒殺腫瘤細胞能力，但於腫瘤微環境中由於免疫功能被抑制，其毒殺腫瘤細胞能力亦被抑制。我們欲了解乳癌病患 TILs 中調節性 T 細胞與 CD8+ T 細胞其免疫標記表現與細胞毒殺能力的關係，尤其是記憶細胞是否能經刺激後再具免疫功能，希望能對未來發展對腫瘤的免疫治療有所幫助。

材料與方法

實驗標本的收集：臨床診斷第一至第三期乳癌的病患，依疾病狀況接受手術治療。病患乳癌腫瘤組織（註記為 TIL）將會被收集成實驗組。同時收集病患的血液（註記為 PBL）為對照組。依據病理報告的結果，記錄每個病例的臨床病理特徵。在分離純化浸潤淋巴細胞與分離出周邊血液中的單核細胞後對細胞表面抗原與細胞內作用分子作測定，運用流式細胞儀分析。探討 TIL 中 CD4+CD25+ 調節性 T 細胞及其上 FOXP3、GITR、CD103 和 CD152（CTLA-4） 的表現，及探討調節性 T 細胞分泌 cytokine 的表現，最後探討調

節性 T 細胞對 CD8+ T 細胞毒殺能力之影響。也分析調節性 T 細胞與 CD8+ 記憶 T 細胞及 effector cell 表現之相關。然後我們將檢測 CD4+CD25+ 調節性 T 細胞分泌 Th1 cytokines（IFN-γ、IL-12 和 TNF-α）與 Th2 cytokines（IL-4 和 IL-10）的狀態，也對細胞內毒殺顆粒（包括 granzyme B 及 perforin）的表現作分析。

結果

共有30位病患列入分析，性別均為女性，平均年齡為56.8歲（28歲至89歲）。CD4+CD25+ 調節性 T 細胞在腫瘤中的比例與血液相比為增加（11.6 ±2 % vs. 37.4 ± 4.4%，P=0.001）。FOXP3、CD103 與 GITR 等活化標記於腫瘤中的調節性 T 細胞表現亦增加（FOXP3，46.7%及71.8%，P=0.033; CD103，11.8% 及 31.5%，P=0.027; GITR，28.9%及80.5%，P=0.04）。而 CD152 在調節性 T 細胞的表現於腫瘤中與週邊血液淋巴細胞內則是相當的（77%及74%，P=0.7）。

週邊血液的 CD8+ T 細胞相較腫瘤內 CD8+ T 細胞明顯有較多組成比例的 CCR7+CD45RO- naive T cells（20.9% 及 4.5%，P<0.001），而 CCR7-CD45RO+ effector memory T cells 的比例（32.3% 及 67.5%，P<0.001）則相反，為減少比例。CCR7+CD45RO+ central memory T cells 在腫瘤內與週邊血液中的比例則是相似無差異（11.9% vs. 16.3%，P=0.175）。腫瘤內的 CD8+ T 細胞相較週邊血液中的 CD8+ T 細胞較多比例會表現 CD69（39.7% vs. 9.6%，P=0.002），CD103（36.3% vs. 2.4%，P<0.001），及 CD152（54.7% vs. 24.1%，P=0.014），而 CD45RA 則較少比例會表現（9.6% vs.52.4%，P<0.001）。所以大部分的腫瘤內的 CD8+ T 細胞的表現是 CD28-CD45RA-CD45RO+CCR7-，顯示著他們是分化良好的一群細胞，而且大部分屬於 CD69+CD103+CD152+。

乳癌病患的腫瘤內的 CD4+CD25+ 調節性 T 細胞顯著地較多比例分泌毒殺顆粒（granzyme B，23.5% vs. 11.5%，P<0.001；perforin，37.8% vs. 17.3%，P<0.001），乳癌病患的CD8+ T 細胞在腫瘤內的與週邊血液中的相比，表現毒殺顆粒比例顯著地減少（granzyme B，10.6% vs. 27.3%，P<0.001；perforin，17.6% vs. 48.9%，P<0.001），乳癌腫瘤內浸潤 CD8+ T 細胞功能明顯受到抑制。

腫瘤內浸潤淋巴球與週邊血液單核細胞球經過 anti-CD3/anti-CD28 刺激後，IL-2，IFN-γ，及 TNF-α 的分泌在兩者是相當的，這表示 anti-CD3/anti-CD28

可誘發出乳癌腫瘤內浸潤淋巴球的 Th1 細胞激素，包括 IL-2，IFN-γ，及 TNF-α 的分泌；而 Th2 細胞激素，包括 IL-4 與 IL-10，於腫瘤內浸潤淋巴細胞與週邊血液淋巴細胞內的分泌並無改變，這表示經過 anti-CD3/anti-CD28 刺激後，腫瘤內浸潤淋巴細胞並沒有分泌較多的 Th2 細胞激素。進一步檢測其 CD8+ T 細胞分泌毒殺顆粒（perforin 和 granzyme B）的表現，我們發現在經 anti-CD3/anti-CD28 刺激後，腫瘤內浸潤 CD8+ T 細胞分泌毒殺顆粒的表現比例，與週邊血液CD8+ T 細胞是相當的。我們的實驗證明腫瘤內浸潤淋巴球的活性是完整的，並且在某些刺激下是可以再回復的。

結論

由本研究發現，乳癌病患的腫瘤浸潤淋巴細胞與周邊血液淋巴細胞確有相關免疫標記表現差異，尤其是調節性 T 細胞（CD4+CD25+）比例會增加。調節性 T 細胞在對乳癌病患的免疫反應上具有重要作用，調節性 T 細胞會干擾毒殺性 T 細胞分泌毒殺顆粒的能力，進一步造成乳癌腫瘤微環境免疫受到抑制。雖然大部分的腫瘤內浸潤 CD8+ T 細胞的表現是分化良好的，但這些細胞缺少毒殺細胞顆粒的表現，使他們喪失細胞毒殺的能力。我們的研究還證明，在適當的體外刺激下，乳癌病患腫瘤內浸潤淋巴球是能夠恢復適當的抗腫瘤免疫反應，這個發現對於日後發展乳癌的免疫療法是非常有幫助。經由這個研究，我們可以對於乳癌病人其腫瘤浸潤淋巴細胞的特性，有進一步的瞭解，此結果將有助於日後發展出對於乳癌有效的免疫治療策略。

Abstract

Background:

To determine the functional attributes of CD4+CD25+regulatory T cells (Tregs) in cancer microenvironment

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Material and Methods:

Triple-color flow cytometry was utilized to study the phenotype expression of CD4+CD25+Tregs and CD8+T-cell in the peripheral blood lymphocytes (PBLs) and tumor infiltrating lymphocytes (TILs) of 30 stage I to III breast cancer.

Results:

The prevalence of CD4+CD25+T cells was significantly higher in the TILs than PBLs. The expression of FOXP3, CD103 and GITR on CD4+CD25+Tregs was lower in PBLs than TILs. Most tumor-infiltrating CD8+T cells were CD28-CD45RA-CD45RO+CCR7-, suggesting good terminal differentiation. Most of them had an activated role with CD69+CD103+CD152+. Functionally, both granzyme B and perforin were scarcely expressed in peripheral Tregs but were highly expressed in Treg cells in the tumor micro-environment. On the contrary, CD8+cytotoxic T cells derived from PBLs expressed both granzyme B and perforin, and were significantly higher than those in TILs. Further functional assays demonstrated that Th1 cytokines and cytotoxic molecules can be synchronously up-regulated in CD8+cytotoxic T cells.

Conclusions:

Tregs in the tumor microenvironment may abrogate CD8+T cell cytotoxicity in a granzyme B-and perforin-dependent conduit. Decreases in both Th1 cytokines and cytotoxic enzymes are relevant for Treg-mediated restraint of tumor clearance in vivo. Of clinical significance, the expression of Tregs in TILs may mediate T-cell immune repression within cancer milieu.

Keywords: Breast cancer, regulatory T cells, CD8+ T cells, tumor-infiltrating lymphocytes, memory T cells

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第一章　緒論

第一節　研究背景

腫瘤微環境與免疫系統

早於 1909 年，Paul Ehrlich 首先提出「免疫監督假說」（immunosurveillance hypothesis），他認為免疫系統會辨識體內的惡性腫瘤細胞並加以清除，在對抗癌細胞的發生與蔓延中，免疫系統扮演一個相當重要的角色[1]。

在腫瘤的微環境（microenvironment）中，腫瘤細胞與宿主免疫細胞之間的交互作用，會產生免疫編輯（immunoediting）的過程。腫瘤細胞與免疫細胞間的消長，最終結果可能為其中三者之一：腫瘤細胞減少（elimination）、或腫瘤細胞與免疫細胞平衡（equilibrium），或腫瘤細胞逃脫（escape）[2]。在平衡期中，腫瘤細胞經與免疫系統作用後，可能會篩選出較少引發免疫反應的腫瘤細胞留存下來，反而抵抗免疫監督機制[3]。

在腫瘤的微環境裡，可以發現有許多類型的免疫細胞存在，其中各種類型的淋巴細胞統稱為腫瘤浸潤淋巴細胞（tumor-infiltrating lymphocytes, TIL)。

調節性T細胞

有一群 T 細胞本身具有負向調節免疫反應的作用，稱為調節性T細胞（regulatory T cell, Treg）。調節性 T 細胞能抑制屬於 Th1 細胞性（cell-mediated）與屬於 Th2 體液性（humoral）的免疫反應，也能抑制 CD4+ 或 CD8+ T 細胞的作用[4]。免疫系統對自身抗原有容忍性，有一部分要靠調節性 T 細胞的抑制作用。調節性 T 細胞已被證實參與宿主的多種免疫反應，如感染症、免疫系統耐受移植器官、自體免疫疾病與腫瘤免疫等[5-8]。

調節性 T 細胞目前已發現分為許多類亞型，但佔最多數的為帶有 CD4+CD25+ 此型的 T 細胞，其 CD25 為 IL-2 receptor 的 α chain。調節性 T 細胞依形成的來源分為：自然產生的調節性 T 細胞（natural occurring Treg），和抗原專一的調節性 T 細胞（antigen-specific Treg）。前者是由胸腺發展而來，後者為經抗原刺激後產生。調節性 T 細胞會表現轉錄因子 FOXP3（Forkhead/winged helix of transcription factor P3），此轉錄因子對調節性 T 細胞的成熟與功能有很大的影響。

調節性 T 細胞在腫瘤的微環境中，已被發現會抑制宿主免疫系統清除腫瘤細胞，其中可能的機轉包括藉由阻止毒殺細胞（cytotoxic cells）的增生，或抑制免疫反應中必要 cytokine 的產生達到抑制免疫系統抗腫瘤的作用[9, 10]。調節性 T 細胞亦會經由分泌 cytokine 抑制免疫系統，如 IL-10（interleukin 10）與 TGFβ1（transforming growth factorβ1）等[9]。

記憶T細胞

Sallusto[11] 首先提出免疫系統中的記憶 T 細胞（memory T lymphocyte）可以依照其細胞表面 CCR7 有無表現，區分為兩種類型：central memory（T_{CM}）及 effector memory（T_{EM}）。前者 T_{CM} 細胞表面具有 CCR7，而擁有返回次級淋巴器官的能力，能分泌 IL-2，但較缺乏 effector 的功能。後者 T_{EM} 細胞表面失去 CCR7，喪失返回次級淋巴器官的能力，較少分泌 IL-2，但能分泌大量的 INFγ，擁有較多較快的 effector 功能。

依據其假說，從 naïve cell 增長為 T_{CM} 細胞，與再成為 T_{EM} 細胞是一種階梯式演變過程[12]。naïve T 細胞在抗原呈現細胞（antigen presenting cells，如樹突狀細胞等）的作用下，移轉到次級的淋巴器官。一旦經刺激活化後將會快速增殖形成 effector cell，在免疫反應後有一部分的 T 細胞會形成記憶細胞，以備他日再後刺激後能再增生為 effector cell。

腫瘤細胞逃脫免疫系統監控後，有可能進一步發生遠處轉移。遠端的轉移是目前癌症治療上棘手的問題。運用免疫治療被視為解決此困境的一項具有潛力的療法。目前文獻所載，對有遠處轉移的黑色素瘤病患，若接受活化過的自身 CD8+ T 細胞治療，已有多起初步報告顯示具有一定的治療效果[13-15]。

第二節　腫瘤浸潤細胞與癌症相關性之文獻回顧

依據之前本實驗室對乳癌病患腫瘤浸潤淋巴球的測定[16]，發現其中的 CD8+ T 細胞比例會增加，而 CD4+ T 細胞的比例會減少，其增加的 CD8+ T 細胞與乳癌疾病期別進展有顯著地增加，即 TIL 中的 CD4/CD8 的比例會顯著的減少。CD4/CD8 比例有明顯相關於淋巴血管侵襲與淋巴結轉移，對疾病的預後有重要的意義。

調節性 T 細胞已在多種腫瘤上被發現與疾病的預後有相關聯，如頭頸部腫

瘤[17]、卵巢癌[18, 19]、胰臟癌[20]、肝癌[21]、胃癌[22]、大腸直腸癌[23]、肺癌[19]、乳癌[16]及黑色素細胞癌[24]等。

Liyanage[25] 取樣35位乳癌病患的周邊血液、腫瘤組織、與有腫瘤侵襲的淋巴結，對照正常志願者的血液與良性乳房疾病病患的組織細胞，分析發現乳癌病患血液中的 CD4+CD25+ T 細胞比例會升高，但在其中表現 CD45RO 與 CTLA-4 的部分則與對照組相同。

Leong[26] 則討論乳癌病患 TIL 中表現 regulatory cell 與 effector cell 的組成，他們取樣自47位乳癌病患的腫瘤組織，發現在 TIL 中 CD8+ 的淋巴球佔大多數（23.4%)，其次為 CD4+ 的淋巴球（12.6%）及 CD56+ 的 NK cell（6.4%）。而 CD4+/CD8+ 的比例在乳癌病患下降為0.8。在 CD8+ 細胞中，有69%為 effector type(CD8+CD28+)，相對的有31%則為 suppressor type。在 CD4+ 細胞中，有59%為 regulatory type（CD4+CD25+），其中更有43%同時表現有 CD152（CTLA-4）及 FOXP3 分子。

Pagès[27] 對大腸直腸癌的患者分析，發現 TIL 的記憶 T 細胞與 effector T 細胞（CD45RO+ 者）的多寡與腫瘤的微小轉移（micrometastases）有相關聯性，分析發現無微小轉移的病患，CD45RO+ 細胞比例較高，預後較佳，五年存活率為46.3%；然而有微小轉移的病患，CD45RO+ 細胞比例較低，預後較差，五年存活率降至23.7%。

第三節　研究目的

腫瘤的生長會伴隨人體免疫系統的反應，若腫瘤細胞得以逃脫宿主免疫系統清除，必是藉由影響腫瘤微環境免疫細胞的作用。我們的研究目的在於找出乳癌細胞如何逃避宿主的免疫監控，進一步希望對於未來發展免疫調節治療有所幫助。

我們以流體細胞儀分析乳癌病患 CD4+CD25+ 調節性 T 細胞及其 FOXP3、GITR、CD103 和 CTLA-4 的表現，另外也會分析調節性 T 細胞與 CD8+ 記憶 T 細胞及 effector cell 表現之相關性。然後我們將檢測CD4+CD25+ 調節性 T 細胞分泌 Th1 cytokines（IFN-γ、IL-12 和 TNF-α）和 Th2 cytokines（IL-4 和 IL-10）的狀態，並分析調節性 T 細胞與毒殺性 T 細胞內毒殺顆粒

（包括 granzyme B 及 perforin）的表現。以期能找出癌細胞影響免疫系統之途徑，進一步能針對腫瘤免疫調控（immuno –regulation)之研究。本研究探討乳癌病患腫瘤浸潤淋巴細胞中 CD4+ T 細胞與 CD8+ T 細胞其相關免疫標記表現差異，包含檢測 T 細胞分化、T 細胞 effector 分子相關免疫標記，檢測 CD8+ T 細胞是否能經體外刺激在產生與細胞毒殺作用相關的 cytokine 與 effector 分子。

第四節　研究假說

我們有興趣的是：乳癌組織腫瘤內浸潤淋巴球的 effector 型態與記憶型態比例為何？是否這些調節性 T 細胞分泌毒殺顆粒 granzyme B 及 perforin？並進一步抑制毒殺性 T 細胞的毒殺反應？

研究的假說是：乳癌組織腫瘤內浸潤淋巴細胞，其表現的免疫標記和周邊血液中的淋巴細胞相異。這些調節性 T 細胞分泌毒殺顆粒 granzyme B 及 perforin，並進一步抑制毒殺性 T 細胞的毒殺反應。

第二章　研究方法與材料

第一節　研究材料

實驗標本的收集

臨床診斷第一至第三期乳癌的病患，依疾病狀況接受手術治療，包含改良式乳房切除手術或乳房保留型手術。

所有病患須符合的標準有：（1）均有病理組織診斷確認為乳癌者、（2）無乳腺發炎、（3）無免疫功能缺乏狀態，非人類免疫缺乏病毒 HIV 感染者、（4）排除曾因乳癌接受過手術或化學治療者、（5）罹病時無懷孕狀態、（6）無其他共存發炎疾病。

（一） 實驗組：病患乳癌腫瘤組織將會被收集成實驗組（註記為 TIL）。收集的腫瘤標本期望至少有一立方公分以上體積。

（二） 對照組：包含病患的血液（註記為 PBL）。血液採集於病患在接受麻醉完成後，手術治療前的靜脈血液標本，至少有 20cc 以上的血液，注入含抗凝劑肝素（heparin）的血液試管中。

病歷基本資料登錄

依據病理報告的結果，記錄每個病例的組織診斷（histologic type）、腫瘤大小、腋下淋巴結轉移數目、癌症分期（stage）、癌症病理分級（histological grade）、賀爾蒙受體表現（ER 與 PR）、HER-2/Neu 受體表現、淋巴或血管侵犯狀態。癌症的病理分級區分以 Nottingham combined histologic grade 為標準，區分為第一級（grade 1）、第二級（grade 2）及第三級（grade 3）。癌症分期依據 2010 年 AJCC 的定義為標準。

第二節　研究方法

分離純化浸潤淋巴細胞

本實驗運用機械式研磨萃取法（Mechanical dispersal technique）分離組織內的淋巴細胞[16, 28-30]。乳癌組織經無菌收集後，用 phosphate-buffered saline（PBS） 將其上的血塊沖洗乾淨，以刀片切成碎片。加入 RPMI-1640 medium（Gibco, Life Technologies, Grand Island, NY, USA）研磨後，先後以孔徑 380μm

及孔徑 45.7μm 的篩網過濾。

濾液先以 400xg 離心15分鐘，再運用不同濃度的 Percoll solution 分層（包含 30%、55%、與 100% 三層濃度)。富含單核細胞（mononuclear cell）的懸浮液層會位於 55% 及 100% Percoll solution 兩層間，而癌細胞的懸浮液層會位於 30% 及 55% Percoll solution 兩層間。以此方法可將乳癌細胞和腫瘤浸潤淋巴細胞分離，正常乳房細胞也可以此方法分離出來。收集的單核細胞再以 RPMI-1640 medium 清洗兩次。再利用 MiniMACS 分離純化出 CD4+ 及CD8+ T 細胞。

運用 trypan blue 染色法來檢測收集細胞存活情形。其原理依據細胞膜的完整性，當細胞受損時，細胞膜的通透性被破壞，細胞膜會破裂不完整，trypan blue 藍色染料就會進入細胞內，使細胞著色。而存活的細胞因細胞膜仍完整，染劑不會進入細胞內，細胞不會被染色，於顯微鏡下可看出細胞的存活比率。

於手術前抽取病患 20 cc 靜脈血，置於含抗凝劑肝素的試管中，運用 Ficoll hypaque（1.077 density，Amersham Biosciences AB, Uppsala, Sweden）分離出周邊血液中的單核細胞（peripheral blood mononuclear cells），分離出的單核細胞球再置於 RPMI-1640 medium，使其濃度為 $1x10^6$ cells/mL。再利用 MiniMACS 分離純化出 CD4+ 及 CD8+ T 細胞。

細胞表面免疫標記測定

運用標定螢光物質（fluorochrome）的單株抗體，對前述方法分離出的周邊血液、正常組織與腫瘤組織浸潤淋巴球表面抗原檢測。使用的螢光物質有 fluorescein isothiocyanate（FITC），phycoerythin（PE）及 Peridinin chlorophyll protein（Per-CP）（Beckton-Dickinson Inc., San Jose, CA, USA）。

使用的單株抗體安排如下:

anti-CD4-FITC/anti-CD8-PE/anti-CD3-PerCP、

anti-CD16+56-FITC/anti-CD19-PE/anti-CD3-PerCP、

anti-CCR7-FITC/anti-CD45RO-PE/anti-CD25-PerCP、

anti-CD69-FITC/anti-CD28-PE/anti-CD25-PerCP、

anti-CD103-FITC/anti-CD152-PE/anti-CD25-PerCP、

anti-CCR7-FITC/anti-CD45RO-PE/anti-CD8-PerCP、

anti-CD103-FITC/anti-CD28-PE/anti-CD8-PerCP、

細胞內細胞激素的染色

為了偵測不同細胞群產生的細胞激素，將分離出來的周邊血液 CD4+ 及 CD8+ T 細胞與腫瘤浸潤 CD4+ 及 CD8+ T 細胞，先加入 20ng/ml phorbol 12-myristate 13-acetate（PMA）和 1μM ionomycin，再同時加入 4μM monensin 一起經4小時刺激，以 PBS 沖洗細胞一次後，加入含螢光標定的單株抗體（anti-CD8- PerCP或anti-CD25-PerCP），混合均勻後，於 4˚C 溫度避光作用30分鐘。再利用 Cytofix/Cytoperm buffer 作用一小時，之後以 permeabilization buffer 連續三次沖洗細胞，使細胞固定及破壞細胞膜讓通透性增加，在染細胞內分子前，先以 2% 牛胚胎血清結合非特異性抗原，再藉由 anti-granzyme B-FITC 及 anti-perforin-FITC 的單株抗體作用。

使用的單株抗體安排如下:

anti-Granzyme B-FITC/anti-FoxP3-PE/anti-CD25-PerCP、

anti-Perforin-FITC/anti-GITR-PE/anti-CD25-PerCP、

anti- Granzyme B -FITC/anti-CD69-PE/anti-CD8-PerCP、

anti- Perforin -FITC/anti-CD152-PE/anti-CD8-PerCP

流式細胞儀分析

將要檢測的細胞懸浮液加入單株抗體均勻混合，在 4˚C 溫度30分鐘後，以含 2% 牛胚胎血清及 0.1%NaN3（sodium azide）的 PBS 液沖洗兩次，以流式細胞儀分析。其原理為當細胞或顆粒被雷射光激發後，產生 0.5˚– 5˚ 之前散射光（forward scatter, FSC）及 15˚– 150˚ 之側散射光（side scatter, SSC）。當顆粒本身帶有螢光物質，或被帶有螢光物質的抗體或被其它螢光物質染上的話，會產生波長不等的螢光。由於入射光的波長小於螢光的波長，因此由光的波長及強度變化，即可測出顆粒的大小（與 FSC 成正比），顆粒性（與 SSC 成正比）與特定抗體或螢光物質結合情形。這些波長及強度，可透過一連串的光學鏡片及偵測器，轉變成電子訊號，並以電腦加以記錄及分析。

細胞收集

以一種間接磁性標籤制度與磁珠 MACS（Miltenyi Biotec, Gladbach,

Germany）用來純化 CD8+ T 細胞。簡單來說，細胞與20微升的 Hepten-Antibody Cocktail(含有 CD4，CD11b，CD16，CD19，CD36，及 CD56 抗體; Miltenyi Biotec, Gladbach, Germany）懸浮培養於低溫下（6°C 至 12°C 間）10分鐘。經過反覆洗滌後，加入每20微升含有10^7個細胞總數的 Hepten-Antibody 磁珠，有磁性標記的細胞會通過一個磁珠分離的磁場，分離流出來的是豐富純化的毒殺性 CD8+ T 細胞。

經由加入 CD4+CD25+ 磁珠（CD4+CD25+ bead selection kit）（Miltenyi Biotec）CD4+CD25+ T 細胞及 CD4+CD25- T 細胞會從乳癌病患的週邊血液分離純化。我們再以流式細胞儀，加入 anti-CD8-FITC，anti-CD4-FITC 及 anti-CD25-PerCP 來檢測細胞的純度，豐富純化的細胞如果純度大於 90%，即可用於細胞增殖分析和細胞毒性分析。

細胞培養

將周邊血液中的單核細胞和腫瘤內浸潤淋巴球培養在含10%人類血清的 RPMI 1640，經過 37℃，5% CO2 之培養4小時後，使細胞數目達到 $1x10^6$/mL。然後以 anti-CD3/anti-CD28 抗體刺激2小時，並培養在 3:1 的比例，使最終濃度為 $1x10^6$/mL，在含 10% 人類血清的 RPMI 1640 且在 5% 二氧化碳 37 °C 共2天。在分析前4小時前加入 Brefeldin A（10mg/mL）。將磁珠分離並培養細胞。以流式細胞儀分析免疫表型為 CD4+ T 細胞，並且以免疫磁珠 MiniMACS（Miltenyi Biotec）富集。使用的螢光單株抗體有 fluorescein isothiocyanate（FITC），phycoerythin（PE）及 Peridinin chlorophyll protein（Per-CP）（Beckton-Dickinson Inc., San Jose, CA, USA）。

使用的單株抗體安排如下:

PE- 染色的單株抗體：anti-CD25, anti-FoxP3, anti-45RO, anti-CD28, anti-CD69, anti-CD152, anti-GITR, anti-IL2, anti-IL4, anti-IL10, anti-IFNγ, anti-TNFα

FITC- 染色的單株抗體:：anti-CD4, anti-CD103, anti-CD45RA, anti-CCR7, anti-granzym B, antiperforin

PerCP - 染色的單株抗體：anti-CD8, anti-CD4, anti-CD25, and anti-CD3.

第三節　分析方法

表現比例的定義

為了比較調節性 T 細胞上各種不同抗原的表現，我們定義表現比例（expression ratio, ER）=（抗原表現為陽性之 CD4+CD25+ 調節性 T 細胞／所有調節性 T 細胞）× 100%，例如: FOXP3 在 CD4+CD25+ 調節性 T 細胞的表現比例為 ER of FOXP3 ＝（percentage of gated CD4+CD25+FOXP3+ T lymphocytes／percentage of all gated CD4+CD25+ T lymphocytes）× 100%。

為了比較 CD8+ T 細胞上各種不同抗原的表現，我們定義表現比例（expression ratio, ER）＝（抗原表現為陽性之 CD3+CD8+ T 細胞／所有 CD3+CD8+ T 細胞）× 100%，例如: CD28 在 CD3+CD8+ T 細胞的表現比例為 ER of CD28＝（percentage of gated CD3+CD8+CD28+ T lymphocytes／percentage of all gated CD3+CD8+ T lymphocytes）× 100%。

分析軟體與統計分析

流式細胞儀檢測收集的資料使用 WinMDI 2.9 版（Joseph Trotter, Scripps Institute, La Jolla, CA）軟體分析。收集的資料使用 SPSS 第12版分析（SPSS Inc., Chicago, IL, USA），各項細胞表面抗原的表現比例視為連續變項，以平均值±標準差（mean±SD）來表示，比較每一病患 TIL 及 PBL 的表現使用成對 t 檢定（paired T test）。$P<0.05$ 視為有統計上顯著差異。

第三章　結果

第一節　比較 Treg 與 CD8+ T 細胞

個案基本資料

共有30位病患列入分析，性別均為女性，平均年齡為56.8歲（28歲至89歲）。接受乳房保留型手術有16位，接受乳房切除手術有14位。無腋下淋巴結轉移有16位，其餘14位有轉移。病理檢查有17位有發現淋巴管或血管的微小侵襲（表一）。

乳癌病患的腫瘤內浸潤淋巴球含有較高比例的 CD4+CD25+調節T細胞

我們以流式細胞儀分析比較乳癌病患的週邊血液淋巴細胞球及腫瘤內浸潤淋巴球內 CD4+CD25+調節性 T 細胞的表現，發現乳癌病患的週邊血液內 CD4+CD25+ 調節性 T 細胞的比例明顯低於腫瘤內浸潤淋巴球內的比例（11.6 ±2.0 % vs. 37.4 ± 4.4%，P=0.001）（圖一）。

乳癌病患的腫瘤內浸潤淋巴細胞與週邊血液淋巴細胞細胞 CD4+CD25+ 調節性T細胞的組成不同

乳癌病患的腫瘤內浸潤淋巴細胞與週邊血液淋巴細胞的調節性 T 細胞均表現 CD45RO，但很少表現為 CCR7+CD45RO-（näive）（8% 及 5%），特別是腫瘤內浸潤的 CD4+CD25+ 調節性 T 細胞幾乎不表現 CCR7（圖二 A）。而週邊血液 CD4+CD25+ 調節性 T 細胞明顯有較高比例的 CCR7+CD45RO+ 的記憶細胞（central memory cells，53.8% 及 34.0%，P=0.018），而 CCR7-CD45RO+ 的記憶細胞（effector memory cells）組成則相反（33.9% 及 59.7%，P=0.04）（圖二 A）。

乳癌病患腫瘤內的CD4+CD25+調節性T細胞表現較高的FOXP3、GITR與CD103

將 CD4+CD25+ 調節性 T 細胞做進一步分析，比較其上 FOXP3，CD152，CD103 及 GITR 等免疫標記分子的表現。FOXP3、CD103 與 GITR 等活化標記於腫瘤中的調節性 T 細胞表現亦增加（FOXP3，46.7% 及 71.8%，

P=0.033; CD103，11.8% 及 31.5%，P＝0.027; GITR，28.9% 及 80.5%，P=0.04）。而 CD152 在調節性 T 細胞的表現於腫瘤中與週邊血液淋巴細胞內則是相當的（77%及74%，P=0.7）（圖二B）。

乳癌腫瘤內各種記憶CD8+ T細胞的表現

乳癌病患的腫瘤內與週邊血液的 CD8+ T 細胞均表現 CD28，CD45RA 及 CD45RO（圖三A，B），但是腫瘤內的 CD8+ T 細胞表現較少的 CCR7（圖三B），週邊血液內的 CD8+ T 細胞有較多組成比例的 CCR7+CD45RO- näive T cells （20.9% 及 4.5%，P<0.001），及較少的 CCR7-CD45RO+ effector memory T cells （32.3% 及 67.5%，P<0.001），而 CCR7+CD45RO+ central memory T cells 在腫瘤內浸潤淋巴球與週邊血液單核細胞球內的 CD8+ T 細胞的表現則是相當的（11.9% vs. 16.3%，P=0.175）（圖三C）。

腫瘤內的CD8+ T 細胞相較於週邊血液中的 CD8+ T 細胞表現較高的 CD69（39.7% vs. 9.6%，P=0.002），CD103 （36.3% vs. 2.4%，P<0.001），及 CD152 （54.7% vs. 24.1%，P=0.014）（圖三C），而 CD45RA 則較少表現（9.6% vs.52.4%，P<0.001）（圖三A）。所以大部分的腫瘤內的CD8+ T 細胞的表現是 CD28-CD45RA-CD45RO+CCR7-，顯示著他們是分化良好的一群細胞，而且大部分為 CD69+CD103+CD152+。

第二節　分析毒殺細胞分子與誘發實驗

乳癌病患腫瘤內CD4+CD25+調節性T細胞表現較多毒殺顆粒

乳癌病患的腫瘤內的 CD4+CD25+ 調節性 T 細胞相比週邊血液中的 CD4+CD25+ 調節性 T 細胞，明顯表現較多的毒殺顆粒（granzyme B，23.5% vs. 11.5%，P<0.001；perforin，37.8% vs. 17.3%，P<0.001）（圖四A）。

乳癌腫瘤內CD8+ T細胞功能被抑制

乳癌病患的腫瘤內的CD8+ T 細胞明顯分泌較少的毒殺顆粒（granzyme B，10.6% vs. 27.3%，P<0.001；perforin，17.6% vs. 48.9%，P<0.001）（圖四B）。

乳癌腫瘤內浸潤淋巴球的Th1 細胞激素分泌可被誘發

為了分析乳癌病患的腫瘤內浸潤淋巴細胞與週邊血液淋巴細胞的活性，我們以 anti-CD3/anti-CD28 刺激淋巴細胞，然後檢測其分泌細胞激素的能力。發現 IL-2，IFN-γ，及TNF-α 的分泌在腫瘤內浸潤淋巴細胞與週邊血液淋巴細胞中是相當的，這表示 anti-CD3/anti-CD28 可誘發出乳癌腫瘤內浸潤淋巴細胞的 Th1 細胞激素即 IL-2，IFN-γ，及 TNF-α 的分泌。

我們也檢測 Th2 相關的細胞激素即 IL-4 與 IL-10 的分泌，發現於腫瘤內浸潤淋巴細胞與週邊血液細胞中分泌的比例均減少，表示經過 anti-CD3/anti-CD28 刺激後，腫瘤內浸潤淋巴細胞並沒有分泌較多的 Th2 細胞激素。我們的實驗證明腫瘤內浸潤淋巴細胞的活性是完整的，並且在某些刺激下是會再回復（圖五）。

乳癌腫瘤內CD8+ T細胞被抑制的功能是可回復的

為了進一步分析 Th1 cytokine 在乳癌病患的腫瘤內浸潤淋巴球與週邊血液單核細胞球造成的影響，我們以流式細胞儀檢測其 CD8+ T 細胞表現細胞毒殺顆粒（perforin 與 granzyme B）的能力。在經過體外刺激後，腫瘤內CD8+ T 細胞分泌細胞毒殺顆粒的表現比例，與週邊血液淋巴細胞是相當的（圖六）。

第四章　討論

根據行政院衛生署國民健康局的資料，在台灣乳癌的的發生率與死亡率逐年增加，從民國87年至91年，年齡標準化發生率由每十萬人口之31.48人增加至43.27人。而96年新增病例約六千五百位乳癌患者，每十萬人年齡標準化發生率49人，已成為台灣地區女性最好發癌症。民國96年台灣地區女性乳癌標準化死亡率為每十萬人口10.7人，已攀升至國人女性主要癌症死因排行榜第四名。民國96年約有一千五百位女性死於乳房惡性腫瘤。目前的治療包含腫瘤切除、化學治療、放射線治療及賀爾蒙治療等，但對於侵襲癌及轉移癌的治療，仍存有未殆之處。因為乳癌之轉移是有階段性的，抗癌的免疫反應對於癌症轉移扮演重要角色。我們的研究在於找出乳癌細胞如何逃避宿主的免疫監控，進一步希望對於未來發展免疫調節治療有所幫助。

以前許多關於腫瘤內浸潤淋巴細胞的研究多是以組織切片染色或免疫組織學的方法進行，而部份學者利用酵素分解法來分離腫瘤內浸潤淋巴球，又可能造成免疫細胞活化狀態的改變。我們利用機械式研磨萃取法[16, 28-30]來獲得乳癌之腫瘤內浸潤淋巴細胞，此方法有別於國外常用之酵素分離法，能有效且不影響活化狀態下分離免疫細胞。

乳癌組織比正常乳房組織含有更多的浸潤 T 細胞，其中又以 CD3+ CD8+ T 淋巴球佔大多數[28]。然而這些 CD8+ T 淋巴細胞卻無法有效發揮其免疫監督的功能，致使乳癌進一步侵襲及惡化。屬於 CD4+ T 細胞的一群細胞，稱之為 CD4+CD25+ 調節性 T 細胞，此類細胞聚集於腫瘤微環境中，減低了免疫系統清除腫瘤的作用。我們目前的研究證實了乳癌病患的腫瘤內浸潤淋巴球比週邊血液淋巴細胞含有較高比例的 CD4+CD25+ 調節性 T 細胞，乳癌病患的 CD4+CD25+ 調節性 T 細胞和 CD8+ T 細胞的抑制是相關的，因此腫瘤微環境中的 CD4+CD25+ 調節性 T 細胞含量的多寡可能和乳癌病患的預後有關。

CD4+CD25+ 調節性 T 細胞的細胞標記包括表現 FOXP3，而這些細胞表現於不論是腫瘤內浸潤淋巴細胞、週邊血液淋巴細胞球或者是局部淋巴結的多寡，均會影響腫瘤的侵犯，並且在有效治療後，其 FOXP3 表現會降低[31]。原因有可能是腫瘤內浸潤淋巴球中的 CD4+CD25+ 調節性 T 細胞會影響腫瘤微

環境的侵犯，而週邊血液的 CD4+CD25+ 調節性 T 細胞相關於腫瘤的全身轉移。

然而調節性 T 細胞抑制免疫的機轉仍待進一步分析，這些有腫瘤特異性的調節性 T 細胞需要被活化，細胞接觸性的誘發他們抑制相關的抗腫瘤T細胞，包括 CD8+ 毒殺性 T 細胞及 CD4+ Th 細胞，並抑制這些 T 細胞的毒殺能力、增生繁殖、及 Th1 細胞激素的分泌能力[31]。在我們目前對乳癌病患的腫瘤內浸潤淋巴球的研究，這些調節T細胞可能依賴溶細胞酶的重要途徑，去抑制腫瘤免疫反應，在我們的研究中，granzyme B 很少在週邊血液的 CD4+CD25+ 調節性 T 細胞表現，而在腫瘤內的 CD4+CD25+ 調節性 T 細胞，granzyme B 表現比例則升高。Perforin 在 CD4+CD25+ 調節性 T 細胞的表現也有類似的結果。

Perforin 為一個使細胞膜穿洞的蛋白質，granzyme 則屬於 serine 蛋白酶，此二者皆為毒殺性細胞的重要作用分子。藉由動物實驗與人類疾病的觀察，可以了解這些分子在免疫作用的重要性。動物實驗發現缺乏 perforin 的 knockout 老鼠和 wild-type 老鼠相比，其清除腫瘤的能力會降低[32, 33]。在人類有一遺傳性 perforin 變異與缺乏疾病，即第二型家族性嗜血症候群，此類病人有半數將會於幼童或青少年時期罹患至少一種以上的血液癌症[34]。

之前有研究指出腫瘤微環境的調節性 T 細胞可依賴 granzyme B 和 perforin 相關的途徑，引發自然殺手細胞與 CD8+ T 細胞的死亡[35]，因此 granzyme B 和 perforin 被認為和調節性 T 細胞抑制免疫的機轉有關。

Grossman 藉由體外實驗（*in vitro*）證明了人類的調節性 T 細胞可利用 granzyme B 和 perforin 相關途徑，去殺死自體的免疫細胞[36]。granzyme B 和 perforin 是自然殺手細胞與 CD8+ T 細胞用來殺死其目標細胞的重要工具，如今發現調節性 T 細胞也可能利用 granzyme B 和 perforin 的途徑，來抑制免疫細胞的反應，進一步使其無法清除腫瘤細胞[37]；另外有研究指出，調節 T 細胞也可能利用 granzyme B 和 perforin 的途徑，來抑制 B 細胞的增殖[38]。Gondek 也報告活化的老鼠調節性 T 細胞可利用 granzyme B 和 perforin 的途徑，去抑制免疫細胞[39]。這些研究均利用體外實驗來活化調節T細胞。

事實上調節性 T 細胞於體內（*in vivo*）是否表現 granzyme，或者這些毒殺

顆粒是否真的是調節性 T 細胞用來抑制免疫細胞的途徑，仍然待證明。在我們的研究中，我們直接利用流式細胞儀來分析腫瘤內浸潤淋巴球的 CD4+CD25+調節性 T 細胞，檢測其表現 granzyme B 和 perforin 的比例，我們發現腫瘤微環境中的調節 T 細胞有很高的比例表現 granzyme B 和 perforin；相反的，腫瘤微環境中的 CD8+ T 細胞則表現 granzyme B 和 perforin 的比例減少。而我們目前的研究也證明了調節性 T 細胞可抑制 CD8+ T 細胞清除腫瘤的能力，我們的研究指出 granzyme B 和 perforin 不只是自然殺手細胞與 CD8+ T 細胞用來殺死其目標細胞的重要工具，同樣的，granzyme B 和 perforin 也被調節性 T 細胞利用來抑制這些免疫細胞反應的重要工具。因此針對調節性 T 細胞的治療方向被認為是腫瘤免疫治療之一重要課題[40]。

藉由刺激 CD28，可達到活化目標的 T 細胞。有研究發現在免疫缺陷的小鼠給予 anti-CD28 的抗體治療，可以治癒轉殖於其上的人類腫瘤及大鼠腫瘤[41]。在我們的研究中，為了分析乳癌病患的腫瘤內浸潤淋巴細胞與週邊血液淋巴細胞的活性，我們在體外實驗以 anti-CD3/anti-CD28 刺激腫瘤內浸潤淋巴球，然後檢測其分泌細胞激素的能力，結果顯示腫瘤內浸潤淋巴細胞有分泌 IL-2，IFN-γ，及 TNF-α 的能力，這些 Th1 細胞激素是自然殺手細胞與 CD8+ T 細胞毒殺能力所必需的，IFN-γ 及 TNF-α 對於腫瘤細胞有直接毒殺的能力，這些細胞激素的增加分泌可能有助於誘導細胞毒性功能，招募和擴增抗原呈現細胞（antigen-presenting cells），並且調控直接性的抗腫瘤作用。綜合以上討論，由目前的研究結果發現這些功能不正常的 T 細胞，在某些環境刺激下是可回復的。但腫瘤細胞藉由多種的免疫機制抑制免疫系統清除腫瘤，從而減弱現今免疫治療的效果，因此在免疫療法要成功應用於腫瘤治療之前，先要扭轉腫瘤誘導的免疫抑制。

在我們的研究中，我們發現大部分的腫瘤內浸潤淋巴球的 CD8+ T 細胞表現是 CD28-CD45RA-CD45RO+CCR7-，顯示著他們是分化良好的一群細胞，而且他們大部分是 CD69+CD103+CD152+，表示著他們在腫瘤微環境中的活躍性。調節性 T 細胞可選擇性的調節 CD8+ T 細胞的終端效應功能，來調節 T 細胞反應，有研究指出，調節性 T 細胞抑制毒殺性 T 細胞的功能是可逆性的[42]，被調節的毒殺性 T 細胞其增殖能力、誘導細胞毒性效應分子和分泌之能力、原位運動、或與目標細胞形成抗原複合物的能力均沒有缺陷；在調節性T細

胞存在下，只有毒殺性 T 細胞分泌毒殺顆粒的能力會受到抑制，調節性 T 細胞也可以選擇性及可逆性地經由 TGF-β 途徑，去干擾毒殺性 T 細胞分泌毒殺顆粒的能力，通過這種方法，調節性 T 細胞可以抑制毒殺性 T 細胞的毒殺作用，而不會影響啟動或分化。

第五章　展望

乳癌已成為台灣地區女性首要的好發癌症，而因為乳癌之轉移是有階段性的，抗癌的免疫反應對於癌症轉移過程有重要角色。我們的數據證明，調節性 T 細胞在控制乳癌抗癌免疫反應上，發揮重要作用，調節性 T 細胞會經由干擾毒殺性 T 細胞分泌毒殺顆粒的能力，進一步抑制免疫系統清除腫瘤細胞。我們期待在不久的將來，可釐清乳癌組織浸潤的調節性 T 細胞在活體內的功能，及其如何受細胞激素之調控，及如何影響毒殺性 T 細胞清除腫瘤細胞，更進一步幫助我們未來治療乳癌免疫治療的發展。

我們希望未來運用細胞增殖分析和細胞毒性分析，深入探討 CD4+CD25+ 調節性 T 細胞可能抑制 CD8+ T 細胞毒殺細胞的之可能機轉；對於免疫系統在腫瘤為環境中受抑制之現象做進一步的分析研究，以了解腫瘤癌細胞與免疫細胞間的互動關係，以期能進行腫瘤免疫調控（immuno-regulation）之研究。

第六章　論文英文簡述

INTRODUCTION

Regulatory T cells (Treg) are a subpopulation of T cells that can inhibit both cell-mediated (Th1) and humoral (Th2) responses. Treg cells have the ability to suppress the responses of both CD4+ and CD8+ T cells [4]. Most of these Treg cells are CD4+ and $CD25^{hi}$, which are naturally occurring and arise directly from the thymus [5, 9]. They have been shown to mediate the process of infection, tolerance of transplantation, autoimmunity, and tumor immunity [6, 7]. They may accumulate in the tumor microenvironment and suppress tumor-specific T-cell responses, thereby impairing the ability of the host immune system to defend against tumor progression. It has been proposed that Treg cells can attenuate anti-tumor immune responses by suppressing the proliferation and cytokine production of effector T cells in cancer milieu [9, 10].

In clinical human studies, an increased proportion of Treg cells in cancer milieu has been recognized in patients with various types of cancers with implication of a poor clinical outcome. Studies about Treg cells in patients with cancer are on the increase and also point to the key role of these cells in related disease progression [18, 43-45]. Both interleukin 10 (IL-10) and transforming growth factor (TGF) β1 produced by Treg cells may mediate immunosuppression in the tumor microenvironment. The detailed mechanisms by which Treg cells suppress antitumor immunity still remain mostly undefined [9].

In the present study, we tried to clarify the essential immune-regulatory roles of Treg cells from patients with breast cancer. We compared the subsets of Treg cells in tumor-infiltrating lymphocytes (TILs) and peripheral blood lymphocytes (PBLs) from

these patients. Furthermore, we measured the expressions of cytokines and cytotoxic molecules of CD8+T cells in functionally associated Treg cells from cancer milieu.

MATERIALS AND METHODS

Patient Recruitment

A total of 30 patients with Stage I–III breast cancer were enrolled prospectively in this study between December 2007 and December 2009. All patients who underwent surgery signed the Institutional Review Board-approved informed consent for release of specimens for research purposes. After the staging operation, the surgical specimens were examined carefully by experienced pathologists to exclude the possibility of coexisting malignancy. Each case of breast cancer was evaluated for clinical and pathological parameters including surgical stage, lymphatic or vascular permeation, lymph node metastatic status, and histologic grade. Surgical staging of each patient was defined according to the 7th edition of American Joint Committee on Cancer for breast cancer.

Collection of Tumor Tissue and Peripheral Blood

Collection of Tumor Tissue and Peripheral Blood Tissue specimens were aseptically excised immediately after surgery from at least four different tumor sites. Tissue fragments were carefully washed and reperfused with phosphate buffered saline to remove contaminated blood. TILs and PBLs were isolated by methods described in our previous study [28-30, 46].

Immunophenotyping Analysis by Flow Cytometry

Immunophenotyping Analysis by Flow Cytometry CD4+T cells were enriched by

magnetic cell sorting using a MiniMACS separator (Miltenyi Biotec). Monoclonal antibodies labeled with FITC, PE, and Per-CP (Immuno-cytometry System; Beckton-Dickinson Inc., San Jose, CA, USA) were used for three-color flow cytometry. The following matchings were arranged: a mixture of PE-coupled specific mAbs: anti-CD25, anti-FoxP3, anti-45RO, anti-CD28, anti-CD69, anti-CD152, anti-GITR, anti-IL2, anti-IL4, anti-IL10, anti-IFNγ, anti-TNFα; a mixture of FITC-coupled specific mAbs: anti-CD4, anti-CD103, anti-CD45RA, anti-CCR7, anti-granzym B, antiperforin; a mixture of PerCP-coupled specific mAbs: anti-CD8, anti-CD4, anti-CD25, and anti-CD3. For intracellular staining, cells were fixed and permeabilized with a BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit according to the manufacturer's instructions. A Simultest control (mouse IgG1-FITC + IgG2a-PE) was used as a background control. Three-color flow cytometry was performed on a FACSCaliber flow cytometer (Beckton-Dickinson Inc., San Jose, CA, USA). The regional gate was set on FL1 (anti-CD3-PerCP). Flow cytometry data was analyzed using WinMDI software.

Cell Culture

PBLs and TILs were thawed and cultured in RPMI 1640 containing 10% human AB sera for 4 h at 37°C in 5% CO2 in multiwell plates or flasks depending upon the cell number at $1x10^6$/ml. Cells stimulated by anti-CD3/anti-CD28-coated beads were first depleted of monocytes by a 2-h adherence in tissue culture flasks. Nonadherent cells were removed and cultured at a 3:1 bead:cell ratio at a final concentration of 1x106/ml in RPMI 1640 containing 10% human AB sera for 2 days at 37°C in 5% CO2. Brefeldin A(10mg/ml) was added 4-h prior to analysis. Cells were harvested and beads were removed by magnetic separation.

Definition of expression ratio (ER)

By considering the expression profiles of CD4+CD25+ T cells, ER was chosen to better reveal the expression level CD4+CD25+ T cells. For example, the ER of CD4+CD25+ T cells on $CD3^{+}CD4^{+}$ T cells fraction should be calculated as:

ER of CD4+CD25+ T cells = (percentage of gated CD3+CD4+CD25+ T cells)/(percentage of all gated $CD3^{+}CD4^{+}$ T cells) x 100%

The ER of CD4+CD25+FoxP3+ T cells on CD4+CD25+ T cells fraction should be calculated as:

ER of CD4+CD25+FoxP3+ T cells = (percentage of gated CD4+CD25+FoxP3+ T cells)/(percentage of all gated CD4+CD25+ T cells) x 100%

Statistical Analysis

The percentage expression of a given marker was obtained for a subset of T lymphocytes. All data were expressed as mean±standard error unless otherwise indicated. The paired t test was used to compare paired samples from the same patients. Statistical significance was defined by a P value less than 0.05.

RESULTS

Compositional differences of CD4+CD25+ Treg cells in PBLs and TILs in breast cancer patients

Samples of the peripheral blood and malignant tissue from BC patients were screened by flow cytometry for CD4+CD25+T cells. The mean ratio of CD4+CD25+Tregs in the PBLs was significantly lower than that in TILs (11.6% vs. 37.4%, respectively, *P*=0.001).The Tregs from both PBLs and TILs expressed membranous CD4, CD25,

and CD45RO, but rarely expressed CCR7+CD45RO- (naive) (8% and 5%, respectively).The Tregs from TILs rarely expressed CCR7. The circulating Treg cells expressed significantly higher CCR7+CD45RO+ (53.8% and 34.0%, respectively, P=0.018) (central memory, CM) and lower CCR7-CD45RO+(34.0% and 59.8%, respectively, P<0.05) (effector memory, EM) than the tumor infiltrating Tregs.

By gating on the CD4+CD25+ Tregs, four additional cell markers could be studied, FOXP3, CD152, CD103 and GITR. High levels of FOXP3, GITR and CD152 expression were detected in CD4+CD25+Tregs in all samples. The circulating Tregs expressed lower level of CD103. The expressions of FOXP3, CD103 and GITR on CD4+CD25+Tregs were lower in those from PBLs than those from TILs (FOXP3, 46.7% and 71.8%, respectively, P=0.033; CD103, 11.8% and 31.5%, respectively, P<0.05; GITR, 28.9% and 80.5%, respectively, P<0.05). However, similar levels of CD152 expression were detected in CD4+CD25+Tregs from blood and tissue (77% and 74%, respectively, P=0.7).

Significant functional compromise of CD8+TILs derived from breast cancer

The majority of TILs were CD4+and CD8+T lymphocytes, although the CD4+/CD8+ cell ratio varied substantially between samples. The expression of intracellular granzyme B and perforin, both markers of cytotoxic potential, was determined on CD4+CD25+Tregs and CD8+T lymphocytes from both peripheral blood and breast cancer tissue. Analysis of the CD4+CD25+Treg cells derived from TILs and PBLs showed that the expressions of granzyme B and perforin could be detected at diverse levels on CD4+CD25+Treg cells from TILs (granzyme B was detected in 23.5% and perforin in 37.8%), and were significantly more prominent than those from PBLs (granzyme B in 11.5% and perforin in 17.3%, respectively).

Analysis of the CD8+T lymphocyte fraction derived from TILs and PBLs showed that lower expression levels of granzyme B and perforin could be detected on gated CD8+T cells from TILs (granzyme B was detected in 10.6% and perforin in 17.6%) (Fig.3B). On the contrary, the expressions of granzyme B and perforin could be detected at diverse levels on CD8+T lymphocytes from PBLs (granzyme B was detected in 27.3% and perforin in 48.9%), and were significantly more prominent than those from TILs.

Treg-mediated immunosuppression of CD8+TILs is reversible

The CD8+ lymphocytes from PBLs and TILs both expressed membranous CD28, CD45RA and CD45RO (Fig.4A,B). The CD8+T lymphocytes from breast cancer express minimal CCR7 (Fig.4B). The circulating CD8+lymphocytes had a significantly more prominent fraction of CCR7+CD45RO- naive T cells (20.9% and 4.5%, respectively, $P<0.001$) and a smaller fraction of CCR7-CD45RO+effector memory T cells (32.3% and 67.5%, respectively, $P<0.001$) than infiltrating Tregs. Similar fractions of CCR7+CD45RO+ central memory T cells were detected in the CD8+T lymphocytes derived from autologous blood and tissue samples (11.9% vs. 16.3%, $P=0.175$). The infiltrating CD8+ T lymphocytes expressed significantly higher CD69 (39.7% vs. 9.6%, $P=0.002$), CD103 (36.3% vs. 2.4%, $P<0.001$), and CD152 (54.7% vs. 24.1%, $P=0.014$) (Fig.4C), but lower CD45RA (9.6% vs.52.4%, $P<0.001$) than circulating Tregs (Fig.4A). Most tumor-infiltrating CD8+T lymphocytes were CD28-CD45RA-CD45RO+CCR7-, suggesting good terminal differentiation. In addition, most of them were CD69+CD103+CD152+, signifying their activated role in the cancer milieu.

Enhanced Th1 Cytokine Production can be achieved in TILs from breast cancer

To assess the functional polarity of TILs, we measured the fractions of T cells secreting cytokines after anti-CD3/anti-CD28 co-stimulation. After stimulation, the percentage of TILs producing IL-2, IFN-γ, and TNF-α was similar to the PBLs. The anti-CD3/anti-CD28-coated beads were able to induce production of IL-2, IFN-γ, and TNF-α in TILs derived from breast cancer. We also measured the levels of the Th2 cytokines, IL-4 and IL-10, in the TILs and found them to be at low levels similar to those in the PBLs. There was no significant production of Th2 cytokines in the TILs after stimulation. Our data demonstrated that the functional polarity of TILs and the responding capacity were intact and that these T cells were able to mount a response against a tumor after appropriate activation stimuli.

Up-regulated expression of Perforin and Granzyme B in TILs

To further study the correlated observation of Th1 cytokine production in TILs and PBLs from breast cancer patients, we assessed the status of various cytotoxic molecules (perforin and granzyme B) on CD8+T cells. The mean percentage of T cells expressing perforin and granzyme B after stimulation was determined by intracellular staining and flow cytometry. After adequate in vitro stimulation, CD8+T cells derived from TILs produced these cytotoxic molecules at levels similar to those in PBLs.

DISCUSSION

In the present results, the proportion of CD4+CD25+Foxp3+ Treg cells among CD4+ cells is increased in the tumor microenvironment. It has been demonstrated that within primary breast tumors, higher numbers of Treg cells are associated with a poor

prognosis.[20, 45] Foxp3 is a transcription factor that is well known to be hallmark of immune suppressive Treg cells, especially thymically derived natural Treg cells.[47] Several studies in murine model have shown that diverse phenotypes of memory Treg cells express variable level of effector molecules and antitumor activities.[48] Yagi et. al. demonstrated that human peripheral CD25+CD4+ T cells are composed of CD45RO+ and CD45RO- cells and only the CD45RO+ Treg cells possess the suppressive activities.[49] Our results also show that most peripheral Treg cells in patient with breast cancer are CD45RO+ rather than naïve (CD45RO-) cells. Moreover, the CCR7-CD45RO+ Treg cells composite the predominant subpopulation in the tumor milieu. The phenotypic heterogeneity of the effector memory subset of Treg cells is responsible for repressing tumor-inhibiting CD8+ T cells and the subsequent loss of concomitant tumor immunity.

The exact mechanisms of Treg-mediated immunosuppression are not fully comprehended and remain investigated. The Treg cells at the tumor site require cytokine-dependent or cell contact-dependent manner to exert their suppressive activity on effector cells (CD8+ cytotoxic T lymphocytes and CD4+Th cells). [31] These Treg cells may impair anti-tumor immune responses through certain cytolytic enzyme-dependent essential pathways. Gondek et al. reported that activated murine Treg cells induced apoptosis of CD4+CD25- T effector cells via a granzyme B dependent mechanism.[39] Grossman et al. demonstrated that activated human Treg cells can use the perforin-granzyme pathway to kill a variety of autologous immune cells in vitro. [37] In a murine model, Treg cells derived from the tumor tissue have found to induce NK and CD8+T cell death in a granzyme B- and perforin-dependent process.[35] Granzyme B and perforin are therefore relevant for Treg cell-mediated suppression of tumor clearance in vivo. In our study, granzyme B was hardly

expressed in peripheral Treg cells but was highly expressed in twofold proportion of CD4+CD25+ Treg cells in the tumor micro-environment. A similar result for perforin was also discovered. It is our postulation that the perforin-granzyme pathway is one of the mechanisms of Treg cells to mediate the immunosupression in the patients with breast cancer.

There have been many studies showing the ability of the immune system to recognize and inhibit experimental tumor cell lines.[33] Cytotoxic function constitutes an important part of the cell-mediated immune system. Cytotoxic T lymphocytes (CTLs) lyse the target cells by means of mechanisms including granule exocytosis or the crosslinking of death receptors.[50-52] Perforin, a pore-forming protein, and granzymes, serine proteases, are key effector molecules of CTLs. The importance of these molecules has been evaluated in observations from animal models or human diseases. Perforin-deficient mice were found to decrease immune surveillance against tumors.[32, 33] Street et. al. demonstrated the importance of perforin to delay the onset of mammary carcinoma and to reduce the number of tumors initially growing in mice.[53] Another mouse model also discloses the involvement of perforin in both the antitumor immune response and the regulation of activity of mucosal inflammation in colitis-associated cancer.[54] Furthermore, the inherited disease resulted from mutation and deficiency of perforin in human, such as type 2 familial hemophagocytic lymphohistiocytosis, is proven a link between defective perforin mediated cytotoxicity and cancer susceptibility. Almost 50% of these patients developed at least one hematological malignancy in childhood or adolescence.[55] In this study, we found that Treg cells in the tumor microenvironment can express significantly higher levels of both granzyme B and perforin. On the contrary, granzyme B and perforin were rarely expressed by CD8+ T cell derived from TILs. The expression ratios of

both effector molecules were significantly down-regulated in the TILs than in the PBLs. Impaired expression of perforin and granzyme B in CD8+ T Cells may contribute to tumor escape in breast cancer.

Some studies have shown that purification and ex vivo culture of TILs can result in the recovery of their cytotoxic function.[56] Therefore, T cells expanded from tumors have been utilized in adoptive T cell transfer therapy to achieve durable clinical regressions in patients with metastatic melanoma.[15, 57] Optimal T cell activation requires two distinct signals. The first signal is mediated by major histocompatibility complex-restricted, antigen-specific triggering of the T-cell receptor-CD3 complex that activates a number of protein tyrosine kinases and thereby controls various downstream signaling pathways. The second signal, termed co-stimulation, relies on an antigen-independent mechanism and through receptors on the T cell surface, inclusive of CD28 as the prototype.[58] The CD28 co-stimulatory pathway plays an important role in anti-tumor responses.[41] To determine the functional capacity of patients' TILs and their potential utility for immunotherapy, we measured intracellular cytokine production after stimulation with anti-CD3 and anti-CD28 antibodies. Our results show that TILs can express significant levels of IL-2, IFN-γ, and TNF-α after in vitro activation with anti-CD3 and anti-CD28 antibodies. All of these cytokines are essential for the potentiation of the cytotoxic activity of CTLs and NK cells. Both IFN-γ and TNF-α possess direct cytotoxic and cytostatic activity towards tumor cells. Increased production of these cytokines may be helpful in inducing cytolytic cell function, recruiting and expanding antigen-presenting cells, and mediating direct antitumor effects. In addition, manipulations of Treg cells in cancers are proposed as possible new strategies for the treatment of cancer by facilitating the loss of tolerance to self-antigens.[59] Our results suggest that the

dysfunction of T cells may be a reversible phenomenon, dependent on the tumor-bearing environment of the patient. It implies a potential application for further adoptive transfer immunotherapy for breast cancer patients. Thus, it is necessary to overcome tumor-related immunosuppression before immunotherapies can successfully be applied.

In conclusion, TILs derived from the patients with breast cancer contain remarkable proportions of CD4+CD25+ Treg cells. Our findings are consistent with the observations from several previous studies and suggest that Treg cells are a part of mechanisms in controlling the anti-cancer immune response to breast cancer. Expression of granzyme B and perforin is mainly constricted in TILs within these cells, which may attribute to immune dysfunction. Furthermore, despite the activated status of CD8+ CTLs, minimal expressions of granzyme B and perforin still render them loss of cytotoxicity. Our study also shows that adequate in vitro activation of TILs is able to restore the appropriate anti-tumor immune response for subsequent immunotherapy.

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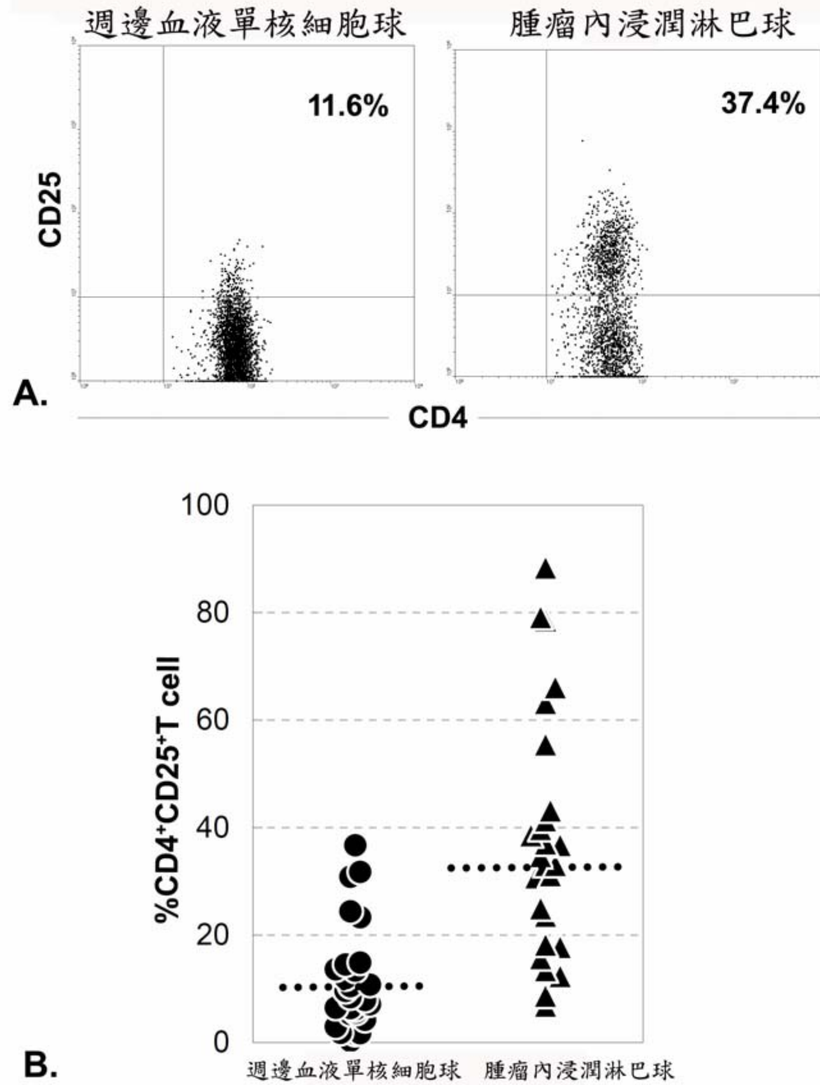
附表

Clinicopathologic characteristics of patients	Number	Percentage (%)
Total (N)	30	
Age (years)	56.8 (28-89)	
Histologic classification		
Infiltrating ductal carcinoma	30	100
T stage		
T1	4	13
T2	23	77
T3	3	10
N stage		
N0	16	53
N1	9	30
N2	4	13
N3	1	3
Stage		
I	2	7
II	21	70
III	7	23
ER		
Positive	17	57
Negative	13	43
PR		
Positive	16	53
Negative	14	47
HER-2		
Negative	27	57
Positive	13	43
Histologic grade		
1	11	38
2	12	40
3	7	23

表一

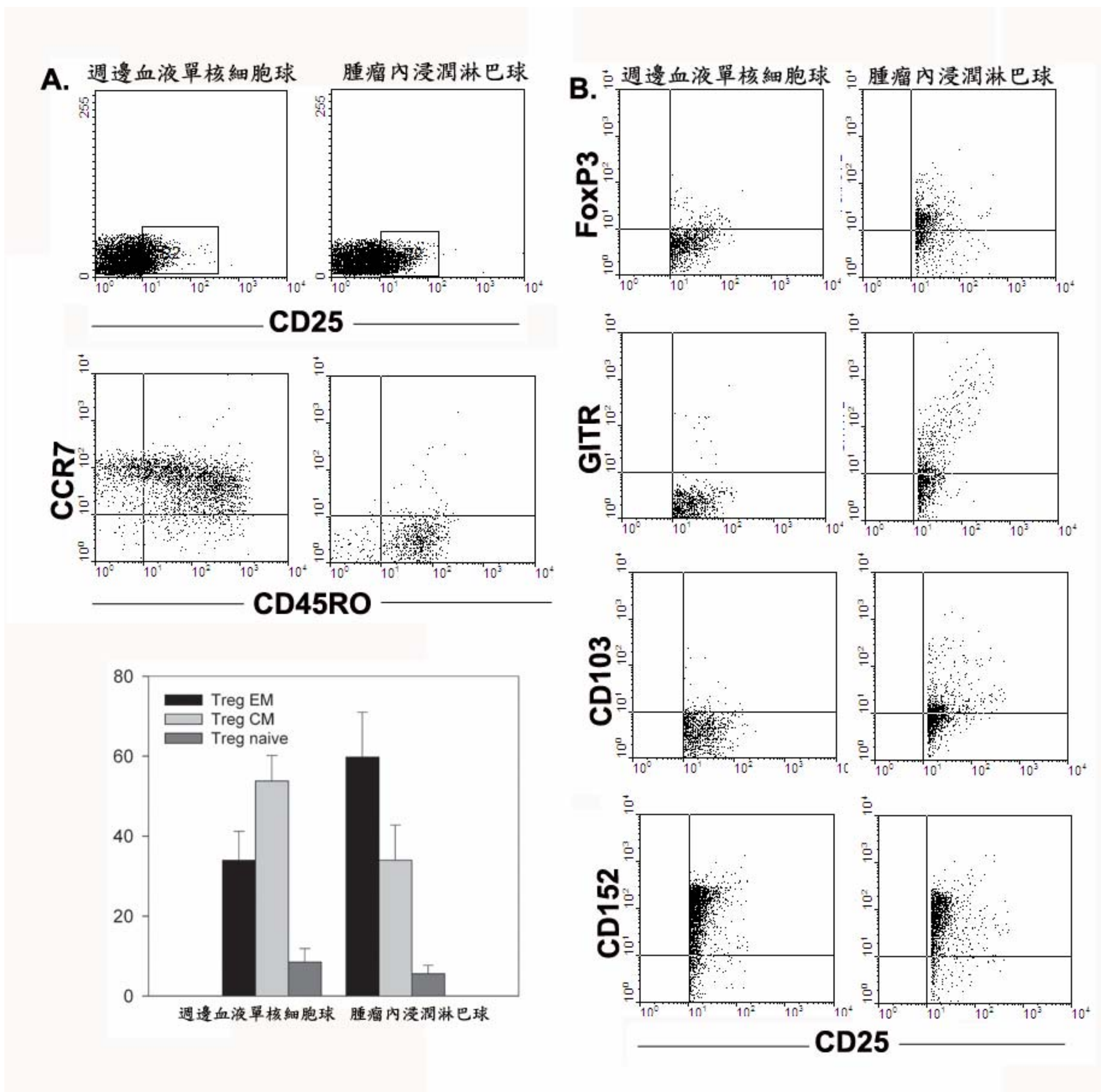
病患臨床基本資料

附圖



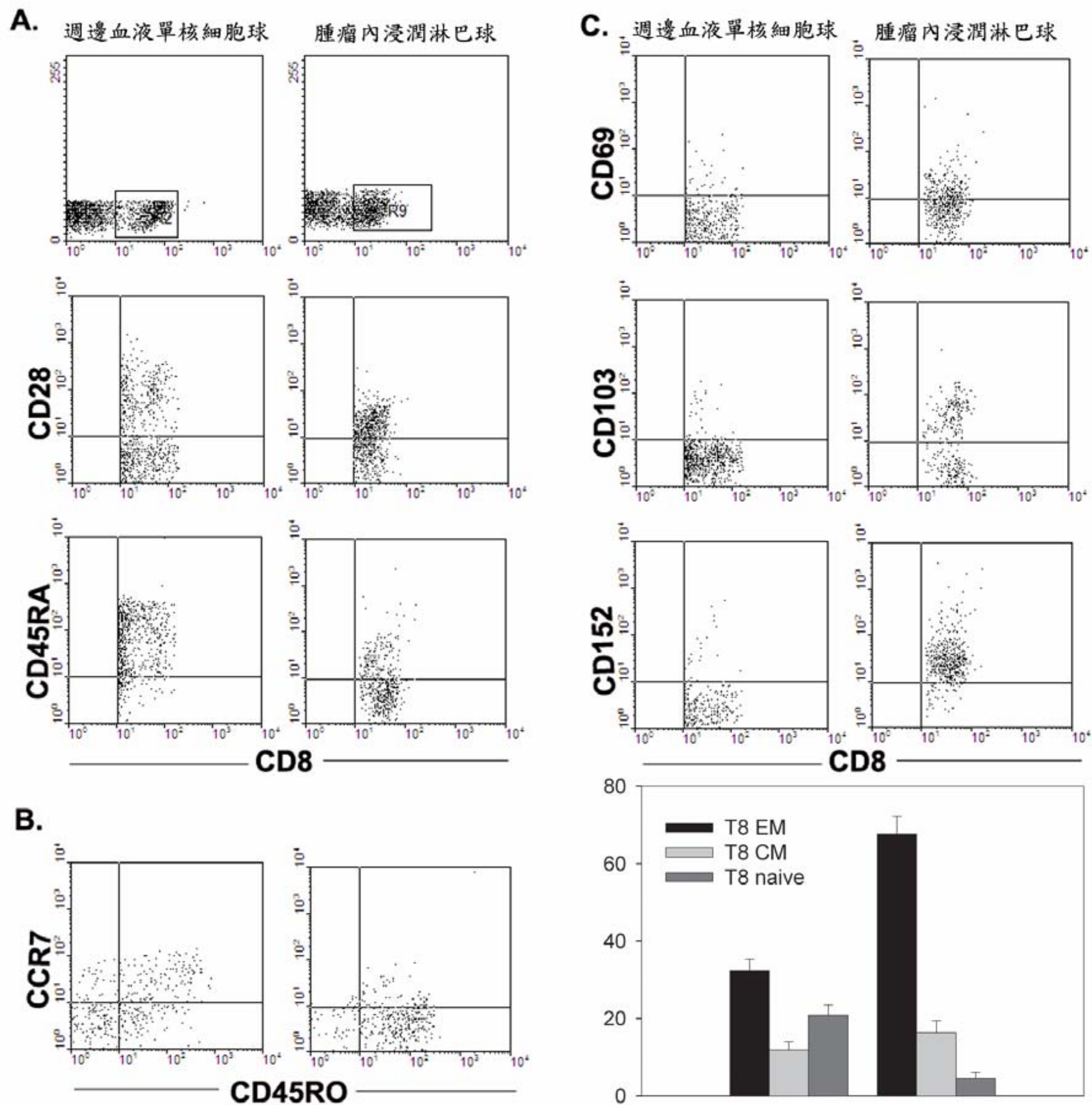
圖一

分析比較乳癌病患的週邊血液單核細胞球及腫瘤內浸潤淋巴球內 CD4+CD25+調節 T 細胞的表現（**圖一 A**）。乳癌病患的週邊血液單核細胞球內 CD4+CD25+調節 T 細胞的表現比例明顯低於腫瘤內浸潤淋巴球內的（11.6 % vs. 37.4%，P=0.001）（**圖一 B**）。



圖二

比較乳癌病患的週邊血液單核細胞球及腫瘤內浸潤淋巴球內CD4+CD25+調節T細胞。週邊血液單核細胞球內CD4+CD25+調節T細胞明顯表現較高比例的CCR7+CD45RO+ (central memory cells)，及較低比例的CCR7-CD45RO+（effector memory cells）（**圖二A**）。進一步分析CD4+CD25+調節T細胞其上FOXP3，CD152，CD103及GITR的表現，發現所有的CD4+CD25+調節T細胞都會表現FOXP3，GITR及CD152，但是週邊血液單核細胞球內的CD4+CD25+調節T細胞明顯表現較低的 FOXP3，CD103及GITR，而CD152 的表現比例在腫瘤內浸潤淋巴球與週邊血液單核細胞球內是相當的（**圖二B**）。

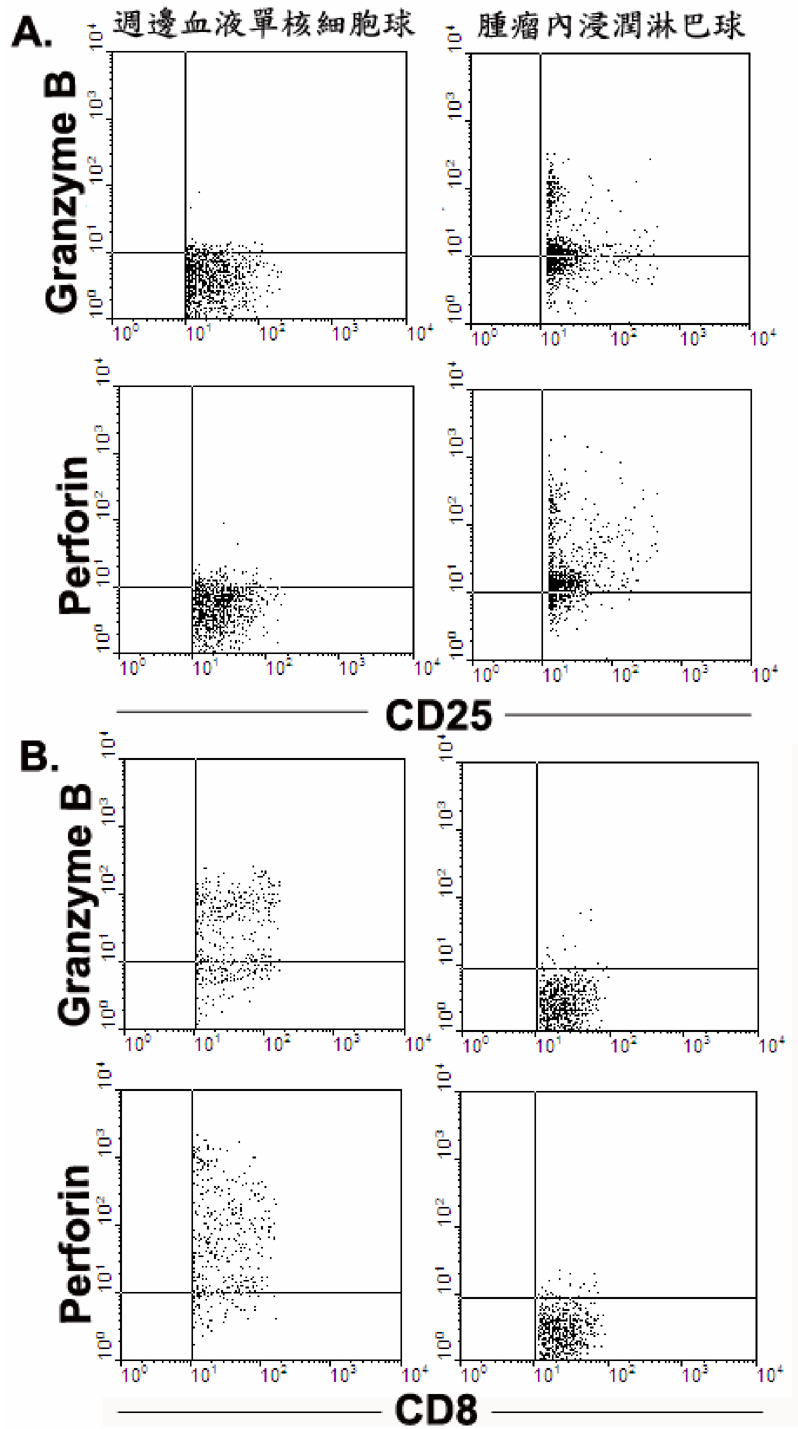


圖三

乳癌病患的腫瘤內浸潤淋巴球與週邊血液單核細胞球內的CD8+ T細胞均表現CD28，CD45RA及CD45RO （**圖三A**），但是腫瘤內浸潤淋巴球的CD8+ T細胞表現較少的CCR7 （**圖三B**），週邊血液單核細胞球內的CD8+ T細胞明顯表現較多的CCR7+CD45RO- näive T cells，及較少的CCR7-CD45RO+effector memory T cells，而CCR7+CD45RO+ central memory T cells 在腫瘤內浸潤淋巴球與週邊血液單核細胞球內的CD8+ T細胞的表現則是相當的（**圖三C**）。

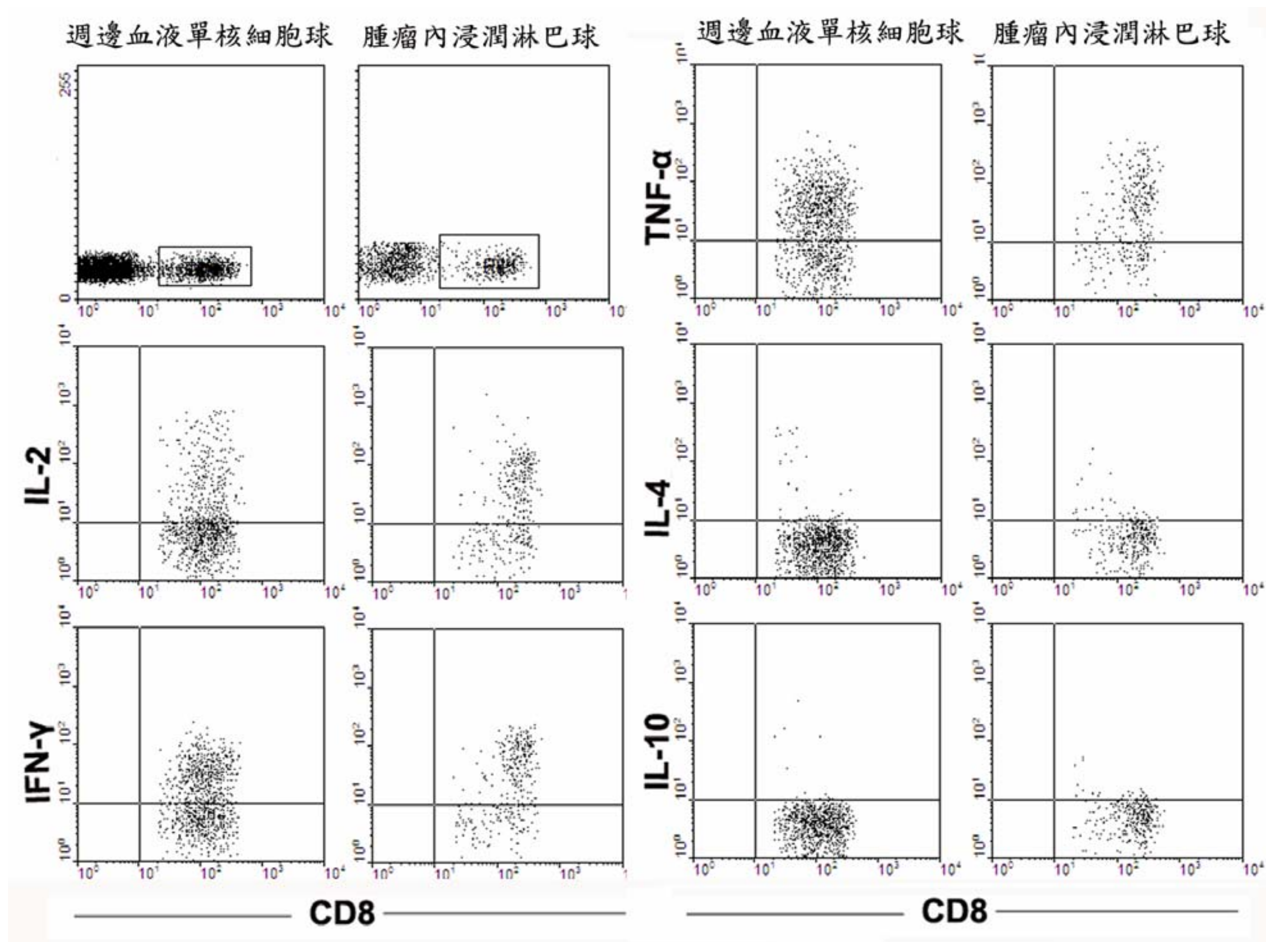
腫瘤內浸潤淋巴球的CD8+ T細胞表現較高的 CD69，CD103，及CD152，但是較少的CD45RA. 所以大部分的腫瘤內浸潤淋巴球的CD8+ T細胞的表現是

CD28-CD45RA-CD45RO+CCR7-，顯示他們是分化良好的一群細胞，而且他們大部分是CD69+CD103+CD152+，表示他們在腫瘤微環境中的活躍性。



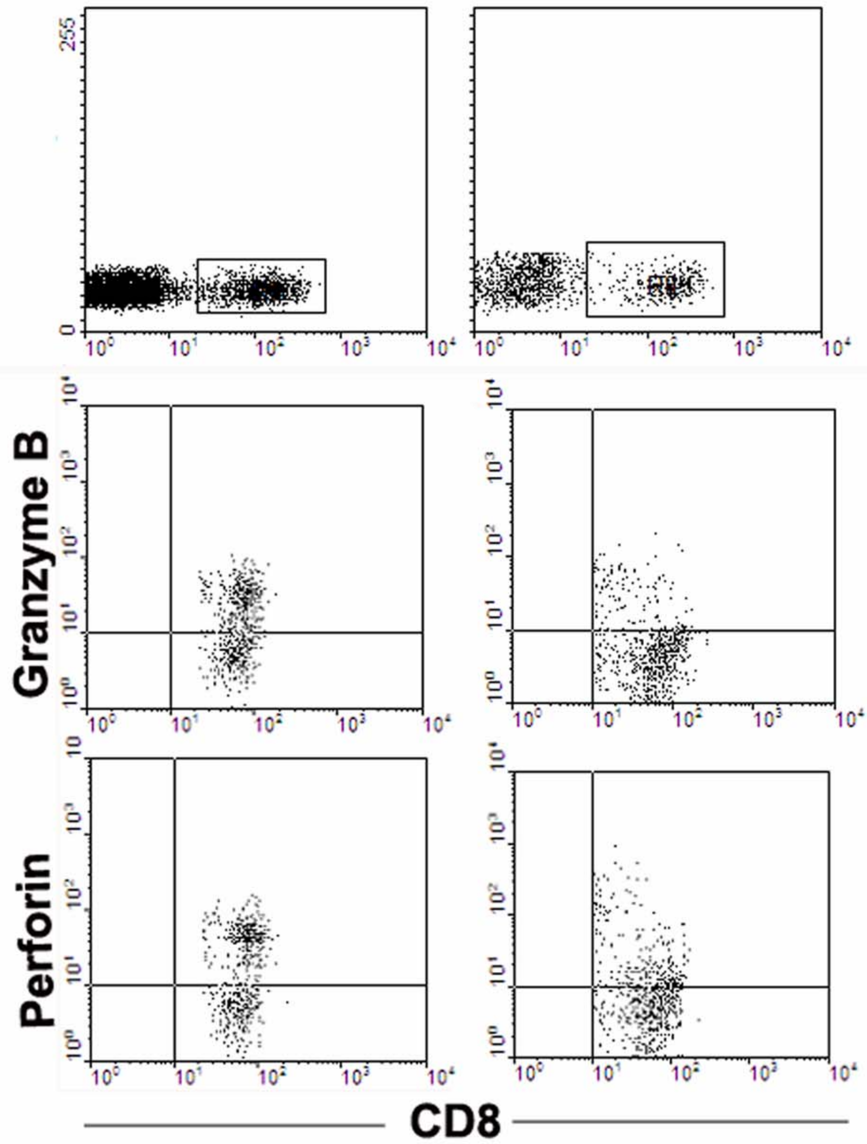
圖四

分析腫瘤內浸潤淋巴球與週邊血液單核細胞球內的CD4+CD25+調節T細胞，發現乳癌病患的腫瘤內浸潤淋巴球的CD4+CD25+調節T細胞明顯分泌較多的毒殺顆粒（granzyme B及perforin）（**圖四A**）。分析腫瘤內浸潤淋巴球與週邊血液單核細胞球內的CD8+ T細胞，乳癌病患的腫瘤內浸潤淋巴球的CD8+ T細胞其毒殺顆粒表現明顯降低（**圖四B**）。



圖五

腫瘤內浸潤淋巴球與週邊血液單核細胞球經過anti-CD3/anti-CD28刺激後，IL-2，IFN-γ，及TNF-α的分泌在兩者是相當的，這表示anti-CD3/anti-CD28可誘發出乳癌腫瘤內浸潤淋巴球的Th1細胞激素即IL-2，IFN-γ ，及TNF-α的分泌；而Th2細胞激素即IL-4及IL-10的分泌，於腫瘤內浸潤淋巴球與週邊血液單核細胞球的分泌均無增加，表示經過anti-CD3/anti-CD28刺激後，腫瘤內浸潤淋巴球並沒有分泌較多的Th2細胞激素。這個實驗證明腫瘤內浸潤淋巴球的活性是完整的，並且在某些刺激下是可以回復的。



圖六

以流式細胞儀檢測其 CD8+ T 細胞分泌毒殺顆粒（perforin and granzyme B）的能力，我們發現在 anti-CD3/anti-CD28 刺激後，腫瘤內浸潤淋巴球 CD8+ T 細胞分泌毒殺顆粒的表現比例，與週邊血液單核細胞球是相當的。