行政院國家科學委員會專題研究計畫　成果報告

蛻膜淋巴球上 IL-2 接受器減少表現的影響因子與可能作用機轉

計畫類別： 個別型計畫
計畫編號： NSC91-2314-B-002-373-
執行期間： 91 年 08 月 01 日至 92 年 07 月 31 日
執行單位： 國立臺灣大學醫學院婦產科

計畫主持人： 趙光漢

計畫參與人員： 蘇紋君

報告類型： 精簡報告

處理方式： 本計畫可公開查詢

中　華　民　國 92 年 10 月 28 日

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

蛻膜淋巴球上 IL-2 接受器減少表現的影響因子與可能作用機轉

計畫類別：■個別型計畫　　□整合型計畫
計畫編號：NSC 91－2314－B－002－373－
執行期間：　91 年 08 月 01 日至　92 年 07 月 31 日

計畫主持人：趙光漢
共同主持人：何弘能
計畫參與人員：蘇紋君

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：國立臺灣大學醫學院婦產科

中　華　民　國　92 年　10 月　28 日

中文摘要

有幾種機轉已被提出來說明母體對胎兒不會發生排斥，其中包括母體與胎兒之間的免疫反應受到抑制。T 細胞是免疫的重要介質，它能導致感染的清除、自體免疫疾病、移植組織的排斥與腫瘤的萎縮；而 T 細胞的活化則需經由 IL-2 與其接受器之作用。我們先前的研究發現正常懷孕時蛻膜中 T 淋巴球會被活化，但是 IL-2 接受器 α 卻選擇性地減少；然而我們在另一研究發現，蛻膜 T 淋巴球中 IL-2 接受器 α 的 mRNA 非但沒有減少，還接近活化時的水平。為了了解 IL-2 接受器 α 減少的機轉，在此研究中我們設立了自體周邊血球與絨毛組織的「體外共同培養」系統。從此系統我們發現絨毛滋養細胞可以增加溶解性 IL-2 接受器 α 之釋出，使 T 淋巴球表面上 IL-2 接受器 α 之表現減少。此外這種釋出過程可以被蛋白質酶抑制劑所改變。從絨毛組織的免疫組織化學染色顯示 MMP 蛋白質酶有明顯表現。因此，MMP 蛋白質酶可能對代謝 IL-2 接受器 α，使其在蛻膜淋巴球上的表現減少，從而降低淋巴球的繁殖活化佔有一重要角色。

Abstract

Several mechanisms are proposed to elucidate the maternal tolerance to fetal allograft in mammalian pregnancies, including suppressed immunologic interaction between mother and fetus. T cells are the critical mediators of immunity that lead to clearance of microbial infections, autoimmune diseases, tissue transplant rejection, and tumor regression. T cell activation is modulated by IL-2 through binding to its corresponding cell surface receptors, such as IL-2 receptor α (IL-2Rα, CD25). In our previous study, we demonstrated that the decidual $CD4^+$ and $CD8^+$ T lymphocytes were activated during normal pregnancies, but the expression of IL-2Rα was selectively decreased. In this study, the amount of IL-2Rα mRNA in decidual T cells did not decrease and approaches the amount in the corresponding activated T cells. For clarifying the mechanism responsible for down-regulation of IL-2Rα, a coculture model of *in vitro* mixed autologous peripheral lymphocytes and villous trophoblasts was established. In this model, we found that the villous trophoblasts could induce the decreased expression of IL-2Rα on T lymphocytes by increased release of soluble IL-2Rα (sIL-2Rα) from the cell surface. This increased release of soluble IL-2Rα could be altered by the protease inhibitors. Immunohistochemical staining revealed prominent matrix metalloproteinase (MMP) expression, including MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9, in the villous tissue. Our findings suggest that the MMPs have another new role in immunomodulation at the fetomaternal interface through the decrease of the expression of IL-2Rα on decidual lymphocytes by proteolytic cleavage of IL-2Rα and therefore down-regulated their proliferative capability.

Keywords

IL-2, IL-2Rα, sIL-2Rα, MMPs

Introduction

The fetal genome is half-paternal and half-maternal. According to transplantation immunology, it is logical to postulate that the maternal immune system should reject it during pregnancy, but this generally dose not occur (1). Although it is still an open question how the conceptus avoids immune rejection, it is now generally accepted that the maternal adaptive immune system is at least aware that the fetus and placenta exist, as certified by alloantigen-specific alterations in maternal T cell phenotypes during pregnancy (2, 3).

In our previous study, we demonstrated that the levels of CD25 on $CD4^+$ and $CD8^+$ T lymphocytes were decreased during pregnancy, although the levels of other activation markers, CD69 and HLA-DR, were markedly increased (4). In our another study about cervical cancer, we also found that the depressed immune responses in cancer patients was due to the down-regulation of the IL-2Rα expression on tumor-infiltrating lymphocytes and MMP system mediated the process by proteolytic cleavage of the IL-2Rα (5). Because the IL-2/IL-2R system plays a crucial role in the generation of cytotoxic lymphocytes, which is necessary for the development of rejective response, the selectively decreased expression of CD25 must be related with the tolerance to the fetoplacental graft. The present study was undertaken to find out the possible mechanisms of depressed IL-2Rα expression on the surface of decidual lymphocytes. Whether there are similar immune modulations in pregnant women, as that in cancer patients, is further investigated.

Materials and Methods

Subjects

Twenty pregnant women (aged 33.0±5.2 years) who had elective abortions of normal pregnancies between 6 and 10 weeks of gestational age due to multiparity, were enrolled in the study with informed consent and under the approval of the investigation review board of our hospital. None of the pregnant subjects was receiving any medication, and all were nonsmokers. Subjects with associated complications or subclinical autoimmune diseases were excluded from this study.

Specimens

Fetal chorionic villi and peripheral blood samples were taken from each pregnant woman at the time of abortion. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS sedimentation. Villous tissue was carefully examined under a dissecting microscope to ensure absence of all maternal tissue and was minced into small pieces. Then the tissue (1.5 gm) was digested in a plastic tube with 6 mL HBSS (Ca^{2+}- and Mg^{2+}-free) containing 0.25% trypsin. The tube was vigorously shaken at 37˚C water bath for 30 minutes and was spun at 1000*g* for 10 minutes. The supernatant was aspirated and the large pellet was resuspended in 6 mL RPMI-1640. The cytotrophoblast cells were separated out using a discontinuous Percoll density gradient and were collected from cell banding between 35% and 55%. Thereafter the cells were transferred to a

plastic culture tube filled with culture medium (RPMI-1640 supplemented with 100IU/mL Penicillin, 100μg/mL Streptomycin, and 10% fetal calf serum).

Autologous Coculture Assay

PBMCs were fully activated with 10 μg/mL of PHA (Life Technologies Co., Rockville, MD USA) in a 37˚C, 5% CO_2- humidified incubator for 24 hours. The activation and coculture procedure outlined below were performed in 12mm × 75mm capped polystyrene test tubes. In the experimental group, 5×10^5 activated T cells were cocultured with 1×10^6 autologous villous cells (1:2) in 3ml of culture medium. In the control group, only 5×10^5 activated T cells were cultured in 3ml of culture medium. The supernatants in both groups were collected on days 1, 3, and 5 of cultures and were stored in -75˚C.

Determination of soluble IL-2Rα by ELISA assay

The shed sIL-2Rα in the supernatants was measured by ELISA assay. They were detected using commercial (R&D Systems, Minneapolis, MN USA; lower limit of sensitivity, <10 pg/mL) matched pairs of anti-human sIL-2Rα monoclonal antibodies (capture antibody and horseradish peroxidase-linked detecting antibody) optimized for use according to the manufacturer's guidelines. The sIL-2Rα concentrations were calculated from a standard curve generated with specific standards provided with each kit.

Protease Inhibition Assay by Different Protease Inhibitors

Protease inhibitors (SIGMA Inc., St. Louis, MO and CHEMICON Inc., Temecula, CA) for a broad spectrum of inhibition were prepared. The selected protease inhibitors included antipain-dihydrochloride (papain, trypsin, cathepsin A and B inhibitor), leupeptin (serine and cysteine protease, plasmin, trypsin, papain, and cathepsin B inhibitor), E-64 (cysteine protease inhibitor), and MMP Inhibitor (a broad spectrum gelatinase inhibitor and a potent inhibitor of collagenase). Titrated concentrations of individual protease inhibitors were prepared according to the recommended working dilution of the reagent suppliers. Drug toxicity was tested by the addition of titrated concentrations of reagents in the lymphocyte culture system for 24 hours with >90% viable cells by the trypan-blue staining method.

Different protease inhibitors were added to the established autologous coculture system. For the maximal expression of IL-2Rα on the surface of T cells, PBMCs were fully activated with 10 μg/mL PHA for 24 hours before coculture. Autologous villous cells (1×10^6) were first cultured in 0.5 mL of culture medium containing protease inhibitors. The final concentration protease inhibitors as recommended in each tube were as follows: (a) antipain-dihydrochloride, 5 and 50 μg/mL; (b) leupeptin, 1 and 10 μg/mL; (c) E-64 , 1 and 10 μg/mL; and (d) MMP-inhibitor, 10 and 100 μM. Constant numbers (1×10^6) of PHA-activated T cells in 0.5 mL of culture medium were then added to the autologous villous cells with specific protease inhibitor. In the control group, including only activated T cells and coculture of activated T cells with villous cells, no protease

inhibitor was added. The supernatant of each culture tube was collected on the third day of coculture, and the concentrations of sIL-2Rα were measured by ELISA method.

Expression of MMPs and TIMPs in Villous Tissue

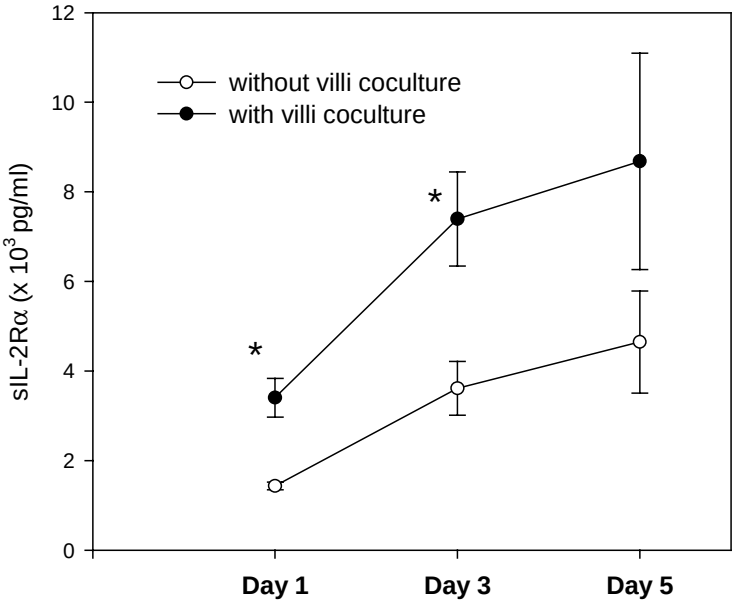
For cryostst sectioning, pieces of villous tissue were frozen in liquid nitrogen. Serial cryostat sections (5 μm) were cut at -21°C and mounted on glass slides. The sections were fixed in absolute acetone (10 min at 4°C). An avidin-biotin-peroxidase complex immunohistochemical staining method was performed for examination of expression patterns of MMP-1, -2, -3, -7, -9, TIMP-1, TIMP-2, and TIMP-3 in the cryostat sections of villous tissue. The antibodies were obtained from CHEMICON Inc. (Temecula, CA), and their specificities were provided by the manufacturer.

Result

Increased sIL-2Rα Release from Activated T Cells by Villous Cells

The IL-2Rα is enzymatically cleaved shed from the surface of expressing cells. It can be measured in the serum by ELISA assay as a 45 kDa soluble IL-2 receptor (sIL-2Rα). In the Figure 1, we analyzed the amounts of sIL-2Rα in the supernatants of PHA-activated PBMCs cocultured with and without villous cells. The mean concentrations of sIL-2Rα were increased about 2.5-fold on day 3 when compared to that on day 1 in both groups. However, the mean concentration of sIL-2Rα in the supernatants of coculture group was significantly higher than that in without-coculture group on both day 1 and 3.

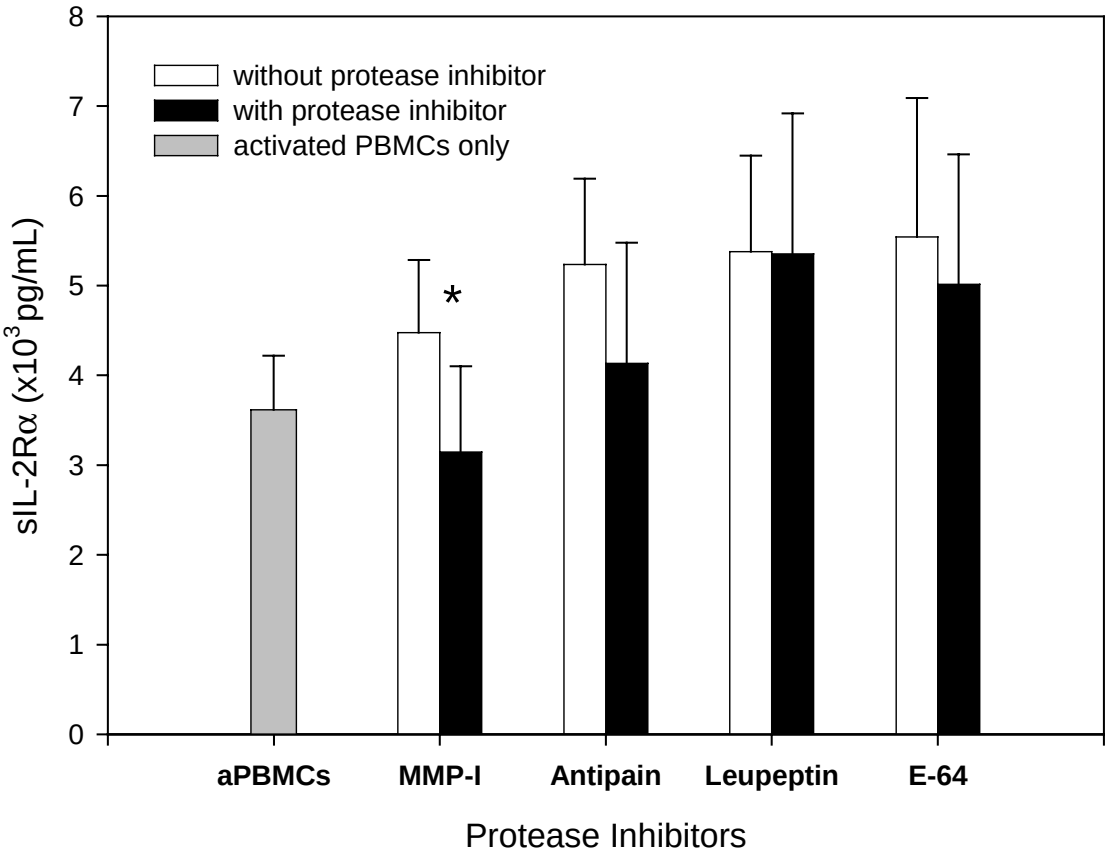
Figure 1. The levels of sIL-2Rα in the supernatants of PHA-activated PBMCs without villous cells cocultured (○, control group) and cocultured with villous cells (●, experimental group).



MMP Inhibitors Block Villus-induced Proteolytic Cleavage of IL-2 Rα

Using the autologous coculture experiments of PHA-activated PBMCs and villous cells, we conduct the protease inhibition assays to determine whether specific inhibitors could reverse or limit the villus-induced proteolytic cleavage of IL-2Rα. For achieving maximal expression of IL-2Rα, PBMCs were fully activated with 10 μg/mL PHA for 24 hours before coculture. After 3 days of coculture, the concentration of IL-2Rα in the supernatant of coculture groups without protease inhibitors added was higher than that of activated PBMCs-only group. Protease inhibitors, such as antipain, leupeptin, and E-64 had no apparent activity in suppressing villus-mediated proteolytic cleavage of IL-2Rα (Fig. 2). However, MMP inhibitor significantly inhibited the villus-induced IL-2Rα cleavage. We also noted that, at a concentration of 100 μM MMP inhibitor, IL-2Rα cleavage was totally inhibited. The finding indicates that an MMP-mediated proteolytic process is likely to be responsible for the IL-2Rα cleavage, and that MMP inhibitors block this process.

Figure 2. The levels of sIL-2α in the supernatants of activated PBMCs (aPBMCs) cocultured with villous tissue in the presence of different protease inhibitors (MMP-I: MMP inhibitor)



The Expression of MMP Families in the Villous Tissue

We then study the expression of various types of MMPs, including MMP-1, -2, -3, -7, -9, and three types of inhibitors (TIMP-1, TIMP-2, and TIMP-3) by immunohistochemical staining in pregnant villous tissues. MMP-1 and MMP-2 are abundantly expressed in most cases, especially in extravillous trophoblast cells. Nevertheless, MMP-3, MMP-7, and MMP-9 were also weakly expressed in the villous tissue. In addition, diffusely weak staining of TIMP-1, -2, and -3 were observed in selected cases. Their immunoreactivities mainly were found in and around extravillous trophoblast cells.

Discussion

In our previous study, we demonstrated that the IL-2Rα was selectively decreased on decidual T lymphocytes (4). As we know, immunologic rejection has been shown to require intact $CD4^+$ helper T cells. Furthermore, the IL-2 plays a crucial role in the generation of immune response against allografts (6, 7). The stimulation of T-cell receptors with antigen or a mitogenic lectin induces a signal transduction pathway involving protein kinase C, which stimulates IL-2 secretion and upregulation of the α subunit of the IL-2 receptor (CD25) (8). Secreted IL-2 functions in an autocrine and paracrine manner by binding to the full IL-2 receptor to initiate T-cell proliferation and differentiation. It has been shown that Induction of IL-2 gene transcription and expression of IL-2R precede acute rejection (9, 10).

To understand how immunity may be suppressed in the uterus during pregnancy and whether the decreased expression of CD25 on the decidual lymphocytes was related with their inert reaction to the fetal antigens, several experiments were performed to determine at what steps T-cell tolerance acts to inhibit lymphocyte proliferation. First, we tested the effects on mitogen-stimulated CD25 expression, mitogen-stimulated IL-2Rα gene expression, and the release pattern of sIL-2Rα. Furthermore, we examined the effects of protease and protease inhibitors on the expression of CD25.

In previous study, decreased expression of CD25 was found on activated $CD4^+$ and $CD8^+$ T lymphocytes derived from decidual tissue, although these lymphocytes expressed high levels of CD69 and HLA-DR antigens. Now we demonstrate that the expression of IL-2Rα on the surface of activated T cells is downregulated when cocultured with villous cells. Together, these finding indicate that villous cells can reduce IL-2Rα expression on encountered T cells in a specific manner. However, the decreased CD25 expression on decidual lymphocytes dose not affect steady-state amounts of IL-2Rα mRNA, which even approaches that in fully-activated T cells. The similar condition is present in tumor-infiltrating lymphocytes from the tissue of cervical cancer and breast cancer (5, 11). The failure of down-regulation of IL-2Rα mRNA in decidual lymphocytes and tumor-infiltrating lymphocytes would suggest that immune suppression during pregnancy and tumor formation did not occur at the transcription level and it might be due to excessive shedding of IL-2Rα from T cells.

Serum concentration of the soluble form of the IL-2Rα has been found to be increased in

patients rejecting allograft. After equilibrium, the increase of sIL-2Rα in the supernatants reflects the increase of IL-2R on the surface of T lymphocytes (12). Nevertheless, in the short term or in the presence of protease, the increased amount of sIL-2Rα in the supernatants means increased shedding of IL-2R from the surface of T lymphocytes, which in turn affects the T-cell proliferation mediated by IL-2. In this study, we used coculture system of activated PBMCs and villous cells and demonstrated that the villous cells could enhance the degradation of IL-2Rα on activated T cells and force the release of sIL-2Rα into the supernatant from the activated T cells (13, 14). However, a proteolytic cleavage of IL-2Rα might mediate the process, because the protease inhibitors, like MMP inhibitor, could block the cleavage of IL-2Rα. Thus, the observed villus-mediated sIL-2Rα down-regulation can be attributed mainly to proteolytic cleavage of MMPs from the villous tissue. Owing to the pivotal role of IL-2Rα in the development and propagation of functional T cells, cleavage of IL-2Rα may lower the proliferative capability of T cells and abolish the development of rejective response.

Like malignant tumor cells, trophoblast cells of the implanting blastocyst and the hemochorial placenta of numerous species, including the human, are highly invasive. This invasiveness is necessary for implantation and subsequent penetration of the endometrial stroma and blood vessels, which in turn is required for proper fetomaternal exchange (15). These cells are considered to be invasive by virtue of their ability to secrete proteases capable of digesting their immediate environments (16). Although cytotrophoblasts produce an array of proteases, only MMPs have the capability of digesting the different components of the basement membrane and the extracellular matrix. Nevertheless, in the immune modulation, these MMPs also have different substrate specificities and cellular sources, with complicated regulation of cytokines and other inflammatory mediators on immune cells. These MMPs may trigger the proteolytic cleavage of cytokines and their receptors, including tumor necrosis factor receptor (17, 18), IL-6 receptor (19, 20), and also IL-2Rα, as shown in the present study. Therefore, due to destruction of the IL-2 receptor, supposed by trophoblast cells through MMPs system, the decidual lymphocytes loss the ability to respond to IL-2, which is the essential cytokine for T-cell activation and the development of rejective phenomenon.

The apparent lack of IL-2Rα expression in the population of decidual T cells may represent the induction of tolerance locally within the uterine microenvironment. However, the mechanism for the inhibition of IL-2Rα expression on activated T cells in decidua is governed by a villus-directed and MMP-dependent cleavage. Through the MMP-proteolytic pathway, The MMPs can inhibit the proliferative function of T cells and therefore their activation. This may explain the relative anergic state of the decidual T lymphocytes, and no rejection found at the fetomaternal interface. Our finding should provide important insights into the understanding of the interaction between fetus and maternal immune system, as well as into the development of anti-rejective strategies in tissue transplantation.

References

1. Clark, D. A., P. C. Arck, and G. Chaouat. 1999. Why did your mother reject you? Immunogenetic determinants of the response to environmental selective pressure expressed at the uterine level. *Am. J. Reprod. Immunol. 41:5*.
2. Geiselhart, A., J. Dietl, K. Marzusch, P. Ruck, M. Ruck, H. P. Horny, E. Kaiserling, and R. Handgretinger. 1995. Comparative analysis of the immunophenotypes of decidual and peripheral blood large granular lymphocytes and T cells during early human pregnancy. *Am. J. Reprod. Immunol. 33:315*.
3. Ho, H. N., K. H. Chao, H. F. Chen, S. U. Chen, M. Y. Wu, and Y. S. Yang. 2001. Distribution of Th1 and Th2 cell populations in human peripheral and decidual T cells from normal and anembryonic pregnancies. *Fertil. Steril. 76:797*.
4. Chao, K. H., M. Y. Wu, J. H. Yang, S. U. Chen, Y. S. Yang, and H. N. Ho. 2002. Expression of the interleukin-2 receptor alpha (CD25) is selectively decreased on decidual CD4+ and CD8+ T lymphocytes in normal pregnancies. *Mol. Hum. Reprod. 8:667*.
5. Sheu, B. C., S. M. Hsu, H. N. Ho, H. C. Lien, S. C. Huang, and R. H. Lin. 2001. A novel role of metalloproteinase in cancer-mediated immunosuppression. *Cancer Res. 61:237*.
6. Nickerson, P., J. Steiger, X. X. Zheng, A. W. Steele, W. Steurer, P. Roy-Chaudhury, and T. B. Strom. 1997. Manipulation of cytokine networks in transplantation: false hope or realistic opportunity for tolerance? *Transplantation 63:489*.
7. Baan, C. C., and W. Weimar. 1998. Intragraft cytokine gene expression: implications for clinical transplantation. *Transpl. Int. 11:169*.
8. Waldmann, T. A. 1993. The IL-2/IL-2 receptor system: a target for rational immune intervention. *Immunol. Today 14:264*.
9. Bugeon, L., M. C. Cuturi, M. M. Hallet, J. Paineau, D. Chabannes, and J. P. Soulillou. 1992. Peripheral tolerance of an allograft in adult rats--characterization by low interleukin-2 and interferon-gamma mRNA levels and by strong accumulation of major histocompatibility complex transcripts in the graft. *Transplantation 54:219*.
10. Dallman, M. J. 1993. Cytokines as mediators of organ graft rejection and tolerance. *Curr. Opin. Immunol. 5:788*.
11. Coventry, B. J., S. C. Weeks, S. E. Heckford, P. J. Sykes, J. Bradley, and J. M. Skinner. 1996. Lack of IL-2 cytokine expression despite Il-2 messenger RNA transcription in tumor-infiltrating lymphocytes in primary human breast carcinoma: selective expression of early activation markers. *J. Immunol. 156:3486*.
12. Junghans, R. P., and T. A. Waldmann. 1996. Metabolism of Tac (IL2Ralpha): physiology of cell surface shedding and renal catabolism, and suppression of catabolism by antibody binding. *J. Exp. Med. 183:1587*.
13. Rubin, L. A., C. C. Kurman, M. E. Fritz, W. E. Biddison, B. Boutin, R. Yarchoan, and D. L. Nelson. 1985. Soluble interleukin 2 receptors are released from activated human lymphoid cells in vitro. *J. Immunol. 135:3172*.

14. Rubin, L. A., and D. L. Nelson. 1990. The soluble interleukin-2 receptor: biology, function, and clinical application. *Ann. Intern. Med. 113:619*.
15. Blankenship, T. N., A. C. Enders, and B. F. King. 1993. Trophoblastic invasion and the development of uteroplacental arteries in the macaque: immunohistochemical localization of cytokeratins, desmin, type IV collagen, laminin, and fibronectin. *Cell Tissue Res. 272:227*.
16. Mullins, D. E., and S. T. Rohrlich. 1983. The role of proteinases in cellular invasiveness. *Biochim. Biophys. Acta 695:177*.
17. Williams, L. M., D. L. Gibbons, A. Gearing, R. N. Maini, M. Feldmann, and F. M. Brennan. 1996. Paradoxical effects of a synthetic metalloproteinase inhibitor that blocks both p55 and p75 TNF receptor shedding and TNF alpha processing in RA synovial membrane cell cultures. *J. Clin. Invest. 97:2833*.
18. Lombard, M. A., T. L. Wallace, M. F. Kubicek, G. L. Petzold, M. A. Mitchell, S. K. Hendges, and J. W. Wilks. 1998. Synthetic matrix metalloproteinase inhibitors and tissue inhibitor of metalloproteinase (TIMP)-2, but not TIMP-1, inhibit shedding of tumor necrosis factor-alpha receptors in a human colon adenocarcinoma (Colo 205) cell line. *Cancer Res. 58:4001*.
19. Gallea-Robache, S., V. Morand, S. Millet, J. M. Bruneau, N. Bhatnagar, S. Chouaib, and S. Roman-Roman. 1997. A metalloproteinase inhibitor blocks the shedding of soluble cytokine receptors and processing of transmembrane cytokine precursors in human monocytic cells. *Cytokine 9:340*.
20. Marin, V., F. Montero-Julian, S. Gres, P. Bongrand, C. Farnarier, and G. Kaplanski. 2002. Chemotactic agents induce IL-6Ralpha shedding from polymorphonuclear cells: involvement of a metalloproteinase of the TNF-alpha-converting enzyme (TACE) type. *Eur. J. Immunol. 32:2965*.